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Citation	北海道大學水産學部研究彙報, 44(2), 80-85
Issue Date	1993-05
Doc URL	https://hdl.handle.net/2115/24113
Type	departmental bulletin paper
File Information	44(2)_P80-85.pdf

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Bull. Fac. Fish. Hokkaido Univ.
44(2), 80-85. 1993.

Effect of Acetic and Citric Acids on the Growth and Activity (VB-N) of *Pseudomonas* sp. and *Moraxella* sp.

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and Haruo SHINANO*

Abstract

The ability of acetic and citric acids to inhibit growth of *Pseudomonas* sp. and *Moraxella* sp. in culture media was investigated. Both acids, at concentrations of 0.02, 0.03, 0.04 and 0.05 % were added to a sterilized nutrient broth that was then inoculated with the above mentioned microorganisms. The media was incubated at 25°C for 8 days. VB-N production of both microorganisms was inhibited by 0.05% of acetic acid, *Moraxella* sp. was found also to be sensitive in the presence of 0.05% of citric acid. The degree of inhibition decreased as the acid concentration decreased. The extent of antimicrobial activity of these acids coincided with their degree of undissociation. Citric acid, with larger dissociation constant, was less detrimental to both microorganisms

Introduction

Since early studies of fish spoilage, it has been known that autolytic activity plays a minor role, i.e., spoilage is caused by bacteria. Pseudomonaceae, *Moraxella*, *Alcaligenes*, *Flavobacterium/Cytophaga*, *Corynebacterium*, Vibrionaceae, *Bacillus* and *Micrococcus* are the microorganisms most frequently isolated[1~3]. Fish handling after catch causes contamination with a flora that is difficult to control[4] unless a rapid and adequate storage is done.

Organic acids whether naturally present in foods or intentionally added to them have been utilized for years to control microbial spoilage[5]. The use of organic acids in raw fish storage after catching may offer interesting prospects for shelf-life extension[6,7]. The efficacy of acetic acid as well as of citric acid[5,9] as antimicrobial is well established[5,8,9]. Current data suggest that the mode of action of organic acids is attributed to direct pH reduction of the substrate, depression of the intracellular pH by ionization of the undissociated acid molecule or disruption of substrate transport by alteration of cell membrane permeability[5], and therefore pH dependent[5,9~11].

The effect of treatment with different concentrations of acetic and citric acids was investigated by using *Pseudomonas* sp. and *Moraxella* sp. strains isolated from brine during sardine *Sardinops melanosticta* storage. The objective of this investigation was to determine whether acetic or citric acid better inhibits *Pseudomonas* and *Moraxella* when inoculated in a "nutrient broth" containing 4% sodium chlo-

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ride; and also sought to determine the minimal antimicrobial acid concentration to cause inhibition.

Materials and Methods

Pseudomonas sp. and *Moraxella* sp. were isolated from brine during storage of sardine, *Sardinops melanosticta*, and cultured on nutrient agar that contained beef extract (Difco Laboratories, Detroit, MI, USA) 5 g/*l*, polypepton (Nihon Seiyaku, Tokyo, Chiyoda, Japan) 10 g/*l*, sodium chloride (Kanto Chemical, Tokyo, Nihonbashi, Japan) 5 g/*l*, and agar (Nihon Seiyaku) 15 g/*l* (pH 7.2) and stored at 5°C. The effect of different concentrations of acetic and citric acids on the growth of *Pseudomonas* and *Moraxella* was tested by adding 0.02, 0.03, 0.04 and 0.05% of each acid to a 4% sodium chloride nutrient broth prepared in the same way that nutrient agar but with not agar addition (pH was not adjusted). Two controls were runned, one, the 4% sodium chloride broth and the second one was the normally containing 0.5% sodium chloride. Inocula for the experiments were prepared by growing the microorganisms in nutrient agar slant at 25°C for 24 h. A loopful of these cells was added to sterile 0.85% physiological saline. Media (100 m*l* in 200-m*l* Erlenmeyer flasks) were inoculated with 1 m*l* of the previously inoculated physiological saline to contain approximately 10^5 cells/m*l*[12,13] and the cultures were incubated aerobically and statically at 25°C for 8 days. Every after two days of the incubation period, *Pseudomonas* sp. and *Moraxella* sp. viability was determined by plating on nutrient

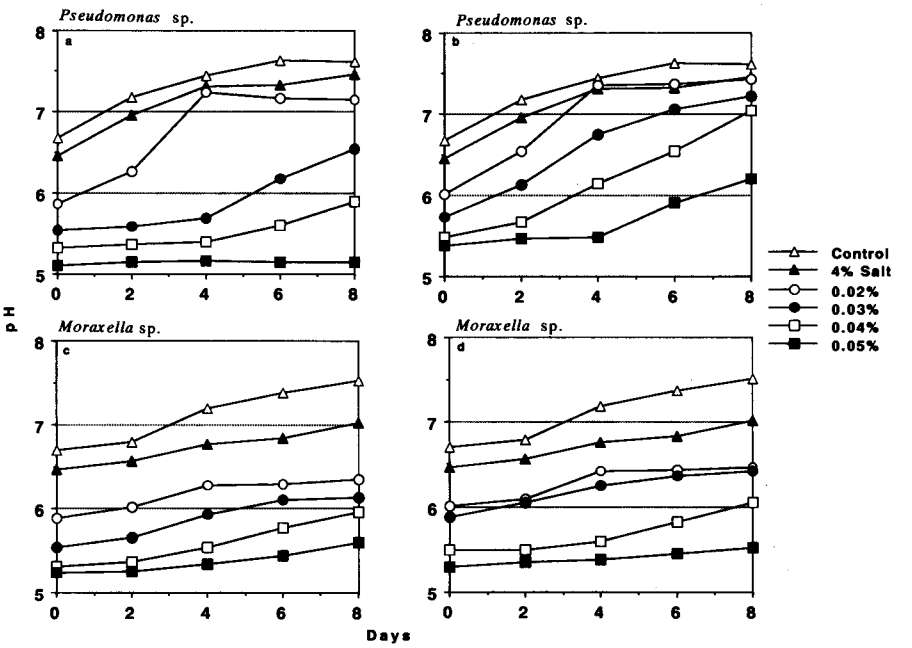

Fig. 1. Effect of acetic (a, c) and citric (b, d) acids on changes in pH values in nutrient broth inoculated with *Pseudomonas* sp. (a, b) and *Moraxella* sp. (c, d).

agar using the standard pour plate technique and duplicate plates.

Activity of the microorganism in the media was tested by observing the changes in volatile basic nitrogen (VB-N) by the Conway method. The pH measurement was performed by a Horiba Compact pH-meter C-1 at the time of plating.

Results

As can be seen in Fig. 1, the pH of the growth media, for both strains, increased throughout the incubation period except for in the case where *Pseudomonas* sp. was inoculated in media containing the highest acetic acid concentration (0.05%, Fig. 1a). The increase in the pH rate for *Pseudomonas* sp. was bigger as the acid concentration decreased.

After 8 days of incubation *Pseudomonas* sp. inoculated to the media containing 0.05% acetic acid, decreased in cell number over 6 days then a small increase occured (Fig. 2a), the other cultures showed growth according to the acid and its concentration (Fig. 2). In the earlier stage (2-4 days) *Moraxella* sp. growing in acetic acid concentrations of 0.03% and higher and citric acid concentrations of 0.04% and higher grew faster but ultimately *Pseudomonas* sp. reached higher numbers in almost all the cases.

VB-N production of the microorganisms (Fig. 3) was comparatively higher for media containing citric acid. Its values increased with the growth of *Pseudomonas*

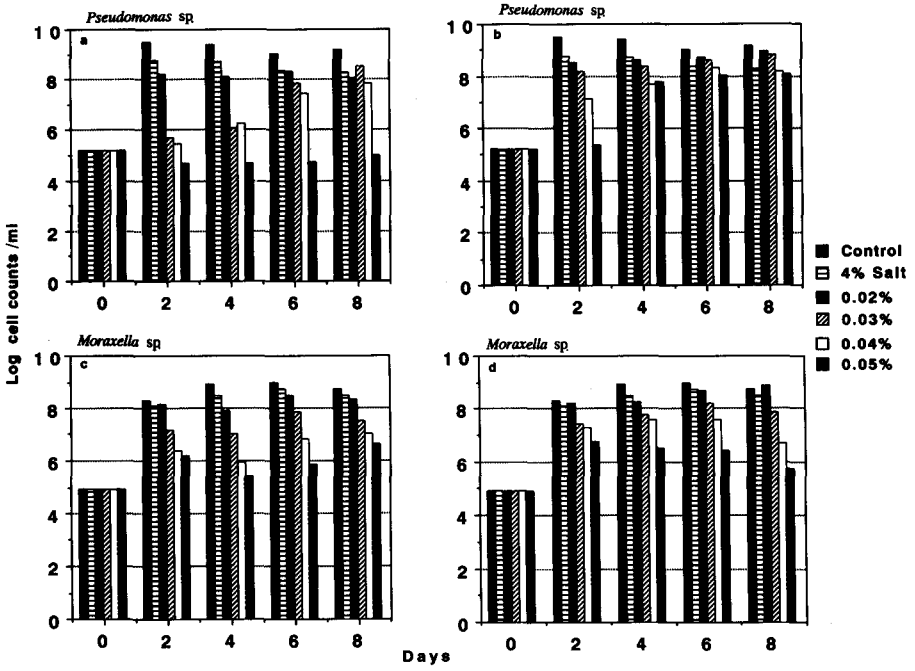

Fig. 2. Effect of acetic (a, c) and citric (b, d) acids on changes in viable bacterial counts of *Pseudomonas* sp. (a, b) and *Moraxella* sp. (c, d) growing in nutrient broth.

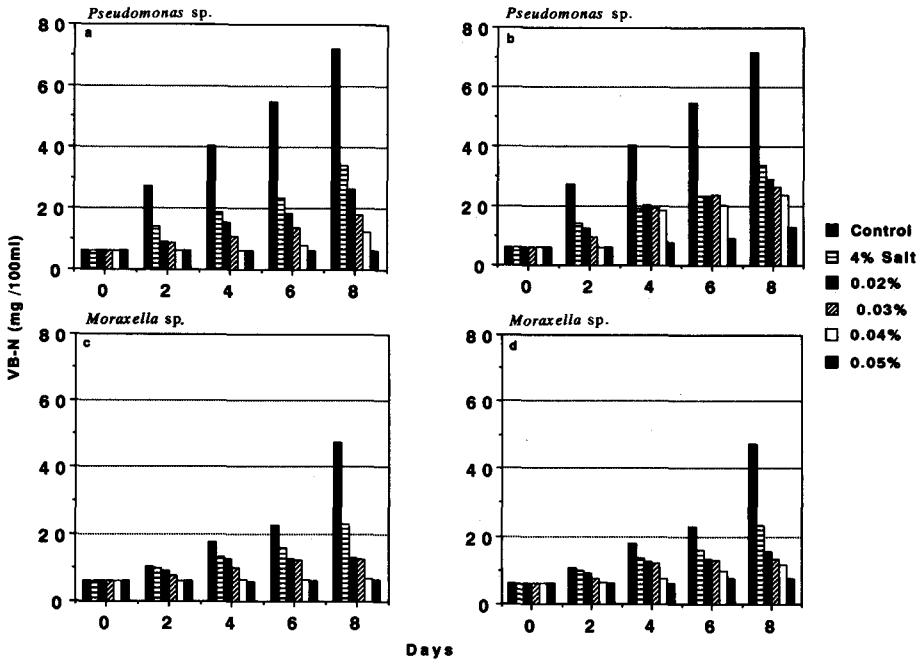

Fig. 3. Effect of acetic (a, c) and citric (b, d) acids on changes in the amount of volatile basic nitrogen (VB-N) in nutrient broth inoculated with *Pseudomonas* sp. (a, b) and *Moraxella* sp. (c, d).

sp. more than with that of *Moraxella* sp. (Fig. 3a and 3c). Here, VB-N production of both microorganisms was inhibited significantly compared with those of the untreated controls.

Discussion

This study was initiated to test to what extent acetic or citric acid inhibits the growth of *Pseudomonas* sp. and *Moraxella* sp. and the degree of inhibition according to acid concentration. The undissociated molecule of the acid is known to be the active antimicrobial[14~16] and also to be responsible for pH values[5,9~11]. When citric acid, with larger dissociation constant[17,18], is contained in the media, the initial value and the rate of increase in pH appeared to be bigger than those reached when acetic acid is present.

Most organic acids are largely ineffective as microbial inhibitors in the pH range of 5.5 to 6.8 within which all food poisoning bacteria and most spoilage bacteria grow[16]. During our investigation, media containing 0.04% and 0.05% of either acetic or citric acid gave pH values below 5.5 (5.1 to 5.4) at the initial stage, under this range the action of the acids was evidently stronger than in any other concentrations tested. However acetic acid gave lower pH values and showed better action against both microorganisms.

Viable cell counts of *Pseudomonas* sp. and *Moraxella* sp. in the presence of acetic acid were always lower than their counterparts in the presence of citric acid, as the former has a smaller dissociation constant[17,18].

A concentration of 0.05% acetic acid in nutrient broth containing 4% NaCl clearly inhibited *Pseudomonas* sp. and to some degree *Moraxella* sp. in nutrient broth containing 4% NaCl. The effect of salt was much greater in the presence of acids than when it works alone. According to Levine et al.[10] a similar effect was observed when 5% NaCl working together with 0.04% acetic acid inhibits *Staphylococcus aureus* growth much more than salt alone. They attributed this effect to the combined action of both, NaCl and acetic acid. Furthermore it is known that the action of organic acids as antimicrobial agents is generally improved by anions which interfere with the dissociation of the acid molecule; certain specific cations may also significantly increase the effectiveness of organic acids by increasing the solubility of the acid in the microbial cell membrane[19].

Many microorganisms use organic acids as metabolizable carbon source[19]. We suppose, therefore, growth at low level, 0.02–0.03% acid concentrations, might show higher values than those samples in which only 4% salt has been added (Fig. 2).

A clearly difference could be seen in VB-N production between *Moraxella* and, *Pseudomonas*, higher for the latter, known to have stronger spoilage activity[1,4,20~22]. Besides even when cell numbers did not show significant differences and in some cases *Moraxella* sp. showed larger numbers, the maximum values of VB-N for *Pseudomonas* are evidently higher (Figs. 2 and 3).

The extent of antibacterial activity of these acids coincided with their degree of undissociation[17,18]. Citric acid, with larger dissociation constant was less detrimental to the tested microorganisms than was acetic acid. Similar results for *Listeria monocytogenes* were found by Ahamad et al.[23] when they were studying inhibition of *L. monocytogenes* CA and V7 by acetic, citric and lactic acids at 7, 13, 21 and 35°C. Moreover, weak lipophilic acids are known to cause leakage of hydrogen ions across the cell membrane, acidifying the cell interior, and inhibiting nutrient transport. Some acids will dissociate to give anions, e.g., lactate, citrate which the cell can transport and whose presence does not therefore inhibit energy yielding metabolism.

Other acids, e.g., acetic and formic are very effective preservatives since they are not only proton conductors but also may yield inhibitory concentrations of their anions within the cell[16]. This might also explain the stronger activity of acetic acid under our experimental conditions.

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