IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 11, Issue 6 Ver. V (Nov. - Dec.2016), PP 01-28
www.iosrjournals.org

Biosynthesis of Prodigiosin and Its Applications

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I. Introduction

A pigment is a material that changes the color of reflected or transmitted light as the result of wavelength selective absorption. This physical process differs from fluorescence, phosphorescence, and other forms of luminescence, in which a material emits light. 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.

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, F. *et al*., 1987). Artificial colourings, those commonly referred as "coal tar dyes" or "food dyes" have been ideal for many years and replaced natural colourants (Santhanakrishnan, T. 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, V. *et al., 1996).*

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).* Coloring of foods using microbial pigments acts as a preservative and also has antioxidant properties to the foods (Sivakumar, 2004).

Some examples of naturally occurring pigment are:

- **Riboflavin** is a yellow water-soluble vitamin produced by many micro-organisms. It is used in baby foods, breakfast cereals, pastas, sauces, processed cheese, fruit drinks, vitamin-enriched milk products, and some energy drinks.
- **Beta-carotene***: Phycomyces* and *Mucor circinelloides (*wild type) are a potential source of beta-carotene (Kushwaha, K. *et al*., 2014). It is used as a colorant in food, beverage and pharmaceutical applications.
- **Canthaxanthin** is produced as the major carotenoid pigment by orange- and dark pink-pigmented bacteriochlorophyll-containing *Bradyrhizobium* (photosynthetic) strains isolated from stem nodules of *Aeschynomene* species and *Halobacterium* spp. Canthaxanthins are potent antioxidants and inhibit the oxidation of lipids in liposomes. It is used for egg yolk and broiler pigmentation as the red component of the yolk color - and in foods and cosmetics requiring a more orange-red hue.
- **Carotenoids** are yellow to orange-red pigments that are ubiquitous in nature. A number of microorganisms produce this pigment such as *Serratia* and *Streptomyces.* Carotenoids are effective antioxidants and are widely used as food colorants.
- **Prodigiosin** is a multipurpose red pigment, produced by various microorganisms such as *Serratia marcescens, Vibrio psychoerythrus, Rugamonas rubra,* actinomycetes, such as *Streptoverticillium rubrireticuli* and other eubacteria. It is known to have antibacterial, anti-malarial, antineoplastic and antibiotic activity.

- **Phycocyanin** is a blue pigment, produced by cyanobacteria which contain chlorophyll *a*. The blue colorant is known by the name spirulina (blue green alga), which is also the name of a dietary supplement rich in proteins and consists of dried cyanobacteria. It is used in coloring of fermented milk products, ice creams, chewing gum, soft drinks, alcoholic drinks, desserts, sweet cake decoration shakes
- **Violacein** is a versatile pigment from a bacterium *Chromobacterium violaceum* that exhibits several biological activities (Kushwaha, K. *et al*., 2014). It has gained increasing importance in industrial markets, such as in medicine, cosmetics, food and textiles.
- **Astaxanthin** (3, 3'-dihydroxy-b, b-carotene-4, 4'- dione) is a orange- red pigment and produced by microorganisms such as red basidiomycetous yeast *Xanthophyllomyces dendrorhous*, green algae *Heamatococcus pluvialis* and *Agrobacterium aurantiacum.* Astaxanthin has been used as a feed supplement and food coloring additive for salmon, crabs, shrimp, chickens, and egg production.

Table 1.1. List of pigments produced by different microorganisms. (Kushwaha, K. *et al*., 2014)

Serratia marcescens is a motile, short rod-shaped, Gram-negative, facultative anaerobe bacterium, classified as an opportunistic pathogen. It was discovered in 1819 by Bartolomeo Bizio in Padua, Italy. Bizio named the genus Serratia in honor of and Italian physicist named Serratia, and chose marcescens for the species name after the Latin word for decay. It is differentiated from other Gram-negative bacteria by its ability to perform casein hydrolysis, which allows it to produce extracellular metalloproteinases which are believed to function in cell-to-extracellular matrix interactions. S. marcescens also exhibits tryptophan and citrate degradation. Another determination of S. marcescens is its capability to produce lactic acid via oxidative and fermentative metabolism. Therefore, it is said that S. marcescens is lactic acid O/F+ .Ubiquitous presence in the environment with a preference for damp conditions. Commonly found growing in bathrooms (especially on tile grout, shower corners, toilet water line, and basin), where it manifests as a pink discoloration and slimy film. Optimally, *Serratia marcescens* grows at 37°C, but it can grow in temperatures that range from 5–40°C. They grow in pH levels that range from 5 to 9. Ten species of *Serratia* have been described, of which only three are capable of producing prodigiosin: *S. plymuthica, S. rubidaea* and some biogroups of *S. marcescens*. *S. marcescens* is a facultative microorganism and therefore the pigment is produced under both aerobic and anaerobic conditions. *Serratia marcescens* is well known for the red pigmentation it produces called prodigiosin. The red pigment production is not present in all strains but in those that it is present, it can resemble blood. Many types of differential and selective media have been used for *Serratia* growth and prodigiosin production.

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Serratia*
Species:	***S. marcescens***

Table 1.2. Scientific classification for Serratia marcescens

Prodigiosin5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl]-2-methyl-3-pentyl-1*H*pyrrole) is a secondary metabolite alkaloid with a unique tripyrrole chemical structure. It is a red pigment isolated from a few species such as *Serratia*, *Pseudomonas* and *Streptomyces*. Prodigiosin has an unusual structure with three pyrrole rings and is a pyrryldipyrrylmethane; two of the rings are directly linked to each other, and the third is attached by way of a methane bridge.

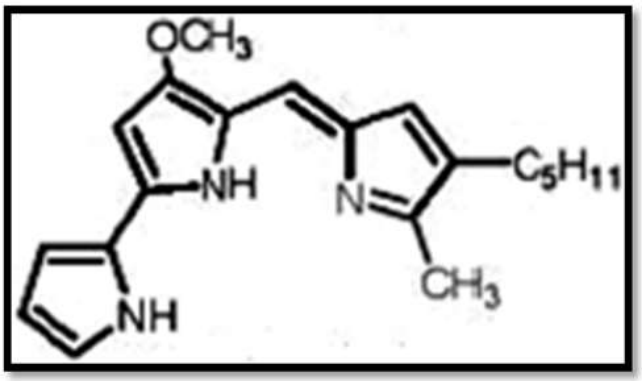


Figure 1.1. Structure of prodigiosin

Prodigiosin forms lustrous, square pyramidal crystals that are dark red with a green reflex; the hydrochloride7, 8, 9. And has a molecular weight of 323.44 Da. The deeply red colored prodigiosin alkaloid contains a 4-methoxy-alpha, alpha bipyrrole moiety which is produced by bifurcated biosynthesis pathway, in which mono-and bipyrrole precursors are obtained separately and then coupled to for the production of linear tripyrrole red pigment during the stationary phase of bacterial growth.

A bifurcated pathway has been proposed for the biosynthesis of prodigiosin culminating in the enzymic condensation of the terminal products of the two pathways, 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC) and 2-methyl-3-n-amyl-pyrrole (MAP) . Although the condensation reaction can occur spontaneously at low pH, in bacteria this reaction occurs by a temperature-sensitive enzymatic reaction. The precursors for prodigiosin were shown to be acetate, serine, alanine, methionine and proline**.** A detailed pathway for the biosynthesis of undecylprodigiosin in *Str. Coelicolor* A3 has been proposed and roles for many of the genes of the red cluster suggested, based mainly on predicted functions of the genes. The mechanism of proline incorporation into one of the pyrrole rings of MBC was then confirmed biochemically. The monopyrrole precursor (2-undecylpyrrole) of undecylprodigiosin is different from the monopyrrole (MAP) of prodigiosin although the MBC moiety is common to both. (Williamson, R**.** *et al*., 2005)

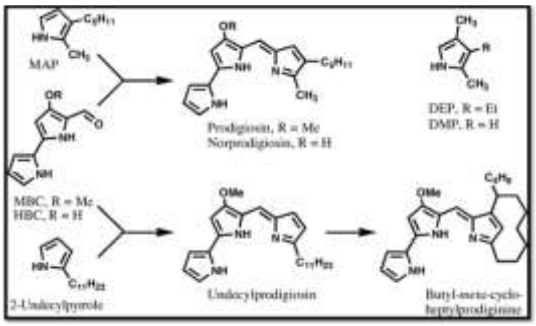


Figure 1.2. Structures of some members of the prodiginines and key prodigiosin biosynthesis intermediates. Prodigiosin and undecylprodigiosin are formed by the condensation of 4-methoxy-2, 2′-bipyrrole-5-carbaldehyde (MBC) and either 2-methyl-3-n-amyl-pyrrole (MAP) or 2-undecylpyrrole respectively. Also shown are the structures of butyl-*meta*-cycloheptylprodiginine a cyclic derivative of undecylprodigiosin and two analogues of MAP, 2,4-dimethyl-3-ethylpyrrole (DEP) and 2,4-dimethylpyrrole (DMP). (Williamson, R. *et al*., 2005)

Bacteria possess huge ability in producing biopigments that are synthesized for producing medicinally important products. These pigments are emerging as a novel group of compounds having distinct biological activities antibacterial, antimalarial, antimycotic, immunomodulating, antitumor and nuclease**.** Prodigiosin has been shown to be active against multidrug resistant cancer cell lines. The production of prodigiosin is highly variable among species and depends on many factors such as species type incubation time, pH, carbon and nitrogen sources and inorganic salts. These pigments are emerging as a novel group of compounds having distinct biological activities antibacterial, antimalarial, antimycotic, immunomodulating, antitumor and nuclease, active against multidrug resistant cancer cell lines. It is sensitive to light and insoluble in water. It is moderately soluble in alcohol and ether, and soluble in chloroform, methanol, acetonitrile and DMSO.

Many artificial synthetic colorants, which have widely been used in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes, comprise various hazardous effects. To counter the ill effect of synthetic colorants, there is worldwide interest in process development for the production of pigments from natural sources. The utilization of natural pigments in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes has been increasing in recent years thanks to the apprehension about the harmful effects of synthetic pigments and their industrial byproducts on humans and the environment. 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. Natural pigments can be obtained from two major sources, plants and microorganisms. The accessible authorized natural pigments from plants have numerous drawbacks such as instability against light, heat or adverse pH, low water solubility and are often non-availability throughout the year. lity of cultivation technology. The advantages of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from

weather conditions and colors of different shades. Hence, microbial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications and could be subjected to biodegradation in the environment (http://express-press-release.com).

II. Literature Survey

There is an increasing interest in biopigment production as an alternative to synthetic pigments, bacterial pigments due to their better biodegradability and higher compatibility with the environment, offer promising avenues for various applications. The industry is now able to produce some bacterial pigments for applications in food, pharmaceuticals, cosmetics and textiles.

2.1. Pigment classification

Pigments can be classified based on their origin as natural, synthetic and organic. 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 (Bauemfeind, 1981).

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

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) (Frick and Meggos,1988)

2.2. Prodigiosin

Prodigiosin (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl]-2-methyl-3-pentyl-1*H*pyrrole) is a secondary metabolite alkaloid with a unique tripyrrole chemical structure. It is a red pigment isolated from a few species such as *Serratia*, *Pseudomonas* and *Streptomyces* (Williamson, R**.** *et al*., 2005). Prodigiosin has an unusual structure with three pyrrole rings and is a pyrryldipyrrylmethane; two of the rings are directly linked to each other, and the third is attached by way of a methane bridge (Qadri and Williams, 1972).

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).

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. *Nocardiamadurae, N. pelletieri,* and *Streptomyceslongisporusruber* 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. *longisporusruber*produce pigments of higher molecular weights (Williams, 1973).

Zooshikellarubidus S1-1produces prodigiosin and cycloprodigiosin as two major metabolites. Both prodigiosin and cycloprodigiosin showed antimicrobial activity against several microbial species. These bacteria were approximately 1.5-fold more sensitive to cycloprodigiosin than to prodigiosin. The metabolites also showed anticancer activity against human melanoma cells, which showed significantly more sensitivity to prodigiosin than to cycloprodigiosin. The secondary metabolite profiles of strain S1-1 and two reference bacterial strains were compared by liquid chromatography-mass spectrometry (Jong, L. *et al*., 2011).

2.3. Microbial production of prodigiosin

S. marcescens TKU011 PG production on squid pen powder (SPP)-containing medium, the effects of phosphate and ferrous ion supplementation, autoclave treatment, and aeration was studied. Autoclave treatment showed positive results for PG productivity (2.48 mg/mL), which increased 2.5-fold when the organism was incubated in 50 mL of 40-min autoclaved medium in a baffle-based flask (250 mL) containing 1.5% SPP at 30

°C for 1 day and then at 25 °C for 2 additional days respectively. Also, the use of pigments including PG and the food colorants Allura Red AC (R40) and Tartrazine (Y4) as insecticides was also investigated. The lethal concentrations causing 50% Drosophila larval mortality (LC50) of PG, Y4, and R40 using a 5-d exposure period were 230, 449, and 30000 ppm, respectively. The studies also revealed that the biopigment PG and the food colorant Y4 were potentially toxic to Drosophila larvae (Liang, T.W *et al*., 2013).

The levels of prodigiosin were studied in peptone glycerol broth and nutrient broth. Influence of aeration is also studied which indicates that higher levels of prodigiosin are found in the media kept for aeration. They found that the prodigiosin production was increased gradually after 48h and was maximal towards 72h thereafter the production was found to decrease towards 92h in both media (Kamble, K. *et al*., 2012).

A new strain of *Serratia marcescens* MBB05 the natural red pigment prodigiosin producing strain was isolated from Western Ghat Ecosystem, Tamil Nadu, and India. The media used were nutrient broth, peptone glycerol broth and production medium. Peanut powder was found to be the best natural substrate at a concentration of 2.0% in distilled water at pH-7; inoculum (5%); temperature (30°C); incubation period of 36 hours. The production was found to be 4.5 times higher (560.4 mg/mL) than the optimized basal medium (Vaikuntavasan, P. *et al*., 2012).

The different media like nutrient broth, peptone glycerol broth, powdered peanut seed broth and powdered sesame seed broth, sugar substrates (maltose and glucose) and different oils like peanut oil, sesame oil, coconut oil at different temperature to study the prodigiosin production in *Serratia marcescens* . Among different oils, peanut oil showed high pigment production (2.72 mg/ml) (Shahitha, S. *et al*., 2012).

The fatty acid form of carbon source has a role to play in enhanced cell growth and prodigiosin production. Also the medium suggested in this work is best suitable from an industrial point of view in being economically feasible, in terms of the higher prodigiosin yield and the extraction of prodigiosin is simple with minimal wastage (Giri, A. *et al*., 2004).

The current technology status and challenges, economics, novel strategies for production of bacterial pigments and metabolic engineering of bacteria with a focus on applications of bacterial pigments in food industry, pharmaceutical industry, dyeing as well as on other application (Chidambaram, V. *et al*., 2013).

S. marcescens y2 grew better and produced more intracellular prodigiosin in darkness than in illumination. The pigment leakage ratio from cells was detected more in light than in darkness conditions. Ethidium bromide uptake assay could visually prove the prodigiosin-related loss of membrane integrity under illumination. A higher concentration of malondialdehyde (MDA) was detected in light-treated culture than in darkness. Tests of different light treatments (red, yellow, blue and green) showed that the maximum extracellular pigment and the minimum biomass formation and intracellular pigment were obtained in green light (Fei, W. *et al*., 2013).

A wild-type of *Serratia marcescens* which could produce prodigiosin was isolated by utilizing the kitchen waste by solid fermentation method. They achieved optimum prodigiosin production when the moisture level was 200%; the ratio of biomass of rice husk to kichen waste material was 1.2 and adding 1.5% level proline to the substrate respectively (Fang, X. *et al*., 2011).

A new strain of *Serratia marcescens*UCP1459 was isolated from a semi-arid soil produced the natural red pigment prodigiosin. Pigment was produced using renewable resources obtained from industrial wastes. *S.marcescens* produced the highest level of prodigiosin (49.5 g/L) at 48 h of cultivation using 6% "*manipueira*" (cassava wastewater) supplemented with mannitol (2%) at pH 7 and 28 °C. The purified pigment extracted from the biomass was analyzed by mass spectrophotometry and showed the expected molecular weight of 324 Da corresponding to prodigiosin (Helvia, W. *et al*., 2010).

Serratia marcescens SM2 was isolated for high productivity prodigiosin which was estimated to be 255.21 (unit/cell). The powder of peanut seeds medium was the best medium which reveals the higher production of pigment at 28 ºC and pH 8 for 72 hr. The pigment was extracted using acetone and ethyl acetate and purified by organic solvents and thin layer chromatography. Prodigiosin pigment was found to be a successful curing agent on plasmids of *E.coli* HB101 and *S.aureas* and failed to cure plasmids of *Proteus mirabilis* and *Enterococcus avium* (Mekhael, R. *et al*., 2009).

Serratia marcescens mutant for prodigiosin production was obtained by U.V. 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-l bioreactor, with a two-step feeding strategy in which glucose was selected as the initial carbon source in the fermentation media and glycerol was fed as a 'prodigiosin inducer', it gave a 7.8 times higher prodigiosin production (583 mg/l) than the parent stain with the original cultivation mode (Tao, J. *et al*., 2005).

The efficacy of Serratia marcescens for pigment production and biological activity was investigated. This study suggests that use of sweet potato powder and casein can be a potential alternative bioresource for commercial production of pigment prodigiosin (Suryawanshi, R. *et al*., 2014).A new strain designated as *S.marcescens* NY1 was isolated. It was observed that maximum amount of prodigiosin was produced at

temperature 30°C and pH 7.0. Among the different sugar substrates tested maltose when amended in the medium yielded 425±40 mg/L of prodigiosin. Among the various oil substrates used the production of prodigiosin was maximum (535±45 mg/L) when the medium was amended with peanut oil (Sundaramoorthy, N. *et al*., 2009).

2.4. Characterization and purification of the prodigiosin

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

- The visible absorption spectrum
- Thin layer chromatogram (Rf)
- Mass Spectrum (Molecular mass)

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 ratios 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).*

Mass spectrometry and nuclear magnetic resonance spectroscopic techniques were studied to elucidate the structures of the pigment and high-performance liquid chromatography to measure pigment purity. They investigated that synthetic fabrics dyed with prodigiosin, such as polyamide and acrylic, have high colorfastness to washing (≥4th grade) and antimicrobial properties (>90%) against *Escherichia coli* and *Staphylococcus aureus.* Antimicrobial properties were found to be different between synthetic and natural fabrics. They also found that the mutant strain Serratia marcescens jx1-1, with high prodigiosin yield and purity, has promising prospects in food, cosmetic, and textile industries (Liu, X *.et al.,* 2013).

A Gram-negative, red-pigment-producing marine bacterial strain, designated S1-1, was isolated from the tidal flat sediment of the Yellow Sea, Korea. Liquid chromatography and mass spectrometry of the red pigments produced by strain S1-1 revealed that the major metabolic compounds were prodigiosin and cycloprodigiosin (Jong, L. *et al*., 2011).A comparative study to check for the effect of UV irradiation on *Serratia marcescens* and *Deinococcus radiodurans.* TSA plates were inoculated with microorganism, irradiate with UV light for specified times, and subsequently score plates for numbers and phenotypes of microorganism. Results indicated that over the range of exposures to UV irradiation tested, *Serratia* is sensitive in a dose-dependent manner and *Deinococcus*is resistant to UV-mediated change (Porter, A., 2011).

The efficacy of Serratia marcescens for pigment production and biological activity was investigated. Extracted pigment was characterized by spectroscopy, Fourier transform infrared (FTIR), and thin layer chromatography (TLC) which confirm production of biological compound prodigiosin (Suryawanshi, R. *et al.,* 2014).

Spray-dried prodigiosin extracted from *Serratia marcescens* using κ-carrageenan and maltodextrin as encapsulation agents. The effect of spray-drying parameters on the encapsulation yield (EY) was calculated. The most intense color was obtained at a 1:1ratio (volume ratio of prodigiosin in ethyl acetate to κ-carrageenan solution) with a200oC inlet temperature, a feed flow rate of 60 m3/h, a pressure of 1.5 bars and a feed rate of 3 mL/min. For confirmation, FTIR and FESEM were used that showed that the particles were regular shaped spheres (Namazkar, S. *et al*., 2013).

2.5. Application

The need to explore various natural sources of food grade colorants and their use potentials was discussed. Use of microbial pigments in processed food is an area of promise with large economic potential. However, microbial pigments offer challenges due to high cost, lower stability and variation in shades due to changes in pH (Malik, K. *et al*., 2012).

The characteristics and potential of prodigiosin pigment from *Serratia* was discussed. The ecological functions and pharmacological activities of prodigiosin such as anticancer activity, immunosuppressive activity and dyeing potential were studied (Chidambaram, V. *et al*., 2009).

Reseachers evaluated prodigiosin pigment as a dyeing agent in rubber latex, paper and polymethyl methacrylate (PMMA) so that it can be considered as an alternative to synthetic pigments. Maximum color shade was obtained in rubber sheet prepared with 0.5 parts per hundred grams of rubber (phr) pigment and PMMA sheet incorporated with 0.08 µg pigment. Results indicate scope for utilization of prodigiosin as dye for

PMMA and rubber and also prodigiosin dyed paper as a pH indicator. Further, being a natural and water insoluble pigment, it is ecofriendly (Jissa, G. *et al*., 2013).

Prodigiosin was isolated with anti-bacterial activity from soil bacteria. Most of the pigments showed better anti-bacterial activity against gram-negative bacteria. Highest zone of inhibition was resulted by pigment no 15 against *Salmonella typhi* and lowest zone of inhibition was observed for pigment 13 against *Staphylococcus aureus*.MIC value of the pigments ranged from 1500-4000 μg/ml and most of the pigments showed lower MIC value against gram-negative organisms (Md. Mamunur, R. *et al*., 2014).

The anticancer property of microbial pigment prodigiosin isolated from waste sample of *Serratia marcescens*, a marine crustacean, against human cervix carcinoma cell (Hela-229 cell line) was analyzed. Prodigiosin showed dose dependent inhibition of cell proliferation. The study suggests that the prodigiosin has strong anticancer and apoptosis activity against human cervical carcinoma cancer (Kavitha, R. *et al*., 2010).

Microorganisms are being employed, since several decades for the large scale production of a variety of bio-chemicals ranging from alcohol to antibiotics and in processing of foods and feeds. Microorganisms have great potential as natural sources of drugs for the treatment and prevention of diseases like cancer, anaemia, diarrhoea, obesity, diabetes, atopic dermatitis, Crohn's disease, etc. They are also potential sources of natural antioxidants, colours, immuno-suppressants, enzyme inhibitors, hypocholesterolemic agents, vitamins, enzymes, and antibiotics (Gupta, C. et al., 2014).Spray-dried prodigiosin extracted from *Serratiamarcescens* using κ-carrageenan and maltodextrin as encapsulation agents. The results suggested that the spray-dried prodigiosin can be useful as a colorant under the above optimum operating conditions (Namazkar, S. *et al*., 2013).The pigments can be used to dye cotton which showed good color tone (Shahitha, S. *et al*., 2012)..

III. Aims And Objectives

3.1. Aim

The aim of this study is to isolate *Serratia marcescens* from soil and to check for various methods for extraction of the prodigiosin, its production and to evaluate it as a coloring agent in various industries.

3.2. Objectives

1. Isolation of the organism from soil.
2. Biochemical characteristics to identify the species of the organism.
3. Presumptive test for presence of prodigiosin.
4. Optimization of various growth parameters for maximum prodigiosin production (media, temperature, pH, static/shaker conditions).
5. Screening for various methods for extraction of prodigiosin to obtain maximum yield.
6. Standardization of procedure for extraction of pigment.
7. Purification of the prodigiosin.
8. Qualitative and quantitative estimation of the prodigiosin.
9. Antibacterial and antifungal activity of the prodigiosin.
10. Applications as colorant in various industries.

IV. Materials And Method

All the following experiments were performed in triplicates and results are represented as average of sets.

4.1. Collection of samples

Soil sample was collected in a sterile zip lock bag aseptically from different parts (Deer park trail, Kanheri caves trail and Shilonda trail) of Sanjay Gandhi National Park (SGNP), Borivali and was transported immediately to the laboratory and processed for bacteriological analysis.

4.2. Preparation of serial dilution of the sample

10g of the sample was added aseptically to 90 ml of sterile physiological saline. After thorough mixing under aseptic condition, the sample was allowed to settle down for 30 min. One ml of the clear supematant was then serially diluted, using 9 ml of physiological saline, and 1 ml of the prepared dilution was used as inoculum for plating purpose.

4.3. Media

Nutrient broth was used for the screening studies.

4.4. Isolation and screening of *Serratia marcescens*.

Pour plate technique was employed for plating the samples. The prepared sterile nutrient agar plates were inoculated with the serially diluted sample and incubated at room temperature for 2-3 days. All the red pigment producing single cell colonies that appeared on the plates were picked, and further isolated on new sterile nutrient agar plate to obtain proper isolation. The colonies were further subcultured on the nutrient agar slants and maintained as stock cultures for further studies (Kavitha *et al*., 2012). Cultures were maintained on nutrient agar slant and stored at 4°C in refrigerator (Gulani *et al*., 2012)

4.5. Biochemical characteristics to identify the species of the organism

Pure cultures of bacterial isolates were identified as *Serratia marcescens* on the basis of their colony morphology and biochemical characteristics according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology.

4.6 . Presumptive test for prodigiosin

The culture broth was centrifuged at 4500 rpm for 15 mins. 10 ml of 95% methanol was added to the cell pellet and centrifuged under the same condition. Debris was removed and the 2ml of the supernatant was taken in two test tubes. The content of one of the test tube was acidified with a drop of concentrated HCl. The tube was observed for color change to red or pink colour in the acidified solution. This gives a positive presumptive test for prodigiosin (Kumar *et al.,* 2014)

4.7. Optimization of various growth parameters for maximum prodigiosin production (pH, Temperature, Static/Shaker conditions, Media)

4.7.1. Effect of media on prodigiosin production

48h old culture was inoculated into each pre-sterilized 100ml nutrient broth, 100ml peptone glycerol broth, 100ml tryptic soy broth, 100ml yeast extract malt extract broth, 100ml box-behnken design and 100ml mannitol medium (Lins *et al*., 2014) .The flasks were incubated at 27°C for 48hrs.The media in which maximum production of prodigiosin was observed was chosen for further studies (extraction, estimation, purification).

4.7.2. Effect of temperature on prodigiosin production

Equal volume of the bacterial isolate was inoculated in sterile nutrient broth and incubated at different temperature viz., -20, 0, 27, 37 and 50 °C for 48 h and then assayed for prodigiosin. The optimum pH achieved by this step was fixed for subsequent experiments.

4.7.3. Effect of initial pH on prodigiosin production

Equal volume of the bacterial isolate was inoculated in sterile nutrient broth with different pH viz., 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 and was incubated at 27°C for 48 hrs and then assayed for prodigiosin. The optimum pH achieved by this step was fixed for subsequent experiments.

4.7.4. Effect of static/shaker conditions on prodigiosin production

Estimation of production of prodigiosin level at static and shaker was carried out to investigate the effect of aeration on production. Levels of prodigiosin were estimated at 27°C after 48h and then assayed for prodigiosin. The optimum incubation period achieved by this step was fixed for subsequent experiments.

4.8. Screening for various methods for extraction of prodigiosin to obtain maximum yield.

The organism was allowed to grow in 3 flasks containing sterile nutrient broth. The flasks were incubated at shaker conditions at 27°C for 48 h. The broth was centrifuged. Extraction was carried out using the below methods to determine the best method for production of prodigiosin.

Method 1- The prodigiosin was extracted by adding 4 volumes of acetone to the cell suspension. The acetone mixture was shaken for 3 hrs at room temperature, and then centrifuged. The sediment cell debris was washed twice by resuspending in 50 ml. of acetone, shaking for 30 min followed by centrifugation at 10,000 rpm for 15 minutes. The washings were combined with the supernatant from the original centrifugation, and the solution was filtered. Prodigiosin was extracted from small portions of the filtrate by mixing thoroughly 1 volume of the acetone solution with 2 volumes of petroleum ether in a separatory funnel. The acetone was removed by adding 10 volumes of water to the funnel, then drawing off the acetone-water phase. This procedure was repeated until the entire filtrate was extracted, and the prodigiosin was in the petroleum ether phase. To obtain dry prodigiosin, the petroleum ether extract was evaporated at 300C. The dry prodigiosin was used for chromatographic or spectral analysis (Bharmal *et al*., 2012).

Method 2- Organism was grown in nutrient broth incubated at 27°C for 48 h on rotary shaker with 250 rpm. The broth was centrifuged at 10,000 rpm for 15 minutes and the supernatant was extracted with ethyl acetate. The prodigiosin from the cell pellet was extracted with acetone and the extraction was centrifuged at 10,000 rpm for 15 minutes and the white pellet was discarded. The prodigiosin extracted acetone fraction was mixed with ethyl acetate fraction and dried with sodium sulphate (Giri *et al*., 2004).

Method 3: Organism was grown in fementation broth incubated at 27°C for 48 h on rotary shaker with 250 rpm. The broth was taken in a centrifuge tube and centrifuged at 10,000 rpm for 10 min. The colourless supematant was discarded. The pellet was washed with distilled water and centrifuged at 10,000 rpm for 10 min .The prodigiosin was extracted from the pellete with methanol repeatedly until the debris turned colorless. The supernatant containing diffused prodigiosin was then allowed to dry.

The wet weight and dry weight of the prodigiosin was determined. The method giving maximum prodigiosin production was chosen for further purification.

4.9. Standardization of procedure for extraction of prodigiosin

4.9.1. Selection of suitable solvent for extracting the bacterial prodigiosin

Initially the solvent that could support maximal yield of prodigiosin on extraction of culture broth was standardized, using different solvents viz; ethanol, acetone, methanol and petroleum ether.

4.9.2. Preparation of culture broth

A pre-culture was developed in 5 ml of Nutrient broth by inoculating the broth with one loop full of selected bacterial strain and incubating at room temperature for overnight. 100ml of freshly prepared nutrient broth taken in 250 mL Erlenmeyer flasks was inoculated with the preculture as inoculums and incubated for 24 h on a rotary shaker at 150 rpm, at room temperature (27 ± 2°C). Extraction of the prodigiosin was done according to **4.6 method no. 3**. This was the general procedure followed for the prodigiosin extraction, unless otherwise specified. All the experiments were done in three independent sets and the mean is expressed.

4.9.3. Detection of λmax

The colored supematant was then analyzed by scanning in a UV-Visible spectrophotometer for detecting the λmax. The scanning range selected was 200-700 nm. Absorbance at the λmax was measured.

4.10. Purification of the prodigiosin.

Prodigiosin was purified using column chromatography. Silica gel column was used for the chromatography for removing impurities from the concentrated prodigiosin after extraction.

4.10.1. Preparation of column

The silica gel, which was used as the stationary phase, was suspended in the methanol, and the fine particles were removed by decantation. The silica gel suspension was carefully poured into the column filled without air bubble and allowed to settle under gravity while maintaining a slow flow rate through the column. The column was stabilized by allowing the solvent methanol to pass though the column bed in descending eluent flow.

4.10.2. Sample preparation and application

The concentrated sample prepared was loaded on to the silica gel column. After the complete entry of sample into the column the sample was eluted with a methanol at a flow rate of 0.1 ml/min. The red colored fraction was collected from the column. The eluted fraction was then analyzed by scanning in UV -Visible spectrophotometer. The selected scanning range was 200-700 nm.

4.11. Quantitative and qualitative estimation of the prodigiosin

4.11.1. Quantitative estimation

4.11.1.1. UV spectrophotometer

The absorption pattern over various wavelengths was initially checked and the absorption maximum was at 499nm where prodigiosin also absorbs maximally. At wavelength the absorptions was recorded.
Isolated prodigiosin was estimated using the formula (Mekhael and Yousif, 2009):

[OD499 – (1.381 x OD 620)] x 1000 /O.D at 620nm = Prodigiosin unit/cell

Prodigiosin unit/cell
OD- Optical density;
OD499 – Prodigiosin absorbance ;
OD620 – Bacterial cell absorbance
1.381 – Constant

4.11.1.2. 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: 4000-500cm^{-1}, Resolution: 0.9 cm^{-1}). The purified prodigiosin was subjected to FT –IR spectroscopic analysis (Shimadzu), equipped with KBr beam splitter with DTGS (Deuterated triglycine sulfate) detector (4000-350 cm^{-1}), at Patkar-Varde college, Goregaon (W) (Jissa, G. *et al* 2008).

4.11.2. Qualitative estimation

4.11.2.1. Paper chromatography

Whatmann filter paper No. 2 was used for paper chromatography. Prodigiosin is dissolved in methanol and is spotted onto the Whatman filter paper and run against the solvent containing acetone: alcohol (3:7), till the solvent front reaches $3/4^{th}$ of the paper. After which the Whatman filter paper is dried, and retardation factor is calculated (Williams *et al*., 1955) (Lins *et al*., 2014).

Retention factor *(Rf)* value was calculated according to the following equation from the chromatogram.

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

4.12. Antibacterial and antifungal activity of the pigment

To examine the antibacterial effect disc diffusion method was used, since the prodigiosin was insoluble. The clinical isolates were swabbed on Mueller Hinton agar plates and disc made up of fabric linen was dipped in prodigiosin and was placed on the plate. The clinical isolates used were *E.coli, S.aureus, P.aeruginosa, C.albicans, A.niger, Penicillium glaucum, S.typhi* and *S. faecalis.*

4.13Applications

In the present study, scope for probable application of the bacterial prodigiosin was evaluated for pH indicator, candle, textile material, paper products, nail paint etc.

4.13.1. pH indicator

Whatman filter paper no: 2 were cut into 7cm by 1 cm strips. Prodigiosin in methanol was used as the stock solution. The stock solution was applied on to the filter paper materials on and allowed to dry at RT for 15 min to impart colour. Paper material without pigment was kept as the control. After dyeing, acidic (pH 2.0), neutral (pH 7.0) and alkaline (pH 10.0) solutions were spotted over all the paper materials to evaluate the dyed paper as probable pH indicators.

4.13.2. Candle

Candles were made by melting wax in a container. The mould was then greased with oil and the melted wax was poured into the mould and 1mL of prodigiosin was added. One control candle was prepared which does not contain the pigment.

4.13.3. Textile Materials

A modified method of (Chandni *et al*., 2012) was carried out using 7cm by 8 cm of each fabric roto, two *2, terrycotton, buter, georgette, cambric, linen and chikan was soaked in 2 mL methanolic extract of prodigiosin taken in different test tubes and incubated for 48 h at room temperature, following which each fabric was dried and cut into 5 smaller pieces. These smaller pieces were then treated with acid, alkali, hot water, cold water and detergent and hot water and detergent for 1 h in respective test tubes. For all the experiments white cloth material were taken as a control.

4.13.4. Paper Products

Eight type of paper with different qualities like tinted paper, B2B paper commercially available in the market were selected for the present study. All the paper materials were cut into equal size of 2 cm. Prodigiosin in methanol was used as the stock solution. This stock solution was applied to the different paper materials and allowed to dry at room temperature for 15 min. Paper material without pigment was kept as control for all paper materials.

4.13.5. Nail Paint

Nail paint was prepared by purchasing transparent color nail paint and then 2 ml of the prodigiosin was added into it. Stability testing and microbiological analysis of the cosmetics were carried out.

4.13.5.1. Physical tests

Stability testing include physical and chemical integrity tests to evaluate color, odor/fragrance, pH value, viscosity, texture, flow and emulsion stability (sign of separation).

4.13.5.1.1. Temperature variation

High temperature testing is now commonly used as a predictor of long-term stability. Most companies conduct their high temperature testing at 37°C and 45°C. If the product is stored at 45°C for three months (and exhibits acceptable stability) then it should be stable at room temperature for two years. Of course, the product must be stored at 25°C for a period of one year. A good control temperature is 4°C where most products will exhibit excellent stability. The product should also be subjected to -20°C for three months.

4.13.5.1.2. Cycle testing

The product should pass the cycles of temperature testing from -10°C to 25°C. Place the product at -20°C for 24 hours and check for physical parameters.

4.13.5.1.3. Centrifuge testing

The dispersed phase has a tendency to separate and rise to the top of the emulsion forming a layer of oil droplets. This phenomenon is called creaming. A good test method to predict creaming is centrifugation. Heat the emulsion at 50°Cand centrifuge it for 30 minutes at 3000rpm.Then inspect the resultant product for the sign of creaming.

4.13.5.1.4. Light testing

Take 5g of the sample into 2 separate tubes. Cover one tube with aluminium foil completely and label it as control and keep another tube in the window such that having exposure to light observe for discolouration.

4.13.5.2. Microbiological testing

Microbiological stability test evaluate the degree of contamination with bacteria, mold and yeast.

1g of the sample was added aseptically to 9 ml of sterile physiological saline. After thorough mixing under aseptic condition, the sample was allowed to settle down for 30 min. One ml of the clear supematant was then serially diluted, using 9 ml of physiological saline, and 1 ml of the prepared dilution was used as inoculum for plating purpose on TSA and Sabouraud's agar medium. Incubate TSA plates at 37°C for 2-5 days and Sabouraud's agar plates at room temperature for 3-7 days.

The number of microorganisms present in the sample is calculated using the following formulae:

N= m*d/V

m=Arithmetic mean of the count obtained from the duplicates.

d=Dilution factor corresponding to the dilution made for the preparation of the initial suspension.

V= Volume of the inoculums applied to each plate in ml

(Protocol as per India Standards, BIS 2011)

V. Results And Discussion

Biopigment produced by bacteria possess enormous efficiency as medicinally important products. Prodigiosin, a red pigment synthesized by *S. marcescens*, belongs to the family of tripyrrole and exhibits antimicrobial, antifungal, antimalarial, immunomodulating and anti-tumor properties. The present investigation focused on formulation of an extraction protocol for effective prodigiosin production and separation of the pigment followed by its antimicrobial activity evaluation and applications.

5.1. Screening, Selection Ad Identification of Potential Prodigiosin Producing Bacterium

5.1.1. Screening and selection of potential strains

One chromogenic bacterium was isolated from three samples. And it was selected for further screening. Pigment production was confirmed by cultivating the isolates in Nutrient broth. The cultures, which showed bright pigmentation in the broth, were selected 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 one isolate produced red pigment and was selected based on the intensity of red pigment produced on agar medium and in liquid broth. The isolate obtained from Shilonda trail showed considerable amount of red pigment production both on the agar medium and in the liquid medium after 24 h of incubation in Nutrient medium. Hence, the strain was selected as the potential strain for pigment production in the present study. It was found to give white coloured colonies on Sabouraud's agar plate no growth on Mac Conkey's agar plate and violet coloured colonies on VRBA.

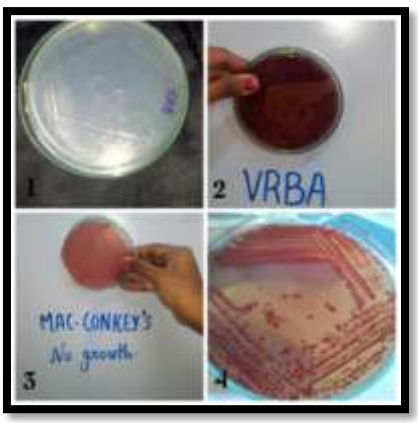


Figure 5.1.1. Isolation of *Serratia marcenses* – 1. Growth on Sabouraud's agar plate, 2. Growth on VRBA, 3. Growth on Mac Conkey's agar and 4. Growth on N.A

5.1.2. Identification of bacterium

The selected bacterial isolate was identified as *Serratia marcescens* according to the morphological and biochemical characteristics (Table 5.1.). The taxonomy of *Serratia marcescens* is given in Table 5.2.

Particulars	Variables	Characters
Colony and cell morphology	Colony shape	Round
	Colony size	1mm
	Edge	Smooth
	Surface	Smooth
	Opacity	Opaque
	Elevation	Convex
	Colour	Red
	Motility	Motile
	Cell shape	Rod
	Gram's nature	Gram Negative rods
Biochemical Characteristics	MOF	Fermentation with gas production
	Arginine decarboxylase test	Negative
	H_2S production	
	Indole	Negative
	Methyl Red	Positive
	Voges-Proskauer	Negative
	Citrate ulilization test	Positive
	Nitrate reduction test	Positive
	Catalase	Positive
	Oxidase	Negative
	Tween 80	Negative
	Urease	Negative
	1% Glucose	Positive
	1% Lactose	Positive
	1% Sucrose	Positive
	1% Mannitol	Positive
	1% Maltose	Positive

Table 5.1. Morphological and biochemical characteristics of *Serratia marcescens.*

Fig 5.1.2. Biochemical characteristics of *Serratia marcescens.*

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

Table 5.2. *Serratia marcescens* taxanomy

5.2. Presumptive Test For Prodigiosin

Presumptive test for prodigiosin was carried out by centrifuging the nutrient broth containing the culture. The cell pellet was then dissolved in methanol and centrifuged. The supernatant was taken in test tubes. It was acidified with a drop of concentrated HCl which changed the colour from red to pink. This gave a positive presumptive test for prodigiosin.

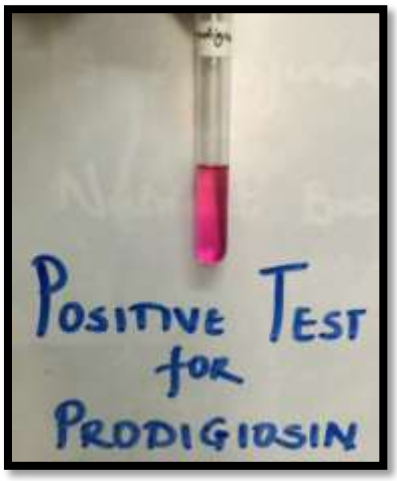


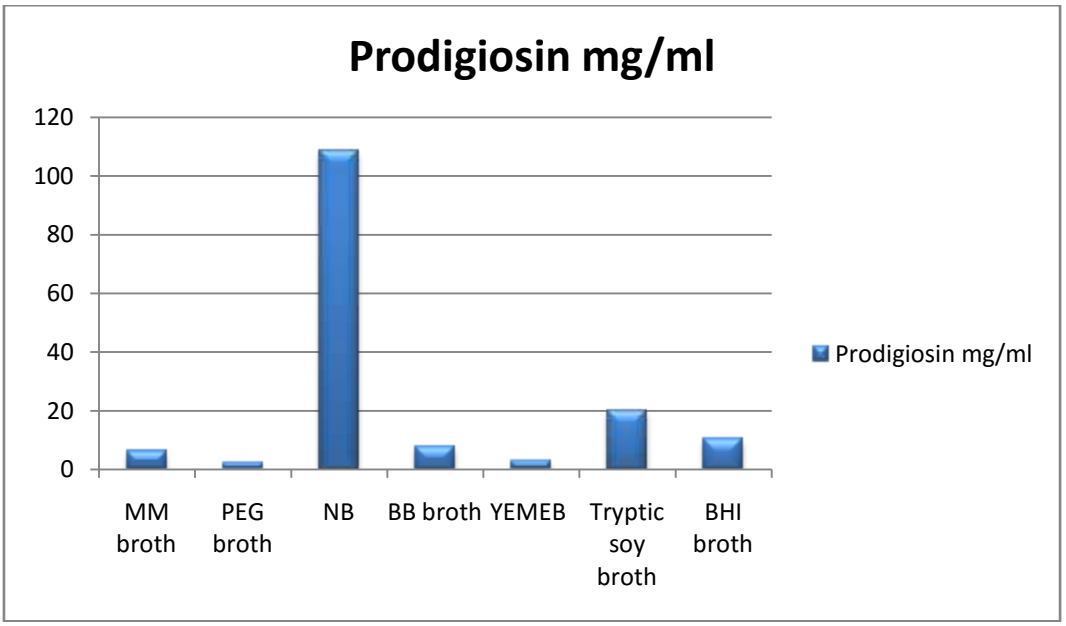
Figure 5.2. Positive presumptive test for prodigiosin

5.3. Optimization of Various Growth Parameters for Maximum Prodigiosin Production

5.3.1 Effect of media on prodigiosin production

Among the 7 media were tested for prodigiosin production, *viz.,* nutrient broth, peptone glycerol broth, tryptic soy broth, yeast extract malt extract broth, box-behnken design, BHI broth and mannitol medium. Comparing the results, nutrient broth showed better prodigiosin production. Hence, the nutrient broth has been selected for further optimization studies. Giri *et al.*, 2004 has reported that the production of prodigiosin was more in nutrient broth (0.52 mg/mL) than peptone glycerol broth (0.302 mg/mL).In our study the potential strains has produced 60.8 and 108.7 mg/mL in MM broth and N.B, which was much higher reported earlier. Both in nutrient broth and peptone glycerol broth the major components were peptone, meat and yeast extract. Peptone is a commercially available digest of a particular plant or animal protein, made available to organisms as peptides and amino acids to help satisfy requirements for nitrogen, sulfur, carbon and energy. Yeast and meat extracts contain eukaryotic tissues (yeast, beef muscle, liver, brain, heart, etc.) that are extracted by boiling and then concentrated to a paste or dried to a powder. These extracts are frequently used as a source of amino acids, vitamins and coenzymes, growth factors by fastidious organisms. In peptone glycerol broth, the glycerol was the carbon source. Giri *et al.,* 2004 has reported that the seeds and oils contain metals; vitamins, saturated and unsaturated fatty acids and the concentration of these components are variable in each kind of seed or oil.

Figure 5.3.1. Effect of MM broth, PGB, N.B, YEMEB broth, BHI broth, tryptic soy broth and BB broth (from left to right) on pigment production.

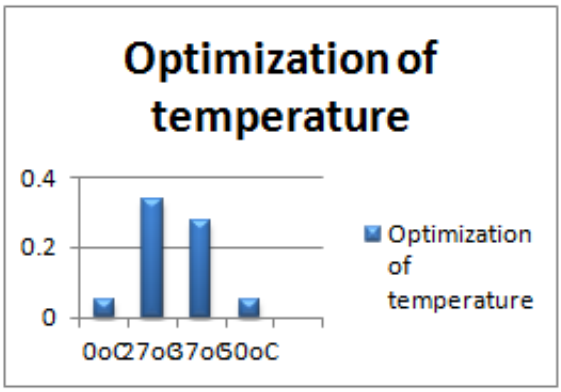


Graph 5.3.1. Pigment production by *Serratia marcescens* in different media (X-axis: media, Y-axis: Prodigiosin mg/ml).

5.3.2 Effect of temperature on prodigiosin production

In this optimization study -20, 0, 27, 37 and 50 °C were the temperature selected. The optimum pH achieved by this step was fixed for subsequent experiments. The optimum temperature of incubation for the selected bacterial strain was 27°C. Giri *et al.*, 2004 has reported that a block in prodigiosin production above 30^{o}C in nutrient broth, and at 37°C *Serratia marcescens* did not show any pigment production in nutrient broth and the culture broth was white in color. These reports are in agreement with the present finding. This might be due to the fact that the terminal step in prodigiosin biosynthesis i.e., condensing of mono and bipyrrole moieties was temperature sensitive. A survey on the role of the temperature and incubation time on the pigment synthesis implies that these are important physical factors which decide the prodigiosin production depending on the type of media. Biosynthesis of prodigiosenes (prodigiosin and prodigiosin like pigments) by *S. marcescens* occurred over a relatively narrow range of temperatures with maximal production being between 24 and 28 ºC, although the bacteria grow over a broad range of temperature (Williams *et al*., 1973). Reduction in the pigment production at elevated temperatures is well documented (Sundaramoorthy *et al*., 2009).

Figure 5.3.2. Effect of temperature on prodigiosin production

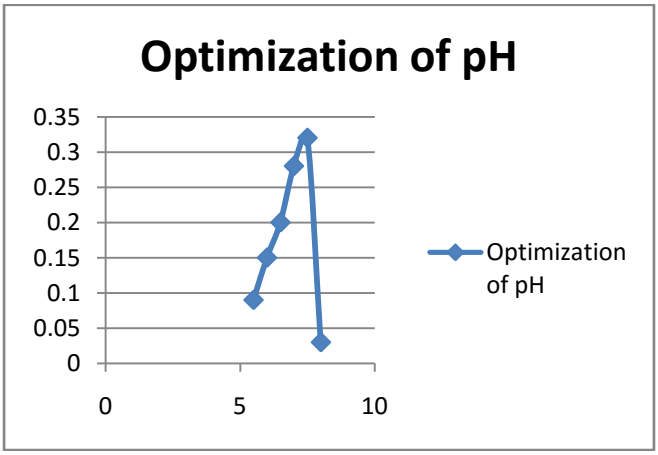


Graph 5.3.2. Optimization of incubation temperature for pigment production by *Serratia marcescens* (X-axis: pH, Y-axis: O.D at 620 nm).

5.3.3 Effect of initial pH on prodigiosin production

The selected potential bacterial strains were subjected to various pH ranging from 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were taken for the study with an interval of pH 0.5. The optimum pH achieved by this step was fixed for subsequent experiments. Less prodigiosin production were noticed in the acidic (5.5to 6.0) and alkaline (8.0) condition. But at the neutral (6.5 to 7.5) range the production was more. Investigation on this study, *S. marcescens* show highest production recorded in the pH 7.5. Hence pH 7.5 was maintained in optimization studies. Maximum amount of prodigiosin by a new strain designated as *S. marcescens* NY1 was produced at pH 7.0 (Sundaramoorthy *et al.*, 2009). This suggests the importance of pH in the media since its altered value can either increase or decrease the amount of prodigiosin. *Serratia marcescens* was considered to grow optimally at pH 6.4-7.4. However, cultures of *S. marcescens* have a powerful buffering capacity. The irrespective of initial pH of media, the final pH was 7.2 to 8.0 as the bacteria grow which has been reported by Williams et al., 1973. In our present study, the maximum prodigiosin was noticed in the medium at the pH of 7.5.

Figure 5.3.3. Effect of initial pH on prodigiosin production for pigment production by *Serratia marcescens*.

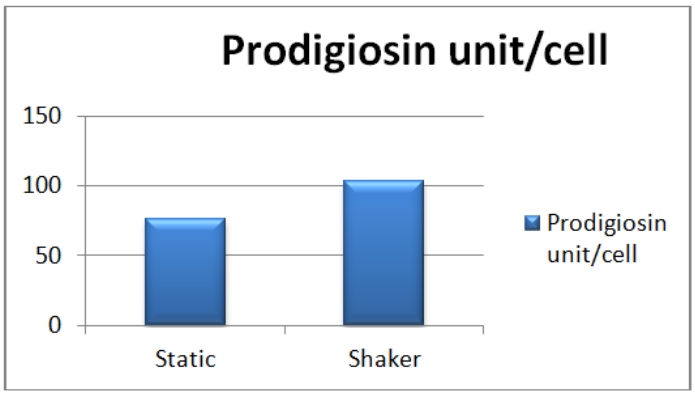


Graph 5.3.3. Optimization of pH on prodigiosin production for pigment production by *Serratia marcescens* (X-axis: pH, Y-axis: O.D at 620 nm).

5.3.4 Effect of static/shaker conditions on prodigiosin production

Estimation of production of prodigiosin level at static and shaker was carried out to investigate the effect of aeration on production. Levels of prodigiosin were estimated at 27°C after 72h and then assayed for prodigiosin. The optimum incubation period achieved by this step was fixed for subsequent experiments.

Figure 5.3.4. Effect of static/shaker conditions on prodigiosin production



Graph 5.3.4. Optimization of shaker and static conditions on prodigiosin production for pigment production by *Serratia marcescens* (X-axis: static /shaker conditions, Y-axis: Prodigiosin units/cell).

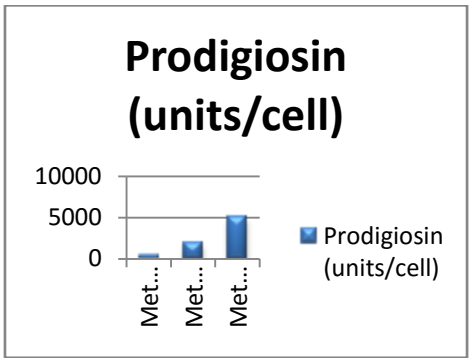
5.4. Screening For Various Methods For Exraction of Prodigiosin To Obtain Maximum Yield

Among the three methods used, method 3 was found to extract maximum amount of the pigment from the culture broth. Table 5.4 indicates the pigment produced by each method in unit/cell.

Figure 5.4. Crude extract obtained after extraction with the three methods.

Method	Prodigiosin unit /cell
Method 1	432.7
Method 2	1970.2
Method 3	4986.9

Table 5.4. Prodigiosin unit/cell produced by method 1, method 2 and method 3 respectively.



Graph 5.4. Indicates amount of prodigiosin produced units/ml by using various methods (X-axis: Methods of extraction, Y-axis: Prodigiosin units/cell).

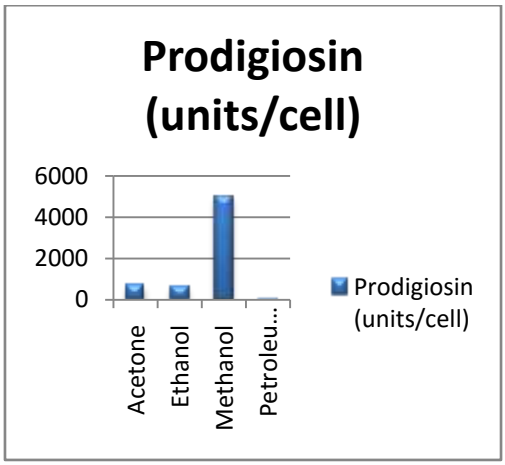
5.5. Standardization of Procedure For Extraction of Prodigiosin

Method 3 was chosen for maximal extraction and isolation of pigment from fcnnentation broth was initially standardized using different organic solvent viz, acetone, ethanol, methanol and petroleum ether. It was noted that this pigment could be assayed at 530 nm where the pigment shows maximum absorption. It is inferred from the results presented in Fig. 5.5, table 5.5 and graph 5.5 that methanol is an ideal solvent for extracting the maximum of the water insoluble membrane bound pigment.

Figure 5.5 Crude extract obtained after extraction with method 3 using acetone, ethanol, methanol and petroleum ether as the organic solvent.

Organic solvent used	Prodigiosin unit/cell
Acetone	692.1
Ethanol	620.0
Methanol	4986.9
Petroleum ether	39.1

Table 5.5. Amount of prodigiosin produced unit/cell by various organic solvents.



Graph 5.5. Indicates amount of prodigiosin produced unit/cell by acetone, ethanol, methanol and petroleum ether (X-axis: Organic solvents, Y-axis: Prodigiosin units/cell).

5.6. Purification of Prodigiosin

The pigment produced by the bacterium, was extracted from the medium and purified by column chromatography using silica gel. The results obtained arc presented below.

5.6.1. Column chromatography

The pigment obtained after the extraction was concentrated and was purified further by column chromatography using silica gel **c**olumn and solvent system comprising pure methanol .The red coloured fragment elutcd was collected and concentrated by evaporation at 40°C water bath. The purified pigment was then used for the characterization studies. The maximum wavelength of the purified sample was found to be 530nm.

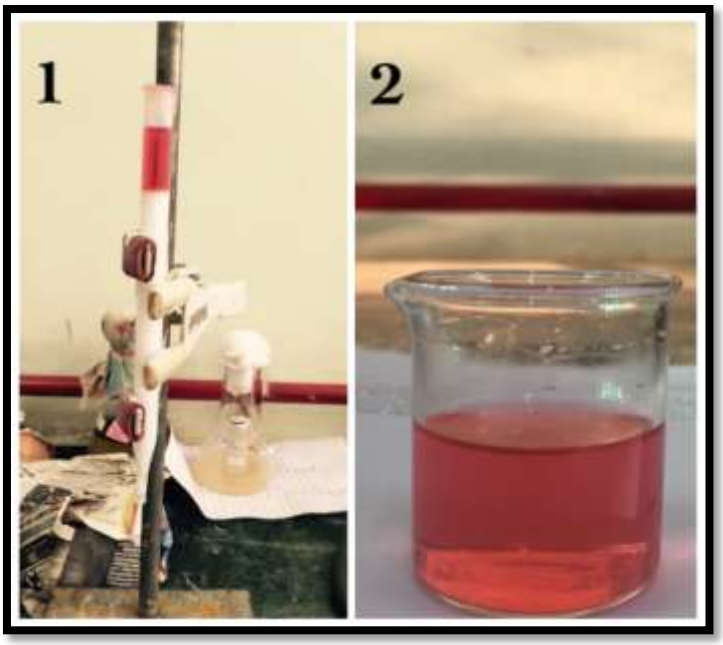


Figure 5.6. Column chromatography – 1. Column chromatography apparatus. 2. The red coloured eluted solution.

5.7. Quantitative And Qualitative Estimation Of Prodigiosin

5.7.1. Paper chromatography

A single band with an Rf value of 0.42 was obtained (Fig. 5.7.1) after paper chromatography with acetone: alcohol (3:7; v/v).

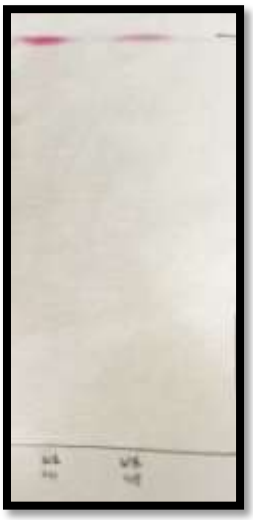


Figure 5.7.1. Paper chromatogram of the pigment isolated from *Serratia marcescens.*

5.7.2. FT-IR

FT-IR absorption in KBr for the red pigment (Fig. 5.7.2.) was dominated by strong bands at 1018.41 cm·1. The spectrum of pure κ- carrageenan showed absorption bands at 1018.41 cm-1 which attributed to C-O stretching. (Namskar,S. *et al*., 2013). These indicate that this pigment's pattern is similar to that of prodigiosin.

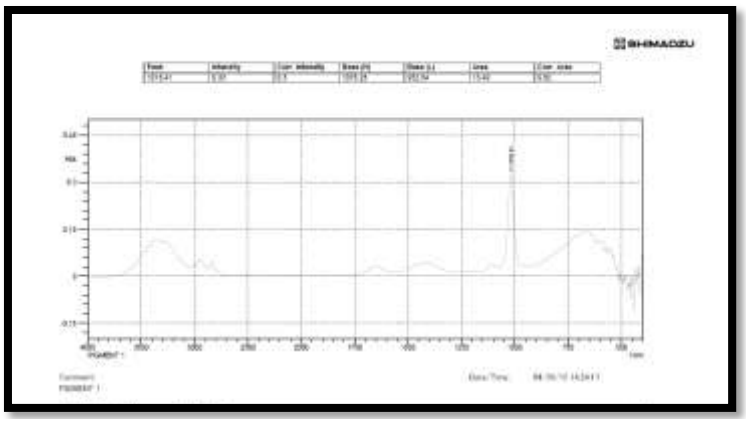


Figure 5.7.2. FT-IR spectrum of the red pigment isolated *Serratia marcenses.*

5.8. Antibacterial and Antifungal Activity of Prodigiosin

The antibacterial activity and antifungal activity of the pigments was tested by Disc Diffusion Method. It was observed that the prodigiosin was able to inhibit majority of the test bacteria. As indicated in Table 5.9, the inhibitory zones for bacteria varied between 10.5±0.47 mm and 18.5 ±0.47 mm, whereas, the inhibitory zones for fungus varied between 15.5 ±0.47 mm and 26.5 ±0.47 mm. *S.typhi* was found to be resistant to the antimicrobial activity of the prodigiosin.

Pathogens	Zone of inhibition in mm
E.coli	15±0.5
S.aureus	18±0.5
S. typhi	Resistant
Strep. coccus	18±0.5
P. aeruginosa	26±0.5
A.niger	22±0.5
C.albicans	16±0.5
P.glaucum	17±0.5
Media control	-

Table 5.8. Antimicrobial activity of prodigiosin

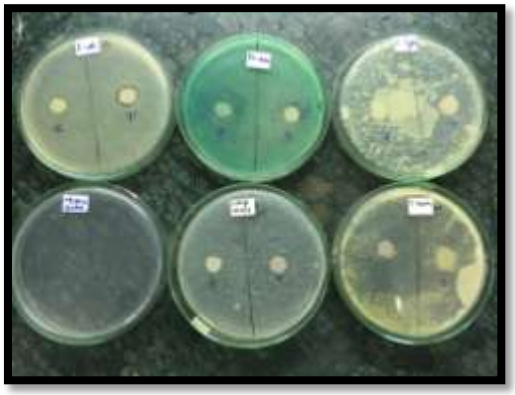


Figure 5.8.1. Antibacterial activity of prodigiosin

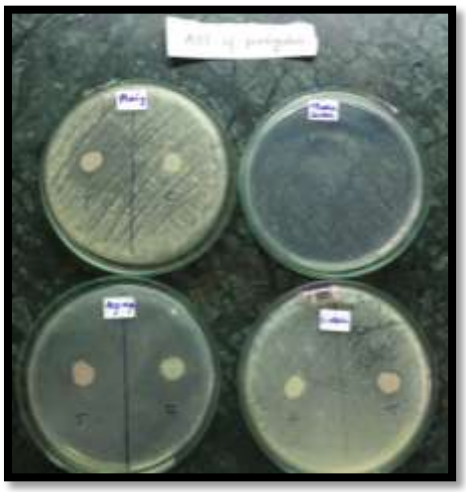


Figure 5.8.2. Antifungal activity of prodigiosin

5.9. Applications

5.9.1. pH indicator

Bacterial pigment in methanol was used as the stock solution; the stock solution was applied on Whatman filter paper no: 2 were cut into 7cm by 1 cm strips and allowed to dry at RT for 15 min. Paper material without pigment was kept as control. After dyeing, acidic (pH 2.0), neutral (pH 7.0) and alkaline (pH 10.0) solutions were spotted over all the paper materials. It was observed that the color of all the prodigiosin dyed paper materials recorded a change from white to red, pink and yellowish orange, respectively with acidic (pH 2.0), neutral (pH 7.0) and alkaline (pH 10.0) solutions strongly suggesting that prodigiosin dyed paper can be used as a pH indicator.

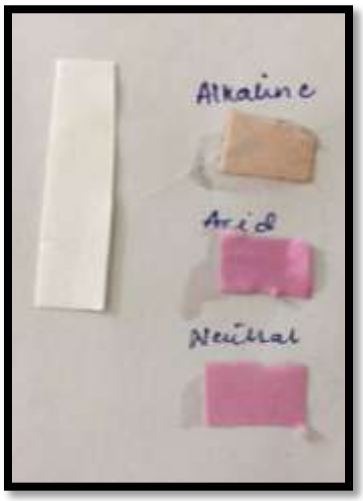


Figure 5.9.1. Prodigiosin as pH indicator.

5.9.2. Candle

Prodigiosin could successfully color the candle. Wax without pigment was kept as a control.

Figure 5.9.2. Candle coloured with prodigiosin on right and control on left.

5.9.3. Textile Materials

Result presented in Fig. 5.9.3. clearly evidence that the pigment produced by *Serratia marcescens* can be effectively used to dye all the textile materials studied.

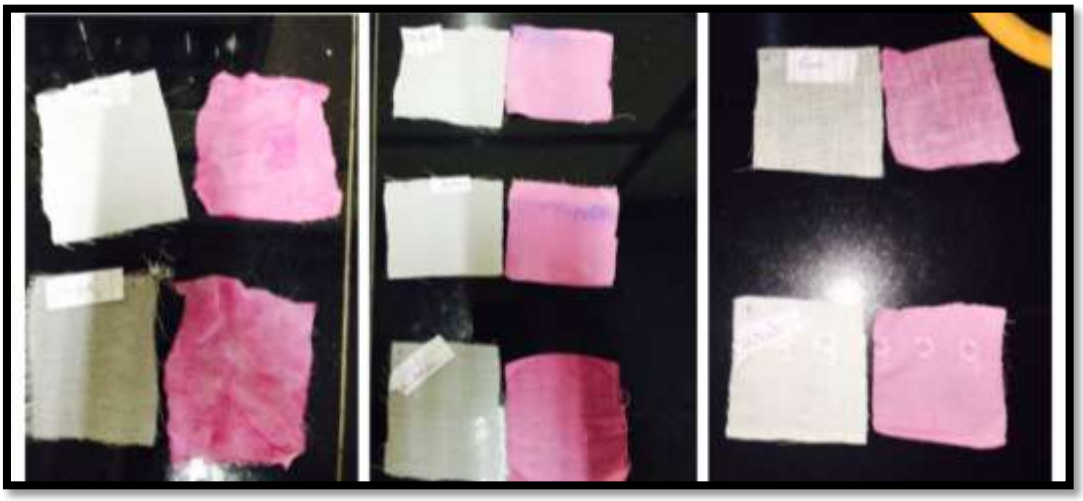


Figure 5.9.3. Cloth materials dyed with prodigiosin pigment

5.9.4. Paper Products

Result presented in Fig. 5.9.4. clearly evidence that the pigment produced by *Serratia marcescens* can be effectively used to paper materials studied.

Figure 5.9.4. Indicates that prodigiosin can effectively dye paper. (left B2B paper and right tinted paper)

5.9.5. Nail Paint

Nail paint was prepared using prodigiosin. Stability testing and microbiological analysis were carried out.

Fig 5.9.5. Nail paint – 1. Control without pigment and 2. Test with pigment

5.9.5.1. Stability testing

Stability test is carried out to ensure that a new or modified product meets the intended physical, chemical and microbiological quality standards as well as functionality and aesthetics when stored under appropriate conditions. Because the development cycle of cosmetic products is relatively short each manufacturer should design their own stability testing program such that it is economically reasonable and efficiently addresses the testing required.

The results of stability testing is tabulated below:

Tests	Procedure	Observation Initial	Inference
Temperature variation	Keep the product at room temperature for 1month and determine its physical characters.	pH - 5.5 colour- pink odour- sweet texture- smooth flow- smooth	Product is stable.
Cycle testing	Keep the product at -20°C for 24hrs and determine its physical characters.	pH - 5.5 colour- pink odour- sweet texture- smooth flow- smooth	Product is stable.
Centrifuge testing	Heat the emulsion at 50°C and centrifuge at 3000rpm for 3 minutes and determine the sign of creaming.	No separate layers of oil droplets formed at the top.	Product is stable.
Light testing	Take 5g of the sample into 2 separate tubes. Cover one tube with aluminium foil completely and label it as control and keep another tube in the window such that having exposure to light observe for discolouration.	The coloured turned peach i.e discolouration was observed.	Product is photosensitive.

Table 5.9.5.1 Indicates results of stability testing of the product (nailpaint made using prodigiosin)

5.9.5.2. Microbiological analysis

Microbiological analysis was carried out to determine the microbial content in the product i.e nail paint produced using prodigiosin. Microbial preservation of cosmetics is important to ensure the microbial safety of cosmetics for the consumer, maintain the quality of the product, and confirm hygienic and high-quality handling. Microbial contamination of cosmetic products may spoil them or seriously reduce the intended quality. Tryptic soy agar plate was used to determine the bacterial count and sabouraud's agar was used to determine the fungal count. The results are tabulated below:

Sets	Dilution	Cfu/plate	Dilution factor	Cfu/ml	Average Cfu/ml
1.	10^{-1} 10^{-2}	19 02	10 10^{2}	190 200	$1.95*10^{2}$
2.	10^{-1} 10^{-2}	07 -	10 10^{2}	70	70

Table 5.9.5.2.1. Indicates the bacterial count obtained from nail paint made using prodigiosin

Sets	Dilution	Cfu/plate	Dilution factor	Cfu/ml	Average Cfu/ml
1.	10^{-1} 10^{-2}	03 -	10 10^{2}	30 -	30
2.	10^{-1} 10^{-2}	- -	10 10^{2}	- -	-

Table 5.9.5.2.2. Indicates the fungal count obtained from nail paint made using prodigiosin

(N)	Arithematic mean (m)	Dilution factor (d)	Volume (ml) (V)	N=m*d/V Cfu/ml
Bacterial count	$1.32*10^{2}$	01	01	$1.32*10^{2}$
Fungal count	30	01	01	30

Table 5.9.5.2.3. Determination of number of micro organisms in the nail paint sample.

VI. Summary And Conclusion

Bacteria isolated from the soil samples of different parts (Deer park trail, Kanheri caves trail and Shilonda trail) of Sanjay Gandhi National Park (SGNP), Borivali was used for the present study. Among the chromogenic bacterial isolates demonstrating different coloured pigments, one isolate was recorded to produce red pigment. This isolate was purified and identified as *Serratia marcescens* .Presumptive test for presence of prodigiosin was carried out and it was found to be positive.

Optimizations of various parameters were carried out. The optimum pH was found to be 7.5 for prodigiosin production. Absorption spectrum of the pigment was dependent on pH value. At pH 7.5, the pigment was red and showed a maximum absorption at 535 nm which is identical to that of prodigiosin hydrochloride. It is therefore concluded that the pigment is a prodigiosin-like pigment. Maximum prodigiosin production was observed at 25°C after 30 h of incubation.

Methanol was found to be an ideal solvent for the maximal extraction of the membrane bound pigment among the different solvents studied using method 3. The pigment recorded maximum absorption at 535 nm.

The pigment was purified by column chromatography using silica gel column. A single red coloured band obtained after column chromatography was eluted. A single band with an *Rf* value of 0.57 was obtained after paper chromatography. FT-IR showed absorption bands at 1018.41 cm-1 which attributed to C-O stretching, confirming the red pigment to be prodigiosin.

Antimicrobial and antifungal potency was determined using disc-agar diffusion technique, it was observed that the prodigiosin was able to inhibit majority of the test bacteria and fungi. *S.typhi* was found to be resistant.

The pigment was evaluated for its application as a pH indicator, candle, textile material, paper products and nail paint. The pigment was take up by the candle and so can be used in making colourful candles. 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 detergent solution. This indicates that a binding agent or mordent is required to prevent the loss of pigment on washing with detergent solution. The pigment was found to successfully dye the paper samples indicating the dyeing potential of the pigment. Also, the pigment can be used in making cosmetics such as nail paint, but the nail paint gave photosensitive test positive indicating that it should be stored in dark. Micro organisms can grow in cosmetic product and may cause spoilage and chemical changes into it which is injurious to the user. Hence, their determination is important. Further research needs to be carried out of use of prodigiosin in cosmetics.

VII. Future Prospects

Recently, prodigiosin has been considered effective as a biological control agent against harmful algae in natural marine environments besides its role in textile dyeing and medicinal uses. Currently pigments of various kinds and forms have been used as additives or supplements in the food industry, cosmetics, pharmaceuticals, and livestock feed and other applications .However, because of the problems of the synthetic pigments that cause toxicity and carcinogenicity in the human body, their use is gradually decreased. Therefore interest in natural pigments that can replace synthetic dyes is increasing. Recently in response to this trend, the tendency to use natural pigments as adding natural materials in the natural dyeing, healthy functional foods, cosmetic products for human health and safety have been gradually expanded.

7.1. Applications as food colorant- The development of foods with an attractive appearance are an important goal in the food industry. Increasingly, food producers are turning to natural food colors, since certain artificial color additives have demonstrated negative health issues following their consumption. Due to the lack of availability of natural food colorants, its demand is much sought especially in the food industry. It is therefore, essential to explore various natural sources of food grade colorants and their potentials. Though many natural colors are available, microbial colorants play a significant role as food coloring agent, because of its production and easy downstreaming process. Bacterial colorants in addition to being environment friendly, can also serve the dual need for visually appealing colors and probiotic health benefits in food products. Microbial colors are in use in the fish industry already, for example to enhance the pink color of farmed salmon.

7.2. Applications in pharmaceutical industry- The genus, Streptomyces or Serratia can produce a red substance of pyrrolylpyromethene skeleton, which is one of following substances: prodigiosin, metacycloprodigiosin, desmethoxy prodi-giosin, and prodigiosin 25-C. These substances have been known to have an antibiotic and antimalarial effect, especially prodigiosin25-C that shows immunosuppressing activity. The useof prodigiosin for treating diabetes mellitus has also been reported by Hwanmook et al. Thus pigments from bacteria offer the wide range of biologically active propertiesand continue to provide promising avenues for applied biomedical research

7.3. Applications in textile industry-. Synthetic dyes are extensively used as a dye in textile, rubber, plastic and paper industries. Nowadays people are concerned about hannful 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 is toxic and cause cnviromnental pollution and hannful to health. The discharge of these waste residues into the enviromncnt eventually poison, damage or affect one or more species in the enviromnent, 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. The textile industry is one amongst the rapidly growing industries worldwide, 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 enviromnent conservation and hence natural pigments have drawn the attention of industry as safe alternative.

7.4. Other aspects - The potential of prodigiosin in coloring, pencil case pouch and as ink in ball pens. Also in making soap and antiseptic wash as it has antimicrobial and antifungal potential. Thus there is an urgent need for alternative colorants that are natural, cost effective and easily degradable and without production of recalcitrant intermediates when they enter the ecosystem. There is an increasing interest involving microorganisms as a possible alternate source of colorants used in food industry. In this direction, microbiology may play a crucial role for large fermentation of natural biocolorants.

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9. Appendix

9.1. Box-behnen design composition

Constituents	Quantity(gm/l)
Sucrose	1%
Peptone	0.4%
Yeast extract	0.1%
Sodium chloride	2.0
Disodium phosphate	9
Potassium chloride	0.41
Sodium monohydrogen carbonate	0.4
Potassium bromide	0.1
Agar Agar	2.5
Distilled Water	100 ml

9.2. Glucose phosphate broth composition

Constituents	Quantity (g/l)
Glucose	5
Potassium hydrogen phosphate	5
Peptone	5
Distilled water	1000ml
pH	7.4

9.3. Indole test composition

Constituents	Quantity (g/l)
Tryptone	20
Sodium chloride	5
Distilled water	1000ml

9.4. Lysine decarboxylase composition

Constituents	Quantity (g/l)
Lysine	10
Peptone	5
Meat extract	5
Glucose	0.5
1% Bromo cresol purple (1:500 diluted solution)	5ml
1% Cresol red (1:500 diluted solution)	2.5ml
Distilled water	1000ml

9.5. Mac Conkey's agar composition

Constituents	Quantity (g/l)
Peptone	3
Gelatin	17
Lactose	10
Sodium chloride	1.5
Crystal violet	0.001
Neutral red	0.030
Agar	14
Distilled water	1000ml
pH	7.1±0.2

9.6. MM composition

Constituents	Quantity (g/l)
Mannitol	20
Peptone	5
Yeast extract	2
Distilled water	1000ml
pH	7.4

9.7. Motility test composition

Constituents	Quantity (g/l)
Peptone	5
Agar	10
Distilled water	1000ml

9.8. Nitrate reduction test composition

Constituents	Quantity (g/l)
Sodium nitrate	0.3
Peptone	5
Distilled water	1000ml

9.9. Nutrient agar composition

Constituents	Quantity (g/l)
Peptone	10
Sodium chloride	5
Meat extract	3
pH	7.4
Agar	15
Distilled water	1000ml

9.10. Peptone glycerol broth composition

Constituents	Quantity (g/l)
Meat extract	1
Peptone	1
Glycerol	1%

Preparation of media: Add 100ml distilled water and 2.5g agar agar.

9.11 Sabourauds agar composition

Constituents	Quantity (g/l)
Dextrose	40
Peptone	10
Agar	15
Distilled water	1000ml
pH	5.6±0.2

9.12 Simmon's citrate broth composition

Constituents	Quantity (g/l)
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15
pH	6.8±0.2

9.13. Tryptic soy broth composition

Constituents	Quantity (g/l)
Tryptone	17
Soyatone	3
Dextrose	2.5
Sodium chloride	5.0
Dipotassium monohydrogen phosphate	2.5
Agar agar	15
pH	7.2
Distilled water	1000ml

9.14. VRBA composition

Constituents	Quantity (g/l)
Peptone	7
Yeast extract	3
Sodium chloride	5
Bile salts mixture	1.5
Lactose	10
Neutral red	0.030
Crystal violet	0.002
Agar	15
Distilled water	1000ml
pH	7.4±0.2

9.15. YEMEB composition

Constituent	Quantity(gm/l)
Peptone	5
Yeast Extract	3
Malt Extract	3
Dextrose	10
pH	6.2
Agar Agar	2.5
Distilled water	100ml