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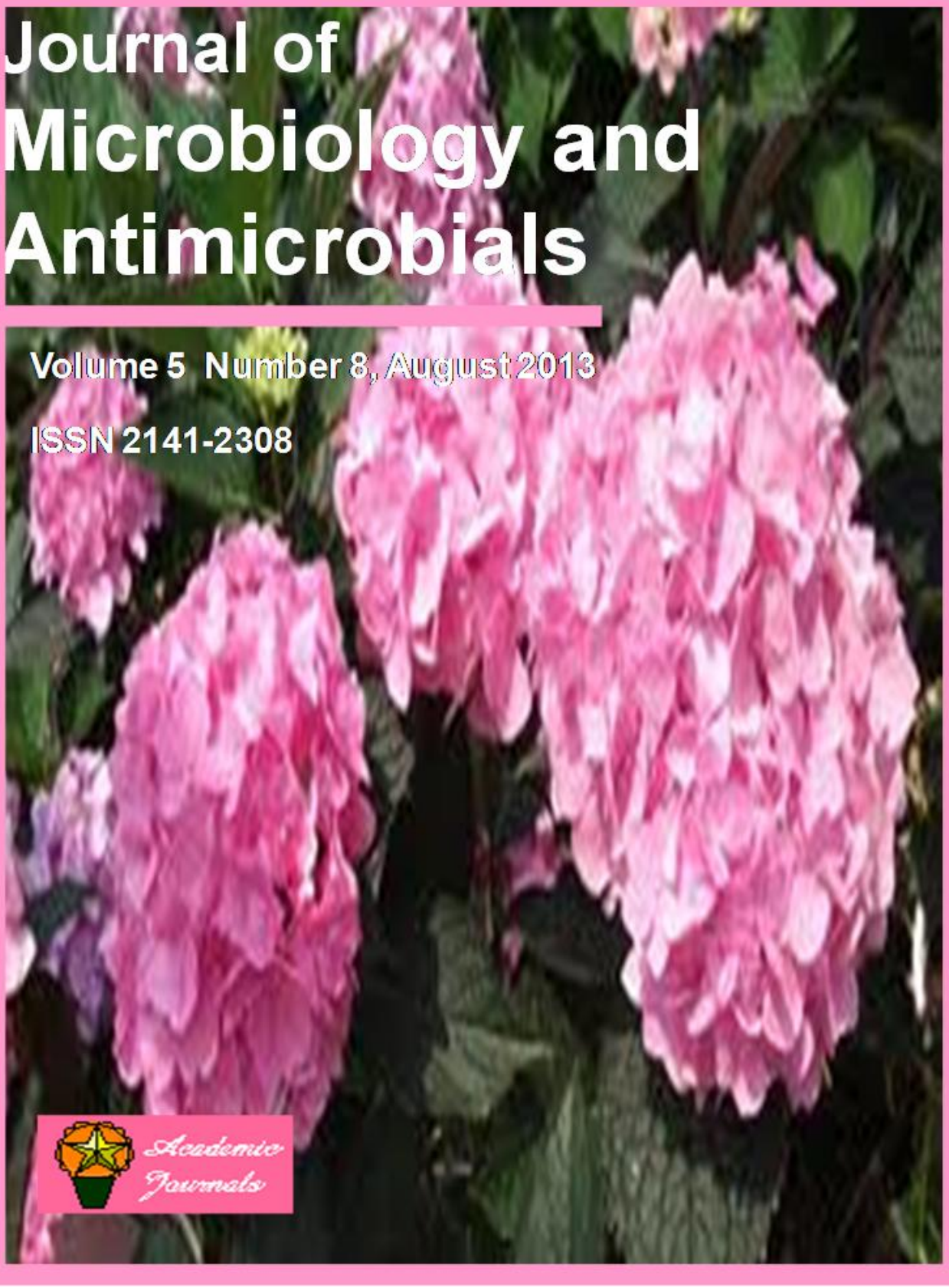

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Journal of Microbiology and Antimicrobials

*Full Length Research Paper*

# Virulence determination of bacterial isolates through culture in India ink including broth

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Accepted 29 July, 2013

**Virulency is the degree of pathogenicity exhibited by most of the pathogens and it is a measure that effectively differentiates pathogenic and non-pathogenic strains. In the present study, culture of bacteria for 10 to 12 h (overnight) at 37°C in 10% India ink included broth that can be used as marker test for initial virulence identification while screening a number of bacterial isolates. These results were further confirmed by the *in vivo* virulence tests. Although, this is the first report, there is no clear explanation to the degradation of India ink by virulent bacteria which was obtained but this could act as a marker for initial screening of isolates for their virulence. Moreover, India ink is used as a suitable capsular stain and capsule act as a virulence factor for most of the bacteria. So virulence test by 10% India ink including broth acts as a simple, low cost and easy method for initial screening of virulent from non virulent strains.**

**Key words:** *Aeromonas hydrophila*, bacteria, India ink, pathogens, virulence.

## INTRODUCTION

Virulence is the degree of pathogenicity exhibited by most of the pathogens and it is a measure that effectively differentiates pathogenic and non-pathogenic strains Russell and Herwald (2005). The degree of virulence depends on several virulence factors (Price, 2008) which includes capsular polysaccharide (Taylor and Roberts, 2005; Lindberg et al., 1979), capsule, DNase, coagulase, fibrinolysin, proteolytic, haemolysin, bacteriocin production, haemagglutination, serum sensitivity, epithelial cells attachment, hydrophobicity (Avila-Campos et al., 2000), lipase, antiphagocytic factor, biofilm (Prasad et al., 2009), extracellular enzymes production, presence of surface layer, lysine decarboxylase production and pili, (Pin et al., 1997) etc. There are several *in vivo* and *in vitro* assays to determine these virulence factors (Calderone et al., 2009). These include biochemical test (Voges-Praskauer reaction or fermentation of arabinose) (Kirov et al., 1998), tissue culture-based method (Yeung et al., 2007), tests using animal model (suckling mouse model) (Wong et al., 2006), molecular characterization by several virulence-associated genetic markers (Ma and Modi, 2002). All these methods though accurate are very tedious; need sophisticated laboratory and equipment, use live animals and time consuming. Some times it becomes difficult to handle quite number of isolates at a time.

Although, the *in vivo* or animal pathogenicity test has been taken for granted as the index for virulence for pretty long time, it is subjected to considerable variation because it reflects the interaction between host and the microorganisms, which is also affected by genetic and environmental factors, the relative susceptibility of the host. Moreover, it involves a lot of ethical issues and most of the countries also ban the use of live animals

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in research. To overcome this problem, the staining method is very useful and cost effective for initial screening of microorganisms as it determines the presence and absence of certain virulence determining characters such as presence of capsules by India ink (Fumiko et al., 2004), A- layer proteins (Udey, 1982), smooth -rough variations using coomassie brilliant blue (Cipriano and Bertolini, 1988) and also differentiate virulent from non-virulent by toluidin blue-iodine (Henry, 1920).

However, their accuracy depends upon various factors such as source of stain, staining methods, requirement of good microscope and visual interpretation. So in this study, we tested a more suitable method for initial screening of virulence from a number of isolates by culturing in India ink inclusion broth and it is the easier method to be practiced. So far, India ink has been used as capsular staining by several workers (Fumiko et al., 2004). Capsules are produced by all the virulent strains during infection but the capsule production and virulence are not correlated (Bennett et al., 2008). So in the present study, we evaluated the use of India ink in culture broth for their virulence.

## MATERIALS AND METHODS

Several bacterial pathogens isolated from different diseases conditions of fish and human food products etc. were used. These include isolates of *Aeromonas hydrophila* (26)*, Flavobacterium branchiophilum* (3), *Flavobacterium columnare*(1), *Edwadsiella tarda* (6), *Pseudomonas fluorescens* (1), *Escherichia coli* (1)*, Streptococcus* sp. (4) and *Salmonella* sp. (1). These isolates were identified and properly characterized through standard microbiological methods (Cruickshank et al., 1975) and kept in slant at 4°C.

Different culture broths such as Brain Heart Infusion (BHI) Broth (Hi-media, India) or in Tryptic Soy Broth (TSB) (Hi-media, India) were prepared. India ink (Dye based Ink with CAMLI-SOL100) (Camlin Ltd., India) was filtered through 0.45 μm (Millipore, India)] and added to the broth at different concentrations (2.5, 5, 10, 15 and 20%). Then 24 h bacterial culture at 50 - 60 µl $ml^{-1}$ was inoculated separately to each broth, incubated at 37°C and checked at definite time interval up to 48 h.

Selected strains were also then streaked on Coomassie brilliant blue (CBB) agar plate to check their virulence, as described by Cipriano and Bertolini (1988), simply adding CBB-R250 MS (s.d.Fine-chem.Ltd., India) to a final concentration of 0.01% in Tryptic soy agar medium and incubated in anaerobic condition for 24 h at 37°C.

*In vivo* virulence test were conducted using fish, *Labeo rohita* juveniles of average body weight ranging from 30 to 40 g. Fish were divided into several groups containing 12 numbers of fish in each group according to the number of selected bacteria taken for virulence study. The 24 h bacterial culture was centrifuged at 5000 xg for 10 min, washed in sterile saline and the final pellet was suspended in sterile PBS solution.

The fishes were injected intraperitoneally with 0.1 ml ($10^6$ - $10^9$ CFU $ml^{-1}$) of bacterial suspension using three fishes per each dilution. Clinical signs and mortalities were recorded up to 7 days. Virulence was determined as the lowest dose, which caused 50% mortality, as calculated using the method of Reed and Muench (1938).

## RESULTS AND DISCUSSION

During growth of these bacteria in India ink including culture broth, some isolates started degrading the ink after 5 to 6 h in only 10% India ink. However in 2.5, 5.0, 15 and 20% ink the degradation of ink color was not so quick, prominently visible and optimum at 10 to 12 h at 37°C unlike that of 10 India ink. Therefore, we have taken 10% India ink as our optimum and standard concentration for further virulence detection.

So keeping the culture in 10% India ink, the dye degradation was optimum at 10 to 12 h at 37°C (Figure 1A and B). By further increasing the culture time for more than 24 up to 48 h, all the isolates and strains degraded the color only in 10% ink. Those bacteria that degraded the color of the dye as early, within 12 h were checked for their growth on CBB agar plate, which showed the blue colonies (Figure 2B) while the other isolates which had no immediate effect on India ink grew as white colonies (Figure 2A). According to Cipriano and Bertolini (1988), CBB agar is a highly effective medium for the *in vitro* screening of virulence because this medium also helps in detection and quantification of the frequency of A-layer protein that acts as a major virulence factor. So this indicates that those bacteria that degraded the color of India ink were virulent unlike that of the other that retains the color as non-virulent. They were also further checked for their virulence by *in vivo* pathogenic trial in fish which showed the $LD_{50}$ less than $10^7$ CFU $ml^{-1}$. So this further confirmed that only the virulent bacteria could degrade the color of India ink in culture broth within 10-12 h which might be differenct in their biochemical and enzymatic processes. So, the present study showed that India ink including culture broth can be used as virulence marker and this method is easier, simpler in use, can be extended to other bacterial species and by this method we can primarily screen quite a number of bacteria at a time with low cost.

Though no clear explanation to the degradation of India ink by virulent bacteria has been obtained but this could act as a marker for initial screening of isolates for their virulence. Moreover, India ink is used as a suitable capsular stain and capsule act as a virulence factor for most of the bacteria. So the difference in culture behavior of bacterial isolates in presence of India ink may act as a marker for initial studying of bacteria, however they have to be further confirmed by different *in vivo* tests.

In conclusion, culture of bacteria for 10 to12 h (overnight) at 37°C in 10% India ink included broth that can be used as marker test for initial virulence identification while screening a number of bacterial isolates.

## ACKNOWLEDGEMENTS

The authors are thankful to the Director and Head, Fish Health Management Division, Central Institute of Fresh-

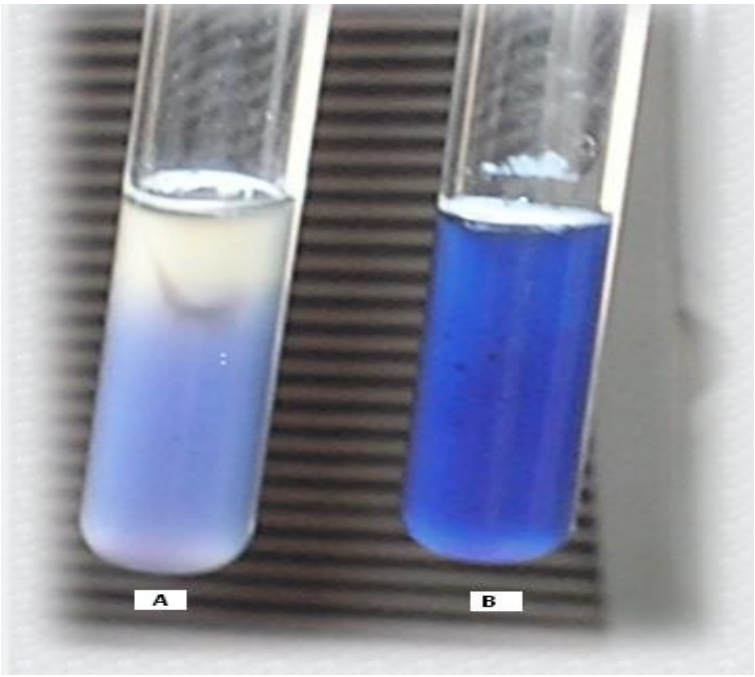


**Figure 1.** Growth of *Aeromonas hydrophila* in 10% India ink incorporated brain heart infusion broth; A: pathogenic strain, B: apathogenic strain.

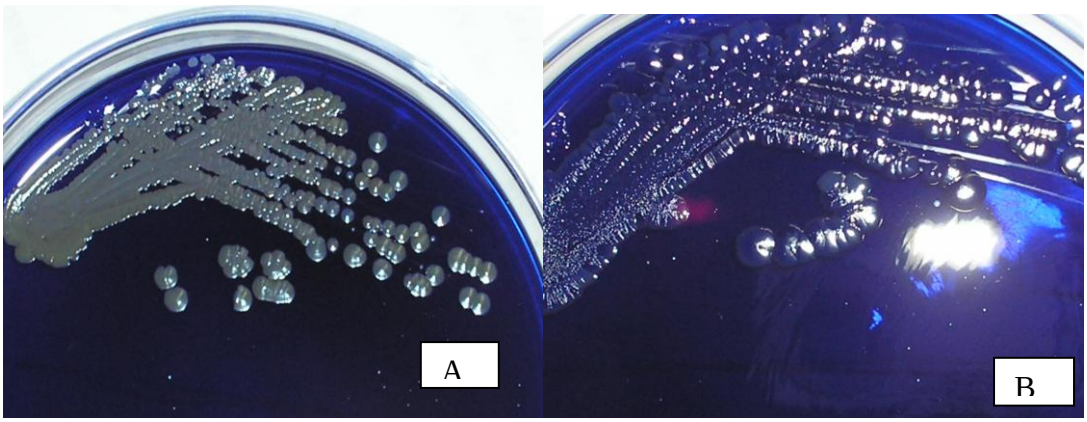


**Figure 2.** Growth of *A. hydrophila* in 10% India ink on coomassie brilliant blue (CBB) agar plate; A: apathogenic strain, B: pathogenic strain.

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*Full Length Research Paper*

# A mathematical approach for describing the dependence between organic matter content and pH value in some waste water

**Chandra Prabha and Shiv Dayal***

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Accepted 29 July, 2013

**Water pollution is a major problem in the world, which can turn our precious earth into abyss. Waste water contains so many pollutants like inorganic and organic matter. Waste water decreases the pH of water. Pollution has high organic matter per-cent, with neutral pH and low salinity. We aimed to explain such a problem mathematically using an exponential method, which can be used to eliminate pollution from earth.**

**Key words:** Thermophilic fungi, heavy metals, organic matter (X) pH in water analysis (Y), mathematic modeling.

## INTRODUCTION

Waste water is enriched with organic and inorganic substances, including heavy metals from domestic areas (Kamaludeen et al., 2003). Rapid industrialization and urbanization all over the country has led to regional and global redistribution of metals with consequent environmental pollution. Organic matter is used as a growth substrate for fungi (Mehra and Jaitly 2003; Kacpizak et al., 2005). Dissolved organic matter is highly heterogenous in nature and ranges from less than 500 to more than 5000Da in molecular weight (Imai et al., 2002).
Microbial population is mostly heterotrophic which grows on and affects solid and liquid waste. Generic composition and size are different for mycoflora. Physical and chemical characteristics vary with environmental condition for mycoflora (Jaitly, 1982). They produce enzymes for the decay of waste/pollutants.

pH and temperature are major abiotic factors which affect fungal growth, development and activity. The term “thermophilic” denotes their affinity for high temperature. That means that organisms can grow optimally above 45°C.They are very few and have been divided into two mains categories: true thermopiles and thermotolerants (Cooney and Emerson, 1964).

One of the prerequisites for a more complete control and exploitation of these fungi is temperature, which is the most important variable in their environment. Fungi are also used as adsorbent/absorbents to remove heavy metals from municipal, sewage (Goomes et al., 1998) and industrial waste water (Kamaludeen et al., 2003). Heavy metals can interact with microbial cells and there are reports that they accumulate these substances as a result of some physico-chemical mechanism and transport systems, depending directly and indirectly on metabolism of the microbes.

## MATERIALS AND METHODOLOGY

Waste water samples were aseptically collected in sterilized bottles from different collecting sites of Bareilly City-Uttar Pradesh (India) listed below:
Pond water sites: Bansinagla, Gosi-gotiya, Madinath -1, Madinath-2, Pashupatinath

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Municipal sites: ADM compound, Chaupla, Civil lines, Madinath, Mission hospital
Sewage sites: Qila, Nakatiya, Sanjay Nagar.

The above sites are around the Bareilly City from U.P. (India). These water bodies' temperature was analyzed at the collection time (6:00am - 8:00am) by thermometer and their pH by systronic µ pH meter 361. Percent of organic matter was analyzed by Walkey and Black's method (1947), using the following formula:

$$X\ (\%) = \frac{6.791}{w}\left(1-\frac{T_1}{T_2}\right), \text{ for every T1 and T2, W} \neq 0$$

Where, X% is organic matter percentage, $T_1$ is titration reading, $T_2$ is Blank reading.

Salinity percentage was calculated by the following formula:

$$\text{Salinity } (\%) = \frac{W2-W}{W1-W}x100, \text{ here W1 can never be equal to W}$$

Where 'W' is pre weighted Petri plate, 'W1' is the weight of water and the Petri plate, 'W2' is the weight of the Petri plate after evaporation of the water sample at $80^0$C (24 hrs).
Direct and indirect method (Warcup 1950) was used for isolation of thermophilic fungi using YPSs (Yeast Extract Soluble Starch) medium at pH 7.0. Three replicates were taken for each sample. Petriplates were incubated at $40^0$C. After appearing in fungal forms, they were isolated and maintained on agar medium slants in the pure forms at $4^0$C. Colonies were identified using microscope and available literature. Fungi use the organic matter as nutrient. Frequency of fungi shows presence of organic matter in water/soil/waste substances.
Now, we would use a mathematical relation for the pH and organic matter.

**Mathematically:** Let us assume a differential equation:

$$\frac{dy}{dx} + y = 0 \qquad \ldots (1)$$

Initially, we will take $y_o = 7$,

Because at pH=7 water is neutral, that is useful for drinking.

$$\frac{dy}{dx} + y = 0$$
$$then:$$
$$\frac{dy}{y} = -dx,$$
$$\Rightarrow \ln y = -x + \ln c$$
$$\Rightarrow \ln y = \ln e^{-x} + \ln c$$
$$\Rightarrow y = ce^{-x}, \text{But when x} = 0, \text{y} = 7 \text{ so c} = 7,$$
$$\Rightarrow y = 7e^{-x} \ldots (2)$$

Where y denotes the pH value and x denotes the organic matter.

**Practically:** We analyzed all the samples for different environmental parameters.

The measured pH value showed extreme alkaline. The range of pH for municipal waste water was 7.5 to 8.9; for sewage, 7.9 to 8.9; for pond water sites, 7.6 to 8.3. The pH value is less in pond water site compared to municipal and sewage sites, indicating a more alkaline nature. The pH is also temperature dependent (Goswami and Sharma, 2007). The overall temperature in the three sites ranged from 9.08 to 33.4°C at the time of sample collection between 6:00AM to 8:00AM.

Organic matter in waste water body decreased with increase of the pH value. It depends on the kinds of solved organic mater from the waste. The organic matter percentage was the highest in the municipal site (0.380 to 2.92%, Table 2), sewage (0.371 to 2.801%, Table 3) and pond water site (0.162 to 2.301%, Table 1). The trend of the total number of fungal colonies was: Municipal sites > Sewage sites > pond water sites.

Highest salinity was noted in the sewage site (10.8%) in June (Table 3). Isolated total number of colonies follows the pH value and organic matter percentage. Better growth (number of colonies) was found if the pH values were low (about neutral) and the organic matter, high. It is clear that fugal populations used organic matter from waste water as nutrient

## RESULTS AND DISCUSSION

Here, in Equation 2, if we increase the value of x then y decreases (Table 4). It is known as exponential decay (Figure 1).

As seen in the above presented data (Table 4 and Figure 1), if the value of x (organic matter) increases Y exponentially decreases**.**

If initially we put x = 0, y = 7, that means that there is no organic matter found in water; and if the pH=7, then mathematically we can say that the water is useful for drinking and free of pollution. If X is greater than 0,y will be less than 7 and so on. In this case, water will be acidic and not useful for drinking. If x goes to infinity, then, from equation (2), y must be 0, which is not practically possible; but mathematically we can say the water is now highly polluted.

## Conclusion

Finally, we would like to say that, in the above theory, if we increase organic matter (x), then pH value of the water decreases exponentially (only in analyzed water sites, not in all – depending on the kind of organic charge).

That means if X increases in a very low quantity, Y decreases very high. If we are trying to decrease the organic mater from the water, then we can save it for drinking, being free of pollution. Otherwise, some day there will be no drinking water and the major problem for life will come easily, for it is well known that life without water is impossible. Pollution will cause destructive lives.

## ACKNOWLEDGEMENT

The author is grateful to the Professor. V. P. Gupta

**Table 1.** Environmental parameters of Pond Sites in Year-2007.

| Collection site | Month | March | June | Sept | Dec |
|---|---|---|---|---|---|
| | Temperature (°C) | 25.5 | 32.0 | 30.9 | 10.10 |
| | pH | 8.1 | 8.0 | 8.3 | 8.0 |
| Madinath 1 | OM (%) | 0.801 | 1.018 | 0.317 | 0.981 |
| | Salinity (%) | 6.6 | 7.9 | 4.6 | 7.3 |
| | TNF | 172 | 210 | 201 | 105 |
| | Temperature (°C) | 28.6 | 32.0 | 28.0 | 9.90 |
| | pH | 8.0 | 8.0 | 7.9 | 8.2 |
| Madinath 2 | OM (%) | 1.162 | 2.232 | 2.301 | 1.10 |
| | Salinity (%) | 6.3 | 5.1 | 5.9 | 4.9 |
| | TNF | 108 | 124 | 130 | 92 |
| | Temperature (°C) | 28.6 | 32.9 | 29.0 | 9.9 |
| | pH | 7.9 | 8.1 | 8.2 | 7.9 |
| Gosi-gotiya | OM (%) | 1.331 | 0.805 | 2.11 | 1.05 |
| | Salinity (%) | 6.8 | 4.1 | 6.0 | 7.7 |
| | TNF | 102 | 102 | 109 | 96 |
| | Temperature (°C) | 26.3 | 32.4 | 29.0 | 9.8 |
| | pH | 7.9 | 8.2 | 8.5 | 8.0 |
| Bansinagla | OM (%) | 1.301 | 1.45 | 0.90 | 1.673 |
| | Salinity (%) | 3.6 | 4.2 | 2.0 | 3.0 |
| | TNF | 147 | 146 | 105 | 167 |
| | Temperature (°C) | 25.0 | 33.3 | 29.0 | 10.1 |
| | pH | 8.2 | 8.3 | 8.2 | 7.6 |
| Pashupatinath temple | OM (%) | 0.301 | 0.219 | 2.613 | 0.740 |
| | Salinity (%) | 2.8 | 5.8 | 3.6 | 2.1 |
| | TNF | 107 | 96 | 122 | 99 |

TNF = Total number of fungal colonies, OM% = organic matter percentage.

**Table 2.** Environmental parameters of municipal water in Year-2007.

| Collection site | Month | March | June | September | December |
|---|---|---|---|---|---|
| | Temperature (°C) | 25.5 | 32.0 | 30.9 | 10.1 |
| | pH | 8.1 | 7.8 | 8.3 | 8.6 |
| Chaupula | OM (%) | 0.901 | 1.018 | 1.117 | 0.981 |
| | Salinity (%) | 3.6 | 3.9 | 2.0 | 2.8 |
| | TNF | 105 | 101 | 240 | 102 |
| | Temperature (°C) | 25.3 | 31.0 | 30.5 | 10.8 |
| | pH | 7.8 | 8.0 | 8.0 | 8.3 |
| City Station | OM (%) | 2.95 | 1.17 | 2.92 | 1.81 |
| | Salinity (%) | 3.0 | 4.0 | 2.0 | 4.5 |
| | TNF | 196 | 87 | 115 | 94 |
| | Temperature (°C) | 25.3 | 31.4 | 29.0 | 9.6 |
| | pH | 7.6 | 8.3 | 8.1 | 7.8 |
| Madinath | OM (%) | 0.464 | 0.319 | 1207 | 0.964 |
| | Salinity (%) | 6.6 | 7.1 | 5.0 | 6.0 |
| | TNF | 208 | 108 | 73 | 128 |

**Table 2. Cont.**

| | | | | | |
|---|---|---|---|---|---|
| Mission | Temperature (°C) | 25.8 | 32.7 | 29.0 | 10.0 |
| | pH | 8.6 | 8.5 | 8.2 | 8.9 |
| | OM (%) | 0.763 | 0.971 | 0.509 | 0.380 |
| | Salinity (%) | 2.0 | 4.9 | 1.3 | 1.6 |
| | TNF | 93 | 102 | 97 | 59 |
| ADM compound | Temperature (°C) | 26.1 | 32.1 | 30.1 | 9.7 |
| | pH | 8.4 | 7.9 | 7.5 | 8.0 |
| | OM (%) | 1.543 | 1.073 | 1.46 | 1.001 |
| | Salinity (%) | 5.4 | 6.8 | 5.0 | 6.7 |
| | TNF | 109 | 205 | 219 | 160 |

TNF=Total number of fungal colonies, OM%= organic matter percentage

**Table 3.** Environmental parameters of sewage sites in Year-2007.

| Collection site | Month | March | June | September | December |
|---|---|---|---|---|---|
| Qila | Temperature (°C) | 30.0 | 32.0 | 31.9 | 11.0 |
| | pH | 8.2 | 8.0 | 7.9 | 8.2 |
| | OM (%) | 0.381 | 1.99 | 2.801 | 0.610 |
| | Salinity (%) | 9.2 | 8.1 | 7.0 | 6.8 |
| | TNF | 207 | 208 | 220 | 179 |
| Nakatiya | Temperature (°C) | 28.4 | 33.4 | 31.0 | 10.8 |
| | pH | 8.3 | 8.5 | 8.2 | 8.9 |
| | OM (%) | 0.747 | 1.071 | 0.447 | 0.689 |
| | Salinity (%) | 6.4 | 9.8 | 8.4 | 9.0 |
| | TNF | 197 | 202 | 96 | 84 |
| Sanjay Nagar | Temperature (°C) | 28.2 | 33.0 | 30.8 | 11.1 |
| | pH | 8.1 | 8.6 | 7.9 | 8.0 |
| | OM (%) | 1.584 | 0.901 | 1.876 | 0.317 |
| | Salinity (%) | 6.5 | 3.0 | 7.3 | 9.3 |
| | TNF | 190 | 182 | 198 | 157 |

TNF = Total number of fungal colonies, OM% = organic matter percentage.

**Table 4.** Organic matter.

| S/N | Organic matter (x) | y=7 Exp(-x) |
|---|---|---|
| 1 | 0 | 1 x 7=7 |
| 2 | 1 | 0.370 x 7=2.59 |
| 3 | 2 | 0.137 x 7=0.959 |
| 4 | 3 | 0.0508 x 7=0.3556 |

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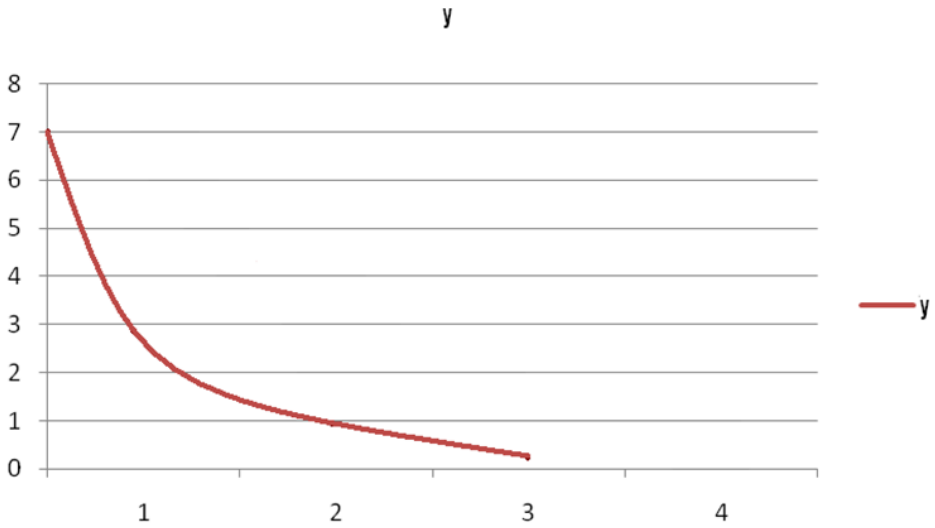


**Figure 1.** The relation between pH and organic matter.

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## *UPCOMING CONFERENCES*

## ICMVM 2013: International Conference on Molecular Virology and Microbiology, Bangkok, Thailand, 24 Dec 2013


## ICBHES 2014: International Conference on Biological, Health and Environmental Sciences, 20 Jan 2014

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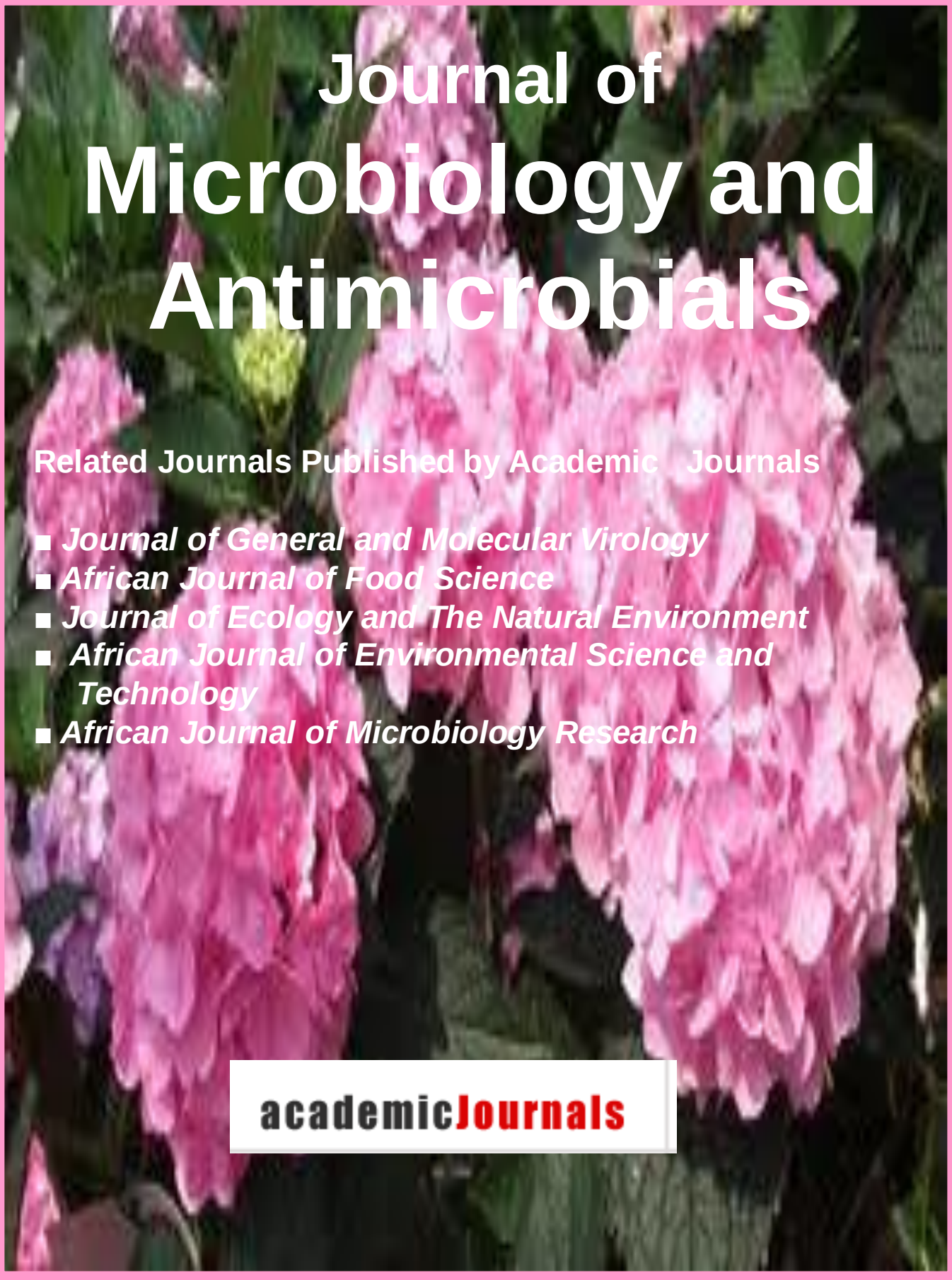
International Conference on Food, Biological and Medical Sciences (FBMS-2014), Bangkok, Thailand, 28 Jan 2014

**February 2014**
Australian Society for Antimicrobials 15th Annual Scientific Meeting, Melbourne, Australia, 20 Feb 2014



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