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## RESEARCH ARTICLE

# SIGNAL TRANSDUCTION OF ATP SYNTHASE BY SULFUR REDUCING METHANOGEN UNDER THE HUMIC ACID SUPPLEMENTATION

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**ABSTRACT**

The present study was the humic acid (HA)-oxidizing and the reduced organisms isolated from different localities of sewage samples in and around the 12 district of Tamil Nadu. The additional electron donating capacity of reduced HA could reasonably be attributed to the oxidation of reduced functional groups. Furthermore, this study indicates that reduced humic acids impact soil geochemistry and the indigenous bacterial community, on the basis of α, β, γ, and σ – Proteobacteria were includes heterotrophs, autotrophs, and methanotrophs. Among them methanotrophic sulfur reducing were isolated and screened by humic acid supplementation under the anaerobic fermentation. Signal transduction of ATP synthase by $H_2$ electron donor and acceptor, the donation $H_2$ molecule during the cell division by inducing the activity of metabolic enzyme binding and non-binding site of the cell wall in the presence of humic acid. However, further studies of metabolic interaction of the bacterial cell under humic acid supplementation traumatically changed in bacterial growth and morphology of the color have become a typical blue/greenish florescent in distinctly appear



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## INTRODUCTION

The archaea is phylogenetically distinct from the eubacteria, and three basic phenotypes are recognized: methanogenic, halophilic, and sulfur-dependent thermophilic types (Woese, C. R., and G. J. Olsen. 1986). Euryarcheota are probably the best known, including many methane-producers (methanogens) and salt-loving archeans (halophiles). Crenarcheota includes those species that live at the highest temperatures of any known living things, though a wide variety which has recently been discovered is growing in soil and water at more moderate temperatures (thermophiles). Not much is known about them Korarcheota except for their DNA sequences as they have only recently been discovered. Structurally diverse, HA contains numerous functional moieties, including carboxylic acid, ketone, quinone, and phenolic/alcoholic hydroxyl groups (Stevenson, 1994; Boyer *et al.,* 1996 and Schulten *et al*., 1991).

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Microbial population plays an important role in the biosphere, particularly in the areas of Meta elements biotransformation and biogeochemical cycling, metal and minerals like humic acid in the soil. All kinds of microbes, including prokaryotes and eukaryotes and their symbiotic associations with each other and 'Archaea', can contribute actively. Interestingly, Archaea too many such geomicrobial processes are transformations of humic acid metals and minerals. Microbes have a variety of properties that can effect changes in the cell wall modification and expression color change based on the mineral dissolution or deterioration. Such mechanisms are important components of natural biogeochemical cycles for metals as well as associated elements in biomass, soil, rocks and minerals, e.g. sulfur and phosphorus, and metalloids, actinides and metal radionuclides. Metals constitute about 75% of the known elements composted of humic contents such as Ag, As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Se, Tl, Zn Sparks, (2005). Apart from being important in natural biosphere processes, metal and mineral transformations can have beneficial or detrimental consequences in a human context.Microbial biofilms can interact with metals and humic content elements (Ferris *et al*., 1989; Polprasert & Charnpratheep, 1989). Biofilms can be

defined as microorganisms and their extracellular products are associated with a substratum (McFeters, 1984). Chemotaxonomic procedures such as cell wall analyses have not been widely used in the identification of methanogens. Cell wall composition (pseudomurein, protein, heteropolysaccharide) can be used to assign as isolate to family and in some cases genus level (Balch *et al.,* 1979), but a more detailed analysis of each cell wall type would be required for this approach to be of general use at genus and species level. Typically 20-30% of membrane associated protein is soluble in water and is loosely associated. The other 70-80% is tightly bound to the membrane, often spanning both sides.

These proteins are also often amphipathic molecules (contain both hydrophobic and hydrophilic portions) with stretches of hydrophilic amino acids and stretches of hydrophobic amino acids. The humic acid transforms from hydrophilic to hydrophophic region of cell wall. Interestingly, metal element goes oxidized into the cell wall region of the electron donor to changes color for the cell morphology. Most of them are placed in the membranes so that the hydrophobic amino acids associate with the lipids in the membrane and the hydrophilic amino acids are outside the membrane interacting with either the cytoplasm or the periplasm contains the majority of purple bacterium is G-bacteria. It is extremely diverse, embracing heterotrophs, chemolithotrophs, and chemophototrophs. Some genera are anaerobic; others are aerobic. The purple non sulfur bacteria is generally photoheterotrophic and anaerobic. Some are photo lithotrophic and it use molecular hydrogen. Others using sulfide are elemental S as the electron donor, with $CO_2$ as the source of cell carbon. Most species, however, are intolerant to Sulfide even at low concentrations; they depend on sulfate for their cell S. The purple non-sulfur bacteria are widely distributed in waters and moist soils. Six genera are recognized; *Rhodospirillum, Rhodopila, Rhodobacter, Rhodopseudomonas, Rhodomicrobium*, and *Rhodocyclus*. Also included in the purple bacteria and of great importance in ecosystem process are the nitrifying bacteria and the Fe and Mn oxidizers.

**Methanogenic soil microbes reducing Sulphur compounds**

Archaea are separable from bacteria both by their molecular phylogeny and by their phenology. Taxonomically the Archaea are divided into two kingdoms and five subgroups. In general the archaea are tolerant in extremely harsh environments. Cell membranes of archaea are unique. Their branch-chained, either-linked lipids differ greatly from those of all other life forms. The basic structure is a 5-C isoprene unit. These are linked to form chains of up to 20 C sometimes as many as 40 C chain. Chains are either linked to glycerol, not steer linked as in bacteria and eucarya. Halophiles have glycerol diether units; methanogens have mixed glycerol- diether and diglycerol-tetraether units. In thermophilic archaea, the tetra ether membrane is predominant. Soil microbes, bacteria, archaea, and fungi play diverse and often crucial roles in these ecosystem services. The vast metabolic diversity of soil microbes means their activities drive or contribute to the cycling of all major elements (e.g. C, N, P), and this cycling affects the structure and the functions of soil ecosystems as well as the ability of soils to provide services to people. *Thermococcus* and *Pyrococcus* are very similar except for a difference in their growth temperatures. *Thermococcus* grows optimally at 83°C and *Pyrococcus* grows at 100°C. Both are obligate anaerobes and chemorganotrophs, using sugars and complex carbon compounds and reducing $S_0$ to $H_2S$. Cells can initiate growth in the absence of $S_0$ but $H_2$ accumulates and inhibits further growth.

**Methanogens**

Anaerobic methanogens are unique in their ability to produce $CH_4$ as a metabolic product. Methane emissions occur from swamps, marshes, and marine sediments; from the intestinal tracts and rumens of animals; and from sludge digesters in sewage plants. In sewage digesters methane is produced in such a quantity that it is commonly harvested for commercial use. Emissions from natural sources escape to the atmosphere. Methanogens do not use sugars as a source of cell C. Carbon dioxide is commonly using the C source. The C atom of $CO_2$ sub is reduced to $CH_4$ by electrons derived from hydrogen.

**Thermophiles**

The extreme thermophiles are *Thermococcus, Archaeoglobus, Thermoplasma,* and *Pyrococcus*. *Archaeoglobus* is strictly anaerobic and chemorganotrophic, catabolizing sugars and simple peptides, using sulfate as the electron acceptor, reducing it to sulfide. *Thermoplasma* is facultative anaerobic and grows best at pH 1.5 and 60°C. Aerobically it grows poorly on sugars. The genus does not have an external cell wall in the cell membrane.

## MATERIALS AND METHODS

**Site description and sampling**

The sewage samples were collected from different localities of all over the urban area of Tamil Nadu. The places where the test are carried outs are Perambalur, Madurai, Tiruchirappalli, Tirunelveli, Nagarkovil, Salem, Coimbatore, Chennai North, Chennai South, Kadalur, Neivally, and Krishnagiri.

**Humic acid**

Humic acid is biopolymers that are formed besides other humic substances (fulvic acid, humin) during the degradation of biological material to supply with appropriate electron donors, during the cell division of bacterial colonies. Soon after their discovery by the German chemist Karl Franz Achard (1753-1821), humic acids were recognized as general and essential constituent of agriculturally utilizable soils.

**Growth medium**

Methanogen organisms were grown in typical composition medium, by using different supplementation such as acetate, lactate and humic acid in (Table-1). The essential chemical compound supplementation was Cysteine hydrochloride, 0.027 g; 0.1% resazurin with sodium acetate 0.041g;

supplementation -2 sodium lactate 11.2ml (10%); supplementation 1 and 2 with 0.001% Humic acid (AGROS ORGANICS; CAS: 68131-04-4, New Jersey, USA) in the media. Cysteine hydrochloride was added to liquid media and the pH was adjusted to 7.5. After sterilization in the autoclave, the pH was 7.0±0.1 (Bryant *et al*. 1968): Methanobacterium strain was a gift of Barathidasan University, Tiruchirappalli, and Tamil Nadu. All other methanogen organisms were isolated from domestic sewage sludge obtained from an anaerobic sewage digester. They were identified on the basis of micro and macroscopic morphology, methane production, and the substrates utilized as precursors for methane biosynthesis.

Archaea build upon the similar structures as other organisms, but they build them from different chemical components. For instance, the cell walls of all bacteria contain the chemical peptidoglycan. Archaeal cell walls do not contain this peptidoglycan compound, though some species contain a similar one. The cell walls of archaea bacteria are distinctive from those of eubacteria. Archaebacterial cell walls are composed of different polysaccharides and proteins like pseudomurin and no peptidoglycan. Humic substances (Humic acid) represent the main carbon reservoir in the biosphere, estimated at 1600 x 101 g C. Due to their crucial role in reductive and oxidative reactions, sorption, complexation and transport of pollutants, minerals and traceelements, plant growth, soil structure and formation, and control of the biogeochemistry of organic carbon in the global ecosystem, HS are then extremely important to environmental processes (Grinhut *et al*, 2007).

## RESULTS AND DISCUSSION

### Identification of methanogen bacteria from sewage samples

Colonies of methanogen bacteria were identified on petri plates by taking advantage of a fluorescent pigment peculiar to this metabolic group of bacteria. Cheeseman *et al*. 1972 (Fig-3a-e). Other potential effects of HAs on microbial communities are structure stabilization: buffering the changes in size or abundance of some microbial groups by chelating unavailable nutrients (thus making them available) and buffering pH (Mackowiak *et al*., 2001; Pertusatti and Prado, 2007). Additionally, HAs may reduce negative effects of direct application of urea and other chemical fertilizers on soil bacteria or fungi.

The buffering of pH is an important determinant of AOB and total bacteria community structure (Frostegård *et al*., 1993; Pennanen *et al*., 1998; Kelly *et al*., 1999; Enwall *et al*., 2007) have shown to buffer pH between 5.5 and 8.0 (Pertusatti and Prado, 2007). So our hypotheses that HAs can buffer the community change caused by increasing or decreasing pH.

**Table. 1. Three different media composition**

**Acetate**

| | |
|---|---|
| Na2CO3 | 100.0mg |
| KH2PO4 | 130.0mg |
| K2HPO4 | 140.0mg |
| NH4Cl | 140.0mg |
| MgSO4 | 50.0mg |
| Na2HCO3 | 35.0mg |
| Fe(NH4)2(SO4)2 | 10.0mg |
| Cystein-Hcl | 27.0mg |
| Yeast extract | 50.0mg |
| Peptone | 50.0mg |
| Agar | 2.0g |
| Sodium acetate | 41.0mg |
| pH | 6.5-6.8 |
| Vitamin tablet | ½ tablet |
| Resarzurin | Pinch |
| Methanol | 5ml |
| Triethanolamine | 5ml |
| Pencillin | 5ug/ml working concentration |
| Na2S.95H2O | 5ml from 30% stock |
| Glycerol | 5ml |
| Good day biscuit extract | 5ml |

**Lactate**

| | |
|---|---|
| Sodium lactate | 11.2ml |
| Na2CO3 | 100.0mg |
| KH2PO4 | 130.0mg |
| K2HPO4 | 140.0mg |
| NH4Cl | 140.0mg |
| MgSO4 | 50.0mg |
| Na2HCO3 | 35.0mg |
| Fe(NH4)2(SO4)2 | 10.0mg |
| Cystein-Hcl | 27.0mg |
| Yeast extract | 50.0mg |
| Peptone | 50.0mg |
| Agar | 2.0g |
| | |
| pH | 6.5-6.8 |
| Vitamin tablet | ½ tablet |
| Resarzurin | Pinch |
| Methanol | 5ml |
| Triethanolamine | 5ml |
| Pencillin | 5ug/ml working concentration |
| Na2S.95H2O | 5ml from 30% stock |
| Glycerol | 5ml |
| Good day biscuit extract | 5ml |

**Humic acid**

| | |
|---|---|
| Sodium lactate | 11.2ml |
| Na2CO3 | 100.0mg |
| KH2PO4 | 130.0mg |
| K2HPO4 | 140.0mg |
| NH4Cl | 140.0mg |
| MgSO4 | 50.0mg |
| Na2HCO3 | 35.0mg |
| Fe(NH4)2(SO4)2 | 10.0mg |
| Cystein-Hcl | 27.0mg |
| Yeast extract | 50.0mg |
| Peptone | 50.0mg |
| Agar | 2.0g |
| Sodium acetate | 41.0mg |
| pH | 6.5-6.8 |
| Vitamin tablet | ½ tablet |
| Resarzurin | Pinch |
| Methanol | 5ml |
| Triethanolamine | 5ml |
| Pencillin | 5ug/ml working concentration |
| Na2S.95H2O | 5ml from 30% stock |
| Glycerol | 5ml |
| Good day biscuit extract | 5ml |
| Humic acid | 0.001% |

**Identification of methanogen bacteria by supplementation of humic acid**

Colonies of methanogenic bacteria isolation and were identified with petri plates by taking advantage of a blue and green fluorescent pigment peculiar to this metabolic group of bacteria which was found in the presence of humic acid (AGROS ORGANICS; CAS: 68131-04-4, New Jersey, USA). Cheeseman *et al.* (1972) reported that strain M.o.H. synthesizes a low-molecular weight compound, F420, which in the oxidized form fluoresces when excited by long-wave of ultraviolet rays. During active metabolism, 80% of F, 20 exists in the oxidized form (A. M. Roberton, personal communication). F420 is cell bound, and therefore its fluorescence is confined to the colony. The large amount of F420 in the cell and its high extinction coefficient prompted us to determine whether it could be visualized in colonies of strain M.o.H. There was ample F420 to permit direct visual observation of the methanogenic colonies when they were in petriplate have florescence blue and green (Fig. 3 a-e). Fluorescence due to F420 is typically blue-green and is readily distinguishable from the plate.

In all cases only colonies containing methanogenic bacteria fluoresced blue-green. This was proven by sub- culturing all fluorescent colonies into liquid media, incubating them anaerobically, and assaying for methane. Fluorescent colonies were not always pure, but they were all methanogenic. *Methanobacterium* strain M.o. H., *Methanospirillum, Methanobacterium formicicum*, and *Methanosarcina* have been identified by this procedure. It is possible to identify colonies of methanogenic bacteria when they are less than 0.5 mm in diameter with the use of a dissecting microscope or hand lens and a white light. This identification technique offers a simple, rapid, and sensitive method for isolation and enumeration of methanogenic bacteria. Methanogenic colonies can be identified in the plate, but the fluorescence as intense as that observed in the petri plates. The δ-proteobacteria contains mainly sulphate and iron reducing bacteria. In soil the sulphate reducer *Desulfovibrio* grows anaerobically with carbon sources such as lactate or ethanol, which occure in soil where oxygen is depleted due to organic matter decomposition *Bdellovibrio*, a bacterial parasite, also belongs to this group. Similarly we have to introduce carbon sources such as lactate, acetate with humic acid.

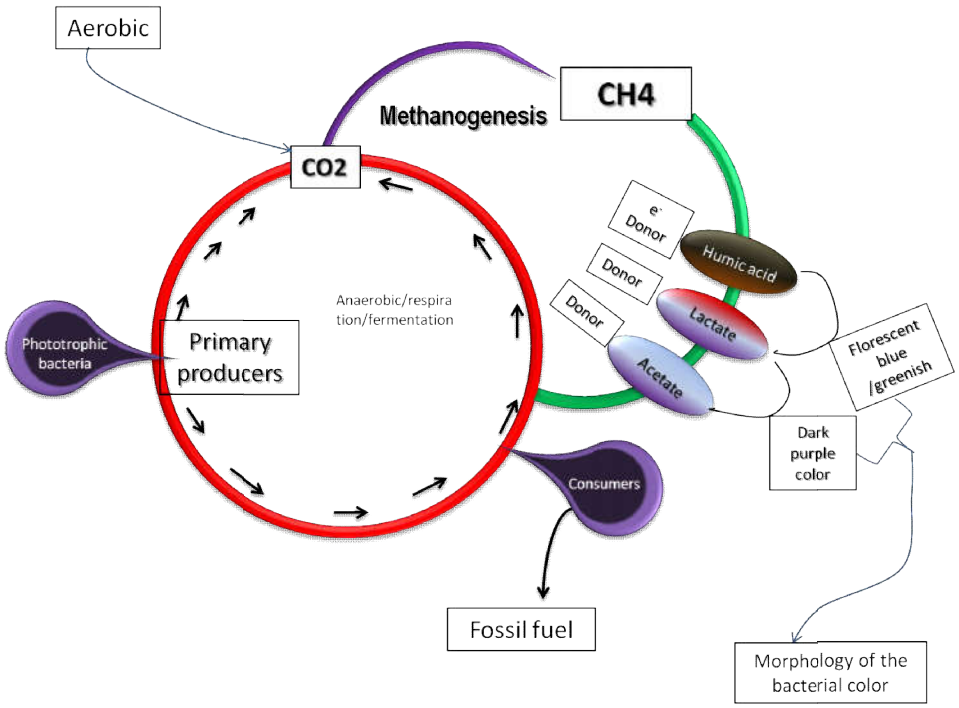


**Fig.1. Role of different $H_2$ electron donor molecule interacts with cell wall metabolism and carbon cycle under the anaerobic methanogen (Draw: Dr.N.Hariram)**

**Acetate supplementation** **Lactate supplementation**

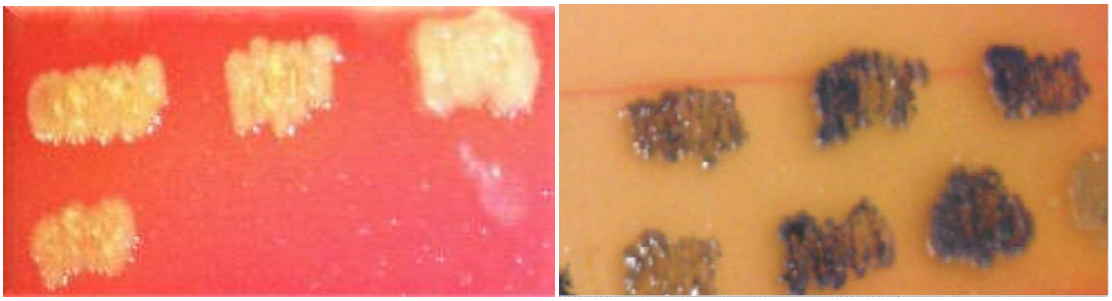

**Fig. 1. 120 hrs Methanogen bacteria**

**Plate-2 120hrs Methanogen bacteria 48hrs culture for supplementation of humic acid with Lactate**

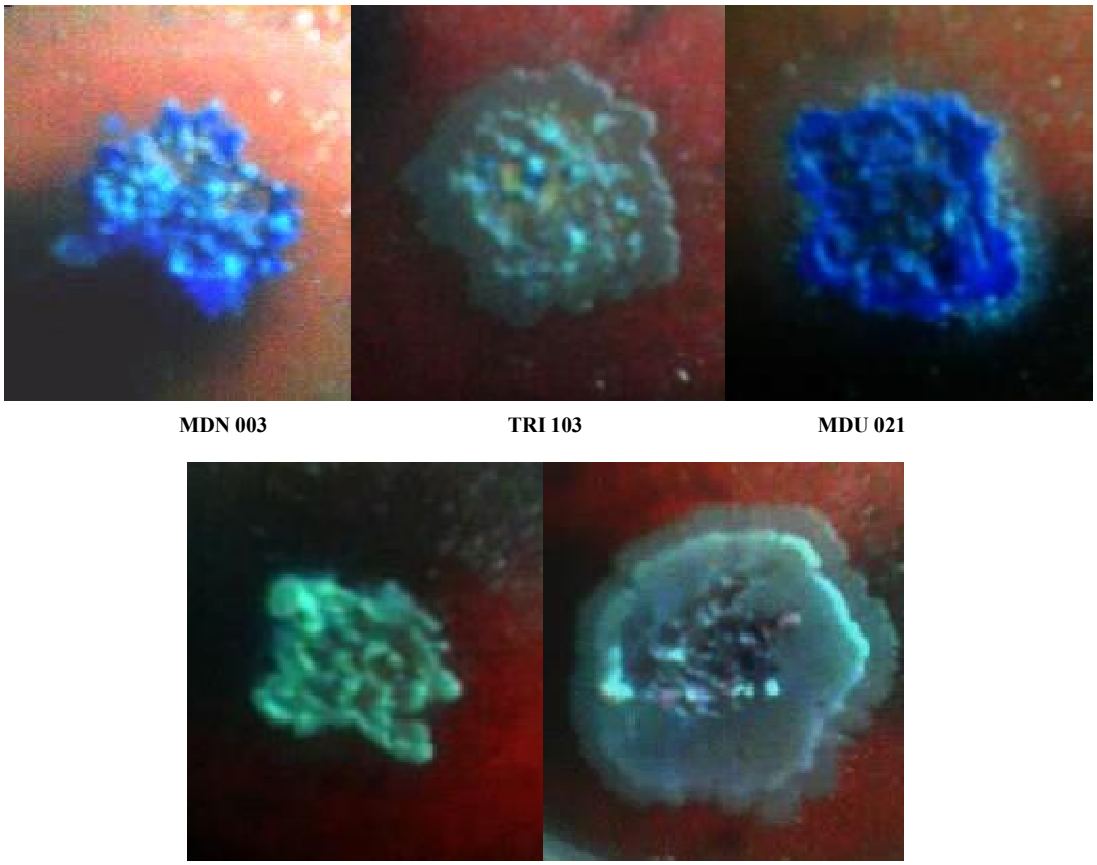


**Fig. 3. Different isolates of methanogen bacteria in Tamil Nadu city (MDN 003, TRI 103, MDU 021, COag019 and SLM011)**

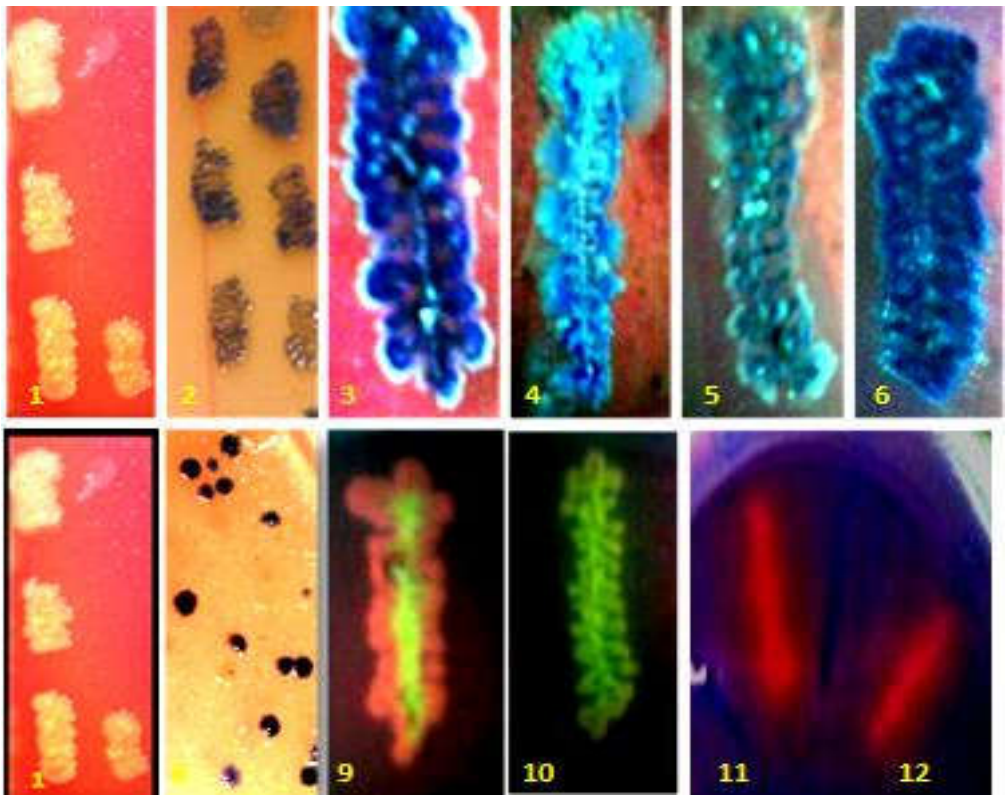


**Fig.4. Humic acid oxidizing and reducing methanogenic bacteria were shown in 1-12 plates. 1 & 7 Acetate supplementation, 2 & 8 Lactate supplementation, 3-6 Humic acid supplemtation with lactate. And 9 & 10 UV light expression compare to Sulphur reducing and oxidizing isolate and 11-12 without humic acid supplementation in the media (no differentiation)**

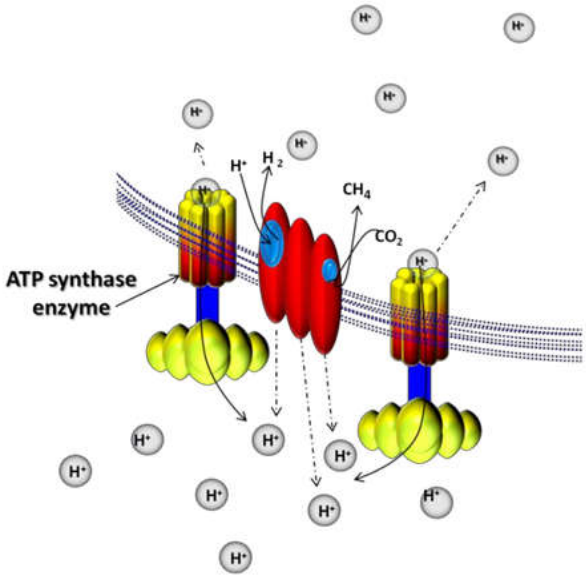


**Fig. 5. Dramatically the $H_2$ electron donor to induce the activity of metabolic enzyme binding and non - binding site of the cell wall under the supplementation of humic acid**

**Table 2. Isolation of methanogen bacteria from sewage samples of urban area of Tamil Nadu city and calculate the percentage of bacterial population**

| S.No | Sources of sample | | Dilutio n Factor | Number colonies/plate | | CFU/ml | Percentage of methanogenic bacterial colony | | Color of the colony (Blue/greenish blue) |
|---|---|---|---|---|---|---|---|---|---|
| | Place | Code | $10^{-7}$ | Methan ogen | Other colonies | $^3$ | Total No. Colo nies | % of Methanog en. colonies | |
| 1 | Perambalur | PLR-001 | $10^{-7}$ | 14±001 | 33±001 | $3.5 \times 10^7$ | 47 | 29.7 | 14±001 |
| 2 | Madurai | MDU-021 | $10^{-7}$ | 19±001 | 19±001 | $3.5 \times 10^7$ | 38 | 50.0 | 19±005 |
| 3 | Trichy | TRI-103 | $10^{-7}$ | 15±003 | 22±005 | $3.5 \times 10^7$ | 37 | 40.5 | 12±003 |
| 4 | Tirunelveli | TRE-004 | $10^{-7}$ | 10±002 | 29±002 | $3.5 \times 10^7$ | 39 | 25.6 | 8±003 |
| 5 | Nagarkovil | NGK-005 | $10^{-7}$ | 29±003 | 18±001 | $3.5 \times 10^7$ | 47 | 61.7 | 19±003 |
| 6 | Salem | SLM-011 | $10^{-7}$ | 19±001 | 12±002 | $3.5 \times 10^7$ | 31 | 61.2 | 12±001 |
| 7 | Coimbatore | COag-019 | $10^{-7}$ | 18±004 | 19±005 | $3.5 \times 10^7$ | 37 | 48.6 | 11±002 |
| 8 | Chennai North | MDN-003 | $10^{-7}$ | 21±001 | 24±003 | $3.5 \times 10^7$ | 45 | 46.6 | 15±005 |
| 9 | Chennai South | MDS-004 | $10^{-7}$ | 20±002 | 19±002 | $3.5 \times 10^7$ | 39 | 51.28 | 18±001 |
| 10 | Kadalur | KAD-005 | $10^{-7}$ | 26±003 | 19±002 | $3.5 \times 10^7$ | 45 | 57.7 | 18±001 |
| 11 | Naively | NVL-006 | $10^{-7}$ | 33±002 | 33±002 | $3.5 \times 10^7$ | 66 | 50.0 | 28±002 |
| 12 | Krishnagiri | KRG-01 | $10^{-7}$ | 27±001 | 20±001 | $3.5 \times 10^7$ | 47 | 57.5 | 10±001 |

** indicate more than 5 times experiment was continued using humic acid supplementation.

This is carbon containing organic molecule as electron donar and acceptor in cell wall metabolism of methanogenic organism (Fig. 5)

## Functions of anaerobic microbes

There are three types of functions in anaerobic microbes under the humic acid supplementation (1). Why color changes take place in bacterial morphology? (2) Why become farmer/nursery people using organic/sewage compost? And (3) given the reasons for bacterial function and uptake of plant system. Interestingly, electron donor and acceptor for the metabolic enzyme activity due to the function of cell wall membrane to express the color pigmentation of blue and greenish of all methanogenic bacterial colonies.

Although, farmer and nursery people were used as compost routinely, because of compost have rich in humic content naturally for the formation of biological, physiological, and geologically changes the pyramid compound by oxidation and reduction. The microbes utilize metabolic compound and changes and expression of enzyme molecule blue/greenish pigmentation.

**Conclusions**

According to the system described it offers a number of practical advantages in culturing methanogenic bacteria. Media are prepared by standard aerobic methodology, thus eliminating time-consuming anaerobic preparation methods. Special culture techniques are not required, and additional sensitivity and speed are gained by superior visibility and accessibility of colonies in high-density cultures. The use of ultra-blue light fluorescence and green fluorescence selection also increases speed and sensitivity. The methanogenic organisms grow rapidly and are detectable sooner than by roll tube methods. Standard genetic procedures such as replica plating can be used to study these organisms. It has been our experience that all fluorescent colonies have been methanogenic and all no fluorescent colonies have been non-methanogenic. However, the possibility exists that there are methanogenic bacteria that have no F420 or only low levels, or, conversely, that there are non-methanogenic bacteria that exhibit similar ultraviolet fluorescence. Fluorescence is presumptive evidence for methanogenic bacteria, but definitive proof requires further characterization of molecular assay.

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