ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Enhancement the recovery of chlorine stressed bacteria from water by using sodium pyruvate

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Manuscript Info

Manuscript History:

Received: 15 October 2015
Final Accepted: 26 November 2015
Published Online: December 2015

Key words:

Chlorine stressed bacteria, bacterial indicator, water, sodium pyruvate.

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Abstract

Although classical methods still used for bacterial indicators detection, but some stressed bacteria cannot determined. Thus this study aimed to improve the recovery of stressed bacteria from water by using sodium pyruvate to culture media. Also, to compare between the standard culture media and modified media supplemented by sodium pyruvate. Total bacterial counts (TBC), total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS) were detected. TC, FC and FC were detected by using two methods including Multiple Tube Fermentation (MTF) and Membrane Filtration (MF). The results showed that, the TBC averages at 37 and 22˚C were 34.7 and 28.7% using media supplemented with 0.03% wt/vol of sodium pyruvate. While the recovery value % increase to 54% at 37˚C, and 45% at 22˚C by using media supplemented with 0.05% wt/vol of sodium pyruvate. The recovery of TC by using MT and MTF methods were 54.9 and 42.4% respectively by using media supplemented with 0.03% wt/vol of sodium pyruvate, While the recovery value % increase to 59.6% by MF method, and 48.9% by MTF, using media supplemented with 0.05% wt/vol of sodium pyruvate. The FC average using selective media was 12.5% using media supplemented with 0.03% wt/vol of sodium pyruvate, While the recovery value % increase to 32% with Selective media using media supplemented with 0.05% wt/vol of sodium pyruvate. It can be concluded that, by addition sodium pyruvate increase capability for stressed bacteria recovery from water samples, these supplemented media with sodium pyruvate are necessary for reducing the threat of false negative results in natural water.

INTRODUCTION

Water treatment plants are designed to provide water continuously that meets drinking water standards for human consumption at the tap. Water at the tap should be free from pathogens and toxic or physiologically undesirable chemicals or any other biological materials (El-Taweel and Shaban, 2001). Though chlorinated water has helped municipal treatment plants fight against pathogens. It is absolutely necessary to maintain chlorine residual in drinking water, to prevent the regrowth of bacteria. Although chlorine is the primary disinfectant of choice in water treatment plant, it is not entirely effective at inactivating all pathogens, many water borne pathogens are resistant to chlorine and are often found in finished water (Rompré et al., 2002; WHO, 2004). Conventionally detection methods for water quality are based on the membrane filtration (MF) and the multiple tube fermentation (MTF) techniques for quantifying fecal bacterial indicator. These techniques owe some limitations such as long analysis times, lack of

sensitivity and accuracy and inability to detect viable but non-culturable bacteria **(**VBNC**)** (Caruso *et al.*, 2002; El-Liethy, 2013). In drinking water treatment processes including disinfection which can cause injury for total bacterial count and coliform bacteria resulting in a damaged cell and convert them in to viable but nonculturable by inability to form a colony on a selective media and increase sensitivity to bile salts or to replacement surface active agents contained in some selective media (Rompré et al., 2002; Kennedy, 2012). Many studies found that, the culturability is reduced after disinfection by chlorination; a substantial part of the bacterial cells is in VBNC because of the inability of these cells to persist under adverse conditions, including chlorination (Catroux et al., 2001; Basaglia et al., 2007; Ibrahim, 2014). This physiological state, in which bacterial cells are still alive, but not able to form colonies, exists in many microorganisms, including total coliforms and *E. coli* (Basaglia et al., 2007; Barcina and Arana, 2009). Such a state was extensively reviewed by Hayes and Low (2009). They recommended that not only determined the number of surviving cells that are able to form colonies on regular growth media, but also estimated the surviving cells using direct counting techniques including the live/dead stain. Based on differences in results between these enumeration techniques, the results indicate that only a fraction of the living bacterial cells are able to form colonies after inoculated into nutrient media suggesting that supplemented with sodium pyruvate, that induces a VBNC state of bacteria to be culturable (Vriezen et al., 2012). Thus, this study was carried out to compare between conventional media and modified media supplemented with sodium pyruvate, to evaluate the microbiological water quality, and determine the proper method for recovery of stressed bacterial indicators from different water sources, to avoid false negative results that may lead to waterborne pathogens that may be reach to the consumer.

Materials and Methods

Sampling Sites

The total numbers of the collected samples were 400 samples from different water treatment plant processes in Fayoum city (80 Km Cairo south), Egypt as following; the thirty water samples were colleceted from Hassn Wasef intake of Old El-Azab drinking water treatment plant from five sites along 20 Km from south to north; El-Lahoon, Hwaret Adlan, Dmshkeen, Hwaret El- Maktaa and El- Mzarea bridge; 60 samples after sedimentation basins (clarified water) from three separated stages, Patrson, Sigma, Bamag of Old El-azab Water treatment plant (OEATP); 240 samples after filtration and post-chlorination; 30 samples from storage tanks and 40 water samples drinking water distribution systems. The samples were collected monthly interval during the period extended from December 2011 to November 2012.

Sampling and preservation

Sampling and preservation procedures were done according to Standard Methods for the Examination of Water and Wastewater (APHA, 2012). All samples were collected in one liter wide mouth sterile sampling bottles contain 5 ml of 10% $Na_2S_2O_3$ that have been cleansed and rinsed carefully, sterilized and autoclaved at 121˚C for 30 minutes, The samples were kept at 4°C in an ice box and were analyzed within 1- 8 h of collection.

Detection of bacterial indicators

Bacterial indicator including; total bacterial counts, total coliforms (TC) and fecal coliforms (FC) were determined according to APHA (2012). Total bacterial counts at both 37 and 22^{o}C were determined by using pour plate method. The TC and FC were determined by using both MTF and MF methods. In parallel to those methods, another set of those media supplemented by different concentration of sodium pyruvate were used. Sodium pyruvate pure (MERCK, German) was added by three concentrations; 0.03, 0.05, and 1.0% (wt/vol) directly to nutrient agar, M-Endo agar, laurayl tryptose broth, BGB and EC broth media prior to boiling and autoclaving. The pH of the sterile media was adjusted according to the specifications of the manufacturer. Media lacking supplements were designated nonamended controls.

Statistical analysis

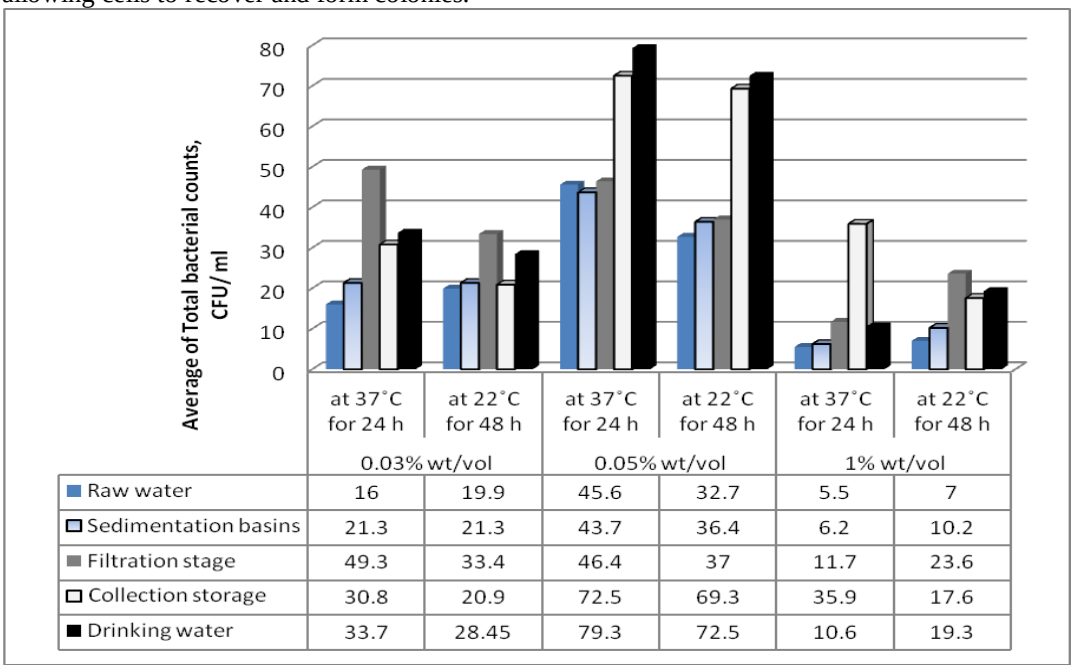
All experiments were performed in triplicate with duplicate samples and being analyzed at each sampling time. Data was subjected to analysis of variance (Paired T-test) using the Statistical Analysis System Program (Minitab). Significant differences ($p<0.05$) between mean values of number of cells in media amended with or without sodium pyruvate were determined by the mean significant difference (MSD) method.

Results and Discussion

Coliform group is considered as a prime indicator organisms used for analysis of treated water; therefore, it is important to ensure that the used media provide high recovery for both stressed and chlorine injured cells (McFeters et al., 1986). The exposure the bacteria to chlorine during and after treatment process makes those injured and loss

those bacteria to be recovered on culture media (Camper and McFeters, 1979). The growth or no growth response on selective media is the traditional means of assessing sublethal injury (Gilbert, 1984; Brashears and Stratton, 2001). The difference in plate counts between selective and nonselective media is used to quantify sublethal injury as a proportion or percentage of the entire population (Brashears and Stratton, 2001). Several investigators have shown that supplementation of various selective media with pyruvate, can improve recovery of stressed *E. coli* (Mackey and Seymour, 1987; Lee and Hartman, 1989). The present investigation evaluates the specificity and selectivity of some media which rely upon media supplemented with sodium pyruvate compared with standard media for detection and enumeration of total bacterial counts (TBC), total coliforms, and fecal coliforms in different aquatic environments. Two methods including MTF and MF were used for determination of coliforms in all collected water samples. Lauryl tryptose broth medium was used as a presumptive test for total coliforms and fecal coliforms; either brilliant green bile broth medium was used as a confirmation test.

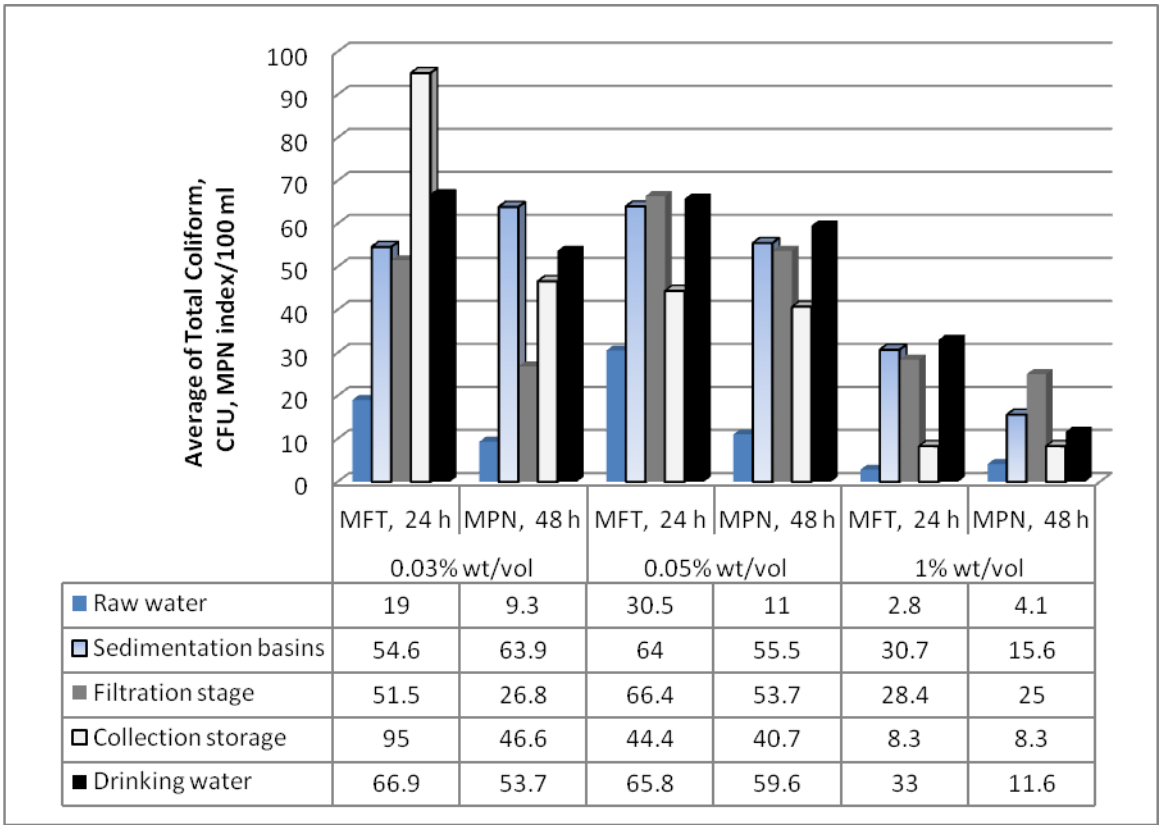
This study found that, all concentrations of sodium pyruvate, 0.03%, 0.05%, 1% wt/vol, which amended with media for determination of TBC, lead to enumeration of total bacterial counts at both 37 and 22˚C, with the average of recovery value 34.7 and 28.7%, respectively by using media supplemented with 0.03% wt/vol of sodium pyruvate, While the recovery value % increase to 54% at 37˚C, and 45% at 22˚C by using media supplemented with 0.05% wt/vol of sodium pyruvate, On the other hand, the recovery value % decrease to 11.6% at 37˚C, and 17.9% at 22˚C by using media supplemented with 1% wt/vol of sodium pyruvate (Figure 1). Statistically in this search, there are a highly significance correlation for the recovery of total bacterial counts at 22˚C, between conventional media and media supplemented with any concentrations of sodium pyruvate, ($p<0.05$). These results matched with Tandon et al. (2007) they demonstrated that, the recovery of bacteria was lower than that, the media supplemented with 0.05 % (wt/vol) sodium pyruvate bacteria and a statistically significant higher mean count ($P < 0.005$) than for aerobic unsupplemented medium. The main function of pyruvate was to degrade H2O2, as opposed to functioning as an additional carbon source for the cells. The enhance recovery of bacterial cells by using supplemented media with sodium pyruvate it may revealed to enhance reactive oxygen species (ROS) generation in mitochondria can initiate the mechanism described as ROS induced ROS-release. Thus, aerobic organisms have developed complex defense and repair systems (Christman et al., 1985). In addition to that, the hydroxyl radicals show strong biochemical activity and can react directly with membrane lipids, proteins and DNA. Reactive oxygen species are normally scavenged by antioxidants and various enzymes; however, elevated concentrations of ROS in microbial cells can result in oxidative stress in microorganisms (VBNC) (Sevcu et al., 2011). Mizunoe et al. (1999) suggested that sodium pyruvate protect nonculturable cells from oxidative stress (i.e., H2O2) when placed on amended media, allowing cells to recover and form colonies.



	0.03% wt/vol		0.05% wt/vol		1% wt/vol	
Raw water	16	19.9	45.6	32.7	5.5	7
Sedimentation basins	21.3	21.3	43.7	36.4	6.2	10.2
Filtration stage	49.3	33.4	46.4	37	11.7	23.6
Collection storage	30.8	20.9	72.5	69.3	35.9	17.6
Drinking water	33.7	28.45	79.3	72.5	10.6	19.3

Figure 1. Comparison between the average counts of total bacterial counts without and with adding 0.03, 0.05, 1% wt/vol. conc. of sodium pyruvate, in all water samples collected from different treatment stages.

The present study showed that, the three selected concentrations of sodium pyruvate, 0.03%, 0.05%, 1% wt/vol have been amended with media for total coliforms, lead to enumeration of total coliforms by using both methods MF and MTF, with the average of recovery value 54.9% and 42.4% respectively by using media supplemented with 0.03% wt/vol of sodium pyruvate, While the recovery value % increase to 59.6% by MF method, and 48.9% by MTF, using media supplemented with 0.05% wt/vol of sodium pyruvate, On the other hand the recovery value % decrease to 27.9% by MF method, and 15% by MTF, using media supplemented with 1% wt/vol of sodium pyruvate (Figure 2). Supplementation of M-Endo, and EC media, with sodium pyruvate resulted in significantly better recovery of coliform bacteria from chlorinated samples. In the present investigation it was found that, there are a highly significance correlation increase of total coliforms by both methods, MF and MTF between conventional media and media supplemented with any concentrations of sodium pyruvate ($p<0.05$). The results of this study were matched with many researchers (Sartory, 1999; Glynis et al., 2001; Ibrahim, 2014). A reduction in catalase activity may give a secondary stress to bacteria, mainly if these cells are pushed to grow under aerobic incubation conditions. One consequence of reduced catalase activity may be the accumulation of toxic hydrogen peroxide (H2O2), to which injured bacteria apparently become increasingly sensitive (Mackey and Seymour, 1987). The adding of exogenous compounds catalase as exogenous enzyme or nonenzyme peroxide-degrading compounds, such as sodium pyruvate, to various selective media has been reported to increase detection of stressed or injured cells of *E. coli* and coliform bacteria (Calabrese and Bissonnette, 1990; Ibrahim, 2014).



	MFT, 24 h	MPN, 48 h	MFT, 24 h	MPN, 48 h	MFT, 24 h	MPN, 48 h
	0.03% wt/vol		0.05% wt/vol		1% wt/vol	
Raw water	19	9.3	30.5	11	2.8	4.1
Sedimentation basins	54.6	63.9	64	55.5	30.7	15.6
Filtration stage	51.5	26.8	66.4	53.7	28.4	25
Collection storage	95	46.6	44.4	40.7	8.3	8.3
Drinking water	66.9	53.7	65.8	59.6	33	11.6

Figure 2. Comparison between the average counts of total coliforms without and with adding 0.03, 0.05, 1% wt/vol. conc. of sodium pyruvate, in all water samples collected from different treatment stages.

In the present study, all the concentrations of sodium pyruvate, 0.03%, 0.05%, 1% wt/vol, that amended with media for fecal coliforms, lead to enumeration of fecal coliforms by using presumptive and selective media, with the average of recovery value 15.4% and 12.5% respectively by using media supplemented with 0.03% wt/vol of sodium pyruvate, While the recovery value % increase to 38% with presumptive media , and 32% with Selective media, using media supplemented with 0.05% wt/vol of sodium pyruvate, On the other hand the recovery value % decrease to 12.5% with presumptive media , and 12.2% with Selective media, using media supplemented with 1% wt/vol of sodium pyruvate (Figure 3). There are a highly significance increase of fecal coliforms by both Presumptive and Selective media, between conventional media and media supplemented with any concentrations of

sodium pyruvate, ($p<0.05$). Sartory (1999) reported that incorporation of sodium pyruvate into M-endo agar resulted in significant improvements on membrane filtration recovery of *E. coli* from chlorinated drinking-water samples. Recovery of total coliforms on a new medium supplemented with 0.05% wt/vol of sodium pyruvate (Grant, 1997). Gurtler and Beuchat (2005) found that, the selective media supplemented with sodium pyruvate, vary greatly in their abilities than conventional media to support resuscitation and colony formation in order to performance of media for recovering stressed cells. Baird-Parker and Davenport (1965) demonstrated that pyruvate enhanced the repair of damaged bacterial cells that were otherwise inhibited in their ability to resist toxic oxidizing substances that were present as a result of the destruction of catalase.

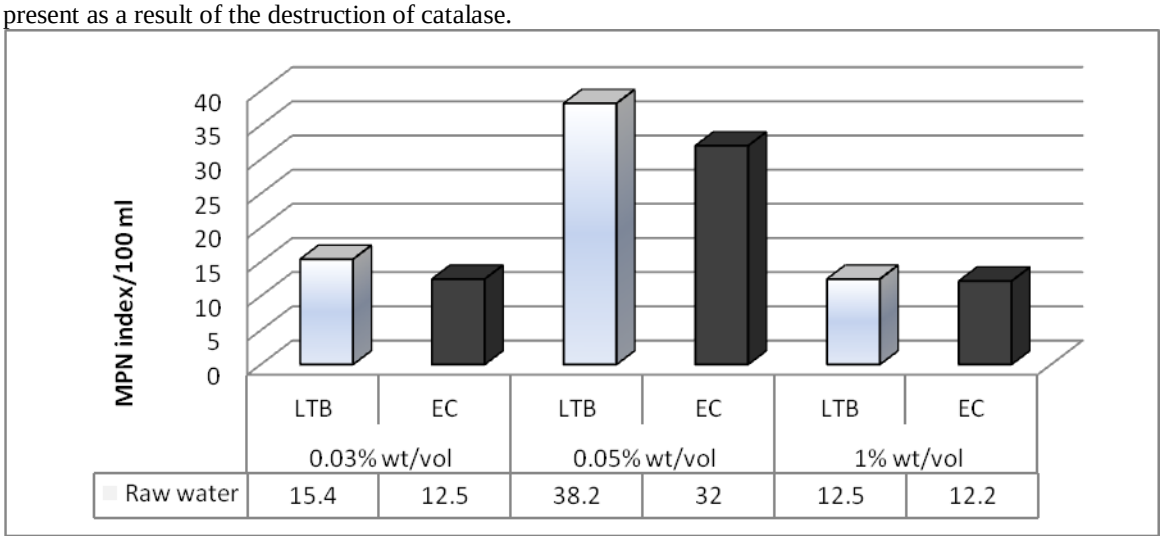


Figure 3. Comparison between the average counts of fecal coliforms without and with adding 0.03, 0.05, 1% wt/vol. conc. of sodium pyruvate, in all water samples collected from different treatment stages.

The observation in this study shows that (i) chlorination induces a VBNC state in bacterial cells, which has not been reported before; (ii) finding methods to avoid a VBNC state may improve the quality of media, used for inoculation in water treatment plants, to avoid false negative results. However, the addition of enzymes to media is not easily accomplished and high cost, It is more convenient to add sodium pyruvate because it can be autoclaved as part of the medium components. The addition of pyruvate alone to selective media increased the recovery of injured cells to many several fold.

In general, microorganisms have developed two principal strategies for oxidative damage defense. The first includes antioxidant enzymes such as superoxide dismutases (SOD), glutathione peroxidases, catalases, and non-enzymatic low molecular mass molecules that include ascorbate, pyruvate, flavonoids, carotenoids and glutathione (Wolfe-Simon et al., 2005). The second strategy of defense is based on repair enzymes which remove and/or repair oxidatively damaged macromolecules (Crawford et al., 1994). Both defense strategies are induced at the lowest level of oxidative stress. During higher levels of oxidative stress, the protective response shifts to a pro-inflammatory response which induces redox sensitive signaling pathways. At the highest level, injury to the electron transfer chain and mitochondrial membranes could lead to acute toxicity and cell apoptosis (Xia et al., 2008; Meng et al., 2009). This study introduces a technique by which enhanced recovery of both coliform and HPC bacteria from chlorinated water can be attained by incorporation of sodium pyruvate, into standard recovery media.

In conclusion, most media that contain selective agents do not permit quantitative recovery of injured bacterial cells, Addition of sodium pyruvate, 0.05% wt/vol. to presumptive or selective media as the best concentration supplemented increased the recovery of injured cells to many several fold. Additionally, in nutrient agar medium, all pyruvate levels except 1.0% provided a 1.7-fold increase of HPC bacteria. The highest recovery of fecal and total coliform bacteria on M-FC media was observed with plates containing 0.05% pyruvate, whereas 0.03 and 0.05% amendments provided optimal recovery on M-Endo medium. Conversely, supplementation of M-Endo with 1.0% pyruvate resulted in a decreased detection of total coliforms compared with the control. A concentration of 0.05% pyruvate is recommended for all media in subsequent experiments.

Overall, from statistical analysis, it was found that, there are a highly significant correlation between the results of conventional media and media supplemented with 0.05% wt/vol. of sodium pyruvate, for recovery of total bacterial counts, total coliforms, and fecal coliforms.

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