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THE EXTRACTION AND ANALYSIS OF ANTIMICROBIAL SECONDARY METABOLITES PRODUCED BY CAVE *STREPTOMYCES* S1, S4, AND PM58B

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by

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ABSTRACT

Antibiotic resistance is a growing problem as microorganisms exhibiting resistance to antibiotics of last resort have been reported in hospitals around the world. To respond to this problem, it is necessary to develop and discover novel antibacterial compounds. A rich reservoir of antibacterial compounds are microbial secondary metabolites, a structurally diverse group of molecules produced by microbes in response to changing environmental conditions providing some advantage to their producers. Particularly prolific producers of these compounds are bacteria of the group *Streptomyces*, these being responsible for a large portion of the naturally sourced antibacterial compounds used today. In order to access novel and untapped chemical diversity, the investigation of unexplored biological niches has become more prevalent, with the study of cave dwelling microorganisms producing promising leads. This study examines the antimicrobial secondary metabolites produced by three cave *Streptomyces* strains S1, S4, and PM58b for their previously described activity against target multi-drug resistant *Escherichia coli* and methicillin resistant *Staphylococcus aureus* with the goal of determining their molecular characteristics. To achieve this, three strains of *Streptomyces* were grown for periods of 10 to 30 days to induce the production of secondary metabolites. Bioactivity was confirmed in S1 and S4 by an agar-plug assay and was observed as a pigmented ring of inhibition after an extended period of incubation. *Streptomyces* strain S1 fermentation broth was extracted and analyzed by matrix assisted laser desorption ionization mass spectrometry for the preliminary identification of the antimicrobial compound present. Antimicrobial activity could not be isolated in a cell free environment from S1 fermentation broths; instead, the antimicrobials were produced on the assay plate. Differences were observed between the fermentation broth and sterile media mass spectra; however, a molecular mass could not be assigned to the putative antimicrobial compound.

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INTRODUCTION

Antibiotic resistance

The progressive spread of antibiotic resistance among pathogenic bacteria is a dire problem to which no clear solution has arisen, to the growing concern of public health authorities, governments, and medical professionals alike. The spread of these antibiotic resistant phenotypes is a clear example of artificial selection. This selective pressure is established as a consequence of the extensive use of antibiotics in agriculture, medicine, and daily life leading to the evolution of resistant strains of bacteria. Shortly after the advent of the widespread therapeutic use of antibiotics with the discovery and application of penicillin, antibiotic resistance has followed close behind with resistant strains becoming more and more prevalent (Davies and Davies 2010). The mechanisms underlying this resistance vary between the intrinsic resistances some groups of pathogenic microbes display to certain classes of antibiotics, to acquired resistances developed over time in response to the selective pressure placed on pathogenic bacteria due to the application of antibiotics (Blair et al 2015). Examples of these acquired resistances are drug efflux systems, antibiotic modifying enzymes, and changes to the cellular target itself (Blair et al. 2015). Adding to the problem of the emergence of antibiotic resistance is its capacity to spread between bacteria through the horizontal transfer of chromosomal and extrachromosomal genetic material leading to the emergence of multi-drug resistant (MDR) strains of bacteria (Cohen et al. 1972; Blahna et al. 2006; Davies and Davies 2010). In order to combat this spread of antibiotic resistance a multifaceted approach must be implemented through new restrictions on the availability and approved uses of existing antibiotics, the development of new analogues of existing antibiotics, and through the exploration of novel chemical space to identify new antibiotic compounds with both unique molecular scaffolds and mechanisms of action (Davies and Davies 2010).

The *Streptomyces* lifecycle and the production of secondary metabolites

Streptomyces have historically been a prolific microbial source of antimicrobial secondary metabolites, producing many of the antibiotics on the market today. These typically soil dwelling bacteria are Gram positive and undergo a complex life cycle, moving between vegetative and aerial hyphal states. *Streptomyces* grow from single spores into branching vegetative hyphae that elongate by apical tip extension forming a substrate mycelium (Sigle et al. 2015, Flardh and

Buttner 2009). Responding to environmental cues such as nutrient depletion, the *Streptomyces* undergo a morphological differentiation producing aerial hyphae during which the secondary metabolism associated with the production of antimicrobial compounds is activated (Flardh and Buttner 2009; Sigle et al. 2015).

The production of antimicrobial secondary metabolites in *Streptomyces* is regulated by a variety of factors, and the synthesis of these compounds can be induced in response to multiple stimuli. The presence of stimulatory precursor molecules can have an inductive effect on the synthesis of these secondary metabolites, as observed in the impact of lysine on the synthesis of cephamycin C in *S. clavuligerus*, or the role of valine in the synthesis of tylosin in *S. fradiae* (Nguyen et al. 1995; Demain 1998; Leite et al. 2013,). The synthesis of these antimicrobial compounds can also be due to the activity of endogenous auto-regulator molecules. These auto-regulatory compounds take several forms one of which are the γ-butytrolactones. These compounds act as ligands to cytoplasmic regulatory proteins such as ArpA in *S. griseus*, binding and deactivating them, preventing their binding of the *Streptomyces* chromosomal DNA and fulfilling their role as repressors (Takano 2006). In *S. griseus* this ArpA deactivation allows for the synthesis of AdpA, a regulatory protein responsible for both the upregulation of the synthesis of secondary metabolism as well as the morphological differentiation of the bacteria (Bibb 2005). The morphological changes observed with activation of AdpA are the formation of aerial hyphae and their subsequent sporulation, a process promoted by lowered ATP levels. (Wolanski et al. 2012). The production of these autoregulatory compounds such as γ-butytrolactones are controlled by biosynthetic genes typically located nearby biosynthetic clusters responsible for secondary metabolites synthesis (Bentley et al. 2002; Liu et al. 2013).

Other regulators in the production of secondary metabolites include the alarmone guanosine pentaphosphate (p)ppGpp, which in response to stresses such as heat shock or nutrient deprivation, modulates cellular metabolism and transcription in a concentration dependant manner initiating the stringent response (Hauryliuk et al. 2015). The cytosolic (p)ppGpp concentration is regulated by the ribosome associated enzyme Re1, which directly monitors the process of translation within the cell. When exposed to stress inducing conditions such as nutrient deprivation (p)ppGpp is synthesized, which through direct or indirect mechanisms, downregulates the production of ribosomal proteins while promoting the biosynthesis of amino acids and genes

associated with stationary phase processes such as secondary metabolism (Hesketh et al. 2007; Hauryliuk et al. 2015).

Accessing novel chemical diversity

Although the chemical diversity of *Streptomyces* secondary metabolism has been the subject of intense study, leading to the discovery of a multitude of anticancer, antifungal, and antibiotic compounds, science still has much to gain from further investigation of this genus. However, in order to achieve this goal scientists must employ new approaches and technologies. Through the development and application of metagenomic approaches to drug discovery, the identification of potential antimicrobial secondary metabolites through the detection and analysis of biosynthetic clusters associated with secondary metabolism progress has led to the discovery of new antimicrobial secondary metabolites (Handelsman 2004, Seipke 2015, Chu et al. 2016). In addition to these wide-net approaches to the discovery of new microbial sources of antibiotics, the exploration of new and understudied microbial niches such as the ocean or caves allows researchers to access new species of *Streptomyces* and potentially novel antibiotic secondary metabolites (Fielder et al. 2005, Cheeptham et al. 2013, Rateb et al. 2011). These understudied niches often represent extreme environments, with the cave environment being a clear example of this; having low levels of readily available organic carbon, little to no light, and high mineral and salt content (Ghosh et al. 2016). In these extreme environments, endogenous bacteria have evolved metabolic processes to access the limited resources available to them, which along with the rich diversity in the cave environment, contributes to the development of unique often species specific secondary metabolites (Bhullar 2012, Cheeptham et al. 2013, Cuezva et al. 2012). This study aims to explore the potentially novel chemical diversity associated with cave *Streptomyces* through the molecular characterization of previously described antimicrobial activity ascribed to *Streptomyces* strains isolated from Iron Curtain Cave near Chilliwack B.C. (S1 and S4) and from Helmcken Falls Cave in Wells Gray Provincial park near Clearwater B.C (PM58b). Helmcken Falls Cave has been extensively studied being identified as volcanic in origin, with subsequent modifications introduced through the activity of the nearby Murtle and Clearwater Rivers. The cave macroenvironment of Helmcken Falls Cave includes basalt walls and ceilings as well as deposits of fine sands and sediments (Cheeptham et al. 2013). Iron Curtain Cave located near Chilliwack B.C. although not nearly as well studied, is also a cave of volcanic origin (Mason et al. 2015).

MALDI as a tool for the preliminary structural elucidation of secondary metabolites

In order to proceed with the drug discovery process, from the identification of an organism exhibiting antimicrobial activity, to the application of the bioactive compound in a clinical environment, the complete structural elucidation of the compound is necessary. A crucial step in this process is the determination of the molecular mass of the compound of interest by mass spectrometry, as this step facilitates further investigation by techniques such as IR and NMR, allowing for complete structural elucidation (Kind and Fiehn 2010). Mass spectrometry in conjunction with elemental analysis techniques such as energy dispersive X-ray spectroscopy, allows for the identification the molecular formula of the compound of interest and thus permits future studies of the connectivity and functionality of the compound by NMR and IR experiments (Kind and Fiehn 2010). In addition to this, dual mass spectroscopy (MS-MS) itself can be used to derive structural information of the compound of interest through its sequential and the prediction of fragmentation patterns (Cabrera 2006).

Matrix assisted laser desorption ionization (MALDI) is a form of mass spectrometry that is typically used to analyze large molecules such as whole proteins or peptides. Although conventionally applied to these larger molecules, MALDI remains a valuable instrument in the analysis of smaller molecules such as those secondary metabolites being produced by *Streptomyces* (Cohen and Gusev 2002). MALDI-MS boasts high sensitivities and high mass resolutions while also being a high throughput technique allowing for the rapid analysis of many samples. In addition to this, MALDI is a soft ionization technique resulting in little to no fragmentation of target compounds. The consequence of this being, instead of producing charged fragments MALDI-MS tends to ionize the whole compound producing a quasi molecular ion (MH^+) peak, providing information on the molecular weight of the compound allowing for greater ease the determination of the compound's molecular formula. Although it benefits from being a high throughput technique capable of achieving high resolutions and sensitivities, MALDI suffers from the detection of matrix peaks at low m/z values such as those examined in this experiment (Cohen and Gusev 2002). In addition to this MALDI cannot be coupled directly to a chromatography column, preventing any separation of the compounds in the sample of interest prior to ionization. These problems can be addressed through the introduction of an ionization

suppressant such as cetyltrimethylammonium bromide (CTAB) and conducting a preparatory separation by column chromatography (Guo et al. 2002).

Experiment goals and approach

Using liquid-liquid extraction and MALDI-MS, this study aims to identify the antimicrobial secondary metabolites of cave *Streptomyces* strains S1, S4, and PM58b by assigning a molecular mass to these compounds. This is achieved by the nutrient deprivation of mature *Streptomyces* cultures through the growth of these bacteria in batch culture until fermentative conditions are achieved. The presence of antimicrobial activity is then to be confirmed through plug diffusion assays against multi-drug resistant and laboratory strains of *S. aureus* and *E. coli*. Once antimicrobial activity is established in the *Streptomyces* fermentation broths, liquid-liquid extraction of the responsible metabolite will be attempted with a variety of organic solvents, after which antimicrobial activity will be confirmed by further bioassays. Low m/z mass spectra will be obtained for these extracts and the original fermentation broths through MALDI-MS and antimicrobial activity will be correlated to the emergence and persistence of novel signals in the MS spectra.

MATERIALS AND METHODS

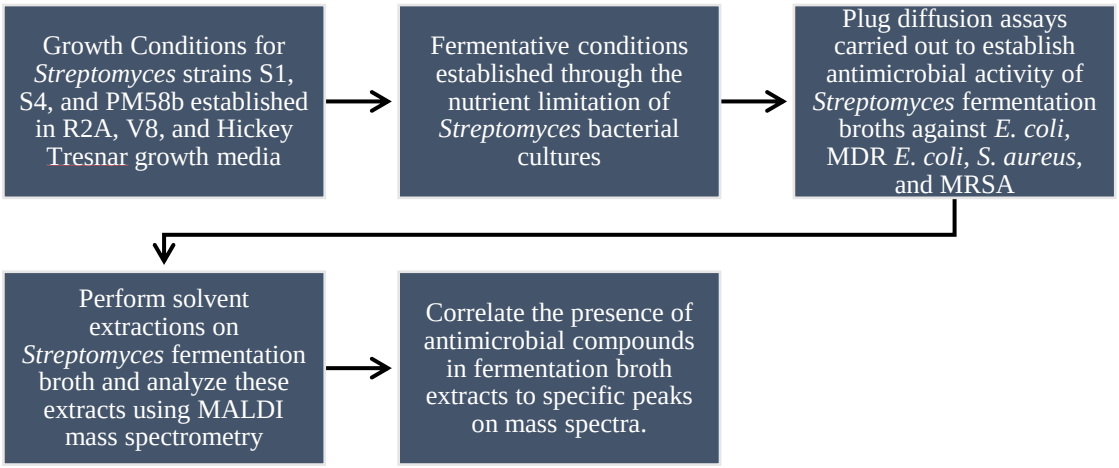

Figure 1. Overview of experimental approach to the production and analysis of antimicrobial activity of cave Streptomyces strains S1, S4, and PM58b.

Growth of Cave *Streptomyces* Cultures

Streptomyces strains S1 and S4 were streaked for isolated colonies from stock plates onto Hickey-Tresnar (HT) Agar using aseptic technique and were then grown at 8°C and 15°C. These HT plates served as stock plates for work done using S1 and S4 in this experiment and were stored under refrigeration at 4°C. Isolated colonies of S1 and S4 were then aseptically transferred from these stock plates into 16x100 mm glass test tubes containing sterile 3 mL of R2A (S1) and V8 (S4) broth. Ten 3 mL cultures of each strain were prepared in this manner. From the twenty S1 and S4 cultures five of each strain were then grown at temperatures of 8°C and 15°C in shaking incubators for a period of 10 days. Following this period, the strains were assessed for antimicrobial activity, and analyzed by MALDI. These cultures were evaluated before conducting bioassays by Gram staining to ensure no contamination had occurred.

Following these growth and antimicrobial activity assays S1 and S4 were grown at 8°C for durations up to 30 days under the above described conditions. These strains were then assessed for antimicrobial activity. Following this a 300 mL volume of sterile R2A broth was inoculated with S1 using 1.00 mL of a 3 mL five-day culture. This culture was incubated at 8°C for a period of 30 days after which extractions, bioassays for antimicrobial activity and mass spectrometry analyses were performed.

Streptomyces strain PM58b was streaked for isolated colonies from a stock culture suspended in 30% glycerol stored at -20°C onto a HT agar plate where it was then grown at 8°C and 15°C. This HT plate served as a stock plate for the work done using PM58b for this study. The plate was stored under refrigeration at 4°C Isolated colonies were then aseptically transferred from the stock plate into16x100 mm glass test tubes containing sterile 3mL HT broth. These PM58b cultures were similarly fermented for durations of 10 to 30 days at temperatures of 8°C and 15°C after which the broth was bioassayed for antimicrobial activity.

Bioassays for Antimicrobial Activity

The *Streptomyces* strains were evaluated for antimicrobial activity using a plug diffusion assay described by S. Ghosh in a personal communication (2016). This assay was prepared using sterilized 225x225 mm polystyrene bioassay plates. These plates were sterilized using 1.5% Peroxyguard solution, 70% ethanol, and UV light exposure for a duration of 20 minutes. Following

this, 250 mL of autoclaved molten nutrient agar maintained at a temperature of 55°C was inoculated to give a concentration of $1x10^6$ cells per mL of the target organism, and was delivered to the plate and allowed to solidify. The target organisms used for these assays were laboratory and drug resistant strains of *S. aureus* (MRSA-43300) and *E. coli* (15-104, 15-124, 15-318) which were provided by LifeLabs (Kamloops B.C.). Isolated colonies of these organisms were aseptically transferred from blood agar stock plates to 3 mL volumes of nutrient broth. These cultures were then incubated with shaking 100 rpm for a duration of 20-24 hours. The concentration of cells in the 3mL culture was then quantified by measuring the culture's optical density (OD). This was done using a Pharmacia NovaSpecIII Spectrophotometer set at a wavelength of 600 nm and Plastibond 1.5 mL cuvettes to which 1.00 mL of culture was added. The spectrophotometer was blanked using sterile nutrient broth, after which OD measurements of the culture being assayed were made in triplicate. An average OD was taken, and using the assumption that an OD of 1.0 corresponds to a cellular concentration of $8x10^8$ cells per mL, a volume of the 3 mL stock culture was delivered using a p1000 VWR micropipette and sterile disposable pipette tips to the molten agar to give a final concentration of $1x10^6$ cells per mL in the nutrient agar. The nutrient agar was then delivered to the plate and allowed to solidify. Once the agar had solidified plugs were bored out of the agar using a flame sterilized bore with a 6 mm ID, producing wells with a volume of 100 µL. To these wells 100 µL of the material being assayed was delivered using a p200 VWR micropipette. As positive controls 100 µL volumes of Peroxyguard and 70% ethanol were used. For drug resistant organisms 6mm diameter diffusion disks impregnated with 30 µg of tetracycline produced by Beeton Dickinson and Company were used also used. Bioassay plates were incubated at 37°C, 25°C, and 15°C for a duration of 7 days, activity was evaluated daily.

Alongside the various growth conditions assayed, assays were performed with fermentation broth supernatant samples, filtrate samples, heat shocked samples, and extracts. Supernatant samples were prepared by transferring 1.00 mL volumes of *Streptomyces* culture to autoclaved 1.5 mL Eppendorf tubes which were subsequently centrifuged at 14,000 rpm for a duration of 10 minutes on a Hermle Z 233 MK-2 Centrifuge. From these Eppendorf tubes 0.500 mL of the supernatant was then transferred to fresh, sterile 1.5 mL Eppendorf tubes from which it was delivered to the assay plate. Heat shocked samples were prepared by delivering 1.00 mL volumes of the *Streptomyces* broth to autoclaved 1.5 mL Eppendorf tubes which were then placed in a boiling water bath for a duration of 10 minutes. Filtered samples were obtained for S1 samples

by first centrifuging 1.00 mL volumes of fermentation broth as described above. The supernatants of these 1.00 mL volumes were then delivered to a sterile 5.0 cc Luer lock syringe using a p1000 VWR micropipette and was subsequently filtered through a 0.45 µm VWR cellulose acetate luer lock filter into sterile Eppendorf tubes from which the filtrate was delivered to the assay plate. Finally extract samples were prepared by centrifuging 30 mL volumes of the *Streptomyces* culture in 50 mL VWR pre-sterilized plastic centrifuge tubes at 5000 rpm in an IEC MP4 Centra centrifuge. The supernatant from these tubes was then poured through a 70µm nylon mesh Fischer Scientific cell strainer in to an autoclaved 250mL volumetric flask. Of this cell free filtrate 50 mL was measured out and transferred using a graduated cylinder into a 250 mL separatory funnel. Several extractions were performed, with the first using three 10 mL volumes of LC-MS grade hexane followed by three 10 mL volumes of LC-MS grade ethyl acetate. The fractions were combined and the solvents were evaporated in evaporating dishes under the fume hood. Upon the evaporation of the solvents the residues were dissolved in 2.00 mL of autoclaved 18 MΩ water, of which 1.5 mL was transferred into autoclaved Eppendorf tubes. Extractions were also attempted on the 70 µm filtrate using three 10 mL volumes of LC-MS grade ethyl acetate which was then similarly evaporated in the fumehood and subsequently dissolved in 2.00 mL of autoclaved 18 MΩ water.

MALDI Sample Preparation

Samples were prepared on a ground steel target plate for analysis by MALDI. The samples were prepared as per the dried droplet protocol described by Bruker Daltonics MALDI preparation protocols. Equal volumes of the fermentation broth sample and TA30 (30:70 (v/v) acetonitrile :0.1% trifluoracetic acid in 18 MΩ water) saturated with α-cyano-4-hydroxy cinnamic acid (HCCA) were premixed in a clean sterile Eppendorf tube. From this Eppendorf tube 1 µL of the sample-matrix solution was delivered directly to the ground steel plate. To ensure the spectra obtained were consistent and representative of the sample solution, fermentation broth samples were run in replicates of 6. The samples analyzed in this manner included, Sterile R2A, and 30 day S1 fermentation broth filtrate

MALDI Sample Analysis

Samples were analyzed using a Bruker MicroFlex LRF MALDI. The method used for this analysis was developed and implemented using Bruker Daltonics flexControl software (version

3.3.108.0). Compounds of m/z's of 0 Da to 4000 Da were selected and evaluated with the response limited to a signal-to-noise threshold of 6.0 and over, a maximal peak number of 300, a maximal peak width of 0.2 m/z and a resolution higher than 100. Noise was reduced using a Tophat baseline subtraction algorithm. The sample and matrix were ionized using a 19.99 kV ion source with the laser set on the random walk setting. Samples were analyzed by summing up 800 satisfactory shots taken in 80 shot steps using a 60 Hz laser starting at 20% power before reaching maximal power at 80% power. The resultant spectra were analyzed using Flex Analysis software. Mass-to-charge values were copied into Microsoft Excel and the m/z values of the 30 day S1 fermentation broth were screened for peaks observed consistently in 2 or more of the 6 replicates analyzed within a difference of 0.005 m/z. These consistently observed peaks were then screened against all peaks observed in any of the R2A background spectra to identify signals present in the 30 day S1 fermentation broth but not present in the R2A background spectra.

RESULTS

Growth of Cave *Streptomyces* Cultures

Strains S1, S4 and PM58b were observed to grow effectively at temperatures of 8°C and 15°C, growing faster at the 15°C condition. On HT agar S1 grew into large white filamentous colonies, S4 grew into flat gray filamentous colonies, and PM58b grew into large raised white filamentous colonies. In R2A broth S1 formed rounded filamentous pellet-like colonies, forming strand like structures on the walls of the test tube. S4 growing in V8 broth was observed to form smaller flocculant, forming filamentous strand like structures on the walls of the test tube. PM58b formed white filamentous colonies in HT broth forming strand like structures on the walls of the test tube. In all three cultures, growth was evaluated visually as the pellet and flocculant nature of the *Streptomyces* strains prevented optical density measurements and made dilution and plating approaches non-representative of the actual number of cells in the culture. As the fermentation broths aged, S1 and S4 cultures developed a brown pigmentation which became more pronounced as the culture grew older; this pigmentation was not observed in PM58b.

Bioassays for Antibacterial Activity

Antimicrobial activity was observed in S1 and inconsistently in S4 against *S. aureus, E. coli,* MRSA and MDR *E. coli*. No antimicrobial activity was observed in the PM58b fermentation

broth. The antimicrobial activity was observed after a 10 day fermentation period up until 30 days of fermentation in S1, beyond which assays were not performed. In S4 activity was only observed after an extended period of nutrient limitation, with the effects being observed after a 30 day fermentation. Antimicrobial activity presented in both strains as a zone of inhibition surrounding the plug to which the fermentation broth was delivered. In addition to being devoid of the cloudiness characteristic of growth of the target organisms, these zones of inhibition were pigmented a dark brown. In the plug holes from which these regions of antimicrobial activity radiated from, *Streptomyces* growth was observed. This antimicrobial activity was observed when the bioassay plates were incubated at 15°C however this activity was not present when higher temperatures (37°C) were used. The antimicrobial activity developed over the duration of the incubation of the bioassay plate with activity first appearing as a small pigmented zone of inhibition after day 2, and developing into a larger clearer pigmented zone of inhibition after day 7. Antimicrobial activity was observed in the supernatants of S1 and S4; however, this activity was accompanied with growth of the *Streptomyces* on the walls of the plug holes. Antimicrobial activity was not observed in heat shocked samples in either strain. Antimicrobial activity was also not observed in the 0.35 µm filtered S1 fermentation broth. Neither the hexane nor the ethyl acetate extracts exhibited antimicrobial activity against the target organisms. Similarly, when the aqueous fraction of the extracted broth was tested, no activity was observed. When assays were performed with the unextracted fermentation broth supernatant, which had previously only been filtered through a 70 µm cell strainer antimicrobial activity was observed along with growth of S1 within the agar plug hole.

Table 1. Compiled observations of antimicrobial activity of S1, S4 and PM58b at 15°C incubation temperatures under varying treatments. (+) designates bioactivity observed, (-) designates no bioactivity observed, and NA indicates that the combination of strains and treatments indicated were not tested.

Strain	8 day	10 day	30 day	Super natant	0.35µm Filtrate	Ethyl Acetate Extract	Hexane Extract	Aqueous Fraction	70µm strained broth	Heat Shock
S1	+	+	+	+	-	-	-	-	+	-
S4	-	-	+	+	NA	NA	NA	NA	NA	-
PM58b	-	-	-	-	NA	NA	NA	NA	NA	-

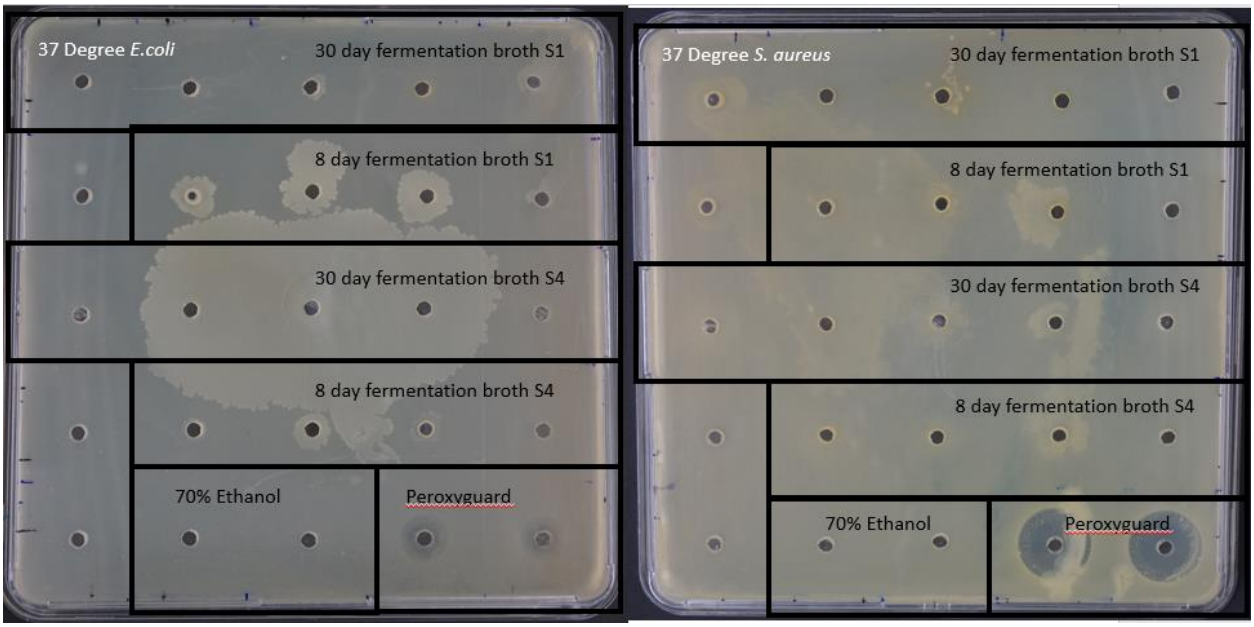

Figure 2. Antimicrobial activity plug assay of S1 and S4 fermentation broths grown for 8 and 30 day durations at 8°C against laboratory strains of E. coli and S. aureus. Plates were incubated at 37°C for a 7 day period

Figure 3. Antimicrobial activity plug assay of S1 and S4 fermentation broths grown for 8 and 30 day durations at 8°C against laboratory strains of E. coli and S. aureus. Plates were incubated at 15°C for a 7 day period

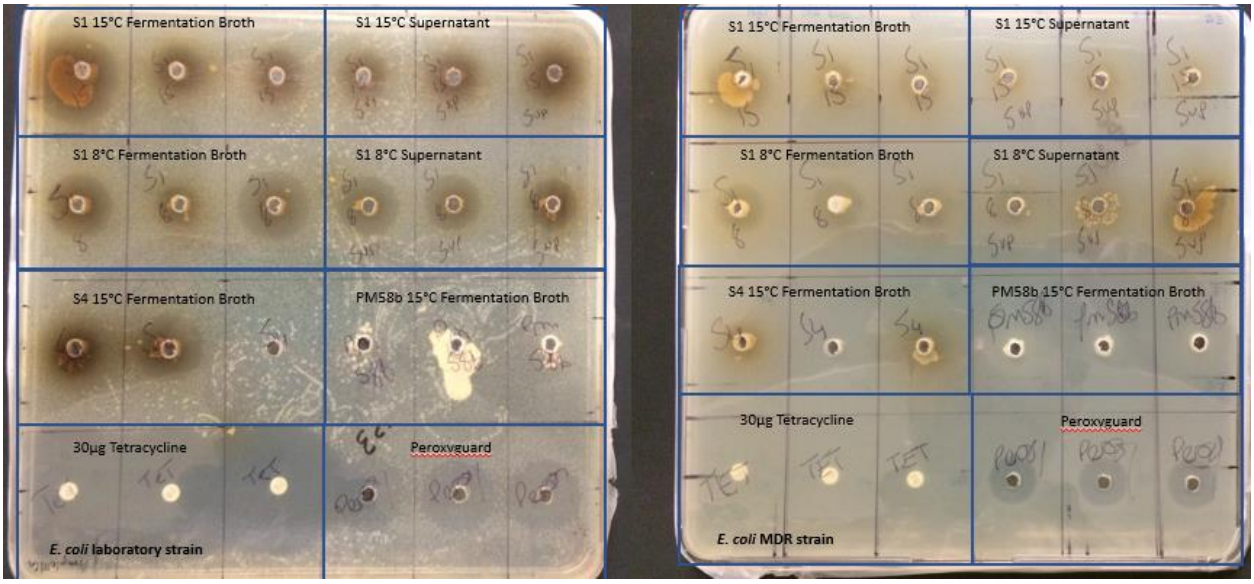

Figure 4. Antimicrobial activity plug assay of S1, S4, and PM58b fermentation broths grown for 10 and 30 day durations at 8°C and 15°C against a laboratory strain and MDR E. coli. Plates were incubated at 15°C for a 7 day period.

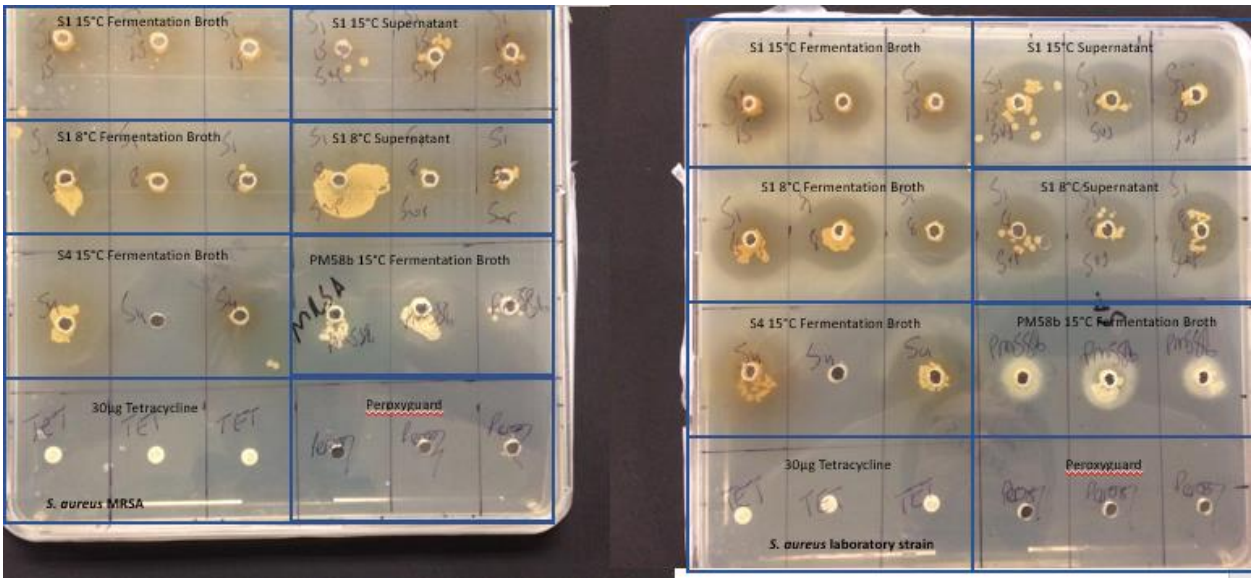

Figure 5. Antimicrobial activity plug assay of S1, S4, and PM58b fermentation broths grown for 10 and 30 day durations at 8°C and 15°C against a laboratory strain of S. aureus and MRSA. Plates were incubated at 15°C for a 7 day period.

MALDI Analysis of Fermentation Broth, and R2A Background

From the comparison of the R2A background and the 30 day S1 fermentation broth 25 consistently observed signals were identified as being present in the fermentation broth but absent in the R2A background spectra. These unique signals ranged from 236.881 to 623.675 m/z. These signals were tabulated in the Appendix.

DISCUSSION

The results of the accumulated bioassays conducted using S1 indicate that the fermentative conditions previously described failed to elicit antimicrobial activity in the cell free fermentation broth. No antimicrobial activity was observed when *Streptomyces* cells were not present or failed to thrive in the plugs of the bioassay plate. This lack of activity in the absence of cells was indicated by the results obtained from filtering the supernatant of the 30 day S1 fermentation broth through a 0.35 µm cellulose acetate filter. This filtration removed the *Streptomyces* cells, leaving an inactive cell free aqueous fermentation broth. This result pointed towards the bioactive compound responsible for the antimicrobial activity observed in bioassays of the cell containing culture being absent or present in concentrations too low to have an observable effect in the cell free broth. This was partially supported by the results of bioassays conducted using heat shocked supernatant. The heat shocking of the *Streptomyces* fermentation broth at 100°C for 10 minutes likely killed the majority of cells persisting in the supernatant leading to no *Streptomyces* growth on the bioassay plate and therefore no antimicrobial activity. However, the treatment that killed the *Streptomyces* present in the supernatant may have also degraded the antimicrobial compound, leading to a loss of bioactivity and making this result inconclusive on its own. More direct support for the conclusion that the antimicrobial compound was not substantially present in solution are the results of assays conducted using the re-dissolved residues of organic extracts and the aqueous component of the organic extract. The extraction of a 50 mL volume of strained S1 fermentation broth supernatant using both hexane and ethyl acetate likely killed any persister cells in the broth, leading to no live *Streptomyces* in the re-dissolved organic residue or in the aqueous fraction. While this treatment likely killed the persisting *Streptomyces* cells it would have had minimal impact on any potentially bioactive compounds in solution, and should have concentrated any that partitioned into the ethyl acetate or hexane fractions. Assaying these re-dissolved extracts, the aqueous fraction they were extracted from and an unextracted portion of the strained fermentation broth supernatant showed no antimicrobial activity but for the unextracted broth after an extended incubation. This indicates that the antimicrobial compound of interest was not produced in solution and was instead being produced on the bioassay plate. The antimicrobial activity exhibited by *Streptomyces* strain S4 presented in a similar manner as S1, showing activity against *E. coli* and *S. aureus*, in conjunction with the spread of dark brown pigment that developed and spread over

time. The antimicrobial activity attributed to S4, however, was obtained inconsistently, occurring in only two separate sets of bioassays in which 30-day old small test tube scale fermentation broths were used. This inconsistency and loss of activity when the process was scaled up to larger volumes prevented further exploration of the antimicrobial activity. PM58b exhibited no activity at all in this experiment, which similarly prevented further exploration.

Observations on the effects of the incubation conditions and duration on the antimicrobial activity of S1 indicate that the antimicrobial compound was produced on the plate. Firstly, the development of antimicrobial activity over the duration of the incubation of the bioassay plates indicates that the compound was produced in increasing concentrations on the bioassay plate as the *Streptomyces* present grew and developed. The degree of antimicrobial activity was observed to progress over time increasing in intensity and in the radius of the zone of inhibition. This observation contrasted sharply with the behaviour of the positive controls; Peroxyguard and tetracycline, which involved finite amounts of each compound, and presented as clearly delineated zones of inhibition that developed shortly after the delivery of the control to the bioassay plate. The lack of antimicrobial activity at 37°C despite the presence of *Streptomyces* cells in the bioassay wells supports the idea that the antimicrobial compound is produced on the plate as a result of *Streptomyces* cells growth. This is because the cave *Streptomyces* S1were unable to grow at the 37°C incubation temperature, and as such the antimicrobial was not produced. Despite not being secreted into the fermentation broth, the progressive diffusion displayed by the antimicrobial compounds produced by S1 suggests that the antimicrobial compound was secreted and not surface bound to the microbe as *Streptomyces* are non-motile organisms. In addition to this, the extent of the diffusion of the antimicrobial compound on the bioassay plates suggests that the compound is water soluble due to its capacity to diffuse through the aqueous agar.

The lack of activity in the cell free broth may indicate that the nutrient depleted conditions in the S1 fermentation broth failed to induce the production of antimicrobial secondary metabolites, the antimicrobial activity observed on the bioassay plates after an extended incubation may have provided these nutrient limited conditions stimulating the production of these metabolites. This achievement of nutrient depleted conditions stimulating the production of secondary metabolism on the bioassay plate but not in the fermentation broth is consistent with the observations of gradual diffusion and development of the antimicrobial compound on the

bioassay plate. Similarly, the observation that no antibacterial activity was exhibited in cell free variations of the fermentation broth also supports this conclusion, as in the absence of cells, the induction of secondary metabolism cannot be achieved. An alternative interpretation of the results above describes an antimicrobial compound being produced by *Streptomyces* S1 in a manner that is attached to the outer cell surface, preventing the isolation of antimicrobial activity in a cell free environment. This interpretation of the experimental data would explain the diffusion of the antimicrobial activity as the result of the growth of hyphae through the agar giving this diffuse zone of inhibition. This interpretation could be tested through the bioassaying of a fermentation broth of S1 after the constituent bacteria's outer cellular surface has been disrupted.

The results of the MALDI-MS analysis of the 30-day fermentation broth produced signals not observed in the R2A background media. This is to be expected as the result of normal microbial metabolism however due to the inability to correlate any antimicrobial activity to the cell free fermentation broth. The spectra obtained appeared quite noisy potentially due to signals detected from the ionized HCCA matrix and matrix adducts of sodium or potassium.

CONCLUSIONS AND FUTURE WORK

The results of this research leave many questions to be answered, and could open up doors to new potential research questions. The observation that antimicrobial activity consistently failed to be observed in the cell free fermentation broth despite being produced on the plate opens the opportunity to further fine-tune fermentation conditions so as to produce the antimicrobial secondary metabolites in solution allowing for their extraction and for further analysis to take place. In future applications of MALDI to the analysis of such complex mixtures blanks should include several replicates of matrix only prepared with hard water to evaluate the presence of any matrix salts to prevent these signals from confounding results. Another addition to the MALDI sample preparation protocol is the matrix suppressor cetyltrimethylammonium bromide (CTAB) which through the suppression of the HCCA matrix could improve resolution (Gou et al. 2002).

LITERATURE CITED

1. Bentley, S. D. et al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 417, 141–147 (2002).
2. Bhullar, K. et al. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS One* 7, e34953 (2012).
3. Bibb, M. J. Regulation of secondary metabolism in streptomycetes. *Current Opinion Microbiology*. 8, 208–215 (2005).
4. Blahna, M. T. et al. The role of horizontal gene transfer in the spread of trimethoprim-sulfamethoxazole resistance among uropathogenic *Escherichia coli* in Europe and Canada. *Journal of Antimicrobial Chemotherapy*. 57, 666–672 (2006).
5. Blair, J. et al.. Molecular mechanisms of antibiotic resistance. *Nature. Reviews. Microbiology.* 13, 42–51 (2015).
6. Cabrera, G. M. Mass spectrometry in the structural elucidation of natural products: Glycosides. *Phytochemistry* 661, 1–22 (2006).
7. Cheeptham, N. et al. Cure from the cave: volcanic cave actinomycetes and their potential in drug discovery. *International Journal of Speleology*. 42, 35–47 (2013).
8. Chu J. et al. Discovery of MRSA active antibiotics using primary sequence from the human microbiome. *Nature Chemical Biology*. 12, 1004-1006. (2016).
9. Cohen, L. H. & Gusev, A. I. Small molecule analysis by MALDI mass spectrometry. *Analytical and Bioanalytical Chemistry*. 571–586 (2002).
10. Cohen, S. N., Chang, A. C. & Hsu, L. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Pnas* 69, 2110–4 (1972).
11. Cuezva, S. et al. The biogeochemical role of Actinobacteria in Altamira Cave, Spain. *FEMS Microbiology Ecology*. 81, 281–290 (2012).
12. Davies, J. & Davies, D. Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews* 74, 417–433 (2010).
13. Fiedler, H. P. et al. Marine actinomycetes as a source of novel secondary metabolites. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. 87, 37–42 (2005).

14. Flärdh, K. & Buttner, M. J. *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nature Reviews Microbiology*. 7, 36–49 (2009).

15. Ghosh, S., Kuisiene, N. & Cheeptham, N. The cave microbiome as a source for drug discovery: reality or pipe dream? *Biochemical Pharmacology*. 134, 18-34 (2017)

16. Ghosh, S. Personal Communication. (2016).

17. Guo, Z., Zhang, Q., Zou, H., Guo, B. & Ni, J. A method for the analysis of low-mass molecules by MALDI-TOF mass spectrometry. *Analytical Chemistry*. 74, 1637–1641 (2002).

18. Handelsman, J. Metagenomics: Application of Genomics to Uncultured Microorganisms. *Microbiology and Molecular Biology Reviews* 68, 669–685 (2004).

19. Hauryliuk, V., Atkinson, G. C., Murakami, K. S., Tenson, T. & Gerdes, K. Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nature Reviews Microbiology* 13, 298–309 (2015).

20. Hesketh, A., Chen, W. J., Ryding, J., Chang, S. & Bibb, M. The global role of ppGpp synthesis in morphological differentiation and antibiotic production in *Streptomyces coelicolor* A3(2). *Genome Biology* 8, R161 (2007).

21. Kind, T. & Fiehn, O. Advances in structure elucidation of small molecules using mass spectrometry. *Bioanalytical Reviews*. 2, 23–60 (2010).

22. Leite, C., Cavallieri, A. P. & Araujo, M. L. G. C. Enhancing effect of lysine combined with other compounds on cephamycin C production in *Streptomyces clavuligerus*. *BMC Microbiology*. 13, 296–307 (2013).

23. Liu, G., Chater, K. F., Chandra, G., Niu, G. & Tan, H. Molecular regulation of antibiotic biosynthesis in *Streptomyces*. *Microbiology and Molecular Biology Reviews*. 77, 112–43 (2013).

24. Mason, C., Randhawa, A., Watson, K., Friedman, C. R. & Cheeptham, N. Using scanning electron microscopy to study microbial communities in speleothem samples collected from Iron Curtain Cave. *Journal of Experimental Microbiology and Immunology*. 2, 1–7 (2016).

25. Nguyen, L. T., Nguyen, K. T., Spfek, J. & Behal, V. The tylosin producer, *Streptomyces fradiae,* contains a second valine dehydrogenase. *Microbiology* 1139–1145 (1995).

26. Rateb, M. E. et al. Diverse metabolic profiles of a *Streptomyces* strain isolated from a hyper-arid environment. *Journal of Natural Products* 74, 1965–1971 (2011).
27. Sadoway, T. The susceptibility of multidrug resistant, Gram-negative pathogens to antimicrobial compounds produced by cave actinomycetes. *Bachelors Science Thesis TRU* (2011)
28. Seipke, R. F. Strain-level diversity of secondary metabolism in *Streptomyces albus*. *PLoS One* 10, 1–14 (2015).
29. Sigle, S., Ladwig, N., Wohlleben, W. & Muth, G. Synthesis of the spore envelope in the developmental life cycle of *Streptomyces coelicolor*. *International Journal of Medical Microbiology*. 305, 183–189 (2015).
30. Takano, E. γ-Butyrolactones: *Streptomyces* signaling molecules regulating antibiotic production and differentiation. *Current Opinion. Microbiology*. 9, 287–294 (2006).
31. Wolański, M., Jakimowicz, D. & Zakrzewska-Czerwińska, J. AdpA, key regulator for morphological differentiation regulates bacterial chromosome replication. *Open Biology*. 2, 120097 (2012).

APPENDICES

Oxoid Nutrient Agar Media

1.0g/L Lab-Lemco powder

2.0g/L Yeast extract

5.0g/L Peptone

5.0g/L Sodium chloride

15g/L Agar

pH 7.4

Oxoid Nutrient Broth Media

1.0g/L Lab-Lemco powder

2.0g/L Yeast extract

5.0g/L Peptone

5.0g/L Sodium chloride

pH 7.4

R2A Broth Media

0.500g/L Casein acid hydrolysate

0.500g/L Yeast extract

0.500g/L Peptone

0.500g/L Dextrose

0.500g/L Starch, soluble

0.300g/L Dipotassium phosphate

0.024g/L Magnesium sulphate

0.300g/L Sodium pyruvate

pH 7.2

V8 Broth Media

200mL reduced salt V8 juice supernatant (centrifuged at 5000rpm for 10 minutes)

3.0g/L Calcium carbonate

pH 7.4

Hickey Tresnar Broth Media

1.0g/L Yeast extract

1.0g/L Beef extract

2.0g/L N2 Amine

10.0g/L Dextrin

pH 7.5

Table 2. Reagents used.

Reagent and Grade	Distributor
HPLC grade hexane	Sigma Aldrich
LC grade ethyl acetate	Sigma Aldrich
Reagent grade trifluoroacetic acid	Sigma Aldrich
Reagent grade acetonitrile	Sigma Aldrich
18MΩ water	-
Reagent grade 2-Propanol	Fischer Scientific
A-Cyano-4-hydroxycinnamic acid	Bruker Daltonics

Table 2. MALDI M/Z signal unique to the S1 30 day fermentation broth, obtained from the systematic comparison with R2A background spectra

Unique 30 Day Broth MS Signals
236.881
244.738
246.72
247.572
250.708
253.578
256.465
262.4
263.463
264.344
274.14
276.246
285.965
293.681
301.795
301.816
311.354
327.263
329.239
338.211
372.196
373.339
420.557
608.043
623.675

Table 3. Tabulated R2A background peaks. Red shaded cells indicate peaks duplicated in more than one replicate.

R2A Background 1	R2A Background 2	R2A Background 3	R2A Background 4	R2A Background 5	R2A Background 6	R2A background Consistent
203.492	203.492	203.492	203.492	203.492	203.492	203.492
219.299	219.352	219.305	219.318	219.437	219.305	219.305
227.819	228.085	227.819	227.819	219.437	228.085	219.437
230.817	230.823	230.822	230.822	230.823	230.823	227.819
231.642	231.918	231.918	231.908	231.918	231.918	228.085
232.746	234.953	232.746	232.746	232.746	232.746	230.822
233.642	236.87	233.756	234.952	234.952	234.953	231.918
234.952	237.727	234.952	235.784	235.784	235.784	232.746
236.616	238.835	236.616	236.696	236.708	236.72	234.952
237.727	239.68	237.728	237.728	237.727	237.727	235.784
238.563	242.755	238.563	238.563	238.68	238.673	236.616
242.483	243.609	239.679	239.679	239.68	239.68	237.727
258.205	258.338	242.483	242.483	242.605	242.593	238.563
259.186	259.368	243.608	243.608	243.608	243.609	239.68
260.243	260.243	254.443	258.205	253.576	258.205	242.483
262.289	262.29	258.205	259.368	254.443	259.368	243.608
273.844	274.138	259.363	260.243	258.205	260.243	254.443
274.842	275.043	260.243	262.29	259.368	262.29	258.205
275.945	275.945	262.29	273.948	260.243	273.977	259.368
276.915	277.148	273.844	275.043	262.29	275.043	260.243
301.504	301.504	275.038	275.945	271.155	275.945	262.29
314.376	314.56	275.945	276.989	273.977	277.148	273.844
315.333	315.532	276.947	278.054	275.043	278.054	273.977
341.567	339.208	281.996	281.996	275.945	301.504	275.043
354.475	341.901	301.504	301.504	276.976	314.56	275.945
384.978	382.289	314.553	314.553	278.054	315.532	277.148
385.746	384.979	315.528	315.523	301.504	341.901	278.054
419.432	386.054	341.891	341.891	314.558	354.475	281.996
422.429	387.128	382.118	354.475	315.528	379.263	301.504
445.716	403.845	384.979	382.119	341.894	382.118	314.56
465.219	406.789	386.053	384.978	354.475	384.979	315.532
466.48	419.432	403.841	386.053	369.035	386.054	341.891
481.272	422.436	419.432	403.844	382.119	392.903	341.901
602.126	446.1	422.435	419.432	384.978	394.715	354.475
657.949	449.984	445.851	445.717	386.052	403.845	382.118
673.766	465.687	465.672	449.978	403.844	406.789	384.979
689.785	466.607	466.48	465.684	406.77	408.636	386.053
	481.273	481.273	466.589	419.432	419.432	403.844
	497.132	483.29	481.273	422.435	422.435	406.789
	658.424	674.22	658.424	446.083	446.083	419.432
	674.248	689.785	674.245	449.98	465.687	422.435
	690.267	705.812	689.785	465.684	466.48	446.083
	706.005		693.188	466.585	481.273	465.684
			706.002	481.273	658.425	465.687
				658.418	674.248	466.48
				674.246	689.785	481.273
				689.785	706.005	658.424
				692.221		674.248
				706.002		689.785
				708.937		706.002

Table 4. Tabulated 30 day fermentation broth peaks. Yellow shaded cells indicate peaks duplicated in more than one replicate.

Broth 1	Broth 2	Broth 3	Broth 4	Broth 5	Broth 6	Consistent Signals
236.887	225.645	230.823	236.881	236.616	236.738	230.823
239.825	230.823	236.883	237.727	237.728	237.728	236.881
242.762	236.888	237.727	239.953	246.714	242.483	236.888
244.738	242.76	239.945	244.737	247.572	246.719	237.727
246.72	246.72	242.756	246.72	258.312	247.572	242.76
250.708	258.374	244.738	247.572	262.29	253.578	244.738
256.465	259.368	246.72	250.708	263.169	256.465	246.72
258.385	262.582	247.572	253.578	264.344	258.205	247.572
259.368	268.182	250.708	256.465	274.142	262.29	250.708
262.443	274.141	253.578	258.325	275.044	264.344	253.578
268.474	275.043	256.465	259.369	276.246	274.13	256.465
274.14	276.246	258.333	262.397	277.15	275.043	259.368
275.044	277.15	259.369	263.463	278.055	276.246	262.29
276.247	281.996	262.4	264.344	293.681	277.15	262.4
277.15	292.129	263.462	270.261	301.504	278.055	263.463
278.055	301.819	264.344	274.138	311.354	293.681	264.344
281.996	311.674	274.14	275.044	314.556	301.504	274.14
285.966	314.567	275.044	276.246	315.523	311.354	275.043
301.816	315.534	276.231	277.15	338.21	314.566	276.246
311.657	317.473	278.055	278.055	338.993	315.534	277.15
314.567	338.211	285.965	285.965	372.196	338.198	278.055
315.534	339.216	301.801	301.795	373.252	372.196	281.996
372.528	372.547	311.526	311.512	403.844	373.31	285.965
373.354	481.273	314.567	314.567	404.58	403.845	293.681
399.084	689.784	315.534	315.534	419.432	409.744	301.504
419.432		327.263	327.263	420.545	419.77	301.795
420.557		329.239	329.239	421.307	420.557	301.816
449.984		372.525	353.789	481.273	481.273	311.354
465.687		373.339	372.519	497.131	497.131	314.567
		608.041	373.339	598.951	623.675	315.534
		624.131	403.845	623.675		327.263
			409.746	631.569		329.239
			419.432	674.249		338.211
			481.273	690.271		372.196
			608.043	706.005		373.339
			624.119	721.928		403.845
			706.005			419.432
						420.557
						481.273
						497.131
						608.043
						623.675
						706.005

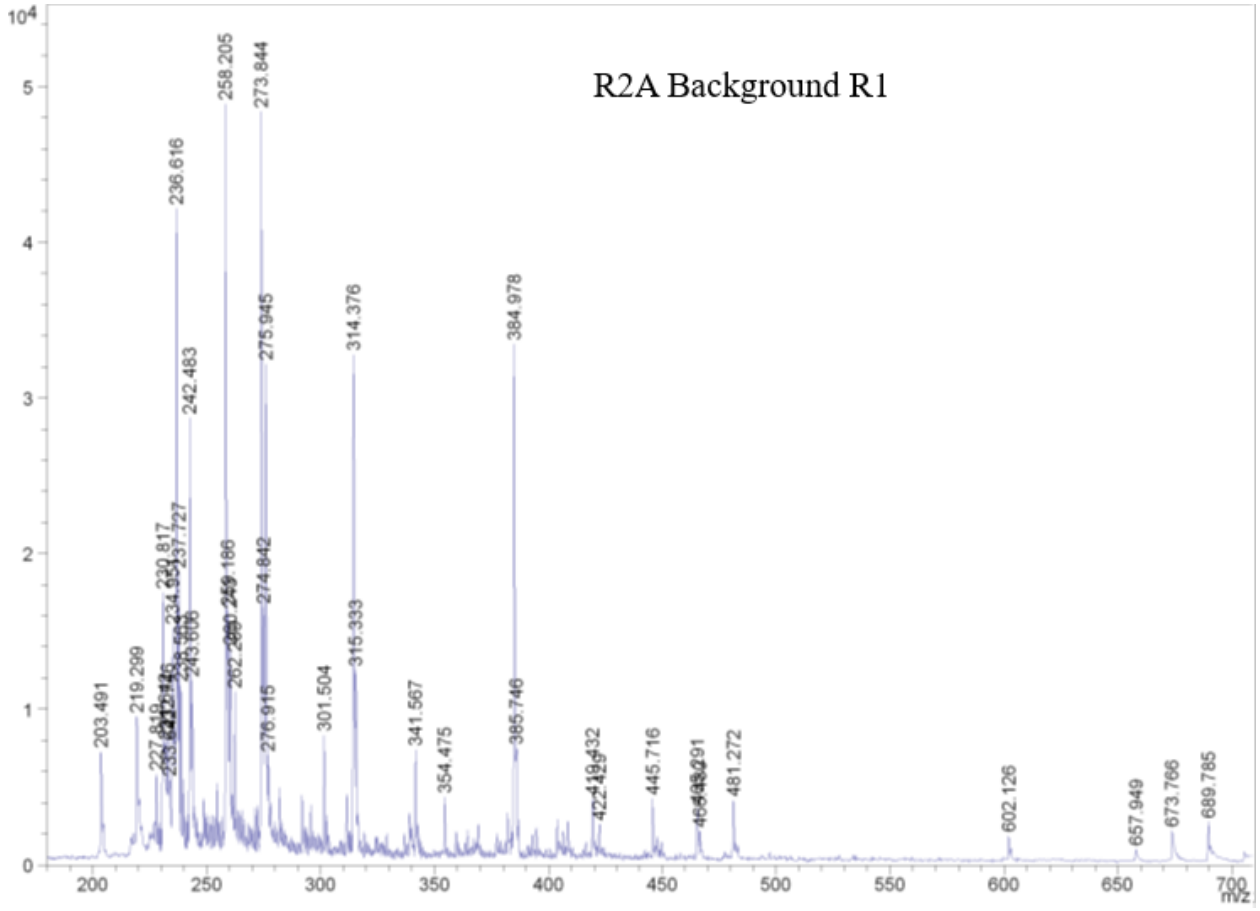

Figure 6. R2A background MS replicate 1

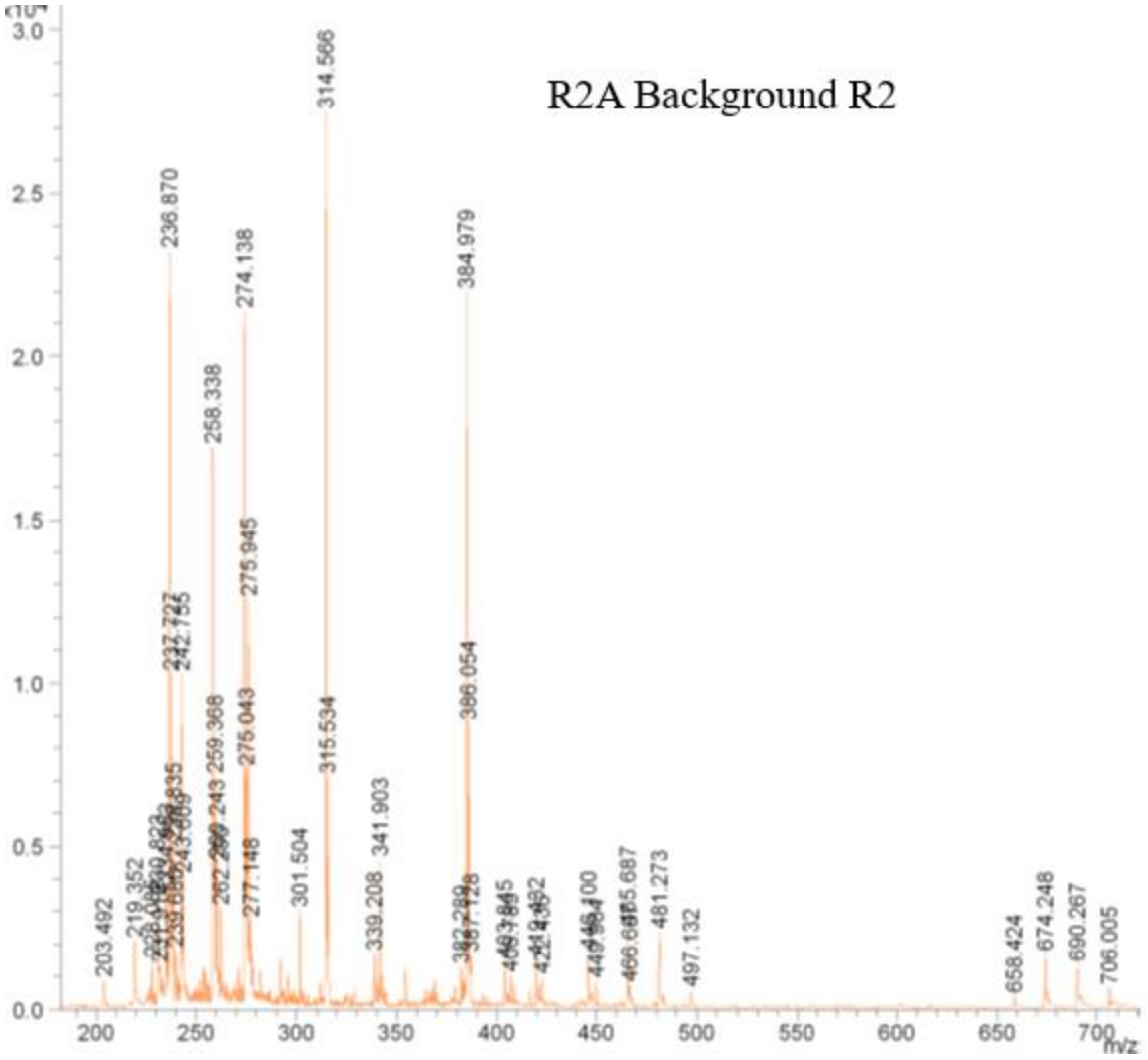

Figure 7. R2A background MS replicate 2

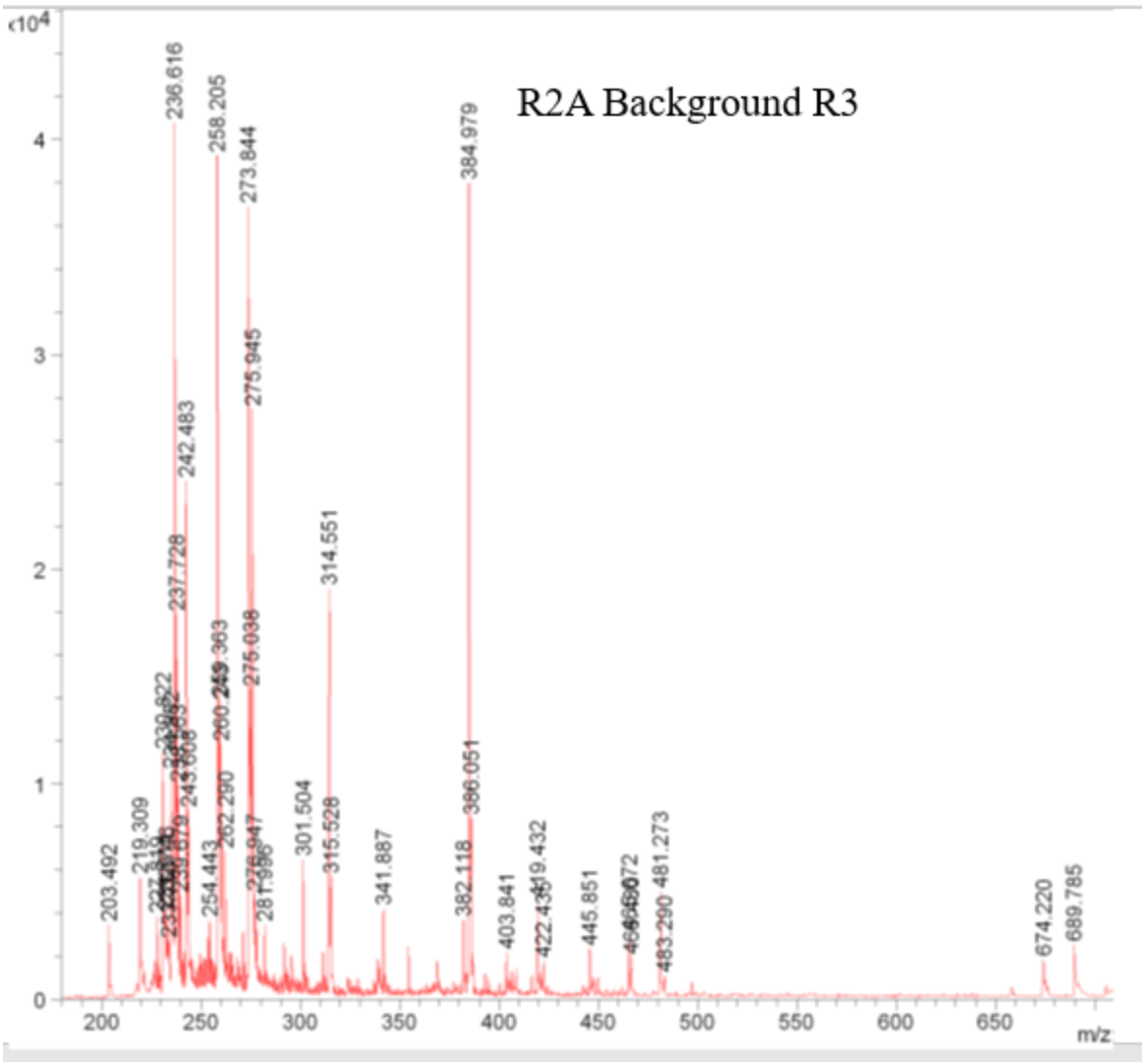

Figure 8 R2A background MS replicate 3

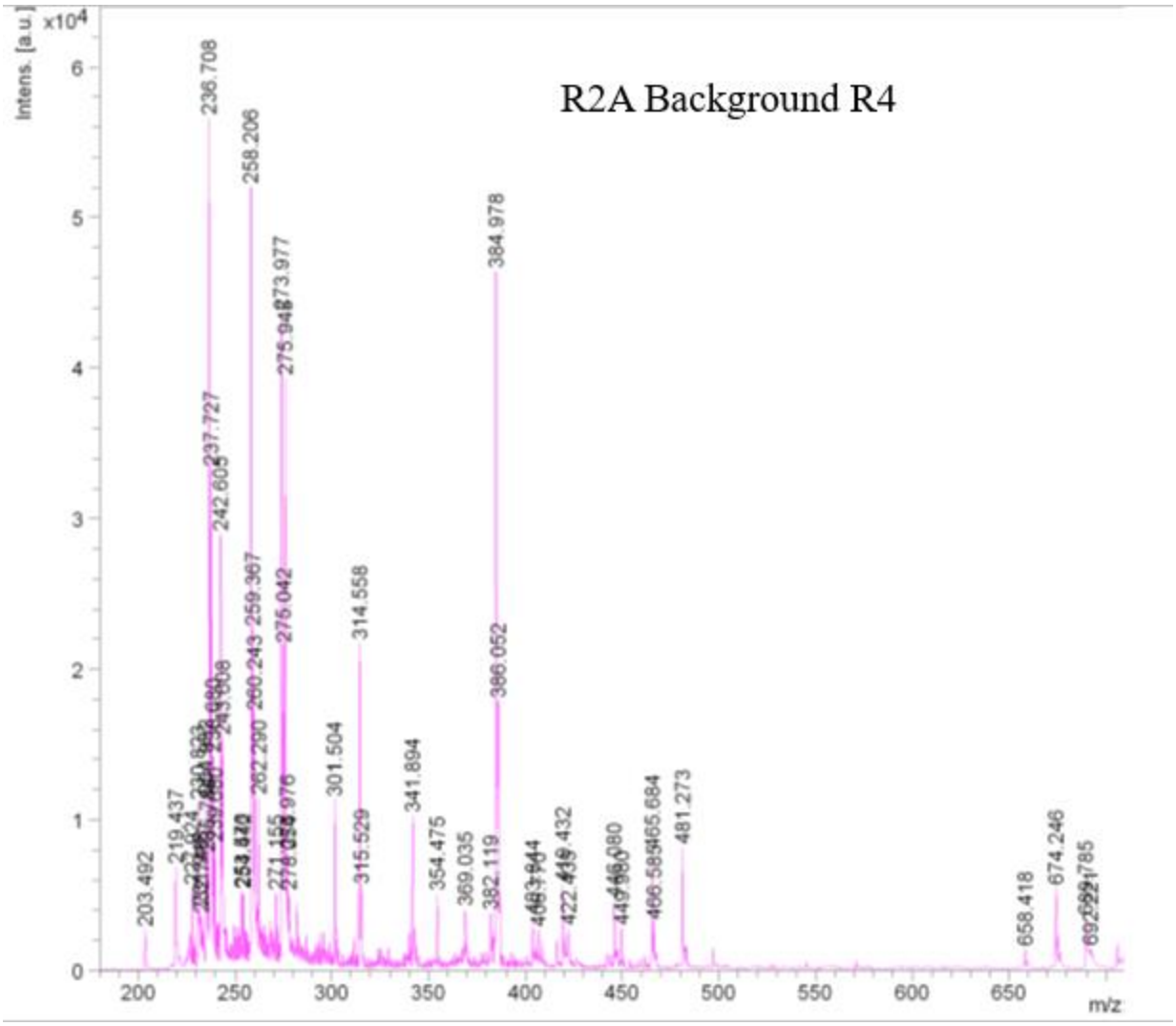

Figure 9 R2A background MS replicate 4

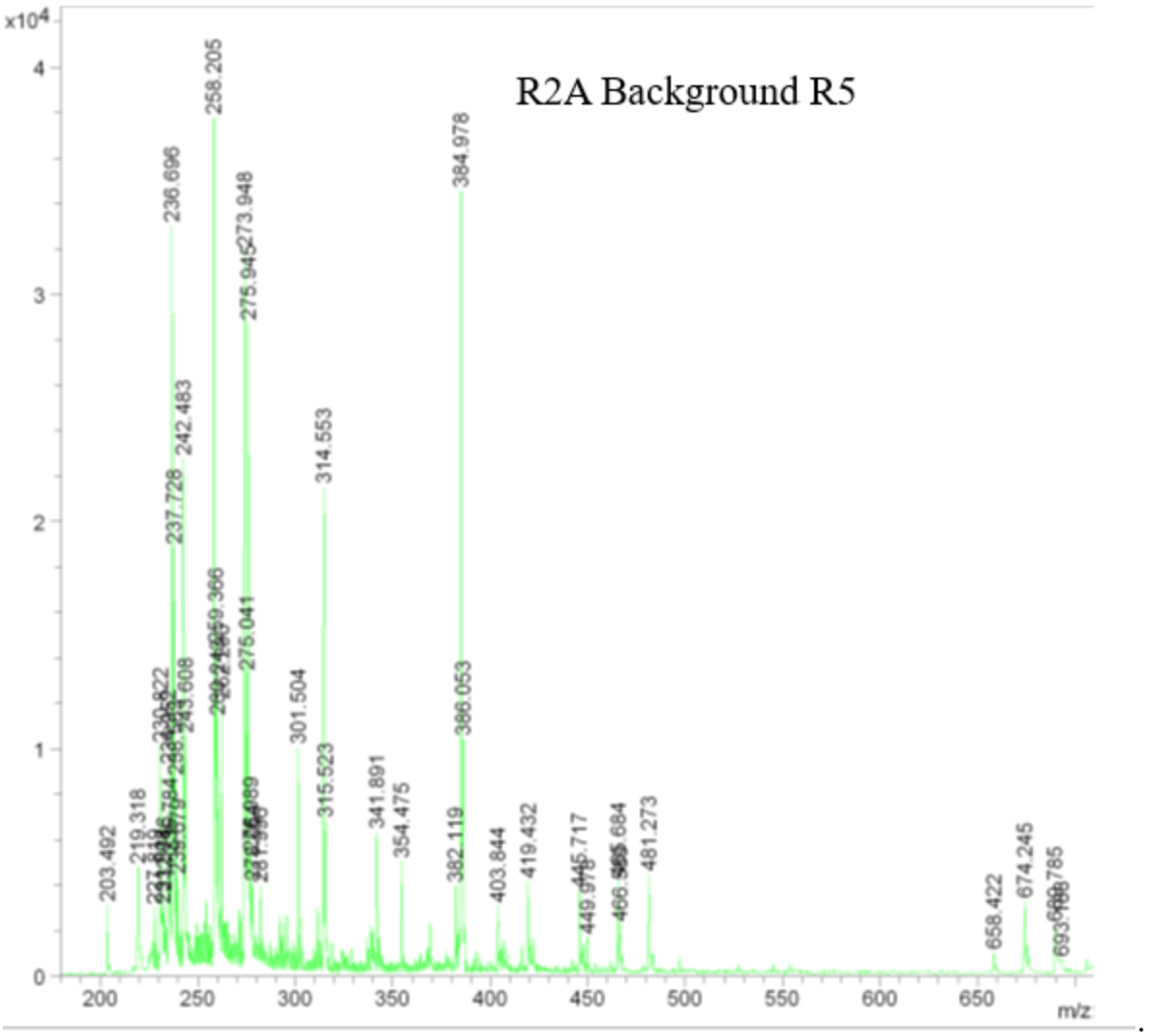

Figure 10 R2A background MS replicate 5

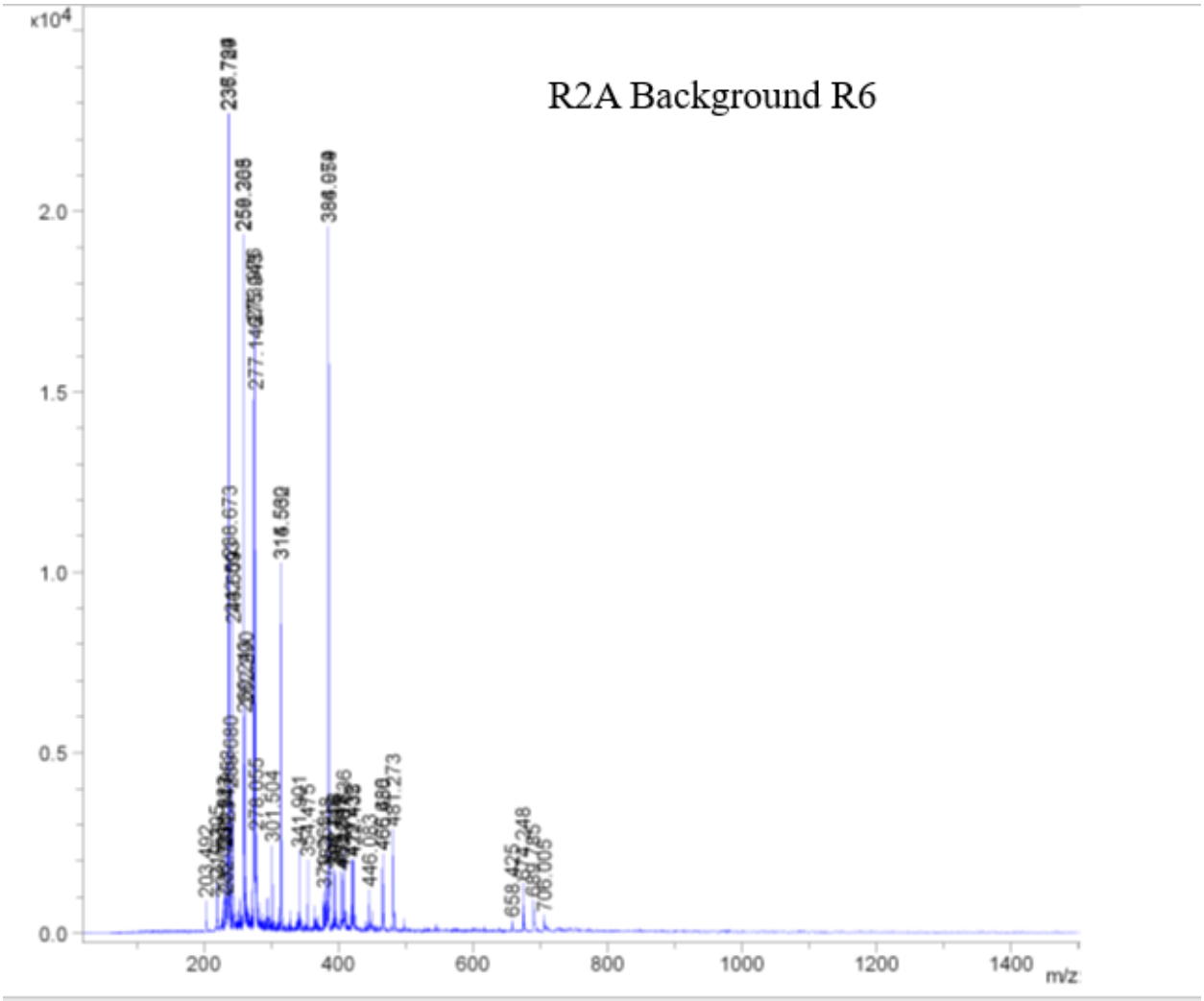

Figure 11 R2A background MS replicate 6

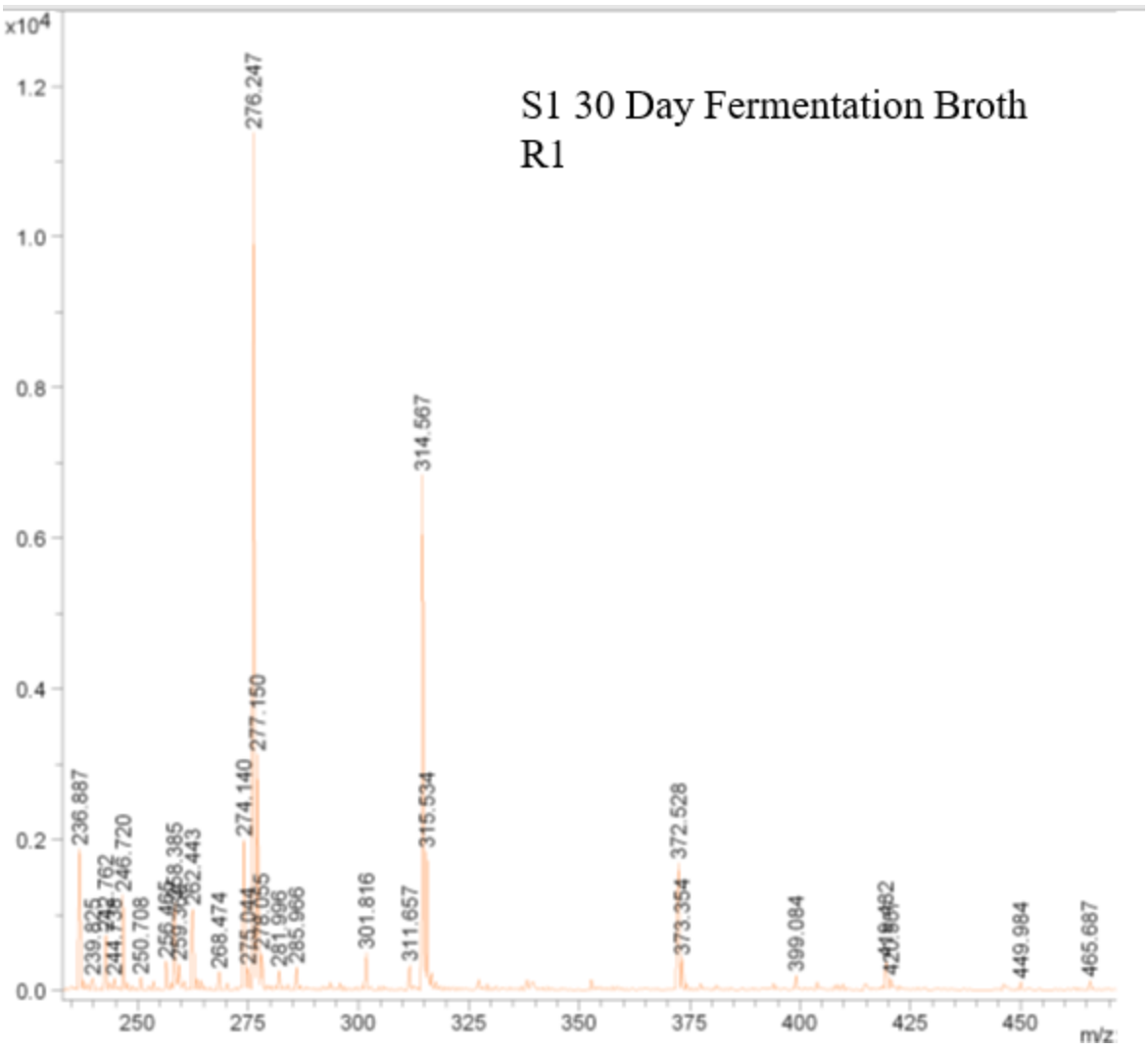

Figure 12 S1 30 day fermentation broth MS replicate 1

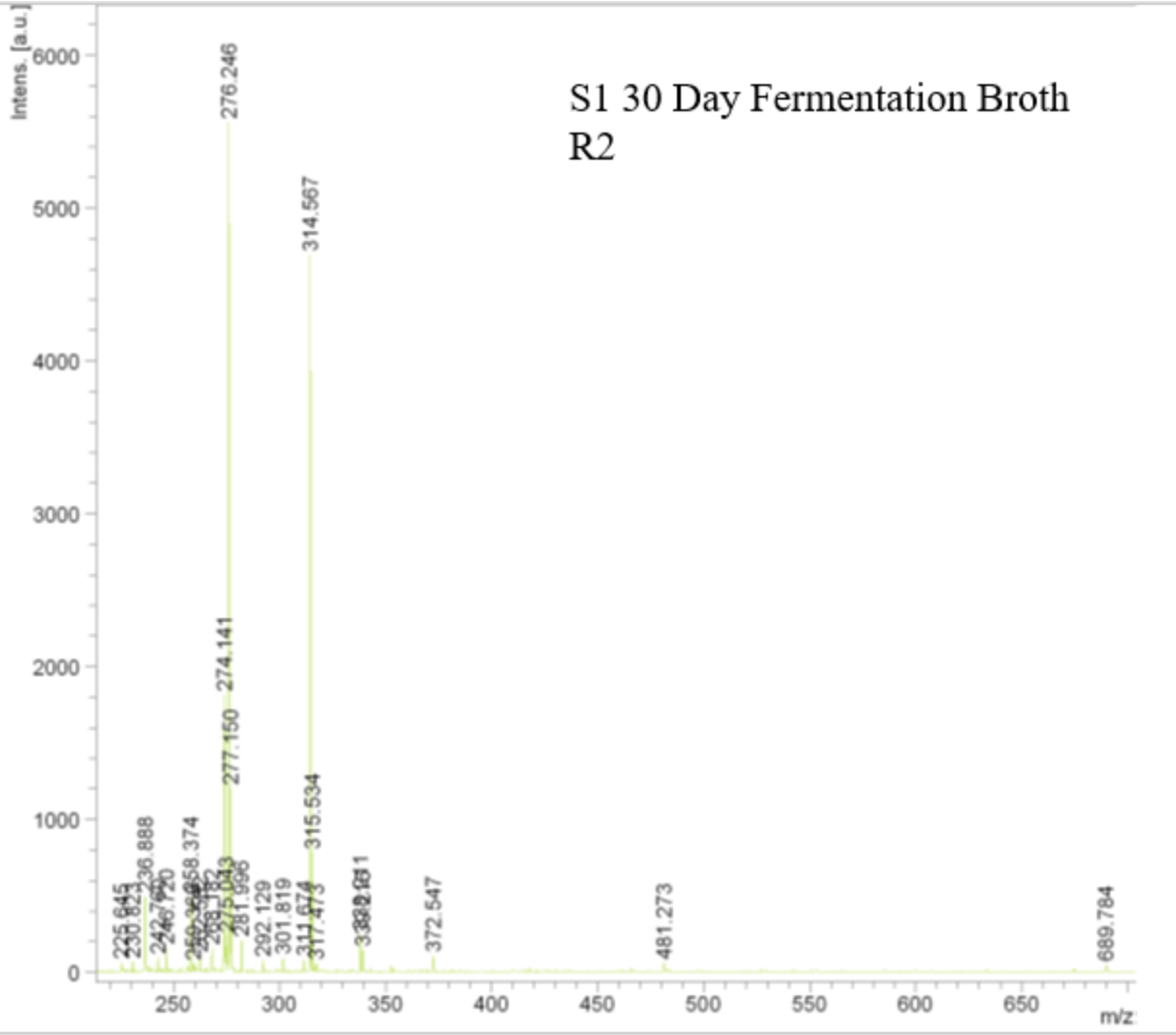

Figure 13 S1 30 day fermentation broth MS replicate 2

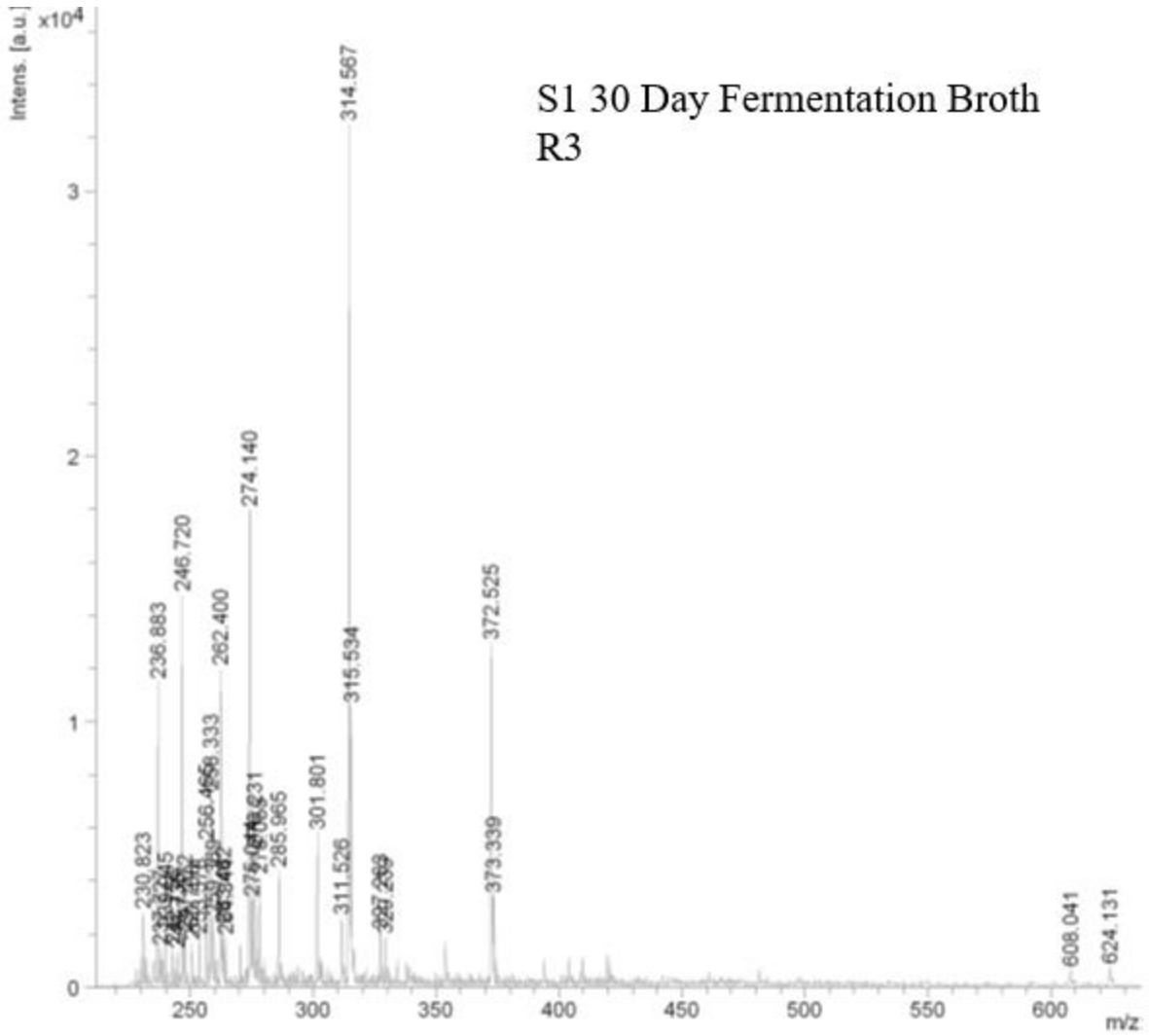

Figure 14 S1 30 day fermentation broth MS replicate 3

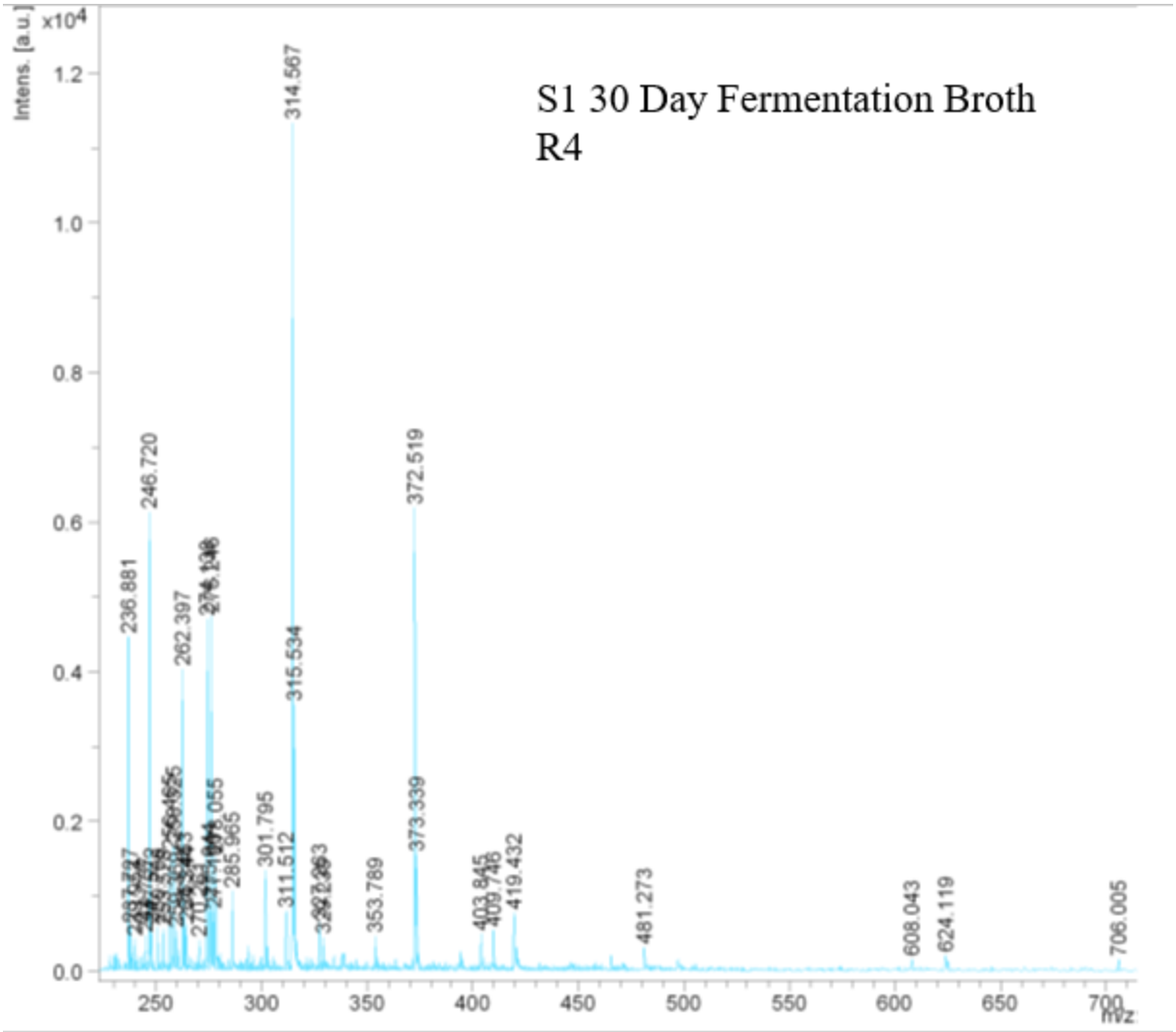

Figure 15 S1 30 day fermentation broth MS replicate 4

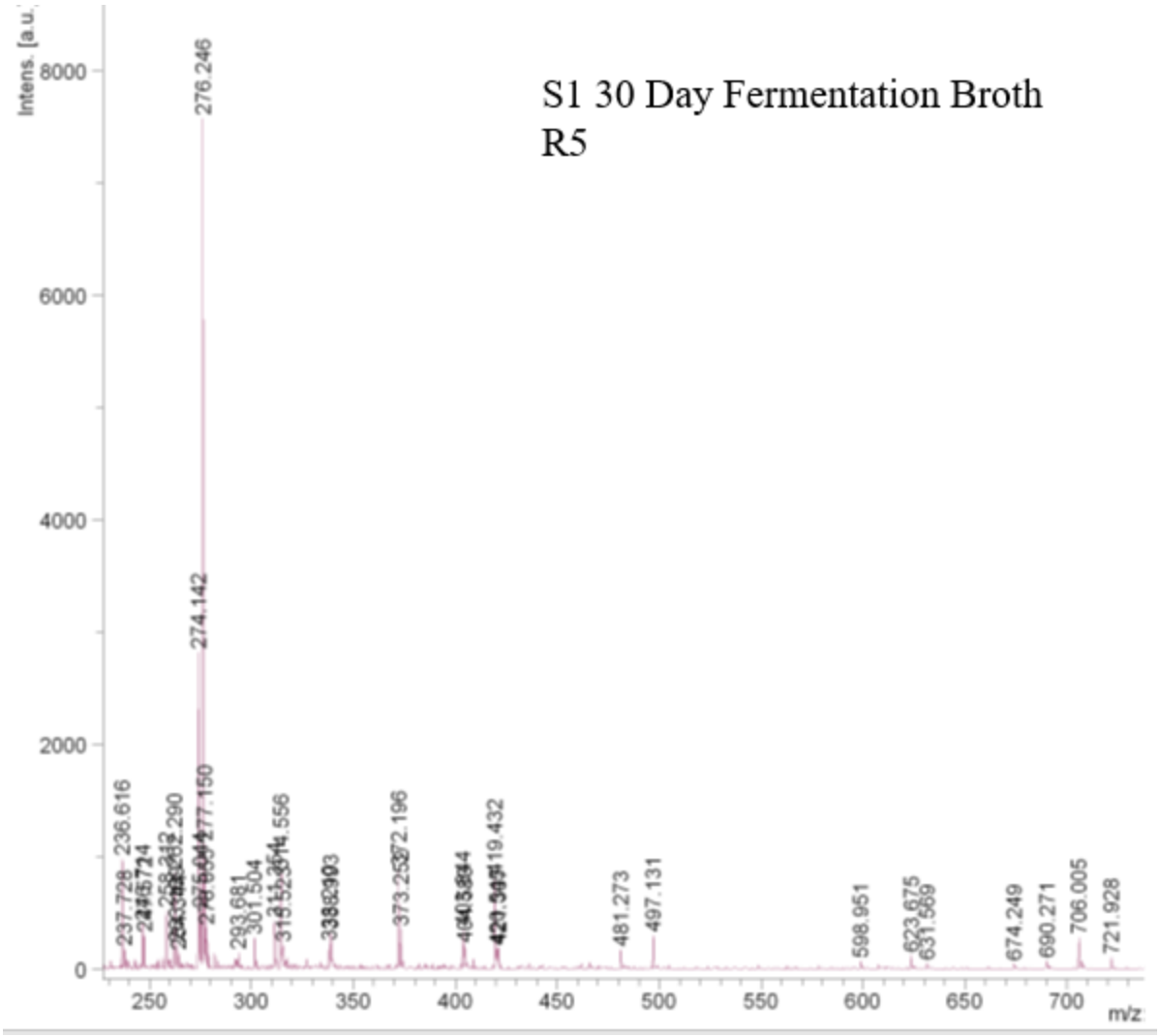

Figure 16 S1 30 day fermentation broth MS replicate 5

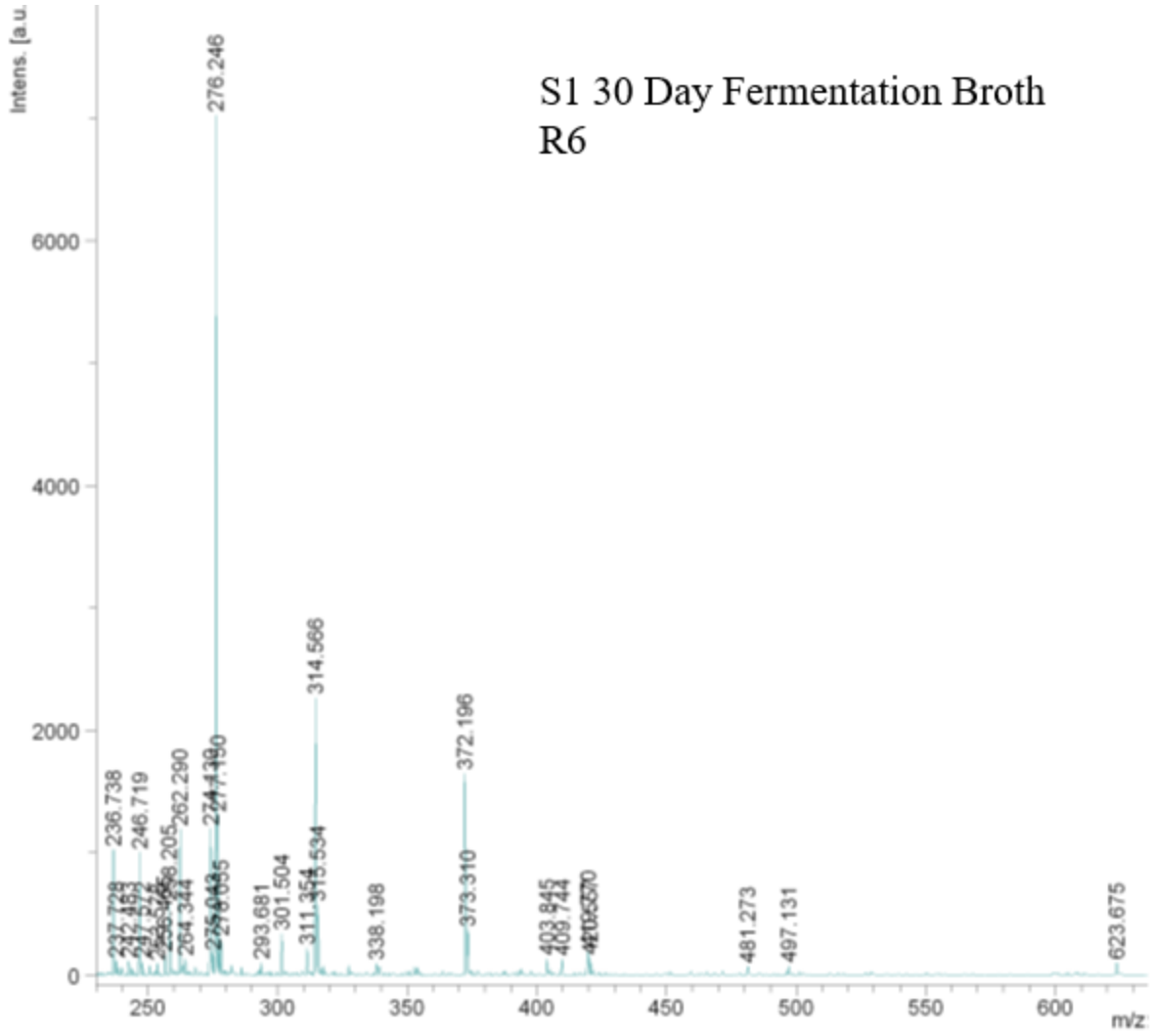

Figure 17 S1 30 day fermentation broth MS replicate 6