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Research Article

Inactivation of *Escherichia coli* and *Salmonella* with Chlorine in Drinking Waters at Various pH and Temperature Levels

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Abstract: Effect of chlorination at pH 6, 7 and 8 at temperatures 15, 25 and 35˚C for maximum inactivation of *Escherichia coli* (*E. coli*) and *Salmonella* in distilled, tap and well water was examined at 0.25, 0.5 and 1.0 mg/L chlorine. Samples were collected at 0.5, 1, 3, 5, 10, 15, 30 and 45 minutes to determine inactivation through spread plate count (SPC). Among the three types of waters, distilled water was found suitable for better chlorine inactivation as no *E. coli* counts were detected at 1.0 mg/L of applied chlorine at pH 7 with a 7 log removal with a survival of 0.001 percent. For pH and temperature effect when observed at 0.5 mg/L of applied chlorine dosage showed that the removal was more at pH 7, which was 6 log removal while for temperatures, 35 ºC was found to be optimum with a final E. coli count of 3.0×10^1 CFU/mL with a survival of 0.00025 percent after 45 minutes contact time. *Salmonella* was found more susceptible to chlorine as compared to *E. coli* with *E. coli* count of 8.1×10^5 and *Salmonella* count of 3.8×10^3 CFU/mL after 45 minutes of contact time at 1 mg/L applied chlorine. *Salmonella*, when tested as monoculture, 7 log removal was observed at 1 mg/L of applied chlorine. So chlorine is found to be an effective disinfectant, provided the optimum temperature of 35ºC and pH 7 for maximum inactivation of *E. coli* and *Salmonella*.

Keywords: Disinfection, *E. coli, Salmonella*, SPC, CFU, residual chlorine, drinking water, inactivation

1. INTRODUCTION

Water pollution is one of the major threats to public health in Pakistan, and the country ranks at number 80 among 122 nations regarding drinking water quality [1]. The availability of safe drinking water to public is only 40% to 60% [2]. Human activities like improper disposal of municipal and industrial effluents and indiscriminate applications of agrochemicals in agriculture are the main factors contributing to the deterioration of water quality. Among all the pollutants, microbial pollutants are the main factors responsible for various public health problems. Microbial contaminants can enter the distribution system through negative pressure and cross connection with other non-potable water pipes [3]. On the other hand, drinking water quality is poorly managed and monitored throughout the country. Waterborne infections such as cholera, typhoid fever and dysentery burden the public health system and impose significant economic losses. One of the causative agents of water borne human diseases is *E. coli* which has fecal origin [4]. The high incidences of waterborne diseases are frequently associated with shiga toxin (STEC) and entero toxin produced by *E. coli* (ETEC) [5]. It is a normal inhabitant of the gastrointestinal tract of warm-blooded animals and is used as an indicator of water quality as they are present in greater number in feaces and they survive longer as compared to other pathogenic bacteria in drinking water. Detection *E. coli* is a major priority in assessing the drinking water quality [6] as their presence in drinking water clearly shows fecal contamination and indicates a possible presence

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of enteric pathogens [7-8] in water. The pathogens present in drinking water like *Salmonella, Shigella*, *Yersinia enterocolitica*, *Campylobacter* and parasites like *Entamaeba histolytica* and *Giardia lambliac*ause serious risk of water borne diseases like cholera, typhoid, dysentery and hepatitis A and E [9]. The increasing bacteriological contamination of drinking water in Pakistan and their consequent effects on human health and environment is an issue of great concern. This contaminated water badly damages natural system of human body and makes it prone to a number of serious illnesses. Clinical manifestations of *E. coli* infection range from asymptomatic excretion, through mild non -bloody diarrhea to hemorrhagic colitis and severe complications as hemolytic uremic syndrome (HUS) with acute renal failure, sometimes resulting in death [10].

Among these infections, 95 % diseases are preventable by applying conventional water treatment practices. Control of microbial growth in drinking water distribution systems, often achieved through the addition of disinfectants, is essential to limit waterborne illnesses, particularly in immune compromised subpopulations [11]. Drinking water supplies are disinfected primarily to inactivate pathogens before water reaches any consumer. Chlorine, as a non-selective oxidant, reacts with both organic and inorganic chemical species in water; therefore, it functions as a highly effective antimicrobial agent to reduce the risk of water-borne infectious diseases. Chlorine also functions as a secondary disinfectant maintaining a disinfectant residual throughout the distribution system, so that a nominated residual is achieved even at the system extremities. Therefore drinking water chlorination is gaining importance for providing its residuals in the form of chloramines in the distribution network at the consumer's end [13]. According to water quality regulations, it is essential to have a minimum of 0.25 mg/L of chlorine residual over the whole distribution system at all times [12].

In Pakistan drinking water chlorination is practiced and this treated water is later on supplied to the consumers through distribution network. But there is no planned chlorination procedure for adequate disinfection process. On the other hand chlorination is affected by different drinking water parameters. These include applied chlorine dose, pH, temperature, total dissolved solids (TDS), electrical conductivity (EC) and contact time [14].

Although chlorine residual greatly contributes to the inactivation and regrowth of indicator bacteria, i.e., fecal coliforms in the pipeline, the question awaiting an answer, is the level of inactivation of other potential pathogens such as *Shigella* and *Salmonella* at the recommended levels of chlorine residual. In addition, there is a considerable gap and knowledge about responses of environmental factors including dose, pH, temperature and contact time and microbial populations to chlorination. Therefore, research in this field regarding improvements in chlorination process and provision of bacteriologically safe drinking water is the need of time which would ultimately have an impact on reduction of the incidence of diarrheal and other waterborne and water related diseases. So this study was designed to observe and determine the disinfection efficiency of chlorine and response of indicator microorganisms like pathogenic microorganisms like *E. coli* and *Salmonella* towards chlorination. The study will help in determining the optimum dose with suitable temperature and pH for maximum inactivation of *E. coli*, as indicator microorganism, and *Salmonella*, as waterborne pathogen.

2. MATERIALS AND METHODS

To examine the disinfection efficacy of chlorine, pure bacterial suspensions in high cell density have often been used. Under these conditions, dose-response behavior may be established for microorganism-disinfectant pairs by analyzing the extent of inactivation. These experiments allow the determination of inactivation to a large extent under highly controlled laboratory conditions so that interference by the complex environment of natural water can be avoided. In most experiments of this type, a pure bacterial culture, from pure bacterial stock has been inoculated in a growth medium for a given set of incubation conditions. Cells are then separated from the growth medium and resuspended in nutrient-free solution. In this manner, the organic materials of the growth medium, which might interfere or otherwise

interact with disinfectants, are separated from the organisms of interest, thereby facilitating analyses of organism-disinfectant interactions [15].

2.1 Characterization of Tap and Well water

The chemical characterization of tap and well water was performed prior to experiment to observe the effect of nature of water on chlorine disinfection efficiency as shown in Table 1.

2.2 Preparation of *E. coli* Culture

For mono culture studies, *E. coli* colonies were taken from EMB plates and streaked on agar slants and incubated at 37ºC for 48 hrs. For washing, the cultures were added to a phosphate buffer (pH 7) and centrifuged at 4000 rotation per minute (rpm) for 15 minutes and pellet was resuspended in 10 mL of phosphate buffer. The process was repeated and pallet was again resuspended in phosphate buffer mentioned above. The optical density of this solution was determined using OD meter.

2.3 Inoculation of the Culture Vessel

Approximately 2 mL of cultured *E. coli* suspension was added to the three 1000 mL reaction vessels each containing different types of water, viz. distilled, tap and well. After inoculating the culture, serial dilutions were made for spread plate count (SPC) before disinfection. This gave the actual number of approximately 10^7 CFU/mL bacteria in the sample before the experiment for chlorine disinfection studies at mesophilic temperatures (30˚C – 35˚C) [16]. The same procedure was repeated for each experiment for pH 6, 7 and 8 with temperature levels of 15, 30 and 35ºC.

2.4 Hypochlorous Acid Challenge Conditions

A freshly prepared free chlorine stock solution (525mg/L) was added to the bacterial suspension to get a final concentration of 0.25, 0.5 and 1.0 mg/L with continuous stirring using magnetic stirrer. Samples were periodically taken at 0.5, 1, 3, 5, 10, 15, 30, 45, 60 minutes and stored at 4 ˚C in one set of test tubes containing 0.1 mL sodium thiosulphate ($Na_2S_2O_3$) for SPC and second set of test tubes for chlorine determination without Na2S2O3. The addition of $Na_2S_2O_3$fixes the excess chlorine and stops its actions on *E. coli* so that it may not interfere with the exact SPC at that time.

2.5 Standard Plate Count (SPC)

For SPC, agar plates were prepared by pouring approximately 20 mL of molten NA (45˚C) into petri plates, evenly distributed and incubated upside down at 37 ˚C for 24 hrs. For getting accurate and countable range of microbial colonies i.e., 30-300 colonies, serial dilutions were made. Each dilution was plated by pipetting out 0.1 mL of serial dilution onto the sterile petriplate containing agar and spreading it gently with a spreader [17-18].

2.6 Residual Chlorine Measurement

Residual free chlorine (hypochlorous acid and hypo chlorite ions) was measured by N, N–diethyl –p-phenylene-di-amine (DPD) methods [19] using Spectroquant Picco colorimeter (Merck SN 059008).

3. RESULTS AND DISCUSSION

The present study was carried out to find the optimized chlorine dosages at pH 7 and room temperature for maximum inactivation of *E. coli* as model microorganisms in different waters, viz. distilled, tap and well. Three different chlorine dosages, i.e., 0.25, 0.5 and 1.0 mg/L were applied to observe the effect of pH and temperature and chlorine concentration on disinfection process.

3.1 Chlorine Disinfection Study in Three Types of Water

With this purpose for maximum inactivation of *E. coli* to meet the WHO Drinking Water Standards, experiments were conducted to determine the inactivation of *E. coli* with chlorine at 25 °C and pH 7.

3.1.1 Comparison of Disinfection Efficiency of Different Types of Water

The disinfection efficiency of chlorine was compared in the three waters, i.e., distilled, tap and well water to observe the behavior of chlorine and its disinfection ability in distilled water (depicting lab conditions) and, i.e., in tap and well water

(depicting field conditions). It is evident from the Fig. 1 that the inactivation of *E. coli* is greater in case of lab conditions, i.e., in distilled water while less evident in tap water and least in case of well water due to the increase chlorine demands (Fig. 1). The chemical analysis of tap and well water is given in Table 1. Due to the presence of salts in tap and well water, the chlorine demand increased, resulted in low inactivation of *E. coli*, respectively.

Similarly, the chlorine residual were also found to be different in three types of waters. In case of distilled water, more chlorine residual were present for the inactivation of *E. coli*, as its chlorine demand is negligible and resulted in greater inactivation of E. *coli* counts but on the other side, the chlorine demand of tap and well water was more so less inactivation occurred in the later cases, i.e., tap and well water respectively (Fig. 2).

3.2 Determination of Optimum pH for Maximum Disinfection of *E. coli*

From the previous experiments conducted, for the disinfection of *E. coli*, three chlorine dosages of 0.25, 0.5 and 1.0 mg/L were applied. Applied chlorine of 1.0 mg/L was determined optimum dosage for complete disinfection. To observe the effect of pH on the disinfection process as well as behavior of residual chlorine with time, the dosage of 0.5 mg/L was taken as test dosage to observe the effect of pH on chlorination process.

To observe the effect of pH, 0.5 mg/L chlorine was applied at three different pH, viz. 6, 7 and 8. Residual chlorine was measured periodically. The initial *E. coli* count, after inoculation of pure culture, was 3.2×107 CFU/mL and residual chlorine of 0.5 mg/L. In the first 30 seconds exposure, disinfection was not profound and the CFU/mL decrease was 1.2×10^7 and $2.0\times10^7$for pH 6 and 8, respectively, giving more disinfection at pH 6 than 8 as shown in Fig. 3. The chlorine residuals at this time were 0.47 and 0.34 mg/L. respectively. Similar results were also shown by Massa et al. [20] , when susceptibility of five *Aeromonas hydrophila* strains and one *E. coli* strain to chlorine was studied under carefully controlled laboratory conditions and it was shown that the rate of inactivation being greater at pH 6 than at pH 8 for both strains. But in case of pH 7, 1 log removal was achieved in this exposure time from 3.2×10^7 to 8×10^6 CFU/mL with a chlorine residual of 0.43 mg/L as shown in Fig. 4. The inactivation rate is slower at pH 6 and 8 but it is a bit more efficient at pH 7. This is due to the fact that the dissociated hypochlorite ion (OCl^{-1}) predominates at higher pH values, while the undissociated hypochlorous acid (HOCl) predominates at lower pH values. Hypochlorous acid is more reactive than the hypochlorite ion, and a much stronger disinfectant. Thus, a lower water pH promotes more efficient disinfection which decreases with increasing pH.

Most research has confirmed that chlorine is more biocidal at low, rather than high pH, and the pH effect is more profound for chlorine than other disinfectants, such as chlorine dioxide, ozone, and even combined chlorine (chloramines) [21]. Early research in the 1940s involving *E. coli, Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella dysenteriae* showed that HOCl is more effective than OCl^- for inactivation of these

Table 1. Chemical analysis of tap and well water as per standard methods [19].

S. No.	Parameter	Values	
		Tap water	Well water
1.	pH	6.71	7.17
2.	Temperature(°C)	18	17.6
3.	Total Dissolved Solids (mg/L)	198	688
4.	Conductivity (µS/cm)	412	1387
5.	Turbidity (NTU)	0.83	0.62
6.	Hardness ($CaCO_3$/L)	212	500

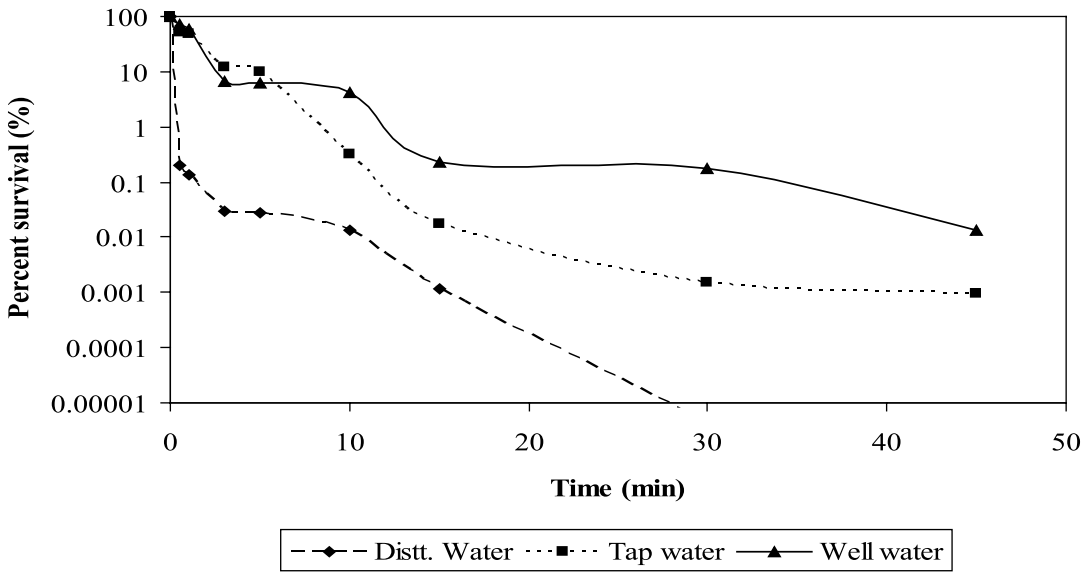

Fig. 1. Comparison of *E. coli* inactivation at 1.0 mg/L of applied chlorine dosage in three types of waters.

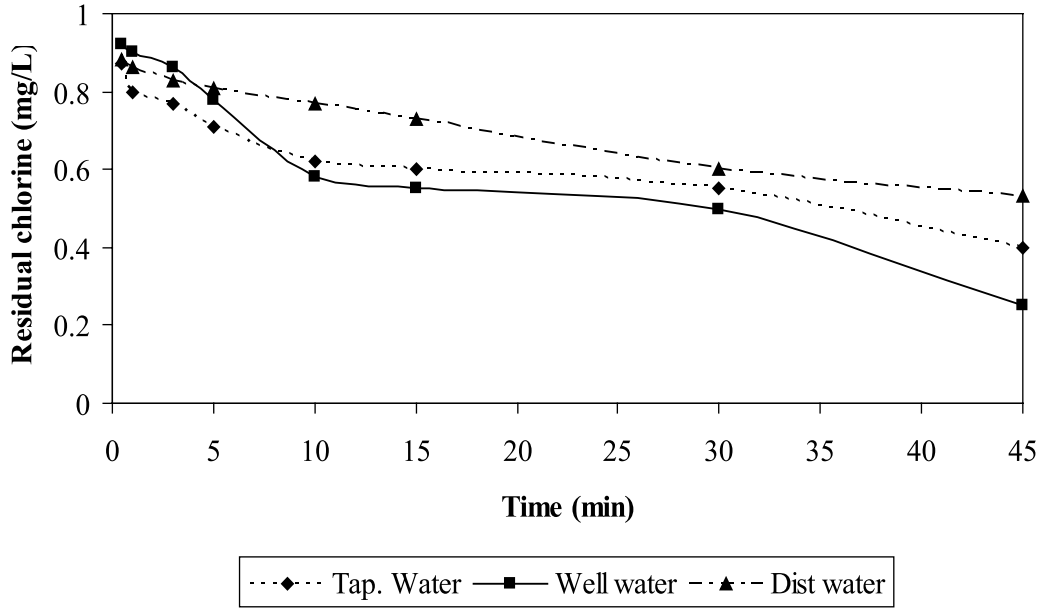

Fig. 2. Variation in chlorine residuals with time at 1.0 mg/L of applied chlorine dosage in three types of waters.

bacteria. Further research showed HOCl to be 70 to 80 times more effective than OCl for inactivating bacteria [22]. At pH of about 7.5, there is an equal distribution of HOCl and OCl^{-}; at pH 6.5, 90 percent of the free chlorine is present as HOCl. These results were in accordance with the results mentioned by Kenyon and Kathryn [23], who studied the kinetic inactivation by Free Available Chlorine (FAC) of the following disaggregated microorganisms, prepared to be free of extraneous chlorine demand. Bacteria tested were *Escherichia coli* (ATTC's 11229 and 23985), *Salmonella typhimurim*, *Shigella boydii*, and *Vibrio cholerae*. They showed that disinfection of these microorganisms was fast at pH 7 than at pH 5. 1 log removal was seen after 3 minutes of exposure time in case of pH 6 with a residual chlorine measurement of 0.33 mg/L. While at pH 8, 1 log removal was not achieved up till 10 minutes

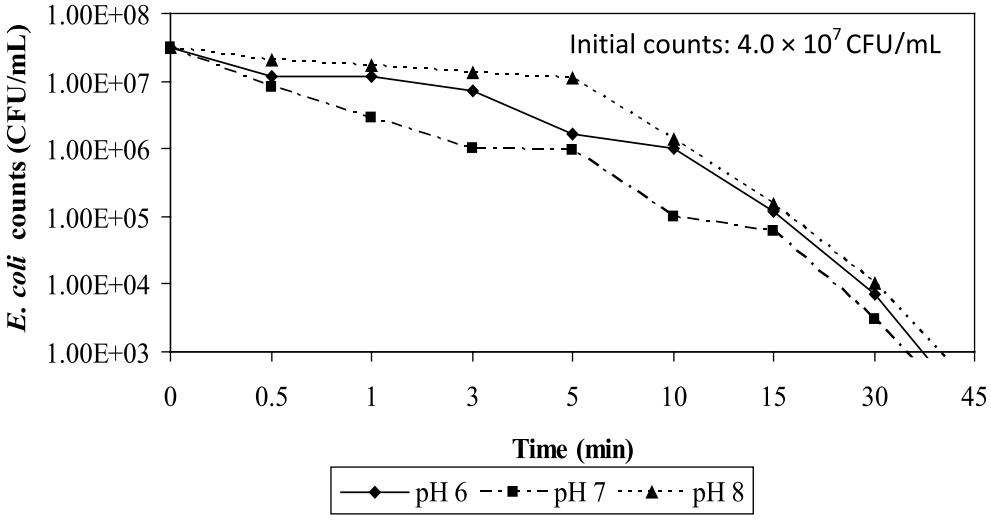

Fig. 3. Effect of different pH levels on survival of *E. coli* at 0.5 mg/L of applied chlorine dosage .

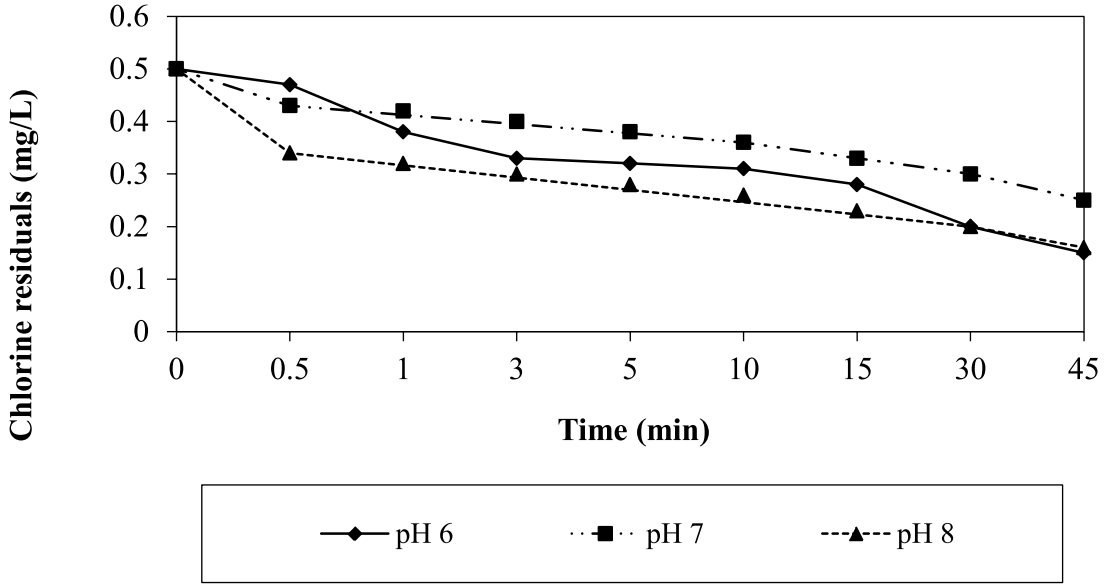

Fig. 4. Variation of chlorine residuals with time at different pH at 25 °C.

of exposure time. After 30 minutes of contact time, there was seen a 4 log removal of E. coli at pH 6 and 7 with 0.2 and 0.3 mg/L of residual chlorine but 3 log removal having residual chlorine 0.2 mg/L at pH 8. The overall inactivation achieved after 45 minutes of contact time was 5 log removals at pH 6 and 8.It is mentioned by Page et al [24] that over a pH range of 6.5 -10, a temperature range of 1 - 30°C in a variety of water types, free chlorine was highly effective against adenovirus type 2. Its disinfection efficacy decreased with increasing pH and decreasing temperature. Driedger et al. [25] found in the study that rate of inactivation decreased with increasing pH in the range of 6.0 –8.5, consistent with hypochlorous acid being primarily responsible for *C. parvum* inactivation within this pH range. Same results were al so mentioned by Churn et al. [26]. In their study the time required for 99 percent inactivation of H-1 parvovirus at pH 7, 20°C and a chlorine dose of 0.2 mg/L free

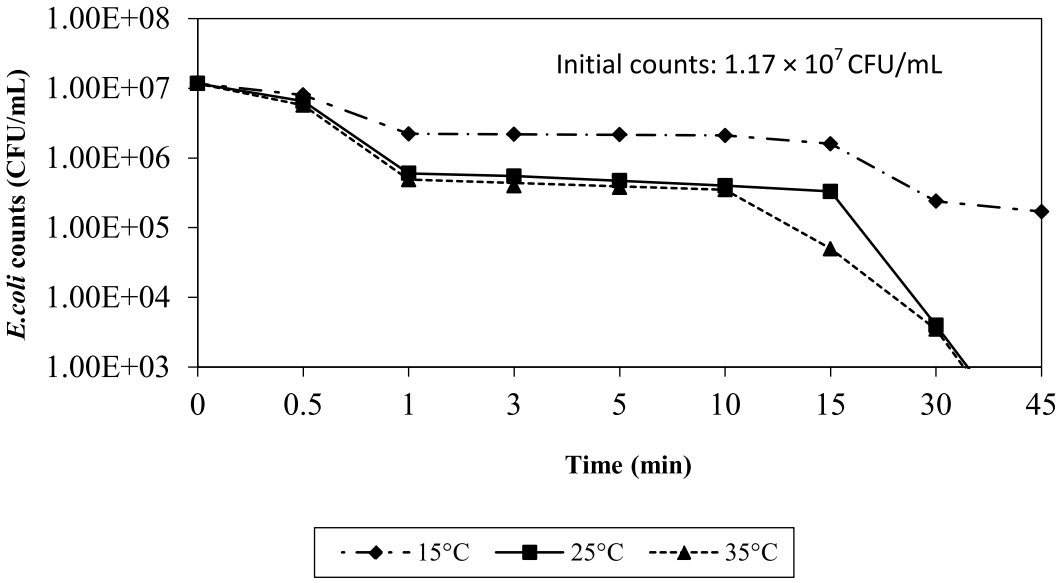

Fig. 5. Survival of *E. coli* at 0.5 mg/L applied chlorine dosages in distilled water at various temperatures.

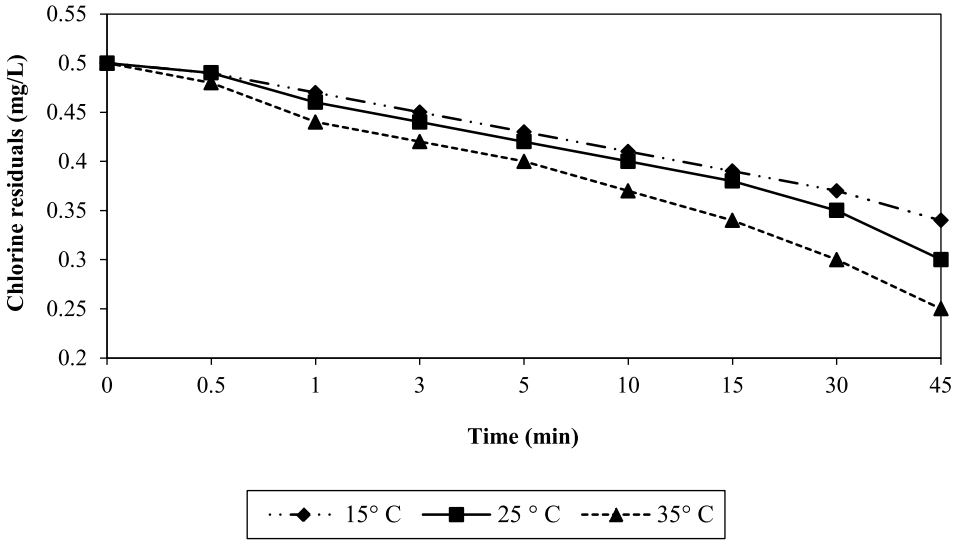

Fig. 6. Variation of chlorine dose with time at various temperatures.

chlorine was 3.2 min.

3.3 Determination of Optimum Temperature for Maximum Disinfection of *E. coli*

From the results of previous set of experiments, it is evident that at pH 7, maximum inactivation of *E. coli* was resulted. Now in this set of experiments, the effect of temperature was studied selecting 0.5 mg/L applied chlorine dosage as used earlier and pH 7 as proved best in the previous experiments.

The initial count applied was 1.17×10^7 CFU/mL at three different temperatures, viz. 15, 25 and 35ºC to observe the disinfection efficiency of chlorine. In the first 30 seconds of exposure time, the disinfection rate was evident and the *E. coli* counts reduced from 8.0×10^7 to 2.21×10^6, 6.5×10^6 and 5.7×10^6 giving 1 log removal at 15, 25 and 35ºC, respectively, as shown in Fig. 5, depicting 35ºC as optimum among the three tested temperatures. At this time the residual chlorine concentration was

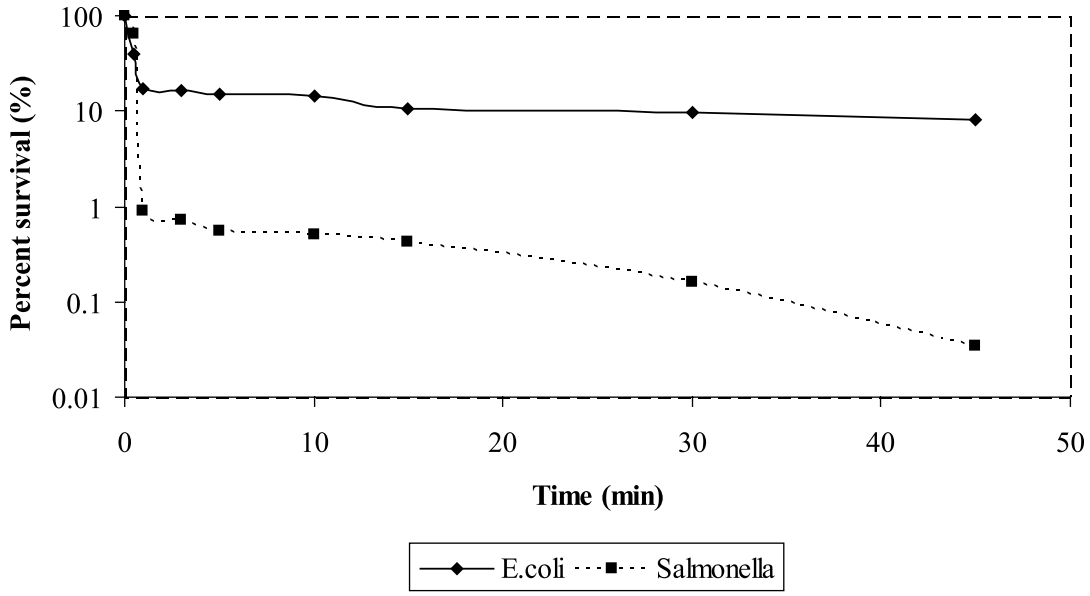

Fig. 7. Comparison of percent survival of *E. coli* and *Salmonella* in mix culture at 1.0 mg/L of applied chlorine dosage in distilled water.

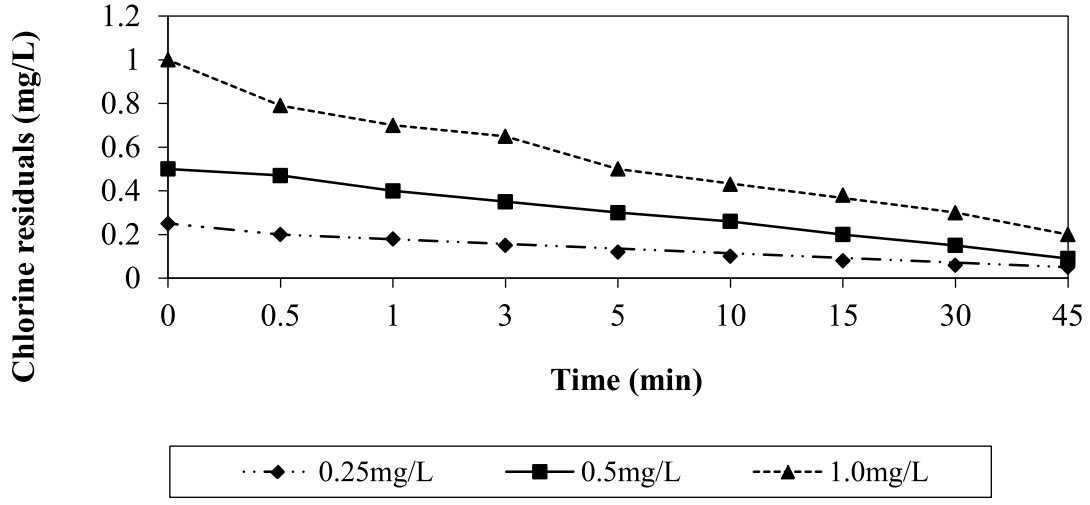

Fig. 8. Variation of residual chlorine with time at 25ºC in mix at various chlorine doses.

determined as 0.49, 0.49 and 0.48 mg/L (Fig. 6). A decrease in residual chlorine was observed with time as *E. coli* number reduced depicting that more chlorine is being used for the removal of microbial count. The increase in temperature enhanced the disinfection efficiency of chlorine, i.e., pathogen inactivation effectiveness increased as water temperature rose as reported previously [21].

3.4 Effect of Chlorine on Mix Culture of *E. coli* and *Salmonella*

Beside monoculture of *E. coli* inactivation studies, mix culture of *E. coli* and *Salmonella* was also used to observe the disinfection behavior of chlorine in distilled water at25º C and pH 7. The initial counts after inoculation of pure culture of *E. coli* and *Salmonella* were 1.0×10^7 and 1.13×10^7CFU/mL,

respectively. In the first 30 seconds of contact time, the disinfection rate was not much higher and the *E. coli* number was reduced from 1.0×10^7 to 9.8×10^6 (1 log removal) 8.6×10^6 and 4.0×10^6 CFU/mL (Fig. 7) and in case of *Salmonella*, the count reduced from $1.13\times10^7$to 9.6×10^6, 9.0×10^6 and 7.2×10^6 at 0.25, 0.5 and 1.0 mg/L of applied chlorine dosages. The residual chlorine concentration was 0.2, 0.47 and 0.79 mg/L for the above three dosages, respectively (Fig. 8).

The inactivation rate of *E. coli* with chlorine was not very high and was different as compared to *Salmonella* when used alone in the previous experiments. The same pace was maintained and in the next 30 seconds, i.e., after 1 minute, the *E. coli* counts reduces to $9.0\times10^{6,}$ 6.7×10^6 and 1.7×10^6 CFU/mL for 0.25, 0.5 and 1.0 mg/L respectively. At the same time the *Salmonella* count was 8.6×10^6, $4.1\times10^5$and 1.0×10^5CFU/mL, with 1 log removal of Salmonella. At this time, the chlorine residual was 0.18, 0.4 and 0.7 mg/L. The disinfection of *E. coli* and *Salmonella* counts and residual count were interrelated. There was seen a gradual decrease in residual chlorine and the disinfection process continues. The disinfection rate of *Salmonella* was more as compared to *E. coli*, which later seemed more resistant than *Salmonella*. At applied chlorine dosage of 0.25 mg/L another log removal was observed after 3 minutes which reduced the *Salmonella* counts to 7.8×10^6CFU/mL. At the contact time 3 minutes, the log removal was 2 and 3 in case of *Salmonella* at 0.5 and 1.0 mg/L, respectively, and the *Salmonella* count reduced to 4.0×10^5 and 8.0×10^4CFU/mL. In the same period of time, the *E. coli* counts were 7.5×10^6,5.9×10^6 and 1.62×10^6CFU/mL with 1 log removal. Another 1 log removal was observed after 30 minutes of exposure time in case of 1.0 mg/L and at this contact time the *E. coli* count decreased to 9.7×10^5CFU/mL. But the in case of 0.5 and 0.25 mg/L of applied chlorine dosage, the count was 2.55×10^6 and 3.0×10^6CFU/mL at this exposure time of 30 minutes, respectively. After 45 minutes it was observed that no decrease in the *E. coli* count for 0.25 mg/L but it reduces to 2.43×10^6CFU/mL. The chlorine residuals were 0.05, 0.09 and 0.2 mg/L after 45 minutes of contact time, respectively.

4. CONCLUSIONS

To meet the goal of clean and safe drinking water with no *fecal coliforms* and *E. coli* in 100 mL of drinking water sample, a multi-barrier approach is required that includes: protecting source water from contamination, appropriately treating raw water and ensuring safe distribution of treated water to consumers' taps. The present study investigated the effect of chlorine dosage, pH, temperature and contact time on survival rate of *E. coli* and *Salmonella*. The study led the following conclusions:

Distilled water was found suitable for chlorination practices due to its negligible chlorine demand. Maximum inactivation observed was 7 log removal at a dose of 1.0 mg/L at 25 °C. The exposure time of 45 minutes was sufficient for maximum inactivation of *E. coli* when 1.0 mg/L chlorine dose was applied however 0.5 and 0.25 mg/L of applied chlorine dosages required more exposure time for complete disinfection. Similarly tap water and well water also required more contact time. Maximum inactivation of *E. coli* was observed at pH 7, which was 6 log removals. High temperature had a profound effect on chlorination process and maximum inactivation was achieved at 35 ºC. In mix culture of *E. coli* and *Salmonella*, the disinfection rate of *Salmonella* was more as compared to *E. coli* being more resistant to chlorine dose.

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