Fakulta zemědělská
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Jihočeská univerzita
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FACULTY OF AGRICULTURE AND TECHNOLOGY

Department of Genetics and Biotechnology

Dissertation Abstract

Enhanced production of daunomycin in *Streptomyces coeruleorubidus*

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Supervisor prof. Ing. Vladislav Čurn, Ph.D.

.

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JIHOČESKÁ UNIVERZITA V ČESKÝCH BUDĚJOVICÍCH

ZEMĚDĚLSKÁ A TECHNOLOGICKÁ FAKULTA

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Autoreferát disertační práce

Zvýšená produkce daunomycinu u *Streptomyces coeruleorubidus*

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Supervisor prof. Ing. Vladislav Čurn, Ph.D.

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Dissertation Abstract	
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The defense of the dissertation will take place on 11/12/2024 at 1:30 p.m. in the meeting and seminar room ZR 01 053, 1st floor at the Department of Genetics and Agricultural Biotechnology, Faculty of Agriculture and Technology, University of South Bohemia in České Budejovice

You are cordially invited to familiarize yourself with the dissertation at the study department of the Faculty of Agriculture and Technology, University of South Bohemia in České Budejovice
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prof. Ing. Vladislav Čurn, Ph.D.
Head of the Board
Agricultural Biotechnology
FAT USB České Budejovice

Autoreferát disertační práce	
Doktorand:	Ing, Baveesh Pudhuvai, Msc.
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Obhajoba disertační práce se koná dne 11.12.2024 v 13:30 hodin v zasedací a seminární místnosti ZR 01 053, 1. patro na katedře genetiky a biotechnologií Fakulty zemědělské a technologické JU v Českých Budějovicích.

S disertační prací se lze seznámit na studijním oddělení Fakulty zemědělské a technologické JU v Českých Budějovicích.

prof. Ing. Vladislav Čurn, Ph.D.
Předseda oborové rady
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Abstract

This Ph.D. thesis is focused on the importance of daunomycin/daunorubicin and a novel method to enhance its production in the *Streptomyces ceoruleorubidus* in combination of cultivation media modifications and counteracting autotoxicity. 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. This work 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. This research successfully induces an autonomous resistance mechanism through biogenic nanoparticle formation (ADBN) by developing a specialised cultivation medium that integrates olive pomace oil and iron. The approach of substituting sugar with oil in culture media has a dual role where it promotes *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 synthesising other therapeutically important compounds.

We anticipate that this work will help researchers working with secondary metabolite production 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-interaction*

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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. 2010a; Vasanthakumar et al. 2013a).

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. 2011a).

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 soilborne 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).

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). They possess a lifecycle similar to filamentous fungi, reproduce through sporulation, and exhibit siderophore activity and produce metabolites like desferrioxamine (iron chelator), geosmin (earthy smell organic compound), streptothricin (antibiotic) and streptomycin (antibiotic) (Zhu et al. 2014; Martín-Sánchez et al. 2019). Approximately 90% of the know antibiotics are obtained from the organisms of *Streptomeces* genera and to date, more than 500 naturally occurring anthracyclines have been isolated from *Streptomyces* sp. (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. 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 (DNR) also called Daunomycin, is an anthracycline antibiotic that was first

discovered in 1964 from *Streptomyces peucetius and* is extensively employed in treating malignant tumours, especially leukemia (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 these 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). 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. 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) and gene regulation

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). 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. 2013b; 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. 2011b).

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. 2013b). The expression and function of drrA and drrB are interdependent 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. Overexpression of *drrAB* has resulted in the overproduction of DNR and promoted self-resistance (Li et al. 2014). Thus, the self-resistance genes also indirectly affect the biosynthetic pathway in DNR/DOX production (Srinivasan et al. 2010b). 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.

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). 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, 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 reduce the side effects of 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). They are key components of the cell membrane that regulate the internal cellular toxic chemicals and elements (metal ions) concentrations through extrusion and also inhibit the re-entry of compounds to evade toxicity (V 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. These efflux pumps comprise transmembrane protein helices facilitating the translocation of produced secondary metabolites (Gaurav et al. 2023). 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).

The ABC (ATP-binding cassette) pumps facilitate the import and export of chemical substances based on their structural architecture and folding (Thomas and Tampé 2024). The ABC efflux pumps in bacteria translocate various compounds like sterols, secondary metabolites and lipids across the membrane through 12 transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). 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 (Kaur and Russell 1998). Several followup 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 (Dong et al. 2024) .

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 strain efficiency, ability to utilise the available nutrients, physical conditions and productivity of the metabolites (Rodrigues da Silva et al. 2021). 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.

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 highest yields are achieved by combining several approaches to strain development, suitable culture media composition, and well-optimised fermentation conditions. 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).

A) 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). 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.

B) 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).

C) 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 (F 2004; Romero-Rodríguez et al. 2018). The limited availability of phosphate results in nutritional stress and initiates the secondary metabolite biosynthetic pathways. 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).

D) 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 a medium for the cultivation of *Streptomyces coelicolor* and reported an increase in activity by 2.5 to 12-fold (Tanaka et al. 2010). Optimization and starndardization 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 article is to prepare culture media for cultivating *Streptomyces* sp. based on binding 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 in the culture medium, converting the DNR/DOX to inactive forms and settling with the DNR-iron-oil complex. Therefore, this hypothesis can safeguard the producer strain from toxicity and avoid inhibiting 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 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. (Olano et al. 2008). The ARC2 similar to the antimicrobial compound triclosan, has been reported to partially inhibit the fatty acid synthesis and utilize the acetyl CoA for polyketide biosynthesis and improve 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 prospective three-way interaction 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. *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 the 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. 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 Mať 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 developing 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 minimising 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. 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.

5. 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 treating 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 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. 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, highlighting the unique origin of the *Streptomyces* strain employed in this study, which was isolated from mosquito larvae, is of the utmost importance. This research not only delineates a novel cultivation process to produce 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 many 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 identifying innovative therapeutic agents, thereby making a significant contribution to the ongoing efforts in pharmaceutical research and development.

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7. List of Publications

Publications Supporting this Thesis Methods and Results.

- Jozová E, Rost M, Rychlá A, Stehlíková D, **Pudhuvai B**, Hejna O, Beran P, Čurn V, Klíma, M 2023. Microsatellite Markers: A Tool to Assess the Genetic Diversity of Yellow Mustard (*Sinapis alba L.*). Plants, 12, 4026. https://doi.org/10.3390/plants12234026

- **Baveesh Pudhuvai**, Karel Beneš, Vladislav Čurn, Andrea Bohata, Jana Lencova, Radka Vrzalova, Jan Barta, Valdimir Matha 2024. Enhancement of Daunomycin Production in Streptomyces sp. By Counteracting Autotoxicity – *A Prospective*. Microrganisms (Under review)

- Karel Beneš, **Baveesh Pudhuvai**, Vladislav Čurn, Andrea Bohata, Jana Lencova, Jan Barta, Valdimir Matha 2024. Dounomycin Production Enhancement in *S.ceoruleorubidus* by Adapting Autonomous Defense Through Biogenic Nanoparticle Formation. Microorganisms (Under preparattion)

Publications during the course of Study.

- **Pudhuvai, B**., Koul, B., Das, R. et al. Nano-Fertilizers (NFs) for Resurgence in Nutrient Use Efficiency (NUE): a Sustainable Agricultural Strategy. Curr Pollution Rep 11, 1 (2025). https://doi.org/10.1007/s40726-024-00331-9

- Ahamed Khan, **Baveesh Pudhuvai**, Ankita Shrestha, Ajay Kumar Mishra, Maulin P. Shah, Bhupendra Koul & Nrisingha Dey (2023) CRISPR-mediated iron and folate biofortification in crops: advances and perspectives, Biotechnology and Genetic Engineering Reviews, DOI: 10.1080/02648725.2023.2205202 (IF 4.2)

- Khan, A.; Nasim, N.; **Pudhuvai, B**.; Koul, B.; Upadhyay, S.K.; Sethi, L.; Dey, N. Plant Synthetic Promoters: Advancement and Prospective. Agriculture 2023, 13, 298. https://doi.org/10.3390/agriculture13020298 (IF 3.4)

- Koul, B.; **Baveesh, P**.; Sharma, C.; Kumar, A.; Sharma, V.; Yadav, D.; Jin, J.-O. *Carica papaya* L.: A Tropical Fruit with Benefits Beyond the Tropics. Diversity 2022, 14, 683. https://doi.org/10.3390/d14080683. (IF 3.03)

Book Chapters:

- Koul, B., **Pudhuvai, B**., Bhanot, M., Tiwari, S. (2024). Updates on Global Status of Transgenic and Genome-Edited Crops. In: Tiwari, S., Koul, B. (eds) Genetic Engineering of Crop Plants for Food and Health Security. Springer, Singapore. https://doi.org/10.1007/978-981-97-3119-0_19 2.

8. Participation in foreign or domestic conferences and internships

- Internship with Oncora S.R.O (2021-2023), Nemanicka 2722, České Budejovice, Czech Republic.
- Internship with VUAB Pharma A.S (2024), Roztoky, Nemanicka 2722, České Budejovice, Czech Republic.
- Conference Participation and Poster Presentation in CPSC conference: Translational agriculture - from model plants to crops, Aug-2024, Copenhagen, Denmark

9. Curriculum vitae

PERSONAL INFORMATION

Name: Pudhuvai Baveesh,
Date of Birth: 18 October 1992. Nationality: Indian.
Current address: Ceske Budejovice, Czech Republic
ORCiD:0000-0002-3563-2142
Email pudhuvaibaveesh@gmail.com Phone: +420-737973975.

EDUCATION AND TRAINING

Master of Science (M.Sc.) in Agriculture (Plant Breeding and Genetics) 2014-2016

Institute Lovely Professional University, Punjab, India.
Thesis Assessment of Genetic variability in Tomato (*Solanum lycopersicum. L*)

Bachelor of Science (B.Sc.) in Agriculture 2010-2014

Institute Faculty of Agriculture, Annamalai University, Tamil Nadu, India.
Thesis The antagonistic potential of various synthetic and non-synthetic agents for controlling the bacterial canker (*Xanthomonas axonopodis pv. citri*) in citrus.

PROFESSIONAL POSITIONS

10/2023 – Present		Research employee (enzymology) (https://www.vuab.cz) (*part-time*) at VUAB Pharma a.s. Ceske Budejovice, Czech Republic.
11/2019-09/2023		Former employee at Oncroa s.r.o - www.oncora.cz Ceske Budejovice, Czech Republic.
	Responsibilities	Isolation of plasmid DNA, competent cell preparation, bioinformatic analysis, gene transformation, recombinant protein production (Phosphorylases: UP, PNP), protein purification, SDS-PAGE, HPLC.
10/2019 – Present		Doctoral researcher, Department of Genetics and Biotechnology, Faculty of Agriculture and Technology, University of South Bohemia, Ceske Budejovice, Czech Republic.
	Responsibilities	Isolation of DNA, RNA, PCR, qPCR analysis for yellow mustard (*Sinapis alba*) and honey bee samples, manuscript writing, and revisions
08/2018 – 09/2019		Research Assistant. Institute of Parasitology, Biology Centre, Czech Academy of Sciences, Czech Republic
	Responsibilities	Isolation of parasitic DNA, RNA, qPCR in carp fish, fish haematology (RBC & WBC Separation), protozoan culturing in fish and handling experimental fish facility.
06/2016 -11/2017		Research Associate & Teaching Assistant, (Regional Agricultural Research Station, Kerala, India) funded by **ICAR** (Indian Council for Agricultural Research)
	Responsibilities	Screening and analysis of newly developed rice varieties (breeder & hybrid seeds) from breeding stations all over India for their abiotic and biotic stress tolerance, nutrient deficiency, yield, and crop duration for further field application and seed certification. Teaching Genetics, Plant Breeding, Physiology and Seed technology subjects for Diploma (polytechnic) Students and tour visiting schools.

TECHNICAL SKILLS

- DNA, RNA, plasmid isolation, Agarose electrophoresis, SDS-PAGE, PCR, qPCR, Flow cytometry, cloning, Bacterial transformation, vector screening, protein isolation, and protein immobilization.
- Recombinant nucleoside enzymes (UDP/PNP) production technology (*E. coli* expression system), bacterial fermentation (lab-scale).
- Planning and handling research plots of rice and field plots of breeding trials, Varietal improvement trials, and Hybrid seed production.
- Basic knowledge and skills in working and maintenance of aquaponics setup.
- Bioinformatics basic tools (Sequence analysis (DNA/protein) and phylogeny, domain/motif analysis).

AGRICULTURAL PROJECTS HANDLED

Rural developmental project – as part of Bachelor's education curriculum

- **Team Lead-** RAWE (Rural Agricultural Work Experience Program), Tamilnadu, 2014.
  Responsibilities: Led a team of 25 members for 90 days, conducted crop awareness campaigns, training and demonstration sessions on various agricultural technologies, rural health camps for farmers and planning village agricultural operations with local leaders.

ICAR Technical projects - as an employee in the National Research Station (RICE mandate)

- **Plant physiology AICRIP** (All India coordinated Rice Improvement Program)
  Supervised six projects of rice varietal trials, planning, execution, pest anddisease management, cultivation practices, data collection, and screening for developing new varieties.
- **Genetics & Plant Breeding AICRIP**- (All India coordinated Rice Improvement Program)
  Supervised thirteen projects of rice varietal trials for breeding traits like resistance, tolerance, stress, and disease factors. Besides responsible for planning, execution, pest/disease management, data collection, and screening for new varieties.

WORKSHOPS/CERTIFICATIONS/ SEMINARS ATTENDED: 9 (major ones listed here)

- Flow-cytometry workshop by Thermofisher, Czech Republic, Sept-2018.
- Biotechnology conference, MEVPIS, Czech Republic, Sept-2018.
- International workshop of Plant genomics and Bioinformatics Feb-2022.
- CPSC conference: Translational agriculture - from model plants to crops, Aug-2024, Copenhagen, Denmark

OUTREACH ACTIVITIES

International Student Ambassador, University of South Bohemia, Ceske Budejovice. Czech Republic.
Responsibilities: 1) Participation in Education fairs and public exhibitions (As a university representative).
2) Contributing to University Web pages (*Blogs* and *articles*),
3) Content creation for university social media handles for research updates/news (Instagram, Facebook and Linkedin)

LANGUAGES: English (B2 FCE), Hindi, Telugu, Tamil, Malayalam, **Czech (A2).**

HOBBIES: Cooking, Travel, Motorbiking, Hiking, photography, tracking company financial reports

LICENSE: **EU** Driver's license "**B**" (issued in Czech Republic), Indian "**LMV/TW**" (Car and motocycle)

REFERENCES

1. Prof. Vladislav Čurn, Department of Genetics and Biotechnology, Faculty of Agriculture and Technology, University of South Bohemia in Ceske Budejovice, 370 05 Ceske Budejovice, Czech Republic.
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2. Dr Andrea Bohata, Vice Dean, Faculty of Agriculture and Technology, University of South Bohemia in Ceske Budejovice, 370 05 Ceske Budejovice, Czech Republic.
   bohata@fzt.jcu.cz

I hereby declare that all the details stated above are true to the best of my knowledge.

Czech Republic. Baveesh Pudhuvai