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Sustainable Synthesis and Characterization of Extracellular Bio-colorants from *Metapseudomonas lalkuanensis*

Gawande S.N.*, Sharma S.M.

Department of Microbiology, Shri R.L.T College of Science, Akola, Maharashtra, India,444001

Affiliated to Sant Gadgebaba Amaravati University, Amaravati

*Corresponding author: Gawande S.N. (Email: sonalingawande@gmail.com)

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Abstract

The escalating environmental and health risks associated with recalcitrant synthetic dyes have catalysed a shift toward sustainable, biodegradable alternative. This study explores the potential of indigenous chromogenic bacteria as biological factories for natural pigment production. Through a systematic screening of soil and sewage samples from diverse ecological niches in Akola, 14 robust pigment-producing bacterial strains were isolated.

Critical growth parameters were optimized to enhance metabolic yield, identifying a temperature of 37°C, a pH of 7.5, and a 0.5% NaCl concentration over a 48-hour incubation period as the physiological optima for maximum pigmentation. Four distinct isolates—GS3B (Orange), SS4A (Yellow), WS2 (Green), and SS3A (Pink)—were selected for detailed characterization. Molecular identification via 16S rRNA gene sequencing confirmed isolate WS2 as *Metapseudomonas lalkuanensis*.

Notably, while most strains produced intracellular pigments, *M. lalkuanensis* demonstrated high-efficiency extracellular secretion. The green pigment from this strain was recovered via solvent extraction and successfully validated as a viable bio-dye for textile applications. These results underscore the feasibility of utilizing local microbial biodiversity for the scalable production of eco-friendly colorants, offering a promising path toward greener industrial dyeing processes.

1. INTRODUCTION

Colors are the most pleasing and first parameter to be noticed about any article by the receptor. Artificial or synthetic colors mostly used by the food processing and cosmetic industries are reliable and economical as compared to the natural colorants which are expensive, less stable, and possess lower intensity (Tuli et al.,2014).

Natural pigments are sourced from ores, insects, plants, animals and as well as from microbes. Microorganism yield secondary metabolites including bacterial pigments (Abdelaziz et.al.,2023). Bacteria that produce pigment are called as Chromogenic bacteria. The pigments production in bacteria is important for protection against ultraviolet radiation,

oxidation stress, extreme temperature and desiccation. Pigments possess anticancer activity, contain pro-vitamin A and have some desirable properties (Sinha et al., 2017).

The industry is interested in natural pigments since they are safer and more biodegradable than synthetic pigments for human use (Abdelaziz et.al.,2023). Production of natural pigments from microbial sources is gaining much importance due to faster recovery, greater intensity of color, higher yield of pigment, and cheaper production methods. Microorganisms such as bacteria, yeast and molds produce several kinds of natural pigments depending on their sources of origin. Pigments such as Quinones, Carotenoids, Violacein, Indigo and Melanin are produced from microbes (Barreto et al.,2023).

Microorganisms are the most important tools in biotechnology to produce variety of molecules like enzymes, antibiotics and pigments. Microorganisms are a promising source for natural colours. The presence of pigments has been reported in entire microbial world including bacteria, fungi, yeast, algae and protozoa. Industrial production of natural pigment by microbial fermentation has several advantages such as cheaper production, easier extraction and higher yields throughout the year. In the food industry they are used as colouring agent and antioxidants. Pigments are present in various colours

Production of intracellular or extracellular pigments is dependent on certain factors like light, pH, temperature and content of medium. Extracellular pigments released in medium while intracellular pigments remain in cell needs additional method of cell lysis for extraction of pigments (Siddiqa et al.,2024).

The microorganism which have ability to produce pigment includes *Monascu, Paecilomyces, Serritia, Cordycepe, Streptomyces, Sarcina, Cryptococcus, Phaffia spp. etc* (Munnefa and Thirumalai,2021). Malik et al., (2012) in their studies culture various pigment producing bacteria like *Serratia marcescens* produce prodigiosin, *Streptomyces coelicolor* produce actinorhodin along with prodigiosin, similarly *Chromobacterium* produce violacien and *Thalkalivibrio versutus* produce natochrome and chloronatochrome which found uniqueness to importance to many industries.

The present study is focused on the isolation and characterization of pigment-producing bacteria from sewage and soil samples collected across various sites in Akola.

2. MATERIALS AND METHODS

2.1 Collection of sample and isolation of pigment producing bacteria:

Total 20 water and sludge samples were collected from different area of Akola city (Sahakar nagar well, MIDC sewage, Gourakshan road side stagnant water). The samples were diluted using ten-fold dilution, where 1ml of sample is the transferred to 9 ml of diluent. After the first tube, each tube is the dilution of the previous dilution tube. The samples were diluted using ten-fold dilution, where 1ml of sample is the transferred to 9 ml of diluent. After the first tube, each tube is the dilution of the previous dilution tube.

2.2Primary screening of pigment producing bacteria:

After incubation for 24-48 hours, bacterial isolates were identified as describe in Bergey's Manual of systematic bacteriology which involves colony characterization, cell morphology and biochemical characterization which includes Indole test (I), methyl red (MR), Voges Proskauer (VP), Simmon's citrate (C) test along and sugar fermentation (Glucose, lactose, mannitol). Nutrient agar plates showing different colored colonies were selected for further sub culturing. These bacterial colonies with color (pink, green, yellow, orange and orange-yellow) were selected for pure culture isolation (Jenitha 2012).

2.3 Premeditated influence of the growth parameters for maximum pigment production (Muneefa and Thirumalai 2021):

1. Effect of Incubation time on pigment production

Different incubation times (24, 48, 72, 96 hours) were employed to study optimum incubation period for the bacterial growth.

2. Effect of temperature on pigment production

Tubes of Nutrient broth with cellulose as the sole source of carbon inoculated with the bacterial cultures and then kept for incubation at different temperatures (30°C and 37°C) in incubator to study the growth at different temperatures

3. Effect of pH on pigment production

The plates were incubated at 37°C in incubator. The bacterial cultures were subjected to incubation at different pH (6, 7, & 8) using phosphate buffer systems.

4. Effect of NaCl concentration on pigment production

The bacterial cultures were subjected to incubation without and with (0.5%) salt concentration respectively.

5. Effect of glycerol on pigment production

To identify whether the bacteria produces more pigment or not, the broths with bacterial culture were incubated with and without glycerol.

2.4 Screening of the pigment producing bacteria and Extraction of pigments:

The isolated colonies of identified pigmented cultures were inoculated in to 100ml sterile Nutrient broth in conical flask. Conical flasks were incubated for 24 to 48 hrs. The extensive growth of pigment producing bacteria was seen in the flasks after 48 hrs of incubation. For more pigment production, they were incubated for 5 to 6 days.

After incubation pigment producing bacteria was extracted by centrifugation method. The pigmented broths were centrifuged at 6000rpm for 15 mins. After centrifugation the supernatants were collected and mixed with equal amount of methanol solvent and filtered through Whattman filter paper no. 1(Sinha et al., 2017).

2.5 Application of extracted pigment to cotton fabric

A pre washed cotton cloth was immersed in 0.5% Nacl solution for 10 mins for fixation. After fixation the cloth was placed on the extracted pigment to absorbing the color. The cloth was kept for 4-5 days for (Muneefa and Thirumalai 2021).

3. RESULTS AND DISCUSSIONS

From 20 samples, 13 pigment producing isolates were screened. Primarily isolates were selected on the basis of pigment producing colonies on the Nutrient agar plates. All these isolates were further purified and sub-cultured for microscopic and biochemical characterization from which, 9 isolates were further tested for physicochemical parameters.

Figure 3.1: Isolation of pigment producing bacteria for dilution method

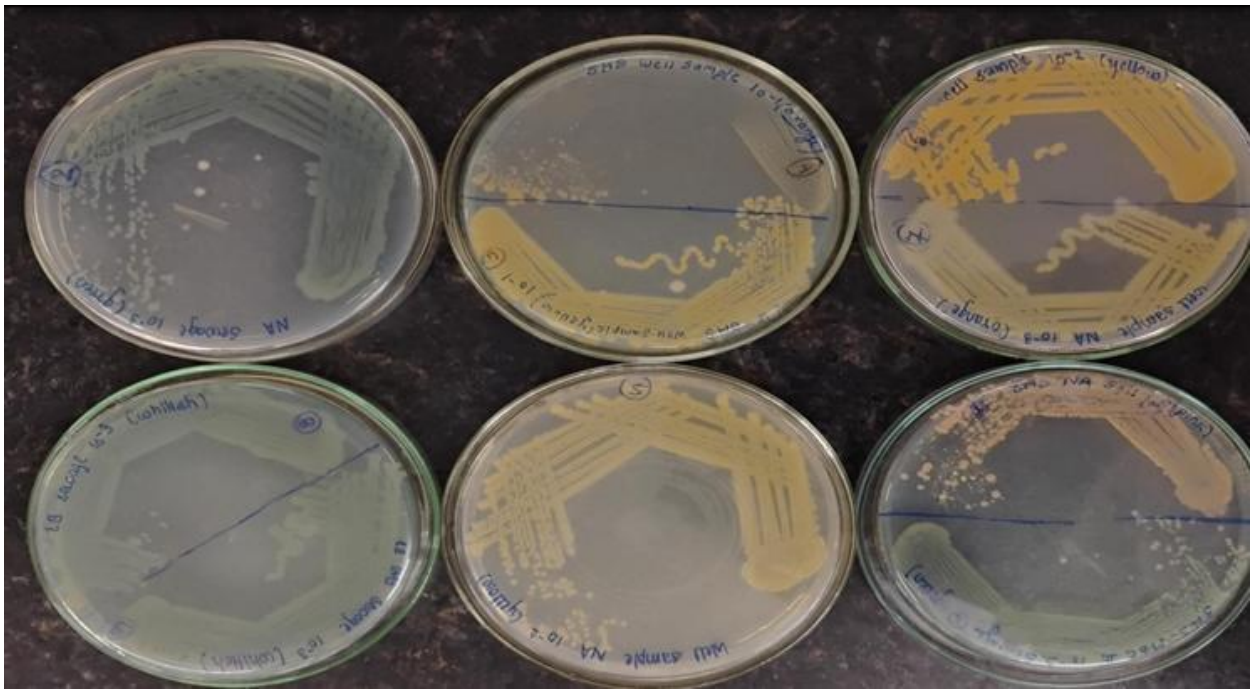


Figure 3.2: Primary screening of different pigment producing bacteria

Identification of pigmented bacteria was carried out, which was based on morphological and biochemical tests.

3.1. Morphological characterization

Morphological study was carried out on the basis of size, shape, color and Gram staining as shown in figure 3.3 and tabulated in table 3.1.

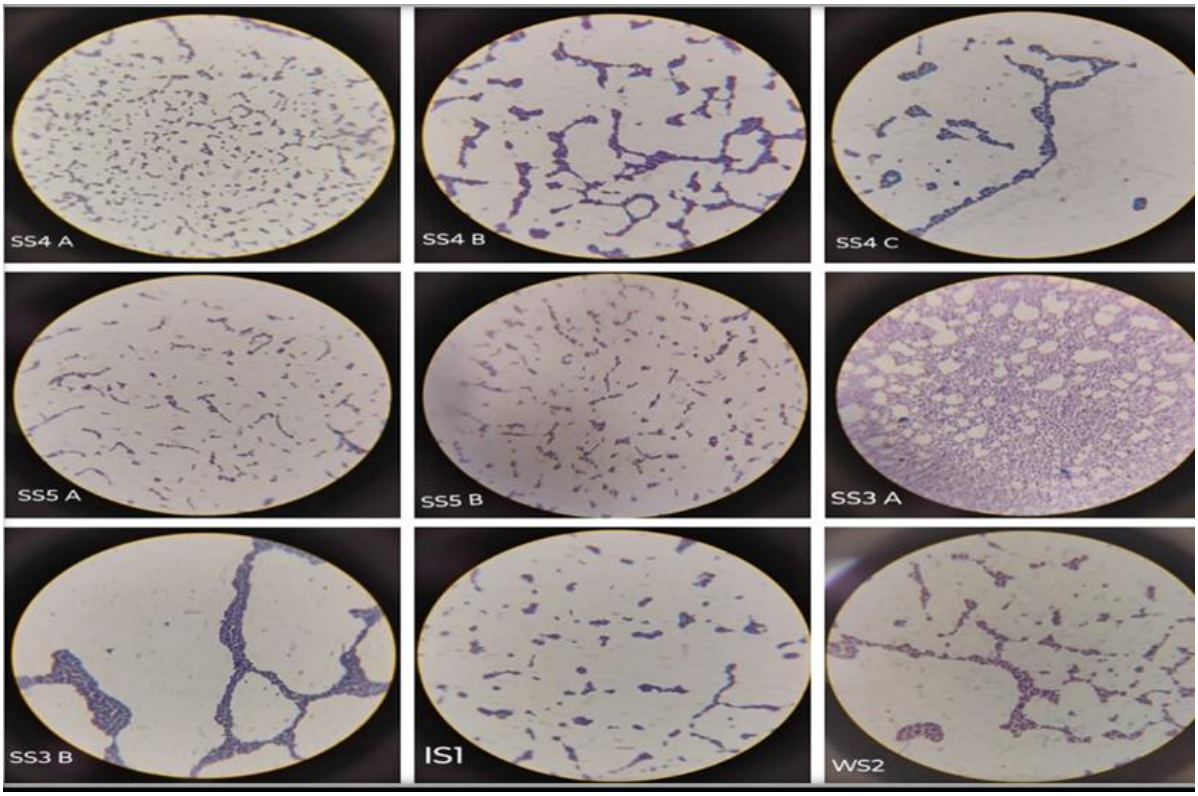


Figure 3.3: Microscopic examination of different pigment producing isolates

3.2. Biochemical characterization

There was no visible change in the solution. Only SS4A showed changes after incubation which indicated that this isolate was indole positive. In MR test the color of the isolates was changed of WS1, WS3 B, GS3B, and SS4 C. So, it indicated that these isolates were MR positive. There were visible changes in the solution. WS3 A, GS3 A, SS4 C changed after incubation which indicated that the isolates were VP positive. Isolates like IS1, WS2, and SS3 A had shown citrate utilization. Carbohydrate utilization tests were observed as isolates like WS2, WS3 A, WS3 B, SS4 A, and SS4 B produced only acid in glucose solution as tabulated in table 3.1.

Table 3.1: Cultural, Morphological and Biochemical characteristic of isolates

Sr. no.	Isolate	Color of pigment	Gram character	Morphology	Biochemical tests									
					Glucose		Lactose		Mannitol		I	MR	VP	C
					acid	gas	acid	gas	acid	gas				
1	WS1	orange yellow	gram positive	short rod	-	-	-	-	-	-	-	+	-	-
2	SS4 A	yellow	gram positive	short rod	+	-	-	-	-	-	+	-	-	-
3	WS3 A	orange	gram negative	short rod	+	-	-	-	-	-	-	-	+	-
4	WS3 B	orange yellow	gram positive	short rod	+	-	-	-	-	-	-	+	-	-
5	IS1	yellow	gram positive	short rod	-	-	-	-	-	-	-	-	-	+
6	GS3 A	green	gram negative	short rod	-	-	-	-	-	-	-	-	+	-
7	WS2	green	gram positive	short rod	-	-	-	-	-	-	-	-	-	+
8	SS3 A	pink	gram negative	short rod	-	-	-	-	-	-	-	-	-	+
9	SS3 B	orange yellow	gram positive	cocci	-	-	-	-	-	-	-	-	-	-
10	GS3 B	orange	gram positive	cocci	+	-	-	-	-	-	-	+	-	-
11	SS4 B	orange yellow	gram positive	coccobacilli	+	-	-	-	-	-	-	-	-	-
12	SS4 C	orange yellow	gram positive	cocci	-	-	-	-	-	-	-	+	+	-

13	**SS5 A**	**orange**	**gram positive**	**short rod**	-	-	-	-	-	-	-	-	-	-

3.3. Physicochemical parameters:

In this study, out of 14 isolates, physicochemical parameters of 10 distinct isolate were studied. These parameters include effect of incubation time, pH, temperature, NaCl concentration, addition of glycerol and heat sensitivity. Significant growth was observed in 24 hrs. but clear development of color and maximum growth was observed at 48 hrs at 37 °C. At pH 7.5 growth was observed to be maximum, followed by moderate growth at pH 8 and minimum growth at pH 6.

Deveikaite and Zvirdauskiene (2023) focused on isolating pigment-producing microorganisms from environmental sources (soil, food waste, etc.), optimizing fermentation conditions, and evaluating the antibacterial activity of extracted pigments. Growth parameters of pigment producing microorganisms such as growth temperature, pH, tryptone and NaCl concentration in the medium were optimized to evaluate pigment production. After fermentation, five types of pigments were isolated. During the study, the optimal conditions for the growth of microorganisms were determined: temperature of 30 °C, pH of 7, concentration of 3% tryptone and 6% NaCl in the culture medium. In the present study, we have isolated nine bacterial species at optimum temperature 37°C, at optimum pH 7.5 with 0.5% of salt concentration.

Muneefa KI and Thirumalai (2021) conducted a study that aimed the isolation and characterization of pigment-producing bacteria from soil and evaluating their potential as natural textile dyes. In this study yellow and orange pigmented bacteria were isolated and developed in to pure culture. Yellow and Orange color bacterial extracts of carotenoids pigments were extracted and used as natural colorants for dying of cloth. The present study indicated that pigment production was influenced by physical factors such as pH, Temperature and NaCl. In this study, orange, yellow, green, and pink pigmented bacteria were isolated. Morphological and biochemical characterization was carried out and these isolates were observed for maximum pigment production at different pH, temperature and NaCl concentrations.

Chaturwedi et al. (2024) conducted a series of examination bacteria from soil and

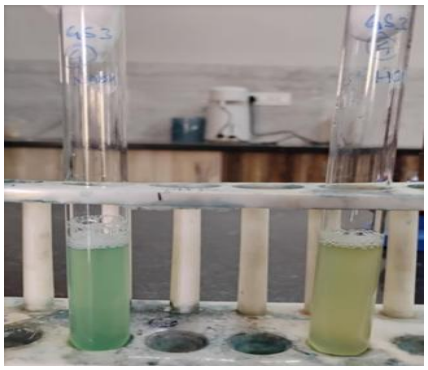
assessed their antibacterial activity. The pigmented bacteria isolates were identified and enriched in nutrient broth. Out of 13 total pigmented bacteria isolates, four different colored pigmented bacterial isolates (S4O, S11Y, S14P and S17G) which produced efficient pigment on nutrient agar were chosen and they were further processed. The present study aimed to isolate 14 different pigmented bacterial isolates, out of which four different colored pigmented bacterial isolates (GS3 B, SS4 A, WS2, and SS3 A).

Isolates showed more pigment production with increase in salt concentration and less growth was observed upon addition of glycerol compared to no glycerol addition besides that isolate WS2, upon heating loose its color from blue green to olive green showing the heat labile nature of the pigment as shown in table 3.2.

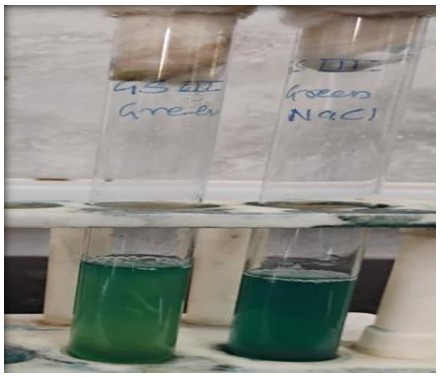
Table 3.2 Effect of various Physicochemical parameters on selected isolate

Sr no.	Isolate	Color of pigment	Temperature (48 hrs)		NaCl concentration (0.5%)		pH			Glycerol	
			30°C	37°C	without	with	6	7.5	8	without	with
1.	WS2	Green	+	++	+	++	+	++	+	++	+
2.	GS3B	Orange	+	++	+	++	+	++	+	++	+
3.	GS3A	Green	++	+	+	++	+	++	+	++	+
4.	SS4A	Yellow	+	++	+	++	+	++	+	++	+
5.	SS3 A	Pink	-	++	+	++	+	++	+	++	+
6.	SS4 C	Orange yellow	+	++	+	++	+	++	+	++	+
7.	SS3 B	Orange yellow	+	++	+	++	+	++	+	++	+
8.	IS1	Yellow	-	++	+	++	+	++	+	++	+
9.	SS4 B	Orange yellow	+	++	+	++	+	++	+	++	+
10.	WS1	Orange yellow	+	++	+	++	+	++	+	++	+

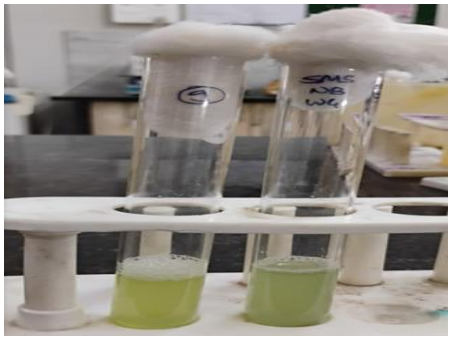
(-): no growth (+): moderate growth (++): maximum growth



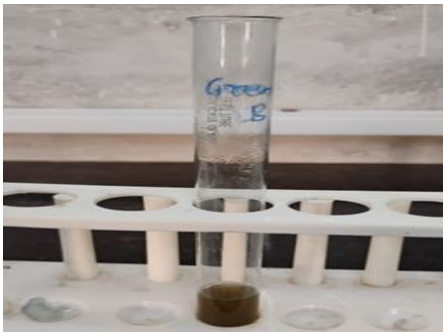
Effect of pH



NaCl concentration



Effect of glycerol

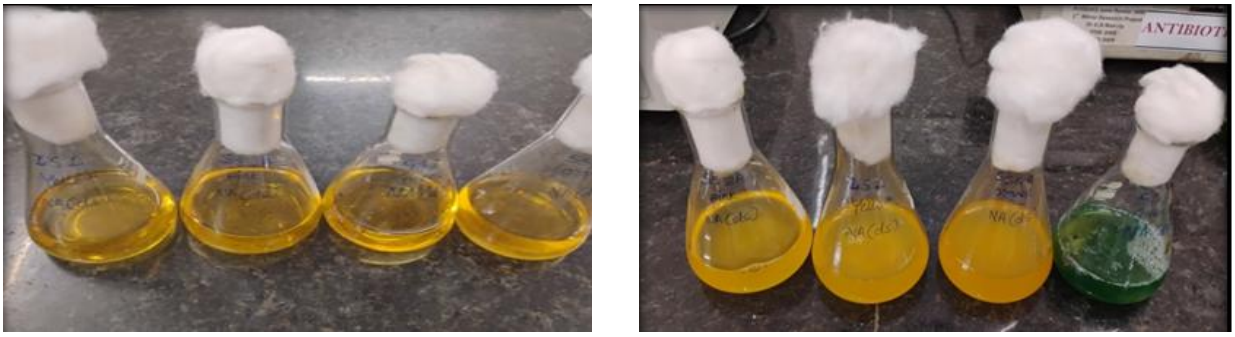


Effect of heat

Figure 3.4: Effect of various physicochemical parameters on isolate WS2

3.4. Screening of pigment producing bacteria and Extraction of pigment

In this study, 4 isolates were further screened to extract pigments, from which WS2 shows extracellular green color in broth whereas SS4 A, GS3B, and SS3 A does not show any color in broth, which means that these isolates produced intracellular pigments which can be extracted by cell lysis. Screening of pigmented bacteria from green colored pigmented broth of WS2. The pigment of isolated pigment producing bacteria was extracted by centrifuging at 6000 rpm for 15 mins and filtered extract with Whatman no:1 filter paper and by the addition of organic solvents.



(a) (b)

Figure 3.5: Pigment Production before incubation (a) and after 4 days of incubation (b)

Figure 3.6: Extraction of green pigment in dry powder form

Dying ability of pigment is checked by using cotton cloth. The pigment extracted from WS2 were mixed with alum to dye cotton fabric and kept for drying. The fabric retained olive green color showing low binding affinity of pigement. Microbial pigment can be extracted by different ways like centrifugation, Filtration method. The extracted pigment can be applied into various industries mainly textile, food, pharmaceuticals (Muneefa KI and Thirumalai, 2021).

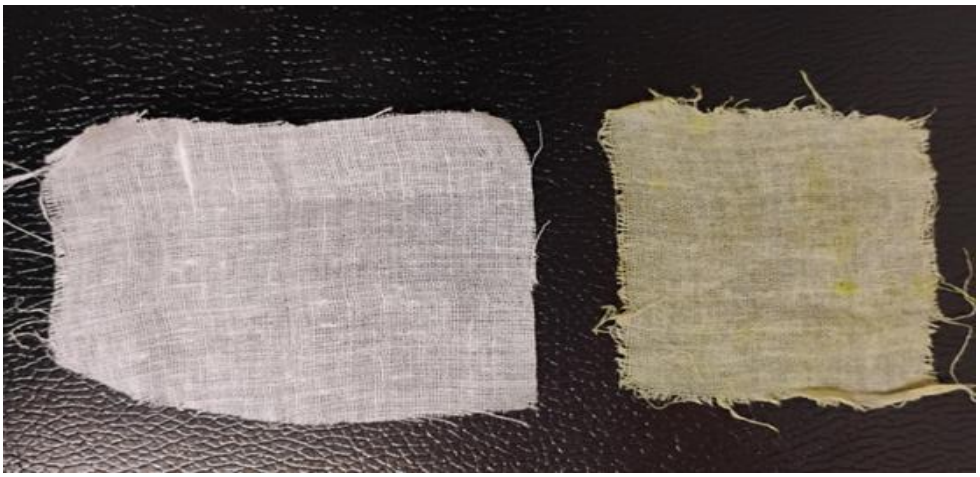


Figure 3.7: Application of extracted pigment of WS2 on to white colored cotton cloth turn slight green

4. CONCLUSION

From the present study, morphological and biochemical characterization was carried out of 14 different pigmented isolates and these isolates were observed for maximum pigment production at different pH, temperature and NaCl concentrations. We have isolated nine bacterial species at optimum temperature 37°C, at optimum pH7.5 with 0.5% of salt concentration. Out of which 4 different colored pigmented bacterial isolates (GS3 B, SS4 A, WS2, and SS3 A) showing orange, yellow, green and pink pigmented bacteria were isolated. The orange and yellow pigments may be carotenoids whereas the pink and green pigments may be prodigiosin and pyocoyanin respectively. The optimum incubation period was 48 hours. Biochemical tests resulted that out of 14 isolates, only 1 isolate showed indole positive, 4 isolates showed MR positive, whereas 3 isolates showed VP and citrate positive. The carbohydrate fermentation resulted in 5 isolates showing only acid positive in glucose solution. Application of these isolates may have several advantages like artificial colors in food, drug and cosmetic items. Microorganisms are known as a potential source for bio-pigment production due to their advantages over plants in terms of availability; stability; cost efficiency; labor; yield and easy downstream processing (Joshi et al.2003). Natural pigments obtained from microbes are more ideal over plants and animals due to the solubility, and stability of pigments and the easy availability of microbes for culturing.

Among these metabolites, pigments are charismatic traits of microorganisms that can be used in several industrial applications. The importance of microbial pigments has been emphasized in different applications, such as cosmetics, food, pharmaceuticals, and textiles, and these compounds are also well-known to exhibit cytotoxic, antioxidant, antimicrobial, antimalarial, anticancer, antitumor, and antifouling activities. Major uses of pigments are in lacquers, oil paints, varnishes, cellulose paints, plastic paints, inks.

The findings suggest that environmentally sourced bacteria are viable candidates for industrial bio-pigment production. By leveraging these microbial sources, the textile industry can transition toward sustainable practices, utilizing colorants that are both aesthetically diverse and ecologically responsible.

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Conflict of Interest: The authors declare that there is no conflict of interest for this study. **5.**

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