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Short Communications

"Comparative study of Disinfectant Efficiency against *Escherichia coli* and *Bacillus subtilis*"

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Abstract

The antimicrobial activities of four disinfectants Sodium Chlorite, Iodine, Tetra butyl ammonium hydrogen and Crystal violet against Escherichia coli and Bacillus subtilis were investigated. These microorganisms were selected to test the Bactericidal activity of Disinfectants as these are frequent surface contaminants among the healthcare settings. Their efficacies were determined using Agar well diffusion method and Broth dilution method (for determination of minimum inhibitory concentration- MIC) at different concentrations of the test disinfectants. The results were recorded as diameter of zone of inhibition (mm) in agar well diffusion method and plate counting was performed in Broth dilution method. Different pathogens responded differently to different concentrations of disinfectants. The test disinfectants used in this study has been confirmed to be very effective, but their rate of efficiency varies due to the differences in their chemical composition and mechanism of action. This study revealed that Crystal Violet and Tetra butyl ammonium hydrogen sulfate have an excellent Bactericidal activity against both Gram positive (Bacillus subtilis) and gram negative (Escherichia coli) bacteria as compared to the others. The use of

Tetra butyl ammonium hydrogen sulfate at 6% and Crystal Violet at 1% against B. subtilis showed strong bactericidal efficacy with a zone of inhibition of 35 mm and 37 mm, respectively as compared to the others. The outcome of this study confirmed that Sodium Chlorite was relatively more effective against both the test organisms than Iodine. The maximum diameter of zone of inhibition observed for Sodium Chlorite against E. coli 40 mm. The least inhibition was seen with Iodine against both bacterial strains. Thus the efficacy of disinfectants in descending order is Crystal Violet >Tetra butyl ammonium hydrogen sulfate > Sodium Chlorite > and Iodine for both cultures. The result of Broth dilution method also show that Crystal violet, Tetra butyl ammonium hydrogen sulfate and sodium chlorite are most effective disinfectants on tested microorganisms, while iodine showed least bactericidal activity even at higher concentrations against both organisms. Thus, to ensure disinfectants efficacy, tests should be carried out on new disinfectant products and also further studies should be carried out about disinfectants.

Key words: Antiseptics, Disinfectants, Bactericidal and Efficacy.

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Introduction

Infectious disease and its physical, physiological and economical impact remains a significant problem in today's society. By limiting the number of infectious agents to which people are exposed, the chances of disease transmission can be reduced [4]. Many methods have been contrived to decrease the population and prevalence of causative agents of infectious diseases. They include chemotherapy, immunization, sterilization and disinfection. Subsequently, decontamination, disinfection and sterilization became main components of any infection control program [11]. One important control measure which helps in the prevention of the spread of infectious diseases is through disinfection.

Antiseptics and disinfectants are used extensively in hospitals and other health care centers to control the growth of microbes on both living tissues and inanimate objects. They are essential parts of infection control practices and aid in the prevention of nosocomial infections [14]. Disinfectants are of different types and may include alcohols, quaternary ammonium compounds, hypochlorides, iodine, bromines, pine oils, peroxides or phenolic compounds. The scope of the organisms controlled and the mechanism of performances varies widely between these agents. Some puncture the cell walls of the microorganisms, allowing the leakage of intracellular constituents, cause destruction either by coagulating or denaturating the protein of the bacteria (e.g. alcohols, aldehydes, phenols), lipids or nucleic acids in the cells, by destroying its cell membrane (inhibition of enzymes, electron transport and oxidative Phosphorylation) or by removal of a sulphonhydric group from the organisms [2, 3, 18, 19], perturbation of cell homeostasis, while others permeate and enters the cell destroying the microorganism from within, although microorganisms differ in their sensitivity to chemical gerimicide [11]. The interaction of disinfectants at the cell surface can produce a significant effect on viability [21] but most antimicrobial agents appear to be active intracellularly [25].

The wide spread use of these agents has promoted some speculation on the development of microbial resistance [5] and this resistance to disinfectants and antiseptics mainly intrinsic in nature whereas antimicrobial resistance is frequently conferred by plasmid or transposons, which have allowed rapid and extensive spread through the globe. Disinfectant resistant bacterial stains have arisen as a result of lack in standardization of some factors such as criteria for use of chemical agents, specification in the label of available products and scarcity of well trained personnel [10]. The prevalence of disinfectant resistant hospital bacteria have increased significantly in the world including Brazil and have become a serious public health problem [16]. An ideal disinfectant to overcome the antimicrobial resistant pathogens should have broad spectrum of antimicrobial activity and the efficacy of these agents may be affected by pH, detergent base, temperature, organic matter, ionic and type of the surfactants [28].

Currently there are a large number of disinfectants and antiseptic products in the local market. However, their antibacterial effectiveness is not always well declared by the manufacturers. Therefore consumers find it difficult to choose the right product according to their needs [33]. Therefore it is necessary to evaluate the effectiveness of an antiseptic or disinfectant against a specific pathogen so that an appropriate agent can be easily selected [27]. It will be continued requirement for new and potent antimicrobial agents together with techniques suitable for control and destruction of microbial pathogens [6]. The aim of this study is to know the effectiveness

of different disinfectants on selected tests microorganisms: *Escherichia coli* and *Bacillus subtilis,* to find out the concentrations at which they were effective; to investigate the susceptibility of the test gram positives and gram negative microorganisms to the test disinfectants, and to help know the most effective disinfections to use for household and hospital cleanings.

Materials and Methods

Bacterial Culture/Source of microorganisms

Cultures of the test organisms *Escherichia coli* and *Bacillus subtilis* were procured from IMTECH (Institute of Microbial Technology, Mohali).

Growth Kinetics of test organisms

To study the growth characteristics of *E. coli* and *B. subtilis*, a loopful of 24 hrs old actively growing, bacterial culture was inoculated into 250 ml Erlenmeyer flasks containing 150 ml of Nutrient Broth. They were incubated at 37^{o}C for 24 hrs to prepare primary inoculums. They were inoculated onto the sterilized plates (separate) containing Nutrient Agar Medium by streaking. Bacterial cultures were maintained at 4^{o}C on Nutrient agar plates and subcultured fortnightly.

Preparation of Disinfectants

Different disinfectants (oxidizing) were used in this study against *Escherichia coli* and *Bacillus subtilis* cultures. Among oxidizing disinfectants category, Sodium Chlorite (1-6% w/v), Iodine (5-100% v/v), Crystal Violet (0.5-1% w/v) and tetra butyl ammonium hydrogen sulfate (1-6% w/v) were used. Different concentrations of each disinfectant were prepared by the following formula: Concentration= RV/O. Where, R is required Concentration, V is required Volume and O is Original Concentration.

Susceptibility Testing

Susceptibility testing against microorganisms was determined by using Agar Well Diffusion Method and Broth Dilution Method.

Agar Well Diffusion Method

Agar well-diffusion method was used to determine the bactericidal activity. Nutrient agar plates were prepared and these plates were homogenously inoculated with 100µl of the test bacterium (*E. coli* and *B. subtilis*) by the spread plate method. Wells were punched into the agar with the help of a sterile well borer. Subsequently, wells were filled with 100µl of the disinfectant at different concentration. The plates were incubated in an upright position at 37^{o}C for 24 hours. Following incubation, the agar plate was examined for zones inhibition (areas of no growth) surrounding the wells. A zone of inhibition is indicative of microbial activity against the organism. Absence of zone of inhibition

indicates that the disinfectant was
ineffective against the test organism [32].

Broth Dilution Method

The Broth dilution method involves subjecting the test organisms to a series of concentrations of disinfectants in a broth environment. At the start of the experiment, the test strains were suspended in normal saline (0.85% NaCl) and turbidity adjusted visually at 2 Mc Farland Standard. The experiment was performed in 2ml tubes, using four different kinds of disinfectants mentioned above. Three different contact times 30 min, 1 hr and 1 ½ hr were tested against micro-organisms (*E. coli* and *B. subtilis*). For each eppendorf tube, 0.1 ml of culture was added into 0.9 ml of disinfectant. After definite contact time, centrifugation was performed at 5000 rpm for 5 min to separate the culture from the solution. Supernatant was discarded and pellet was resuspended in distilled water followed by the spread plating on nutrient agar plate from each eppendorf tube. After

24 hr of incubation at 37°C, numbers of colonies were counted on each plate.

Statistical Analysis

To calculate the average and the standard deviation of the diameter of zone of inhibition for each disinfectant, method used was from [12]

Results and Discussion

A wide divergence was observed in the responses of disinfectant agents among the test organisms *E. coli* and *Bacillus subtilis*. Results of Agar well Diffusion method indicate that different pathogens acquired resistance to different disinfectants. The results also suggested that the bactericidal effects of disinfectants are not only dependent on their types but also on their concentrations. The test microorganisms differ in their susceptibilities to the disinfectants.

Susceptibility testing against E. coli using Agar Well Diffusion Method

The effect of four disinfectants (Sodium Chlorite, Iodine, tetra butyl ammonium hydrogen sulfate and Crystal Violet) against *E. coli* is given below (Table 1) showing the different concentrations of disinfectant used and their respective zones of inhibitions. The results are presented in terms of Resistant, Intermediate and susceptible.

Table 1 *Escherichia coli* response to Oxidizing Disinfectant: Sodium Chlorite, Iodine, Tetra butyl ammonium hydrogen sulfate and Crystal violet

Disinfectant used	Concentration (%)	Diameter of zone of inhibition (mm)	Response	Standard Deviation
Sodium Chlorite (w/v)	1	No Zone of Inhibition	R	20
	2	No Zone of Inhibition	R	
	3	No Zone of Inhibition	R	
	4	30	S	
	5	38	S	
	6	40	S	
Iodine (v/v)	5	No Zone of Inhibition	R	6.12
	10	No Zone of Inhibition	R	
	25	No Zone of Inhibition	R	
	50	No Zone of Inhibition	R	
	75	No Zone of Inhibition	R	
	100	15	I	
Tetra butyl ammonium hydrogen sulfate (w/v)	1	15	I	4.3
	2	20	S	
	3	23	S	
	4	25	S	
	5	26	S	
	6	26	S	
Crystal violet (w/v)	0.5	30	S	1.86
	0.6	31	S	
	0.7	31	S	
	0.8	34	S	

Where R is Resistant, I is Intermediate and S is susceptible

Standard values for the determination of susceptibility of microorganisms against Disinfectant were used [12]

In the present experiment evaluation of the susceptibility of *E. coli* to the Sodium Chlorite (1-6% w/v) disinfectant demonstrated that 4% w/v, 5% w/v and 6% w/v concentrations were effective against it with 30mm, 38mm and 40mm of zone of inhibition, respectively (Fig. 1). But *E. coli* showed resistant towards Sodium Chlorite

at 1% w/v, 2% w/v and 3% w/v concentrations as no zone of inhibition was seen on the plates. Thus these concentrations were poor in its bactericidal action. [20] tested three concentrations of NaOCl- 0.1%, 0.2% and 0.4% to evaluate its antibacterial activity against *E. coli* by disc diffusion method and found that NaOCl at 0.4% concentration presented strong antimicrobial activity against *E. coli* that suppressed the growth of *E. coli* by 100%.

Contrary to these findings, Iodine (5-100% v/v) was found to be least effective. It was effective only at 100% v/v concentration with an intermediate diameter of zone of inhibition (15mm) (Fig. 2). [1] Used 10% concentration of Povidone-iodine against *E. coli* and observed 12mm diameter of zones of inhibition after 24 hrs of incubation.

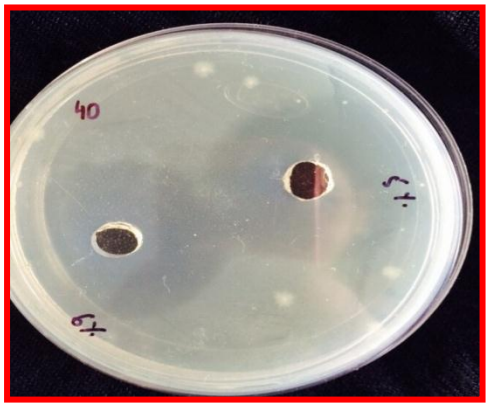

Fig 1 Effect of Sodium Chlorite on *E. coli* at Concentrations 5% & 6% (w/v) by Agar well Diffusion method.

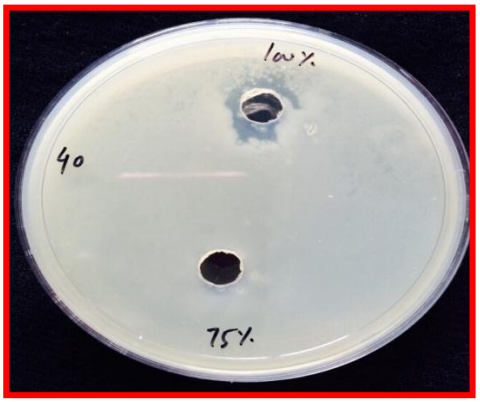

Fig 2 Effect of Iodine on *E. coli* at 75 % &100% (v/v) concentration by Agar Well Diffusion.

The results of using Tetra butyl ammonium hydrogen sulfate (1-6% w/v) disinfectant against *E. coli* demonstrated that it was amazingly effective at all the concentrations (1-6% w/v) showing an increase in a zone of inhibition with an increase in concentration of disinfectant. Similarly, Kortenbout [13] in that the higher the concentration of the solution, the more potent and effective it would be.

At 1% w/v concentration of the disinfectant, an intermediate zone of inhibition was observed with 15mm diameter. At 2%, 3%, 4%, 5% and 6% concentration, the zone of inhibition observed was 20mm, 23mm, 25mm, 26mm and 28mm,respectively (Fig. 3). In addition to this, Crystal Violet also (0.5%, 0.6%, 0.7% and 0.8% w/v) showed strong inhibitory activity against *E. coli* (Fig. 4).

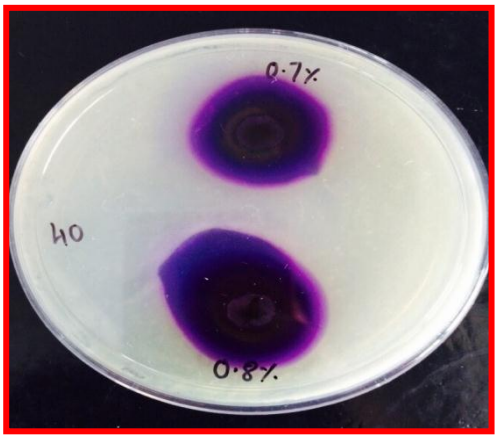

Fig. 3 Effect of Tetra butyl ammonium Sulfate on *E. coli* at Concentration 5% & 6% (w/v).

Fig. 4 Effect of Crystal violet on *E. coli* at 0.7% & 0.8% (w/v) concentrations.

Susceptibility testing against E. coli using Broth Dilution Method

The results were expressed as the bacterial colony count after contact time of 0.5hr, 1 hr, and 1.5 hr. In the present experiment different concentrations of disinfectants were used (50% and 75%). It was clear that when the concentration of disinfectants was increased, bacterial growth declined drastically. Among the Oxidizing Disinfectants, Sodium Chlorite (w/v) at 75% concentration presented strong antimicrobial activity against *E. coli* that suppressed the growth by 100% at contact time of 1.5 hr. But it showed moderate activity at 50% concentration (Table 2). Consistent with these results, [29] found that 1% NaOCl and 5% NaOCl eliminated *E. coli* at the time periods of 5min, 15min and 30min. On the other hand, Iodine (75 %v/v) was found to be least effective against *E. coli* (Table 2). [34] Studied the anti-bacterial effect of Iodine at different concentrations (50µM - 500µM). All the concentrations except 500µM were not effective against *Escherichia coli*. 100% growth suppression of *E. coli* was observed at 500µm. According to [15], revealed that iodine (0.5%) had the lowest lethal effect against all tested bacterial isolates of *E. coli* after 15 seconds exposure time that increased gradually with increased time of exposure (30 & 60 sec) to be moderately lethal against *E. coli* isolates.

Table 2 Bactericidal activity of Oxidizing Disinfectants on *Escherichia coli* by Broth Dilution method

S.No.	Disinfectant used	Concentration (%)	Contact Time (hr.)	No. of Colonies
1.	Sodium Chlorite (w/v)	50	0.5	60
			1	32
			1.5	20
		75	0.5	14 (TFTC)
			1	3 (TFTC)
			1.5	NG
2.	Iodine (v/v)	75	0.5	92
			1	60
			1.5	20
3.	Tetra butyl ammonium hydrogen sulfate (w/v)	50	0.5	12 (TFTC)
			1	10 (TFTC)
			1.5	NG
		75	0.5	8 (TFTC)
			1	1 (TFTC)
			1.5	NG
4.	Crystal Violet (w/v)	50	0.5	36
			1	10 (TFTC)
			1.5	NG

TMTC=Too Many To Count, TFTC=Too Few To Count

While, Tetra butyl ammonium hydrogen sulfate (50% and 75% w/v) were amazingly effective even at the shortest contact time of 0.5 hr. and 100% bacterial growth was suppressed at the highest contact time of 1.5 hr. Moreover, 50% concentration of Crystal

Violet (w/v) showed successful bactericidal activity at the highest contact time of 1 hr and 1.5 hr (Table 2).

Suceptibility testing against Bacillus subtilis using Agar well Diffusion Method

The different concentrations of Sodium Chlorite (1-6% w/v) were used for susceptibility testing against *B. subtilis.* Among these concentrations 3%, 4%, 5% and 6% were found to be effective against the organism with 20mm, 25mm, 28mm and 35mm of a zone of inhibition, respectively (Table 3 & Fig. 5). [9] experimented that 5.25% NaOCl was effective in eliminating the *B. subtilis* spores. When Iodine was used as a disinfectant, it was observed that iodine at 5-50% concentration showed no zone of inhibition. While moderate activity was shown by iodine at 75% (11mm) & 100% (12mm) (Table 3 & Fig. 6). [30] evaluated and compared the anti-bacterial activity of 3% NaOCl, 2% Chlorhexidine, 1% Paracetic acid and 10% Povidone iodine after contact time of 1 min and 5 min against *B. subtilis*. They reported the least antibacterial activity of Povidone iodine against *Bacillus subtilis*.

Table 3 *Bacillus subtilis* response to Oxidizing Disinfectants - Sodium Chlorite, Iodine, Tetra butyl ammonium hydrogen sulfate and Crystal Violet

Disinfectant used	Concentration (%)	Diameter of zone of inhibition (mm)	Response	Standard Deviation
Sodium Chlorite (w/v)	1	No Zone of Inhibition	R	14.76
	2	No Zone of Inhibition	R	
	3	20	S	
	4	25	S	
	5	28	S	
	6	35	S	
Iodine (v/v)	5	No Zone of Inhibition	R	5.94
	10	No Zone of Inhibition	R	
	25	No Zone of Inhibition	R	
	50	No Zone of Inhibition	R	
	75	11	I	
	100	12	I	
Tetra butyl ammonium hydrogen sulfate (w/v)	1	15	I	24.1
	2	18	S	
	3	20	S	
	4	27	S	
	5	30	S	
	6	35	S	
Crystal Violet (w/v)	0.5	25	S	31.7
	0.6	25	S	
	0.7	31	S	
	0.8	36	S	
	0.9	36	S	
	1	37	S	

Where R is Resistant, I is Intermediate and S is susceptible

Standard values for the determination of susceptibility of microorganisms against Disinfectant were used [12]

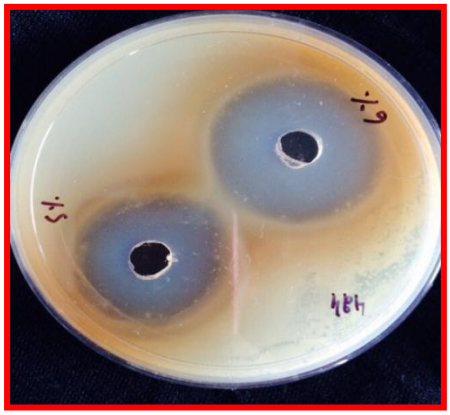

Fig 5 Effect of NaOCl on *B. subtilis* at concentration 5% & 6% (w/v)

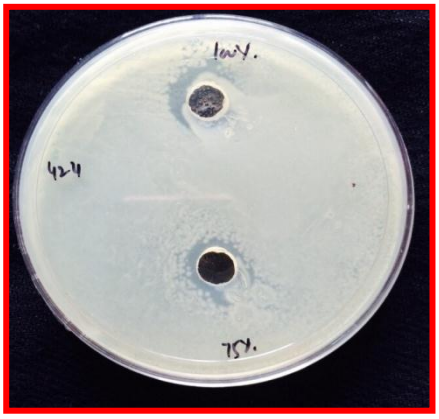

Fig 6 Effcet if Iodine on *B. subtilis* at concentration 75% & 100%.

Tetrabutyl ammonium hydrogen sulfate (1-6%) showed effective bactericidal activity against *B. subtilis*. The zones of inhibition were 15 mm, 18 mm, 20 mm, 27 mm, 30 mm and 35 mm at 1%, 2%, 3%, 4%, 5% and 6% w/v, respectively (Table 3 & Fig 7). From the results, it was predicted that higher the concentration of disinfectant used against test organism greater is the bactericidal activity. [15] Observed the antibacterial effects of QUATS against *Bacillus subtilis*. The zones of inhibition were observed ranging between 12mm-26mm. Crystal Violet exhibited strong bactericidal activity against *B. subtilis*. It was found to be readily effective at all concentrations used (0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1% w/v) (Fig 8) with highest zone of inhibition (25 mm, 25 mm, 31 mm, 36 mm, 36 mm & 37 mm, respectively). The lowest concentration of Crystal Violet that inhibited the growth of the bacteria, which is considered as the MIC was 0.5% (Table 3).

Fig 7 Effect of Tetra butyl ammonium hydrogen sulfate on *B. subtilis* at concentration 5% & 6%

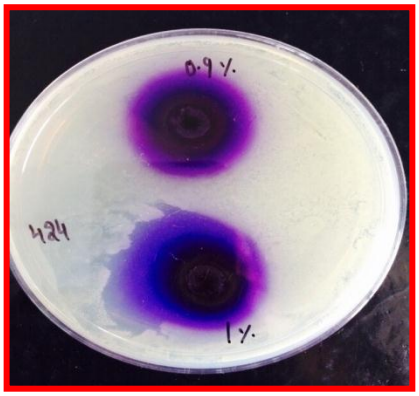

Fig 8 Effect of CV on *B. subtilis* at concentration 0.9% & 1%

Generally, Gram–positive bacteria observed in this study to be more susceptible to antimicrobial agents. This can be attributed to the contents of the cell wall, since it is composed of peptidoglycan and teichonic acid and neither of these appears to act as effective barrier to the entry of antiseptics and disinfectants, therefore high molecular weight substances can readily pass in to the *S. aureus* and vegetative cell of *Bacillus* spp. [24]

Susceptibility testing against B. subtilis by Broth Dilution Method

Among the oxidizing disinfectants used against *Bacillus subtilis* culture, Tetra butyl ammonium hydrogen sulfate (75% w/v and 50% w/v concentration) showed highest bactericidal action at different contact time used (0.5 hr, 1 hr and 1.5 hr) (Table 4). Some previous studies, as parallel with our results, [26] proposed that Quaternary ammonium compounds are good antibacterial and antifungal at relatively low concentration within shorter contact time. Contrary to this, Iodine showed the least antibacterial activity at 75% v/v concentration (Table 4). Similar results for Iodine were reported by [22] that 1% Iodine complex was not effective against *Bacillus subtilis* at different contact times of 15 sec, 30 sec, 1min, 5 min and 15 min. The growth of *B. subtilis* appeared at all the contact times which showed the least activity of Iodine. [30] evaluated and compared the anti-bacterial activity of 3% NaOCl, 2% Chlorhexidine, 1% Paracetic acid and 10% Povidone iodine after contact time of 1 min and 5 min against *B. subtilis*. They reported the least antibacterial activity of Povidone iodine against *Bacillus subtilis*.

Table 4 Bactericidal activity of Oxidizing Disinfectants on *Bacillus subtilis* by Broth Dilution method

S. No.	Disinfectant used	Concentration (%)	Contact time (hr.)	No. of Colonies
1.	Sodium Chlorite (w/v)	50	0.5	120
			1	40
			1.5	20
		75	0.5	2 (TFTC)
			1	1 (TFTC)
			1.5	NG
2.	Iodine (v/v)	75	0.5	TMTC
			1	80
			1.5	40
3.	Tetra butyl ammonium hydrogen sulfate (w/v)	50	0.5	10 (TFTC)
			1	3 (TFTC)
			1.5	NG
		75	0.5	6 (TFTC)
			1	4 (TFTC)
			1.5	NG
4.	Crystal Violet (w/v)	50	0.5	20
			1	18 (TFTC)
			1.5	15 (TFTC)

Where, TMTC=Too Many To Count, TFTC=Too Few To Count, NG = No Growth

However it was observed that Sodium Chlorite was more effective against the organism at 75% w/v concentration as compared to 50% w/v concentration at 0.5 hr, 1 hr and 1.5 hr (Table 4). This results corresponds with the findings of [8] who proposed that 2% NaOCl was effective against *B. subtilis* after contact time of 5min, 10min and 15min. [22] found that 1% NaOCl was effective against *B. subtilis* after the contact time of 30sec, 1min, 5min and 15min but the growth appeared after contact time of 15sec. Similarly in another report, [30] evaluated and compared the antibacterial activity of 3% NaOCl, 2% Chlorhexidine, 1% Paracetic acid and 10% Povidone iodine after contact time of 1 min and 5 min against *B. subtilis*. Among these disinfectants, 3% NaOCl showed better disinfection for 5 min duration and then 1 min duration. [9] Experimented that 5.25% NaOCl was effective in eliminating the *B. Subtilis* spores after 1 minute of disinfection. [23] Tested the efficiency of 5.25% concentration of Sodium Hypochlorite after 1 min, 3 min and 5 min against *Bacillus subtilis*. It was reported that

5.25% concentration of NaOCl at the contact time of 5 min showed less growth against *Bacillus subtilis*.

While Crystal Violet at 50% w/v concentration showed effective bactericidal activity against *B. subtilis* at 0.5 hr, 1 hr and 1.5 hr (Table 4). Consistent to our findings, the inhibition of *Bacillus subtilis* growth by certain basic dyes was earlier reported by [17]. They observed that both the mean growth rate of the cell population at the logarithmic phase and the cell concentration at the stationary phase decreased with the addition of dyes. It is also reported that triphenylmethane dyes (Crystal Violet and Methyl Violet) strongly inhibited cell growth.

It has been concluded that gram positive *B. subtilis* was more susceptible towards all the disinfectants used comparatively to gram negative *E. coli*. In gram–negative bacteria, the outer membrane acts as a selective permeability barrier in limiting or preventing the entry of many unnecessary or harmful chemical compounds into the bacterial cell [26]. The changes in permeability system may lead to acquire resistance to biocidal compounds [31]. The use of these disinfectants may be means to reduce cases of acquired diseases caused by the test microorganisms.

Conclusion

Using antiseptic and disinfectants components are regarded as an essential strategy for fighting with microorganisms. The potency of disinfectants is very important to enhance the antimicrobial activity of these disinfectants towards controlling microbial population which includes prevention of diseases transmission and infection. Disinfectants used in hospital and laboratories must be tested periodically to ascertain its potency and efficacy. Otherwise disinfectants of Questionable quality and efficacy will prolong to be used and mistreated in clinical settings where their microbicidal activity is erroneously assumed to be effective against all organisms that contaminate surfaces. With regard to the widespread use of disinfectant products, the development of resistance to antimicrobial agents, particularly cross resistance to antibiotics, study on disinfectants seems to be a very important topic. The proper exploitation of currently available antimicrobial agents as well as efforts to minimize the spread of resistant bacteria through appropriate infection control would be quite significant, and may represent a first step in solving the issue of resistant microorganisms. Our findings emphasizes that there is necessitate to test the superiority of disinfectants consistently supplied to the laboratory or hospital to ensure suitable control of infections by using accurate disinfectant in precise concentration for an exact contact time.

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