Inactivation of *Cronobacter* spp (*Enterobacter sakazakii)* in infant formula using lactic acid, copper sulfate and monolaurin

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Abstract

Cronobacter spp. (*Enterobacter sakazakii*) is an opportunistic pathogen that poses a health risk to neonates. *E. sakazakii* is a known contaminant of infant formula (IF), and has been associated with cases of necrotizing enterocolitis and infant meningitis. The purpose of this study was to investigate the use of lactic acid (LA) (0.1, 0.2 and 0.3% v/v) and copper sulfate (10, 50, and 100 ppm), as natural antimicrobials against *E. sakazakii* in IF stored at room temperature for 6 h. Also, the effect of monolaurin (1000, 2000, and 3000 μg ml^{-1}) suspended into tween-80 (TW) or dissolved in ethanol was investigated. Reconstituted infant formula (RIF) and powdered infant formula (PIF) were inoculated with five strains of *Cronobacter* spp (*E. sakazakii*) at the levels of ca 1×10^6 CFU $ml^{-1}$and 1×10^3 CFU g^{-1}, respectively. LA at 0.1% v/v only delayed the growth of *E. sakazakii* in RIF. LA at o.2% v/v had a bacteriostatic effect on *E. sakazakii* growth, whereas 0.3% LA v/v resulted in ca 3 logs reduction. Copper sulfate showed a dose-dependent effect on *E. sakazakii* growth, where 10 ppm did not result in a noticeable reduction in *E. sakazakii* count, however, 50 ppm and 100 ppm, elicited ~ 1 and 2 logs reduction at the end of the storage period, respectively. Surprisingly, the combination of 0.2% LA and 50 ppm copper sulfate resulted in a complete elimination of the organism form RIF by the end of the storage time. Monolaurin exhibited a slight inhibitory activity against *E. sakazakii* in RIF and the effect was more pronounced when ethanol was used to deliver monolaurin in RIF. Approximately, 1.5 logs difference between the control and the monolaurin-treated samples was observed at the end of the storage period (6 h). The use of the synergistic interactive combination of LA and copper sulfate could be beneficial to control *E. sakazakii* in the infant formula industry.

1. Introduction

Enterobacter sakazakii is an emerging human pathogen that contaminates powder infant formula (PIF) (Iversen and Forsythe, 2003), and has been associated with various cases of neonatal meningitis (Bar-Oz et al., 2001; CDC, 2002). The microorganism was first implicated in neonatal meningitis in 1958. Although the incidence rate of the infection is low, yet mortality rate ranges from 40-80% among infected infants, and those who survive the infection usually develop irreversible neurological sequelae (Bowen and Braden, 2006). Recently, *Cronobacter* has been proposed as a new genus to replace *E. sakazakii* as a single species. *Cronobacter* currently encompasses six different species that are potentially pathogenic to neonates (Iversen et al, 2007).

Contaminated powdered infant formula (PIF) is a recognized source of *E. sakazakii* as demonstrated in a survey conducted by Muytjens et al. (1988), where the organism was detected in about 14% of 141 samples of PIF, which came from 35 countries. The problem with infant formula is that it is not sterile, and its nutritional characteristics provide excellent conditions for bacterial growth following reconstitution (Drudy et al., 2006, Mullane et al., 2006). *E. sakazakii* does not survive the heat of pasteurization used in the production of infant formula, therefore, the organism mostly emanates from the processing environment or form heat-sensitive ingredients added after pasteurization despite rigorous hygienic practices (Kandhai et al., 2004). Therefore, an end-product control measure should be available to prevent further presence of the organism in the formula.

Preservatives are commonly used in the production of food to ensure safety and stability, Nonetheless, there is a growing awareness of consumers about food-related diseases and

mounting demand to produce pathogen free food with minimal use of artificial preservatives (Brul and Coote, 1999). Hence, the food industry has turned to the use of natural ingredients to ensure the safety of the products. Many research papers have been conducted on survival conditions and inactivation of *E. sakazakii* (Breeuwer et al., 2003; Edelson-Mammel et al., 2004; Perez et al., 2007; Gurtler and Beuchat, 2007; Arku, 2008; Osaili et al., 2008), but little attention was given to the use of antimicrobials that are perceived as more "natural" to inhibit *E. sakazakii* in infant formula and baby foods.

Lactic acid (LA) is a weak-organic acid that has been widely used to control growth of spoilage and pathogenic bacteria in food. LA also acts as a permeabilizer of the Gram-negative bacterial outer membrane, and this is of considerable consequence, because the weak acid could be used to potentiate the antimicrobial activity of other cantimicrobials against Gram-negative bacteria (Alakomi et al., 2000).

All living organisms including bacteria require copper at low concentrations. Copper is used as cofactor for metalloproteins and enzymes, but at high concentrations, inhibition of growth in bacteria can be accrued as well as intoxication of most microorganisms (Faundez et al., 2004). Additionally, fatty acids and there esters may pose antimicrobial activity against certain foodborne pathogens. The monoglyceride of lauric acid (monolaurin) demonstrated a considerable antibacterial activity against *Listeria monocytogenes* in beef emulsion and hotdog and against *E. coli* O157:H7 in UHT milk (Branen et al., 2004; Mbandi et al., 2004).

The objective of this study was to investigate the effect of both lactic acid and copper sulfate alone and in combination on the inactivation of *Cronobacter* spp (*Enterobacter sakazakii)* in infant formula. Additionally, the effect of monolaurin against *E. sakazakii* in infant formula was also explored.

2. Materials and Methods

2.1 Bacterial Strains

Five strains of *E. sakazakii* (*Cronobacter* spp) (*C. sakazakii* (ATCC 29004, ATTC 12868, ATTC 29544), *C. muytjensii* ATTC 51329, and *Cronobacter* isolate FSM 287) were used in this study. The strains were obtained from the culture collection in the Department of Food Science and Human Nutrition, at Washington State University. Strains were kept refrigerated in tryptic soy agar (TSA) (Difco, Becton Dikinson, Spark, MD) slants, prior to the experiment, the strains were individually inoculated into sterilized test tubes containing 10 ml of Brain Heart Infusion Broth (BHI) broth (Difco), and incubated at 37 ^{o}C for 24 h. After incubation, the five strains were transferred to a 50-ml sterilized centrifugal tube, creating a bacterial cocktail. The tubes were then centrifuged at room temperature for 15 min at 4000 rpm (Fisher Scientific, Fisher AccuSpin^{TM} Model 400 Benchtop Centrifuge, Pittsburgh, PA, USA) to harvest bacterial cells. The pellet was re-suspended into 9 ml of sterile 0.85% saline solution and centrifuged as described earlier. The supernatant was discarded, and the pellet was re-suspended into 9 ml of a sterile 0.1% peptone water solution, to give an approximate concentration of 1×10^{9} CFU ml^{-1}. Ten-fold serial dilutions using 0.1% peptone water were carried out to obtain the required concentration of inoculum to be used for inoculation of the RIF milk.

2.2 Inoculation of PIF milk with E. sakazakii

Milk-based infant formula was bought from a local grocery store. *E. sakazakii* cells were desiccation-stressed by inoculating 24-h old cultures of the cocktail of the five strains of *E. sakazakii* (*Cronobacter* spp) listed earlier (section 2.1) onto the surface of the PIF. The PIF was

placed into 1000 ml sterile beaker. The inoculum (100 µl) was sprinkled onto the formula in a drop-wise manner. After inoculation, the IFM with the inoculum was mixed vigorously with a sterile spatula for 3 min. The inoculated formula was kept in a sterile beaker for three months at room temperature in a desiccator. The water activity (a_w) of the powdered IFM was measured before and after the inoculation at day zero, and after 3 months of storage at room temperature in a desiccator using an Aqua lab water activity meter (Model 3TE, Aqualab, Pullman, WA). The initial level of *E. sakazakii* was determined by the overlay method (spread plating on TSA (2 h incubation at 37 ^{o}C) followed by applying another layer of VRBL and incubation at 37 ^{o}C for additional 22 h) (Al-Holy et al., 2008). The initial level of *E. sakazakii* was ca 1×10^6 CFUg^{-1}. The desiccated cells in PIF were further diluted with PIF to obtain a final concentration of ca 1000 CFUg^{-1}.

2.3 Inoculation of reconstituted infant formula (RIF) with E. sakazakii

Commercial infant milk powder was reconstituted with sterile distilled water according to the manufacturer's instructions. One scoop (approximately 7.7 grams) was mixed with 60 ml of water under aseptic conditions and mixed with a sterile spatula. Ten milliliters of the RIF were placed into a sterile test tube, and inoculated with 100 µl of the inoculum solution to give approximately 1×10^6 CFU ml^{-1} of RIF

2.4 Effect of copper sulfate and lactic acid on the growth of E. sakazakii in RIF

The effect of copper sulfate penta-hydrate (Fisher Scientific, Pittsburgh, PA) and LA on the growth of *E. sakazakii* inoculated into RIF was studied. Concentrations of 10, 50, and 100 ppm of copper sulfate were used. The effect of 0.1%, 0.2% and 0.3% v/v of LA (Acros Organics, NJ,

1 USA) on the growth of *E. sakazakii* in RIF was also investigated. In a separate experiment, the
2 effect of LA (0.2% v/v) or copper sulfate (50 ppm) alone or in combination on the growth of *E.*
3 *sakazakii* in 10-ml samples of RIF was studied. RIF inoculated with *E. sakazakii* but without
4 treatment, was used as a control. All treatments were carried out in triplicate.
5
6 *2.5 Effect of lactic acid and copper on the growth of E. sakazakii in PIF*
7 An aliquot of 800 µl of the 10% v/v solution of LA were added to 15.5 g of PIF milk, the
8 powder was reconstituted according to manufacturer's instruction and mixed with a sterile
9 spatula to make a final concentration of 0.2% v/v of LA. For the copper treatment, copper sulfate
10 powder was added to inoculated PIF and rehydrated according to manufacturer's instruction to
11 make a final concentration of copper equivalent to 50 ppm. The combined effect of 0.2% v/v of
12 lactic acid and 50 ppm of copper on the growth of *E. sakazakii* in PIF was also studied. *E.*
13 *sakazakii*- inoculated but untreated PIF was used as a control. All treatments were carried out in
14 triplicate.
15
16 *2.6. Effect of monolaurin on the growth of E. sakazakii in RIF*
17 The effect of monolaurin (Tokyo Chemical Industry, Tokyo, Japan) at different concentrations
18 (1000, 2000, and 3000 µg ml^{-1}) on the growth of *E. sakazakii* was investigated. Stock solutions
19 of monolaurin were prepared either as a suspension in tween 80 (TW) (Fisher Scientific) or as a
20 solution in 96% ethanol (Fisher Scientific), thereafter, 100 µl of monolaurin solutions were
21 added to RIF and the number of *E. sakazakii* was determined at 0, 2, 4 and 6 h. Untreated RIF
22 (control) or samples with 100 µl of TW or ethanol without monolaurin were used for comparison
23 purposes.

2.7 Enumeration of E. sakazakii survivors

Ten-fold serial dilutions in 0.1% peptone water were performed at intervals of 0, 2, 4 and 6 h for the RIF and the PIF. The enumeration of *E. sakazakii* was performed using a minor modification of the overlay method reported by Al-Holy et al. (2008). All samples were spread plated on TSA, instead of TSA supplemented with 0.1% (w/v) of sodium pyruvate. The plates were incubated for 2 hours at 37 ^{o}C, and then a thin layer of violet red bile agar (VRBA) (Difco) was overlaid onto TSA. The plates were incubated for an additional 22 hours at 37 ^{o}C. This method was specifically used to enumerate intact and injured *E. sakazakii* cells.

2.8 Statistical Analysis

At least three independent replicate trials were conducted and standard deviations were determined. *E. sakazakii* counts were log transformed and data were analyzed with a computer software package (SAS Institute, Cary, NC) using analysis of variance and Fisher's least significant difference (LSD) test for mean separations ($P \leq 0.05$).

3. Results and Discussion

The effect of different concentrations of LA on the growth of *E. sakazakii* in RIF is shown in Fig. 1. Lactic acid (0.1% v/v) apparently does not have an inhibitory effect on the growth of the microorganism, especially during the first four hours of incubation at room temperature. Nonetheless, the number of *E. sakazakii* (6.70 logs) was significantly ($P \leq 0.05$) lower compared to the control (7.56 logs) at the end of the storage period. LA at 0.2% v/v imparted a bacteriostatic effect on the growth of *E. sakazakii*. Only a subtle increase (0.14 logs) in the number of *E. sakazakii* was noticed after 6 h of storage at room temperature, which was

significantly ($P \leq 0.05$) lower compared to the control and 0.1% v/v LA. In contrast, LA at 0.3% v/v elicited the most pronounced bactericidal effect against *E. sakazakii* compared to the other concentrations (0.1 and 0.2% v/v). A noticeable gradual reduction in the numbers of the organism was noticed during the whole storage period. In this treatment, the numbers of *E. sakazakii* were significantly ($P \leq 0.05$) lower compared to the other LA concentrations (0.1 and 0.2% v/v). LA is a weak-organic acid, which presents antimicrobial activity and has been used as an antimicrobial agent in foods. LA in the un-dissociated form can penetrate the cytoplasmic membrane, which results in reduced intracellular pH and disruption of the transmembrane proton motive force, which accounts for a significant part of its antibacterial action. (Alakomi et al., 2000; Brul and Coote, 1999). Besides its antimicrobial activity, LA is a strong outer membrane disintegrating agent. LA permeabilizes the outer membrane of Gram negative bacteria, a property that could help other antimicrobials penetrate bacterial cells, and produce a toxic effect. The permeabilizing properties of LA make it both an antimicrobial, and a possible potentiator for other antimicrobial substances (Alakomi et al., 2000).

The antibacterial activity of copper sulfate against *E. sakazakii* in RIF is shown in Fig. 2. Three different levels (10, 50, and 100 ppm) of copper sulfate were used. A dose-dependent response was shown. As the concentration of copper sulfate increased, the extent of inhibition accordingly increased. At a concentration of 10 ppm, a slight but a significant decrease in the number of *E. sakazakii* cells was observed after 2 h, but a re-growth occurred and the number of *E. sakazakii* reached about 6.4 logs after 6 h. At 50 ppm of copper sulfate, a slight but steady decrease in the number of *E. sakazakii* cells was observed during the 6-h storage period. Copper sulfate at 100 ppm exerted the most remarkable antimicrobial activity against *E. sakazakii*, where

about 1.8 logs reduction at the end of the storage period was noticed. The toxic effect of copper on bacteria is possibly attributed to displacement of essential ions, hence inactivating enzymes and obstructing functional groups of proteins, producing hydroperoxide free radicals and affecting membrane integrity (Nies, 1999).

The antimicrobial effect of the combination of LA and copper sulfate against *E. sakazakii* was investigated. Fig. 3 shows the effect of either 0.2% LA or copper sulfate (50 ppm) alone or in combination against *E. sakazakii* in RIF. Interaction between antimicrobials can either be synergistic, additive, or antagonistic in nature. As shown in Fig. 3, a synergistic effect of LA and copper sulfate against *E. sakazakii* was observed. More than 5-log reduction of the organism was obtained after 2 h, and a total elimination was rendered at the end of the storage period. In comparison, either of LA or copper sulfate treatment alone only resulted in < 1 log reduction of *E. sakazakii* and the number of *E. sakazakii* in the untreated samples (control) increased to about 7.6 logs after 6 h. The practical validity of the combined treatment (LA and copper sulfate) against *E. sakazakii* was further explored in PIF. Samples of PIF milk were seeded with *E. sakazakii* and kept under dry conditions at room temperature to simulate the dry stress conditions that *E. sakazakii* may encounter under normal storage conditions. The water activity of the inoculated PIF milk after 3 months of storage was 0.21. As shown, in Fig. 4, after applying the combined treatment (0.2% LA and 50 ppm copper sulfate), a synergistic effect was obtained, where a complete elimination of the organism was obtained after 2 h and till the end of the storage period. In contrast, the cells increased slightly but significantly ($P \leq 0.05$) in the LA treatment alone (~0.5 log increase) and by about 0.15 logs in the copper sulfate treatment. The numbers in the untreated samples (control) increased by about 1 log after 6 h of storage at room temperature.

Metallic copper surfaces inhibited the growth of *Salmonella enterica* and *Campylobacter jejuni*, two of the most common foodborne pathogen, suggesting its possible use as a bacterial inhibitor in food processing (Faundez et al., 2004). But the ions have to enter the cell in order to have a physiological or toxic effect (Faundez et al., 2004). It is possible that at concentrations as low as 10 and 50 ppm, no enough ions are penetrating the cell membrane to produce a toxic effect. The permeabilizer properties of LA could have facilitated the entry of copper ions into the *E. sakazakii* cells and produce the toxic effect, as well as producing a toxic effect itself. The combination of inhibitory activity and disruption of the outer membrane of *E. sakazakii* by LA may have lead to potentiation of the antimicrobial activity of copper sulfate and consequently resulted in the inhibition of *E. sakazakii* growth. Furthermore, the toxic effect of copper on microorganisms can occur due to displacement of essential metals from their native binding sites or by ligand interactions. Additionally, copper may also change the conformational structure of nucleic acids and proteins, and interfere with oxidative phosphorylation and osmotic balance (Borkow and Gabbay, 2005). The combination of copper sulfate and LA was effective in inhibiting the growth of *Salmonella* spp. and *Escherichia coli* O157:H7. LA and copper sulfate were added alone and in combination to laboratory medium and carrot juice inoculated with the pathogens. A significant inhibition of the pathogens was only obtained when a combination of both was used (Ibrahim et al., 2008).

The impact of different concentrations of monolaurin (1000, 2000 and 3000 μg ml^{-1}) as a suspension in tween-80 (TW) (Fig. 5A) or dissolved in absolute ethanol (Fig. 5B) on the growth of *E. sakazakii* in RIF stored at room temperature was investigated. Surprisingly, TW containing no monolaurin posed a slight (0.3 log) but significant ($P \leq 0.05$) reduction in the count of *E.*

sakazakii compared to the control after 2 h of storage at room temperature. The effect of monolaurin in TW at all of the tested levels was comparable to TW alone, where a slight inhibition of *E. sakazakii* growth took place during the first two hours of storage followed by an increase in *E. sakazakii* count till the end of storage period (6 h). Nonetheless, there was a 1-log difference between the control form one hand and the TW with or without monolaurin on the other hand throughout the 6-h storage period, revealing the inhibitory activity of TW on *E. sakazakii* growth. Fig. 5B presents the effect of manolaurin dissolved in ethanol on the growth of *E. sakazakii* in RIF. Monolaurin at different levels exhibited an inhibitory activity against *E. sakazakii* compared to the control. At the end of the storage period, there was about 1.5 log difference between monolaurin treatments and the control. Although ethanol posed an inhibitory activity against *E. sakazakii* in RIF, the extent of inhibition was more pronounced in the monolaurin treatments. Increasing monolaurin concentration from 1000 to 3000 μg ml^{-1} did not result in further inhibition. Additionally, it is worthwhile to mention that monolourin in the form of TW suspension exhibited less inhibitory activity against *E. sakazakii* (Fig. 5 A) compared to monolaurin presented soluble in ethanol (Fig. 5 B).

Monolaurin is a monoglyceride of lauric acid that has been shown to have antimicrobial properties. Monolaurin was shown to pose some inhibitory activity against *E. coli* O157:H7 in UHT milk (Branen and Davidson, 2004). In other study, monolaurin and lauric acid at the level of 500 μg g^{-1} exhibited a remarkable antilisterial activity in hotdog emulsion stored at 5 ^{o}C (Mbandi et al., 2004). Additionally, a combination of monolaurin and lactoperoxidase system resulted in a synergistic inhibitory activity against *E. coli* O157:H7 and *Staphylococcus aureus* that was not achievable when using either agent alone (McLay et al., 2002). The mechanism of inhibition of bacteria by monolaurin is not known, but the cell membrane is thought to be the

target. Monoalurin may disrupt membrane integrity and consequently interferes with membrane activities such as transport of amino acids, resulting in cell starvation (Kabara, 1993)

LA and copper sulfate showed a noticeable inhibitory activity against the growth of *E. sakazakii* in RIF. Using a combination of sub-lethal concentrations of lactic acid (0.2%) and copper sulfate (50 ppm) resulted in a synergistic inhibitory activity against *E. sakazakii* in RIF stored at room temperature; where a complete elimination of inoculated strains of *E. sakazakii* was obtained. Additionally, monolaurin showed a slight but important inhibitory activity against *E. sakazakii*. Using monolaurin in the form of ethanol solution was more effective compared to a suspension of monolaurin in TW. The use of the synergistic interactive combination of lactic acid and copper sulfate could be beneficial to control *Cronobacter* spp (*E. sakazakii*) in the infant formula industry.

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Fig. 1. Effect of lactic acid (LA) concentration on the growth of *Enterobacter sakazakii* (Log CFU ml^{-1}) in reconstituted infant formula stored at room temperature (21 ^{o}C).

Fig. 2. Effect of copper sulfate (Cu) concentration on the growth of *Enterobacter sakazakii* (Log CFU ml^{-1}) in reconstituted infant formula stored at room temperature (21 ^{o}C).

Fig. 3. The combined effect of lactic acid (0.2% LA) and copper sulfate (50 ppm Cu) on the growth of *Enterobacter sakazakii* (Log CFU ml^{-1}) in reconstituted infant formula stored at room temperature (21 ^{o}C).

Fig. 4. The combined effect of lactic acid (0.2% LA) and copper sulfate (100 ppm Cu) on the growth of *Enterobacter sakazakii* (Log CFU g^{-1}) in powdered infant formula (PIF) stored at room temperature (21 ^{o}C). The PIF was reconstituted and kept at room temperature for a 6-h period to determine *Enterobacter sakazakii* count.

Fig. 5. Effect of monolaurin (μg ml^{-1}) suspended in tween 80 (TW) (**A**) or dissolved in absolute ethanol (Et-OH) (**B**) on the growth of *Enterobacter sakazakii* (Log CFU ml^{-1}) in reconstituted infant formula stored at room temperature (21 ^{o}C).

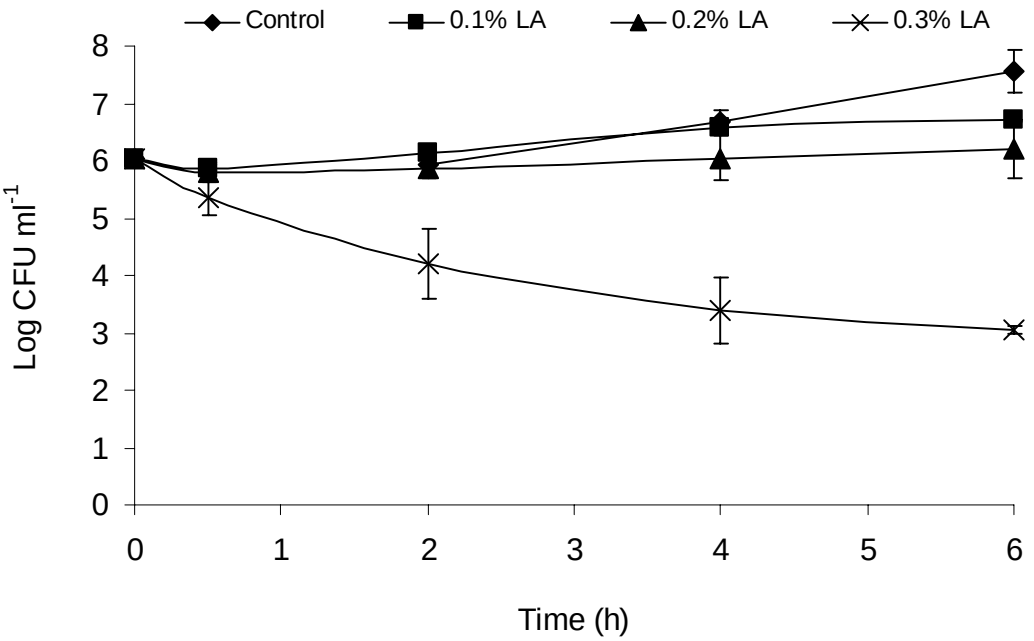
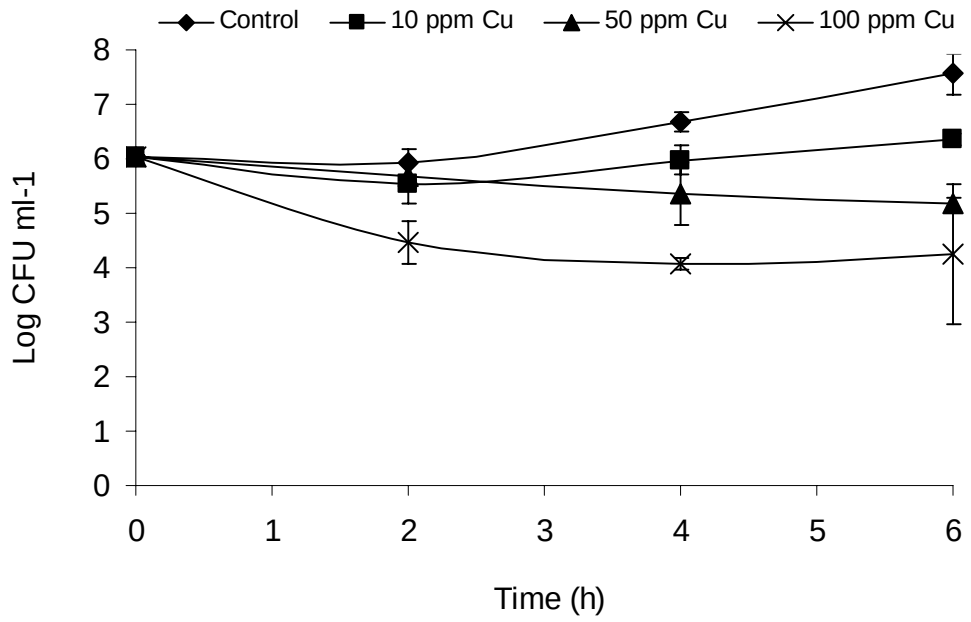


Fig. 1.


Control
10 ppm Cu
50 ppm Cu
100 ppm Cu
Log CFU ml-1
Time (h)
0
1
2
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8

Fig. 2.

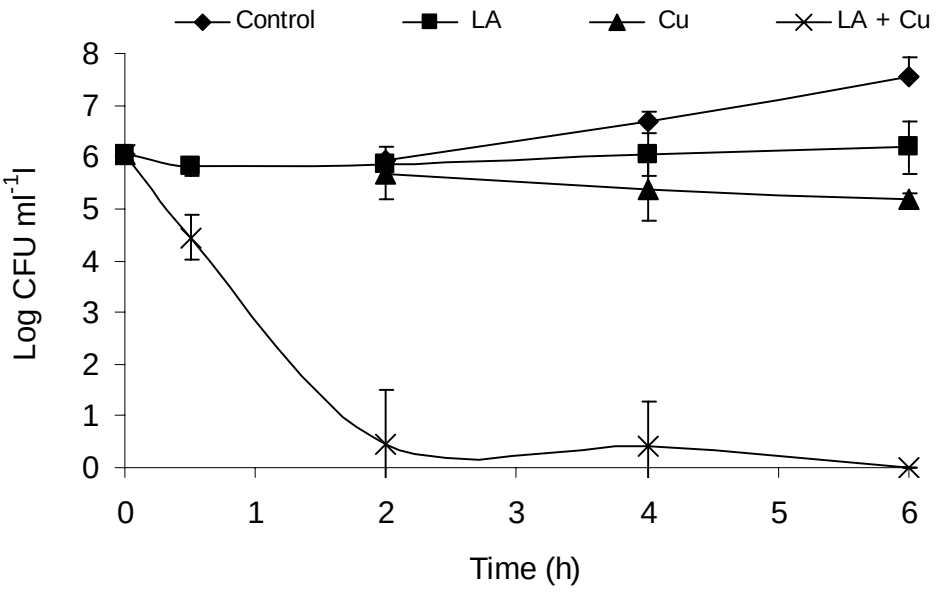

Fig. 3.

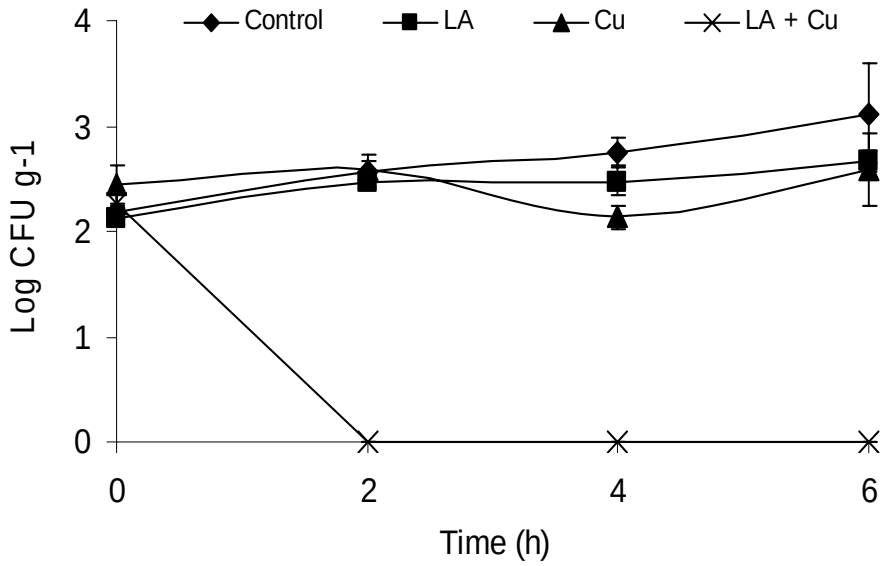

Fig. 4.

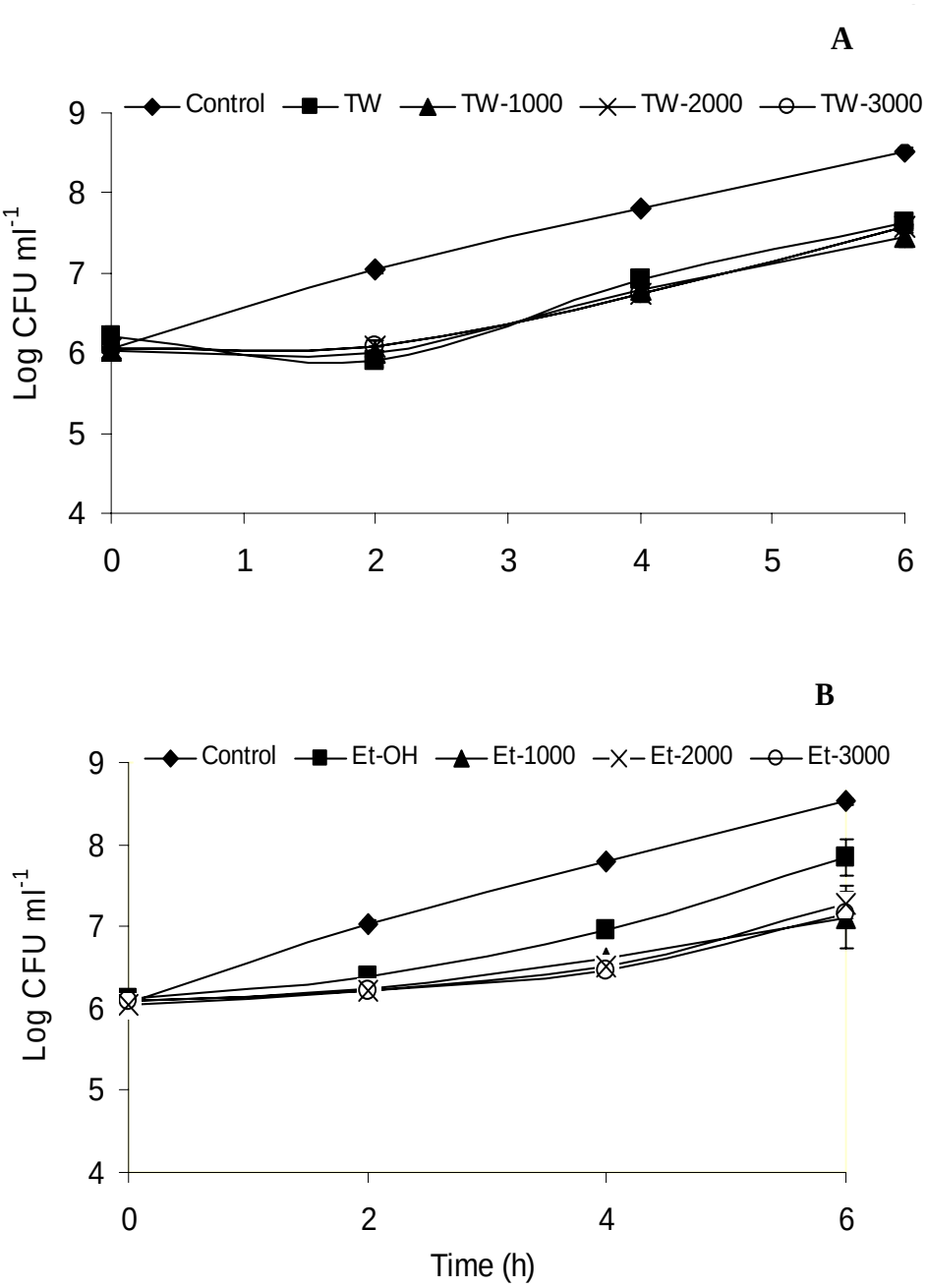
A
Control
TW
TW-1000
TW-2000
TW-3000
Log CFU ml-1
9
8
7
6
5
4
0
1
2
3
4
5
6
B
Control
Et-OH
Et-1000
Et-2000
Et-3000
Log CFU ml-1
9
8
7
6
5
4
0
2
4
6
Time (h)

Fig. 5.