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ORIGINAL ARTICLE

Synthesis and biocidal activity of modified poly(vinyl alcohol)

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Abstract Functionalized polymers and their polymer nature give them more advantages than the corresponding small molecules. In this respect, polymeric ammonium and phosphonium salts were prepared by chemical modifications of poly(vinyl alcohol) (PVA) aiming to explore their antimicrobial activities against pathogenic bacteria and fungi. The modifications were performed by chloroacetylation with chloroacetyl chloride. Incorporation of the ammonium and phosphonium salts was conducted by the reaction of chloroacetylated poly(vinyl alcohol) (CPVA) with triethylamine (TEA), triphenylphosphine (TPP), and tributylphosphine (TBP). The antimicrobial activity of the polymers against variety of test microorganisms was examined by the cut plug and viable cell counting methods of shake cultures of 10 times dilute nutrient broth and Sabouraud's media, seeded with the test microorganisms. It was found that the immobilized polymers exhibited antimicrobial activity against the Gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Shigella* sp. *and Salmonella typhi*) and Gram positive bacteria (*Bacillus subtilis and B. cereus*) and the dermatophyte fungus (*Trichophyton rubrum*). The growth inhibition of the test microorganisms (ratio of surviving cell number, M/C) varied according to the composition of the active group in the polymer and the test organism. It increased by increasing the concentration of the polymer. Triphenyl phosphonium

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salt of the modified poly(vinyl alcohol) exhibited the most biocidal activity against both Gram-negative and Gram-positive bacteria after 24 h.

1. Introduction

Recently, much attention has been paid to the problems of environmental pollution and health. Infection by pathogenic microorganisms is of great concern in many fields, particularly in medical devices, drugs, health care products and hygienic applications, water purification systems, hospital and hospital furniture, dental surgery equipment, textiles, food packaging, food storage, etc. (Kenawy et al., 2007a, 2009)

Antimicrobial agents are used for many applications, such as water sterilization, drugs, soil sterilization, biomedical-device sterilization, and prevention of the microbial contamination of shipboard, water compensated, and hydration fuel tanks (Kenawy et al., 1998, 2002, 2005, 2006, 2011). They are also in common use in the areas of health care and hygienic applications such as sterile bandages and clothing (e.g., antimicrobial surgical gowns and antifungal athletic socks). Furthermore, antimicrobial agents are commonly used in coating of surfaces such as ship hulls, shower walls, and many kinds of tubing to minimize the problems of biofouling (Kenawy et al., 2006; Worley and Sun, 1996). The use of antimicrobial polymers offers promise for enhancing the efficacy of some existing antimicrobial agents and minimizing the environmental problems accompanying conventional antimicrobial agents by reducing the residual toxicity of the agents, increasing their efficiency and selectivity, and prolonging the lifetime of the antimicrobial agents. Also, polymeric antimicrobial agents have the advantage that they are nonvolatile and chemically stable and do not permeate through skin. Therefore, they can reduce losses associated with volatilization, photolytic decomposition, and transportation. In the field of biomedical polymers, infections associated with biomaterials represent a significant challenge to the more widespread application of medical implants (Kenawy et al., 2007b; Acharya et al., 2005; Jiang et al., 2004).

Considerable interest has been focused on functional polymers and on their diverse applications in many fields such as biomedical applications including drug delivery and antimicrobial polymers. Functional polymers have the potential advantages over small analogous molecules. Their usefulness is related to both the functional groups and to their polymeric nature whose characteristic properties depend on the extraordinarily large size of the molecules (El-Newehy et al., 2012b; Kenawy et al., 1998; Akelah and Moet, 1990).

Poly(vinyl alcohol) (PVA) is a hydrophilic polymer, has unique properties. It absorbs water and swells easily, but the swelling is inhibited by salts. Its physico-chemical properties depend on the degree of poly(vinyl acetate) hydrolysis. The solubility of PVA in water increases greatly as the degree of acetate group hydrolysis increases. Moreover, PVA can be used for releasing biological and medical materials in a controlled way (Kenawy et al., 2007; Zhang et al., 2005; Gimenez et al., 1996). PVA was used in some modern technologies, such as hydrogels, polyelectrolytes, optics, and biomaterials including soft contact lenses, implants, and artificial organs. This is due to their inherent non-toxicity, non-carcinogenicity, good biocompatibility, and high degree of swelling in aqueous solutions. The PVA hydrogels were used as drug delivery matrices or in the form of powders added to mixtures of other excipients for tablet formation (Kenawy et al., 2007; Zhang et al., 2005; Gimenez et al., 1996; Colombo et al., 1985; Carstensen et al., 1981). In our previous work, we have reported the use of PVA as a controlled release device for drugs using the freezing and thawing technique (Kenawy et al., 2010) and the electrospinning technique (El-Newehy and Alamri, 2012a; Kenawy et al., 2007)

In this study, we prepared immobilized polycationic biocides by modification of poly(vinyl alcohol) via chemical modification with chloroacetyl chloride followed by immobilization of onium groups onto the chloroacetylated polymer. Antimicrobial activities of the resultant polymers were explored *in vitro* by cut plug and the viable cell counting methods against strains of appropriate microorganisms.

2. Experimental

2.1. Materials and instruments

Poly(vinyl alcohol) (PVA) (M_w 13k–23k, 98% hydrolyzed), tributylphosphine (TBP), triphenylphosphine (TPP), and chloroacetyl chloride were purchased from Aldrich. Triethylamine (TEA) was of purest grade available from Merck-Schuchardt and was used without further purification. Pyridine was dried before use. All solvents were dried and distilled before use.

IR Spectroscopy: IR spectra were recorded on a PERKIN–ELMER 1430 Ratio Recording Infrared Spectrophotometer from KBr pellets.

Elemental microanalyses were determined on Heraeus (Microanalysis Center, Cairo University, Egypt) and Elemental Analyzer Mode 1106 Carlo Erba Strumentazione (Pisa University, Pisa, Italy).

2.2. Test microorganisms and media used

The test microorganisms included were Gram negative bacteria; *Escherichia coli, Pseudomonas aeruginosa, Shigella sp.* and *Salmonella typhi*, and the Gram positive bacteria; *Bacillus subtilis* and *B. cereus*, in addition to the dermatophyte fungus *Trichophyton*. The test microorganisms were obtained from the culture collection of the Bacteriology Unit, Botany Department, Faculty of Science, Tanta University, Egypt. Nutrient and Sabouraud's broths and nutrient and Sabouraud's agar were used for growing and maintenance of the test bacteria and fungi.

2.3. Chloroacetylation of poly(vinyl alcohol)

In a two-neck round-bottomed flask, pyridine (55 mL, 681.8 mmol) was added to a suspension of PVA (I) (10 g, 227.3 mmol) in chloroform and the mixture was cooled to 0 °C in an ice-salt bath. To the cold mixture, chloroacetyl chloride (55 mL, 681.8 mmol) was added dropwise with vigorous

stirring. After the addition, the reaction mixture was stirred at 0 °C for 3 h and at room temperature for further 3 days.

The insoluble part was removed by filtration (5%) and the filtrate was collected and was concentrated on a Rotavapor. The product (II) was precipitated by adding to distilled water. The product (II) was filtered off, washed with water, and was dried under vacuum at 40 °C for two days (Scheme 1).

2.4. Immobilization of ammonium and phosphonium salts onto the chloroacetylated poly(vinyl alcohol) (CPVA) (II)

2.4.1. Triethyl ammonium salt (III)

To a suspension of CPVA (II) (2 g, 16.6 mmol) in dry benzene (20 mL), TEA (9.2 mL, 66.4 mmol) was added. The reaction mixture was stirred at 80 °C for four days. The product (III) was filtered off, washed with excess dry benzene and was dried at 40 °C under vacuum for two days to give a yield of 82% (Scheme 2).

2.4.2. Triphenyl phosphonium salt (IV)

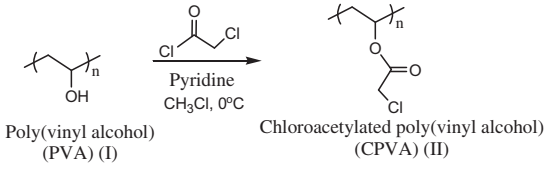
The titled compound was prepared as described earlier for triethyl ammonium salt (III) using the following quantities: CPVA (II) (1.0 g, 8.3 mmol) and TPP (8.7 g, 33.2 mmol) in 15 ml dry benzene to give 63.1% yield (Scheme 2).

2.4.3. Tributyl phosphonium salt (V)

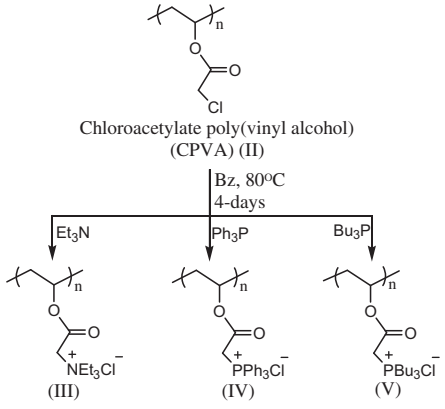
The titled compound was prepared as described earlier for triethyl ammonium salt (III) using the following quantities: CPVA (II) (1.0 g, 8.3 mmol) and TBP (8.3 mL, 33.2 mmol) to give 37.5% yield (Scheme 2).

2.5. Evaluation of antimicrobial activity

The antimicrobial spectrum of the prepared polymers was determined against the tested bacteria on powdery samples



Scheme 1 Chloroacetylation of poly(vinyl alcohol) (PVA).



Scheme 2 Immobilization of ammonium and phosphonium salts onto chloroacetylated poly(vinyl alcohol) (CPVA).

by the cut plug method (Pridham et al., 1956) on nutrient agar which contained per liter: 10 g peptone, 5 g sodium chloride; 5 g beef extract; 20 g agar; pH 7. The assay plates were seeded with the test bacteria, after solidification the wells were made and each was filled with 20 mg powdery polymer. The plates were incubated at 30 °C for 24 h, after which the diameters of inhibition zones were measured, and the compounds which produced inhibition zones were further assayed at different concentrations in aqueous suspension.

In order to quantify the inhibitory effects of the active compounds, the representative microorganisms; *E. coli*, *B. subtilis*, and *Trichophyton rubrum* were tested. A loopful of each culture was placed in test tubes containing 10 mL of 10 times dilute nutrient or Sabouraud's broth. The tube cultures were then incubated overnight at 30 °C. At this stage, the cultures of the test bacteria were adjusted to 1.5×10^5 cells/mL and that of the test fungus was adjusted to 7.2×10^4 spores/mL, which were used as inoculants for the antimicrobial test.

Since the polymers were not completely soluble neither in water nor in different solvents, they were suspended in sterile 10 times dilution of the above nutrient broth media to make 0.05 g/mL, and 0.5 mL were transferred to flasks containing sterile 10 times dilute nutrient broth to give the final concentrations of 10, 5 and 2.5 mg/mL. Exposure of bacterial cells or fungal spores to biocides started when 0.2 mL of the culture containing 1.5×10^5 bacterial cells/mL or 7.2×10^4 fungal spores/mL was added to 9.3 mL of the above biocide suspension which was preequilibrated and shaken at 30 °C as recommended by (Nakashima et al., 1987). The starting number of cells was counted by the spread plate method. After 24 h contact, 1.0 mL portions were removed and mixed with 9.0 mL of 10 times dilute broths and then decimal serial dilutions were prepared from these dilutions, and the surviving bacteria or fungi were counted by the spread plate method. After inoculation, the plates were inoculated at 30 °C, and the number of colonies was counted after 24 h for bacteria and 48 h for fungi. The ratio was carried out in triplicate every time. The ratio of the colony numbers for the media containing the polymers (M) and those without these compounds (C) was taken as surviving cell number and by this value the antimicrobial activity was evaluated.

3. Results and discussion

3.1. Chloroacetylation of poly(vinyl alcohol)

Chloroacetylated poly(vinyl alcohol) (CPVA) (II) was synthesized by treatment of PVA (I) with excess chloroacetyl chloride in the presence of pyridine as an acid acceptor under dehydrating conditions. Similar chloracetylation methods were described by F. Arranz et al. for amylase and cellulose (Martin et al., 1999; Arranz and Sanchez-Chaves, 1995). Elemental microanalysis showed that the chloroacetylation of PVA was in good agreement with the calculated values in which: Cl%: calc. (found): 29.4% (29.0%) as shown in (Table 1). This indicated the high percentage conversion of PVA (I) to CPVA (II) (Scheme 1).

The IR spectrum of the product (II) showed peaks at: 745 cm^{-1} and 1200 cm^{-1} (–CH_2Cl), 1719 cm^{-1} (–C=O), 2856 cm^{-1} (CH), (CH_2), 1486 cm^{-1} and 2925 cm^{-1} (CH_3).

Table 1 Elemental microanalysis for polymers (I–V).

Polymer code	Microanalysis									
	%C		%H		%Cl		%N		%P	
	Calc.*	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found
I	54.5	52.9	9.1	5.0	–	–	–	–	–	–
II	54.5	40.5	9.1	5.0	29.5	29.0	–	–	–	–
III	54.2	46.7	9.0	8.0	16.0	16.2	6.3	9.5	–	–
IV	69.0	46.6	5.2	5.6	9.3	9.2	–	–	8.1	3.9
V	59.5	52.2	9.9	5.4	11.0	11.0	–	–	9.6	6.7

* Calculated.

3.2. Immobilization of ammonium and phosphonium salts onto CPVA (III-V)

Biocidal polymers (III–V) were synthesized to study the influence of the nature of the active group on the antibacterial activity of the polymer. Quaternization of chloroacetylated polymers were carried out in dry benzene by reacting CPVA (II) with TEA, TPP and TBP, respectively at room temperature followed by heating at 80 °C for 4 days (Scheme 2).

Generally, TEA, TPP and TBP were quaternized with chloromethylated polymer at room temperature. To confirm quaternization of the polymers, the reaction mixtures were heated at 80 °C (under benzene refluxing). This method favored the formation of ammonium and phosphonium salts. The amount of TEA, TPP and TBP was little excess than the chloromethyl group content, hence it was reported earlier that in stoichiometric conditions, the yield of quaternization was limited (Hazziza-Lasker et al., 1993). When polymer (IV) was retreated with TPP the yield increased from 33.44% to 63.1%, while when polymer (V) was retreated with TBP, there is no increase in the yield (37.5%). The nitrogen and phosphorus contents of the biocidal polymers (III–V) were determined by elemental microanalysis as shown in (Table 1) and the results were in good agreement with the calculated values.

Also the IR spectra of the polymers (III–V) showed the presence of peaks at 1455–1494 cm^{-1}, (P-CH_2) and at 1494–1402 cm^{-1} (P-Ph) which confirmed the formation of ammonium and phosphonium salts.

3.3. Antibacterial assessment of polymers (III–V)

The antimicrobial activities of biocidal polymers (III-V) against *E. coli, P. aeruginosa, Shigella sp, B. subtilis,* and *B. cereus* were explored by the cut plug method and the viable cell counting methods as described before in materials and methods. The capability of the prepared polymers to inhibit the growth of the tested microorganisms on solid media is shown in (Table 2). It was found that the diameter of inhibition zone varied according to the polymer microstructure and the test bacteria.

The triphenyl phosphonium salt of the modified poly(vinyl alcohol) (IV) was found to be the most effective polymer on both Gram negative and Gram positive bacteria (diameters of inhibition zones ranged between 30 and 80 mm) after 24 h.

This means that the triphenyl phosphonium salt is better than other effective groups compared with salts from triethylamine and tributylphosphine. These results are similar to what is previously reported (Kenawy et al. 1998). The growth inhibiting effect was quantitatively determined by the ratio

Table 2 Diameters of inhibition zones (mm) produced by 20 mg powdery polymers of modified poly(vinyl alcohol) against different test bacteria after 24 h by the cut plug method on nutrient agar at 30 °C.

Organism	Polymer code		
	III	IV	V
Escherichia coli	26	30	20
Pseudomonas aeruginosa	40	30	60
Shigella sp.	30	50	60
Salmonella typhi	34	60	40
Bacillus subtilis	30	80	50
Bacillus cereus	40	32	32

(M/C) of the surviving cell number. As shown in Fig. 1, the growth inhibitory effect of polymer (III) differed among the bacterial and fungal strains. The inhibition becomes stronger in the order *P. aeruginosa* < *E. coli* < *T. rubrum* < *B. subtilis*. The results also show that the inhibitory effect increased by increasing the concentration of the polymer. Fig. 2 shows the inhibitory effect of polymer (IV). The results show that the inhibition becomes stronger in the order *E. coli* < *T. rubrum* < *B. subtilis* < *P. aeruginosa* and the inhibition was increased by increasing the concentration of the polymer. Figs. 3 and 4 show the inhibitory effect of polymer (V). The results showed that the inhibition becomes stronger in the order *P. aeruginosa* < *E. coli* < *T. rubrum* < *B. subtilis* and the former organism is the most sensitive even at lower concentrations, compared to other organisms, starting from 0.5 mg/ml. In general, the inhibition increased by increasing the concentration of the polymer and the potency of inhibition varied according to the polymer and the test strain.

3.4. Mode of action

The mode of action of cationic biocides is interpreted in terms of the following sequence of elementary processes (Kanazawa et al., 1993; Franklin and Snow, 1981): (1) Adsorption onto the bacterial cell surface; (2) Diffusion through the cell wall; (3) Binding to the cytoplasmic membrane; (4) Disruption of the cytoplasmic membrane; (5) Release of cytoplasmic constituents such as K^+ ions, DNA, and RNA; (6) Death of the cell.

It is now generally accepted that the mode of action of the polycationic biocides based on quaternary ammonium salts can be interpreted on the basis of each elementary process described above, since the same physiological events as in

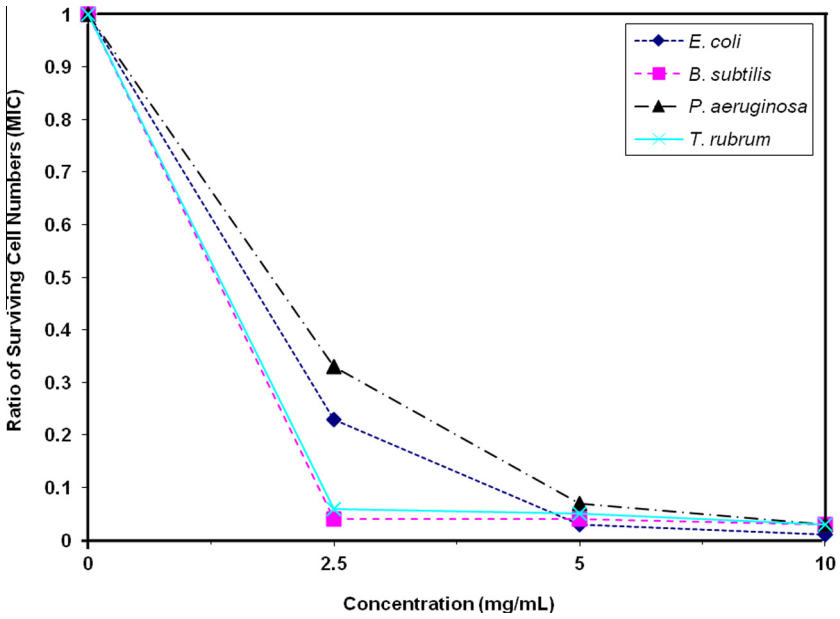


Figure 1 Growth inhibition of different concentrations of triethyl ammonium salt (III). Cultures were inoculated with 1.5×10^5 cells/ml of each test bacterium and 7.2×10^4 spores/ml of the test fungus.

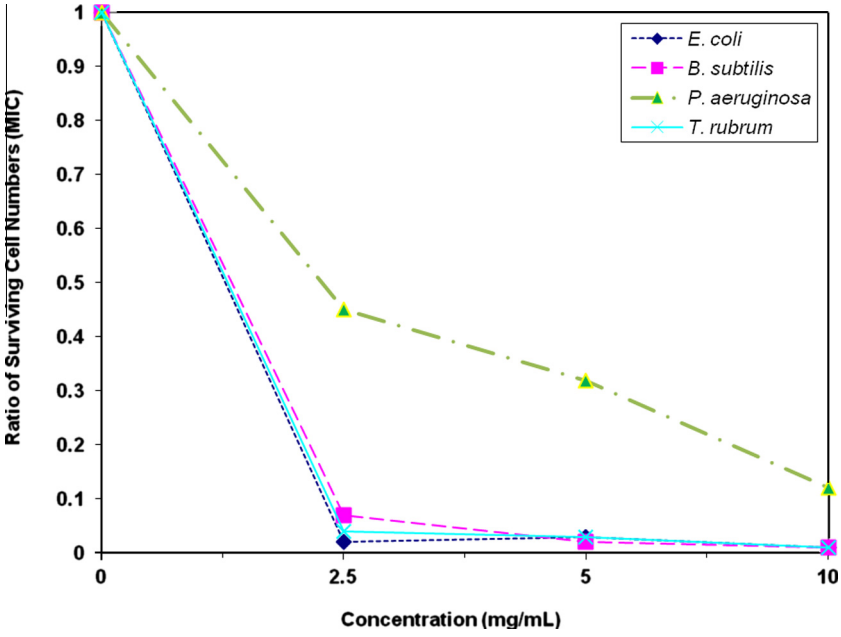


Figure 2 Growth inhibition of different concentrations of triphenyl phosphonium salt (IV). Cultures were inoculated with 1.5×10^5 cells/ml of each test bacterium and 7.2×10^4 spores/ml of the test fungus.

processes 1, 3 and 5 have been observed for the polycationic biocides (Hugo and Longworth, 1964, 1966). It is well known that the bacterial cell surfaces are negatively charged. Then, adsorption onto the negatively charged cell surface (process 1) is expected to be enhanced with increasing charge density of the cationic biocides. Therefore, it is reasonable to assume that process 1 is much more enhanced for polymers, compared with that for model compounds with similar mode of action as explained by Kanazawa et al. (1993a) and Katchalsky (1964). A similar situation can also be expected in process 3, since there are many negatively charged species present in the cytoplasmic membrane, such as acidic phospholipids and some membrane proteins (Gel'Man et al., 1975; Ikeda et al., 1984; Kanazawa et al., 1993b). The disruption of the membrane (process 4) is a consequence of interaction of the bound polymers with the membrane, thus, it is expected to be facilitated with increasing amounts of the bound polymers.

Hydrophobicity of the substituent affected the antibacterial activity of the phosphonium salts. It is expected that with increasing hydrophobicity of the foreign compounds, they

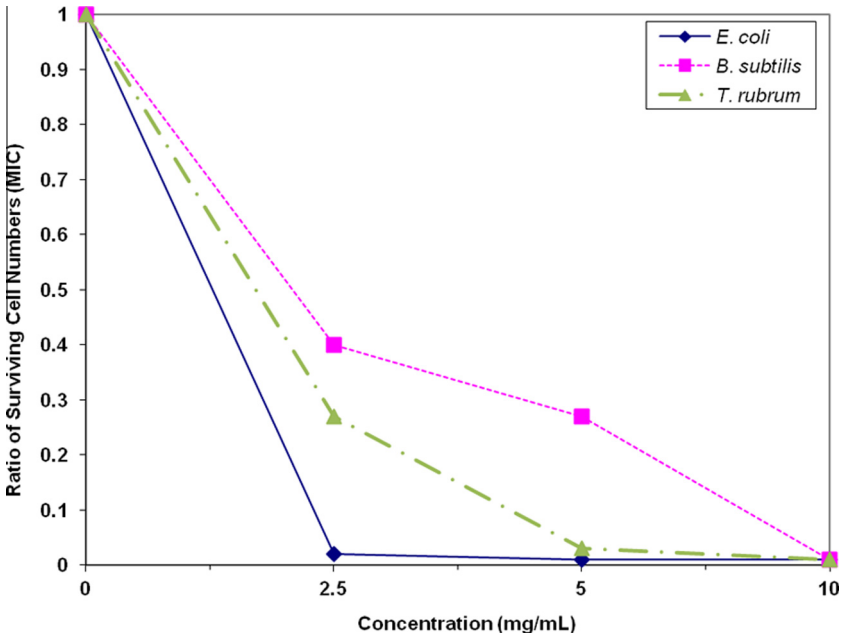


Figure 3 Growth inhibition of different concentrations of tributyl phosphonium salt (V). Cultures were inoculated with 1.5×10^5 cells/ml of each test bacterium and 7.2×10^4 spores/ml of the test fungus.

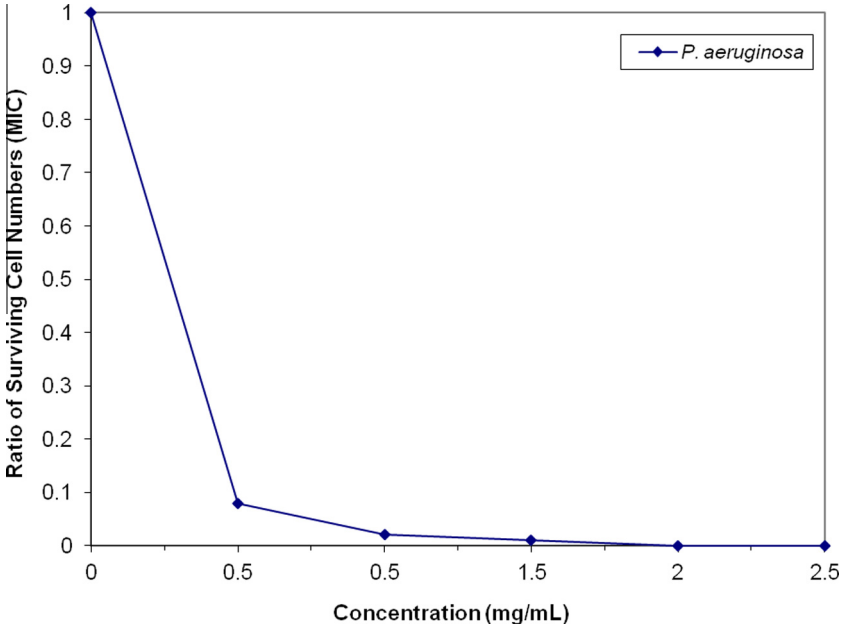


Figure 4 Growth inhibitions of different concentrations of tributyl phosphonium salt (V). Culture was inoculated with 1.5×10^5 cells/ml of the test bacterium.

become more active to interact with the cytoplasmic membranes (processes 3 and 4) (Kanazawa et al., 1993).

It is known that there is much difference in the structure of cell walls between Gram-positive and Gram-negative bacteria. The former cells have a simple cell wall structure, in which outside the cytoplasmic membrane there is only a rigid peptidoglycan layer. The peptidoglycan layer, though relatively thick, is composed of networks with plenty of pores which allow foreign molecules to come into the cell without difficulty. This explains the different effects of the polymers on various microorganisms. Our results are supported by a similar proposal by Kanazawa et al., and Casterton and Cheng. On the other hand, Gram-negative bacteria have very complicated cell walls. There is another membrane outside the peptidoglycan layer, which is called the outer membrane, and has a structure similar to that of the cytoplasmic membrane. Because of the bilayer structure, the outer membrane is a potential barrier against foreign molecules with high

molecular weight (Kanazawa et al., 1993; Casterton and Cheng, 1975).

Consequently, the overall activity would be determined by two factors: one is favored for polymers (process 1, 3 and 4) and the other is unfavored for the polymers (process 2) (Kanazawa et al., 1993).

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