Microbial Inactivation by Chlorine in the Presence and Absence of a Chlorine-Demanding Substrate and Its Effect on ß-D-Galactosidase in Egypt

Rasha Y. Mahmoud[1*], M. S. Moussa[2] and Tareq Youssef[3]

[1] Department of Microbiology and Immunology, Faculty of Pharmacy, Helwan University, Ain Helwan, P.O.11795, Cairo – Egypt
[2]Faculty of engineering, El Matareia, Helwan University
[3] National Institute of Laser Enhanced Sciences – Cairo University, Gamma St., P. O. 12613, Giza - Egypt.
rasha_kotab@pharm.helwan.edu.eg

Abstract: Chlorine is a powerful antimicrobial substance due to its potential oxidizing capacity. However, decrease of the actual chlorine level through the oxidation of organic matter contaminated water sources is observed. This study reveals that the germicidal effect (GE) of chlorine doses in aqueous contact media is significantly higher than that in organic matter containing contact media. The effect of chlorine on cultivability and ß-D-Galactosidase (GALase) activity of *Escherichia coli* isolates were compared and revealed that GALase activities were more resistant to chlorine than their cultivability. Therefore, the determination of enzymatic activity as a method for quantification of the waterborne pathogens is more suitable with the chlorinated water sources that may have stressed pathogens (can be called viable but are nonculturable (VBNC) bacteria), which do not have the ability to grow properly on the solid media.

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Key words: ß-D-Galactosidase, chlorine, Germicidal effect, viable but nonculturable cells.

1. Introduction

The process of water purification requires disinfectants such as chlorine, which affects the cytoplasmic membranes of microbial cells contaminated water sources, disrupts protein synthesis, affects reactions of nucleic acids, and compromises nutrient transport (Brenner *et al*., 1996). The increased sensitivity of stressed coliforms to selective media can result in the failure to detect them or underestimation of their concentration in drinking water. Therefore, this can result in the use of unsafe water by the public with its attendant health risks. However, under proper conditions of temperature and nutrients, injured organisms can repair cellular lesions and other forms of sub-lethal injury (Brenner *et al.*, 1996). Chlorination is the most common method of disinfection. In sufficient doses, chlorine kills microorganisms within 30 minutes. In addition to killing microorganisms, chlorine reacts with organic compounds, oxidizing and effectively neutralizing them. Therefore, since most taste- and odor-producing compounds are organic in nature, chlorine treatment also improves water taste and smell. Chlorine is added to water either from a concentrated solution of sodium or calcium hypochlorite or as a gas from pressurized tanks. The latter method is used most commonly in large water treatment plants because it is most amenable to automatic control (Madigan, 2003).

Chlorine is consumed when it reacts with organic materials. Therefore, sufficient quantities of chlorine must be added to water so that a residual amount remains to react with the microorganisms after all reactions with organic materials have occurred. The effectiveness of disinfection is commonly determined by monitoring culturable coliform bacteria. However, the absence of culturable bacteria may not adequately reflect the state of the original population. For example, after exposure to hypochlorous acid (HOCl), an *E. coli* K-12 population will form three subpopulations: (i) Culturable bacteria, which are able to form colonies on solid medium; (ii) VBNC bacteria, which are unable to form colonies but which still display respiratory or metabolic activity detectable by direct viability assays such as direct viable count tests; and (iii) Dead bacteria (Dukan *et al*., 1997).

Numerous bacteria, such as *E. coli*, *Vibrio cholerae*, *Vibrio vulnificus*, *Salmonella enteritidis*, *Shigella sonnei*, *Shigella flexneri*, and *Campylobacter jejuni*, can enter the VBNC state after exposure to adverse environmental conditions. Therefore, it is important to know the physiological capacities of bacteria once they have entered the state of nonculturability. Whether the increase in the number of cfu results from true resuscitation of VBNC cells or from re-growth of a few culturable cells at the expense of dead or moribund cells is controversial. Therefore, it is highly urgent and significant to investigate other methods to determine the pathogenic contamination in the chlorinated water. Tryland *et al.* (Tryland *et al*., 1998) approved the applicability of using the rapid GALase assay for recovery of viable target bacteria from water and wastewater.

2. Materials and Methods

2.1. Bacterial isolates and culture conditions.

E. coli isolates were obtained from Zeneen station, Abu Rawash station for wastewater purification and Embaba station for drinking water purification, at Cairo, Egypt. All isolates were identified microscopically and by biochemical tests.

2.2. Standardization of chlorine reagent.

In a stoppered flask, 50 ml distilled water, 1gm of potassium iodide and 12.5 ml of 2 molar acetic acid were added. 10 ml of the chlorine solution being examined was diluted to 100 ml in a volumetric flask using distilled water. Then 10 ml from the diluted chlorine solution was added to the stoppered flask and the titration was performed against 0.1 molar sodium thiosulphate solution using 1 ml of starch solution as indicator. Each 1 ml of 0.1 M sodium thiosulphate will be equivalent to 3.546 mg of active chlorine (Pharmacopeia, 1998).

2.3. Chlorination of pure bacterial cultures using aqueous solution as a contact medium "The quantitative suspension test".

The isolated pure cultures were grown in 10% TSB and the bacterial concentration was adjusted to 10^7 cfu ml^{-1} suspended in PBS solution pH (6.8). The decimal log reduction rate can be determined through addition of 10 µl of the previously prepared bacterial suspension to 1ml of Na-hypochlorite solutions of concentrations (0.05, 0.1, 0.5, 1, 2, 4, and 6 mg l^{-1}). Samples were collected after specific contact times of (10, 20, 30 and 40 minutes) after which the effect of chlorination was quenched by addition of 100 µl to 900 µl sodium thiosulphate solution. Control series were prepared by the same procedure except that the chlorine solutions were replaced by sterile distilled water (Russell, 1999). Then the count of the residual surviving cells using appropriate serial dilutions was determined by surface viable count by spreading method using tryptic soy agar with yeast extract (TSAYE) as a growth medium and incubated at 37ºC for 24 hours (Virto, 2005a). It was previously checked that TSAYE neutralized the remaining chlorine in the corresponding dilutions.

The germicidal effect (GE) can be calculated using the following relation;

$$GE = \log N_C - \log N_D$$

GE = Germicidal effect

N_C = No. of cfu in control series

N_D = No. of cfu after chlorine exposure (Russell, 1999).

2.4. Chlorination of pure bacterial cultures using TSB as a contact medium.

The experiment was performed as mentioned above, except that the bacterial suspension (10^7 cfu ml^{-1}) would be suspended in TSB instead of PBS (1,500 ppm, corresponding to 1,120 ppm of organic load) (Virto *et al*., 2005b). The used Na-hypochlorite solutions were of concentrations (0.05, 0.5, 2, 4, and 6 mg l^{-1}). In addition, control series were prepared by the same procedure except that the chlorine solutions were replaced by sterile distilled water.

2.5. Comparison between GALase activity and cfu as a measurement of chlorine GE.

10 µl of the previously prepared bacterial suspension (10^7 cfu ml^{-1}) was added to 1ml of Na-hypochlorite solutions of concentrations (0.05, 0.5 and 1 mg l^{-1}) for specific contact time of (10 minutes) after which the effect of chlorination was quenched by addition of 100 µl to 900 µl sodium thiosulphate solution. Control series were prepared by the same procedure except that the chlorine solutions were replaced by sterile distilled water. The residual surviving cells using appropriate serial dilutions was determined by surface viable count by spreading method using TSAYE as growth medium , incubated at 37ºC for 16-18 hours and also through their enzymatic activity using the fluorometric method (Tryland *et al.*, 1998).

Comparison was achieved by calculating (log [N/N◦]) of both GALase activity and cfu of the same bacterial isolate ,when N represents cell numbers and enzyme activities after chlorination, N◦ represents cell numbers and enzyme activities before chlorination (Tryland *et al.*, 1998).

2.6. Measurement of GALase activity.

The GALase assay was performed by placing 100µl of each bacterial concentration in a 250-ml flask containing 20 ml of PB (pH 7.2) supplemented with 0.7 gm of MUGal per liter, 0.2 gm of sodium lauryl sulfate per liter, and 0.1% nutrient broth. The flasks were incubated in a shaking water bath at 44.5 º C, The fluorescence intensities of sample aliquots (2.5 ml of sample and 100 µl of 10 M NaOH) were measured every 5 minutes for 35 minutes with A Perkin Elmer model LS55 fluorometer with excitation at 365 nm and emission at 440 nm (Tryland and Fiksdal, 1998).

2.7. Statistical analyses.

ANOVA and other tests were developed using GraphPad Prism 5 (For Windows, © 1992- 2007 Graphpad software Inc., V 5.01, USA).

3. Results

Results displayed in Table (1) and Fig. (1-4) illustrate the comparison between the behavior of chlorine dosages using PBS and TSB as contact media with eight *E. coli* isolates using ANOVA. When using PBS solutions as a contact medium, the mean values of the GEs of chlorine concentrations on all isolates show an extremely significant different from control group (show zero GEs) at $P < 0.0001$, high R-squared values were observed which ware at all contact times > 0.85. When using TSB as a contact medium, the mean values of the GEs of chlorine concentrations on all isolates show an extremely significant different from control

group at $P < 0.0001$ only at contact time of 10 minutes. While, $P < 0.001$ was observed at contact time of 20 minutes and $P < 0.01$ at contact times of 30 and 40 minutes and R-squared values were observed which ware at all contact times < 0.5.

The decimal log reduction rate of selected eight *E. coli* isolates was again determined when they were suspended in PBS pH (6.8), using chlorine dosage range (0.05, 0.1 and 0.5) mg l^{-1} at contact time of 10 minutes. Comparison was achieved by calculating (log [N/N◦]) of both GALase activity and cfu of the same bacterial isolate ,when N represents cell numbers and enzyme activities after chlorination, N◦ represents cell numbers and enzyme activities before chlorination. Enzyme activities were measured using the fluorometric method mentioned before and cfu was calculated through surface viable count using TSAYE media and growth conditions of (37º C, 16-18 hours) by spreading method.

The mean value was taken to the results of the eight isolates and XY relationship was performed between chlorine concentration, and (log [N/N◦]) of enzyme activity and that of cfu. The results displayed in Fig. (5) demonstrate that the resulted relationships had minimum values - 0.042 and -1.35 and median values - 0.022 and - 0.22 for (log [N/N◦]) of enzyme activity and that of cultivability (cfu) respectively. Moreover, (log [N/N◦]) of enzyme activity had (Mean ± SEM: - 0.02 ± 0.009) and (log [N/N◦]) of cultivability (cfu) had (Mean ± SEM : -0.44 ± 0.312).

Table 1. Summary of the statistical results of decimal log reduction rate of *E. coli* isolates suspended in PBS pH (6.8) and TSB using chlorine dosage range (0.05 to 6) mg/l (contact time = 10, 20, 30 and 40 minutes)

Chlorine dosage	Contact time	Number of tested isolates	*P value*	*P value* meaning	R square
In saline solution (Chlorine dosage = 0.05, 0.1, 0.5, 1, 2, 4 and 6 mg l^{-1})	10 minutes	8	< 0.0001	Extremely significant	0.9269
	20 minutes		< 0.0001	Extremely significant	0.8635
	30 minutes		< 0.0001	Extremely significant	0.9435
	40 minutes		< 0.0001	Extremely significant	0.9012
In T.S.B. media (Chlorine dosage = 0.05, 0.5, 2, 4 and 6 mg l^{-1})	10 minutes	8	< 0.0001	Extremely significant	0.4109
	20 minutes		< 0.001	Highly significant	0.3484
	30 minutes		< 0.01	Significant	0.2866
	40 minutes		< 0.01	Significant	0.2692

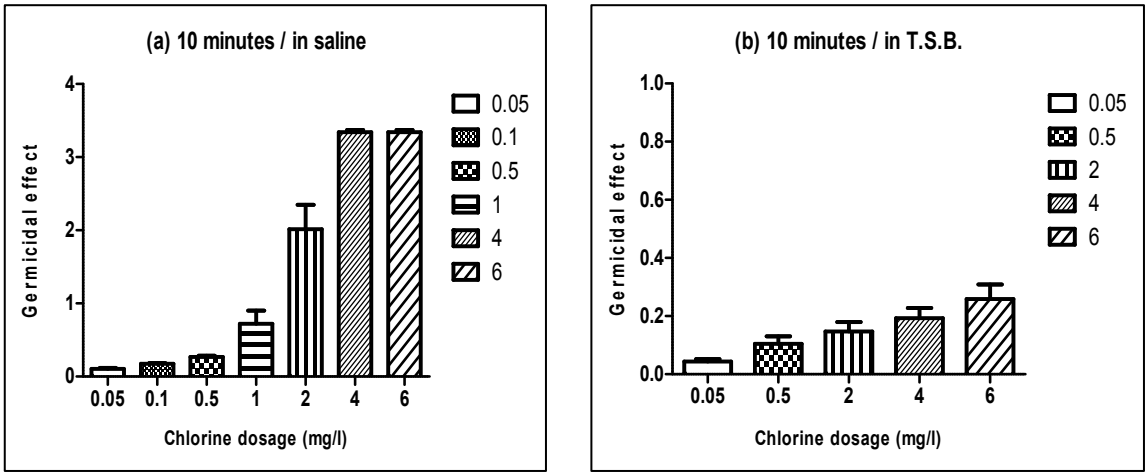

Fig.1. The column analysis of the decimal log reduction rate of 8 *E. coli* isolates suspended in PBS pH (6.8) and TSB using chlorine dosage range (0.05 to 6) mg/l (contact time = 10 minutes)

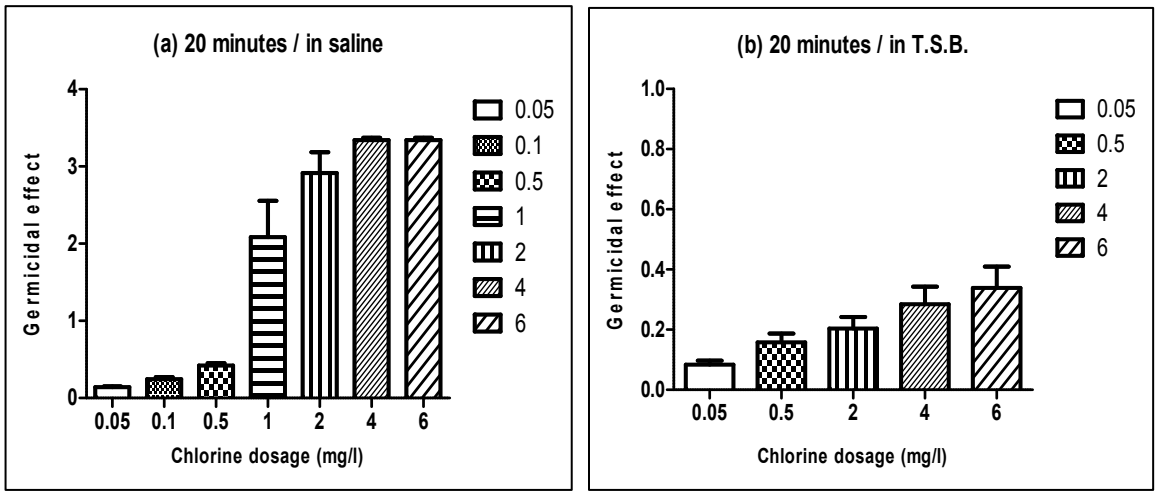

Fig.2. The column analysis of the decimal log reduction rate of 8 *E. coli* isolates suspended in PBS pH (6.8) and TSB using chlorine dosage range (0.05 to 6) mg/l (contact time = 20 minutes)

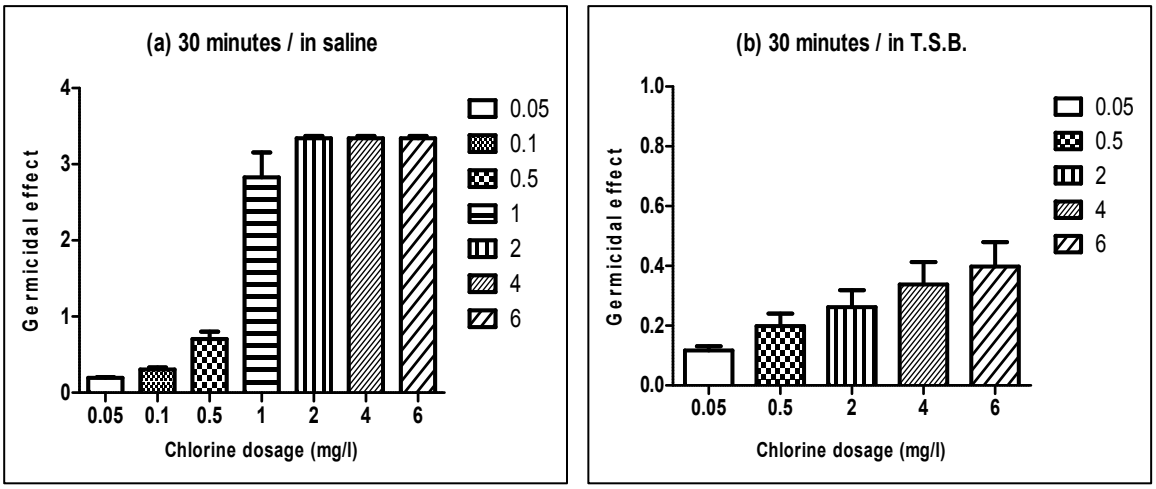

Fig.3. The column analysis of the decimal log reduction rate of 8 *E. coli* isolates suspended in PBS pH (6.8) and TSB using chlorine dosage range (0.05 to 6) mg/l (contact time = 30 minutes)

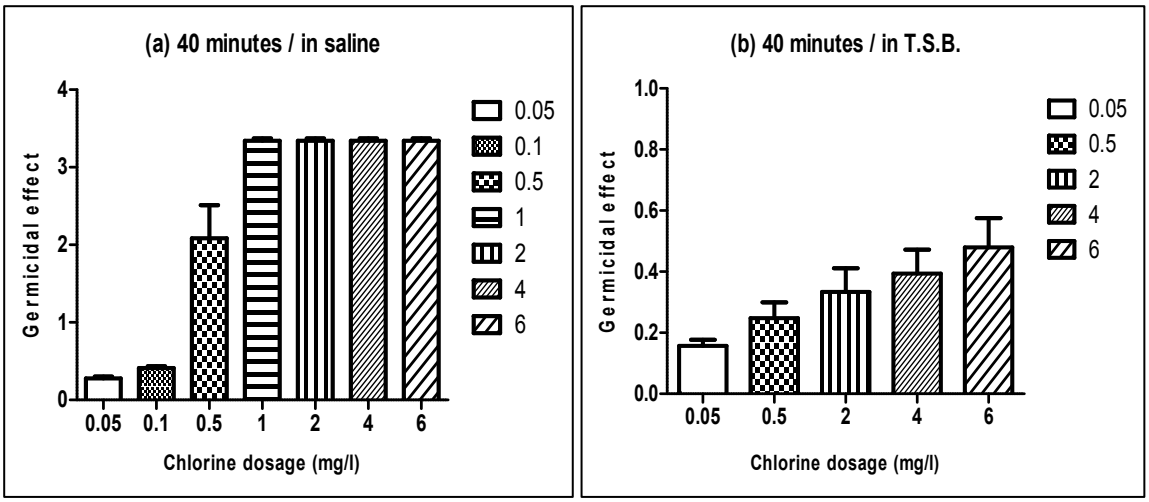

Fig.4. The column analysis of the decimal log reduction rate of 8 *E. coli* isolates suspended in PBS pH (6.8) and TSB using chlorine dosage range (0.05 to 6) mg/l (contact time = 40 minutes).

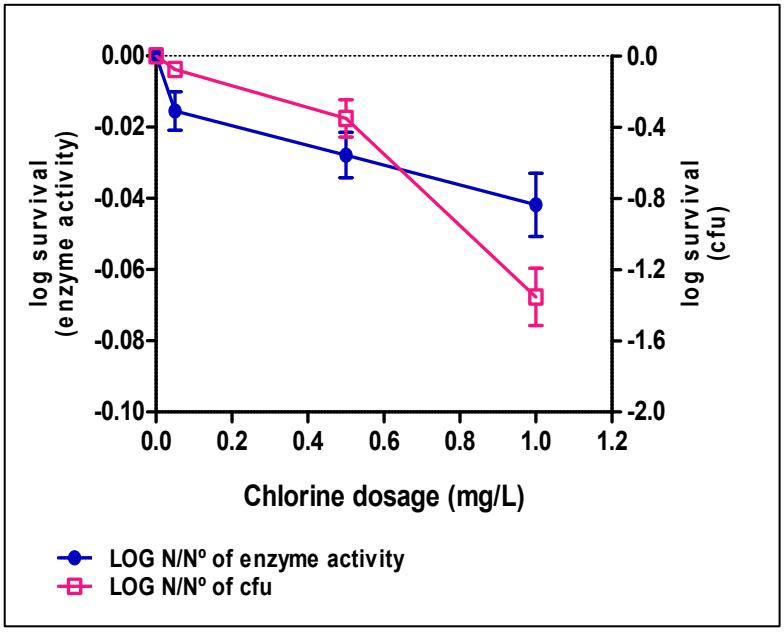

Fig.5. Statistical analysis of log survival of all *E. coli* isolates (log [N/Nº]) represented as GALase activity as well as cfu on TSA after exposure to chlorine concentrations (0.05, 0.5 and 1 mg/l) at contact time 10 minutes. "N represents cell numbers and enzyme activities after chlorination and Nº represents cell numbers and enzyme activities before chlorination". Error bars represent SEM of mean values based on duplicate experiments

4. Discussion

Effective microbial control by chlorine requires appropriate disinfection design criteria to ensure protection of public health and to minimize unwanted effects of the chlorination process. A better understanding of the way that chlorine kills cells would help in defining effective chlorine treatments and in optimizing strategies for chlorination.

The GE of chlorine solutions can be easily increased by increasing the chlorine concentrations even more than increasing the contact time, *i.e.* the GE is a matter of chlorine concentration and contact time. When using TSB as a treatment medium, the mean values of the GEs of chlorine concentrations on all isolates show an extremely significant difference from control group at $P < 0.0001$ through 10 minutes. On the other hand, the P value decreased while the contact time increased, and R-squared values were < 0.5. Those results clearly indicate the weak relationship between the GE and chlorine concentrations. Moreover, the worse the relationship becomes, the more the contact time increases.

The death of the bacterial cell depends on the penetration of chlorine into the interior of the cell. Treatments that increase the cell's permeability to chlorine should increase the death rate and reduce the time required to inactivate the target microorganism. The pH of the test solution is one of the most important factors affecting chlorine sanitizer activity. Formation of hypochlorous acid (HOC1) and of hypochlorine ion (OC1-), both responsible for antimicrobial chlorine activity, is a function of the pH value solution (Penna *et al*., 1996). It was found after initial trials that the bactericidal effectiveness of total available chlorine increased as pH decreased due to formation of hypochlorous acid form. The hypochlorous acid form has greater antimicrobial activity and an elevated ability to diffuse through the cellular membrane if it is compared to the hypochlorine ion form produced at higher pH.

The antimicrobial effect of available chlorine was inhibited by mixing with sodium thiosulfate neutralizer. Populations of viable *E. coli* cells exposed to 0.1 N sodium thiosulfate for up to 15 min did not differ significantly from cells in water. The sodium thiosulfate was therefore assumed to have no effects on *E. coli* cell population viability.

The effect of using TSB as a contact medium during chlorination of pure bacterial cultures.

There were large differences in chlorine resistance depending on the composition of the treatment medium. In aqueous media, *E. coli* isolates were high chlorine sensitive. The microbial resistance drastically increased when TSB was present in the treatment medium. The protective effect of organic matter against chlorine has been attributed to the higher chlorine demand of organic compounds, which results in a rapid decline in the available free chlorine. However, it was observed that, the residual chlorine concentration in the presence of organic matter was

much higher than the chlorine concentration necessary to completely inactivate the microbial population in distilled water. Therefore, the protective effect cannot be attributed solely to the chlorine demand of TSB but may be due to effective stabilization of some cellular structures and that was aligned with Virto *et al.* (Virto *et al.*, 2005b).

Chlorine is generally considered a nonselective oxidant, which reacts avidly with a variety of cellular components and affects metabolic processes. The cytoplasmic membrane has been proposed to be a possible key target involved in bacterial inactivation by chlorine, since alterations in its permeability after chlorination have frequently been described. For gram-negative cells and for cells treated in the presence of organic matter, damage to the membrane was either irreparable or nonexistent (Virto *et al.*, 2005b). However, we cannot disregard the possibility that the cell wall plays a role in the inactivation of cells by chlorine since the presence of organic matter in the treatment medium protected cell membranes against permeabilization and simultaneously increased the free chlorine concentration needed to attain cell killing.

For bactericides to be effective, they must be able to penetrate the cell envelope and attain a concentration at the target site high enough to exert their antimicrobial action. Results obtained in this investigation suggest that the presence of organic matter could stabilize the envelopes and thus slow the penetration of chlorine into the cell.

Comparison between GALase activity and cfu as a measurement of chlorine effect.

Obviously, transient exposure to HOCl in nutrient-free buffer leads to a significant loss of culturability, even though a large fraction of the cells remains viable. The apparent recovery of culturability without addition of nutrients is largely due to regrowth of a few culturable cells at the expense of the damaged cells. The level of recovery appears to be dependent on the initial stressed-cell density and is independent of the number of surviving cells after the stress. Postgate and Hunter (Postgate and Hunter, 1962) calculated that approximately 50 cells need to die to support the growth of 1 cell, interestingly, filtrates of stressed cells supported the growth of untreated cells as well as the crude extracts did, suggesting that essentially all nutrients are released immediately upon stress. Stress conditions may induce response pathways that permit mildly injured VBNC cells to repair or bypass injury. Previous studies (Dukan and Touati, 1996), (Dukan *et al*., 1996) suggested that exposure to HOCl triggers an adaptive response(s). Recovery (resuscitation) of VBNC cells with different lag periods produces a characteristic growth pattern with an increased apparent growth rate. This was not related to a lack of nutrient, since 2 x 10^6 stressed cells supplemented with the filtrate of 2 x 10^8 stressed cells grew at the same rate as unstressed cells. This result indicates that when there are fewer than 10^7 stressed cells, the number of recoverable VBNC cells is too low to be detectable by our experimental methods. To characterize these growth patterns, a deterministic model was constructed. Some HOCl-stressed *E. coli* organisms are capable of switching between the VBNC and culturable states in PB. This recovery of culturability under particular environmental conditions constitutes a challenge for microbiologists attempting to detect these indicator organisms by currently accepted methods.

We can generally observe that, at low dosage of chlorine (0.05 mg l^{-1}), neither cultivability (cfu) nor enzyme activity of *E. coli* suspensions was severely impaired *i.e.* the maximum-recorded value of (log [N/N◦]) of cultivability (cfu) was - 0.17 and that of enzyme activities was - 0.04. Besides, in rare cases we can observe that the enzyme activity of *E. coli* suspensions increased with low chlorine concentration that was clearly interpreted by Tryland *et al.* (Tryland *et al.*, 1998) as the small increase in enzyme activity of *E. coli* observed at small chlorine dosages indicates cell membrane damage that could facilitate the transport of enzyme or substrate across the membrane and so apparently increase the enzyme activity even more than the control groups.

When the chlorine dosage increases to (0.1 mg l^{-1}), the differences between the cultivability and enzyme activity become higher and significant, *i.e.* the maximum-recorded value of (log [N/N◦]) of cultivability (cfu) was – 0.98 and that of enzyme activities was – 0.058. Finally when the chlorine dosage further increased to (0.5 mg l^{-1}), cultivability nearly indicates the death of the bacterial cells while, enzyme activity still indicates an impairment in the biological activity of the cells but not death.

The mean value was taken to the results of the eight isolates and XY relationship was performed between chlorine concentration & (log [N/N◦]) of enzyme activity and that of cultivability (cfu). The results displayed in figure 5 demonstrated that (log [N/N◦]) of enzyme activity had (Mean ± SEM: - 0.02 ± 0.009) and (log [N/N◦]) of cultivability (cfu) had (Mean ± SEM: -0.44 ± 0.312). From those results, we can easily observe that the mean value obtained from log [N/N◦] of cultivability (cfu) was more than twenty times lower than log [N/N◦] of enzyme activity.

George *et al.* (George *et al*., 2000)**,** offers a possible explanation for higher enzymatic activity per culturable coliform in less contaminated waters that it could be a stronger underestimation of the number of coliforms when enumerated by plate count. This is due to a higher proportion of active (cells with a detectable GALase activity) but nonculturable coliforms in these waters. Indeed, several studies have shown that bacteria could be metabolically active even if they were not detected by the cultivation techniques

commonly used (Roszak and Colwell, 1987), (Barcina *et al*., 1989), (Garcia-Lara *et al*., 1993), (Davies *et al*., 1995) and (Pommepuy *et al*., 1996).

The smaller and dissimilar decrease observed when enzyme activity was compared with decrease in cultivability (cfu) after chlorination, therefore demonstrated the presence of non-cultivable *E. coli* cells, which retained assayable GALase activity. The chlorine oxidized more highly reactive sites first. The amount of chlorine, however, was insufficient to cause a measurable decrease in GALase activity in whole cells, although it decreased activity of crystalline enzyme. The results suggest that enzyme activity damage induced by chlorine was not the primary lethal event in inactivation of *E. coli* at this low dosage where cultivability (cfu), but not enzyme activity, was severely impaired (Tryland *et al.*, 1998).

Conclusion:

Under Egyptian conditions, our study has resulted in that chlorine should be added to water supply free from any organic matter that could reduce its potential and so decrease its efficacy. Chlorine germicidal effect should be measured using the enzymatic activity not conventional culture based methods to avoid the underestimation of water contamination.

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