Antioxidant potential of marine pigmented bacteria from the central west coast of India

A thesis submitted to Goa University for the award of degree of

Doctor of Philosophy

In

MICROBIOLOGY

By

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Under the guidance of

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Declaration

As required under the University ordinance, I hereby state that the present thesis for Ph.D. degree entitled "**Antioxidant potential of marine pigmented bacteria from the central west coast of India**" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/grade/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

The literature related to the problem investigated has been cited. Due acknowledgement have been made whenever facilities and suggestions have been availed of.

RAVINDRA PAWAR

Certificate

Certified that the research work embodied in this thesis entitled "**Antioxidant potential of marine pigmented bacteria from the central west coast of India**" submitted by Mr. Ravindra Tanaji Pawar for the award of Doctor of Philosophy degree in Microbiology at Goa University, Goa, is the original research work carried out by the candidate himself under my supervision and guidance.

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RAVINDRA PAWAR

Dedicated to My Beloved Father

and Family members

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List of abbreviations

AO: Antioxidant

FR: Free radical

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

CWCI: Central west coast of India

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

ABTS: 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic Acid Ammonium Salt)

FRAP: Ferric Reducing Antioxidant Power

RP: Reducing power

LPO: Lipid peroxidation

BHT: Butylated hydroxytoluene

TPTZ: 2,4,6-Tri(2-pyridyl)-s-triazine

SOD: Superoxide dismutase

CAT: Catalase

GST: Glutathione-S-transferase

AsA: Ascorbic acid

GAE: Gallic acid equivalents

TPC: Total phenolic content

CFU: Colony forming unit

PIGB: Pigmented bacteria

ZMA: Zobell Marin agar

NA: Nutrient agar

SWNA: Seawater nutrient agar

LB agar: Luria-Bertani agar

PP: Process parameters

MH agar: Mueller Hinton agar

Eq. : Equivalent

R_f: Retention factor

GC: Gas chromatography

LC: Liquid chromatography

MS: Mass spectroscopy

NMR: Nuclear magnetic resonance

TLC: Thin layer chromatography

ELISA: Enzyme-linked immunosorbent assay

MIC: Minimum inhibitory concentration

IC: Inhibitory concentration

EC: Effective concentration

TGI: Total growth inhibition

NCIM: National collection of industrial microbes

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Free radicals and antioxidants

Free radicals (FR) are the atoms or molecules that have lost an electron and as a result possess net charge or unpaired electron in their outermost shell. These free radicals are evidenced to be highly reactive, unstable and capable of independent existence (Halliwell and Gutteridge 2006). Free radicals/ oxidants are commonly generated during metabolic processes in the body and kept under controlled state (Gulcin et al. 2003). They perform certain necessary defensive functions e.g. neutralization of viruses and bacteria. However, it becomes a problem when these radicals are produced too many and remains present in the body for a longer time (Sies 1999).

Mitochondria, macrophages and peroxisomes are considered to be the primary sources of free radicals in the body of a living organism. However, the exposure to harsh external conditions such as pollution, radiation, industrial chemicals, cigarette smoke, herbicides, pathological metabolism or stressful modern lifestyle can also lead to an excessive production of free radicals (Del Mastero et al. 1980; Ebadi 2001).

1.1.1. Reactive species and their classification

The free radicals act either as reducing or oxidizing agents. In a reducing reaction, FR donates a single electron to non-radical species [$X^{\cdot} + Y \rightarrow X^{+} + Y^{\cdot -}$] whereas, in oxidation reaction it takes single electron from non-radical species [$PR + OH^{\cdot} \rightarrow PR^{\cdot +} + OH^{-}$]. In both cases, unstable reactive species are generated. To stabilize themselves, reactive species try to steal electrons/ hydrogen from surrounding biomolecules like proteins, carbohydrates, lipids and nucleic acids, etc. which interns initiates cascade of chain reactions in biological systems (Halliwell and Gutteridge 2006). It affects the normal functioning of bio-molecules and causes deleterious effects

on cell e.g. Damage to the cell membranes by free radical mediated lipid peroxidation results in homeostatic disruption and death of a living cell (Dekkers et al. 1996).

These reactive radicals categorized into two major forms i.e. the oxygen-derived reactive oxygen species (ROS) and nitrogen derived reactive nitrogen species (RNS). In living system ROS and RNS are formed either from enzymatic and non-enzymatic reactions or induced by endogenous and exogenous sources (Pham-Huy et al. 2008). Besides this the anthropogenic activities like exposure to chemicals, solvents and pesticides are also documented to generate reactive bromine species (RBS) and reactive chlorine species (RCS) causing adverse effects on human health. The nature of various reactive species and their sources are summarized in Table 1.1.

1.1.2. Oxidative stress and associated health risks

The overproduction of free radicals and their imbalance in a biological system termed as oxidative stress (OS). Under this state, organisms are unable to cope up with toxic effects of excessively produced reactive species. This cause imbalance of a tissue and leads to form peroxide and other related radicals that damage the cell components. Oxidative stress is known to cause deleterious effects on human health as well. The etiology of many human diseases (>100) is reported to implicate various free radical reaction e.g. premature aging, inflammatory, cardiovascular and other stress-associated disorders (Fig. 1.1). To counteract with such harmful oxidative reactions, the human body has developed certain defensive mechanisms. This majorly includes antioxidant defense systems that neutralize the excessively produced reactive species (ROS/RNS) and helps in prevention of disease (Pham-Huy et al. 2008).

Table 1.1. Types of reactive species, their nature and source.

Type of reactive species		Stability and reactivity nature	Source/ Reason of origin
ROS	Superoxide anions ($^{\bullet}O_2^-$)	Unstable	Mitochondria; cellular enzymes e.g. Oxidase etc.
	Peroxyl radical ($ROO^{\bullet}$)	Moderately stable and reactive	Proteins, lipids, sugars and nucleotides exposed to oxidative stress
	Hydroxyl ($OH^{\bullet}$)	Very highly unstable and reactive	Enzymatic reactions; phagocytic enzymes
	Hydrogen peroxide (H_2O_2)	Moderately stable	Mitochondria; cytoplasm; microsomes; enzymatic reactions
	Singlet oxygen ($^1O_2$)	Highly unstable and reactive	Skin; eyes; lungs etc.
RNS	Nitric oxide ($NO^{\bullet}$)	Stable	Mitochondria; phagocytes; exposure from polluted air
	Nitrogen dioxide ($NO^{\bullet}_2$)	Moderately stable	Environmental anthropogens
	Nitrate ($NO^{\bullet}_3$)	Moderately stable	Contaminated ground water
RBS	Atomic bromine ($Br^{\bullet}$)	Unstable	Environmental anthropogens
RCS	Atomic chlorine ($Cl^{\bullet}$)	Unstable	Environmental anthropogens

(Devasagayam et al. 2004; Halliwell and Gutteridge 2006)

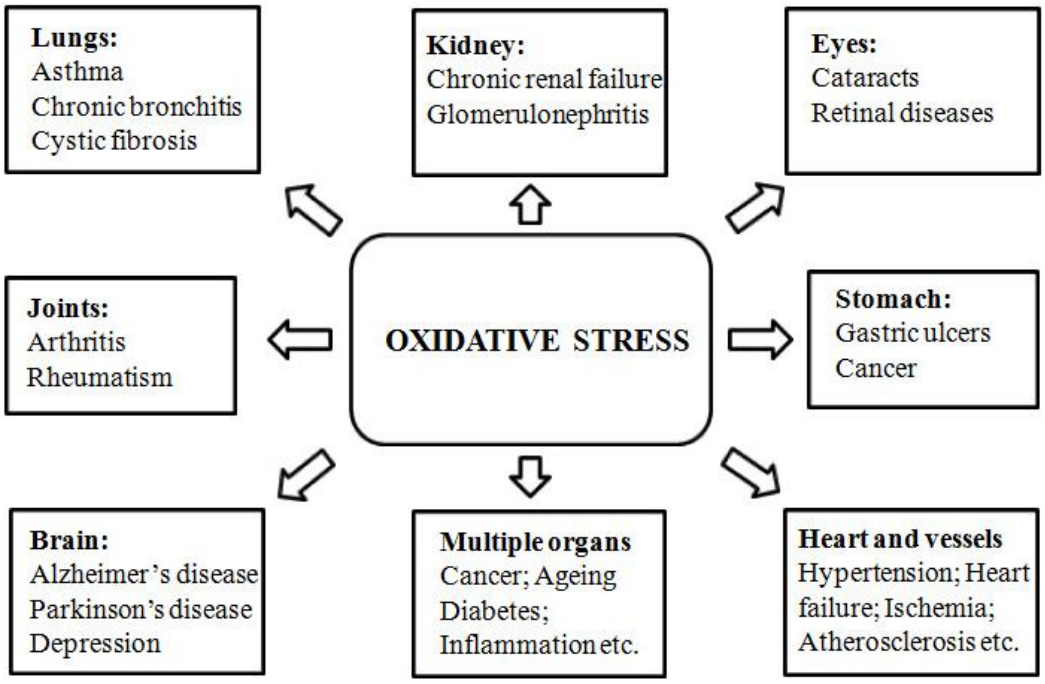


Fig. 1.1. Diseases associated with the implication of oxidative stress in human health.

(Adopted from Pham-Huy et al. 2008; Devasagayam et al. 2004)

1.1.3. Antioxidants

Antioxidant denotes the chemical term "against oxidation". Antioxidants (AO) are known to neutralize free radicals (reactive species) and impart protection to the living organisms against stress associated conditions. At the molecular level, AO gives up its electron to the reactive radicals and stops further chain reactions (Dekkers et al. 1996). Some of the mechanisms involved during radical neutralization process are (1) Scavenging of reactive species, (2) Minimizing localized production of reactive species to stop oxidation, (3) Chelation of metal ions to obstruct the formation of free radicals, (4) Quenching of superoxide ($^{\bullet}O_2^-$) radicals to avoid oxidation of biomolecules and (5) Breaching of radical associated chain reactions (Halliwell and Gutteridge 2006; Brewer 2011).

The antioxidant defense system is categorized into enzymatic and non-enzymatic types. The enzymatic antioxidant includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST). These enzymes play an important role *in vivo* as they directly involve in the catalysis of detoxification steps by converting toxic oxidants to less harmful products. The non-enzymatic AO refers to a variety of vitamins (A: Retinol, C: Ascorbic acid, E: Tocopherol), minerals and microelements that impart protective functions to the vital organelles of a cell (Fig. 1.2).

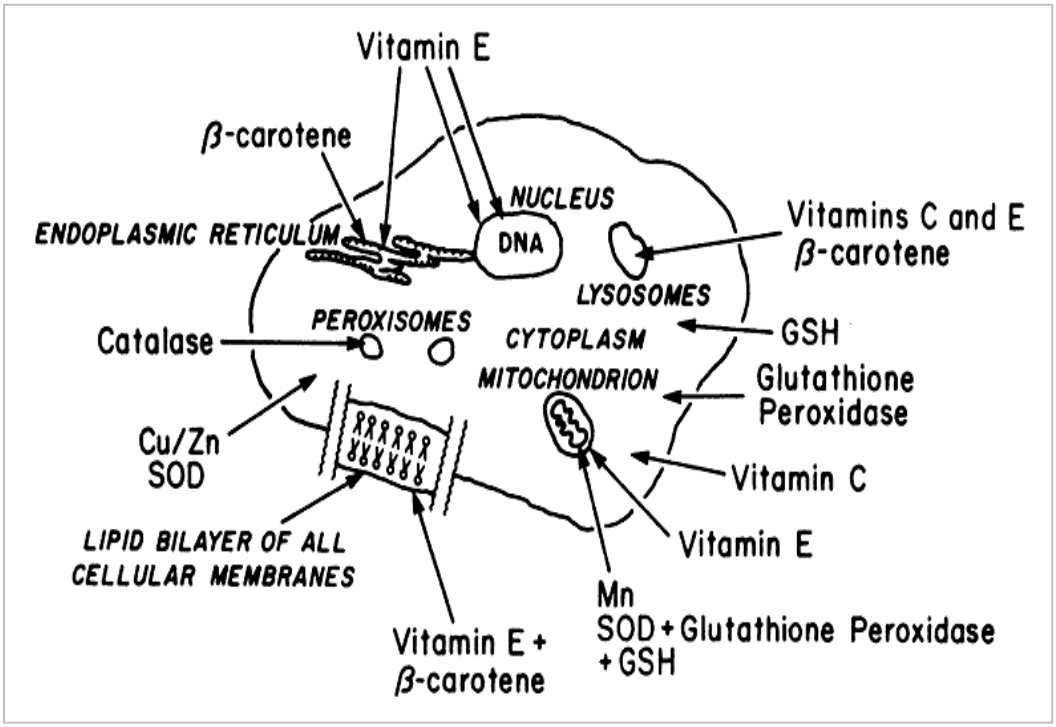


Fig. 1.2. Protective role of antioxidants in various cell organelles.

(Adopted from Freeman and Crapo 1982; Machlin and Bendich 1987)

1.1.4. Source and biological role of antioxidants

Antioxidants are normally synthesized within the body endogenously as well as obtained exogenously from dietary food contents. Different types of naturally occurring antioxidants, their source and its importance are summarized in Table 1.2. The

endogenous AO's broadly includes physiological enzymes like SOD, CAT, GPx and GST as well as some of the minor molecules. The minor molecules include metal chelating proteins, lipoic acid, ubiquinol, L-arginine, coenzyme Q10 and uric acid that are produced during normal metabolism by our body (Pham-Huy et al. 2008). On the other hand, the non-enzymatic essential antioxidants e.g. micronutrients/ vitamins are not synthesized *in vivo* by our body and that need to be supplied exogenously through diet or medicines.

Conversely, over last two decades, the synthetic AO's have also been used for instant relief of stress associated pathophysiological conditions, anti-aging and food preservation (Race 2009). However, later it was realized that the long term use or consumption of such artificial AO's causes an ill effect on human health. For example, butylated hydroxyanisole, butylated hydroxytoluene and more recently the tertiary butyl hydroquinone (TBHQ) are reported to be toxic, allergic and carcinogenic in animal studies and have been banned from human use in many countries (Llaurado 1983; Race 2009; Halliwell 2012).

This awareness is increasing the demand of natural antioxidants in health, food and personal care continuum at an exponential rate. However, the production specific antioxidative metabolites of targeted use and their isolation from plant source seem to be time-consuming and expensive. Hence to meet these demands it is of prime necessity to discover alternative resources of natural antioxidants for their continued supply for the human use. Targeting microbial products can be of beneficial interest due to their easy and fast product recovery, low space requirements and less cost of production.

Table 1.2. Biological importance of natural antioxidants.

Antioxidant		Source	Biological role
Classification	Name		
Enzymatic	Superoxide dismutase (SOD)	Endogenous: Mitochondria, cytoplasm, extracellular fluids	Dismutation of harmful superoxide radical ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2)
	Catalase (CAT)	Endogenous: Produced in almost all organs exposed to excess oxygen	Conversion of high concentration of H_2O_2 to water and oxygen
	Glutathione peroxidase (GPX)	Endogenous: Mitochondria and cytosol	Breakdown of H_2O_2 to water and oxygen
	Glutathione-S-transferase (GST):	Endogenous: Mitochondria, cytosol and microsomes.	Metabolism of hydroperoxides and prevents formation of hydrogen peroxide
Non-enzymatic	Vitamin A (Retinol)	Exogenous: Carrots, broccoli, sweet potatoes, tomatoes, peaches and apricots.	Quenching of singlet oxygen
	Vitamin C (Ascorbic acid)	Exogenous: Fruits like oranges and lime etc. Green leafy vegetables and tomatoes.	Protection against cardiovascular, DNA damage anti-carcinogenic
	Vitamin E (Tochopherol)	Exogenous: Nuts, seeds, grains, green leafy vegetables and oils	Avoid cell membrane damages, cancer, neurological and eye disorders
	Selenium	Exogenous: Seafood, nuts, garlic and meat	Imparts anti-carcinogenic and immunomodulatory effects
	Polyphenolic flavonoids, lycopene, etc.	Exogenous: Plants or microbial pigments	Anti-aging, anti-proliferative, helps to reduce inflammation and infections

(Pham-Huy et al. 2008; Lobo et al. 2010, Halliwell 2012)

1.2. Marine bacteria, pigments and biological significance

Bacteria living in association with higher organisms are receiving increased importance due to their ability to produce novel compounds of human importance. Metabolites, particularly from seaweed associated bacteria find their unique potentials. It is mainly because such plants provide more surface area, growth nutrients and protective environments for the invading microbes. This attracts an incredibly diverse number of organisms to adhere the algae surfaces. However, once the bacterial colonies established individual bacteria competes and try to avoid other organisms to occupy same inhabiting places by producing strong antagonistic molecules (Burke et al. 2011). Isolation and characterization of such molecules have shown practical applicability in bio-controlling agents and other bioactivities.

Conversely, the surfaces loaded bacterial comminutes are also recognized to play a major role in the life cycle of the host organisms. For example, Singh and Reddy (2014) reviewed the influencing role of associated bacteria in the morphogenesis and development of seaweeds. Besides this, reports also documented the surface attached bacteria during their symbiotic interaction attains similar genes from their host organisms that are responsible for biosynthesis of secondary metabolites (Schmidt 2005). Remarkably, the seaweed surfaces though known to harbor diverse forms of heterotrophic, phototrophic and chemotrophic bacteria only a small percentage of them have explored in context to their biological potentials.

During last decade, researchers are keenly engaged in drug screening programs to demonstrate the bio-pharmaceutical potential of marine organisms for the betterment of human health (e.g. ‘Drugs from Sea’). Among different forms of organisms present in marine environments, the bacteria with eye-catching pigments are one of such organisms

that have diverse physiological and biological role in the ecosystem. Investigating a range of such untapped pigmented bacteria (free-living and attached) for their bioactive potential is expected to reveal novel targets of biomedical importance.

Pigment 'the colouring substance' is considered to be one of the most conspicuous traits in live forms. Their diverse nature helps them to take part in various physiological and molecular processes like colouration, photosynthesis and protection against cell damage. Plants and animals are well known for pigment production. Moreover, many microorganisms like algae, fungi and bacteria also synthesize a variety of pigments (Venil and Lakshmanaperumalsamy 2009). Pigmentation is widespread among bacteria and observed to play potential roles in their life cycle. They are present in all phototrophic bacterial cell membranes and carry out the photosynthetic function for utilization of energy as a part of their physiology and growth. Besides this, the protection against environmental stressors, oxidative damage, UV radiation, resistance to heavy metals, anthropogenic pollutants and antimicrobials are some of the most important biological roles conferred by pigments to the host organisms (Cardona-Cardona et al. 2010).

Since ancient time, pigment as an attributive character of a particular genus has been utilized as a reference taxonomic tool for identification of various microorganisms like algae, fungi and bacteria (Szczepanowska and Lovett 1992). For example, the organisms belonging to *Xanthomonas* genus express yellow pigment with typical chromatographic and absorption spectra that intern utilized for their categorization (Bhawsar 2011).

Bacteria belonging to *Cytophaga*, *Chromobacteria*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia* genera are most commonly known for the production of pigments (Fig. 1.3). They produce diverse range of carotenoid,

phycobilins, melanins, betacarotene, quinines, xanthophylls, phycoerythrin, flavins and phycocyanin pigments with various colour properties (Logan 1994; Shindo and Misawa 2014). Among these, carotenoids are the most diverse forms of pigments in nature. Bacterial pigments vary in their nature due to differences in chemical structure, compositions and the type of chromatophores. Chemotrophic organisms produce carotenoids and over 750 different structures have been identified till the date (Britton et al. 2004). They appear yellow, orange, brown or red. Because of their color and biological properties they are used commercially as food colorants, animal feed supplements and more recently, as nutraceuticals for cosmetic/ pharmaceutical purposes (Stafsnes et al. 2010).

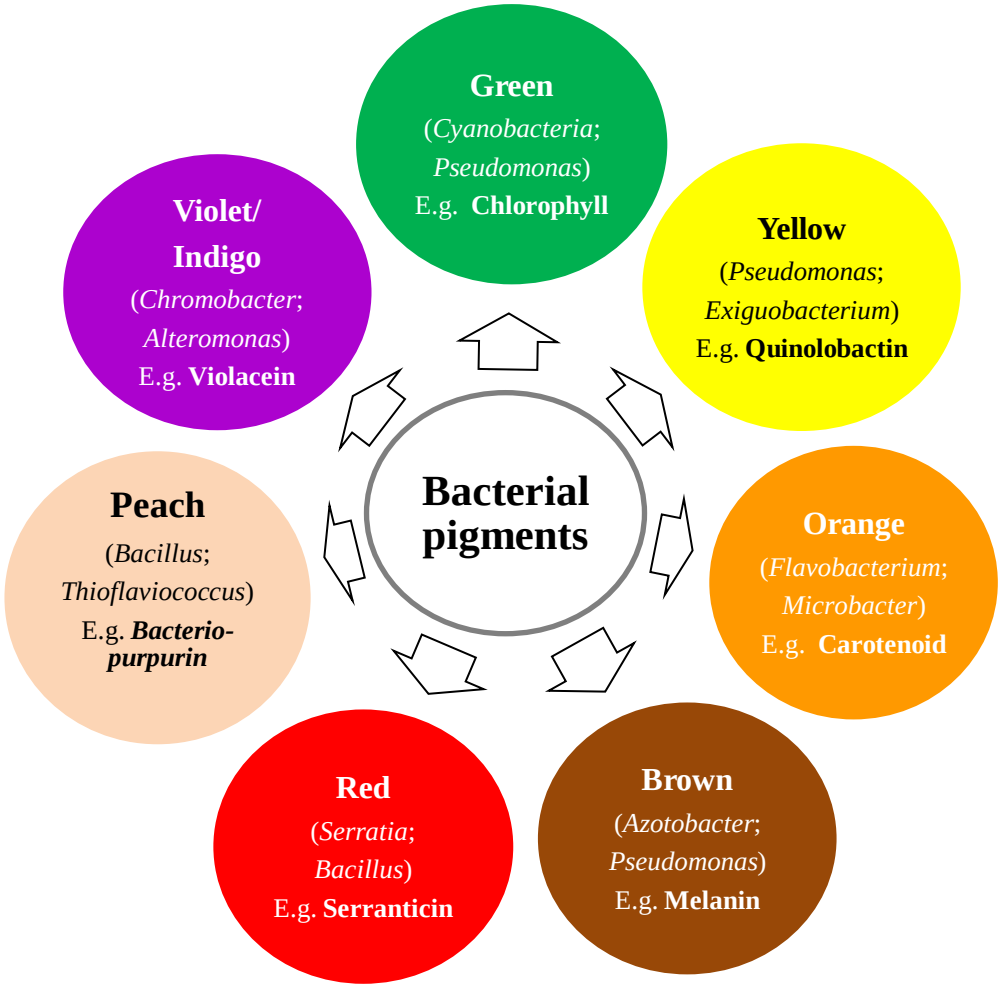


Fig. 1.3. Biological pigments produced by bacterial species.

Bacteria can produce soluble as well as insoluble pigments either as primary or secondary metabolites and this production is dependent on different stages of their growth (Venil and Lakshmanaperumalsamy 2009; Bhawsar 2011). As discussed earlier most of the pigments are produced as part physiology or induced to overcome environmental stress. Recent findings have proved that marine bacteria utilize pigments as a defensive agent to scavenge or nullify the harmful effects of stress associated free radicals in natural environments (Dieser et al. 2010; Correa-Llanten et al. 2012).

Various physiological and molecular properties attributed to pigments are summarized as below (Cardona-Cardona et al. 2010; Soliev et al. 2011; Correa-Llanten et al. 2012; Pawar et al. 2015)

- ✓ It helps in acquisition of energy by photosynthesis
- ✓ Maintains membrane integrity and stability
- ✓ Protects host cell under extremes heat and cold temperatures
- ✓ Protection against Ultra-Violet (UV) radiations
- ✓ Protection against oxidative reactions
- ✓ Acquisition of nutrients such as iron
- ✓ Resistance to antibacterial agents and heavy metals

Among various kinds of pigments produced by bacterial species, some are observed to impart beneficial effects while some are reported to be harmful to humans. For example 'Violacein' a violet coloured pigment produced by *Chromobacterium violaceum* shown to have tumoricidal, bactericidal, trypanocidal and anti-viral activities of human use (Mendes et al. 2001). While, on the other hand, *Pseudomonas aeruginosa* produces pyocyanin and pyoverdin pigments that are recognized to facilitate pathogenesis (Kipnis et al. 2006). Looking from the beneficial point of view pigments of

bacteria especially those of marine origin can be of great importance for their prospective utilization in animal and human health.

More recently the pigmented microorganisms have awakened the interest of the scientific community, because of their biotechnological perspective role in industrial processes like fermentation, bioprocess engineering and pharmacological applications (Szczepanowska and Lovett 1992; Chatoopadhyay et al. 2008; Soliev et al. 2011). Bacterial pigments like prodigiosin, phenazine and serranticin derivatives are documented to show antiplasmodial, antibiotic, antitumor and inhibition of lipid peroxidation activities (Lavy et al. 2005; Kuo et al. 2011). More recently, Horta et al. (2014) and Pawar et al. (2015) have demonstrated the seaweed associated pigmented bacteria possess significant antimicrobial and antioxidant properties.

1.3. Review on antioxidant potential of bacterial pigments

Smith and Alford (1970) first time emphasized the presence of antioxidant compounds in bacteria. Later, Scwartz and Shklar (1988) reviewed the importance of pigmented compounds from the marine origin and their potential pharmacological applications. However, since then very limited studies have taken efforts to investigate pigmented bacteria for their antioxidant potentials. Some of such studies revealing antioxidative pigmented compounds and associated derivatives from the bacterial source summarized in Table 1.3.

To our knowledge, studies in context to antioxidative metabolites from bacteria are largely contributed by pigment producers. On the other hand, though researchers have demonstrated that more than half percentage of bacteria available in nature are

pigmented there has been a big knowledge gap between what is available and what is explored for antioxidant search (Courington and Goodwin 1955; Du et al. 2006).

Researchers have recognized the role of these pigments and derivative metabolites in providing relief from stress associated pathological conditions including inflammation, tumor, cancer and cardiovascular diseases (Venil and Lakshmanaperumalsamy 2009; Shindo and Misawa 2014). As discussed earlier, most of the pigments synthesized by bacteria are a large number of carotenoids in nature and proven to exhibit potent singlet oxygen ($^1O_2$) and reactive radical scavenging properties (Fiedor and Burda 2014).

A red pigment carotenoid derivative obtained from *Dienococcus radiophilus* has been evidenced to protect the bacterial cell from the oxidation of membrane proteins. Later, this radical scavenging (antioxidant) mechanism is evidenced to impart radioresistant property to the host organism (Lavy et al. 2005). Recently, this pigment was elucidated as Deinoxanthin and attributed for it's strong radioresistant and antioxidant properties as well (Ji 2010). Similarly, other bacterial pigments like violacein, serranticin, zeaxanthin are also documented to be involved in obstruction of free radical reactions and act as significant antioxidants (Kuo et al. 2011; Prabhu et al. 2013).

Interestingly, as represented in Table 1.3, the pigmentary metabolites possessing antioxidant properties are also reported to exhibit one or more other beneficial bioactivities of human importance. For example, Konzen et al. (2006) isolated *Chromobacterium violaceum* and reported that these bacteria have the ability to produce a purple coloured pigment called as violacein with high antioxidant efficiency against ROS and RNS. Later, this pigment was also observed to exhibit anti-inflammatory,

anticancer, anti-protozoan, antimicrobial and Gastroprotective activities as well (Duran and Menck 2001; Antonisamy et al. 2014).

Thses evidences emphasize that the pigments of bacterial origin do play multipotent functions in human health. Here we hypothesize that diverse biological activities of such colourful bacteria are must be assigned by the antioxidative pigmentary metabolites secreted by them. Some of the above-discussed pigments e.g. astaxanthin and zeaxanthin are even referred as ‘super antioxidants’ due to their better radical scavenging functions. However, the production of such antioxidant-rich pigmentary metabolites is still at the level of research projects or developmental stage. Thus, it determines the need of more elegant studies on marine bacterial pigments to include novel metabolites in the antioxidant therapy.

Table 1.3. List of antioxidants and its biological activity from the pigmented bacteria.

Pigment type (Colour)	Biological activity	Microbial source (Origin)	Scale up status	Reference
Astaxanthin (Pink)	Anti-oxidation	*Agrobacterium aurantiacum* (Marine)	IP	Misawa et al. 1995
Violacein (Violet)	Antioxidant, antibacterial, antiviral and antitumor	*Chromobacterium violaceum*, *Pseudoalteromonas luteoviolacea* (Marine)	IP	Konzen et al. 2006; Duran and Menck 2001; Yang et al. 2007
Carotenoid (Red)	Antioxidant	*Planococcus maritimus* (Marine)	RP/DS	Shindo et al. 2008
Deinoxanthin (Red)	ROS scavenger; Inhibition of LDL lipid peroxidation	*Dienococcus radiophilus* (Marine)	RP/DS	Lavy et al. 2005; Ji 2010
Serranticin (Pink/ Red)	Antioxidant + antitumor	*Serratia ureilytica* (Terrestrial)	RP/DS	Kuo et al. 2011
Zeaxanthin (Yellow)	Antioxidant	*Flavobacter* sp. (Marine)	RP/DS	Prabhu et al. 2013

Unknown (Orange)	Antioxidant + Antimicrobial	*Shewanella* sp. (Marine)	RP/DS	Horta et al. 2014
Carotenoids (Yellow/ Red)	Antioxidant	*Rubritalea*, *Planococcus*, *Flavobacter* sp. (Marine)	RP/DS	Shindo and Misawa 2014
Unknown (Peach)	Free radical scavenging	*Exiguobacterium* sp. (Marine)	RP/DS	Balraj et al. 2014
Unknown (Red)	Antioxidant + antimicrobial	*S. marcescens* (Terrestrial)	RP/DS	Nongkhlaw and Joshi 2015
Unknown (Brown)	Antioxidant	*Pseudomonas koreensis* (Marine)	RP/DS	Pawar et al. 2015; Pawar et al. 2016
Unknown (Yellow)	Antioxidant	*P. argentinensis*, *Vibrio* sp. (Marine)		
Unknown (Red)	Antioxidant	*S. rubidaea* (Marine)		

Industrial production (IP), research project/ development stage (RP/DS).

1.4. Why marine pigmented bacteria for antioxidants?

Ocean harbors a large number of chromogenic organisms possessing photosynthetic pigments. As stated by Courington and Goodwin (1955), more than 50% of bacteria present in marine ecosystem are pigmented in nature. In a very early survey from a marine source has shown among thousands of bacteria isolated, 61.8% are pigmented (ZoBell and Feltham 1934). Du et al. (2006) had also shown vast endurance of heterotrophic pigmented bacteria in coastal and shelf waters.

The antioxidant property of bacteria can be explained depend upon the environmental conditions in which bacteria live or adapt (Horta et al. 2014). Most of the marine bacteria live in association with higher organisms and lack their prominent structural defense mechanisms to overcome ecological stressors. However, to survive extreme vicinity environments, they are totally dependent on chemical defense system where they produce unique secondary metabolites those helps them to overcome the stress conditions (Debbab et al. 2010).

In a marine ecosystem, the formation of ROS is obvious due to frequently changing physicochemical conditions. The dissolved organic matter in ocean normally absorbs solar rays including UV radiations and causes various photochemical reactions. It further generates a variety of reactive radical species (Okuyama et al. 2008). Bacterial communities residing in the close vicinity may not have the ability to challenge such environmental changes. However, they produce certain stress relieving secondary metabolites (i.e. antioxidant) or attain enzymatic mechanisms to conquer excessively produced free radicals.

Recent investigations have significantly correlated the protective role of bacterial pigments against environment-induced free radicals and oxidative stress conditions (Shindo and Misawa 2014). For example, Dieser and coworkers (2010) demonstrated the Antarctic heterotrophic bacteria like *Arthrobacter*, *Flavobacterium* and *Sphingomonas* sp. utilize carotenoid pigments as a strategy to withstand environmental stresses. They exposed these bacterial species to freeze-thaw cycles and solar radiation and found out that pigmented bacteria have more resilience power against environmental stressors. Similarly, Godinho and Bhosle (2008) and Correa-Llanten et al. (2012) have also documented the defensive role of bacterial pigments in overcoming the stressed marine habitats.

As illustrated in Table 1.3, the carotenoid pigments of marine bacterial origin are known to impart radical quenching and antioxidant effects (Shindo et al. 2008; Prabhu et al. 2013). Researchers from Marine Biotechnology Institute (Japan) have evidenced that marine bacteria do own gene clusters required for biosynthesis of antioxidative carotenoids. This group has isolated the pigmented carotenoid derivatives astaxanthin glucoside, 2-hydroxyastaxanthin and 4-ketonostoxanthin-3′-sulfate from *Agrobacterium*, *Brevundimonas* and *Erythrobacter* sp. and demonstrated to exhibit radical scavenging action (Yokoyama et al. 1995; Yokoyama et al. 1996). Recently, they also showed that the novel species of marine pigmented bacteria to produces rare types of acyclic C_{30} and monocyclic C_{40} carotenoids with characteristic antioxidant properties (Shindo and Misawa 2014).

These investigations indicate that marine pigmented bacteria do naturally own stress relieving (radical scavenging) capacity. Hence, it is indeed of immense relevance

to utilize this antioxidant property of marine bacterial pigments for their implications in stress associated diseases.

Some of the major reasons for bio-prospecting marine pigmented bacteria towards antioxidant search can be summed up as below,

- Pigments of marine bacteria possess natural stress relieving (radical scavenging) property.
- Availability of incredibly diverse flora of pigmented bacteria in marine ecosystems.
- Diverse pigments with distinct chemical properties.
- Significant source of bio-pigments (e.g. carotenoids).
- Reduced downstream processing and easy product recovery due to colour property.
- Requirement of minimal growth medium, less space, faster growth rates and prominent cheap source of natural antioxidants.

Thus, as discussed above and the data collected from recent literature indicate that researchers of this decade are keenly interested in the exploration of marine pigments towards their antioxidant and other biological properties (Table 1.3). Conversely, except our first report (Pawar et al. 2015), very limited information is available exclusively on ‘antioxidants’ from ‘associated pigmented bacterial flora’. This evidence establishes the need of exploring pigmented bacterial flora from the Indian coasts for their radical scavenging and antioxidant potentials.

1.5. Objective and work plan

Objective:

Marine pigmented bacteria from the central west coast of India for its potential towards antioxidant properties.

Work plan:

1. Isolation of marine pigmented bacteria from seawater, sediment and seaweed associates.
2. Checking the bacterial pigments for its properties over free radical scavenging and antioxidant activities.
3. Molecular and biochemical characterization of potential antioxidative bacterial strains.
4. Structural analysis of antioxidant materials and its potential for biotechnological applications.

CHAPTER 2

SCREENING, ISOLATION AND CHARACTERIZATION OF MARINE PIGMENTED BACTERIA

2.1. Introduction

Marine ambiance is a complex system. It nurtures diverse types of bacteria by supplying a variety of nutrients for their growth, survival and adaptation. The isolation of different types of bacteria (heterotrophic, oligotrophic, chemotrophic, etc.) from marine environments in laboratory conditions has been a controversial topic since a long time (Schut et al. 1997; Stewart 2012). It's documented that many of the marine bacteria fail to grow on artificial media unless prepared in seawater (Fisher 1894). Since it's almost impossible to imitate similar oceanic conditions *in vitro*, it's a crucial step to select the suitable media for primary isolation of marine bacteria.

Concerning isolation of pigmented bacteria researchers has employed various synthetic, semi-synthetic and organic media for their primary retrieval *in vitro*. Among these some of the commonly used media such as R2A agar (Reasoner and Geldreich 1985), Difco sporulation medium (Khaneja et al. 2010), Zobell Marine Agar (ZMA: Zobell and Feltham 1934; Jafarzade et al. 2013), Luria-Bertani (LB) and sea water yeast extract-peptone (SYEP) medium (Balraj et al. 2014) have been documented to support the growth and differentiation of diverse pigment producing bacteria. Similarly, general isolation studies with nutrient agar supplemented with seawater also suggested its use for maximum growth of marine bacteria particularly the pigmented ones (Ahmad et al. 2012).

Zobell and his colleagues (1944) explored 60 novel species of marine bacteria and found that 19 of them belong to pigmented family. Likewise, heterotrophic PIGB isolated from China coast had shown wide retrievability in terms of colour property and genetic basis (Du et al. 2006). They showed the supportive role of ZMA for isolation of diverse coloured bacteria belonging to Alphaproteobacteria, Gammaproteobacteria,

Actinobacteria, Bacilli, Flavobacteria and Sphingobacteria groups. Further, recent studies emphasize the use of ZMA in addition to commonly available SWNA for isolation of marine PIGB from different sources like seawater, sediment, sponges, seaweeds, mangrove and other higher animals (Jafarzade et al. 2013, Horta et al. 2014).

Looking at the Arabian Sea in particular, it has been found to endure a wide range of marine microbes. This is mainly because of the geographical variations and environmental conditions e.g. monsoon, nutrients/mineral variability, upwelling, etc. and presence of specialized niches like Oxygen Minimum Zone (Khandeparker et al. 2011; Jain et al. 2014). Microbes from this area particularly the bacteria are witnessed to play diverse ecological and physiological functions and have demonstrated their potentials in industrial applications as well.

Above studies though highlights the vastness of useful bacteria, pigmented bacteria and their potentials are not well understood exclusively. Few of the recent studies on the biological activity tested, have shown the pharmaceutical importance of pigmented organisms (Jayanth et al. 2002; Balraj et al. 2014; Pawar et al. 2015). It emphasizes the need of comprehensive investigations on exploring marine PIGB from different locations and sources. Hence, we explored a part of CWCI (i.e. Maharashtra and Goa coasts) for pigmented bacteria using ZMA and SWNA for antioxidant studies.

2.2. Materials and methods

2.2.1. Description of sampling locations

India has a stretch of about total 7516.6 km coastline divided into the east coast, west coasts and island chains. The west coast, as a part of Indian Ocean is situated along the

Arabian Sea and extends right from Gujarat to Kerala including the coral atolls of Lakshadweep Islands. Among which the central west coast of India (CWCI) covers coastal locations from the states of Maharashtra and Goa. As discussed earlier, these sites have received a prime importance due to south-west monsoon, appearance of oxygen minimum zones, coastal upwelling and high nutrient availability.

As shown in Fig. 2.1 we selected four stations, two from Maharashtra and two from Goa along the CWCI. Station Kunkeshwar (Site: Kunkeshwar temple: 16˚ 20' 02.4" N; 73˚ 23' 31.3" E) and Malvan (Site: Rock garden coast: 16˚ 03' 47.5" N; 73˚ 27' 22.5" E) are from the state of Maharashtra. These sites primarily composed of an open coastal ecosystem with dominated sedimentary rocky outcrops over long stretches and sandy beach.

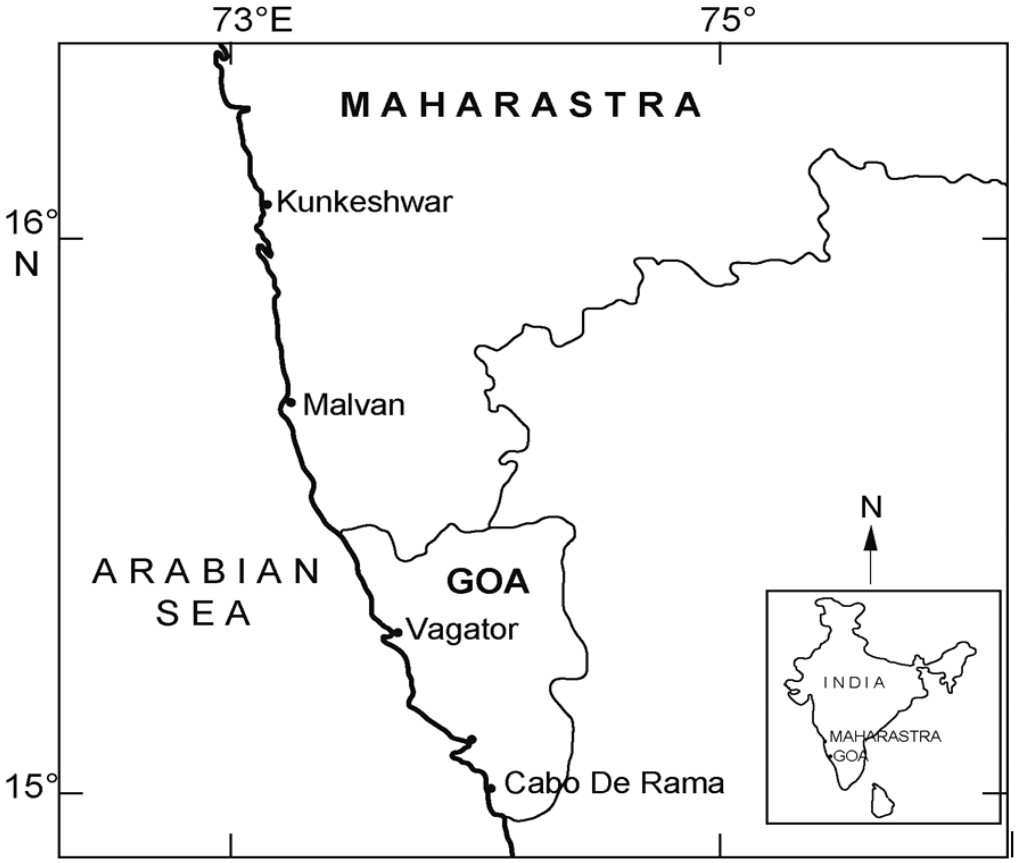


Fig. 2.1. Sampling locations along the central west coast of India (CWCI).

The other two locations, Vagator (15˚ 35’ 54.4” N; 73˚ 44’ 14.6” E) and Cabo De Rama (15˚ 05’ 25.37” N; 73˚ 55’ 15.15” E) are situated along the Goa coast. The Vagator has rocky and sandy ecosystems where numbers of tidal pools have formed due to tidal fluctuations. While, the Cabo De Rama coast has narrowed submerged and exposed rocky beach that continues to the Cabo De Rama fort. All these selected locations support the rich diversity of seaweeds and form suitable locations for isolating the associated bacteria (Fig. 2.2).

2.2.2. Sample collection strategy

Seawater, sediment and seaweeds were collected bimonthly during March-2010 to January-2011 from the selected sites along the central west coast of India (Fig. 2.1). To cross check the occurrence and diversity of pigmented bacteria, we also repeated the sampling during March 2012 to January- 2013 seasonally.

Seawater sampling was carried out by collecting water samples directly just below the surface using sterile plastic bottles. Sediment samples were collected with the help of core. Seaweed samples (4-5 dominant species) from each site were picked with sterile forceps during ebb tide. Seaweed samples were added with little amount of seawater from the same vicinity to maintain the viability of associated bacterial flora. All collected samples were stored in ice cold condition and transported to the laboratory at the earliest for further analysis. To obtain maximum numbers and diverse forms of pigmented bacteria, we have collected 28 species of seaweeds from different locations. These algae belonging to the major groups like Chlorophyta (green algae), Phaeophyta (brown algae) and Rhodophyta (red algae) (Fig. 2.3).

Fig. 2.2. Geographical overview and seaweed vegetation along the sampling sites.

Fig. 2.3. Representative seaweed species from the CWCI [Chlorophyta: (A) *Ulva* sp., (B) *Valoniopsis pachynema*, (C) *Caulerpa peltata*, (D) *Caulerpa scalpelliformis*, (E) *Chaetomorpha* sp.; Phaeophyta: (F) *Sargassum* sp., (G) *Padina tetrastromatica*, (H) *Dictyota dichotoma*, (I) *Sphacelaria* sp., (J) *Spatoglossum asperum*; Rhodophyta: (K) *Gelidium micropterum*, (L) *Acanthophora spicifera*, (M) *Gracilaria* sp., (N) *Polysiphonia* sp., (O) *Porphyra vietanamensis*].

2.2.3. Isolation of pigmented bacteria

2.2.3.1. Analysis of seawater and sediment samples

The seawater and sediment samples were plated preferably by spread plate method. Seawater samples depending upon the turbidity were serially diluted from 10^{-4} to 10^{-6} with sterile seawater. Conversely, in the case of sediment approximately 1 gm samples was suspended in 9 ml of sterile seawater and serial dilutions were made up to 10^{-7} dilution. 100 µl of diluted samples were inoculated in duplicates on Zobell Marine Agar (ZMA) and 50% Seawater Nutrient Agar (SWNA) (Hi- Media, Mumbai). Plates were at 28 ± 2 °C for 24-72 hours (h) and observed for the appearance of pigmented colonies.

2.2.3.2. Analysis of epiphytic bacteria

(A) Primary observation by scanning electron microscopy (SEM)

The primary observational studies on surface attached bacteria were carried out with scanning electron microscopy from few of the representative seaweed samples by following standard protocol (Underwood 1991).

1. The seaweed was cut into small (< 1 cm) pieces, washed with sterile seawater and preserved in 5% (v/v) phosphate-buffered glutaraldehyde (pH 7.2) at 4 °C.
2. Before use, the preservative was removed by washing the sample for a short time (10 min) by sodium cacodylate buffer solution (0.05 M; pH 7.2).
3. Each sample passed through a graded ethanol series (30%, 50%, 70%, 90%, and 100%) and finally dehydrated in 100% acetone till critical point dried.
4. The pieces of dried samples were then stuck on aluminium specimen holder (stub), coated with a very thin layer of gold in a sputter coater and finally viewed under electron microscope (JEOL JSM-5200LV, Japan).

(B) Isolation of epiphytic bacteria

The isolation of epiphytic bacteria from seaweed samples was carried out by shake flask method (Lakshmanaperumalsamy and Purushothaman 1982). First, the seaweed samples were rinsed with sterile seawater to remove debris and unattached bacteria. Approximately 10 gm of seaweed sample was suspended in 100 ml of sterile seawater in a conical flask. This consequently kept for shaking at 120 rpm for 20 minutes on shaker incubator (MaxQ 4000, Thermo Scientific). Finally, the supernatant was serially diluted and plated in duplicates by spread plate method on ZMA and 50% SWNA. Incubation and further processing were carried in a similar fashion as mentioned in the earlier section.

2.2.3.3. Determination of viable counts

The total viable counts (TVC) in seawater, sediment and seaweed samples were determined by standard plate count method. Counts expressed in terms of CFU ml^{-1} for seawater and CFU $gram^{-1}$ for sediment samples. In the case of seaweed samples, viable counts were measured as CFU $gram^{-1}$ weight of wet samples.

2.2.4. Purification and maintenance of bacterial cultures

Distinctly formed pigmented bacterial colonies on primary isolation were marked and purified separately. Each pigmented colony was suspended in sterile saline water (0.85% NaCl) re-inoculated on separate ZMA plate to obtain pure cultures. Obtained isolates labeled with acronym ‘PIGB’ indicating ‘pigmented bacteria’ followed by strain number in sequential order from start to the end of defined samplings. Each pure isolate was

grown in Zobell Marine broth (ZMB; HiMedia Laboratories 2216, M385) separately till the density reaches 1 OD at 600 nm (approx. 10^6 cells ml^{-1}). Liquid cultures (0.5 ml each) transferred to sterile cryovials containing 25% glycerol. A total of 190 pure pigmented isolates obtained from the above collections were stored at –80 ºC for further use.

2.2.5. Characterization of pigmented bacteria

2.2.5.1. Cultural characteristics

Each pure colony of pigmented bacterial isolates (PIGB 1 - 190) was recorded for its cultural properties based on their growth on marine agar plates. Characteristics such as size, shape, margin, elevation and opacity was recorded by following respective attributions given Bergey's manual of determinative bacteriology while the colour property was noted based on the isolate specific pigmentation.

2.2.5.2. Morphological characteristics

The cellular morphology of individual isolate was noted with reference to standard parameters. The gram nature determined by Gram staining (Gram 1884) and potassium hydroxide (KOH) string test (Halebian et al. 1981). Bacterial shapes recorded as rod, cocci and coccobacilli forms. The presence/absence of spores in the individual bacterial cell was observed using spore stain assay kit (Schaeffer and Fulton's; HiMedia K006) while the motility was determined by air bubble/ hanging drop test.

2.2.5.3. Biochemical and physiological properties

All the pure isolates of pigmented bacteria were characterized biochemically by assessing their abilities towards the production of physiological enzymes. These enzymes were categorized into two subtypes which include oxidative (catalase and oxidase) and substrate specific hydrolytic enzymes (MOF: marine oxidation fermentation, DNase, amylase, casease, lipase and gelatinase). All biochemical activities were recorded qualitatively (+/-ve) as a part of taxonomic identification.

(A) Oxidative enzymes

Catalase test: An aliquot of the hydrogen peroxide (H_2O_2: 3% v/v) solution was placed on clean glass slide. Later, a well grown isolated colony of pure culture was picked up aseptically with a sterile loop and mixed with H_2O_2 solution. Observation of bubble formation (O2 + water = bubbles) considered as a positive test (Smibert and Krieg 1994).

Cytochrome oxidase: Oxidase test was performed with a smear of a loopful bacterial cell mass on commercial tetramethyl-p-phenylenediamine dihydrochloride (TPPD: Himedia, DD018) discs. The development of dark purple colour within 10 to 15 seconds was considered as a positive test while others recorded as negative.

(B) Hydrolytic enzymes

Marine oxidation fermentation (MOF) test: Oxidative or fermentative metabolic nature of each isolate was assessed by following Hugh and Leifson's fermentative test (1983). Broth suspension cultures were stab inoculated in MOF media (HiMedia, M379) prepared in test tubes and grown for 48-72 h. Formation of yellow coloration (top: oxidative, bottom: fermentative) in culture butt considered as positive test whereas blue coloration of the medium was recorded as an alkaline/negative/inert reaction.

Amylase test: An aliquot of 2 µl cell suspension of individual pigmented isolate was drop inoculated on SWNA plate containing 1 % starch and incubated at 28 ± 2 ºC for 48-72 h. Later, plates were flooded with Lugol's iodine solution (3 % potassium iodide, 0.3 % iodine) over the culture. The presence of clear zone surrounding the colony considered as a positive test for amylase production.

Casease test: Sterile casein agar plates (1% casein + SWNA) were drop inoculated with 2 µl of culture and incubated at 28 ± 2 ºC for 48-72 h. Later, plates were flooded with 15% trichloroacetic acid (TCA) solution. Observation of clear zone surrounding the colony was considered as a positive test (Gerhardt et al. 1981).

Lipase test: An aliquot of 2 µl cell suspension of each pigmented bacterial isolate was spot inoculated on agar plates (SWNA containing 1 % Tween-20). Plates were incubated at 28 ± 2 °C for up to 48-72 h and later the formations of calcium precipitated zone surrounding the spot of growth was considered as lipase positive test (Gerhardt et al. 1981).

Deoxyribonuclease (DNase) test: Bacterial cultures were spot inoculated on DNase test agar (M1041, Himedia) media and incubated at 28 ± 2 °C for up to 48-72 h. Later, plates were flooded with 1N hydrochloric acid (HCl) solution sparingly and kept as such for approximately 15-20 min. Formation of the non-opaque clear zone surrounding the colony was considered as a positive test.

Gelatin hydrolysis test: Gelatin liquefaction (Gelatinase) ability of each pigmented isolate was estimated by inoculating a drop (2 µl) of test organisms on gelatin agar plate (SWNA containing 1 % gelatin). Plates were incubated at 28 ± 2 °C for up to 48-72 h and later flooded with 15 % TCA. The formation of clear gelatin liquefaction zone around the grown colony was considered as a positive test (Smibert and Krieg 1994).

2.2.5.4. Identification and diversity studies

Based on cultural, morphological and biochemical parameters each pigmented bacterium was assessed for its identity using Bergey's manual of systematic bacteriology by Holt et al. (1994). Taxonomic methods and other detailed literature available on biochemical characterization of marine bacteria were also followed to cross-check the identity (Oliver 1982; Austin 1988; Jensen and Fenical, 1995; Das et al. 2007). Molecular characterization was carried out for potent antioxidant strains and discussed in consecutive chapter no. 3.

2.3. Results and Discussion

2.3.1. Primary observation by SEM

The first step in the isolation of epiphytic bacteria from any source is the keen observation of type and density of associated flora. It helps in better understanding and devising further steps of isolation. In the present study as an initial step of isolating epibiotic pigmented bacteria, we assessed some of the dominant seaweeds for their associated microflora under SEM (Fig. 2.4).

Electron microscopic observations on selected samples indicated that different seaweed species were characterized by variable density of associated bacteria. *Sargassum*, *Spatoglossum* and *Padina* sp. were seen to be covered by diverse biofilms with maximum populations of bacterial forms such as rods, cocci and coccobacilli. Conversely, the *Ulva* and *Gracilaria* sp. were observed to endure fewer numbers of associated bacteria. This indicated the bacterial frequencies living in the association were dependent on the type and morphology of host seaweed species.

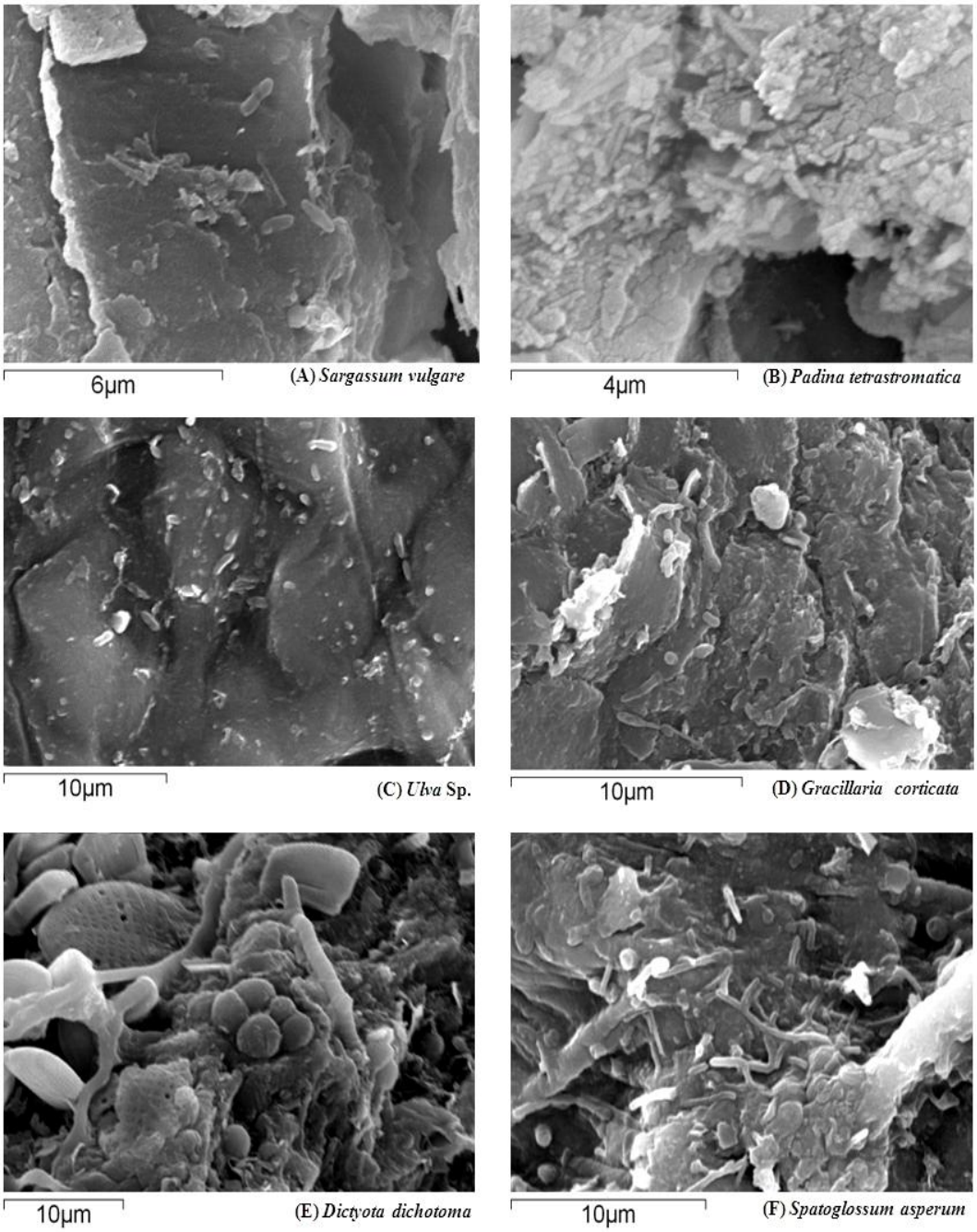


Fig. 2.4. Scanning electron microscopic observation of epiphytic bacteria in association with seaweed surfaces.

The seaweed-bacterial association is known to be affected by factors like type of algal species, depth of algal occurrence, surface area and morphological properties (Goecke et al. 2010). As discussed earlier seaweeds with more surface area and nutrient availability attracts more number of organisms where the rate of bioinvasion is directly proportional to surface area (Burke et al. 2011). *Sargassum*, *Spatoglossum* and *Padina* sp. are composed of broad and more number of holdfasts, stripes and fronds and provide a wider place for the attachment of bacteria. This might be the reason behind the high density of bacteria over the above seaweed species.

Similarly, the surface properties of seaweed also play an important role in attachment of invading bacteria. Singh et al. (2011) demonstrated that seaweed *Ulva* sp. as a part of morphological adaption produces oily substances that coat host body and provides self-protection against transpiration/ desiccation during tidal fluctuations. However, such smooth surfaces, on the other hand, render the attachment of other organisms including microbes. Less density of associated bacteria observed on *Ulva* and *Gracillaria* sp. surfaces might have been affected by similar factors where the slimy surfaces of seaweeds not allowed more number of bacteria to invade.

Morris et al. (1997) even demonstrated the tiny biofilms of 30 to 445 µm on leaf surfaces can hold up to 4,000 culturable CFUs. Plethora of studies on epiphytic seaweed bacteria has demonstrated the role of associated bacteria in the food web as well as in the production of bioactive metabolites (Kim 2015). Thus, overall primary microscopic observations in the present study made us realize that seaweed collected from selected locations along CWCI harbors diverse bacterial forms and consequently strengthened our next possible step i.e. isolation of epiphytic pigmented bacteria for antioxidant search.

2.3.2. Retrievability and enumeration of culturable bacteria

2.3.2.1. Viability from seawater and sediment samples

The total viable counts retrieved from seawater and sediment samples were in the range 10^3-10^5 ml^{-1} and 10^4-10^7 gm^{-1} respectively (Fig. 2.5). Water samples were observed with high counts during monsoon months (July – September = 5.3 ± 1.2 to 315 ± 77.8 × 10^3 ml^{-1}). Sediment samples were in the order of 10^5-10^7 gm^{-1} during this period. Low counts were observed in both water and sediment samples during January (Seawater = 1.55 ± 0.49 × 10^2 ml^{-1}; Sediment = 1.25 ± 0.25 × 10^3 gm^{-1}).

Water samples of Kunkeshwar showed two orders higher values during monsoon (July-2010) in comparison with other collection sites. Contrastingly, the Malvan waters had very high counts during November-2010. In the case of Goa waters, stations Vagator and Carbo De Rama expressed high numbers during May-2010. In case of sediment samples, the similar trend was observed towards retrievability of viable bacteria where the counts were exponentially increased from pre-monsoon to monsoon and decreased in post-monsoon season during both the years (Fig. 2.5). Counts obtained from some of the stations like Cabo De Rama (Goa) and Kunkeshwar (Maharashtra) exceptionally remained same during pre and post-monsoon seasons.

Determination of culturable bacteria by enumerating total viable count gives the overall idea about the abundance of heterotrophic bacteria in a particular ecosystem. Higher bacterial retrievability observed at Goa (Vagator) and Maharashtra (Kunkeshwar) coasts during monsoon season could be due to the mixing of rainwater and organic/domestic discharges. This adds an extra nutrient to the water body thus helps to increase the microbial growth and activity in coastal waters (Albright 1983). This could

be the possible reason behind higher CFU's experienced in the present study. Earlier investigations on bacterial diversity from the coastal locations have reported higher bacterial populations during heavy rainfall (Crowther et al. 2001; Ramaiah et al. 2001). We observed similar trend on bacterial retrievability from the selected sampling locations along the CWCI during monsoon season.

Contrastingly, few of the sampling locations as stated above endured near about similar population during pre-monsoon and post-monsoon seasons. Seasonal variation in CFU count from seawater and sediment along the same coast have documented such observations earlier (Nagvenkar and Ramaih 2010; Rodrigues et al. 2011). Overall observations on the seawater and sediment viable count supported maximum CFU in monsoon season.

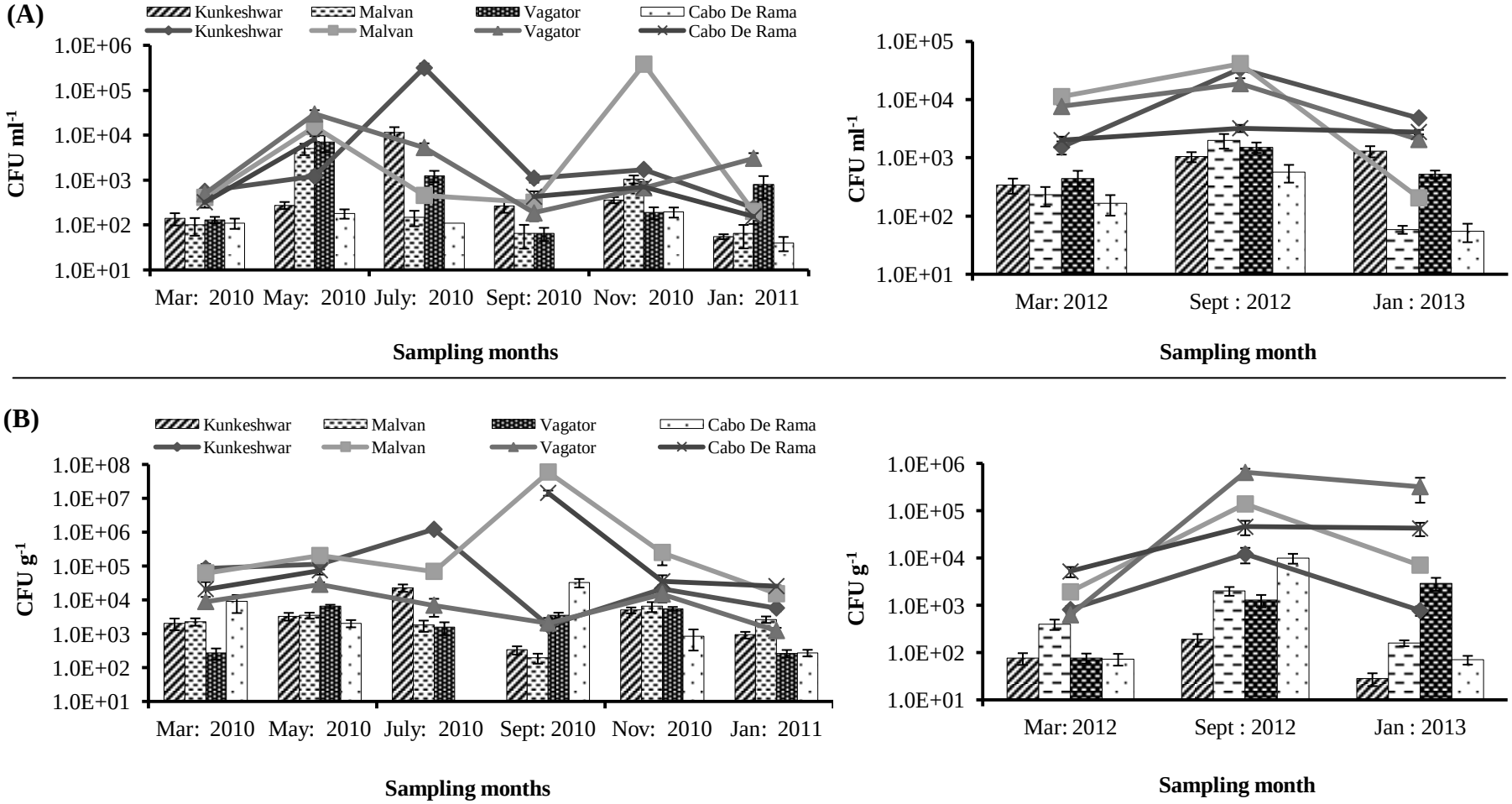


Fig. 2.5. Total viable bacteria in (A) seawater and (B) sediment samples. Line graph represents viable counts and bar graphs demonstrate pigmented counts. Sampling was not carried out at Cabo De Rama during July- 2010 due to heavy rain and inaccessibility of site.

2.3.2.2. Viability of seaweed-associated bacteria

Epiphytic viable counts retrieved from diverse seaweed species are presented as colony forming units (CFU) in Table 2.1. These counts noticed to vary at greater range irrespective of station or seasons of isolation. They ranged as low as in the order of 10^3 CFU and reached up to 10^6 with some seaweed species. *Sargassum*, *Caulerpa*, *Valoniopsis*, *Padina* and *Sphacelaria* were observed to hold the maximum numbers (8.3 – 363 × 10^4 g^{-1}) whereas *Stechospermum*, *Gelidium* and *Spatoglossum* retrieved with (1.8 – 65.5 × 10^4 g^{-1}) epibiotic bacteria. Notably, the *Chaetomorpha*, *Hypnea*, *Porphyra* and *Ceramium* sp. were observed to hold low numbers of viable bacteria as compared to all other seaweed species (Table 2.1).

Conversely, enumeration data when examined with reference to algal groups showed clear demarcation in terms of associated bacterial flora. Seaweeds belonging Phaeophyta found to hold maximum numbers of viable bacteria (*Padina* = 326 × 10^4 g^{-1}; *Spacillaria* = 363 × 10^4 g^{-1}) which was followed by Chlorophyta (*Valoniopsis* = 238 × 10^4 g^{-1}; *Caulerpa* = 128 × 10^4 g^{-1}) and Rhodophyta (*Gelidium* = 59 × 10^4 g^{-1}; *Acanthophora* = 17.6 × 10^4 g^{-1}) groups respectively. Moreover, comparing overall data on estimated total viable bacteria made us realize that the seaweeds are lodged with 2-3 fold higher counts than seawater and sediment samples.

Sea plants including macroalgae provide a distinct inhabiting place for diverse types of heterotrophic bacteria (Bengtsson et al. 2010). Isolation, determination of viability and diversity studies forms the first step in understanding seaweed–bacteria associations and their interactive roles in marine environments. Marine bacteria inhabiting living surfaces of higher organisms in a marine ecosystem are remarked to

form complex and highly dynamic communities (Holmstrom et al. 2002; Zheng et al. 2005).

In the present study, a total of 28 different species of seaweeds (9: Chlorophyta, 12: Phaeophyta and 7: Rhodophyta) assessed for epiphytic bacteria resulted in retrieval of viable densities in the range of 10^3 to 10^6 g^{-1}. These results are concurrent with earlier studies on surface associated heterotrophic bacteria which demonstrated the seaweeds *Enteromorpha*, *Hypnea*, *Chaetomorpha*, *Ulva*, *Gracilaria* and *Porphyra* sp. to hold 10^2 to 10^8 CFU g^{-1} (Lakshmanaperumalsamy and Purushothaman 1982; Armstrong et al. 2000; Karacalar and Turan 2008; Bengtsson et al. 2010; Singh et al. 2015).

Exceptionally, few of the seaweeds were witnessed to lodge either too low or high number of viable bacteria (Table 2.1). This indicates that associated bacterial populations among the seaweed samples even from same locations vary in their abundance and are influenced by the vicinity parameters and geographical locations as noticed by earlier researchers (Lachnit et al. 2011). Moreover, the associated flora is reported to vary based on type or morphology of host organisms. For example, thallus tips of *Ulva* sp. are noticed with 10^6 cells while the base held 10^7 cells of epibiotic bacteria per cm^2 (Tujula 2006).

Conversely, we observed the greater strength of viable counts on primary isolation from seaweed samples in comparison with seawater and sediment samples. As discussed before the macro-algae with more surface area exudates variety of nutrients that are rapidly metabolized by omnipotent bacteria from surrounding water body (Goecke et al. 2010). This results in colonization and formation of biofilms with diverse bacteria on surfaces of seaweeds. Similar effects might have contributed the retrieval of more number of bacteria from seaweed samples in the present study.

This investigation not only adds the information on the abundance of epibacterial flora on various unreported seaweeds from CWCI but also marks up the *Sargassum*, *Padina* and *Spacillaria* sp. (Phaeophyta) as prospective sources for maximum recovery of diverse organisms (Table 2.1). In addition, we observed that most of the seaweed species are season specific and not occurred throughout the year. Hence to stick with the purpose of present investigation i.e. to isolate a maximum number of pigmented bacteria for antioxidant search, the viable count data not further stressed based on the associated population of individual algae vs seasonal change. Instead, much effort was taken to look the effects of source samples and seasons on retrievability, distribution and diversity of pigmented bacteria and discussed elaborately in following sections.

Table 2.1. Seaweed samples collected for the isolation of epiphytic pigmented bacteria from selected sampling locations.

Sr. No.	Seaweed species	Month, year and site of sample collection*	Viable counts (CFU × 10^4 g^{-1})	Pigmented bacterial counts (CFU × 10^2 g^{-1})
Chlorophyta (Green algae)				
1.	*Caulerpa racemosa*	Sep-10-Cabo De Rama; Nov-10-Cabo De Rama; Nov-10-Kunkeshwar; Jan-11-Malvan	5.1 – 7.5	11.3 – 16.3
2.	*Caulerpa peltata*	Jan-11-Cabo De Rama; Feb-12-Malvan; Jan-13-Kunkeshwar; Jan-13-Malvan	1 – 9.5	8 – 17
3.	*Caulerpa scalpelliformis*	Jan-11-Kunkeshwar; Sep-12-Kunkeshwar; Jan-13-Kunkeshwar	50.3 – 128	19 – 63.3
4.	*Chaetomorpha media*	Mar-10-Malvan; Mar-10-Vagator; Mar-10-Kunkeshwar; Mar-10-Cabo De Rama; May-10-Cabo De Rama; July-10-Malvan	0.61 – 0.78	0 – 3.67
5.	*Chaetomorpha antennina*	July-10-Kunkeshwar; Sep-10-Malvan; Sep-10-Vagator; Nov-10-Malvan; Sep-12-Cabo De Rama; Sep-12-Malvan; Sep-12-Vagator	0.2 – 1	0 – 4.67
6.	*Enteromorpha clathrata*	Mar-10-Kunkeshwar; July-10-Kunkeshwar	5.4 – 7.7	ND
7.	*Ulva* sp./*Ulva lactuca*	Mar-10-Malvan; Sep-10-Vagator; Sep-10-Kunkeshwar; Nov-10-Kunkeshwar; Jan-11-Kunkeshwar; Feb-12-Vagator; Sep-12-Kunkeshwar; Sep-12-Vagator; Jan-13-Cabo De Rama; Jan-13-Vagator; Jan-13-Kunkeshwar; Jan-13-Malvan	1.2 – 4.8	113.3 – 156.7
8.	*Ulva fasciata*	July-10-Malvan; July-10-Kunkeshwar; Sep-10-Malvan	0.3 – 7.3	0 – 113.3
9.	*Valoniopsis pachynema*	Nov-10-Cabo De Rama; Feb -12-Kunkeshwar	38 – 238	0 – 14.6

Phaeophyta (Brown algae)				
10.	*Dictyota sp./ D. dichotoma*	Nov-10-Malvan; Jan-11-Vagator; Jan-11- Cabo De Rama; Feb -12-Kunkeshwar	3 – 7.6	11.3 – 25.0
11.	*Padina boergesenii*	Sep-10-Kunkeshwar; Sep-10-Cabo De Rama	0.5 – 1	10.3 – 16.3
12.	*Padina tetrastromatica*	Mar-10-Cabo De Rama; Nov-10-Vagator; Jan-11-Malvan; Jan-11-Kunkeshwar; Jan-11- Cabo De Rama; Feb-12-Vagator; Jan-13-Vagator; Jan-13-Kunkeshwar; Jan-13-Malvan	14.7 – 326	16.6 – 22.3
13.	*Padina gymnoriza*	Mar-10-Malvan; Feb-12-Malvan	5 – 5.3	12.7 – 19.3
14.	*Padina boryana*	Sep-10-Malvan; Nov-10-Malvan; Jan-11-Vagator	8.3 – 47	9.33 – 16.0
15.	*Sargassum* sp.	Mar-10-Malvan; Mar-10-Vagator; May-10-Cabo De Rama; July-10-Malvan; Sep-10-Cabo De Rama; Feb-12-Vagator; Sep-12-Cabo De Rama; Jan-13-Malvan	15 – 25.3	10.9 – 86.7
16.	*Sargassum vulgare*	Sep-10-Malvan; Sep-10-Kunkeshwar; Nov-10-Kunkeshwar; Nov-10-Vagator; Nov-10-Cabo De Rama; Jan-11-Vagator	9.3 – 45	19.0 – 22.67
17.	*Sargassum ilicifolium*	May-10-Vagator; Jan-13-Cabo De Rama; Jan-13-Vagator	22 – 94.3	11.6 – 17.7
18.	*Sargassum cinnaurm*	Mar-10-Cabo De Rama; Jan-11-Cabo De Rama; Feb-12-Malvan; Sep-12-Kunkeshwar	12.6 – 38	5.34 – 13.6
19.	*Spatoglossum asperum*	Mar-10-Kunkeshwar; May-10-Vagator; Jan-11-Malvan; Feb -12-Kunkeshwar	10.3 – 65.5	4 – 26.3
20.	*Sphacelaria* sp.	Mar-10-Cabo De Rama; July-10-Vagator; Sep-10-Kunkeshwar; Nov-10-Vagator; Sep-12-Malvan; Jan-13-Cabo De Rama	273 – 363	12.3 – 13
21.	*Stechospermum marginatum*	Nov-10-Vagator; Jan-11-Kunkeshwar	1.8 – 31.6	7.30 – 11

Rhodophyta (Red algae)				
22.	*Acanthophora spicifera*	Sep-12-Kunkeshwar	17.6	83.0 – 25.1
23.	*Ceramium* sp.	Mar-10-Kunkeshwar	4.7	15.6 – 4.50
24.	*Gelidium micropterum*	Mar-10-Malvan; May-10-Cabo De Rama; May-10-Vagator; July-10-Malvan; Sep-10-Vagator; Nov-10-Malvan; Nov-10-Kunkeshwar; Jan-11-Malvan; Jan-11-Vagator; Feb-12-Vagator; Sep-12-Cabo De Rama; Sep-12-Vagator; Jan-13-Malvan	3.2 - 59	8 – 19.5
25.	*Gracilaria corticata/* *Gracilaria* sp.	Mar-10-Malvan; July-10-Kunkeshwar; July-10-Vagator; Sep-10-Malvan; Sep-10-Kunkeshwar; Sep-10-Cabo De Rama; Jan-11-Malvan; Jan-11-Cabo De Rama; Jan-11-Vagator; Feb-12-Vagator; Jan-13-Cabo De Rama	1 – 1.6	4 – 6.5
26.	*Hypnea musciformis*	Mar-10-Kunkeshwar; Mar-10-Vagator; Sep-12-Malvan	2 – 3.6	0 – 1
27.	*Polysiphonia* sp.	Sep-12-Kunkeshwar; Sep-12-Malvan	7 – 10.4	ND
28.	*Porphyra vietanamensis*	Sep-12-Cabo De Rama; Sep-12-Vagator	1.5 – 4.1	0 – 1.5

Example: 'Feb-12-Malvan' indicates sample was collected from station Malvan during February 2012; the other short forms can be read in a similar fashion.

2.3.2.3. Pigmented bacterial counts and strain collection

The viable pigmented counts were enumerated separately from seawater, sediment and seaweed samples (Table 2.1; Fig. 2.5). Chromogenic bacteria were retrieved 2-3 orders lower (i.e. 10^2-10^3) than the non-pigmented ones (10^4-10^6) (Fig. 2.6). These results were in match with the findings of Jayanth et al. (2002), who has reported the pigmented bacteria 2-3 order lower as compared to the total population along the east coast of India. As discussed in introduction section few of the studies on pigmented bacteria and their distribution in the marine environment have documented up to 15-50 % contribution of the chromogenic population (ZoBell and Feltham 1934; ZoBell and Upham 1944; Du et al. 2006).

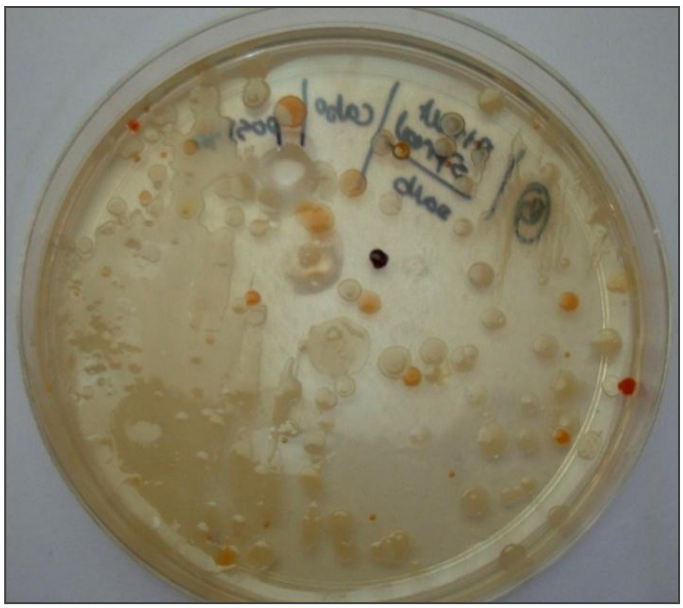


Fig. 2.6. Plate showing retrievability of pigmented and non-pigmented bacteria from seaweed sample plated in ZMA.

Seawater and sediments samples evidenced to hold maximum pigmented population of 10^4 CFU during monsoon season, which were consequently reduced to 10^1 during late post-monsoon (January) and early pre-monsoon (March). Interestingly, the

sediments though had higher viable count (10^7 gm^{-1}), it supported low numbers of pigment producers (10^2 - 10^4 gm^{-1}). On the other hand the overall population of TVC is lower in seawater when compared to sediment samples but the fractions of pigmented ones were found to be more. Fig. 2.5 demonstrates the station wise variability among PIGB. Kunkeshwar, Malvan, Cabo De Rama represented high PIGB counts during monsoon season. The other location Vagator experienced near about similar counts during pre-monsoon and post-monsoon seasons.

In the case of macro-algae uneven viable pigmented counts were retrieved from different seaweed species (Table 2.1). The overall associated pigmented flora was in the order of 4 to 156 × 10^2 g^{-1}. Seaweeds species like *Ulva*, *Caulerpa* and *Sargassum* were observed to hold more density (10^4 cells g^{-1}) whereas *Hypnea, Porphyra* and *Valoniopsis* harboured low population (10^2 cells g^{-1}) of chromogenic bacteria. Seaweeds collected during post-monsoon months (Sept.-Jan.) were lodged with greater numbers as compared to monsoon and pre-monsoon seasons. Interestingly, we also observed that same species of seaweed collected from different sampling sites possess different CUF of pigmented bacteria. These findings emphasized the quantum or endurance of pigmented forms are not only affected by the season and sample source but also influenced by the locality and vicinity parameters.

Conversely, the isolation and pure culturing of pigmented bacteria from CWCI resulted in the consolidated collection of total 190 isolates (Table 2.2). Among these 87 isolates were obtained from seaweed surfaces alone while the seawater could give 64 strains. The seawater and seaweed resources together contributed 79.47 % in overall PIGB population retrieved during this study.

Table 2.2. Number of pigmented bacteria isolated from seawater, sediment and seaweed samples.

Source	Number of pigmented bacteria isolated	Percentage (%) contribution
Seawater	64	33.68
Sediment	39	20.52
Seaweed	87	45.79
Total	190	100.00

Values are based on consolidated data of two-year sampling (2010-2011 and 2012-2013).

The abundance and distribution of chromogenic bacteria in marine ecosystem is accounted to be highly influenced by associated environmental parameters. For example, an amount of light and nutrient in a particular ecosystem are the responsible factors that determine the overall existence of pigmented organisms to that locality. Du et al. (2006) studied pigmented heterotrophic bacteria from oceanic environments and showed that such bacteria endure more in surface waters than sub-surface levels. This group of researchers observed decreased frequencies of pigmented bacteria with increasing depth.

The distinct population of PIGB experienced from algal species during this study reveals that the occurrence of PIGB depends on the morphology of the seaweeds as well the seasons. These factors were discussed earlier in determining the population of seaweed associated bacteria (Burke et al. 2011). For example, we observed *Chaetomorpha*, *Sphacelaria* and *Gelidium* with less surface area supported low number of PIGB than *Padina* and *Sargassum* (Table 2.1). Moreover, high recovery of pigmented

organisms from seaweed resource collected from Malvan, Kunkeshwar and Vagator indicates that these coasts must provide optimum environments for the growth of algae as well as the associated chromogens. Hence, these sites can be spotlighted as suitable places for isolation of pigmented bacteria at greater scale.

2.3.4. Pigmented bacteria: Identification, diversity and distribution

2.3.4.1. Diversity with reference to colour property

The overall chromogenic population at first step categorized based on colour property. These colors are mainly yellow, orange, brown, creamy, red, pink and violet (Fig. 2.7). Distinctly coloured bacteria (PIGB 1-190) when assessed based on their contribution to overall density, the yellow coloured bacteria with 40% occurrence dominated the overall population. This was followed by orange (26.32%), creamy (21.05%), pink (5.26%), brown (4.74%), red (1.58%), and violet (1%) (Fig. 2.8).

Our results matched with an early investigation of Zobell and Feltham (1934) who showed the contribution of yellow, orange, brown and red coloured bacteria in the order of 31.3 > 15 > 9.9 > 5.5 % respectively. Likewise, more recent studies had noted the similar trend in occurrence coloured bacteria in marine ecosystems (Jafarzade et al. 2013; Balraj et al. 2014). They isolated pigmented strains from seawater, sediment, sponge, mangrove associates and demonstrated that the yellow and orange bacteria cumulatively dominate up to 72-90% of the coloured population. Moreover, except these few reports, there is no much information available on diversity and distribution of pigmented bacteria in marine environments.

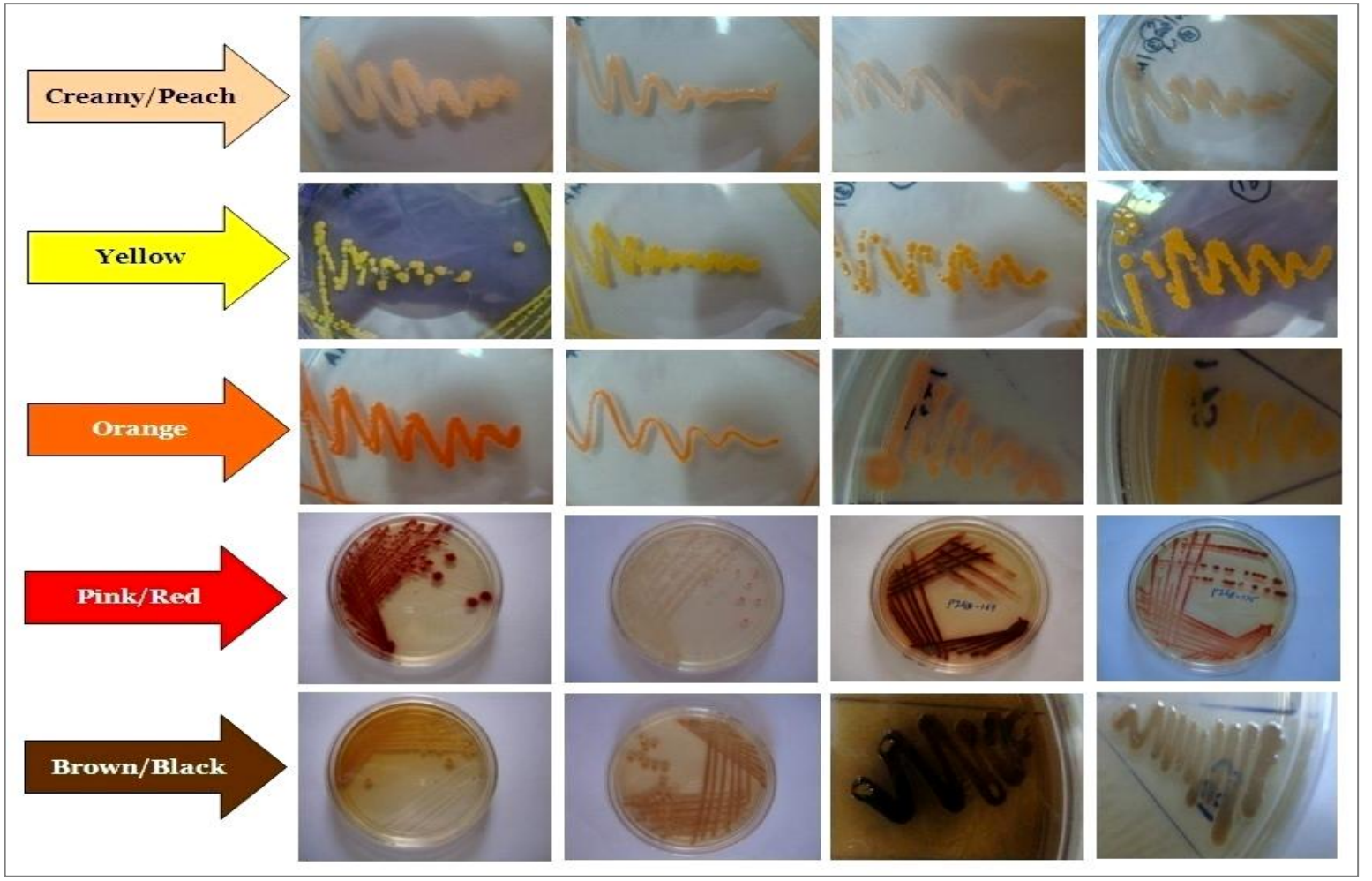


Fig. 2.7. Pictorial representation of various types of pigmented isolates obtained from CWCI.

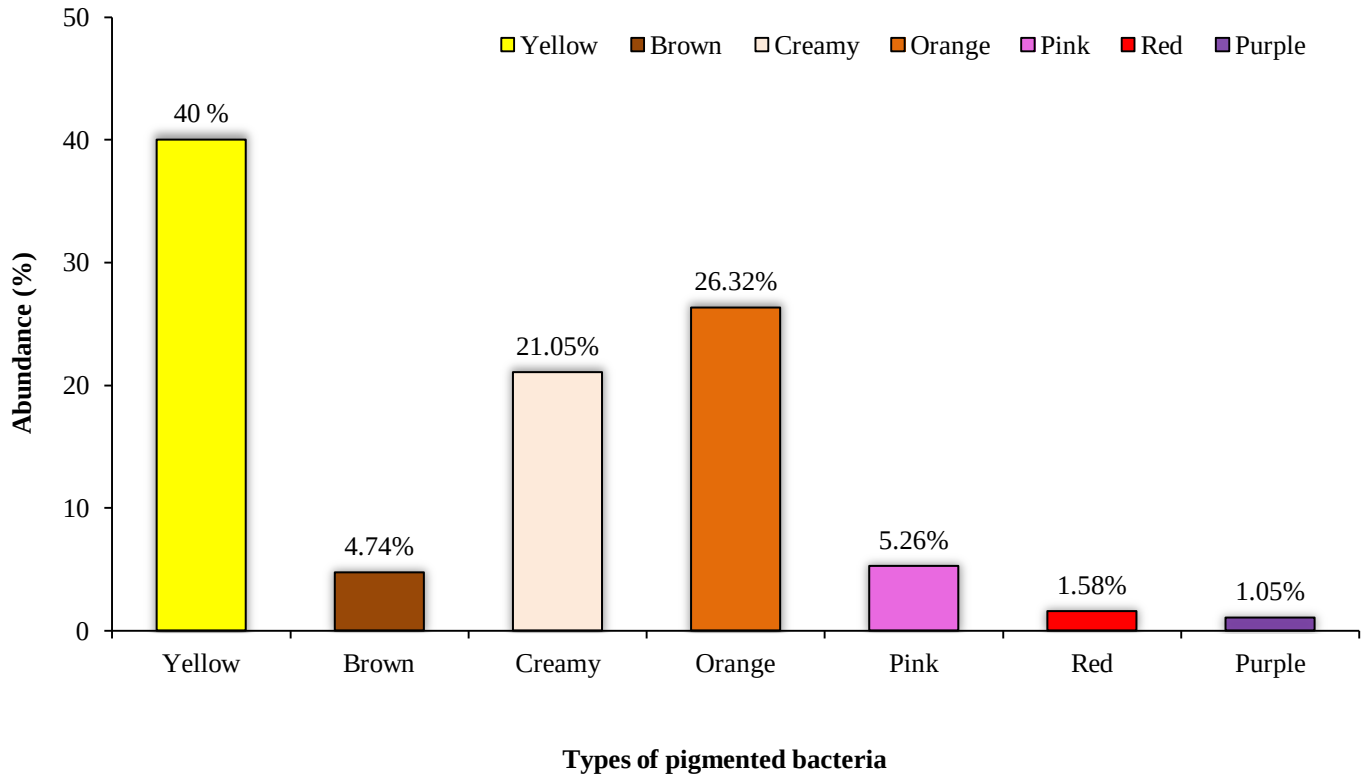


Fig. 2.8. Percentage distributions of pigmented bacteria along the CWCI.

Notably, in addition to common yellow, brown, creamy, red and pink isolates we also come across isolation of some of the rare green, violet and dark red coloured bacteria. However, they failed to either subculture or produce pigments after brief storage at –80 ºC and hence not included in the present report. Stafsnes et al. (2010) experienced similar results where they isolated about 3500 pigmented isolates at first step out of which 2000 could not grow in subsequent step. This indicates the bacteria loose pigment property and cell viability after few cell cycles or revivals.

Isolation studies witnessed with considerable differences in retrievability of pigmented bacteria and their chromogenic appearance was observed to be affected by source sample and seasons as well (Fig. 2.9). Isolates bearing yellow tints were retrieved in a high number (45%) from water samples than sediment and seaweeds. Similarly, the season wise grouping of these yellow bacteria showed greater abundance during pre-monsoon (59%) which was consequently decreased in monsoon (46%) and post-monsoon (24%) seasons respectively. High recovery of these organisms in seawater samples and during pre-monsoon months indicates that such pigments must help the host bacteria to overcome the high temperature and salinities as more common during this season.

Interestingly, the orange isolates though recovered with near about equal numbers irrespective of source samples; their occurrence was observed to be influenced by seasonal shifts. They appeared more or less equivalent during pre-monsoon and monsoon months (21%), while the post-monsoon season it got elevated the numbers by 13% (i.e. 21 to 34%). In contrast, the creamy isolates showed exact opposite trend where monsoon and post-monsoon seasons supported equal densities while the pre-monsoon was observed to suppress it by 12%. It indicates the pre and post-monsoon shifts in the

population of orange and creamy coloured bacteria controlled by respective environmental conditions where the occurrence of one hampers the other. Moreover, these results clearly attest the months July-September and September-January to be more suitable for endurance as well as retrieval of isolates bearing creamy and orange colour properties respectively.

The red and pink coloured bacteria were retrieved from the sample sources of seawater and sediment in 5% and it got elevated to 9 % in seaweeds. Seasonal changes on the red pigmented organisms indicate its occurrence by 2% in pre-monsoon, 5% in monsoon and 10% during post-monsoon. Notably, we also come across the rare chromogens like violet in late monsoon from the seaweeds. Few of the reports on diversity of bacteria with reference to pigment property though have documented related findings (Jayanth et al. 2002; Du et al. 2006) there are no much investigations have been carried out with intention studying seasonal or source wise distribution of PIGB in marine environments especially from the Arabian Sea/ Indian coasts. Our detailed report forms a base study and contributed significant data on available density and endurance of diverse type coloured bacteria along CWCI.

(A)

(B)

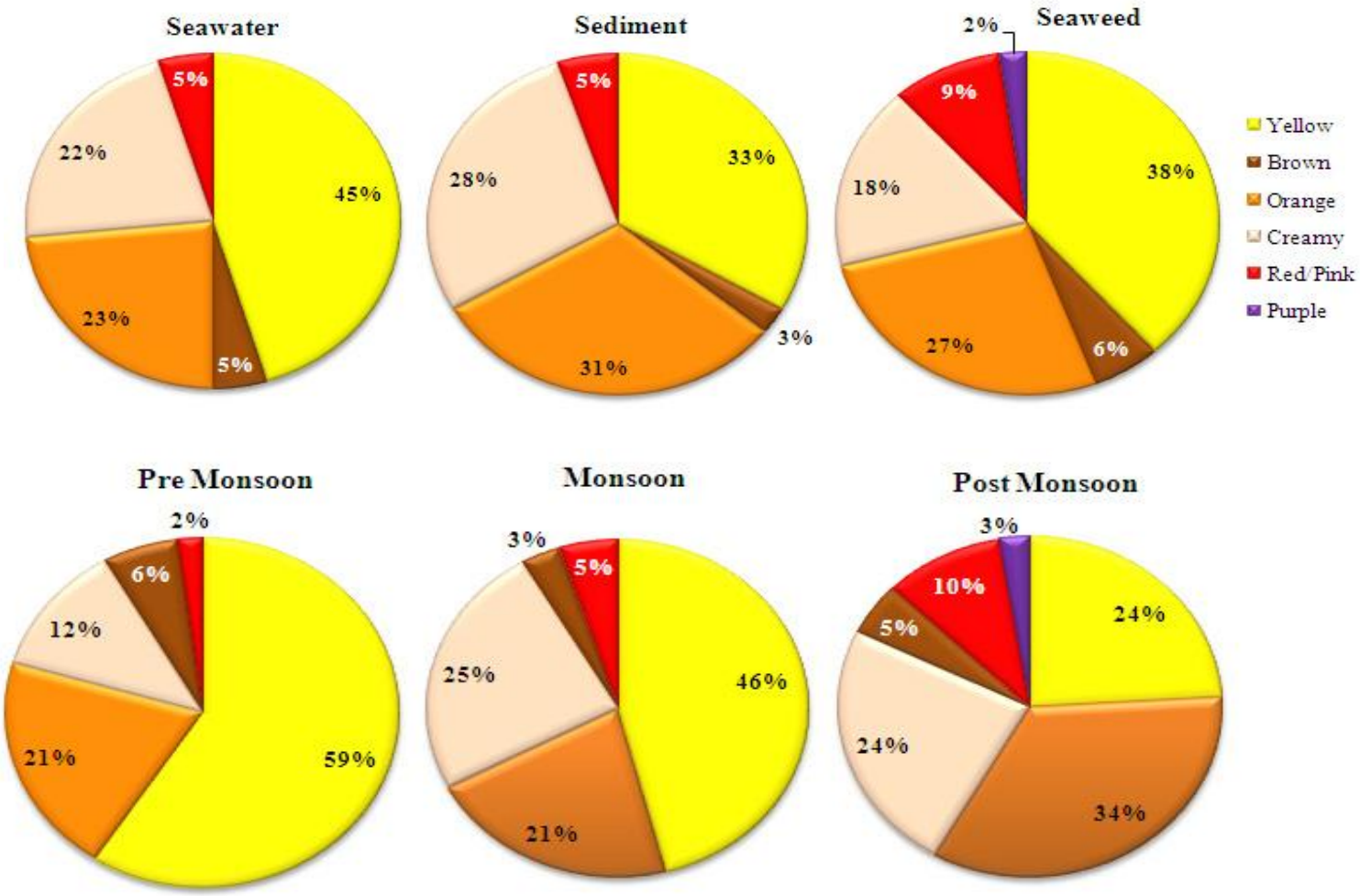


Fig. 2.9. Sample source (A) and the seasonal shifts (B) on the distribution of pigmented bacteria.

Individually pigmented strains (PIGB 1 – 190) were characterized by morphological, biochemical and physiological properties and results are presented in Table 2.3.

2.3.4.2. Morphological grouping of PIGB

Microscopic phenotype observations on pigmented bacteria grouped overall population into two morphotypes viz. Rods and Cocci forms. Results showed more contribution of rod shaped bacteria (75.8%) in comparison with coccoid forms. Observations on total pigmented flora attributed Gram-negative property to 132 (69.5%) isolates whereas the remaining 58 (30.5%) belonged Gram-positive family. Similarly, looking at motility nature 62 (32.6%) isolates found motile while 128 (67.4%) were non-motile (Table 2.3).

Marine environments are well known to endure large population of Gram-negative bacteria than Gram-positive ones (Lakshmanaperumalsamy and Purushothaman 1982; Ramaiah, 2004). We observed similar results where the Gram-negative pigment producers dominated the selected coastal locations along the Indian shoreline. Studies by Jayanth et al. (2002), Stafsnes et al. (2010) and Cardona-Cardona (2010) had independently explored the pigmented bacteria from different coastal locations and showed the analogous contribution of 50-75% Gram-negative organisms.

Concerning morphotypic distribution results of the current study are in accord with earlier findings of Weinbauer and Peduzzi (1994) who documented >80% abundance of rods in the marine ecosystem. The possible reason attributed here is cylindrical bacteria may acquire nutrients more actively as compared to cocci that result in increased abundance of rods (Herndl 1988). These evidences clearly indicate morphology and cell wall composition plays a crucial role in stability and survival of

Gram-negative bacteria against constantly changing marine environments and hence determines their dominant existence.

2.3.4.3. Physiological grouping of PIGB

Pigmented flora of water, sediment and seaweeds collected from CWCI assigned to different physiological groups based on their biochemical properties (Table 2.3). It emphasized high numbers of PIGB (84.7%) to secrete enzyme catalase while only half population exhibited the oxidase property. Utilization of energy source (glucose) with MOF test indicated the oxidative (34.2%) organisms exist double to that of fermentative ones (15.3%). Interestingly, 36.8% of bacteria expressed proteolytic and amylolytic activities in common. Among overall pigmented bacteria assessed only 59, 53 and 43 isolates showed positive responses towards lipolytic, DNase and gelatinase productions. These results on PIGB though were utilized for identification purpose in the present study it additionally highlighted the biological role of such flora in the marine ecosystem.

Bacteria overcome the environmental stress conditions by regularized synthesis of diverse physiological enzymes mainly through catalase and oxidase (Thompson et al. 2003; Yumoto et al. 2000). This enzyme helps the organisms to convert the hydrogen peroxide (a harmful by-product of normal metabolic process) to less dangerous form. Similarly, enzyme oxidase catalyzes the reduction of molecular oxygen to water and use the free energy to pump protons across the membrane for their growth and survival (Iwata 1998). Significant production of catalase and oxidase by pigmented bacteria in the

current study could have helped them to conquer the stress in natural environments as well.

Determination of oxidative or fermentative nature of pigmented bacteria in the present investigation witnessed more percentage of organisms prefer aerobic (oxidative) utilization of energy source glucose by MOF test. It has been documented that most of the aerobic bacteria are tend to produce pigments and fall in the category of aerobic phototrophic bacteria (Perez-Jimenez et al. 1996; Rathgeber et al. 2004). This not only supports our results on more occurrences of oxidative organisms but also strengthens the earlier views on oxidative nature of pigmented bacteria.

Conversely, the heterotrophic bacteria are proficient in generating certain hydrolytic end products as a part of their metabolism. These products (enzymes) are helpful in initiating degradation of structurally complex organic matter (i.e. carbohydrates, proteins, lipids etc.) and hence taking an important role in nutrient cycling (Valdemarsen et al. 2010). We did not intend to look such bio-environment studies on pigmented bacteria and therefore, the qualitative data utilized for identification purpose only. However, an interesting observation to be noted here is that the seawater and seaweed-associated PIGB exhibits hydrolytic activities at greater strength as compared to those which nurtures in sediment samples. These results additionally signify considerable contribution of pigmented bacteria in biological processes as well.

Table 2.3. Morphological, biochemical and physiological attributions on the overall population of pigmented bacteria.

Sample source	No. of PIGB	Morphological properties						Catalase	Oxidase	Biochemical properties						
		Shape		Motility		Gram nature				MOF		Proteolytic	Amylolytic	Lipolytic	DNase	Gelatinolytic
		Rods	Cocci	M	NM	+	–			Oxid.	Ferm.					
Seawater	64	45 (70.3%)	19 (29.7%)	17 (26.6%)	47 (73.4%)	24 (37.5%)	40 (62.5%)	56 (84.8%)	39 (59.1%)	27 (40.9%)	7 (10.6%)	27 (40.9%)	25 (37.8%)	19 (28.7%)	15 (22.7%)	14 (21.1%)
Sediment	39	30 (76.9%)	9 (23.1%)	11 (28.2%)	28 (71.8%)	11 (28.2%)	28 (71.8%)	36 (97.3%)	20 (54.1%)	16 (43.2%)	6 (16.2%)	10 (27.02%)	17 (45.9%)	14 (37.8%)	9 (24.3%)	6 (16.2%)
Seaweeds	87	62 (71.3%)	25 (28.7%)	34 (39.1%)	53 (60.9%)	23 (26.4%)	64 (73.6%)	69 (79.3%)	49 (56.3%)	22 (25.3%)	16 (18.4%)	33 (37.9%)	28 (32.2%)	26 (29.9%)	29 (33.4%)	23 (26.4%)
Total	190	137 (72.1%)	53 (27.9%)	62 (32.6%)	128 (67.4%)	58 (30.5%)	132 (69.5%)	161 (84.7%)	108 (56.8%)	65 (34.2%)	29 (15.3%)	70 (36.8%)	70 (36.8%)	59 (31.1%)	53 (27.9%)	43 (22.6%)

MOF: Marine oxidation fermentation test. M: Motile, NM: Non-motile, +: Positive test, -: Negative test, Oxid.: Oxidative, Ferm.: Fermentative.

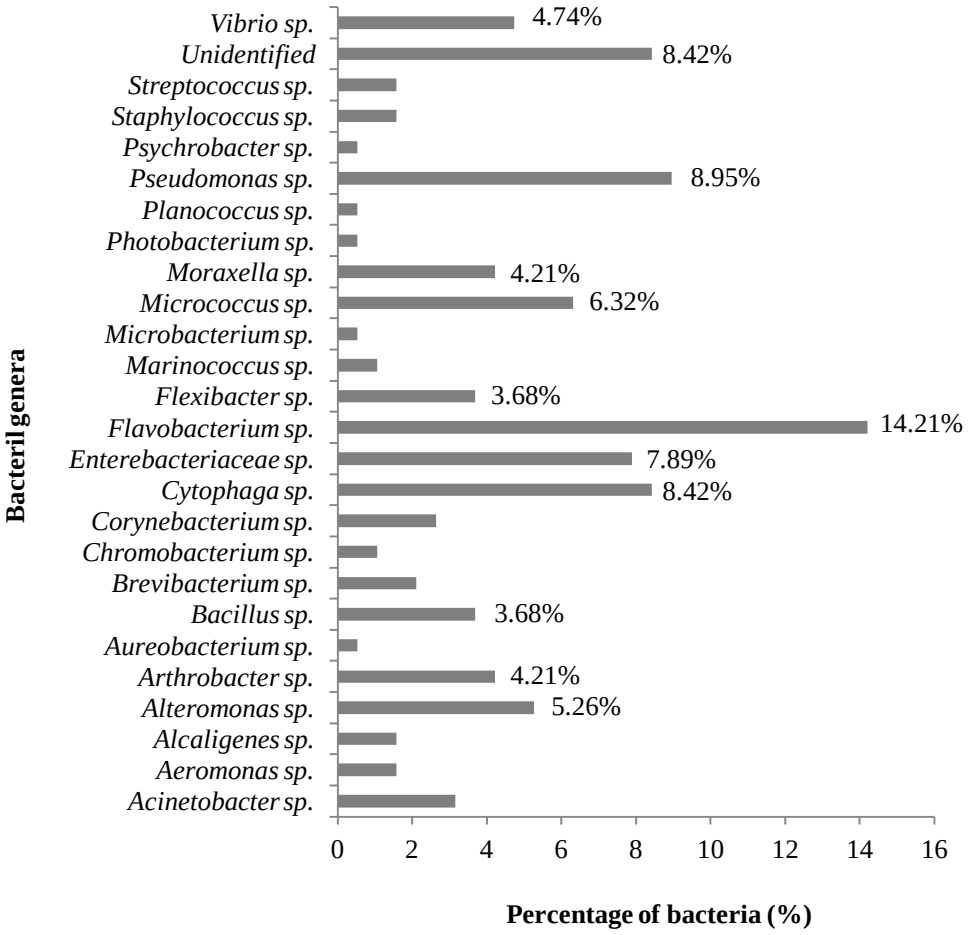
2.3.4.4. Diversity and distribution with reference to identity

Each strain of pigmented bacteria was characterized for its identity and results are presented in Table 2.4. Total community composition structure analysis witnessed the endurance of 25 chromogenic genera along the CWCI coast (Fig. 2.10A). It mainly includes *Flavobacterium* (14.21%), *Pseudomonas* (8.95%), *Cytophaga* (8.42%) and *Enterobacteriaceae* sp. (7.89%). These four genera dominated ~40% of the chromogenic population.

Pigmented bacteria affiliating *Acinetobacter*, *Alteromonas*, *Arthrobacter*, *Bacillus*, *Flexibacter*, *Micrococcus*, *Moraxella* and *Vibrio* genera formed a second most abundant group. The *Aeromonas*, *Alcaligenes*, *Aureobacterium*, *Brevibacterium*, *Chromobacterium*, *Corynebacterium*, *Marinococcus*, *Microbacterium*, *Photobacterium*, *Planococcus*, *Psychrobacter*, *Staphylococcus* and *Streptococcus* sp. obtained in very low numbers. It indicated the sparse occurrence of these genera in coastal waters of India.

Looking at higher order classification, the overall chromogenic bacterial community was affiliated to 5 major phyla i.e. β-Proteobacteria, γ-Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Fig. 2.10B). The Proteobacteria (γ: 37.9% + β: 3.2%) alone contributed 41.1 % of pigmented isolates. Within Proteobacteria group the γ-Proteobacteria dominated with genera *Pseudomonas*, *Enterobacter*, *Vibrio*, *Acinetobacter*, *Aeromonas* sp. whereas β-Proteobacteria was obtained with 1-3 isolates of *Alcaligenes, Chromobacterium and Planococcus* sp. Bacteroidetes (26.3%), the second largest group obtained during this investigation grouped three genera *Flavobacterium*, *Cytophaga* and *Flexibacter*. The *Flavobacterium-Cytophaga* together obtained 43 pigmented isolates whereas *Flexibacter* represented by seven isolates.

(A)



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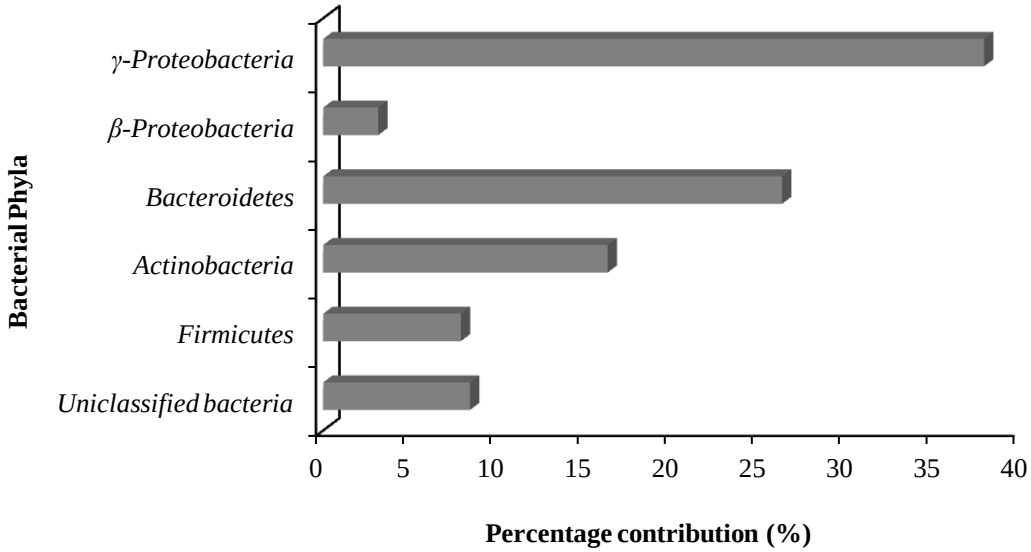


Fig. 2.10 (A) Taxonomic affiliations on the pigmented bacterial genera.

(B) Percentage contribution of major phyla on the pigmented bacteria.

Among the other bacterial groups obtained in the present study, Actinobacteria (*Brevibacterium*, *Corynebacterium* and *Micrococcus* sp.) and Firmicutes (*Bacillus*, *Marinococcus*, *Staphylococcus*, *Streptococcus* sp.) contributed 16.3 and 7.9% of organisms respectively. Interestingly, one could note here that pigmented bacteria belonging γ-proteobacteria and Bacteroidetes are the predominant inhabitants at studied locations along the west coast of India.

The interest in oceanic heterotrophic pigmented organisms is increasing the attention of researchers due to their potential roles in host physiology, environmental and biological processes (Cardona-Cardona et al. 2010; Stafsnes et al. 2010; Pawar et al. 2015). However, the essential prerequisite towards development and utilization of such microbes is to culture and depict their available diversity/ distribution at a particular location. To our knowledge, studies on diversity of bacteria in the Arabian Sea though have reported presence of above mentioned groups of bacteria (Divya et al. 2010; Singh and Ramaiah 2011; Jain et al. 2014) there are very few reports on *in situ* distribution of pigmented bacteria specifically along the Indian coasts.

The dominance of *Flavobacterium*, *Pseudomonas* and *Cytophaga* as seen in the present study were in alignment with an earlier (Lakshmanaperumalsamy and Purushothaman 1982) and recent studies (Nithya and Pandian 2010). They documented coincident findings on prevailing endurance of *Pseudomonas* (16.67%) and *Flavobacterium-Cytophaga* (10%) in marine environments. Similarly, other studies on oceanic bacteria have demonstrated the linear abundances of *Flavobacterium*, *Cytophaga*, *Vibrio*, *Enterobacter* and other Gram-positive organisms (Nakanishi et al. 1996). Thus, it highlights above bacterial genera as the common inhabitants in marine ecosystems. We observed analogous trend on dominant bacterial genera

(*Flavobacterium*, *Pseudomonas* and *Cytophaga*) which together contributed 31.58% of organisms. However, in the case of *Vibrio* and *Enterobacteriaceae* sp. results showed little deviations from CWCI (Fig. 2.10).

Contrastingly, there is limited information on the diversity of seaweed epibiotic pigmented bacteria both at local (Arabian Sea/ Indian coasts) and global level. This investigation in addition to seawater and sediment flora explored and strengthened data on the available diversity of pigmented bacteria specifically to algae associates along the Indian coast. Also, the distribution studies on phylogenetic groups of pigmented bacteria demonstrated wide variation and heterogeneity towards the isolation sources, seasons and sampling sites (Fig. 2.11). Influence of these factors on the individual bacterial group was assessed and discussed to understand which source/season/location is suitable for retrieval particular type of pigmented bacteria.

γ-Proteobacteria

The predominant group γ-Proteobacteria obsessed seawater and seaweed samples with near about similar occurrence (43-45%) whereas in the case of sediments the abundance was reduced by 11-13% (Fig. 2.11A). Looking at seasonal change, this group showed exponential trend in its seasonal endurance with increasing values from 35 % in pre-monsoon to 40 % in monsoon and 43 % during post-monsoon (Fig. 2.11B). Noticeably, γ-Proteobacteria did not show much variation at different sampling locations along the CWCI except Cabo De Rama with the lowest occurrence (Fig. 2.11C).

β-Proteobacteria

β-Proteobacteria the least occurred group during this study. The seawater and sediment samples showed an equal number. Most of the organisms belonging to this group

occurred only during pre and post-monsoon seasons. Interestingly, this group of organisms did not appear from the clear and less contaminated waters from the station Cabo De Rama. Station Vagator indicate high counts of β-Proteobacteria.

Bacteroidetes

The second most abundant group observed during this study was Bacteroidetes. The population almost doubled in sediment samples (46%) compared with seawater and seaweeds (21-28%). The seasonal shifts from monsoons to post-monsoon did not affect much endurance on this group whereas in pre-monsoon, the numbers were increased by 9-13%. Location wise distribution did not show any differences between Kunkeshwar and Cabo De Rama but in Vagator this group found to be less.

Actinobacteria

Frequency distribution of Actinobacterial PIGB showed a similar trend to that of β-Proteobacteria. Their numbers increased from seawater (6%) to sediments (8%) and seaweeds 11%. It indicates that the organisms belonging to this group prefers to attach with some surfaces for its survival. Moreover, high retrieval of this group during monsoon indicates its low temperature preference.

Firmicutes

Pigmented organisms affiliating this group were recovered at a frequency of 18 – 21% from seawater and seaweed samples whereas in sediments it was only 11%. Seasonal variations on isolation sites did not have much influence on the frequency distribution of this group. The exception was observed only from Vagator where the numbers are little high.

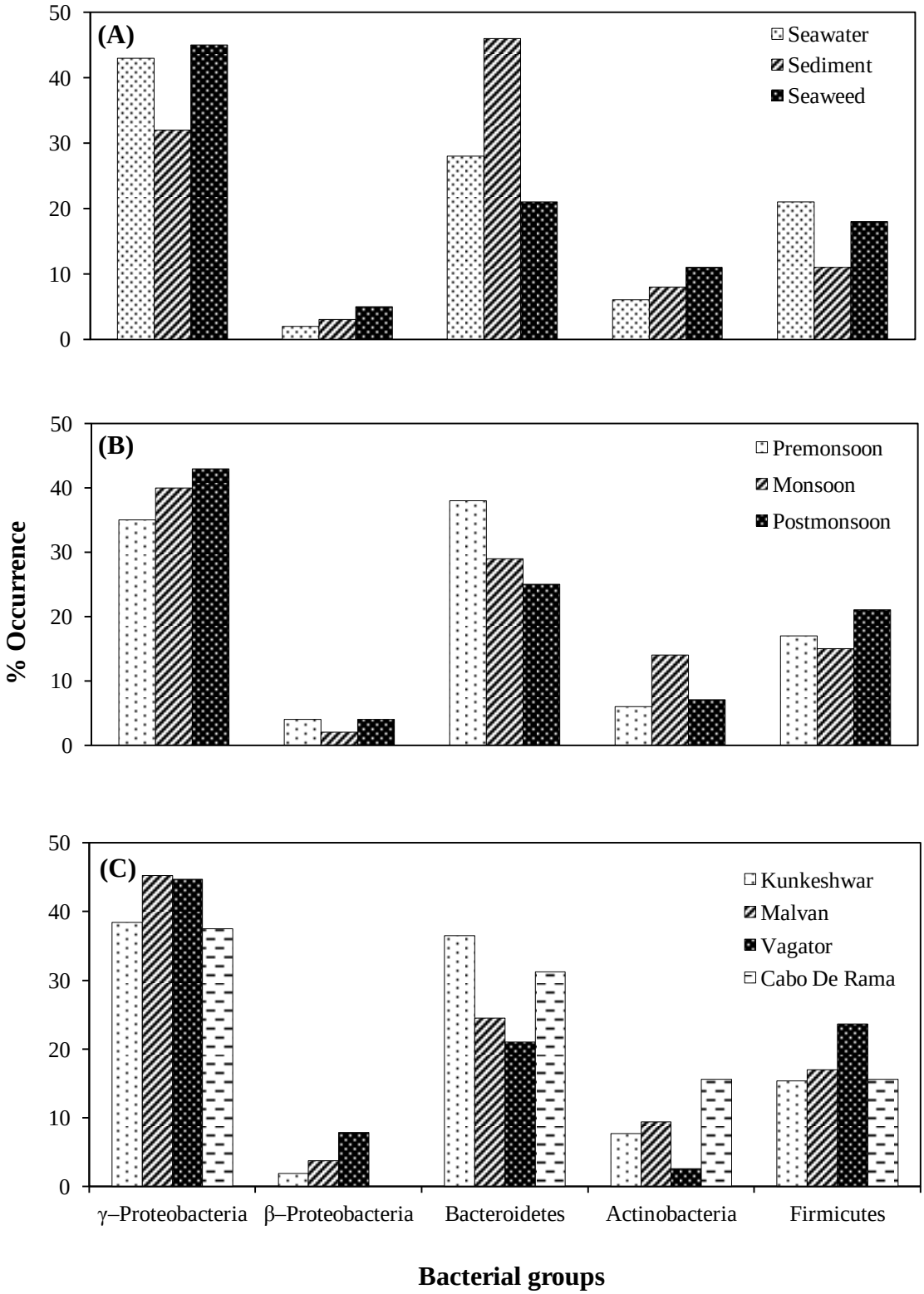


Fig. 2.11. Percentage occurrence of pigmented bacteria affiliated to various phylogenetic groups retrieved from different (A) sources, (B) seasons and (C) sampling stations.

Studies on marine pigmented bacteria from various parts of the world indicate the dominance of γ-Proteobacteria in the oceanic waters. For example, Kinko Bay (Japan) showed the predominance *Pseudomonas* and *Flavobacterium* sp. (Kakimoto et al. 1980). Du et al. (2006) reported supremacy of γ-Proteobacteria (78.7%) in tropical North Pacific Oceanic waters. Our study from coastal locations of India also confirms the predominance of γ-Proteobacteria.

An investigation exploring the diversity of PIGB by Du and co-workers (2006) documented in a total of 247 strains, 37 belong to Actinobacteria and 62 were Firmicutes. In our collections also we got around 15 strains of Actinobacteria and 32 of Firmicutes. It shows that the Actinobacteria were relatively lower in contribution towards pigmented family.

Comprehensive studies on community composition and phylogenetic relationship of surface associated bacteria are still scarce (Burke et al. 2011). Concerning seaweed-epibiotic bacteria along the Indian coasts; JanakiDevi et al. (2013) isolated 126 seaweed epiphytic strains (irrespective of colour property) from the east coast of India and found the γ-Proteobacteria to dominate overall diversity. We came across similar observations along the west coast but with reference to pigmented organisms.

One of the contrasting findings here is that we did not come across any α-Proteobacteria from the CWCI with the property of pigmentation. It indicates organisms belonging to this group must be either non-chromogenic in nature or present at very low numbers in the coastal ecosystem. The current study reports presence of β-Proteobacteria and Actinobacteria along the west coast which is not reported along the east coast. This also brings out a conclusion that both of these coasts differ in their marine bacterial diversity.

Research on bacterial density and community composition of seaweed associated bacteria is even can be seen as contradictory with reference to earlier findings. Some of the studies have revealed that algal species belonging to different group shows variation among the associated bacterial flora (Lachnit et al. 2011). In some cases 80% of the population and their occurrence specified with seaweeds are the same (Viszwapriya et al. 2015). Moreover, evidences have documented that the bacterial community composition of seaweeds differs as compared to that of surrounding water and other inanimate substrata (Burke et al. 2011; Grossart et al. 2013).

We noticed substantial differences among the population of seaweed associated flora in the case of pigmented bacteria as well. These observations are in alignment with other investigations where the algae are evidenced to manage the selective population of surface attached bacteria (Steinberg et al. 1997; Saha et al. 2011). Hence, the pigmented bacterial communities obtained in the present investigation are not much stressed with reference to individual seaweed species rather than assessed all together for antioxidant search by aiming selection of potent strain for next level of study.

Also, few of the bacterial species *Bacillus*, *Corynebacterium* (Firmicutes), and *Psychrobacter* (γ-Proteobacteria) were found more in seawater whereas *Planococcus* and *Chromobacterium* (β-Proteobacteria) strictly retrieved from seaweed surfaces. These results support the global conclusion that the bacterial community composition is specific for specific samples and may drive overall differences in the bacterial structural composition of each sample (Aravindraja et al. 2013). During this study, we come across some of the bacteria (8.42%) which couldn't classify based on their distinct biochemical properties. This might represent novel flora and are need to be studied in more details by molecular means. It forms a prospective objective for the future study.

Diversity and distribution of planktonic (free-living) or sessile (attached) colonizers including bacterial types are not much affected by sampling stations rather than influenced by host and season of isolation at a particular location (Lachnit et al. 2009). We observed similar findings at most of the assessed stations on PIGB where the seasonal change or sample type from different sites along CWCI gave more or less similar population of bacterial groups (Fig. 2.11).

Table 2.4. Characterization and phylogenetic affiliations of marine pigmented bacteria isolated from the central west coast of India.

Strain code	Sampling station/Month-Year of collection	Isolation source	Bacterial genus	Phylogenetic group/class	Phylum
PIGB 1	CDR/Mar-2010	*G. micropterum*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 2	CDR/Mar-2010	*Chaetomorpha* sp.	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 3	Mal./Mar-2010	Seawater	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 4	Mal./Mar-2010	Seawater	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 5	Mal./Mar-2010	Seawater	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 6	Mal./Mar-2010	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 7	Mal./Mar-2010	Sediment	Unidentified	Unidentified	Unidentified
PIGB 8	Mal./Mar-2010	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 9	Mal./Mar-2010	*Ulva* sp.	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 10	Mal./Mar-2010	*G. corticata*	*Bacillus* sp.	Bacilli	Firmicutes
PIGB 11	Mal./Mar-2010	*G. corticata*	*Arthrobacter* sp.	Actinobacteria	Actinobacteria
PIGB 12	Vaga./Mar-2010	*Chaetomorpha* sp.	*Alcaligenes* sp.	β -Proteobacteria	Proteobacteria
PIGB 13	Vaga./Mar-2010	*Chaetomorpha* sp.	*Arthrobacter* sp.	Actinobacteria	Actinobacteria
PIGB 14	Kunk./Mar-2010	Seawater	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 15	Kunk./Mar-2010	Seawater	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 16	Kunk./Mar-2010	*Ceramium* sp.	*Acinetobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 17	Kunk./Mar-2010	*Ceramium* sp.	Unidentified	Unidentified	Unidentified
PIGB 18	Kunk./Mar-2010	*E. clatharata*	*Arthrobacter* sp.	Actinobacteria	Actinobacteria
PIGB 19	Kunk./Mar-2010	*Ceramium* sp.	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 20	Kunk./Mar-2010	*Ceramium* sp.	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 21	Kunk./May-2010	Seawater	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 22	Kunk./May-2010	Seawater	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 23	Kunk./May-2010	Sediment	*Flexibacter* sp.	Sphingobacteria	Bacteroidetes
PIGB 24	Kunk./May-2010	*E. tubulosa*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 25	Kunk./May-2010	*E. tubulosa*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 26	CDR/May-2010	Seawater	*Marinococcus* sp.	Bacilli	Firmicutes

PIGB 27	CDR/May-2010	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 28	CDR/May-2010	*Chaetomorpha* sp.	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 29	CDR/May-2010	*Sargassum* sp.	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 30	CDR/May-2010	*Gelidium* sp.	*Vibrio* sp.	γ–Proteobacteria	Proteobacteria
PIGB 31	Vaga./May-2010	Seawater	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 32	Vaga./May-2010	Sediment	*Photobacterium* sp.	γ–Proteobacteria	Proteobacteria
PIGB 33	Vaga./May-2010	*S. ilicifolium*	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 34	Mal./May-2010	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 35	Mal./July-2010	Seawater	*Psychrobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 36	Mal./July-2010	Seawater	*Moraxella* sp.	γ–Proteobacteria	Proteobacteria
PIGB 37	Mal./July-2010	Seawater	Unidentified	Unidentified	Unidentified
PIGB 38	Mal./July-2010	Sediment	*Aeromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 39	Mal./July-2010	Sediment	*Arthrobacter* sp.	Actinobacteria	Actinobacteria
PIGB 40	Mal./July-2010	Sediment	*Alcaligenes* sp.	β -Proteobacteria	Proteobacteria
PIGB 41	Mal./July-2010	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 42	Mal./July-2010	Sediment	*Aeromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 43	Mal./July-2010	*G. micropterum*	*Bacillus* sp.	Bacilli	Firmicutes
PIGB 44	Mal./July-2010	*U. Fasciata*	Unidentified	Unidentified	Unidentified
PIGB 45	Mal./July-2010	*U. Fasciata*	Unidentified	Unidentified	Unidentified
PIGB 46	Mal./July-2010	*C. media*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 47	Mal./July-2010	*C. media*	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 48	Mal./July-2010	*C. media*	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 49	Vaga./July-2010	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 50	Kunk./July 2010	Seawater	Unidentified	Unidentified	Unidentified
PIGB 51	Kunk./July 2010	Seawater	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 52	Kunk./July 2010	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 53	Kunk./July 2010	Sediment	*Streptococcus* sp.	Bacilli	Firmicutes
PIGB 54	Kunk./July 2010	*G. corticata*	*Acinetobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 55	Kunk./July 2010	*C. media*	Unidentified	Unidentified	Unidentified
PIGB 56	Kunk./July 2010	*C. media*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 57	Kunk./July 2010	*U. fasciata*	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 58	Mal./Sep.-2010	Seawater	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 59	Mal./Sep.-2010	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 60	Mal./Sep.-2010	Seawater	*Aeromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 61	Mal./Sep.-2010	*G. corticata*	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 62	Mal./Sep.-2010	*C. antennina*	*Aureobacterium* /sp.	Actinobacteria	Actinobacteria
PIGB 63	Mal./Sep.-2010	*C. antennina*	*Flexibacter* sp.	Sphingobacteria	Bacteroidetes
PIGB 64	Kunk./Sep.- 2010	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 65	Kunk./Sep.- 2010	Seawater	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 66	Kunk./Sep.- 2010	Seawater	*Bacillus* sp.	Bacilli	Firmicutes
PIGB 67	Kunk./Sep.- 2010	Seawater	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 68	Kunk./Sep.- 2010	Seawater	*Moraxella* sp.	γ–Proteobacteria	Proteobacteria
PIGB 69	Kunk./Sep.- 2010	Seawater	*Flexibacter* sp.	Sphingobacteria	Bacteroidetes
PIGB 70	Kunk./Sep.- 2010	Seawater	*Moraxella* sp.	γ–Proteobacteria	Proteobacteria
PIGB 71	Kunk./Sep.- 2010	Sediment	*Enterobacteriaceae* sp.	γ–Proteobacteria	Proteobacteria

PIGB 72	Kunk./Sep.- 2010	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 73	Kunk./Sep.- 2010	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 74	Kunk./Sep.- 2010	Sediment	*Staphylococcus* sp.	Bacilli	Firmicutes
PIGB 75	Kunk./Sep.- 2010	Sediment	*Vibrio* sp.	γ–Proteobacteria	Proteobacteria
PIGB 76	Kunk./Sep.- 2010	*S. vulgare*	*Corynebacterium* sp.	Actinobacteria	Actinobacteria
PIGB 77	Kunk./Sep.- 2010	*S. vulgare*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 78	Kunk./Sep.- 2010	*G. corticata*	Unidentified	Unidentified	Unidentified
PIGB 79	Kunk./Sep.- 2010	*P. boergesenii*	*Flexibacter* sp.	Sphingobacteria	Bacteroidetes
PIGB 80	Kunk./Sep.- 2010	*P. boergesenii*	*Arthrobacter* sp.	Actinobacteria	Actinobacteria
PIGB 81	Vaga./Sep.-2010	Seawater	*Moraxella* sp.	γ–Proteobacteria	Proteobacteria
PIGB 82	Vaga./Sep.-2010	Seawater	*Brevibacterium* sp.	Actinobacteria	Actinobacteria
PIGB 83	Vaga./Sep.-2010	Sediment	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 84	Vaga./Sep.-2010	Sediment	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 85	Vaga./Sep.-2010	Sediment	Unidentified	Unidentified	Unidentified
PIGB 86	Vaga./Sep.-2010	*Ulva* sp.	*Staphylococcus* sp.	Bacilli	Firmicutes
PIGB 87	Vaga./Sep.-2010	*Ulva* sp.	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 88	Vaga./Sep.-2010	*Ulva* sp.	*Enterobacter* (*Serratia*) sp.	γ–Proteobacteria	Proteobacteria
PIGB 89	CDR/Sep.-2010	Seawater	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 90	CDR/Sep.-2010	Seawater	*Streptococcus* sp.	Bacilli	Firmicutes
PIGB 91	CDR/Sep.-2010	Seawater	*Flexibacter* sp.	Sphingobacteria	Bacteroidetes
PIGB 92	CDR/Sep.-2010	*G. corticata*	*Photobacterium* sp.	γ–Proteobacteria	Proteobacteria
PIGB 93	CDR/Sep.-2010	*Sargassum* sp.	*Streptococcus* sp.	Bacilli	Firmicutes
PIGB 94	Vaga./Nov.-2010	Seawater	*Arthrobacter* sp.	Actinobacteria	Actinobacteria
PIGB 95	Vaga./Nov.-2010	Seawater	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 96	Vaga./Nov.-2010	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 97	Vaga./Nov.-2010	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 98	Vaga./Nov.-2010	Sediment	*Moraxella* sp.	γ–Proteobacteria	Proteobacteria
PIGB 99	Vaga./Nov.-2010	Sediment	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 100	Kunk./Nov.- 2010	Seawater	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 101	Kunk./Nov.- 2010	Seawater	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 102	Kunk./Nov.- 2010	Seawater	*Alcaligenes* sp.	β -Proteobacteria	Proteobacteria
PIGB 103	Kunk./Nov.- 2010	Sediment	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 104	Kunk./Nov.- 2010	Sediment	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 105	Kunk./Nov.- 2010	Sediment	*Marinococcus* sp.	Bacilli	Firmicutes
PIGB 106	Kunk./Nov.- 2010	Sediment	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 107	Mal./Nov.- 2010	Seawater	*Moraxella* sp.	γ–Proteobacteria	Proteobacteria
PIGB 108	Mal./Nov.- 2010	Seawater	*Moraxella* sp.	γ–Proteobacteria	Proteobacteria
PIGB 109	Mal./Nov.- 2010	*G. micropterum*	*Alteromonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 110	Mal./Nov.- 2010	*G. micropterum*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 111	Mal./Nov.- 2010	*G. micropterum*	*Flexibacter* sp.	Sphingobacteria	Bacteroidetes
PIGB 112	Mal./Nov.- 2010	*G. micropterum*	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 113	CDR/Nov.-2010	Seawater	*Brevibacterium* sp.	Actinobacteria	Actinobacteria
PIGB 114	CDR/Nov.-2010	Sediment	*Brevibacterium* sp.	Actinobacteria	Actinobacteria
PIGB 115	CDR/Nov.-2010	*S. vulgare*	*Vibrio* sp.	γ–Proteobacteria	Proteobacteria
PIGB 116	CDR/Nov.-2010	*S. vulgare*	*Micrococcus* sp.	Actinobacteria	Actinobacteria

PIGB 117	CDR/Nov.-2010	*S. vulgare*	*Vibrio* sp.	γ–Proteobacteria	Proteobacteria
PIGB 118	CDR/Nov.-2010	*C. racemosa*	*Vibrio* sp.	γ–Proteobacteria	Proteobacteria
PIGB 119	CDR/Nov.-2010	*C. racemosa*	*Moraxella* sp.	γ–Proteobacteria	Proteobacteria
PIGB 120	Kunk./Jan.- 2011	Sediment	*Acinetobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 121	Kunk./Jan.- 2011	Sediment	*Brevibacterium* sp.	Actinobacteria	Actinobacteria
PIGB 122	Kunk./Jan.- 2011	Sediment	*Flexibacter* sp.	Sphingobacteria	Bacteroidetes
PIGB 123	Kunk./Jan.- 2011	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 124	Kunk./Jan.- 2011	*C. taxifolia*	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 125	Kunk./Jan.- 2011	*C. marginatum*	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 126	Kunk./Jan.- 2011	*C. marginatum*	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 127	CDR/Jan.-2011	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 128	CDR/Jan.-2011	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 129	CDR/Jan.-2011	*C. peltata*	*Bacillus* sp.	Bacilli	Firmicutes
PIGB 130	CDR/Jan.-2011	*D. cervicornis*	Unidentified	Unidentified	Unidentified
PIGB 131	Mal./Jan.-2011	Seawater	*Microbacterium* sp.	Actinobacteria	Actinobacteria
PIGB 132	Mal./Jan.-2011	Seawater	*Bacillus* sp.	Bacilli	Firmicutes
PIGB 133	Mal./Jan.-2011	Seawater	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 134	Mal./Jan.-2011	Seawater	*Acinetobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 135	Mal./Jan.-2011	Sediment	*Vibrio* sp.	γ–Proteobacteria	Proteobacteria
PIGB 136	Mal./Jan.-2011	Sediment	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 137	Mal./Jan.-2011	*P. tetrastomatica*	*Arthrobacter* sp.	Actinobacteria	Actinobacteria
PIGB 138	Mal./Jan.-2011	*P. tetrastomatica*	*Bacillus* sp.	Bacilli	Firmicutes
PIGB 139	Mal./Jan.-2011	*P. tetrastomatica*	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 140	Mal./Jan.-2011	*P. tetrastomatica*	*Staphylococcus* sp.	Bacilli	Firmicutes
PIGB 141	Vaga./Jan.-2011	Seawater	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 142	Vaga./Jan.-2011	Seawater	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 143	Vaga./Jan.-2011	Sediment	*Acinetobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 144	Vaga./Jan.-2011	*S. vulgare*	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 145	Vaga./Jan.-2011	*D. serrata*	*Chromobacterium* sp.	β -Proteobacteria	Proteobacteria
PIGB 146	Vaga./Jan.-2011	*G. micropterum*	*Chromobacterium* sp.	β -Proteobacteria	Proteobacteria
PIGB 147	Vaga./Jan.-2011	*G. corticata*	*Acinetobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 148	Vaga./Mar.- 2012	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 149	Vaga./Mar.- 2012	Seawater	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 150	Vaga./Mar.- 2012	Seawater	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 151	Vaga./Mar.- 2012	*U. lactuca*	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 152	Vaga./Mar.- 2012	*P. tetrastromatica*	*Arthrobacter* sp.	Actinobacteria	Actinobacteria
PIGB 153	Mal./ Mar.- 2012	Seawater	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 154	Mal./ Mar.- 2012	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 155	Mal./ Mar.- 2012	*C. peltata*	*Cytophaga* sp.	Bacteroidetes	Bacteroidetes
PIGB 156	Mal./ Mar.- 2012	*C. peltata*	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 157	Mal./ Mar.- 2012	*U. lactuca*	*Planococcus* sp.	β -Proteobacteria	Proteobacteria
PIGB 158	CDR/ Mar.-2012	Seawater	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 159	CDR/ Mar.-2012	Seawater	*Corynebacterium* sp.	Actinobacteria	Actinobacteria
PIGB 160	CDR/ Mar.-2012	Sediment	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 161	CDR/ Mar.-2012	*C. media*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 162	CDR/ Mar.-2012	*Gelidium* sp.	*Bacillus* sp.	Bacilli	Firmicutes

PIGB 163	Vaga./Sept.- 2012	Seawater	*Enterobacter* (*Serratia*) sp.	γ–Proteobacteria	Proteobacteria
PIGB 164	Vaga./Sept.- 2012	*C. media*	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 165	Vaga./Jan.- 2013	Seawater	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 166	Vaga ./Jan.- 2013	*Gelidium* sp.	*Vibrio* sp.	γ–Proteobacteria	Proteobacteria
PIGB 167	Vaga ./Jan.- 2013	*C. peltata*	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 168	Vaga ./Jan.- 2013	*C. peltata*	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 169	CDR/Jan.- 2013	Sediment	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 170	CDR/Jan.- 2013	*B. composita*	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 171	CDR/Jan.- 2013	*D. dichotoma*	Unidentified	Unidentified	Unidentified
PIGB 172	CDR/Jan.- 2013	*D. dichotoma*	Unidentified	Unidentified	Unidentified
PIGB 173	CDR/Jan.- 2013	*D. dichotoma*	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 174	CDR/Jan.- 2013	*P. tetrastromatica*	Unidentified	Unidentified	Unidentified
PIGB 175	CDR/Jan.- 2013	*U. fasciata*	Unidentified	Unidentified	Unidentified
PIGB 176	CDR/Jan.- 2013	*U. fasciata*	*Corynebacterium* sp.	Actinobacteria	Actinobacteria
PIGB 177	CDR/Jan.- 2013	*U. fasciata*	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 178	Kunk./Jan. - 2013	Seawater	*Micrococcus* sp.	Actinobacteria	Actinobacteria
PIGB 179	Kunk./Jan. - 2013	Seawater	*Flavobacterium* sp.	Flavobacteria	Bacteroidetes
PIGB 180	Kunk./Jan. - 2013	Seawater	*Acinetobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 181	Kunk./Jan. - 2013	Seawater	Unidentified	Unidentified	Unidentified
PIGB 182	Kunk./Jan. - 2013	Seawater	*Corynebacterium* sp.	Actinobacteria	Actinobacteria
PIGB 183	Kunk./Jan. - 2013	Seawater	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 184	Mal./Jan. - 2013	Seawater	*Vibrio* sp.	γ–Proteobacteria	Proteobacteria
PIGB 185	Mal./Jan. - 2013	Seawater	*Corynebacterium* sp.	Actinobacteria	Actinobacteria
PIGB 186	Mal./Jan. - 2013	*P. tetrastromatica*	*Pseudomonas* sp.	γ–Proteobacteria	Proteobacteria
PIGB 187	Mal./Jan. - 2013	*Gelidium* sp.	Unidentified	Unidentified	Unidentified
PIGB 188	Mal./Jan. - 2013	*C. peltata*	*Acinetobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 189	Mal./Jan. - 2013	*C. peltata*	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria
PIGB 190	Mal./Jan. - 2013	*S. tenerrimum*	*Enterobacter* sp.	γ–Proteobacteria	Proteobacteria

Unidentified: Biochemical characteristics were found to be diverse and did not to match the identification keys given in the Bergey's manual of determinative bacteriology.

All the identified pure cultures of pigmented bacteria maintained at –80 ºC as 25% glycerol stocks for further qualitative and quantitative antioxidant testing. Molecular (16S rRNA) characterization and phylogenetic studies carried out on potent antioxidant strains are discussed in consecutive chapter no. 3.

2.4. Conclusion

An elegant study on isolation and characterization of marine pigmented bacteria from the distinct locations, sample sources and season evidenced their diverse endurance along the CWCI. Interestingly, the chromogenic population retrieved 2-3 orders lower (i.e. 10^2-10^3) than the non-pigmented ones (10^4-10^7). The pure culturing led isolation of 190 PIGB where the seawater and seaweed resources together contributed 79.47% of isolates. Categorization based on colour property demonstrated the yellow bacteria (~40%) to dominate the overall population. It followed by 26.32% orange, 21.05% creamy, 5.26% pink, 1.58% red, 5% brown and 1% violet tint organisms.

Bacterial characterization of individual strain affiliated overall population to 5 major phyla i.e. β-Proteobacteria, γ-Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes. It included 25 bacterial genera of which *Flavobacterium, Pseudomonas, Cytophaga* and *Enterobacteriaceae* communally contributed ~40% of the chromogens. PIGB belonging *Acinetobacter, Alteromonas, Arthrobacter, Bacillus, Flexibacter, Micrococcus, Moraxella and Vibrio* genera formed a second most abundant group, while notably the *Aureobacterium, Microbacterium, Photobacterium* and *Planococcus* sp.were represented by single isolates.

Also, the retrieval of PIGB and bacterial communities were experienced to be influenced by source sample and seasons of isolation with no much considerable differences at sampling locations. The results of present study made us realize that the CWCI waters are rich in pigmented forms and need to be explored for prospective biological activities of human interest. The organisms obtained from the CWCI belonging to the group α-Proteobacteria did not show the property of pigmentations thus they may be either non-chromogenic in nature or present at very low numbers in the study area.

CHAPTER 3

ANTIOXIDANT POTENTIAL OF MARINE PIGMENTED BACTERIA

3.1. Introduction

Pigment, the universal trait is receiving increased attention of researchers due to its diverse biological properties of environmental and human health importance (Matthews and Wurtzel 2007; Venil and Lakshmanaperumalsamy 2009; Dieser et al. 2010). One among such property is its antioxidant attribute which plays an important role in radical scavenging and reduction of associated health risks. In context, studies on terrestrial pigmented metabolites obtained from bacteria (e.g. prodigiosin from *Serratia* sp.) have been evidenced to exhibit prominent antioxidant effects (Kuo et al. 2011; Gulani et al. 2012).

Few of the recent studies have revealed the antioxidant role of rare carotenoid pigments from marine bacteria and stated that such metabolites do obstructs free radical associated stress reactions in biological systems (Shindo and Misawa 2014; Balraj et al. 2014). In support, our recent report on seaweed associated pigmented bacteria documents significant outcomes where the pigmented metabolites are evidenced to have comparable activities as good as to that of known commercial antioxidants (Pawar et al. 2015).

Research intended to determine the antioxidant effects of any biological material need to estimate various tests. This includes various methods among which DPPH and ABTS are the most commonly used to determine the anti-radical effects. The contents of antioxidant compounds can be quantified with reference to standard compounds using reducing reactions like FRAP and reducing powers. In addition to radical scavenging molecules, determination of enzymatic antioxidants is another key criterion to predict the endogenous potential of an organism to withstand vicinity stress. The role of such antioxidant enzymes has been devised to protect the host organisms against organic acids and environmental changes (Bruno-Barcena et al. 2010; Correa-Llanten et al. 2012). Few

of the studies have even utilized these enzymes to enhance the biodegradation of anthropogenic material and some have been employed in biomedicines as well (Kang et al. 2007; Allocati et al. 2009; Zhang et al. 2012).

The initial step in the development of any bioactive compounds involves selection of appropriate bacteria from a natural source, activity check and optimization of process parameters. As discussed earlier, except few compounds like Astaxanthin, Zeaxanthin, Violacein and carotenoids the research on antioxidants specifically from bacterial origin is still at the laboratory or developmental stage (Dufosse 2006; Venil and Lakshmanaperumalsamy 2009). Thus, it represents the need of comprehensive studies on pigmented antioxidants from the marine bacteria that could give cost effective alternatives.

In context to the same, initial screening and isolation studies on pigmented bacteria demonstrated retrieval of 190 diverse organisms (Chapter 2). In this chapter we are directed to understand the antioxidant potential of them. Qualitative and quantitative study on such chromogens from the potent strains and their antioxidative properties are also discussed.

3.2. Materials and methods

3.2.1. Primary screening for bacterial antioxidants

The simple primary DPPH-antioxidant screening method of Takao et al. (1994) was followed for initial antioxidant testing. Pure culture suspension broth (2 µl; OD_{600} ~1.0) of individual pigmented bacteria was drop inoculated on ZMA plates and incubated at 28 ± 2 ºC for 24 h. Later, a sterilized filter paper (Whatman no. 1) placed on the agar plate

carefully, so that colony and its extracellular metabolites get replicated on the paper. Incubation was further continued for another 24 h. Later, the filter paper was removed, dried and consequently sprayed with DPPH solution (80 µg ml^{-1} in methanol). Strains showing a yellowish/white zone on the purple background considered antioxidant positive. Colony size and the AO zones were recorded for each of the analyzed cultures.

3.2.2. Selection of potent strains and optimization

The primarily antioxidant screened bacteria based on the activity zone categorized into high potent (>3.5 cm), potent (2-3.5 cm) and low (<2 cm) active strains. Five of such high potent strains i.e. three from seaweeds (PIGB 46, 77, 88) and two from seawater (PIGB 163 and 184) samples were selected for optimization and quantitative antioxidant analysis.

3.2.2.1. Growth studies on antioxidant bacteria

A loopful culture of individual bacteria was inoculated into 250 ml flask containing 100 ml of sterile marine broth and incubated (28 ± 2 ºC) until growth reached 10^6 cell ml^{-1}. Later 10 µl cell suspension of each strain was inoculated in microtiter plate wells containing 190 µl of sterile marine broth in triplicates. Controls (200 µl ZMB) were maintained without any bacterial cultures. Optical density values were recorded at 600 nm with an interval of every 5 h and used for determination of initial growth curve.

3.2.2.2. Optimization of process parameters

Antioxidative strains were assessed for its growth with reference to various process parameters (PP) like media concentration, pH, temperature and various carbon and

nitrogen sources (Fig. 3.1). Maximum growth, pigment productions and antioxidant activity obtained at particular process parameter were considered optimum and used for further analysis.

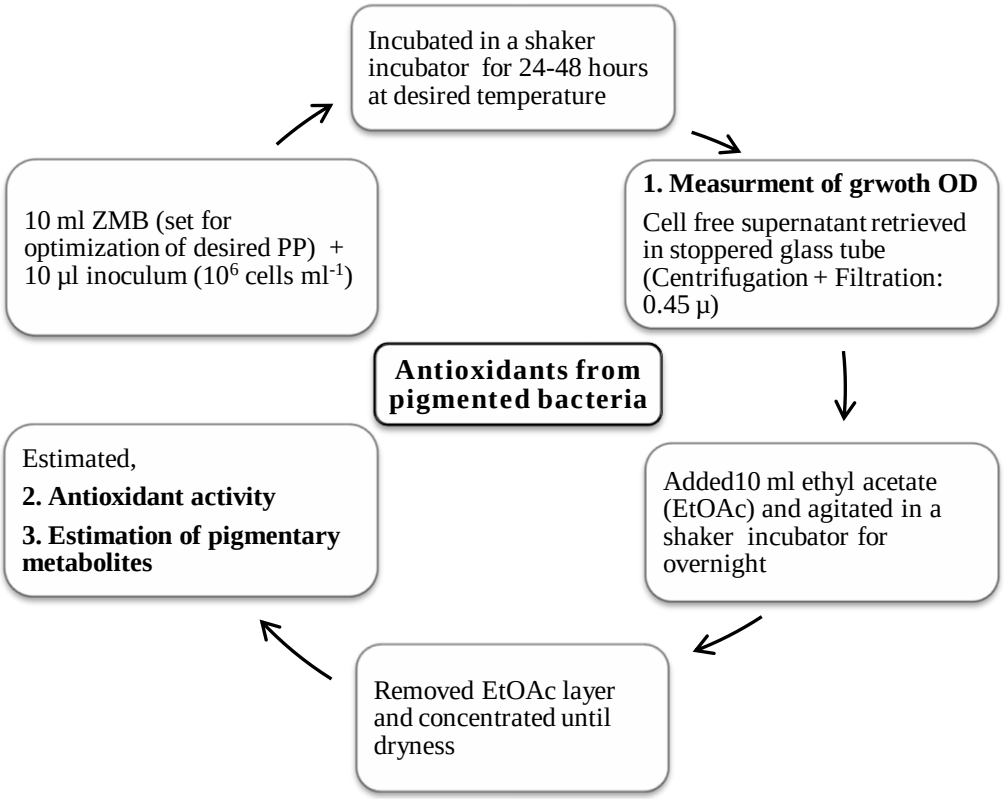


Fig. 3.1. Schematic representation of optimization method for enhanced production of antioxidants from pigmented bacteria.

Effect of pH: Sterile marine broth 10 ml, in 50 ml test tubes adjusted to a range of pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using 0.1 N HCl or NaOH. Media with respective pH were inoculated with 10 µl of selected bacterial cultures. Tubes were incubated at 28 ± 2 ^oC (120 rpm) for 24 h. Growth, antioxidant and the quantity of the pigments were estimated as discussed in the above section (Fig. 3.1).

Effect of temperature: Sterile marine broth 10 ml, in 50 ml test tubes adjusted to optimized pH values for respective bacteria and sterilized. It was inoculated with 10 µl of

selected bacteria. Tubes were incubated at 10, 20, 30, 40 and 50 ℃ (120 rpm) for 24 h in shaking incubator (Bio-Technics, BTI India). Growth, antioxidant activities and the quantity of the pigments were estimated as discussed in the above section.

Effect of media strength: Marine broth media was prepared in similar fashion as discussed earlier with different strengths ranging from 25-100 % with an interval of 25 percentile. Bacterial inoculation, growth study and antioxidant estimations were carried out in a similar manner as discussed in previous sections.

Effect of carbon and nitrogen source: Influence of additional energy source on growth and antioxidant activity analyzed by supplementing media with 2 % different carbon (glucose, sucrose, maltose, dextrose, lactose, xylose etc.) and nitrogen (casein, ammonium sulfate, peptone, tryptone, yeast extract etc.) components. Further growth, pigment recovery and AO estimations were carried out as discussed above.

3.2.3. Biosynthesis and extraction of AO pigmentary metabolites

An aliquot, 100 µl pure culture of selected isolates were inoculated into separate flasks containing sterile 500 ml marine broth. It was incubated in a shaker incubator at 28 ± 2 ºC, 120 rpm for 48 h. The media was then centrifuged (10000 × g, 10 ºC at 10 min) and the cell-free supernatant collected in another sterile collection tube. The supernatant further filtered (0.45 µm) to remove all the bacterial cells from broth. Later, 10 ml of cell-free supernatant added with 10 ml solvent in a stoppered glass bottle and kept for overnight shaking. Different solvents like ethyl acetate, petroleum ether, dichloromethane and n-hexane were employed to assess the most suitable extraction system. The solvent layer was separated, concentrated with a rotary evaporator

(Equitron, Roteva (63) R-V) and samples finally retained in a minimum quantity of HPLC grade methanol.

Dry weight determination of the sample extract was carried out as per the standard protocol of Ekanayake et al. (2004). Briefly, 1.0 ml concentrated EtOAc extract of potent strain was allowed to dry in a pre-weighed aluminium foil and weight of the samples was determined. The solvent giving maximum recovery of pigmentary products was utilized for mass extraction procedure as discussed above. The known concentrations of samples viz. 0.10, 0.50, 1.00, 5.00, 10.00 and 15.00 mg ml^{-1} were prepared in HPLC-methanol and used for quantitative antioxidant analysis.

3.2.4. Quantitative estimation of antioxidants

3.2.4.1. Non-enzymatic studies

The non-enzymatic studies included free radical scavenging tests, reducing ability check and determination of total phenolic contents in the extracts of selected pigmented bacteria.

A. DPPH radical scavenging activity

Radical scavenging activity of PIGB against DPPH radicals assessed according to the method of Larrauri et al. (1998). Briefly, 0.8 ml (0.05 mM) of the DPPH-methanol solution mixed with different concentrations of sample and final volume made up to 1.0 ml. The reaction mixture then incubated for 30 min (25 ºC) in dark and absorbance was measured at 517 nm. Control maintained with DPPH solution while, the blank contained only methanol throughout the experiment. Butylated hydroxytoluene (BHT) used as

positive control. The DPPH activity was calculated according to the equation, DPPH scavenging (%) = [1-(Absorbance of the sample/ Absorbance of the control)] × 100.

B. ABTS total antioxidant power assay

The $ABTS^{+}$ radical decolourization assay was employed to evaluate the total antioxidant power of sample extracts (Re et al. 1999). Primarily, the ABTS was dissolved in water to a 7 mM concentration. ABTS radical cations were produced by reacting ABTS stock solution with 2.45 mM potassium persulphate. The mixture allowed to stand in dark (12-16 h) prior use. The stock solution diluted in ethanol (1:89 v/v) to an absorbance of 0.70 ± 0.02) at 734 nm. Reaction components equilibrated at 30°C exactly 6 min after initial mixing and used for further analysis. To start with reaction, 1 ml of diluted ABTS solution was mixed with 10 µl of sample extracts of different strengths (0.1 to 15 mg ml^{-1}). The decrease in absorbance was noted at 734 nm and antioxidant capacity expressed in percent inhibition (%I) values. Quercetin was used as a standard reference antioxidant compound and IC_{50} values were calculated from the regression analysis.

C. Determination of reducing power

Reducing abilities of pigmented extracts determined by following the method of Ferreira (2007). Briefly, 0.25 ml sample mixed with 2.5 ml of 200 mM Na_2PO_4 buffer (pH 6.6). To this 2.5 ml 1% potassium ferricyanide was added with slow mixing. The whole reaction mixture incubated at 50 ºC (20 min). Later, 2.5 ml of 10% trichloroacetic acid (w/v) added to the reaction mixture. Whole reaction mixtures centrifuged at 4000 × g for 10 min. Later, 5 ml reaction aliquot from the upper layer mixed with 5 ml of distilled water (DW). To this 1.0 ml of 0.1% ferric chloride added and absorbance of the solution

measured at 700 nm. The reducing abilities were determined based on the change in absorbance values.

D. Ferric reducing antioxidant power (FRAP) assay

FRAP analysis was carried out with a modified method of Benzie and Strain's (1996). The working FRAP reagent prepared by mixing 300 mM (pH 3.6) acetate buffer, 10 mM TPTZ solution (prepared in 40 mM HCl) and 20 mM $FeCl_3 6H_2O$ in a 10:1:1 ratio. The reaction components mixed just before the use, heated to 37°C and cooled to room temperature. An aliquot, 150 µl working FRAP reagent added to each of the well in a microtiter plate and a blank read performed using ELISA plate reader (Biorad, 680 XR, Biosciences) at 595 nm. A total of 20 µl of sample added to each well and a second reading was taken after 8 min of incubation. The antioxidant content in the sample extracts were expressed in terms of ascorbic acid equivalents (AsA Eq. µg ml^{-1}).

E. Determination of total phenolic contents

Total phenolic compounds in extracellular pigmented extracts of selected bacteria were quantified by following Folin-ciocalteu's colorimetric method (Shan et al. 2005). Different concentrations of sample extracts (0.2 ml) were initially oxidized with 1 ml 0.5 M Folin-ciocalteu's reagent for a period of 4 min. The reaction mixture then neutralized with 1.0 ml of saturated sodium carbonate (Na_2CO_3: 75 g l^{-1}). The incubation continued for 30 min and absorbance (OD= 760 nm) values for the resulting blue color were recorded. Quantification of phenolics carried out from the standard gallic acid curve and results were expressed as mg of Gallic Acid Equivalents (GAE) in sample extract.

3.2.4.2. Enzymatic studies

A) Exposure to stressor

In order to observe the endogenous enzymatic AO potential of selected chromogenic bacteria, the cultures were exposed to an oxidizing agent hydrogen peroxide (H_2O_2). At an initial step different concentrations of H_2O_2 (0-40 mM with an interval of 2 mM in a 96 well microtiter plate) were tested to determine the growth and sensitivity values for selected bacteria. Later, the individual PIGB was exposed to a particular H_2O_2 concentration. Two experimental (treated) sets were maintained to have a better understanding of activation of endogenous antioxidants. Set one (T1) was treated right from initial growth/inoculation to observe the effect on growing cells while in other set (T2) the fully grown cells were exposed to H_2O_2. Control was maintained without any addition of stressor. Bacterial cells were retrieved after 12 h incubation from both experimental and control sets and utilized for estimation of stress enzymes.

B) Sample preparation and enzyme extraction

Treated and untreated bacterial cells were collected by centrifugation (10,000 × g for 10 min at 4°C) immediately after H_2O_2 exposure. Ten milligram wet cell mass of each selected bacteria was homogenized by sonication (22% power with 7 sec exposure and 5 sec interval) in homogenizing buffer [50 mM phosphate buffer, pH 7.4 containing 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.15 M potassium chloride (KCl) and 0.01% w/v phenylmethylsulfonyl fluoride (PMSF)]. Cell homogenate further centrifuged (10,000 × g for 10 min at 4°C) and the resulting supernatant collected separately. This extract was used for protein (Lowry et al. 1951) as well as enzyme estimations. All the procedures were carried out in cold conditions. Enzyme

quantifications were carried out immediately after cell homogenization using readymade kit or preset standard protocols.

C) Estimation of stress enzymes

i) Catalase (CAT) activity: Enzymatic stress index in selected strains in terms of catalase activity was carried out using ELISA plate microtiter plate assay method as per the EnzyChrom catalase assay kit protocol (BioAssay Systems, CA, USA). To start with reaction, an aliquot of 10 µl of the sample (prepared as explained in above section) mixed with 90 µl 50 µM H_2O_2 (substrate). After incubation (30 min at RT), an aliquot of 100 µl detection dye reagent was added into each well (sample, control and standard). OD values were recorded at 570 nm using Microplate reader (BMG LABTECH, Pvt. Ltd., Victoria). Catalase activity was expressed as Unit of enzyme produced per milligram protein (U mg^{-1}).

ii) Superoxide dismutase (SOD) activity: Estimation of SOD in individual pigmented bacteria was carried out by using EnzyChrom Superoxide Dismutase Assay kit (BioAssay Systems, USA) as per the manufacturer instructions. Briefly, 20 µl of the appropriately diluted sample, blank (assay buffer) and standard (SOD enzyme) were added into separate wells of a microtiter plate. Later, 160 µl working reagent (standardized assay buffer, xanthine and water-soluble tetrazolium WST dye) and xanthine oxidase (XO) enzyme solution added to each well, mixed and initial (0 min) readings were taken immediately at 440 nm using microplate reader. Reaction plate was allowed to incubate at RT for another 60 min and second reading (60 min) was noted. SOD activities in the sample (U mg^{-1} protein) were estimated based on a standard curve.

iii) Glutathione-S-transferase (GST) activity: GST activity of each sample was estimated by the modified method of Habig et al. (1974). Briefly, 10 µl of appropriately diluted sample mixed with 990 µl of phosphate buffer (pH 6.9, 100 mM) containing 1 mM Glutathione (GSH) and 1 mM 1-Chloro-2,4-dinitrobenzene (CDNB). The absorbance and delta absorbance readings were recorded at 340 nm for 5 min reaction with an interval of 30 seconds using UV-VIS Spectrophotometer (Shimadzu UV-1800). Reaction control was maintained in a similar fashion where the sample was replaced with phosphate buffer. GST activities were expressed in terms of U mg^{-1} protein.

3.2.5. Molecular identification and phylogenetic studies on potent bacteria

3.2.5.1. 16S rRNA gene identification

Genomic DNA was extracted from the potent isolates by using DNeasy Blood and Tissue kit (QIAGEN India Pvt. Ltd., New Delhi) according to manufacturer's instructions. The extracted DNA was subjected to polymerase chain reaction (PCR) with universal primers forward 27F and reverse 1492R (Lane 1991). The PCR reaction components were purified using PCR purification kit.

The DNA sequencing was carried out with an automatic sequencer (Applied Biosystems 3130xl Genetic Analyzer) using bacterial primers 27F, 515R and 1492R. Molecular (16S rRNA) gene sequences assessed with the BLASTn algorithm of National Center for Biotechnology Information (NCBI). Identity results submitted to the GenBank and accession numbers were obtained.

3.2.5.2. Phylogenetic analysis of AO strains

The phylogenetic tree was devised by retrieving gene sequences of earlier reported AO bacteria from the existing database and compared with our potent bacteria (Table 3.1). Formed clades were observed for phylogenetic distances and bootstrap significance.

Table 3.1. 16S rRNA gene sequences of AO bacteria utilized for constructing phylogenetic tree on selected strains of PIGB.

Bacteria	Closest reported AO strains		Acc. No.	Reference
P. argentinensis (PIGB46) and *P. koreensis* (PIGB77)	1.	*Pseudomonas* sp. HAV-1	JN172106.1	Dua et al. 2014
	2.	*Pseudomonas* sp. AS1	KP076214.1	Kang et al. 2007
	3.	*Pseudomonas* sp. PF-6	GU944677.1	Ye et al. 2012
	4.	*Pseudomonas* sp. SLI-02-4	KC865054.1	Yang et al. 2014
	5.	*P. fluorescens* CrN6	KF359766.1	Sirajunnisa et al. 2015
S. rubidaea (PIGB88) and *S. nematodiphila* (PIGB163)	1.	*Serratia* sp.	LG045291.1	Bhattarai et al. 2013
	2.	*S. ureilytica*	HE612873	Kuo et al. 2011
	3.	*S. marcescens*	KM035849.1	Gulani et al. 2012; Vora et al. 2014
	4.	*S. marcescens* cenA	JN613282.1	Nongkhlaw and Joshi 2015
Vibrio sp. (PIGB184)	1.	*Vibrio* sp. P1Ma8	JX477113	Dupont et al. 2013
	2.	*Vibrio* sp. P1Ma5	JX477119	
	3.	*Vibrio* sp. SKMARSP9	HE798514.1	Shiva Krishna et al. 2015
	4.	*Vibrio* sp. 13B	GQ406756.1	Horta et al. 2014

3.2.6. Statistical analysis

All biochemical tests performed in triplicates (n=3) for statistical analysis. Results were recorded as a mean ± standard deviation. Variations among the biochemical parameters studied cross tested with one-way ANOVA and post hoc tests (Newman-Keuls) to discriminate between means of values. The observed differences considered statistically significant when $p < 0.05$. Antioxidant parameters were correlated against phenolic compounds of selected test organisms and the significance coefficient (R^2) values were recorded for each assessed parameter.

3.3. Results and Discussion

3.3.1. Antioxidant potential of PIGB: Qualitative properties

Antioxidant screening DPPH decolorization test on total 190 PIGB strains demonstrated 44.21 % of the organisms to secrete antioxidant metabolites in their extracellular environment (Table 3.2; Fig. 3.2). Around 7.37 % of the isolates showed greater than 2 cm while 3.68 % isolates retained with > 3 cm AO zones. Organisms with 0-1 cm zones were considered as low active. Strains isolated from seaweed (36 isolates) and seawater (34 isolates) samples contributed more than three fourth of the positive population as compared to that of sediments (14 isolates). Interestingly, maximum and minimum activity values were recorded with bacteria isolated from seaweed and sediment samples.

Conversely, the positive strains in terms of their pigment colour and AO zones were observed to follow the order pink (4.9 cm) > yellow/brown (4.3 cm) > Creamy (3.7 cm) > orange (2.8 cm) > red (2.3 cm) (Table 3.2; Fig. 3.3). Isolates with highest activity zones i.e. PIGB 46, 77, 88, 163 and PIGB 184 considered as potent strains and below that were excluded from further quantitative analysis.

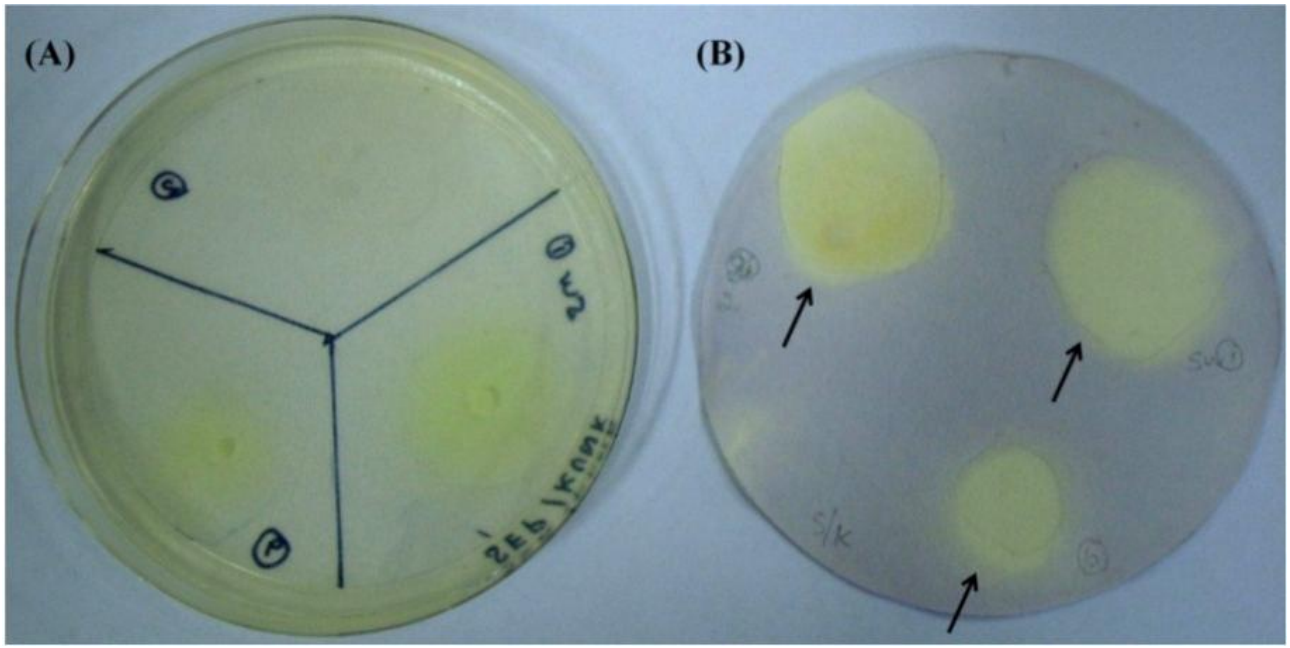


Fig. 3.2. Antioxidant screening test by DPPH decolorization assay.

(A) Bacterial colonies grown in plates. (B) Whatman filter paper tested with DPPH reagent.

Table 3.2. Qualitative analysis of antioxidants tested in various bacterial strains.

S. No.	Cultue code	Pigment type	Antioxidant zone in cm*			Source	Bacterial identity
			a	b	b-a		
1.	PIGB 1	Orange	0.4	3.1	2.7	Algae	*Pseudomonas* sp.
2.	PIGB 4	Yellow	0.4	1.5	1.1	Seawater	*Pseudomonas* sp.
3.	PIGB 5	Pale yellow	0.4	1.7	1.3	Seawater	*Pseudomonas* sp.
4.	PIGB 6	Red	0.4	1.0	0.6	Seawater	*Flavobacterium sp.*
5.	PIGB 10	Yellow	0.3	0.9	0.6	Algae	*Bacillus* sp.
6.	PIGB 17	Pale yellow	0.5	0.7	0.2	Algae	Unidentified
7.	PIGB 18	Red	0.6	2.5	1.9	Algae	*Arthrobacter sp.*
8.	PIGB 22	Creamy	0.5	0.7	0.2	Seawater	*Pseudomonas* sp.
9.	PIGB 23	Yellow	0.4	1.0	0.6	Seawater	*Flexibacter* sp.
10.	PIGB 28	Pale yellow	0.3	0.9	0.6	Algae	*Flavobacterium sp.*
11.	PIGB 61	Creamy	0.7	1.3	0.6	Algae	*Alteromonas* sp.
12.	PIGB 29	Pale yellow	0.2	1.1	0.9	Algae	*Flavobacterium* sp.
13.	PIGB 30	Pale yellow	0.4	0.13	0.9	Algae	*Vibrio* sp.
14.	PIGB 31	Orange	0.4	0.6	0.2	Seawater	*Pseudomonas* sp.
15.	PIGB 32	Pale brown	0.4	0.6	0.2	Sediment	*Photobacterium* sp.
16.	PIGB 33	Pale yellow	0.4	0.9	0.5	Algae	*Alteromonas* sp.
17.	PIGB 34	Orange	0.5	0.7	0.2	Sediment	*Flavobacterium* sp.
18.	PIGB 35	Creamy	0.5	0.7	0.2	Seawater	*Psychrobacter* sp.
19.	PIGB 37	Pale yellow	0.6	1.2	0.6	Seawater	Unidentified
20.	PIGB 44	Pale yellow	1.0	2.6	1.6	Algae	Unidentified
21.	PIGB 45	Yellow	0.3	0.8	0.5	Algae	Unidentified
22.	**PIGB 46**	**Pale yellow**	**0.5**	**4.0**	**3.5**	**Algae**	***Pseudomonas* sp.**
23.	PIGB 47	Red/orange	0.5	2.8	2.3	Algae	*Micrococcus* sp.
24.	PIGB 50	Pale yellow	0.5	1.0	0.5	Seawater	Unidentified
25.	PIGB 51	Pale yellow	0.6	1.2	0.6	Seawater	*Alteromonas* sp.
26.	PIGB 58	Pale yellow	0.8	2.9	2.1	Seawater	*Alteromonas sp.*
27.	PIGB 60	Creamy	0.9	2.0	1.1	Seawater	*Aeromonas* sp.
28.	PIGB 64	Orange	0.4	0.7	0.3	Seawater	*Flavobacterium sp.*
29.	PIGB 68	Pale orange	0.7	1.0	0.3	Seawater	*Moraxella sp.*
30.	PIGB 69	Yellow	0.5	1.9	1.4	Seawater	*Flexibacter sp.*
31.	PIGB 70	Creamy	0.5	2.3	1.8	Seawater	*Moraxella* sp.
32.	PIGB 71	Orange	0.4	1.0	0.6	Sediment	*Enterobacter* sp.
33.	PIGB 73	Pale yellow	0.3	0.6	0.3	Sediment	*Flavobacterium* sp.
34.	PIGB 75	Yellow	0.4	0.6	0.2	Sediment	*Vibrio sp.*
35.	PIGB 76	Creamy	0.4	1.5	1.1	Algae	*Corynebacterium* sp.
36.	**PIGB 77**	**Brown**	**0.7**	**5.0**	**4.3**	**Algae**	***Pseudomonas* sp.**
37.	PIGB 79	Orange	0.7	3.5	2.8	Algae	*Flexibacter* sp.
38.	PIGB 80	Orange/Red	0.3	1.7	1.4	Algae	*Arthrobacter* sp.
39.	PIGB 81	Yellow	0.4	0.9	0.5	Sediment	*Moraxella* sp.
40.	PIGB 82	Orange	0.6	1.0	0.4	Seawater	*Brevibacterium* sp.
41.	PIGB 83	Creamy	0.5	0.8	0.3	Sediment	*Alteromonas* sp.
42.	PIGB 84	Yellow	0.6	1.1	0.5	Sediment	*Micrococcus* sp.
43.	PIGB 87	Creamy	0.5	1.3	0.8	Algae	*Pseudomonas* sp.

44.	**PIGB 88**	**Pink**	**0.5**	**5.4**	**4.9**	**Algae**	***Enterobacter* (*Serratia*) sp.**
45.	PIGB 91	Pale orange	0.8	1.2	0.4	Seawater	*Flexibacter* sp.
46.	PIGB 92	Orange	0.6	1.8	1.2	Algae	*Photobacterium* sp.
47.	PIGB 93	Pale yellow	0.4	1.5	1.1	Algae	*Streptococcus* sp.
48.	PIGB 96	Red	0.5	1.0	0.5	Seawater	*Flavobacterium* sp.
49.	PIGB 99	Orange	0.6	1.2	0.6	Sediment	*Cytophaga* sp.
50.	PIGB 100	Creamy	0.5	0.7	0.2	Seawater	*Micrococcus* sp.
51.	PIGB 101	Yellow	0.5	0.7	0.2	Seawater	*Pseudomonas* sp.
52.	PIGB 102	Orange	0.6	1.0	0.4	Seawater	*Alcaligenes* sp.
53.	PIGB 103	Yellow	0.5	1.0	0.5	Sediment	*Cytophaga* sp.
54.	PIGB 109	Creamy	0.5	0.8	0.3	Seawater	*Alteromonas sp.*
55.	PIGB 114	Pale orange	0.6	0.8	0.2	Sediment	*Brevibacterium sp.*
56.	PIGB 117	Creamy	0.4	2.3	1.9	Algae	*Vibrio sp.*
57.	PIGB 119	Brown	0.2	2.8	2.6	Algae	*Vibrio sp.*
58.	PIGB 122	Orange	0.8	2.4	1.6	Sediment	*Flexibacter sp.*
59.	PIGB 125	Pale orange	0.4	0.7	0.3	Algae	*Cytophaga* sp.
60.	PIGB 132	Orange	0.5	1.0	0.5	Seawater	*Bacillus* sp.
61.	PIGB 140	Pale orange	0.5	1.0	0.5	Algae	*Staphylococcus* sp.
62.	PIGB 149	Brown	0.4	0.6	0.2	Seawater	*Micrococcus* sp.
63.	PIGB 150	Pale yellow	0.3	0.7	0.4	Seawater	*Cytophaga* sp.
64.	PIGB 152	Creamy	0.4	0.8	0.4	Algae	*Arthrobacter* sp.
65.	PIGB 153	Orange	0.4	0.8	0.4	Seawater	*Cytophaga* sp.
66.	PIGB 155	Orange	0.3	0.6	0.3	Algae	*Cytophaga* sp.
67.	PIGB 156	Brown	0.4	0.7	0.3	Algae	*Micrococcus* sp.
68.	PIGB 159	Yellow	0.3	0.6	0.3	Seawater	*Corynebacterium* sp.
69.	PIGB 160	Pale orange	0.4	1.0	0.6	Sediment	*Flavobacterium* sp.
70.	PIGB 162	Pale orange	0.2	3.3	3.1	Algae	*Bacillus* sp.
71.	**PIGB 163**	**Pink**	**0.7**	**5.6**	**4.9**	**Seawater**	***Enterobacter* (*Serratia*) sp.**
72.	PIGB 166	Creamy/Yellow	1.3	5.0	3.7	Algae	*Vibrio* sp.
73.	PIGB 167	Orange	0.6	3.0	2.6	Algae	*Micrococcus* sp.
74.	PIGB 168	Orange	0.3	0.6	0.3	Algae	*Flavobacterium* sp.
75.	PIGB 169	Pink	0.5	1.0	0.5	Sediment	*Enterobacter* sp.
76.	PIGB 174	Pink	0.3	0.5	0.2	Algae	Unidentified
77.	PIGB 177	Pink	1.0	1.2	0.2	Algae	*Flavobacterium* sp.
78.	PIGB 179	Yellow	0.4	0.8	0.4	Seawater	*Flavobacterium* sp.
79.	PIGB 180	Pale brown	0.5	2.0	1.5	Seawater	*Acinetobacter* sp.
80.	PIGB 181	Yellow	0.3	0.6	0.3	Seawater	Unidentified
81.	PIGB 183	Brown	0.4	3.2	2.8	Seawater	*Pseudomonas* sp.
82.	**PIGB 184**	**Pale yellow**	**0.5**	**3.7**	**3.2**	**Seawater**	***Vibrio* sp.**
83.	PIGB 186	Orange	0.5	1.0	0.5	Algae	*Pseudomonas sp.*
84.	PIGB 190	Pale brown	0.5	1.2	0.7	Algae	*Enterobacter sp.*

*a: Colony size, b: DPPH decolorization Zone, b-a: Antioxidant zone. Text highlighted in bold represents high potent strains.

Fig. 3.3. (A)

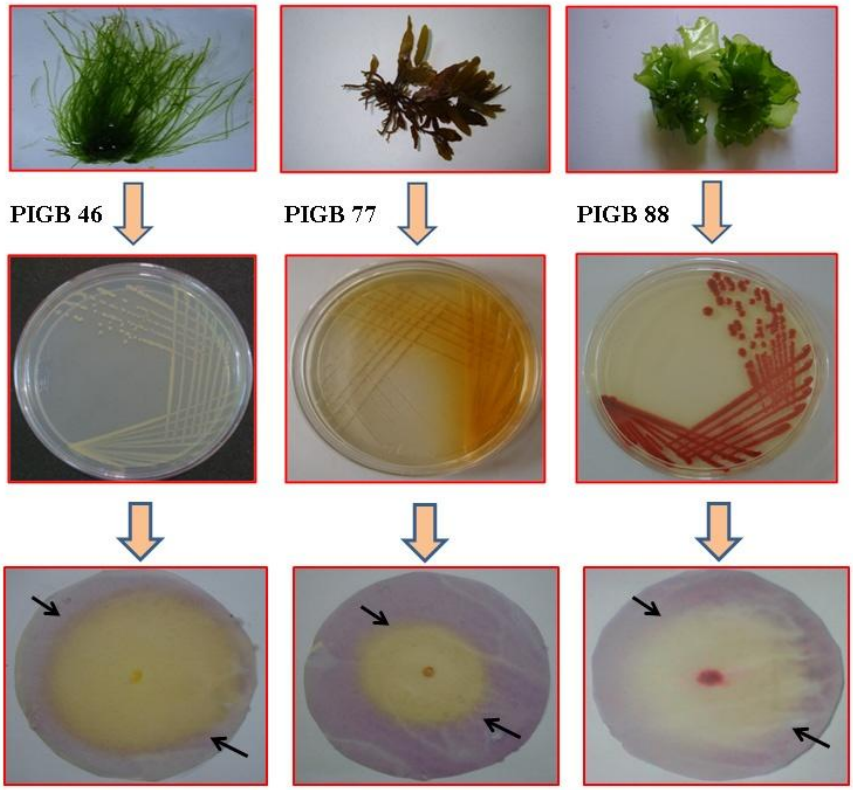


Fig. 3.3. (B)

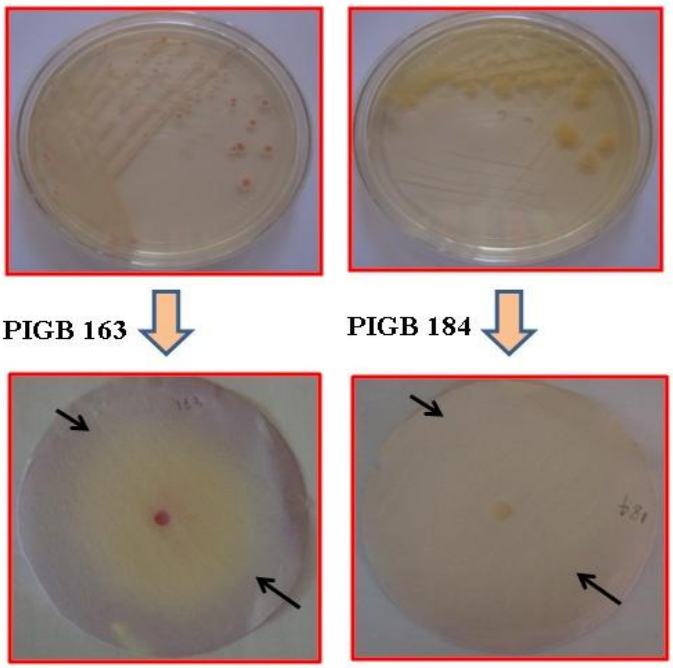


Fig. 3.3. (A) Pictorial representation of antioxidant potent strains from seaweed and (B) Seawater samples.

3.3.2. Optimization of pigmentary antioxidants

Bacterial strains PIGB 46, 77, 88, 163 and 184 selected based on primary screening further optimized for their growth process parameters by aiming enhanced production of antioxidative metabolites.

3.3.2.1. Growth studies on the selected strains

All the selected chromogenic bacteria were observed to remain in the lag phase for first 5-10 h. It followed by an exponential (10-20 h) and a long stationary phase up to 36 h (Fig. 3.4). The growth of PIGB 46 and 184 was declined after 25 h while 88 declined after 36 h. PIGB 163 and PIGB 77 showed a long exponential phase of 48 and 60 h respectively. Simultaneously, antioxidants are also responded a linear relation with bacterial growth (21.1 – 41.13% DPPH scavenging). AO activity was started increasing from 20 h and found to be in the steady state till 36-48 h. Pigment production also followed a similar fashion.

Primary experimental observations, we were able to track the incubation time required for each of the strain to reach stationary phase and consequent production of secondary metabolites (Fig. 3.4A; 3B). Based on this we assigned the incubation period 25-36 h for the strains PIGB 46 and 184. Longer incubation time of 36-48 h was chosen for PIGB 77, 88 and PIGB 163.

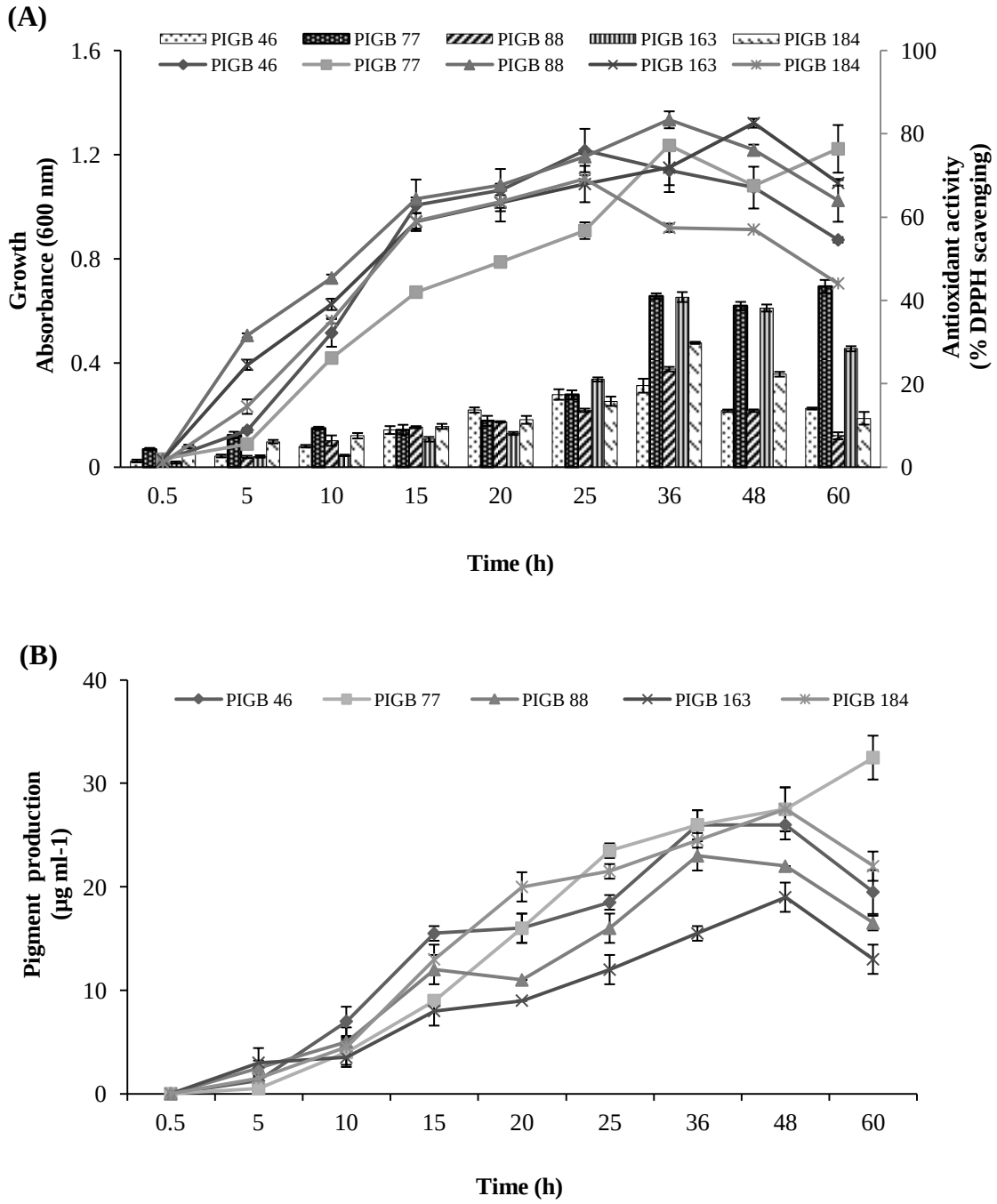


Fig. 3.4. (A) Growth and innate property of marine pigmented bacteria for antioxidant. Line graph represents absorbance while bar graph represents AO activity values.

(B) Pigment production from the selected strains at various time periods.

3.3.2.2. Effect of process parameters (PP)

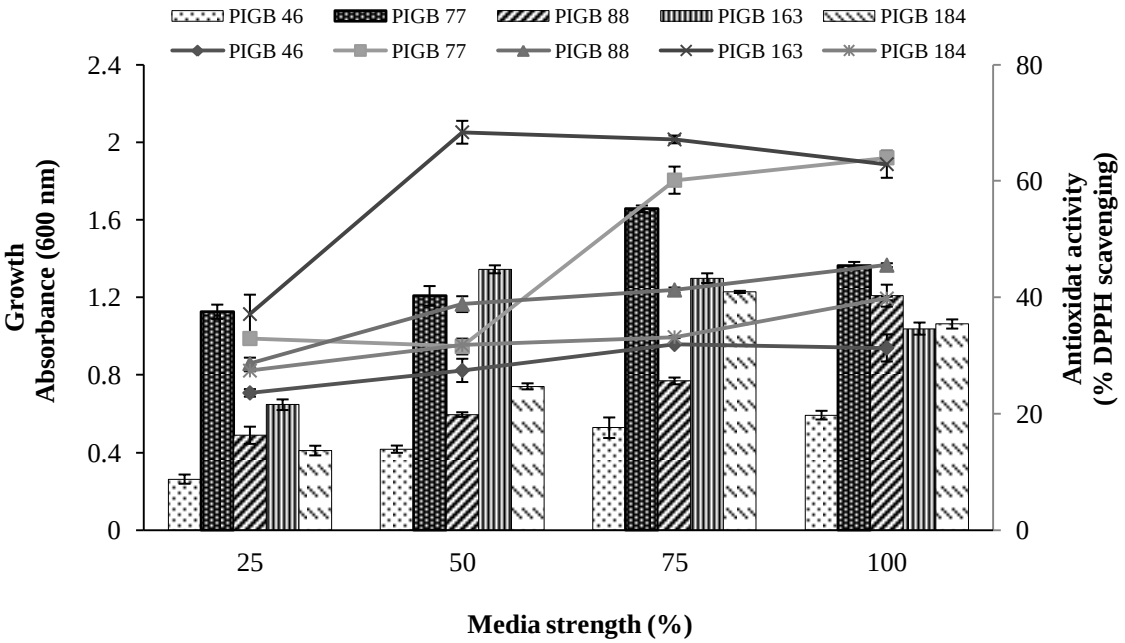
The effect of each PP like media strength, pH, temperature, carbon and nitrogen source on selected bacteria is summarized in Table 3.3.

Media strength:

An effect of media concentration on growth and AO activity is depicted in Fig. 3.5A. *Pseudomonas* strain (PIGB46: *P. argentinensis*) showed equal growth rate and antioxidant activity in 75 (OD= 0.96; 17.6% DPPH activity) and 100 % (OD= 0.94; 19.8% DPPH activity) media strengths. In contrast, the other yellowish brown pigmented *Pseudomonas* strain PIGB 77 though expressed higher OD values in 100 % Zobell media the antioxidant activity was found to be lower while comparing with the media strength of 75%.

The pink coloured bacterial isolates PIGB 88 and PIGB 163 even though belonging to the same genera *Serratia* the growth and antioxidant activity differs. PIGB 88 had higher growth OD= 1.07; AO activity: 40.3% in 100% media strength whereas the PIGB 163 gave prominent results (43.3-44.8 % DPPH scavenging) in 50-75%.

When we are looking at the pigment production against the media strength, PIGB 163 expressed more amounts of pigment at 50 and 75% concentrations (Fig. 3.5B). In case of PIGB 184 (*Vibrio* sp.), media strength 75 % supported the considerable recovery of the pigmented products. Considering overall observation on growth and recovery of pigmented metabolites, most of the yellow coloured bacteria gave their maximum in full strength media whereas the pink chromogens (PIGB 88 and 163) preferred diluted media.



(B)

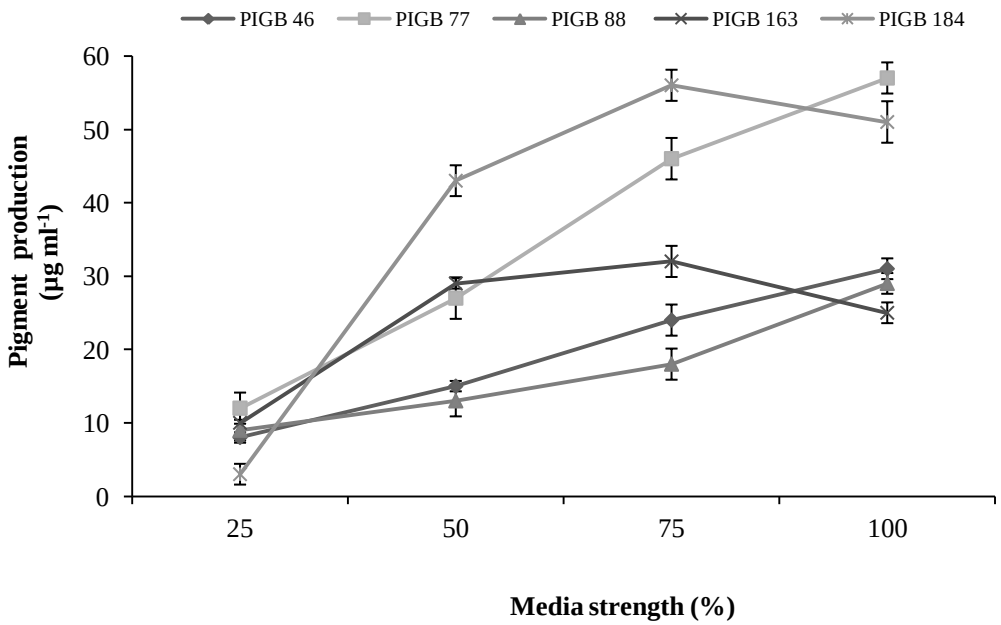


Fig. 3.5. (A) Effect of media strength on growth and antioxidant activity.

Line graph represents growth while bar graph represents AO activity.

(B) Effect of media strength on pigment recovery.

pH:

Influence of core factor pH (ionic potential) on bacterial growth and antioxidant productions is summarized in Fig 3.6. Among 5 studied bacteria *Pseudomonas* strain PIGB 46 was observed to be more active (growth and antioxidant) at pH 6.0 whereas the PIGB 77 was found to exhibit near about similar effects in the pH range 5.0 to 6.0. Likewise, the PIGB 163 also demonstrated optimum results in the pH range of 6.0 to 7.0. Strains PIGB 88 and 184 were found to be more active at pH 7.0.

An interesting observation we would like to make here is PIGB 77 though had higher growth at pH 5.0 (OD= 1.54 ± 0.01) it showed prominent AO activity in the pH range 6.0-7.0. Similarly, the pink coloured organisms had higher growth values at pH 4.0 and 5.0 however; they produced more pigmentary compounds at pH 6.0 and 7.0 (Fig. 3.6A; 3.6B). This trend in pigment synthesis and AO activity further reduced with increasing pH above 7.0. These results emphasize that bacterial growth may not always have a direct relation to the antioxidant activity. However, the AO property may be related to the compounds secreted by individual species at a particular pH value.

Results on the recovery of pigmented metabolites demonstrated the acidic pH range to impart good support as compared to a basic range (Fig. 3.6B). The pigment secretion was found to be low (6.25 to 34 µg ml^{-1}) at pH 4.0 and kept increasing up to pH 6.0-7.0. Further, increase in pH values (9.0 to 10.0) had decremented effect on pigment production (3 to 22 µg ml^{-1}). Pink chromogens (PIGB 88 and 163) recorded with the highest recovery (28.5-37.5 µg ml^{-1}) whereas, the yellow (PIGB 46)/ brown (PIGB 77) coloured organisms had little lower secretions (25-26.5 µg ml^{-1}) at neutral pH (7.0). Interestingly, in few cases the growth and pigment recovery had an unambiguous relation where higher OD concurred with less production of pigmentary metabolites and vice versa e.g. PIGB 163 and 77.

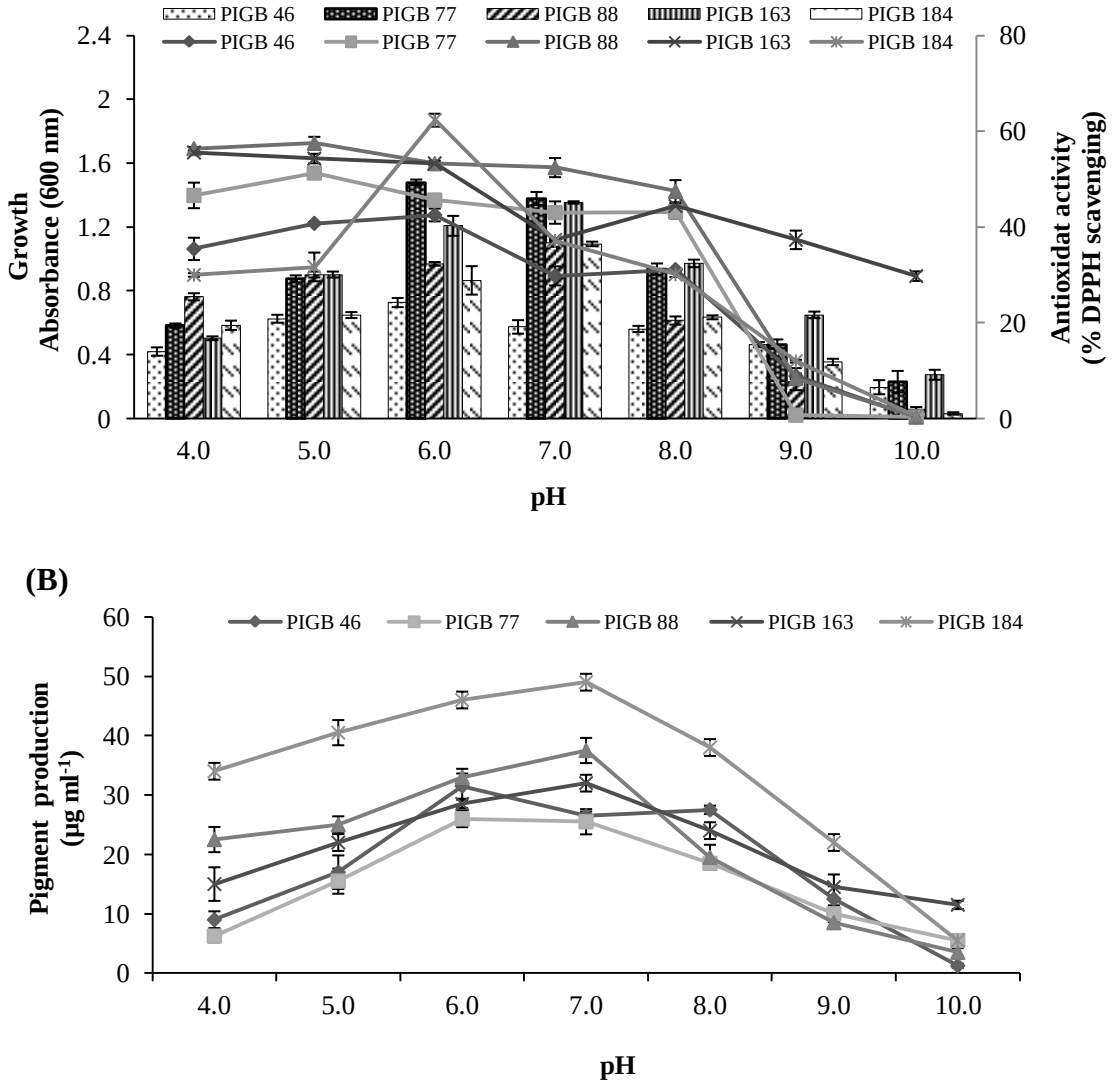


Fig. 3.6. (A) Effect of pH on growth and antioxidant activity.

Line graph represents growth while bar graph represents AO activity.

(B) Effect of pH on pigment recovery.

Temperature:

Effect of temperature on targeted AO parameters concerning pigmented bacteria presented in Fig. 3.7. Pigmented bacteria showed greater growth, pigment recovery and prominent antioxidant activity at optimum temperature 30 ºC. Notably, the *Serratia* sp. (PIGB 88 and PIGB 163) though showed excelled growth at 40 ºC they had low pigment productions and antioxidant activity at this temperature (Fig. 3.7A and 3.7B).

The effect of temperature had a direct relation with pigment production (Fig. 3.7B). All the bacteria under current study secreted low quantity of pigment (6 to 34 µg ml^{-1}) at lower temperatures (10 and 20 °C). Pigment productions were optimum at 30 °C (22-40 µg ml^{-1}). Increased temperatures i.e. 40 and 50 °C also brought down the pigment production (1-3 µg ml^{-1}). This is due to the suppressed growth at these temperatures which consequently resulted in lower secretion of pigmented metabolites. Among selected strains PIGB 184 evidenced with highest production of AO metabolites (40 ± 1.4 µg ml^{-1}) whereas PIGB 46 could give the lowest value (22 ± 1.4 µg ml^{-1}) at 30 ºC.

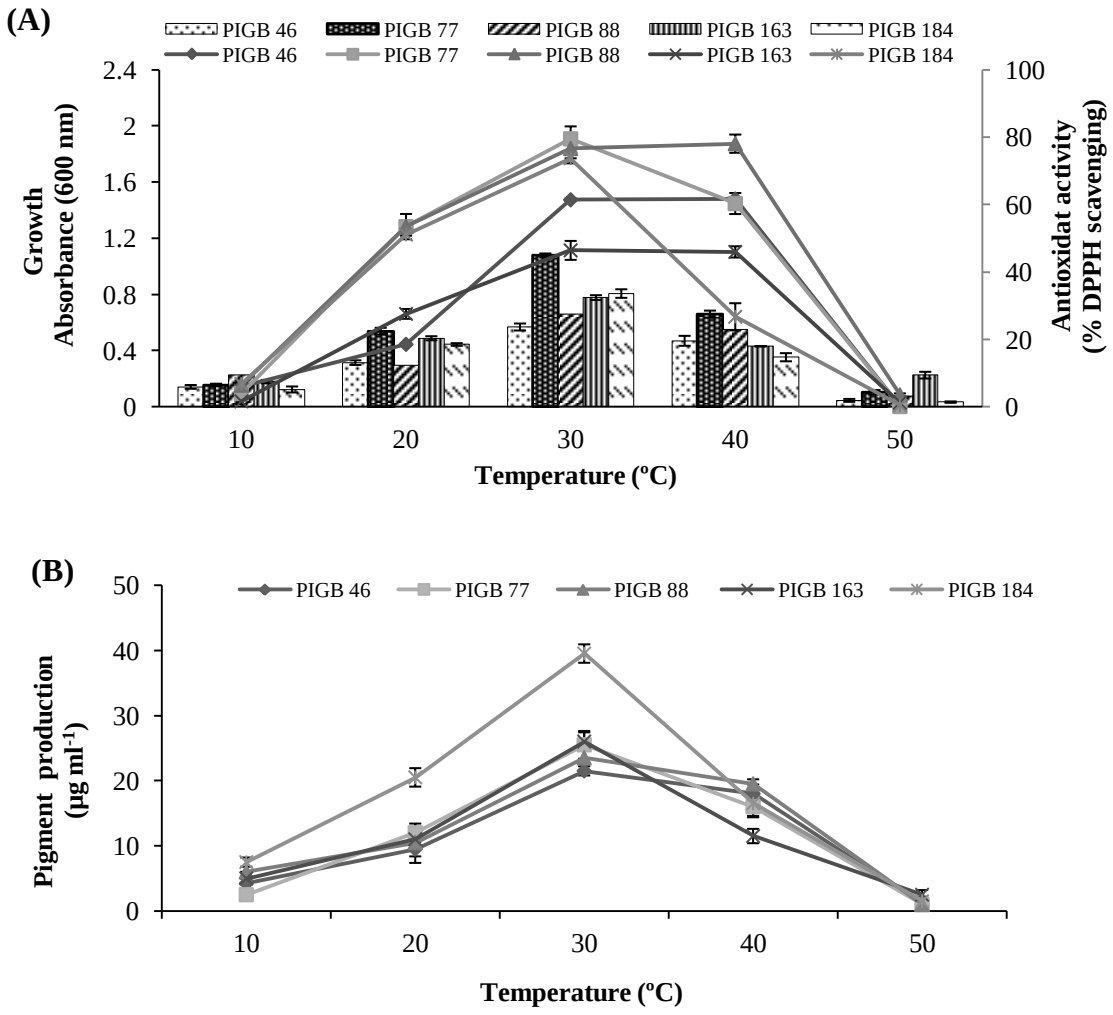


Fig. 3.7. (A) Effect of temperature on growth and antioxidant activity. Line graph represents growth while bar graph represents AO activity. **(B)** Effect of temperature on pigment recovery.

Carbon source:

Results presented in Table 3.3 and Fig 3.8 demonstrates all the pigmented bacteria required distinct types of carbon energy source for their growth and biological activity. PIGB 46, 77, 88, and 184 were found to give optimum results with xylose, fructose, maltose and dextrose respectively. PIGB 163 witnessed more or less similar affiliations towards carbon source lactose and maltose. In addition, some bacterial species did not support growth vs. antioxidant production trend e.g. PIGB 77 showed higher growth (OD=1.63) in media supplemented with dextrose but had more AO activity (44.4% DPPH scavenging) with substrate fructose (Fig 3.8A).

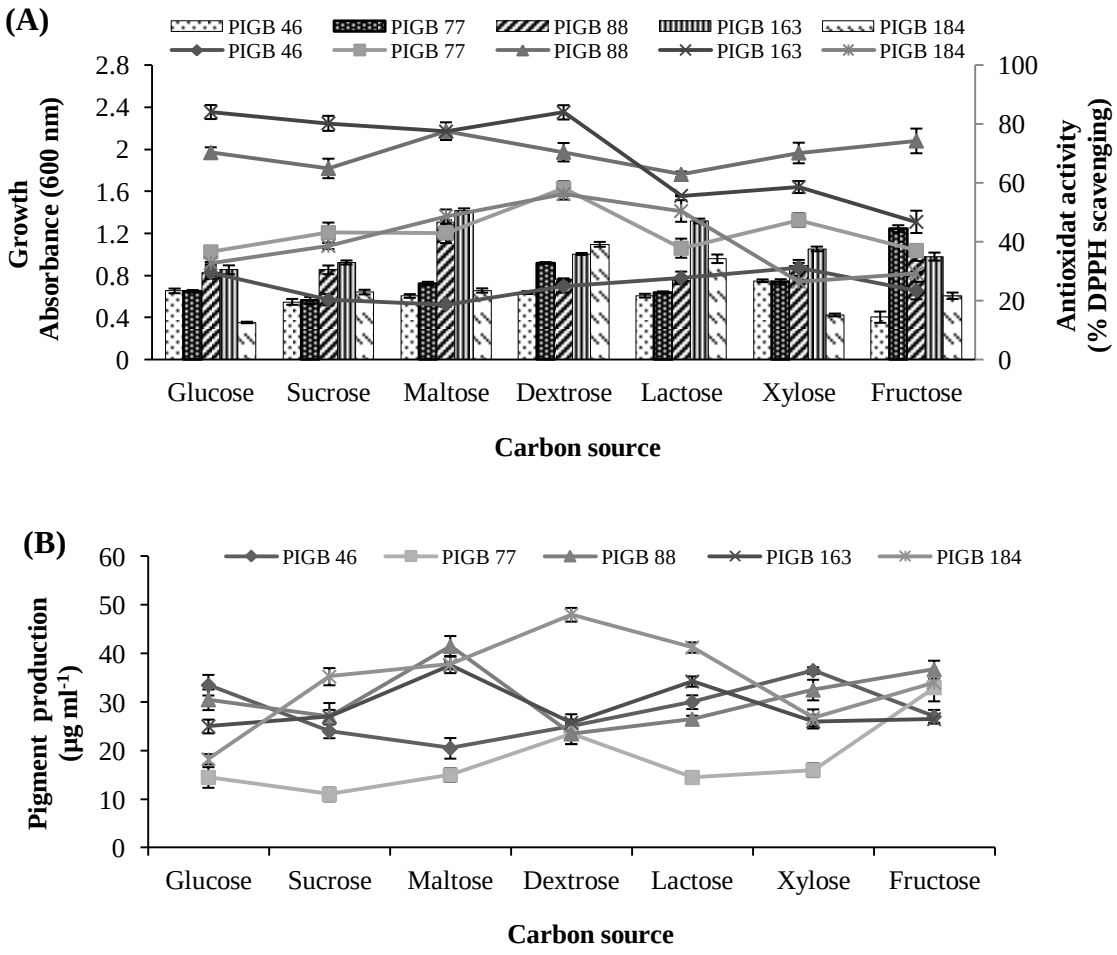


Fig. 3.8. (A) Effect of carbon source on growth and antioxidant activity. Line graph represents growth while bar graph represents AO activity.

(B) Effect of carbon source on pigment recovery.

The carbon source had variable effect on pigment production (Fig. 3.8B). Each bacterium preferred their one likes. Among the five bacteria studied PIGB 184 gave higher pigment production (48 ± 1.4 µg ml^{-1} µg ml^{-1}) with the carbon source dextrose. PIGB 46 secreted considerable amount of AO pigmentary metabolites (36.5 ± 0.7 µg ml^{-1}) by utilizing xylose whereas the PIGB 77 had optimum results with fructose. The PIGB 88 and PIGB 163 preferred maltose for its maximum secretion 37.5-41.5 µg ml^{-1}.

Nitrogen source:

Nitrogenous source peptone preferred by pigmented strains PIGB 46 and 88 for growth. The PIGB 184 found to utilize either peptone or tryptone in a more efficient way as it supported higher growth and AO activity (Fig. 3.9). Tryptone had a considerable effect on growth and antioxidant in strain PIGB 77. PIGB 163 had lowest growth rate (OD= 0.84) towards casein among all the studied nitrogen sources (Fig. 3.9 and 3.10).

In case of PIGB 46, 88 and 184 media supplemented with N substrates peptone evidenced to boost the synthesis of pigmented metabolite (40-53 µg ml^{-1}) and antioxidant activity (Fig. 3.9A; 3.9B). The other organisms PIGB 77 and 163 utilized tryptone and casein respectively for its optimum (33-46 µg ml^{-1}). This indicates the nitrogenous elements are the key factors for multiplication of bacteria where they primarily act as building blocks for nucleotide and protein synthesis. However, in some cases the addition of nitrogen source was evidenced to have decremented effects on antioxidant activity.

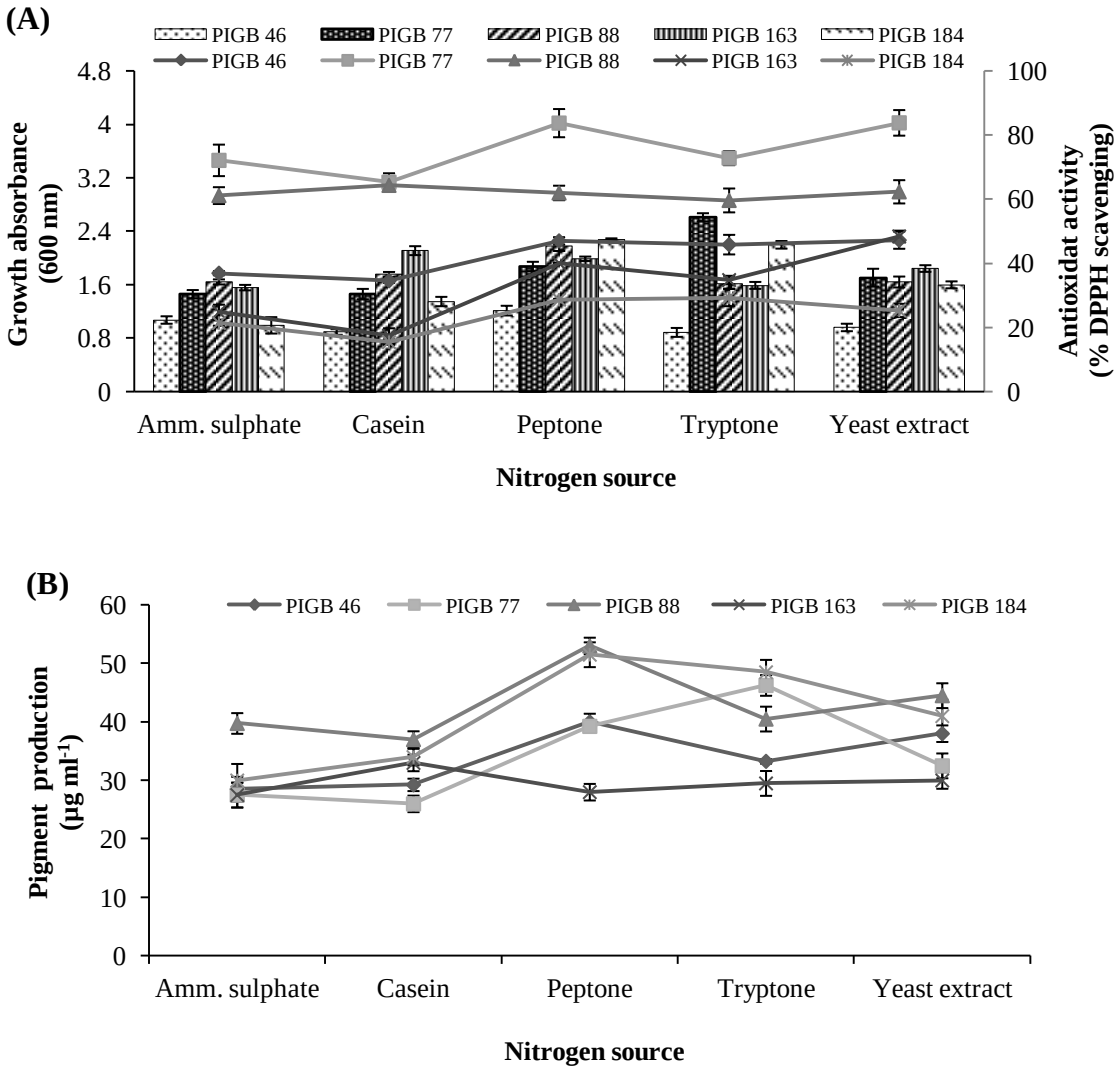


Fig. 3.9. (A) Effect of nitrogen source on growth and antioxidant activity. Line graph represents growth while bar graph represents AO activity.

(B) Effect of nitrogen source on pigment recovery.

Gulani et al. (2012) optimized the physicochemical parameters on the growth of terrestrial bacterium *S. marcescens* and recorded pH 7.0 at 25 °C to support better growth and pigment recovery. One of our *Serratia* strains isolated from coastal location, *S. rubidaea* demonstrated similar affiliation towards pH 7.0. Likewise, the optimization results on carbon (maltose) and nitrogen (peptone) source matched equally with results of Gulani et al. (2012). It indicates that organisms belonging *Serratia* genera require

maltose and peptone as selective energy sources. Contrastingly, the other *Serratia* strain under present study i.e. *S. nematodiphila* (PIGB 163) had distinct affiliations towards PP and emphasized that individual bacterial species even belonging from same genera requires different requirements for optimal growth and synthesis of pigmentary metabolites.

A very recent study on sponge-associated bacteria (irrespective of pigment property) documented the essentiality of PP optimization (pH/time duration) for boosting the growth and antioxidant activity (Balakrishnan et al. 2015). This group of researchers studied the effect of pH (2.0-10.0) with an interval of 2 units and observed greater activity in the range of 6-8. In contrast, we studied the effect of pH (3.0 to 10.0) with a narrow interval of 1 unit and found out the optimum pH values for an each individual (Table 3.3).

Earlier studies concerning the influence of C and N source on the growth and pigment production have reported variable results. Sucrose, lactose and fructose are reported to hamper the pigment production in *Serratia* sp. (Gulani et al. 2015; Sundaramoorthy et al. 2009). We experienced the same effect on our isolates PIGB 88 and PIGB163 (Fig. 3.10). Similarly, glucose a primary energy source though known to have a critical role in the growth of bacteria, it has been reported to limit the production of pigments (Gulani et al. 2012). None of our strains gave a considerable amount of pigment production with glucose. This suggests that glucose may be the limiting factor in pigment production. Overall results on optimization of PP suggest that each bacterium may have specific growth requirements for its regulated physiology and secretion of active metabolites. One must study the primary need and effect of growth components to have significant retrieval of pigmented antioxidant metabolites from a bacterial source.

Table 3.3. Optimized process parameters for selected antioxidant strains.

Parameters	Pigmented bacteria				
	PIGB 46 (*P. argentinensis*)	PIGB 77 (*P. koreensis*)	PIGB 88 (*S. rubidaea*)	PIGB 163 (*S. nematodiphila*)	PIGB 184 (*Vibrio* sp.)
Media strength	100 % ZMB	75 % ZMB	100 % ZMB	50-75 % ZMB	75 % ZMB
pH	6.0	5.0 - 6.0	7.0	6.0 - 7.0	7.0
Temperature	30 ℃	30 ℃	30 ℃	30 ℃	30 ℃
Carbon Source	Xylose	Fructose	Maltose	Maltose	Dextrose
Nitrogen source	Peptone	Tryptone	Peptone	*Casein	Peptone/ Tryptone
Incubation time	2-3 days	5 days	2 - 3 days	2 - 3 days	2 - 3 days

*Not used for mass production.

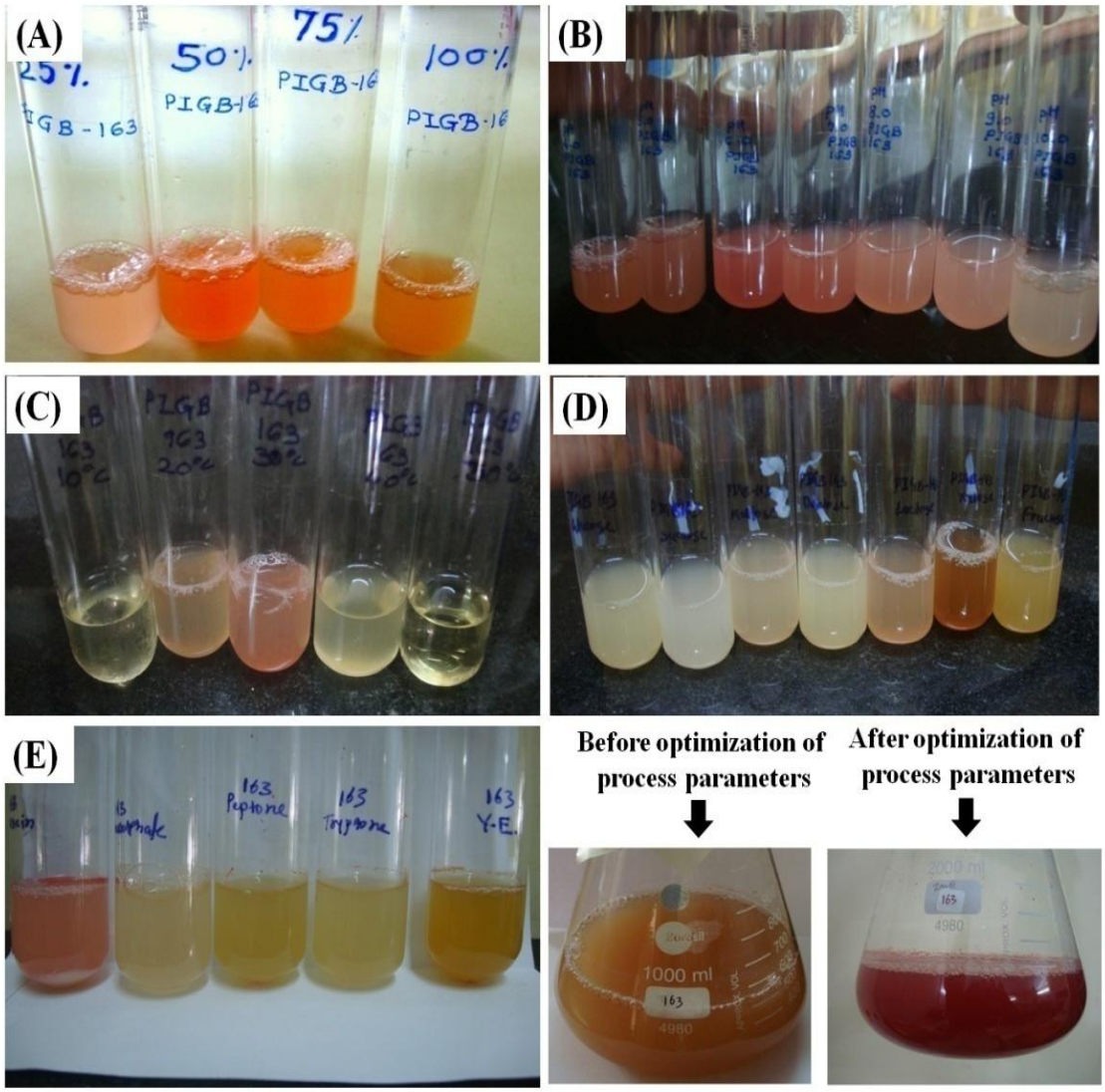


Fig. 3.10. Pictorial depiction of process parameter on pigment production.

(A) Media strength, (B) pH, (C) Temperature, (D) Carbon source, (E) Nitrogen source.

3.3.2.3. Mass culture and extraction

Bacteria optimized for growth conditions as discussed in previous sections were consequently assessed for maximum recovery of antioxidative pigmentary metabolites using different solvent systems and results are presented Fig. 3.11. All studied organisms had the highest recovery of extracellular pigmented metabolites with ethyl acetate (33-50 µg ml^{-1}). Solvent dichloromethane (DCM) could retrieve medium level of pigmentary compounds whereas the nonpolar hexane and petroleum ether had near about equal and lowest recovery (3-8 µg ml^{-1}) from the culture supernatant.

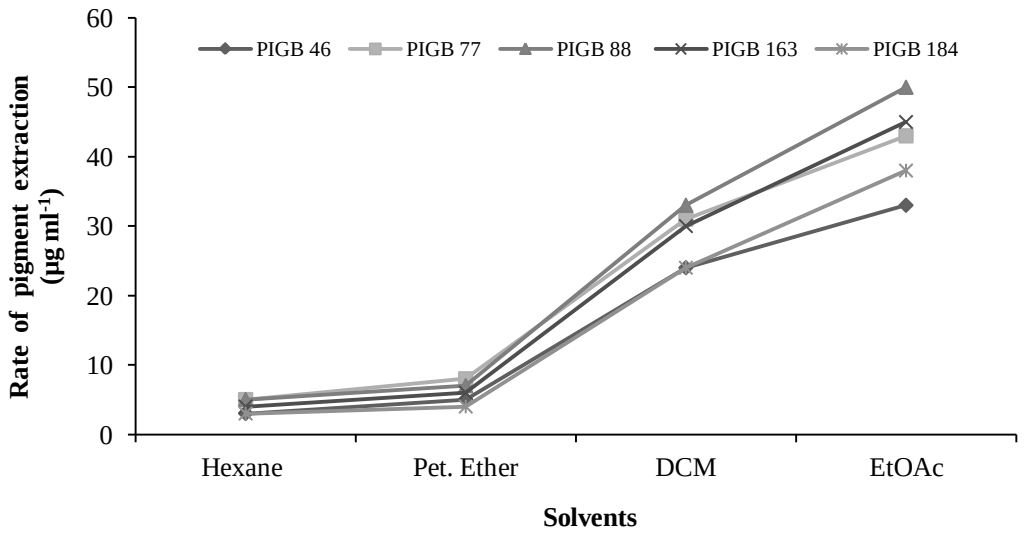


Fig. 3.11. Extraction of pigments using various solvent systems.

The first step in the isolation of pigmented metabolites involves solvent extraction. Selection of solvent system always based on the type of interested compound (Kim 2013). However, it is necessary to take a precautionary measure to avoid maximum exposure to oxygen, light and heat because these factors affect the activity and chemical nature of active components. The aqueous: solvent system overcomes this phenomenon by avoiding exposure to oxygen as the compounds get separated at the liquid-liquid interface.

The literature on the extraction of AO metabolites as well as pigments from culture supernatant had documented the advantages of employing ethyl acetate (Kuo et al. 2011 and 2012; Saravanan et al. 2012; Horta et al. 2014). This includes faster and greater recovery of active metabolites in its native form. We observed a similar trend in the retrieval of pigmented metabolites from the culture supernatant of selected bacteria. The maximum recovery with EtOAc in the present study indicates polar/ medium polar nature of the metabolites secreted by selected organisms.

The reason behind less solubility and retrievability of pigmented compounds in the solvents like n-hexane, chloroform and petroleum ether during the present study could be due to unavailability/ low secretion of nonpolar compounds by selected bacteria. It further supported by increasing trend in metabolite recovery with nonpolar to polar solvent systems tested (Fig. 3.11). Hence, the most appropriate solvent ethyl acetate (EtOAc) was employed for mass scale extraction procedures *(Mass biosynthesis, recovery of EtOAc extracted metabolites; product yield and purification for individual PIGB are explained in detail in consequent Chapter no. 4).

3.3.3. Quantitative antioxidant properties of PIGB

Antioxidant investigations on metabolites from living organisms involve determinations of both enzymatic and non-enzymatic activities. The non-enzymatic determinations are necessary to estimate the radical neutralization strength and the enzymatic studies give an insight of endogenous mechanisms where the activation of various physiological reactions plays an important role in conquering stress conditions (Halliwell and Gutteridge 2006).

3.3.3.1. Non-enzymatic AO activities of PIGB

A) DPPH scavenging activity

The ability of PIGB extracts to scavenge the DPPH free radicals assessed in comparison with known synthetic antioxidant BHT summarized in Table 3.4A and 3.4B. All the tested samples showed elevating AO activity with increased sample concentrations. *P. koreensis* exhibited 23.6 ± 2.32% of scavenging activities for 0.1 mg ml^{-1} of sample extract and reached to 95.6 ± 5.61% when the concentrations increased to 15 mg ml^{-1}.

S. rubidaea and *P. argentinensis* exhibited 57.5 ± 3.03 and 28.7 ± 0.92% of DPPH scavenging at highest sample concentration. Radical scavenging effects by strain PIGB163 at 0.1 to 15 mg ml^{-1} of sample extract were in the order of 22.88 ± 0.86 to 94.8 ± 0.29 % respectively (Table 3.4A). The other potent strain PIGB 184 though had little lower activities than PIGB 163, it found to increase the scavenging activity in relation to increased sample concentration.

On comparing EC_{50} concentrations by selected strains, the PIGB 163 noticed to impart efficient activity at the lowest value of 1.82 ± 0.09 mg ml^{-1}. It stood the most efficient strain in terms of DPPH radical scavenging property. At second level, this trend was followed by PIGB 77 (EC_{50} = 2.56 ± 0.10 mg ml^{-1}) and 184 (EC_{50} = 3.78 ± 0.13 mg ml^{-1}) whereas the PIGB 46 and 88 took higher sample concentrations to scavenge DPPH radicals at efficient way. From the above results it is highlighted that pink, brown and yellow coloured pigments have good role in scavenging free radicals.

DPPH activity is a quantification of the reactivity where the decrease in absorbance by sample indicates antioxidant progress. Purified bacterial pigments from *Pseudomonas* sp. are reported to scavenge 40-98% of DPPH radicals at 0.1 mg sample

concentration (Kumar et al. 2014). The crude pigmented metabolites of our strain *Pseudomonas koreensis* (PIGB 77) could scavenge around 23% of DPPH radicals at 0.1 mg sample and rose up to 93.4% with 10 mg concentration. The strain PIGB 77 may be a better option to yield high scavenging after purification.

Considering antioxidants of *Serratia* sp., a recent study by Nongkhlaw and Joshi (2015) demonstrated a plant associated strain *S. marcescens* requires 17 mg ml^{-1} of the compound to reach its DPPH EC_{50} value. In contrast, our seaweed associated strain *S. rubidaea* (PIGB 88) took only 10.6 mg ml^{-1} (EC_{50}) extract to show a similar effect. It emphasizes the greater AO potential of marine strains as compared to that of terrestrial ones. Seaweed associated bacteria *Serratia* and *Vibrio* sp. evidenced to possess DPPH EC_{50} values at 1 mg ml^{-1} (Horta et al. 2014). Our strains *S. nematodiphila* and *Vibrio* sp. also evidenced EC_{50} more or less close to this values i.e. 1.82 to 3.78 mg ml^{-1}.

Observed scavenging effects can be attributed towards donation of hydrogen atoms by pigmented compounds that quenched the free radicals. Kahkonen et al. (1999) documented the same kind of quenching effects by the antioxidant sample. Pigment carotenoids of bacterial origin are well documented to possess DPPH scavenging properties (Shindo et al. 2008; 2014). Conversely, the pigments and related bioactive's from marine *Serratia* sp. (Kuo et al. 2011) and *Pseudomonas* (Ye et al. 2012) are also ascribed to exhibit AO activities. Thus, as we employed extracellular pigmentary extracts in this test, the overall DPPH scavenging attributed towards the combined radical quenching by pigments and associated metabolites secreted by the potent bacteria. Moreover, the significant correlation with reducing agents like phenolic contents additionally supported our view on radical scavenging by marine PIGB.

Table 3.4. (A) Antioxidant activities of pigmented bacterial extract against DPPH and ABTS radical scavenging. Values are mean ± SD (n=3).

Test organism	Sample conc. (mg ml^{-1})	DPPH Scavenging assay (%)	EC_{50} (mg ml^{-1})	ABTS scavenging assay (%)	EC_{50} (mg ml^{-1})
P. argentinensis (PIGB 46)	0.1	5.75 ± 2.30	29.55 ± 1.97	0.4 ± 0.1	47.0 ± 3.38
	0.5	8.05 ± 1.15		1.4 ± 0.5	
	1.0	10.9 ± 0.28		1.8 ± 0.01	
	5.0	13.7 ± 0.39		4.7 ± 0.01	
	10	22.4 ± 0.33		8.4 ± 0.03	
	15	28.7 ± 0.92		15.7 ± 0.22	
P. koreensis (PIGB 77)	0.1	23.6 ± 2.32	2.56 ± 0.10	6.8 ± 0.6	9.73 ± 0.06
	0.5	37.7 ± 1.35		10.6 ± 0.1	
	1.0	47.3 ± 0.77		19.2 ± 0.05	
	5.0	76.1 ± 2.61		38.9 ± 1.98	
	10	93.4 ± 6.54		53.4 ± 2.75	
	15	95.6 ± 5.61		69.5 ± 1.00	
S. rubidaea (PIGB 46)	0.1	07.5 ± 1.80	10.60 ± 0.26	4.2 ± 0.89	67.02 ± 4.64
	0.5	13.6 ± 2.31		4.8 ± 0.52	
	1.0	18.0 ± 0.17		7.2 ± 0.03	
	5.0	35.5 ± 0.32		9.3 ± 0.03	
	10	53.0 ± 0.56		11.3 ± 0.03	
	15	57.5 ± 3.03		15.2 ± 0.09	
S. nematodiphila (PIGB 163)	0.1	22.9 ± 0.86	1.82 ± 0.09	25.1 ± 0.70	0.38 ± 0.01
	0.5	41.7 ± 0.85		76.4 ± 0.57	
	1.0	56.9 ± 0.27		90.2 ± 0.73	
	5.0	78.7 ± 2.76		95.5 ± 0.15	
	10	92.3 ± 1.17		96.7 ± 0.17	
	15	94.8 ± 0.29		98.7 ± 0.08	
Vibrio sp. (PIGB 184)	0.1	20.5 ± 1.32	3.78 ± 0.13	25.3 ± 0.42	0.36 ± 0.02
	0.5	34.5 ± 1.06		68.7 ± 0.92	
	1.0	54.1 ± 0.51		90.9 ± 0.42	
	5.0	62.4 ± 0.92		96.7 ± 0.17	
	10	76.8 ± 0.94		98.1 ± 0.15	
	15	83.1 ± 0.27		98.9 ± 0.14	

Table 3.4. (B) Antioxidant activities of known commercial synthetic antioxidants.

BHT Conc. (µg ml^{-1})	DPPH scavenging assay (%)	EC_{50} (µg ml^{-1})	Quercetin Conc. (µM ml^{-1})	ABTS scavenging assay (%)	EC_{50} (µM ml^{-1})
40	56.6 ± 1.63		1.0	78.0 ± 1.14	
80	80.3 ± 0.61	24.33 ± 1.82	5.0	97.6 ± 1. 25	5.74 ± 1.26
120	82.8 ± 0.72		10	99.6 ± 1. 18	
160	84.3 ± 1.34		15	99.5 ± 1. 06	

B) Total antioxidant power: ABTS assay

Estimation of total antioxidant powers of pigmented bacterial extracts demonstrated significant dose-dependent quenching of $ABTS^{\cdot+}$ radicals (Table 3.4A and 3.4B). Metabolites of PIGB 163 and 184 exhibited highest antiradical effects (~98.9%) with the lowest amount of sample (EC_{50}: 0.36-0.38 mg ml^{-1}) among all the tested bacteria. These activities were comparable to 1 µM of standard pure commercial AO pigment quercetin (88.75%).

P. argentinensis (PIGB 46) and *S. rubidaea* (PIGB 88) exhibited poor ABTS scavenging activity 0.4 to 15.7 % and 4.2 to 15.2 % at 1 to 15 mg ml^{-1} of sample concentrations respectively. These two strains though had different EC_{50} values they were able to exhibit near about same ABTS scavenging effects. Notably, the potent strain *P. koreensis* which had considerably prominent activities towards DPPH scavenging (95.6 ± 5.61%; EC_{50} 2.56 ± 0.10 mg ml^{-1}) showed intermediate responses towards ABTS scavenging (69.5 %; EC_{50} 9.73 ± 0.06 mg ml^{-1}). Most of the samples tested with higher concentrations i.e. 10 and 15 mg ml^{-1} were recorded with prominent and saturated AO effect towards quenching of free radicals in the system.

Determination of total antioxidant capacity is a prime requisite measure to know the efficacy of natural or synthetic AO compounds that are intimated for therapeutic applications. Dua et al. (2014) recently studied Indigo pigment from *Pseudomonas* sp. HAV-1 for its ABTS quenching and showed that the activity of this pigment is equivalent to 2.2 µM of standard ascorbic acid. This study did not intend to compare the observed ABTS effects concerning the non-pigmented standard like ascorbic acid. Instead, to have a more appropriate interpretation of results we assessed the ABTS activities of pigmented extracts exclusively in comparison with standard pigment (quercetin). In context, results presented in Table 3.4A and 3.4B highlighted the brown (*P. koreensis*) pigmented metabolites to impart equimolar ABTS obstruction to standard pigment quercetin (EC_{50} 5.74 ± 1.26 µM ml^{-1}). The pink (*S. nematodiphila*) and yellow (*Vibrio* sp.) even had more prominent ABTS scavenging (EC_{50} 0.38 ± 0.1 µM ml^{-1}).

Literature with reference to ABTS scavenging potentials on pigmented bacteria like *Serratia* and *Vibrio* sp. is scarce. However, our strains *S. nematodiphila* and *Vibrio* sp. showed 90.2-90.9% of ABTS scavenging at 1.0 mg ml^{-1} concentration. Also, as mentioned in result section some of our strains observed to show unambiguous variation among ABTS and DPPH scavenging effects. For example *S. rubidaea* extracts showed increased DPPH and decreased ABTS scavenging whereas, *P. koreensis* showed strong antiradical effects towards both DPPH and ABTS radicals. Similarly, radical scavenging of PIGB 184 had high DPPH EC_{50} values (3.78 ± 0.13 mg ml^{-1}) as compared to that of ABTS total antioxidant power (0.36 ± 0.02 mg ml^{-1}). An early study by Wang et al. (1998) has evidenced that the compounds showing high ABTS activity may not exhibit similar DPPH activity and vice versa. Hence, variations occurred in DPPH and ABTS results may be in relation to biochemical properties of a compound excreted by an individual bacterium.

Various factors like stereoselectivity of radicals, the presence of functional groups in bioactive compounds, the solubility of the extract and solvent polarity are reported to demonstrate the overall radical scavenging effect (Yu et al. 2002). Efficient scavenging of such proton radicals is an important attribute of natural antioxidants. ABTS radical is known to be reactive towards most of the natural antioxidants including phenolics, thiols and vitamin C (Walker et al. 2009). Thus, the efficient ABTS radical scavenging by selected species of marine bacteria and its significant correlation with phenolic contents (r^2= 0.790–0.986) indicates the possible existence of relatable bioactive's in pigmentary extracts.

Furthermore, looking at the considerable contribution of the present study, the bacterial pigments observed to exhibit comparable ABTS scavenging properties over the standard antioxidant quercetin. The pigment quercetin (a plant-derived flavonoid) is being used as a dietary supplement in food and beverage items due to its significant antioxidant and anti-inflammatory properties (Martin and Appel 2010). Our marine bacterial pigments possessing excellent anti-radical powers would be used as an antioxidant rich natural substitute for similar applications in human health.

C) Reducing power assay

The reducing capacities of pigmented extracts are represented in Fig. 3.12. *P. koreensis* showed prominent reducing activity 0.65 ± 0.04 at 0.1 mg ml^{-1} sample concentration. RP value has increased to 3.19 ± 0.01 when we increased the sample concentration to 15 mg ml^{-1}. This activity was found to be much higher than that of pure standard compound i.e. ascorbic acid (160 µg ml^{-1}). The other two strains (PIGB 46 and 88) tested had a similar

trend in their RP and sample concentrations. The rise in absorbance directly indicates more reducing power of a particular compound. This property is based on the presence of antioxidant compounds with reducing power (Duh et al. 1999; Ye et al. 2012).

Pigmented metabolites of marine *Pseudomonas* sp. have been recorded to show increased RP values against increased sample concentration. Ye et al. (2012) obtained the RP value of 0.5 with a sample concentration of 1.5 mg. In our case the strain PIGB 77 contributed the RP value of 1.31 with a sample concentration of 1.0 mg. This shows the supremacy of PIGB 77 while comparing with the earlier work.

Conversely, the other strain *Pseudomonas* PIGB 46 needs a concentration of 10 mg ml^{-1} to reach the OD of 0.5. These variations in RP observed from two different *Pseudomonas* species in the present study suggest that the RP values mainly depend on the constituents present in the pigments. These results can be interpreted such a way that the two pigments produced by *Pseudomonas* species may not be the same.

At the molecular level, active metabolites present in sample carry out various reactions to contribute the overall RP effect. This includes obstruction of free radical chain reactions, decomposition of the peroxides and radical scavenging (Diplock 1997). Investigation on AO potential of eukaryotic organisms e.g. *Sargassum* sp. at 10 mg ml^{-1} have been reported to exhibit highest reducing potential (Cho et al. 2007) and until now there are no reports on associated bacteria which can yield similar results. Interestingly, one of our pigmented bacterium *P. koreensis* (PIGB 77) obtained from *Sargassum* surface showed greater reducing potential at a sample concentration of 5 mg ml^{-1}. This result suggests that reducing capabilities of a sample are enhanced by the associated bacteria and could be used as an alternative source for antioxidative compounds.

Similarly, Kuo et al (2011) explored *Serratia ureilytica* and demonstrated that this pink coloured strain to exhibit a linear trend between sample concentration and RP activity. Our strains *S. rubidaea* and *S. nematodiphila* during the current investigation also behave in a similar fashion.

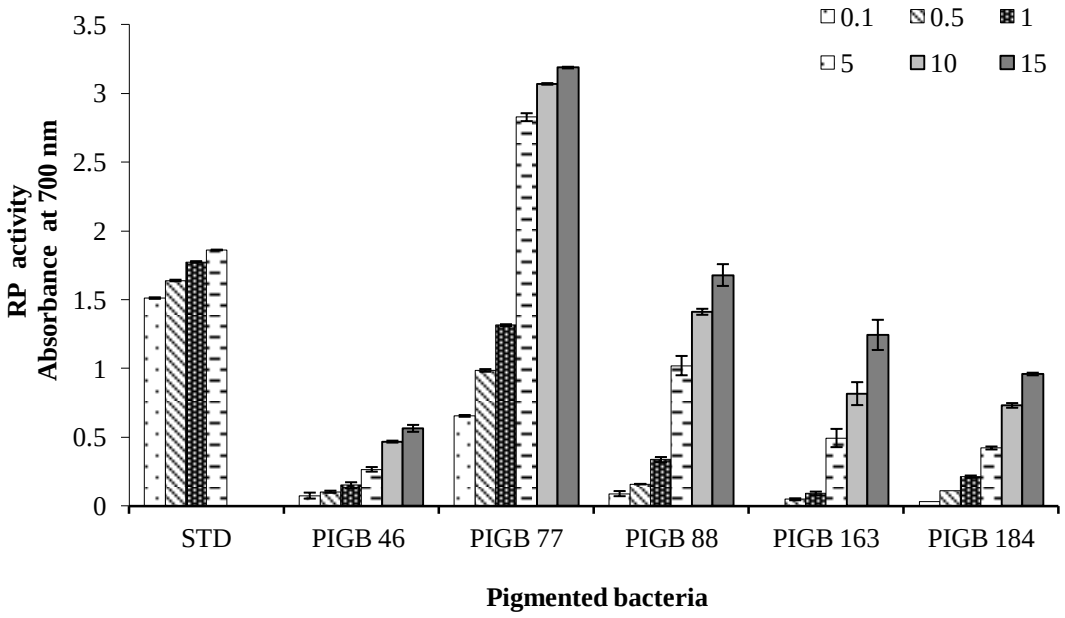


Fig. 3.12. Reducing potential of pigmented bacteria.

Values are mean ± SD (n=3). PIGB 46: *P. argentinensis*, PIGB 77: *P. koreensis*, PIGB 88: *S. rubidaea*, PIGB 163: *S. nematodiphila* and 184: *Vibrio* sp. Samples concentrations = mg ml^{-1}; Standard (STD) concentrations = 40, 80, 120 and 160 µg ml^{-1} ascorbic acid.

D) FRAP assay

The ferric reducing antioxidant power of pigmentary extracts assessed in terms of their Fe^{3+} to Fe^{2+} transition ability and results are summarized in Table 3.5. Increasing orders of FRAP values (i.e. µg AsA Eq.) were observed in *P. koreensis* (PIGB 77) extracts from

0.57 for 0.1 mg to 47.01 for 15 mg of sample respectively. *S. nematodiphila* (PIGB 163) witnessed significant FRAP activity (55.48 ± 7.05 µg ml^{-1} AsA Eq.) while PIGB 184 gave maximum up to 41.11 ± 0.17 µg ml^{-1} AsA Eqs. at highest sample concentrations (15 mg ml^{-1}).

P. argentinensis (PIGB 46) with similar exponential results had an intermediate level of FRAP powers. *S. rubidaea* (PIGB 88) expressed low levels of antioxidant equivalents in comparison with all the other strains studied. Significant correlations were found with FRAP the phenolic compounds. Looking at the maximum sample concentration (15 mg ml^{-1}) strains PIGB 163, 184 and 77 expressed considerable amounts of FRAP powers.

A recent investigation on bacterial metabolites (indigo pigment) obtained from a soil isolate *Pseudomonas* sp. is witnessed to exhibit ferric reducing antioxidant powers (Dua et al. 2014). FRAP results on *Pseudomonas* strain (PIGB 77) with yellow-brown pigments in the present study also contributed similar activities. This emphasizes that the pigmentary metabolites irrespective of colour property have the tendency to act on ferric compounds.

Likewise, FRAP estimations on *S. marcescens* (terrestrial source) is recorded to produce a maximum of 22.05 µg of AsA antioxidant equivalents (Gulani et al. 2012). In the current study, the organism belonging to *Serratia* genera i.e. PIGB 163 (marine source) is observed to excrete more amount of AsA equivalents (26.70 ± 0.70). It shows that the biological activities may differ from species to species and are affected by the environment in which the organism resides and or retrieved.

Table 3.5. FRAP and total phenolic contents from the pigmented bacterial extract of the selected strain. Values are mean ± SD (n=3).

Sample	Sample Conc. (mg ml^{-1})	Antioxidant contents	
		FRAP (µg ml^{-1} AsA Eq.)	TPC (mg ml^{-1} GAE)
P. argentinensis (PIGB 46)	0.1	0.27 ± 0.02	0.06 ± 0.00
	0.5	4.34 ± 0.18	0.07 ± 0.00
	1.0	7.97 ± 0.12	0.09 ± 0.01
	5.0	10.80 ± 0.13	0.17 ± 0.01
	10	14.11 ± 2.3	0.24 ± 0.02
	15	18.70 ± 1.84	0.31 ± 0.00
P. koreensis (PIGB 77)	0.1	0.57 ± 0.02	0.07 ± 0.00
	0.5	2.06 ± 0.17	0.12 ± 0.00
	1.0	5.90 ± 0.13	0.21 ± 0.01
	5.0	23.26 ± 4.96	0.34 ± 0.02
	10	35.17 ± 0.36	0.65 ± 0.02
	15	47.01 ± 0.27	0.83 ± 0.05
S. rubidaea (PIGB 88)	0.1	0.27 ± 0.01	0.05 ± 0.00
	0.5	0.64 ± 0.02	0.07 ± 0.00
	1.0	1.81 ± 0.01	0.08 ± 0.01
	5.0	4.76 ± 0.05	0.21 ± 0.01
	10	7.46 ± 0.12	0.32 ± 0.01
	15	9.35 ± 0.98	0.48 ± 0.07
S. nematodiphila (PIGB 163)	0.1	1.20 ± 0.07	0.01 ± 0.00
	0.5	10.90 ± 0.79	0.023 ± 0.002
	1.0	26.70 ± 0.70	0.052 ± 0.002
	5.0	35.65 ± 3.79	0.25 ± 0.005
	10	45.26 ± 1.68	0.32 ± 0.01
	15	55.48 ± 7.05	0.49 ± 0.01
Vibrio sp. (PIGB 184)	0.1	0.39 ± 0.08	0.004 ± 0.001
	0.5	9.22 ± 0.24	0.035 ± 0.005
	1.0	22.14 ± 1.21	0.059 ± 0.002
	5.0	29.82 ± 0.36	0.27 ± 0.001
	10	36.43 ± 4.98	0.32 ± 0.001
	15	41.11 ± 0.17	0.41 ± 0.001

E) Total phenolic contents in pigmentary extracts

Phenolic compounds in extracellular extracts obtained from pigmented bacteria are estimated in terms of gallic acid equivalents (GAE) and summarized in Table 3.5. All the strains in this study were observed to express increasing levels of TPCs with increasing sample concentrations. Quantified GAE values indicated high secretions of phenolic compounds from brown coloured *Pseudomonas* (PIGB 77) followed by pink *Serratia* sp. (PIGB 88 and 163) and yellow coloured *P. argentinensis* (PIGB 46) and *Vibrio* sp. (PIGB 184). Interestingly, extracts of two *Serratia* species had near about equal quantity of phenolics i.e. ~0.49 mg ml^{-1} GAE's at highest sample concentrations tested (15 mg ml-1). The yellow pigmented extracts from PIGB 46 and 184 expressed dose-dependent manner in the production phenolic compounds.

Plants are the known source of phenolic and flavonoid compounds. However, till now limited amount of information is available on their presence in bacteria. The current investigation on marine bacteria shows a positive indication of expressing significant levels of phenolic metabolites that can be directly proportional to the pigment content in the sample extracts. Polyphenols, carotenoids and their derivatives appear to function as good electron and hydrogen donors and therefore, terminate radical chain reaction by converting FR to more stable or neutral products (Shahidi and Wanasundara 1992).

Horta et al. (2014) studied antioxidants from marine bacteria demonstrated the radical scavenging role of *Vibrio* sp. In order to find the possible reason behind the AO activity they quantified and ascribed the radical scavenging effects towards phenolic metabolites secreted by this species. PIGB184 in the current study also showed a dose-dependent production of phenolic compounds with concurrent AO activity. This result

further supported by our recent reports on marine PIGB wherein the phenolics had a significant contribution towards antioxidant effect (Pawar et al. 2015; 2016).

Kuo et al. (2011) assessed *S. ureilytica* extracts for possible antioxidant compounds and concluded that the fraction of phenolic substance gives higher activities than those with deprived ones. Further, they utilized squid pen fermented supernatant of this strain and retrieved 1.7 mg ml^{-1} GAE phenolics. In contrast, Wang et al. (2010) when utilized culture supernatant of the same *S. ureilytica* strain but grown in shrimp shell substrates noted the lower level of phenolic expressions (0.196 mg ml^{-1} GAE). Our *Serratia* strains PIGB 88 and 163 grown in marine broth expressed comparable levels of phenolics i.e. 0.49 mg ml^{-1} GAE. These results indicate the use of different growth substrates induces the secretion of active metabolites including phenolics or pigments.

F) Relationship between antioxidant parameters and phenolic contents

Results of correlation analysis among AO tests and total phenolic contents gave linear curves with significant R^2 values (Table 3.6). Parameter wise relation on pigmented bacterial species PIGB 46 (*P. argentinensis*), 163 (*S. nematodiphila*) and 184 (*Vibrio* sp.) indicate the phenolic metabolites to have a prominent contribution towards DPPH activity. In contrast, the strain PIGB 77 had a reverse trend, it's phenolic attributions which supported ABTS activity more than DPPH.

Similarly, in case of PIGB 163 and PIGB 184 the R^2 were significant towards AO test RP (0.9938 and 0.9768) followed by FRAP (0.9698 and 0.9971), DPPH (0.973 and 0.978) and ABTS (0.9861 and 0.8449). The PIGB 184 stood an outstanding strain as it demonstrated the most linear relation against all the assessed AO activities ($R^2 \geq 0.969 – 0.994$; $p < 0.01$).

As discussed in the earlier section, the phenolic compounds are well documented for their powerful radical scavenging properties. Koren et al. (2009) demonstrated bacteria when coated with polyphenols acquire potent oxidant scavenging powers and helps the host cells to withstand against oxidative cell damage. Likewise, recently the phenolic compounds have been strongly correlated with the antioxidant potential of marine bacteria (Horta et al. 2014; Pawar et al. 2015, 2016).

Significant productions of phenolics and their correlation with AO parameters in the present report highlight the protective function of bacterial pigmentary metabolites towards obstruction of free radical reactions. Also, this forms a first ever report that correlates 'pigmented bacterial phenolics' towards their 'antioxidant response' from a marine source.

Table 3.6. Relation of total phenolic contents and antioxidant parameters.

Antioxidant parameters	Pigmented bacteria (PIGB)				
	PIGB 46	PIGB 77	PIGB 88	PIGB 163	PIGB 184
DPPH	0.976	0.741	0.855	0.978	0.973
ABTS	0.872	0.964	0.790	0.845	0.986
RP	0.979	0.725	0.943	0.976	0.994
FRAP	0.971	0.962	0.975	0.997	0.969

Values demonstrated represents correlation coefficient R^2. The analysis was considered significant at $p < 0.01$.

3.3.3.2. Enzymatic activities of PIGB

A) Bacterial sensitivity and survival under stress

Pigmented bacteria exposed to different concentrations of chemical stressor at initial step demonstrated isolate wise variable sensitivity towards hydrogen peroxide. Pink coloured bacteria PIGB 163 and 88 had the highest stability and were able to grow up to 48 mM of H_2O_2 in the growth medium. Notably, the brown (PIGB77) and yellow (PIGB 46 and 184) chromogens were found to be very sensitive even to the minimum concentration 2-4 mM.

The growth of selected bacteria in presence and absence of oxidative stressor H_2O_2 was investigated and summarized in Fig. 3.13. All pigmented bacteria had suppressed growth under exposure to H_2O_2. PIGB 88 and 163 was not much affected by the chemical stressor as they had only ~0.2 OD difference among treated and control cells. Isolates PIGB 46, 77 and 184 were more sensitive and evidenced with suppressed growth curve with prolonged lag phase up to 15 h of incubation period. Furthermore, these three isolates had more than 0.6 OD difference in exponential phase among treated and untreated samples.

Hydrogen peroxide is an oxidizing agent. When bacteria come in contact with hydrogen peroxide it oxidizes the cell membrane proteins, lipids and nucleic acids. Thus it affects the normal physiology, growth and multiplication which consequently results in suppressed growth of bacteria. For example *Pseudomonas, Bacillus, Escherichia coli* and *Salmonella* sp. exposed to various anthropogens have been evidenced to show reduced growth rates as compared to unexposed bacteria (Kang et al. 2007; Nur et al. 2014). Also, Zhang et al. (2012) and Peters et al. (2014) observed that bacteria under stress remain undivided for a long time before entering into exponential phase. We observed similar trend among the growth of pigmented bacteria under exposure to hydrogen peroxide. Here, almost all the strains remained in lag phase up to first 10-15 h.

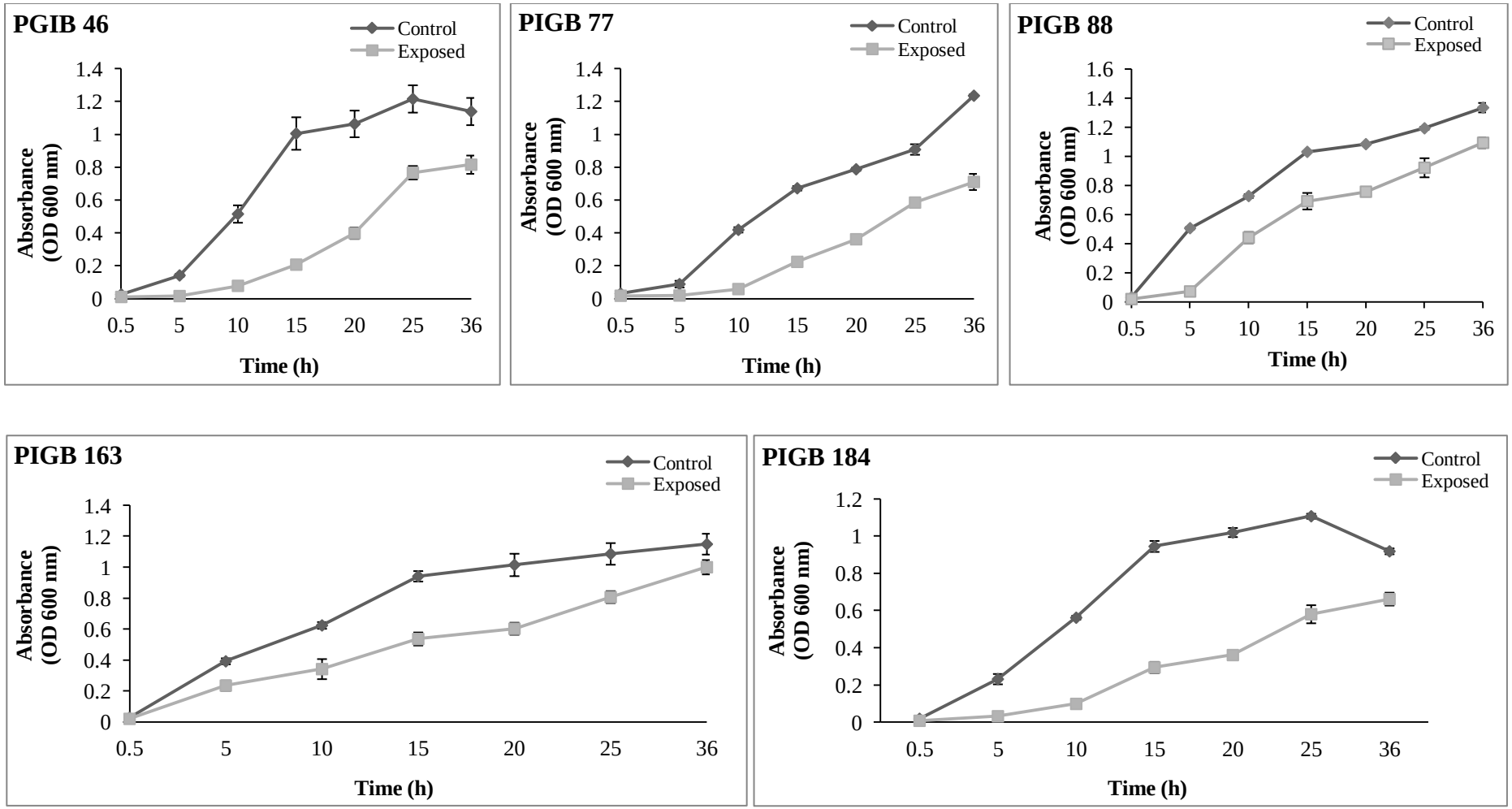


Fig. 3.13. Growth curves of pigmented bacteria in presence and absence of stressor hydrogen peroxide.

The higher tolerance of pink pigmented *Serratia* species (PIGB 88 and 163) towards elevated concentrations of hydrogen peroxide in the present study can be attributed towards their endogenous biochemical and physiological properties. Yamazaki et al. (2006) studied the effect of increased salt concentrations on *Serratia* strain *S. rubidaea* and noted that this bacterium can tolerate higher salt concentrations up to 2 Mm. Likewise, *Serratia* retrieved from sludge containing H_2O_2 is also reported to survive under high-stress conditions (Zeng et al. 2011). These evidences indicate that bacteria belonging to genera *Serratia* are naturally stress tolerant. This might be the reason for high tolerance of our *Serratia* sp. towards H_2O_2.

B. Enzymatic response of PIGB towards oxidative stress

Catalase activity

Catalase activities measured in pigmented bacteria exposed to hydrogen peroxide are shown in Fig. 3.14. Grown cells had higher activity than the growing cells. Bacterial strains PIGB 88 and 163 showed 93.1 and 97.6 U catalase activity respectively. Among the marine pigmented bacteria it is noted that PIGB 184 (*Vibrio* sp.) may produce maximum catalase (115.7 U) in the grown cells. Exceptionally, the catalase activity in PIGB 46 was found more in growing cells than in the grown cells.

Pseudomonas sp. strains As1 and HF-1 exposed to nicotine and naphthalene were reported to express elevated activity of the enzyme catalase during biodegradation processes (Kang et al. 2007; Shao et al. 2009). The *Pseudomonas* sp. As1 is evidenced to show up to 25-40 U of CAT activity per mg of protein. In comparison, our strains belonging to the same genera exposed to H_2O_2 had a concurrent trend in their CAT

productions and were observed to produce 40.2 - 52.5 U catalase. Conversely, the variable CAT response in case of yellow bacterial isolates (PIGB 46 and 184) with reference to growing and control organisms remains unpredicted. This needs much more inclusive studies to conclude the actual response by employing different concentrations of stressor to have a fitted trend in their activities.

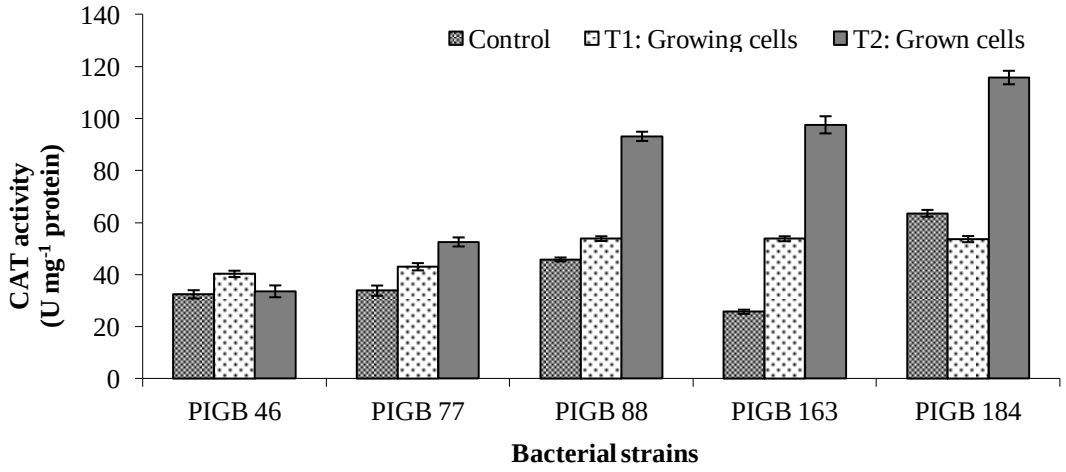


Fig. 3.14. Catalase activity in pigmented bacteria exposed to hydrogen peroxide.

The experimental arms T1 and T2 in Fig. 3.14 represent the production of catalase in growing and grown cells respectively. Most of the grown cells had higher activity (52.5-115.7 U) than the growing cells (46-53.8 U). This might be due to the innate capability of the organisms to produce the catalase enzyme as a defensive mechanism to withstand the stress. Extracts obtained from a mesophilic strain *Serratia marcescens* is evidenced to exhibit novel catalase with 745.7 U activity per mg of protein (Zeng et al. 2010). Also, the active secretion of catalase by *Serratia* sp. has been supported by earlier findings of Williams and Sessums (1959). The lower CAT activity by *Serratia* strains in the present study could not be understand at this stage unless a detailed observations made with various other parameters.

Likewise, Yumoto and his colleagues (1998) isolated a *Vibrio* strain from drain pool effluent containing high concentraitons of H_2O_2 and revealed that this bacterium

express extraordinary CAT activity (7276 U mg^{-1} protein). Biochemical estimations on selected bacteria also showed a maximum production of catalase (115.7 U) by *Vibrio* sp. (PIGB 184). However, the variations in catalase activity here can be attributed towards the concentration of stressor to which the organism exposed.

Superoxide dismutase activity

Effect of an oxidizing stressor on induction of AO enzyme SOD in marine pigmented bacteria summarized in Fig. 3.15. Endogenous cell extracts obtained from PIGB 88, 163 and 184 showed prominent differences among SOD activities similar to CAT response. These isolates under stress expressed elevated SOD (growing cells: 126.8 - 227.8 U; grown cells: 227.6 - 476.3 U) as compared to that of control (107.4-184.7 U) organisms. PIGB 46 exposed to stress during growth evidenced with lowered activities whereas the grown cells had significantly increased 4 fold higher SOD values. PIGB 77 had least productions of the enzyme SOD. The SOD production was directly proportional to the number of bacteria in the current study. In T1 arm the bacteria are exposed to hydrogen peroxide thus by the growth of the bacteria and production of SOD is limited. In T2 arm the bacterial cultures allowed to grow more and then exposed to the stressor that resulted in enhanced SOD production.

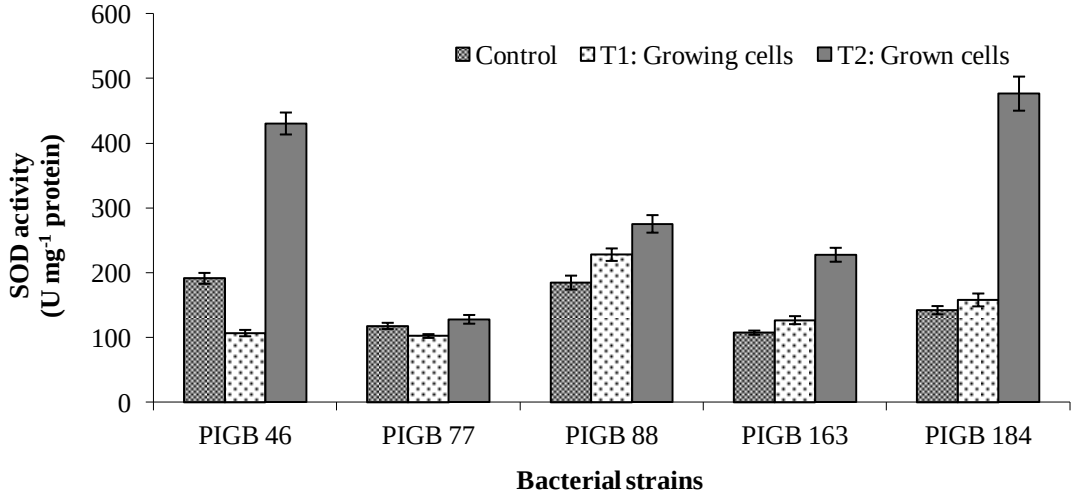


Fig. 3.15. SOD activity in pigmented bacteria exposed to hydrogen peroxide.

SOD activity studies on bacteria like *Pseudomonas*, *E. coli* and *Bacillus* sp. exposed to anthropogenic stressors reported ~50% elevated production of AO enzymes (Shao et al. 2009; Zhang et al. 2012). Likewise, the direct analysis of expressed SOD enzyme from naphthalene exposed *Pseudomonas* species have also been reported increased activity response in stressed cells (Kang et al. 2007). One of our *Pseudomonas* strain PIGB 46 had consistent effect with earlier reports where the production of stress enzyme SOD increased 1-5 fold under stress.

An early study on cell extract acquired from *S. marcescens* reported 90 U SOD activity per mg of protein (Maejima et al. 1983). Our pink isolates *S. rubidaea* (PIGB 88) and *S. nematodiphila* (PIGB 163) showed 2-fold activities (227.6-275.3 U mg^{-1} protein) when exposed to chemical stressor hydrogen peroxide. Kim et al. (2005) studied acid tolerance in *Vibrio vulnificus* with wild and recombinant type strains. They demonstrated maximized induction of cytosolic SOD in bacteria retrieved from stationary growth phase. The SOD response in our *Vibrio* sp. (PIGB 184) gave concomitant results, where the grown stationary phase culture also had higher activity. These results suggest bacteria that are already grown and competent may express more amount of stress enzyme.

GST activity

Effect of hydrogen peroxide on activation of stress enzyme GST tested in two different sets of bacteria i.e. cell exposed right from initial growth (T1) and grown cells (T2) is depicted in Fig. 3.16. Surprisingly, bacteria that are grown for 12 h and exposed to stressor gave comparatively higher GST than growing cells. PIGB 46 (*Pseudomonas*) and 184 (*Vibrio*) strains responded significantly by expressing 136 - 409 U of GST mg^{-1} of protein. Pink chromogens PIGB 88 and 163 (*Serratia* sp.) had intermediate activities

whereas the PIGB 77 could not show much enzymatic response towards GST. Except PIGB 77, the GST results obtained for the remaining bacteria were either in equal or slightly lower than the control.

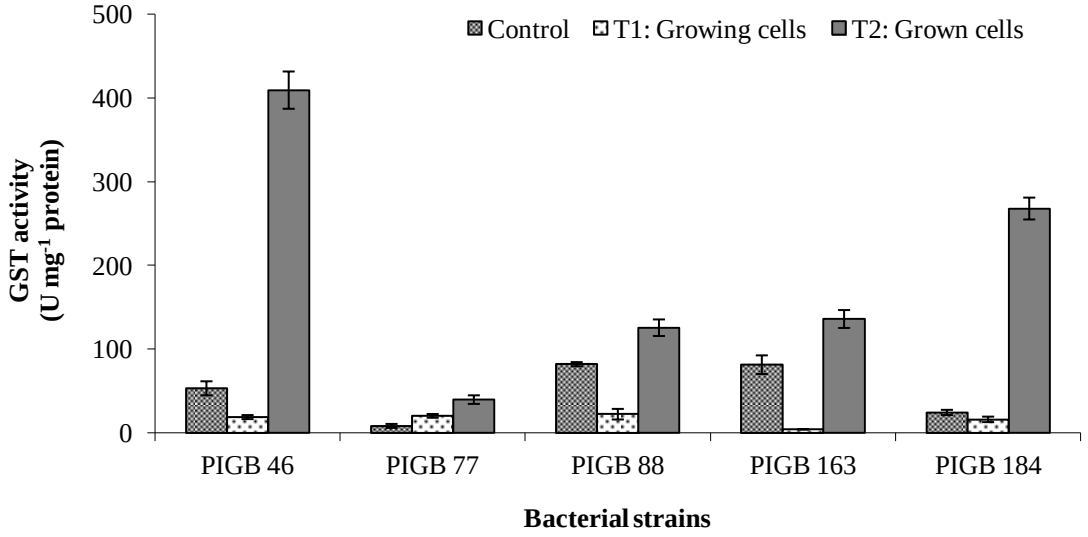


Fig. 3.16. GST activity in selected pigmented bacteria exposed to hydrogen peroxide.

Peters et al. (2014) explored two *Pseudomonas* strains (CC07 and 4C07) for differential stress response towards herbicides. They showed that GST antioxidant defense system helps the host to withstand and overcome the stress. A recent investigation on *Pseudomonas* species also showed that this bacterium can tolerate up to 6 mM of hydrogen peroxide (Nur et al. 2014). However, this group of researcher could not give any firm reason behind such tolerance. Our *Pseudomonas* strains though found to be sensitive to a lower dose (2 mM) of hydrogen peroxide one of them (PIGB 46) had significant productions of AO enzyme GST. This reveals the biochemical basis and the reason for their survival under stress conditions. *Pseudomonas* sp. is known to withstand diverse conditions including the exposure of anthropogenic stressors (Nur et al. 2014; Peters et al. 2014). In the present study the PIGB 46 also belongs to *Pseudomonas* sp. had more GST activity that could be related with the quantum of stress overcome by the organism.

Serratia sp. from terrestrial source has been explored for isolation of GST and evidenced to possess significant AO properties (Di Ilio et al. 1991). Two of our *Serratia* strains PIGB 88 and 163 also showed the production of GST with good AO properties. These results support the bacterial species *Serratia* to possess an endogenous defensive mechanism through enzymatic actions depending upon the necessity.

In this study, superior AO enzyme response in the grown cells as compared to growing cells suggests bacteria that are physiologically competent have the ability to overcome the stress condition quickly. Low activity in growing cells might be the causes of hampered cell division under the influence of chemical stress.

Overall observation on enzymatic AOs demonstrated that the bacteria exposed to H_2O_2 regulate the production of endogenous enzymes to overcome the toxicity. It points out that bacteria living in natural environments like marine ecosystems might produce AO enzymes to withstand the stress conditions.

Looking the application point of view the antioxidative enzymes are used in a variety of biotechnological process. The chimeric/fused AO enzyme like (CAT + GST) used in medicines and biosensor fabrications (Allocati et al. 2009). Catalases obtained from *Pseudomonas* species have been reported to provide protection to planktonic organisms against hydrogen peroxide and singlet oxygen species (Elkins et al. 1999). Similarly, the SOD obtained from *Serratia* sp. has been patented for its anti-inflammatory effect (P. No.: EP0172577A1). Considerable productions of GST, CAT and SOD as evidenced in our pigmented strains may be used for mass utilization and find similar applications in future.

3.3.4. Molecular and phylogenetic relatedness

3.3.4.1. 16S rRNA identity

The molecular (16S rRNA) gene sequences of five antioxidant potent pigmented bacteria assessed for their closest match using NCBI BLASTn database and obtained results are presented in Table 3.7. Among that two of them belonging to *Pseudomonas* (PIGB 46 and 77), one affiliated to *Vibrio* (PIGB 184) and the remaining are *Serratia* (PIGB 88 and 163). Interestingly all the above strains belong to the group Gammaproteobacteria.

Bacteria belonging Gammaproteobacteria group have been recently reported to exhibit excellent antioxidant activities. For example, Horta et al. (2014) and Pawar et al. (2015) independently studied seaweed associated marine bacteria from different coasts and demonstrated that more than 80% of AO active bacteria affiliates to Gammaproteobacteria. This information highlights the significance of Gammaproteobacteria group for its contributions towards AO activity.

3.3.4.2. Phylogenetic relatedness among AO bacteria

In a distinctive approach, we assessed the phylogenetic relatedness of our antioxidant potent pigmented strains with their closest genera that are reported for antioxidant productions (Fig. 3.17). The high potent bacterium PIGB 77 (*P. koreensis*) branched on a distinct node away from all the other *Pseudomonas* sp. with a significant Bootstrap value of 64%. The other *Pseudomonas* strain PIGB 46 (*P. argentinensis*; JX915781) did not show much variation in its sequence and had close similarity to *Pseudomonas* strains PF-6 (GU944677.1) and HAV-1 (JN172106.1).

Table 3.7. 16S rRNA identity for the high potent antioxidant PIGB strains.

Isolate	Closest match (NCBI accession number)	Accession number	Similarity	Phylogenetic group
PIGB 46	*Pseudomonas argentinensis* (JX915781)	JX915781	99%	Gammaproteobacteria
PIGB 77	*Pseudomonas koreensis* (JX915782)	JX915782	100%	Gammaproteobacteria
PIGB 88	*Serratia rubidaea* (JX915783)	JX915783	99%	Gammaproteobacteria
PIGB 163	*Serratia nematodiphila* (KJ396267)	KJ396267	99.85%	Gammaproteobacteria
PIGB 184	*Vibrio* sp. (KJ396268)	KJ396268	99.9%	Gammaproteobacteria

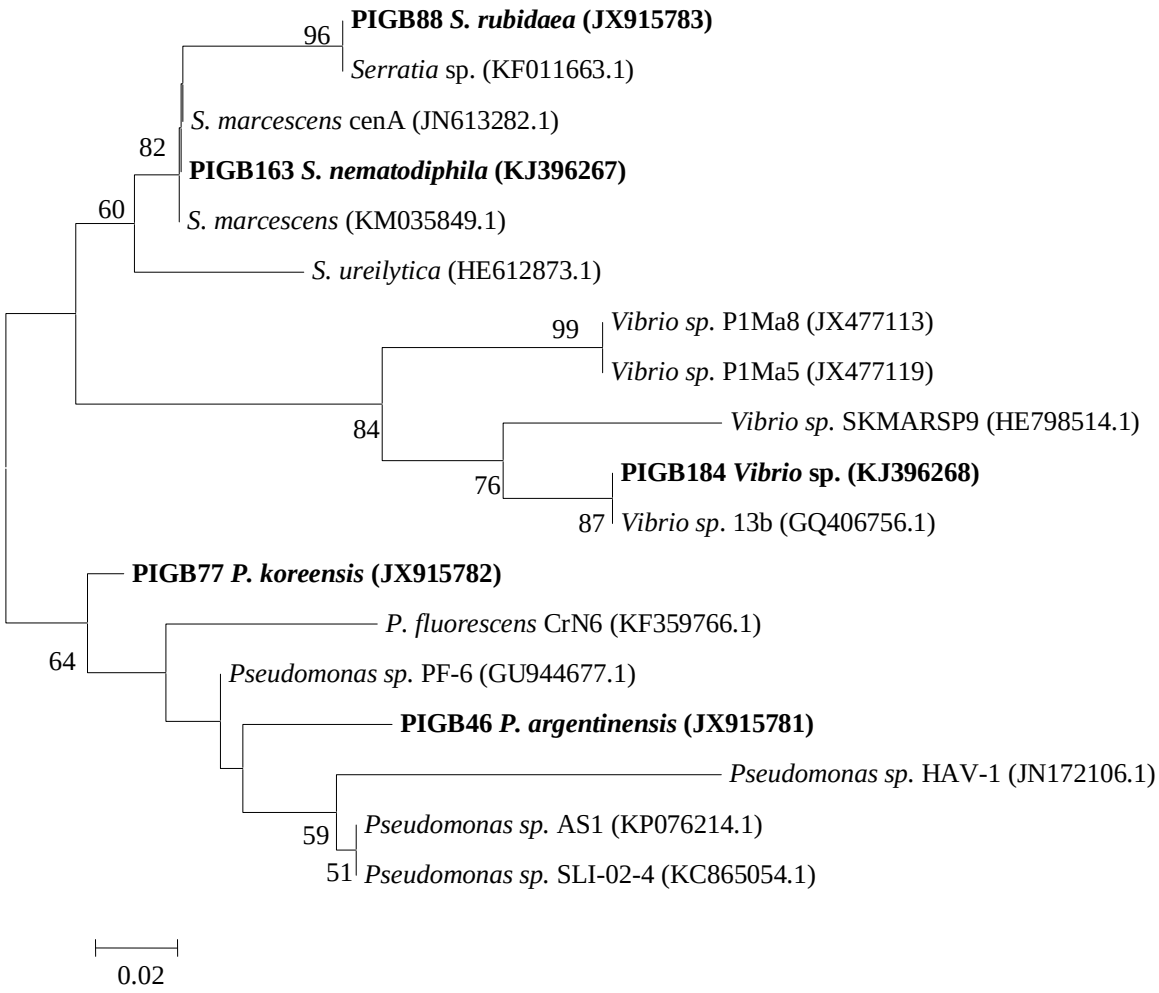


Fig. 3.17. Neighbour-joining phylogenetic tree based on 16S rRNA genes of pigmented strains with closely related antioxidant bacteria.

Bootstrap values ≥ 50 % are shown in the nodes.

P. koreensis (PIGB 77) branching on a separate node indicates that this bacterium has comparatively distinct genome sequence from the reported antioxidant species. This novel bacterium reported by Kwon et al. in the year 2003 has not yet much explored for its bioactive properties. First time this bacterium was isolated from seaweed surfaces and reported for its significant contribution towards antioxidant potential (Pawar et al. 2015).

Organisms belonging *Serratia* genera i.e. PIGB 88 (*S. rubidaea*) had close affiliations with other antioxidative strains *Serratia* sp. (Bhattarai et al. 2013). The PIGB 163 (*S. nematodiphila*) clustered closely with *S. marcescens* (Gulani et al. 2012; Nongkhlaw and Joshi 2015). This indicates both of our *Serratia* species though belonged same genera they formed different nodes due to their varied genome sequence. *S. nematodiphila* (PIGB163) and *S. marcescens* as characterized with close similarities they may excrete similar kind of metabolites e.g. pigments that responsible for AO activities. Since, our *Serratia* strains (PIGB 88 and 163) forms separate nodes from the recently reported AO terrestrial strain *S. ureilytica* (Kuo et al. 2011) it may expect to produce different AO metabolites.

Phylogenetic grouping on *Vibrio* sp. (PIGB 184) made us realize that this bacterium has very close similarity with other *Vibrio* sp. 13b (GQ406756.1) that has been recently retrieved with significant AO property by Horta et al. (2014). A similar biological and phylogenetic attribution on this particular species supports our earlier statement on the species-specific secretion of AO metabolites.

3.4. Conclusion

Investigation on AO potential of 190 pigmented bacterial isolates revealed 44.21% of the organisms were able to secrete active metabolites, in that 3.68% of the organisms were highly potent.

Optimization of process parameters like pH, temperature, media strength, carbon and nitrogen sources demonstrated that these parameters are not common with the tested organisms for pigment productions and AO activity. At the same time optimization of the above parameters on individual organisms could yield 2-3 fold increase in pigment production and ~20% enhanced AO activity.

Quantitative AO estimations of PIGB 77 gave good radical scavenging properties (95.6%) in comparison with synthetic BHT (84.3%). Strains PIGB 184 and PIGB 163 expressed high scavenging against ABTS (~98.9%) similar to that of standard pigmented compound quercetin (99.5%).

Molecular characterization and phylogenetic analysis interestingly showed all the antioxidant potent bacterial strains (PIGB 46, 77, 88, 163 and 184) belonging to a single group Gammaproteobacteria. It indicates the organisms belonging to this phylum would have better insights for prospective antioxidant search.

CHAPTER 4

STRUCTURAL ELUCIDATION OF ANTIOXIDANT MATERIALS

4.1. Introduction

Interest in renewable natural products mainly the antioxidant compounds is increasing the attention among the researchers in the present era. The radical scavenging property is one of the major concerns of antioxidant activity in human health (Halliwell 2012). However, the first step in the utilization of such putative therapeutic antioxidants involves isolation and characterization of metabolites from a natural source (Pawar et al. 2015). Knowing the chemical nature would give prospective insights of a biological compound for targeted drug delivery and other biotechnological applications.

The literature on the characterization of antioxidant compounds from marine bacteria demonstrates the use of various biochemical and molecular techniques. It includes fractionation of crude samples based on size and charge with column chromatography followed by spectrometric purifications and confirmation. For example, Shindo et al. (2014) characterized antioxidative carotenoid pigments from novel bacteria using series of chromatographic (partition, silica gel), high-performance liquid chromatography (HPLC), high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) and spectroscopic (UV-Vis, NMR) methods. Similarly, Balakrishnan et al. (2015) recently studied the possible antioxidant metabolites from sponge associated bacterium *Bacillus* sp. using high throughput GC-MS techniques. We intended to employ similar techniques for purification and structural elucidation of AO components from selected marine pigmented bacteria.

Conversely, the information of renewable antioxidants specifically of marine bacterial origin is limited (Horta et al. 2014). The characterization of related metabolites is not only expected to reveal the novel compounds but also will strengthen the

knowledge on such metabolites for their prospective utilization. To accomplish so, we assessed active metabolites from our potent antioxidant marine pigmented bacteria using various bio-analytical techniques. Results on nature of compounds produced by the individual bacterium and their chemical properties are discussed in details.

4.2. Materials and methods

4.2.1. Mass extraction and quantification of AO pigmented metabolites

Based on the primary AO screening (Chapter 3), an aliquot (100 µl pure culture) of selected strains PIGB 46, 77, 88, 163 and 184 was inoculated separately in four 2 liter (L) Erlenmeyer flasks containing 800 ml marine broth in each flask (total media inoculated per bacterium: 4 × 800 = 3.2 L). Culture flasks were incubated in a shaking incubator at optimized temperatures and incubation period for each bacterium (Chapter 3; Table 3.3). Later, the supernatant separation, extraction (ethyl acetate) and concentration were carried out as per the standard methods described elsewhere in details (Chapter 3; methodology section).

4.2.2. Purification of antioxidants from potent strains

Purification of antioxidative metabolites from selected strains was carried out by series of standard systematic steps as depicted in Fig. 4.1.

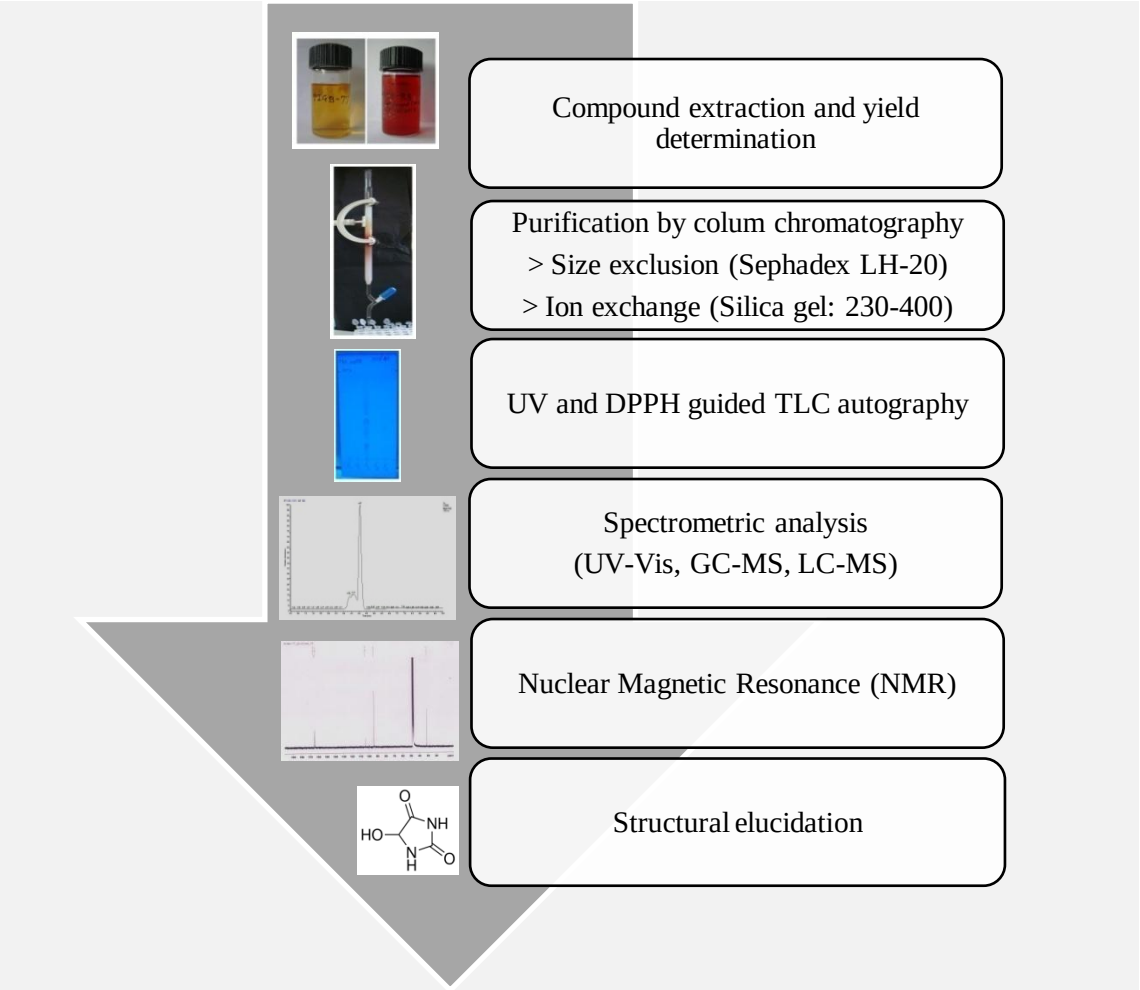


Fig. 4.1. Schematic representation of various methods employed for purification and characterization of antioxidant metabolites from selected pigmented bacteria.

4.2.2.1. Column chromatography

A. Size exclusion chromatography

The crude pigmentary extract (PIGB 46: 485.7 mg, PIGB 77: 743.4 mg, PIGB 88: 569.4, PIGB 163:864.2, PIGB 184: 227.3 mg) was primarily subjected to Sephadex-LH20 size exclusion chromatography (Sigma-Aldrich; 2.0 × 50 cm). Fractions were eluted using HPLC grade methanol at the rate of 0.5 ml min^{-1} in separate glass vials. Obtained

fractions were checked for antioxidant activity qualitatively as well as quantitatively (Explained in consecutive sections).

B. Ion exchange chromatography

The most potent fraction devised in earlier step was completely dried along with a little amount of silica using rotary vacuum concentrator. This step coated sample on silica beads and helped to retrieve maximum yield. Dried sample was then applied on top of the ion exchange silica gel (230-400 mesh) chromatography column. Sub-fractions were eluted based on the polarity of the compounds using petroleum ether: ethyl acetate (PE: EtOAc) solvent system. Ion gradient was shifted by changing the ratio of (PE 100: EtOAc 00 to PE 00:100 EtOAc) in a stepwise manner with a ten-fold volume increase of ethyl acetate right from the beginning of elution. Obtained fractions were checked intermittently for elution of intended compounds with UV detector and DPPH spot test (Explained in consecutive sections).

4.2.2.2. Thin Layer Chromatography

A. DPPH-TLC spot test

Each of the chromatography (Sephadex LH-20; Silica gel) eluted fraction was a spot (2 µl) inoculated on silica gel chromatography plate (Si60 F254, Merck, Germany). Sample spots were then allowed to dry and sprayed with DPPH solution (0.01 mM in methanol). Fractions showed yellow spots against purple background considered as antioxidant

positive. Related fractions were pooled together into 5-7 major fractions (F1-F7) and checked for quantitative AO (%DPPH) activity.

B. DPPH-TLC autography bioassay

The AO components from most active fraction (as determined in earlier steps) were run on the preparative TLC plates in duplicates in same chromatography chamber (Mobile phase - 10:1 PE: MeOH and or Chloroform: MeOH). Both TLC plates were first visualized under UV detector and separated compounds were marked. Later, one of the TLC plate was sprayed with DPPH solution (0.01 mM in methanol) to visualize and differentiate the mere AO compounds. Retention factor (R_f) values were recorded for each of the components and the corresponding AO compounds were retrieved from other replicate TLC plate in HPLC methanol.

4.2.3. Spectrometric studies on antioxidative metabolites

4.2.3.1. UV-Visible spectrophotometric analysis

The crude sample extracts, as well as purified antioxidant fractions, were subjected to UV-Visible spectrophotometric (Carry 300 UV-Vis, Agilent Technologies) analysis for determination of spectral properties. UV-Vis spectrum was recorded in the absorbance range of 200-800 nm for each sample. Absorption wavelength peak data utilized as supporting information for prediction of possible compounds present in the samples based on the earlier literature.

4.2.3.2. LC-MS/MS spectral analysis

AO active fractions (PIGB 77: F4II and F4III) were analyzed with high-resolution LC-MS (HR-MS), Thermo scientific Hybrid Quadrupole-Orbitrap Mass Spectrometer (Q-Exactive) attached with C-18 Hypersil Gold Reverse Phase-HPLC column (150 × 4.6 mm, 8 micron). A complete mass spectrum (MS) was split based on mass and mass fragmentation patterns (MS/MS) analysis. The retention time (RT) as well as mass/charge (m/z) ratio values were recorded for the MS and MS/MS peaks. This data was utilized for devising mass and consequent structure of the compound.

4.2.3.3. GC-MS spectral analysis

Purified AO components obtained from PIGB 46, 88, 163 and 184 were subjected to gas chromatography mass spectrometry (GC-MS: Agilent 7890A) analysis. GC-MS spectra obtained with instrument specifications: Column HP-5 capillary column (30 m × 0.32 mm × 0.25 mm, J and W Scientific), carrier gas Nitrogen (flow rate 1 ml/min), sample injection port temperature 250 ℃, split ratio 8:1, oven temperature 40 to 280 ℃ (raise rate: 5 ℃/min up to 150 degrees and then 10 ℃/min) with final hold at 280 ℃ for 3 min.

Compound identity was confirmed by comparing retention time (RT). Mass fragmentation patterns of the standards and the major antioxidant constitutes compared using acquired mass spectra and RT values from the NIST (National Institute of Standards and Technology)/ NBS (National Bureau of Standards) as well as the Wiley mass spectral library (Software Version 2.0).

4.2.4. Nuclear Magnetic Resonance (NMR) studies

Structural confirmation of purified compounds was carried out with nuclear magnetic resonance (NMR: Bruker 300 or 500 MHz) spectrometric studies. The sample was first dissolved in D_2O (deuterated water) completely and then analyzed by standard NMR techniques such as (1) proton nuclear magnetic resonance (^{1}HNMR), (2) carbon-13 nuclear magnetic resonance (^{13}CNMR) and (3) distortionless enhancement by polarization transfer (DEPT) experiments. Chemical shifts noted in parts per million (δ) for each sample. Recorded NMR spectra and mass information retrieved from LC-MS analysis were used to elucidate the structures (ChemDraw Ultra software; Version 11.0).

4.2.5. Confirmatory tests for biosynthesis of hydantoinase

A) Selective agar plate assay

Selective agar plate assay was employed for primary detection of hydantoinase productions (Kim et al. 1997). A pure culture of *P. koreensis* (PIGB 77) was streaked on LB agar medium (pH 7.0) containing 1% hydantoin and 0.005% phenol red indicator. It was then incubated for 24-48 h at 30 °C. Conversion of hydantoin to hydantoic acid estimated by the production of yellow coloured halo zone around colonies. Secretion of hydantoinase converts hydantoin to hydantoic acid. It results in a decrease in pH and consequently the colour of phenol red changes to yellow.

B) Hydantoin hydrolysis bio-catalytic resting cell assay

Resting cell biocatalytic assay on PIGB 77 was performed according to the method of Hartley et al. (2001). Briefly, an aliquot of 100 µl PIGB 77 stationary phase culture was inoculated in 200 ml of sterile marine broth containing 1% Hydantoin or 0.1% of 2-Thoiurea inducers. Cultures were grown at 25 ± 2 ºC for 36 h and harvested by centrifugation at 6000 × g for 10 min. Later, the whole cell mass was washed and resuspended (20 mg/ml) in 0.1 M phosphate buffer (pH 8.0) containing substrate 25 mM Hydantoin and 50 mM 5-Methylhydantoin separately. The reaction mixture was incubated at 60 ºC for 6 h in shaking condition. The end product amino acids were quantified by standard ninhydrin assay (Plummer 1987).

4.3. Results and Discussion

4.3.1. Quantification of antioxidant metabolites

Cell free media used for antioxidant metabolites were extracted with an equal quantity of the solvent gave distinct quantities of pigmented products from the individual organism (Table 4.1). PIGB163 (*S. nematodiphila*) and PIGB 77 (*P. koreensis*) stood the most prominent strains as they yielded with higher quantities of pigmentary compounds 270.1 and 232.3 mg L^{-1} respectively.

A yellow PIGB 46 (*P. argentinensis*) and another pink isolate PIGB 88 (*S. rubidaea*) gave moderate yields. The *Vibrio* sp. (PIGB 184) had the lowest production of pigmented metabolites (71.03 % L^{-1}) among the selected bacteria.

Table 4.1. Recovery of pigmented metabolites from the potent marine bacteria.

Quantity of pigmented metabolites	Pigmented strains				
	PIGB 46 (*P. argentinensis*)	PIGB 77 (*P. koreensis*)	PIGB 88 (*S. rubidaea*)	PIGB 163 (*S. nematodiphila*)	PIGB 184 (*Vibrio* sp.)
Total recovery (mg)*	485.7	743.4	569.4	864.2	227.3
Yield (mg L^{-1})	151.8	232.3	177.9	270.1	71.03

*Total recovery from 3.2 L.

Earlier studies on recovery of pigmented metabolites from marine bacteria have evidenced similar variations among bacterial genera as well as the species. For example, as discussed earlier Kuo et al. (2011) extracted 4.5 L of *Serratia* supernatant (squid pen waste) with ethyl acetate and yielded 317.5 mg of pigmented compound. On the other hand, Gulani et al. (2012) extracted pigmented metabolites from same *Serratia* species grown in peptone glycerol broth and could retrieve 425 ± 40 mg L^{-1} of the compound. These evidences indicate that the production of pigment and associated secondary metabolites is highly influenced by factors such as the type of bacteria (genera/species) and the growth media. Thus, the variable quantity of pigmented metabolites obtained in the present study is due to species specific production.

4.3.2. Antioxidant metabolites of *P. argentinensis* (PIGB 46)

4.3.2.1. Purification of PIGB 46 AO compounds

Primary UV-Visible spectrophotometric analysis on PIGB 46 crude sample extract indicated presence of aromatic compounds with prominent peaks at around 207 nm (Fig. 4.2). Peak at 212 nm represents the presence of extracellular polymeric substances (EPS) containing derivatives of polyhydroxy ketones/ polyhydroxy aldehyde (Faust 1997). Another peak at 278 nm indicates the presence of phenolic derivatives (Engida et al. 2015). Sephadex LH-20 size exclusion chromatography loaded with 371.3 crude extract yielded a total of 126 methanolic fractions (0.5 ml each). DPPH-TLC spot test confirmed fraction no. 44 to 98 to possess the AO metabolites (Fig. 4.3).

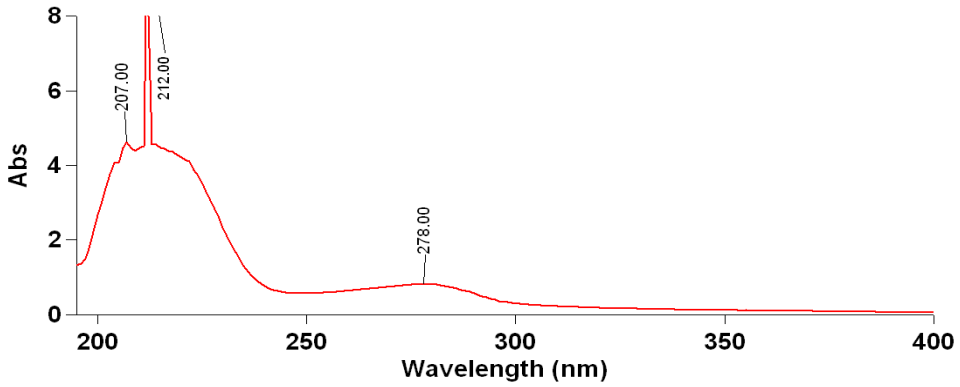


Fig. 4.2. UV-Vis spectrum for the antioxidative metabolites isolated from pigmented strain PIGB 46.

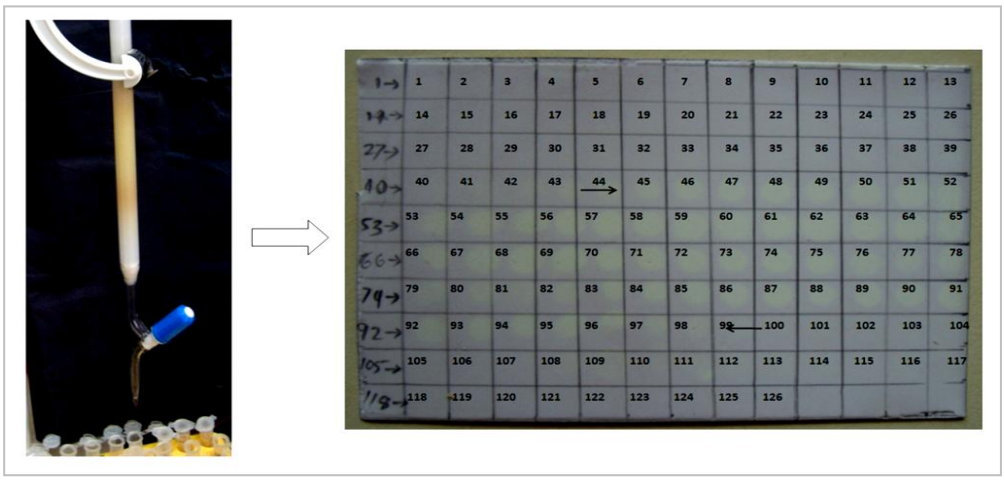


Fig. 4.3. Chromatographic purification and qualitative DPPH-TLC spot test for PIGB 46 antioxidant metabolites.

Primary AO activity (DPPH-TLC spot test) tested fractions pooled into major fractions F1-F5. Results on yield recovery and quantitative antioxidant activity (% of DPPH scavenging) demonstrated the fraction number F3 to contain potent compounds (Table 4.2). It had 16.81 ± 0.30% of DPPH scavenging and gave 49.77 % of compound recovery.

Table 4.2. Quantification of antioxidant yield and activity on PIGB 46.

Experimental stage	**Sample weight (mg)**	**Antioxidant activity (% of DPPH scavenging)**	**Yield (%)**
Initial crude sample	371.3	22.4 ± 0.33	100
Fraction F1	7.4	1.53 ± 0.22	1.99
Fraction F2	15.6	4.19 ± 0.12	4.20
Fraction F3*	184.8	16.81 ± 0.30	49.77
Fraction F4	23.5	5.13 ± 0.19	6.32
Fraction F5	57.3	3.86 ±0.03	15.43

*Represents potent fraction.

UV and DPPH guided autography test showed that the Fraction F3 with more AO activity. Fractions F3 and F5 though resolved with UV active compounds on TLC, except the F3 none of the other fractions showed the presence of AO compounds (Fig. 4.4). Separation of active components from F3 on silica gel plate indicated the presence of 7 sub-components a-g (Table 4.3). Compounds with retention factors (R_f) values 0.34, 0.55 and 0.68 showed prominent decolorization of DPPH reagent.

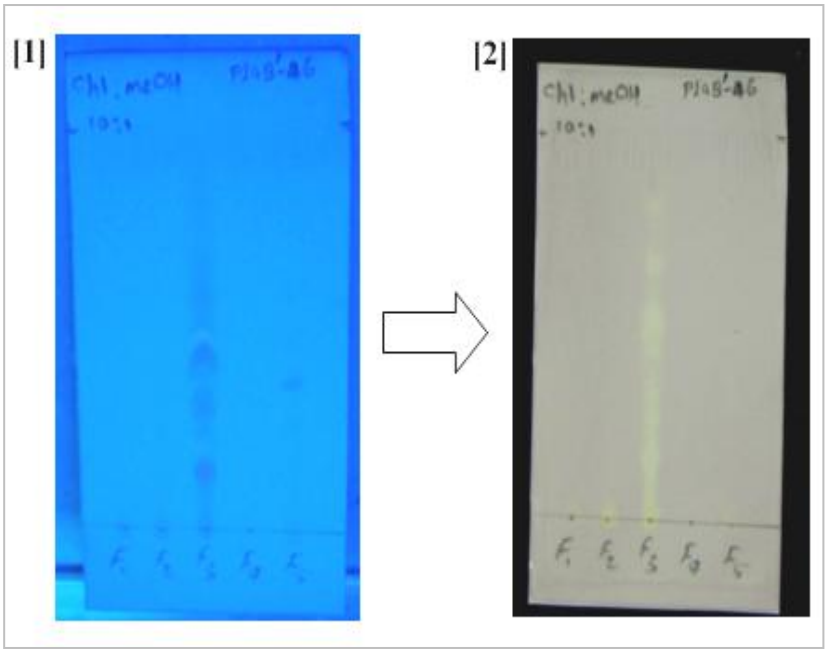


Fig. 4.4. UV and DPPH guided TLC autography for separation of AO metabolites from PIGB46. TLC plates exposed to UV [1] and DPPH reagent [2].

Table 4.3. Antioxidant components from fraction F3 and their properties.

Pigmented strain	**Antioxidant fraction**	**Number of components in active fraction**	**A**	**B**	**R_f = A/B**	**Antioxidant property**
PIGB 46	Fraction F3	a	0.8	4.4	0.18	+
		b	1.3	4.4	0.29	+
		c	1.5	4.4	0.34	++
		d	1.7	4.4	0.39	NA
		e	2.4	4.4	0.55	++
		f	3.0	4.4	0.68	++
		g	3.6	4.4	0.82	+

A: Distance traveled by solute, B: solvent front, +: antioxidant active, NA: no activity.

4.3.2.2. Structural and chemical properties of PIGB 46 antioxidants

GC-MS analysis on AO fraction F3 revealed the nature of active components as, (1) Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- {MW – 210; RT – 29.4 min} (2) 3,6-Diisopropylpiperazin-2,5-dione {MW – 198; RT – 29.8 min} and (3) n-Hexadecanoic acid {MW – 256; RT – 31.09 min} (Table 4.4). Former two compounds (1 and 2) demonstrated to be aromatic in nature whereas the latter (compound 3) was found to have aliphatic symmetry. Compound 1 was found to be more dominant and frequently occurred during GC-MS analysis at retention times 26.4, 29.4, 30.6, 30.8 and 30.9 min (Fig. 4.5).

Earlier studies have documented production of Pyrrolo type of compounds in marine bacteria. For example, Pandey et al. (2011) documented secretion of Pyrrolo-(1,2-a) pyrazine-1,4-dione,hexahydro-3(2-methylpropyl) and Pyrrolo-(1,2-a)pyrazine-1,4-dione,hexahydro-3(2-methyl phenyl) by marine bacterium *Aeromonas* sp. Similarly, Synthesis of Pyrrolo- derivatives by marine *Pseudomonas* sp. has been reported by Burkholder et al. (1996). Conversely, production of another similar type of metabolite hexahydropyrrolo[1,2-a]pyrazine-1,4-dione is reported from marine *P. aeruginosa* and *P. fluorescens* species.

Also, a recent study by Wang et al. (2014) documented the secretion of 3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione by *Pseudomonas* sp. NJ-011 which is synonymous to a compound Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- produced by our strain. It confirmed active secretion or widespread presence of such secondary metabolites in organisms belonging *Pseudomonas* genera. In the present study, the isolation of this particular compound with antioxidant activity forms a first ever report on its synthesis by marine *Pseudomonas* species.

Hitherto, there is only one report on the synthesis of compound 3,6-Diisopropylpiperazin-2,5-dione by marine *Pseudomonas* sp. NJ6-3-1 (Zheng et al. 2005). This report additionally revealed the biological importance (anticancer) of such indole derived compound. Our findings on the secretion of 3,6-Diisopropylpiperazin-2,5-dione by *P. argentinensis* forms second report on its occurrence in *Pseudomonas* sp. while records novelty on its antioxidant property.

Considering n-Hexadecanoic acid studies on *Pseudomonas* species, Bae et al. (2007) demonstrated a terrestrial strain *P. aeruginosa* (PR3) to produce a dihydroxy hexadecenoic acid (DHD) with value-added properties. Likewise, *P. pseudomallei* also evidenced to secrete similar metabolite with a biodegradable polymer property (Das et al. 2005). Hexadecanoic acid is one among the industrially important hydroxy fatty acids (HFA) because of its high viscosity and reactivity. It has been used as fabricant in lubricants, biodegradable plastics, waxes, cosmetics and as an additive in paints. Active secretion of this particular compound by PIGB 46 (*P. argentinensis*) emphasizes its industrial importance for similar applications.

Moreover, the n-Hexadecanoic acid has been documented to express the antioxidant properties in biological samples (Thomas et al. 2010; Patra et al. 2015). The antioxidant nature of n-Hexadecanoic acid as evidenced in the present investigation provides additional information on its biological property for efficient implementation in various industrial processes mentioned above.

Table 4.4. Antioxidant compounds from pigmented strain *P. argentinensis* (PIGB 46).

Compounds name	**Chemical formula**	**Molecular weight**	**Synonyms**
Pyrrolo-[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	1.3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione
3,6-Diisopropylpiperazin-2,5-dione	$C_{10}H_{18}N_2O_2$	198	1.3,6-Diisopropyl-2,5-piperazinedione
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	Hexadecanoic acid; n-Hexadecanoic acid; Palmitic acid etc.

Fig. 4.5. (A)

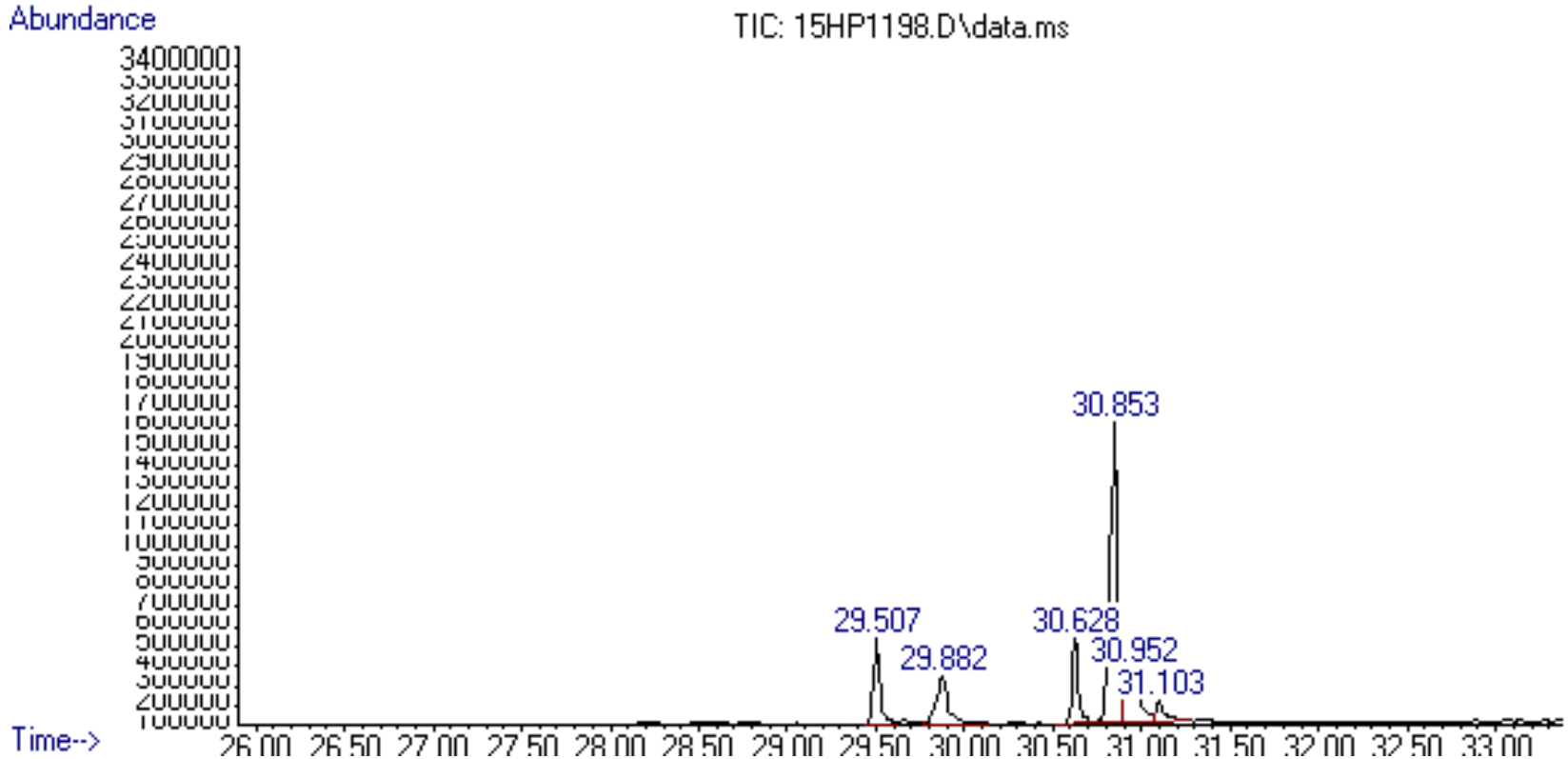
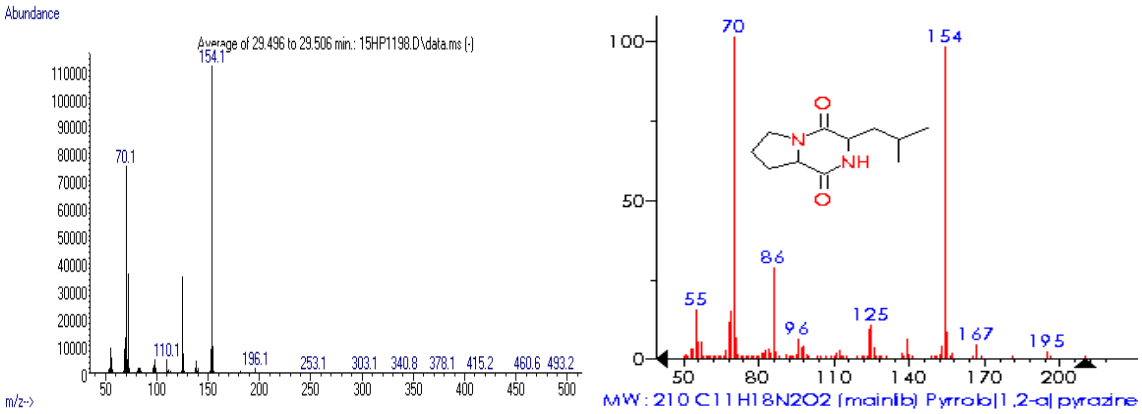


Fig. 4.5. (A) GC-MS spectra on fraction F3 of PIGB 46.

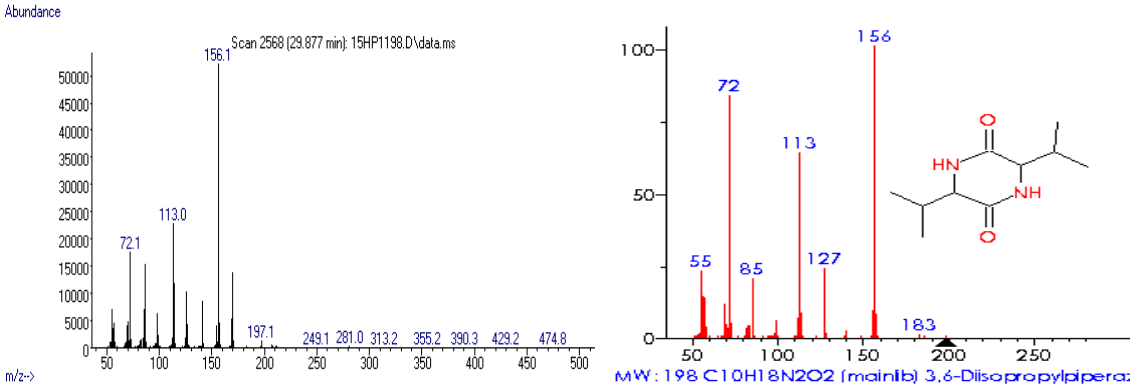
Cont...

Fig. 4.5. (B)

Compound I: Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- {RT: 29.4 min}



Compound II: 3,6-Diisopropylpiperazin-2,5-dione {RT: 29.8 min}



Compound III: n-Hexadecanoic acid {RT: 31.09 min}

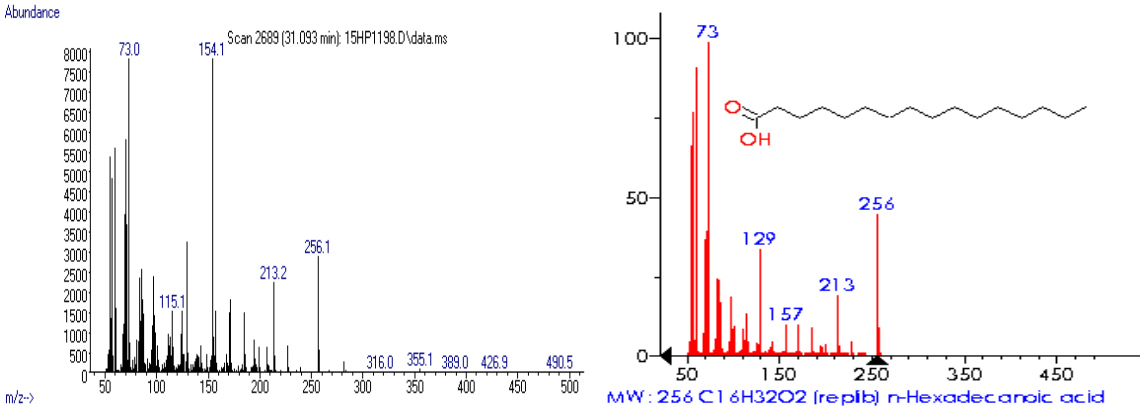


Fig. 4.5. (B) MS/MS spectra on Fraction F3 of PIGB 46.

4.3.3. Antioxidant metabolites of *P. koreensis* (PIGB 77)

4.3.3.1. Purification of PIGB 77 AO compounds

Crude pigmented extract (458.7 mg) obtained from *P. koreensis* (PIGB 77) passed through Sephadex LH-20 column yielded 134 (0.5 ml each) fractions (Fig. 4.6). Individual fractions assessed on its qualitative AO property by DPPH spot TLC test showed fraction no. 19 – 59 to possess active antioxidant compounds as they showed immediate decolorization spots. These fractions were visually observed to retrieve pigments from Sephadex column. Fractions 9-15 and 62-124 also showed activity zones, but they had comparatively less intense DPPH decolorization spots. Based on pigment separation and antioxidant test, related fractions were pooled into seven major fractions i.e. F1-F7.

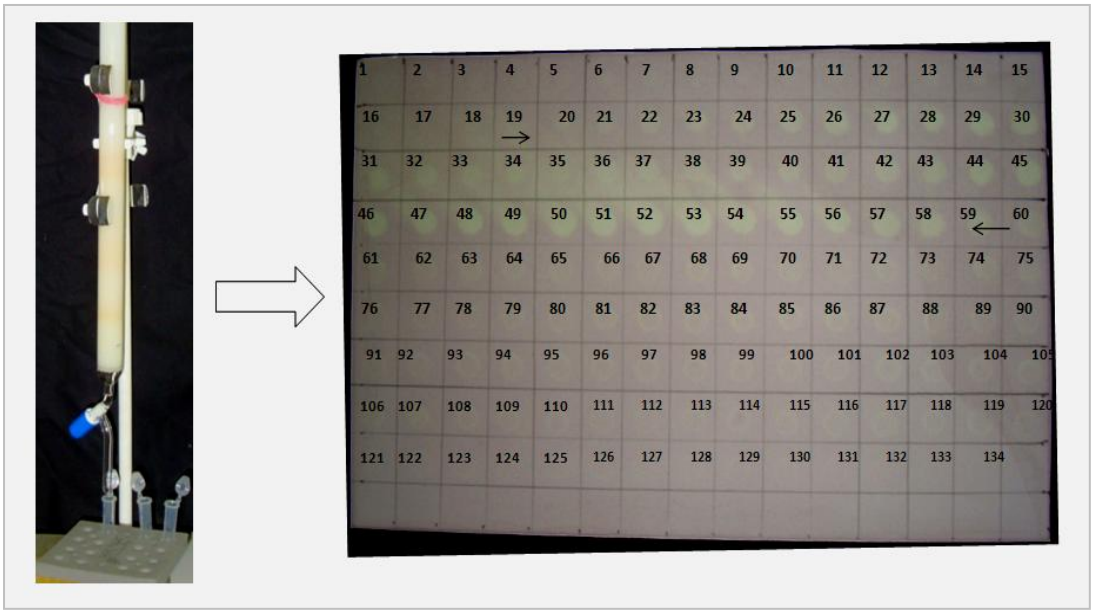


Fig. 4.6. Chromatographic purification and qualitative DPPH-TLC spot test for PIGB 77 antioxidant metabolites.

Quantitative antioxidant and yield retrieval check on the main fractions (F1-F7) demonstrated the fraction numbers F2, F3 and F4 to possess active compounds (Table 4.5). Fraction F4 exhibited higher DPPH radical scavenging activity (41.13 ± 3.56%). Notably, F4 though had lower recovery of metabolites (22 %) it gave higher antioxidant activity than the F3 with greater yield (25.75 %) and lower activity (18.21 ± 0.56%).

Table 4.5. Quantification of antioxidant yield and activity on PIGB 77.

Experimental stage	**Sample weight (mg)**	**Antioxidant activity (% of DPPH scavenging)**	**Yield (%)**
Initial crude sample	458.7	93.4 ± 6.54	100
Fraction F1	6.2	7.03 ± 0.30	1.35
Fraction F2	95.5	11.23 ± 0.43	20.82
Fraction F3	118.1	18.21 ± 0.56	25.75
Fraction F4*	100.9	41.13 ± 3.56	22.00
Fraction F5	3.3	2.63 ± 0.09	0.72
Fraction F6	1.9	0.52 ± 0.00	0.41
Fraction F7	1.3	0.61 ± 0.00	0.28

*Represents potent fraction.

AO active fraction F4 separated by silica gel chromatography resulted 3 sub-compounds viz. F4(1), F4(2) and F4(3). The UV/ DPPH autography and spectrophotometric analysis confirmed the purity of the above compounds (Fig. 4.7[1]; Fig. 4.8). But the DPPH staining demonstrated, fractions F4(2) (R_f = 0.33) and F4(3) (R_f = 0.13) could exhibit positive AO activity (Fig. 4.7[2]). At the same time fraction F4(1) with R_f = 0.55 did not show AO activity in DPPH staining (Fig. 4.7[2]; Table 4.6).

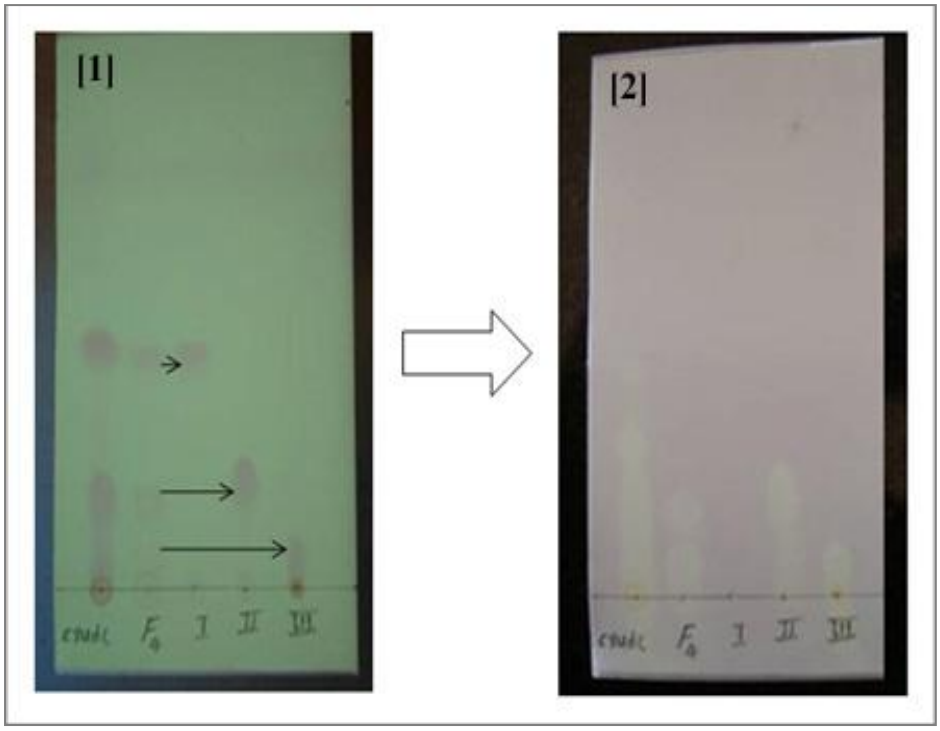


Fig. 4.7. UV and DPPH guided TLC autography for separation of AO metabolites from PIGB77. TLC plates exposed to UV [1] and DPPH reagent [2].

Table 4.6. Antioxidant components from fraction F4 and their properties.

Pigmented strain	**Antioxidant fraction**	**Number of components in active fraction**	**A**	**B**	**R_f = A/B**	**Antioxidant property**
PIGB 77	Fraction F4	(1)	2.2	4.0	0.55	NA
		(2)	1.3	4.0	0.33	+++
		(3)	0.5	4.0	0.13	++

A: Distance traveled by solute, B: Solvent front, +: AO active, NA: no activity.

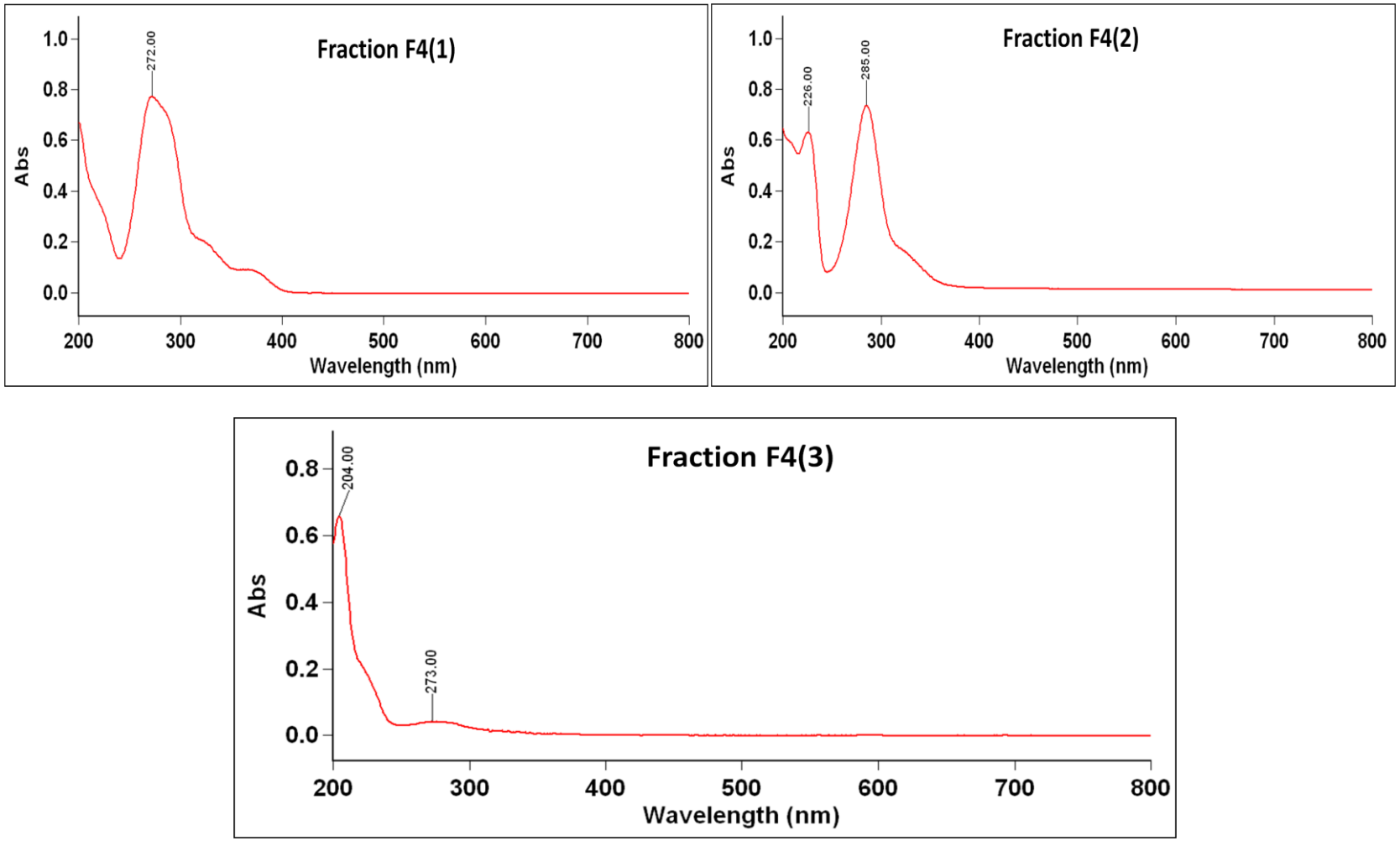


Fig. 4.8. UV-Visible spectra for the strain PIGB 77 and its fractions F4 (1), F4(2) and F4(3).

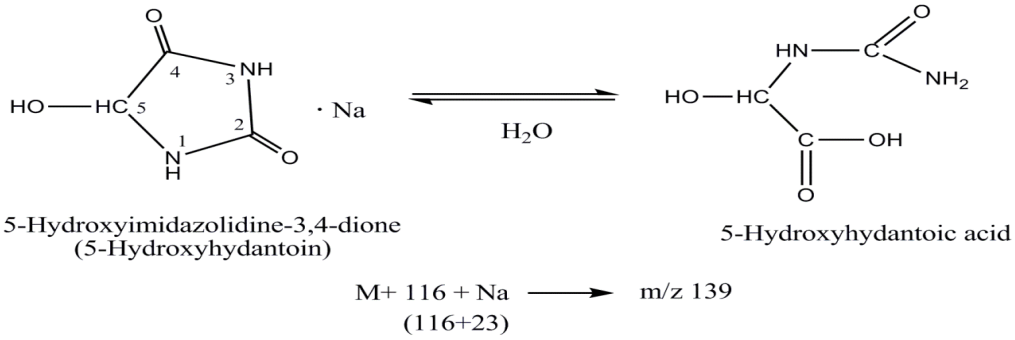
4.3.3.2. Structural and chemical properties of PIGB 77 antioxidants

(A) Fraction F4(3):

Structural and chemical properties of Fraction F4(3) are given in Fig. 4.9; Fig. 4.10 and Table 4.7. The LC/MS analysis on F4(3) showed presence of 3 components with RT of 3.71, 4.06 and 5.4 min (Fig. 4.9.A). Each peak was further subjected to ESI-MS in positive ionization mode and fragmentation patterns are represented in Fig. 4.9.B.

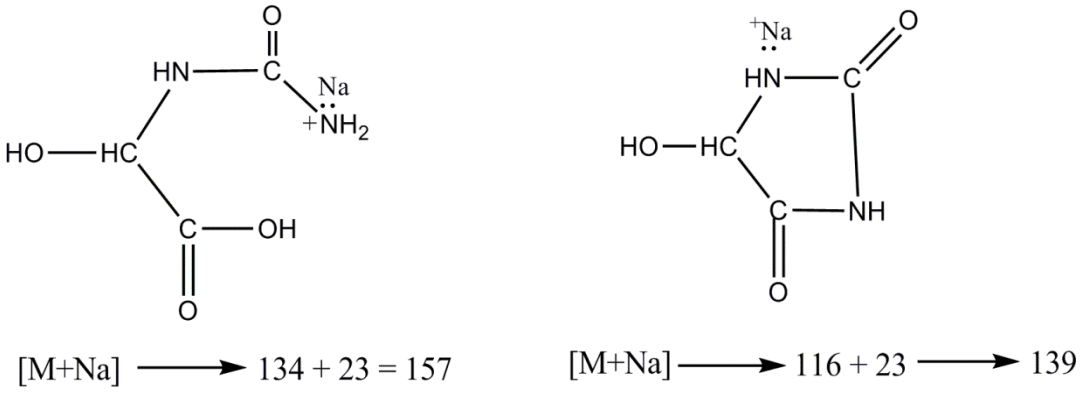
(i) The peak with retention time of 3.71 displayed the most intense signal at m/z 139 (Fig. 4.9.B). It's MS/MS showed the presence of a signal with lower intensity at m/z 157, suggestive that m/z 139 must have derived from a molecule with $[M+H]^+$ at m/z 157 by the elimination of water.

Its ^{1}HNMR in D_2O showed only two singlet's at δ 4.68 due to residual water proton and at δ 5.875 due to CH-OH methane proton of hydroxyl methane, -OH and -NH proton not observed as they got exchanged with deuterium of D_2O (Fig. 4.10[1]). The ^{13}C NMR showed peaks at 95.12 ppm due to methane (-CH) carbon and two quaternary carbon (C) at 99 ppm and 157.76 (ureido carbonyl/peptide) (Fig. 4.10[2]). The multiplicity of carbons was evident by DEPT experiments (Fig. 4.10[3]). Based on the spectral data the following structure was assigned to the compound with sodiated molecular ion $[M^+$+Na] at m/z 139:



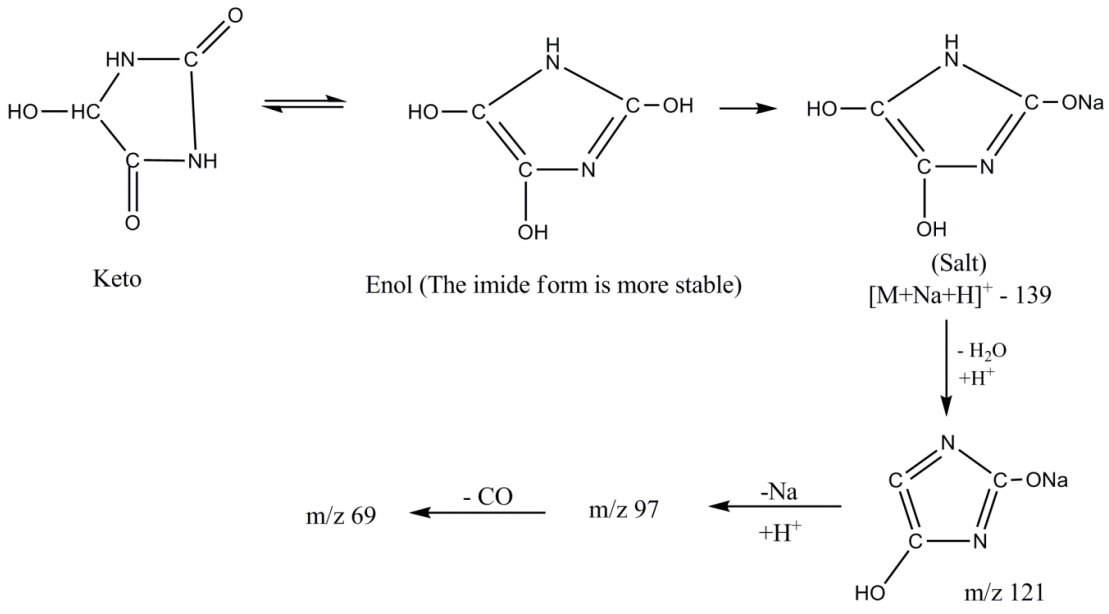
(Hydrolysis of 5-OH-hydantoin to 5-hydroxy hydantoic acid is reversible reaction)

5-Hydroxy imidazolidine-2,4-dion, also known as 5-Hydroxy hydantoin has a molecular mass (M+) of 116. The peak at m/z 139 results from the formation of sodium adduct with the two lone pair of an electron on nitrogen. Thus, the formation of the peaks at m/z 139 and m/z 157 $[M+Na]^+$ could be explained as follows:



Fragmentation observed in the ESI-MS/MS of m/z 139 (Fig. 4.9.B) are: m/z 121, 97 and 69 (Scheme - 1).

Hydantoin behaves as tautomeric substance; the enol form is acidic and form salts,

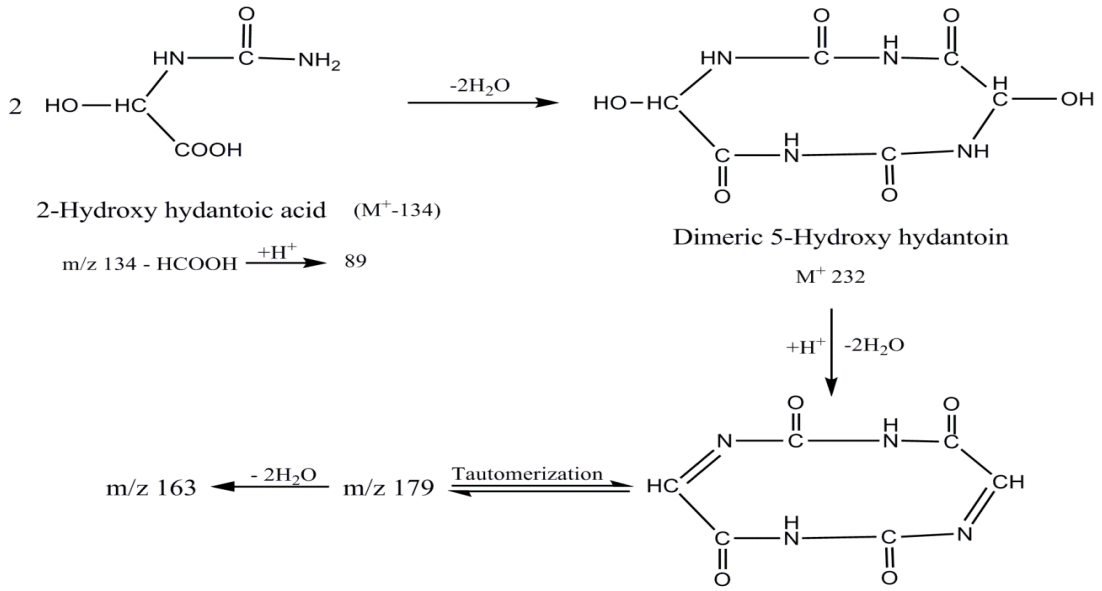


Scheme – 1. Fragmentation observed in ESI-MS/MS of m/z 139.

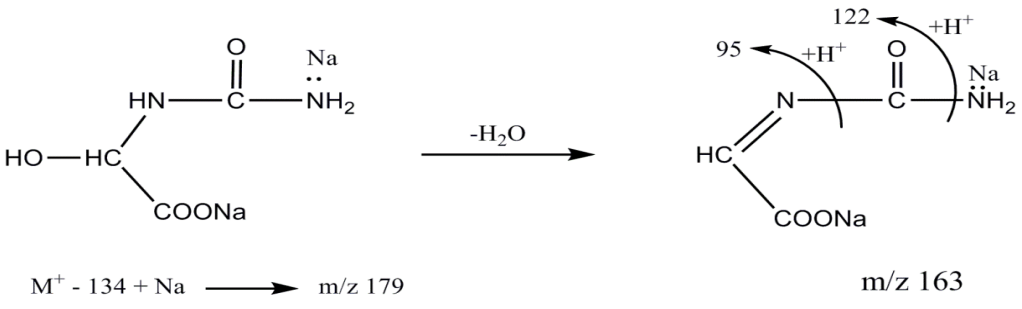
(ii) ESI-MS profile (Fig. 4.9.B) of the peak in the LC-MS chromatogram (Fig. 4.9.A) with the retention time of 4.05 revealed the presence of a molecular species with the most intense signal at m/z 179 and the relatively peak of lower intensity at m/z 301, 139, 99 and 73.

ESI-MS/MS (tandem mass spectrum) of the signal at m/z 179 (Fig. 4.9.B) showed the presence of fragments of lower intensity including a peak at m/z 197 of negligible intensity. The fragmentation path explained as follows:

(1)



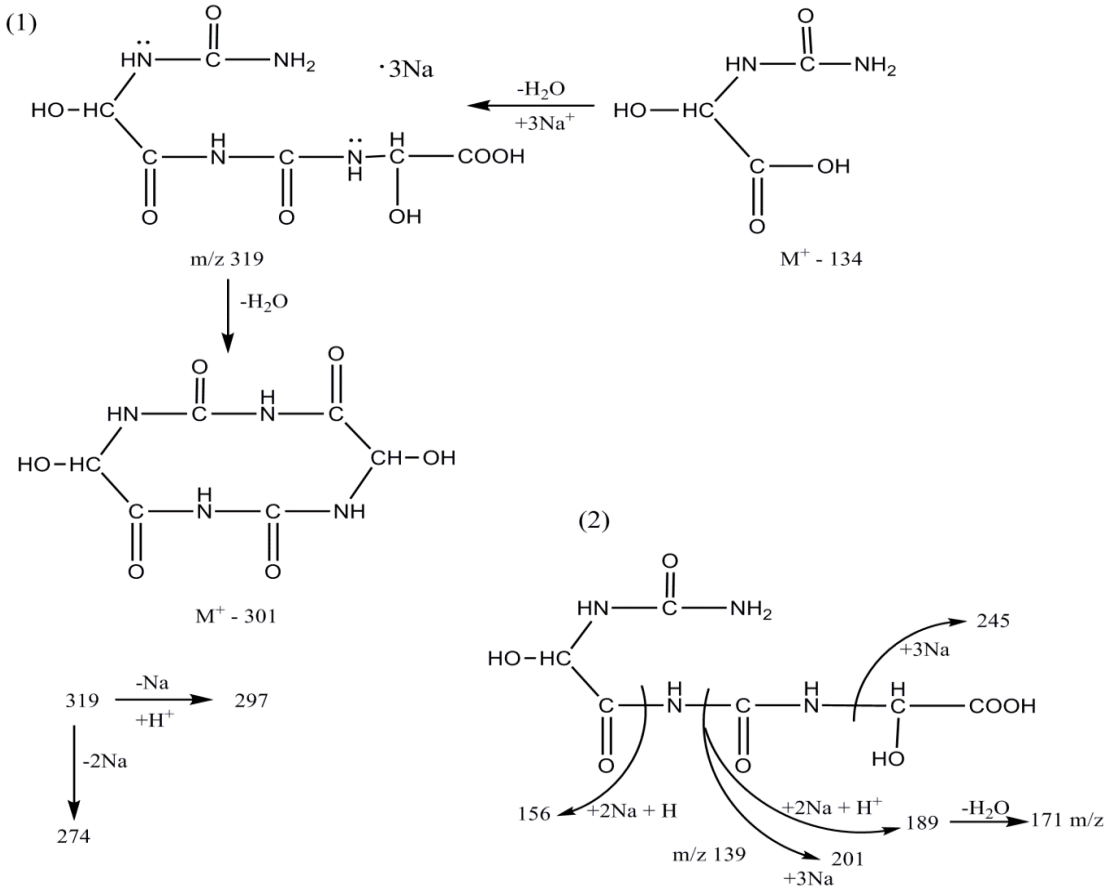
(2)



$163 \rightarrow Na{:}NH_2 + COONa \rightarrow 57$

$\rightarrow 163 - (39 + 67) \rightarrow 57$

(iii) ESI-MS (Fig. 4.9.A) of the third peak observed at RT 5.4 min showed the most intense signal at m/z 301 with a pseudomolecular ion at m/z 319. Tandem mass spectrum (Fig. 4.9.B) of this signal (m/z 301) showed fragmentation that could be clarified depending on the basis of the structure assigned to the molecular species. It has probably resulted by condensation of two molecules of hydantoic acid.



It is to be noted that dimeric 5-hydroxyhydantoin (m/z 301) is more stable than the dimeric 5-hydroxy hydantoic acid. Hence, carboxylic carbonyl (-COOH) resonance at ~ 174 ppm is not observed. Similarly, carbonyl resonance of –COONa usually observed at ~181 ppm is also not observed neither in ^{13}C NMR nor in $^{1}HNMR$ (between δ 10-13.5).

All these data summed up in Table 4.7 which suggests that compound F4(3) is a mixture of,

1. 5-OH-hydantoin +Na (m/z 139)/ hydantoic acid +Na (m/z 157)
2. i) Cyclic dimeric 5-OH-hydantoin → m/z 197 → m/z 179

   or

   ii) Sodium salt adduct of 5-OH-hydantoic acid (m/z 179)
3. i) Cyclic dimeric 5-OH-hydantoin +3Na (m/z 301)

   or

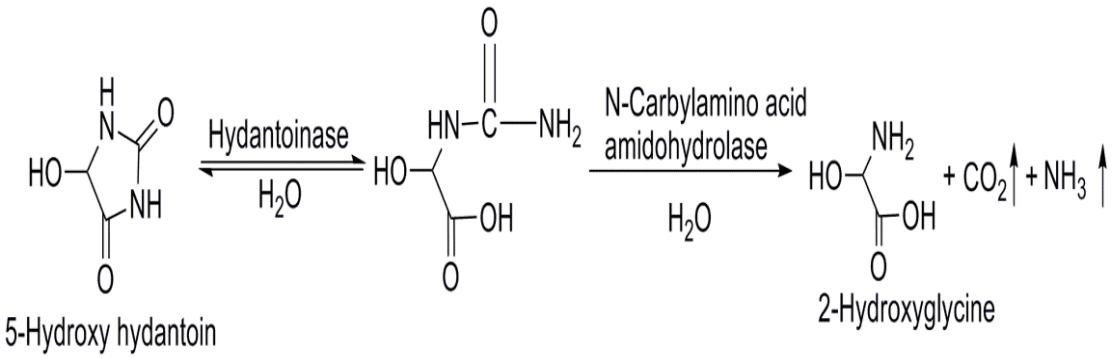
   ii) Dimeric 5-OH-hydantoic acid +3Na-H_2O (m/z 319).

(B) Fraction F4(2):

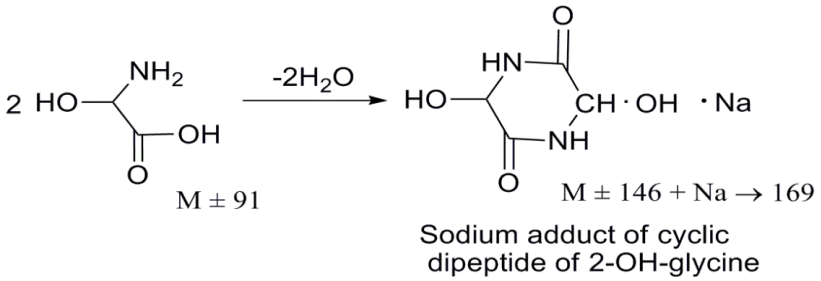
LC-MS (Fig. 4.11.A) and MS/MS (Fig. 4.11.B) spectrum of the purified fraction F4(2) (recorded homogeneous on TLC; Fig. 4.7) showed the presence of 3 sub-components with retention time (RT) = 3.86, 4.00 and 4.36 min.

i) ^{1}HNMR of F4(2) showed absorption at δ 2.615 due to –OH protons, a singlet at δ 5.8 was assigned to methane proton of hydroxymethine (-CH-OH) (Fig. 4.12[1]). A broad signal at δ 4.6 was attributed to –NH protons. ^{13}C NMR (Fig. 4.12[2]) with DEPT experiment (Fig. 4. 12[3]) corroborated the presence of –CH methane carbonate 94.2 ppm and signal for ureido as well as peptide carbonyl at 164.4 and 164.89 ppm respectively. This data also indicated that compound F4(2) also must contain hydantoin/ hydantoin derivatives.

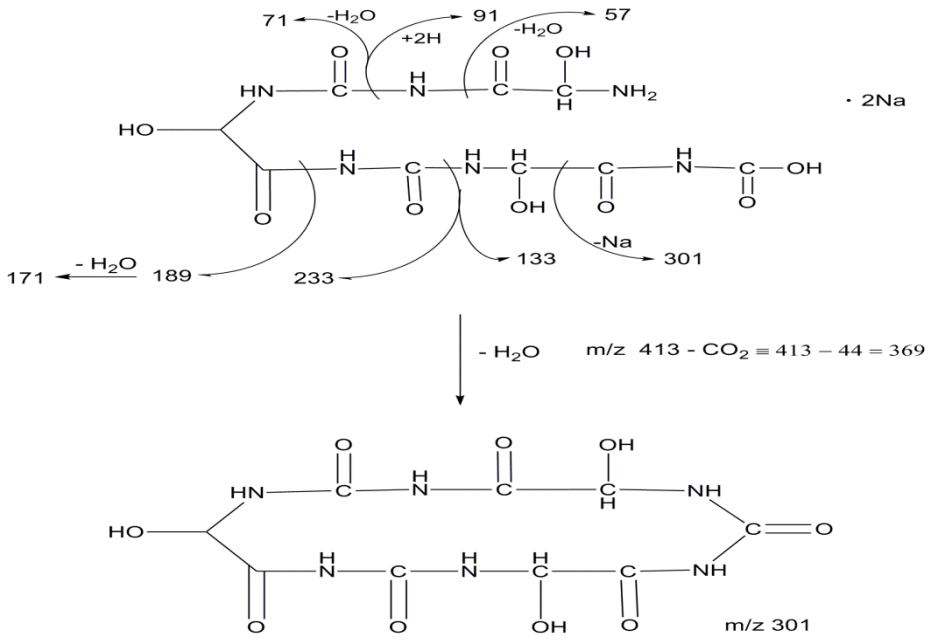
Since it was clear from the spectral data for compound F4(3) that chemical constituents of F4(3) are derived from 5-hydroxy hydantoin, literature survey revealed that hydrolysis of hydantoin proceeds as follows:



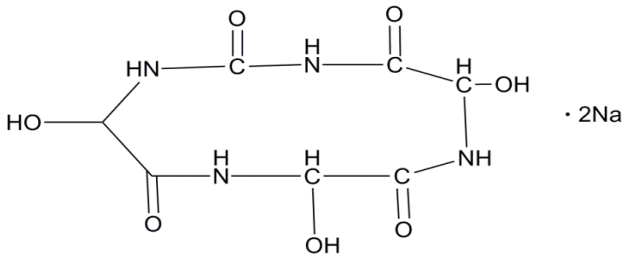
i) ESI-MS (Fig. 4.11.B) of the component with RT = 3.86 showed besides the base peak at m/z 163, peaks of considerable intensity at m/z 151 and m/z 123. The fragmentation pathways could be explained as follows:



ii) The component with RT = 4.00 was found to be molecular species with a molecular mass of 413 amu. ESI-MS/MS of this component with pseudomolecular ion (Fig. 4.11.B) which is in agreement with the structure of disodium adduct of hydroxyl homotrimer, a linear tripeptide of 5-hydroxy hydantoin.



iii) The component in purified fraction F4III (PF3) with RT = 4.36 and a pseudomolecular ion at m/z 308 was identified as disodium adduct of the heterotrimeric cyclic peptide. It formed by condensation of 1 molecule of 5-hydroxy hydantoic acid and 2 molecules of 2-hydroxy glycin. Fragmentation pathways observed in the ESI-MS/MS of the molecule (Fig. 4.11.B) is explained on the structure. Scheme-



m.z 308 (M^+) ® [M + 2Na] = 262+ 46

Scheme

(M^+) 308 – NH → 293

(M^+) 308 – CO → 280

M+ – H_2O → 260 – 18 $\xrightarrow{+H^+}$ 243

Enzyme hydantoinase act on substrate hydantoin and converts it into amino acid mainly the D types. These D-amino acids have industrial importance where it's been utilized for the synthesis of hormone peptides, semi-synthetic antibiotics and pesticides (Syldatk et al. 1999; Durr et al. 2006). Secretion of hydantoinase in bacteria is though

well known, reports on the ones with D stereospecificity are scarce as well as less occurred in nature. Bacterial members belonging *Pseudomonas* genera for example, *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. desmolyticum* and *Pseudomonas* sp. are reported for production of hydantoinase and or hydantoin derived enzymes (Janssen et al. 1982; Morin et al. 1986; Ogawa et al. 1995; Ishikawa et al. 1997; Gokhale et al. 1996; Ranjan and Dave 2000). On the other hand, a recombinant hydantoinase biosynthesized from *Pseudomonas* sp. has also been patented for *in vitro* production of D-amino acids (Ikenaka et al. 1997). A positive result in the case of our strain PIGB 77 (*P. koreensis*) is a first report on the production of hydantoinase.

Concerning hydantoinase activity, an earlier study by LaPointe et al. (1994) demonstrated the bioconversion of 5'-monosubstituted hydantoin substrate into N-carbamyl-D-amino acid using *P. putida*. Likewise, *P. desmolyticum* (Gokhale et al. 1996) and *Pseudomonas* sp. NCIM 5109 (Sudge et al. 1998) employed for synthesis of D(-)*N*-carbamoylphenylglycine amino acids using DL-5-phenylhydantoin substrate. In the present investigation, PIGB 77 evidenced the isolation of bio-converted 2-hydroxyglycine products. Its chemical properties are distinct from earlier reports.

Interestingly, a metabolite '5-Hydroxy-1-methylimidazoline-2,4-dione' similar to our PIGB 77 constituent '5-Hydroxy imidazolidine-2,4-dione' (i.e. 5-Hydroxy hydantoin) has been intrinsically tested on rats and documented to provide antioxidant protection to the host animal (Ienaga and Yokozawa 2011). Finding natural renewable sources of such hydantoinase could in turn help for larger scale production of D-amino acids with better prospective in industrial applications. Thus, PIGB 77 isolated by us from the seaweed samples with hydantoinase and antioxidant activity will have better application in biotechnological industries.

Table 4.7. Antioxidant compounds from pigmented strain *P. koreensis* (PIGB 77).

Fraction	Compounds identity	Retention time (RT)	Chemical formula	Molecular weight
F4(2)	i) Na adduct of cyclic dipeptide of 2-OH-glycine	i) 3.86	i) $C_4H_6N_2O_4 \cdot Na$	i) 169 (146+ Na)
	ii) Disodium adduct of hydroxyl homotrimer/ tripeptide of 5-Hydroxy hydantoin	ii) 4.00	ii) $C_9H_{14}N_6O_{10} \cdot 2Na$	ii) 413
	iii) Disodium adduct of heterotrimeric cyclic peptide of one 5-hydroxy hydantoic acid and two 2-hydroxyglycine	iii) 4.36	iii) $C_7H_{10}N_4O_7 \cdot 2Na$	iii) 308 (262 + 2Na)
F4(3)	i) 5-Hydroxy hydantoin/ 5-Hydroxy imidazolidine-2,4-dione	i) 3.71	i) $C_3H_4N_2O_3$	i) 116
	ii) Cyclic dimeric 5-OH-hydantoin	iii) 4.05	ii) $C_6H_8N_4O_6$	ii) 232
	iii) Dimeric 5-OH-hydantoic acid · 3Na	iii) 5.04	iii) $C_6H_8N_4O_6 \cdot 3Na$	iii) 301 (232 +3Na)

Fig. 4.9. (A)

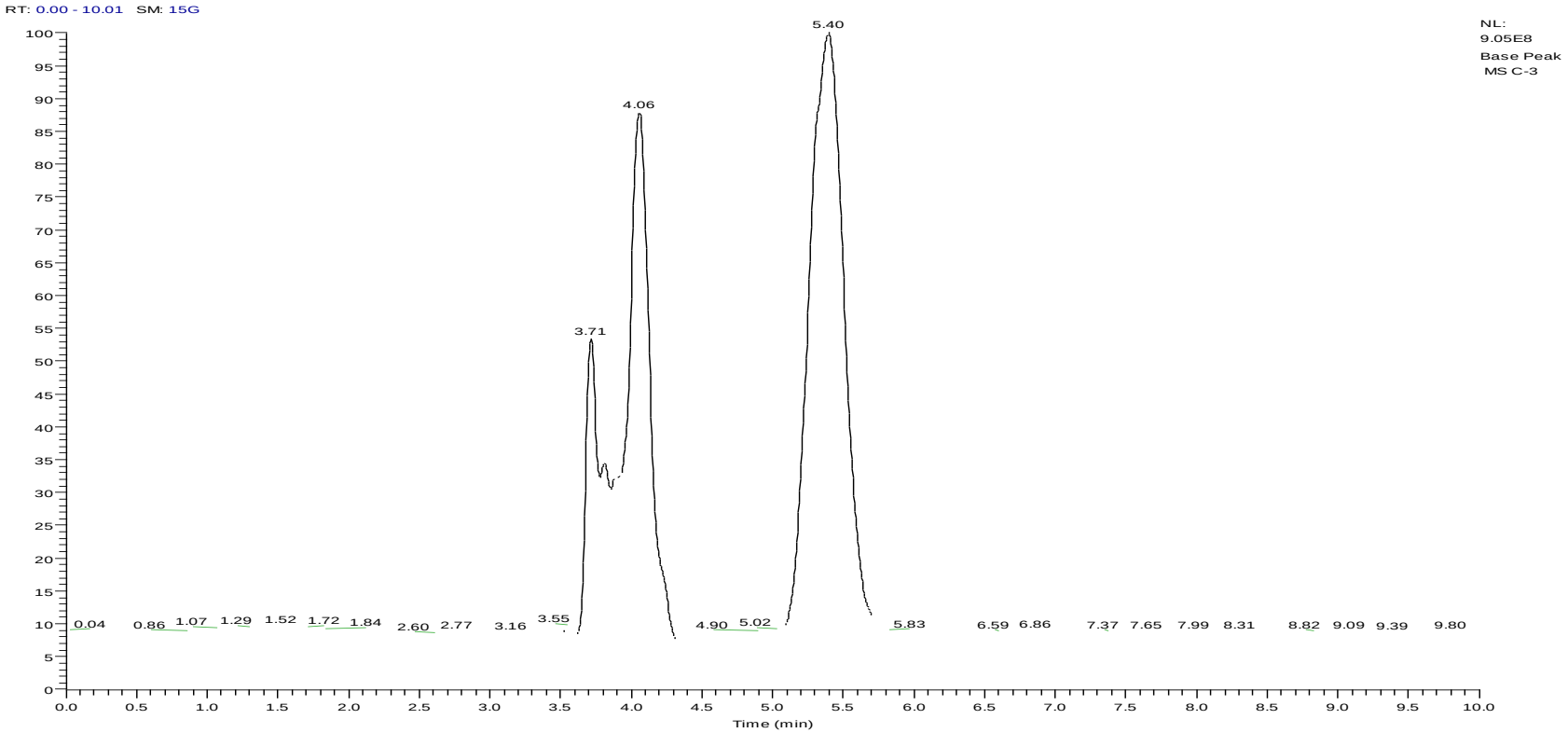


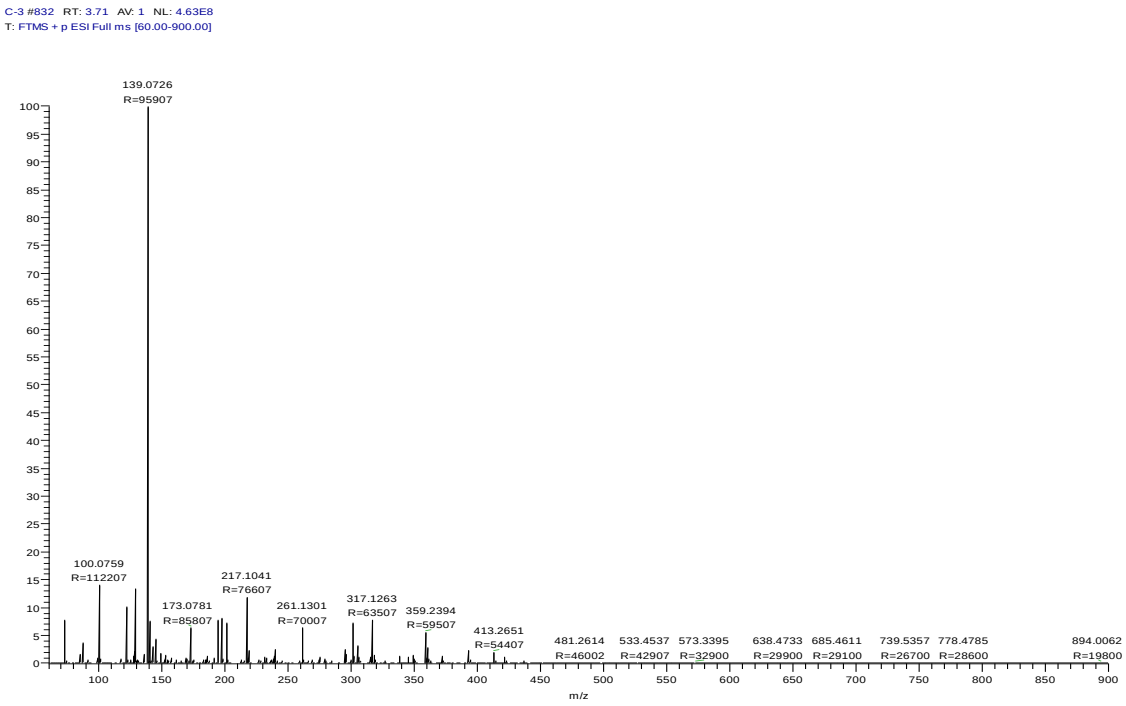
Fig. 4.9. (A) LC-MS spectra for purified antioxidant Fraction F4(3).

Cont…

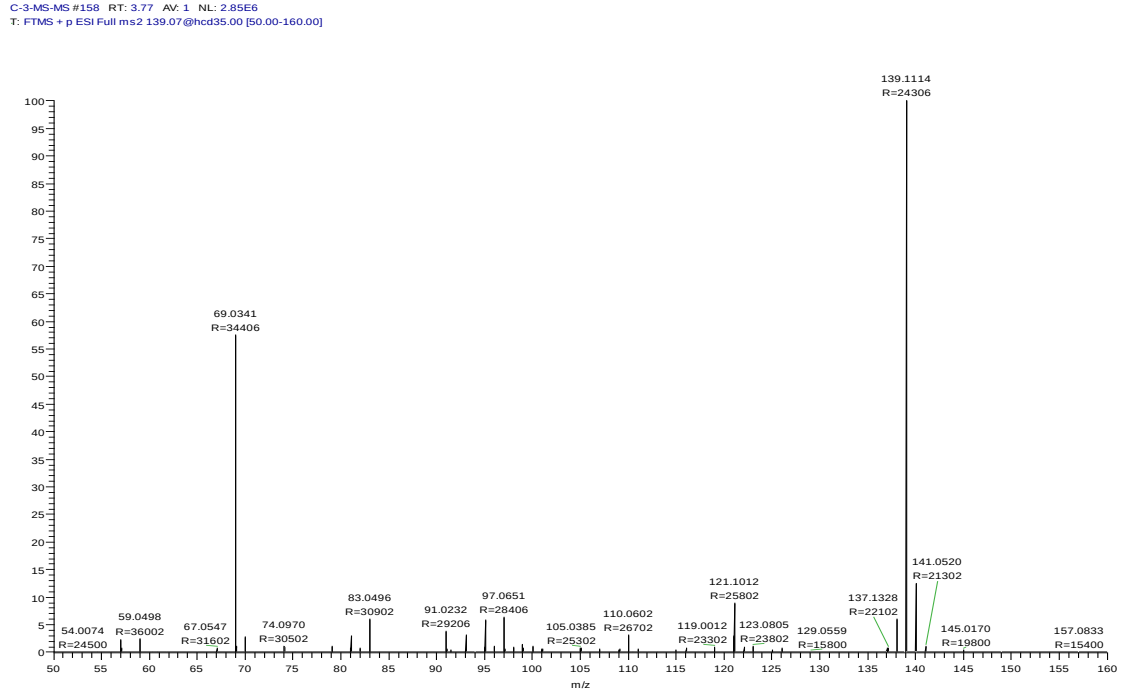
Fig. 4.9. (B)

i) Peak at RT = 3.71 min

m/z 139 MS



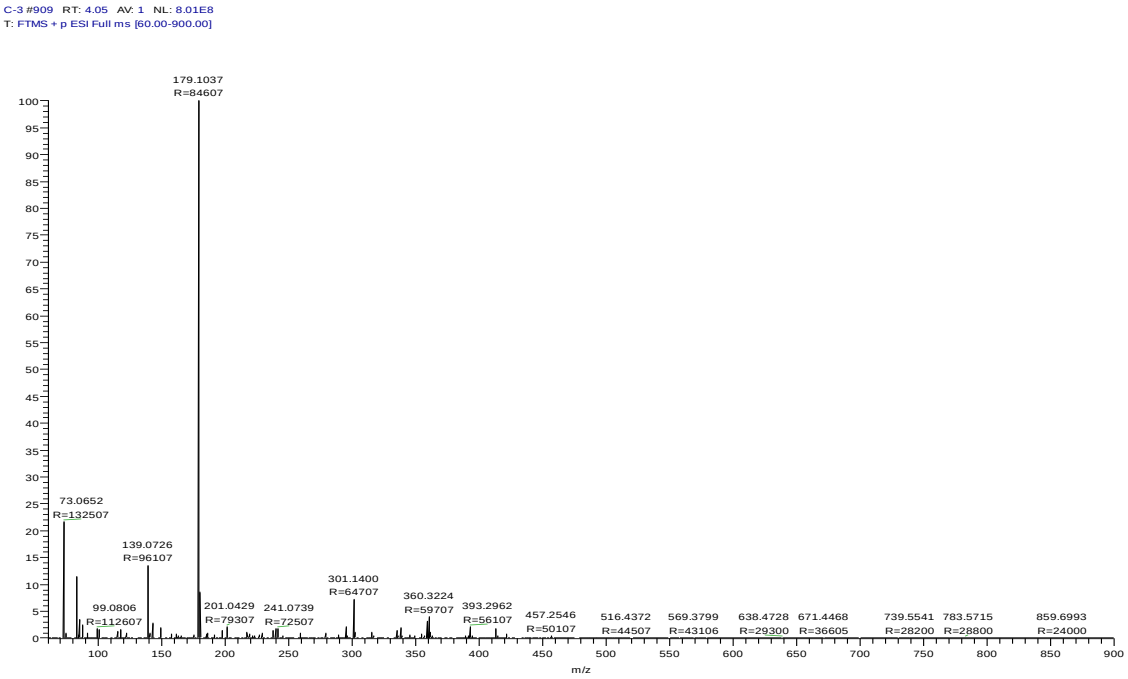
m/z 139 MS/MS



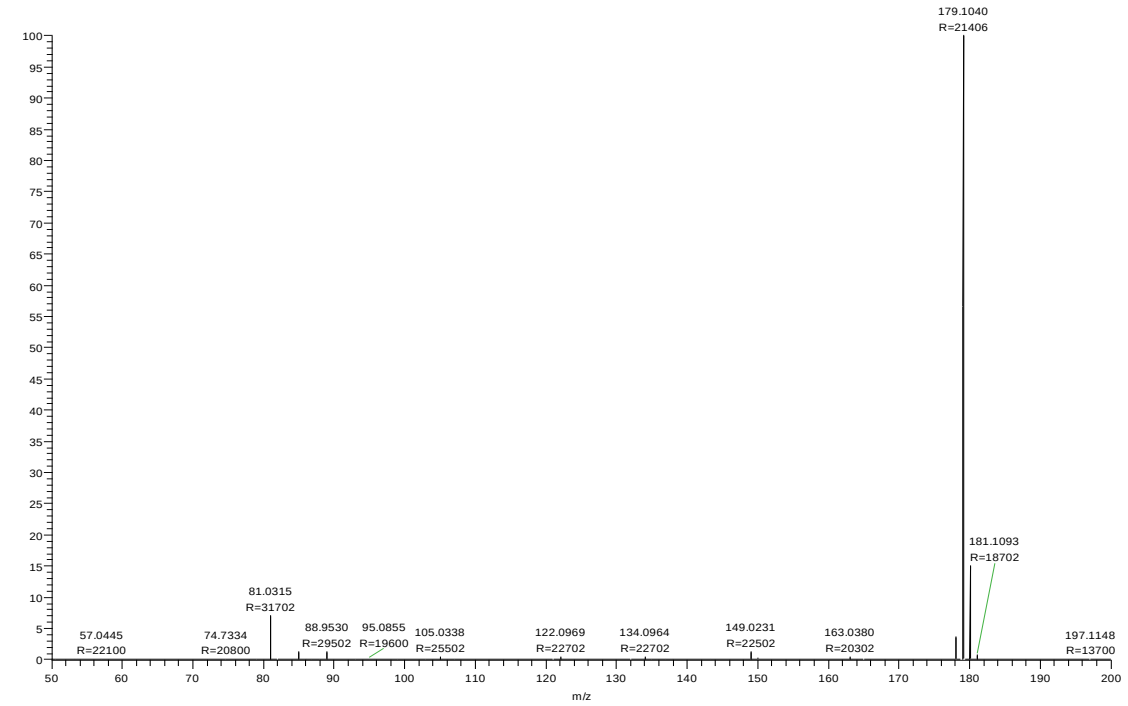
Cont…

ii) Peak at RT = 4.06 min

m/z 179 MS



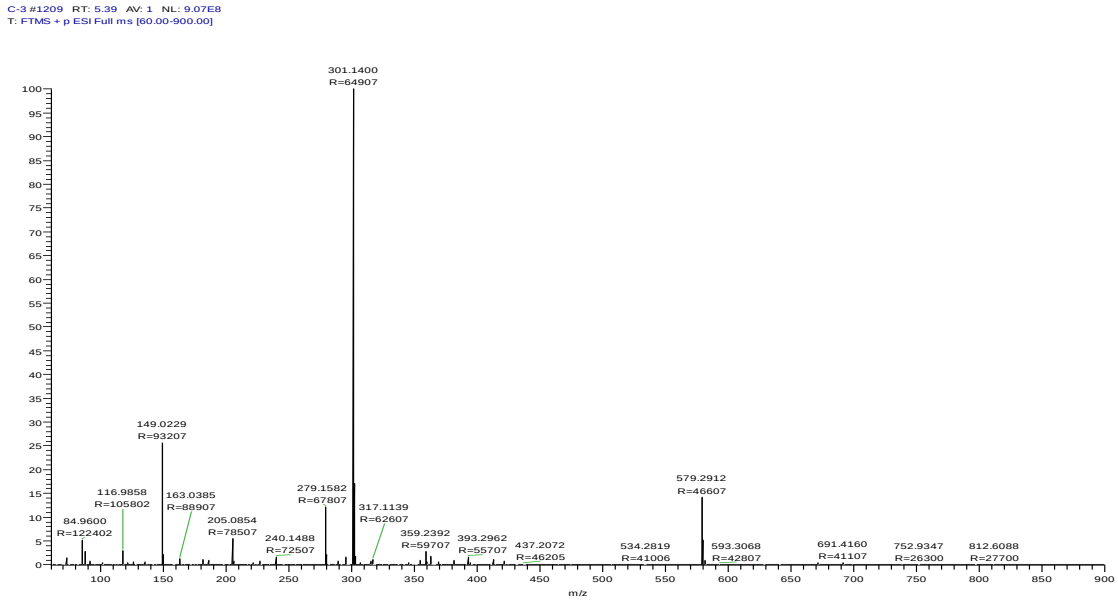
m/z 179 MS/MS



Cont…

iii) Peak at RT = 5.40 min

m/z 301 MS



m/z 301 MS/MS

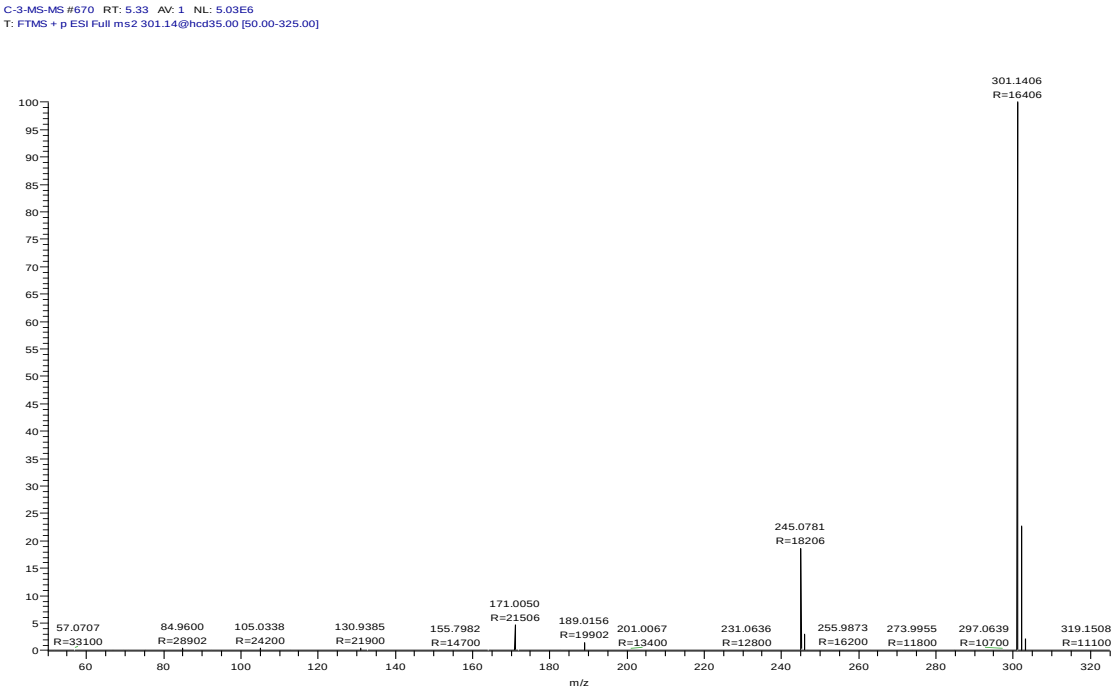


Fig. 4.9. (B) LC-MS and MS/MS spectra for Fraction F4(3) subcomponents.

Fig. 4.10.

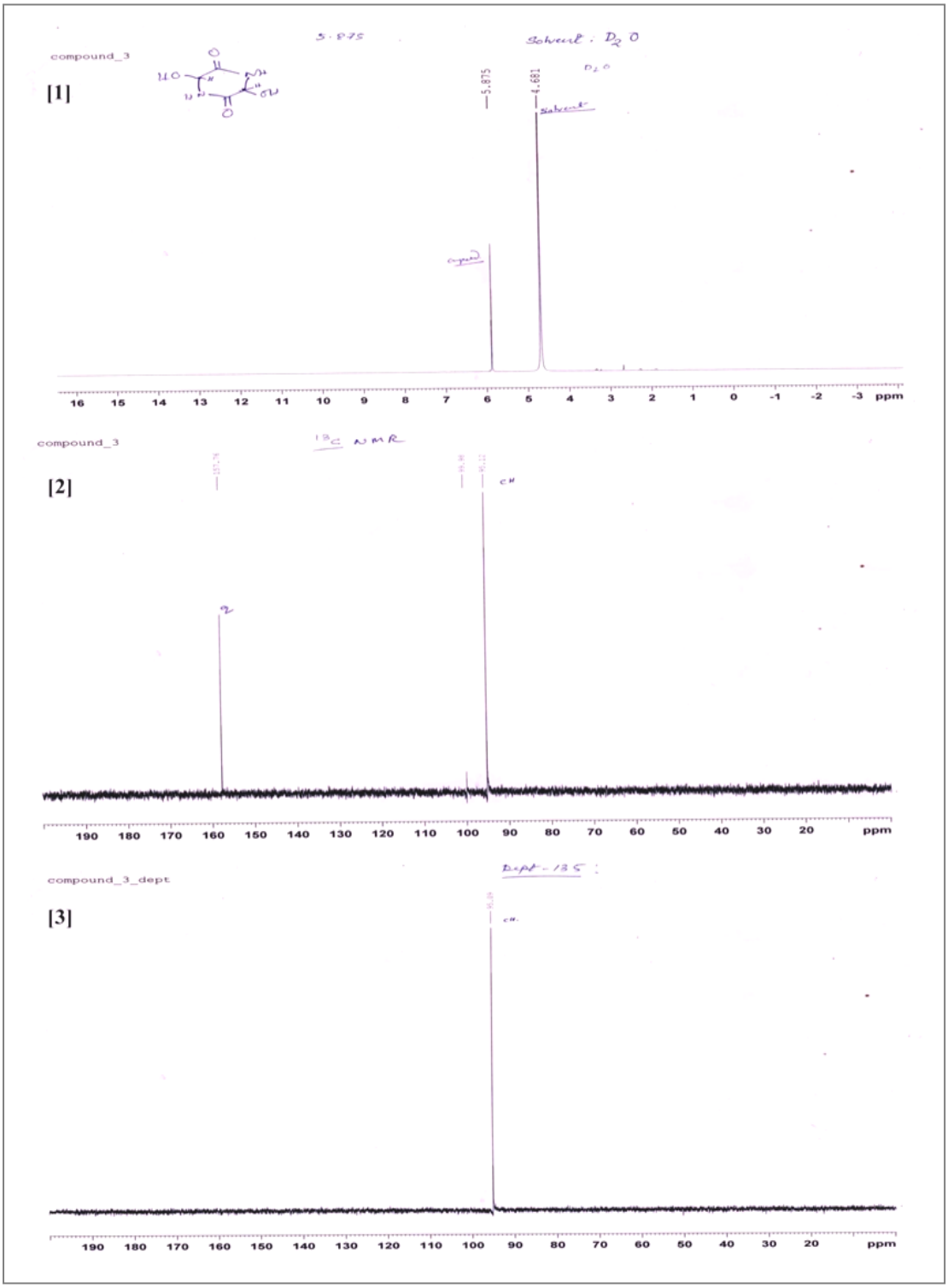


Fig. 4.10. NMR chromatograms for PIGB 77 Fraction F4(3).

[1] ^{1}HNMR [2] ^{13}C NMR and [3] DEPT experiments.

Fig. 4.11. (A)

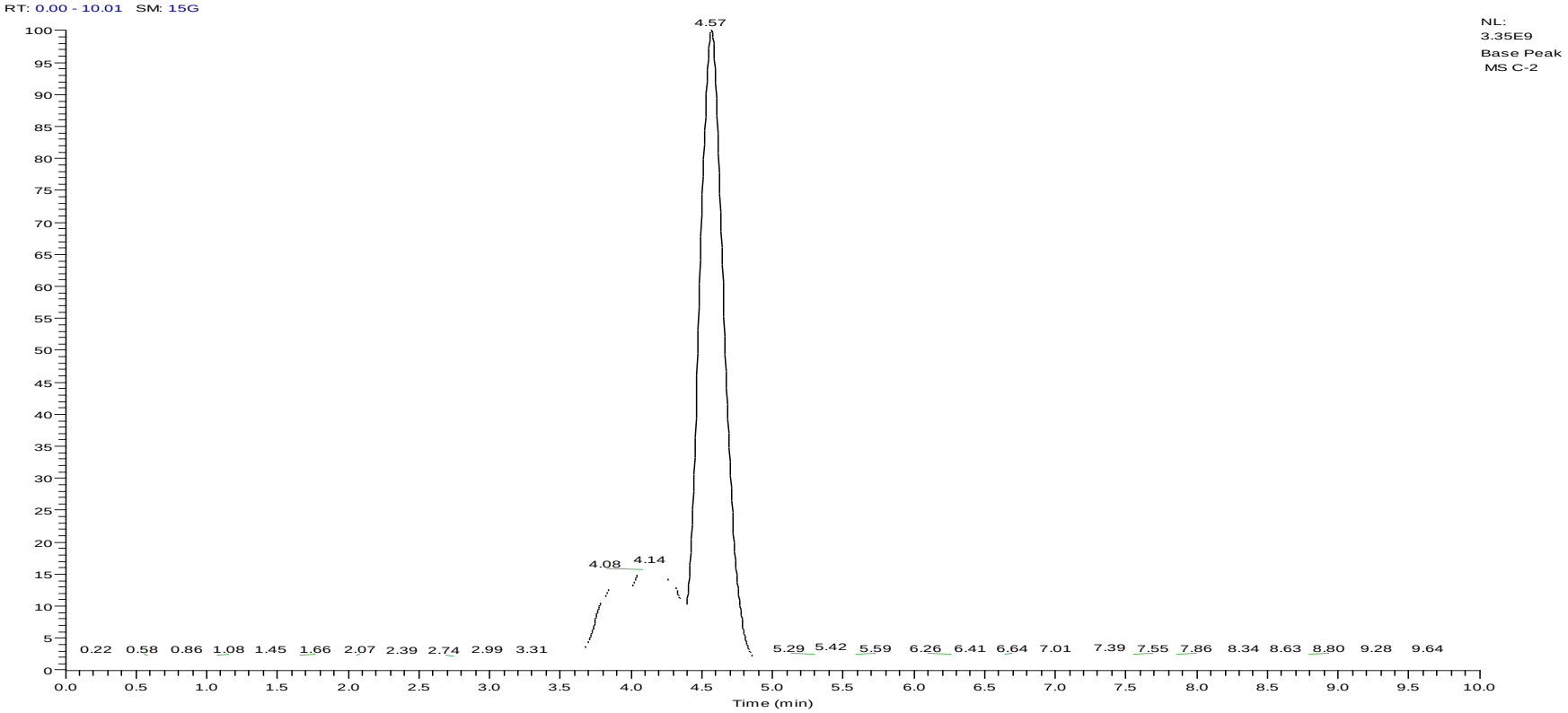


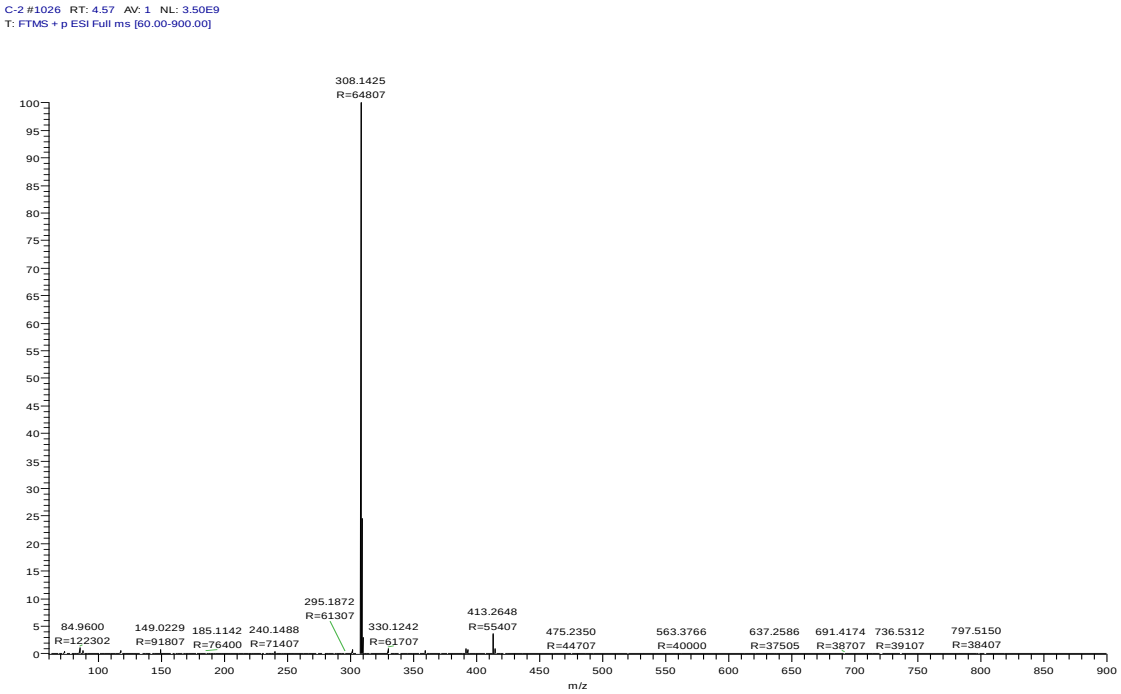
Fig. 4.11. (A) LC-MS spectra for purified antioxidant Fraction F4(2).

Cont…

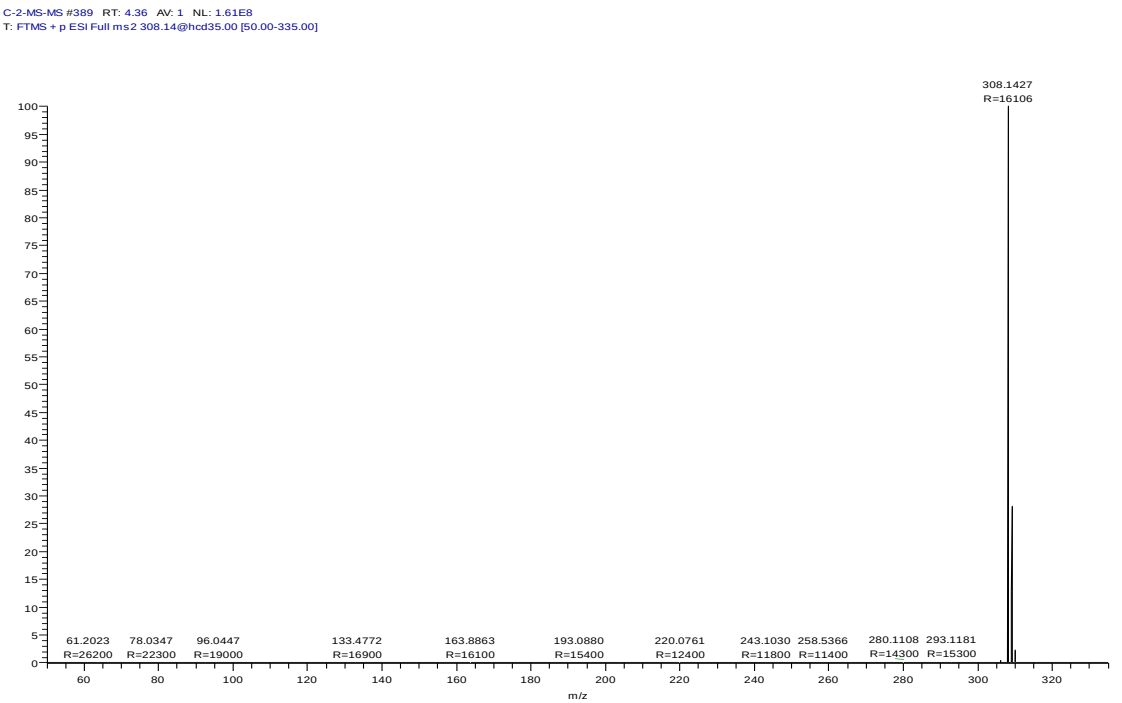
Fig. 4.11. (B)

i) Peak at RT = 4.57 min

m/z 308 MS



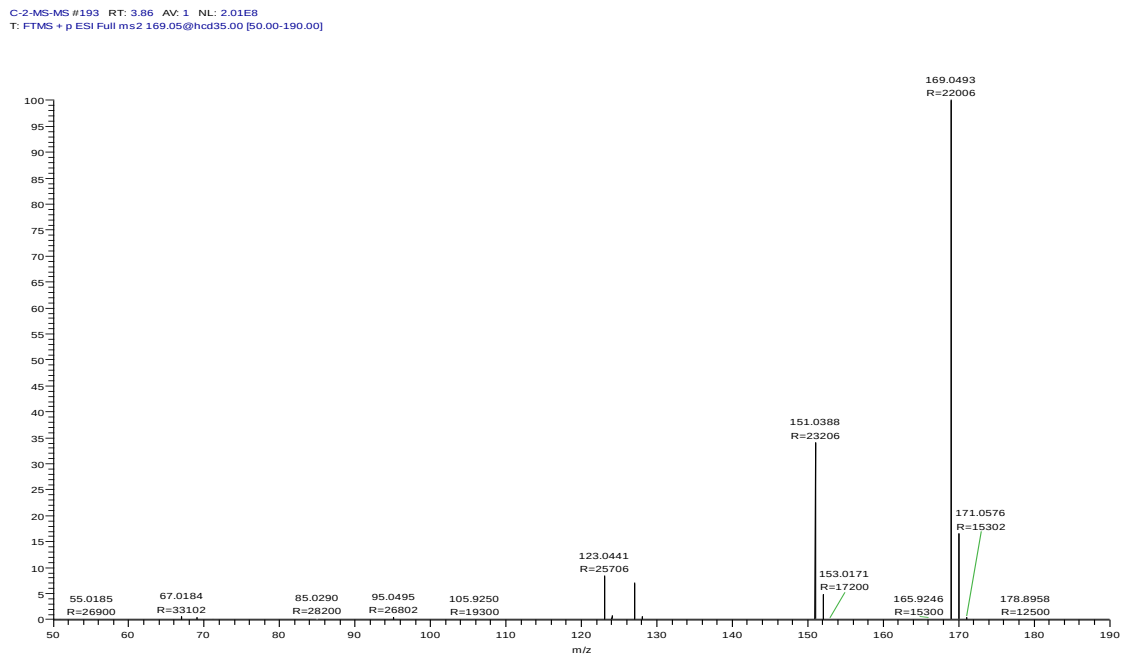
m/z 308 MS/MS



Cont…

ii) Peak at RT = 3.86 min

m/z 169 MS/MS + m/z151 and 123



iii) Peak at RT = 4.00 min

m/z 413 MS/MS

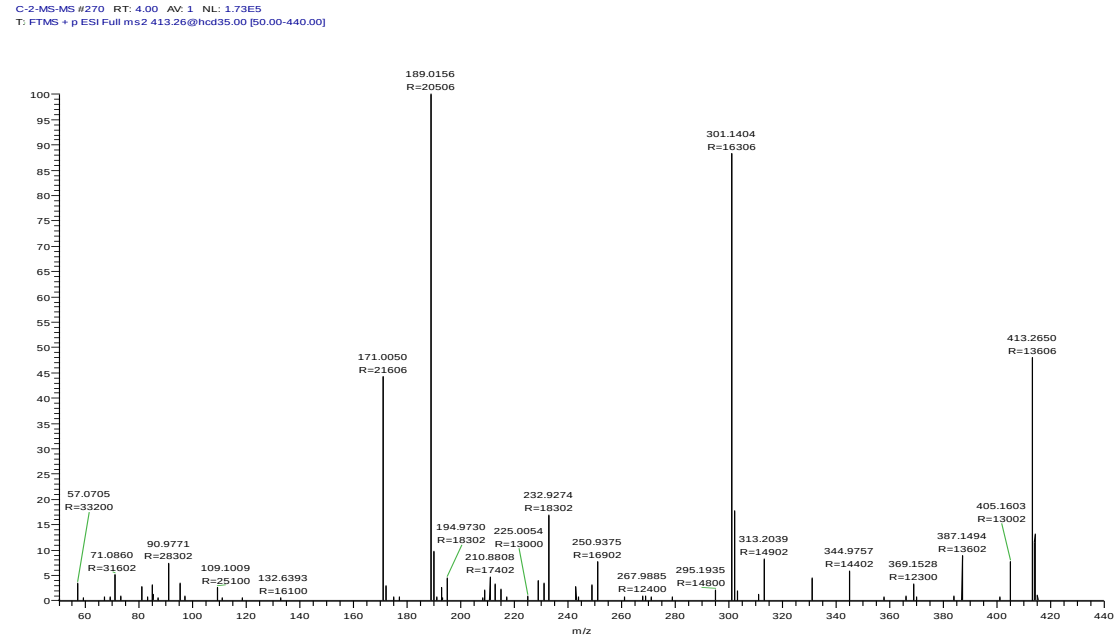


Fig. 4.11. (B) LC-MS and MS/MS spectra for purified antioxidant Fraction F4(2).

Fig. 4.12.

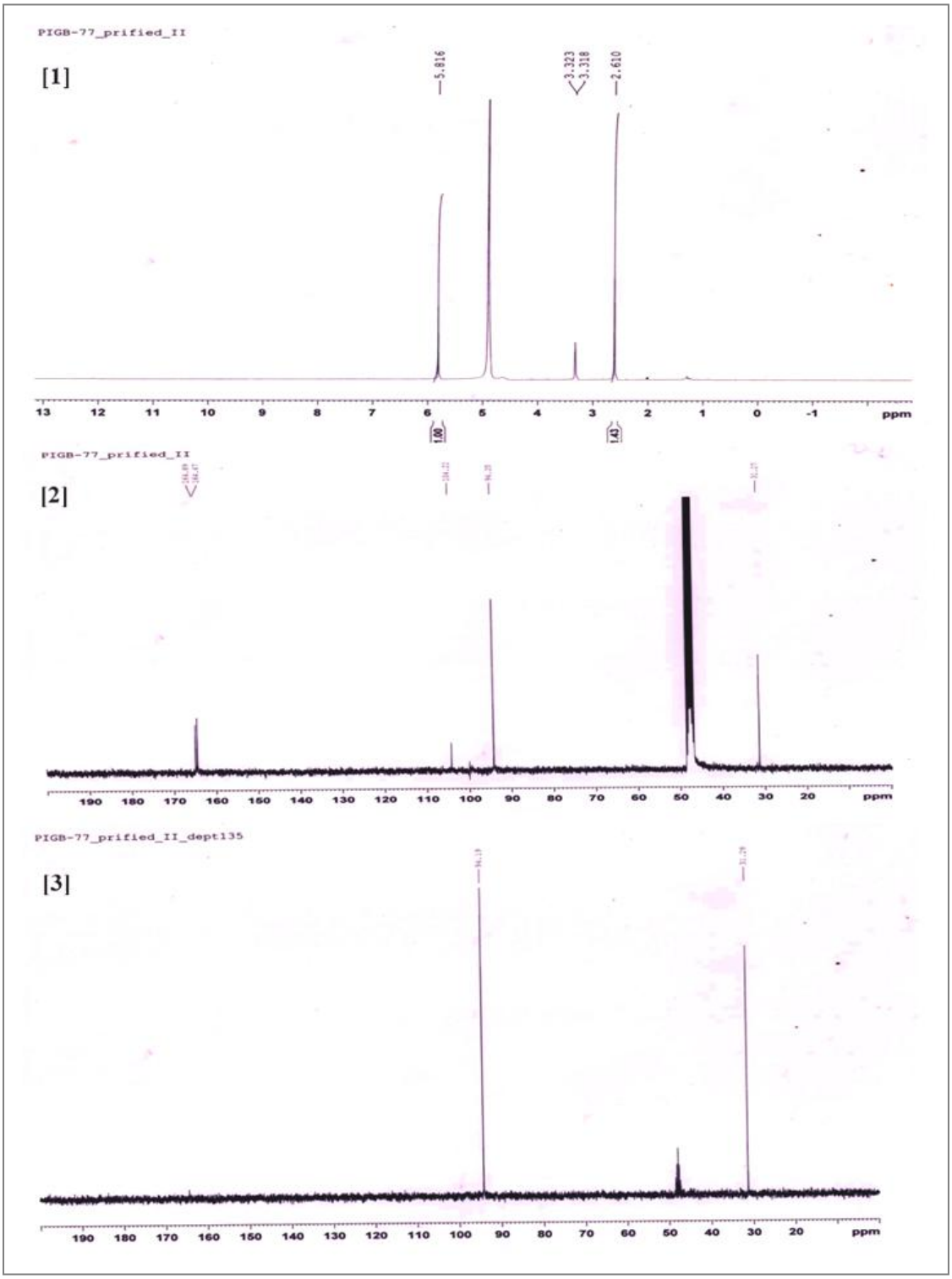


Fig. 4.12. NMR chromatograms for PIGB 77 Fraction F4(2).

[1] ^{1}HNMR, [2] ^{13}C NMR and [3] DEPT experiments.

4.3.3.3. Hydantoinase detection and confirmation of D-amino acid production

i) Plate assay: The formation of above identified active components (Table 4.7) may be confirmed by the production of 5-hydroxy hydantoin and the hydrolyzing enzyme hydantoinase. The positive activity observed by changing the colour from red to yellow by PIGB 77 (*P. koreensis*) within 48 h of the incubation period confirmed the active production of hydantoinase (Fig. 4.13).

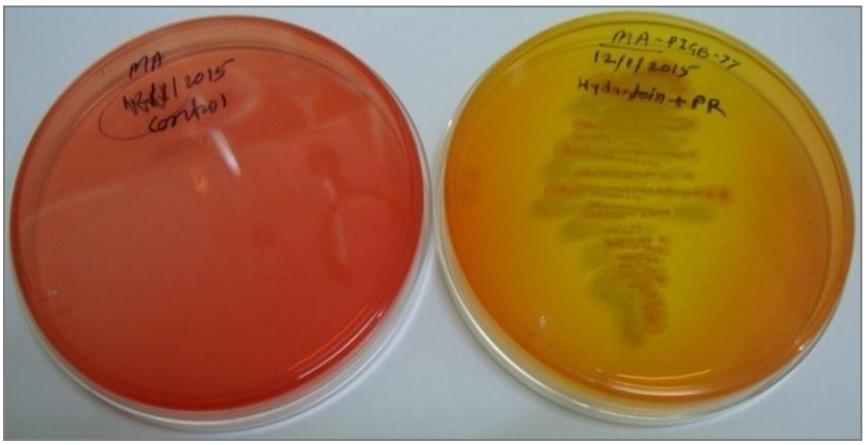


Fig. 4.13. Agar plate assay showing production of hydantoinase by PIGB 77.

ii) Hydantoin hydrolysis bio-catalytic resting cell assay: Hydantoin hydrolysis assay carried out with two different substrates showed more production of amino acids by utilizing the substrate 5–Methylhydantoin (2.44 ± 0.05 µg mg^{-1} protein) than with plane hydantoin (1.08 ± 0.02 µg mg^{-1} protein). In addition the effects of inducers tested, hydantoin showed to induce more production of hydantoinase as compared to that of 2-Thiouracil (Table 4.8). These confirmatory results supported the active production of hydantoinase by PIGB 77.

Table 4.8. Hydantoin-hydrolyzing activity of *P. koreensis* (PIGB 77).

Substrates	Inducer			
	Hydantoin		2-Thiouracil	
	AA µg ml^{-1}	AA µg mg^{-1}	AA µg ml^{-1}	AA µg mg^{-1}
Hydantoin	21.60 ± 0.49	1.08 ± 0.02	14.73 ± 0.52	0.74 ± 0.03
5-Methylhydantoin	48.82 ± 0.95	2.44 ± 0.05	24.89 ± 0.02	1.24 ± 0.00

AA: Amino acids.

4.3.4. Antioxidant metabolites of *Serratia* sp. (PIGB 88 and 163)

4.3.4.1. Purification of AO compounds

UV-Vis spectra of *Serratia* strains gave maximum absorption at 537 nm in visible range and 274 nm at UV range (Fig. 4.14A and 4.14B). The UV range peak (274 nm) affiliated may be due to the presence of pigment like antibiotics (Boudjella et al. 2007). More prompt peak in the visible region by PIGB 163 indicated the production of pigment prodigiosin.

Serratia species is known to produce hallmark pigment called 'prodigiosin' that imparts pink or red colour property to the host organisms. Slater et al. (2003) obtained prodigiosin pigment from *Serratia* sp. ATCC 39006 and demonstrated it to exhibit UV-Vis peak at 534 nm. Likewise, a recent report by Lapenda and coworkers (2014) recorded similar spectrophotometric findings on *S. marcescens* pigments. Concurrent observation on our *Serratia* strains PIGB 88 and 163 in present investigation supported the active production of prodigiosin in the same wavelength of 534 nm.

Fig. 4.14.

(A)

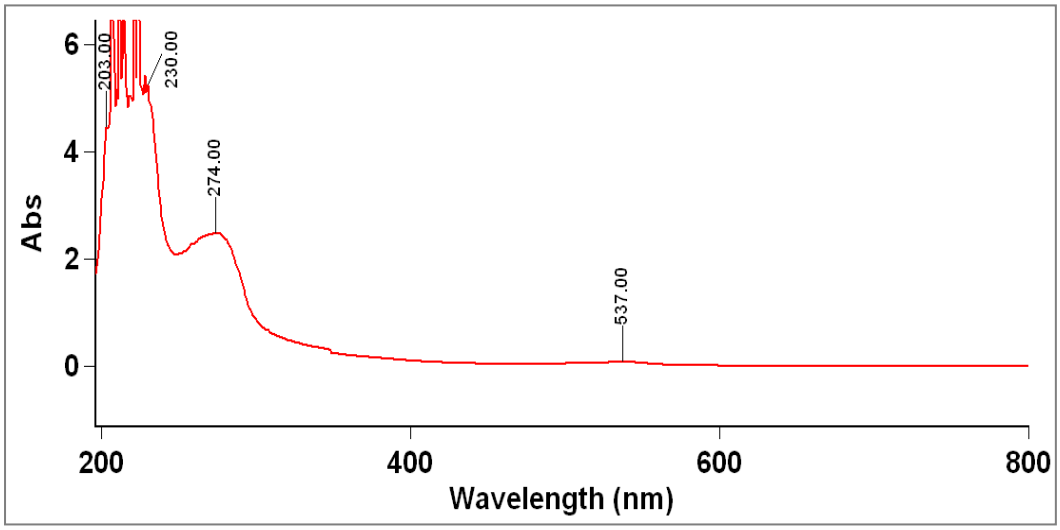


Fig. 4.14. (A) UV-Vis spectrum for the antioxidative metabolites isolated from pigmented strain PIGB 88.

(B)

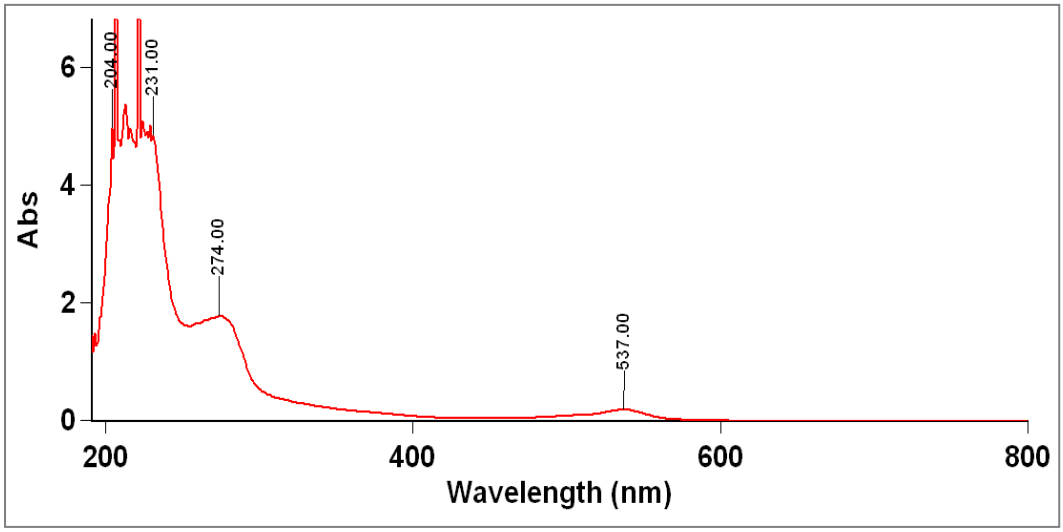


Fig. 4.14. (B) UV-Vis spectrum for the antioxidative metabolites isolated from pigmented strain PIGB 163.

Crude pigmented metabolites of PIGB 88 (400.6 mg) and PIGB 163 (241.6 mg) employed separately to Sephadex LH20 column yielded total 96 and 117 fractions (0.5 ml each) respectively. Qualitative DPPH-TLC check demonstrated the eluted fraction numbers 21-68 (PIGB 88; Fig. 4.15) and 16-47 (PIGB 163) to contain active AO metabolites.

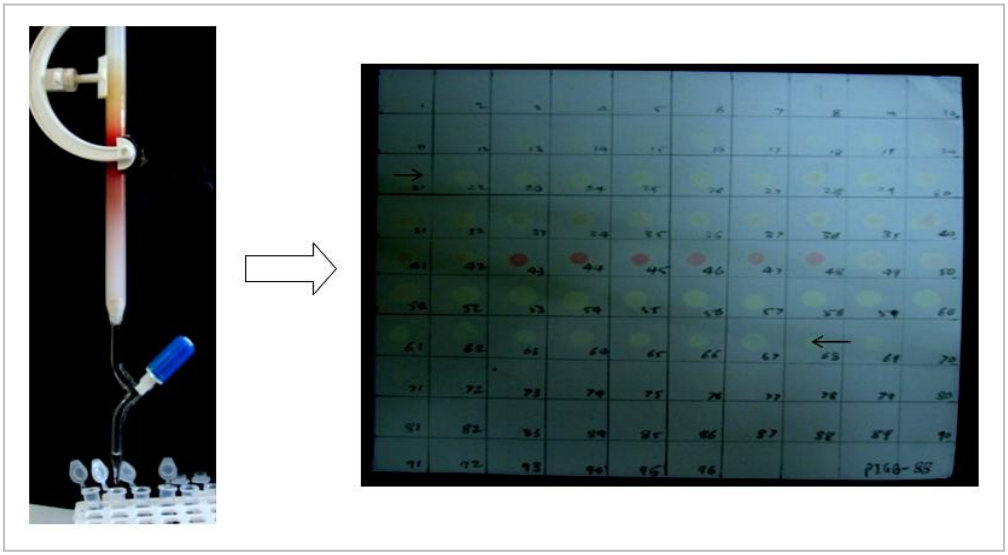


Fig. 4.15. Chromatographic purification and qualitative DPPH-TLC spot test for PIGB88 antioxidant metabolites.

Fractions pooled together based on chromatographic separation and primary DPPH-spot test yielded a total of 6 (F1-F6) for PIGB 88 and 5 (F1-F5) fractions for PIGB 163. Subsequent quantitative AO estimation demonstrated the PIGB88 F5 and PIGB163 F3 to exhibit prominent radical scavenging properties (Table 4.9). PIGB 88 fraction number F5 yielded higher quantity (49.1 mg) of active metabolites with considerable AO activity (32.77 ± 2.42**%**). Contrastingly, the PIGB 163 F3 though had a lower yield (27.9 mg) it showed higher AO activity (39.03 ± 1.44%) than F4 with more yield (79.8 mg) and lower activity (6.42 ± 0.04%). Active fractions were further assessed for AO components.

Table 4.9. Quantification of antioxidant yield and activity on PIGB 88 and PIGB 163.

Strain code	Crude sample	Chromatographic fractions					
		Fraction F1	Fraction F2	Fraction F3	Fraction F4	Fraction F5	Fraction F6
PIGB 88							
Sample wt. (mg)	a: 400.6	4.9	28.8	77.6	32.2	49.1*	17.6
Activity (% DPPH)	b: 53.0 ± 0.56	3.39 ± 0.02	0.91 ± 0.01	10.97 ± 0.40	6.31 ± 0.15	32.77 ± 2.42	4.95 ± 0.21
PIGB 163							
Sample wt. (mg)	a: 241.6	6.2	5.7	27.9*	79.8	35.1	--
Activity (% DPPH)	b: 56.89 ± 0.27	0.22 ± 0.00	3.13 ± 0.09	39.03 ± 1.44	6.42 ± 0.04	7.98 ± 0.39	--

*Represents potent fraction.

Active fractions of F5 (PIGB 88) showed 8 components and F3 (PIGB 163) 5 components analyzed through UV and DPPH guided TLC autography (Table 4.10). Among these three components from PIGB 88 and two components from PIGB163 showed AO property. These components were purified and characterized by GC-MS.

Table 4.10. Antioxidant components from active fractions and their properties.

Pigmented strain	Antioxidant fraction	Number of components in active fraction	A	B	R_f =A/B	Antioxidant property
PIGB 88	Fraction F5	a	0.7	4.4	0.16	NA
		b	1.8	4.4	0.41	++
		c	2.1	4.4	0.48	++
		d	2.4	4.4	0.55	NA
		e	2.7	4.4	0.61	NA
		f	3.1	4.4	0.70	+
		g	3.5	4.4	0.80	NA
		h	3.7	4.4	0.84	NA
PIGB 163	Fraction F3	a	0.5	4.4	0.11	+
		b	0.8	4.4	0.18	+
		c	1.9	4.4	0.43	+
		d	2.2	4.4	0.5	NA
		e	2.5	4.4	0.57	NA

A: Distance traveled by solute, B: solvent front, +: antioxidant active, NA: no activity.

4.3.4.2. Structural and chemical properties of *Serratia* antioxidants

GC-MS enabled characterization of antioxidant components is depicted in Table 4.11; Fig. 4.16 and Fig. 4.17. PIGB 88 and 163 belongs to same genera *Serratia* also contributed similar types of compounds. Both of these strains evidenced to produce aromatic metabolite Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (Compound 1, MW: 210, RT: 30.6 min) and 3,6-Diisopropylpiperazin-2,5-dione

(Compound: 2, MW: 198, RT: 29.8 min). Repeated peaks of compound 1 at RT = 29.4, 29.5, 30.6, 30.8 and 30.9 min etc. showed its dominance in the active fraction and consecutively emphasized its higher production by *Serratia* sp.

Biochemical, spectrophotometric and GC-MS data indicated the *Serratia* sp. (PIGB 88 and 163) to produce Pyrrolo-, Prodigiosin derived pigments. Results also recognized these pigments to exhibit antioxidant and other biological activities. Hitherto, there are no reports on the production of heterocyclic Pyrrolo- type of compounds by marine *S. rubidaea* (PIGB 88) and *S. nematodiphila* (PIGB 163). First time we document the secretion of such metabolites with AO properties from *Serratia* sp.

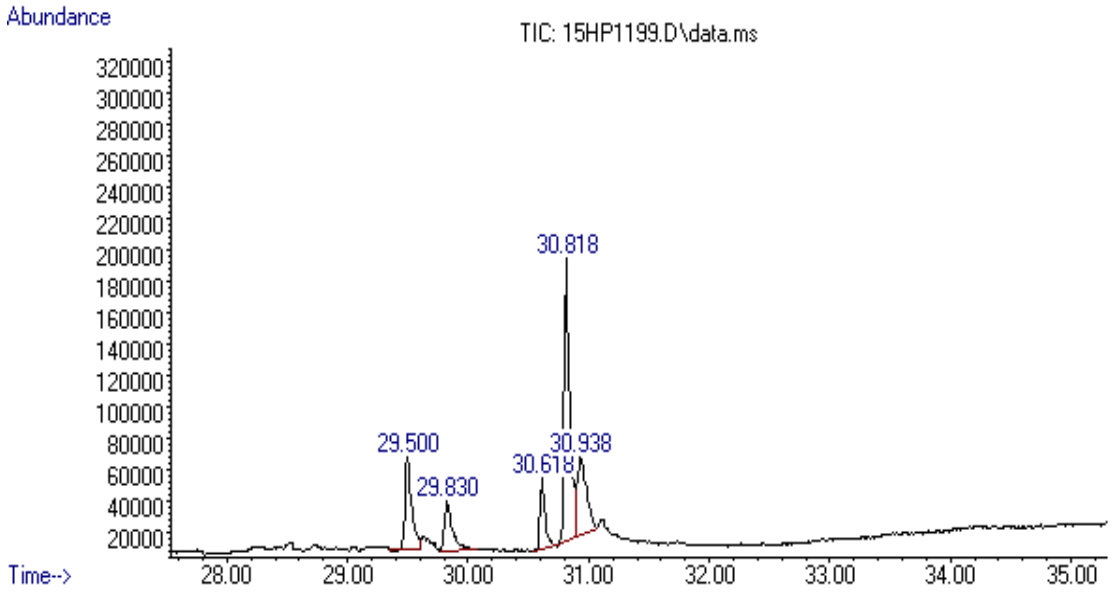
Prodigiosin, a pink/red pigment is ubiquitous in most of the *Serratia* species (Gulani et al. 2012). Chemically this pigment is a bi-pyrrole derivative complex. Studies on isolation, characterization and bioactivity check on this particular pigment have documented its radical scavenging, antioxidant and other biological potentials (antimicrobial, anticancer etc.; Darshan and Manonmani 2015). We also come across Pyrrolo- derived metabolites from our *Serratia* strains and they may be expected to have similar properties and applications.

Table 4.11. Antioxidant compounds from pigmented strains PIGB 88 and PIGB 163.

Compounds name	**Chemical formula**	**Molecular weight**	**Synonyms**
Pyrrolo-[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	1.3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione
3,6-Diisopropylpiperazin-2,5-dione	$C_{10}H_{18}N_2O_2$	198	1.3,6-Diisopropyl-2,5-piperazinedione
Prodigiosin: (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl]-2-methyl-3-pentyl-1H- pyrrole)	$C_{20}H_{25}N_3O$	323	4-Methoxy-5-((5-methyl-4-pentyl-2H-pyrrol-2-ylidene)methyl)-2,2'-bipyrrole

Fig. 4.16. (A)

GC-MS spectra for PIGB 88 Fraction F5



GC-MS spectra for PIGB 163 Fraction F3

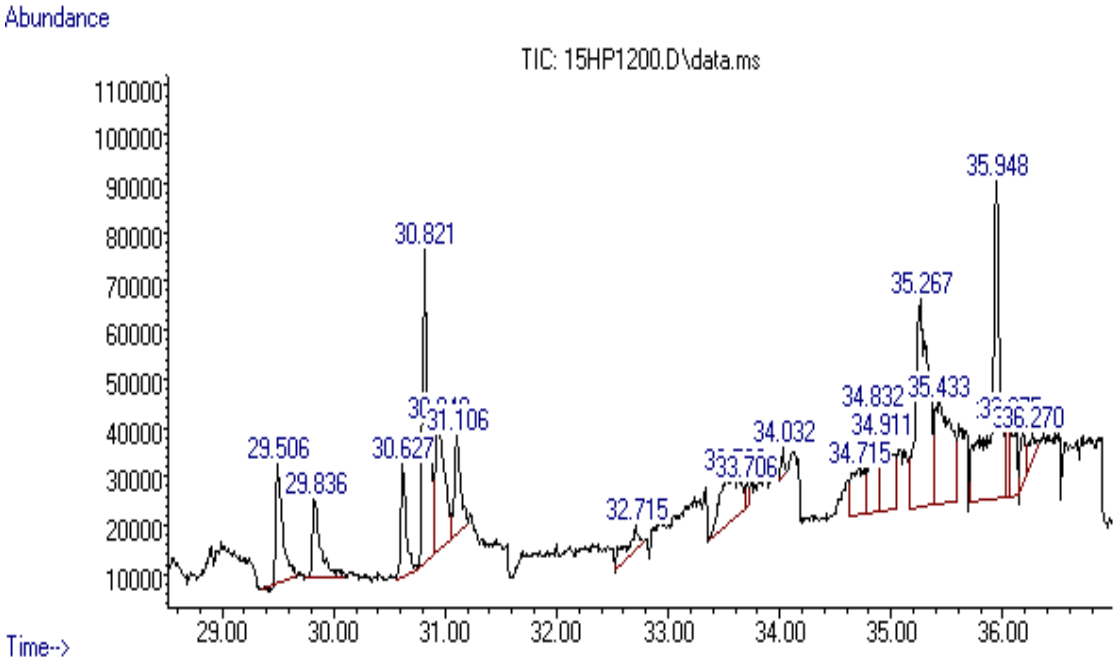
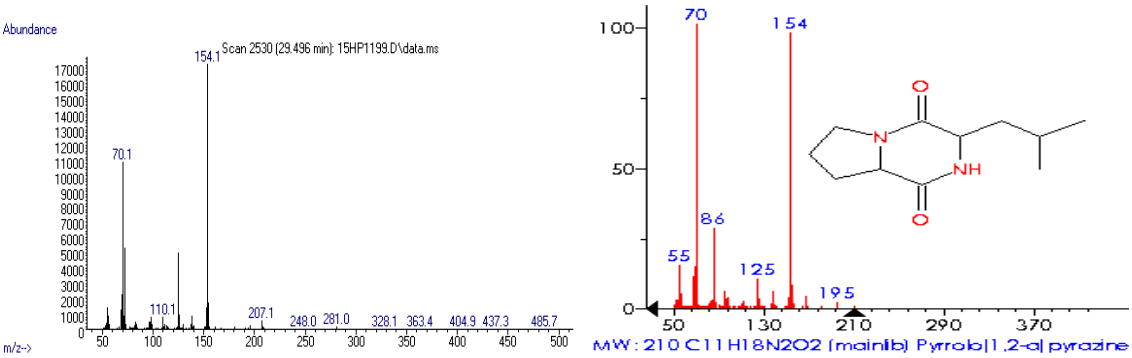


Fig. 4.16. (A) GC-MS spectra on Fraction F5 of PIGB 88 and Fraction F3 of PIGB 163.

Fig. 4.16. (B)

Compound I: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- {RT : 29.49 min}.



Compound II: 3,6-Diisopropylpiperazin-2,5-dione {RT: 29.82 min}.

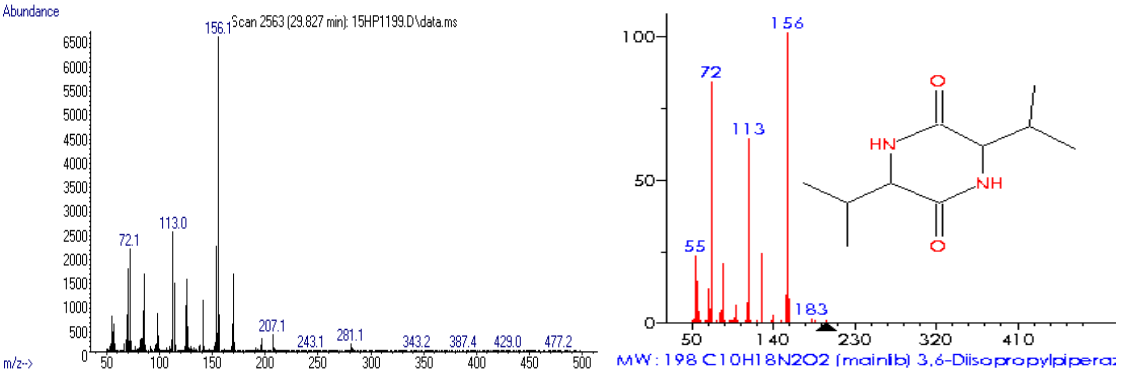


Fig. 4.16. (B) MS/MS spectra on active fractions of *Serratia* sp. PIGB88 and 163.

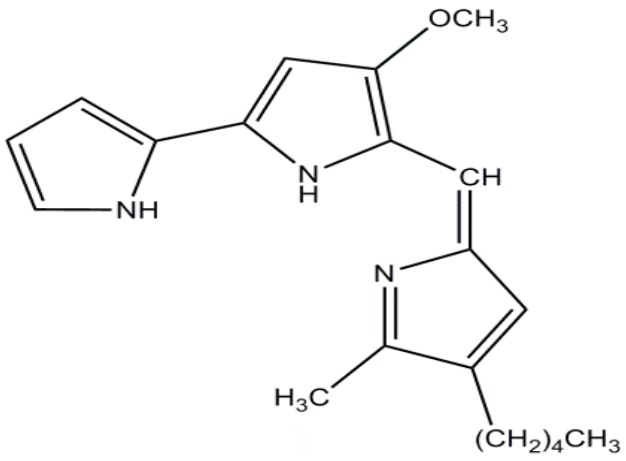


Fig. 4.17. The structure of prodigiosin produced by *Serratia* sp. PIGB 163.

4.3.5. Antioxidant metabolites of *Vibrio* sp. (PIGB 184)

4.3.5.1. Purification of PIGB 184 AO compounds

The PIGB 184 UV-Vis absorption spectrum gave four prominent peaks at 204, 213, 270 and 287 nm (Fig. 4.18). It indicated the presence of poly-unsaturated/ aromatic compounds (E.g. acetylpyrrole or furan; λ= 287 nm). Such pyrrole/ furan compounds and their analogs have been reported to mediate protection to the host cell by either imparting or increasing the antioxidant property (Kitts et al. 2012; Balakrishnan et al. 2015). Conversely, metabolites like acetylfuran and acetylpyrrole are also reported to exhibit other biological activities of human importance (e.g. bactericidal).

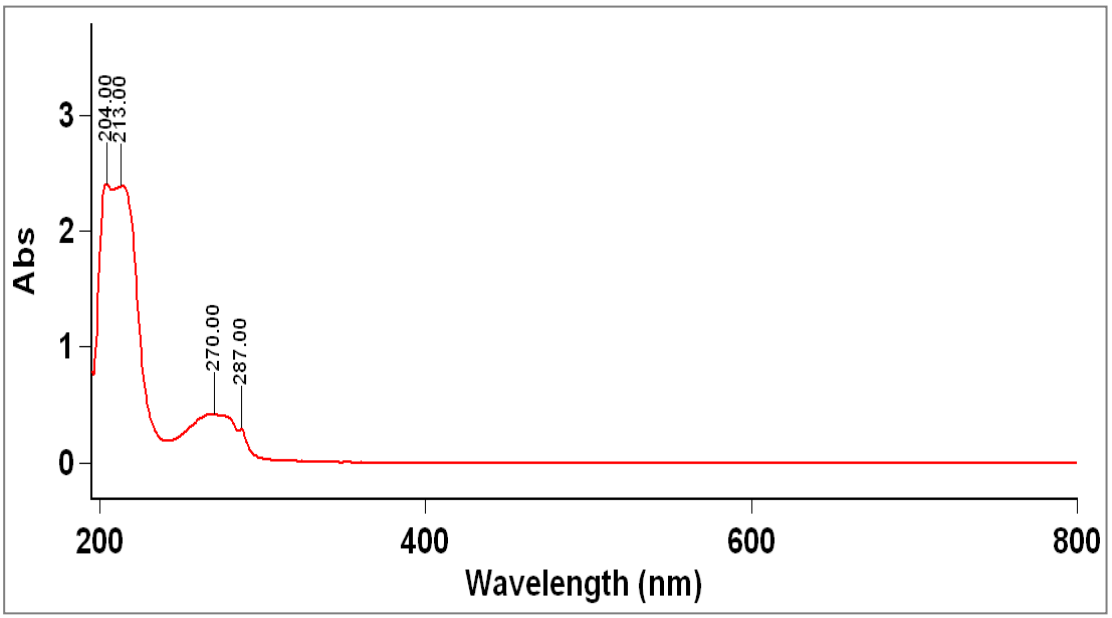


Fig. 4.18. UV-Vis spectrum for the antioxidative metabolites isolated from pigmented strain PIGB 184.

The crude antioxidative PIGB 184 pigmentary metabolites (Total weight: 227.3 mg) subjected to Sephadex-LH20 size exclusion chromatography yielded 114 fractions (0.5 ml each). Resulted fractions tested for antioxidant activity check by DPPH spot TLC test indicated fraction 8-52, 54-61 and 72-79 to contain AO active components (Fig. 4.19).

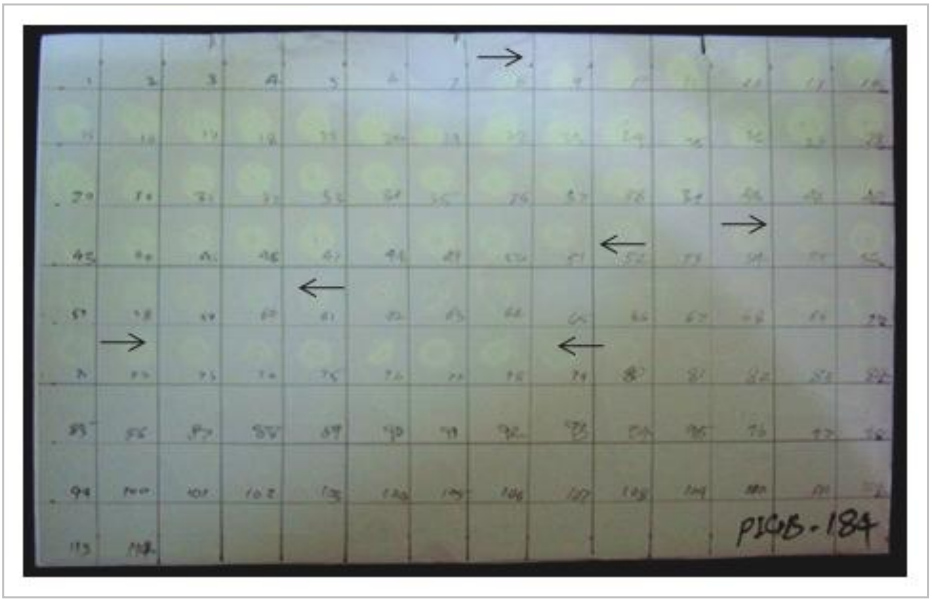


Fig. 4.19. Qualitative DPPH-TLC spot test for PIGB 184 antioxidant metabolites.

Fractions showing positive test pooled together to yield seven main fractions (F1-F7). Quantitative AO activity results on each of these fractions are summarized in Table 4.12. Fraction number F3 exhibited prominent DPPH scavenging (30.89 ± 1.88%) with a total yield of 13.9%. This fraction F3 run on preparative TLC indicated that it's composed of 8 sub-components. UV and DPPH guided detection retrieved 6 AO active components from preparative TLC with increasing order of R_f values 0.2, 0.32, 0.49, 0.62, 0.66 and 0.82. These components further analyzed by GC-MS.

Table 4.12. Quantification of antioxidant yield and activity on PIGB 184.

Experimental stage	Sample weight (mg)	Antioxidant activity (% of DPPH scavenging)	Yield (%)
Initial crude sample	227.3	54.04 ± 0.51	100.00
Fraction F1	3.2	1.83 ± 0.03	1.41
Fraction F2	10.5	6.52 ± 0.06	4.62
Fraction F3*	31.7	30.89 ± 1.88	13.9
Fraction F4	5.2	3.07 ± 0.07	2.19
Fraction F5	19.3	6.60 ± 0.05	8.5
Fraction F6	45.4	12.12 ± 0.14	19.8
Fraction F7	2.1	0.82 ± 0.00	0.92

*Represents potent fraction.

4.3.5.2. Structure and chemical properties of PIGB 184 antioxidants

GC-MS analysis demonstrated the active compounds present in F3 fraction as (1) Phenol, 2,4-bis(1,1-dimethylethyl) [$C_{14}H_{22}O$; MW – 206; RT – 24.8 min], (2) Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- [$C_7H_{10}N_2O_2$; MW – 154; RT – 28.8 min] and (3) Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) [$C_{11}H_{18}N_2O_2$; MW – 210; RT – 29.5 min] (Table 4.13; Fig. 4.20A and 4.20B).

Biological compounds with similar chemical properties from bacterial sources have been reported for antioxidant and antimicrobial properties by earlier workers. For

example, the compound Pyrrolo[1,2-a]pyrazine-1,4-dione derivatives from the marine bacterium *B. subtilis* have been recently attributed to its significant radical scavenging property (Balakrishnan et al. 2015).

GC-MS analysis of antimicrobial components from another marine bacterium *Aeromonas hydrophila* documented the active contribution of Phenol- compounds towards antagonistic activity (Pandey et al. 2011). Padmavathi and co-workers (2014) realized that the Phenol, 2,4-bis(1,1-dimethylethyl) derivative of *V. alginolyticus* exhibit biological (anti-fouling) activities of human importance as well. Similarly, PIGB 184 of our strain also indicates the production of Pyrrolo- and Phenol derivatives with significant antioxidant activity.

Conversely, information retrieved from NCBI PubChem Compound database (CID=7311) on Phenol,2,4-Bis(1,1-dimethylethyl) and its polymer Phenol,2,4-Bis(1,1-dimethylethyl)-,1,1,1-Phosphite represents its industrial importance (Lee 2010). This compound also called as antioxidant No. 33 currently employed in various industrial processes as intermediate oxidizing/reducing agents.

In addition, PIGB 184 compound Phenol, 2,4-bis(1,1-dimethylethyl)- also had a close similarity in its structural and functional (radical scavenging) properties to 2,6-Bis(1,1-dimethylethyl)-4-methylphenol (i.e. BHT). BHT used as a synthetic antioxidant in food, cosmetics and medicines. However, the overdose of BHT is reported to have adverse effects on animal health (Halliwell and Gutteridge 2006). Although we did not test our compound for similar applications, the secretion of related phenolic metabolites (Phenol, 2,4-bis(1,1-dimethylethyl)-) from our strain could form a natural alternative source of antioxidant compounds (Pawar et al. 2016).

Table 4.13. Antioxidant compounds from pigmented strain *Vibrio* sp. (PIGB 184).

Compounds name	**Chemical formula**	**Molecular weight**	**Synonyms**
Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	1. Phenol, 2,4-di-tert-butyl- 2. 2,4-Di-tert-butylphenol 3. 2,4-di-t-Butylphenol 4. 1-Hydroxy-2,4-di-tert-butylbenzene 5. Antioxidant No. 33 6. Prodox 146
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_{10}N_2O_2$	154	1. Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione 2. Pyrrolidino[1,2-a]piperazine-3,6-dione
Pyrrolo-[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	1.3-Isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione

Fig. 4.20. (A)

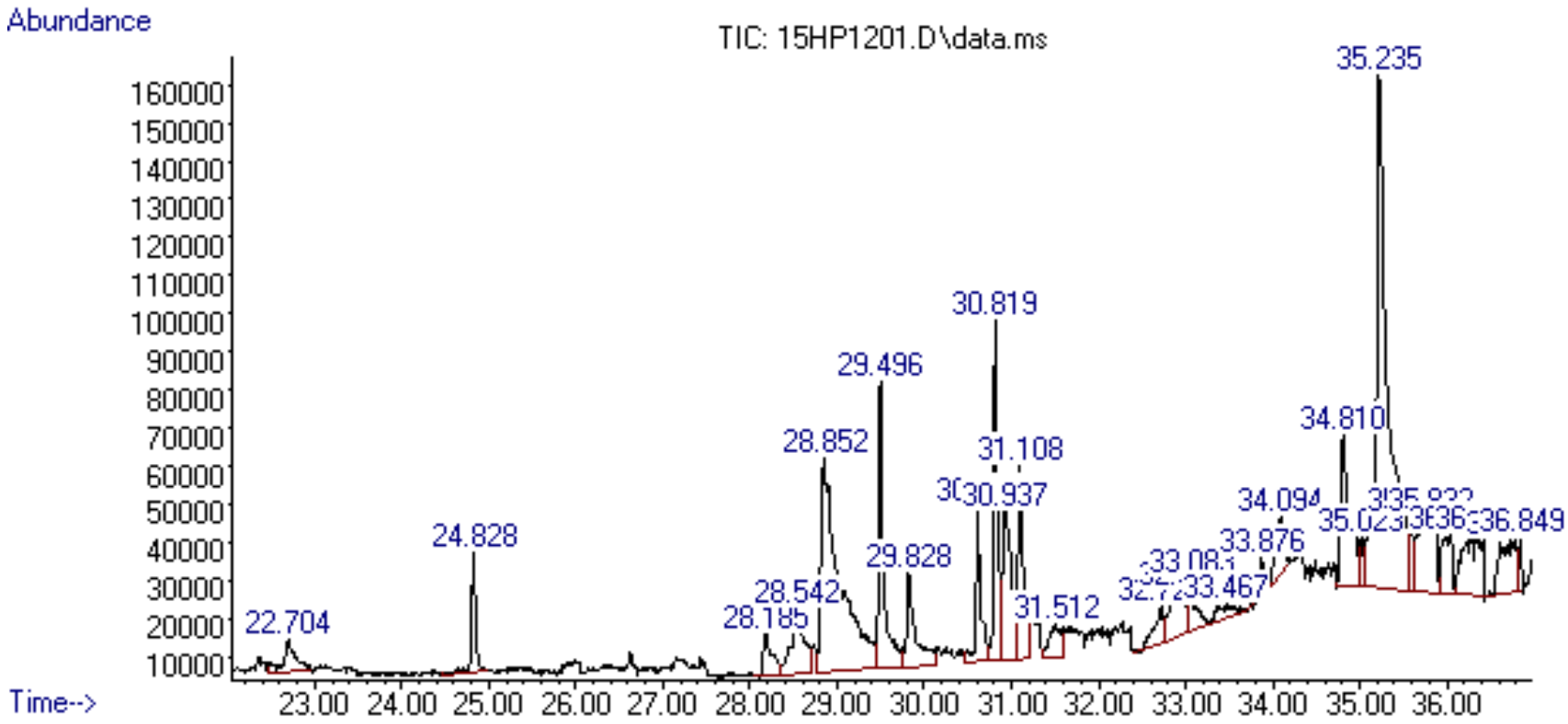
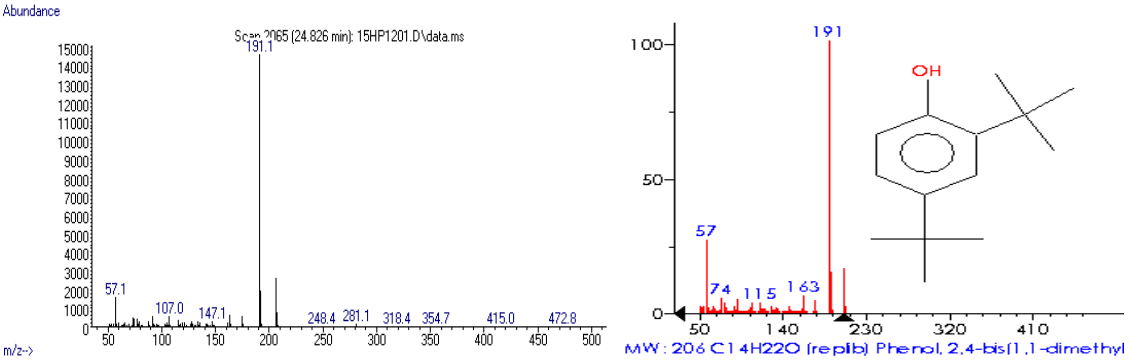


Fig. 4.20. (A) GC-MS spectra on Fraction F3 of PIGB 184.

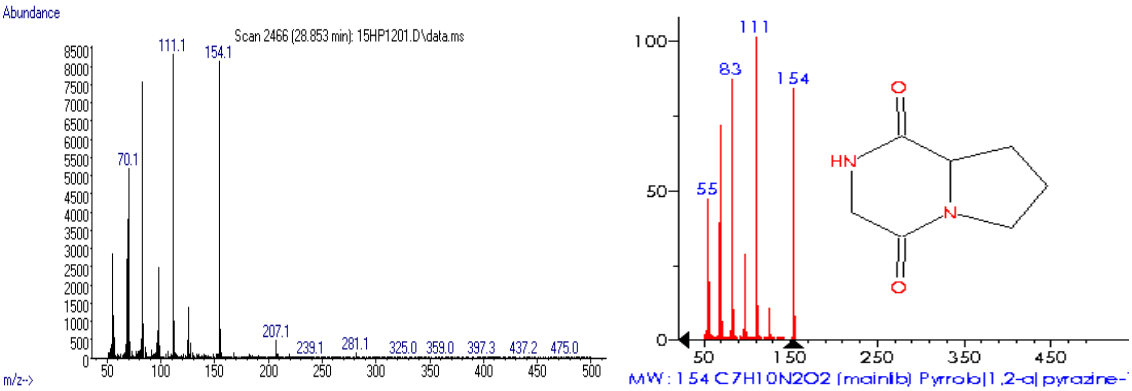
Cont…

Fig. 4.20. (B)

Compound I: Phenol, 2,4-bis(1,1-dimethylethyl)- {RT: 24.8 min}.



Compound II: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- {RT: 28.8 min}.



Compound III: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- {RT: 29.5 min}.

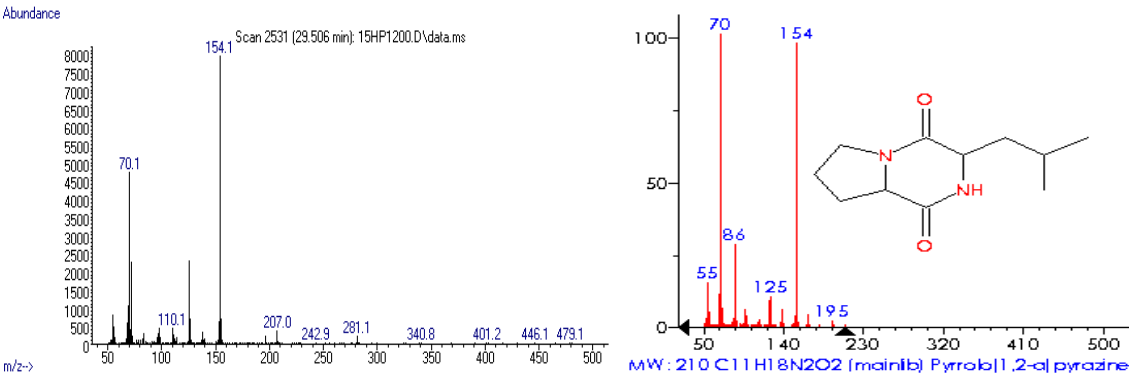


Fig. 4.20. (B) MS/MS spectra on Fraction F3 of PIGB 184.

4.4. Conclusion

The isolation, purification and characterization of antioxidative metabolites studied here demonstrated the most potent strain PIGB 77 as hydantoinase and its related aromatic derivatives. These compounds find applications in the production of D-amino acids with industrial importance.

Purified pigmented compounds from PIGB 46 (*P. argentinensis*) were revealed to be (1) Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-, (2) 3,6-Diisopropylpiperazin-2,5-dione and (3) n-Hexadecanoic acid.

PIGB 184 was evidenced to produce (1) Phenol, 2,4-bis(1,1-dimethylethyl)-, (2) Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- and Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-.

Conversely, the pink coloured bacteria PIGB 88 (*S. rubidaea*) and PIGB 163 (*S. nematodiphila*) demonstrated to secrete antioxidative pigment 'prodigiosin' in addition to common pyrrolo- compounds. Almost all isolates demonstrated to synthesize one or more pyrrolo- derivatives.

Significant radical scavenging property expressed by the pyrrolo-, phenol- and prodigiosin derived from our marine pigmented bacteria may form alternatives to the synthetic antioxidant compounds. Utilization of pigmented bacteria for large scale production of such antioxidants would give a better natural resource for industrial applications.

CHAPTER 5

BIOTECHNOLOGICAL POTENTIAL OF ANTIOXIDATIVE PIGMENTARY METABOLITES

5.1. Introduction

Defensive characteristic of antioxidant against oxidative stress has a significant role in animal physiology and overall health. It plays important functions in nutrition, ageing and disease therapy (Halliwell and Gutteridge 2006). Conversely, it's also been used as an additive in food and in other inanimate articulates to avoid oxidation reactions e.g. rubbers, paints, pesticides etc. (Bieleman 2000). Any compound once determined for antioxidant activity it's necessary to test their putative applications of industrial significance.

The recent advances in search of novel antioxidants from marine organisms are one among the emerging area of modern science (Shindo et al. 2014, Balakrishnan et al. 2015). Oceanic bacteria in order to withstand natural stress are evidenced to produce antioxidative pigmented secondary metabolites (Correa-Llanten et al. 2012; Pawar et al. 2015). Extruding this knowledge and testing such metabolites for diverse bioactive property would give better insights for their *in vitro* and *in vivo* utilization in biological system.

Literature survey over last decade point out that pigmented metabolites having antioxidant property also express one or more other biotechnological activities (Darshan and Manonmani 2015; Shindo et al. 2014). For example, a red pigment prodigiosin and its associated derivatives earlier reported for antioxidant activities are recently recorded to possess clinical properties like antimalarial, antipathogenic and antineoplastic (Kuo et al. 2011). On the other hand, microbial pigments due to their anti-oxidation and their stable properties they are employed as a natural colouring agent in the textile industry

(Alihosseini et al. 2008; Siva et al. 2012). Some of such attributive biological roles on pigmented metabolites are briefly summarized in the introduction section (Chapter 1).

As mentioned earlier, studies on antioxidant and other biological properties of pigmented bacterial compounds specifically of marine origin from the Indian coasts are scarce. In context, a brief investigation on biomedical and industrial properties of such metabolites from the Indian Ocean is expected to reveal novel and cost effective natural sources. Thus, in this chapter we are anticipated to bio-prospect the antioxidative pigmented metabolites from selected CWCI bacteria for their attributive biological activities.

5.2. Materials and methods

5.2.1. Human cancer cell lines and anticancer test

i) Selection of human cancer cell lines:

Distinct types of human cancer cells like lung (A549), breast (MCF7), colon (colo205), melanoma (SK-MEL-2) and nasopharyngeal (KB) were targeted to test the anticancer effect of pigmented metabolites. Selection of distinct cell lines originating and responsible for the particular type of cancer in human was carried out in order to get the fundamental idea of cell inhibition by pigmented metabolites.

ii) Estimation of anticancer activity by pigmented compounds:

The anticancer activity of antioxidant rich fractions from selected pigmented strains (PIGB 46, 77, 88, 163 and 184) was analyzed using Sulforhodamine B (SRB) microtiter

plate assay (Skehan et al. 1990). In details, an aliquot of 90 µl of selected cancer cell suspension was inoculated in microtiter well plate at predetermined densities. Plates were primarily incubated at standard growth conditions (5% CO_2, 95% air, 100% relative humidity) at 37 ºC for 24 h. Later, the known concentration of drug (pigmented sample/ standard) i.e. 10 - 80 µg ml^{-1} was added into each well. The incubation was further continued for 48 h. Later, SRB dye (0.4% in 1% acetic acid) was added to an individual microtiter well and allowed to bind the cells. Unbound stain was washed with 10 mM Tris solution and absorbance was recorded using ELISA plate reader at λ= 540 nm and reference λ= 690 nm. Inhibitory effects were observed in the presence of PIGB sample and activities were compared with known anticancer drug Adriamycin (ADR).

Anticancer activity parameters were calculated as below

a) GI50: Growth inhibition by 50 %. Values were calculated from the formula [(Ti-Tz)/(C-Tz)] × 100 = 50. Where, Tz is the time zero, C is the growth of control cells and Ti represents growth in presence of PIGB sample or the standard drug.

b) The total growth inhibition (TGI) drug concentration was calculated from Ti = Tz.

c) LC_{50}: Concentration of drug that cause 50% reduction in the assessed protein at the end of the treatment in comparison to that at the beginning i.e. indicating a net loss of 50% cells following treatment was calculated from [(Ti-Tz)/Tz] × 100 = -50.

5.2.2. Antimicrobial activity by pigmented bacteria

5.2.2.1. Antibacterial activity

i) Pathogenic organisms: Bacterial pathogens such as *Bacillus megaterium* (NCIM:2087), *Staphylococcus epidermidis* (NCIM:2493), *B. cereus* (NCIM:2156), *Staphylococcus aureus* (NCIM:2079), *Escherichia coli* (NCIM:2065), *Salmonella typhimurium* (NCIM:2501), *Klebsiella pneumoniae* (NCIM:2706), *Pseudomonas aeruginosa* (NCIM:5089), *P. cepacia* (NCIM:2200), *Micrococcus luteus* (NCIM:2169) and *Acinetobacter baumannii* (NCIM:5152) were obtained from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory, Pune.

ii) Antibacterial activity test: The antibacterial potential of antioxidant active fractions from selected bacteria was evaluated by well diffusion assay. Various human pathogenic bacteria obtained from NCIM were initially grown in Mueller Hinton broth (MHB; M391-HiMedia Laboratories, India) until the cell density reached 10^6 cells ml^{-1}. Later, the individual culture was spread plated on Mueller Hinton agar. Immediately wells (0.5 mm) were punched with a sterile cork borer and saturated with 1 mg sample. The standard broad spectrum and third generation antibiotics discs (HiMedia Laboratories, India) were placed on the plates. Plates were first kept at 10 °C to allow complete diffusion of sample drug and then consequently incubated at 37 °C for 24-48 h. Plates were observed after every 6 h of the incubation period for growth and antibacterial zones were recorded.

iii) MIC and IC_{50}: The MIC and IC_{50} values were estimated by microtiter well plate assay against selected pathogens by the method of Horta et al. (2014). Samples were taken with the concentrations of 0.97, 1.95, 3.9, 7.8, 15.6, 31.5, 62.5, 125, 250, 500 and

1000 µg ml^{-1} (just by doubling). Samples were added in separate wells of the microtiter plate and allowed to evaporate the solvents completely under sterile conditions. Each well was added with 198 µl of MHB plus 2 µl of pathogenic bacterial culture. Later, the microtiter plate was incubated at 37 °C for 24 h and the readings were recorded using ELISA plate reader (FLUOstar Omega, BMG LABTECH) at λ = 600 nm. Controls processed in a similar fashion except the addition of sample.

5.2.2.2. Antifungal activity

Fungal pathogens *Aspergillus flavus* (NCIM 538), *A. Niger* (NCIM 545), *Cladosporium* sp. (NCIM 901) and *Fusarium oxysporum* (NCIM 1281) were collected from NCIM resource center, CSIR-NCL, Pune. Cultures were revived on potato dextrose agar (PDA) medium slants. Resulting spores were suspended in saline water (0.85% NaCl) and spread plated on PDA. Antifungal activity was tested by well diffusion method explained in earlier section 5.2.2.1.

5.2.2.3. Anti-yeast activity

Pathogenic yeast cultures *Candida albicans* (NICM 3471), *C. parapsilosis* (NCIM 3323), *C. krusei* (NICM 3515), *C. tropicalis* (NCIM 3118), *Filobasidium uniguttulatum* (NICM 3444), *Cryptococcus laurentii* (NICM 3373) were obtained from NCIM resource center, CSIR-NCL, Pune. Cultures were primarily revived in malt extract-glucose-yeast extract-peptone (MGYP) broth and obtained pure isolates were stored on MGYP agar slants at 4 ˚C for further use. Antiyeast activity was tested by well diffusion method explained in earlier section 5.2.2.1.

5.2.3. Anti-lipid peroxidation activity of pigmented metabolites

i) Sample preparation

Freshly excised sheep liver (SL) and brain (SB) samples were brought from consumer market of Goa, India (Fig. 5.1). Samples were washed with ice-cold potassium chloride (1.15% KCl) and 20% tissue homogenate was prepared in KCl (Sigma-Aldrich, Bangalore). It was further filtered and centrifuged at 10,000 × g for 10 minutes at 4 °C. The post-mitochondrial fractions (PMF) were obtained as per the standard procedure (Pawar et al. 2013). The lipoprotein concentrations in SL and SB samples were determined by Lowry's (1951) method.

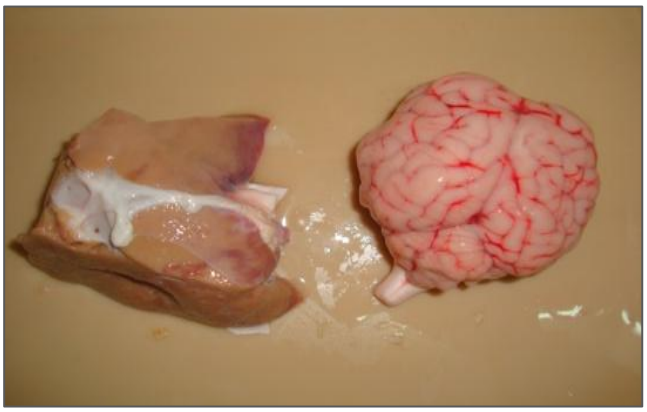


Fig. 5.1. Sheep liver (SL) and brain (SB) samples used for extraction of membrane lipoproteins.

ii) Anti-LPO test

The lipid peroxidation inhibitory efficacy of antioxidative pigmented metabolites on SL and SB membrane lipoproteins was evaluated by modified TBARS method (Ruberto et al. 2000). Briefly, the post-mitochondrial lipid fractions of SL and SB (0.5 ml, 10% v/v) were taken in separate test tubes. An aliquot of 0.1 ml pigmented sample (100, 500 and

1000 µg ml^{-1}) was added. The reaction volume was adjusted to 1 ml with distilled water. Lipid peroxidation reaction was induced by adding oxidizing agent $FeSO_4$ (0.05 ml; 0.07 M). After 30 min of incubation, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH), 1.5 ml TBA (0.8% w/v, prepared in 1.1% sodium dodecyl sulfate) and 20% TCA was added to the reaction mixture. The whole content was vortexed and heated at 95°C for 60 min. Later, 5 ml of butan-1-ol was added to each tube and centrifuged at 2500 × g for 10 min. The OD was measured for the separated organic layer at 532 nm. LPO inhibition was calculated using formula,

$$\text{Anti LPO effect (\%)} = (1 - E/C) \times 100$$

Where, C: absorbance value of the fully oxidized control; E: absorbance in the presence of sample. The results were presented as relative % activity in comparison with commercial antioxidant ascorbic acid (100 µg ml^{-1}).

5.3. Results and Discussion

5.3.1. Antineoplastic effects of pigmented metabolites

The *in vitro* antiproliferative action of pigmentary metabolites from PIGB 46, 77, 88, 163 and 184 tested against 5 distinct human cancer cell lines and their comparison with known drug Adriamycin summarized in Table 5.1 and Fig 5.2. Results demonstrated PIGB 77 (*P. koreensis*) and PIGB 163 (*S. nematodiphila*) to exhibit substantial growth inhibitions on lung (A549), breast (MCF7), melanoma (SK-MEL-2) and nasopharyngeal (KB) cancer cells.

The significant inhibitory values highlight the potential of PIGB 77 (TGI: 54.0 µg ml^{-1}) and PIGB 163 (TGI: 63.4 µg ml^{-1}) against the human lung cancer cell lines A549. Further, as shown in Fig. 5.3 the microscopic observations showed clear differences between treated and non-treated cells. The treated cancer cells had reduced cell numbers, retarded/ amorphous growth and were morphologically shrunken than the normal cancer cells. It emphasized the antineoplastic potential of pigmented compounds from these two strains.

PIGB 88 (*S. rubidaea*) had a considerable anticancer effect on lung A549 cells (GI50: 52.4 µg ml^{-1}). Conversely, the other pigmented strains PIGB 46 and 184 though exhibited the anticancer effect in high doses like 40 and 80 µg ml^{-1} their activities were comparatively less in lower concentrations. All pigmented bacterial samples had LC_{50} values >80 µg ml^{-1}.

Above results on the antiproliferative actions by pigmented compounds were either concurrent or overwhelming the earlier reports of marine bacteria. Ganesh Kumar and co-worker's (2013) isolated melanin pigment from *P. stutzeri* and demonstrated that it inhibits the growth of A549 lung cells up to 80%. Pigmented metabolites from our strain *P. koreensis* with ~100% inhibition of A549 cells supported the anticancer potential of pigmented compounds.

Conversely, Sachdeva et al. (2014) synthesized novel amino acids via green chemistry root and showed such molecules to exhibit anticancer effect with GI_{50} values 41.8 – 67.2 µg ml^{-1}. In contrast, our biological samples supported similar or litter overwhelmed activity with GI_{50} values 25.6 – 63.7 µg ml^{-1}. In addition to above report Shaaban et al. (2008) and Farag et al. (2010) has indicated that the compounds with aromatic nature having heteroatoms (N/S/O) will exhibit significant anticancer effects.

PIGB 77 as explained in chapter no. 4 was evidenced to produce aromatic compounds (e.g. hydantoic acid, hydantoinase and related derivatives) with N and O moieties in their structures. As shown in Table 5.1, the PIGB 77 in comparison with other strains under study showed prominent antiproliferative effects against 3 out of 5 cancer cell lines. Thus, the high potent anticancer activities of this bacterium can be attributed towards heterocyclic compounds expressed by it.

Secondary metabolites from *Serratia* group of organisms are well established for their multifarious biological properties. One of such important characteristic lies in their anticancer potential. A terrestrial strain *S. ureilytica* with a new compound (Serranticin) is demonstrated to inhibit the growth of MCF7 (breast adenocarcinoma) cells by ~51 % at 40 µg ml^{-1} sample concentration (Kuo et al. 2011). Pigmented metabolites from one of our marine bacterium *S. nematodiphila* during the current investigation had remarkable effects on same types of cancer cell line. It allowed the growth of MCF7 only 29.2 % (i.e. 70.8% inhibition) (Fig. 5.2).

Likewise, prodigiosin pigment from another *Serratia* sp. is recently reported to inhibit ~80% growth of multi-drug resistant cancer cells with 100 µM of sample concentration (Elahian et al. 2013). Similar results were recorded on HeLa and KB cancer cell where the cell growth was inhibited up to 80-95% with 7.0 µM concentration of prodigiosin pigment (Sumathi et al. 2014). *S. nematodiphila* (PIGB 163) pigmented compound was observed to exhibit a similar effect on the growth of cancer cell lines with 40-80 µg. Thus, a more inclusive study on isolation of individual compounds from PIGB 163 may reduce the required concentration.

Montaner et al. (2000) obtained supernatant metabolites from *S. marcescens* along with its pigment and reported its apoptosis potential over haematopoietic cancer

cells. Similarly, in another study Montaner and Perez-Tomas (2001) even demonstrated the anticancer potential of prodigiosin towards adenocarcinoma cells of colon cancer. Significant growth inhibition of MCF7, MEL-2 and A549 cells (90-100%) by *Serratia* strain PIGB 163 under present investigation corroborate the anticancer potential of prodigiosin and associated metabolites. Concerning *Vibrio* sp., bioactive compounds isolated from this genus are also documented to exhibit anticancer effects (Prabhu and Chandrasekaran 1999; Al-Zereini et al. 2010). Unfortunately, our *Vibrio* strain (PIGB184) though had a good amount of antioxidant activity it did not give considerable results on the anticancer test.

Fig. 5.2.

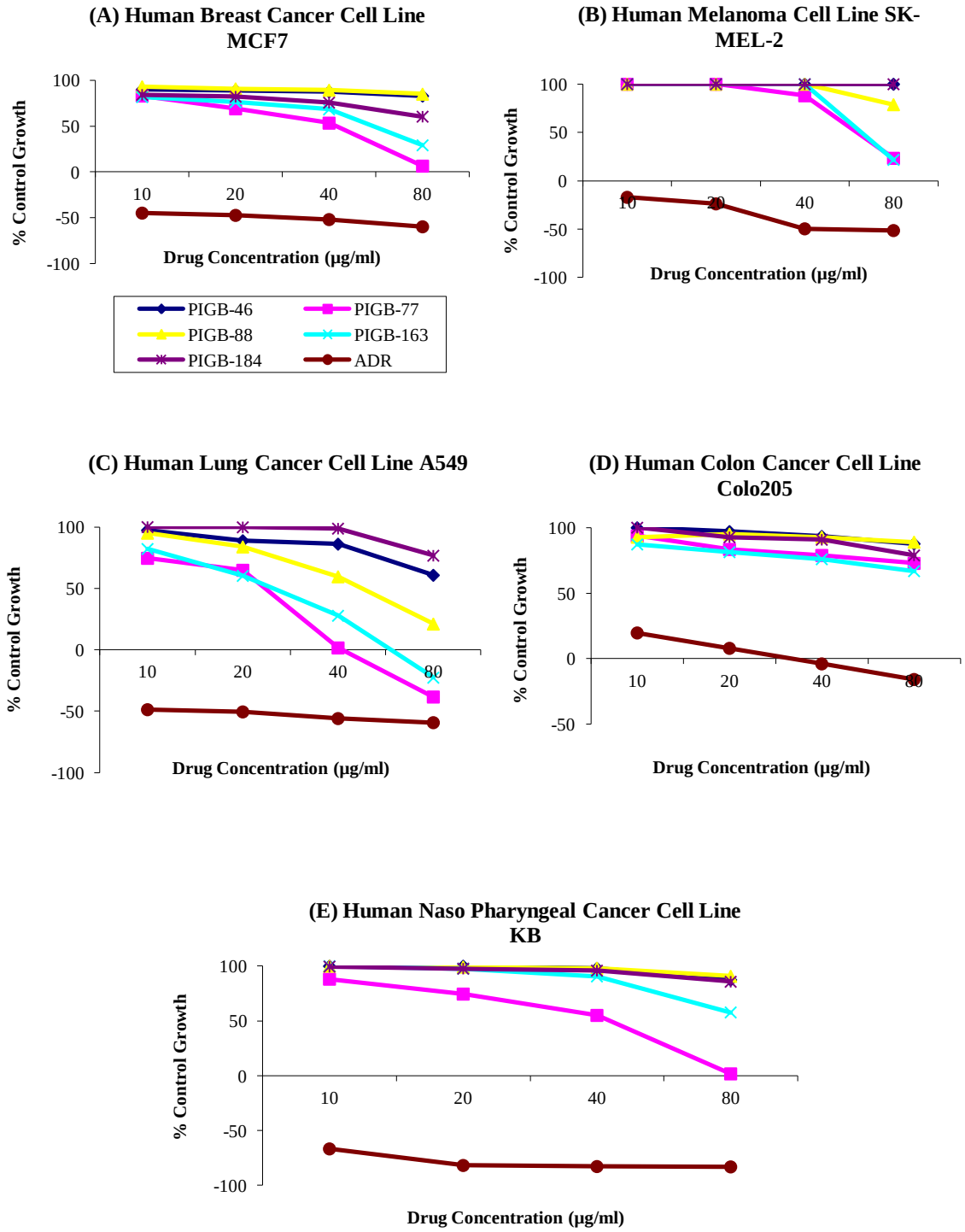


Fig. 5.2. Growth inhibition of human cancer cells by antioxidative pigmented metabolites.

Table 5.1. Anticancer activities of antioxidative pigmented metabolites.

Compound	Cancer cell lines and drug concentrations (μg ml^{-1})														
	MCF7			MEL-2			A549			Colo205			KB		
	LC_{50}	TGI	GI_{50}	LC_{50}	TGI	GI_{50}	LC_{50}	TGI	GI_{50}	LC_{50}	TGI	GI_{50}	LC_{50}	TGI	GI_{50}
PIGB 46	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
PIGB 77	>80	>80	**42.0**	>80	>80	**63.7**	>80	**54.0**	**25.6**	>80	>80	>80	>80	>80	>80
PIGB 88	>80	>80	>80	>80	>80	>80	>80	>80	**52.4**	>80	>80	>80	>80	>80	>80
PIGB 163	>80	>80	**57.0**	>80	>80	**66.1**	>80	**63.4**	**30.7**	>80	>80	>80	>80	>80	>80
PIGB 184	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
ADR	53.8	14.8	<10	61.4	24.9	<10	52.5	12.8	<10	>80	51.1	<10	35.8	<10	<10

ADR: Adriamycin, GI_{50}: growth inhibition by 50%, TGI: total growth inhibition, LC_{50}: Lethal concentration that causes a net loss of 50% cell protein after drug treatment. Letters represented in bold demonstrates potent activity.

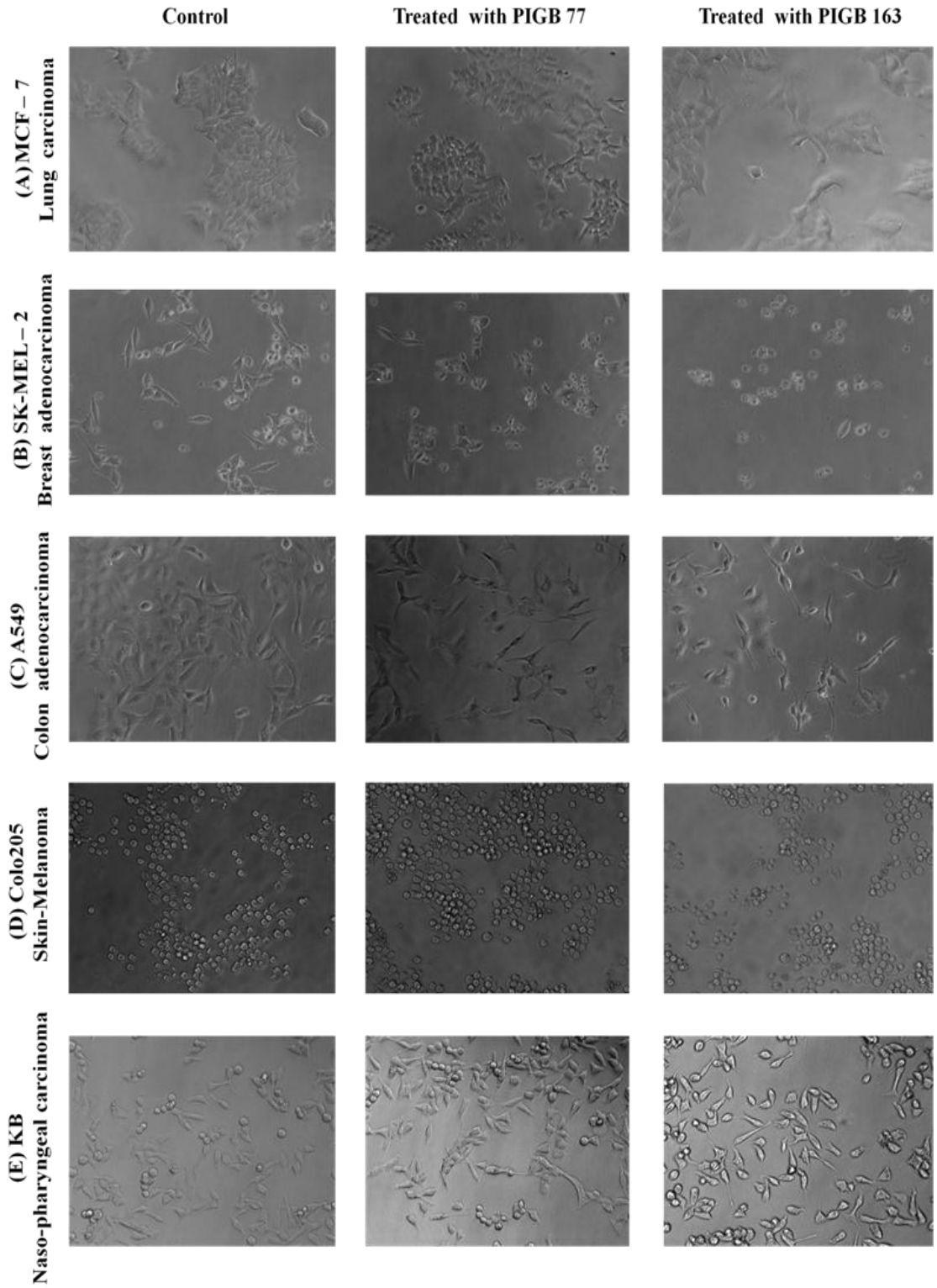


Fig. 5.3. Pictorial representation depicting the inhibitory effects on human cancer cells lines.

5.3.2. Antimicrobial potential of pigmented compounds

Pigmented metabolite tested for their ability to hamper the growth of human pathogenic microorganisms against standard antibiotics is summarized in Table 5.2A; 5.2B and Fig. 5.4A; 5.4B. Bacterial strains PIGB 77, 163 and 184 had considerable antimicrobial effects while the PIGB 46 and 88 had limited activity against particular types of pathogens.

5.3.2.1. Antibacterial properties

i) PIGB 77 (*P. koreensis*)

Antioxidative metabolites from the brown pigmented bacterium (PIGB77) demonstrated positive activity against all the pathogenic bacteria tested. It showed higher antibacterial zones (16-21 cm) against gram-positive *B. cereus*, *B. megaterium*, *S. aureus* and *S. epidermidis* and required lowest sample concentrations (MIC: 3.91-7.81; IC_{50}: 0.31-0.95 µg ml^{-1}) to inhibit the growth. Interestingly, the metabolites of PIGB 77 not only showed 2-3 fold higher activities than other pigmented strains tested under this study but also showed to inhibit the growth of more pathogens (Table 5.2A and Fig. 5.4A). Concerning Gram-negative pathogens, *K. pneumoniae* and *S. typhimurium* were comparatively more sensitive than *E. coli* and *P. aeruginosa*. Broad spectrum and third generation antibiotics tested with our compound, we realized that the pigmented metabolites express more or less similar inhibitory potential like Chloramphenicol (22 ± 2 mm) towards *B. cereus* and Tetracycline/Ceftazidime (18 ± 2 mm) towards other pathogenic bacteria (Table 5.2A and 5.2B).

Bacteria belonging *Pseudomonas* genera are well documented to exhibit diverse antagonistic activities (Mandryk et al. 2007; Cardozo et al. 2013). Concerning studies on antibacterial pigments, Darabpour et al. (2010) tested ethyl acetate extract from a brown pigmented *Pseudomonas* sp. against various human pathogens. Among different pathogens studied, they showed it to express higher antibacterial zone (30 mm) against methicillin-resistant *S. aureus* (MRSA) at 100 mg ml^{-1} sample concentration. Likewise, an antibacterial substance i.e. pigmented Siderophore from *P. fluorescens* is demonstrated to exhibit maximum of 14 mm inhibition zone against pathogen *S. aureus* (Waleed 2009). Pigmented metabolites from our bacterium *P. koreensis* excelled over this activity by showing a zone of 21 mm at a much lower sample concentration of 1 mg ml^{-1}.

We also reviewed antibacterial studies irrespective of pigmented compounds on *Pseudomonas* sp. For example, Haba et al. (2003) isolated a Rhamnolipids from *P. aeruginosa* and reported it to obstruct the growth of human pathogens *B. Cereus, M. luteus, S. epidermidis* and *S. aureus* (MIC: 32-64 µg ml^{-1}). In comparison, metabolites from PIGB 77 exhibited positive results at much lower sample concentration against same pathogens (MIC: 3.91 – 7.81 µg ml^{-1}). In another study, El-Sheshtawy and Doheim (2014) demonstrated bio-surfactant isolated from terrestrial strain *P. aeruginosa* ATCC-10145 inhibit the growth of pathogenic *Bacillus* and *Micrococcus* species with inhibitory zones 20 and 21 mm respectively. Pigmented metabolites from seaweed associated marine bacterium (PIGB 77) during present study evidenced to express consistent results (AM zones: 20 and 16 mm) against such pathogens. Similarly, the antibacterial zone of PIGB 77 surpassed the results of Subramaniyan and Saravanan (2015) who showed *Pseudomonas* metabolites to exhibit inhibitory zones of *S. aureus* (10 mm), *S. epidermidis* (10 mm) and *P. aeruginosa* (13 mm) respectively.

ii) PIGB 163 (*S. nematodiphila*)

The selected strain PIGB 163 had ~ 2 to 2.5 fold higher inhibitory zones (10 - 18 mm) against most of the pathogens and stood the second most potent strain in terms of their antibacterial effects. Metabolites from this bacterium were also observed to excel antibacterial property (*B. cereus*: 18 ± 4 mm zone) over the standard antibiotics CPD, CAZ, AMP, CTR and TE (2 - 16 ± 1 mm). It also actively inhibited the growth of various other pathogens in the order of *P. cepacia* (20 ± 3) > *P. aeruginosa* (18 ± 2) > *S. aureus* (17 ± 2) > *S. epidermidis* (16 ± 3) > *B. megaterium* (10 ± 2) respectively.

Recent studies indicate metabolites of *Serratia* do play a significant role in inhibition of human pathogens (Gulani et al. 2012; Ugras et al. 2013). They demonstrated *S. marcescens* to inhibit the growth of *B. Cereus* and *S. epidermidis* with 10-20 mm antibacterial zones. However, none of these reports could record activity towards Gram-negative *P. aeruginosa*. Our bacterium PIGB 163 gave not only similar inhibitory zones against Gram-positive pathogens (9-18 mm) but also showed a marked inhibition of *P. aeruginosa* (AM zone: 18 ± 2). Ugras et al. (2014) explored the actual components responsible for antibiotic activity and confirmed as bacteriocin. It indicates antagonistic activities are determined by the type of compound secreted by individual bacteria.

Serratia is one of the most studied bacterium for its biotechnological applications. Its metabolites are reported to possess potential antimicrobial, antioxidant, anticancer and antimalarial activities. Antimicrobial properties of *Serratia* attributed towards pigment prodigiosin, bacteriocin-like antibiotics and aromatic compounds (Ugras et al. 2014; Sumathi et al. 2014; Lapenda et al. 2014). Conversely, the synthetic heterocyclic aromatic compounds like pyrrole scaffolds are also recently acknowledged

for antibacterial and antifungal effects (Joshi et al. 2013). In concern, the antibacterial activity shown by PIGB 163 could be ascribed by the production of prodigiosin and aromatic pyrrolo- compounds.

iii) PIGB 184 (*Vibrio* sp.)

Antioxidative pigmented metabolites of another potent strain PIGB 184 (*Vibrio* sp.) significantly inhibited the growth of 5 Gram-positive and 3 Gram-negative pathogens (Table 5.2 A and 5.2 B). It exhibited maximum activities against *A. baumannii* (10 ± 3 mm) and *P. aeruginosa* (8 ± 2 mm) in terms of antibiotic zones at 1000 µg sample concentration. However, the *S. aureus* (6 ± 1 mm) and *B. megaterium* (5 ± 1 mm) were less prone to the inhibitory actions of PIGB 184.

Commercial antibiotic sensitivity discs of broad spectrum Ampicillin and the third generation (CAZ- Ceftazidime, CPD- Cefpodoxime, CTR- Ceftriaxone) tested with PIGB 184 showed ~ 1.5 to 2.5 fold increases against the human pathogen *P. aeruginosa* (Table 5.2A). The MIC tested with the PIGB 184 inhibited Gram-positive bacteria more efficiently (MIC: 31.25 – 62.5 µg ml^{-1}) than the Gram-negative bacteria (MIC: ≥1000 µg ml^{-1}).

The literature on the antibacterial property of pigmented compounds from marine *Vibrio* sp. is limited. A novel 'nitro maleimide' aromatic metabolites from sponge associated *Vibrio* sp. is reported to exhibit antibacterial property against *M. luteus* with MIC 6.25-100 µg ml^{-1} (Al-Zereini et al. 2010). MIC of *Vibrio* sp. (PIGB 184) in current investigations demonstrated near about 31.25 µg ml^{-1} against *M. luteus*. Further, these results were comparable with the *Vibrio* species isolated from the east coast of India (Anand et al. 2006) as well as Mediterranean Sea (Dupont et al. 2013).

Phenolic derivatives of *Vibrio* sp. (Horta et al. 2014) have been evidenced to exhibit antibacterial properties against *B. subtilis* (IC_{50}: 58.86 µg ml^{-1}) and *S. aureus* (IC_{50}: >1000 µg ml^{-1}). PIGB 184 pigmented metabolites in the present study had better inhibitions against *Bacillus* (EC_{50}: 9.36-23.69 µg ml^{-1}) and *Staphylococcus* pathogen (EC_{50}: 26.82 µg ml^{-1}). Hence, antagonistic compounds produced by our *Vibrio* sp. PIGB184 also underline the potential of bacterial phenolics towards the development of novel antibiotic agents.

Notably, an interesting observation we made was that the available literature mainly reports the antibacterial resistance towards Gram-negative organisms, but our results with pigmented compounds support more action against Gram-positive pathogens. Darabpour et al. (2010) documented similar effects where the pigmented compounds had more inhibition of Gram-positive pathogens than Gram-negative one. These evidenced highlights the importance of pigmented compounds towards control of Gram-positive human pathogenic bacteria. Antibiotic sensitivity and resistance may depend on the metabolites produced by the organisms and also its bioactive properties. Here in this study, it may be seen that the marine pigmented bacteria produces mainly phenolic compounds which help to control the growth of most of the clinical pathogens.

Table 5.2. (A) Antibacterial activities of pigmentary metabolites against opportunistic human pathogens.

Pathogenic strains (NCIM code)	Pigmented bacteria (PIGB)														
	PIGB 46			PIGB 77			PIGB 88			PIGB 163			PIGB 184		
	AB zone	MIC	IC_{50}	AB zone	MIC	IC_{50}	AB zone	MIC	IC_{50}	AB zone	MIC	IC_{50}	AB zone	MIC	IC_{50}
B. cereus (2156)	5 ± 2	>1000	1190	20 ± 1	3.91	0.95	4 ± 1	>1000	515	18 ± 4	62.5	13.6	7 ± 1	31.25	9.4
B. megaterium (2087)	6 ± 2	>1000	1030	21 ± 2	3.91	0.57	NA	NT	NT	6 ± 1	>1000	1395	5 ± 1	62.5	23.7
M. luteus (2169)	3 ± 1	>1000	780	16 ± 2	7.81	1.57	NA	NT	NT	10 ± 2	7.81	1.07	7 ± 1	31.25	18.6
S. aureus (2079)	5 ± 2	>1000	1138	21 ± 2	3.91	0.31	NA	NT	NT	17 ± 2	125	52.9	6 ± 1	62.5	26.8
S. epidermidis (2493)	NA	NT	NT	21 ± 1	7.81	1.29	NA	NT	NT	9 ± 3	>1000	702	NA	NT	NT
A. baumannii (5152)	NA	NT	NT	6 ± 1	500	157	NA	NT	NT	4 ± 2	>1000	1213	10 ±3	62.5	21.1
E. coli (2065)	NA	NT	NT	4 ± 1	>1000	NT	NA	NT	NT	NA	NT	NT	NA	NT	NT
K. pneumoniae (2706)	NA	NT	NT	3 ± 1	1000	207	NA	NT	NT	NA	NT	NT	NA	NT	NT
P. aeruginosa (2200)	5 ± 2	500	176	19 ± 2	>1000	1974	7 ± 1	>1000	674	18 ± 2	1000	493	8 ± 2	1000	464.3
S. typhimurium (2501)	NA	NT	NT	8 ± 1	1000	221	NA	NT	NT	NA	NT	NT	4 ± 1	1000	273.4

Clinical pathogens: Sr. No. 1-5 Gram-positive and 6-10 Gram-negative bacteria. AB zone: antibacterial zone in millimeter (mm). Inhibitory concentrations IC_{50} and MIC = µg ml^{-1}. MIC values above highest test concentation (i.e. 1000 µg ml^{-1}) is represeted as >1000. IC_{50} values were determined from regression curve. NA: No activity, NT: Not tested.

Table 5.2. (B) Antibacterial activities tested against standard antibiotics.

Pathogenic strains (NCIM code)	**Inhibition zones** (mm)					
	Broad spectrum antibiotics			Third generation antibiotics		
	TE	AMP	CL	CAZ	CPD	CTR
B. cereus (2156)	16 ± 2	2 ± 1	22 ± 2	3 ± 1	2 ± 1	10 ± 1
B. megaterium (2087)	30 ± 2	15 ± 4	32 ± 3	27 ± 2	11 ± 1	26 ± 2
M. luteus (2169)	18 ± 2	NA	13 ± 2	18 ± 2	6 ± 2	5 ± 2
S. aureus (2079)	26 ± 2	29 ± 2	25 ± 1	12 ± 2	8 ± 2	24 ± 2
S. epidermidis (2493)	30 ± 2	9 ± 2	28 ± 3	22 ± 1	10 ± 1	29 ± 1
A. baumannii (5152)	19 ± 2	NA	11 ± 1	23 ± 2	14 ± 1	29 ± 2
E. coli (2065)	16 ± 1	11 ± 1	21 ± 2	28 ± 2	14 ± 2	20 ± 1
K. pneumoniae (2706)	16 ± 2	NA	16 ± 1	25 ± 1	12 ± 1	29 ± 2
P. aeruginosa (2200)	25 ± 3	6 ± 2	25 ± 3	4 ± 1	NA	8 ± 1
S. typhimurium (2501)	18 ± 2	21 ± 2	27 ± 3	27 ± 2	17 ± 2	31 ± 2

Standards: TE- Tetracycline (10 µg), AMP- Ampicillin (25 µg), CL- Chloramphenicol (25 µg), CAZ- Ceftazidime (30 µg), CPD- Cefpodoxime (10 µg) and CTR- Ceftriaxone (30 µg).

Fig. 5.4. (A)

Pathogens (NCIM Code)	Pigmented bacteria				
	PIGB 46	PIGB 77	PIGB 88	PIGB 163	PIGB 184
2156					
2087					
2169					
2079					
2493					
5152					
2065					
2706					
2200					
2501					

Fig. 5.4. (A) Pictorial representation of antibacterial zones by pigmented metabolites.

Fig. 5.4. (B)

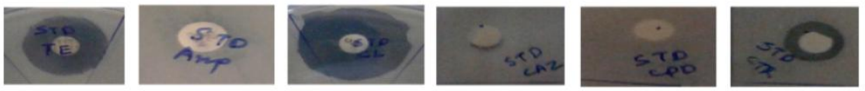
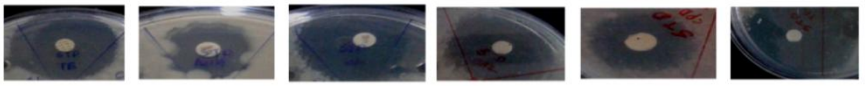
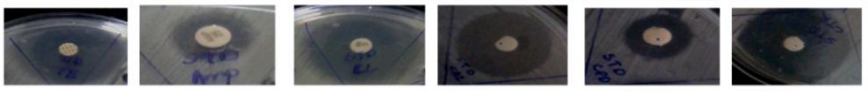
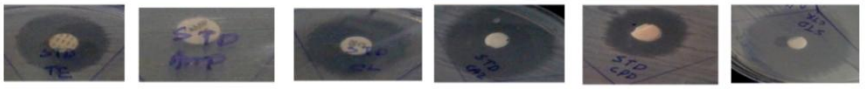
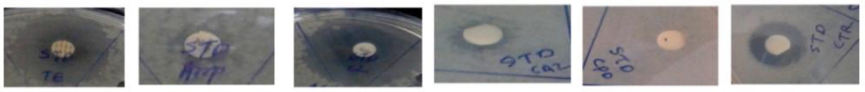
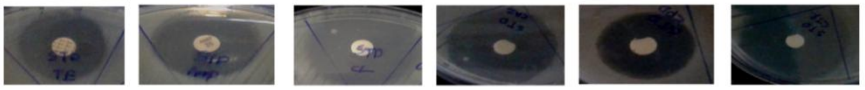
Pathogens (NCIM Code)	Broad spectrum antibiotics			Third generation antibiotics		
	TE	AMP	CL	CAZ	CPD	CTR
2156						
2087						
2169						
2079						
2493						
5152						
2065						
2706						
2200						
2501						

Fig. 5.4. (B) Pictorial representation of antibacterial zones by standard antibiotics.

5.3.2.2. Antifungal and anti-yeast properties

The antimicrobial activities tested against pathogenic fungi and yeast unfortunately did not support much inhibition by pigmented compounds (Table 5.3). Only the potent strain PIGB 77 had meaningful activities against both types of pathogens i.e. fungi and yeast (Inhibitory zones: 4-8 and 16-21 mm respectively). None of the other species show anti-yeast activity. PIGB 88 and 184 showed positive inhibitions towards fungi *A. flavus* and *F. oxysporum* while the later could additionally inhibit yeast pathogens *C. albicans* and *Cryptococcus laurentii*. Overall results on this suggest PIGB 77 metabolites can be considered for further antifungal/anti-yeast prospective.

Mandryk et al. (2007) studied large pool of antimicrobial compounds from *P. aurantiaca* S-1 and showed alkyl quinolinol derivatives to exhibit antifungal activity against phytopathogens *F. oxysporum* (AM zone: 12 ± 0.9 mm). Pigmented compounds from our *Pseudomonas* strain (PIGB 77) overwhelmed this action by giving 21 ± 3 mm inhibition zone against *F. oxysporum*. It demonstrates the metabolites of marine bacteria have better activity than the terrestrial one.

Pseudomonas sp. have been documented for its importance in biocontrolling the yeast pathogens. Buck and Mayer (1965) employed marine *Pseudomonas* sp. for studying the antagonistic effect on pathogenic strains of many *Candida* species. They retrieved maximum anti-yeast zones up to 1-2 mm. In comparison, metabolites from our strain PIGB 77 (*P. koreensis*) exhibited two-fold higher inhibitory zones (4 ± 1 mm) against same pathogenic yeast. This result emphasizes that the bioactive compound could contribute greater activity than the whole cells.

In another study, Anand et al. (2006) experienced the marine *Pseudomonas* sp. SC11 to impart 7 mm zone against *C. albicans* whereas he could not observe any activity in *Vibrio* spp. The pigmented metabolites of *Pseudomonas* and *Vibrio* tested by us against *C. albicans* even though showed growth inhibitions but the activity zones were comparatively lower (4 ± 2 mm) than the previous study (Anand et al. 2006). Additionally, researchers have reviewed various types of antifungal and anti-yeast metabolites from bacterium *Pseudomonas* (Ligon et al. 2000; Morales et al. 2010). It mostly includes aromatic compounds like phenazines and pyrrolnitrin derivatives. Similarly, the phenolics and pyrrolo- compounds produced from our strains *Pseudomonas* and *Vibrio* sp. might have contributed the antagonistic effect against fungal and yeast pathogens.

As discussed in previous sections, the bacterial aromatic pyrrole, pyrrolo-, phenol compounds and prodigiosin pigments have been reported to possess anticancer and antimicrobial effects (Burkholder et al. 1966; Montaner and Perez-Tomas 2001; Ligon et al. 2000; Ganesh Kumar et al. 2013). Notably, results obtained with our pigmented bacteria PIGB 77, PIGB 163 and PIGB 184 was also able to synthesize the above compounds in equivalent with the existing reports or slightly more in some cases. Further, various antimicrobial and anticancer activities tested with the bacterially derived compounds were also contributed significantly. The above results from our strains highlight the potential of bioactives and its future applications in biopharmaceutical industries.

Table 5.3. Antimicrobial properties tested by marine pigmented bacteria.

Pathogen type	**Pathogenic strains**		**Pigmented bacteria** Zones of inhibition (mm)				
	Pathogen	NCIM code	PIGB 46	PIGB 77	PIGB 88	PIGB 163	PIGB 184
Yeast	*C. albicans*	3471	NA	4 ± 1	NA	NA	4 ± 2
	C. parapsilosis	3323	NA	4 ± 1	NA	NA	NA
	F. uniguttulatum	3444	NA	3 ± 1	NA	NA	NA
	C. krusei	3515	NA	6 ± 1	NA	NA	NA
	C. tropicalis	3118	NA	6 ± 1	NA	NA	NA
	Cryptococcus laurentii	3373	NA	8 ± 2	NA	NA	5 ± 2
Fungi	*A. flavus*	538	NA	16 ± 2	13 ± 3	NA	9 ± 3
	F. oxysporum	1281	10 ± 4	21 ± 3	8 ± 3	NA	9 ± 4

Inhibition zones are the mean values of triplicate analysis (n ± 3). NA: No activity.

5.3.3. Protective role of PIGB on oxidative deterioration of membrane lipids

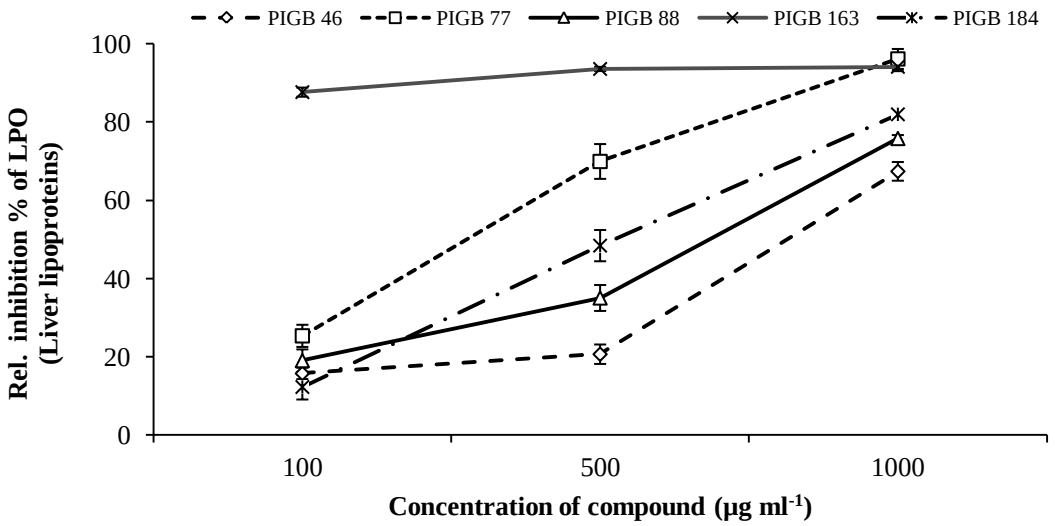
The ability of marine pigmented bacterial metabolites to prevent oxidation reactions in mammalian (sheep) brains and liver lipoproteins is demonstrated in Fig. 5.5. Pigmented compounds showed exponential inhibitory effect with increasing sample concentration. PIGB 77 showed 96.15% inhibition of lipid peroxidation in sheep liver and 87.95 % in sheep brain with the sample concentration of 1000 µg ml^{-1}.

Likewise, PIGB 88 and 163 also demonstrated exponential anti-LPO activities. Inhibitory effect by PIGB 88 was reached to 75.81 ± 0.87% at highest sample concentration 1000 µg ml^{-1} whereas PIGB 163 could give a similar effect (87.66 ± 1.17%) at 100 µg ml^{-1}. Similarly, in case of *Vibrio* sp. (PIGB 184), pigmented metabolites inhibited LPO reactions in the order of 12.25 > 48.41 > 81.98% and 31.44 > 46.34 > 63.03% in liver and brain samples respectively. *P. argentinensis* (PIGB 46) had least LPO inhibitory effects in comparison with other pigmented strains studied. Overall observations also demonstrate that antioxidative pigmented compounds inhibited LPO reactions in liver more prominently (67.40 ± 2.39 to 96.15 ± 2.56%) than the brain (55.89 ± 0.69 to 87.95 ± 1.19%) (Fig. 5.5A and 5.5B).

Studies with marine *Pseudomonas, Serratia* and *Vibrio* sp. on LPO reactions are limited. A recent report by Correa-Llanten et al. (2012) revealed pigmented extract obtained from Antarctic bacterium *Pedobacter* sp. intercalates the lipid peroxidation reactions. They estimated anti-LPO values in terms of malondialdehyde (MDA) equivalents. It was noted that formation of MDA is significantly decreased in presence antioxidative pigments. Similarly, our results were also showing the relative % LPO inhibitions in the presence of antioxidative pigments.

Fig. 5.5.

(A)



(B)

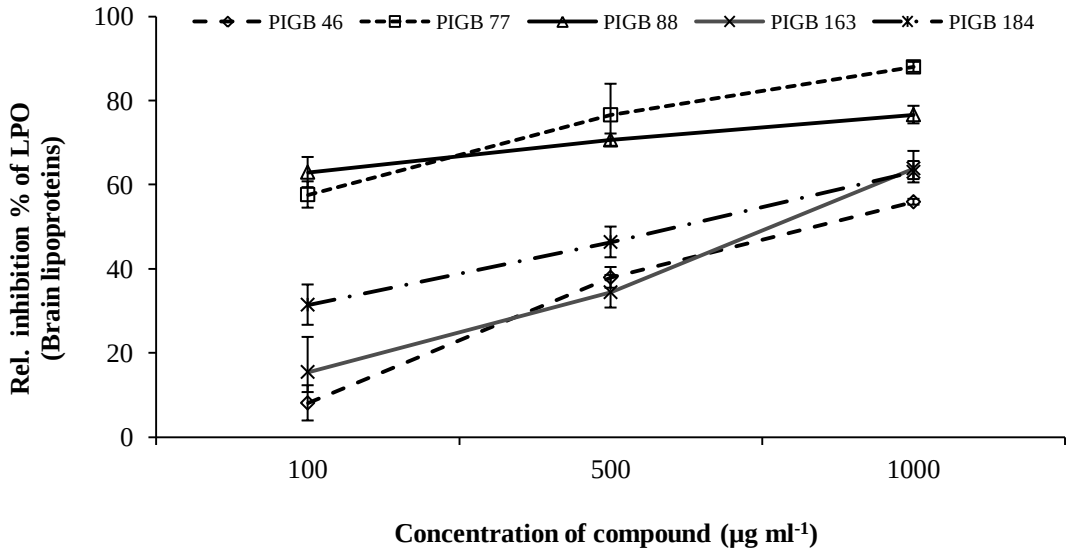


Fig. 5.5. Anti-LPO activities tested with marine pigmented antioxidant compound.

(A) Sheep liver and **(B)** Sheep brain lipoproteins. Relative inhibition activities correspond to standard ascorbic acid (100 µg ml^{-1}).

Conversely, in another approach Iwamoto et al. (2000) demonstrated pigmented compound (Astaxanthin) to impart promising *in vitro* and *ex vivo* inhibitory action on oxidation of low density lipids (LDL). Similar results were also obtained by Sy et al. (2013), who documented the pigmented compounds from the marine *Bacillus* to inhibit LPO reactions in lab models imitating gastric conditions of animals. Though we did not study *in vivo,* the significant results obtained during *in vitro* studies on sheep liver and brain confirms the inhibitory effect of pigmented compound on LPO reactions. Outputs of this study not only support the recent findings on Anti-LPO pigments but also records first ever report on marine bacteria *P. argentinensis, P. koreensis, S. rubidaea, S. nematodiphila* and *Vibrio* sp.

The literature on the anti-LPO potential of secondary metabolites from different sources has correlated possible activities with phenolic contents (Cetkovic et al. 2007; Alencar et al. 2015). In the present investigation, the anti-LPO results when correlated with phenolic contents of selected bacteria gave significant correlation values (R^2 ~0.997; P<0.05-0.1) (Fig. 5.6). PIGB 77 and 184 anti-LPO effects highly correlated with phenolic compounds supported by R^2 values in the range of 0.905-0.997. Correlation results on other pigmented strains PIGB 46, 88 and 163 also supported the similar trend with phenolics and LPO reactions (Fig. 5.6). Lavy et al. (2005) showed bacterium *D. radiophilus* with antioxidant property have more ability to attenuate the LPO reactions. Similarly, a recent investigation by Horta et al. (2014) evidenced marine bacterial extracts containing significant amounts TPCs do take part in radical scavenging processes. However, these studies could not point out the actual reason behind their observed bioactivities.

In the present investigation, an observation on the antioxidative pigments, phenolics and anti-LPO properties of bacterial metabolites are directly related to each other. For example, the potent strain PIGB 77 with higher antioxidant activities gave prominent anti-LPO activity. On the other hand, the PIGB 46 with lower production of pigmentary antioxidant exhibited minimum anti-LPO effects. This clearly suggests that biological material that owns AO property would contribute greater inhibitions of LPO. Here, for the first time we document the protective role of antioxidative pigmentary bacterial phenolics towards inhibition of oxidative reactions in membrane lipids.

Fig. 5.6.

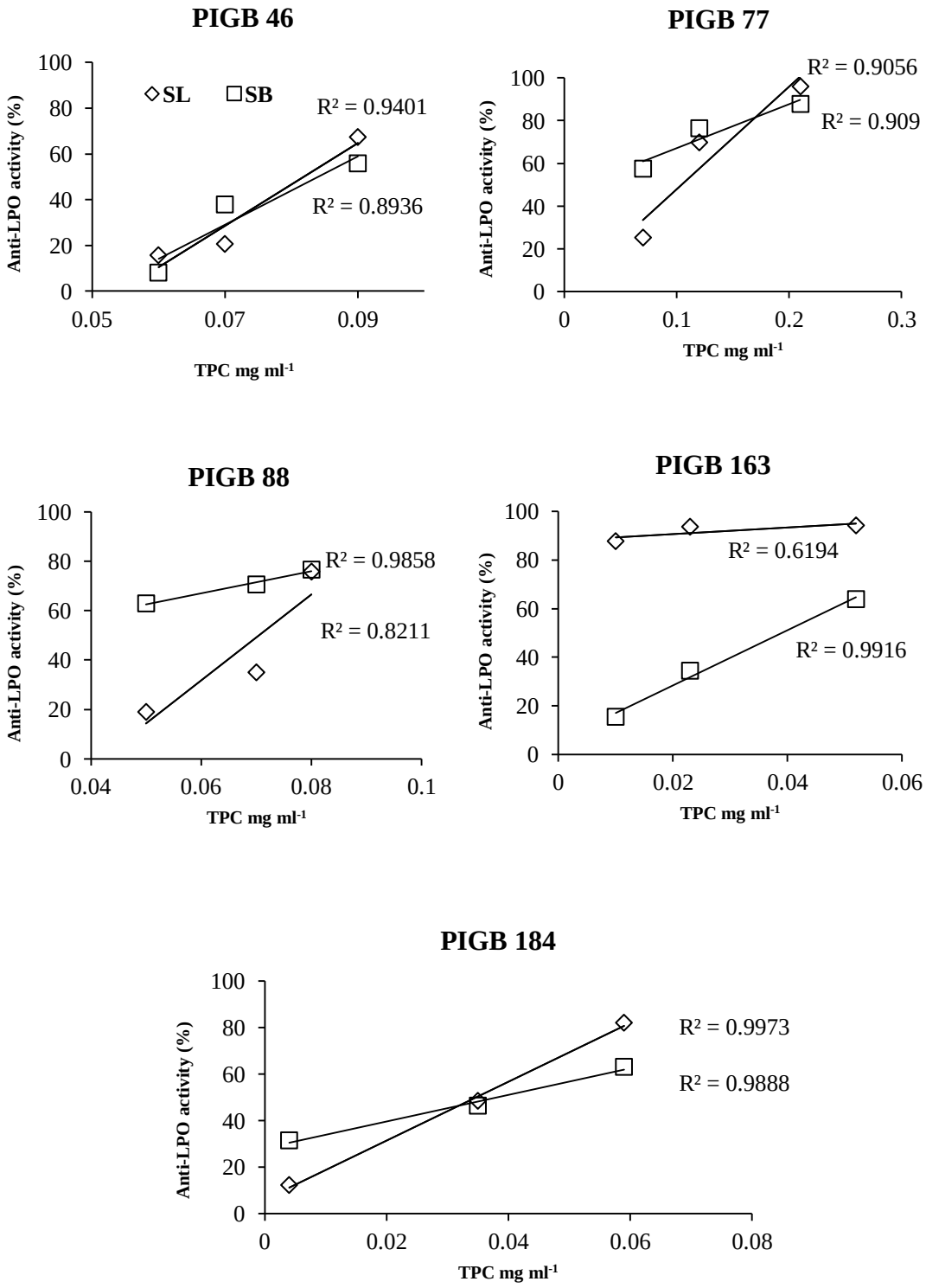


Fig. 5.6. Relation of total phenolic contents with anti-LPO activities tested with PIGB.

SL: Sheep liver, SB: Sheep brain.

5.4. Conclusion

Antioxidative pigmented metabolite tested for biotechnological potentials showed significant antiproliferative effects on human cancer cell lines, antimicrobial effects on human microbial pathogens and protective action on stress associated lipid peroxidation in mammalian cells.

Results on anticancer activity on human cancer cells showed pigmented compounds of PIGB 77 and PIGB 163 to exhibit substantial inhibition of lung cancer cells (A549). Metabolites of these two bacteria also had considerable anticancer effects on the breast (MCF7), melanoma (SK-MEL-2) and nasopharyngeal neoplastic cells (KB).

Antimicrobial test on pigmentary metabolites evidenced marked inhibition of Gram-positive pathogens. The PIGB 77 stood most potent strain as it inhibited near about all studied bacterial, yeast, and fungal pathogens.

Antimicrobial effects tested with broad spectrum and third generation antibiotics expressed the inhibition zones in equivalent with the standard antibiotics or slightly more. It highlights the significance of marine pigmented compounds and its potential in developing bio-based antibiotics for future use.

Pigmented metabolites of marine bacteria demonstrated significant inhibitions of oxidative deterioration of membrane lipids in mammalian cells (87-96%). These activities were comparable with artificial standard antioxidant ascorbic acid. It indicates marine bacterial pigmented compounds could be putatively harnessed as protective agents in lipid biology.

CHAPTER 6

SUMMARY AND CONCLUSIONS

- Free radicals and stress are the key factors responsible for the implication of deadly diseases and ageing process in human health. Antioxidants play various complex defense mechanisms and provide relief from stress associated health risks. However in order to have balance among stress and defense, it's of prime necessity to supply the body with enough quantity of antioxidants either from external diet or medicines.

- Realizing importance of antioxidants in health and need of finding novel natural resources we explored pigmented bacterial isolates and their metabolites for antioxidant and other biotechnological properties.

- Systematic 2-year sampling from 4 different environmentally diverse locations, two from the states of Maharashtra (Kunkeshwar and Malvan) and Goa (Cabo De Rama and Vagator) was carried out. It resulted in the isolation of 190 distinct pigmented isolates. This includes 64 seawater, 39 sediment and 87 seaweed associated bacteria.

- Overall observations on chromogenic bacteria represented the diverse occurrence along central west coast of India (CWCI). Based on the color property, they ordered in the range of yellow (40%), orange (26.32%), creamy (21.05%), pink (5.26%), brown (4.74%), red (1.58%) and violet (1%). The endurance of chromogens is highly influenced by seasons and sample source. Pink and violet pigmented bacteria show their dominant occurrence during post monsoon seasons.

- Bacterial community structure analysis witnessed endurance of 25 chromogenic genera affiliating five major phyla (β-Proteobacteria, γ-Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes) along the CWCI coast.

- Initial antioxidant screening test on the total of 190 pigmented isolates demonstrated 44.2 % organisms to secrete radical scavenging metabolites in their extracellular

environment. Of these 3.68% were highly potential with an expression of >3 cm primary AO zones. Isolates expressing highest antioxidant zones PIGB 46, 77, 88, 163 and 184 were considered as high potent and selected for quantitative studies.

- Optimization of process parameters for selected 5 pigmented strains demonstrated the media strength of 50-75% ZMB, pH 6.0 - 7.0, temperature ~30 ºC and the carbon (fructose, maltose , dextrose) / nitrogen components tryptone and peptone enhances 2-3 fold increase in production of antioxidant metabolites including pigments.

- Pink pigmented bacteria PIGB 163 and PIGB 88 AO activity was hampered with additions of nitrogenous components specifically in *Serratia* species.

- *P. koreensis* PIGB 77 excel in its DPPH scavenging (95.6%) over standard AO compounds BHT (Butylated hydroxytoluene: 84.3%). Chromatographic purification by Sephadex-LH20 size exclusion, Silica gel ion exchange and the structural elucidation by LC-MS and NMR affiliated to the above active components belonging to hydantoinase, hydantoic acid and related 5-methyl hydantoin derivatives. These compounds have an additional importance in the industrial application for producing D-amino acids.

- Metabolites of a pink chromogenic bacterium PIGB 163 (*S. nematodiphila*) gave concurrent activity towards ABTS radicals (1 mg = 90.2 %) in comparison with standard pigment quercetin (160 µM: 99.5%). It emphasized the importance of bacterial pigments as an alternative source of AO compound. The spectroscopic, DPPH guided active separation and GC-MS analysis revealed the AO compounds to belong to (a) Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- (b) 3,6-Diisopropylpiperazin-2,5-dione and (c) prodigiosin.

- A yellow pigmented strain PIGB 184 (*Vibrio* sp.) also gave considerable AO results towards DPPH, ABTS, FRAP and Reducing power in comparison to that of standard antioxidant ascorbic acid (AsA). Structural elucidation of AO active components showed secretion of Phenol, 2,4-bis(1,1-dimethylethyl) and Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl) by this strain. These compounds and its related derivates are used as intermediate oxidizing/reducing agents in the production of light-protecting and phenolic antioxidant compounds.

- Statistical analysis between antioxidant properties and the phenolic compounds gave linear relationship. Significant correlations were observed in case of PIGB 163 and PIGB 184 (R^2 = 0.845 – 0.997). The PIGB 184 stood an outstanding strain as it demonstrated the most linear relation against the AO activities ($R^2 \geq 0.969 – 0.994$, $p < 0.01$).

- 16S rRNA identification and phylogenetic classification affiliated antioxidative strains to bacterial genera *Pseudomonas* (PIGB 46; PIGB 77), *Serratia* (PIGB 88; PIGB 163) and *Vibrio* (PIGB 184) species. Interestingly all the potent bacteria belonged to a single major group Gammaproteobacteria. These results indicate the other bacterial groups belonging Gammaproteobacteria may also possess significant AO properties and needs to be explored perceptively.

- Activity check and large extent bioactivity screening on antioxidant rich metabolites additionally emphasized the biotechnological potential of marine pigmented bacteria. PIGB 77 (*P. koreensis*) and PIGB 163 (*S. nematodiphila*) significantly obstructed the growth of human lung (A549: IC_{50} 25.6 – 30.7 mg ml^{-1}), breast (MCF7: IC_{50} 42.0 – 57.0 mg ml^{-1}), melanoma (SK-MEL-2: 63.7 – 66.1 mg ml^{-1}) cancer cell lines. It highlights the importance of pigmented metabolites in cancer cell therapy.

- PIGB 77, PIGB 163 and PIGB 184 had significant inhibitions of opportunistic human pathogens in comparison with standard 'broad spectrum' and 'third generation' antibiotics. *P. koreensis* (PIGB 77) metabolites showed substantial antagonistic activity against all types of pathogenic microbes (bacteria, fungi and yeast etc.) tested.
- Anti-lipid peroxidation check on radical induced oxidation of mammalian liver and brain lipoproteins furthermore affirmed 60-92% protection against stress associated damage of vital cell components.
- Results of present investigation revealed the potential of marine pigmented bacteria towards the reduction of free radicals and their protection over stress associated health risks.

In conclusion, the pigmented bacteria explored for their antioxidant potential revealed novel activities on pyrrolo, phenol and Diisopropylpiperazin compounds. Results of this study form a foundation on the availability of pigmented bacteria coastal waters, their distribution, diversity and variation with respect sample source, season and antioxidant capabilities from the Indian coast. The PIGB 77 (*P. koreensis*) with its excellent antioxidant and other attributive biological properties stood candidate species for the production of hydantoinase. PIGB 163 (*S. nematodiphila*) and 184 (*Vibrio* sp.) also represents their potential towards antioxidant and anticancer research. This study opens a new way for studying more and more PIGB's from not only the coastal sites but also from the deep sea and other environments to obtain the candidate species for their applications in biopharmaceutical research.

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PUBLICATIONS

Research papers published

Pawar R, Mohandass C, Dastager SG, Kolekar YM, Malwankar R (2016) Antioxidative metabolites synthesized by a marine *Vibrio* sp. (PIGB 184) and its antimicrobial activities against opportunistic pathogens. Applied Biochemistry and Biotechnology DOI:10.1007/s12010-016-1985-z.

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Manuscripts under review/to be communicated

Pawar RT, Mohandass C, Dastager SG, Kolekar YM (2016) Antioxidant derivatives Pyrrolo-, Diisopropylpiperazin- and Prodigiosin from the marine pigmented *Serratia nematodiphila* and its potential towards human cancer (Communicated: J Ind Microbiol Biotech).

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Pawar RT, Mohandass C, Singh K, Wahidulla S, Dastager SG, Kolekar YM. Hydantoinase derivatives from *P. koreensis* and its chemical properties on the production of D-amino acids (to be submitted for peer review).

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Pawar R, Mohandass C, Jagtap TG (2013) Seaweed associated marine pigmented bacteria: A potential source for antioxidative compounds. In Asean-India Marine Biotechnology workshop-2013 (March 19-22), CSIR-NIO, Goa, India.