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Enhanced production of daunomycin in *Streptomyces coeruleorubidus*

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Declaration

I declare that I am the author of the thesis entitled "Enhanced production of daunomycin in *Streptomyces coeruleorubidus*" and that I used only sources and literature displayed in the list of references in its preparation.

In České Budějovice on ....

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Abstract

Daunorubicin (DNR) is an anthracycline antibiotic originating from soil-dwelling actinobacteria extensively used to treat malignant tumors. Over the decades, extensive attempts were made to enhance the production of anthracyclines by introducing genetic modifications and mutations in combination with media optimisation, but the target production levels remain comparatively low. Developing an appropriate culture medium to maximise the yield of DNR and preventing auto-toxicity for the producing organism remains a challenge. Our prospective review sheds light on a method involving perturbation that enhances the precursors to regulate PKS II biosynthesis, enhancing cells' capacity to increase secondary metabolite production. The suggested method also entails the preparation of culture media for the cultivation of *Streptomyces* sp. and enhanced yield of DNR and making it inactive with iron or its reduced forms following efflux from the producer. The iron or iron-DNR complex is encapsulated by oleic acid or lipid micelle layers in the culture media, finally resulting in the generated inactive DNR and the DNR-iron-oil complex. This idea has the potential to protect the producer organism from autotoxicity and prevent the inhibition of metabolite production. By developing a specialized cultivation medium that integrates olive pomace oil and iron, this research successfully induces an autonomous resistance mechanism through biogenic nanoparticle formation (ADBN). The approach of substituting sugar with oil in culture media has a dual role where it promotes the Streptomyces growth by utilizing lipids as an energy source and encapsulating the generated DNR-iron complex in the medium. The amphiphilic properties of olive pomace oil not only serve as a carbon source but also facilitate the stabilization of nanoparticles, thereby enhancing the efficacy of the synthesis process due to its rich phenolic content, which promotes crucial redox reactions. The optimization of the medium composition through empirical methods resulted in a marked increase in daunomycin production, achieving yields between 5.5 and 6.0 g/L, which demonstrates a significant advancement relative to prior methodologies. This research not only contributes to the field of microbial fermentation and antibiotic production but also emphasizes the importance of minimizing environmental impacts through the production of insoluble daunomycin precipitates that can be efficiently recovered from the cultivation medium. Overall, these findings present promising avenues for further investigation into the mechanisms underlying biogenic nanoparticle formation and the

optimization of cultivation processes. Such explorations may not only refine microbial production systems for daunomycin but also broaden the potential application of similar strategies for the synthesis of other therapeutically important compounds.

We anticipate that our work help researchers working with secondary metabolites production and decipher a methodology that would enhance DNR yield and facilitate the extraction of the resulting DNR by lowering costs in large-scale fermentation.

Keywords: *Streptomyces, daunomycin, autotoxicity, enhanced production, iron-lipid interaction*

Table of Contents

1. Introduction

Daunomycin, an anthracycline antibiotic originally isolated from the bacterium *Streptomyces peucetius*, plays a pivotal role in treating various cancers, particularly leukemia. Its therapeutic efficacy is primarily attributed to its ability to intercalate into DNA, disrupting essential replication and transcription processes in rapidly dividing cells. Additionally, daunomycin serves as a precursor for synthesising more advanced anthracyclines (Thomas 1990). However, its clinical utility is often compromised by inherent cytotoxic effects (Thirumaran et al. 2007), including toxicity to the microbial strains used for its production, posing significant challenges for industrial-scale synthesis (Srinivasan et al. 2010; Vasanthakumar et al. 2013).

A critical aspect governing daunomycin's biological activity is its interaction with iron, particularly through the Fenton reaction. This process illustrates how iron can catalyze the production of reactive oxygen species (ROS) from hydrogen peroxide, leading to oxidative stress and cellular damage (Taatjes et al. 1997). While iron is vital for numerous physiological functions, excessive concentrations can exacerbate oxidative effects. The therapeutic use of iron chelators to mitigate daunomycin toxicity (Kaiserová et al. 2007) underscores the need for a deeper understanding of the interplay between these two elements, as it significantly influences the compound's pharmacological properties and therapeutic efficacy in fermentation contexts.

While most research has traditionally focused on the toxicological effects of daunomycin in eukaryotic models, its implications for prokaryotic organisms remain only partially explored nowadays (Cai et al. 2023). Investigations have largely centered on the biosynthesis of daunomycin and the molecular mechanisms of resistance, particularly involving efflux pumps (Malla et al. 2010; Yuan et al. 2011).

This study focuses on the development of a cultivation medium specifically designed to induce auto-resistance through Autonomous Defense Through Biogenic Nanoparticle Formation (ADBN). Utilizing a fermentation medium enriched with olive pomace oil and iron, this research aims to explore the inherent affinity of daunomycin for both iron and oil. Our strategy leverages the combination of olive pomace oil and bacteria to facilitate the formation of iron nanoparticles (NPs) (Afonso et al. 2024).

Furthermore, it seeks to bridge the existing knowledge gap by investigating the effects of daunomycin on eukaryotic cells in conjunction with the underexplored data from prokaryotic organisms, especially those involved in the production of daunomycin. This dual approach aims to enhance our understanding of daunomycin's mechanisms of action and its potential applications in both eukaryotic and prokaryotic systems.

2. Literature Review

2.1. Anthracyclines

Anthracyclines are chemical compounds derived from soil-dwelling gram-positive actinobacteria that have been used in antibiotics and as anticancer medication agents (Dinis et al. 2023). They are a class of chemotherapeutic drugs that have been widely utilized to treat leukaemia and cancer in adults and paediatrics since their discovery in *Streptomyces peucetius* in the 1960s (Weiss 1992; Shapiro and Recht 2001; Minotti et al. 2004; McGowan et al. 2017; Murabito et al. 2023).

Cancer refers to a group of diseases characterized by the uncontrolled proliferation and spread of abnormal cells, varying in sizes and types, responsible for nearly 10 million deaths globally in 2020 (Fischer 2020; Sung et al. 2021; Kaur et al. 2022). The most prevalent types of cancer include carcinomas, lymphomas, sarcoma, leukaemia and melanoma, which are incurred by tobacco use, chemical and pollutant exposure, infectious agents, viruses, radiations, ageing and inherited genetic abnormalities (Wu et al. 2018; Kaur et al. 2022). Despite the fact that chemotherapy is incapable of effectively eliminating all types of cancer in a short duration, it remains an extremely viable treatment option, particularly for cancers complicated by metastasis (Behranvand et al. 2022).

Chemotherapy drugs cause accelerated senescence in tumour cells via inducing apoptosis, DNA alkylation and damage, autophagy, activating MAPK (mitogen-activated proteins kinases), promoting antimetabolites, inhibiting topoisomerases, and other mechanisms that result in cell death or cell cycle arrest (Bagnyukova et al. 2010; Sui et al. 2013; Demaria et al. 2017; Xiao et al. 2017; Behranvand et al. 2022). The class of Anthracyclines and their derivatives, including doxorubicin, epirubicin, daunorubicin, and idarubicin, are the most potent anticancer drugs ever discovered, having the ability to alter mitochondrial dynamics by intercalating with DNA helix and cause cytotoxicity. Anthracyclines exert their cytotoxic effects primarily through their interaction with DNA. By intercalating into DNA strands, they inhibit proper DNA replication and transcription, inducing double-strand breaks and impairing DNA repair mechanisms. Additionally, anthracyclines generate reactive oxygen species (ROS) through redox cycling, further contributing to DNA damage and apoptosis (Dinis et al. 2023).

The anthracyclines are tetracyclic aromatic polyketides that are produced by the PKS II (polyketide synthase type II) pathway and are structurally composed of an anthraquinone (aglycone) moiety and an amino sugar (carbohydrate unit) at the C_7 or C_{10} or at both positions. The absence of sugar at C_{10} is substituted by a carbo-methoxyl group or a hydroxyl group through processes like glycolisation and hydroxylation (Fujiwara et al. 1985; Hortobágyi 1997; Dinis et al. 2023). The term "Anthracyclines" was introduced to denote the colour (red to yellow-red optical dyes) of the chemical derivatives 7,8,9,10-tetrahydro-5,12-naphtacenoquinones (Brockmann and Brockmann Jr. 1963; Metsä-Ketelä et al. 2008). In the 1970s, chemists were driven to create anthracycline derivatives with reduced toxicity because even small structural modifications might significantly affect the bioactivity of the anthracyclines (Hortobágyi 1997). The interest and progress in synthesis pathway engineering (synthetic and semi-synthetic *via* site-directed mutations, gene alterations) of anthracyclines and their analogues were carried out after the 1990s due to their exciting catalysing properties (Metsä-Ketelä et al. 2008).

2.2. The Anthracyline Producers

The anthracycline compounds occurring in nature are the secondary metabolites produced by the Actinobacteria, especially in the genus *Streptomyces* (Ait et al. 2015). Streptomycetes are gram-positive bacteria, thriving in aquatic and terrestrial habitats and known for their capacity to synthesize a variety of secondary metabolites such as antibiotics, enzymes, and pigments (Fenical and Jensen 2006; Sarkar and Suthindhiran 2022). They possess a lifecycle similar to filamentous fungi, reproduce through sporulation, and exhibit siderophore activity (breakdown of macromolecules in the environment). The common metabolites produced by nearly all *Streptomyces* sp. are siderophore desferrioxamine (iron chelator), geosmin (earthy smell organic compound), streptothricin (antibiotic) and streptomycin (antibiotic) (Zhu et al. 2014; Martín-Sánchez et al. 2019). The organisms contain a multitude of biosynthetic gene clusters (BCGs) and transcription units in their genome, which are responsible for the production of secondary metabolites according to the cues in their natural habitat or environment (Zhu et al. 2014). Approximately 90% of the know antibiotics are obtained from the organism of the genus *Streptomyces*. To date, more than 500 naturally occurring anthracyclines have been isolated from *Streptomyces* sp., which are widely considered medically important (Elshahawi et al. 2015; Hulst et al. 2022).

Streptomyces ceoruleorubidus is a potentially important streptomyces bacteria that employs the synthesis of antifungal, antibacterial, immunosuppressive, and antitumor compounds such as Doxorubicin and Daunorubicin (Kandula and Terli 2013; Bundale et al. 2015; Li and Zhang 2021). This proliferative ability for synthesizing such metabolites can be altered by influencing variables such as nitrogen and carbon sources, culturing conditions such as temperature, pH, and incubation period, which can play a critical role in the economic dynamics involved in secondary metabolite production. Despite the fact that the mutagenesis method is the earliest option, it remains one of the most effective methods for producing high-yield strains in experimental models. The use of physical and chemical mutations in the *Streptomyces* species has been reported to have the enhanced production of secondary metabolites, especially anthracyclines (Oki et al.; Blumauerova et al. 1978; Zhang et al. 2018).

2.3. Daunorubicin/Daunomycin

Daunorubicin, also called Daunomycin, is an anthracycline antibiotic that was first discovered in 1964 from *Streptomyces peucetius and* is extensively employed in treating malignant tumours, ovarian cancer, breast cancer and AML (acute myeloid leukaemia) (Arcamone et al. 1964; Drevin et al. 2022; Bayles et al. 2023). Despite the demonstrated cardiotoxicity of daunomycin in Guinea pigs, rats, and humans (Ainger et al.1976; Bossa et al. 1977; von Hoff et al. 1977), the cumulatively reduced dosages administered during chemotherapy allows the mitigation of this cardiovascular risks (Swain et al. 2003; Hegazy et al. 2023). It was approved in 1974 as an anti-cancer drug worldwide for commercial use.

The daunomycin (DNR) and doxorubicin (DOX) share tetracyclic alycone and daunosamine sugar moieties. The only difference between DNR and DOX is that the side chain of DOX terminates with primary alcohol and DNR with a methyl group (Minotti et al. 2004). However, the clinical use of DOX and DNR was hampered due to their increased concerns and deaths due to cardiotoxicity and the development of resistance in tumour cells (Minotti et al. 2004; van der Zanden et al. 2021). The quest to find a better alternative with reduced toxicity has led to thousands of analogues with many substitution reactions in the anthraquinone moiety (tetracyclic structure) (Minotti et al. 2004). Out of which currently, six semi-synthetic derivatives, including DOX, idarubicin, epirubicin, pirarubicin and valurubicin, are under clinical use.

2.4. Biosynthesis of DNR in Streptomyces

The production of secondary metabolites occurs through two phases: trophophase (normal growth phase), followed by idiophase (capacity to produce metabolites), where, at times, both phases can be regulated, overlapped and changed with the alterations in media and growth conditions. The enhancement in the secondary metabolite production can be possible with the development of resistance in the cells as the produced compounds are autotoxic. But in the course of any selection event over the past decades, focusing on improved resistance did not impact or lead to enhanced production. This led to the works on biosynthetic gene cluster alterations and expression enhancement of activator genes, transcription factors and increased mutations in promotor genes (Ohnuki et al. 1985; Minotti et al. 2004).

2.4.1. Biosynthetic Gene Clusters (BGCs)

The importance and biosynthesis of Daunorubicin and its gene clusters have been characterized by two BGCs in different strains (Grimm et al. 1994; Dickens et al. 1995). The majority of BGCs share homologous genes encoding monofunctional enzymes for the assembly of aglycone units, and the BGC for DNR (daunorubicin) and DOX (doxorubicin) was sequenced from the *Streptomyces peuctius* ATCC 27952 (Parajuli et al. 2004). They identified a 40kb sequence encoding the BGC for DNR, DOX consisting of 37 ORFs (open-reading frames). The distinctive characteristics among the BCGs include a high abundance of glycosyl transferases, gene sets involved in deoxysugar production and a repertoire of tailoring genes for secondary metabolite. The DNR/DXR biosynthesis is completed in three steps: (A) Formation of Aglycone (ε-rhodomycinone), (B) Formation of an active sugar moiety (thymidine diphosphate daunosamine), (C) Glycosylation of ε-rhodomycinone and post polyketide modifications (decarboxylation, methylation and hydroxylation) (Grimm et al. 1994; Hutchinson 1997).

(A) Formation of Aglycone (ε-rhodomycinone)

The biosynthesis of DNR starts with the formation of aglycone ε-rhodomycinone, which is the important intermediate, synthesised by the PKS-II synthase by the genes *dpsA, dpsB, dpsC, dpsD, dpsE, dpsF, dpsG* and *dpsY*. The nine Malonyl-CoA units with a propionyl CoA starter unit undergo serial condensations to form a 21-carbon

decaketide, where the multiple reactions are facilitated through the polyketide synthase enzymes. The enzymes are produced by the genes *dpsA* (3-oxoacyl ACP synthase), *dpsB* & *dpsC* (ketosynthases), *dpsD* (acyltransferase) and *dpsG* (acyl carrier protein) to form a decaketide compound (Shrestha et al. 2019). The *dpsE* (ketoreductase) carries out the ketoreduction of the decaketide, followed by an aldol condensation and ring cyclization (3 steps) through catalyzing enzymes produced by *dpsF*, *dpsY* and forms a 12-deoxy alkanoic acid (Wohlert et al. 2001). The intermediate undergoes a *dnrG* (monooxygenase) mediated reaction, which adds a keto group to form alkalonic acid and is further transformed to aklaviketone by *dnrC* (alkanoic acid-S-adenosyl-1-methionine methyl ester transferase) a homodimeric protein (Grimm et al. 1994; Madduri and Hutchinson 1995). The formed aklaviketone intermediate undergoes cyclization by *dnrD* (alkanoic acid methyl ester cyclase) to form a 7-oxo moiety, which is further reduced to a hydroxy group of aglycone ε-rhodomycinone by *dnrH* (aklaviketone reductase) and *dnrF* (hydroxylase) (Madduri and Hutchinson 1995).

(B) Formation of an active sugar moiety (thymidine diphosphate daunosamine)

The biosynthesis of dTDP-L-Daunosamine is initiated from D-glucose-1-phosphate, which is carried out by the BGC, including seven genes *dnmL*, *dnmM*, *dnmU*, *dnmT*, *dnmJ* and *dnmV* (Shrestha et al. 2019). The *dnmL* (transferase) and *dnmM* (dehydratase) catalyse the reaction to generate the intermediate TKDG (thymidine diphosphate-4-6-deoxy-D-glucose). The epimerase produced by *dnmU* converts TKDG to TKLG (thymidine diphosphate-4-6-deoxy-L-glucose) through epimerization (Gallo et al. 1996). The *dnmT* (hydratase) and *dnmJ* (aminotransferase) facilitate the addition of a keto and an amino group at the C-3 position of the TKLG intermediate. The *dnmV* (ketoreductase) reduces the ketone to the hydroxyl group at the C-4 position to produce dTDP-L-Daunosamine (Otten et al. 1997).

(C) Tailoring reactions

The ε-rhodomycinone undergoes glycosylation with dTDP-L-Daunosamine in the presence of enzyme from *dnrS/dnrQ* to generate Rhodomycin D. The *dnrP* (esterase) converts Rhodomycin D to 13-deoxy-carminomycine, which undergoes an *O*-methylation by *dnrK* (methyltransferase) to generate 13-deoxy-daunorubicin (Furuya

and Richard Hutchinson 1998). The intermediate undergoes a C-13 oxidation by *DoxA* (cytochrome P450 enzyme) in two steps to produce 13-dihydrodaunorubicin and DNR (daunorubicin)(Dickens et al. 1997). Daunorubicin is hydroxylated later at the C-14 position through *DoxA* to form DOX (Doxorubicin) (Walczak et al. 1999; Shrestha et al. 2019).

Since their discovery, the DNR and DOX have been extensively employed for treating solid tumours but have faced significant drawdown due to their toxic properties. Anthracyclines enter cells through cation transport and passive diffusion, eventually leading to alterations in the proteasome and nucleosome (Mattioli et al. 2023).

2.4.2. Gene regulation in DNR/DOX biosynthesis

The BGC responsible for the biosynthesis of polyketide and sugar moieties in DNR/DOX also includes the regulatory genes for the initiation, regulation and termination of the entire synthesis pathway. The production pathway is regulated by the genes including *dnrO*, *dnrN* and *dnrI*, the transcription factors, where *dnrO* holds a significant importance in initiating the pathway. The *dnrO* produces a DNA helix binding domain, which is a key transcriptional regulator that activates the *dnrN* transcriptional activator, which finally leads to the activation of *dnrI*. The *dnrI* encoding enzyme binds to several polyketide synthases and facilitates the activation of efflux regulatory genes and initiation of DNR biosynthesis. The BGC also includes a transcriptional repressor *drrD*/*dnrW*, which promotes the transcriptional control by coherent feed-forward loop, self-resistance and feedback regulation (Vasanthakumar et al. 2013; Shrestha et al. 2019). The *drrD*/*dnrW* regulates the master transcription factor *dnrI*, which is crucial for the DNR/DOX biosynthesis. Deleting *dauW* (ortholog of *drrD*/*dnrW* in *S. ceoruleorubidus*) has increased the production of DNR by 8 folds (Yuan et al. 2011).

The maintenance of the produced DNR requires regulation inside the producing organism as the compound exhibits toxicity by intercalating with cellular DNA and eventually leading to cell death. The regulation of the lethal concentrations of produced DNR inside the cell is conferred by the *drrAB* locus, which includes the drrA and drrB proteins necessary for the efflux of the finished product (Guilfoile and Hutchinson 1991; Vasanthakumar et al. 2013). The expression and function of drrA and drrB are interdependent on each other at an ATP-driven pump, where drrA is a

peripheral membrane protein acting as an energy-transducing unit inside the cell when bound to the ATP in a DOX-dependent manner and drrB is the internal protein with hydrophobicity and helps in the efflux of produced DNR/DOX (Kaur 1997; Kaur et al. 2005). A mutant strain without the *drrAB* has exhibited a decline in DNR production and resulted in cell death, and overexpression of *drrAB* has resulted in the overproduction of DNR and promoted self-resistance (Li et al. 2014). Thus, the self-resistance genes also have an indirect effect on the biosynthetic pathway in DNR/DOX production (Srinivasan et al. 2010). Another resistance gene is *drrC*, which functions in the presence of ATP and DNR by binding to the DNR intercalated DNA and propelling it outside of the cell. This self-resistance gene maintains cell viability and regulates the lethal concentrations of DNR in a dependent manner, which relies on *dnrN* and *dnrI* in the biosynthetic pathway (Furuya and Richard Hutchinson 1998).

The entire pathway and its regulation decide the fate of DNR/DOX quantity production in *Streptomyces* sp. Thus, over the past decades, researchers have considered engineering the genes involved in the biosynthesis of aglycone, sugar moiety, tailoring reactions, transcriptional factors, transcriptional repressor and self-resistance to improve DNR/DOX production at an industrial level for commercial uses in cancer medication. The present techniques of modifying genes to enhance the production of DNR/DOX are not effective due to the complex cellular enzymatic reactions involved. These approaches have yet to provide a clear understanding of the entire mechanism and also could not participate in significant improvement of the DOX production.

2.5. Mode of Action of DNR/DOX

Since their discovery, the DNR and DOX have been extensively employed for treating solid tumours but have faced significant drawdown due to their toxic properties. Anthracyclines enter cells through cation transport and passive diffusion, eventually leading to alterations in the proteasome and nucleosome (Mattioli et al. 2023).

2.5.1. DNA intercalation

Anthracyclines exhibit a strong affinity for DNA by inserting their aglycone moieties between the base pairs, causing the separation of the existing base pairs, and positioning their sugar components in the minor groove of the DNA (Comings and

Drets 1976). DNR and DOX have a preferential ability to bind to DNA at GC base pairs of both mitochondrial and nuclear DNA by establishing hydrogen bonding between the hydroxyl group on the C-9 position at aglycone moiety and N2, N3 of guanine (Chaires et al. 1990; Nunn et al. 1991; Ashley and Poulton 2009). This inhibits cellular DNA transcription, replication, recombination and repair, which creates torsional stress. The torsional stress alters the structure (disassociation of H2A/H2B dimers from histone core) and dynamics of nucleosomes (Gupta et al. 2009; Martins-Teixeira and Carvalho 2020). The histone eviction caused by DOX/DNR (in H3 due to rich GC base pairs) majorly due to the sugar moiety binding to DNA critically causes chromatin damage, which leads to epigenomic aberrations and transcriptional alterations (Pang et al. 2015; Mattioli et al. 2023).

2.5.2. Topoisomerase II (Topo II) poisoning

The Topoisomerase II (Topo II) induces double-stranded breaks (DSBs), releases torsional stress and re-ligates the DNA breaks, ensuring the proper DNA transcription, replication and repair (Nitiss 2009). Anthracyline interacts with the Topo II enzyme to form an anthracycline-topoisomerase-DNA quarternary complex. It induces irreversible DNA damage by preventing the regeneration of phosphodiester bonds between the DNA strands (Mattioli et al. 2023). DNR/DOX intercalates the Topo II DNA with their cyclohexane ring A in aglycone moiety and 4-methoxy group in sugar moiety. The changes in the functionality of Topo II to a DNA nuclease generate genomic instability, activation of DNA damage response and TP53 pathways, eventually leading to cell death (van der Zanden et al. 2021). In mammals, the Topo II enzyme is distinguished into isoforms Topo IIα (generate replication forks during mitosis in actively diving cells) and Topo IIβ (expressed in most cell types devoid proliferation status), where the DOX interacts with Topo IIβ in cardiomyocytes and lead to cardiotoxicity (Lyu et al. 2007; Zhang et al. 2012).

2.5.3. Formation of DNA adducts

Anthracyclines form DNA adducts between the two strands through covalent and hydrogen bonds with aglycone and sugar moieties, respectively. The DOX-DNA covalent bond in the cancerous cell is facilitated by the cellular formaldehyde, produced due to free radicle reactions with polyamines and lipids is responsible for the block in transcription, DSBs and replication (Kato et al. 2001). *In vitro* studies using

DOX by pre-activated formaldehyde resulted in the formation of transcriptional blocks through the formation of inter-strand adduct (G-DOX-G cross-linking), inhibiting the transcription process (Cullinane and Phillips 1992). The treatment of mice cancer cell lines with DOX leads to the disruption of the replication process and cell cycle arrest through the blocks in [8H]-thymidine (Bilardi et al. 2012; Forrest et al. 2012). The investigations involving DOX and DOX-formaldehyde conjugate on colorectal cancer cell lines for DNA repair mechanisms resulted in DNA adduct-induced damage. The studies also prove the damage (apoptosis) caused by DOX-DNA adducts is independent and does not rely on the Topo II activity (Swift et al. 2006; Spencer et al. 2008; Barthel et al. 2016).

2.6. Self-Resistance in non-target species/microbial factories

The microbial cell factories of antibiotics, anthracyclines, and related cytotoxic compounds like filamentous *Actinobacteria* and *Streptomyces* are programmed to deal with the cytotoxic compounds made by them (Hopwood 2007; Julian and Dorothy 2010). These resistance mechanisms include the expression of resistance genes, efflux systems to pump out anthracyclines, the inactivation of anthracyclines through enzymatic modifications and interaction with other metal elements.

2.6.1. Resistance genes

The microbial cell factories of antibiotics, anthracyclines, and related cytotoxic compounds like filamentous *Actinobacteria* and *Streptomyces* are programmed to deal with the cytotoxic compounds made by them (Hopwood 2007; Julian and Dorothy 2010). The self-resistance developed by the bacteria through the expression of resistance genes is a prerequisite to its survival against the produced toxic (DNA intercalating majorly) compounds. Similar to the antibiotic pathway-synthesising genes on BGCs, the resistance genes are also encoded in the BGCs, which initiates the process of self-resistance through time-space co-ordinated expression or intermediate-dependent (compound produced) expression (Mak et al. 2014). The resistance mechanisms are variable according to BGCs or product type, which include target protection, compound inactivation, modification, sequestration and efflux.

In *Streptomyces peucetius*, the genes encoding resistance for DNR/DOX are *drrA*, *drrB*, and *drrC* unravelled when expressed in *E. coli* and *S. lividans.* The *drrA* and *drrB* proteins act as drug-efflux complexes produced during the idiophase,

whereas the *drrC* is transcribed earlier and facilitates the efflux through drug binding (Guilfoile and Hutchinson 1991; Lomovskaya et al. 1996; Kaur 1997). A detoxification strategy of *Streptomyces* by reducing the DOX to 7-deoxydoxorubicinolone via deglycosylation using NADH: ubiquinone oxidoreductases was reported (Westman et al. 2012). Developing and employing natural microbiome inhibitors against toxicity for drug delivery in oncological medicine would result in reduced side effects of the anthracyclines.

2.6.2. Efflux pumps

Efflux pumps play a pivotal role in conferring multidrug resistance in bacteria by facilitating the expulsion of toxic compounds either produced by the organism or acquired from the external environment (Webber and Piddock 2003). Efflux pumps are the key components of the cell membrane that regulate the internal cellular concentrations of toxic chemicals and elements (metal ions) through extrusion and also inhibit the re-entry of compounds to evade toxicity (Piddock 2006; Bazzi et al. 2020). The efflux pumps utilize energy by hydrolysing the ATP and can use the electrochemical or ionic gradient inside the bacterial cells to efflux the toxic compounds. The efflux systems found in bacterial cells are categorised into six families: ABC (ATP-binding cassette), MATE (multidrug and toxic compound extrusion), PACE (proteo-bacterial antimicrobial compound efflux), MFS (major facilitator superfamily), SMR (small multidrug resistance family) and RND (resistance nodulation cell division) (Du et al. 2018). These efflux pumps comprise transmembrane protein helices facilitating the translocation of produced secondary metabolites outside the producer organisms (Gaurav et al. 2023). However, despite their varied structural differences, substrate redundancy is prevalent across all the efflux pump families. The DOX/DNR is extruded out by the AbeM efflux pump of the MATE family (using antiporters H^+ and Na^+) in *Acinetobacter baumannii,* whereas the ABC pumps (generally hydrolyse ATP) perform the extrusion in *Streptomyces* sp. (Abdi et al. 2020; Zack et al. 2024).

2.6.3. ABC efflux pumps

The ABC (ATP-binding cassette) pumps constitute the most prominent protein families and are widely present in all living organisms, facilitating the import and export of chemical substances based on their structural architecture and folding

(Thomas and Tampé 2024). The ABC efflux pumps in bacteria use the energy by hydrolysing ATP and translocating various chemical compounds like sterols, secondary metabolites and lipids across the membrane through 12 transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) (Jacek et al. 2007; Li et al. 2014). The TBDs aid in substrate binding, whereas the NBDs carry the translocation of compounds hydrolysing ATP. The detailed mechanism and structural diversity of ABC pumps and their activity depending on the arrangement of helices, loops and protein domains are reviewed in Thomas and Tampé (2024). The *drrAB* transporter system encodes for the efflux of DNR/DOX in *S. peucetius*, where *drrA* (peripheral membrane protein) binds to ATP and *drrB* (hydrophobic membrane protein) enables the translocation acting as resistance mechanism. The subcloning of these *drrAB* genes in *E. coli* resulted in similar expression (Kaur and Russell 1998). Several follow up studies conferred the resistance mechanism of *drrAB* transporter system and the co-dependence of both proteins in efflux activity (Méndez and Salas 2001; Li et al. 2013). A recent study by Dong et al. (2024) conducted on ABC transporter in *Streptomyces ceoruleorubidus* yielded significant findings, indicating that the *drrAB* genes of the DNR BGC facilitate the efflux of excess DNR/DOX within the cell. Additionally, the two-component ABC transporters, encoded by drrAB2 and drrAB3 and situated outside the cluster, regulated by the TetR family regulator DrrR1, were identified as playing a complementary role in the efflux of daunorubicin in *S. coeruleorubidus* in response to the intracellular accumulation of daunorubicin (Dong et al. 2024).

2.6.4. Inactivation of drug by enzymatic reaction

Resistance mechanisms to evade autotoxicity in microorganisms also include inactivating or modifying the produced metabolites or antibiotics through enzyme activity. The β-lactamases inhibit the production of penicillin derivatives and cephalosporins as a resistance mechanism (Essack 2001; Bush 2013). Likewise, chloramphenicol undergoes modification by acetylation, *O*-phosphorylation of erythromycin, acetyl, and phosphor transferases modify the amino-glycoside antibiotics (kanamycin, gentamycin, streptomycin) as a resistance mechanism (O'Hara et al. 1989; Murray and Shaw 1997). The activation of repressor gene *dnrH* in *S. peucetius* carries out the glycosylation reaction of the daunosamine sugar to baumycin-like glycosides, thereby preventing the formation of DNR/DOX (Scotti and

Hutchinson 1996). Similarly, the *doxA* gene encoding the cytochrome P450 oxidase, crucial for three oxidation steps in DNR/DOX development, gets downregulated by the excess concentration of produced daunorubicin inside the *Streptomyces* sp. (Dickens et al. 1997; Walczak et al. 1999). A detoxification strategy of *Streptomyces* by reducing the DOX to 7-deoxydoxorubicinolone via deglycosylation using NADH: ubiquinone oxidoreductases was reported (Westman et al. 2012). Thus, employing the activation of products through enzymes aids in the inhibition of the intercalation of DNA and evades autotoxicity in the producer organisms.

2.7. Interaction of DNR/DOX with iron

Daunomycin is the chelator of iron, where ionic forms of iron (Fe^{2+} and Fe^{3+}) bind to specific functional groups of anthraquinone moiety and form stable complexes (Zweier et al. 1986). The quinone group at position 5 and hydroxy group at position 6 on the aglycone part of DNR acts as the binding sites for iron by donating electrons. The DNR also has a side chain with hydroxyl groups which can donate lone pair of electrons and bind to iron (Zweier et al. 1986). Both ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms of iron bind to daunomycin, where Fe^{2+} is highly reactive and readily participate in redox cycling and alter between ionic states and Fe^{3+} is less reactive and form stable complexes (Fiallo and Garnier-Suillerot 1986; Fiallo et al. 1993). This stabilization activity can be employed for the therapeutic purposes. The first tri-ferric doxorubicin compound, named Quelamycin, a metallic derivative of the Adriamycin prepared was through chelation in the presence of Fe (III) (Gosálvez et al. 1978). The compound has been reported to be highly stable in phase I clinical trials and P 388 leukaemia cells, where the cytosolic components do not degrade the compound, and it also inhibits the free flow of electrons from NADH to oxygen molecules in cells (Cortés-Funes et al. 1980; Beraldo et al. 1985; Fiallo and Garnier-Suillerot 1986). The bond strength of the iron-DNR complex is high and the chelation activity can be reversible or disassociated in high acidic pH (lower) conditions and in presence of iron binding compounds like transferrin, ferritin.

2.8. Interaction of DNR/DOX with oil

The anthracycline compounds daunorubicin and idarubicin are lipophilic and their interaction mechanisms with the lipids are studied using various experiments (Ribeiro

et al. 2013; Alves et al. 2017; Matyszewska 2020). Oils and the oleic acid being non-polar, bind to the hydrophobic regions on the anthraquinone moiety, which is often used in therapeutic formulation. The liposome-associated doxorubicin was reported to have reduced systemic and cardiotoxicity in clinical trials for humans and mice (Gabizon et al. 1986, 1989; Amselem et al. 1992). The daunorubicin is encapsulated by the liposomes (phospholipid vesicles) and exploited for drug delivery mechanism (Juliano and Stamp 1978; Mussi et al. 2014).

2.9. Culture Media for metabolites production in *Streptomyces* sp.

The production of antibiotics at a large scale is a combinatorial effect which relies upon the (Rodrigues da Silva et al. 2021) strain efficiency, ability to utilise the available nutrients, physical conditions and productivity of the metabolites. The primary nutrients like carbon, nitrogen and phosphorus, along with minor mineral elements, remain the major constituents of the culturing media responsible for the growth and production of necessary chemical compounds in *Streptomyces* sp. The carbon serves as a prominent energy source, nitrogen is responsible for cell growth and metabolism, and phosphates assist in the production of the metabolites (Rokem et al. 2007). To date, many investigations over the decades have concentrated on improving secondary metabolites using strain engineering *via* genetic alterations. However, the culmination of improved levels of metabolite production through extensive genetic research remains unpromising due to the intricate metabolic mechanisms involved as reviewed in the biosynthesis and gene regulation sections. Additional investigations employing the modifications in media have the potential to result in more streamlined and economical techniques for manufacturing daunomycin and other crucial antibiotics in *Streptomyces*.

The DNR/DOX compounds are produced in the late growth phase through a multitude of enzymatic reactions by the *Streptomyces* strains, utilizing nutrients (Sánchez et al. 2010). The host cells synthesizing the secondary metabolites in nature (µg/L) are not sufficient to achieve the harvest at the desired quantities (g/L) on an industrial fermentation scale (Bilyk and Luzhetskyy 2016). So, the efficient native strains are screened and improved through metabolic engineering (contemporary), mutations (traditional) and selections. However, the highest yields are achieved through the combination of several approaches in strain development, suitable culture media composition and well-optimised fermentation conditions. The complete

genomic sequencing of the model actinomycete *Streptomyces coelicolor* revealed the presence of multiple genes in the *Streptomyces* species' genome that can break down complex carbohydrates and proteins (Bentley et al. 2002). This facilitates in utilising various carbon and nitrogen sources to optimise culture media. The production of metabolites is also linked to factors like nutrients available in culture media and fermentation conditions (temperature, light, oxygen and pH) (Sánchez et al. 2010; Bilyk and Luzhetskyy 2016). The optimisation of media and the source of nutrients remained a major variable factor in the growth of strains and the production of metabolites for several decades.

2.9.1. Carbon source

Glucose or sugars are the most often utilized carbon sources in industrial fermentation due to their low cost and high availability, even though they inhibit secondary metabolite synthesis (Rokem et al. 2007; Ruiz et al. 2010; Hulst et al. 2022). The carbon source serves as the vital controlling agent for secondary metabolite production in *Streptomyces*, as transcriptional activation or carbon catabolite repression (CCR) is dependent on the source and concentration of carbon (Hodgson 2000; Rokem et al. 2007; Ruiz et al. 2010). Carbon from sugars like glucose, maltose, glycerol, sucrose, mannose, and xylose has been reported to interfere with the production of more than 30 types of secondary metabolites (mostly antibiotics) in *Streptomyces* sp. (Ruiz et al. 2010; Romero-Rodríguez et al. 2017). The synthesis of Doxorubicin in *S. peucetius* has been impeded by the utilization of glucose and galactose as the carbon source in the culture medium (Escalante et al. 1999). Sugar carbon in the media at an industrial level leads to an increase in acidification and triggers feedback inhibition through produced intermediates.

The erythromycin yield in *Saccharopolyspora erythraea* at the industrial level using oil and soy flour has been improved to 3.5g/L compared to the dextrin control (Hamedi et al. 2004). Clavulanic acid production in *Streptomyces clavuligerus* has been improved using olive oil as a sole carbon source (Efthimiou et al. 2008). Employing soybean oil as a source of carbon has enhanced the production of FK506 (tacrolimus) - an immunosuppressant polyketide by 88.8% in *Streptomyces tsukubaensis* (Wang et al. 2017). Enhanced production of DOX (1100mg/L) was achieved by mutation treatment (UV and ART-plasma) and soybean oil as a carbon source in *Streptomyces peucetius* SIPI-11 (Wang et al. 2018). Increased levels of

salinomycin production and improved TAG (triacylglycerol) metabolism were observed in *Streptomyces albus* ZD11 using soybean oil as a carbon source in culture media (Han et al. 2020). Oil utilisation has also benefited from imparting the activity as an antifoam at the industrial scale of fermentation. The breakdown of oils supports the activity of malonyl Co-A and Acetyl Co-A, which are essential for the biosynthesis of secondary metabolites. Thus, employing an oil-based carbon source instead of sugar in combination with optimised fermentation conditions and selection would enhance DNR/DOX production.

2.9.2. Nitrogen source

The source and concentration of nitrogen in the media also remain a vital factor for secondary metabolite production. Nitrogen in the form of ammonia is mostly preferred by microorganisms, and the genera *Streptomyces* naturally possess a constant nitrogen acquisition and metabolism to ensure their survival (Tiffert et al. 2011; Romero-Rodríguez et al. 2017). *Streptomyces* assimilate ammonia through glutamate dehydrogenase in ammonia-rich conditions and glutamine synthetase pathways in ammonia-deficient conditions (Rokem et al. 2007). The influence of various regulatory mechanisms of nitrogen in *Streptomyces* has been clearly reviewed in (Krysenko 2023). The forms or sources of nitrogen, like ammonium, nitrate, amino acids, and polyamines, have an impact on the production of secondary metabolites in *Streptomyces* (Romero-Rodríguez et al. 2017; Krysenko 2023). Specific concentrations of various nitrogen sources can be used to quantify the yield of different actinobacterial compounds in *Streptomyces* according to their industrial relevance.

2.9.3. Phosphorus source

Phosphorus, in the form of inorganic phosphate, is the crucial element acting as the building blocks for nucleotides, proteins, and several regulatory signalling cascades (van Wezel and McDowall 2011). The concentration of phosphate in cells significantly impacts the production of secondary metabolites in *Streptomyces* and related actinobacteria (Barreiro and Martínez-Castro 2019). Increased concentrations of phosphates in media (>10mM) have resulted in decreased yields of antibiotic production, whereas the lower concentration (<0.1mM) has positively increased the secondary metabolite production, implying the significance of phosphates on biosynthetic pathways (Martín 2004; Romero-Rodríguez et al. 2018). The limited

availability of phosphate results in nutritional stress and initiates the secondary metabolite biosynthetic pathways. In phosphate-rich conditions, the *Streptomyces* convert phosphate to polyphosphate and accumulate, which aids in cellular metabolism. The regulation of phosphate in *Streptomyces* is carried out through a two-component mechanism, PhoR-PhoP, clearly reviewed elsewhere (Allenby et al. 2012; Martín and Liras 2020).

2.9.4. Other elements

A well-established culture media including all these macro components together with the essential microelements like Fe, Ca, Zn, S, etc, results in the enhancement of secondary metabolites yield. The use of rare earth elements in the culture medium for *Streptomyces* sp. is reported to activate the BCG's cluster for secondary metabolite production (Hosaka et al. 2009). Tanaka et al. (2010) used scandium and lanthanum in medium for the cultivation of *Streptomyces coelicolor* and reported the increase of 2.5 to 12 folds expression of genes belonging to secondary metabolite-biosynthetic gene clusters. Optimization and standardization of culture media considering pH, combinations of nutrients, agitation, temperature, had resulted in enhanced production of daunomycin in *Streptomyces* sp. (Bundale et al. 2015; Wang et al. 2018).

2.10. Engineering culture media – in Prospect for improved production.

Over the past decades, genetic alterations have been frequently used to enhance the production of metabolites in *Streptomyces*, improving regulatory gene expression, modifying resistance, developing efflux mechanisms, and possible combinations with strain development. However, modifications to the culture media can also potentially improve production yields. A considerable amount of research is lacking in this area, but strategies employed for other polyketide synthesis in *Streptomyces* relevant to daunomycin can provide promising insights into the enhancement of production devoid of complex and expensive gene editing methods.

The prominent effect of DOX/DNR is its autotoxicity by intercalating with the DNA in the producers when the concentration increases. The prospective idea of this project is to prepare culture media for cultivating *Streptomyces* sp. based on binding of daunomycin with iron or reduced forms of iron after effluxed from the producer. The iron or iron-DNR complex is encapsulated by the oleic acid or lipid micelle layers present in the culture medium converting the produced DNR/DOX to inactive forms

and settled with DNR-iron-oil complex. Therefore, this hypothesis can safeguard the producer strain from toxicity and avoid the inhibition of metabolite production.

2.10.1. Perturbation of metabolite biosynthesis in Streptomyces sp.

The over expression of regulatory genes in BGCs and downregulation of repression genes and factor always remained as prominent approaches in the metabolic engineering of *Streptomyces* for metabolite production (Méndez and Salas 2005; Shrestha et al. 2019; Hulst et al. 2022). On the contrary, the availability of biosynthetic precursors also serves as a key factor that are generated primarily by carbon catabolism in the organisms (Nielsen 1998; Tanaka et al. 2017). Perturbation is the supply of precursors for modulating the biosynthesis to improve the ability of cells in enhancing the secondary metabolite production. The ARCs (antibiotic remodelling compounds) screened from *Streptomyces coelicolor* A3(2) are known to stimulate the metabolite production acting as precursors (Olano et al. 2008). The ARC2 similar to the antimicrobial compound triclosan, has reported to partially inhibit the fatty acid synthesis and utilize the acetyl CoA for polyketide biosynthesis and improved the actinorhodin yield in *S. coelicolor* (Olano et al. 2008; Craney et al. 2012). The overproduction of metabolites like oligomycin, salinomycin, erythromycin and actinorhodin have been reported by the using the triclosan as an elicitor of polyketide biosynthesis in *Streptomyces* sp. (Norimasa et al. 2003; Yukinori et al. 2013; Tanaka et al. 2017). The aim of this discussion is to propose an equivalent approach for the strains of *Streptomyces ceoruleorubidus* to enhance daunorubicin production capacity, rather than adhering to usual genetic engineering methods.

2.10.2. Media construction for three-way interaction (DOX/DNR-iron-oligolipid)

The achievement of the prospective three-way interaction from the can be achieved from distinctive methods under a single hood with critical optimization of conditions like pH, temperature, pressure, incubation time and initial components like natural chelators, metal salts and nutrient sources. The biosynthesis of FeO particles from their salts like $FeCl_3$ using phytoextracts are being employed in nanoparticles synthesis over decades (Singh et al. 2018; Pudhuvai et al. 2024). The phytic acid present in plants, cereals, and legumes has a tremendous metal chelation potential (Graf and Eaton 1990). The phytate-metal complex is stable and cannot be liberated in wide pH ranges. Phytates from soybean or soy-derived products have high iron binding ability, which

is considered a major drawback in diet and nutrition (Hurrell et al. 1992; Gupta et al. 2015). Thus, utilizing soybean phytates in the culture medium facilitates iron binding and chelation.

As discussed in the above carbon sources section, the oil source of carbon in the culture media for *Streptomyces* describes its prominence in the improved production in several instances, including erythromycin (Hamedi et al. 2004), clavulanic acid (Efthimiou et al. 2008), doxorubicin (Wang et al. 2018), salinomycin (Han et al. 2020) and josamycin (Eiki et al. 1988). Employing crude oils, including the raw plant parts with phytic acid contents, will deliver the nutrient carbon source and act as a reducing agent for iron in the media. Crude oils of soybean and pomace have enhanced clavulanic acid production in *Streptomyces*, which is also a waste-to-value strategy (Efthimiou et al. 2008; Young et al. 2020). The crude plant oil substrate used for the cultivation media form micelles due to elevated temperature and pressure during autoclaving and encapsulate the FeO particles. After inoculation of the perturbated *Streptomyces ceoruleorubidus* culture to the cultivation media, the production of daunorubicin takes place and is effluxed out into cultivation media.

Considering the lipophilic nature of daunorubicin, the produced, effluxed DOX/DNR into the medium can interact with the oligolipid surface layer with FeO particles from the oil-based medium (Alves et al. 2017). The interaction between anthracycline and metal ions, especially iron, has the potential to form complexes demonstrate high stability constants in the medium (Seke et al. 2019). The produced and effluxed DOX/DNR by the *Streptomyces* strain interacts directly with the FeO-micelle to form a DOX-Fe-micelle complex (Calendi et al. 1965; Cortés-Funes et al. 1980; Xu et al. 2005). Thus, capturing the produced metabolite in an inactive form helps avoid toxicity to the producer organism.

Streptomycetes are also well-known for their metal resistance, which involves their intricate intracellular iron homeostasis mechanisms (Yaqing et al. 2018). As a defensive strategy, the reduced iron entering the cells is segregated and secreted into the external environment through ABC pumps. Streptomyces' defensive strategy of effluxing the excess DNR/DOX re-initiates the production of new DNR/DOX molecules inside the cells, resulting in improved productivity. Therefore, the enhancement of the daunomycin production in *Streptomyces* using this media construction approach can be established with reduced costs and negligible metabolic engineering of strains.

3. Aims and Hypothesis

The main aim of thesis was to develop a daunomycin-producing strain and optimise an efficient medium to enhance the DNR production, reduce costs and simplify downstream processing. Studies were focused on:

- Isolation and cultivation of the *Streptomyces ceoruleorubidus* strain and optimisation of its growth on R2A (Reasoner's 2A agar), PDA, and M2 (Melanocyte growth media).
- Strain identification using 16S RNA.
- Performing protoplast fusion with the wild strain of *S. ceoruleorubidus* by triclosan treatment to achieve a high DNR-producing strain.
- Screening and selecting strains by regulatory genes *dnrN*, *dnrO*, and dnrI, daunomycin resistance genes *drrA*, *drrB*, and *drrC*.
- Observational recording of the strain morphology of the improved strains for sporulation behaviour and homogenous growth.
- Testing various C (sugar, oils) and N sources individually or in combination, including different mineral supplements, e.g., rare earth elements, copper, zinc, vitamins, antibiotics, etc., for enhanced DNR production.
- Optimization of the culture media to encapsulate the DNR together with reduced iron as complex with oil and make it inactive.

The dissertation hypothesis was (a) whether a specific iron-containing medium would lead to the formation of daunomycin-iron complexes that could reduce the solubility and bioavailability of daunomycin and (b) whether this approach would lead to reduced toxic impacts during fermentation while simultaneously increasing the yield of the target metabolite and overall production efficiency, all while minimizing the ecological footprint of the process.

4. Material and Methods

4.1. Bacterial Strain

The original bacterial strain *S. coeruleorubidus* was isolated from mosquito larvae collected from the Vltava River pool during Spring 2019. Identification was confirmed through morphological assessments, phenotypic characterization, and genomic sequencing. PCR amplification revealed the presence of daunomycin resistance genes *drrA, drrB,* and *drrC*, which are significant for potential applications in developing antimicrobial media.

4.2. Triclosan-Induced Mutation

To enhance the production of daunomycin, triclosan was employed to induce mutations in *S. coeruleorubidus* due to its known effect on lipid synthesis, especially on enoyl-acyl carrier protein reductase a key enzyme involved in fatty acid synthesis (McMurry et al. 1998). Solid Potato Dextrose Agar (PDA) medium was prepared, to which triclosan was added aseptically at final concentrations of 0.1, 0.5, 1, and 10 µM. Subcultures were maintained for ten passages to stabilize the mutant strains, which were subsequently evaluated for increased productivity on both standard and modified media.

4.3. Olive Pomace Oil-Resistant Strain Development

To develop a strain capable of growth on olive pomace oil, ultraviolet (UV) irradiation was employed, following methods described by (Hopwood and Wright 1979; 1981). The strains were exposed to UV light at a dose rate of approximately 10 W/m^2 for about 30 minutes and subsequently cultivated in solid and liquid media containing 10% olive pomace oil as the sole carbon source.

4.4. Protoplast Formation

Protoplasts of *S. coeruleorubidus* were prepared using a modified method adapted from Okanishi et al. (1974) and Hopwood and Wright (1978). Briefly, spores were inoculated in Yeast Extract-Malt Extract (YEME) medium supplemented with 0.5% glycine and 5 mM $MgCl_2 \cdot 6H_2O$, followed by incubation at 28°C and 220 RPM for 48 hours. Protoplasts were generated from two distinct strains: one resistant to triclosan

and another capable of growing on olive pomace oil. The cultures were centrifuged at 4000 RPM for 10 minutes and washed with a 10.3% sucrose solution. The cell pellet was treated with Medium P (Hopwood and Wright, 1978) for washing, then resuspended in a lysozyme solution (2 mg/ml) for cell wall disruption. After lysis, protoplasts were washed and resuspended in a 40% PEG 4000 solution. Darkening of the agar around colonies was used as a selective criterium.

4.5. Optimization of the Production Medium

The cultivation process was maintained at a speed of 220 RPM (with a shaker arm length of 25 mm) and a temperature of 28 °C. The culture was deemed ready for inoculation after 36 to 40 hours (±2 hours) in a basal medium composed of the following ingredients (g/l): 30 g of soy flour, 5 g of starch, 5 g of glucose, 5 g of yeast extract, 1 g of K_2HPO_4, and 1 g of NaCl. The pH was adjusted to 7.0 using either 2 M NaOH or 2 M HCl.

The production medium was inoculated with 2.5 ml of the actively grown culture from the vegetative medium (constituting a 5% inoculum) into 50 ml of the production medium, also contained in a 250 ml Erlenmeyer flask. The culture was incubated at 28 °C with agitation at 220 RPM. The composition of the production was as follows (g/L): 10 g of bakery yeast, 20 g of soy flour, 100 g of pomace olive oil, 5 g of yeast extract, 5 g of glycerol, 2 g of K_2HPO_4, 1 g of magnesium sulfate heptahydrate, 3 g of calcium carbonate, and 3.6 g of ferrous sulfate heptahydrate. The pH of the medium was adjusted to a range of 5.9 to 6.1 using 2 M NaOH or 2 M HCl.

4.6. Effect of Carbon and Nitrogen Sources on Daunomycin Production

The influence of various carbon sources on daunomycin metabolite production was evaluated by substituting starch in the production medium with alternatives such as soybean oil, refined rapeseed oil, crude rapeseed oil, virgin olive oil, pomace olive oil, mineral oil, and crude cannabis oil. Similarly, the impact of different nitrogen sources was assessed by replacing soy flour in the production medium with casein, yeast extracts, defatted soy flour, soy grits, peptones, ammonium sulfate, and potassium nitrate. Following the incubation period, the impact of carbon and nitrogen sources on daunomycin production was analyzed by HPLC.

4.7. Effects of Iron on Daunomycin Production

To investigate the effects of minerals on daunomycin production, the medium containing optimized carbon and nitrogen sources was supplemented with various iron sources, including $FeCl_3$, $FeSO_4$, and $Fe_2(SO_4)_3$. These were added at final concentrations of up to 1.44 g per liter of Fe^{2+} and 2.79 g per liter of Fe^{3+}, which corresponded to the following additions: 2.1 g/L of $FeCl_3$; 3.6 to 7.2 g/L of $FeSO_4$; and 5.2 to 10 g/L of $Fe_2(SO_4)_3$.

4.8. Monitoring of daunomycin production

Samples were collected from the flasks at 120, 168, 216, and 264 hours, with the possibility of extending to 312 hours. For each sampling point, 1 ml of homogeneous medium was extracted from each flask. The samples were processed according to a rapid isolation protocol using oxalic acid and daunomycin concentration measured by HPLC.

4.9. Changes in Media Color and Physical Parameters

Media color and physical parameters were assessed at 24-hour intervals throughout the cultivation period, beginning at 24 hours and continuing up to 264 hours. At each designated time point, a sample of the cultivated media was visually inspected to determine its color and observed physical parameters, such as the formation of precipitate. This information was recorded using subjective visual assessment. Specific colors observed—such as yellow, green, gray, and black—were noted, along with any relevant changes in intensity or hue. This consistent monitoring allowed for the documentation of color transitions and physical parameters of the culture over time.

4.10. Sediment friability

To evaluate the friability of the sediment, samples were dispersed in water containing 0.1% SDS as a surfactant at a temperature of 20°C. Control samples were left untreated, while the examined samples were mixed using a mixer at 5-minute intervals. The characterization of the samples included an analysis of particle size distribution, which was determined using laser diffraction with a Mastersizer 2000 equipped with a Hydro 2000G dispersion unit (Malvern Instruments, UK). The mean particle size relative to volume was chosen as the primary criterion for particle size assessment. To

ensure the accuracy and reliability of the results, two parallel measurements were conducted.

4.11. Measurement of pH, O_2, and Eh During Fermentation

To monitor the fermentation process, pH, dissolved oxygen (O_2), and redox potential (Eh) were measured at regular intervals throughout the cultivation period.

pH Measurement: The pH of the fermentation media was measured using a calibrated pH meter with a glass electrode. At each sampling interval (e.g., every 24 hours), a small volume of the media was taken aseptically and transferred to a clean, appropriate container. The pH probe was immersed in the sample, and the reading was recorded after stabilization. The pH meter was calibrated before measurements using standard buffer solutions at pH 4.00 and 7.00 to ensure accuracy.

Dissolved Oxygen Measurement: Dissolved oxygen levels were assessed using a portable dissolved oxygen meter equipped with an appropriate probe. Samples were taken at the same intervals as pH measurements. The probe was inserted into the sample, allowing for direct measurement of O_2 concentration. The oxygen sensor was calibrated beforehand according to the manufacturer's instructions, ensuring accurate readings throughout the fermentation process.

Redox Potential Measurement: The redox potential (Eh) was measured using a portable redox meter with a suitable combination electrode. Similar to the previous measurements, samples were taken at regular intervals. The redox electrode was placed in the sample, and stabilization was allowed before recording the Eh value. Calibration of the redox meter was performed using standard reference solutions to ensure reliable measurements.

All measurements were conducted under controlled conditions to minimize the impact of external factors, and each parameter was recorded systematically to analyze the fermentation dynamics over time.

4.12. Methods for Observing Morphological Changes During Fermentation

Optical Microscopy: To observe morphological changes in the fermentation medium, samples were taken at designated intervals throughout the fermentation period. Each sample was placed on a clean glass slide and covered with a coverslip. The slides were examined under an optical microscope Nikon Eclipse Ni-E at varying magnifications

(e.g., 100x, 400x) to assess cell morphology, size, and aggregation. Digital images were captured for further analysis and comparison over time.

Scanning Electron Microscopy (SEM): For surface morphology analysis, samples were prepared for scanning electron microscopy. Initially, samples were fixed in an appropriate fixative and then dehydrated through a series of graded alcohol solutions. The samples were mounted onto conductive stubs and coated with a thin layer of gold-palladium to enhance conductivity. The SEM JSM-7401F was calibrated, and samples were examined under high vacuum conditions. Secondary electron imaging was used to visualize the surface features of the cells, and images were captured for analysis of morphological changes during fermentation.

Transmission Electron Microscopy (TEM): For detailed structural analysis, samples were prepared for transmission electron microscopy following standard fixation and embedding protocols. Initially, samples were fixed in a solution of 2.5% glutaraldehyde buffered with phosphate buffer for 2 hours at 4°C. Following fixation, the samples were washed in phosphate buffer and post-fixed in 1% osmium tetroxide for 1 hour. Then, samples were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%) and embedded in an epoxy resin. Thin sections (approximately 70 nm) were cut using an ultramicrotome and placed on copper grids. The grids were stained with uranyl acetate and lead citrate to enhance contrast. Finally, sections were examined using a transmission electron microscope JEM-1400 Jeol at an appropriate accelerating voltage, with images captured for analysis of cellular structures and morphological features.

4.13. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) was performed using SDS-PAGE (Laemmli) and native PAGE techniques, following Davis's procedures. SDS-PAGE was utilized to determine protein molecular weights and to cut out specific bands for subsequent analysis by TOF MALDI mass spectrometry. The SDS gel was stained with Coomassie Brilliant Blue, while in the native PAGE, proteins of interest were naturally labeled with daunomycin. This approach allowed proteins to maintain their native conformations, specifically for identifying daunomycin-pigmented proteins.

4.14. Extraction Procedures

Two primary methods can be employed for extraction: oxalic acid and phosphoric acid. The oxalic acid method is suitable for monitoring the cultivation process due to its low volume requirement, albeit it does not capture the full extent of daunomycin production. In contrast, the phosphoric acid method is more reliable and reproducible, effectively disrupting the iron-daunomycin complex. For oxalic acid extraction, transfer 400 µl of the sample to a 2 ml tube, acidify with 180 µl of 1M oxalic acid, and vortex thoroughly. Add 2 x 600 µl of acetone, vortex again, and then add 300 µl of distilled water. Mix well and centrifuge at 14,000–15,000 RPM for 6 minutes, then take 400 µl of the supernatant for HPLC analysis. For phosphoric acid extraction, shake the culture and take 10 ml, transferring it to a 50 ml Falcon tube. Add 40 ml of 0.1M H_3PO_4 (1:4 dilution) and mix thoroughly. Measure the pH, which should be between 1.8 and 1.9, before heating the sample in a 50°C water bath for 30 minutes, mixing periodically. After heating, check that the pH remains between 1.4 and 1.8; if it exceeds 2, yield may be compromised. Take a 400 µl sample and transfer it to a 2 ml tube, then centrifuge the Falcon tube for 6–7 minutes at 4000–4700 RPM. Discard the supernatant and resuspend the sediment (approximately 5 ml) in 40 ml of 0.05M H_3PO_4 (changing the dilution ratio to 1:8), mixing until homogeneous. Measure the pH again, ensuring it is ≤ 1.7, then heat the resuspended sample for another 30 minutes at 50°C, mixing periodically. Following this step, the pH should not fall below 1.3; take a final 400 µl sample for further analysis.

4.15. HPLC Sample Preparation

The HPLC sample preparation procedure is identical for both oxalic and phosphoric acid samples. Begin by transferring 400 µl of the acidified sample into a 2 ml tube. Next, add 180 µl of 1M oxalic acid and vortex the mixture thoroughly. Subsequently, add 1200 µl of acetone and vortex again, followed by the addition of 300 µl of distilled water and another vortex to ensure homogeneity. After mixing, spin the tube in a centrifuge at 15,000 RPM for 6 minutes. Finally, take 400 µl of the supernatant to serve as the HPLC sample.

4.16. Chromatography Conditions

The analysis utilizes a ProntoSIL C18AQ column (150 x 4.6 mm, 5 µm) maintained at ambient temperature, with a flow rate of 1 ml/min and UV/Vis detection at a

wavelength of 254 nm. The mobile phase consists of two components: Mobile Phase A (MPA) containing H_2O at pH 2.7 (adjusted with H_3PO_4) and 1 g/L of SDS, and Mobile Phase B (MPB) comprising acetonitrile, in an isocratic flow ratio of 50:50 (A:B). The retention time for daunorubicin is approximately 7 minutes, and the total analysis length is 25 minutes.

5. Results and Discussion

Optimization of the Production Medium

The original strain showed considerable variability in mycelium, colony, and spore morphology, with dwarfed spore chains formed by rudimentary spines or smooth spores, as noted by Blumauerová et al. (1978). In contrast, the producing strain Ret2210, developed through a process of repeated protoplast fusion involving triclosan-resistant strains and those capable of utilizing olive pomace oil, supplemented by an adaptive laboratory evolution technique, demonstrated significant morphological homogeneity. In the optimization process, the daunomycin production of the initial strain in a sugar-based medium was approximately 2.0 g/L. After establishing stable production, we replaced the strain with a new fusant specifically developed for oil-based media. Various ingredients were tested in the sugar medium to assess their influence on production and evaluated using High-Performance Liquid Chromatography (HPLC).

Substitution of Glucose with Oils

The addition of pomace olive oil, rapeseed oil, and soybean oil to the sugar medium resulted in a 20 % decrease in production compared to the sugar-only medium. Similarly, Perlman and Wagman (1952) reported that the glucose utilization rate was significantly higher than the soybean oil utilization rate and streptomycin production was lower when cultured on lipid medium. To optimize oil concentration, supplementation with yeast was necessary, with yeast quality significantly affecting the yield. The source and quality of oil profoundly influenced both media parameters and daunomycin output. Notably, rapeseed oil led to significant declines in production despite its fatty acid composition being similar to that of olive oil. In addition, cannabis oil produced under 5 % of the yield compared to the pomace oil medium. Fresh pomace oil, rich in these compounds, provides antioxidant benefits that mitigate oxidative stress (Čepo et al. 2018), particularly important for sensitive strains during cultivation. Incorporating olive pomace oil as a carbon source is a novel approach, serving as an alternative carbon source and offering potential antioxidant properties that mitigate oxidative stress from high iron concentrations. This dual functionality promotes nutrient supply and stress alleviation, a combination rarely observed in standard

growth media. Our experiments indicated that chemical changes over time in the same oil sample, represented by specific measurements, can significantly influence oil quality and media output. Media containing trivalent iron (Tsylents et al. 2024) demonstrated increased sensitivity to variations in oil source and quality.

Influence of Soy Source on Daunomycin Production

Soy grits may preserve more intact proteins and fibers from whole soybeans compared to the highly processed soy flour, significantly impacting metabolic pathways. As a potential source of phytic acid, soy grits can chelate excess iron (Zhang et al. 2014), reducing its bioavailability and minimizing toxicity (Cruz et al. 2020; Malik et al. 2022). This characteristic is particularly beneficial for maintaining stable growth environments for sensitive strains, an aspect often overlooked in standard cultivation protocols. Additionally, glycinins—an important component of soy proteins—are known to play a role in iron metabolism (Lynch et al. 1994). In contrast, replacing soy grits with a high-fat soy source has been shown to result in significant decreases in both media parameters and daunomycin production.

Influence of Iron on Production

Adding iron to the sugar medium caused a pronounced rust coloration after autoclaving, indicating the conversion of iron to its oxide form, which did not enhance daunomycin production. However, supplementing the original medium with yeast led to iron stabilization and increased production levels upon the addition of pomace olive oil, maintaining an olive-green-grey hue post-autoclaving. Incorporating both bivalent (Fe^{2+}) and trivalent (Fe^{3+}) iron sulfates—spontaneously formed during autoclaving—was critical for managing iron concentration. While many formulations typically use only one form of iron, providing both allows for better control over iron bioavailability and uptake, potentially enhancing metabolic processes without toxicity (Mancy et al. 1975).

Optimal Composition for Daunomycin Production

The optimal composition for sustained daunomycin production, identified through empirical methods, was determined to be as follows (g/L): 100 g pomace oil, 5.6 g $FeSO_4$, 10 g yeast, 20 g soy grits, 5 g yeast extract, 5 g glycerol, 2 g K_2HPO_4, 3 g

$CaCO_3$, and 1 g $MgSO_4$, with the pH adjusted to 6.0. Under these conditions, cultivation of the original strain achieved stable production levels of 3.0 to 3.5 g/L.
The newly developed fusion strain was subsequently tested in this optimized medium, yielding daunomycin production levels of 5.5 to 6.0 g/L post-optimization. To demonstrate the independent repeatability of production, 11 cultivations were conducted by different team members, confirming consistent daunomycin production in the developed medium.

Table 1. Production of daunomycin in optimized medium

Time of cultivation [h]	N [samples]	Production [mg/L]		pH	
		Average	SD	Average	SD
120	11	1389.0	350.7	6.40	0.17
168	11	3070.2	724.6	6.62	0.13
216	11	4728.2	726.1	6.72	0.10
264	11	5466.4	681.4	7.24	0.41

Monitoring of Daunomycin Production

Different approaches were employed to monitor and characterize daunomycin production in the newly developed medium, including assessments of media physical parameters, process monitoring, and ultrastructural morphology of the daunomycin-containing particles and producing microorganisms.

Changes in Media Color and Physical Parameters

A non-sterile medium that starts off as a specific colour—such as light brown—may experience alterations upon autoclaving owing to Maillard reactions or the breakdown of certain compounds. This initial transformation sets the stage for how microbial metabolism can further impact the visual characteristics of the medium. As the cultivation progresses, the medium transitions from a grey-olive green to a dark grey hue, indicative of the accumulation of metabolic byproducts produced by *Streptomyces* species. The 24 hours post-inoculation, it darkens to a deeper grey, continuing to evolve in color over time. By 48 hours post-inoculation, the color shifts further to dark grey, with visible green oily droplets emerging. Black particles, remnants of soy grits bound to iron, become apparent. The oil within the medium is not fully dispersed, creating bubbles along the sides of the flask. At approximately 72 hours after inoculation, the medium transforms into a dark grey-black colour. A layer of oil forms

on the surface, which may take on a slight orange hue as microorganisms begin to produce daunomycin in significant amounts. During the cultivation period (ranging from 72 to 264 hours), the medium undergoes notable changes in color: it transitions to a complete brown-red-black hue, and the number of visible particles at the bottom of the tilted flask increases. The oil becomes fully dispersed in the medium, exhibiting a significant orange color, indicative of daunomycin presence. When the flask is left undisturbed, a slight but discernible orange layer containing daunomycin may form at the top of the medium due to the oil. The initial color of the medium post-autoclaving, characterized by an olive grey/green hue, suggests the presence of oxidized compounds and iron in solution (Fe^{3+}), indicating that iron is in an oxidized state. This coloration is often indicative of specific iron compounds and potentially other components within the complex medium. Additionally, the olive or grey tint may arise from supplementary elements such as phytates from soy flour or phenolic compounds found in olive pomace oil, which contribute to this initial hue. As the substrate is utilized and the culture transitions into a more reduced state during cultivation, the medium darkens significantly. This color change correlates with increased anaerobic or reducing conditions, likely due to reduction processes facilitated by the metabolic activities of strain. The darkening indicates the formation of reduced iron species (Fe^{2+}) as Fe^{3+} is reduced during microbial respiration and other metabolic processes. Moreover, the accumulation of other metabolites or degradation products from microbial growth may further contribute to the overall darkening. At the peak of daunomycin production, the medium exhibits a complete black coloration, signifying a substantial shift in the chemical composition. This extensive reduction of ferric iron to ferrous iron, along with the production of complex organic molecules such as daunomycin and possibly other metabolites, results in this dark appearance. The black color may also indicate the formation of complex compounds, either through the aggregation of iron with organic matter or through the precipitation of iron complexes, particularly in systems where high concentrations of organic acids and phenolic compounds are present. The changes in color of the culture medium correlate closely with the redox potential (Eh) and the microbiological activity of strain. The darkening indicates the reduction of oxidized iron species and possibly the production of additional organic compounds (e.g., daunomycin and other metabolites), which uniquely alter the optical properties of the medium. The presence of black, hard precipitates at the end of fermentation mixed with inactive daunomycin that can be

liberated by strong acid calls for a deeper exploration of the chemical interactions occurring during the fermentation of *Streptomyces coeruleorubidus*. Here are some potential explanations for this phenomenon, implications for your process, and suggestions for further exploration.

Chemical Nature of formed Precipitate Complexes

The black precipitate is likely composed of complexed iron (Fe^{2+} or Fe^{3+}) in conjunction with organic matter produced during the fermentation process, including metabolites such as daunomycin. Precipitation occurs when iron ions interact with specific organic compounds or when pH levels shift due to microbial metabolism. Often, phenolic compounds and other secondary metabolites can bind with iron, resulting in the formation of insoluble complexes that aggregate into larger precipitates.

Inactive Daunomycin

The inactivity of the daunomycin in the precipitate could be due to its binding to iron or other components. When daunomycin complexes with iron or other molecules, it may lose its bioactivity (Abdella and Fisher 1985). This situation can arise due to structural changes in the drug or steric hindrance (Matzanke et al. 1992). The black precipitate likely represents a combination of iron-daunomycin complexes and other organic materials. The binding of daunomycin may render it unavailable in its active form.

Measurement of pH, DO, and Eh During Fermentation

To confirm the hypothesis that color changes in the culture medium result from alterations in redox potential (Eh) and iron reduction during fermentation, we conducted precise measurements of pH, dissolved oxygen (DO), and Eh throughout the fermentation process. The results presented aim to elucidate how these parameters interact and influence one another, thus providing insights into the biochemical processes during microbial growth. However, our results indicated that pH and DO were inadequate metrics for this purpose. While pH experienced minimal fluctuations, DO levels significantly declined within the first 24 hours, ultimately dropping to near-zero values. In contrast, the measurement of redox potential emerged as a highly

informative parameter. Our findings revealed a marked increase in Eh immediately after autoclaving, likely due to the oxidation of organic compounds and trivalent iron (Fe^{3+}) present in the medium. This observation suggests that redox potential is a more reliable indicator for monitoring fermentation processes and understanding the biochemical changes linked to color variations in the culture medium. Notably, we found an inverse relationship between Eh values and daunomycin production: as Eh decreased during fermentation, daunomycin production correspondingly increased. This correlation underscores the potential for utilizing redox potential as a tool to optimize conditions for enhanced secondary metabolite production.

Sediment friability

According to the proposed idea, sediment may consist of aggregates formed from smaller particles containing daunomycin and iron. To validate this hypothesis, we assessed the sediment's friability using a Malvern 2000 particle size analyzer. When the sediment was mixed with 0.1% SDS in water, the aggregates initiated a time-dependent disaggregation process characterized by an increase in the number of smaller, free particles. These particles seem to undergo spontaneous aggregation throughout the fermentation process. This phenomenon may be partially explained by changes in the consistency of the growth medium, as well as a potential increase in precipitate formation during fermentation. The interaction between these factors likely facilitates the aggregation process, forming the observed sediment. The data obtained significantly enhance our understanding of the nature of the sediments. To further clarify the formation and morphology of the sediment-forming particles, it was crucial to observe their morphology during the aggregation process.

Observation of Morphological Changes During Fermentation

The morphological diversity observed during the fermentation process highlights various particle sizes and structures formed throughout synthesis. The medium contains a multitude of particles exhibiting different shapes and sizes, ranging from nanoparticles to more organized structures, which likely correspond to different stages of fermentation and detoxification. The presence of nanoparticles indicates successful iron reduction and the formation of stable iron-containing particles (Afonso et al. 2024). These nanoparticles are characterized by their small size, which contributes to unique properties, including increased surface area and potential bio-reactivity. Micro-

sized particles, ranging from approximately 3 to 5 microns, likely represent intermediate structures developed during fermentation. These particles are composed of membrane-bound, optically dense materials, which may indicate the aggregation of nanoparticles, and are covered by a shell formed by dark oval structures. Moreover, the identification of larger sediment pieces, greater than 100 microns, suggests the aggregation of smaller particles, potentially facilitated by interactions with extracellular polymeric substances (EPS) produced during microbial growth or the settling of heavier complexes that form throughout the fermentation process. Scanning Electron Microscopy (SEM) analysis of the native samples reveals that these large particles are covered by a layer of inorganic material, identified by X-ray Diffraction (XRD) analysis as gypsum. This inorganic surface layer may stabilize the particles and prevent the release of daunomycin into the medium. Exposure particles to hydrogen peroxide resulted in the bleaching of darker pigmentation, liberating reddish vesicles. Extended exposure led to an increase in the number of vesicles formed. High-Performance Liquid Chromatography (HPLC) analysis of the collected vesicles confirmed the presence of daunomycin. The release of pigmented vesicles or vacuoles suggests that daunomycin is likely localized within membrane-bound structures inside the larger particles rather than existing in a freely accessible form. This finding implies that the optically dense structures identified by Transmission Electron Microscopy (TEM) may serve as reservoirs for daunomycin, effectively isolating it from the surrounding environment. The presence of iron in the particles was proven by a combination of SEM and EDS.

Detection and identification of proteins in the daunomycin-iron-organic matter complex

To determine the daunomycin binding or transporting protein homogenate from the washed sediment was prepared in a sample buffer and analyzed on 10% gel. The proteins involved in daunomycin interaction were stained directly by a metabolite and control visually. Samples for SDS-PAGE were prepared in a reducing buffer, boiled, and separated on a 10% gel, followed by staining with Coomassie Blue. After destaining, the visible proteins were excised and analyzed by TOF MALDI to determine the proteins present in the complex. Only results with more than three matches and greater than 20% sequence coverage were considered positive. Under conditions characterised by high iron content and chemical stress, two distinct groups

of proteins exhibit critical functional similarities in managing oxidative stress and enhancing cellular resilience.

Table 2. Proteins detected in daunomycin-iron-protein particles.

GroupOne:	**Group Two:**
• Bacterioferritin • Catalase • Oxidoreductase • Glycinin G1 • Glycinin G4 • β-Conglycinin	• Chemical-damaging agent resistance protein C • Major outer membrane lipoprotein • Xanthine dehydrogenase • Molybdopterin dehydrogenase • Aklanon acid methyl ester cyclase (Dau D) • Superoxide dismutase • Nickel-containing superoxide dismutase • Glutamate-binding protein

In a high iron content medium, proteins such as bacterioferritin (Rivera 2017), catalase, (Mahaseth and Kuzminov 2016; Kim et al. 2021), oxidoreductase (Toone 2006; Sirivech et al. 1977; Martelin et al. 2002), glycinin G1, glycinin G4, and β-conglycinin exhibit functional similarities that are crucial for managing iron homeostasis and mitigating oxidative stress related to elevated iron levels (Schnepf 1984; Lynch et al. 1994; Yang et al. 2021). Bacterioferritin plays a vital role in sequestering excess iron to prevent toxicity by storing it in a biologically accessible form while facilitating its release when needed for cellular processes (Rivera 2017). Catalase is essential in protecting cells from oxidative damage that can arise from increased iron, particularly by catalyzing the formation of reactive oxygen species, including hydrogen peroxide. Oxidoreductases contribute to redox balance in the presence of high iron by facilitating necessary metabolic reactions and detoxifying harmful compounds. Additionally, storage proteins like glycinin G1, G4, and β-conglycinin provide essential amino acids and may participate in cellular stress responses, modulating protein synthesis and repair processes in response to elevated iron levels (Kim et al. 2011). Together, these proteins work synergistically to maintain iron homeostasis, protect against oxidative stress, and support overall metabolic functions, reinforcing resilience in high iron environments.

Although not quantified, phytic acid, which is present in soy grits (Deak and Johnson 2007), serves as a multifunctional component in high iron content systems . It supports iron chelation, acts as an antioxidant, regulates mineral absorption, and may

influence cellular signaling and gene expression. Its presence can synergize with the aforementioned proteins, bolstering iron homeostasis and enhancing the organism's overall resilience under stress conditions (Aguree et al. 2020; Minihane and Rimbach 2002).

The second group of proteins—including chemical-damaging agent resistance protein C, major outer membrane lipoprotein, xanthine dehydrogenase (YagS FAD-binding subunit), molybdopterin dehydrogenase, aklanonic acid methyl ester cyclase (Dau D), putative flavoprotein, superoxide dismutase, nickel-containing superoxide dismutase, and glutamate-binding protein—exhibits notable functional similarities related to stress response. Specifically, these proteins contribute to the organism's ability to withstand chemical toxicity and oxidative stress. The chemical-damaging agent resistance protein C provides protection against harmful compounds, while the major outer membrane lipoprotein helps maintain membrane integrity under stress (Cascales et al. 2002). Xanthine dehydrogenase (Ohe and Watanabe 1979) and molybdopterin dehydrogenase (Leimkühler and Iobbi-Nivol 2016) are critical for redox balance, managing reactive oxygen species. Although the aklanonic acid methyl ester cyclase is primarily associated with secondary metabolite biosynthesis of anthracyclines (Kantola et al. 2000), it also participates in metabolic pathways essential for stress resilience. Additionally, superoxide dismutase and nickel-containing superoxide dismutase directly combat oxidative damage, converting harmful superoxide radicals into less toxic molecules (Youn et al. 1996). Finally, glutamate-binding protein belongs to ABC transporters and may support cellular homeostasis indirectly under stress conditions (Le Maréchal et al.2013). Collectively, these proteins enhance the strain’s survival and adaptability amidst chemical stresses, underscoring their importance in the strain’s metabolic and stress response networks.

It is of the utmost importance to highlight the unique origin of the Streptomyces strain employed in this study, which was isolated from mosquito larvae. This research not only delineates a novel cultivation process for the production of daunomycin but also illuminates the significant potential of insect-derived microorganisms as valuable assets in pharmaceutical research. Insects, representing one of the most diverse taxa on the planet, are hosts to a vast array of microorganisms, which may yield a wealth of bioactive compounds relevant to drug development. Recent studies have increasingly recognized these insect-associated bacteria as promising sources for

pharmaceutically active substances (Chevrette et al. 2019; Van Moll et al. 2021; De Smet et al. 2021; Diarra et al. 2024).

The metabolic diversity inherent in these insect-associated microbes, cultivated over millions of years of co-evolution with their hosts, presents a significant opportunity for advancing drug discovery, particularly in the context of rising antimicrobial resistance and the emergence of novel diseases (Dettner 2011; Bode 2011). It is therefore evident that the exploration of this hitherto underutilized microbial reservoir has the potential to pave the way for the identification of innovative therapeutic agents, thereby making a significant contribution to the ongoing efforts in pharmaceutical research and development.

6. Overview of the Obtained Results

The results achieved during the dissertation were prepared in two manuscripts:

Pudhuvai B., Beneš K., Čurn V., Bohata A., Lencova J., Vrzalova R., Barta J. and Mat'ha V.: Enhancement of Daunomycin Production in Streptomyces sp. By Counteracting Autotoxicity – A Prospective. Microorganisms (submitted to edition).

This review is focused on the issue of daunorubicin (DNR) and the possibility of increasing the production of this metabolite using a specific culture medium. Our prospective review sheds light on a method involving perturbation that enhances the precursors to regulate PKS II biosynthesis, enhancing cells' capacity to increase secondary metabolite production. The suggested method also entails the preparation of culture media for the cultivation of *Streptomyces* sp. and enhanced yield of DNR and making it inactive with iron or its reduced forms following efflux from the producer. The iron or iron-DNR complex is encapsulated by oleic acid or lipid micelle layers in the culture media, finally resulting in the generated inactive DNR and the DNR-iron-oil complex. This idea has the potential to protect the producer organism from autotoxicity and prevent the inhibition of metabolite production. The approach of substituting sugar with oil in culture media has dual role where in promotes the Streptomyces growth by utilizing lipids as an energy source and encapsulating the generated DNR-iron complex in the medium. In this review, we discussed aspects like anthracycline producers, biosynthesis pathways and gene regulation, side effects of DNR, mechanism for autotoxicity evasion and culture media components for enhancement of DNR production in Streptomyces sp. We anticipate that our work help researchers working with secondary metabolites production and decipher a methodology that would enhance DNR yield and facilitate the extraction of the resulting DNR by lowering costs in large-scale fermentation.

Beneš K., Pudhuvai B., Čurn V., Motis J., Rost M., Michalcova Z., Vilcinskas A. and Mat'ha V.: Autonomous Defense Through Biogenic Nanoparticle Formation in Daunomycin Producing Streptomyces. Microorganisms (submitted to edition).

This study focuses on the development of a cultivation medium specifically designed to induce auto-resistance through Autonomous Defense Through Biogenic Nanoparticle Formation. Utilizing a fermentation medium enriched with olive pomace oil and iron, this research aims to explore the inherent affinity of daunomycin for both iron and oil. Our strategy leverages the combination of olive pomace oil and bacteria to facilitate the formation of iron nanoparticles (NPs). Furthermore, it seeks to bridge the existing knowledge gap by investigating the effects of daunomycin on eukaryotic cells in conjunction with the underexplored data from prokaryotic organisms, especially those involved in the production of daunomycin. This dual approach aims to enhance our understanding of daunomycin's mechanisms of action and its potential applications in both eukaryotic and prokaryotic systems. By systematically optimizing iron levels, we aim to facilitate the formation of daunomycin-iron complexes that can reduce the solubility and bioavailability of daunomycin. This transformation not only alleviates its cytotoxic effects on microbial production strains but also has the added ecological benefit of producing daunomycin as an insoluble precipitate. This precipitate can be easily separated from the cultivation medium by filtration or centrifugation, concentrated into a smaller volume, and extracted using phosphoric acid, followed by a final extraction of the dissolved daunomycin using significantly reduced volumes of organic solvents. Thus, this approach targets the reduction of toxic impacts during fermentation while enhancing compound yield and overall production efficiency, all while minimizing the environmental footprint of the proces. This research not only contributes to the field of microbial fermentation and antibiotic production but also emphasizes the importance of minimizing environmental impacts through the production of insoluble daunomycin precipitates that can be efficiently recovered from the cultivation medium. Overall, these findings present promising avenues for further investigation into the mechanisms underlying biogenic nanoparticle formation and the optimization of cultivation processes.

Pudhuvai B., Beneš K., Čurn V., Bohata A., Lencova J., Vrzalova R., Barta J. and Mať ha V.: Enhancement of Daunomycin Production in Streptomyces sp. By Counteracting Autotoxicity – A Prospective. Microorganisms (submitted to edition).

microorganisms

MDPI

Review

Enhancement of Daunomycin Production in *Streptomyces sp.* By Counteracting Autotoxicity – *A Prospective*

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Abstract: Daunorubicin (DNR) is an anthracycline antibiotic originating from soil-dwelling actinobacteria extensively used to treat malignant tumors. Over the decades, extensive attempts were made to enhance the production of anthracyclines by introducing genetic modifications and mutations in combination with media optimisation, but the target production levels remain comparatively low. Developing an appropriate culture medium to maximise the yield of DNR and preventing autotoxicity for the producing organism remains a challenge. Our prospective review sheds light on a method involving perturbation that enhances the precursors to regulate PKS II biosynthesis, enhancing cells' capacity to increase secondary metabolite production. The suggested method also entails the preparation of culture media for the cultivation of Streptomyces sp. and enhanced yield of DNR and making it inactive with iron or its reduced forms following efflux from the producer. The iron or iron-DNR complex is encapsulated by oleic acid or lipid micelle layers in the culture media, finally resulting in the generated inactive DNR and the DNR-iron-oil complex. This idea has the potential to protect the producer organism from autotoxicity and prevent the inhibition of metabolite production. The approach of substituting sugar with oil in culture media has dual role where in promotes the *Streptomyces* growth by utilizing lipids as an energy source and encapsulating the generated DNR-iron complex in the medium. In this review, we discussed aspects like anthracycline producers, biosynthesis pathways and gene regulation, side effects of DNR, mechanism for autotoxicity evasion and culture media components for enhancement of DNR production in *Streptomyces* sp. We anticipate that our work help researchers working with secondary metabolites production and decipher a methodology that would enhance DNR yield and facilitate the extraction of the resulting DNR by lowering costs in large-scale fermentation.

Keywords: Daunomycin; Anthracyclines; *Streptomyces*; Efflux; Autotoxicity; Enhancement, Oil-based medium

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1. Introduction

Anthracyclines are a class of chemotherapeutic compounds derived from soil-dwelling gram-positive actinobacteria that have been used as anticancer medication agents [1]. They have been widely utilized to treat leukemia and cancer in adults and paediatrics since their discovery in *Streptomyces peucetius* in the 1960s [2–6]. The term "Anthracy-

clines" was introduced to denote the colour (red to yellow-red op-tical dyes) of the chemical derivatives 7,8,9,10-tetrahydro-5,12-naphtacenoquinones [7,8]. The anthracyclines are tetracyclic aromatic polyketides that are produced by the PKS II (polyketide synthase type II) pathway and are structurally composed of an anthraquinone (aglycone) moiety and an amino sugar (carbohydrate unit) at the C7 or C10 or at both positions. The absence of sugar at C10 is substituted by a carbo-methoxy or hydroxyl group through processes like glycosylation and hydroxylation [1,9,10].

Daunorubicin (DNR), also called daunomycin, is an anthracycline antibiotic that has been extensively employed in treating malignant tumours, ovarian cancer, breast cancer and AML (acute myeloid leukemia) since its discovery in 1964 from *Streptomyces peucetius* [11–13]. DNR was initially derived, successfully isolated from *Streptomyces peucetius* sp. (strain FI 1762-B-101) and found to possess antitumor activities. During the same period, French researchers isolated a similar substance from *Streptomyces ceoruleorubidus*, naming it rubidomycin [14]. The successful characterization of the compound and clinal use case has led to the development of several hundreds of anthracycline analogues through synthetic chemistry and modified bacteria [15]. The latter research in 1969 on a mutant of *S. peucetius* has led to the identification and isolation of a homologue (related compound) named Doxorubicin (DOX) with better efficiency in solid tumours [16]. The DNR and DOX share tetracyclic aglycone and daunosamine sugar moieties but differ in their side chains, where DOX terminates with primary alcohol and DNR with a methyl group, as shown in **Figure 1**.

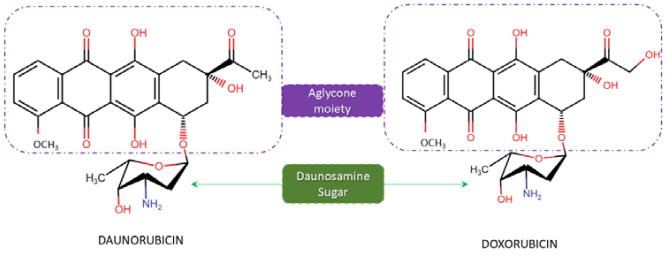

Figure 1. Structure of daunorubicin (DNR) and doxorubicin (DOX) with the aglycone sugar moieties

The clinical use of DOX and DNR was hampered due to their increased concerns and deaths due to cardiotoxicity and the development of resistance in tumour cells [5,15]. Despite the demonstrated toxicity of daunomycin in Guinea pigs, rats, and humans [17–19], the cumulatively reduced dosages administered during chemotherapy allow the mitigation of these risks [20,21]. A minor structural modification of anthracyclines significantly affected the bioactivity, which had driven chemists in the 1970s to synthesise anthracycline derivatives with reduced toxicity [10]. The interest and progress in synthesis pathway engineering (synthetic and semi-synthetic via site-directed mutations and gene alterations) of anthracyclines and their analogues were carried out after the 1990s due to their exciting catalysing properties [8]. The quest to find a better alternative with reduced toxicity has led to the development of thousands of analogues with many substitution reactions in the anthraquinone moiety (tetracyclic structure) [5]. Out of which currently, six semi-synthetic derivatives, including DOX, idarubicin, epirubicin, pirarubicin and valrubicin, are under clinical use. The class of anthracyclines and their derivatives, including doxorubicin, epirubicin, daunorubicin, and idarubicin, are the most potent anticancer drugs ever discovered, having the ability to alter mitochondrial dynamics by intercalating with DNA helix and cause cytotoxicity. These drugs cause accelerated senescence in tumour cells via inducing apoptosis, DNA alkylation and damage, autophagy, activating

MAPK (mitogen-activated proteins kinases), promoting antimetabolites, inhibiting topoisomerases, and other mechanisms that result in cell death or cell cycle arrest [22–26].

Over the past decades, numerous efforts have been made to generate anthracyclines through multiple initiatives, which have led to the creation of several strains with genetic modifications, mutations, and changes to culture conditions and media. Nevertheless, despite the accomplishment of numerous intricate investigations and genetic modifications, the desired output production numbers by fermentation have still to be attained. Engineering of suitable culture media in a way to sufficiently achieve the enhanced production of DNR/DOX and make them inactive to evade the self-toxicity to the producer organism still remains void. The alteration of strain for enhanced production and culture media, conditions in order to entrap the produced DNR/DOX compounds in media and turning them non-toxic for the producer proves as an ideal strategy. Taking into account the complex gene functions and metabolic intricacies in the PKS-II pathway, the alterations towards the efflux systems, metabolic pathway inducers, and interaction of DNR/DOX with other elements and substances should be considered to design a hypothetical culture media and conditions.

In this review, we have discussed several aspects of the production of DNR/DOX by *Streptomyces*, their activity, biosynthesis, gene regulation, toxicity, and how the culture media could be designed for production enhancement based on the interaction of daunomycin with iron and oil and self-resistance property of bacteria. Our prospective idea of strain development and culture media optimisation would benefit the investigators in the microbiology, bioprocessing, therapeutic and industrial biotechnology fields to enhance production with reduced expenses at a commercial level.

2. The Anthracycline Producers

The anthracycline compounds occurring in nature are the secondary metabolites produced by the Actinobacteria, especially in the genus *Streptomyces* [27]. Streptomycetes are gram-positive bacteria, thriving in aquatic and terrestrial habitats, possessing a lifecycle similar to filamentous fungi, reproduce through sporulation, and exhibit siderophore activity (breakdown of macromolecules in the environment) and are known for their capacity to synthesize a variety of secondary metabolites such as antibiotics, enzymes, and pigments [28,29]. The common metabolites produced by nearly all *Streptomyces sp.* are siderophore desferrioxamine (iron chelator), geosmin (earthy smell organic compound), streptothricin (antibiotic) and streptomycin (antibiotic) [30,31]. The organisms contain a multitude of biosynthetic gene clusters (BCGs) and transcription units in their genome, which are responsible for the production of secondary metabolites according to the cues in their natural habitat or environment [31]. Approximately 90% of the know antibiotics are obtained from the organism of the genus *Streptomyces*. To date, more than 500 naturally occurring anthracyclines have been isolated from *Streptomyces sp.*, which are widely considered medically important [32,33]. *Streptomyces ceoruleorubidus* is a potentially important *Streptomyces* bacteria that employs the synthesis of antifungal, antibacterial, immunosuppressive, and antitumor compounds such as doxorubicin and daunorubicin [34–36]. This proliferative ability for synthesizing such metabolites can be altered by influencing variables such as nitrogen and carbon sources and culturing conditions such as temperature, pH, and incubation period, which can play a critical role in the economic dynamics involved in secondary metabolite production. Although the mutagenesis method is the earliest option, it remains one of the most effective methods for producing high-yield strains in experimental models. The use of physical and chemical mutations in the *Streptomyces* species has been reported to have enhanced the production of secondary metabolites, especially anthracyclines [37–39].

3. Synthesis of Daunomycin

The production of secondary metabolites occurs through two phases: trophophase (normal growth phase), followed by idiophase (capacity to produce metabolites), where,

at times, both phases can be regulated, overlapped and changed with the alterations in media and growth conditions [5]. Enhancing secondary metabolite production can be possible with the development of resistance in the cells as the produced compounds are autotoxic. But in the course of any selection event over the past decades, focusing on improved resistance did not impact or lead to enhanced production. This led to the works on biosynthetic gene cluster alterations and expression enhancement of activator genes, transcription factors and increased mutations in promotor genes [5,40].

3.1. Biosynthetic Gene Clusters (BGCs)

The importance and biosynthesis of daunorubicin and its gene clusters have been characterized by two BGCs in different strains [41,42]. The majority of BGCs share homologous genes encoding monofunctional enzymes for the assembly of aglycone units, and the BGC for DNR (daunorubicin) and DOX (doxorubicin) was sequenced in *Streptomyces peuctius* ATCC 27952, and a 40kb sequence encoding the BGC for DNR, DOX consisting of 37 ORFs (open-reading frames) was identified [43]. The distinctive characteristics among the BCGs include a high abundance of glycosyl transferases, gene sets involved in deoxysugar production and a repertoire of tailoring genes for secondary metabolite. The DNR/DXR biosynthesis is completed in four steps: (A) formation of aglycone (ε-rhodomycinone), (B) formation of an active sugar moiety (thymidine diphosphate daunosamine), (C) glycosylation of ε-rhodomycinone and (D) post polyketide modifications (decarboxylation, methylation and hydroxylation) [41,44].

3.1.1. Formation of ε-rhodomycinone

The biosynthesis of DNR starts with the formation of aglycone ε-rhodomycinone, which is the important intermediate, synthesised by the PKS-II synthase by the genes *dpsA*, *dpsB*, *dpsC*, *dpsD*, *dpsE*, *dpsF*, *dpsG* and *dpsY*. The nine malonyl-CoA units with a propionyl CoA starter unit undergo serial condensations to form a 21-carbon decaketide, where the multiple reactions are facilitated through the polyketide synthase enzymes. The enzymes are produced by the genes *dpsA* (3-oxoacyl ACP synthase), *dpsB* & *dpsC* (ketosynthases), *dpsD* (acyltransferase) and *dpsG* (acyl carrier protein) to form a decaketide compound [45]. The *dpsE* (ketoreductase) carries out the ketoreduction of the decaketide, followed by an aldol condensation and ring cyclization (3 steps) through catalyzing enzymes produced by *dpsF*, *dpsY* and forms a 12-deoxy alkanoic acid [46]. The intermediate undergoes a *dnrG* (monooxygenase) mediated reaction, which adds a keto group to form alkalonic acid and is further transformed to aklaviketone by *dnrC* (alkanoic acid-S-adenosyl-1-methionine methyl ester transferase)- a homodimeric protein [41,47]. The formed aklaviketone intermediate undergoes cyclization by *dnrD* (alkanoic acid methyl ester cyclase) to form a 7-oxo moiety, which is further reduced to a hydroxy group of aglycone ε-rhodomycinone by *dnrH* (aklaviketone reductase) and *dnrF* (hydroxylase) [47]. The biosynthesis of aglycone ε-rhodomycinone, including the genes involved, is shown in the **figure (2A).**

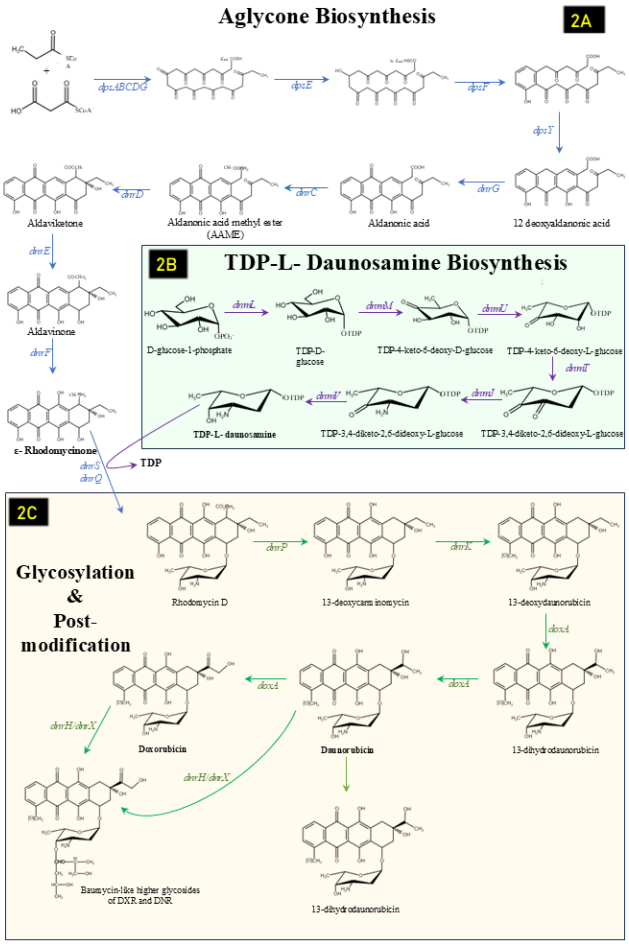

Figure 2. Biosynthesis pathway and involved genes of daunorubicin (DNR) and doxorubicin (DOX) in *Streptomyces* with A) the aglycone moiety synthesis, B) the sugar moiety, and C) the glycosylation and post-modification steps in DNR/DOX synthesis.

3.1.2. Formation of a sugar moiety (Thymidine diphosphate-L-daunosamine)

The biosynthesis of dTDP-L-Daunosamine is initiated from D-glucose-1-phosphate, which is carried out by the BGC, including seven genes *dnmL, dnmM, dnmU, dnmT, dnmJ* and *dnmV* [45]. The *dnmL* (transferase) and *dnmM* (dehydratase) catalyse the reaction to generate the intermediate TKDG (thymidine diphosphate-4-6-deoxy-D-glucose). The epimerase produced by *dnmU* converts TKDG to TKLG (thymidine diphosphate-4-6-deoxy-

L-glucose) through epimerization [48]. The *dnmT* (hydratase) and *dnmJ* (aminotransferase) facilitate the addition of a keto and an amino group at the C-3 position of the TKLG intermediate. The *dnmV* (ketoreductase) reduces the ketone to the hydroxyl group at the C-4 position to produce dTDP-L-Daunosamine [49]. The biosynthesis of an active sugar moiety (Thymidine diphosphate-L-daunosamine), including the genes involved, is shown in figure **(2B).**

3.1.3. Tailoring reactions/modifications in DNR/DOX biosynthesis

The ε-rhodomycinone undergoes glycosylation with dTDP-L-Daunosamine in the presence of enzyme from *dnrS*/*dnrQ* to generate rhodomycin D. The *dnrP* (esterase) converts rhodomycin D to 13-deoxy-carminomycine, which undergoes an *O*-methylation by *dnrK* (methyltransferase) to generate 13-deoxy-daunorubicin [50]. The intermediate undergoes C-13 oxidation by *DoxA* (cytochrome P450 enzyme) in two steps to produce 13-dihydro daunorubicin and DNR (daunorubicin)[51]. Daunorubicin is hydroxylated later at the C-14 position through *DoxA* to form DOX (Doxorubicin)[45,52]. The biosynthesis of DNR and DOX, including the genes involved, is shown in **figure (2C).**

3.2. Gene regulation in DNR/DOX biosynthesis

The BGC responsible for the biosynthesis of polyketide and sugar moieties in DNR/DOX also includes the regulatory genes for the initiation, regulation and termination of the entire synthesis pathway. The production pathway is regulated by the genes including *dnrO*, *dnrN* and *dnrI*, the transcription factors, where *dnrO* holds a significant importance in initiating the pathway. The *dnrO* produces a DNA helix binding domain, which is a key transcriptional regulator that activates the *dnrN* transcriptional activator, which finally leads to the activation of *dnrI*. The *dnrI* encoding enzyme binds to several polyketide synthases and facilitates the activation of efflux regulatory genes and initiation of DNR biosynthesis. The BGC also includes a transcriptional repressor *drrD*/*dnrW*, which promotes transcriptional control by coherent feed-forward loop, self-resistance and feedback regulation [45,53]. The *drrD*/*dnrW* regulates the master transcription factor *dnrI*, which is crucial for the DNR/DOX biosynthesis. Deleting *dauW*(ortholog of *drrD*/*dnrW* in *S. ceoruleorubidus)* has increased the production of DNR by 8 folds [54].

The maintenance of the produced DNR requires regulation inside the producer organism as the compound exhibits toxicity by intercalating with cellular DNA and eventually leading to cell death. The regulation of the lethal concentrations of produced DNR inside the cell is conferred by the *drrAB* locus - includes the drrA and drrB proteins necessary for the efflux of the finished product [53,55]. The expression and function of drrA and drrB are interdependent on each other at an ATP-driven pump, where DrrA is a peripheral membrane protein acting as an energy-transducing unit inside the cell when bound to the ATP in a DOX-dependent manner and drrB is the internal protein with hydrophobicity and helps in the efflux of produced DNR/DOX [56,57]. A mutant strain without the *drrAB* has exhibited a decline in DNR production and resulted in cell death, and overexpression of *drrAB* has resulted in the overproduction of DNR and promoted self-resistance [58]. Thus, the self-resistance genes also have an indirect effect on the biosynthetic pathway in DNR/DOX production [59]. Another resistance gene is *drrC*, which functions in the presence of ATP and DNR by binding to the DNR intercalated DNA and propelling it outside of the cell. This self-resistance gene maintains cell viability and regulates the lethal concentrations of DNR in a dependent manner, which relies on *dnrN* and *dnrI* in the biosynthetic pathway [50].

The entire pathway and its regulation decide the fate of DNR/DOX quantity production in *Streptomyces sp.* Thus, over the past decades, researchers have considered engineering the genes involved in the biosynthesis of aglycone, sugar moiety, tailoring reactions, transcriptional factors, transcriptional repressor and self-resistance to improve DNR/DOX production at an industrial level for commercial uses in cancer medication. The present

techniques of modifying genes to enhance the production of DNR/DOX are not effective due to the complex cellular enzymatic reactions involved. These approaches have not provided a clear understanding of the entire mechanism and also could not participate in significant improvement of DOX production.

4. Daunomycin Mode of Action

Since their discovery, the DNR and DOX have been extensively employed for treating solid tumours but have faced significant drawdown due to their toxic properties. Anthracyclines enter cells through cation transport and passive diffusion, eventually leading to alterations in the proteasome and nucleosome [60].

4.1. DNA intercalation

Anthracyclines exhibit a strong affinity for DNA by inserting their aglycone moieties between the base pairs, causing the separation of the existing base pairs, and positioning their sugar components in the minor groove of the DNA [61]. DNR and DOX have a preferential ability to bind to DNA at GC base pairs of both mitochondrial and nuclear DNA by establishing hydrogen bonding between the hydroxyl group on the C-9 position at aglycone moiety and N2, N3 of guanine[62–64]. This inhibits cellular DNA transcription, replication, recombination and repair, which creates torsional stress. The torsional stress alters the structure (disassociation of H2A/H2B dimers from histone core) and dynamics of nucleosomes [65,66]. The histone eviction caused by DOX/DNR (in H3 due to rich GC base pairs) majorly due to the sugar moiety binding to DNA critically causes chromatin damage, which leads to epigenomic aberrations and transcriptional alterations [60,67].

4.2. Topoisomerase II (Topo II) poisoning

The Topoisomerase II (Topo II) induces double-stranded breaks (DSBs), releases torsional stress and re-ligates the DNA breaks, ensuring the proper DNA transcription, replication and repair [68]. Anthracyline interacts with the Topo II enzyme to form an anthracycline-topoisomerase-DNA quarternary complex. It induces irreversible DNA damage by preventing the regeneration of phosphodiester bonds between the DNA strands [60]. DNR/DOX intercalates the Topo II DNA with their cyclohexane ring A in aglycone moiety and 4-methoxy group in sugar moiety. The changes in the functionality of Topo II to a DNA nuclease generate genomic instability, activation of DNA damage response and TP53 pathways, eventually leading to cell death [15]. In mammals, the Topo II enzyme is distinguished into isoforms Topo IIα (generate replication forks during mitosis in actively diving cells) and Topo IIβ (expressed in most cell types devoid proliferation status), where the DOX interacts with Topo IIβ in cardiomyocytes and lead to cardiotoxicity [69,70].

4.3. Formation of DNA adducts

Anthracyclines form DNA adducts between the two strands through covalent and hydrogen bonds with aglycone and sugar moieties, respectively. The DOX-DNA covalent bond in the cancerous cell is facilitated by the cellular formaldehyde, produced due to free radicle reactions with polyamines and lipids is responsible for the block in transcription, DSBs and replication [71]. *In-vitro* studies using DOX by pre-activated formaldehyde resulted in the formation of transcriptional blocks through the formation of inter-strand adduct (G-DOX-G cross-linking), inhibiting the transcription process [72]. The treatment of mice cancer cell lines with DOX leads to the disruption of the replication process and cell cycle arrest through the blocks in [8H]-thymidine [73,74]. The investigations involving DOX and DOX-formaldehyde conjugate on colorectal cancer cell lines for DNA repair mechanisms resulted in DNA adduct-induced damage. The studies also prove the damage (apoptosis) caused by DOX-DNA adducts is independent and does not rely on the Topo II activity [75–77].

5. Side effects of DNR/DOX

Over the past five decades, DNR/DOX has been significant in cancer treatment; however, its application is associated with adverse effects predominantly affecting the bone marrow and cardiac muscle, resulting in bone marrow suppression and cardiotoxicity [15]. Despite efficiently targeting tumour cells, their associated side effects on healthy cells during treatment adversely affect their functionality. The associated effects include acute and reversible chemotherapy-related symptoms such as nausea, vomiting, diarrhoea, stomatitis, mucositis, alopecia, gastrointestinal problems, rash, and bone marrow suppression [60]. The long-term effects include cardiotoxicity, nephrotoxicity, gonadotoxicity, and several therapy-related malignancies, which impact the patient's quality of life and severely limit the usage of anthracyclines. Factors like dosage, treatment length, and the patient's individual risk factors determine the possibility of developing anthracycline-related adverse effects.

5.1.Cardiotoxicty

Cardiotoxicity is a well-documented adverse effect of anthracycline chemotherapy. It encompasses both acute and chronic detrimental impacts on the heart, ranging from myocardial changes, impaired contraction ability, cardiomyopathy, arrhythmias and heart failure necessitating heart transplantation [78,79]. The mechanisms involving the DNR/DOX-induced cardiotoxicity are due to the inhibition of Topoisomerase [80], mitochondrial dysfunction (membrane permeability and transcription enzymes) [81,82], iron ion metabolism, imbalance in calcium homeostasis in cardiac muscles [83], oxidative stress and ROS generation [84], loss of ATP production and cell apoptotic pathways [85,86]. The molecular mechanisms explaining the detailed functioning effects of individual DNR/DOX-induced pathways regarding mitochondrial activity and apoptosis were explained here [82,87].

The enzymatic pathways leading to free radical formation in cells are mediated by mitochondria, which hold abundant quantities of phospholipid in their internal membranes called cardiolipin. DNR/DOX has a high affinity for cardiolipin, which makes its penetration inside the mitochondrial membranes easier [88]. The mitochondrial numbers in cardiomyocytes are typically higher compared to other cellular organelles. Under normal conditions, cardiomyocytes hold negligible amounts of active free iron, and most iron is bound to cellular proteins. The DOX/DNR also possess a strong affinity for iron and disrupts the Iron haemostasis in the cells through a redox reaction, reducing the cellular iron and cyclically forming the DOX-Fe complexes between Fe^{2+} and Fe^{3+} [89].

The derivatives of DNR/DOX can chelate iron from cellular iron-sulfur clusters of cytoplasmic iron regulatory protein 1(IRP-1), making it inactive and diminishing the synthesis of ferritin (iron storage protein). The inactive IRP-1 leads to elevated levels of free intracellular iron [5]. The daunomycin under abundant iron conditions leads to increased cellular ROS (reactive oxygen species) and induces oxidative stress in cardiomyocytes [82,90].

The anthraquinone moiety in anthracyclines can be reduced by NADPH (nicotinamide adenine dinucleotide phosphate dehydrogenase) to semiquinone radicals, which further undergo an oxidation reaction to generate anthraquinone radicals and superoxide anions (O^{2-}). The enzyme superoxide dismutase (SOD) converts O^{2-} to hydrogen peroxide (H_2O_2). The accumulated H_2O_2 undergoes the Fenton reaction and produces toxic hydroxyl (-OH) radicals, increasing intracellular ROS levels and oxidative stress, leading to cardiac impairment [91].

The prevention of anthracycline-induced cardiotoxicity involves the co-administration of cardio-protectant compounds like dexrazoxane, neuregulin, β-blockers (carvedilol & nebivolol), aldosterone antagonists, atorvastatin, angiotensin receptor blockers (ARBs), ascorbic acid, and sodium-glucose transport protein-2 inhibitors (SGLT-2) [92,93]. The natural or herbal-derived compounds like polyphenols, alkaloids, saponins, terpenoids

and various polysaccharides, along with their positive impacts in model studies for evading anthracycline-induced cardiotoxicity, have been reviewed in detail here [93]. The iron chelator dexrazoxane reduces anthracycline-dependent ROS generation, oxidative stress, and DNA double-strand breaks. It has significant clinical efficacy, decreasing cardiac toxicity without reducing anthracycline activity or enhancing secondary malignancies. Dexrazoxane is the only FDA and EMA-approved cardioprotective treatment for anthracycline cardioprotection.

The early mechanisms of using Fe-(HDOX/HDNR)$_3$ complex compared to the parent compound have higher antitumour activity in P 388 leukaemia cells with reduced cardiotoxicity [94]. Unlike its parent compound, the tri-ferric DNR does not catalyse the electron's free flow from the NADH to molecular oxygen, thereby reducing the superoxide formation in cells by about 65% [94]. The in-vitro results obtained were in accordance with the Quelamycin [Fe-(HDNR)$_3$], phase I clinical trials [94,95]. Thus, employing the anthracycline drug delivery using the Fe-anthracyline compounds would result in effective antitumour activity with reduced cardiotoxicity.

5.2. Redox mechanisms and Oxidative stress

Anthracyclines cause apoptosis in cells through alteration of the iron-dependent lipid peroxidation. The iron levels increase in the cells through interaction with iron regulation proteins (IRP1,2). The process is mediated by glutathione peroxidase 4 (GP4), where DOX-Fe^{2+} and DOX-Fe^{3+} adducts are formed and cause the accumulation of lipid-based reactive oxygen species (ROS) [96]. The DOX downregulates the GP4 activity and interacts with genomic and mitochondrial DNA, accumulating iron-DOX adducts and inhibiting the *ABCB8* efflux transporter pump. The inhibition or downregulation of the *ABCB8* efflux transporter increases DOX-induced toxicity, ROS levels, and cardiomyocyte apoptosis [97].

5.3. ROS alleviation and mitochondrial dysfunction

Cardiomyocytes have a substantially greater number of mitochondria than regular cells to obtain more energy (ATP) for the contraction function. The DOX accumulation in mitochondria is significantly higher than in the cytosol, which causes DOX-induced mitochondrial impairment increase in ROS in cells [98]. An increase in ROS, a peculiar anthracycline toxicity condition, leads to deformity in cell organelles and membranes and induces cell death. In H9C2 cardiac cells, the DOX-induced mitochondrial dysfunction, together with ROS production and cytochrome C release, activated the Capsase -3 and initiated cell death [99]. DOX stimulates the generation of superoxide anions in Ehrlich cancer cells in a dose-dependent manner. The cyclic process is mediated by NADPH: cytochrome P-450 reductase, which includes the transfer of electrons from NADPH to DOX to convert to semi-quinone (DOX-SQ), eventually forming an O_2 molecule, superoxide anion ($O_2^{\cdot-}$) and a DOX molecule. The superoxide dismutase (SOD) converts superoxide anion to hydrogen peroxide (H_2O_2), which then undergoes the Fenton reaction to produce hydroxyl radicals [100]. A similar investigation revealed the production of reactive species through the interference of DOX with the iNOS enzyme (nitric oxide cofactor), leading to reactive nitrogen species (RNS) and causing cell death [101]. The DOX alters the ROS production process through interferences in the electron transport system [100,102]. DOX exhibits a high affinity towards cardiolipin in the mitochondrial membrane, transforming the cardiolipin's attachment ability for cytochrome c and other mitochondrial proteins and altering the normal function [103]. The DOX can impart irregularities in the mitochondrial function by creating imbalances in regular fusion and fission, causing mitophagy and mitochondrial fragmentation.

5.4. Lipid dysfunction and cell membrane alterations

DOX has the ability to disrupt the lipid organization in the cell due to its lipophilic nature, where the interaction will the cell and mitochondrial membrane is high. The localization of DOX in the mitochondria enables it to interact with the inner mitochondrial membrane due to lipid peroxidation, and the resultant lipid aglycone is hard to diffuse out of the membrane to the cytosol. Especially in cardiomyocytes, the mitochondrial dysfunction leading to the proteotoxic burden is due to this DOX lipid interaction [104,105]. Dox hinders the activity of the phosphatidylserine decarboxylase enzyme (catalytic enzyme for phosphatidylserine to phosphatidylethanolamine), a crucial element of cell membranes, thus leading to cellular membrane dysfunctioning in HeLa cells [106].

5.5. Immune modulation

Anthracycline can promote antitumour activity (ability to gain immunity against cancers) through immunogenic cell death (ICD) by increasing the production of interleukins (ILs), interferon (IFN) gamma protein, DAMs (danger-associated molecules) and T-cells [15,107]. The DAMs can recognise tumour-inducing cells, recruit immune cells and generate inflammatory responses. DOX treatment in mouse colon carcinoma model has resulted in increased tumour-specific $CD8^+$ T cell response [108].

6. Self-Resistance in microbial factories/non-target species

The microbial cell factories of antibiotics, anthracyclines, and related cytotoxic compounds like filamentous *Actinobacteria* and *Streptomyces* are programmed to deal with the cytotoxic compounds made by them [109,110]. These resistance mechanisms include the expression of resistance genes, efflux systems to pump out anthracyclines, the inactivation of anthracyclines through enzymatic modifications and interaction with other metal elements.

6.1. Resistance genes

The self-resistance developed by the bacteria through the expression of resistance genes is a prerequisite to its survival against the produced toxic (DNA intercalating majorly) compounds. Similar to the antibiotic pathway-synthesising genes on BGCs, the resistance genes are also encoded in the BGCs, which initiates the process of self-resistance through time-space co-ordinated expression or intermediate-dependent (compound produced) expression [111]The resistance mechanisms are variable according to BGCs or product type and include target protection, compound inactivation, modification, sequestration, and efflux.

In *Streptomyces peucetius*, the genes encoding resistance for DNR/DOX are *drrA*, *drrB*, and *drrC* unravelled when expressed in *E. coli* and *S. lividans*. The drrA *and* drrB proteins act as drug-efflux complexes produced during the idiophase, while the drrC is produced earlier and facilitates the efflux through drug binding [55,56,112].

6.2. Efflux pumps

Efflux pumps play a pivotal role in conferring multidrug resistance in bacteria by facilitating the expulsion of toxic compounds either produced by the organism or acquired from the external environment [113]. Efflux pumps are the key components of the cell membrane that regulate the internal cellular concentrations of toxic chemicals and elements (metal ions) through extrusion and also inhibit the re-entry of compounds to evade toxicity [114,115]. The efflux pumps utilize energy by hydrolysing the ATP and can use the electrochemical or ionic gradient inside the bacterial cells to efflux the toxic compounds. The efflux systems found in bacterial cells are categorised into six families: ABC (ATP-binding cassette), MATE (multidrug and toxic compound extrusion), PACE (proteobacterial antimicrobial compound efflux), MFS (major facilitator superfamily), SMR (small multidrug resistance family) and RND (resistance nodulation cell division) [116]. These

efflux pumps comprise transmembrane protein helices facilitating the translocation of produced secondary metabolites outside the producer organisms [117]. However, despite their varied structural differences, substrate redundancy is prevalent across all the efflux pump families. The DOX/DNR is extruded out by the AbeM efflux pump of the MATE family (using antiporters H^+ and Na^+) in *Acinetobacter baumannii*, whereas the ABC pumps (generally hydrolyse ATP) perform the extrusion in *Streptomyces sp*. [118,119].

6.2.1 ABC Efflux pumps

The ABC (ATP-binding cassette) pumps constitute the most prominent protein families and are widely present in all living organisms, facilitating the import and export of chemical substances based on their structural architecture and folding [120]. The ABC efflux pumps in bacteria use energy by hydrolysing ATP and translocating various chemical compounds like sterols, secondary metabolites and lipids across the membrane through 12 transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) [58,121]. The TBDs aid in substrate binding, whereas the NBDs carry the translocation of compounds hydrolysing ATP. The detailed mechanism and structural diversity of ABC pumps and their activity depending on the arrangement of helices, loops and protein domains are reviewed by Thomas et al. (2024) [120]. The *drrAB* transporter system encodes for the efflux of DNR/DOX in *S. peucetius*, where *drrA* (peripheral membrane protein) binds to ATP and *drrB* (hydrophobic membrane protein) enables the translocation acting as resistance mechanism. The subcloning of these *drrAB* genes in *E. coli* resulted in similar expression [122]. Several follow-up studies conferred the resistance mechanism of *drrAB* transporter system and the co-dependence of both proteins in efflux activity [123,124]. A recent study by Dong et al. (2024) conducted on ABC transporter in *Streptomyces ceoruleorubidus* yielded significant findings, indicating that the *drrAB* genes of the DNR BGC facilitate the efflux of excess DNR/DOX within the cell. Additionally, the two-component ABC transporters, encoded by *drrAB2* and *drrAB3* and situated outside the cluster, regulated by the TetR family regulator *DrrR1*, were identified as playing a complementary role in the efflux of daunorubicin in *S. coeruleorubidus* in response to the intracellular accumulation of daunorubicin [125].

6.3. Inactivation of drug by enzymatic reaction

Resistance mechanisms to evade autotoxicity in microorganisms also include inactivating or modifying the produced metabolites or antibiotics through enzyme activity. The β-lactamases inhibit the production of Penicillin derivatives and cephalosporins as a resistance mechanism [126,127]. Likewise, chloramphenicol undergoes modification by acetylation, *O*-phosphorylation of erythromycin, acetyl, and phosphor transferases modify the amino-glycoside antibiotics (kanamycin, gentamycin, streptomycin) as a resistance mechanism [128,129]. The activation of repressor gene *dnrH* in *S.peucetius* carries out the glycosylation reaction of the daunosamine sugar to baumycin-like glycosides, thereby preventing the formation of DNR/DOX [130]. Similarly, the *doxA* gene encoding the cytochrome P450 oxidase, crucial for three oxidation steps in DNR/DOX development, gets downregulated by the excess concentration of produced daunorubicin inside the *Streptomyces* sp. [51,52]. A detoxification strategy of *Streptomyces* by reducing the DOX to 7-deoxydoxorubicinolone via deglycosylation using NADH: ubiquinone oxidoreductases was reported [131]. Thus, employing the activation of products through enzymes aids in the inhibition of the intercalation of DNA and evades autotoxicity in the producer organisms.

6.4. Alteration of Drug Targets

Conferring to resistance towards self-toxicity in microorganisms also involves the alteration of the drug targets. Such modifications inhibit the interaction of produced me-

tabolites- cellular components like ribosomes, DNA and topoisomerases [53]. Upregulation of genes responsible for methylation for DNA and Topo II alteration in response to evade self-toxicity from produced DNR/DOX is a resistance mechanism exhibited by *Streptomyces sp*. Thus, deciphering such systems and enhancing their activity can lead to improved production by the strain in industrial settings [53,132].

7. Interaction of DNR/DOX with iron

Daunomycin is the chelator of iron, where ionic forms of iron (Fe^{2+} and Fe^{3+}) bind to specific functional groups of anthraquinone moiety and form stable complexes [133]. The quinone group at position 5 and hydroxy group at position 6 on the aglycone part of DNR act as the binding sites for iron by donating electrons. The DNR also has a side chain with hydroxyl groups which can donate lone pair of electrons and bind to iron [133]. Both ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms of iron bind to daunomycin, where Fe^{2+} is highly reactive and readily participate in redox cycling and alter between ionic states and Fe^{3+} is less reactive and form stable complexes [134,135]. This stabilization activity can be employed for the therapeutic purposes. The first tri-ferric doxorubicin compound, named Quelamycin, a metallic derivative of the Adriamycin prepared was through chelation in the presence of Fe (III) [136]. The compound has been reported to be highly stable in phase I clinical trials and P 388 leukaemia cells, where the cytosolic components do not degrade the compound, and it also inhibits the free flow of electrons from NADH to oxygen molecules in cells [94,95,135]The bond strength of the iron-DNR complex is high, and the chelation activity can be reversible or disassociated in high acidic pH (lower) conditions and in the presence of iron-binding compounds like transferrin and ferritin.

8. Interaction of DNR/DOX with oil

The anthracycline compounds daunorubicin and idarubicin are lipophilic and their interaction mechanisms with the lipids are studied using various experiments [137–139]. Oils and the oleic acid being non-polar, bind to the hydrophobic regions on the anthraquinone moiety, which is often used in therapeutic formulation. The liposome-associated doxorubicin was reported to have reduced systemic and cardiotoxicity in clinical trials for humans and mice [140–142]. The daunorubicin is encapsulated by the liposomes (phospholipid vesicles) and exploited for drug delivery mechanism [143,144]. The interactions and their practical implications are clearly reviewed here [145].

9. Culture Media for metabolites production in *Streptomyces* sp.

The production of antibiotics at a large scale is a combinatorial effect which relies upon the strain efficiency, ability to utilise the available nutrients, physical conditions and productivity of the metabolites. The primary nutrients like carbon, nitrogen and phosphorus, along with minor mineral elements, remain the major constituents of the culturing media responsible for the growth and production of necessary chemical compounds in *Streptomyces* sp. Carbon serves as a prominent energy source, nitrogen is responsible for cell growth and metabolism, and phosphates assist in the production of metabolites [146]. To date, many investigations over the decades have concentrated on improving secondary metabolites using strain engineering *via* genetic alterations. However, the culmination of improved levels of metabolite production through extensive genetic research remains unpromising due to the intricate metabolic mechanisms involved, as reviewed in the biosynthesis and gene regulation sections. Additional investigations employing modifications in media have the potential to result in more streamlined and economical techniques for manufacturing daunomycin and other crucial antibiotics in *Streptomyces*.

The DNR/DOX compounds are produced in the late growth phase through a multitude of enzymatic reactions by the *Streptomyces* strains, utilizing nutrients [147]. The host cells synthesizing the secondary metabolites in nature (μg/L) are not sufficient to achieve

the harvest at the desired quantities (g/L) on an industrial fermentation scale [148]. So, the efficient native strains are screened and improved through metabolic engineering (contemporary), mutations (traditional) and selections. However, the highest yields are achieved through the combination of several approaches in strain development, suitable culture media composition and well-optimised fermentation conditions. The complete genomic sequencing of the model actinomycete *Streptomyces coelicolor* revealed the presence of multiple genes in the *Streptomyces* genome that can break down complex carbohydrates and proteins [149]. This facilitates utilizing various carbon and nitrogen sources to optimise culture media. The production of metabolites is also linked to factors like nutrients available in culture media and fermentation conditions (temperature, light, oxygen and pH) [147,148]. The optimisation of media and the source of nutrients remained a major variable factor in the growth of strains and the production of metabolites for several decades.

9.1. Carbon source

Glucose or sugars are the most often utilized carbon sources in industrial fermentation due to their low cost and high availability, even though they inhibit secondary metabolite synthesis [32,146,150]. The carbon source serves as the vital controlling agent for secondary metabolite production in *Streptomyces*, as transcriptional activation or carbon catabolite repression (CCR) is dependent on the source and concentration of carbon [146,150,151]. Carbon from sugars like glucose, maltose, glycerol, sucrose, mannose, and xylose has been reported to interfere with the production of more than 30 types of secondary metabolites (mostly antibiotics) in *Streptomyces sp.* [150,152]. The synthesis of Doxorubicin in *S. peucetius* has been impeded by the utilization of glucose and galactose as the carbon source in the culture medium [153]. Sugar carbon in the media at an industrial level leads to an increase in acidification and triggers feedback inhibition through produced intermediates.

The erythromycin yield in *Saccharopolyspora erythraea* at the industrial level using oil and soy flour has been improved to 3.5g/L compared to the dextrin control [154]. Clavulanic acid production in *Streptomyces clavuligerus* has been improved using Olive oil as a sole carbon source [155]. Employing soybean oil as a source of carbon has enhanced the production of FK506 (tacrolimus), an immunosuppressant polyketide by 88.8% in *Streptomyces tsukubaensis* [156]. Enhanced production of DOX (1100mg/L) was achieved by mutation treatment (UV and ART-plasma) and soybean oil as a carbon source in *Streptomyces peucetius* SIPI-11 [157]. Increased levels of salinomycin production and improved TAG (triacylglycerol) metabolism were observed in *Streptomyces albus* ZD11 using soybean oil as a carbon source in culture media [158]. Oil utilisation has also benefited from imparting the activity as an antifoam at the industrial scale of fermentation. The breakdown of oils supports the activity of malonyl Co-A and Acetyl Co-A, which are essential for the biosynthesis of secondary metabolites. Thus, employing an oil-based carbon source instead of sugar in combination with optimised fermentation conditions and selection would enhance DNR/DOX production.

9.2. Nitrogen source

The source and concentration of nitrogen in the media also remain a vital factor for secondary metabolite production. Nitrogen in the form of ammonia is mostly preferred by microorganisms, and the genera *Streptomyces* naturally possess a constant nitrogen acquisition and metabolism to ensure their survival [152,159]. *Streptomyces* assimilate ammonia through glutamate dehydrogenase in ammonia-rich conditions and glutamine synthetase pathways in ammonia-deficient conditions [146]. The influence of various regulatory mechanisms of nitrogen in *Streptomyces* has been clearly reviewed in [160]. The forms or sources of nitrogen, like ammonium, nitrate, amino acids, and polyamines, have an

impact on the production of secondary metabolites in *Streptomyces* [152,160]. Specific concentrations of various nitrogen sources can be used to quantify the yield of different actinobacterial compounds in *Streptomyces* according to their industrial relevance.

9.3. Phosphorus source

Phosphorus, in the form of inorganic phosphate, is the crucial element acting as the building blocks for nucleotides, proteins, and several regulatory signalling cascades [161]. The concentration of phosphate in cells significantly impacts the production of secondary metabolites in *Streptomyces* and related actinobacteria [162]. Increased concentrations of phosphates in media (>10mM) have resulted in decreased yields of antibiotic production, whereas the lower concentration (<0.1mM) has positively increased the secondary metabolite production, implying the significance of phosphates on biosynthetic pathways [163,164]. The limited availability of phosphate results in nutritional stress and initiates the secondary metabolite biosynthetic pathways. In phosphate-rich conditions, the *Streptomyces* convert phosphate to polyphosphate and accumulate, which aids in cellular metabolism. The regulation of phosphate in *Streptomyces* is carried out through a two-component mechanism, PhoR-PhoP, clearly reviewed elsewhere [165,166].

9.4. Other elements

A well-established culture media including all these macro components together with the essential microelements like Fe, Ca, Zn, S, etc, results in the enhancement of secondary metabolites yield. The use of rare earth elements in the culture medium for *Streptomyces* sp. is reported to activate the BCG's cluster for secondary metabolite production [167]. Tanaka et al. (2010) used scandium and lanthanum in medium for the cultivation of *Streptomyces coelicolor* and reported an increase in activity by 2.5 to 12-fold [168]. Optimization and standardization of culture media, considering pH, combinations of nutrients, agitation, and temperature, have resulted in enhanced production of daunomycin in *Streptomyces sp.* [36,157]

10. Engineering culture media – in Prospect of improved DNR/DOX production

Over the past decades, genetic alterations have been frequently used to enhance the production of metabolites in *Streptomyces*, improving regulatory gene expression, modifying resistance, developing efflux mechanisms, and possible combinations with strain development. However, modifications to the culture media can also potentially improve production yields. A considerable amount of research is lacking in this area, but strategies employed for other polyketide synthesis in *Streptomyces* relevant to daunomycin can provide promising insights into the enhancement of production devoid of complex and expensive gene editing methods.

The prominent effect of DOX/DNR is its autotoxicity by intercalating with the DNA in the producers when the concentration increases. The prospective idea of this article is to prepare culture media for cultivating *Streptomyces* sp. based on binding DNR with Iron or reduced forms of iron after effluxed from the producer. The iron or iron-DNR complex is encapsulated by the oleic acid or lipid micelle layers present in the culture medium, converting the produced DNR/DOX to inactive forms and settling with the DNR-iron-oil complex, as shown in Figure **3**. Therefore, this hypothesis can safeguard the producer strain from toxicity and avoid the inhibition of metabolite production.

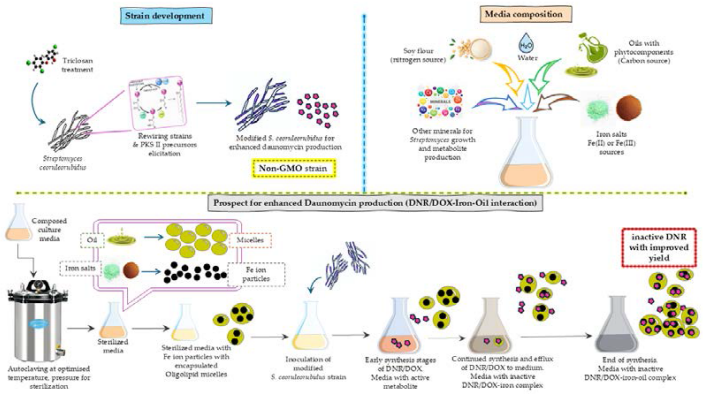

Figure 3. Schematic representation of enhancement of daunorubicin in *Streptomyces* based on strain alterations (boosting elicitors by triclosan) and tailor-made media composition (oil, iron salts and phytomaterials).

10.1. Perturbation of metabolite biosynthesis in Streptomyces sp.

The overexpression of regulatory genes in BGCs and downregulation of repression genes and factors have always remained as prominent approaches in the metabolic engineering of *Streptomyces* for metabolite production [32,45,169]. On the contrary, the availability of biosynthetic precursors also serves as a key factor that is generated primarily by carbon catabolism in the organisms [170,171]. Perturbation is the supply of precursors for modulating biosynthesis to improve cells' ability to enhance secondary metabolite production. The ARCs (antibiotic remodelling compounds) screened from *Streptomyces coelicolor* A3(2) are known to stimulate metabolite production by acting as precursors [172]. The ARC2, similar to the antimicrobial compound triclosan, has been reported to partially inhibit fatty acid synthesis, utilize the acetyl CoA for polyketide biosynthesis and improve the actinorhodin yield in *S. coelicolor* [172,173]. Using triclosan as an elicitor of polyketide biosynthesis in Streptomyces sp. has been reported to overproduce metabolites like oligomycin, salinomycin, erythromycin, and actinorhodin. [171,174,175].

10.2. Media construction for three-way interaction (DOX/DNR-iron-oligolipid).

The achievement of prospective three-way interaction from figure 3 can be achieved from distinctive methods under a single hood with critical optimization of conditions like pH, temperature, pressure, incubation time and initial components like natural chelators, metal salts and nutrient sources. The biosynthesis of FeO and Fe ion particles from their salts like $FeCl_3$ using phytoextracts has been employed in nanoparticle synthesis over decades [176,177]. The phytic acid present in plants, cereals, and legumes has a tremendous metal chelation potential [178]. The phytate-metal complex is stable and cannot be liberated in wide pH ranges. Phytates from soybean or soy-derived products have high iron binding ability, which is considered a major drawback in diet and nutrition [179,180]. Thus, utilizing soybean phytates in the culture medium facilitates iron binding and chelation.

As discussed in the above carbon sources section, the oil source of carbon in the culture media for *Streptomyces* describes its prominence in the improved production in several instances, including erythromycin [154], clavulanic acid [155], doxorubicin [156], salinomycin [158] and josamycin [181]. Employing crude oils, including the raw plant parts with phytic acid contents, will deliver the nutrient carbon source and act as a reducing

agent for iron in the media. Crude oils of soybean and pomace have enhanced clavulanic acid production in *Streptomyces*, which is also a waste-to-value strategy [155,182]. The crude plant oil substrate used for the cultivation media forms micelles due to elevated temperature and pressure during autoclaving and encapsulation of Fe^{2+}/Fe^{3+} particles. After inoculation of the perturbated *Streptomyces ceoruleorubidus* culture to the cultivation media, the production of daunorubicin takes place and is effluxed out into the cultivation media.

Considering the lipophilic nature of daunorubicin, the produced, effluxed DOX/DNR into the medium can interact with the oligolipid surface layer with Fe ion particles from the oil-based medium [137]. The interaction between anthracycline and metal ions, especially iron, has the potential to form complexes that demonstrate high stability constants in the medium [183]. The produced and effluxed DOX/DNR by the Streptomyces strain interacts directly with the Fe-micelle to form a DOX-Fe-micelle complex [90,95,184]. Thus, the catchment of the produced metabolite in an inactive form helps in evasion of the toxicity to the producer organism.

Streptomycetes are also well-known for their metal resistance, which involves their intricate intracellular iron homeostasis mechanisms [185]. As a defensive strategy, the reduced iron entering the cells is segregated and secreted externally through ABC pumps. The defensive strategy of *Streptomyces* in effluxing the excess DNR/DOX re-initiates the production of new DNR/DOX molecules inside the cells, resulting in improved productivity. Therefore, the enhancement of the daunomycin production in Streptomyces using this media construction approach can be established with reduced costs and negligible metabolic engineering of strains.

Our group intends to perform protoplast fusion with the wild strain of *Streptomyces coeruleorubidus* followed by triclosan treatment to achieve high DNR-producing strain and optimize efficient medium to reduce downstream processing and expenses. After considering various C and N sources, a final combination of crude olive oil, defatted soya flour, dried yeasts, yeast extract, calcium carbonate, basic salts, and iron sulfate was selected as the production medium for enhancing the production of daunomycin. Iron, either in bivalent or trivalent form, was identified as the crucial element influencing the production and purification processes significantly. The use of an iron-based medium simplified the whole process; no sequential feeding was required. Daunomycin is not present free in the medium, just in particles (pellets) or adhered to mycelial structures. About 95% can be removed by centrifugation of about 14,000 g or by filtration. Daunomycin is produced as an intact dark violet sediment from which it can be extracted by acids. The outcome of the study holds a significant future for therapeutic advancements and strategies in both oncology and microbiology in terms of the production of various other secondary metabolites of similar or related characteristics as daunomycin (Unpublished data).

Moreover, the complex of iron and anthracyclines is known to be less cardiotoxic than their original counterparts [94,134]. A similar interaction has been reported in Adriamycin: iron complex with phosphatidylcholine in the presence of oxygen to form a compound similar to cardiolipin [186]. The liposome-associated doxorubicin was reported to have reduced systemic and cardiotoxicity in clinical trials for humans and mice [140–142] . Thus, the three-way compound can also be employed for a liposomal drug delivery approach after extensive trials. Additional investigations in this field could result in more streamlined and economically feasible techniques for manufacturing daunomycin and other crucial secondary metabolites.

11. Conclusion

The authors conclude that this review is fabricated with the aim to use a traditional method of switching in cultivation media sugar to oil-based and develop the *Streptomyces ceoruleorubidus* strain in a contemporary way to favour the active usage of lipids as a source of energy and also entrap the produced DNR/DOX with iron present in the medium. This approach would enhance the production of DNR/DOX by the strain, and the

produced metabolite does not interact with the producing strain and evades self-toxicity. However, over the past decades, the efforts of various groups working with the enhancement of daunomycin, and doxorubicin production have involved the engineering of the strain (gene regulation, resistance genes development, metabolic pathway regulation) and using sugar-based media; our prospective approach sheds light on the topic in a different approach which remains first report in the context of daunomycin production. Adapting this strategy would improve yield and also benefit the extraction of the derived compound (DNR/DOX) by reducing the expenses at a large-scale fermentation.

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microorganisms

MDPI

Article

Autonomous Defense Through Biogenic Nanoparticle Formation in Daunomycin Producing *Streptomyces ceoruleorubidus*.

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Abstract: This study focuses on the development of a cultivation medium specifically designed to induce auto-resistance through Autonomous Defense Through Biogenic Nanoparticle Formation. Utilizing a fermentation medium enriched with olive pomace oil and iron, this research aims to explore the inherent affinity of daunomycin for both iron and oil. Our strategy leverages the combination of olive pomace oil and bacteria to facilitate the formation of iron nanoparticles (NPs). Furthermore, it seeks to bridge the existing knowledge gap by investigating the effects of daunomycin on eukaryotic cells in conjunction with the underexplored data from prokaryotic organisms, especially those involved in the production of daunomycin. This dual approach aims to enhance our understanding of daunomycin's mechanisms of action and its potential applications in both eukaryotic and prokaryotic systems. By systematically optimizing iron levels, we aim to facilitate the formation of daunomycin-iron complexes that can reduce the solubility and bioavailability of daunomycin. This transformation not only alleviates its cytotoxic effects on microbial production strains but also has the added ecological benefit of producing daunomycin as an insoluble precipitate. This precipitate can be easily separated from the cultivation medium by filtration or centrifugation, concentrated into a smaller volume, and extracted using phosphoric acid, followed by a final extraction of the dissolved daunomycin using significantly reduced volumes of organic solvents. Thus, this approach targets the reduction of toxic impacts during fermentation while enhancing compound yield and overall production efficiency, all while minimizing the environmental footprint of the process. This research not only contributes to the field of microbial fermentation and antibiotic production but also emphasizes the importance of minimizing environmental impacts through the production of insoluble daunomycin precipitates that can be efficiently recovered from the cultivation medium. Overall, these findings present promising avenues for further investigation into the mechanisms underlying biogenic nanoparticle formation and the optimization of cultivation processes.

Keywords: daunomycin; streptomyces; redox potential; oil-based cultivation; triclosan

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1. Introduction

The Daunomycin, an anthracycline antibiotic originally isolated from the bacterium *Streptomyces peucetius*, plays a pivotal role in the treatment of various cancers, particularly leukemia. Its therapeutic efficacy is primarily attributed to its ability to intercalate into DNA, disrupting essential replication and transcription processes in rapidly dividing cells. Additionally, daunomycin serves as a precursor for the synthesis of more advanced anthracyclines [1]. However, its clinical utility is often compromised by inherent cytotoxic effects [2], including toxicity to the microbial strains used for its production, posing significant challenges for industrial-scale synthesis [3,4].

A critical aspect governing daunomycin's biological activity is its interaction with iron, particularly through the Fenton reaction. This process illustrates how iron can catalyze the production of reactive oxygen species (ROS) from hydrogen peroxide, leading to oxidative stress and cellular damage [5]. While iron is vital for numerous physiological functions, excessive concentration can exacerbate oxidative effects [6]. The therapeutic use of iron chelators to mitigate daunomycin toxicity underscores the need for a deeper understanding of the interplay between these two elements, as it significantly influences the compound's pharmacological properties and therapeutic efficacy in fermentation contexts.

While most research has traditionally focused on the toxicological effects of daunomycin in eukaryotic models, its implications for prokaryotic organisms remain only partially explored [7]. Investigations have primarily centred on the biosynthesis of daunomycin and altering the molecular mechanisms of resistance, particularly involving efflux pumps [8,9].

This study focuses on developing a cultivation medium specifically designed to induce auto-resistance through Autonomous Defense Through Biogenic Nanoparticle Formation (ADBN). Utilizing a fermentation medium enriched with olive pomace oil and iron, this research aims to explore the inherent affinity of daunomycin for both iron and oil. Our strategy leverages the combination of olive pomace oil and bacteria to facilitate the formation of iron nanoparticles (NPs) [10].

Furthermore, it seeks to bridge the existing knowledge gap by investigating the effects of daunomycin on eukaryotic cells in conjunction with the underexplored data from prokaryotic organisms, especially those involved in the production of daunomycin. This dual approach aims to enhance our understanding of daunomycin's mechanisms of action and its potential applications in both eukaryotic and prokaryotic systems.

Olive pomace oil is a valuable source of both lipids and polyphenols [11]. The unique amphiphilic nature of these lipids—characterized by hydrophilic heads and hydrophobic tails—enables them to form a protective shell around the NPs [12]. The hydrophilic heads interact with the aqueous environment, while the hydrophobic tails orient themselves towards the nanoparticle core. This self-assembly leads to the formation of a stable bilayer, which effectively prevents the uncontrolled aggregation of the NPs [13].

Moreover, the lipids in the reaction mixture promote controlled growth and morphology of the synthesized NPs [14]. Polyphenols further enhance the synthesis process by initiating redox reactions when mixed with metal ions. Acting as electron donors, they facilitate the reduction of metal ions to their metallic state, a crucial step in nanoparticle formation [15]. The versatile nature of polyphenols also allows them to influence the size, shape, and characteristics of the synthesized NPs [16,17]. Beyond their reducing capabilities, polyphenols stabilize nanoparticles by forming a protective layer that prevents agglomeration and ensures colloidal stability [18,19]. This dual functionality positions polyphenols as key contributors to green synthesis.

Additionally, *Streptomyces* can efficiently serve as biofactories for nanoparticle production (Moslamy et al. 2023). Their enzymatic processes convert metal ions into NPs, offering precise control over size and shape while ensuring excellent stability, thus making this biological synthesis route a preferred choice for various applications [20,21].

By systematically optimizing iron levels, we aim to facilitate the formation of daunomycin-iron complexes that can reduce the solubility and bioavailability of daunomycin. This transformation alleviates its cytotoxic effects on microbial production strains and has the added ecological benefit of producing daunomycin as an insoluble precipitate. This precipitate can be easily separated from the cultivation medium by filtration or centrifugation, concentrated into a smaller volume, and extracted using phosphoric acid, followed by a final extraction of the dissolved daunomycin using significantly reduced volumes of organic solvents. Thus, this approach targets the reduction of toxic impacts during fermentation while enhancing compound yield and overall production efficiency, all while minimizing the environmental footprint of the process.

Given the complexity of the problem and the interplay of various factors, we adopted an empirical approach to optimization. Rather than sequentially addressing adjustments, we approached optimization as a holistic challenge, accounting for interdependencies among key variables to inform our adjustments and improve both performance and efficiency. Ultimately, the multifactorial nature of our optimization process—measured through daunomycin yield as a key indicator—highlights its intricacy and reinforces our objective to maximize production. Our findings not only validate our initial hypotheses but also offer valuable insights for future research efforts aimed at optimizing production processes in this field.

2. Materials and Methods

2.1. Chemicals

Defatted soy grits were supplied by MP Biochemicals. Potato starch, G-glucose, yeast extract, NaCl, magnesium sulfate, iron sulfates ($FeSO_4$ and $Fe_2(SO_4)_3$), and glycerol were sourced from VWR. Dried bakery yeasts from Lallemand (Spain) were provided by Instaferm, while brewery yeast was obtained from a local brewery (Budvar, České Budějovice). Organic solvents and buffer salts were also provided by VWR. Cannabis oil was supplied by a local producer, rape oil by a local supplier, and extra virgin and pomace olive oils by Ondoliva.

2.2. Bacterial Strain and Isolation

The original bacterial strain, *S. coeruleorubidus*, was isolated and collected from the Vltava River pool during the spring of 2019. The identified strain was confirmed through morphological assessments, phenotypic characterization, and genomic sequencing. PCR amplification revealed the presence of daunomycin resistance genes *drrA*, *drrB*, and *drrC*, which are significant for potential applications in developing antimicrobial media. The original strain showed high variability in mycelium and colony morphology, precisely as described by [22].

2.3. Triclosan induced mutation

To enhance the production of daunomycin, triclosan was employed to induce mutations in *S. coeruleorubidus* due to its known effect on lipid synthesis, especially on enoyl-acyl carrier protein reductase a key enzyme involved in fatty acid synthesis [23]. Solid Potato Dextrose Agar (PDA) medium was prepared, to which triclosan was added aseptically at final concentrations of 0.1, 0.5, 1, and 10 μM. Subcultures were maintained for ten passages to stabilize the mutant strains, which were subsequently evaluated for increased productivity on both standard and modified media.

2.4. Olive Pomace Oil-Resistant Strain Development

To develop a strain capable of growth on olive pomace oil, ultraviolet (UV) irradiation was employed, following methods described by [24,25]. The strains were exposed to UV light at a dose rate of approximately 10 W/m^2 for about 30 minutes and subsequently cultivated in solid and liquid media containing 10% olive pomace oil as the sole carbon source.

2.5 Protoplast Formation

The protoplasts of *S. coeruleorubidus* were prepared using a modified method adapted from [26] and [27]. Briefly, spores were inoculated in Yeast Extract-Malt Extract (YEME) medium supplemented with 0.5% glycine and 5 mM $MgCl_2 \cdot 6H_2O$, followed by incubation at 28°C and 220 RPM for 48 hours. Protoplasts were generated from two distinct strains: one resistant to triclosan and another capable of growing on olive pomace oil. The cultures were centrifuged at 4000 RPM for 10 minutes and washed with a 10.3% sucrose solution. The cell pellet was treated with Medium P [27], for washing and then resuspended in a lysozyme solution (2 mg/ml) to disrupt the cell wall. After lysis, protoplasts were washed and resuspended in a 40% PEG 4000 solution to Darken the agar around colonies, which was used as a selective criterium.

The producing strain Ret2210 was developed through repeated protoplast fusion of triclosan-resistant and olive pomace oil-utilizing strains, combined with an adaptive laboratory evolution technique. Unlike the original strain, the resulting mutants from protoplast fusion exhibited highly homogeneous cultures characterized by prolific sporulation and fully developed chains of spores.

2.6. Working Bank Preparation

Five millilitres (5 ml) of a 25% aqueous solution of glycerol were placed on the surface of a Petri dish. The surface mycelium was scrubbed to create a cell suspension. This suspension was then transferred to a PTFE-glass homogenizer and homogenized for 15 to 20 minutes until achieving a homogeneous liquid. Six hundred microliters (600 µl) of the suspension were transferred into Eppendorf tubes or cryotubes and stored at -20 °C. The potential contamination was checked after three days by inoculating Potato Dextrose Agar (PDA) with 5 µl of the cell suspension.

2.7. Inoculum Preparation

The fermentation inoculum was prepared from the working bank. Inoculation of the vegetative medium was carried out using 2,500 µl of the working bank solution, introduced into 50 ml of cultivation medium contained within a 250 ml Erlenmeyer flask. The cultivation process was maintained at a speed of 220 RPM (with a shaker arm length of 25 mm) and a temperature of 28 °C. The culture was deemed ready for inoculation after 36 to 40 hours (±2 hours) in a basal medium composed of the following ingredients (g/l): 30 g of soy flour, 5 g of starch, 5 g of glucose, 5 g of yeast extract, 1 g of K_2HPO_4, and 1 g of NaCl. The pH was adjusted to 7.0 using either 2 M NaOH or 2 M HCl.

2.8. Production Medium

The production medium was inoculated with 2.5 ml of the actively grown culture from the vegetative medium (constituting a 5% inoculum) into 50 ml of the production cultivation medium, also contained in a 250 ml Erlenmeyer flask. The culture was incubated at 28 °C with agitation at 220 RPM. The composition of the production medium

was as follows (g/L): 10 g of bakery yeast, 20 g of soy flour, 100 g of pomace olive oil, 5 g of yeast extract, 5 g of glycerol, 2 g of K_2HPO_4, 1 g of magnesium sulfate heptahydrate, 3 g of calcium carbonate, and 3.6 g of ferrous sulfate heptahydrate. The pH of the medium was adjusted to a range of 5.9 to 6.1 using 2 M NaOH or 2 M HCl.

2.8.1. Effect of Carbon and Nitrogen Sources on Daunomycin Production

The influence of various carbon sources on daunomycin metabolite production was evaluated by substituting starch in the production medium with alternatives such as soybean oil, refined rapeseed oil, crude rapeseed oil, virgin olive oil, pomace olive oil, mineral oil, and crude cannabis oil. Similarly, the impact of different nitrogen sources was assessed by replacing soy flour in the production medium with casein, yeast extracts, defatted soy flour, soy grits, peptones, ammonium sulfate, and potassium nitrate. Following the incubation period, the impact of carbon and nitrogen sources on daunomycin production was analyzed by HPLC.

2.8.2. Effects of Iron on Daunomycin Production

To investigate the effects of minerals on daunomycin production, the medium containing optimized carbon and nitrogen sources was supplemented with various iron sources, including $FeCl_3$, $FeSO_4$, and $Fe_2(SO_4)_3$. These were added at final concentrations of up to 1.44 g per litre of Fe^{2+} and 2.79 g per litre of Fe^{3+}, which corresponded to the following additions: 2.1 g/L of $FeCl_3$; 3.6 to 7.2 g/L of $FeSO_4$; and 5.2 to 10 g/L of $Fe_2(SO_4)_3$.

2.9. Method for Direct Detection of Iron Using SEM and EDS

The samples were mounted onto conductive stubs, and a thin conductive coating was applied if necessary. The Scanning Electron Microscope (SEM) was calibrated, and the accelerating voltage was set, typically between 10-20 kV. Secondary electron imaging was employed to visualize the samples and identify areas of interest. The Energy Dispersive X-ray Spectroscopy (EDS) detector was activated to capture X-ray emissions from the samples when exposed to the electron beam. The resulting X-ray spectrum was analyzed for characteristic iron peaks, particularly the Fe Kα peak around 6.4 keV, and elemental mapping was conducted to assess spatial distribution. Finally, the detected iron was quantified using the SEM-EDS software, and the results were validated with standard reference materials, providing insights into the iron dynamics in the microbial samples.

2.10. Monitoring of daunomycin production

Samples were collected from the flasks at 120, 168, 216, and 264 hours, with the possibility of extending to 312 hours. For each sampling point, 1 ml of homogeneous medium was extracted from each flask. The samples were processed according to a rapid isolation protocol using oxalic acid and daunomycin concentration measured by HPLC.

2.10.1. Changes in Media Color and Physical Parameters

Media colour and physical parameters were assessed at 24-hour intervals throughout the cultivation period, beginning at 24 hours and continuing up to 264 hours. At each designated time point, a sample of the cultivated media was visually inspected to determine its colour and observed physical parameters, such as precipitate formation. This information was recorded using a subjective visual assessment. Specific colours observed—such as yellow, green, grey, and black—were noted, along with any relevant changes in intensity or hue. This consistent monitoring allowed for the documentation of colour transitions and physical parameters of the culture over time.

2.11. Sediment friability

To evaluate the friability of the sediment, samples were dispersed in water containing 0.1% SDS as a surfactant at a temperature of 20°C. Control samples were left untreated, while the examined samples were mixed using a mixer at 5-minute intervals. The characterization of the samples included an analysis of particle size distribution, which was determined using laser diffraction with a Mastersizer 2000 equipped with a Hydro 2000G dispersion unit (Malvern Instruments, UK). The mean particle size relative to volume was chosen as the primary criterion for particle size assessment. Two parallel measurements were conducted to ensure the accuracy and reliability of the results.

2.12. Methods for Observing Morphological Changes During Fermentation

Optical Microscopy: To observe morphological changes in the fermentation medium, samples were taken at designated intervals throughout the fermentation period. Each sample was placed on a clean glass slide and covered with a coverslip. The slides were examined under an optical microscope Nikon Eclipse Ni-E at varying magnifications (e.g., 100x, 400x) to assess cell morphology, size, and aggregation. Digital images were captured for further analysis and comparison over time.

Scanning Electron Microscopy (SEM): For surface morphology analysis, samples were prepared for scanning electron microscopy. Initially, samples were fixed in an appropriate fixative and then dehydrated through a series of graded alcohol solutions. The samples were mounted onto conductive stubs and coated with a thin layer of gold-palladium to enhance conductivity. The SEM JSM-7401F was calibrated, and samples were examined under high vacuum conditions. Secondary electron imaging was used to visualize the surface features of the cells, and images were captured for analysis of morphological changes during fermentation.

Transmission Electron Microscopy (TEM): Samples were prepared for transmission electron microscopy following standard fixation and embedding protocols for detailed structural analysis. Initially, samples were fixed in a solution of 2.5% glutaraldehyde buffered with phosphate buffer for 2 hours at 4°C. Following fixation, the samples were washed in phosphate buffer and post-fixed in 1% osmium tetroxide for 1 hour. Then, samples were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%) and embedded in an epoxy resin. Thin sections (approximately 70 nm) were cut using an ultramicrotome and placed on copper grids. The grids were stained with uranyl acetate and lead citrate to enhance contrast. Finally, sections were examined using a transmission electron microscope JEM-1400 Jeol at an appropriate accelerating voltage, with images captured for analysis of cellular structures and morphological features.

2.13. Polyacrylamide Gel Electrophoresis (PAGE):

PAGE was performed using SDS-PAGE (Laemmli) and native PAGE techniques, following Davis's procedures. SDS-PAGE was utilized to determine protein molecular weights and to cut out specific bands for subsequent analysis by TOF MALDI mass spectrometry. The SDS gel was stained with Coomassie Brilliant Blue, while in the native PAGE, proteins of interest were labeled with daunomycin. This approach allowed proteins to maintain their native conformations, specifically for identifying daunomycin-pigmented proteins.

2.14. Measurement of pH, O_2, and Eh During Fermentation

To monitor the fermentation process, pH, dissolved oxygen (O_2), and redox potential (Eh) were measured at regular intervals throughout the cultivation period.

pH Measurement: The pH of the fermentation media was measured using a calibrated pH meter with a glass electrode. At each sampling interval (e.g., every 24 hours), a small volume of the media was taken aseptically and transferred to a clean, appropriate container. The pH probe was immersed in the sample, and the reading was recorded after stabilization. To ensure accuracy, the pH meter was calibrated before measurements using standard buffer solutions at pH 4.00 and 7.00.

Dissolved Oxygen Measurement: Dissolved oxygen levels were assessed using a portable dissolved oxygen meter equipped with an appropriate probe. Samples were taken at the same intervals as pH measurements. The probe was inserted into the sample, allowing for direct measurement of O_2 concentration. The oxygen sensor was calibrated beforehand according to the manufacturer's instructions, ensuring accurate readings throughout the fermentation process.

Redox Potential Measurement: The redox potential (Eh) was measured using a portable redox meter with a suitable combination electrode. Similar to the previous measurements, samples were taken at regular intervals. The redox electrode was placed in the sample, and stabilization was allowed before recording the Eh value. The redox meter was calibrated using standard reference solutions to ensure reliable measurements.

All measurements were conducted under controlled conditions to minimize the impact of external factors, and each parameter was recorded systematically to analyze the fermentation dynamics over time.

2.15. ***Extraction Procedures***

Two primary methods can be employed for extraction: oxalic acid and phosphoric acid. The oxalic acid method is suitable for monitoring the cultivation process due to its low volume requirement, albeit it does not capture the full extent of daunomycin production. In contrast, the phosphoric acid method is more reliable and reproducible, effectively disrupting the iron-daunomycin complex. For oxalic acid extraction, transfer 400 µl of the sample to a 2 ml tube, acidify with 180 µl of 1M oxalic acid, and vortex thoroughly. Add 2 x 600 µl of acetone, vortex again, and then add 300 µl of distilled water. Mix well and centrifuge at 14,000–15,000 RPM for 6 minutes, then take 400 µl of the supernatant for HPLC analysis. For phosphoric acid extraction, shake the culture and take 10 ml, transferring it to a 50 ml Falcon tube. Add 40 ml of 0.1M H_3PO_4 (1:4 dilution) and mix thoroughly. Measure the pH, which should be between 1.8 and 1.9, before heating the sample in a 50°C water bath for 30 minutes, mixing periodically. After heating, check that the pH remains between 1.4 and 1.8; if it exceeds 2, yield may be compromised. Take a 400 µl sample and transfer it to a 2 ml tube, then centrifuge the Falcon tube for 6–7 minutes at 4000–4700 RPM. Discard the supernatant and resuspend the sediment (approximately 5 ml) in 40 ml of 0.05M H_3PO_4 (changing the dilution ratio to 1:8), mixing until homogeneous. Measure the pH again, ensuring it is ≤ 1.7, then heat the resuspended sample for another 30 minutes at 50°C, mixing periodically. Following this step, the pH should not fall below 1.3; take a final 400 µl sample for further analysis.

2.16. HPLC Sample Preparation

The HPLC sample preparation procedure is identical for both oxalic and phosphoric acid samples. Begin by transferring 400 µl of the acidified sample into a 2 ml tube. Next, add 180 µl of 1M oxalic acid and vortex the mixture thoroughly. Subsequently, add 1200 µl of acetone and vortex again, followed by the addition of 300 µl of distilled water and another vortex to ensure homogeneity. After mixing, spin the tube in a centrifuge at 15,000 RPM for 6 minutes. Finally, take 400 µl of the supernatant to serve as the HPLC sample.

2.17. Chromatography Conditions

The analysis utilizes a ProntoSIL C18AQ column (150 x 4.6 mm, 5 µm) maintained at ambient temperature, with a flow rate of 1 ml/min and UV/Vis detection at a wavelength of 254 nm. The mobile phase consists of two components: Mobile Phase A (MPA) containing H_2O at pH 2.7 (adjusted with H_3PO_4) and 1 g/L of SDS, and Mobile Phase B (MPB) comprising acetonitrile, in an isocratic flow ratio of 50:50 (A:B). The retention time for daunorubicin is approximately 7 minutes, and the total analysis length is 25 minutes.

3. Results and Discussions

3.1. Producing strain

The original strain showed considerable variability in mycelium, colony, and spore morphology, with dwarfed spore chains formed by rudimentary spines or smooth spores, as noted by Blumauerová et al. (1978) [22]. In contrast, the producing strain Ret2210, developed through a process of repeated protoplast fusion involving triclosan-resistant strains and those capable of utilizing olive pomace oil, supplemented by an adaptive laboratory evolution technique, demonstrated significant morphological homogeneity. The resulting cultures from this fusion were characterized by prolific sporulation and well-formed chains of spiny spores, representing a marked contrast to the variability observed in the original strain.

3.2. Optimization of the Production Medium

In the optimization process, the daunomycin production of the initial strain in a sugar-based medium was approximately 2.0 g/L. After establishing stable production, we replaced the strain with a new fusant specifically developed for oil-based media. Various ingredients were tested in the sugar medium to assess their influence on production and evaluated using High-Performance Liquid Chromatography (HPLC).

3.2.1. Substitution of Glucose with Oils

The addition of pomace olive oil, rapeseed oil, and soybean oil to the sugar medium resulted in a 20% decrease in production compared to the sugar-only medium. To optimize oil concentration, supplementation with yeast was necessary, with yeast quality significantly affecting the yield. The high-quality bakery yeast yielded the best results; substituting it with brewery yeast resulted in reduced daunomycin production.

The source and quality of oil profoundly influenced both media parameters and daunomycin output. Notably, rapeseed oil led to significant declines in production, despite its fatty acid composition being similar to that of olive oil. In addition, cannabis oil produced under 5% of the yield compared to the pomace oil medium. The importance of oil quality became evident: expired oil did not support the process. Specifically, expired

pomace oil failed to produce the desired green-grey medium and instead formed a rusty emulsion. It is essential for oils to be characterized by their peroxide value before use in cultivation media.

Recent observations regarding the ineffectiveness of expired pomace oil underscore the critical role that phenolic compounds play in microbial growth and activity. Fresh pomace oil, rich in these compounds, provides antioxidant benefits that mitigate oxidative stress, particularly important for sensitive strains during cultivation. The degradation of phenolic content in expired oils suggests that factors such as light exposure, temperature, and air contribute to oxidation, emphasizing the need for proper storage conditions to preserve these beneficial components. This experience highlights the necessity of careful selection and monitoring of media components, especially oils, in cultivation protocols. Overall, these insights stress the importance of using fresh and stable growth-promoting agents to enhance the productivity and viability of sensitive microbial strains in future experiments.

3.2.2. *Use of Olive Pomace Oil*

Incorporating olive pomace oil as a carbon source is a novel approach, serving not only as an alternative carbon source but also offering potential antioxidant properties that mitigate oxidative stress from high iron concentrations. This dual functionality promotes nutrient supply and stress alleviation, a combination rarely observed in standard growth media.

The oil must be characterized at least by its peroxide value before being incorporated into the cultivation medium. Our experiments indicated that chemical changes over time in the same oil sample, represented by specific measurements, can significantly influence oil quality and media output. Media containing trivalent iron demonstrated increased sensitivity to variations in oil source and quality.

3.2.3. *Influence of Soy Source on Daunomycin Production*

Because soy grits are coarsely ground and undergo less processing than soy flour, they are likely to retain higher levels of naturally occurring phytic acid. The differences in physical structure and starch granule size between soy grits and soy flour may influence moisture retention, microbial accessibility, and the gradual release of nutrients over time, which could enhance performance in growth dynamics. Moreover, soy grits may preserve more intact proteins and fibres from whole soybeans compared to highly processed soy flour, significantly impacting metabolic pathways.

As a potential source of phytic acid, soy grits can chelate excess iron, reducing its bioavailability and minimizing toxicity. This characteristic is particularly beneficial for maintaining stable growth environments for sensitive strains, an aspect often overlooked in standard cultivation protocols. Additionally, glycinins—an important component of soy proteins—are known to play a role in iron metabolism.

In contrast, replacing soy grits with a high-fat soy source has been shown to result in significant decreases in both media parameters and daunomycin production. This further emphasizes the importance of soy source selection in cultivation protocols. The processing methods used to produce soy flour—especially heat treatment, such as roasting—can significantly reduce both phytic acid and soluble protein contents, further complicating their use as a cultivation medium.

3.3. *Influence of Iron on Production*

Adding iron to the sugar medium caused a pronounced rust coloration after autoclaving, indicating the conversion of iron to its oxide form, which did not enhance daunomycin production. However, supplementing the original medium with yeast led to iron stabilization and increased production levels upon the addition of pomace olive oil, maintaining an olive-green-grey hue post-autoclaving. Incorporating both bivalent (Fe^{2+}) and trivalent (Fe^{3+}) iron sulfates—spontaneously formed during autoclaving—was critical for managing iron concentration. While many formulations typically use only one form of iron, providing both allows for better control over iron bioavailability and uptake, potentially enhancing metabolic processes without toxicity.

3.4. Optimal Composition for Daunomycin Production

The optimal composition for sustained daunomycin production, identified through empirical methods, was determined to be as follows (g/L): 100 g pomace oil, 5.6 g $FeSO_4$, 10 g yeast, 20 g soy grits, 5 g yeast extract, 5 g glycerol, 2 g K_2HPO_4, 3 g $CaCO_3$, and 1 g $MgSO_4$, with the pH adjusted to 6.0. Under these conditions, cultivation of the original strain achieved stable production levels of 3.0 to 3.5 g/L.

The newly developed fusion strain was subsequently tested in this optimized medium, yielding daunomycin production levels of 5.5 to 6.0 g/L post-optimization. To demonstrate the independent repeatability of production, 11 cultivations were conducted by different team members, confirming consistent daunomycin production in the developed medium (**Table 1**).

Table 1. Production of daunomycin in optimized medium

Time of cultivation [h]	N [samples]	Production [mg/L]		pH	
		Average	SD	Average	SD
120	11	1389.0	350.7	6.40	0.17
16.8	11	3070.2	724.6	6.62	0.13
216	11	4728.2	726.1	6.72	0.10
264	11	5466.4	681.4	7.24	0.41

3.5. Monitoring of Daunomycin Production

Different approaches were employed to monitor and characterize daunomycin production in the newly developed medium, including assessments of media physical parameters, process monitoring, and ultrastructural morphology of the daunomycin-containing particles and producing microorganisms.

3.5.1. Changes in Media Color and Physical Parameters

The medium in the flasks exhibits distinct color changes throughout the cultivation process. Prior to autoclaving, the color of the microbial growth medium undergoes significant changes throughout the cultivation process, beginning with its initial appearance. A non-sterile medium that starts off as a specific color—such as light brown—may experience alterations upon autoclaving, owing to Maillard reactions or the breakdown of certain compounds. This initial transformation sets the stage for how microbial metabolism can further impact the visual characteristics of the medium. The autoclaved medium remains stable without any color changes for weeks when stored even at room temperature.

As the cultivation progresses, the medium transitions from a grey-olive green to a dark grey hue, indicative of the accumulation of metabolic byproducts produced by Streptomyces species.

Twenty-four hours post-inoculation, it darkens to a deeper grey, continuing to evolve in color over time. By 48 hours post-inoculation, the color shifts further to dark grey, with visible green oily droplets emerging. Black particles, remnants of soy grits bound to iron, become apparent. The oil within the medium is not fully dispersed, creating bubbles along the sides of the flask. At approximately 72 hours after inoculation, the medium transforms into a dark grey-black color. A layer of oil forms on the surface, which may take on a slight orange hue as microorganisms begin to produce daunomycin in significant amounts.

During the cultivation period (ranging from 72 to 264 hours), the medium undergoes notable changes in color: it transitions to a complete brown-red-black hue, and the number of visible particles at the bottom of the tilted flask increases. The oil becomes fully dispersed in the medium, exhibiting a significant orange color, indicative of daunomycin presence. When the flask is left undisturbed, a slight but discernible orange layer containing daunomycin may form at the top of the medium due to the oil.

The initial color of the medium post-autoclaving, characterized by an olive grey/green hue, suggests the presence of oxidized compounds and iron in solution (Fe^{3+}), indicating that iron is in an oxidized state. This coloration is often indicative of specific iron compounds and potentially other components within the complex medium. Additionally, the olive or grey tint may arise from supplementary elements such as phytates from soy flour or phenolic compounds found in olive pomace oil, which contribute to this initial hue.

As the substrate is utilized and the culture transitions into a more reduced state during cultivation, the medium darkens significantly. This color change correlates with increased anaerobic or reducing conditions, likely due to reduction processes facilitated by the metabolic activities of strain. The darkening indicates the formation of reduced iron species (Fe^{2+}) as Fe^{3+} is reduced during microbial respiration and other metabolic processes.

Moreover, the accumulation of other metabolites or degradation products from microbial growth may further contribute to the overall darkening. At the peak of daunomycin production, the medium exhibits a complete black coloration, signifying a substantial shift in the chemical composition.

This extensive reduction of ferric iron to ferrous iron, along with the production of complex organic molecules such as daunomycin and possibly other metabolites, results in this dark appearance. The black color may also indicate the formation of complex compounds, either through the aggregation of iron with organic matter or through the precipitation of iron complexes, particularly in systems where high concentrations of organic acids and phenolic compounds are present.

The changes in color of the culture medium correlate closely with the redox potential (Eh) and the microbiological activity of strain. The darkening indicates the reduction of oxidized iron species and possibly the production of additional organic compounds (e.g., daunomycin and other metabolites), which uniquely alter the optical properties of the medium.

The presence of black, hard precipitates at the end of fermentation mixed with inactive daunomycin that can be liberated by strong acid calls for a deeper exploration of the chemical interactions occurring during the fermentation of Streptomyces coeruleorubidus. Here are some potential explanations for this phenomenon, implications for your process, and suggestions for further exploration.

3.6. Chemical Nature of Precipitates

Formation of Precipitated Complexes: The black precipitate is likely composed of complexed iron (Fe^{2+} or Fe^{3+}) in conjunction with organic matter produced during the fermentation process, including metabolites such as daunomycin. Precipitation occurs when iron ions interact with specific organic compounds or when pH levels shift due to microbial metabolism. Often, phenolic compounds and other secondary metabolites can bind with iron, resulting in the formation of insoluble complexes that aggregate into larger precipitates.

Inactive Daunomycin: The inactivity of the daunomycin in the precipitate could be due to its binding to iron or other components. When daunomycin complexes with iron or other molecules, it may lose its bioactivity. This situation can arise due to structural changes in the drug or steric hindrance. The black precipitate likely represents a combination of iron-daunomycin complexes and other organic materials. The binding of daunomycin may render it unavailable in its active form.

3.7. Measurement of pH, DO, and Eh During Fermentation

To confirm the hypothesis that color changes in the culture medium result from alterations in redox potential (Eh) and iron reduction during fermentation, we conducted precise measurements of pH, dissolved oxygen (DO), and Eh throughout the fermentation process. The results presented aim to elucidate how these parameters interact and influence one another, thus providing insights into the biochemical processes during microbial growth. By systematically correlating the data from these measurements with the observed color variations, we sought to identify the most effective indicators for monitoring the cultivation process.

However, our results indicated that pH and DO were inadequate metrics for this purpose. While pH experienced minimal fluctuations, DO levels significantly declined within the first 24 hours, ultimately dropping to near-zero values. This decline was further exacerbated by calibration limitations of the oxygen electrode and only became evident at the point of process collapse.

In contrast, the measurement of redox potential emerged as a highly informative parameter. Our findings revealed a marked increase in Eh immediately after autoclaving, likely due to the oxidation of organic compounds and trivalent iron (Fe^{3+}) present in the medium. This observation suggests that redox potential is a more reliable indicator for monitoring fermentation processes and understanding the biochemical changes linked to color variations in the culture medium. Notably, we found an inverse relationship between Eh values and daunomycin production: as Eh decreased during fermentation, daunomycin production correspondingly increased. This correlation underscores the potential for utilizing redox potential as a tool to optimize conditions for enhanced secondary metabolite production.

Based on the obtained data, the changes related to Eh during cultivation can be generally divided into three phases:

1. *Eh Measurement Before and After Autoclaving:* Measurements of redox potential (Eh) provide critical insights into the state of the culture medium before and after autoclaving. Prior to autoclaving, Eh reflects the initial conditions of the medium, which may contain a mix of organic substrates, dissolved oxygen, and potentially microbial metabolites or contaminants that influence redox potential. Following autoclaving, a marked increase in Eh is observed, attributable to several key factors. First, the breakdown of organic compounds during autoclaving releases oxidizing agents, such as hydrogen peroxide, and modifies the solubility of metal ions, collectively enhancing the overall redox potential. Additionally, autoclaving effectively sterilizes the medium, allowing any residual dissolved oxygen to remain, further contributing to the elevation of Eh values. These changes underscore the significance of monitoring redox potential, illustrating how autoclaving not only sterilizes but also transforms the oxidation-reduction environment of the culture medium.

2. *During Microbial Growth:* As the growth of Streptomyces coeruleorubidus progresses, we observe a notable decline in redox potential (Eh), driven by intrinsic biological processes. This decrease is primarily attributed to microbial metabolism, where the breakdown of carbon sources generates reducing equivalents, such as NADH. These reducing equivalents are utilized in various biochemical pathways, resulting in a reduced oxidation state of the medium. Moreover, the aerobic growth of the bacteria consumes dissolved oxygen, further promoting reducing conditions. The accumulation of reductive metabolites, including organic acids and other reducing agents, exacerbates the decline in redox potential. Collectively, these factors highlight the dynamic interplay between microbial activity and redox potential, emphasizing how the metabolic processes of Streptomyces coeruleorubidus significantly alter the redox environment as the culture develops.

3. *The Irreversible End of Cultivation:* At the irreversible end of microbial cultivation, we observe a significant increase in redox potential (Eh), signalling critical phenomena within the culture medium. One major contributor to this rise in Eh is nutrient depletion; as available substrates become scarce, the metabolic processes of microorganisms may shift, potentially halting or reversing reduction processes due to resource lack. This transition can create oxidative conditions as the medium recovers from previous reduced states. Additionally, cell lysis releases various intracellular compounds that can be complex with metal ions, such as iron, which may lead to cytotoxic effects that inhibit bacterial growth, further influencing the redox state. Moreover, as the culture nears the end of its viability, remaining cells may begin to oxidize any residual substrates, contributing to the increase in Eh. Together, these factors illustrate how the microbial community's dynamics evolve toward the conclusion of cultivation, culminating in a notable shift in the redox potential of the medium as resources dwindle and cellular structures break down.

3.8. Sediment friability

As previously described, we propose that the sediment may consist of aggregates formed from smaller particles containing daunomycin and iron. To validate this hypothesis, we assessed the friability of the sediment using a Malvern 2000 particle size analyzer. When the sediment was mixed with 0.1% SDS in water, the aggregates initiated

a time-dependent disaggregation process characterized by an increase in the number of smaller, free particles.

These particles seem to undergo spontaneous aggregation throughout the fermentation process. This phenomenon may be partially explained by changes in the consistency of the growth medium, as well as a potential increase in precipitate formation during fermentation. The interaction between these factors likely facilitates the aggregation process, leading to the formation of the observed sediment. The data obtained significantly enhance our understanding of the nature of the sediments. To further clarify the formation and morphology of the sediment-forming particles, it was crucial to observe their morphology during the aggregation process.

3.9. Observation of Morphological Changes During Fermentation

The morphological diversity observed during the fermentation process highlights various particle sizes and structures formed throughout synthesis. As illustrated in the accompanying photographs, the medium contains a multitude of particles exhibiting different shapes and sizes, ranging from nanoparticles to more organized structures, which likely correspond to different stages of fermentation and detoxification.

The presence of nanoparticles indicates successful iron reduction and the formation of stable iron-containing particles. These nanoparticles are characterized by their small size, which contributes to unique properties, including increased surface area and potential bio-reactivity.

Micro-sized particles, ranging from approximately 3 to 5 microns, likely represent intermediate structures developed during fermentation. These particles are composed of membrane-bound, optically dense materials, which may indicate the aggregation of nanoparticles (see Figures 3b and 3c), and are covered by a shell formed by dark oval structures.

Moreover, the identification of larger sediment pieces, greater than 100 microns, suggests the aggregation of smaller particles, potentially facilitated by interactions with extracellular polymeric substances (EPS) produced during microbial growth or the settling of heavier complexes that form throughout the fermentation process.

Scanning Electron Microscopy (SEM) analysis of the native samples reveals that these large particles are covered by a layer of inorganic material, identified by X-ray Diffraction (XRD) analysis as gypsum (see Figures 5d and 5e). This inorganic surface layer may stabilize the particles and prevent the release of daunomycin into the medium.

The photographs also indicate the presence of highly variable particles or aggregates, which might interact with or store daunomycin. Most particles consist of iron-organic water-insoluble precipitates covered by a mineral gypsum layer. To ascertain the presence of daunomycin within these particles, it was necessary to remove the protective layers using hydrogen peroxide.

Exposure to hydrogen peroxide resulted in the bleaching of darker pigmentation, liberating reddish vesicles. Extended exposure led to an increase in the number of vesicles formed. High-Performance Liquid Chromatography (HPLC) analysis of the collected vesicles confirmed the presence of daunomycin.

The release of pigmented vesicles or vacuoles suggests that daunomycin is likely localized within membrane-bound structures inside the larger particles rather than

existing in a freely accessible form. This finding implies that the optically dense structures identified by Transmission Electron Microscopy (TEM) may serve as reservoirs for daunomycin, effectively isolating it from the surrounding environment.

The presence of iron in the particles was proven by a combination of SEM and EDS. The results from the energy-dispersive X-ray spectroscopy (EDS) analysis reveal the presence of iron (Fe) in the analyzed sample. The Fe L emission lines were not detected, indicating that the EDS system did not identify any significant contributions from the L shell transitions for iron. In contrast, the Fe K emission lines exhibited a measurable intensity of 5.27 counts, with an uncertainty of ±0.79, suggesting a notable presence of iron in the sample. This intensity implies that iron is a significant component within the analyzed material.

3.10. Detection and identification of proteins in the daunomycin-iron-organic matter complex

To determine the daunomycin binding or transporting protein homogenate from the washed sediment was prepared in a sample buffer and analyzed on 10% gel. The proteins involved in daunomycin interaction were stained directly by a metabolite and control visually.

Samples for SDS-PAGE were prepared in a reducing buffer, boiled, and separated on a 10% gel, followed by staining with Coomassie Blue. After destaining, the visible proteins were excised and analyzed by TOF MALDI to determine the proteins present in the complex. Only results with more than three matches and greater than 20% sequence coverage were considered positive.

Under conditions characterized by high iron content and chemical stress, two distinct groups of proteins exhibit critical functional similarities in managing oxidative stress and enhancing cellular resilience.

Group One	Group Two
➢ **Bacterioferritin** ➢ **Catalase** ➢ **Oxidoreductase** ➢ **Glycinin G1** ➢ **Glycinin G4** ➢ **β-Conglycinin**	➢ Chemical-damaging agent resistance protein C ➢ Major outer membrane lipoprotein ➢ Xanthine dehydrogenase ➢ Molybdopterin dehydrogenase ➢ Aklanon acid methyl ester cyclase (Dau D) ➢ Superoxide dismutase ➢ Nickel-containing superoxide dismutase ➢ Glutamate-binding protein

In a high iron content medium, proteins such as bacterioferritin [28], catalase, [29,30], oxidoreductase [31–33], glycinin G1, glycinin G4, and β-conglycinin exhibit functional similarities that are crucial for managing iron homeostasis and mitigating oxidative stress related to elevated iron levels [34–36]). Bacterioferritin plays a vital role in sequestering excess iron to prevent toxicity by storing it in a biologically accessible form while

facilitating its release when needed for cellular processes [28]. Catalase is essential in protecting cells from oxidative damage that can arise from increased iron, particularly by catalyzing the formation of reactive oxygen species, including hydrogen peroxide. Oxidoreductases contribute to redox balance in the presence of high iron by facilitating necessary metabolic reactions and detoxifying harmful compounds. Additionally, storage proteins like glycinin G1, G4, and β-conglycinin provide essential amino acids and may participate in cellular stress responses, modulating protein synthesis and repair processes in response to elevated iron levels. Together, these proteins work synergistically to maintain iron homeostasis, protect against oxidative stress, and support overall metabolic functions, reinforcing resilience in high-iron environments.

Although not quantified, phytic acid, which is present in soy grits, serves as a multifunctional component in high-iron content systems. It supports iron chelation, acts as an antioxidant, regulates mineral absorption, and may influence cellular signaling and gene expression. Its presence can synergize with the aforementioned proteins, bolstering iron homeostasis and enhancing the organism's overall resilience under stress conditions.

The second group of proteins—including chemical-damaging agent resistance protein C, major outer membrane lipoprotein, xanthine dehydrogenase (YagS FAD-binding subunit), molybdopterin dehydrogenase, alkanoic acid methyl ester cyclase (Dau D), putative flavoprotein, superoxide dismutase, nickel-containing superoxide dismutase, and glutamate-binding protein—exhibits notable functional similarities related to stress response. Specifically, these proteins contribute to the organism's ability to withstand chemical toxicity and oxidative stress. The chemical-damaging agent resistance protein C provides protection against harmful compounds, while the major outer membrane lipoprotein helps maintain membrane integrity under stress [37]. Xanthine dehydrogenase [38] and molybdopterin dehydrogenase [39] are critical for redox balance and managing reactive oxygen species. Although the alkanoic acid methyl ester cyclase is primarily associated with secondary metabolite biosynthesis of anthracyclines [40], it also participates in metabolic pathways essential for stress resilience. Additionally, superoxide dismutase and nickel-containing superoxide dismutase directly combat oxidative damage, converting harmful superoxide radicals into less toxic molecules [41]. Finally, glutamate-binding protein belongs to ABC transporters and may support cellular homeostasis indirectly under stress conditions [42]. Collectively, these proteins enhance the strain's survival and adaptability amidst chemical stresses, underscoring their importance in the strain's metabolic and stress response networks.

It is of the utmost importance to highlight the unique origin of the Streptomyces strain employed in this study, which was isolated from mosquito larvae. This research not only delineates a novel cultivation process for the production of daunomycin but also illuminates the significant potential of insect-derived microorganisms as valuable assets in pharmaceutical research. Insects, representing one of the most diverse taxa on the planet, are hosts to a vast array of microorganisms, which may yield a wealth of bioactive compounds relevant to drug development. Recent studies have increasingly recognized these insect-associated bacteria as promising sources for pharmaceutically active substances [43–45].

The metabolic diversity inherent in these insect-associated microbes, cultivated over millions of years of co-evolution with their hosts, presents a significant opportunity for advancing drug discovery, particularly in the context of rising antimicrobial resistance and the emergence of novel diseases [46,47]. It is, therefore, evident that the exploration of this hitherto underutilized microbial reservoir has the potential to pave the way for the identification of innovative therapeutic agents, thereby making a significant contribution to the ongoing efforts in pharmaceutical research and development.

5. Conclusions

In conclusion, this study presents significant advancements in understanding the biogenic nanoparticle formation mechanism, particularly in the context of daunomycin production, an anthracycline antibiotic pivotal for the treatment of cancers such as leukemia. The inherent cytotoxicity of daunomycin and the challenges associated with its industrial-scale synthesis due to microbial toxicity have limited its therapeutic applicability. By developing a specialized cultivation medium that integrates olive pomace oil and iron, this research successfully induces an autonomous resistance mechanism through biogenic nanoparticle formation (ADBN). The amphiphilic properties of olive pomace oil not only serve as a carbon source but also facilitate the stabilization of nanoparticles, thereby enhancing the efficacy of the synthesis process due to its rich phenolic content, which promotes crucial redox reactions.

The optimization of the medium composition through empirical methods resulted in a marked increase in daunomycin production, achieving yields between 5.5 and 6.0 g/L, which demonstrates a significant advancement relative to prior methodologies. Characterization of the nanoparticles confirmed the successful incorporation of iron and daunomycin, underscoring the potential of this approach to mitigate cytotoxicity while improving yield. The presence of specific proteins associated with iron homeostasis and oxidative stress response further illustrates the organism's ability to adapt to high iron concentrations, highlighting the intricate biochemical pathways at play. Moreover, the observed inverse correlation between redox potential (Eh) and daunomycin production suggests that monitoring Eh could serve as a valuable indicator for optimizing fermentation conditions.

This research not only contributes to the field of microbial fermentation and antibiotic production but also emphasizes the importance of minimizing environmental impacts through the production of insoluble daunomycin precipitates that can be efficiently recovered from the cultivation medium. Overall, these findings present promising avenues for further investigation into the mechanisms underlying biogenic nanoparticle formation and the optimization of cultivation processes. Such explorations may not only refine microbial production systems for daunomycin but also broaden the potential application of similar strategies for the synthesis of other therapeutically essential compounds.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, V.M. and K.B.; methodology, V.M.; software, M.R.; validation, B.P., A.V., V.C., and J.M.; formal analysis, J.M.; investigation, K.B.; resources, V.M.; data curation, B.P.; writing—original draft preparation, V.M., V.C., and B.P.; writing—review and editing, V.M., B.P., V.C., A.V.,; visualization, K.B., B.P.; supervision, V.M and V.C. All authors have read and agreed to the published version of the manuscript."

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7. Conclusion

In conclusion, this study presents significant advancements in understanding the biogenic nanoparticle formation mechanism, particularly in the context of daunomycin production, an anthracycline antibiotic pivotal for the treatment of cancers such as leukemia. The inherent cytotoxicity of daunomycin and the challenges associated with its industrial-scale synthesis due to microbial toxicity have limited its therapeutic applicability. By developing a specialized cultivation medium that integrates olive pomace oil and iron, this research successfully induces an autonomous resistance mechanism through biogenic nanoparticle formation (ADBN). The amphiphilic properties of olive pomace oil not only serve as a carbon source but also facilitate the stabilization of nanoparticles, thereby enhancing the efficacy of the synthesis process due to its rich phenolic content, which promotes crucial redox reactions.

The optimization of the medium composition through empirical methods resulted in a marked increase in daunomycin production, achieving yields between 5.5 and 6.0 g/L, which demonstrates a significant advancement relative to prior methodologies. Characterization of the nanoparticles confirmed the successful incorporation of iron and daunomycin, underscoring the potential of this approach to mitigate cytotoxicity while improving yield. The presence of specific proteins associated with iron homeostasis and oxidative stress response further illustrates the organism's ability to adapt to high iron concentrations, highlighting the intricate biochemical pathways at play. Moreover, the observed inverse correlation between redox potential (Eh) and daunomycin production suggests that monitoring Eh could serve as a valuable indicator for optimizing fermentation conditions.

This research not only contributes to the field of microbial fermentation and antibiotic production but also emphasizes the importance of minimizing environmental impacts through the production of insoluble daunomycin precipitates that can be efficiently recovered from the cultivation medium. Overall, these findings present promising avenues for further investigation into the mechanisms underlying biogenic nanoparticle formation and the optimization of cultivation processes. Such explorations may not only refine microbial production systems for daunomycin but also broaden the potential application of similar strategies for the synthesis of other therapeutically important compounds.

In conclusion, it is of the utmost importance to highlight the unique origin of the Streptomyces strain employed in this study, which was isolated from mosquito larvae. This research not only delineates a novel cultivation process for the production of daunomycin but also illuminates the significant potential of insect-derived microorganisms as valuable assets in pharmaceutical research. Insects, representing one of the most diverse taxa on the planet, are hosts to a vast array of microorganisms, which may yield a wealth of bioactive compounds relevant to drug development. Recent studies have increasingly recognized these insect-associated bacteria as promising sources for pharmaceutically active substances (Piel 2006; Chevrette et al. 2019; Van Moll et al. 2021; Diarra et al. 2024).

The metabolic diversity inherent in these insect-associated microbes, cultivated over millions of years of co-evolution with their hosts, presents a significant opportunity for advancing drug discovery, particularly in the context of rising antimicrobial resistance and the emergence of novel diseases (Bode 2011; Dettner 2011). It is therefore evident that the exploration of this hitherto underutilized microbial reservoir has the potential to pave the way for the identification of innovative therapeutic agents, thereby making a significant contribution to the ongoing efforts in pharmaceutical research and development.

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