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ISOLATION, ENZYME PRODUCTION AND COMPARISON OF DIFFERENT PIGMENTS FROM *ACTINOBACTERIA*

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Abstract: *Actinobacteria* are a diverse group of Gram-positive, filamentous microorganisms known for their remarkable ability to produce industrially valuable enzymes and natural pigments. The present investigation aimed to isolate, characterize, and evaluate pigment-producing Actinobacteria from soil and sediment samples collected from the Kumbharwada wetland region of Bhavnagar, Gujarat, India. Isolation was carried out using Gause's Synthetic Agar (GSA), resulting in a total of 23 actinobacterial isolates, of which 13 exhibited distinct pigment production. Morphological and microscopic observations were confirmed by typical morphological characteristics. Biochemical characterization using the Triple Sugar Iron (TSI) test revealed carbohydrate fermentation without gas or hydrogen sulphide production. Enzymatic screening demonstrated significant extracellular enzyme activity, including amylase, gelatinase and pectinase production, highlighting the biocatalytic potential of the isolates. Pigments were extracted using ethyl acetate and analysed using spectrophotometrically in the range of 400-800nm, revealing diverse absorption maxima indicative of different pigment types. The findings suggest that the Kumbharwada wetland is a rich reservoir of pigment-producing Actionbacteria with potential applications in food, textile, pharmaceutical, and biotechnological industries. The study emphasizes the potential of naturally derived microbial pigments and enzymes as eco-friendly alternatives to synthetic counterparts.

Keywords - *Actinobacteria*, pigment production, extracellular enzymes, amylase, gelatinase, pectinase, TSI test, UV-Vis spectroscopy.

I. INTRODUCTION

Actinobacteria are omnipresent and a diversified group of Gram-positive bacteria with a high G+C (Guanine + Cytosine) content in their DNA. *Actinobacteria* are members of the order Actinomycetales that form a bridge between fungi and bacteria in terms of their morphological growth patterns and ecological roles. The ability to produce spores makes them highly environmental stress resistant and helps to survive in adverse conditions. *Actinobacteria* have a great physiological and biotechnological potential for synthesizing numerous primary and secondary metabolites. It possesses an extensive ability to produce antibiotics as a secondary metabolite than any other group of microorganisms. *Actinobacteria*, especially from the genus Streptomyces, are responsible for producing more than two-thirds of all known naturally derived antibiotics. The defining and most specific property of *Actinobacteria* is their unparalleled ability to produce a vast array of bioactive secondary metabolites, which makes them invaluable in medicine and

pharmaceutical research. While antibiotic production is the hallmark, it also shows other distinct traits; one of them is pigment production, which is noteworthy. The pigment production often serves as an important tool for the growth of bacteria by protecting them from UV exposure, spore formation or oxidative stress. Some of the popularly produced pigments of *Actinobacteria* are yellow (*S. venezuelae*), red (*S. antibioticus*), and light orange (*S. albus*). Some species of *Streptomyces* and *Nocardia* produce dark brown to black pigment, i.e., melanin. The pigment production depends on several factors such as nutrient availability, temperature, light exposure and oxygen levels. Besides its application in industries, it holds biological activities such as anti-cancer, anti-inflammatory, anti-oxidant, and anti-biofilm activity. Pigments from *Actinobacteria* represent a sustainable, biologically active, and environmentally friendly alternative to traditional synthetic dyes. They align perfectly with current consumer preferences for natural, safe, and eco-conscious products, while offering potential health benefits. As technology advances, the commercial use of these pigments will likely continue to grow, offering a promising future for both industry and the environment. *Actinobacteria* are also a vast source of biocatalysts. The production of enzymes in *Actinobacteria* is a multi-faceted process that involves metabolic pathways, environmental conditions and genetic regulation. The enzymes produced are of great importance and have immense value across multiple industries, from that too pharmaceuticals to agriculture and also the process of bioremediation. It produces many extracellular enzymes that improve the industrial needs. *Actinobacteria* isolated from extreme environments synthesize such novel enzyme that fulfils the industrial demand. Amylase is the most prominent enzyme produced by *Actinobacteria*, which holds great industrial application. It is produced extracellularly into the medium and breaks down the starch into simpler sugars. Enzymes catalyse the hydrolysis of starch, which plays a crucial role in food processing, textiles, etc. The zone of hydrolysis is clearly observed around the growth of the colony which indicates starch hydrolysis by producing amylase. Along with amylase, some species of *Actinobacteria* also produce gelatinase which is an important characteristic for further identification of these organisms. The gelatinase enzyme breaks down or hydrolyses gelatin by breaking its peptide bond. It also hydrolyses other proteins to obtain smaller peptides or amino acids; which is often secreted extracellularly. The production of gelatinase enzyme is often induced by providing bacteria with a gelatin-rich medium or a protein-rich medium. It is applicable in the industrial and medical fields. Certain species, such as Streptomyces, Mycobacterium, etc., can produce gelatinase. One more enzyme, pectinase, is produced by *Actinobacteria* and is of significant interest due to its application in agriculture, food industry and the biotechnology field. It is also applicable in breaking down pectin-rich organic waste, which is a valuable tool in waste management. Species like *Nocardia*, *Micromonospora*, etc., are responsible. The extracellular secretion of pectin- degrading enzymes helps to access complex molecules and break them into simple molecules. *Actinobacteria* are also known to ferment different types of sugars and also the ability to produce H_2S by fermenting these sugars. The colour change in the slant indicates the fermentation of a particular sugar, and change in the pH indicates the acid production, which can be detected by performing the TSI (Triple Sugar Iron) biochemical test.

II. MORPHOLOGY OF *ACTINOBACTERIA*

The filamentous, dense, branched and flat colony on the substrate represents the characteristic of that of fungi. The rest of the features of it are common to that of bacteria than of fungi. *Actinobacteria* is placed in a group of bacteria that includes Corynebacterium, Mycobacterium, etc. The colony diameter of *Actinobacteria* is much smaller than that of a true fungal filament. It is aerobic and produces spores through its filamentous form which produces spores singly or in chain. The colony formation is slightly raised and appears to be a powdery mass with a specific odor, and is pigmented due to aerial spore formation. *Actinobacteria* when cultivated on a solid-agar medium, it forms a network of branches and forms a hyphal structure. The hypha is produced in and on the substrate; the hypha that is on or above the substrate is an aerial hyphae and the hypha that penetrates inside the substrate is a substrate hyphae.

III. CELL WALL COMPOSITION

The unique cell wall structure of *Actinobacteria* contributes to the exclusive properties including antibiotic resistance and harsh environmental conditions. The uniqueness of cell wall structure gives them an important identification among the different groups of bacteria. The major components of cell wall consist of:

-Peptidoglycan (provides rigidity and shape); more complex layer due to presence of unique sugars.
-Mycolic acids (Mycobacterium genus); long-chain fatty acids that adds a waxy layer, and provides hydrophobicity to cell wall.
-Polysaccharides (mannose, galactose, glucose) presence of these sugars can vary differently among species.
-Teichoic acids (made of repeating units of glycerol phosphate or ribitol phosphate)
-Liporabinomannan (LAM); it is a glycolipid. It inhibits process of phagocytosis and also acts as a virulence factor.
-Peptidoglycan Binding Proteins (PBPs); interacts with peptidoglycan layer. During cell growth and cell division; these proteins are involved in maintenance and remodeling of peptidoglycan.

IV. MATERAILS AND METHODS

4.1 Sampling Site

The samples were collected from a locality Kumbharwada that falls near the Bhavnagar district shown in "Fig.1"(a), (b), (c); situated in the western part of Gujarat, India. It is a wetland that covers shallow lakes, marshes that supports a wide range of aquatic life. The coordinates of the study site are approximately latitude: 21.7871°N, longitude: 71.8764°E. The wetland is rich in diversity and is a home for many migratory birds.

4.2 Collection of Samples

The samples were collected from different areas of Kumbharwada wetland; one each was from soil and sediment shown in "Fig.2". Furthermore, the sediment sample was obtained from the depths of the water bodies and the soil sample was collected beneath the trees and was dug a few centimeters from the ground surface. The collected samples were stored in sterile 10 ml screw cap bottles and were stored aseptically for further experiments for obtaining pigment producing *Actinobacteria*.

4.3 Isolation of *Actinobacteria*

1g of soil sample and 1ml of sediment sample was weighed and measured respectively and was added to 10ml of sterile distilled water test tubes to make a uniform suspension and follow the serial dilution technique (Etinosa O. Igbinosa et. al., 2017). The particulate was allowed to settle down and from the supernatant 1ml of sample was withdrawn and added to 9ml of sterile distilled water test tubes. The dilutions were prepared from 10^{-1}, 10^{-2},10^{-3}…..10^{-7} and from each diluent 0.1ml of the sample was inoculated in GSA (Gause's Synthetic Agar) plates containing (Starch-20.0g, KNO_3-1.0g, NaCl-0.5g, $MgSO_4.7H_2O$-0.5g, $FeSO_4$-0.01g, $K_2Cr_2O_7$-0.01%, Agar-3%, Distilled water-1000ml) by spread plate technique. All the plates were incubated at 30°C for 7 days (Dholakiya et al., 2017). The plates were daily observed for the growth of isolates and pigment production. To maintain the culture, it was further subculture and preserved in GSA slants and glycerol stocks.

4.4 Characterization of Isolates

The isolated colony was studied in detail by observing its colony characteristics such as its edge, margin, optical characteristics, texture, odor, surface, and its morphology was studied by performing Gram's staining method. The microscopy of each isolate was performed individually. Biochemical test such as TSI (Triple Sugar Iron) was performed to observe the fermentation of sugars by the isolates (Dholakiya et al., 2017). The change in the color of TSI slant and change in the pH was observed. The enzymatic test was performed to detect the production of enzymes by the isolates.

V. ENZYMATIC TESTS

5.1 Starch Hydrolysis test

All the isolates were inoculated on Starch Agar Plate containing (Peptone-5.0g, Sodium chloride-5.0g, Yeast Extract-1.5g, Starch soluble-2.0g, Agar-15.0g, Distilled water-1000ml). After inoculation, the plates were incubated at 30°C for 7 days. Further, the plates were flooded with Lugol's Iodine to observe the zone of clearance or zone of hydrolysis around the growth of colony.

5.2 Gelatin Hydrolysis test

All the isolates were inoculated in a test tube containing Nutrient Gelatin medium (Bacteriological gelatin-5.0g, Nutrient broth-100ml). The tubes were incubated at 30°C for 7 days. After incubation, the tubes were further placed in refrigerator for 30 minutes to observe the liquefaction of the medium.

5.3 Pectin Hydrolysis test

All the isolates were inoculated on Pectin Agar Plate containing (Pectin-1.0g, KH_2PO_4-0.2g, K_2HPO_4-0.3g, $MgSO_4$-0.01g, $(NH_4)_2PO_4$-0.3g, Agar-3%, Distilled water-100ml) and incubated at 30°C for 7 days. After incubation, the plates were observed and zone of clearance or pectin hydrolysis was observed around the growth of colony.

VI. BIOCHEMICAL TEST

6.1 TSI (Triple Sugar Test)

It is a biochemical test performed to identify the bacteria that can ferment sugars, produce acid and also H_2S. The TSI slant(Yeast Extract-3.0g, Peptone-15.0g, Lactose-10.0g, Glucose-1.0g, Sucrose-10.0g, ferrous sulphate-0.2g, Sodium thiosulphate-0.3g, Sodium chloride-5.0g, Agar-20.0g, Phenol red-0.24g, distilled water-1000ml) is stabbed in the butt and streaked in the slant with the isolates and incubated at 30°C for 7 days. The slants were observed for the growth and color changes in the slant.

VII. PIGMENT EXTRACTION USING ETHYL ACETATE

5ml ethyl acetate was added to plate containing pigmented colonies

↓

Plate was scraped and the mixture was added to mortar pestle to get the intracellular pigment

↓

The mixture was thoroughly grind and was collected in centrifuge tubes

↓

Centrifugation at 8000rpm for 10mins

↓

Collect the supernatant and discard the pellets

↓

Preserve the solvent containing pigment into refrigerator for further purification

7.1 Spectrum Measurement

The extracted pigment was used to determine the range of maximum wavelength. The sample was measured between the range of 400-800nm, and the highest peak was obtained and further studied.

VIII. RESULTS

Actinobacteria were successfully isolated from soil and sediment samples collected from Kumbharwada wetland, Bhavnagar, using Gause's Synthetic Agar (GSA). After incubation at 30°C for 7 days, well-developed colonies appeared on the plates. The isolated colonies showed slow growth and exhibited typical actinomycetal characteristics such as dry, rough, chalky or powdery texture with irregular margins. Pigment production was observed in several isolates, showing different colors. Microscopic examination following Gram's staining revealed that all the isolates were Gram-positive and filamentous in nature. A

total of 23 isolates were obtained by performing serial dilution of the samples, out of which 13 isolates were pigment-producing shown in Fig.3. *Actinobacteria* is a source of biocatalyst-producing extracellular enzymes. Different enzymatic tests were performed so as to identify the enzyme production. All the isolates were screened for the production of amylase and inoculated on starch agar medium. After flooding with Lugol's iodine solution, clear zone of hydrolysis were observed around the colonies of several isolates, indicating starch degradation. Isolates from AD1 to AD14 and AD17 to AD20 showed the presence of a distinct zone of clearance, confirming the extracellular production of amylase enzyme by these isolates shown in Fig.4. The rest of the isolates did not show any clear zone and were considered negative for starch hydrolysis. Gelatin hydrolysis was assessed by observing liquefaction of nutrient gelatin after refrigeration. A number of isolates AD1 to AD8 and AD15 to AD22 showed complete liquefaction of gelatin, indicating positive gelatinase activity shown in Fig.5 (a), (b), (c). Partial liquefaction or no liquefaction was recorded as gelatinase negative. On pectin agar plates, clear zones around the growth of isolates AD1 and AD2 were observed, indicating pectin degradation shown in Fig.6. These results confirmed the production of extracellular pectinase enzyme by these two isolates, while the rest of the isolates were pectinase negative. Table 1 depicts the extracellular activity of all the isolates. The Triple Sugar Iron Test (TSI) was performed to study sugar fermentation patterns and H_2S production. All the isolates from AD1 to AD23 were able to ferment sugars shown in Fig.7. No prominent black precipitate and gas production was observed which resulted in pH and color change of the medium. Pigment-producing *Actinobacteria* were selected for pigment extraction. The pigments were successfully extracted using ethyl acetate shown in Fig.8. The extracted pigments exhibited distinct coloration corresponding to the colony pigmentation observed on GSA plates. The solvent extracts were clear and stable, indicating effective extraction of intracellular pigments. Spectrophotometric analysis of the extracted pigments was carried out in the wavelength range 400-800nm. Each pigment extract showed a characteristic absorption peak within the visible region. The variation in maximum absorbance peaks among isolates suggested the presence of different types of pigments produced by *Actinobacteria*. On the basis of wavelength and its absorbance the graph was plotted shown in Chart 1.

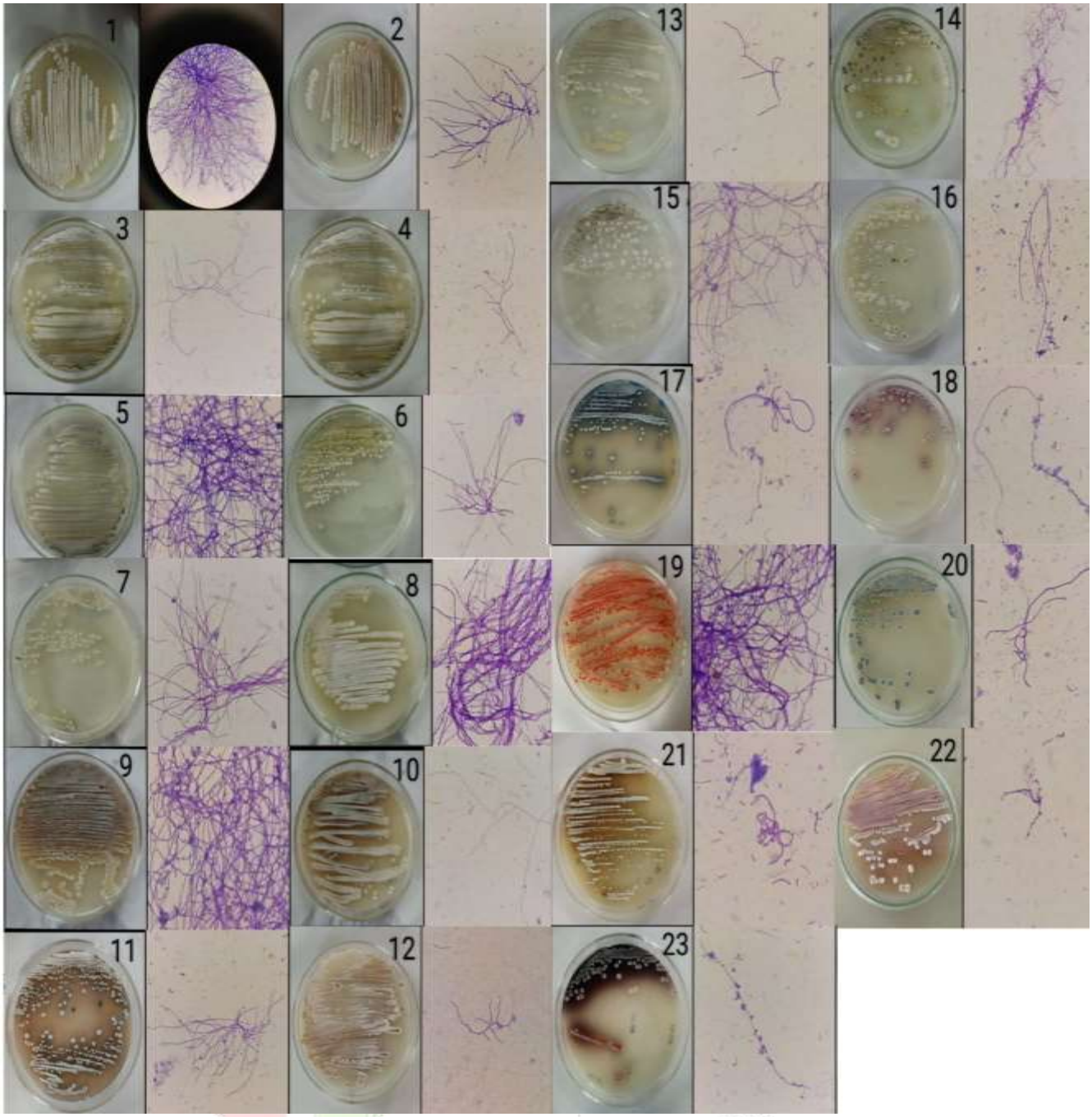
(a)

(b)

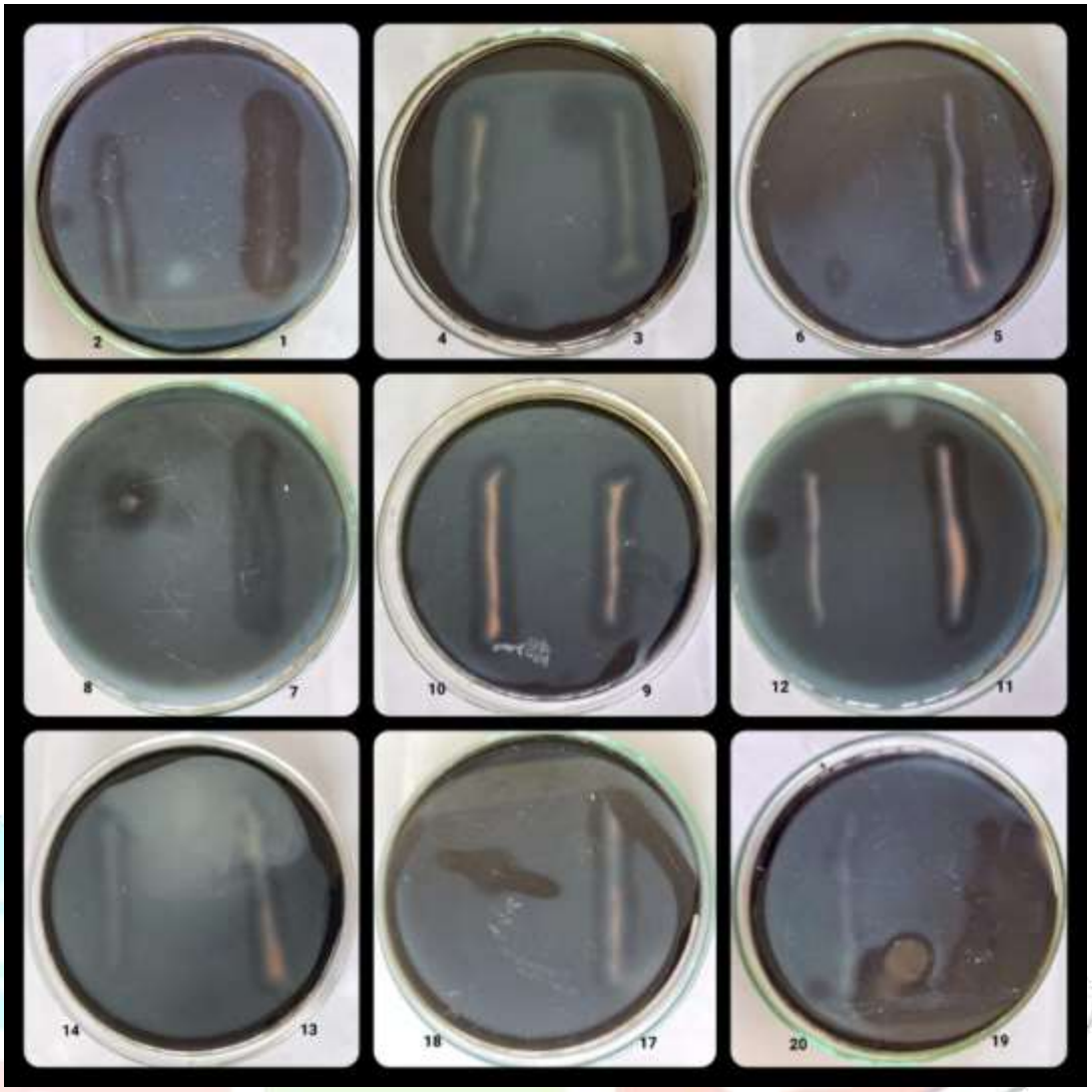
(c)

"Fig.1" (a), (b), (c): sampling sites, Kumbharwada, Bhavnagar, Gujarat.

"Fig.2": collection of samples



"Fig.3": colony morphology and its microscopic structure



"Fig.4": starch hydrolysis test

(a) **(b)**

(c)

"Fig.5" (a), (b), (c): gelatin hydrolysis test

"Fig.6": pectin hydrolysis test

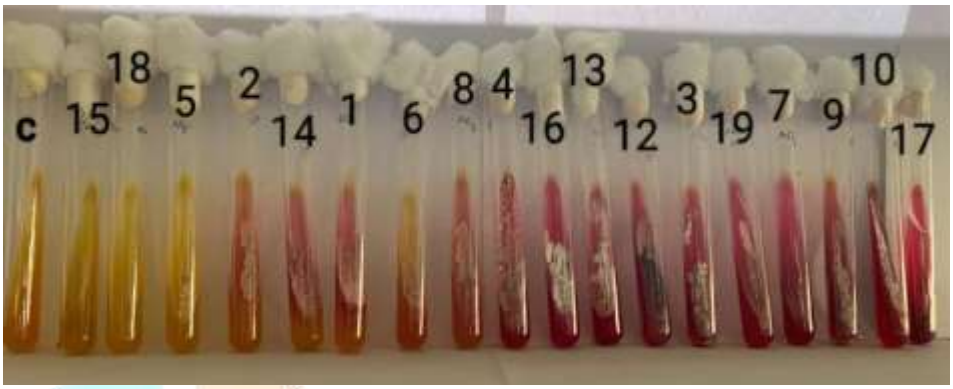
Table 1 extracellular enzymatic activity of all the isolates

Isolates	Amylase	Gelatinase	Pectinase
AD1	+	+	+
AD2	+	+	+
AD3	+	+	-
AD4	+	+	-
AD5	+	+	-
AD6	+	+	-
AD7	+	+	-
AD8	+	+	-
AD9	+	-	-
AD10	+	-	-
AD11	+	-	-
AD12	+	-	-
AD13	+	-	-
AD14	+	-	-
AD15	-	+	-
AD16	-	+	-
AD17	+	+	-
AD18	+	+	-
AD19	+	+	-
AD20	+	+	-

AD21	-	+	-
AD22	-	+	-
AD23	-	-	-

(+) = Positive enzymatic activity

(−) = Negative enzymatic activity



"Fig.7": TSI slants

Fig.8: pigment extractions of different isolates

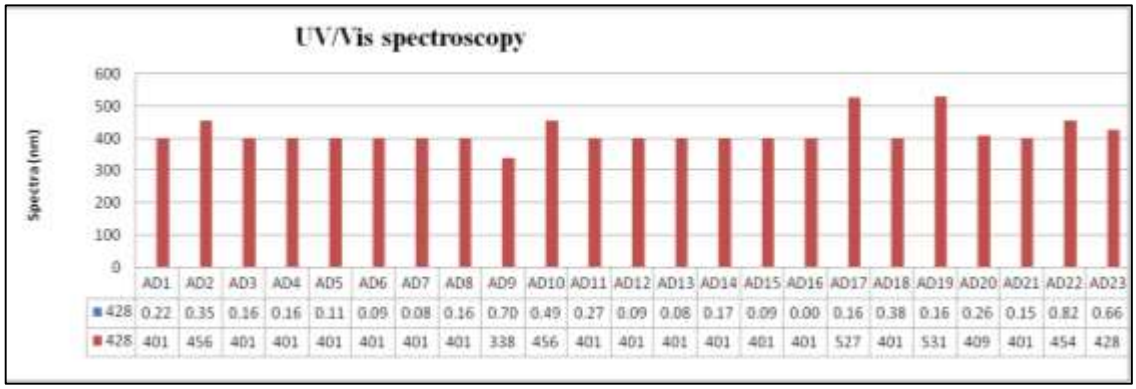


Chart 1 UV/Vis spectroscopy: highest peak at maximum wavelength (400-800nm)

IX. DISCUSSION

The present study indicates the successful isolation of pigment-producing *Actinobacteria* from soil and sediment samples collected from the Kumbharwada wetland, Bhavnagar. Wetland ecosystems are known to support diverse microbial communities due to their high organic matter content and fluctuating environmental conditions. Morphological and microscopic examinations revealed that the isolates exhibited characteristic actinomycetal features, including Gram-positive staining, filamentous and branched mycelia, and powdery colonies with a typical earthy odour. These observations are consistent with earlier reports on *Actinobacteria*, particularly members of the genus Streptomyces. The production of pigments by several isolates further supported their actinomycetal identity, as pigment formation is a common phenotypic trait of this group (Dholakiya et al., 2017). The isolates exhibited the wide range of pigment colours, indicating grey, brown, red, blue, and dark shades. Such variation in pigment production may be attributed to differences in genetic composition, nutrient utilization, and metabolic pathways among the isolates. Actinobacterial pigments are known to play protective roles against environmental stresses such as ultraviolet radiation and oxidative damage. The growing demand for eco-friendly and natural pigments has increased interest in microbial pigments, highlighting *Actinobacteria* as a promising alternative to synthetic dyes. Enzymatic screening demonstrated that a considerable number of isolates were capable of producing extracellular enzymes, including amylase, gelatinase, and pectinase. Amylase activity, indicated by starch hydrolysis, reflects the ability of *Actinobacteria* to degrade complex carbohydrates, a property of significant importance in the food, textile, and fermentation industries. Similar amylolytic activities have been reported previously in Streptomyces species. Gelatin hydrolysis observed in several isolates confirmed proteolytic activity through gelatinase production. Gelatinase-producing *Actinobacteria* contribute to protein degradation in natural ecosystems and have industrial relevance in leather processing, pharmaceutical applications, and waste management. Moreover, gelatinase activity serves as an important biochemical marker for the characterization of *Actinobacteria*. Pectin hydrolysis was detected in selected isolates, indicating their ability to degrade complex polysaccharides such as pectin. Pectinase production is particularly valuable in fruit juice clarification, textile processing and agricultural waste management. The presence of pectinolytic *Actinobacteria* in wetland samples suggests their ecological role in the breakdown of plant-derived organic matter and nutrient recycling within the ecosystem. The TSI biochemical test revealed variable sugar utilization patterns among the isolates. Acid production resulting from the fermentation of one or more sugars was indicated by changes in the slant colour, while the absence of black precipitate confirmed the lack of H_2S production. These variations in carbohydrate metabolism further highlight the physiological diversity of the isolated *Actinobacteria* and their adaptability to diverse nutrient conditions. Pigment extraction using ethyl acetate proved to be an effective method for recovering intracellular pigments from pigmented colonies. The use of an organic solvent solvent facilitated efficient extraction suggesting that the pigments are likely non-polar or moderately polar secondary metabolites. Spectrophotometric analysis conducted in the wavelength range of 400-800nm revealed distinct absorption peaks, confirming the presence of chromophoric compounds. Variations in maximum absorbance wavelengths among the pigments indicate chemical diversity, which may correspond to different functional properties and potential industrial applications. Similar to the findings of Parmar et al., (2016), the present study also reports pigment-producing *Actinobacteria* and is also in higher proportion. According to Dholakiya et al., (2017), pigmented Actinobacteria were isolated from marine sediments of the Gulf of Khambhat, emphasizing their antibacterial and antioxidant potential. In contrast, present study focuses on *Actinobacteria* isolated from wetland soil and sediment samples, along with significant extracellular activities. This difference may be attributed to variations in environmental conditions between marine and wetland ecosystems, influencing metabolic diversity and functional capabilities. Fernandes et al., (2021) reported the isolation of a single dominant Saccharomonospora azurea producing a greenish-blue pigment with bioactive properties. In contrary, *Actinobacteria* isolated from wetland soil and sample exhibits a wider range of pigment colours which highlights the greater functional and metabolic diversity of Kumbharwada wetland ecosystems. Kazi et al., (2022) reported pigment production by *Streptomyces* spp. with a strong focus on antimicrobial activity of the extracted pigments. In contrast, the present study emphasizes not only pigment production by multiple *Actinobacteria* isolated from wetland soil and sediment samples but also their extracellular enzyme production. This broader relevance of wetland derived *Actinobacteria* beyond pharmaceutical applications. Chart 2 depicts the comparative interpretation of previous studies with the present study.

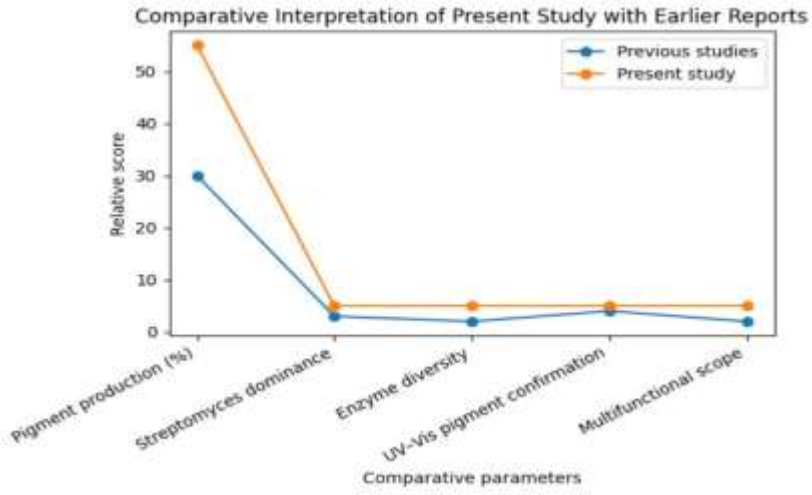


Chart 2 Comparative interpretation

X. ACKNOWLEDGEMENT

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