Pigment production by marine *Serratia* sp. BTWJ8

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by

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Dedicated to my Parents,
Chandrasekaran Sir,
Nampoothiri Sir,
&
My Uncle Jayaprasad

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CERTIFICATE

This is to certify that the research work presented in this thesis entitled **"Pigment production by marine *Serratia* sp. BTWJ8"** is based on the original research work carried out by Ms. Jissa G Krishna under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

M. CHANDRASEKARAN

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Pigment production by marine *Serratia* sp. BTWJ8"** is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin under the guidance of Dr. M. Chandrasekaran, Professor, Cochin University of Science and Technology and the thesis or no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles or recognition.

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ABBREVIATIONS

%	-	Percentage
°C	-	degree Celsius
ANOVA	-	Analysis of Variance
BLAST	-	Basic Local Alignment Search Tool
bp	-	base pairs
$CDCl_3$	-	deuterated chloroform
cm	-	centimeter
Da	-	Dalton
DNA	-	Deoxyribo Nucleic Acid
dNTP	-	deoxy Nucleotide tri phosphate
DW	-	Distilled water
Fig.	-	Figure
FT-IR Spectroscopy	-	Fourier Transform Infrared Spectroscopy
FT-Raman Spectroscopy	-	Fourier Transform Raman Spectroscopy
g	-	gram
GAB	-	Glycerol Asparagine Broth
g/L	-	grams per liter
h	-	hours
HPLC	-	High Performance Liquid Chromatography
kb	-	kilobase
KBr	-	Potassium bromide
LB	-	Luria Bertani
LC-MS	-	Liquid Chromatography Mass Spectroscopy
L	-	Liter
M	-	Molar
mg	-	milligram
mg/ml	-	milligram per milliliter
µg	-	microgram
µg/L	-	microgram per liter

µl	-	microliter
µm	-	micrometer
min.	-	minutes
ml	-	milliliter
ml/min.	-	milliliter per minute
mm	-	millimeter
mM	-	milliMolar
MSTS	-	Mineral Salts Tryptone Sucrose
MOF	-	Marine Oxidative Fermentative
mV	-	milliVolt
NB	-	Nutrient Broth
NCBI	-	National Center for Biotechnology Information
ng	-	nano gram
NJ	-	Neighbour joining
nm	-	nanometer
NMR Spectroscopy	-	Nuclear Magnetic Resonance Spectroscopy
OD	-	Optical Density
PB	-	Plackett-Burman
PCR	-	Polymerase Chain Reaction
PG	-	Prodigiosin
phr	-	parts per hundred gram rubber
PMMA	-	Poly methyl methacrylate
pNP	-	paranitrophenyl
R_f	-	Retention factor
RNA	-	Ribonucleic acid
rpm	-	revolutions per minute
rRNA	-	ribosomal RNA
RSM	-	Response Surface Methodology
sec.	-	seconds
SmF	-	Submerged Fermentation

sp.	-	species
SW	-	Seawater
SWYPB	-	Seawater Yeast extract Peptone Broth
Taq	-	*Thermus aquaticus*
TLC	-	Thin Layer Chromatography
U/ml	-	Units per milliliter
UV	-	Ultraviolet
V	-	Volt
v/v	-	volume per volume
w/v	-	weight per volume
λ_{max}	-	Absorption maxima
ZMB	-	Zobell Marine Broth

CONTENTS

Chapter 1

INTRODUCTION

Natural organic pigments were a significant part of historical pigments before the modern era, particularly for bodily ornamentation, cosmetics and textile dyeing. Natural organic pigments are generally extracted from fruits, vegetables, seeds, roots and microorganisms and they are sometimes called biocolours because of their biological origin (Pattnaik *et al.*, 1997). Natural pigments have important functions other than the imparted beauty, such as the photosynthesis and probably life all over the world without chlorophylls and carotenoids. In animals oxygen and carbon dioxide could be transported with hemoglobin or myoglobin. Under stress conditions plants show the synthesis of flavonoids; the quinones are very important in the conversion of light into chemical energy (Frick and Meggos, 1988; Hari *et al.*, 1994; Koes *et al.*, 1994; Mol *et al.*, 1996). Although natural pigments based on herbs and other plant sources were used as dyes for imparting colours in clothes, ink, paper, and paints since ancient times synthetic dyes have replaced the herbal dyes for they are preferred for their colour variation, easy availability as they are manufactured in large scale, as well as cost of synthetic dye is less than herbal dye.

Colour is one of the most important qualities of foods. In the past, consumers did not care about the kind of pigments used in food colouring (natural or synthetic). But with reference to food colourants recently there is an aversion towards synthetic pigments owing to the belief such as "synthetic pigments are associated with several illness" and "natural pigments have pharmacological benefits". However, the natural pigments that are permitted for human foods are very limited, and there is difficulty in getting approval for new sources for the

reason that the U.S. Food and Drug Administration (FDA) considers the pigments as additives, and consequently pigments are under strict regulations (Clydesdale, 1993; Wissgot and Bortlik, 1996; Wodicka, 1996). Recently there have been changes in the legislation also causing a significant reduction in number of synthetic colours used in foods (Downham and Collins, 2000).

Interest in use of natural colourants is increasing worldwide. Different from artificial synthetic colours, they are much more reliable due to their excellent stability. In addition, they can be mixed in numerous ways due to their various colour tones. Natural food colourings are more desirable than artificial dyes for both nutritional and marketing reasons. Synthetic colours are responsible for the hypersensitivity reaction in humans (Francis, 1987). Artificial colourings, those commonly referred as "coal tar dyes" or "food dyes" have been ideal for many years and replaced natural colourants (Santhanakrishnan, 1981). Synthetic colours are found technically more suitable than natural colours because the former are known for their fastness, availability in a wide range of colours, low cost even at high concentration in low volumes and they are devoid of aroma and taste. However, in the world market a number of permitted synthetic colourants are known to cause allergies in human. Many are carcinogenic and teratogenic in nature. With the increasing concern for health, people avoid using synthetic colours. Orange dyes caused local tumour in the lumen of the bladder of mouse and intestinal gut flora was affected due to the intake of synthetic colours in rats (Aeris *et al.*, 1996).

Consumer pressure, sociological changes, and technological advances leading to more advances in the food processing industry have increased the overall food colour market. Percentage market share of food colours is shown in Fig. 1.1. The most significant growth has been in naturally derived colours owing to the improvements in stability as well as the food industries with an aim to meet

the increasing consumer perception that 'natural is best'. Future growth is going to be large for naturally derived colours with a predicted annual growth rate of 5-10 %. Synthetic colours are still forecast to grow but at a lower rate of between 3 and 5 % (Alison and Paul, 2000).

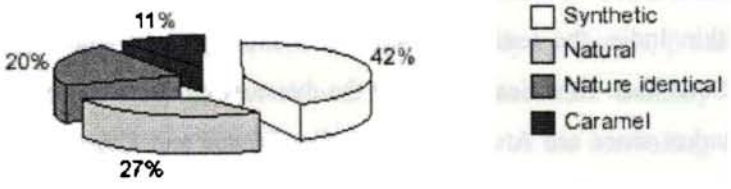


Fig.1.1 Percentage market share of food colourants

According to a study on dyes and organic pigments, the worldwide demand for organic colourants is projected to increase from 4.9 % in 2003 to $10.6 billion in 2008. Generally, the dyestuff industry comprises three sub-segments, *viz;* dyes, pigments, and intermediates. The dye intermediates are petroleum downstream products, which are further processed into finished dyes and pigments. These are important additives in major industries like textiles, plastics, paints, paper and printing inks, leather, packaging sector etc.

In India the dyestuff industry supplies its majority of the production to the textile industry. Huge amounts of dyes and pigments from India are also exported to the textile industry in Europe, South East Asia and Taiwan. India has a share of approximately 6 % of the world production in dyestuff products. The hold of polyester and cotton in the global market has positively created the demand for some kinds of dyestuffs. The Asian region has seen the highest development in textile production, followed by North America, Latin America and Western Europe. Asia tops dyestuff production both in terms of volume and value, with about a 42 %

share of the global production followed by the US with 24 % and Europe around 22 %. Due to a wide use of polyester and cotton-based fabrics, there has been changes towards reactive dyes, applied in cotton-based fabrics, and disperse dyes used in polyester. These two dyes have been leading in all the three regional global market, particularly Asia.

Within India, the leading producers in the pigments industry are Colour Chem and Sudarshan Chemicals while in the dyestuff industry the major players in terms of market share are Atul, Clariant India, Dystar and Ciba Specialities. The Indian companies together account for nearly 6 % of the world production. The trend in trade has shifted from supplying mere products to colour package solutions and greater importance is given to innovation, production range, quality and environmental friendly products (Doshi, 2006).

Although there are a number of natural pigments, only a few are available in sufficient quantities to be useful for industry because they are usually extracted from plants (Lauro, 1991). In spite of the availability of variety of pigments from fruits and vegetables, there is an ever growing interest in microbial pigments due to several reasons like their natural character and safety to use, production being independent of seasons and geographical conditions, controllable and predictable yield (Francis, 1987). The rapid growth of microbes reduces the production time to a matter of days. Compared to plant or animal sources, the production is flexible and can easily be controlled (Taylor, 1984).

A large number of different species of bacteria, molds, yeasts and algae are used to produce pigments. The ability to produce desired products cost efficiently through fermentation is a benefit of the microbial system. In addition, the collection of microbial organisms is sustainable and has no negative impact on the environment. Improvement in stability, safety and solubility can certainly make

widespread use of microbial pigments in the food industry (Joshi *et al.*, 2003). Colouring of foods using microbial pigments acts as a preservative and also has antioxidant properties to the foods (Sivakumar, 2004).

Now a days some fermentative food grade pigments are on the market: *Monascus* pigments, astaxanthin from *Xanthophyllomyces dendrorhous*, Arpink Red from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, β-carotene from *Blakeslea trispora*. The interest shown by the aquaculture industry for natural sources of astaxanthin has been growing as a result of the increasing demand for fish feed with natural pigments (Guerin and Hosokawa, 2001). Many species of *Monascus* have attracted special attention because they have the capability of producing different coloured pigments showing high chemical stability (Hajjaj *et al.*, 2000; Hamdi *et al.*, 1997).

The red pigments, produced in solid-state cultures by several species of the genus *Monascus,* have been traditionally used in many Asian countries for colouring and securing a number of fermented foods (Dziezak, 1987; Francis, 1987). Furthermore, their therapeutic properties and their relatively high stability with respect to pH and temperature are interesting features, which favour their use as substitutes for synthetic colourants. Only a few mild side effects have been associated with administration of these microbial pigments. *Monascus* pigment production by submerged and solid-state cultures in complex media has been thoroughly studied (Johns and Stuart, 1991). During the submerged culture of various edible mushrooms for the production of bioactive compounds, several fungi produced pigments at relatively high yields (Park and Kim, 2005). Single cell algae and fungi are better options for new biotechnologically derived colourants. One recent development has been with the β-carotene from the fungus *Blakeslea trispora*. This is currently being marketed as a natural food colour by Gist

Brocades (DSM Gist Brocades Delft, Heerlen, The Netherlands) (Pattnaik *et al.*, 1997).

In addition to bacteria, fungi and yeasts, several species of microalgae accumulate high concentrations of carotenoids such as β-carotene, astaxanthin and canthaxanthin. The first of these carotenoids to be commercialized was β-carotene from the green halophilic flagellate, *Dunailiella salina.* Another microalgae of interest is *Haematococcus pluvialis,* which are used for the production of astaxanthin. Phycocyanin (blue) and Phycoerythrin (red) are the two main natural pigments commercially produced from algae (Nelis and Leenheer, 1991).

Cyanotech Corporation is the world's leading producer of high-value products from microalgae and the largest producer of natural astaxanthin. The products are BioAstin® a powerful antioxidant with health benefits mainly protection from sunburn and enhancing skin, muscle and joint health, and also has the ability to support and enhance natural inflammatory response. NatuRose® is a red pigment used primarily in the aquaculture industry to impart colour to the flesh of fish and shrimp (http://www.cyanotech.com/bioastin.html). Aquapharm Bio-Discovery Ltd. is one of the first UK Marine Biotechnology companies and provides new approaches to 'bio-prospecting' to source novel natural products from marine microbes. Currently Aquapharm has naturally high producer organisms of both carotenoids such as astaxanthin and the flavanoid anthocyanin. Synthetic astaxanthin is added to commercial aquaculture diets to ensure the colour of the flesh product, and the market for the same currently worth $250 million a year and growing at 8 % per year (http://www.aquapharm.co.uk).

Prodigiosins are a family of naturally occurring tripyrrole ring-containing red pigments produced by microorganisms. They have a common pyrrolyldipyrrolylmethene skeleton. These pigments are emerging as a novel group

of compounds having distinct biological activities like antibacterial, antimycotic, immunomodulating, anti-tumor, antimalarial and nuclease (Azuma *et al.*, 2000; Bennett and Bentley, 2000; D'Alessio and Rossi, 1996; Melvin *et al.*, 2000). Hence, they are desired for several medical applications and consequently there is more interest in this pigment among the researchers.

A wide variety of bacterial taxa, including *Serratia rubidaea*, *Vibrio gazogenes*, *Alteromonas rubra*, *Rugamonas rubra*, *Streptoverticillium rubrireticuli* and *Streptomyces longisporus ruber* produces prodigiosin and/or derivatives of this molecule (Austin and Moss, 1986). Prodigiosin was first isolated from *S. marcescens* in pure form in 1929. Its name, used by early researchers, was retained but the pigment was not characterized and its main structural features elucidated until 1934. One strain of *S. marcescens*, which produced prodigiosin like pigment, was also isolated long back from marine environment (Lewis and Corpe, 1964).

In recent times there is an escalation in the use of synthetic dyes for example in the ever-growing textile colouration industry and consequently effluent of dyes and associated chemicals are now serious concern of environmental pollution. Wastewater from printing and dyeing units is often rich in colour, containing residues of reactive dyes and chemicals. The toxic effects of dyestuffs and other organic compounds, as well as acidic and alkaline contaminants in these dye effluents have reached a stage where they are not treated effectively before their disposal into environment. Hence, due to the harmful effect of chemical dye on environment pollution, a number of countries have issued stricter regulations so as to preserve our environment. As a consequence there is a revived interest in the use of natural pigments as dyes, which could be subjected to biodegradation in the environment (http://express-press-release.com).

In this context, there is a resurgence in the search for good sources of natural pigments which are ecofriendly. Accordingly several investigators have started screening natural pigments from various sources. The marine environment, which covers three quarters of the surface of the planet, is estimated to be home to more than 80 % of life and yet it remains largely unexplored. Marine microorganisms are an untapped source for pigments that can have wide range of applications in industries including food industry and textile industry. More than half the bacteria occurring in the sea are chromogenic. In a general survey of several thousand colonies isolated from marine sources it was found that 31.3 % were yellow, 15.2 % orange, 9.9 % brown and 5.4 % red or pink (Zobell and Feltham, 1934). However, there are not many reports available on investigation on marine microorganisms as source of natural pigments. Hence this study was desired.

OBJECTIVES OF THE PRESENT STUDY

The marine microorganisms are yet to be exploited as a source of natural pigments for probable utilization in various industries. Hence, in this study focus was made only on pigment producing marine bacteria for pigment production and evaluation of the same for some application besides development of an ideal bioprocess for subsequent indigenous production of the pigment using the same organism towards ultimate industrial application.

Specific objectives of the present study included the following:

1. Screening of potential pigment producing bacteria from the marine and mangrove environments of Kerala.
2. Purification and characterization of the pigment.
3. Optimization of bioprocess variables for the production of pigment employing statistical approach.
4. Application studies of the pigment.

Chapter 2

REVIEW OF LITERATURE

Natural colours are generally defined as materials extracted, isolated or otherwise derived from plants, animals, or microorganisms that are capable of imparting a distinguishing colour when added to foodstuffs.

2.1 Pigments – Classification

2.1.1. Based on origin

Pigments can be classified based on their origin as natural, synthetic, or inorganic. Natural pigments are produced by living organisms such as plants, animals, and microorganisms. Natural and synthetic pigments are organic compounds. Inorganic pigments are found in nature or reproduced by synthesis (Bauernfeind, 1981).

2.1.2. Based on chemical structure of the chromophore

Pigments can be classified according to the chemical structure of the chromophore as: (i) Chromophores with conjugated systems (carotenoid, anthocyanin, betalain, caramel and synthetic pigment) and (ii) Metal-coordinated porphyrins (myoglobin, chlorophyll, and their derivatives) (Wong, 1989).

2.1.3. Based on the structural characteristics of the natural pigments

Natural pigments are also classified based on their structural characteristics as: Tetrapyrrole derivatives (chlorophylls and heme colours), Isoprenoid derivatives (carotenoids and iridoids), N-heterocyclic compounds different from tetrapyrroles (purines, pterins, flavins, phenazines, phenoxazines, and betalains), Benzopyran derivatives (anthocyanins and other flavonoid pigments), and Quinones (benzoquinone, naphthoquinone, anthraquinone and melanins). (Bauernfeind, 1981; Hari *et al.*, 1994).

2.1.4. Based on application as food additives

Based on the application of the pigments as food additives, the Food and Drug Administration (FDA) has classified pigments into two types. (i) Certifiable (synthetic pigments and lakes) and (ii) Exempt from certification (pigments derived from natural sources such as vegetables, minerals, or animals, and synthetic counterparts of natural derivatives) (FNB, 1971; Frick and Meggos, 1988; Wong, 1989).

2.2. Characteristics of major pigments

2.2.1. Tetrapyrrole Derivatives

Tetrapyrrole derivatives have pyrrole rings in linear or cyclic arrays. Linear array is very common in algae Rhodophyta and Cryptophyta. In the cyclic compounds, the heme group (the porphyrin ring bonded to an iron atom) is present in hemoglobin and myoglobin of animals, in cytochromes, peroxidases, catalases, and vitamin B_{12} as a prosthetic group. However, chlorophylls (Fig. 2.1) constitute the most important subgroup of pigments within the tetrapyrrole derivatives. They are mainly present in the chloroplasts of higher plants and in other groups such as

algae and bacteria (Britton, 1991; Counsell *et al.*, 1979; Hari *et al.*, 1994; Lichtenhaler, 1987; Rudiger and Schoch, 1988).

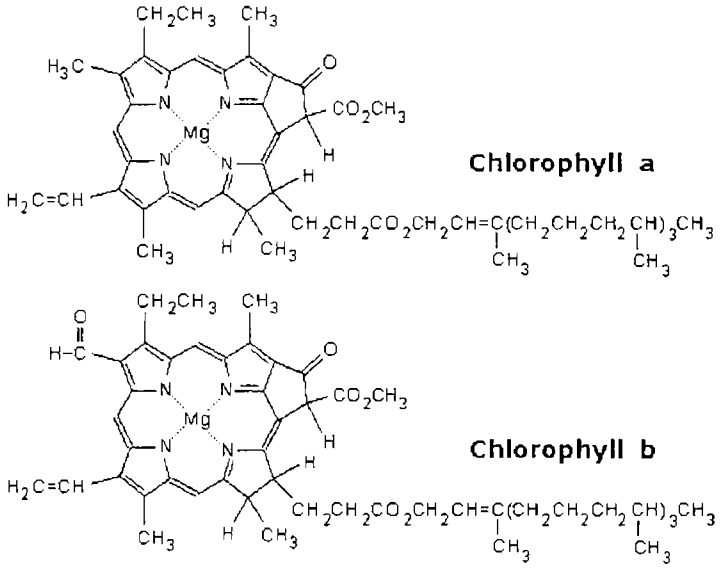


Fig. 2.1. Structure of chlorophyll

2.2.2. Isoprenoid Derivatives

Among the isoprenoid derivatives the most common and most important natural pigments are the carotenoids. They have drawn the attention of chemists, biochemists, biologists, food science and technologists, and pharmacists, for more than a century.

Carotenoids are a class of fat soluble pigments responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers, as well as the colours of some birds, insects, fish, and crustaceans. In plants, algae and photosynthetic bacteria these pigments play a critical role in the photosynthetic process. They also occur in some non-photosynthetic bacteria, yeasts, and molds,

where they may carry out a protective function against damage by light and oxygen. Although animals appear to be incapable of synthesizing carotenoids, many animals incorporate carotenoids from their diet. Within animals, carotenoids provide bright colouration, serve as antioxidants, and can be a source for vitamin A activity (Britton, 1991; Ong and Tee, 1992). Some familiar examples of carotenoid colouration are the oranges of carrots and citrus fruits, the reds of peppers and tomatoes, and the pinks of flamingoes and salmon (Pfander, 1992). Some 600 different carotenoids are known to occur naturally (Ong and Tee, 1992), and new carotenoids continue to be identified (Mercadante, 1999). It is estimated that nature produces about 100 million tons of carotenoids annually (http://www.industrialorganica.com/carotenoids.html).

Carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives are known as xanthophylls. They consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1, 6-position relationship and the remaining non terminal methyl groups are in a 1, 5-position relationship. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure, having a long central chain of conjugated double bonds by hydrogenation, dehydrogenation, cyclization or oxidation, or any combination of these processes (Fig. 2.2).

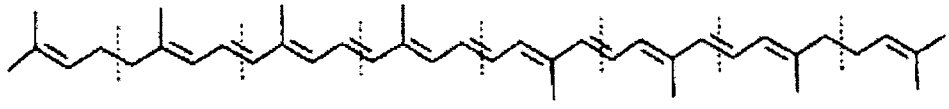


Fig. 2.2. Acyclic $C_{40}H_{56}$ structure

In Greek, "xantho" means yellow, and "phylls" stands for leaves, which is comparable to "chlorophylls" (green leaves). Structures of some important carotenoids are given in Fig. 2.3.

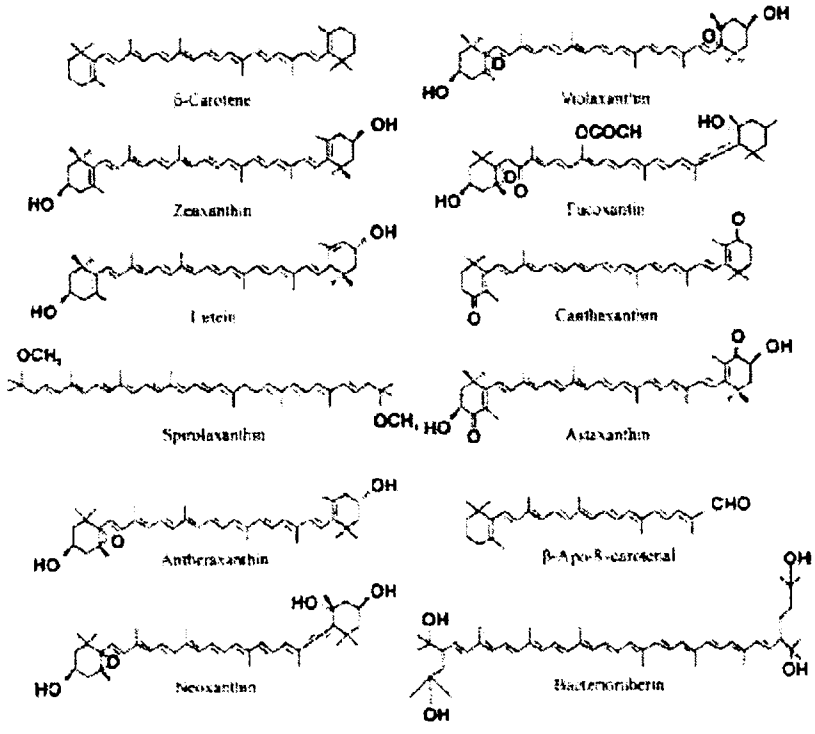


Fig. 2.3. Structures of some important carotenoids

Xanthophylls were found in algae, and lutein, a component of xanthophylls were found in egg yolks. Lutein is the carotenoid pigment found in amaranth, red paprika, mustard green, and marigold flower petals. Important carotenoids are the orange, carotenes of carrot, apricot, peach, citrus fruits; the red lycopene of watermelon and apricot; the yellow orange xanthophylls of peach (Pfander, 1992).

Some carotenoids serve as precursors for vitamin A synthesis. A molecule of orange beta-carotene is converted into two molecules of colourless vitamin A with in the body. Alpha-carotene, gamma-carotene and cryptoxanthin are also known as precursors for vitamin A synthesis. However, because of minor difference in chemical structure one molecule of each of these pigments yields only one molecule of vitamin A (Britton, 1991; Ong and Tee, 1992).

Recently, iridoids, a group of plant isoprenoid compounds, have acquired some relevance. Saffron (*Crocus sativus* L.) and Cape jasmine fruit (*Gardenia jasminoids* Ellis) are the best-known iridoid-containing plants, but their colours are influenced by carotenoids (Sacchettini and Poulter, 1997).

2.2.3. N-Heterocyclic compounds different from tetrapyrroles

a. Pterins

Pterins were first isolated as the colouring agents from the butterfly wings (hence the origin of their name, from the Greek "pteron", wing) and they are known to perform many roles in colouration in the biological world (Smith and Ramfrez, 1960). The pterin ring system (Fig. 2.5) is probably present in every form of life. They are responsible for colour in some insects, in vertebrate eyes, human urine, and bacteria (*Lactobacillus casei* and *Streptomyces faecalis* R) (Forrest, 1962; Hari *et al.*, 1994).

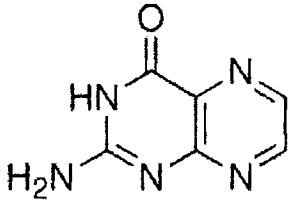


Fig. 2.5. Structure of Pterin

b. Flavins

Flavins are wide spread compounds, synthesized by all living cells of microorganisms and plants. Flavin is a tricyclic heteronuclear organic ring (Fig. 2.6) whose biochemical source is the vitamin riboflavin. The flavin moiety is often attached with an adenosine diphosphate to form flavin adenine dinucleotide (FAD), and in other circumstances, is found as flavin mononucleotide (FMN), a phosphorylated form of riboflavin. It is in one or the other of these forms that flavin is present as a prosthetic group in flavoproteins. Other sources are a wide range of leafy vegetables, meat and fish (Counsell *et al.*, 1979; Forrest, 1962; Hari *et al.*, 1994).

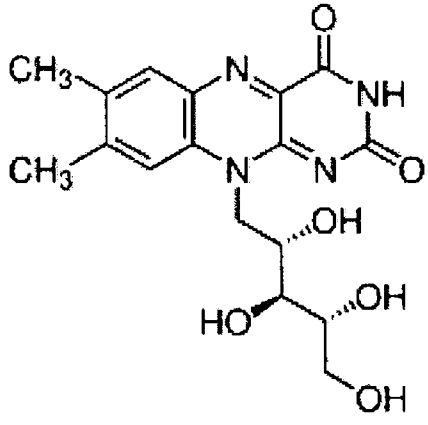


Fig. 2.6. Structure of flavin

c. Phenazines

Many phenazine compounds (Fig. 2.7) are found in nature and are produced by bacteria such as *Pseudomonas* sp., *Streptomyces* sp., and *Pantoea agglomerans*. These phenazine natural products have been implicated in the virulence and competitive fitness of producing organisms. For example, the

phenazine pyocyanin produced by *Pseudomonas aeruginosa* contributes to its ability to colonise the lungs of cystic fibrosis patients. Similarly, phenazine-1-carboxylic acid, produced by a number of *Pseudomonas*, increase survival in soil environments and has been shown to be essential for the biological control activity of certain strains (McDonald and Mavrodi, 2001; Turner and Messenger, 1986).

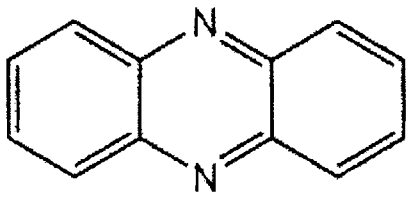


Fig. 2.7. Structure of phenazine

d. Phenoxazines

Phenoxazines are found in fungi and insects (Fig. 2.8) and are structurally related with phenazines (Hari *et al.*, 1994). They impart yellow, golden yellow, and dark brown colours. In invertebrate animals, phenoxazines are represented by the group called ommochromes. Interestingly some microorganisms produce this pigment that show antibiotic activities. *Streptomyces* sp. produces the pink-red phenoxazine "actinomycin", a chromopeptide antibiotic of commercial importance (Delgado-Vargas *et al.*, 2000).

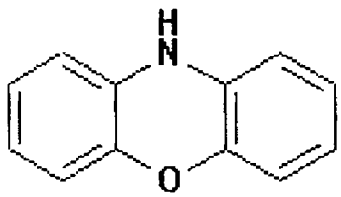
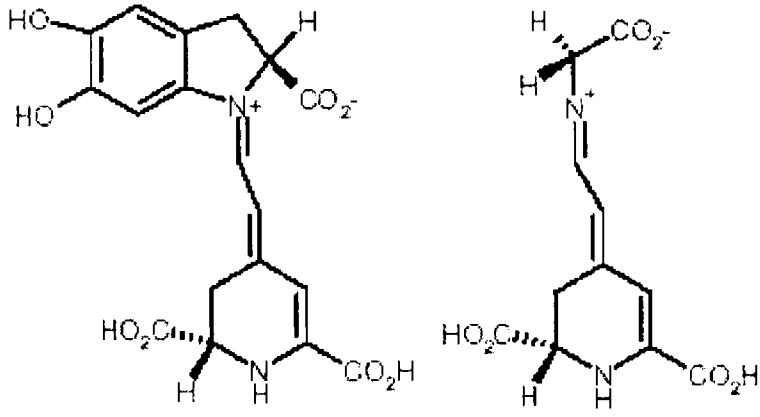


Fig. 2.8. Structure of phenoxazine

e. Betalaines

Betalain pigments are water soluble vacuolar yellow (betaxanthins) and violet (betacyanins) pigments (Fig. 2.9) that replace anthocyanins in most plant families of the order Caryophyllales. They are also found in some fungal species of *Amanita* and *Hygrocybe*. Betalains are conjugates of the chromophore betalamic acid which are derived from dihydroxyphenylalanine by an oxidative 4, 5-extradiol ring opening mechanism (Wohlpart and Mabry, 1968).



Betacyanin **Betaxanthin**

Fig. 2.9. Structure of betalaines

2.2.4. Benzopyran derivatives

The most studied benzopyran derivatives are the flavonoids (Fig. 2.10). These are phenolic compounds with two aromatic rings bonded by a C_3 unit (central pyran ring) and divided in to 13 classes based on the oxidation state of the pyran ring and on the characteristic colour: anthocyanins, aurons, chalcones,

yellow flavonols, flavones, uncoloured flavonols, flavanones, dihydroflavonols, dihydrochalcones, leucoanthocyanidins, catechins, flavans, and isoflavonoids. Each type of flavonoid can be modified by hydroxylation, methylation, acylation, and glycosylation to obtain a great natural diversity of compounds. Flavonoids are water soluble and are widely distributed in vascular plants. More than 5000 flavonoids have been chemically characterized (Harborne, 1993; Koes *et al.*, 1994).

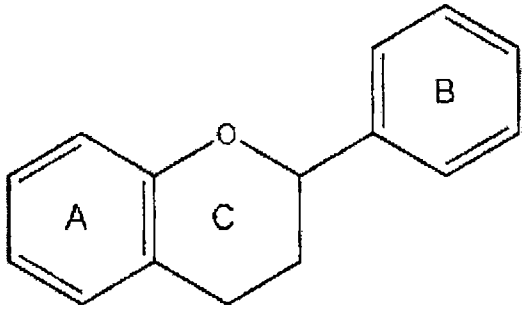


Fig. 2.10. Structure of a typical flavanoid

2.2.5. Quinones

Quinones include large number of structural variants and have a great number of colouring compounds. Their basic structure (Fig. 2.11) consists of a desaturated cyclic ketone that is derived from an aromatic monocyclic or polycyclic compound. Coenzyme Q, represented by the ubiquinone has the quinone structure. This group is widely present in animals, plants and microorganisms and plays an important role in electron transport system in their cells.

Quinones can be classified based on their structure as benzoquinones, naphthoquinones, anthraquinones, dibenzoquinones, dianthraquinones, and dinaphthoquinones. Large number of quinines is found due to their variability in the kind and structure of substituents. Quinones are found in plants: plastoquinones are found in chloroplasts of higher plants and algae; ubiquinones are ubiquitous in living organisms; menaquinones are found in bacteria; naphthoquinones in animals; and anthraquinones in fungi, lichens, flowering plants, and insects. Many quinones are byproducts of the metabolic pathways and a few organisms (fungi) produce large quantities. In general, quinones produce yellow, red, or brown colourations, while quinine salts show purple, blue, or green colours (Hari *et al.*, 1994; Thomson, 1962b).

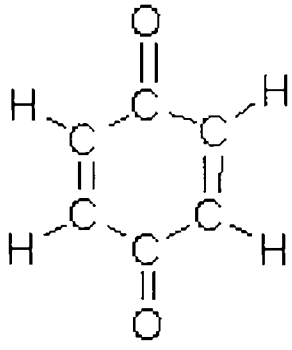


Fig. 2.11. Basic structure of quinone

2.2.6. Melanins

Melanins are nitrogenous polymeric compounds and exist as a mixture of macromolecules with indole ring. They are responsible for the black, grey and brown colouration in animals, plants and microorganisms. There are three different types of melanin (Brown and Salvo, 1994; Hari *et al.*, 1994; Thomson, 1962a; Thomson, 1962b).

(i) Eumelanins - widely distributed in vertebrate and invertebrate animals.

(ii) Phaemelanins - macromolecules in mammals and birds.

(iii) Allomelanins - present in seeds, spores and fungi.

Neuromelanin is the dark pigment present in pigment bearing neurons. The loss of pigmented neurons from a specific nucleus is seen in a variety of degenerative diseases. Dark skin protects against ultraviolet light which causes mutations in skin cells, which in turn cause skin cancers. Light-skinned persons have about a tenfold greater risk of dying from skin burn under equal sun conditions. Furthermore, dark skin prevents UV-A radiation from destroying the essential B vitamin folate, which is needed for the synthesis of DNA in dividing cells. The melanins (Fig. 2.12) act as a protective screen in humans and other vertebrates and in some fungi melanins are essential for their vital cycle.

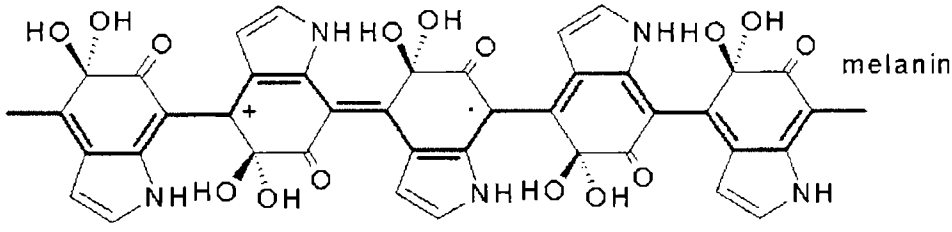


Fig.2.12. Structure of melanin

2.3. Microbial pigments

Among the natural sources of colourants, microorganisms offer great scope and hope. The ease of cultivation, extraction, the genetic diversity in microbes and sophistication of technology has made their choice more feasible (Juailova *et al.*, 1997). Among the different organisms bacteria, yeast, algae, fungi, and actinomycetes appear more efficient and attractive sources of biocolourants. List of some of the microorganisms and their pigment is given in Table 2.1.

There are a number of microorganisms, which have the ability to produce pigments in high yields, including species of *Monascus* (Hajjaj *et al.*, 2000) and *Serratia* (Williams *et al.*, 1971a). The red pigments, produced in solid-state cultures by several species of the genus *Monascus*, have been traditionally used in many Asian countries for colouring and securing a number of fermented foods (Francis, 1987).

Table 2.1. List of some of the pigment producing microbes

Organism	Pigment	Colour	Reference
Janthinobacterium lividum	Violacein	Purple	Matz *et al.*, 2004
Xanthomonas oryzae pv. oryzae	Xanthomonadin	Yellow	Rajagopal *et al.*, 1997
Staphylococcus aureus	Zeaxanthin	Yellow	Hammond and White, 1970
Pseudomonas aeruginosa	Pyocyanin	Blue-green	Baron and Rowe, 1981
Phaffia rhodozyma	Astaxanthin	Red	Florencio *et al.*, 1998
Haematococcus pluvialis	Astaxanthin	Red	Kobayashi *et al.*, 2001
Serratia rubidaea	Prodigiosin like pigment	Red	Moss, 2002
Vibrio gazogenes	Prodigiosin like pigment	Red	Moss, 2002
Alteromonas rubra	Prodigiosin like pigment	Red	Moss, 2002
Rugamonas rubra	Prodigiosin like pigment	Red	Gerber, 1975
Streptoverticillium rubrireticuli	Prodigiosin like pigment	Red	Gerber, 1975
Bradyrhizobium	Canthaxanthin	Orange	Lorquin *et al.*, 1997
Corynebacterium insidiosum	Indigoidine	Blue	Starr *et al.*, 1966
Micrococcus roseus	Canthaxanthin	Orange-pink	Cooney *et al.*, 1966
Dunaliella salina	β-carotene	Orange	Jacobson and Wasileski, 1994

The microorganisms such as *Monascus*, *Rhodotorula*, *Bacillus*, *Achromobacter*, *Yarrowia* and *Phaffia* produce a large number of pigments. Commonly found microbial pigments are carotenoids and astaxanthin. Carotenoids are yellow, orange and red pigments, which are widely distributed in nature. They are utilized as food or feed supplements and as antioxidants in pharmaceutical formulations (Miura *et al.*, 1998).

Several microorganisms have been shown to produce astaxanthin. They include *Agrobacterium aurantiacum* (Misawa *et al.*, 1995), *Phaffia rhodozyma* and *Haematococcus pluvalis* (Johnson and An, 1991). Among them *Phaffia rhodozyma* is a potential candidate for commercial production due to its high astaxanthin content (Andrews *et al.*, 1972). Astaxanthin added to poultry feed can improve the colour of both egg yolks and flesh (Johnson *et al.*, 1980). Astaxanthin possesses an unusual antioxidant activity, which has caused a surge in the nutraceutical market for the encapsulated product. Astaxanthin has potent antioxidant activity and may have a role in decaying or preventing degenerative diseases in human and animals (Schroeder and Johnson, 1993). Research on the health benefits of astaxanthin is very recent and has mostly been performed *in vitro* or at the pre-clinical level with humans (Higuera-ciapara *et al.*, 2006). The medical literature suggests that β-carotene may exhibit anticancer activities and aid in reducing the incidence of cardiovascular diseases (Sies and Krinski, 1995). Further, health benefits such as cardiovascular disease prevention, immune system boosting, bioactivity against *Helycobacter pylori*, and cataract prevention, have been associated with astaxanthin consumption (Ciapara *et al.*, 2006).

The species of *Monascus* produces several natural pigments, including primary colour, red and the secondary colour, orange during the solid state fermentation. These colours are widely used in Asia as food colourants. *Monascus* are traditionally used in oriental countries, originally in China and Thailand, to

prepare fermented rice with strong red colour, which finds several applications ranging from conferring colour to products such as wine, cheese and meat, to medicinal uses and as a meat preservative (Wong and Koehler, 1981).

A maroon dye was extracted from the rhizome of *Arnebia nobilis* (Indrayan *et al.*, 2004). Phycoerythrin pigment was isolated from the cyanobacterium, *Nostoc muscorum* (Ranjitha and Kaushik, 2005). The nutritional requirements for pyoverdine production by *Pseudomonas aeruginosa* was reported by Barbhaiya and Rao, (1985b). The pigment Xanthomonadin produced by the genus *Xanthomonas* has a role against photo damage (Rajagopal *et al.*, 1997).

2.4. Prodigiosin

2.4.1. Occurrence and structure

Prodigiosin is a tripyrrole first characterized from *Serratia marcescens*, which forms beautiful pillar box red colonies. Its name is derived from "prodigious" - something marvellous. The prodigiosin tripyrrole was shown to be localized in extracellular and cell-associated vesicles and in intracellular granules (Kobayashi and Ichikawa, 1991). Secondary metabolites related to prodigiosins are produced by a wide variety of bacteria including *Serratia marcescens*, *Serratia rubidaea*, *Vibrio gazogenes*, *Vibrio psychroerythrous*, *Pseudomonas magneslorubra*, *Alteromonas rubra*, *Rugamonas rubra*, *Streptomyces longisporus*, *Streptomyces spectabilis* and *Streptoverticillium rubrireticuli* (Variyar *et al.*, 2002). Prodigiosin, $C_{20}H_{25}N_3O$, has an unusual structure with three pyrrole rings and is a pyrryldipyrrylmethene; two of the rings are directly linked to each other, and the third is attached by way of a methene bridge (Gerber, 1975; Qadri and Williams, 1972). The highly conjugated system of seven double bonds (Fig. 2.13) presumably accounts for the intense pigmentation.

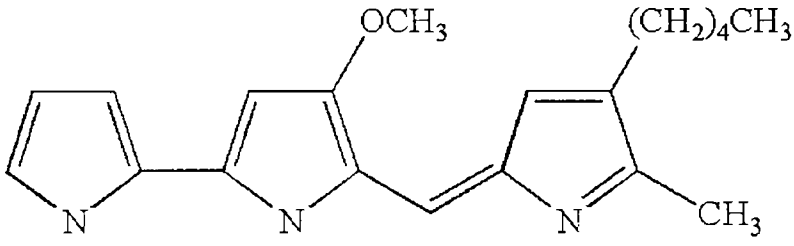


Fig. 2.13. Structure of prodigiosin

A novel endospore forming *S. marcescens* subsp. *sakuensis*, isolated from activated sludge (Ajithkumar *et al.*, 2003) and two novel halophilic bacterial strains named *Zooshikella ganghwensis*, isolated from Korean tidal flats (Yi *et al.*, 2003), have been reported to produce prodigiosin. Further, a novel red pigment, 2, 2V-[3-methoxy-1' amyl-5'-methyl-4-(1"-pyrryl)] dipyrryl-methene (MAMPDM), was isolated from an alkalophillic *Micrococcus* sp. (Variyar *et al.*, 2002).

A family of natural red pigments called prodigiosins is synthesized from *Serratia marcescens* (Han *et al.*, 1998). The members of this family include prodigiosin, cycloprodigiosin hydrochloride (cPrG-HCl), uncedylprodigiosin, metacycloprodigiosin and desmethoxyprodigiosin. Immunosuppressive properties have been attributed to members of the prodigiosin family, which includes undecylprodigiosin, metacycloprodigiosin and cycloprodigiosin hydrochloride (cPrG.HCl), with a mechanism of action different from that of other well known immunosuppressants such as cyclosporin A, FK506 and rapamycin (Kataoka *et al.*, 1995; Songia *et al.*, 1997; Tsuji *et al.*, 1990).

Several bacteria produce metabolites similar to prodigiosin and there has been considerable confusion with respect to naming them. To some extent, "prodigiosin" is used in the literature in a generic sense to include a family of similar materials. In devising trivial names for a group of related compounds it is useful to define a basic nucleus. Two such possibilities have been used for the prodigiosin-like materials (Gerber, 1975). The completely stripped down nucleus, devoid of all substituents, is termed "prodigiosene," while the portion common to most of the natural products, and containing a 6-methoxy substituent, is termed "prodiginine". Hence, prodigiosin could also be referred to as either 2-methyl-3-pentylprodiginine or 2-methyl-3-pentyl-6-methoxyprodigiosene. Prodigiosenes are synthesized by members of two families of Actinomycetales, Actinomycetaceae and Streptomycetaceae. *Nocardia madurae*, *N. pelletieri*, and *Streptomyces longisporus ruber* each synthesize two pigments, one of which has a cyclic side chain attached to the prodigiosene nucleus. Ability to produce these cyclic compounds may be characteristic of species of Actinomycetales. Pigments of lower molecular weight are produced by *S. marcescens*. Whereas, *N. madurae*, *N. pelletieri*, and *S. longisporus ruber* produce pigments of higher molecular weights (Table 2.2) (Williams, 1973).

Four structural types based on the prodiginine nucleus can be recognized:

1. Presence of straight chain alkyl substituents:

1A. Alkyl substituents at both positions 2 and 3.

The prototype is prodigiosin itself with a methyl group at position 2 and a pentyl group at position 3. Higher homologues with methyl at position 2 and either hexyl or heptyl at position 3 (along with prodigiosin) have been isolated from *Pseudomonas magnesiorubra,* the marine psychrophilic bacterium *Vibrio psychroerythreus,* a sewage bacterium (Gerber, 1975). A river bacterium, *Rugamonas rubra* produced prodigiosin (Austin and Moss, 1986). Norprodigiosin

(2-methyl-3-pentyl-norprodiginine) is formed by the *Serratia marcescens* mutant OF (Qadri and Williams, 1973).

IB. Alkyl substituents at position 2 only.

Prodigiosin-like materials with an undecyl chain at position 2 were first fully characterized from *Streptomyces longisporus ruber* (Harashima *et al.*, 1967; Wasserman *et al.*, 1966) and with a nonyl sidechain from *Actinomadura madurae* (Gerber, 1975).

2. Ring formation between positions 2 and 4

A structure with a cyclononyl ring linked to positions 2 and 4, and carrying an additional ethyl substituent was isolated from *Streptomyces longisporus ruber* generally known as metacycloprodigiosin, is probably identical with streptorubin A from *Streptomyces rubrireticuli* var. *pimprina* (Gerber, 1975). Some organisms like *Streptomyces hiroshimensis,* produce both metacyclo-prodigiosin and undecylprodiginine. This is also true for an actinomycete isolated from leek roots and belonging to the *Streptoverticillium baldaccii* cluster (Brambilla *et al.*, 1995).

3. Ring formation between positions 3 and 4

The only example of this structural type is a cyclized form of prodigiosin itself, usually known as cycloprodigiosin; originally isolated from a marine bacterium, *Alteromonas ruber* (Gerber and Gauthier, 1979). Cycloprodigiosin was also found, together with prodigiosin itself, in the anaerobic marine bacterium *Vibrio gazogenes* (Gerber, 1983; Harwood, 1978; Laatsch and Thomson, 1983) and as its hydrochloride in *Pseudoalteromonas denitrificans,* isolated from the sea near Japan (Kawauchi *et al.*, 1997).

4. Ring formation between position 2 of the monopyrryl unit and position 10 of the dipyrryl unit.

Compounds described generically as "macrocyclic prodiginines" have been isolated from *Actinomadura pelletieri* (formerly *Nocardia pelletieri)* and *Actinomadura madurae* (formerly *N. madurae)* (Gerber, 1975). These structures contain a bridge with several $-CH_2-$ groups between the first and third pyrrole rings. These "macrocyclic prodiginines" are apparently unique to the two organisms named. Finally, there has been unfortunate nomenclature confusion between the red pigment prodigiosin and a material referred to in Russian literature as "prodigiosan". The latter is a polysaccharide or lipopolysaccharide also isolated from *Serratia marcescens.* Moreover, in some cases, the Russian word for prodigiosan was translated as prodigiosin to take only one example, "activation of mononuclear phagocytes by a lipopolysaccharide (prodigiosin)" (Panin *et al.*, 1996).

Table 2.2. Structurally identified prodigiosenes (Williams, 1973)

Order	Genus and species	Trivial name of pigment	Prodigiosene nomenclature	Molecular weight (Da) and Formula	Reference
Eubacteriales	*Serratia marcescens*	Prodigiosin	2-Methyl-3-amyl-6-methoxyprodigiosene	323.4 $C_{20}H_{25}N_3O$	Rapoport and Holden., 1962
	S. marcescens	Norprodigiosin	2-Methyl-3-amyl-6-hydroxyprodigiosene	309.4 $C_{19}H_{23}N_3O$	Hearn *et al.*, 1970
	S. marcescens	Dipyrrolyl-dipyrromethene prodigiosin	2-(2-Pyrryl)-4,6-dimethoxypyrodigiosene	334.4 $C_{19}H_{18}N_4O_2$	Wasserman *et al.*, 1968
Actinomycetales	*Nocardia (Actinomadura) madurae*	Nonylprodigiosin	2-Nonly-6-methoxyprodigiosene	363.5 $C_{23}H_{31}N_3O$	Gerber, 1969
	N. (A) madurae	Cyclononylprodigiosin	2,10-Nonano-6-methoxyprodigiosene	365.5 $C_{23}H_{29}N_3O$	Gerber, 1970
	N.(A.) pelletieri	Methylcyclodecylprodigisin	2,10-(10-Methyldecano)-6-methoxyprodigiosene	391.5 $C_{25}H_{33}N_3O$	Gerber, 1971
	Streptomyces longisporusruber and also *N. pelletieri*	Undecylprodigiosin	2-Undecyl-6-methoxyprodigiosene	393.6 $C_{25}H_{35}N_3O$	Harashima *et al.*, 1967; Wasserman *et al.*, 1966
	S. longisporus-ruber	Metacycloprodigiosin	2,4-(9-Ethylnonano)-6-methoxy-prodigiosene	391.6 $C_{25}H_{33}N_3O$	Wasserman *et al.*, 1969

2.5. Microbial production of Prodigiosin

2.5.1. Submerged Fermentation (SmF)

Prodigiosin (5-[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene) methyl]-2-ethyl-3-pentyl-1H-pyrrole) was isolated from *Serratia*, *Pseudomonas* and *Streptomyces* (Giri *et al.*, 2004), actinomycetes and a few other bacteria (Grimont and Grimont, 1978) and was observed as a typical alkaloid compound produced as a secondary metabolite. It has a unique structure consisting of three pyrrole rings and a pyrrolylpyrromethene skeleton with a C-4 methoxy group (Bennett and Bentley, 2000). Prodigiosin appeared only in the later stages of bacterial growth (Williams *et al.*, 1971b). The actinomycete *Streptomyces coelicolour* A3(2) produced a closely related linear tripyrrole, undecylprodigiosin, and a cyclic derivative, butyl-meta-cycloheptylprodiginine in a 2 : 1 ratio (Tsao *et al.*, 1985).

A bifurcated pathway has been proposed for the synthesis of prodigiosin culminating in the enzymic condensation of the terminal products of the two pathways, 4-methoxy-2, 2-bipyrrole-5-carboxyaldehyde and the monopyrrole, 2-methyl-3-n-amyl-pyrrole (MAP). The precursors for prodigiosin were shown to be acetate, serine, alanine, methionine and proline (Williams, 1973). Recently, the mechanism of proline incorporation into a pyrrole moiety has been shown biochemically and a pathway for synthesis of undecylprodigiosin proposed (Cerdeno *et al.*, 2001; Thomas *et al.*, 2002). Several investigations indicated that the pyrrole groups of prodigiosin arise from amino acids (Shrimpton *et al.*, 1963; Stavri and Marx, 1961; Tanaka *et al.*, 1972; Williams *et al.*, 1971a) and acetate (Cushley *et al.*, 1971).

Many studies were carried out to improve prodigiosin production. A classical syntrophic cross-feeding method showed that prodigiosin was formed *via* a bifurcated pathway, which depended upon a number of genes coding for the

enzymes involved (Block, 1961). Many factors, such as temperature, pH, dissolved oxygen level, light and medium composition influenced the production of prodigiosin (Heinemann *et al.*, 1970; Rjazantseva *et al.*, 1995; Sole *et al.*, 1994). Prodigiosin secretion was enhanced on addition of SDS into the cultures (Feng *et al.*, 1982). An integrated fermentation-separation system was employed to eliminate the toxic effects of metabolites on cell growth and alleviate the feedback repression by end product (Bae *et al.*, 2001).

Species of *Serratia marcescens* are the major producers of prodigiosin (Furstner, 2003). Secondary metabolites may be located in the cell envelope of microorganisms, as is true of prodigiosin (Purkayastha and Williams, 1960), although prodigiosin is not released into the medium, as is characteristic of many secondary metabolites. The production of prodigiosin in *S. marcescens* is susceptible to temperature and is substantially inhibited at temperatures higher than 37°C (Giri *et al.*, 2004). Conventional media used for the biosynthesis of prodigiosin by *S. marcescens* strains are complex media that are rich in a variety of nutrients (Furstner, 2003; Giri *et al.*, 2004; Yamashita *et al.*, 2001). Certain nutrients, such as thiamine (Goldschmidt and Williams, 1968) and ferric acid (Silverman and Munoz, 1973), are particularly crucial for prodigiosin production, whereas phosphate (Witney *et al.*, 1977), adenosine triphosphate, and ribose (Lawanson and Sholeye, 1975) have inhibitory effects on prodigiosin yield. It was observed that novel peanut seed broth gave rise to a significant enhancement of prodigiosin production (Giri *et al.*, 2004). Moreover, it was reported that the addition of silica-gel carriers to a liquid culture of *S. marcescens* led to marked increases in cell growth and the production of prodigiosin (Yamashita *et al.*, 2001). In addition, since prodigiosin is often located on the cell envelope, the addition of surfactants, such as sodium dodecyl sulphate (SDS), could also enhance the recovery efficiency for prodigiosins (Feng *et al.*, 1982). Identification of optimized medium composition to achieve more efficient production of a prodigiosin-like

pigment (PLP) from *S. marcescens* SMΔR, which is a SpnR-defective isogenic mutant of *S. marcescens* SS-1 was reported (Wei and Chen, 2005). LB broth was shown to be an effective growth medium for *S. marcescens* SMΔR, leading to the production of a biosurfactant and also a prodigiosin-like-pigment (Horng *et al.*, 2002; Wei *et al.*, 2004). The components of LB broth (tryptone, NaCl, and yeast extract) were examined individually for their effects on prodigiosin production. The effect of vegetable oil supplementation on prodigiosin production was also reported (Wei and Chen, 2005).

A *Serratia marcescens* mutant for prodigiosin production was obtained by UV mutation with rational screening methods and a two-step feeding strategy was used to increase its productivity. In flasks, the mutant strain B6 gave a 2.8-fold higher prodigiosin production than that of the parent strain with glycerol as a carbon source. In a 5 liter bioreactor, with a two-step feeding strategy in which glucose was selected as the initial carbon source in the fermentation media, glycerol was fed as a 'prodigiosin inducer' (Tao *et al.*, 2005).

2.6. Purification and characterization of Prodigiosin

Water insoluble, lipophilic pigments are usually extracted with water-miscible organic solvent, such as acetone, methanol, ethanol, or mixtures thereof, to allow better solvent penetration. Dried materials can be extracted with water-immiscible solvents. The extract usually contains a substantial amount of water, which can be removed by partition to hexane, petroleum ether, diethyl ether, or dichloromethane or mixtures of these solvents. The chromatographic behavior and the ultraviolet and visible absorption spectrum provide the first clues for the identification of pigments. Both the position of the absorption maxima (λ_{max}) and the shape (fine structure) of the spectrum reflect the chromophore. Spectra is taken, stored, and subsequently compared with those of standards (Amaya, 2001).

It is now recommended that the following minimum criteria be fulfilled for identification of an unknown compound (Liaaen-Jensen, 1971; Pfander *et al.*, 1994).

- The visible absorption spectrum (λ_{max})
- Thin layer chromatogram (R*f*)
- High performance liquid chromatogram (Retention time)
- Mass Spectrum (Molecular mass)
- NMR (Chemical shift data)

Prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosene) was extracted from a 72 h culture of *Serratia marcescens* in Nutrient broth with absolute ethanol and petroleum ether; followed by drying in a boiling water bath; and dissolution of the residue in 50 % ethanol (Rosenzweig and Stotzky, 1980).

The pigment from the cell pellet of *Serratia marcescens* isolated from soil was extracted with acetone, mixed with ethyl acetate fraction, and dried with sodium sulphate. The extracts were evaporated and a wave length scan was done from 200 to 700 nm. A solvent mixture in 2.5:2.5:0.5 ratio of dichloromethane, chloroform and acetone was used for effective separation of the impurities extracted along with the pigment by thin layer chromatography. Silica column of mesh size 80–100 was used for separation of the non coloured impurity from the pigment. The purified sample showed a single peak absorbance at 535 nm in the UV spectrophotometer. It was further analysed for determination of molecular weight using mass spectrophotometer. The pure prodigiosin pigment analysed by mass spectrophotometry showed a molecular weight of 324 Da (Giri *et al.*, 2004).

Prodigiosin was extracted by shaking the environmental isolate *Serratia marcescens* 2170 cells with acidic methanol (1 ml of 1N HCl: 24 ml of methanol) and the supernatant was evaporated under vacuum. Atmospheric pressure liquid

chromatography of the extract was performed on silica gel with chloroform and methanol as solvents. The isolated pigment was redissolved in methanol and analyzed by electrospray ionization mass spectrometry (ESI-MS) using a VG-Quattro triple quadrupol mass spectrometer. The isolated pigment was repurified by subsequent semipreparative HPLC. A Nucleosil C_{18} reversed-phase column (250x4 mm, 10 μm) was used with a 0 % to 100 % linear gradient in 30 minutes (A: 10 mM ammonium acetate, pH 7.0, B: 100 % acetonitrile). The elution was monitored both using diode-array UV detector and by ESI-MS (Montaner and Perez-Tomas, 2001; Tomas and Montaner, 2003).

After fermentation of *Serratia marcescens* from the soil, the broth was drained from the internal adsorbent bioreactor (IAB), and then 1 l of a 95 % (v/v) acidified ethanol (pH 3.0) solution was added to the IAB. Attached pigments were extracted by circular desorption using an impeller in the IAB at 200 rpm for 5 h, and then concentrated and centrifuged. It was then isolated using phase separation with water and chloroform. Red prodigiosins were placed in a down-filled chloroform phase and then concentrated by evaporation. Prodigiosin was separated by silica gel column chromatography (2.5×30 cm; Kieselgel 60; Merck, Darmstadt, Germany). It was eluted with a mixture of hexane: ethyl acetate (2:1; v/v). The concentrated pigment was separated by development in a 95:5 (v/v) mixture of chloroform: methanol using TLC. Single red prodigiosin (*Rf* value: 0.43) was then collected and dissolved in acetone. The pigment was repurified by further TLC (chloroform: methanol: diethyl ether = 6:2:2), and finally purified by preparative HPLC (with a C_{18} column (2.5×10 cm). It was isocratically eluted with a mixture of methanol: water (7:3, v/v) that was adjusted to pH 3.0 with 0.1 N HCl at a flow rate of 20 ml/min. A large open stainless steel grid with a pore size of 0.5 cm was used as the support of the internal adsorbent. The concentration of the red prodigiosin produced was estimated by measuring the absorbance at 535 nm using a double beam UV-visible spectrophotometer in acidified methanol (0.01 N HCl

4 ml + methanol 96 ml) (Goldschmidt and Williams, 1968). The molecular mass of the pigment purified was determined using an electrospray-ionization mass spectrometer. The ^{1}H- and ^{13}C-NMR spectra of the pigment sample were recorded after dissolved in $CDCl_3$. The chemical shifts were referenced to an internal TMS (trimethylsilyl) signal. A FT-IR spectrum of the pigment was recorded (Song *et al.*, 2006).

Prodigiosin from the environmental isolate 2170 was extracted from pigmented suspensions with acidic methanol (1 ml of 1N HCl: 24 ml of methanol) and then centrifuged (6,800x*g* for 15 minutes). The solvent of the supernatant was then evaporated under vacuum. Atmospheric pressure liquid chromatography of the extract was performed on silica gel with chloroform and methanol as solvents. The eluted fractions were pooled and the chloroform/methanol extract was vacuum evaporated, redissolved in H_2O and lyophilized. (Montaner *et al.*, 2000). The isolated pigment was redissolved in methanol and analyzed by electrospray ionization mass spectrometry (ESI-MS) using a VG-Quattro triple quadrupol mass spectrometer. The isolated pigment was repurified by subsequent semipreparative HPLC. A Nucleosil C_{18} reversed-phase column was used with a 0 % to 100 % linear gradient in 30 minutes (A: 10 mM ammonium acetate, pH 7.0, B: 100 % acetonitrile). Prodigiosin was purified from *S. marcescens* 2170 by methanol/HCl extraction followed by silica gel chromatography and semipreparative reverse-phase HPLC. ESI-MS gave a molecular weight of 323.4 Da, consistent with the expected value for prodigiosin ($C_{20}H_{25}N_3O$). The structure of prodigiosin was further confirmed by high-field ^{1}H-NMR spectroscopy (Montaner *et al.*, 2000).

The pigment produced by *Serratia marcescens* SMΔR (an SpnR-defective isogenic mutant of *S. marcescens* SS-1 (Horng *et al.*, 2002) was purified and characterized by NMR and mass spectrometry to determine its chemical structure (Wei and Chen, 2005). The pigment was extracted from the fermentation broth

with methanol and then purified by using a hexane-balanced silica gel column to trap the target product within the column (Cang *et al.*, 2000; Montaner *et al.*, 2000). The loaded column was eluted with 10 M ethyl acetate to liberate the adsorbed product. The orange eluate was harvested and dried in a vacuum drier at 45°C to obtain the purified product (red powder). The pigment obtained in their study, reported as undecylprodigiosin based on the NMR and MS analyses, also known as prodigiosin 25-C, is one of the red pigment produced by *Serratia* sp. and *Streptomyces* sp. It possesses immunosuppressive and apoptosis-inducing activities similarly to prodigiosin, but has been less studied (Tomas *et al.*, 2003).

2.7. Molecular biology and genetics

The genes for prodigiosin biosynthesis in *Serratia* lie in a large operon. The organization of the prodigiosin biosynthetic gene (pig) clusters in *Serratia* 39006 and in an *S. marcescens* strain, ATCC 274 (Sma 274) was reported (Cerdeno *et al.*, 2001).

The *Serratia* 39006 pig gene cluster contains an additional gene, tentatively designated pigO. RT-PCR and primer extension has confirmed that there is transcriptional read through between pigN and pigO, consistent with pigO being part of the pig operon in that strain (Slater *et al.*, 2003).

Many strains of *Serratia marcescens* produce pigment *via* a bifurcated pathway in which 2-methyl-3-amylpyrrole (MAP) and 4-methoxy-2, 2'-bipyrrole-5-carboxyaldehyde (MBC) are enzymatically condensed into 2-methyl-3-amyl-6-methoxypyrodigiosene, or prodigiosin. Several mutants of *S. marcescens* have been identified as being blocked in either the MAP or MBC pathway. However, little is known about the precursors accumulated by these mutants, and nothing is known about the enzymes or gene products involved (Williams and Qadri, 1980).

Dauenhauer *et al.*, (1984) isolated a *Serratia marcescens* genomic clone capable of condensing the two prodigiosin precursors, MAP and MBC, to form prodigiosin. However, no sequence data were reported for this clone. The pig gene cluster from *Serratia* sp. ATCC 39006 (*Serratia* 39006) was expressed in *Erwinia carotovora* subsp. *carotovora* (Ecc; 25 out of 36 strains tested), though it was not expressed in several other members of the Enterobacteriaceae, including *E. coli* (Thomson *et al.*, 2000). In *Serratia* 39006 the production of prodigiosin is regulated by multiple factors, including a quorum-sensing system, *via* the LuxIR homologues, SmaI and SmaR (Slater *et al.*, 2003; Thomson *et al.*, 2000). Interestingly, prodigiosin production became N-(3-oxohexanoyl)-L-homoserine lactone (OHHL) dependent when expressed in Ecc, despite the latter producing a different signaling molecule from that made by *Serratia* 39006 (Thomson *et al.*, 2000).

Prodigiosin is an easily assayed secondary metabolite of *S. marcescens* and may be useful as a model system to study the mechanism of expression of secondary metabolites in bacteria. Isolation of recombinant molecules encoding the prodigiosin biosynthetic pathway would provide an approach to identifying gene products and understanding the enzymology and the genetics of prodigiosin biosynthesis. The isolation of DNA sequences encoding part of the prodigiosin biosynthetic pathway by use of a cosmid vector-*Escherichia coli* cloning system was reported (Dauenhauer *et al.*, 1984).

A cosmid containing <35 kb of the *Serratia* chromosome encodes synthesis of the pigment in the heterologous host, *Erwinia carotovora*, demonstrating, for the first time, that the complete prodigiosin biosynthetic gene cluster had been cloned and functionally expressed (Thomson *et al.*, 2000). Prior to this study the biosynthetic cluster of the related pigment undecylprodigiosin (Red),

produced by *Streptomyces coelicolour* A3(2), had also been cloned (Tsao *et al.*, 1985). Because *S. coelicolour* A3(2) Red2 mutants were able to cross-feed defined *S. marcescens* prodigiosin mutants (Feitelson and Hopwood, 1983), undecylprodigiosin is thought to be produced along a similar biosynthetic route to that proposed for the biosynthesis of prodigiosin by *S. marcescens*. The red biosynthetic cluster of *S. coelicolour* A3(2) was cloned from a cosmid based chromosomal library on a 35.7 kb chromosomal insert (Malpartida *et al.*, 1990) and is thought to comprise at least 18 genes (Coco *et al.*, 1991; Narva and Feitelson, 1990).

2.8. Application

Prodigiosin has several biological activities such as immunomodulatory, antibacterial, antimycotic and antimalarial activities and so on (Lazaro *et al.*, 2002; Pandey *et al.*, 2003). It has been reported that prodigiosin could induce apoptosis in various kinds of cancer cells, such as haematopoietic, colorectal and gastric cancer cells. (Diaz-Ruiz *et al.*, 2001; Montaner *et al.*, 2000; Montaner and Perez-Tomas, 2001). However, the inhibitory effects of prodigiosin on metastasis and invasion, and the underlying mechanism have not been elucidated. The effects of prodigiosin isolated from *Hahella chejuensis* on the production of inflammatory cytokines and nitric oxide (NO) in lipopolysaccharide (LPS)-activated murine macrophage was studied (Huh *et al.*, 2007).

The cytotoxic properties of prodigiosins, tripyrrole red pigments, have been recognised for some times. Fullan *et al.*, (1977) observed the antitumour activity of prodigiosin in mice. Although apoptotic mechanisms of prodigiosins are still to be fully determined (additional *in vivo* assays are necessary), it is clear that prodigiosin are a new class of anticancer drugs, which hold out considerable

promise as a therapeutic agent (Tomas *et al.*, 2003). List of some patents related to prodigiosin is given in Table 2.3.

Prodigiosins also possess anti-cancer properties (Perez-Tomas *et al.*, 2003), with the methoxy group playing a critical role (Boger and Patel, 1988) and their anti-cancer effects have been observed in several human cancer cell lines *in vitro* (Campas *et al.*, 2003; Diaz-Ruiz *et al.*, 2001; Kawauchi *et al.*, 1997; Montaner *et al.*, 2000; Montaner and Perez-Tomas, 2001; Yamamoto *et al.*, 2000a; Yamamoto *et al.*, 2000b) and in human primary cancer cells (Campas *et al.*, 2003) with no marked toxicity toward non-malignant cell lines (Montaner *et al.*, 2000; Montaner and Perez-Tomas, 2001).

Prodigiosin induces apoptosis in certain cancer cells (Montaner *et al.*, 2000; Montaner and Perez-Tomas, 2001; Perez-Tomas *et al.*, 2003). Owing to these characteristics, prodigiosin may have potential for medical applications, for instance, it may be used to develop antitumor drugs (Furstner, 2003; Perez-Tomas *et al.*, 2003). In light of its potential commercial values, there is a demand to develop high-throughput and cost effective bioprocesses for prodigiosin production. The molecular mechanisms of the antitumorigenic potential of prodigiosin are affected by multiple events, giving rise to apoptosis (Soto-Cerrato *et al.*, 2007a).

Prodigiosins and synthetic derivatives have been shown to have potent and specific immunosuppressive activity, with novel targets clearly distinct from other drugs (D'Alessio *et al.*, 2000; Mortellaro *et al.*, 1999; Tsuji *et al.*, 1990). Montaner *et al.*, (2000) showed that prodigiosin extracted from *S. marcescens* could induce apoptosis in haematopoietic cancer cell lines including acute T-cell leukaemia, myeloma and Burkitt's lymphoma, with little effect on non-malignant cell lines. Prodigiosin has also been shown to induce apoptosis in human primary cancer cells

in the case of B and T cells in B-cell chronic lymphocytic leukaemia samples (Campas *et al.*, 2003). The anticancer agent prodigiosin has been shown to act as an efficient immunosuppressant, eliciting cell cycle arrest at non-cytotoxic concentrations, and potent proapoptotic and antimetastatic effects at higher concentrations (Soto-Cerrato *et al.*, 2007b).

The apoptotic effects of prodigiosin have been observed in several human cancer cell lines in tissue culture (Azuma *et al.*, 2000; Yamamoto *et al.*, 2001; Yamamoto *et al.*, 2000a; Yamamoto *et al.*, 2000b), in hepatocellular carcinoma xenografts (Yamamoto *et al.*, 1999). Prodigiosin was found to be a potent anticancer agent which could induce apoptosis of several cancer cell lines *in vitro* including haematopoietic cancer cells, colon cancer cells, B-cell and chronic lymphocytic leukemia cells (Campas *et al.*, 2003).

Furthermore, several pharmaceutically relevant prodigiosins (PGs) such as undecylprodigiosin, metacycloprodigiosin, roseophilin and nonylprodigiosin, in addition to prodigiosin, are thought to have potential for antibacterial, antimalerial, anticancer, cytotoxic and immunosuppressive activities (D'Alessio *et al.*, 2000; Diaz-Ruiz *et al.*, 2001; Han *et al.*, 1998; Montaner and Perez-Tomas, 2001).

Table 2.3. Prodigiosin patents

Patent No	Abstract	Inventors	Published date
United States Patent 6638968	A novel use of prodigiosin for treating diabetes mellitus without any side effect. Link: http://www.freepatentsonline.com/6638968.html	Kim, Hwanmook (Taejon, KR) Han, Sangbae (Cheongju-si, KR) Lee, Changwoo (Taejon, KR) Lee, Kihoon (Taejon, KR) Park, Sehyung (Taejon, KR) Kim, Youngkook (Taejon, KR)	10/28/2003
United States Patent 6645962	The prodigiosin from *Serratia marcescens* is useful as an immunosuppressive in various fields, including the treatment of the diseases requiring immunosuppression and the basic research for the diseases, the transplantation of the organs or tissues, and the immune cells. Link: http://www.freepatentsonline.com/6645962.html	Kim, Hwanmook (Taejon, KR) Kim, Youngkook (Taejon, KR) Han, Sangbae (Choongcheongbuk-do, KR) Yoo, Sungrak (Seoul, KR)	11/11/2003

United States Patent 4266028	Prodigiosin, an antibiotic, is effectively produced by culturing a novel *Serratia marcescens* R-2 strain. A synthetic culture medium is also provided, which contains a higher fatty acid having 12 to 18 carbon atoms, a salt thereof or an ester thereof as the sole or main source of carbon and in which a strain of *Serratia marcescens* having the abilities to assimilate the source of carbon and to produce prodigiosin can be cultivated to obtain prodigiosin. Link: http://www.freepatentsonline.com/4266028.html	Nakamura, Katsumi (Takasaki, JP) Kitamura, Kumpei (Takasaki, JP)	05/05/1981
United States Patent 20040127547	The invention relates to novel use of prodigiosin for the treatment of Rheumatic arthritis and can provide excellent treatment effect to Rheumatic arthritis by treating composition including prodigiosin isolated from *Serratia marcescens* as an active principle to DBA-1 mouse of collagen-induced Rheumatic arthritis animal model and thereby inhibiting production of internal cytokine which is a important pathogen of Rheumatic arthritis. Link:http://www.freepatentsonline.com/20040127547.html	Kim, Hwan-Mook (Taejon, KR) Han, Sang-Bae (Chungju, KR) Lee, Chang-Woo (Taejon, KR) Lee, Ki-Hoon (Taejon, KR) Park, Se-Hyung (Taejon, KR) Kim, Hyoung Chin (Taejon, KR) Kim, Young-Kook (Taejon, KR)	07/01/2004

2.9. Prodigiosin from marine bacteria

The marine microorganisms are yet to be tapped as a source of prodigiosin in spite of few reports. *Serratia marinorubra*, a species producing prodigiosin isolated originally from Pacific Ocean water was described by ZoBell and Upham, (1944). The purple pigment in *Serratia marinorubra* was easily extracted from the bacterial biomass with ethanol (Courington and Goodwin, 1955). In acidified ethyl ether it is purplish-pink, and in alkaline ether yellowish-brown; the absorption spectra of these two forms were identical with those of prodigiosin (Hubbard and Rimington, 1950). The pigment produced by two aerobic, Gram negative, rod-shaped bacteria, was compared morphologically and physiologically with *Serratia* sp., was shown to be similar to prodigiosin, the red pigment of *S. marcescens* (Lewis and Corpe, 1964).

The red pigment of *Vibrio psychroerythrus* (formerly marine psychrophile NRC 1004) was identified as prodigiosin by comparison of its mass spectrum, absorption spectrum in the visible range, and chromatographic behavior with prodigiosin isolated from *Serratia marcescens* (D' Aoust and Gerber, 1974). Kim *et al.*, (2007) reported that the main red-metabolite of *Hahella chejuensis* KCTC 2396 as prodigiosin. *Serratia rubidaea* N-1, which was isolated from the Ariake Sea, a bay located in the Kyusyu region, Japan, is a producer of red pigment prodigiosin (Yamazaki *et al.*, 2006).

Three strains of red, antibiotic-producing marine bacteria, which are commonly found in Mediterranean coastal waters during the autumn months (Gauthier *et al.*, 1975), were described and named *Alteromonas rubra* (Gauthier, 1976). The absorption spectra at pH 4.0 and 9.0 of ethanolic extracts of the red pigments resembled closely those of a similar *S. marcescens* extract. However, high pressure liquid chromatography of chloroform methanol extracts showed

considerable heterogeneity for each extract, with some similarities and some differences in peak positions. Thus, the marine pigments were judged to be similar, but not identical, to the "prodigiosin" of *S. marcescens* (Gauthier, 1976).

2.10. Indian Scenario

In general, the scenario on research on microbial pigments at national level is limited to very few reports over the years. The role of pigment Xanthomonadin produced by the genus *Xanthomonas* against photodamage was reported (Rajagopal *et al.*, 1997). Barbhaiya and Rao, (1985a) optimized the nutritional requirements for pyoverdine production by *Pseudomonas aeruginosa*. Studies on a new generation biocolours from *Monascus* sp. and related fungi for use as a colourant to the foods was reported (Tamilselvan, 2001). Cellulose acetate membrane was used for the purification and concentration of natural pigments (Chaudhuri *et al.*, 2004). A maroon dye was extracted from the rhizome of *Arnebia nobilis* (Indrayan *et al.*, 2004). Effect of hexoses and di-hexoses on the growth, morphology and pigment synthesis in the transformed root cultures of red beet was studied (Ravishankar, 2004). A novel medium for enhanced cell growth and production of prodigiosin from *Serratia marcescens*, isolated from soil was optimized (Giri *et al.*, 2004).

Chapter 3

MATERIALS AND METHODS

3.1 SCREENING AND SELECTION OF POTENTIAL PIGMENT PRODUCING BACTERIUM

3.1.1. Source of Microorganism

Bacterial strains, capable of pigment production were isolated from the water and sediment samples from the Coastal areas of Kerala and the mangrove environments of Cochin. Cultures isolated from various marine environments, as part of the ongoing Council of Scientific and Industrial Research (CSIR) sponsored Drug Discovery Project and available as stock cultures in the Microbial Technology Laboratory, DBT, Cochin University of Science and Technology were also screened for pigment production.

3.1.2. Collection of samples

Surface water from a depth of about 50 cm and sediment samples were collected in sterile polythene bags aseptically, transported immediately to the laboratory in iced condition, and processed for bacteriological analyses.

3.1.3. Preparation of serial dilution of the sample

a) In the case of water samples, the collected water sample was homogenized aseptically and 1 ml of the sample was subjected to serial dilution using 9 ml of physiological saline. One ml of the prepared dilution of appropriate dilution (randomly selected) was used as inoculum for plating purpose.

b) In the case of sediment samples 10 g of the sample was added aseptically to 90 ml of sterile physiological saline. After thorough mixing in an electric blender under aseptic condition, the sample was allowed to settle down for 30 min. One ml of the clear supernatant was then serially diluted, using 9 ml of physiological saline, and 1 ml of the prepared dilution was used as inoculum for plating purpose.

3.1.4. Medium

Zobell Marine Broth 2216 (HiMedia) was used for the screening studies. The composition is as given below.

Zobell Marine Broth	:	40.25 g
pH	:	7.0 ± 2.0
Distilled water	:	1000 ml

The prepared medium was autoclaved and used. When used as solid agar medium, 2.0 % agar (w/v) was added to the prepared broth and autoclaved before use.

3.1.5. Isolation of pigment producing bacteria

Pour plate technique was employed for plating the samples. The prepared Zobell Marine agar plates were inoculated with the serially diluted sample and incubated at room temperature for 2-3 days. All the pigment producing single cell colonies that appeared on the plates were picked, purified by streak plate method on the same medium, subcultured on the Zobell Marine agar slants and maintained as stock cultures for further studies. Working stock cultures were maintained at 4°C and subcultured once in two weeks. Permanent stock cultures were maintained by employing paraffin overlay method.

Paraffin overlay method: A single cell derived colony was inoculated onto Zobell Marine agar slant in glass stocking vial and was incubated at room temperature (28 ± 2°C). After 18 h of incubation sterile liquid paraffin was overlaid on the top of the culture and the vials were covered with sterile rubber cap. The culture vials were maintained at room temperature (28 ± 2°C).

3.1.6. Selection of potential strain

Potential strain that produce economically valuable pigment was selected based on the colour of the pigment. In the first phase all chromogenic cultures that showed bright pigmentation were short listed. During the second phase, those cultures that produced red pigment were selected and subjected to further studies towards selection of potential strain. The criteria employed for selection of potential strain included production of intense red pigmentation in solid agar medium and in the broth. After screening 60 chromogenic cultures, the isolate with Code No. **BTWJ8** which recorded considerable amount of red pigmentation on the agar as well as in the broth was selected.

3.2. IDENTIFICATION OF THE BACTERIUM

The selected strain BTWJ8 was identified based on the morphological and biochemical characteristics, as outlined in Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974). In addition molecular ribotyping was also performed in order to confirm the identity as detailed under section 3.2.1.

3.2.1. Molecular ribotyping

Molecular ribotyping of the selected strain was carried out using the partial gene sequence of 16S rRNA.

3.2.1.1. Isolation of genomic DNA (Sambrook *et al.*, 2000)

1) Mid-log phase culture of the bacteria (40 ml) was taken in a sterile oakridge tube and centrifuged at 5000 rpm for 10 min. at 4°C.
2) The supernatant was discarded and the pellet blot dried.
3) The cell pellet was dissolved in 8.75 ml of TE buffer.
4) To the content, 50 µl of Proteinase K (10 mg/ml) and 10 % SDS (1 ml) were added, mixed gently, and incubated at 37°C for 1 h.
5) To this equal volume of phenol-chloroform mixture (1:1) was added, mixed gently, and kept for 10 min. at 4°C.
6) This was centrifuged at 10,000 rpm for 10 min. at 4°C, and the supernatant was transferred to a fresh sterile tube using sterile cut tip.
7) The steps 5 and 6 were repeated three times.
8) To the aqueous phase taken in a sterile 50 ml beaker, 0.1 volume of 5 M sodium acetate (pH 5.2) and 20 ml of isopropanol were added.
9) The precipitated DNA was gently collected by spooling on a glass rod and then washed with 70 % ethanol.
10) The DNA was dissolved in 1 ml TE buffer.

3.2.1.2. Agarose gel electrophoresis (Sambrook *et al.*, 2000)

The agarose gel electrophoresis was carried out in order to check the quality of the DNA prepared.

a) Agarose gel with a concentration of 0.8 % (w/v) was prepared for electrophoresis.
b) Ten µl of the DNA was loaded on to the gel and electrophoresis was carried out at 80 V for 1 h or until the migrating dye (Bromophenol blue) had traversed two-thirds distance of the gel. Lambda DNA cut with *Eco*R1 and *Hind*III (Bangalore Genei) was used as the marker.

c) The gel was stained in a freshly prepared 0.5 mg/ml ethidium bromide solution for 20 min.

d) The gel was viewed on a UV transilluminator, and image captured with the help of Gel Doc system (Biorad).

3.2.1.3. Ribotyping

Ribotyping was performed using universal primer pair for 16S rDNA. A portion of the 16S rRNA gene (1.5 kb) was amplified from the genomic DNA (Reddy *et al.*, 2000; Reddy *et al.*, 2002a, 2002b; Shivaji *et al.*, 2000). The sequences of forward (16SF) and reverse (16SR) primers used for amplifying 16S rDNA were as follows:

Sequence	Reference
16SF 5' AGTTTGATCCTGGCTCA 3'	(Shivaji *et al.*, 2000)
16SR 5' ACGGCTACCTTGT TACGACTT 3'	(Reddy *et al.*, 2002a, 2002b)

3.2.1.4. Polymerase Chain Reaction (PCR)

PCR was performed in a thermal cycler (Eppendorf master cycler personal) under the following conditions standardized in our laboratory.

PCR mix composition

Deionized water	-	12.5 µl
Enyme buffer	-	2.5 µl
dNTP	-	10 mM
Forward primer	-	10 picomol
Reverse primer	-	10 picomol
Sample DNA	-	50 ng
Taq polymerase (1/10)	-	0.6 U

PCR conditions

1. Initial denaturation - 94°C (2 min.)
2. Denaturation - 94°C (45 sec.)
3. Annealing - 59°C (1 min.)
4. Primer extension - 72°C (2 min.)
5. Repeat step 2, 3 and 4 for 29 times
6. Final extension - 72°C (10 min.)
7. Hold - 4°C (5 min.)

3.2.1.5. DNA sequencing

Nucleotide sequences of the PCR amplicon was determined by using the ABI Prism 310 genetic analyzer, using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. The identity of the sequences determined was established by a comparison with the sequences obtained with the gene sequences available in the database using BLAST software (Altschul *et al.*, 1980).

3.2.1.6. Phylogenetic tree construction

Phylogenetic tree was constructed using the neighbour joining method implemented in CLUSTALW. Tree was constructed using nucleotide evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. Tree was visualized using the CLUSTALW N-J tree.

3.3. Enzyme profile of the culture

Enzyme profile of the selected strain was determined as described below.

3.3.1. Protease

The proteolyitic activity was measured according to Nitkowski *et al.*, (1977). The proteolytic medium consisted of 0.3 % beef extract, 0.5 % peptone, 3 % NaCl and 1.5 % agar in distilled water. Casein was provided by adding 1:1 volume to volume solution of evaporated, skimmed milk diluted aseptically in sterile distilled water, the final concentration of milk was 1.5 %. The mixture was swirled gently and poured into Petriplates, which were subsequently dried at room temperature. A loopfull of bacterial culture was spot inoculated on the solidified skimmed milk agar plate. The plate was incubated at room temperature (28 ± 2°C) for 24 h. An uninoculated media was kept as control. Formation of a clear halo around the bacterial colony was considered as positive for casein hydrolysis and hence proteolytic.

3.3.2 Lipase

Lipase assay was done according to Kim and Hoppe, (1986) on agar plates supplemented with lipid substrate. To the Zobell Marine agar medium (section 3.1.4), 1 % Tween 80 (sorbitol monooleate; w/v) was added as lipase substrate, autoclaved and poured into sterile Petriplates. After solidification of the agar a loopfull of bacterial culture was spot inoculated on to the top of agar plates and incubated at room temperature (28 ± 2°C) for 24 h. An uninoculated media was kept as control. Appearance of dense opacity around the colony was considered positive indicating production of extracellular lipase.

3.3.3. Alpha amylase

Alpha amylase was assayed according to Kim and Hoppe, (1986) on Zobell Marine agar added with starch as substrate. To the Zobell Marine agar medium (section 3.1.4), 1 % of soluble starch (w/v) was added, autoclaved and poured to presterilized Petriplates. A loopfull of bacterial culture was spot inoculated on the solidified agar plates and incubated at room temperature (28 ± 2°C) for 24 h. After incubation the plate was flooded with Iodine reagent. Iodine reagent reacts with starch and forms a blue coloured complex. A clearing zone around the bacterial colony is an indication of extracellular alpha amylase production. An uninoculated media was kept as control.

3.4. STANDARDIZATION OF PROCEDURE FOR EXTRACTION OF PIGMENT

3.4.1. Selection of suitable solvent for extracting the bacterial pigment

Initially the solvent that could support maximal yield of pigment on extraction of culture broth was standardized, using different solvents *viz*; ethanol, acetone, methanol, petroleum ether, ethyl acetate, chloroform, hexane, diethyl ether and distilled water.

3.4.1.1. Preparation of culture broth

(1) A preculture was developed in 5 ml of Zobell Marine broth by inoculating the broth with one loopfull of selected bacterial strain and incubating at room temperature for overnight.

(2) Fifty ml of freshly prepared Zobell Marine broth taken in 250 ml Erlenmeyer flasks was inoculated with the preculture as inoculum at 1 % (v/v) level and

incubated for 24 h on a rotary shaker at 150 rpm, at room temperature (28 ± 2°C).

(3) Extraction of the pigment was done according to Slater *et al.*, (2003) with slight modification. The standardized extraction procedure is as follows:

(i) One ml of the fermentation broth was taken in a microfuge tube and centrifuged at 10,000 rpm for 10 min. at 4°C.

(ii) The colourless supernatant was discarded. The coloured cell pellet was resuspended in one ml of solvent.

(iii) The microfuge tubes with the suspended cell pellet was then kept in a water bath, at 60°C, for 20 min. and again centrifuged for 10 min. at 4°C at 10,000 rpm (Sigma-laboratory Centrifuge, Germany).

This was the general procedure followed for the pigment extraction, unless otherwise specified. All the experiments were done in three independent sets and the mean is expressed.

3.4.2. Detection of λ_{max}

The coloured supernatant was then analyzed by scanning in a UV-Visible spectrophotometer (Shimadzu, Japan) for detecting the λ_{max}. The scanning range selected was 400-600 nm. Absorbance at the λ_{max} was measured.

3.5. PURIFICATION OF THE PIGMENT

Pigment produced by the bacterium was purified according to Song *et al.*, (2006) with some modification.

3.5.1. Phase separation

Equal volume of petroleum ether was added to the methanol extract taken in a separatory funnel and mixed well. Equal volume of distilled water and concentrated solution of sodium chloride was then added to the separatory funnel in order to enhance the phase separation. Slowly the pigment got transferred to the epiphase (petroleum ether phase). The hypophase with methanol and water soluble impurities was removed. The petroleum ether phase is washed 4 or 5 times with distilled water to remove residual methanol. The pigment collected from the hypophase was treated with 1 N HCl (9:1; v/v) and concentrated by evaporating the solvent in a 40°C water bath.

3.5.2. Column chromatography

Silica gel column was used for the chromatography for removing impurities from the concentrated pigment after phase separation.

3.5.2.1. Preparation of the column

(1) The silica gel, which was used as the stationary phase, was suspended in the n-hexane, and the fine particles were removed by decantation.
(2) The suspension was degassed under vacuum to remove the air bubbles.

(3) The silica gel suspension was carefully poured into the column filled with n-hexane without air bubble and allowed to settle under gravity while maintaining a slow flow rate through the column.

(4) The column was stabilized by allowing the solvent mixture n-hexane and ethyl acetate (2:1; v/v) to pass though the column bed in descending eluent flow.

3.5.2.2. Sample preparation and application

The concentrated sample prepared as described in section 3.5.1 was loaded on to the silica gel column. After the complete entry of sample into the column the sample was eluted with a mixture of n-hexane: ethyl acetate (2:1; v/v) at a flow rate of 1 ml/min. The red coloured fraction was collected from the column. The eluted fraction was then analyzed by scanning in UV-Visible spectrophotometer (Shimadzu, Japan). The selected scanning range was 400-600 nm.

3.6. CHARACTERIZATION AND IDENTIFICATION OF THE PIGMENT

Generally chromatographic behaviour and the ultraviolet and visible spectrum provide the first clues for the identification of pigments. Pigment produced by the *Serratia* sp. was characterized according to Song *et al.*, (2006) with some modification. The pigment purified after phase separation and column chromatography was characterized by Liquid chromatography-Mass spectrometry (LC-MS), Nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infra red spectroscopy (FT-IR), and Fourier transform-Raman spectroscopy (FT-Raman). Absorption pattern of the purified pigment in different pH was also studied as part of the characterization studies.

3.6.1. Thin-Layer Chromatography (TLC)

The purified pigment was analysed by thin-layer chromatography with silica gel G-60 F_{254} (Merck, Mumbai, India). The solvent system consists of chloroform: methanol (95:5; v/v). The chromatography chamber with the solvent was kept for 20 min. for the equilibration. The sample was spotted on the silica gel sheet using a capillary tube and air dried. The TLC sheet was then dipped in the solvent system. After 45 min. the TLC sheet was carefully removed and the Retention factor (R_f) value was calculated according to the following equation from the chromatogram.

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

3.6.2. Liquid Chromatography-Mass Spectroscopy (LC-MS)

Liquid chromatography-Mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (HPLC) with the mass analysis capabilities of mass spectrometry. 5 µl aliquot sample dissolved in methanol was injected into LC-MS equipped with turbo-ion spray source. Parameter settings used in the analysis were as follows: Ion spray voltage: 5500 V, Curtain gas (CUR): 25.0 lb/in^2, Collision gas (CAD): 6.0 lb/in^2, Ion source gas 1: 20.0 lb/in^2 , Ion source gas 2: 30.0 lb/in^2, Polarity: positive. The LC-MS analysis (Schimadzu Scientific) was done at Spices Board, Govt. of India, Palarivattom, Kochi, Kerala.

3.6.3. Fourier-Transform Infrared Spectroscopy (FT-IR Spectroscopy)

Fourier transform infrared spectroscopy, or simply FT-IR analysis, is a technique that provides information about the chemical bonding or molecular structure of materials, whether organic or inorganic. The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. A molecule that is exposed to infrared rays absorbs infrared energy at frequencies, which are characteristic to that molecule. During FT-IR analysis, a spot on the specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of the infrared rays at different frequencies is translated into an IR absorption plot consisting of reverse peaks. The resulting FT-IR spectral pattern is then analyzed and matched with known signatures of identified materials.

The parameters used in the FT-IR analysis were: spectral range: 4000-500 cm^{-1}, Resolution: 0.9 cm^{-1}. The purified pigment sample was subjected to FT-IR spectroscopic analysis (Thermo Nicolet, Avatar 370), equipped with KBr beam splitter with DTGS (Deuterated triglycine sulfate) detector (7800-350 cm^{-1}), at Sophisticated Test and Instrumentation Centre, Cochin University of Science and Technology, Kochi, Kerala.

3.6.4. Fourier-Transform Raman Spectroscopy (FT Raman Spectroscopy)

Fourier transform (FT) Raman spectroscopy is shown to be a very powerful and valuable technique for the analysis of brightly coloured dyestuffs and pigments. Raman instrumentation virtually eliminates fluorescence from the dye, thus allowing high-quality Raman spectra to be collected. Excitation with infrared wavelengths prevents electronic absorptions, which give rise to fluorescence. In addition, the obtained spectra are completely nonresonant, allowing detection of

vibrational modes of all parts of the molecule including the chromophore (Hallmark *et al.*, 1988).

The parameters used in the analysis were: Raman laser power: 150 mV, Resolution: 4 cm^{-1} and Temperature of measurement: 22°C. The purified pigment sample was analysed by FT Raman Spectroscopy (BRUKER RFS100/S) equipped with standard InGaAs (Indium gallium arsenide) Detector at Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojappura, Thiruvananthapuram, Kerala.

3.6.5. ^{1}H-Nuclear Magnetic Resonance Spectroscopy (NMR Spectroscopy)

Nuclear magnetic resonance spectroscopy most commonly known as NMR spectroscopy is the technique which exploits the magnetic properties of certain nuclei. When placed in a magnetic field, NMR active nuclei absorb at a frequency characteristic of the isotope. The resonant frequency, energy of the absorption and the intensity of the signal are proportional to the strength of the magnetic field. Since this frequency shift is proportional to the strength of the magnetic field, it is converted into a field-independent dimensionless value known as the chemical shift. By understanding different chemical environments, the chemical shift can be used to obtain some structural information about the molecule in a sample. The conversion of the raw data to this information is called assigning the spectrum. The ^{1}H-NMR spectra of the purified pigment was analysed at National Institute for Interdisciplinary Science and Technology (NIST), Thiruvananthapuram, Kerala.

3.6.6. Effect of pH on the absorption spectrum of the purified pigment

The pH of the purified pigment dissolved in methanol was pH 7.0, the pH was adjusted to 2.0 using 0.01 N HCl, and pH 10.0 was adjusted using 0.01 N

NaOH. Absorption spectrum of the purified pigment at different pH was determined using a UV-Visible spectrophotometer (Shimadzu, Japan). The scanning range selected was 300-800 nm.

3.7. PRODUCTION OF PIGMENT BY SERRATIA sp. BTWJ8 UNDER SUBMERGED FERMENTATION

Bioprocess variables that influence the production of pigment by *Serratia* sp. BTWJ8 under submerged fermentation was optimized towards determining the ideal bioprocess. Initially the medium suitable for pigment production was standardized and the medium that supported maximal pigment was used in the subsequent studies.

3.7.1. Selection of media for pigment production

Zobell Marine broth (ZMB), Nutrient broth (NB), Glycerol asparagine broth (GAB), Seawater yeast extract peptone broth (SWYPB) and Mineral salts tryptone sucrose medium (MSTS) with peptone as nitrogen source were evaluated for the impact of media components on pigment production. The composition of media is as follows:

(a) Zobell Marine broth (ZMB): As described in section 3.1.4

(b) Nutrient broth (NB)

Peptic digest of animal tissue	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
NaCl	5.0 g
pH	7.4
Distilled water	1000 ml

(c) Glycerol asparagine broth (GAB)

Glycerol	10.0 g
Asparagine	1.0 g
K_2HPO_4	1.0 g
NaCl	10.0 g
pH	7.0
Distilled water	1000 ml

(d) Sea water yeast extract peptone broth (SWYPB)

Peptone	20.0 g
Yeast extract	12.0 g
Aged sea water	750 ml
pH	7.8
Distilled waster	250 ml

(e) Mineral salts tryptone sucrose medium (MSTS)

Tryptone	10.0 g
Sucrose	20.0 g
$MgSO_4$	4.0 g
$CaCl_2$	7.0 g
K_2HPO_4	0.08 g
KH_2PO_4	0.07 g
NaCl	10.0 g
$FeCl_3$	5.0 mg
$MnCl_2$	5.0 mg
Na_2MoO_4	5.0 mg
$ZnSO_4$	5.0 mg
pH	6.0
Distilled water	1 L

The effect of seawater on pigment production by the bacterium was also studied using the MSTS medium. Bacterial pigment was isolated according to the procedure described earlier in sections 3.4.1 and 3.4.2.

3.7.2. Medium preparation

MSTS medium was prepared as 50 ml broth in 250 ml Erlenmeyer flasks. All the components were dissolved in distilled water, pH adjusted to 6.0 and then autoclaved at 15 lbs for 15 min.

3.7.3. Inoculum preparation and incubation

A loopfull of the agar slope culture was transferred onto 5 ml of autoclaved MSTS medium taken in a boiling tube and incubated for 18 h at room temperature (28 ± 2°C), at 150 rpm in an orbital shaker. Using 1 % (v/v) level of this preculture as inoculum, 50 ml of MSTS medium taken in an Erlenmeyer flask was inoculated and incubated at room temperature (28 ± 2°C) in an orbital shaker for 18 h. Later, one ml of this bacterial culture (Optical density 1.00) was used as inoculum for all the experiments unless otherwise mentioned.

3.7.4. Extraction of pigment

Bacterial pigment produced in the fermented medium was extracted as described earlier in sections 3.4.1 and 3.4.2.

3.7.5. ANALYTICAL METHODS

3.7.5.1. Pigment analysis

The total pigment was estimated according to the following formula (Chen *et al.*, 2006; Williams *et al.*, 1960).

$$TP\ (\mu g/L) = \frac{ADV_1}{7.07 \times 10^4 V_2}$$

Where 'TP' denotes the total pigment yield (μg/L), 'A' the absorbance of the methanol extract at 535 nm, 'D' is the dilution ratio, 'V_1' the volume of methanol added, $7.07x10^4$ is extinction coefficient of prodigiosin and 'V_2' is the volume of the fermentative liquid.

3.7.5.2. Biomass estimation (Vazquez, 2001)

Ten ml aliquot of the sample was withdrawn from the fermentation medium and centrifuged for 10,000 rpm for 10 min. Sedimented cell pellet was washed twice with sterile distilled water and centrifuged again. The cell pellet was then resuspended in 1 ml sterile distilled water and kept for drying in a hot air oven at 100°C until a constant weight (24 h) of biomass was obtained. Biomass was expressed as g/L.

3.7.5.3. Protease assay

Protease in the culture supernatant was assayed by caesinolytic method (Kunitz, 1947) with minor modification. Hammerstein casein, used as substrate for the assay, was measured by the increase in absorbance at 280 nm.

The method followed for the enzyme assay was as described below:

1) Two ml of 1 % (w/v) Hammerstein casein prepared in 0.05 M phosphate buffer (pH 7.0) added with 0.5 ml of the same buffer were preincubated at 40°C for 10 min.
2) To the above solution, 0.5 ml of the diluted enzyme solution was added and incubated at 40°C for 30 min. Appropriate control/blanks were also incorporated.

3) The reaction was arrested with 2.5 ml of 0.44 M Trichloroacetic acid (TCA) solution. To the control, TCA was added before the addition of enzyme sample.
4) The reaction mixture was then transferred to fresh centrifuge tubes and the precipitated protein was removed by centrifugation at 10,000 rpm for 15 min. at 4°C.
5) The absorbance of the clear supernatant was measured at 280 nm in a UV-Visible spectrophotometer against suitable blanks. The TCA soluble fractions of protein formed were quantified by comparison with a standard graph plotted with tyrosine as standard.
6) One unit of protease activity was defined as the amount of enzyme liberated 1 µg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions described.
7) Enzyme activity was expressed as Units per ml (U/ml).

3.7.5.4. Lipase assay

Lipase in the culture supernatant was assayed using pNP Caprylate-pNPC8 (Fluka-Chemie, Germany) as the substrate according to the modified method of Prim *et al.*, (2003) in a microtitre plate.

3.7.5.4.1. Substrate preparation

Solution A (Stock substrate solution)

pNP Caprylate was dissolved in isopropanol and sonicated for 6 min. in a continuous mode for proper emulsification. A concentration of 1.5 % (v/v) of substrate was prepared in isopropanol.

Solution B

Phosphate buffer (0.05 M; pH 7.0) contained 0.1 % (w/v) gum arabic (Sigma-Aldrich, USA) and 0.4 % (v/v) Triton X-100 (SRL, Mumbai).

Buffered substrate (1:10 dilution of the substrate stock solution A in Solution B)

Buffered substrate was prepared by adding 1 ml of solution A to 9 ml of continuously stirred solution of B.

3.7.5.4.2. Procedure

1) An aliquot of 230 µl of buffered substrate was taken in a flat bottom microtiter plate and incubated at 37°C for 10 min. in the microtitre plate reader (BIO-RAD, Model 680 series Microplate reader, USA).
2) To the preincubated buffered substrate, 20 µl of appropriately diluted enzyme solution was added and incubated at 37°C for 30 min.
3) The released pNP was determined by immediate measurement of the absorbance at 415 nm against suitable blanks.
4) One unit of enzyme activity was defined as the amount of enzyme that released one µmol of pNP per minute under the assay conditions described.
5) Enzyme activity was expressed as Units per ml (U/ml).

3.7.5.5. Alpha amylase assay

Alpha amylase in the culture supernatant was assayed according to Medda and Chandra, (1980) as detailed below.

1) One ml of the cell free extract was incubated with 1 ml of 1 % (w/v) soluble starch and 1 ml of phosphate buffer (0.1 M; pH 7.0) at 40°C for 10 min.
2) The reaction was arrested by the addition of 100 µl of 1 N HCl.

3) One ml of the above sample was added with 0.1 ml of Iodine reagent and the volume was made up to 15 ml with distilled water.
4) The blue colour developed was measured at 650 nm in a UV-Visible spectrophotometer (Shimadzu, Japan).
5) One unit of alpha amylase activity was defined as the amount of enzyme required to bring about the hydrolysis of 0.1 mg of starch at 40°C in 10 min. at pH 8.0.
6) Enzyme activity was expressed as Units per ml (U/ml).

3.7.5.6. Protein estimation

Protein content was estimated according to the method of Lowry *et al.*, (1951), using Bovine Serum Albumin (BSA) as the standard. Optical density was measured at 750 nm and expressed in milligram per milliliter (mg/ml).

Reagent

(a) Sodium carbonate in 0.1 N sodium hydroxide 2.0 % (w/v)
(b) Cupric sulphate in distilled water 0.5 % (w/v)
(c) Solution of sodium potassium tartarate in distilled water 1.0 % (w/v)
(d) Working reagent: 100 ml of reagent (a) was added with 1 ml each of reagent (b) and reagent (c).
(e) 1:1 Folin & Ciocalteu's phenol reagent diluted with distilled water
* (d) and (e) prepared freshly before use

Estimation

An aliquot of 200 µl sample was made up to 2 ml with distilled water and added to 5 ml of freshly prepared working reagent (d), mixed thoroughly, and incubated for 10 min. 0.5 ml of reagent (e) was added and incubated for 30 min.

followed by measuring the absorbance at 750 nm in a UV-Visible spectrophotometer (Schimadzu, Japan).

3.7.6. Growth curve

Growth curve of the bacterium *Serratia* sp. BTWJ8 was estimated using MSTS medium. Preparation of medium, inoculum preparation and inoculation were performed as detailed under sections 3.7.2 and 3.7.3 respectively. The prepared medium (50 ml), taken in 250 ml conical flasks was inoculated (1% v/v) and incubated at room temperature (28 ± 2°C) for a total period of 48 h. At regular intervals samples were drawn and growth of the bacteria was measured at 600 nm in a UV-Visible spectrophotometer (Shimadzu, Japan). Uninoculated MSTS medium was kept as control.

3.7.7. Optimization of bioprocess variables for pigment production by *Serratia* sp. BTWJ8

Various physico-chemical and bioprocess variables that influence pigment production by bacterium under submerged fermentation were optimized towards achieving maximal pigment production using MSTS medium. Strategy adopted for the optimization was to evaluate individually the effect of different parameters, through "one-variable-at-a-time" approach, on pigment production under SmF, conduct statistical optimization, and perform a time course experiment under optimized conditions.

The parameters optimized included incubation time, incubation temperature, initial pH of the medium, agitation, effect of different carbon and nitrogen sources, concentration of calcium chloride, trace salts, sodium chloride and inoculum. Preparation of medium, preparation of inoculum, inoculation and incubation and extraction of the pigment were done as described under sections

3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively unless otherwise mentioned. In each case, pigment analysis and biomass estimation were done as detailed under sections 3.7.5.1 and 3.7.5.2 respectively.

3.7.7.1. Incubation time

Optimal incubation time for maximal pigment production was determined by incubating the inoculated media for a total period of 48 h at room temperature (28 ± 2°C) and analyzing the samples at regular interval of 6 h for pigment production. Medium preparation, inoculum preparation and inoculation and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.2. Incubation temperature

Optimal incubation temperature for maximal pigment production was evaluated by incubating the inoculated media at various temperatures *viz*;15°C, 20°C, 25°C, 30°C, 40°C and 50°C, and determining the pigment production after 30 h of incubation (optimized time). Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.3. Initial pH of the medium

Initial pH of the medium that could support maximal pigment production was determined by adjusting the pH of the medium to various levels (i.e., 2, 3, 4, 5, 6, 7, 8, 9,10,11,12 and 13) with either 1 N HCl or 1 N NaOH and determining the pigment production after 30 h of incubation. Medium preparation, inoculum

preparation and inoculation and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.4. Effect of agitation

Effect of agitation on pigment production was studied by incubating the inoculated media taken in the conical flasks on an orbitary shaker at different rpm (50, 100, 150 and 200 rpm) and the pigment production was determined after 30 h of incubation. Control experiment was done by incubating the inoculated media at static condition. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.5. Effect of carbon sources

Effect of carbon sources on pigment production was studied by the addition of dextrose, fructose, galactose, xylose, mannose, glycerol, starch, mannitol and maltose at 2 % (w/v) level, replacing sucrose, which was the original carbon source in the MSTS medium. Control experiment had sucrose as carbon source 2 % (w/v). Pigment production was determined after 30 h of incubation. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.5.1. Effect of different concentrations of dextrose

Effect of different concentrations of dextrose on pigment production was studied by the addition of 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 100 mM, 200 mM and 300 mM concentration of dextrose into the medium, replacing

sucrose the original carbon source. Medium without carbon source was used as the control. Pigment production was determined after 30 h of incubation. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.6. Effect of inorganic nitrogen sources

Effect of inorganic nitrogen sources on pigment production was studied by the addition of 1 % (w/v) ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate and potassium nitrate, replacing tryptone, which was the original nitrogen source. Control medium did not contain any nitrogen source. Pigment production was determined after 30 h of incubation. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.7. Effect of organic nitrogen sources

Effect of organic nitrogen sources on pigment production was studied using 1 % (w/v) yeast extract, beef extract, peptone, malt extract, and urea, and tryptone. Medium without any nitrogen source was used as control. Pigment production was determined after 30 h of incubation. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.7.1. Effect of different concentrations of yeast extract

Effect of different concentrations of yeast extract on pigment production was studied by the addition of 0.2 %, 0.6 %, 0.8 %, 1.0 %, 1.5 %, 2.0 %, 2.5 % and 3.0 % (w/v) yeast extract, replacing tryptone which was the original nitrogen

source in medium. Control medium did not contain any nitrogen source. Pigment production was determined after 30 h of incubation. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.8. Inoculum concentration

Optimal inoculum concentration that supports maximal pigment production was evaluated using different concentrations of inoculum (0.5 %, 1 %, 2 %, 3 %, 4 % and 5 %; v/v) prepared as mentioned under section 3.7.3. Pigment production was determined after 30 h of incubation. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.9. Effect of calcium chloride

Effect of different concentrations of $CaCl_2$ was studied by the addition of 2 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 100 mM and 200 mM of $CaCl_2$ in the medium. Control medium did not contain $CaCl_2$. Pigment production was determined after 30 h of incubation. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.10. Effect of trace salts

Effect of different trace salts was studied by the addition of $FeCl_3$, $MnCl_2$, Na_2MoO_4 and $ZnSO_4$ (0.03 mM) in the media. Control medium contained all trace salts. Pigment production was determined after 30 h of incubation. Medium

preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.7.11. Effect of sodium chloride

Effect of different concentrations of NaCl was studied by the addition of 10 mM, 20 mM, 40 mM, 60 mM, 100 mM, 200 mM, 400 mM, 600 mM, 800 mM and 1000 mM of NaCl in the media. Control medium was maintained without NaCl. Pigment production was determined after 30 h of incubation. Medium preparation, inoculum preparation and inoculation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.7.8. Optimization of bioprocess variables for pigment production by statistical method

Optimization of medium for maximal enzyme production by bacterium through statistical approach was carried out employing Plackett-Burman (PB) Design and Response surface Methodology (RSM) as detailed below. The statistical software package used was Design-Expert® 6.0 (Stat Ease Inc., Minneapolis, U.S.A).

3.7.8.1. Plackett-Burman Design (PB Design)

Optimization of process variables using statistical approach for maximal pigment was carried out using Plackett-Burman design (Haaland, 1990) with selected eleven factors after studying the effect of different parameters by "one-variable-at-a-time" method. The eleven factors included the following: incubation period, inoculum, yeast extract, dextrose, NaCl, $CaCl_2$, K_2HPO_4, KH_2PO_4, trace salts, pH and incubation temperature. The parameters were varied over two levels

and the minimum and maximum ranges selected for the parameters are given in Table 3.1.

Table 3.1: Minimum and maximum ranges for the parameters selected in Plackett-Burman Design for optimization of pigment production by *Serratia* sp. BTWJ8

Sl. No.	Factors	Level	
		Minimum	Maximum
1.	Incubation (h)	12	36
2.	Inoculum (%)	0.50	2
3.	Yeast extract (%)	0.50	1
4.	Dextrose (mM)	10	100
5.	NaCl (mM)	100	200
6.	$CaCl_2$ (mM)	50	100
7.	K_2HPO_4 (mM)	0	0.45
8.	KH_2PO_4 (mM)	0	0.5
9.	Trace salts (mM)	0	0.03
10.	pH	5	7
11.	Temperature (°C)	20	30

The statistical software package Design-Expert® 6.0 (Stat Ease Inc., Minneapolis, U.S.A) was used to generate a set of 12 experimental designs. Production was set up by inoculating the media with respective inoculum percentages as suggested by the model and incubated for specified incubation period (12-36 h), at specified temperature (20-30°C), at 150 rpm. For each experiment, the pigment production was calculated in terms of µg/L. The experiments were done in triplicate. Regression analysis of the experimental data was conducted using statistical software.

Based on the results obtained from the Plackett-Burman design, the fitted first-order model is:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i$$

Where 'Y' is the predicted response, 'β_0' is the model intercept, 'βi' is the linear coefficient and 'x_i' is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response.

Effect of each variable on the production was determined by calculating their respective E-values (Gupta *et al.*, 2004).

$$E = \frac{\text{(Total of responses at high level)–(Total of responses at low level)}}{\text{Number of trials}}$$

3.7.8.2. Response Surface Methodology (RSM)

The significant factors affecting pigment production by *Serratia* sp. BTWJ8 were optimized using a response surface type Box-Behnken (Box and Behnken, 1960) model experimental design. The treatments considered in the design were concentration of inoculum, sodium chloride, calcium chloride, and incubation temperature.

Based on the results of the "one-variable-at-a-time" experiments and PB Design, the effect of five factors *viz*; concentration of inoculum (A), concentration of sodium chloride (B), concentration of calcium chloride (C) and incubation temperature (D) was studied on pigment production using Response Surface Methodology. Other components of the medium which were kept constant included yeast extract: 1 % (w/v), dextrose: 10 mM, K_2HPO_4: 0.45 mM, KH_2PO_4: 0.5 mM, pH: 6.0, incubation time: 22 h.

3.7.8.2.1. Box-Behnken Design

Box-Behnken design model is a second-order design that allows estimation of quadratic effects, and is based on combining a two-level factorial design with an incomplete block design. This design was used for creating the quadratic response model.

Each factor in the design was studied at three different levels. All the variables were taken at a central coded value, considering as zero. A design model with 30 runs in 3 blocks of 10 cases was used as exhibited in Table 3.2, and each independent variable was tested at three levels. The levels were coded in standardized units with the values -1, 0 and +1 representing the lower, middle and higher levels respectively.

Design-Expert® 6.0 (Stat-Ease, Inc., Minneapolis, U.S.A) was used to analyze the experimental design. The average of maximum pigment production values recorded was taken as the dependent variable or response (Y). Regression analysis was performed on the data obtained. The results of the Box-Behnken design were then used to fit a quadratic equation by multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

The following quadratic model was chosen to represent the relationship fitted between the above four variables.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=1}^{4} \beta_{ij} x_i x_j$$

Or in the expanded form,

$$Y= \beta_0 +\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4$$

In this model, Y represents the dependent variable-pigment yield; X_1, X_2, X_3 and X_4 are the independent variables denoting inoculum concentration, concentration of sodium chloride, concentration of calcium chloride and incubation temperature respectively. β_1, β_2, β_3 and β_4 are linear coefficients, β_{12} β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are second order interaction coefficients or cross product coefficients and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients. The design of experiments in terms of actual factors is given in Table 3.3.

The levels tested were inoculum concentration 0.5, 1.25 and 2.0 %; concentration of sodium chloride 100, 150 and 200 mM; concentration of calcium chloride 50, 75 and 100 mM, and incubation temperature 20, 25 and 30°C. Analysis of Variance (ANOVA) was performed and 3-dimensional response surface curves were plotted by Design Expert® software to study the interaction among various physico-chemical factors.

Table 3.2. Box-Behnken design for 4 variables at 3 levels-3 blocks and 30 runs for optimization of pigment production by *Serratia* sp. BTWJ8

BLOCK	RUN	Inoculum (A) X_1 (%)	Sodium chloride (B) X_2 (mM)	Calcium chloride (C) X_3 (mM)	Incubation temperature (D) X_4 (°C)
1	1	0	0	-1	+1
1	2	0	0	-1	-1
1	3	0	0	+1	+1
1	4	+1	-1	0	0
1	5	0	-1	0	0
1	6	+1	+1	0	0
1	7	0	0	0	0
1	8	0	0	+1	0
1	9	-1	+1	0	-1
1	10	-1	-1	0	0
2	11	+1	0	0	0
2	12	0	-1	-1	-1
2	13	0	+1	+1	0
2	14	+1	0	0	+1
2	15	0	0	0	0
2	16	0	+1	-1	0
2	17	-1	0	0	+1
2	18	0	-1	+1	0
2	19	-1	0	0	-1
2	20	0	0	0	0
3	21	0	0	0	0
3	22	0	+1	0	+1
3	23	-1	0	+1	0
3	24	0	-1	0	+1
3	25	+1	0	-1	0
3	26	+1	0	+1	0
3	27	-1	0	-1	0
3	28	0	-1	0	-1
3	29	0	+1	0	-1
3	30	0	0	0	0

Table 3.3. Box-Behnken design for pigment production by *Serratia* sp. BTWJ8

BLOCK	RUN	Inoculum (A) X_1 (%)	Sodium chloride (B) X_2 (mM)	Calcium chloride (C) X_3 (mM)	Incubation temperature (D) X_4 (°C)
1	1	1.25	150	50	30
1	2	1.25	150	50	20
1	3	1.25	150	100	30
1	4	2.00	100	75	25
1	5	1.25	150	75	25
1	6	2.00	200	75	25
1	7	1.25	150	75	25
1	8	1.25	150	100	20
1	9	0.50	200	75	25
1	10	0.50	100	75	25
2	11	2.00	150	75	20
2	12	1.25	100	50	25
2	13	1.25	200	100	25
2	14	2.00	150	75	30
2	15	1.25	150	75	25
2	16	1.25	200	50	25
2	17	0.50	150	75	30
2	18	1.25	100	100	25
2	19	0.50	150	75	20
2	20	1.25	150	75	25
3	21	1.25	150	75	25
3	22	1.25	200	75	30
3	23	0.50	150	100	25
3	24	1.25	100	75	30
3	25	2.00	150	50	25
3	26	2.00	150	100	25
3	27	0.50	150	50	25
3	28	1.25	100	75	20
3	29	1.25	200	75	20
3	30	1.25	150	75	25

3.7.8.2.2. Validation of the model

In order to validate the response surface model, a random set of experiments was set up according to the conditions predicted by the model. The responses obtained from the trials conducted as mentioned above, following the Box-Behnken design model for four variables, was used to estimate the coefficients of the polynomial model using standard regression techniques. The estimate of "Y" was used to generate an optimal combination of factors that can support maximal pigment production using predictive models from response surface methodology. The software Design-Expert® 6.0 (Stat Ease, Minneapolis, U.S.A) was used to fit the response surface-Box-Behnken model to the experimental data. All the experiments were carried out independently in triplicates.

3.7.9. Time course study under optimized condition

Time course experiment was conducted with the optimized conditions determined after statistical optimization of various variables. The conditions selected included the following:

Yeast extract	1 % (w/v)
Dextrose	10 mM
K_2HPO_4	0.45 mM
KH_2PO_4	0.5 mM
pH	6.0
Incubation time	22 h
Inoculum concentration	1.64 % (v/v)
Incubation temperature	28°C
$CaCl_2$	75 mM
NaCl	196 mM

Medium preparation, inoculum preparation, and extraction of pigment were performed as detailed under sections 3.7.2, 3.7.3, 3.4.1 and 3.4.2 respectively.

3.8. APPLICATION STUDIES

In the present study, scope for probable application of the bacterial pigment was evaluated for textile, rubber, paper and plastic industries.

3.8.1. Textile Materials

Different types of textile materials like 'Cotton', 'Chiffon', 'Poplene', '2 by 2', 'Pure silk', 'Century cotton', 'Dupoil silk', '2 by 1', 'Organdi', 'Polyester', 'Terrycotton' and 'Nylon' which were commercially available were selected for the experiment. Each material was cut into equal size of 2 cm^2. Bacterial pigment in methanol (40 µg/L) was used as the stock solution. From this stock solution 100 µl (0.004 µg; w/v), 200 µl (0.008 µg; w/v) and 300 µl (0.012 µg; w/v) were applied to the cloth material in a warm surface. The cloth material was allowed to dry at room temperature for about 1 h.

One set of experiment was done with the application of thiourea as a mordant (Shirata *et al.*, 2000). The dyed cloth materials were dipped in thiourea solution (1 %; w/v) for 30 min. at 70°C. For all the experiments white cloth material were taken as a control.

3.8.1.1. Wash performance of the textile materials at room temperature

All the dyed textile materials were washed with soap solution (sunlight 0.7 %; w/v) for 30 min. at room temperature. After 30 min. (random selection) the cloths were washed with tap water and allowed to dry at room temperature (28 ± 2°C). Absorbance of the soap solution after washing was measured at 535 nm in a UV-Visible spectrophotometer. Appropriate blank was also used for

the experiment. The same procedure was repeated for the dyed textile material treated with thiourea as described under section 3.8.1.

3.8.1.2. Wash performance of the textile materials at 40°C

All the dyed textile materials were washed with soap solution (sunlight 0.7 %; w/v) for 30 min. at 40°C. After 30 min. the cloths were washed with tap water and allowed to dry at room temperature. Absorbance of the soap solution after washing was measured at 535 nm in a UV-Visible spectrophotometer. Appropriate blank was also used for the experiment. The same procedure was also repeated with the dyed textile material treated with thiourea as described in section 3.8.1.

3.8.2. Rubber Products

Aqueous dispersion (1 %) of pigment was prepared by means of sonicating for 30 min. Other compounding ingredients were prepared by ball milling. Compounding recipe is given in the Table 3.4.

Table 3.4. List of compounding ingredients

Ingredients	Mix 1	Mix 2	Mix 3	Mix 4
	Parts per hundred gram rubber (phr)			
Natural rubber latex (60 % Dry rubber content)	100.0	100.0	100.0	100.0
Sulphur (50 %)	1.5	1.5	1.5	1.5
ZnO (50 %)	0.9	0.9	0.9	0.9
Accelerator (50 %)	0.7	0.7	0.7	0.7
Antioxidant (50 %)	0.5	0.5	0.5	0.5
Pigment dispersion (1 %)	0.0	0.16	0.3	0.5

The three mixes were casted on to Petriplates and kept for 24 h at room temperature (28 ± 2°C). The rubber sheets with 1 mm thickness were then kept at 70°C for 2 h in a hot air oven for proper vulcanization of the rubber. Mix 1 was kept as the control.

3.8.3. Paper Products

Eight type of paper with different qualities like 'art paper', 'JK paper', 'sunlight', '6.9 SPB', '7.8 SPB', '11 Kg JK', '21.3 Kg JK', '18.6 Kg SPB' commercially available in the market were selected for the present study. All the paper materials were cut into equal size of 2 cm^2. Bacterial pigment in methanol (40 µg/L) was used as the stock solution. From this stock solution 200 µl (0.008 µg; w/v) was applied to the different paper materials on a warm surface and

allowed to dry at room temperature for 15 min. (random selection). Paper material without pigment was kept as control for all paper materials.

3.8.4. Plastic products

Bacterial pigment in methanol (40 µg/L) was used as the stock solution. 10 % solution of polymethyl methacrylate (PMMA) was prepared in chloroform. 250 µl (0.01 µg; w/v), 500 µl (0.02 µg; w/v), 1ml (0.04 µg; w/v) and 2 ml (0.08 µg; w/v) of bacterial pigment were added to PMMA solution from the stock solution separately. Mixed well and poured into a watch glass and kept for 3 h at room temperature (28 ± 2°C). Adequate care was taken to cover the watch glass well in order to prevent air contact.

Chapter 4

RESULTS

4.1. SCREENING, SELECTION AND IDENTIFICATION OF POTENTIAL PIGMENT PRODUCING BACTERIUM

4.1.1. Screening and selection of potential strain

One hundred chromogenic bacteria were isolated from various samples. Among them 60 chromogenic cultures, which showed different morphological characteristics, were selected for further screening. Pigment production was confirmed by cultivating the isolates in Zobell Marine broth. The cultures, which showed bright pigmentation in the broth, were shortlisted based on visual observation.

Further studies were restricted to red pigment producing isolates since red colour is known to be produced by only a few groups of microbes and it is used as an appealing colouring agent for various applications. Only six isolates produced red pigment and from among them the potential strain was selected based on the intensity of red pigment produced on agar medium and in liquid broth. The isolate BTWJ8 obtained from Puthuvypin, Kochi showed considerable amount of red pigment production both on the agar medium and in the liquid medium after 6 h of incubation in Zobell Marine medium compared to other isolates which produced red pigment only after 24 h besides recording rapid growth. Hence, the strain **BTWJ8** was selected as the potential strain for pigment production in the present study.

4.2. IDENTIFICATION OF BACTERIUM

The selected bacterial isolate BTWJ8 was identified as *Serratia* sp. according to the morphological and biochemical characteristics (Table 4.1). The taxonomy of *Serratia* sp. BTWJ8 is given in Table 4.2.

Table 4.1. Morphological and biochemical characteristics of *Serratia* sp. BTWJ8

	Variable	Characteristics
Colony and cell morphology	Colony shape	Round
	Colony size	Medium
	Edge	Smooth
	Surface	Smooth
	Opacity	Opaque
	Elevation	Convex
	Colour	Red
	Motility	Motile
	Cell shape	Rod
	Cell size	Small
Biochemical characteristics	Gram staining	Negative
	MOF	Fermentation with gas production
	H_2S production	Negative
	Indole	Negative
	Methyl Red	Negative
	Voges-Proskauer	Positive
	Citrate ulilization test	Positive
	Catalase	Positive
	Oxidase	Negative
	Glucose	Positive
	Lactose	Negative
	Sucrose	Positive
	Mannitol	Positive
	Urease	Negative
	Enzyme profile	
	Protease	Positive
	Lipase	Positive
	Alpha amylase	Positive

Table 4.2. *Serratia* sp. BTWJ8-Taxonomy (Hejazi and Falkiner, 1997)

Rank	Name
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Serratia*

4.2.1. Ribotyping using 16S rRNA gene

The identity of the strain was further confirmed by ribotyping using partial gene sequence of 16S rRNA, which was amplified and sequenced. The amplicon with size of approximately 1500 bp obtained after PCR is shown in Fig. 4.1 and the partial nucleotide sequence is presented in Fig. 4.2.

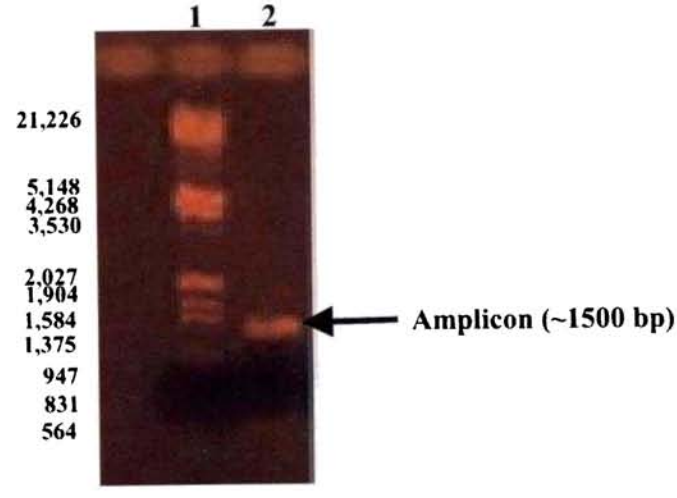


Fig. 4.1. The PCR amplicon of 16S rRNA gene obtained from *Serratia* sp. BTWJ8

Lane 1 Lamda DNA/*Eco*R1- *Hind* III double digest marker
Lane 2 Amplified 16S rRNA gene

TATCCGACTCTCCCTTGCCCTCGCCGTGCTCGCCATGATGGGATCGA
CGCTCATACCGGTCGATGGCTATGGGTAACAAGGGTGCCTTCGGG
TGTCCCGCTTCTCGATATGGGCATGATGATTTGCTAGCTGCTGACG
TTCGGCGGTTTATGAGAGGCTGGCTACCTGTCTTACCCACATGATC
TGCTTCAGTGGAACTTTAAGTGTTTCAATAGTGCCTAGGGGAGGAC
GCAGCGTTGAATATTGCACCCTGGCAACACCCCTGATGCACCCGCC
TCAGAGCTCCCAAACGTCAGGGCAATCTCATCCGTTTTCACAGAAA
CGCAAAAAATAAGTTTGGCATATTGCTGTTTGAAATTAAAATAGTA
CCGCAGGGGGCCTCCCCCCCCTTTTTGTTTGAGTGGAGCTAGAACGT
AATCGGACGGGAGGACAAAAAACAATGTGGCGGCGCG

Fig. 4.2. The partial nucleotide sequence (452 bp) of the amplicon of 16S rRNA gene obtained from *Serratia* sp. BTWJ8

The partial sequence of the 16S rRNA gene obtained for *Serratia* sp. BTWJ8 was submitted to Genbank (Accession number EU239958) through BankIt programme, at NCBI site (http:/ www.ncbi.nlm.nih.gov/BankIt). The identity of the *Serratia* sp. BTWJ8 could be confirmed by comparing the sequences with Genbank entries, by BLAST programme (Altschul *et al.*, 1980). The sequences, which showed similarity to the amplified gene sequence, are given in Fig. 4.3.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF693770.1	Serratia rubidaea isolate H415 16S ribosomal RNA gene, partial sequence	41.0	41.0	12%	0.023	75%
JX904583.1	Serratia sp. 621GF 16S ribosomal RNA gene, partial sequence	41.0	41.0	12%	0.023	75%
AY730005.1	Serratia marcescens strain SI-20 16S ribosomal RNA gene, partial sequence	41.0	41.0	12%	0.023	75%
AY178556.1	Serratia fonticola 16S ribosomal RNA gene, partial sequence	41.0	41.0	12%	0.023	75%
EU137875.1	Serratia sp. LJ-1 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
CP000826.1	Serratia proteamaculans 568, complete genome	39.2	274	12%	0.079	75%
EU104734.1	Serratia proteamaculans strain Q42-3 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
AB244453.1	Serratia marcescens gene for 16S rRNA, partial sequence, strain: An17-1	39.2	39.2	12%	0.079	75%
AB244433.1	Serratia marcescens gene for 16S rRNA, partial sequence, strain: A19-1	39.2	39.2	12%	0.079	75%
AB244291.1	Serratia marcescens gene for 16S rRNA, partial sequence, strain: A5-1	39.2	39.2	12%	0.079	75%
EU100391.1	Serratia sp. 48P 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
EU100389.1	Serratia sp. D5 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
EF693785.1	Serratia rubidaea isolate H319 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
EF693784.1	Serratia rubidaea isolate H506 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
EF693783.1	Serratia rubidaea isolate H505 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
EF693782.1	Serratia rubidaea isolate H516 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
EF693781.1	Serratia rubidaea isolate H420 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
EF693780.1	Serratia rubidaea isolate H518 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%
EF693778.1	Serratia rubidaea isolate H514 16S ribosomal RNA gene, partial sequence	39.2	39.2	12%	0.079	75%

Fig. 4.3. The sequence similarity score of the partial 16S rRNA partial gene sequence of *Serratia* sp. BTWJ8 with other known sequences.

4.2.2. Phylogenetic tree construction

The partial gene sequence of 16S rRNA of *Serratia* sp. BTWJ8 showed only 75 % similarity with the other reported 16S rRNA gene sequences of *Serratia* sp. With the sequence data generated for the partial gene sequence of 16S rRNA of *Serratia* sp. BTWJ8, a phylogram was constructed using CLUSTALW N-J programme (Fig. 4.4).

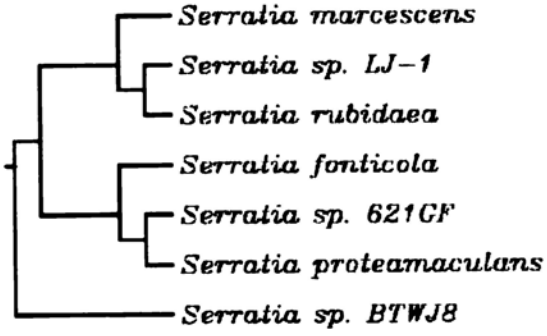


Fig. 4.4. Phylogram of *Serratia* sp. BTWJ8

4.3. STANDARDIZATION OF PROTOCOL FOR ISOLATION OF PIGMENT

Suitable protocol for maximal extraction and isolation of pigment from fermentation broth was initially standardized using different solvent system as explained under the sections 3.4.1 and 3.4.2. It was noted that this pigment could be assayed at 535 nm where the pigment shows maximum absorption, λ_{max} (Fig. 4.5). It is inferred from the results presented in Fig. 4.6 that methanol is an ideal solvent for extracting the maximum of the water insoluble membrane bound pigment.

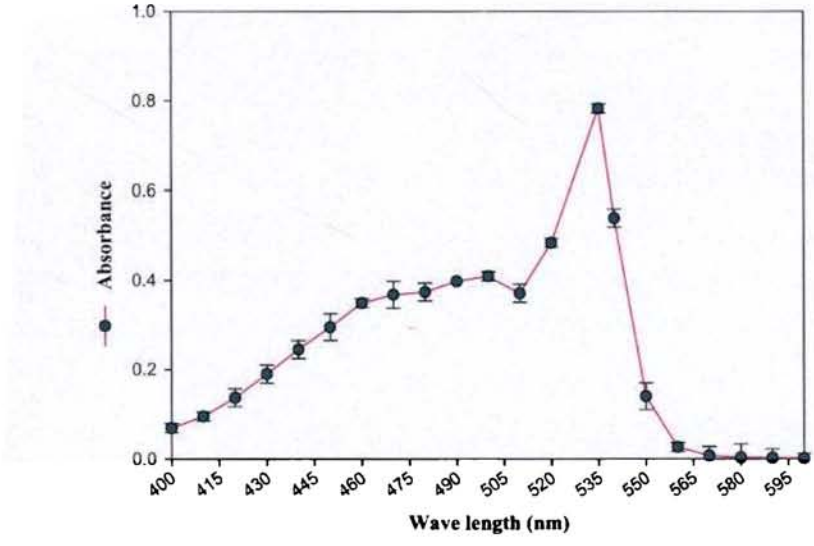


Fig. 4.5. Detection of λ_{max} of the pigment produced by *Serratia* sp. BTWJ8: Fermentation was conducted using 1 % inoculum, incubated at room temperature (28 ± 2°C) and activity was measured after 24 h of incubation. Scanning range selected was 400-600. Extraction was done by suspending the coloured cell pellet in methanol and incubated at 60°C for 20 min.

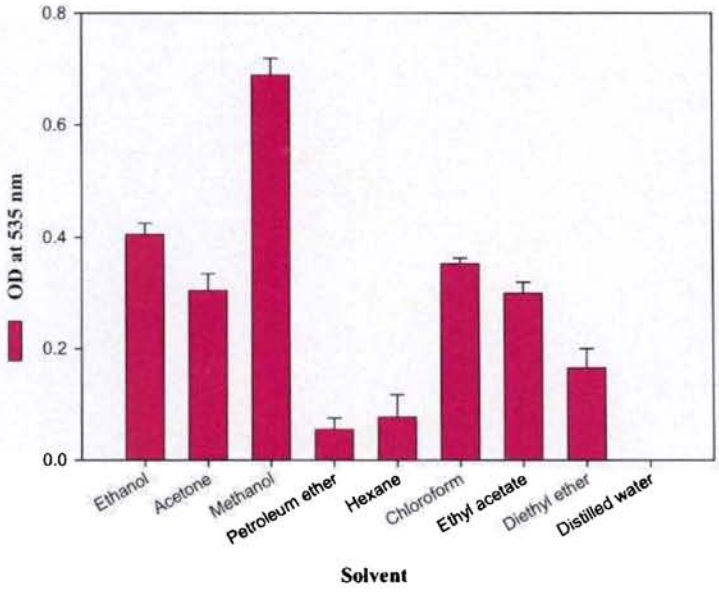


Fig. 4.6. Selection of suitable solvent for extracting the pigment from *Serratia* sp. BTWJ8: Fermentation was conducted using 1 % inoculum, incubated at room temperature (28 ± 2°C) and activity was measured after 24 h of incubation. Extraction was done by suspending the coloured cell pellet in solvents and incuabted at 60°C for 20 min.

4.4. PURIFICATION OF THE PIGMENT

The pigment produced by the bacterium, was extracted from the medium and purified employing solvent extraction-phase separation followed by column chromatography. The results obtained are presented below.

4.4.1. Phase separation

Pigment in methanol was purified by extracting with petroleum ether. After phase separation the hypophase with methanol and water soluble impurities were removed. The residual methanol from the petroleum ether epiphase was

removed by repeated washing with distilled water. The petroleum ether epiphase with pigment (Fig. 4.7) was collected and concentrated by evaporation of the solvent at 40°C water bath for further purification by column chromatographic method.

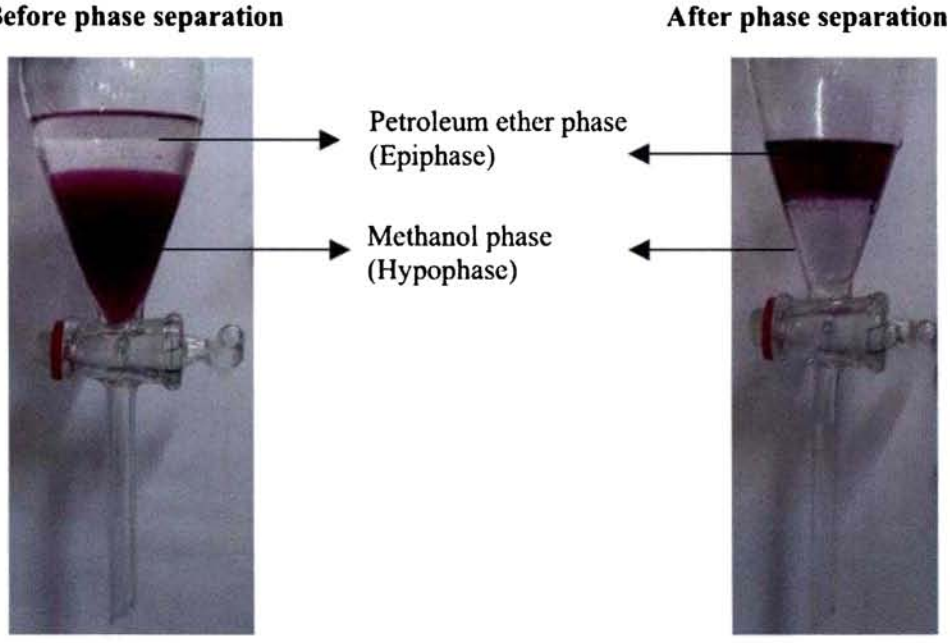


Fig. 4.7. Phase separation of pigments isolated from *Serratia* sp. BTWJ8: Hypophase contains methanol and water insoluble impurities and the epiphase contains pigment.

4.4.2. Column chromatography

The concentrated pigment in the petroleum ether epiphase collected after phase separation was purified further by column chromatography using silica gel column and solvent system comprising n-hexane and ethyl acetate (2:1; v/v). The red coloured fragment eluted was collected and concentrated by evaporation at

40°C water bath. The absorption pattern of the purified pigment dissolved in methanol is shown in Fig. 4.8. A reduction of absorbance in the range of 430 to 490 nm wavelength indicates the removal of impurities. The purified pigment was then used for the characterization studies.

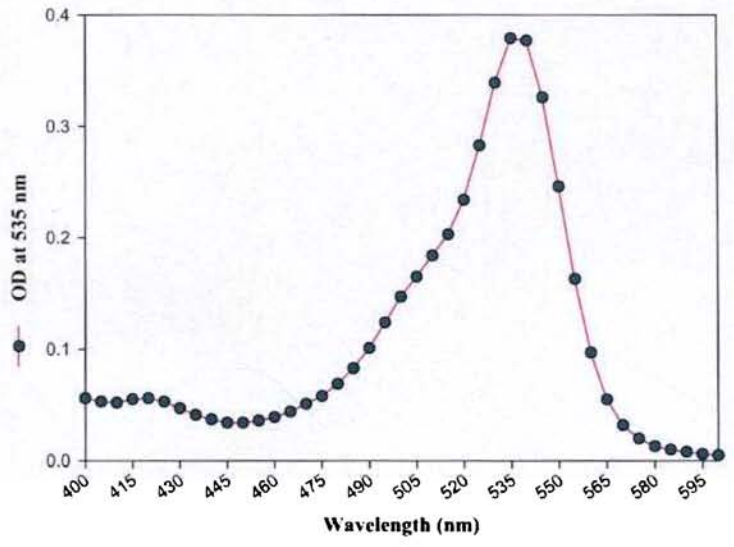


Fig. 4.8. Absorption pattern of purified pigment, isolated from *Serratia* sp. BTWJ8, dissolved in methanol after phase separation and column chromatography

4.5. CHARACTERIZATION AND IDENTIFICATION OF THE PIGMENT

4.5.1. Thin-Layer Chromatography (TLC)

A single band with an R_f value of 0.42 was obtained (Fig. 4.9) after thin-layer chromatography with chloroform: methanol (95:5; v/v).

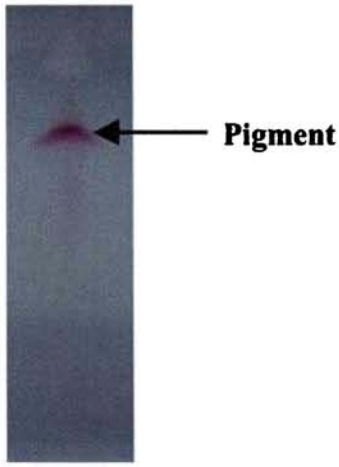


Fig. 4.9. Thin layer chromatogram of the pigment isolated from *Serratia* sp. BTWJ8

4.5.2. Liquid Chromatography-Mass Spectroscopy (LC-MS)

The molecular mass of the sample pigment was 324.2 Da, as shown in Fig. 4.10, which corresponds to that of prodigiosin ($C_{20}H_{25}N_3O$). It is therefore concluded that the pigment isolated from *Serratia* sp. BTWJ8 is prodigiosin-like pigment.

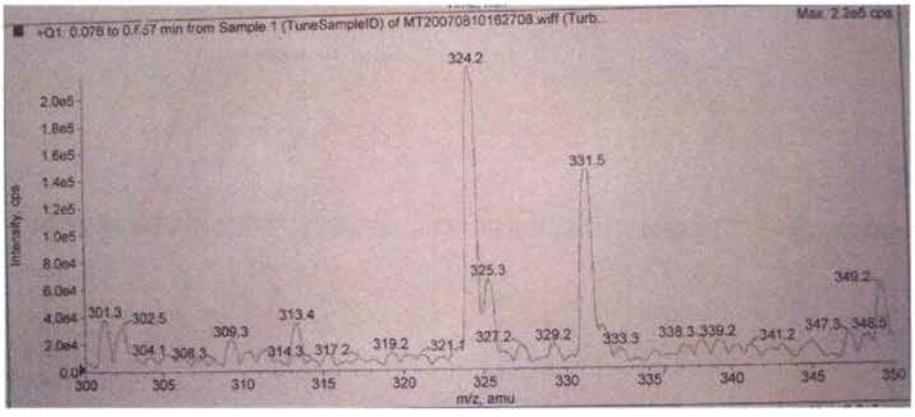


Fig. 4.10. Liquid chromatography-Mass spectrum of the red pigment isolated from *Serratia* sp. BTWJ8

4.5.3. Fourier-Transform Infrared Spectroscopy (FT-IR Spectroscopy)

FT-IR absorption in KBr for the red pigment (Fig. 4.11) was dominated by strong bands at 2924.78 $cm^{-1}$and 2853.67 cm^{-1} (aromatic CH), 1736.21 cm^{-1} and 1710.44 cm^{-1} (C=O), 1611.15 cm^{-1} (aromatic C=C), 1548.24 cm^{-1} (N-H), 1459.18 cm^{-1} (C-H), 1264.91 cm^{-1} (C-N). These indicate that this pigment's pattern is similar to that of prodigiosin.

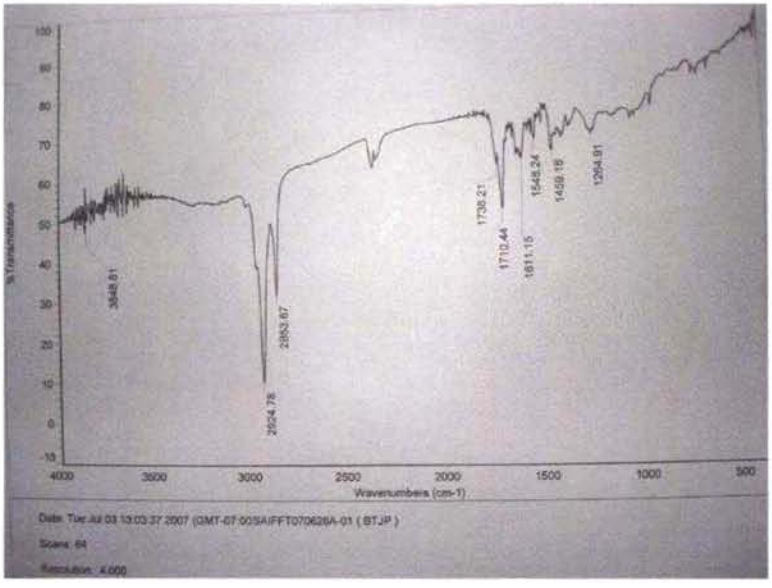


Fig. 4.11. FT-IR spectrum of the red pigment isolated from *Serratia* sp. BTWJ8

4.5.4. Fourier-Transform Raman Spectroscopy (FT Raman Spectroscopy)

The spectrum obtained after the analysis showed strong bands at 2924.79 cm^{-1} and 2834.96 cm^{-1} very similar to that of FT-IR spectrum which indicates the presence of aromatic CH bonds in the pigment molecule (Fig. 4.12). This spectrum is unique to the particular structural properties of prodigiosin.

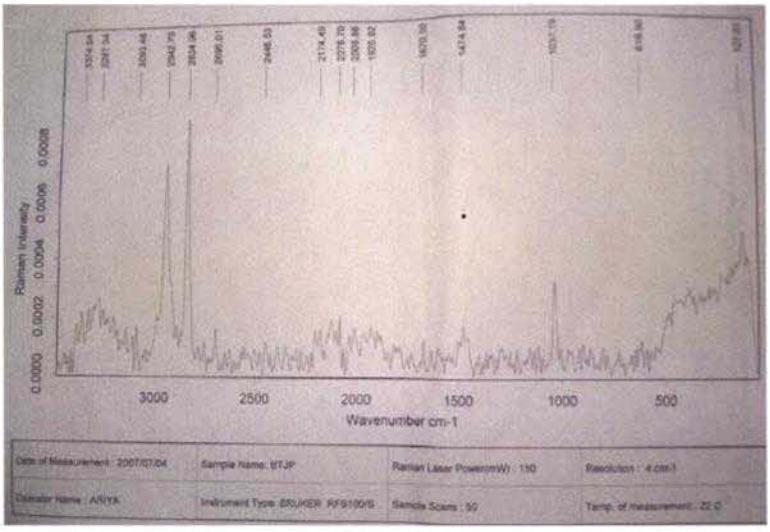


Fig. 4.12. FT-Raman spectrum of the red pigment isolated from *Serratia* sp. BTWJ8

4.5.5. ^{1}H-Nuclear Magnetic Resonance Spectroscopy (NMR Spectroscopy)

NMR spectral analysis presented a distinct spectrum, which indicates the position of each proton in the sample molecule (Fig. 4.13). In the spectrum, a chemical shift of the methoxy group in the molecule exhibited δ 4.012 ppm as a single peak. In addition, the chemical shift (in $CDCl_3$) of NH protons in pyrrole ring was δ 12.72 ppm. It is therefore concluded that the identity as prodigiosin-like pigment.

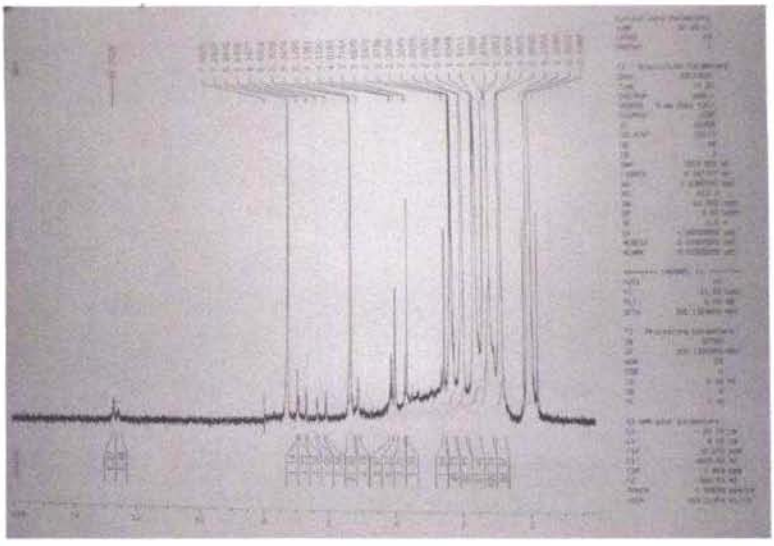


Fig. 4.13. NMR spectrum of the red pigment isolated from *Serratia* sp. BTWJ8

4.5.6. Effect of pH on the absorption spectrum of the purified pigment

Results obtained for the study on effect of pH on the absorption spectrum of the purified pigment is presented in Fig. 4.14. At pH 2.0, the pigment was red and showed a maximum absorption at 535 nm, which is identical to that of prodigiosin hydrochloride. Under neutral condition (pH 7.0), its absorption intensity was decreased and it changed to pink. However, in alkaline condition (pH 10.0) the colour was yellowish orange and its absorption spectrum shifted to 470 nm.

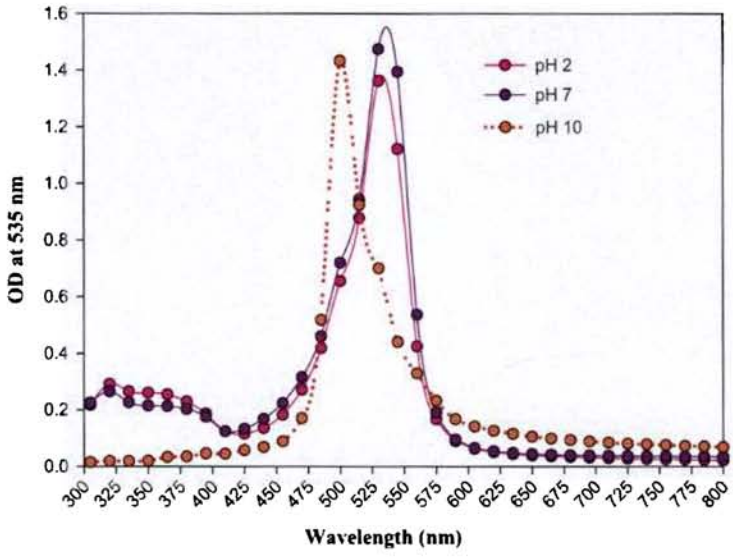


Fig. 4.14. Effect of pH on the absorption pattern of pigment isolated from *Serratia* sp. BTWJ8 in methanol: Fermentation was conducted using 1 % inoculum, incubated at room temperature (28 ± 2°C) and activity was measured after 24 h of incubation. Extraction was done by suspending the coloured cell pellet in methanol and incuabted at 60°C for 20 min.

4.6. PRODUCTION OF PIGMENT UNDER SUBMERGED FERMENTATION

4.6.1. Selection of media for pigment production

Among the five different media evaluated for pigment production, MSTS medium supported maximal pigment production (OD 0.453) when compared to Zobell Marine broth (OD 0.228), Nutrient broth (OD 0.008), Seawater yeast extract peptone broth (OD 0.348) and Glycerol asparagine broth (OD 0.213) (Fig. 4.15).

Further studies were conducted to evaluate the impact of seawater concentration and sodium chloride concentration in the medium on pigment production during fermentation. From the results presented in Fig. 4.16 it is inferred that the pigment production was more in media prepared in 50-100 % seawater. During the course of this experiment it was also observed that in spite of considerable level of pigment production in medium prepared with various

concentration of seawater there was loss of pigments during recovery from the fermentation broth. It was observed that the pigments got bound to secreted proteins and thus escaped during the solvent extraction. When secreted proteins from the culture supernatant was precipitated with ammonium sulphate the culture supernatant remained clear indicating that there was more secondary proteins in the culture supernatant when bacteria was grown in the presence of seawater (Fig. 4.17). Hence, MSTS medium prepared in distilled water and with 1 % sodium chloride (OD 0.453) was selected for the further optimization studies, since there was appreciable level of pigment production compared to the combination 25 % seawater + 75 % distilled water (OD 0.270) and there was minimal loss of pigment during harvesting of the pigment.

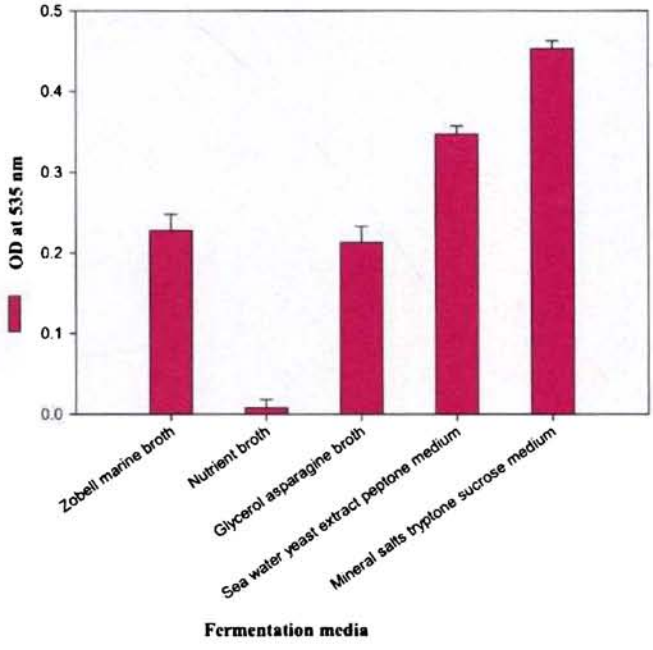


Fig. 4.15. Pigment production by *Serratia* sp. BTWJ8 in different media: Fermentation was conducted using 1 % inoculum, incubated at room temperature and activity was measured after 24 h of incubation. Extraction was done by suspending the coloured cell pellet in methanol and incuabted at 60°C for 20 min.

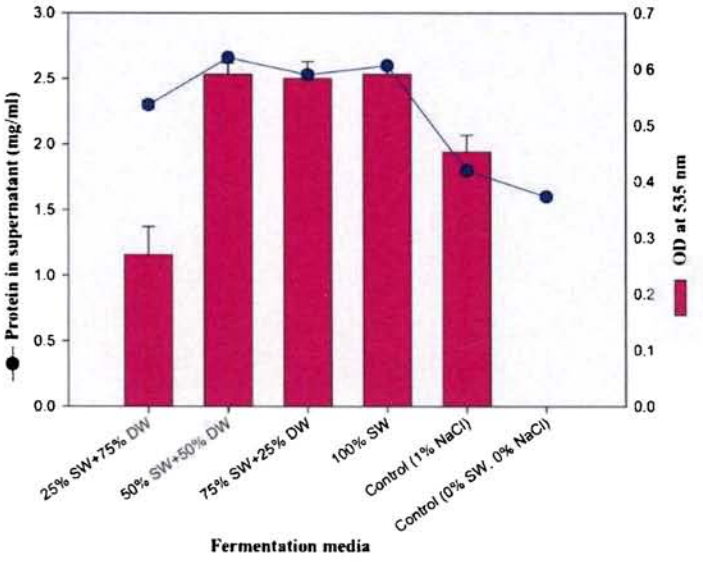


Fig. 4.16. Pigment production by *Serratia* sp. BTWJ8 in MSTS media prepared in different concentration of seawater: Fermentation was conducted using 1 % inoculum, incubated at room temperature (28 ± 2°C) and activity was measured after 24 h of incubation. Extraction was done by suspending the coloured cell pellet in methanol and incuabted at 60°C for 20 min.

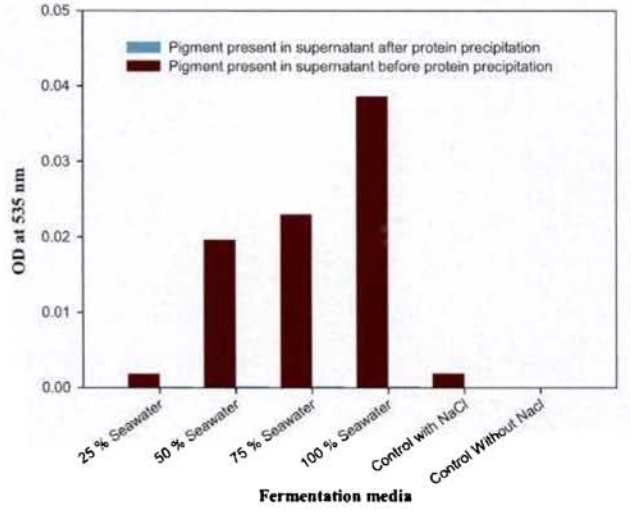


Fig. 4.17. Pigment present in the media supernatant before and after protein precipitation: Ammonium sulphate (1 %; w/v) was used for precipitating the protein from the media supernatant.

Enzyme profile of the culture supernatant after fermentation was performed after 24 h of incubation. Protease (39.9 U/ml) and alpha amylase (44.2 U/ml) activity were recorded in the culture supernatant while lipase activity was very negligible (2.3 U/ml).

4.6.2. Growth curve

The growth curve of the *Serratia* sp. BTWJ8 was estimated by incubating the MSTS medium for 36 h at room temperature. From the data presented in Fig. 4.18 it was concluded that the bacteria is fast growing. The bacterium has its exponential phase/log phase during 6 to 12 h and after 12 h of incubation the bacteria enters into the stationary phase.

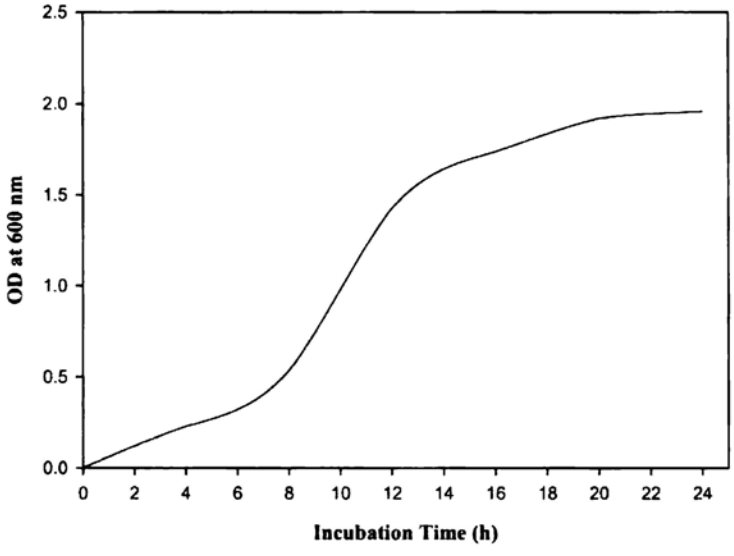


Fig. 4.18. Growth curve of *Serratia* sp. BTWJ8: Fermentation was carried out in MSTS media using 1 % inoculum, incubated at room temperature (28 ± 2°C) and the growth was measured at regular intervals of 2 h.

4.6.3. Optimization of bioprocess variables for pigment production by *Serratia* sp. BTWJ8

4.6.3.1. Optimization of incubation time

Results documented in Fig. 4.19 very clearly testify that the bacterium produced maximum level of pigment after 30 h of incubation (21.13 µg/L) although pigment production was observed from 6 h of incubation onwards (7.69 µg/L). However, the pigment production declined after 36 h of incubation. The biomass was also maximum at 30 h of incubation (2.15 g/L) although a gradual decrease in biomass was observed during the late hours of incubation. Hence, 30 h of incubation was considered as optimum for maximal production of pigment by *Serratia* sp. BTWJ8. Further the results presented in the Fig. 4.20 also suggest that the pigment production could be growth associated since there was increase in pigment level along with increase in the level of biomass during the incubation period up to 30 h.

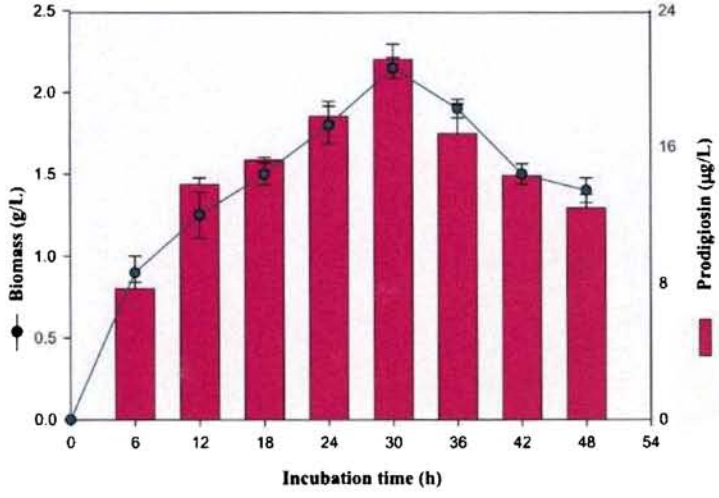


Fig. 4.19. Optimization of incubation time for pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, incubation temperature 28 ± 2°C and pH 6.0. Extraction was done in methanol at 60°C for 20 min.

4.6.3.2. Optimization of incubation temperature

Results presented in Fig. 4.20 clearly evidence that pigment production by *Serratia* sp. BTWJ8 was maximum at 25°C (22.44 µg/L) followed by 20°C (20.56 µg/L). Whereas, pigment production decreased proportionately along with increase in incubation temperature from 30°C (9.87 µg/L) onwards. In general, it was observed that *Serratia* sp. BTWJ8 could produce the pigment at all the incubation temperatures tested and the values recorded for 15°C was almost similar to that of 30°C. Incubation temperatures above 30°C led to a rapid decline in pigment production. Biomass production was maximum at 25°C (2.5 g/L) and a gradual decrease was recorded after 30 h of incubation.

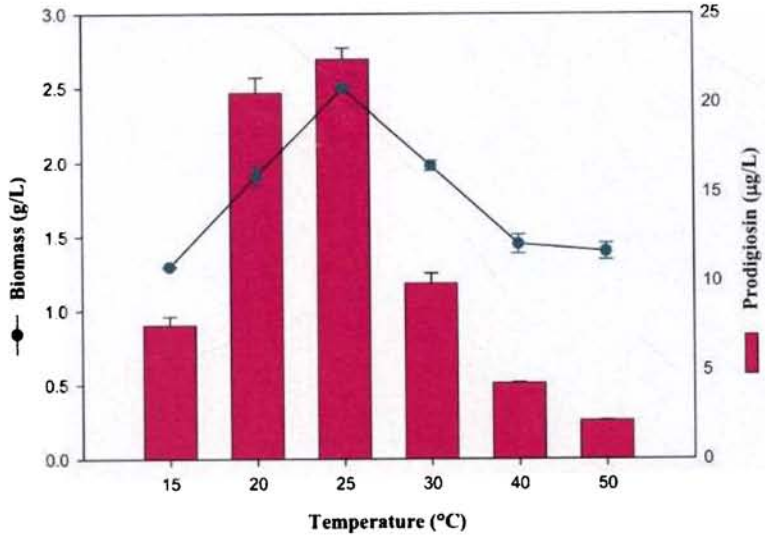


Fig. 4.20. Optimization of incubation temperature for pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, 30 h of incubation and pH 6.0. Extraction was done in methanol at 60°C for 20 min.

4.6.3.3. Optimization of initial pH of the fermentation media

Studies conducted for optimization of initial pH indicated that the bacterium could produce pigment over a range of pH from 5.0 to 9.0 although maximal pigment production was recorded at pH 6.0 (21.41 µg/L). It was also noted that pigment production declined along with increase in pH (Fig. 4.21). Media with high acidic (pH 2.0 - 4.0) and high alkaline (pH 10.0 - 13.0) did not support pigment production. Biomass was maximum at pH 6.0 (2.5 g/L). Both pigment production and biomass showed a direct relationship, both in their increase as well as decrease, in response to variations in pH.

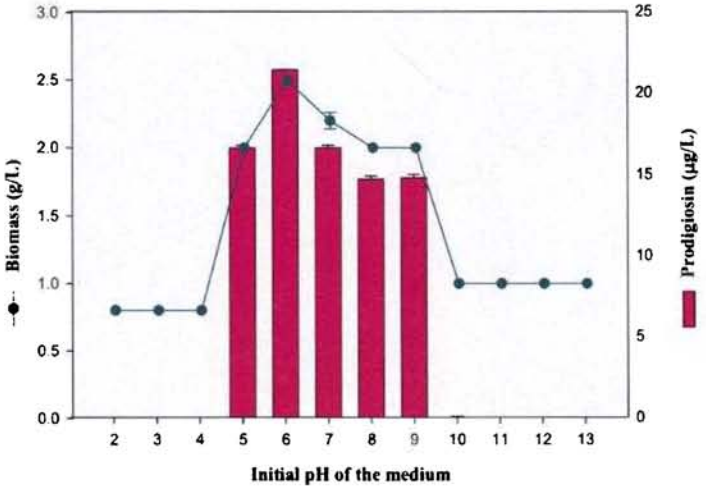


Fig. 4.21. Optimization of initial pH for pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, 30 h of incubation and incubation temperature 28 ± 2°C. Extraction was done in methanol at 60°C for 20 min.

4.6.3.4. Effect of agitation

Agitation of the culture medium is required for pigment production by *Serratia* sp. BTWJ8 since there was no pigment production at static condition (Fig. 4.22). From the results it is also inferred that maximum pigment production was recorded at 150 rpm (22.296 µg/L) and the rate of agitation above and below 150 rpm led to a decrease in pigment production. Biomass also increased gradually during incubation from static condition to 150 rpm (2.9 g/L), which later decreased at 200 rpm. Hence, it is concluded that agitation at the rate of 150 rpm is required for maximal pigment production by this bacterium.

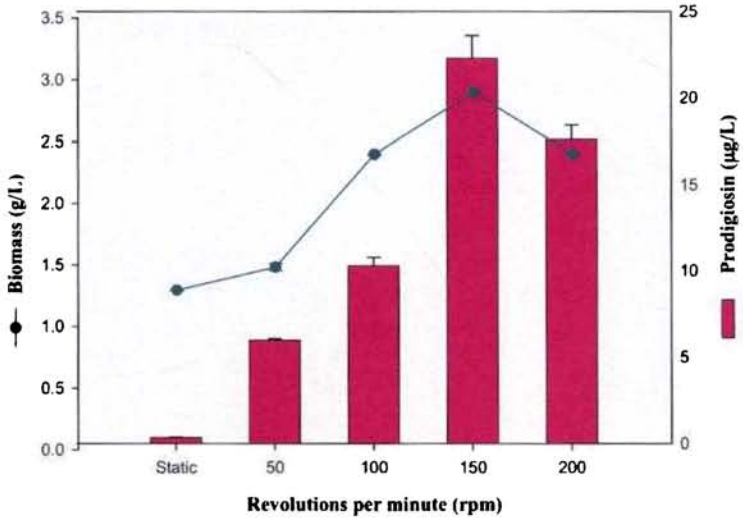


Fig. 4.22. Effect of agitation on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 30 h of incubation, incubation temperature 28 ± 2°C and pH 6.0. Extraction was done in methanol at 60°C for 20 min.

4.6.3.5. Effect of carbon sources

Data obtained for the studies conducted on the effect of carbon sources on pigment production clearly testify that among the various carbon sources tested only dextrose enhanced pigment production (23.75 µg/L). All the other sugars led to a reduction in the level of pigment production when compared with that of the control that contained sucrose as carbon source (Fig. 4.23).

Whereas, with respect to biomass dextrose supported enhanced level of biomass (2.93 g/L) when compared to sucrose as control, and maltose led to a decrease in biomass compared to control. All other carbon sources recorded almost a similar level of biomass as that of control although there was negligible pigment production. Hence, dextrose was selected as the ideal carbon source for pigment production by *Serratia* sp. BTWJ8.

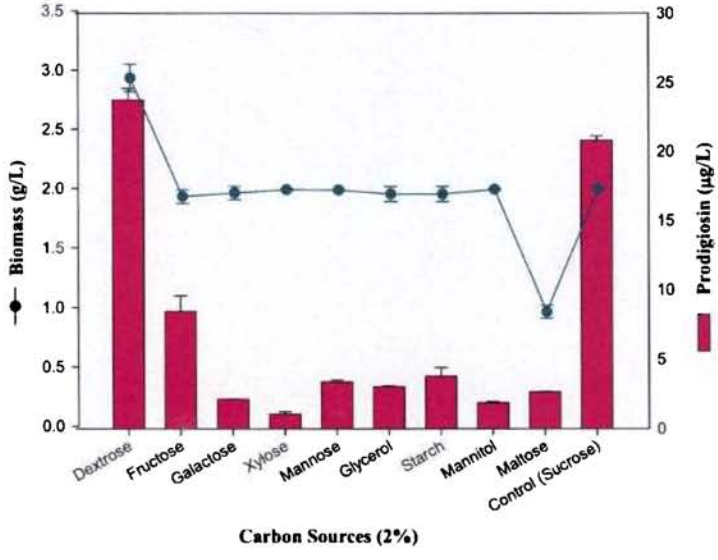


Fig. 4.23. Effect of carbon sources on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.3.5.1. Effect of different concentrations of dextrose on pigment production

Since dextrose was recorded as the suitable carbon source for maximal pigment production further studies were conducted to optimize the ideal concentration of dextrose. Results presented in Fig. 4.24 indicate that 30 mM of dextrose supported maximum pigment production (27.51 µg/L) followed by 20, 40 and 10 mM, which gave 27.12, 26.99 and 25.26 µg/L pigment respectively. In general maximal level of pigment production in the range of 25.26 µg/L to 26.99 µg/L for dextrose at concentration 10 mM to 40 mM was recorded. Biomass gradually increased at 10-30 mM concentration of dextrose (10 mM: 1.3 g/L; 20 mM: 1.95 g/L; 30 mM: 2.45 g/L) and remained almost stable at 40-300 mM concentration of dextrose.

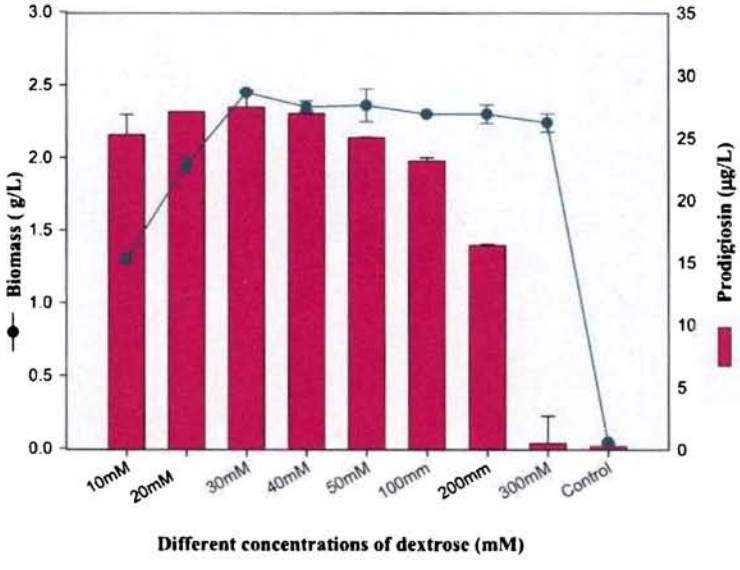


Fig. 4.24. Effect of different concentration of dextrose on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.3.6 Effect of inorganic nitrogen sources

Data obtained for the studies conducted on the effect of inorganic nitrogen sources on pigment production (Fig. 4.25) evidence that the inorganic nitrogen source could enhance pigment production by the bacterium compared to the control medium, which was devoid of nitrogen source. However, in the presence of both ammonium sulphate and sodium nitrate there was no pigment production while other sources supported negligible amount of pigment. It was also observed that inorganic nitrogen source provided as potassium nitrate (0.48 µg/L), ammonium nitrate (0.35 µg/L) and ammonium chloride (0.2 µg/L) could support very low level of pigment. Nevertheless, all the inorganic nitrogen sources led to a reduced biomass. Maximum biomass was observed in media with potassium nitrate (0.99 µg/L).

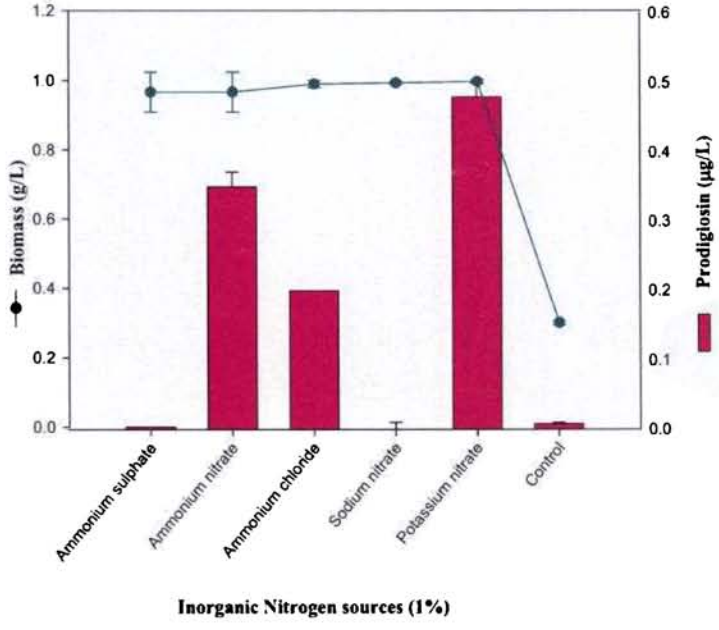


Fig. 4.25.Effect of inorganic nitrogen sources on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.3.7. Effect of organic nitrogen sources

Data documented in Fig. 4.26 indicated that among the organic nitrogen sources tested, yeast extract supported maximum pigment (28.75 µg/L) and biomass production (3.93 g/L). From the results it is also clear that beef extract and malt extract also supported pigment production (23.03 µg/L and 22.63 µg/L respectively) next to yeast extract. Peptone led to a decreased level of pigment (9.15 µg/L). Addition of urea did not support pigment as well as biomass production.

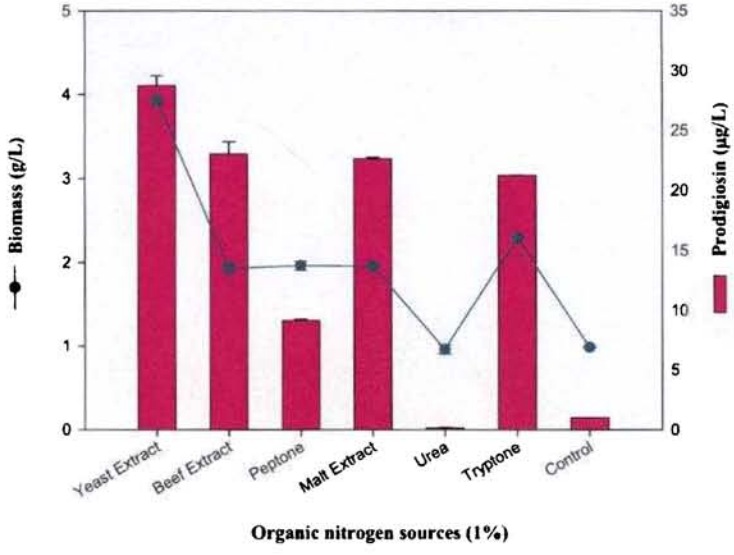


Fig. 4.26. Effect of organic nitrogen sources on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.3.7.1. Effect of different concentrations of yeast extract

Since yeast extract was observed to support enhanced level of pigment production, further studies were conducted in order to optimize the concentration of yeast extract required for maximal production of pigment. Results presented in Fig. 4.27 indicate that 1.0 % of yeast extract supported maximum pigment production (28.42 µg/L). Nevertheless considerable level of pigment production was recorded at all the levels of yeast extract evaluated in the range of 0.2 to 3.0 %. While there was a gradual increase in the pigment yield along with increase in concentration of yeast extract from 0.2 to 1.0 %, there was marginal decline in pigment level for the yeast extract concentration that varied from 1.5 to 3.0 %. Biomass production of the bacterium in medium supplemented with 0.4 - 3 % was found to be almost same.

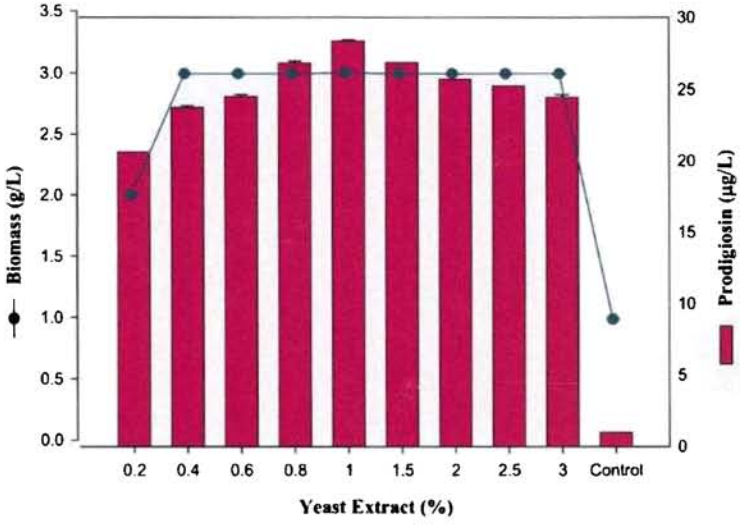


Fig. 4.27.Effect of different concentrations of yeast extract on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.3.8. Inoculum concentration

Studies conducted for the optimization of inoculum concentration indicated that 1.0 % inoculum supported maximum pigment and biomass production (22.40 µg/L and 3.2 g/L respectively). Of course very similar level of pigment production could be recorded at 0.5 % inoculum concentration. However, the pigment production marginally declined in a gradual fashion for the higher concentration of inocula in the range 2.0 to 5.0 %. In general, considerable level of pigment could be recorded at all the concentration of inocula tested. Increased concentration of inoculum led to decreased biomass and a slight decrease was also observed in the case of pigment production (Fig. 4.28).

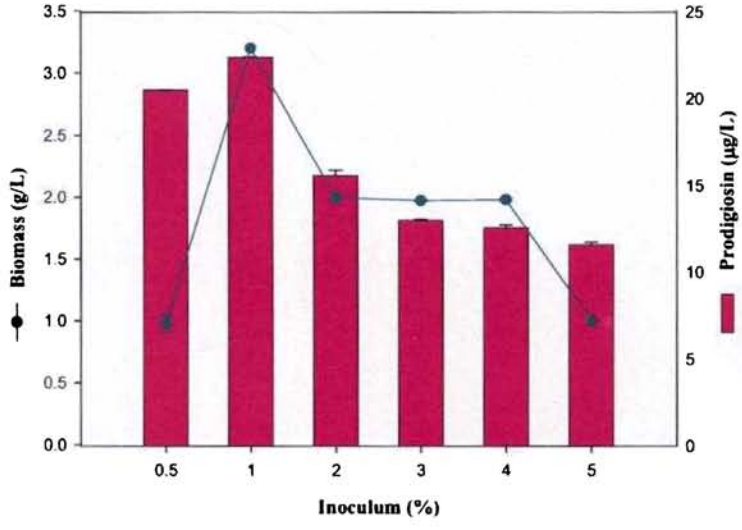


Fig. 4.28.Effect of different concentrations of inoculum on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.3.9. Effect of calcium chloride

Results presented in Fig. 4.29 clearly evidence that 100 mM of calcium chloride supported maximum pigment (22.65 µg/L) and biomass (1.98 g/L) production when compared to control. In general considerable level of pigment production was observed at all the levels of $CaCl_2$ concentration tested. The increase in pigment production, although only marginal, was along with increase in $CaCl_2$ concentration from 2-60 mM. Whereas, the increase in pigment production was rapid at 50-100 mM. There was a steep decline in pigment when $CaCl_2$ concentration was further increased to 200 mM. Increase in biomass production was gradual and marginal as the concentration of calcium chloride varied from 2-100 mM.

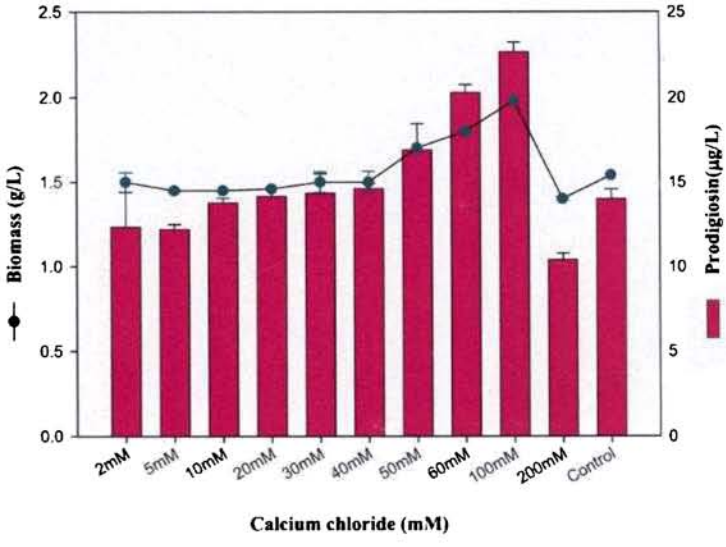


Fig. 4.29.Effect of different concentrations of calcium chloride on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.3.10. Effect of trace salts

An attempt was made to study the impact of selective trace salts like $FeCl_3$, $MnCl_2$, Na_2MoO_4 and $ZnSO_4$ on pigment production by preparing the MSTS media in different combinations devoid of the desired salt. Results presented in Fig. 4.30 suggest that the selected trace salts $FeCl_3$, $MnCl_2$, Na_2MoO_4 and $ZnSO_4$ influence pigment production in the MSTS medium significantly. Thus, when all but one is added or not all added there was reduction in the level of pigment production when compared to control that contained all the four salts. Interestingly, even in the absence of these trace salts the bacterium could produce pigment and when they are added to the medium they supported enhanced level of pigment production (21.58 µg/L). Of the four salts $ZnSO_4$ had relatively a higher influence compared to other salts since in its absence there was more decrease in the pigment level. Whereas, biomass production by the bacterium was observed more in media without $ZnSO_4$ (2.5 g/L).

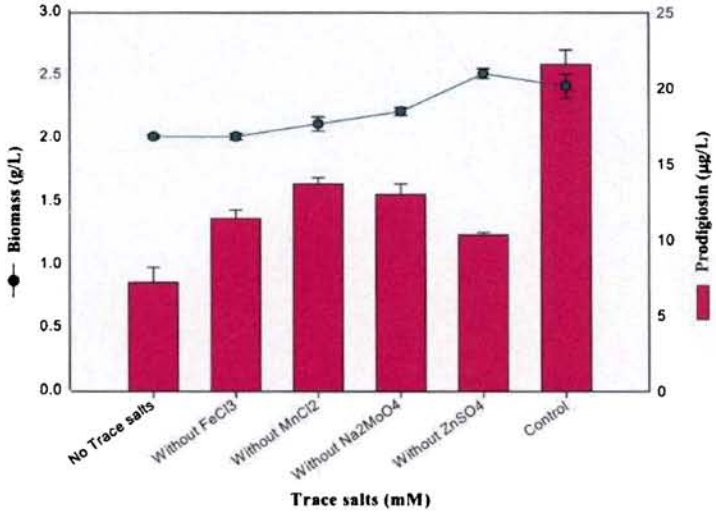


Fig. 4.30. Effect of trace salts on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.3.11. Effect of sodium chloride

Results presented in Fig. 4.31 clearly evidence that pigment production was influenced significantly not only by its absence but also by the varying concentration of sodium chloride. Pigment and biomass production gradually increased along with increase in concentration of NaCl from 10 mM and maximal pigment (21.80 µg/L) and biomass (3.99 g/L) was recorded with 200 mM sodium chloride. Similarly a gradual decrease in pigment and biomass was observed along with further increase in sodium chloride. Another interesting observation was that the bacterium did not produce pigment in media without sodium chloride, indicating the mandatory requirement of sodium chloride for pigment production.

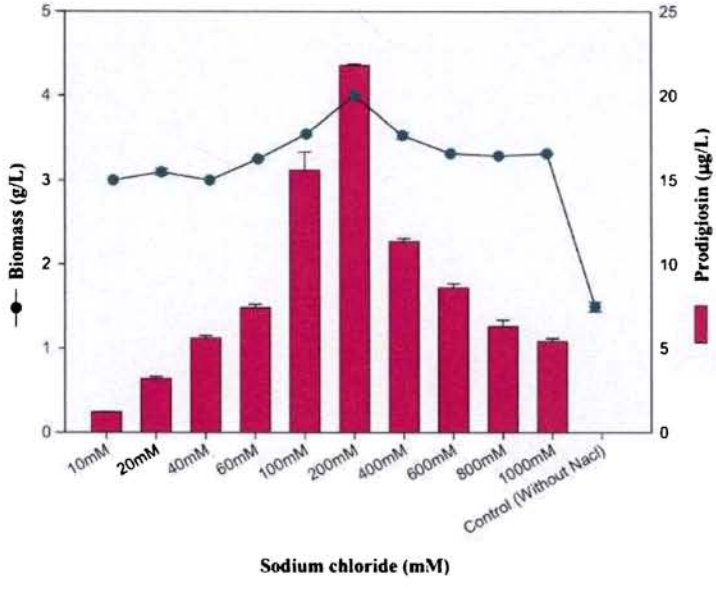


Fig. 4.31.Effect of sodium chloride on pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1 % inoculum, 150 rpm, pH 6.0, incubation temperature 28 ± 2°C and 30 h of incubation. Extraction was done in methanol at 60°C for 20 min.

4.6.4. Optimization of bioprocess variables employing statistical approach for pigment production by *Serratia* sp. BTWJ8

Statistical approach was used for optimizing the medium that could support maximum pigment production. Initially process variables were optimized using Plackett-Burman design and in the second stage Response Surface Methodology was adopted towards selection of optimal variables and understanding the interrelationship among significant variables.

4.6.4.1. Plackett-Burman Design (PB Design)

Data obtained for the studies conducted on optimization of medium for pigment production by *Serratia* sp. BTWJ8 using Plackett-Burman design was analysed by Design expert software and a first-order model was fitted to the data obtained from the experiment.

The experimental results of pigment production by Plackett-Burman design are shown in Table 4.3. With the help of the software the results were analysed and it is inferred that out of the eleven variables screened during PB design, four factors *viz*; inoculum (X_1), NaCl (X_2), $CaCl_2$ (X_3), and temperature (X_4) were found to be the most significant variables.

First-order model equation

$$\text{Pigment } (\mu g/L) = 9.43 + 6.52\ X_1 + 4.97\ X_2 - 5.84\ X_3 + 5.15\ X_4$$

The statistical significance of the model equation was evaluated by the *F*-test analysis of variance (ANOVA), which revealed that this regression is statistically significant (Table 4.4). The Model F-value of 9.15 implied that the model is significant. Values of "Prob > F" less than 0.05 indicated that the model terms are significant. "Adequate precision" measures the signal to noise ratio.

A ratio greater than 4 is desirable. Adequate precision ratio of 8.379 indicated an adequate signal. Thus, this model could be used to navigate the design space.

The effect of individual parameters studied in PB design is presented as Pareto chart in Fig. 4.32. The data evidence that inoculum, sodium chloride and temperature had a positive effect in enhancing pigment production along with their increase in concentration. Whereas, calcium chloride had a negative effect on pigment production along with increase in concentration.

Table 4.3. The matrix of the Plackett-Burman design experiment, together with the observed experimental data for pigment production by *Serratia* sp. BTWJ8

Run	Incubation period (h) X_1	Inoculum (%) X_2	Yeast extract (%) X_3	Dextrose (mM) X_4	NaCl (mM) X_5	$CaCl_2$ (mM) X_6	K_2HPO_4 (mM) X_7	KH_2PO_4 (mM) X_8	Trace salts (mM) X_9	pH X_{10}	Temperature (°C) X_{11}	**Pigment (µg/L) (Y)**
1	12	0.50	0.50	10	100	50	0.00	0.0	0.00	5	20	0.014
2	36	0.50	1.00	100	100	100	0.00	0.0	0.00	7	30	0.89
3	12	0.50	1.00	100	200	50	0.45	0.5	0.00	7	20	0.084
4	36	2.00	0.50	100	200	50	0.45	0.0	0.00	5	30	36.48
5	36	2.00	1.00	10	200	100	0.00	0.5	0.00	5	20	14.99
6	12	2.00	0.50	10	100	100	0.45	0.5	0.00	7	30	2.927
7	12	2.00	1.00	10	200	50	0.00	0.0	0.03	7	30	32.48
8	12	2.00	1.00	100	100	100	0.45	0.0	0.03	5	20	0.367
9	36	0.50	1.00	10	100	50	0.45	0.5	0.03	5	30	14.13
10	12	0.50	0.50	100	200	100	0.00	0.5	0.03	5	30	0.565
11	36	2.00	0.50	100	100	50	0.00	0.5	0.03	7	20	8.458
12	36	0.50	0.50	10	200	100	0.45	0.0	0.03	7	20	1.78

Table 4.4. ANOVA for the experiments with Plackett-Burman design for pigment production by *Serratia* sp. BTWJ8

Term	Pigment yield
F-value	9.15
Prob > F	0.0065
Mean	9.43
R-squared	0.8394
Adjusted R-squared	0.7476
Coefficient of variance	68.66
Adequate precision	8.379

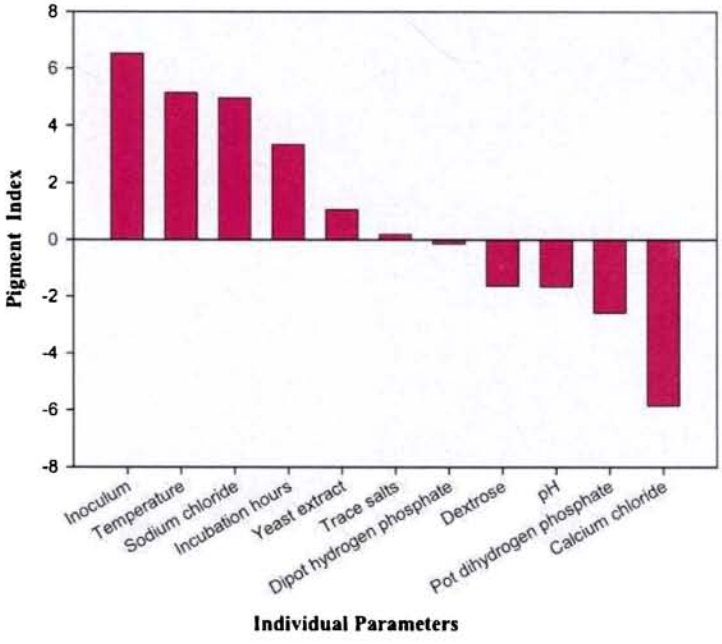


Fig. 4.32.Pareto Chart showing the effect of individual factors on pigment production by *Serratia* sp. BTWJ8

4.6.4.2. Response surface methodology

Response surface methodology (using Box-Behnken design experiment) was adopted towards selection of optimal level of the significant variable *viz*; inoculum (X_1), NaCl (X_2), $CaCl_2$ (X_3), and temperature (X_4) which were identified based on the PB design experiment. The design matrix (Box-Behnken) and the corresponding experimental data obtained are shown in Table 4.5.

The results obtained for the Box-Behnken design experiment were analyzed by ANOVA, which yielded the following regression equation for the level of pigment production (Y):

$$\text{Pigment, Y } (\mu g/L) = 33.14 + 7.38 X_1 + 7.26 X_2 + 2.52 X_3 - 2.91 X_4 - 3.61 X_1^2 - 4.36 X_2^2 + 0.22 X_3^2 - 9.39 X_4^2 - 1.36 X_1X_2 + 0.058 X_1X_3 + 0.85 X_1X_4 - 9.06 X_2X_3 + 6.99 X_2X_4 - 15.00 X_3X_4$$

Table 4.5. Effect of individual variable on pigment production by *Serratia* sp. BTWJ8 studied using Box-Behnken design experiment

BLOCK	RUN	Inoculum (%) X_1	Sodium chloride (mM) X_2	Calcium chloride (mM) X_3	Temperature (°C) X_4	*Pigment (µg/L) Y
1	1	1.25	150	50	30	36.71
1	2	1.25	150	50	20	7.84
1	3	1.25	150	100	30	0.865
1	4	2.00	100	75	25	26.83
1	5	1.25	150	75	25	34.23
1	6	2.00	200	75	25	38.32
1	7	1.25	150	75	25	22.57
1	8	1.25	150	100	20	31.98
1	9	0.50	200	75	25	17.0
1	10	0.50	100	75	25	0.084
2	11	2.00	150	75	20	36.73
2	12	1.25	100	50	25	2.842
2	13	1.25	200	100	25	36.49
2	14	2.00	150	75	30	20.71
2	15	1.25	150	75	25	28.18
2	16	1.25	200	50	25	37.62
2	17	0.50	150	75	30	1.29
2	18	1.25	100	100	25	37.95
2	19	0.50	150	75	20	20.71
2	20	1.25	150	75	25	37.95
3	21	1.25	150	75	25	37.95
3	22	1.25	200	75	30	38.32
3	23	0.50	150	100	25	35.33
3	24	1.25	100	75	30	11.62
3	25	2.00	150	50	25	33.85
3	26	2.00	150	100	25	37.95
3	27	0.50	150	50	25	31.46
3	28	1.25	100	75	20	24.24
3	29	1.25	200	75	20	22.97
3	30	1.25	150	75	25	37.95

* Pigment production (Experimental value) is considered as the Response (Y)

The ANOVA analysis of pigment production (Table 4.6) showed that Prob > F value was less than 0.05, which indicate that the model is significant. Two linear coefficient, inoculum (X_1), NaCl (X_2); one quadratic term, temperature (X_4^2) interaction coefficient and two interaction coefficient, NaCl and $CaCl_2$ (X_2X_3); $CaCl_2$ and temperature (X_3X_4) were found to be significant model terms.

Table 4.6. ANOVA for the response surface experiments conducted using Box-Behnken design for pigment production by *Serratia* sp. BTWJ8

Term	Pigment yield
F-value	3.98
Prob > F	0.008
Mean	26.29
R-squared	0.8107
Adjusted R-squared	0.6069
Coefficient of variance	30.70
Adequate precision	6.693

The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with coefficient of determination (R^2) of 0.8107. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 81.07 % of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for comparing models with different numbers of independent variables, was 0.6069. All the selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on pigment production. The coefficient of variance was 30.70. A ratio greater than 4 is desirable as it indicates an adequate signal. Thus, this model could be used to navigate the design space.

4.6.4.2.1. Analysis of factors influencing pigment production

Three-dimensional response surface curves were plotted to study the interaction among various physicochemical factors, and to determine the optimum concentration of each individual factor for maximum pigment production. The model predicted maximum pigment production up to 40 μg/L that could be achieved using 1.64 % (v/v) inoculum, 75 mM $CaCl_2$, 196 mM NaCl, incubation temperature 28°C and incubation period for 22 h.

4.6.4.2.2. Interactions between factors

The pair wise interaction among the factors in terms of pigment production in the optimized set was assessed by examining the response surfaces. Three dimensional response surfaces were generated holding three factors constant at a time and plotting the response obtained for varying levels of the other two.

4.6.4.2.2.1. Interaction between concentrations of inoculum and sodium chloride

There was a parabolic change in pigment production pattern with respect to inoculum and sodium chloride concentration and maximum pigment yields were recorded over 1.75-2.00 %. Sodium chloride concentration showed maximum response over the range of 175-200 mM (Fig. 4.33). The concentrations of calcium chloride and the incubation temperature were held at their optimum levels 75 mM and 28°C respectively.

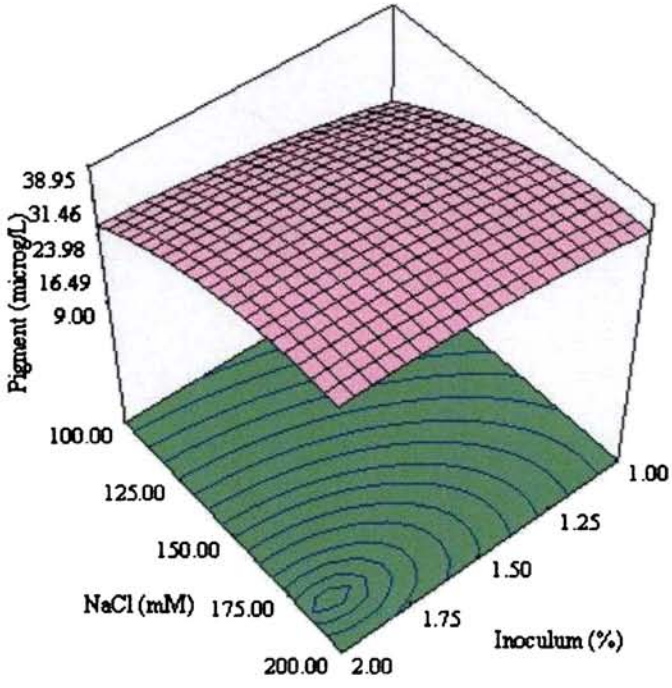


Fig. 4.33.Effect of concentrations of inoculum and sodium chloride on pigment production by *Serratia* sp. BTWJ8: Extraction was done by suspending the coloured cell pellet in methanol and incubated at 60°C for 20 min.

4.6.4.2.2.2.Interaction between concentrations of inoculum and calcium chloride

From the results presented in Fig. 4.34, it is inferred that there was no prominent interaction between concentrations of inoculum and calcium chloride in influencing pigment production. The concentrations of sodium chloride and the incubation temperature were held at their optimum levels 196 mM and 28°C respectively.

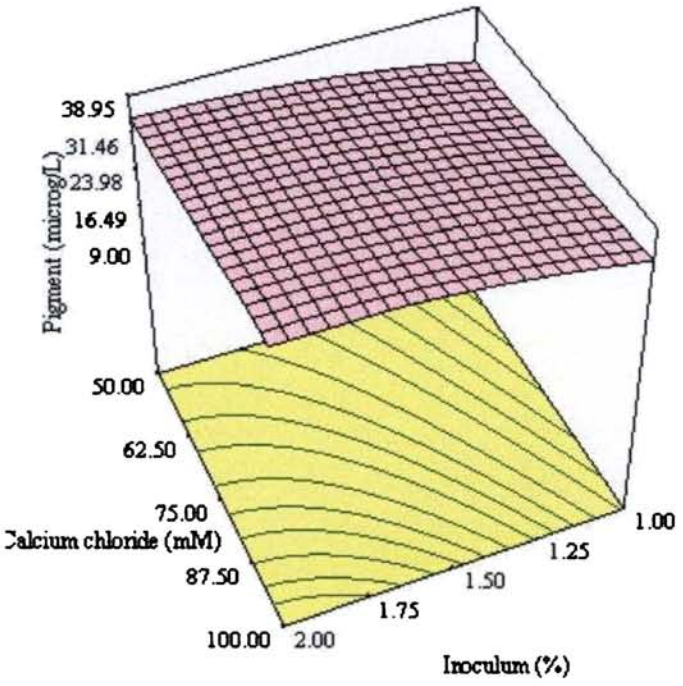


Fig. 4.34.Effect of concentrations of inoculum and calcium chloride on pigment production by *Serratia* sp. BTWJ8: Extraction was done by suspending the coloured cell pellet in methanol and incubated at 60°C for 20 min.

4.6.4.2.2.3. Interaction between concentration of inoculum and incubation temperature

The maximum pigment yield was obtained at a temperature range between 23-28°C and inoculum concentration in the range between 1.5-2.0 %. There was a positive interaction between inoculum concentration and incubation temperature as it is evidenced by the data presented in the Fig. 4.35. The pigment yield was reduced at temperature below 23°C and above 29°C. The concentrations of sodium chloride and calcium chloride were at their optimum levels, 196 mM and 75 mM respectively.

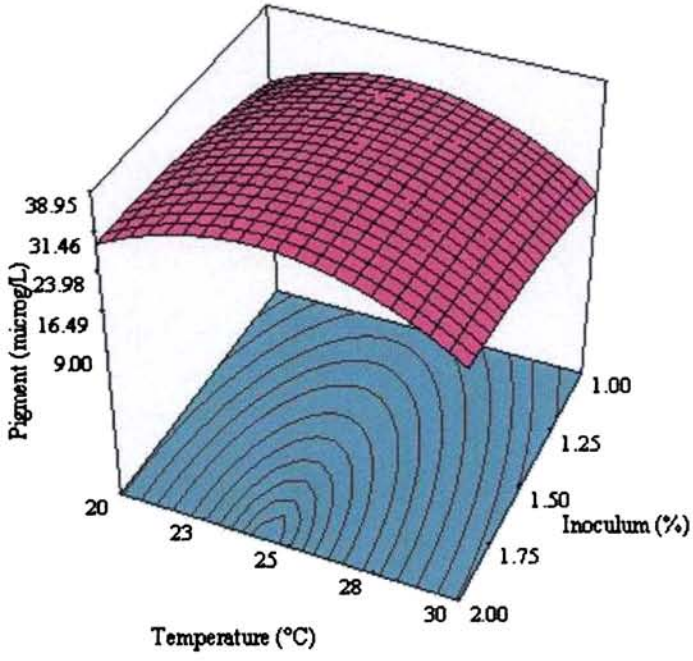


Fig. 4.35. Effect of concentration of inoculum and incubation temperature on pigment production by *Serratia* sp. BTWJ8: Extraction was done by suspending the coloured cell pellet in methanol and incubated at 60°C for 20 min.

4.6.4.2.2.4. Interaction between concentrations of calcium chloride and sodium chloride

Results presented in Fig. 4.36 indicate a very clear interaction between concentration of calcium chloride and sodium chloride in their cumulative effect on pigment production. Maximum pigment yield was obtained in the range of 175-200 mM sodium chloride concentration and 50-62 mM calcium chloride concentration. It is also evident from the data that concentrations lower than 175 mM led to reduction in the pigment yield. The concentrations of inoculum and incubation temperature were at their optimum levels, 1.64 % (v/v) and 28°C respectively.

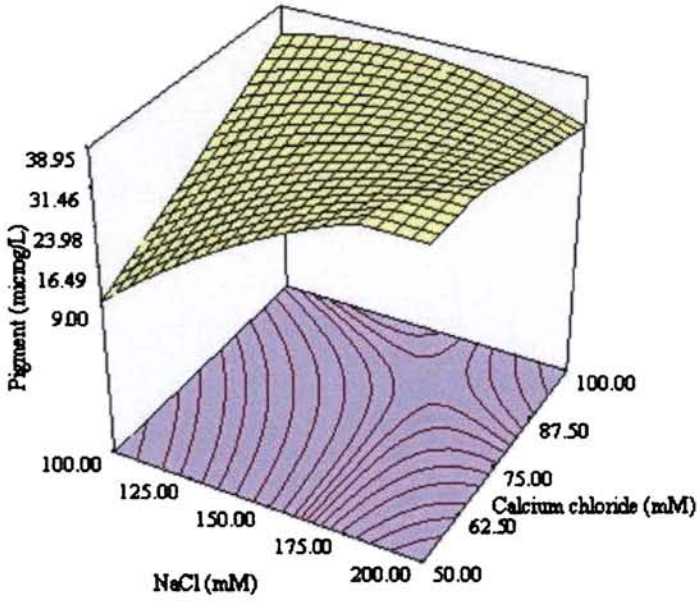


Fig. 4.36. Effect of concentration of calcium chloride and sodium chloride on pigment production by *Serratia* sp. BTWJ8: Extraction was done by suspending the coloured cell pellet in methanol and incubated at 60°C for 20 min.

4.6.4.2.2.5.Interaction between concentration of sodium chloride and incubation temperature

Pigment production showed a parabolic trend in response to variation in incubation temperature from 25-28°C and 175-200 mM sodium chloride concentration (Fig. 4.37). Pigment production decreased at sodium chloride concentration below 120 mM and incubation temperature below 23°C. Concentrations of calcium chloride and inoculum were at their optimum levels, 75 mM and 1.64 % (v/v).

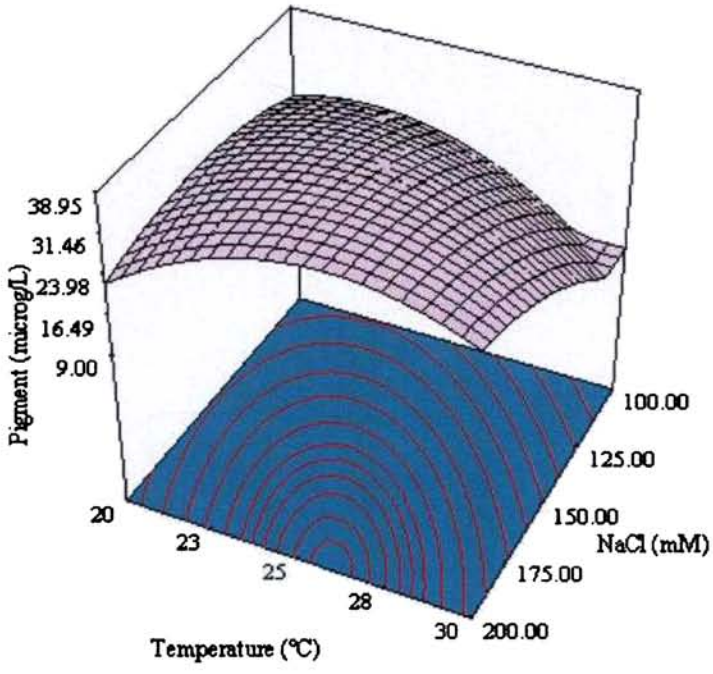


Fig. 4.37. Effect of concentration of sodium chloride and incubation temperature on pigment production by *Serratia* sp. BTWJ8: Extraction was done by suspending the coloured cell pellet in methanol and incubated at 60°C for 20 min.

4.6.4.2.2.6.Interaction between concentration of calcium chloride and incubation temperature

Pigment yields were higher at a temperature range between 20-25°C and calcium chloride concentration from 87.5-100 mM (Fig. 4.38). Incubation temperature above 25°C and calcium chloride concentration below 87.5 mM led to decreased pigment production. Concentrations of inoculum and sodium chloride were at their optimum levels, 1.64 % (v/v) and 196 mM respectively.

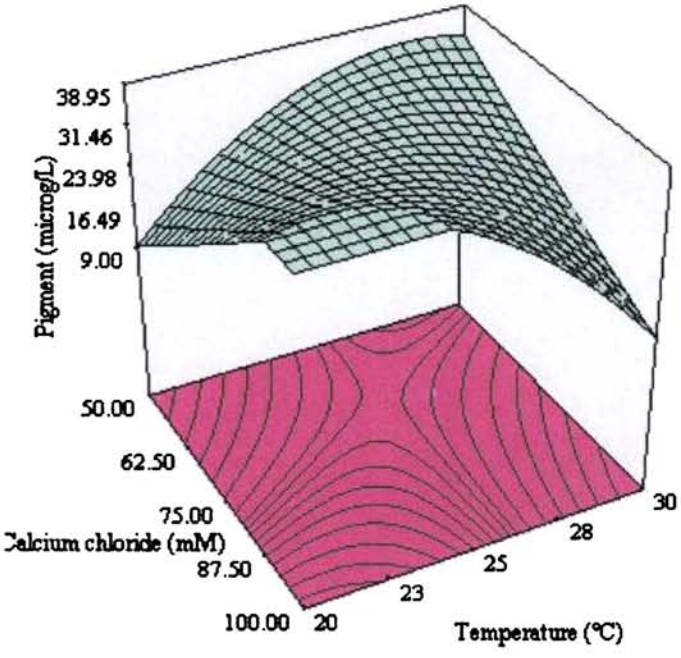


Fig. 4.38. Effect of concentration of calcium chloride and incubation temperature on pigment production by *Serratia* sp. BTWJ8: Extraction was done by suspending the coloured cell pellet in methanol and incubated at 60°C for 20 min.

4.6.4.3. Validation of the response surface model

Validation of the deduced response surface model was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated (Table 4. 7 & Fig. 4.39).

The optimized conditions for pigment production were as follows:

Yeast extract-1 % (w/v); dextrose-10 mM; K_2HPO_4-0.45 mM; KH_2PO_4-0.5 mM; calcium chloride-75 mM; sodium chloride-196 mM; inoculum-1.64 % (v/v); pH-6.0; incubation temperature-28°C and incubation period of 22 h.

Table 4.7. Predicted and experimental values of pigment production obtained for validation of response surface model

Sl. No.	Inoculum (%)	Sodium chloride (mM)	Calcium chloride (mM)	Temperature (°C)	Pigment yield (µg/L)	
					Predicted	Experimental
1	1.96	171	75	27	39.89	37.46
2	1.44	184	68	27	39.20	39.13
3	1.33	132	96	25	39.43	37.95
4	1.50	135	97	25	38.67	35.84
5	**1.64**	**196**	**75**	**28**	**40.09**	**39.14**
6	1.41	145	96	23	39.12	37.24

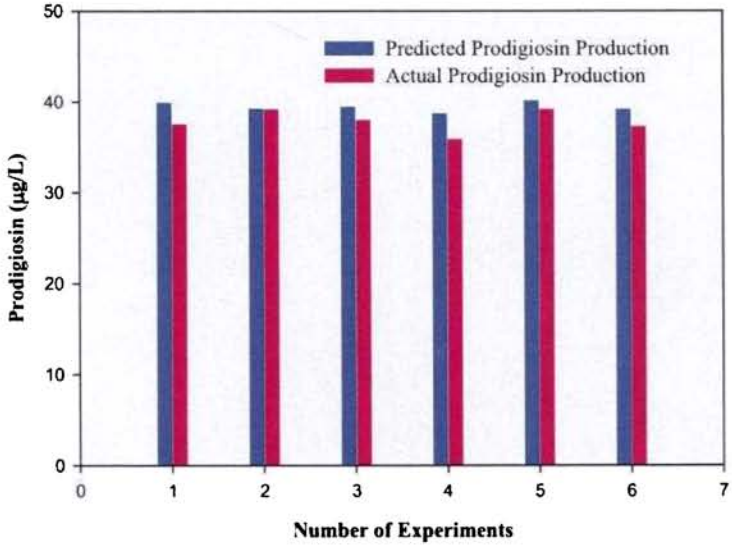


Fig. 4.39. Predicted and experimental values of pigment production by *Serratia* sp. BTWJ8 for validation of the response surface model

4.6.5. Time course study under optimal conditions

Data obtained for the time course experiment conducted over a period of 48 h under optimized condition (Fig. 4.40) testify that pigment production commenced after 8 h of incubation and reached a peak at 24 h of incubation (39.95 µg/L). Further incubation period beyond 24 h resulted in a slight decline in pigment production. Maximum biomass was attained within 24 h of incubation and was found to be decreased during the late hours of incubation. An overall two fold increase in pigment production was achieved after statistical optimization compared to the MSTS media before optimization (21.06 µg/L) (Fig. 4.41).

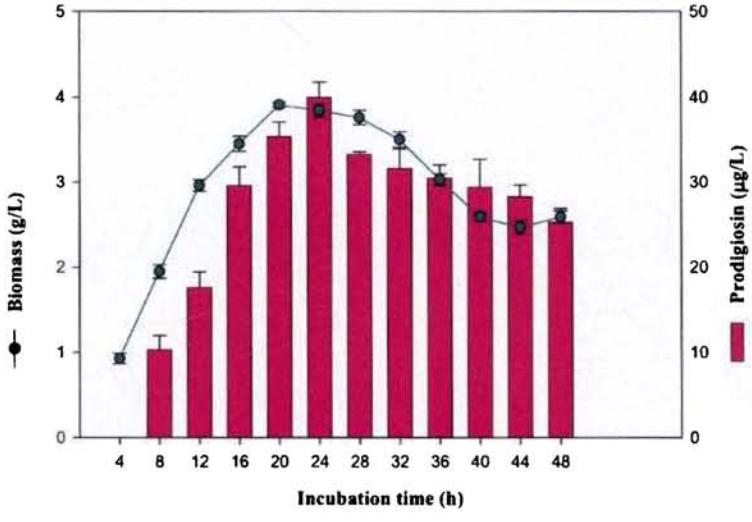


Fig. 4.40. Time course study under optimized condition for pigment production by *Serratia* sp. BTWJ8: Fermentation conditions: 1.64 % inoculum, 150 rpm, pH 6.0, incubation temperature 28°C and 48 h of incubation. Extraction was done in methanol at 60°C for 20 min.

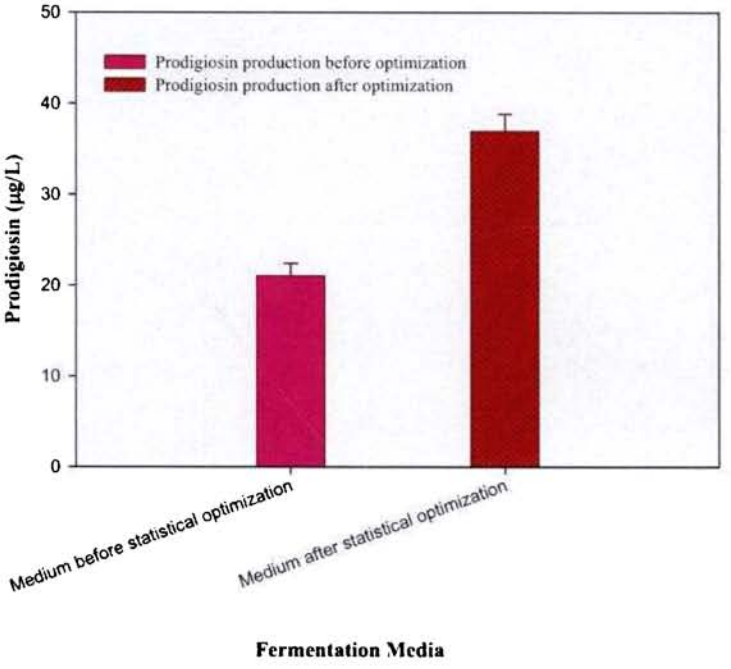
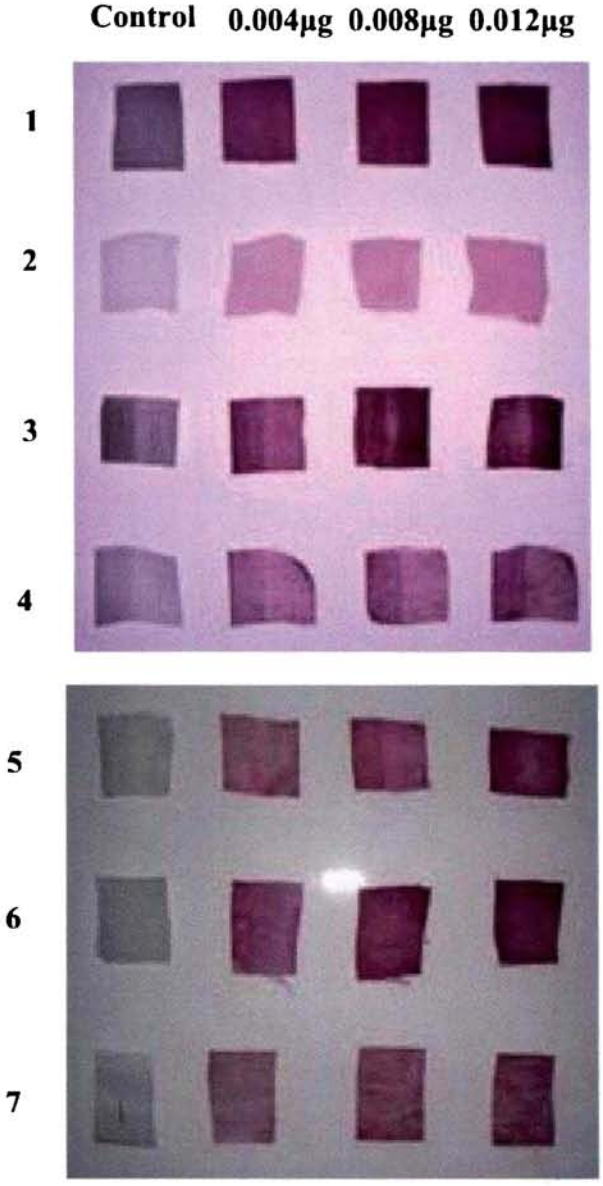


Fig. 4.41. Comparison of pigment production by *Serratia* sp. BTWJ8 before and after statistical optimization: Extraction was done by suspending the coloured cell pellet in methanol and incuabted at 60°C for 20 min.

4.7. APPLICATION STUDIES

4.7.1. Textile materials

Results presented in Fig. 4.42 clearly evidence that the pigment produced by *Serratia* sp. BTWJ8 can be effectively used to dye all the textile materials studied.



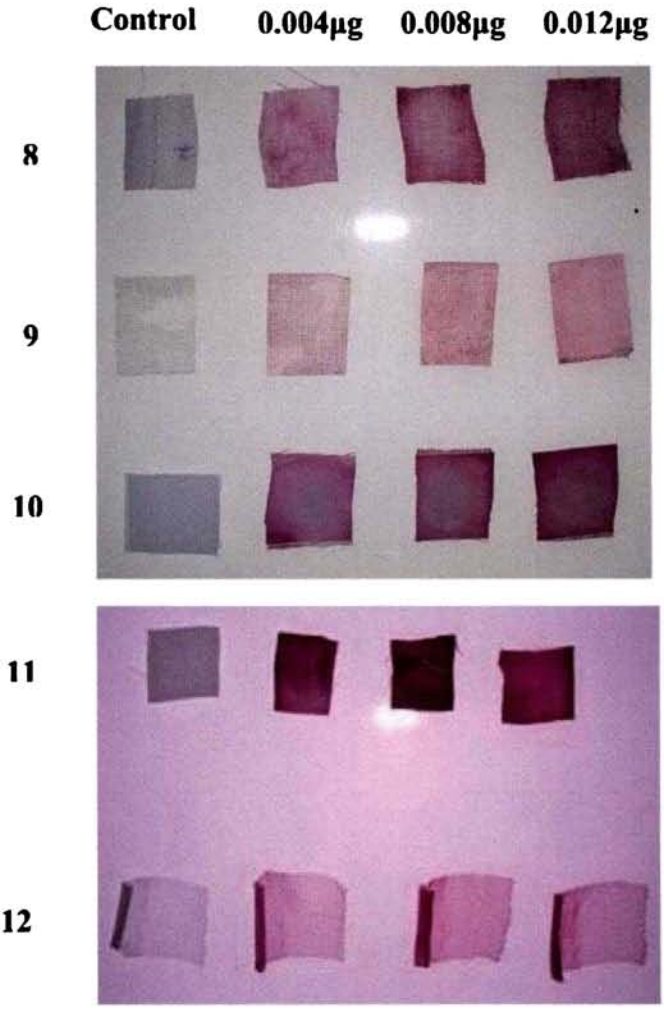


Fig. 4.42 Cloth materials dyed with prodigiosin pigment: 100 µl (0.004 µg), 200 µl (0.008 µg) and 300 µl (0.012 µg) of pigment were applied in a warm surface to the cloth material from a stock solution of 40 µg/L. The cloth material was allowed to dry at room temperature for about 1 h.

1-	Cotton	5-	Pure silk	9-	Organdi
2-	Chiffon	6-	Century cotton	10-	Polyester
3-	Poplene	7-	Dupoil silk	11-	Terrycotton
4-	2 by 2	8-	2 by 1	12-	Nylon

4.7.1.1. Wash performance of the textile materials at room temperature

During the wash performance studies with the textile materials treated with pigment, it was found that the pigment is lost from the cloth after washing in soap solution at room temperature (28 ± 2°C). Whereas, the loss of pigment from the same textile materials treated with mordant was found to be less. So it is inferred that thiourea is an effective mordant for treating the dyed textile materials (Fig. 4.43).

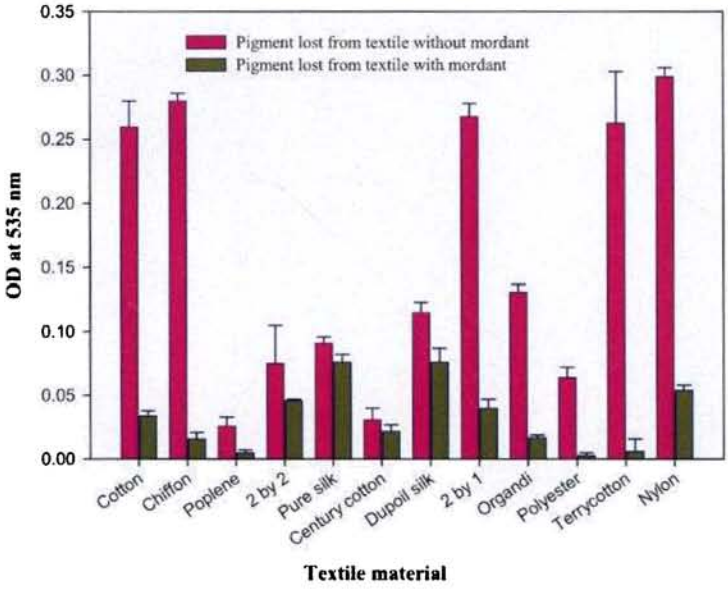


Fig. 4.43. Wash performance of textile materials in soap solution (room temperature; 28 ± 2°C): Dyed textile materials were washed with soap solution (sunlight 0.7 %; w/v) for 30 min. Absorbance of the soap solution after washing was measured at 535 nm.

4.7.1.2. Wash performance of the textile materials at 40°C

The pigment was found to be lost from the cloth after wash performance in soap solution at 40°C. Whereas, the loss of pigment from the same textile materials treated with mordant was found to be less. So this experiment also adds evidence that thiourea is an effective mordant for dyeing the textile materials with this pigment and it can withstand at hot wash conditions (Fig. 4.44).

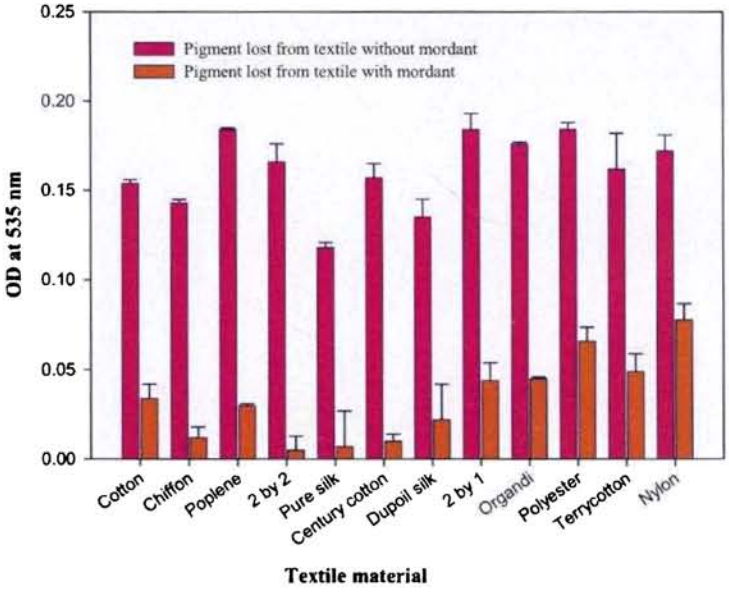


Fig. 4.44. Wash performance of textile materials in soap solution (at 40°C): Dyed textile materials were washed with soap solution (sunlight 0.7 %; w/v) for 30 min. Absorbance of the soap solution after washing was measured at 535 nm.

4.7.2. Rubber products

From the results presented in Fig. 4.45, the rubber sheets dyed with pigment indicate that prodigiosin pigment produced by *Serratia* sp. BTWJ8 is an effective natural dye for rubber products. The maximum colour obtained in rubber sheet prepared with Mix 4 that contained 0.5 phr pigment followed by 0.3 phr and the minimum colour shade got in Mix 2 incorporated 0.16 phr pigment. Hence, it is inferred that different colour shades can be produced by varying the concentrations of pigment incorporated into rubber latex.

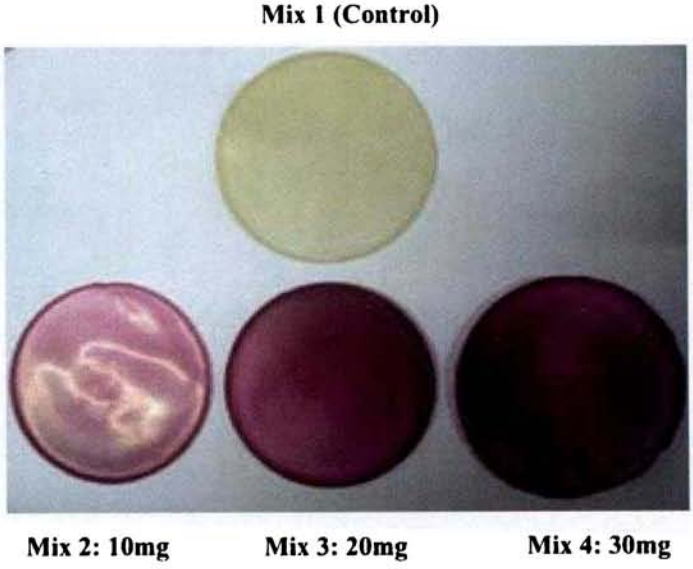


Fig. 4.45. Rubber sheets dyed with bacterial pigment: The mixes were casted on to Petriplates and kept for 24 h at room temperature (28 ± 2°C). The rubber sheets with 1 mm thickness were then kept at 70°C for 2 h in a hot air oven for proper vulcanization of the rubber.

4.7.3. Paper products

Data presented in Fig. 4.46 evidence that the pigment produced by *Serratia* sp. BTWJ8 can be used to dye different grades of paper.

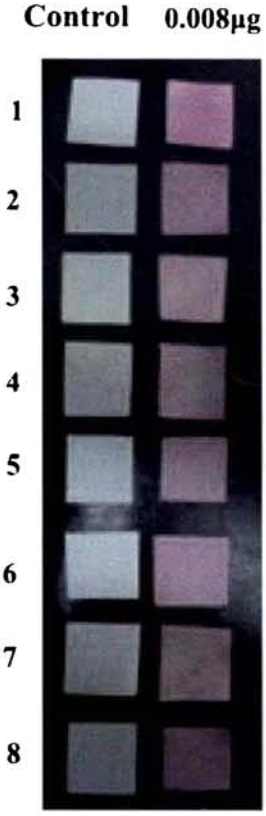


Fig. 4.46. Papers dyed with bacterial pigment: 200 µl (0.008 µg) was applied to the different paper materials on a warm surface and allowed to dry at room temperature (28 ± 2°C) for 15 min.

1-	Art paper	5-	7.8 SPB
2-	JK paper	6-	11 Kg JK
3-	Sunlight	7-	21.3 Kg JK
4-	6.9 SPB	8-	18.6 Kg SPB

4.7.4. Plastic products

Results presented in Fig. 4.47 shows that the bacterial pigment can be used as a colouring agent for plastic products. The maximum colour intensity was noticed with polymethylmethacrylate sheet incorporated 0.08 µg pigment followed by plastic sheet with 0.04 µg and 0.02 µg pigment. The least colour shade was observed with plastic sheet integrated 0.01 µg of pigment. So it is assumed that different colour shades can be produced by incorporating varying concentrations of the pigment produced by *Serratia* sp. BTWJ8 in polymethylmethacrylate solution.

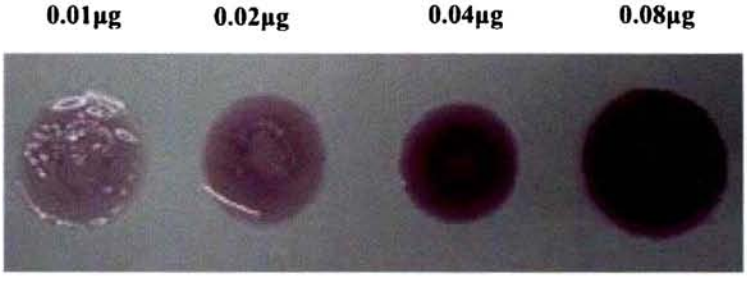


Fig. 4.47.Polymethylmethacrylate dyed with bacterial pigment: 250 µl (0.01 µg), 500 µl (0.02 µg), 1ml (0.04 µg) and 2 ml (0.08 µg) of bacterial pigment were added to 10 % PMMA solution in chloroform. Mixed well and poured into a watch glass and kept for 3 h at room temperature (28 ± 2°C).

Chapter 5

DISCUSSION

5.1. SCREENING, SELECTION AND IDENTIFICATION OF POTENTIAL PIGMENT PRODUCING BACTERIUM

Serratia sp. occurs in water, soil, plants, insects and vertebrates, and it has various characteristics including the pigment prodigiosin (Hejazi and Falkiner, 1997). *Serratia* sp. BTWJ8, isolated from seawater was identified as a potential strain that produced bright red pigment. The identity of the bacterium was further confirmed by ribotyping using partial 16S rRNA gene. From the phylogram it was inferred that this bacterium is closely related to *S. marcescens* (76 %), *S. rubidea* (75 %), *S. fonticola* (75 %), *S. proteamaculens* (75 %).

The pigment produced by *Serratia* sp. BTWJ8 is water insoluble and methanol was found to be an ideal solvent for the maximal extraction of the pigment among the different solvents studied. The results obtained for spectrophotometric and chromatographic analysis indicate that the pigment produced by the strain is prodigiosin or a close derivative. It has been reported that certain strains belonging to genus *Serratia* as well as other genera of marine bacteria produce prodigiosin, red antibiotic pigment (Cang *et al.*, 2000; D'Aoust and Gerber, 1974), which is insoluble and accumulates in the cells (Allen *et al.*, 1983).

Serratia sp. is reported to produce cell associated red colour pigment prodigiosin (Carbonell *et al.*, 2000; Ding and Williams, 1983; Singlton and Sainsbury, 2001). Microscopic observation of *S. marcescens* colonies showed that

prodigiosin pigment was localized in vesicles (extracellular and cell associated) or as intracellular granules (Matsuyama *et al.*, 1986).

In the present study, the pigment could be completely transferred to the solvent after incubation at 60°C in a water bath testifying that the pigment produced by *Serratia* sp. BTWJ8 is membrane bound. Prodigiosin was reported to display a characteristic absorption spectrum in ethanol, with a maximum at 534 nm (Slater *et al.*, 2003) and single peak absorbance at 535 nm (Giri *et al.*, 2004; Song *et al.*, 2006). Montaner *et al.*, (2000) extracted prodigiosin by shaking the *S. marcescens* 2170 cells with a mixture of methanol/1N HCl in the ratio 24:1. Pigment produced by *Serratia* sp. BTWJ8 recorded maximum absorption at 535 nm suggesting that this pigment is prodigiosin.

5.2. PURIFICATION AND CHARACTERIZATION OF THE PIGMENT

Pigments from *S. marcescens* were purified by extraction with acetone followed by transfer to petroleum ether and the petroleum ether extract was evaporated *in vacuo* at 30 to 40°C in order to obtain dry pigment. A single red coloured band was obtained after column chromatography using silica gel column and the same was eluted with n-hexane and ethyl acetate (2:1; v/v) and concentrated by evaporation (Williams *et al.*, 1960; Williams *et al.*, 1956). In the present study, the red pigment from the *Serratia* sp. BTWJ8 was purified by extraction with methanol followed by transfer to petroleum ether and dry pigment was obtained by evaporation of the solvent at 40°C. A single band with an R_f value of 0.42 was obtained after thin-layer chromatography with chloroform: methanol (95:5; v/v) solvent system. Song *et al.*, (2006) reported single red prodigiosin band with R_f value 0.43. The molecular mass of the pigment produced by *Serratia* sp. BTWJ8 was 324.2 Da, which corresponds to that of the molecular mass of

prodigiosin ($C_{20}H_{25}N_3O$). Similar result was reported earlier for *Serratia* sp. (Giri *et al.*, 2004; Song *et al.*, 2006).

In the present study, the data obtained for the spectroscopic analyses of the red pigment with LC-MS, FT-IR, FT-Raman and ^{1}H-NMR very clearly testify that the pigment produced by *Serratia* sp. BTWJ8 is prodigiosin-like pigment.

Absorption spectrum of the pigment produced by *Serratia* sp. BTWJ8 was dependent on pH value similar to that reported earlier (Song *et al.*, 2006). Thus at pH 2.0, the pigment was red and showed a maximum absorption at 535 nm, which is identical to that of prodigiosin hydrochloride. Under neutral condition (pH 7.3), its absorption intensity decreased and the colour of the pigment changed to pink. However, in alkaline condition (pH 10.0) the colour was orange and its absorption spectrum shifted to 470 nm. It has been suggested that the nitrogen of the three conjugated pyrrole rings are protonated by NaOH (Rizzo *et al.*, 1999). Prodigiosin has long been known to respond to pH change since the addition of acid causes a bright red colour and the addition of alkali produces an orange shade (Lewis and Corpe, 1964).

5.3. PRODUCTION OF PIGMENT UNDER SUBMERGED FERMENTATION

Various nutrients influence the rate and amount of pigment production by bacteria. Hence, in order to increase the potentiality of the bacteria to synthesize maximal quantities of the pigment a suitable medium was developed after conducting a comparative study using different media that are known to be used commonly for carotenoid production. Among the different media evaluated Mineral salts tryptone sucrose medium (MSTS) supported maximal pigment production when compared to Zobell Marine broth, Nutrient broth, Seawater yeast extract peptone broth and Glycerol asparagine broth. Since MSTS medium led to

enhanced red pigment production by *Serratia* sp. BTWJ8, this medium was optimized further towards maximizing pigment production.

It was noted that in spite of more pigment production in MSTS medium prepared in 50-100 % seawater, there was large pigment loss from the cells into the culture supernatant during the recovery of pigments when compared to the medium prepared in distilled water. Hence, an attempt was made to precipitate the culture supernatant with ammonium sulphate in order to ascertain whether the pigment was bound to soluble proteins and hence removed along with supernatant. It was observed that after precipitation the supernatant became colourless denoting that the pigments got bound to the secreted proteins and got removed in the culture supernatant. Probably in the presence of various salts that are natural components of seawater, there was forced binding of the pigment to the proteins and hence removed along with proteins. Further studies to this effect may throw more light on this property of pigment. In an earlier study a water soluble pigment composed of prodigiosin, carbohydrate and protein excreted from *Serratia marcescens* has been purified by precipitation with ammonium sulphate and dialysis (Parachuri and Harshey, 1987).

S. marcescens is reported to produce extracellular metalloproteases which are believed to function in cell-to-extracellular matrix interactions (Hejazi and Falkiner, 1997). Serrapeptase, also known as Serratiapeptidase, is a proteolytic enzyme isolated from the *Serratia* sp. Clinical studies show that Serrapeptase induces fibrinolytic, anti-inflammatory and anti-edemic activity in a number of tissues, and that its anti-inflammatory effects are superior to other proteolytic enzymes. Besides reducing inflammation, one of Serrapeptase's most profound benefits is reduction of pain, owing to its ability to block the release of pain-inducing amines from inflamed tissues. Serrapeptase has also been used in the successful treatment of fibrocystic breast disease (Mazzone *et al.*, 1990).

Consequently greater attention is paid by researchers and industries in hunting for Serratiapeptidase, a protease, towards harnessing the enzyme for therapeutic applications. In this context the observation made in the present study that *Serratia* sp. BTWJ8 produce protease holds scope for further study in developing this enzyme as a therapeutic enzyme.

All proteins denature, or lose their optimal shape for carrying out a specific purpose, when heated. In some cases proteins that regulate the expression of other genes may not fully denature but lose their ability to bind DNA when a certain temperature is exceeded. If the loss is reversible, these regulators regain the ability to bind DNA and activate or inhibit gene expression at lower temperatures. Another possibility is that temperature affects the activity of one or more enzymes involved in the synthesis of prodigiosin. At temperatures over 30°C a particular enzyme in the pathway to synthesize prodigiosin may lose activity (http://www.madsci.org). In the present study maximum prodigiosin production by *Serratia* sp. BTWJ8 was observed at 25°C followed by 20°C and the production decreased proportionately along with increase in incubation temperature from 30°C to 50°C. Maximum yield of prodigiosin from *S. marcescens* was observed at 28°C, while at 37°C no pigment production was observed and the culture broth was white in colour (Giri *et al.*, 2004). Maximal level of prodigiosin was synthesized by *S. marcescens* at 27°C while no pigment was formed at 16 or 32°C (Williams *et al.*, 1971b). Further, it has been reported that the production of undecylprodigiosin from *Serratia marcescens* was inhibited when the temperature was lower than 20°C or higher than 37°C (Furstner, 2003; Giri *et al.*, 2004). Whereas, some strains of *S. marcescens* produced pigment when incubated at 37°C (Davis *et al.*, 1957).

Extremes of pH, either acidic range (below pH 3.0) or alkaline range (above pH 10.0), prevented pigmentation by *S. marcescens* (Williams *et al.*,

1971b). While the optimum growth of all strains of *Serratia* has been observed at pH 9.0 (Giri *et al.*, 2004), the optimum pH for undecylprodigiosin production by *Serratia marcescens* was observed at 8.0 (Wei *et al.*, 2005). In the present study, it was observed that the media pH influenced the pigment production by *Serratia* sp. BTWJ8. Thus there was considerable level of pigment production over a range of pH from 5.0 to 9.0 and maximal pigment was recorded at pH 6.0 in spite of a decline in pigment production along with increase in pH. Highly acidic (2.0-4.0) and alkaline (10.0 - 13.0) media did not support pigment production.

The agitation rate which influences the mass transfer of both oxygen gas and medium components in the medium was a crucial factor in undecylprodigiosin synthesis by *S. marcescens* which recorded maximum pigment production at 200 rpm (Wei *et al.*, 2005). Prodigiosin biosynthesis by the nonproliferating cells was maximum when *S. marcescens* strain was incubated under aeration (Qadri and Williams, 1972). Mass transfer of medium components into the cells might play an important role in the relationship between agitation rate and undecylprodigiosin production. Meanwhile higher rate of agitation led to a slight decrease in pigment production, probably due to the problem of high shear force (Wei *et al.*, 2005). In the present study also it was observed that agitation significantly led to increase in the growth and pigment production by *Serratia* sp. BTWJ8. Maximum pigment production was recorded at 150 rpm and there was a decrease in pigment production at agitation rates above and below 150 rpm. Biomass also increased from static condition to 150 rpm.

Dextrose supported highest level of pigment and biomass production when it was used as the sole source of carbon when compared to all the other sugars, which led to a reduction in the level of pigment production. Hence, dextrose was selected as the ideal carbon source for pigment production by *Serratia* sp. BTWJ8. Further, it was noted that 30 mM of dextrose supported maximum pigment

production. The results clearly testify that dextrose support cell growth and thereby prodigiosin production. Since no similar reports are available in the literature no comparative evaluation could be done.

Data obtained for the studies conducted on the effect of inorganic nitrogen sources on pigment production evidence that the inorganic nitrogen source could enhance pigment production by the bacterium compared to the control medium, which was devoid of nitrogen source. However, when the inorganic nitrogen source was provided as ammonium sulphate and sodium nitrate, there was no pigment production. Interestingly both nitrate and ammonium in other combinations supported pigment production. Hence, it is possible that sodium and sulphate could have blocked the induction role of nitrogen for pigment production in their respective combination. Further studies are required to understand this induction/repression role of these salts.

A stimulatory effect of nitrogen source on pigment formation has been reported by Hamdi *et al.*, (1997). Among the organic nitrogen sources evaluated yeast extract enhanced maximal pigment production by *Serratia* sp. BTWJ8 followed by beef extract and malt extract. Yeast extract, extracted by boiling from eukaryotic tissues, is frequently used as a source of amino acids, vitamins, coenzymes and as growth factors by fastidious organisms. Cell growth increased with increasing yeast extract concentration whereas maximum undecylprodigiosin production occurred at a yeast extract concentration of 5 g/L (Wei *et al.*, 2005). In the present study, 1 % of yeast extract supported maximum pigment production although considerable level of pigment production was recorded at all the levels of yeast extract. While there was gradual increase in the pigment yield along with increase in concentration of yeast extract from 0.2 to 1 %, there was marginal decline in pigment level for the yeast extract concentration that varied from 1.5 to 3 %.

Lewis and Corpe, (1964) reported that calcium ions are necessary for the production of prodigiosin by two Gram negative marine bacteria. The present study adds evidence to this observation that calcium ions do influence pigment production. Thus maximum pigment production was obtained with 100 mM of calcium chloride and concentrations of $CaCl_2$ above 40 mM led to enhanced pigment production compared to medium devoid of $CaCl_2$.

It has been reported that trace elements are important factors that affect pigment production in several microorganisms (An *et al.*, 2001; Bau and Wong, 1979; Fogarty and Tobin, 1996; Kim *et al.*, 1998; Oshima *et al.*, 1981) and there is an interaction between pigment and metal ions (Fogarty and Tobin, 1996). Zinc ions were reported to have detrimental effect on *Monascus* pigment production (Bau and Wong, 1979). Iron was responsible for decreased astaxanthin production and its composition in *Phaffia rhodozyma* (An *et al.*, 2001). Whereas, in the present study the selected trace salts namely $FeCl_3$, $MnCl_2$, Na_2MoO_4 and $ZnSO_4$ were observed to enhance the pigment production significantly in the MSTS medium when they were included in the medium. A reduction in the level of pigment production was observed in the medium prepared without the said trace salts and also at situations when one of the salts was deleted among the four and retaining all the other three, when compared to that of control, which contained all the four salts. Interestingly, even in the absence of all the four salts the bacterium could produce pigment and when they are added to the medium they supported enhanced level of pigment production compared to control. Of the four salts $ZnSO_4$ had relatively a greater influence compared to other salts. Whereas, biomass production by the bacterium was observed more in media without $ZnSO_4$. It is inferred that this bacterium prefers zinc for pigment production and not for biomass yield.

To maintain the activities of permeases for the uptake of organic and inorganic growth factors marine bacteria require inorganic ions (MacLeod and Onofrey, 1963). In the present study, it was noted that pigment production was influenced significantly by the varying concentrations of sodium chloride. The bacterium did not produce pigment in media without sodium chloride indicating the requirement for NaCl for pigment production. Whereas, both pigment and biomass production gradually increased along with increase in concentration of NaCl from 10 mM and maximal pigment and biomass was recorded with 200 mM sodium chloride. *Serratia* sp. BTWJ8 can be considered as slighty halophilic since it can tolerate NaCl concentration from 10 mM to 1000 mM (0.05 to 5.8 %). In an earlier investigation, the effect of sodium ions on growth and prodigiosin production by *V. gazogenes* ATCC 29988 was studied by varying the concentration of NaCl in defined medium from 0 to 600 mM. Optimal cell growth occurred in the presence of 100 mM NaCl and decreased at higher sodium ion levels whereas, prodigiosin synthesis was not suppressed by increased levels of sodium ions in the medium (Allen *et al.*, 1983). Although *Serratia* sp. displayed satisfactory growth in distilled water media, slightly better growth occurred after 24 h at 30°C in a medium of low salinity. The salinity optimum was 1 % for *S. marcescens* and 3 % for *S. marinorubra*, a marine organism (Lewis and Corpe, 1964). In the present study, *Serratia* sp. BTWJ8 was recorded to tolerate NaCl concentration from 10 mM-1000 mM even though maximal pigment production was recorded at 200 mM. Whereas, it is also reported that NaCl may be an inhibitor of undecylprodigiosin by *Serratia marcescens* (Wei *et al.*, 2005). The optimal growth of *Beneckea gazogenes* was recorded in the presence of 300 mM NaCl (Harwood, 1978). The inhibitory effect of 300 to 500 mM Na^+ on some marine bacteria has been reported (Reichelt and Baumann, 1974). Whereas Wei *et al.*, (2005) reported that prodigiosin like pigment production was enhanced in the absence of sodium chloride. The higher-level of production of pigment by *S. rubidaea* N-1 was observed at 0.6-1.0 M NaCl when the cells were incubated for 48 h in LB media

with 0-2.0 M NaCl, indicating that it is a salt-tolerant bacterium of which prodigiosin is produced dependent on the salt concentration (Yamazaki *et al.*, 2006).

Medium optimization by statistical methods has proved to be a powerful and useful tool of biotechnology. Plackett-Burman design offers good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component. Only the most effective factors with positive significance would be selected for further optimization studies, while those showing high negative effect on the bioprocess may be dropped in all further experiments (Plackett and Burman, 1946). This indicates the effectiveness of Plackett-Burman design as a tool for elucidating the most important variables affecting the response (Abdel-Fattaha *et al.*, 2005). In the present study, the process variables were statistically optimized initially using Plackett-Burman design and Response Surface Methodology in the second stage for the maximum pigment production. Among the eleven factors namely incubation period, inoculum, yeast extract, dextrose, NaCl, $CaCl_2$, K_2HPO_4, KH_2PO_4, trace salts, pH and incubation temperature evaluated with Plackett-Burman Design for pigment production. Inoculum (X_1), sodium chloride (X_2), calcium chloride (X_3), and Temperature (X_4) were alone found to be the most significant variables. The statistical significance of the model equation evaluated by the *F*-test analysis of variance (ANOVA) revealed that this regression is statistically significant. The Model F-value of 9.15 implied that the model is significant.

The data obtained evidence that inoculum, sodium chloride, and temperature had a positive effect in enhancing pigment production along with their increase in concentration. Whereas, calcium chloride had a negative effect on pigment production along with their increase in concentration

Applying Box-Behnken design is an efficient method to optimize the selected factors for maximal production that tests the effect of factors interaction. Besides, it converts the bioprocess factor correlations into a mathematical model that predicts where the optimum is likely to be located. It is worthwhile to advise the microbial industry sponsors to apply these experimental designs to maintain high efficiency and profit bioprocesses (Abdel-Fattaha *et al.*, 2005). The results obtained for the Box-Behnken design experiment were analyzed by ANOVA and the result showed that Prob > F value was less than 0.05, which indicate that the model is significant. Two linear coefficient, inoculum (X_1), sodium chloride (X_2); one quadratic term, temperature (X_4^2) interaction coefficient and two interaction coefficient, sodium chloride and calcium chloride (X_2X_3); calcium chloride and temperature (X_3X_4) were found to be significant model terms.

The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with coefficient of determination (R^2) of 0.8107. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 81.07 % of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for comparing models with different numbers of independent variables, was 0.6069. All the selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on pigment production.

Three-dimensional response surface curves were plotted to study the interaction among various physicochemical factors, and to determine the optimum concentration of each individual factor for maximum pigment production. The model predicted maximum pigment production up to 40 μg/L that could be

achieved using 1.64 % (v/v) inoculum, 75 mM calcium chloride, and 196 mM sodium chloride, at incubation temperature 28°C and incubation period for 22 h.

Validation of the deduced response surface model based on the previous experiments was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. The optimized conditions for pigment production were as follows: Yeast extract-1 % (w/v); Dextrose-10 mM; K_2HPO_4-0.45 mM; KH_2PO_4-0.5 mM; $CaCl_2$-75 mM; NaCl-196 mM; Inoculum-1.64 % (v/v); pH-6.0; Incubation temperature-28°C and incubation period of 22 h in shake flasks.

Data obtained for the time course experiment conducted over a period of 48 h under optimized conditions testify that pigment production commenced after 6 h of incubation and reached a peak after 24 h of incubation. Further incubation period beyond 24 h resulted in a slight decline in pigment production. The results further suggest that pigment production by *Serratia* sp. BTWJ8 is growth associated. Maximum biomass was attained within 24 h of incubation and was found to be decreased during the late hours of incubation. An overall 2 fold increase in pigment production was achieved after statistical optimization compared to the MSTS media before optimization at the time of the experiment. So the medium can be successfully optimized for supporting the enhanced growth and simultaneously a high yield of prodigiosin by statistical approach.

Medium composition was optimized for high level production of astaxanthin by *Xanthophyllomyces dendrorhous* mutant JH1 using statistical experimental designs (Kim *et al.*, 2005). Statistical experimental design was employed to enhance carotenoid production from sugar cane molasses in the yeast *Rhodotorula glutinis* (Park and Kim, 2005). The effect of the major media

constituents of *Porphyridium* sp. was studied using response surface methodology on the production of phycoerythrin (Kathiresan *et al.*, 2006). Based on a three level Box Behnken design involving the variables pH (X_1), incubation temperature (X_2), and fermentation time (X_3), a response surface methodology for the production of carotenoid by a mutant *Aspergillus carbonarius* CFTRI-UV10046 was standardized (Sanjay *et al.*, 2007).

Prodigiosin, a multifaceted secondary metabolite, is produced by *Serratia marcescens*, *Pseudomonas magneslorubra*, *Vibrio psychroerythrous* and other bacteria (Gerber, 1975; Parachuri and Harshey, 1987). The prodigiosin groups of natural products are a family of tripyrrole red pigments that contain a common 4-methoxy, 2-2 bipyrrole ring system. The biosynthesis of the pigment is a bifurcated process in which mono and bipyrrole precursors are synthesized separately and then assembled to form prodigiosin (Boger and Patel, 1988). Pigmentation by *S. marcescens* was recorded during the exponential phase, and maximal production occurred in the stationary phase (Williams *et al.*, 1971a). These data testify the fact that prodigiosin can be regarded as a secondary metabolite (Williams and Hearn, 1967).

Stavri and Marx, (1961) argued that if the pigment were of no use to the bacterium the ability to synthesize prodigiosin would have been lost. Synthesis of secondary metabolites such as prodigiosin that have no demonstrable function in the bacteria also offers a paradox in which useful cellular macromolecules (genes and proteins) are involved in synthesis of a useless product. The paradox can be explained by Woodruff's hypothesis (Woodruff, 1966) that as cells enter the late phases of growth they face death by accumulation of toxic precursors. Biosynthesis of secondary metabolites converts there toxic substances to an end product that has no specific function for the cell but does prolong life by removal of the lethal substances. Thus, secondary metabolism is of value because removal of the

primary metabolites may prolong survival of the microorganisms. During senescence of the bacteria, synthesis of prodigiosin and its intermediates may function indirectly in *S. marcescens* by removing toxic accumulations of metabolites such as aminoacids (Williams, 1973). Investigations of prodigiosin biosynthesis by nonproliferating cells, in which cellular growth and multiplication are separated from formation of pigment and its precursors, may provide clues as to the function of secondary metabolites in senescent cells. An understanding of the induction and regulation of the biosynthesis may provide information pertinent to the process of aging in living cells.

After conventional "one-variable-at-a-time" approach the optimized conditions for maximal pigment (21.06 µg/L) included the following: Incubation time-30 h; incubation temperature: 25°C; initial pH of the media-6.0; agitation-150 rpm; dextrose-30 mM; yeast extract -1 % (w/v); inoculum-1 % (v/v); $CaCl_2$ - 100 mM; trace salts ($FeCl_3$, $MnCl_2$, Na_2MoO_4 and $ZnSO_4$)-0.03 mM; NaCl-200 mM. Whereas, almost two fold increase in pigment production (36.95 µg/L) was recorded after statistical optimization of the variables which included: Yeast extract-1 % (w/v); dextrose-10 mM; K_2HPO_4-0.45 mM; KH_2PO_4-0.5 mM; $CaCl_2$-75 mM; NaCl-196 mM; inoculum-1.64 % (v/v); initial pH of the media-6.0; incubation temperature-28°C and incubation period of 22 h. It was observed that optimal levels of some of the critical factors such as NaCl, $CaCl_2$ and yeast extract were almost very close for effecting enhanced pigment production, despite the fact they were required at a slightly lesser level, except yeast extract. Reduced level of dextrose (10 mM) could have contributed to enhanced pigment production. Statistical optimization demonstrated almost an accurate picture of the actual requirements of the strain for maximal pigment production. So this study strongly supports the statistical method of media optimization for enhancing pigment production by *Serratia* sp. BTWJ8.

5.4. APPLICATION STUDIES

Synthetic dyes are extensively used as a dye in textile, rubber, plastic and paper industries. Now a days people are concerned about harmful effect of using synthetic dye and going for natural dye in spite of synthetic dye. The effluent released from the dyeing of the synthetic dyes are toxic and cause environmental pollution and harmful to health. The discharge of these waste residues into the environment eventually poison, damage or affect one or more species in the environment, with resultant changes in the ecological balance. There are several attempts being made to evolve ideal processes for safe and effective disposal of dye effluent from industries that use dyestuff. The harmful effects of synthetic dye and chemicals used at the time of dyeing have forced us to concern about the alternative preparation of dye using natural sources. With concern for environment protection becoming so important, there is a challenge to evolve environment friendly technologies and yet be competitive on a global level. So in this study an attempt was made to explore the probable use of natural pigment produced by *Serratia* sp. BTWJ8 for dyeing purpose in textiles, rubber, paper and plastic.

The textile industry is one amongst the rapidly growing industries world wide, which utilizes enormous amounts of synthetic dyes. Consequently, the effluent from these textile industries poses serious threat to the environment, which is often very difficult to treat and dispose. This has become a very grave problem in environment conservation and hence natural pigments have drawn the attention of industry as safe alternative.

Results obtained in the present study strongly evidence that the pigment produced by *Serratia* sp. BTWJ8 has the dyeing property and could be used to dye different grades of textile materials. Further, the wash performance studies with the textile materials treated with pigment and thiourea, which is generally considered

as a safe and effective mordant, suggest that there is ample scope for using this pigment as a dye in textile industry. In an earlier study the blue pigment from *Janthinobactreium lividum* was used to dye natural fibers and the colour shade depending on the material. Dyeing was performed by a simple procedure consisting of either dipping in the pigment extract or boiling with the bacterial cells. It was found that when the dyed material was subjected to post-treatment with thiourea solution, the fading of the bluish-purple colour to light was considerably reduced (Shirata *et al.*, 2000).

The impact of rubber and its products has been growing in our day-to-day life and the use of rubber is widespread, ranging from household to industrial products, entering the production stream at the intermediate stage or as final products. The rubber plantations and rubber goods manufacturing industries in India have achieved steady growth and development throughout the post-independence era. India is today the fourth largest producer of natural rubber, and has the highest productivity among major producers (http://planningcommission.nic.in). The significant uses of rubber are door and window profiles, hoses, belts, matting, rubber bands and balloons. Attempt made in the present study to evaluate the probable use of this pigment as a colouring agent suggest that the prodigiosin-like pigment produced by *Serratia* sp. BTWJ8 is an effective dye for rubber products. It is also inferred that different colour shades can be produced by varying the concentration of pigment in rubber latex.

Paper continues to remain as a popular medium for printing and writing. Main grades of paper for dyes/pigments are printings and writings, towel, napkin, facial, copier papers, cover papers/boards, decorative laminates, packaging materials, envelope grades, and specialty papers like poster. Further, paper remains a very competitive packaging material. New innovations and applications help maintain its position against other materials and systems. So colourants for paper

industry have a bright but challenging future. Results presented in the study advocates use of the pigment produced by *Serratia* sp. BTWJ8 as a dye for paper material.

Polymethyl methacrylate (PMMA) or poly (methyl 2-methylpropenoate) is the synthetic polymer of methyl methacrylate. This thermoplastic and transparent plastic is sold by the tradenames Plexiglas, Limacryl, R-Cast, Perspex, Plazcryl, Acrylex, Acrylite, Acrylplast, Altuglas, Polycast and Lucite and is commonly called acrylic glass or simply acrylic. PMMA is widely used in the lenses of exterior lights of automobiles. Plastic optical fibre used for short communication is made from PMMA. In the present study it was observed that different colour shades can be produced by varying the concentration of the bacterial pigment in polymethylmethacrylate solution in chloroform. It may be noted that red pigment produced by *Serratia* sp. and recognized as prodigiosin or prodigiosin-like pigment have been reported only for their pharmaceutical applications. Whereas, this is the first time, to the best of our knowledge the pigment was evaluated as a dyeing agent for use in textile, rubber, paper and plastic and could record satisfactory performance. Being a natural pigment it is definitely harmless and would be ecofriendly.

Chapter 6

SUMMARY AND CONCLUSIONS

Bacteria isolated from the seawater and sediment samples of Kerala coast was used for the present study. Among the sixty chromogenic bacterial isolates demonstrating different coloured pigments, six isolates were recorded to produce red pigments. Among them one isolate BTWJ8 was recognized to produce red pigment abundantly. This isolate was purified, characterized and identified as *Serratia* sp. The identity of the strain was confirmed by ribotyping using partial 16S rRNA gene. Partial sequence of the 16S rRNA gene was submitted to GenBank (Accession number EU239958) through BankIt programme, at NCBI site. The identity of the *Serratia* sp. could be compared with the sequences available in the GenBank, by BLAST programme. None of the *Serratia* sp. available in the database showed more than 90 % sequence similarity, and hence the bacterium *Serratia* sp. BTWJ8 isolated during the present study is considered as a new species not reported earlier.

The red pigment produced by *Serratia* sp. BTWJ8 was isolated and characterized. Methanol was found to be an ideal solvent for the maximal extraction of the membrane bound pigment among the different solvents studied. The pigment recorded maximum absorption at 535 nm.

The pigment was purified by phase separation followed by column chromatography. A single red coloured band obtained after column chromatography was eluted and concentrated by evaporation for the characterization of the pigment.

A single band with an R_f value of 0.42 was obtained after thin-layer chromatography. The molecular mass of the pigment was 324.2 Da, which corresponds to that of the molecular mass of prodigiosin ($C_{20}H_{25}N_3O$). Spectroscopic analyses of the pigment using LC-MS, FT-IR, FT-Raman and ^{1}H-NMR strongly indicate that this red coloured pigment is a prodigiosin-like pigment.

Absorption spectrum of the pigment was dependent on pH value. At pH 2.0, the pigment was red and showed a maximum absorption at 535 nm, which is identical to that of prodigiosin hydrochloride. Under neutral condition (pH 7.0), its absorption intensity was decreased and the colour of the pigment changed to pink. However, in alkaline condition (pH 10.0) the colour was orange and its absorption spectrum shifted to 470 nm. It is therefore concluded that the pigment is a prodigiosin-like pigment.

Various bioprocess parameters affecting pigment production by *Serratia* sp. BTWJ8 under submerged fermentation were optimized towards maximal pigment production using Mineral salts tryptone sucrose (MSTS) medium. Strategy adopted for the optimization was to evaluate individually the effect of different parameters ("one-variable-at-a-time" method) on pigment production under SmF, conduct statistical optimization, and perform a time course experiment under optimized condition.

Results obtained for the studies on optimization of incubation time indicate that the bacterium is fast growing. The log phase of the culture was recorded during 6 to 12 h of incubation. Pigment production was observed from 6 h of incubation onwards (7.69 µg/L). However, maximum level of pigment was recorded after 30 h of incubation (21.13 µg/L). Maximum biomass was also

recorded at 30 h (2.15 g/L). Hence, it is inferred that the pigment production by *Serratia* sp. BTWJ8 is growth associated.

Maximum prodigiosin production was observed at 25°C (22.44 µg/L) followed by 20°C (20.56 µg/L). Pigment production decreased proportionately along with increase in incubation temperature from 30°C (9.87 µg/L) onwards. Biomass production was also maximal at 25°C (2.5 g/L), which declined gradually after 30 h of incubation.

There was considerable level of pigment production over a range of pH from 5.0 to 9.0 and maximal pigment was recorded at pH 6.0 (21.41 µg/L). However, pigment production declined along with increase in pH. Highly acidic (2.0-4.0) and alkaline (10.0-13.0) media did not support pigment production. Nevertheless pigment production and biomass showed a direct relationship in response to variations in pH.

Maximum pigment production was recorded at 150 rpm (22.296 µg/L). However, there was a decrease in pigment production at agitation rates above and below 150 rpm. Biomass also recorded increased along with increase in agitation rate from static condition to 150 rpm (2.9 g/L), which however declined later at 200 rpm.

None of the sugars supplied as carbon sources in the production medium enhanced pigment production when compared to the control. Nevertheless, dextrose supported maximal pigment production (23.75 µg/L) and biomass (2.93 g/L) when used as the sole source of carbon. It was also observed that 30 mM of dextrose supported maximum pigment production (27.51 µg/L) followed by 20, 40 and 10 mM (27.12, 26.99 and 25.26 µg/L respectively).

Among the inorganic nitrogen sources evaluated for pigment production, ammonium sulphate and sodium nitrate did not support pigment production. However, both nitrate and ammonium in other combinations supported pigment production. Among the organic nitrogen sources tested yeast extract was recorded as a better source of organic nitrogen source in enhancing pigment (28.75 µg/L) and biomass production (3.93 g/L) followed by beef extract (23.03 µg/L) and malt extract (22.63 µg/L). Addition of urea did not support production of pigment as well as biomass. 1.0 % of yeast extract supported maximum pigment production (28.42 µg/L).

It was observed that 1 % inoculum supported maximum pigment and biomass production (22.40 µg/L and 3.2 g/L respectively) and higher concentrations of inoculum led to decreased pigment production and biomass.

Maximum level of pigment (22.65 µg/L) and biomass (1.98 g/L) production was supported by 100 mM of calcium chloride.

Data obtained in the present study indicate that $FeCl_3$, $MnCl_2$, Na_2MoO_4 and $ZnSO_4$ influence the pigment production by their presence and absence in the MSTS medium. All the four trace elements led to decline in the level of pigment production in their absence compared to control that contained all the four salts. Another interesting point observed was that the bacterium could produce pigment even in the absence of these salts. However, in the presence of salts in the medium they supported enhanced level of pigment production (21.58 µg/L). Among these salts $ZnSO_4$ had relatively a greater influence compared to other salts since there was more decline in pigment level in its absence. Whereas, biomass production by the bacterium was observed to be more in media without $ZnSO_4$ (2.5 g/L).

Pigment and biomass production gradually increased along with increase in the concentration of NaCl from 10 mM and maximal pigment (21.80 μg/L) and biomass (3.99 g/L) could be recorded with 200 mM sodium chloride. Similarly, gradual decrease in pigment and biomass was observed along with further increase in sodium chloride. Another interesting observation was that the bacterium did not produce pigment in media without sodium chloride, indicating the mandatory requirement of sodium chloride for pigment production.

Four factors *viz.* inoculum (X_1), sodium chloride (X_2), calcium chloride (X_3) and temperature (X_4) greatly influenced pigment production, as it is evident from the PB design. Further analysis using RSM showed that inoculum, sodium chloride, and temperature were significant model terms for the response. Three-dimensional response surface curves were plotted to study the interaction among various physicochemical factors, and to determine the optimum concentration of each individual factor for maximum pigment production. The model predicted that maximum pigment production, up to 40 μg/L, could be achieved using 1.64 % (v/v) inoculum, 75 mM calcium chloride and 196 mM sodium chloride at incubation temperature 28°C for 22 h. Validation of the deduced response surface model was carried out in shake flasks under conditions predicted by the model. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. The optimized conditions for pigment production were as follows: Yeast extract-1 % (w/v); dextrose-10 mM; K_2HPO_4-0.45 mM; KH_2PO_4-0.5 mM; calcium chloride-75 mM; sodium chloride-196 mM; inoculum-1.64 % (v/v); pH-6.0; incubation temperature-28°C, and incubation period of 22 h in shake flasks.

Data obtained for the time course experiment conducted over a period of 48 h under optimized conditions testify that pigment production commenced after 6 h of incubation and reached a peak after 24 h of incubation (39.95 μg/L). Further

incubation beyond 24 h resulted in a slight decline in pigment production. Maximum biomass was attained within 24 h of incubation and was found to be decreased during the late hours of incubation. An overall two-fold increase in pigment production was achieved after statistical optimization compared to the MSTS media before optimization (21.06 μg/L) at the time of the experiment.

Enzyme profile of the culture supernatant after fermentation was performed after 24 h of incubation. Protease and α-amylase were recorded in the culture supernatant, 39.9 U/ml and 44.2 U/ml respectively while lipase enzyme was at negligible level (2.3 U/ml). So the medium can be successfully optimized for supporting the enhanced growth and high yield of prodigiosin by statistical approach.

The pigment was evaluated for its application as a dye in textile, paper and rubber industry. The pigment was taken up by all the textile samples evaluated indicating the dyeing property of the pigment. During the wash performance studies with the textile materials treated with pigment it was found that the pigment is lost from the cloth after wash in soap solution at room temperature (28 ± 2°C) and at 40°C. Whereas, the loss of pigment from the same textile materials treated with thiourea as mordant was found to be less at both the incubation temperatures. So it is inferred that thiourea is an effective mordant for treating the dyed textile materials.

Studies conducted with rubber products also suggested that prodigiosin-like pigment is an effective dye for inclusion in rubber products. The maximum colour obtained in rubber sheet prepared with Mix 4 that contained 0.5 phr pigment, followed by 0.3 phr and the minimum colour shade got in Mix 2 incorporated 0.16 phr pigment. The results indicate that different colour shades can be produced by varying the concentration of pigment in rubber latex.

Similarly the results obtained for the studies conducted with plastic and paper materials indicate that the pigment can be used to dye paper material as well as plastic products. The topmost colour intensity was noticed with polymethylmethacrylate sheet incorporated with 0.08 µg pigment followed by plastic sheet with 0.04 µg and 0.02 µg pigment. The least colour shade was observed with plastic sheet integrated 0.01 µg of pigment. Hence, it is proposed that different colour shades can be produced by varying the concentration of pigment in polymethylmethacrylate solution in chloroform.

Based on the results obtained from the present study it is concluded that there is ample scope for the probable utilization of marine bacterium *Serratia* sp. BTWJ8 for the indigenous production of prodigiosin-like pigment. The observations made on the production of protease by *Serratia* sp. BTWJ8 holds scope for further study in developing this enzyme as a therapeutic enzyme as Serratiapeptidase produced by several species of *Serratia* is well known for its therapeutic applications. Further studies regarding the protein binding capacity of the pigment may throw more light on the property of pigment. To the best of our knowledge this is the first report on statistical modeling of media optimization towards industrial production of prodigiosin-like pigment from marine *Serratia* sp. This study also highlights the scope of this pigment for application in rubber, paper, textile and plastic industries as a colouring agent. A detailed study on the genetics of this bacterium could throw more light on the intriguing observations made with respect to varying levels of pigment production in response to trace elements, inorganic nitrogen sources and sodium chloride concentration besides understanding gene expression pattern in this marine bacterium.

Chapter 7

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