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Title: Effects of Propionibacterial Metabolites On Spoilage and Pathogenic Bacteria in Dairy Products

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William E. Sandine

Microgard™ (MG) is the patented trade-name for pasteurized fermented milk containing an antimicrobial substance produced by *Propionibacterium freudenreichii* ssp. *shermanii* . The active ingredient in MG is inhibitory for Gram-negative bacteria such as *Pseudomonas putida, Pseudomonas aeruginosa* and *Escherichia coli* . The inhibitory spectrum has been extended to Gram-positive food-borne pathogens (*Listeria monocytogenes*) and food contaminating yeasts such as *Saccharomyces cerevisiae*. This was accomplished by the addition of sodium citrate or EDTA chelating agents.

MG is stable to acid and base hydrolysis. It was found to be thermal stable when hydrolysed for 60 minutes at 121 °C at pH 2.00. Also, there was a 40% enhancement in the antimicrobial activity of MG when hydrolyzed at a pH 1.00 at 121 °C for 15 minutes .

Gas-chromatographic analysis of MG showed that acetic and propionic acids were the predominant acids. Concentrations of these acids normally found in MG (4425.8 µg/g and 1171.7 µg/g, respectively) were not inhibitory

for test organisms. Furthermore, removal of acetic and propionic acids from crude non-dairy MG did not eliminate its inhibitory ability.

Active fractions from ultra-filtration, anion exchange chromatography, gel filtration and high performance liquid chromatography (HPLC) exhibited maximum UV absorbance between 214 and 275 nm. The proteineous nature of the active ingredient was confirmed by the ninhydrin and Lowry tests.

The active agent was identified as a bacteriocin. A purified single-peak fraction from reversed-phase HPLC using a Bio-Sil ODS-5S C-18 column which was acid-free, proteineous (0.046 mg/ml), showed an increased specifc acitivity of 434.78 AU/mg. The molecular weight of the bacteriocin was estimated to be 860 daltons by gel filtration. Data from mass spectroscopy showed two peaks in a series, 295 and 441.7, suggesting charges of 3 (MH^{+3}) and 2 (MH^{+2}), respectively. The molecular weight determined by mass spectroscopy was 881.7 daltons . Amino-acid analysis by Edman degradation method indicated that the peptide may consist of 4 residues of methionine, 2 of valine, 1 of glutamic acid, and possibly 1 of glycine. The molecular weight from this composition was estimated to be 926 daltons.

Effects of Propionibacterial Metabolites On Spoilage and Pathogenic Bacteria in Dairy Products

by

Alex Yeow-Lim, Teo

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Typed by researcher for Alex Yeow-Lim, Teo

Dedicated with love ...

To my beloved wife Susan Lim, my parents Teo Kheng Kim and Pik Ah Hwa, my siblings Sock Hong, Yeow Koon, Seok Yan and Yeow Sim, and my mother-in-law Tan Ah Moy, people from the Republic of Singapore and finally, last but not least, my Lord Jesus Christ.

The fear of the Lord is the
beginning of wisdom;
all who follow his precepts have
good understanding.
To him belongs eternal praise.

Psalm 111:10

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GENERAL INTRODUCTION

Propionibacteria have a long history of use in the fermentation and dairy industries. In the dairy industry, propionibacteria play important roles in the development of the characteristic flavor, aroma, texture and eye formation in Swiss cheese (Langsrud and Reinbold 1973a,b; Biede and Hammond 1979; Rehberger and Glatz 1990). They are also well known for their ability to synthesize vitamin B_{12} (Perlman 1978).

Propionibacteria are Gram-positive rods, nonspore-forming, pleomor-phic, non-motile, mostly slow-growing aerobic to aerotolerant mesophiles that form small yellow, orange, cream, and dark red colonies (Glatz 1992). The optimum pH range for growth is between 6 and 7. However, there is no growth when the pH is less than 4.5 (Hettinga and Reinbold 1972a; Hsu and Yang 1991). Their ability to produce inhibitory metabolites has been studied from as early as 1972 (Hettinga and Reinbold 1972a, b,c).

Propionibacteria have become increasingly important to the fermentation industries (Caulsen and Gaddy 1981; Datta 1981; Calvin et al., 1985; Hendricks et al., 1985; Blanc and Goma 1987a; Bodies et al., 1987; Border et al., 1987; Boyaval and Corre 1987; Crespo et al., 1990; Emde and Schink 1990; Hsu and Yang 1991; Lewis 1991; Lewis and Yang 1992). Fermentation of glucose or lactate as carbon sources produces propionate, acetate and carbon dioxide. Typically, 3 moles of lactate result in the production of a 2:1:1 molar ratio of propionic acid; acetic acid; and carbon dioxide, respectively (Ray and Daeschel 1992). Propionic and acetic acids are the two major products derived from the

fermentation of lactose. The yield of propionic acid changes from approximately 33% (w/w) at pH 6.1-7.1 to about 63% at pH 4.5-5.0 . However, acetic acid remains within the range of 9%-12% at all pH values. The succinic acid yield decreases from 17% at pH 7.0 to 8% at pH 5.0 . In contrast, pyruvic acid produced during fermentation of lactose is reconsumed (Hsu and Yang 1991). The production of acetic acid is closely associated with cell growth, whereas propionic acid can be both growth and non-growth associated (Hsu and Yang 1991).

In the production of Swiss cheese, the inhibitory effects of the undissociated forms of propionic and acetic acids are potentiated against Gram-negative bacteria (Baird-Parker 1980) by the low cheese pH. However, the extent of the fermentation also is restricted by the acidic pH (Neronova et al., 1967; Ibragimova et al., 1969; Hettinga and Reinbold 1972; Herrero 1983; Blanc and Goma 1987). In addition, propionic acid is also known as an important mold inhibitor. As producers of propionic and acetic acids, the propionibacteria could serve as alternative sources of these organic acids when chemical synthesis becomes costly and environmentally prohibitive.

The concentration of diacetyl produced by strains of *Propionibacterium freudenreichii* ssp. *shermanii* in milk (10 µg/ml) was found to be non-inhibitory to yeast and non-lactic acid bacteria (Lee et al., 1970; Jay 1982; Perez-Mendoza et al.,1983; Ro et al., 1979; and Marwaha et al., 1983). Both propionic acid and diacetyl have attained GRAS (generally recognized as safe) status as food additives in the United States (Basler et al., 1987; Daeschel 1989). Other volatile compounds such as sesquitrepenes have also been reported to be produced by propionibacteria (Dumont et al. 1978; Crow and Turner. 1986; Crow 1987).

Bovine lactoferrin, an antimicrobial component of colostrum and milk, helps in the protection of infants (Bullen et al., 1972; Brock 1980; Bullen 1981) from gastrointestinal infections. Porcine pepsin cleavage of native lactoferrin produces low molecular weight peptides inhibitory for some Gram-negative and Gram-positive bacteria (Tomita et al., 1991). Also, hydrolysis of native lactoferrin at pH 2 and 120 °C produces active peptides which are bactericidal (Saito et al., 1991).

Microbial peptides with pronounced antimicrobial activity are commonly isolated from animals, plants, microbes (Sahl, 1985) and in non-sterile food (Muriana, 1993). They are small and cationic with molecular masses between 3000 to 6000 (Roller 1991). Post-translational modification of precursor peptides has been shown to introduce intramolecular thioether bridges to cationic peptides such as Pep 5, nisin, and subtilin (Gross and Morell 1971; Kordel and Stahl 1986; Kordel et al., 1989;). Although these peptides offer an important potential safety advantage over chemically synthesized preservatives when incorporated into food, many peptides are not suitable owing to the pathogenic nature of the producer-strains. Peptides such as colicins (Konisky 1982) , epidermin and Pep 5 (Ersfeld-Dressen, H. et al., 1984; Horner et al., 1989) may be useful in topical application in creams and salves, but are unlikely to be approved for use in foods because of the nature of the producer-strains (Roller 1991).

Propionibacteria are also important in the production of antimicrobial substances including bacteriocins. Propionin A, an antiviral peptide isolated from cellular extracts of *P. freudenreichii* was found to be inhibitory against vaccinia viruses (Ramanathan et al., 1966). Propionins B and C have demonstrated both *in vivo* as and *in vitro* activity against Columbia SK virus.

The sizes of both peptides are between 1000 and 2000 Da. (Ramanathan et al., 1968).

Crude lactic cultures are known to inhibit some psychrotrophs in milk and ground beef (Gilliand and Speck, 1974). A large number of lactic acid bacteria, singly or in combination, display varying degrees of antimicrobial activity. Methanol-acetone extracts of lyophilized fermented milk contain ninhydrin-postive materials. These proteineous materials were reported not to lose antimicrobial activity when exposed to 100 °C for 10 minutes (Pulusani et al., 1979). The methanol-acetone extract was active at pH 5.4 and not inactivated by pepsin treatment (Pulusani et al., 1979). In another study, purified cationic, low molecular weight material isolated from *Streptococcus diacetylactis* was found heat stable and active towards several *Pseudomonas* species at pH 6.0 and lower (Branen et al., 1975). Acidophilin, a low molecular weight (approximately 700 daltons) nitrogenous compound from *Streptococcus diacetylactis* and *Leuconostoc citrovorum* cultures was reported by Branen et al., (1975) and Shahani et al., (1976, 1977) to have potent antimicrobial activity. Acidophilin was also reported to be an antibiotic produced by *Lactobacillus acidophilus* (Shahani et al., 1977). Antimicrobial compound(s) from *Streptococcus thermophilus* are likely to be amines of low molecular weights which are heat stable (Pulusani et al., 1979). An antimicrobial substance from *Lactobacillus* sp. strain GG was found inhibitory to Gram-postive as well as Gram-negative bacteria but not lactobacilli (Silva et al., 1987). It was active between pH 3 and 5 but not pH 5.3 . The substance was also found to be heat stable and resistant to proteinase K, a-chymotrypsin, trypsin, and carboxypeptidase A (Silva et al., 1987) . It could not be considered a bacteriocin but it does resemble a microcin from some of the characteristics studied (Asensio et al., 1976; Baquero and Asensio 1979; Baquero and Moreno

1984). The antimicrobial substance from *Lb.* sp. strain GG has a molecular weight of less than 1000 daltons and is soluble in acetone-water (Silva et al., 1987).

Microcins are low molecular weight amino-acid-oligopeptide antibiotics which are resistant to some proteases, extreme pH values and heat, and soluble in methanol (Baquero and Moreno 1984). Bacteriocins are larger proteins which can be inactivated by proteases. Microcin A15 (< 500 daltons) is a bacteriostatic compound with a methionine moiety. Microcin B17, with a molecular weight of 4000 daltons, is sensitive to pronase, subtilisin, and thermolisin. Microcin C7, about 900 daltons, is senstive to trypsin and subtilisin but loses little activity when exposure to 100 °C for 30 minutes. Microcin C7 is a linear structure consisting of acetyl-methionine, arginine, threonine, glycine, asparagine, alanine and a supposed ethanolamine entity at the carboxyl-terminal end (Garcia-Bustos et al., 1984). Microcins D15, D93, and D140 are highly hydrophilic, basic and small peptides (Baquero and Moreno 1984).

Bacteriocins are proteineous compounds usually with bactericidal activity against species closely related to the producer bacterium (Tagg et al., 1976; Barefoot and Klaenhammer 1983). Diplococcin (Whitehead 1933; Davey and Richardson 1981) and nisin (Hurst 1966,1981; Hurst and Dring 1968; Delves-Broughton 1990) are well-known bacteriocins produced by *Lactococcus cremoris* and *Lc. lactis*, respectively. Nisin contains a total of 34 amino-acids and unusual lanthionines (Gross and Morell, 1971). Since 1969, nisin has been approved as a preservative in processed cheese, cheese spread, pasteurized milk (Delves-Broughton 1990) and dairy desserts (Roller 1991). Ethylenediaminetetraacetic acid (EDTA), Triton X-100 and Tween 20 have been used to potentiate nisin activity against Gram-negative organisms including

Salmonella typhimurium and *Escherichia coli* (Stevens et al., 1991; Stevens et al., 1992a, 1992b).

Other bacteriocins include pediocin PA-1 from *Pediococcus acidilactici* (Gonzales and Kunka 1987; Lozano et al., 1992), found effective in preventing spoilage of salad dressing (Gonzales 1989). Pediocin A from *Pediococcus pentosaceus* has a broader-range of activity against food spoilage pathogens (Fleming et al., 1975; Daeschel and Klaenhammer 1985; Daeschel 1989).

Although numerous bacteriocins from Gram-positive bacteria have been isolated, characterized, and purified (Hale and Hinsdill 1973; Barefoot and Klaenhammer 1984; Torreblanca et al., 1989; Kato et al., 1991; Mortvedt et al., 1991; Muriana and Klaenhammer 1991; Fitzgerald et al., 1992; Guomundsdottir et al., 1992; Hechard et al., 1992; Henderson et al., 1992; Hill and Parente 1992; Holck et al., 1992; Jiménez-Díaz et al., 1993; Mathieu et al., 1993; Piard et al., 1992; Stoffels et al., 1992; Tahara et al., 1992) , very few from the propionibacteria are documented. *Propionibacterium acnes* from the cutaneous group, produces a bacteriocin bacteriostatic against both Gram-positive and Gram-negative anaerobes (Fukushima and Nakamura 1978; Paul and Booth 1988). The classical dairy propionibacteria also have been shown to produce bacteriocins (Lyon and Glatz 1991; Glatz 1992; Lyon et al., 1993; Grinstead and Barefoot 1992).

Propionibacteria grown in milk or cheese whey, including the medium, cells, acids and any other antimicrobial substances produced, may be dried and used as a natural preservative. For example, Microgard™ (Ayres et al., 1987), a grade A skim milk product (Wesman Foods, Inc., Beaverton, Oregon) fermented by *P. freudenreichii* ssp. *shermanii* and pasteurized is used as a biopreservative in cottage cheese (Weber and Broich 1986). Approved by the Food and Drug Administration (Sandine et al., 1992), it has been estimated that

30% of the cottage cheese produced in the United States has Microgard™ (MG) incorporated (Daeschel 1989).

Initial studies of MG showed that it prolonged the shelf life of cottage cheese by inhibiting psychrotrophic spoilage bacteria (Weber and Broich 1986; Salih and Sandine 1990). Subsequent studies (Al-Zoreky et al., 1988) have shown that MG is antagonistic toward most Gram-negative bacteria except *Escherichia coli* V517. Certain yeasts and molds, but not Gram-positive bacteria, are inhibited by MG (Al-Zoreky et al., 1988). Non-dairy MG is also commercially available for use with non-dairy products. The antimicrobial substance in MG is proteineous since it can be inactivated by a-chymotrypsin, papain, and pepsin (Al-zoreky 1988). MG is not inactivated by trypsin, suggesting the presence of trypsin inhibitors. Such activity was reported in some food systems (Konishi et al., 1985). Unlike the antimicrobial substances from lactic acid bacteria (Anderson 1986; Gonzalez et al., 1987), the active ingredient in MG is heat stable.

Propionic, acetic and lactic acids present in MG could be responsible for the inhibtory action against Gram-negative psychotrophs. The ratio of propionic acid and acetic acid in MG is about 1:3.5 (Al-Zoreky 1988). The concentration of diacetyl in MG is insufficient to be inhibitory to bacteria. The metabolism of lactate by *P. freudenreichii* ssp. *shermanii* ATCC 9616 did not produce succinic acid, contrary to the findings of Crow (1987). Furthermore, the addition of catalase did not cause bubble formation indicating that hydrogen peroxide was not the inhibitory substance in MG (Al-Zoreky 1988). Vorob"eva et al. (1987) and Hitchner (1934) reported that *P. shermanii* has the ability to produce catalase and peroxidase.

Bovine albumin was found to be antagonistic toward MG, confirming reports that albumin and other substances can antagonize those antimicrobial

compounds produced by bacteria (Schales, 1951; Shibasaki et al., 1978; Kabara 1978). EDTA did not interfere with the antimicrobial ability of MG (Al-Zoreky 1988). However, EDTA in itself could be inhibitory to bacterial growth (Bulgarelli et al., 1985). The active ingredient in MG was determined by gel filtration to be 700 daltons in molecular weight. However, purified fractions were found to have lost activity when stored under refrigeration temperature for a period of 2 months (Al-Zoreky 1988). This is in contrast to a non-proteineous antimicrobial substance, isolated from *Lactobacillus bulgaricus*, which was stable at 6°C for only 18 days (Abdel-Bar et al., 1987).

The research reported in this thesis involved further characterization of the active components in MG. It was desirable that the organic acids be removed during the purification process of MG. In Chapter 1, organic acids in MG were quantified by gas-liquid chromatography (Mitruka 1975; Drucker 1981; Thornhill and Cogan 1984), and the known concentrations of these acids were used to simulate inhibitory activity against *Ps. putida* (Figure 1.7). In addition, the active ingredient in crude dairy MG was quantified and examined for its stability when hydrolyzed under acidic or basic conditions. In this study, we are interested to know if the antimicrobial substance(s) in MG resembles the active precursors of lactoferrin upon heat and pH treatments (Saito et al., 1991).

In Chapter 2, the purification process of bacteriocin involved ultra-filtration, anion exchange chromatography, gel-filtration and reverse-phase high performance liquid chromatography. The low molecular weight peptide was then analysed and sequenced for its amino-acid contents. Mass spectroscopy was used to characterize its charge-groups and molecular weight.

The effect of chelating agents on nisin have been studied by Stevens et al. (1992a, 1992b). In Chapter 3, sodium citrate and EDTA were used to potentiate the antimicrobial effect of MG against Gram-positive and Gram-

negative bacteria and yeasts. In this study, the effect of chelating agents and MG were tested in broth and milk system.

CHAPTER 1

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS AND EXTRACTION OF VOLATILE COMPOUNDS FROM MICROGARD™

ABSTRACT

Various methods were investigated to remove volatile acids from Microgard™ (MG) . It was found that the predominant organic acids were acetic and propionic acids. There were also traces of lactic acid and diacetyl. Lyophilization was employed to remove all volatile acids through low temperature (liquid nitrogen) evaporation. Gas-liquid chromatographic analysis of the lyophilized samples showed a reduction in the amount of acetic and propionic acids. Although the residual acetic and propionic acids were 76.3% and 74.5% of that originally present, respectively, the use of ethyl-acetate was found to be efficient in removal of the remaining acids. Gas-liquid chromatography detected approximately 37.3% acetic acid and 20.3% propionic acid remaining in the aqueous phase. Anion exchange chromatography was found effective in removal of essentially all acetic and propionic acids from crude MG. Gas-liquid chromatography quantitated 6.3% acetic acid and 2.98% propionic acid from the anion exchanged eluates.

Disk-activity assays showed that lyophilized (pH 6.34), ethyl-acetate extracted bottom fraction and eluates from the anion exchanger remained inhibitory toward the indicator-organism. This provides evidence that the antimicrobial activity of MG could not be solely due to acetic or propionic acids.

INTRODUCTION

Previous studies by Al-Zoreky (1988, 1991) showed that the antimicrobial activity of MG is due to acid presence as well as a proteineous substance. It is possible that the latter material could be a low molecular weight bacteriocin and to ensure that MG activity was due to both acids and bacteriocin the study in this chapter was undertaken.

It is well known that organic acids such as acetic, propionic, lactic acids and diacetyl are bacteriocidal at low pH values (Hettinga and Reinbold 1972 a, b, c; Hsu and Yang 1991). Therefore, it would not be surprising that a portion of MG activity was due to these metabolic products. However, publications dealing with bacteriocins produced by Propionibacteria are few in number and only recently, reports being made by Lyon and Glatz (1991), Lyon et al. (1993), and Grinstead and Barefoot (1992).

The present study was carried out to further characterize the active components of the commercially available MG. It was desirable that the organic acids be removed during the purification process of MG. Complete removal of organic acids from MG would be necessary to show the presence of a proteineous bacteriocin. In this chapter, organic acids in MG were quantified by gas-liquid chromatography (Mitruka 1975; Drucker 1981; Thornhill and Cogan 1984), and the known concentrations of these acids were used to simulate inhibitory activity against *Pseudomonas putida*.. In addition, the active ingredient(s) in crude dairy MG was quantified and examined for its stability when hydrolyzed under acidic or basic conditions. After hydroysis at various pH values, the antimicrobial substance(s) in MG was examined for any similarity with the active precursors released by lactoferrin (Saito et al., 1991).

MATERIALS AND METHODS

Preparation of Microgard™

Liquid dairy Microgard™ (pH 6.38) was obtained from Wesman Foods, Inc., Beaverton, OR. The crude MG was adjusted to pH 4.00 with anhydrous tartaric acid to precipitate casein. All pH adjustments were made with a Corning 125 pH meter. The sample was then centrifuged at 10,000 x g for 30 minutes. Supernatnant was decanted into another container and back-neutralized to pH 5.30 with 1.0 N NaOH. The supernatant was then filter-sterilized using a 0.45 μm pore diameter filter (Nalge Co., Rochester, NY) and stored at -40 °C.

Preparation of growth media

Rehydrated Plate Count agar (Difco Laboratories, Detroit, MI) was prepared according to manufacturers' instructions and aliquoted (100 ml) into dilution bottles. The pH of medium was adjusted with 10% tartaric acid solution to pH 5.30 before autoclaving . Lactose broth (Difco Laboratories, Detroit, MI.) was prepared and aliquoted (10 ml) in screw-capped tubes for the cultivation of indicator-bacteria. The pH of lactose broth was 7.00. Both media were autoclaved at 121°C for 15 minutes.

Activity detection assay

Disk-activity assay (Branen et al., 1975; Pulusani et al., 1979; Reddy et al., 1983) was the method of choice used in the comparison of relative activity of various MG fractions with the untreated controls. *Ps. putida* ATCC 12633 was

inoculated into 10 ml of lactose broth and incubated at 30°C for 18 hours. The overnight culture was used as the indicator-bacterium. A 1.0% stock solution of 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co., St. Louis, MO) was prepared and filter-sterilized as for the MG supernatant. A total of 0.5 ml of *Pseudomonas* culture was inoculated into molten plate count agar (100 ml) after tempering at 45 °C for 30 minutes. A total of 2.0 ml of 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) stock solution was added to the molten agar. TTC was used as a colony coloring agent as the indicator-bacteria reduced the compound to an insoluble red formazan (Figure 1.6). A total of 18 ml of the bacterial-TTC mixture was aliquoted onto sterile disposable petric plates (100 x 15 mm, VWR Scientific Inc.). Analytical paper disks (0.5 inch Schleicher & Schuell Inc., Keene, NH) were saturated with 100 µl of sample on the surface of solidified PCA. Activity-assays were conducted at pH 5.30, the optimum pH value for MG. All culture-plates were incubated agar-side down at 30 °C for 18 hours.

Lyophilization of crude Microgard™

One of the main concerns in this project was to completely remove all organic acids found in dairy MG. It has been established that the predominant organic compounds found in MG are acetic, propionic, and lactic acids as well as diacetyl (Al-Zoreky 1988). The proposed method was intended to remove the volatiles at a vacuum of 150 millitorrs and a temperature of -40 °C. It was reasoned that lyophilization under such conditions would cause the acids to volatilize and evaporate from crude MG.

A volume of 100 ml of crude dairy MG was titrated with 10% tartaric acid solution or 0.1 N NaOH solution to pH values of 5.00, 5.30, 6.34 or 8.00.

Titrated samples were lyophilized overnight to complete dryness. All dried samples were rehydrated to their original volumes with 0.1 M citrate buffer, pH 5.30 . The pH values were measured with a Corning 125 pH meter. Treated samples were assayed for antimicrobial activity at pH 5.30 as described above.

Quantitation of protein in ultra-filtrated fractions

The objective of these experiments was to isolate the antimicrobial substance from crude MG which was estimated to have a molecular weight of 700 daltons (Al-Zoreky 1988). Pre-treated dairy MG samples were filtered through various molecular weight cut-off membranes at high pressure under nitrogen gas as described below. All retentate and filtrate samples were assayed by the disk-activity method for antimicrobial activity at pH 5.30. Active molecular weight cut-off filtrate or retentate were analyzed for total protein concentration by the Lowry Protein Assay (Sigma Protein Assay Kit P-5656).

Crude dairy MG (100 ml) was titrated with anhydrous tartaric acid to pH 4.00. The sample was then centrifuged at 10,000 x g for 30 minutes. The supernatant was decanted into another container and adjusted to pH 5.30 with 0.1 N NaOH. A total of 250 ml of this sample was placed into an Amicon filtration cell unit #8050 (Amicon, Inc., Beverly, MA) with molecular weight (MW) cut-off membrane of 10,000 daltons (labeled by manufacturer as YM 10 > 10000 MW) at a pressure of 30 psi. A total of 247 ml of 10,000 MW ultra-filtrate (10,000-F) was collected. The retentate (10,000-R) was washed with 0.1 M citrate buffer, pH 5.30 and the remaining 225 ml ultra-filtrate (10,000-F) was placed onto another MW membrane of 5000 daltons (YM 5 > 5000 MW) at a pressure of 40 psi. All retaintates (5000-R & 1000-R) collected were washed with 0.1 M citrate buffer (pH 5.30). A volume of 222 ml of 5000 MW ultra-filtrate

(5000-F) was filtered through a 1000 daltons MW membrane (YM 2 > 1000 MW) at 45 psi. A 500 daltons membrane (YC05 > 500 MW) was used to filter the 1000 MW ultra-filtrate . All collected samples were tested for antimicrobial activity by the disk-assay. Active fractions were also quantified for total protein concentration by the Lowry Assay. The protocols used in this assay were as established by Sigma Protein Assay Kit No. P5656.

Protein separation by Isoelectric-focusing

Isoelectric focusing (Egen et al., 1984) is a sensitive technique for protein separation based on the principle that protein migration in an electrically charged field will cease at its isoelectric point. The isoelectric point of a protein is defined as the point of electrical neutrality (zero-charge). Operationally, the Rotofor Preparative Isoelectric Focusing Cell Model 3000 Xi (Bio-Rad Laboratories, Richmond, CA) requires a column stabilized with an anticonvection agent and a pH gradient within this stabilized column. Hence, there is a tubular system containing an anticonvection system within a linear gradient. This gradient must contain the macromolecules (proteins) to be analyzed along with ampholytes. Ampholytes are small polymers containing randomly distributed weak acids and bases.

This system is connected to dilute acid and alkali electrode pools and a voltage is applied. Applied voltage initiates the current or ion flow. In the absence of added salts or buffers, the ampholytes are the major source of ions in the system. These ampholytes migrate according to their charge distributions; that is, those with lower isoelectric points (more carboxyls) migrate toward the acidic electrode pole, and those with higher isoelectric points (more amines) move to the alkaline electrode pole. Eventually, a pH gradient of

ampholyte molecules that have migrated to their isoelectric points is produced. The proteins of filtrated dairy MG when injected into the system also act like ampholytes in that they migrate as a function of their (+) and (-) groups. As the ampholyte-dependent pH gradient forms, protein molecules migrate to the pH regions in the pH gradient where the attractive forces on protein molecules are equal. This is the pH at which the number of (+) and (-) charges on any protein are equal at its isoelectric point. Once the pH gradient forms and the isoelectric focusing of individual proteins into specific bands have taken place, the applied current must be turned off.

The active filter-sterilized supernatant from MG was isoelectric focused by the Rotofor Preparative Isoelectric Focusing unit (BioRad). The apparatus has cathode and anode termini which consist of membranes saturated with 50 ml 0.1 M phosphoric acid or 0.1 M NaOH, respectively. A mixture consisting of 2.5 ml of 2% ampholytes (pH range 3-10), 2 ml of 1000 M.W. filtrate and 45.5 ml of distilled water was prepared before injecting into the apparatus. The voltage was set at 1000 V and power at 12 W. After 4 hours of separation, 20 1ml-fractions were collected from the fractionator column and assayed for antimicrobial activity at pH 5.3 .

Ethyl-acetate extraction of organic acids from Microgard™

Acetic and propionic acids were thought to contribute to the inhibitory activity of MG. However, it was established that the principal antimicrobial substance in MG was proteineous. Therefore, to prove that a portion of the activity of MG was a protein (bacteriocin), removal of all organic acids was necessary. Organic solvent extraction was attempted to remove acetic and propionic acids from MG.

Crude dairy MG was acidified to pH 4.00 so that the casein proteins were precipitated. The treated sample was centrifuged at 10,000 x g for 30 minutes and the supernatant neutralized to pH 5.30. A total of 20 ml of active supernatant (pH 5.30) was extracted with an equal volume of ethyl-acetate solvent for 1 hour. The top fraction (T1) and bottom fraction (B1) were separated and allowed to evaporate under vacuum to dryness using a rotary-evaporator. The temperature of the rotary-evaporator was set at 70 °C to remove the organic solvent. Upon evaporation to dryness, T1 was rehydrated with distilled water and along with B1 subsequently extracted with equal volumes of ethyl acetate for ten more times. All samples from T1 (1^{st} time extraction), and B1 (1^{st} time extraction) to T10 (10^{th} time extraction) and B10 (10^{th} time extraction) were assayed for activity. All fractions were analyzed for the presence of acetic and propionic acids by gas-liquid chromatography (GLC).

A volume of 20 ml of 1000 MW ultra-filtrate (1000-F) was extracted with an equal amount of ethyl-acetate for 1 hour. The top fraction and bottom fraction were separated and allowed to evaporate under vacuum to dryness using a rotary-evaporator. Both fractions were rehydrated with distilled water and analyzed for the presence of acetic and propionic acids by GLC.

Anion Exchange Chromatography

Amberlite IRS-400 anion-exchanger (hydroxyl-form) was found to be effective in binding organic acids, resulting in the elution of acid-free active ingredient(s) from crude MG.

After washing with deionized water, the resin was packed into a glass column (2.5 cm x 60.0 cm) and then equilibrated with 1.0 M sodium carbonate

overnight. After equilibration, the resin was washed with deionized water to remove the excess sodium carbonate and hydroxyl-residues. The resin pH after washing was within the range of 5.30 to 6.00. A total of 60 ml of crude MG was loaded onto the column and the flow-rate was adjusted to 1.0 ml/min. After the sample was completely immersed in the resin-bed, another 60 ml of deionized water was used to eluate the non-acid materials from the column. Eluates from the anion exchanger were collected in 4.0-ml fractions. A reducing agent, D-L dithiothreitol (1 mM), was added to each of the fractions. After the wash, 60 ml of 1.0 M sodium carbonate was used to regenerate the resin.

Only active fractions were combined and passed through another anion-exchange column (0.7 cm x 25.5 cm). Active fractions from anion exchange chromatography were analysed by gas-liquid chromatography for the presence of organic acids.

Organic acids quantitation by gas-liquid chromatography

Concentrations (µg/g) of acetic and propionic acids were determined by GLC on a Hewlett Packard 5710-A Gas Chromatograph & Integrator 3390-A. The standard curve was determined with known concentrations of acetic and propionic acids (Thornhill and Cogan 1984). The column (solid phase) used was 80/120 Carbopack B-DA/4% Carbowax 20 M (Supelco Chromatography Products, Inc. Bellefonte, PA). Inert nitrogen gas was used as the mobile phase. The flow-rate of nitrogen gas was established at 24 ml per minute (60 psi). The column temperature was 175 °C while the temperature of the injection port and detector were set at 200 °C . The increase in temperature with respect to time was 4 °C per minute. During combustion, hydrogen and compressed gases were set at 38 and 20 psi, respectively.

A standard curve for acetic and propionic acids was used to quantitate the amount of these acids found in treated MG. Oxalic acid (0.03 M) was used as buffer and internal standard. Lyophilized ethyl-acetate-extracted and anion exchanged fractions were suspended in equal volumes of 0.03 M oxalic acid. A total of 1 µl of mixture was injected into the GLC column. The column was cleaned and equilibrated with 0.03 M oxalic acid.

Acid hydrolysis of Microgard™

Lactoferrin when hydrolyzed at acidic pH and high temperature has been found to increase in activity (Saito et al. 1991). The acidity and thermal stability is similar to that of MG and therefore, in this study we attempted to confirm that the active ingredient in MG is not due to the presence of lactoferrin.

Crude dairy MG was adjusted to pH 4.00 to precipitate casein proteins. It was centrifuged at 10,000 x g for 30 minutes. The supernatant was collected and aliquots adjusted with 1.0 N HCl to pH 1.00, 2.00, 3.00, and 4.00, respectively. A total of 5 ml of each sample was placed in screw-capped tubes. Temperatures of 50 °C, 60 °C, 70 °C, 80 °C, 100 °C, & 121 °C were administrated to each sample. Each sample was heated at these temperatures for 5, 15, or 60 minutes. After hydrolysis, all samples were back-neutralized to pH 5.30 and then assayed for activity.

Base hydrolysis of Microgard™

Crude dairy MG was adjusted to pH 4.00 to precipitate casein. The titrated sample was centrifuged at 10,000 x g for 30 minutes, after which the supernatant was removed and aliquots adjusted with 1.0 N NaOH to pH 9.00,

10.00, 11.00 and 12.00, respectively . A total volume of 5 ml of each sample were placed into screw-capped tubes. Triplicate samples then were placed in a water bath at 50 °C, 60 °C, 70 °C, 80 °C, 100 °C or 121 °C. for 5, 15 or 60 minutes. All samples were assay for activity at pH 5.3 .

RESULTS

Gas-liquid chromatography analysis of lyophilized Microgard™

The inhibitory activity of lyophilized MG was retained for all samples (Table 1.1). However, there was a decrease in activity for samples at pH 6.34, 8.00, 5.30, and 5.00 . There was no change in activity between the pH range of 5.30 to 8.00 . GLC analysis showed the ratio of acetic to propionic acid in untreated MG was 1:3.8 (Table 1.6). Also, there was a small amount of diacetyl detected by the GLC, estimated to be 10 µg/g. Lyophilized MG showed a substantial reduction in the concentrations of acetic and propionic acids (Figure 1.3 & Table 1.6). The percent reduction of acetic acid at these same pH values. and 8.00 were calculated to be 40.4%, 56.7%, 76.3%, and 69.6% respectively. The percent reduction of propionic acid at pH 5.00, 5.30, 6.34, and 8.00 were calculated to be 43.4%, 61.8%, 74.5%, and 67.7% respectively. The concentrations of acetic and propionic acids were extrapolated from the standard curves (Figure 1.1). A new standard curve was generated when an analysis was made and figure 1.1 present typical data which were highly repeatable. The chromatogram of untreated crude MG is presented in Figure 1.2.

Gas-liquid chromatography analysis of ethyl-acetate extracted Microgard™

Ethyl-acetate was partially effective in the removal of organic acids from 1000 MW ultra-filtered MG (Figure 1.4) . The extraction of acetic and propionic acids from 1000 MW ultra-filtrate showed that the concentration of acetic acid remaining in the top (organic-phase) and bottom (aqueous-phase) fractions

were 0.905% and 59.82% respectively (Table 1.8). The concentration of propionic acid in the top and bottom fractions from 1000 MW filtrate were 2.542% and 29.13%, respectively (Table 1.8). The concentration of acetic and propionic acids in the top-fraction (organic-phase) of crude MG were 0.884% and 1.813%, respectively (Table 1.7). The acetic and propionic acids in the bottom fraction (aqueous-phase) were calculated to be 37.26% and 20.25%, respectively (Table 1.7). The disk-activity assay showed that the top fraction was more inhibitory than the bottom fraction (Table 1.4). There was no activity in the middle fraction. Ethyl-acetate extraction of 1% non-fat milk used as a control generated fractions which produced no zone of inhibition.

Total protein concentration and activity of ultra-filtered fractions

During ultra-filtration, the antimicrobial substance(s) of MG could diffuse through the 10,000 MW (10000 MW), 5000 MW, 1000 MW, and 500 MW cut-off membranes (Figure 1.7 - 1.10). All filtrates were found to possess inhibitory activity when assayed against, *Ps. putida* ATCC 12633. The retentate (brown precipitate) from 10,000 (10000-R), 5000 (5000-R), 1000 (1000-R), & 500 (500-R) molecular weight cut-off membranes revealed no antimicrobial activity (Figure 1.11 - Figure 1.14 and Table 1.2).

Lowry total protein assay measured the amount of proteins and amino acids in the 10,000, 5,000, 1,000 and 500 molecular weight cut-off filtrates. Concentration decreased from 1695 ug/ml in 10,000-filtrate to 687 ug/ml in 500-filtrate (Table 1.2).

Separation of active protein by isoelectric focussing

The active protein(s) from the 1000-molecular weight cut-off filtrate (1000-F) was separated by the isoelectric focussing technique. Bands of macromolecules (proteins) generated from the ampholyte-dependent pH gradient were collected into twenty 1-ml fractions. Fractions 1 to 6 (pH 2.21 - pH 5.13) were found to be inhibitory for *Ps. putida*. However, fraction 1 (pH 2.21) was most inhibitory (Table 1.3). Control experiments with ampholytes (pH range 3-10) fractions did not produce any inhibitory activity toward the indicator-organism (data not shown).

Removal of acetic and propionic acids from crude Microgard by anion-exchange chromatography

Fractions 5 to 16 from the anion-exchange column were found to be inhibitory for *Ps. putida* (Table 1.10). Fraction 8 had the highest activity, showing a 1.80 cm zone of inhibition toward the indicator-organism. Combining fractions 5 to 16 did not increase the overall activity, but actually decreased it to 1.60 cm (Table 1.11). A second anion-exchange chromatography of the combined samples yielded two active fractions (2 and 3). There was increased activity in both fractions (Table 1.12). Also GLC showed an improvement in the removal of acetic and propionic acids from crude MG (Figure 1.5). After anion-exchange chromatography, the percent recovery of acetic and propionic acids from crude MG was 6.30% and 2.98%, respectively (Table 1.9).

Lactoferrin determination by acid-hydrolysis of crude Microgard™

The acid-hydrolysis of MG at pH 1.00, 2.00, 3.00, or 4.00 resulted in an increase in antimicrobial activity (Table 1.13 - Table 1.16). The increase was corelated with an increase in hydrolysis temperature from 50 °C to 121 °C. It was seen, therefore, that the treated MG became more active after fifteen minutes of heating at pH values from 1.00 to 4.00.

Lactoferrin determination by base-hydrolysis of crude Microgard™

Base-hydrolysis of MG at pH 9.00, 10.00, 11.00, and 12.00 showed a slight increase in activity at 121 °C (Table 1.17 - Table 1.20). However, progressive increased in temperature did not seem to enhance the activity of MG. Overall, the data showed that a 15-minutes hydrolysis time was sufficient to see slight increase in activity at 121 °C. The optimum temperature for a short period of hydrolysis was 80 °C. With 5-minutes of hydrolysis, there was an increase in activity of MG treated at pH 9.00, 10.00, and 12.00.

DISCUSSION

Although propionic and acetic acids have been identified as the predominant propionibacterial fermentation products (Hsu and Yang 1991), concentrations of these acids in MG could not contribute significantly to inhibition of food spoilage bacteria by this product. GLC analysis of crude MG showed that the concentration of acetic acid is greater than propionic acid. This finding is in contrast to that of Glatz et al. (1991, 1992, and 1993) who claimed that the predominant acid in MG was propionic acid. It also contradicts the observations of Marcoux et al. (1992) who reported that a higher concentration of acetic acid was produced when fermented ammoniated condensed milk permeate was used to supplement a fermentation carried out by *P. freudenreichii* ssp. *shermanii*. The concentrations of lactic acid and diacetyl also were too negligible to be inhibitory. Nonetheless, reports exist (Grinstead and Barefoot 1992; Glatz 1992; and Lyon et al., 1993) indicating that the inhibitory activity of MG parallels the concentration of propionic acid. In the present studies, the acid and base hydrolysis of MG have taken into account the pKa-values of acetic (4.757) and propionic (4.874) acids. Undissociated forms of these acids are inhibitory to Gram-negative bacteria (Tang et al., 1989; and Baird-Parker 1980). However, the ionized forms of these acids and other volatile substances should be mostly destroyed at 121 °C for 15 minutes.

Heat treatment of MG at low pH values caused an enhancement in its activity against *Ps. putida*. Since GLC analysis (data not shown) showed no sign of acetic and propionic acids, it was reasoned that these acids were destroyed and the activity of MG was likely due to a stable metabolite. This stable substance was sensitive to a-chymotrypsin, pepsin and papain (Al-Zoreky 1989), inferring that it was proteineous in nature. Bacteriocins from

propionibacterial species are known to be thermal stable and resistant to a wide range of pH levels (Grinstead and Barefoot 1992; Glatz 1992; and Lyon et al., 1993). We found that alkaline-hydrolysis was slightly inhibitory toward the indicator-bacteria. This could be due to the release of active precursor(s) from MG when hydrolysed under high temperature and pH conditions.

Hydrolysis of MG under acidic conditions did not produce the same pattern of antimicrobial activity known to activate lactoferrin precursors. Saito et al. (1991) indicated that bovine lactoferrin, upon heat treatment under low pH conditions, had antimicrobial activity. In their studies, an enhancement of antimicrobial activity was observed when lactoferrin was heat-treated at pH 2 and 3. The inhibitory activity of MG after acid-hydrolysis could be due to the release of active precursors different from lactoferrin molecules.

The antimicrobial activity of crude MG was only slightly reduced when it was lyophilized at pH 5.00 . GLC analysis of this lyophilized product showed a 59.6% reduction in the concentration of acetic acid and a 56.6% reduction of propionic acid. Lyophilization at higher pH values did not significantly reduce amounts of these acids. The decrease in activity of the pH 5.00 dry product inferred that the remaining undissociated acetic and propionic acids could still contribute to some of the overall antimicrobial activity of MG. However, simulated concentrations of the acetic and propionic acids at this pH 5.00 in the lyophilized fraction were found not to be inhibitory to *Ps. putida* either individually or when combined.

Ethyl-acetate extraction of crude MG followed by evaporation of the organic phase under vacuum, caused the concentrations of acetic and propionic acids to diminished significantly. When the organic phase (top fraction), organic-aqueous interface (middle fraction) and aqueous phase (bottom fraction) were evaporated under vacuum, the total recoverable acids

were less than present in crude MG. Compared to the original acid concentrations in crude MG, 37.26% of acetic acid and 20.25% of propionic acids were still recoverable from the aqueous fraction after ethyl-acetate extraction. However, only 0.88% of acetic acid and 1.8% of propionic acids remained in the top extracted fraction. From these data, it appeared that organic solvent extraction and evaporation resulted in volitilization of the organic acids causing their loss from the treated MG. Since some amino acid-oligopeptide antibiotics are known to be soluble in organic solvents (Baquero and Moreno 1984; García-Bustos et al., 1984; Silva et al., 1987), it was not surprising that the top fractions of extracted MG and the 1K molecular weight cut-off filtrate were more inhibitory than the bottom fraction. Although the concentrations of organic acids were greater in the aqueous fraction, it is our belief that the antimicrobial substance(s) from MG must be soluble in organic solvent.

The concentrations of acetic and propionic acids in the ethyl-acetate extracted fractions (1000 MW filtrate) were found to be non-inhibitory to *Ps. putida*. Also the same concentrations of propionic and acetic acids in 11% non-fat dry milk (40.05 ppm of acetic acid and 29.79 ppm of propionic acids) produced no inhibitory effect against Gram-negative bacteria. Since the ethyl-acetate extracted fraction (1000 MW filtrate) remained active, these acids could not be responsible for the antimicrobial activity of MG.

Anion-exchange chromatography (Bryant and Overell 1953) has proved to be successful in removal of acetic and propionic acids from MG. GLC showed that the original concentration of acetic acid in crude MG was reduced to 6.30%. The concentration of propionic acid was reduced to 2.98%. Successful reduction of organic acids should dispel any speculation that the inhibitory nature of MG was mainly due to an acid-effect. (Glatz 1992; Lyon et

al., 1993). The data herein indicate that repeated anion-exchange chromatography can ensure complete removal of all organic acids thus paving the way for the purification of the antimicrobial substance(s) remaining in acid-free MG.

From the ampholyte-dependent pH gradient, bands of macromolecules in MG were generated after isoelectric focussing. The inhibitory activity of eluted bands increased as the pH decreased from pH 5.46 to pH 2.95 . It is known that macromolecules with lower isoelectric points (more carboxyls) will migrate toward the acidic electrode pole, while those with higher isoelectric points (more amines) move to the alkaline electrode pole (Egen et al., 1984). In this experiment, the antimicrobial substance was focused at the lower pH-range. From this, it was evident that the antimicrobial substance found in MG is a low molecular weight acidic peptide (Hurst 1981; Delves-Broughton 1990).

The molecular weight of the antimicrobial substance was previously estimated by gel filtration to be 700 daltons (Al-Zoreky 1988). In the present study, ultra-filtration showed that the enhancement of the substance in the 1000 MW filtrate was greater than that in either the 5000 MW or the 500 MW filtrate. The overall decrease in activity from crude MG to the 500 MW filtrate could be due to losses after each stage of filtering. Since there was no establishment of activity-units in this experiment, the decrease in overall activity did not necessarily mean a decrease in specific-activity for each fraction. However, all active fractions were shown to contain protein.

From all these studies of MG, we believe that the antimicrobial substance is a low molecular weight (<1000 daltons) acidic peptide that resembles bacteriocins or, more appropriately, microcins. The presence of organic acids complicates matters since acetic and propionic acids, as well as diacetyl, could be inhibitory when present in sufficient concentration (Figure 1.7). However,

we were able to establish that these organic substances do not contribute exclusively to the antimicrobial activity of MG (Figure 1.7). Although propionates do not inhibit Gram-positive bacteria (Internat. Comm. Microbiol. Spec. Foods, 1980; Barefoot and Nettles 1993), the antimicrobial spectrum of MG was shown to include both Gram-negative and Gram-positive bacteria. The inhibitory substance was shown to be thermal stable at low pH ranges and to be slightly soluble in organic solvent. The thermal stability is similar to bacteriocins from *Lactococcus lactis* strains FS 84, FS 90, FS 91-1, and FS 97. The bacteriocin produced by strain FS 84 demonstrated the greatest stability at pH 4.00 after 2 hours at 100 °C (Muriana, 1993). The thermal stability is also comparable to some bacteriocins which remain active after heating at 120 °C for 20 minute (Toba et al., 1991a; Toba et al., 1991b) or 100 °C for 30 minutes (Joerger and Klaenhammer 1986). The difference in thermal stability could be attributed to the differences in degree of secondary structure of the bacteriocins. It could also be due to the specificity of certain amino acids which may include side chains that are interactive at various pH values (Muriana 1993). The antimicrobial activity of this peptide is similar to microcin A15, from which a methionine moiety is released when hydrolysed under mild acid conditions (Baquero and Moreno 1984). The mechanism of action of microcin A15 involves L-methionine, which inhibits homoserine-O-transsuccinylase, hence blocking protein synthesis (Baquero and Moreno 1984; Aquilar et al., 1982). In addition, the inhibitory activity of crude MG was observed to be enhanced by sodium citrate salts.

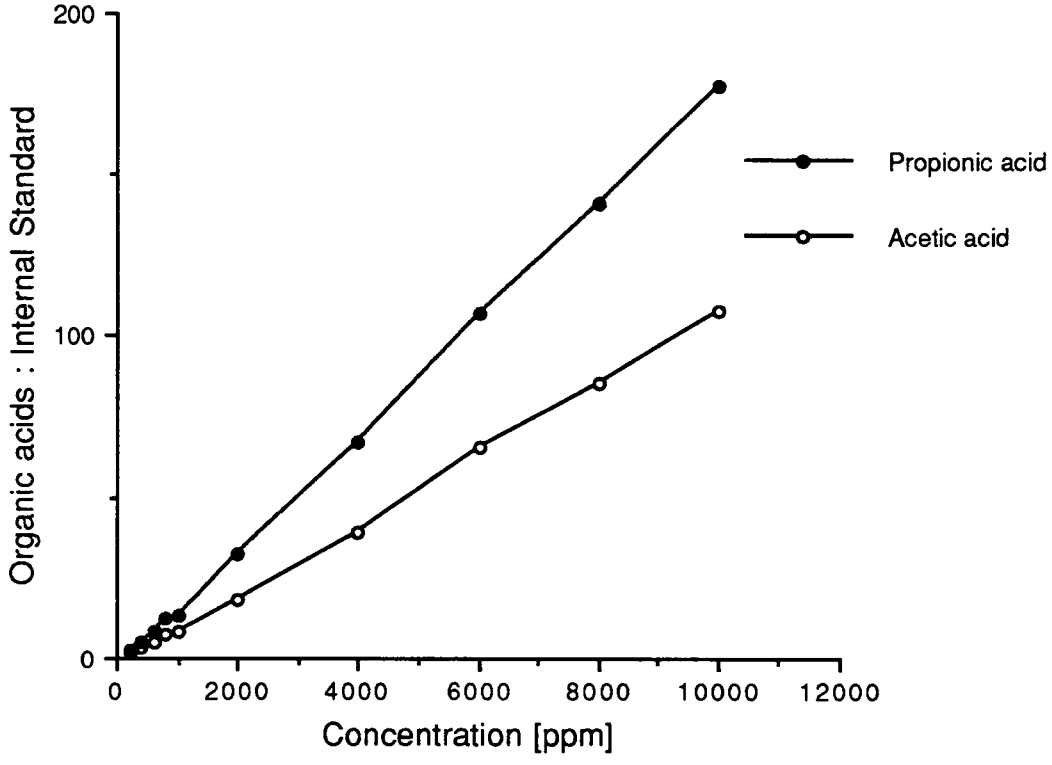

Figure 1.1 Gas-liquid chromatography standard curves for acetic and propionic acids as determined using 200, 400, 600, 800, 1000, 2000, 4000, 6000, 8000, and 10,000 µg/g of each acid.

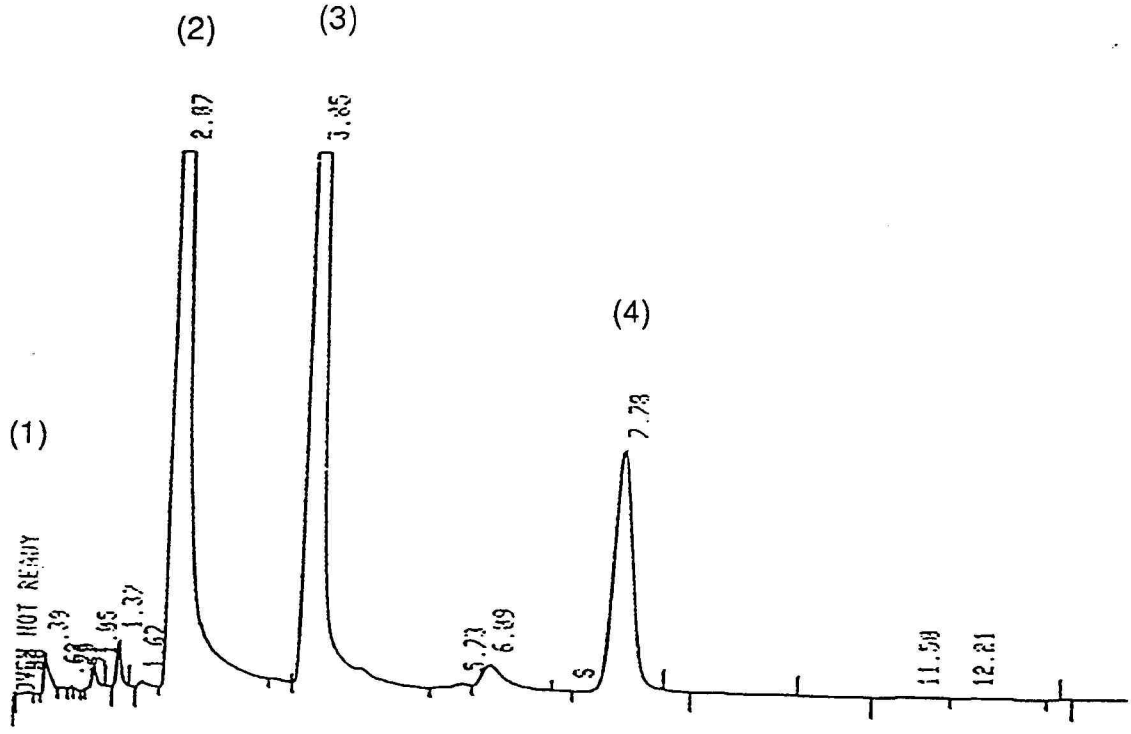

Figure 1.2 Gas-liquid (GC) chromatogram of crude Microgard™. GC peaks were identified as (1) 0xalic acid, (internal standard); retention time=0.39, (2) acetic acid; retention time=2.07, (3) propionic acid; retention time 3.85, (4) butyric acid; retention time=7.78. Attenuation level for the instrument was 8.

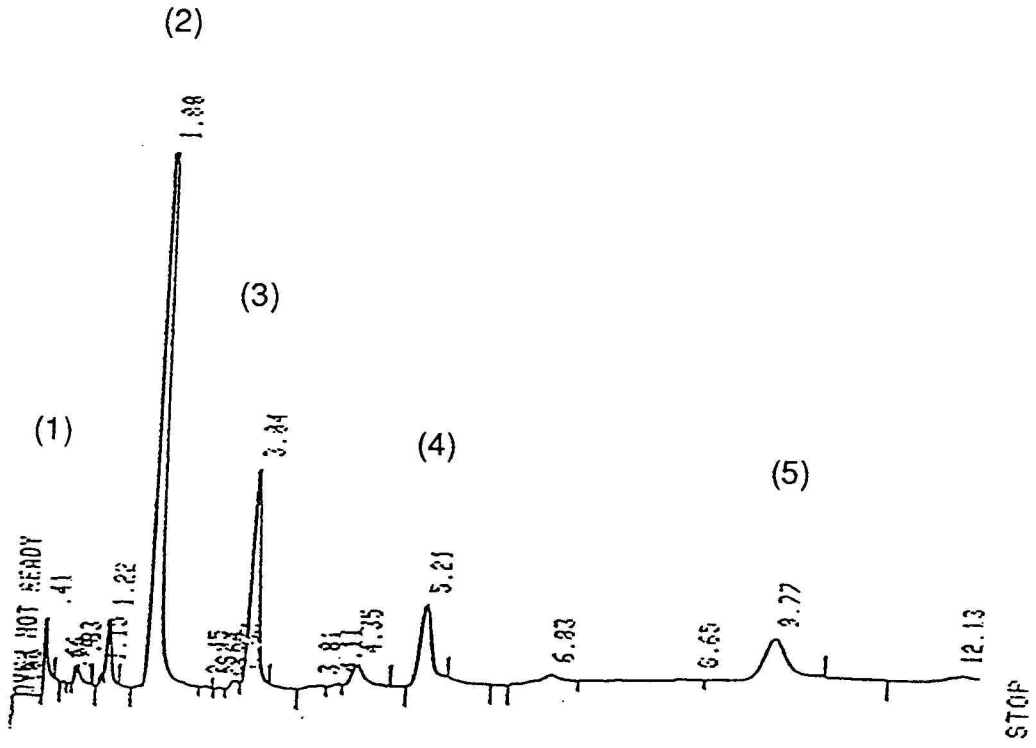

Figure 1.3 Gas-liquid (GC) chromatogram of lyophilized Microgard™, pH 5.00. GC peaks were identified as (1) oxalic acid (internal standard); retention time=0.41, (2) acetic acid; retention time=1.80, (3) propionic acid; retention time=3.04, (4) isobutyric; retention time=5.21, (5) trimethylacetic acid; retention time=9.77. Attenuation level for the instrument was 8.

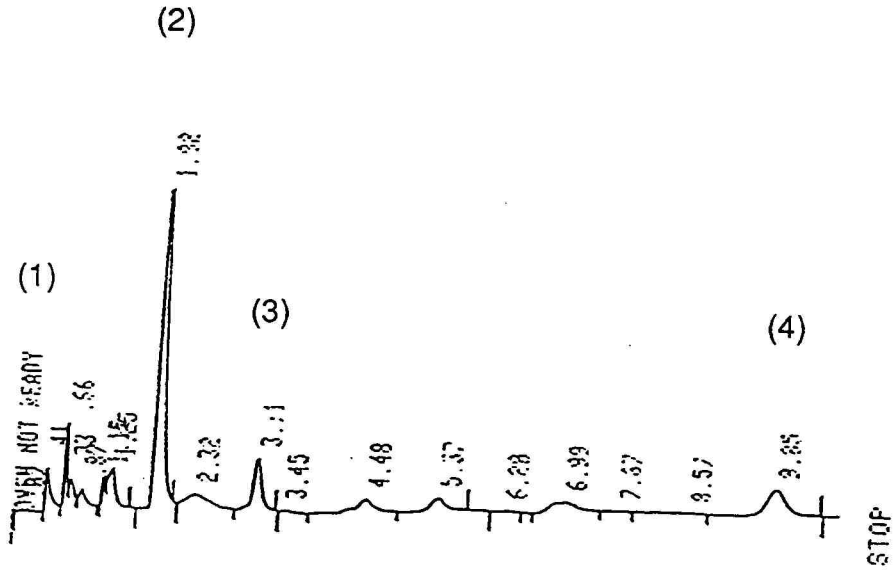

Figure 1.4 Gas-liquid (GC) chromatogram of ethyl-acetate extract from 1000 ultra-filtrate. GC peaks were identified as (1) oxalic acid (internal standard); retention time=0.41, (2) acetic acid; retention time=1.82, (3) propionic acid; retention time=3.11, (4) trimethylacetic acid; retention time=9.85. Attentuation level for the instrument was 8.

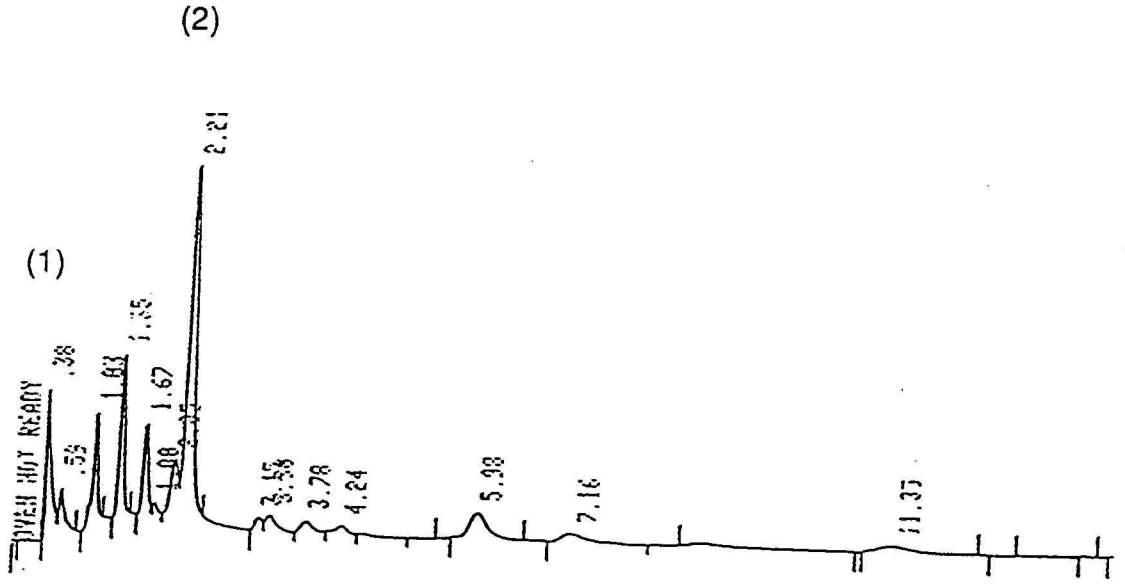

Figure 1.5 Gas-chromatogram (GC) of Microgard™ after anion exchange chromatography. GC peaks were identified as (1) oxalic acid (internal standard) retention time=0.38; (2) acetic acid; retention time=2.21. No propionic acid was detected. Attenuation level for the instrument was 2.

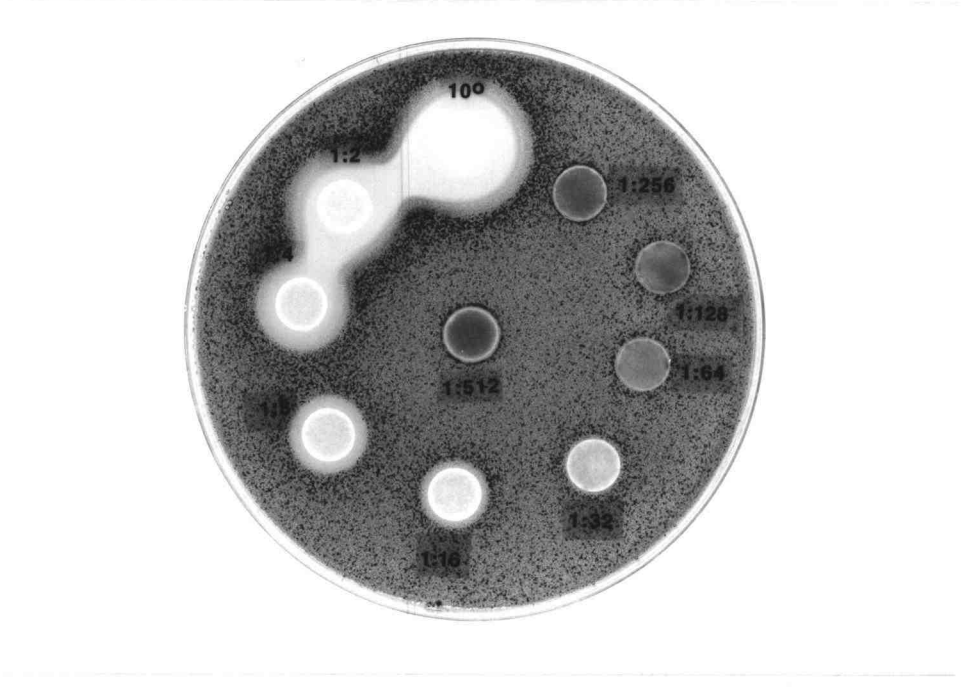

Figure 1.6 Disk assay inhibitory activity against *Pseudomonas putida* found in fractions from crude Microgard™. The diameter of inhibition zones were distinctive in fractions 10°, 1:2, 1:4, 1:8, 1:16, and 1:32 . The activity of crude Microgard™ used in this experiment was 320 AU/ml .

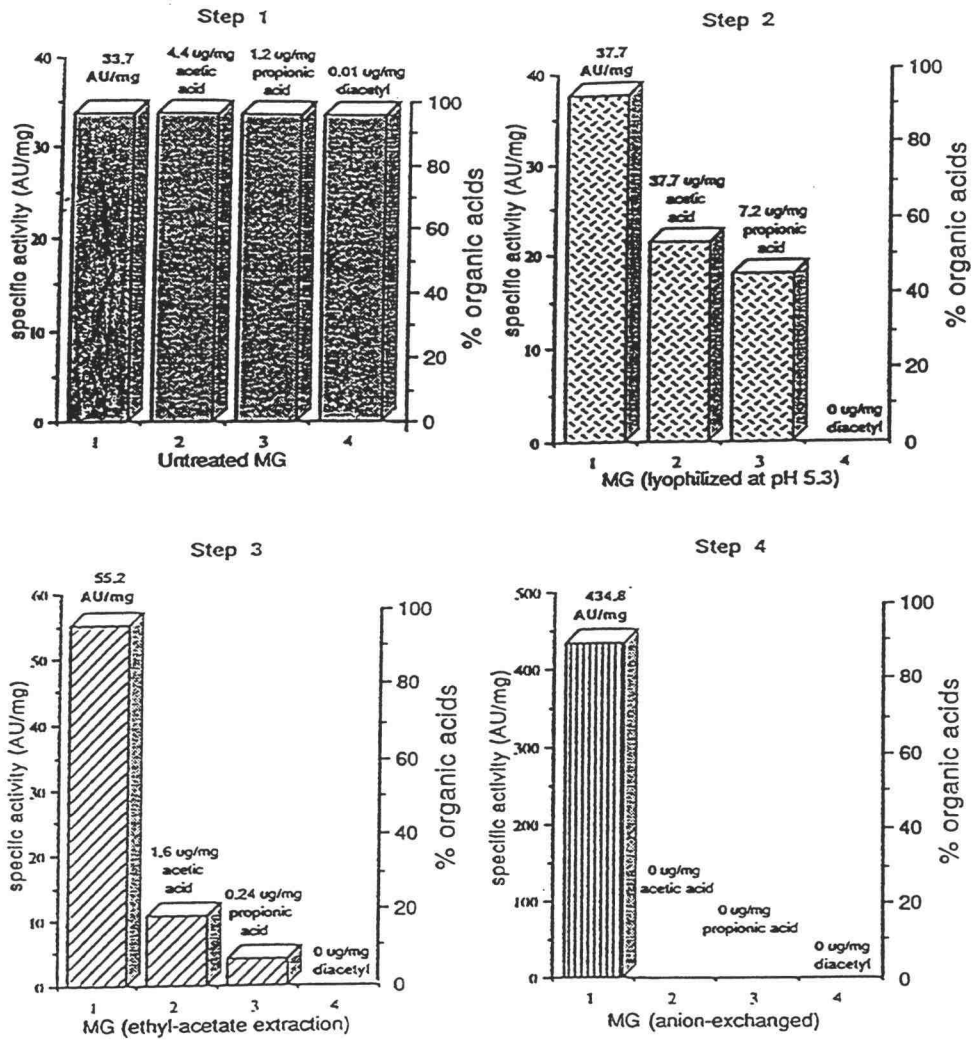

Figure 1.7 Influence of different purification steps on the inhibitory activity of Microgard™ against *Pseudomonas putida* in relation to amounts of acetic and propionic acids and diacetyl present after each step.

Table 1.1 Results of disk assay inhibitory activity against *Pseudomonas putida* of lyophilized[4] Microgard™ at pH 5.00, 5.30, 6.34, and 8.00.

Zones of inhibition[3] (diameter-cm)

pH 5.00 [1]	pH 5.30[1]	pH 6.34	pH 8.00[2]
3.50 cm	3.70 cm	3.70 cm	3.80 cm

[1] Crude Microgard™ adjusted with 10 % tartaric acid to pH 5.00 and 5.30 before lyophilization

[2] Crude Microgard™ adjusted with 0.1 N NaOH solution to pH 8.00 before lyophilization

[3] Size of analytical paper disk : 1.30 cm (1/2"); diameter of inhibition zone measured in cm

[4] All samples were resuspended in 0.1 M citrate buffer, pH 5.30 after lyophilization at the appropriate pH

Table 1.2 Disk inhibitory activity & total protein assay results with ultra-filtered Microgard™ ; inhibition was measured against *Pseudomonas putida*

Sample	Zone of Inhibition[3] (cm)	Total Protein (ug/ml)
Crude Microgard™	4.00	NA[4]
Supernatant	3.60	NA
Pellet	0.00	NA
10000 Mol. Wt Retaintate	1.60	39598.80
10000 Mol[1]. Wt Filtrate[2]	3.30	1695
5000 Mol. Wt Retaintate	1.50	4050.69
5000 Mol. Wt Filtrate	2.40	1500
1000 Mol. Wt Retaintate	1.60	3247.28
1000 Mol. Wt Filtrate	2.50	1070
500 Mol. Wt Retaintate	1.50	NA
500 Mol. Wt Filtrate	2.30	687

[1]molecular weight cut-off membranes were supplied by Amicon Co. Inc.

[2]all sample were filtered under 30 - 80 psi

[3]size of analytical paper disk: 1.30 cm; diameter of inhibition zone measured in cm

[4]NA denotes not analyzed

Table 1.3 Disk assay inhibitory activity results against *Pseudomonas putida* of isoelectric focussed[1] (IEF) fractions from 1000 dalton ultra-filtrate of Microgard™

Fractions	Ampholyte[2]-3/10 pH (Control)	Fractionated pH (Sample)	Inhibition Zone[3,4] (cm)
1	2.95	2.21	3.40
2	3.87	3.33	2.70
3	4.30	4.17	1.80
4	4.68	4.40	1.70
5	5.24	4.78	1.55
6	5.46	5.13	1.40
7	6.04	5.61	0.00
8	6.44	6.01	0.00
9	6.74	6.41	0.00
10	7.16	6.76	0.00

[1]Rotofor fractionator was used for the IEF experiment .

[2]Commercial ampholyte used was 3/10 (Bio-Rad Laboratories, Richmond, CA)

[3] Size of analytical paper disk: 1.30 cm

[4] Diameter of inhibition zone measured in cm

Table 1.4 Disk[1] assay inhibitory activity results against *Pseudomonas putida* using from ethyl-acetate extracted crude Microgard™ , 1000 molecular cutoff filtrate, and 1% nonfat milk control

Extracted Fractions[2]	Crude Microgard™	1K Molecular Wt. Cut-Off Filtrate	1% Non-Fat Milk Control
Top	1.70[3] cm	2.20 cm	No Zone of Inhibit.
Middle	No Zone of Inhibit.	No Zone of Inhibit.	No Zone of Inhibit.
Bottom	1.60 cm	1.80 cm	No Zone of Inhibit.

[1] Size of analytical paper disk- 1.30 cm

[2] All fractions were purged with nitrogen gas to dryness and then rehydrated to original volume with 0.1 M citrate buffer (pH 5.30)

[3] Diameter of inhibition zone measured in cm

Table 1.5 Disk assay inhibitory activity results against *Pseudomonas putida* found in ethyl-acetate extracted Microgard™

Dilut. Samp	0	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
T1[1]	4.20	2.90	2.50	1.90	1.60	NI[3]	NI	NI	NI	NI	NI	NI
B1[2]	6.55	3.60	2.50	2.15	1.60	1.50	1.50	1.45	1.40	1.40	1.40	1.40
B2	6.50	4.50	3.50	2.70	2.35	2.10	2.00	1.50	1.45	1.45	1.45	1.45
B3	5.75	3.20	2.80	2.10	2.05	1.60	1.50	NI	NI	1.55	1.60	2.10
B4	3.30	3.00	2.70	2.50	2.00	1.70	1.55	NI	NI	1.60	1.70	2.50
B10	3.60	2.75	2.50	1.75	NI	NI	NI	NI	NI	NI	NI	NI

[1] T denotes top-fraction or organic phase

[2] B denotes bottom-fraction or aqueous phase

[3] NI denotes non-inhibitory to *Pseudomonas putida*

Table 1.6 Gas-liquid chromatography analysis data for acetic and propionic acids in lyophilized Microgard™

Sample	Acetic acid[1]		Propionic acid[1]	
	µg/g	% remaining	µg/g	% remaining
Crude Microgard™ pH 6.34	4425.82	100	1171.72	100
Lyophilized pH 5.00	1788.00	40.4	508.19	43.4
Lyophilized pH 5.30	2507.60	56.7	724.20	61.8
Lyophilized pH 6.34	3378.44	76.3	873.31	74.5
Lyophilized pH 8.00	3080.77	69.6	793.45	67.7

[1] Concentration of organic acids were derived from standard curve (Figure 1.1)

Table 1.7 Gas-liquid chromatography analysis data for acetic and propionic acids in ethyl-acetate extracted Microgard™ (crude)

Sample	Acetic acid[1]		Propionic acid[1]	
	µg/g	% remaining	µg/g	% remaining
Untreated Microgard™	4426	100	1172	100
Ethyl-acetate extracted Microgard™ (top-fraction)	39.12	0.884	21.25	1.813
Ethyl-acetate extracted Microgard™ (middle-fraction)	191.3	4.322	50.65	4.322
Ethyl-acetate extracted Microgard™ (bottom-fraction)	1649	37.26	237.3	20.25

[1] Concentration of organic acids were derived from standard curve (Figure1.1)

Table 1.8 Gas-liquid chromatography analysis data for acetic and propionic acids in ethyl-acetate extracted Microgard™ (1000 MW ultra-filtrate)

Sample	Acetic acid[1]		Propionic acid[1]	
	µg/g	% remaining	µg/g	% remaining
Untreated Microgard™	4426	100	1172	100
Ethyl-acetate extracted 1000 ultra-filtrate (F) top-fraction	40.05	0.905	29.79	2.542
Ethyl-acetate extracted 1000 ultra-filtrate (F) bottom-fraction	2647.5	59.82	341.4	29.13

[1] Concentration of organic acids were derived from standard curve (Figure1.1)

Table 1.9 Gas-liquid chromatography analysis of ethyl-acetate extracted and anion exchange treated Microgard™

Sample	Acetic Acid (µg/g)	Propionic Acid (µg/g)	Acetic Acid % Recovery	Propionic Acid % Recovery
Crude Microgard	12206.7	5393.16	100	100
10K* (F) B-10	11217.30	2645.40	91.9	49.1
Anion-Exchange (active fraction)	770.00	160.80	6.30	2.98

*10K (F) B-10 denotes filtrate of 10,000 daltons, extracted by ethyl-acetate solvent for a total of 10 times

Table 1.10 Disk assay inhibitory activity results against *Pseudomonas putida* found in fractions from initial anion exchange chromatography of Microgard™

Fraction #	Serial Dilution	Zone of Inhibition (cm)
1	undiluted	NI[1]
2	undiluted	NI
3	undiluted	NI
4	undiluted	1.45
5[2]	undiluted	1.60
6	undiluted	1.70
	1:2	1.45
7	undiluted	1.80
	1:2	1.45
8	undiluted	1.80
	1:2	1.45
9	undiluted	1.65
	1:2	1.40
10	undiluted	1.65
	1:2	1.40
11	undiluted	1.60
12	undiluted	1.60
13	undiluted	1.60
14	undiluted	1.55
15	undiluted	1.50
16	undiluted	1.50
17	undiluted	1.45
18	undiluted	1.45
19	undiluted	NI
20	undiluted	NI

[1] NI denotes non-inhibitory

[2] fractions 5 to 16 were combined (6.00 ml), assayed for activity and passed through a 2nd anion exchange column

Table 1.11 Disk assay inhibitory activity results against *Pseudomonas putida* found in the combined active fractions 5 to 16 from anion-exchange chromatography of Microgard™

Serial Dilution	Zone of Inhibition (cm)
10 ° (undiluted)	1.60

Table 1.12 Disk assay inhibitory activity results against *Pseudomonas putida* found in fractions from 2nd anion-exchange chromatography of Microgard™

Fraction	Zone of Inhibition (cm)
1	NI*
2	1.75
3	1.65
4	NI
5	NI

* NI denotes non-inhibitory to the indicator-bacteria

Table 1.13 Activity data for Microgard™ hydrolyzed at pH 1.00 with respect to time and temperature. After hydrolysis, samples were adjusted to pH 5.3 with 1 N HCl and assayed against *Pseudomonas putida*.

Hydrolysis Time (min)	50 °C	60 °C	70 °C	80 °C	100 °C	121 °C
0 min	2.65 cm*	3.10 cm	3.30 cm	2.95 cm	2.65 cm	2.15 cm
5 min	2.65 cm	3.10 cm	3.30 cm	2.95 cm	2.65 cm	2.15 cm
15 min	2.70 cm	2.90 cm	3.25 cm	3.75 cm	3.80 cm	4.40 cm
60 min	3.45 cm	2.95 cm	2.55 cm	2.65 cm	2.90 cm	2.65 cm

* Diameter of inhibition zone measured in cm

Table 1.14 Activity data for Microgard™ hydrolyzed at pH 2.00 with respect to time and temperature. After hydrolysis, samples were adjusted to pH 5.3 with 1 N HCl and assayed against *Pseudomonas putida*.

Hydrolysis Time (min)	50 °C	60 °C	70 °C	80 C°	100 °C	121 °C
0 min	2.45 cm*	2.10 cm	2.00 cm	2.60 cm	2.00 cm	2.80 cm
5 min	2.45 cm	2.10 cm	2.00 cm	2.60 cm	2.00 cm	2.80 cm
15 min	2.45 cm	2.35 cm	2.60 cm	2.55 cm	2.60 cm	3.10 cm
60 min	2.60 cm	2.80 cm	2.80 cm	3.40 cm	3.00 cm	3.00 cm

* Diameter of inhibition zone measured in cm

Table 1.15 Activity data for Microgard™ hydrolyzed at pH 3.00 with respect to time and temperature. After hydrolysis, samples were adjusted to pH 5.3 with 1 N HCl and assayed against *Pseudomonas putida*.

Hydrolysis Time (min)	50 °C	60 °C	70 °C	80 °C	100 °C	121 °C
0 min	2.65 cm[1]	2.95 cm	3.35 cm	3.15 cm	NI[2]	NI
5 min	2.60 cm	2.90 cm	3.30 cm	3.10 cm	NI[2]	NI
15 min	3.00 cm	3.15 cm	3.40 cm	3.15 cm	3.10 cm	3.30 cm
60 min	2.10 cm	3.05 cm	2.00 cm	1.50 cm	1.50 cm	1.50 cm

[1] Diameter of inhibition zone measured in cm

[2] NI denotes non-inhibitory to the indicator-bacteria

Table 1.16 Activity data for Microgard™ hydrolyzed at pH 4.00 with respect to time and temperature. After hydrolysis, samples were adjusted to pH 5.3 with 1 N HCl and assayed against *Pseudomonas putida*.

Hydrolysis Time (min)	50 °C	60 °C	70 °C	80 °C	100 °C	121 °C
0 min	NI[2]	2.10 cm[1]	2.20 cm	2.90 cm	3.35 cm	2.45 cm
5 min	NI	2.10 cm	2.20 cm	2.90 cm	3.35 cm	2.45 cm
15 min	3.40 cm	3.65 cm	3.65 cm	4.80 cm	5.40 cm	4.10 cm
60 min	2.70 cm	3.15 cm	2.35 cm	2.20 cm	2.60 cm	2.50 cm

[1] Diameter of inhibition zone measured in cm

[2] NI denotes non-inhibitory to the indicator-bacteria

Table 1.17 Activity data for Microgard™ hydrolyzed at pH 9.00 with respect to time and temperature. After hydrolysis, samples were adjusted to pH 5.3 with 1 N HCl and assayed against *Pseudomonas putida.*

Hydrolysis Time (min)	50 °C	60 °C	70 °C	80 °C	100 °C	121 °C
0 min	1.70 cm*	1.75 cm	1.70 cm	2.10 cm	1.65 cm	1.90 cm
5 min	1.70 cm	1.75 cm	1.70 cm	2.10 cm	1.65 cm	1.90 cm
15 min	1.60 cm	1.80 cm	1.80 cm	1.75 cm	1.90 cm	2.00 cm
60 min	1.60 cm	1.60 cm	1.60 cm	1.60 cm	1.60 cm	1.60 cm

* Diameter of inhibition measured in cm

Table 1.18 Activity data for Microgard™ hydrolyzed at pH 10.00 with respect to time and temperature. After hydrolysis, samples were adjusted to pH 5.3 with 1 N HCl and assayed against *Pseudomonas putida*.

Hydrolysis Time (min)	50 °C	60 °C	70 °C	80 °C	100 °C	121 °C
0 min	2.20 cm*	2.20 cm	2.20 cm	2.20 cm	2.20 cm	1.80 cm
5 min	2.20 cm	2.20 cm	2.20 cm	2.20 cm	2.20 cm	1.80 cm
15 min	2.10 cm	2.00 cm	2.10 cm	1.60 cm	1.65 cm	1.90 cm
60 min	1.65 cm	1.70 cm	1.70 cm	1.70 cm	1.70 cm	1.70 cm

* Diameter of inhibition zone measured in cm

Table 1.19 Activity data for Microgard™ hydrolyzed at pH 11.00 with respect to time and temperature. After hydrolysis, samples were adjusted to pH 5.3 with 1 N HCl and assayed against *Pseudomonas putida*.

Hydrolysis Time (min)	50 °C	60 °C	70 °C	80 °C	100 °C	121 °C
0 min	2.40 cm*	1.95 cm	1.90 cm	1.80 cm	2.00 cm	1.65 cm
5 min	2.40 cm	1.95 cm	1.90 cm	1.80 cm	2.00 cm	1.65 cm
15 min	1.80 cm	1.80 cm	1.80 cm	1.85 cm	2.10 cm	2.15 cm
60 min	1.85 cm	1.80 cm	1.60 cm	1.60 cm	1.60 cm	1.60 cm

* Diameter of inhibition zone measured in cm

Table 1.20 Activity data for Microgard™ hydrolyzed at pH 12.00 with respect to time and temperature. After hydrolysis, samples were adjusted to pH 5.3 with 1 N HCl and assayed against *Pseudomonas putida*.

Hydrolysis Time (min)	50 °C	60 °C	70 °C	80 °C	100 °C	121 °C
0 min	1.75 cm*	1.80 cm	1.75 cm	2.00 cm	1.80 cm	2.45 cm
5 min	1.75 cm	1.80 cm	1.75 cm	2.00 cm	1.80 cm	2.45 cm
15 min	1.65 cm	1.65 cm	1.85 cm	1.85 cm	1.85 cm	1.90 cm
60 min	1.65 cm	1.70 cm	1.70 cm	1.70 cm	1.85 cm	1.90 cm

* Diameter of inhibition zone measured in cm

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CHAPTER 2

PURIFICATION OF A BACTERIOCIN FROM MICROGARD™

ABSTRACT

The bacteriocin in crude Microgard™ (MG) was isolated and purified to homogeneity by reverse-phase high performance liquid chromatography. Acetic and propionic acids present were successfully removed by a strong anion exchanger. Isolation of the antimicrobial substance involved ultra-filtration and gel filtration. The bacteriocin was found to be most active in the filtrate from the 1000 molecular weight cut-off membrane (< 1000 daltons). The 500 molecular weight cut-off retaintate was found to be more inhibitory when compared to other retaintates. The molecular weight of the bacteriocin was estimated to be 860 daltons by gel filtration. Data from mass spectroscopy showed two peaks that are in a series, 295 and 441.7 suggesting charges of 3 (MH^{+3}) and 2 (MH^{+2}), respectively. The molecular weight determined by mass spectroscopy was 881.7 daltons . Amino-acid analysis by Edman degradation method confirmed that it is a peptide consisting of 4 residues of methionine, 2 of valine, 1 of glutamic acid, and possibly 1 of glycine. The molecular weight by this process was estimated to be 926 daltons. The psychotrophic bacterium *Pseudomonas putida* ATCC 12633 was sensitive to all partial and fully purified fractions of MG. The final product from high performance liquid chromatography was stable when stored at -40 ^{o}C in sodium acetate buffer (pH 5.3).

INTRODUCTION

Bacteriocins are an extremely heterogeneous group of substances. The original definition of bacteriocins referred to proteins of the colicin type produced by *Escherichia coli* (Jacob et al., 1953). Produced by strains of Gram-positive and Gram-negative bacteria, they were characterized by lethal biosynthesis, intraspecific activity, and adsorption to specific receptors (Tagg et al., 1976). Tagg et al. (1976) defined bacteriocins as active macromolecules possessing a narrow inhibitory spectrum of activity, protein in nature, plasmid encoded and without effect on producer cells. Reports have shown that, unlike most bacteriocins produced by Gram-negative bacteria which act on closely related species, bacteriocins from Gram-positive bacteria have been shown also to inhibit Gram-negative organisms (Wolff and Duncan 1974; Silva et al., 1987; Brunia et al., 1988; Lyon and Glatz 1991; Lewus et al., 1991). On the other hand, Klaenhammer (1988) pointed out that inhibition of Gram-negative bacteria has not been clearly demonstrated by purified bacteriocins from Gram-positive organisms. In this regard, the presence of a lipoteichoic acid receptor for pediocin AcH in the host cell wall suggested that the bacteriocins from Gram-positive bacteria may not be inhibitory to Gram-negative organisms since they do not possess cell wall teichoic acid (Bhunia et al., 1991).

Many strains of Gram-positive fermentation starter culture bacteria produce bacteriocins such as Nisin, Diplococcin, Leuconocin Lcm, Mesenteroicin 5, Pediocin AcH, Pediocin PA1, Pediocin A, Lactacin A, Lactacin F, Acidophilucin A, Plantacin B, Plantaricin A, Lacticin, Lactocin 27, Helveticin J, Brevicin 37, Sakacin A, Lactocin S, Bifidin, and Propionicin PLG-1 (Ray and Daeschel 1992). These bacteriocins possess narrow or wide spectra of activity. However, it is those with the wider range of activity that are desirable as food

biopreservatives. Characteristics that defined a bacteriocin have been expanded to include their chemical nature, stability, mode of action, genetic determination, inhibitory spectrum, sensitivity to various proteases, mode of synthesis, and toxicity level to animals (Ray and Daeschel 1992). The molecular weights of bacteriocins also vary greatly. They have been classified into small or large molecular-weight groups. In general, low molecular weight bacteriocins are sensitive to trypsin digestion but insensitive to heat inactivation (Bradley 1967). Caseicin 80, a large bacteriocin is produced by *Lactobacillus casei* and has a molecular weight of 40,000 daltons (Rammelsberg and Radler 1990). A well characterized low molecular weight bacteriocin (2500 daltons) is produced by *Lactobacillus acidophilus* (Muriana and Klaenhammer 1991).

Another group of amino acid-peptide antimetabolites of low molecular weight (< 5000 daltons) produced by enterobacteria has been reported. Microcin C7 is a bacteriostatic antibiotic of about 900 daltons in size. It is resistant to heat treatment (100 ^{o}C, 30 minutes) but sensitive to trypsin and subtilisin (Baquero and Moreno 1984). Amino acid analysis of Microcin C7 showed a linear octapeptide of moderate polarity. The structure consists of acetyl-methonine, arginine, threonine, glycine, asparagine, and alanine with an ethanolamine at its carboxyl terminus. Microcin D140 (~500 daltons) is a highly hydrophilic basic peptide with sensitivity to trypsin and subtilisin (Garcia-Busto et al., 1984). The spectrum of activity of these microcins includes inhibition for *Escherichia* spp. *Salmonella* spp., *Shigella* spp., *Citrobacter* spp., *Klebsiella* spp., and *Enterobacter* spp. However, *Pseudomonas* spp. and *Acinetobacter* spp. are resistant (Baquero and Moreno 1984).

In the United States, nisin is the only bacteriocin that is permitted to be incorporated into food systems (Anonymous 1988). It was first recognized and characterized by Rogers and Whittier (1928). Nisin is an basic compound

(anode migrating) produced by *Lactococcus lactis* ssp. *lactis* and is stable under very acidic conditions (Delves-Broughton 1990). Structurally, nisin consists of 34 amino-acids, including lanthionine and b-methyllanthionine, with a molecular weight of 3510. Nisin is inhibitory for Gram-positive but not Gram-negative bacteria, yeast or fungi. The use of nisin as a food preservative was first suggested by Hirsch et al. (1951) and the incorporation of nisin in canned food was first permitted in Britain (Anonymous 1959). In 1969, the Joint FAP/WHO Expert Committee on Food Additives gave approval for its use in food (Hurst 1966; Hurst and Dring 1968; Hurst 1981; Roller 1991). Currently, nisin is used as a preservative in cheese spread, processed cheese, and dairy desserts in some middle-eastern countries. It has been shown that nisin can be used to kill *Listeria monocytogenes* in cottage cheese and yogurt (Benkerroum and Sandine 1988).

Bacteriocins from various *Propionibacterium* species have been isolated and purified. Propionicin PLG-1, produced by *Propionibacterium thoenii*, has a wide inhibitory spectrum. It was found to be inhibitory against Gram-positive and Gram-negative bacteria (Lyon and Glatz 1991; Lyon et al., 1993). Jenseniin G, a bacteriocin isolated from *Propionibacterium jensenii* P126 has a narrow inhibitory spectrum, though it inhibits activity closely related classical propionibacteria, lactobacilli and lactococci (Grinstead and Barefoot 1992). Microgard™ (MG) is a product of *Propionibacterium freudenreichii* ssp. *shermanii* fermentation of grade A skim milk (Wesman Foods, Inc. Beaverton, Oregon). It has been demonstrated to prolong the shelf-life of cottage cheese against food spoilage psychotrophs (Salih 1990; Weber and Broich 1986). The proteineous antimicrobial ingredient in MG was shown to be active over a wide pH range, with optimum activity at pH 5.30 (Al-Zoreky 1991). First patented in Canada (Ayres et al., 1987) and later in the United States (Sandine et al.,

1992), MG has been approved by the Food and Drug Administration as a biopreservative and now is present in about 30% of the cottage cheese produced in the United States (Sandine et al., 1992; Sandine, 1993). Partial purification of MG has revealed a low molecular weight protein of ~700 daltons (Al-Zoreky 1988). Recent studies also showed that the bacteriocin in MG is proteineous in nature since it is inactivated by a-chymotrypsin, papain, and pepsin. However, it is not inactivated by trypsin, suggesting the presence of anti-trypsin inhibitors in MG. It is antagonistic toward Gram-positive bacteria, yeasts and some molds (Al-Zoreky 1988).

Despite their great potential, there are several limitations to use of bacteriocins as biopreservatives in food. Most of the well characterized bacterocins from lactic acid bacteria do not inhibit Gram-negative bacteria, which are common causes of food-borne illnesses. Furthermore, the level of bacteriocins production can be low thus ineffective in most food systems. Under plasmid control, some of these bacteriocins are also known to be unstable (Kim 1993).

It was the objective of this study to purify the bacteriocin from crude MG to homogeneity. It was desirable that all organic acids and non-active macromolecules be removed. Purified bacteriocin was then hydrolysed for determination of its amino-acid composition and its sequence studied by Edman degradation and mass spectroscopy.

MATERIALS AND METHODS

Bacterial Strains and Media

Bacteriocin producer *Propionibacterium freudenreichii* ssp. *shermanii strain 9616* was used in the fermentation of grade A skim milk, at 30 °C, neutralized to pH 6.30 and then pasteurized. The product MG was supplied by Wesman Food, Inc, Beaverton, OR. *Pseudomonas putida* ATCC 12633 was grown in lactose broth for 18 hours at 30 °C (OD_{700} 0.1). Plate count agar (Difco Laboratories, Detroit, Mich.) was adjusted with a 10% tartaric acid solution to pH 5.30 . 100 ml of dissolved Plate Count Agar was aliquoted into each dilution bottle. All media were autoclaved at 121 °C for 15 minutes.

Bacteriocin Detection and Activity Assay

A modified agar-well diffusion method (Tagg and McGiven 1971)) was used to examine the activity of the bacteriocin extracts from MG. Overnight culture of *Pseudomonas putida* ATCC 12633 was used as indicator strain for the routine bacteriocin assays. Plate count agar was tempered to 45 °C before being inoculated with 0.5 ml of overnight culture of indicator strain. This was followed by the addition of 2 ml of 2,3,5-triphenyltetrazolium chloride (1% stock) solution. A 100 ml of this mixture was poured onto each sterile petri-plate (VWR Scientific Company, Inc., West Chester, PA) and allowed to solidify at room temperature for 1 hour. The wells (0.75 cm diameter x 1.0 cm depth) in the plate count agar were aseptically created by a hole-borer. A volume of 100 ul sample was placed in each well. Bacteriocin titers were determined by a one to one serological dilution, i.e., one volume of bacteriocin diluted in one volume of

diluent (1:2). Diluent of 0.1% peptone water was used in the critical dilution assay. Activity was defined as the reciprocal of the last serial dilution demonstrating inhibitory activity and presented as activity units (AU) per milliliter. All culture plates were incubated at 30 °C for 18 hours.

Protein Determinations

Absorbance at 217 nm was used to monitor the protein in fractions from gel filtration chromatography. Active fractions from ultra-filtration, gel filtration, anion exchange, and HPLC were assayed for their protein contents by the method of Lowry et al. (1951) using bovine serum albumin as the standard (700 nm). The procedures for this assay are described in the Lowry Total Protein Assay Kit, No. 5656 (Sigma Chemical Co., St. Louis, MO).

Purification of Microgard™

All purification procedures were performed at 4 °C. unless otherwise stated.

Step 1. pH adjustment and Centrifugation

Crude dairy MG (50 ml) was adjusted to pH 4.00 with anhydrous tartaric acid (Sigma Chemical Co., St. Louis, MO) and centrifuged at 10,000 x g for 1 hour. The supernatant was decanted and back-neutralized to pH 5.30 with 10% tartaric acid solution. The supernatant was concentrated to a volume of 24 ml with a concentrator RC-1009 (Jouan, Inc. Winchester, VA). The conditions for concentrating the supernatant were set at 150 millitorr vacuum and a

temperature of -45°C for 1 hour. The supernatant (pH 5.30) was assayed for activity and total protein concentration.

Step 2. Ultra-Filtration

A total volume of 24 ml of concentrated supernatant was ultra-filtered using an Amicon filtration cell unit #8050 (Amicon Division, W.R. Grace & Co. Beverly, MA) An initial molecular weight cut-off membrane was used (YM 10 > 10000 MW) at a pressure of 30 psi. A total of 9.2 ml 10,000 MW ultra-filtrate (10,000-F) was collected. The retaintate (10,000-R) was washed with 0.01 M sodium acetate buffer (pH 5.3) and 9.2 ml ultra-filtrate (10,000-F) was placed onto another membrane of 5000 daltons (YM 5 > 5000 MW) to be filtered at a pressure of 40 psi. All retaintates (5000-R & 1000-R) collected were washed with 0.01 M sodium acetate buffer (pH 5.3). A volume of 6.2 ml of 5000 MW ultra-filtrate (5000-F) was filtered through a 1000 daltons cut-off membrane (YM 2 > 1000 MW) at 45 psi. This 1000 ultra-filtrate (5.0 ml) was filtered through a 500 daltons cut-off membrane (YC05 > 500 MW). A volume of 2.0 ml of 500 MW ultra-filtrate was collected. All collected samples were assayed for antimicrobial activity and total protein concentration.

Step 3. Amberlite IRA-400 (OH) Column Chromatography

An Amberlite-OH anion exchanger column (7 x 250 mm) was equilibrated with 1.0 M $NaCO_3$. After equilibrating with the $NaCO_3$ solution , the column was washed and re-equilibrated with 0.01 M sodium acetate buffer (pH 5.3) before loading of 1000 MW ultra-filtrate. Eluates from anion-exchanger

were collected and assayed for activity and protein concentration. GLC was used to monitor the active fractions for presence of organic acids.

Step 4. Sephedex G-10 Column Chromatography

The active fractions from step 3 were put onto a Sephedex G-10 column (10 x 400 mm) after equilibration with 0.01 M sodium acetate buffer, pH 5.30. The eluated active fractions were combined and concentrated by another Sephedex G-10 chromatography step. A single fraction was assayed for activity and protein concentration. L-Tyrosine (217.7 MW), Leu-Pro-Pro-Ser-Arg peptide(568.7 MW), and His-Pro-Phe-His-Leu-Leu-Val-Tyr peptide (1025.2 MW) were used (Sigma Chemical Co., St. Louis, MO) to establish a standard curve.

Step 5. Reversed-Phase Chromatography

The concentrated active fraction (250 ul) from step 4 was injected into a reversed-phase C-18 column (4 x 250 mm, Bio-Sil ODS-5S) equilibrated with 0.1% trifluoroacetic acid (TFA). The collection of HPLC fractions was done at room temperature with a linear gradient from 0 to 100% acetonitrile in 0.1% TFA with a flow-rate of 2.0 ml/min. All eluates were evaporated to dryness under vacuum (150 millitorr) at -45 °C for 1 hour and then rehydrated with cold 0.01 M sodium acetate buffer (pH 5.30) to a final volume of 500 ul. The eluates from reversed-phase high performance liquid chromatography were assayed for activity and total protein concentration.

Step 6. Amino acid composition and N-terminal amino acid sequence

Active single-peak fraction in sodium acetate buffer (pH 5.30) from the reversed-phase high performance liquid chromatography was sent to Central Services Laboratory, OSU (Corvallis, OR) and AAA Laboratory (Mercer Island, WA) for amino acid hydrolysis and sequencing. Amino acid sequence was determined by Edman degradation and mass spectroscopy.

RESULTS

Purification of Microgard™

Results of the purification process are summarized in Table 2.1 . The 500 MW ultra-filtrate (320 AU/ml) and retentate (160 AU/ml) were less active when compared to the 1000 MW (640 AU/ml) and 5000 MW ultra-filtrates (640 AU/ml). All retentates except 500 MW's (Figure 2.3 and 2.4) were less active when compared to the ultra-filtrates (Figure 2.1 and 2.2). The reduction in activity also corresponded to a lesser protein concentration in the retentates. Therefore, the 1000 MW ultra-filtrate was extracted for further purification by anion-exchange chromatography, gel filtration, and reverse-phase high performance liquid chromatography. The specific activities of fractions from the anion-exchanger (17.39 AU/mg) and gel filtration increased greatly (108.7 AU/mg). Reversed-phase high performance liquid chromatography, the final step of purification, produced a single peak of bacteriocin activity (Figure 2.5), which was recovered in about 4.4-6.0% acetonitrile (Figure 2.6). The overall purification process resulted in about 25.8-fold recovery and a low yield of 0.065% (Table 2.1).

Molecular weight determination

The molecular weight of purified bacteriocin was estimated to be 859.68 daltons by gel filtration (Figure 2.7). The eluting profile from gel filtration showed two active peaks between absorbance 0.80 and 1.30, respectively (Figure 2.8). The single and active fraction from gel filtration was also found to be inhibitory to indicator-organism.

The m/z data from mass spectroscopy showed two peaks that are in a series, 295 and 441.7 (Figure 2.9). They suggest charges of 3 (MH^{+3}) and 2 (MH^{+2}), respectively. The molecular weight was determined to be 881.7 (Figure 2.10) .

Amino acid composition

Initial amino acid hydrolysis of the single-peak fraction from HPLC (Central Services Laboratory, Oregon State Univ., Corvallis) revealed the presence of methionine, valine, glutamate, glycine, serine, and arginine in a decreasing order of concentration. Analysis from another laboratory (AAA Laboratory, Mercer Island, Washington) suggested that the peptide consists of 4 residues of methionine, 2 of valine, 1 of glutamic acid, and possibly 1 of glycine. The molecular weight was calculated to be 926 daltons.

DISCUSSION

The proteineous nature of the active and acid-free HPLC purified fraction confirmed that the antimicrobial agent present in MG was a low molecular weight bacteriocin. The presence of peptide bonds and proteineous nature of this inhibitory substance was also confirmed by a positive Biruet test with maximum absorbance at 210 nm (Barefoot and Klaenhammer 1984; Al-Zoreky 1988). In addition, the antimicrobial agent in MG was sensitive to a-chymotrypsin, pepsin, and papain but not trypsin or catalase (Al-Zoreky 1988). The trypsin-resistant characteristic was similar to Linecin A, a bacteriocin produced by *Brevibacterium linens* ATCC 9175. The low content of basic amino acids probably explains why Linecin A is resistant to trypsin (Kato et al., 1991).

The antimicrobial spectrum of MG is similar to Propionicin PLG-1, a bacteriocin produced by *Propionibacterium thoenii*; the latter also inhibts some Gram-negative bacteria, yeasts and molds (Lyon and Glatz 1991; Lyon et al., 1993). On the contrary, Jenseniin G produced by *Propionibacterium jensenii* has a narrower spectrum, being active against closely related classical propionibacteria, some lactococci and lactobacilli but not Gram-negative bacteria (Grinstead and Barefoot 1992). However, the molecular weight of the bacterocin in MG is smaller than Propionicin PLG-1 (10,000 daltons) and Jenseniin G (12,000 daltons). Initial studies by Al-Zoreky (1988) estimated the molecular weight of the partially purified antimicrobial substance at 700 daltons. This is consistent with the active fractions from ultra-filtration and gel filtration chromatography. Compared to other molecular weight cut-off filtrates and retentates, the fraction occurring between the 1000 MW and 500 MW cut-off membranes was the most active. This suggested that the molecular weight of

the antimicrobial agent in MG was less than 1000 daltons but greater than 500 daltons. Gel filtration estimated the size of this antimicrobial substance to be within the vicinity of 860 daltons. The molecular weight of this bacteriocin is similar to another antibiotic peptides isolated by Garcia-Bustos et al. (1984).

Active single-peak fraction from reversed-phase HPLC was recovered between the range 4.4-6.0% acetonitrile solvent with a retention time of 3.29 minutes. The eluting of active agent from the C_{-18} column at a low solvent concentration suggested a hydrophillic nature (Shugar and Dean 1990). In this aspect, MG is different from the hydrophobic Lacticin 481 (*Lactococcus lactis* ssp. *lactis* CNRZ 481) which required a higher concentration of organic solvent to elute from a reversed-phase HPLC column (Piard et al., 1992).

Overall, the purified bacteriocin in MG showed an increase in specific activity. Although there was a 25.8-fold recovery of the inhibitory substance, the yield was quite low (0.0065%), despite concentrating and scale-up injections of the original sample. It was suggested that a preparatory reversed-phase HPLC column be used to improve the total yield of the purified product (Ayres 1993).

Preliminary analysis of amino acid hydrolysate revealed the presence of methionine, valine, glutamate, glycine, serine, and arginine in decreasing order of concentration. The presence of methionine, valine, glutamic acid and glycine was confirmed by another laboratory in the state of Washington. Interestingly, a total of 4 methionine residues was recovered from the amino acid hydrolysate. Initial amino acid sequencing of the bacteriocin (Central Services Laboratory, OSU) yielded a methionine residue at the N-terminus. Other than valine from the second cycle of degradation, no amino acid was detectable upon direct N-terminal sequencing, indicating the possibility of presence of unusual amino acids or a cyclic oligopeptide (Mørtvedt et al., 1991). The release of a terminal

methionine moiety was seen during the Edman degradation of a microcin (Aquilar et al., 1982).

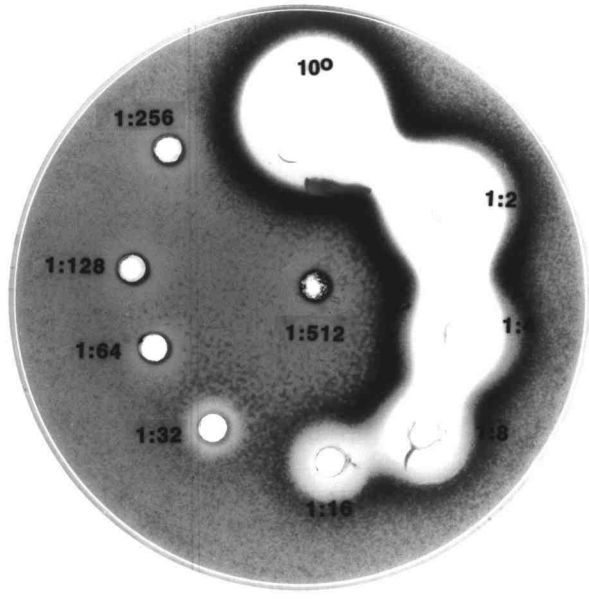
The antimicrobial activity of the purified bacteriocin from MG is similar to Microcin A15 (~500 daltons). The release of a methionine moiety under acidic conditions was also observed in Microcin A15 (Baquero and Moreno 1984). The antimicrobial mechanism of Microcin A15 was related to L-methionine in which homoserine-O-transsuccinylase was inhibited. The inihibition of homoserine-O-transsuccinylase blocks protein synthesis in the methionine biosynthesis pathway (Aquilar et al., 1982; Brush and Paulus; 1971; Lee et al., 1966). The bacteriocin from MG cannot resemble any fatty acids found at the amino termini of certain proteins and peptide antibiotics, because that will require much larger concentrations of methanol or acetonitrile to elute from the reversed-phase high performance liquid chromatography column (Alastair et al., 1982; Kimura et al., 1981; Carr et al., 1982).

Since there were blank cycles with the Edman degradation of purified MG, the possibility of unusual amino acids should not be ruled out. Nisin (Gross and Morell, 1971), subtilin (Gross and Kiltz, 1973) epidermin (Allgaier et al.,1985), Pep5 (Kellner et al., 1989), and gallidermin (Kellner et al., 1988) have been shown to contain unusual amino acids such as lanthionine or methyllanthionines (Stoffels et al., 1992). Some bacteriocins are known to contain modified amino-acids such as S-(2-aminovinyl)-D-cysteine, lysino-alanine, dehydroalanine, and dehydrobutyrine, the latter two being the precursors of lanthionine and 3-methyl-lanthionine (Kellner and Jung 1989). Incorporation of a lanthionine residue introduces a monosulfur bridge which results in unique peptide ring structures (Kellner et al., 1988 & 1989).

The molecular weight determined by gel filtration (859.7 daltons), amino acid analysis (926 daltons) and mass spectroscopy (881.7 daltons) confirmed that the bacteriocin in MG was in the vicinity of 700 daltons (Al-Zoreky, 1988).

Various examples on the mode of action of oligopeptides have been suggested. In one instance, an oligopeptide was thought to be involved in the depolarization of the membrane of sensitive cells (Aguilar et al., 1982). In another case, a methionine moiety was shown to inhibit methionine biosynthesis in treated cells (Duro et al., 1979). In any case, bacteriocins with some degrees of hydrophobicity were able to interact with cell membranes by forming pores, thus disrupting the bacterial transport functions (Muriana and Klaenhammer 1991). The uncertainty about the structure of the antibiotic peptide in MG is due to the fact that, to date, the recoverable yields of purified sample remain low.

(a)



(b)

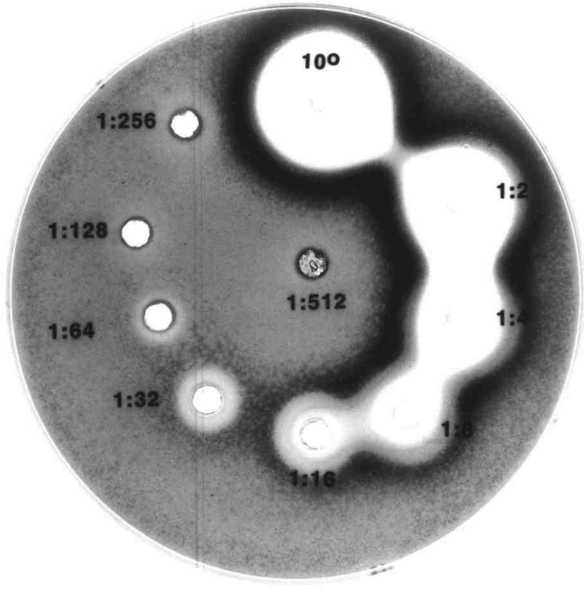
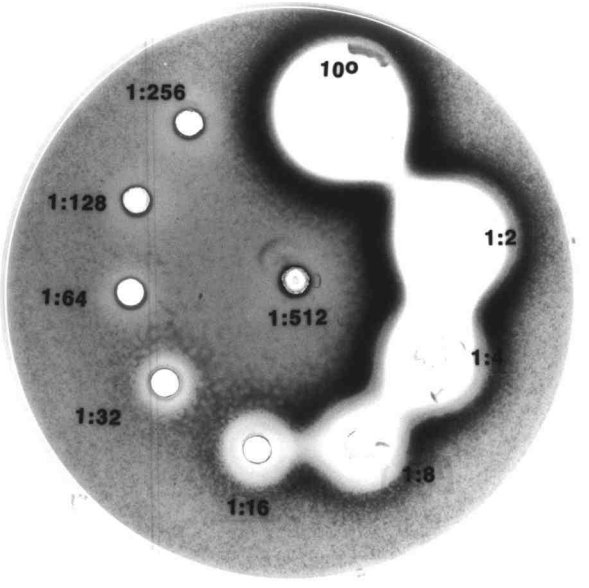


Figure 2.1 Serological dilutions of (a) 10,000 MW filtrate with an activity unit of 640 AU/ml (b) 5,000 MW filtrate with an activity of 640 AU/ml. Indicator organism was *Pseudomonas putida*.

(a)



(b)

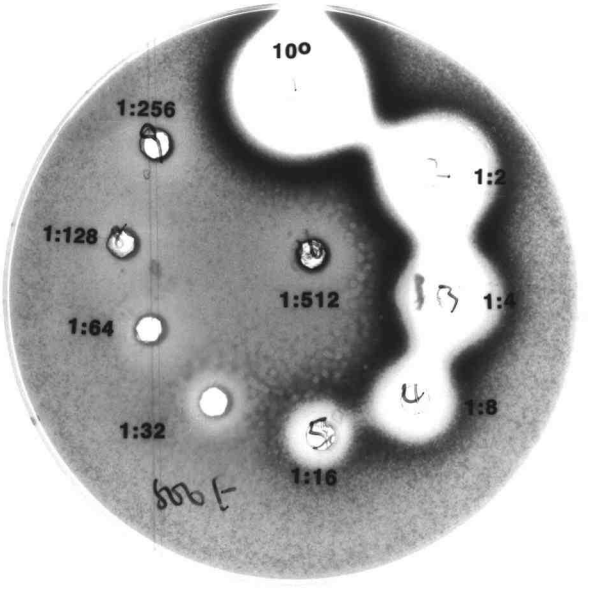
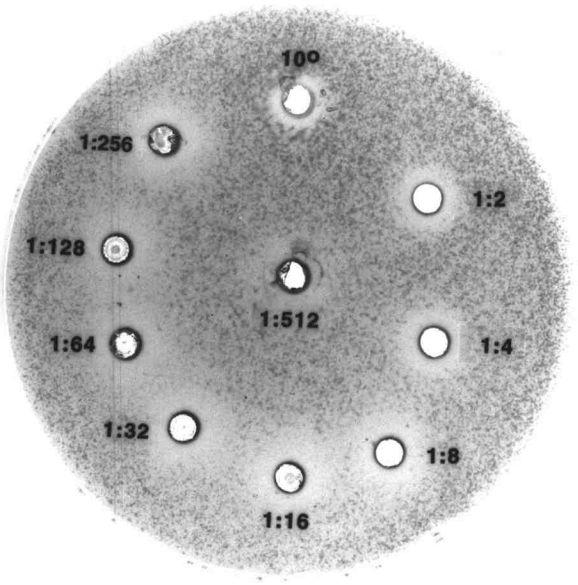


Figure 2.2 Serological dilutions of (a) 1,000 MW filtrate with an activity unit of 640 AU/ml (b) 500 MW filtrate with an activity of 320 AU/ml. Indicator organism was *Pseudomonas putida*.

(a)



(b)

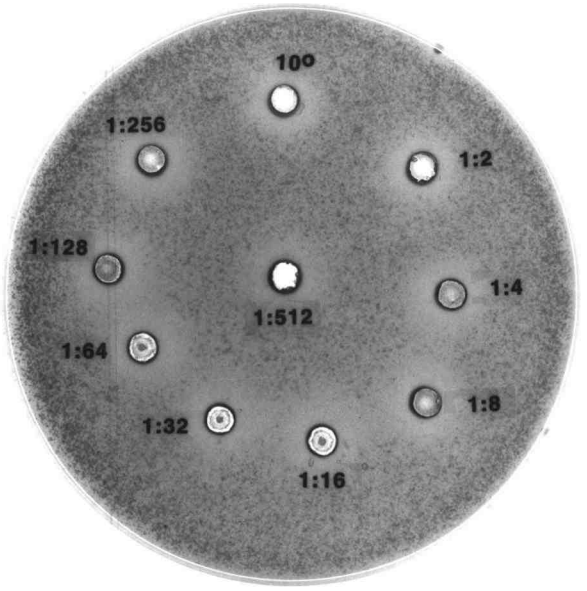
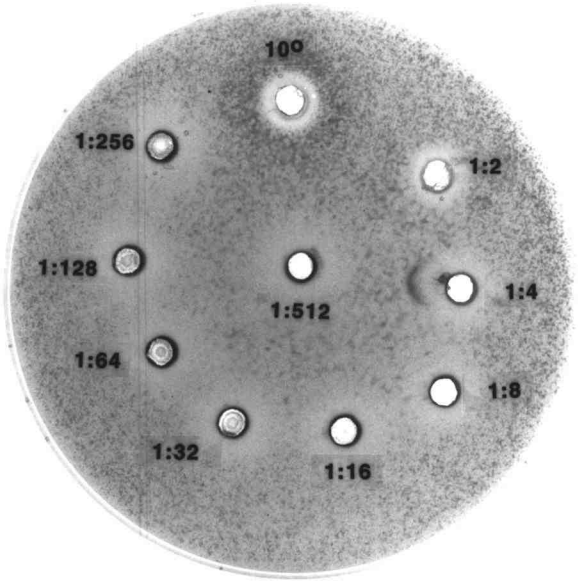


Figure 2.3 Serological dilutions of (a) 10,000 MW retentate with an activity unit of 40 AU/ml (b) 5,000 MW retentate with an activity of 1 AU/ml. Indicator organism was *Pseudomonas putida*.

(a)



(b)

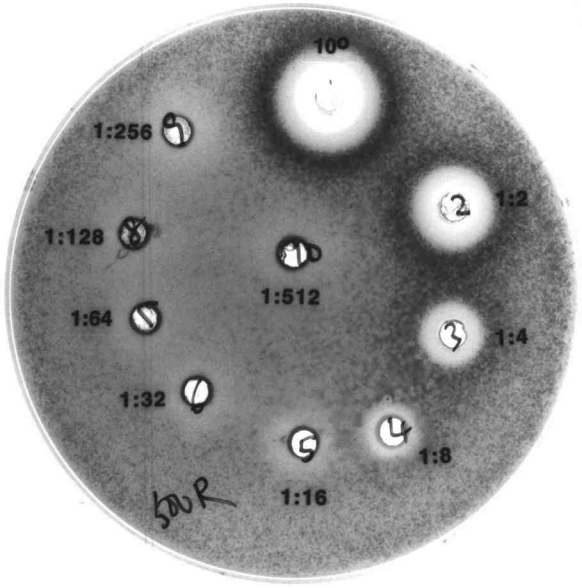


Figure 2.4 Serological dilutions of (a) 1,000 MW retentate with an activity unit of 10 AU/ml (b) 500 MW retentate with an activity of 160 AU/ml. Indicator organism was *Pseudomonas putida*.

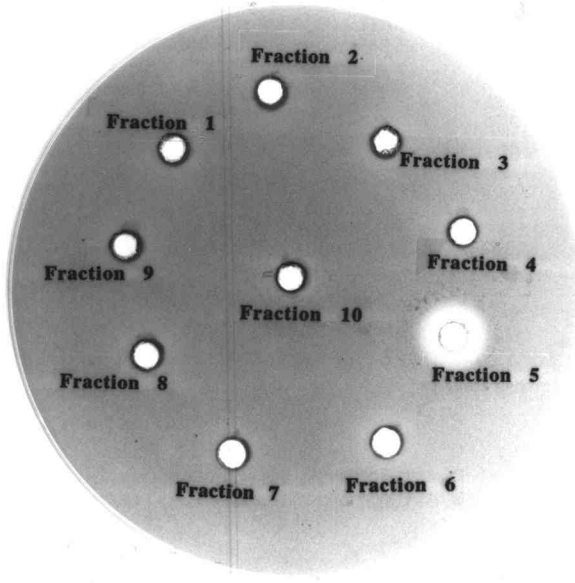


Figure 2.5 Well activity assay of fractions from reversed-phase high performance liquid chromatography (Bio-Sil ODS-5S, C_{18} -column); fraction 5 was active against indicator-organism,*Pseudomonas putida*.

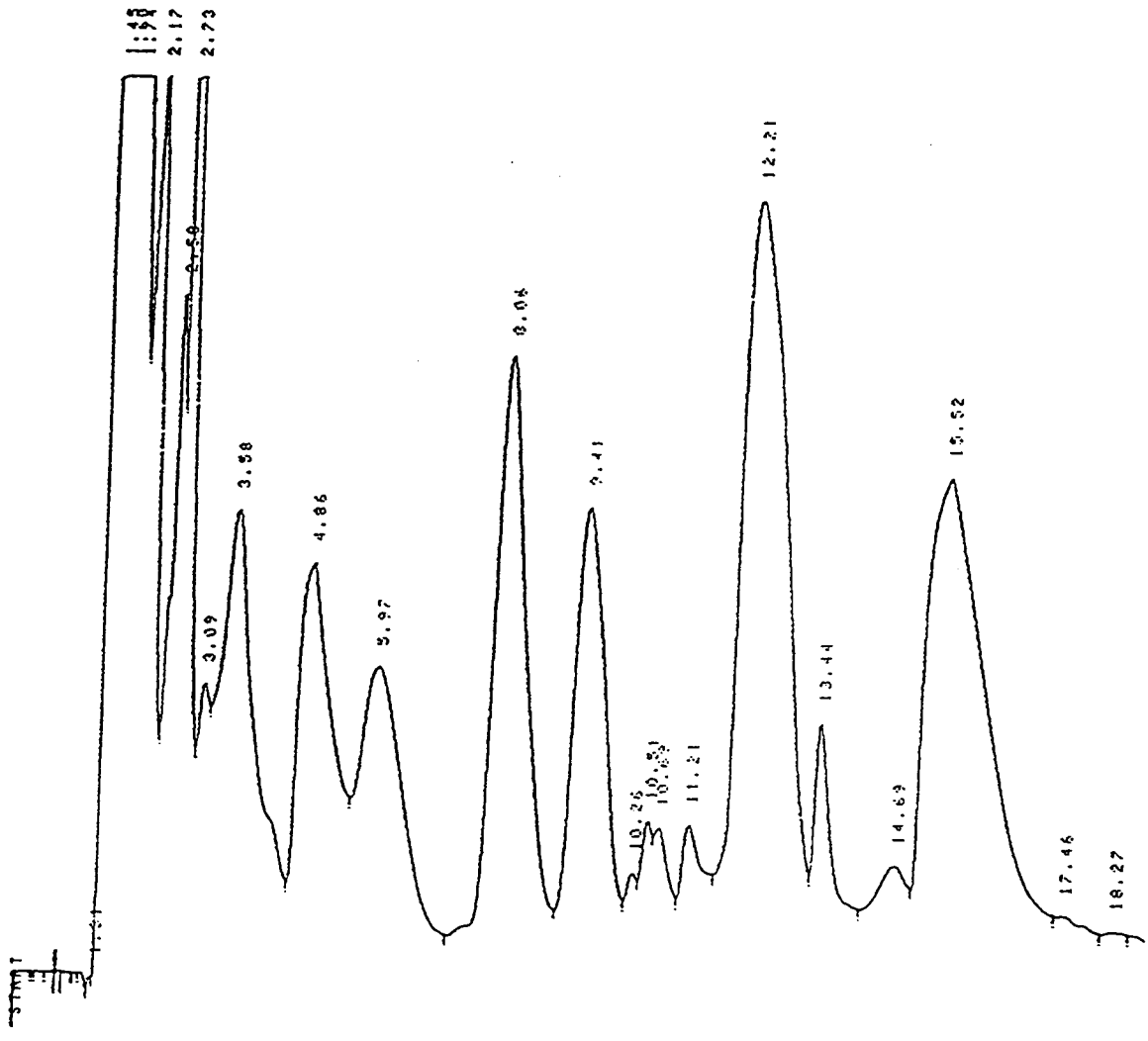


Figure 2.6 Isolation of bacteriocin from reversed-phase high performance liquid chromatography (Bio-Sil ODS-5S, C_{18}-column); retention time 3.58. The attenuation level for instrument was 4

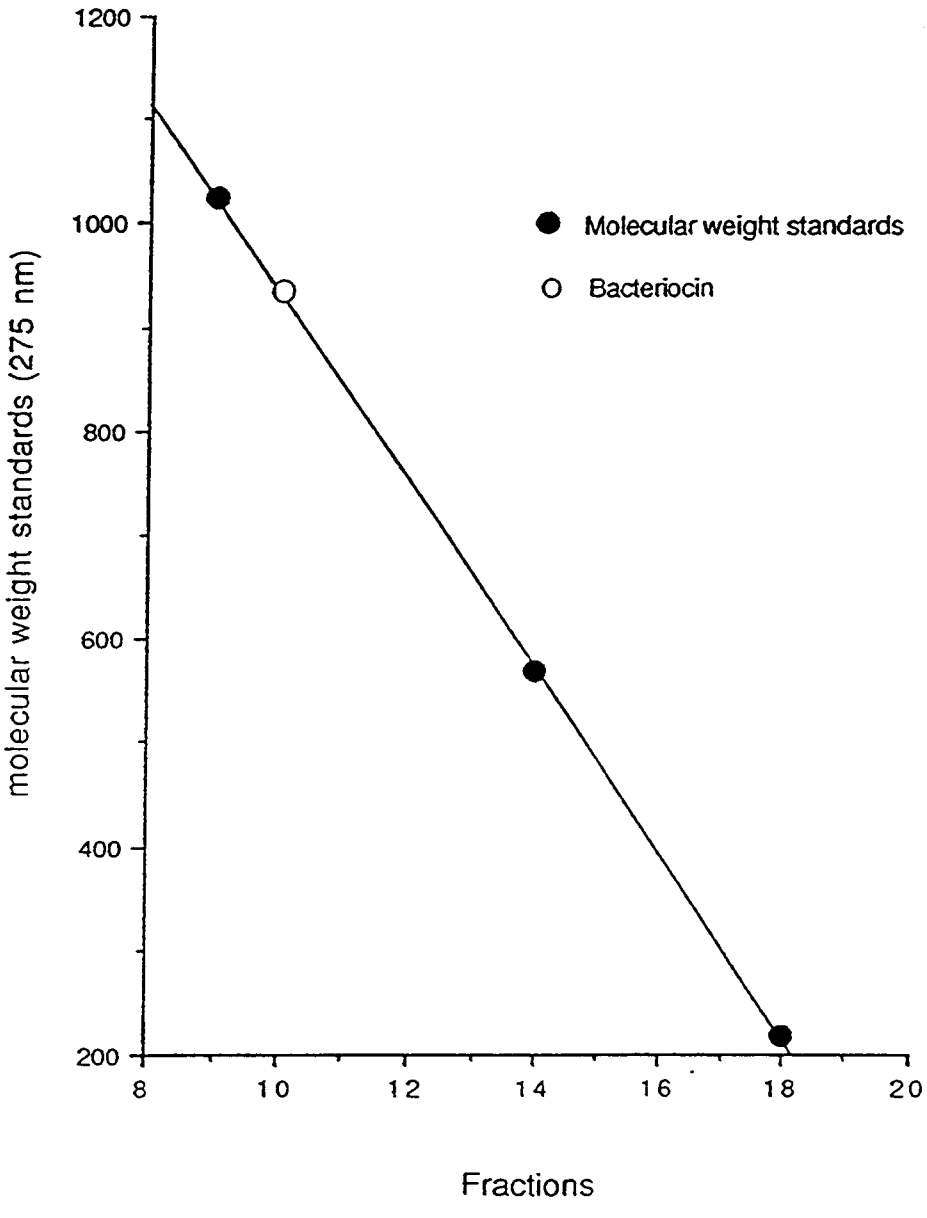


Figure 2.7 Molecular weight determination (859.68 daltons) of Microgard™ bacteriocin by gel filtration, Sephedex G-10

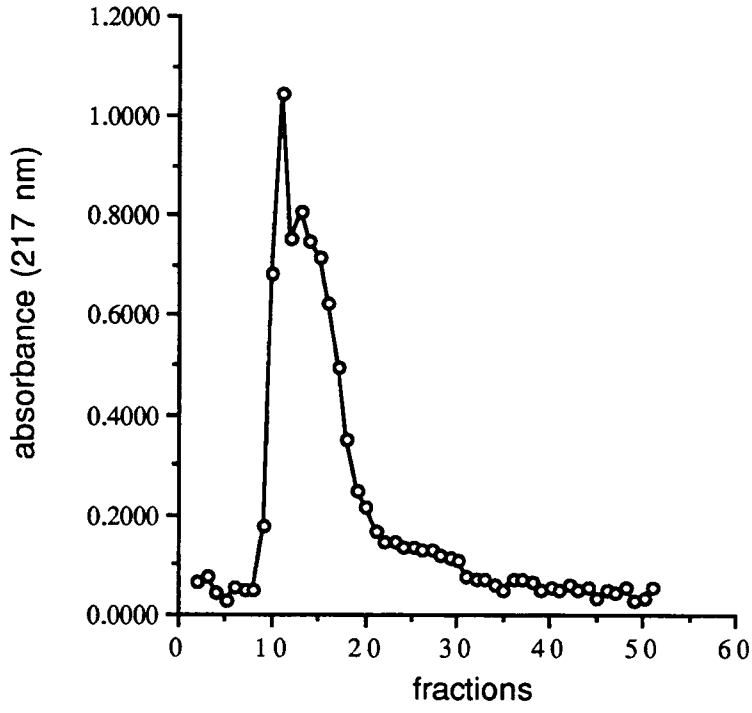


Figure 2.8 Eluting profile of combined active anion-exchanged Microgard™ fractions from Sephedex G-10 column

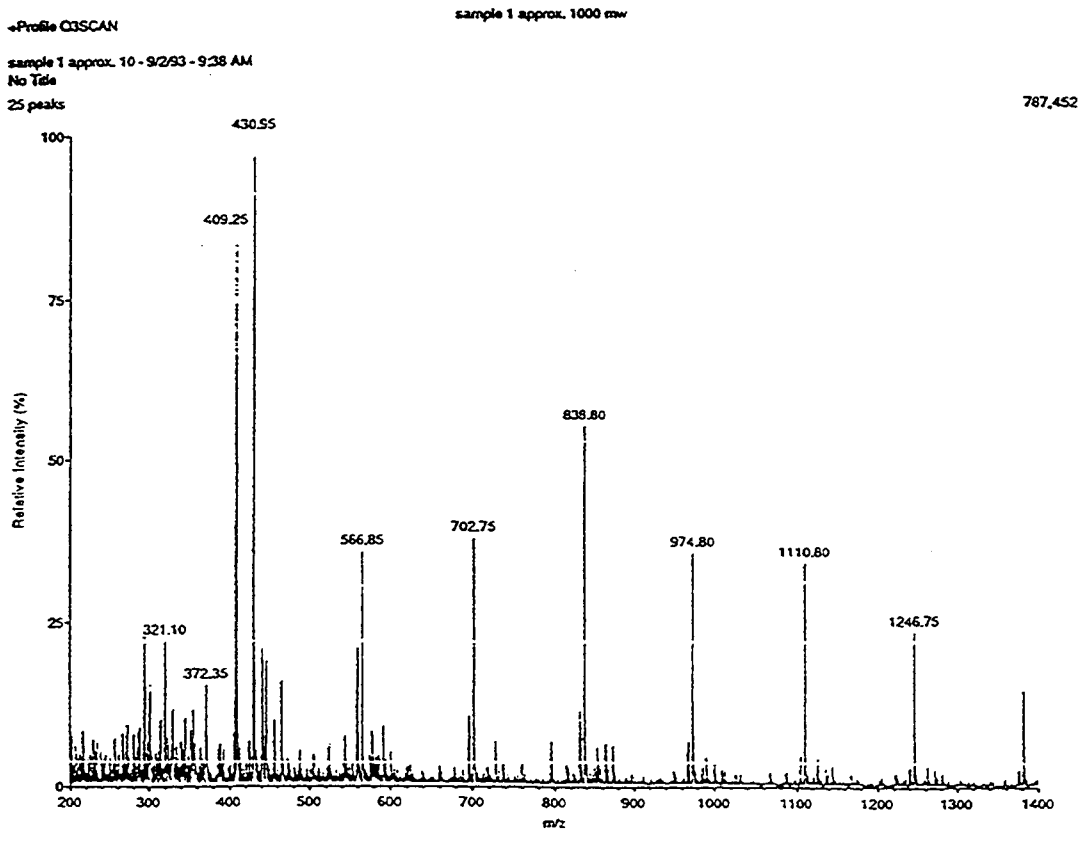


Figure 2.9 Mass spectrometric analysis (ESMS) of Microgard™ active peaks from reversed phase HPLC C_{18} -column (Bio-Sil ODS-5S)

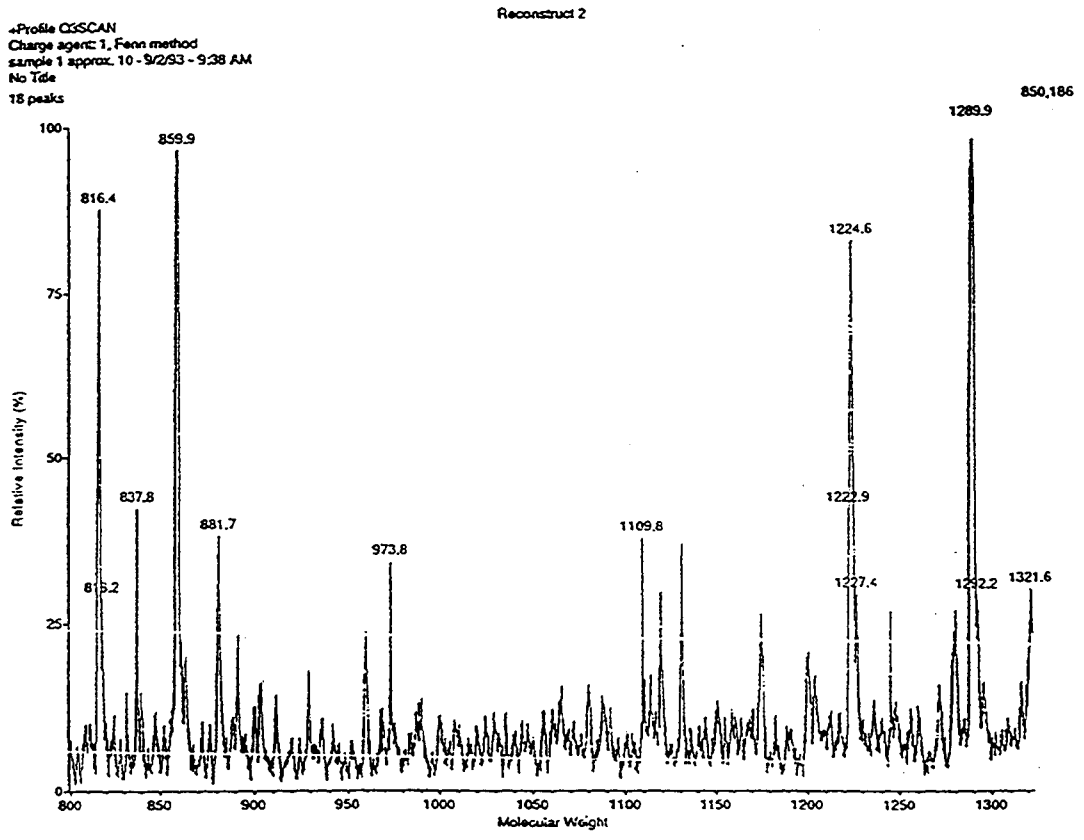


Figure 2.10 Molecular weight determination of active Microgard™ HPLC fraction by mass spectroscopy (ESMS)

Table 2.1 Purification of Microgard™

Sample	ml (vol)	AU/ml	Total AU	Total Protein mg/ml	Specific Activity AU/mg	Activity recovered (%)	Fold Purification
Crude Microgard	24	640	15360	38.01	16.84	100	1.00
10 K (F)	9.2	640	5888	26.91	23.91	38.3	1.42
10 K (R)	14	40	560	3.12	12.82	3.65	-
5 K (F)	6.2	640	3968	23.97	26.70	25.8	1.59
5 K (R)	9.2	1	9.2	0.038	26.04	0.06	-
[a]1 K (F)	5.0	640	3200	14.74	43.42	20.83	2.58
1 K (R)	1.0	10	10	16.14	0.620	0.065	-
500 (F)	2.0	320	640	8.48	37.74	4.167	2.24
500 (R)	1.0	160	160	1.45	110.34	1.042	-
[a]Anion Exchange -Amberlite	1.2	40	48	2.30	17.39	0.313	1.03
[a]Gel Filtration -G 10	1.0	10	10	0.092	108.7	0.065	6.45
[a]HPLC -reversed phase C_{18}	0.250	20	5	0.046	434.78	0.033	25.8

Note: "a" indicates that 1K-(F) was used for further purification

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CHAPTER 3

ENHANCEMENT OF MICROGARD™ ACTIVITY AGAINST FOOD SPOILAGE AND PATHOGENIC MICROORGANISMS

ABSTRACT

The antimicrobial spectrum of Microgard™ (MG) was extended to Gram-positive and additional Gram-negative bacteria. Application of MG in combination with the chelating agents ethylenediaminetetraacetate (EDTA) or sodium citrate resulted in the inhibition of *Listeria monocytogenes* ATCC 894, *Salmonella typhimurium* ATCC 79631, *Escherichia coli* O157:H7, and *Pseudomonas aeruginosa* ATCC 10145. The chelating agents were found to further potentiate the activity of MG against *Pseudomonas putida* ATCC 12633, the orginal assay organism. Chelating agents also increased the activity of MG against contaminating yeasts such as *Saccharomyces cerevisiae* var *ellipsoideus* 1B15 and *Candida albicans* ATCC 18804. The concentrations of chelating agents that were required for the enhancement of the activity of MG varied with different bacteria. However, it was established that 2.0% sodium citrate and 0.74% EDTA were sufficient in enhancing the inhibitory activity of MG. Further increase in the concentrations of chelating agents alone were found to be inhibitory to *Ps. putida*. In the present study, the inhibition of Gram-negative bacteria by a combination of MG and citrate was found to be a time-dependent phenomenon.

Unlike sodium citrate, increasing concentrations of EDTA alone were found to exert some inhibitory activity against tested bacteria. The time period

necessary for MG to kill sensitized cells (221 x 10^2 CFU/ml) was determined to be 30 minutes.

INTRODUCTION

A fundamental difference among Gram-positive and Gram-negative prokaryotes concerns the structure of the cell wall. In Gram-negative bacteria the wall is distinctively different from Gram-positive bacteria in that the former have an outer membrane layer consisting of a thin underlying peptidoglycan layer (Murray et al., 1965; Glauert and Thornley, 1969). The membrane itself consists of phospholipids, proteins and lipopolysaccharides (Nikaido and Nakae, 1979). It is the lipopolysaccharide molecules that account for the large number of negative charges within the outer membrane (White et al., 1972). These molecules are anchored in the outer membrane, through hydrophobic interactions with Lipid A (Yamada and Mizushima, 1980).

In *Escherichia coli* and *Salmonella typhimurium*, these outer membranes have been studied extensively in recent years. The strong electrostatic repulsion between polyanionic molecules and acidic proteins on the outer portion of the membrane is the major destabilizing force in the membrane (Schmitges and Henning, 1976). Therefore, stabilization of this membrane comes from divalent cations, which probably play an important role in neutralization and bridging between adjacent lipopolysaccharide molecules (Nikaido and Nakae, 1979; Lugtenberg, B., and L. van Alphen, 1983; Nikaido and Vaara, 1987).

Magnesium (Mg^{2+}) and calcium (Ca^{2+}) ions have been shown to stabilize disrupted outer membranes of *E. coli* (Nikaido and Nakae, 1979) and *Pseudomonas aeruginosa* (Gray and Wilkinson, 1965). The combination of negative charges and divalent cation cross-bridging of lipopolysaccharide molecules provide Gram-negative bacteria with resistance to lysozyme, hydrophobic antibiotics (Smit and Nikaido, 1978; Hancock 1984; Nikaido and

Vara, 1987), bile salts, lipases, proteases, and detergents (Nikaido and Nakae, 1979; Lugtenberg, B., and L. van Alphen, 1983). The addition of Ca^{2+} and Mg^{2+} helps to restore the resistance of cells injured by lysozyme (Ray, 1993). The strong association between divalent cations and the outer membrane also excludes large hydrophillic and most hydrophobic and amphiphillic molecules (Nikaido and Nakae, 1979; Hancock, 1984; Nikaido and Vara, 1987). It has been hypothesized by Nikaido and Nakae (1979) that when hydrophillic molecules pass through the outer membrane, they do so by means of water-filled pores and carrier-mediated transport.

The removal of divalent cations by EDTA from the outer membranes of *E. coli* and *Ps. aeruginosa* results in the release of lipopolysaccharide (Leive, 1965; Rogers et al., 1969; Roberts et al., 1970). By chelating Mg^{2+} from the outer membrane sites, there is an enhancement of killing by polycationic antibiotics (Sykes and Morris, 1975) as well as the uptake of lysozyme and b-lactams. The presence of divalent cations indicates that the lipopolysaccharide has a single class of divalent cation-binding sites that are responsible for the permeability alterations of the outer membrane (Coughlin et al., 1981; Schinder and Osborn, 1979). It has been estimated that EDTA causes the loss of 30% and 67% of the total lipopolysaccharide in *E. coli* at 37 °C or 4 °C, respectively (Leive et al., 1968; Graham et al., 1979). The alteration of lipopolysaccharide molecules in Gram-negative bacteria also enables bacteriocins to come in contact with the cytoplasmic membrane (Ray, 1993). However, even in the absence of lipopolysaccharide synthesis, injured cells can regain barrier function by reorganizing exisiting molecules in the outer membrane (Ray, 1993).

It has been observed that when *Pseudomonas fluorescens* was grown on succinate, the bacteria became sensitive to the permeabilizing effects of

EDTA (Walker and Durham, 1975). Also, the incorporation of phosphate has been shown to cause the outer membrane of *P. fluorescens* to bind to polymyxin B (Dorrer and Teuber, 1977).

In Gram-positive bacteria, the sulfhydryl groups of *Bacillus cereus* spores are critical for proper membrane function. These groups are potential sites to which antibiotics and bacteriostatic agents are attracted. Inhibition of outgrowing *B. cereus* spores is a consequence of interfering with specific sulfhydryl groups since they exist in a sterically unhindered environment, offering accessibility to antibiotic agents (Morris et al., 1984). Dehydroalanine residues in nisin are thought to interact with these membrane sulfhydryl groups, resulting in the loss of cell viability (Gross and Morrell, 1967, 1971; Liu and Hansen, 1990). Inactivation of cells also could be due to disruption of the proton motive force or loss of membrane integrity (Kordel et al., 1986; Liu and Hansen, 1990). Use of EDTA as chelating agent to alter Gram-positive bacterial cell walls has not been reported.

Nisin, once thought to be inhibitory for only Gram-positive bacteria , has been demonstrated to inhibit *Salmonella* spp. and other Gram-negative organisms when used with the chelating agent EDTA (Blackman et al., 1989; Stevens et al., 1991). Cells treated with either EDTA or nisin were not inhibited (Stevens et al., 1991). The inhibition of *Salmonella* spp. by a nisin-EDTA combination is a time dependent phenomenon (Stevens et al., 1991). It was also demonstrated that cellular contents were released (Hancock 1984; Nikaido and Vaara, 1987) when *Salmonella* spp. were treated with the combination of nisin and EDTA (Stevens et al.,1992a). Combination of nisin with ethylenebis (oxyethylene-nitrilo) tetraacetic acid, citric acid monohydrate, and sodium phosphate dibasic was examined by Stevens et al. (1992b) for effect on Gram-negative bacterial cells. It was found that the inhibitory effect from the use of

citrate was comparable to the EDTA treatment. Polyphosphates could be used effectively but only at a low pH (Stevens et al., 1992b).

In this study, sodium citrate and EDTA were used to potentiate the activity of MG against selected Gram-positive bacteria, Gram-negative bacteria and yeasts. Various combinations of sodium citrate and MG were used to demonstrate the enhancement against common food spoilage organisms in cottage cheese. The effects of treatment conditions for various organisms were evaluated. Both EDTA and sodium citrate were used to sensitize bacteria and yeasts before the application of MG. In addition, the period of exposure to citrate-MG was compared to the rate of cell death.

MATERIALS AND METHODS

Preparation of Microgard™

Liquid dairy MG (pH 6.38) and non-dairy MG (pH 6.32) were obtained from Wesman Foods, Inc., Beaverton, OR. Initially all MG was adjusted to pH 4.00 with sterile anhydrous tartaric acid (Sigma Chemical Co., St. Louis, MO) to precipitate casein, other milk proteins and dead bacterial cells. The acidified sample was centrifuged at 10,000 rpm for 30 minutes. Supernatnant was decanted into another container and back-neutralized with 1.0 N NaOH to pH 5.30 . The final supernatant was filter-sterilized using a 0.45 μm pore diameter filter (Nalge Co., Rochester, NY). All pH adjustments were measured with a Corning 125 pH meter at room temperature.

Preparation of growth media for bacteria

Plate count agar (PCA) was prepared and aliquoted (100 ml) into dilution bottles. The pH of medium was adjusted with 10% tartaric acid solution to pH 5.30 . Lactose broth, pH 7.00 (Difco Laboratories, Detroit, MI) was used to cultivate *Pseudomonas* strains and *Escherichia coli* .

Brain and heart infusion agar, pH 5.30 (Difco Laboratories, Detroit, MI), was prepared and aliquoted (100 ml) into dilution bottles. Brain heart infusion broth was used to grow *Listeria monocytogenes* ATCC 894 and *Salmonella typhimurium* ATCC 79631 at 30 °C. All media were autoclaved at 121°C for 15 minutes.

Preparation of growth media for yeasts

Potato dextrose agar (Difco Laboratories, Detroit, MI) was autoclaved at 121 °C for 15 minutes before adjustment to pH 3.50 with 10% sterile tartaric acid solution (Schwab, 1984). Mycophil broth (Difco Laboratories, Detroit, MI) was used to cultivate *Saccharomyces cerevisiae* var *ellipsoideus* 1B15 and *Candida albicans* ATCC 18804 at 30 °C.

Preparation of buffers and chelating agents

Citrate buffer (0.1 M), pH 5.30, was prepared by titrating 0.1 M citric acid with 0.1 M sodium citrate. HEPES (Sigma Chemical Co., St. Louis, MO) buffer was titrated to pH 5.30 with 0.1 N HCl solution. Sodium acetate (0.01 M) buffer, pH 5.30 was prepared by titrating with 0.1 M acetic acid.

The composition of cell buffer (CB) was 50 mM Tris-HCl (pH 5.30), 1mM $MgSO_4$, 4mM $CaCl_2$, and 0.1% gelatin (Sigma Chemical Co., St. Louis, MO & Difco Laboratories, Detroit, MI). Final pH of 5.30 was achieved with 1N HCl or 1N NaOH. Stock solutions of 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% sodium citrate were prepared in CB. All sodium citrate solutions were titrated to pH 5.30. Stock solutions of 0.037% (1 mM), 0.1861% (5 mM), 0.37% (10 mM), 0.56% (15 mM), and 0.74% (20 mM) ethylenediaminetetraacetate (EDTA) were prepared in CB, pH 5.30. Peptone water (0.1%) was used as diluent (Difco Laboratories, Detroit, MI). All buffers and diluents were filter-sterilized by 0.45 μm pore diameter filters (Nalge Co., Rochester, NY).

The effect of Microgard™ in various buffers

Dairy MG was diluted with different amounts of 0.1M citrate buffer (pH 5.30), HEPES buffer (pH 5.30) and distilled water. A seriological dilution scheme was adopted. Each dilution was assayed for activity at pH 5.30. The disk-activity assay involved adding of 0.5 ml *Pseudomonas putida* ATCC 12633 to 100-ml of PCA (Difco Laboratories, Detroit, MI). The staining reagent, 1% 2,3,5-triphenyltetrazolium chloride (2 ml), was added to the molten agar. A total of 18 ml was aliquoted into 100 x 15 mm sterile petri dishes (VWR Scientific Inc., West Chester, PA). Upon solidification, 1/2-inch diameter (13 mm) sterilized filter paper disks (Schleicher & Schuell Inc., Keene, NH) were aseptically placed on each agar. A volume of 100 µl sample was placed on the surface of the paper disks. Plates were incubated at 30° C, agar-side down, for 18 hours.

Enhancing effect of sodium citrate on the inhibitory activity of diluted Microgard™

All procedures pertaining to disk-activity assay in this experiment remain the same as have been described above, except for the further addition of different sodium citrate concentrations (0.05%, 0.1%, and 0.2%) into 100-ml PCA (Difco Laboratories, Detroit, MI). Non-dairy MG was serologically diluted and assayed for activity. The final pH of PCA was adjusted to 5.30 before assaying against *Pseudomonas putida* ATCC 12633 . Plates were incubated at 30° C, agar-side down, for 18 hours.

Effect of chelating agents with Microgard™ and incubation time on bacterial growth

Overnight culture (OD_{600} 0.1) of *Pseudomonas putida* ATCC 12633 was centrifuged at 13,000 X g for 5 minutes. The havested cells were washed twice with CB, pH 5.30, before resuspending (1 ml) in various concentrations of sodium citrate or EDTA solutions. All sodium citrate/EDTA treated cells were incubated at 30 °C for 30 minutes. After incubation, the treated cells were washed twice with CB, pH 5.30. Subsequently, cell pellets were suspended in non-dairy MG at 30 °C for 15, 30, 60, 90, 120, or 150 minutes. After each incubation period, each batch of samples was washed and resuspended in CB before plating for viable cell counts. All culture plates were incubated at 30 °C for 18 hours.

Effect of chelating agents combined with acetic and propionic acids against Gram-negative bacteria

Acid-mixtures consisting of 12,206.7 ppm (12.21 µg/ml) acetic acid and 5393.16 ppm (5.4 µg/ml) propionic acid were used to simulate the concentrations of these acids in crude MG. Various concentrations of citrate and EDTA, in CB (pH 5.30), were used to sensitize the Gram-negative organisms for a period of 30 minutes. After 30 minutes of incubation, and washing in CB, pH 5.30, the acid-mixtures were used instead of MG to interact with the bacteria for another 30 minutes. After the incubation period, the cells were retrieved for viable cell counts. Two acid-mixtures were used in this experiment; the original preparation and a 1:2 diluted acid-mixture.

Effect of chelating agents on the activity of Microgard™ against Gram-negative & Gram-positive bacteria and yeasts

Overnight cultures (OD_{600} 0.1) of *Pseudomonas putida* ATCC 12633 , *Pseudomonas aeruginosa* ATCC 10145, *Escherichia coli* O157:H7, *Salmonella typhimurium* ATCC 79631, *Listeria monocytogenes* ATCC 894, *Saccharomyces cerevisiae* var *ellipsoideus* 1B15, and *Candida albicans* ATCC 18804 were harvested by centrifuging at 13,000 X g for 5 minutes. The cells were washed twice with CB, pH 5.30, and then resuspended (1 ml) in various concentrations of sodium citrate or EDTA solutions. The suspensions were incubated in a 30 °C water bath for 30 minutes. After incubation, cells were centrifuged at 13,000 X g for 5 minutes, and then washed twice with CB, pH 5.30. The cell-pellets were resuspended in undiluted MG (1 ml) and then re-incubated at 30 °C for another 30 minutes. After the incubation period, all cells were centrifuged and washed twice with CB, pH 5.30. The cell-pellets were resuspended in a final 1 ml-volume of CB before plating for viable cell counts. The controls included untreated cells, cells treated with sodium citrate or EDTA alone, and MG-treated cells alone. All culture, except *E.coli* (37°C) were incubated at 30 °C for 18 hours.

The effect of sodium citrate on Microgard™ activity against Gram-negative bacteria and yeasts in cottage cheese

Cottage cheese and half and half cream (12% milk fat) were obtained from Albertson's Food Center. A 100-ml volume of half and half cream was aliquoted into steriled 250-ml Eryermeyer flasks. A total of 0.25 grams of spray-dried dairy MG was added to each flask and mixed evenly. Various

concentrations of sodium citrate (0.1%, 0.5%, 1.0%, 1.5%, and 2.0%) were incorporated into the half and half cream. The pH of this mixture was adjusted to 5.30 before incorporating into cottage cheese. A total of 1 ml overnight *Pseudomonas putida* (10^7 CFU/ml) culture was inoculated before any addition of cottage cheese (150 grams) into each flask. Negative controls were set up for various concentrations of sodium citrate and MG. Positive controls involved the inoculation of bacteria without the addition of sodium citrate or MG. All experimental samples were incubated at 8°C (Bodyfelt and Davidson, 1975) for 2,3,4,7,10,15, or 21 days.

For viable cell counts, 11 grams of cottage cheese were added to a stomacher with 99 ml 0.1% peptone water diluent. All samples were diluted appropriately and 0.1 ml of the suspension was plated for bacterial cell counts. The pH for all inoculated samples were measured by a Corning 125 pH-meter at room temperature. The pH for all samples was adjusted to 5.30 when necessary.

Effect of sodium citrate on the activity of Microgard™ against Gram-negative bacteria and yeasts in half and half cream

Volumes of 100 ml half and half cream (12% milk fat) were aliquoted into sterile 250-ml Eryermeyer flasks. Net weight of 0.25 grams spray-dried dairy MG was mixed evenly into the cream. Concentrations of 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% of sodium citrate were incorporated into the half and half cream. The pH of this mixture was adjusted to 5.30. Overnight cultures of *Pseudomonas putida* , *Saccharomyces cerevisiae* var *ellipsoideus* 1B15 , or *Candida albicans* ATCC 18804 were diluted to approxmately 100 cells per ml before inoculating the half and half cream. Negative controls were set up for

various concentrations of sodium citrate and MG. Positive controls involved the inoculation of bacteria or yeasts without the addition of sodium citrate or MG. The samples containing *Ps. putida*, *S. cerevisiae*, and *C. albicans* were incubated at 8 °C (Bodyfelt and Davidson, 1975) for 28, 26, and 33 days respectively.

All samples were diluted appropriately and 0.1 ml aliquots plated for bacterial cell counts. The pH for all inoculated samples was measured using a Corning 125 pH-meter at room temperature. The pH for all samples was adjusted to 5.30 when necessary.

RESULTS

Effect of Microgard™ activity in distilled water, HEPES and citrate buffers on bacterial cells

Serological dilutions of MG in citrate buffer (pH 5.30) produced an enhancement in activity (Table 3.1). There were two active fractions resulting from citrate-diluted MG (1:128 and 1:8192 dilutions). An activity unit (AU) is defined as recipocal of the highest dilution yielding a definite inhibition zone on the indicator-bacterial lawn. Therefore, the active fractions were calculated to be 1280 activity units per ml (AU/ml) and 81920 AU/ml, respectively. However, dilution with distilled water and HEPES buffer, pH 5.30, produced no active fraction after 1:64 dilution. The activity units for MG in water and HEPES buffer were calculated to be only 640 AU/ml.

Enhancing effect of sodium citrate on the inhibitory activity of diluted Microgard™

Compared to MG controls, an increase in citrate concentrations was shown to enhance the inhibitory activity of MG (Figure 3.1 and Figure 3.2). Furthermore, with 0.1% citrate, the activity of diluted MG (1:64) was shown to be enhanced (Figure 3.3). The citrate controls were not inhibitory to the assay organism.

Effects of chelating agents with Microgard™ and incubation time on bacterial growth

Results indicated that increases in citrate concentration potentiated the activity of MG (Figure 3.4 - Figure 3.8). It was also observed that after an incubation period of 60 minutes, the bacteria were completely inhibited by MG, with or without the presence of citrate. In addition, the 1.0% citrate-MG combination, after 30 minutes of incubation, produced the optimum inhibitory effect on *Pseudomonas putida* . However, an increase in citrate concentration beyond 1.0% did not further enhance the activity of MG. Controls showed that the increase in the citrate concentrations alone did not inhibit *Ps. putida* .

After 15 minutes of incubation, the activity of MG was shown to be enhanced by the increase in the concentrations of EDTA (Figure 3.9 - 3.13). However, with 0.037% and 0.1861% EDTA, MG was enhanced only after 15 minutes but not 30 minutes of incubation (Figure 3.9 & Figure 3.10). Results also indicated that with 0.56% or higher concentrations, the combination of EDTA-MG completely inihibited the bacteria after 30 minutes of incubation (Figure 3.12 & Figure 3.13). However, the EDTA controls showed that progressive increments in EDTA concentration also resulted in inhibitions of *Ps. putida* .

Effect of chelating agents on acetic and propionic acids against Gram-negative bacteria

The increase in concentrations of sodium citrate did not cause the activity of acid-mixture (undiluted) to be more active against *Pseudomonas putida* (Figure 3.14). On the contrary, as the percent concentrations of citrate

increased, the acid-mixture was rendered less effective in inhibition of the Gram-negative organism. The effect of EDTA on acetic and propionic acids resulted in an approximately 2-$\log_{10}$ reduction of *Ps. putida* (Figure 3.15).

The sodium citrate in diluted acid-mixture (1:2) caused more than a 2-$\log_{10}$ reduction in bacterial cell counts (Figure 3.16). EDTA was shown to enhance the activity of diluted acid-mixture but less effectively than the undiluted mixture (Figure 3.17).

Enhancement by chelating agents of Microgard™ activity against Gram-negative bacteria

The effect of MG on *Pseudomonas putida* ATCC 12633 was further enhanced by the incorporation of sodium citrate (Figure 3.18). Citrate concentration of 0.5% was found to be adequate for the enhancement against Gram-negative bacteria. However, it took 0.370% EDTA to cause a similar effect against this bacterium (Figure 3.19). It was also noted that the citrate controls were different from EDTA controls, in that the latter was inhibitory at higher concentrations.

Sodium citrate, at a concentration of 0.5% or greater, was found to enhance the activity of MG against *Escherichia coli* O157:H7 (Figure 3.20). On the contrary, increased in concentrations of EDTA did not potentiate the activity of MG against this organism. Results also pointed out that the EDTA-MG combination was not effective against Gram-negative bacteria (Figure 3.21).

Although MG was effective (2-$\log_{10}$ reduction) against *Salmonella typhimurium* ATCC 79631, the addition of sodium citrate did not produce further enhancement in its activity (Figure 3.22). Similarly, the application of EDTA and MG combination did not enhance inhibition of *S. typhimurium* (Figure 3.23). On

the contrary, in both cases, the incorporation of chelating agents had an antagonizing effect on the activity of MG.

Originally, MG was not effective against *Pseudomonas aeruginosa* ATCC 10145 (Figure 3.24). However, with the incorporation of 0.1% sodium citrate, a 2-$\log_{10}$ reduction in bacterial cell counts was observed. In addition, the rate of cell death also increased with the increase in concentrations of the chelating agent in MG. Interestingly, citrate controls showed no sign of inhibitory activity against *Ps. aeruginosa* . With EDTA as the chelating agent, MG was shown to be highly active against Gram-negative organisms. A concentration of 0.03722% EDTA was sufficient to cause a 4-$\log_{10}$ reduction in bacterial cells (Figure 3.25).

Effect of chelating agents on the activity of Microgard™ against Gram-positive bacteria

MG was found to be inhibitory against *Listeria monocytogenes* ATCC 894. After a 30-minutes exposure to MG, there was a 2-$\log_{10}$ reduction in bacteria cell counts (Figure 3.26). Incorporation of 1.5% sodium citrate resulted in slight enhancement of MG against this bacterium. However, further increases in concentrations of citrate did not promote further enhancement of MG.

The use of EDTA was also successful in potentiation of the activity of MG and increases in concentration enhanced the effect (Figure 3.27). It was found that 0.5583% EDTA was the optimum.

Effect of chelating agents on the activity of Microgard™ against yeasts

Saccharomyces cerevisiae var *ellipsoideus* 1B15 was found to be sensitive to MG. Results showed a 1-$\log_{10}$ reduction in viable cell counts (Figure 3.28). The use of 0.1% sodium citrate with MG was optimum for the inhibition of *S. cerevisiae* . Similarly, 0.1861% EDTA was found to enhance the activity of MG against the yeast cells (Figure 3.29). However, there was no further change in the activity of MG when treated with higher concentrations (> 0.1861%) of EDTA.

Candida albicans ATCC 18804 was found to be sensitive to MG. An increase in concentrations of sodium citrate, from 1.0% to 2.0% resulted in an enhancement of MG activity against the yeast cells (Figure 3.30). The use of EDTA as chelating agent, however, did not potentiate the activity of MG against this organism (Figure 3.31). However, controls showed that the yeast cells were inhibited by increasing EDTA concentrations.

The effect of sodium citrate on Microgard™ activity against Gram-negative bacteria and yeasts in cottage cheese

Due to the high inoculation rate of *Pseudomonas putida* ATCC 12633 (10^7 bacteria/ml), the effect of citrate on MG activity was less pronounced. During the first 5 days, the citrate-MG combination was able to somewhat enhance inhibition on *Ps. putida*. A 2.0% citrate-MG combination was consistent in the inhibition of the bacteria (Figure 3.32). Further incubation produce erratic results. The efficiency of MG-citrate did not affect growth of the bacteria after 10 days (data and graphs not presented). However, 2.0% citrate in MG, after the tenth day, was useful in inhibition of yeasts. It was also

observed that the lower citrate concentrations in MG did not inhibit the growth of yeasts in cottage cheese (Figure 3.33).

Effect of sodium citrate on the activity of Microgard™ against Pseudomonas putida in half and half cream

The growth of *Pseudomonas putida* ATCC 12633 in the half and half cream was inhibited by MG alone for a period of 7 days (Figure 3.34). Citrate concentrations of 0.5% and more were found to enhance its activity against the Gram-negative bacterium. Furthermore, a 1.0% citrate-MG combination was active against *Ps. putida* for 30 days (100% inhibition).

Effect of sodium citrate on the activity of Microgard™ against yeasts in half and half cream

It was shown that the 0.5% citrate-MG combination was effective for inhibition of *Candida albicans* ATCC 18804 for up to 33 days (Figure 3.35). At 2% citrate concentration, MG was found to exert a lesser inhibitory effect on the yeast. *Saccharomyces cerevisiae* var *ellipsoideus* 1B15 was less sensitive to MG than *C. albicans* (Figure 3.36). However, after 20 days of incubation, a concentration of 1.0% citrate-MG was found to be optimum for inhibition of *S. cerevisiae*. Similar to *C. albicans*, higher citrate concentrations did not enhance the activity of MG.

DISCUSSION

When crude MG was diluted with citrate buffer (pH 5.30), two enhanced activities were observed, one at 1:128, and the other at 1:8192 dilution; in between, citrate was without effect. It was evident that the active ingredient(s) in MG was potentiated by citrate. The inhibitory activity from the lower dilutions could be attributed to the presence of organic acids. However, the higher dilutions produced a more active fraction inferring that it could be due to a bacteriocin. It seemed that a trace amount of the bacteriocin in citrate buffer was sufficient to cause inhibition of *Pseudomonas putida*. However, in distilled water and HEPES buffer (pH 5.30), the antimicrobial substance in MG was considerably less active. In both diluents, only one type of activity was detectable. This indicated that trace amounts of MG antimicrobial substance(s) in water or HEPES buffer was not potentiated against the assay organisms under these conditions. Also, the citrate-enhancing phenomenon could serve as a rapid and sensitive method of detecting bacteriocin in a chelator-sensitized environment.

The inhibitory effect on Gram-negative bacteria by the citrate or EDTA-MG combinations was a time-dependent phenomenon (Stevens et al.,1991). The inhibitory effect against *Ps. putida*, also increased with the incubation period. In addition, different concentrations of chelating agents exerted varying effects on the activity of MG. In general, the optimum effect was achieved when further increases in chelator concentrations did not enhance activity. Data herein reported also showed that organisms responded differently to various chelating agents. This could be due to the fact that the outer membranes of different Gram-negative bacteria are stabilized by different amounts or types of divalent ions in the lipopolysaccharide layer (Nikaido and Nakae, 1979). The

structures of EDTA and citric acid make them good chelating agents because of the surface charges they carry (Windholz et al.,1983). EDTA was more effective because it strongly binds a variety of divalents cations, including calcium and magnesium (Nikaido and Vaara, 1985). The removal of magnesium ions by chelators disrupts the cell wall lipopolysaccharide, resulting in damage to the permeability of the bacterial membrane (Nikaido and Vaara, 1987). Nisin is believed to permeate disrupted cell outer membranes, inactivating bacteria at the cytoplasmic membrane (Morris et al., 1984; Stevens et al.,1991). In the present study, it was found that the permeability of acidic metabolites through the cell membrane was facilitated by EDTA or citrate. Findings showed that mixture of acetic and propionic acids has a greater inhibitory effect when used with sodium citrate. Under the same conditions, it was observed that the acidic mixture was more potent when diluted by water. EDTA was more effective in concentrated organic acid mixture than a diluted one. It is obvious that EDTA works efficiently in an acidic environment.

The effective incubation temperature for cells treated with either citrate-MG or EDTA-MG appeared to be between 30 and 37 °C. This is similar to the effective temperature range of between 30 and 42 °C for nisin-EDTA combinations against *Salmonella* spp. (Stevens et al., 1992b). The effect of exposure temperature may be important for citrate or EDTA interactions with MG in food systems.

In cottage cheese and cream, the effect of chelating agents on MG was less pronounced. The MG activity was probably affected by the protein and lipid content within the cheese and milk media. In this regard, the presence of fat in food has been found to cause a decrease in the activity of nisin (Daeschel, 1990). However, citrate-MG interaction in CB (pH 5.30) was effective in the inhibition of *Pseudomonas putida* ATCC 12633 and *Escherichia coli O157:H7*

but not *Pseudomonas aeruginosa* ATCC 10145 or *Salmonella typhimurium* ATCC 79631 (Table 3.2 and Table 3.3). Unlike the effect of nisin-EDTA (Blackburn et al., 1989; Roller, 1991; Stevens et al., 1991,1992a,1992b) the EDTA-MG combination was not effective against *E. coli* or *S. typhimurium*. The differences in the inhibitory effects for both bacteriocins could infer that there is variability among different lipopolysaccharide layers of Gram-negative bacteria (Hancock, 1984; Nikaido and Nakae, 1979).

Unlike previous works on MG (Al-Zoreky, 1988), the bacteriocin was found to be inhibitory against *E. coli* and *S. typhimurium* . Also in the present study, MG was found to be inhibitory against *L. monocytogenes* , a Gram-positive bacterium. Moreover, sodium citrate and EDTA were shown to be effective in the enhancement of MG against this organism. Recently, nisin also has been shown to exert a bactericidal effect on *L. monocytogenes* (Mohamed et al.,1984; Benkerroum and Sandine, 1988; Asperger et al.,1989; Carminati et al., 1989; Harris et al., 1989; Maisnier-Patin et al.,1992). Citrate-MG has potential for use in food safety applications since *L. monocytogenes* is common in food and has been isolated from silage, milk, cheese, vegetables and meat (El-Gazzar and Marth, 1991; Skovgaard, 1990).

The peptidoglycan matrix of Gram-positive bacteria consists of a variety of polysaccharides and polyphosphates known as teichoic acids (Stanier et al.,1986). These teichoic acids constitute the major antigens of these bacteria and are found on the outer surface of the peptidoglycan. The acids are charged and could affect the passage of ions through the outer surface layers. The competitve binding of Cl^- and phosphate anions to the lipoteichoic acid of sensitized cells has been shown to cause a decrease in the activity of pediocin AcH (Bhunia et al., 1991; Kim, 1993). Although the mode of action of MG remains unknown, studies of nisin and other bacteriocins could help to shed

some light on its inhibitory nature against Gram-positive bacteria. Beside disruption of the outer membrane by chelating agents, several possible mechanisms of membrane destabilization by MG have been hypothesized. The loss of viability of Gram-positive organisms could be attributed to (1) disruption of membrane potential, (2) the damaging influx of MG into the cells, (3) the presence of specific receptors on which bacteriocin molecules could be adsorbed or bound, and (4) loss of a specific membrane constituent(s) as a result of cell lysis.

Bacterial cell membrane potential was shown to be dramatically decreased when amino acids, ions and ATP were released from nisin-treated cells and cytoplasmic vesicles (Ruhr and Sahl, 1985; Kordel and Sahl, 1986; Kordel et al.,1989; Barefoot and Nettles, 1993; Garcerá et al.,1993; Bruno et al., 1993). The disruption of outer membrane by physical means will cause a structural discontinuity, resulting in permeability of bacteriocins across the outer membrane. The presence of specific antibiotic-binding receptors, such as sulfhydryl groups, has been shown by Morris et al. (1984). These sulfhydryl groups exist in a highly intereactive environment and therefore are easily inhibited by sulfhydryl agents or possibly bacteriocins. In addition, the sulfhydryl groups appeared to be in communication with one or more histidines, and covalent modification of either sulfhydryl group or histidine has a profound recipocal effect on the reactivity of the other (Morris et al., 1984). Similar to nisin, it is possible that MG may have a specific capability to alter, either directly or indirectly, the membrane sulfiydryl groups causing bacteriostasis in bacteria. It is also possible that interferences by chelating agents on these specific receptors enhance the effect of bacteriocins. The affinity of nisin and Pep 5 is strongly dependent on the presence of negatively charged phospholipids and electrostatic interactions (Kordel et al.,1989). Therefore, the removal of divalent

cations by chelating agents could produce effective binding of bacteriocins to the cell membrane. The end product of bacteriocins could be seen in the collapse of the proton motive force in *L. monocytogenes* and*Clostridium sporogenes* PA 3679 after treating with pediocin JD (Christensen and Hutkins, 1992) or nisin (Okereke and Montville, 1992).

The inhibitory effect of citrate-MG or MG alone has been extended to yeast cells. The use of EDTA remains uneffective against *S. cerevisiae* or *C. albicans* (Table 3.2 and Table 3.3). Presently, the inhibitory mechanism of MG against yeasts and molds is still unclear. However, it is obvious that MG and citrate-MG combinations do inhibit yeasts as well as a wide spectrum of bacteria, and therefore has potential application in food systems.

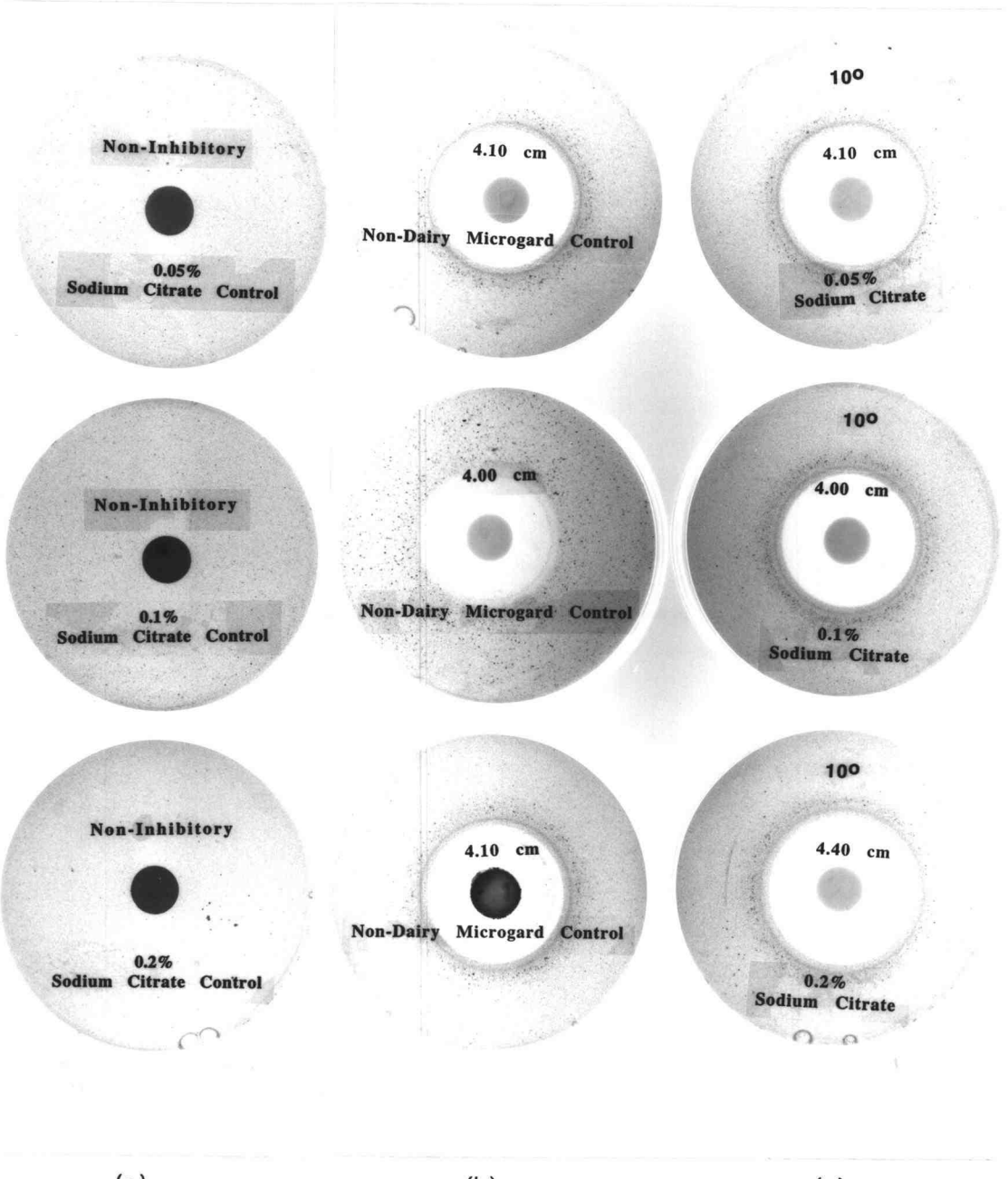

Figure 3.1 Enhancement of sodium citrate in non-dairy Microgard™ (MG) against *Pseudomonas putida* ATCC 12633. (a) sodium citrate controls, (b) non-dairy MG controls, and (c) effect of citrate in undiluted MG

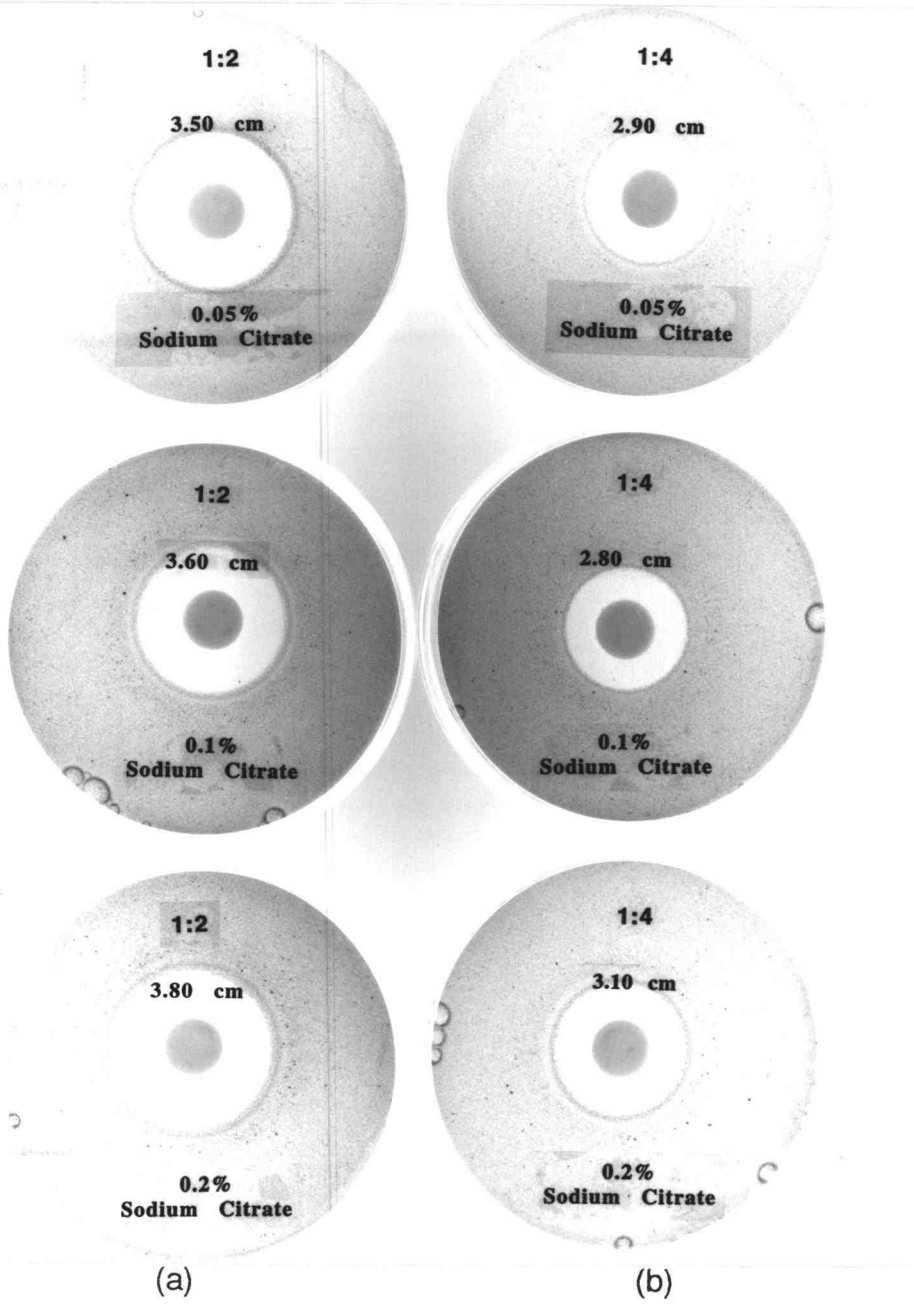

Figure 3.2 Enhancement of sodium citrate in non-dairy Microgard™ (MG) against *Pseudomonas putida* ATCC 12633. (a) effect of citrate in diluted (1:2) MG (b) effect of citrate in diluted (1:4) MG

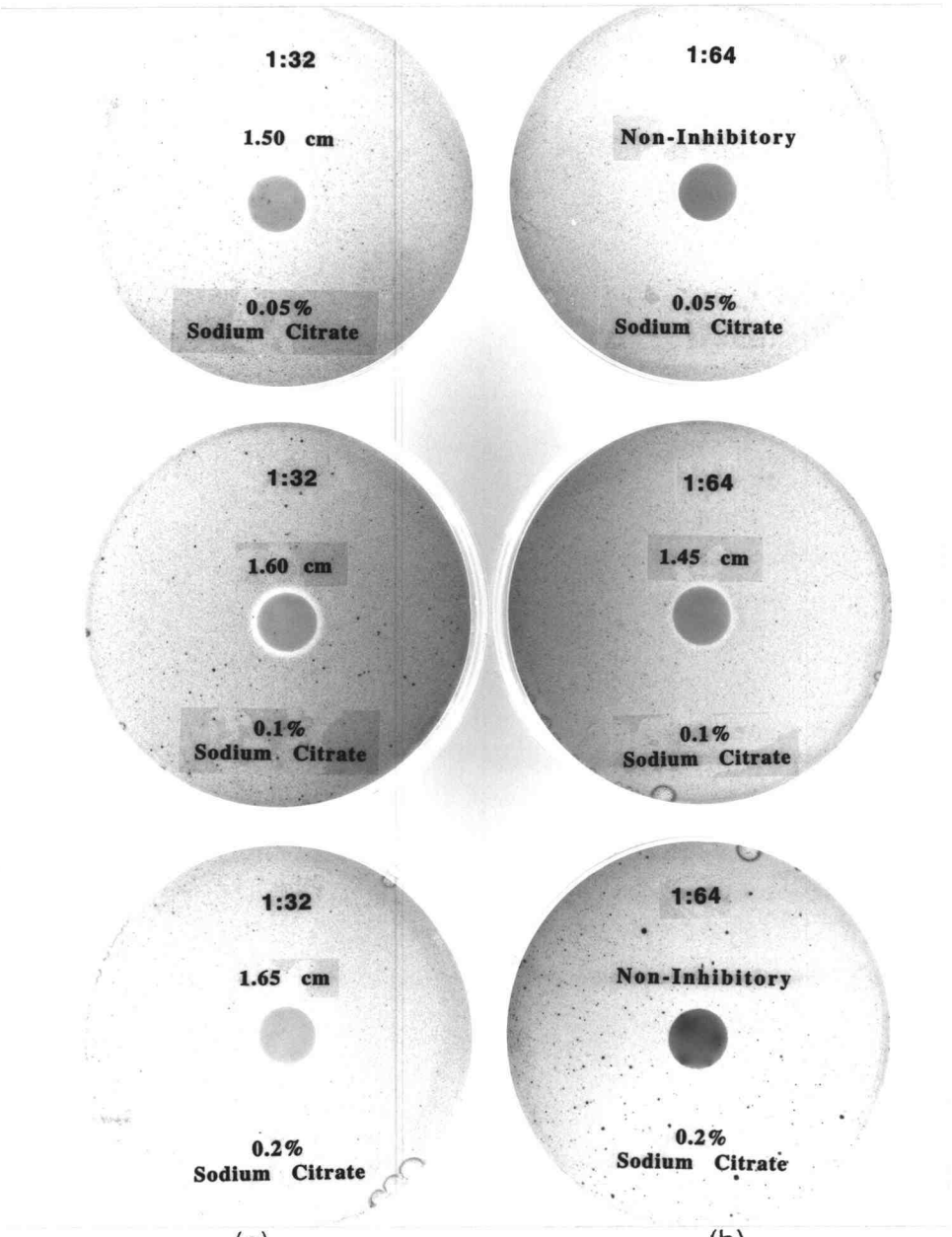

Figure 3.3 Enhancement by sodium citrate of activity of non-dairy Microgard™ (MG) against *Pseudomonas putida* ATCC 12633. (a) effect of citrate in diluted (1:32) MG (b) effect of citrate in diluted (1:64) MG

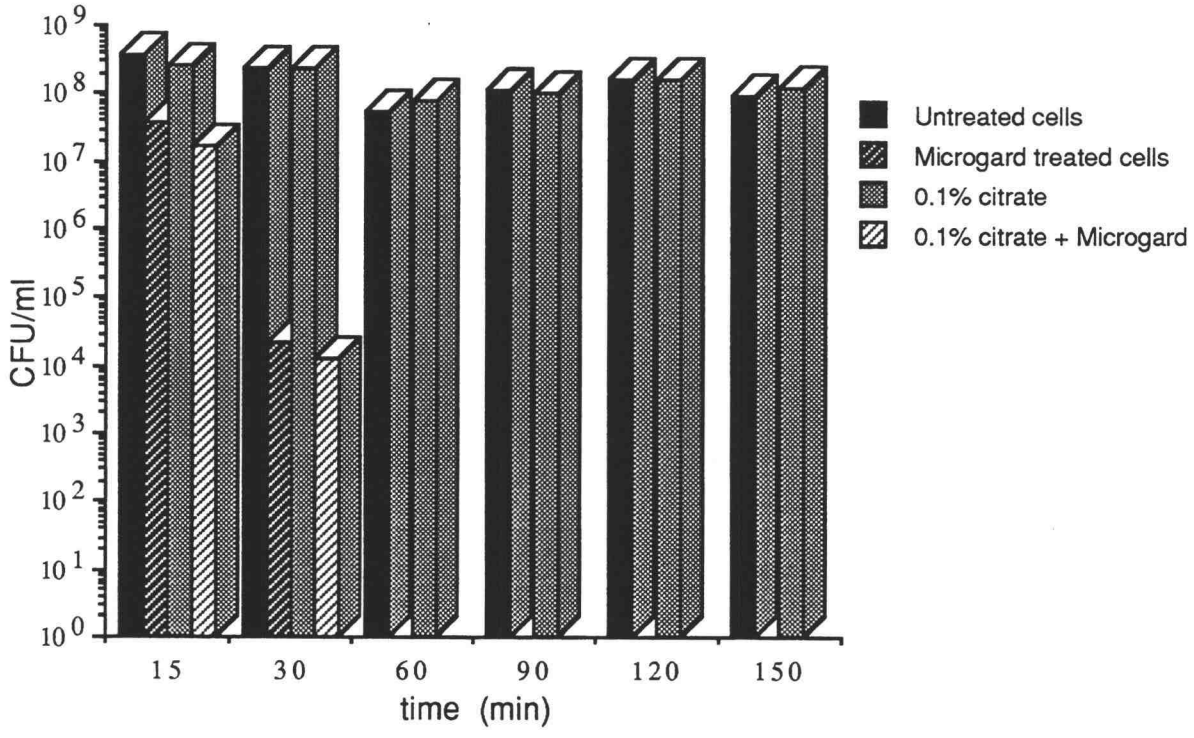

Figure 3.4 Effect of 0.1% citrate in Microgard on growth of Pseudomonas putida ATCC 12633 with respect to time

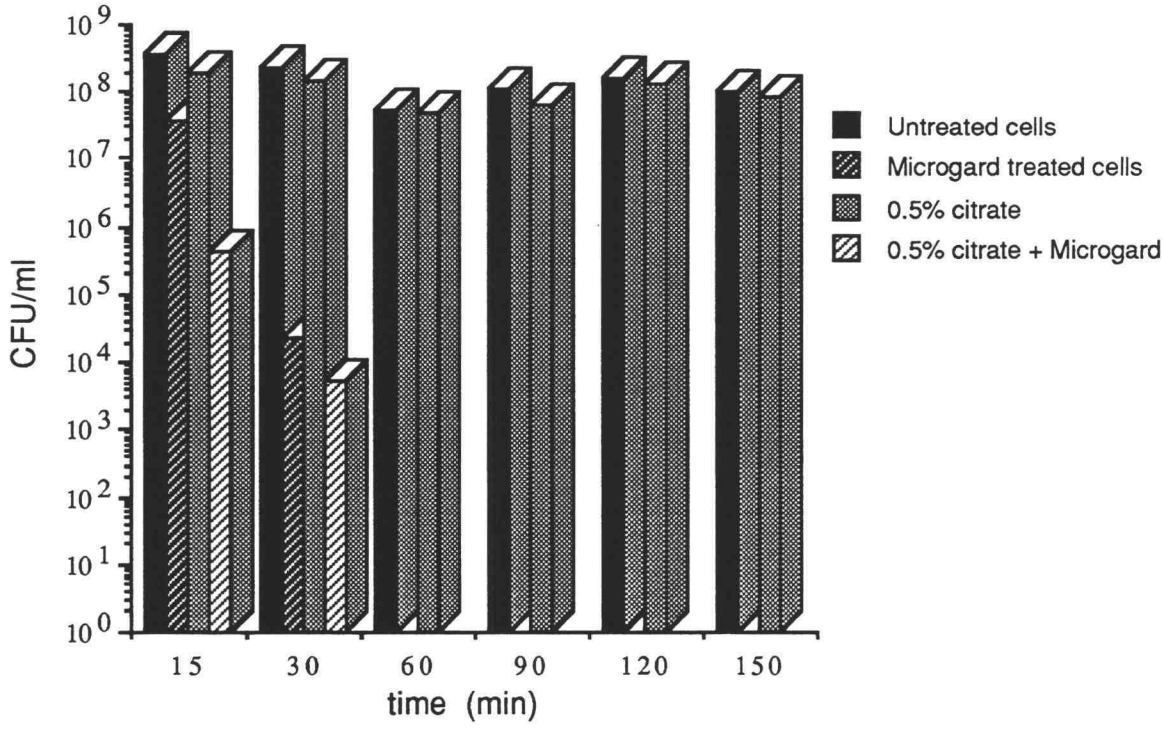

Figure 3.5 Effect of 0.5% citrate in Microgard on growth of Pseudomonas putida ATCC 12633 with respect to time

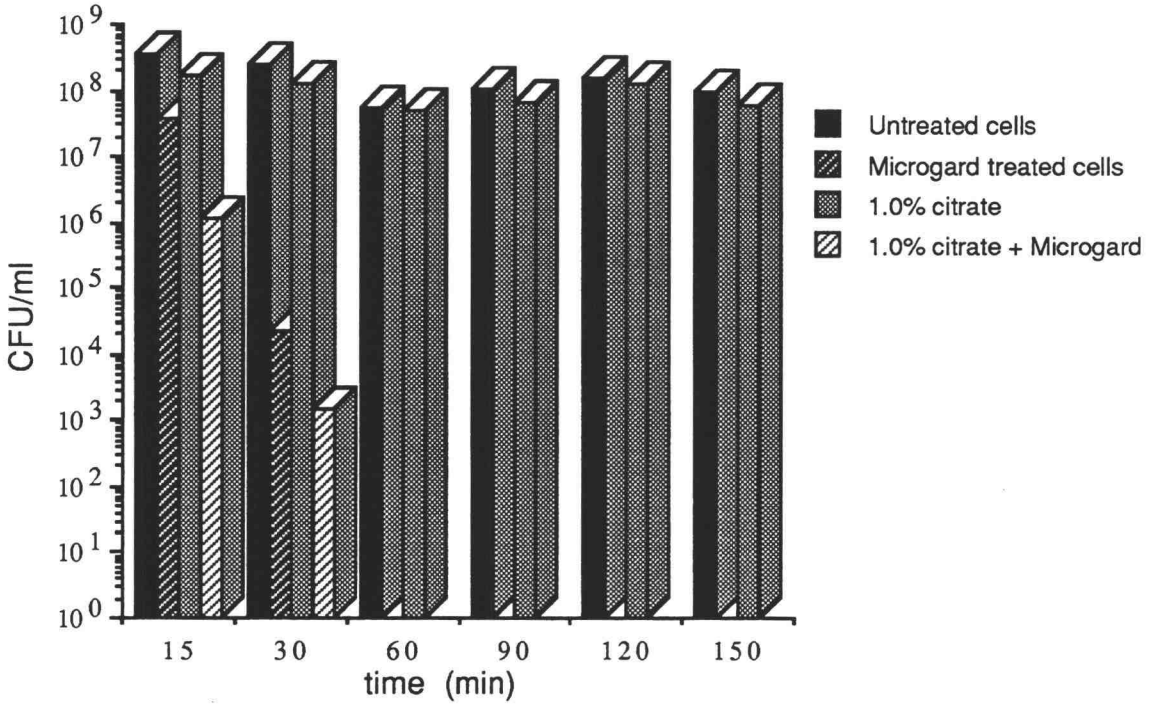

Figure 3.6 Effect of 1.0% citrate in Microgard on growth of Pseudomonas putida ATCC 12633 with respect to time

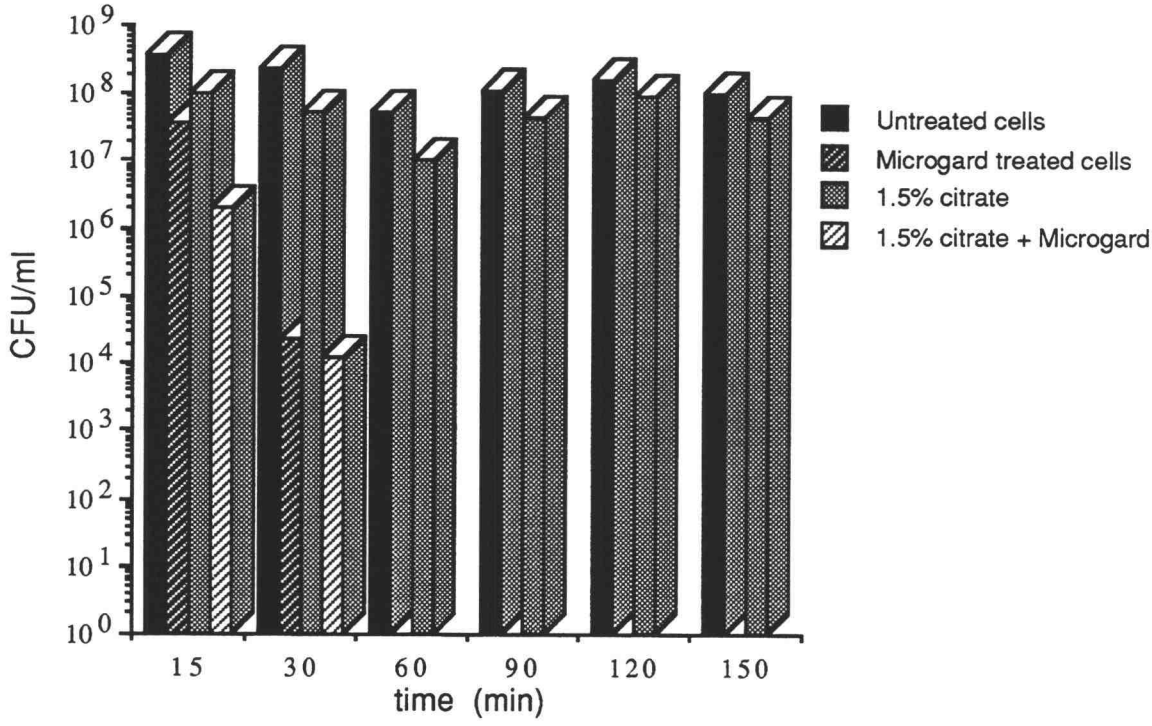

Figure 3.7 Effect of 1.5% citrate in Microgard on the growth of Pseudomonas putida ATCC 12633 with respect to time

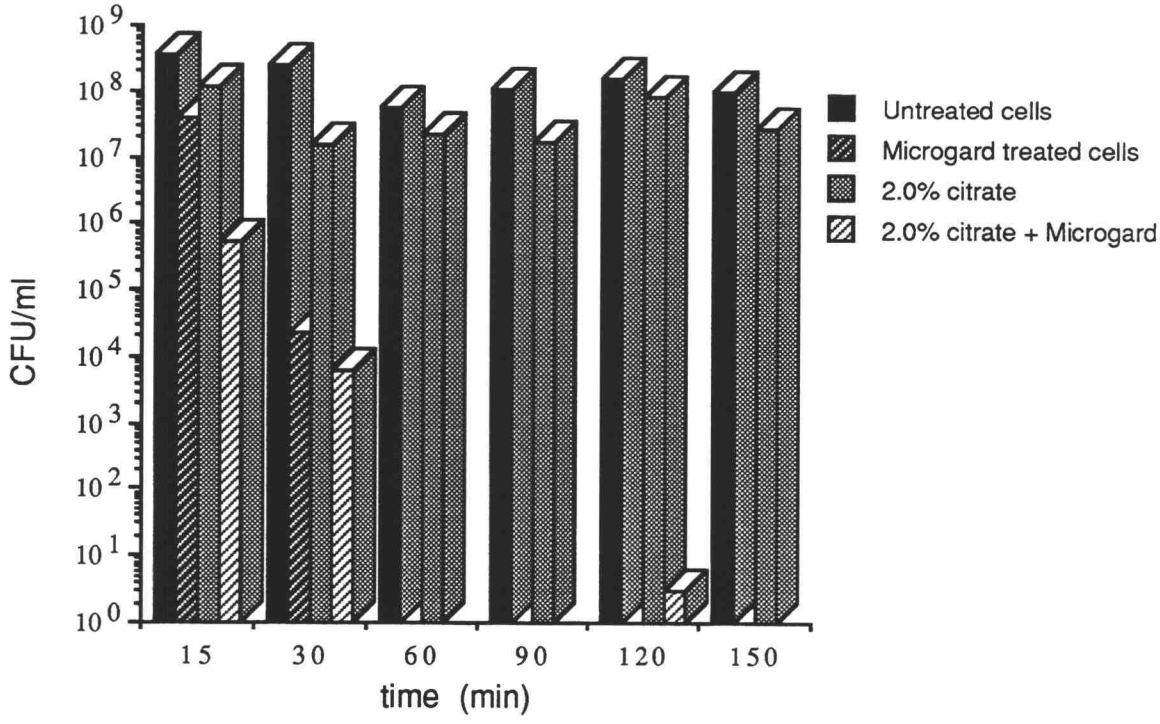

Figure 3.8 Effect of 2.0% citrate in Microgard on growth of Pseudomonas putida ATCC 12633 with respect to time

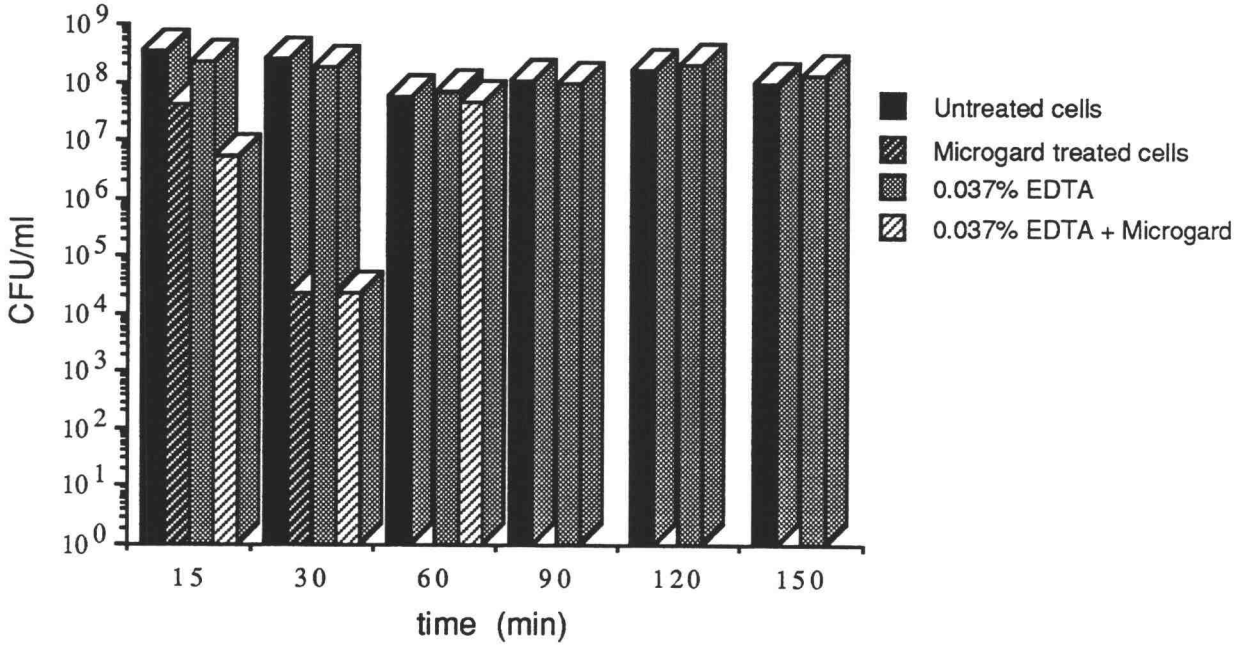

Figure 3.9 Effect of 0.037% EDTA in Microgard on the growth of Pseudomonas putida ATCC 12633 with respect to time

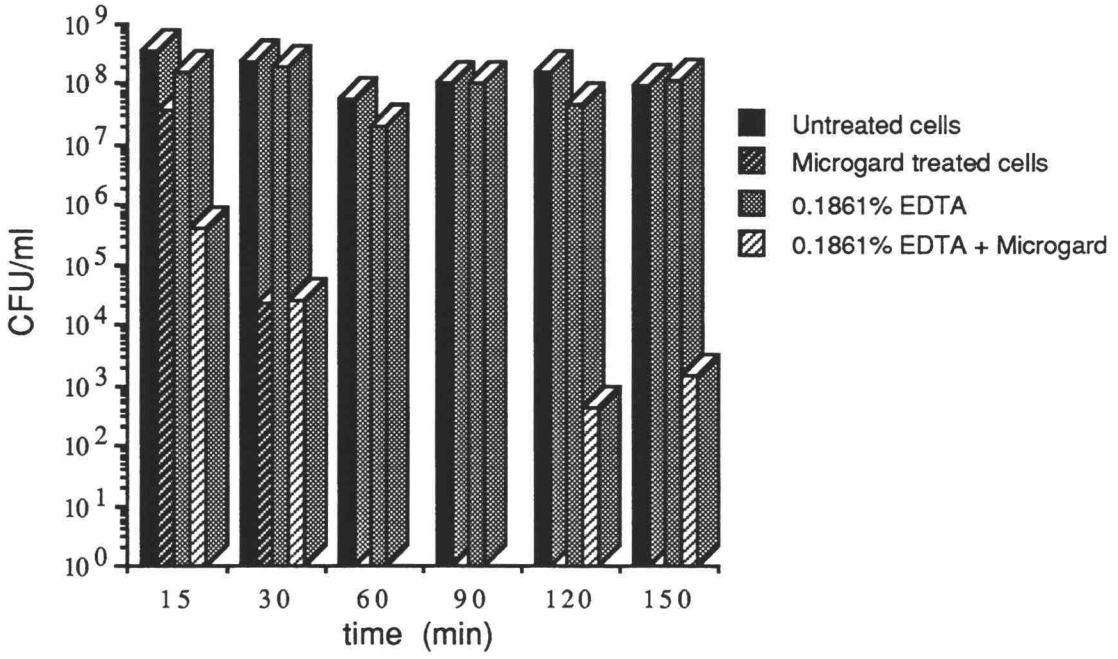

Figure 3.10 Effect of 0.1861% EDTA in Microgard on the growth of Pseudomonas putida ATCC 12633 with respect to time

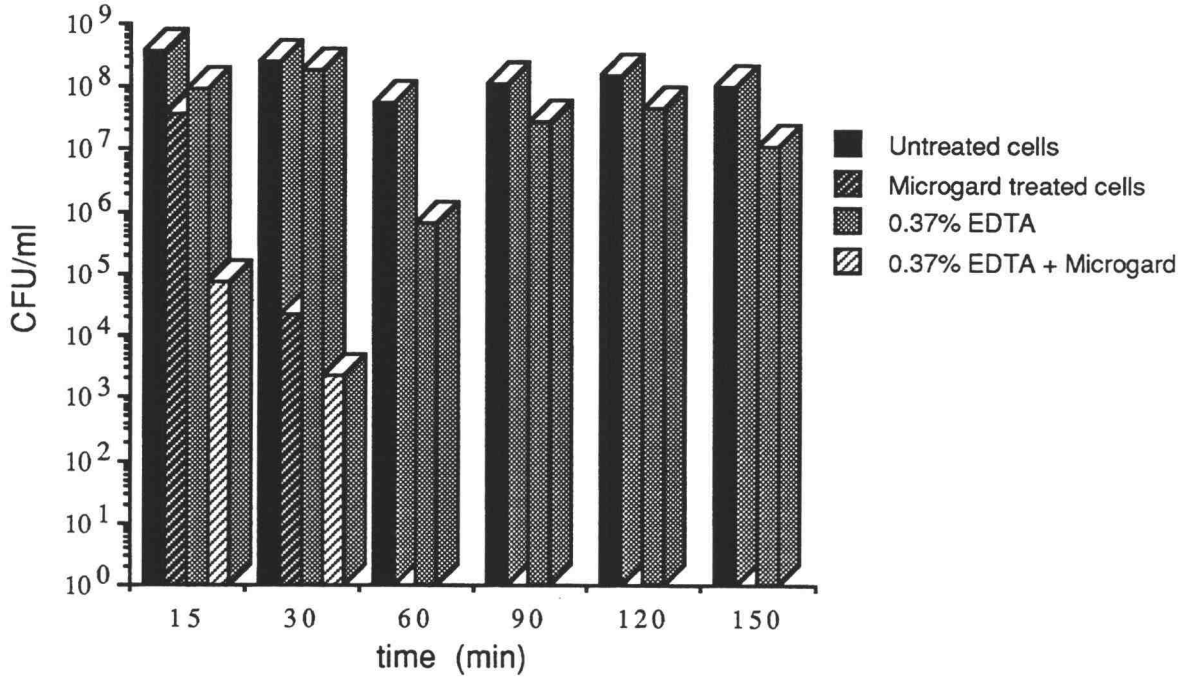

Figure 3.11 Effect of 0.37% EDTA in Microgard on the growth of Pseudomonas putida ATCC 12633 with respect to time

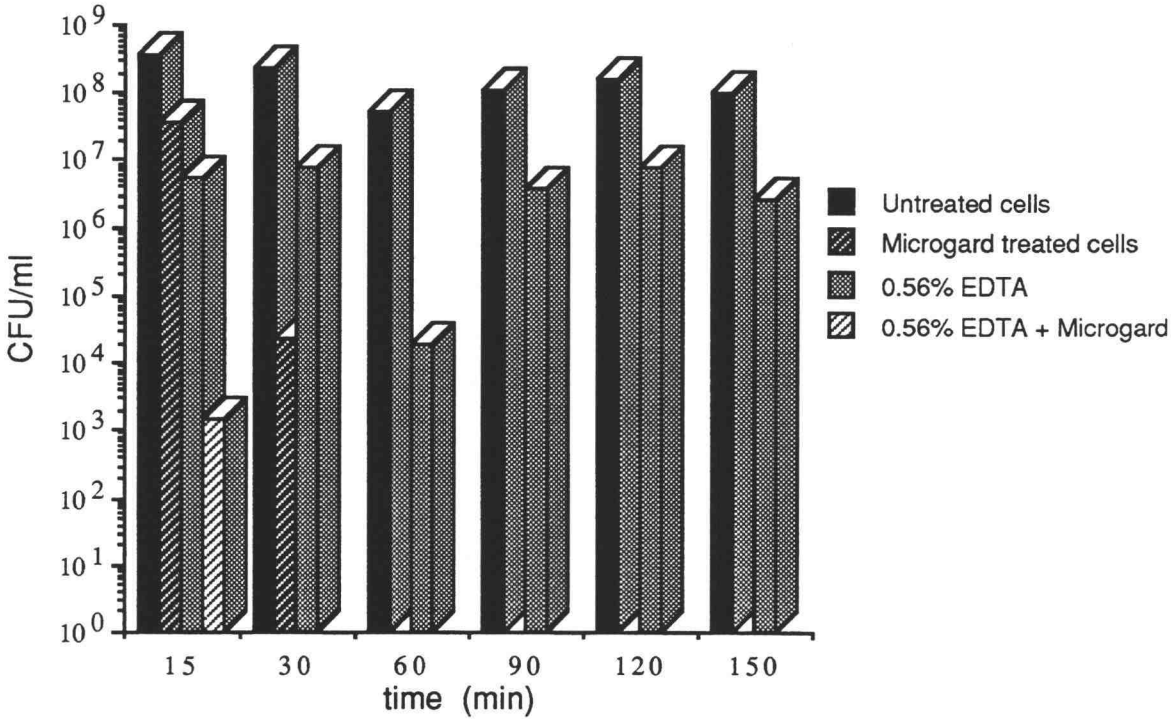

Figure 3.12 Effect of 0.56% EDTA in Microgard on growth of Pseudomonas putida ATCC 12633 with respect to time

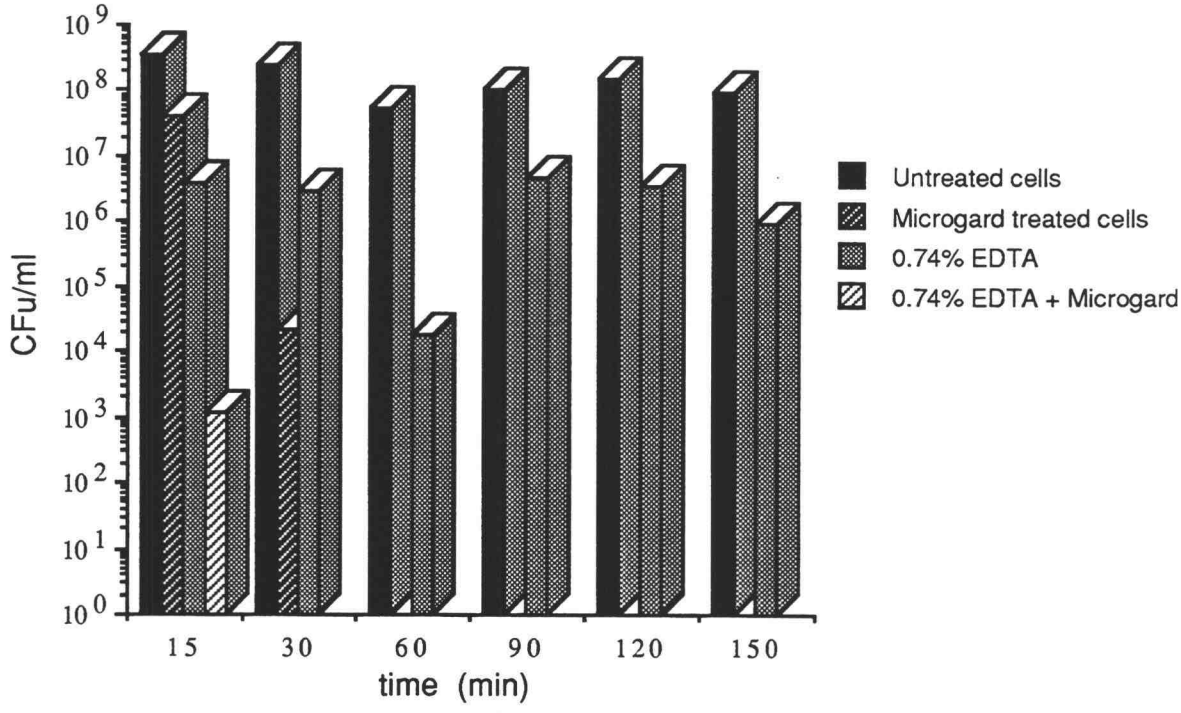

Figure 3.13 Effect of 0.74% EDTA in Microgard on growth of Pseudomonas putida ATCC 12633 with respect to time

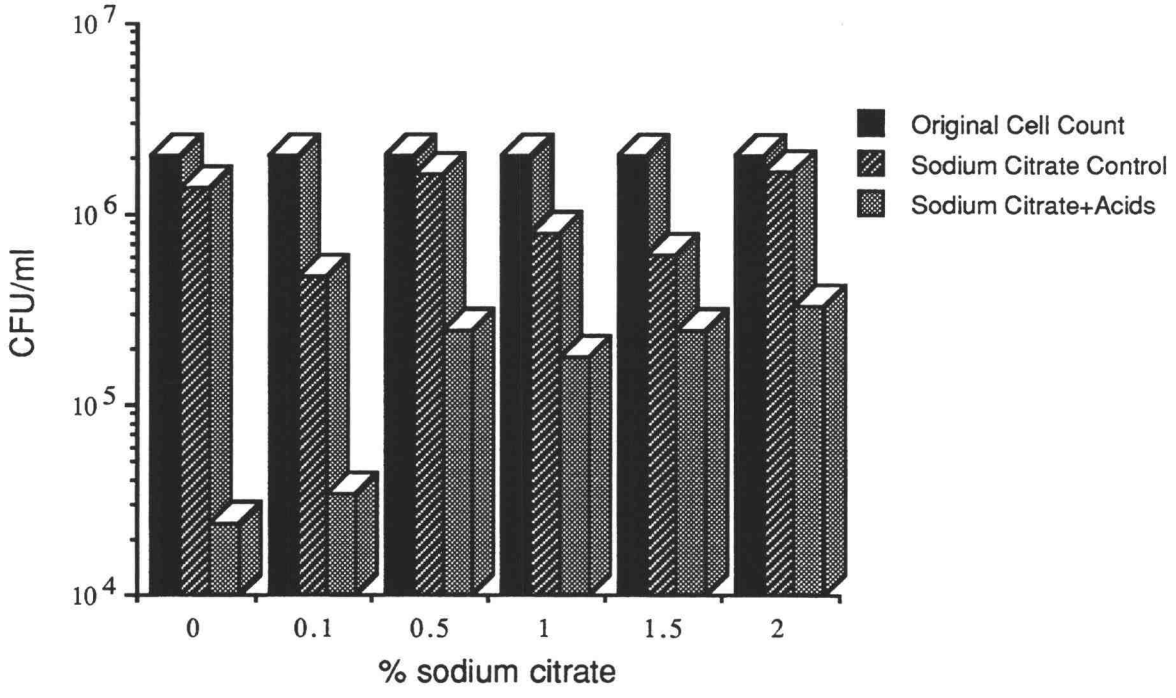

Figure 3.14 Effect of sodium citrate in concentrated acetic and propionic acids on inhibition of Pseudomonas putida ATCC 12633

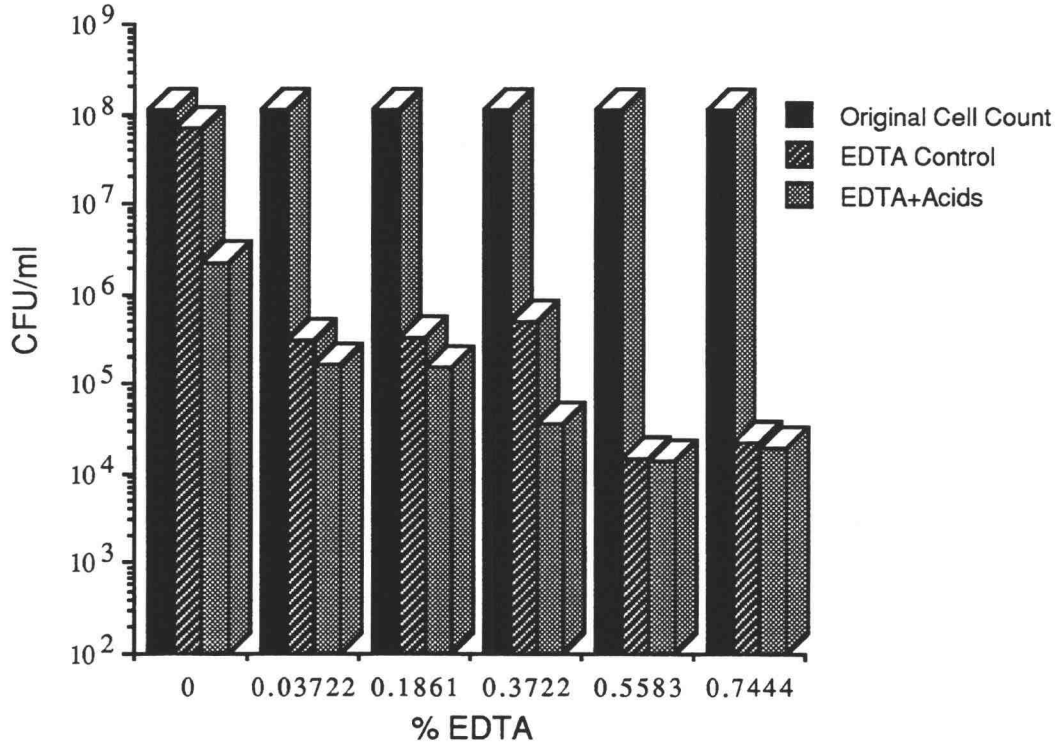

Figure 3.15 Effect of EDTA in concentrated acetic and propionic acids on inhibition of Pseudomonas putida ATCC 12633

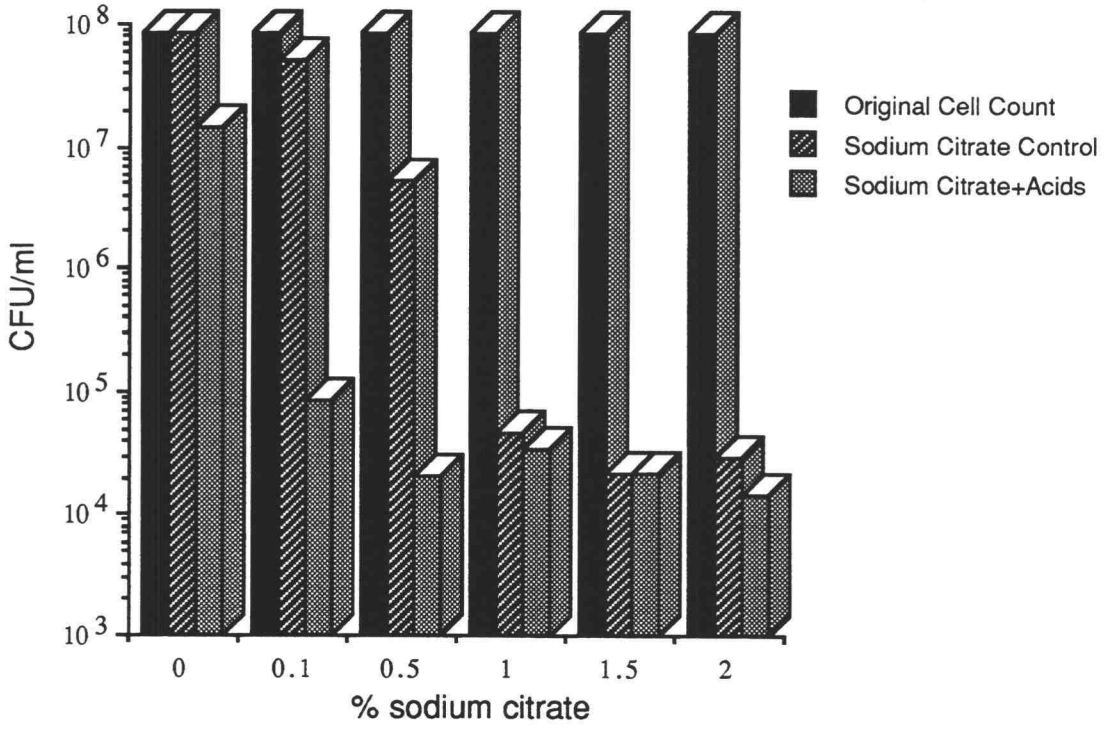

Figure 3.16 Effect of sodium citrate in diluted acetic and propionic acids on inhibition of Pseudomonas putida ATCC 12633

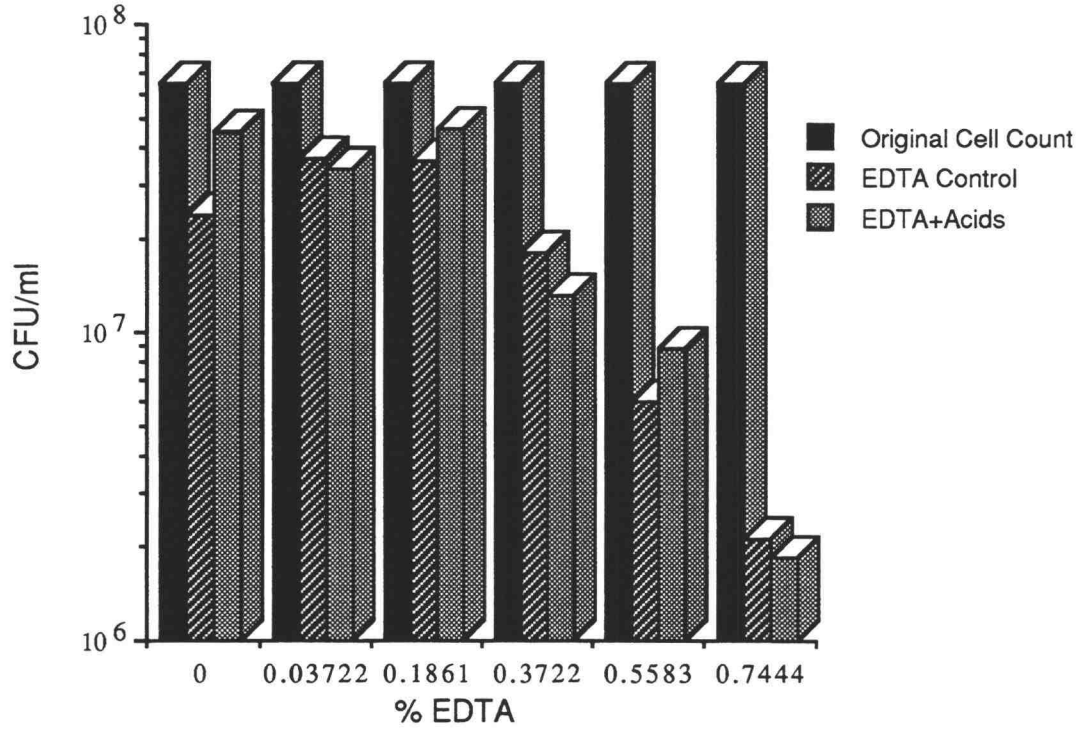

Figure 3.17 Effect of EDTA in diluted acetic and propionic acids on inhibition of Pseudomonas putida ATCC 12633

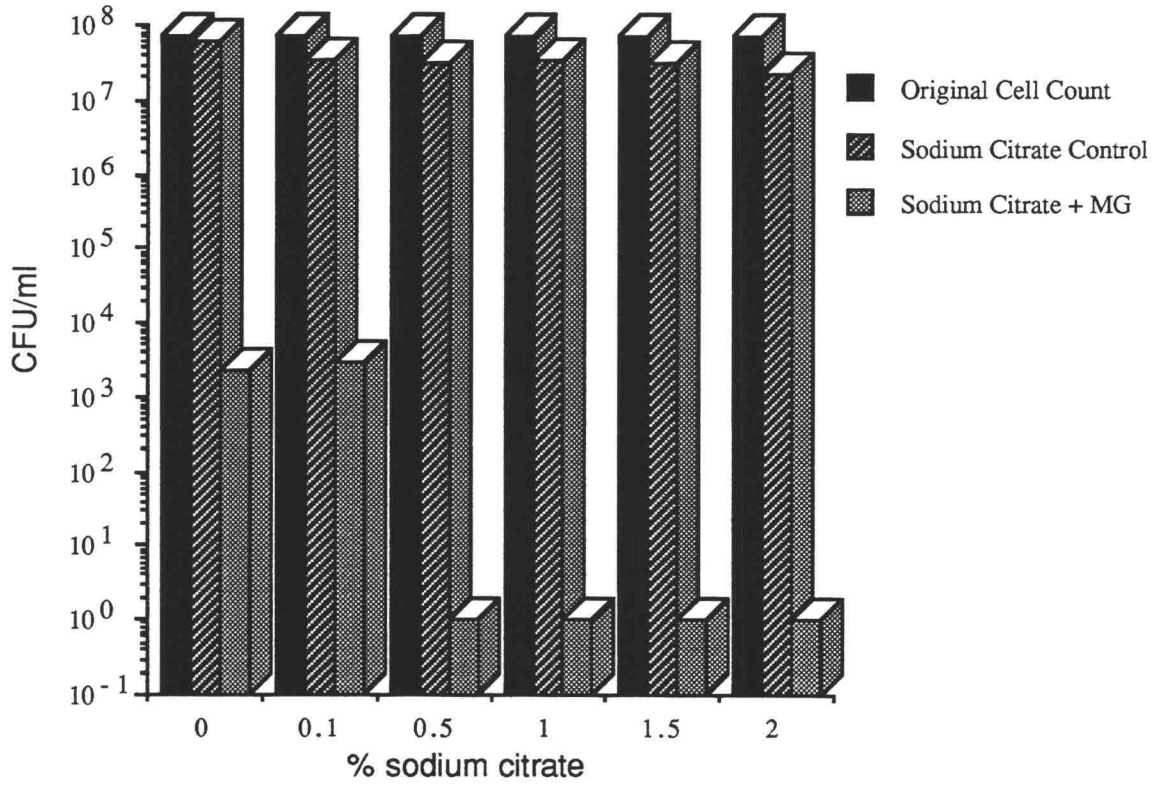

Figure 3.18 Effect of sodium citrate on inhibitory activity of non-dairy Microgard against Pseudomonas putida ATCC 12633

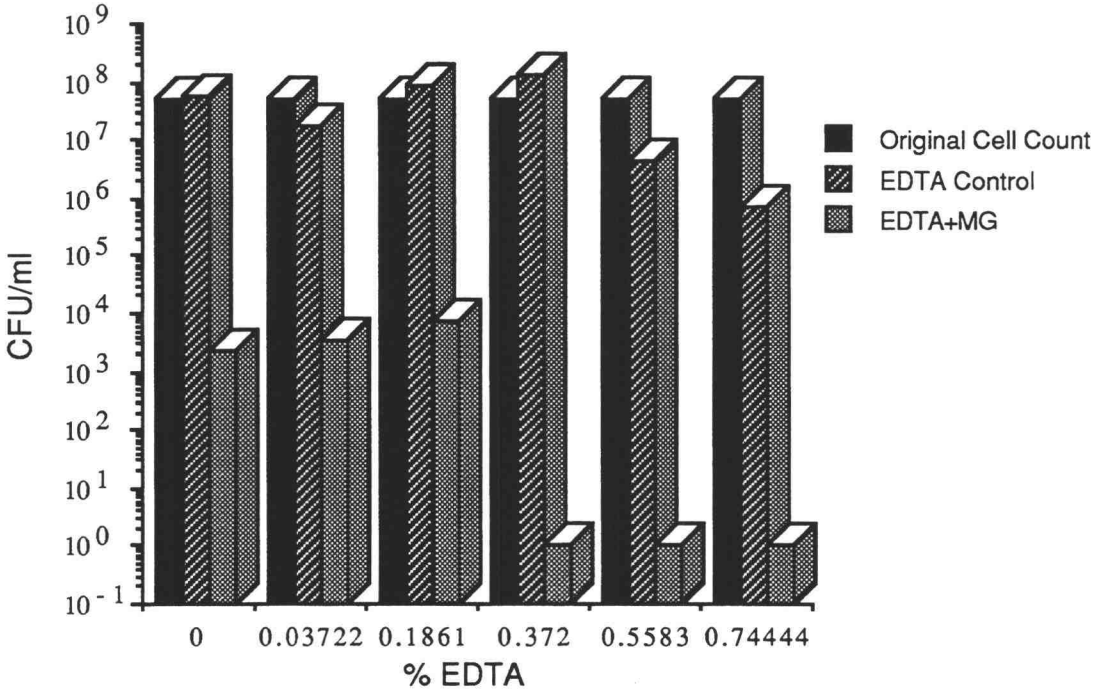

Figure 3.19 Effect of EDTA on inhibitory activity of non-dairy Microgard against Pseudomonas putida ATCC 12633

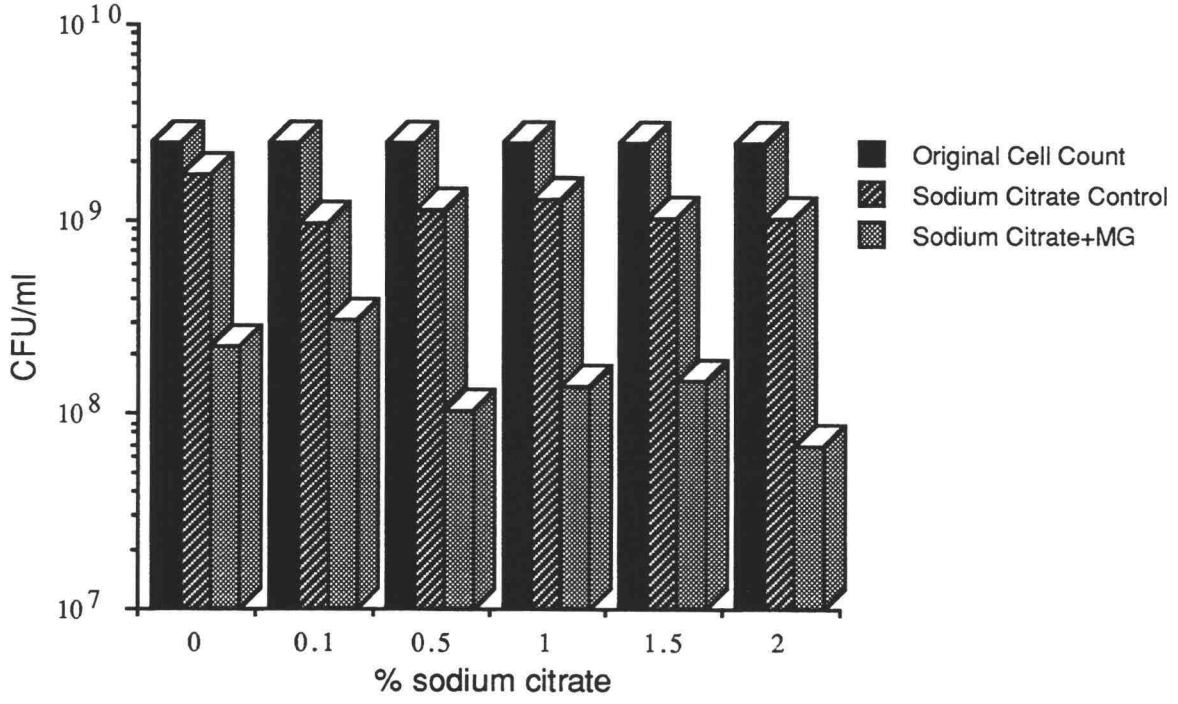

Figure 3.20 Effect of sodium citrate on inhibitory activity of non-dairy Microgard against Escherichia coli O157:H7

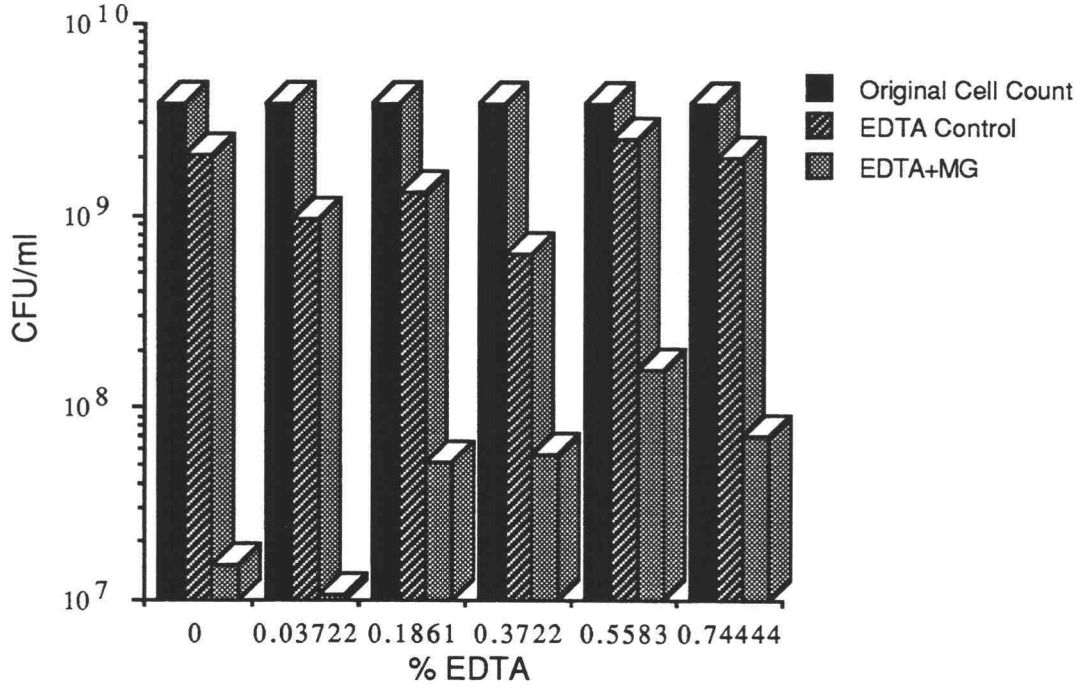

Figure 3.21 Effect of EDTA on inhibitory activity of non-dairy Microgard against Escherichia coli O157:H7

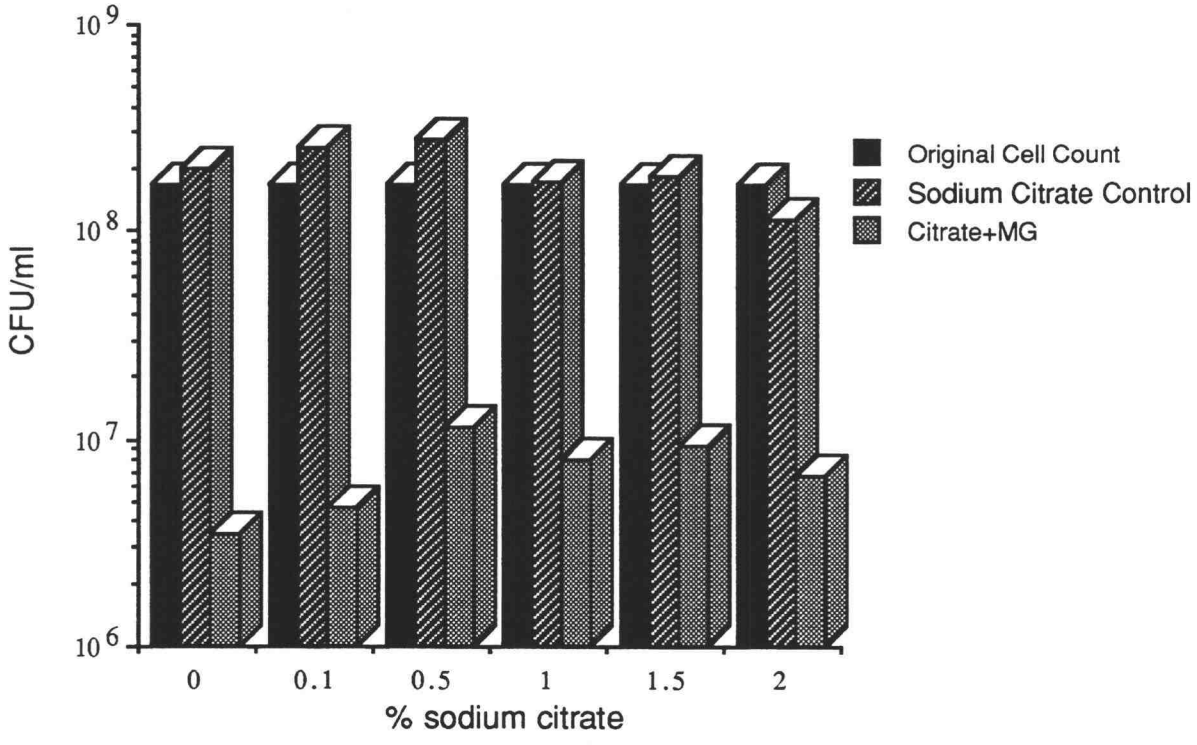

Figure 3.22 Effect of sodium citrate on inhibitory activity of non-dairy Microgard against Salmonella typhimurium ATCC 79631

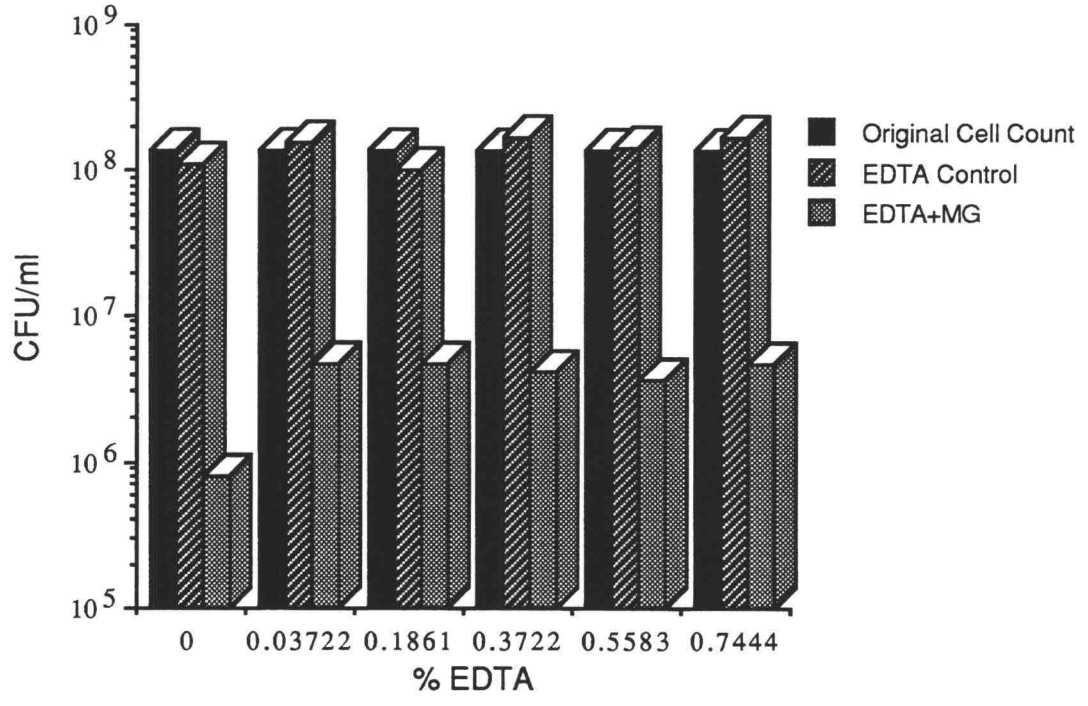

Figure 3.23 Effect of EDTA on inhibitory activity of non-dairy Microgard against Salmonella typhimurium ATCC 79631

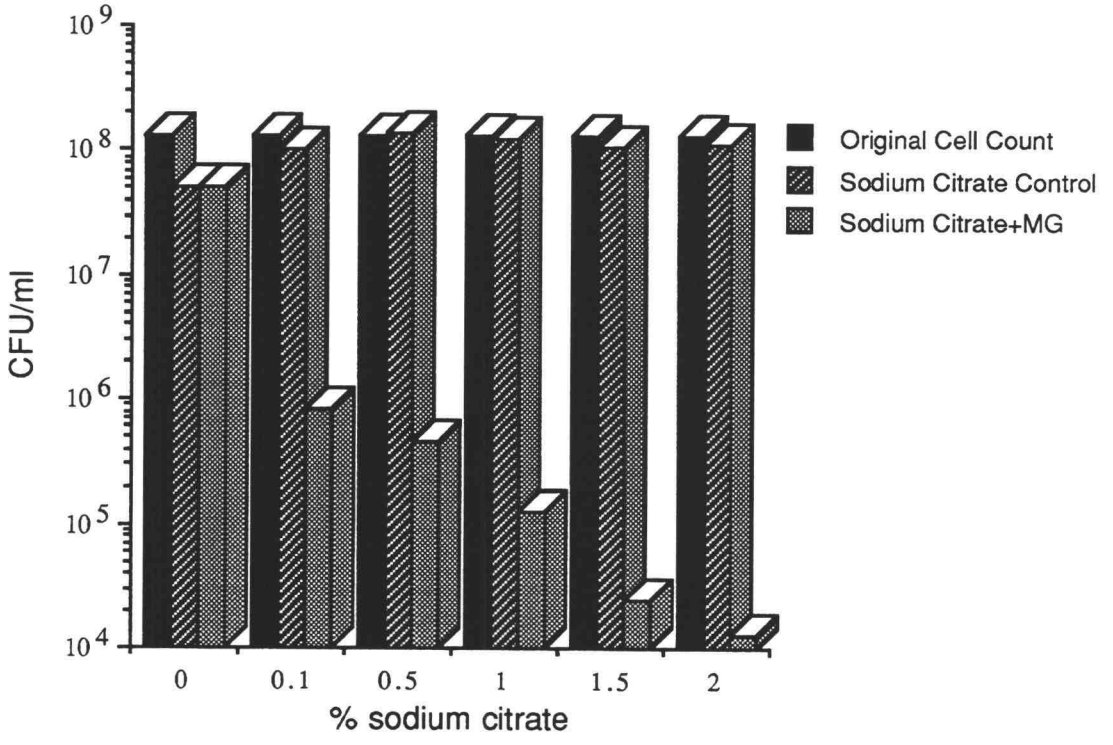

Figure 3.24 Effect of sodium citrate on inhibitory activity of non-dairy Microgard against Pseudomonas aeruginosa ATCC 10145

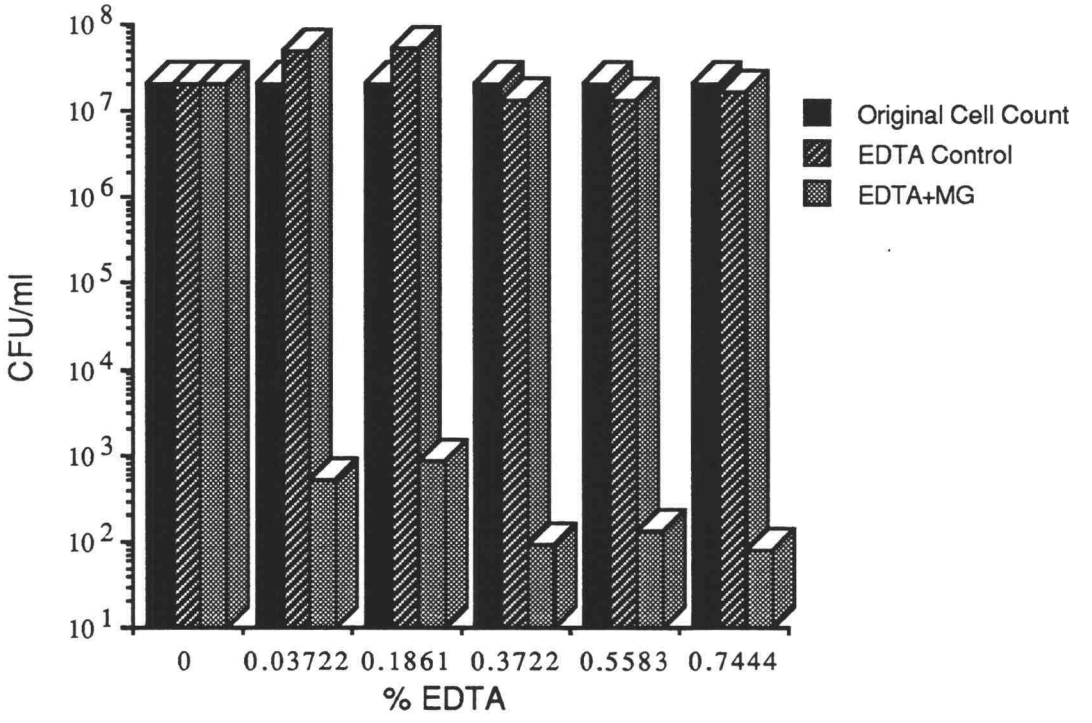

Figure 3.25 Effect of EDTA on inhibitory activity of non-dairy Microgard against Pseudomonas aeruginosa ATCC 10145

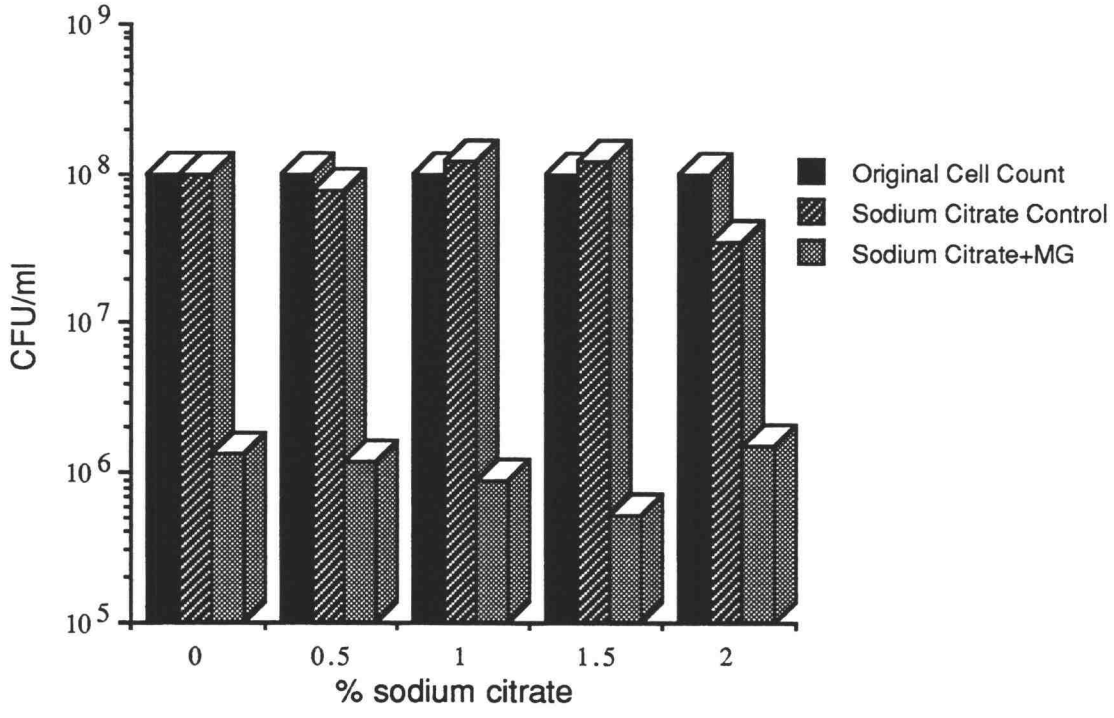

Figure 3.26 Effect of sodium citrate on inhibitory activity of non-dairy Microgard against Listeria monocytogenes ATCC 894

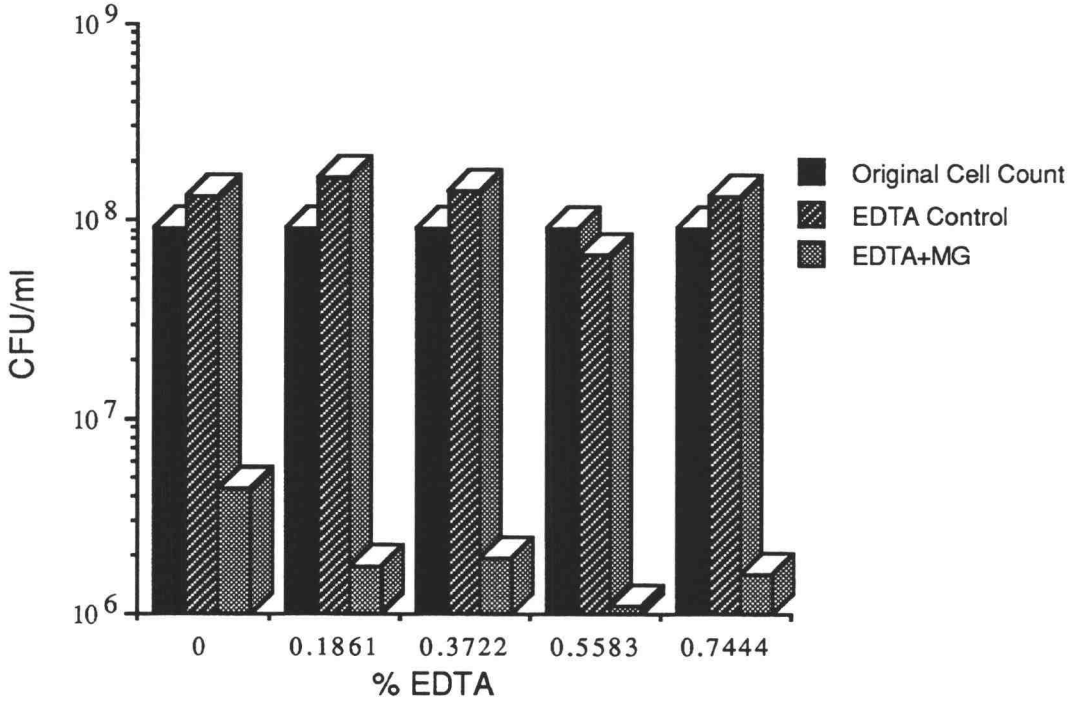

Figure 3.27 Effect of EDTA on inhibitory activity of non-dairy Microgard against Listeria monocytogenes ATCC 894

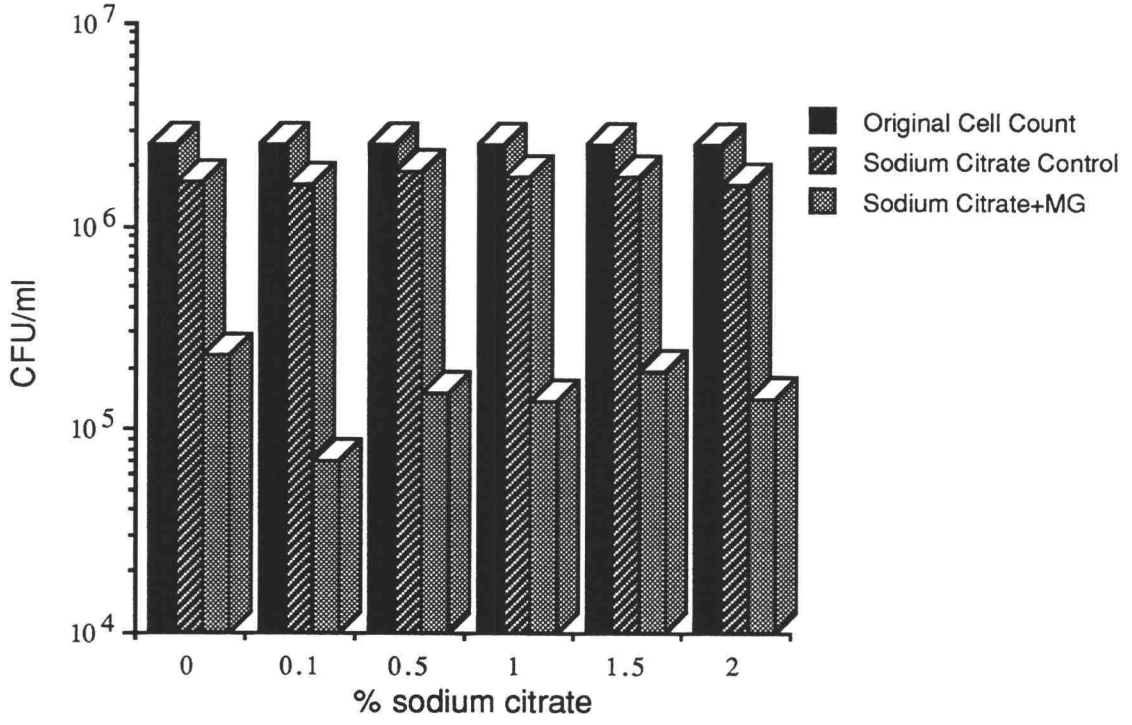

Figure 3.28 Effect of sodium citrate on inhibitory activity of non-dairy Microgard against Saccharomyces cerevisiae var ellipsoideus 1B15

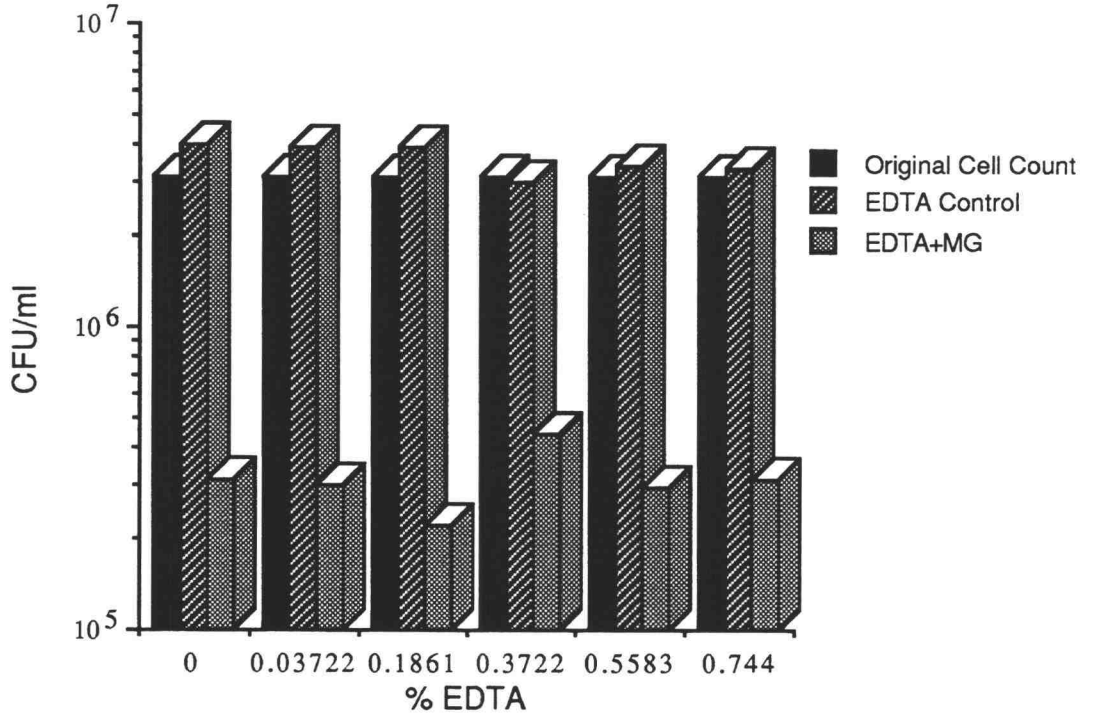

Figure 3.29 Effect of EDTA on inhibitory activity of non-dairy Microgard against Saccharomyces cerevisiae var ellipsoideus 1B15

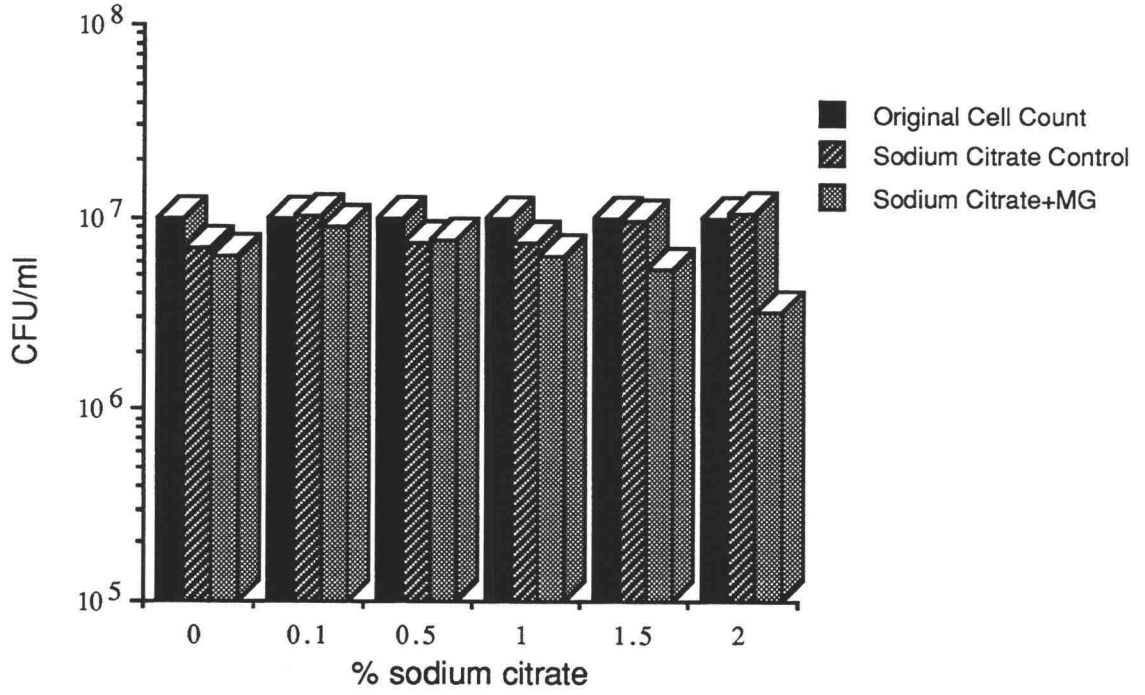

Figure 3.30 Effect of sodium citrate on inhibitory activity of non-dairy Microgard against Candida albicans ATCC 18804

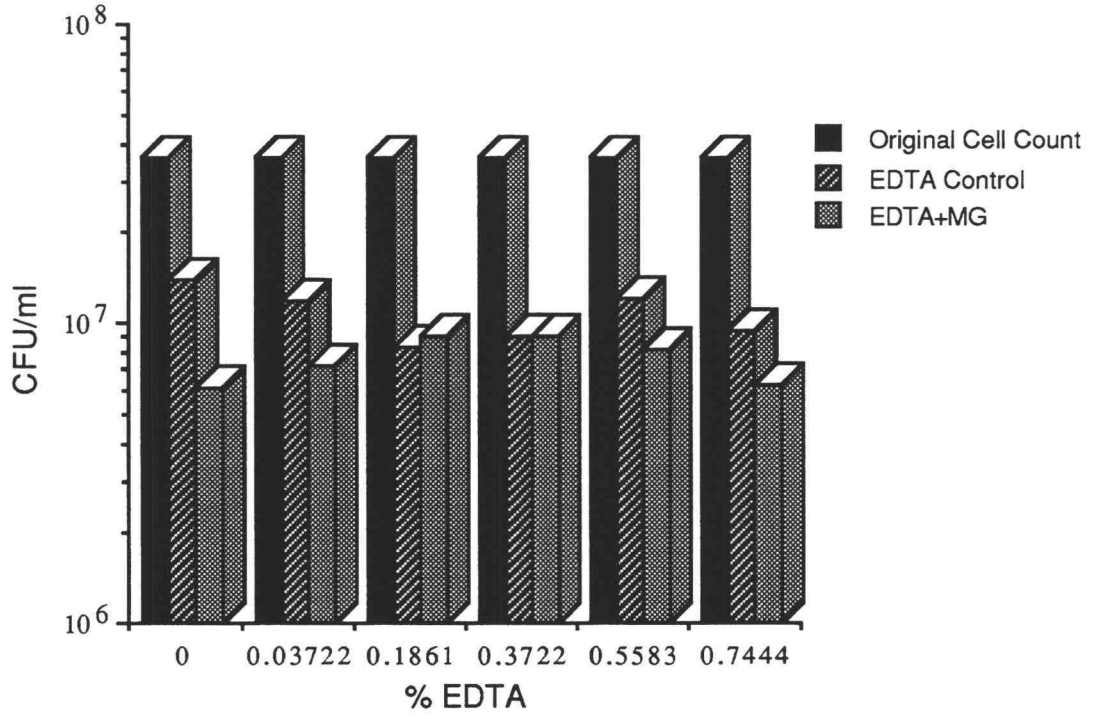

Figure 3.31 Effect of EDTA on inhibitory activity of non-dairy Microgard against Candida albicans ATCC 18804

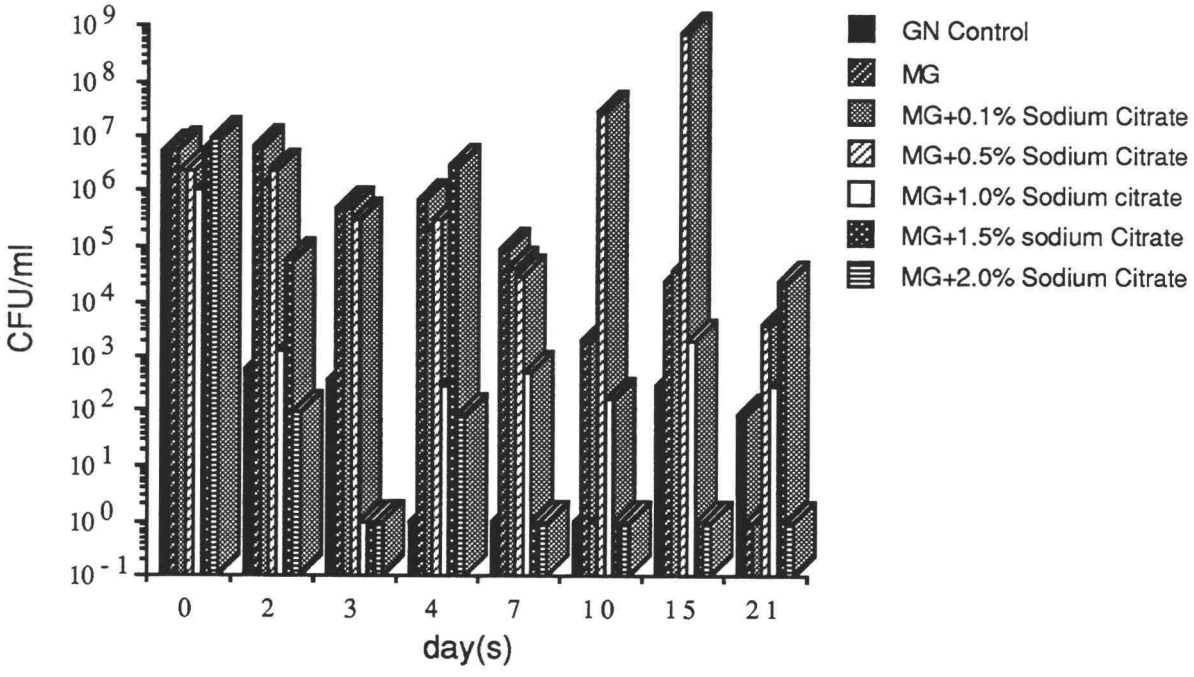

Figure 3.32 Effect of citrate on inhibitory activity of dairy Microgard against Pseudomonas putida in cottage cheese

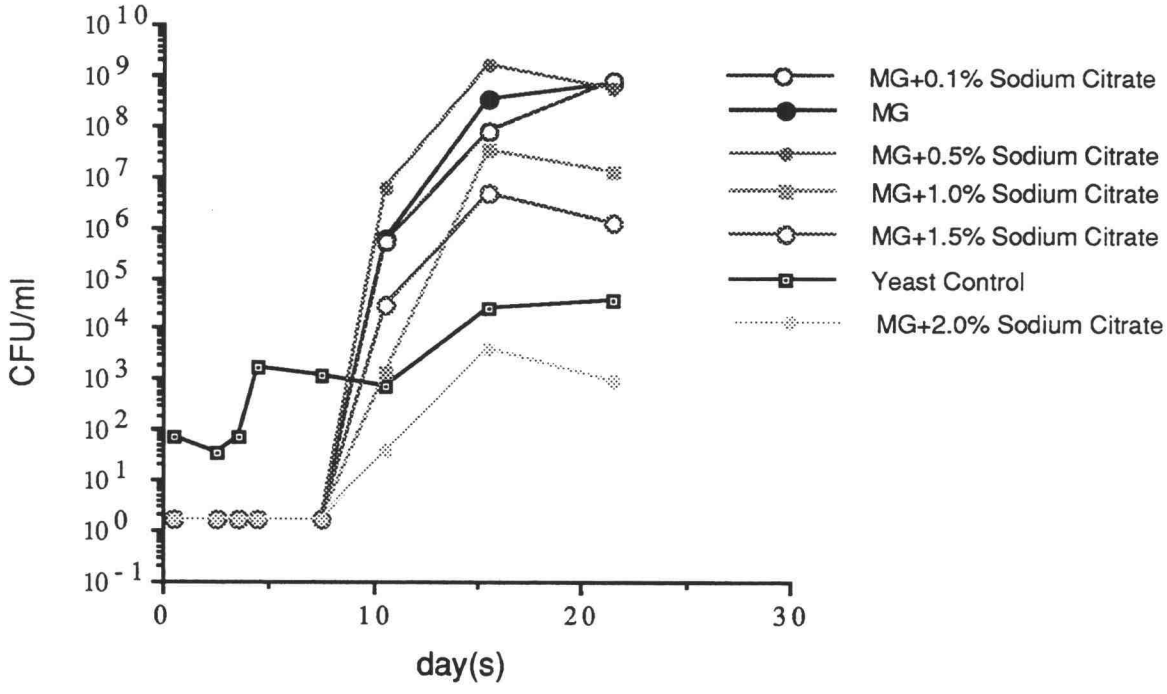

Figure 3.33 Effect of citrate on inhibitory activity of dairy Microgard against Saccharomyces cerevisiae in cottage cheese

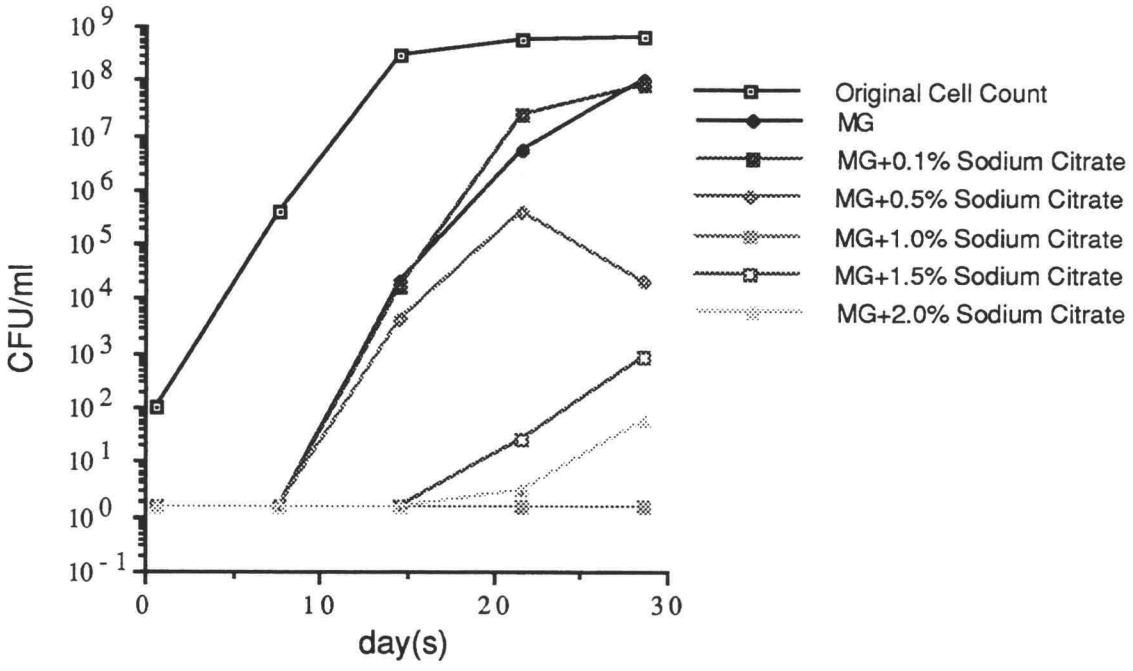

Figure 3.34 Effect of citrate on inhibitory activity of dairy Microgard against Pseudomonas putida in half and half cream

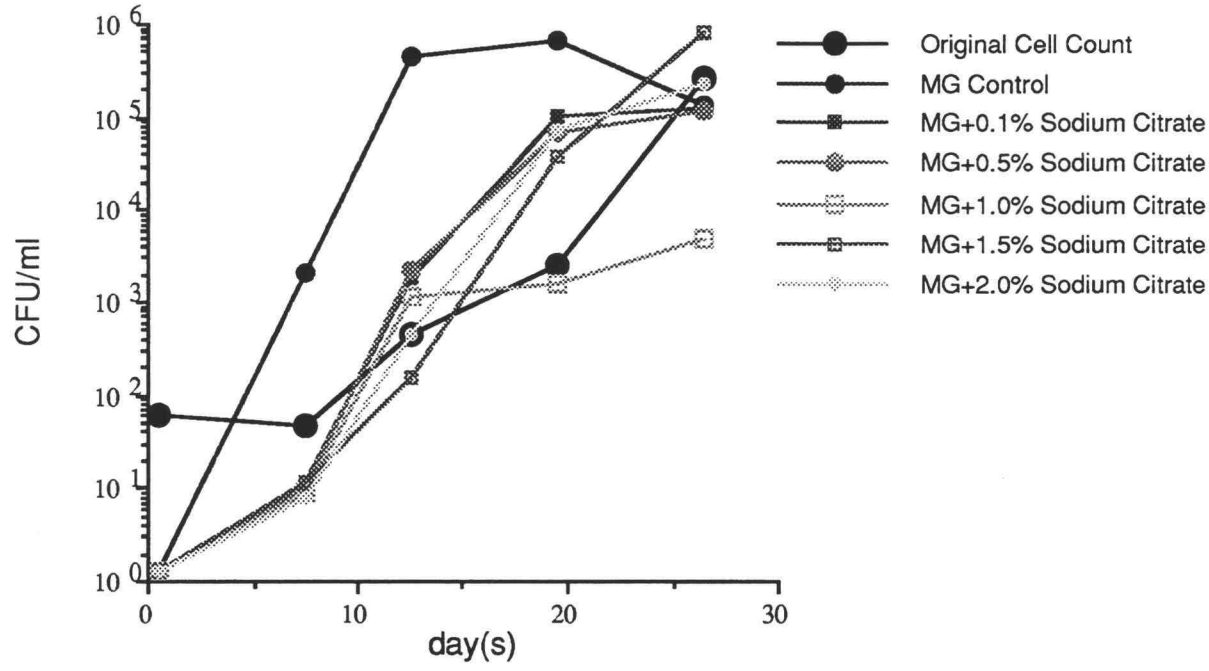

Figure 3.35 Effect of citrate on inhibitory activity of Microgard against Saccharomyces cerevisae in half and half cream

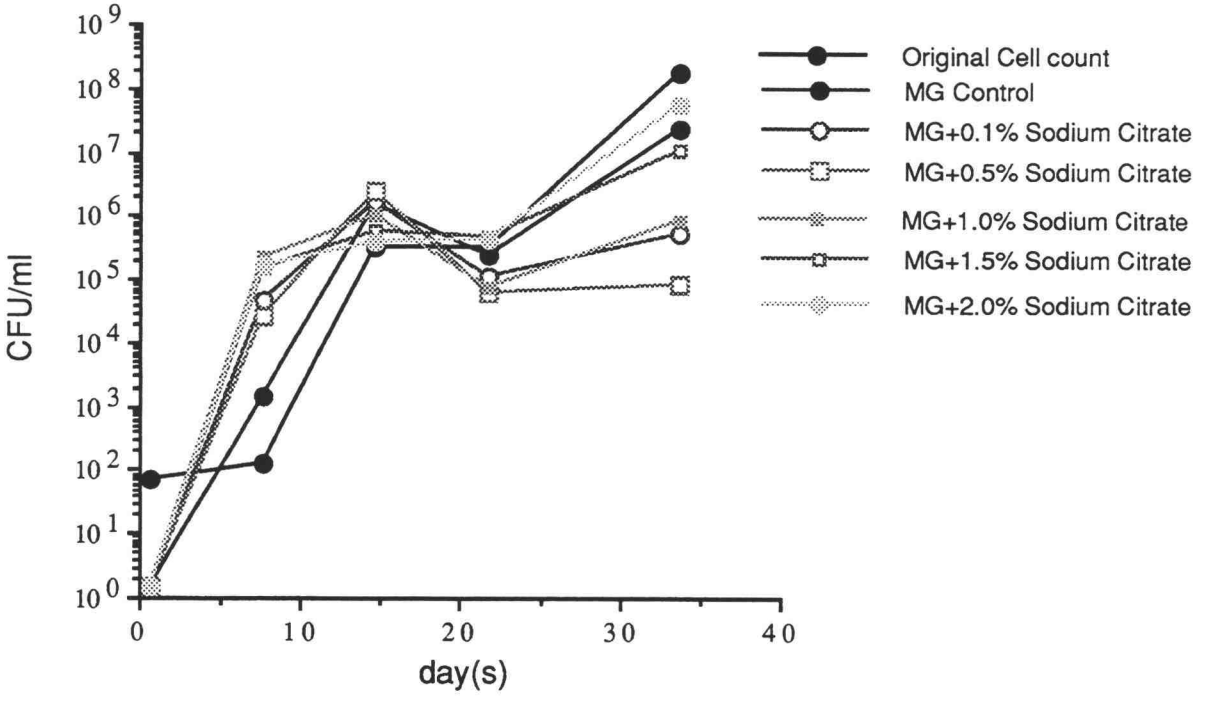

Figure 3.36 Effect of citrate on inhibitory activity of dairy Microgard against Candida albicans in half and half cream

Table 3.1 Effect of diluting Microgard™ (MG) in various diluents on inhibitory activity against *Pseudomonas putida* ATCC 12633. MG was serologically diluted with 0.1 M citrate buffer (pH 5.30), 0.1 M HEPES buffer (pH 5.30), or distilled water. Disk-activity assay (pH 5.30) was performed for each diluted fraction against *Ps. putida.* Plates were incubated at 30 °C for 18 hours.

dilu. / samp	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1024	2048	4096	8192	6384
Citr[b]	3.30[e]	2.80	2.50	2.00	1.55	1.55	2.10	1.60	1.75	2.00	2.00	2.40	3.30	1.80
H_2O[c]	3.40	3.10	2.65	2.50	1.75	1.45	NI[a]	NI	NI	NI	NI	NI	NI	NI
HEP[d]	3.75	3.25	2.40	1.75	1.45	1.45	NI	NI	NI	NI	NI	NI	NI	NI

a. "NI" denotes non-inhibitory
b. "Citr" denotes citrate-buffer used as diluent
c. "H_2O" denotes water used as diluent
d. "HEP" denotes HEPES-buffer used as diluent
e. All inhibitory zones are measured in cm-diameter
f. Blank disk diameter is 1.3 cm

Table 3.2 The effect of Microgard™ -citrate treatment on Gram-negative and Gram-postive bacteria and yeasts

Organism	Log reduction of bacterial[a] and yeast[b] population after treatment at 30 °C for 30 minutes	
	Microgard™ treatment	Microgard™-citrate treatment
Pseudomonas putida ATCC 12633[c]	2.48[e]	6.84
Pseudomonas aeruginosa ATCC 10145[c]	4.04[f]	3.59
Escherichia coli O157:H7[d]	1.06[f]	1.55
Salmonella typhimurium ATCC 79631[c]	1.62[e]	1.56
Listeria monocytogenes ATCC 894[c]	1.87[f]	2.29
Saccaromyces cerevisiae var *ellipsoideus* 1B15[d]	0.86[f]	1.38
Candida albicans ATCC 18804[c]	0.046[f]	0.34

[a] Untreated bacterial cell populations ~6.9 $X10^7$/ml for *Ps. putida,* ~5.0$X10^7$/ml for *Ps. aeruginosa*, ~2.5 $X10^9$/ml for *E. coli*, ~1.7 $X10^8$/ml for *S. typhimurium*, and ~1.01 $X10^8$/ml for *L. monocytogenes*,
[b] Untreated yeast cell populations ~1.66 $X10^6$/ml for *S. cerevisiae*, and ~7.0 $X10^6$/ml for *C. albicans*.
[c] American Type Culture Collection, Rockville, Md.
[d] Oregon State University Culture Collection, Corvallis, Or.
[e] Diluted Microgard™ (1:2) from Wesman Foods, Inc., Beaverton, Or.
[f] Undiluted Microgard™ from Wesman Foods, Inc., Beaverton, Or.

Table 3.3 The effect of Microgard™ -EDTA treatment on Gram-negative and Gram-postive bacteria and yeasts

Organism	Log reduction of bacterial[a] and yeast[b] population after treatment at 30 °C for 30 minutes	
	Microgard™ treatment	Microgard™-EDTA treatment
Pseudomonas putida ATCC 12633[c]	2.33[e]	4.16
Pseudomonas aeruginosa ATCC 10145[c]	4.66[f]	5.42
Escherichia coli O157:H7[d]	2.41[f]	1.88
Salmonella typhimurium ATCC 79631[c]	2.25[e]	1.84
Listeria monocytogenes ATCC 894[c]	1.33[f]	1.93
Saccaromyces cerevisiae var *ellipsoideus* 1B15[d]	1.11[f]	1.25
Candida albicans ATCC 18804[c]	0.18[f]	0.17

[a] Untreated bacterial cell populations ~5.3 $X10^7$/ml for *Ps. putida*, ~2.1$X10^7$/ml for *Ps. aeruginosa*, ~3.85 $X10^9$/ml for *E. coli*, ~1.4 $X10^8$/ml for *S. typhimurium*, and ~9.3 $X10^7$/ml for *L. monocytogenes*,
[b] Untreated yeast cell populations ~4.0 $X10^6$/ml for *S. cerevisiae*, and ~9.2 $X10^6$/ml for *C. albicans.*
[c] American Type Culture Collection, Rockville, Md.
[d] Oregon State University Culture Collection, Corvallis, Or.
[e] Diluted Microgard™ (1:2) from Wesman Foods, Inc., Beaverton, Or.
[f] Undiluted Microgard™ from Wesman Foods, Inc., Beaverton, Or.

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CONCLUSIONS

Research reported in this thesis showed that the inhibitory activity of Microgard™ (MG) could not solely be due to acetic, propionic and lactic acids or diacetyl. The removal of organic acids corresponded to an increase in the specific activity of MG. The antimicrobial substance(s) in MG is thermal stable and does not resemble the active precursors of lactoferrin when hydrolyzed under acidic or basic conditions. Also, the active compound is proteineous in nature resembling the characteristics of bacteriocins or microcins. The active peptide was eluted from reverse-phase HPLC by a low concentration of organic solvent (6-10% acetonitrile) suggesting hydrophillicity of the compound.

Analysis by mass spectroscopy showed that the peptide has a molecular weight of 881.7 daltons. The result agrees with previous estimations from ultra-filtration (between 500 and 1000 daltons), gel-filtration (860 daltons) and amino-acid analysis (926 daltons). The peptide was found to contain eight residues consisting of 4 methionine, 2 of valine, 1 of glutamine/glutamate, and possibly 1 of glycine.

Chelating agents such as sodium citrate or EDTA have been shown to potentiate the antimicrobial activity of MG against a wider range of bacteria and yeasts. The incubation time for chelating agents may be crucial in that organisms possessing different membrane structures have varying amounts of stabilizing cations on their surfaces. Incorporation of food-grade chelating agents and MG holds promise for use in the extension of shelf-life of various foods.

Future work on MG remains exciting. The peptide will be sequenced and synthesized *in -vitro*. After which, probes can be made from the synthesized peptide and its nucleotide bases determined and cloned into lactic acid bacteria

to induce the production of this bacteriocin. Numerous strains of various species can be produced in this manner for use in food preservation. The active Microgard™ peptide likely also will find use in human medicine.

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