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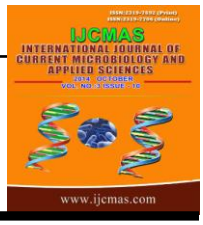

Original Research Article

Study of Membrane Fluidity of *Acidithiobacillus ferrooxidans* on Different Ores (Pyrite and Hematite)

R.Sharmila, T.Nathiya, CH.VK Pruthvi, R.S.Abhishek and Anand Prem Rajan*

*Research Officer, CO2 and Green Technology, School of Bio Sciences and Technology, VIT University, Vellore- 632014, India
**Corresponding author*

Keywords

Membrane fluidity; *A. ferrooxidans*; Fatty acids; Phospholipids; FTIR; Spectro-fluorometer

A B S T R A C T

The fluidity refers to the viscosity of the lipid bilayer of cell membrane. The mosaic characteristic of the plasma membrane, the nature of lipids and phospholipids, fatty acids helps the membrane to retain the fluid. Phospholipids with unsaturated fatty acid tails make membranes more fluidic while saturated fatty acids make the membranes more rigid and less fluidic. *A. ferrooxidans* is an obligate, iron-oxidizing acidophile isolated from the acid mining drainage environments. The work was carried out by culturing *A. ferrooxidans* on different ore (Pyrite and Hematite) substrates under favourable environmental conditions. The *A. ferrooxidans* grown in ferrous sulphate, maintained as control. The membrane fluidity was determined from the fluorescence polarization of 1, 6 - diphenyl-1, 3, 5 – hexatriene (DPH) which indicated the pyrite and hematite has a high membrane fluidity compared to ferrous sulphate. Further results of FTIR indicated the presence of similar functional groups in pyrite and hematite (liquid) with differences in comparison with the Ferrous Sulphate grown media. The soul purpose of this study was to determine whether changes in the membrane characteristics contributed to the ability of *A. ferrooxidans* to tolerate and maintain the effect of changes in membrane fluidity.

Introduction

Cell membranes are key structures existing in all living organisms in the world. Their importance in biology and medicine has been clear for many decades, and an extensive body of research for all aspects of their structure and function. They are ubiquitous in biology and are central to understanding key problems in biology and medicine (Lodish *et al.*, 2000). All biological membranes are fluid in nature which is the function of lipids and phospholipids (Mathews *et al.*, 2000). In general, membrane holds majority of 75% lipids with 20% proteins and consists of 5% carbohydrates by dry weight (Powar and Chatwal, 1994).

A biological membrane or cell membranes are dynamic and fluid structures, and majority of the molecules move within the plane of the membrane which is two dimensional

orientations of lipids and phospholipids. The lipid bilayer is the basic, fundamental structure of all cells, organelles of membranes and the basis of cell biology. Many membrane lipids and proteins tend to move freely within the plane of the membrane, among which lipids move faster because they are smaller than proteins (Becker *et al.,* 2000). An important aspect in function of membranes is the fluidity of the lipid bilayer and diffusion of the membrane components**.** The term "fluidity" which is the reciprocal of viscosity determines the extent of disorder and the motion of molecules within lipid bilayer (Cossins, 1994). Fluid properties of biological membranes are involved for many cell functions. Even small changes in membrane fluidity can cause alterations within them and in processes. Fluid properties are established mainly by the occurrence of polyunsaturated fatty acids in phospholipids molecules located in both sides of the lipid bilayer (Angel 2011). Further membrane fluidity can be estimated by several physical methods, such as fluorescence polarization, electron-spin resonance, and Fourier transform IR spectroscopy (Murata and Los, 1997).

The objective of the work is to study the membrane fluidity of bacteria using different ores (Pyrite and Hematite). *A. ferrooxidans* grown in 9K media with ferrous sulphate were used as control. The fluorescence was analysed by using Polarization Spectrofluorometer. The present work examines the result both in emission and excitation wavelength along with the adjustments made in the width length. The amplification at the fluorescence polarization is set at 400 V (Mykytczuk *et al*. 2010), the florescence dye used in the instrument is DPH dye which inserts the probe into the membrane to be examined for its fluidity and the data obtained are recorded in the TURBO MASS software, which gives the result in peak graph, intensity in y – axis and wavelength in x – axis. Further results of Fourier Transform Infrared Spectroscopy (FTIR) were performed to determine the structure of a compound, chemical bonds and functional groups present in it.

Materials and Methods

Sample Collection and Acclitimization

The pure culture of *A. ferrooxidans* used in the study, was provided by Department of Materials Engineering, Indian Institute of Science, Bangalore. Bacteria were inoculated in 9K culture media containing basal salts (ammonium sulfate - 3g/L, dipotassium phosphate - 0.5 g/L, magnesium sulfate - 0.5 g/L, potassium chloride - 0.1 g/L, calcium nitrate - 0.01 g/L and ferrous sulphate - 44.7g/L) of pH 2.3. The culture was incubated in temperature control orbital shaker (Orbitek – LE, Refrigerated Orbital Shaker Scigenic Make) at 30°C and maintained at 260 rpm. For every 15 days, subculture was done to maintain pure culture (Devasia *et al*. 1996). The experiment was started after 30 days of acclimatization to the laboratory conditions. The ores (pyrite and hematite) samples were collected from Almin Rock Pvt. Ltd, Bangalore.

Experimentation procedure

A. ferrooxidans grown in 9K media with ferrous sulphate (control) $5x10^5$ cell/ml of *A. ferrooxidans* were inoculated in 9K culture media containing basal salts (ammonium sulfate - 3g/L, dipotassium phosphate - 0.5 g/L, magnesium sulfate - 0.5 g/L, potassium chloride - 0.1 g/L, calcium nitrate - 0.01 g/L and ferrous sulphate - 44.7g/L) of pH 2.3. The culture was incubated in temperature control orbital shaker (Orbitek – LE, Refrigerated Orbital Shaker Scigenic Make) at 30°C and maintained at 260 rpm.

A. ferrooxidans grown in 9K media with Pyrite:

5 grams of grounded pyrite (75µ size) was added to sterile 250ml conical flask containing 1 liter $9K^-$ media [$(NH_4)_2SO_4$ -3g, K_2HPO_4-0.5g, $MgSO_4$-0.5g, Kcl -0.1g, $Ca(No_3)_2$-0.01g] and the pH was set to 2.0. To that $5x10^5$ cell/ml of *A.ferrooxidans* culture was inoculated and the flask was incubated at 30°C in orbital shaker at 160 RPM (Wang *et al.*, 2008).

A. ferrooxidans grown in 9K media with Hematite:

5 grams of ground hematite was added to sterile 250ml conical flask containing 1 liter $9K^-$ media [$(NH_4)_2SO_4$ -3g, K_2HPO_4-0.5g, $MgSO_4$-0.5g, Kcl -0.1g, $Ca(No_3)_2$-0.01g] and pH was set to 2.0. To that $5x10^5$ cell/ml of *A.ferrooxidans* culture was inoculated and the flask was incubated at 30°C in orbital shaker at 260 RPM (Wang *et al.*, 2008).

Removal of *A. ferrooxidans* grown in indirect (ferrous sulphate) substrates

$5x10^5$cells/ml of *A. ferrooxidans* grown in ferrous sulphate and sodium thiosulphate was filtered through 0.22µ cellulose filter to remove all finer precipitates. The cells are retained and centrifuged at 10,000 rpm for 10min at 5°C. The obtained pellet was washed three times in 0.01N H_2SO_4. The pellets were resuspended in wash solution to an OD 600nm of 0.2. 3ml of aliquot of the resuspend culture were transferred to a polarization curvet. To that 1µl of 12mM stock solution DPH (1, 6-diphenyl-1, 3, 5- hexatriene) in tetrahydrofuran was added and incubated in dark for 15 min at room temperature. Measurement of the fluorescence polarization was performed using Spectrofluorometer (Mykytezuk *et al*. 2010).

Removal of *A. ferrooxidans* grown in direct (pyrite and hematite) substrate

$5x10^5$cells/ml of *A. ferrooxidans* grown in sulfur was centrifuged at 10,000 rpm. The obtained pellet was three times in 0.01N H_2SO_4 and it was dissolved by triton X-114 phase separation technique (Adam, 1995) (which has ability to remove bacteria attached in sulfur, pyrite and hematite surface). After dissolving pellet bacteria were settled in aqueous layer. Add 0.01N H_2SO_4 to the aqueous solution containing bacteria. 3ml of aliquot of the resuspend culture were transferred to a polarization curvet. To that 1µl of 12mM stock solution DPH (1, 6-diphenyl-1, 3, 5- hexatriene) in tetrahydrofuran was added and incubated in dark for 15 min at room temperature. Measurement of the fluorescence polarization was performed using Spectrofluorometer (Mykytezuk *et al*. 2010).This is done to allow the probe incorporation into the cytoplasmic membrane. Measurement of the fluorescence polarization and FTIR was performed.

Fluorescence polarization

The Spectrofluorometer is used to measure the florescence polarization; the polarizing equipment used for this experiment was Hitachi F7000. The emission wavelength was set to 358nm and the excitation wavelength was set to 428nm (Mykytczuk *et al.*, 2011). Samples were analysed using ultraviolet light which analyses the fluorescence and excites the electrons to higher states causing them to emit light. The emission mode was set with start wavelength of 380nm and the stop wavelength of 700nm. In the excitation mode, the start and stop wavelength was set to 200 to 400 nm. The scan rate was 1200 nm per minute and the amplification of the fluorescence polarization is set at 400 V. The respective slits present in the instrument were set at the width of 5nm. The Ferrous Sulphate

found to excite at 443 nm and Pyrite at 410nm, Hematite with the excitation range of 435nm. The data produced by the Spectrofluorometer is recorded in the TURBO MASS software.

Fourier Transform Infrared Spectra

The work was carried out as described by (Kamnev *et al*., 1999). The obtained pellet were resuspended in a Tris-Hcl buffer solution (pH 7.0) and cooled on ice using ultrasonic disintegrator. The resulting cell debris was washed and centrifuged as described earlier. It was then air dried for 4 hours at 40^{o} C. To determine the functional groups and compounds present in the sample, FTIR was used (Shimadzu Model No-IR Affinity-1). KBr pellet was made to a diameter of 13mm using KBr press. A drop of liquid sample was added to it followed by drying for few minutes.

Results and Discussion

Polarization Spectrofluorometer

The membrane fluidity of *A. ferrooxidans* grown on indirect (ferrous sulphate) and direct (pyrite and hematite) substrates was determined by polarization spectrofluorometer using the fluorescent dye DPH (1, 6 Diphenyl 1, 3, 5- Hexatriene). The peak of emission and excitation in the graph represents the fluidity of the organism. The membrane fluidity is inversely proportional to the fluorescence polarization, which indicates that the highest peak value represents lower membrane fluidity and the lowest peak value represents the higher membrane fluidity. The maximum membrane fluidity can be determined by the ratio given below (Shinitzky and Barenholz, 1978).

$$P= (Ivv-Ivh\ G)/(Ivv+Ivh\ G)$$

Where,

Ivv - Light intensities emitted from vertical directions

Ivh - Light intensities emitted from horizontal directions

G- Grating factor which is (G=Ihv/Ihh) – Is the account of the different sensitivity of the photomultipliers to the polarized light measured from horizontal and vertical directions (G= 0.01).

Ferrous Sulphate

The average peak of Ferrous sulphate was obtained at the intensity value of 4 nm and the peak value was calculated to be 1.0.

Pyrite

The average peak value of Pyrite was obtained at the intensity value of 9nm and the peak value was calculated to be 0.99.

Hematite

The average peak of Hematite **was** obtained at the intensity value of 11nm and the peak value **was** calculated to be 0.98.

The peak value represents the fluorescence polarization which is inversely proportional to the membrane fluidity. As the peak value of the control (*A. ferrooxdians* grown in ferrous sulphate) obtained is 1.0, while the value of *A. ferrooxdians* grown in pyrite was 0.99 and the hematite value was 0.98. Compared to the control value, the peak value of the *A. ferrooxdians* grown in pyrite and hematite were containing least values. Hence as per Mykytczuk *et al*. 2010a, the membrane fluidity of the direct (pyrite and hematite) substrates is higher when compared to the indirect (ferrous sulphate) substrate, while the least value shows the higher membrane fluidity mechanism in them. The peak value is very least in the control sample (pyrite and hematite), as the pyrite and hematite was

oxidized by the bacteria, since no ore particle is present on the membrane and the peak formation is low.

Fourier Transform Infrared Spectra

***A. ferrooxidans* grown in ferrous sulphate**

There spectrum shows the presence of broad weak band at 3410cm^{-1} which is assignable to the O-H group. A sharp bending at 1625cm^{-1} can be attributed to the C=O groups. The bending of the band 1395 cm^{-1} shows the occurrence of C-H group. A weak stretching of the band is seen at 1097 cm^{-1} attributing to the presence of C-N group. The group of bands between 500- 700cm^{-1} are attributable to C-Cl, C=C-H, N-HH respectively.

***A. ferrooxidans* grown in Pyrite**

The presence of O-H group is indicated by the weak bending of the band between 3420 cm^{-1} and 3442 cm^{-1} in contrast to the 3410 cm^{-1} band in the ferrous sulphate. A weak bending of the band is seen near 2040cm^{-1} suggesting C=C group. The spectrum also shows a sharp bending at 1657cm^{-1} indicating the presence of N-H group. The group of bands between 1000cm^{-1} and 1500cm^{-1} indicates C-O, C-N, C-H, C-C groups respectively. The minor bending of the bands near 500cm^{-1} to 5200cm^{-1} shows the occurrence of C-Br and C-Cl groups.

***A.ferrooxidans* grown in Hematite**

The spectral pattern of hematite is similar to pyrite in the bending of the bands (O-H, C=C, N-H, C-H). But there is an absence of band bending at 1141cm^{-1} and 1240cm^{-1} indicating the absence of C-N and C-O as seen in pyrite.

In the FTIR spectrum shown above (*A. ferrooxidans* grown in 9k Media with Ferrous sulphate, Pyrite and Hematite substrate), the common functional group present was found to be O-H which is an Alcohol with the frequency range 3640–3610cm^{-1}. The next functional group was found to be N-H which is Primary and Secondary amines with a frequency range 3400–3250cm^{-1}. The commonly found functional group in Pyrite and Hematite was C=O which are aldehydes with a frequency range 1740–1720cm^{-1}. The functional Group C-Br is **an** Alkyl halide with a frequency range of 690-515cm^{-1}, was found in solid (pyrite and hematite) substrates but was absent in ferrous sulphate. The functional group alkanes C-H is found in both indirect and direct substrates.

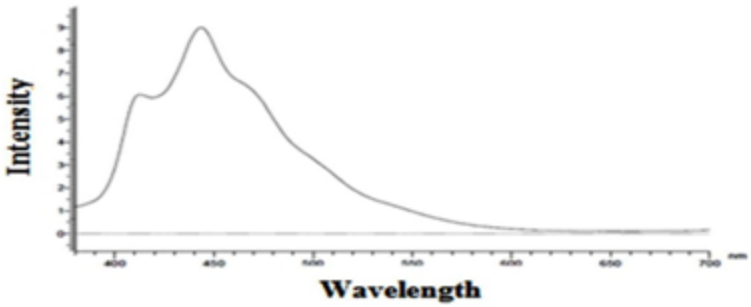

Figure.1.1 Graph showing the Fluorescence Peak of *Acidithiobacillus ferrooxidans* grown on ferrous sulphate substrate

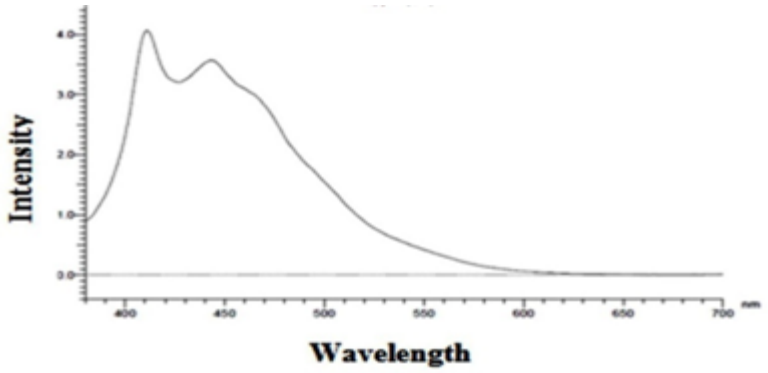

Figure.1.2 Graph showing the Fluorescence peak of *A.ferrooxidans* grown on pyrite substrate

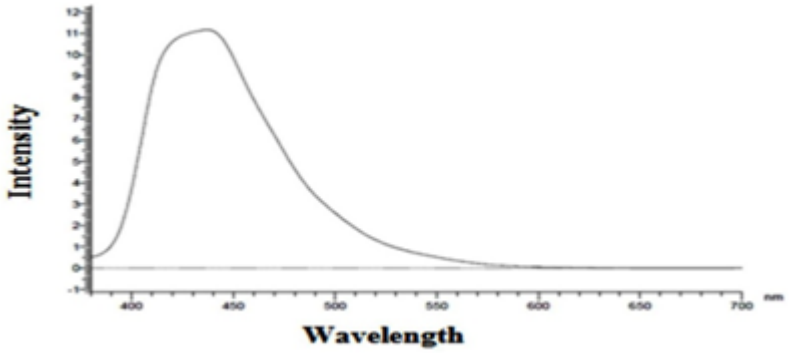

Figure.1.3 Graph showing the Fluorescence peak of *A. ferrooxidans* grown on hematite substrate

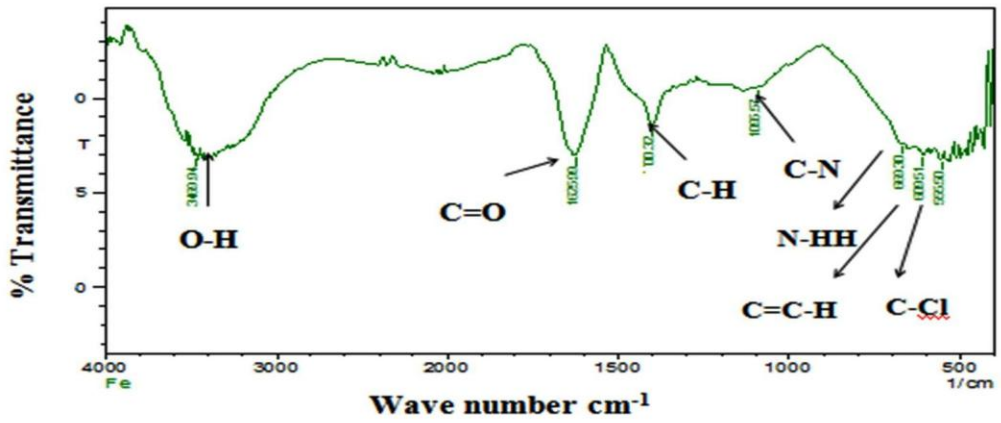

Figure.2.1 Fourier transform infrared spectra of vacuum-dried cell membrane samples obtained from *A.ferrooxidans* cells grown in Ferrous Sulphate

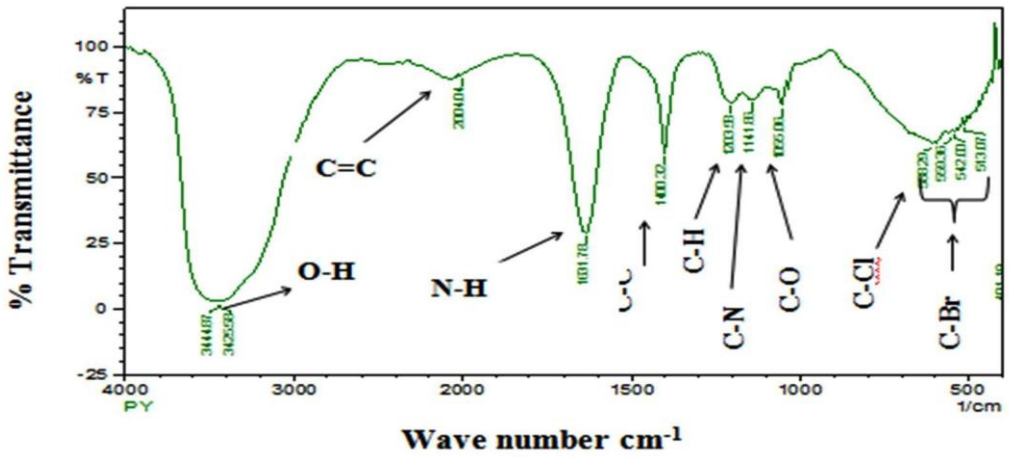

Figure.2.2 Fourier transform infrared spectra of vacuum-dried cell membrane samples obtained from *A.ferrooxidans* cells grown in Pyrite

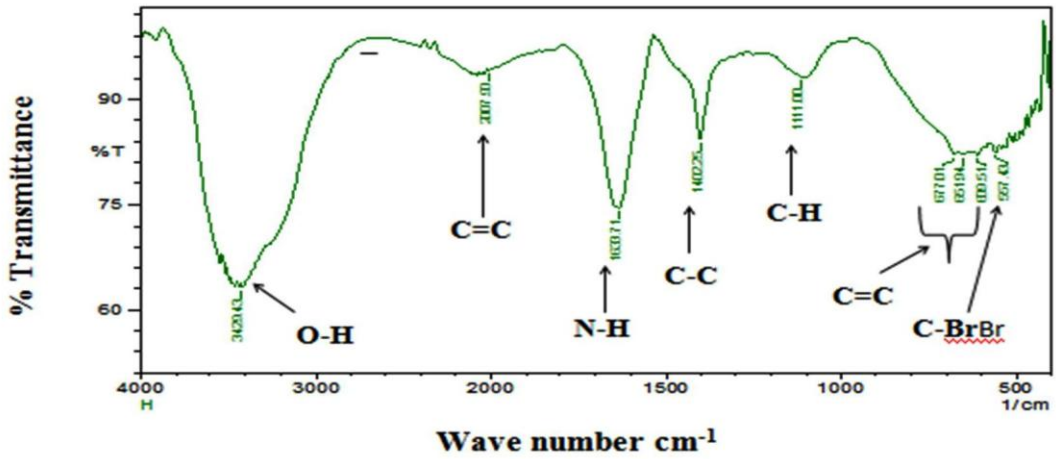

Figure.2.3 Fourier transform infrared spectra of vacuum-dried cell membrane samples obtained from *A.ferrooxidans* cells grown in Hematite

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References

Alvaro Orell, Claudio A. Navarro, Rafaela Arancibia, Juan C. Mobarec, Carlos A. Jerez2010 'Life in blue: Copper resistance mechanisms of bacteria and Archaea used in industrial biomining of minerals' Biotechnology Advances 28 (2010) 839–848.

Angel Catala., 2011. 'Lipid peroxidation modifies the picture of membrane from the fluid mosaic model to the lipid whisker model' Biochimie 94 (2012) 101-109.

Becker W. M., Kleinsmith L.J., and Hardin J.,2000 'The World of the Cell', 4th ed. (San Francisco, CA: Addison Wesley Longman© Addison Wesley Longman, Inc.

Cossins A.R., 1994. 'Temperature adaptations in Biological Membranes and its functional significance' London: Portland Press.

Devasia P, Natarajan K.A, and Rao G.R 1996 'Role of bacterial growth conditions and adhesion in the bioleaching of chalcopyrite by *Thiobacillus ferrooxidans'* Minerals and Metallurgical Processing.95-643

FoziaAnjum , Muhammad Shahid , Ata Akcil2012 'Biohydrometallurgy techniques of low grade ores: A review on black shale' Hydrometallurgy 117–118 (2012) 1–12.

Francesca Mapellia, Ramona Marascoa, Annalisa Balloi , EleonoraRolli , Francesc Cappitelli, Daniele Daffonchioa, Sara Borina, 2012 'Mineral–microbe interactions: Biotechnological potential of bioweathering' Journal of Biotechnology 157 (2012) 473–481.

Harvey Lodish, Arnold Berk, S Lawrence Zipursky, Paul Matsudaira, David Baltimore, and James Darnell.2000. 'Molecular Cell Biology', 4th edition. New York: W.H Freeman .

Inez J.T. Dinkla , Paula Gonzalez-Contreras, Chandra SekharGahan, Jan Weijma, Cees J.N. Buisman, Maurice J.C. Henssen , ÅkeSandström.2013 'Quantifying microorganisms during biooxidation of arsenite and bioleaching of zinc sulfide' Minerals Engineering 48 :25–30.

Kamnev A. A, Antonyuk L.P, Matora L, Serebrennikova O.B, Sumaroka M.V, Colina M, Renou-Gonnord M.F, Ignatov V.V 1999. 'Spectroscopic characterization of cell membranes and their constituents of the plant-associated soil bacterium *Azospirillumbrasilense.*Journal of Molecular Structure 480–481 (1999) 387–393.

Kelly, D.P and Wood A.P. 2000 'Reclassification of some species of Thiobacillus to the newly designated genera Acidithiobacillus gen.nov., Halothobacillu gen. nov' Microbiol. 50 (2): 511-6.

Mathews, C.K., K.E. van Holde, and K.G. Ahern. 2000 '*Biochemistry'* 3d ed. Menlo Park, CA: Benjamin/Cummings.

Mykytczuk N.C.S, Trevors J.T, Ferroni G.D, Leduc L.G 2010. 'Cytoplasmic membrane fluidity and fatty acids composition of Acidithiobacillus ferrooxidans in response to pH stress. 14:427-441.

Norio Murata and Dmitry A. Los. 1997. 'Membrane Fluidity and Temperature Perception' Plant Physiology 115(3): 875–879.

Powar C.B and G.R. Chatwal. 1994 'Biochemistry'3rd edition; Himalaya Publishing House.

Rawlings D.E 2002 'Heavy metal mining using microbes' Annu. Rev. Microbial 56;65-91.

Rohwerder T, Sand W. 2003 'The sulfane sulfur of persulfides is the actual substrate of the sulfur-oxidising from Acidithiobacillus and Acidiphilium spp. Microbiology 149:1699-1709.

Shinitzky M, Barenholz Y 1978. Biochim.Biophys. Acta, Biomembr, 515, 367.

Trevors J T. 2003 'Fluorescent probes for bacterial cytoplasmic membrane research' J Biochem Biophys Methods 57;87-103.

Wang Jun, Qin Wen-qing, Zhang Yan-sheng, Yang Cong-ren, Zhang Jian-wen, Nai Shao-shi, Shang e, Qui Guan-zhou. 2008 'Bacterial leaching of chalcopyrite and bornite with native bioleaching microorganis' . Trans. nonferrous.Met.Soc. china 18: 1468-1472.