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Isolation and identification of pigment-producing bacteria from a mangrove ecosystem

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Abstract
The present study reports on the isolation of several pigment-producing marine bacteria from a mangrove ecosystem in the state of Goa. The samples were collected from the core sediments from the estuarine region of Chorao Island, Dr. Salim Ali Bird Sanctuary.
Four pigment-producing bacteria (M1 to M4) were isolated among which one of the bacterial strains M1 was capable of producing a red pigment, M2 produced yellow pigment, M3 produced pink pigment and M4 produced pale yellow colored pigments respectively. The red pigment-producing bacterium (M1) was of interest to us and hence we took up this culture for further study. Laboratory conditions were optimized for the growth of M1 using cheaply available agro materials to increase the yield of pigment production. Instead of using readymade media provided by companies, we used cereals to obtain the best yield economically for commercial production of the red pigment. Optimal growth of M1 was obtained by studying its growth at pH-6, 7, and 8, temperature-25° C, 30° C, and 35° C, and salinity of 8.5 ppt, 17 ppt, and 34 ppt respectively for optimum production of the red pigment. The cereals used were *Triticum* (wheat), *Glycine max* (soya bean), *Arachis hypogaea* (groundnut), and *Pennisetum glaucum* (Bajra) which was used as a carbon source. In addition to cereals, peptone, yeast extract and, agar was used to form the media. The bacterial growth curve for M1 was plotted by reading O.D. at 270nm on a UV-Vis Spectrophotometer and presented in this report. Gram staining and DNA extraction were performed for the identification of bacterial species.

Keywords: mangroove, boactive molecule, pigments, bacteria

Introduction

Microbes from mangrove sediments

Mangroveeco systems are highly specialized, which are characterized by salt-resistant plants growing in the intertidal areas along sheltered seacoasts and estuaries in the tropical and subtropical regions (Sappal *et.al*., 2012) [17]. Microorganisms associated with sediments are important components of mangrove ecosystems (Kristensen *et.al*. 2008; Thatoi *et.al*. 2012) [9, 22]. They assist in the decomposition of organic matter and are critical for the cycling of nutrients and water (Kathiresan and Bingham 2001; Lovelock, 2008) [7, 12]. In tropical mangrove soil, bacteria and fungi constitute 91% of the total microbial biomass, whereas algae and protozoa represent only 7% and 2%, respectively (Alongi 1988) [1]. Research has revealed a close relationship between soil microorganisms, nutrients, and plants in the recycling and conserving of nutrients in mangrove ecosystems (Lovelock, 2008) [12]. The highly productive and diverse microbial community living in mangrove sediments continuously transforms nutrients bound in dead mangrove vegetation into nutrients that can be used by the living plants. In turn, root exudates serve as a food resource for the microorganisms. Soil microorganisms are also essential for protists and invertebrates, forming the base of benthic food webs and perhaps acting as a sink for carbon in estuaries (Alongi, 1994) [2]. Some plant growth-promoting rhizobacteria that aggressively colonize mangrove roots could be used in mangrove reforestation or restoration (Bashan, & Holguin 2002) [3]. Finally, microorganisms often play an essential role in the bioremediation of polluted mangrove ecosystems (Krishnan, *et.al*. 2007) [8]

Importance of microorganisms from mangrove ecosystem in biotechnological applications

The microbial diversity of mangrove ecosystems can also provide information on their ecological role and unique biotechnological potential in the field of agriculture, industry, medicine, and pharmaceuticals (Lageiro *et al*. 2007) [11]. Mangrove microorganisms have proven to be an important source of food, feed, medicine, enzymes, and antimicrobial substances (Lin *et al*. 2001; Maria *et al*. 2005) [11, 14]. Both halotolerant and halophilic bacteria and other microbes from the mangrove ecosystem have large numbers of industrial applications in terms of their unique enzymes (Sabu 2003) [16] that are capable of producing bio surfactants (Yakimov *et al*. 1999) [23], bioplastics (Steinbüchel and Fuchtenbusch 1998) [21], compatible solutes (Margesin and Schinner, 2001) [13], natural bio products and other commercially important products. Filamentous fungi-the

principal commercial sources of xylanolytic enzymes-have many industrial uses, such as in paper manufacturing, animal feed, bread making, juice preparation, the wine industry, and in xylitol production (Polizeli *et al*. 2005) [15]. Actinomycetes isolated from mangrove habitats are a potentially rich source of anti-infection and anti-tumor compounds and of agents for treating neurodegenerative diseases and diabetes (Hong *et al*. 2009) [6].

Importance of pigment-producing bacteria

Marine bacterial communities possess enormous potentiality to produce diverse bioactive molecules such as pigment molecules. On usual microbial culture media, several marine Gram-positive and Gram-negative bacteria appear to produce an array of pigments (Chatragadda *et.al* 2019) [4]. Several marine bacterial pigments have demonstrated various biological activities such as antimicrobial, anticancer, and immunosuppressive activities (Soliev *et.al* 2011) [20]. Recently, studies on natural products and microbial autecology science have increased the demand for novel resources of eco-friendly natural products such as bacterial pigments for different biomedical and industrial applications. Natural pigment molecules of microbial origin have a great demand in the industry due to their functional attributes such as nontoxic nature, easier gene manipulation, a large volume of biomass production, and environmental acceptability. Therefore, exploration, exploitation, and identification of novel or rare types of pigment compounds from marine-pigmented bacteria (MPB) are necessary for a wide range of biomedical and industrial applications (Shindo and Misawa 2014) [19].

Materials and Methods

Sampling and isolation of microbes

Collection of samples

The soil sample was collected in sterile polythene bags from Chorao Island at Dr. Salim Ali Bird Sanctuary in the vicinity of mangroves *Rhizophora mucronata*, around the Mandovi estuary of Goa. The samples were transported to the laboratory in an icebox immediately after collection for further processing.

Isolation of microbial isolates

On reaching the lab, the soil sample was rinsed in sterile seawater (SSW) followed by the addition of a known volume of SSW. Thereafter, saline suspension ranging from 10^{-0}, 10^{-1}, and 10^{-2} dilutions were prepared, and the same was spread in plates containing Zobell Marine agar (ZMA) and Nutrient agar (NA). Both the agar plates were prepared using 34ppt saline seawater. The plates were incubated at 30°C for 24h until visual growth of cultures was observed. Individual colonies were picked based on size, color, texture, and nature of growth.

Purification of the Microbes

Bacterial isolates

The isolated bacterial cultures were purified by repeated sub-culturing on Nutrient agar plates and preserved on slants, plates, and in NB media, and stored at 4°C until further use.

Characterization of the Microbial Isolates

Gram staining

Gram staining was performed to study cell morphology to distinguish between Gram-positive and Gram-negative microbes using the Gram's method of staining. The results were observed under 100x oil immersion.

Morphological characteristics

Microbial characterization was done for the differentiation of the culture isolates. Characterization was done based on size, shape, texture, margin, and color. The results were observed and tabulated.

Optimization of the Cultural Conditions

Pigment production using different substrates

a. Various substrates were used as carbon sources as a substitute for the production of commercial media such as *Triticum* (wheat), *Glycine max* (soya bean), *Arachis hypogaea* (groundnut), and *Pennisetum glaucum* (bajra). Briefly, 1.5 gram of dried powder, 0.2 gram of Yeast extract, and 1 gram of Peptone in theabove-mentioned substrates were added to 100ml of seawater at 34ppt salinity. The media was autoclaved at 121 psi for 20 minutes. The media was then inoculated with the culture and incubated for 24 hrs at 30ºC temperature and checked for pigment production.
b. After maximum growth of culture M1, was evident in Glycine max medium, further optimization was carried out in *Glycinemax* (soya bean) broth medium using the below-listed parameters:

pH optimization

The optimum pH and growth conditions required were checked for the isolate. The culture broth was subjected to different pH ranges (6, 7, and 8) and checked for optimum growth.

Effect of temperature on pigment production and bacterial growth

The culture broth was subjected to temperatures such as 25°C, 30°C, and 35°C and checked visually for maximum growth and pigment production.

Salinity optimization

The culture broth was subjected to salinities of 8.5 ppt, 17 ppt, and 34 ppt and checked visually for maximum growth and pigment production.

Growth Curve

When a bacterial culture is placed in a suitable medium that provides all the conditions and nutrients necessary for its growth, then the live bacterial cells in the population over a while exhibit four main phases of growth. The initial lag phase, the exponential or log phase, the stationary phase, and finally the death or decline phase. In the initial lag phase, the bacteria are metabolically live and active but not dividing. In the exponential or log phase, there is exponential growth taking place. The third stationary phase is characterized by the growth reaching a plateau as the number of dying cells equals the number of dividing cells. Finally, the death phase is characterized by an exponential decrease in the number of living cells.

1. The isolated M1 colony was inoculated into 15 ml nutrient broth and kept at 30ºC for 24 hours of the incubation period.
2. 1ml of this culture was inoculated in *Glycine max* broth prepared in 1.5 gram of dried powder of *Glycine max*, 0.2 gram of Yeast extract, 1 gram of Peptone, and 100ml of seawater.
3. Spectrophotometer was used for measuring UV for measuring the growth of the bacterium M1.
4. Initially media was scanned to see at which wavelength max absorption was obtained.
5. The selected wavelength was obtained at 270nm which was later used for measuring the growth at different periods. Five ml of uninoculated sterile media was added to a clean cuvette, which was blank. The machine was set to zero ABS with this sample. This step standardizes the turbidity of the media without any cells in it so that further calculations can quantitate growth due to changes in the turbidity.
6. Immediately after inoculating, a 5 mL sample of the inoculated media was taken and pipetted into a clean cuvette. Placed in the blanked spectrophotometer, the OD reading was recorded. This reading should be recorded at a time "0".
7. The UV reading was taken every 2 hours for 48h.
8. The readings were plotted on a graph with time as the X-axis and OD as the Y-axis.

Molecular Identification

DNA extraction by CTAB protocol

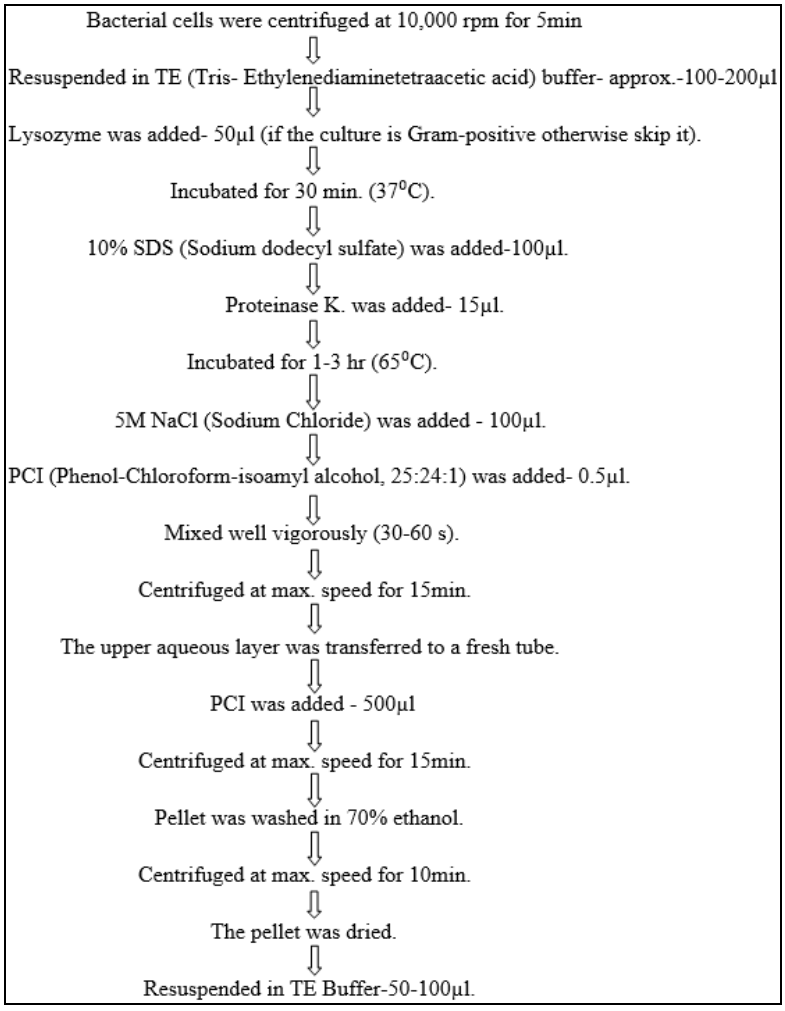
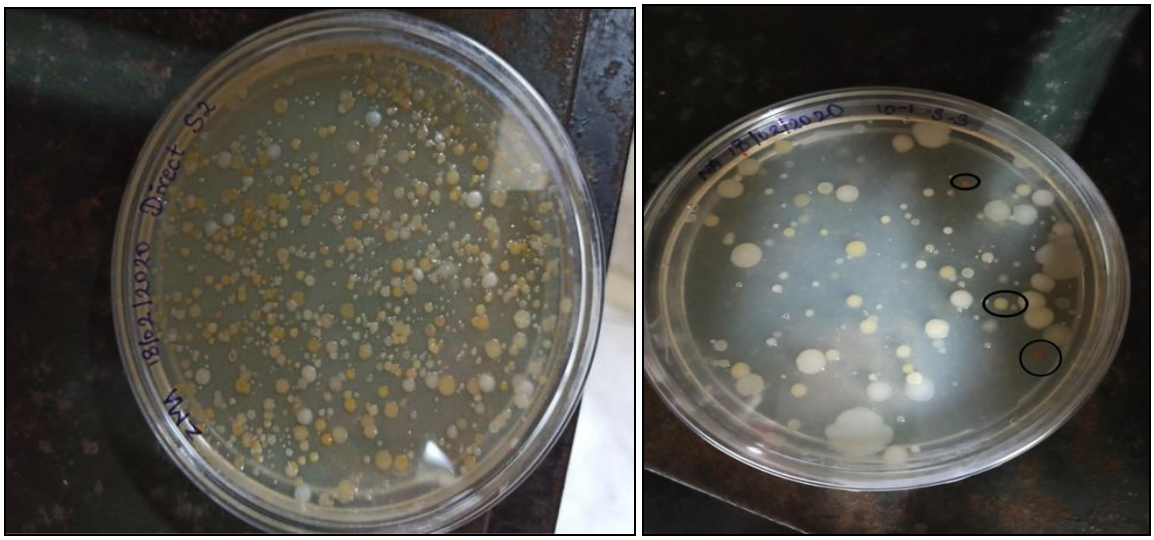


Fig 1

Results and Discussion

Isolation and identification were carried out using standard techniques as per Bergey's Manual. Pure cultures were obtained by repeated subculturing on NA plates/slants after incubating the master plates for 24-48 hours at 30ºC. The above procedure yielded 4 bacterial isolates from the sediment soil sample. One (M1) of the four bacterial cultures was selected based on the basic characterization and used for further studies.



(a) **(b)**

Fig 2: (a) and (b) are isolated pigmented bacteria

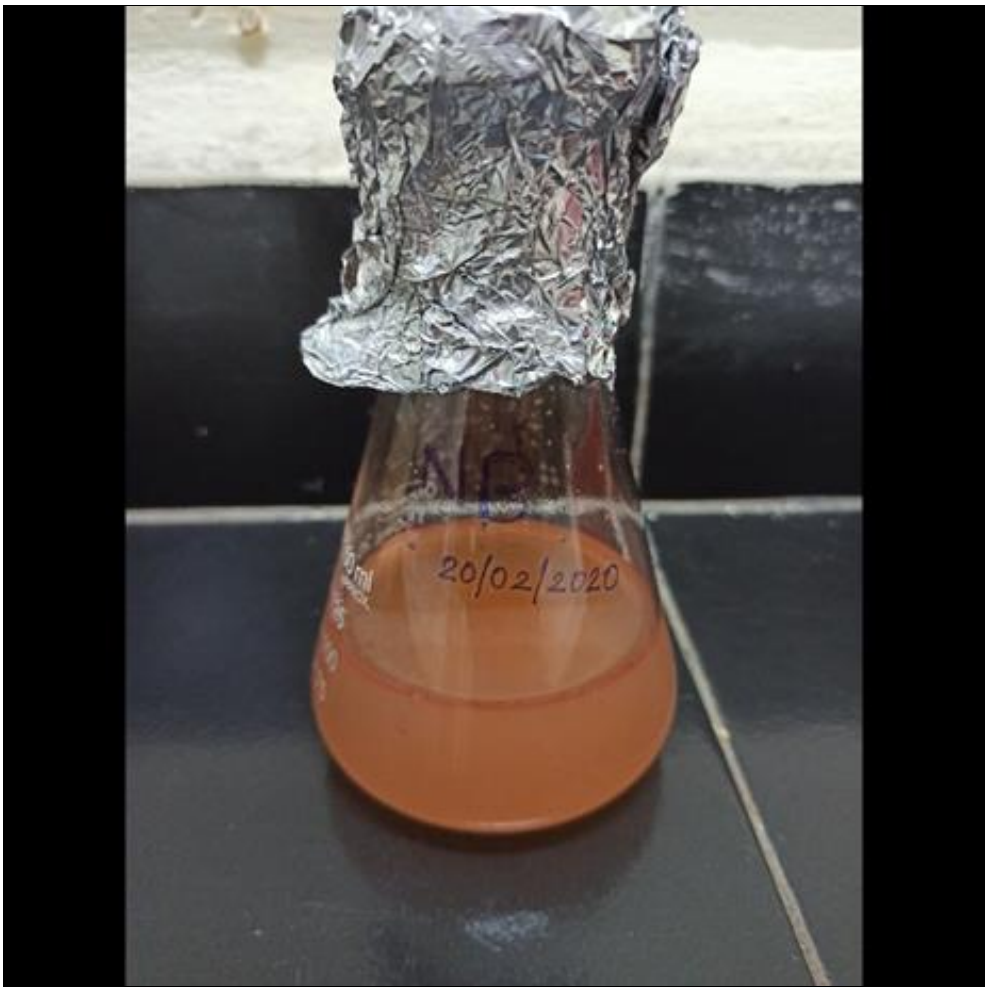


Fig 3: Red pigmentation in nutrient broth

After the growth of red pigment in Nutrient Broth 0.1µl of M1 was streaked on Nutrient agar prepared in salinity ranges of 8.5 ppt, 17 ppt, and 25.5 ppt, seawater and incubated at 30ºC for 24-48 hours. Maximum growth was observed at 8.5pptsalinity as shown in figure 5.

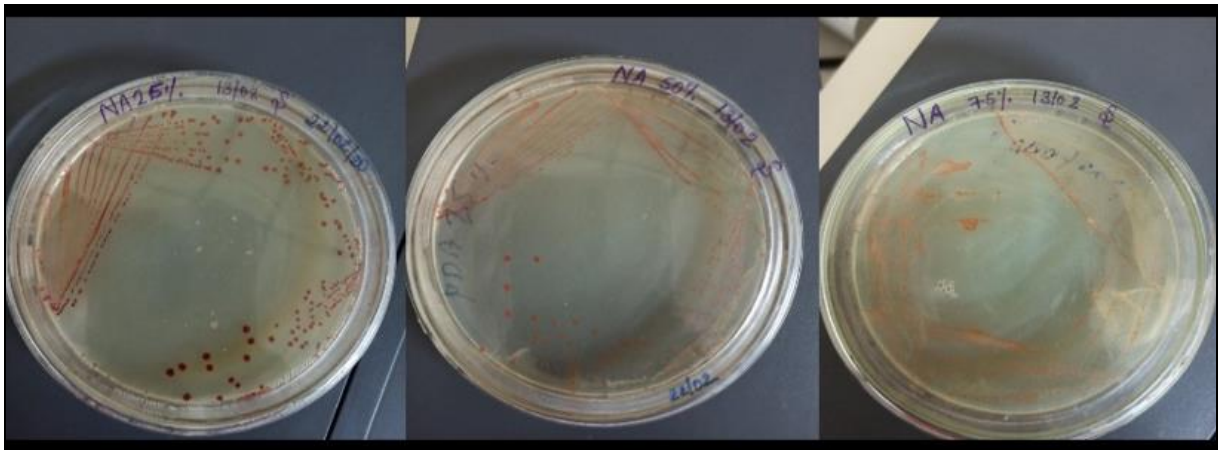


Fig 4: Growth of red-pigmented bacterial culture at 8.5ppt, 17ppt, and 25.5 ppt salinity.

Microbial characteristics

Three Gram-positive and one Gram-negative bacterial culture were observed under 100x oil immersion.

Gram's character

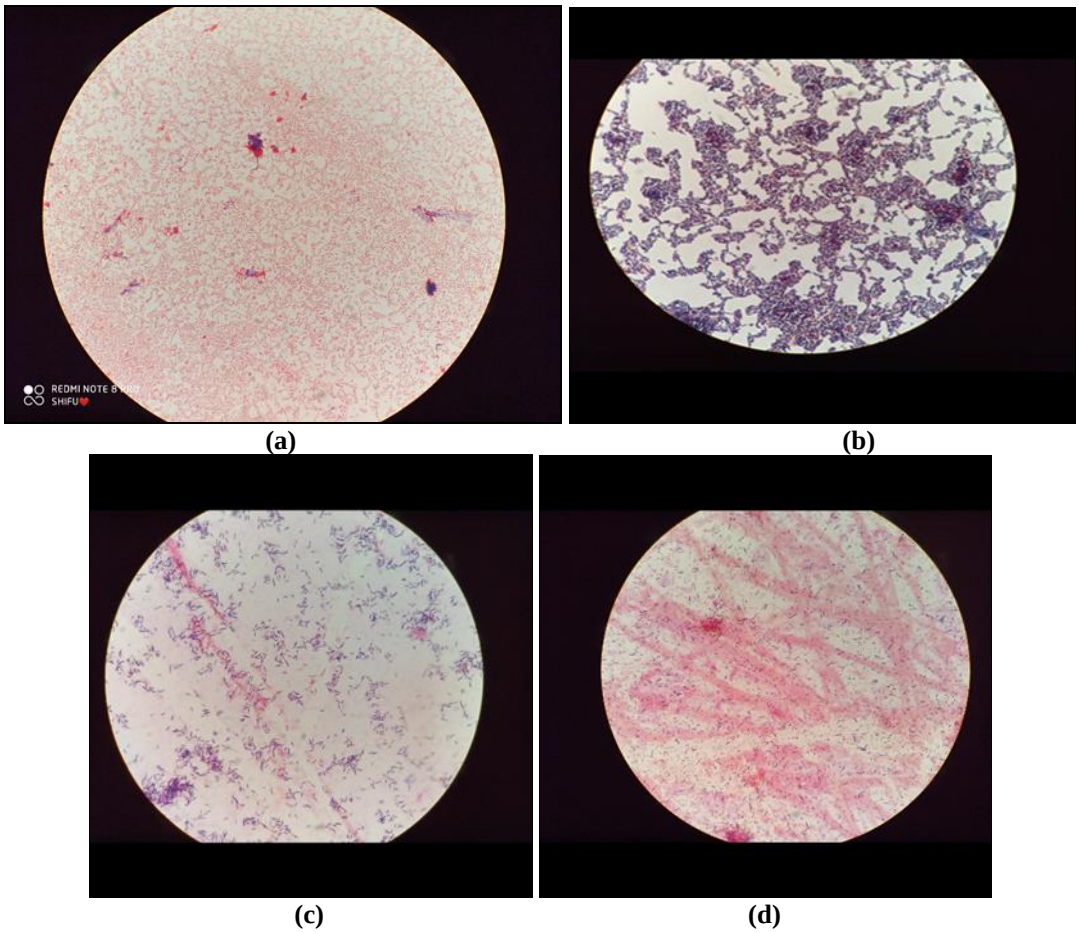


Fig 5: (a) M1 bacterial culture, (b) M2 bacterial culture, (c) M3 bacterial culture, (d) M4 bacterial culture

The analysis of the gram character was done using the gram method of staining. The results are given in Table 1.

Table 1: Gram character of the bacteria isolated from the mangrove soil.

Culture	Gram character	Shape
M1	Negative	Short rods
M2	Positive	Bacillus
M3	Positive	Streptococci
M4	Positive	Diplobacilli

Morphological characteristics

The isolates obtained were observed for their morphology and other colony characteristics as listed in table 2.

Table 2: Colony characteristics of bacteria isolated from the mangrovesediments

Isolates	Size	Form	Pigmentation	Margin	Opacity	Elevation	Texture
M1	Small	Circular	Red	Entire	Opaque	Flat	Smooth
M2	Moderate	Circular	Yellow	Undulate	Opaque	Raised	Smooth
M3	Moderate	Circular	Pink	Entire	Opaque	Raised	Smooth
M4	Moderate	Irregular	White	Undulate	Opaque	Flat	Rough

Growth of red-pigmented bacterial culture M1 in different substrates

Pigment production using different substrates

The maximum growth of M1 and red pigment production was observed in*Glycine max* (soya bean) broth as shown in Figure 5.

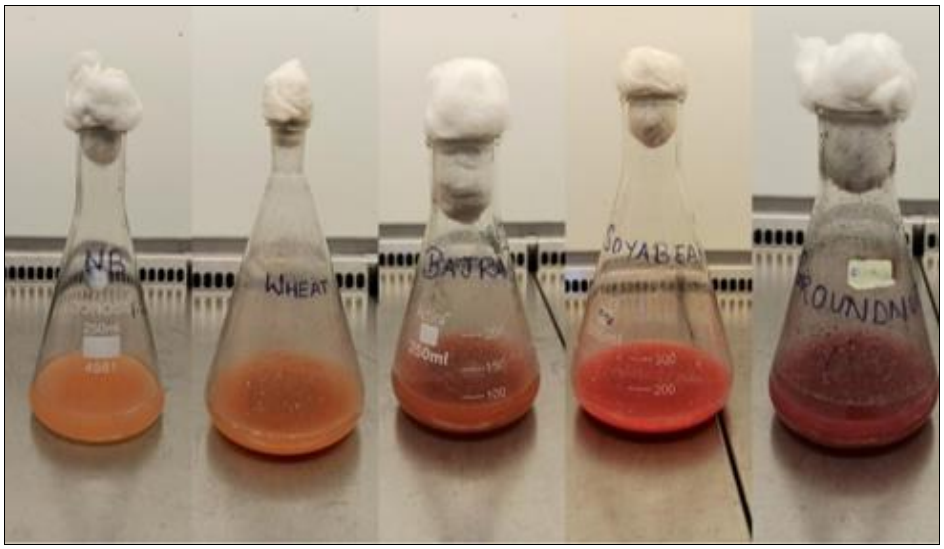


Fig 6: Growth of red-pigmented bacterial culture in different media.

Optimization of growth conditions of M1 (pH, temperature, salinity)

After the maximum growth of M1 culture and production of red pigment in *Glycine max* was observed among all the substrates it was further optimized for different pH, temperature, and salinity.

M1 grown in *Glycine max* broth showed maximum growth at pH 6, temperature 25ºC, and salinity 8.5 ppt after 48-72 hours of the incubation period. At a temperature of 35ºC, pH 6, 7, and 8, and salinity 8.5 ppt, no color change was observed after 48-72 hours of incubation period as shown in Figures 6, 7, and 8 and Table 3.

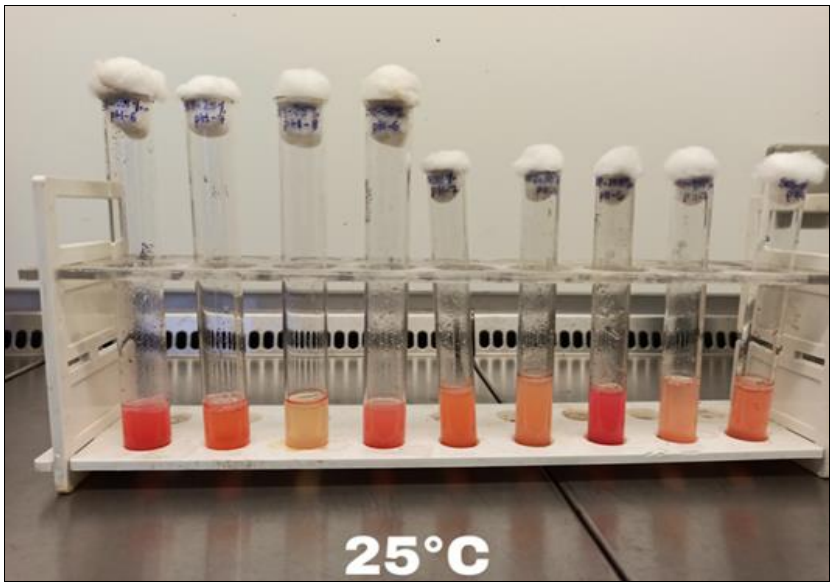


Fig 7: Red pigmentation max at 25ºC, pH 6 in 8.5 ppt in *Glycine max* broth

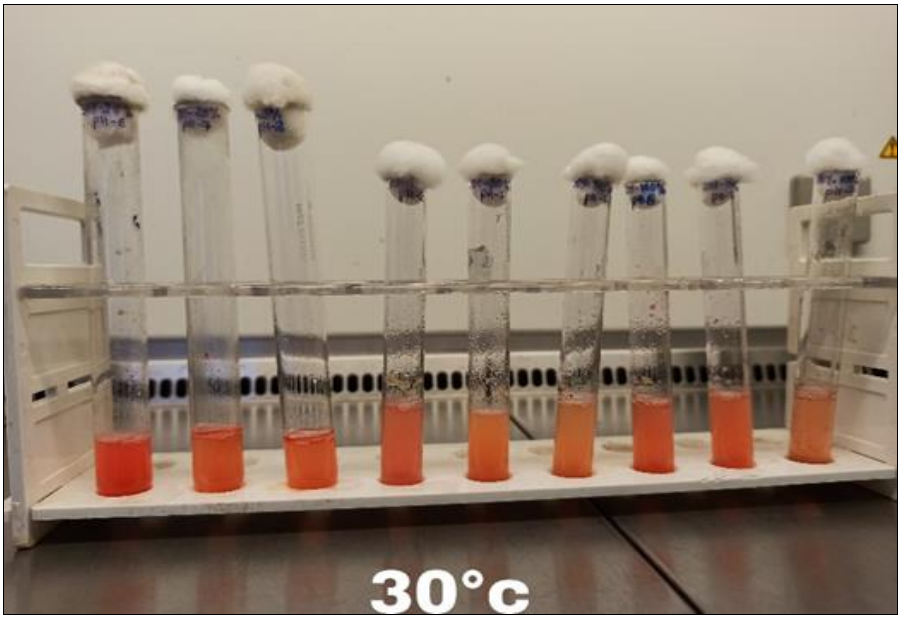


Fig 8: Red pigmentation max in pH 6, salinity 8.5ppt and temp 30ºC in *Glycine max* broth

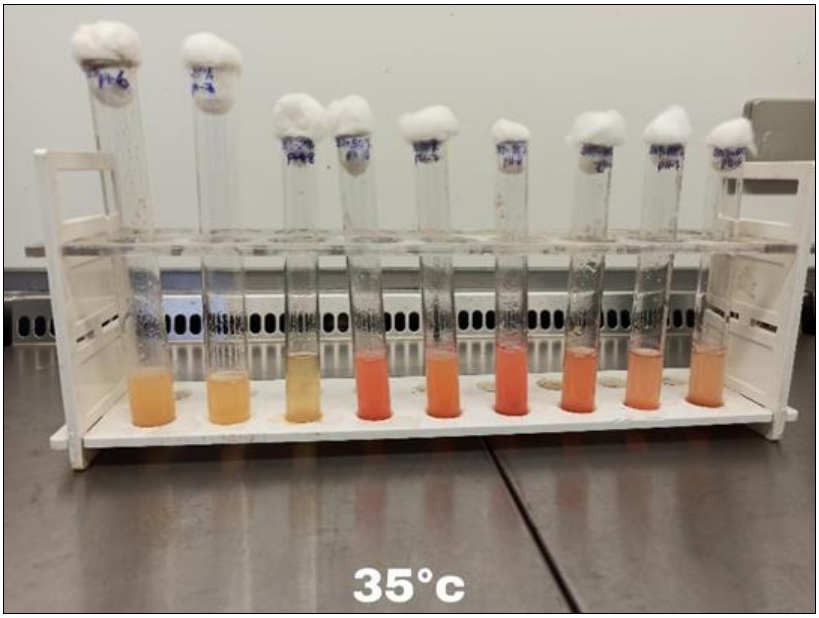


Fig 9: Red pigmentation max in pH 6 and 8, salinity 17 ppt and at temp 35ºC in *Glycine max* broth.

Table 3: Conditions required for maximum pigment production. (+++ = Maximum growth), (++ = Average growth), (- = Minimum growth)

Culture	Substrates	Media	pH	Temperature (in ºC)	Salinity (ppt)		
					8.5	17	34
M1	*Glycine max*	Nutrient Broth	6	25	+ + +	+ +	+ +
				30	+++	++	++
				35	-	++	++
			7	25	++	++	+
				30	++	+	+
				35	-	+	+
			8	25	+	+	+
				30	++	+	+
				35	-	++	+

Growth curve

After optimization of growth conditions of bacterium M1 to produce maximum red pigment in the growth medium (*Glycine max* broth) measured by using the UV-Vis Spectrophotometer is recorded and shown in Table 4.

Table 4: Measured optical density of M1 culture in *Glycine max* using UV Spectrophotometer

Time	Wavelength	Absorbance
0 min	270nm	0.22
2 hours	270nm	0.44
4 hours	270nm	0.67
6hours	270nm	0.7
8hours	270nm	0.74
10hours	270nm	0.86
12hours	270nm	0.89
14hours	270nm	0.92
16hours	270nm	0.98
18hours	270nm	1.08
20hours	270nm	1.12
22hours	270nm	1.17
24hours	270nm	1.19
26 hours	270nm	1.16

The curve thus obtained is sigmoid as shown in Figure 9.

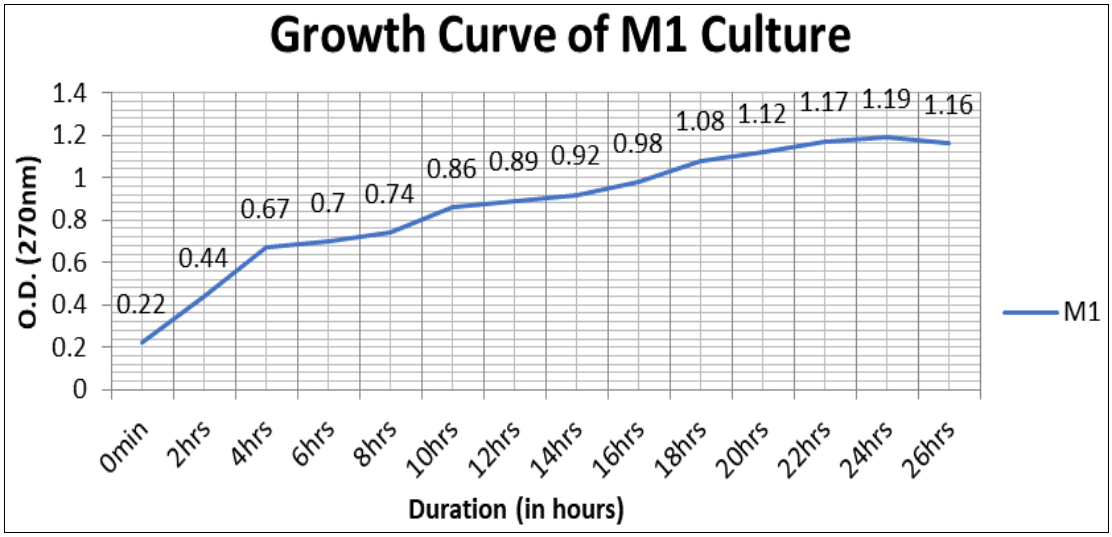


Fig 10: Growth curve of M1 bacterial culture in *Glycine max* broth.

Conclusion

The Mandovi mangrove estuarine ecosystems are associated with biotechnologically useful marine pigmented bacteria with important industrial applications. The present study reports on the isolation of bacterium M1 to M4 capable of producing pigments. Among them, Bacterium M1 was most significant and capable of producing bright red colored pigment which could have application in textiles, food, paper, and leather dying industries. This study attempts to optimize its (M1) growth and increase the production of the pigment using cheaply available cereals and legumes. Such studies may be significant in the commercial production of industrially important compounds economically.

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