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Extraction of pink pigment (Prodigiosin) from *Serratia marcescens* MTCC 10774 bacteria and their application in Industries

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Abstract

A pigment producing bacterium from coir retting effluent was isolated and it was identified as *Serratia marcescens MTCC 10774*. The pigment production of the bacterium was tested under different culture conditions. The bacteria were isolated in King B agar media. The pigment production and growth were also observed in liquid culture of King B medium. Pigment production was found maximum at 25°C, neutral pH and 0.25% sodium chloride. Compared to the wild strain, pigment production was reduced in UV and ethidium bromide treated mutants. Pink pigment prodigiosin from the bacteria was more soluble in ethanol than other solvents verified by this study. The pigment was not toxic to higher organisms especially aquatic species. Dyeing was observed at its maximum when the fabrics and polypropylene plastic were immersed in dye bath at 160°C. The dyeing efficiency was high in polyester. The fading of fabrics against washing with detergents and rubbing was found to be low. The maximum colour fastness against sunlight was observed in cotton. Among the adjuvant used, phosphoric acid was found to suppress fading by sunlight. It was found that pre-treatment with thiourea solution considerably reduced the fading of the pink colour due to sunlight. Moreover, since mass culture of the bacteria is possible, the dyeing pigment can be produced cheaply.

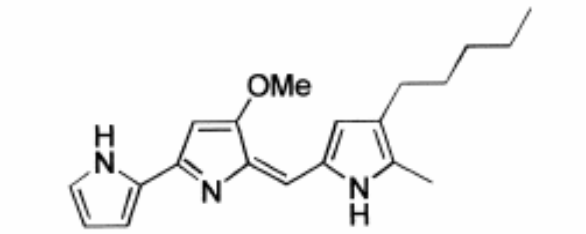
Keywords: Pigment, prodigiosin, dyeing, *Serratia marcescens*, King B etc.

INTRODUCTION

Colour has been the source of attraction to man and animals alike. Colour also represents the cultural, geographical, sociological and economical status among the population. The word ‘pigment’ is Latin in origin (pigmentum) and originally denoted a colour in the sense of a colouring matter, but was later extended to indicate coloured decoration (e.g. makeup). Pigments can be characterized by their chemical composition, and by their optical properties. Most dyes, except natural dyes are highly poisonous in nature. The following properties of the pigments are important to determine the pigment quality. General chemical and physical properties: chemical composition, moisture and salt content, content of water-soluble and acid-soluble matter, particle size, density and hardness. Stability properties: resistance towards light, weather, heat and chemicals, anti-corrosive properties, retention of gloss. Behaviour in binders: interaction with the binders, compatibility, and solidifying effect.

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Natural dyes can exhibit better biodegradability and generally have a higher compatibility with the environment. Recently, the potentiality of using natural dyes in textile colouration as anti-UV and anti-microbial has been reported. Man has been using chemicals or synthetic colourants in foodstuff, cosmetic, textile and pharmaceutical manufacturing processes which are having side effects, searching for alternative pigment sources has led to natural sources like microbes and plants which can yield pigments (Kang *et al*.,1996). These natural pigments do not have side effects. There is a great demand for microbial pigments due to their natural characters, safe to use, medicinal properties and easy production (Carels and Shephard, 1997). Microorganisms would therefore seems to offer great potential for the direct production of novel textile dyes or dye intermediates by controlled fermentation techniques replacing chemical synthesis which has inherent waste disposal problems (e.g. toxic heavy metal compounds) (Hamlyn, 1995). At present the textile industry virtually exclusively uses synthetic dyes which are based on petrochemical production. However, natural pigments are still valuable because of their natural colour tones (Shirata *et al*., 1997). Natural dyes can be used for majority types of fibre, but the success in terms of fastness and clarity of colour differs significantly. Industries are continuously looking for cheaper, more environment friendly alternatives to existing dyes. Therefore, it is advantageous to produce natural pigments from microorganisms. There are a number of microorganisms which have the ability to produce pigments in high yields, including species of *Monascus* (Yongsmith *et al*., 1994; Hajjaj *et al*., 2000), *Serratia* (Williams *et al*., 1971) and *Streptomyces*(Oshima *et al*., 1981; Ryu *et al*., 1989). Members of the bacterial species, *Serratiamarcescens*, are characterized by the production of red or pink pigments. These pigments are described as being insoluble, or sparingly soluble in water, and to be associated with the bacterial cell (Williams *et al.*,1958). Prodigiosin (5-[3-methoxy-5-pyrrol-2- ylidenepyrrol- 2- ylidene)-2-methyl-3-pentyl-1H pyrrol) is a red pigment isolated from a few species such as *Serratia,Pseudomonas, Streptomyces.* The prodigiosin group of natural products is a family of tripyrrole red pigments that contains a common 4-methoxy, 2-2 bipyrrole ring system.



It is thought to have potential for antibacterial, antimalarial, anticancer, cytotoxic and immunosuppressive activities (Song *etal.*, 2006). Alihosseini *et al.* (2008) characterized the bright red pigment prodigiosin from *Vibrio* sp. And suggested that it could be used to dye many fibers including wool, nylon, acrylics and silk. The synthetic dye molecule which is released to the aquatic fields from

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the dyeing industry causing some serious problems to aquatic flora and fauna. But in this study the natural dye from the bacteria, *Serratia marcescens* did not cause any problems to the aquatic animals.

MATERIALS AND METHODS

Sample collection

Water samples which are mixed with sea water were collected from the coir pith effluent canal, Pannaiyoor, Kanyakumari district, Tamil Nadu. Samples were brought to the laboratory for further studies.

Screening and isolation of pigment producing bacterium

The serially diluted sample was used for the isolation of the pigment producing bacteria. 0.1 ml of the sample was spread plated on nutrient and King B agar media. After 24 hours of incubation at 37°C, only the pigment producing bacterial colonies were taken for pure culturing. Fresh bacteria that had been cultured for one day were used for identification.

Identification of the bacteria

The identification of the bacteria was done using morphological, biochemical and sequence analysis.

Phylogenetic analysis

The phylogenetic study was carried out in MTCC (Microbial Type Culture Collection). The 16s rRNA sequences were analysed using this study.

Pigment production by the isolated bacterium under different culture conditions

Pink colour pigment production was verified against different media and culture conditions.

Culture medium

Pigment production was verified using nutrient agar and King B agar and broth.

Nutrient agar medium: 5g of peptone, 3g of beef extract 5g of sodium chloride, 15g of agar and 1000ml of distilled water.

King B agar medium: 20g of proteoe peptone No:3, 1.5g of K2HPO4, 1.5g of MgSO4.7H2O,10ml of glycerol, 15g of agar and 1000 ml of distilled water.

Nutrient broth medium: 5g of peptone, 3g of beef extract, 5g of sodium chloride and 1000ml of distilled water.

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King B broth medium: 20g of proteoe peptone No:3, 1.5g of K2HPO4, 1.5g of MgSO4.7H2O, 10ml glycerol and 1000ml of distilled water.

Culture temperature

The bacterium was cultured at temperatures of 5^{o}C, 15^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C, 50^{o}C and 55^{o}C to verify the pigment production.

Culture pH

The bacterium was cultured in different pH 3 to 10 for verifying high pigment production.

Mutation

The bacterial culture was subjected to both physical and chemical mutation. The physical mutation was carried out by exposure to UV rays at different length of time, from 30 seconds to 5 minutes. Chemical mutation was carried out using different strength of ethidium bromide, from 0.1 to 1%.

Effect of Sodium chloride

Sodium chloride is one of the component of the nutrient agar medium. Pigment production was tested by adding sodium chloride in various concentrations to the liquid media, from 0.25 to 4%.

Antibiotic sensitivity test

The antibiotic discs of streptomycin, chloramphenicol, penicillin, ampicillin, rifampicin, ciprofloxacin were used against the bacteria.

Extraction of pigments

The bacterium was inoculated into the King B broth medium and cultured for one week. The bacterial culture that had become dark pink was centrifuged at 10000rpm for 15 minutes. The pellet was homogenized with various solvents like ethanol, methanol, N and iso butanol, diethyl ether, hexane, propanol, phenol and water.

Toxicity test

The toxicity of the pink colour pigment was verified using selected freshwater fish *Chanos chanos* (milk fish). Fish was reared with various concentrations of bacterial pigment. The toxicity was determined by estimation of fish's whole body protein, carbohydrate and lipid content in control and pigment treated fishes. Estimation of the protein, carbohydrateand lipid by Lowry *et al*., (1951), Roe, (1955) and Folch *et al*., (1957) methods respectively.

Dyeing

Wool, silk, nylon, cotton and polyester fabrics were dyed by boiling with the bacterial liquid culture (broth) from 120 to 160^{o}C.

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Colour fastness

The colour fastness of the dyed fabrics was verified by dipping the fabrics in commercial detergent solutions for 48 hours and by rubbing. It was also determined by exposure to sunlight at different time scale i.e., from 1 to 8 days.

Improvement of colour fastness

To improve the colour fastness, the fabrics were boiled with adjuvant like sodium chloride, sodium sulphate and phosphoric acid in bacterial culture and also post treated with thiourea solution, that is, the dyed material was then immersed in a saturated thiourea solution for about 1 minute at room temperature. It was then drained and air-dried, washed with distilled water for 2 minutes, dipped in saturated thiourea solution for 1 minute at room temperature, drained and air- dried. The fading of colour under light was suppressed even more with repeated thiourea treatment.

Dyeability in plastics

The poly propylene tubes were filled with liquid culture of pigmented bacteria and the tubes boiled at 160ºC.

RESULTS

Identification study

Several pink coloured bacterial colonies were observed on the King B and nutrient agar media after 24 hrs of incubation. These cultures were subjected to pure culturing on the same media from which they were collected. Pink colour colonies of various shades were observed. The bacterial culture was also maintained in broth media. The bacteriological and sequential characteristics of strain were agreed with the reported properties of *Serratia marcescens*.

Sequence analysis

The 16s rRNA sequence of *Serratia marcescens* MTCC 10774 is in the following form

1 cgagcggtag cacaggggag cttgctccct gggtgacgag cggcggaggg agcgtaatgt
61 ctgggaaact gcctgatgga gggggataac tactggaaac ggtagctaat accgcataac
121 gtcgcaagac caaagagggg gaccttcggg cctcttgcca tcagatgtgc ccagatggga
181 ttagctagta ggtggggtaa tggctcacct aggcgacgat ccctagctgg tctgagagga
241 tgaccagcca cactggaact gagacacggt ccagactcct acgggaggca gcagtgggga
301 atattgcaca atgggcgcaa gcctgatgca gccatgccgc gtgtgtgaag aaggccttcg
361 ggttgtaaag cactttcagc gaggaggtag gtggtgagct taatacgctc atcaattgac
421 gttactcgca gaagaagcac cggctaactc cgtgccagca gccgcggtaa tacggagggt
481 gcaagcgtta atcggaatta ctgggcgtaa agcgcacgca ggcggtttgt taagtcagat
541 gtgaaatccc cgggctcaac ctgggaactg catttgaaac tggcaagcta gagtctcgta

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601 gaggggggta gaattccagg tgtagcggtg aaatgcgtag agatctggag gaataccggt
661 ggcgaaggcg gccccctgga cgaagactga cgctcaggtg cgaaagcgtg gggagcaaac
721 aggattagat accctggtag tccacgctgt aaacgatgtc gatttggagg ttgtgcccttt
781 gaggcgtggc ttccggagct aacgcgttaa atcgaccgcc tggggagtac ggccgcaagg
841 ttaaaactca aatgaattga cgggggcccg cacaagcggt ggagcatgtg gtttaattcg
901 atgcaacgcg aagaacctta cctactcttg acatccagag aactttccag agatggattg
961 gtgccttcgg gaactctgag acaggtgctg catggctgtc gtcagctcgt gttgtgaaat
1021 gttgggttaa gtcccgcaac gagcgcaacc cttatccttt gttgccagcg gttcggccgg
1081 gaactcaaag gagactgcca gtgataaact ggaggaaggt ggggatgacg tcaagtcatc
1141 atggcccttа cgagtagggc tacacacgtg ctacaatggc gtatacaaag agaagcgacc
1201 tcgcgagagc aagcggacct catatagttt gtttttagtc cggattggag tctgcaactc
1261 gactccatga agtcggaatc gctagtaatc gtagatcaga atgctacggt gaatacgttc
1321 ccgggccttg tacacaccgc ccgtcacacc atgggagtgg gttgcaaaag aagtaggtag
1381 cttaaccttc gggagggcgc ttaccacttt gtgattcatg actggggtga agtc //

Culture analysis

The production of the pink pigment differed considerably depending on the medium used. Pigment production was higher in King B agar than nutrient agar medium (Fig 1). When shake flask culture in a liquid nutrient and King B medium were compared with solid agar culture, pigment production was found better in liquid culture. Extraction of the pigment was also easier from the bacterium in liquid medium. The same results were obtained in this study also. The bacterium grew well in the range of 15 - 40^{o}C, with optimum growth and maximum pigment production occurred at 25oC for 48 hours of incubation. Above and below this temperature pigment production became very low. Both the bacterial growth and pigment production were better at pH 7.

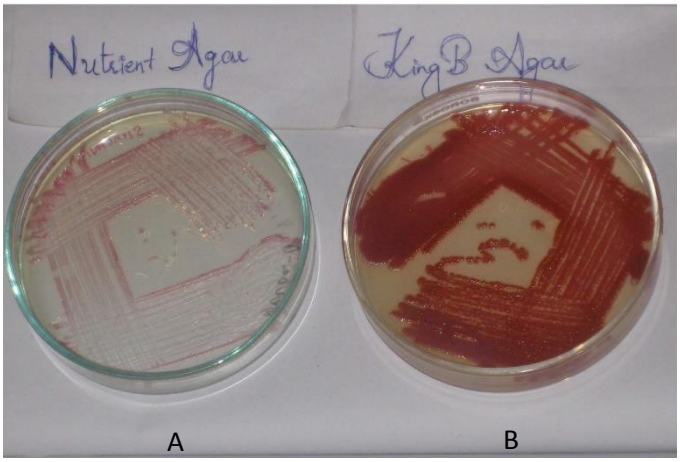


Fig.1. Isolated Bacterial culture on A. Nutrient agar B. King B Agar Media

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There was no pigment production at below pH 5 and above 10.5. Sole *et al*., (2003) revealed that at pH 7, pigment production in *Serratia marcescens* increased in media with repressing carbon sources. Wild bacterial colonies grew well and produced higher amounts of pigment than the physically and chemically mutated colonies. Maximum pigment production was observed at concentration of 0.25% sodium chloride. The antibiotics like ampicillin and penicillium showed resistance whereas the streptomycin, ciprofloxacin and chloramphenicol showed good sensitive results in Muller Hinton agar media.

Pigment

The pigment produced by *Serratia marcescens* MTCC 10774 is prodigiosin.

Pigment extraction

Ethanol was found to be the most efficient solvent for extracting the pigment followed by methanol, acetone, ether and water. No pigment extraction in hexane, propanol and phenol.

Toxicity test

When the effect of the pigment on cultured milk fish (*Chanos chanos*) was studied, no adverse effect was detected. The protein, carbohydrate and lipid content of the fish were not much varied between the control and pigment treated fish as shown in Table 1. It was thus concluded that the pigment was not toxic to aquatic organisms.

Table 1: Toxicity Test Results

Biomolecules (tested in milk fish)	Control	Sample
Protein (mg/g)	5.326	5.581
Carbohydrate (mg/g)	2.970	2.664
Lipid (mg/g)	0.072	0.069

Dyeing analysis

Various pink shades of the fabrics were obtained by immersing the fabrics in boiled dye bath containing the pigment. The shade of the dyed fabric varied depending on the amount of pigment or the dye present in the broth. It was found that, the pigment was capable of dyeing not only natural fibres but also synthetic fibres. Dyeing performance on five different fibres was compared. The dyeing performance differed depending on the type of fibres. Maximum dyeing efficiency was observed in wool followed by silk, polyester, nylon and cotton.

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Among the various temperatures tested, deep colour was developed on the fibre heated at 160^{o}C. The colour fastness against sunlight was maximum in polyester followed by nylon, silk, wool and cotton. It was very low in cotton material. No observable change in the colour fastness against washing with detergents and rubbing. Among various adjuvants used, phosphoric acid was found to highly improve the colour fastness of the fabrics against sunlight. It was also improved by repeated post-treatment of fabrics with thiourea solution (Fig.2).

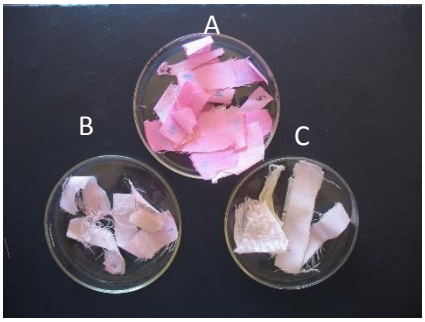


Fig. 2. Colour fastness against sunlight A. Control (Before exposure to sun light) B. Treated with adjuvants and thiourea after exposure C. Untreated fabrics after exposure

Like the fabric, materials like poly propylene plastic materials also got good colouration after boiling with the pigment (Fig.3).



Fig. 3. Dyeing ability of the pigments in polypropylene plastic.

DISCUSSION

The regular liquid media currently being used for prodigiosin biosynthesis are nutrient broth (0.52 mgml^{-1}) (Pryce and Terry, 2000), peptone glycerol broth (0.302 mg ml^{-1}) (Hiroaki *et al*., 1996).

Shirata *et al*., (1997) isolated a blue-purple pigment called violacein producing bacteria *Janthinobacterium lividum* (Eisenberg) on King B agar medium. The King B agar medium is rich in peptone or amino acid than nutrient medium. So it may lead to good pigment production. For industrial applications of microbial pigments, higher production of pigment yield, chemical and light

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stability are essential features. These characteristics obtained in submerged cultivation because of high aeration and agitation (Gunasekaran and Poorniammal, 2008).

In this study the pigment production occurred maximum at lower temperature (8 to 25^{o} C. Below and above this temperature did not produce good pigmentation. Innis and Mayfield (2005) suggested that prevention of synthesis of a violet pigment violacein at 0^{o}C. At this temperature condition, the carbon sources not acted as growth sources for violacein. Some of the carbon sources actually inhibited pigment production at more than 25^{o}C, preventing violacein synthesis in the presence of pyruvate which was shown to allow pigmentation. So from this we can confirm that the pigmentation require some moderate temperature for their high productivity. The lower and higher temperature will prevent the production of pigment.

De Ley (1964) suggested that DNA of bacteria drastically changed by spontaneous and induced mutations. Melting points of pure DNA are taken as a criterion to indicate possible changes. DNA from dark blue *Chromobacteria* indicated very small over all change in its base composition that leads to lower production of pigments.

It was proved that *Serratia marcescens* produce prodigiosin pigment. It was analysed in many works (William *et al*., 1958, Venil and Lakshmanaperumalsamy, 2009). So in this study also it was confirmed that the pigment produced by this bacteria (*Serratia marcescens*) is surely prodigiosin. Shirata (1999) suggested that the blue-purple pigment violacein from *Janthinobacterium lividum* can be extracted efficiently with ethanol than other solvents like our bacterium. The prodigiosin pigment from *Serratiamarcescens* was extracted clearly by using ethanol.

Nystrom *et al*. (1999) conducted a toxicity test of colourants for a large number of aquatic organisms. In this study the toxicity test was conducted in the fresh water fish such as *Chanos chanos* (milk fish).

Kato (1998) suggested that the colour fastness against sun light was increased by post treatment of fabrics with thiourea solution. Here also the experiment conducted in this procedure. Gamma radiation also used to improve the dyeing characteristics from fair to good (Bhatti *et al*., 2010).

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