IDENTIFICATION OF NOVEL *STREPTOMYCES* STRAINS WITH ANTIMICROBIAL AND ANTITUMOUR ACTIVITY ISOLATED FROM TERRESTRIAL HABITATS

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Abstract

Streptomyces, Gram positive filamentous bacteria, are very important organisms due to their capability of producing a variety of useful secondary metabolites with many biological activities and applications such as antibiotics, anticancer drugs and antioxidants. The increase in resistant pathogens, emergence of new diseases and toxicity of drugs that are currently used makes the urgent search to find new bioactive compounds in the medical industry to be of major importance. UAE is an under explored habitat that may have the potential to harbour novel *Streptomyces* strains producing diverse natural products. The main aim of the study is to isolate novel *Streptomyces* species from terrestrial habitats. Six Streptomyces strains (CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1) were found to produce antimicrobial metabolites against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces cerevisiae* and *Escherichia coli*-ESBL using the well diffusion method. Phylogenetic analysis of the 16S rRNA sequence confirmed the identification and novelty of the six isolates to the genus of *Streptomyces.* The cytotoxicity of the extracts was investigated on HeLa cancer cell line by MTT assay and DAPI staining where some of the isolates inhibited the growth of HeLa cells in a concentration-dependent manner and caused apoptosis observed by DNA fragmentation and chromatin condensation. Cultivating the isolates with *Bacillus subtilis* and *N*-acetylglucosamine resulted in one isolate acquiring cytotoxic activity against HeLa cells which was not observed in the monoculture. Cultivation with γ-Butyrolactone led to either enhancement or inhibition of antimicrobial and cytotoxic activity. Genomic sequencing and liquid extraction surface analysis mass spectrometry (LESA-MS) techniques were performed to identify biosynthetic genes produced by the isolates. The genome of the isolates was sequenced and a variety of biosynthetic gene clusters were predicted by antiSMASH analysis. Finally, LESA-MS was performed as a novel technique of direct sampling from the bacterial colonies. Distinct changes in mass profiles were generated between the uninduced and induced isolates which was observed as appearance, disappearance or increase of intensity in the produced peaks. The masses of the biosynthetic gene clusters produced by antiSMASH were additionally confirmed by LESA. Based on the results of this study, we conclude that these isolates are capable of producing antimicrobial and anticancer metabolites, varied and distinct biosynthetic gene clusters and they represent novel species of the genus *Streptomyces*.

Declaration

Acknowledgments

I have to start with thanking “Allah” for all his blessings, Alhamdulillah.

What a journey! I must say that being a “distance learning” student was not easy for me, not to be able to physically communicate and interact with other students and people in the field made me feel ironically very distant. Yet, there are a number of people that I would like to thank for their help and support.

I would like to express my extreme gratitude to my supervisors Debbie Cunningham, Luke Alderwick and Neil Hotchin for their great guidance, support and encouragement. Special thanks to my UOS co-supervisor Prof. Ismail Saadoun for his support and introducing me to the world of *Streptomyces*. I would, also, like to thank all members at the department of Applied Biology at the University of Sharjah for their support. Many thanks to Klaudia Kocurek and Rian Griffiths in Helen Cooper group for running my samples with LESA and their prompt response for all the inquiries I had.

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List of Abbreviations

°C	degrees centigrade
ASW	artificial sea water
ATCC	american type culture collection
BGC	biosynthetic Gene Cluster
Bp	base pairs
DAPI	4′,6-diamidino-2-phenylindole
ddH_2O	double distilled water
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EtOAc	ethyl acetate
ESBL	Extended-spectrum beta lactamase
FBS	foetal bovine serum
G	grams
GBL	Ɣ-Butyrolactone
GlcNAc	N-acetylglucosamine
Hrs	Hours
KEGG	kyoto encyclopaedia of genes and genomes
L	litre
LB	luria bertani broth
LESA	liquid extraction surface analysis
MIC	minimum inhibitory concentration
min	minutes
ml	millilitre
mM	millimolar
MS	mass spectrometry
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
PFA	paraformaldehyde

ppm	parts per million
RED	undecylprodigiosin
SCA	starch casein agar
V/V	volume/volume
ZOI	zone of Inhibition
µg	microgram
µl	microlitre
µM	micromolar

Chapter 1

Introduction

1. Introduction

Streptomyces are Gram positive filamentous bacteria, capable of forming spores and aerial mycelium. *Streptomyces* belongs to family *Streptomycetaceae* and they are of major interest due to their ability to produce bioactive compounds with antimicrobial activity (Goodfellow & Fiedler, 2010). About 40-50% of the actively produced secondary metabolites come from actinomycetes and about 70-80% of these are produced by *Streptomyces* (Berdy, 2012), which makes it a significant genus. More than 6,000 bioactive metabolites produced by different species of *Streptomyces* possess some form of inhibitory activity that has been utilized in antibiotics, antifungals, antiviral or anticancer agents (Berdy, 2005).

Streptomyces are known as producers of medicine as they are the source for the production of many secondary metabolites with clinical applications some of which are antimicrobial (erythromycin, streptomycin) and anticancer (bleomycin and doxorubicin) (Demain and Sanchez, 2009) which signifies their importance and interest in isolating them. The emergence of multidrug-resistant pathogenic bacteria has increased the search for new antibiotics from natural sources with low or no side effects (Malapaka et al., 2007). *Streptomyces* that have inhibitory activity may produce many antibiotics that can be different in their mode of action, toxicity, chemical nature and their therapeutic potentials. Some *Streptomyces* may produce more than one antibiotic such as *S. griseus* which produces streptomycin and dihydrogranticin (Procópio et al., 2012) or different species of *Streptomyces* may produce the same antibiotic (e.g., actiomycin and streptothricin) (Harir et al., 2018). In addition, some antibiotics have more than one mode of application such as arenimycin which is used as antibacterial and anticancer compound. *Streptomyces* have many other applications such as production of enzymes (e.g., lipases, cellulases and amylases) (Hiramatsu and Ouchi, 1963), herbicides (e.g. herbicidine) (Shelton et al., 1996) and production of vitamin B12 (Hall et al., 1953), and therefore playing an important role in bioremediation and degradation of aromatic compounds.

Streptomyces can be found in soil, marine sediment and freshwater. Yet, most of the antibiotics that are found in the market were produced from terrestrial *Streptomyces*, when in contact with a surface *Streptomyces* reproduce by spores that are formed by specific aerial mycelium which is synchronized with antibiotic production (Zhu et al., 2014). Their existences and dominance depend on many factors such as temperature, pH, nutrients, climate, season, soil type and moisture (Saadoun and Gharaibeh, 2003).

Searching for secondary metabolites from natural habitat is still of great interest, even with the emergence of the synthesis of synthetic antibiotics, as it is a rich and unlimited source for finding new antimicrobial agents with new structures and chemical properties (Baltz, 2006). Multidrug resistance of pathogenic organisms, resistance and toxicity of chemotherapeutic drugs and unfavourable side effects are all playing a vital part in the importance of finding novel bioactive compounds.

1.1. Characteristics and Life Cycle of *Streptomyces*

Streptomyces are aerobic microorganisms with DNA containing high GC content. They have many morphological, biochemical and physiological properties that make them significantly different from other microorganisms indicating formation of spores and 16S rRNA partial sequences (Korn-Wendisch and Kutzner, 2006). They are found in many environments especially soil and marine habitats. Morphologically, they are similar to fungi as they grow by tip extension which leads to the formation of branched hyphae. Under nutrient rich conditions, *Streptomyces* produce heavy vegetative mycelium and cell division takes place by the formation of cross walls separating each hyphae into individual compartments (Anderson and Wellington, 2001). As such, this makes *Streptomyces* multicellular organisms as each compartment have many copies of the chromosome (Claessen et al., 2014).

Streptomyces have a complex life cycle in which they reproduce asexually by sporing. The life cycle begins when a spore is inoculated into a nutrient medium using starch as the primary source of carbon and casein as the nitrogen source leading to the germination of the spore from the dormant state into one or two germ tubes forming the aerial hyphae (Figure 1.1). Under environmental stresses such as lack of nutrients they move from vegetative state (substrate mycelium) into sporulation phase in the form of aerial multinucleated mycelium. The germ tubes expand and form branches of vegetative mycelium. Growth of the colony leads to the hyphae breaking through the medium, extending into the air (Flardh and Buttner, 2009). The substrate hyphae have a diameter of about 0.5-1.0 µm. Wavy, straight or helical chains of sporophores come out into the surface (Chater, 1993).

In the environment, soil microorganisms go into a dormant state under harsh conditions where growth stops, replication ceases and they become metabolically inactive and they differentiate into spores known as either arthrospores or exospores depending on the microorganism producing them (Bobek et al., 2017). Endospores produced by *Bacillus* and

Clostridia are resistant to ultraviolet radiation, heat and desiccation (Setlow, 2007; Galperin et al. 2012). These organisms have very thick surface structures and low water levels that allows them to survive harsh conditions (Henriques and Moran, 2007). However, the streptomycete arthrospores have thick coat (Neiman, 2005), heat shock proteins and macromolecules protected by sugars such as trehalose (Wyatt et al., 2013). The main function of the spores in *Streptomyces* is to shield or protect genetic information during stressful conditions and then spread it into other areas by air or water. Nutrient depleted conditions cause the cells to down regulate processes that require energy and upregulates stress response and survival genes (Sivapragasam and Grove, 2019) leading to the production of secondary metabolites.

Streptomyces use many extracellular hydrolytic enzymes such as amylase, cellulase and chitinase to decompose decaying or dead organic compounds (Kieser et al., 2000) to get the needed nutrients for growth. Yet, when nutrient conditions are scarce aerial mycelium is formed and then differentiates into the formation of spores. This morphological change results in metabolic changes leading to the production of secondary metabolism. Many studies have shown the direct link between sporulation and the production of secondary metabolites such as the production of actinorhodin in *Streptomyces coelicolor* A3(2) (Bibb, 2005).

Streptomyces are slow-growing microorganisms and have an earthy odour due to the presence of geosmin, a volatile metabolite, produced by the bacteria (Juttner and Watson, 2007). Colonies are powdery (velvety) and leathery in texture. The pigments produced by the bacteria give the vegetative and aerial mycelium its colour (Flardh and Buttner, 2009). In this project, the *Streptomyce*s that were isolated have colonies with leathery texture, earthy smell and with grey, black and dark brown aerial mycelium.

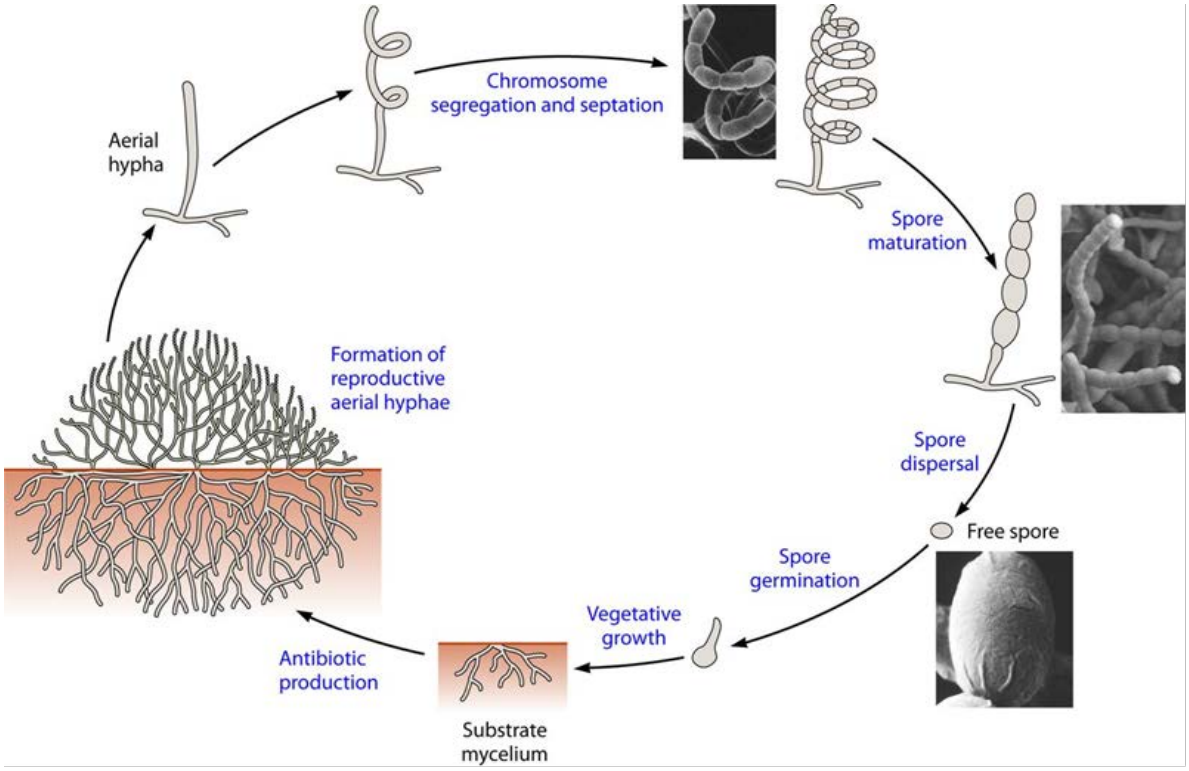


Figure 1. 1 Life Cycle of *Streptomyces*

Sporulation in the life cycle of *Streptomyces*. Adapted from Berdy, 2016.

1.2. *Streptomyces* Genome

The size of the *Streptomyces* genome is approximately between 6 and 10 Mb, such a large bacterial genome is due to the complex life cycle of the organism and the variation in size is due to the diversity of biosynthetic gene clusters in the genome of *Streptomyces* species. Studies have shown that *Streptomyces* contain accessory secondary metabolites that are strain specific which could have been obtained by intraspecies horizontal gene transfer (Sottorff et al., 2019; Vicente et al., 2018). The genetic information of *Streptomyces* is located on a linear chromosome associated with either a linear or circular plasmid depending on the species. This linear chromosome is made of a core (central) region and arms which end by terminal inverted repeats (TIRs) with terminal proteins binding to the 5' end of the DNA. All essential genes that are responsible for replication, transcription, translation and protein synthesis are located in the core along with genes involved in primary metabolism while all the non-essential genes such as bioactive compounds and hydrolytic exoenzymes are located in the variable arms (Bentley et al., 2002). In addition, transposases and insertion sequences (mobile sequences)

are also in the arms (Choulet et al., 2006). More than 10% of the *Streptomyces* genome is involved with the synthesis of bioactive compounds (Carlson et al., 2015). Among the sequenced *Streptomyces* genomes, it is found that about half of the chromosome comprises a core region which is the most conserved region within *Streptomyces* species (Choulet et al., 2006; Ikeda et al., 2003; Ohnishi et al., 2008).

Streptomyces have a highly complex regulatory systems that causes morphological and metabolic pathways changes, and regulate growth rates in response to environmental changes (De Bruijn, 2016) including secondary metabolites production which are strongly regulated at the transcription level in *Streptomyces* (Sun et al., 2017). These regulatory systems act based on the transcription of specific genes (Rebets et al., 2018). There are many regulatory proteins in *Streptomyces*, many of which are sigma factors (factors that initiate transcription in bacteria) along with other proteins that are involved in transport, decomposition of nutrients and production of bioactive compounds (Bentley et al., 2002). RNA polymerase (RNAP) plays an essential role in transcription. Sigma factors in RNAP determines specific promoter elements allowing the transcription initiation of specific sets of genes (Kang et al., 1997). All bacteria have essential sigma factors that designates the expression of housekeeping genes and non-essential sigma factors with many functions in response to cell differentiation and stresses (Paget, 2015). This explains why *Streptomyces* genomes have many genes coding for RNA polymerase sigma factors and many systems of transcriptional regulators (Rückert et al., 2015). For example, *S. coelicolor* contains 66 chromosomal sigma factor genes while *E. coli* contains 7 only (Paget et al., 2002; Gruber and Gross, 2003).

Advances in genomic sequencing along with the decrease in its cost has allowed us to identify biosynthetic gene clusters of specific *Streptomyces* species. Only, 3% of the bioactive compounds have been discovered to date and this is mainly due to the in-vitro culturing conditions leading to reduced production (Gontang et al., 2010). Genomic sequencing has shown that *Streptomyces* are capable of producing many secondary metabolites but only few show activity in the laboratory (Hoshino et al., 2015).

1.3. Secondary Metabolites in *Streptomyces*

Metabolites are small molecules that are produced from a variety of living things (Krause and Tobin, 2013). These molecules are produced either by primary metabolism or by secondary metabolism (Berdy, 2005). Primary metabolites are chemicals that are usually found in all

biological processes such as nucleic and fatty acids and polysaccharides, while secondary metabolites are molecules with low molecular weights that are produced as a defence mechanism to protect the cells from other surrounding organisms that compete for nutrients or in morphological differentiation (Thirumurugan et al., 2018) and these secondary metabolites are increasingly referred to as "specialised metabolites" in the literature. Therefore, microbial secondary metabolites are produced as an adaptation mechanism to the surrounding environment and they act at as signalling or defence molecules (Chadwick and Whelan, 2008). Therefore, an organism's secondary metabolites profile can be different according to the habitat they occupy.

The most distinct property of *Streptomyces* species is the existence of biosynthetic gene clusters, these clusters encode to proteins leading to the production of secondary metabolites (Nett et al., 2009). They are known as "Secondary" because they are not involved in cell division and don't affect the growth of the organism. Most secondary metabolites are produced during the *Streptomyces* life cycle when they progress from a vegetative state into sporulation and the formation of aerial multinucleated mycelium (Dyson, 2011). This morphological differentiation takes place due to stressful environmental conditions such as nutrient depletion, space, competition with other organism, temperature, pH and salt changes or as a reaction to physiological signalling molecules (Bibb, 2005). Secondary metabolism in *Streptomyces* usually happens in the stationary phase of the organism's life cycle.

Most of the secondary metabolites produced by *Streptomyces* have medicinal properties which are largely antibiotics (e.g. erythromycin) plus antifungal (e.g. amphotericin B) and anticancer (e.g. Doxorubicin) (Newman and Cragg, 2007). Most of the discovered secondary metabolites come from soil *Streptomyces*. This terrestrial habitat has been a useful and important source for many years. Although the discovery of new *Streptomyce*s species and bioactive compounds has decreased considerably in recent years, there are still unexplored areas such as the Middle East and specifically UAE that could still be a potential source for discovering novel secondary metabolites.

The marine environment is also a rich resource for bioactive compounds, the extreme environment of salinity and pressure causes *actinomyces* to adapt and produce natural compounds. Marine *Streptomyces* have been of great interest as an unusual source of chemically diverse secondary metabolites and novel bioactive compounds (Juhnke et al., 1987).

Recently, the search for marine *actinomyces* have been of great interest because they have the ability to endure severe habitats (Subramani and Aalbersberg, 2012). They have rare metabolic

and physiological abilities making them capable of producing novel bioactive compounds (Williams, 2009). However, a major concern involving marine *actinomyces* is the difficulty to culture these organisms, as these organisms are halophiles and require sea salts for culturing (Thykaer and Nielsen, 2003). It has been actually a major challenge in this project to grow marine *Streptomyces* that could be resolved by optimizing their isolation, growth (Busscher et al., 2005; O'Driscoll et al., 2008; Wang et al., 2005) and fermentation process for the production of bioactive metabolites (Thykaer and Nielsen, 2003; Tsai et al., 2013).

1.4. Biosynthesis of Secondary Metabolites

The processes of biosynthesis from microbial secondary metabolites clusters are divided into: supply of building blocks, building blocks assembly and precursor molecule tailoring to produce the final product (Flickinger, 2010). Some of the biosynthetic gene clusters (BGCs) present in the *Streptomyces* genome have been found to play a major role in the production of secondary metabolites. They are produced when the organism shifts from the substrate mycelium phase to the sporulation phase. A gene cluster is composed of genes that are located next to each other in the organism's chromosome and are important in the production of an inhibitory agent. A BGC contains genes that are involved in the precursor biosynthesis, tailoring steps, gene regulation and resistance and transport of the antibiotic (Adegboye and Babalola, 2012; Figure 1.2). The antibiotic backbone can be modified by a number of tailoring steps such as chlorination, glycosylation, methylation and others. Then, there are many regulatory genes in the cluster, overexpressing positive regulators and deleting repressor genes which may contribute to increase antibiotic production. Resistance genes are used to protect the organism from suicide and finally transport genes are used to take the antibiotic out of the cell.

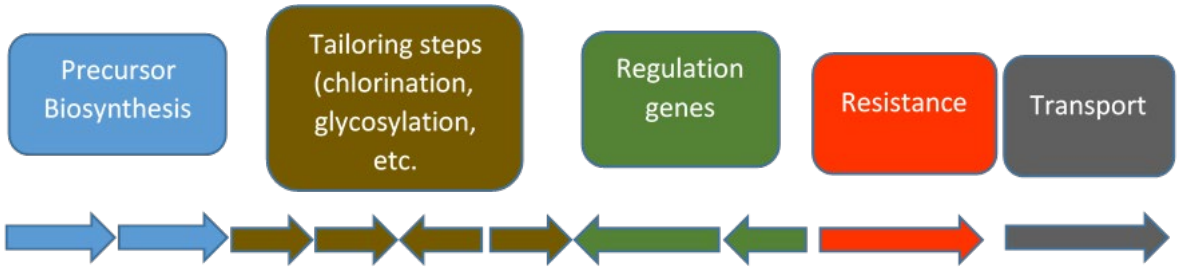


Figure 1. 2 Biosynthetic Gene Cluster

Scheme of a biosynthetic Gene Cluster.

In these mega-enzymatic complexes, each gene encodes certain modules and each module is divided into domains. The actions of enzymes in the domains will lead to the activation and attachment of extender unit. The complexity in secondary metabolites biosynthesis depends on variations in the three processes.

1.4.1. Polyketides

Many secondary metabolites have been produced as polyketides and used as antibiotics (erythromycin, tetracycline), immunosuppressant (rapamycin) and antitumor agents (doxorubicin). Spore pigments have been also been known to be synthesized by polyketide synthases (Davis and Chater, 1990). Depending on the construction of the domains, PKSs are grouped into three types which can be modular (multifunctional proteins) known as Type I PKS (T1PKS) or iterative (small monofunctional repetitive proteins) and these are identified as Type I, II and III PKSs (T1PKS, T2PKS, T3PKS; Sattely et al., 2008). The biosynthesis of polyketides is very similar to the biosynthesis of fatty acids in that the acetyl and malonyl are condensed to give a long carbon chain under the action of the polyketide synthase (PKS; Figure 1.3). For this to happen three proteins are required ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) (Böhm et al., 1998). The main difference between the two biosynthetic routes is that the polyketides use a variety of carbohydrates units as starter units and therefore leading to compounds with different structures and chemical functions (Revill et al., 1996). The biosynthesis begins with acyltransferase (AT) which recognizes a suitable substrate and transfers it to ACP, once the substrate is attached to the ACP the formation of C-C between the synthesized precursor and growing polyketide chain is catalysed by ketosynthase (KS; Bibb et al., 1989). Several modifications take place during assembly of the long chain governed by other enzymes such as ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), cyclase (CYC) and aromatase (ARO; Zhan, 2009). PKS choose among the different modifications in each step of elongation which results in greater variation if the final product. In addition, PKS requires additional proteins involved in chain initiation and termination. AT and ACP are responsible for chain initiation while thioterase (TA) is required for chain termination by catalyzing the excretion of the polyketide chain from the enzyme, this is usually associated with cyclization (Keating and Walsh, 1999). The release of the synthesized polyketide backbone is followed by modifications by other tailoring enzymes such as glycosylation, methylation etc. (Weissman, 2009).

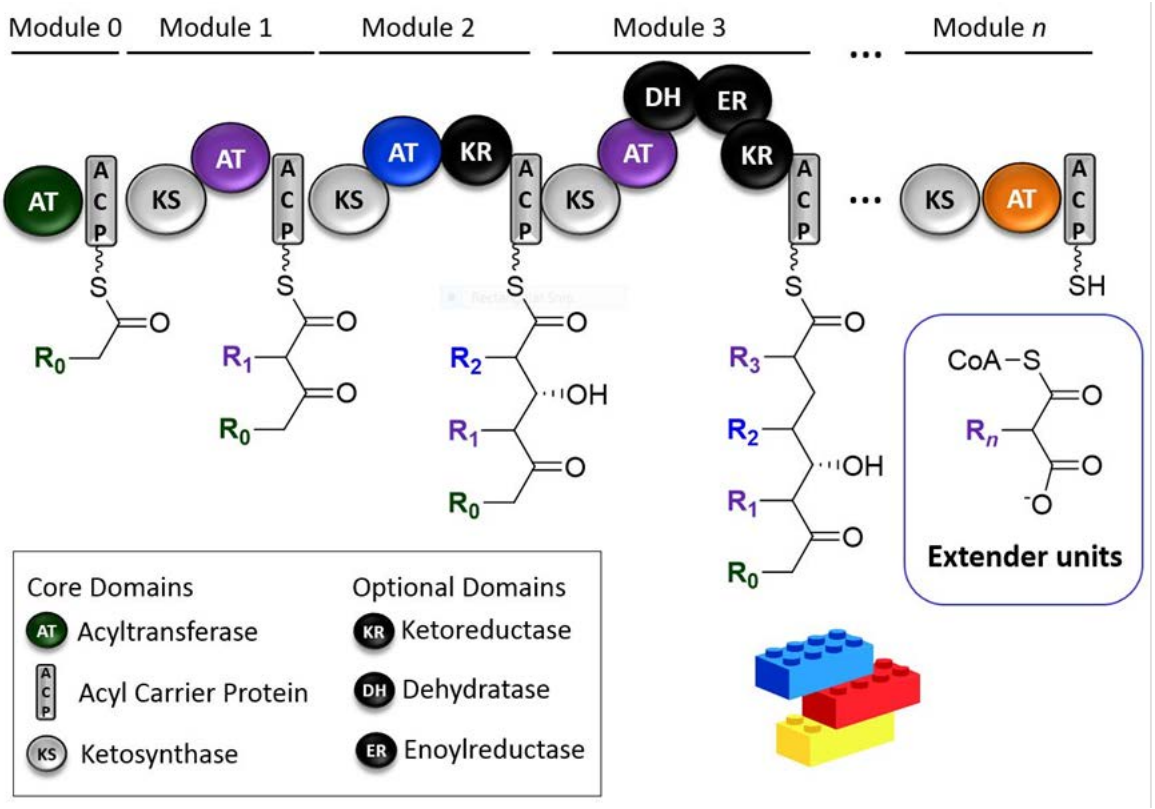


Figure 1. 3 Backbone of Polyketides

Polyketide backbone assembly. Adapted from Hertweck, 2009.

1.4.2. Non-ribosomal Peptides

Peptide BGCs are made of a large family of compounds with variety of chemical structure and biological activity. The peptide compounds can be synthesized by large multienzyme complexes (NRPSs) or by the classical ribosomal pathways (lantibiotics) (Widdick et al., 2003). Peptide compounds synthesized by non-ribosomal peptide synthetases (NRPSs) are composed of proteogenic (natural) and non-proteogenic (unnatural) amino acids leading to distinct and diverse peptide structures (Sieber and Marahiel, 2005). The synthesis of non-ribosomal peptide can take place with a single NRPS which often happens in fungi or by several NRPS proteins as it is the case in most bacteria (Finking and Marahiel, 2004) resembling the synthesis of T1PKS in which the proteins are attached by interpolypeptide linkers. NRPSs require 3 proteins acting as core domains: adenylation domain (A) which selects distinct amino acid and activates it into acyl adenylate using ATP, this amino acid is

then transported to peptidyl carrier protein (PCP) into which it hold the activated protein followed by the c-domain catalysing the formation of C-C bond (Figure 1.4; Keating et al., 2000). NRPSs are made of modules, each one is responsible for the recognition and incorporation of one amino acid into the extender polypeptide chain (Marahiel, 1997). In addition to the core domains, the NRPS polypeptide chain have tailoring enzymes that are either essential in the NRPS and act in *cis* or separate enzymes that act in trans along the way to the final NRPS product (Finking and Marahiel, 2004). The two most common modifications in the NRPS polypeptide chain are: 1- epimerisation (E) in which the cis-acting E-domain converts L-amino acids attached to PCP domain into D-isomers before transferring it to the next module and 2- methyltransferase (MT) domains responsible for the N-methylation of the NRPS residues (Walsh, 2016).

The module for the initiation differs from the termination in that it does not contain C-domain and the last domain of the termination module is usually a thioesterase (TE) domain which is responsible to the release of the synthesized final product (Schneider and Marahiel, 1998).

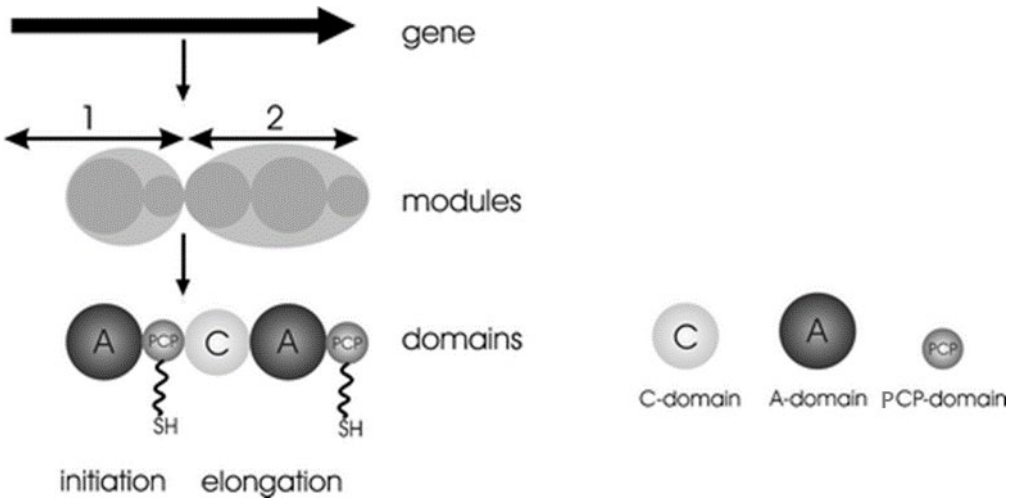


Figure 1. 4 NRPS modular organization

Modules are divided into core domains which are: adenylation (A-domain), peptidyl carrier protein (CP-domain) and condensation (C-domain). Adapted from Finking and Marahiel, 2004.

1.4.3. Hybrid PKS-NRPS

Hybrid PKS-NRPS are secondary metabolites that are produced as a result of the fusion of PKSs and NRPSs together. These hybrid assembly lines make complex polyketide-amino acid-peptide hybrids that use acyl and aminoacyl building blocks in their final products. An example of a hybrid BGC is the immunosuppressant ascomycin (FK520) which consists of 11 PKS modules spread on 3 proteins (FkbB, FkbC, FbkA) and one NRPS on FkbP protein (Gatto et al., 2005). Bleomycin (anticancer compound), another example, consists of ten NRPS modules and one PKS module. The biochemistry of the PKS and NRPS proteins in the hybrid seem to be similar to the non-hybrid molecules (Du et al., 2014); then how do these two enzymes interact to produce a hybrid molecule? The PKS-NRPS hybrid system can have 4 different approaches (Figure 1.5): 1- tethered hybrid modular type where both the PKS and NRPS modules are found in the same polypeptide, 2- separate hybrid modular type where the PKS and NRPS are found as separate units. 3- Partially stand-alone hybrids use non-modular NRPS proteins with modular PKS system and 4- Stand-alone hybrids where the PKS and NRPS enzymes exist as individual proteins.

In the tethered hybrid modular type, PKS-NRPS are formed by modules that are located in the same polypeptide. For example, leinamycin is an anticancer drug produced from *S. atroolivaceus* which consists of two NRPS modules and six PKS modules (Pan et al., 2017; Figure 1.5 A). One example of the separate hybrid modular approach is the biosynthesis of myxobacterial epothilons (Julien et al., 2000) where it consists of nine PKS modules that are divided over 5 proteins EpoA, EpoC, EpoD, EpoE and EpoF and one NRPS found as a separate protein EpoB (Figure 1.5B). In the third approach, partially stand-alone hybrids, the biosynthetic machinery involves type II NRPS (a dissociated NRPS) system with a modular PKS. The type II NRPS contains an A-domain and CP-domain and it makes an amino acid as an extender unit for the modular PKS. The generated aminoacyl unit moves from the CP domain of the type II NRPS to the CP/KS domain of the modular PKS. The modular PKS is not covalently bonded to the CP domain of the type II NPRST. The stand-alone AT domain recognizes the acyl building block and facilitates its transfer from the stand-alone CP to the fixed CP domain of the PKS module in the vicenistatin biosynthesis (Shinohara et al., 2011, Figure 1.5C). Finally, andrimid biosynthesis is an example of the fully stand-alone approach where the assembly line is very fragmented with a stand-alone transglutaminase and type II PKS and Type II NRPS (Figure 1.5D). Andrimid synthetase contain 11 proteins, none of the proteins have more than three domains (Jin et al., 2006). All the chain elongation happens

between intermediates targeted to different proteins because none of the proteins have more than one T domain. AdmA is a stand-alone CP in the type II PKS system and AdmI is a stand-alone in the type II NRPS system. In the condensation reaction, the stand-alone transglutaminase (AdmF) recognizes and uses an acyl unit from AdmA and an aminoacyl unit from AdmI (Miyanaga et al., 2018).

1.4.4. Ribosomally Synthesized and Post-translationally Modified Peptides

Genome sequencing have led to the identification of ribosomally synthesized and post-translationally modified peptides (RiPPs), it is a large group of natural products with many unique chemical structures and variety of biological activities. The biosynthesis of RiPPs starts with a precursor peptide containing a core peptide connected to a leader peptide, a follower peptide or both which aids biosynthetic enzymes for the post-translational modification (PTM) on the core peptide. After the modification of the core peptide, the leader and/or follower peptides are removed by proteases leading to the final product. Due to their ribosomal origin, RiPPs chemical structure can be easier to predict than other natural products making them more appealing for genome driven discoveries of natural products. Some RiPPs such as lantipeptide duramycin to fight cystic fibrosis (Zeitlin et al., 2004) and labyrinthopeptin A2 for neuropathic treatment (Teichert and Olivera, 2010) progressed into clinical trials and therapeutic use.

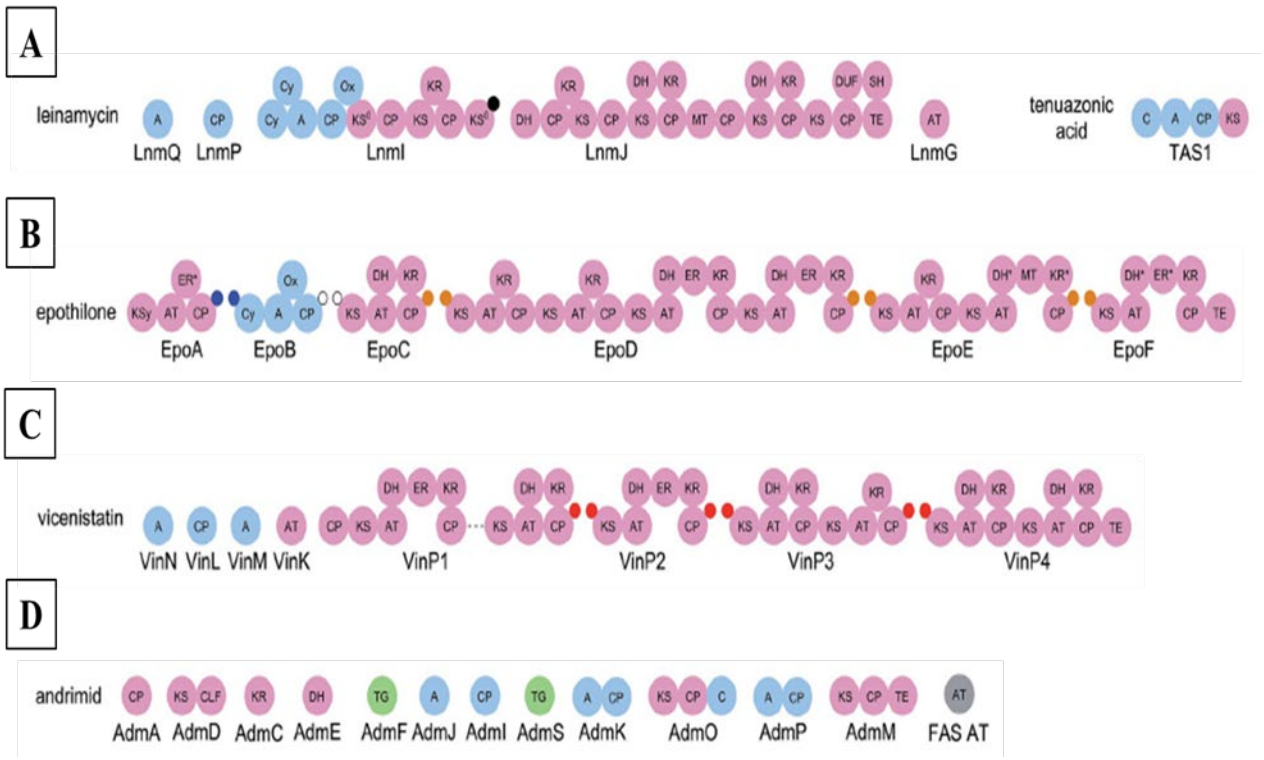


Figure 1. 5 Types of PKS-NRPS hybrids

PKS-NRPS hybrids can be classified into 4 types according to their biosynthetic lines. Tethered hybrid modular type (A), separate hybrid modular type (B), partially stand-alone type (C) and fully stand-alone type (D). PKS and NRPS are shown as pink and blue colours, respectively. Transglutaminase is shown in green colour and type II FAS AT in grey circle. Abbreviations: Cdd: C-terminal docking domain; CLF: chain-length factor; Cy: cyclization; DH: dehydratase; E: epimerase; ER: enoylreductase; KR: ketoreductase; KS^{0}: ketosynthase-like transacylase; KSy: ketosynthase-like decarboxylase; MT: methyltransferase; Ndd: N-terminal docking domain; Ox: oxidase; TE: thioesterase; TG: transglutaminase Docking domains are shown as small circles. Docking domains are shown as small circles with red colour for class II docking domains, orange for class II docking domains, white for EpoB-Cdd/EpoC-Ndd-type docking elements, blue for EpoB-Ndd-type docking domains and black for DHD domains. Adapted from Miyanaga et al., 2018.

1.4.5. Terpenes

Terpenes are the most diverse natural compounds discovered with more than 65,000 different compounds (Jansen and Shenvi, 2014) with a variety of biological functions such as signaling molecules, antibiotics, odors or pigments. Terpenes are composed of two or more C5 isoprene classified on the basis of the number of the isoprene units. Terpenoids are derivatives of terpenes which involves the addition or removal of the methyl groups or the addition of an oxygen.

The biosynthesis of terpenes falls into three stages: 1- formation of isopentenyl diphosphate (IPP), 2- condensation of IPP and 3- cyclization and modification of the linear isoprenoids that are synthesized (Takahashi and Koyama, 2006). The IPP can be made either by the acetyl-CoA using acetate/mevalonate (MVA) pathway or the pyruvate and glyceraldehyde 3-phosphare using nonmevalonate or methyl-D-erythriol-4 phosphate (MEP) pathway (Rohmer et al. 1993). Most of *Streptomyces* use the MEP pathway (Dairi, 2005). Geosmin is an earthy smelling terpene and is the most commonly produced in *Streptomyces*. Another example is albaflavenone isolated from *S. albidoflavus* with an antibacterial activity (Zhao et al., 2008).

1.4.6. Aminoglycosides

Aminoglycosides are antibacterial drugs produced by actinobacteria. The first antibiotic produced by this family was streptomycin isolated from *Streptomyces griseus* and used for tuberculosis treatment (Wehmeier and Piepersber, 2009). Neomycin, kanamycin and gentamicin are other examples of aminoglycoside antibiotics. The name of the aminoglycosides is based on the structure of the antibiotics which involves an amino group attached to glycosides. The biosynthesis of aminoglycosides starts with monofunctional enzymes in which each activates one step in the pathway.

All aminoglycosides have similar structures with cyclitols as the core components and different sugars such as deoxysugars and aminosugars (Kudo and Eguchi, 2009). They bind to the bacterial 30S ribosome and inhibit protein synthesis.

1.5. Regulation of Secondary Metabolites

Environmental stresses, especially nutrient depletion, along with primary metabolic responses leads to antibiotic production in *Streptomyces* (Martín and Liras, 2010). Therefore, the capability of *Streptomyces* to produce secondary metabolites depends on primary metabolic changes and the presence of precursors and nutrients required for their production (van Wezel and McDowall, 2011). The genes responsible for the biosynthesis of secondary metabolites are clustered on the chromosome or on the plasmid in *Streptomyces*. These clusters are usually large (about tens of kilobases) and have numerous operons (Chater, 1985). Most of the genes in the cluster code for enzymes that catalyse amino acids as building blocks leading to the production of the secondary metabolites (Niu and Tan, 2013). Genomic sequencing has allowed to study secondary metabolites biosynthesis at the molecular level, this has shed light on the complex development of the association of secondary metabolites with morphological differentiation. Most genomes contain more than 15 biosynthetic gene clusters for secondary metabolites but many of these metabolites are undetected under lab culturing conditions. Therefore, to identify these clusters we need to find ways of activating their expression by studying their environmental niches and regulatory mechanisms (van Wezel and McDowall, 2011; Medema and Fischbach, 2015). The production of secondary metabolites is determined by many physiological and environmental factors such as nutrient depletion or the existence of signalling molecules. Most of the Streptomyces that have secondary metabolism are located in competitive and nutrient depleted environments, where the bacteria may produce inhibitory agents such as antibiotics to kill surrounding competitors (Stubbendieck and Straight, 2016).

In *Streptomyces*, antibiotic production is governed by many regulatory proteins (Bibb, 2005). Cluster-situated regulators (CSRs) have an effect on the levels of antibiotic production by controlling neighbouring gene transcription within a biosynthetic gene cluster (Liu et al., 2013). There are other regulators that are functional at higher levels, such as pleiotropic regulators which are found outside the BGCs and effect production of multiple antibiotics and morphological development (Wei et al., 2018), and global regulators which are found in various locations on the chromosome and control CSR genes and central metabolic genes (Liu et al., 2013). Many of the global and pleiotropic regulators send signals from changes in the environment or cellular physiology to CSR genes. Gamma butyrolactone (GBL) is a signalling molecule that was identified in many *Streptomyces* species which act by binding receptor proteins to activate CSRs (Nodwell, 2014).

Studies have shown that *Streptomyces* have cryptic pathways with unexpressed BGCs and that it is crucial to discover new methods to activate these pathways (Li and Tan, 2017). These methods include optimization of culture and fermentation conditions, co-culturing to activate silent gene clusters, genomic sequencing to identify novel compounds, and activation of silent genes by inducing signalling molecules such as Ƴ-butyrolactone A-factor or with the amino sugar *N*-acetylglucosamine (GlcNAc) as a physiological signal. For example, In *Streptomyces griseus* A-factor regulates many genes that are involved in morphological differentiation and secondary metabolism (Demain, 1998). A-factor induces many proteins that control the production of Streptomycin and its deletion leads to loss of transcription of the Streptomycin gene cluster (Mansouri et al., 1989). Many *Streptomyces* produce A-factor or related γ-butyrolactones that induce the production of other antibiotics (Gräfe et al., 1984). Another group of important regulators is GntR-family such as DasR (GntR-like repressor) which has been shown to control gene expression in secondary metabolism and morphogenesis in response to environmental conditions (Aigle and Corre, 2012). DasR changes the levels of gene expression in secondary metabolites and antibiotic production depending on the levels of chitin monomer *N*-acetylglucosamine (GlcNAc) in the environment (Martín et al., 2011).

1.5.1. Culturing Conditions

Culturing conditions for primary metabolites, which are involved in growth and division, are different than conditions required for secondary metabolites. Therefore, changes in culturing conditions may increase or decrease secondary metabolite production. The effect of carbon, nitrogen, phosphate, light, temperature, oxygen concentration and pH play important roles in the production of secondary metabolites. These different environmental factors can also be different from one species to another depending on the source of habitat where the organism was isolated from.

The carbon source has been identified to play a major part in the production of both primary and secondary metabolites. The carbon source to be used by *Streptomyces* at the molecular level can either inhibit genetic activation or repression (Uguru et al., 2005). Gene inhibition by a carbon source is known as carbon catabolic repression (CCR) (Hodgson, 2000) and this event has been observed by glucose in organisms such as *E. coli* and *Salmonella* (Deutscher, 2008) but few studies have been done in *Streptomyces*. Yet, there are many secondary metabolites that have been repressed by the presence of carbon source, for instance

glucose represses the activation of biosynthetic enzymes in the production of aminoglycoside antibiotics (Demain, 1989). In nature, nutrients are found in a variety of forms from simple to complex and *Streptomyces* have the capability of producing extracellular hydrolytic enzymes to degrade complex polymers (McCarthy and Williams, 1992). In recent studies, *N*-acetylglucosamine (GlcNAc) has been found to increase the production of actinorhodin antibiotic in *Streptomyces coelicolor*, GlcNAc increased antibiotic production in poor conditions and inhibited production in rich conditions (Świątek et al., 2012). This phenomenon is not observed for all species, for example GlcNAc has no effect on Tetracycline antibiotic production in *S. rimosus* (Rigali et al., 2008). The source of GlcNAc in nature could be as peptidoglycan in the bacteria's cell wall under poor conditions or as chitin under rich conditions (Sanchez et al., 2010).

Studies have shown that nitrogen present in simple organic and inorganic forms may decrease lincomycin antibiotic production in an industrial strain of *S. lincolnensis* (Young et al., 1985). However, using complex nitrogen sources such as soya bean, yeast extract, peptone and casein increased secondary metabolites production in *S. albidoflavus* (Narayana and Vijayalakshmi, 2008). Factors such as incubation period, temperature and pH have also been found to influence the production of secondary metabolites. Most studies based on different *Streptomyces* species conclude that exponential growth is observed after 4-7 days of incubation and stationary phase starts at about 10 days. Therefore, secondary metabolism is usually observed during the end or near the stationary phase of growth; the ultimate temperature observed for *Streptomyces* growth and secondary metabolites production is 28-30 °C with a neutral pH. Minerals such as $MgCl_2$ and KNO_3 seem to have positive effect on some *Streptomyces* species while $ZnCl_2$ and $MnSO_4$ had a negative effect (Bundale et al., 2015).

Phosphate is another signal molecule that affects morphological differentiation and secondary metabolism. Inorganic phosphate (Pi) is the preferred sources of phosphorous in bacteria and it is carried to the cell either by high affinity phosphate transporter (Pst) or low affinity inorganic transporter (Pit) (Santos-Beneit et al., 2008). Organic phosphate compounds (sugar phosphatase and phospholipids) are hydrolysed to phosphatases and phosphodiesterases by *Streptomyces* before they enter the cell (Apel et al., 2007). Phosphate has been identified as a repressor for the biosynthesis of some secondary metabolites. The level of the extracellular phosphate is influenced by the PhoR/PhoP two component system. When Pi is under scarce conditions PhoR, which is a membrane bound phosphate sensor protein, is autophosphorylated leading to the activation of PhoP response regulator. Phop expresses genes that are involved in the biosynthesis of secondary metabolites (Santos-Beneit et al., 2008).

In summary, the culturing conditions for each strain of *Streptomyces* has to be optimized as each species may require different carbon, nitrogen and mineral sources for positive initiation and production of secondary metabolites.

1.5.2. Co-culturing with Other Organism

Co-culturing is another approach that can be used to increase the production of known antibiotics, produce novel secondary metabolites or increase the yield of secondary metabolites (Pettit, 2009). In nature organisms exist together which leads to interspecies interaction and initiation of bioactive compound production (Oh, et al., 2005). These interspecies interactions may induce production of secondary metabolites which otherwise would not be detected in the culture. In the laboratory, organisms can be co-cultured together to activate production of secondary metabolites, for example co-culturing *Streptomyces sp.* with mycolic acid-containing bacteria has led to the production of alchivemycin A antibiotic (Onaka et al., 2011). Cellular physiology and signalling molecules are highly affected when two organisms are co-cultured together (Hogan et al., 2004). Studies of co-culturing *S. coelicolor* and *B. subtilis*, spore forming soil bacteria, have shown that aerial hyphae were inhibited in *S. coelicolor* as a result of the production of surfactant lipopeptide surfactin by *B. subtilis* (Straight et al., 2006). This surfactant which is required for aerial development in *B. subtilis* has antagonized the activity of surfactant SapB, needed for aerial hyphae production in *S. coelicolor* (Kodani et al., 2004), causing loss of aerial structures production. Co-culturing experiments of *S. tenjimariensis*, which produces istamycin antibiotic, with other organisms has led to a 2-fold increase in the production of the antibiotic (Slattery et al., 2001). Undecylprodigiosin is an anticancer compound produced by *S. coelicolor*, a 6-fold increase in the production of this compound was observed when *S. coelicolor* was co-cultured with E.coli (Luti and Mavituna, 2011). An increase in this compound was also found when heat-inactivated *Bacillus subtilis* was added to the *S. coelicolor* culture (Luti and Mavituna, 2011). Competition that happens in co-culturing leading to the production of bioactive compounds may not take place in a monoculture.

1.5.3. Effect of Signalling Molecules

An important factor that has been found to effect bioactive metabolites production are signalling molecules such as γ-butyrolactones (GBL) as they are permeable through the cell

membrane of *Streptomyces* which facilitates their entrance to the cell. They bind to receptor proteins which act as repressors for antibiotic synthesis. This interaction results in structural change in the repressor protein hindering it from binding to the targeted DNA of the antibiotic cluster (Takano, 2006), leading to the synthesis of the antibiotic. A-factor is the first GBL to be explored in *Streptomyces* and found to be associated with the production of Streptomycin antibiotic and sporulation in *S. griseus* (Khokhlov et al., 1967). Cultivation with γ-butyrolactones may lead to signal-receptor binding and production of otherwise repressed secondary metabolites.

Searching for secondary metabolites from natural habitats is still of great interest, even with the emergence of the synthesis of synthetic antibiotics, as it is a rich and unlimited source for finding new antimicrobial agents with new structures and chemical properties (Baltz, 2006). The aims of this project are to explore the isolation of novel antimicrobial and/or antitumor bioactive compounds from terrestrial and/or marine habitats. Potential *Streptomyces* isolates showing inhibitory activity against Gram-positive, Gram-negative, yeast and fungus will be characterized physiologically, morphologically and molecularly. Cytotoxicity of these strains on cancer cells will be assessed. The effects of elicitation experiments will be studied to observe any novel production of secondary metabolites or increase the yield of existing ones. Genomic sequencing and organic extraction will be performed to isolate the presence of any bioactive compounds.

1.5.4. Effect of *N*-acetylglucosamine (GlcNAc)

Terrestrial *Streptomyces* can respond to changes in the environment and contain sigma factors which regulate gene expression during vegetative growth and sporulation in poor nutrient conditions and they, also, have a two-component system that makes them capable of sensing and responding to environmental stimuli (Martín and Liras, 2010). In terrestrial habitats, *Streptomyces* evolved about 400 million years ago with the main role of solubilizing cell walls of fungi, insects and plants (Chater et al., 2010). There is a great partnership between *Streptomyces* and fungi with fungal cell walls being an important source of nutrition. Chitin is a major component of fungal cell walls and the most dominant, *Streptomyces* produce hydrolytic enzyme chitinase to degrade chitin in the soil (Nazari et al., 2011). *N*-acetylglucosamine (GlcNAc) is an amino sugar that has many roles in cell signalling pathways that is related to virulence in microbes. GlcNAc is an important component of the bacterial cell

wall peptidoglycan and a monomer of chitin. It is the preferred source of carbon and nitrogen in *Streptomyces* and acts as a signalling molecule in the bacteria (Rigali et al., 2006). A change in the morphological differentiation and antibiotic production has been observed when there is an increase in the concentration of GlcNAc around the *Streptomyces* colonies. GlcNAc has been observed to induce development and antibiotic production in famine (poor nutritional conditions) while inhibition occurs in feast (high nutritional conditions) (Rigali et al., 2008). This observation of antibiotic inhibition on rich nutrient medium is common but not universal in the *Streptomyces* (Colson et al., 2008).

S. coelicolor has been well studied for the effect of GlcNAc on nutritional conditions, studies showed that the master regulator DasR, GntR family repressor, plays a crucial role in metabolism and transport of GlcNAc (Rigali et al., 2004). The binding affinity of DasR is regulated by ligands produced from GlcNAc-6P and GlcN-6P. GlcN-6P is an intermediate of carbon and nitrogen metabolism and cell wall synthesis and also acts as an effector on DasR antibiotic production system; therefore, it plays an important role between primary and secondary metabolism (van der Heul et al., 2018) (Figure 1.6). DNA-binding capability of DasR depends on environmental changes. In minimal medium, high concentration of GlcNAc leads to inactivation of DasR (inhibiting the repressing action of DasR) and its targets which results in increased antibiotic production while rich nutrient media leads to decrease in GlcNAc leading to inhibition of antibiotic production (Rigali et al., 2006). Therefore, addition of GlcNAc to a culture media may induce production of silent BGCs (Rigali et al., 2008). This simple method may result to the discovery of novel natural products.

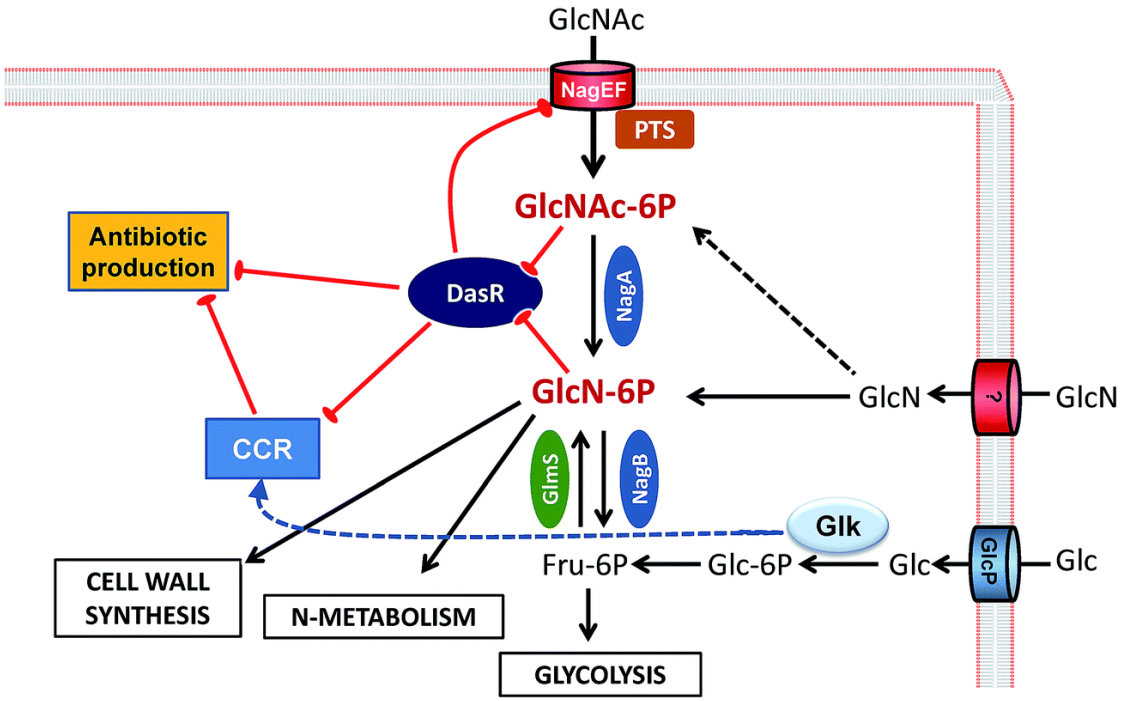


Figure 1. 6 DasR regulatory cascade by *S. coelicolor*

GlcNAc is transported to the cell by phosphotransferase system (PTS) where it is phosphorylated to GlcNAc-6P. NagA deacetylates GlcNAc-6P to glucosamine-6-P (GlcN-6P) which can be used for nitrogen metabolism, cell wall synthesis or glycolysis. Adapted from van der Heul et al., 2018.

1.6. Genomic Sequencing

The rapid increase in the sequencing technologies and easy access to bacterial genomes has brought back the focus on antibiotic producing organisms. Genetic data of these organisms, especially for *Streptomyces*, are showing that their metabolic potential has been underestimated (Corre and Challis, 2009). Genomics, bioinformatics, synthetic biology and mass spectrometry has allowed special attention to be given to underexplored organisms with secondary metabolism capabilities (Brady et al., 2009). The association of secondary metabolites to the genes that produce them has allowed the discovery of novel molecules through genome sequencing data (Bergmann et al., 2007). Genome sequences have uncovered many gene clusters that encode for bioactive molecules.

Genome sequencing uses shotgun sequencing strategy where the DNA is cut into small fragments which are separately sequenced. Computer algorithms are used to assemble the generated reads into longer regions of sequence known as “contigs” and this process is called *de novo* assembly. For appropriate assembly there should be enough overlap between the reads. Contigs are stitched together into scaffolds (Figure 1.7).

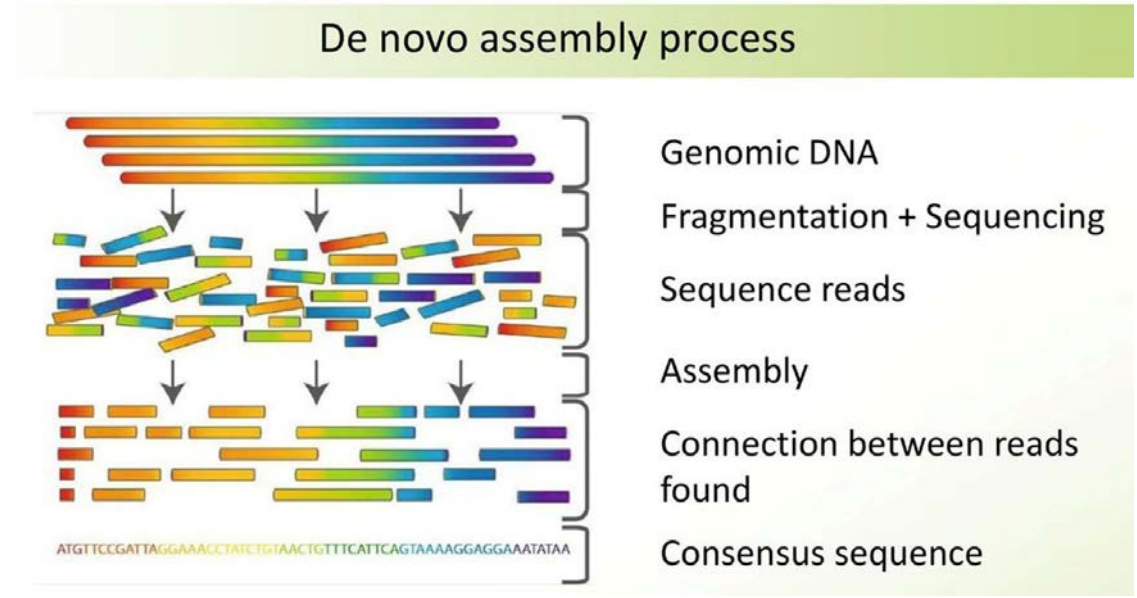


Figure 1. 7 *De novo* assembly of Genomic sequencing

In Genomic sequencing DNA is fragmented into small pieces that are sequenced separately which are known as sequence reads. Overlaps between reads are identified and assembled into contigs and then they are assembled into scaffolds and a consensus sequence is generated. Adapted from Seemann, Melbourne University

Genome mining technique has become a very popular and encouraging with the improvement of whole genome sequencing methods and the availability of the data. The genome sequence is analysed for the identification of genes encoding for enzymes that are associated in the production of secondary metabolites. *S. coelicolor* sequencing has uncovered the presence of cryptic BGCs (Bentley et al., 2002) with more than 20 BGCs identifying a variety of secondary metabolites (Challis and Hopwood, 2003). A number of actinobacterial genomes may carry up to 50 BGCs but most of them are silent under laboratory conditions or

produced in small amounts (Cao et al., 2016). This has promoted the sequencing and analysis of many species to identify new BGCs and how to develop ways to enhance or induces production of natural products in the lab (Bibb, 2005; Ling et al., 2015; Scheffler et al., 2013). There are a variety of bioinformatics tools that can be used to identify BGCs such as AntiSMASH and PRISM. These databases rely on known or conserved biosynthetic genes; although secondary metabolites are very diverse yet many of the biosynthetic intermediates are conserved (Ziemert et al., 2016). AntiSMASH does not only identify BGCs but also compares submitted sequences to massive databases of other organisms and MIBiG database to identify similar pathways (Medema et al., 2015). AntiSMASH and PRISM software are also capable of predicting the structure of the polyketide and peptide enzymes. Clusters identified as inactive are known as cryptic or silent clusters.

One of the main reasons why *Streptomyces* are not able to produce the secondary metabolites they are capable of producing in laboratory conditions is due to the tightly regulated metabolic pathways (van Wezel and McDowall, 2011). In *Streptomyces*, the genes required for production of natural products are clustered together. Regulatory elements and self-resistance genes are usually co-located with the biosynthetic genes (Bentley et al., 2002).

These silent genes are not expressed in the lab conditions or they are expressed but they have some physio-chemical properties that are not allowing their identification. If we have some information about some features of the structure and their physio-chemical properties, then analytical techniques can be utilized to focus on identifying these metabolites with these specific characteristics. Conserved regions such as PKSs and NRPSs have distinct peptide sequences. Acetyl transferase (AT) domain in PKSs and adenylation (A) domain in NRPSs have highly conserved sequences and it is possible to estimate substrate specificity from the amino acid sequence only. This was used in the identification of coelichin siderophore in *S. coelicolor* (Lautru et al., 2005) (Figure 1.8A).

Most of the bioinformatics tools related to analysis of genomic sequencing data can mainly identify the enzymes involved in a biosynthetic cluster and in some cases the structure. Thus, in order to link natural products to a specific pathway we will need to perform genetic manipulation. In cases where it is not possible to predict the structure then universal approach has to be taken. One of these approaches is to express the BGC of interest into a heterologous host, secondary metabolites produced by the wild type and non-producing mutant are compared to discover novel secondary metabolites and associate their biosynthesis to the BGC introduced in the host organism (Figure 1.8B). This approach was used in the discovery of 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (AHFCA) compounds from *S. coelicolor*

(Corre et al., 2008). M1146 and M1152 are heterologous hosts of *S. coelicolor* where large gene clusters such as actinorhodin, coelimycin and calcium-dependent antibiotic were deleted.

Another approach is to knock out the gene of interest and replace it by a resistant marker (Figure 1.8C). A gene from the BGC is inactivated, then extracts from both the wild type and mutant are compared using analytical methods such as liquid chromatography-mass spectrometry (LC-MS). Natural products found in the wild type but not in the mutant are correlated to the products of the silent cluster. Mass spectrometry is then used to characterize this portion. This approach was used in the identification of germicidins (Song et al., 2006). Overexpression of activator and inactivation of repressors is another model that can be used for the production of silent BGCs. Normally, regulatory genes influence the production of metabolites in clusters (Bibb, 2005; van Wezel and McDowall, 2011). In *S. coelicolor*, actII-orf4 and red are transcriptional activators that are located within the gene cluster for the biosynthesis of actinorhodin and streptorubin B respectively (Aigle and Corre, 2012; O'Rourke et al., 2009). Hence, regulatory genes in a targeted biosynthetic pathway may be used to activate silent pathways (Aigle and Corre, 2012; Laureti et al., 2011) (1.8D and E). An example of this model is overexpression of the transcriptional activator samR0484 in *S. ambofaciens* by positioning it under the ermE promoter which led to the discovery of stambomycins (51 macrolactones members; Laureti et al., 2011).

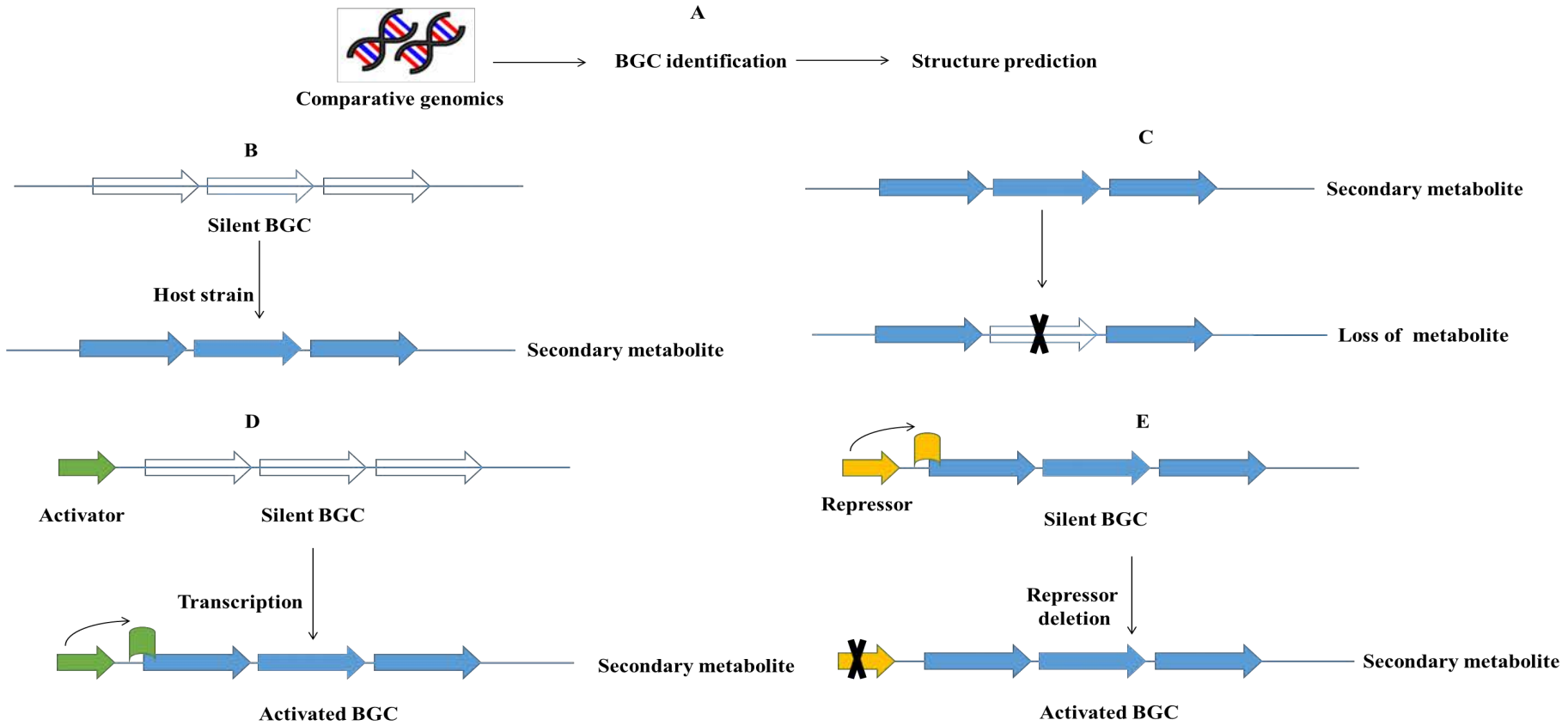


Figure 1. 8 Genomic Mining Methods

A variety of strategies can be used for the discovery of secondary metabolites. Some of the strategies are: (A) bioinformatics tools used to predict biosynthetic genes by BGCs such as antiSMASH, (B) expressing genes in host strains, (C) knocking out genes to predict their roles as biosynthetic genes, (D) identify activators to overexpress the BGC of interest and (E) identify repressors to be deleted to increase the expression of a BGC

Heterologous expression is a promising technique to identify unknown BGCs. The advances in synthetic biology along with CRISPR/Cas techniques have allowed us to successfully clone and remodel BGCs. Heterologous expression is affected by the position of the BGC to be integrated, copy number, regulatory elements and promoters (Huo et al., 2019). About 30% of the BGCs taken from a native streptomycetes genome will be expressed in another Streptomyces host, this "refactoring" of BGCs allows the replacement of the native regulatory elements with synthetic regulatory elements for the successful heterologous expression of the secondary metabolite (Myronovsky and Luzhetskyy, 2019). Most of the BGCs in Streptomyces are regulated at the transcription level. Therefore, strong promoters are required to ensure expression of the biosynthetic genes, *ermEP1*-semisynthetic promoters were used to activate the silent lazarimide BGC leading to the identification of lazarimide A, B and C with high anti-cancer activity (Siegl et al., 2013). Another important factor in heterologous expression is the host. Streptomyces hosts are the most prominent to use as they contain the appropriate precursors and they provide proper environment for the successful expression of the refactored BGC (Gomez-Escribano and Bibb, 2014). Optimally, expression of active BGCs would be in the original producer organisms but for silent BGCs an alternative host may provide regulatory and biosynthetic elements for better heterologous expression (Myronovsky and Luzhetskyy, 2019). Nowadays, the most commonly used hosts for the production of secondary metabolites are *S. avertimitis*, *S. coelicolor*, *S. albus*, and *S. lividans* (Huo et al., 2019). Although, the type of host is highly important for heterologous expression yet it could produce low yields as a result of the supply of precursors, availability of cofactors or differentiation of metabolic and transcriptional regulation (Huo et al., 2019). Heterologous expression has been well-developed for silent biosynthetic genes but these hosts require improvement in their characteristics in order to increase cluster expression (Myronovsky and Luzhetskyy, 2019).

1.7. Liquid Extraction Surface Analysis

Genomics development has revealed that many organisms are capable of producing many secondary metabolites. Many of these secondary metabolites are only predicted by bioinformatics analysis and are not produced under standard laboratory conditions or are produced under very low concentrations to be detected. In section 1.6, we have shown that genetic manipulations can be used to identify these silent secondary metabolites.

Metabolomics plays a key role in identifying metabolites at the metabolic level by measuring many low molecular weight compounds (Berg et al., 2013). Analytical methods are the most commonly used in secondary metabolites studies, you can cluster microbial strains based on the metabolites they produce (Hou et al., 2012). Mass spectrometry has allowed us to identify novel compounds when biosynthetic gene clusters are activated by genetic manipulation, different growth condition, co-culturing with other organisms or induced with other external factors such as γ-butyrolactone as a signalling molecule.

A direct surface sampling technique known as liquid extraction surface analysis (LESA; Kertesz and Berkel, 2010) mass spectrometry is a new significant technique for the analysis of many biological samples such as lipids (Griffiths et al., 2013), proteins (Kocurek et al., 2017) and drugs (Eikel et al., 2011). Extraction of analytes from the surface takes place through liquid microjunction by using TriVersa NanoMate automated pipette tip system (Kertesz and Van Berkel, 2010). The LESA technique is based on the solvent ejected by a pipette tip to the surface of the sample (colonies) where it stays for few seconds to form a liquid microinjection, then the analyte diffuses into the solvent and then the droplet is retrieved into the pipette tip where it is injected by nanoelectrospray ionization into the mass spectrometer (Figure 1.9). The main advantage of the LESA technique is the ability to analyse a variety of analytes from one location directly from the sample surface (in our case from the colonies) without advance preparation. The main drawback of the technique is the extraction of a variety of molecules which can result in a complex spectra (Tomlinson et al., 2014).

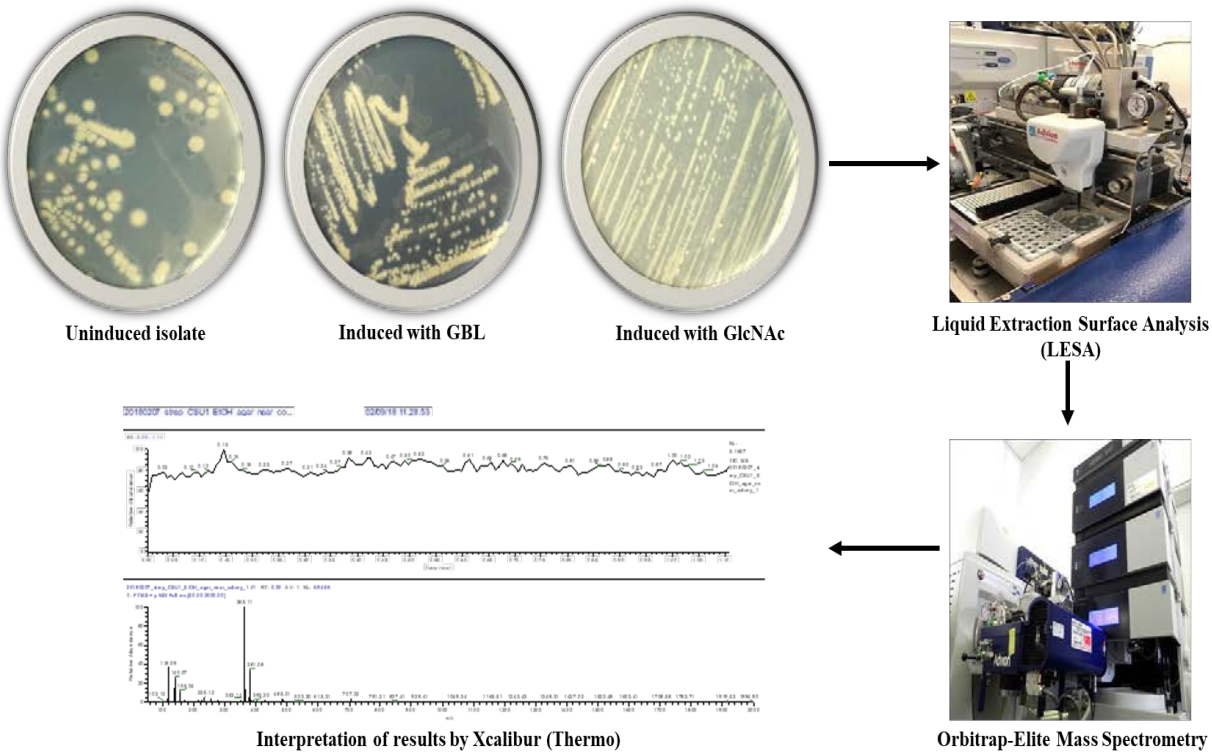


Figure 1. 9 Screening of Streptomyces isolates by LESA

All *Streptomyces* isolates were cultured on ISP1 agar medium, ISP1 agar medium induced with 0.7% GBL and ISP1 agar medium induced with 50 mM GlcNAc. Uninduced and induced bacterial colonies were screened by LESA-MS system and generated peaks were analyzed using Xcalibur software Thermofisher scientific).

1.8. Project aims

The aim of this project is to identify novel *Streptomyces* strains isolated from UAE terrestrial habitats with the ability to produce antimicrobial and antitumour agents. This will be achieved by studying the antibacterial antifungal and antitumour activity of the isolated strains followed by elicitation studies with *B. subtilis* lysate, γ-Butyrolactone and *N*-acetylglucosamine. Finally, whole genome sequencing and metabolomics using the novel liquid extraction surface analysis will be applied to identify secondary metabolites. The principle aims to achieve the above were:

1- Isolate *Streptomyces* from soil samples and test antimicrobial/antitumor inhibitory activity of the isolated *Streptomyces* strains and culture extracts.
2- Physiological, biochemical and molecular characterization of the *Streptomyces* isolates.
3- Optimization of cultivation parameters by elicitation to increase secondary metabolite production or produce novel secondary metabolites.
4- Explore cytotoxic activity of the culture extracts against HeLa cancer cell line.
5- Genomic sequencing of the *Streptomyce*s strains to identify biosynthetic gene clusters (BGCs).
6- Analyse produced metabolites directly from colonies by liquid extraction surface analysis (LESA)

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. General reagents, bacterial strains and culture media

Genomic DNA extraction kit was purchased from Promega. All chemical reagents used including DAPI stain were purchased from Sigma-Aldrich (USA). Bacterial and cancer cells were purchased from American Type Culture Collection (Rockville, US). Strains used in this study are: *Escherichia coli* (ATCC® 25922), *Staphylococcus aureus* (ATCC® 29213), *Candida albicans* (ATCC® 66027), *Saccharomyces cerevisiae* (ATCC® 2601) and *Bacillus subtilis* (ATCC® 6051) and human cervical adenocarcinoma cell line (HeLa). *E. coli*-ESBL (extended spectrum Beta-Lactamase) obtained from the microbiology lab at the University of Sharjah Hospital, Sharjah, UAE.

Most forms of microbial media used in this study were purchased from HiMedia Laboratories (India) and prepared according to manufacturer's recommendation unless mentioned otherwise. Luria Bertani Broth, Miller (LB, cat# M1245), Luria Bertani agar, Miller (cat# M1151), Starch casein agar (SCA, cat# M801), Mueller hinton agar (cat# M173), Oatmeal agar (cat# M397), Tryptone yeast extract (ISP1, cat# M356), Zobell marine agar (cat# M384), Glycerol asparagine (ISP5, cat# M360), Yeast malt broth (cat# M425), Tryptone glucose yeast extract broth (cat# M952), Peptone yeast extract iron agar (ISP6, cat# M361), Tyrosine agar (ISP7, cat# M362)

Dulbecco's modified eagle medium (DMEM) was supplied from Sigma Aldrich (USA). Foetal bovine serum (FBS), penicillin-streptomycin and Trypsin-EDTA were purchased from Gibco (Life technologies, UK).

2.2. Sample collection and location

2.2.1. Terrestrial sample collection

Fifteen soil samples were collected from 8 cultivated central locations (Figure 2.1) namely Al Khawaneej, Al Mizhar, Al Warqa, Al Qusais, Oud Al Mateena, University of Sharjah Campus, Al Jurain and Al Dhaid. After removing the top 3 cm of soil, the samples were collected with a shovel down to a depth of about 10 cm (Figure 2.2) and placed in polystyrene bags. Samples were stored at 4°C prior to processing. The names of the samples were abbreviated according to their source locations (Table 2.1).

Figure 2. 1 Locations of the Terrestrial Samples

Map of Sharjah and Dubai cities with highlighted boxes marking the collection of the terrestrial samples

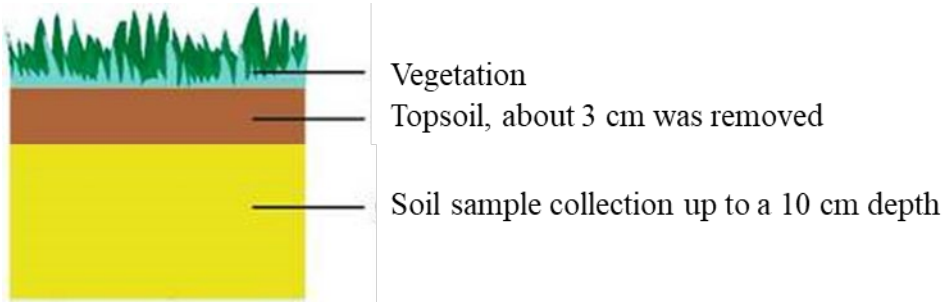


Figure 2. 2 Soil Sample Collection

Soil samples were collected to a depth of 10 cm after removing 3 cm of the topsoil

Table 2. 1 Sample Labeling

Samples were labelled according to their collection location.

Soil Sample ID	Location	GPS Coordinates
CSK	Al Khawaneej	25.22739, 55.517345
CSM	Al Mizhar	25.256970, 55.40414
CSW	Al Warqa	25.183040, 55.443815
CSQ	Al Qusais	25.264058, 55.398643
CSO	Oud Al Mateena	25.266124, 55.453377
CSU	University of Sharjah	25.287253, 55.480456
CSG	Al Gharayen	25.302647, 55.509504
CSD	Al Dhaid	25.287258, 55.895499

2.2.2. Marine sediment sample collection

Seventeen marine samples, were collected from the coastline shore of the Arabian Gulf (Figure 2.3) from Ajman to Tarif (near the border of Saudi Arabia), samples were labelled according to names of collection location (Table 2.2). The samples were collected by removing some of the marine sediment upper layer with a shovel and then scooping a sample of the sediment with overlying water using a wide neck glass bottle and placing it in polystyrene bags at 4°C until processing time (van veen grab was not available for sample collection). Sample collection was done under the water for all samples except for Ras AlKhaima/Sha'am and Hamim locations where samples were collected after the tide had gone out.

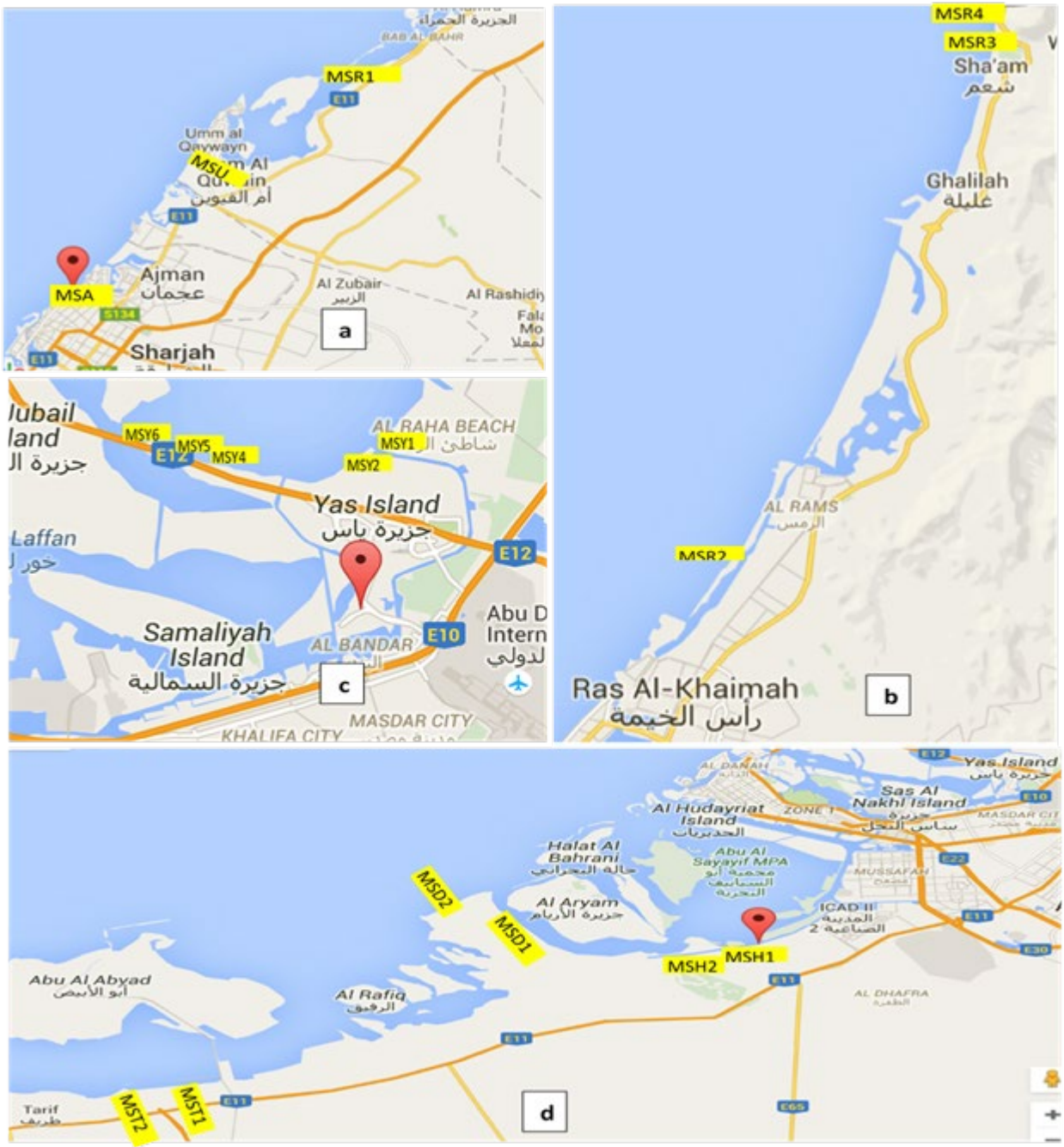


Figure 2. 3 Maps of the aquatic sample collection locations

Samples were collected from different locations along the coast of the Arabian Gulf starting from Ajman (2) to the border of Saudi Arabia (d).

Table 2. 2 Marine sample labeling

The names of the samples were abbreviated according to sample's location

Marine Sample ID	Location	GPS Coordinates
MSA	Ajman	25.3946409, 55.4202767
MSU	Umm Al Quwain	25.317231, 55.5719047
MSR1	Ras Al Khaima	25.6565362, 55.7468665
MSR2	Ras Al Khaima	25.8508129, 55.9973148
MSR3	Ras Al Khaima / Sha'am	26.0326448, 56.0837734
MSR4	Ras Al Khaima / Sha'am	26.0405369, 56.0846891
MSY1	Yas Island	24.5153439, 54.6053472
MSY2	Yas Island	24.5162698, 54.6017886
MSY3	Yas Island	24.5167566, 54.6004619
MSY4	Yas Island	24.5128709, 54.5495888
MSY5	Yas Island	24.5170577, 54.5374062
MSY6	Yas Island	24.5181504, 54.527784
MSH1	Hamim	54.3850158, 54.3850158
MSH2	Hamim	-
MSD1	Dabiya	24.2783542, 54.1816004
MSD2	Dabiya	24.3143852, 54.1224658
MST1	Tarif	24.0653839, 53.8766946
MST2	Tarif	24.0644673, 53.8759449

2.2.3. Sample treatment

The terrestrial and marine samples were air dried in a chemical hood overnight to facilitate the isolation of *Streptomyces*. The samples were then heat treated by placing the samples in a water bath at 56 °C for 30 min to prevent the growth of vegetative cells (Saadoun et al., 1999). All samples were diluted up to 10^{-5} with sterile water for terrestrial samples and 50% artificial seawater (Table 2.3) for marine sediment samples.

Table 2. 3 Artificial sea water recipe (adapted from Cold Spring Harbor Protocols)

The quantities are for 1 L

Reagent	Weight (grams)
NaCl	26.29
KCl	0.74
$CaCl_2$	0.99
$MgCl_2.6H_2O$	6.09
$MgSO_4.7H_2O$	3.94
Adjust to pH 7.8 and autoclave	

2.3. Isolation of *Streptomyces*

2.3.1. Culture conditions

One gram of soil sample was suspended in 9 ml of either sterile water or sterile artificial sea water respectively. Each sample was serially diluted 10^{-1} to 10^{-5}, 0.1 ml of the last three dilutions (10^{-3} to 10^{-5}) were spread on Starch Casein Agar (SCA) plates made with sterile water. The plates were incubated at 28°C for 7-14 days and observed for the growth of *Streptomyces* colonies that were identified by their firm, chalky and rough textures. These colonies were picked and streak purified on SCA and oatmeal agar plates for morphological characterization and refrigerated as working stocks. Pure cultures were inoculated into 50 ml of Tryptone yeast extract medium (ISP1) and incubated at 28 °C for 7-10 days in a rotary shaker (125 rpm) before screening.

2.3.2. Selective isolation of *Streptomyces* from marine sediment samples

Streptomyces could not be isolated from marine sediment samples using the above standard method; therefore, samples were subjected to different pretreatment methods as follows: (1) Ten grams of marine sediment sample was suspended in 90 ml artificial sea water (ASW) incubated at room temperature for 1 hr in a shaker at 180 rpm; (2) 10 g of marine

sediment was suspended in 90 ml of ASW and enriched by shaking at 180 rpm for 14 days at RT; (3) 0.1 g of $CaCO_3$ was added to 1g of marine sediment sample and incubated at 28 °C for 7-14 days, then samples were serially diluted and spread on SCA and Zobell marine agar medium; (4) SCNA medium was constituted using artificial sea water (ASW) instead of de-ionized water and (5) Decreasing ASW concentration to 25% and using it to constitute SCA.

2.4. Screening *Streptomyces* for antimicrobial activity

2.4.1. Disk diffusion method

Inhibitory activity of pure isolates recovered from terrestrial samples was assessed against *E. coli* and *S. aureus* by using the disk diffusion method (Kirby Bauer method; Bauer et al., 1966)) on Mueller Hinton agar medium. From the *Streptomyces* purified agar stocks (working stock), disks of 10 mm in size of *Streptomyces*' colony mass were prepared by using sterile cork borers. The disks were transferred aseptically to Mueller Hinton agar plates spread with fresh overnight culture of the target microorganism with an OD between 0.02 and 0.05. Plates were incubated at 28 °C for 24 – 72 hrs and zones of inhibition were measured in mm (Figure 2.4). Strains that showed inhibitory effect against both *E. coli* and *S. aureus* were further tested by the disk diffusion method against *C. albicans* and *S. cerevisiae* to assess their antifungal effect.

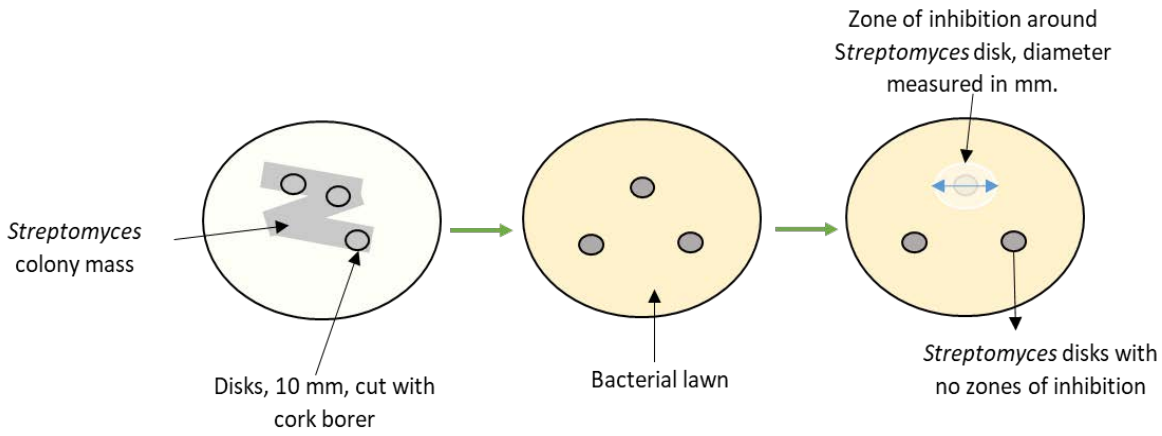


Figure 2. 4 Disk Diffusion Method Diagram

Disks were cut from *Streptomyces* colony mass and placed on a bacterial lawn. After incubation at 28 °C for 24 hrs some isolates exhibited zones of inhibition (clearance of bacterial growth) around the disks while others did not have any inhibitory properties towards the tested organism and therefore no zone of inhibition was observed.

2.4.2. Well diffusion method

Well diffusion method was used to test antimicrobial activity of the filtrates against *E. coli*, *S. aureus*, *C. albicans* and *S. cerevisiae*. Spores from pure cultures were inoculated into 50 ml of tryptone yeast extract medium (ISP1) and incubated at 28 °C for 7-14 days in a rotary shaker (125 rpm), mycelium and supernatant were separated by centrifugation at 4,000 rpm for 10 minutes. The filtrate was collected and filtered with 0.22 µm filters. A lawn culture of the target microorganism was spread on Mueller hinton agar plates, well of 5 mm in diameter were made with the end of a sterile pasteur pipette, the discs from the wells were removed and 50 µl of the *Streptomyces* strains' filtrates were added into the well (Figure 2.5). Antagonism was determined by measuring the diameter of the inhibition zone around the well in millimetres.

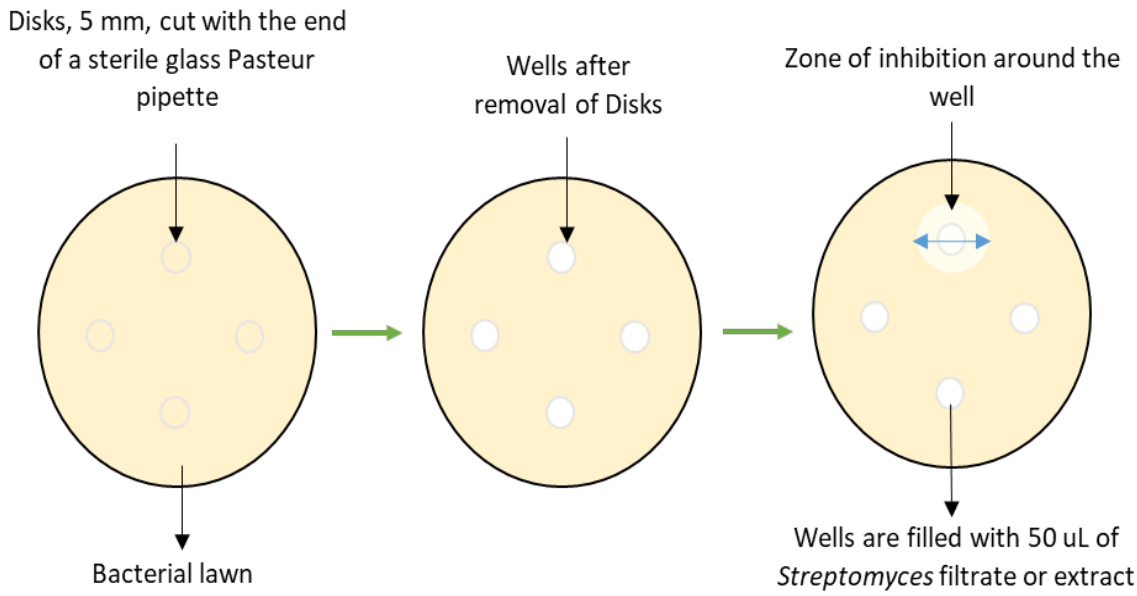


Figure 2. 5 Well Diffusion Diagram

Wells were made on mueller hinton agar plates inoculated with the tested organism using the end of sterile Pasteur pipette. *Streptomyces* organic extracts were added into the wells. Zones of inhibition were measured based on the diameter (d) in mm of the clearance of growth surrounding the well.

2.5. Physical and biochemical characterization

Streptomyces isolates that produced microbial inhibition were characterized physically, biochemically and morphologically in reference to the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Locci, 1989). Physiological and biochemical tests included gram staining, carbon utilization, melanin synthesis and hydrogen sulphide production.

2.5.1. Macroscopic and microscopic morphological characterization

Morphological identification was based on the growth patterns of the isolates on oatmeal agar and starch casein agar (SCA). The colour of the aerial mycelium, substrate mycelium and soluble pigment production was observed by the naked eye. Bacterial smear of each isolate was prepared and gram stained to observe under the light microscope in order to assess spore chain morphology.

2.5.2. Melanin production

Production of melanoid pigments were observed on peptone iron agar (ISP6) and Tyrosine agar (ISP7) agar slants streaked with the culture inoculum after 2 and 4 days of culturing. Inoculated slants were compared to uninoculated control. Formation of green to brown to black diffusible colours is considered as positive.

2.5.3. Carbon source utilization

Four carbon sources (inositol, L-Arabinose, xylose and sorbitol) were assessed. Ten percent solution (w/v) of each carbon solution was filter sterilized and added to basal mineral salts agar medium to make a final concentration of 1%. The mixture was poured into petri dishes and incubated at 4°C. A washed inoculum was prepared by transferring 1 ml of turbid suspension of spores into 5 ml of ISP1 medium and incubated at 28°C for 48 hrs. After incubation, the mixture was vortexed for 30-60 seconds, the suspension was centrifuged at 11,000 rpm for 10 minutes and resuspended in sterile 0.85% saline. The centrifugation and washing steps were repeated twice. Finally, 50 µl of the washed inoculum was used to streak the prepared carbon plates. Glucose was used as a positive control and no carbon source was negative control.

2.5.4. Hydrogen sulphide production

Peptone-iron agar slants were prepared as manufacturer's instructions. The slants were streaked with culture inoculum of each isolate and observed after incubation at 28°C for 4 to 15 hrs. Bluish-black coloration of the medium surrounding the colonies signifies production of hydrogen sulphide. Isolates that do not produce hydrogen sulphide will result in no change of the medium.

2.6. Preparation of organic extracts

Streptomyces isolates' cultures were grown in 25 ml of yeast tryptone extract medium (ISP1) for 21 days at 28°C. The cultures were centrifuged at 5,000 rpm for 10 minutes. The supernatant was filtered with 0.22 µM syringe filters. An equal amount of ethyl acetate

(EtOAc) was added to the filtered isolates. The mixture was allowed to shake for 30 minutes before adding it to a funnel to allow the separation of the two phases. The organic phase layer was evaporated to dryness in an oven at 50°C. The residue was then weighed and resuspended in 1-3 ml ddH_2O (depending on the weight, if it is low or high) and filtered with 0.2 µM syringe filters. These organic extracts were used to assess the "Minimum Inhibitory Concentration" (MIC) on microbes and cytotoxicity on HeLa cells.

2.7. Minimum Inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) for all isolates (organic extracts purified by ethyl acetate) was determined by resazurin-based turbidometric (TB) assay (Elshikh et al., 2016). A 96 well microtiter plate was set up with 50 µl of mueller hinton broth added to all wells from column 2 to column 12, column 1 contained only 100 µl of mueller hinton as a control for sterility. The isolates were prepared by diluting the organic extracts of CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1 in mueller hinton to 2x the top concentration required in the test (30 µg/µl) and the tested bacteria (*E. coli*, *S. aureus*, *C. albicans*, *S. cerevisiae*, *B. subtilis* and *E. coli*-ESBL) was standardized to optical density of 0.05 at 600 nm. A volume of 50 µl of the 2x organic extract of the isolate was added to column 2, a twofold step dilution was performed up to column 11 with extracts concentration ranging from 30 to 0.12 µg/µl. The standardized bacterial suspension was added to columns 2 to 12 to a final volume of 100 µl. Column 12 contained only the tested organism as a control for growth of the bacterium. After incubation at 37 ºC for 24 hrs, 10 µl of 0.1% resazurin was added to all wells and incubated in the dark for 3 hrs. Absorbance was measured after incubation at 630 nm with an ELISA reader (BioTek ELx808, USA). The test was performed in triplicates for each isolate against each of the tested bacteria.

The MIC values were plotted using GraphPad prism (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The curve is defined into three main areas: a- area where the isolate has no effect on the tested bacteria in relation to the control growth, b- area of increased inhibition of growth and c- an area where there is no growth in comparison with the control (Figure 2.6). The MIC value marks the concentration above which no bacterial growth is observed in comparison to the control.

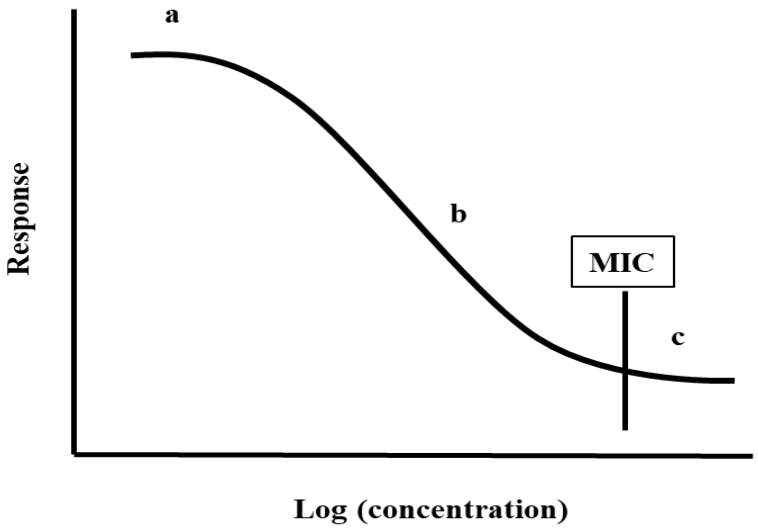


Figure 2. 6 Sigmoid-shaped curve

Assessing the inhibition of organisms by measuring minimum inhibitory concentration using Gompertz model.

2.8. Cell culture

The human cervical adenocarcinoma cell line (HeLa) was grown as monolayer in Dulbecco's modified eagle medium (DMEM) supplemented with high glucose (4.5g/L) and L-glutamine (Sigma-Aldrich) containing 10% Foetal bovine serum (FBS) and 100 units/ml penicillin/Streptomycin, in a 5% humidified CO_2 incubator at 37°C. Cells were subcultured at 70%-80% confluency using 0.25% Trypsin-EDTA.

2.9. Cytotoxic activity

2.9.1. MTT assay

The antiproliferative effect of the extracts was measured with MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reagent using three independent replicates. Hela cells were seeded in 96 well plate at a density of $4x10^5$ cells/well in 100 µl culture medium. After incubation for 24 hrs, extracts were added at 5, 10, 15 and 20 µg/µl concentration to the cells and incubated for 24 hrs. Non-treated HeLa cells were used as control. The culture medium was removed after incubation and replaced with 100 µl of growth medium. A volume of 15 µl of MTT reagent (5 mg/ml) was added to the cells and incubated

for 3 hrs at 37°C. Thereafter, the supernatant was removed and replaced with 100 µl of DMSO to dissolve the coloured formazan crystals produced by MTT. Optical density was measured at 570 nm with an ELISA reader (BioTek ELx808, USA). The concentrations were transformed to log concentration values and the absorbance values were normalized to percentage of inhibition (PI) using the Graphpad Prism 7.04 statistical software (GraphPad Software Inc., USA) in order to calculate the IC_{50} values through non-linear regression using dose-response-inhibition analysis.

2.9.2. DAPI (DNA-specific fluorochrome) staining

Hela cells were treated with 5 and 20 µg/µl concentrations of the organic extract of each isolate in triplicates after 24 hrs of seeding. Non treated cells were used as a control. After incubation for 24 hrs, cells were fixed with 4% Paraformaldehyde (PFA) for 8 minutes. The fixed cells were washed in PBS and permeabilized with 0.1% Triton-X-100 for 10 minutes. After washing, the cells were stained with 1 µg/ml of DAPI (4′,6-diamidino-2-phenylindole) for 5 minutes at room temperature (RT) in the dark. After washing with PBS, stained cells were observed under the fluorescent microscope (IX53 Olympus, Tokyo, Japan). Apoptotic nuclear changes, based on chromatin condensation and nuclear fragmentation, in DAPI stained cells were used to quantify apoptotic cells. Images were captured at different fields and counting was done in three independent replicates of each sample.

2.10. Elicitation of isolates

2.10.1. Effect of *Bacillus subtilis*

To prepare *B. subtilis* cell lysate, a loopful of an overnight culture of *B. subtilis* was inoculated into a flask containing 25 ml of Luria Bertani broth and incubated at 37°C for 24 hrs. The cells were centrifuged at 13,000 rpm for 5 min and then washed and resuspended in 25 ml of sterile saline. To lyse the bacterial cells, the cell suspension was placed in boiling water for 30 min. As for the *Streptomyces* isolates, a vegetative inoculum was prepared as previously reported by Elibol et al., 1995. An inoculum of 1.2 x 10^8 spores/ml of each isolate were inoculated into 25 mL of ISP1 broth medium and cultured for 3 days in an orbital shaker at 180 rpm at 28 °C which was then used as a vegetative inoculum for the elicitation experiments. Each vegetative inoculum was added at 3% (v/v) of the 3 day culture and induced

with 0.3% (v/v) of lysed *B. subtilis* cells. Inhibitory activity of the filtrates was assessed using well diffusion method against *E. coli, S. aureus* and *B. subtilis*. Organic extraction was performed on isolate CSK1 cultivated with *B. subtilis* lysate only and antimicrobial activity was assessed using the well diffusion method.

2.10.2. Effect of Ɣ-Butyrolactone (GBL)

A vegetative inoculum of 1.2 x 10^8 spores/ml of each isolate was inoculated into 25 ml of ISP1 medium and cultivated with 0.2, 0.5, 0.7 and 1 % of Ɣ-Butyrolactone respectively and incubated at 28°C for 14 days. Ethyl acetate extracts was prepared as described previously in section 2.6. Antimicrobial activity of the organically extracted elicitation cultures were assessed with *E. coli*, *S. aureus, C. albicans* and *S. cerevisiae* on Mueller-Hinton agar medium using the disk diffusion method (Kirby Bauer method). MTT assay was performed as in section 2.8.2, HeLa cells were treated with 5 and 20 µg/µl concentrations of organic extracts to assess cytotoxicity.

2.10.3. Effect of *N*-acetylglucosamine (GlcNAc)

Streptomyces isolates were inoculated into 25 ml of ISP1 medium supplemented with 50 mM of GlcNAc and incubated at 28°C for 14 days. Antimicrobial and cytotoxic activity were assessed as in section 2.9.2.

2.11. Genomic sequencing and bioinformatics analysis

Genomic DNA of all positive isolates was isolated with promega Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's recommendation for Gram positive organisms. The quality and quantity of the isolated DNA was verified by 1% Agarose gel electrophoresis and NanoDrop (Thermo Fisher Scientific). Genomes were sequenced using Illumina MiSeq platform (MicrobesNG centre, University of Birmingham).

The reads were trimmed using Trimmomatic and the quality was assessed using in-house scripts along with Samtools, BedTools and bwa-mem software. Genome reads were de novo assembled using QUAST. Sequences were blasted to NCBI (National Centre for Biotechnology Information) for identification. Gene annotation was performed by Prokka for prokaryotic

genome annotation. Genetic classification based on biological systems and KEGG Pathways were analysed by RAST system. Biosynthetic Gene Clusters (BGCs) were identified by antiSMASH (version 4.1.0) and Prism 3 online platforms.

2.12. Liquid Extraction Surface Analysis (LESA)

Three types of *Streptomyces* colonies were analysed with LESA: colonies produced from inoculation of strains on ISP1 agar medium (un-induced), colonies produced from inoculation of strains on ISP1 agar medium induced with 0.7% GBL and colonies produced from the inoculation of strains into ISP1 agar medium supplemented with 50 mM GlcNAc. LESA-MS spectra were obtained using the TriVersa NanoMate^{TM} robotic pipette nanoelectrospray system (Advion, Ithaca, USA) attached to an Orbitrap Elite mass spectrometer (ThermoFisher Scientific, Germany). For lipid/small molecule analysis, the robotic pipette tip of the TriVersa NanoMate^{TM} aspirated 3 µl from a well of a 96-well plate containing 1:1 ethanol-water, dispensed 2 µl onto the colony and then reaspirated 2.5 µl before nanoelectrospray ionisation. For protein/peptide analysis 3 µl of acetonitrile-water-formic acid (50:45:5) extraction solvent were aspirated; 2 µl of the solvent were dispensed whilst the pipette tip was held in contact with the surface of the colony. Contact was kept for 10 s followed by reaspiration of the solution into the pipette tip and infused into the mass spectrometer at 1.7 kV with a gas pressure of 0.3 psi. Most of the MS spectra were obtained in the 50 – 1500 *m/z* range and visualized by Xcalibur software (ThermoFisher scientific, USA).

2.13. Statistical Analysis

The statistics of the study was performed using the student's t-test. MIC concentrations were assessed using PRISM 7.04 (Graphpad software). The mass spectra data generated by LESA was analyzed by Xcalibur (ThermoFisher scientific).

Chapter 3

Isolation and Activity of *Streptomyces*

3. Isolation and Activity of *Streptomyces*

3.1. Introduction

Streptomyces have been isolated from many terrestrial habitats around the world but their abundance and ability to produce bioactive metabolites depend on their habitat geographical and environmental conditions (Dolotkeldieva and Totubaeva, 2006). Each bacterial community differs in their microbial flora based on ecological differences (Stubbendieck et al., 2016). A specific ecosystem is defined by the species it occupies. The higher the biodiversity in an ecosystem (more niches occupied) demands higher usage of resources and vice versa (Hunting et al., 2015). *Streptomyces* were discovered from cultivated soil with antagonism potential against some bacteria and were capable of producing novel antibiotics (Oskay et al., 2004). Diversity of *Streptomyces* can be influenced by the different plant species as these bacteria may grow in the humus layer (Jeffrey, 2008). In addition, plants can produce toxic chemical compounds against the *Streptomyces* and in return resulting in the Streptomyces producing their own secondary metabolites.

Bacterial neighbourhoods are governed by competition which is a vital part of communication between bacteria (Foster and Bell, 2012). Bacteria communicate and interact with other species while responding to external environmental conditions. Bacteria's ability to distinguish interspecies signals and environmental changes is reflected in their bacterial genome. For example, *Bacillus subtilis* has many genes that are considered "non-essential", yet these genes can play an important role in their defence against competitors (Sansinenea and Ortiz, 2011). *Streptomyces* produce a variety of important enzymes as an outcome of their interactions with other microorganisms in biodiverse communities. These secondary or specialized metabolites are produced in response to environmental changes. Soil bacteria are governed by external stresses such as heat, drought and UV (Van der Meij et al., 2017). Extreme habitats are becoming major interest for scientist in the isolation of novel *Streptomyces*. Novel strains imply new gene clusters synthesizing new secondary metabolites. Extreme environments such as extreme temperature, pH and salinity may lead to the identification of new strains of *Streptomyces.*

UAE is an underexplored habitat with a hot desert climate, the climate is harsh with temperatures ranging between 40 and 50 °C for most of the year with lowest of 20 °C reached for not more than 2 months of the year. Rainfall is uncommon in the UAE and does not last for long, it is in the form of downpours during the winter season. Another important factor to

consider is the sandstorms that are common during the summer season and it may last for several days which can allow horizontal gene transfer between bacterial communities. This climate is considered an extreme habitat that should be explored to identify novel *Streptomyces* containing new gene clusters. In this study, isolation and screening of *Streptomyces* species from UAE terrestrial habitat were performed. The ability of the isolated strains to produce inhibitory bioactive compounds was explored. We identified some species to possess antimicrobial and cytotoxic activities.

3.2. Results

3.2.1. Isolation of *Streptomyces* from Terrestrial Habitats

Streptomyces spores are more resistant to desiccation than other bacteria, therefore the collected soil samples were air dried for 24 hrs before processing to eliminate the growth of other bacteria in the sample. In addition, *Streptomyces* flora was enriched by heating the samples in a 55 °C water bath for 30 minutes with shaking to free the spores that can be trapped by the soil particles and the heat to eliminate the growth of pathogenic bacteria.

The above pretreatment methods resulted in the isolation of 15 *Streptomyces* isolates (Table 3.1) after 7-10 days of plating 0.1 ml of the serially diluted samples on starch casein agar (SCA). Isolates were identified according to the genus description of Shirling and Gottlieb (Shirling and Gottlieb, 1966) indicated by chalky, discrete, powdery and velvety aerial mycelium which is a unique feature of the genus (Figure 3.1). The colony sizes ranged between small to medium with white, yellow, brown and grey in colour. Each isolate was streak purified on SCA medium and oatmeal agar medium for further morphological characterization and antimicrobial activity (Figure 3.2).

Table 3. 1 Identified isolates and their locations

Fifteen isolates were identified from the 8 cultivated samples taken from different locations in UAE

Isolates	Location
CSK1	Al Khawaneej
CSK2	Al Khawaneej
CSK3	Al Khawaneej
CSM1	Al Mizhar
CSM2	Al Mizhar
CSW1	Al Warqa
CSW2	Al Warqa
CSQ1	Al Qusais
CSQ2	Al Qusais
CSO1	Oud Al Mateena
CSU1	University of Sharjah
CSU2	University of Sharjah
CSU3	University of Sharjah
CSG1	Al Gharayen
CSD1	Al Dhaid

Figure 3. 1 *Steptomyces* isolated from terrestrial samples

Soil sample isolated from Al Khawaneej area was serially diluted soil (10^{-1} to 10^{-5}), each dilution was plated on starch casein agar (SCA) to isolate *Streptomyces*. Dilution 10^{-3} showed colonies showed distinct features of *Streptomyces* colonies resembled by chalky and powdery colonies of various colours ranging from white, beige, yellow, brown and greyish-black (shown as red circles in the graph).

Figure 3. 2 Streak purified *Streptomyces* isolated from soil.

Isolates exhibiting small chalky and velvety colonies were streak purified on starch casein and oatmeal agar medium for further characterization. The above isolates (CSK1, CSK3, CSG1 & CSM1), observed on starch casein medium, show distinct features for *Streptomyces* as reported by Shirling and Gottlieb (Shirling and Gottlieb, 1966) with earthy smell due to the presence of the volatile metabolite, geosmin, produced by *actinomyces*.

3.2.2. Screening for antimicrobial activity of isolated *Streptomyces* strains

The antimicrobial activity was assessed for all of the isolates using the disk diffusion method (Bauer et al., 1966) against *E. coli* and *S. aureus*. Positive isolates showed Zones of inhibitions (clearance of growth) around the disks against both *E. coli* and *S. aureus* (Figure 3.3) or against one of them. The isolates that showed inhibitory effect against both *E. coli* and *S. aureus* (CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1) were tested against *C. albicans* and *S. cerevisiae* to assess their antifungal capabilities by the disk diffusion method (Figure 3.4).

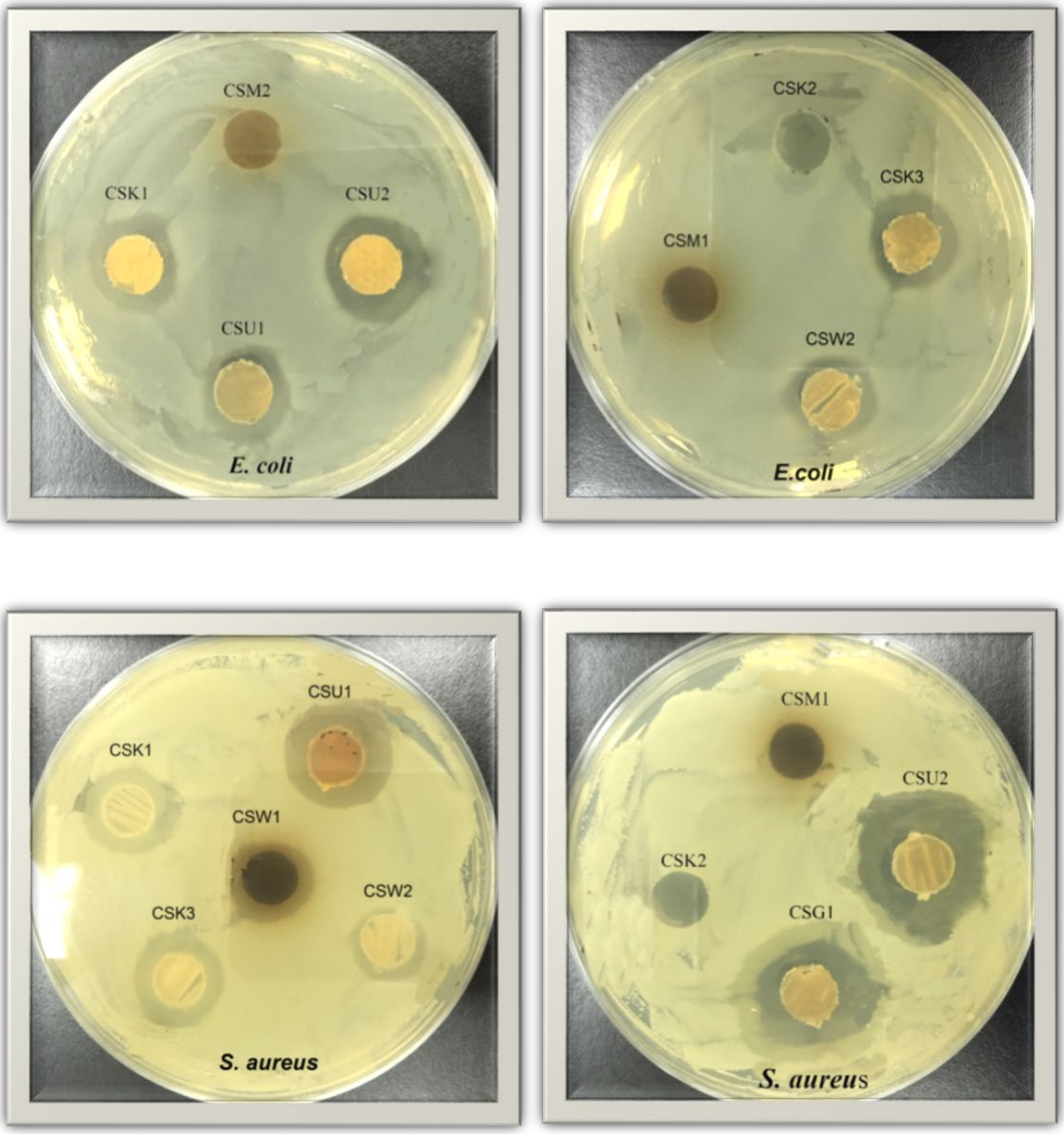


Figure 3. 3 Inhibitory activity of *Streptomyces* isolates against bacteria

Disks of 10 mm in size of the *Streptomyces* isolates were cut from streak purified starch casein agar (SCA) medium and placed on mueller hinton agar plates that were spread with either *E. coli* or *S. aureus* to assess the inhibitory effect of the isolates by observing clearance of growth surrounding the disks.

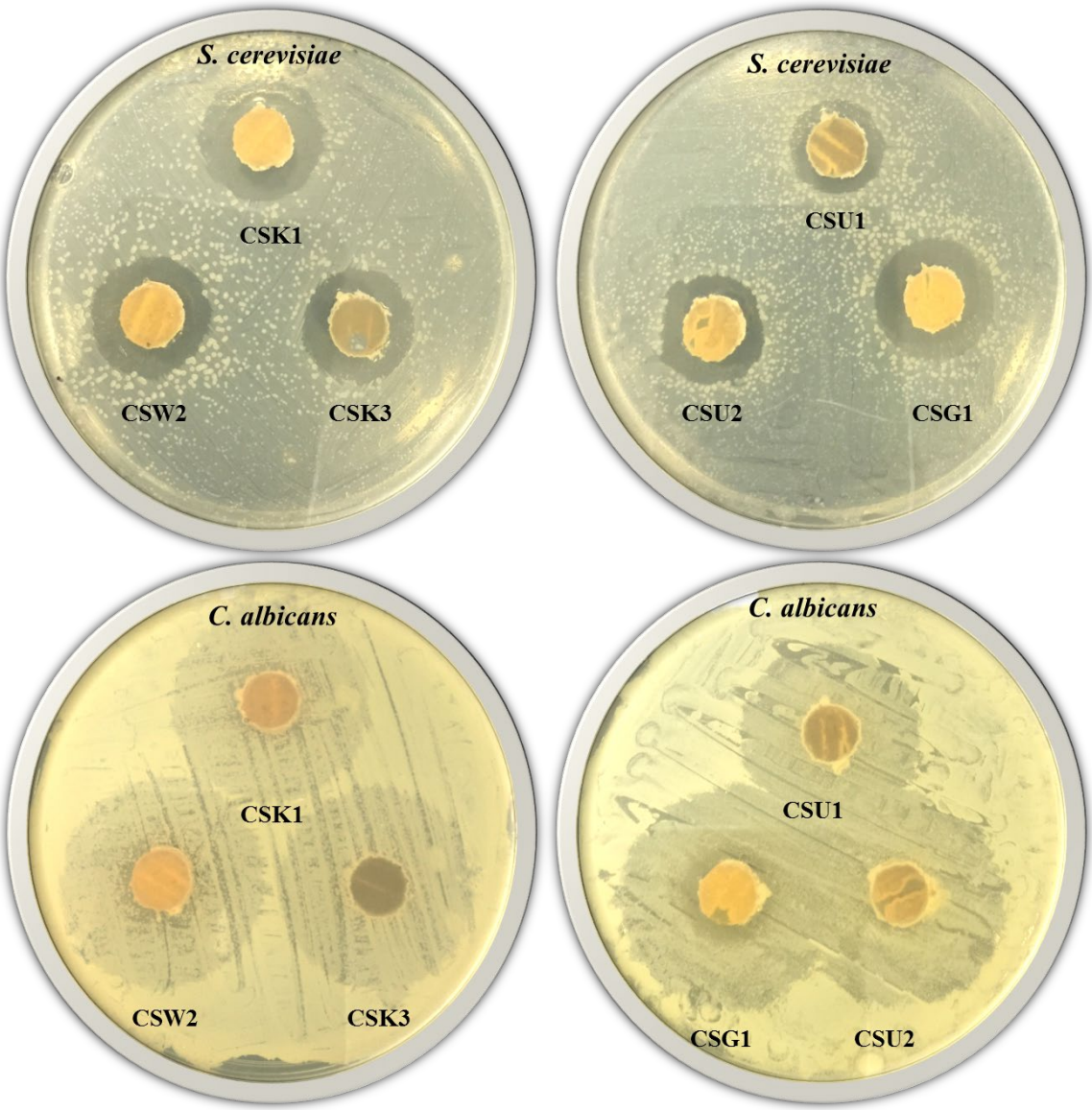


Figure 3. 4 Inhibitory activity of *Streptomyces* isolates against fungus

Ten millimetre disks were cut from streak purified *Streptomyces* isolates (CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1) on SCA and placed on mueller hinton agar plates that were spread with either *S. cerevisiae* or *C. albicans*. Inhibitory activity of the tested isolates was observed against both organisms.

Positive isolates that showed inhibitory activity against *E. coli, S. aureus, C. albicans* and *S. cerevisiae* by the disk diffusion method were inoculated into ISP1 medium for 10-14 days and production of secondary metabolites was monitored. The culture filtrate (filtered supernatant) of each isolate was assessed by the well diffusion method (as described in section 2.4.2) using *E. coli, S. aureus, C. albicans* and *S. cerevisiae*. Table 3.2 shows the average values of the zones of inhibitions (ZOI) of 3 independent experiments and the standard deviation was calculated for each sample. Six (CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1) out of the 15 isolates showed inhibitory activity against *E. coli* and *S. aureus* with zones of inhibition ranging between 16 and 26 mm including the 5mm disks. The strains showed greater antifungal activity when tested against *S. cerevisiae* and *C. albicans* with zones of inhibition ranging from 17 to 35 mm (Figure 3.5). Isolate CSM1 showed no antimicrobial activity against any of the above organisms and was used as a negative control in the project.

Table 3. 2 Zones of inhibition measured by well diffusion method

Well diffusion method was used and zone of inhibition measured for the six positive strains in triplicates and standard deviation calculated

Isolate	***E. coli*, mm ± SD**	***S. aureus*, mm± SD**	***S. cerevisiae*, mm ± SD**	***C. albicans*, mm ± SD**
CSK1	18 ± 2.08	20 ± 0.58	22 ± 1.73	35 ± 2.31
CSK3	16 ± 0.76	14 ± 0.36	19 ± 0.58	34 ± 0.58
CSW2	16 ± 0.58	18 ± 0.58	20 ± 2.08	35 ± 1.15
CSU1	16 ± 2.89	25 ± 1.15	17 ± 1.53	30 ± 2.08
CSU2	17 ± 0.58	26 ± 1.53	17 ± 0.58	27 ± 0.14
CSG1	20 ± 1.15	17 ± 0.58	20 ± 1.73	34 ± 3.46

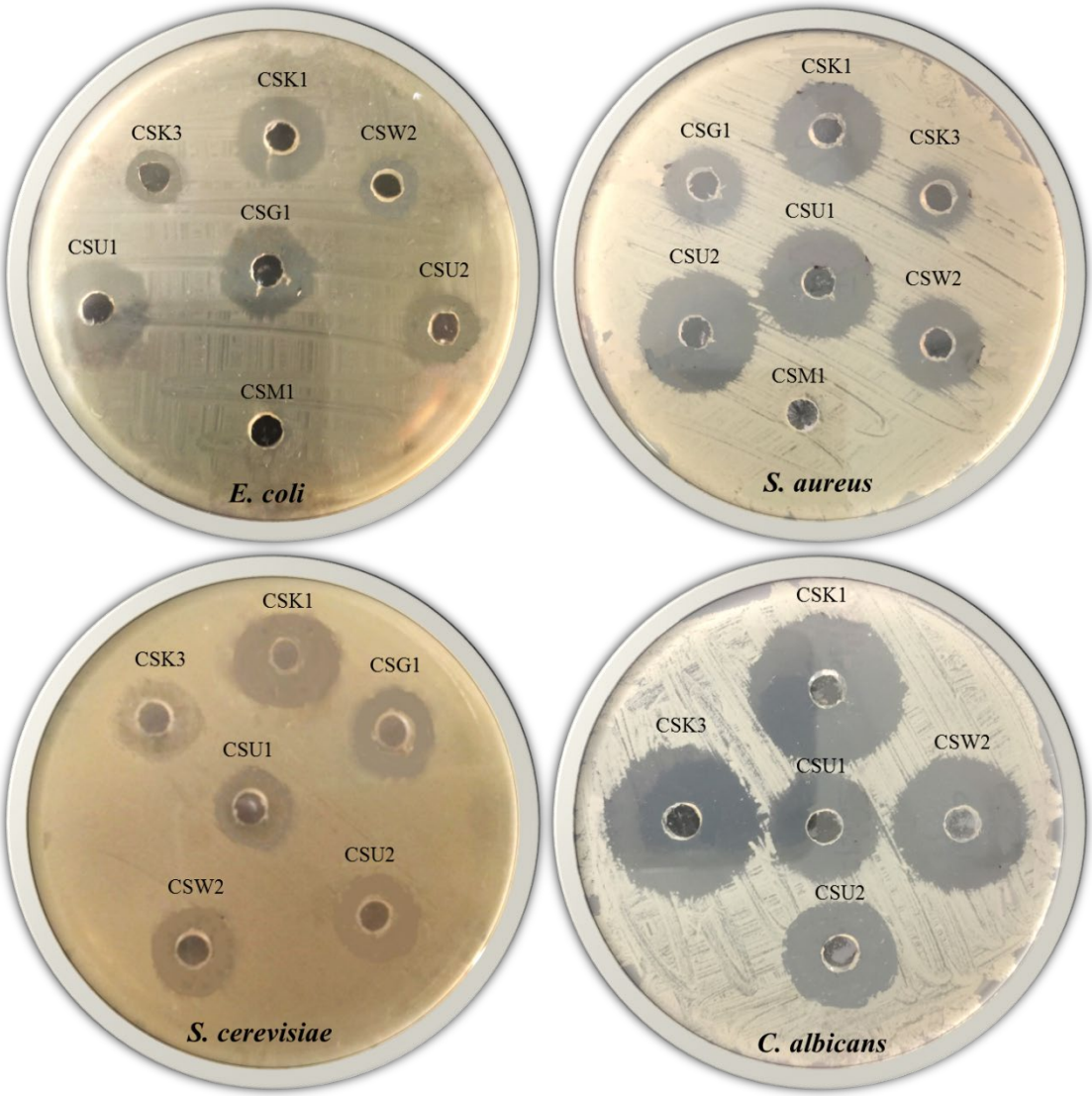


Figure 3. 5 Inhibitory effect of *Streptomyces* isolates by well diffusion method.

Filtrates of each Streptomyces strain was isolated from fermented culture broth. The filtrates were tested by well diffusion method against *E. coli*, *S. aureus*, *S. cerevisiae* and *C. albicans* using Mueller hinton agar medium. All the isolated strains' filtrates (CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1) showed inhibitory activity against the tested organisms except for CSM1 which is the negative isolate. Zones of inhibition is summarized in Table 3.2.

3.2.3. Phenotypic characterization

The selected six isolates were observed as Gram positive filamentous bacteria with branching hyphae from aerial mycelium under the light microscope. Morphological characterization on SCA (Figure 3.6) and oatmeal agar medium showed aerial mycelium colour of beige for CSK1, CSW2 and CSG1, brown for CSK3, CSU1 and CSU2 and Yellow for CSM1.

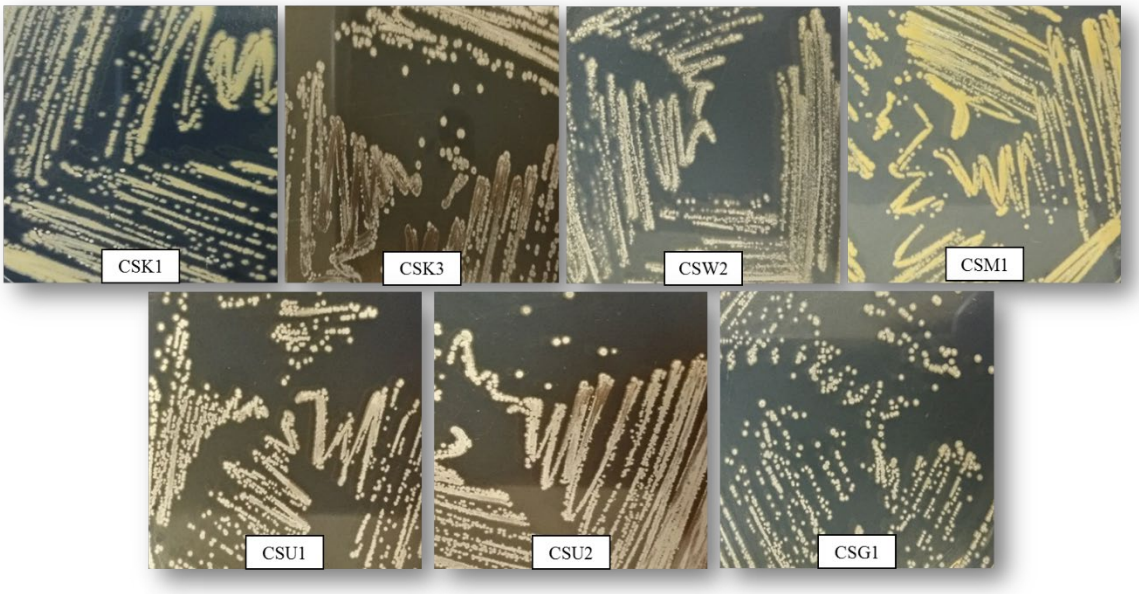


Figure 3. 6 Morphological characterization of positive *Streptomyces* isolates

Isolates obtained from soil samples were streak purified on starch-casein agar medium to further identify and characterize the isolates morphologically.

A range of phenotypic properties were tested such as colour of aerial and substrate mycelium (Table 3.3). Melanoid pigments of light brown were produced by CSK1 and CSK3 and black for CSM1 on ISP7 (Tyrosine agar) medium only. All isolates were negative for Hydrogen Sulphide production and positive for catalase. The ability of the isolates to utilise different carbon sources such as inositol, L-arabinose, xylose and sorbitol (carbon sources were chosen based in their availability in the lab) was assessed using glucose as positive control.

Inositol being utilised by all isolates except CSM1 (negative control) while the least utilized by all isolates was xylose and sorbitol.

Table 3. 3 Phenotypic & biochemical characterization of *Streptomyces* strains

Isolates were characterized according to the colour of their aerial and substrate mycelium. Data analysis was recorded based on + (positive) or – (negative) on observance. Production of brown colour pigment was observed for CSK3, CSU2 and CSG1. Isolates CSW2 and CSM1 were positive for utilization of inositol, L-arabinose, xylose and sorbitol while CSU1 was negative for the utilization of these carbon sources.

	CSK1	**CSK3**	**CSW2**	**CSU1**	**CSU2**	**CSG1**	**CSM1**
Colour of aerial mycelium	White	White	White	White	White	White	Yellow
Colour of substrate mycelium	Yellow	Grey	Yellow	Grey	Grey	Yellow	Yellow
Pigment	-	Brown	-	-	Brown	Brown	-
Melanoid production (ISP7)	Light brown	Light brown	-	-	-	-	Black
Hydrogen sulphide	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
Carbon source utilization							
Inositol	+	+	+	-	+	+	+
L-Arabinose	+	+	+	-	-	-	+
xylose	-	-	+	-	-	-	+
Sorbitol	-	-	+	-	-	-	+

The characterization of spores has been an important identifying feature in the taxonomy of *Streptomyces* as they form when the organism undergoes stressful conditions leading to morphological differentiation which is linked to secondary metabolite production. Scanning electron microscopy examination showed spores with short chains of rod-shaped morphology (Figure 3.7). Spore surface was observed to be smooth for CSK1 and CSK3 and spiny for the other isolates.

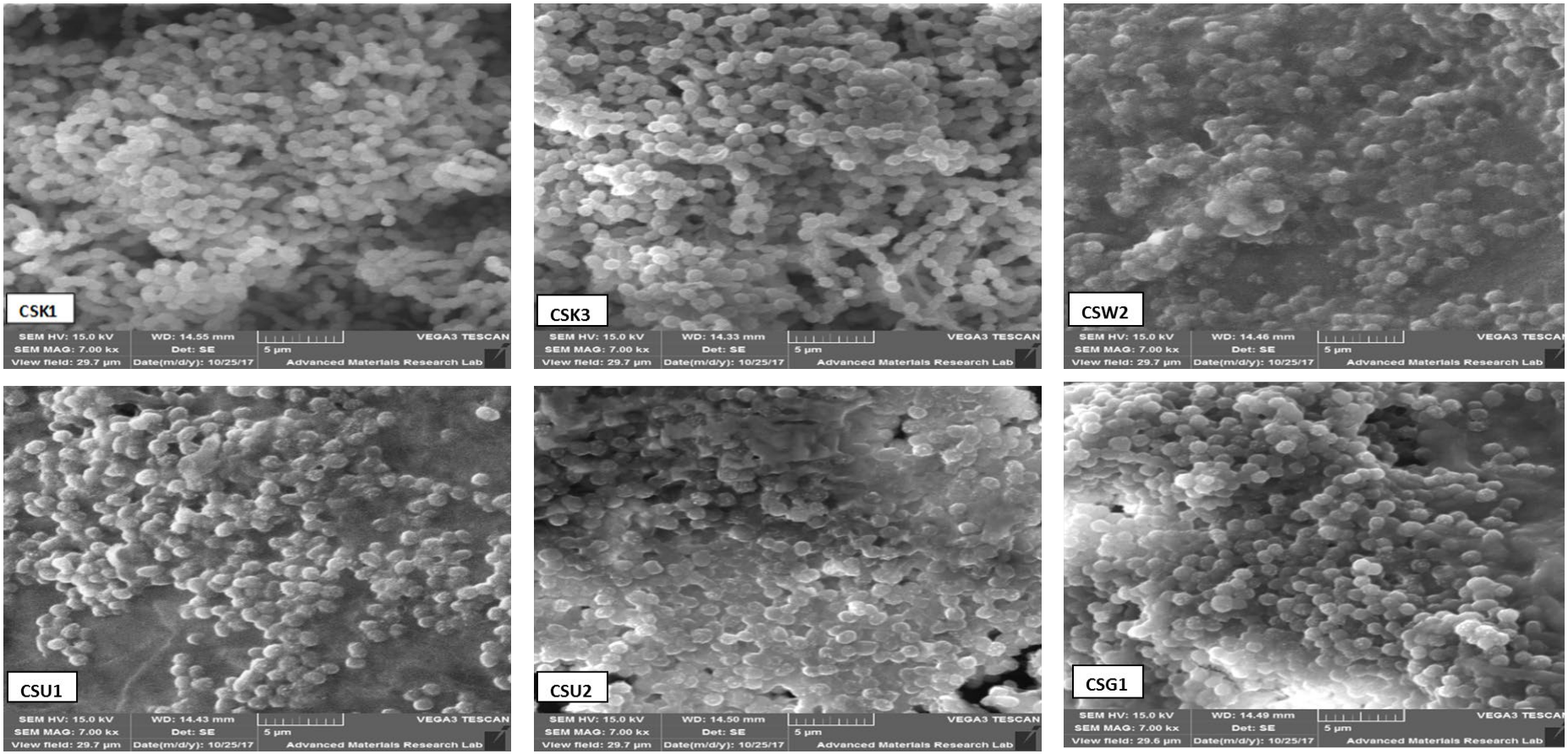


Figure 3. 7 Electron Microscopy of *Streptomyces*' spore surfaces

Spore surface morphology of *Streptomyces* isolates was examined by Scanning electron microscopy at x 7000. Spiral chains of spores with smooth surface were observed in isolates CSK1 & CSK3 while isolates CSW2, CSU1 and CSU2 had a spiny spore surfaces observed by projections of different length and thickness (as described by Tresner et al., 1961).

3.2.4. Minimum Inhibitory Concentration (MIC)

The susceptibility of each isolate was determined by broth microdilution method against *E. coli*, *S. aureus*, *C. albicans*, *S. cerevisiae*, *E. coli*-ESBL and *B. subtilis*. The minimum inhibitory concentration (MIC) for all isolates (organic extracts purified by ethyl acetate) was determined by resazurin-based turbidometric (TB) assay (Elshikh et al., 2016). A 96 well microtiter plate was set up with the tested bacteria being standardized to optical density of 0.05 at 600 nm and extracts ranging in concentration from 0.12 to 30 µg/µl in twofold step dilutions. MIC values were calculated for each isolate with 3 independent replicates using Gompertz model (Lambert & Pearson, 2000) by Graphpad prism 4 software (Table 3.4). The values were quantified by plotting the OD of the bacterial growth on a logarithmic scale resulting in a sigmoid-shaped curve using Graphpad prism (Appendix A).

It is concluded from the MIC values that all isolates were highly inhibiting Gram-positive *S. aureus* bacterium with MIC values ranging between 0.21 and 0.29 µg/ml. Meanwhile, lowest antagonism was observed for *E. coli*-ESBL by all isolates of MIC values ranging between 6.16 and 10.91 µg/ml except for CSK3 which showed lowest antagonism against *S. cerevisiae* at 12.17 µg/ml.

Table 3. 4 Minimum inhibitory concentration assessment of the isolates

As described in section 2.7 of chapter 2. Values were quantified by the graphpad Prism 4 software based on the Gompertz model.

	Concentration µg/ml ± SD					
Isolate	***E. coli***	***S. aureus***	***C. albicans***	***S. cerevisiae***	***B. subtilis***	***E. coli*-ESBL**
CSK1	4.03 ± 0.01	0.21 ± 0.01	1.97 ± 0.01	7.32 ± 0.01	2.54 ± 0.01	9.07 ± 0.02
CSK3	1.91 ± 0.04	0.23 ± 0.02	4.61 ± 0.01	12.17 ± 0.01	6.53 ± 0.01	6.16 ± 0.02
CSW2	4.72 ± 0.04	0.29 ± 0.02	4.13 ± 0.01	9.33 ± 0.01	3.58 ± 0.003	8.60 ± 0.03
CSU1	1.97 ± 0.02	0.24 ± 0.02	3.98 ± 0.01	3.36 ± 0.01	7.36 ± 0.01	10.64 ± 0.03
CSU2	1.97 ± 0.02	0.21 ± 0.01	4.25 ± 0.01	7.10 ± 0.02	10.91 ± 0.02	5.69 ± 0.01
CSG1	1.95 ± 0.01	0.23 ± 0.01	4.67 ± 0.01	9.35 ± 0.03	5.69 ± 0.02	10.91 ± 0.01

3.2.5. Cytotoxic Activity

3.2.5.1. MTT Assay

The antiproliferative effects of the extracts were assessed on HeLa cervical cancer cells using the MTT and DAPI staining. In the MTT assay Doxorubicin was used as a positive control isolate while CSM1 was used as negative controls. The growth of cancer cells was inhibited in a concentration-dependent manner (concentrations ranging between 5-20 µg/µl) for most isolates except CSK1 and CSU2 (Figure 3.8). The IC50 values were determined for isolates that showed cytotoxicity effect against HeLa cells using graphpad prism using Dose-response inhibition (Table 3.5). The IC_{50} values ranged from 3.46 µg/µl for CSK3 to 9.74 µg/µl for CSU1.

3.2.5.2. DAPI Staining

The morphological changes induced by apoptosis were assessed by inducing Hela cells with 5 and 20 µg/µl of the *Streptomyces* organic extracts for 24 hrs. After fixing and staining the cells with DAPI, apoptosis was examined using nuclear DAPI staining. Apoptotic features such as DNA fragmentation and chromatin condensation were clearly observed in CSK1, CSW2, CSU1 and CSU2 specifically at 20 µg/µl (Figure 3.9), unlike the control (untreated HeLa cells) and the negative extract CSM1 which showed normal nuclei. Apoptosis was not observed for CSK1 and CSU2 isolates. Hela cells were counted to assess percentage of nuclear fragmentation, as shown in Figure 3.10 higher dosage of the extracts induces greater nuclear alterations.

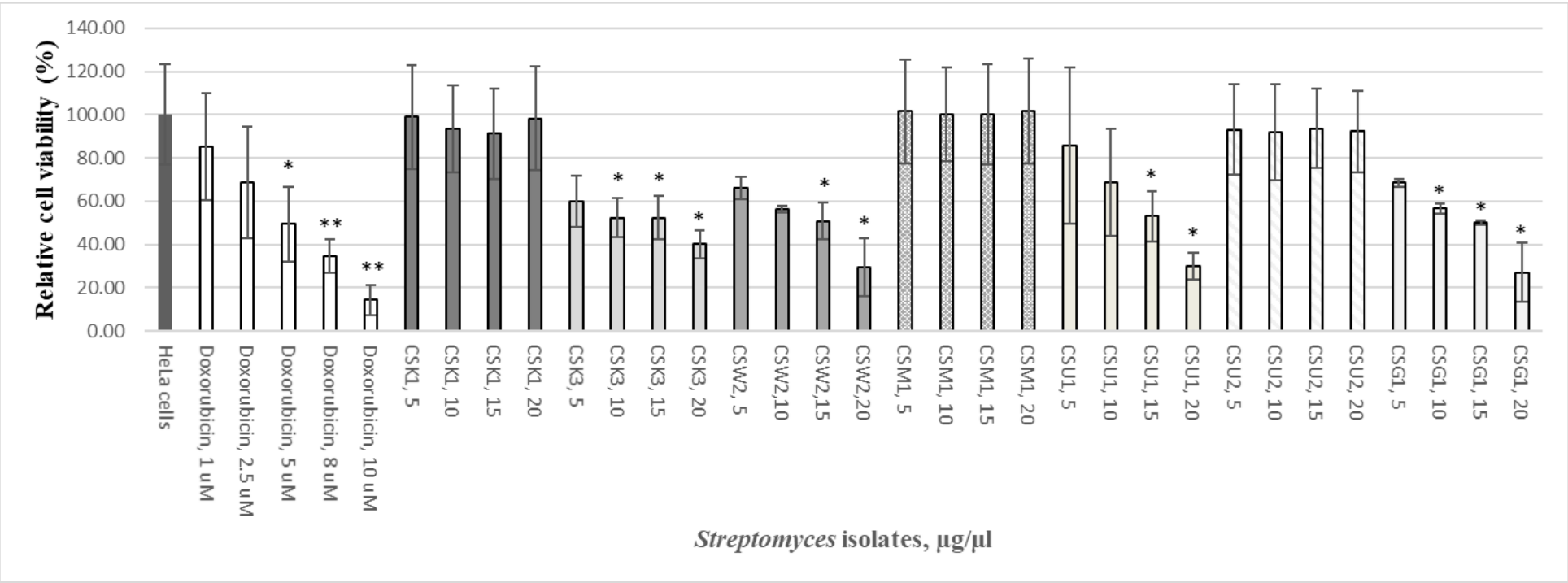


Figure 3. 8 Cytotoxicity of *Streptomyces* extracts on HeLa cells.

HeLa cells were treated with different concentrations (5-20 µg/µl) of *Streptomyces* organic extracts. Cytotoxicity on the cancer cells was assessed with MTT assay with absorbance measured at 570 nm. Doxorubicin was used as a positive control and isolate CSM1 as negative control. The presented results are means of standard deviation for three independent experiment. Data was normalized to the mean, T-test was used to analyze two sample comparison assuming equal variance (*P<0.05 and **P<0.005).

Table 3. 5 Dose response (IC_{50}) values

IC50 values for each isolate was determined by MTT assay using Graphpad prism software (n=3)

Streptomyces isolates	IC_{50} (µg/µl)(mean & error ± SEM)
CSK3	3.46 ± 0.19
CSW2	5.73 ± 0.09
CSU1	9.74 ± 0.08
CSG1	6.34 ± 0.07

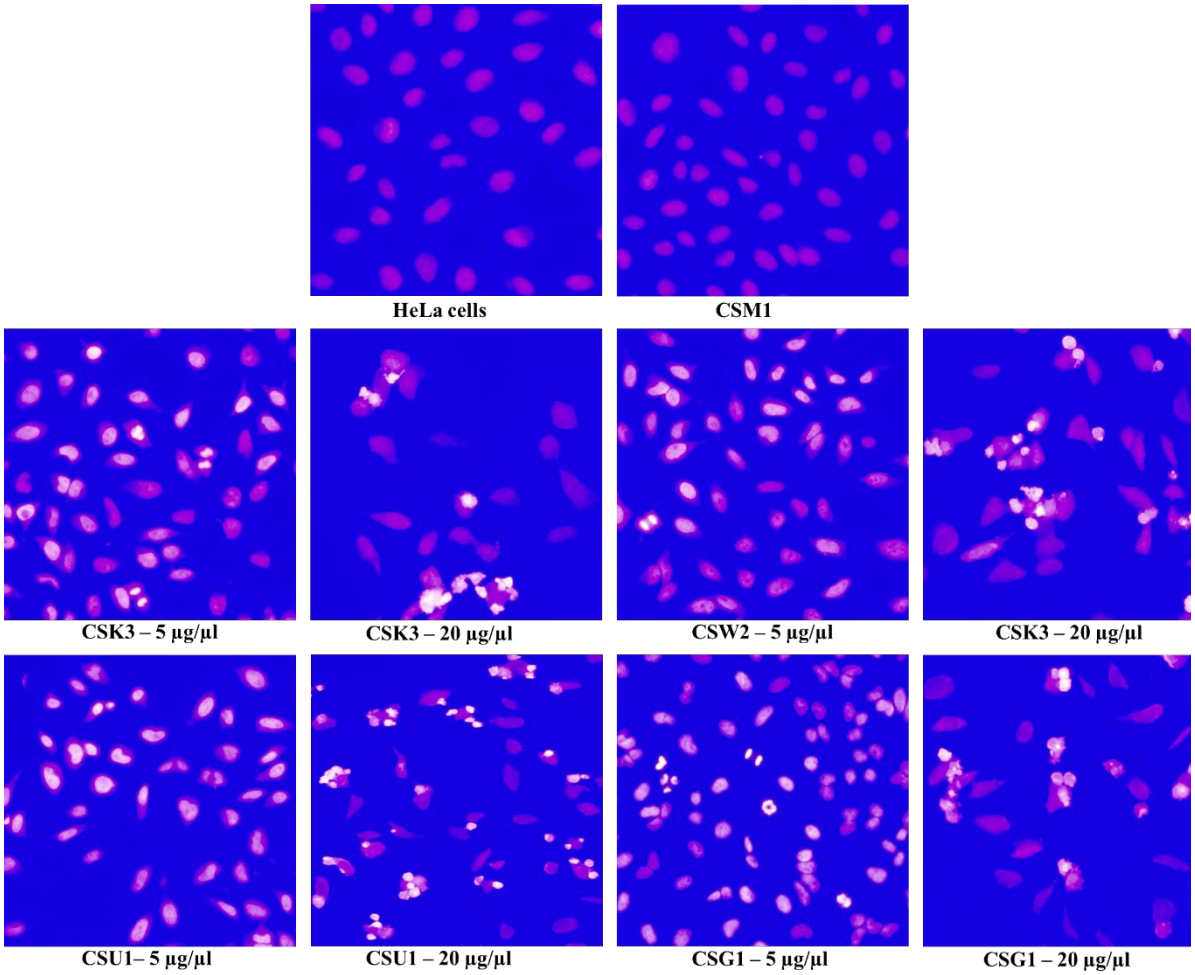


Figure 3. 9 Apoptotic Induction of *Streptomyces* organic extracts on HeLa cells.

HeLa cells were cultured in the absence of extract (control) and in the presence of 5 and 10 µg/µl of the *Streptomyces* ethyl acetate organic extracts including the CSM1 negative control isolate. After 24 hrs, the cells were fixed and stained with DAPI. Nuclear fragmentation was observed for CSK3, CSW2, CSU1 and CSG1 isolates under the fluorescent microscope.

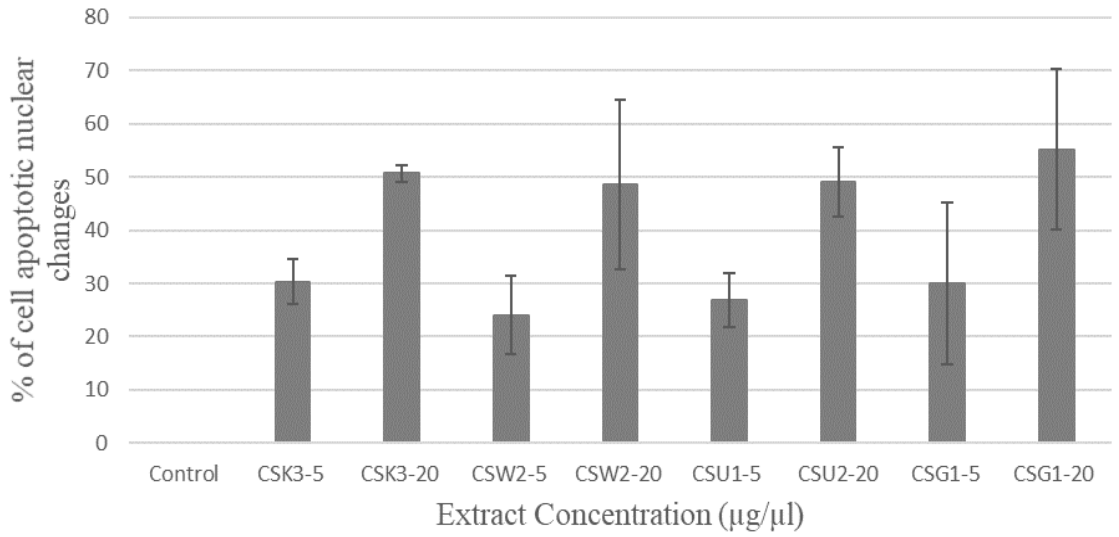


Figure 3. 10 Quantification of DAPI staining

Morphological changes of Hela cells induced with Streptomyces extracts (CSK3, CSW2, CSU1 and CSG1) were observed and quantified. Apoptotic nuclear changes are expressed as mean ± STDEV of three experiments.

3.2.6. Isolation of *Streptomyces* from Marine Habitats

Culturing of marine sediment samples did not produce any *Streptomyces* colonies. Yet, these samples which were collected from a variety of marine sediment areas along the coastline of the Arabian Gulf may have a great potential for the isolation of novel strains of *Streptomyces*. A variety of pretreatment and culturing conditions are required before we can conclusively state that these samples do not contain any *Streptomyces* species.

3.3. Discussion

Streptomyces are widely found in both terrestrial and aquatic habitats, predominantly affected by, nutrients, temperature, pH and moisture (Gilbert et al., 1995). *Streptomyces* are known for the isolation of a variety of bioactive compounds such as anti-viral, anti-tumour, modulator of immune response, various enzymes inhibitors, insecticide, anti-parasitic and bioherbicides (Sanglier et al., 1993; Thompson et al., 2002). These important bioactive compounds are strain-specific and are produced by *Streptomyces* secondary metabolites (Thompson et al., 2002). These secondary metabolites are formed in stress surroundings such as nutrient depletion and waste accumulation, usually in the stationary phase of their life cycle, making them highly dependent on environmental conditions.

A total of 15 *Streptomyces* isolates were obtained from 8 soil samples collected from different locations in the eastern region of UAE. The disk and well diffusion method (also known as Kirby-Bauer method) measuring the zone of inhibition in diameter (mm) was used to assess the antimicrobial activity of the *Streptomyces* isolates against *E. coli* (Gram negative bacterium) and *Staphylococcus aureus* (Gram positive bacterium). More than 70% (11 out 15) of the isolates showed activity against one or both of the tested bacterium. Six isolates (40%) showed bioactivity against both organisms with zones of inhibition ranging between 16-26mm in diameter as shown in Table 3, with maximal inhibition observed for CSK1, CSU1, CSU2 and CSG1 isolates. In addition, the six positive isolates showed zones of inhibition ranging from 17-35 mm when tested on *Candida albicans* and *Saccharomyces cerevisiae*. However, these isolates showed higher activity against *C. albicans* than *S. cerevisiae*. This Zone of inhibition which is detected by clearance of growth of the microbe surrounding the tested isolate is due to the secondary metabolites produced by the extracted *Streptomyces* isolates. CSM1 isolate obtained from Al Mizhar area in Dubai showed no activity against any microbial organism and alternatively was used as a negative control in this project.

The use of phenotypic characteristics plays an important part in the identification of the *Streptomyces* genus, in particular the distinct colony morphology exhibiting chalky, powdery and velvety aerial mycelium which were evidently observed in the isolated strains (Figure 12). All the isolates were positive for catalase capable of breaking down toxins produced from oxygen metabolism and negative for Hydrogen sulphide production but they exhibited different patterns for carbon utilization which could be an implication that they are different strains.

The antimicrobial properties of the isolates have also been demonstrated by the resazurin-based turbidometric (TB) assay to determine the MIC values against a variety of pathogenic bacteria. The results showed that all isolates secrete wide spectrum of antimicrobial agents which could inhibit the growth of both bacteria and fungus. The isolates exhibited high activity against Gram-positive bacterium *S. aureus*, moderate activity against *E. coli* and *C. albicans* and low activity against *S. cerevisiae* and multidrug resistant isolate *E. coli*-ESBL. These results correlate with many studies (Cwala et al., 2011; Nurkanto et al., 2012) which show that *actinomyces* exhibit good activity against Gram-positive bacteria and low or no activity against Gram-negative bacteria and this could be due to the biochemical structure of the outer membrane of the Gram-negative bacteria being surrounded by an outer membrane containing LPS (lipopolysaccharide) which make the cell wall impermeable to lipophilic compounds.

The highest rate of deaths in the world is attributed to cancer with an estimation of 9.6 million in 2018 (World health organization; Bray et al., 2018). Many studies have shown the ability of *Streptomyces* to produce secondary metabolites as an anti-cancer agent (Berdy, 2005; Demain and Sanchez, 2009). Toxicity assay of extracts (secondary metabolites) was initially examined by the change of morphology on HeLa cells, most of the cells were observed as adherent with angular and spindle shape (epithelial) but most of the cells lost these characters after treatment with increasing concentration of the extracts. Cell shrinkage, rounding and detaching of the cells and apoptotic bodies were observed indicating the toxic effect of the extracts on HeLa cells.

The earliest changes noticed in apoptosis are attributed to shrinkage of cells and pyknosis which are the result of chromatin condensation. The apoptotic cell contains dark purple chromatin fragments in the nucleus, this is followed by the formation of apoptotic bodies which is the separation of cell fragments (Elmore, 2007). In order to assess apoptosis, induced by the isolated *Streptomyces* strains, DNA fragmentation and cell viability measurements of the extracts on HeLa cells by MTT assay and DAPI staining were demonstrated. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay is used to assess cell viability by measuring the mitochondrial activity of viable cells by reducing the yellow tetrazolium MTT reagent into purple formazan crystal with the addition of DMSO. The quantity of formazan produced is presumed to be proportional to the number of viable cells (Twentyman and Luscombe, 1987). Viability of the HeLa cells decreased with the increase in the concentration of the extracts from 5 to 20 µg/µl specifically for cells treated with CSK3, CSW2, CSU1 and CSG1 extracts. Finally, morphological changes such as DNA fragmentation, nuclear condensation and inhibition of growth was evident after staining and

quantifying the treated HeLa cells with DAPI specifically for CSK3, CSW2, CSU1 and CSG1 compared to the cells treated with CSM1 (negative control) and non-treated cells.

As for the marine sediment samples, we believe that there is a great potential to find novel strains of marine *Streptomyces* as it is a promising underexplored source for secondary metabolites production. A possible explanation could be that most of the samples were collected under the water and the right depth for sample collection was not reached; nevertheless we could not isolate *Streptomyces* even from the samples that were collected after the tide had gone out. These samples will be processed again with a variety of nutritional and environmental conditions which may lead to the isolation of new strains of marine *Streptomyces*.

Chapter 4

Elicitation of Isolated *Streptomyces* Strains

4. Elicitation of Isolated *Streptomyces*

4.1. Introduction

In nature, all microbes co-exist with each other competing for nutrition and survival and therefore they develop defence systems that mainly rely on the production of secondary metabolites (Bertrand et al., 2014). In vitro, co-culturing microbes with each other may force this defence mechanism to increase the production of secondary metabolites that are already present or lead to the expression of silent biosynthetic gene clusters and therefore the production of new secondary metabolites (Vargas-Bautista, 2014). Elicitation of secondary metabolites is an arising approach to help in the production of new secondary metabolites. It is based on triggering the bacterial cells with external stimuli known as "elicitors" to induce stress resulting in the activation of genes involved in defence or inactivation of genes that are not involved in defence production of new secondary metabolites (Abdelmohsen et al., 2015).

The exact mechanism for the elicitation of secondary metabolites by other microbes is not fully understood but it could occur in a number of ways (Marmann et al., 2014): physical interaction of the producer organism with other bacterial cells may stimulate production (Figure 4.1-A); production of siderophores or small signalling molecules such as ϒ-Butyrolactone hormones by other bacteria which may produce them for quorum sensing (Figure 4.1-B); production of enzymes by the inducer that leads to activation in the producer (Figure 4.1-C); and finally by gene transfer which could result in the activation or repression of the secondary metabolite as a permanent change in the producer genome (Figure 4.1-D).

Elicitation mechanism was implemented in this study by culturing the *Streptomyces* isolates with *B. subtilis* lysate, γ-Butyrolactone and *N*-acetylglucosamine. Antimicrobial and cytotoxic activities were assessed. Culturing CSK1 isolate with *B. subtilis* lysate and *N*-acetylglucosamine resulted in change of the culturing medium to red which could indicate the production of undecylprodigiosin (RED). We, also, observed that culturing with GBL negatively affected antimicrobial activity against *E. coli*, *C. albicans* and *S. cerevisiae* for most of the tested isolates. Yet, increased inhibitory activity was observed against *S. aureus* for all isolates.

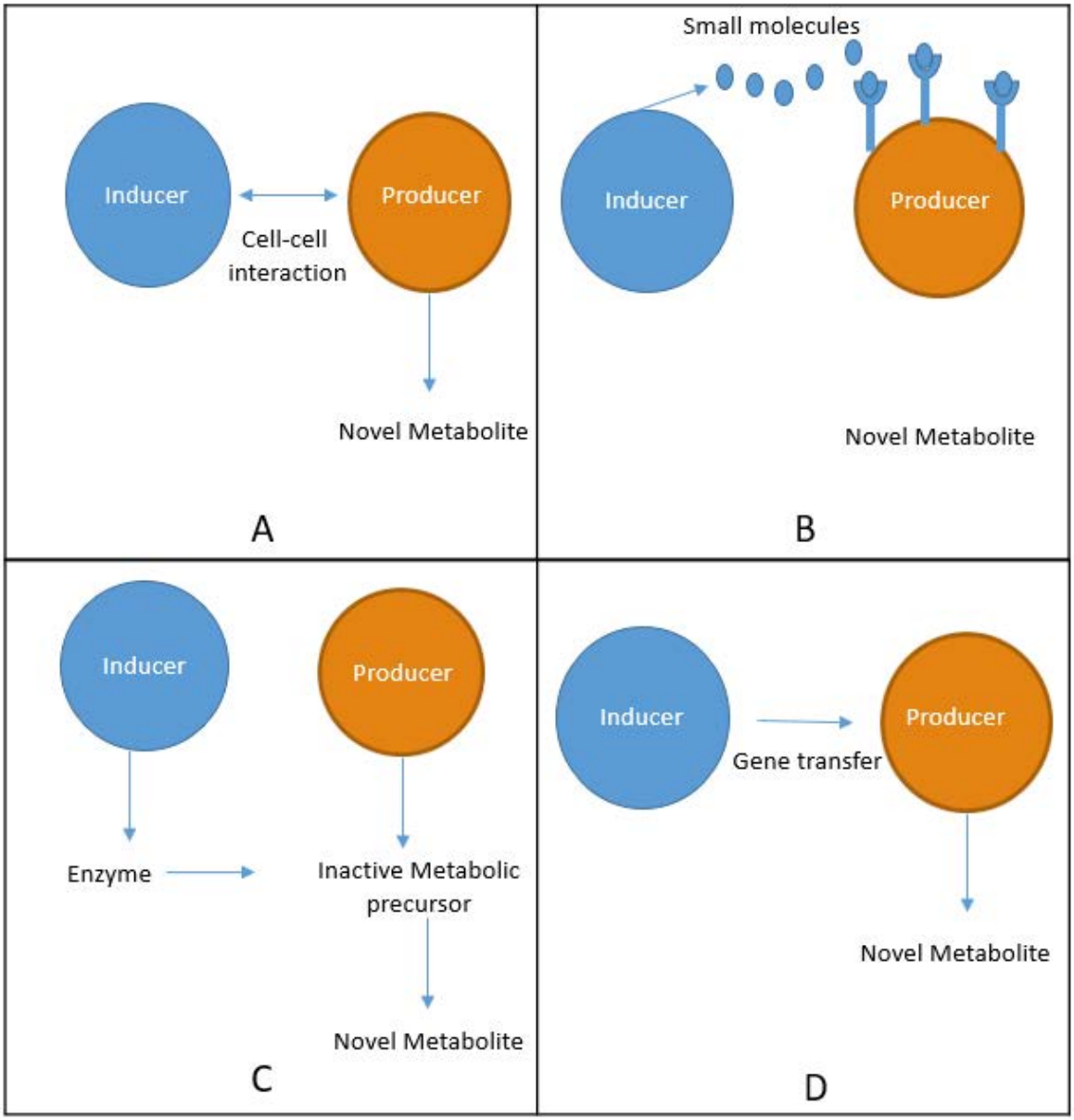


Figure 4.1 Mechanisms for Biological elicitation of novel secondary metabolites

Production of new secondary metabolites or the expression of silent genes may be caused by cell to cell interaction (A), production of small molecules (B), production of enzymes (C) or gene transfer based on evolution (D).

4.2. Results

Elicitation based on cultivation with hormones (Gamma Butyrolactone, GBL), signalling molecules (*N*-acetylglucosamine, GlcNAc) and another microorganism (*B. subtilis* lysate) to induce silent biosynthetic genes or enhance the production of existing ones was studied based on the well diffusion method.

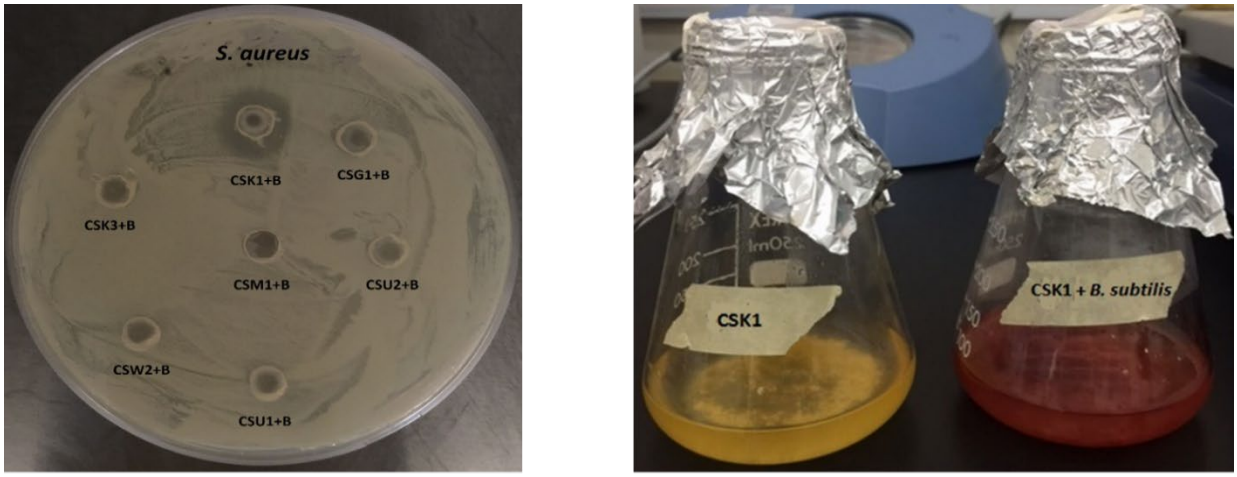
4.2.1. Effect of *Bacillus subtilis* lysate on *Streptomyces* isolates

The interaction between the two spore-forming bacteria *Streptomyces sp.* and *Bacillus subtilis* was studied by cultivating the two organisms as an attempt to induce a diverse production of secondary metabolites. Each *Streptomyces* isolate was induced with *B. subtilis* cell lysate and antimicrobial activity against *E. coli* and *S. aureus* using the well diffusion method was assessed after 6 days of culture. CSK1 isolate, only, induced with *B. subtilis* was able to cause inhibitory effect against *S. aureus* (Table 4.1; Figure 4.2A) compared to the control which showed no activity as expected (the isolated *Streptomyces* strains monocultures show activity between 14-21 days of culturing). Furthermore, the elicited culture physically changed to red in colour after 14 days of culturing with no such change observed in the monoculture (Figure 4.2B).

Table 4. 1 Effect of *B. subtilis* lysate on Streptomyces isolates

Zone of inhibition diameter measured in mm for Streptomyces isolates after 6 days of cultivation with B. subtilis lysate. Standard deviation (SD) was calculated for three independent replicates

Sample	*E. coli* mm ± SD	*S. aureus* mm ± SD	Observation
CSK1-C	-	-	-
CSK1-*B. subtilis* lysate	-	13.0±0.41	Red colour
CSK3-C	-	-	-
CSK3-*B. subtilis* lysate	-	-	-
CSW2-C	-	-	-
CSW2-*B. subtilis* lysate	-	-	-
CSU1-C	-	-	-
CSU1-*B. subtilis* lysate	-	-	-
CSU2-C	-	-	-
CSU2-*B. subtilis* lysate	-	-	-
CSG1-C	-	-	-
CSG1-*B. subtilis* lysate	-	-	-
CSM1-C	-	-	-
CSM1-*B. subtilis* lysate	-	-	-



A B

Figure 4.2 *Streptomyces* isolates cultivated with *B. subtilis*.

Isolates were separately cultivated with 3% (v/v) *B. subtilis* lysate (as reported by Elibol et al., 1995) in ISP1 medium and inhibitory activity of the cultivation was assessed against *S. aureus* by the well diffusion method, Inhibition of growth was observed for CSK1 only. Isolate CSK1 was monocultured in ISP1 medium and cultivated with *B. subtilis* lysate (B). After 14 days of incubation at 28 °C, red pigmentation was observed in the elicitation culture (n=3).

4.2.2. Effect of ϒ-Butyrolactone (GBL) on *Streptomyces* isolates

GBL is a microbial hormone that has been identified to cause secondary metabolism in *Streptomyces*. The hormone was co-cultivated with the *Streptomyces* isolates (CSK1, CSK3, CSW2, CSU, CSU2 and CSG1) at different concentrations of 0.2, 0.5, 0.7 and 1 %. After 14 days of elicitation, extracts were tested against *E. coli*, *S. aureus*, *C. albicans* and *S. cerevisiae*. Well diffusion method was used to assess activity and zones of inhibition were measured in mm (Table 4.2).

Table 4. 2 Antimicrobial zones of inhibition of *Streptomyces* isolates cultivated with GBL.

The diameter of the zones of inhibition was measured in mm for the *Streptomyces* isolates induced with various concentrations of GBL (0.2, 0.5, 0.7 and 10 %). The standard deviation (SD) was calculated for three independent replicates.

Sample	*E. coli* mm ± SD	*S. aureus* mm ± SD	*C. albicans* mm ± SD	*S. cerevisiae* mm ± SD
CSK3-C	14.0±0.31	13.0±0.67	15.5±0.25	13.0±0.52
CSK3-0.2% GBL	0	0	0	0
CSK3-0.5% GBL	22.0±0.15	16.0±0.64	0	0
CSK3-0.7% GBL	23.5±0.31	17.0±0.12	0	0
CSK3-1% GBL	21.5±0.35	15.0±0.42	0	0
CSW2-C	14.0±0.35	12.0±0.2	11.5±0.42	12.0±0.34
CSW2-0.2% GBL	0	13.5±0.58	0	0
CSW2-0.5% GBL	13.5±0.42	17.0±0.40	0	0
CSW2-0.7% GBL	15.5±0.20	18.0±0.61	0	0
CSW2-1% GBL	18.0±0.20	15.5±0.15	0	0
CSU1-C	13.0±0.16	12.0±0.06	15.0±0.22	12.5±0.6
CSU1-0.2% GBL	0	0	0	0
CSU1-0.5% GBL	0	14.0±0.04	0	0
CSU1-0.7%GBL	0	14.0±0.10	0	0
CSU1-1% GBL	0	13.5±0.25	0	0
CSU2-C	15.0±0.32	14.0±0.32	14.0±0.15	11.5±0.72
CSU2-0.2% GBL	0	0	0	0
CSU2-0.5% GBL	0	14.0±0.17	14.0±0.32	0
CSU2-0.7% GBL	0	14.0±0.76	13.5±0.25	0
CSU2-1% GBL	0	15.0±1.00	14.5±0.38	0
CSG1-C	15.0±0.23	20.0±0.36	20.0±0.25	15.0±0.32
CSG1-0.2% GBL	0	13.0±0.50	0	0
CSG1-0.5% GBL	0	22.0±0.25	0	17.0±0.5
CSG1-0.7% GBL	0	23.0±0.35	0	17.0±0.25
CSG1-1% GBL	0	18.0±0.58	0	11.0±0.42

Each isolate exhibited different inhibitory activities after induction against the tested organisms. All GBL induced *Streptomyces* isolates except for CSK1 have resulted in higher inhibitory activity against *S. aureus* compared to the monoculture (Figure 4.3 & 4.4). Same trend of higher inhibitory activity of the elicited culture was observed for CSK3 and CSW2 against *E. coli*, with the highest zone of inhibition observed with 0.7% induction of GBL with CSK3 at 23.5 mm and 18.0 mm for CSW2 induced with 1% GBL compared to the non-induced with a zone of inhibition of 14.0 mm (Figure 4.3). In addition, inhibitory activity of the isolates' elicited cultures ceased against *C. albicans* with the exception of isolate CSU2 which showed higher inhibitory effect when induced with 1% GBL (Figure 4.4B). The same effect of loss of inhibitory activity was also observed against *S. cerevisiae* except for isolate CSG1 which showed higher inhibitory activity when induced with 0.5% and 0.7% GBL (4.4C). Finally, CSK1-GBL cultivation failed to produce any inhibitory activity against all the tested organisms. The data is, also, represented as an increase or decrease in activity based on the measured zones of inhibitions compared to the control (non-induced isolate), where the control is taken as a baseline with a value of 0 against each of the tested organism (Figure 4.5). As shown in the figure, an increase in inhibitory effect was clearly observed of all the isolates' elicitations (based on all or one of the induced GBL concentrations) against *S. aureus*, CSK3 and CSW2 against *E. coli*, CSU2 against *C. albicans* and CSG1 against *S. cerevisiae*.

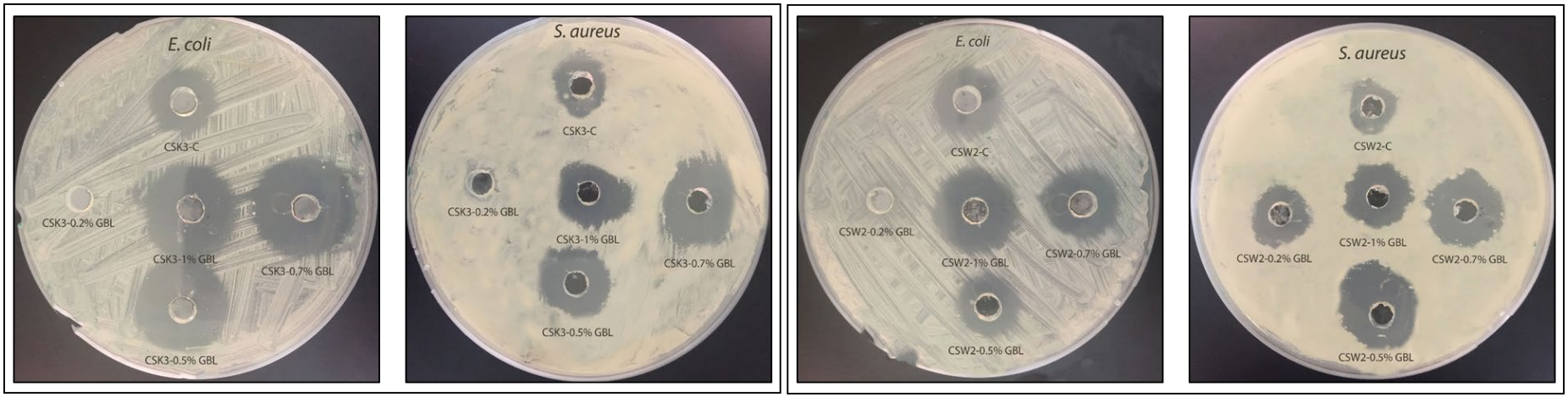


Figure 4.3 Induction of isolates CSK3 and CSW2 with γ-Butyrolactone (GBL)

Well diffusion method was used to assess inhibitory activity of isolates CSK3 and CSW2 induced with GBL at different concentrations (0.2, 0.5, 0.7 & 1%) against *E. coli* and *S. aureus*. Zones of inhibition, observed as clearance of growth, were measured. For CSK3, zones of inhibitions were observed in the uninduced and induced isolate except for CSK3 induced with 0.2% GBL (A). As for CSW2, zones of inhibition were observed for all tested samples except for CSW2-0.2% GBL against *E. coli*.

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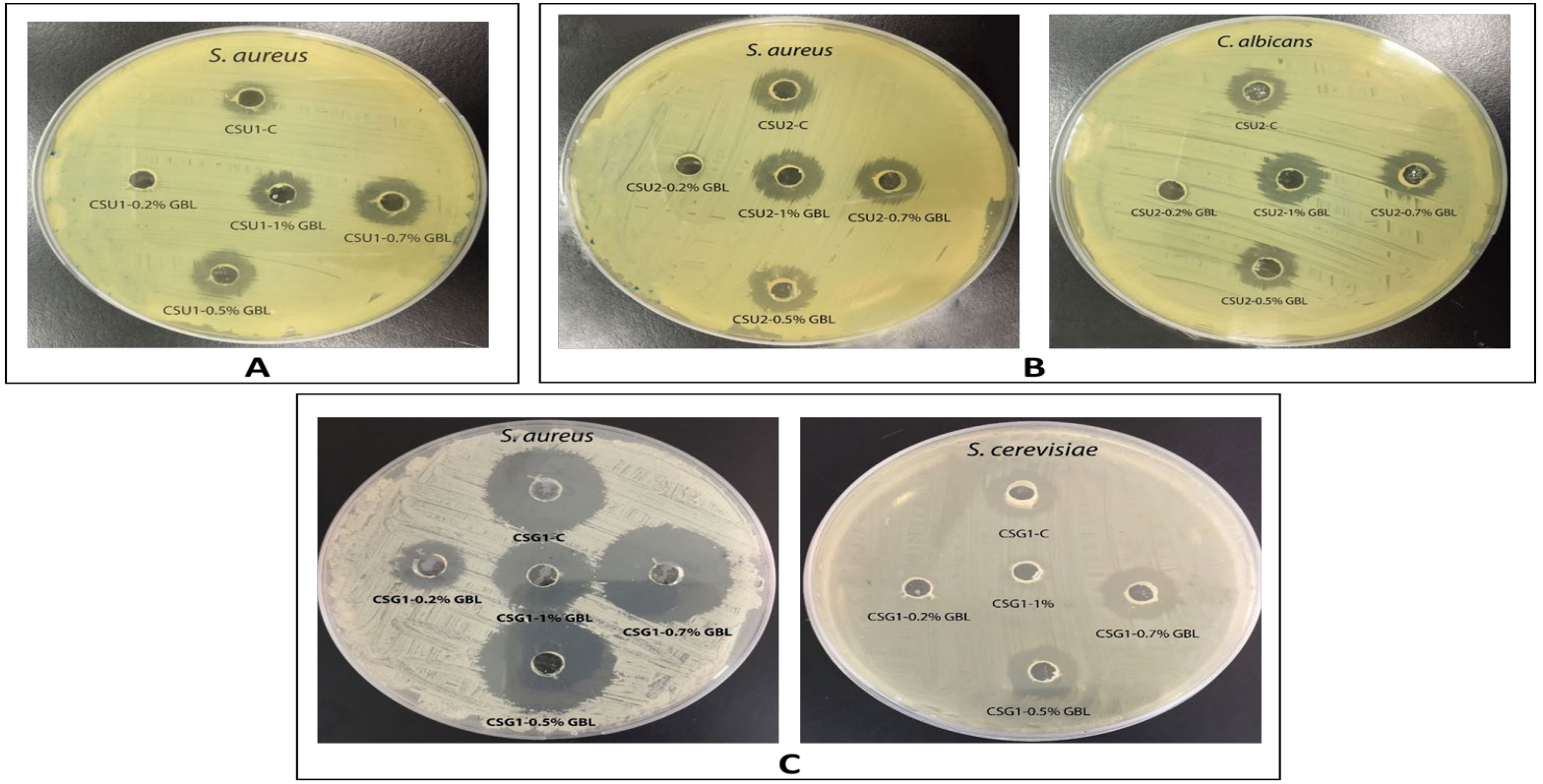
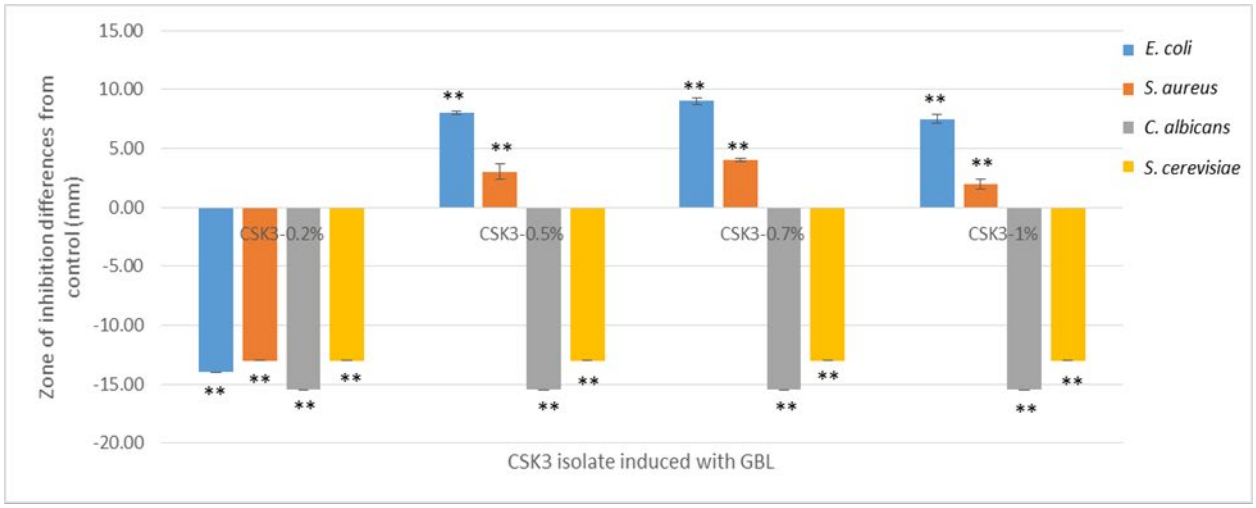
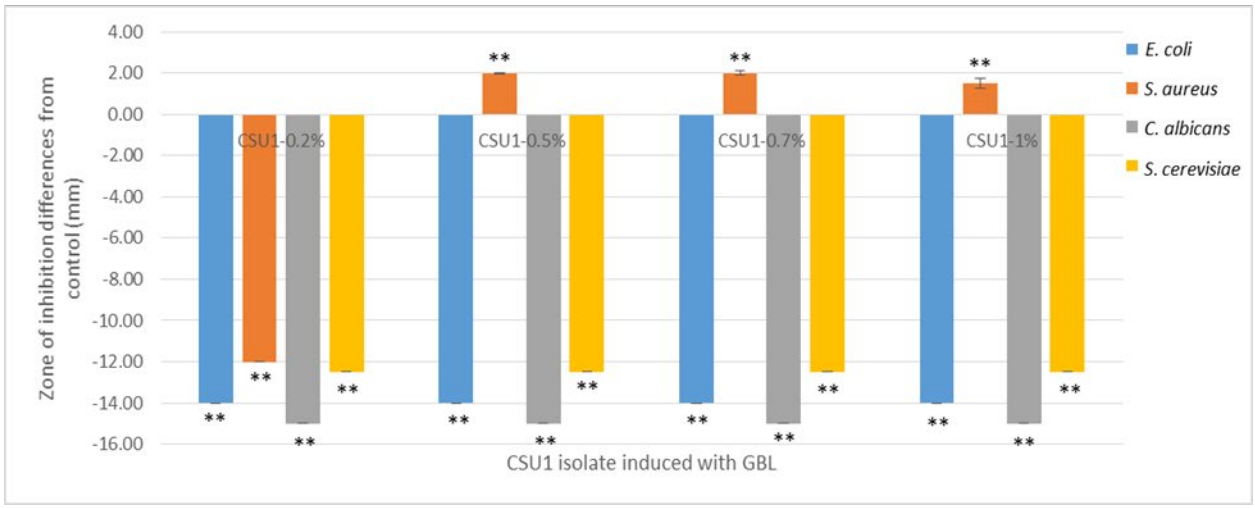


Figure 4.4 Induction of isolates CSU1, CSU2 and CSG1 with γ-Butyrolactone (GBL).

Isolates CSU1, CSU2 and CSG1 were induced with GBL at different concentrations (0.2, 0.5, 0.7 & 1%) and well diffusion method was used to assess inhibitory activity against *E. coli* and *S. aureus*. Zones of inhibition were observed for uninduced and induced CSU1 isolate against *S. aureus* except for CSU1-0.2% (A). The same trend was observed for CSU2 against *S. aureus* and *C. albicans* (B). Uninduced and induced CSG1 isolate caused inhibitory effect against *S. aureus* and *S. cerevisiae* (except foe CSG1-0.2% GBL against *S. aureus*).


15.00
10.00
5.00
0.00
-5.00
-10.00
-15.00
-20.00
Zone of inhibition differences from control (mm)
CSK3-0.2%
CSK3-0.5%
CSK3-0.7%
CSK3-1%
E. coli
S. aureus
C. albicans
S. cerevisiae
CSK3 isolate induced with GBL


10.00
5.00
0.00
-5.00
-10.00
-15.00
-20.00
Zone of inhibition differences from control (mm)
CSW2-0.2%
CSW2-0.5%
CSW2-0.7%
CSW2-1%
E. coli
S. aureus
C. albicans
S. cerevisiae
CSW2 isolate induced with GBL


4.00
2.00
0.00
-2.00
-4.00
-6.00
-8.00
-10.00
-12.00
-14.00
-16.00
Zone of inhibition differences from control (mm)
CSU1-0.2%
CSU1-0.5%
CSU1-0.7%
CSU1-1%
E. coli
S. aureus
C. albicans
S. cerevisiae
CSU1 isolate induced with GBL

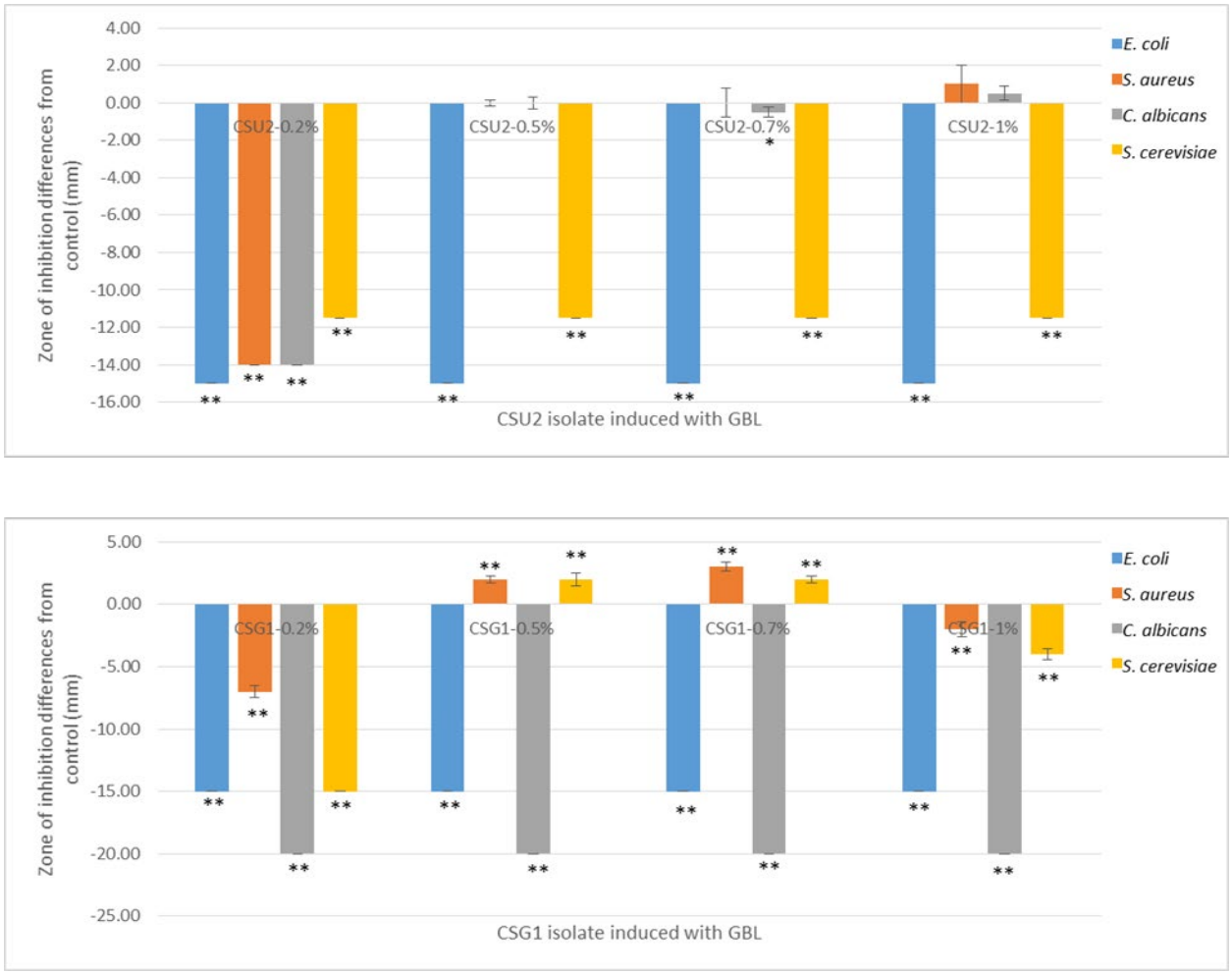


Figure 4.5 Differences in inhibitory activity of isolates induced with GBL

Main effect of GBL induction on the inhibitory activity of the isolates against *E. coli*, *S. aureus*, *C. albicans* and *S. cerevisiae* was generated based on the produced zones of inhibitions, and the zone of inhibition measurement for the non-induced isolate was taken as the baseline. An increase of inhibitory activity was observed for all isolates against S. aureus, for CSK3 and CSW2 against E. coli, for CSU2 against *C. albicans* and finally CSG1 against *S. cerevisiae*. The presented results are standard deviation for three independent experiment. T-test was used to analyze two sample comparison assuming equal variance (**P<0.005).

4.2.3. Cytotoxic Activity of *Streptomyces* isolates cultured with GBL

HeLa cells were treated with non-induced CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1, and isolates induced with 0.2, 0.5, 0.7 and 1 % GBL at two concentrations of 5 and 10 µg/µl of the extracts to assess cell viability using the MTT assay (Figure 4.6). After 24 hrs of treatment, isolate CSK1 induced with 0.2% GBL showed significant decrease in cell viability for both 5 and 10 µg/µl concentrations although the control had no effect on viability. Induced CSK3 showed not cytotoxic activity on both concentrations of the extracts compared to the non-induced which showed significant cytotoxic effect for the concentrations. Isolate CSW2 induced with 0.2, 0.5, 0.7 and 1 % GBL showed significant decrease in cell viability at concentration of 10 µg/µl only compared to the non-induced where a decrease in cell viability was observed for both 5 and 10 µg/µl concentrations. A decrease in viability was observed for HeLa cells treated with non-induced CSU1 at 5 and 10 µg/µl extract concentration and when the cells were treated with 10 µg/µl extracts induced with 0.2 and 0.7 % GBL. Viability of HeLa cells was also decreased when they were treated with CSG1 induced with 0.5 % of GBL at extract concentration of 10 µg/µl. Induced CSK3 and CSU2 showed no effect on the viability of the HeLa cells. The same trend of results was observed for the non-induced isolates when they were previously assessed by MTT assay (Figure 3.8).

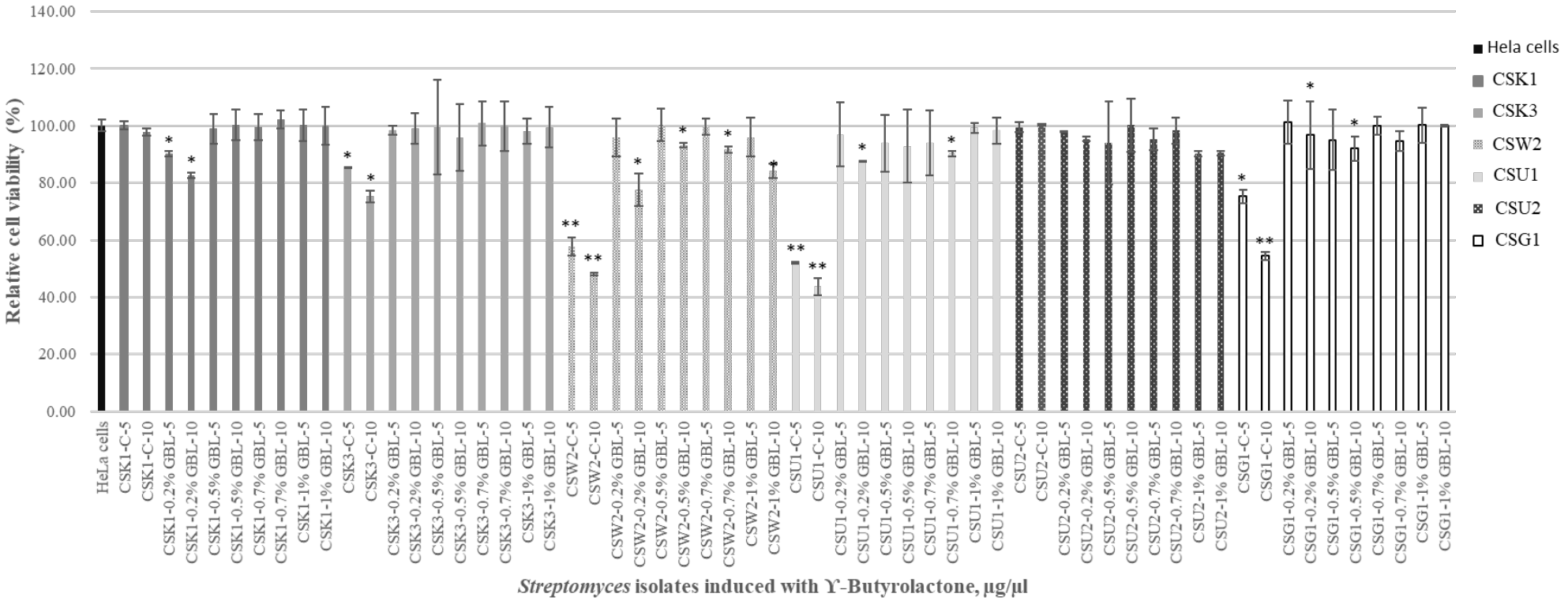


Figure 4.6 Cytotoxic activity of *Streptomyces* isolates induced with GBL against HeLa cells.

Cell viability of HeLa cells was measured using MTT assay. The graph represents cytotoxic effect of the isolates (CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1) induced with different concentrations (0.2, 0.5, 0.7 and 1%) of Gamma Butyrolactone compared to the non-induced isolate. The Hela cells were treated with 5 and 10 µg/µl of the non-induced and induced isolates for 24 hrs. Data is expressed as means of standard deviation (n=2) and analysed using T-test two sample comparison assuming equal variance (*P<0.05 and **P<0.005).

4.2.4. Effect of *N*-acetylglucosamine (GlcNAc) on *Streptomyces* isolates

Streptomyces isolates (CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1) were cultured with 50 mM of *N*-acetylglucosamine (GlcNAc) as a signalling molecule to assess changes in the inhibitory effect and cultural changes. After 7 days of culturing, GlcNAc had a stimulating effect on CSK1 antibiotic production where a zone of inhibition of 16.5 mm and 17 mm was observed against *S. aureus* and *B. subtilis* respectively (Figure 4.7-A) compared to no activity of the non-induced isolate as expected (*Streptomyces* isolates usually show activity after 14-21 days of culturing). Furthermore, red-pigmented culture was observed after 14 days of culturing (Figure 4.7-B) compared to no colour change in the monoculture.

It is worth mentioning that the physical characteristics of the colonies on the agar plate change to smaller in size and lose their chalky and powdery appearance when isolates are induced with GlcNAc, this effect is shown in Figure 4.7-C for CSK1 isolate. The same effect is also observed of the mycelium in broth culture changing from big in size of the non-induced to smaller mycelium size of the induced isolates. This change was consistent for all of the isolates.

The red colour produced from CSK1 cultivated with *B. subtilis* and induction with GlcNAc may imply the production of Prodigiosins (PGs), a family of red pigmented secondary metabolites that are produced by *Streptomyce*s and other bacteria and found to have anticancer, antimalarial and antimicrobial activity (Fürstner, 2003). Therefore, cytotoxicity of the ethyl acetate extracts of CSK1 cultivated with *B. subtilis* and induced with GlcNAc was assessed by MTT assay. Figure 4.8 clearly shows the antiproliferative activity of the induced CSK1 isolate compared to uninduced with a statistically significant decrease with the extracts at concentration of 20 µg/µl.

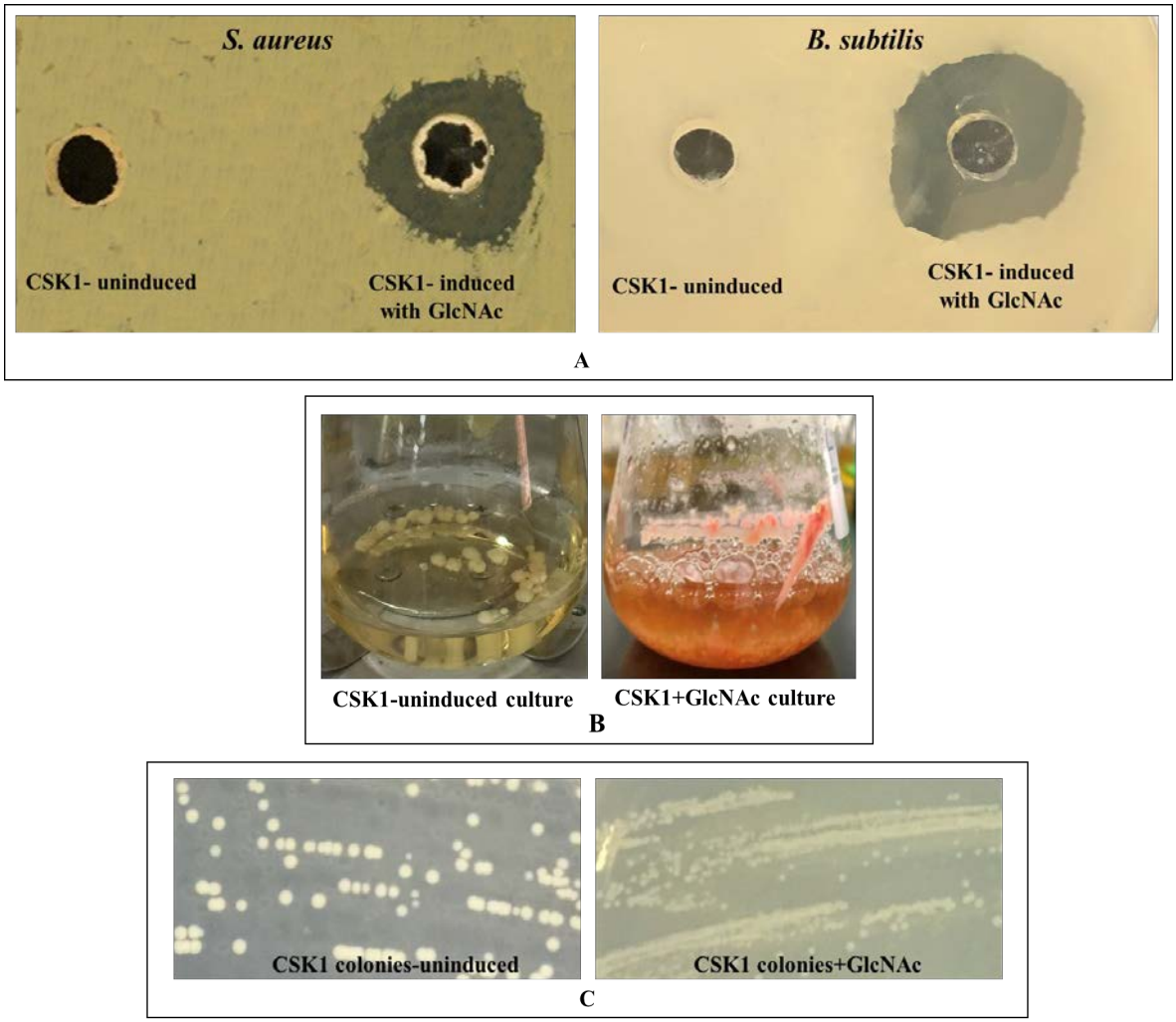


Figure 4.7 CSK1 isolate induced with GlcNAc

Inhibitory activity of CSK1 induced with 50 mM of GlcNAc resulted in a clear zone of inhibition of against *S. aureus* and *B. subtilis* after 7 days of culturing compared to uninduced which showed activity after 14 days of culturing (A). The CSK1-GlcNAc culture changed to red colour after 14 days of culturing compared to no change in colour of the uninduced isolate (B). In addition, colony morphology has changed from white and chalky appearance of the uninduced to colourless and smaller in size colonies of the CSK1 colonies induced with GlcNAc.

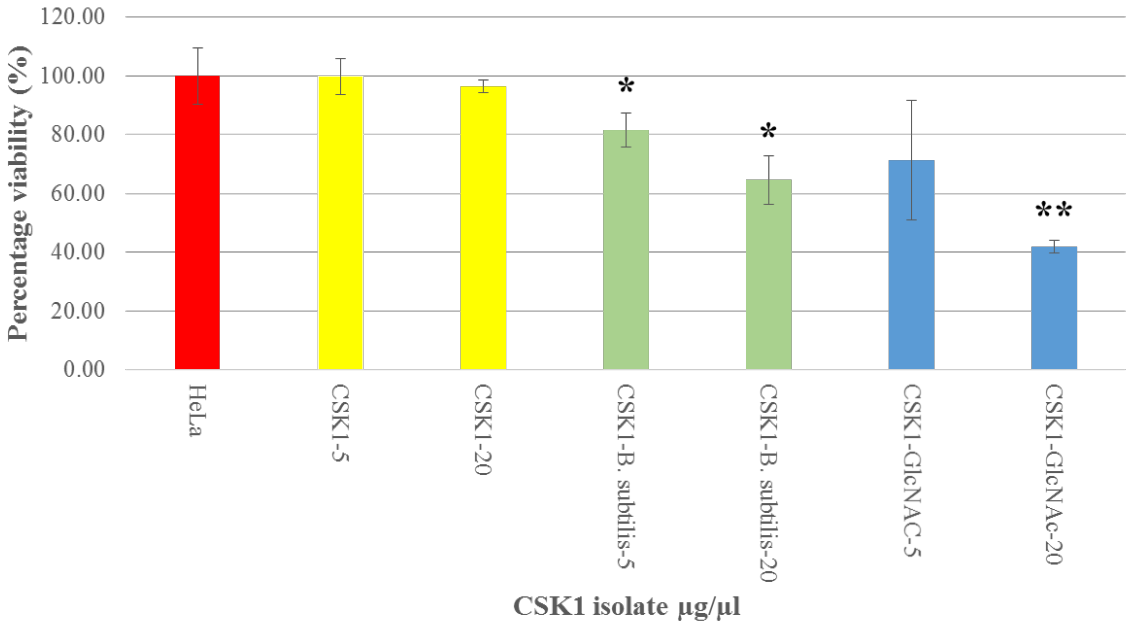


Figure 4.8 Effect of induced CSK1 isolate on HeLa cells

HeLa cells were treated with uninduced CSK1, CSK1 cultivated with *B. subtilis* lysate and CSK1 induced with 50 mM GlcNAc at 5 and 20 µg/µl concentrations of the ethyl acetate extracts. Antiproliferative effect of the treatments was assessed by MTT assay. A decrease in the viability of HeLa cells was observed in the elicited culture and induced extracts compared to the treatment of the uninduced extract and non-treated HeLa cells. The presented results are means of standard deviation for two independent experiment. Data was normalized to the mean, T-test was used to analyze two sample comparison assuming equal variance (*P<0.05 and **P<0.005).

4.3. Discussion

The isolated *Streptomyces* strains are terrestrial Gram positive microorganisms. They exist in natural soil habitat that is rich with other microbes. This co-existence usually results in competition for survival triggered by the depletion in nutrients which leads to the production of secondary metabolites. Growing *Streptomyces* in vitro may result in inactivating or silencing the production of these secondary metabolites. Genome sequencing results have shown that *Streptomyces* contain many inactive biosynthetic gene clusters (Bentley et al., 2002; Nett et al., 2009) may be due to the in vitro cultural conditions which lacks the inter and intra-species communication mediated by the transfer of small signalling molecules between species. One way of eliciting or stimulating the expression of silent biosynthetic genes or increase the production of secondary metabolites is co-culturing.

Many studies showed that co-culturing organisms together can lead to the effect of one organism affecting secondary metabolism in the second organism. *B. subtilis* is a Gram positive bacterium that is commonly found in soil and it has been identified in plant rhizosphere (Val et al., 2009). Since the *Streptomyces* strains in this study were isolated from cultivated soil in farms then most probably the two bacteria co-existed in nature. *B. subtilis* cell lysate was used in this study as a strategy to study elicitation where 3% of the culture was from *Streptomyces* isolates and 0.3% from *B. subtilis*. We have showed that cultivating the two organisms resulted in a red pigmentation (colour change) in the fermentation medium of CSK1 isolate after 14 days of culturing which was not observed in the cultivation of the monoculture. In addition, cytotoxicity in Hela cells was induced when they were treated with the elicited extract compared to the monoculture extract. This result may suggest the production of undecylprodigiosin (RED), an antimicrobial antibiotic produced by *Streptomyces coelicolor* (White and Bibb, 1997) that also has a cytotoxic effect on breast cancer cells (Ho et al., 2007). Studies have shown that co-culturing *S. coelicolor* with *B. subtilis* increased the production of RED (Luti and Mavituna, 2011) in *S. coelicolor*. In addition, inhibitory activity of the CSK1 isolate against *S. aureus* and *B. subtilis* was observed after 6 days of the elicitation compared to inhibitory activity between 14-21 days for the monoculture. The cultivation of CSK1 with *B. subtilis* may indicate the activation of silent biosynthetic genes and production of new secondary metabolites as the antimicrobial activity was observed about 8-15 days earlier than usual. Furthermore, RED is probably one of the produced secondary metabolites in the co-culture indicated by the change of culture to red and induction of cytotoxicity on HeLa cells.

Another approach to activate the silent biosynthetic gene clusters is cultivating the isolates with ϒ-Butyrolactone (GBL). GBL is produced by most *Streptomyces* (Horinouchi and Beppu, 1992) and appear to be mainly involved in production of secondary metabolites (Horinouchi and Beppu, 1994). GBL are signalling molecules that are also known as autoregulators, they bind to structural receptors that are mainly involved in the expression of biosynthetic clusters. The results show that there was significant increase of inhibitory activity for CSK3 and CSW2 isolates when induced with different concentrations of GBL against *E. coli* and *S. aureus* compared to the control (the monoculture). The same increased effect was also observed for CG1 against *S. aureus* and *S. cerevisiae*. This increased activity could be due to the diffusion of the GBL through the cell membrane and regulating the expression of the biosynthetic genes and production of secondary metabolites by either activating or suppressing genes that encode for enzymes involved in secondary metabolism (Niu et al., 2016).

Cytotoxicity effect of isolates induced with GBL on HeLa cells was examined. The results revealed that CSK1 isolate induced with 0.2% GBL had a toxic effect on the breast cancer cells compared to the non-toxic effect of CSK1 isolate alone. In addition, there was no increased cytotoxicity effect of GBL induced CSK3, CSW2, CSU1 and CSG1 isolates; on the contrary, there was a decrease in toxicity of the induced isolates on the HeLa cells. These results may imply that GBL has induced activation of silent biosynthetic clusters with antimicrobial and not anti-tumour properties or turned off repressor genes.

Studies have shown that high concentrations of GlcNAc keeps *S. coelicolor* in the vegetative state and that progression to sporulation happens only when the environmental conditions changes such as a decrease in GlcNAc (Rigali et al., 2006). Rigali group has also shown that GlcNAc inhibits antibiotic production in highly nutritive media but it activates red-pigmented antibiotic undecylprodigiosin (Red) production in poor nutritive media in *S. coelicolor* (Rigali et al., 2008; Chater, 2001) bld genes which are produced at the beginning of aerial development with a loss of the aerial mycelium resulting in a “bald” phenotype. In this study, the culturing of ISP1 medium with 50 mM GlcNAc resulted in antimicrobial inhibition of all the isolates except for CSK1 which could be due to keeping the organism in the vegetative state and inhibiting antibiotic production. In addition, induced isolates showed colonies with a change of phenotype from chalky and powdery appearance due to the formation of aerial hyphae to small size colonies with “bald” appearance due to the lack of aerial hyphal formation. To the contrary from the other isolates, CSK1 isolate has resulted in early activity (after 7 days only) against *S. aureus* and *B. subtilis* and a red colour change in the induced culture which could be an indication for the production of RED. CSK1-GlcNAc extract

resulted in reduction of proliferation in HeLa cells and this could be due to the production of RED in the elicited culture. In order to have a marked effect of GlcNAc on the isolated *Streptomyces* strains the culturing media needs to be further examined and to see the difference between nutrient rich and poor media on the production of RED in the elicited cultivation.

Chapter 5

Genomic Sequencing &

Liquid Extraction Surface Analysis

5. Genomic Sequencing & Liquid Extraction Surface Analysis (LESA)

5.1. Introduction

Genome analysis of bacteria have revealed that BGCs are clustered together and that they possess cryptic or silent secondary metabolites that had not been reported before (Corre and Challis, 2009). These silent BGCs are of interest to us as they may have inhibitory effect and may be potential to the discovery of natural products. Also, these BGCs are of interest because they possess genes that code for the modular enzymes PKS and NRPS which are involved in the production of secondary metabolites (Walsh and Fischbach, 2010). In addition, techniques such as HPLC and TLC have been used to identify BGCs, in this project a novel technique known as Liquid Extraction Surface Analysis (LESA) based on liquid microinjection combined with Mass Spectrometry (MS) will be used to identify peptides.

Genome sequencing of each isolate was performed by using Illumina MiSeq platform and BGCs were identified by antiSMASH and Prism software. Then, Liquid Extraction of Surface Analysis was used to assess the differences between the mono-cultured strains and isolates that were cultured with GBL and GLcNAc. The data from the two systems were correlated to identify the BGCs that were common in both systems.

5.1.1. Genomic Sequencing

All the genes responsible for enzymes encoding secondary metabolism pathway are clustered together in a narrow locus known as biosynthetic gene cluster (BGC) (Cimermancic et al., 2014). Sequencing and metagenomics will allow us to discover a consortium of novel BGCs (Medema et al., 2015) that are capable of producing a variety of natural products that could be missed in the classical methods of fermentation and crude extraction (Winter et al., 2011).

One of the main breakthroughs in the identification of natural products is linking classical isolation and analytical analysis with genome mining. Genomic sequencing results have shown that *Streptomyces sp.* have larger genomes than other bacteria and about 5-10% of it is used for the production of silent secondary metabolites (Baltz, 2008). One very promising approach that can be used to identify these silent BGCs is to use genomic sequencing data to identify BGCs and compare them to known clusters based on pathway types such as PKS I,

PKS II, NRPS or RiPP, chemical structure classes, domain structure and conserved motifs of the synthesized natural product (Ward and Allenby, 2018).

Many bioinformatics tools are now available for genomic identification of secondary metabolite biosynthetic gene clusters. “Antibiotics and Secondary Metabolite Analysis Shell” antiSMASH and Prism are the most common and comprehensive bioinformatics programs that can identify and annotate biosynthetic gene clusters (Niu and Tan, 2013). About 1346 BGCs have been detected by antiSMASH; some of the products are conserved among *Streptomyces* such as ectoine, hopene, siderophores, spore pigment, geosmin, butyrolactones and melanin.

Whole genome annotation and comparative genomic analyses was performed on the seven *Streptomyces* strains including the control strain (CSM1). Several biosynthetic gene clusters were identified and other putative secondary metabolites were not identified. Phylogenetic analysis revealed the identification of novel *Streptomyces* isolates producing natural products that can be beneficial to the pharmaceutical industry.

5.1.2. Liquid Extraction Surface Analysis (LESA)

Environmental changes in the cultivation of *Streptomyces* such as composition of the media, pH, temperature and oxygen supply can lead to the production of various profiles of secondary metabolites for the same organism (Bode et al., 2002). More complex changes involving signalling molecules, communication and predators can also lead to the production of other secondary metabolites (Plaga et al., 1998). Typically, isolation of antibiotics involves solvent extraction, chromatographic separation and then using mass spectrometry for structure elucidation which is time consuming and does not always work as sometimes the spectra is too complicated and a relevant signal may be missed. Liquid extraction surface analysis (LESA) using Triversa Nanomate technology is a novel technique which is based on liquid microinjection method combined with high resolution mass spectrometry. LESA has been used to detect lipids, proteins and small molecules from a variety of surfaces such as plants, blood spots, tissue sections and food surfaces (Kai et al., 2012).The extraction system in LESA involves applying a droplet of solvent onto the surface via a robotic pipette tip, the droplet is kept for few seconds to form a liquid microinjection to allow the dispersion of the analyte molecules to the solvent. The sample is then transferred to the Orbitrap-elite mass spectrometer through the ionization of the analytes by a nano electrospray as explained previously in section 1.7 and shown in Figure 1.9.

Strains grown on ISP1 broth produced antimicrobial and anticancer inhibitory agents as shown in chapter 3 of this thesis. Therefore, liquid extraction surface analysis (LESA) was utilized to distinguish and identify metabolites' profiles directly from the bacterial colonies of the seven *Streptomyces* strains on ISP1 medium (un-induced), ISP1 medium induced with GBL and ISP1 medium induced with GlcNAc.

5.2. Results

5.2.1. Genomic Sequencing and Annotation

The isolated *Streptomyces* strains (CK1, CSK3, CSW2, CSU1, CSU2, CSG1 and CSM1) were sequenced using Illumina HiSeq at the microbesNG sequencing center of the University of Birmingham. A summary of the main principle features of the sequencing data is summarized in table 5.1 supplemented by the Microbe NG centre. The genome size of the strains ranged between 7.2 Mb to 7.7 Mb and GC content of all the strains was more than 72%, these assembly metrics were calculated by QUAST (quality assessment tool for genome assemblies; Gurevich et al., 2013). Taxonomic distribution of the isolates was calculated by Kraken (taxonomic sequence classification system), by assigning taxonomic labels to DNA sequences with high sensitivity and speed. Kraken has a database that contains *k*-mer records and the lowest common ancestor (LCA) of all organisms containing *k*-mers in their genomes. Classification is based on searching the database for each *k*-mer in a sequence. The LCA taxa generated was used to assign a suitable label for the sequence. Sequences with no *k*-mers in the databases are labelled “unclassified” which reduces false positive identification. Kraken database is built with 31 *k*-mers. Kraken has high sensitivity to identify the genus but sensitivity drops tremendously when the species are not known this is because Kraken relies on matches of long *k*-mers. Therefore, with novel organisms Kraken can accurately classify them at the genus level but not the species. Hence, the sequencing data identified the strains to belong to the *Streptomyces* genus with percentage similarity ranging between 56 to 62 % and very low contamination of 0.3 and 0.36 % with other genus. All of the strains had 12-13 % similarity with *S. coelicolor* species except for CSM1 (the control strain in the study) had 13.82 % similarity with *S. davawensis*. Number of coding sequences and RNAs for each isolate was estimated by RAST (Rapid Annotation using Subsystem Technology, Aziz et al., 2008).

Table 5. 1 Genome features of the *Streptomyces* isolates

Assembly metrics data generated using QUAST for # of contigs, total length and GC content parameters of the *Streptomyces* isolates sequenced by Illumina. As for the taxonomic distribution which shows the top genera that the reads map to was calculated by Kraken software. Annotation of the result was provided by the Microbes NG center at the University of Birmingham. The Number of coding sequences and RNAs were estimated by RAST system.

Streptomyces strain	# of contigs	Total length (bp)	GC content (%)	Streptomyces genus similarity (%)	Streptomyces species similarity (name & %)	# of coding sequences	# of RNAs
CSK1	534	7,367,906	72.25	61.86	coelicolor, 13.55	6997	73
CSK3	740	7,429,274	72.30	56.20	coelicolor, 12.33	7167	73
CSW2	874	7,296,860	72.34	57.54	coelicolor, 12.34	7038	74
CSU1	597	7,443,916	72.16	56.32	coelicolor, 12.32	7507	74
CSU2	843	7,238,480	72.31	59.10	coelicolor, 12.92	6904	72
CSG1	578	7,366,039	72.83	62.91	coelicolor, 13.84	7127	75
CSM1	580	7,728,272	72.20	65.40	davawensis, 13.82	7454	75

The genomes of the isolated strains were annotated using PROKKA (Seemann, 2014) for prokaryotic genome annotation to predict the main features of the generated sequences which will be publicly available upon request. 16S rRNA was extracted from the annotated data and phylogenetic tree was constructed based on evolutionary relationship of the isolates with other species of *Streptomyces* deposited at the National Centre for Biotechnology Information (NCBI) using the Mega X software (Figure 5.1). The phylogenetic analysis showed that the isolates shared distinct, but related, phyletic lines with other *Streptomyces* species. CSK1, CSW2, CSU2 and CSG1 were closely clustered together and to *Streptomyces sp.* E2N17. Isolates CSK3 and CSU1 showed close relationship in the phylogenetic dendrogram to each other and to *Streptomyces sp.* ETH927. CSM1 which is the negative strain in this project was closely related to CSK3 and CSU1 and to *S. albogriseolus*. The complete genomic sequencing data of each isolate when blasted to NCBI has revealed a 99 to 97%

identity with *Streptomyces sp.* ETH927 and *Streptomyces sp.* 4F with a Query coverage of 87% to 83%. The percentage identity then decreases to below 80% for the rest of the blasted *Streptomyces* species (Appendix B).

RAST system was used to identify the open reading frames where the genes are classed into subsystems (based on gene and RNA sequence databases) (Table 5.2). The number of genes for certain subsystems was identified for each isolate, the most observed pattern deduced from the table is that in general, with few exceptions, the highest number of genes for each subsystem belonged to CSM1 which is the negative strain in the project followed by CSU1 isolate. The highest number of genes are found in the "Amino Acids and Derivatives" and "Carbohydrates" subsystems followed by "Cofactors, Vitamins, Prosthetic Groups, Pigments" and "Protein Metabolism" subsystems. The lowest number of genes are found in "Phages, Prophages, Transposable elements, Plasmids", "Motility and Chemotaxis" and "Secondary metabolism" subsystems. RAST identified 8 genes for CSU1 belonging to "Secondary Metabolism" followed by 7 genes for CSM1, 5 genes for CSK3, 2 genes for CSK1 and 1 gene for CSU2. KEGG metabolic pathways of the isolates was analyzed by RAST in comparison with *S. coelicolor* (most studied species in the *Streptomyces* genus) and to most identical species *Streptomyces sp.* ETH9427, as identified by complete genome BLAST (Table 5.3). CSU1 had the highest percentage of annotated proteins involved in various metabolic pathways alongside *S. coelicolor* followed by CSG1, CSK1, CSK3 and CSW2 with the lowest percentage correlation found with CSU1.

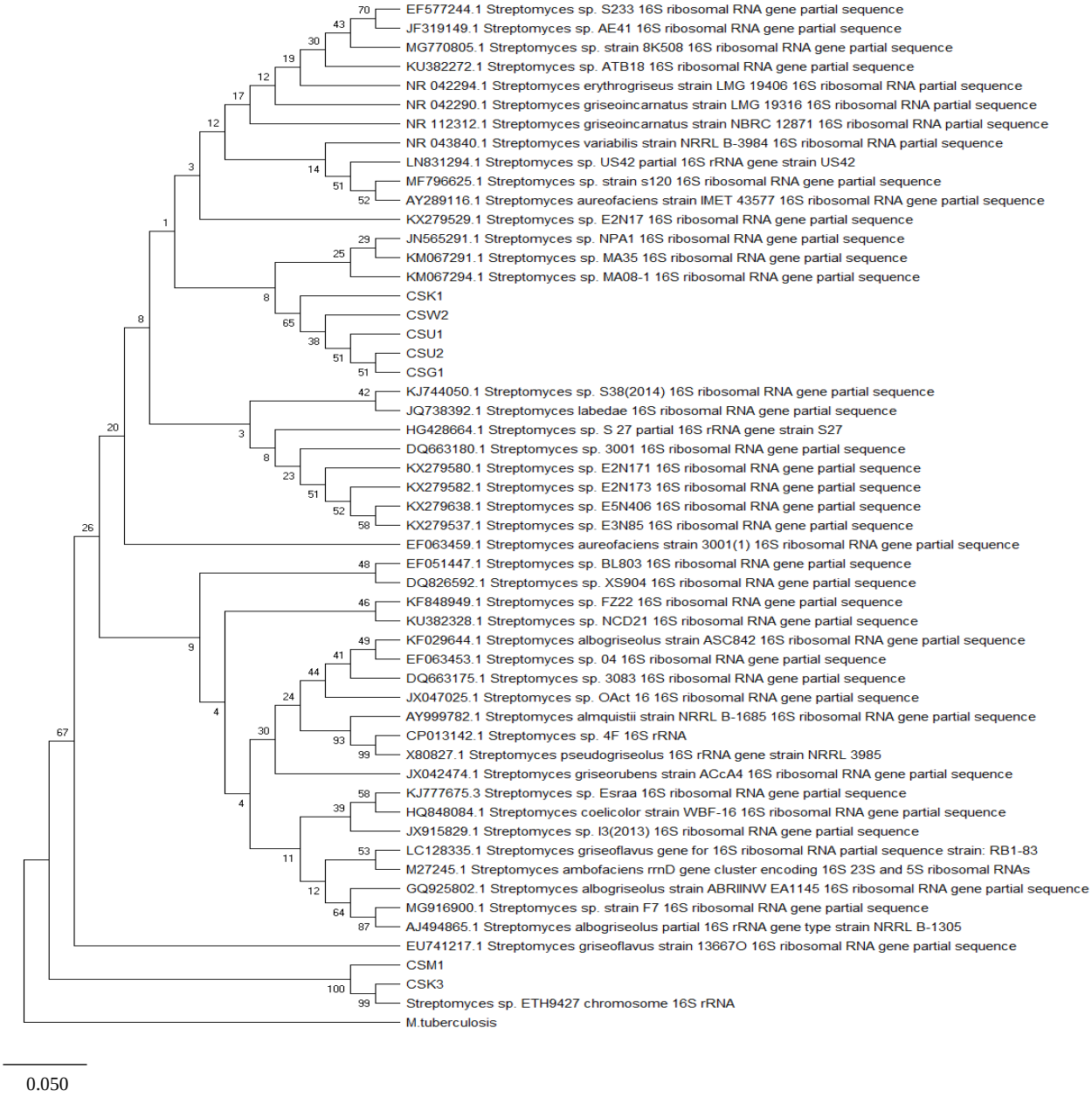


Figure 5. 1 Phylogenetic tree constructed by neighbor-joining method

The evolutionary tree of the seven isolates' 16S rRNA gene sequences to other *Streptomyces* specie was constructed. The tree shows the distant relationship between the CSM1 (negative control isolate in the project) and the rest of the isolates. Isolate CSK3 showed to be closely related to *Streptomyces sp.* ETH927. All the other isolates (CSK1, CSW2, CSG1 and CSU2) shared close distances with each other and to *Streptomyces sp*. E2N17. The phylogenetic tree of the 16S rRNA for the Bootstrap values based on 500 replicates are shown next to the branches. Jukes-Cantor method was used to calculate the evolutionary distances which is based on the number of base substitutions per site represented by 5% nucleotide sequence difference (scale bar). Evolutionary analysis was performed using MEGA X. The tree is rooted to *M. tuberculosis* as an outgroup.

Table 5. 2: Classification of isolates by RAST system

The genes for each isolate were grouped into subsystems where the highest number of genes for a specific subsystem is denoted by green and the lowest by red. *Streptomyces sp.* ETH9427 was included as it has the highest identity to all isolates when blasted to NCBI but it has the lowest number of genes for each subsystem. *S. coelicolor* is the most characterized *Streptomyces* isolate and has the highest number of genes as shown in the table. As for the *Streptomyces* isolates, the highest number of genes for each subsystem was observed for isolate CSM1 followed by CSU1 and the lowest number of genes was observed for isolate CSU2.

RAST Subsystems	CSK1	CSK3	CSW2	CSU1	CSU2	CSG1	CSM1	*Streptomyces sp.* ETH9427	*Streptomyces coelicolor*
Cofactors, Vitamins, Prosthetic Groups, Pigments	217	215	220	245	212	213	221	203	382
Cell Wall and Capsule	36	35	36	43	37	37	57	30	110
Virulence, Disease and Defense	55	59	54	77	57	56	64	55	87
Potassium metabolism	9	8	10	9	9	10	9	10	24
Miscellaneous	29	31	28	31	31	29	39	27	55
Phages, Prophages, Transposable elements, Plasmids	2	3	2	2	2	2	4	2	0
Membrane Transport	83	77	79	101	77	80	74	76	72
Iron acquisition and metabolism	34	34	33	27	27	32	40	30	43
RNA Metabolism	57	57	59	60	55	58	57	53	142
Nucleosides and Nucleotides	107	109	104	129	113	108	111	106	149
Protein Metabolism	262	267	262	267	253	265	275	251	244
Motility and Chemotaxis	2	0	0	0	2	0	0	0	1
Regulation and Cell signaling	38	34	38	47	37	38	27	32	84
Secondary Metabolism	2	5	0	8	1	0	7	4	17
DNA Metabolism	107	105	107	106	101	106	106	90	132
Fatty Acids, Lipids, and Isoprenoids	161	162	160	207	166	166	207	114	258
Nitrogen Metabolism	21	21	20	25	20	22	27	13	45
Dormancy and Sporulation	11	12	11	12	12	11	16	12	12
Respiration	132	124	128	144	122	127	129	124	147
Stress Response	80	69	80	81	76	76	89	74	132
Metabolism of Aromatic Compounds	23	21	23	35	25	23	55	21	42
Amino Acids and Derivatives	415	408	413	498	413	430	450	412	484
Sulfur Metabolism	9	10	9	20	9	9	28	9	35
Phosphorus Metabolism	33	36	33	43	35	35	43	37	45
Carbohydrates	394	371	383	450	397	374	393	326	629

Table 5. 3 KEGG pathway comparisons of the *Streptomyces* isolates

KEGG metabolic analysis of the isolates in comparison with published *Streptomyces* species was executed by using RAST system. EC number is used to link genomes to metabolic pathways, each enzyme is identified by an EC number that is organism specific. The percentage of ECs for a specific pathway is represented in the table for each isolate in comparison with *Streptomyces sp.* ETH9427 and *S. coelicolor*.

KEGG map	Distinct ECs	CSK1	CSK3	CSW2	CSU1	CSU2	CSG1	CSM1	*Streptomyces sp.* ETH9427	*S. coelicolor*
Amino sugar and nucleotide sugar metabolism	94	39.4%	38.3%	39.4%	38.3%	38.3%	37.2%	39.4%	37.2%	39.4%
Anthocyanin biosynthesis	9	11.1%	11.1%	11.1%	22.2%	11.1%	11.1%	11.1%	11.1%	33.3%
beta-Lactam resistance	1	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Biosynthesis of alkaloids derived from terpenoid and polyketide	62	51.6%	51.6%	51.6%	51.6%	50.0%	51.6%	51.6%	50.0%	51.6%
Biosynthesis of ansamycins	2	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Biosynthesis of siderophore group nonribosomal peptides	7	28.6%	42.9%	28.6%	28.6%	14.3%	42.9%	42.9%	28.6%	42.9%
Biosynthesis of terpenoids and steroids	108	36.1%	36.1%	36.1%	36.1%	35.2%	37.0%	36.1%	34.3%	33.3%
Biosynthesis of type II polyketide backbone	3	33.3%	33.3%	33.3%	33.3%	33.3%	33.3%	33.3%	33.3%	66.7%
Biosynthesis of type II polyketide products	2	50.0%	50.0%	50.0%	50.0%	50.0%	50.0%	50.0%	50.0%	50.0%
Biosynthesis of unsaturated fatty acids	15	53.3%	53.3%	53.3%	53.3%	53.3%	53.3%	53.3%	53.3%	66.4%
Biosynthesis of vancomycin group antibiotics	1	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	100.0%
Citrate cycle (TCA cycle)	22	68.2%	68.2%	68.2%	63.6%	63.6%	63.6%	68.2%	68.2%	63.6%

Table 5.3 continued

KEGG map	Distinct ECs	CSK1	CSK3	CSW2	CSU1	CSU2	CSG1	CSM1	*Streptomyces sp.* ETH9427	*S. coelicolor*
Drug metabolism - cytochrome P450	9	11.1%	11.1%	11.1%	22.2%	11.1%	22.2%	11.1%	11.1%	22.2%
Drug metabolism - other enzymes	22	40.9%	40.9%	40.9%	45.5%	40.9%	40.9%	40.9%	40.9%	36.4%
Fatty acid biosynthesis	21	47.6%	47.6%	47.6%	52.4%	47.6%	47.6%	47.6%	47.6%	38.1%
Fatty acid metabolism	29	34.5%	27.6%	27.6%	34.5%	27.6%	31.0%	27.6%	27.6%	27.6%
Flavonoid biosynthesis	23	8.7%	8.7%	8.7%	13.0%	8.7%	4.3%	8.7%	4.3%	13.0%
Fructose and mannose metabolism	65	27.7%	27.7%	26.2%	29.2%	27.7%	27.7%	26.2%	27.7%	21.5%
Galactose metabolism	37	37.8%	40.5%	37.8%	40.5%	40.5%	37.8%	43.2%	37.8%	35.1%
Glycolysis / Gluconeogenesis	41	51.2%	51.2%	51.2%	53.7%	51.2%	51.2%	51.2%	51.2%	48.8%
Isoflavonoid biosynthesis	18	5.6%	5.6%	5.6%	5.6%	5.6%	5.6%	5.6%	0.0%	0.0%
Lipopolysaccharide biosynthesis	22	31.8%	31.8%	22.7%	22.7%	27.3%	27.3%	27.3%	31.8%	4.5%
Metabolism of xenobiotics by cytochrome P450	7	14.3%	14.3%	14.3%	42.9%	14.3%	42.9%	14.3%	14.3%	28.6%
Naphthalene and anthracene degradation	22	4.5%	4.5%	4.5%	9.1%	4.5%	9.1%	4.5%	4.5%	27.3%
Novobiocin biosynthesis	6	66.7%	66.7%	66.7%	66.7%	66.7%	66.7%	66.7%	66.7%	66.7%
Penicillin and cephalosporin biosynthesis	14	21.4%	21.4%	28.6%	21.4%	21.4%	21.4%	21.4%	28.6%	28.6%

Table 5.3 continued

KEGG map	Distinct ECs	CSK1	CSK3	CSW2	CSU1	CSU2	CSG1	CSM1	*Streptomyces sp.* ETH9427	*S. coelicolor*
Peptidoglycan biosynthesis	18	66.7%	66.7%	66.7%	66.7%	66.7%	66.7%	66.7%	66.7%	77.8%
Polyketide sugar unit biosynthesis	8	25.0%	25.0%	25.0%	25.0%	25.0%	62.5%	25.0%	25.0%	37.5%
Puromycin biosynthesis	5	20.0%	20.0%	20.0%	0.0%	0.0%	20.0%	20.0%	20.0%	40.0%
Pyruvate metabolism	64	43.8%	43.8%	43.8%	43.8%	42.2%	42.2%	43.8%	43.8%	39.1%
Starch and sucrose metabolism	71	35.2%	35.2%	35.2%	35.2%	35.2%	33.8%	35.2%	33.8%	32.4%
Streptomycin biosynthesis	18	38.9%	38.9%	38.9%	38.9%	38.9%	50.0%	38.9%	38.9%	38.9%
Terpenoid backbone biosynthesis	27	48.1%	48.1%	48.1%	48.1%	44.4%	48.1%	48.1%	44.4%	59.3%
Tetracycline biosynthesis	3	33.3%	33.3%	33.3%	33.3%	33.3%	33.3%	33.3%	33.3%	33.3%
Tryptophan metabolism	67	17.9%	17.9%	17.9%	22.4%	17.9%	23.9%	17.9%	17.9%	16.4%
Ubiquinone and other terpenoid-quinone biosynthesis	25	28.0%	28.0%	28.0%	36.0%	24.0%	28.0%	28.0%	24.0%	32.0%

5.2.2. Identification of Biosynthetic Gene Clusters (BGCs)

The Number and type of identified clusters for each isolate was analysed by antiSMASH version 4.1.0. The BGCs ranged between 25 to 29 with 27 cluster types identified for CSK1, 27 clusters for CSK3, 29 clusters for CSW2, 26 clusters for CSU1, 29 clusters for CSU2, 27 clusters for CSG1 and finally for CSM1 25 clusters were generated. A phylogenetic tree of the isolates with each other confirmed that CSM1 is distantly related from the other isolates and that CSK3 and CSU1 are closely related to each other. Figure 5.2 shows phylogenetic tree of the seven different isolates mapped with metabolites identified by antiSMASH with known cluster types, the highest number of clusters was observed for CSW2 and CSU2 with 29 clusters and the lowest for CSM1 with 25 clusters. As observed from the figure, the distribution of the BGCs is very distinct for each isolate even when the isolates are closely related. The modular enzymes identified by antiSMASH of the isolated strains were between 10 and 15, with 10 modular enzymes identified for CSU1, CSG1 and CSM1, 13 for CSU2, 14 for CSW2 and 15 for CSK1 and CSK3.

For CSK1 isolate, 27 clusters were identified in total with fifteen clusters consisting of modular enzyme coding genes for polyketide synthase (PKS) or non-ribosomal peptide synthetase (NRPS) or a hybrid of both were identified by antiSMASH. Six of the clusters were NRPS/PKS hybrids identified as polyoxypeptin_A (is a gene was identified to induce apoptosis against human pancreatic carcinoma cells; Du et al., 2014), antimycin (an antibiotic produced by *Streptomyces sp.* and has been annotated by antiSMASH to have antifungal and cytotoxic molecular activity), lipomycin (an antibiotic that inhibits the growth of Gram positive bacteria), meridamycin (is a non-immunosuppressant polyketide that plays a great role in the treatment of neurological disorders; Sun et al., 2006), chlorizidine_A (an anti-tumour agent with cytotoxicity effect on human colon cancer cells; Alvarez-Mico et al., 2013) and splenocin (anti-inflammatory antibiotics that inhibit a wide range of cytokines and therefore considered useful in the treatment of asthma). Five clusters were classified as NRPS type, two were identified as JBIR_34 (has no known function) and naphthyridinomycin (antimicrobial and antitumor activity; Pu et al., 2013) while the other three were not correlated to any known clusters. As for the PKSs: T1PKS as meilingmycin (an antiparasitic macrolide produced by *S. nanchangensis* and is used as a pesticide in agriculture; Wang et al., 2010), T2PKS as spore pigment and two T3PKSs identified as herboxidiene (has antitumor activity; Lagisetti et al., 2014) and alkylresorcinol (provides Penicillin Resistance in *Streptomyces griseus*; Funabashi

et al., 2008). A cluster classified as “other” was correlated to arginomycin (an antibiotic produced by *S. arginensis* that inhibits Gram positive bacteria and fungi; Argoudelis et al., 1987). The other clusters belonged to non-modular enzymes.

Six hybrid clusters were identified for CSK3 as Polyoxypeptin_A, antimycin, lipomycin, chlorizidine_A, sanglifehrin_A (an immunosuppressant; Zhang and Liu, 2001) and griseoviridin/viridogrisein (antibiotics used against infections produced by multi-drug resistant bacteria synthesized by *Streptomyces griseoviridis*; Xie et al., 2017). Three NRPS clusters similar to naphthyridinomycin, coelibactin (plays a role in antibiotic regulation in *S. coelicolor*; De Montellano, 2005) and “unknown” clusters. T1PKS were correlated to 3 clusters with similarity to Isofuranonaphthoquinone (exhibits antibacterial and antioxidant properties in *Streptomyces sp.*; Fotso et al., 2003), Borrelidin (has antibacterial, antiviral and antimalarial properties isolated from *S. rochei*; Darna et al., 2011) and Nystatin-like-pseudonocardia-polyene (antifungal compound; Kim et al., 2018). T2PKS was identified as spore pigment and T3PKS as herboxidiene and alkylresorcinol as observed with CSK1. Also, cluster identified as “other” was generated as arginomycin.

CSW2 generated thirteen modular enzymes. Four hybrid clusters belonging to polyoxypeptin_A, antimycin, splenocin and sanglifehrin_A. NRPS clusters were identified as naphthyridinomycin, polyoxypeptin and three uncorrelated clusters. The non-ribosomal peptides were identified as naphthrydinomycin, and four “unknown” clusters. As for the PKSs clusters, T1PKS was reported as chlacomycin, T2PKS as spore pigment and T3PKS as Herboxidiene and alkylresorcinol.

Isolate CSU1 was identified by 10 modular enzymes. Four NRPS/PKS hybrids similar to antimycin, sanglifehrin_A, splenocin and polyoxypeptin_A clusters. Three NRPS clusters identified as naphthyridinomycin, berninamycin (an antibiotic that inhibits protein synthesis in Gram positive bacteria produced by *S. bernesis*; Reusser, 1969) and “unknown” clusters. Three PKS classified as T2PKS with resistomycin (a pentacyclic polyketide produced by *Streptomyces resistomycificus*, it has cytotoxic effect against cancer cell lines and an antioxidant activity; Vijayabharathi et al., 2011) and spore pigment and T3PKS with alkylresorcinol.

Sanglifehrin_A and naphthyridinomycin are the two hybrid clusters identified for CSU2. Piercidin_A1 (inhibits mitochondrial NADH dehydrogenase with antibacterial and antifungal activity; Hall et al., 1966), aurantimycin (exhibits inhibitory effect against Gram positive bacteria and antitumour activity; Gräfe et al., 1995), neocarzilin (antitumor compound; Otsuka et al., 2004), Antimycin and two “unknown” clusters were identified as NRPS clusters.

T1PKS clusters were identified as polyoxypeptin and antimycin; T2PKS as Spore pigment and resistomycin and T3PKS as alkylresorcinol. Finally, a cluster type classified as “other” was identified as polyoxypeptin.

Ten modular enzymes were identified for CSG1 with polyoxypeptin, antimycin and naphthyridinomycin as the hybrid clusters; antimycin, glycopeptidolipid and three “unknown” clusters as NRPS type; spore pigment as T2PKS cluster and alkylresorcinol as the T3PKS cluster.

Finally, CSM1had only one hybrid cluster belonging to sanglifehrin_A; four NRPS clusters in which two are identified as coelichelin (siderophore isolated from *S. coelicolor*; Tierrafria et al., 2011) and two as “unknown clusters”; one T1PKS classified as “unknown” cluster, two T2PKS identified as spore pigment and rabelomycin (angucycline antibiotic isolated from *S. olivaceus* with antibacterial activity against Gram positive bacteria; Kharel et al., 2010), one T2PKS-Butyrolactone cluster identified as fluostatins M-Q (angucyclins with special 6-5-6-6 carbon ring structure with unknown function; Jin et al., 2018). and one T3PKS cluster identified as germicidin (has inhibitory effect on spore germination; Petersen et al., 1993).

The biosynthetic assembly and predicted core structure were analysed for each cluster and the similarity of the cluster to other known genes and its presence in other *Streptomyces* species was identified using both Prism3 and antiSMASH databases (Appendix C). Appendix C shows the clusters that were identified by both tools only (more clusters were identified by antiSMASH than Prism3 as shown above).

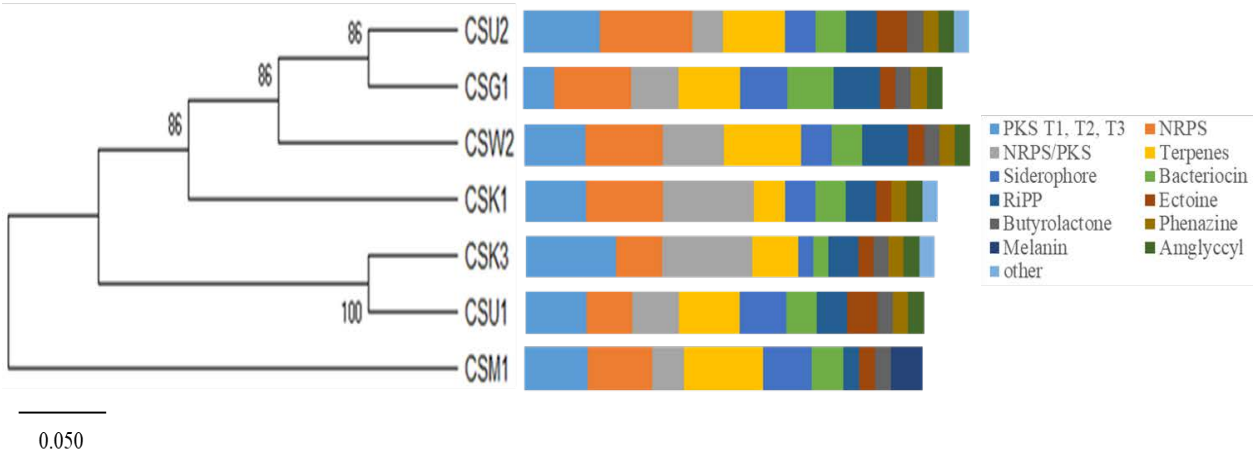


Figure 5. 2 Phylogenetic tree and secondary metabolite gene clusters

Neighbor-joining method was used to construct an evolutionary tree of the seven isolates using MEGA X. The phylogenetic tree of the 16S rRNA for the Bootstrap values based on 500 replicates are shown next to the branches. Jukes-Cantor method was used to calculate the evolutionary distances which is based on the number of base substitutions per site represented by 5% nucleotide sequence difference (scale bar). The bar plot represents the secondary metabolite gene clusters for each isolate identified by antibiotics and secondary metabolite analysis shell (antiSMASH). Different colours corresponding to different BGC types as shown in the key next to the bar plot.

5.2.3. Liquid Extraction Surface Analysis (LESA)

LESA-MS technology was used as a novel technique of direct sampling from the isolates' bacterial colonies to determine and identify the metabolite profiles of each isolate using acetonitrile-water-formic acid solvent system. The mass spectra generated by LESA was classified or identified according to regions (Figure 5.3). The spectra were divided into 4 main regions and comparisons between the uninduced and induced spectra were based on these regions. The regions are colour coded based on their locations on the spectra. First region represents the peaks generated with the highest signals and generally they are between *m/z* 0-550. Second region is between *m/z* 550-950 and denoted by a brown box and magnified brown spectrum. The next region is represented by blue box between *m/z* 950-1250 and blue magnified spectra and finally the fourth region is identified by green box with magnified green spectrum between *m/z* 1250-1500.

The mass spectra of uninduced CSK1 were analysed and Figure 5.4 represents the full scan where four distinct regions were identified. The first region between *m/z* 0-600 with charge of one and had the highest signals between *m/z* 0-300. The second region denoted by a brown box which is magnified as the brown spectrum shows peaks with doublet and triplet charges between *m/z* 650-1010. More intense peaks were observed in the *m/z* 1050-1120 region (blue box) with doublet charges (magnified blue spectrum) and the fourth region was marked by the green box (*m/z* 1100-1500) as shown by the magnified green spectrum with charges ranging between 6 and 9. The profile of the CSK1 isolate induced with GBL (Figure 5.5) changed from the uninduced, showing intense peaks with higher signals in the first region *m/z* 0-600. The region between *m/z* 630 and 950 (brown box) had the same doublet charges but with peaks of higher signals than the uninduced spectra as shown by the brown coloured spectrum. Similar profile was observed for region *m/z* 1050-1120 (blue box) with doublet charges but with a lower number of produced molecules (blue magnified spectra). Peaks with charges between 6-9 (green box region in uninduced CSK1) was not observed in the in CSK1 induced with GBL. Another change of profile was observed for CSK1 isolate when induced with GlcNAc (Figure 5.6), lower signal of peaks was observed for the region between *m/z* 0-600 than CSK1 induced with GBL, yet it is higher than the uninduced CSK1. The doublet region denoted by brown box and spectrum was between *m/z* 650 and 880 with similar signal intensity as the uninduced and finally no doublet region was observed between *m/z* 1050-1100 and the signal decreased tremendously. In addition, no region with charges between 6 to 9 in mass peaks greater than *m/z* 1200 as observed in the uninduced.

As for isolate CSK3, the spectral signal between *m/z* 0-450 was low and no distinct regions of peak charges higher than 1 was observed (Figure 5.7) while CSK3 induced with GBL (Figure 5.8) generated dense peaks with high signals between *m/z* 0-600 and a distinct region of double charged peaks between *m/z* 725-770. As for CSK1 induced with GlcNAc (Figure 5.9), the peaks between *m/z* 0-450 had higher signals than the uninduced but lower than CSK1 induced with GBL. A distinct region of peaks with doublet charge was also observed but with masses of *m/z* 300-400, lower than mass region generated by CSK1-GBL. The individual peak for all the above signal masses are found in Appendix D.

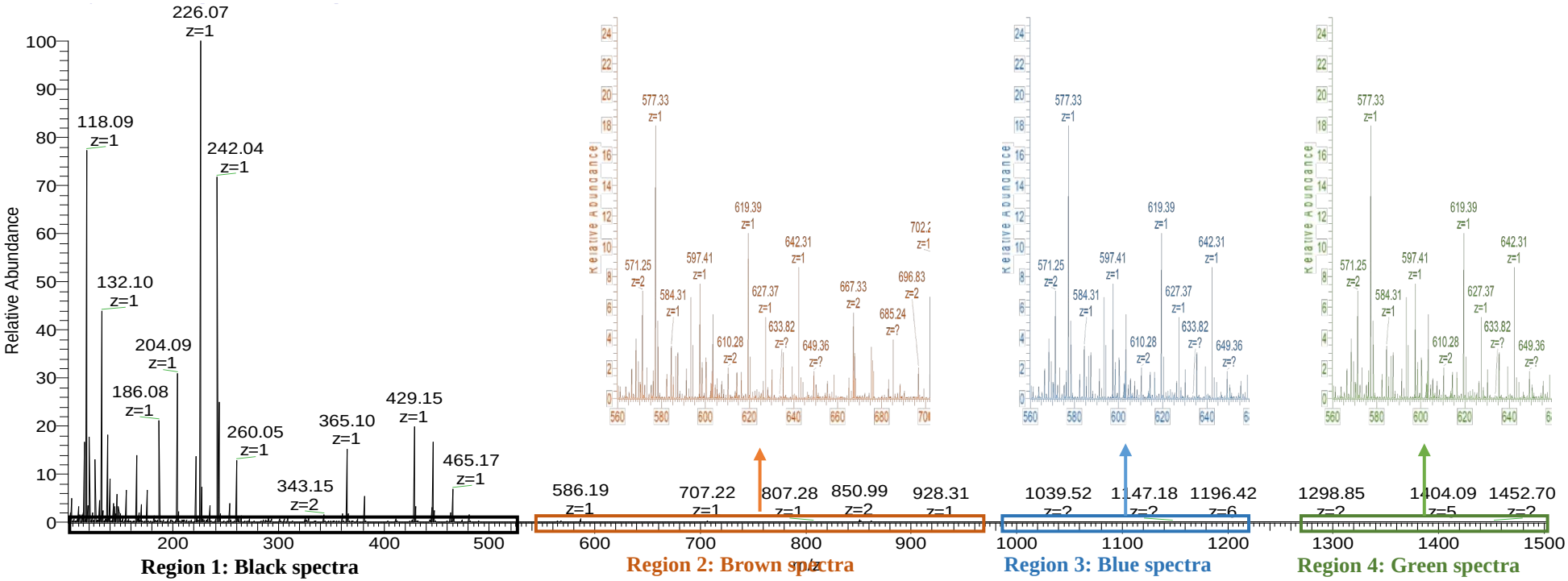


Figure 5. 3 Identification of mass spectra generated by LESA

The spectra generated by LESA was divided into 4 distinct regions to analyze and compare the results between the uninduced and induced isolates systematically. The four regions are colour coded based on the location they represent. Main region of the spectra which represents the peaks with the highest signal is commonly between *m/z* 0-550 and represent the black coloured spectra. Second region is denoted by brown box and brown magnified spectra between *m/z* 550-950, third region between *m/z* 950-1250 is highlighted with a blue box and blue magnified spectra and the fourth region with green box and green magnified spectra is between *m/z* 1250-1500.

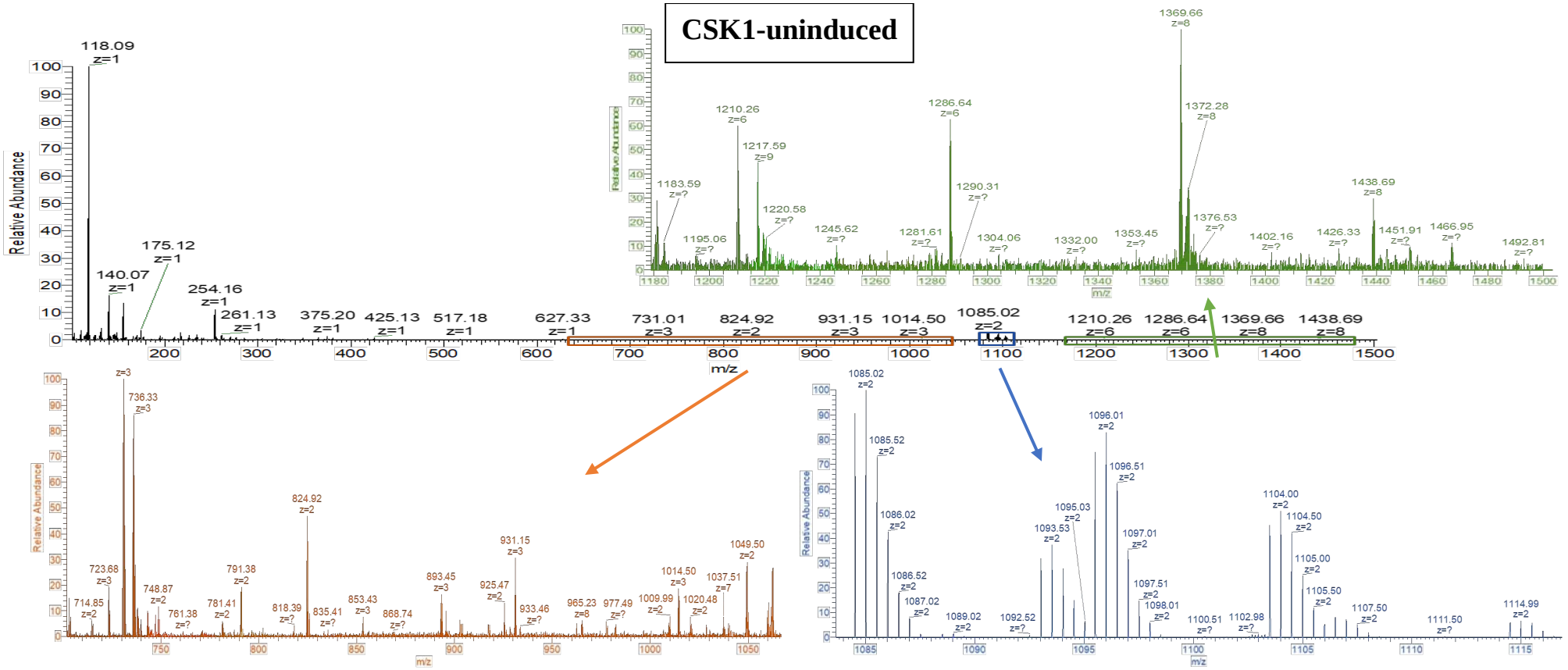


Figure 5. 4 LESA mass spectrum of isolate CSK1

CSK1 colonies on ISP1 agar medium were analysed with LESA-MS system to identify potential metabolites using acetonitrile-water-formic acid (50:45:5) solvent, the spectra showed up to 15 kDa of different molecules. Doublet and triplet regions denoted by brown and blue boxes were identified (magnified regions of B &C) in the spectrum between *m/z* 600 and 1100. Region D identified peaks between *m/z* 1200 and 1500 (green box and spectrum) with charges between 6 and 9.

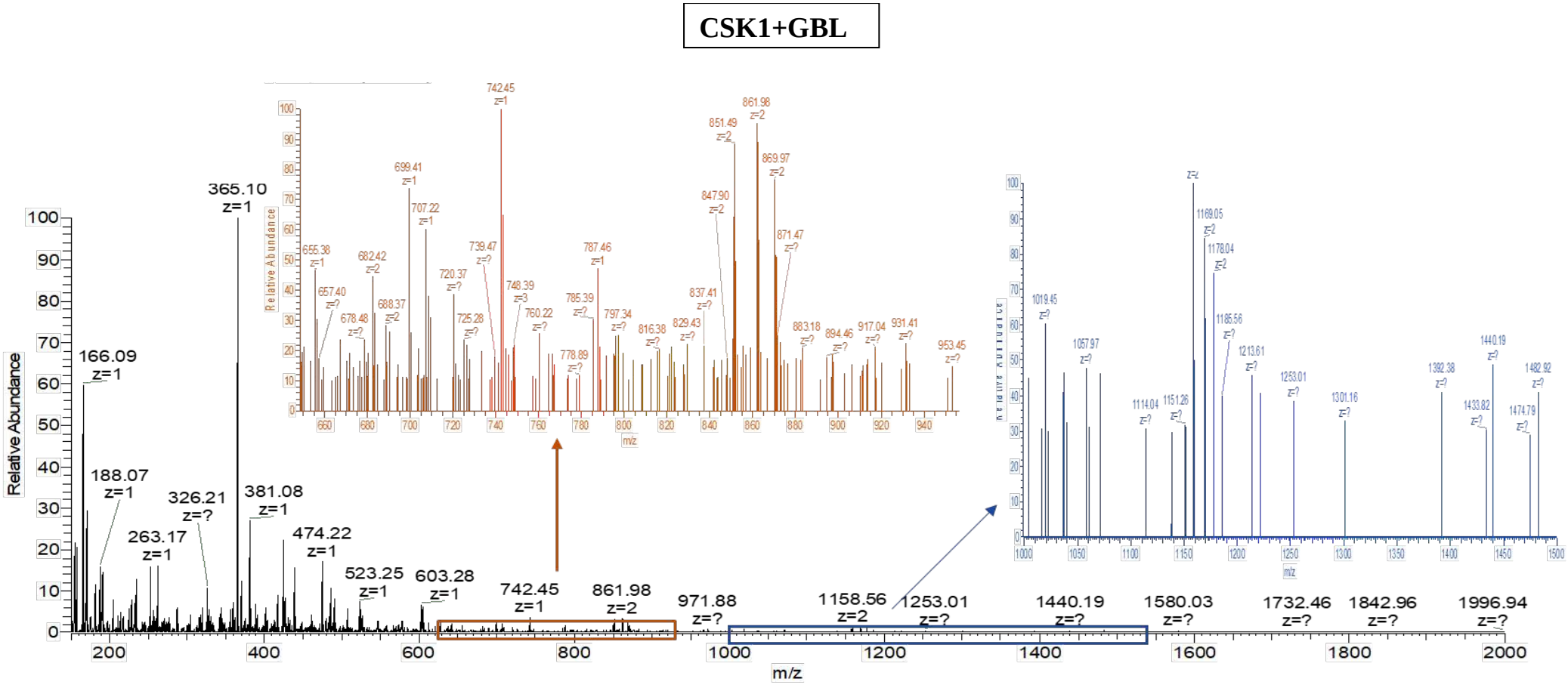


Figure 5. 5 LESA mass spectrum of CSK1 induced with GBL

Colonies produced from the cultivation of CSK1 isolate on ISP1 agar medium induced with 0.7% GBL were analyzed by LESA-MS using acetonitrile-water-formic acid (50:45:5) solvent to identify metabolites. Doublet regions were identified between *m/z* 630-950 and *m/z* 1050-1120.

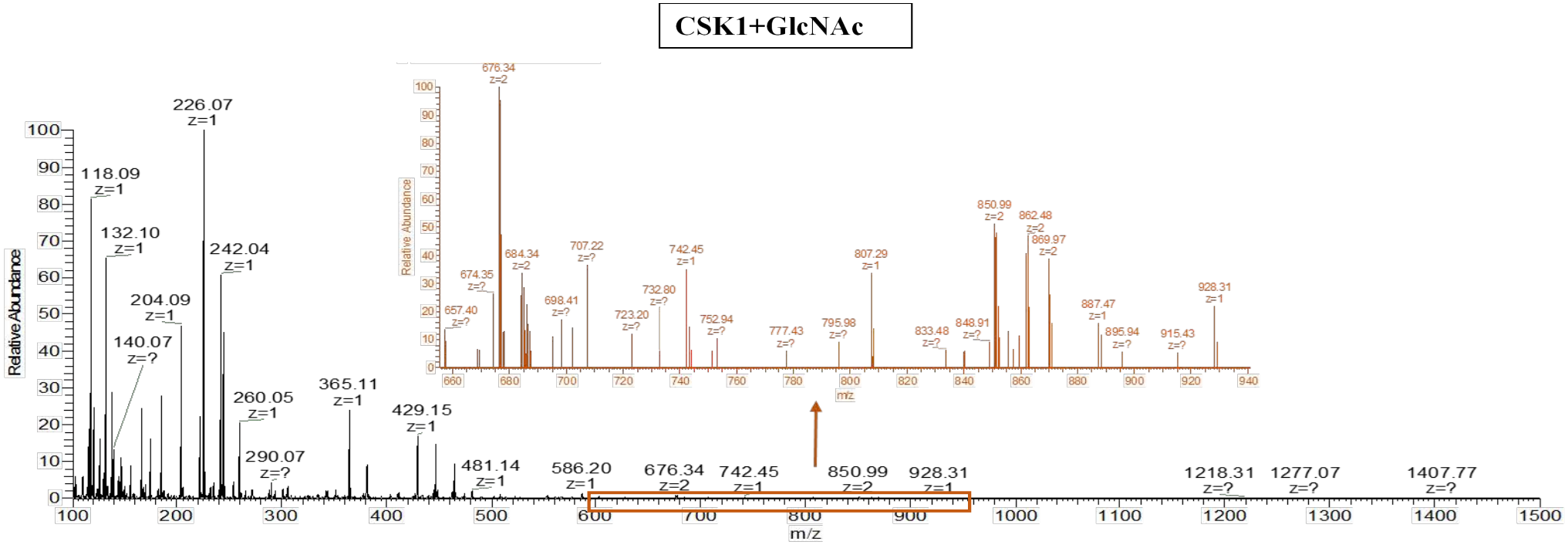


Figure 5. 6 LESA mass spectrum of CSK1 induced with GlcNAc

CSK1 isolate was cultivated in ISP1 agar medium induced with 50 mM GlcNAc. Colonies were analyzed by LESA-MS system using acetonitrile-water-formic acid (50:45:5) solvent to identify metabolites. Doublet regions (brown box) were identified between *m/z* 650 and 850 (magnified brown spectrum).

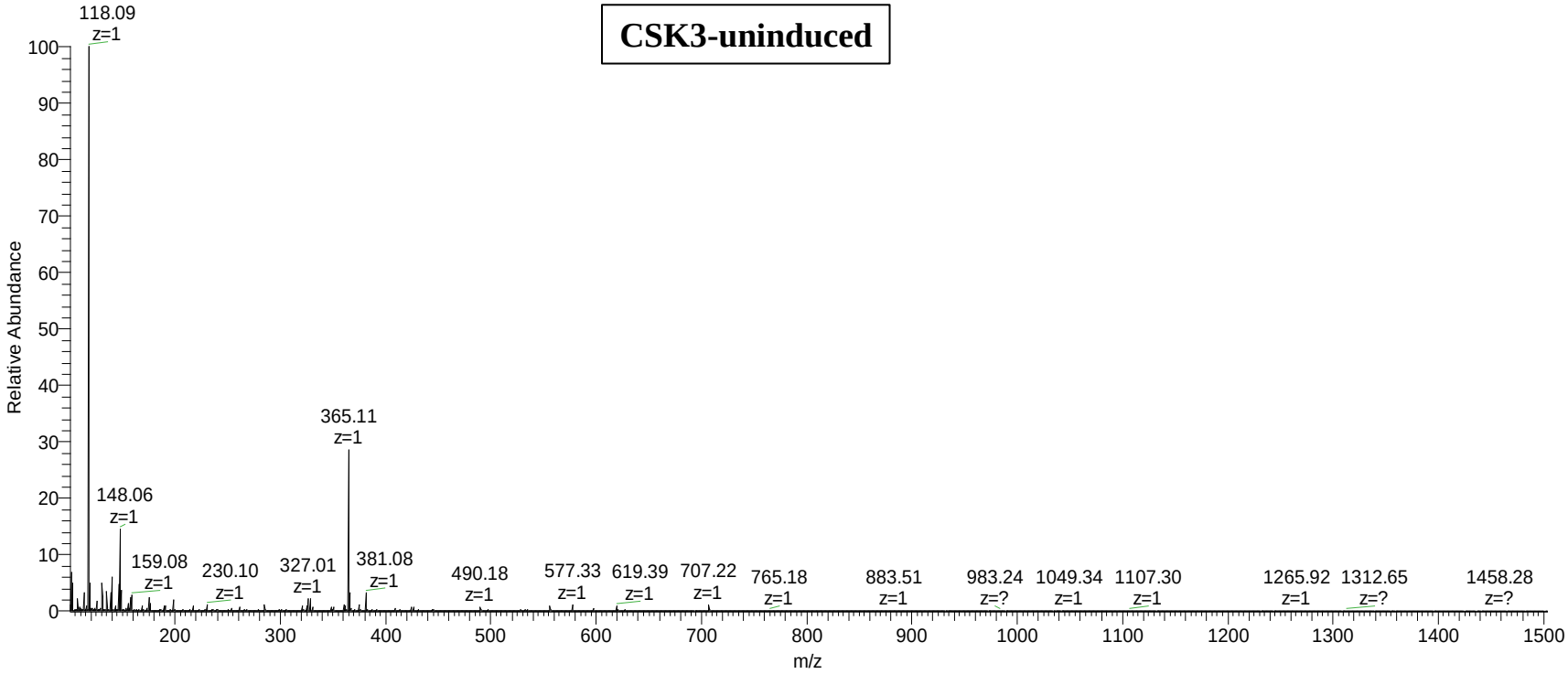


Figure 5. 7 LESA spectrum of isolate CSK3

CSK3 colonies on ISP1 agar medium were analysed with LESA-MS system to identify potential metabolites using acetonitrile-water-formic acid (50:45:5) solvent, the spectra showed up to 15 kDa of different molecules. Peaks with the highest signals were observed between *m/z* 0-450. Doublet peaks were observed at different regions of the spectrum.

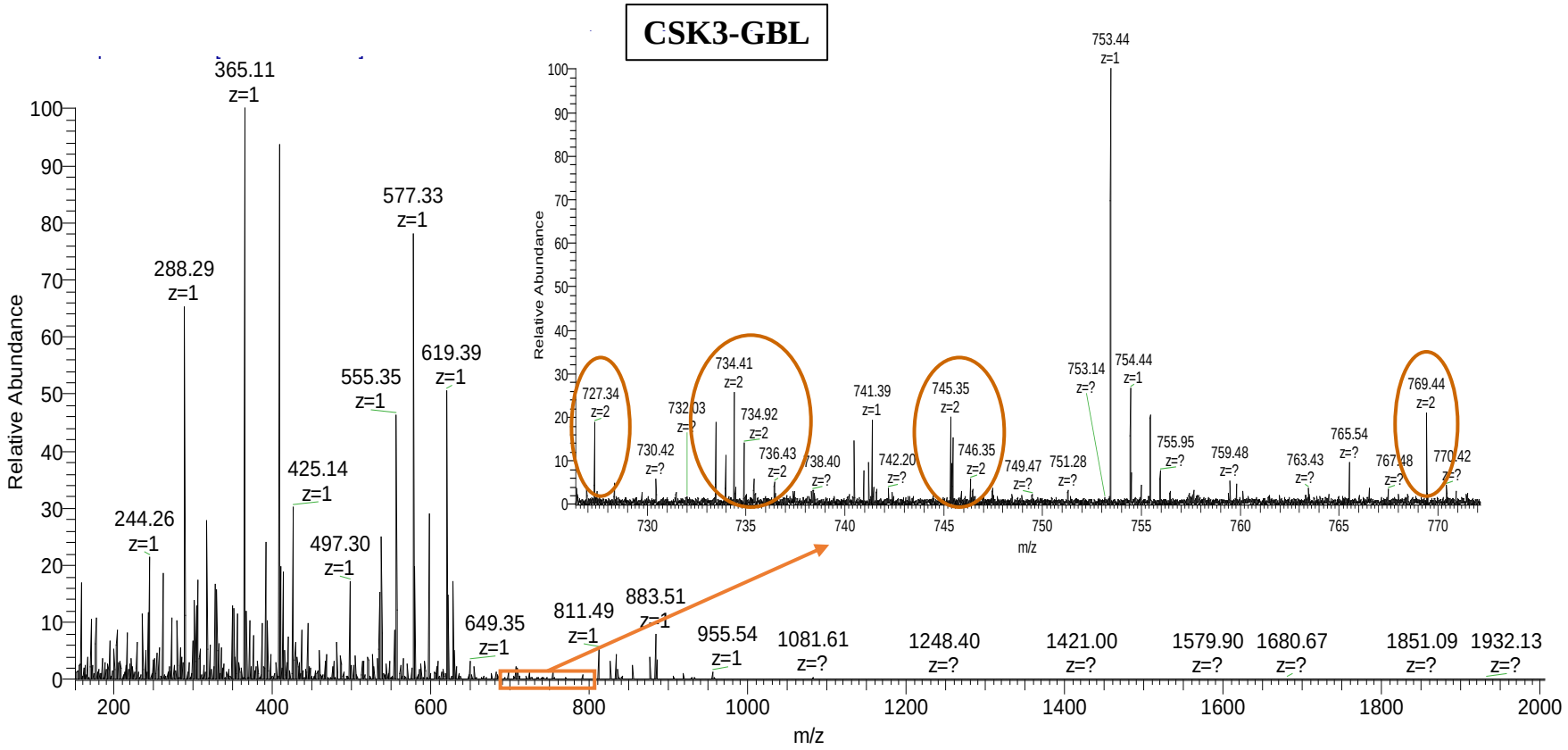


Figure 5. 8 LESA mass spectrum of CSK3 induced with GBL

CSK3 was cultivated on ISP1 medium induced with 0.7% GBL. Colonies were analyzed by LESA using acetonitrile-water-formic acid solvent. A region between *m/z* 725-750 (brown box) had various doublet peaks as shown in the magnified spectrum denoted with brown ovals.

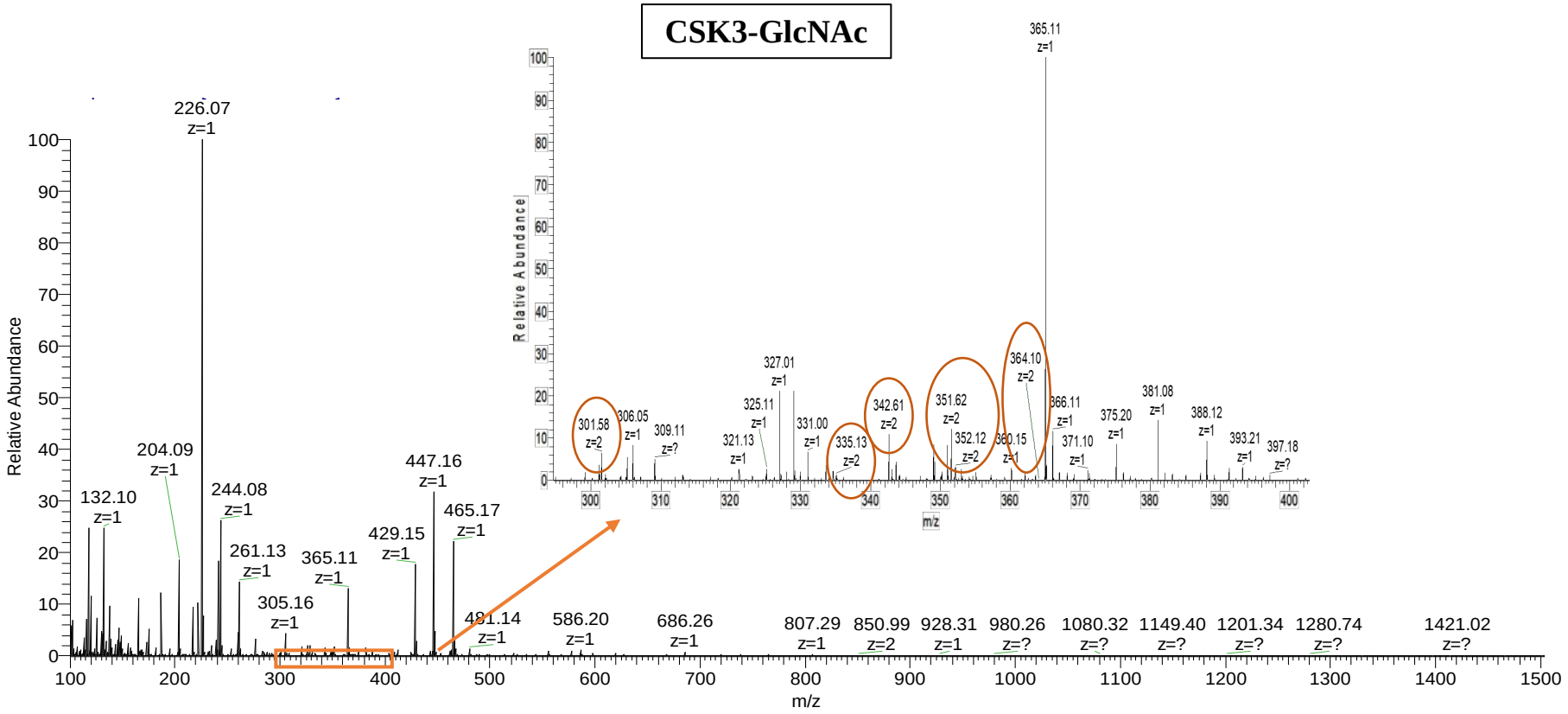


Figure 5. 9 LESA mass spectrum of CSK1 induced with GlcNAc

CSK3-GlcNAc colonies produced from CSK3 cultivated on ISP1 medium supplemented with 50 mM GlcNAc. The brown box region represents peaks produced of *m/z* 300-400 with doublet charge highlighted on the magnified spectrum by brown ovals.

Uninduced CSW2 mass spectra generated 3 distinct regions (Figure 5.10). The first region with the highest observed signal between *m/z* 0-450 with single charge. The next region produced doublet peaks between *m/z* 1060-1200 and the last region produced peaks with charge of 9 found between *m/z* 1300-1305 and another location of peaks with a charge of 8 found between *m/z* 1465-1470. As for the mass spectra generated from CSW2 colonies induced with GBL a black coloured spectrum with dense and high signal peaks Figure 5.11) were observed between *m/z* 0-650 with a doublet region between *m/z* 680-690 denoted by magnified brown spectra, another region of doublet peaks was observed between *m/z* 1070-1180 (blue spectra). As for CSW2 induction with GlcNAc resulted in a spectrum lower in intensity (black peaks) than CSW2-GBL but higher than the uninduced CSW2 with single charge peaks (Figure 5.12).

LESA analysis of CSU1 isolate generated a spectrum of doublet charged peaks between *m/z* 650-855 (brown spectrum) and another region between *m/z* 1300-1470 with charges between 8-9 shown in the green magnified spectrum (Figure 5.13). Low signal peaks between *m/z* 0-550 were observed for CSU1-GBL spectrum (Figure 5.14) compared to uninduced isolate, all peaks were of single charge. Another change in the spectrum profile was observed for CSU1-GlcNAc where the peaks between *m/z* 0-500 were denser and higher in signal (Figure 5.15) compared to both the uninduced and CSU1-GBL with a region of doublet charge between *m/z* 750-900.

Although there were only few high signals in CSU2 and they were concentrated between *m/z* 0-160 as shown by the black peaks of figure 5.16, yet a region between *m/z* 1090-1190 had peaks with a charge of 6 (brown magnified spectrum). CSU2-GBL spectrum resulted in denser spectrum with a wider range between *m/z* 0-700 (Figure 5.17). As for CSU2-GlcNAc it generated a spectrum of high signal peaks between *m/z* 0-500 and a region with doublet peaks between *m/z* 600-900 (Figure 5.18).

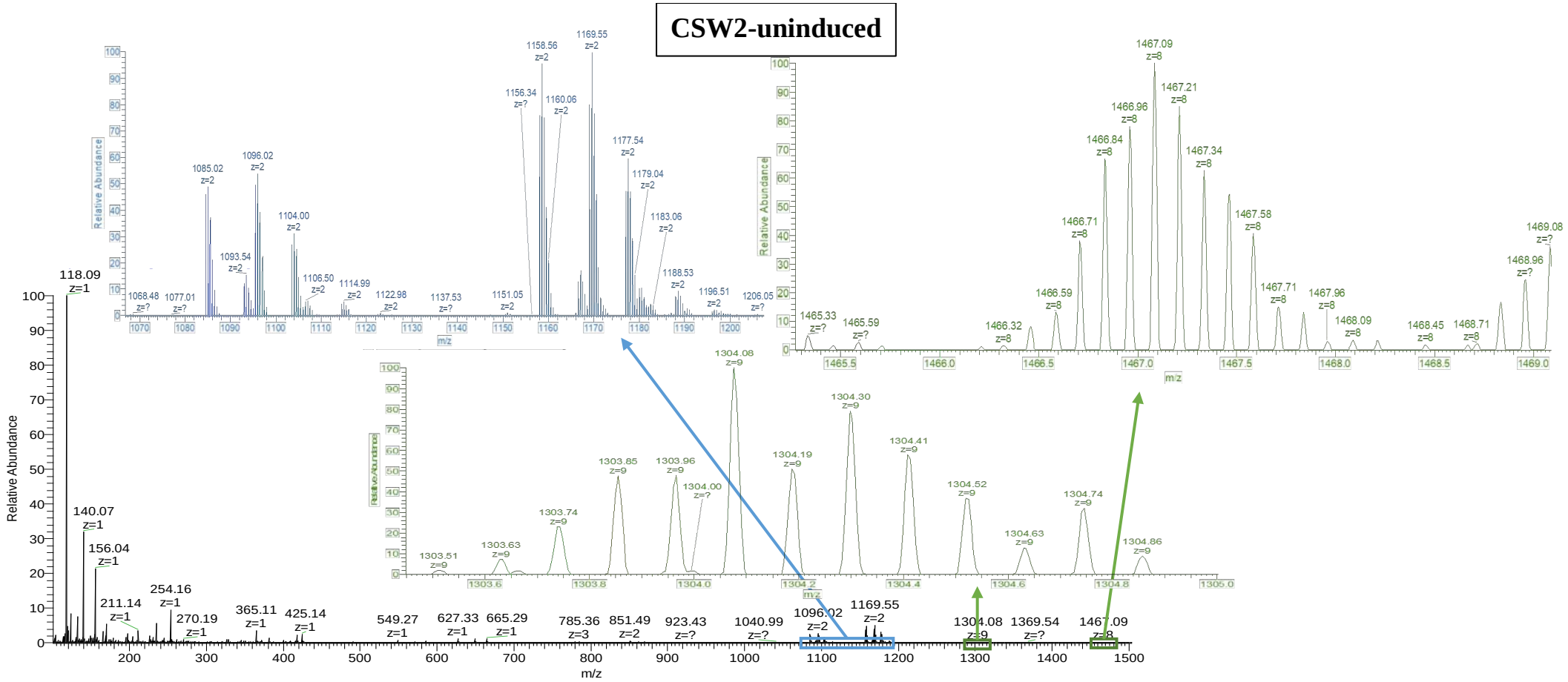


Figure 5. 10 LESA mass spectrum of isolate CSW2

CSW2 was cultivated on ISP1 agar medium and colonies were analyzed by LESA. Two distinct regions were produced, a region between *m/z* 1070-1200 (blue box) with doublet peaks (blue spectrum) and a spectrum region between *m/z* 1300-1470 region with charges between 8-9 specifically the peaks in the spectra region between *m/z* 1300-1305 and 1465-1470 (green boxes), magnified regions shown as green spectra.

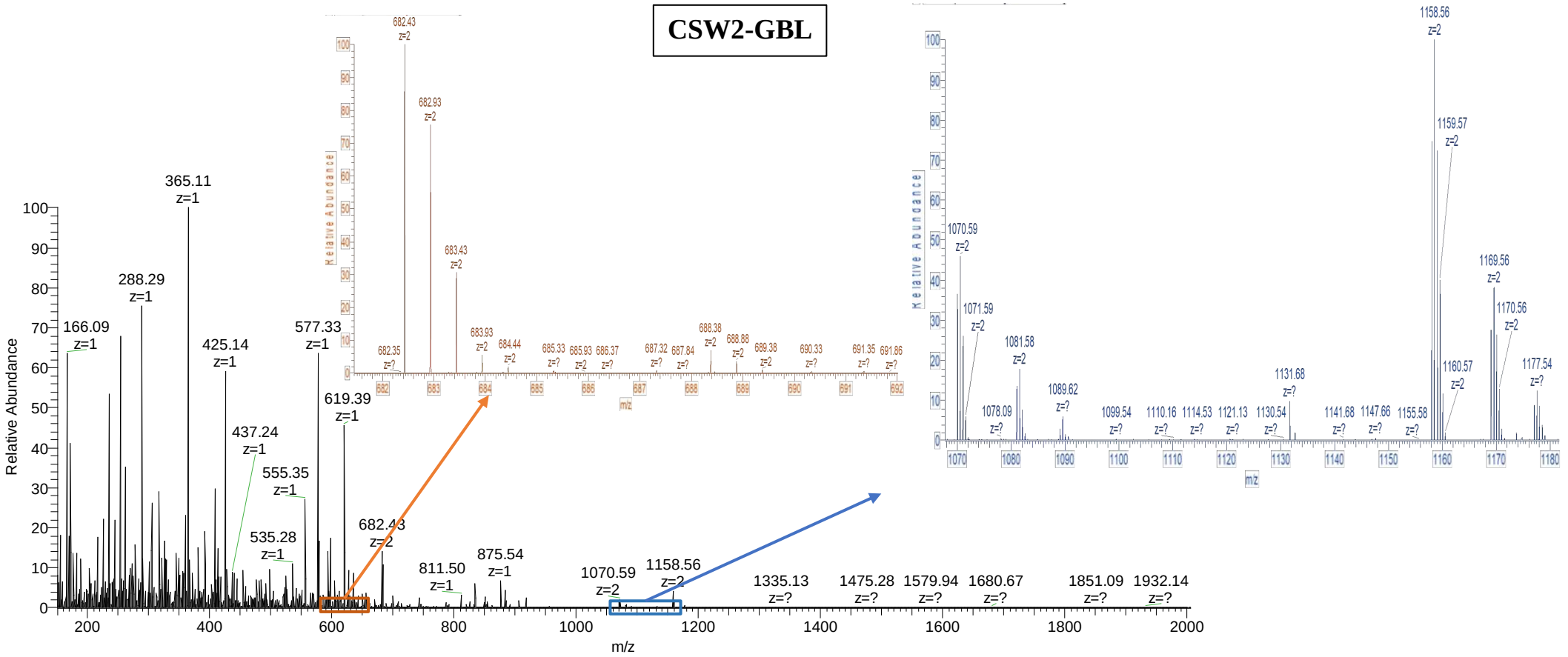


Figure 5. 11 LESA analysis of CSW2 induced with GBL

CSW2 induced with GBL colonies mass spectrum shows three distinct regions of doublet charges. The first region was a dense spectrum between *m/z* 0-650 with high signal peaks. The second region represented by brown box on the main spectra and magnified as a brown spectrum with peaks of doubled charges was between *m/z* 680-690. The last region with, also, doublet charges are between *m/z* 1070-1175 and shown as the blue spectra.

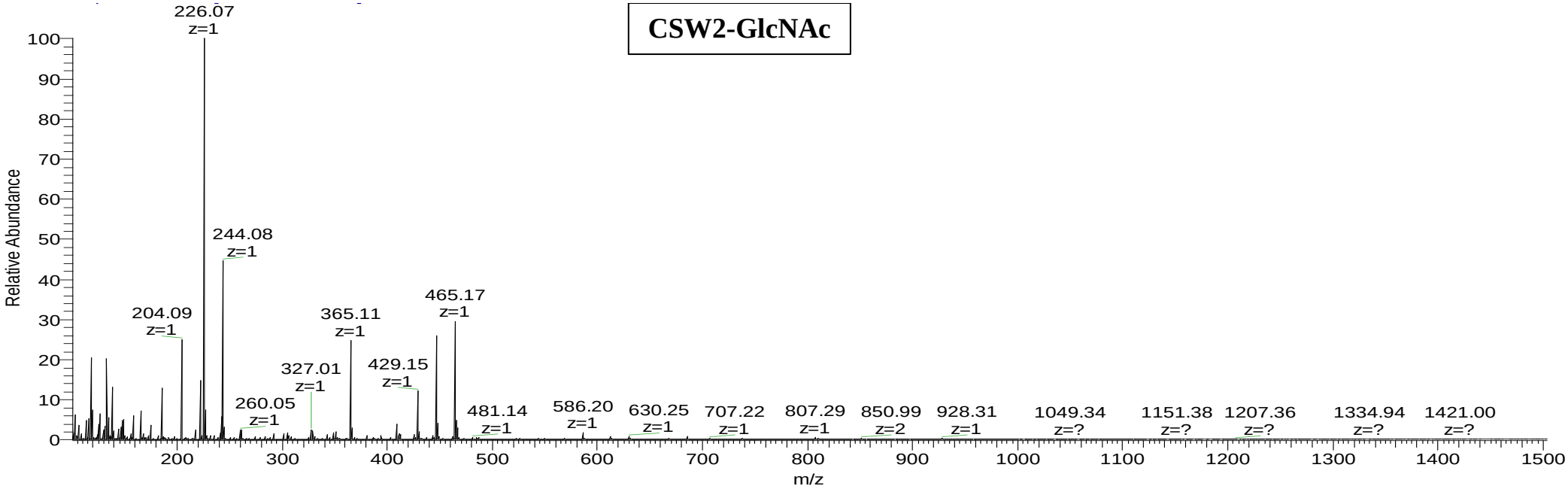


Figure 5. 12 LESA mass spectrum of CSW2 induced with GlcNAc

Colonies of CSW2 induced with GlcNAc were analyzed by LESA. Peaks with high signal were observed between *m/z* 0-470. All peaks were with a charge of 1.

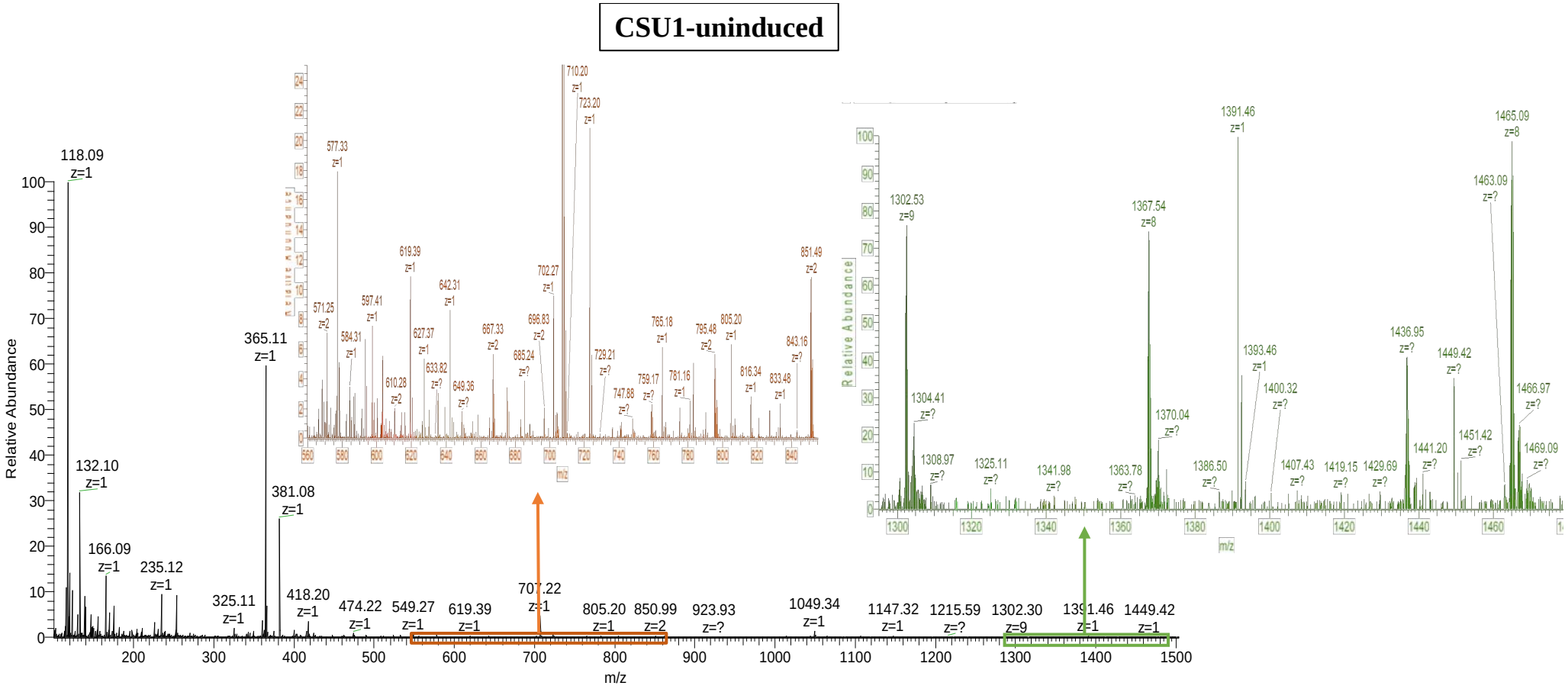


Figure 5. 13 LESA mass spectrum of isolate CSU1

The highest signals were observed in the region between *m/z* 0-450 of the spectrum produced from the LESA analysis of colonies generated from the cultivation of isolate CSU1 on ISP1 agar medium. Two distinct regions were observed (brown and green boxes), a doublet region between *m/z* 560 and 850 (brown spectra) and a region of peaks with charges between 8 and 9 (green spectra).

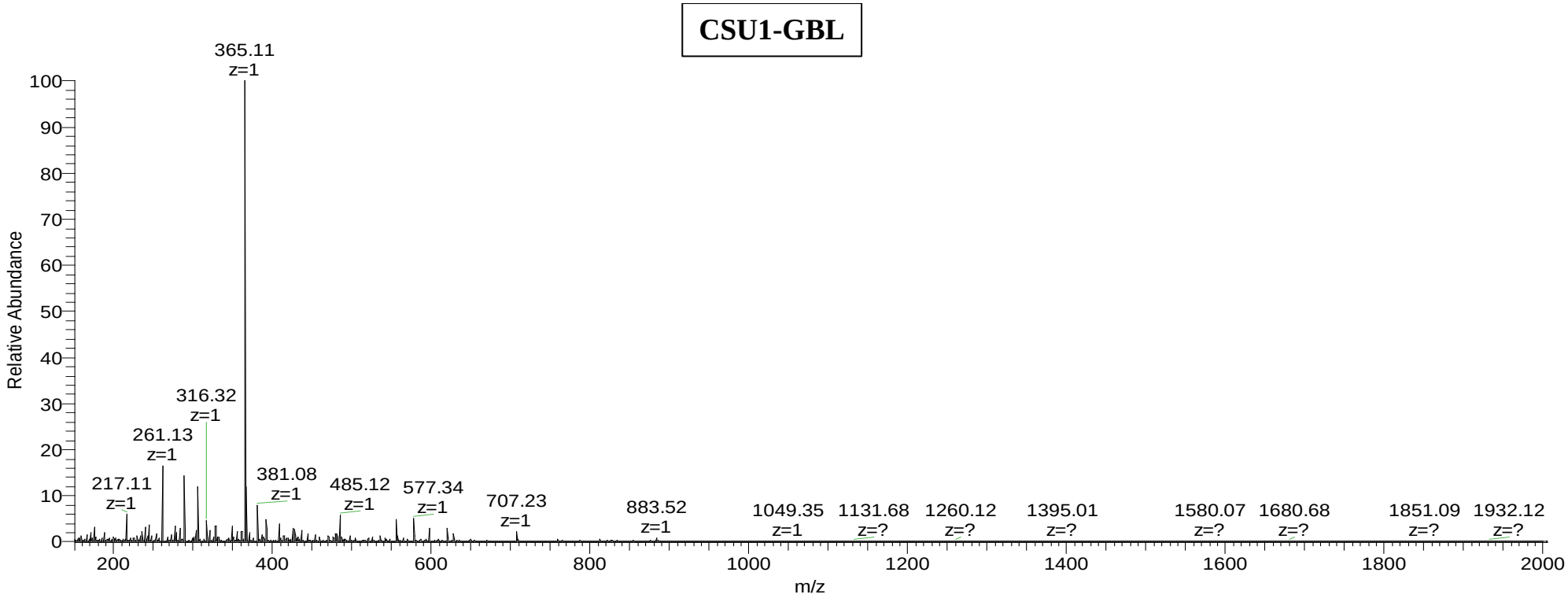


Figure 5. 14 LESA mass spectrum of CSU1 induced with GBL

CSU1 isolate cultivated on medium induced with 0.7% GBL resulted in colonies that were analyzed by LESA. Peaks with the highest signals were produced between *m/z* 0-500 with charge of 1.

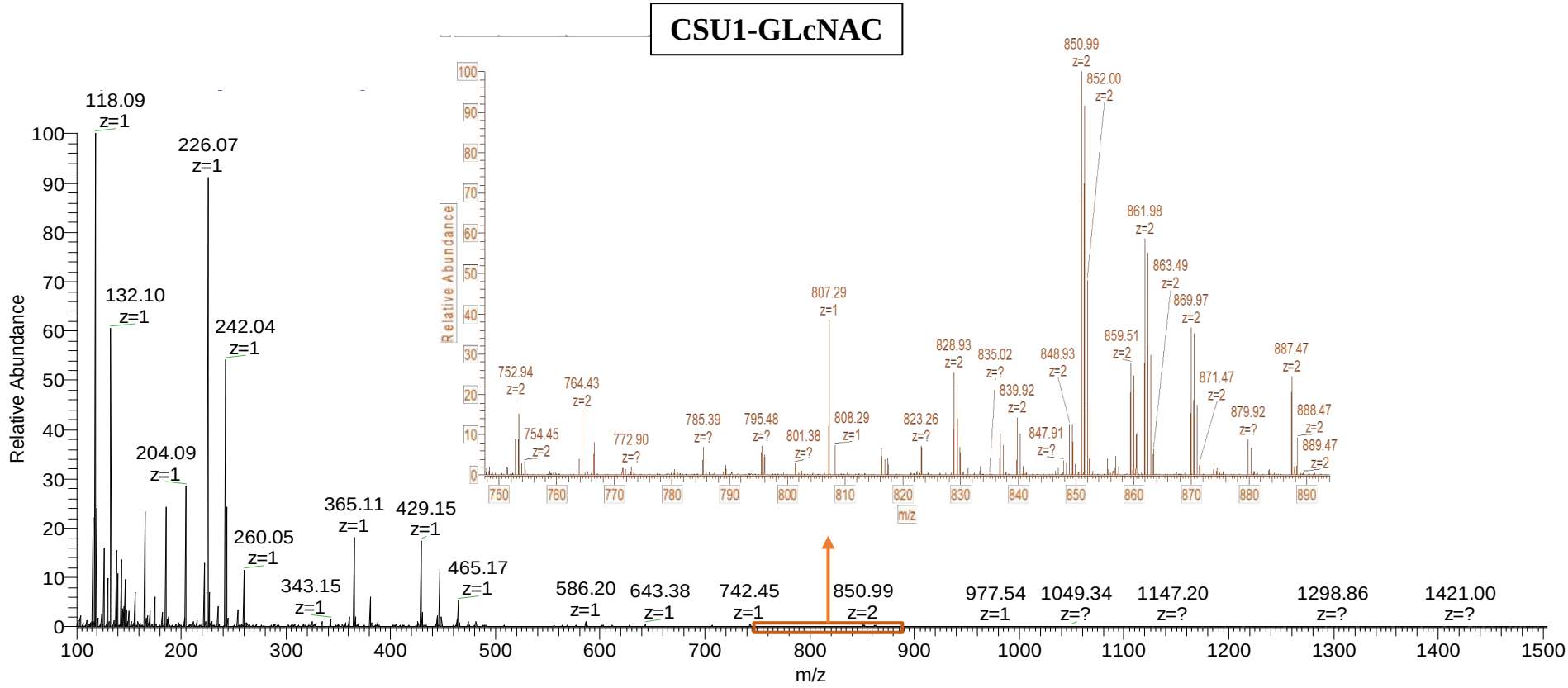


Figure 5. 15 LESA mass spectrum of CSU1 induced with GlcNAc

Colonies of CSU1 isolate induced with GlcNAc were analyzed by LESA, spectrum had doublet peaks in various locations along the spectrum even between *m/z* 100-500 but doublet peaks (brown box) were mainly concentrated in the region of *m/z* 750-900 (brown spectra).

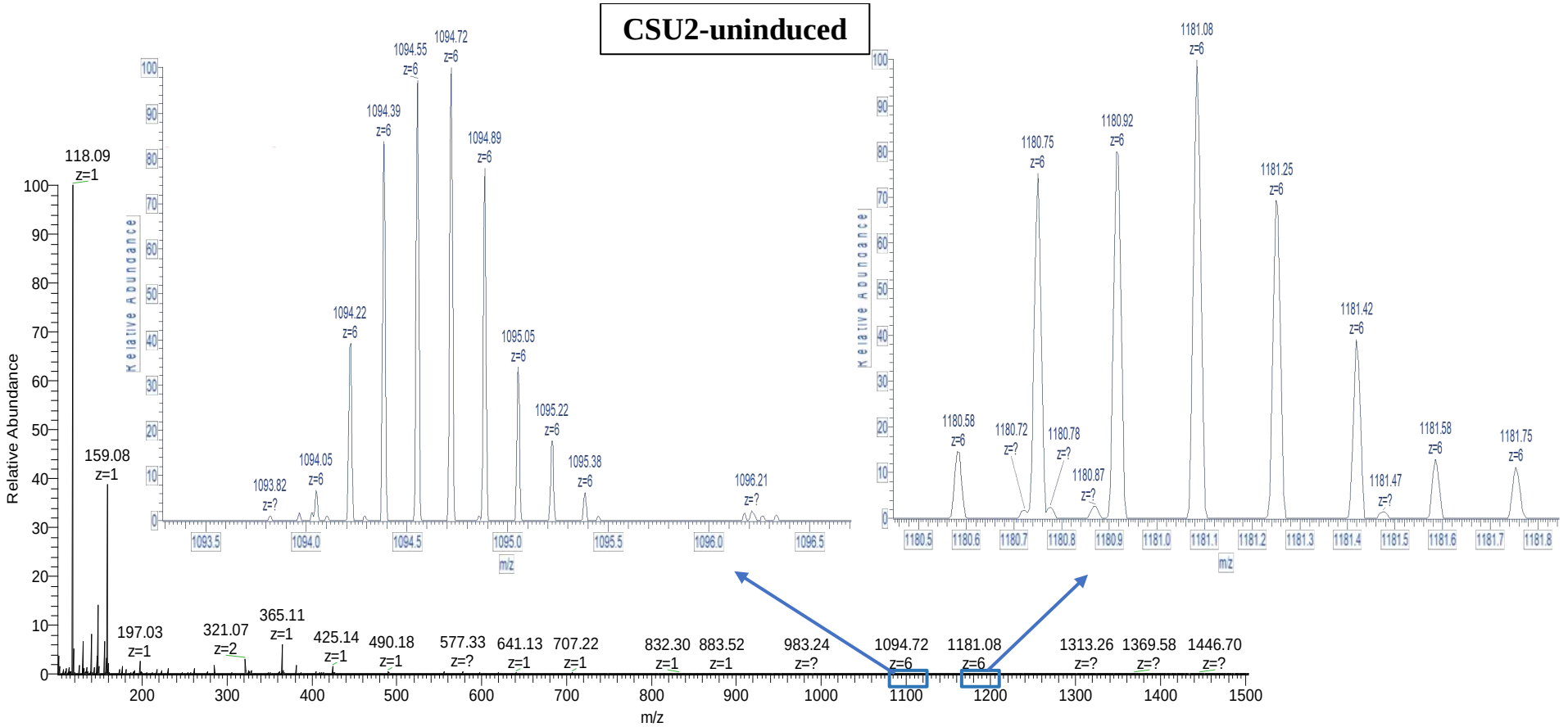


Figure 5. 16 LESA mass spectrum of isolate CSU2

Colonies produced from CSU2 isolate on ISP1 agar medium generated a distinct spectrum (blue boxes) between *m/z* 10930-1190 when analyzed by LESA, these regions with charge of 6 are shown as blue spectra.

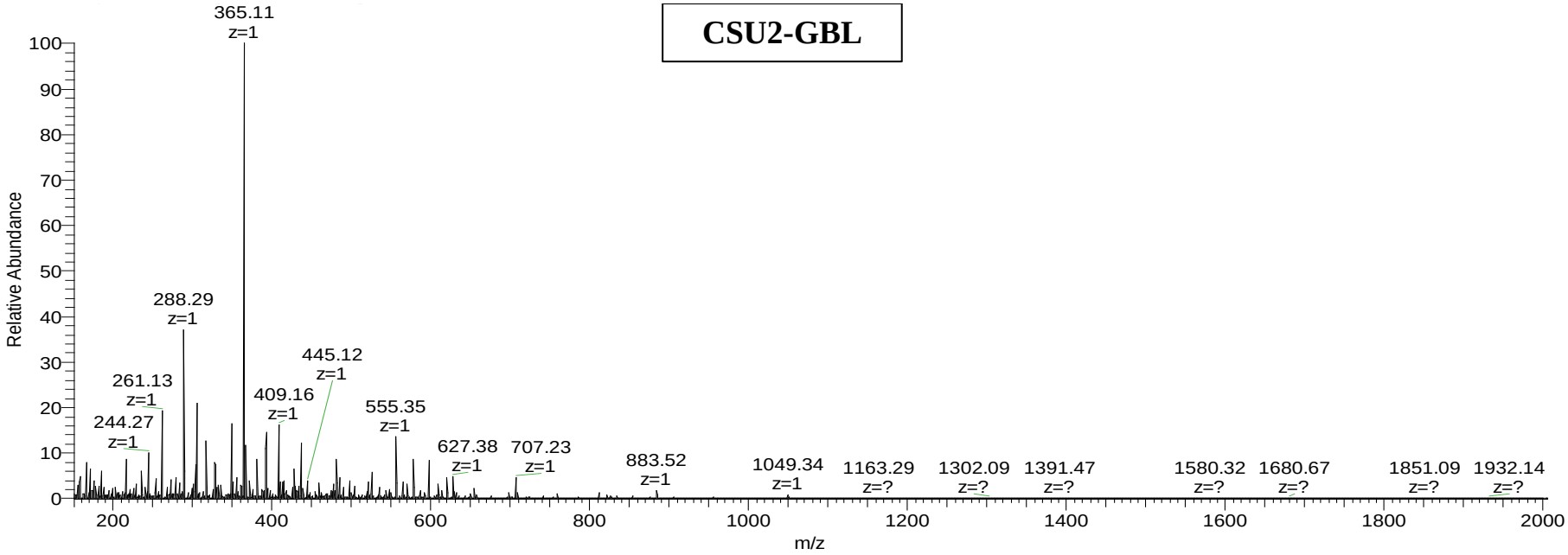


Figure 5. 17 LESA mass spectrum of CSU2 induced with GBL

A mass spectrum was generated by LESA-MS from colonies of CSU2 induced with GBL on ISP1 agar medium. Peaks of the spectrum were produced with a charge of 1 and highest signals were observed in the region between *m/z* 0-700.

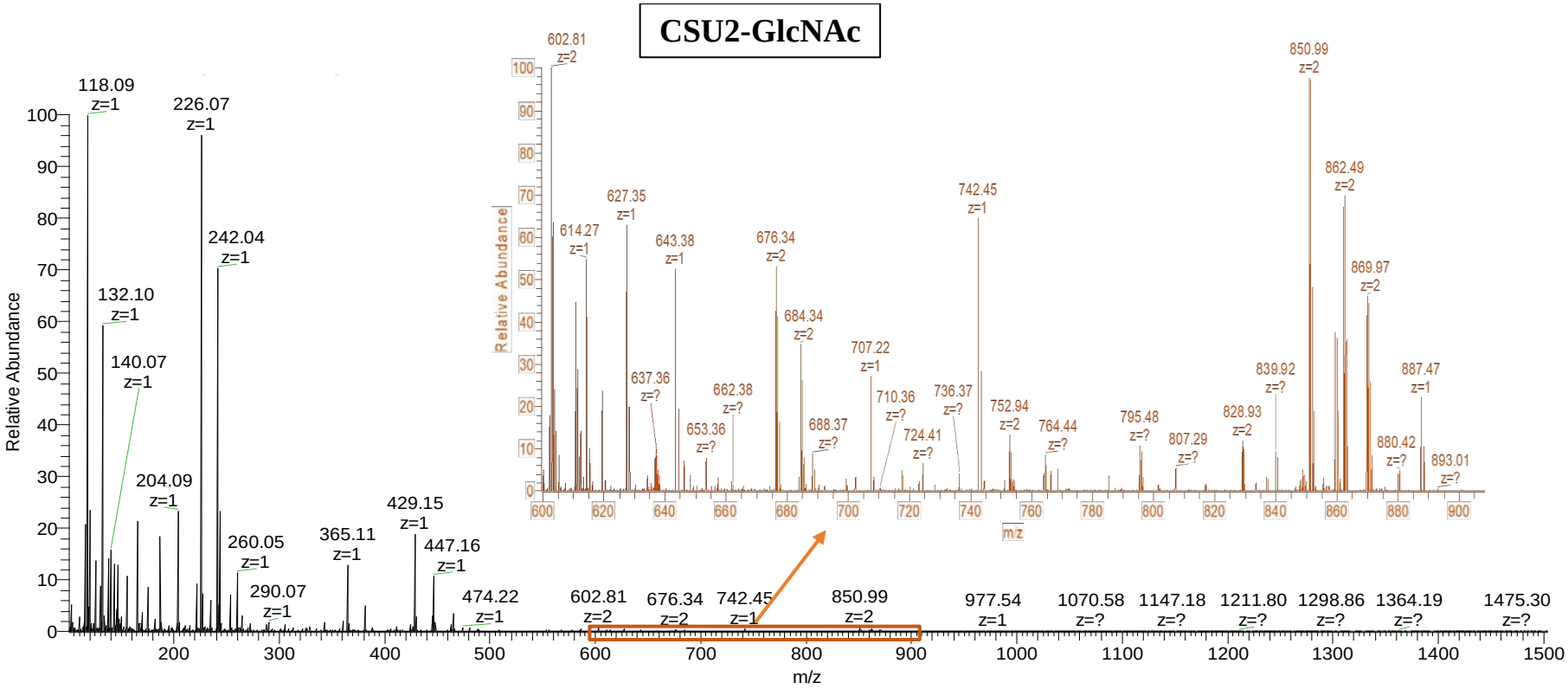


Figure 5. 18 LESA spectrum of CSU2 induced with GlcNAc

CSU2 isolate was cultivated in ISP1 agar medium induced with 50 mM GlcNAc. The generated spectrum by LESA revealed a doublet region (brown box) between *m/z* 600-900. All other peaks were produced with a charge of 1.

CSG1 mass spectrum generated from LESA analysis revealed four distinct regions. The first region was shown as black dense peaks between *m/z* 0-450 (Figure 5.19). Another region between *m/z* 720-740 of peaks with triplet charge was identified (brown spectrum) followed by a region of doublet peaks denoted by blue spectrum in the figure ranging between *m/z* 1080-1100. Finally, the fourth region had distinctive peaks with charge of 8 observed between *m/z* 1365-1375. Denser peaks were observed for CSG1-GBL spectrum between *m/z* 0-650 (Figure 5.20). Doublet peaks were observed at two different regions in the CSG1-GBL spectrum, with the first between *m/z* 680-870 and the second between *m/z* 1150-1195. As for CSG1-GlcNAc spectrum, 4 distinctive regions were identified with the high signal peaks region between *m/z* 0-450 (Figure 5.21); peaks with charges between 2-5 were observed as brown spectrum in the figure between *m/z* 625-965 followed by a region with peaks of charges 4 and 5 (magnified as blue spectrum) between *m/z* 1090-1150 and the final region shown as green spectrum in the figure was a region of peaks between *m/z* 1370-1380 with peaks of charge 4.

The last analyzed isolate CSM1 generated low signal peaks between *m/z* 0-500 by surface sampling which is commonly used on bacterial colonies and where there is no direct contact of the pipette tip containing the solvent (Figure 5.22A). Another sampling procedure was examined where the pipette tip was in direct contact with the colony which generated a high mass spectrum region between *m/z* 600-850 with doublet charges and another region between *m/z* 1100-1650 with peaks ranging in charge between 2 and 10 (Figure 5.22B). CSM1-GBL spectrum had low signal peaks in the region between *m/z* 0-450 and a doublet region between *m/z* 680-860 magnified as a brown spectrum (Figure 5.23). CSM1 colonies induced with GlcNAc generated spectrum with three regions (Figure 5.24); the first region was high signal peaks between *m/z* 0-450 followed by a region of doublet peaks (brown spectrum) and then a region with peaks containing a charge of 5 and 6 between *m/z* 1200-1410.

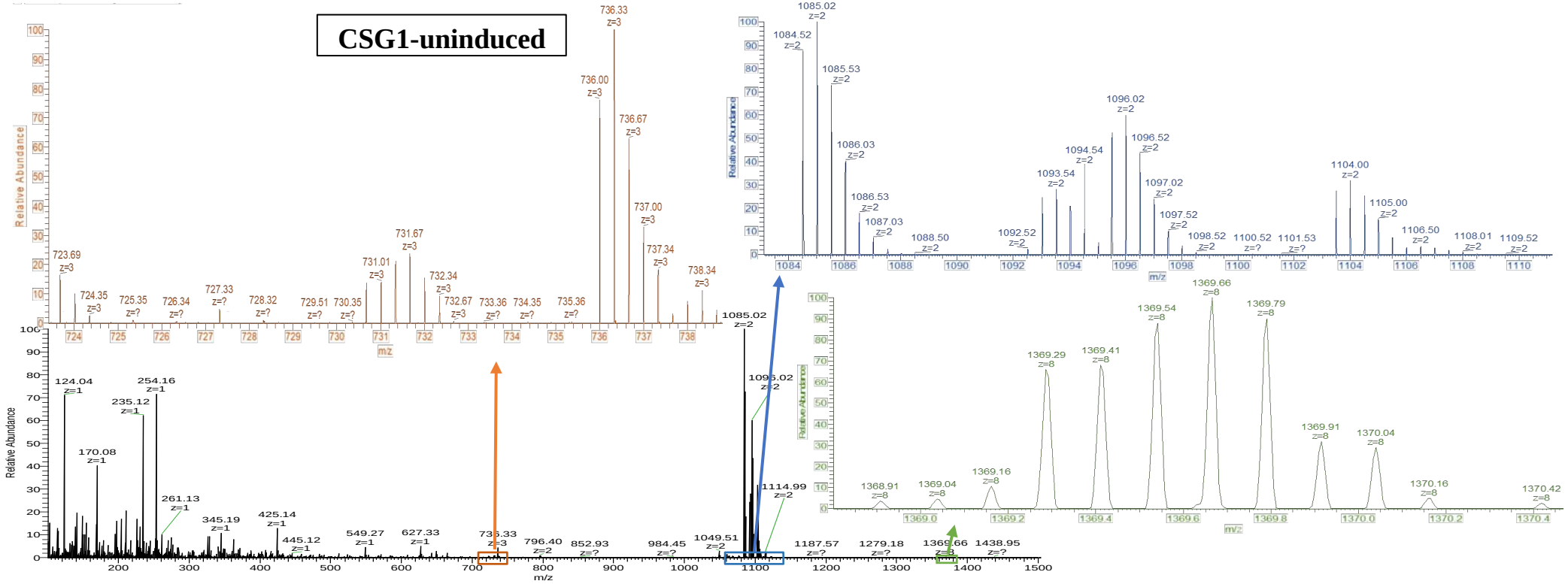


Figure 5. 19 LESA spectrum of CSG1 isolate

Colonies of CSG1 grown on ISP1 agar medium generated a spectrum with four distinct regions when analyzed by LESA. High signal peaks were observed between *m/z* 0-450 with charge of 1. A region between *m/z* 670 and 810 (brown box) with doublet and triplet charged peaks (brown spectra). Another region of doublet charges (blue box) between *m/z* 1030-1150 (blue spectra) and a region with peaks of charge 8 (green box) between *m/z* 1365-1371 (green spectra).

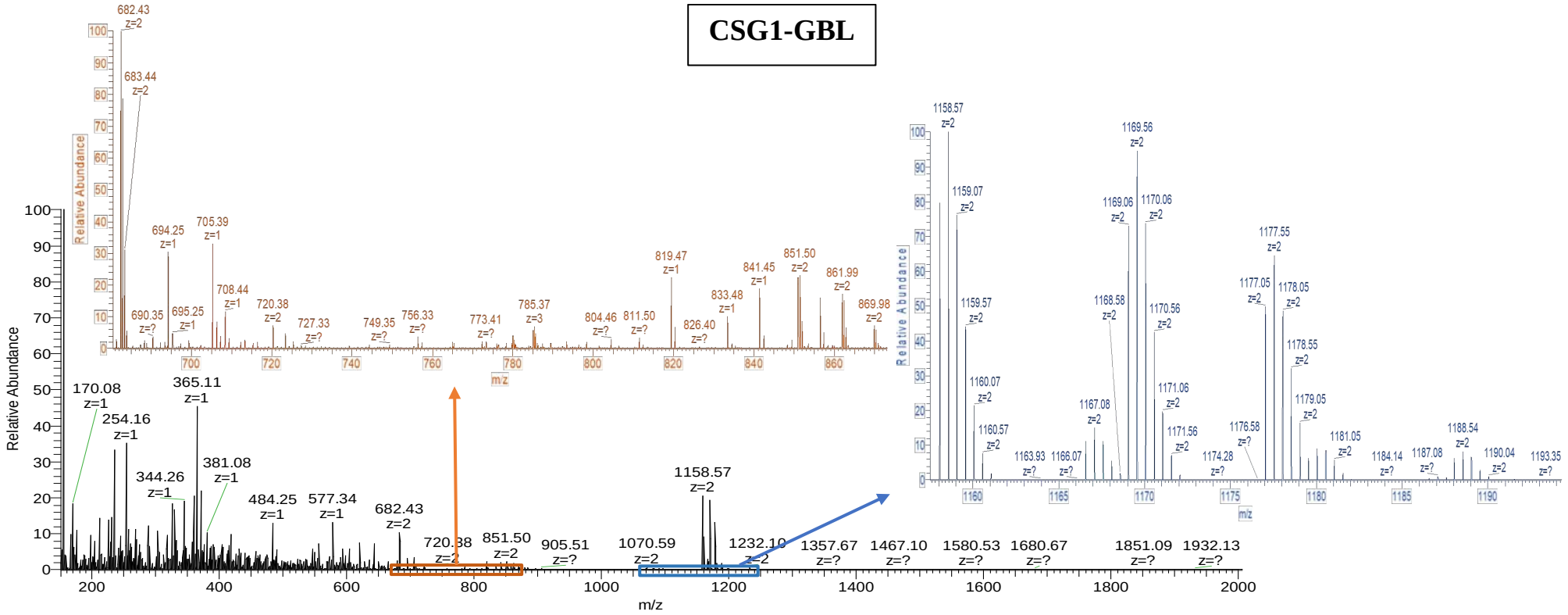


Figure 5. 20 LESA mass spectrum of CSG1 induced with GBL

Mass spectrum of CSG1 colonies induced with 0.7% GBL produced three distinct regions; a very dense region of *m/z* 0-600 peaks with charge of 1, another region between *m/z* 680-870 with doubled and triplet charged peaks and the final distinct region between *m/z* 1150-1195 with doublet charge peaks.

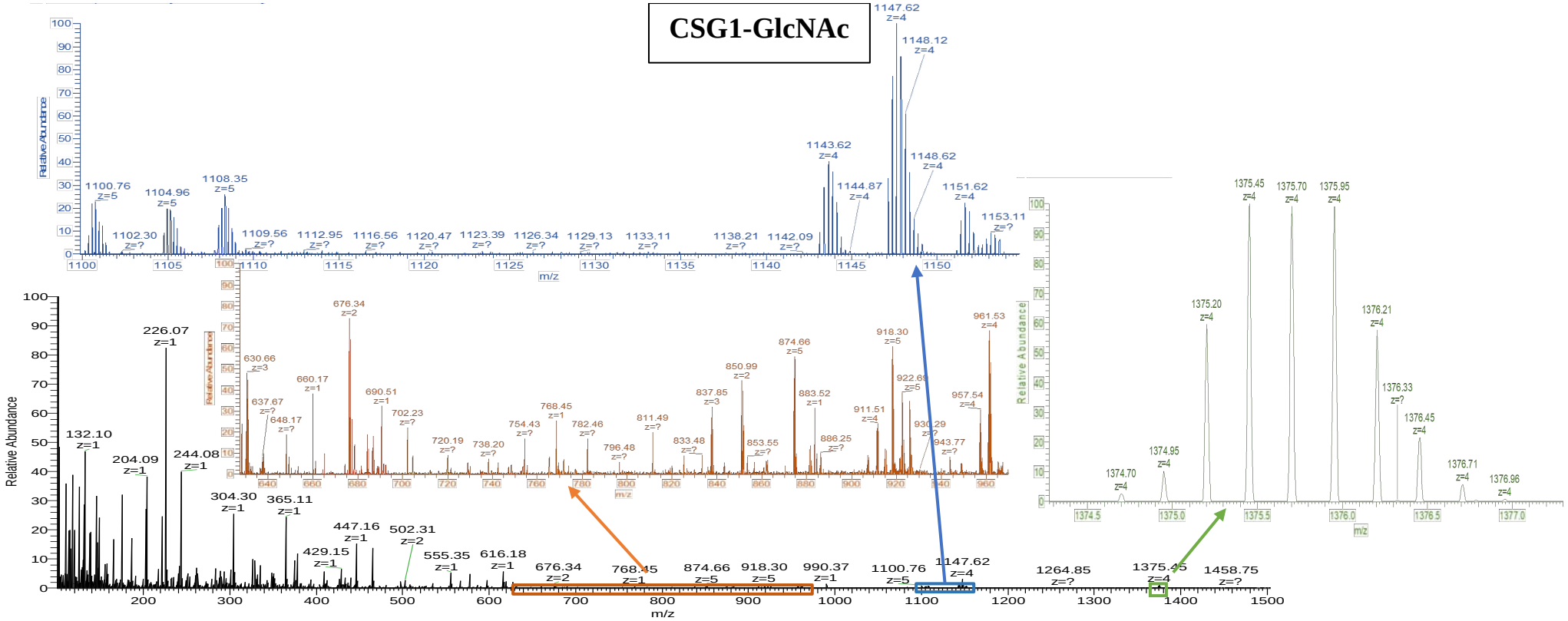


Figure 5. 21 LESA mass spectrum of CSG1 induced with GlcNAc

The spectrum generated by LESA of CSG1 induced with 50 mM GlcNAc had 4 distinct regions. A region with high signal and dense peaks between *m/z* 0-450 (black peaks of the main spectrum), a region between *m/z* 620-970 (brown box) with peaks of charges between 2-5 (brown spectra), another region between *m/z* 1050-1155 (blue box) containing peak spectra of charges 4-5 and a final region of *m/z* 1370-1380 with peaks of a charge of 4.

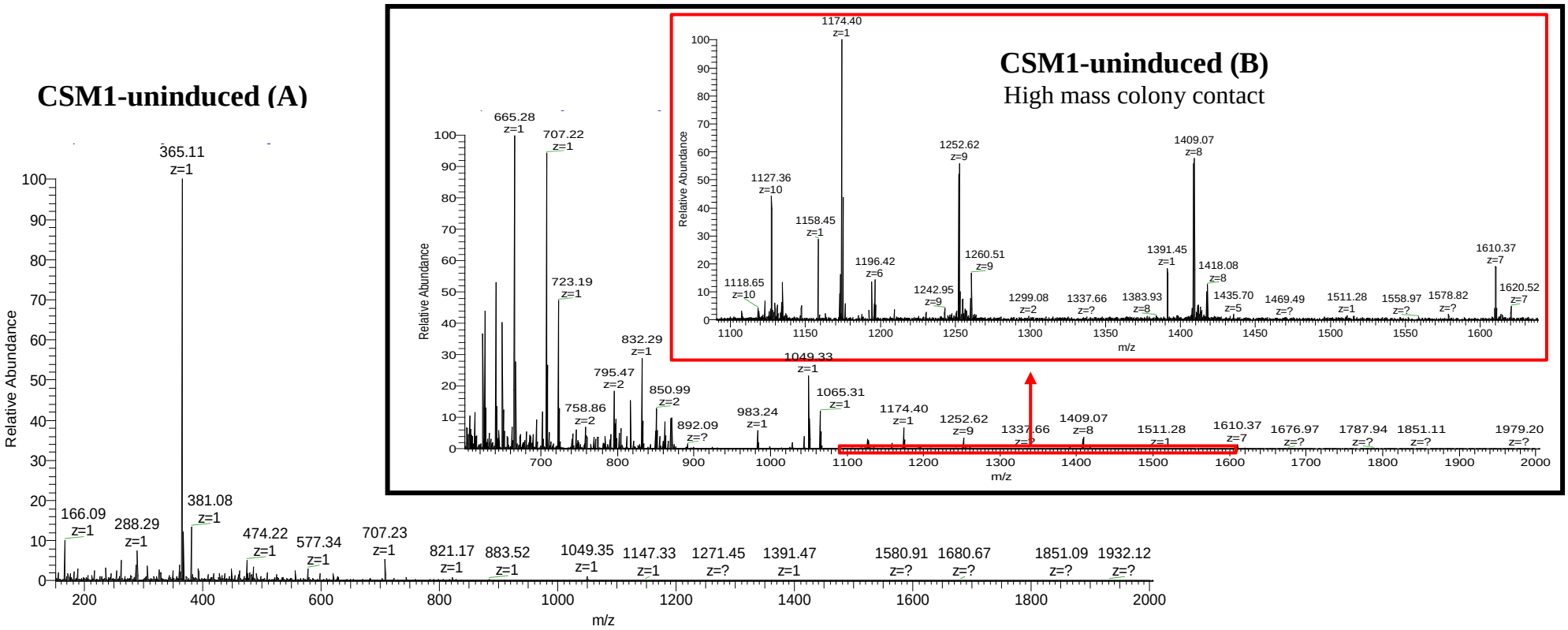


Figure 5. 22 LESA mass spectrum of CSM1 isolate

Two spectra were generated for CSM1 colonies, the first spectra (A) was generated by surface sampling where there was no direct contact of the pipette tip in the LESA system with the colony which resulted in low signal peaks between *m/z* 0-600 and all the peaks in the spectra contained a charge of 1. The second spectra (B) was generated by direct contact of the pipette tip with the colony which gave a better signal of the overall spectra and produced a region between *m/z* 1100-1650 (red box) with peak charges ranging between 2-10 (magnified spectra outlined with red box).

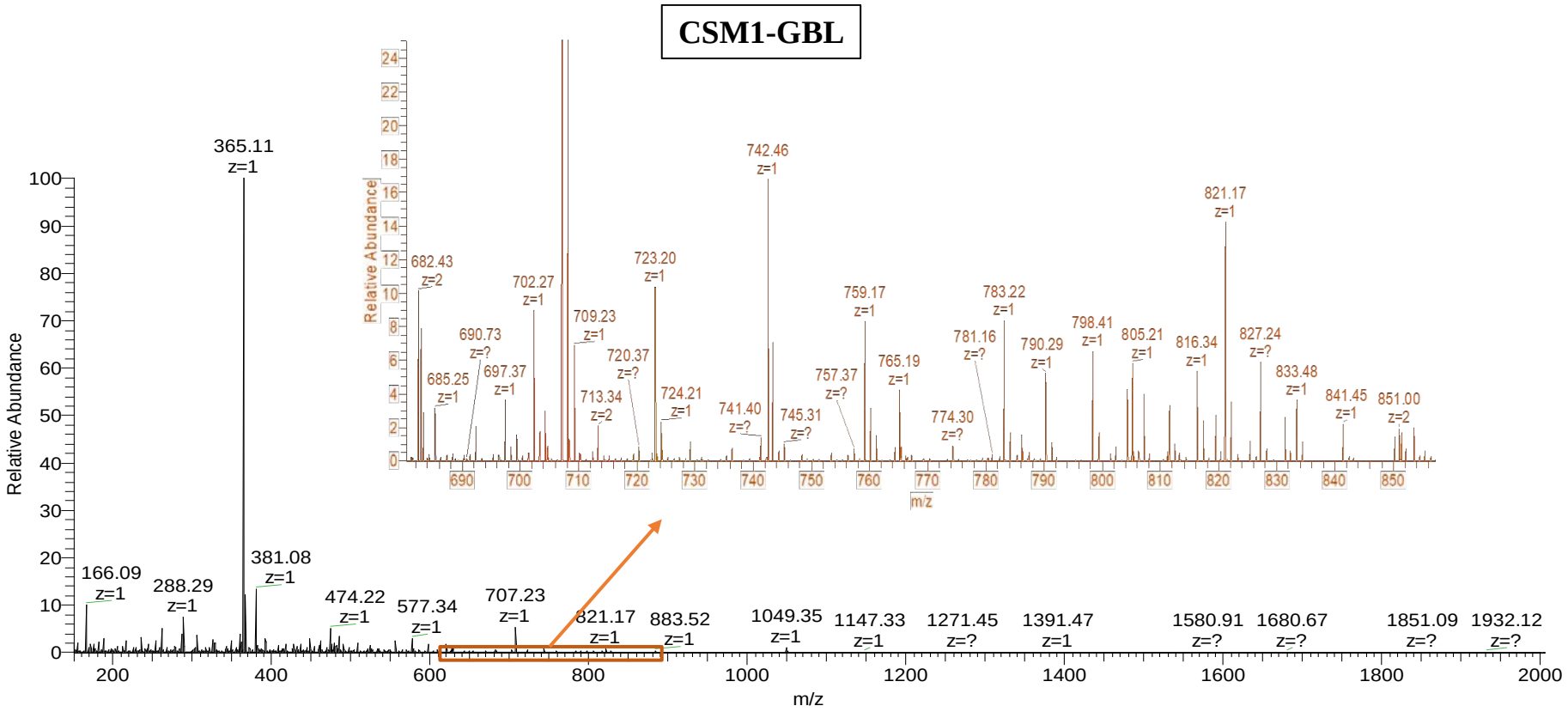


Figure 5. 23 LESA spectrum of CSM1 induced with GBL

The colonies of CSM1 induced with GBL resulted in low signal peaks between *m/z* 0-500 but there was a region between *m/z* 680-860 (brown box) with peaks of doublet charges as shown in the magnified brown spectrum.

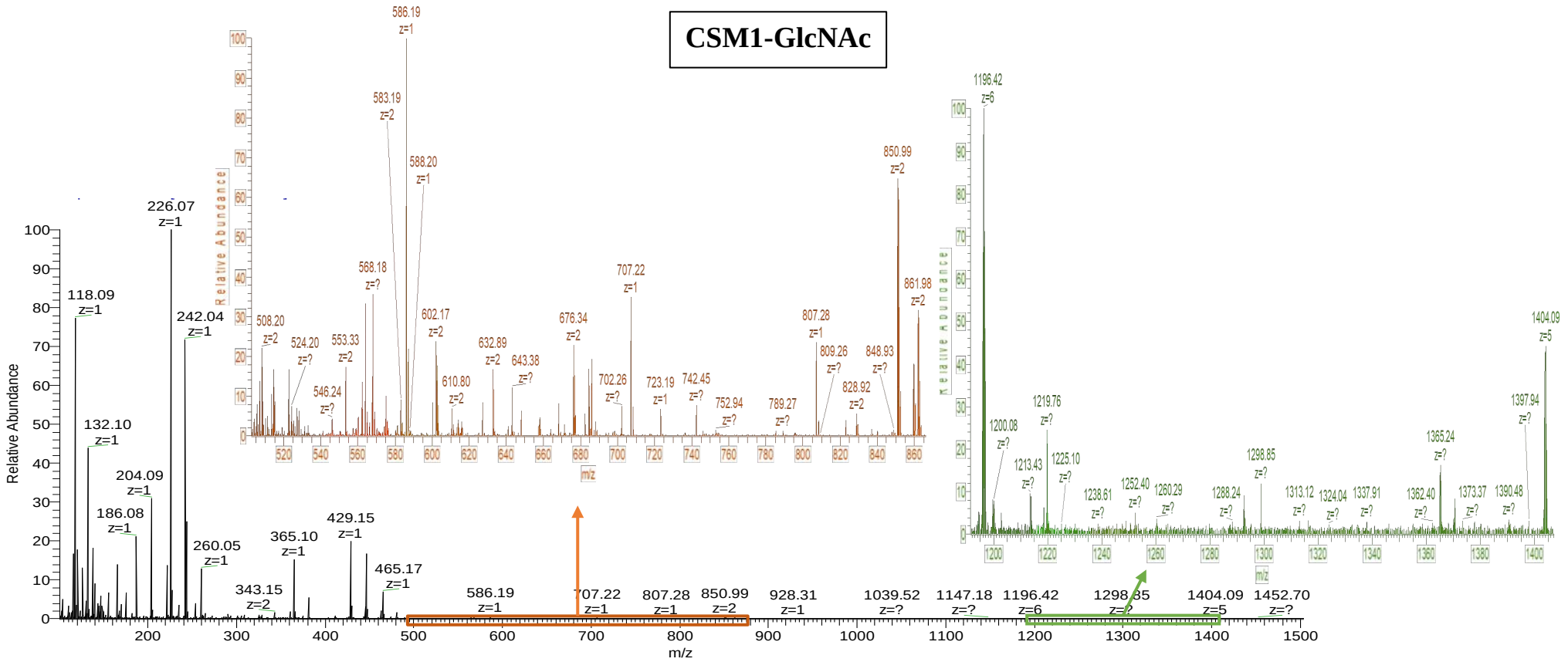


Figure 5. 24 LESA mass spectrum of CSM1 induced with GlcNAc

CSM1 colonies induced with GlcNAc generated dense peaks with high signal between *m/z* 0-450. In addition, two distinct regions were observed, one between *m/z* 500-865 with peaks of doublet charges and another region between *m/z* 1150-1410 with peaks of charge 6.

5.2.4. AntiSMASH and LESA BGC mass comparison

The LESA mass spectrum for each isolate was analysed for the presence of biosynthetic gene clusters (BGC) that were identified by antiSMASH. Tables 5.4-5.10 summarizes the masses, ID and structure of the BGCs identified for each isolate with Δppm of less than 5.000 (The generated mass peak for each compound are found in Appendix D). Cetoniacytone_A was identified in all isolates except for CSM1 (Appendix D: CSK1_213.063, CSK3_213.064, CSW2_213.064, CSU1_213.064, CSU2_213.064, CSG1_213.064 *m/z*). Desferrioxamine_B was identified in all isolates except for CSW2 (Appendix D: CSK1_560.686, CSK3_560.682, CSU1_560.682, CSU2_560.686, CSG1_560.686, CSM1_560.687 *m/z*).

BGCs with "unknown" cluster type, as classified by antiSMASH, were: meilingmycin identified in CSK1 and CSU1 (Appendix D: CSK1_626.775 and CSU1_626.777 *m/z*); meridamycin in CSK1, CSW2 and CSU2 (Appendix D: CSK1_822.092, CSW2_820.078 and CSU2_820.075 *m/z*); arginomycin in CSK1 and CSK3 (Appendix D: CSK1_436.463 and CSK3_436.466 *m/z*); herboxidiene in CSK3 and CSW2 (Appendix D: 438.607 and 438.604 *m/z*); JBIR-34 CSG1 (Appendix D_CSG1_480.897 *m/z*) ; lipomycin in CSK3 and CSU1 (Appendix D: 587.701 and 578.701 *m/z*); chlorozidine in CSW2, CSG1 and CSM1 (Appendix D: CSW2_444.087, CSG1_444.087 and CSM1_444.089 *m/z*); herbimycin found in CSU1 (Appendix D_CSU1_574.661 *m/z*).

Seven modular enzymes were identified; in isolate CSK3 polyoxypeptin_A (T1PKS-Butyrolactone, Appendix D_CSK3_969.145 *m/z*) was determined, in CSW2 splenocin (NRPS, Appendix D_CSW2_470.472 *m/z*), in CSU1 naphthyridinomycin (KS-NRPS, Appendix D_CSU1_417.550 *m/z*) and antimycin (T1PKS-NRPS, Appendix D_CSU1_534.600 *m/z*), in CSU2 polyoxypeptin_A, resistomycin (T2PKS) and splenocin (Appendix D: CSU2_969.141, CSU2_376.357 and CSU2_470.471 *m/z*) were found and finally isolate CSM1 had rabelomycin (T2PKS-Butyrolactone, Appendix D_CSM1_338.310 *m/z*) and coelichin (NRPS, Appendix D_CSM1_565.583 *m/z*) modular enzymes.

The rest of the BGCs were terpenes such as albaflavenone found in CSK3, CSG1 and CSM1 (Appendix D: CSK3_218.335, CSG1_218.335 and CSM1_218.336 *m/z*); hopene in CSK1, CSK3 and CSM1 (Appendix D: CSK1_410.717, CSK3_410.719 and CSM1_410.712 *m/z*); and carotenoid in CSK3, CSW2 and CSG1 (Appendix D: CSK3_213.064, CSW2_213.064 and CSG1_213.064 *m/z*). Informatipetin (bacteriocin) was identified in CSW2 only (Appendix D_CSW2_1065.573 *m/z*). Ectoine was determined in CSK1, CSK3, CSG1 and CSM1 (Appendix D: CSK1_142.155, CSK3_142.155, CSG1_142.155 and

CSM1_142.155 *m/z*). Finally, BGC sanglifehrin (other) was determined in CSK1 (Appendix D_CSK1_1090.393 *m/z*).

Table 5.11 summarizes the BGCs that were identified by antiSMASH and masses observed by LESA-MS. We can observe that the isolates indeed produce different metabolites. All isolates except for CSM1 (the negative control isolate) produced a BGC cluster similar to cetoniacytone_A which is an antitumour agent. The siderophore desferrioxamine_B is an iron chelator and is a highly conserved gene cluster in *Streptomyces* was found in all isolates except for CSW2 which instead showed similarity to informatipeptin cluster which was, also, unique to that isolate. Informatipeptin is identified as a class III lantipeptide formed through the post ribosomal peptide synthesis. Unique cluster similarities were observed to each isolate. Sanglifehrin A is an immunosuppressant that belongs to the immunophilin-binding ligands was found in CSK1 isolate only; CSU1 isolate identified BGCs similar to naphthyridinomycin (a broad spectrum antibiotic), antimycin (inhibits mitochondrial electron transport chain complex III and suppresses tumorigenesis in lung cancer cells) and herbimycin (an antibiotic that inhibits protein tyrosine kinase and inhibitor of angiogenesis); CSU2 to resistomycin (an antibiotic that has an antioxidant activity and can be cytotoxic against cancer cell lines); CSG1 to JBIR-34 (a nonribosomal peptide with uncommon 4-methyloxazoline moiety); and CSM1 to rabelomycin (belongs to angucyclines antibiotics with antibacterial activity against gram positive microorganisms). All other clusters were common to two or three isolates.

Three NRPS clusters were not identified to any known compounds by antiSMASH in CSK1, CSK3 and CSU2. NORINE, a nonribosomal peptide database (Caboche et al., 2010) was used to identify peptides through their annotations or monomeric structures. In CSK1, region 191.1, a polymer was predicted based on core scaffold assuming PKS/NRPS collinearity with (thr-val) in antiSMASH. A structure search in NORINE database of thr-val generated a list of peptides that were identified according to their similarity to the searched polymer (Caradec et al., 2014). N-coronafacoyl-L-valine peptide with mass of 307.178 and "toxin" activity was also observed by a peak at 307.175 *m/z* in LESA-MS of CSK1-GBL colonies and not in CSK1 colonies (Figure 5.25A). CSK1 NRPS regions 350.1 and 471.1 did not match any peptides in Norine database. For region 350.1, the predicted amino acid is: asp, asn, glu, gln, aad and it is not similar to any known compounds. As for region 471.1, the adenylation domain was not annotated and therefore amino acids can't be predicted.

As for CSU2, region 615.1 with mass of 487.304 was identified, based on (ala) polymer prediction, as beauverolide H cyclic peptide with unknown activity in NORINE which was observed as a peak of 487.303 *m/z* in CSU2-GBL LESA-MS (Figure 5.25B). The other two

unknown NRPSs in CSU2 are regions 405.1 and 500.1. Region 405.1 was predicted with val monomer identified to amycolamycins A and B (newly discovered enediyne-derived compounds with cytotoxic activity to the M231 breast cancer cell line.; Ma et al., 2017) but the compound could not be confirmed by LESA. As for region 500.1 the adenylation domain was not annotated and therefore amino acids can't be predicted.

As for CSK3, region 476.1 in CSK3 was identified with two peptides under the name of tokaramide A (mass of 476.274) as a protease inhibitor and pepstatin (mass of 685.462) with unknown activity based on (val) polymer prediction and were observed by LESA as peaks of 476.272 and 685.461 *m/z* respectively (Figure 5.26A). Additionally, geodiamolide C (mass of 549.262) with antitumour activity and halipeptin B (mass of 612.356) with anti-inflammatory activity were identified by NORINE for region 547.1 based on (ala) polymer prediction and were determined with peaks of 549.26 *m/z* and 612.358 *m/z* by LESA respectively (Figure 5.26 B).

Table 5. 4 CSK1 BGCs identified by antiSMASH and LESA

The masses of BGCs identified by antiSMASH were correlated to spectrum masses generated by LESA. The below molecules were identified by both systems for CSK1 isolate with Δ ppm less than 5.

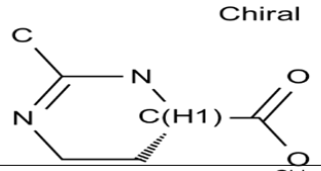
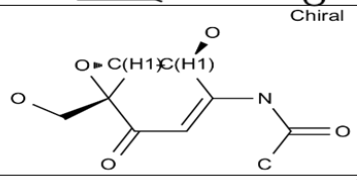
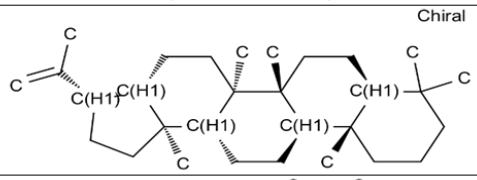
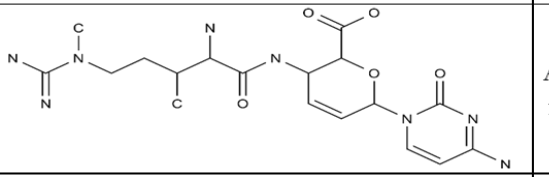
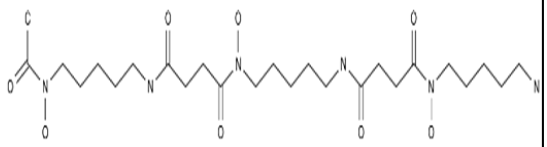
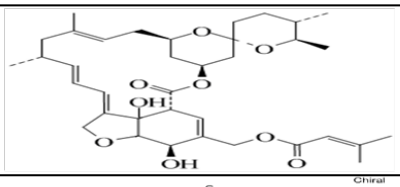
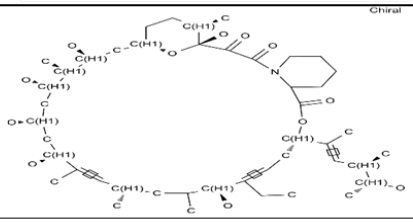
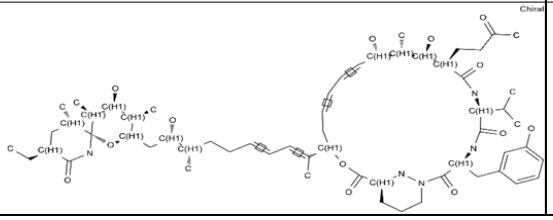
antiSMASH mass	LESA mass spectra, m/z	Δ, ppm	Structure	Molecular ID
142.155	142.155	0.000		Ectoine: osmolyte
213.063	213.063	0.000		Cetoniacytone_A: anti-tumour
410.718	410.717	2.435		Hopene: Terpenes
436.465	436.463	4.582		Arginomycin: peptidyl nucleoside antibiotic
560.684	560.686	-3.567		Desferrioxamine_B: Iron chelator
626.777	626.775	3.191		Meilingmycin: macrolide antibiotic
822.090	822.092	-2.433		Meridamycin: non-immunosuppressant
1090.390	1090.393	-2.751		Sanglifehrin_A: immunosuppressant

Table 5. 5 CSK3 BGCs identified by antiSMASH and LESA

The masses of BGCs identified by antiSMASH were correlated to spectrum masses generated by LESA. The below molecules were identified by both systems for CSK3 isolate with Δ ppm less than 5.

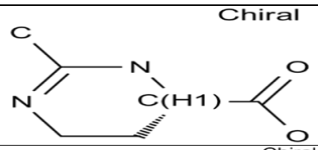
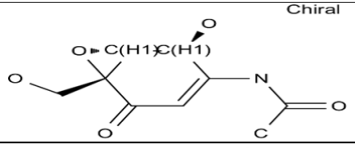
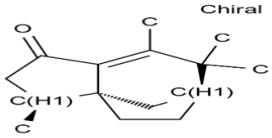
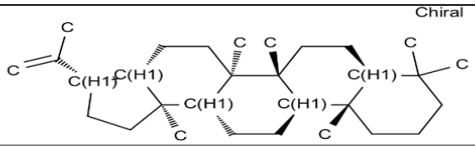
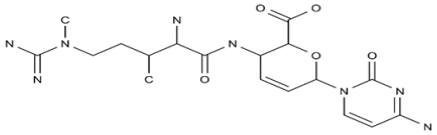
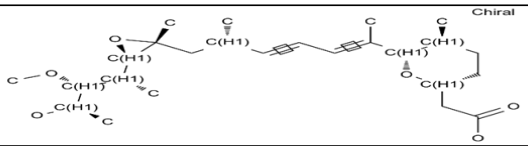
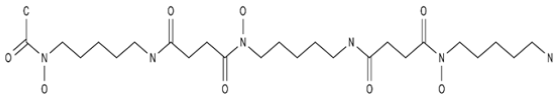
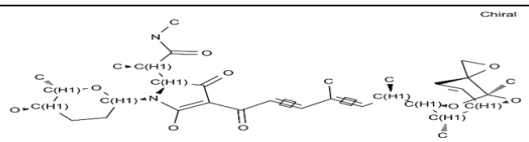
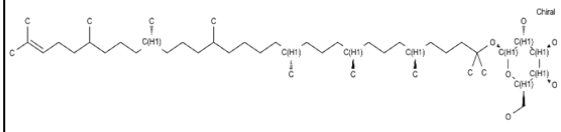
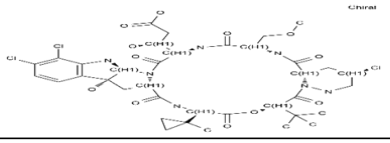
antiSMASH mass, m/z	LESA mass spectra, m/z	Δ, ppm	Structure	Molecular ID
142.155	142.155	0.000		Ectoine
213.063	213.064	-4.693		Cetoniacytone_A: anti-tumour
218.335	218.335	0.000		Albaflavenone
410.718	410.719	-2.435		Hopene: Terpenes
436.465	436.466	-2.291		Arginomycin: peptidyl nucleoside antibiotic
438.605	438.607	-4.560		Herboxidiene: polyketide molecule
560.684	560.682	3.567		Desferrioxamine_B
587.701	587.701	0.000		Lipomycin
568.871	568.871	0.000		Carotenoid: organic pigment
969.144	969.145	-1.032		Polyoxypeptin A

Table 5. 6 CSW2 BGCs identified by both antiSMASH and LESA analysis

The masses of BGCs identified by antiSMASH were correlated to spectrum masses generated by LESA. The below molecules were identified by both systems for CSW2 isolate with Δ ppm less than 5.

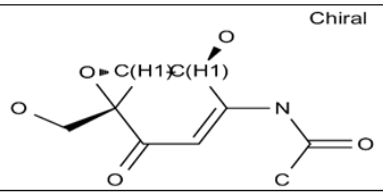
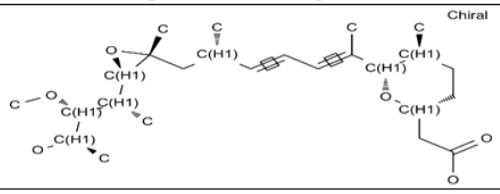
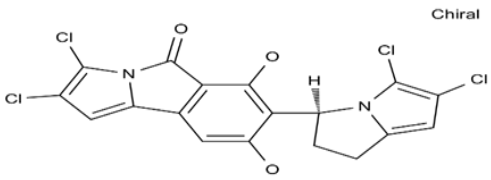
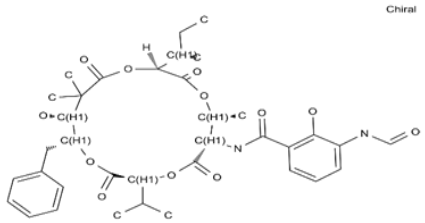
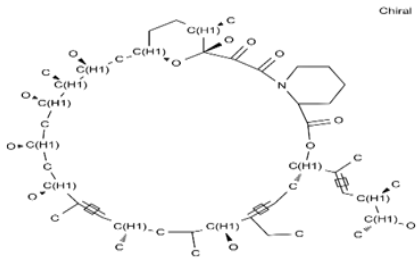
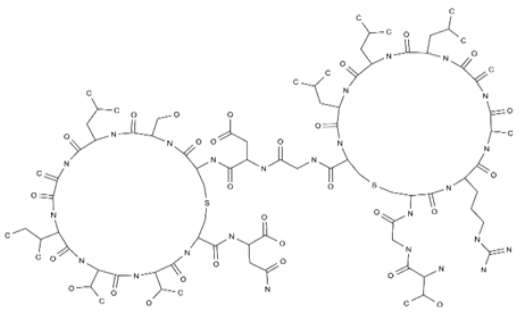
antiSMASH mass	LESA mass spectra	Δ, ppm	Structure	Molecular ID
213.063	213.064	-4.693		Cetoniacytone_A: anti-tumour
438.605	438.604	2.280		Herboxidiene: polyketide molecule
444.089	444.087	4.504		chlorizidine: immunosuppressive macrocyclic natural product
470.472	470.472	0.000		Splenocin
820.074	820.078	-4.878		Meridamycin: non-immunosuppressant
1065.57	1065.573	-2.815		Informatipeptin

Table 5. 7 CSU1 identified by both antiSMASH and LESA analysis

The masses of BGCs identified by antiSMASH were correlated to spectrum masses generated by LESA. The below molecules were identified by both systems for CSU1 isolate with Δ ppm less than 5.

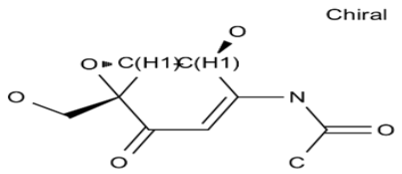
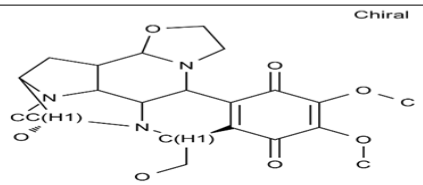
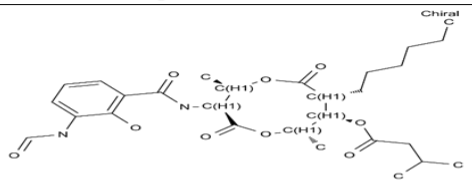
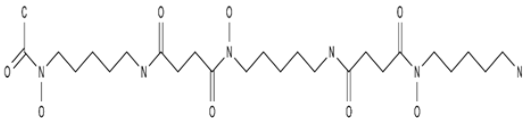
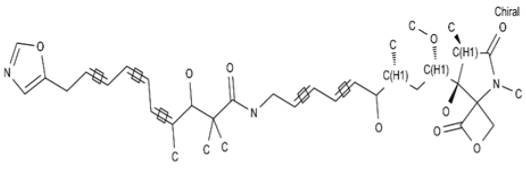
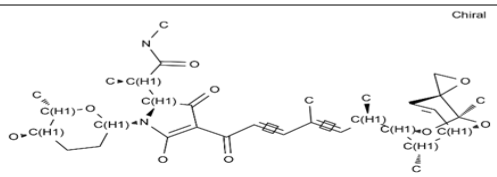
antiSMASH mass	LESA mass spectra, m/z	Δ, ppm	Structure	Molecular ID
213.063	213.064	-4.693		Cetoniacytone_A
417.456	417.455	2.395		Naphthyridinomycin
534.599	534.600	-1.871		Antimycin
560.684	560.682	3.567		Desferrioxamine_B
574.662	574.661	1.740		Herbimycin
587.701	587.700	1.702		Lipomycin

Table 5. 8 CSU2 BGCs identified by both antiSMASH and LESA analysis

The masses of BGCs identified by antiSMASH were correlated to spectrum masses generated by LESA. The below molecules were identified by both systems for CSU2 isolate with Δ ppm less than 5.

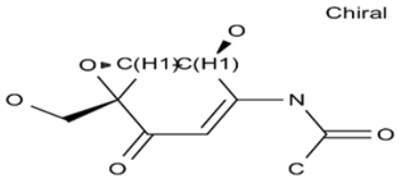
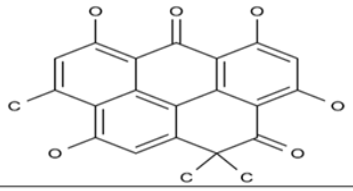
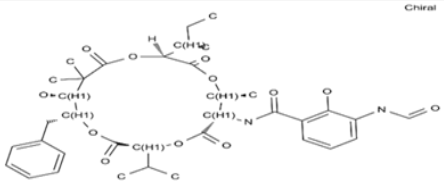
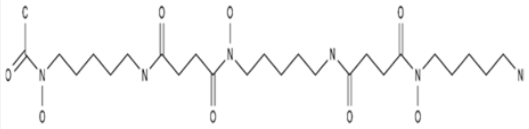
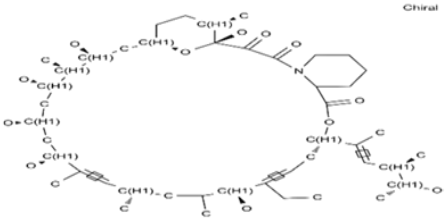
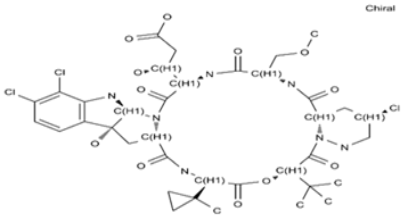
antiSMASH mass	LESA mass spectra, m/z	Δ, ppm	Structure	Molecular ID
213.063	213.064	-4.693		Cetoniacytone_A
376.359	376.357	5.314		Resistomycin
470.472	470.471	2.126		Splenocin
560.684	560.686	-3.567		Desferrioxamine_B
820.074	820.075	-1.219		Meridamycin: non-immunosuppressant
969.144	969.141	3.096		Polyoxypeptin A

Table 5. 9 CSG1 BGCs identified by both antiSMASH and LESA analysis

The masses of BGCs identified by antiSMASH were correlated to spectrum masses generated by LESA. The below molecules were identified by both systems for CSG1 isolate with Δ ppm less than 5.

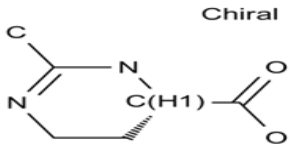
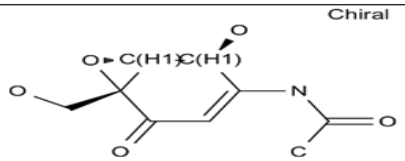
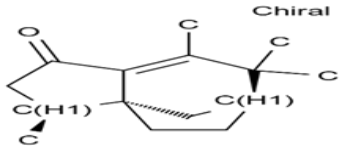
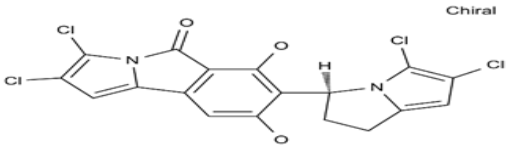
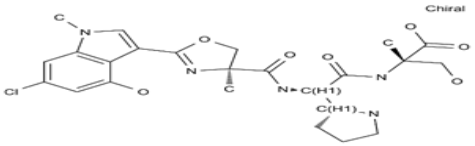
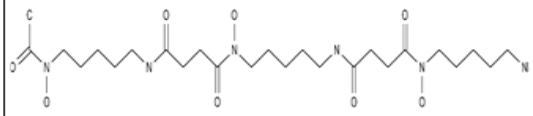
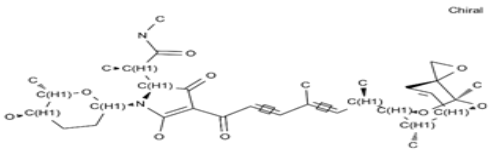
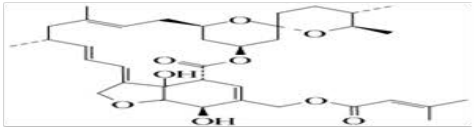
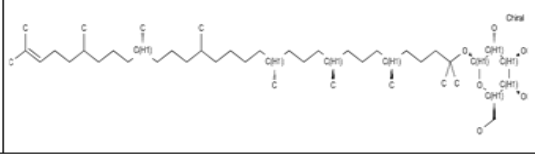
antiSMASH mass	LESA mass spectra, m/z	Δ, ppm	Structure	Molecular ID
142.155	142.155	0		Ectoine
213.063	213.064	-4.693		Cetoniacytone_A
218.335	218.335	0		Albaflavenone
444.089	444.087	4.5036		chlorizidine: immunosuppressive macrocyclic natural product
480.899	480.897	4.1589		JBIR-34
560.684	560.686	-3.567		Desferrioxamine_B
587.701	587.701	0		Lipomycin
626.777	626.777	0		Meilingmycin
739.203	739.204	-1.353		Carotenoid

Table 5. 10 CSM1 BGCs identified by both antiSMASH and LESA analysis

The masses of BGCs identified by antiSMASH were correlated to spectrum masses generated by LESA. The below molecules were identified by both systems for CSM1 isolate with Δ ppm less than 5.

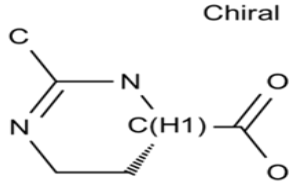
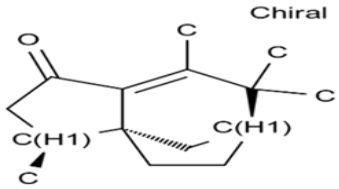
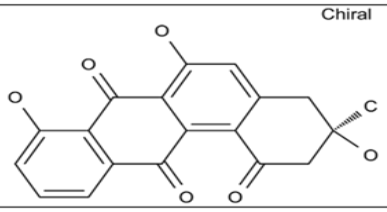
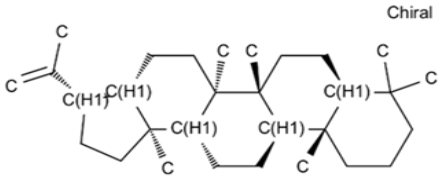
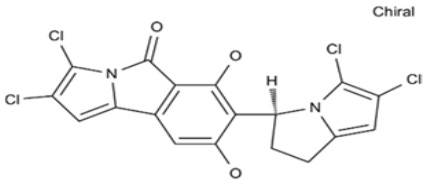
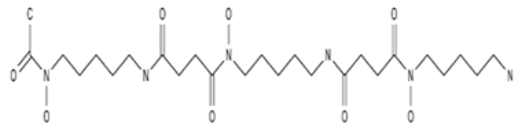
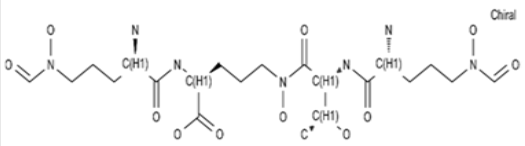
antiSMASH mass	LESA mass spectra, m/z	Δ, ppm	Structure	Molecular ID
142.155	142.155	0.000		Ectoine
218.335	218.336	4.580		Albaflavenone
338.311	338.310	-2.956		Rabelomycin
410.710	410.712	4.870		Hopene
444.089	444.089	0.000		chlorizidine: immunosuppressive macrocyclic natural product
560.684	560.687	5.351		Desferrioxamine_B
565.581	565.583	3.536		Coelichelin

Table 5. 11 Summary of BGCs identified for each isolate

The BGCs identified by both antiSMASH and LESA-MS analysis are summarized in the below table

BGC	CSK1	CSK3	CSW2	CSU1	CSU2	CSG1	CSM1
Ectoine	X	X				X	X
Cetoniacytone_A	X	X	X	X	X	X	
Hopene	X	X					X
Arginomycin	X	X					
Desferrioxamine_B	X	X		X	X	X	X
Meilingmycin	X			X		X	
Meridamycin	X		X		X		
Sanglifehrin_A	X						
Albaflavenone		X				X	X
Herboxidiene		X	X				
Lipomycin		X		X		X	
Carotenoid		X				X	
Polyoxypeptin_A		X			X		
Chlorizidine			X			X	X
Splenocin			X		X		
Informatipeptin			X				
Naphthyridinomycin				X			
Antimycin				X			
Herbimycin				X			
Resistomycin					X		
JBIR-34						X	
Rabelomycin							X

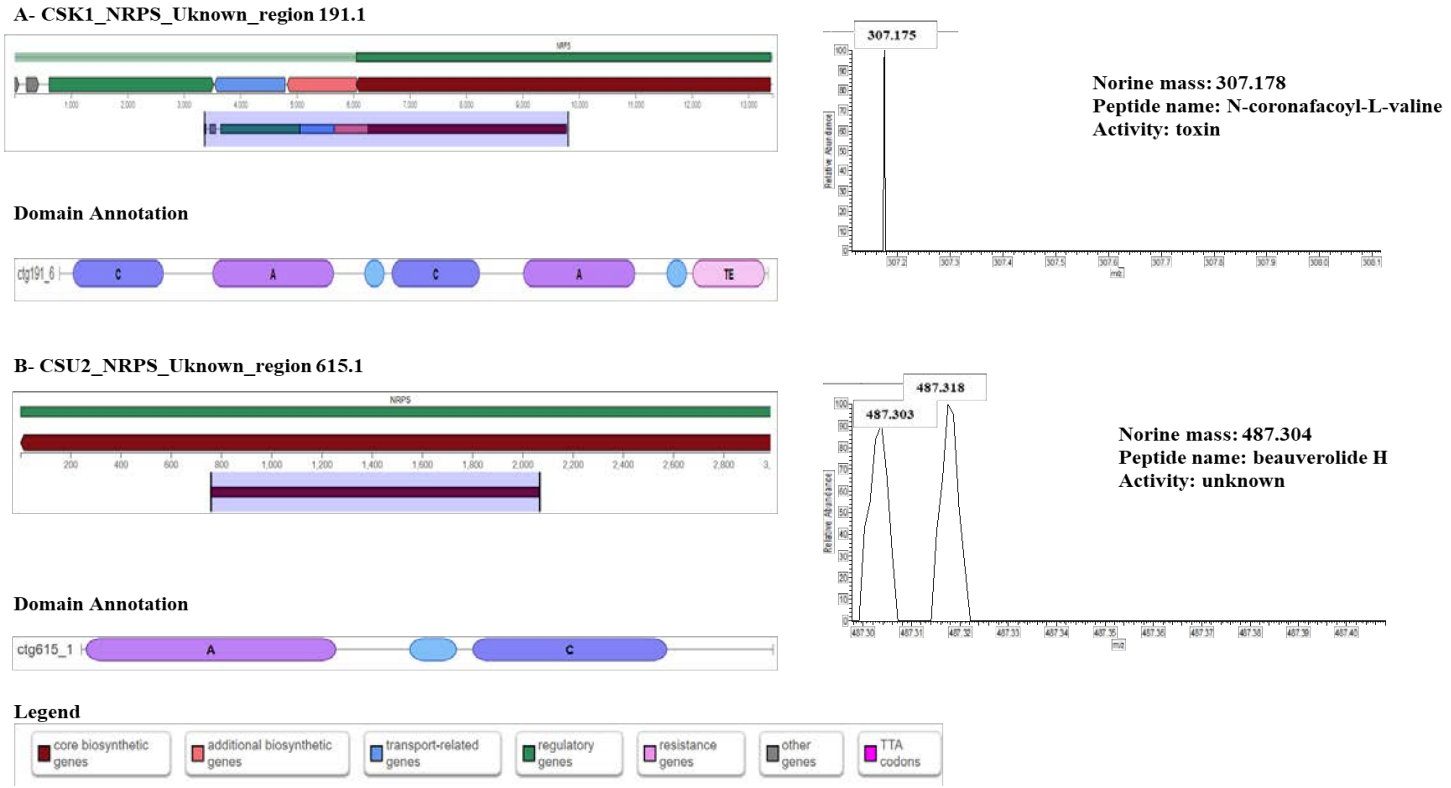


Figure 5. 25 Biosynthetic gene clusters encoding "unknown" NRPS product for CSK1 and CSU2

NRPS clusters encoded in the CSK1 region 191.1 (A) and CSU2 region 615.1 (B) genomes and their protein domains as identified by antiSMASH with unpredicted product. Predicted domains and nonribosomal peptides are coloured by general function. Domain key: A-adenylation, C- condensation and TE- thioesterase. NORINE database (Caboche et al., 2010) was used to estimate peptides based on antiSMASH polymer prediction. Masses of the peptides were confirmed by LESA-MS peaks as observed in the above spectra.

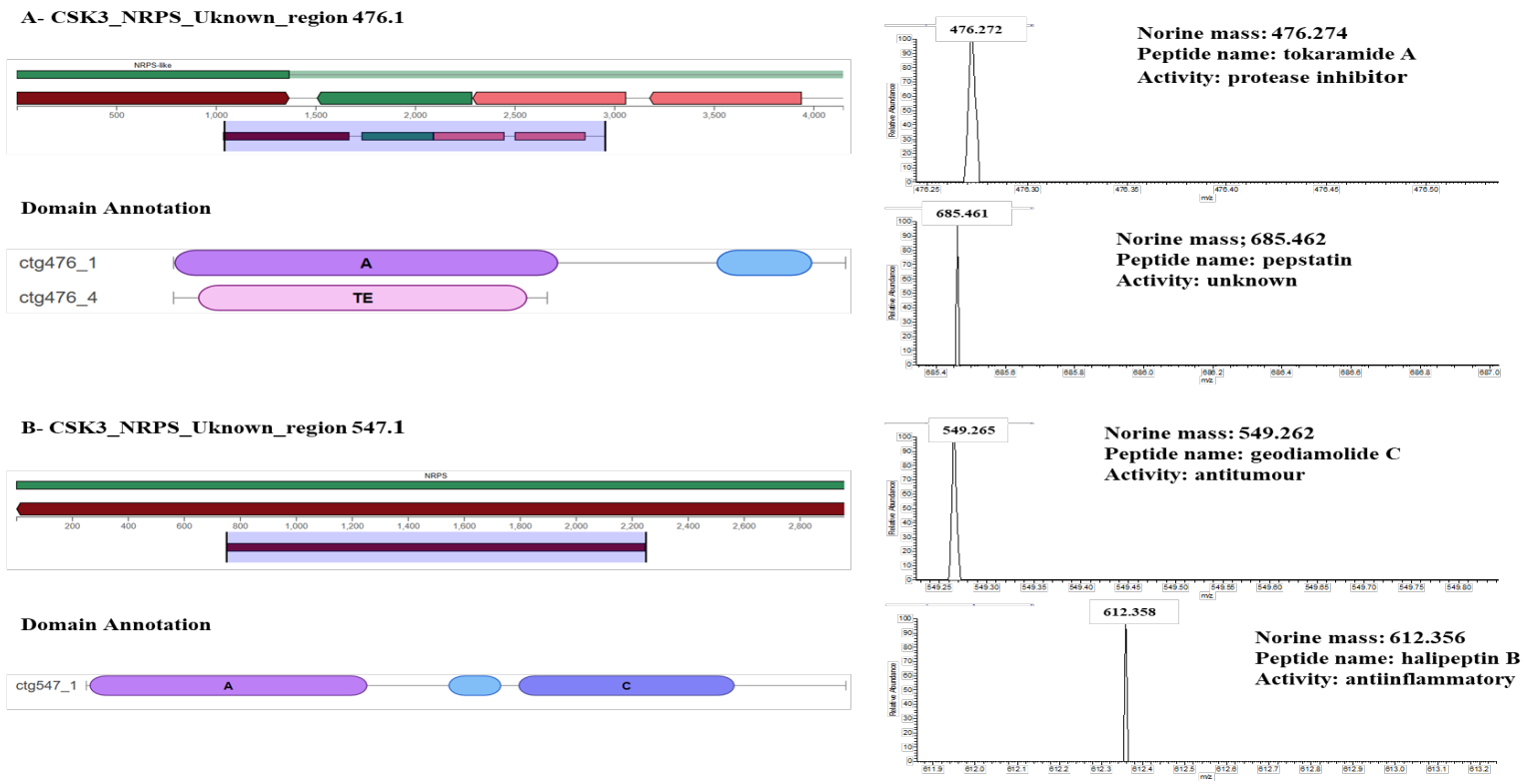


Figure 5. 26 Biosynthetic gene clusters encoding "unknown" NRPS product for CSK3

NRPS clusters encoded in the CSK3 region 476.1 (A) and 547.1 (B) genome and their protein domains as identified by antiSMASH with unpredicted product. Predicted domains and nonribosomal peptides are coloured by general function. Domain key: A-adenylation, C- condensation and TE- thioesterase. NORINE database (Caboche et al., 2010) was used to estimate peptides based on antiSMASH polymer prediction. Masses of the peptides were confirmed by LESA-MS peaks as observed in the above spectra.

5.3. Discussion

In this study, we report the use of genomics combined with metabolomics to characterize and identify secondary metabolites from seven strains of *Streptomyces*. Strains CSK1, CSK3, CSW2, CSU1, CSU2, CSG1 and CSM1were isolated from different cultivated terrestrial areas in UAE and its genomes were sequenced. Main features of the genomes such as size, GC content and coding sequences (Table 5.1) were typical of other *Streptomyces*. The 16S rRNA analyses distinctly showed that the isolates correlated to the *Streptomyces* genus (Figure 5.2). CSK1, CSW2, CSU2 and CSG1 were found to be closely related to each other and to *Streptomyces sp.* E2N17. *Streptomyces sp.* E2N17 is an unpublished strain with antimicrobial activity isolated from Fetzara Lake in the north eastern region of Algeria. Isolates CSK3 and CSU1 were closely related to *Streptomyces sp.* ETH9427 (Thibessard et al., 2018), this strain was previously described as *Streptomyces ambofaciens* ETH9427 (Hütter, 1967). As for CSM1, the negative isolate in the study was found to be closely related to *S. albogriseolus* ABRIINW EA1145 strain (unpublished strain). Whole genome sequence blast search revealed that all sequences were closest to *Streptomyces sp.* ETH9427 with 98% to 99% identity and query cover ranging between 34 to 91 % for the different isolates (Appendix B) except for CSM1 which was found closest to *S. niger* strain 452 with 98% identity and 82% query coverage.

The seven isolates along with *S. coelicolor* (most studied and representative model of the genus) and *Streptomyces sp.* ETH9427 (closely related to all the isolates by whole genome blast) were genetically classified into subsystems by RAST system (Table 5.2). The highest number of correlated genes to a subsystem were identified in *S. coelicolor* followed by CSM1 and CSU1 isolates with small differences in number. *Streptomyces sp.* ETH9427 had the lowest number of genes for most subsystems. Seventeen genes correlating to secondary metabolism were identified for *S. coelicolor* followed by 8 for CSU1 and, interestingly, 7 for CSM1. In KEGG pathway analysis, same trend was observed where the highest percentage of enzymes for the various pathways were observed in *S. coelicolor* followed by CSU1 which was very closely correlated and then CSM1 isolate (Table 5.3). In general, all isolates have demonstrated their involvement in the biosynthesis of antibiotics, sugars, siderophores, polyketides and terpenoids.

We have found and demonstrated that all isolates were very similar to BGCs with antimicrobial and antitumor properties such as polyoxipeptin_A, arginomycin, meilingmycin,

antimycin, lipomycin, chlorizidine, herboxidiene, meridamycin, rabelomycin, herbimycin and splenocin.

Direct sampling of the colonies generated by the isolates uninduced or induced with GBL or GLcNAc using acetonitrile, water and formic acid solvent was performed with liquid extraction surface analysis (LESA) to generate mass spectra of the produced metabolites. We have shown that the spectral profiles between the uninduced and induced isolates were different. This change was either in the appearance of new peaks, disappearance of others or increase of intensity in some. In this study, we have shown that some BGCs generated by antiSMASH were produced in the LESA analysis such as arginomycin, meilingmycin, meridamycin, herboxidiene, JBIR-34, lipomycin, antimycin, polyoxypeptin_A, cetoniacytone_A, chlorizidine, splenocin, naphthyridinomycin, herbimycin, resistomycin, coelichin and rabelomycin in addition to the commonly found core BGCs such as carotenoid, informatipeptin, ectoine, hopene, albaflavenone and desferrioxamine_B.

Some of the putative NRPS-encoding clusters in CSK1, CSK3 and CSU2 showed no similarity to any reference BGCs by antiSMASH. The number of amino acids and molecular weight for these peptides were predicted by NORINE database and were further confirmed by LESA (Table 5.12). N-coronafacoyl-L-valine is a toxin that was predicted by NORINE database for CSK1 and a peak was observed in CSK1-GBL LESA spectra. NORINE database has determined beauverolide H peptide with unknown activity for one of CSU2 NRPS cluster blast and a peak was observed in CSU2-GBL LESA spectra. These results confirm that using GBL as an elicitor produced molecules that were not generated in the non-elicited strain. Finally, four peptides were identified for CSK3 as tokaramide A (protease inhibitor), pepstatin (unknown activity), geodiamolide C (antitumour) and halipeptin B (anti-inflammatory) and confirmed by the LESA spectra. Finally, the most interesting results were the three BGCs encoding for NRPS (CSK1-350.1, CSK1 471.1 and CSU2 500.1) that were not predicted to any known peptides by both antiSMASH and NORINE database and thus may have novel biological activity.

Table 5. 12 Properties of novel BGCs and Peptides

NRPS Biosynthetic gene clusters, that were not correlated to known compounds by antiSMASH, were identified by NORINE database based on structure search. Predicted peptides were generated based on the monomer structure. LESA-MS was analysed to confirm the presence of these peptides.

Strain	Cluster region	Predicted monomer	Peptide	Activity	LESA-MS *m/z*
CSK1	NRPS 191.1	Thr-Val	N-coronafacoyl-L-valine	toxin	307.175
CSK1	NRPS 350.1	asp, asn, glu, gln, aad	unknown	-	-
CSK1	NRPS 471.1	-	-	-	-
CSU2	NRPS 615.1	ala	Beauverolide H	unknown	487.304
CSU2	NRPS 405.1	val	Amycolamycins A and B	cytotoxicity	-
CSU2	NRPS 500.1	-	-		-
CSK3	NRPS 476.1	val	Tokaramide A	protease inhibitor	476.272
			Pepstatin	unknown	685.462
CSK3	NRPS 547.1	ala	Geodiamolide C	antitumour	549.260
			Halipeptin B	anti-inflammatory	612.358

Chapter 6

General Discussion

6. General Discussion

Streptomyces are Gram positive bacteria that have been known as a rich source of producing secondary metabolites which have been used in clinical, biotechnological and agricultural aspects (Harir et al., 2018). They are found in environments with a variety of nutrients and they are capable of utilizing different carbon, nitrogen and phosphate sources. Typically, in scarce conditions cell growth ceases and aerial mycelium is formed which then differentiates into spores. This morphological change along with metabolic changes leads to the initiation of secondary metabolism. Spores are formed to resist the unfavourable environmental conditions and spread the bacterium. Under these famine conditions, the bacteria produce degradative enzymes such as lipases and proteases and form dry resistant exospores (Rigali et al., 2008).

Screening for antibiotics has been done in the last 50 years and the urge to find new antibiotics has increased especially with the emergence of multi-drug resistant bacteria (Li and Webster, 2018). New approaches are required that should aim on selecting microorganisms that possess novel and potent antibiotics. The biosynthetic genes that encode for the production of secondary metabolites are located in clusters known as biosynthetic gene clusters (BGCs) along with resistance gene and transcriptional gene regulators to control the biosynthesis process (Adegboye and Babalola, 2012).

Genome sequencing has revealed that *Streptomyces* contain many biosynthetic gene clusters that are poorly expressed or silent and therefore their corresponding secondary metabolites are not detectable (Cao et al., 2016). These species have the potential to produce more natural products than initially detected. This has stimulated researchers to sequence and analyse the genomes of species to identify novel BGCs (Gross, 2009; Medema et al., 2011) and study the conditions required to induce the production of their corresponding metabolites in vitro (Tyc et al., 2014).

The main aim of this thesis was to isolate novel *Streptomyces* isolates with antibacterial and antitumour activity and to identify the secondary metabolites they produce. In this study, we have isolated and characterized seven *Streptomyces* isolates from different areas of the UAE (unexplored habitat). Six isolates (CSK1, CSK3, CSW2, CSU1, CSU2 and CSG1) showed an inhibitory effect against *E. coli*, *S. aureus*, *C. albicans*, *S. cerevisiae* and *E. coli*-ESBL as determined using minimum inhibitory concentration and well diffusion methods. The seventh isolate, CSM1, did not show any inhibitory effect against any of the tested bacteria. We also showed that some of the isolates had cytotoxic activity when tested against HeLa

cancer cells. Isolates CSK3, CSW2, CSU1 and CSG1 inhibited the growth of HeLa cells with IC_{50} values ranging from 3.46 µg/µl for CSK3 to 9.74 µg/µl for CSU1.

In nature, bacteria are never found as single species but as part of a community where they are consistently interacting with their own species or other organisms (Braga et al., 2016). Soil contains a diverse and dense microbial community with prokaryotic population between 10^7 to 10^{10} cells/g soil (Uroz *et al.*2010). This heterogeneous and nutrient poor soil environment involves the exchange of molecular and genetic information between organisms in a variety of mechanisms and molecules such as secondary metabolites, siderophores and quorum sensing in response to an environmental stimulus (Braga et al., 2016). The production of secondary metabolites by bacteria in the soil is a survival strategy to defend their host, compete for nutrients or fight off predators (Demoling et al., 2007). As part of communication, *Streptomyces* are able to produce and sense chemical signals with neighbouring organisms. Chemical compounds such as γ-butyrolactones (GBL, signalling molecule) and *N*-acetylglucosamine (GlcNAc, an amino sugar) that are found in *Streptomyces* habitat but not in the culture media in vitro may play an important role as signals in the activation of silent metabolites (Niu et al., 2016). In addition, *Streptomyces* secondary metabolism can be stimulated by elicitation which is based on introducing cell extracts of a competing species (such as heat killed cells of *B. subtilis*) in order to mimic the interspecies interactions in nature and results in the production of antimicrobial compounds (Radman et al., 2003).

Cultivation with heat killed cells of *B. subtilis*, ϒ-Butyrolactone or *N*-acetylglucosamine was performed on the six isolates to enhance the activity of the produced secondary metabolites or activate the expression of cryptic secondary metabolite biosynthetic gene clusters. Interestingly, the colour of cultured CSK1 changed to red when cultivated with *B. subtilis* and *N*-acetylglucosamine and activity against *S. aureus* was observed after 6 days of culturing compared to the monoculture which normally shows inhibitory activity after 14 days of culturing. In addition, cytotoxicity of the extracts isolated from cultivating CSK1 isolate with *B. subtilis* and *N*-acetylglucosamine caused inhibition of growth of HeLa cells compared to no inhibition of the CSK1 monoculture extract. This clearly shows a change in the bioactive genes produced from the CSK1 monoculture and CSK1 cultures with elicitors.

The generation of biosynthetic genes from elicitation could be due to microbial competition and thus activation of production of cryptic bioactive molecules as a means of survival or antagonism (Abdelmohsen, 2015). The change in colour to red could be due to the production of undecylprodigiosin an antibiotic known to be produced by *S. coelicolor* (White and Bibb, 1997). But, based on antiSMASH results, the BGC encoding for undecylprodiginines was not

detected for CSK1 isolate. The colour change may be due to a pH change and this needs to be investigated further. Also, the earlier inhibitory effect of the isolate can be due to the environmental changes that take place in the cultivation because of changes in growth, signalling molecules or from changes in nutrition (Marmann et al., 2014). The results could also imply that the elicitation of the biosynthetic genes could be due to the bacterial cell wall. *B. subtilis* as a Gram positive bacterium contains a cell wall with thick peptidoglycan which maintains the cell shape of the bacteria. Changes or interruption in the cell that could have occurred due to cell lysis or heating can lead to the peptidoglycan acting as a signalling molecule (Peterson et al., 2006) as peptidoglycan fragments have been found to play an important role in interspecies interactions (Koropatnick et al., 2004) in which it can act as a carbon and nitrogen source (Peterson et al., 2006) to the *Streptomyces* species. Rigali et al., 2006 have shown that *N*-acetyl glucosamine, which is one of the main components of the peptidoglycan, can trigger antibiotic production in *Streptomyces*. Addition of this monomer to the CSK1 culture could have stimulated the production of undecylprodigiosin observed by the red colour in the medium.

Genomic sequencing analysis of the seven isolates (CSK1, CSK3, CSW2, CSU1, CSU2, CSG1 and CSM1) revealed a genome size, GC content and other features that are typical of *Streptomyces*. The 16S rRNA clearly show that the isolates belong to the *Streptomyces* genus that were isolated from soil. Based on whole genome blast, the isolates were closely related to *Streptomyces sp.* ETH9427 (draft genome sequence; accession number: NZ_CP029624.1; Thibessard et al., 2018) which was previously identified as *S. ambofaciens*. *S. ambofaciens* bacterium is found in soil, it produces the antitumour oligopeptide netropsin and macrolide antibiotic spiramycin (Juguet et al., 2009). AntiSMASH results of *S. ambofaciens* showed 25 BGCs with many silent clusters in its genome such as kinomycins, stambomycins and antimycins (Aigle et al., 2014). As for the 16S rRNA blast, it showed CSK1, CSW2, CSU2 and CSG1 to be closely correlated with *Streptomyces sp.* E2N17 (accession number: KX279529.1), a strain deposited into NCBI but with no studies in the literature; CSU1 and CSK3 with *Streptomyces sp.* ETH9427 which is also not studied (draft genome sequence) and CSM1 with *S. albogriseolus* strain ABRIINW EA1145 (draft genome sequence; accession number GQ925802.1). *S. albogriseolus* is a bacterium isolated from soil and produces neomycin B and neomycin C (Benedict *et al.*, 1954) but ABRIINW EA1145 strain is not identified in the literature. Since there is no available literature on the strains that are closely related to our isolates, it was hard to correlate the BGCs of the isolated strains to known strains.

The number of BGCs, predicted by antiSMASH, in the genomes ranged between 25 to 29 and some NRPS clusters in CSK1, CSK3 and CSU2 did not appear to encode production of any known molecules which may be of particular interest as this may indicate potential to produce novel secondary metabolites. Genomic analysis predicted that all isolates carry biosynthetic gene clusters that are capable of producing a variety of secondary metabolites and some of these metabolites were identified by LESA analysis. The variety of BGCs in each isolate confirms the potential of these isolates to produce a diverse range of compounds (Sekurova et al., 2019). All isolates, except for CSM1, carried clusters encoding production of antimycin (antifungal) and naphthyridinomycin (antimicrobial and antitumour) metabolites. Chlorizidine_A and herboxidiene, both antitumour agents, were identified in CSK1. Interestingly, CSK1 extract did not show any cytotoxic activity against HeLa cells but when cultivated with *B. subtilis* lysate, γ-Butyrolactone and GlcNAc an anti-proliferative effect was observed. This may imply that silent BGCs were activated by the effect of these different elicitors.

Siderophores are small molecules that form stable compounds with ferric iron (Miethke and Marahiel, 2007). In iron deficient environments microorganisms produce siderophores to chelate ferric iron. Biosynthetic genes for siderophores such as desferrioxamine_B were also identified in the isolates. More than 10 *Streptomyces* species have been found to be capable to produce desferrioxamine B, E and G (Challis and Hopwood, 2003). In addition, *in silico* analysis of *S. coelicolor* sequence implied that many nonribosomal peptide synthetase (NRPS) pathways are used to assemble the structure of many siderophores (Bentley et al., 2002) such as the ones encoding for coelichelin (isolate CSM1; Challis and Ravel, 2000).

All Streptomyces genomes were found to have a single ectoine biosynthesis gene cluster (Remali et al., 2017). The presence of ectoines in nature implicates important applications such as therapeutic uses and protective agents for cellular components (Pastor et al., 2010). The ectoine biosynthetic gene cluster was identified in all isolates. In addition, other post-translationally modified peptides such as bacteriocin was predicted by antiSMASH in the isolates.

A variety of molecules were separated using Liquid Extraction Surface Analysis (LESA) system (Kertesz and Berkel, 2010), a novel approach to detect metabolites directly from the *Streptomyces* colonies. The technique was also used for the assessment of metabolites in the non-induced colonies versus colonies induced with GBL and GLcNAc. A clear change in the spectral profile of the uninduced vs induced isolates was observed, indicating that the produced metabolites are different between the uninduced, induced with GBL and induced with GlcNAc

colonies. Some of the metabolites predicted by antiSMASH were confirmed by LESA including ectoine, cetoniacytone, hopene, arginomycin, desferrioxamine_B, meilingmycin, meridamycin and sanglifehrin_A for CSK1. Tables 5.4-5.10 summarizes the secondary metabolites predicted by antiSMASH and confirmed with LESA for all the isolates. These results affirm the potential of these isolates to produce a variety of natural products.

In conclusion, effective secondary metabolites are required especially with the increased emergence of drug resistant organisms and toxicity of some drugs. The present study indicates that six different isolates with antimicrobial and anticancer activity were isolated from terrestrial habitats in UAE. The genomic sequencing and LESA results have predicted and identified respectively that these organisms are capable of producing a variety of secondary metabolites. We have, also, showed that exposing these isolates to biological or chemical elicitors such as *B. subtilis*, γ-butyrolactone and *N*-acetylglucosamine is an excellent way to induce the expression of genes encoding bioactive products which can lead to the production of novel secondary metabolites.

6.1. Future Work

There are many approaches that can be used to activate silent secondary metabolite-biosynthetic gene clusters (SM-BGCs). For example, studying and optimizing culture conditions such as carbon, nitrogen and phosphorus (Nazari et al., 2013) based on the one strain many compounds (OSMAC) approach. Changes in the composition of the growth media may result in the production of new molecules, although this may be a strenuous approach it is still commonly used. Genetic engineering techniques such as promoter exchange, ribosome engineering, inactivation of negative regulatory genes or activation of positive regulatory genes have also been used to activate silent SM-BGCs (Ochi, 2017). Another cost-effective and simple approach is co-culturing the isolates with other microorganisms mimicking the microbial interactions that are happening in the natural habitat that can trigger the expression of silent biosynthetic gene clusters encoding for seconding metabolite production (Antoraz et al., 2015).

The focus for future experimental work based on Genome sequencing and LESA-MS results would be to isolate and characterize the bioactive molecules that may have novel biological activity. In order of priority, I would first identify the peptides identified by Norine database but not by antiSMASH such as CSK1_region 191.1, CSU2_region 615.1,

CSK3_region 476.1 and CSK3_region 5471.1. Then, further identify the peptides that do not show any homology to known peptides by both Norine and antiSMASH databases such as CSK1_region 350.1, CSK1_region 471.1 and CSU2_region 570.1.

Connecting genes to a compound is one of the approaches that can be used to activate the expression of silent biosynthetic gene cluster where you can identify unknown peptides by using the sequence of the genes encoding its synthesis (Challis and Ravel, 2000). A product of a novel BGC can be identified by inactivating a specific biosynthetic gene along with comparative metabolic analysis of the wildtype and mutant strains using HPLC (Sekurova et al., 2019). This approach does not require the metabolite, encoded by the silent BGC, to be active and primarily relies on the comparison of the two metabolic profiles. This approach was used to identify coelichelin from *S. coelicolor*, a silent NRPS gene cluster was inactivated and the correlation of the BGC to its metabolite was made by comparative metabolomics (Lautru et al., 2005).

Studies have shown that many pleiotropic regulators affect secondary metabolism in *Streptomyces* (van Wezel and McDowall, 2011). The *afsQ* two-component system is one of these regulators (Liu et al., 2019). These genes are conserved in many *Streptomyces* and studies showed that it can regulate the production of secondary metabolites (McKenzie et al., 2010). A study done by Daniel-Ivad et al. (2017) showed that expressing an activated allele of afsQ1, generated from *S. coelicolor*, lead to the production of a variety of secondary metabolites in many *Streptomyces* strains with antibacterial activity. A similar approach can be used on our isolated *Streptomyces* strains to activate and generate the unknown compounds.

Another important approach to enhance or activate SM-BGCs is by overexpressing the *abrC3* response regulator. Studies have shown that *abrC3* can positively control the production of actinorhodin and undecylprodiginine production in addition to morphological differentiation in *S. coelicolor* (Rico et al., 2014). A construct containing *abrC3* can be introduced into the isolate of interest and the recombinant strain can be grown in appropriate media. Organic extracts can then be analysed by HPLC-MS and compared to the control strain (empty vector).

Future studies at the molecular level must be implemented in the discovery of novel secondary metabolites since we don't have enough information about the signals that are required for their production. One important molecular tool is the CRISPR-Cas9 (clustered regulatory interspaced short palindromic repeats and CRISPR-associated protein 9) system as a tool for genome engineering in *Streptomyces* isolates. The system provides adaptive immunity in many bacteria and archaea. Studies have shown that engineered CRISPR-Cas9-

based technology can be used to edit biosynthetic gene clusters by deleting genes or gene clusters and conduct gene replacements (Cobb et al., 2014). Engineered CRISPR-Cas9 can be used to knock-in constitutive promoters upstream of known biosynthetic gene clusters in a well characterized model of *Streptomyces* strains (Cobb et al., 2014). This method can, also, be used to produce novel natural products by knocking in active promoter upstream of the cluster to induce production of possibly new secondary metabolites (Zhang et al., 2017).

Changing the host for heterologous expression is another tool that has many advantages such as not using the natural regulatory system of the native host (as some genetic editing tools are not easy to use in some *Streptomyces* hosts) and surpasses the challenging genetic manipulation of the native host (Xu and Wright, 2019). The best host for heterologous expression is the *Streptomyces* species for the production of natural products, because they have a variety of cofactors, enzymes and precursors; they have complicated post modification arrangements such as acetylation, phosphorylation and glycosylation; they have resistance and tolerance genes; and appropriate protein folding to process multi-enzyme complexes (Liu et al., 2018). The most commonly used hosts include *S. coelicolor*, *S. lividans* and *S. avermitilis* (Zhao et al., 2019). The genome minimizing approach has, previously, played a major role in the discovery and overexpression of natural products. For example, a 1.4 Mb region was deleted from *S. avermitilis*, by Komatsu et al., 2010, to remove nonessential genes and produce a series of specific deletion mutants that did not produce any of the secondary metabolites found in the wild type strain. SUKA17 and SUKA22 generated mutants were tested for their high efficiency heterologous expression of unknown metabolites such as cephamycin C from *S. clavuligerus* and streptomycin from *S. griseus* (Komatsu et al., 2010). BGCs can be cloned into these mutants' model host and transformants carrying the gene cluster may produce metabolites due to the expression of the introduced biosynthetic gene clusters (Komatsu et al., 2013).

Another approach that can be used to activate silent BGCs is high-throughput elicitor screening (HiTES) to identify small molecule signals in order to induce BGCs. This can be done by inserting a reporter gene into the biosynthetic gene cluster of interest which provides read-out of its expression. Then, small molecule libraries can be screened for potential elicitors (Okada and Seyedsayamdost, 2017).

Ribosome engineering is an emerging approach to discover organisms with specific spontaneous mutation in their ribosome or RNA polymerase through screening antibiotic-resistant mutants. Selected mutants may have changes in secondary metabolite production or produce new natural products with unusual biological activities (Zhu et al., 2019). Ochi's

group in 2009 screened 1068 actinomycetes from soil grown on antibiotics to select antibiotic resistance strains (Hosaka et al., 2009). They showed that they were able to isolate a number of novel macrocyclic piperidamycin from one soil strain with either a single or double mutation in *rpoB* (encoding the RNA polymerase (RNAP) β-subunits) and *rspl* (encoding the ribosomal protein S12). This confirms that mutations in ribosomal proteins or RNAP changes bacterial gene expression leading to the improvement of production strains or inducing silent biosynthetic gene clusters (Okada and Seyedsayamdost, 2017).

In summary, the results of our Next Generation Sequencing (NGS) and Liquid Extraction Surface analysis (LESA) have revealed that these isolates have a great potential to produce novel secondary metabolites. We can make tremendous progress in activating silent genes or discovering cryptic gene products if we adopt one of the above synthetic biology approaches on these strains which can serve as an important source of pharmaceutical compounds.

Chapter 7

References

7. References

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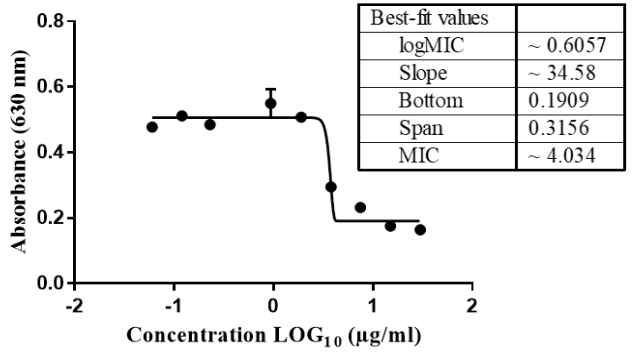
Chapter 8

Appendices

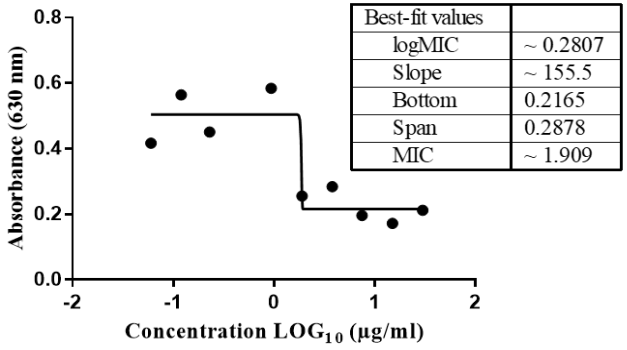
8. Appendices

Appendix A
Minimum Inhibitory Concentration of the isolates against *E. coli*

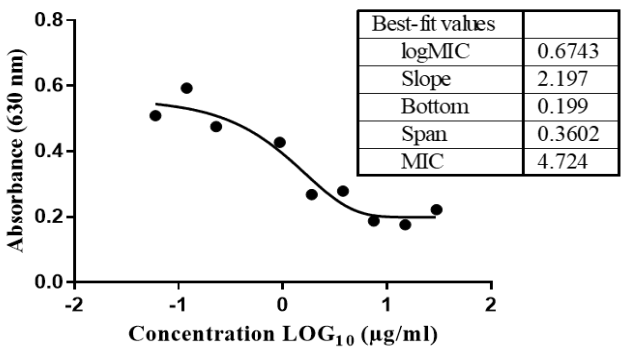
CSK1-*E. coli*



CSK3-*E. coli*



CSW2-*E. coli*



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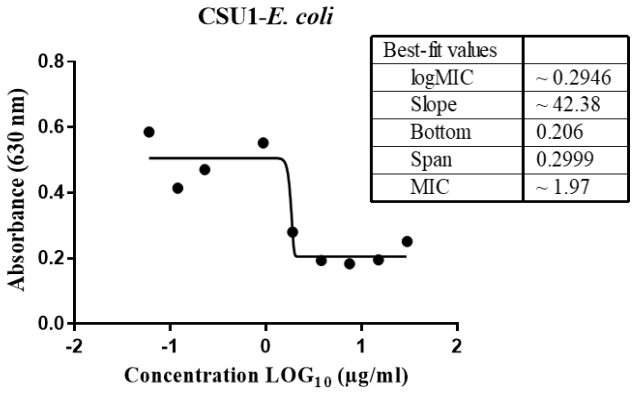
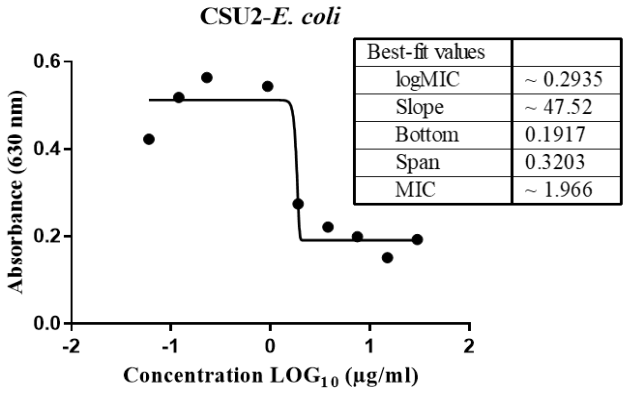
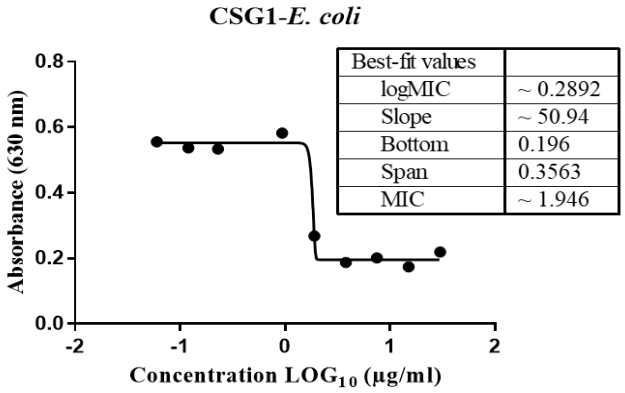


CSU2-*E. coli*

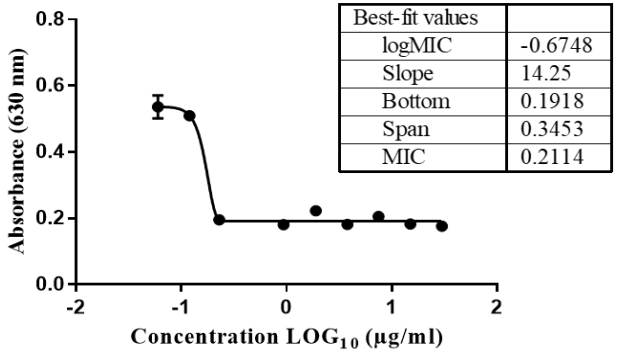


CSG1-*E. coli*



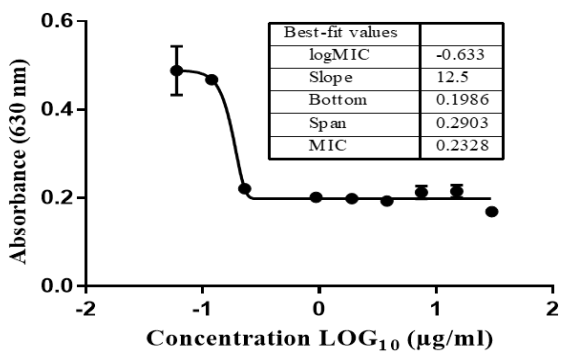
Minimum Inhibitory Concentration of the isolates against *S. aureus*

CSK1-*S. aureus*



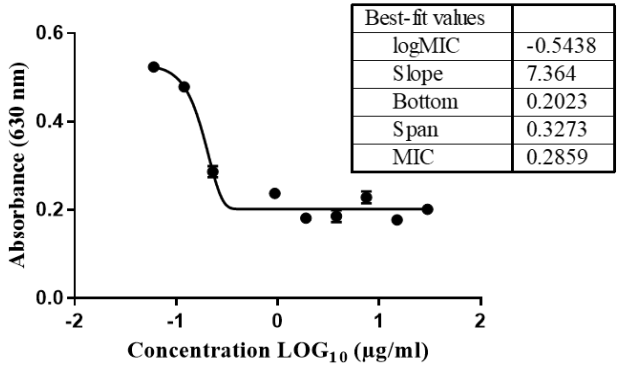
Best-fit values	
logMIC	-0.6748
Slope	14.25
Bottom	0.1918
Span	0.3453
MIC	0.2114

CSK3-*S. aureus*



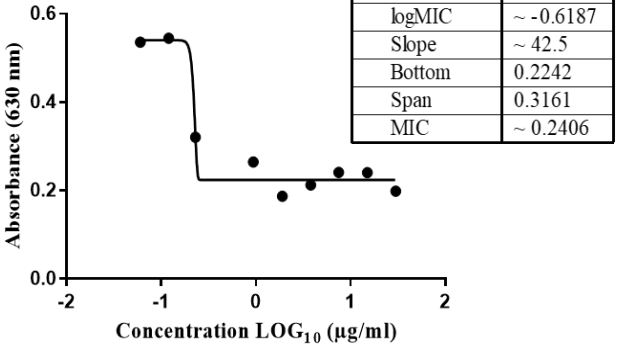
Best-fit values	
logMIC	-0.633
Slope	12.5
Bottom	0.1986
Span	0.2903
MIC	0.2328

CSW2-*S. aureus*



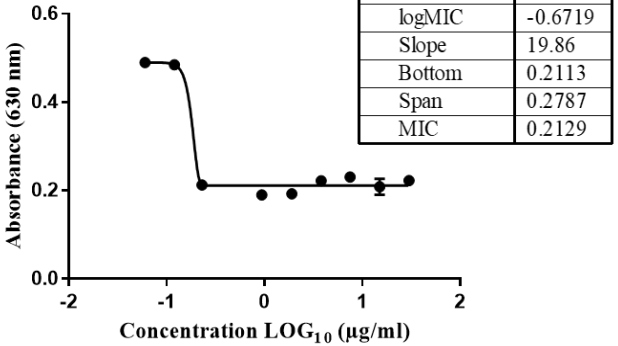
Best-fit values	
logMIC	-0.5438
Slope	7.364
Bottom	0.2023
Span	0.3273
MIC	0.2859

CSU1-*S. aureus*



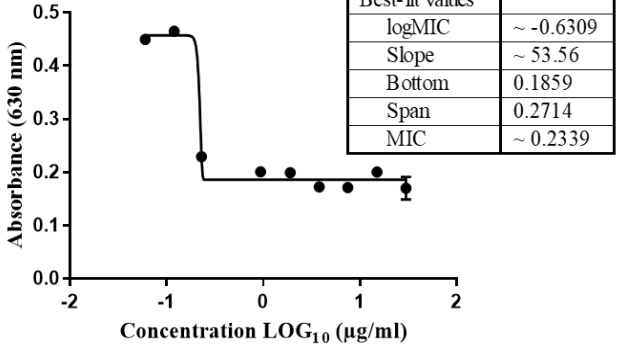
Best-fit values	
logMIC	~ -0.6187
Slope	~ 42.5
Bottom	0.2242
Span	0.3161
MIC	~ 0.2406

CSU2-*S. aureus*



Best-fit values	
logMIC	-0.6719
Slope	19.86
Bottom	0.2113
Span	0.2787
MIC	0.2129

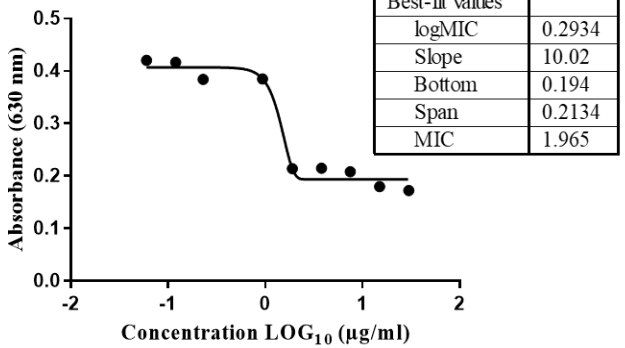
CSG1-*S. aureus*



Best-fit values	
logMIC	~ -0.6309
Slope	~ 53.56
Bottom	0.1859
Span	0.2714
MIC	~ 0.2339

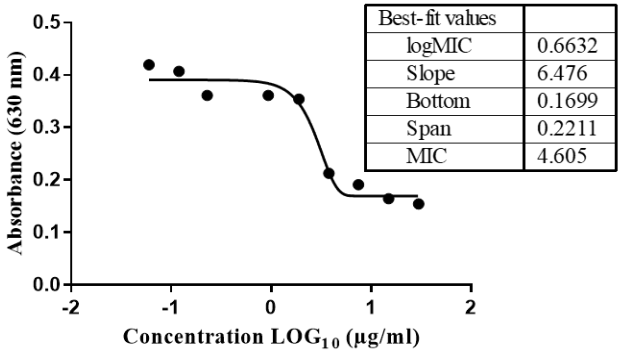
Minimum Inhibitory Concentration of the isolates against *C. albicans*

CSK1-*C. albicans*



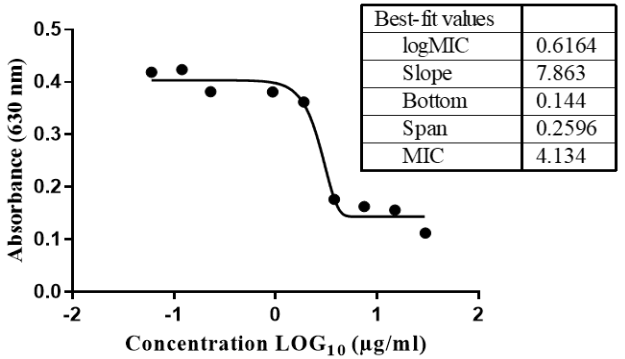
Best-fit values	
logMIC	0.2934
Slope	10.02
Bottom	0.194
Span	0.2134
MIC	1.965

CSK3-*C.albicans*



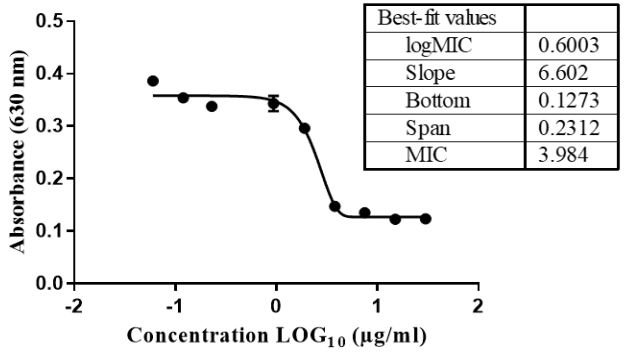
Best-fit values	
logMIC	0.6632
Slope	6.476
Bottom	0.1699
Span	0.2211
MIC	4.605

CSW2-*C. albicans*



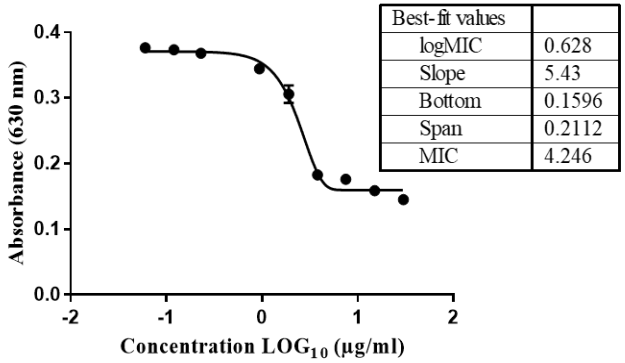
Best-fit values	
logMIC	0.6164
Slope	7.863
Bottom	0.144
Span	0.2596
MIC	4.134

CSU1-*C. albicans*



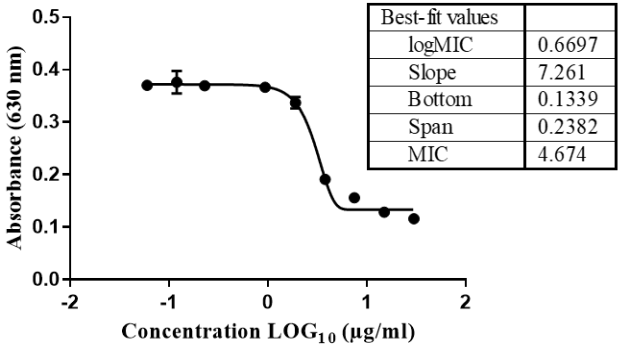
Best-fit values	
logMIC	0.6003
Slope	6.602
Bottom	0.1273
Span	0.2312
MIC	3.984

CSU2-*C. albicans*



Best-fit values	
logMIC	0.628
Slope	5.43
Bottom	0.1596
Span	0.2112
MIC	4.246

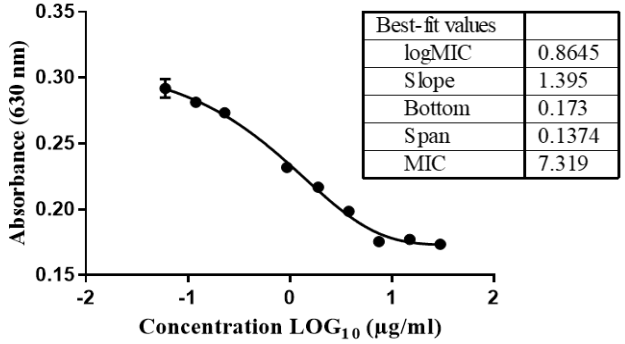
CSG1-*C. albicans*



Best-fit values	
logMIC	0.6697
Slope	7.261
Bottom	0.1339
Span	0.2382
MIC	4.674

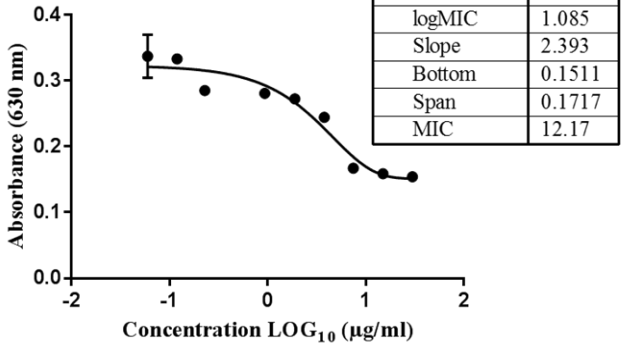
Minimum Inhibitory Concentration of the isolates against *S. cerevisiae*

CSK1-*S. cerevisiae*



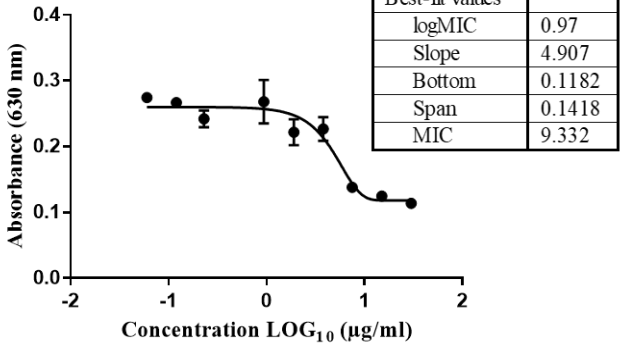
Best-fit values	
logMIC	0.8645
Slope	1.395
Bottom	0.173
Span	0.1374
MIC	7.319

CSK3-*S.cerevisiae*



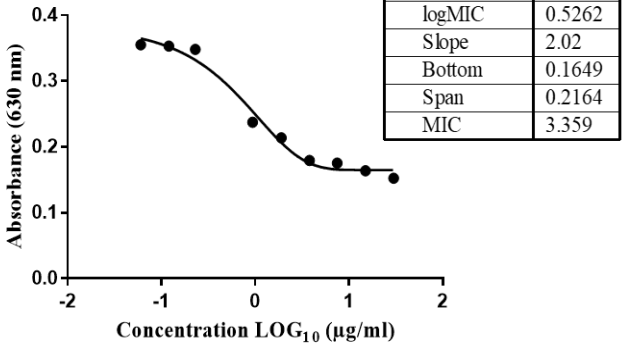
Best-fit values	
logMIC	1.085
Slope	2.393
Bottom	0.1511
Span	0.1717
MIC	12.17

CSW2-*S. cerevisiae*



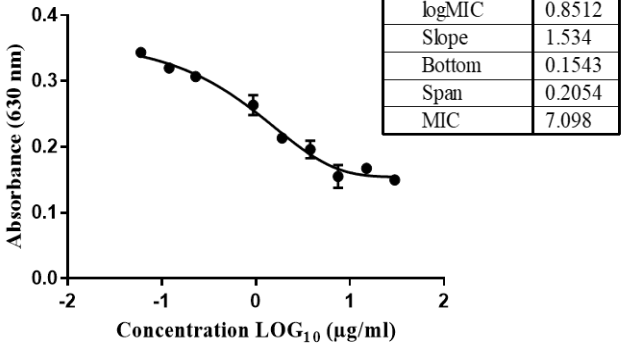
Best-fit values	
logMIC	0.97
Slope	4.907
Bottom	0.1182
Span	0.1418
MIC	9.332

CSU1-*S. cerevisiae*



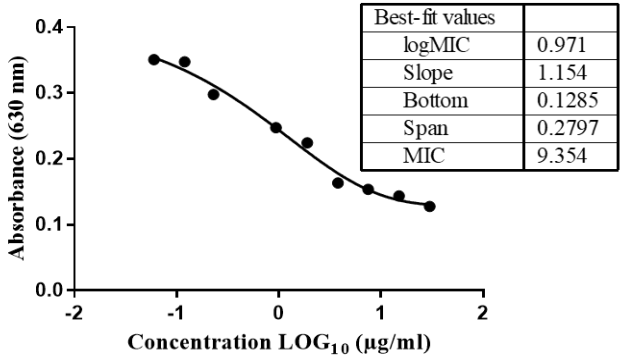
Best-fit values	
logMIC	0.5262
Slope	2.02
Bottom	0.1649
Span	0.2164
MIC	3.359

CSU2-*S. cerevisiae*



Best-fit values	
logMIC	0.8512
Slope	1.534
Bottom	0.1543
Span	0.2054
MIC	7.098

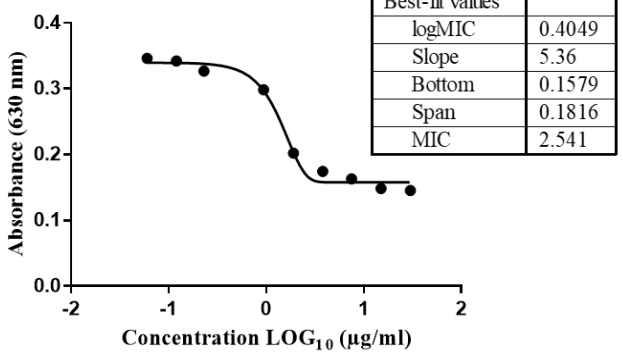
CSG1-*S. cerevisiae*



Best-fit values	
logMIC	0.971
Slope	1.154
Bottom	0.1285
Span	0.2797
MIC	9.354

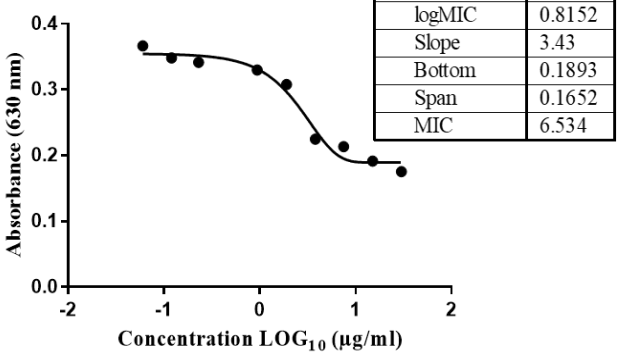
Minimum Inhibitory Concentration of the isolates against *B. subtilis*

CSK1-*B. subtilis*



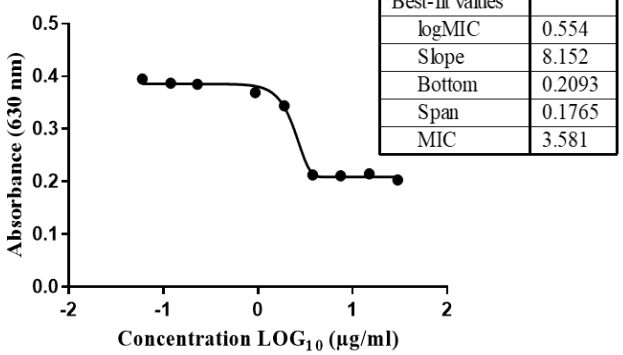
Best-fit values	
logMIC	0.4049
Slope	5.36
Bottom	0.1579
Span	0.1816
MIC	2.541

CSK3-*B. subtilis*



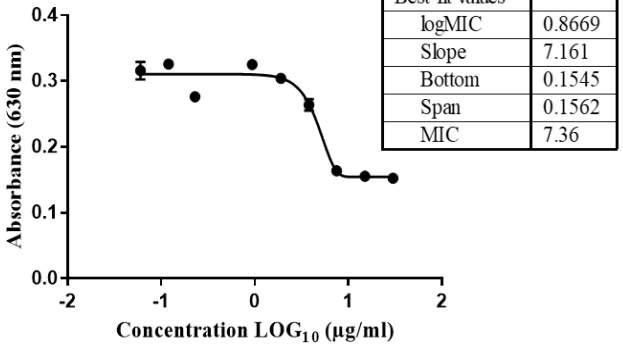
Best-fit values	
logMIC	0.8152
Slope	3.43
Bottom	0.1893
Span	0.1652
MIC	6.534

CSW2-*B. subtilis*



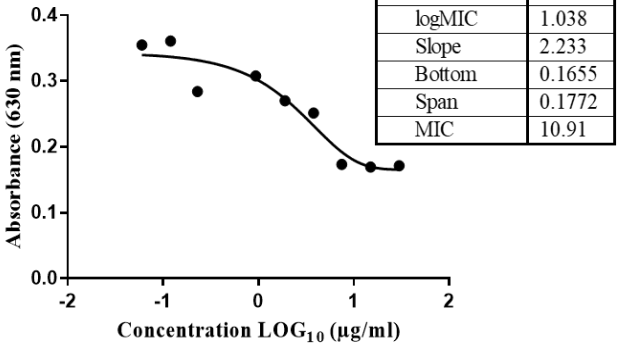
Best-fit values	
logMIC	0.554
Slope	8.152
Bottom	0.2093
Span	0.1765
MIC	3.581

CSU1-*B. subtilis*



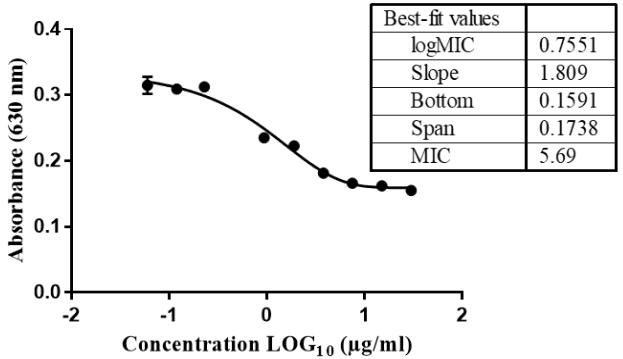
Best-fit values	
logMIC	0.8669
Slope	7.161
Bottom	0.1545
Span	0.1562
MIC	7.36

CSU2-*B. subtilis*



Best-fit values	
logMIC	1.038
Slope	2.233
Bottom	0.1655
Span	0.1772
MIC	10.91

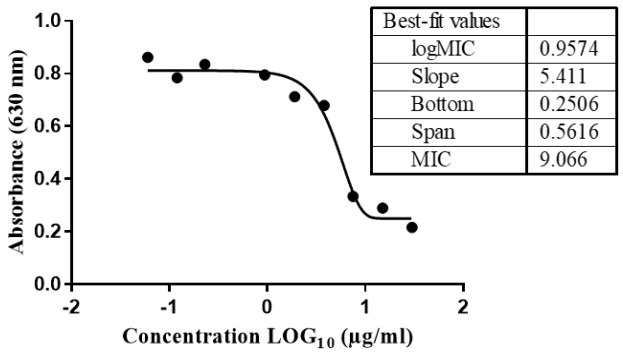
CSG1-*B. subtilis*



Best-fit values	
logMIC	0.7551
Slope	1.809
Bottom	0.1591
Span	0.1738
MIC	5.69

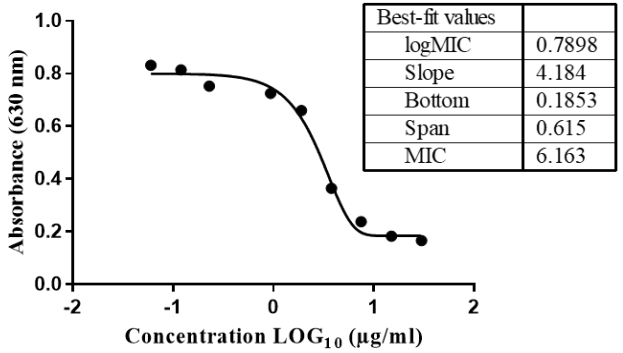
Minimum Inhibitory Concentration of the isolates against *E. coli*-ESBL

CSK1-*E. coli*-ESBL



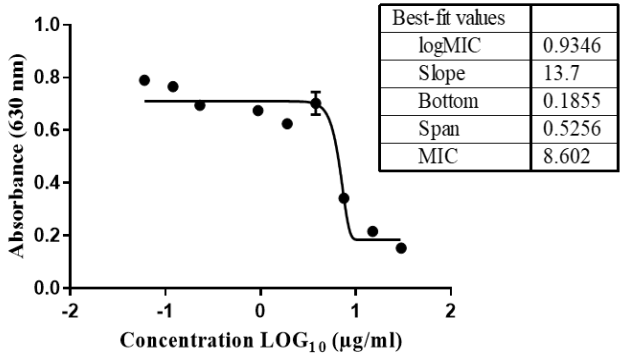
Best-fit values	
logMIC	0.9574
Slope	5.411
Bottom	0.2506
Span	0.5616
MIC	9.066

CSK3-*E. coli*-ESBL



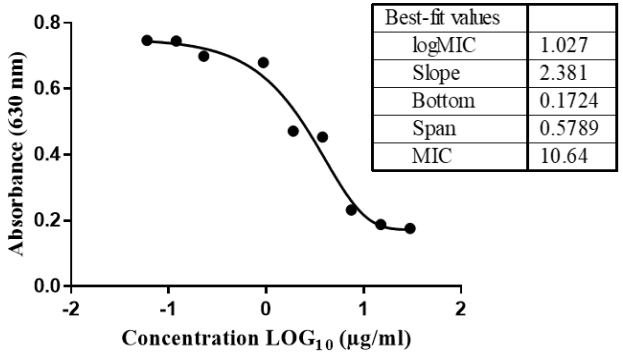
Best-fit values	
logMIC	0.7898
Slope	4.184
Bottom	0.1853
Span	0.615
MIC	6.163

CSW2-*E. coli*-ESBL



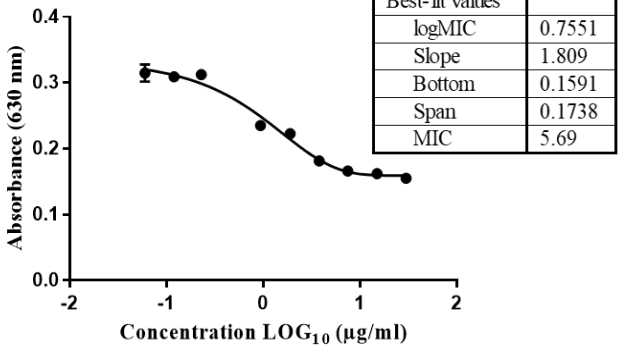
Best-fit values	
logMIC	0.9346
Slope	13.7
Bottom	0.1855
Span	0.5256
MIC	8.602

CSU1-*E. coli*-ESBL



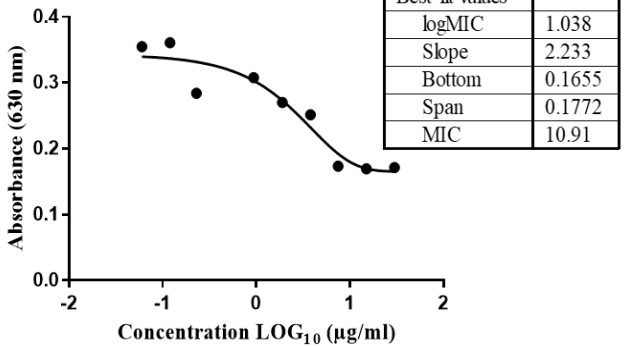
Best-fit values	
logMIC	1.027
Slope	2.381
Bottom	0.1724
Span	0.5789
MIC	10.64

CSU2-*E. coli*-ESBL



Best-fit values	
logMIC	0.7551
Slope	1.809
Bottom	0.1591
Span	0.1738
MIC	5.69

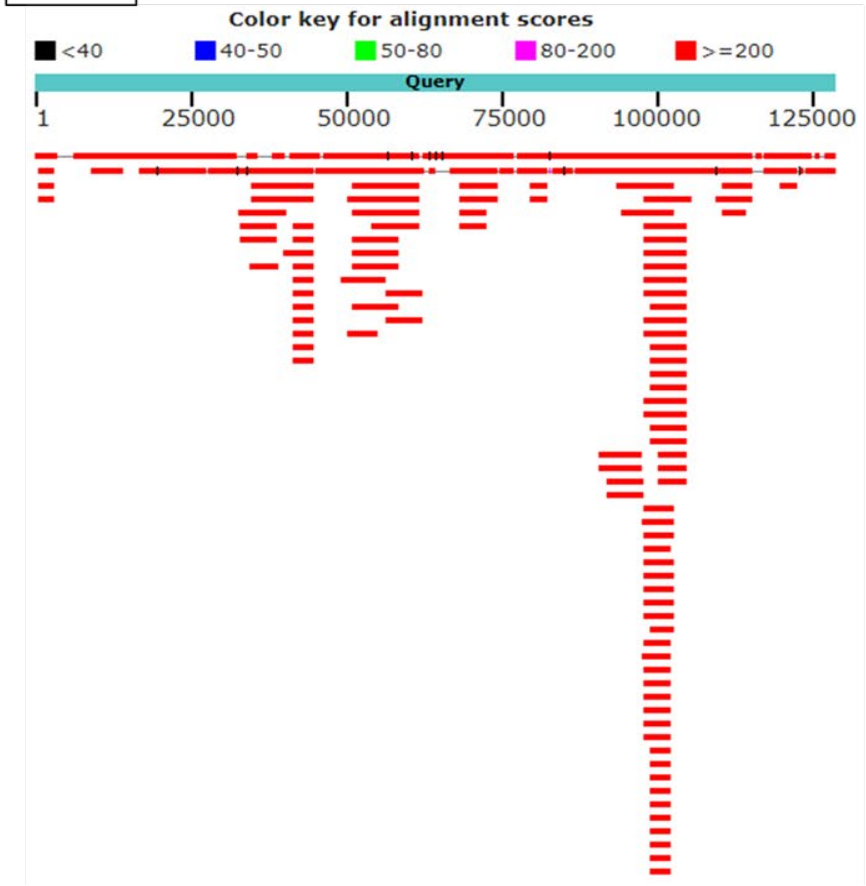
CSG1-*E. coli*-ESBL



Best-fit values	
logMIC	1.038
Slope	2.233
Bottom	0.1655
Span	0.1772
MIC	10.91

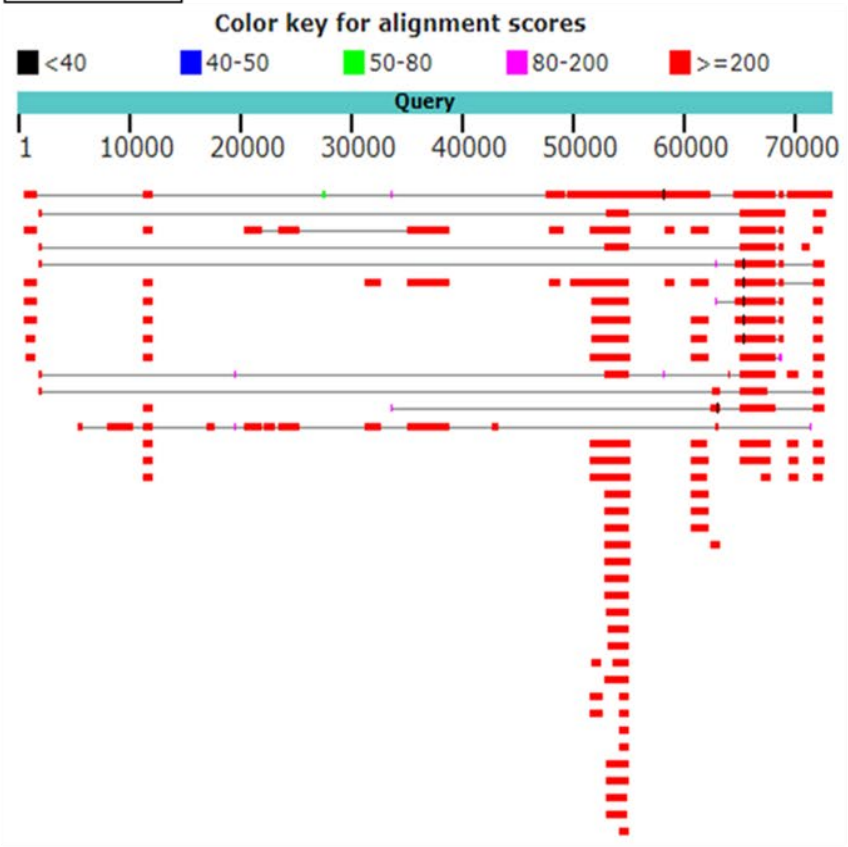
Appendix B
Whole Genome Sequence Blast of isolates by NCBI

CSK1



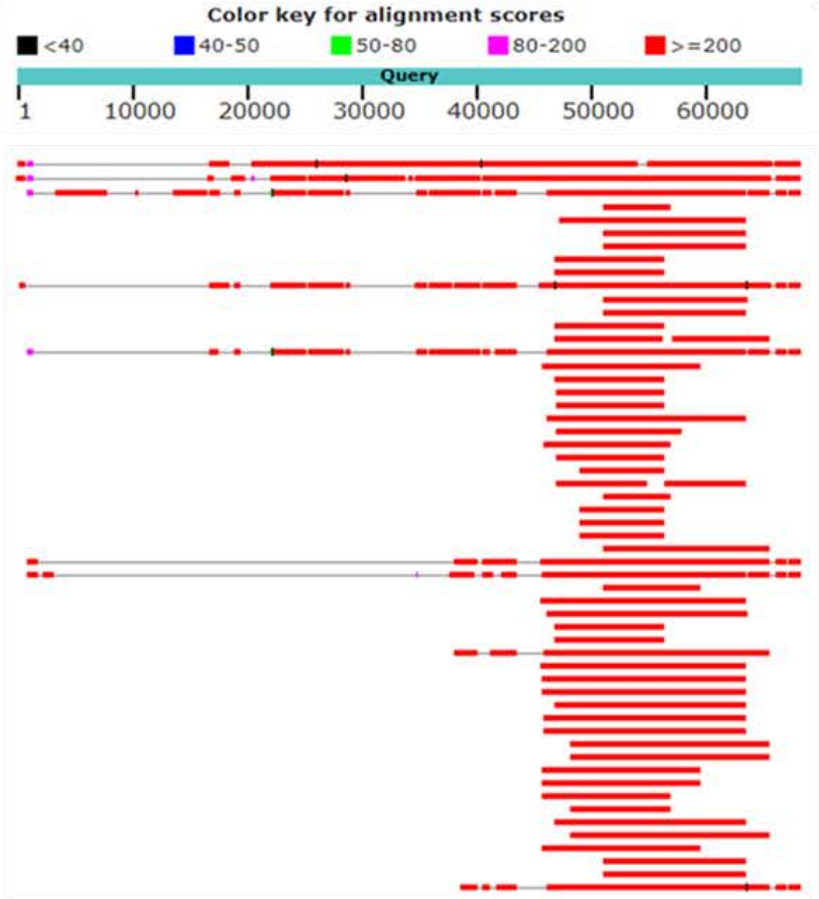
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces sp. ETH9427 chromosome	59110	2.145e+05	91%	0.0	99%	CP029624.1
Streptomyces sp. 4F, complete genome	37537	1.702e+05	81%	0.0	97%	CP013142.1
Streptomyces sp. WAC 01438 chromosome, complete genome	11335	1.123e+05	77%	0.0	87%	CP029601.1
Streptomyces glaucescens strain GLA.O, complete genome	5088	94964	73%	0.0	85%	CP009438.1
Streptomyces sp. S10(2016), complete genome	9380	92641	72%	0.0	84%	CP015098.1
Streptomyces sp. Go-475 chromosome, complete genome	7456	92504	72%	0.0	84%	CP026121.1
Streptomyces cyaneogriseus subsp. noncyanogenus strain NMWT 1, complete genome	8580	90018	71%	0.0	89%	CP010849.1
Streptomyces chartreusis NRRL 3882 isolate NRRL3882 genome assembly, chromosome: I	6220	92056	71%	0.0	86%	LT963352.1
Streptomyces chartreusis NRRL 3882 isolate NRRL3882 genome assembly, chromosome: I	6220	92056	71%	0.0	86%	LT962942.1
Streptomyces collinus Tu 365 chromosome, complete genome	5725	88886	71%	0.0	84%	CP006259.1
Streptomyces reticuli genome assembly TUE45, chromosome : I	5371	88997	71%	0.0	84%	LN997842.1
Streptomyces leeuwenhoekii genome assembly sleC34, chromosome	8599	89020	70%	0.0	89%	LN831790.1
Streptomyces sp. CdTB01, complete genome	7066	86256	70%	0.0	88%	CP013743.1
Streptomyces sp. PBH53 genome	5339	88515	70%	0.0	84%	CP011799.1
Streptomyces fungicidicus strain TXX3120 chromosome, complete genome	9945	1.018e+05	69%	0.0	90%	CP023407.1

CSK3



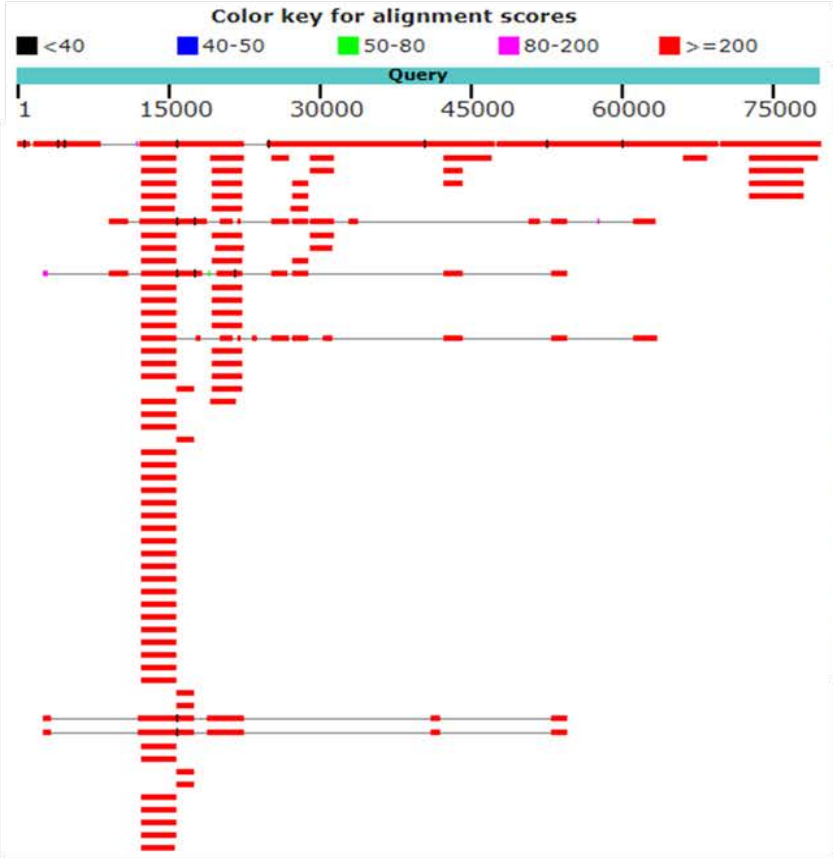
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces sp. ETH9427 chromosome	14958	87456	34%	0.0	98%	CP029624.1
Streptomyces hygroscopicus subsp. jinggangensis TL01, complete genome	2481	18093	27%	0.0	79%	CP003720.1
Streptomyces hygroscopicus subsp. jinggangensis 5008, complete genome	2481	18440	27%	0.0	79%	CP003275.1
Streptomyces hygroscopicus subsp. limoneus strain KCTC 1717 chromosome II, complete sequence	2490	14562	20%	0.0	79%	CP013220.1
Streptomyces davawensis strain JCM 4913 complete genome	2281	6435	11%	0.0	75%	HE971709.1
Streptomyces fungicidicus strain TXX3120 chromosome, complete genome	1626	3145	9%	0.0	76%	CP023407.1
Streptomyces sp. CC0208 chromosome, complete genome	900	4707	9%	0.0	84%	CP031969.1
Streptomyces sp. CCM_MD2014 chromosome, complete genome	4968	6189	8%	0.0	89%	CP009754.1
Streptomyces sp. 2114.2 genome assembly, chromosome: I	3995	5505	8%	0.0	89%	LT629768.1
Streptomyces olivaceus strain KLBMP 5084 chromosome, complete genome	3583	5961	8%	0.0	87%	CP016795.1
Streptomyces sp. Go-475 chromosome, complete genome	1306	4935	8%	0.0	90%	CP026121.1
Streptomyces bacillaris strain ATCC 15855 chromosome, complete genome	1118	3712	8%	0.0	86%	CP029378.1
Streptomyces ambofaciens ATCC 23877, complete genome	3951	24679	7%	0.0	89%	CP012382.1
Streptomyces ambofaciens ATCC 23877 left chromosomal arm	3951	10929	7%	0.0	89%	AM238663.1
Streptomyces sp. SAT1, complete genome	2377	3996	7%	0.0	79%	CP015849.1

CSW2



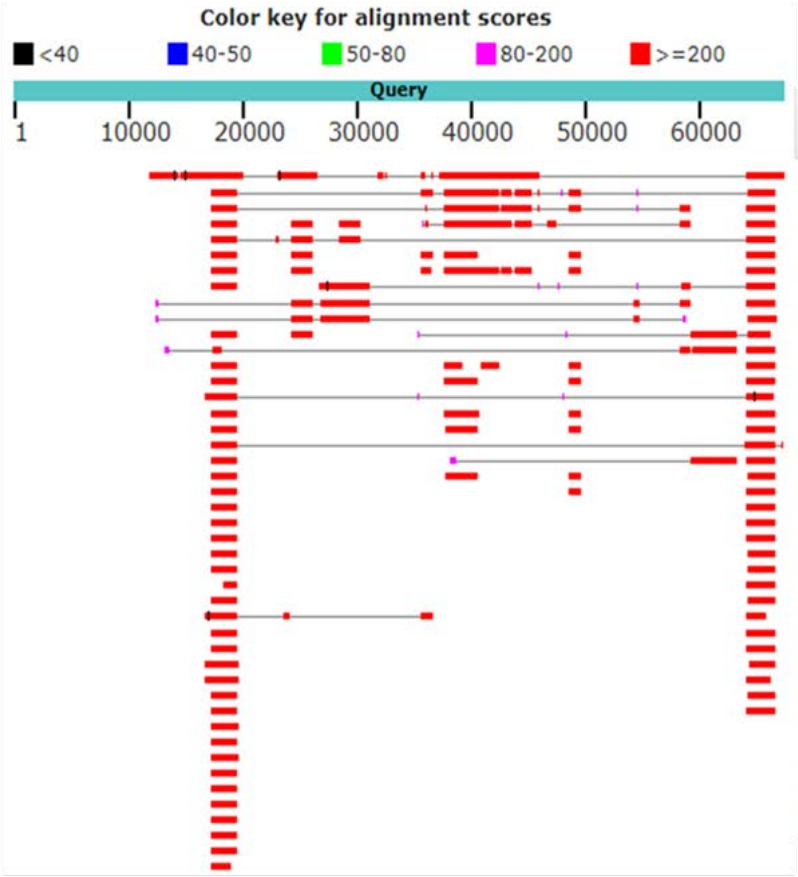
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces sp. ETH9427 chromosome	25909	87451	72%	0.0	99%	CP029624.1
Streptomyces sp. 4F, complete genome	41779	77779	70%	0.0	97%	CP013142.1
Streptomyces ambofaciens strain DSM 40697, complete genome	22663	50160	67%	0.0	90%	CP012949.1
Streptomyces davawensis strain JCM 4913 complete genome	7046	46630	66%	0.0	88%	HE971709.1
Streptomyces parvulus strain 2297, complete genome	21741	46639	64%	0.0	91%	CP015866.1
Streptomyces chartreusis NRRL 3882 isolate NRRL3882 genome assembly, chromosome: I	16105	46450	62%	0.0	90%	LT963352.1
Streptomyces chartreusis NRRL 3882 isolate NRRL3882 genome assembly, chromosome: I	16105	46450	62%	0.0	90%	LT962942.1
Streptomyces coelicolor A3(2) complete genome; segment 17/29	12054	45423	62%	0.0	89%	AL939120.1
Streptomyces sp. 2114.2 genome assembly, chromosome: I	12024	45313	62%	0.0	89%	LT629768.1
Streptomyces fungicidicus strain TXX3120 chromosome, complete genome	24088	52360	59%	0.0	93%	CP023407.1
Streptomyces sp. S10(2016), complete genome	15588	44314	58%	0.0	89%	CP015098.1
Streptomyces brunneus strain CR22 chromosome, complete genome	15119	40879	56%	0.0	89%	CP034463.1
Streptomyces lividans TK24, complete genome	12096	42753	56%	0.0	90%	CP009124.1
Streptomyces sp. CB09001 chromosome, complete genome	12094	42533	56%	0.0	90%	CP026730.1
Streptomyces ambofaciens ATCC 23877, complete genome	22657	43646	55%	0.0	90%	CP012382.1

CSU1



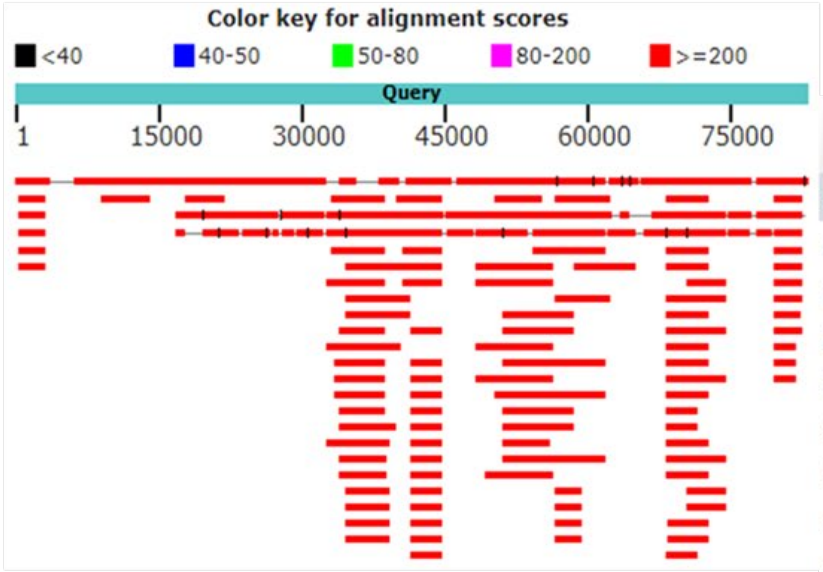
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces sp. ETH9427 chromosome	26600	1.313e+05	91%	0.0	98%	CP029624.1
Streptomyces griseorubiginosus strain 3E-1 chromosome, complete genome	4726	21231	30%	0.0	92%	CP032427.1
Streptomyces pactum strain ACT12, complete genome	3615	28744	30%	0.0	94%	CP019724.1
Streptomyces sp. CdTB01, complete genome	4889	29702	29%	0.0	92%	CP013743.1
Streptomyces scabiei 87.22 complete genome	4964	24158	27%	0.0	93%	FN554889.1
Streptomyces brunneus strain CR22 chromosome, complete genome	4833	23429	27%	0.0	92%	CP034463.1
Streptomyces hundungensis strain BH38 chromosome, complete genome	4621	21305	27%	0.0	91%	CP032698.1
Streptomyces actuosus strain ATCC 25421 chromosome, complete genome	5535	26639	26%	0.0	95%	CP029788.1
Streptomyces sp. Go-475 chromosome, complete genome	5326	20496	25%	0.0	95%	CP026121.1
Streptomyces xinghaiensis S187 chromosome, complete genome	5204	22489	25%	0.0	94%	CP023202.1
Streptomyces sp. P3 chromosome, complete genome	5108	22675	24%	0.0	94%	CP028369.1
Streptomyces sp. Z022 chromosome, complete genome	5432	26041	22%	0.0	95%	CP033073.1
Streptomyces sp. S10(2016), complete genome	5206	23625	22%	0.0	94%	CP015098.1
Streptomyces alfalfae strain ACCC40021 chromosome, complete genome	5064	21502	22%	0.0	94%	CP015588.1
Streptomyces glaucescens strain GLA.O, complete genome	5378	25714	21%	0.0	95%	CP009438.1

CSU2



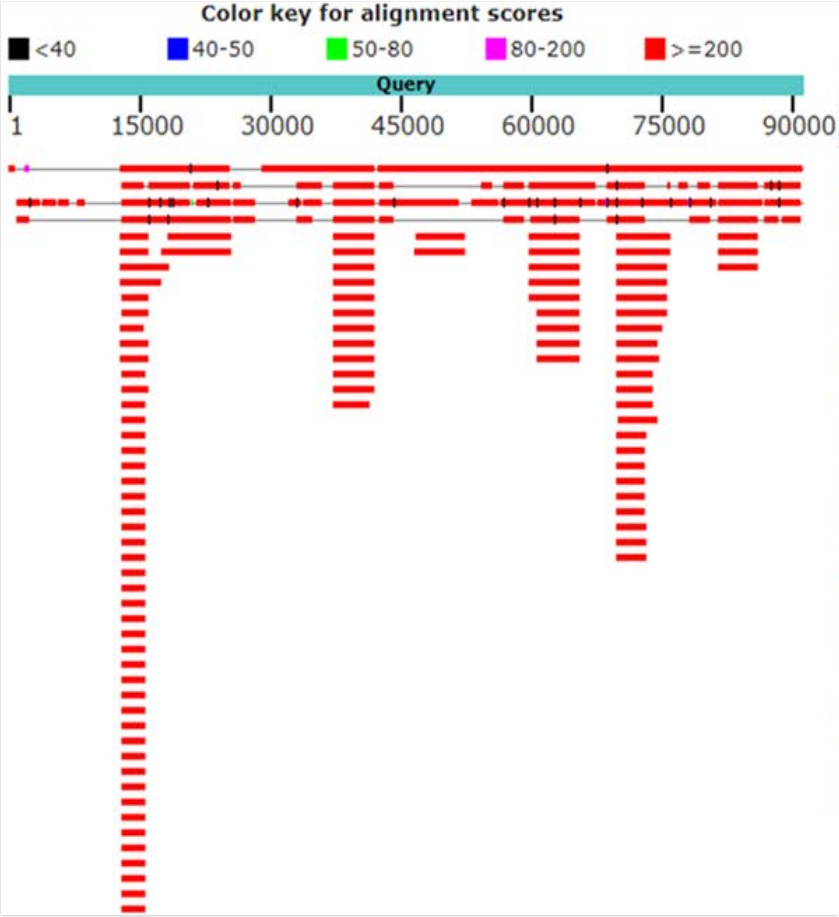
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces sp. ETH9427 chromosome	15477	82422	37%	0.0	99%	CP029624.1
Streptomyces ambofaciens ATCC 23877, complete genome	4580	17154	21%	0.0	84%	CP012382.1
Streptomyces ambofaciens strain DSM 40697, complete genome	4597	11794	18%	0.0	84%	CP012949.1
Streptomyces lividans TK24, complete genome	2294	14054	18%	0.0	85%	CP009124.1
Streptomyces sp. 2114.2 genome assembly, chromosome: I	2806	13304	17%	0.0	86%	LT629768.1
Streptomyces hygroscopicus strain XM201, complete genome	3059	10798	15%	0.0	88%	CP018627.1
Streptomyces hygroscopicus subsp. jinggangensis TL01, complete genome	3003	19356	15%	0.0	87%	CP003720.1
Streptomyces hygroscopicus subsp. jinggangensis 5008, complete genome	3003	19826	15%	0.0	87%	CP003275.1
Streptomyces sp. KPB2 chromosome, complete genome	2193	9476	15%	0.0	84%	CP034353.1
Streptomyces fungicidicus strain TXX3120 plasmid p1, complete sequence	6501	10213	14%	0.0	87%	CP023408.1
Streptomyces sp. CCM_MD2014 chromosome, complete genome	3024	9550	14%	0.0	85%	CP009754.1
Streptomyces leeuwenhoekii genome assembly sleC34, chromosome	3022	15824	13%	0.0	88%	LN831790.1
Streptomyces sp. S10(2016), complete genome	4706	9852	12%	0.0	90%	CP015098.1
Streptomyces rapamycinicus NRRL 5491 genome	2409	7293	12%	0.0	85%	CP006567.1
Streptomyces lincolnensis strain NRRL 2936, complete genome	6156	9557	11%	0.0	92%	CP016438.1

CSG1



Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces sp. ETH9427 chromosome	46557	1.354e+05	89%	0.0	99%	CP029624.1
Streptomyces collinus Tu 365 chromosome, complete genome	5725	59640	75%	0.0	84%	CP006259.1
Streptomyces glaucescens strain GLA.O, complete genome	4765	60062	74%	0.0	86%	CP009438.1
Streptomyces sp. 4F, complete genome	27242	99256	73%	0.0	95%	CP013142.1
Streptomyces sp. WAC 01438 chromosome, complete genome	11335	64730	71%	0.0	87%	CP029601.1
Streptomyces reticuli genome assembly TUE45, chromosome : I	5371	56023	70%	0.0	84%	LN997842.1
Streptomyces sp. PBH53 genome	5339	56216	70%	0.0	84%	CP011799.1
Streptomyces leeuwenhoekii genome assembly sleC34, chromosome	4490	54342	69%	0.0	85%	LN831790.1
Streptomyces cyaneogriseus subsp. noncyanogenus strain NMWT 1, complete genome	4429	55082	69%	0.0	84%	CP010849.1
Streptomyces sp. S10(2016), complete genome	9380	53031	66%	0.0	84%	CP015098.1
Streptomyces sp. Go-475 chromosome, complete genome	7456	52359	66%	0.0	84%	CP026121.1
Streptomyces brunneus strain CR22 chromosome, complete genome	4649	48451	64%	0.0	84%	CP034463.1
Streptomyces fungicidicus strain TXX3120 chromosome, complete genome	6499	57871	63%	0.0	86%	CP023407.1
Streptomyces chartreusis NRRL 3882 isolate NRRL3882 genome assembly, chromosome: I	4320	51739	63%	0.0	85%	LT963352.1
Streptomyces chartreusis NRRL 3882 isolate NRRL3882 genome assembly, chromosome: I	4320	51739	63%	0.0	85%	LT962942.1

CSM1



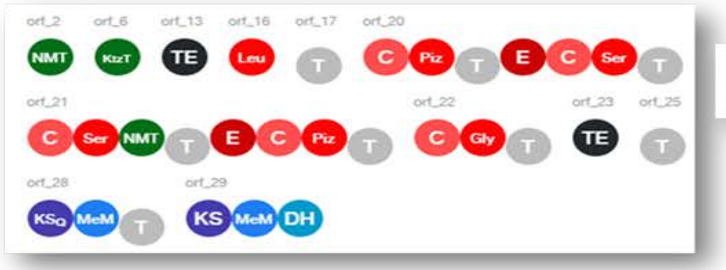
Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces nigra strain 452 chromosome, complete genome	44802	1.310e+05	82%	0.0	98%	CP029043.1
Streptomyces sp. MK45 chromosome, complete genome	7180	79530	81%	0.0	85%	CP034539.1
Streptomyces davawensis strain JCM 4913 complete genome	5384	63201	67%	0.0	84%	HE971709.1
Streptomyces chartreusis NRRL 3882 isolate NRRL3882 genome assembly, chromosome: I	6508	57390	62%	0.0	83%	LT963352.1
Streptomyces chartreusis NRRL 3882 isolate NRRL3882 genome assembly, chromosome: I	6508	57390	62%	0.0	83%	LT962942.1
Streptomyces avermitilis MA-4680 = NBRC 14893 DNA, complete genome	5007	54220	62%	0.0	85%	BA000030.4
Streptomyces sp. CC0208 chromosome, complete genome	6185	54852	59%	0.0	86%	CP031969.1
Streptomyces sp. Go-475 chromosome, complete genome	5805	57524	59%	0.0	87%	CP026121.1
Streptomyces lincolnensis strain LC-G chromosome, complete genome	5254	55740	59%	0.0	87%	CP022744.1
Streptomyces lincolnensis strain NRRL 2936, complete genome	5254	55740	59%	0.0	87%	CP016438.1
Streptomyces sp. S10(2016), complete genome	5121	55820	59%	0.0	87%	CP015098.1
Streptomyces griseorubiginosus strain 3E-1 chromosome, complete genome	6410	53639	58%	0.0	86%	CP032427.1
Streptomyces puniciscabiei strain TW1S1, complete genome	5605	51259	58%	0.0	85%	CP017248.1
Streptomyces brunneus strain CR22 chromosome, complete genome	5101	53263	58%	0.0	86%	CP034463.1
Streptomyces griseochromogenes strain ATCC 14511, complete genome	5736	52899	57%	0.0	85%	CP016279.1

Appendix C
Modular enzymes identified by antiSMASH and Prism 3 Databases

CSK1_Cluster 4_T1Pks/Nrps

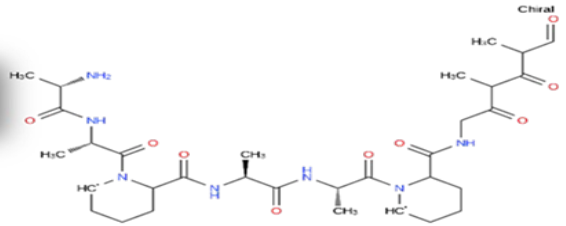
A

Biosynthetic assembly

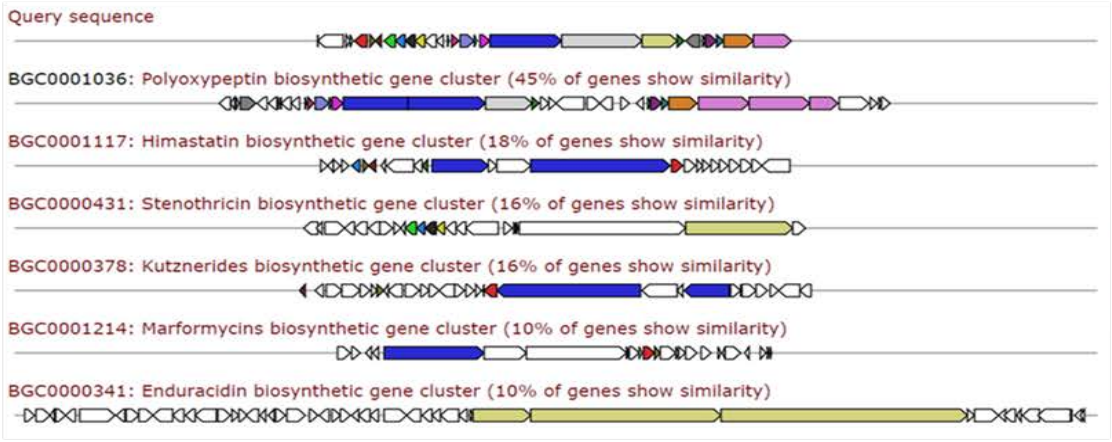


Cluster

Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

CSK1_Cluster 6_T3Pks

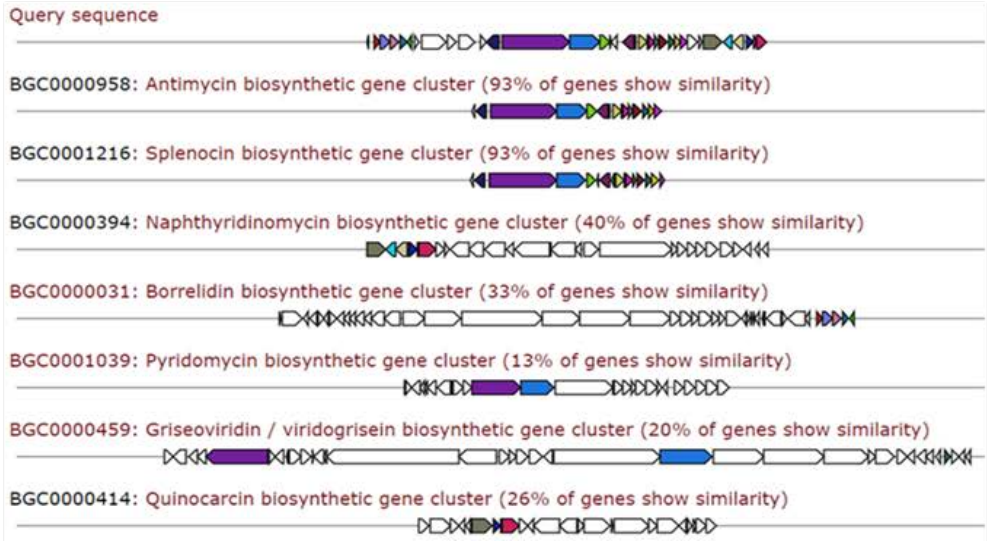
Biosynthetic assembly

Cluster

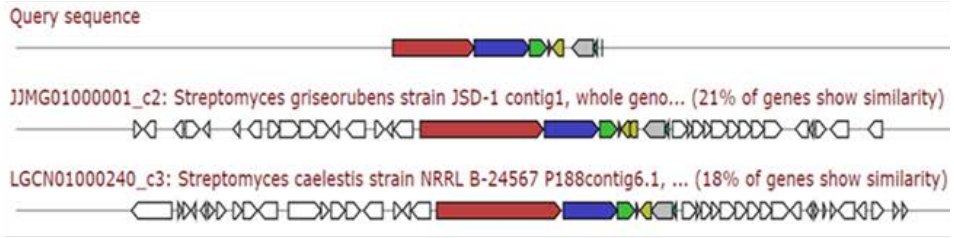
Predicted core structure

- **No structure was predicted**

Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

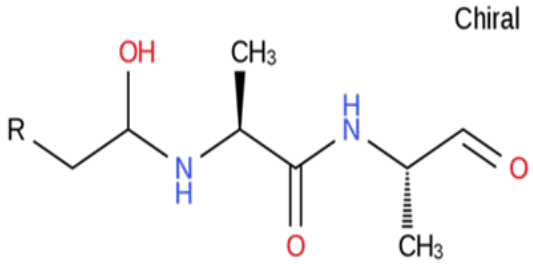


CSK1_Cluster 13_Other Nrps

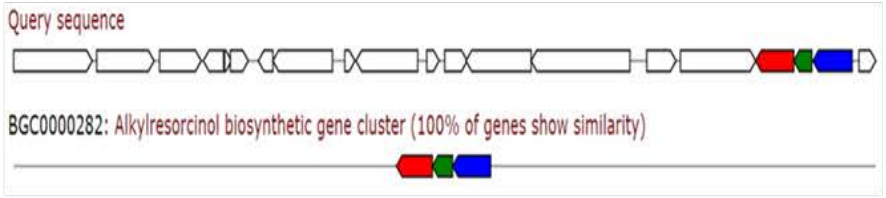
Biosynthetic assembly

Cluster

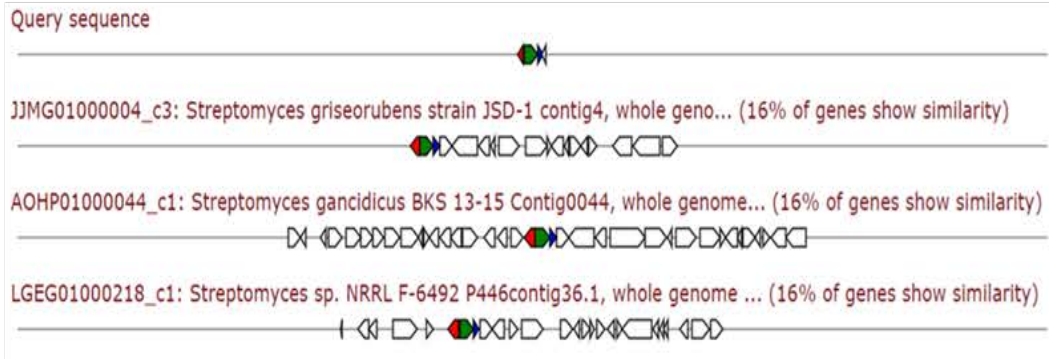
Predicted Structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

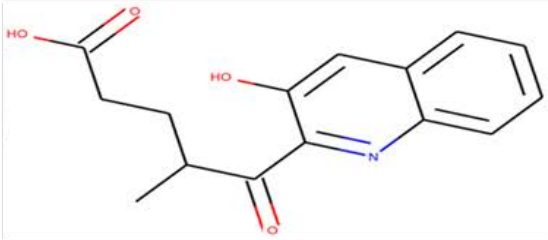


CSK1_Cluster 15_Pks

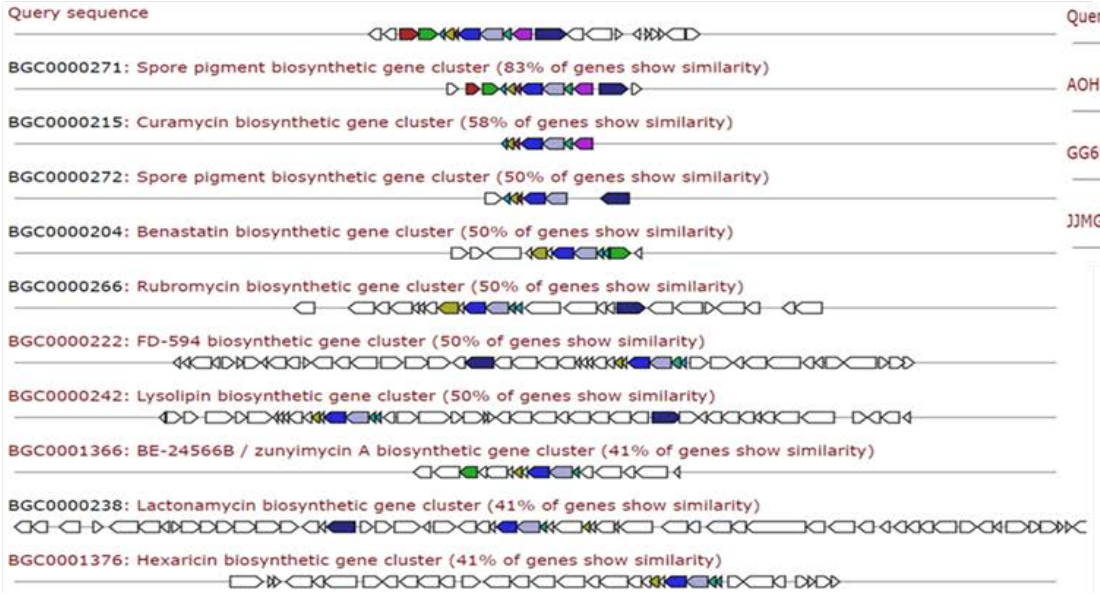
Biosynthetic assembly

Cluster

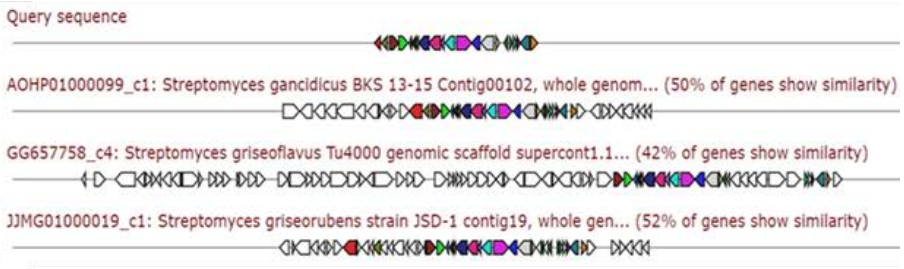
Predicted core structure



Cluster similarity to known genes

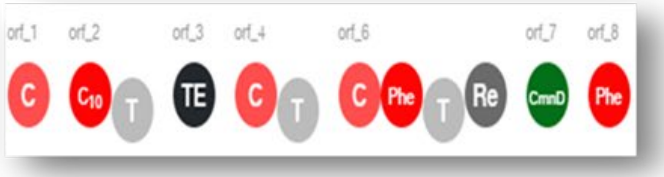


Cluster similarity in *Streptomyces* species



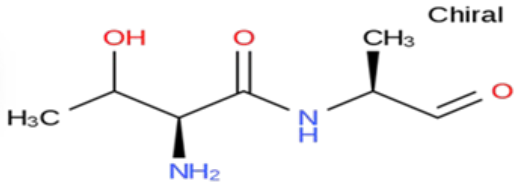
CSK1_Cluster 17_NRPS

Biosynthetic assembly



Cluster

Predicted core structure



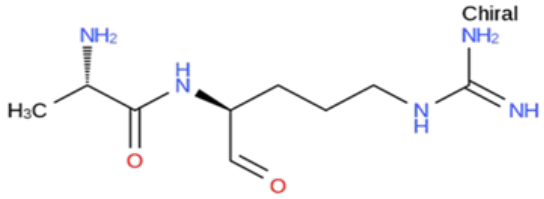
- No similar gene clusters are identified in the database

CSK1_Cluster 22_NRPS

Biosynthetic assembly

Cluster

Predicted core structure



- No similar gene clusters are identified in the database

CSK1_Cluster 24_NRPS

Biosynthetic assembly

Cluster

- No similar gene clusters are identified in the database
- No predicted structure

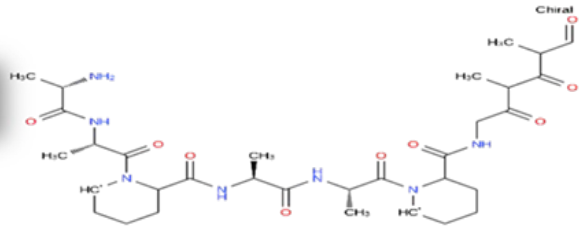
B

CSK3_Cluster 3_T1Pks/Nrps

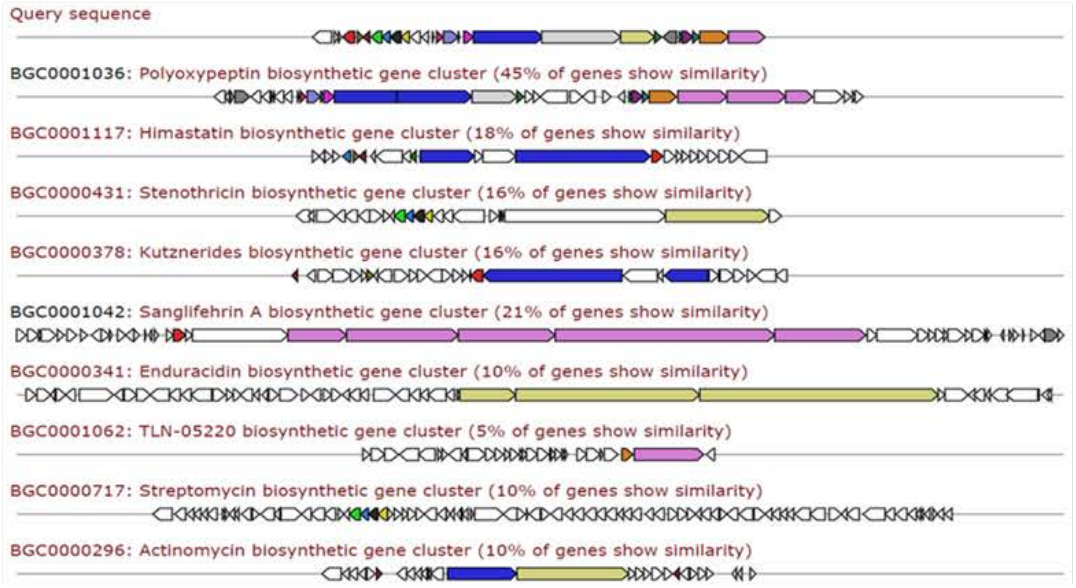
Biosynthetic assembly

Cluster

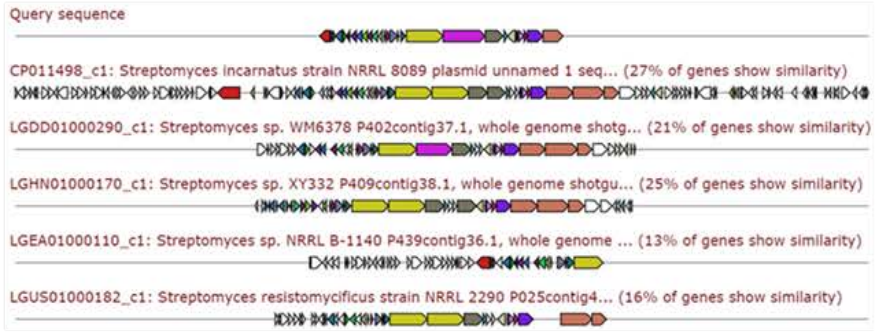
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

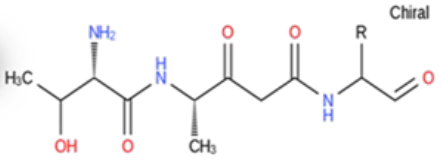


CSK3_Cluster 6_T1Pks/Nrps

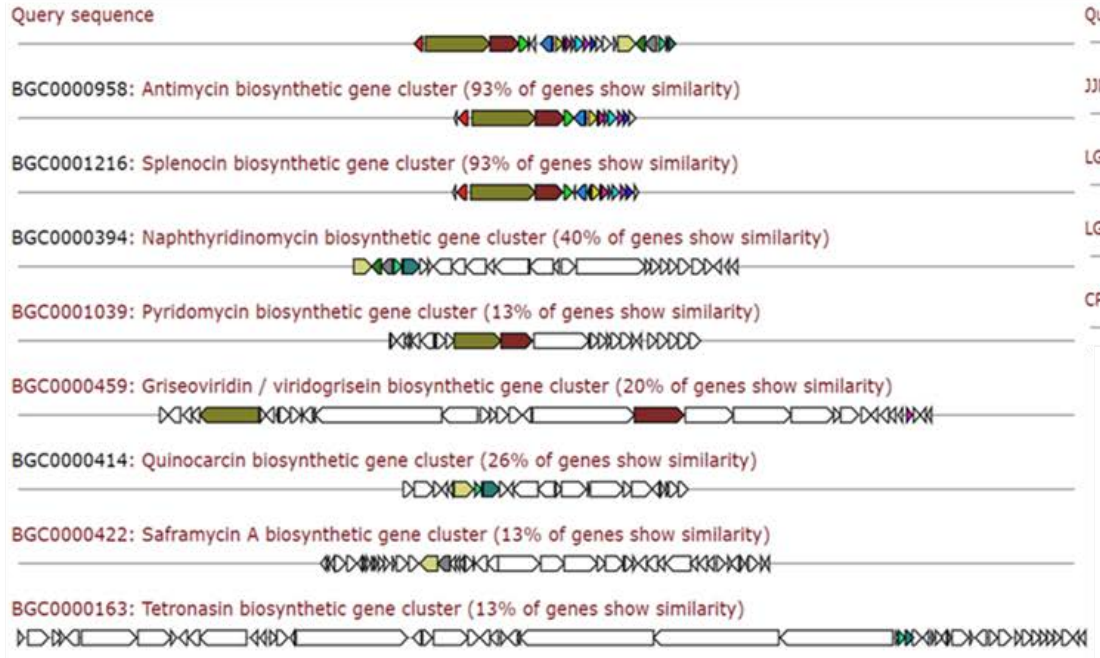
Biosynthetic assembly

Cluster

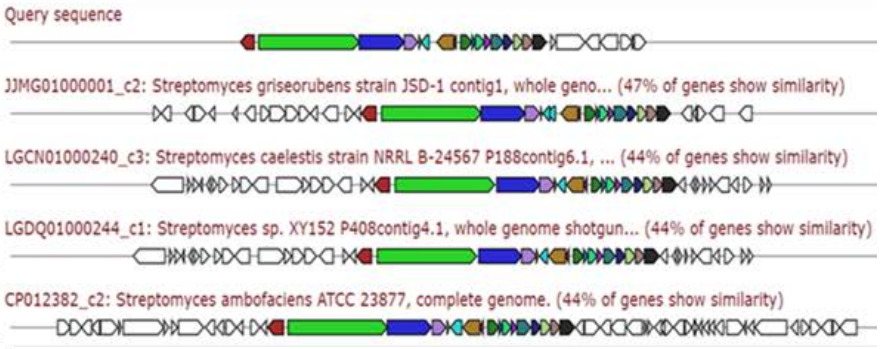
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

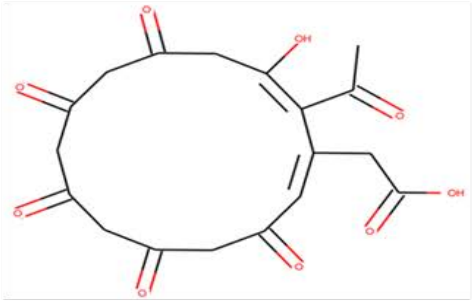


CSK3_Cluster 8_T2Pks

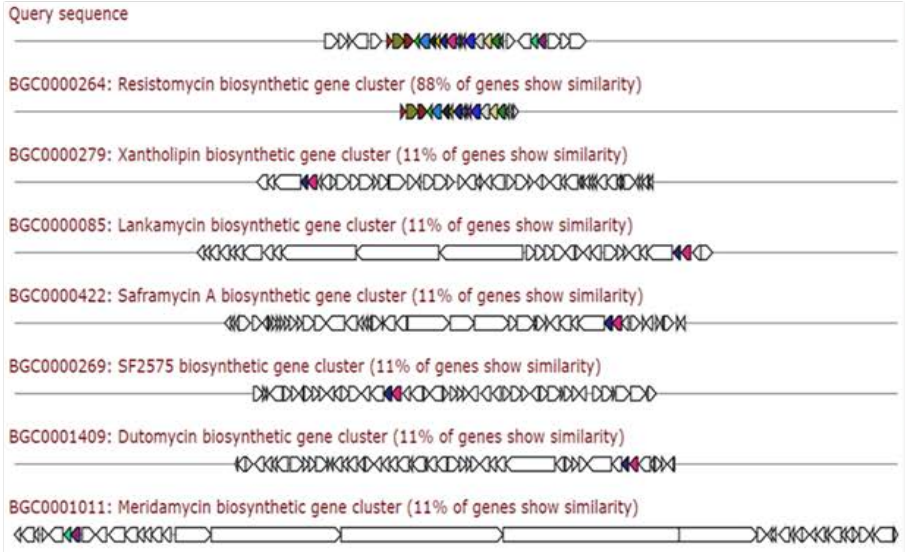
Biosynthetic assembly

Cluster

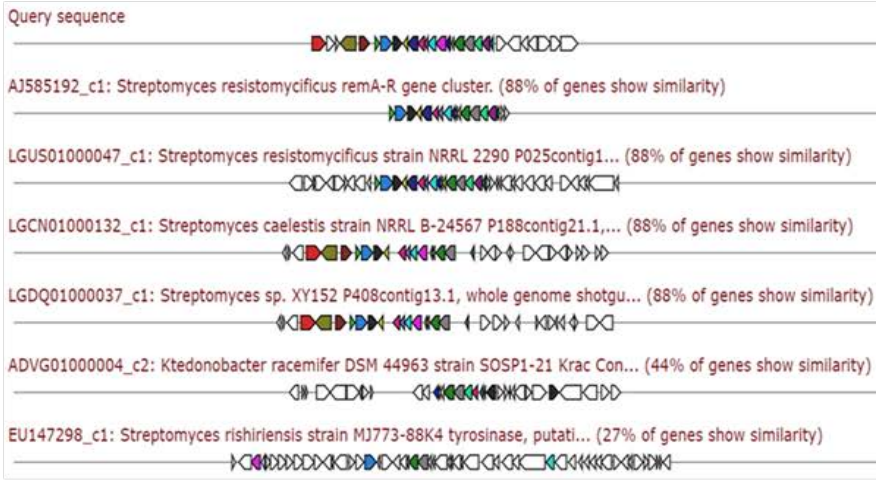
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

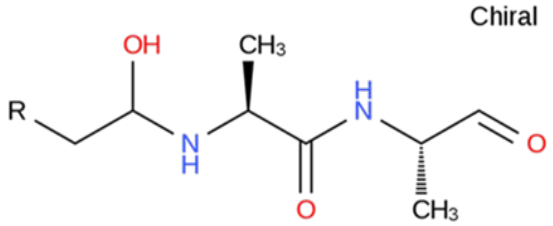


CSK3_Cluster 12_Nrps_Other Ks

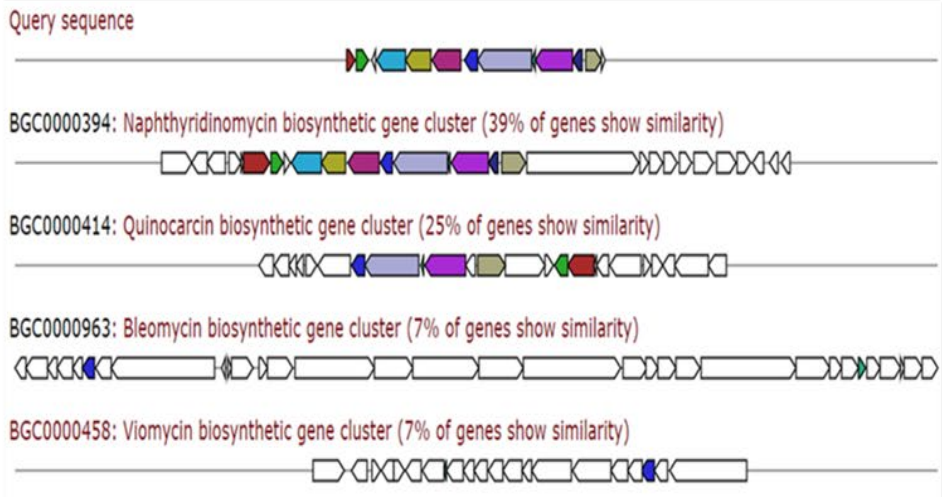
Biosynthetic assembly

Cluster

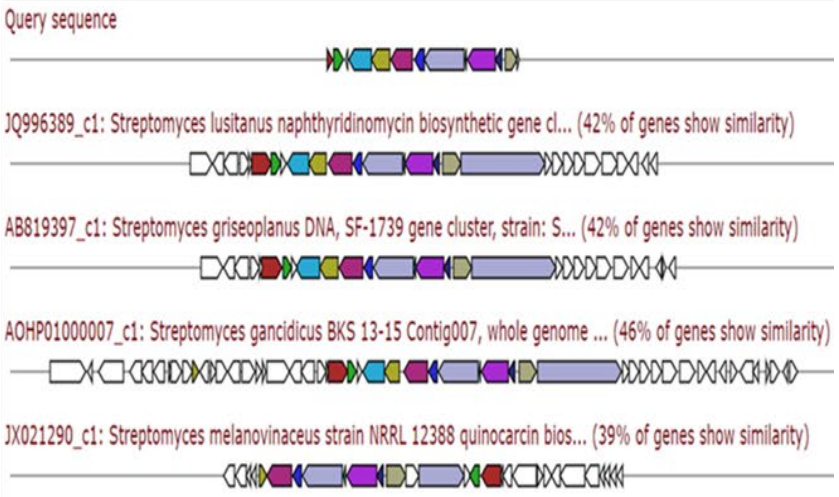
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

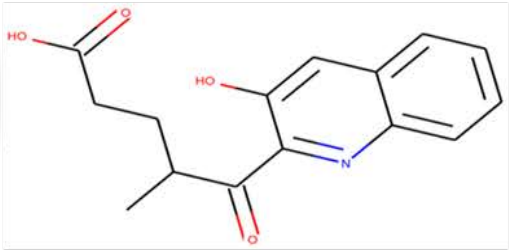


CSK3_Cluster 13_T2Pks

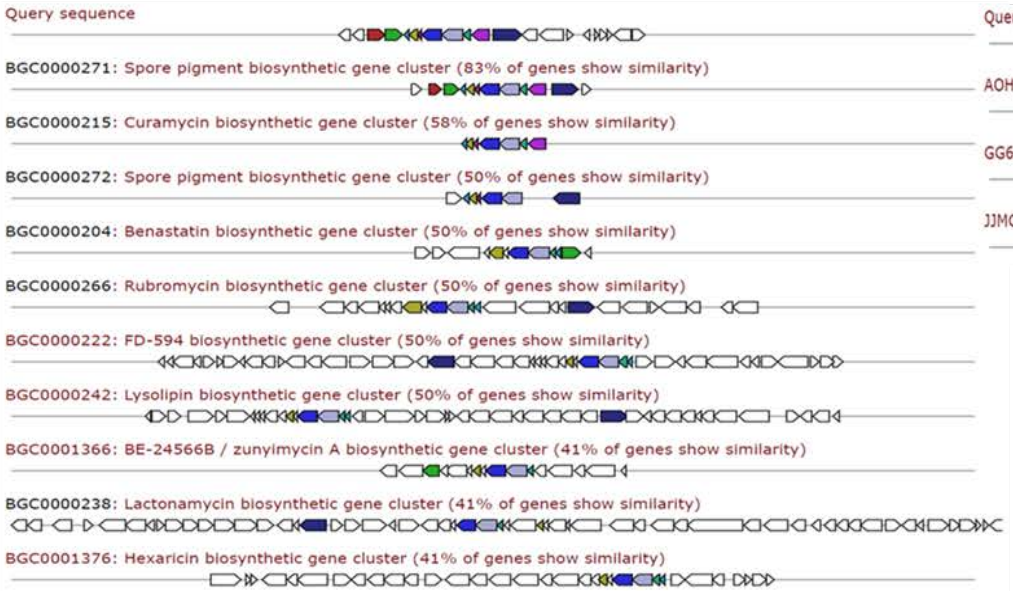
Biosynthetic assembly

Cluster

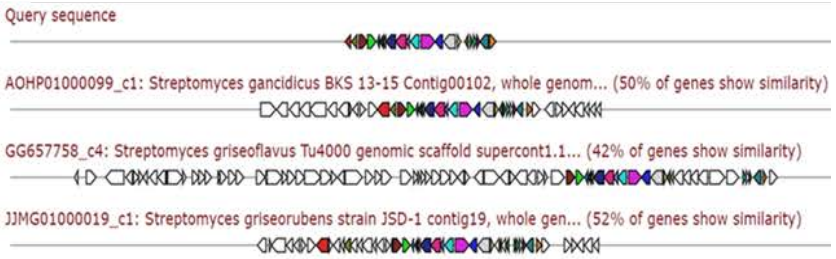
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

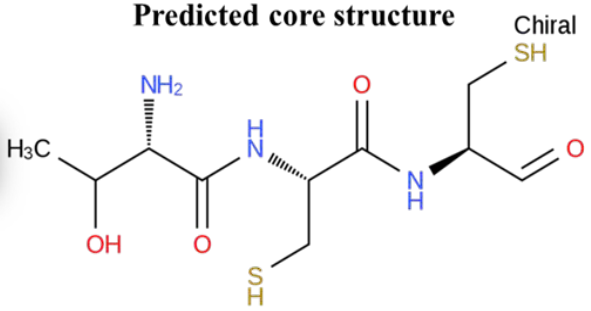


CSK3_Cluster 15_Nrps

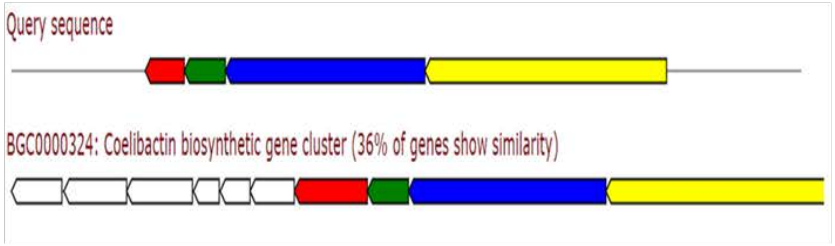
Biosynthetic assembly

Cluster

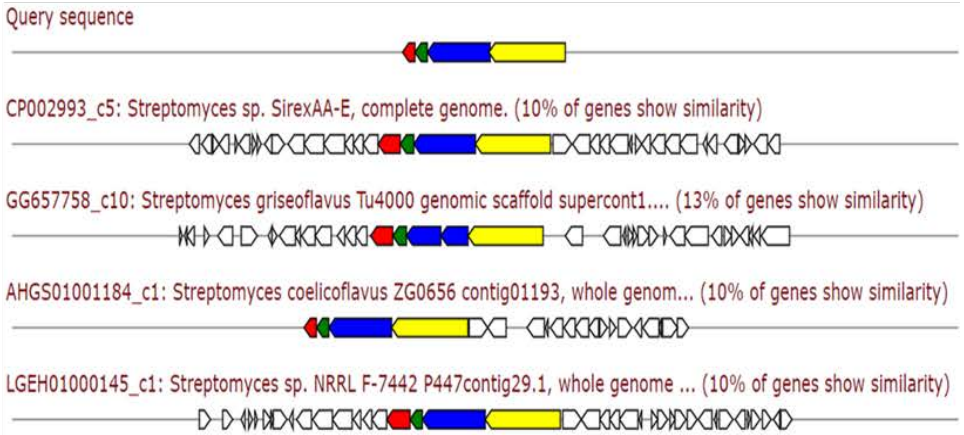
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

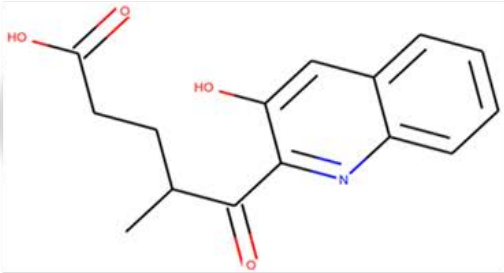


CSK3_Cluster 18_T1Pks/Nrps

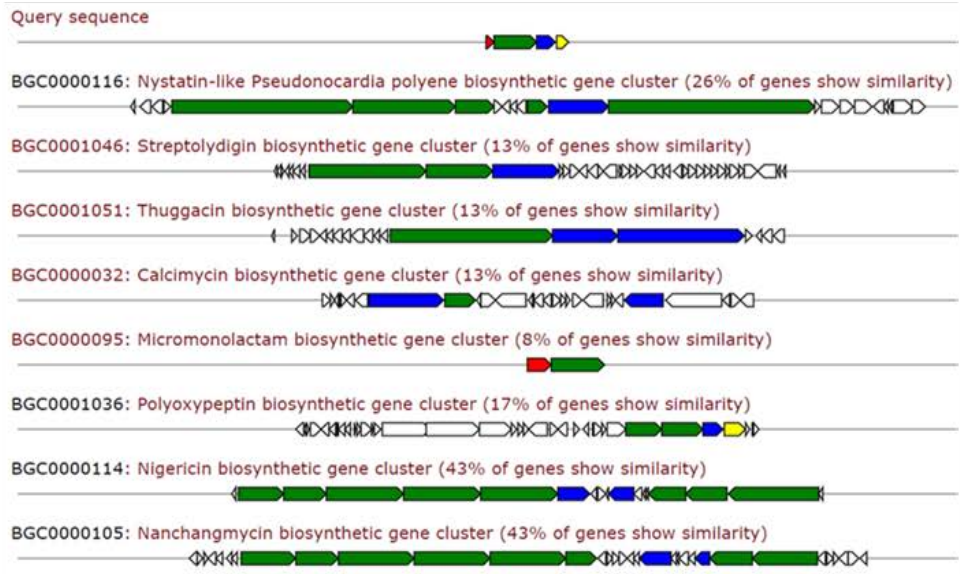
Biosynthetic assembly

Cluster

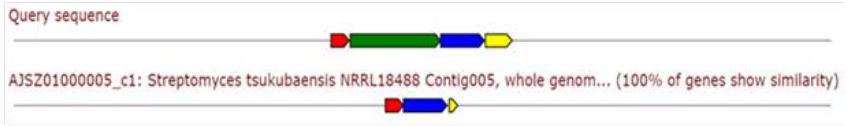
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSK3_Cluster 23_Nrps

Biosynthetic assembly

Cluster

Predicted core structure

- **No similar gene clusters are identified in the database**
- **No predicted structure**

CSK3_Cluster 26_T3Pks

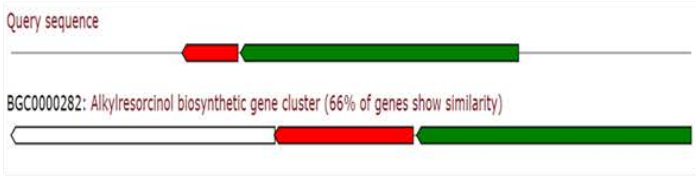
Biosynthetic assembly

Cluster

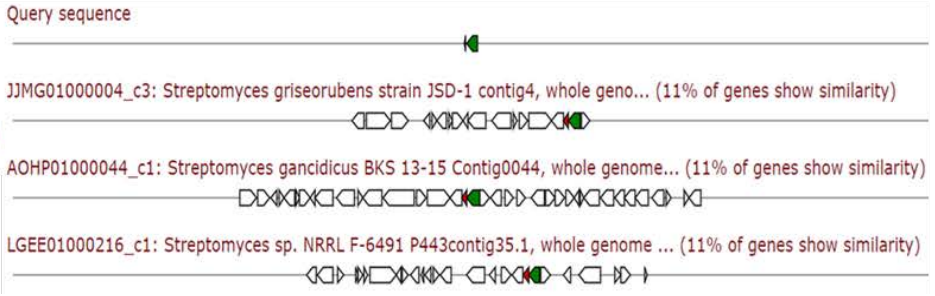
Predicted core structure

- **No predicted structure**

Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



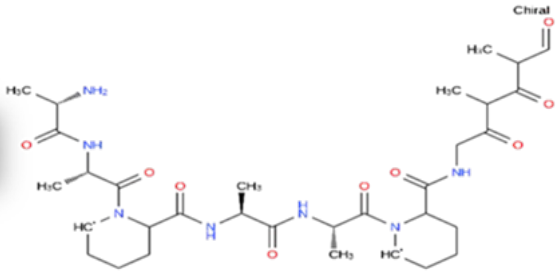
CSW2_Cluster 3_Nrps

C

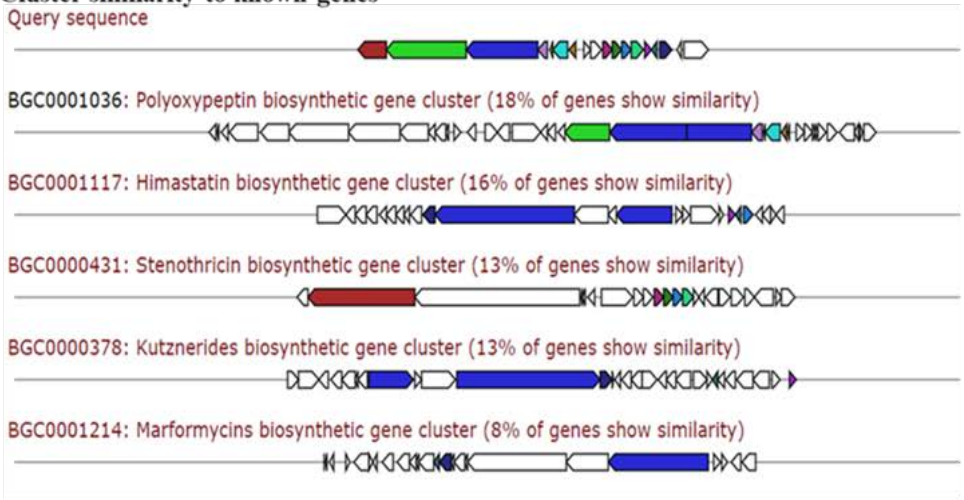
Biosynthetic assembly

Cluster

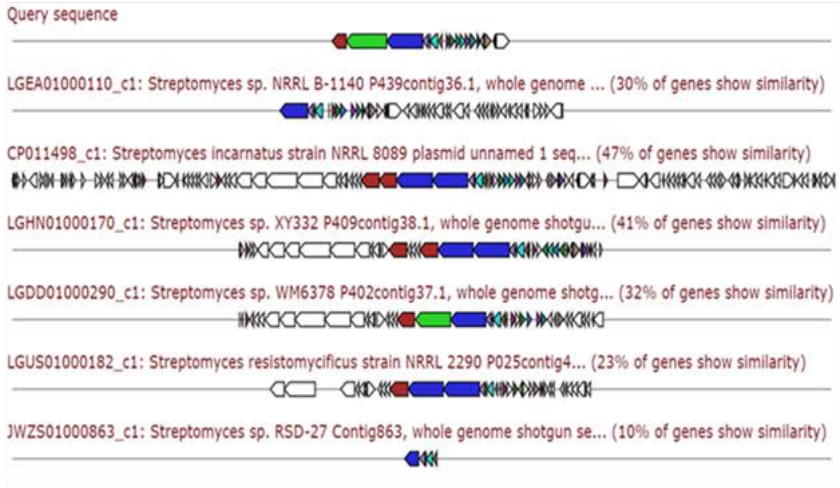
Predicted core structure



Cluster similarity to known genes

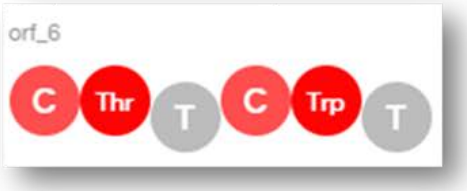


Cluster similarity in *Streptomyces* species



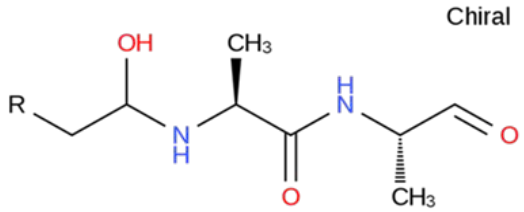
CSW2_Cluster 8_Nrps_Other Ks

Biosynthetic assembly

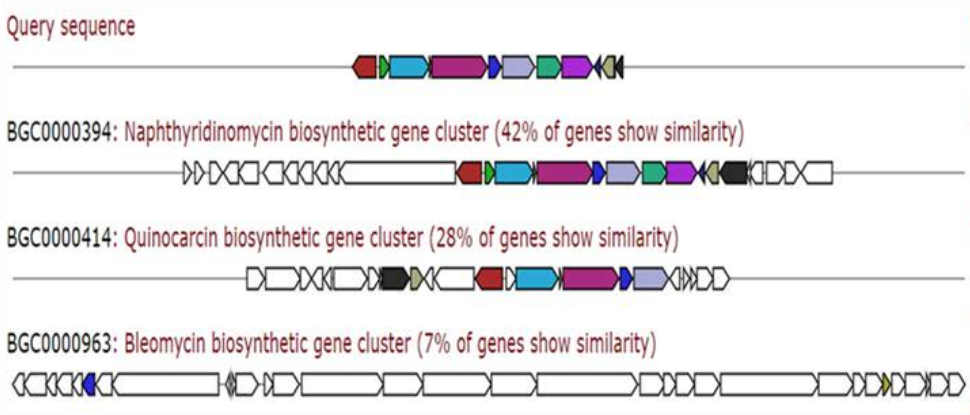


Cluster

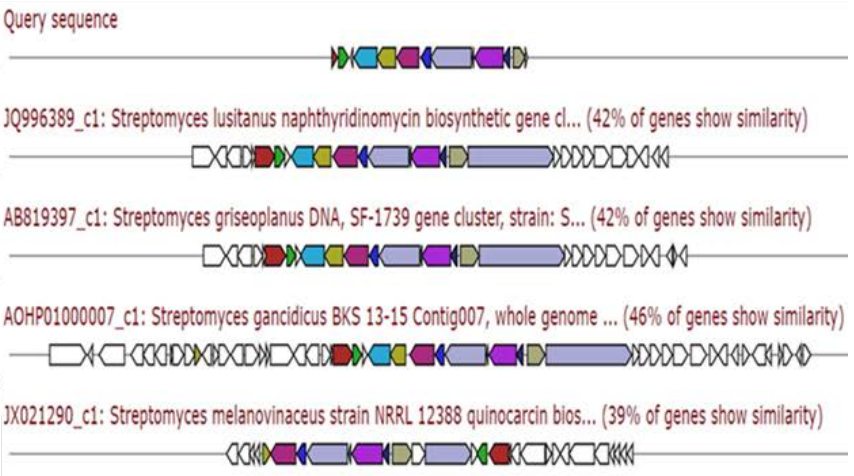
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

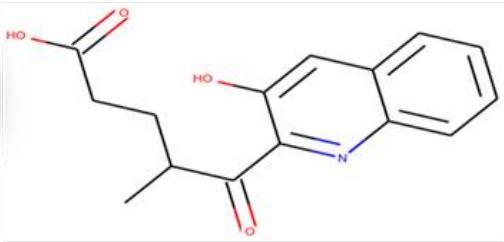


CSW2_Cluster 9_T2Pks

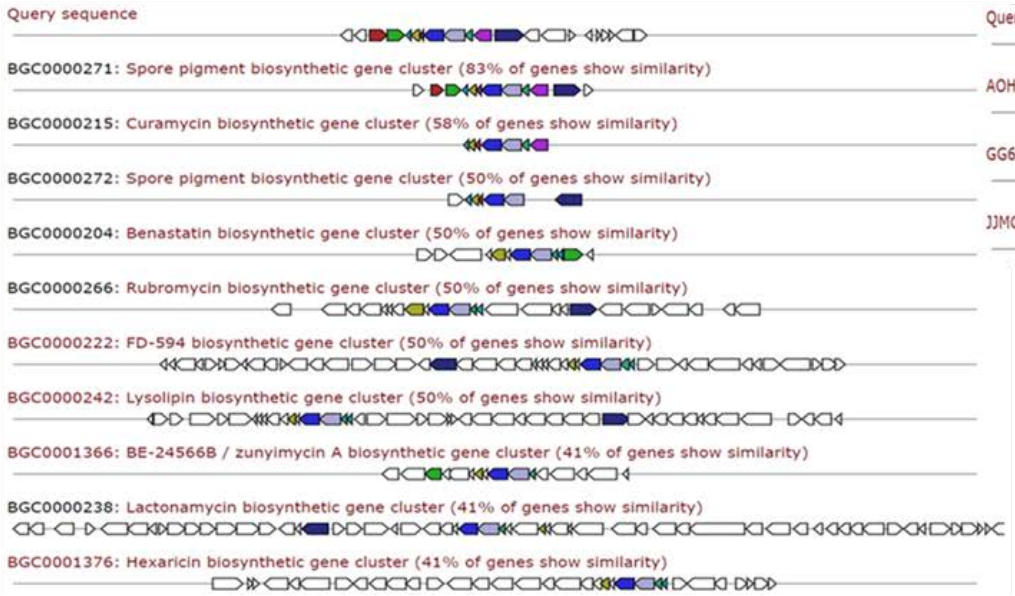
Biosynthetic assembly

Cluster

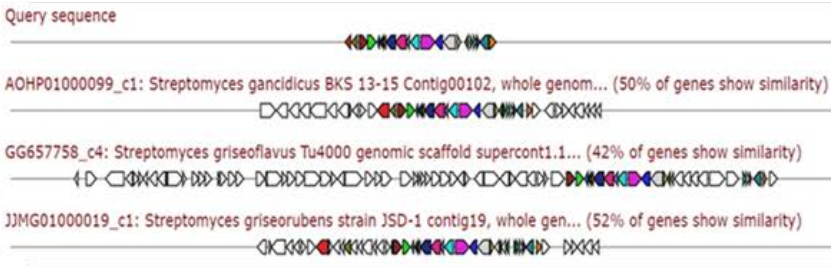
Predicted core structure



Cluster similarity to known genes

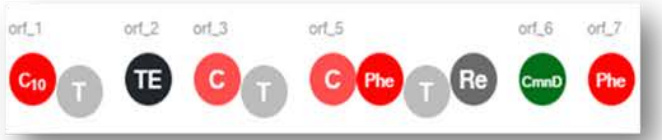


Cluster similarity in *Streptomyces* species



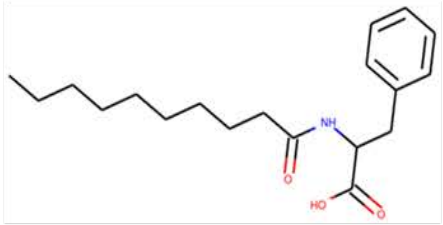
CSW2_Cluster 11_Nrps

Biosynthetic assembly

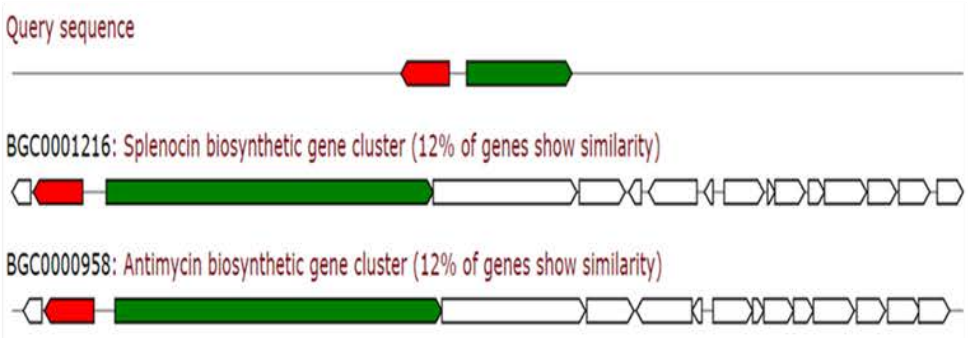


Cluster

Predicted core structure



Cluster similarity to known genes



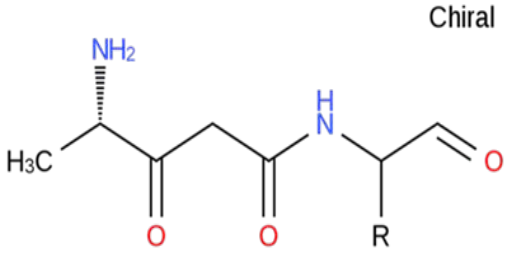
Cluster similarity in *Streptomyces* species

CSW2_Cluster 14_T1Pks/Nrps

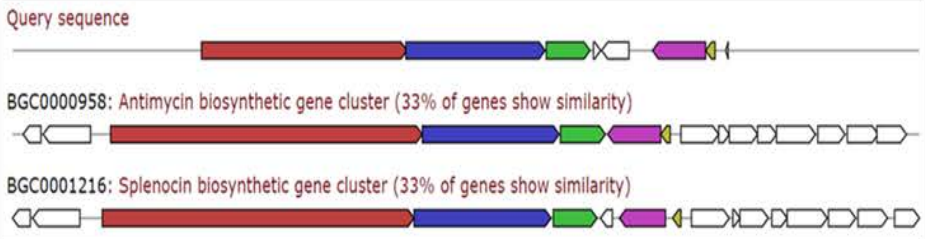
Biosynthetic assembly

Cluster

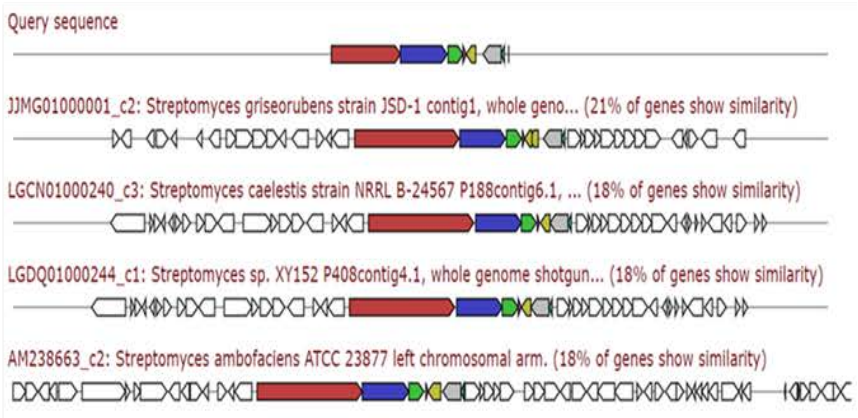
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

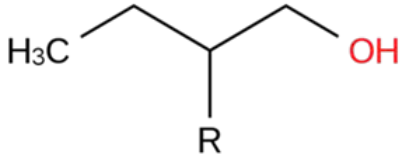


CSW2_Cluster 15_T1Pks_Nrps

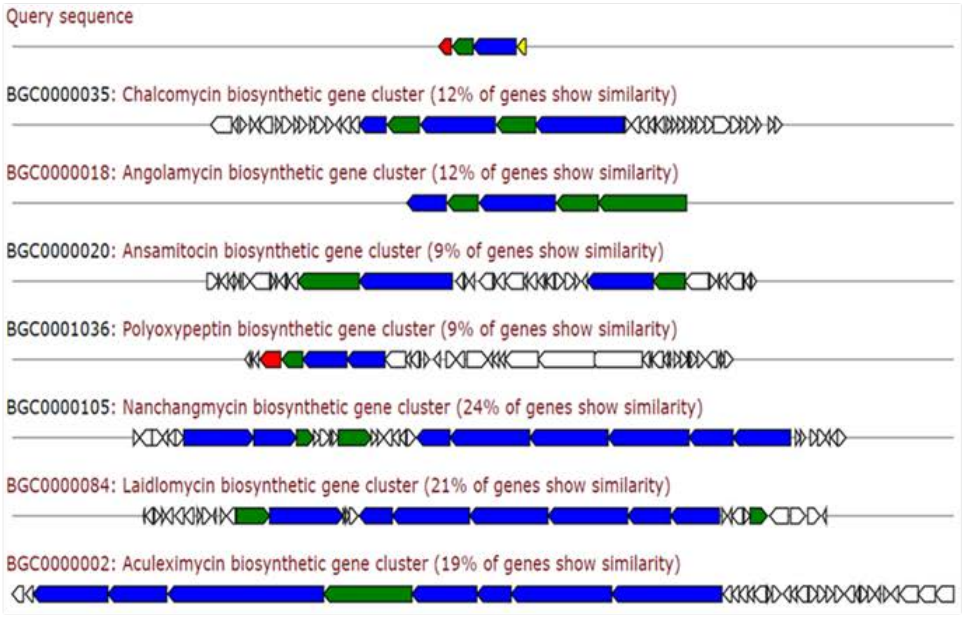
Biosynthetic assembly

Cluster

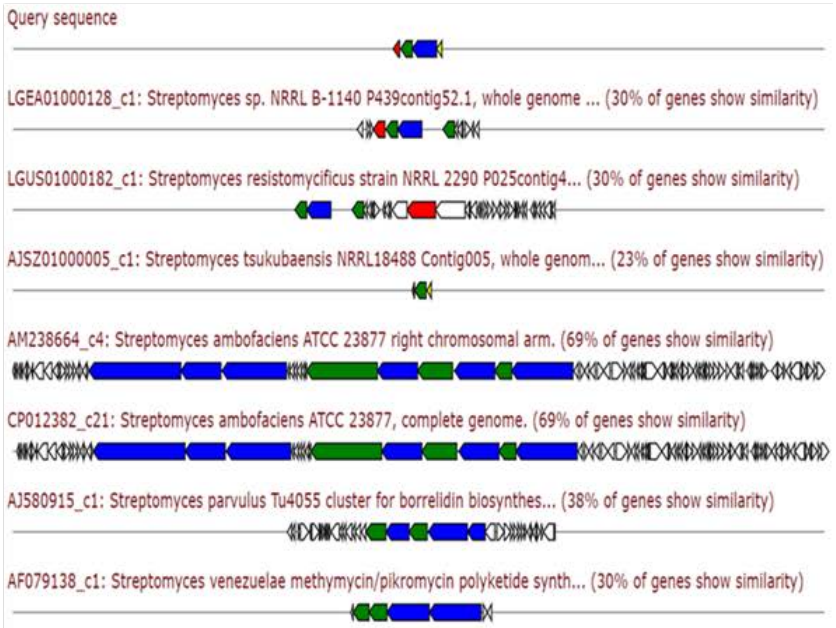
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

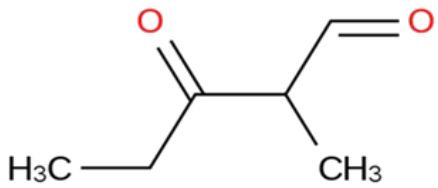


CSW2_Cluster 16_T1Pks

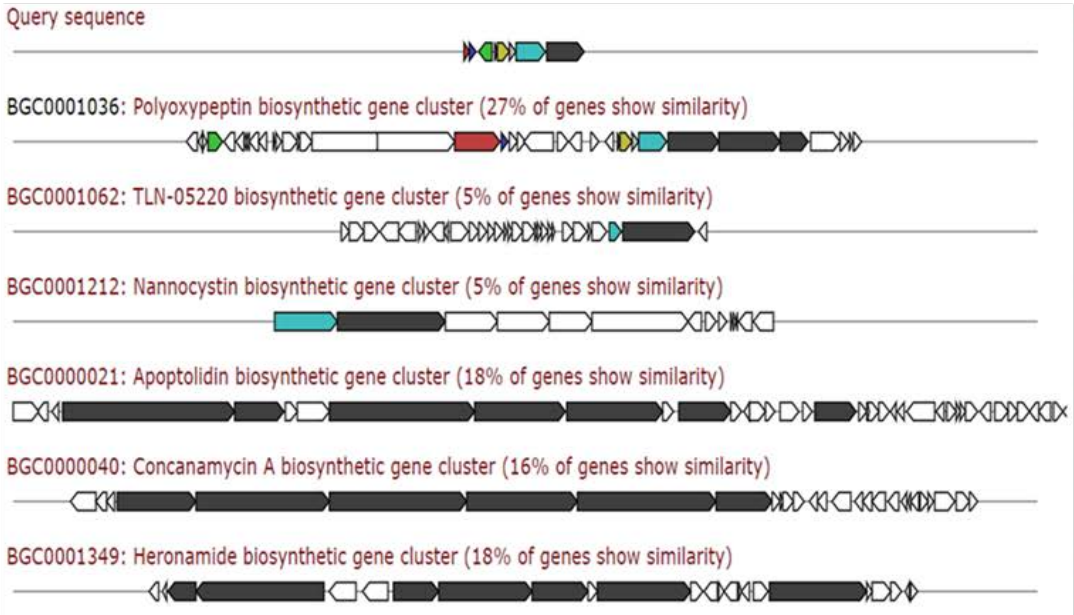
Biosynthetic assembly

Cluster

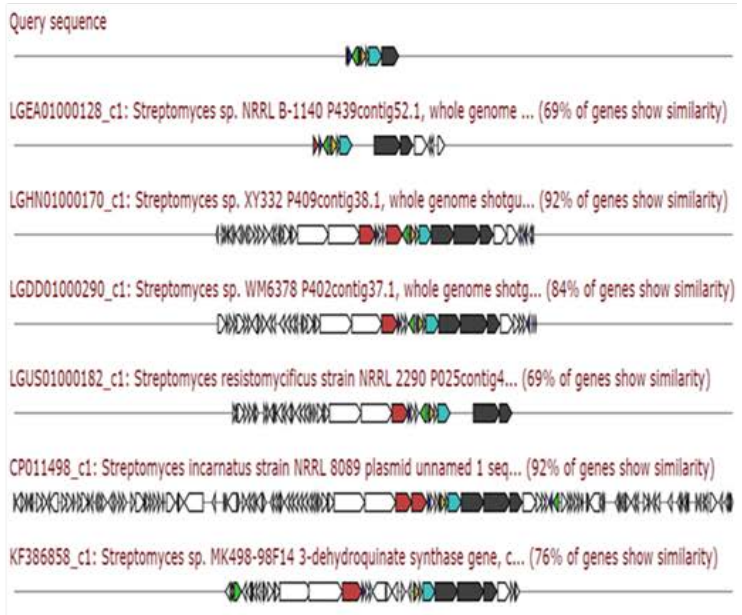
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSW2_Cluster 23_Nrps

Biosynthetic assembly

Cluster

CSW2_Cluster 24_Nrps

Biosynthetic assembly

Cluster

- **No predicted structure in these clusters**
- **No similar gene clusters are identified in the database**

CSW2_Cluster 26 _Nrps

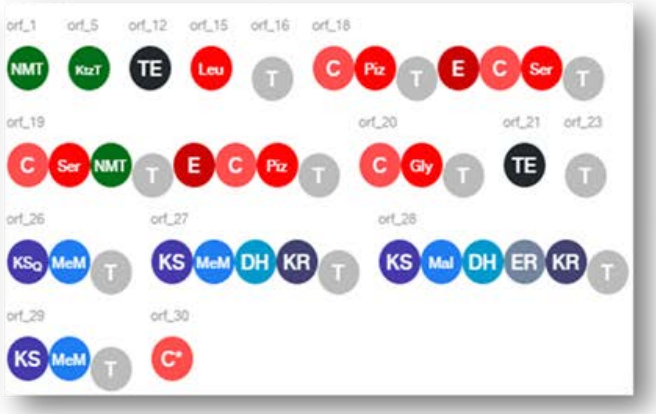
Biosynthetic assembly

Cluster

D

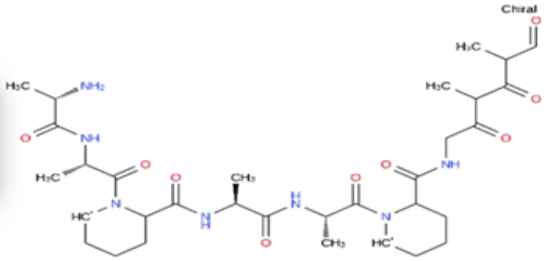
CSU1_Cluster 4_T1Pks/Nrps

Biosynthetic assembly

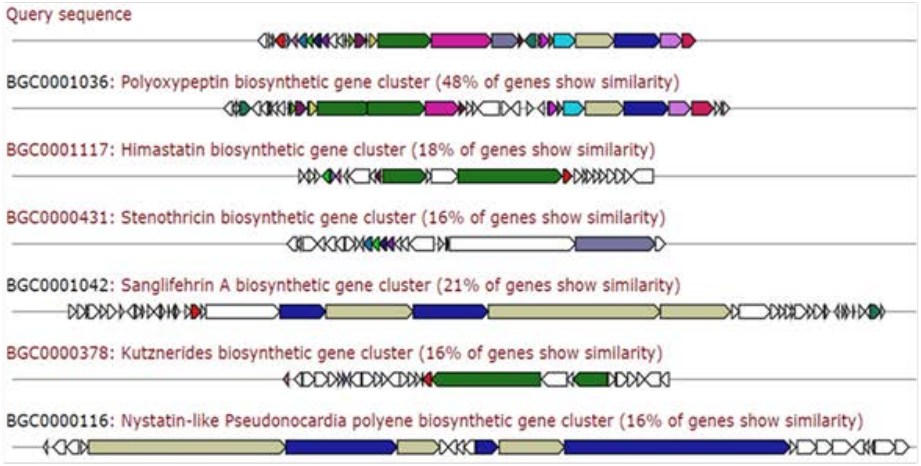


Cluster

Predicted core structure



Cluster similarity to known genes



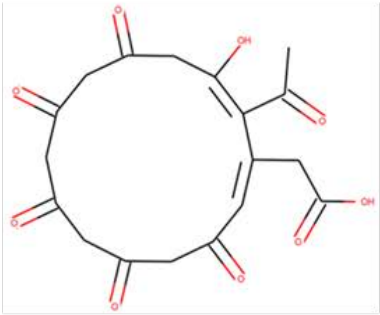
Cluster similarity in *Streptomyces* species

CSU1_Cluster 7_T2Pks

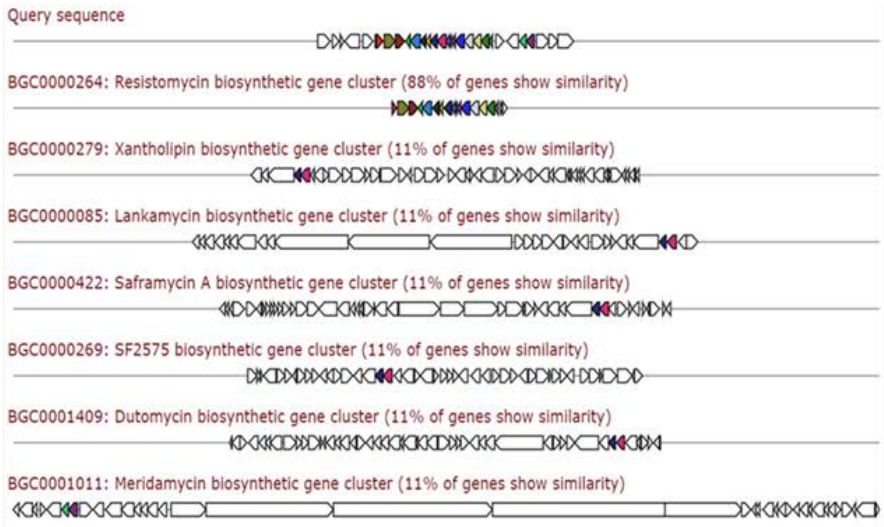
Biosynthetic assembly

Cluster

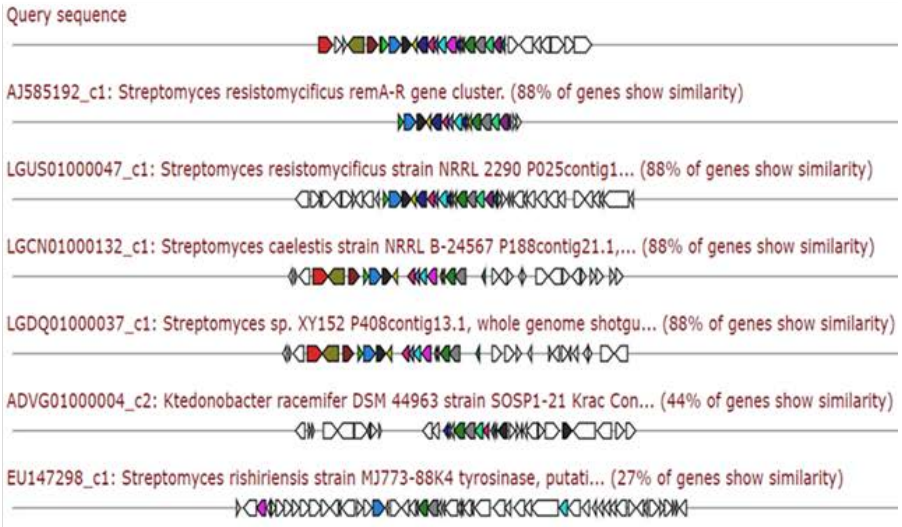
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

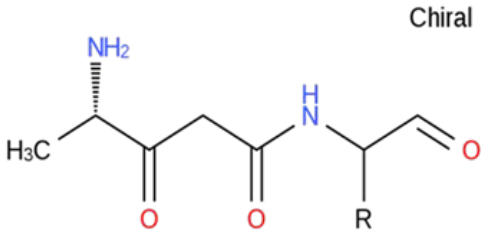


CSU1_Cluster 8_T1Pks/Nrps

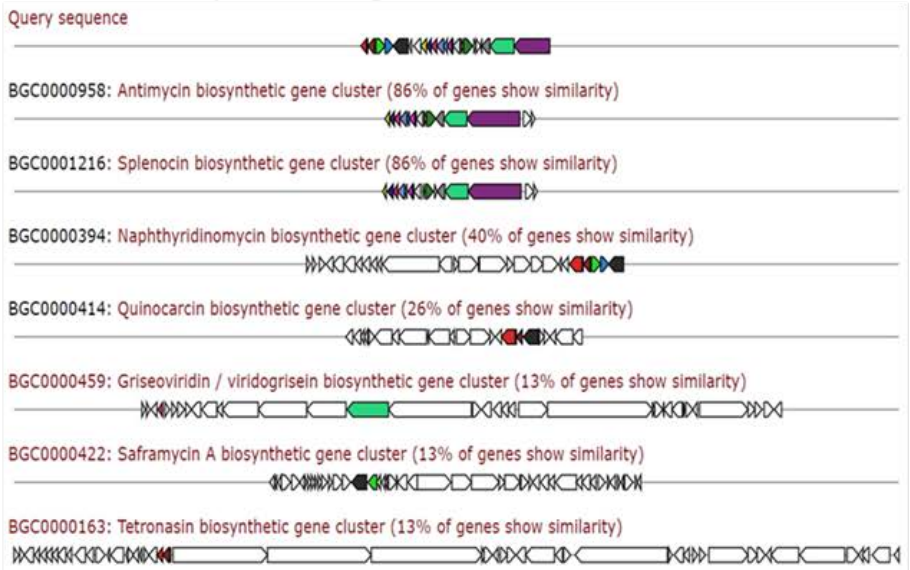
Biosynthetic assembly

Cluster

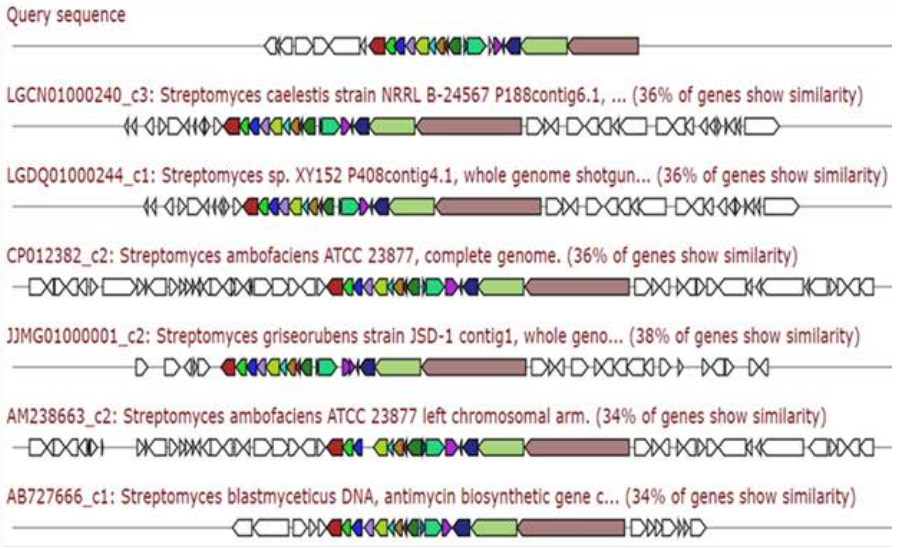
Predicted core structure



Cluster similarity to known genes

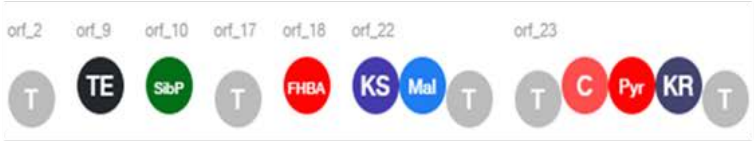


Cluster similarity in *Streptomyces* species



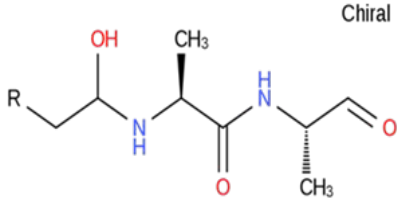
CSU1_Cluster 12_Other_Ks/Nrps

Biosynthetic assembly

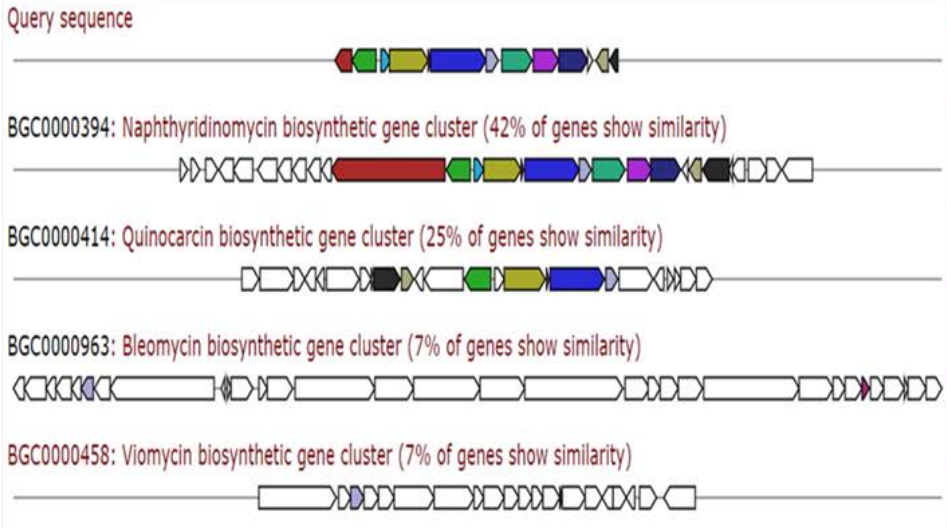


Cluster

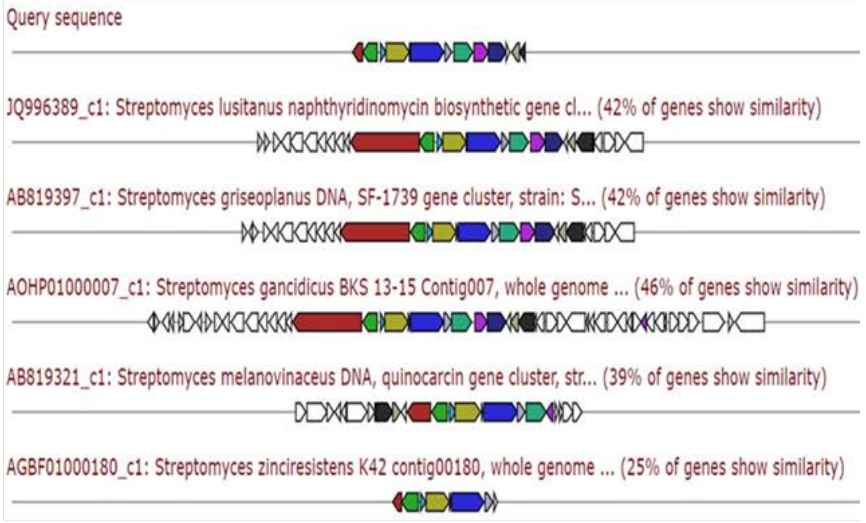
Predicted core structure



Cluster similarity to known genes

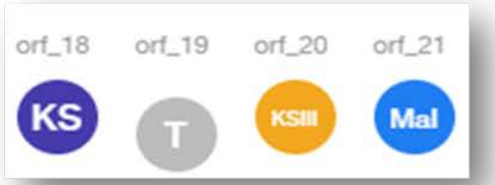


Cluster similarity in *Streptomyces* species



CSU1_Cluster 16_T3Pks

Biosynthetic assembly

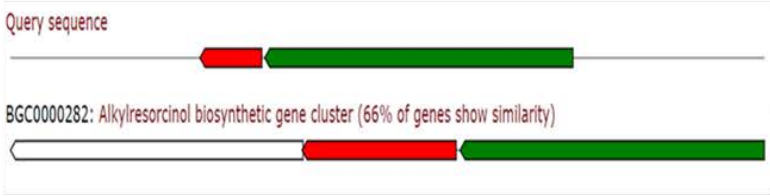


Cluster

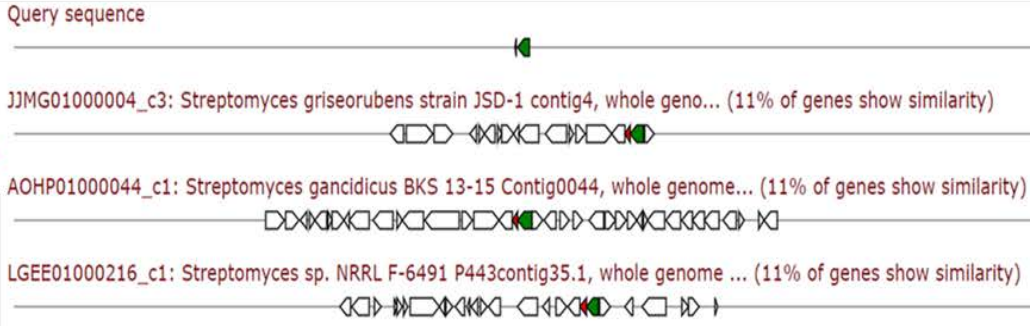
Predicted core structure

- **No predicted structure**

Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSU1_Cluster 21_Nrps

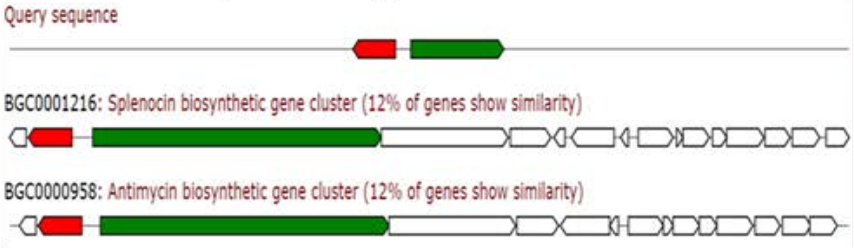
Biosynthetic assembly

Cluster

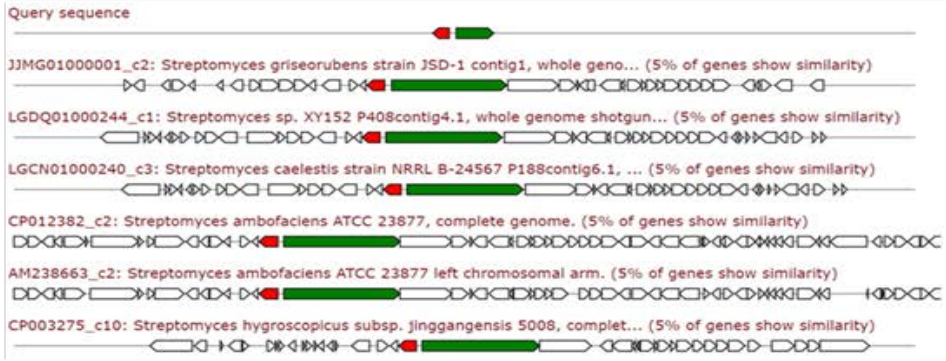
Predicted core structure

- No predicted structure

Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSU1_Cluster 23_Nrps

Biosynthetic assembly

Cluster

Predicted core structure

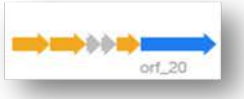
- No predicted structure
- No similar gene clusters are identified in the database

E

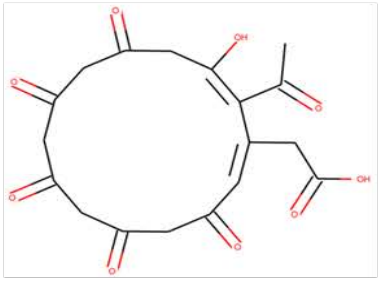
CSU2_Cluster 4_T2Pks

Biosynthetic assembly

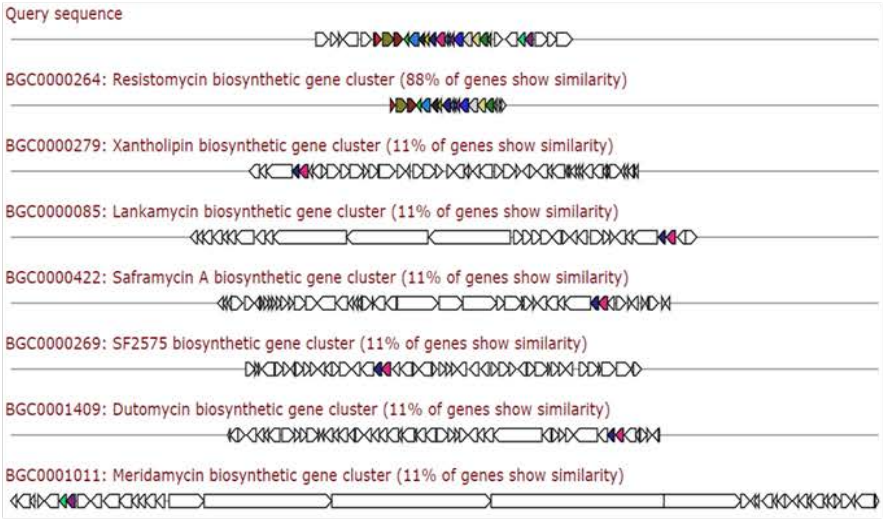
Cluster



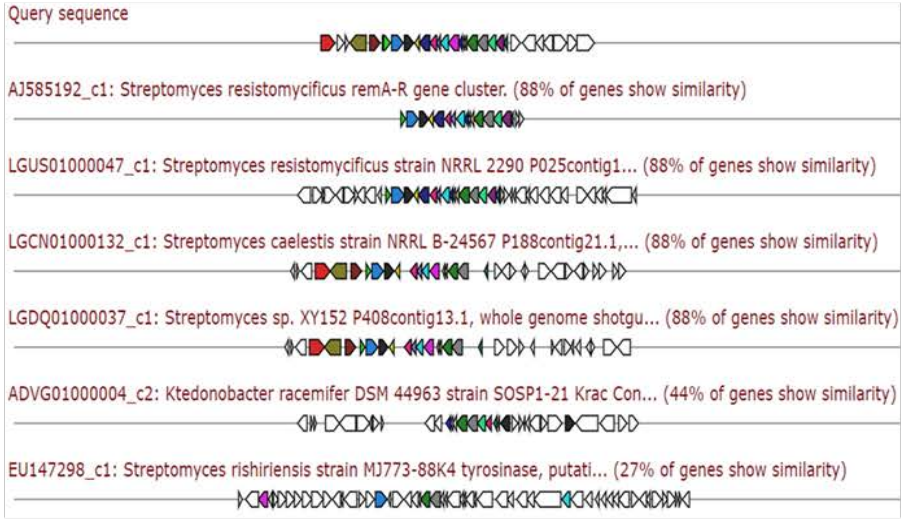
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

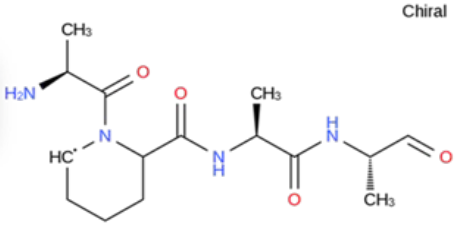


CSU2_Cluster 7_Nrps

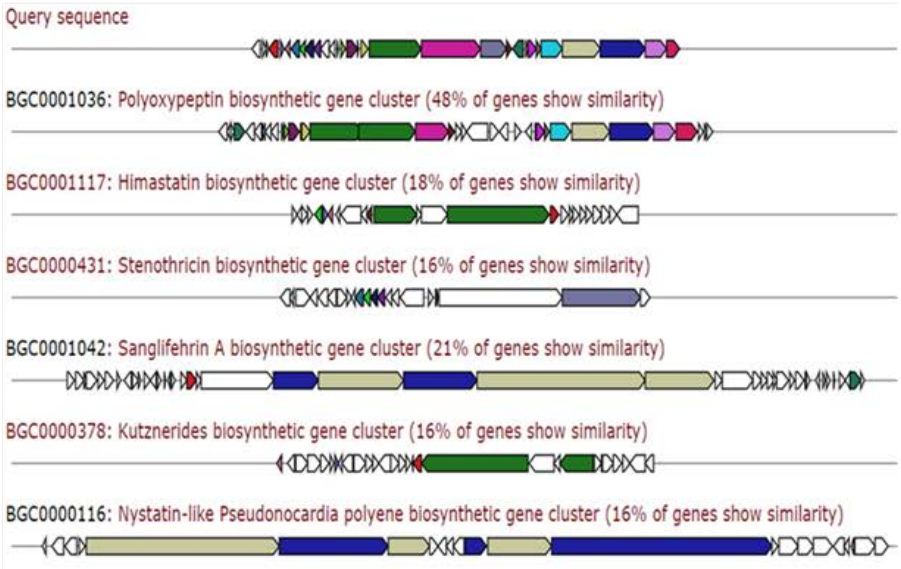
Biosynthetic assembly

Cluster

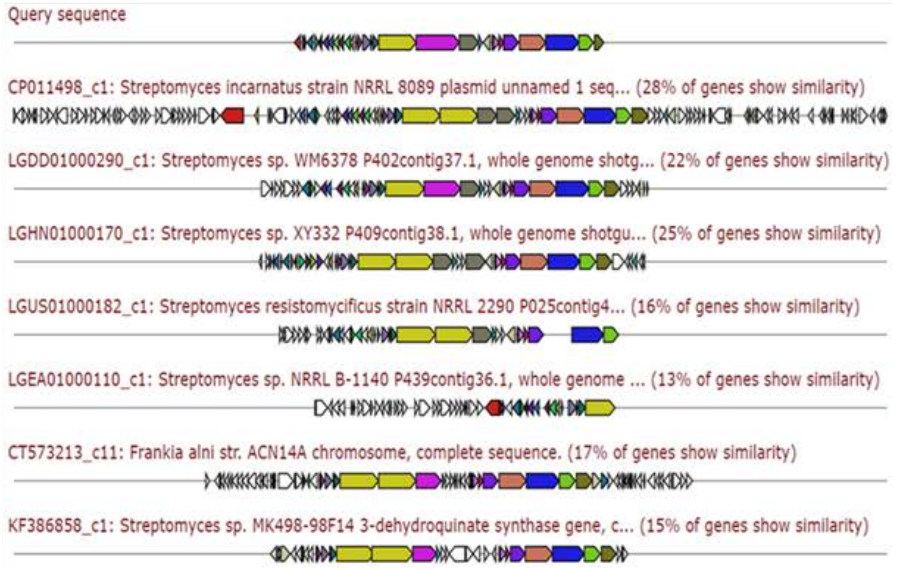
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

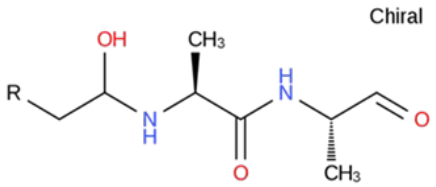


CSU2_Cluster 9_Other_Ks/Nrps

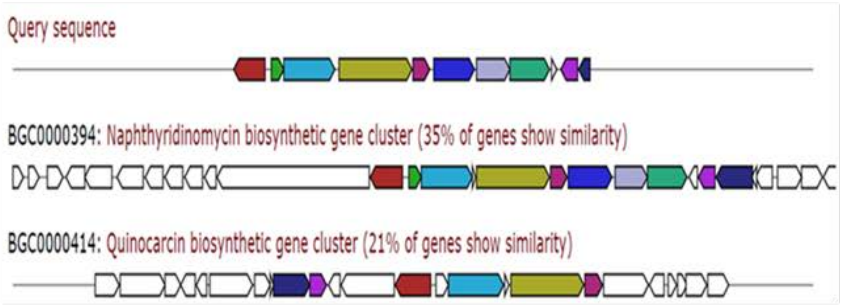
Biosynthetic assembly

Cluster

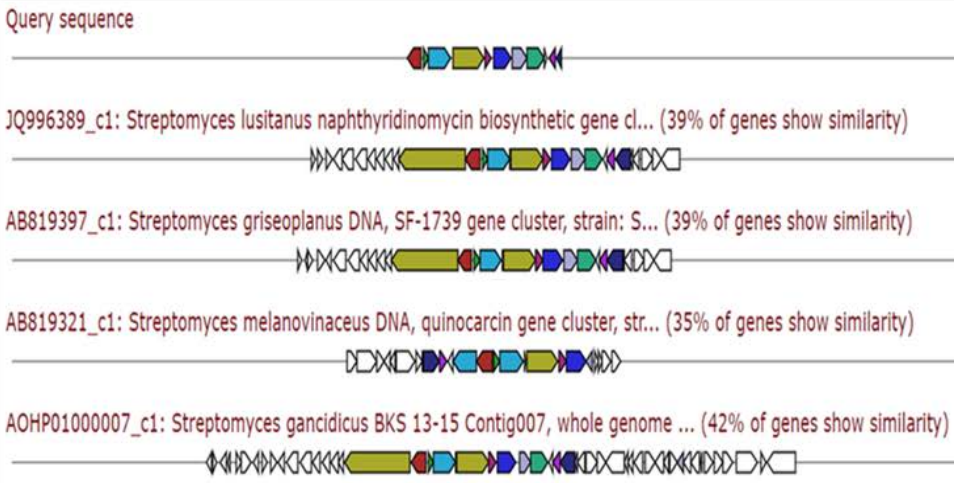
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSU2_Cluster 10_T2Pks

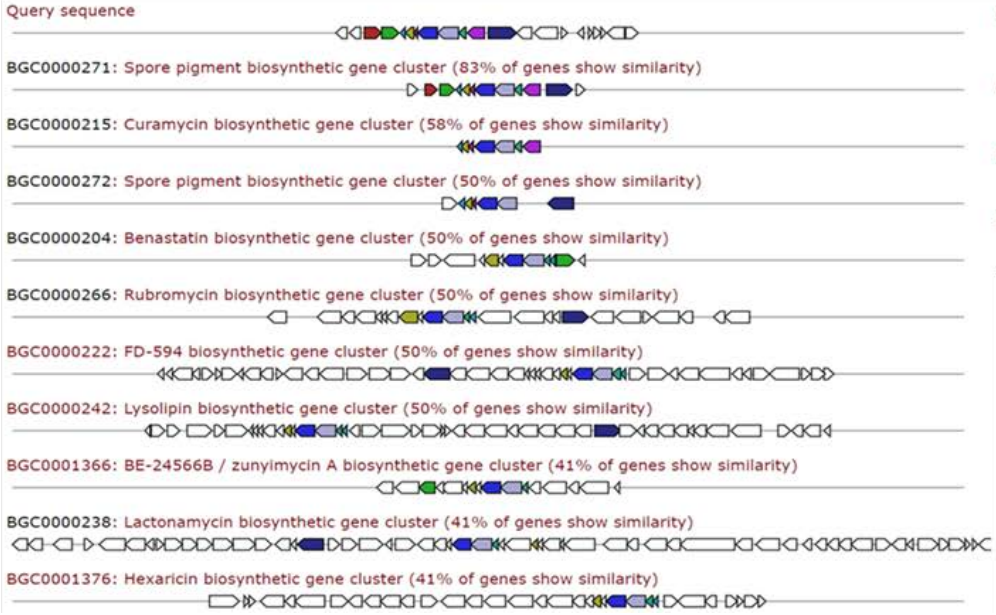
Biosynthetic assembly

Cluster

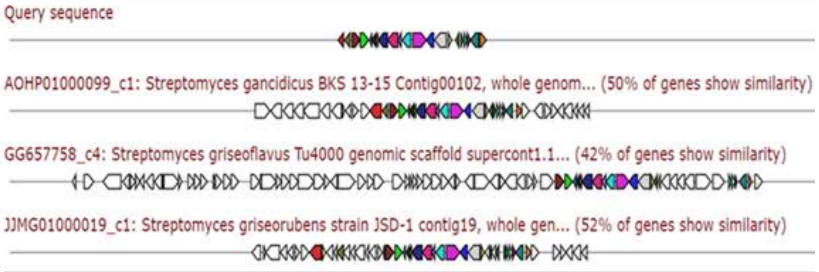
Predicted core structure

- No predicted structure

Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

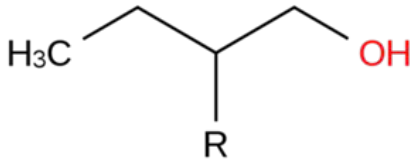


CSU2_Cluster 14_T1Pks/Nrps

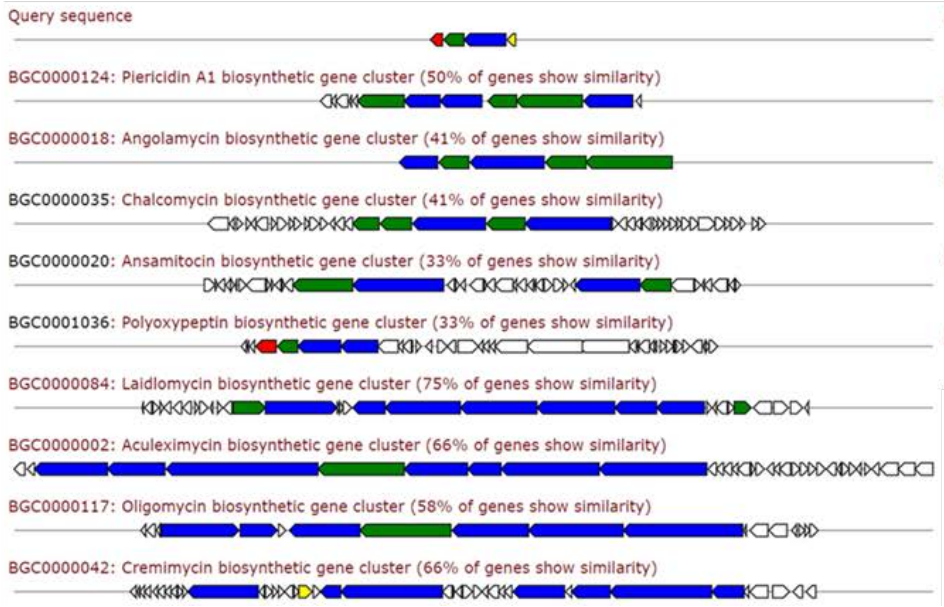
Biosynthetic assembly

Cluster

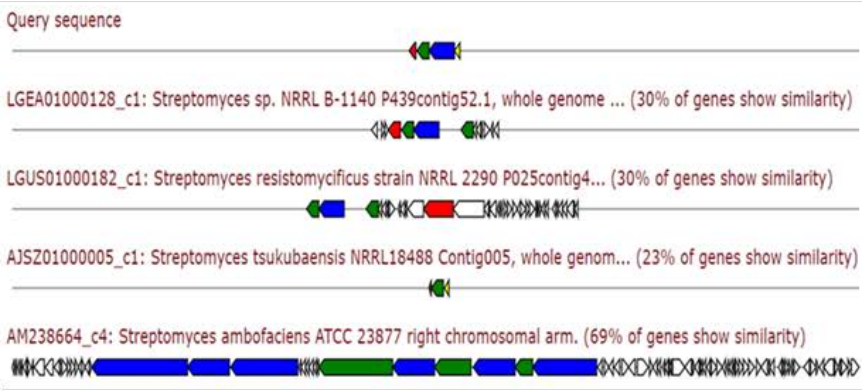
Predicted core structure



Cluster similarity to known genes

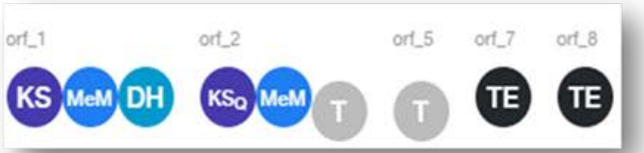


Cluster similarity in *Streptomyces* species



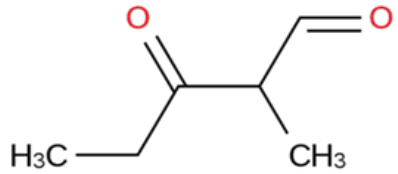
CSU2_Cluster 15_T1Pks

Biosynthetic assembly

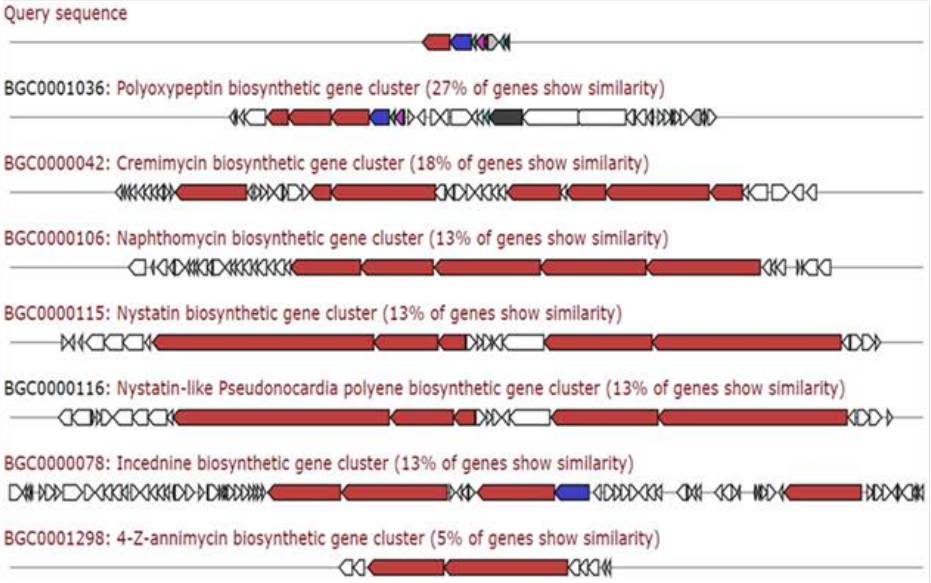


Cluster

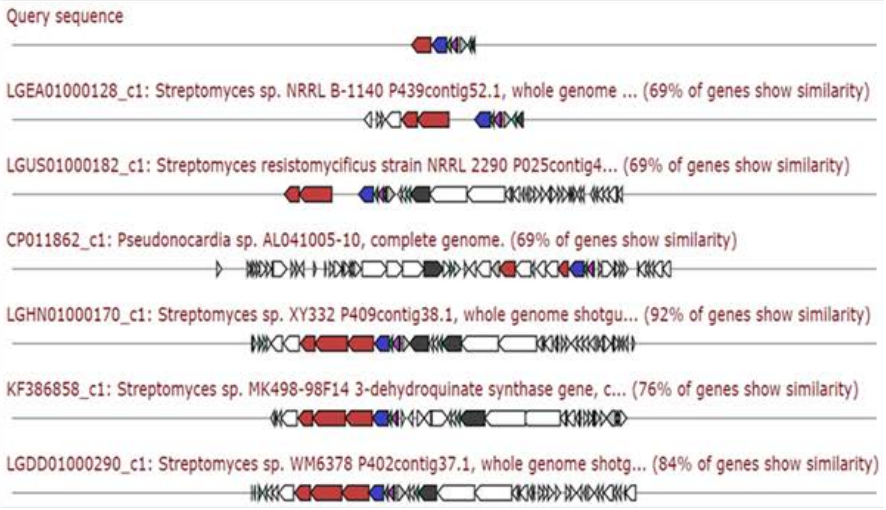
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

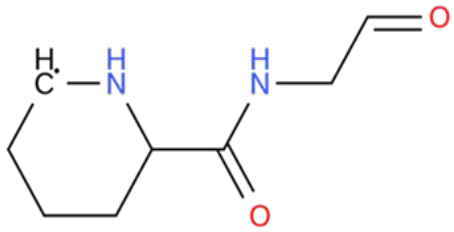


CSU2_Cluster 19_Nrps

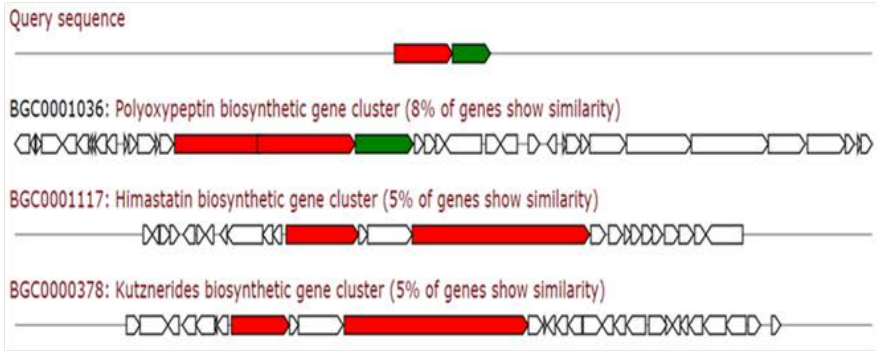
Biosynthetic assembly

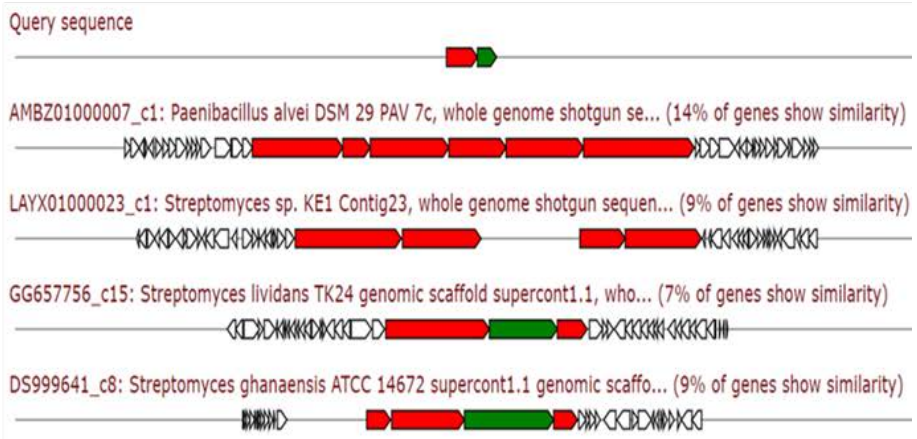
Cluster

Predicted core structure



Cluster similarity to known genes





CSU2_Cluster 22_Nrps

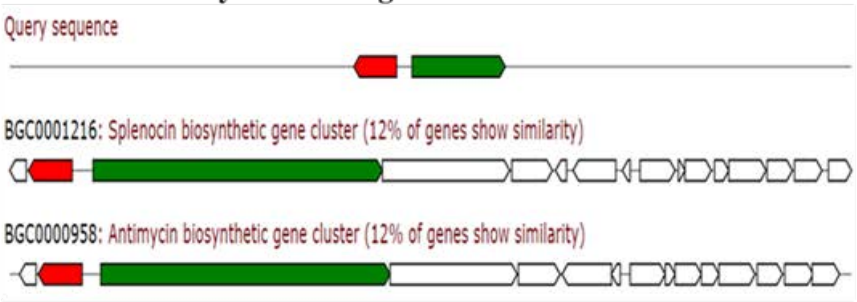
Biosynthetic assembly

Cluster

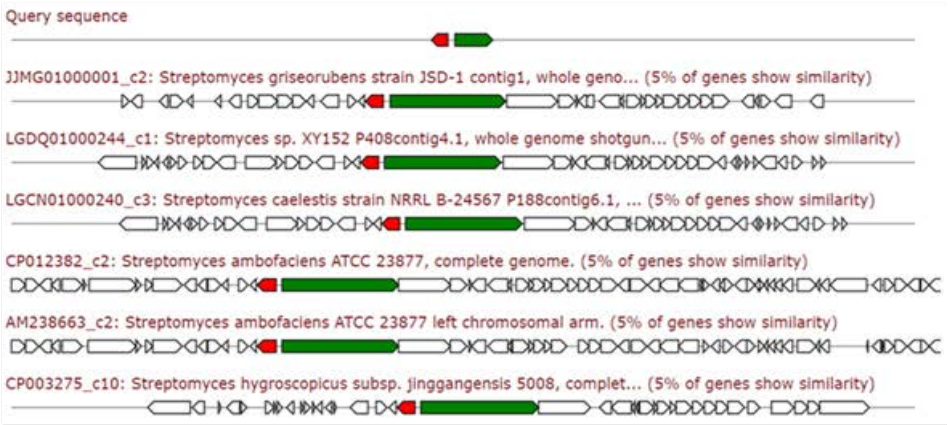
Predicted core structure

- **No predicted structure**

Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSU2_Cluster 23_Nrps

Biosynthetic assembly

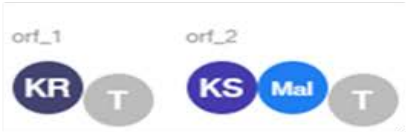
Cluster

Predicted core structure

- **No predicted structure**
- **No similar gene clusters are identified in the database**

CSU2_Cluster 24_T1Pks

Biosynthetic assembly

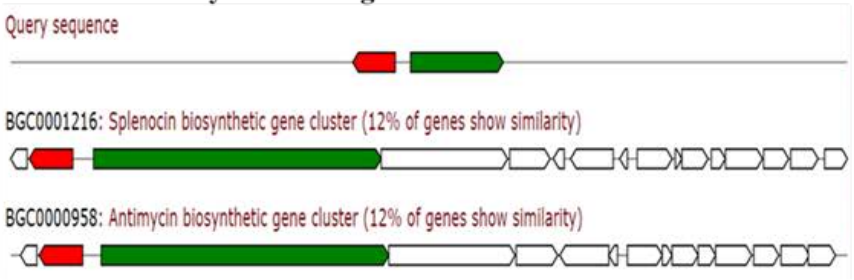


Cluster

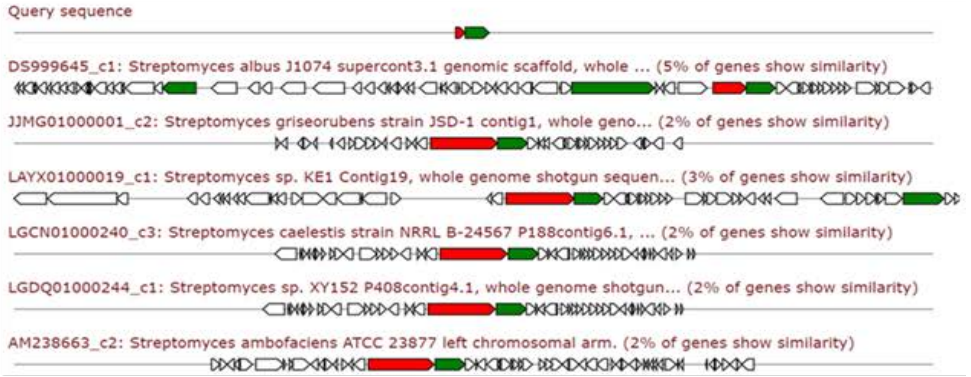
Predicted core structure

- **No predicted structure**

Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSU2_Cluster 26_Nrps

Biosynthetic assembly

Cluster

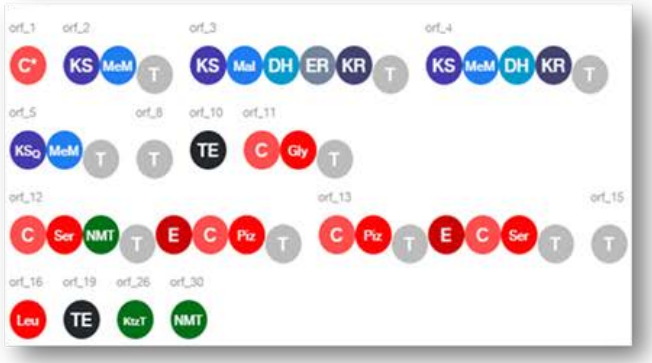
Predicted core structure

- **No predicted structure**
- **No similar gene clusters are identified in the database**

F

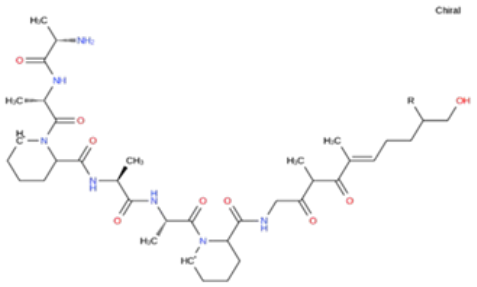
CSG1_Cluster 4_T1Pks/Nrps

Biosynthetic assembly

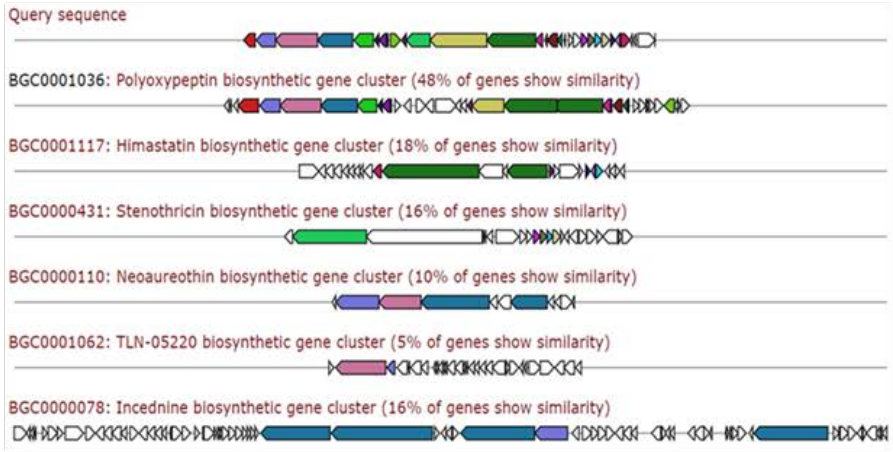


Cluster

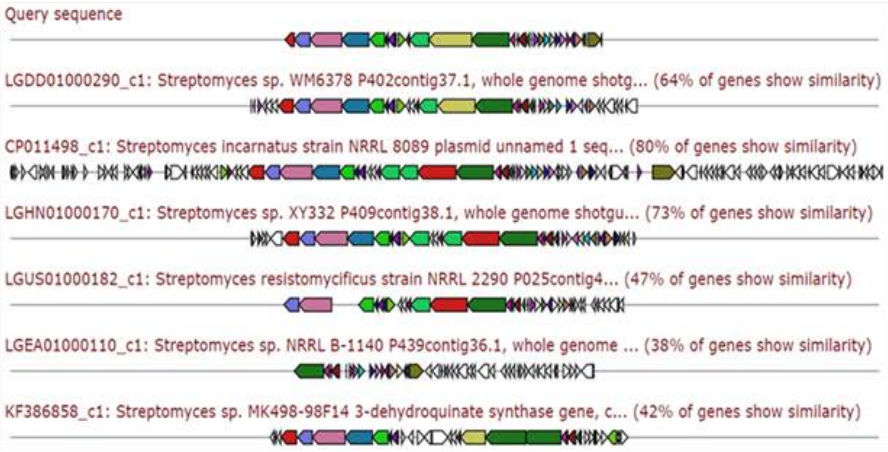
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

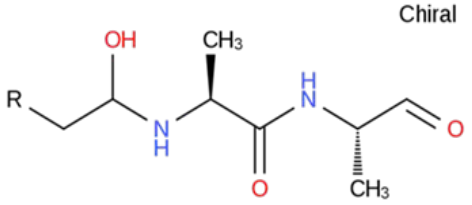


CSG1_Cluster 12_Nrps/Other Ks

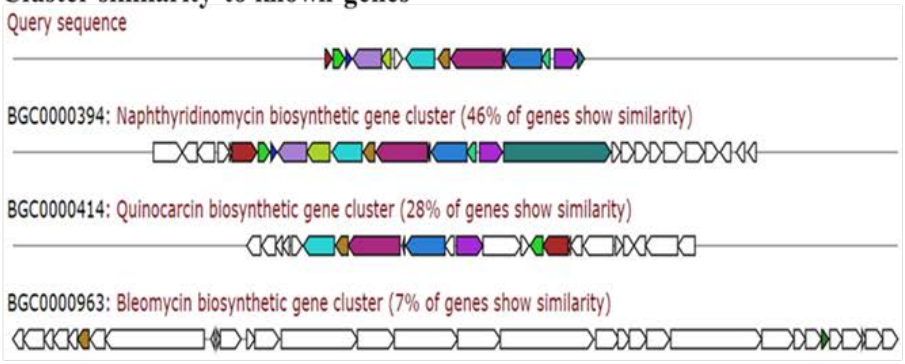
Biosynthetic assembly

Cluster

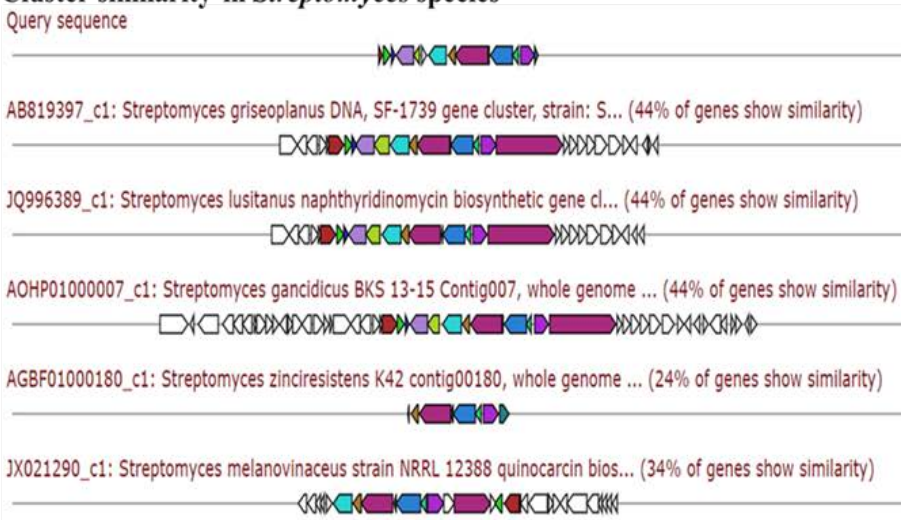
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSG1_Cluster 16_T2Pks

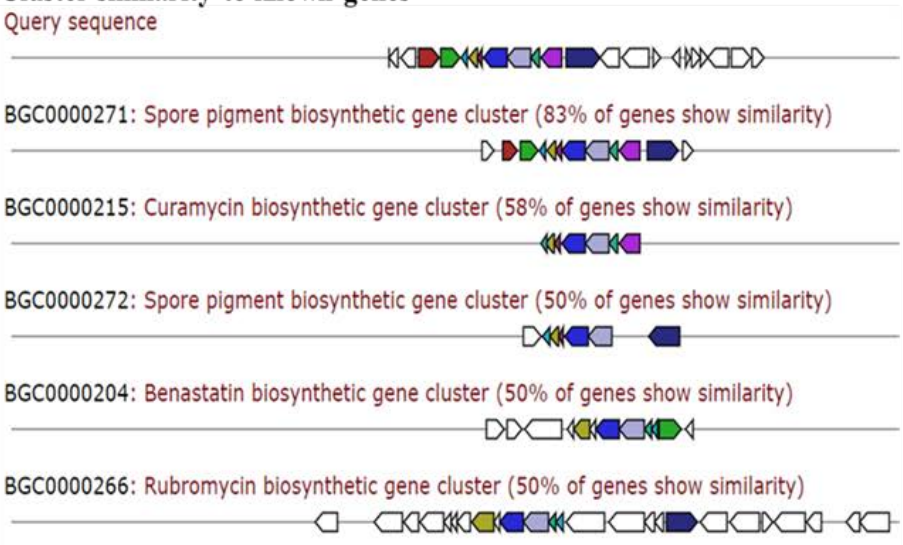
Biosynthetic assembly

Cluster

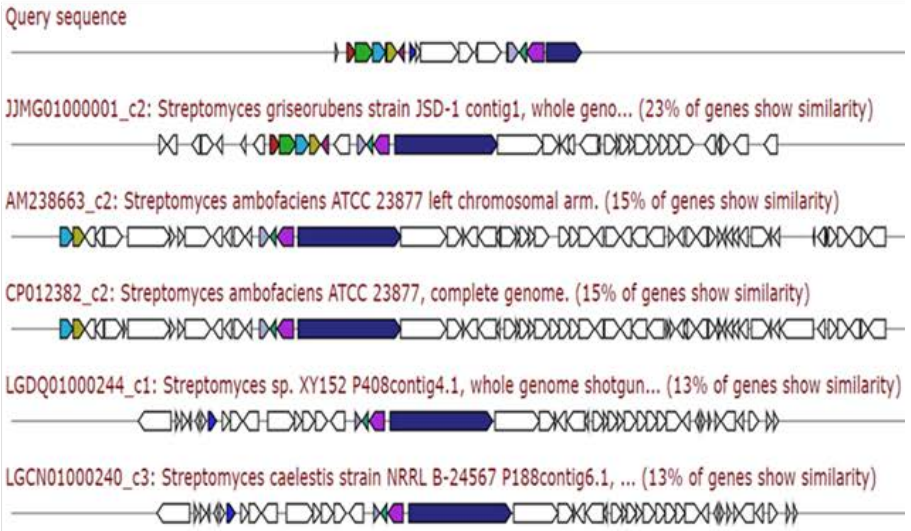
Predicted core structure

- No predicted structure

Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSG1_Cluster 23_Nrps

Biosynthetic assembly

Cluster

CSG1_Cluster 23_Nrps

Biosynthetic assembly

Cluster

- **No predicted structure for these clusters**
- **No similar gene clusters are identified in the database**

CSG1_Cluster 23_Nrps

Biosynthetic assembly

Cluster

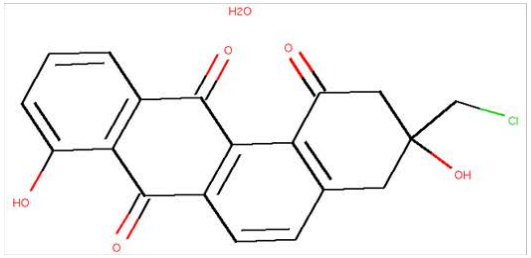
CSM1_Cluster 5 _T2PKS/Butyrolactone

G

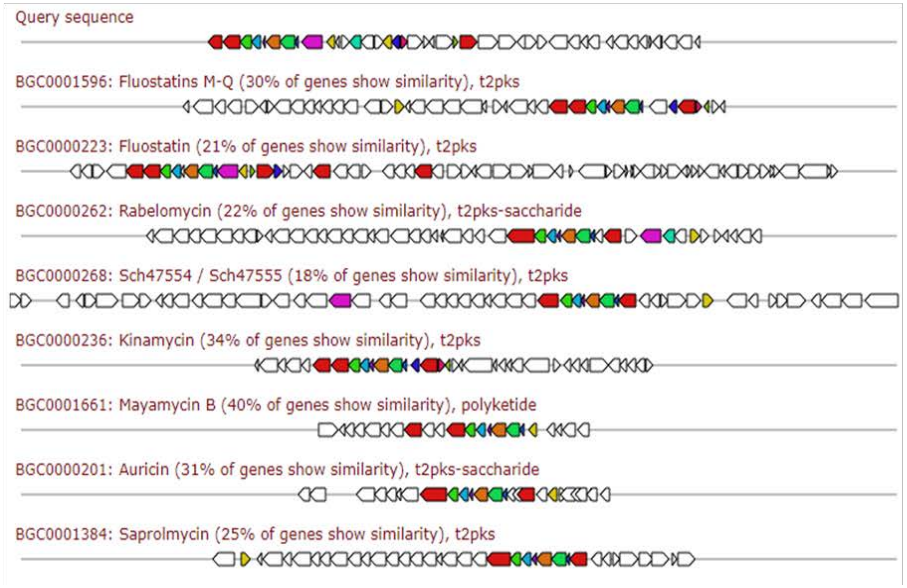
Biosynthetic assembly

Cluster

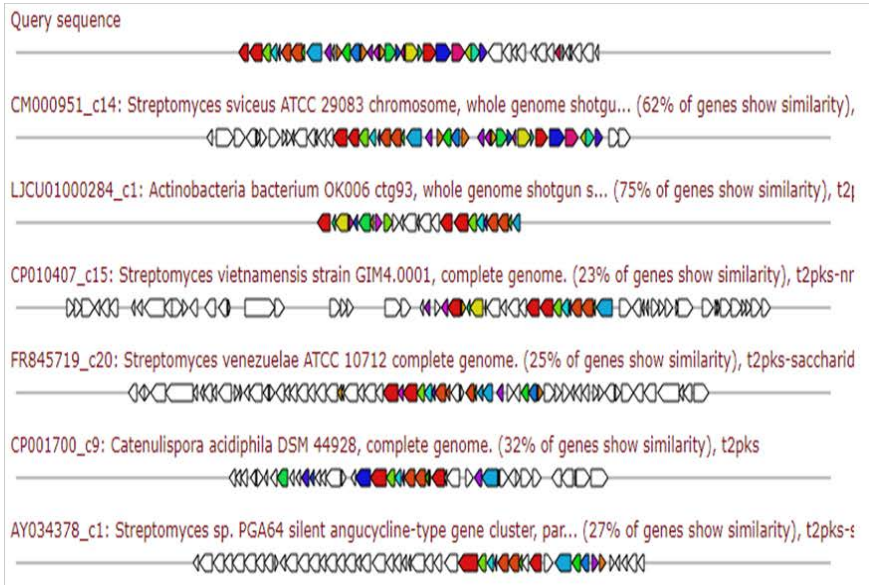
Predicted core structure



Cluster similarity to known genes

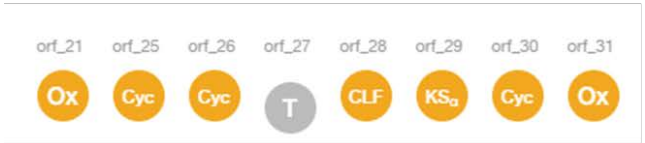


Cluster similarity in *Streptomyces* species



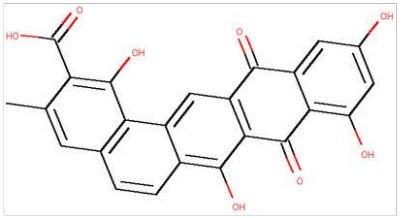
CSM1_Cluster6 _T2PKS

Biosynthetic assembly

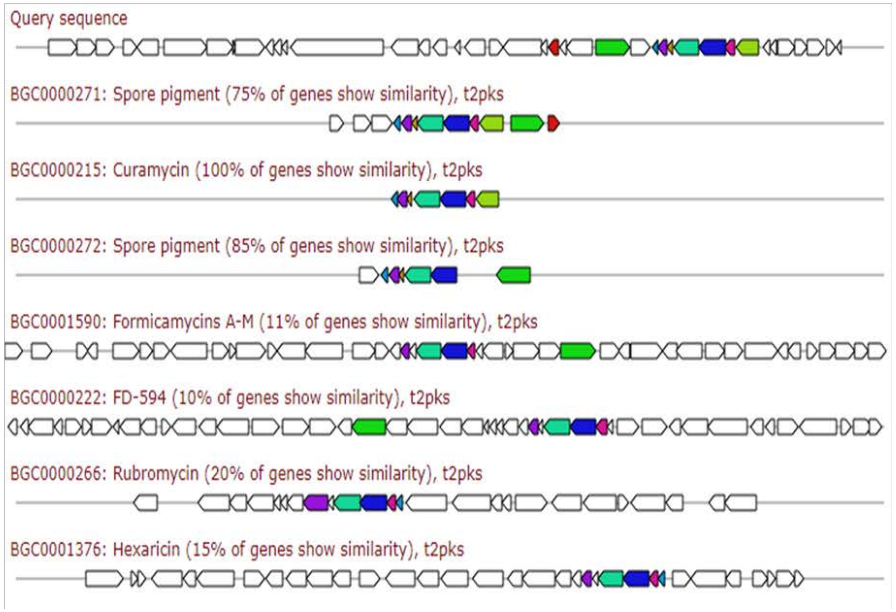


Cluster

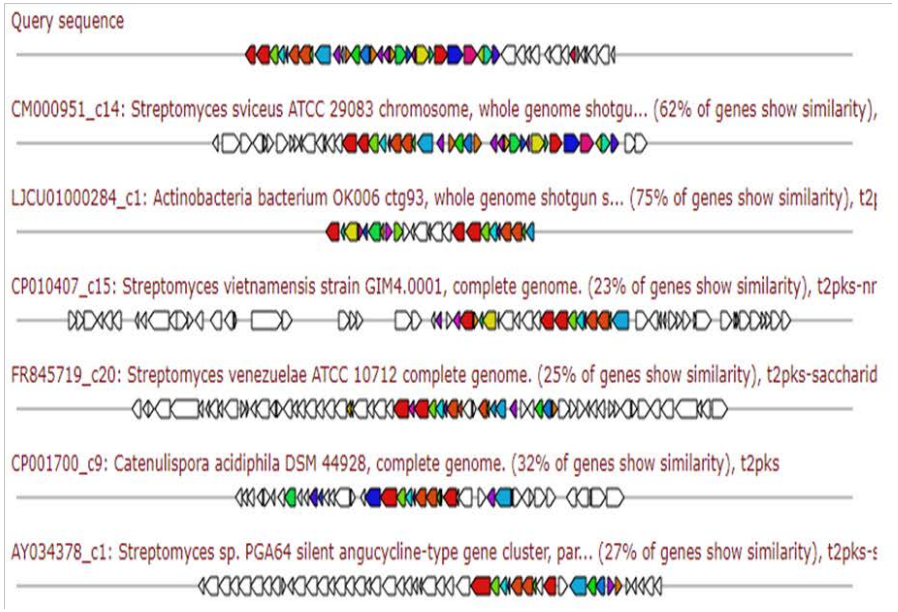
Predicted core structure



Cluster similarity to known genes

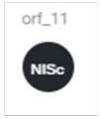


Cluster similarity in *Streptomyces* species



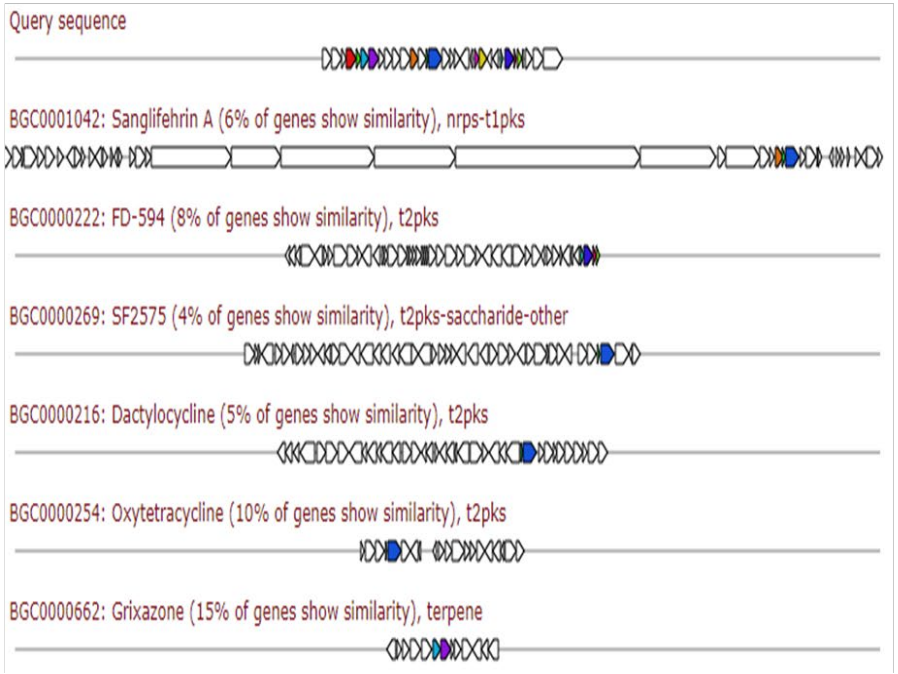
CSM1_Cluster 8_PKS-like

Biosynthetic assembly **Cluster**

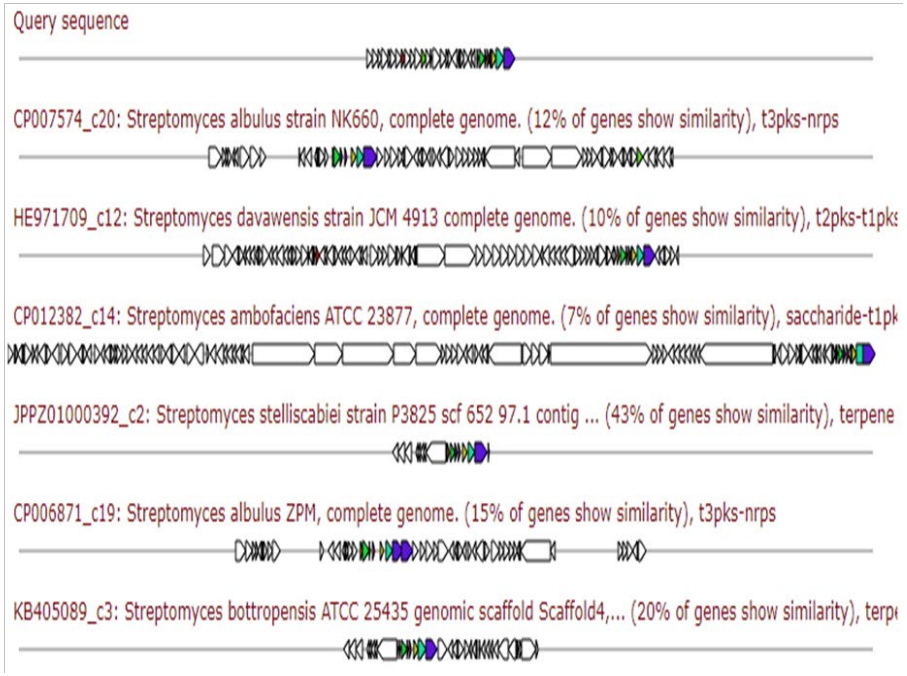


No Predicted core structure

Cluster similarity to known genes



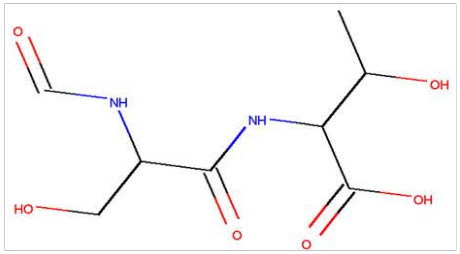
Cluster similarity in *Streptomyces* species



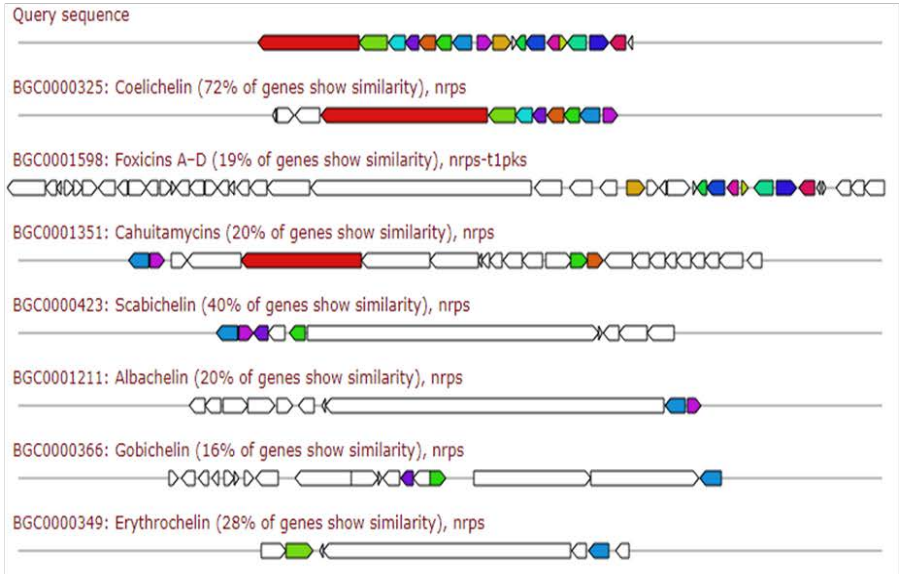
CSM1_Cluster 11 _NRPS

Cluster

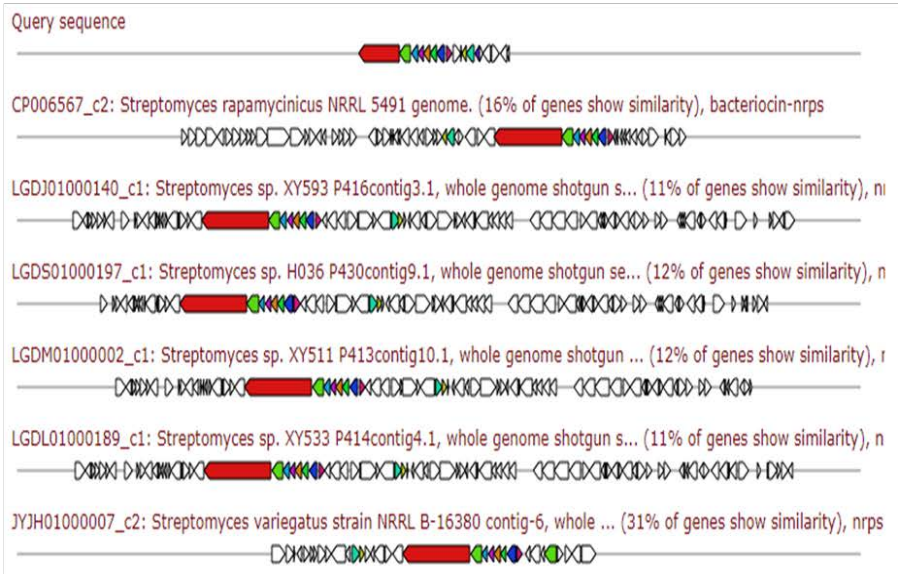
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

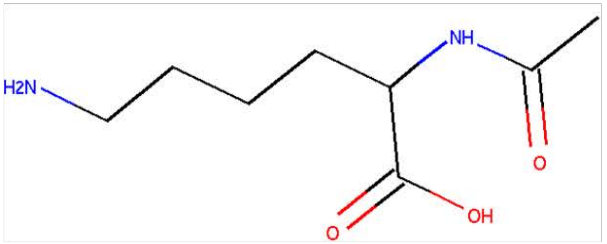


CSM1_Cluster 14 _NRPS-PKS

Biosynthetic assembly

Cluster

Predicted core structure



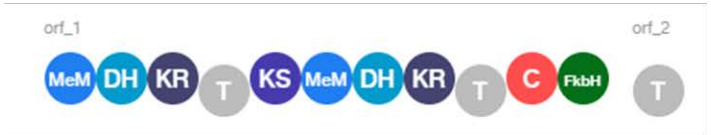
Cluster similarity to known genes

No genes similarity

Cluster similarity in *Streptomyces* species

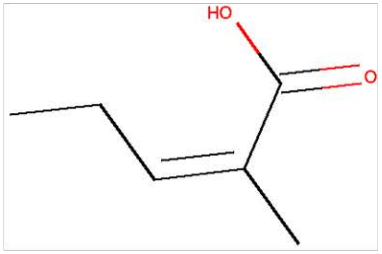
CSM1_Cluster 17_T3PKS

Biosynthetic assembly

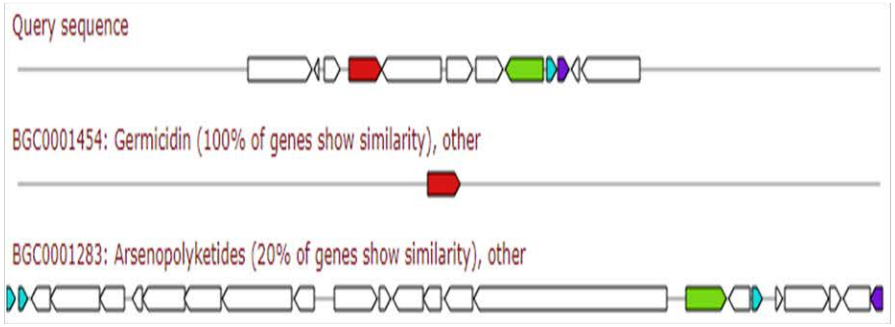


Cluster

Predicted core structure



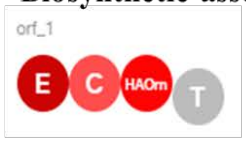
Cluster similarity to known genes



Cluster similarity in *Streptomyces* species

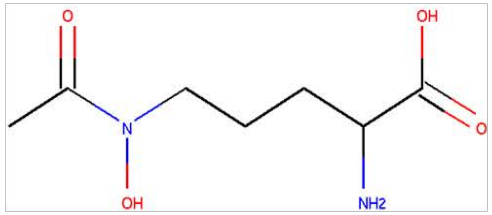
CSM1_Cluster 23 _NRPS

Biosynthetic assembly

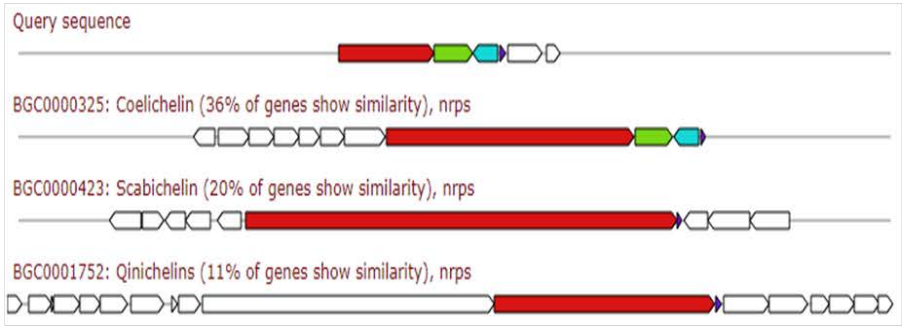


Cluster

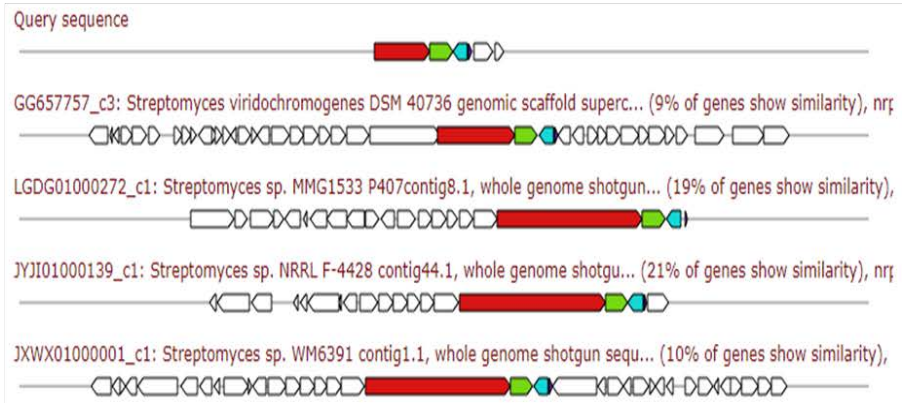
Predicted core structure



Cluster similarity to known genes



Cluster similarity in *Streptomyces* species



CSM1_Cluster 25 _NRPS-like

Biosynthetic assembly

Cluster

No Predicted core structure

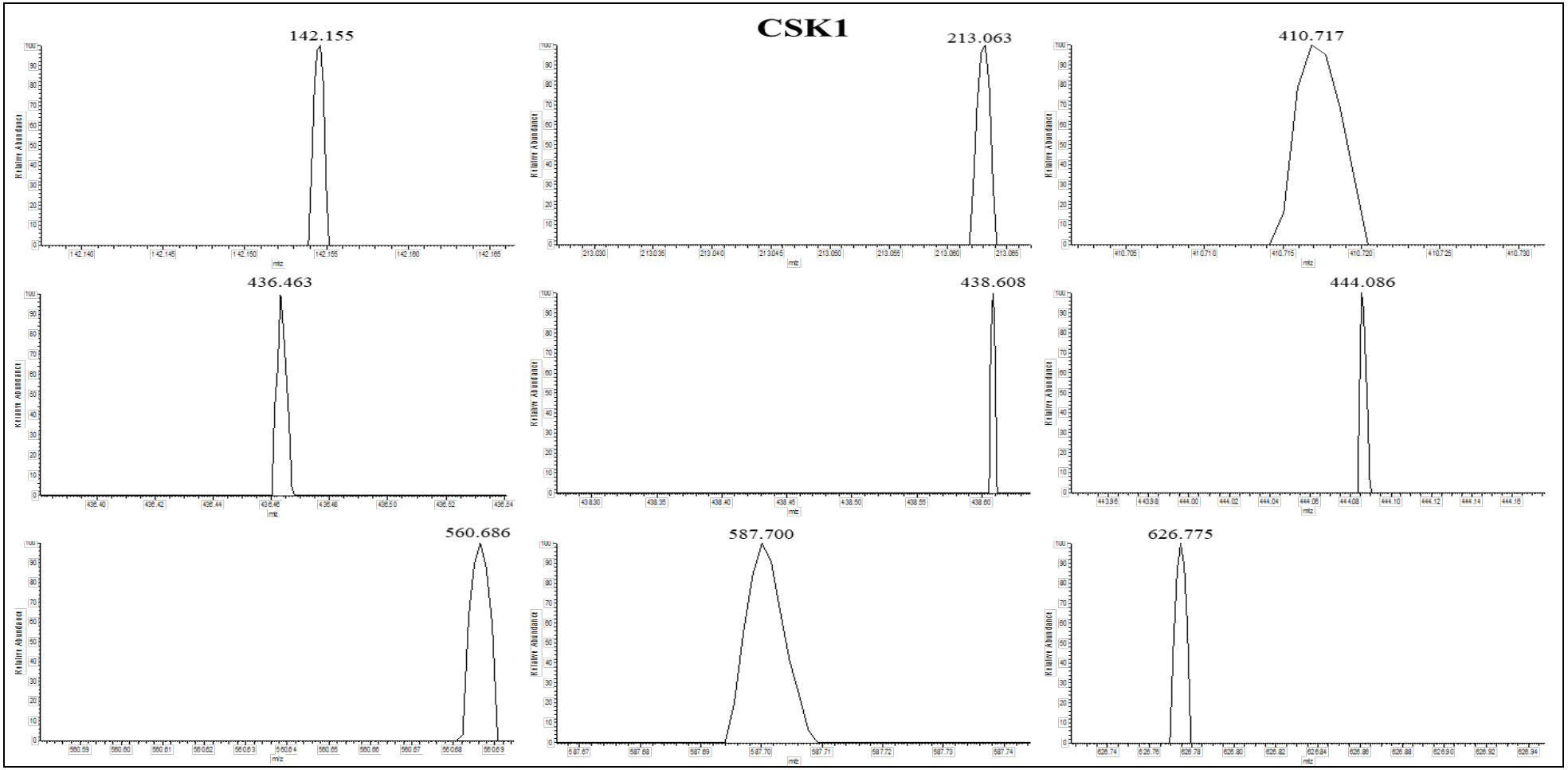
Cluster similarity to known genes

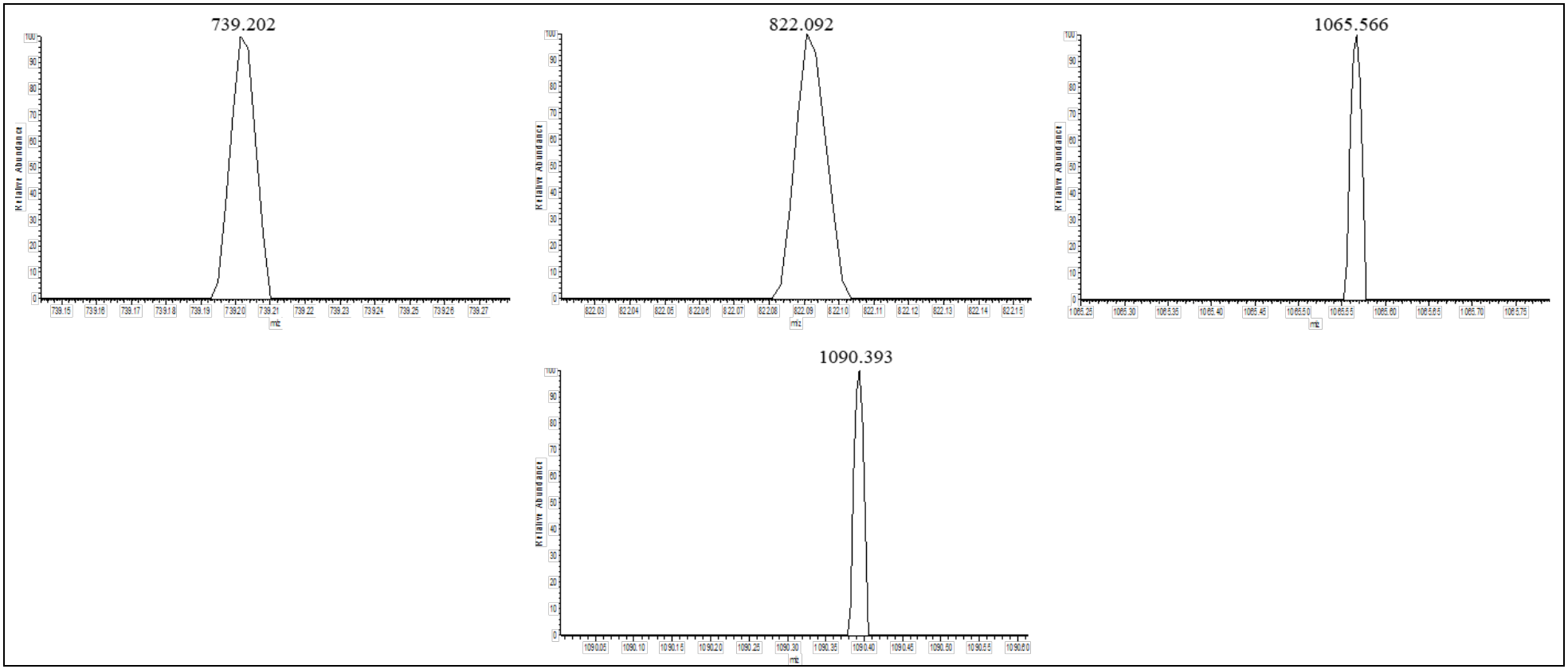
Cluster similarity in *Streptomyces* species

No gene and *Streptomyces* similarity

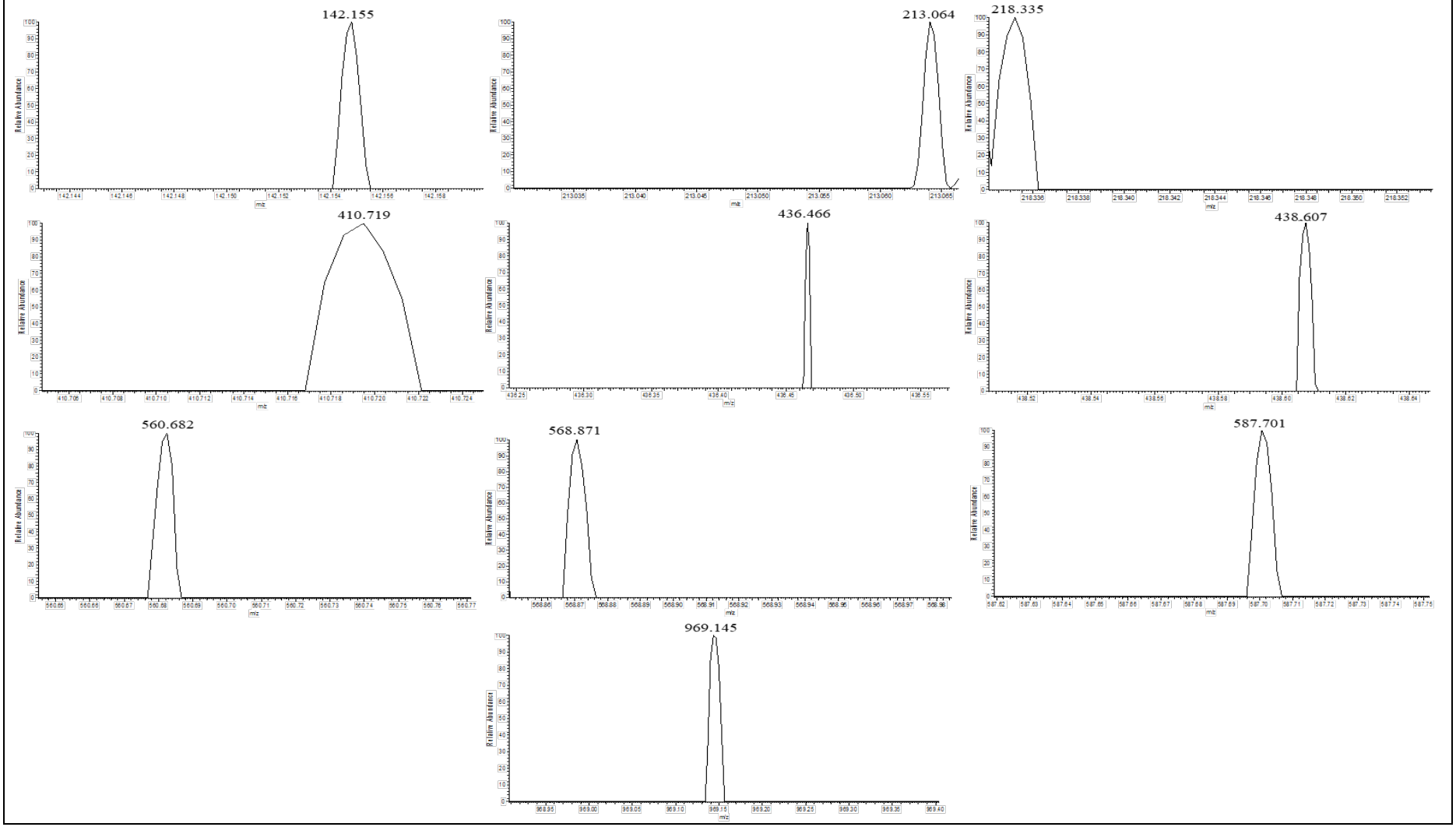
Appendix D

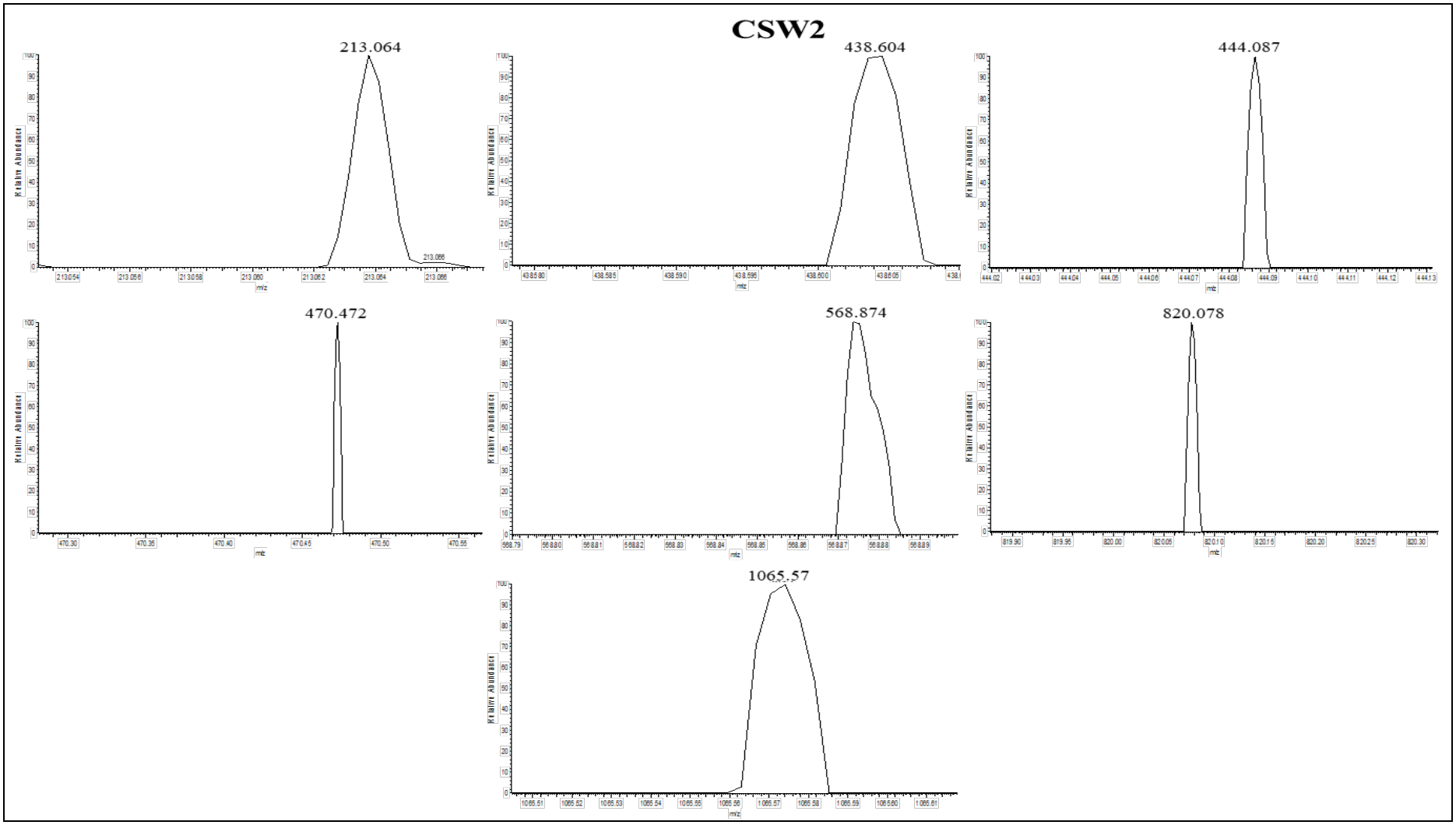
BGCs identified by antiSMASH and confirmed with LESA spectra (in reference to Tables 5.4-5.10)

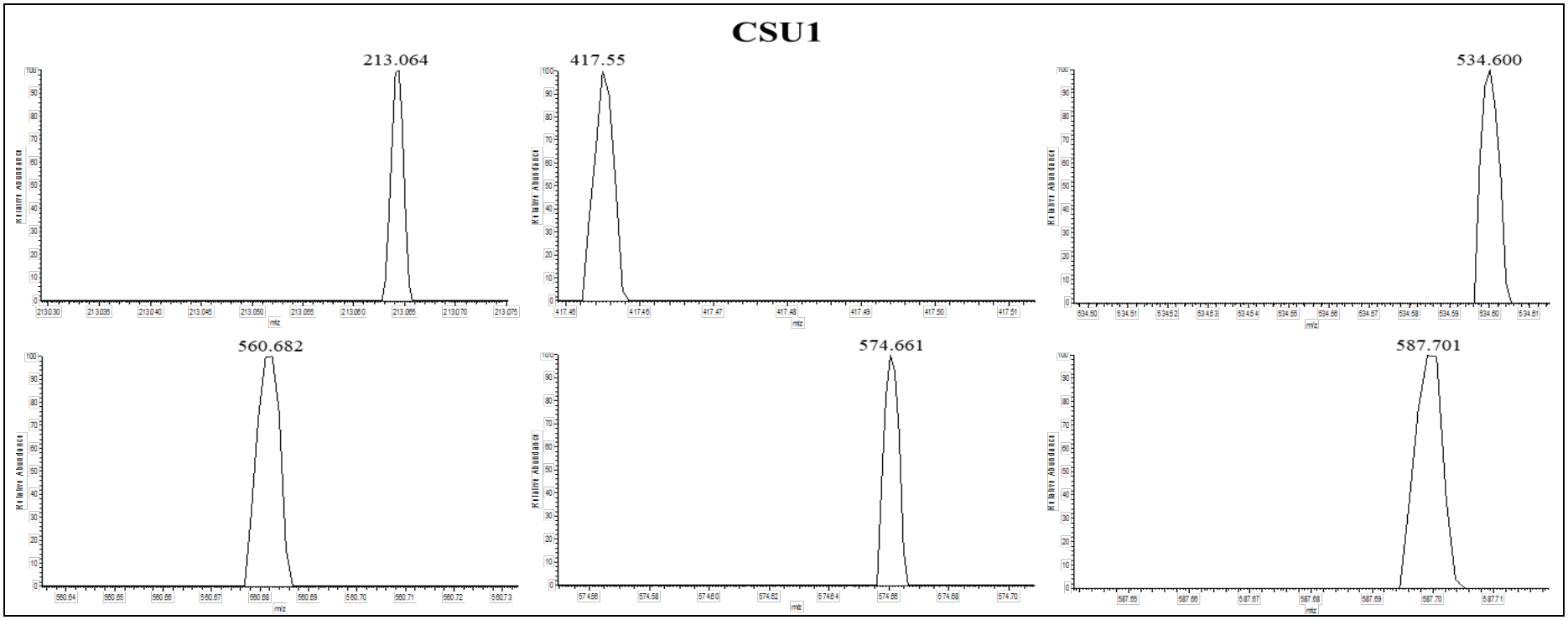



739.202
822.092
1065.566
1090.393
Relative Abundance
m/z

CSK3

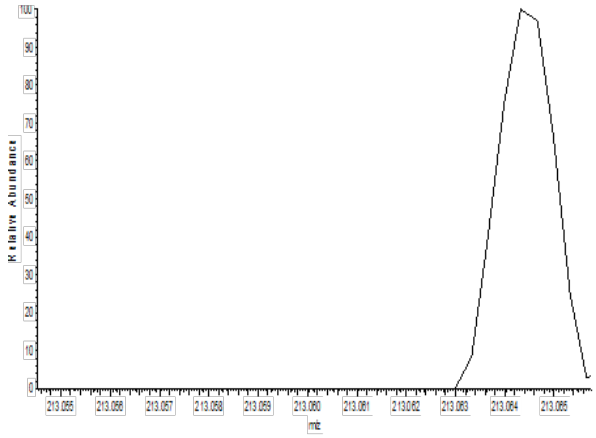

142.155
213.064
218.335
410.719
436.466
438.607
560.682
568.871
587.701
969.145
Relative Abundance
m/z


CSW2
213.064
438.604
444.087
470.472
568.874
820.078
1065.57
Relative Abundance
m/z

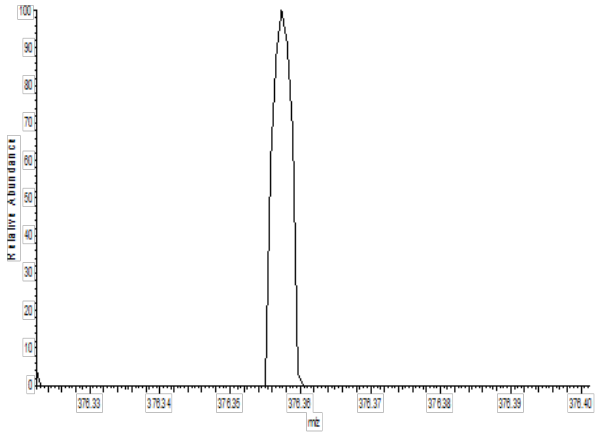

CSU1
213.064
417.55
534.600
560.682
574.661
587.701

CSU2

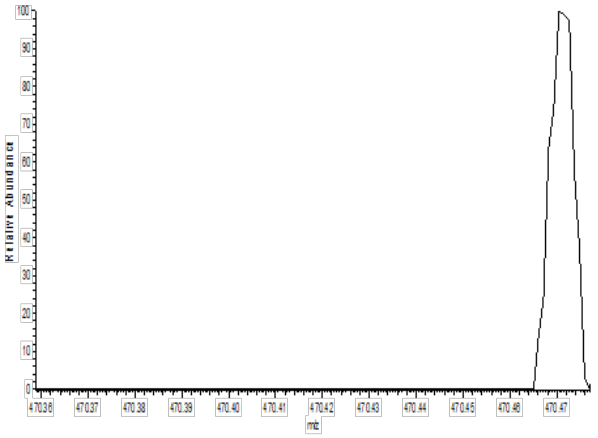
213.064



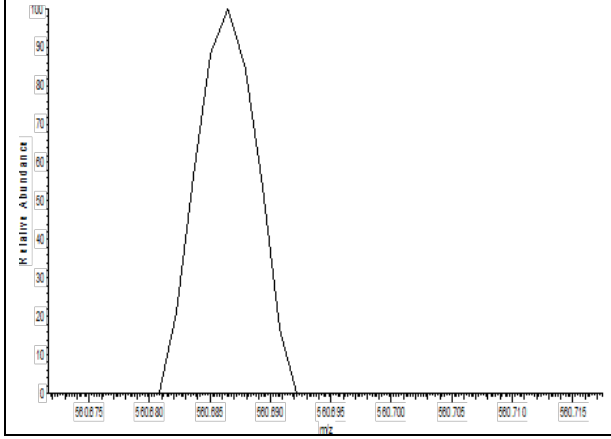
376.357



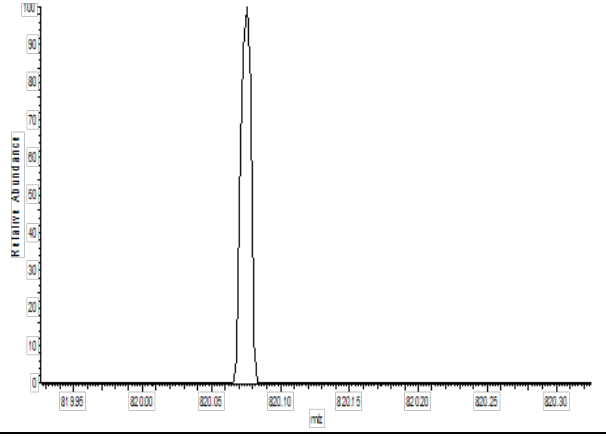
470.471



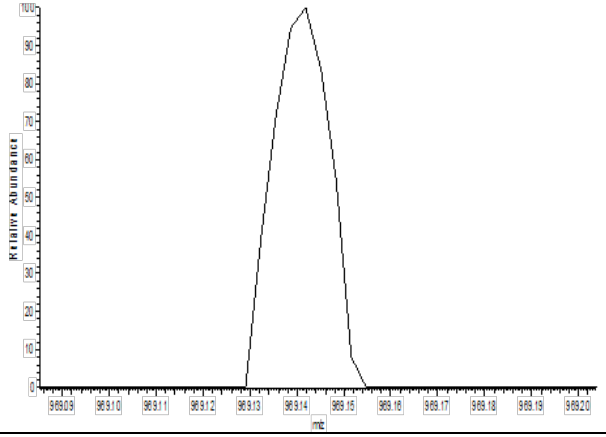
560.686

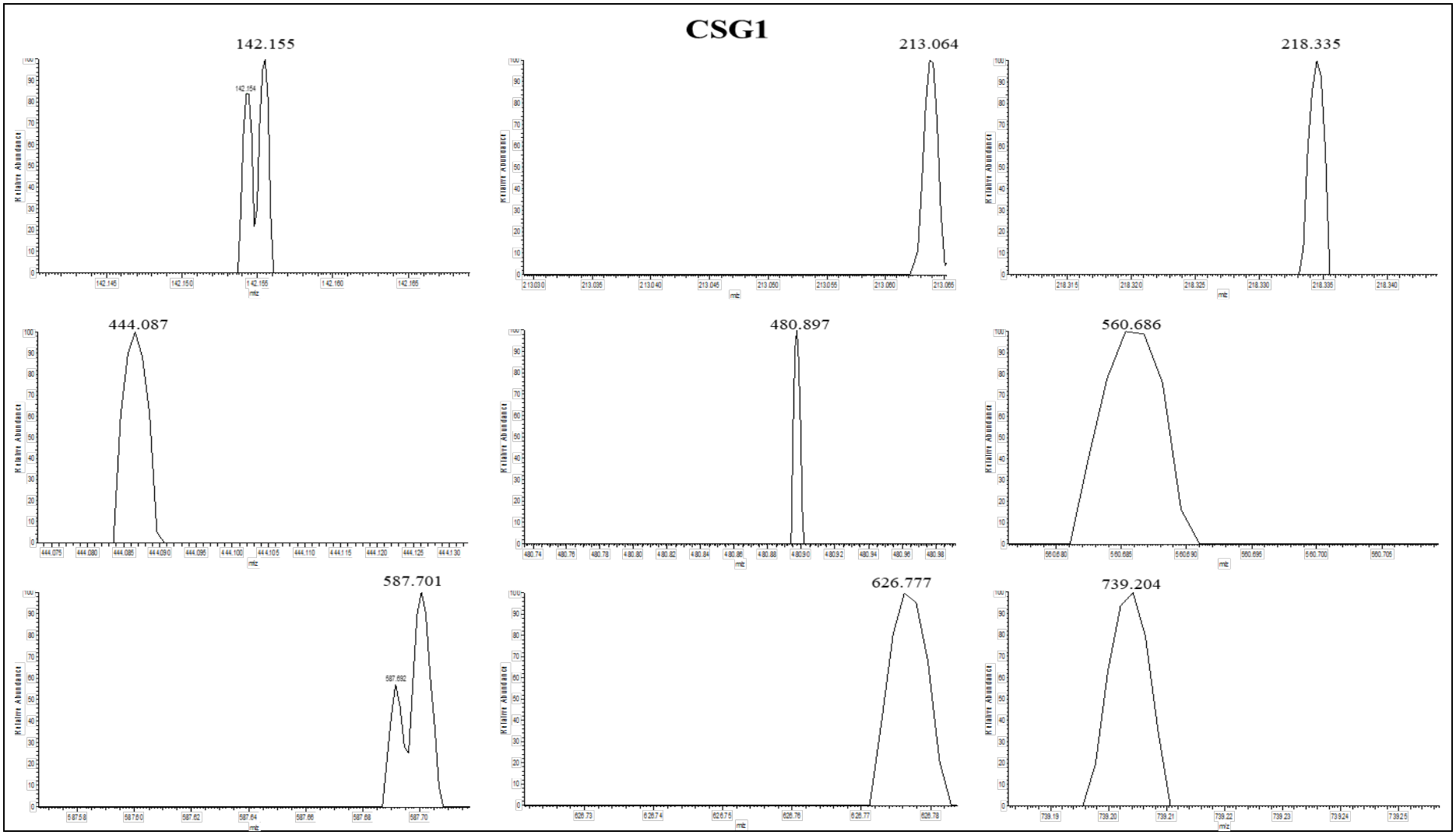


820.075



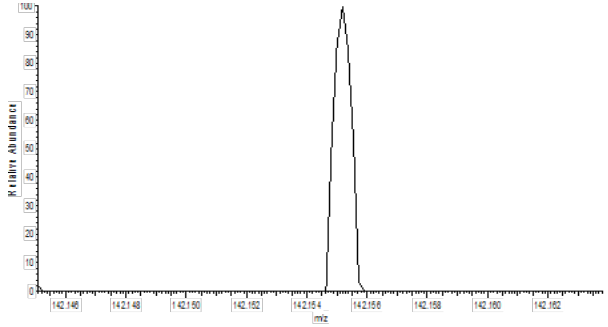
969.141



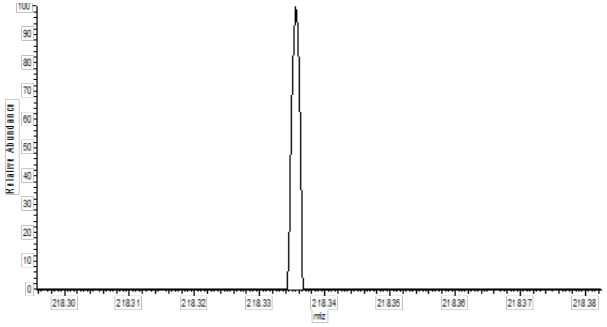

CSG1
142.155
213.064
218.335
444.087
480.897
560.686
587.701
626.777
739.204
Relative Abundance
m/z

CSM1

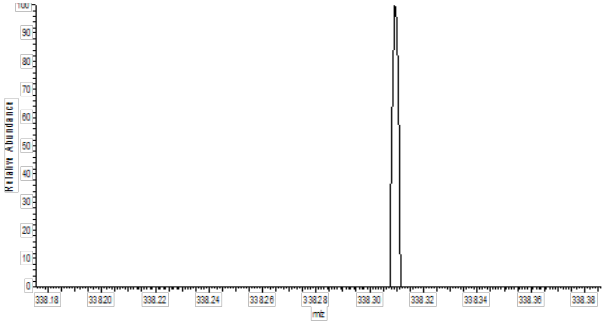
142.155



218.336



338.310



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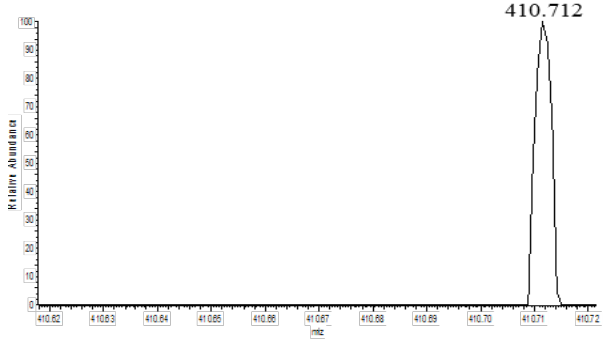

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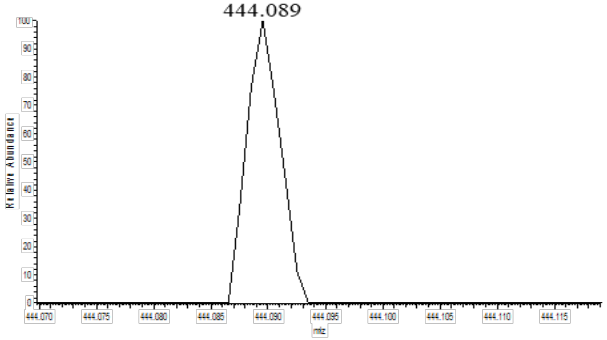

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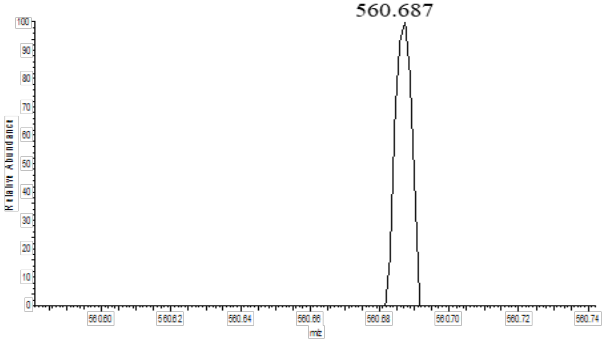

565.583