# Anthracycline biosynthesis in Streptomyces: engineering, resistance and antimicrobial activity

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# Integrated proteomics and metabolomics analysis of small-scale *Streptomyces peucetius* cultures

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## Abstract

The cultivation and characterisation of *Streptomyces* strains in a high-throughput manner is important to screen for natural products under a range of culturing conditions. However, small-scale cultivation of mycelial microorganisms faces major challenges such as high biological variability and reduced mixing efficiency. Here we present an integrated quantitative proteomics and metabolomics workflow to analyse natural product biosynthesis in small-scale *Streptomyces* cultures. Wild-type *Streptomyces peucetius* and an industrial production strain were cultivated in 1 mL complex media in a microbioreactor (BioLector) with continuous non-invasive monitoring of growth data, pH and dissolved oxygen concentrations. Cultures were subjected to paired omics data analysis, with protein expression analysed by quantitative proteomics on the biomass, and metabolomics analysis on the spent media. Despite the inherent challenges of reproducibility of growing *Streptomyces* pellets in submerged cultures, the technical reproducibility of the paired omics data was sufficiently high to allow proper interpretation of the data. This shows that our methodology holds promise for automated high-throughput multi-omics analysis of *Streptomyces* strains. This approach will aid scientists in the study of biosynthetic gene cluster expression in combination with metabolite analysis.

# Introduction

Streptomycetes are prolific producers of bioactive natural products with various activities including antibacterial, antifungal, insecticidal, antiviral and anticancer[2–4]. This industrially important genus of Gram-positive bacteria exhibits a distinctive multicellular lifestyle[21]. The life cycle of *Streptomyces* starts with the formation of spores, which disperse for survival in challenging environments. Following germination, the spores develop into a complex network of vegetative hyphae[43,44]. In response to nutrient depletion or environmental stresses, a programmed cell death process is activated within the vegetative hyphae[57,353]. This process releases essential nutrients for the formation of aerial hyphae, which ultimately develop into spore chains[21]. The autolytic degradation of the vegetative hyphae is temporally correlated with the production of natural products, which is tightly regulated by a complex signalling system[5,45,354]. The genes involved in the production of these bioactive metabolites are typically clustered on the genome in so-called biosynthetic gene clusters (BGCs). Recent advances in genome mining have revealed the presence of numerous and highly diverse BGCs in the genomes of *Streptomyces* bacteria[46,168,355]. This discovery holds promising implications for addressing the current antibiotic crisis[338,339].

Systematic screening efforts are required to active and annotate the BGCs that often remain silent under standard laboratory conditions. Identification of the corresponding metabolites can be accelerated through the application of various omics techniques, such as metabolomics, transcriptomics and proteomics analysis. The combination of multiple omics techniques presents a particularly promising strategy, providing insights into the correlation of BGC expression and metabolite production[52,356–358]. For screening purposes, small-scale cultivation in submerged environments is favoured over solid cultures, because it provides a more rapid and economically feasible approach to study extensive strain collections under diverse experimental conditions[359]. Microbioreactor (MBR) systems are of particular interest in this context. These systems not only provide extensive bioprocess monitoring, but present opportunities for integration into liquid handling robots[360]. Achieving reproducible small-scale cultivation of *Streptomyces* is challenging, yet it is feasible with tightly controlled inoculation strategies and well-characterised cultivation methods[361,362]. In submerged environments, *Streptomyces* grows as mycelial networks, typically forming dense pellets or mycelial mats[363]. This leads to diffusion gradients of nutrients within the pellets, which results in increased culture heterogeneity[363]. Previous work from our laboratory indicates that the model strains *Streptomyces lividans* and *Streptomyces coelicolor* can be cultured in low volumes of 100 µL in microtiter plates while maintaining enzyme and antibiotic production by optimising the agitation rate[362]. In another study, *S. lividans* was successfully cultivated in a 1 mL volume by optimising the preculturing strategy[361]. Their workflow involved the BioLector, an automated cultivation device that allows for non-invasive measurement of biomass formation, pH and dissolved oxygen (DO) concentration and fluorescence in a 48-well format. A recent study developed an automated workflow for multi-omics screening of microbial model organisms[364]. Their work focused on *Escherichia coli*, *Saccharomyces cerevisiae* and *Pseudomonas putida*, which are all unicellular organisms without complex morphologies.

In this study, we present a workflow for integrated quantitative proteomics and metabolomics analysis of 1 mL MBR cultures of *Streptomyces peucetius*, producer of the anticancer compounds daunorubicin and doxorubicin. Our results indicate that the technical reproducibility highly depends on the quality of the biological replicates. We show that it is feasible to analyse both the metabolome and proteome of small-scale *Streptomyces* cultures in a high-throughput manner, although further development of the method is required to reduce biological variance.

# Results

## Inoculum strategy for microbioreactor cultivation

*Streptomyces* strains are typically cultivated in shake flasks or on agar plates to generate proteomics and metabolomics datasets. In this study, we aimed to assess the feasibility of generating such datasets from microbioreactor (MBR) cultures. To achieve this, we used the BioLector, which has the advantage of non-invasive monitoring of growth, pH and dissolved oxygen (DO) concentration in real time. The device makes use of 48-wells plates with a working volume of 800 to 2400 µL. In this study, we used a wild-type and an industrial strain of *S. peucetius*. *S. peucetius* var. *caesius* ATCC 27952 (further referred to as WT) produces the anticancer compounds daunorubicin and doxorubicin[8,266]. The industrial strain *S. peucetius* G001 produces more than 100-fold more daunorubicin in production media[268]. Strictly controlled inoculation procedures are key to generate reliable growth data from complex mycelial cultures. We applied a relatively simple inoculation method that involves the fragmentation of mycelium into small fragments for direct inoculation of microtiter plates (Figure 1A).

WT and G001 were grown on SFM agar plates, and subsequently transferred to shake flasks containing TSB. The shake flasks were equipped with metal coils to facilitate dispersion of the mycelium. The submerged cultures were homogenised using an IKA tube drive. Small mycelial fragments were collected using a sterile syringe filled with cotton wool, and stocked in glycerol. The colony forming unit (CFU) concentration of the fragmented mycelial stocks was determined (see Materials and Methods for details). The optimal homogenising parameters were determined based on the morphology of the strains. For 48 h TSB cultures of both strains, homogenising for 2 min at 6000 rpm was sufficient to obtain small mycelial fragments at a high CFU concentration. Using this method, 20 mL of TSB culture would typically generate 5 mL glycerol stock of approximately $1{\cdot}10^{8}$ CFU·mL$^{-1}$.

## Microbioreactor cultivation of *S. peucetius*

MBR cultivation was performed in a 48-well FlowerPlate with 1 mL working volume in a BioLector device with non-invasive measurement of growth, pH and DO concentration (Figure 1B). The flower-shaped wells provide a baffle-like structure, promoting dispersed mycelial growth. Modified R5 medium (R5*) was used as the cultivation medium, consisting of 50% diluted liquid R5 medium supplemented with TES buffer of pH 7.2 to a final concentration of 20 mM. Fragmented mycelial stocks of WT and G001 were inoculated into 1 mL R5* media, achieving

a concentration of $1{\cdot}10^6$ CFU·mL$^{-1}$ with ten replicates. The cultures were agitated at 1200 rpm and online measurements were recorded at a 15 min interval. After 68 h, five replicate cultures were sacrificed for proteomics and metabolomics analysis ($t_1$, $n$=5). The remaining five replicates cultures were sampled after 115 h ($t_2$, $n$=5).

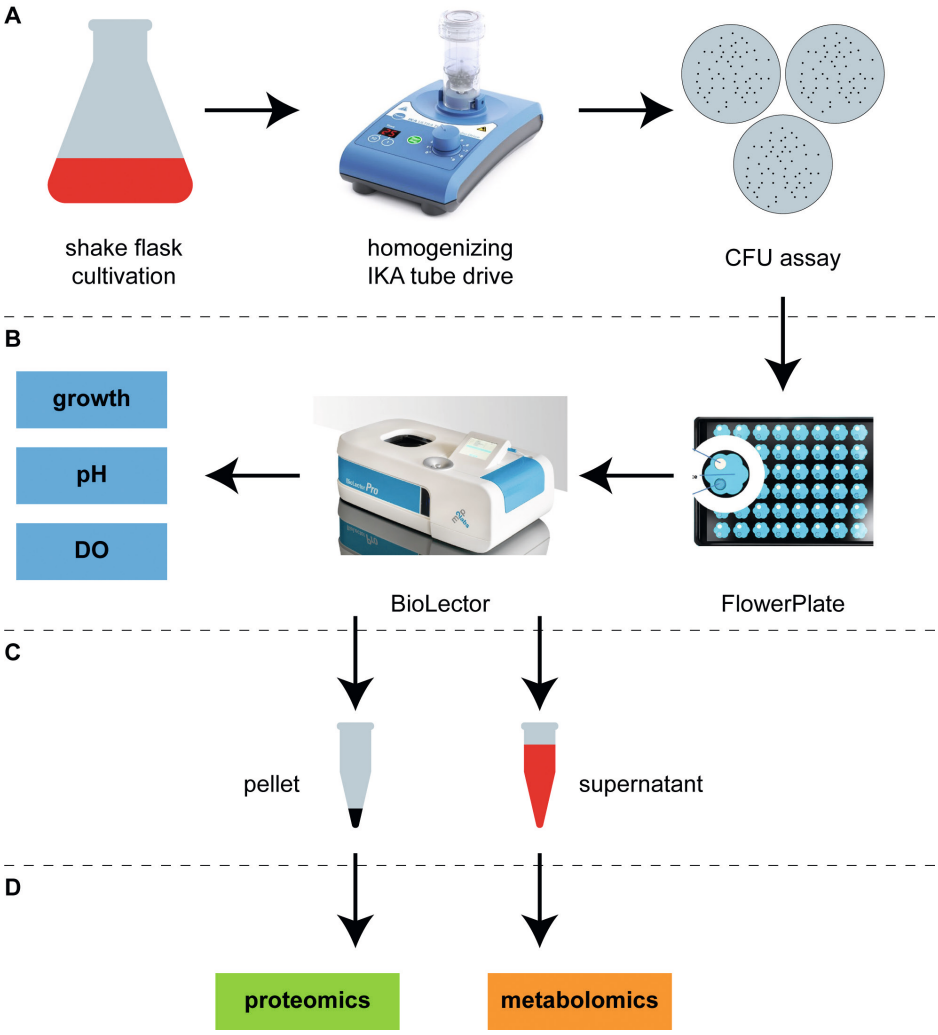


**Figure 1. Workflow for integrated proteomics and metabolomics analysis of microbioreactor cultures.** **(A)** To prepare the inoculi, precultures were homogenised and the CFU of the mycelial fragments were determined. **(B)** FlowerPlates were filled with 1 mL media and inoculated with fragmented mycelial stock. The plates were incubated in a BioLector with online measurement of growth, pH and dissolved oxygen (DO) concentration. **(C)** Sample preparation involved separation of pellet and supernatant via centrifugation. **(D)** The samples were processed for quantitative proteomics and metabolomics analysis, resulting in a paired dataset for each culture. Images: https://www.ika.com, https://www.m2p-labs.com/.

The growth profiles of the replicate cultures overlapped consistently during both the lag (about 0–15 h) and the growth phase (about 15–30 h) for both strains (Figure 2A). However, the values were more different following the onset of the stationary phase at about 30 h (Figure 2A).

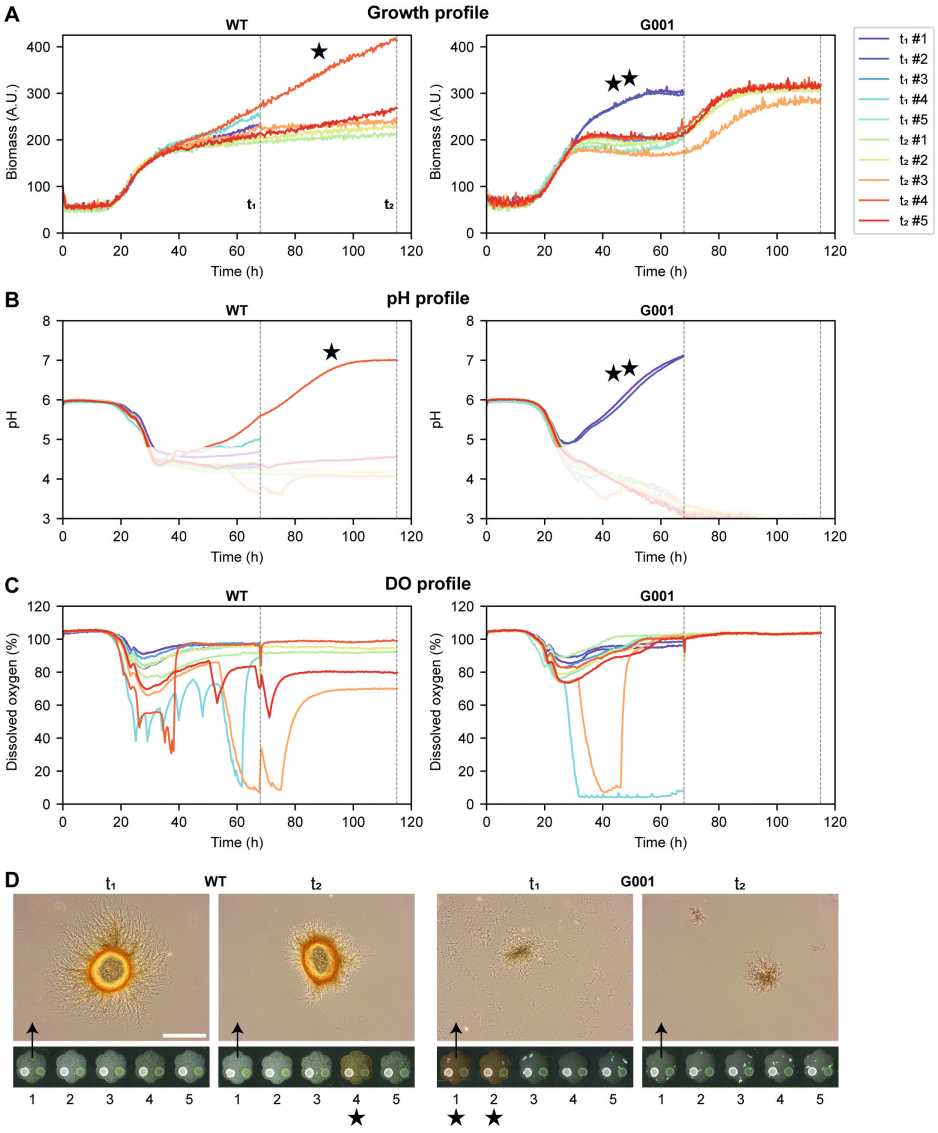


**Figure 2. Online monitoring of growth, pH and dissolved oxygen (DO) of *S. peucetius* microbioreactor (MBR) cultures.** *S. peucetius* WT and G001 were cultivated in 1 mL modified R5 medium (R5*) in an MBR for 68 h ($t_1$, $n$=5) or 115 h ($t_2$, $n$=5). **(A)** Growth profile based on scattered light measurements. **(B)** pH based on optical sensor data. The accurate measurement range lies between pH 4.8–7.2. **(C)** DO based on optical sensor data. **(D)** Morphology of WT and G001 at $t_1$ and $t_2$ (culture 1 for both strains). Outliers in growth, pH and phenotype are indicated by a star and were discarded for proteomics and metabolomics analyses. Scale bar: 200 µm.

The average biomass-specific growth rate during mid-growth phase (20–25 h) was 0.098±0.006 $h^{-1}$ for WT and 0.082±0.008 $h^{-1}$ for G001. The pH profiles overlapped consistently until the values dropped below the measurement range after about 30 h (Figure 2B). The DO concentration indicated that the stationary phase occurred at about 30 h when the DO concentration increased back to 100% saturation (Figure 2C). In six out of twenty wells, the DO data demonstrated irregular patterns, suggesting erroneous measurements in those particular wells. In terms of morphology, while WT formed mycelial pellets, G001 had a more fragmented morphology with a smaller pellet size (Figure 2D). WT sample 4 at $t_2$ and G001 samples 1 and 2 at $t_1$ exhibited increased red pigment production compared to the other cultures (Figure 2D, indicated with a star). The growth and pH data of these cultures also differed from the remaining replicates. The pH of the cultures turned basic during the stationary phase, and the growth measurements had higher final values (Figure 2A and 2B, indicated with a star). Based on the phenotype and cultivation data, we decided to omit these three cultures from the proteomics and metabolomics analyses.

In parallel to the MBR cultivations, the fragmented mycelial stocks of WT and G001 were inoculated in triplicate to the same CFU concentration in 10 mL R5* media in shake flasks. After 68 h ($t_1$) and 115 h ($t_2$), 950 µL of culture was collected from both the MBR plates ($n$=5) and the shake flasks ($n$=3). For MBR cultures, a single well was sacrificed for each sample, whereas each shake flask was sampled at both timepoints. The samples were centrifuged (Figure 1C), the pellets were used for proteomics analysis, and the supernatants for metabolomics analysis (Figure 1D).



## Quantitative proteomics analysis of *S. peucetius* microbioreactor cultures

The biomass samples of *S. peucetius* WT and G001 MBR and shake flask cultures were processed for quantitative proteomics. As describe above, we discarded three MBR samples because of their highly divergent phenotypes as compared to all other samples, resulting in five samples of WT at $t_1$ ($n$=5), four samples of WT at $t_2$ ($n$=4), and five samples of G001 at $t_2$ ($n$=5), and three samples of G001 at $t_1$ ($n$=3). Additionally, three shake flasks cultures of each strain were sampled at both timepoints ($n$=3). The proteins were extracted and analysed using liquid chromatography-mass spectrometry (LC-MS) analysis (see Materials and Methods for details). The data were normalised and filtered using MaxQuant software (v2.2.0.0)[289].

In total, 3135 proteins were detected across all samples, and at least 2432 proteins were detected in each individual sample. The *S. peucetius* genome contains 6592 putative coding sequences, so we could detect 48% of the proteins in at least one sample. Venn diagrams were created using the web-based tool InteractiVenn[365]. Most proteins (89%) were detected in both strains under all experimental conditions (Supplementary Figure S1A). Principle component analysis (PCA) was used to visualise the similarities between the individual samples. The samples separated by strain and cultivation method in the PCA score plot, indicating that the two cultivation methods resulted in strong differences in the proteomes of the two strains (Figure 3A). Furthermore, the two strains showed strongly different proteomes, which may be

expected when comparing a wild-type and industrial strain. The MBR samples of WT showed more variability between the individual samples than the G001 samples. Proteins with a minimum two-fold difference in abundance and a *p*-value ‹0.05 in a two-sample *t*-test were considered differentially expressed.

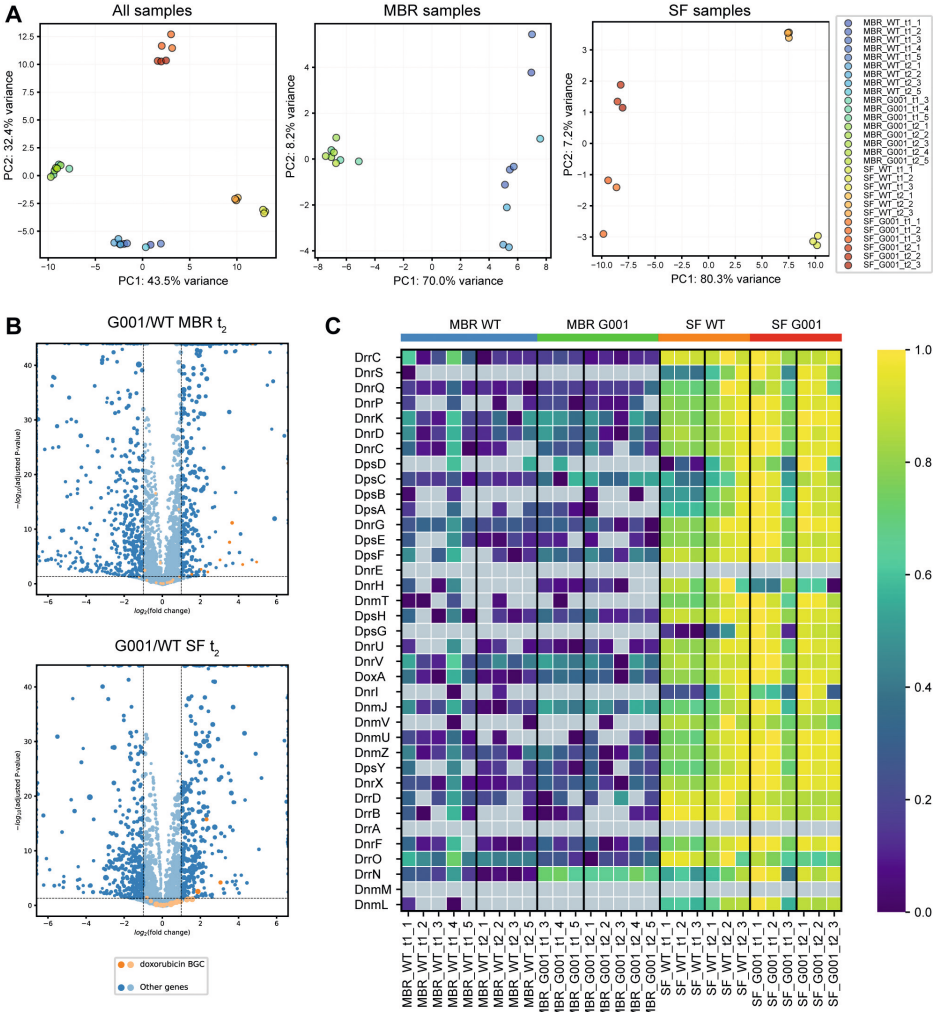


**Figure 3. Proteomics analysis of *S. peucetius* grown in a microbioreactor (MBR) and in shake flasks (SF).** **(A)** PCA score plots of proteomics data of G001 and WT grown in R5* in a MBR or in shake flasks for 68 h ($t_1$) or 115 h ($t_2$). Left panel: all samples, middle panel: only MBR samples, right panel: only shake flask samples. **(B)** Volcano plots of proteomics data of MBR (upper panel) and shake flask (lower panel) cultivation of G001 and WT at $t_2$. Proteins with an FDR-adjusted *p*-value ›0.05 and fold change ‹2 are represented in a lighter shade. Positive $\log_2$ fold change values represent overexpression in G001. Proteins derived from the doxorubicin BGC are indicated in orange. **(C)** Heatmap showing the relative abundance of the proteins encoded by the doxorubicin BGC in each sample. Grey tiles indicate that the protein was not detected.

Volcano plots were generated to compare the protein expression in the two strains at the second timepoint in each cultivation method. Differentially expressed proteins are visualised in darker shade, and proteins that belong to the doxorubicin BGC were indicated in orange. For both MBR and shake flask cultures, several proteins encoded by the doxorubicin BGC were upregulated in G001 as compared to WT (Figure 3B). A heatmap was generated of the relative abundance that shows the detected proteins that belong to the doxorubicin BGC. This revealed that the expression of the doxorubicin BGC was relatively high in the shake flask cultures as compared to the MBR cultures (Figure 3C). In the MBR cultures, not all proteins encoded by the doxorubicin BGC could be detected.

Taken together, we could detect about half of the proteins encoded by the genome of *S. peucetius* in both shake flask and MBR cultures using this integrated workflow. PCA analysis indicated a strong correlation among individual samples from the same strain, and distinct separation between samples from the different strains.

## Metabolomics analysis of *S. peucetius* microbioreactor cultures

The supernatants of *S. peucetius* WT and G001 cultures grown in R5* in either MBR or shake flasks were processed for metabolomics analysis (see Materials and Methods for details). As describe above, we discarded three MBR samples with differing phenotype. Metabolites were extracted using LC-18 SPE tubes, eluted using methanol, and analysed using LC-MS. The LC-MS data was processed using MZmine 3[366], resulting in a list containing all the mass features and their peak areas detected in each crude extract.



Venn diagrams were created using the web-based tool InteractiVenn[365]. Of all detected metabolites, 20% were detected only in MBR extracts, and 28% only in shake flask extracts (Supplementary Figure S1B). Furthermore, 22% of the metabolites were only detected in WT extracts and 8.6% only in G001 extracts (Supplementary Figure S1B). PCA analysis was performed using MetaboAnalyst 6.0 with log transformation and pareto scaling[367]. The samples separated by strain and cultivation method, but the timepoints were not clearly separated in PC1 and PC2 (Figure 4A). To study the abundance of the individual metabolites, a heatmap with hierarchical clustering was generated using MetaboAnalyst 6.0[367]. The anthracyclines doxorubicin, daunorubicin and 13-dihydrodaunorubicin were annotated using a previously described method[293]. The anthracyclines were highly abundant in the extracts of G001 grown in shake flasks (Figure 4B). In contrast, the anthracyclines could not be detected in the extracts of MBR-grown cultures. This is in accordance with the protein levels that relate to the doxorubicin BGC, which were relatively low in the MBR samples. Interestingly, the heatmap indicated that different metabolite groups were detected in MBR and shake flask cultures. The processed data obtained from MZMine 3 were exported to the Feature-Based Molecular Networking (FBMN) workflow[368] on GNPS[369] to create a molecular network (see Materials and Methods for details). Interestingly, the spectral families were distinctly separated between the two cultivation methods (Figure 5). Spectral families 5, 6 and 9 were only detected in the MBR-derived extracts (in blue and green). Other spectral families were overrepresented in the shake

flask-derived extracts (in red and orange). Spectral family 8 comprised the anthracyclines 13-dihydrodaunorubicin, daunorubicin and doxorubicin. Three features in spectral family 1 were annotated as dehydrorabelomycin, a non-glycosylated angucycline polyketide, by comparison of MS/MS data against GNPS spectral libraries[369,370]. No compounds could be dereplicated in the spectral families that were overrepresented in the MBR cultures.

Taken together, we detected differences in the chemical space between MBR and shake flask cultures. The MBR cultures provided consistent and reproducible metabolome profiles. High expression of doxorubicin biosynthetic proteins corresponded to high abundance of anthracyclines in the crude extracts.

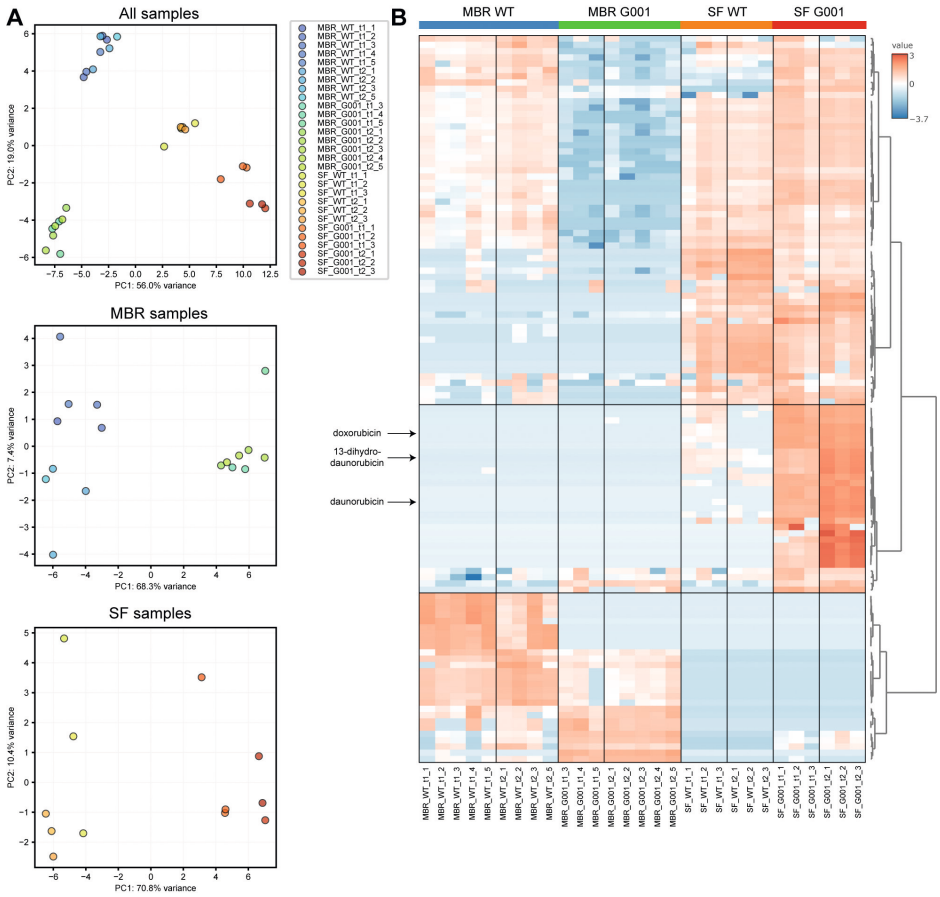


**Figure 4. Metabolomics analysis of *S. peucetius* grown in a microbioreactor (MBR) and in shake flasks (SF).** **(A)** PCA score plots of metabolomics data of G001 and WT grown in R5* in a MBR or in shake flasks for 68 h ($t_1$) or 115 h ($t_2$). **(C)** Heatmap with hierarchical clustering showing the relative abundance of the mass features detected in each sample. Mass features with relatively high peak areas are presented in red. The anthracyclines doxorubicin, daunorubicin and 13-dihydrodaunorubicin are indicated.

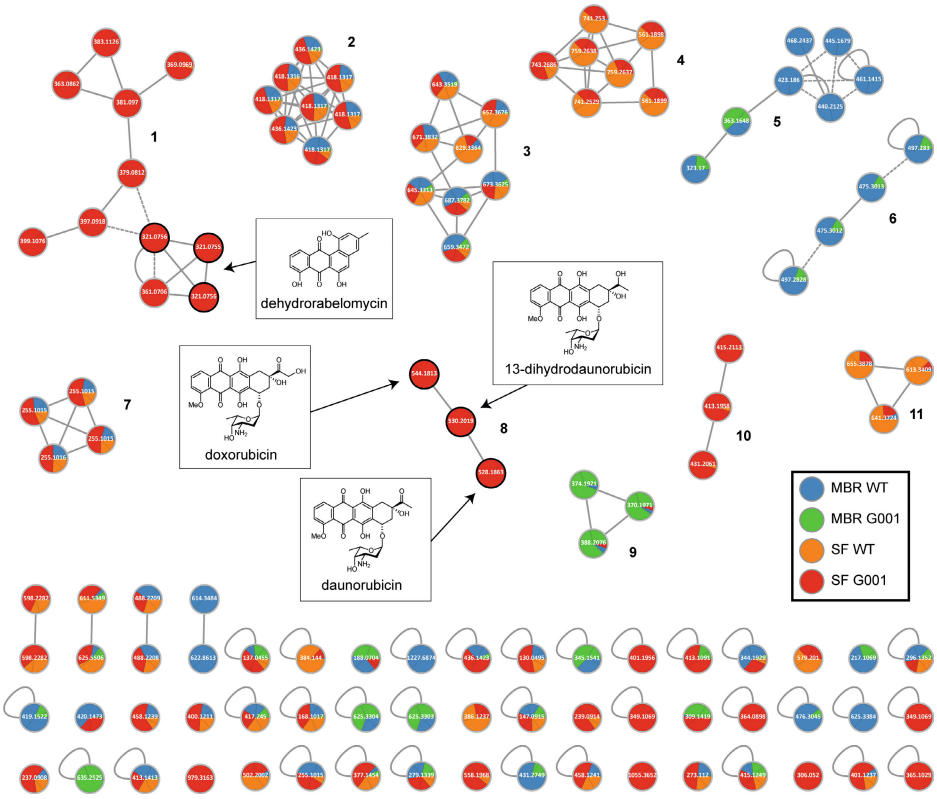


**Figure 5. Molecular network of the ions detected in the crude extracts of *S. peucetius* grown in a microbioreactor (MBR) and in shake flasks (SF).** A pie chart was mapped to the nodes which represents the relative intensities of the ion in the MBR extracts of WT (blue) and G001 (green), and in the shake flask extracts of WT (orange) and G001 (red). The nodes are labelled by the precursor mass of their ions. The dashed edges connect different ions of the same molecule and solid edges connect nodes based on their MS/MS similarity. Nodes highlighted with a black stroke represent annotated compounds: 13-dihydrodaunorubicin, daunorubicin, doxorubicin and dehydrorabelomycin.

# Discussion

Streptomycetes harbour a wealth of BGCs with yet unidentified natural products[2,371]. As a promising strategy to accelerate the discovery of these potentially bioactive metabolites, we developed a high-throughput multi-omics workflow for characterisation of *Streptomyces* strain collections at diverse experimental conditions. To achieve this, a workflow was developed for integrated quantitative proteomics and metabolomics analysis of small-scale *Streptomyces* cultures. As test strains for the system, *S. peucetius* WT and its industrial variant G001 were selected and grown in 1 mL volume, followed by proteomics and metabolomics analyses. The results of these paired omics experiments indicated that reproducible data with high sensitivity can be obtained from *S. peucetius* grown in small culture volumes.

Optimisation of the cultivation method is required to reduce biological variability. MBR cultures of *S. peucetius* WT and G001 showed high biological variability, with two of the ten G001 cultures and one of the ten WT cultures showing a significantly different phenotype with increased red pigmentation. These samples had to be removed from further analysis via metabolomics and proteomics. Optimisation of the inoculum procedure may reduce the biological variability between the replicate cultures. For instance, the reproducibility of MBR cultures of *S. lividans* was optimised by applying two pre-cultures in shake flasks and subsequently inoculating a MBR based on optical density[361]. When disregarding the three cultures with increased red pigmentation, the remaining replicates were of good quality in terms of growth and pH data. However, the DO concentration measurements did not consistently align across all replicate samples. The measurement of DO concentration relies on fluorescence measurement conducted via an optical sensor positioned inside the cultivation chamber. Consequently, the production of fluorescent metabolites, such as anthracyclines, could potentially interfere with the DO measurements[372]. Alternatively, the irregularity of the DO curves may also have resulted from a technical error with the sensor. The consistent growth and pH profiles observed between the replicate cultures confirm that this cultivation method is suitable for process control applications with *Streptomyces*. Integrating the BioLector device with the corresponding liquid robot handling system (RoboLector) provides opportunities for more complex cultivation strategies[373]. For instance, elicitors may be added to the cultures at a specific timepoints or growth phase to study the effect on natural product biosynthesis, or a pH control strategy may be considered, by the addition of acid or base solutions based on online measurements.

Proteomics and metabolomics analysis of the MBR cultures yielded promising results. PCA analysis of both datasets indicated distinct separation among samples based on strain, and strong correlation among samples of the same strain. The shake flask samples demonstrated better separation between the two timepoints as compared to the MBR-derived samples. These results indicate that the metabolic state between the timepoints varies more in shake flask-grown cultures than in well-controlled MBR-grown cultures. Furthermore, the expression of the proteins encoded by the doxorubicin BGC was relatively high in shake flask cultures, which corresponded to a higher abundance of anthracyclines in the associated crude extracts. Molecular networking of the mass features identified in each extract indicated that the metabolite profiles in the shake flask and MBR cultures were very different. These results indicate that the metabolite extraction method has good sensitivity to detect differences in the chemical space, and that MBR cultivation can result in the detection of different (and hence probably also new) metabolites than what is produced in shake flasks. It would be of interest to expand this study to other streptomycetes, and analyse more replicate cultures for better statistical analysis. Additionally, the proteomics and metabolomics sample preparation could be automated using liquid handling robotics. Both methods are highly dependent on sample preparation procedures and quenching of biochemical reactions at the time of sampling, posing challenges for automation. To study the extracellular metabolome, we used solid-phase extraction with methanol, which is a fast method but reduced sensitivity[374]. Recent developments the proteomics field have provided sensitive label-free quantitative mass-

spectrometry methods that we applied in this workflow[52]. In the future, new developments in mass spectrometry-based analytical procedures may provide more sensitive multi-omics data from small sample sizes.

In conclusion, we developed a workflow for integrated quantitative proteomics and metabolomics analysis of small-scale *Streptomyces* cultures. The workflow provided good quality data from a technical perspective, although biological reproducibility should be optimised in future work.

# Materials and Methods

## Bacterial strains and culture media

Two *Streptomyces peucetius* strains were used in this study. *S. peucetius* var. *caesius* ATCC 27952[8] is a wild-type strain that produces daunorubicin and doxorubicin (designated WT). *S. peucetius* G001[268] is derived from ATCC 27952 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis and has increased production of daunorubicin. All media and routine *Streptomyces* techniques have been described previously[1]. Soy flour mannitol (SFM) agar plates were used for phenotypical characterisation. Luria-Bertani (LB) agar plates were used for viability assays. Tryptone soy broth (TSB) was used for preparation of mycelial stocks. Modified R5 medium (R5*) was used for shake flask and microbioreactor (MBR) cultivation. R5 liquid medium was prepared following the agar recipe[1] without the addition of agar. R5* was prepared by diluting the R5 liquid medium to 50% in a TES buffer of pH 7.2 to a final concentration of 20 mM TES.



## Preparation of mycelial stocks

Strains were streaked on SFM agar plates and incubated at 30 °C for 7 days. Five single colonies were inoculated in 20 mL of TSB medium in 100 mL Erlenmeyer flasks equipped with metal coils. The cultures were incubated in an orbital shaker at 200 rpm (1 inch orbit) at 30 °C for 48 h. Cultures were centrifuged (10 min, 3000 rpm), resuspended with 15 mL of 10.3% (w/v) sucrose and centrifuged again (10 min, 3000 rpm). For preparation of mycelial stocks, the washed biomass was resuspended in 2–5 mL of 20% (w/v) glycerol and stored in aliquots of 1 mL at -80 °C. For preparation of fragmented mycelial stocks, the washed biomass was resuspended in 15 mL of 10.3% (w/v) sucrose. The mycelial suspensions were homogenised using a ULTRA-TURRAX Tube Drive (IKA, Germany) in a 15 mL dispersing tube (DT-20 eco, IKA, Germany). The mycelium suspensions were homogenised at 6000 rpm for 1 min, cooled down on ice for 1 min, and homogenised again at 6000 rpm for 1 min. The mycelium suspensions were collected from the dispersing tube using two volumes of 10.3% (w/v) sucrose. The large mycelial fragments were filtered out using a sterile syringe filled with cotton wool. The mycelium suspensions were centrifuged (10 min, 3000 rpm) and resuspended in 2–5 mL 20% glycerol. Fragmented mycelial stocks were stored in aliquots of 1 mL at -80 °C. The concentration of viable mycelial fragments was determined using a colony forming units (CFU) assay. A dilution series was prepared in 10.3% (w/v) sucrose and plated on LB agar plates. CFUs were counted after 2 days incubation at 30 °C.

## Shake flask cultivation

Shake flask cultivations were performed in 50 mL Erlenmeyer shake flasks without metal coils. Fragmented mycelial stocks were centrifuged (2 min, 6000 rpm), and resuspended in R5* medium to $1{\cdot}10^8$ CFU·mL$^{-1}$. Subsequently, 9.9 mL of R5* medium was inoculated with 1 mL of mycelium suspension to a final concentration of $1{\cdot}10^6$ CFU·mL$^{-1}$. Shake flasks were incubated in an orbital shaker at 200 rpm (1 inch orbit) at 30 °C for 5 days. All experiments were performed in triplicate.

## Microbioreactor cultivation

MBR cultivation was performed in an automated cultivation device (BioLector II, Beckman Coulter). Strains were cultivated in 1 mL working volume in a 48-well FlowerPlate (MTP-48-BOH2, Beckman Coulter, USA) covered with an adhesive gas-permeable membrane for reduced evaporation (F-GPR48-10, Beckman Coulter). Fragmented mycelial stocks were centrifuged (2 min, 6000 rpm), and resuspended in R5* medium to $1{\cdot}10^8$ CFU·mL$^{-1}$. Subsequently, 990 µL of R5* medium was inoculated with 10 µL of mycelium suspension to a final concentration of $1{\cdot}10^6$ CFU·mL$^{-1}$. The shaking frequency was set to 1200 rpm (3 mm orbit), temperature was controlled at 30 °C, and relative humidity was controlled at 85%. Online measurements were recorded at a 15 min interval. Growth was measured using the scattered light filter module at gain 6 (E-OP-401). The pH and dissolved oxygen (DO) concentration were measured using the corresponding optical sensors (optodes) integrated in the microtiter plates. The data was obtained using the LG1 (E-OP-421) and RF (E-OP-428) filter modules, for pH and DO respectively. All experiments were performed with five replicates.

## Sample collection

After 68 h and 115 h incubation, 950 µL samples were obtained from shake flask ($n$=3) or MBR cultures ($n$=5). For MBR cultures, a single well was sacrificed for each sample, whereas each shake flask was sampled at both timepoints. Samples were cooled down on ice and centrifuged (10 min, 13,000 rpm, 4 °C). The supernatant was transferred to a fresh tube. Biomass and supernatant samples were snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

## Proteomics sample preparation and LC-MS/MS analysis

Frozen biomass samples were washed using washing buffer [100 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$]. Subsequently, the biomass was lysed using a Bioruptor Plus (Diagenode SA) and proteins were extracted using lysis buffer [4% SDS, 100 mM Tris-HCl (pH 7.6), 50 mM EDTA]. Sample preparation for LC-MS/MS measurement was performed as described previously[286]. Briefly, total protein was precipitated using the chloroform-methanol method[287] and dissolved in 0.1% RapiGest surfactant (Waters Crop.) at 95 °C. The protein concentration was determined using the BCA method. Protein samples were reduced by adding 5 mM DTT and incubated in the dark at 60 °C for 30 min, followed by thiol group protection using 21.6 mM iodoacetamide and incubation in the dark at room temperature for 30 min. Subsequently, 0.1 µg of trypsin (recombinant, proteomics grade, Roche) per 10 µg of protein was added, and samples were digested overnight at 37 °C. After digestion, trifluoroacetic acid was added to a concentration of 0.5%. The samples were incubated at 37 °C for 30 min, followed by centrifugation to degrade

and remove the RapiGest surfactant. 4 µg of peptides was cleaned and desalted using the StageTips method[288]. Briefly, 4 µg of peptides was loaded onto a conditioned StageTip (premade SPE tips, cat No. C18.T1.10.960, Affinisep), washed twice using a 0.5% formic acid solution, and eluted with elution solution (80% acetonitrile and 0.5% formic acid). Acetonitrile was evaporated using a SpeedVac. The final peptide concentration was adjusted to 40 ng·µL$^{-1}$ using sample solution (3% acetonitrile and 0.5% formic acid) for analysis.

Quantitative proteomics was performed as described previously[286]. Briefly, the desalted peptide solution was separated using an UltiMate 3000 RSLCnano system (Thermo Scientific) set in a trap-elute configuration, coupled with a QExactive HF mass spectrometer (Thermo Scientific). The liquid chromatography system used a Waters nanoEase M/Z Symmetry $C_{18}$ trap column (5 µm, 100 Å, 180 µm × 20 mm) for peptide loading and retention, and a Waters nanoEase M/Z HSS T3 $C_{18}$ analytical column (1.8 µm, 100 Å, 75 µm × 250 mm) for peptide separation. The mass spectrometer was operated in positive mode with data-dependent acquisition.

## Proteomics data analysis

Raw LC-MS/MS files were analysed using MaxQuant software v2.2.0.0[289] using the label-free quantification (LFQ) method. Only proteins that were detected in at least 30% of the replicates of one treatment group were included in the analysis. LFQ data was imported to DESeq2 v1.38[303] and differential expression analysis was performed with the function logarithmic fold change shrinkage[375]. PCA plots were generated from the variance stabilising transformed data in Python (v3.10) with matplotlib (v3.7.1). Volcano plots were generated from the differential expression analysis data. LFQ data was log-transformed and normalised by protein to the value of 1 using scikit-learn (v1.3.0), and heatmaps were generated using seaborn (v0.12.2).

## Metabolomics sample preparation and LC-MS/MS analysis

For metabolite extraction, supernatant was loaded onto LC-18 SPE tubes (Supelco, 100 mg), previously conditioned with 1 mL of methanol and 1 mL of water. The resin was then washed with 1 mL of water to remove salts and polar media components, and the metabolites were eluted using 1 mL of methanol.

LC-MS/MS acquisition was performed using Shimadzu Nexera X2 UHPLC system, with attached photodiode array detector (PDA), coupled to Shimadzu 9030 QTOF mass spectrometer (MS), equipped with a standard electrospray ionisation (ESI) source unit, in which a calibrant delivery system (CDS) was installed. A total of 2 µL were injected into a Waters Acquity HSS $C_{18}$ column (1.8 µm, 100 Å, 2.1 × 100 mm). The column was maintained at 30 °C, and run at a flow rate of 0.5 mL·min$^{-1}$, using 0.1% formic acid in $H_2O$ as solvent A, and 0.1% formic acid in acetonitrile as solvent B. A gradient was employed for chromatographic separation starting at 15% B for 1 min, then 15–60% B for 9 min, 60–100% B for 1 min, and finally held at 100% B for 3 min. The column was re-equilibrated to 5% B for 3 min before the next run was started. The PDA acquisition was performed in the range 200–600 nm, at 4.2 Hz, with 1.2 nm slit width. The flow cell was maintained at 40 °C.

All the samples were analysed in positive polarity, using data dependent acquisition mode. In this regard, full scan MS spectra (*m/z* 100–2000, scan rate 20 Hz, ID disabled) were followed by three data dependent MS/MS spectra (*m/z* 100–2000, scan rate 20 Hz, ID disabled) for the three most intense ions per scan. The ions were selected when they reach an intensity threshold of 1500, isolated at the tuning file Q1 resolution, fragmented using collision induced dissociation at fixed collision energy of 20 eV, and excluded for 0.01 s before being re-selected for fragmentation. The parameters used for the ESI source were: interface voltage 4 kV, interface temperature 300 °C, nebulising gas flow 3 $L \cdot min^{-1}$, and drying gas flow 10 $L \cdot min^{-1}$. Samples were randomised before injection and pooled QC were injected.

## Metabolomics data analysis

LC-MS raw data files were converted to mzXML centroid files using Shimadzu LabSolutions Postrun Analysis. The files were then imported into MZmine 3 (v3.9.0) for data processing[366]. For all the modules unless stated differently, *m/z* tolerance was set to 0.002 *m/z* or 10.0 ppm, RT tolerance was set to 0.05 min and minimum scans were set to 6. For mass detection in positive polarity and when using the algorithm centroid the noise was set to 1000 for MS1 and 10 for MS2, the option of detecting isotopes signals below noise level was selected. For the module ADAP chromatogram builder to minimum intensity for consecutive scans was set to 3000 and the minimum absolute height to 10000. For peak deconvolution the local minimum resolver algorithm was used with a chromatographic threshold of 90%, a minimum search range RT of 0.05, a minimum absolute hight of 1000, and a minimum ratio peak top/edge of 1.80. The 13C isotope filter module was used with RT tolerance of 0.03 min, and maximum charge of 3. The isotopic peaks finder module was set using the chemical elements C, H, N, O, and S, and a maximum charge isotope *m/z* of 3. To align the peak lists, the weight of *m/z* was set at 4 and the weight of RT was set at 1. The aligned feature list was filtered using the duplicate filter module in the filter mode new average, and the feature list row filter features present in at least 2 samples. The peak finder module was used, the intensity tolerance was set at 10% and the RT tolerance at 0.1 min. In order to build the ion identity network, the correlation group module was used with a minimum feature height of 10000 and an intensity threshold for correlation of 1000. For the ion identity network module, the ion identity library parameters were a maximum charge of 3, maximum molecules per cluster of 3, and adducts $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, modifications $[M-H_2O]^+$, $[M-2H_2O]^+$, and $[M-3H_2O]^+$. The resulted feature list was exported to be used for GNPS feature based molecular networking analysis. In addition, the quantitative table was filtered to remove features from the media or with low technical repeatability by removing features which average intensity in the media samples was more than 30% of the average in the samples, or the relative standard deviation (RSD) in the pooled QC was higher than 30%. PCA analysis was performed using MetaboAnalyst 6.0[367]. Log transformation with pareto scaling was applied to the data. PCA plots were generated in Python (v3.10) with matplotlib (v3.7.1). Venn diagrams were created using the web-based tool InteractiVenn[365].

The processed data obtained from MZMine 3 were exported to the Feature-Based Molecular Networking (FBMN) workflow[368] on GNPS[369] to create a molecular network. In addition, the MS/

MS data was filtered and all fragment ions within +/- 17 Da of the precursor *m/z* were removed. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50 Da window throughout the spectrum. The tolerance for precursor ion mass and MS/MS fragment ion were set to 0.01 Da. To generate the network, only edges with cosine values above 0.7 and more than 6 matched fragment peaks were taken into account. Moreover, to keep the edges between two nodes they must be in each other's top 10 most similar nodes. Lastly, the maximum size of a molecular family was set to 100. The MS/MS spectra were compared against GNPS spectral libraries[369,370]. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. Additional edges obtained from the ion identity network analysis in MZmine3 were also included in the network[376]. For visualisation of the molecular network Cytoscape (v3.10.1) was used[377]. Edges were coloured according to the edge type, cosine edges were coloured grey and MS1 IIMN edges were coloured red. A heatmap was generated from the doxorubicin related nodes using MetaboAnalyst 6.0[367] using with settings.

# Supplemental Information

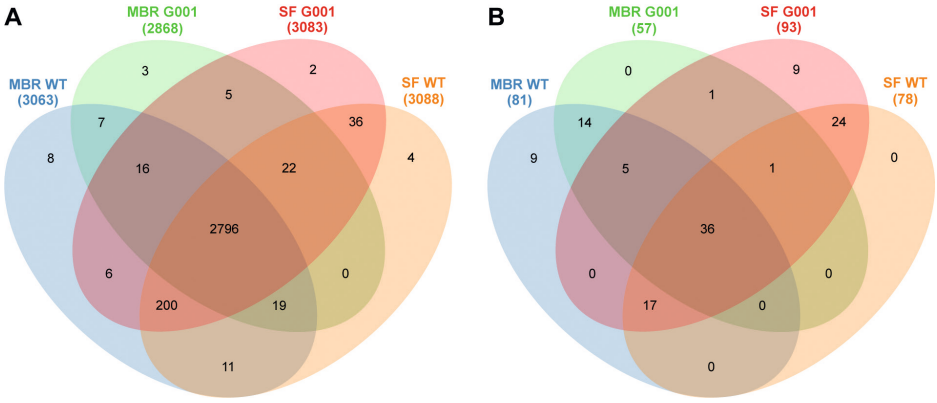


**Figure S1. Venn diagrams of *S. peucetius* proteins and metabolites detected in microbioreactor (MBR) and in shake flask (SF) cultures. (A)** Total number of proteins detected across all samples of WT from MBR (blue), G001 from MBR (green), G001 from shake flask (red) and WT from shake flask (orange). Most proteins (89%) were detected in both strains under all experimental conditions. **(B)** Total number of mass features detected across all samples of WT from MBR (blue), G001 from MBR (green), G001 from shake flask (red) and WT from shake flask (orange). Of the 116 detected mass features, 20% were detected only in MBR cultures, and 28% only in shake flask cultures.