Studies on *Achromobacter denitrificans* strain SP1 regarding production of prodigiosin upon utilizing hazardous phthalate

Thesis submitted to the
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DOCTOR OF PHILOSOPHY IN BOTANY

by

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This is to certify that both the adjudicators have not mentioned any corrections in the thesis entitled "Studies on *Achromobacter denitrificans* strain SP1 regarding production of prodigiosin upon utilizing hazardous phthalate" submitted by **Mr. Hareesh, E.S.,** Research scholar, Department of Botany, University of Calicut. The thesis is hereby submitted to the University of Calicut vide reference no. 20182/RESEARCH-C-ASST-1/2021/Admn. dated 22/05/2021

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No part of the present work has formed the basis for the award of any other degree or diploma, previously.

University of Calicut
30th January 2021

Dr. C. C. Harilal
(Supervising Teacher)

DECLARATION

I, **Hareesh, E. S.** do hereby declare that this thesis entitled **"Studies on *Achromobacter denitrificans* strain SP1 regarding production of prodigiosin upon utilizing hazardous phthalate"** is the summary of the research work carried out by me under the guidance and supervision of **Dr. C. C. Harilal**, Professor, Division of Environmental Science, Department of Botany, University of Calicut in partial fulfillment of the requirement for the award of Ph.D. degree in Botany of the University of Calicut. I also declare that no part of this thesis has been submitted by me for the award of any other degree or diploma.

University of Calicut
30th January 2021

Hareesh, E. S.

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Hareesh, E. S.

Dedicated to

Prof. Sailas Benjamin

CONTENTS

ABBREVIATIONS

BBP	:	butyl benzyl phthalate
BB	:	blood storage bag
BSM	:	basal salt medium
ddH_2O	:	double distilled water
DAPs	:	dialkyl phthalates
DBP	:	di-n-butyl phthalate
DEP	:	di-ethyl phthalate
DEHP	:	di(2-ethylhexyl)phthalate
DEIP	:	di-ethyl isophthalate
DETP	:	diethyl terephthalate
DIBP	:	di-isobutyl phthalate
DIDP	:	di-isodecyl phthalate
DOP	:	di-octyl phthalate
DMIP	:	dimethyl isophthalate
DMP	:	di-methyl phthalate
DMTP	:	di-methyl terephthalate
DINP	:	di-isononyl phthalate
DNHP	:	di-n-hexyl phthalate
DNOP	:	di-n-octyl phthalate
DNPP	:	di-n-pentyl phthalate
DPP	:	di-n-propyl phthalate
FL	:	fluorescent lamb
g	:	gram
g/L	:	gram per litre

HSL	:	homoserine lactone
IA	:	isophthalic acid
L	:	litre
MEHP	:	mono(2-ethylhexyl)phthalate
mg	:	milligram
mg/ml	:	milligram per millilitre
mL	:	milli litre
MW	:	molecular weight
PA	:	phthalic acid
PAEs	:	phthalic acid esters
PVC	:	polyvinyl chloride
QS	:	quorum sensing
RSM	:	response surface methodology
TA	:	terephthalic acid
w/v	:	weight per volume
α	:	alpha
β	:	beta
γ	:	gamma
ε	:	epsilon
µg	:	microgram
µL	:	microlitre

EQUIPMENTS USED

Item	Brand	Country
Compound microscope	Magnus	India
Refrigerated centrifuge	Remi	India
Chromatography column	Magnum	India
Digital pH meter MK-VI	Systronics	India
Double distillation unit	Borosil	India
Environmental shaker	Orbitek	India
FT-IR	Jasco	Japan
Heating mantle	Kemi	India
Incubator	Inlabco	India
Laboratory oven	Labline	India
Laminar air flow cabinet	Labline	India
LC-Q-ToF-MS	Waters Acquity	USA
Magnetic stirrer	Kemi	India
Micropipettes (0.5-1000 µL)	Accupipete, Thermo Scientific	India, Finland
Refrigerator	LG	India
Sonicator	Qsonica,LLC	USA
UV-Visible spectrophotometer	Shimadzu	Japan
Multi scanner	Thermo Scientific	Finland
Digital weighing balance	Shimadzu	Japan
Deep freezer (-40°C)	Haier Biomedical	China
Vortex mixer	Grant-bio	England
Modular baby fermenter	Murhopye Scientific	India
Lyophilizer	Labogene (ScanVac)	Denmark
Autoclave	Labline	India

CHAPTER 1

INTRODUCTION

"Necessity is the mother of invention" and is substantially true. But certain necessities that led to the invention of plastics makes us rethink about the responsible use of such inventions. The word plastic is derived from the Greek word *plastikos*, which refers to the ability to adopt to any shape or form (Shah *et al*., 2008). Appropriate polymers (polyvinyl chloride, polyethylene, polystyrene, etc.) with adequate proportions of plasticizers, inert fillers and coloring agents are blended to make plastics. Plasticizers are colorless and odorless chemicals that determine the physical properties of polyvinyl chloride. In order to impart flexibility and strength, plasticizers like phthalates are incorporated into polyvinyl chloride (PVC) plastics. Phthalates are esters of 1, 2-benzene dicarboxylic acid. They are not chemically integrated into the polymer mesh and hence leach out to the environment. Owing to the hepatoxic, teratogenic and carcinogenic properties, phthalates and their metabolites are found to be potentially harmful to the humans and the environment (Pak *et al*., 2011). PVC is linked to significant quantities (40-50 %) of phthalate esters, that serve as plasticizers for the polymer to make it durable and appropriate for all uses, including medical.

Due to particular concerns regarding pediatric exposure, two phthalates, DEHP and diisononyl phthalate (DINP), have gained significant attention in recent times. DEHP and DINP are pervasive pollutants in food, indoor air, soil and sediment, like all phthalates. DEHP is widely used in toy-making and for the manufacturing of blood and urine storage bags in hospitals (Heudorf *et al*., 2007). DEHP has detrimental consequences that can be passed onto future generations by epigenetic modifications, such as affecting the endocrine system, hormonal, metabolic and behavioral disorders (Benjamin *et al*., 2017).

Microorganisms play a lead role in the biodegradation of various contaminants in heterogeneous environmental conditions by aerobic or anaerobic processes (Pradeep *et al*., 2013). Various studies have shown the degradation of phthalates in soil, natural water and wastewater, under aerobic conditions (Zhu *et al*., 2019; Roslev *et al*., 2007). Microorganisms that degrade phthalates can be aerobic (Roslev *et al*., 2007), anaerobic (Junghare *et al*., 2016), or facultative (Liang *et al*., 2008). A number of gram-negative bacteria such as *Pseudomonas* sp. *Acinetobacter* sp. and gram-positive bacteria such as *Gordonia* sp. *Micrococcus* sp. *Bacillus* sp. etc. are the key degraders of phthalates. Phthalate degradation in gram-negative and gram-positive bacteria is different. Only a few studies have been reported from India regarding the microbial remediation of free phthalates (i.e., *ex situ* approach). If plastic materials with bound phthalates are used in bioremediation studies, the phthalates released from them would be utilized by the novel microbes as the sole source of carbon and energy (*i.e*., *in situ* approach). Only a few studies in the world addressed this *in situ* remediation of phthalates by microbes.

Achromobacter denitrificans, formerly known as *Alcaligenes denitrificans,* is a gram-negative bacterium and these bacteria are present in soil, water and rarely cause human infections (Coenye *et al*., 2003; Weitkamp *et al*., 2000). *A. denitrificans* SP1 used in this study (GenBank Accession No.HQ645935; MTCC No.5710) is isolated from the heavily plastic polluted sewage sludge of Canoly canal, flowing through the city Kozhikode, Kerala (Pradeep *et al*., 2014, 2015).

Secondary metabolites such as pigments produced by microbes are used as therapeutic agents, food additives, coloring agents etc. Prodigiosin is a tripyrrole structural cell-associated red pigment, shown to be produced by various microbes, especially *Serratia* sp. (Pradeep *et al*., 2014). The word came from ‘prodigious’ which means something miraculous. Prodigiosin has been known to be a natural compound for many decades but of late, many of

its natural as well as synthetic analogs are also available. Due to its drug ability like anti-bacterial, anti-fungal, anti-cancer, immunosuppressive, anti-diabetic, anti-rheumatic, and anti-parasitic properties, prodigiosin is a useful secondary metabolite of great interest. It could also be used as a colorant in the textile industry (Kim and Choi, 2015). The 25-C prodigiosin analog reported by Benjamin *et al*. (2012) was produced by *A. denitrificans* strain SP1 (MTCC No. 5710). Interestingly, the carbon chain (numbered 6-10) is unique, which makes it different from other analogs.

In light of the above, the present study has been undertaken to assess an optimum conditions for the bioremediation of DEHP (both *in situ* and *ex situ)* with simultaneous production of prodigiosin. Such innovations can contribute to industrially-viable technology in a cost effective manner. The specific objectives outlined in the study are listed below:

Objectives

- Influence of light conditions favouring prodigiosin production in *A. denitrificans* strain SP1.
- Response surface optimization of prodigiosin production from *A. denitrificans* strain SP1 using blood bag as the carbon source.
- Response surface optimization of prodigiosin production from *A. denitrificans* strain SP1 using free DEHP as the carbon source and scale-up in bioreactor.
- Quorum sensing mediated response of *A. denitrificans* SP1 regarding prodigiosin production under phthalate stress.
- *In vitro* studies for the drugability of 25-C prodigiosin.

CHAPTER 2

REVIEW OF LITERATURE

Part I: Phthalates are a bitter pill to swallow

2.1. Introduction

The term plastic, which implies ‘flexible and simply shaped’ is recently generalized for all polymers. The word polymer means "of many parts" and they are composed of large molecules that are made of smaller chemical units called monomers. Polymers are of two types; natural and synthetic. Natural polymers occurs in nature and examples of this kind are cellulose, chitin, carbohydrates, proteins, DNA, RNA, rubber, etc. (Benjamin *et al*., 2015). Synthetic polymers are man-made, derived mainly from petroleum oil. Plastic, which is the topic of research in the present study is a synthetic one. They are mainly of four types; thermoplastics, thermosets, elastomers and synthetic fibers. The backbone of very common synthetic polymers is made of carbon-carbon bonds; whereas heterochain polymers have other types of elements like oxygen, sulfur, nitrogen, etc. along with the carbon-carbon interactions (Peters, 2002). Thermoplastics are a type of polymers that are moldable and malleable upon heating and harden upon cooling. So, the material can be reused upon repeated heating and cooling. Some of the most common types are polyvinylchloride, polyethylene, polystyrene, polypropylene, polycarbonate, etc. Due to this property, they are an important part of everyday life like drinking bottles, grocery bags, CDs and DVDs, food storage containers, lenses, etc. (Olabisi and Adewale, 2016). Thermosetting polymers are irreversibly becoming rigid when heated, cannot change the shape once they have set; for that reason, they are mainly used as adhesives. Common examples are epoxy resins, melamine-formaldehyde, polyester

resins, urea-formaldehyde, phenol-formaldehyde, etc. They are widely been used in the manufacturing of electrical insulators, circuit boards, car bodies, control knobs, kettle handles, etc. (Pascault *et al*., 2002). Elastomers are polymers that are having both viscosity and elastic properties. They have been held together with weak intermolecular bonds. Examples of such kinds are synthetic polyisoprenes, polybutadiene, chloroprene rubber, neoprene, butyl rubber, ethylene-propylene rubber, epichlorohydrin rubber, silicone rubber, etc. (Bhowmick and Stephens, 2000). Synthetic fibers are developed by chemically improving the natural fibers obtained from plants and animal origin. Common examples are rayon, nylon, polyester, acrylic, and spandex (Fourne, 1999).

2.2. Phthalates

In the 1920s, phthalates were introduced to the world market, which replaced the old used plasticizers like camphor. In the 1930s, Polyvinyl chlorides and Di-Ethylhexyl phthalate (DEHP) plasticizers were introduced in the plastic industry and they literally changed the world of plastics and thereby the lifestyle of human beings. Now, these phthalates have become a bitter pill to swallow.

Phthalates or phthalic Acid Esters (PAEs) are compounds that are used in the manufacturing of plastics for importing flexibility, durability, longevity and transparency. Phthalates are produced by the reaction of phthalic anhydride with suitable alcohols (typically 6-13 carbons). They are not chemically bonded with the polymeric mess and hence can easily leach out to the environment due to various physicochemical factors (Sarath Josh *et al*., 2012). Phthalates are esters of 1, 2-benzene dicarboxylic acid and have three isomeric forms, i.e., the ortho-isomer (phthalic acid (PA), para-isomer (terephthalic acid (TA), and meta-isomer (isophthalic acid (IA) (Benjamin *et al*., 2015). Esters of phthalic acids are mainly incorporated in the polyvinyl

chloride containing plastic products. Esters of terephthalic acids are used in the manufacturing of products such as polyester fibers and polyethylene plastics. Esters of isophthalic acids are relatively less in use and have been used in resin manufacturing (Liang *et al*., 2008). The basic structure of phthalic acid ester and some of their isomers and their esters are depicted in **Figure 1**.

Phthalate production was reported to be increasing from 2 to 9 million tons per year in the global market from 1980 to 2018 (Rhowdhwal and Chen, 2018). According to molecular weight, phthalates are classified into two groups; low molecular weight and high molecular weight phthalates. DEP (Diethyl phthalate), DMP (Dimethyl phthalate), DBP (Dibutyl phthalate), etc. are the commonly used low molecular weight phthalates and are mainly used in cosmetic products like nail polishes, shampoo, lotions, perfumes, soups and also in adhesives and insecticides. DEHP (Diethylhexyl phthalate), DOP (Dioctyl phthalate), DINP (Diisononyl phthalate), etc. are examples of high molecular weight phthalates and they are commonly incorporated in the making of plastic containers, toys, food packagings and in medical devices like blood storage bags, urine bags and other medical tubings (NRC, 2009). Among the phthalates, DEHP is the most widely used one with harmful effects, both for humans and the environment (Benjamin *et al*., 2017). DEHP is a colorless, odorless and viscous liquid, with a chemical formula $C_{24}H_{38}O_4$ and a molecular weight of 390.56 g mol^{-1}. It is not covalently bonded with the plastic, and hence leach out from the products after repeated use (Nakamiya *et al*., 2005). It has become an inevitable ingredient of many major medical devices such as blood bags, nutrition feeding bags, dialysis bags, and also in toys and infant products. Due to overuse, they can be found in air, soil, and water as a pollutant and thereby causing serious problems to human health. Through inhalation, ingestion, diffusion, and direct contact, they enter human body and cause serious incurable health issues to the kidney, liver, lungs, endocrine system, heart, and reproductive system (Heudorf *et al*., 2007). **Table 1** shows a detailed list of different phthalates and their products.

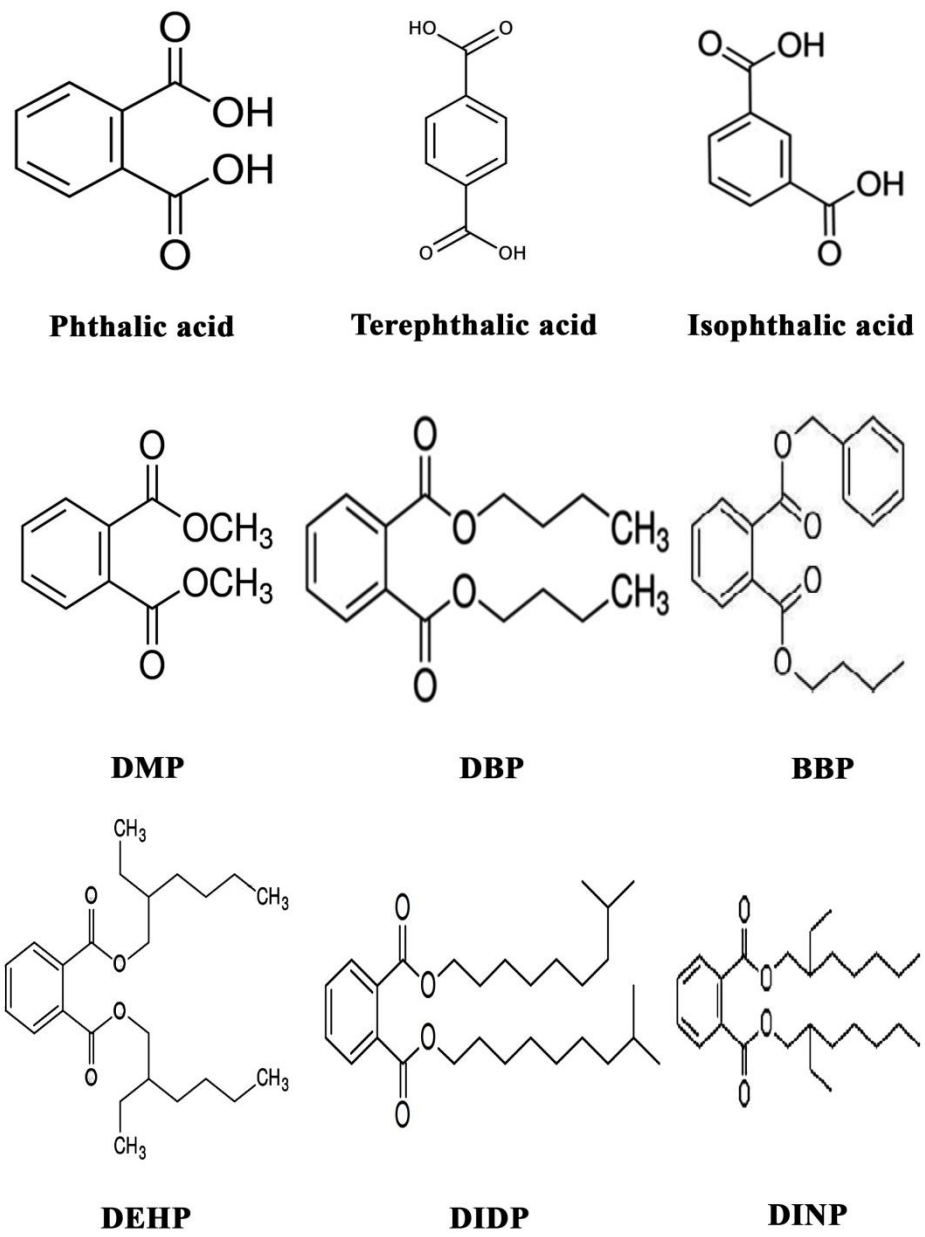

Figure 1. Few representative chemical structures of different phthalate isomers and their esters.

Table 1. Details of commonly used phthalates.

Common Name	IUPAC Name	Molecular formula	Molecular weight
Di-methyl phthalate (DMP)	Dimethyl benzene-1,2-dicarboxylate	$C_{10}H_{10}O_4$	194.18
Di-ethyl phthalate (DEP)	Diethyl benzene-1,2-dicarboxylate	$C_{12}H_{14}O_4$	222.24
Di-allyl phthalate (DAP)	Diallyl benzene-1,2-dicarboxylate	$C_{14}H_{14}O_4$	246.26
Di-n-propyl phthalate (DPP)	Dipropyl benzene-1,2-dicarboxylate	$C_{14}H_{18}O_4$	250.29
Di-n-butyl phthalate (DBP)	Dibutyl benzene-1,2-dicarboxylate	$C_{16}H_{22}O$	278.34
Di-isobutyl phthalate (DIBP)	Bis(2- methylpropyl) benzene-1,2-dicarboxylate	$C_{16}H_{22}O_4$	278.35
Di-n-pentyl phthalate (DNPP)	Dipentyl benzene-1,2-dicarboxylate	$C_{18}H_{26}O_4$	306.40
Butyl benzyl phthalate (BBP)	Benzyl butyl benzene-1,2-dicarboxylate	$C_{19}H_{20}O_4$	312.40
Di-n-hexyl phthalate (DNHP)	Dihexyl benzene-1,2-dicarboxylate	$C_{20}H_{30}O_4$	334.40
Di(2-ethylhexyl) phthalate (DEHP)	Bis(2-ethylhexyl) benzene-1,2-dicarboxylate	$C_{24}H_{38}O_4$	390.56
Di-n-octyl phthalate (DNOP)	Dioctyl benzene-1,2-dicarboxylate	$C_{24}H_{38}O_4$	390.60
Di-isooctyl phthalate (DIOP)	Bis(6-methylheptyl) benzene-1,2-dicarboxylate	$C_{24}H_{38}O_4$	390.55
Di-isononyl phthalate (DINP)	Bis(7-methyloctyl) benzene-1,2-dicarboxylate	$C_{26}H_{42}O_4$	418.60
Di-isodecyl phthalate (DIDP)	Bis(7-methylnonyl) benzene-1,2-dicarboxylate	$C_{28}H_{46}O_4$	446.67
Di-undecyl phthalate	Diundecyl benzene-1,2-dicarboxylate	$C_{30}H_{50}O_4$	474.70
Di-tridecyl phthalate	Ditridecyl benzene-1,2-dicarboxylate	$C_{34}H_{58}O_4$	530.80

Figure 2. Commercially available phthalate containing products.

2.3. Negative impact of phthalates on environment and human health

2.3.1. Environmental issues

Due to the non-chemical bondage, phthalates are easily detached from the polymeric matrix and contaminate the surrounding air, water, and soil, leading to pollution. Phthalic acid esters pollute the environment by leaching, migration, and oxidation during the manufacturing of plastics and through the daily usage of plastic goods (Przybylinska and Wyszkowski, 2016). In India, only a few studies have been conducted on the occurrence of phthalates in surface, ground, and

industrial waste waters and other aquatic sources. The inefficient removal of phthalates from wastewater is the main cause of their occurrence in aquatic environments. Gani and Kazmi (2016), studied the occurrence of phthalates (DEP, DBP, BBP, and DEHP) in twenty-five wastewater treatment plants in North India and concluded that DEHP is the highest contaminant in both untreated and treated wastewater (28.4 µg L^{-1} and 4.9 µg L^{-1}, respectively). Selvaraj *et al*. (2015) studied phthalate contamination in the Kaveri River, which flows through the Karnataka and Tamil Nadu regions of South India. His study reported the presence of six phthalates (DMP, DEP, DBP, BBP, DEHP, and DOP) from 16 sampling sites in the Kaveri River. Among the six, 57% of total measured concentration was that of DEHP, followed by DEP (22%) and DBP (11%). The total concentration range reported was 21.3 ng L^{-1} (DEP) to 514 ng L^{-1} (DEHP).

Srivastava *et al*. (2009) studied the occurrence of phthalate in the sediments from 30 locations of Gomti River and found five prominent phthalic acid esters like DMP, DEP, DBP, DEHP, and DOP. The mean concentrations of DMP, DEP, DBP, DEHP and DOP were found to be 10.54, 4.57, 10.41, 31.61, and 5.16 µg Kg^{-1}. 93.3% of the samples showed the presence of DEHP and DOP was detected only in 36.7% of samples. Khalid *et al*. (2018) studied the contaminant levels of phthalate in Periyar River, Kerala. He has analyzed samples from six locations and reported the presence of a single phthalic acid ester i.e., Diisobutylphthalate (DIBP) in about 29.4 ng L^{-1} concentration. Ramzi *et al*. (2020) conducted a study on the occurrence of phthalic acid esters in Cochin estuary, India, and checked the presence of six phthalates (DMP, DEP, DnBP, BBP, DEHP, and DnOP). His report concluded that in dissolved state, DnBP has the highest occurrence of 20.368 µg L^{-1} and DnOP has the least occurrence of 0.388 µg L^{-1} and in the case

of particulate PAE concentration, DEHP has the highest level of 594.15 µg g^{-1} and BBP has the lowest value of 41.22 µg g^{-1}.

Dargnat *et al*. (2008) reviewed the studies conducted in relation to Marne Aval Station, a wastewater treatment plant (WWTP), situated in a highly populated area of Paris (France). His study reported the occurrence of phthalate in the station and found DMP, DEP, DnBP, BBP, DEHP, and DnOP as the main phthalate pollutant in the WWTP. Here also, DEHP was the major pollutant observed (9 to 44 µg L^{-1}), followed by DEP (1.6 to 25 µg L^{-1}) and other phthalates with an average of 1 µg L^{-1}. According to Rank (2005), in the entire life cycle of DEHP incorporated products, around 72% of DEHP is leached out to the soil, 21% is released to the water, and rest (7%) is evaporated to the air column. Yuan *et al*. (2002) detected the DEHP concentration in surface water in Taiwan Rivers as 18.5 µg L^{-1}. Fromme *et al*. (2002) detected 97.8 µg L^{-1} of DEHP in surface waters of Germany. Lopez-Roldan *et al*. (2002) observed 5.661 µg L^{-1} of DEHP in groundwater samples from Spain. DEHP has also been detected from the drinking water sources around the world (China 3.47 µg L^{-1}; USA 0.55 µg L^{-1}; Greece 0.93 µg L^{-1}; Germany and Poland 0.05-0.06 µg L^{-1}) (Liu *et al*., 2013; Huerta-Fontela and Ventura, 2008; Kavlock *et al*., 2006; Psillakis and Kalogerakis, 2003). Rivers like Xiangjiang and Songhua in China are heavily polluted with DEHP concentrations ranging from 0.62 µg L^{-1} (min) and 15.23 µg L^{-1} (max) in Xiangjiang River and 2.26 µg L^{-1} (min) and 11.55 µg L^{-1} (max) in Songhua River (Zhu and Qiu, 2011; Gao *et al*., 2014). From nation to nation, the DEHP concentration in the aquatic environments is varying, in accordance with the intensity of usage of DEHP products and the regulations in force in the chosen country.

Many studies have been conducted in China during the past three decades on the occurrence of phthalic acid esters (PAEs) in the soil column and also on the associated risks. In comparison to other countries, higher levels of PAEs are seen in the soils of China. The use of fertilizers, plastic films and various processes and products associated with electronic industries are reported to be the major sources of phthalates in soil (Lu *et al*., 2018). Li *et al*. (2006) reported the presence of 6.5 mg kg^{-1} DEHP and 3.8 mg kg^{-1} DBP from the urban soils of North China. Kong *et al*. (2012) conducted a study in the Tianjin provinces of North China for evaluating the PAE occurrence in agricultural soils and found DEHP concentration as 4.17 mg kg^{-1} and DBP as 0.28 mg kg^{-1} in the soils. Wu *et al*. (2015) tested the soil from an electronic manufacturing area in Xiangyang (East China) and found a higher level of DEHP (153 mg kg^{-1}) and DBP (31.2 mg kg^{-1}). Zhang *et al*. (2013) detected 35.8 mg kg^{-1} of DEHP from the agricultural soils of the Qingdao province of East China. Only a few studies have been reported regarding the soil contamination by phthalates in Indian region. Tiwari *et al*. (2016) conducted a study on the estuarine sediments from Mumbai, India for the detection of endocrine-disrupting chemicals and pointed out the presence of 14 phthalate esters, and among them, Di-n-butyl phthalate (DBP) had the highest level of occurrence. Sinha *et al*. (2019) investigated the phthalate contamination in soils of 22 municipal wards of Patna, Bihar, India. Around eight phthalic acid esters were identified using LCMS analysis and DEP was observed in maximum municipal wards. Zornikova *et al*. (2014) studied the presence of DEHP and DEP in crop plants like *Triticum aestivum*, *Brassica napus*, *Zea mays*, and found their presence in the underground and aboveground plant parts. From this study, it is clear that along with nutrients, plants absorb poisonous phthalates through their root system.

Phthalates in plastics are also making severe contaminations to the aerosol. The literature reveals that only isolated studies have been conducted in India on phthalate contamination in the atmosphere. Giri *et al*. (2013) observed the presence of phthalate concentration from 2 to 926 ng m^{-3} during the winter seasons in the aerosol of Raipur, India. Fu *et al*. (2010) conducted a study to evaluate the organic molecular composition in the aerosols of Chennai, India. His study identified a total of five phthalates i.e., dimethyl phthalate (DMP), diethyl phthalate (DEP), diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP), and di (2-Ethylhexyl) phthalate (DEHP) and concluded that DEHP was the most abundant pollutant (171 ng m^{-3}) and DMP (0.04 ng m^{-3}) was found in minimum concentration in the aerosols. Gupta and Gadi (2018), conducted a detailed evaluation of atmospheric matter in North Delhi, India, and reported the presence of phthalic acid esters of concentration 703.1 ±36.2 ng m^{-3}, which is slightly higher in winter than summer. From their study also, DEHP was found to be in higher concentration, followed by DEP. Das *et al*. (2014) studied phthalate concentration in the atmosphere of South Delhi, India. Area adjoining Jawaharlal Nehru University campus and Okhla, an industrial area was selected for the study and estimated around 15 different phthalate types. Among them, DEHP showed a higher concentration in all the study area. Sampath *et al*. (2017) evaluated the status of pollutants in the air from Tamil Nadu, India. He selected six major cities of Tamil Nadu and studied the spatial distribution of six major phthalates in summer, pre-monsoon, and monsoon seasons and it was 52 ng m^{-3}, 61 ng m^{-3}, and 17 ng m^{-3} respectively. This study also pointed out the major share of DEHP within the air pollutants.

2.3.2. Health issues

As we are aware, phthalates are not chemically attached to the polymeric plastic mess and slight variation in the environmental conditions, like changes in pH, temperature, pressure, microwaving, or interaction with appropriate solvents would speed up their leaching to the environment and finally reaches humans, leading to incurable illness. Phthalates have enormous opportunities to trespass the human system through plastic containers used for food and drinking purposes, drinking water from contaminated sources, atmospheric inhalation, from infected mother to infants through breast milk and through medical devices (plastic tubings, blood bags, etc.). Several *in vitro, in silico,* preclinical, and clinical studies were carried out to show the ill effects of phthalates on human health. Some of the major health issues related to phthalates are detailed in the following session.

2.3.2.1. Health issues in children

The National Centre for Health Statistics (NCHS), United States, conducts National Health and Nutrition Examination Survey (NHNES) in every two years from 1999. They test the urine samples of children in the age group 6-17 for the detection of phthalates like DEHP, DBP, and BBzP. The study from 2001-2008 reported an increasing trend in the concentration of DEHP (387 µg/L to 564 µg/L), DBP (166 µg/L to 191 µg/L), and BBzP (104 µg/L to 107 µg/L) in the urine samples. Contrary to this, study from 2008-2014 showed a decrease in the concentration of all detected phthalates (DEHP from 564 µg/L to 69 µg/L, DBP from 191 µg/L to 103 µg/L, and BBzP from 107 µg/L to 56 µg/L). Children in the age group of 3 - 14 in Germany also reported a higher level of phthalate (5OH-MEHP and 5oxo-MEHP) in their urine samples (Becker *et al*., 2009). In

Germany, among 111 primary school students (5-6 years old), the three most critical phthalates (DEHP, DnBP, and DiBP) are seen in 24%. Apart from this, 54% of the children are exposed to phthalates, which are above 50% of the cumulative Tolerable Daily Intake (TDI_{cum}) (Koch *et al*., 2011).

Trasande *et al*. (2013) studied the cross-sectional data of 766 fasting children in the age group 12-19 years from the Department of Health and Human Services (NHANES) of the United States of America using homeostatic model assessment of insulin resistance (HOMO-IR) to find out the relation between phthalate metabolites and insulin resistance. They concluded that with 21.6% prevalence, DEHP was significantly related to insulin resistance. A study from China on school children (aged 8-15), revealed a strong correlation between Body mass index (subgroups- normal weight, overweight, and obesity), waist circumference and phthalate metabolites in urine samples. The mean level of phthalates like MEHP, MiBP, MEP, and DBP showed an increasing trend with BSI (Wang *et al*., 2013). Zhang *et al*. (2014) studied the urine samples of 247 boys and 246 girls of school age and reported that low molecular weight phthalates have a positive association with the obesity of boys, while in the case of girls, phthalates like MEHP, MEHHP and DEHP have a negative correlation with obesity. Taiwanese children at the age range of 2-6 are also reported to be exposed to poisonous phthalates like DEHP, DiBP, DnBP, BBP, DiNP, etc. (Lin *et al*., 2011). Phthalate metabolites in the form of dust, vapors, and air borne particles turn to trigger allergy and serious lung disorders like asthma and associated symptoms in human beings (Benjamin *et al*., 2017). Studies carried out to establish the relationship between phthalate exposure and lung disorder like asthma are limited. A recent study carried out among children in Europe put forwarded a direct link between phthalate exposure and asthma (Beko *et al*.,

2013). Bertelsen *et al*. (2013) conducted a study on 623 children (10-year-old) in Norway to find out the relationship between asthma and exposure to phthalate metabolites. Around 11 metabolites, 8 phthalates were identified and among them mono (carboxyoctyl) phthalate (MCOP), mono (carboxynonyl) phthalate (MCNP) and two higher molecular weight phthalates; DiNP and DiDP were found to be directly linked with asthma- related issues.

Studies suggest that exposure of children to both low and high molecular weight phthalates adversely affects the neuro reflexes and leads to unusual behavioral patterns and even leads to autism-like mental disorders and reduced cognitive development. DEHP metabolites like 5-OH- and 5-oxo-MEHPs were found to have a direct correlation between autism spectrum disorders (Testa *et al*., 2012). Huang *et al*. (2015) studied the neurocognitive function and intelligence of 2-12 year-old children from Taiwan using Bayley and Wechsler test and they observed a persistent exposure caused by adverse effects and have a direct association with the cognitive development in children. Only limited studies have been carried out on the relationship between phthalate exposure and Attention Deficit Hyperactivity Disorders (ADHD). A study conducted among 261 children (8-11 years-old) in Korea reported that the exposure to DBP has a direct association with ADHD and DEHP metabolites like MEHP and MEOP adversely affect the ADHD (Kim *et al*., 2009). Chopra *et al*. (2014) carried out a detailed study on Attention Deficit Disorder (ADD) and Learning Disability (LD) of American children (6-15 years) in connection with phthalate exposure and concluded a strong relationship between the DEHP metabolites and ADD.

2.3.2.2. Health issues in women

Assessment of phthalate exposure in women can carried out by testing any one of the fluids like blood, urine, amniotic fluid, cord blood, milk, and follicular fluid. (Silva *et al*., 2004; Lin *et al*., 2011; Du *et al*., 2016). The exposure of phthalates to pregnant women and breastfeeding mothers directly affects the future generations and so many studies are focused on this direction.

According to Environmental Protection Agency (US EPA), phthalate metabolites like DEHP, DBP, etc. have shown an increasing trend in the urinary samples of women from 1999 to 2008. Tranfo *et al*. (2014) conducted a valuable study on the exposure of phthalates in pregnant women. They collected the amniotic fluid and urine samples from pregnant women and found that MnBP and MEHP are the prominent metabolites in these samples. A significant conclusion they have drawn from the results is that the concentration of metabolites in amniotic fluid was higher than the maternal urine, indicating the fetal exposure of phthalates. Hines *et al*. (2009) analyzed the concentration of phthalate metabolites in the lactating women in the US. They tested saliva, serum, urine, and milk for the detection of mono (3-carboxypropyl) phthalate, mono (2-ethyl-5-carboxypentyl) phthalate (MECPP), mono (2-ethyl-5-hydroxyhexyl) phthalate, mono (2-ethyl-5-oxohexyl) phthalate and found that urine samples have a higher concentration of metabolites than serum, milk and saliva.

Latini *et al*. (2003) evaluated the serum and cord blood of 84 newborns and found that the DEHP / MEHP are abundant in the samples. They suggested that phthalate metabolites have a significant association with shorter pregnancy duration, and mainly DEHP has been associated with the reduced gestational age of the fetus. Frederiksen *et al*. (2012) determined the relation between phthalate

exposure and pubertal development (breast and pubic hair stage) of 725 healthy Danish girls (5.6-19.1 years) and they determined 12 phthalate metabolites from the urine samples. They concluded that youngest girls with less advanced pubertal development have an increased concentration of monobutyl phthalate isoforms, monobenzyl phthalate, and DEHP metabolites in their urine samples. They have also noticed that there is no significant relationship between phthalate concentration and breast development.

Endometriosis is another type of health issue that severely affects women at the reproductive stage. According to Upson *et al*. (2013), greater urinary concentration of MBzP and MEP has shown an increased risk of endometriosis. Cobellis *et al*. (2003) also worked on DEHP exposure in women to endometriosis. They found that women affected by endometriosis have a higher plasma DEHP concentration than the normal women. Mu *et al*. (2015) conducted a study to compare the urinary phthalate concentration and clinical pregnancy loss in women from China. They observed the case of 132 women having clinical pregnancy loss with those of 172 healthy pregnant women. After the study, they concluded that the exposure to MEP, MiBP, and MnBP is directly related to the increased risk of clinical pregnancy loss. Hauser *et al*. (2016) studied the urinary phthalate concentration in 256 women enrolled in the Environmental and Reproductive Health (2004-2012) programme of Massachusetts General Hospital, USA and studied the reproductive outcome and concluded that DEHP and DiDP were associated with decreased oocyte yield and only metabolites of DiDP and DiNP were associated with reduced fertilization rate.

2.3.2.3. Health issues in men

Literature suggests that exposure to phthalates contributes to numerous health issues in men, particularly in the reproductive system, like decreased anogenital distance (AGO), hypospadias, increased sterility, reduction in quality and quantity of sperm production, cryptorchidism, and reproductive tract malformation (NRC, 2009). According to a pioneer study, 134 boys (2-36 months of age) were tested for the correlation between anogenital distance and penile volume and the proportion of boys with incomplete testicular descents. The study concluded that four phthalates; MEP, MBP, MBzP, and MiBP, are inversely related to the anogenital index and suggests that phthalate exposure in the prenatal stage at environmental levels can adversely affect male reproductive development in humans (Swan *et al*., 2005). Another study from Japan analyzed 111 pregnant women to find out the correlation between prenatal phthalate exposure and anogenital distance in male newborns and identified that DEHP and its metabolite MEHP adversely affected the anogenital distance in male newborns (Suzuki *et al*., 2012).

Joenson *et al*. (2012) studied 881 healthy Danish men to estimate phthalate exposure on reproductive hormone levels and semen quality. They evaluated DEHP, DiNP, and their primary metabolites and found that these metabolites significantly changed the hormone levels and also decreased the semen quality. Lambrot *et al*. (2009) studied the impact of MEHP phthalate on the development of human fetal testis and found that MEHP reduced the number of germ cells by increasing the apoptosis. Sperm count, motility, and morphology are directly related to male sterility. Duty *et al*. (2003) studied 168 subfertile married US males to find out the semen parameters like sperm concentration, motility, and morphology and also studied and correlated with phthalate concentration in their

body fluids. Their study showed that 17% had a sperm concentration less than the preferred levels, 44% had less than 50% sperm motility and 26% had less than 4% normal shaped sperm. Their urinary samples showed phthalate levels ranging from 153 ng/ml of MEP and 6.3 ng/ml of MEHP, which are quite above the preferred levels. The study suggested that phthalate monoesters have a direct correlation with lower sperm concentration, lower motility, and increased percentage of sperm with abnormal morphology in human males. Hauser *et al*. (2006) also detailed that MBP concentration decreases the sperm concentration and motility in human males and their study also concluded that there is no direct relationship between MEP and MMP with semen quality and quantity. Another study by Hauser *et al*. (2007) reported that urinary concentration of MEP and MEHP increasingly damages the DNA of sperms.

2.4. Degradation of phthalic acid esters (PAEs)

Phthalic acid degradation in nature happens both by biotic and abiotic means. Non-biological (abiotic) degradation processes of PAEs like hydrolysis of PAEs, TiO_2 mediated photocatalysis, UV-light photolysis is very much slow. Short-chain phthalate derivatives have a half-life of 100 days to 3 years and long-chains have a half-life up to 2000 years in the abiotic means of degradation (Staples *et al*., 1997). Biotic degradation (mainly microbial degradation) is a very effective, fast, and environmentally friendly, compared to abiotic destruction. Hydrolysis of PAEs result in the formation of an acid and an alcohol. It is a two-step hydrolytic process, in which the first step produces a monoester and an alcohol moiety and the second step results in the production of phthalic acid and a second alcohol. The catalyzation process is mainly mediated by acid or base and sometimes employing metal ions, anions, and organic compounds (Huang *et al*., 2013). An advanced oxidative process such as heterogeneous photocatalysis

using titanium dioxide (TiO_2) is one of the effective methods of removing a large variety of organic and inorganic pollutants in an aqueous environment through the generation of highly reactive radicals to react with the pollutants. Photo-excitation, diffusion, trapping, recombination, and oxidation are the key steps involved in the TiO_2 mediated photo-catalysis (Sin *et al*., 2012). Direct UV photolysis and hydrogen peroxide (H_2O_2) mediated UV photolysis is an effective method which employs advanced oxidation process for the degradation of organic pollutants. H_2O_2 mediated process is mainly used for the removal of toxic organic matter in aquatic environments (Chen *et al*., 2010).

2.4.1. Biological degradation

Unscientific disposal of plastic wastes into the soil, sludge, and water bodies pave a way for the evolution of new microbial strains, which are good in the utilization of phthalic acid wastes. All over the world, scientists are investigating on new microbial isolates that are capable of using the toxic PAEs as sole carbon and energy source and succeeded in completing the degradation in a short period. Aerobic and anaerobic are the two means of degradation methods employed by the microbes. A lot of bacterial strains, few fungi, and some algae have been reported to be effective in the degradation of phthalates (Boll *et al*., 2020).

2.4.1.1. Bacterial degradation

Due to the persistence of phthalate isomers in nature, bacterial strains eventually developed new pathways for the degradation of these compounds. Both aerobic and anaerobic phthalate degradation is associated with bacterial strains. Many reports have been published, which highlights the use of bacterial strains that have the ability to degrade phthalate isomers (**Table 2)**.

2.4.1.1.1. Anaerobic degradation

There are several reports on the degradation of phthalic acid esters under anaerobic conditions, mainly by methanogenic consortia. Through the anaerobic mode of degradation, bacteria degrade all the three phthalate isomers and their esters and finally produce an end product of acetate and methane. The anaerobic degradation pathway is shown in **Figure 3.** The initial and the most important step in the degradation of phthalate isomers is the production of corresponding esters of CoA by an enzyme acyl CoA synthetase and the further decarboxylation of these esterified phthalates transformed into benzoyl-CoA by decarboxylase (Vamsee-Krishna and Phale, 2008). Kleerebezem *et al*. (1999) reported a case of direct decarboxylation of phthalate isomers by specific decarboxylases, followed by esterification with CoA. This benzoyl-CoA is then converted to cyclohex-1-ene-carboxyl-CoA by reductase and then transform to 2-hydroxycyclohexane-1-carboxyl-CoA by hydrolase and finally form pimelyl-CoA via 2-ketocyclohexane-1-carboxyl-CoA by hydrolase and it enters the β-oxidation pathway as the final step. PAEs with shorter side chains are easily been degraded, compared to longer-chain ones. The rate of degradation is better in aerobic mode, compared to anaerobic degradation (Chen *et al*., 2003).

Table 2. List of bacterial strains that have the ability to degrade phthalate isomers

Type of phthalate utilized	Name of Bacteria	Reference
DBP	*Delftia* sp.TBKNP-05	(Patil *et al*., 2006)
	Acinetobacter sp.	(Ogawa *et al*.,2009)
	Ochrobactrum sp.	(Wu *et al*., 2010)
	Agrobacterium sp.	(Wu *et al*., 2011)
	Pseudomonas sp. V21b	(Kumar *et al*., 2017)
	Comamonas sp. 51F	
	Arthrobacter sp.	(Nandi *et al*., 2020)
	Arthrobacter nicotianae ZM05	(Wang *et al*., 2020)
	Bacillus subtilis	(Kolb *et al*., 2019)
BBP	*Acinetobacter* sp.	(Yang *et al*., 2013)
	Arthrobacter sp.	(Nandi *et al*., 2020)
	Gordonia sp. MTCC 4818	(Chatterjee and Dutta, 2003)
	Bacillus subtilis	(Kolb *et al*., 2019)
DEHP	*Acinetobacter* sp.	(Hashizume *et al*., 2002)
	Bacillus subtilis No.66	(Quan *et al*., 2005)
	Microbacterium sp. CQ0110	(Chen *et al*., 2007)
	Micrococcus luteus	(Baek *et al*., 2009)
	Achromobacter sp.	(Li *et al*., 2018)
	Bacillus sp.	(Li *et al*., 2018)
DMP	*Arthrobacter* sp.	(Vega and Bastide, 2003)
	Bacillus sp.	(Niazi *et al*., 2001)
	Micrococcus sp. strain 12B	(Eaton and Ribbons, 1982)
	Pseudomonas fluorescens, Pseudomonas aureofaciens, Sphingomonas paucimobilis, Xanthomonas maltophilia	(Wang *et al*., 2004)
	Comamonas testosterone	(Li *et al*., 2017)
DMTP	*Comamonas acidovorans* D-4	(Patel *et al*., 1998)
	Pseudomonas sp.	(Tserovska and Dimkov, 2002)
	Pasteurella multocida, Sphingomonas paucimobilis	(Li *et al*., 2005)
DMIP	*Rhodococcus erythropolis* 5D,	(Aleshchenkova *et al*., 1997)
	Rhodococcus rubber 1B	
	Variovorax paradoxus strain T4	(Wang and Gu, 2006)

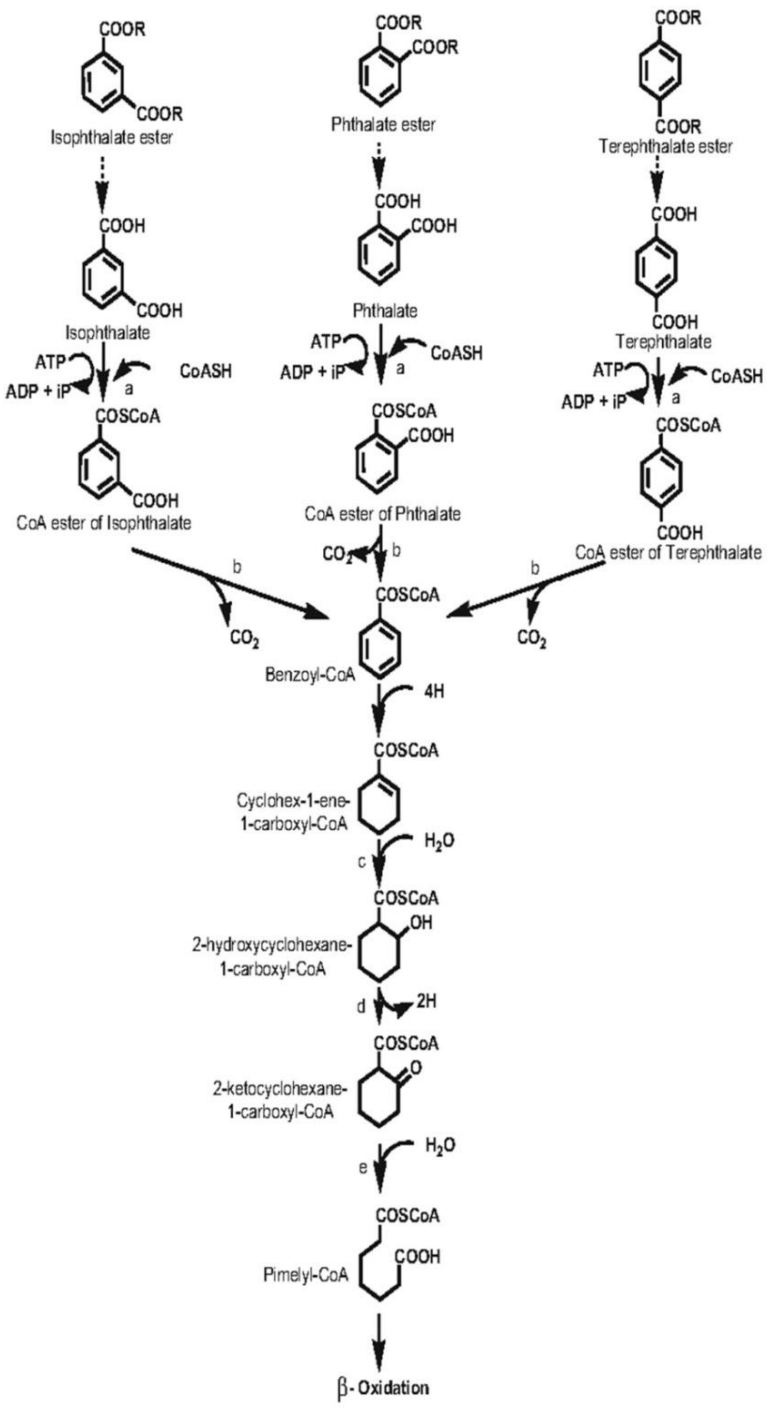

Figure 3. Anaerobic degradation pathway of phthalate esters (Vamsee-Krishna and Phale, 2008)

2.4.1.1.2. Aerobic degradation

Bacterial degradation of PAEs by the aerobic pathway was first reported by Evans *et al*. (1965). The initial steps involved in the aerobic degradation of phthalate isomer are different in gram-negative and gram-positive bacteria. In gram-negative bacteria, phthalate 4, 5-dioxygenase acts upon phthalate isomer to yield cis-4, 5 –dihydroxy-4, 5- dihydro phthalate, which is then dehydrogenated by cis-dihydrophthalate dihydrodiol dehydrogenase to 4,5-dihydroxy phthalate. By the action of 4,5-dihydroxyphthalate decarboxylase, this dihydroxylated phthalate undergoes decarboxylation to yield 3,4-dihydroxybenzoate. In Gram-positive bacteria, phthalate 3,4-dioxygenase acts upon phthalate isomer to yield cis-3,4-dihydroxy-3,4-dihydrophthalate, which is then converted to 3,4-dihydroxyphthalate and subsequently decarboxylated to yield 3,4-dihydroxybenzoate by 3,4-dihydroxyphthalate decarboxylase. Metabolism of terephthalate is initiated by terephthalate 1,2-dioxygenase to yield 2-hydro-1,-dihydroxy terephthalic acid, which is then converted to 3,4- dihydroxy benzoate. Metabolism or microbial degradation of isophthalate is difficult due to the structural resistance imparted by the isomer. Isophthalate 3,4-dioxygenase acts on isophthalate to form 4-hydro 3, 4-dihydroxy isophthalate and then decarboxylated to form 3,4-dihydroxybenzoate.

Every phthalate isomer has a 3,4-dihydroxybenzoate as a central metabolic intermediate. Protocatechuate 4,5-dioxygenase (meta-pathway) acts on 3,4-dihydroxybenzoate to yield 4-carboxy-2-hydroxy-muconic semialdehyde and then oxidized to pyruvate and oxaloacetate by 4-oxalocitramalate. Protocatechuate 3,4- dioxygenase (ortho pathway) acts on 3,4-dihydroxybenzoate to form β-carboxy-cis, muconic acid and then oxidized to form succinyl CoA and acetyl-CoA by β-keto adipate **(Figure 4)**

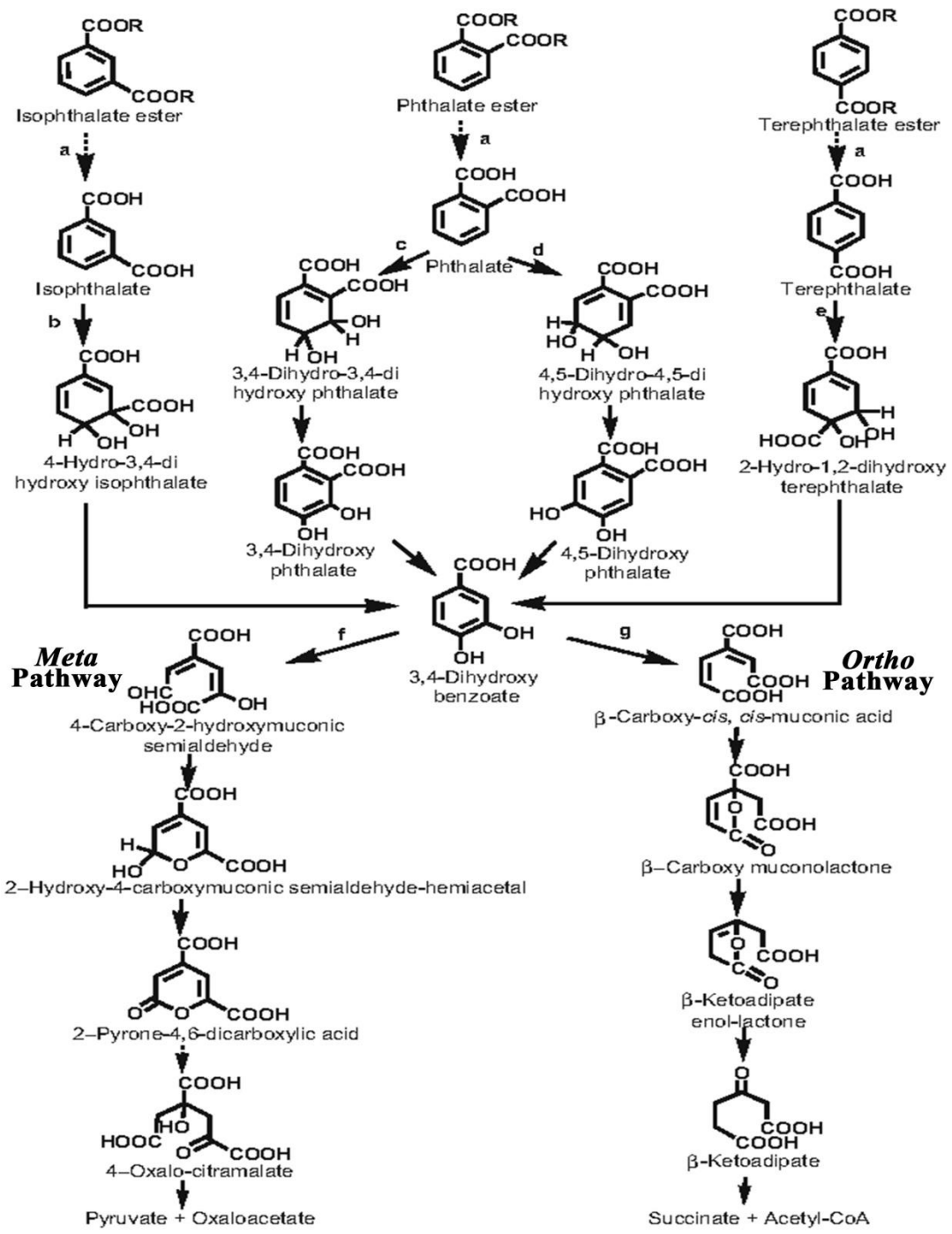

Figure 4. Aerobic degradation pathway of phthalate esters (Vamsee-Krishna and Phale, 2008)

2.4.1.2. Fungal degradation

Degradation of PAEs by fungi received little attention, compared to bacterial degradation. Studies on fungal degradation of PAEs are little been reported and available literature is on degradation through pure fungal cultures and by purified fungal enzymes. Sivamurthy *et al*. (1991) reported that the fungus *Sclerotium rolfsii* has the ability to degrade dimethyl-terephthalate to terephthalate with the formation of monomethyl terephthalate as the intermediate. Also, phthalate esterase was the main enzyme which took part in the degradation, and is detected in the mycelia of *Sclerotium rolfsii.* Complete degradation of Dibutyl phthalate by three fungal isolates (*Phanerochaete chrysosporium, Trametes versicolor, Daldinia concentrica*) was reported by Lee *et al*. (2004). Lee *et al*. (2007) also reported the degradation of Dibutyl phthalate using *Polyponus brumalis,* with phthalic acid anhydride as the end product through trans-esterification and de-esterification pathways. *Aspergillus versicolor,* a filamentous fungus isolated from deep-sea sediments, degrade dimethyl isophthalate and dimethyl terephthalate to monomethyl isophthalate and terephthalic acid, respectively (Wang *et al*., 2017). Hwang *et al*. (2008) studied the degradation of DMP, DEP, and BBP using 10 different species of white-rot fungi. They reported that all the fungi showed the ability to degrade all the three phthalates and among them, *Pleurotus ostreatus* showed higher degradation ability. Chai *et al*. (2008) reported the degradation of DEHP by 14 fungal strains (*Aspergillus oryzae, A. sydowii*, 2 strains of *A. ustus*, *Fusarium graminearum*, *F. morniforme*, *F. sporotrichioides, Penicillium citrinum, P. expansum*, *P. frequentans*, *Curvularia lunata*, *Trichoderma viride*, *T. viride* PHF-2, *Rhizopus stlonifer*) and among these fungi, 3 strains of *Fusarium* showed better performances in the degradation of DEHP.

Aspergillus niger completely metabolizes dimethyl phthalate through MMP, phthalic acid, and protocatechuate as intermediates; through ortho and meta cleavage pathways (Sharanagouda and Karegoundar, 2000; Ganji *et al*., 1995). Degradation of PAEs by yeasts is not extensively studied and hence only a few reports are documented. Gartshore *et al*. (2003) studied the yeast *Rhodotorula rubra* and found that it has partially degraded bis-2-ethylhexyl adipate, dioctyl phthalate, and dioctyl terephthalate. But, the degradation process resulted in two toxic metabolites; dipropylene glycol monobenzoate and diethylene glycol monobenzoate. Begum *et al*. (2003) reported that *Saccharomyces cerevisiae* has the ability to metabolize DEP, BBP, and DBP. Much of studies have already been attempted for the utilization of purified fungal enzymes for PAEs degradation. Enzymes cutinases and esterases are extensively utilized for such purposes. Fungal cutinases have a greater advantage over esterases due to their higher stability and production of non-toxic intermediates (Ahn *et al*., 2006; Kim *et al*., 2003).

2.5. *Ex situ* and *In situ* degradation of PAEs

Ex-situ utilization of PAEs by microbes under well-defined laboratory conditions is the major type of degradation study conducted in the field of phthalate degradation. Several studies highlighted the *ex-situ* degradation of PAEs by microbes isolated from different sources like river water / sediment (Kolb *et al*., 2019; Zhang *et al*., 2018; Yuan *et al*., 2002; Hashizume *et al*., 2002), soil (Chao *et al*., 2006; He *et al*., 2018), mangrove habitat (Luo *et al*., 2009; Li *et al*., 2005), sludge (Roslev *et al*., 2007; Jianlong *et al*., 2000; He *et al*., 2013), etc. Immobilization techniques using microbes, mainly bacterial cell immobilization, gets higher rates of PAE degradation than non-immobilized cells. Hu and Yang (2014) investigated the microbial degradation of DBP by immobilized

Micrococcus sp. using polyvinyl alcohol (PVC) as an immobilizing agent. They used PVA-boric acid beads, PVA-sodium nitrate beads, and PVA-orthophosphate beads and reported that PVA-orthophosphate immobilized cells have a higher rate of DBP degradation. *Burkholderia* sp. immobilized with corncob and sodium alginate showed the highest DOP removal rate, compared to corncob and free cells respectively (Zhang *et al*., 2017).

Limited numbers of studies have been conducted in the field of *in situ* degradation of PAEs. Roberts and Davidson (1989) are the pioneers in this field. They inoculated the spores of nine species of fungi in the medium containing plasticized polyvinyl chloride film as the only carbon source and confirmed that only two species, *Aspergillus fischeri,* and *Phecilomyces* sp. have the ability to utilize the components of PVC film, without adding nutrients. Kirbas *et al*. (1999) studied the degradability of PVC using several white-rot fungi and found that all the species have the potentiality to cleave the C-H, C-Cl, and C=O bonds in the PVC film. Nakamiya *et al*. (2005) reported that 90% of DEHP degradation in PVC sheets were achieved by using *Mycobacterium* sp., within 3 days. Ali *et al*. (2014) isolated 4 fungi (*Panerochaete chrysosporium* PV1, *Leotinus tigrinus* PV2, *Aspergillus niger* PV3, *Aspergillus sydowii* PV4) from the soil, which have the potential to degrade PVC film and showed that *Phanerochaete chrysosporium* PV1 significantly reduced the molecular weight of PVC film, than other members. Deepika and Jaya (2015) conducted an interesting study using low-density polyethylene / LDPE (polythene granule) to find out the degradability using *Pseudomonas* sps., *A. niger*, *A. flavus*, *Streptomyces* sps. and revealed that after 6 months incubation, *Streptomyces sps*. reduced 46.7% of LDPE, which is higher compared with other isolates studied [*A. flavus* (16.45%), *A. niger* (26.17%), *Pseudomonas* sps. (24.00%)]. Six novel fungi (*Aspergillus*

japonicas BP9, *Aspergillus parasiticus* BP10, *Fusarium subglutinans* BP8, *Paecilomycetes lilacium* BP13, *Penicillium funiculosum* BP7, *Penicillium brocae* BP6) and a bacterium (*Achromobacter denitrificans* SP1) were introduced by Pradeep and Benjamin (2012) and Pradeep *et al*. (2013), which are effective in degrading DEHP blended in commercial blood bag. No other research work has earlier utilized a chemically defined PVC - plastic material, like the blood bag. Considering its novelty, blood bag is utilized in the present study.

Part II: Microbial prodigiosin and its potential applications

2.6. Introduction

Colors are the most noticeable entity that easily catches human attention. In Latin, the word 'pigment' means the 'coloring matter'. Pigments are of two types; synthetic and natural. Synthetic pigments are man-made compounds that are widely used in various industries including textiles, food, cosmetics, and pharmaceuticals (Akilandeswari and Pradeep, 2017**).** High toxicity issues have arisen as a result of the usage of synthetic pigments, which pave way for intensive research in the field of natural pigments and dyes, which have additional features like antioxidant, antibacterial, and anticancer properties. Due to the slow growth rate of plants and animals, the pigment availability from them are practical difficulty. Due to short life cycle, stability, yield, cost-effectiveness and easy downstream processing, microorganisms mainly bacteria are an effective competitor towards the production of natural pigments **(**Tuli *et al*., 2015). Compared to other microorganisms, pigment production from bacteria has now become an appealing and relevant field of research. Pigment producing microorganisms are enlisted in **Table 3**.

Table 3. List of pigment producing microorganisms and their proposed bioactivities

Pigment	**Microorganism**	**Colour**	**Function**	**Reference**
BACTERIA				
Astaxanthin	*Agrobacterium aurantiacum, Paracoccus carotinifaciens*	Pink-Red	Antioxidant, Anticancer, Anti-inflammatory, Antioxidant	Yokoyama and Miki, 1995; Reyes *et al*., 1996
β- Carotene	*Flavobacterium*	Yellow	Food additive	Bhosale and Bernstein, 2004
Canthaxanthin	*Bradyrhizobium* spp. *Lactobacillus pluvalis.*	Orange	Antioxidant, Anticancer	Lorquin *et al*., 1997; Mathews-Roth, 1982; Chew *et al*., 1998; Duffose, 2006
Flexirubin	*Chryseobacterium*	Yellowish-orange	Food additive	Venil *et al*., 2014
Granadaene	*Streptococcus agalactiae*	Orange-red	Antioxidant, Detoxify ROS	Liu and Nizet, 2009; Rosa-Fraile, 2006
Heptyl prodigiosin	*Proteobacteria*	Red	Antiplasmodial	Lazaro *et at*., 2002
Indigoidine	*Streptomyces aureofaciens*	Blue-green	Antibacterial, Antifungal and Antimalarial	Navakova *et al*., 2010; Karniawan *et al*., 2016
Melanin	*Streptomyces neoformans*	Black	Antimicrobial, Antibiofilm and antioxidant	Vasanthabharathi *et al*., 2011
Prodigiosin	*Serratia marcescens, Pseudoalteromonas rubra*	Red	Anticancer, DNA Cleavage, Immunosuppressant	Williams, 1973; Kalivoda *et al*., 2010; Feher *et al*., 2008
Phycocyanin	*Pseudomonas* spp.	Blue, green	Cytotoxicity, Neutrophil apoptosis, Ciliary dysmotility, Proinflammatory	Baron and Rowe, 1981

Riboflavin	*Bacillus subtilis*	Yellow	Used in food	Richter *et al*., 1997; Vogl *et al*., 2007
Rubrolone	*Streptomyces echinoruber*	Red	Antimicrobial	Palleroni *et al*., 1978; Yan *et al*., 2016
Staphyloxanthin	*Staphylococcus aureus*	Golden	Antioxidant, Detoxify ROS	Pelz *et al*., 2005; Leejae *et al*., 2013
Tryptanthrin	*Cytophaga/Flexibacteria* AM13	Light-dark Yellow	Antioxidant, Anticancer	Soliey *et al*., 2011
Undecylprodigiosin	*Streptomyces* sp.	Red	Antibacterial, Antioxidant, UV-protective, Anticancer	Stankovic *et al*., 2012; Kang *et al*., 1998; Rioseras *et al*., 2014
Violacein	*Janthinobacterium lividum, Pseudoalteromonas tunicate, Pseudoalteromonas spp. Chromobacterium violaceum*	Purple	Antioxidant, Detoxify ROS	Pantanella *et al*., 2007; Huang *et al*., 2011; Zhang *et al*., 2011; McClean *et al*., 1997
Zeaxanthin	*Staphylococcus aureus*, F*lavobacterium* spp., *Paracoccus zeaxanthinifaciens, Sphingobacterium multivorum*	Yellow	Photoprotectant, Antioxidant	Taylor and Davies, 1983; Masetto *et al*., 2001; Berry *et al*., 2003; Rosa *et al*., 2001
Xanthomonadin	*Xanthomonas oryzae*	Yellow	Antimicrobial	Poplawsky and Chun, 1997
FUNGI				
Ankaflavin	*Monascus* sp.	Yellow	Antitumor, Anti-inflammatory	Hsu *et al*., 2011
Anthraquinones	*Penicillium oxalicum*	Red and other hues	Antifungal, Virucidal	Wang *et al*., 2014; Andersen *et al*., 1991; Agarwal *et al*., 2000

		Known as Arpink red or Natural Red		
Aspergillin	*Aspergillus niger*	Black	Food colorant	Ray and Eakin, 1975; Nielsen *et al*., 2009
Atronenetin	*Paecilomyces sinclairii*	Red	Food and Pharmaceuticals	Mukherjee *et al*., 2017
Azaphilones	*Talaromyces atroroseus, Penicillium purpurogenum*	Red	Antioxidant, Anticancer, Antioxidant	Padmapriya and Murugesan, 2016; Frisvad *et al*., 2013; Dufosse, 2017
b- carotene	*Blakeslea trispora, Fusarium sporotrichioides, Mucor circinelloides, Neurospora crassa, Phycomyces blakesleeanus*	Yellow-orange	Anticancer, Antioxidant, Suppression of cholesterol synthesis	Kim *et at*., 1997; Jones *et al*., 2004; Navarro *et al*., 1995; Hausmann and sandman, 2000; Cerda-Olmedo, 2001
Canthaxanthin	*Monascus* spp.	Orange, pink	Antioxidant, Anticancer	Malik *et al*., 2012
Cycloprodigiosin	*Pseudoalteromonas denitrificans*	Red	Antiplasmodial, Anticancer	Kim *et al*., 1999; Yamamoto *et al*., 1999
Fusarubin	*Fusarium solani*	Dark red	Food colorant	Parisot *et al*., 1990
Lycopene	*Fusarium sporotrichioides, Blakeslea trispora*	Red	Antioxidant, Anticancer	Jones *et al*., 2004
Mitorubin	*Penicillium rubrum*	Orange to yellow	Pharmaceuticals	Midland *et al*., 1982
Monascorubramin	*Monascus* spp.	Red	Antioxidant, Anticancer	Shi and Pan, 2011
Naphtoquinone	*Cordyceps unilateralis*	Deep blood red	Anticancer, Antibacterial, Trypanocidal	Prathumbai *et al*., 2006; Nematollahi *et al*., 2012; Ventura *et al*., 2009

Riboflavin	*Eremothecium gossypi*	Yellow	Anticancer, Antioxidant, Protection against cardiovascular diseases	Unagul *et al*., 2005; Hong *et al*., 2008; Powers, 2003
Rubropunctatin	*Monascus* spp.	Orange	Anticancer	Wong *et al*., 1977; Zheng *et al*., 2010
Viridin/Viridol	*Trichoderma viride, Trichoderma virens*	Yellow to orange	Antifungal activity	Singh *et al*., 2018
Xanthomonadin	*Xanthomonas oryzae*	Yellow	Protection against photo damage	Rajagopal *et al*., 1997; Goel *et al*., 2002
YEAST				
Astaxanthin	*Xanthophyllomyces dendrorhous*	Pink-red	Antioxidant, Photoprotectant, Anticancer, Anti-inflammatory	Florencio *et al*., 1998; Niklitschek *et al*., 2008
Melanin	*Saccharomyces neoformans*	Black	Antimicrobial, Antibiofilm and Antioxidant	Vinarov *et al*., 2003
Torularhodin	*Rhodotorula* spp.	Orange-red	Antioxidant, Antimicrobial	Yadav and Prabha, 2017; Kot *et al*., 2013

In the European middle ages, there are so many documentations and reports for the appearance of blood like coloration in the communion wafers, and these bleeding wafers are labeled as witchcraft. There are so many prodigious events that occur behind the discovery of the so-called pigment 'Prodigiosin'. Work conducted by Bizio and Sette during the 1820s found that these bleeding wafers are due to the action of the bacterium *Serratia marcescens.* After the 1960s, development in the field of separation chemistry and spectroscopy leads to the discovery of the true structural constitution of prodigiosin pigment (Bennett and Bentley, 2000). Prodigiosins are a family of tripyrrole red pigment, which has gained interest in research due to their antifungal, antibacterial, antimalarial, antiprotozoal, and immunosuppressive (Venil *et al*., 2009) properties. Prodigiosins are red pigmented secondary metabolites produced by both Gram-negative and Gram-positive bacteria like *Serratia marcescens, Pseudomonas megneslorubra, Hahella chejuensis, Vibrio psychroerythrous, Serratia rubidaea, Vibrio gazogenes, Alteromonas rubra, Streptoverticillium rubriverticuli* and *Streptomyces coelicolor.* Major prodigiosin and their derivatives produced by bacteria are enumerated in **Table 4.**

2.7. Chemistry of Prodigiosin

Wrede and Hettche (1929) isolated the pure form of prodigiosin for the first time from *Serratia marcescens*. Through degradation studies, Wrede discovered that prodigiosin contains three pyrrole groups. Santer and Vogel (1956) isolated a compound with the formula $C_{10}H_{10}O_2N_2$ from *Serratia marcescens* and he concluded that this compound could be a part of prodigiosin biosynthesis. In 1960, Wasserman *et al*. reported the synthesis of prodigiosin, following some treatment to the Vogels compound. Rapoport and Wilson (1962) finally identified the exact chemical structure of prodigiosin

with common pyrrolyl dipyromethane skeleton consisting of a common 4-methoxy, 2-2 bipyrrole ring system.

Bacterial prodigiosins are commonly divided into linear and cyclic derivatives. Prodigiosin and undecylprodigiosin are the representatives of linear derivatives and cyclic analogs include cyclononylprodigiosin, streptorubin B, and cycloprodigiosin (**Figure 5**). 25-carbon prodigiosin is a rare analog of prodigiosin, that are produced by *A. denitrificans* SP1 having a chemical formula of $C_{25}H_{33}N_3O$, with a molecular weight of 391 (Pradeep *et al*., 2014) (**Figure 6**). The nomenclature for prodigiosin is 2- methyl-3-amyl-6-methoxy prodigiosin and a molecular weight of 323.432 g/mol with a chemical formula of $C_{20}H_{25}N_3O$ (Hu *et al*., 2016). Prodigiosin is hydrophobic in nature and due to its particular chemical structure, it supports several cellular interactions. 4-methoxypyrrolic center of prodigiosin carries a cationic charge at physiological pH, which was found to be critical for DNA binding without discriminating between AT and CG sites (Melvin *et al*., 1999). The greater affinity of the three pyrroles enables the prodigiosin to bind to metal ions, such as copper, thereby prodigiosin-copper complex facilitates DNA cleavage (Kimyon *et al*., 2016).

The cellular role of prodigiosin in the producer organism is still undefined. The precise location of prodigiosin in the cell is also vague, as it is detected on both the intracellular and extracellular fractions. So many reports have suggested that the prodigiosin add an advantage or a protective function in competition with other microbes (Gulani *et al*., 2012) and resistance to the natural and chemical stresses (Stankovic *et al*., 2014) as well as to disperse in different ecological conditions by increasing the hydrophobicity (Song *et al*., 2006). Although the physiological function of prodigiosin in the producer organism is still a debated one.

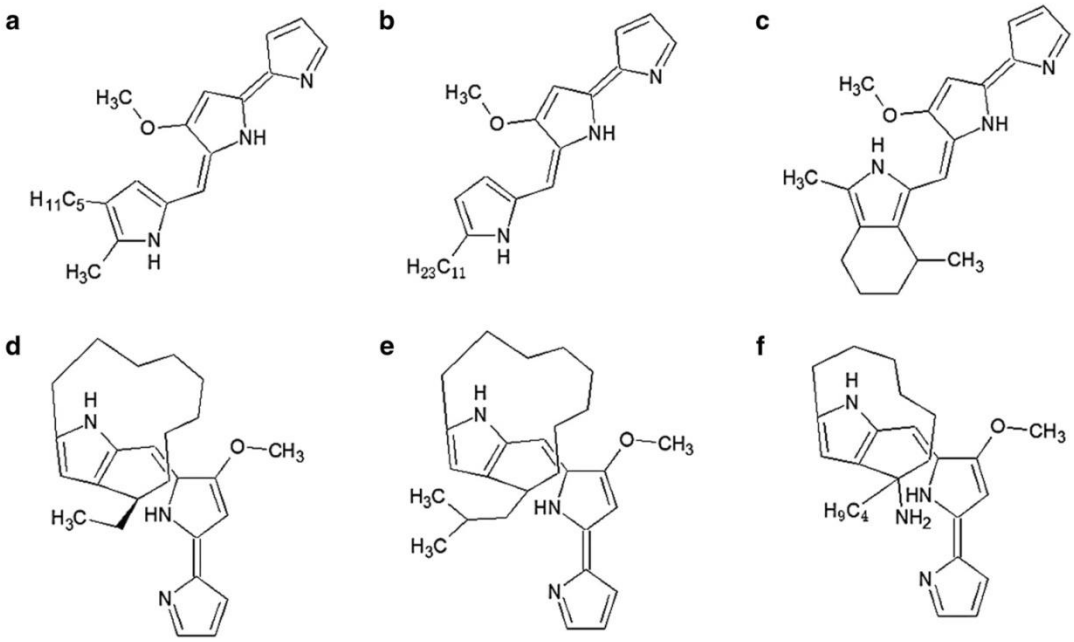

Figure 5. Members of prodigiosin family. **a)** Prodigiosin **b)** Undecylprodigiosin **c)** Cycloprodigiosin **d)** Metacycloprodigiosin **e)** Prodigiosin R1 **f)** Streptorubin B.

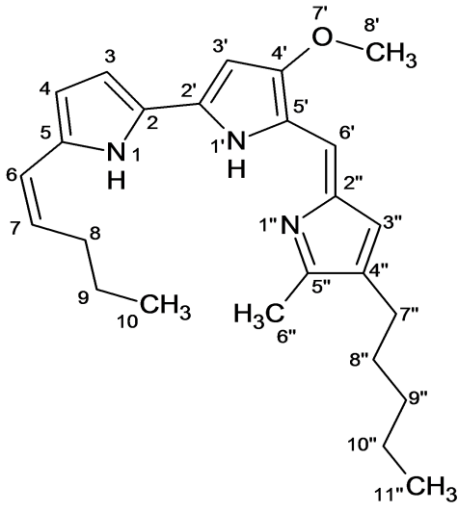

Figure 6. Structure of the 25-C prodigiosin analog, showing two 5C hydrophobic tails (Pradeep *et al*., 2014).

Table 4. Bacterial prodigiosins and their analogs.

Type of Prodigiosin	Organism	Reference
Prodigiosin	*Serratia marcescens* C3	(Chen *et al*., 2013)
	Serratia marcescens P-125	(Su *et al*., 2011)
	Serratia rubidaea N-1	(Yamazaki *et al*., 2006)
	Serratia plymuthica	(Berg, 2000)
	Hahella chejuensis KCTC 2396	(Kim *et al*., 2008)
	Streptoverticillium baldaccii	(Brambilla *et al*., 1995)
	Streptomyces griseoviridis 2464-S5	(Kawasaki *et al*., 2008)
	Vibrio psychroerythrus	(Daoust and Gerber, 1974)
	Vibrio gazogenes	(Allen *et al*., 1983)
	Vibrio sp DSM 14379	(Boric *et al*., 2011)
	Pseudomonas magnesiorubra	(Gerber, 1975)
	Zooshikella ganghwensis	(Yi *et al*., 2003)
Undecylprodigiosin	*Serratia marcescens* SMΔR	(Wei and Chen, 2005a)
	Serratia marcescens SS-1	(Wei and Chen, 2005b)
	Streptomyces sp JS520	(Stankovic *et al*., 2012)
	Streptomyces coelicolor A3(2)	(Cerdano *et al*., 2001)
	Streptomyces lividans	(Rossa *et al*., 2002)
	Saccharopolyspora sp nov	(Liu *et al*., 2005)
Cycloprodigiosin	*Pseudoalteromonas denitrificans*	(Sertan de Guzman *et al*., 2007)
	Alteromonas rubra	(Gauthier, 1976)
Nonyl prodigiosin	*Actinomadura madurae*	(Gerber, 1969)
	Actinomadura pelletieri	(Gerber, 1969)
Metacycloprodigiosin	*Streptomyces spectabilis* BCC 4785	(Isaka *et al*., 2002)
	Streptomyces longisporus ruber	(Wasserman *et al*., 1969)

2.8. Biosynthesis of Prodigiosin

Biosynthesis of prodigiosin proceeds in a bifurcated pathway, culminating in the enzymatic condensation of the terminal products of the two pathways, 2-

methyl-3-pentylpyrrole (MAP) and 4-methoxy-2,2-bipyrrole-5-carbaldehyde (MBC) to form prodigiosin (Williamson *et al*., 2006). The precursors for the biosynthesis of prodigiosin are shown to be alanine, acetate, proline, methionine, and serine (Williams *et al*., 1973). Prodigiosin production in bacteria was controlled by a cluster of genes called *pig* cluster. The *pig* cluster comprise of 14 genes with a size of 20,960 bp. The *pig* cluster from *Serratia* sp. ATCC39006 was expressed in *Erwinia carotovora* subsp. *Carotovora* however it was not expressed in other species like *E. coli*. *Serratia* ATCC39006 regulates the production of prodigiosin through multiple ways, including quorum-sensing.

In *Serratia* sp. these *pig* genes are arranged in the order: *pigA, pigB, pigC, pigD, pigE, pigF, pigG, pigH, pigI, pigJ, pigK, pigL, pigM,* and *pigN* (Venil and Lakshmanaperualsamy, 2009). Among these, eight gene (*pigA, pigF, pigG, pigH, pigI, pigJ, pigM,* and *pigN)* products are responsible for the synthesis of MBC and three genes (*pigB, pigD,* and *pigE*) for the MAP production. Williamson *et al*. (2005) reported that *pigC* encodes for the terminal condensing enzyme, that condenses both MAP and MBC to produce prodigiosin. Functions of two genes (*pigK* and *pigL*) are still undefined. Williamson *et al*. (2005) suggested that *pigK* act as a molecular chaperone, which helps in the folding of other enzymes in the biosynthetic pathway of MBC and *pigL* may involve in the phosphopantetheinylation reaction in the MBC pathway.

Serratia ATCC39006 has an additional gene named *pigO*. The only difference between Sma 274 and ATCC39006 was in the gap between *pigC* and *pigD*. *Serratia* ATCC39006 has a 184 bp gap and Sma 274 has a 64 bp gap. The huge gap between *pigC* and *pigD* in *Serratia* ATCC39006 having a possibility of two translational units (Crow, 2001). *Streptomyces coelicolar* producing undecylprodigiosin was controlled by a cluster of 23 genes called *Red* cluster. Unidirectional transcription was seen in *Serratia* sp. and four transcriptional units regulated by *RedZ* and *RedD* are seen in *Streptomyces* sp.

(Slater *et al*., 2003). Between *RedQ* and *RedP*, there is a *RedD* dependent promotor region that contains two divergent promotors that transcribe in the opposite direction. A response regulator *RedZ*, is thought to bind upstream of the *RedD* gene. This dissimilarity in the arrangement of homologous genes, which even produces chemically similar products, put up some evolutionary questions regarding gram-positive and gram-negative bacteria. Some genes like *pigD* and *pigE* have no homologues in the *Red* cluster. The twelve *Red* cluster proteins, with their homologues encoded in the *pig* cluster are similar in size to their *pig* counterpart. The only exception was seen in *pigB*, whose homologue *RedS* is smaller in size (Harris *et al*., 2004). An overview of prodigiosin biosynthetic pathway is illustrated in **Figure 7**.

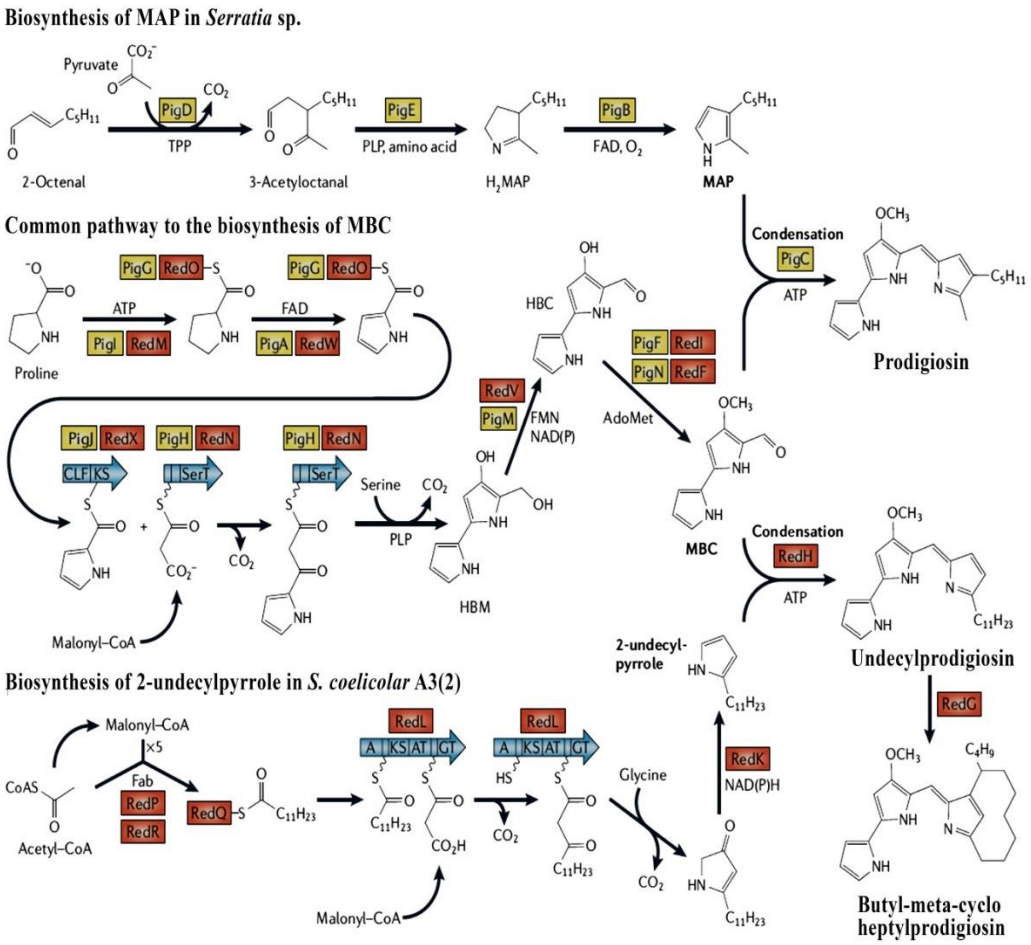

Figure 7. The biosynthetic pathway of prodigiosin (Williamson *et al*., 2006)

2.9. Major factors influencing prodigiosin production

In the life cycle of bacteria, like all the bioactive secondary metabolites, prodigiosin is also produced in the later stages (stationary phase) of bacterial growth (Darshan and Manonmani, 2015). As a major producer of prodigiosin, *Serratia* spp. is widely studied in this context and the physiological regulation of prodigiosin is well understood. But the specific role of prodigiosin in the producer organism is still unclear and the maximum production of prodigiosin is under the nutrient depletion or stress, when the cells enter the stationary phase (Williamson *et al*., 2006). There are so many factors influencing the production of prodigiosin like, medium composition (carbon, nitrogen, and other natural substrates), temperature, pH, light, availability of inorganic phosphate, salts, signaling molecules, and various environmental factors (Yip *et al*., 2019).

2.9.1. Medium Composition

Many types of differential and selective media have been formulated for the production of prodigiosin from various microorganisms. Carbon and nitrogen sources are the major components for any medium composition. Among the carbon sources, glucose is widely used in most of the fermentation strategies, but in the case of prodigiosin production, glucose plays an inhibitory role in the biosynthesis pathway. Earlier studies formulated that the inhibition was pH-dependent and there was no involvement for cyclic AMP (cAMP). Recent studies proved that prodigiosin inhibition by glucose was controlled by quinoprotein glucose dehydrogenase (GDH) located in multiple gene loci, including pyrroloquinoline quinone and ubiquinone genes and their enzymatic products (D-glucono-1,5-lactone and D-gluconic acid) was also involved in the inhibition (Fender *et al*., 2012). cAMP phosphodiesterase is inhibited by glucose and which leads to the increase of cAMP in cell and which in turn induce the prodigiosin pathway (Zang *et al*., 2014; Sole *et al*., 1997).

According to Zang *et al*. (2014), *Serratia marcescens* N10612 utilize sucrose effectively and contributed higher prodigiosin yield (459.13 mg/L) other than glucose, fructose, soluble starch, and lactose in the medium.

Gondil *et al*. (2017) studied the effect of different carbon sources on the production of prodigiosin from *Serratia nematodiphila* RL2. The sources studied were sucrose, galactose, glucose, maleic acid, ammonium acetate, citric acid, glycerol, sodium oxalate, and lactose. The study reported maximum prodigiosin production (0.52 mg/ml), while using lactose as the carbon source. Comparing the studies of Zang *et al*. (2014) and Gondil *et al*. (2017), it is clear that prodigiosin production is varying in the species level among the *Serratia* itself. Giri *et al*. (2004) reported a complete decrease in production by *S. marcescens*, while adding glucose or maltose to sesame seed broth, due to catabolite repression.

Sundaramoorthy *et al*. (2009) tested the effect of maltose, glucose, lactose and sucrose on prodigiosin production in *Serratia marcescens* NY1 and concluded that maximum production (425 mg/L) was in the presence of maltose. Gulani *et al*. (2012) also reported a maximum production of prodigiosin (784.15 unit/cell) using maltose as carbon source and moderate levels of production using lactose, fructose, sucrose, and mannitol. Prasad (2018) reported the highest impact of maltose on prodigiosin production (1381 unit/cell), followed by sucrose, fructose, and glucose in *S .marcescens* (MTCC 4822). Khadir *et al*. (2015) also established that culture supplemented with maltose has better yield of prodigiosin from *Paeonia anomala***,** an endophytic bacterium. Elkenawy *et al*. (2017) reported that crude glycerol obtained from the biodiesel industry supported an enhanced prodigiosin production (560 unit/cell) from *S. marcescens*, compared to other substrates like cottonseed cake, soybean cake, and black seed cake. Bae *et al*. (2001) conducted a bioreactor study with an internal absorbent for pigment absorption and the

maximum prodigiosin production was 13 mg/ml using dextrose and casein in the culture medium using *Serratia* sp. KH-95. Cang *et al*. (2000) studied a medium containing different carbon sources like ethanol, glucose, galactose, fructose, rhamnose, xylose, arabinose, glycerol, sucrose, maltose, and lactose. It has been concluded that ethanol has promoted a high yield of prodigiosin, about 3 mg/ml, from *S. marcescens* strain S389 and low production was noticed in galactose and fructose-containing medium.

Srimathi *et al*. (2017) attempted on *S. marcescens* and found that mannitol favored maximum prodigiosin yield than other carbon sources such as dextrose, fructose, lactose, and sucrose. Mannitol also favored a better yield (277.74 mg/l) from *S. marcescens* MO-1, other than glucose and maltose, in the study conducted by Kurbanoglu *et al*. (2015). The study conducted by Araujo *et al*. (2010) reported that mannitol supplemented with cassava wastewater was a good promoter of prodigiosin production from *S. marcescens* UCP 1549. Mahmoud *et al*. (2015) studied the effect of arabinose, cellulose, fructose, glucose, lactose, maltose, mannitol, mannose, methylcellulose, rhamnose, starch, and sucrose on the production of prodigiosin from *S. marcescens* strain S23 and found that culture supplemented with sucrose have shown a better yield (147.9 mg/l). According to Mathlom *et al*. (2018), *S. marcescens* produced high prodigiosin yield while using starch as a carbon source other than sucrose, fructose, mannitol, maltose, and xylose in the culture medium. From the above-mentioned literature, it is well understood that the effect of carbon source on the prodigiosin production was different for different bacterial strains.

Studies have been carried out to check the efficiency of various nitrogen sources on the yield of prodigiosin. Zang *et al*. (2014) conducted a study using nitrogen sources like yeast extract, peptone, malt extract, ammonium chloride, ammonium sulfate, and sodium nitrate to check the yield of

prodigiosin from *S. marcescens* N10612. Among the nitrogen sources, yeast extract and peptone showed maximum prodigiosin production and sodium nitrate exhibited an inhibitory effect on the production. Kurbanoglu *et al*. (2015) conducted an interesting study using different peptone sources like ram horn peptone, tryptone, bacto peptone, and fish peptone as nitrogen sources to maximize the prodigiosin yield and found that ram horn peptone contributed more to the production from *S. marcescens* MO-1. Su *et al*. (2011) observed that prodigiosin production was maximum while using glycine as a nitrogen source other than yeast extract and ammonium sulphate by using *S. marcescens starin* P-125. Elkenawy *et al*. (2017) investigated the effect of five nitrogen sources; peptone, tryptone, urea, ammonium sulphate, and ammonium nitrate in the culture medium and found that peptone showed the highest yield of 610 unit/cell from *S. marcescens.*

Gondil *et al*. (2017) analyzed the effect of nitrogen sources such as yeast extract, peptone, tryptophan, beef extract, glycine, and ammonium sulphate on the production of prodigiosin from *S. nematodiphila* RL2 and found that yeast extract contributes more to the production of prodigiosin (0.6 mg/ml). Zang *et al*. (2014) reported that yeast extract supported a better yield of prodigiosin from *S. marcescens*, compared to peptone, malt extract, ammonium sulphate, and sodium nitrate. According to Cang *et al*. (2000) pharmamedia (commercial protein source) and polypepton enhanced the prodigiosin production in *S. marcescens*, other than nitrogen sources like ammonium nitrate, ammonium chloride, ammonium sulphate, urea, soybean meal, and dried yeast, which was also tested in this study. Srimathi *et al*. (2017) tested the effect of nitrogen sources like urea, tryptone, peptone, beef extract powder and yeast extract powder, and concluded that peptone supported the maximum pigment production and the yield was 2006 mg/l from *S. marcescens*. In this study, urea showed the least prodigiosin production. Mahmoud *et al*. (2015) studied the effect of nitrogen sources like

peptone, urea, ammonium nitrate, ammonium sulphate, ammonium chloride and estimated that peptone supported high prodigiosin yield (149.1 mg/l) from *S. marcescens* S23. Suryawanshi *et al*. (2014) studied nitrogen sources like sodium nitrate, ammonium sulphate, casein, yeast extract, and peptone on prodigiosin production from *S. marcescens* and found that casein enhanced the yield of prodigiosin.

2.9.2. Temperature

Temperature plays a crucial role in the biosynthesis of prodigiosin. Maximum prodigiosin synthesis was recorded mainly in the lower temperatures (20°C - 30°C). Biosynthesis of prodigosin is genetically controlled by a group of genes, called *pig* cluster, which comprised of 14 genes (*pigA, pigB, pigC, pigD, pigE, pigF, pigG, pigH, pigI, pigJ, pigK, pigL, pigM and pigN*) (Yip *et al*., 2019). Biosynthesis of prodigiosin is a bifurcated pathway, followed by enzymatic condensation of MAP (2-methyl-3-n-amyl-pyrrole) and MBC (4-methoxy-2,2-bipyrrole-5-carbaldehyde) to become prodigiosin. This condensation reaction is very much influenced by temperature. In the *pig* cluster, *pigC* contribute to the enzymatic condensation of MAP and MBC. In higher temperatures (> 35 °C), the enzymatic condensation of MAP and MBC does not occur, which leads to the non-formation of prodigiosin pigment (Williamson *et al*., 2006). Elkenawy *et al*. (2017) reported that *S. marcescens* MN5 at 22 °C produced maximum prodigiosin and reported that gamma-irradiated strain *S. marcescens* MN200 showed prodigiosin pigmentation at 32 °C, 34 °C and 36 °C, but has gradually decreased at increasing temperatures. Gulani *et al*. (2012) reported that the maximum amount of prodigiosin was produced at temperature 25 °C by *S. marcescens* growing in a synthetic medium comprising of maltose, peptone, NaCl, and glycerol. Giri *et al*. (2004) studied the production of prodigiosin in three different media; nutrient broth, peptone glycerol broth and sesame seed broth

at different temperatures (28°C, 30°C and 37 °C) and reported that nutrient broth and sesame seed broth enhanced the prodigiosin production at 28 °C and *S. marcescens* grown in peptone glycerol broth showed maximum prodigiosin production at 30 °C. All the media at 37 °C showed a drastic decrease in prodigiosin yield. Lapenda *et al*. (2015) reported the maximum prodigiosin production from *S. marcescens* (UFPEDA 398) at 30 °C on mannitol agar medium. Pradeep *et al*. (2016) also studied and found that 30 °C favoured the maximum prodigiosin production from *S. marcescens* MBBO2. Picha *et al*. (2015) tested different temperatures (28 °C, 32 °C, 37 °C, and 42 °C) on prodigiosin production from *S. marcescens* and found that 28 °C favoured the highest prodigiosin yield. Shahitha and poornima (2012) and Samrot *et al*. (2011) also established that 28 °C enhances the maximum prodigiosin production from *S. marcescens.* Wang *et al*. (2012) tested different temperatures (25 °C, 30 °C, and 37 °C) on prodigiosin production and found that 25 °C contributed higher prodigiosin yield from *S. marcescens* TKU011.

2.9.3. pH

pH is another important parameter that influences the biosynthesis of prodigiosin. The final condensation of MAP and MBC to form prodigiosin can occur in low pH (3-5) (Williamson *et al*., 2006). Prodigiosin production occurs mainly when the culture has grown in a pH range of 4-10. pH below 3 and above 10 inhibited the growth of the organism and the prodigiosin production (Yip *et al*., 2019). Lapenda *et al*. (2015) worked on *S. marcescens* (UFPEDA 398) and found that neutral pH 7 contributed more to prodigiosin production. Giri *et al*. (2004) also found that neutral pH of 7 enhanced the yield of prodigiosin from *S. marcescens*. Gulani *et al*. (2012) and Suryawanshi *et al*. (2014) also studied the effect of pH on prodigiosin production from *S. marcescens* and concluded that neutral pH 7 showed

maximum production. Tao *et al*. (2005) maintained two stages of pH for the production of prodigiosin from *S. marcescens* mutant. He maintained pH 7 as the first stage for cell growth in the bioreactor and increased to 7.4 in the second stage for the secretion of prodigiosin. Picha *et al*. (2015) inoculated *S. marcescens* in peanut broth maintained with different pH (6, 7, 7.5, and 8) to study the production of prodigiosin and found that pH 7 enhanced the pigment production, than other pH tested. Prodigiosin production by *S. marcescens* TKUOO1 was found to be suitable in the pH range 7-9 (Wang *et al*., 2012). Williams *et al*. (1971) maintained a pH range of 8.0 - 8.3 for the maximum production of prodigiosin from *S. marcescens*. Krishna *et al*. (2014) tested the production prodigiosin from *Vibrio* sp. at different temperatures (4, 5, 6, 7, 8, and 9) and found that the pH range of 7-8 showed a high range of pigmentation, particularly the alkaline condition at pH 8 showed maximum pigment yield. pH range 4-5 limited the pigment production. Siva *et al*. (2012) studied the production of prodigiosin in *S. marcescens* and *S. rubidaea* and found that both the species produced maximum prodigiosin in the pH 4.5, which gradually decreased at pH 10. Elkenawy *et al*. (2017) found that alkaline pH 9 has better prodigiosin production in *S. marcescens.*

2.9.4. Light Sources

Light is a major influencing factor that has the ability to induce morphological, physiological and behavioral changes in the organism. Phatake and Dharmadhikari (2016), conducted studies using *S. marcescens* to evaluate the effect of different types of light on the production of prodigiosin. They used CFL (Compact fluorescent lamp) as source of light and colored gelatin papers (red, blue, green, yellow, and black) to get different wavelengths. They also maintained a flask without gelatin paper to study the effect of white light. The study revealed that white light supported high prodigiosin yield (816 unit/cell), followed by blue (650 unit/cell) and yellow

light (624 unit/cell). Black (602 unit/cell), Red (521 unit/cell), and Green (607 unit/cell) showed lesser yield, compared to other colors of light.

Wang *et al*. (2013) studied the effect of different light sources on the intracellular and extracellular prodigiosin production in *S. marcescens* y2. The study used colored glass paper to get different wavelengths, like red, yellow, blue, green, and also maintained cultures in continuous dark and white light. In this study, cultures under white light decreased the intracellular pigment and more pigments are released to the extracellular space. But in dark condition, minimum extracellular pigment was reported. Among the red, green, blue, and yellow wavelengths, red light contributed more to intracellular pigment production and green light has a minimum effect. Extracellular pigment production was maximum in green than red light. Someya *et al*. (2004) studied the effect of different light sources like white fluorescent tube, Red LED, Blue LED, Far-red LED and also the continuous dark condition on the production of prodigiosin from *S. marcescens* strain B2. In this report, they showed that a high yield of prodigiosin was accumulated in cultures under dark, red and far-red light. Prodigiosin production under white and blue light was lesser than the red and far-red light**.** Velmurugan *et al*. (2010) studied the effect of light on intracellular and extracellular pigment production from five pigment-producing filamentous fungi. They also used colored glass papers for obtaining different wavelengths like red, green, blue, and yellow. Their investigation proved that all the fungi showed high pigment production in the cultures incubated under complete darkness and the cultures under white light showed an inhibiting effect on pigment production. Ryazantseva *et al*. (2012) also studied the effect of illumination conditions (light and darkness) on the biosynthesis of prodigiosin in *S. marcescens* and found that continuous illumination promoted the prodigiosin production with in the first 24 hr incubation, after that the rate of prodigiosin accumulation decreased, compared to the cultures grown in dark condition.

As a secondary metabolite, prodigiosin production was during the stationary phase of the growth curve and observed a long incubation period for the higher yield of prodigiosin. The incubation period for the maximum production of prodigiosin from *S. marcescens* was also strain-dependent and ranged with 24 hr (Siva *et al*., 2012), 36 hr (Giri *et al*., 2004), 48 hr (Suryawanshi *et al*., 2014, Kurbanoglu *et al*., 2015, Wang *et al*., 2012), 72 hr (Samrot *et al*., 2011) and 96 hr (Ramani *et al*., 2014). In most of the literature, the effect of inorganic salts on prodigiosin production was also investigated. Su *et al*. (2011) studied the effect of inorganic salts like magnesium sulphate, calcium chloride and dipotassium phosphate on the production of prodigiosin from *S. marcescens* strain P-125 and found that dipotassium phosphate supported the maximum pigment yield. In another study, Suryawanshi *et al*. (2014) studied the effect of inorganic salts like calcium carbonate, ferrous sulphate, dipotassium phosphate, magnesium sulphate, sodium, and potassium chloride from *S. marcescens* and found that dipotassium phosphate supported a high yield of pigment. Gulani *et al*. (2012) studied the effect of different concentrations of inorganic salts like sodium chloride, ferric chloride and dipotassium phosphate and put forward that NaCl supported maximum production from *S. marcescens.* Cang *et al*. (2000) also supported the fact that dipotassium phosphate contributed higher prodigiosin yield from *S. marcescens*.

2.10. Quorum sensing mechanism in bacteria

Contrary to the earlier belief, researchers around the world have discovered the social behavioral pattern of bacteria. Through quorum sensing, the ways and means of communication in bacteria, they can count their local population density through small diffusible signal molecules, called autoinducers (Mukherjee and Bassler, 2019). To overcome changing and challenging environmental conditions, both gram-negative and gram-positive bacteria

produces various types of signaling molecules (Ortori *et al*., 2019; Banerjee and Ray, 2017). Several physiological functions like the formation of biofilm, secondary metabolites, antibiotics, bio-surfactants, bioluminescence, and swarming motility are regulated by quorum sensing signaling systems and systems are doubtful in their completion when only one individual bacterium undertakes the mission; therefore, the QS system has a significant role in initiating the co-behavioral patterns in both gram-negative and gram-positive bacteria (Morohoshi *et al*., 2007; Hammer and Bassler, 2003; Zhang and Pierson, 2001; Shrout *et al*., 2006; Zhang and Li, 2016). When the amount of signaling molecules surpasses the threshold level, they influences the signaling pathways and this leads to an alteration in gene expression in a uniform mode all over the population (Coulthurst *et al*., 2004).

In gram-negative bacteria, signaling through N-acyl homoserine lactone (HSL) molecules are predominant; but other interactions are a little bit recondite (Thomson *et al*., 2000). The basic structure of N-acyl homoserine lactone is similar in all gram-negative bacteria, but differs in their R-group. It varies from 4-18 carbons. The third carbon moiety which holds hydrogen, oxo, or hydroxyl group, acts as a defensive mechanism for identifying their own signaling molecules from other organisms (Frederix and Downie, 2011). Chemical structures of representative HSL members are illustrated in **Figure 8**. LuxI/R protein families are the two proteins that are responsible for the production and controlling of HSL molecules. In the HSL mediated quorum sensing, LuxI helps in the synthesis of HSLs. When the amount of HSLs surpasses the threshold level, HSLs binds with the transcriptional activator LuxR at its amino terminus, meanwhile the carboxyl terminus of transcriptional activator binds with a specific gene promoter sequence called lux boxes. luxI gene which placed in the lux boxes are the genes that produce the protein, Luxl (Van Houdt *et al*., 2007). In Gram-positive bacteria, cell-population density-dependent regulations are employed by small post-

translationally modified peptides called autoinducing peptides (AIPs) (Miller and Bassler, 2001).

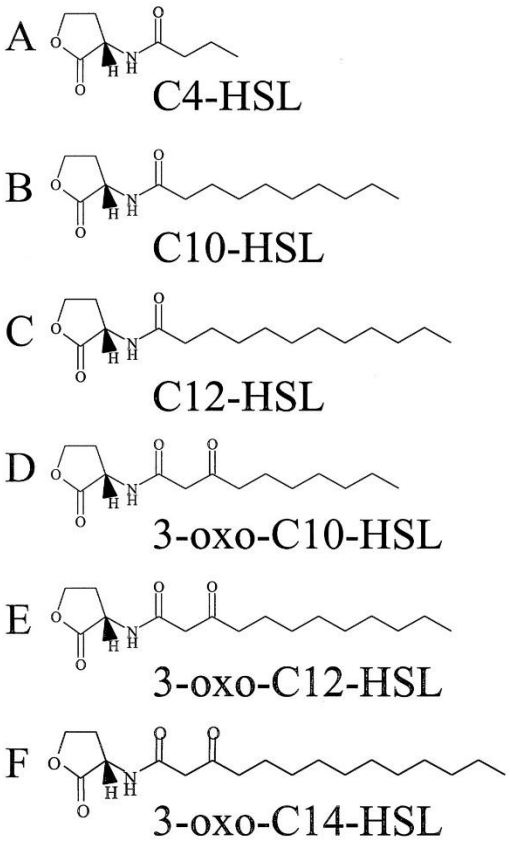

Figure 8. Chemical structures of different homoserine lactone molecules (Swift *et al*., 1999).

The regulation and synthesis of HSLs produced may vary among bacterial species. Some species produce a single type of HSL like 3-oxo-C6-HSL in *Pantoea stewartii* and 3-oxo-C8-HSL in *Agrobacterium tumifaciens*, respectively. In some species (*Burkholderia* and *Silicibacter*), multiple HSL synthase genes are present and result in the production of very complex mixtures of HSLs. *Burkholderia* sp. produces two HSL genes, which produce C8-HSL and 3-hydroxy-C8-HSL (Churchill and Chen, 2011). Only fewer studies are reported, which substantiate the relationship between quorum sensing and prodigiosin production. A research team led by George Salmond of the Department of Biochemistry, University of Cambridge, UK, are only

known to work on the genetic relationship between quorum sensing mechanism and prodigiosin production. Thomson *et al*. (2000), from the team of George Salmond, first published an article that explains the biosynthesis of prodigiosin under quorum sensing in *Serratia* sp. The study explained the relationship by inserting the prodigiosin gene locus from *Serratia* sp. ATCC 39006 to a heterologous host, *Erwinia carotovora* sp. They also observed a high level of prodigiosin production in *E. carotovora* sp. while adding external HSLs in the medium.

2.10.1. Extraction of HSLs

There are technical difficulties in extracting HSLs from bacteria, as they are produced in very low quantities and also affected by the interference of growth media and other extracellular products while extracting the HSLs. To avoid all these hurdles, stationary growth phase is recommended for the extraction of HSLs (Wang *et al*., 2011). Liquid-Liquid extraction is one of the most common and easiest methods to extract HSLs (Brelles-Mario and Bedmar, 2001). This method uses organic solvents for extraction and after that, these solvents are removed by evaporation method. The commonly used solvents are ethyl acetate (Chu *et al*., 2011), hexane (Pomini *et al*., 2006), dichloromethane (Morin *et al*., 2003), ethyl ether (Pearson *et al*., 1994) and chloroform. The polarity of homoserine lactones highly influences the yield of extraction (Morin *et al*., 2003). Solid-phase extraction is not a common extraction method but also used for an additional pre-concentration after the liquid-liquid extraction method. In this method, the dried HSLs, after LLE are passed through different solid phases in the SPE column like silica, neutral, basic, and acidic aluminum (Schupp *et al*., 2005).

2.10.2. Detection and quantification of HSLs

Bacterial biosensors are the most widely used detection method to know the presence of HSLs in the organism (Chu *et al*., 2011). Biosensors don't have the ability to produce HSLs but have the potential to produce noticeable phenotypes like light emission, pigment production, and β-galactosidase activity incorporation with active external HSLs (Saurav *et al*., 2017). *Chromobacterium violaceum* CV026 is a type of bacterial biosensor that produce violacein (pigment) with a mutation of one transposon that is inserted into the *cvil* HSL synthase gene and the other inserted in violacein repressor locus. This CV026 strain produces a deep purple pigmentation, when it interacts with HSLs. CV026 strain is capable of detecting HSLs like C6-HSL, C6-3-oxo-HSL, C8-HSL, C8-3-oxo-HSL, and C4-HSL (Steindler and Venturi, 2007). *Agrobacterium tumifaciens* NT1 is another type of biosensor that contains a plasmid with a lacZ fusion to traG. When this strain exposes to an exogenous HSL, it induces the traG::lacZ reporter gene and produces a blue-colored zone, wherever the HSL molecules migrate on the plate. This blue color is achieved through the hydrolysis of Xgal in the medium by the β-galactosidase. Strain NT1 detects the HSLs like 3-oxo, 3-hydroxy side chains of all length, expect C4-HSL (Fuente *et al*., 2015). Biosensor techniques can detect the presence of HSLs but it has its limitations, as it is difficult to determine its structure and concentrations.

TLC biosensor is an improved method of a bacterial biosensor for the quantification of HSLs by additional incorporation of the TLC plate. In this method, the unknown supernatant samples are spotted on the TLC plate and this plate is overlaid on the biosensor streaked plate and incubated. The fluorescent light will be emitted on the spot, where HSL is present. The quantification is done through the calibration curve obtained from the standard HSLs (Yang *et al*., 2006). Scott (2007), put forward a method called

sulphuric acid spray method, which can be used with the TLC method, with a complete separation of HSLs in a very short time. The only limitation of this method is that HSLs cannot be recovered after this method and cannot be used for further purification. Due to lesser amount of HSL production and difficulty in detecting it in biofilm-producing cultures, Schaefer *et al*. (2000) developed a radiolabeled assay for HSL detection. In this method, first the test bacterium is grown in a methionine-free medium and in the early stationary phase, 1% of radiolabelled methionine is incubated with the cells. After that, radiolabelled methionine bounded with HSLs will be detected using a scintillation cocktail with standard HSLs. Yang *et al*. (2006) developed a colorimetric method that needs only a small amount of sample with high precision, which is quite comparable with high end instrumentation methods. Nowadays HPLC is vastly used to determine the concentration of HSLs in the sample. Methods discussed previously have limitations to detect the HSLs in the structural levels. Developments in the field of spectroscopic methods like infrared spectroscopy, GCMS, HPLC-MS, LCMS, magnetic resonance spectroscopy (NMR) etc. makes the detection of ultralow concentration of HSL at the molecular level.

2.11. Applications of prodigiosin

2.11.1. Antioxidant activity

Nowadays the red pigment prodigiosin gained a lot of attention from the pharmaceutical and health industry due to their inherent bioactivity against the pathogenic strains of bacteria, fungi, algae, and parasites (Ramesh *et al*., 2020; Balasubramaniam *et al*., 2019). Like every bacterial pigment, prodigiosin also showed antioxidant properties. Arivizhivendhan *et al*. (2018) conducted a detailed study on the antioxidant potential of prodigiosin produced by *Serratia marcescens* using DPPH (2,2-diphenyl 1-1-picrylhydrazyl) and ABTS (2,2-azino-bis 3-ethylbenthiazoline-6-sulfonic

acid) radical scavenging assays. They got a significant decrease in ABTS at all concentrations of prodigiosin. They studied a total of five prodigiosin concentrations (2, 4, 6, 8, and 10 µg/ml). Prodigiosin concentration of 2 µg/ml observed a scavenging activity of 25%, 51% for 4 µg/ml, 74% for 6 µg/ml, 92% for 8 µg/ml, and 99% scavenging activity for 10 µg/ml. Their DPPH assays also showed positive results. DPPH is one of the most stable forms of hydroxyl and superoxide radicals, which are used in antioxidant determination. DPPH free radical scavenging activity of prodigiosin was tested over 2, 4, 6, 8 and 10 µg/ml concentrations and got a scavenging percentage of 29, 58, 65, 86, and 99% respectively. Like ABTS, a directly proportional relationship between scavenging activity and prodigiosin concentration was noticed. Sajjad *et al*. (2018) studied the antioxidant potential of prodigiosin isolated from *Streptomyces* sp. strain WMA-LM31 and found that the prodigiosin has a good inhibition percentage of 60.5% for 10 µg/ml prodigiosin under DPPH assay. They also got an increase in quenching capability with an increase in prodigiosin concentration. Gulani *et al*. (2012) reported a promising result of prodigiosin antioxidant activity of 22.05 µg ascorbic acid equivalent/ml of extract. Othman *et al*. (2019) studied the antioxidant activity of 6 different concentrations (25, 50, 100, 200, 400, and 1000 µg/ml) of prodigiosin extracted from *Serratia* sp. They found the DPPH free radical scavenging activity be 51.79, 59.82, 60.49, 62.05, 63.17 and 92.63 % at 25, 50, 100, 200, 400, and 1000 µg/ml of prodigiosin concentration. Renukadevi and Vineeth *et al*. (2017) reported the antioxidant property of prodigiosin and found that 600 µg/ml prodigiosin concentration showed 94 % scavenging activity and also noticed an increasing activity with increasing concentration.

2.11.2. Antimicrobial activity

Prodigiosin and its analogs are well known for their antibacterial and antifungal properties (Sakai-Kawada *et al*., 2019). Prodigiosin isolated from *Serratia marcescens* strain RMN1 and RMN2 showed antibacterial activity against the oxacillin resistant *Staphylococcus aureus* and in the tested prodigiosin concentrations (500, 250, 125, and 62.5 mg/ml) 500 mg/ml was found to be effective (Akin-Osanaiye *et al*., 2019). Prodigiosin extracted from *Serratia marcescens* had a bacteriostatic effect on *E.coli* cells and it did not kill the cells, instead inhibited their division and metabolic activity (Davevcic *et al*., 2016). Prodigiosin from *Serratia nematodiphila* darsh1 was found to be an effective antibacterial agent against the food born bacterial pathogens like *Bacillus cereus*, *Pseudomonas aeroginosa*, *Staphylococcus aureus* and *Escherichia coli*. Prodigiosin inhibits bacterial growth by programmed cell death through DNA fragmentation, ROS generation, and expression of substrate-specific proteins (Darshan and Manonmani, 2016). Suryawanshi *et al*. (2017) reported the prodigiosin pigment extracted from *Serratia marcescens* with high antimicrobial activity of 30% growth reduction on *Candida albicans, E.coli*, and *Staphylococcus aureus* at 0.3, 100 and 0.18 µg/ml of pigment concentration. They also studied the ways of inhibitory action in pathogenic strains and found that prodigiosin acts as a hydrophobic stressor, which induces the disruption of the plasma membrane. Gulani *et al*. (2012) tested the antimicrobial activity of prodigiosin isolated from *Serratia - marcescens* and concluded that the prodigiosin has a potent inhibitory effect on gram positive bacteria like *Staphylococcus aureus* and *Bacillus cereus* and against fungal strains like *Candida albicans, C. parapsilosis,* and *Cryptococcus* sp. Sumathi *et al*. (2014) evaluated the antimicrobial activity of prodigiosin and found that it is good against human pathogenic bacteria like *Pseudomonas aeroginosa* and *E. coli,* and comparatively least inhibition on

Klebsiella pneumonia. It is also reported to have antifungal activity on *Aspergillus niger*, *Trichoderma viridae*, and *Trichoderma rubrum*.

2.11.3. Antimalarial activity

Antimalarial activity of prodigiosin was studied in 1960s by Castro (1967). Further investigations were in a slow phase until recently, when extensive research was undertaken in the area of structural biology of prodigiosin influencing the antimalarial property by Papireddy *et al*., (2011) and Mahajan *et al*., (2012, 2013). Papireddy *et al*. (2011) studied the in-vitro antimalarial activity of four natural and three sets of synthetic prodigiosin against *Plasmodium falciparum*. A potent in-vitro activity (IC_{50}= 0.9-16 nM) was observed in alkyl and aryl substituents of prodigiosin. They tested the antimalarial activity of metacycloprodigiosin and synthetic analogs against *Plasmodium yoelii* and observed a reduced parasitemia by more than 90% after 25 mg/day dosing. Lazaro *et al*. (2002) isolated a heptyl prodigiosin from an α-proteobacteria and found a strong antimalarial activity for the prodigiosin, which extended the survival period of *Plasmodium berghei* ANKA strain-infected mice, but observed a sclerotic lesion at the site of injection. Strong in-vitro, together with an in-vivo antimalarial activity against chloroquine-sensitive D6 and multidrug-resistant Dd2 strains of *Plasmodium falciparum* has also been reported (Papireddy *et al*., 2011). *Serratia marcescens* NMCC46 produces a prodigiosin, which is effective against the larvae of mosquito species, *Aedes aegypti* and *Anopheles stephensi* (Patil *et al*., 2012).

2.11.4. Antitrypanosomal activity

McRacy *et al*. (1953) first studied the antitrypanosomal activity of prodigiosin against *Trypanosoma cruzi* and reported a lethal concentration of 10µg/ml. Prodigiosin from *Serratia marcescens* leads to cell lysis of trypanosomal

members like *Trypanosoma cruzi* and *Leishmania chagasi* (Moraes *et al*., 2008; Azambuja *et al*., 2004). Genes *et al*. (2011) conducted a detailed study and observed that prodigiosin extracted from *Serratia marcescens* interacts with the oxidative phosphorylation processes in the mitochondria of *Trypanosoma cruzi*, leading to cell apoptosis without damaging the human tissues.

2.11.5. Antialgal activity

Gerber *et al*. (1975) reported the antialgal effect of prodigiosin on *Prototheca zopfi*, at an inhibitory concentration of 25 µg/ml. *Hahella chejuensis* KCTC 2394, a marine isolated bacterial strain produces a prodigiosin that having a potent antialgal effect though it acts as a biocontrol for marine environmental disturbances like harmful algal blooms (Jeong *et al*., 2005). Kim *et al*. (2008) and Priya *et al*. (2013) reported that the prodigiosin isolated from *Hahella chejuensis* can effectively be used for the mitigation of red tide-causing microalgae. Liu *et al*. (2010) isolated an antialgal prodigiosin from *Serratia marcescens* and observed a complete reduction of harmful algae like *Nitzschia closterium*, *Skeletonema costatum*, *Anabaena flosaquae*, and *Merismopedia* spp. at a prodigiosin concentration of 5 µg/ml. Harmful blooms caused by *Microcystis aeroginosa* are a serious marine issue and Yang *et al*. (2013) isolated a prodigiosin from *Serratia marcescens*, which is found to be effective in *Microcystis*-lysis activity, by damaging the cell membrane.

2.11.6. Anticancer activity

Anticancer activity of prodigiosin specifically induces apoptosis in tumor cells, without disturbing the normal ones (Williamson *et al*., 2007). Prodigiosin specifically attacks the multidrug-resistant cells and multiple cellular targets, using different molecular mechanisms, depending on the type

of cancer (Chang *et al*., 2011). Prodigiosin causes alkalinization of organelles like lysosomes and also induces the cell cycle arrest by the acidification of the cytoplasm. Prodigiosin can also interfere with DNA, causing fragmentation, leading to cancer cell death (Melvin *et al*., 2000). Mainly prodigiosin interferes with the MAPK (mitogen-activated protein kinase) signaling pathways and leads to the apoptosis of cancer cells. Prodigiosin and its related compounds are tested over sixty cancer cell lines with a minimum inhibitory concentration of 2µM (Paul *et al*., 2020).

A study conducted by Zhao *et al*. (2020) showed that prodigiosin along with 5-fluorouracil inhibits the autophagy of colorectal cancer cell lines by employing caspase-dependent apoptosis under in vivo condition. They also found that prodigiosin interacts and repressed the autophagosome-lysosome fusion and cathepsin-dependent maturation of lysosomes in the colorectal cancer cells. El-Batel *et al*. (2017) conducted an in vitro study and reveals that prodigiosin conjugation with silver nanoparticles has potent anticancer activity against human liver cancer cells (HepG2).

2.11.7. Immunosuppressive activity

Nakamura *et al*. (1986) reported that the prodigiosin isolated from *Serratia marcescens* has the immunosuppressive activity as a selective inhibition of the polyclonal proliferation of T cells. *In vitro* and *in vivo* experiments employing prodigiosin, undecylprodigiosin at non-cytotoxic concentration found that it inhibits the murine T cell proliferation (Han *et al*., 1998). Prodigiosin hinders both T cell receptor-dependent and independent proliferation of T cells because it suppresses the lymphocytic proliferation along with the stimulation of concanavalin A, ionomycin and CD 3 (Pandey *et al*., 2007). Undecylprodigiosin showed a divergent type of action compared with the well-known immunosuppressive drugs like FK506 and cyclosporine A (Stankovic *et al*., 2014). Due to toxicity issues in effective doses, natural

prodigiosin is still not clinically used, but synthetical prodigiosin derivatives are found to be an effective immunosuppressant (Stepkowski *et al*., 2001).

2.11.8. Dyeing properties

Due to the dependence on non-renewable resources, environmental toxicity and human health issues, the usage of synthetic colorants is not that much appreciated. In such a situation, bio-colorants, especially bacterial pigments, have an appreciable position (Venil *et al*., 2013). Researches are going on and exploring the dyeing ability of prodigiosin in textile, food and cosmetic industries. Shen and Yang (2013) developed a suitable prodigiosin pigment dyeing technology and studied the thermodynamic properties of polyester dyeing with prodigiosin. Prodigiosin from *Serratia marcescens* has proved to be a colorant on different textile fabrics like cotton, silk, polyester, acrylic fiber, with minimum color fading (Ahmad *et al*., 2012). Kim and Choi, (2015) studied the dyeing properties of prodigiosin extracted from *Zooshikella rubidus* on silk fabrics and concluded that by incorporating the mordant (Al and Ti) with prodigiosin, the grade level of bio-colorant increases more than one. Some researchers applied the prodigiosin on cotton fabrics and found an excellent color tone even after continuous washing (Shahitha and Poornima, 2012). Prodigiosin from *Vibrio* sp. has dyeing ability on a wide range of fabrics like wool, nylon, acrylics, silk, and also showed excellent color stability (Alihosseini *et al*., 2008). Ryazantseva and Andreyeva (2004), tested the dyeing ability of prodigiosin on polyolefines (Polyethylene and Ultratene) and concluded that prodigiosin can be considered as a good colorant on polyethylene. Dyeing properties prodigiosin has also been tested over soap, candles, papers, and ink making processes (Shahid *et al*., 2013).

In the light of above, it is clear that phthalates are detrimental to the health of human beings and associated life. The effective role of microbes in the degradation of phthalates and the strategic influence of prodigiosin as a

secondary metabolite was also explained. Considering all these, the present study focused on the optimization of cultural parameters that influence the production of prodigiosin from *A. denitrificans* SP1 using blood bag and free DEHP as the only carbon source. Attempt has also been carried out to determine the quorum sensing mechanism on the production of prodigiosin and its *in vitro* applications.

CHAPTER 3

INFLUENCE OF LIGHT CONDITIONS FAVOURING PRODIGIOSIN PRODUCTION IN *ACHROMOBACTER DENITRIFICANS* STRAIN SP1

[Hareesh, E.S., & Harilal, C.C. (2020). Response of *Achromobacter denitrificans* SP1 towards prodigiosin production to different light conditions. ***World Journal of Microbiology and Biotechnology, Springer*** (Communicated)]

3.1. Aim: To study the effect of various light conditions towards the production of prodigiosin and to find out the suitable light source which supports maximum prodigiosin yield.

3.2. Materials and Methods

3.2.1. Chemicals

Analytical and bacteriological grade chemicals from HiMedia (India) and Merk (India) were used for the preparation of the medium for the bacterial culture and solvents for prodigiosin extraction.

3.2.2. Bacterium and Medium

Achromobacter denitrificans strain SP1 (GenBank Accession No. HQ645935 and MTCC No.5710) was employed in this study. The bacterium, *A. denitrificans* SP1 was isolated from plastic polluted sewage sludge from Canoly Canal (11.2500 N; 75.7667 E), a water body flowing through Kozhikode City, Kerala, India. This was cultured in Basal Salt Medium (BSM) containing (g/L) K_2HPO_4, 1.0; NaCl, 1.0; NH_4Cl, 0.5 and $MgSO_4$, 0.4. The initial pH was maintained at 7.2 and supplemented with DEHP as inducer come sole carbon source.

3.2.3. Light treatment

Bacterial culture plates were placed under five different light conditions like Cool white fluorescent lamp (FL, 36W. Philips make), Red, Green, Blue and White LED strips, each with 30 individual units. One set of culture plate was completely wrapped using aluminium foil and kept in dark condition to avoid light.

3.2.4. Experimental Setup

A. denitrificans SP1 was inoculated in BSM, supplemented with DEHP (2.5 mM) as the sole carbon source and cultured for 24 h in an environmental shaker and then 100µL (~3×10^8 CFU) of the cultures were spread uniformly along with DEHP (20 µL) over the surface of solid BSM in petri plates (inner diameter of 88 mm and 92.4 mm of outer lid diameter). Two sets of culture plates were prepared and one set was kept under cool, white fluorescent lamp (36W, 33 µmol m^{-2} s^{-1}) and the other set was completely under continuous darkness at an equivalent distance of 15 cm at different temperatures (20, 25, 30, 35, 40 & 45°C) for 4 days. After getting an optimum temperature, another set of culture plates were kept at different LEDs (i) red (direct light of 30 LED-63 µmol m^{-2} s^{1}), (ii) green (direct light of 30 LED-70 µmol m^{-2} s^{-1}), (iii) blue (direct light of 30 LED-53 µmol m^{-2} s^{-1}) and (iv) white (direct light of 30 LED-63 µmol m^{-2} s^{-1}) for 4 days. To provide LED treatment, each culture plate was kept in black colored chambers. **Figure 9** shows the box type black chamber setup designed to provide different LED light conditions. The direct light intensity from each LED was maintained at ~ 35 µmol m^{-2} s^{-1} (near to FLs intensity). Prodigiosin production and biomass estimation associated with each culture condition was estimated after 4 days of incubation. All the experiments were conducted in triplicates.

3.2.5. Biomass estimation

After 4 days of incubation at different light conditions, biomass was scraped off from the culture plates and the air dried biomass was weighed accurately using a digital weighing balance (Shimadzu AX200).

3.2.6. Prodigiosin extraction and quantification

The extraction of prodigiosin was carried out as reported by Pradeep *et al*. (2014). 3.0 ml of acetone was added to the collected air dried pigmented cell pellet and sonicated (output wattage 15 for 3 min) until the entire pigment was removed from the cells into the solvent and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant containing pigment was evaporated and the dry weight of prodigiosin (crude) was quantified using a digital weighing balance (Shimadzu AX200).

3.2.7. Statistical analysis

All the experiments were carried out in triplicates. Results were calculated as mean ± standard error. Statistical Package for the Social Sciences (SPSS version 16) for windows was employed throughout the study.

Figure 9. Box type-black chamber setup for providing different LED light conditions

3.3. Results

3.3.1. Prodigiosin production and biomass formation in *A. denitrificans* SP1 at different temperatures under light and dark conditions.

In the present study, prodigiosin production and biomass formation of *A. denitrificans* SP1 at different temperatures under light (fluorescent lamp) and dark conditions were assessed. **Figure 10** and **Table 5** shows the production of prodigiosin at different temperatures and **Figure 11** and **Table 6** depicts the formation of biomass at different temperatures under light and dark conditions. Culture plates under both light and dark conditions showed maximum prodigiosin and biomass formation at 25 °C. Maximum prodigiosin production of 25.4 mg was observed under the fluorescent lamp and 10.1 mg was noted in culture plates kept under complete dark conditions. The lowest prodigiosin production observed under the fluorescent lamp and dark condition was 0.3 mg and 0.2 mg, respectively at 45 °C. Maximum biomass formation of 210.7 mg was observed under the fluorescent lamp and 200.5 mg was recorded on culture plates under dark condition. The lowest biomass formation observed under the fluorescent lamp and dark condition was 10.1 mg and 8.3 mg respectively at 45 °C. A drastic decrease in prodigiosin production and biomass formation was noticed above 35 °C in culture plates under both fluorescent and dark conditions. From **figure 12,** it is clear that the culture plates under the fluorescent lamp at 25 °C provided a better environment for prodigiosin production. **Figures 12** and **13** show the visible color difference in culture plates at different temperatures under fluorescent light and complete dark conditions.

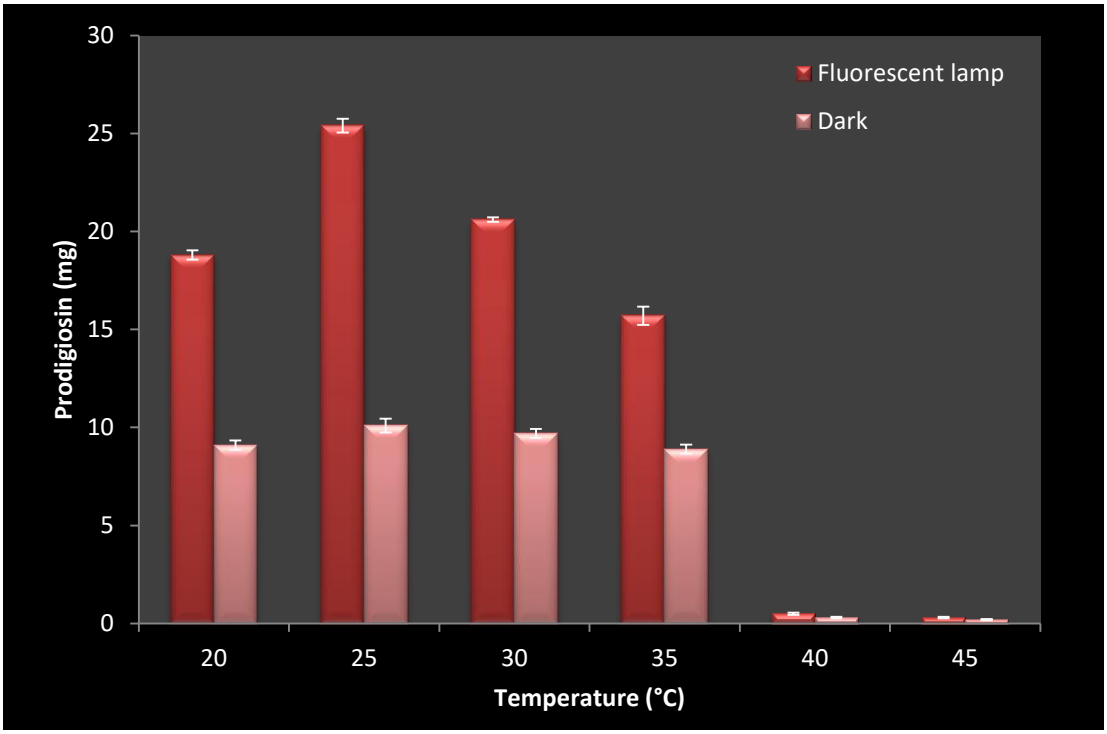

Figure 10. Prodigiosin production by *A. denitrificans* SP1 at different temperatures under fluorescent light and complete dark conditions.

Table 5. Prodigiosin production by *A. denitrificans* SP1 at different temperatures under fluorescent lamp and complete dark conditions.

Temperature (°C)	Prodigiosin (mg)	
	Fluorescent lamp	Dark
20	18.8 ±0.230	9.1 ±0.230
25	25.4 ±0.346	10.1 ±0.346
30	20.6 ±0.115	9.7 ±0.230
35	15.7 ±0.461	8.9 ±0.230
40	0.5 ±0.057	0.3 ±0.033
45	0.3 ±0.033	0.2 ±0.033

Note: Values are mean of three replications (±SE)

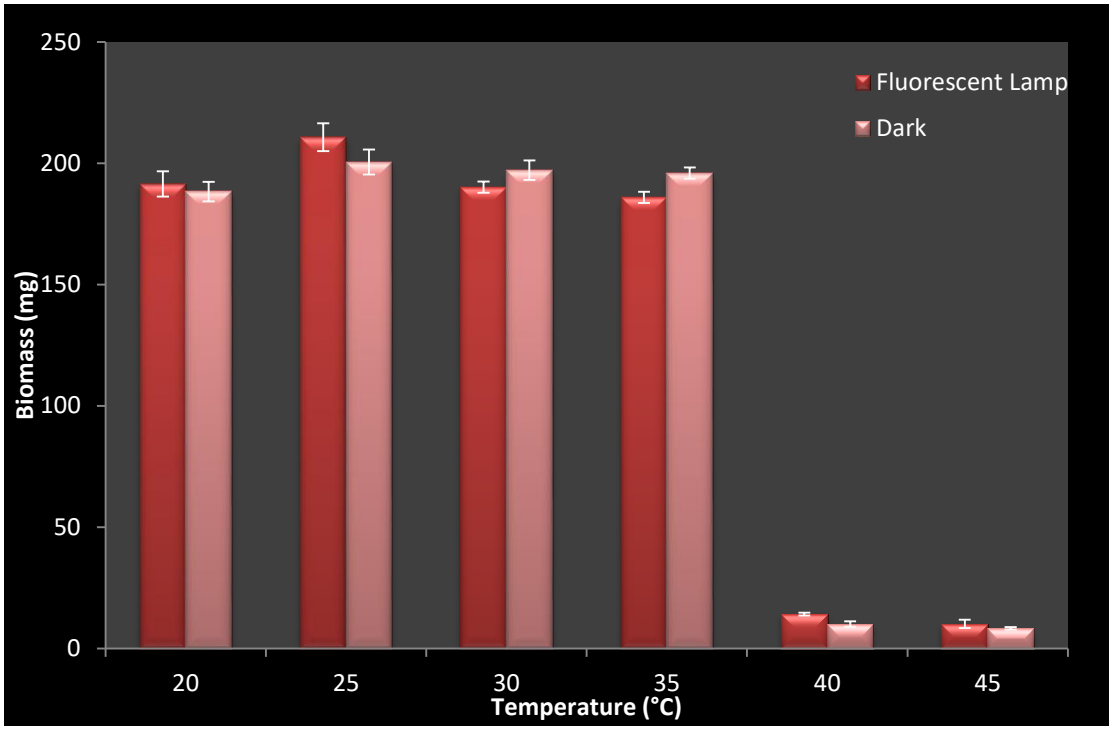

Figure 11. Biomass production by *A. denitrificans* SP1 at different temperatures under fluorescent light and complete dark conditions.

Table 6. Biomass formation by *A. denitrificans* SP1 at different temperatures under fluorescent light and complete dark conditions.

Temperature (°C)	Biomass (mg)	
	Fluorescent lamp	Dark
20	191.5 ±5.19	188.3 ±4.04
25	210.7 ±5.77	200.5 ±5.19
30	190.2 ±2.30	197.1 ±4.04
35	185.9 ±2.30	195.9 ±2.30
40	14.2 ±0.57	10.1 ±1.15
45	10.1 ±1.73	8.3 ±0.57

Note: Values are mean of three replications (±SE)

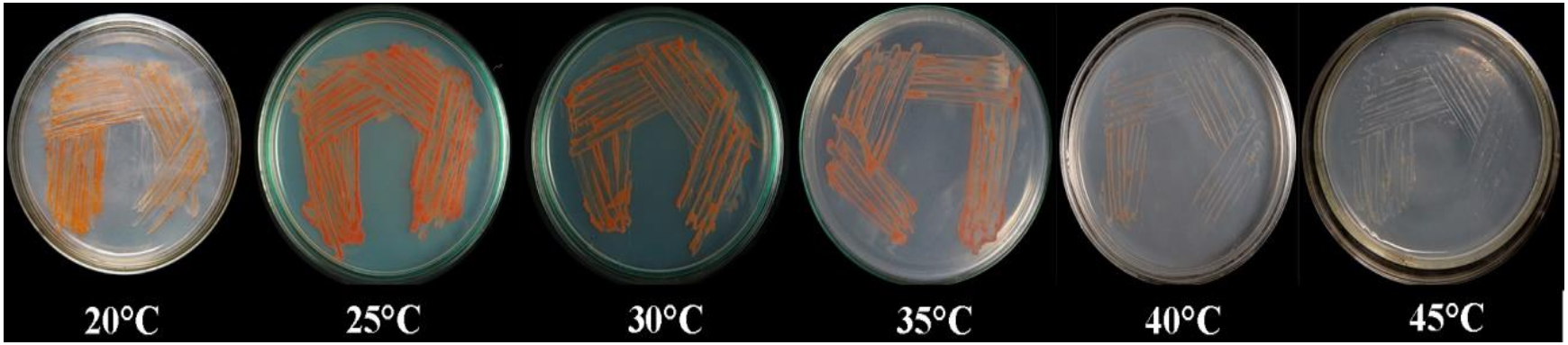

Figure 12. Culture plates of *A. denitrificans* SP1 kept under fluorescent lamp at various temperatures.

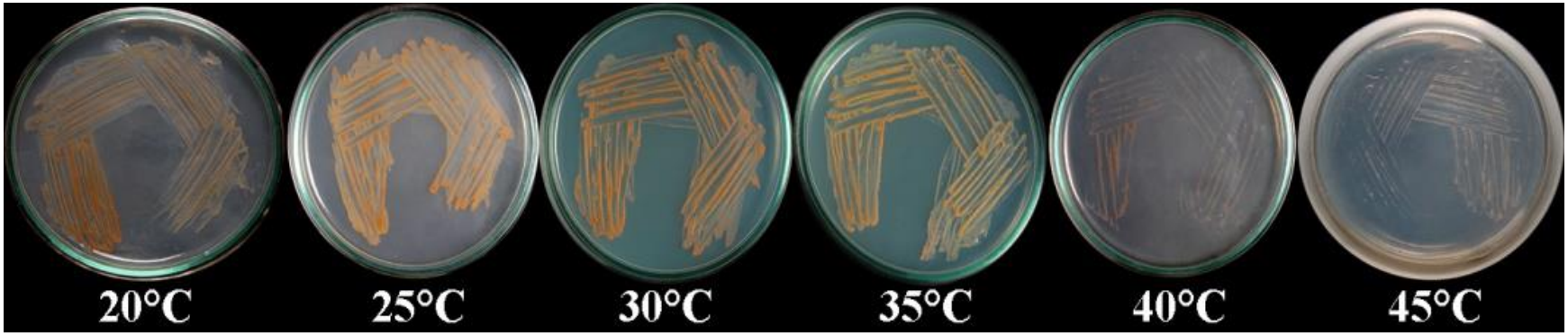

Figure 13. Culture plates of *A. denitrificans* SP1 kept under complete darkness at various temperatures.

3.3.2. Prodigiosin production and biomass formation of *A. denitrificans* SP1 under various LED light sources.

After selecting 25 °C as an optimum temperature for the growth of *A. denitrificans*, culture plates were kept under different LED sources like red, green, blue, and white, for a period of 4 days. Among the LEDs, culture plates under red contributed maximum prodigiosin production (20.9 mg) and biomass formation (204.7 mg) (**Figures 14 & 15)**. The lowest production of prodigiosin (0.2 mg) and biomass formation (9.1 mg) was noted in culture plates under white LEDs. All the four LEDs showed limited production of prodigiosin (below 3 mg) expect the red LED (**Table 7)**. **Figure 16** shows the difference in culture plates under various LED light sources.

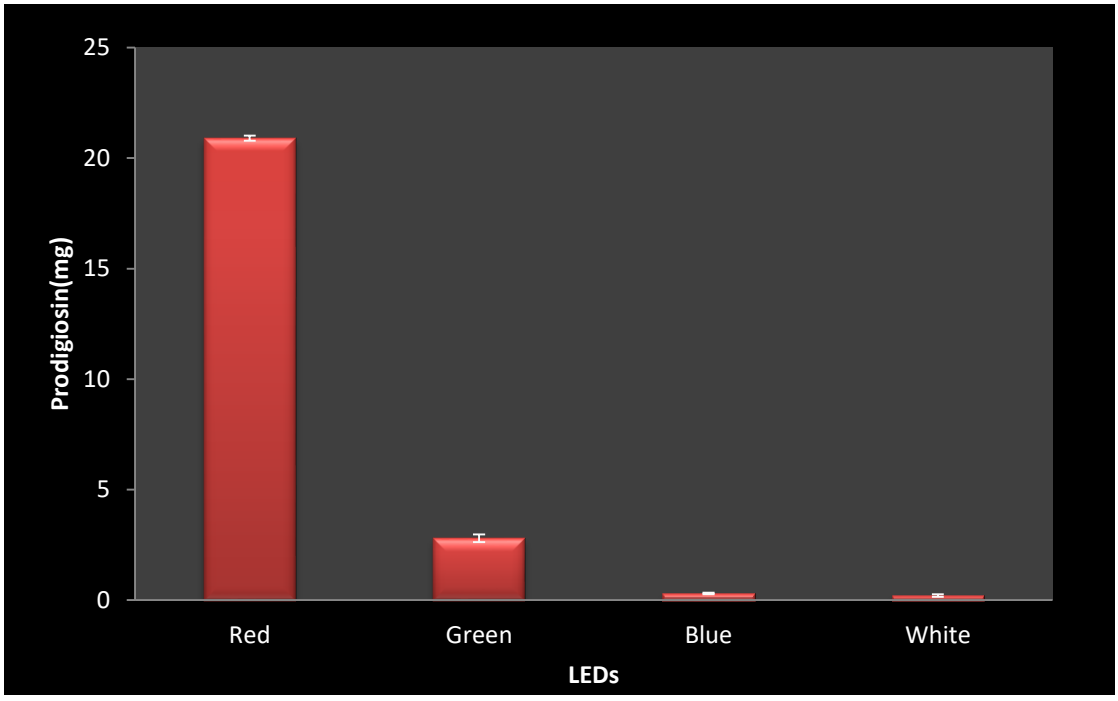

Figure 14. Prodigiosin production by *A. denitrificans* SP1 under different LED light sources

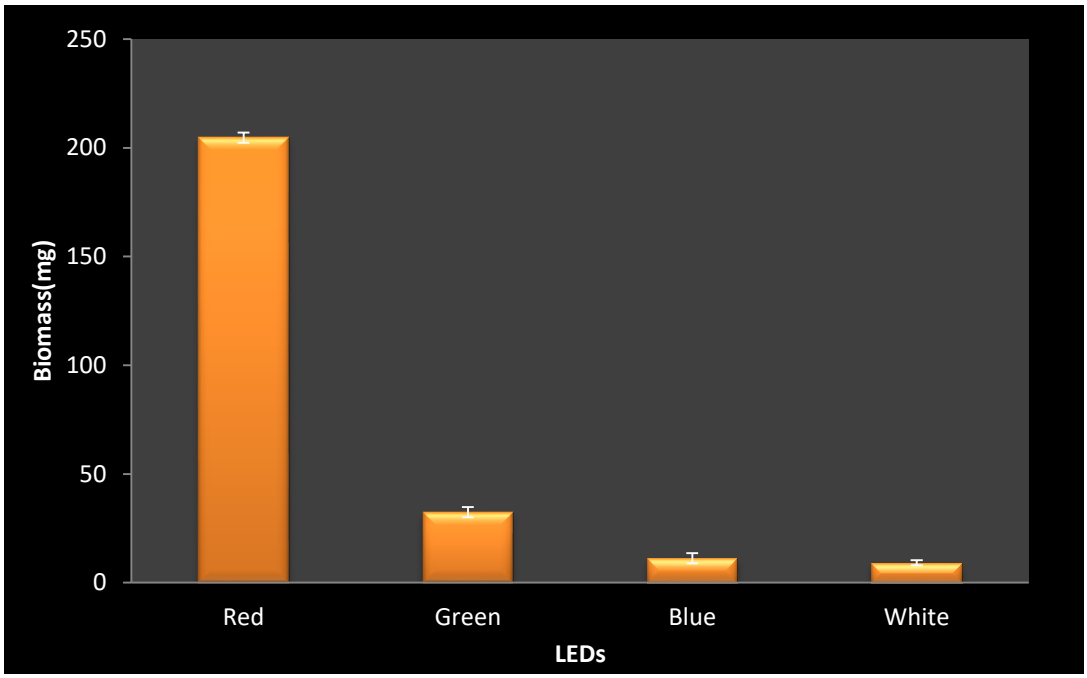

Figure 15. Biomass production by *A. denitrificans* SP1 under different LED light sources

Table 7. Prodigiosin and biomass production by *A. denitrificans* SP1 under different LED light sources .

LEDs	Prodigiosin (mg)	Biomass (mg)
Red	20.9 ±0.115	204.7 ±2.30
Green	2.8 ±0.173	32.4 ±2.30
Blue	0.3 ±0.033	11.2 ±2.3
White	0.2 ±0.057	9.1 ±1.15

Note: Values are mean of three replications (±SE)

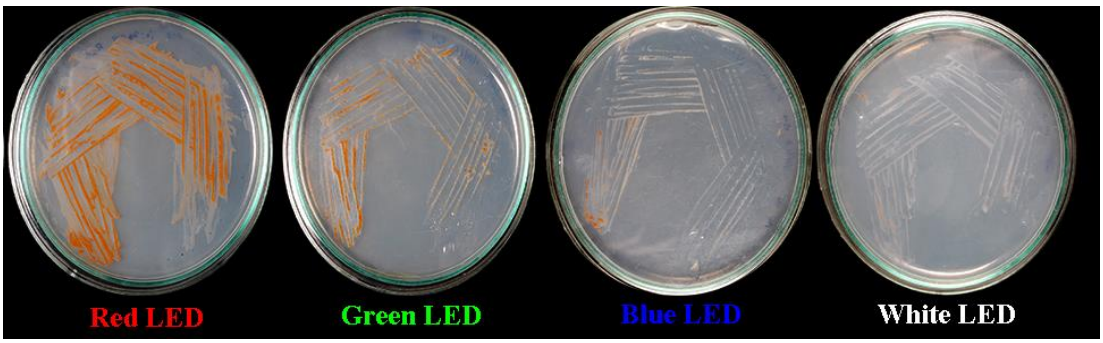

Figure 16. Culture plates under various LED light sources.

3.4. Discussion

The bacterium, *A. denitrificans* SP1 (GenBank accession no. HQ645935 and MTCC no. 5710) employed throughout this study was isolated from the heavily plastic polluted sewage sludge of canoly canal, Kozhikode city, Kerala. *A. denitrificans* SP1 is an efficient degrader of poisonous phthalates (mainly DEHP) and the use of DEHP in the culture medium as a carbon source imparted stress to the bacteria and result in the production of a 25-carbon prodigiosin. As a bacterial secondary metabolite, prodigiosin is appearing in the stationary phase of bacterial growth. The exact role of prodigiosin in the producer organism is still a debated one. As stated in various literature, secondary metabolites (pigments) produced by bacteria is influenced by a lot of physiological and multiple environmental factors like temperature, light, pH, oxygen supply, nutrient stress, ionic strength, etc. (Williamson *et al*., 2006).

The present study discuss about the influence of light on prodigiosin production in *A. denitrificans* SP1. The bacterium, *Serratia marcescens* was extensively studied and largely exploited for the production of prodigiosin (Elkenawy *et al*., 2017). Majority of the microorganisms, including *Serratia*, which produces prodigiosin is a 20 carbon compound. These 20 carbon prodigiosins are not stable under light conditions (Someya *et al*., 2004**)**. *A. denitrificans* SP1 is an interesting candidate in this regard, as it produces a rare 25 carbon prodigiosin, which is insensitive to light. Due to these interesting reasons, an attempt has been carried out to study the influence of different light sources on the production of prodigiosin and to find out the most suitable light source, which maximizes its production in *A. denitrificans* SP1. Only limited studies are reported on the influence of light on prodigiosin production.

Among various temperatures studied, cultures of *A. denitrificans* SP1 kept under 25 °C have the maximum production of biomass and prodigiosin. Several scientific studies reported that a temperature below 30 °C is ideal for prodigiosin production (Gulani *et al*., 2012; Sumathi *et al*., 2014). Here the influence of temperature on prodigiosin production is not discussed in detail as a dedicated statistical optimization section on growth parameters is depicted in Chapter 4. Among the six light sources (including dark condition), culture plates under fluorescent lamp showed maximum prodigiosin (25.4 mg) and biomass (210 mg) production at 25 °C (**Figure 10 and Table 5**). The light sources which supports maximum prodigiosin production was in the order of Fluorescent lamp>Red LED>Dark>Green LED>Blue LED>White LED. Also a drastic decrease in prodigiosin production in cultures under light and dark, kept above 30°C was noticed (**Tables 5 and 6**). The nature and magnitude of prodigiosin produced are evident in **Figures 12, 13, and 16**. Compared to other light sources, an enhancement in biomass production has been noticed in dark condition at a temperature ranging from 30 and 35°C. Ryazantseva *et al*. (2012) studied the response of *Serratia marcescens* 9986S to varying levels of illumination and resultant prodiosin production. As per reports, a higher level of prodigiosin production was noticed in 24h of light cultures and after 48h, the rate of pigment accumulation decreased as compared to the cultures kept in the dark. Studies on *Serratia marcescens* TKU011 showed that under continuous lighting of 2 days, during the exponential growth phase, prodigiosin production was slightly higher than that in the dark (Wang *et al*., 2012). In the present study, *A. denitrificans* SP1 produced high prodigiosin under fluorescent lamp, than dark conditions, after 4 days of incubation.

Andreyeva and Ogorodnikora (2015), studied the photosensitivity of prodigiosin and also explained how pigment-protein interactions influence the light sensitivity in *Serratia marcescens*. They showed that the production of

prodigiosin under light condition is very low, compared to dark. Here, in the present study, 25-C prodigiosin is insensitive to light condition and it may be due to the native protein that bounds with the prodigiosin in *A. denitrificans* SP1. Among the LEDs, red showed the maximum production of prodigiosin (20.9 mg) and biomass (204.7 mg), but other LEDs like green, blue and white decreased the production of prodigiosin to 2.8, 0.3, and 0.2 mg, respectively (**Table 7**). Someya *et al*. (2004) studied the effect of light conditions on prodigiosin stability in *Serratia marcescens* strain B2. They incubated the culture plates at 25 °C under continuous dark, white (using fluorescent lamp), blue, red, and far-red light (LED). Prodigiosin production in *Serratia marcescens* strain B2 was inhibited by the white and blue light sources, but red and far-red LED light sources enhanced the production and showed a large amount of prodigiosin accumulation. These data are in agreement with the present results, that the red LED provides a better environment than other LEDs. In *A. denitrificans* SP1, white LED showed minimum prodigiosin and biomass production (**Table 7**). Like all other *Serratia*, strain B2 also produced maximum prodigiosin under dark conditions.

Some unique results are also reported in *Serratia* sp. regarding the photosensitivity of prodigiosin. Phatake *et al*. (2016) reported a *Serratia* sp. which is capable of producing prodigiosin under white light (CFL). They used gelatin papers for providing different colored wavelengths. According to them, maximum prodigiosin and biomass production was observed in white light, followed by blue and yellow light and comparatively less yield in black, red, and green light. According to Wang *et al*. (2013), *Serratia marcescens* y2 produces maximum prodigiosin under dark conditions. They used colored glass papers for providing red, yellow, blue, and green wavelengths of light and the source of light used is a white fluorescent lamp. They also concluded that white light reduces the prodigiosin production and among the colored

lights, red showed maximum and green showed minimum prodigiosin production.

3.5. Conclusions

The present study reveals the effects of different light sources on to the production of prodigiosin from DEHP degrading novel bacterium *A. denitrificans* SP1 (MTCC 5710). Among the different light sources studied (white fluorescent lamp, red, green, blue, white LEDs, and dark conditions), cultures under white fluorescent lamp showed maximum prodigiosin (25.4 mg) and biomass (210.7 mg) at 25 °C. Among the LEDs, red showed maximum prodigiosin (20.9 mg) and biomass (204.7 mg) yield. Cultures incubated under red LEDs at 25 °C showed a quick response in prodigiosin productivity, compared to other light sources. These results revealed that both fluorescent lamp and red LEDs at 25 °C play a crucial role in maximizing the biosynthesis of prodigiosin in *A. denitrificans* SP1.

CHAPTER 4

RESPONSE SURFACE OPTIMIZATION OF PRODIGIOSIN PRODUCTION FROM *ACHROMOBACTER DENITRIFICANS* STRAIN SP1 USING BLOOD BAG AS THE CARBON SOURCE

[Hareesh, E.S., Harilal, C.C., & Pradeep, S. (2020). Response surface optimization of prodigiosin production by phthalate degrading *Achromobacter denitrificans* SP1 and exploring its antibacterial activity. ***Preparative Biochemistry and Biotechnology, Taylor & Francis***, 50(6), 564-571. (Published)]

4.1. Aim: To apply response surface methodology to optimize the growth parameters for the production of prodigiosin from *A. denitrificans* SP1 using blood bag as the only carbon source.

4.2. Materials and Methods

4.2.1. Chemicals

Analytical and bacteriological grade chemicals from Merk (India) and HiMedia (India) were used for the preparation of the medium for the bacterial culture and solvents for pigment extraction.

4.2.2. Bacterium and Medium

Achromobacter denitrificans strain SP1 (GenBank Accession No. HQ645935 and MTCC No.5710) was employed in this study. The bacterium, *A. denitrificans* SP1 was isolated from plastic polluted sewage sludge from Canoly Canal; flowing through Kozhikode City, Kerala, India (11.2500 N; 75.7667 E). This was cultured in a simple basal salt medium (BSM) containing (g/L) K_2HPO_4, 1.0; NaCl, 1.0; NH_4Cl, 0.5 and $MgSO_4$, 0.4 (initial pH, 7.2). This was supplemented with DEHP blended Blood Bag (BB) (*in situ* cultivation). Commercially available HL haemopack BB (Batch No. HO

30419B), manufactured by the Hindustan Latex Ltd., Thiruvananthapuram, India, was used as model PVC plastics. This commercial product was plasticized with 33.5% (w/w) DEHP (Sarath Josh *et al*., 2012). Intact pieces of BB (~ 10 mm^2) were used throughout the study.

4.2.3. Experimental Setup

Initially, the bacterium was cultured in BSM supplemented with 2.5 mM DEHP on a 2% agar plate (incubated for 3 days), and a loop full of cells were taken for further inoculation. *A. denitrificans* strain SP1 inoculum (100 µl; ~ 3×10^8 CFU) was cultured in 10 ml of BSM and incubated in a temperature-controlled shaker, has set at different conditions as described in **Tables 8 and 9**. Prodigiosin production was estimated according to the trials predicted by the RSM software. All the experiments were conducted in triplicates.

4.2.4. Prodigiosin extraction and quantification

The prodigiosin extraction was done as follows. The pigmented cell pellet was harvested after the required time period by centrifugation (10,000 rpm for 10 min at 4°C). 3ml acetone was added to the collected pellet, sonicated (output wattage 15 for 3 min) until the entire pigment was removed from the cells into the solvent and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant containing pigment was evaporated and the dry weight of prodigiosin (crude) was quantified. **Figure 19** shows the eppendorf tube containing prodigiosin after acetone vaporization.

4.2.5. Statistical optimization for prodigiosin production

4.2.5.1. Plackett-Burman experimental design

Five independent variables were selected for the initial screening studies; i.e., temperature (20 & 30 **°C)**, pH (4 & 10), agitation (100 & 200 rpm), Blood bag (1 & 5 g) and incubation time (10 & 15 days). Twenty different

combinations were generated to estimate the combined effects of these parameters on prodigiosin production (**Table 8**). Minitab version 17 was used to generate the experimental design with high (+1) and low (-1) levels for each variable selected.

Table 8. Plackett-Burman experimental design for the production of prodigiosin from *A. denitrificans* SP1. The parameters selected were temperature (20 and 30 °C); pH (4 and 10); agitation (100 and 200 rpm); blood bag concentration (1 and 5 g); incubation time (10 and 15 days).

Run order	Temperature (°C)	pH	Agitation (rpm)	Blood bag (g)	Incubation (days)
1	30	10	100	1	10
2	30	10	100	5	15
3	30	4	100	1	10
4	20	4	100	1	15
5	20	4	200	5	10
6	30	10	200	1	10
7	20	10	200	1	15
8	20	10	100	5	10
9	30	4	100	5	15
10	30	10	200	5	10
11	30	10	100	1	15
12	30	4	200	5	10
13	30	4	200	1	15
14	20	10	100	5	15
15	20	10	200	5	15
16	20	4	100	5	10
17	20	4	200	1	15
18	30	4	200	5	15
19	20	10	200	1	10
20	20	4	100	1	10

4.2.5.2. Box-Behnken model and response surface methodology

Based on the results of Plackett-Burman experiment, three parameters (pH of the medium, Blood bag concentration and temperature) were found significant for the production of prodigiosin by *A. denitrificans* SP1. Employing these three parameters, different combinations of experiments were designed

(**Table 9**). Each variable was analyzed at low (-1), medium (0) and high (+1) levels, and the results are analyzed by applying second order polynomial equation.

4.2.5.3. Validation Experiments

Validation experiments for the quadratic model was conducted as predicted by the point prediction software Minitab 17 (**Table 10**). The difference between the estimated and predicted values of prodigiosin production was compared.

Table 9. Box-Behnken design for the production of prodigiosin from *A. denitrificans* SP1. The parameters selected were pH of the medium (6.5, 8.5 and 10.5); temperature (22, 25 and 28 °C); blood bag concentration (0.5, 1 and 1.5 g).

Run order	pH	Temperature (°C)	Blood bag (g)
1	10.5	22	1
2	10.5	28	1
3	8.5	28	1.5
4	8.5	22	0.5
5	6.5	22	1
6	6.5	25	1.5
7	8.5	25	1
8	6.5	25	0.5
9	10.5	25	0.5
10	10.5	25	1.5
11	8.5	22	1.5
12	6.5	28	1
13	8.5	28	0.5

Table 10. Validation of experimental design for the production of prodigiosin from *A. denitrificans* SP1.

Sl. No.	pH	Temperature (°C)	Blood Bag (g)
1	8.8	24	1
2	8.5	22	1
3	10.5	25	1
4	10.5	28	1.5

4.3. Results

4.3.1. Statistical optimization of prodigiosin production

The results of Plakett-Burman experimental design are given in **Table 11**. The maximum prodigiosin production (0.79 mg/ml) was attained at 20 °C, pH 10, 200 rpm, 1 g (Blood bag) and 15 days of incubation, while the minimum production (0.04 mg/ml) was observed at 6 trials and are given in **Table 11**. Based on the results of Plackett-Burman experimental design, a pareto chart was constructed (**Figure 20**). The effects of the standardized parameters (Temperature, pH, Agitation, Blood bag concentration, Incubation time) on prodigiosin production were estimated at 5% level of significance. **Figure 17** shows the growth pattern of *A. denitrificans* SP1 on blood bag tubings in basal salt medium.

Table 11. Results of Plackett-Burman experimental design for the production of prodigiosin by *A. denitrificans* strain SP1.

Run order	Temperature (°C)	pH	Agitation (rpm)	Blood bag (g)	Incubation (days)	Prodigiosin (mg/ml)
1	30	10	100	1	10	0.15
2	30	10	100	5	15	0.04
3	30	4	100	1	10	0.04
4	20	4	100	1	15	0.08
5	20	4	200	5	10	0.05
6	30	10	200	1	10	0.15
7	20	10	200	1	15	0.79
8	20	10	100	5	10	0.08
9	30	4	100	5	15	0.04
10	30	10	200	5	10	0.04
11	30	10	100	1	15	0.16
12	30	4	200	5	10	0.04
13	30	4	200	1	15	0.04
14	20	10	100	5	15	0.14
15	20	10	200	5	15	0.13
16	20	4	100	5	10	0.05
17	20	4	200	1	15	0.08
18	30	4	200	5	15	0.04
19	20	10	200	1	10	0.77
20	20	4	100	1	10	0.07

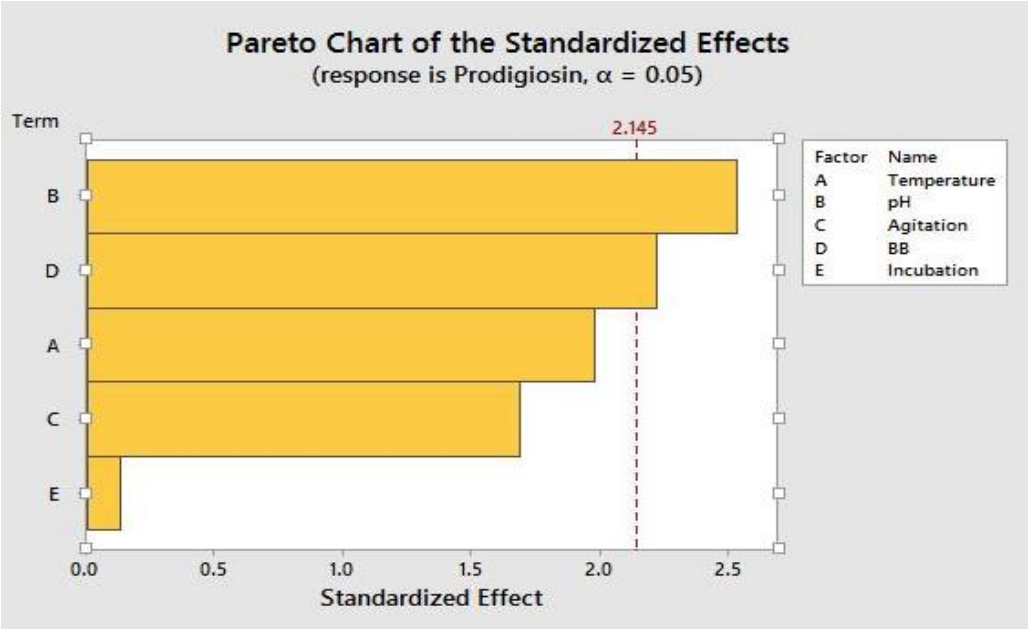

Figure 20. Pareto chart showing the standardized effects on the production of prodigiosin by *A.denitrificans* strain SP1. The parameters found relevant were in the order: pH> Blood bag> Temperature, while agitation and incubation were found to have negligible effects.

From the pareto chart, three highly influential parameters (pH, Blood bag, Temperature) were selected. Eventually, these three parameters were considered for Box-Behnken experimental design by RSM to identify the optimized conditions for the maximum production of prodigiosin by *A. denitrificans* SP1. Regression coefficient was estimated for the production of prodigiosin (Prodigiosin versus Blood bag concentration, pH and Temperature) (**Table 12**). The regression model for the production of prodigiosin was highly significant ($p < 0.05$) with an acceptable value of determination coefficient ($R^2 = 99.33\%$). **Figure 18** shows the growth of *A. denitrificans* SP1 on the pieces of blood bag after 15 days of incubation.

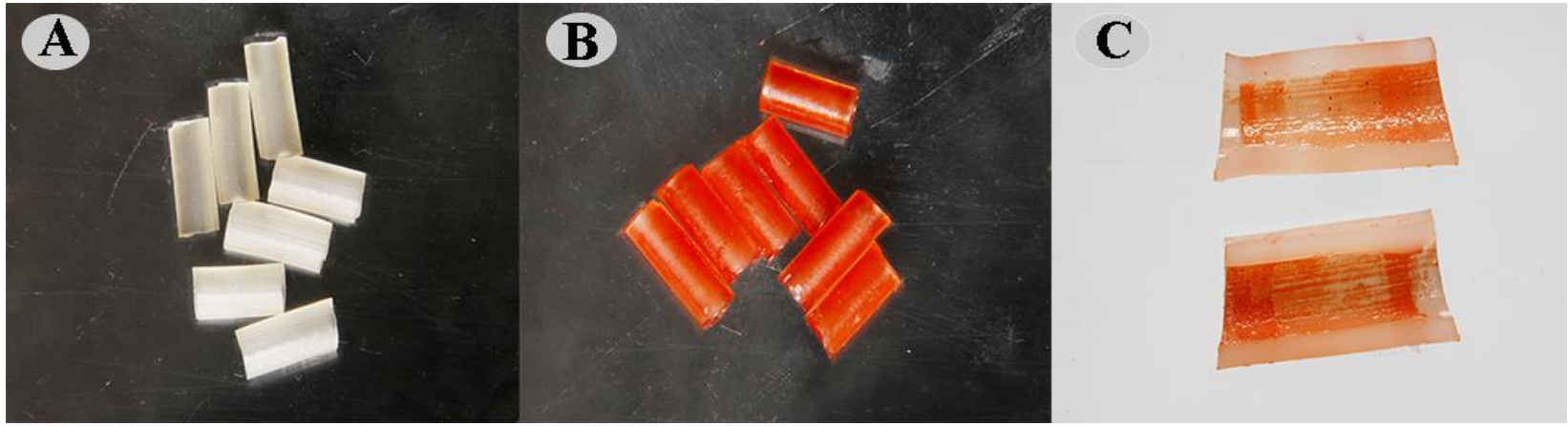

Figure 17. *A. denitrificans* SP1 growing inside the blood bag tubings. **A)** Blood bag tubings before inoculating *A. denitrificans* SP1**. B&C)** Blood bag tubings after 15 days of incubation.

Figure 18 *A. denitrificans* SP1 growing on the pieces of blood bag (1 g) in flask containing basal salt medium (Digital image on 15th day)

Figure 19. Prodigiosin after acetone vaporization

The regression equation obtained for prodigiosin production was:

$Y = -2.340 + 0.3802X_1 + 0.0593X_2 + 0.1788X_3 – 0.01991X_1^2 – 0.001012X_2^2 – 0.0699X_3^2 – 0.001167X_1X_2 – 0.00240X_1X_3 – 0.00062X_2X_3$; where X1, X2, X3 are pH, temperature and blood bag, respectively.

The quadratic regression equation was used to generate two dimensional contour as well as three dimensional surface plots to depict the interactive effects of the selected parameters **(Figures 21, 22 & 23)**.

Table 12. Results of Box-Behnken design for the production of prodigiosin by *A. denitrificans* strain SP1.

Run order	pH	Temperature (°C)	Blood bag (g)	Observed prodigiosin (mg/ml)	Predicted prodigiosin (mg/ml)
1	10.5	22	1	0.780	0.736
2	10.5	28	1	0.490	0.487
3	8.5	28	1.5	1.030	0.982
4	8.5	22	0.5	1.010	1.058
5	6.5	22	1	0.185	0.187
6	6.5	25	1.5	0.156	0.160
7	8.5	25	1	1.295	1.295
8	6.5	25	0.5	0.130	0.79
9	10.5	25	0.5	0.540	0.535
10	10.5	25	1.5	0.470	0.520
11	8.5	22	1.5	1.117	1.109
12	6.5	28	1	0.175	0.218
13	8.5	28	0.5	0.960	0.967

Three parameters (pH, temperature, blood bag) selected from the validation experiments falling within the range determined from Box-Behnken were assessed in detail for the fitness of the model. The summary of the analysis of variance (ANOVA) for the selected quadratic model is shown in **Table 13**.

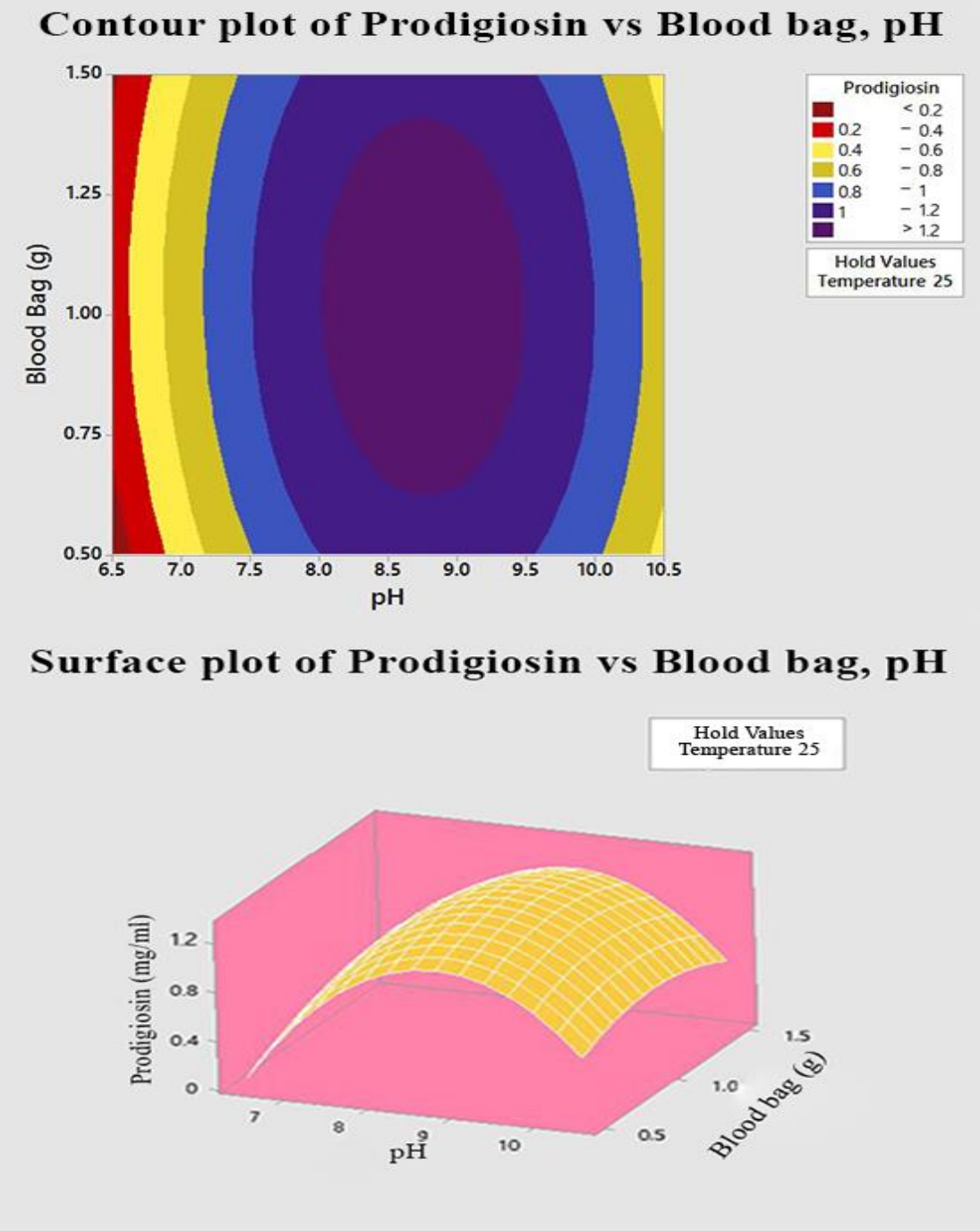

Figure 21. Contour and three dimensional surface plot showing the prodigiosin production *vs* blood bag (g), pH. Here the temperature (25 °C) was kept constant.

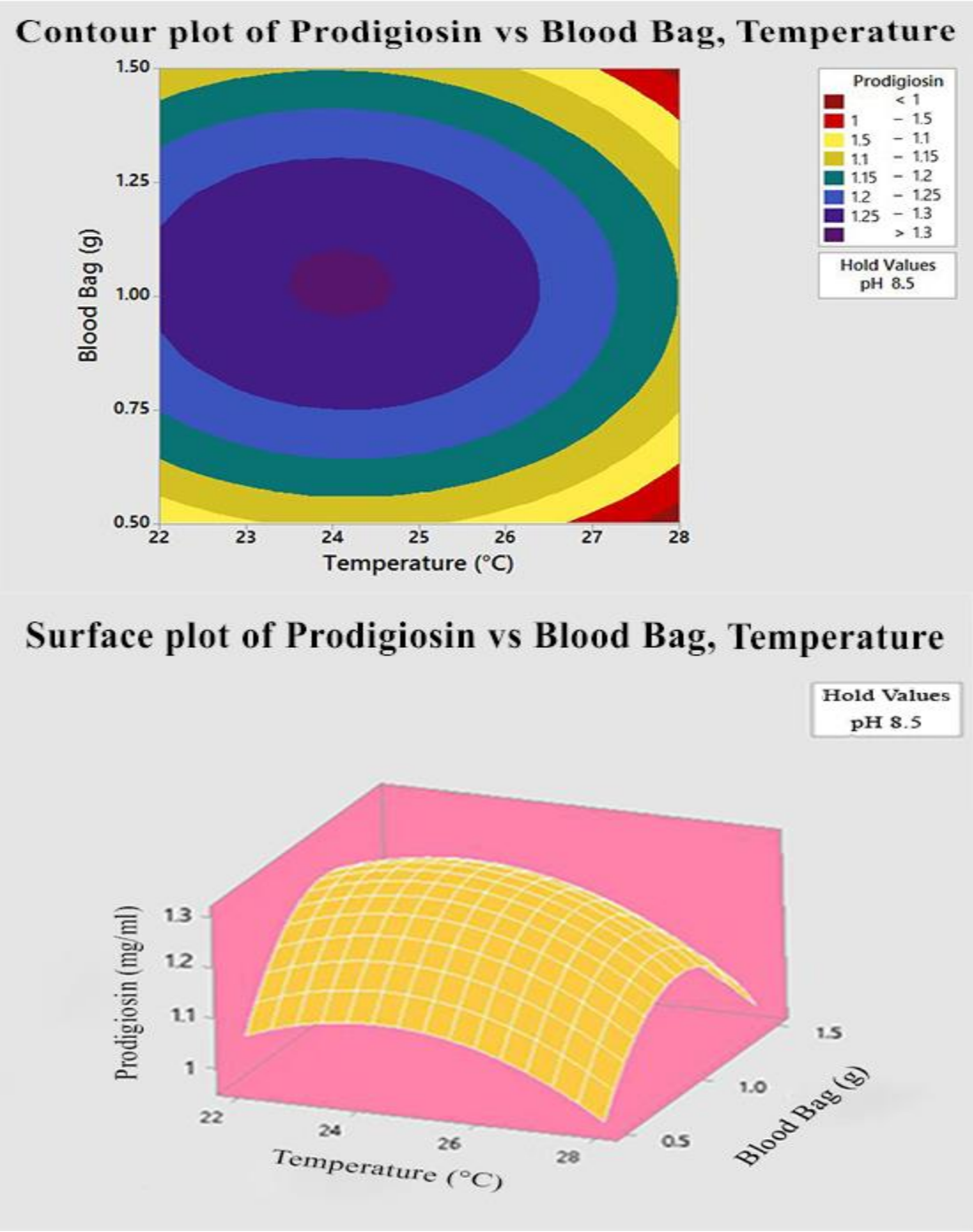

Figure 22. Contour and three dimensional surface plot showing the prodigiosin production *vs* blood bag (g), temperature (°C). Here the pH 8.5 was kept constant.

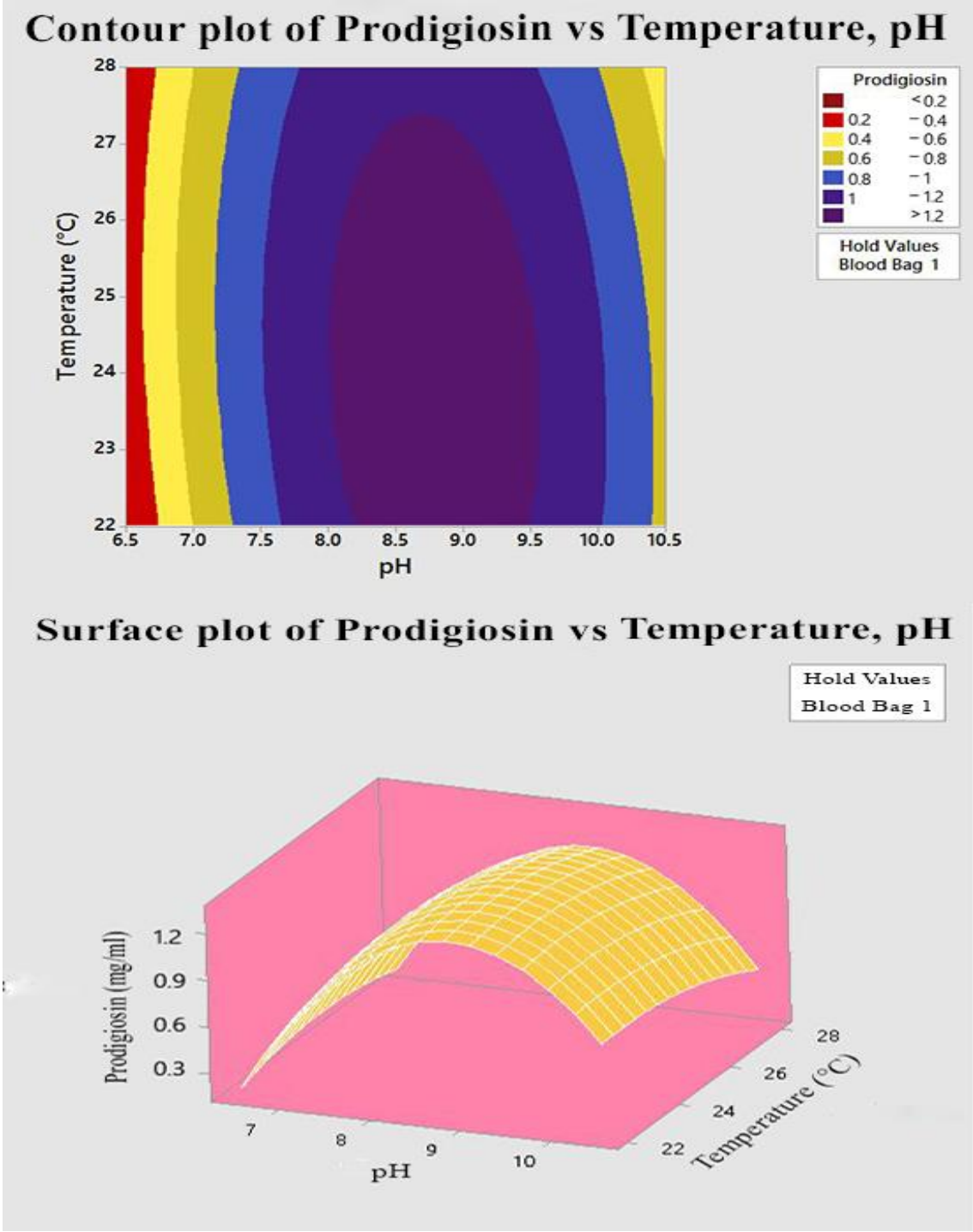

Figure 23. Contour and three dimensional surface plot showing the prodigiosin production *vs* temperature (°C), pH. Here the blood bag concentration 1 g was kept constant.

Table 13. Analysis of variance (ANOVA) for the fitted quadratic model of prodigiosin production

Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Regression	9	2.03278	0.22586	49.28	0.000
Linear	3	0.35983	0.11994	26.17	0.000
Square	3	1.65071	0.55024	120.05	0.000
Interaction	3	0.02225	0.00742	1.62	0.003
Error	3	0.01375	0.00458		
Total	12	2.04654			

Table 14. Validation experiments

Sl. No.	**pH**	**Temperature (°C)**	**Blood Bag (g)**	**Prodigiosin (mg/ml) Observed**	**Prodigiosin (mg/ml) Predicted**
1	8.8	24	1	1.314	1.31962
2	8.5	22	1	1.277	1.26178
3	10.5	25	1	0.654	0.70206
4	10.5	28	1.5	0.451	0.29588

Based on these results, the model was utilized to generate response surfaces for the analysis of the variable effects on the production of prodigiosin. The correlation coefficient of the result was 0.987 **(Table 14),** which was in good agreement with the predicted and experimental values. The optimum prodigiosin production was 1.314 mg/ml at 24 °C, pH 8.8 and 1.0 g (BB).

4.4. Discussion

A. denitrificans SP1 (MTCC No. 5710) is a promising phthalate degrading bacterium, which was from plastic contaminated sewage sludge. This bacterium utilizes the hazardous DEHP, both *in situ* (blended in PVC blood bag) and *ex situ*

(in liquid form), supplemented in the BSM as the only carbon source and energy and produce 25-C prodigiosin as a by-product (Pradeep *et al*., 2015). Like many secondary metabolites, prodigiosin biosynthesis is dependent on the growth phase, having maximum production when the cell enters the stationary phase. As with various secondary metabolites, the true physiological role of prodigiosin in the producer organisms is quite disputed. Prodigiosin synthesis responds to multiple environmental and physiological cues, which can include temperature, pH, light, and ionic strength (Bennett and Bentley, 2000). This is the first report, which gives a detailed account of the application of RSM for prodigiosin production from a phthalate degrading bacterium.

Plackett-Burman, followed by Box-Behnken are widely employed in the field of optimization of pigment production parameters, as they evaluate the interactive effects of physico-chemical parameters, which are being ignored in the conventional *one-at-a-time* strategy (Hareesh *et al*., 2016). Statistical designs demand the least number of experimental trials and generally less laborious and time-saving (Karimifard and Moghaddam, 2018). For reducing the number of process parameters and to find out the most influencing factors, Plackett-Burman experimental design was attempted. It generated 20 different combinations of the five parameters attempted (Temperature, pH, Blood bag, Agitation, Incubation time). For each variable, high and low levels were fixed, according to previous preliminary screening. From the Plakett-Burman results, it is well understood that pH, blood bag and temperature are the most influencing factors in the production of prodigiosin.

The pH of the medium has been reported to play a crucial role in the synthesis of secondary metabolites and therefore regulates the biosynthesis of prodigiosin (Gulani *et al*., 2012). Pareto chart also showed pH as the most influencing factor.

Here, in the present study, low pH level was set at 4 and high at 10 and maximum production was observed at 10. This gives support to the earlier findings that acidic pH below 3.0 and alkaline pH above 10 prevents pigmentation (Sumathi *et al*., 2014). Gulani *et al*. (2012) reported that alkaline pH supported the maximum production of prodigiosin.

In Plackett-Burman, 5.0 g blood bag was selected as high and 1.0g as low level. From the Plackett-Burman results, it is understood that low level (1.0g) prefer pigment production. Supplementation of 5.0 g blood bag resulted in a higher level DEHP leach out to the medium, which may leads to a stress for the log phase and finally went to a decrease in prodigiosin production.

In the case of temperature, high level is selected as 30 °C and low level as 20 °C. Many reports from S*erratia* sp. stated that the production of prodigiosin is highly sensitive to temperature (Suryawanshi *et al*., 2014). Previous reports have shown that the temperature required for the production of prodigiosin varied from 20 to 27°C with a significant reduction at higher temperatures (Giri *et al*., 2004). From the Plakett-Burman results, it is clear that the strain used in this work prefer the lower temperatures (maximum production at 20 °C). Applying the Plackett-Burman design reduced the number of parameters from five to three; thereby reduced the experimental trails needed for RSM analysis.

Most influencing factors like temperature, pH, and blood bag concentration were taken into consideration in Box-Behnken design. The R^2 value was 0.99 – close to unity, which indicates the accuracy of the model. The validation data were statistically analyzed, so as to correlate the observed and predicted values. In all these cases, model prediction was in good agreement with the experimental data, and correlation coefficient was found to be 0.98, suggesting the significance of

the model. The optimum prodigiosin production was found to be 1.314 mg/ml culture medium i.e., 1314 mg/L at 24 °C, pH of 8.8, and blood bag supplementation of 1.0 g). Sole *et al*. (1994) reported the highest yield of prodigiosin in *Serratia* sp., when the pH of the medium was about 8-8.5. Giri *et al*. (2004) optimized the temperature as 25°C for the maximum production of prodigiosin from *Serratia marcescens*. These observations showed strong agreement with the statistical findings of the present work. Su *et al*. (2011) reported 2423 mg/L prodigiosin from *Serratia marcescens*, after RSM optimization. Wang *et al*. (2012) reported an insecticidal prodigiosin having maximum production (978 mg/L) in *Serratia marcescens* TKU011, using squid pen containing medium. Venil and Lakshmanaperumalsamy (2009), described a prodigiosin from *Serratia marcescens* SWML08 with 1397.96 mg/L after statistical optimization of culture medium. Zang *et al*. (2014) reported the highest prodigiosin yield as 1303 mg/L from *Serratia marcescens* N10612. These results are more or less comparable with the present work, stating that the bacterium can be used as a commercial competitor for the production of prodigiosin and also as an efficient bio remedy for plastic degradation. While applying classical statistical tools, only the response of a single parameter to the production of yield (prodigiosin) was obtained. By employing modern statistical tools like RSM, it paves a better way to know the combined effect of parameters on to the response (Lynch *et al*., 2018)

4.5. Conclusions

This study describes the optimal conditions for the production of prodigiosin from *A. denitrificans* SP1 using blood bag as the only carbon source. The study also highlights the significance of Response Surface Methodology as an effective tool for improving the physicochemical parameters, leading to higher prodigiosin

production. Using RSM analysis, higher prodigiosin production (1.314 mg/ml) was identified at 24 °C, pH 8.8, and 1.0 g (BB). The present study proved that *A. denitrificans* SP1 can be exploited further in a pharmaceutical perspective with regard to the production of prodigiosin, and in an industrial perspective as a bio remedy for phthalate (plastic) contaminated environments.

CHAPTER 5

RESPONSE SURFACE OPTIMIZATION OF PRODIGIOSIN PRODUCTION FROM *ACHROMOBACTER DENITRIFICANS* STRAIN SP1 USING FREE DEHP AS THE CARBON SOURCE AND SCALE UP IN BIOREACTOR

[Hareesh, E.S., and Harilal, C.C. (2020). Response surface optimization of prodigiosin production from *Achromobacter denitrificans* SP1 using free DEHP as the only carbon source and investigating its antifungal properties. ***Indian Journal of Microbiology, Springer*** (Communicated)]

5.1. Aim: To apply response surface methodology to optimize the growth parameters for the production of prodigiosin from *A. denitrificans* SP1 using free DEHP as the only carbon source and scale up using bioreactor.

5.2. Materials and Methods

5.2.1. Chemicals

Analytical and bacteriological grade chemicals from Merk (India) and HiMedia (India) were used for the preparation of the medium and DEHP were purchased from BDH Prolabo chemicals (India).

5.2.2. Bacterium and Medium

Achromobacter denitrificans strain SP1 (GenBank Accession No. HQ645935 and MTCC No.5710) was employed in this study. The bacterium, *A. denitrificans* SP1 was isolated from plastic polluted sewage sludge from Canoly Canal (11.2500 N; 75.7667 E), a water body flowing through Kozhikode City, Kerala, India. This was cultured in Basal Salt Medium (BSM) containing (g/L) K_2HPO_4, 1.0; NaCl, 1.0; NH_4Cl, 0.5 and $MgSO_4$, 0.4. The initial pH was

maintained at 7.2 and supplemented with DEHP as inducer cum sole carbon source.

5.2.3. Experimental Setup

Initially, the bacterium was cultured in BSM supplemented with 2.5 mM DEHP on a 2% agar plate (incubated for 3 days), and a loop full of cells were taken for further inoculation. *A. denitrificans* strain SP1 inoculum (100 µl; ~ 3×10^8 CFU) was cultured in 10 ml of BSM and incubated in a temperature-controlled shaker (equipped with fluorescent lamp, which is found to be the best light source for prodigiosin production), has set at different conditions as described in **Tables 15 and 16**. Prodigiosin production was estimated according to the trials predicted by the RSM software. All the experiments were conducted in triplicates. RSM optimized cultural conditions were provided in the bioreactor (2L) for the large scale production of prodigiosin. After column purification, the purity of prodigiosin was rechecked.

5.2.4. Prodigiosin extraction and quantification

The prodigiosin extraction was done as follows. The pigmented cell pellet was harvested after the required time period by centrifugation (10,000 rpm for 10 min at 4°C). 3ml acetone was added to the collected pellet, sonicated (output wattage 15 for 3 min) until the entire pigment was removed from the cells into the solvent and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant containing pigment was evaporated and the dry weight of prodigiosin (crude) was quantified.

5.2.5. Statistical optimization for prodigiosin production

4.2.5.1. Plackett-Burman experimental design

Five independent variables were selected for the initial screening studies; i.e., DEHP, pH, temperature, incubation time and agitation. Twenty different

combinations were generated to estimate the combined effects of these parameters on prodigiosin production (**Table 15**). Minitab version 17 was used to generate the experimental design with high (+1) and low (-1) levels for each selected variable.

Table 15. Plackett-Burman experimental design for the production of prodigiosin from *A. denitrificans* SP1. The parameters selected were DEHP concentration (2.5 and 25 mM); pH (4 and 10); temperature (20 and 30 °C); incubation time (4 and 8 days); agitation (100 and 200 rpm).

Run order	DEHP (mM)	pH	Temperature (°C)	Incubation (days)	Agitation (rpm)
1	2.5	4	20	4	100
2	2.5	10	20	8	200
3	2.5	10	30	8	200
4	25	4	30	8	200
5	2.5	10	30	4	100
6	2.5	4	20	4	200
7	2.5	4	20	8	100
8	25	4	20	8	200
9	25	10	20	4	100
10	25	4	30	4	200
11	2.5	4	30	4	200
12	25	10	30	4	100
13	25	10	30	8	100
14	2.5	10	20	8	100
15	2.5	4	30	8	100
16	2.5	10	30	4	200
17	25	4	30	8	100
18	25	4	20	4	100
19	25	10	20	4	200
20	25	10	20	8	200

5.2.5.2. Box-Behnken model and response surface methodology

Based on the results of Plackett-Burman experiment, three parameters (pH of the medium, temperature and DEHP concentration) were found significant for the production of prodigiosin by *A. denitrificans* SP1. Employing these three parameters, different combinations of experiments were designed (**Table 16**). Each variable was analyzed at low (-1), medium (0) and high (+1) levels, and the results are analyzed by second order polynomial equation.

5.2.5.3. Validation Experiments

Validation experiments for the quadratic model was conducted as predicted by the point prediction software Minitab 17 (**Table 17**). The difference between the estimated and predicted values of prodigiosin production was compared.

Table 16. Box-Behnken design for the production of prodigiosin from *A. denitrificans* SP1. The parameters selected were pH of the medium (6.5, 8.5 and 10.5); temperature (22, 25 and 28 °C); and DEHP concentration (2.5, 7.5 and 12.5 mM).

Run order	pH	Temperature (°C)	DEHP(mM)
1	10.5	25	2.5
2	6.5	28	7.5
3	8.5	22	2.5
4	8.5	25	7.5
5	6.5	25	12.5
6	10.5	28	7.5
7	8.5	28	2.5
8	8.5	22	12.5
9	8.5	25	7.5
10	10.5	22	7.5
11	6.5	22	7.5
12	10.5	25	12.5
13	6.5	25	2.5
14	8.5	25	7.5
15	8.5	28	12.5

Table 17. Validation of experimental design for the production of prodigiosin from *A. denitrificans* SP1.

Sl. No.	pH	Temperature (°C)	DEHP(mM)
1	9.2	24.9	8
2	8	24.9	8
3	10	22	7
4	6.5	22	7

5.2.6. Scale up studies in bioreactor

Large scale production of prodigiosin was carried out in a 2L lab-scale bioreactor (Murhopye Scientific, India) equipped with pH and temperature electrodes. Fluorescent lamp light was also fixed externally as the light source. RSM optimized cultural conditions are maintained in the bioreactor. The agitation was controlled at 400 rpm. Prodigiosin was harvested after 8 days of incubation.

5.2.7. Column chromatography

After the large-scale extraction of prodigiosin from the bioreactor, the concentrated pigment in acetone was purified by the column chromatography method as described by Aruldass *et al*. (2014) using a column of a specific size (31×1 cm) and silica gel (60-120 mesh) as absorbent. For washing of the column, *n*-hexane was used and 5 % ethyl acetate in *n*-hexane was used for eluting the pigment from the column.

5.2.8. Thin layer chromatography (TLC)

Prodigiosin fractions obtained after column chromatography were pooled and air-dried. Then a minimum amount of acetone was used to dissolve the prodigiosin pigment. From this, 5 µL was spotted on a silica gel coated glass plate and developed with the solvent system of methanol: chloroform (2:1).

5.2.9. Rechecking the purity of prodigiosin

5.2.9.1. Spectrophotometry

The absorption spectrum of prodigiosin in acetone was scanned with double beam spectrophotometer in the range of 400-800 nm. Characteristic peak at λ_{max} was measured.

5.2.9.2. Fourier-transform infrared spectroscopy (FT-IR)

Prodigiosin pigment was pelleted with potassium bromide (KBr) and analyzed using FT-IR. The relative intensity of transmitted light was measured against the wavelength of absorption in the region of 400-4000 cm^{-1}

5.3. Results

5.3.1. Statistical optimization of prodigiosin production

The results of Plakett-Burman experimental design are given in **Table 18**. The maximum prodigiosin production (0.525 mg/ml) was attained at 20 °C, pH 10, 100 rpm, 2.5 mM (DEHP) and 8 days of incubation, while the minimum production (0.03 mg/ml) was observed at 2 trials and are given in **Table 18**. Based on the results of Plackett-Burman experimental design, a pareto chart was constructed (**Figure 25**). The effects of the standardized parameters (Temperature, pH, Agitation, DEHP concentration, Incubation time) on prodigiosin production were estimated at 5% level of significance. **Figure 24** shows the bacterial clump formation after 8 days of incubation in DEHP containing BSM.

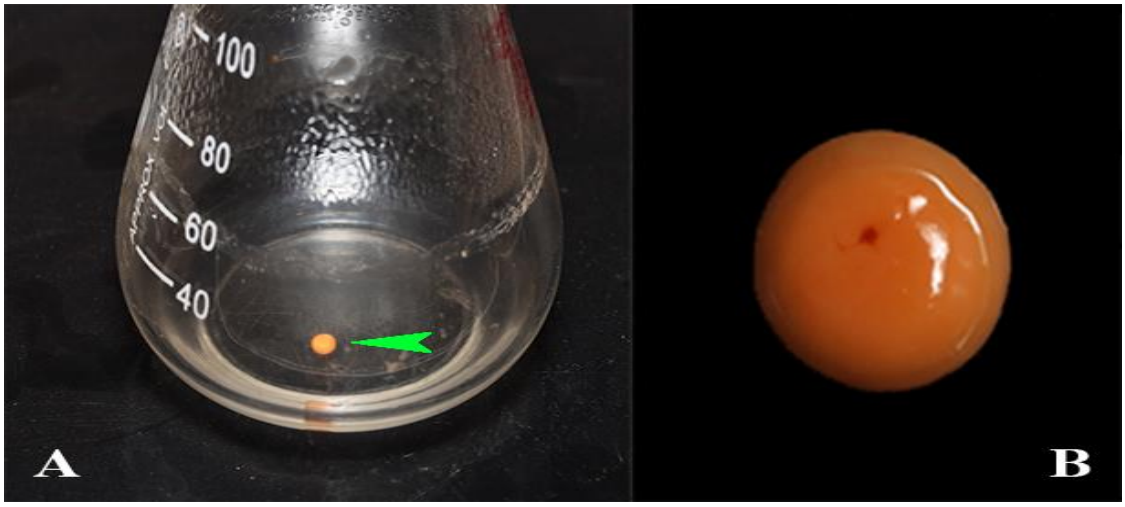

Figure 24. A) Bacterial clump formation in *A. denitrificans* SP1 after 8 days of incubation in DEHP containing BSM. **B)** Enlarged view of prodigiosin containing bacterial clump.

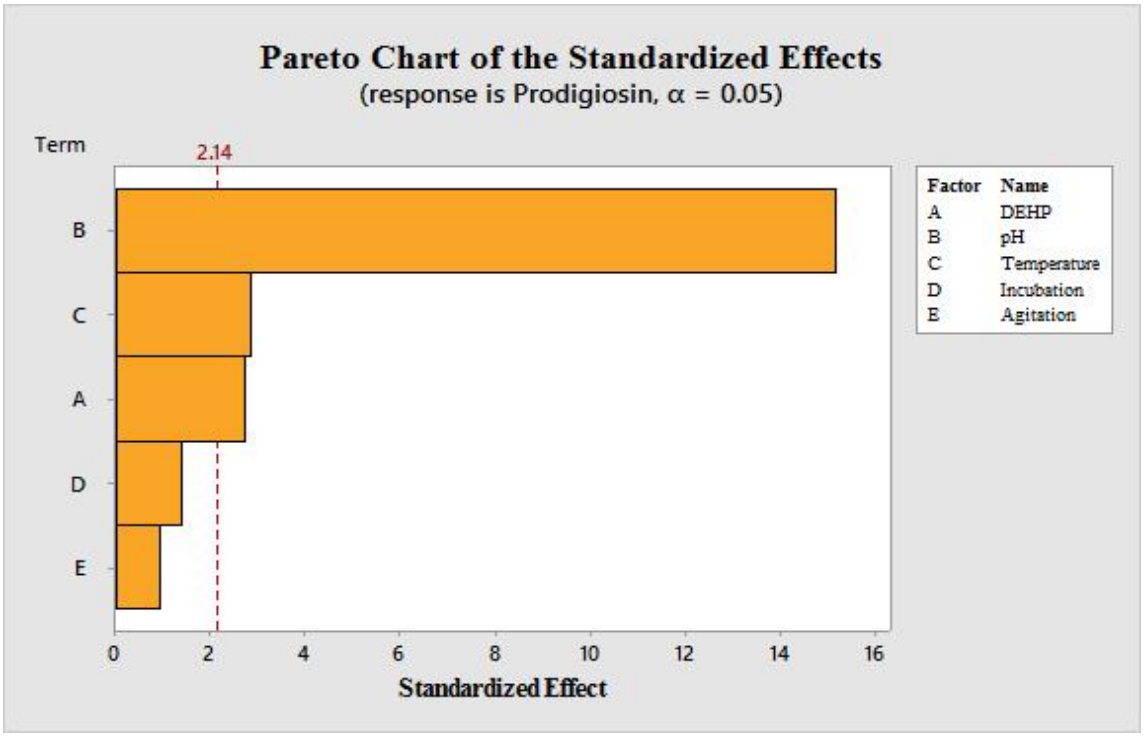

Figure 25. Pareto chart showing the standardized effects on the production of prodigiosin by *A.denitrificans* strain SP1. The parameters found relevant are in the order: pH> Temperature > DEHP; while agitation and incubation are found to have negligible effects.

Table 18. Results of Plackett-Burman experimental design for the production of prodigiosin by *A. denitrificans* strain SP1.

Run order	DEHP (mM)	pH	Temperature (°C)	Incubation (days)	Agitation (rpm)	Prodigiosin (mg/ml)
1	2.5	4	20	4	100	0.050
2	2.5	10	20	8	200	0.520
3	2.5	10	30	8	200	0.385
4	25	4	30	8	200	0.030
5	2.5	10	30	4	100	0.380
6	2.5	4	20	4	200	0.050
7	2.5	4	20	8	100	0.060
8	25	4	20	8	200	0.050
9	25	10	20	4	100	0.370
10	25	4	30	4	200	0.040
11	2.5	4	30	4	200	0.040
12	25	10	30	4	100	0.250
13	25	10	30	8	100	0.245
14	2.5	10	20	8	100	0.525
15	2.5	4	30	8	100	0.040
16	2.5	10	30	4	200	0.360
17	25	4	30	8	100	0.030
18	25	4	20	4	100	0.060
19	25	10	20	4	200	0.360
20	25	10	20	8	200	0.380

From the pareto chart, three highly influential parameters (pH, Temperature and DEHP) were selected. Eventually, these three parameters were considered for Box-Behnken experimental design by RSM to identify the optimized conditions for the maximum production of prodigiosin by *A. denitrificans* SP1. Regression coefficient was estimated for the production of prodigiosin (Prodigiosin versus DEHP concentration, pH, Temperature) (**Table 19**). The regression model for the production of prodigiosin was highly significant ($p < 0.05$) with an acceptable value of determination coefficient ($R^2 = 99.89\%$). The regression equation obtained for prodigiosin production was:

$Y = -17.746 + 1.8977X_1 + 0.7775X_2 + 0.0862X_3 – 0.10490X_1^2 – 0.015648X_2^2 – 0.007583\ X_3^2 + 0.000208X_1X_2 + 0.003750X_1X_3 + 0.000083X_2X_3$; where X1, X2, X3 are pH, temperature and DEHP, respectively.

The quadratic regression equation was used to generate two dimensional contour as well as three dimensional surface plots to depict the interactive effects of the selected parameters **(Figures 26, 27 & 28)**.

Table 19. Results of Box-Behnken design for the production of prodigiosin by *A. denitrificans* strain SP1.

Run order	pH	Temperature (°C)	DEHP (mM)	Observed prodigiosin (mg/ml)	Predicted prodigiosin (mg/ml)
1	10.5	25	2.5	0.590	0.598
2	6.5	28	7.5	0.110	0.116
3	8.5	22	2.5	0.630	0.628
4	8.5	25	7.5	0.980	0.981
5	6.5	25	12.5	0.080	0.071
6	10.5	28	7.5	0.720	0.710
7	8.5	28	2.5	0.610	0.610
8	8.5	22	12.5	0.690	0.689
9	8.5	25	7.5	0.985	0.981
10	10.5	22	7.5	0.730	0.723
11	6.5	22	7.5	0.125	0.134
12	10.5	25	12.5	0.730	0.737
13	6.5	25	2.5	0.090	0.082
14	8.5	25	7.5	0.980	0.981
15	8.5	28	12.5	0.675	0.676

Three parameters (pH, temperature and DEHP) selected from the validation experiments falling within the range determined from Box-Behnken were assessed in detail for the fitness of the model. The summary of the analysis of variance (ANOVA) for the selected quadratic model is shown in **Table 20**.

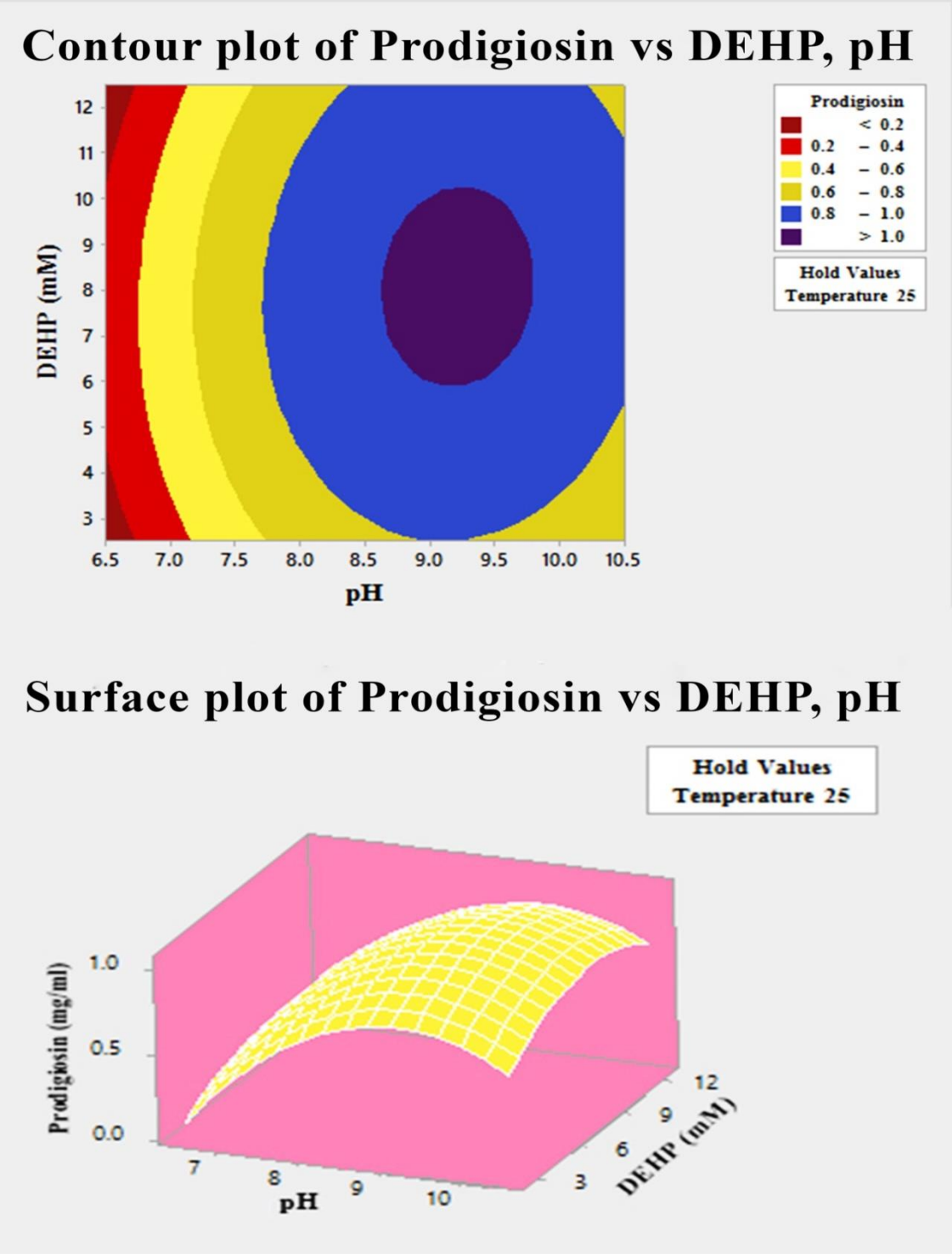

Figure 26. Contour and three dimensional surface plot showing the prodigiosin production *vs* DEHP (mM), pH. Here the temperature (25°C) was kept constant.

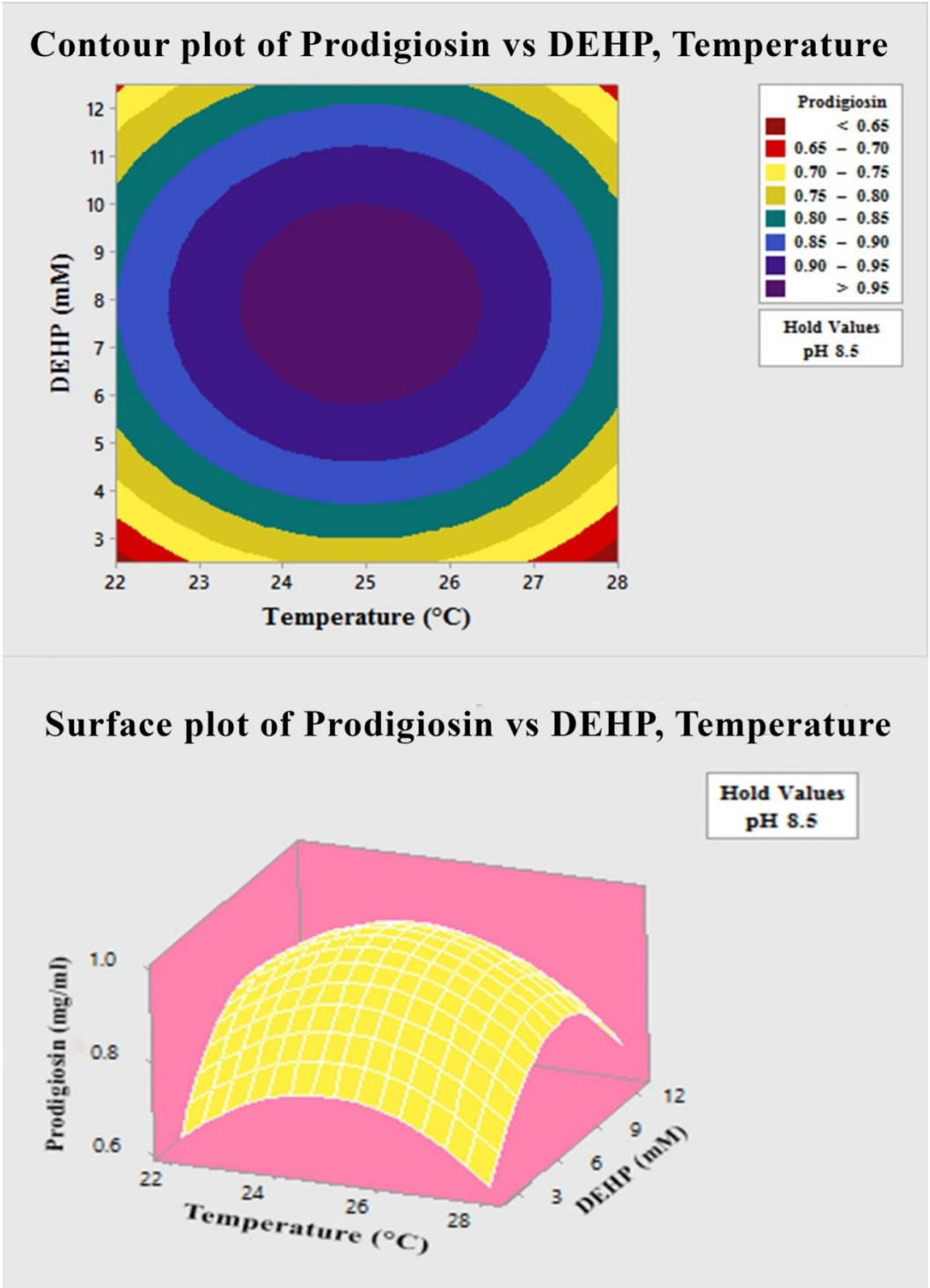

Figure 27. Contour and three dimensional surface plot showing the prodigiosin production *vs* DEHP (mM), temperature (°C). Here the pH 8.5 was kept constant.

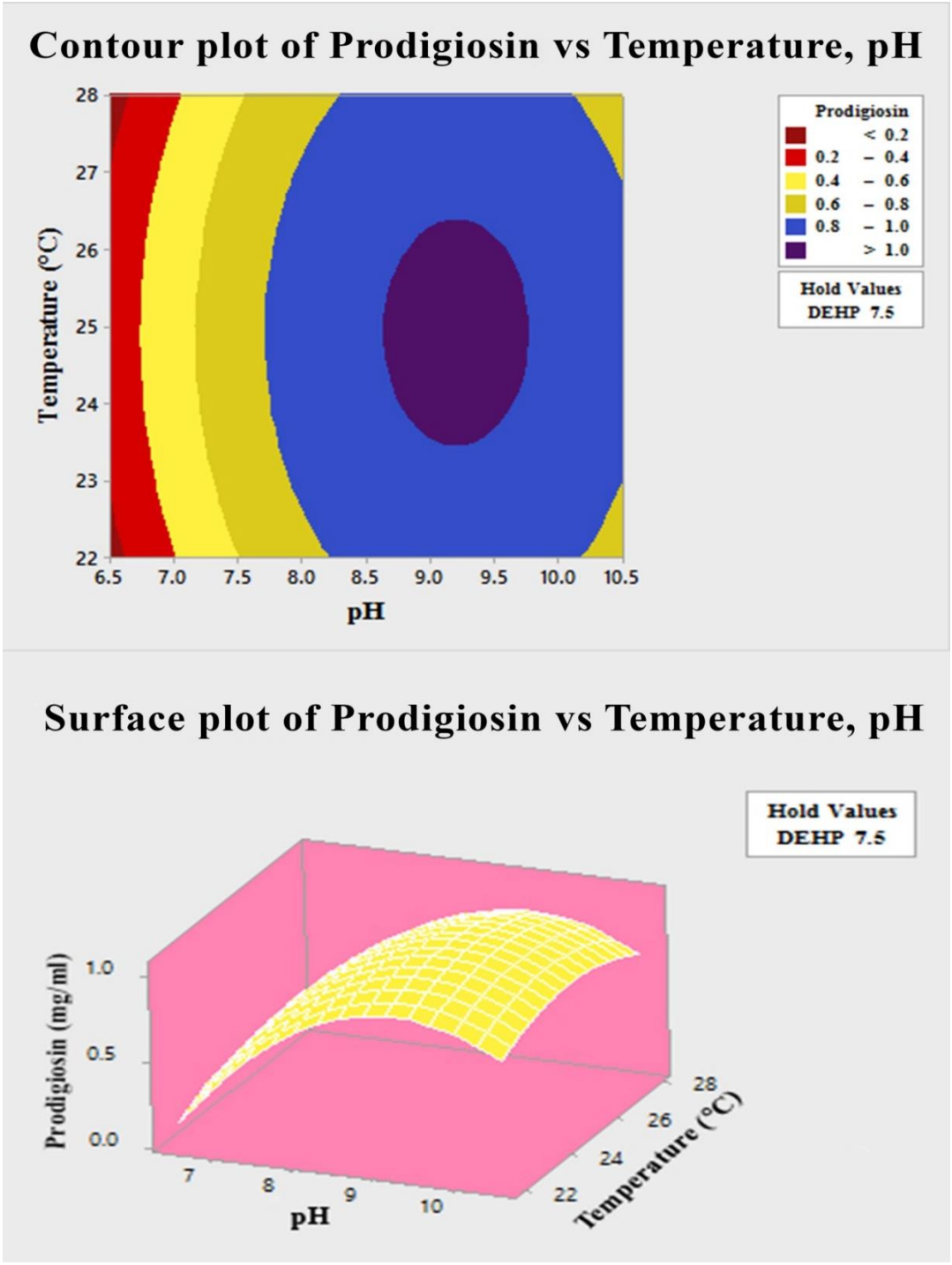

Figure 28. Contour and three dimensional surface plot showing the prodigiosin production *vs* temperature (°C), pH. Here the DEHP concentration 7.5 mM was kept constant.

Table 20. Analysis of variance (ANOVA) for the fitted quadratic model of prodigiosin production.

Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Regression	9	1.49067	0.16563	1477.74	0.000
Linear	3	0.70773	0.23591	2104.78	0.000
Square	3	0.77730	0.25910	2311.69	0.000
Interaction	3	0.00564	0.00187	16.77	0.005
Error	5	0.00056	0.00011		
Total	14	1.49123			

Table 21. Validation experiments

Sl. No.	**pH**	**Temperature (°C)**	**DEHP (mM)**	**Prodigiosin (mg/ml) Observed**	**Prodigiosin (mg/ml) Predicted**
1	9.2	24.9	8	1.008	1.0365
2	8	24.9	8	0.869	0.8885
3	10	22	7	0.791	0.8226
4	6.5	22	7	0.098	0.1334

Based on these results, the model was utilized to generate response surfaces for the analysis of the variable effects on the production of prodigiosin. The correlation coefficient of the result was 0.999 **(Table 21),** which was in good agreement with the predicted and experimental values. The optimum prodigiosin production was 1.008 mg/ml at 24.9 °C, pH 9.2 and 8 mM (DEHP).

5.3.2. Scale up studies in bioreactor

The maximum amount of prodigiosin recovered was 889.1 mg/L at RSM optimized cultural conditions (pH 9.2, Temperature 24.9 °C, DEHP 8 mM) along with fluorescent lamp as the light source. **Figure 29** shows the bioreactor setup for the scale up production of prodigiosin.

Figure 29. Bioreactor setup for the large scale production of prodigiosin having DEHP as the only carbon source for *A. denitrificans* SP1. **A)** Bioreactor setup before inoculation. **B) & C)** After 8 days of incubation at RSM optimized cultural conditions. **D)** Extracted prodigiosin.

5.3.3. Column chromatography

The solvent system was of *n*-hexane and ethyl acetate. Initial elution was done with 50 mL of *n*-hexane. The polarity of the solvent was increased by adding ethyl acetate. The pigment was separated in a 5 % ethyl acetate fraction (**Figure 30)**.

Figure 30. Silica gel (60-120 mesh) column loaded with the crude pigment extract. Purified fraction was eluted with 5 % ethyl acetate in *n*-hexane.

5.3.4. Thin layer chromatography

TLC profile of the purified pigment in acetone showing a single spot with an R_f value 0.67 in the solvent system methanol: chloroform (2:1) (**Figure 31**).

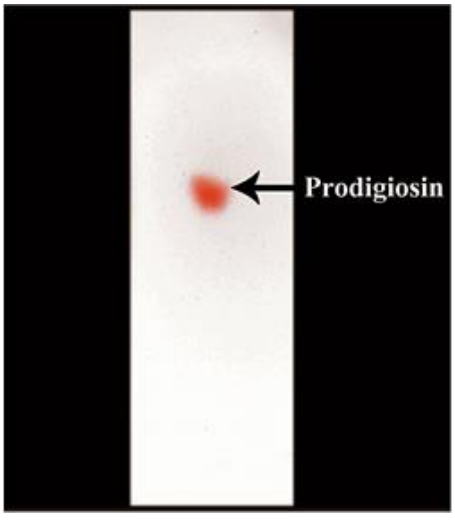

Figure 31. TLC chromatogram of column purified pigment in acetone showing single spot with an R_f value 0.67 in the solvent system methanol: chloroform (2:1).

5.3.5. Rechecking the purity of prodigiosin

5.3.5.1. Spectrophotometry

Prodigiosin dissolved in acetone was scanned with spectrophotometer in the range of 400-800 nm, and the maximum peak was obtained at λ_{480} (**Figure 32**)

5.3.5.2. Fourier transform-infrared spectroscopy (FT-IR)

Prodigiosin (air dried) compound was pelleted with potassium bromide (KBr) and analyzed using FT-IR (**Figure 33**). FT-IR characterization showed wave numbers (cm^{-1}) with characteristic vibrations at 3289 (N-H str aromatic), 2823 and 2852 (C-H str aliphatic) 1650 (N-H str aromatic) and 1539 (C-O str ether in $–OCH_3$).

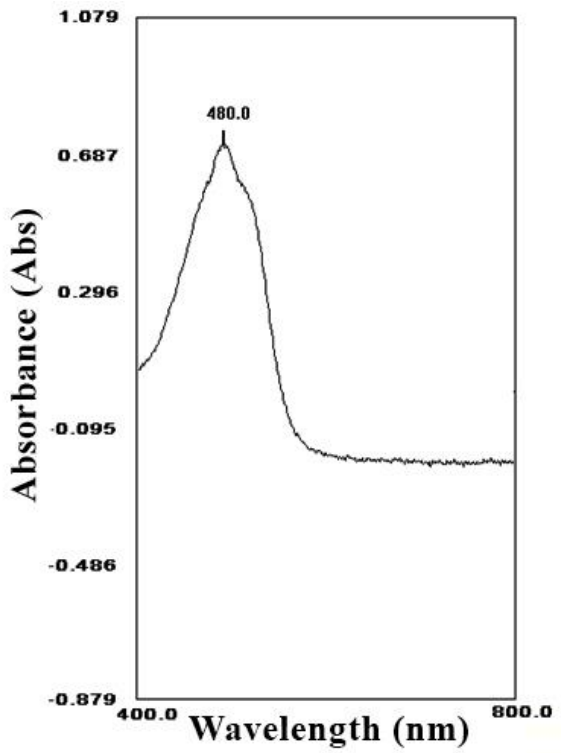

Figure 32. Spectrophotometric profile of prodigiosin produced by A. denitrificans SP1, extracted in acetone. Sharp peak at λ_{480} is evident.

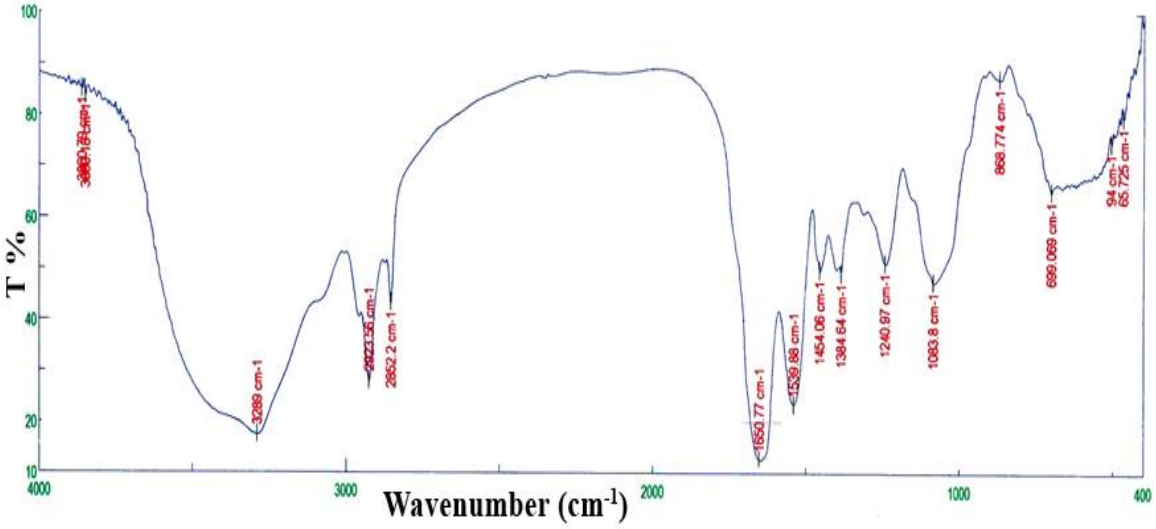

Figure 33. FTIR profile of prodigiosin produced by *A. denitrificans* SP1.

5.4. Discussion

In chapter 4, the response surface optimization of prodigiosin production from *A. denitrificans* SP1 using blood bag as the only carbon source was attempted. The study helped in recognizing the importance of the RSM technique and the optimization of the combined effect of parameters on the production of prodigiosin. The present study utilized DEHP (Di (2-Ethylhexyl) phthalate) as the only carbon source and for checking the combinational effect of parameters (Temperature, pH, DEHP concentration, agitation and incubation) on the production of prodigiosin in *A. denitrificans* SP1. The Plackett-Burman method was used to reduce the number of parameters and to find out the most influencing factors. Plackett-Burman followed by Box-Behnken was widely employed in the field of optimization of pigment production parameters as they evaluate the interactive effects of physico-chemical parameters, which are being ignored in the conventional *one-at-a-time* strategy (Hareesh *et al*., 2016). Plackett-Burman experimental design generates 20 different combinations of five parameters

(Temperature, pH, DEHP concentration, agitation, and incubation time). For each variable, high and low levels were fixed according to previous preliminary screening studies. From the Plackett-Burman results, it was clear that pH, DEHP concentration and temperature are the most influencing factors towards the production of prodigiosin.

During the biosynthesis of prodigiosin, the pH of the medium plays an important role (Sumathi *et al*., 2014). Pareto chart results of Plackett-Burman studies highlighted pH as the most influencing parameter, as discussed in chapter 4. In Plackett-Burman studies, 25 mM DEHP was selected as higher level and 2.5 mM as lower. From the Plackett-Burman results, it was clear that low levels of DEHP (2.5 mM) and lower temperatures prefer pigment production.

Plackett-Burman suggested pH, DEHP concentration and temperature as the most influencing factors and they were taken into consideration in Box-Behnken designs. The R^2 value was 0.99 - close to unity, which indicates accuracy of the model. The validation data was statistically analyzed to correlate the observed and predicted values. In all these cases, the model prediction was in good agreement with the experimental data and the correlation coefficient was found to be 0.99, suggesting the significance of the model. The optimum prodigiosin production was found to be 1.008 mg/ml culture medium (at 24.9 °C, pH 9.2 and DEHP 8 mM).

Maximum prodigiosin production (889.1 mg/L) by *A. denitrificans* SP1 in bioreactor setup was higher than that reported by Tao *et al*. (2005), who got maximum prodigiosin production (583 mg/L) using fed-batch approach from *Serratia marcescens* mutant. They used glucose as the initial carbon source and glycerol as a prodigiosin inducer. The purity of 25-C prodigiosin was rechecked

and confirmed by a spectrophotometer and FPLC. The absorbance maximum (λ_{480}), R_f value (0.67) and the functional groups present in the FTIR profile substantiated the purity of 25-C prodigiosin. With the help of IR, LCMS and NMR spectral characterization, Pradeep *et al*. (2014) confirmed that the orange-red pigment produced by *A. denitrificans* SP1 during the degradation of hazardous DEHP was 25-carbon prodigiosin with a molecular formula of $C_{25}H_{33}N_3O$. The main highlight of the present study was the utilization of a toxic DEHP as the only carbon source for *A. denitrificans* SP1 and the production of a unique 25-C prodigiosin analog, which is industrially and pharmaceutically significant.

5.5. Conclusions

This study describes the optimal conditions for the production prodigiosin by *A. denitrificans* SP1 using DEHP as the only carbon source. The study also highlights the significance of RSM tool for improving the physicochemical parameters that leads to higher prodigiosin production. Using RSM analysis, higher prodigiosin production (1.008 mg/ml) was identified at 24.9 °C, pH 9.2 and 8 mM DEHP concentration. In bioreactor setup, maximum prodigiosin was observed under optimized RSM condition (889.1 mg/L). This work concludes that the unique and promising 25-C prodigiosin can be a better contribution to the pharmaceutical industry, whose applications can be exploited further.

CHAPTER 6

QUORUM SENSING MEDIATED RESPONSE OF *ACHROMOBACTER DENITRIFICANS* SP1 REGARDING PRODIGIOSIN PRODUCTION UNDER PHTHALATE STRESS

[Hareesh, E.S., Harilal, C.C., Pradeep, S., & Julia Garvasis (2020). Quorum sensing mediated response of *Achromobacter denitrificans* SP1 regarding prodigiosin production under phthalate stress. ***Journal of Basic Microbiology, Wiley***, 60, 758-767. (Published)]

6.1. Aim: To study the influence of quorum sensing mechanism in *Achromobacter denitrificans* SP1 towards prodigiosin production under DEHP stress.

6.2. Materials and Methods

6.2.1. Chemicals

Analytical and bacteriological grade chemicals from Merk (India) and HiMedia (India) were used for the preparation of the medium for the bacterial culture and solvents for HSLs extraction.

6.2.2. Bacterium and Medium

Achromobacter denitrificans strain SP1 (GenBank Accession No. HQ645935 and MTCC No.5710) was employed in this study. The bacterium, *A. denitrificans* SP1 was isolated from plastic polluted sewage sludge from Canoly Canal (11.2500 N; 75.7667 E), a water body flowing through Kozhikode City, Kerala, India. This was cultured in Basal Salt Medium (BSM) containing (g/L) K_2HPO_4, 1.0; NaCl, 1.0; NH_4Cl, 0.5 and $MgSO_4$, 0.4. The initial pH was

maintained at 7.2 and supplemented with DEHP as inducer come sole carbon source.

6.2.3. Experimental design

In order to obtain sufficient amount of HSLs, *A. denitrificans* SP1 was cultured in petri plates containing BSM agar supplemented with DEHP (20 µl) as the sole source of carbon and incubated the cultures at 28°C. In accordance with the referred protocols, culture plates were taken at regular intervals for various assays and for the detection of HSLs.

6.2.4. Detection of homoserine lactones by colorimetric method

Primary detection of homoserine lactone activities of *A. denitrificans* SP1 was undertaken by colorimetric method, as described by Yang *et al*. (2006). *A. denitrificans* SP1 was cultured in BSM supplemented with DEHP at 28 °C. HSLs productions were detected after 6 h intervals up to 4 days. All the experiments were carried out in triplicates. For the extraction of HSLs, after required incubation, cultures were aseptically transferred and centrifuged at 12,000 rpm at 4°C for 15 min. The cell pellets were discarded and the supernatant was collected for the extraction of HSLs. The supernatant was then mixed with an equal amount of chloroform (HPLC grade) and vortexed for 10 min and the upper layer was collected in sterile glass bottles after 3 min. Extraction was repeated twice, with the lower layer for complete elucidation. 40 µl of upper layer of each sample was poured into wells of 96-well polystyrene flat-bottom tissue culture micro plates and 50 µl of 1:1 mixture of hydroxylamine (2M): NaOH (3.5M) was aliquoted and mixed with the sample, the same amount of 1:1 mixture of ferric chloride (10% in 4M HCl): 95% ethanol was added and OD was measured at 520 nm by using a multi scanner (Thermo Fisher Scientific, Finland). Samples with lactone compounds showed dark brown color, an indication of HSL production.

6.2.5. Determination of HSLs functional groups by FT/IR

To identify the HSLs functional groups, the strain was grown in BSM supplemented with DEHP at 28°C. HSLs were extracted after 12 h intervals up to 4 days. For the extraction of HSLs, after required incubation, cultures were aseptically transferred and centrifuged at 12,000 rpm at 4°C for 15 min. The cell pellets were discarded and the supernatant was collected for the extraction of HSLs. The supernatant was mixed with an equal amount of ethyl acetate (HPLC grade) and vortexed for 10 min., allowed to stand for 3 min and the upper layer was collected in sterile glass bottles. Single drop of each sample was placed on a KBr pellet and subjected to Fourier transform infrared spectroscopy (FT/IR) (JASCO, FTIR 4100)

6.2.6. Identification of HSLs by LC-Q-ToF-MS

To identify the molecular weight of HSLs, the samples collected for FT/IR analysis were subjected to Liquid Chromatography-Quadrupole Time-of-Flight (LC-QToF) (Waters Acquity H class UPLC system coupled with a Waters Xevo G2 Quadrupole-Time-of-Flight) high-resolution mass spectrometer (MS) and electron spray ionization (ESI) for ionization. The column used for mass spectrometry was BEH C18 (50 mm * 2.1 mm* 1.7 µm). LC-Q-ToF-MS helped to confirm the type of HSLs produced by *A. denitrificans* SP1.

6.2.7. DEHP degradation studies

Percentage utilization of DEHP

DEHP utilization percentage was quantified by spectrophotometric method as described by Pradeep *et al*. (2015). *A. denitrificans* SP1 was cultured in BSM supplemented with DEHP (20 µl) at 28°C. DEHP utilization percentage was

detected at regular intervals of 6 h up to 4 days. All the experiments were carried out in triplicates. At regular intervals, the residual DEHP in the medium was extracted with an equal volume of *n*-hexane. The spent medium was centrifuged at 12,000 rpm at 4°C for 15 min to separate the cell pellet from the medium. The cell-free supernatant was mixed with *n*-hexane and vortexed for 15 min and kept to stand for phase separation and the upper layer was collected in sterile glass bottles. OD was measured at 275 nm using a UV-Visible-Spectrophotometer (Thermo Fisher Scientific, Finland). The DEHP utilization percentage was calculated from these data, by fitting it to the standard graph of DEHP.

6.2.8. Quorum sensing inhibition studies in relation to prodigiosin production

To find out the relation between quorum sensing and prodigiosin production in *A. denitrificans* SP1, vanillin was used as an inhibitor in normal BSM+DEHP medium. After 4 days incubation, prodigiosin production was detected.

6.2.9. Statistical analysis

All the experiments were carried out in triplicates. Results were calculated as mean ± standard error. Statistical Package for the Social Sciences (SPSS) version 16 for windows was employed to analyze the statistical significance using one way ANOVA and post hoc comparisons of means were made using Duncan's multiple range test (DMRT) at 0.05 significance level.

6.3. Results

6.3.1. Primary detection of QS molecules

6.3.1.1. Detection of Homoserine lactones activity by Colorimetric method

In the present study, HSL activity of *A. denitrificans* SP1 in BSM up to 4 days at 6 h interval was monitored. **Table 22** and **Figure 34** shows the HSL activity of *A. denitrificans* SP1 detected by colorimetric method. As seen in graph (**Figure 34**), there was a sharp increase in the HSL activity after 18 h incubation and reached a maximum at 60 h and showed a sharp decline after 72 h. However, the HSL activities at OD 520 showed an absorbance below 0.1 during 84 h to 96 h, similar to the initial incubation periods of 6 h to 18 h.

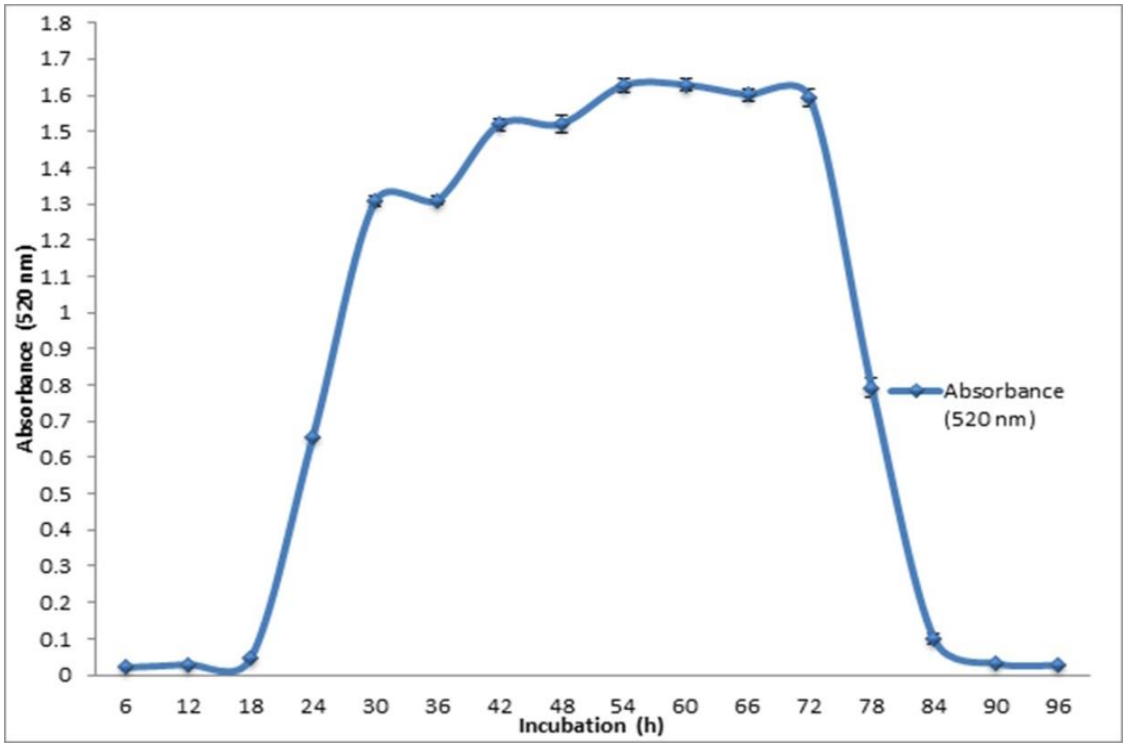

Figure 34. HSL activity of *A. denitrificans* SP1 upto 4 days at 6 h intervals.

Table 22. HSL activity of *A. denitrificans* SP1 upto 4 days at 6 h intervals. Values are mean of three replications (± SE). Means followed by the same letter are not significantly different at $p<0.05$ [ANOVA followed by Duncan multiple range test (DMRT)]

Hour (h)	Absorbance (520 nm)
6	$0.021^{H} \pm 0.002$
12	$0.029^{H} \pm 0.004$
18	$0.045^{H} \pm 0.007$
24	$0.655^{F} \pm 0.012$
30	$1.306^{D} \pm 0.024$
36	$1.431^{C} \pm 0.022$
42	$1.519^{B} \pm 0.031$
48	$1.521^{B} \pm 0.039$
54	$1.627^{A} \pm 0.036$
60	$1.629^{A} \pm 0.033$
66	$1.601^{A} \pm 0.031$
72	$1.593^{A} \pm 0.041$
78	$0.791^{E} \pm 0.046$
84	$0.099^{G} \pm 0.022$
90	$0.031^{H} \pm 0.011$
96	$0.026^{H} \pm 0.007$

6.3.2. Identification of Homoserine lactone molecules

6.3.2.1. FT/IR Analysis

Samples collected at 12 h intervals up to 4 days were subjected to FT/IR analysis (**Figure 35A, 35B, 35C and 35D**). In FT/IR, the spectrum showed bands at 1738, 1645, 1542, 1175 and 1025 cm^{-1}, which can be assigned to characteristic vibrations of the amide and lactone functional groups, which are the basic structural moieties of homoserine lactones. The band at 1738 cm^{-1} correspond to the C=O stretch of the lactone ring. The band at 1645 and 1542 cm^{-1} must be

assigned as the key vibrations of amide groups, dominated by C=O stretch and N-H bend. The 1175 and 1025 cm^{-1} band can probably be assigned to lactone C-O type stretching vibrations, dominated by OC-O and O-CH_2 stretches. From 24 to 72 h showed a significant peak height at 1738 cm^{-1} wavelength.

6.3.2.2. LC-Q-ToF-MS Analysis

FTIR analysis confirmed the presence of HSLs and for the actual identification of HSLs, samples of 12 h, 48 h and 96 h were subjected to LC-Q-ToF-MS analysis. Four HSL molecules were identified with the help of LC-Q-ToF-MS analysis, like 3-oxo-C6 HSL, 3-oxo-C8 HSL, C-10 HSL and 3-oxo-C12 HSL at different time intervals (**Figure 36, 37, 38 and 39**). List of identified HSLs are illustrated in **Table 23**.

6.3.3. Studies on DEHP degradation

Initially 20 µL of DEHP was added to the basal salt medium and was assumed as 100 percent. Percentage utilization of DEHP by *A. denitrificans* SP1 was analyzed in every 6 h intervals up to 96 h. It was observed that 57.2 % DEHP was utilized at 30 h incubation and 100 percent degradation was recorded within 72 h (**Figure 40 and Table.24).**

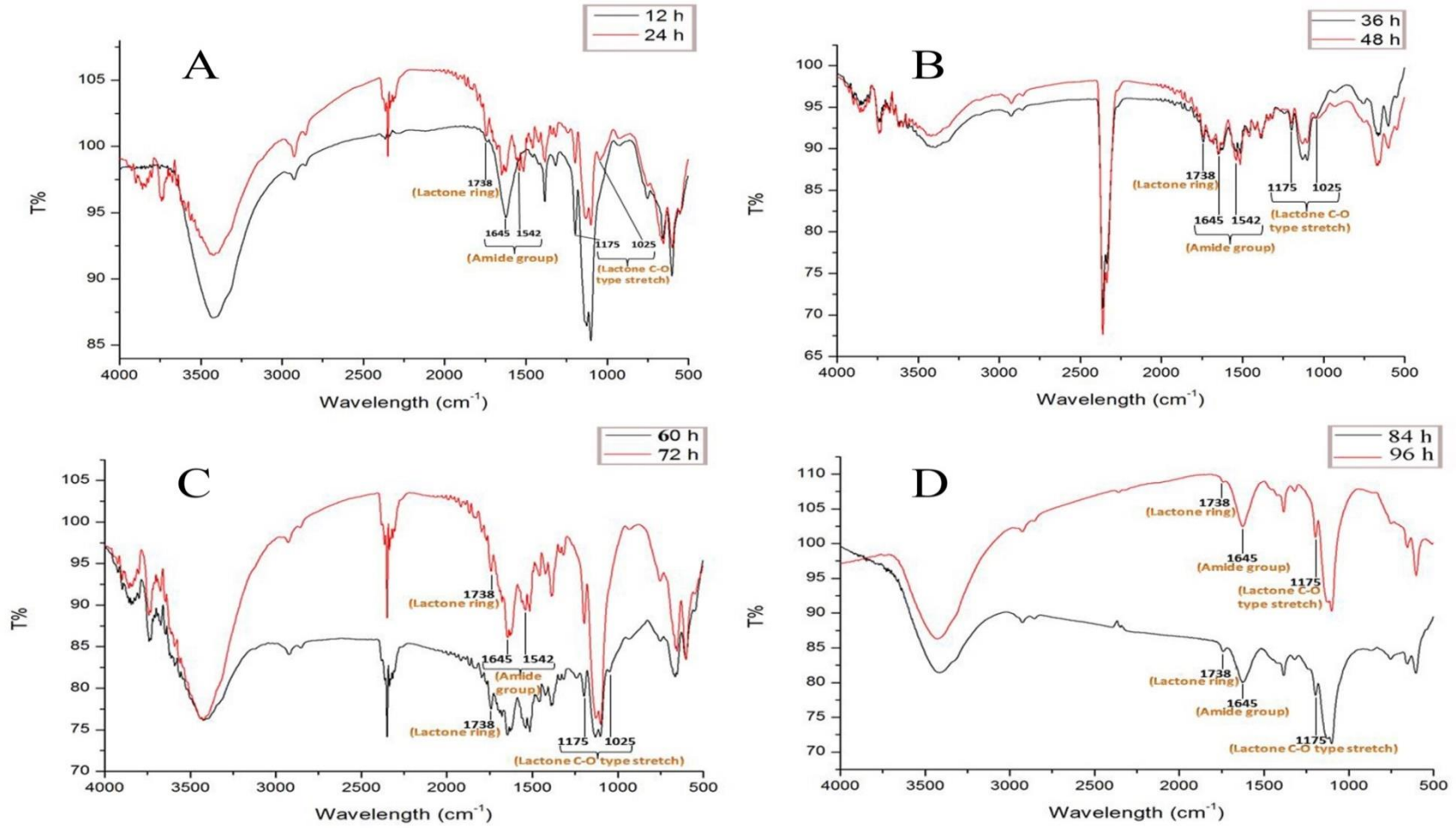

Figure 35. FTIR of sample extract at different time intervals showing the presence of HSLs. **A)** 12 h and 24 h **B)** 36 h and 48 h **C)** 60 h and 72 h **D)** 84 h and 96 h.

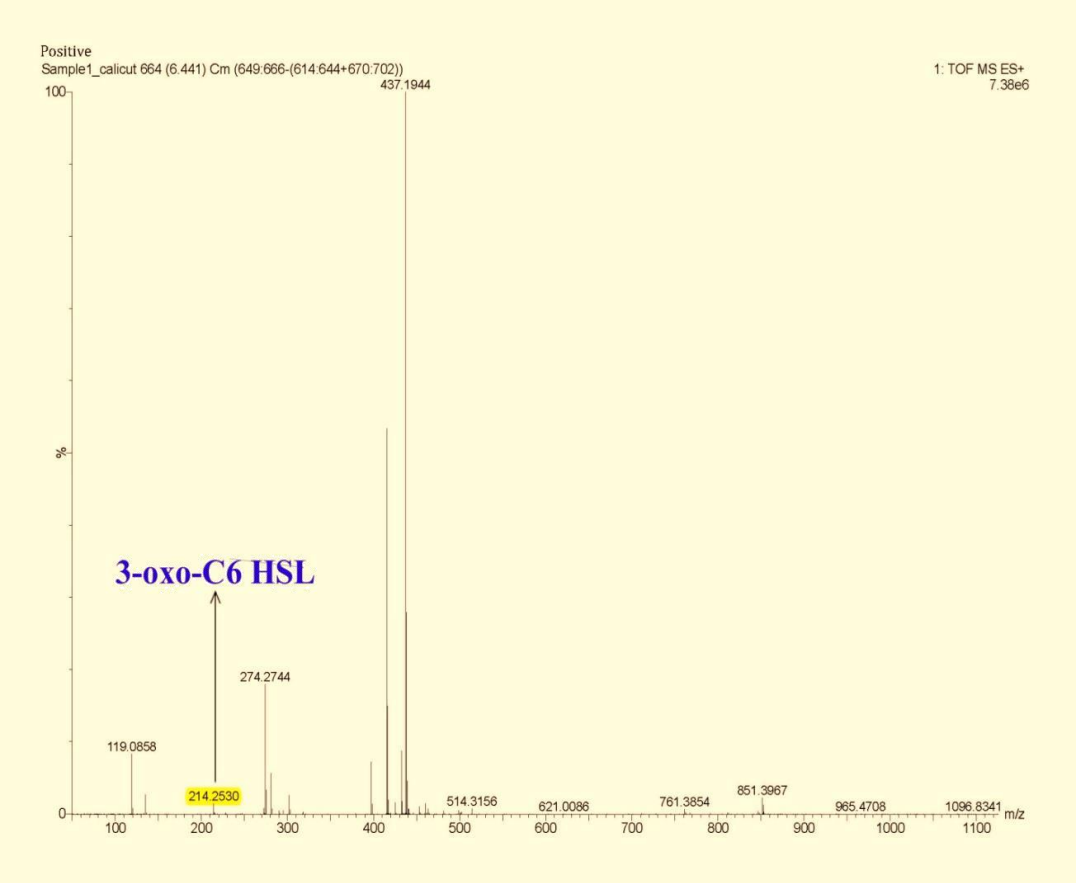

Figure 36. Liquid chromatography-quadrapole time-of-flight analysis of the 12 h sample showing the presence of 3-oxo-C6-HSL.

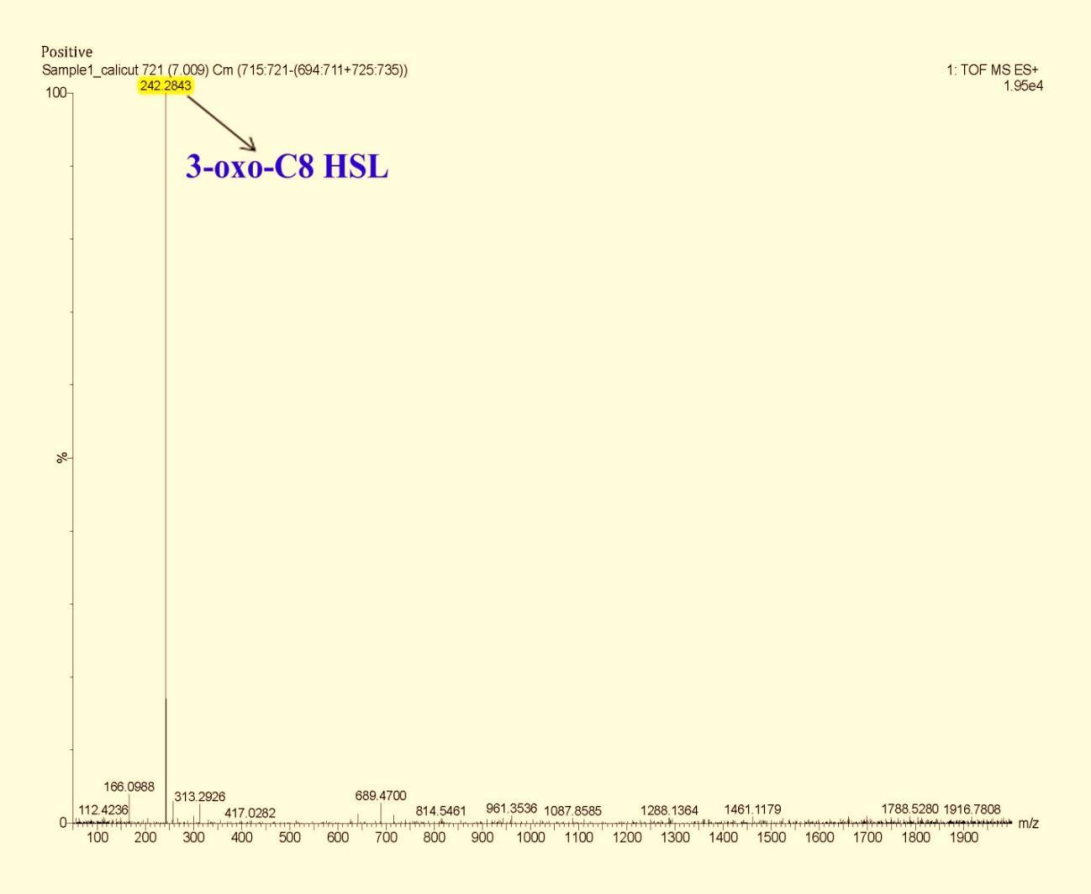

Figure 37. Liquid chromatography-quadrapole time-of-flight analysis of the 48 h sample showing the presence of 3-oxo-C8-HSL.

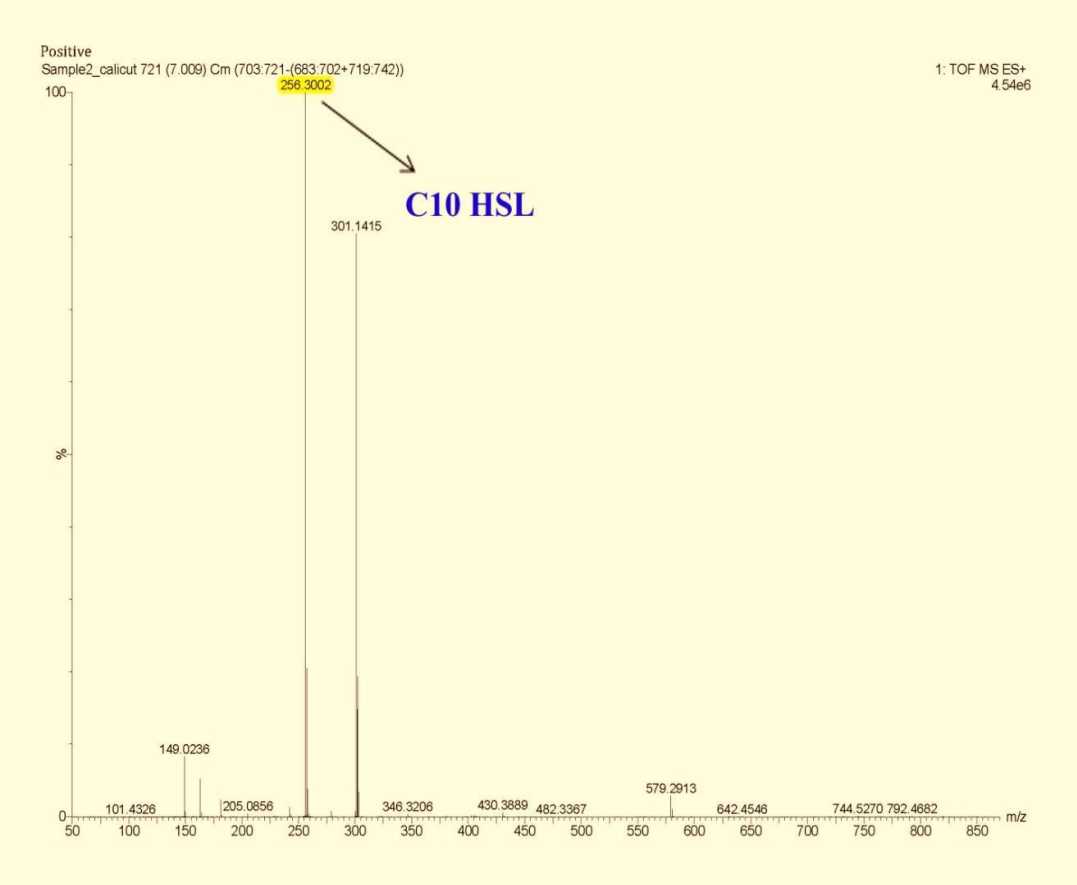

Figure 38. Liquid chromatography-quadrapole time-of-flight analysis of the 48 h sample showing the presence of C10-HSL.

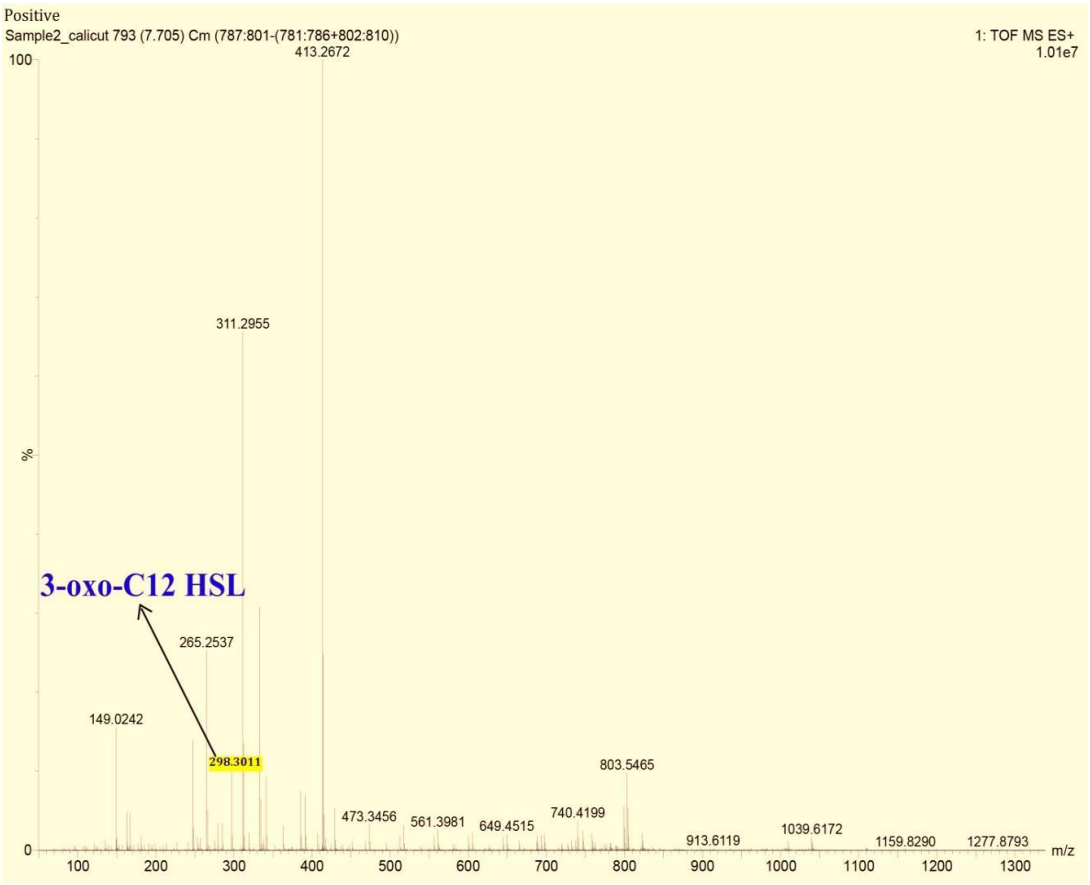

Figure 39. Liquid chromatography-quadrapole time-of-flight analysis of the 96 h sample showing the presence of 3-oxo-C12-HSL.

Table 23. List of HSLs identified through LC-Q-ToF-MS analysis

Sl.No.	HSLs	m/z	Retention time (min)	Reference
1	3-oxo-C6-HSL	214.2530	6.45	Hu *et.al.*,2016-RSC Advances
2	3-oxo-C8-HSL	242.2843	7.00	Hu *et.al.*,2016-RSC Advances
3	C10-HSL	256.3002	7.00	Hu *et.al.*,2016-RSC Advances
4	3-oxo-C12-HSL	298.3011	7.70	Hu *et.al.*,2016-RSC Advances

Table 24. Percentage Utilization of DEHP by *A. denitrificans* SP1

Hour (h)	Percentage Utilization (DEHP)
6	24.1 ± 5.7
12	33.8 ± 4.65
18	39.9 ± 3.1
24	42.7 ± 5.4
30	57.2 ± 2.9
36	76.9 ± 4.7
42	93.4 ± 4.1
48	94.1 ± 3.6
54	97.2 ± 1.3
60	99.4 ± 0.6
66	99.8 ± 0.3
72	100 ± 0
78	100 ± 0
84	100 ± 0
90	100 ± 0
96	100 ± 0

Values are the mean of three replications (± SE).

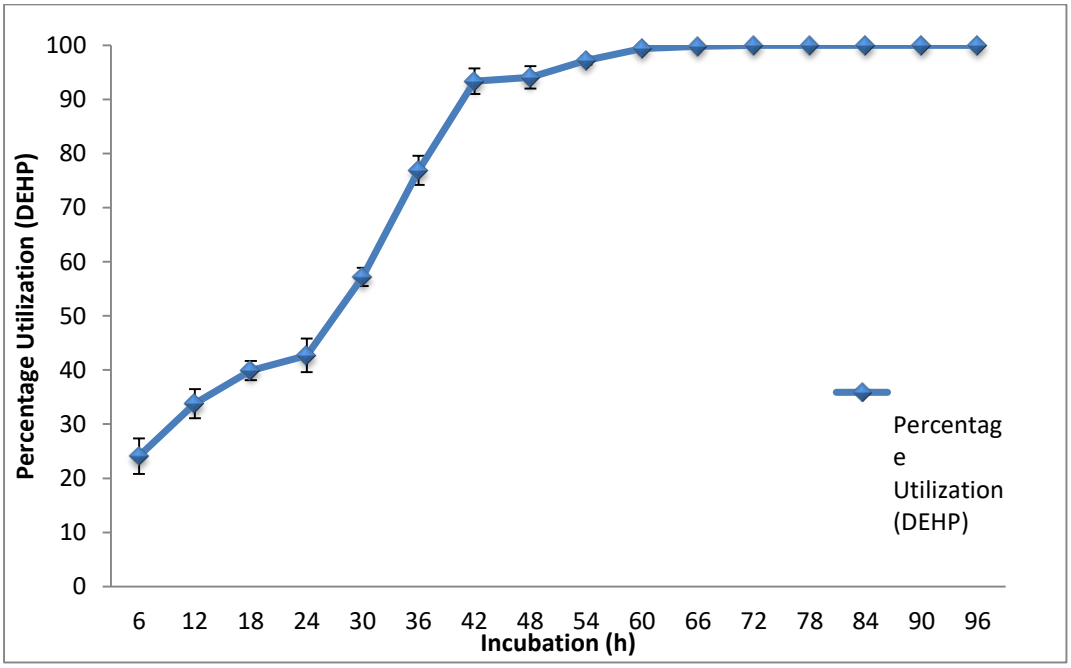

Figure 40. Percentage utilization of DEHP by *A. denitrificans* SP1.

6.3.4. Quorum sensing inhibition studies in relation to prodigiosin production

The present study demonstrates how quorum sensing inhibition affects prodigiosin production in *A. denitrificans* SP1. Petri plate having medium with HSL inhibitor (vanillin) showed the inhibition of prodigiosin production in *A. denitrificans* SP1. A reduced bacterial growth rate was also noticed in the vanillin containing plates. Petri plate having medium without vanillin showed orange red prodigiosin pigmented colonies (**Figure 41**).

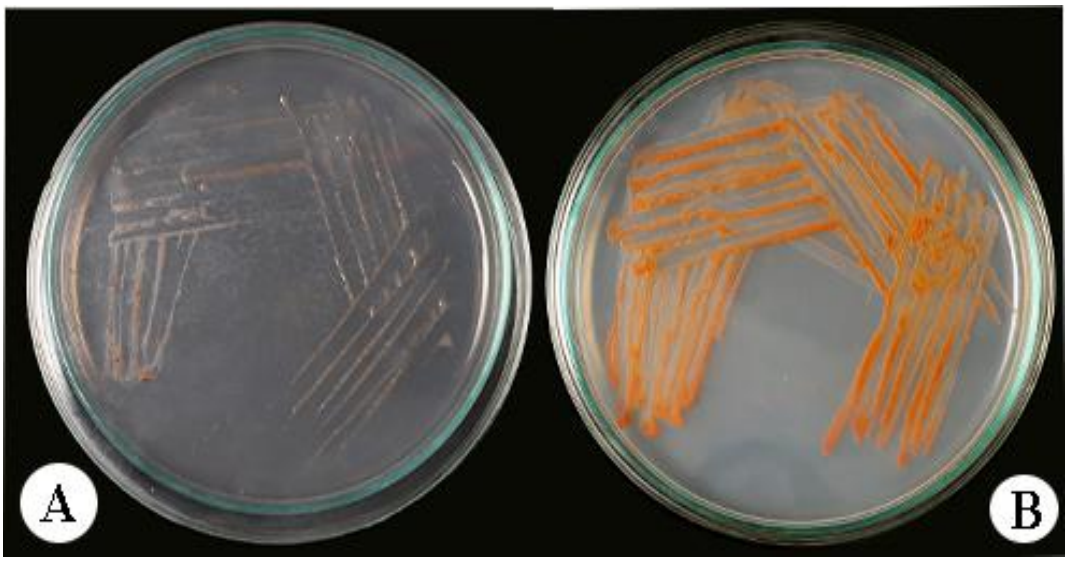

Figure 41. *A. denitrificans* SP1 (4 day culture). **A)** Vanillin containing BSM+DEHP medium showing no prodigiosin production. **B)** Normal BSM+DEHP medium showing prodigiosin production.

6.4. Discussion

Quorum sensing is an environmental sensing mechanism of bacteria to count their own population density with the help of small signaling molecules. Bacterial community mainly uses this co-operative behavior during the time of physico-chemical or environmental stresses (Wang *et al*., 2020). Detection and characterization of HSL signal molecules received great attention as it provide better understanding of cell-to-cell communication (Wang *et al*., 2011). Our earlier research work reported that *A. denitrificans* SP1 is an

efficient degrader of poisonous phthalates (mainly DEHP), a plasticizer, which is globally used in the manufacturing of PVC of plastics. Furthermore, the use of DEHP in the culture medium as carbon source imparted stress to the bacteria and resulted in the production of a 25-C prodigiosin. However, the presence of quorum sensing molecules facilitating the degradation of DEHP and accompanied by the concomitant prodigiosin generation property of *A. denitrificans* SP1 was not addressed yet.

Colorimetric detection of QS molecules by Yang *et al*. (2006) is the most simple and easy to use method, which analyses the amount of lactone compounds in the sample. This detection strategy, which has already been demonstrated to be operational (Barbey *et al*., 2018; Chane *et al*., 2019) and here ester group of HSLs react with the hydroxylamine leads to the formation of hydroxamic acid in alkaline solution, then it reacts with the ferric ions and forms a dark brown colored complex. Initially (6 h, 12 h and 18 h), the HSL activity has seemed to be low, compared to the increased activity showed up to the third day. A higher range of HSL activity has been noticed from 30 h to 72 h; which is also the peak time of DEHP degradation. The highest HSL activity (1.629 ± 0.033) was observed at 60 h of incubation.

While analyzing the IR spectrum of every 12 h intervals, it seems that the presence of lactone C=O stretch bands at 1738 cm^{-1} and amide vibrations at 1645 and 1542 cm^{-1} further reveals the presence and role of HSLs in the making up of *A. denitrificans* SP1 towards the biodegradation of DEHP followed by prodigiosin production. FT/IR analysis confirmed the compound to be homoserine lactone with reference to Solomons and Fryhle, (2007) and Silverstein and Webster, (1998). FT/IR of the sample extract at 12 h showed only a minute peak height at 1738 cm^{-1} wavelength, a key vibration of lactone C=O stretch, which is the basic unit of every HSLs (**Figure 35A**). FT/IR of the sample extract from 24 h to 72 h showed a significant peak height at 1738

cm^{-1} wavelength, which indicates the abundance of HSLs in the specified period (**Figure 35A, 35B and 35C**). Sample extracted at 84 h and 96 h showed a negligible peak height (**Figure 35D**), compared to the HSLs lactone bands obtained from 24 h to 72 h. FT/IR analysis confirmed the presence of lactone compounds (indirectly the presence of HSLs) in the sample and further confirmed that the maximum accumulation was from 24 h to 72 h. LC-Q-ToF-MS analyses of the samples at 12 h incubation showed $[M+H]^+$ value of 214.2, corresponding to the presence of 3-oxo-C6 HSL (Hu *et al*., 2016) (**Figure 36**). Sample at 48 h incubation showed $[M+H]^+$ values of 242.2 and 256.3 corresponding to the presence of 3-oxo-C8 HSL and C10 HSL (**Figure 37 and 38**) and DEHP percentage utilization profile also showed 48 h as the peak time of DEHP degradation. Samples at 96 h incubation showed $[M+H]^+$ values of 298.3 which confirmed the presence of 3-oxo-C12 HSL (**Figure 39**) which appeared after the complete degradation of DEHP.

Our research team has already reported that *A. denitrificans* SP1 is an efficient degrader of DEHP. The present study discusses the relationship of DEHP degradation with quorum sensing. A degradation of 33.8 % at 12 h incubation was noticed and LC-Q-ToF-MS analysis showed the presence of 3-oxo-C6-HSL in the same period. The role of 3-oxo-C6-HSL during the initial stages of DEHP degradation is yet to be identified. 42.7 % degradation was noted at 24 h incubation. 24 to 72 h was the peak time of DEHP degradation and LC-Q-ToF-MS analysis showed the presence of 3-oxo-C8-HSL and C10-HSL in the same period. From the above results, it is clear that 3-oxo-C8-HSL and C10-HSL plays a major role in the degradation of DEHP. Antony and Jayachandran, (2016) reported the presence of C8-HSL during phenol degradation by *Pseudomonas putida* strain JMQS1 and in the absence of phenol. Similarly, complete degradation of DEHP was observed from 72 h incubation and 3-oxo-C12-HSL was identified at 96 h incubation. Ponnusamy *et al*. (2009) reported that vanillin is a good QS inhibitor in the medium and

hence it is used in the present study. **Figure 41** showed that the culture plate containing vanillin has not produced any prodigiosin, whereas the culture plate without vanillin appears red due to the presence of prodigiosin. Teplitski *et al*. (2000) suggested a direct interaction of vanillin with HSL receptors for the QS inhibition. According to Ponnusamy *et al*. (2009) vanillin may interact with LuxR protein and also interfere with the binding of short chain HSLs to their cognate receptor and therefore inhibiting the HSL activity. In the present study, the researchers hypothesize that vanillin would have imparted some structural changes to the HSL signal molecules for the QS inhibition. This experiment reveals the role of QS molecules in both DEHP degradation and prodigiosin production in *A. denitrificans* SP1.

6.5. Conclusions

The present study reveals that the cellular responses of *A. denitrificans* SP1 is associated with quorum sensing and four HSL compounds have been identified during the DEHP degradation process and further investigation is required to explore the actual role of these HSL molecules in the DEHP degradation and regulation of prodigiosin synthesis. Moreover, the addition QS molecules in the growth medium will enhance the DEHP degradation property and concomitant production of prodigiosin by *A. denitrificans* SP1. Thus the results derived from this study could be exploited further for the treatment of large scale DEHP wastes generated during plastic manufacturing as well as its disposal.

CHAPTER 7

IN VITRO STUDIES FOR THE DRUGABILITY OF 25-C PRODIGIOSIN

7.1. Aim: To investigate the antibacterial, antifungal and the antioxidant activity of 25-C prodigiosin.

7.2. Materials and Methods

7.2.1. Chemicals

Analytical and bacteriological grade chemicals from Merk (India) and HiMedia (India) were used for the preparation of the medium for the culturing of microbes.

7.2.2. Disc diffusion method for antibacterial activity

Six pathogenic bacterial strains i.e., *Proteus mirabilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeroginosa*, *Escherichia coli* and *Acinetobacter baumannii* were used to test the antibacterial activity of the 25-C prodigiosin produced from *A. denitrificans* SP1. Nutrient agar media (NaCl- 5 g/L, Peptone- 5 g/L, Yeast extract- 3 g/L, Meat extract- 3 g/L and Agar- 2 %) plates were swabbed uniformly with 24 h cultures of pathogenic bacterial strain inoculums of ~2×10^7 CFU/ml. Whatman No.1 filter paper discs (6 mm in diameter) containing prodigiosin (0.5 mg/disc) were placed on the top of the agar plates. Ampicillin (10 mcg) was used as positive control and disc without prodigiosin was maintained as the negative control. The plates were incubated for 24 h and the zone of inhibition was measured on the millimeter scale.

7.2.3. Broth dilution method for antifungal activity

Seven fungal strains i.e., *Candida tropicalis*, *Purpureocillium lilacinum*, *Trichoderma viridae*, *Trichoderma harzianum*, *Aspergillus flavus*, *Aspergillus heteromorphus* and *Penicillium verruculosum* were used to test the antifungal activity of the 25-C prodigiosin from *A. denitrificans* SP1. In broth dilution method (Sumathi *et al*., 2014), first the prodigiosin is prepared in various concentrations (50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300 µg/ml) to find out the minimum inhibitory concentrations (MIC). 100 µl inoculum (~3×10^5 CFU) of each above mentioned fungal strains and 100 µl potato dextrose broth (PDB) media were poured into wells of 96-well polystyrene flat-bottom tissue culture microplates and added different prodigiosin concentration in each well and incubated for seven days, except *Candida* sp. (24 h) to test the minimum inhibitory concentration. Control wells were maintained without adding prodigiosin. OD was measured at 600 nm using a multi scanner (Thermo Fisher Scientific, Finland).

7.2.4. DPPH radical scavenging assay for antioxidant activity

The free radical scavenging activity of 25-C prodigiosin was evaluated using DPPH radical scavenging assay (Arivizhivendhan *et al*., 2018). Various concentrations of prodigiosin (50, 75, 100, 125, 150 µg/ml) dissolved in ethanol were prepared. 4.3 mg of DPPH (1,1-Diphenyl-2-picrylhydrazyl) was dissolved in 3.3 ml ethanol (protected it from light by covering it with aluminum foil). 150 µl DPPH solution was added to 3 ml ethanol and absorbance was taken immediately at 517 nm for control reading. Various prodigiosin concentrations were diluted with ethanol up to 3 ml and to each, 150 µl DPPH was added. Absorbance was taken after 15 min at 517 nm using ethanol as blank on UV-Visible spectrometer (Shimadzu UV-1601). The scavenging efficiency of prodigiosin on free radicals (DPPH) was calculated according to the following equation:

$$\text{Scavenging percentage} = \frac{(\text{Absorbance of control} - \text{Absorbance of test sample})}{\text{Absorbance of control}} \ 100$$

7.3. Results

7.3.1. Antibacterial activity of prodigiosin

The antibacterial activity of prodigiosin was assessed against six pathogenic bacterial strains, compared to conventional antibiotic compounds. The antibacterial effect of prodigiosin was assessed based on the zone of inhibition in the agar disc diffusion method. Prodigiosin showed the maximum zone of inhibition against *Klebsiella pneumoniae* (18 mm) and the least zone of inhibition against *Escherichia coli* (11 mm). Ampicillin also showed a greater zone of inhibition against *Klebsiella pneumoniae* and the least zone of inhibition against *Escherichia coli*. The negative control (disc without prodigiosin) showed no zone of inhibition against any bacterial strain. Antibacterial activities of prodigiosin against tested strains are shown in **Table 25** and **Figure 42**.

Table 25. Antibacterial activity of prodigiosin against pathogenic bacterial strains.

Bacterial Strain	**Zone of Inhibition (mm)**	
	Prodigiosin	**Ampicillin**
Klebsiella pneumoniae	18	23
Proteus mirabilis	16	21
Staphylococcus aureus	15	20
Pseudomonas aeroginosa	15	21
Acinetobacter baumannii	12	21
Escherichia coli	11	19

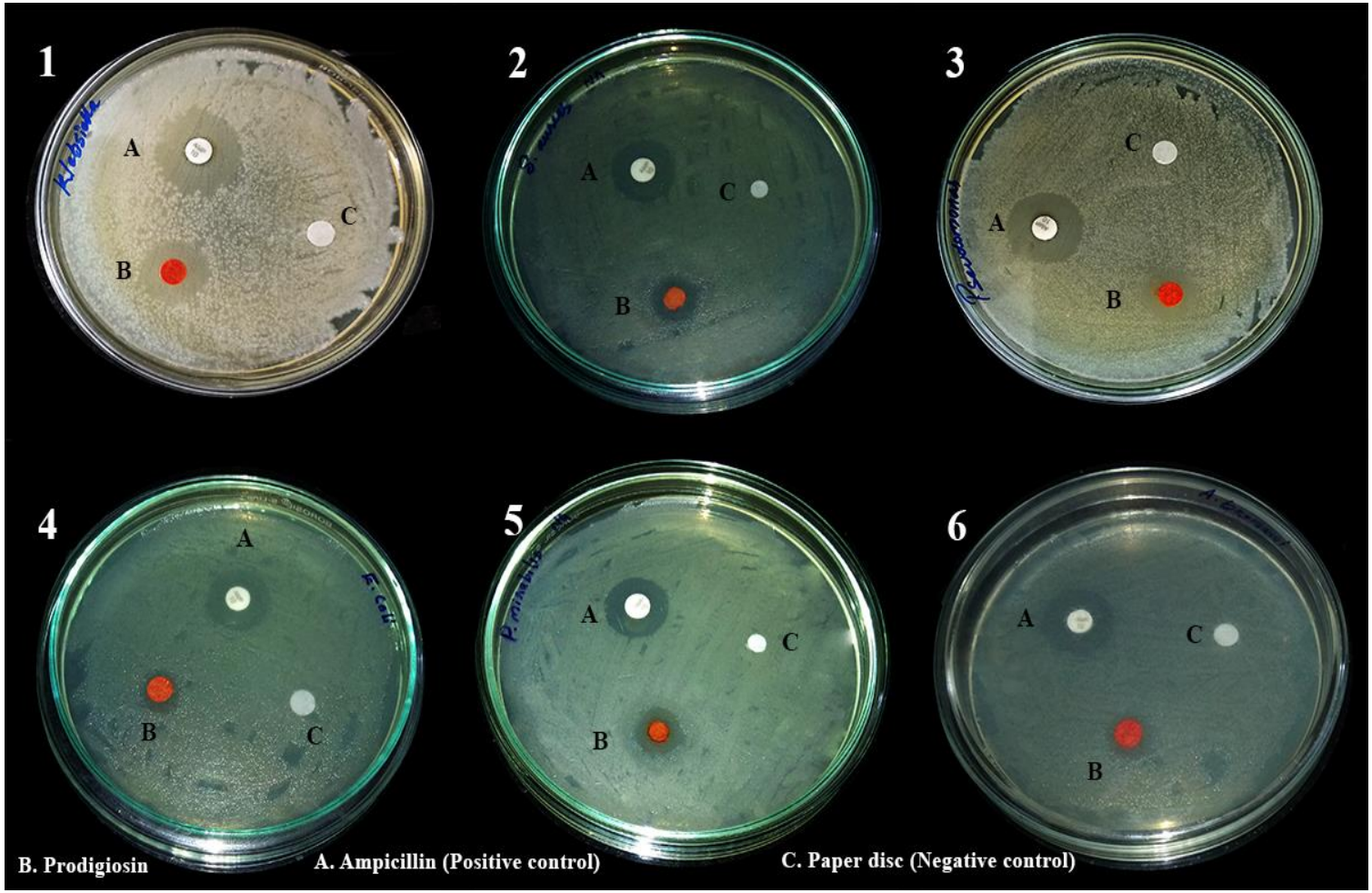

Figure 42. Antibacterial activity of prodigiosin against pathogenic bacterial strains **1)** *Klebsiella pneumonia* **2)** *Staphylococcus aureus* **3)** *Pseudomonas aeroginosa* **4)** *Escherichia coli* **5)** *Proteus mirabilis* **6)** *Acinetobacter baumannii*

7.3.2. Antifungal activity of prodigiosin

Prodigiosin exhibited marked antifungal activity against all the tested fungal strains (**Table 26 and Figure 43**). Maximum activity was seen against *C. tropicalis* (minimum inhibitory concentration- 75 µg/ml) and least activity against *P. lilacinum* (MIC- 275 µg/ml). Both *Trichoderma* sp. showed similar MIC (125 µg/ml). In the case of *Aspergillus*, *A. flavus* (MIC- 100 µg/ml) and *A. heteromorphus* (150 µg/ml) showed different inhibitory concentrations.

Table 26. Minimum inhibitory concentration (MIC) of prodigiosin against fungal strains.

Fungal strain	Prodigiosin (MIC µg/ml)
Candida tropicalis	75
Trichoderma viridae	125
Trichoderma harzianum	125
Aspergillus heteromorphus	150
Aspergillus flavus	100
Penicillium verruculosum	150
Purpureocillium lilacinum	275

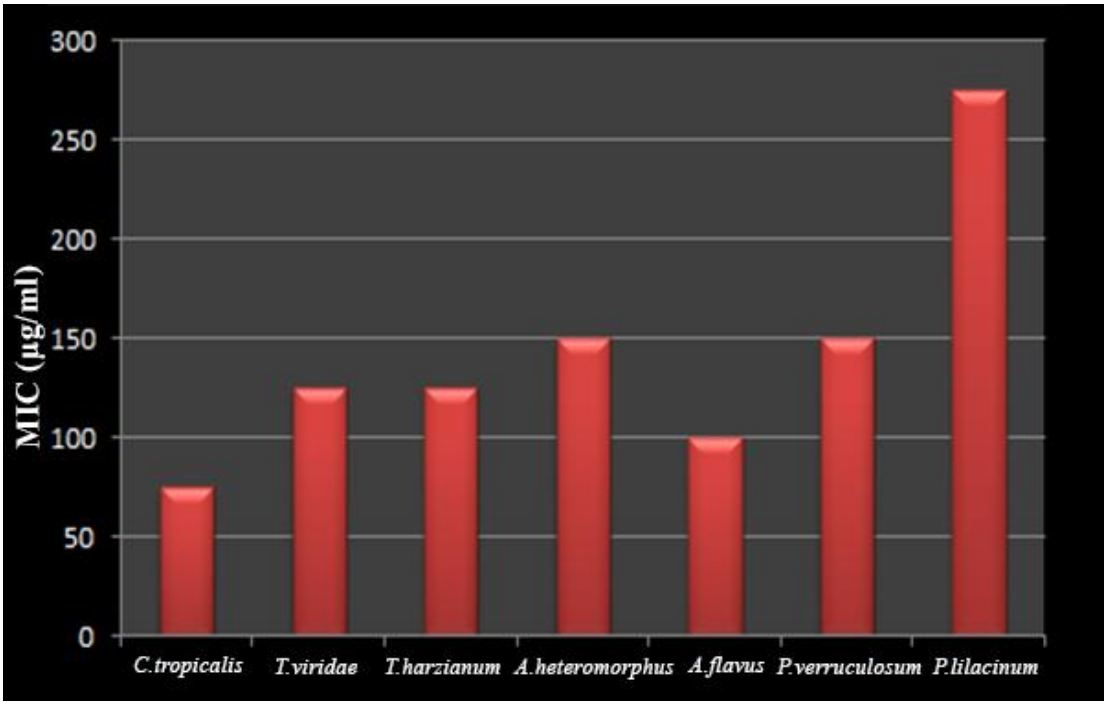

Figure 43. Minimum inhibitory concentration (MIC) of prodigiosin against fungal strains.

7.3.3. Antioxidant activity of prodigiosin

Different concentrations of prodigiosin (50, 75, 100, 125 and 150 µg/ml) were tested for the radical scavenging effect against DPPH. The DPPH radical scavenging activities of prodigiosin were found to be 19, 52, 78, 91 and 99 % at 50, 75, 100, 125 and 150 µg/ml of prodigiosin respectively (**Figure 44**).

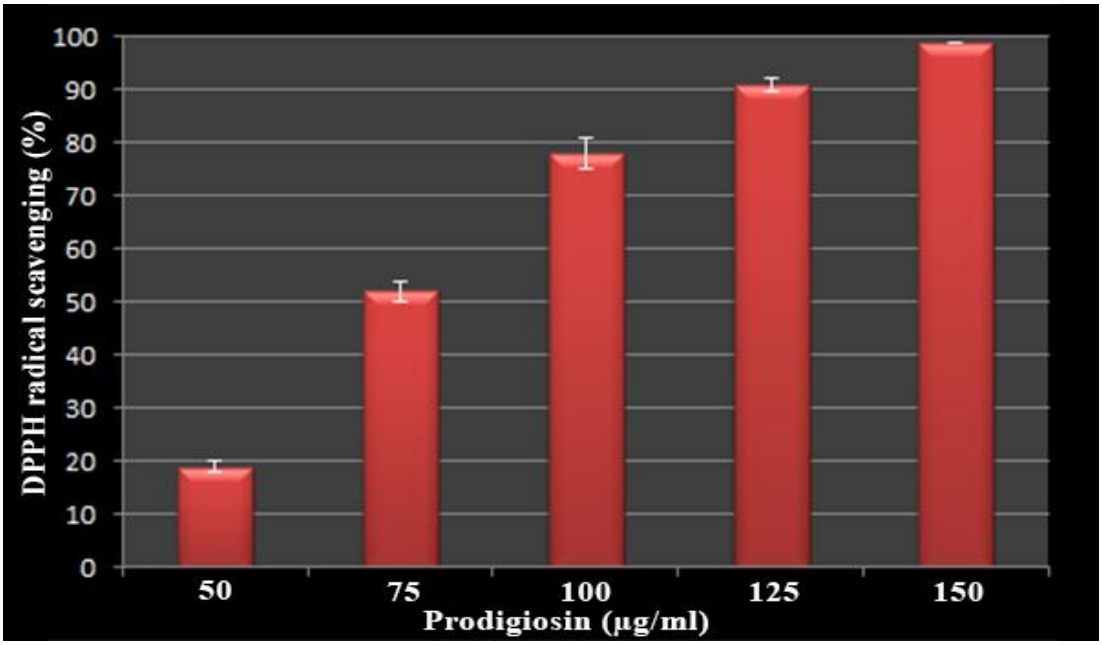

Figure 44. DPPH radical scavenging activity of prodigiosin (scavenging (%) *vs* prodigiosin concentration)

7.4. Discussion

The tripyrrole alkaloid, prodigiosin and its synthetic analogs are now an interesting candidate in the pharmaceutical research because of their vast properties like antibacterial, antifungal, antioxidant, antimalarial, anticancer, antidiabetics, immunosuppressive and antiparasitic properties (Stankovic *et al*., 2014). The commonly occurring prodigiosin are 20-carbon compounds. Interestingly, the carbon atom (numbered 6-10 in the present structure) of the present 25-C prodigiosin is unique and this makes it different from other

prodigiosin analogs. In the present study, the antibacterial, antifungal and antioxidant activity of 25-C prodigiosin was evaluated.

Suryawanshi *et al*. (2014), Sumathi *et al*. (2014) and Gulani *et al*. (2012) reported the antibacterial activity of prodigiosin against a range of pathogens. Suryawanshi *et al*. (2014) reported that prodigiosin from *Serratia marcescens* induces the leakage of intracellular substances, interrupts the activities of proteases, catalases, oxidases and changes the surface appearance of pathogenic bacterial cells. *Serratia nematodiphila* produces a prodigiosin, which induces programmed cell death by DNA fragmentation and expression of caspase-like proteins in bacterial cells (Darshan and Manonmani, 2016). The antibacterial activity of prodigiosin due to the reduced respiration activity and inhibition of protein and RNA synthesis leads to bacterial cell death (Danevcic *et al*., 2016). The present study also showed that the 25-C prodigiosin produced by *A. denitrificans* SP1 is a good antibacterial agent against pathogenic strains like *Proteus mirabilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeroginosa*, *Escherichia coli* and *Acinetobacter baumannii*.

Parani and Saha (2008), Sumathi *et al*. (2014) and Jimtha *et al*. (2017) studied the antifungal activity of prodigiosin against a range of fungal strains and supported the antifungal behavior of prodigiosin. The 25-C prodigiosin showed marked antifungal activity against *Candida tropicalis*, *Purpureocillium lilacinum*, *Trichoderma viridae*, *Trichoderma harzianum*, *Aspergillus flavus*, *Aspergillus heteromorphus* and *Penicillium verruculosum*. Sumathi *et al*. (2014) tested the antifungal activity of prodigiosin against *Aspergillus niger*, *Trichoderma viridae*, *Penicillium chrysogenum*, *Microsporum canis*, *Candida albicans*, *Fusarium moniliforme*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Fusarium oxyzporum*, *Aspergillus flavus* and they found that prodigiosin exhibit better antifungal activity against

A. niger, *T. viridae* and *T. rubrum*. Parani and Saha, (2008) studied the antifungal activity of prodigiosin against 6 fungal pathogens (*Helminthosporium sativum*, *Curvularia lunata*, *Alternaria alternate*, *Fusarium oxysporum*, *Cercospora apii* and *Rhizoctonia solani*) and found that prodigiosin is very effective against *H. sativum*, *C. lunata* and *A. alternate*. Prodigiosin produced by *Serratia marcescens* strain B2 was found to be very effective against gray mold pathogen, *Botrytis cinerea* (Someya *et al*., 2001).

The antioxidant potential of prodigiosin was studied by estimating the free radical scavenging percentage. Through DPPH radical scavenging assay, it is clear that 25-C prodigiosin produced by *A. denitrificans* SP1 have a good antioxidant capacity. The DPPH radical scavenging percentage was increased by increasing the concentration of prodigiosin; the hydrogen donation by the antioxidant prodigiosin changes the color of DPPH from violet to yellow. Arivizhivendhan *et al*. (2018) studied the antioxidant activity of prodigiosin from *Serratia marcescens* through DPPH and ABTS radical assays and showed that prodigiosin is a potent antioxidant that increased the scavenging percentage by increasing the concentration. Gulani *et al*. (2012) also studied and proved that prodigiosin from *Serratia marcescens* has a potent antioxidant capability. Prodigiosin produced by radio-resistant *Streptomyces* sp. strain WMA-LM31 was found to be a good antioxidant (Sajjad *et al*., 2018).

7.5. Conclusions

The present study describes the antibacterial, antifungal and antioxidant properties of 25-C prodigiosin, produced by *A. denitrificans* SP1. Disc diffusion method confirms that the 25-C prodigiosin was a good antibacterial agent against pathogenic strains like *Proteus mirabilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeroginosa*, *Escherichia coli* and *Acinetobacter baumannii*. Broth dilution method evaluated the antifungal

property and concluded that 25-C prodigiosin was a potential agent against fungal strains like *Candida tropicalis*, *Purpureocillium lilacinum*, *Trichoderma viridae*, *Trichoderma harzianum*, *Aspergillus flavus*, *Aspergillus heteromorphus* and *Penicillium verruculosum*. DPPH free radical scavenging assay confirms that 25-C prodigiosin is a good antioxidant agent. Thus the 25-C prodigiosin from *A. denitrificans* SP1 could be exploited further for pharmaceutical purposes.

CHAPTER 8

SUMMARY AND CONCLUSIONS

8.1. Title

Studies on *Achromobacter denitrificans* strain SP1 regarding production of prodigiosin upon utilizing hazardous phthalate.

8.2. Principal goal

The principal goal of this study was to statistically optimize the cultural parameters for the production of 25-C prodigiosin, utilizing hazardous di (2-ethylhexyl) phthalate – one of the high molecular weight plasticizer by *in situ* (DEHP bounded blood bag) and *ex situ* (DEHP in liquid form) means using *A. denitrificans* SP1. Upon the aforesaid background, the principal goal of this study was subdivided as follows:

1. Influence of light conditions favouring prodigiosin production in *Achromobacter denitrificans* strain SP1.

2. Response surface optimization of prodigiosin from *Achromobacter denitrificans* strain SP1 using blood bag as the carbon source.

3. Response surface optimization of prodigiosin from *Achromobacter denitrificans* strain SP1 using free DEHP as the carbon source and scale-up in bioreactor.

4. Quorum sensing mediated response of *Achromobacter denitrificans* SP1 regarding prodigiosin production under phthalate stress.

5. *In-vitro* studies for the drugability of 25-C prodigiosin.

8.3. Introduction

Plastic has created the modern world and modified the quality of life. Plastic is existing to be essential to the modern world because of their appreciating properties such as malleability and versatility. Characteristics such as lightweight, strength, durability and inexpensiveness made them suitable for the manufacture of a wide range of products. Plasticizers are used in plastics to add softness to the polymer (polyethylene, polyvinyl chloride *etc.*). Phthalic acid esters (phthalates) are the most widely used plasticizers in the world, especially blended in the commonly used products, including toys of children, health and beauty supplies (*e.g.* personal care products and cosmetics), medical equipments (*e.g.* storage bags, tubings and intravenous bags), and the capsular coating of some pharmaceuticals. These plasticizers are not chemically bounded with the PVC mesh, so that it easily leaches out and cause serious health problems in humans.

Di-methyl phthalate (DMP), Di-ethyl phthalate (DEP), Di-n-octyl phthalate (DNOP), Di-isononyl phthalate (DINP), Di (2-ethylhexyl) phthalate (DEHP) etc., are the widely used phthalates. Among them, DEHP is the dominant plasticizer used globally. It is used in various soft polymer products like lifesaving medical devices (such as medical tubes, blood bags, etc.), footwear, packing materials, and tarpaulins for trucks, flooring stationary and roofings. DEHP is not chemically bound to polymeric matrices and can mobilize from plastics. It is characterized under the category of endocrine disrupting chemicals (EDCs) and is regulated under the list of environmental priority pollutants. The toxicity of DEHP has raised serious talks on using them in lifesaving medical devices.

Microorganisms have proved to have a major role in the degradation of hazardous phthalates. Many bacterial strains utilize phthalates in defined mineral salt medium. Apart from bacteria, few fungi, yeast and algae have

been reported to degrade phthalates. If plastic materials with bound phthalates are used in bioremediation studies, the phthalate released from them would be utilized by the microbes as the sole source of carbon and energy. The predominant phthalate present in commercial PVC-blood bag (DEHP) was used as a model plastic in this venture to investigate the response of the bacterium *A. denitrificans* strain SP1 toward the production of a secondary metabolite known as 25-C prodigiosin.

8.4. Summary

The major findings emerged out of this study are summarized under the following sections.

8.4.1. Influence of light conditions favouring prodigiosin production in *Achromobacter denitrificans* strain SP1.

To know the influence of different light sources on the production of prodigiosin, *A. denitrificans* SP1 (100 µL (~3×10^8 CFU) was spread uniformly along with DEHP (20 µL) over the surface of solid BSM in petriplates. Two sets of culture plates were prepared and one set was kept under a cool white fluorescent lamp (33 µmol m^{-2} s^{-1}) and the other set was kept completely under continuous darkness at an equivalent distance of 15 cm at different temperatures (20, 25, 30, 35, 40 and 45°C). In each culture plate, prodigiosin production and biomass were calculated after 4 days of incubation. After selecting the optimum temperature as 25 °C, another set of culture plates were kept at different LEDs such as red (direct light of 30 LED-63 µmol m^{-2} s^{-1}) green (direct light of 30 LED-70 µmol m^{-2} s^{-1}), blue (direct light of 30 LED-53 µmol m^{-2} s^{-1}) and white (direct light of 30 LED-63 µmol m^{-2} s^{-1}) for 4 days. To maintain a constant light intensity to the culture plates, the number of LEDs in each chamber was adjusted. The direct light intensity from each LED was maintained at ~ 35 µmol m^{-2} s^{-1} (near to FLs intensity). A

total of six light conditions were tested to know the ideal one which maximizes the production of prodigiosin. Culture plates under fluorescent lamp showed maximum prodigiosin (25.4 mg) and biomass production (210.7 mg) and white LEDs showed minimum prodigiosin (0.2 mg) and biomass production (9.1 mg). Among the LEDs, red showed maximum prodigiosin (20.9 mg) and biomass production (204.7 mg). This study has proved that both fluorescent lamp and red LEDs at 25 °C play a crucial role in maximizing the biosynthesis of prodigiosin in *A. denitrificans* SP1.

8.4.2. Response surface optimization of prodigiosin production from *Achromobacter denitrificans* strain SP1 using blood bag as the carbon source.

Commercially available PVC blood storage bag (BB) from Hindustan Latex Ltd, Thiruvananthapuram, Kerala, India was used as the only carbon source, while optimizing the growth parameters of *Achromobacter denitrificans* strain SP1 for the production of prodigiosin using Response surface methodology (RSM). To reduce the number of parameters and to find out the most influencing one, the Plackett-Burman experimental design was followed. In Plackett-Burman experimental design, five independent variables were selected such as Blood bag concentration (1 and 5 g), temperature (20 and 30°C), pH (4 and 10), agitation (100 and 200 rpm) and incubation (10 and 15 days). Twenty different combinations were suggested by the software to estimate the combined effect. Based on the results of the Plackett-Burman experimental design, the Pareto chart was constructed. The Pareto chart showed three of the parameters (pH, blood bag concentration and temperature) as the most influencing ones for the production of prodigiosin. These three parameters were further taken into the Box-Behnken design. Based on Box-Behnken experimental results, contour and three-dimensional surface plots were generated to explain the optimized conditions for the

biosynthesis of prodigiosin. Regression coefficients were estimated for the production of prodigiosin (prodigiosin production *versus* blood bag, temperature and pH). The regression model for prodigiosin production was highly significant ($p < 0.05$) with the acceptable value of determination coefficient (R^2 = 99.33 %). Validation experimental trails for the quadratic model was predicted by the point prediction method and found that pH 8.8, temperature 24°C, and blood bag (1.0g) as the optimized condition for prodigiosin production. Experimentally observed prodigiosin production was 1.314 mg/ml, while the predicted production was 1.319 mg/ml. The correlation coefficient between the experimental and predicted was 0.987 *i.e*., highly significant and fit to the predicted model. This study showed the optimum conditions for the production of prodigiosin by *A. denitrificans* SP1, using blood bag as the carbon source.

8.4.3. Response surface optimization of prodigiosin production from *A. denitrificans* strain SP1 using free DEHP as the carbon source and scale up in bioreactor

In this study, scaling up of prodigiosin production in a bioreactor and rechecking the purity of 25-C prodigiosin after optimizing the culture parameters using free DEHP as the only carbon source by Response surface methodology (RSM) was attempted. Similar to the optimization studies with blood bag, the Plackett-Burman experimental design with five independent variables such as temperature (20 and 30 °C), pH (4 and 10), DEHP (2.5 and 25 mM), incubation (4 and 8 days) and agitation (100 and 200 rpm) were attempted. Based on the results of the Plackett-Burman experimental design, the Pareto chart was constructed. Considering the three parameters (pH, temperature and DEHP concentration) observed to be relevant from the Pareto chart results, Box-Behnken design with different combinations of experiments was designed. Based on the results obtained in this experimental design,

contour and three dimensional surface plots were generated to explain the optimized conditions for the production of prodigiosin. Regression coefficients were estimated for the production of prodigiosin (Prodigiosin production *versus* pH, temperature and DEHP). The regression model for the production of prodigiosin was very much significant ($p < 0.05$) with the satisfactory value of determination coefficient (R^2 = 99.89 %). The point prediction method of validation optimized the process parameters such as DEHP concentration (8 mM), pH (9.2) and temperature (24.9 °C). The experimental results showed the optimum prodigiosin production (1.008 mg/ml) when supplemented with 8 mM DEHP, pH 9.2 and temperature 24.9 °C, where as the predicted value was 1.0365 mg/ml. The correlation coefficient value (0.99) revealed that the experimental values were highly compatible with those of the predicted values, proving the precision of the model. These optimized conditions were provided in a bioreactor setup and obtained a maximum prodigiosin production of 889.1 mg/L. The crude prodigiosin was further column purified with 5% ethyl acetate in *n*-hexane. TLC profile of the purified prodigiosin showed a single spot with an R_f value of 0.67 in the solvent system, methanol: chloroform (2:1). The purity of 25-C prodigiosin was rechecked and confirmed using a spectrophotometer and FTIR and the absorption maximum (λ_{480}) and the functional groups present in the FTIR profile substantiated the purity of 25-C prodigiosin.

8.4.4. Quorum sensing mediated response of *A. denitrificans* SP1 regarding prodigiosin production under phthalate stress.

Here, the influence of quorum sensing molecules in *A. denitrificans* SP1 towards the production of prodigiosin under DEHP stress was attempted. Homoserine lactone signal molecules are the key molecules involved in the cell to cell communication in bacteria. The bacterial community mainly uses this co-operative behavior during the time of physico-chemical or

environmental stress. Colorimetric method was used for the primary detection of lactone molecules produced by *A. denitrificans* SP1. HSLs were extracted from the culture plates using chloroform and performed the assay. HSLs productions were detected after 6 h intervals up to 4 days. A sharp increase in the HSL activity after 18 h incubation, reached a maximum at 60 h and showed a sharp decline after 72 h. The functional groups in the samples collected at 12 h intervals up to 4 days were subjected to FTIR analysis. The FTIR spectrum showed bands at 1738, 1645, 1542, 1175 and 1025 cm^{-1}, which can be assigned to characteristic vibrations of the amide and lactone functional groups, which are the basic structural moieties of homoserine lactones. 24 to 72 h showed a significant peak height at 1738 cm^{-1}, which corresponds to the C=O stretch of the lactone ring. FTIR analysis confirmed the presence of HSLs and for the actual identification of HSLs, samples of 12, 48, and 96 h were subjected to LC-Q-ToF-MS analysis. Four HSL molecules were identified with the help of LC-Q-ToF-MS analysis such as 3-oxo-C6 HSL, 3-oxo-C8 HSL, C10 HSL and 3-oxo-C12 HSL. DEHP degradation study also substantiates the LC-Q-ToF-MS results. 24 to 72 h was the peak time of DEHP degradation and LC-Q-ToF-MS analysis showed the presence of 3-oxo-C8 HSL and C10 HSL in the same period. Complete degradation of DEHP was observed from 72 h incubation and 3-oxo-C12 HSL was identified at 96 h incubation. To demonstrate the production of prodigiosin in *A. denitrificans* SP1 as quorum sensing mediated, vanillin was applied to the medium as an inhibitor, which eventually showed that the vanillin containing plates are having no prodigiosin production. The study also made clear that the addition of quorum sensing molecules in the growth medium will enhance the DEHP degradation property and concomitant production of prodigiosin by *A. denitrificans* SP1.

8.4.5. In vitro studies for the drugability of 25-C prodigiosin

The antibacterial, antifungal and antioxidant activity of 25-C prodigiosin from *A. denitrificans* SP1 was assessed. Disc diffusion method was performed to check the antibacterial activity of 25-C prodigiosin and found that it is effective against the tested pathogenic bacterial strains such as *Proteus mirabilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeroginosa*, *Escherichia coli* and *Acinetobacter baumannii* with a zone of inhibition of 16, 15, 18, 15, 11 and 12 mm, respectively. Broth dilution method was used to evaluate the antifungal activity and observed a strong antifungal activity against the fungal strains such as *Candida tropicalis*, *Purpureocillium lilacinum*, *Trichoderma viridae*, *Trichoderma harzianum*, *Aspergillus flavus*, *Aspergillus heteromorphus* and *Penicillium verruculosum* with a minimum inhibitory concentration of 75, 275, 125, 125, 100, 150 and 150 µg/ml respectively. DPPH free radical scavenging assay revealed that 25-C prodigiosin is a good antioxidant agent with 99 % scavenging in 150 µg/ml prodigiosin concentration. This study highlights the application of 25-C prodigiosin at the pharmaceutical level.

8.5. Conclusions

The influence of different light sources on the prodigiosin production was analyzed. Statistically optimized the cultural parameters along with blood bag (*in situ*) and free DEHP (*ex situ*) for maximizing the production of prodigiosin through Response Surface Methodology. Identified and characterized the quorum sensing molecules that are indirectly controlling the degradation of DEHP and the prodigiosin production in *A. denitrificans* SP1. Prodigiosin production was scaled up using a bioreactor and the purity of 25-C prodigiosin was rechecked. Antibacterial, antifungal and antioxidant activity of 25-C prodigiosin was evaluated.

8.6. Major outcomes/Deliverables

- Influence of six different light conditions on the production of 25-C prodigiosin was identified
- Statistically optimized the cultural parameters for maximizing the production of prodigiosin by utilizing blood bag (*in situ*) as the only carbon source.
- Statistically optimized the cultural parameters for maximizing the production of prodigiosin, utilizing free DEHP (*ex situ*) as the only carbon source.
- Identified and demonstrated the quorum sensing mechanism involved in the degradation of DEHP and the concomitant production of prodigiosin from *A. denitrificans* SP1.
- Confirmed the pharmaceutical importance of 25-C prodigiosin through antibacterial, antifungal and antioxidant assays.

BIBLIOGRAPHY

Agarwal, S. K., Singh, S. S., Verma, S., & Kumar, S. (2000). Antifungal activity of anthraquinone derivatives from *Rheum emodi*. *Journal of Ethnopharmacology*, 72(1-2), 43-46.

Ahmad, W. A., Ahmad, W. Y. W., Zakaria, Z. A., & Yusof, N. Z. (2012). Application of bacterial pigments as colorant. 57-74. Springer, Berlin, Heidelberg.

Ahn, J. Y., Kim, Y. H., Min, J., & Lee, J. (2006). Accelerated degradation of dipentyl phthalate by *Fusarium oxysporum* f. sp. *pisi cutinase* and toxicity evaluation of its degradation products using bioluminescent bacteria. *Current Microbiology*, 52(5), 340-344.

Akilandeswari, P., & Pradeep, B. V. (2017). Microbial pigments: potential functions and prospects. *Bio-pigmentation and Biotechnological Implementations*, 241-261.

Akin-Osanaiye, B. C., Aruwa, I. N., & Olobayotan, I. W. (2019). Isolation of *Serratia marcescens* from the Soil and *in vitro* prodigiosin production as source of antibiotic, active against oxacillin-resistant *Staphylococcus aureus*. *South Asian Journal of Research in Microbiology*, 1-9.

Aleshchenkova, Z. M., Samsonova, A. S., Semochkina, N. F., Baikova, S. V., & Tolstolutskaya, L. I. (1997). Utilization of isophthalic acid esters by *Rhodococci*. *Microbiology (New York, NY)*, 66(5), 515-518.

Ali, M. I., Ahmed, S., Robson, G., Javed, I., Ali, N., Atiq, N., & Hameed, A. (2014). Isolation and molecular characterization of polyvinyl chloride

(PVC) plastic degrading fungal isolates. *Journal of Basic Microbiology*, 54(1), 18-27.

Alihosseini, F., Ju, K. S., Lango, J., Hammock, B. D., & Sun, G. (2008). Antibacterial colorants: characterization of prodiginines and their applications on textile materials. *Biotechnology Progress*, 24(3), 742-747.

Allen, G. R., Reichelt, J. L., and Gray, P. P. (1983). Influence of environmental factors and medium composition on Vibrio gazogenes growth and prodigiosin production. *Applied and Environmental Microbiology*, 5, 1727–1732.

Andersen, D. O., Weber, N. D., Wood, S. G., Hughes, B. G., Murray, B. K., & North, J. A. (1991). In vitro virucidal activity of selected anthraquinones and anthraquinone derivatives. *Antiviral Research*, 16(2), 185-196.

Andreyeva, I. N., & Ogorodnikova, T. I. (2015). Pigmentation of *Serratia marcescens* and spectral properties of prodigiosin. *Microbiology*, 84(1), 28-33.

Antony, M., & Jayachandran, K. (2016). Regulation of acyl homoserine lactone synthesis in *Pseudomonas putida* JMQS1 under phenol stress. *Water, Air & Soil Pollution*, 227(9):338.

Arivizhivendhan, K. V., Mahesh, M., Boopathy, R., Swarnalatha, S., Mary, R. R., & Sekaran, G. (2018). Antioxidant and antimicrobial activity of bioactive prodigiosin produces from *Serratia marcescens* using agricultural waste as a substrate. *Journal of Food Science and Technology*, 55(7), 2661-2670

Aruldass, C. A., Venil, C. K., Zakaria, Z. A., & Ahmad, W. A. (2014). Brown sugar as a low-cost medium for the production of prodigiosin by locally isolated *Serratia marcescens* UTM1. *International Biodeterioration & Biodegradation*, 95, 19-24.

Bae, J., Moon, H., Oh, K. K., Kim, C. H., Lee, D. S., Kim, S. W., & Hong, S. I. (2001). A novel bioreactor with an internal adsorbent for integrated fermentation and recovery of prodigiosin-like pigment produced from *Serratia* sp. KH-95. *Biotechnology Letters*, 23(16), 1315-1319.

Balasubramaniam, B., Alexpandi, R., & Darjily, D. R. (2019). Exploration of the optimized parameters for bioactive prodigiosin mass production and its biomedical applications in vitro as well as in silico. *Biocatalysis and Agricultural Biotechnology*, 22, 101385.

Banerjee, G., & Ray, A. K. (2017). Quorum-sensing network-associated gene regulation in Gram-positive bacteria. *Acta microbiologica et immunologica Hungarica*, 64(4), 439-453.

Barbey, C., Chane, A., Burini, J. F., Maillot, O., Merieau, A., Gallique, M., & Latour, X. (2018). A rhodococcal transcriptional regulatory mechanism detects the common lactone ring of AHL quorum-sensing signals and triggers the quorum-quenching response. *Frontiers in Microbiology*, *9*, 2800.

Baron, S. S., & Rowe, J. J. (1981). Antibiotic action of pyocyanin. *Antimicrobial Agents and Chemotherapy*, 20(6), 814-820.

Begum, A., Katsumata, H., Kaneco, S., Suzuki, T., & Ohta, K. (2003). Biodegradation of phthalic acid esters by bakery yeast *Saccharomyces cerevisiae*. *Bulletin of Environmental Contamination and Toxicology***,** 70,255–261.

Beko, G., Weschler, C. J., Langer, S., Callesen, M., Toftum, J., & Clausen, G. (2013). Children's phthalate intakes and resultant cumulative exposures estimated from urine compared with estimates from dust ingestion, inhalation and dermal absorption in their homes and daycare centers. *PloS one*, 8(4).

Benjamin, S., Masai, E., Kamimura, N., Takahashi, K., Anderson, R. C., & Faisal, P. A. (2017). Phthalates impact human health: epidemiological evidences and plausible mechanism of action. *Journal of Hazardous Materials*, 340, 360-383.

Benjamin, S., Pradeep, S., Balachandran, S., Sudhadevi, R., Sarath Josh, M. K., Sadasivam, R., & Thirugnanam, P.E. (2012). A preparation of novel pharmaceutically active compounds through bio-remediation. *Indian Patent Application No. 3958/CHE/2012***.**

Benjamin, S., Pradeep, S., Sarath Josh, M. K., Kumar, S., & Masai, E. (2015). A monograph on the remediation of hazardous phthalates. *Journal of Hazardous Materials*, 298, 58-72.

Bennett, J. W., & Bentley, R. (2000). Seeing red: the story of prodigiosin. *Advances in Applied Microbiology*, 49, 1-32

Berg, G. (2000). Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *Journal of Applied Microbiology*, 88(6), 952-960.

Berry, A., Janssens, D., Humbelin, M., Jore, J. P., Hoste, B., Cleenwerck, I., & Shanmugam, B. (2003). *Paracoccus zeaxanthinifaciens* sp. nov., a zeaxanthin-producing bacterium. *International Journal of Systematic and Evolutionary Microbiology*, 53(1), 231-238.

Bertelsen, R. J., Carlsen, K. C. L., Calafat, A. M., Hoppin, J. A., Haland, G., Mowinckel, P., Carlsen,K. H., & Lovik, M. (2013). Urinary

biomarkers for phthalates associated with asthma in Norwegian children. *Environmental Health Perspectives*, 121(2), 251-256.

Bhosale, P., & Bernstein, P. S. (2004). β-Carotene production by *Flavobacterium multivorum* in the presence of inorganic salts and urea. *Journal of Industrial Microbiology and Biotechnology*, 31(12), 565-571.

Bhowmick, A. K., & Stephens, H. (Eds.). (2000). *Handbook of Elastomers*. CRC Press, New York.

Boll, M., Geiger, R., Junghare, M., & Schink, B. (2020). Microbial degradation of phthalates: biochemistry and environmental implications. *Environmental Microbiology Reports*, 12(1), 3-15.

Boric, M., Danevcic, T., & Stopar, D. (2011). Prodigiosin from *Vibrio* sp. DSM 14379; a new UV-protective pigment. *Microbial Ecology*, 62(3), 528.

Brambilla, U., Nasini, G., Petrolini, B., Quaroni, S., Saracchi, M., & Fedeli, L. (1995). Prodigiosin-like and other metabolites produced by a *Streptoverticillium* strain. *Actinomycetes*, 6, 63-70.

Brelles-Marino, G., & Bedmar, E. J. (2001). Detection, purification and characterisation of quorum-sensing signal molecules in plant-associated bacteria. *Journal of Biotechnology*, 91(2-3), 197-209.

Cang, S., Sanada, M., Johdo, O., Ohta, S., Nagamatsu, Y., & Yoshimoto, A. (2000). High production of prodigiosin by *Serratia marcescens* grown on ethanol. *Biotechnology Letters*, 22(22), 1761-1765.

Castro, A. J. (1967). Antimalarial activity of prodigiosin. *Nature*, 213(5079), 903-904.

Cedano, M., Villasenor, L., & Guzman-Davalos, L. (2001). Some Aphyllophorales tested for organic dyes. *Mycologist*, 2(15), 81-85.

Cerda-Olmedo, E. (2001). Phycomyces and the biology of light and color. *FEMS Microbiology Reviews*, 25(5), 503-512.

Chai, W., Suzuki, M., Handa, Y., Murakami, M., Utsukihara, T., Honma, Y., & Horiuchi, C. A. (2008). Biodegradation of di-(2ethylhexyl) phthalate by fungi. *Report of National Food Research Institute*, 72, 83-87.

Chane, A., Barbey, C., Bourigault, Y., Maillot, O., Rodrigues, S., Bouteiller, M., Merieau, A., Konto-Ghiorghi, Y., Beury-cirou, A., Gattin, R., & Latour, X. (2019). A flavor lactone mimicking AHL quorum-sensing signals exploits the broad affinity of the QsdR regulator to stimulate transcription of the rhodococcal qsd operon involved in quorum-quenching and biocontrol activities. *Frontiers in Microbiology*, 10, 786.

Chang, C. C., Chen, W. C., Ho, T. F., Wu, H. S., & Wei, Y. H. (2011). Development of natural anti-tumor drugs by microorganisms. *Journal of Bioscience and Bioengineering*, *111*(5), 501-511.

Chao, W. L., Lin, C. M., Shiung, I. I., & Kuo, Y. L. (2006). Degradation of di-butyl-phthalate by soil bacteria. *Chemosphere*, 63(8), 1377-1383.

Chatterjee, S., & Dutta, T. K. (2003). Metabolism of butyl benzyl phthalate by *Gordonia* sp. strain MTCC 4818. *Biochemical and Biophysical Research Communications*, 309(1), 36-43.

Chen, C. Y. (2010). The oxidation of di-(2-ethylhexyl) phthalate (DEHP) in aqueous solution by UV/H 2 O 2 photolysis. *Water, Air, & Soil Pollution*, 209(1-4), 411-417.

Chen, J. A., Li, X., Li, J., Cao, J., Qiu, Z., Zhao, Q., & Shu, W. (2007). Degradation of environmental endocrine disruptor di-2-ethylhexyl phthalate by a newly discovered bacterium, *Microbacterium* sp. strain CQ0110Y. *Applied Microbiology and Biotechnology*, 74(3), 676-682.

Chen, W. C., Yu, W. J., Chang, C. C., Chang, J. S., Huang, S. H., Chang, C. H., & Wei, Y. H. (2013). Enhancing production of prodigiosin from *Serratia marcescens* C3 by statistical experimental design and porous carrier addition strategy. *Biochemical Engineering Journal*, 78, 93-100.

Chen, Y. C., Zhan, H. Y., Chen, Z. H., Fu, S. Y., & Zhang, X. Y. (2003). Coupled anaerobic/aerobic biodegradation of 2, 4, 6 trichlorophenol. *Journal of Environmental Sciences*, 15(4), 469-474.

Chew, B. P., Park, J. S., Wong, M. W., & Wong, T. S. (1999). A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. *Anticancer Research*, 19(3A), 1849-1853.

Chopra, V., Harley, K., Lahiff, M., & Eskenazi, B. (2014). Association between phthalates and attention deficit disorder and learning disability in US children, 6–15 years. *Environmental Research*, 128, 64-69.

Chu, W., Vattem, D. A., Maitin, V., Barnes, M. B., & McLean, R. J. (2011). Bioassays of quorum sensing compounds using *Agrobacterium tumefaciens* and *Chromobacterium violaceum*. *Quorum Sensing* (3-19). Humana Press.

Churchill, M. E., & Chen, L. (2011). Structural basis of acyl-homoserine lactone-dependent signaling. *Chemical Reviews*, 111(1), 68-85.

Cobellis, L., Latini, G., Felice, C. D., Razzi, S., Paris, I., Ruggieri, F., & Petraglia, F. (2003). High plasma concentrations of di-(2-ethylhexyl) -

phthalate in women with endometriosis. *Human Reproduction*, 18(7), 1512-1515.

Coenye, T., Vancanneyt, M., Cnockaert, M. C., Falsen, E., Swings, J., & Vandamme, P. (2003). *Kerstersia gyiorum* gen. nov., sp. nov., a novel *Alcaligenes faecalis*-like organism isolated from human clinical samples, and reclassification of *Alcaligenes denitrificans* Ruger and Tan 1983 as *Achromobacter denitrificans* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 53(6), 1825-1831.

Coulthurst, S. J., Kurz, C. L., & Salmond, G. P. (2004). luxS mutants of *Serratia* defective in autoinducer-2-dependent 'quorum sensing'show strain-dependent impacts on virulence and production of carbapenem and prodigiosin. *Microbiology*, 150(6), 1901-1910.

Crow, M. A. (2002). The genetic regulation of pigment and antibiotic synthesis in *Serratia* sp. (Doctoral dissertation, University of Cambridge).

Danevcic, T., Boric Vezjak, M., Tabor, M., Zorec, M., & Stopar, D. (2016). Prodigiosin induces autolysins in actively grown *Bacillus subtilis* cells. *Frontiers in Microbiology*, 7, 27.

Danevcic, T., Boric Vezjak, M., Zorec, M., & Stopar, D. (2016). Prodigiosin-A multifaceted *Escherichia coli* antimicrobial agent. *PLoS One*, 11(9), e0162412.

Daoust, J. Y., & Gerber, N. N. (1974). Isolation and purification of prodigiosin from *Vibrio psychroerythrus*. *Journal of Bacteriology*, 118(2), 756-757.

Dargnat, C., Teil, M. J., Chevreuil, M., & Blanchard, M. (2009). Phthalate removal throughout wastewater treatment plant: case study of Marne Aval station (France). *Science of the Total Environment*, 407(4), 1235-1244.

Darshan, N., & Manonmani, H. K. (2015). Prodigiosin and its potential applications. *Journal of Food Science and Technology*, 52(9), 5393-5407.

Das, M. T., Ghosh, P., & Thakur, I. S. (2014). Intake estimates of phthalate esters for South Delhi population based on exposure media assessment. *Environmental Pollution*, 189, 118-125.

Deepika, S., & Madhuri, R. J. (2015). Biodegradation of low density polyethylene by micro-organisms from garbage soil. *Journal of Experimental Biology and Agricultural Sciences*, 3(1), 15-21.

Du, Y. Y., Fang, Y. L., Wang, Y. X., Zeng, Q., Guo, N., Zhao, H., & Li, Y. F. (2016). Follicular fluid and urinary concentrations of phthalate metabolites among infertile women and associations with in vitro fertilization parameters. *Reproductive Toxicology*, 61, 142-150.

Dufosse, L. (2006). Microbial production of food grade pigments. *Food Technology and Biotechnology*, 44(3), 313-323.

Duty, S. M., Silva, M. J., Barr, D. B., Brock, J. W., Ryan, L., Chen, Z., Herrick, R. F., Christiani, D.C., & Hauser, R. (2003). Phthalate exposure and human semen parameters. *Epidemiology*, 269-277.

Eaton, R. W., & Ribbons, D. W. (1982). Metabolism of dimethylphthalate by *Micrococcus* sp. strain 12B. *Journal of Bacteriology*, 151(1), 465-467.

El-Batal, A. I., El-Hendawy, H. H., & Faraag, A. H. (2017). In silico and in vitro cytotoxic effect of prodigiosin-conjugated silver nanoparticles on liver cancer cells (HepG2). *BioTechnologia. Journal of Biotechnology Computational Biology and Bionanotechnology*, 98(3).

Elkenawy, N. M., Yassin, A. S., Elhifnawy, H. N., & Amin, M. A. (2017). Optimization of prodigiosin production by *Serratia marcescens* using crude glycerol and enhancing production using gamma radiation. *Biotechnology Reports*, 14, 47-53.

Evans, W. C., Fernley, H. N., & Griffiths, E. (1965). Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads. The ring-fission mechanism. *Biochemical Journal*, 95(3), 819-831.

Feher, D., Barlow, R. S., Lorenzo, P. S., & Hemscheidt, T. K. (2008). A 2-substituted prodiginine, 2-(p-hydroxybenzyl) prodigiosin, from *Pseudoalteromonas rubra*. *Journal of Natural Products*, 71(11), 1970-1972.

Fender, J. E., Bender, C. M., Stella, N. A., Lahr, R. M., Kalivoda, E. J., & Shanks, R. M. (2012). *Serratia marcescens* quinoprotein glucose dehydrogenase activity mediates medium acidification and inhibition of prodigiosin production by glucose. *Applied and Environmental Microbiology*, 78(17), 6225-6235.

Florencio, J. A., Soccol, C. R., Furlanetto, L. F., Bonfim, T. M. B., Krieger, N., Baron, M., & Fontana, J. D. (1998). A factorial approach for a sugarcane juice-based low cost culture medium: increasing the astaxanthin production by the red yeast *Phaffia rhodozyma*. *Bioprocess Engineering*, 19(3), 161-164.

Fourne, F. (1999). *Synthetic fibers: machines and equipment, manufacture, properties*. Hanser.

Frederiksen, H., Sorensen, K., Mouritsen, A., Aksglaede, L., Hagen, C. P., Petersen, J. H., Shakkebaek, N. E., Anderson, A., & Juul, A. (2012). High urinary phthalate concentration associated with delayed pubarche in girls. *International Journal of Andrology*, 35(3), 216-226.

Frederix, M., & Downie, J. A. (2011). Quorum sensing: regulating the regulators. *Advances in Microbial Physiology* 58, 23-80.

Fromme, H., Kuchler, T., Otto, T., Pilz, K., Muller, J., & Wenzel, A. (2002). Occurrence of phthalates and bisphenol A and F in the environment. *Water Research*, 36(6), 1429-1438.

Fu, P. Q., Kawamura, K., Pavuluri, C. M., Swaminathan, T., & Chen, J. (2010). Molecular characterization of urban organic aerosol in tropical India: contributions of primary emissions and secondary photooxidation. *Atmospheric Chemistry and Physics*, 10(6), 2663-2689.

Fuente, M. D. L., Miranda, C. D., Jopia, P., Gonzalez-Rocha, G., Guiliani, N., Sossa, K., & Urrutia, H. (2015). Growth inhibition of bacterial fish pathogens and quorum-sensing blocking by bacteria recovered from Chilean salmonid farms. *Journal of Aquatic Animal Health*, 27(2), 112-122.

Gani, K. M., & Kazmi, A. A. (2016). Comparative assessment of phthalate removal and risk in biological wastewater treatment systems of developing countries and small communities. *Science of the Total Environment*, 569, 661-671.

Ganji, S. H., Karigar, C. S., & Pujar, B. G. (1995). Metabolism of dimethylterephthalate by *Aspergillus niger*. *Biodegradation*, 6(1), 61-66.

Gao, D., Li, Z., Wen, Z., & Ren, N. (2014). Occurrence and fate of phthalate esters in full scale domestic wastewater treatment plants and their impact on receiving waters along the Songhua River in China. *Chemosphere***,** 95, 24-32.

Gartshore, J., Cooper, D. G., & Nicell, J. A. (2003). Biodegradation of plasticizers by *Rhodotorula rubra*. *Environmental Toxicology and Chemistry: An International Journal*, 22(6), 1244-1251.

Gauthier, M. J. (1976). *Alteromonas rubra* sp. nov., a new marine antibiotic-producing bacterium. *International Journal of Systematic and Evolutionary Microbiology*, 26(4), 459-466.

Genes, C., Baquero, E., Echeverri, F., Maya, J. D., & Triana, O. (2011). Mitochondrial dysfunction in *Trypanosoma cruzi*: the role of *Serratia marcescens* prodigiosin in the alternative treatment of Chagas disease. *Parasites & vectors*, 4(1), 1-8.

Gerber, N. N. (1969). Prodigiosin-like pigments from *Actinomadura* (Nocardia) *pelletieri* and *Actinomadura madurae*. *Applied and Environmental Microbio*logy, 18(1), 1-3.

Gerber, N. N. (1975). Prodigiosin-like pigments. *CRC Critical Reviews in Microbiology*, 3(4), 469-485.

Giri, A. V., Anandkumar, N., Muthukumaran, G., & Pennathur, G. (2004). A novel medium for the enhanced cell growth and production of prodigiosin from *Serratia marcescens* isolated from soil. *BMC Microbiology*, 4(1), 11.

Giri, B., Patel, K. S., Jaiswal, N. K., Sharma, S., Ambade, B., Wang, W., Simonich, S. L. M., & Simoneit, B. R. T. (2013). Composition and sources of organic tracers in aerosol particles of industrial central India. *Atmospheric Research*., 120-121, 312–324.

Goel, A. K., Rajagopal, L., Nagesh, N., & Sonti, R. V. (2002). Genetic locus encoding functions involved in biosynthesis and outer membrane localization of xanthomonadin in *Xanthomonas oryzae* pv. *oryzae*. *Journal of Bacteriology*, 184(13), 3539-3548

Gondil, V. S., Asif, M., & Bhalla, T. C. (2017). Optimization of physicochemical parameters influencing the production of prodigiosin from *Serratia nematodiphila* RL2 and exploring its antibacterial activity. *3 Biotech*, 7(5), 338.

Gulani, C., Bhattacharya, S., & Das, A. (2012). Assessment of process parameters influencing the enhanced production of prodigiosin from *Serratia marcescens* and evaluation of its antimicrobial, antioxidant and dyeing potentials. *Malaysian Journal of Microbiol*ogy, 8(2), 116-122.

Gupta, S., & Gadi, R. (2018). Temporal variation of phthalic acid esters (PAEs) in ambient atmosphere of Delhi. *Bulletin of Environmental Contamination and Toxicology*, 101(2), 153-159.

Hammer, B. K., & Bassler, B. L. (2003). Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Molecular Microbiology*, 50(1), 101-104.

Han, S. B., Kim, H. M., Kim, Y. H., Lee, C. W., Jang, E. S., Son, K. H., & Kim, Y. K. (1998). T-cell specific immunosuppression by prodigiosin

isolated from *Serratia marcescens*. *International Journal of Immunopharmacology*, 20(1-3), 1-13.

Hareesh, E. S., Faisal, P. A., & Benjamin, S. (2016). Optimization of parameters for the production of cellulase from *Achromobacter xylosoxidans* BSS4 by solid-state fermentation. *Electronic Journal of Biology*, 12(4), 432-437.

Harris, A. K., Williamson, N. R., Slater, H., Cox, A., Abbasi, S., Foulds, I., Simonsen, H. T., Leeper, F. J., & Salmond, G. P. (2004). The *Serratia* gene cluster encoding biosynthesis of the red antibiotic, prodigiosin, shows species-and strain-dependent genome context variation. *Microbiology*, 150(11), 3547-3560.

Hashizume, K., Nanya, J., Toda, C., Yasui, T., Nagano, H., & Kojima, N. (2002). Phthalate esters detected in various water samples and biodegradation of the phthalates by microbes isolated from river water. *Biological and Pharmaceutical Bulletin*, 25(2), 209-214.

Hauser, R., Gaskins, A. J., Souter, I., Smith, K. W., Dodge, L. E., Ehrlich, S., & Earth Study Team. (2016). Urinary phthalate metabolite concentrations and reproductive outcomes among women undergoing in vitro fertilization: results from the EARTH study. *Environmental Health Perspectives*, 124(6), 831-839.

Hauser, R., Meeker, J. D., Duty, S., Silva, M. J., & Calafat, A. M. (2006). Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. *Epidemiology*, 682-691.

Hauser, R., Meeker, J. D., Singh, N. P., Silva, M. J., Ryan, L., Duty, S., & Calafat, A. M. (2007). DNA damage in human sperm is related to

urinary levels of phthalate monoester and oxidative metabolites. *Human Reproduction*, 22(3), 688-695.

Hausmann, A., & Sandmann, G. (2000). A single five-step desaturase is involved in the carotenoid biosynthesis pathway to β-carotene and torulene in *Neurospora crassa*. *Fungal Genetics and Biology*, 30(2), 147-153.

He, L., Fan, S., Müller, K., Wang, H., Che, L., Xu, S., Song, Z., Yuan, G., Rinklebe.J., Tsang, D., Ok, Y.S., & Bohan,N.S. (2018). Comparative analysis biochar and compost-induced degradation of di-(2-ethylhexyl) phthalate in soils. *Science of the Total Environment*, 625, 987-993.

He, Z., Xiao, H., Tang, L., Min, H., & Lu, Z. (2013). Biodegradation of di-n-butyl phthalate by a stable bacterial consortium, HD-1, enriched from activated sludge. *Bioresource Technology*, 128, 526-532.

Heudorf, U., Mersch-Sundermann, V., & Angerer, J. (2007). Phthalates: toxicology and exposure. *International journal of hygiene and environmental health*, 210(5), 623-634.

Hines, E. P., Calafat, A. M., Silva, M. J., Mendola, P., & Fenton, S. E. (2009). Concentrations of phthalate metabolites in milk, urine, saliva, and serum of lactating North Carolina women. *Environmental Health Perspectives*, 117(1), 86-92.

Hong, M.Y., Seeram, N. P., Zhang, Y., & Heber, D. (2008). Anticancer effects of Chinese red yeast rice versus monacolin K alone on colon cancer cells. *The Journal of Nutritional Biochemistry*. 19, 448–58.

Hsu, L. C., Hsu, Y. W., Liang, Y. H., Kuo, Y. H., & Pan, T. M. (2011). Anti-tumor and anti-inflammatory properties of ankaflavin and monaphilone

A from *Monascus purpureus* NTU 568. *Journal of Agricultural and Food Chemistry*, 59(4), 1124-1130.

Hu, H., He, J., Liu, J., Yu, H., Tang, J., & Zhang, J. (2016). Role of N-acyl-homoserine lactone (AHL) based quorum sensing on biofilm formation on packing media in wastewater treatment process. *Rsc Advances*, 6(14), 11128-39.

Hu, D. X., Withall, D. M., Challis, G. L., & Thomson, R. J. (2016). Structure, chemical synthesis, and biosynthesis of prodiginine natural products. *Chemical Reviews*, 116(14), 7818-7853.

Hu, J., & Yang, Q. (2015). Microbial degradation of di-n-butyl phthalate by *Micrococcus* sp. immobilized with polyvinyl alcohol. *Desalination and Water Treatment*, 56(9), 2457-2463.

Huang, J., Nkrumah, P. N., Li, Y., & Appiah-Sefah, G. (2013). Chemical behavior of phthalates under abiotic conditions in landfills. *Reviews of Environmental Contamination and Toxicology*, 224, 39-52.

Huang, Y. L., Li, M., Yu, Z., & Qian, P. Y. (2011). Correlation between pigmentation and larval settlement deterrence by *Pseudoalteromonas* sp. sf57. *Biofouling*, *27*(3), 287-293.

Huang, H. B., Chen, H. Y., Su, P. H., Huang, P. C., Sun, C. W., Wang, C. J., Chen, H. Y., Hsiung, C. A.,& Wang, S. L. (2015). Fetal and childhood exposure to phthalate diesters and cognitive function in children up to 12 years of age: Taiwanese maternal and infant cohort study. *PloS one*, 10(6).

Huerta-Fontela, M., & Ventura, F. (2008). Traceability of Emerging Contaminants from Wastewater to Drinking Water. *The Handbook of Environmental Chemistry*. Springer, Berlin, Heidelberg.

Hwang, S. S., Lee, S. J., Kim, H. K., Ka, J. O., Kim, K. J., & Song, H. G. (2008). Biodegradation and saccharification of wood chips of *Pinus strobus* and *Liriodendron tulipifera* by white rot fungi. *Journal of Microbiology Biotechnol*ogy, 18, 1819-1825.

Isaka, M., Jaturapat, A., Kramyu, J., Tanticharoen, M., & Thebtaranonth, Y. (2002). Potent in vitro antimalarial activity of metacycloprodigiosin isolated from *Streptomyces spectabilis* BCC 4785. *Antimicrobial Agents and Chemotherapy*, 46(4), 1112-1113.

Jeong, H., Yim, J. H., Lee, C., Choi, S. H., Park, Y. K., Yoon, S. H., & Park, K. H. (2005). Genomic blueprint of *Hahella chejuensis*, a marine microbe producing an algicidal agent. *Nucleic acids Research*, 33(22), 7066-7073.

Jianlong, W., Lujun, C., Hanchang, S., & Yi, Q. (2000). Microbial degradation of phthalic acid esters under anaerobic digestion of sludge. *Chemosphere*, 41(8), 1245-1248.

Jimtha, C. J., Jishma, P., Sreelekha, S., Chithra, S., & Radhakrishnan, E. K. (2017). Antifungal properties of prodigiosin producing rhizospheric *Serratia* sp. *Rhizosphere*, 3, 105-108.

Joensen, U. N., Frederiksen, H., Jensen, M. B., Lauritsen, M. P., Olesen, I. A., Lassen, T. H., Andersson, A. M. & Jorgensen, N. (2012). Phthalate excretion pattern and testicular function: a study of 881 healthy Danish men. *Environmental Health Perspectives*, *120*(10), 1397-1403.

Jones, J. D., Hohn, T. M., & Leathers, T. D. (2004). Genetically modified strains of *Fusarium sporotrichioides* for production of lycopene and β-carotene. *Society of Industrial Microbiology Annual Meeting, San Diego, USA* (Vol. 91).

Junghare, M., Spiteller, D., & Schink, B. (2016). Enzymes involved in the anaerobic degradation of ortho-phthalate by the nitrate-reducing bacterium *Azoarcus* sp. strain PA01. *Environmental Microbiology*, 18(9), 3175-3188.

Kalivoda, E. J., Stella, N. A., Aston, M. A., Fender, J. E., Thompson, P. P., Kowalski, R. P., & Shanks, R. M. (2010). Cyclic AMP negatively regulates prodigiosin production by *Serratia marcescens*. *Research in Microbiology*, 161(2), 158-167.

Kang, S. G., Jin, W., Bibb, M., & Lee, K. J. (1998). Actinorhodin and undecylprodigiosin production in wild-type and rel A mutant strains of *Streptomyces coelicolor* A3 (2) grown in continuous culture. *FEMS Microbiology Letters*, 168(2), 221-226.

Karimifard, S., & Moghaddam, M. R. A. (2018). Application of response surface methodology in physicochemical removal of dyes from wastewater: a critical review. *Science of the Total Environment*, 640, 772-797.

Kavlock, R., Barr, D., Boekelheide, K., Breslin, W., Breysse, P., Chapin, R., Gaido, K., Hodgson, E., Marcus, M., Shea, K., & Williams, P., (2006). NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. *Reproductive Toxicology*, 22, 291-399.

Kawasaki, T., Sakurai, F., & Hayakawa, Y. (2008). A prodigiosin from the roseophilin producer *Streptomyces griseoviridis*. *Journal of Natural Products*, 71(7), 1265-1267.

Khadir, B. U. Z. O. H. R. E. G. U. L., Zunun, G. U. L. Z. I. R. A., & Mijit, G. H. O. P. U. R. (2015). Optimization of fermentation condition for

prodigiosin by *Paeonia anomala* endophytic bacteria XJU-PA-6. *Journal of Anhui Agricultural Sciences*, 2015(18), 10.

Khalid, N. K., Devadasan, D., Aravind, U. K., & Aravindakumar, C. T. (2018). Screening and quantification of emerging contaminants in Periyar River, Kerala (India) by using high-resolution mass spectrometry (LC-Q-ToF-MS). *Environmental Monitoring and Assessment*, 190(6), 370.

Kim, B. N., Cho, S. C., Kim, Y., Shin, M. S., Yoo, H. J., Kim, J. W., Yang, Y. H., Kim, H. W., Bhang, S. Y., & Hong, Y. C. (2009). Phthalates exposure and attention-deficit/hyperactivity disorder in school-age children. *Biological Psychiatry*, 66(10), 958-963.

Kim, D. K., Kim, J. H. F., Yim, J. H., Kwon, S. K., Lee, C. H., & Lee, H. K. (2008). Red to red-the marine bacterium *Hahella chejuensis* and its product prodigiosin for mitigation of harmful algal blooms. *Journal of Microbiology and Biotechnology*, 18(10), 1621-1629.

Kim, H. S., Hayashi, M., Shibata, Y., Wataya, Y., Mitamura, T., Horii, T., & Moriyama, Y. (1999). Cycloprodigiosin hydrochloride obtained from *Pseudoalteromonas denitrificans* is a potent antimalarial agent. *Biological and Pharmaceutical Bulletin*, 22(5), 532-534.

Kim, S. J., Lee, H. K., Lee, Y. K., & Yim, J. H. (2008). Mutant selection of *Hahella chejuensis* KCTC 2396 and statistical optimization of medium components for prodigiosin yield-up. *The Journal of Microbiology*, 46(2), 183-188.

Kim, S. W., Seo, W. T., & Park, Y. H. (1997). Enhanced production of b-carotene from *Blakeslea trispora* with Span 20. *Biotechnology Letter*, 19(6), 557-560.

Kim, S. Y., Lee, S. E., Kim, Y. R., Kim, C. M., Ryu, P. Y., Choy, H. E., Chung, S. S., & Rhee, J. H. (2003). Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. *Molecular microbiology*, 48(6), 1647-1664.

Kim, Y. H., Lee, J., & Moon, S. H. (2003). Degradation of an endocrine disrupting chemical, DEHP [di-(2-ethylhexyl)-phthalate], by *Fusarium oxysporum* f. sp. pisi cutinase. *Applied Microbiology and Biotechnology*, 63(1), 75-80.

Kim, Y., & Choi, J. (2015). Dyeing properties of microbial prodiginine from *Zooshikella rubidus* for silk fabrics. *Fibers and Polymers*, 16(9), 1981-1987.

Kimyon, O., Das, T., Ibugo, A. I., Kutty, S. K., Ho, K. K., Tebben, J., & Manefield, M. (2016). *Serratia* secondary metabolite prodigiosin inhibits *Pseudomonas aeruginosa* biofilm development by producing reactive oxygen species that damage biological molecules. *Frontiers in Microbiology*, 7, 972.

Kirbaş, Z., Keskin, N., & Guner, A. (1999). Biodegradation of polyvinylchloride (PVC) by white rot fungi. *Bulletin of Environmental Contamination and Toxicology*, 63(3), 335-342.

Kleerebezem, R., Pol, L. W. H., & Lettinga, G. (1999). The role of benzoate in anaerobic degradation of terephthalate. *Applied and Environmental Microbiology*, 65(3), 1161-1167.

Koch, H. M., Wittassek, M., Brüning, T., Angerer, J., & Heudorf, U. (2011). Exposure to phthalates in 5–6 years old primary school starters in Germany—a human biomonitoring study and a cumulative risk

assessment. *International Journal of Hygiene and Environmental Health*, 214(3), 188-195.

Kolb, S. A., OLoughlin, E. J., & Gsell, T. C. (2019). Characterization of phthalate-degrading bacteria from Asian carp microbiomes and riverine sediments. *International Biodeterioration & Biodegradation*, 143, 104727.

Kong, S., Ji, Y., Liu, L., Chen, L., Zhao, X., Wang, J., Bai, Z., & Sun, Z. (2012). Diversities of phthalate esters in suburban agricultural soils and wasteland soil appeared with urbanization in China. *Environmental Pollution*, 170, 161–168.

Kot, A. M., Błazejak, S., Kurcz, A., Gientka, I., & Kieliszek, M. (2016). *Rhodotorula glutinis*-potential source of lipids, carotenoids, and enzymes for use in industries. *Applied Microbiology and Biotechnology*, 100(14), 6103-6117.

Krishna, P. S., Kumar, B. S., Raju, P., Murty, M. S. R., Rao, T. P., Charya, M. S., & Prakasham, R. S. (2015). Fermentative production of pyranone derivate I from marine *Vibrio* sp. SKMARSP9: isolation, characterization and bioactivity evaluation. *Indian Journal of Microbiology*, 55(3), 292-301.

Kumar, V., Sharma, N., & Maitra, S. S. (2017). Comparative study on the degradation of dibutyl phthalate by two newly isolated *Pseudomonas* sp. V21b and *Comamonas* sp. 51F. *Biotechnology Reports*, 15, 1-10.

Kurbanoglu, E. B., Ozdal, M., Ozdal, O. G., & Algur, O. F. (2015). Enhanced production of prodigiosin by *Serratia marcescens* MO-1 using ram horn peptone. *Brazilian Journal of Microbiology*, 46(2), 631-637.

Kurniawan, Y. N., Kitani, S., Iida, A., Maeda, A., a Nijeholt, J. L., Lee, Y. J., & Nihira, T. (2016). Regulation of production of the blue pigment indigoidine by the pseudo γ-butyrolactone receptor FarR2 in *Streptomyces lavendulae* FRI-5. *Journal of Bioscience and Bioengineering*, 121(4), 372-379.

Lambrot, R., Muczynski, V., Lecureuil, C., Angenard, G., Coffigny, H., Pairault, C.,& Rouiller-Fabre, V. (2009). Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. *Environmental Health Perspectives*, 117(1), 32-37.

Lapenda, J. C., Silva, P. A., Vicalvi, M. C., Sena, K. X. F. R., & Nascimento, S. C. (2015). Antimicrobial activity of prodigiosin isolated from *Serratia marcescens* UFPEDA 398. *World Journal of Microbiology and Biotechnology*, 31(2), 399-406.

Latini, G., De Felice, C., Presta, G., Del Vecchio, A., Paris, I., Ruggieri, F., & Mazzeo, P. (2003). In utero exposure to di-(2-ethylhexyl) phthalate and duration of human pregnancy. *Environmental Health Perspectives*, 111(14), 1783-1785.

Lazaro, J. E., Nitcheu, J., Predicala, R. Z., Mangalindan, G. C., Nesslany, F., Marzin, D., & Diquet, B. (2002). Heptyl prodigiosin, a bacterial metabolite, is antimalarial in vivo and non-mutagenic in vitro. *Journal of Natural Toxins*, 11(4), 367-377.

Lee, S. M., Lee, J. W., Koo, B. W., Kim, M. K., Choi, D. H., & Choi, I. G. (2007). Dibutyl phthalate biodegradation by the white rot fungus, *Polyporus brumalis*. *Biotechnology and Bioengineering*, 97(6), 1516-1522.

Lee, S. M., Koo, B. K., Lee, S. S., Kim, M. K., Choi, D. H., Hong, E. J., Jeung, E. B., & Choi, I. G. (2004). Biodegradation of dibutylphthalate by white rot fungi and evaluation on its estrogenic activity. *Enzyme and Microbial Technology,* 35 (5), 417-423.

Leejae, S., Hasap, L., & Voravuthikunchai, S. P. (2013). Inhibition of staphyloxanthin biosynthesis in *Staphylococcus aureus* by rhodomyrtone, a novel antibiotic candidate. *Journal of Medical Microbiology*, 62(3), 421-428.

Li, F., Liu, Y., Wang, D., Zhang, C., Yang, Z., Lu, S., & Wang, Y. (2018). Biodegradation of di-(2-ethylhexyl) phthalate by a halotolerant consortium LF. *PloS one*, 13(10), e0204324.

Li, J., Gu, J. D., & Yao, J. H. (2005). Degradation of dimethyl terephthalate by *Pasteurella multocida* Sa and *Sphingomonas paucimobilis* Sy isolated from mangrove sediment. *International Biodeterioration & Biodegradation*, 56(3), 158-165.

Li, J., Luo, F., Chu, D., Xuan, H., & Dai, X. (2017). Complete degradation of dimethyl phthalate by a *Comamonas testosterone* strain. *Journal of Basic Microbiology*, 57(11), 941-949.

Li, X. H., Ma, L. L., Liu, X. F., Fu, S., Cheng, H. X., & Xu, X. B. (2006). Phthalate ester pollution in urban soil of Beijing, People's Republic of China. *Bulletin of Environmental Contamination and Toxicology*, 77, 252–259.

Liang, D. W., Zhang, T., Fang, H. H., & He, J. (2008). Phthalates biodegradation in the environment. *Applied Microbiology and Biotechnology*, 80(2), 183.

Lin, L. C., Wang, S. L., Chang, Y. C., Huang, P. C., Cheng, J. T., Su, P. H., & Liao, P. C. (2011). Associations between maternal phthalate exposure and cord sex hormones in human infants. *Chemosphere*, 83(8), 1192-1199.

Lin, S., Ku, H. Y., Su, P. H., Chen, J. W., Huang, P. C., Angerer, J., & Wang, S. L. (2011). Phthalate exposure in pregnant women and their children in central Taiwan. *Chemosphere*, 82(7), 947-955.

Liu, B., Wei, D., Lu, S., Zhou, W., Shen, Y., Xu, R., & Wang, J. (2010). Algicidal activity of prodigiosin against harmful algae. *China Environmental Science*, 30(4), 477-482.

Liu, G. Y., & Nizet, V. (2009). Color me bad: microbial pigments as virulence factors. *Trends in Microbiology*, 17(9), 406-413.

Liu, R., Cui, C. B., Duan, L., Gu, Q. Q., & Zhu, W. M. (2005). Potent *in vitro* anticancer activity of metacycloprodigiosin and undecylprodigiosin from a sponge-derived actinomycete *Saccharopolyspora* sp. nov. *Archives of Pharmacal Research*, 28(12), 1341-1344

Liu, Y., Chen, Z., & Shen, J. (2013). Occurrence and removal characteristics of phthalate esters from typical water sources in Northeast China. *Journal of Analitical Methods in Chemistry***,** 2013, 1-8.

Lopez-Roldan, P., De Alda, M. L., & Barcelo, D. (2004). Simultaneous determination of selected endocrine disrupters (pesticides, phenols and phthalates) in water by in-field solid-phase extraction (SPE) using the prototype PROFEXS followed by on-line SPE (PROSPEKT) and analysis by liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry. *Analytical and Bioanalytical Chemistry*, 378(3), 599-609.

Lorquin, J., Molouba, F., & Dreyfus, B. L. (1997). Identification of the carotenoid pigment canthaxanthin from photosynthetic *bradyrhizobium* strains. *Applied and Environmental Microbiology*, 63(3), 1151-1154.

Lu, H., Mo, C. H., Zhao, H. M., Xiang, L., Katsoyiannis, A., Li, Y. W., Cai, Q.Y., & Wong, M. H. (2018). Soil contamination and sources of phthalates and its health risk in China. a review. *Environmental Research*, 164, 417-429.

Luo, Z. H., Pang, K. L., Gu, J. D., Chow, R. K. K., & Vrijmoed, L. L. P. (2009). Degradability of the three dimethyl phthalate isomer esters (DMPEs) by a *Fusarium* species isolated from mangrove sediment. *Marine Pollution Bulletin*, 58(5), 765-768.

Lynch, S. A., Alvarez, C., O'Neill, E. E., Keenan, D. F., & Mullen, A. M. (2018). Optimization of protein recovery from bovine lung by pH shift process using response surface methodology. *Journal of the Science of Food and Agriculture*, 98(5), 1951-1960.

Mahajan, D. T., Masand, V. H., Patil, K. N., Hadda, T. B., & Rastija, V. (2013). Integrating GUSAR and QSAR analyses for antimalarial activity of synthetic prodiginines against multi drug resistant strain. *Medicinal Chemistry Research*, 22(5), 2284-2292.

Mahajan, D. T., Masand, V. H., Patil, K. N., Hadda, T. B., Jawarkar, R. D., Thakur, S. D., & Rastija, V. (2012). CoMSIA and POM analyses of anti-malarial activity of synthetic prodiginines. *Bioorganic & Medicinal Chemistry Letters*, 22(14), 4827-4835.

Mahmoud, S. T., Luti, K. J. K., & Yonis, R. W. (2015). Enhancement of prodigiosin production by *Serratia marcescens* S23 via introducing

microbial elicitor cells into culture medium. *Iraqi Journal of Science*, 56(3A), 1938-1951.

Malik, K., Tokkas, J., & Goyal, S. (2012). Microbial pigments: a review. *International Journal of Microbial Resource Technology*, 1(4), 361-365.

Masetto, A., Flores-Cotera, L. B., Diaz, C., Langley, E., & Sanchez, S. (2001). Application of a complete factorial design for the production of zeaxanthin by *Flavobacterium* sp. *Journal of Bioscience and Bioengineering*, 92(1), 55-58.

Mathews-Roth, M. M. (1982). Antitumor activity of β-carotene, canthaxanthin and phytoene. *Oncology*, *39*(1), 33-37.

Mathlom, G. S., Hayder, N. H., & Mahmood, M. S. (2018). Synergistic effect of biosurfactant and prodigiosin produced by *Serratia marcescens* as antimicrobial agent. *Current Research in Microbiology and Biotechnology*, 6(2), 1601-1615.

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., & Stewart, G. S. (1997). Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology*, 143(12), 3703-3711.

McRary, W. L., Beaver, E. L., & Noble, E. R. (1953). In Vitro Effects of Prodigiosin and other Antibiotics on *Trypanosoma cruzi*. *Experimental Parasitology*, 2(2), 125-8.

Melvin, M. S., Tomlinson, J. T., Saluta, G. R., Kucera, G. L., Lindquist, N., & Manderville, R. A. (2000). Double-strand DNA cleavage by copper

prodigiosin. *Journal of the American Chemical Society*, 122(26), 6333-6334.

Midland, S. L., Izac, R. R., Wing, R. M., Zaki, A. I., Munnecke, D. E., & Sims, J. J. (1982). Melleolide, a new antibiotic from *Armillaria mellea*. *Tetrahedron Letters*, 23(25), 2515-2518.

Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Reviews in Microbiology*, 55(1), 165-199.

Moraes, C. S., Seabra, S. H., Castro, D. P., Brazil, R. P., de Souza, W., Garcia, E. S., & Azambuja, P. (2008). *Leishmania* (Leishmania) *chagasi* interactions with *Serratia marcescens*: ultrastructural studies, lysis and carbohydrate effects. *Experimental Parasitology*, 118(4), 561-568.

Morin, D., Grasland, B., Vallee-Rehel, K., Dufau, C., & Haras, D. (2003). On-line high-performance liquid chromatography–mass spectrometric detection and quantification of N-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices. *Journal of Chromatography A*, 1002(1-2), 79-92.

Morohoshi, T., Shiono, T., Takidouchi, K., Kato, M., Kato, N., Kato, J., & Ikeda, T. (2007). Inhibition of quorum sensing in *Serratia marcescens* AS-1 by synthetic analogs of N-acylhomoserine lactone. *Applied and Environmental Microbiology*, 73(20), 6339-6344.

Mu, D., Gao, F., Fan, Z., Shen, H., Peng, H., & Hu, J. (2015). Levels of phthalate metabolites in urine of pregnant women and risk of clinical pregnancy loss. *Environmental Science & Technology*, *49*(17), 10651-10657.

Mukherjee, G., Mishra, T., & Deshmukh, S. K. (2017). Fungal Pigments: An Overview. *Developments in Fungal Biology and Applied Mycology*, 525-541

Mukherjee, S., & Bassler, B. L. (2019). Bacterial quorum sensing in complex and dynamically changing environments. *Nature Reviews Microbiology*, 17(6), 371-382.

Nakamiya, K., Takagi, H., Nakayama, T., Ito, H., Tsuruga, H., Edmonds, J. S., & Morita, M. (2005). Microbial production and vaporization of mono-(2-ethylhexyl) phthalate from di-(2-ethylhexyl) phthalate by microorganisms inside houses. *Archives of Environmental & Occupational health*, *60*(6), 321-325.

Nakamura, A., Nagai, K., Ando, K., & Tamura, G. (1986). Selective suppression by prodigiosin of the mitogenic response of murine splenocytes. *The Journal of Antibiotics*, 39(8), 1155-1159.

Nandi, M., Paul, T., Kanaujiya, D. K., Baskaran, D., Pakshirajan, K., & Pugazhenthi, G. (2020). Biodegradation of benzyl butyl phthalate and di-butyl phthalate by *Arthrobacter* sp. via micellar solubilization in a surfactant-aided system. *Water Supply*. doi: 10.2166/ws.2020.347

National Research Council. (2009). *Phthalates and cumulative risk assessment: the tasks ahead*. National Academies Press.

Navarro, E., Sandmann, G., & Torres-Martínez, S. (1995). Mutants of the carotenoid biosynthetic pathway of *Mucor circinelloides*. *Experimental Mycology*, *19*(3), 186-190.

Nematollahi, A., Aminimoghadamfarouj, N., & Wiart, C. (2012). Reviews on 1, 4-naphthoquinones from *Diospyros L*. *Journal of Asian Natural Products Research*, 14(1), 80-88.

Niazi, J. H., Prasad, D. T., & Karegoudar, T. B. (2001). Initial degradation of dimethylphthalate by esterases from *Bacillus* species. *FEMS Microbiology Letters*, 196(2), 201-205.

Nielsen, K. F., Mogensen, J. M., Johansen, M., Larsen, T. O., & Frisvad, J. C. (2009). Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Analytical and Bioanalytical Chemistry*, 395(5), 1225-1242.

Niklitschek, M., Alcaíno, J., Barahona, S., Sepúlveda, D., Lozano, C., Carmona, M., & Cifuentes, V. (2008). Genomic organization of the structural genes controlling the astaxanthin biosynthesis pathway of *Xanthophyllomyces dendrorhous*. *Biological Research*, 41(1), 93-108.

Novakova, R., Odnogova, Z., Kutas, P., Feckova, L., & Kormanec, J. (2010). Identification and characterization of an indigoidine-like gene for a blue pigment biosynthesis in *Streptomyces aureofaciens* CCM 3239. *Folia Microbiologica*, 55(2), 119-125.

Ogawa, G., Ishida, M., & Urano, N. (2009). Isolation and identification of dibutyl phthalate-degrading bacteria from hydrospheres in Tokyo. *The Journal of General and Applied Microbiology*, 55(4), 261-265.

Olabisi, O., & Adewale, K. (Eds.). (2016). *Handbook of Thermoplastics* (Vol. 41). CRC press, New York.

Ortori, C. A., Dubern, J. F., Chhabra, S. R., Camara, M., Hardie, K., Williams, P., & Barrett, D. A. (2011). Simultaneous quantitative profiling of N-acyl-L-homoserine lactone and 2-alkyl-4 (1H)-quinolone families of quorum-sensing signaling molecules using LC-MS/MS. *Analytical and Bioanalytical Chemistry*, 399(2), 839-850.

Othman, M. A., El-Zamik, F. I., Hegazy, M. I., & Salama, A. S. A. (2019). Isolation and identification of egyptian strains of serratia marcescens producing antibacterial and antioxidant prodigiosin pigment. *Zagazig Journal of Agricultural Research*, 46(5), 1573-1582.

Padmapriya, C., & Murugesan, R. (2016). Characterization of methanolic extract of red pigment from *Penicillium purpurogenum* and its antioxidant activity. *Journal of Pure Applied Microbiology*, 10, 1505–11.

Pak, V. M., McCauley, L. A., & Pinto-Martin, J. (2011). Phthalate exposures and human health concerns: A review and implications for practice. *AAOHN Journal*, 59(5), 228-235.

Palleroni, N. J., Reichelt, K. E., Mueller, D., Epps, R., Tabenkin, B., Bull, D. N., & Berger, J. (1978). Production of a novel red pigment, rubrolone, by *Streptomyces echinoruber* sp. nov. *The Journal of Antibiotics*, 31(12), 1218-1225.

Pandey, R., Chander, R., & Sainis, K. B. (2007). Prodigiosins: a novel family of immunosuppressants with anti-cancer activity. *Indian Journal of Biochemistry and Biophysics*, 44, 295-302.

Pantanella, F., Berlutti, F., Passariello, C., Sarli, S., Morea, C., & Schippa, S. (2007). Violacein and biofilm production in *Janthinobacterium lividum*. *Journal of Applied Microbiology*, 102(4), 992-999.

Papireddy, K., Smilkstein, M., Kelly, J. X., Salem, S. M., Alhamadsheh, M., Haynes, S. W., & Reynolds, K. A. (2011). Antimalarial activity of natural and synthetic prodiginines. *Journal of Medicinal Chemistry*, 54(15), 5296-5306.

Parani, K., & Saha, B. K. (2008). Optimization of prodigiosin production from a strain of *Serratia marcescens* SR1 and screening for antifungal activity. *Journal of Biological Control*, 22(1), 73-79.

Parisot, D., Devys, M., & Barbier, M. (1990). Naphthoquinone pigments related to fusarubin from the fungus *Fusarium solani* (Mart.) Sacc. *Microbios*, 64(258), 31-47.

Pascault, J. P., Sautereau, H., Verdu, J., & Williams, R. J. (2002). *Thermosetting polymers* (Vol. 64). CRC press, New York.

Patel, D. S., Desai, A. J., & Desai, J. D. (1998). Biodegradation of dimethylterephthalate by *Comamonas acidovorans* D-4. *Indian Journal of Experimental Biology*, 36(3), 321-324.

Patil, N. K., Kundapur, R., Shouche, Y. S., & Karegoudar, T. B. (2006). Degradation of a plasticizer, di-n-butylphthalate by *Delftia* sp. TBKNP-05. *Current Microbiology*, 52(3), 225-230.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H., & Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proceedings of the National Academy of Sciences*, 91(1), 197-201.

Pelz, A., Wieland, K. P., Putzbach, K., Hentschel, P., Albert, K., & Gotz, F. (2005). Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. *Journal of Biological Chemistry*, 280(37), 32493-32498.

Peters, E. N. (2002). *Plastics: thermoplastics, thermosets, and elastomers.* Wiley-Interscience, New York.

Phatake, Y. B., & Dharmadhikari, S. M. (2016). Physical parameters optimization for enhancement of prodigiosin production by using *Serratia* spp. *World Journal of Pharmaceutical and Medical Research*, 2(6), 40-48.

Picha, P., Kale, D., Dave, I., & Pardeshi, S. (2015). Comparative studies on prodigiosin production by *Serratia marcescens* using various crude fatty acid sources—its characterization and applications. *International Journal of Current Microbiology and Applied Sciences*, 2, 254-267.

Pomini, A. M., Araujo, W. L., & Marsaioli, A. J. (2006). Structural elucidation and biological activity of acyl-homoserine lactones from the phytopathogen *Pantoea ananatis Serrano* 1928. *Journal of Chemical Ecology*, 32(8), 1769-1778.

Ponnusamy, K., Paul, D., & Kweon, J. H. (2009) Inhibition of quorum sensing mechanism and *Aeromonas hydrophila* biofilm formation by vanillin. *Environmental Engineering Science*, 26(8), 1359-63.

Poplawsky, A. R., & Chun, W. (1997). pigB determines a diffusible factor needed for extracellular polysaccharide slime and xanthomonadin production in *Xanthomonas campestris* pv. campestris. *Journal of Bacteriology*, 179(2), 439-444.

Powers, H. J. (2003). Riboflavin (vitamin B-2) and health. *The American Journal of Clinical Nutrition*, 77(6), 1352-1360.

Pradeep, B. V., Akilandeswari, P., Usharani, V. & Palaniswamy, M. (2016). Application of multifactorial experimental design for the optimization of prodigiosin production using *Serratia marcescens* MBB01, MBB02 and MBB05. *Asian Journal of Pharmaceutical and Clinical Research*, 9(3), 408-416

Pradeep, S., & Benjamin, S. (2012). Mycelial fungi completely remediate di (2-ethylhexyl) phthalate, the hazardous plasticizer in PVC blood storage bag. *Journal of Hazardous Materials*, 235, 69-77.

Pradeep, S., Faseela, P., Sarath Josh, M. K., Balachandran, S., Devi, R. S., & Benjamin, S. (2013). Fungal biodegradation of phthalate plasticizer *in situ*. *Biodegradation*, 24(2), 257-267.

Pradeep, S., Sarath Josh, M. K., Balachandran, S., Devi, R. S., Sadasivam, R., Thirugnanam, P. E., Doble, M., Anderson, R. C. & Benjamin, S. (2014). *Achromobacter denitrificans* SP1 produces pharmaceutically active 25C prodigiosin upon utilizing hazardous di (2-ethylhexyl) phthalate. *Bioresource Technology*, 171, 482-486.

Pradeep, S., Sarath Josh, M. K., Binod, P., Devi, R. S., Balachandran, S., Anderson, R. C., & Benjamin, S. (2015). *Achromobacter denitrificans* strain SP1 efficiently remediates di (2-ethylhexyl) phthalate. *Ecotoxicology and Environmental Safety*, 112, 114-121.

Prasad, B. (2018). Effect of various substrate and process parameters on the production of prodigiosin by *Serratia marcescens* MTCC 4822. *International Journal of Advanced Microbiology and Health Res*earch 2(1), 17-23.

Prathumpai, W., Phimmakong, K., Srikitikulchai, P., & Wongsa, P. (2006). Kinetic study of naphthoquinone and key metabolite production of *C. unilateralis* BCC1869. *Thai Journal of Biotechnology*, 7(2), 39-43.

Priya, K. A., Satheesh, S., Ashokkumar, B., Varalakshmi, P., Selvakumar, G., & Sivakumar, N. (2013). Antifouling activity of prodigiosin from estuarine isolate of *Serratia marcescens* CMST 07. *Microbiological Research in Agroecosystem Management* (11-21). Springer, India.

Przybylinska, P. A., & Wyszkowski, M. (2016). Environmental contamination with phthalates and its impact on living organisms. *Ecological Chemistry and Engineering S*, 23(2), 347-356.

Psillakis, E., & Kalogerakis, N. (2003). Hollow-fibre liquid-phase microextraction of phthalate esters from water. *Journal of Chromatography A*, 999(1-2), 145-153.

Quan, C. S., Liu, Q., Tian, W. J., Kikuchi, J., & Fan, S. D. (2005). Biodegradation of an endocrine-disrupting chemical, di-2-ethylhexyl phthalate, by *Bacillus subtilis* No. 66. *Applied Microbiology and Biotechnology*, 66(6), 702-710.

Rajagopal, L., Sundari, C. S., Balasubramanian, D., & Sonti, R. V. (1997). The bacterial pigment *xanthomonadin* offers protection against photodamage. *FEBS Letters*, 415(2), 125-128.

Ramani, G., Nair, A., & Karthika, K. (2014). Optimization of cultural conditions for the production of prodigiosin by *Serratia marcescens* and screening for the antimicrobial activity of prodigiosin. *International Journal of Pharma and Bio Sciences*, 5(3), 383-392.

Ramesh, C., Vinithkumar, N. V., Kirubagaran, R., Venil, C. K., & Dufosse, L. (2020). Applications of Prodigiosin Extracted from Marine Red Pigmented Bacteria *Zooshikella* sp. and Actinomycete *Streptomyces* sp. *Microorganisms*, 8(4), 556.

Ramzi, A., Gireeshkumar, T. R., Rahman, K. H., Balachandran, K. K., Shameem, K., Chacko, J., & Chandramohanakumar, N. (2020). Phthalic acid esters–A grave ecological hazard in Cochin estuary, India. *Marine Pollution Bulletin*, 152, 110899.

Rank, J. (2005). Classification and risk assessment of chemicals: the case of DEHP in the light of REACH. *The Journal of Transdisciplinary Environmental Studies*, 4(3), 1-15.

Rapoport, H., & Willson, C. D. (1962). The Preparation and Properties of Some Methoxypyrroles. *Journal of American chemical Society*, 84, 630.

Ray, A. C., & Eakin, R. E. (1975). Studies on the biosynthesis of aspergillin by *Aspergillus niger*. *Applied Microbiology*, 30(6), 909-915.

Renukadevi, K. P., & Vineeth, M. (2017). Antimicrobial, antioxidant and cytotoxicity activity of pigment produced from *Serratia marcescens*. *European Journal of Biomedical and Pharmaceutical Sci*ences 4(2), 265-269.

Reyes, F. G. R., Valim, M. F. C. F. A., & Vercesi, A. E. (1996). Effect of organic synthetic food colours on mitochondrial respiration. *Food Additives & Contaminants*, 13(1), 5-11.

Richter, G., Fischer, M., Krieger, C., Eberhardt, S., Lüttgen, H., Gerstenschläger, I., & Bacher, A. (1997). Biosynthesis of riboflavin: characterization of the bifunctional deaminase-reductase of *Escherichia coli* and *Bacillus subtilis*. *Journal of Bacteriology*, 179(6), 2022-2028.

Rioseras, B., Lopez-Garcia, M. T., Yague, P., Sánchez, J., & Manteca, A. (2014). Mycelium differentiation and development of *Streptomyces coelicolor* in lab-scale bioreactors: programmed cell death, differentiation, and lysis are closely linked to undecylprodigiosin and actinorhodin production. *Bioresource Technology*, 151, 191-198.

Roberts, W. T., & Davidson, P. M. (1986). Growth characteristics of selected fungi on polyvinyl chloride film. *Applied and Environmental Microbiology*, *51*(4), 673-676.

Rosa-Fraile, M., Rodriguez-Granger, J., Haidour-Benamin, A., Cuerva, J. M., & Sampedro, A. (2006). Granadaene: proposed structure of the group B *Streptococcus* polyenic pigment. *Applied and Environmental Microbiology*, 72(9), 6367-6370.

Rosa-Putra, S., Hemmerlin, A., Epperson, J., Bach, T. J., Guerra, L. H., & Rohmer, M. (2001). Zeaxanthin and menaquinone-7 biosynthesis in *Sphingobacterium multivorum* via the methylerythritol phosphate pathway. *FEMS Microbiology Letters*, 204(2), 347-353.

Roslev, P., Vorkamp, K., Aarup, J., Frederiksen, K., & Nielsen, P. H. (2007). Degradation of phthalate esters in an activated sludge wastewater treatment plant. *Water Research*, 41(5), 969-976.

Rossa, C. A., White, J., Kuiper, A., Postma, P. W., Bibb, M., & de Mattos, M. T. (2002). Carbon flux distribution in antibiotic-producing chemostat cultures of *Streptomyces lividans*. *Metabolic Engineering*, 4(2), 138-150.

Rowdhwal, S. S. S., & Chen, J. (2018). Toxic effects of di-2-ethylhexyl phthalate: an overview. *BioMed Research International*, 2018.

Ryazantseva, I., & Andreyeva, I. (2014). Application of prodigiosin as a colorant for polyolefines. *Advances in Biological Chemistry*, *2014*.

Sajjad, W., Ahmad, S., Aziz, I., Azam, S. S., Hasan, F., & Shah, A. A. (2018). Antiproliferative, antioxidant and binding mechanism analysis of prodigiosin from newly isolated radio-resistant *Streptomyces* sp. strain WMA-LM31. *Molecular Biology Reports*, 45(6), 1787-1798.

Sakai-Kawada, F. E., Ip, C. G., Hagiwara, K. A., & Awaya, J. D. (2019). Biosynthesis and Bioactivity of Prodiginine Analogues in Marine Bacteria, *Pseudoalteromonas*: A Mini Review. *Frontiers in Microbiology*, 10, 1715.

Sampath, S., Selvaraj, K. K., Shanmugam, G., Krishnamoorthy, V., Chakraborty, P., & Ramaswamy, B. R. (2017). Evaluating spatial distribution and seasonal variation of phthalates using passive air sampling in southern India. *Environmental Pollution, 221*, 407-417.

Samrot, A. V., Chandana, K., Senthilkumar, P., & Narendra, K. G. (2011). Optimization of prodigiosin production by *Serratia marcescens* SU-10 and evaluation of its bioactivity. *International Research Journal of Biotechnology*, 2(5), 128-133.

Santer, U. V., & Vogel, H. J. (1956). Prodigiosin synthesis in *Serratia marcescens*: isolation of a pyrrole-containing precursor. *Biochimica et Biophysica Acta*, 19(3), 578.

Sarath Josh, M. K., Pradeep, S., Balachandran, S., Devi, R. S., Amma, K. V., & Benjamin, S. (2012). Temperature-and solvent-dependent migrations of di (2-ethylhexyl) phthalate, the hazardous plasticizer from commercial PVC blood storage bag. *Journal of Polymer Research*, 19(7), 9915.

Saurav, K., Costantino, V., Venturi, V., & Steindler, L. (2017). Quorum sensing inhibitors from the sea discovered using bacterial N-acyl-homoserine lactone-based biosensors. *Marine Drugs*, 15(3), 53.

Schaefer, A. L., Taylor, T. A., Beatty, J. T., & Greenberg, E. P. (2002). Long-chain acyl-homoserine lactone quorum-sensing regulation of

Rhodobacter capsulatus gene transfer agent production. *Journal of Bacteriology*, 184(23), 6515-6521.

Schupp, P. J., Charlton, T. S., Taylor, M. W., Kjelleberg, S., & Steinberg, P. D. (2005). Use of solid-phase extraction to enable enhanced detection of acyl homoserine lactones (AHLs) in environmental samples. *Analytical and Bioanalytical Chemistry*, 383(1), 132-137.

Scott, R. P. W. (2007). Thin layer chromatography. *URL http://www. chromatography-online.org/TLC/Detection-and-Quantification/Sulfuric-Acid-Spray.*

Selvaraj, K. K., Sundaramoorthy, G., Ravichandran, P. K., Girijan, G. K., Sampath, S., & Ramaswamy, B. R. (2015). Phthalate esters in water and sediments of the Kaveri River, India: environmental levels and ecotoxicological evaluations. *Environmental geochemistry and health*, 37(1), 83-96.

Sertan-de Guzman, A. A., Predicala, R. Z., Bernardo, E. B., Neilan, B. A., Elardo, S. P., Mangalindan, G. C., & Concepcion, G. P. (2007). *Pseudovibrio denitrificans* strain Z143-1, a heptylprodigiosin-producing bacterium isolated from a Philippine tunicate. *FEMS microbiology Letters*, 277(2), 188-196.

Shah, A. A., Hasan, F., Hameed, A., & Ahmed, S. (2008). Biological degradation of plastics: a comprehensive review. *Biotechnology advances*, 26(3), 246-265.

Shahid, M., & Mohammad, F. (2013). Recent advancements in natural dye applications: a review. *Journal of Cleaner Production*, 53, 310-331.

Shahitha, S., & Poornima, K. (2012). Enhanced production of prodigiosin production in *Serratia marcescens*. *Journal of Applied Pharmaceutical Science*, 2(8), 138.

Sharanagouda, U., & Karegoudar, T. B. (2002). Degradation of 2-methylnaphthalene by free and immobilized cells of Pseudomonas sp. strain NGK1. *World Journal of Microbiology and Biotechnology*, 18(3), 225-230.

Shen, J. J., & Yang, Y. (2013). Kinetics and thermodynamics studies of prodigiosin dyeing on polyester. *Advanced Materials Research*, 779, 156-160.

Shi, Y. C., & Pan, T. M. (2011). Beneficial effects of *Monascus purpureus* NTU 568-fermented products: a review. *Applied Microbiology and Biotechnology*, 90(4), 1207.

Shrout, J. D., Chopp, D. L., Just, C. L., Hentzer, M., Givskov, M., & Parsek, M. R. (2006). The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. *Molecular Microbiology*, 62(5), 1264-1277.

Silva, M. J., Reidy, J. A., Herbert, A. R., Preau, J. L., Needham, L. L., & Calafat, A. M. (2004). Detection of phthalate metabolites in human amniotic fluid. *Bulletin of Environmental Contamination and Toxicology*, 72(6), 1226-1231.

Silvertein, R. M., & Webster, F. X. Spectrometric identification of organic compound (6^{th} ed. 1998). New York: John Wiley and Sons, Inc.

Sin, J. C., Lam, S. M., Mohamed, A. R., & Lee, K. T. (2012). Degrading Endocrine Disrupting Chemicals from Wastewater by TiO_2 Photocatalysis: A Review. *International Journal of Photoenergy*, 2012.

Singh, A., Shukla, N., Kabadwal, B. C., Tewari, A. K., & Kumar, J. (2018). Review on plant-*Trichoderma*-pathogen interaction. *International Journal of Current Microbiology and Applied Sciences*, 7(2), 2382-2397.

Sinha, P., Anshu, A. K., Tiwari, M., & Ahmad, M. Y. (2019). Determination of PAEs in soil of municipal wards of Patna, India by microwave assisted extraction and LC-MS/MS. *Soil and Sediment Contamination: An International Journal*, 28(2), 162-170.

Siva, R., Subha, K., Bhakta, D., Ghosh, A. R., & Babu, S. (2012). Characterization and enhanced production of prodigiosin from the spoiled coconut. *Applied Biochemistry and Biotechnology*, 166(1), 187-196.

Sivamurthy, K., Swamy, B. M., & Pujar, B. G. (1991). Transformation of dimethylterephthalate by the fungus *Sclerotium rolfsii*. *FEMS Microbiology Letters*, 79(1), 37-40.

Slater, H., Crow, M., Everson, L., & Salmond, G. P. (2003). Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and-independent pathways. *Molecular Microbiology*, 47(2), 303-320.

Sole, M., Francia, A., Rius, N., & Loren, J. G. (1997). The role of pH in the 'glucose effect'on prodigiosin production by non-proliferating cells of *Serratia marcescens*. *Letters in Applied Microbiology*, 25(2), 81-84.

Soliev, A. B., Hosokawa, K., & Enomoto, K. (2011). Bioactive pigments from marine bacteria: applications and physiological roles. *Evidence-Based Complementary and Alternative Medicine*, 2011.

Solomons, T. W. G., & Fryhle, C. Organic chemistry (10th ed. 2007). New York: John Wiley.

Someya, N., Nakajima, M., Hamamoto, H., Yamaguchi, I., & Akutsu, K. (2004). Effects of light conditions on prodigiosin stability in the biocontrol bacterium *Serratia marcescens* strain B2. *Journal of General Plant Pathology*, 70(6), 367-370.

Song, M. J., Bae, J., Lee, D. S., Kim, C. H., Kim, J. S., Kim, S. W., & Hong, S. I. (2006). Purification and characterization of prodigiosin produced by integrated bioreactor from *Serratia* sp. KH-95. *Journal of Bioscience and Bioengineering*, 101(2), 157-161.

Srimathi, R., Priya, R., Nirmala, M., & Malarvizhi, A. (2017). Isolation, identification, optimization of prodigiosin pigment produced by *Serratia marcescens* and its applications. *International Journal of Latest Engineering and Management Research*, 2(9), 11-21.

Srivastava, A., Sharma, V. P., Tripathi, R., Kumar, R., Patel, D. K., & Mathur, P. K. (2010). Occurrence of phthalic acid esters in Gomti River Sediment, India. *Environmental Monitoring and Assessment,* 169(1-4), 397-406.

Stankovic, N., Radulovic, V., Petkovic, M., Vuckovic, I., Jadranin, M., Vasiljevic, B., & Nikodinovic-Runic, J. (2012). *Streptomyces* sp. JS520 produces exceptionally high quantities of undecylprodigiosin with antibacterial, antioxidative, and UV-protective properties. *Applied Microbiology and Biotechnology*, 96(5), 1217-1231.

Staples, C. A., Peterson, D. R., Parkerton, T. F., & Adams, W. J. (1997). The environmental fate of phthalate esters: a literature review. *Chemosphere*, 35(4), 667-749.

Steindler, L., & Venturi, V. (2007). Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiology Letters*, 266(1), 1-9.

Stepkowski, S. M., Nagy, Z. S., Wang, M. E., Behbod, F., & Erwin-Cohen, R. (2001). PNU156804 inhibits Jak3 tyrosine kinase and rat heart allograft rejection. *Transplantation Proceedings*, 33(7-8), 3272-3273.

Su, W. T., Tsou, T. Y., & Liu, H. L. (2011). Response surface optimization of microbial prodigiosin production from *Serratia marcescens*. *Journal of the Taiwan Institute of Chemical Engineers*, 42(2), 217-222.

Sumathi, C., MohanaPriya, D., Swarnalatha, S., Dinesh, M. G., & Sekaran, G. (2014). Production of prodigiosin using tannery fleshing and evaluating its pharmacological effects. *The Scientific World Journal*, 2014.

Sundaramoorthy, N., Yogesh, P., & Dhandapani, R. (2009). Production of prodigiosin from *Serratia marcescens* isolated from soil. *Indian Journal of Science and Technology*, 2(10), 32-34.

Suryawanshi, R. K., Patil, C. D., Koli, S. H., Hallsworth, J. E., & Patil, S. V. (2017). Antimicrobial activity of prodigiosin is attributable to plasma-membrane damage. *Natural Product Research*, 31(5), 572-577.

Suzuki, Y., Yoshinaga, J., Mizumoto.Y., Serizawa,S., & Shiraishi, H. (2012). Foetal exposure to phthalate esters and anogenital distance in male newborns. *International Journal of Andrology***,** 35 (3), 236-244.

Swan, S. H., Main, K. M., Liu, F., Stewart, S. L., Kruse, R. L., Calafat, A. M., Mao, C .S., Redmon, J. B., Ternand, C. L., Sullivan, S., & Teague, J. L. (2005). Decrease in anogenital distance among male infants with

prenatal phthalate exposure. *Environmental Health Perspectives*, 113(8), 1056-1061.

Swift, S., Lynch, M. J., Fish, L., Kirke, D. F., Tomás, J. M., Stewart, G. S., & Williams, P. (1999). Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. *Infection and Immunity*, 67(10), 5192-5199.

Tao, J. L., Wang, X. D., Shen, Y. L., & Wei, D. Z. (2005). Strategy for the improvement of prodigiosin production by a *Serratia marcescens* mutant through fed-batch fermentation. *World Journal of Microbiology and Biotechnology*, 21(6-7), 969-972.

Taylor, R. F., & Davies, B. H. (1983). The triterpenoid carotenoids and related terpenoids in *Staphylococcus aureus* 209P. *Canadian Journal of Biochemistry and Cell Biology*, 61(8), 892-905.

Teplitski, M., Robinson, J. B., & Bauer, W. D. (2000). Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Molecular Plant-Microbe Interactions*, 13(6), 637-648.

Testa, C., Nuti, F., Hayek, J., De Felice, C., Chelli, M., Rovero, P. Latini, G., & Papini, A. M. (2012). Di-(2-ethylhexyl) phthalate and autism spectrum disorders. *ASN Neuro*, 4(4), AN20120015.

Thomson, N. R., Crow, M. A., McGowan, S. J., Cox, A., & Salmond, G. P. C. (2000). Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. *Molecular Microbiology*, 36(3), 539-556.

Tiwari, M., Sahu, S. K., & Pandit, G. G. (2016). Distribution and estrogenic potential of endocrine disrupting chemicals (EDCs) in estuarine sediments from Mumbai, India. *Environmental Science and Pollution Research*, 23(18), 18789-18799.

Tranfo, G., Paci, E., Pigini, D., Bonanni, R. C., Capanna, S., De Carolis, C., & Iavicoli, S. (2014). Phthalate metabolites in amniotic fluid and maternal urine samples. *Journal of Environmental Protection*, 5(14), 1411.

Trasande, L., Spanier, A. J., Sathyanarayana, S., Attina, T. M., & Blustein, J. (2013). Urinary phthalates and increased insulin resistance in adolescents. *Pediatrics*, 132(3), 646-655.

Tserovska, L., & Dimkov, R. (2002) Dimethylterephthalate catabolism by *Pseudomonas sp*. *Journal of Culture Collections*, 3(1), 33-37.

Tuli, H. S., Chaudhary, P., Beniwal, V., & Sharma, A. K. (2015). Microbial pigments as natural color sources: current trends and future perspectives. *Journal of Food Science and Technology*, 52(8), 4669-4678.

Unagul, P., Wongsa, P., Kittakoop, P., Intamas, S., Srikitikulchai, P., & Tanticharoen, M. (2005). Production of red pigments by the insect pathogenic fungus *Cordyceps unilateralis* BCC 1869. *Journal of Industrial Microbiology and Biotechnology*, 32(4), 135-140.

Upson, K., Sathyanarayana, S., De Roos, A. J., Thompson, M. L., Scholes, D., Dills, R., & Holt, V. L. (2013). Phthalates and risk of endometriosis. *Environmental Research*, 126, 91-97.

Vamsee-Krishna, C., & Phale, P. S. (2008). Bacterial degradation of phthalate isomers and their esters. *Indian Journal of Microbiology*, 48(1), 19.

Van Houdt, R., Moons, P., Aertsen, A., Jansen, A., Vanoirbeek, K., Daykin, M., Williams, P., & Michiels, C. W. (2007). Characterization of a luxI/luxR-type quorum sensing system and N-acyl-homoserine lactone-dependent regulation of exo-enzyme and antibacterial component production in *Serratia plymuthica* RVH1. *Research in Microbiology*, 158(2), 150-158.

Vasanthabharathi, V., Lakshminarayanan, R., & Jayalakshmi, S. (2011). Melanin production from marine Streptomyces. *African Journal of Biotechnology*, 10(54), 11224-11234.

Vega, D., & Bastide, J. (2003). Dimethylphthalate hydrolysis by specific microbial esterase. *Chemosphere*, 51(8), 663-668.

Velmurugan, P., Kamala-Kannan, S., Balachandar, V., Lakshmanaperumal samy, P., Chae, J. C., & Oh, B. T. (2010). Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather. *Carbohydrate Polymers*, 79(2), 262-268.

Venil, C. K., & Lakshmanaperumalsamy, P. (2009). An insightful overview on microbial pigment, prodigiosin. *Electronic Journal of Biology*, 5(3), 49-61.

Venil, C. K., Zakaria, Z. A., Usha, R., & Ahmad, W. A. (2014). Isolation and characterization of flexirubin type pigment from *Chryseobacterium* sp. UTM-3T. *Biocatalysis and Agricultural Biotechnology*, *3*(4), 103-107.

Ventura Pinto, A., & Lisboa de Castro, S. (2009). The trypanocidal activity of naphthoquinones: a review. *Molecules*, 14(11), 4570-90.

Vinarov, A., Robucheva, Z., Sidorenko, T., & Dirina, E. (2003). Microbial biosynthesis and making of pigment melanin. *Communications in Agricultural and Applied Biological Sciences*, 68(2), 325.

Vogl, C., Grill, S., Schilling, O., Stülke, J., Mack, M., & Stolz, J. (2007). Characterization of riboflavin (vitamin B2) transport proteins from *Bacillus subtilis* and *Corynebacterium glutamicum*. *Journal of Bacteriology*, 189(20), 7367-7375.

Wang, F., Luo, H., Song, G., Liu, C., Wang, J., Xu, J., & Ma, X. Y. (2013). Prodigiosin found in *Serratia marcescens* y2 initiates phototoxicity in the cytomembrane. *Electronic Journal of Biotechnology*, 16(4), 7-7.

Wang, H., Zhou, Y., Tang, C., He, Y., Wu, J., Chen, Y., & Jiang, Q. (2013). Urinary phthalate metabolites are associated with body mass index and waist circumference in Chinese school children. *PloS One*, 8(2).

Wang, J. W., Xu, W., Zhong, T. H., He, G. Y., & Luo, Z. H. (2017). Degradation of dimethyl phthalate esters by a filamentous fungus *Aspergillus versicolor* isolated from deep-sea sediments. *Botanica Marina*, 60(3), 351-359.

Wang, J., Quan, C., Wang, X., Zhao, P., & Fan, S. (2011). Extraction, purification and identification of bacterial signal molecules based on N-acyl homoserine lactones. *Microbial Biotechnology*, 4(4), 479-490

Wang, M., Zhu, P., Jiang, J., Zhu, H., Tan, S., & Li, R. (2020). Signaling molecules of quorum sensing in bacteria. *Reviews in Biotechnology and Biochemistry*, 1(1).

Wang, P. L., Li, D. Y., Xie, L. R., Wu, X., Hua, H. M., & Li, Z. L. (2014). Two new compounds from a marine-derived fungus *Penicillium oxalicum*. *Natural Product Research*, 28(5), 290-293.

Wang, S. L., Wang, C. Y., Yen, Y. H., Liang, T. W., Chen, S. Y., & Chen, C. H. (2012). Enhanced production of insecticidal prodigiosin from

Serratia marcescens TKU011 in media containing squid pen. *Process Biochemistry*, 47(11), 1684-1690.

Wang, X., Wu, H., Wang, X., Wang, H., Zhao, K., Ma, B., & Lu, Z. (2020). Network-directed isolation of the cooperator *Pseudomonas aeruginosa* ZM03 enhanced the dibutyl phthalate degradation capacity of *Arthrobacter nicotianae* ZM05 under pH stress. *Journal of Hazardous Materials*, 124667.

Wang, Y. P., & Gu, J. D. (2006). Degradation of dimethyl isophthalate by *Viarovorax paradoxus* strain T4 isolated from deep-ocean sediment of the South China Sea. *Human and Ecological Risk Assessment: An International Journal*, 12(2), 236-247.

Wang, Y., Fan, Y., & Gu, J. D. (2004). Dimethyl phthalate ester degradation by two planktonic and immobilized bacterial consortia. *International Biodeterioration & Biodegradation*, 53(2), 93-101.

Wasserman, H. H., McKeon, J. E., Smith, L., & Forgione, P. (1960). Prodigiosin. Structure and partial synthesis. *Journal of the American Chemical Society*, 82(2), 506-507.

Wei, Y. H. and Chen, W. C. (2005 a). Enhanced production of prodigiosin-like pigment from *Serratia marcescens* SMΔR by medium improvement and oil-supplementation strategies. *Journal of Bioscience and Bioengineering*, 99, 616–622

Wei, Y. H., Yu, W. J., and Chen, W. C. (2005b). Enhanced undecylprodigiosin production from *Serratia marcescens* SS-1 by medium formulation and amino-acid supplementation. *Journal of Bioscience and Bioengineering*, 100, 466–471

Weitkamp, J. H., Tang, Y. W., Haas, D. W., Midha, N. K., & Crowe Jr, J. E. (2000). Recurrent *Achromobacter xylosoxidans* bacteremia associated with persistent lymph node infection in a patient with hyper-immunoglobulin M syndrome. *Clinical Infectious Diseases*, 31(5), 1183-1187.

Williams, R. P. (1973). Biosynthesis of prodigiosin, a secondary metabolite of *Serratia marcescens*. *Applied Microbiology*, 25(3), 396-402.

Williams, R. P., Gott, C. L., Qadri, S. H., & Scott, R. H. (1971). Influence of temperature of incubation and type of growth medium on pigmentation in *Serratia marcescens*. *Journal of Bacteriology*, 106(2), 438-443.

Williamson, N. R., Fineran, P. C., Gristwood, T., Chawrai, S. R., Leeper, F. J., & Salmond, G. P. (2007). Anticancer and immunosuppressive properties of bacterial prodiginines. *Future Microbiology*, 2(6), 605-618.

Williamson, N. R., Fineran, P. C., Leeper, F. J., & Salmond, G. P. (2006). The biosynthesis and regulation of bacterial prodiginines. *Nature Reviews Microbiology*, 4(12), 887-899.

Williamson, N. R., Simonsen, H. T., Ahmed, R. A., Goldet, G., Slater, H., Woodley, L., & Salmond, G. P. (2005). Biosynthesis of the red antibiotic, prodigiosin, in *Serratia*: identification of a novel 2-methyl-3-n-amyl-pyrrole (MAP) assembly pathway, definition of the terminal condensing enzyme, and implications for undecylprodigiosin biosynthesis in *Streptomyces*. *Molecular Microbiology*, 56(4), 971-989.

Wong, H. C., & Bau, Y. S. (1977). Pigmentation and antibacterial activity of fast neutron-and X-ray-induced strains of *Monascus purpureus* Went. *Plant Physiology*, 60(4), 578-581.

Wrede, F., & Hettche, O. (1929). About prodigiosin, the red dye of *Bacillus prodigiosus* (I. Commun.). *Reports of the German Chemical Society (A and B Series)* , 62 (9), 2678-2685.

Wu, W., Hu, J., Wang, J., Chen, X., Yao, N., Tao, J., & Zhou, Y. K. (2015). Analysis of phthalate esters in soils near an electronics manufacturing facility and from a non-industrialized area by gas purge microsyringe extraction and gas chromatography. *Science of Total Environ*ment, 508, 445–45.

Wu, X. L., Wang, Y. Y., Liang, R. X., Dai, Q. Y., & Chao, W. L. (2010). Degradation of di-n-butyl phthalate by newly isolated *Ochrobactrum* sp. *Bulletin of Environmental Contamination and Toxicology*, 85(3), 235-237.

Wu, X., Wang, Y., Liang, R., Dai, Q., Jin, D., & Chao, W. (2011). Biodegradation of an endocrine-disrupting chemical di-n-butyl phthalate by newly isolated *Agrobacterium* sp. and the biochemical pathway. *Process Biochemistry*, 46(5), 1090-1094.

Yadav, K. S., & Prabha, R. (2017). Production of intracellular carotenoid pigment from wild strains of *Rhodotorula*. *International Journal of Current Microbiology and Applied Sciences*, 6(5), 679-683.

Yamamoto, C., Takemoto, H., Kuno, K., Yamamoto, D., Tsubura, A., Kamata, K., & Inoue, K. (1999). Cycloprodigiosin hydrochloride, a new H+/Cl− symporter, induces apoptosis in human and rat hepatocellular cancer cell lines in vitro and inhibits the growth of

hepatocellular carcinoma xenografts in nude mice. *Hepatology*, 30(4), 894-902.

Yamazaki, G., Nishimura, S., Ishida, A., Kanagasabhapathy, M., Zhou, X., Nagata, S., & Ikeda, T. (2006). Effect of salt stress on pigment production of *Serratia rubidae*a N-1: a potential indicator strain for screening quorum sensing inhibitors from marine microbes. *The Journal of General and Applied Microbiology*, 52(2), 113-117.

Yan, Y., Ma, Y. T., Yang, J., Horsman, G. P., Luo, D., Ji, X., & Huang, S. X. (2016). Tropolone ring construction in the biosynthesis of rubrolone B, a cationic tropolone alkaloid from endophytic *Streptomyces*. *Organic Letters*, 18(6), 1254-1257.

Yang, F., Wei, H. Y., Li, X. Q., Li, Y. H., Li, X. B., Yin, L. H., & Pu, Y. P. (2013). Isolation and characterization of an algicidal bacterium indigenous to Lake Taihu with a red pigment able to lyse *Microcystis aeruginosa*. *Biomedical and Environmental Sciences*, 26(2), 148-154.

Yang, X., Zhang, C., He, Z., Hu, X., Guo, J., Zhong, Q., Wang,J., Xiong, li., & Liu, D. (2013). Isolation and characterization of two n-butyl benzyl phthalate degrading bacteria. *International Biodeterioration & Biodegradation*, 76, 8-11.

Yang, Y. H., Lee, T. H., Kim, J. H., Kim, E. J., Joo, H. S., Lee, C. S., & Kim, B. G. (2006). High-throughput detection method of quorum-sensing molecules by colorimetry and its applications. *Analytical Biochemistry*, 356(2), 297-299.

Yi, H., Chang, Y. H., Oh, H. W., Bae, K. S., & Chun, J. (2003). *Zooshikella ganghwensis* gen. nov., sp. nov., isolated from tidal flat

sediments. *International Journal of Systematic and Evolutionary Microbiology*, 53(4), 1013-1018.

Yip, C. H., Yarkoni, O., Ajioka, J., Wan, K. L., & Nathan, S. (2019). Recent advancements in high-level synthesis of the promising clinical drug, prodigiosin. *Applied microbiology and Biotechnology*, *103*(4), 1667-1680.

Yokoyama, A., & Miki, W. (1995). Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum*. *FEMS Microbiology Letters*, 128(2), 139-144.

Yuan, S. Y., Liu, C., Liao, C. S., & Chang, B. V. (2002). Occurrence and microbial degradation of phthalate esters in Taiwan river sediments. *Chemosphere*, 49(10), 1295-1299.

Zang, C. Z., Yeh, C. W., Chang, W. F., Lin, C. C., Kan, S. C., Shieh, C. J., & Liu, Y. C. (2014). Identification and enhanced production of prodigiosin isoform pigment from *Serratia marcescens* N10612. *Journal of the Taiwan Institute of Chemical Engineers*, 45(4), 1133-1139.

Zhang, H. G., Sun, G. S., Sun, L., Zhou, Z. F., Zhang, S. K., & Sui, H. (2013). Preliminary study on phthalic acid esters pollution of typical plastic mulched crops soils. *Environmental Monitoring and Assessment*, 29, 60–63.

Zhang, K., Liu, Y., Luo, H., Chen, Q., Zhu, Z., Chen, W., Chen,J., Ji,L., & Mo, Y. (2017). Bacterial community dynamics and enhanced degradation of di-n-octyl phthalate (DOP) by corncob-sodium alginate immobilized bacteria. *Geoderma*, 305, 264-274.

Zhang, W., & Li, C. (2016). Exploiting quorum sensing interfering strategies in gram-negative bacteria for the enhancement of environmental applications. *Frontiers in Microbiology, 6*, 1535.

Zhang, X., & Enomoto, K. (2011). Characterization of a gene cluster and its putative promoter region for violacein biosynthesis in *Pseudoalteromonas* sp. 520P1. *Applied Microbiology and Biotechnology*, 90(6), 1963-1971.

Zhang, Y., Meng, X., Chen, L., Li, D., Zhao, L., Zhao, Y., Li, L., & Shi, H. (2014). Age and sex-specific relationships between phthalate exposures and obesity in Chinese children at puberty. *PloS One*, 9(8).

Zhang, Z. M., Zhang, H. H., Zhang, J., Wang, Q. W., & Yang, G. P. (2018). Occurrence, distribution, and ecological risks of phthalate esters in the seawater and sediment of Changjiang River Estuary and its adjacent area. *Science of the Total Environment*, 619, 93-102.

Zhang, Z., & Pierson, L. S. (2001). A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Applied and Environmental Microbiology*, 67(9), 4305-4315.

Zhao, C., Qiu, S., He, J., Peng, Y., Xu, H., Feng, Z., & Nie, Y. (2020). Prodigiosin impairs autophagosome-lysosome fusion that sensitizes colorectal cancer cells to 5-fluorouracil-induced cell death. *Cancer Letters*, 481, 15-23

Zheng, Y., Xin, Y., Shi, X., & Guo, Y. (2010). Anti-cancer effect of rubropunctatin against human gastric carcinoma cells BGC-823. *Applied Microbiology and Biotechnology*, 88(5), 1169-1177.

Zhu, F., Zhu, C., Zhou, D., & Gao, J. (2019). Fate of di (2-ethylhexyl) phthalate and its impact on soil bacterial community under aerobic and anaerobic conditions. *Chemosphere*, 216, 84-93.

Zhu, X. J., & Qiu, Y. Y. (2011). Measuring the phthalates of Xiangjiang river using liquid-liquid extraction gas chromatography. *Advanced Material Research*, 301, 752-755.

Zornikova, G., Jarosova, A., & Hrivna, L. (2014). Distribution of phthalic acid esters in agricultural plants and soil. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 59(3), 233-238.

APPENDIX

LIST OF PUBLICATIONS

A. List of Published Research Papers

1. **Hareesh, E. S.,** Harilal, C. C., Pradeep, S., & Julia G. (2020). Quorum sensing mediated response of *Achromobacter denitrificans* SP1 towards prodigiosin production under phthalate stress. *Journal of Basic Microbiology (Wiley)*, 60(9), 758-767**.** IF- 1.909

2. **Hareesh, E. S.,** Harilal, C. C., & Pradeep, S., (2020). Response surface optimization of prodigiosin production by phthalate degrading *Achromobacter denitrificans* SP1 and exploring its antibacterial properties. *Preparative Biochemistry and Biotechnology (Taylor & Francis)*, 50(6), 564-571. IF-1.415

3. **Hareesh, E. S.,** Faisal, P. A., & Sailas Benjamin (2016). Optimization of parameters for the production of cellulose from *Achromobacter xylosoxidans* BSS4 by solid-state fermentation. *Electronic Journal of Biology*, 12(4), 443-448.

4. Pradeep, S., Sarath Josh, M. K., **Hareesh, E. S.,** Sunil, K., & Sailas Benjamin (2015). *Achromobacter denitrificans* strain SP1 produces an intracellular esterase upon utilizing di(2-ethylhexyl) phthalate. *International Biodeterioration and Biodegradation (Elsevier)*, 105, 160-167. IF- 4.074

5. Unni, K. N., Faisal, P. A., Priji, P., Sajith, S., Sreedevi, S., **Hareesh, E. S.,** Nidheesh Roy, T. A., & Sailas Benjamin (2015). Rubber seed kernel as potent solid substrater for the production of lipase by

Pseudomonas aeroginosa strain BUP2. *Advances in Enzyme Research,* 3, 31-38.

6. Faisal, P. A., **Hareesh, E. S.,** Priji, P., Unni, K. N., Sajith, S., Sreedevi, S., Sarath Josh, M. K., & Sailas Benjamin (2014). Optimization of parameters for the production of lipase from *Pseudomonas* sp. BUP6 by solid state fermentation. *Advances in Enzyme Research*, 2, 125-133.

B. Conferences/Seminar Presentations

1. **Hareesh, E. S.** and Harilal, C. C. Selection of ideal medium and DEHP concentration for the enhanced production of prodigiosin by *Achromobacter denitrificans* SP1 and an *in-vitro* assessment of its antibacterial properties. National conference organized by Department of Botany, Periyar University, Salem, Tamil Nadu on 1-2 February 2018.

2. **Hareesh, E. S.,** Harilal, C. C. and Ajayan, K. V. Response of *Achromobacter denitrificans* strain SP1 on prodigiosin production to different light sources and temperatures. National seminar organized by SAFI Institute of Advanced Study, Ramanattukara, Kerala on 15-16 March 2018.

3. **Hareesh, E. S.** and Harilal, C.C. Profiling of quorum sensing signaling molecules (N-acyl-Homoserine Lactones) produced by *Achromobacter denitrificans* SP1 using LC-Q-ToF-MS analysis and its role in prodigiosin production. International conference organized by Department of Microbiology, Periyar University, Salem, Tamil Nadu on 19-21 December 2018.