# Polymer producing bacteria showing siderophore activity with chrome azurol S (CAS) agar plate assay

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***Abstract-*** Biological waste water are characterized by the exposure of microorganism to transient conditions, where biomass is submitted to alternating periods of high and low substrate concentration and aerobic and anaerobic environments which make some microorganisms respond the production of polymers and various biochemical agents. Bacteria utilize siderophores to help the process of ferric iron uptake in environment. This process can found across three domains and is necessary for microorganisms to obtain iron needed for essential process. The present investigations were undertaken to check the siderphore producing activity of bacteria which produces polymer. A total of 640 bacteria were isolated from waste water pond. Among them 198 bacterial strains showing polymer production wee isolated. Out of which 12 potential strains were selected and investigated for siderphore activity. The result showed that out of 12 bacteria some of them showed siderophore activity.

***Index Terms***- Bacteria; polymer; Siderophore

## I. INTRODUCTION

Iron is most essential microelements for virtually all living cells and is usually abundant in the environment, particularly in soils and natural aquifers. However, its bioavailabitily is relatively low and with a dramatically decreased solubility of ferric species under physiological pH values owing to their complete hydrolysis. This results in the development of special biological regulated mechanism of Fe (III) solubilization e.g., involving specific naturals low-molecular weight chelating agents such as siderophore, which transport Fe (III) to the cell surface in the form of a complex with further Fe (III) release from the latter in the course of its reductive assimilation (kamnev et al., 2000). Many microorganism possess high affinity iron uptake system mediated by the action of low molecular weight iron chellators termed as siderophores (lankford, 1973; neilands 1981). Bacteria utilize siderophores to help the process of ferric iron uptake present in environment. This process is found in all three domains and is necessary for many microorganisms to obtain the environmental iron needed for various processes.

Dissolution of Fe minerals liberate two soluble forms of iron these are $Fe^{2+}$ and $Fe^{3+,}$ which can be used by microorganism and plants. Dissolution occurs depending upon water redox potential and PH. Under aerobic conditions at a neutral to alkaline PH, iron is present in the form of Fe(III) mineral, $Fe(OH)_3$, which is essentially insoluble. (Lindsay and schwab 1982). Under these conditions, organism that depend on the soil and water as their sole source of mineral nutrients are susceptible to Fe deficiency. Siderophores are classified by ligands used to chelate ferric iron. These include catecholates, hydroxamates, and carboxylates (6). Various assays has been developed to detect different phenotypes of siderophores (2–4). These assays are useful for the identification of various siderophores, numerous assays would has to be performed to detect all possible forms of siderophores. Schwyn and Neiland (8) developed a universal siderophore assay using chrome azurol S(CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators. chrome azurol S (CAS) agar effectively differentiated bacteria that are capable of exereting large amounts of siderophore. In the current investigation, we focused on performing siderophore activity for the all 12 isolates which were isolated based on polymer character.

## II. MATERIALS AND METHODS

### Sampling

The present investigation was under taken with an objective to select a promising native polymer producing strains. The samples were collected at four different sites of polluted pond and screened for potential PHA accumulators. The collection of samples and survey for PHA accumulating bacteria was done for two consecutive years. The screening was done regularly on monthly intervals to determine the variation in bacterial flora and the PHA accumulators simultaneously.

### Isolation, screening and maintenance of bacteria

The water samples 1 ml**,** were measured and mixed vigorously for 10 min. Samples were serially diluted ten folds before plating. A 0.1 ml sample of each dilution was surface spread on sterile Luria Bertani agar medium. After incubation of 48 h at room temperature, the colony forming units (CFU) were counted to check the total viable count. After the initial sampling, the incubated plates were used to estimate CFU/ ml sample. The colonies formed on these plates were also checked for pigment production. These pigments have much value due to their natural origin and industrial use.

All isolates accumulating PHA were maintained on Luria Bertani agar slopes as working cultures. Culture stocks were also maintained on Luria Bertani agar slopes, by sealing the tubes with paraffin wax. Preservation of cultures at 4°C was achieved by growing the isolates in 0.5 ml half strength Luria Bertani

broth in sterile capped vials. Glycerol was sterilized and 0.5 ml was added to the grown culture as a cryoprotectant and the vials were preserved at 4°C.

**Morphological characterization and microscopic observation**

The selected bacterial isolates were examined for their morphological features. The morphological characteristics were examined on their respective agar plates. The pure cultures from the slants were placed on the agar plates. After the growth of colonies morphological characters of the colonies like the colour, shape, size, surface, pigment production and gram staining etc. were recorded. All the 196 bacterial isolates were screened for their biochemical and enzymatic activities. Out of which 12 prominent PHA producers are accumulated.

**Preparation of glassware**

All the glassware used was cleaned with 3mol/L HCl to remove iron and rinsed in deionized water without exception (Cabaj and Kosakowska, 2007).

**Preparation of chrome (CAS)**

**The procedure given for making CAS agar by Schwyn and Neiland's original paper was followed:**

CAS agar plates were prepared by mixing a dye made of CAS, Fe, and hexadecyl-trimethyl-ammonium bromide (HDTMA) with M9-based growth media. For 1 L of CAS-agar, 100 mL of CAS-Fe-HDTMA dye was mixed with 900 mL of freshly prepared growth media. The CAS-Fe-HDTMA dye was prepared in advance as follows, for 1L: 10 mL of a 10 mM ferric chloride (FeCl3) in 100 mM hydrochloric acid (HCl) solution was mixed with 590 mL of a 1-mM aqueou solution of CAS. The Fe-CAS solution was then added to 400 mL of a 2-mM aqueous solution of HDTMA. The resulting CAS-Fe-HDTMA solution was autoclaved for 25 min in a polycarbonate bottle that had previously been soaked overnight in 10% (vol/vol) HCl then rinsed five times with MilliQ water. The CAS-Fe-HDTMA dye was stored at room temperature covered from light until use.

**The growth media was prepared as follows, for 1L of CAS-agar:** 30.24 g of 1,4-piperazine-diethanesulfonic acid (Pipes), together with 1 g of ammonium chloride (NH4Cl), 3 g potassium phos- phate (KH2PO4), and 20 g sodium chloride (NaCl) was dissolved into MilliQ water by adjusting the pH with 10 M NaOH to 6.8. As a solidifying agent, 9 g of agar noble (Difco) were added to the solution. We found that the more commonly used solidifying agents, agarose, and agar also led to a discoloration of the CAS dye, likely owing to higher phosphate content. The volume was adjusted to 860 mL, and the solution was autoclaved. After cooling, 30 mL of a sterile 10% (wt/vol) Casamino acids (Difco) aqueous solution and 10 mL of a sterile 20% (wt/vol) glucose aqueous solutions were added. Finally, the 100 mL of CAS-Fe- HDTMA were added to the growth media. The final concentrations of the CAS-agar components are as follows: 100 mM pipes,18mm $NH_4Cl$, 22mm $KH_2PO_4$. 2% (wt/vol) glucose, 10mm $FeCl_3$, 58mm CAS, 80mm HDTMA.

Conventional paper-disc agar diffusion assays were used for investigating, Sterilized filter paper discs, 10 mm in diameter, were placed on overnight agar plate in sterile conditions. A 10 µL supernatant achieved by centrifuging of fermentation broth of strain QM3 at 11,000 g for 15 min was diffused onto a paper disc. The uninoculated plates of CAS-agar as control were incubated under the same conditions as described previously. All these experiments were made at least three times with three replicates for each one. The CAS reaction was determined by measuring the position or distance of the advancing color-change front (in centimeter) in the CAS-blue agar, starting from the center of the paper-disc after incubation times. The clone diameter (CD) of strain QM3 (growth reaction) and orange halo diameter (HD) (CAS reaction) .

## III. Results and Discussion

Twelve isolates were selected to check the siderophore activity, the strains were grown in LB media. These isolates were then sub-cultured for isolation in pure culture form and CAS assay was performed using these different strains.

When the strains were incubated on CAS agar plates the following three responses were observed: no growth, growth but no halos surrounding colonies, and growth and small to large orange halos surrounding the colonies illustrated in figure-1. Results were visually distinct in terms of halo formation, because there was a contrast of orange halos against the blue medium. Very few polymer bacteria showed siderophore activity, Consequently in the presence of HDTMA, CAS is competitive in chelating the metal below neutral pH, while ferric hydroxide seems to have a higher stability at pH values above 7. When orange halos from isolates were compared with pure strain of Rhizobium meliloti it was found that the diameter of orange halos from all isolates were less than the pure strain.

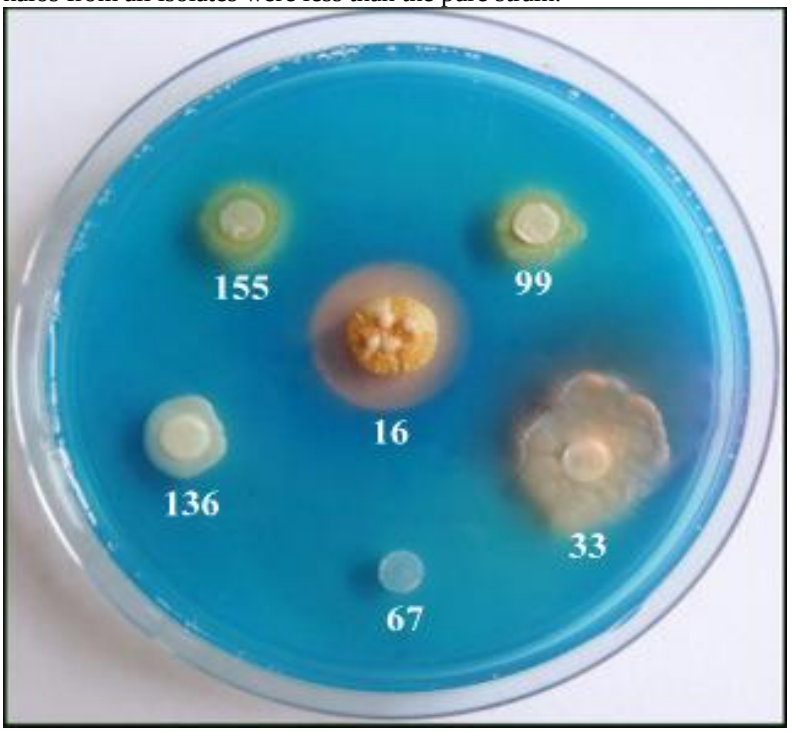


**Figure 1: Isolates showing siderophore activity by producing orange colour.**

Since the assay is based on the competitive exchange of iron (III), potential chelators are detectable corresponding to their affinity for the metal i.e., strong chelators like siderophores react in a 1: 1 ratio, while weaker ones need to be present in an excess. Hence, it is conceivable that, at least at higher concentrations, transition metal binding metabolites, especially antitumor antibiotics such as bleomycin, Adriamycin, and streptonigrin could also be detected.

However, it is difficult to grow fastidious microorganisms on the CAS agar plate and some ingredients of the CAS agar have innate antibacterial activity. The detergent HDTMA used in the preparation of CAS medium proved to be toxic to some bacteria as indicated by small halo formation in case of mixed rhizobial culture.

## IV. Conclusion

In conclusion, the CAS assay on solid medium can be a useful tool for screening siderophore producer microorganisms. Although very little polymer bacteria showed siderophore production. It is simpler and cheaper but can offset the effect of inoculation on siderophore by using paper-disc diffusion. We have shown that the choice of substrate is crucial for the evaluation of siderophore production of microorganisms. Particularly, strain QM3, a potential use in biocontrol, is evaluated to improve of siderophore production.

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