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Evaluation of Antimicrobial Activity of Buforin I and Nisin and Synergistic Effect of the Combination of them as a Novel Antimicrobial Preservative

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Research Paper

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Evaluation of Antimicrobial Activity of Buforin I and Nisin and Synergistic Effect of the Combination of them as a Novel Antimicrobial Preservative

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Key words: Antimicrobial effects, Synergistic effects, Buforin I, Nisin

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Abstract

One of the most effective methods for increasing the antimicrobial activity of a substance is to combine it with one or more other antimicrobial agents. The aim of the present study was to evaluate the antimicrobial effect of buforin I and nisin alone and investigate the synergistic action of these compounds against the most important food spoilage microorganisms in clouding *B. subtilis, S. epidermidis, L. innocua, E. coli, S.* Enteritidis, *A. oryzae, R. glutinis* and *G. candidum*. The results of MIC and MBC/MFC examinations showed that buforin I had higher antimicrobial activity than nisin on all the microbial strains used in this study ($p \leq 0.5$). *E.coli* was the most resistant to both antimicrobial agents, while *Listeria innocua* and *Staphylococcus epidermidis* were the most sensitive to nisin and buforin I, respectively. The results of synergistic interaction between buforin I and nisin indicated that the combination of buforin I and nisin on *B. subtilis, S. epidermidis* and *A. oryzae* showed synergistic effect, while it had no effect on *S.* Enteritidis and *Geotrichum candidum*. The combination of buforin I and nisin showed partial synergistic effect on *Listeria innocua, Escherichia coli, Rhodotorula glutinis*. Assessment of viability of the microorganisms under the antimicrobial agents alone and in combination with each other at MICs and FICs indicated that use of these antimicrobial agents in combination enhances antimicrobial activity at lower concentrations of both agents. The present study investigated the antimicrobial properties of buforin I against food spoilage microorganisms for the first time and suggests that its use alone or in combination with nisin may provide a clear horizon for the application of antimicrobial peptides as natural preservatives. Thus, the combination of antimicrobial peptides and traditional antimicrobial food preservative could be a promising option for the prevention of contamination, spoilage, and infestation of food and beverage products.

HIGHLIGHTS

- Buforin I had higher antimicrobial activity than nisin.
- The combination of nisin and bofurin eliminates the limitation of the antimicrobial effect of nisin.
- Buforin I may provide a clear horizon for the application of antimicrobial peptides as natural preservatives.

Despite significant advances in food production and preservation methods, food safety remains a global challenge *(14)*. Although global health levels have been upgraded, the rates of foodborne diseases and poisoning continue to be high and unsafe food causes 600 million cases of food borne diseases and 420,000 deaths annually. 30% of food borne deaths occur among children under 5 years of age *(75)*. On the other hand, the world health organization (WHO), has many recommendations for reducing salt consumption to reduce the incidence of cardio-vascular diseases *(74)*. Since salt has antimicrobial properties, its removal or reduction in food products that it has a protective role in, lead to decreased shelf life, hence, other antimicrobial agents may be needed to maintain the safety of foods *(26)*. In addition, increased concern over synthetic preservatives, the prevalence of foodborne pathogens with resistance to classical antibiotics, restrictions or prohibition of the use of some chemical preservatives in some countries, and the increased consumer tendency for fresh or minimally processed foods have created many technological challenges in the food industry *(58)*. Therefore, it is necessary to study and investigate new natural antimicrobial compounds with a broad spectrum of antimicrobial activity *(62)* or to find ways to increase the effectiveness of the compounds or methods currently used *(38)*.

Antimicrobial peptides (AMPs) are produced as an important part of the immune system in all aspects of life, antimicrobial activity of cationic antimicrobial peptides (CAPs) has been extensively studied *(1, 4, 9, 35, 39)*. The major benefit of using AMPs as new natural food preservatives, is that they preserve the food without changing its quality and they are not harmful *(68)*. They are short amino acid sequences (less than 50) with positive charge (in general +2 to +9 due to basic amino acids, such as lysine and arginine) *(21, 51)* and contain more than 30% of hydrophobic amino acids. Currently, nisin is the only antimicrobial peptide that is widely utilized in the preservation of food commercially *(61)*. Nisin, due to its non-toxic nature, flavorless, heat

stability and tolerance of low pH is the most commonly used bacteriocin *(41, 63)*. Nisin is a polycyclic antibacterial peptide comprising 34 amino acid residues, with an overall positive charge and amphipathic properties *(41)*. It exerts its antimicrobial activity through impaired membrane function and permeability period. Buforin I is a 39-amino acid CAP that was first isolated from the stomach tissue of the Asian toad *Bufo bufo gargarizans* which shows strong antimicrobial activities against a wide range of microorganisms including Gram-positive (*Bacillus subtilis, Staphylococcus aureus, Streptococcus mutans* and *Streptococcus pneumoniae*) and Gram-negative (*Escherichia coli, Salmonella* Typhimurium, *Pseudomonas putida* and *Serratia* species) bacteria and fungi (*Candida albicans, Cryptococcus neoformans* and *Saccharomyces cerevisiae*) *(54)*.

Buforin I belongs to the great buforins family that all share N-terminal region of histone H2A as a common trait. This region specifies the protein's DNA binding activity and in fact, this family are Histone- derived peptides *(15)*. Histone-derived peptides do not have any role in replication; this extracellular histone derivatives have a substantial antimicrobial property *(54)*. Although the mode of action of the buforin family is still unknown, reports indicate that they kill a microorganism by translocating into the cell and binding to nucleic acids and cross lipid bilayers via the transient formation of a peptide–lipid supramolecular complex pore *(15)*. Thus, the purpose of this research was to assess the impact of nisin and buforin I and their combination on food spoilage microorganisms including Gram-negative and Gram-positive bacterial and fungal isolates.

B. cereus is a psychrotroph and sporogenic microorganism that proved able to grow in products at cold-storage temperatures and even cause alimentary diseases (*28*).*L. monocytogenes* is the only species of the genus Listeria that has been involved in known food-borne outbreaks of listeriosis (*29*). Due to the difficulty and risks of detection of *L. monocytogenes, L. innocua,* which appears to be present together with *L. monocytogenes,* often used as an indication for the presence

of this bacteria *(18)*. *Bacillus subtilis*, *Staphylococcus epidermidis* and *Listeria innocua* were studied as the most important representatives of gram-positive bacteria in this study. Gram negative bacteria are not only associated with foodborne illness and poisoning, but also important factors in food spoilage. In this study, *Escherichia coli* and *Salmonella* Enteritidis were used as representatives of gram-negative bacteria. The combination of buforin I and nisin was evaluated against the growth of the spoilage fungal strain including *Aspergillus oryzae*, *Rhodotorula glutinis*, and *Geotrichum candidum* which are known to be responsible for the production of allergenic and toxic compounds (*31*).

MATERIALS AND METHODS

Microorganisms, Media, and Antimicrobial Agents. *Bacillus subtilis* (PTCC 1023), *Staphylococcus epidermidis* (PTCC 1114), *Listeria innocua* (ATCC 33090), *Escherichia coli* (ATCC 25922), *Salmonella* Enteritidis (PTCC 1735), *Aspergillus oryzae* (PTCC 5164), *Rhodotorula glutinis* (PTCC 5257) and *Geotrichum candidum* (ATCC 34614), were procured from microbial collection, Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad. Bacterial strains were cultured 24 h in Mueller Hinton Broth (MHB) (Sigma-Aldrich) at 37˚C and fungal strains were cultured 48 hours in Potato Dextrose Broth (PDB) (Sigma-Aldrich) at 25˚C, before the antimicrobial tests were performed. A 0.5 McFarland standard was used to prepare microbial suspension, which was equivalent to 10^6-10^8 CFU/ml of microorganism *(3)*.

The amino acid sequence of buforin I was determined by the National Center of Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) with accession number: P55897,

then synthesized by the Mimotopes Company, Mulgrave,Australia. The purity of buforin I was 96%. One mg of buforin I was dissolved in one ml water and dimethyl sulfoxide (DMSO) solution (80:20 v/v) and filter sterilized (0.22µm) to prepare a 1 mg ml^{-1} stock solution *(64)*. One gram of Nisin (2.5% from *Lactococcus lactis*, CAS Number: 1414-45-5, Sigma-Aldrich) was dissolved in 25 ml of 0.05% acetic acid solution, centrifuged at 4000g for 20 min. The supernatant was then filter sterilized to prepare a 1 mg/ml stock solution *(63)*.

Minimum Inhibitory Concentration (MIC). MICs were obtained using the micro broth dilution method. Serial dilutions (1, 2, 4, 6, 8, 10, 12, 14 and 16µg/ml) of the buforin I and (1, 2, 4, 8, 16, 32, 64, 128, 256 and 512µg/ml) of the nisin in MHB for bacterial strains and in PDB for fungal strains were prepared. 10µl of microbial suspensions with an optical density at 630nm (OD_{630}) equal to 0.08-0.13, were added to 90µl each dilution in the 96 micro-well plates. The micro-well plates were incubated at 37˚C for 24 h for bacterial strains and 25˚C for 48 h for fungal strains *(8)*. To determine the MICs, the absorbance was measured at 630nm by ELISA reader (BioTek ELx808). Since the increase in turbidity is a sign of the growth of microorganisms, MICs were determined as the lowest concentrations that prevented visible growth. Growth medium without inoculum was used for negative control (*36*).

Minimum Bactericidal/Fungicidal Concentration (MBC/MFC).100 µl from each well, that growth was not seen, were pour-plated cultured in Mueller Hinton Agar (MHA) (Sigma-Aldrich) for bacterial strains and surface plate cultured on Potato Dextrose Agar (PDA) (Sigma-Aldrich) for fungal strains. The plates were incubated at 37°C for 24 h for bacterial strains or 25°C for 48 h for

fungal strains and the lowest dilution that yielded complete inhibition of growth was taken as MBC or MFC *(70)*. Thus MBC or MFC was the lowest concentration of antimicrobial agents that prevented visible growth on the subculture plate (*32*).

Synergistic Interaction between Buforin I and Nisin by Checkerboard Assay. The synergistic interaction between nisin and buforin I, was assessed using the checkerboard method *(27, 44, 53)*. Thus, seven two-fold serial dilutions (from 2MIC to MIC/32) of nisin and buforin I, in accordance with obtained MIC in the previous section for each microorganism, were prepared. Equal amount (25µl) of each dilution was poured into 96-well microplates to obtain a fixed amount of both antimicrobial peptides, so that each row (and column) contained a fixed amount of the first agent and increasing amounts of the second one. A total of 50µl of fresh bacteria and fungi suspension (10^8 CFU/ml) was added to each well and cultured at 37˚C for 24 h for bacterial strains and 25˚C for 48 h for fungal strains.

The Fraction Inhibitory Concentration Index (FIC_I) was calculated using the following formula:

$$FIC_I = \frac{MIC_{A/B}}{MIC_A} + \frac{MIC_{B/A}}{MIC_B}$$

where MIC_A is the MIC of compound A, MIC_B is the MIC of compound B and $MIC_{A/B}$ is the MIC of compound A in combination with compound B. Total synergism ($FIC_I \leq 0.5$), partial synergism ($0.5 < FIC_I \leq 0.75$), indifference ($0.75 < FIC_I \leq 2$) or antagonism ($FIC_I > 2$) between the two compounds was reduced using the FIC_I *(46)*.

Survival Curve. The effect of nisin and buforin I was evaluated separately and in combination on the growth of microbial strains through the construction of a survival curve *(48)*. The final concentration of suspension of the strain (adjusted to 10^6-10^8 CFU/ml) was added to the wells of 96-well micro-plates, and 50µl of the antimicrobial agent (at MICs or FIC_s concentrations), was added to each well. The bacterial strains were cultured at 37˚C for 30 h and fungal strains were cultured at 25˚C for 50 h. After incubating for 0, 6, 12, 18, 24 and 30 h for bacterial strains and 0, 10, 20, 30, 40 and 50 h for fungal strains, a 50µl liquid from each dilution was spread on the surface of the agar plates and incubated at 37°C for 24 h or 25˚C for 48 h; for bacterial and fungal strains, respectively. Then, the number of CFU/ml was counted. 50µl of the microbial suspensions without antimicrobial agents was used as a control group. Thereafter, survival curves were constructed by plotting the log number of CFU/ml against time (h).

Statistical analysis. In order to confirm the results, the experiments were repeated three times. Results of the study were analyzed by Minitab version 18.0 and differences among the means were determined by one-way ANOVA for significance at $p < 0.05$.

RESULTS

Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal/Fungicidal Concentration (MBCs/ MFCs). MICs and MBCs of antimicrobial agents were evaluated and reported in Table 1. Buforin I and nisin showed different antimicrobial effect against the tested

strains. The range of obtained MICs for nisin and buforin I was 32 – 512 µg/ml and 4 – 16 µg/ml, respectively.

In general, Gram-negative bacteria were more resistant to both nisin and buforin I than Gram-positive bacteria. Also, the antimicrobial effect of buforin I on the microbial strains was - higher than nisin. The results of MIC and MBC/MFC tests show that *E. coli* was the most resistant strain to both antimicrobial agents, while *Listeria innocua* and *Staphylococcus epidermidis* were the most sensitive strains to nisin and buforin I, respectively. The results also indicated that nisin showed bactericidal or fungicidal effect on *Listeria innocua* and *Geotrichum candidum* at 256µg/ml and on *Bacillus subtilis*, *Staphylococcus epidermidis*, *Salmonella* Enteritidis and *Rhodotorula glutinis* at 512µg/ml. buforin I showed fungicidal effect on all fungal strains, used in this study and bactericidal effect on *Listeria innocua* and *Staphylococcus epidermidis* at 16 and 10µg/ml, respectively.

Synergistic Interaction between Buforin I and Nisin by Checkerboard Assay. The results of synergistic interaction between buforin I and nisin are shown in Table 2. Results indicated that the combination of buforin I and nisin on *Bacillus subtilis*, *Staphylococcus epidermidis* and *Aspergillus oryzae* showed synergistic effect, while it had no effect on *Salmonella* Enteritidis and *Geotrichum candidum*. The combination of buforin I and nisin showed partial synergistic effect on other microorganisms including *Listeria innocua*, *Escherichia coli*, *Rhodotorula glutinis*. As shown in the Table 2, smaller amounts of both antimicrobial agents are used to inhibit the growth of microorganisms when viewing the synergistic or partial synergistic effect.

Survival Curve: The survival curve shows the effect of nisin, buforin I and their combination against the growth of the microbial strains used in this study (Figure 1). The survival and activity curves showed overlap and confirm the results of each other. For all microbial strains except *Salmonella* Enteritidis and *Geotrichum candidum*, the curves represent FIC in synergism or partial synergism state, placed lower than the curves of buforin I and nisin alone, indicating higher bacteriostatic or fungistatic of the combined use of both agent. For all microbial strains of buforin I antimicrobial activity was higher than nisin.

DISCUSSION

The antibacterial activity of various cationic peptides on foodborne and food spoilage microorganisms, like *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Shigella sonnei*, *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* were investigated in many studies *(12, 19, 20, 61)*. Nisin is one of the most attractive cationic peptides in the field of food microbiology and its antimicrobial effect on many Gram-positive foodborne and food spoilage microorganisms including *Staphylococcus aureus (11), Listeria innocua (65), Listeria monocytogenes* and *Bacillus subtillis (44)* with MIC equal to 7.8, 1, 250 and 125µg/ml, respectively, has been investigated. Nisin inhibitory effect on the outgrowth of spores of *Bacillus* species and *Clostridium* species, was recorded *(25)*. Ashari *et al*. (2019) reported MICs of nisin for *Eschericia coli* FNCC 0091, *Pseudomonas fluorescens* FNCC 0070 and *Aspergillus niger* FNCC 6080, were 500, 500 and 250 IU, respectively *(5)*. Yosef-Ahmad *et al*. (1980) reported nisin can inhibit growth of *Aspergillus parasiticus* and accumulation of aflatoxin B1 and G1 over 3 days; with continued incubating for 10 days, growth inhibitory effect of nisin was decreased, while its inhibitory effect on toxin production was still observed *(77)*.

Similar results were reported by Gourama and Bullerman (1995), when they had investigated inhibition of growth and aflatoxin production of *Aspergillus flavus* subsp. Parasiticus by *Lactobacillus* species. Not only did growing *Lactobacillus* spp. cells inhibit germination of mold spores, even culture supernatant broth from the mixture of strains inhibited mold growth. These authors reported that *Lactobacillus* species prevented mold growth because of low pH and a microbial competition effect; however the inhibition of aflatoxins in this study was probably due to a low-molecular-weight bacterial metabolite(s) *(34)*. Numerous studies have shown that these low-molecular-weight bacterial metabolites are bacteriocin with molecular weight about 3.4 kDa (*7*) and nisin is the main metabolite produced by *Lactobacillus* spp (*23, 30*).Lay *et al.* (2008) recorded that nisin reduced *Candida albicans* proliferation and nisin inhibited *C. albicans* growth beginning at 500 µg/ml *(42)*.

Many studies have confirmed and reported that nisin bind to lipid-II (the peptidoglycan precursor), just like other lantibiotics, and leads to pore formation and inhibition of cell wall biosynthesis. *(24, 63, 71, 72, 73)*. Gram-negative bacteria due to their lipopolysaccharides in the outer membrane, show less sensitivity or resistance to nisin *(63)*. Reports of the impact of the nisin effect on Gram- negative bacterial due to their outer membrane and fungal strains due to their rigid cell wall (a complex structure consisting of glucan cross-linked with chitin and cell wall protein) *(25)* show variable results that range from ineffective *(44)* to meaningful effects *(50)*.

The MICs of isolated buforin I from the stomach tissue of *Bufo bufo gargarizans*, an Asian toad, on the growth of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Candida albicans* and *Saccharomyces cerevisiae* were recorded 4, 8, 4, 4, 4 and 4 µg/ml respectively *(54, 55)*. These results demonstrated that buforin I and nisin exhibited different effects against different microorganisms, reflecting potential differences in the inoculum level,

experimental temperature, the physiological condition of the microorganism *(44)* and the culture mediaand methods *(53)* that were used for evaluating antimicrobial effect in different studies. Also, the origin of the antimicrobial agents that can be extracted from nature or synthetically made, must be considered *(6)*.It is worth mentioning, although consumption of nisin for concentrations less than 83.25mg/kg has not shown obvious effects on human health, and usually one gram of food in the United States and other countries may contain 250mg/kg nisin, or it can be found up to 300 mg/kg in mouthwashes, many studies recorded that 300–400mg/kg nisin has a contraceptive effect in humans *(41, 47, 76)*. Although the FDA recommends a maximum of 250µg/ml of nisin in the finished product in 2017, the European Food Safe Authority re-evaluated the toxic potential of nisin and approved an acceptable daily intake of up to 1 mg nisin/kg body weight per day for use in certain food products. Therefore, the combination of nisin with another CAPs due to their broad-spectrum activity (Gram-positive and Gram-negative bacteria, fungi, and viruses) *(9, 13)*, low level induced resistance *(66)*, improvement in nutrient digestibility and modulation of gut microbiota *(61)*, can increase its effect on food spoilage or pathogenic microorganisms. Churklam *et al*. (2020) recorded the synergistic interaction with FIC_I values ranging from 0.375 to 0.5 for carvacrol and nisin combination against *Literia monocytogenes* 10403S and three food isolates. In addition, they examined the survival of *L. monocytogenes* 10403S under the synergistic effect of carvacrol and nisin during storage of sliced bologna sausages at 4°C and reported the presence of carvacrol combined with nisin resulted in significant growth rate reductions compared to those of controls *(16)*. Ashari *et al*. (2019) recorded that the combination of nisin and essential oil had synergistic effect against *Bacillus cereus*, *Aspergillus niger*, and *Salmonella* Typhimurium *(5)*. Liu *et al*. (2015) recorded that the combination of ε-polylysine and nisin against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* had synergistic effect and against *Micrococcus luteus* and *Hansenula anomala* had partial synergic effect. Their result showed that the combination of ε-

polylysine and nisin did not have any effect on *Aspergillus niger (44)*. In addition to the mentioned examples, many studies have been conducted to investigate the synergistic effect of different compounds to enhance the antimicrobial effect of nisin *(22, 43, 48, 60)*. What is common among all reports is the low effect of nisin on some microorganisms, especially Gram-negative bacteria and fungal strains, is due to nisin restriction on the passage of the outer membrane or rigid membrane, respectively *(50, 52)*. Reports indicate that if a factor can facilitate the passage of nisin through these layers, its inhibitory effect on such microorganisms will also be visible *(10, 59, 69)*.

Although buforin I was first identified in the stomach of Asian toads by Park *et al.* (1996) (*54*); but Kim *el. al* (2000) showed that histone H2A was precursor of Buforin I, and the H2A non-acetylated histone was converted to Buforin I after the secretion from stomach cells and exposure to pepsin. They also investigated the presence of Buforin I in human, cow and pigs secretions *(40)*. Another studies, like Minn *et. al* (1998), recorded that peptides derived from pepsinogen action in the stomach on some proteins, such as buforin I, have a strong antimicrobial activity and are found in most vertebrates, including humans *(49, 67)*. A combination with a similar amino acid sequence was found on the lung of the sheep's lungs *(49)*. The broad-spectrum activity of buforin I, its high activity as well as its minimum inhibitory concentration at low concentrations and the absence of cell cytotoxicity and hemolytic effect, demonstrate the potential of this compound for use as a food preservative. The results also indicated that the use of buforin I and nisin in combination enhances antimicrobial activity (bacteriostatic or fungistatic effect) at lower concentrations of both agents. Although there are many studies on the safe use of cationic peptides at MIC concentrations (*17, 33, 37, 56*), they need to be legally approved by a regulatory body.

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FIGURE LEGEND

FIGURE 1. The survival curves of the antimicrobials agents. The microbial suspensions were treated with the antimicrobial agents at MICs concentrations of nisin and buforin I separately and FIC_s concentrations in combination with nisin and buforin I. The microbial suspensions without antimicrobial agents was used as a control group. A: *Listeria innocua*, B*: Bacillus subtilis*, C: *Staphylococcus epidermidis*, D: *Escherichia coli*, E: *Salmonella* Enteritidis, F: *Aspergillus oryzae*, G: *Rhodotorula glutinis*, and H: *Geotrichum candidum*. Vertical error bars represent standard deviation of tree replications.

TABLE 1. MICs and MBCs/MFCs of nisin and buforin I against some food spoilage bacterial and fungal strains

Microorganisms	Nisin		Buforin I	
	MIC (µg/m1)	MBC/MFC (µg/m1)	MIC (µg/m1)	MBC/MFC (µg/m1)
Listeria innocua	64	256	10	16
Bacillus subtilis	256	512	14	>16
Staphylococcus epidermidis	256	512	4	10
Escherichia coli	512	>512	16	>16
Salmonella Enteritidis	128	512	8	>16
Aspergillus oryzae	256	>512	8	16
Rhodotorula glutinis	512	512	8	16
Geotrichum candidum	128	256	10	16

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; MFC: Minimum Fungicidal Concentration.

TABLE 2. FIC index and FIC concentrations of nisin combination with buforin I against some food spoilage bacterial and fungal strains

Microorganisms	**FIC_I**	**FIC concentrations Nisin + Buforin I (µg/ml)**	**Synergistic interaction**
Listeria innocua	0.75	64+3.5	partial synergism
Bacillus subtilis	0.5	128+4	synergism
Staphylococcus epidermidis	0.5	128+1	synergism
Escherichia coli	0.75	256+3.5	partial synergism
Salmonella Enteritidis	1.25	32+8	no effect
Aspergillus oryzae	0.375	32+2	synergism
Rhodotorula glutinis	0.625	256+1	partial synergism
Geotrichum candidum	2	128+10	no effect

FIC_I: Fraction Inhibitory Concentration Index.

Figure 1

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