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# Use of the Modified Winogradsky Microcosm Technique to Help in Building an Indigenous Culture Collection of Iron and Sulphur Bacteria Valuable in Green Synthesis of Gold Nanoparticles

**Dabolkar Sujata[1]* and Kamat Nandkumar[2]**

***[1]Mycological Laboratory, Department of Botany, Goa University, Panjim, Goa, India***

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**ABSTRACT**

This paper describes an easy to use modified winogradsky microcosm (MWMC) technique for enrichment and isolation of useful chemolithotrophic bacteria which are increasingly employed in green synthesis of Gold nanoparticles. The technique was necessitated due to non availability of pure cultures of Iron and Sulphur bacteria in India and find out a way to help the scientists to build an indigenous culture collection of such useful chemolithotrophic bacteria. Winogradsky column is an example of an interdependent microbial ecosystem and is an excellent tool to determine the major bacterial communities in a sample. Winogradsky columns have been used extensively to demonstrate microbial nutrient cycling and metabolic diversity. This work was aimed at application of the Winogradsky Microcosm (WGMC) by modification using simple, low cost PET bottles to enrich microbial biofilms of Iron and Sulphur bacteria from different local mineral samples in order to isolate useful cultures of particularly *Acidithiobacillus, Leptospirillum* and *Sulfobacillus* spp. After prolonged incubation in dark followed by exposure to light, interesting coloured zones were identified in 24 columns from six samples indicating differential colonization of biofilms of Iron and Sulphur bacteria. The biofilms were carefully sampled, microscopically analysed, aseptically processed, enriched and pure cultures of iron and sulphur bacteria were successfully obtained on specific hyperacidic media. These cultures have excellent potential in bioleaching of gold sulphides and green synthesis of pure Gold nanoparticles. Considering the dependence of our country on foreign nations to acquire such rare, expensive and patented cultures, our technique is claimed to be potentially helpful to Indian researchers to build an indigenous collection of industrially useful, diverse and chemically creative strains of Iron and Sulphur bacteria. The results are presented and discussed.

***Key words :*** *Modified Winogradsky Microcosm (WGMC), Iron and Sulphur Bacteria, Green Synthesis, Gold*

## Introduction

The Winogradsky column was developed and named after Sergei Winogradsky (1856-1953), a Russian microbiologist. It is a miniature ecosystem that illustrate microbial succession of several groups of microbes. He studied the complex interactions between environmental conditions and microbial activities using soil enrichment to isolate pure bacterial cultures. The Winogradsky column is a miniature and nutrients interact over time. As oxygen diffuses downward from the surface, fermentation products from the breakdown of cellulose and hydrogen sulfide diffuse upward from the reduced lower zone (Prescott *et al.*, 1996). As microbial metabolites migrate within the column in response to various sul-

fate enrich various) are used by sulfate-reducing organisms such as Desul-fovibrio to produce hydrogen sulfide. The development of sulfate-reducing bacteria appear as blackened areas in the lower portion of the column and may even blacken zones throughout the sediment if sufficient aerobic bacteria are present to deplete the oxygen supply.

The sulfides are then used by anaerobic sulphides such as green sulfur, Chlorobium, and purple sulfur, Chromatium *Chlorobium* of this is seen *Chromatium* green patches in areas throughout the column as these phototrophs respond to gradients of light and sulfide. In nature, purple and green sulfur bacteria may be found in any fresh or marine waters as long as there is a sufficient supply of hydrogen sulfide and the water is clear enough so that light penetrates to the anoxic (anaerobic) zone (Madigan *et al.,* 1997). Ferric compounds may precipitate and appear as brown or greenish-gray deposits. The predominant iron-reducing bacteria within gleyed soils appear to be *Bacillus* and *Pseudomonas* (Atlas and Bartha 1993). *Bacillus* and *Pseudomonas* microaerophilic zone, rust-colored patches will appear, generally from the photoheterotrophs, such as the purple nonsulfur bacteria (*Rhodospirillum* and *Rhodopseudomonas*). These organisms trap light energy and use organic molecules as both electron and carbon sources. At the mud-water interface, where both hydrogen sulfide and oxygen are found, various aerobic, sulfide-oxidizing organisms such as Beggiatoa, Thiothrix and Thiobacillus may colonize. *Beggiatoa, Thiothrix* and *Thiobacillus*. Algae and cyanobacteria appear quickly in the upper portion of the water column, where sunlight is abundant. By producing oxygen, these organisms help to keep this zone aerobic. This watery top layer contains an aerobic of microbes-green algae, cyanobacteria, various aerobic bacteria, fungi and protozoa.

This paper describes an easy to use modified winogradsky microcosm (MWMC) technique for enrichment and isolation of useful chemolithotrophic bacteria which are increasingly employed in green synthesis of Gold nanoparticles. Winogradsky columns consist of sediment and water added to a clear container. They may also contain sources of carbon and sulfur. Naturally occurring microbes establish geochemical gradients inside the column based on their metabolic strategies. Algae and cyanobacteria which are oxygenic photosynthetic microorganisms grow at the surface. At the lower levels are anaerobic green and purple sulphur bacteria while obligately anaerobic heterotrophs appear at the lowest levels of the column. *Acidithiobacillus* obtain their energy through the oxidation of ferrous to ferric iron or through the reduction of inorganic sulphur compound to sulfate. *Acidithiobacillu s*is an autotrophic, acidophilic, *Acidithiobacillus* is single cells or occasionally in pairs or chains, depending on growth conditions (Bellenberg *et al.,* 2012; Khan *et al.,* 2012). Biofilms formed by chemolithotrophi cleaching microorganisms on metal sulfides surfaces have an important influence on dissolution of the substratum and consequently for bioleaching efficiency (Rohwerder *et al.,* 2003). It was found that this culture was able to show biofilm formation. This paper describes an easy to use MWMC technique for enrichment and isolation of useful chemolithotrophic bacteria which are increasingly employed in green synthesis of Gold nanoparticles. The technique was necessitated due to non availability of pure cultures of Iron and Sulphur bacteria in India and find out a way to help the scientists to build an indigenous culture collection of such useful chemolithotrophic bacteria. These cultures have excellent potential in bioleaching of gold sulphides and green synthesis of pure Gold nanoparticles. Considering the dependence of our country on foreign nations to acquire such rare, expensive and patented cultures, our technique is claimed to be potentially helpful to Indian researchers to build an indigenous collection of industrially useful, diverse and chemically creative strains of Iron and Sulphur bacteria.

## Methodology

### Sample collection

Samples were collected from the mining and non-mining areas in Goa. Samples were surface sterilized using 70% alcohol treatment on the exolithic surface of 70% (v/v) and flame sterilized near the spirit lamp. Samples were scrapped using sterilized scrapper till sufficient amount was obtained and collected on a sterile aluminium foil.

### Preparation of Winogradsky column

Sterile plastic bottles were filled with 1.5-3 inches with PVC bottles which included 3.81 cm to 7.62 cm of shredded filtre paper(sterilized), 10 g of each calcium sulphate, calcium carbonate and 1g of unsterilized soil sample. Column was filled carefully

with the sterile D/W to about ½ inches from the top and kept the bottle in dark for 1.27 cm away from the light. After two weeks the columns were kept in light and incubated for several weeks (Hairston, 1999).

**Sampling of the biofilm**

The sampling of the biofilm was carried out under sterile condition. The biofilms were carefully sampled by using alcohol sterilized plastic tube which were used to stipend out the liquid by applying suction and was transferred to another bottle without disturbing the biofilm. Alcohol sterilized scissor was used to cut the bottle to make two semi cylindrical parts which were further cut into coverslip size. Mounted on a slides and sealed with cover slip size, mounted on slides.

**Suspension preparation and enrichment**

The scrapped biofilm from each bottle of each sample were suspended into 10 ml tubes of 9k broth medium, i.e. The *Thiobacillus* cultures were isolated on *Thiobacillus ferroxidans* medium containing metal salts (g/100ml distilled water) : $FeSO_4.7H_2O$,4.42; $(NH_4)_2SO_4$, 0.30g; Kcl, 0.010 g; $K_2HPO_4$, 0.050g; $MgSO_4$,0.050 g; $CaNO_3$, 0.0010 g; agar, 3.5 g and pH (1.8-2.0)-dil $H_2SO_4$ (Starkey, 1935) and grown at 25-30 ˆC. *Leptospirillium* cultures were grown on medium containing metal salts (g/100 ml D/W): $FeSO_4.7H_2O$,1.4 g; Ammonium sulphate, 0.02g; $MgSO_4$, 0.04 g; $K_2HSO_4$, 0.010 g; Agar, 3.5 g; pH(1.9-2.4) and grown at 25-30 °C. *Sulphobacillus* cultures were grown on medium containing metal salts (g/100ml D/W): $FeSO_4.7H_2O$, 2.210 g; $(NH_4)_2SO_4$, 0.050 g; Sodium thiosulphate, 0.0248 g; $MgSO_4$, 0.030 g; Nacl, 0.02 g; $K_2HSO4$, 0.02 g; $CaNO_3$, 0.0070 g; Yeast extract; 0.002 g, pH (1.9) and grown at 25-30 °C. It was carried out under shacking condition at 1500 rpm for three weeks.

**Obtaining pure cultures**

Isolation of pure cultures of iron and sulphur bacteria were successfully obtained on specific hyperacidic media (9k medium) after two weeks. The pure cultures were studied by monochrome staining and microscopically crystal violet staining was carried out under light microscope examined using Nikon Eclipse E200 microscope with Nikon DS-fi2 camera and NIS elementmicroscope imaging software.

## Results and Discussion

Total 6 samples were used for the study from mining and non-mining areas in Goa. We were successful in use of Winogradsky Microcosm (WGMC) by modification using simple, low cost PET bottles. After prolong incubation in dark followed by exposure prolonged, interesting coloured zones were identified in 24 columns from six samples indicating differential colonization of biofilms of Iron and Sulphur bacteria (Fig 1a-f and Fig 1 g ,h) and as shown in Table 1. The Winogradsky column is a miniature ecosystem in which microorganisms and nutrients interact over time. As oxygen diffuses downward fromthe surface, fermentation products from the breakdown of cellulose and hydrogen sulfide diffuse upward from the reduced lower zone (Prescott *et al*. 1996).

The development of biofilms is initiated by microorganisms forming a monolayer attachment on the surface. Over time, this film becomes more complex with layers of organisms ofdifferent types colonizing the surface. Depending on the energy sources available, photosynthetic microbes, facultative chemo-organotrophs, or sulfate-reducing microorganisms may be present (Prescott *et al.*, 1996). *Acidithiobacillus* obtain their energy through the oxidation of ferrous to ferric iron or through the reduc-

**Table 1.** Differential colonization of biofilms of Iron and Sulphur bacteria

| Sample | Sterile mixture | Sterile mixture + sterile sample | Sterile mixture + Unsterile sample | Unsterile mixture + Sterile water |
|---|---|---|---|---|
| Banded Haematite Quartzite (BHQ) | - | - | + + | ++ |
| Banded magnetite Quartzite (BMQ) | - | - | ++ | ++ |
| Mining rejects | - | - | +++ | +++ |
| Laterite | - | - | +++ | +++ |
| Vermicast | - | - | +++ | +++ |
| Stream sediment | - | - | ++ | ++ |

tion of inorganic sulphur compound to sulfate. Similar studies were carried out by Janet and Holmes 1988. *Acidithiobacillus thiobacillus* is an autotrophic, acidophilic, mesophile occurring in single cells or occasionally in pairs or chains, depending on growth conditions (Bellenberg *et al.*, 2012; Khan *et al.*, 2012). Biofilms are interface-associated colonies of microorganisms embedded in a matrix of extra-

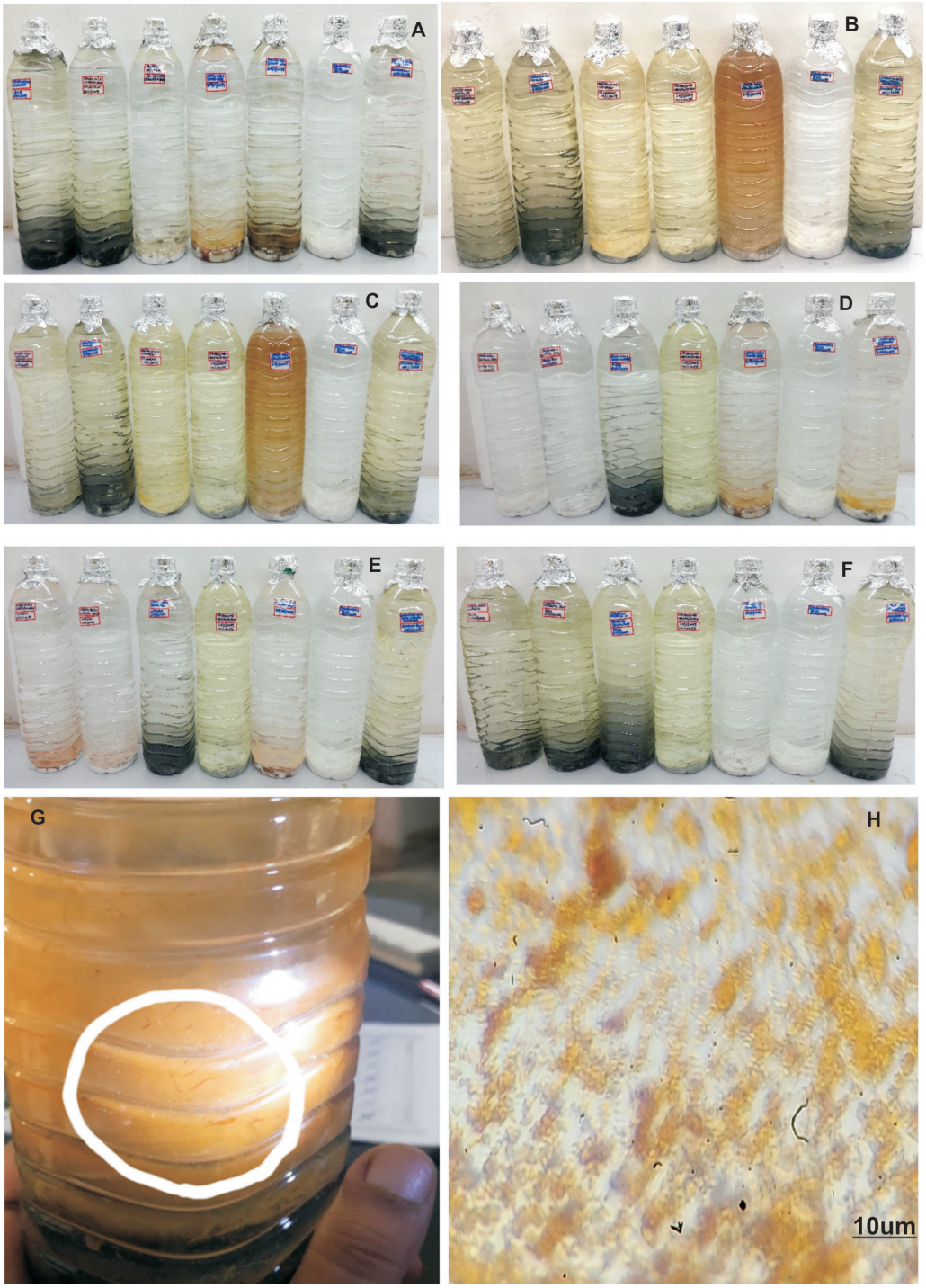


**Fig. 1(a-h).** a-f: Several weeks after incubation and formation of Biofilms in different samples; g: Differential colonization of biofilms of Iron and Sulphur bacteria. h: Micromorphology of the g zone

cellular polymeric substances (EPS) consisting of biopolymers like polysaccharides, proteins, lipids and nucleic acids (Costerton *et al.,* 1995; Flemming and Wingender, 2010). Biofilms formed by chemolithotrophic leaching rowing on the *lepstopirillium* microorganisms on metal sulfides surfaces have an important influence on sulphides of the substratum and consequently for bioleaching efficiency (Rohwerder *et al.,* 2003). It was found that this culture was able to show biofilm formation as seen in Fig. 2a. The Gram negative, rod shape bacteria can be seen in Fig. 2e. Spiral cells of *Leptospirillum* (Fig. 2c) and *sulphobacillus* cells (Fig. 2d) were observed. *Sulphobacillus* colonies

We were successful in use of Winogradsky in research experiments (Hairston, 1999; Loss *et al.,* 2013; Parks, 2015). Successful in enrichment of microbial biofilms of Iron and Sulphur bacteria from different local mineral samples. Similar studies were carried out previously (Rawlings *et al.,* 2013; Olson *et al.,* 2003; Brierley and Brierley, 2013; Gottschal and Kuenen, 1980). This is the first report on isolation of iron and sulphur bacteria from mining and non-Iron Sulfur. Interesting coloured zones were identified in 24 columns from six samples indicating differential colonization of biofilms of Iron and Sulphur bacteria. we were successful to isolate useful cultures of particularly *Acidithiobacillus, Leptospirillum* and *Sulfobacillus* spp. These cultures have excellent potential in bioleaching of gold sulphides and green synthesis of pure Gold (Johnson, 2014; Hedrich *et al.,* 2020). The abilities of acidophilic chemolithotrophic bacteria and archaea to accelerate the oxidative dissolution of sulfide minerals have been harnessed in the development and application of a biotechnology for extracting metals from sulfidic ores and concentrates. Biomining is currently used primarily to leach an oxidative pretreatment for refractory gold ores. Recent developments have included using acidophiles to process electronic wastes, to extract metals from oxidized ores, and to selectively recover metals from process waters and waste streams (Johnson, 2013; Ehrlich, 2001; Van Aswegen *et al.,* 2007; Rawlings and Johnson, 2007). Our technique is claimed to be potentially helpful to Indian researchers to build an indigenous collection of industrially useful, diverse and chemically creative strains of Iron and Sulphur bacteria.

## Conclusion

Biomining is well established as a niche technology for extracting metals from low-grade and polymetallic base metal ores, and refractory gold ores. As energy and environmental constraints become more demanding, there will be greater need

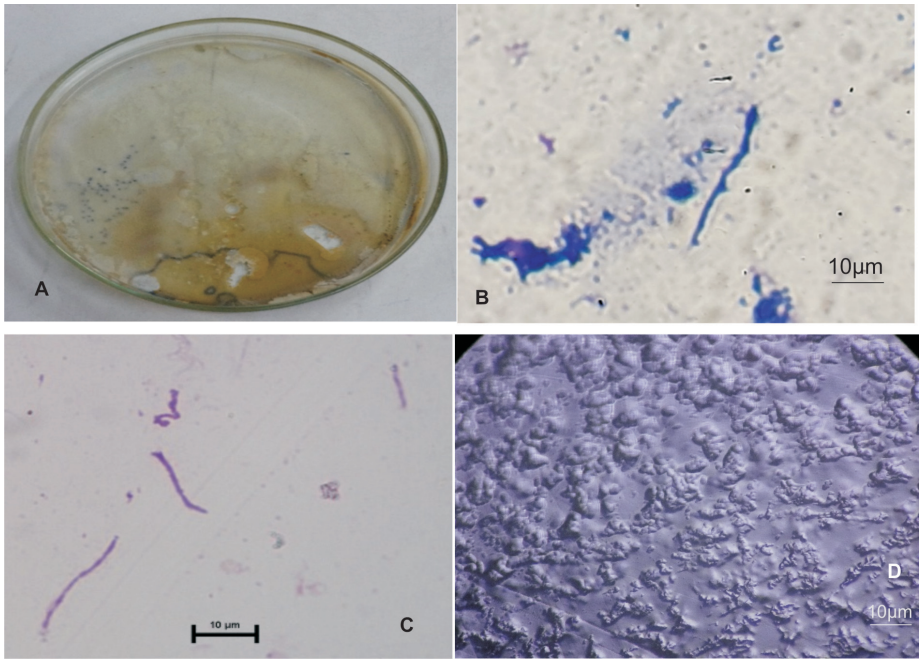


**Fig. 2**. a. Representative plate of presumtive *Acidithiobacillus* culture; b. *Acidithiobacillus* wild type cells; c. *Leptospirillum* cells; d. *Sulphobacillus* colonies

and incentives to reprocess relic mine wastes (which often contain greater concentrations of metals than readily accessible ore bodies) and to recover and recycle metals from electronic and other metallic wastes and metal-laden waste waters, while in situ biomining could allow deeply buried ore bodies to be economically exploited. Although a number of unresolved challenges in conventional biomining remain, such as bioleaching chalcopyrite and using brackish and saline water for bio-processing minerals, recent innovations, such as the reductive bioprocessing of oxidized metal deposits, suggest that new opportunities for developing biotechnologies in the mining and mineral sectors will emerge in the near future.

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