AN ABSTRACT OF THE THESIS OF

MOHAMMED ALI SALIH for the degree of Ph.D. in MICROBIOLOGY presented on April 29, 1985.

Title: STUDIES ON GROWTH METABOLITES PRODUCED BY PROPIONIBACTERIUM SHERMANII

Abstract approved: _____ William E. Sandine

The ability of Propionibacterium shermanii to produce growth metabolites which were inhibitory to food spoilage indicator organisms was investigated. Aspergillus niger, a Pseudomonas species and Kluyveromyces fragilis were the spoilage indicators chosen to represent molds, gram negative psychrotrophic bacteria and yeasts, respectively. Supplementation of milk or whey based media was attempted to optimize synthesis of the inhibitory growth metabolites by P. shermanii. Production of the propionibacterial growth metabolites (PGM) was stimulated by addition of cobalt (II) nitrate (16 mg/L) or disodium fumarate (0.4%) to milk or whey growth media. PGM was heat sensitive, bacteriostatic and fungistatic.

The antimicrobial effect of PGM was utilized in preservation of commercially produced cottage cheese and

strawberry-flavored yogurt. The incorporation of freeze dried PGM (FDPM) into yogurt and liquid PGM (LPM) into cottage cheese caused significant reductions in spoilage organisms and extension of shelf life. Cottage cheese containing LPM (1% in the dressing) exhibited 6 to 9 days extension in the cheese shelf life over the controls. Ten percent FDPM completely preserved a commercial brand of yogurt against spoilage organisms for 82 days at 5°C.

PGM components were separated by gel filteration, ultrafilteration and cationic exchange. Activity of fractions was assayed against the indicator bacterium (Pseudomonas sp.). Fractions showing high activity were assayed for fatty acid content and uv absorption (200-400 nm). Several lines of evidence suggested the existence of open structured tetrapyrroles (bactobilin) and their degradation products. Bactobilin was thought to function as an ionophore in the inhibitory action of PGM and also as a natural buffering agent protecting the organism against its own toxic metabolites.

The ability of P. shermanii to utilize and accumulate elemental selenium was studied. Utilization was influenced by the presence of additional sugars in a tryptic soy broth medium. Swiss cheese starter cultures formed a muddy pink color in growth media containing

sodium selenite. Pink color defects in cheeses were thought to depend on the ability of starter bacteria to accumulate elemental selenium. Extracellular rennet-like protease(s) was observed to occur in the presence of selenite in P. shermanii strains and Lactobacillus bulgaricus strain R-5. The enzyme appeared to be an oxygen sensitive selenoprotein.

Differences in selenite and tellurite tolerance by L. bulgaricus and Streptococcus thermophilus were utilized as a basis for development of a selective differential medium for isolation and enumeration of the two organisms. Growth of L. bulgaricus was selectively promoted at high selenite concentrations (above 160 μ g/L). Streptococcus thermophilus growth was inhibited by the same selenite concentrations. Conversely, S. thermophilus was stimulated to grow at high tellurite concentrations (above 100 μ g/L) in contrast to the inability of L. bulgaricus to grow under the same conditions.

STUDIES ON GROWTH METABOLITES PRODUCED BY *PROPIONIBACTERIUM SHERMANII*

by

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Typed by Deborah K. Safley for Mohammed Ali Salih

To Assya whose love, care, patience and understanding has been of great importance to me. To our loved son "Hanni" we dedicate this too.

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STUDIES ON GROWTH METABOLITES PRODUCED BY *PROPIONIBACTERIUM SHERMANII*

Chapter I

Antimicrobial Growth Metabolites Produced by *Propionibacterium Shermanii*

Abstract

Various approaches were investigated to stimulate *Propionibacterium shermanii* to produce antimicrobial growth metabolites in milk or whey based media. Activity of the propionibacterial growth metabolites (PGM) was assayed against three indicator organisms. Each was chosen to represent an important group of food spoilage microorganisms, i.e., gram negative psychrotrophic bacteria (*Pseudomonas* sp.), yeasts (*Kluyveromyces fragilis*) and molds (*Aspergillus niger*). Supplementation of the milk or whey based media with additional carbon and nitrogen sources was least needed to improve inhibitory PGM production by *P. shermanii*. Fortification of whey based media with cobalt (II) nitrate (16 mg/L) depressed proteolytic activity and improved effeciency of *P. shermanii* to synthesize active PGM. The addition of a mixture of trace minerals to whey based media elevated the proteolytic activity and decreased the production of active PGM by *P. shermanii* in all tested combinations.

The addition of disodium fumarate (0.1%, 0.2%, and 0.4%) to propionibacterial growth medium increased the potency of metabolites active against the indicator mold. PGM produced by continuous neutralization and slow agitation of P. shermanii cultures, offered no improvement in PGM activity over that produced during static unneutralized fermentations. PGM was found to be heat sensitive, bacteriostatic and fungistatic in nature.

Introduction

Propionibacterium shermanii has been utilized for decades in the commercial production of vitamin B_{12} and for the manufacture of Swiss cheese. A sizable body of the literature on propionibacteria has been devoted to the nutrition and growth of these organisms (19). However, the continual display of unusual and unique physiological and biochemical characteristics by propionibacteria, has continued to stimulate interest in their growth and metabolism. Recently, Charakhchyan et al. (7) demonstrated the peculiar assimilation and excretion of sulfate by P. shermanii. The sulfate accumulation and excretion process was recognized to be of an unusual oscillatory nature. Other interesting observations include the ability of P. shermanii to accummulate polyphosphates in response to induction by diammonium sulfate (15) and the ability of polyphosphate kinase to direct polyphosphate synthesis (16). Propionibacterium shermanii also has been characterized as a unique organism by virtue of its ability to synthesize either iron- or manganese-containing superoxide dismutase with almost identical protein moieties (31).

Numerous efforts have been made to create optimal growth conditions for P. shermanii, utilizing different organic carbon sources, in the over-production of vitamin B_{12} (5, 27, 28). Also, some investigators have manipulated growth conditions to maximize the production of propionic acid and acetic acid for preservation of silage (6, 29). Inspite of all the optimization efforts, basic questions are yet to be answered: Why has the production of propionate or acetate by the organism not yet become a successful commercial process? What cellular control mechanisms regulate the production of these acids? How does the organism accummulate and excrete toxic undissociated acids, especially in consideration of evidence regarding the toxicity of these acids to the organism (33)?

Different metabolites produced by P. shermanii such as propionates, acetates, diacetyl and propionin have demonstrable antimicrobial activities (20). Furthermore, Dumon and Adda (10) identified 9 sesquiterpenes in mountain type Gruyere cheese volatiles using gas chromatographic-mass spectrometry analyses. These sesquiterpenes were found to be antimicrobial agents (30).

The mechanism of antimicrobial action of weak organic acids has been studied by several investigators (12, 13,

14, 38). Parabens were found to inhibit transport across membrane vesicles by reduction of the internal pH, a component of the proton motive force. Also, the potency of weak acids as food preservatives was found to reside in their capacity to reduce the microbial intracellular pH (homeostasis). Though both the undissociated and dissociated acid forms will cause the intracellular pH to fall, the growth inhibition of E. coli was concluded to result from the predominance of undissociated acid forms.

The present study was undertaken to identify and characterize the antimicrobial nature of growth metabolites produced by P. shermanii in milk against yeasts, molds and bacteria.

Materials and Methods

Organisms used:

Propionibacterium shermanii American Type Culture Collection (ATCC) strain 9616 was grown in either sodium lactate broth or TGY broth. The later contained in g/L Tryptone, 10; glucose, 5; yeast estract, 5 with incubation at 30°C for 2 days. The activity of propionibacterial growth metabolites was assayed against the following indicator organisms:

1) Representative yeast: Kluyveromyces fragilis was from the culture collection maintained in the Department of Microbiology, Oregon State University (OSU). The organism was repeatedly propagated in sterile Difco Lactose broth at 30°C with overnight incubation.

2) Representative mold: Aspergillus niger (OSU culture collection) inoculum was prepared by transfer of the organism into acidified Difco yeast malt extract broth (pH 3.5) with incubation at 25°C for 3 days.

3) Representative gram negative bacterium: A gram negative psychrotrophic bacterium was isolated from cottage cheese supplied by HP Hood Dairy, Inc., Boston, MA. The organism was identified to genus as a Pseudomonas species by virtue of possessing monotrichous

flagella as seen in electron photomicrographs. The organism was propogated in sterile Difco lactose broth with overnight incubation at 30°C.

The ability of P. shermanii to produce growth inhibitory metabolites was investigated as follows:

1) Supplementation of milk and whey based media with additional nitrogen and carbon sources:

A basal medium of the following composition was made up (w/v) using either 10% nonfat dry milk [NFDM] or fresh cheddar cheese whey supplemented with 0.2% yeast extract). It was autoclaved at 121°C for 12-13 minutes. Filter sterilized fructose (2.5%) or autoclaved glycerol (3%) was added to separate flasks of the basal medium, which was then aseptically acidified with lactic acid (85%) to pH 5.3. The propionibacterial inoculum was 3% and incubation was carried out statically at 30°C for 5 days. Samples were taken every 24 hours and inhibitory activity was assayed against the three indicator organisms. Diammonium phosphate (4%, w/v) ammonium acetate (1%, w/v) or ammonium citrate (1%, w/v) also were added to the basal milk or whey medium as additional nitrogen sources (pH 6.9-7.0). The media were then pasteurized at 85°C for 45 minutes and inoculated with 3% P. shermanii and incubated statically

at 30°C for 5 days. Sampling and activity assays were conducted as before.

2) Effect of Trace Elements:

Five liters of the basal whey medium were prepared and dispensed in 500 ml aliquots into 1-liter flasks. To each of four flasks, 8 mg of cobalt (II) nitrate were added. Into three of these flasks the following concentrations of chemicals were added: Diammonium phosphate (0.4%) or ammonium acetate (1%) or glycerol (3%). A fifth flask was a whey control. To another four flasks the following chemical (g/L) were added: $CuSo_4$, 60; NaCl, 135; $FeSO_4$, 662; $ZnCl_2$, 284, Na_2SeO_3, 166 and cobaltous nitrate, 20. To three flasks the same supplements as above were added (diammonium phosphate or ammonium acetate or glycerol). As for the cobalt-containing flasks, a second whey only control was run with this set. The pH of all flasks was adjusted to 6.8-7.0 and all flasks were then pasteurized at 85°C for 45 minutes in a water bath. Propionibacterium shermanii inoculum was 3% and incubation was carried out at 30°C (static) for 4 days. Samples were withdrawn every 24 hours to measure the pH, proteolytic (21) and the inhibitory activities against the three indicator organisms.

3) Effect of the addition of propionate intermediates:

Six liters of tryptone-yeast extract-lactose TYL medium were prepared with the following w/v composition: Tryptone, 1%, yeast extract 0.5% and lactose 1% - pH adjusted to 5.70. The medium was dispensed into four 1.5 liter flasks and autoclaved at 121^{o}C for 20 minutes.

Disodium fumarate - tomato juice solution: Into a filtered tomato juice solution, 10% (w/v) disodium fumarate was dissolved and the pH of the solution adjusted to 5.70. The tomato juice fumarate solution was then filter-sterilized and 0.1%, 0.2% and 0.4% fumarate concentrations achieved in three flasks of TYL medium. To the fourth flask, filter sterilized tomato juice only, at the same strength, was added and the flask designated as control. The tomato juice solution in all flasks was maintained at 6.6% (v/v). All flasks were inoculated with 3.3% P. shermanii and incubated statically at 30^{o}C over a period of 6 days. Samples were taken every 24 hours for pH and dry weight measurement and assayed against the three indicator organisms. Dilution of the growth metabolites was done in phosphate buffer (pH 5.0).

Cell dry weight determinations: Five ml samples from all inoculated and uninoculated (control) flasks were

centrifuged at 10,000 rpm (at 4°C) for 15 minutes. Cellular pellets were washed twice by resuspension in 5 ml of phosphate buffer (pH 6.5) after centrifugation. The final pellets were freeze dried and weighed. The dry weight determinations were plotted against time.

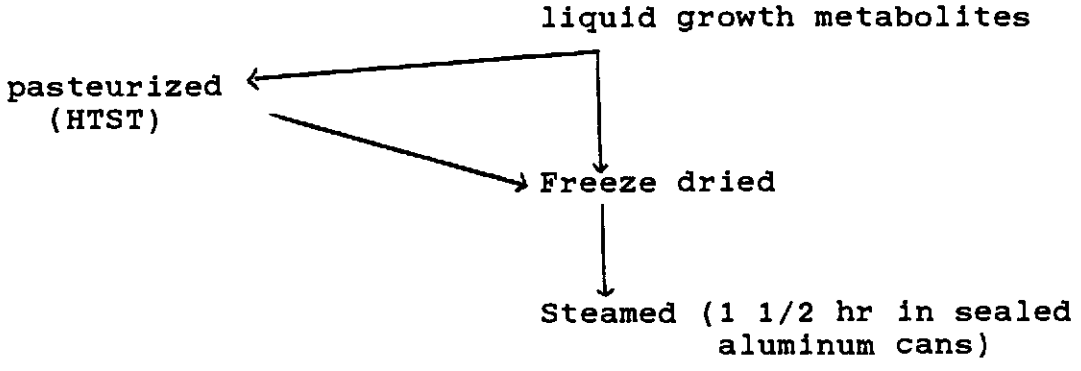
4) Static and Continuous Neutralization Effects:

Whey or milk based media w/v, 10% NFDM or whey +0.2% yeast extract, were divided into 2 equal 0.9-liter amounts. Each 800 ml was added to a presterilized 2-liter flask or a fermentor flask (Fermentation Design, Inc., Allentown, PA). Both flasks were pasteurized at 85°C for 45 minutes, cooled to 30°C and acidified with lactic acid (85%) to pH 5.3 under aseptic conditions. Both flasks were then inoculated with 5% P. shermanii cultures. Fermentations in both flasks were manipulated as follows:

a) The fermenter flask was positioned on the fermenter base and a sterile ingold pH electrode was immersed in the culture medium. The automatic pH control was set at 4.8 and the pH was maintained with an ammonium hydroxide solution (20% v/v). The cultures were stirred (50-60 rpm) and maintained at 30°C over a period of 5 days.

b) The other flask was incubated statically at 30°C and shaken only when taking samples. The five day old growth liquid metabolites were treated according to scheme 1, below, and each sample was assayed for its inhibitory activity against the three indicator organisms:

Scheme (1)



Assay proceedures against indicator organisms:

a) Pseudomonas sp.:

One ml of an overnight culture was diluted in sterile 0.1% peptone water. Dilutions 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} were commonly utilized except for a few cases when only the 10^{-6} and 10^{-7} dilutions were used. Crystal violet (1 ppm) plate count agar (pH 7.1) dispensed 100 ml per bottle was autoclaved at 121°C for 15 minutes. The medium was then acidified with 10% sterile tartaric acid solution to pH 5.3 and 50 ppm filter sterilized; 2, 3, 5

triphenyl tetrazolium chloride was achieved in each bottle. Different concentrations of the propionibacterial growth metabolites (percentages) were then made into the crystal violet-tetrozolium (CVT) agar plates which were then poured in duplicate for each dilution of the organism. Control plate were prepared excluding the growth metabolites from the CVT agar. All plates were incubated at 30°C for 48 hours and emergent red colonies were counted. The percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{control counts} - \text{sample counts}}{\text{control counts}} \times 100$$

b) Assay against the yeast and the mold:

Difco Yeast Extract Malt (YMA) medium was prepared and dispensed in 100 ml volumes and autoclaved at 121°C for 15 minutes. The medium was then acidified with 10% sterile tartaric acid (1.4 ml/100 ml YMA) to pH 3.5. The yeast indicator organism (K. fragilis) was diluted and dilutions of 10^{-4}, 10^{-5} and 10^{-6} were used. The fermentation metabolites were then mixed with the acidified YMA and plates were poured in duplicate. All plates were incubated at room temperature (25°C). Yeast counts were used to deduce the inhibition percentage as

with the Pseudomonas bacterial assay. Molds were scored on the same medium after 4 days of incubation as follows: Abundant growth as for the control was given a 4; good growth = 3; moderate growth = 2; poor growth = 1 and no growth = 0.

5) Some properties of the fermentation growth metabolites:

a) Heat stability:

Propionibacterium shermanii was grown in an acidified (pH 5.3) liquid milk (10% FDPM + 0.2% yeast extract) for 72 hours. The resulting fermentation metabolites (FM) were investigated for their inhibitory activities against the indicator bacteria following different heat treatments. The FM were dispensed 100 ml each into 7 screw cap bottles. One bottle was left untreated and used as a control. The other 6 bottles were divided into 3 groups and each group received one of the following heat treatments: 62.5^{o}C for 30 min, 70^{o}C for 15 min or 80^{o}C for 10 min. One bottle from each group was used to assay for the stability of the metabolites and the other bottle was repasteurized again at its same temperature. The effect of the first and second heat treatment in each group was determined by measuring the loss in inhibitory activity

against the gram negative indicator bacteria (Pseudomonas sp.).

b) Bacteriostatic Effect:

In 9 ml of sterile Difco lactose broth, different percentage concentrations of the FM (96 hr) were made. From an overnight Pseudomonas sp. culture, 0.2 ml was transfered into each of the lactose test tubes including a control tube with no FM. One ml samples were withdrawn from each test tube after 3 days of incubation at 30°C. The samples were diluted and plates were poured with CVT agar. Incubation was at 30°C for 48 hr. The difference between the final bacterial inhibition (after 3 days) and the initial (zero time) inhibition was calculated as percentage recovery. The bacterial inhibition was calculated as in the assay section above. Also, recovery of the survival organism from the surface of the CVT agar, which was pre-supplemented with the fermentation metabolites, was attempted.

c) Fungistatic Effect:

1. Effect of calcium propionate, sodium propionate and propionic acid versus time:

A mineral medium was made with the following composition in g/L: $(NH_4)_2H\ PO_4$, 1.5; KH_2PO_4, 1.0; $MgSO_4\ 7H_2O$, 0.2; dextrose, 0.5) and pH adjusted to 5.5. The

medium was dispensed 9 ml each into test tubes and autoclaved at 121°C for 15 minutes. Three groups each consisting of 5 tubes, were supplemented with different concentrations of sodium propionate, calcium propionate or propionic acid. Two tubes with only the mineral medium were used as controls. Each tube was then inoculated with 0.2 ml of A. niger culture (3 days old). Incubation was at 30°C and 0.1-ml samples were withdrawn every 2 hr for the first 8 hr. Twenty hour samples were also included. One ml of each sample was mixed and poured with an acidified YMA agar (pH 3.5)into sterile petri plates. Mold plates were counted after 4 days of incubation at 25°C. Scoring was done as before.

2) Effect of the fermentation metabolites versus time:

Similar experimental proceedures were utilized to assay freeze dried P. shermanii metabolites grown in milk (5 days old).

3) Inhibitory effects of acetic and propionic acid in milk:

The combined inhibitory actions of acetic and propionic acid were investigated in a milk medium (w/v: 10% NFDM + 0.2% yeast extract). Three hundred ml of the milk medium were prepared in 500 ml flasks and steamed for

20 minutes. Acetic acid and propionic acid (Sigma grades) (1: 2 ratio) were added for a total final concentration (v/v) of 0.3%, 0.6% and 0.9%. Different concentrations of the liquid amended milk medium which was freeze dried were assayed against A. niger using the acidified YMA plate technique. The mold plates were scored as before.

Results

Different approaches were attempted to stimulate *P. shermanii* growth metabolite (PGM) production in milk and whey based media. The inhibitory activity of PGM was assayed against three spoilage indicator organisms. The spoilage organisms chosen were a gram negative psychrotrophic bacterium (*Pseudomonas* sp.), a mold (*A. niger*) and a yeast (*K. fragilis*). The supplementation of milk or whey based media with various additional carbohydrate or nitrogen compounds was futile and no significant increase in the ability of *P. shermanii* to synthesize inhibitory metabolites was observed (data not shown). By contrast, the addition of trace elements triggered dramatic differences in the proteolytic activity of *P. shermanii* (Figure 1.1). The increased proteolytic activity of the organism resulted in a relatively high pH in all the media supplemented with the trace element mixture solution. Such a condition however depressed the ability of *P. shermanii* to synthesize more inhibitory metabolites active against the indicator organisms. The presence of cobaltous nitrate in the basal milk or whey medium promoted the ability of *P. shermanii* to produce the inhibitory metabolites and drastically reduced its

Figure 1.1 A-D: Effect of trace elements on the proteolytic activity and pH of P. shermanii. The organism was grown on different whey based media supplemented with the following combinations.

A: 1) whey based media alone (w), 2) whey (w) + cobaltous nitrate (co), 3) whey (w) + trace element mixture solution (TEM).

B: 1) whey (w) + cobalt (co) + diammonium phosphate, 2) whey (w) + trace element mixture solution (TEM) + diammonium phosphate (Amm. Phosph.).

C: 1) whey (w) + trace element mixture (TEM) + ammonium acetate.

D: 1) whey (w) + cobaltous (co) + glycerol, 2) whey (w) + trace element mixture solution + glycerol.

In most combinations the presence of trace element solution resulted in an increased proteolytic activity and a reduction in amount of acids produced by the organism.

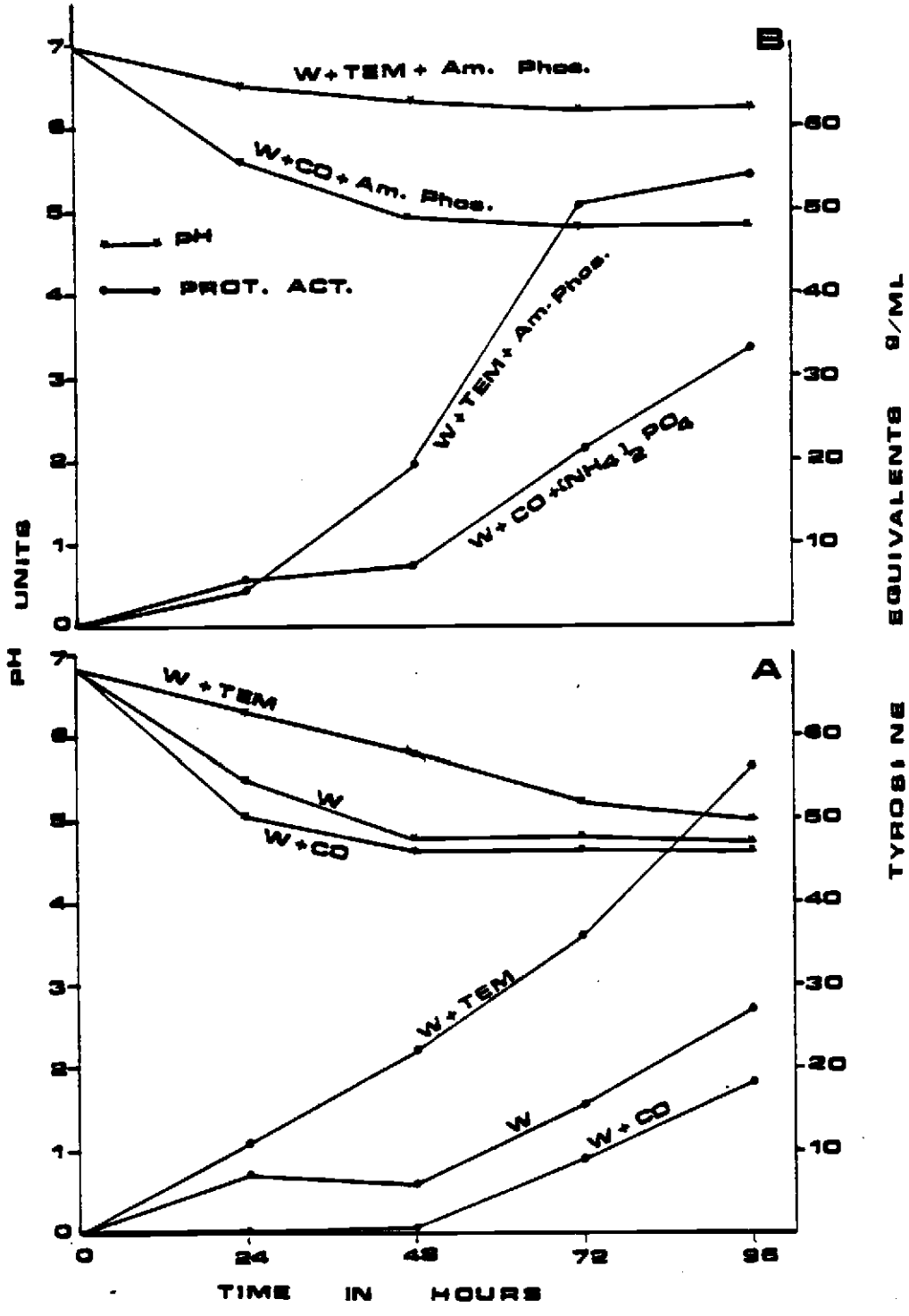

B
W+TEM + Am. Phos.
W+CO+ Am. Phos.
pH
PROT. ACT.
W+TEM+ Am.Phos.
W+CO+(NH4)2PO4
pH UNITS
TYROSINE EQUIVALENTS g/ML
A
W+TEM
W
W+CO
W+TEM
W
W+CO
0
24
48
72
96
TIME IN HOURS

Figure 1.1

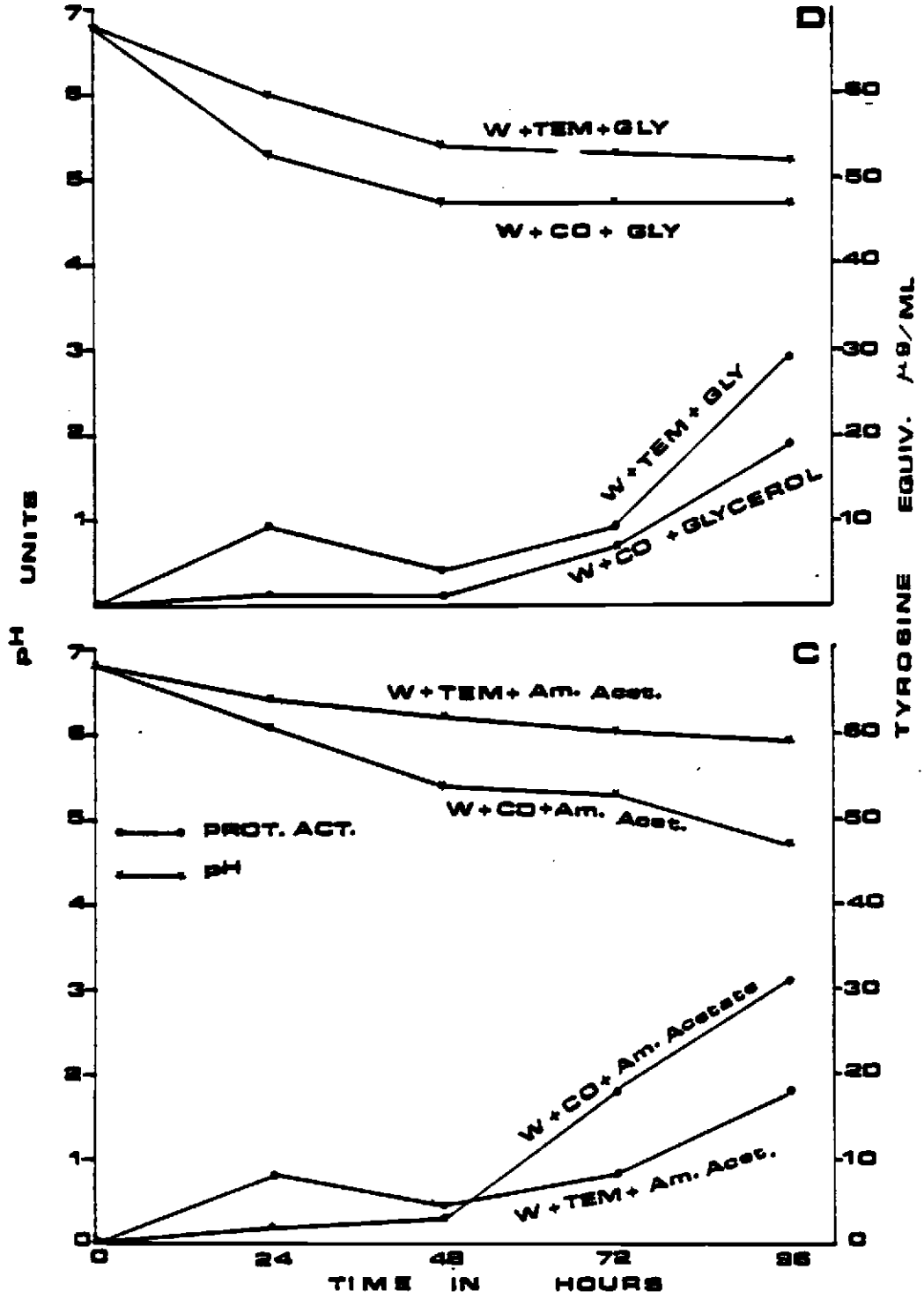


Figure 1.1 (continued)

Table 1.1 Effect of different cations on the production of Propionibacterial inhibitory metabolites against Pseudomonas sp. and Aspergillus niger.

Major ingredients in a medium	% inhibition against Pseudomonas using 1% LPCM	Assay against Aspergillus [a] using 1%, 3%, and 5% LPCM																		
	Time in hrs				24 hrs				48 hrs				72 hrs				96 hrs			
	24	48	72	96	1%	3%	5%	Con	1%	3%	5%	Con	1%	3%	5%	Con	1%	3%	5%	Con
Whey	100	93	100	100	4	4	4	4	4	4	4	4	4	3	2	4	3	2	1	4
Whey + Co^{++}	100	100	100	100	4	4	4	4	4	4	4	4	4	3	2	4	3	2	1	4
Whey + mix. trace elements	65	75	93	100	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Whey + mix. + $(NH_4)_2 Po_4$	100	100	100	100	4	4	4	4	4	4	4	4	4	3	2	4	3	2	1	4
Whey + mix. + $(NH_4)_2 Po_4$	0	N.D.	100	100	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Whey + Co^{++} + Ammonium acetate	100	100	100	100	4	4	4	4	4	4	4	4	4	3	2	4	3	2	1	4
Whey + mix. + Ammonium acetate	35	95	97	100	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Whey + Co^{++} + glycerol	100	100	100	100	4	4	4	4	4	4	4	4	4	3	2	4	3	2	1	4
Whey + mix. + glycerol	0	93	100	100	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

[a] The mold assay plates were scored as follows: 4 - no inhibition (excellent growth as the control), 3 - good growth, 2 - poor growth, 1 - very poor growth.

NOTE: Con = Control

proteolytic activity (Table 1.1). The insignificant effect of additional sources of carbohydrates and nitrogen was even more obvious during the trace element study.

The addition of fumarate, as a propionate pathway intermediate, induced P. shermanii to produce more inhibitory metabolites. The slow agitation of P. shermanii cultures with continuous neutralization yielded products similar in activity to the unneutralized, statically-incubated cultures. The concentration effect of the metabolites was obvious with the freeze dried and the freeze dried, steamed PGM (Figures 1.2, 1.3, 1.4, and 1.5). Though the steaming of the freeze dried PGM was undertaken to destroy viable P. shermanii cells, the enhanced activity of the product was noteworthy. Surprisingly, PGM concentrations obtained from all treatments was least effective against the yeast indicator organism (K. fragilis). Nevertheless, the freeze dried steamed PGM exhibited remarkable inhibition against all the indicator organisms (Table 1.5). The temperature effect on the PGM revealed the heat liability of the metabolites (Figure 1.6). The recovery of the indicator Pseudomonas sp. (at a rate of 8×10^6) after exposure to low levels of PGM (1 g/100 ml), showed the bacteriostatic nature of the metabolites. PGM exerted fungistatic

effects similar to the propionate salts and propionic acid (Tables 1.6 and 1.7). Also, pH 3.1 was the lowest value that permitted growth of the mold (A.niger) with the propionic acid additions. The simulated inhibitory effects of propionic and acetic acid (1.86) in milk showed that 488.5 mm total acid concentration distinctly slowed the mold growth without completely eliminating it (Table 1.8).

Table 1.2. Propionibacterium growth metabolites, obtained from a medium that contained different concentrations of disodium fumarate[a] assayed against Pseudomas sp.

Time in hours	Control (no fumarate)				0.1% Fumarate				0.2% Fumarate				0.4% Fumarate			
	0.25[b]	0.5	1.0	2.0	0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0
24	0	38	50	80	0	30	50	78	0	36	84	84	0	80	90	100
48	0	80	87	100	0	83	100	100	18	85	100	100	29	85	100	100
72	0	87	90	100	0	90	100	100	27	72	100	100	59	88	100	100
96	54	99	100	100	90	100	100	100	91	100	100	100	92	100	100	100
120	93	96	100	100	91	100	100	100	94	100	100	100	95	100	100	100
144	90	100	100	100	88	100	100	100	92	100	100	100	93	100	100	100

[a] material and methods

[b] % concentration of growth metabolites (w/v) in the assay medium g/100 ml

Table 1.3 The assay of PGM concentrations (obtained from a medium that contained disodium fumarate) against the indicator mold A. niger.[a]

Time in hours	Control[b]	Control (no fumerate)			0.1% Fumarate			0.2% Fumarate			0.4% Fumarate		
		1%	3%	5%	1%	3%	5%	1%	3%	5%	1%	3%	5%
24	4	4	4	4	4	4	4	4	4	4	4	4	4
48	4	4	4	4	4	4	4	4	4	4	4	4	4
72	4	4	4	4	4	4	4	4	4	4	4	4	4
96	4	3	3	2	3	3	2	2	1	1	2	1	1
120	4	3	2	1	3	1	1	2	1	1	2	1	1
144	4	2	1	1	2	1	1	2	1	1	2	1	1

[a] See footnote [a], Table 1
[b] Only plate assay medium (acidified YMA) with no more additions.

Figure 1.2 Effect of PGM produced in milk (static) against the indicator (Pseudomonas) bacterium. A density of 1.0 was assumed in the liquid metabolites when assaying them for activity as shown in the figure.

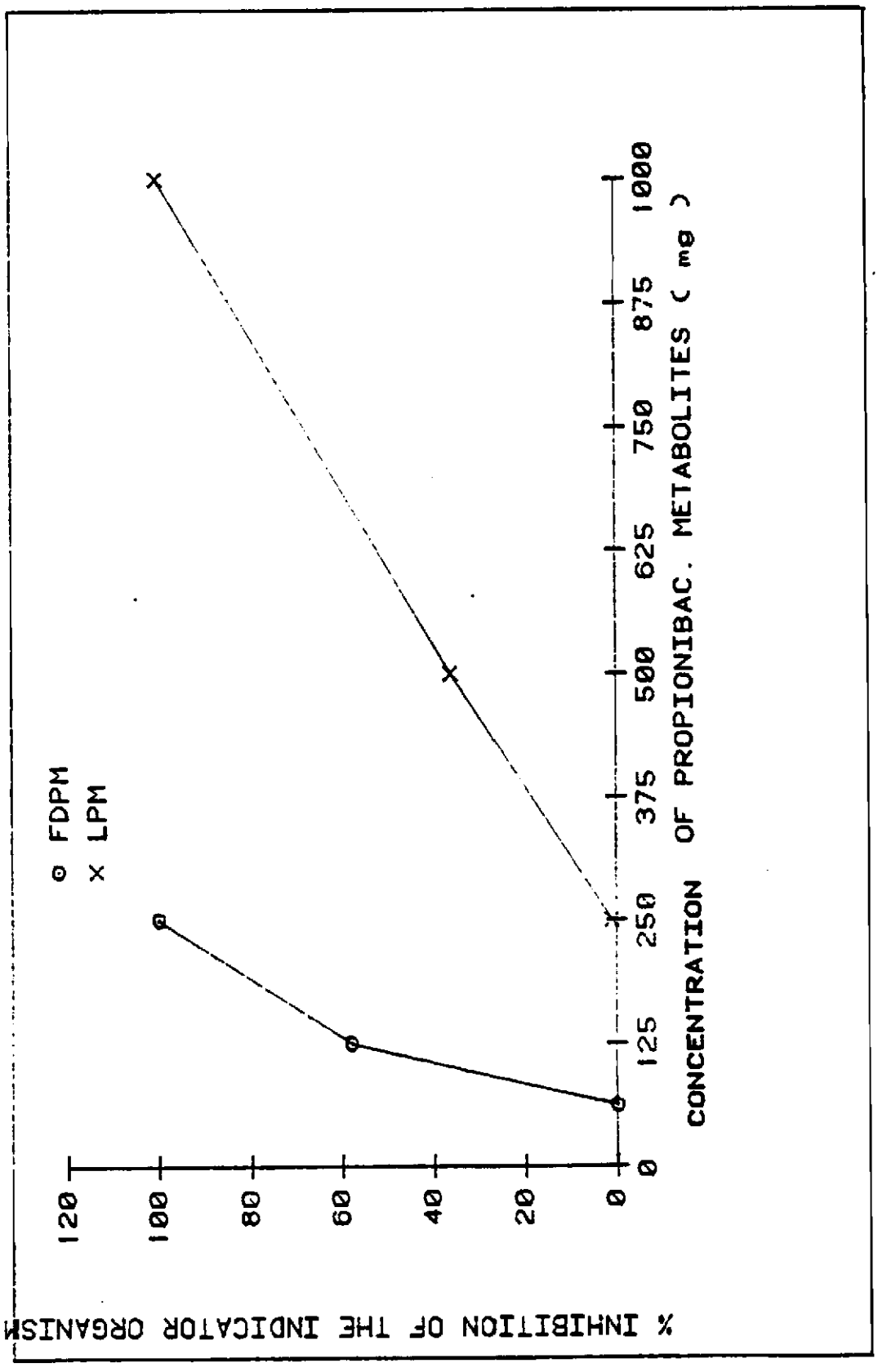

% INHIBITION OF THE INDICATOR ORGANISM
120
100
80
60
40
20
0
0
125
250
375
500
625
750
875
1000
CONCENTRATION OF PROPIONIBAC. METABOLITES (mg)
o FDPM
x LPM

Figure 1.2

Figure 1.3 Effect of PGM produced in milk (neutralized) against the indicator (Pseudomonas) bacterium. A density of 1.0 was assumed in the liquid metabolites when assaying them for activity as shown in the figure.

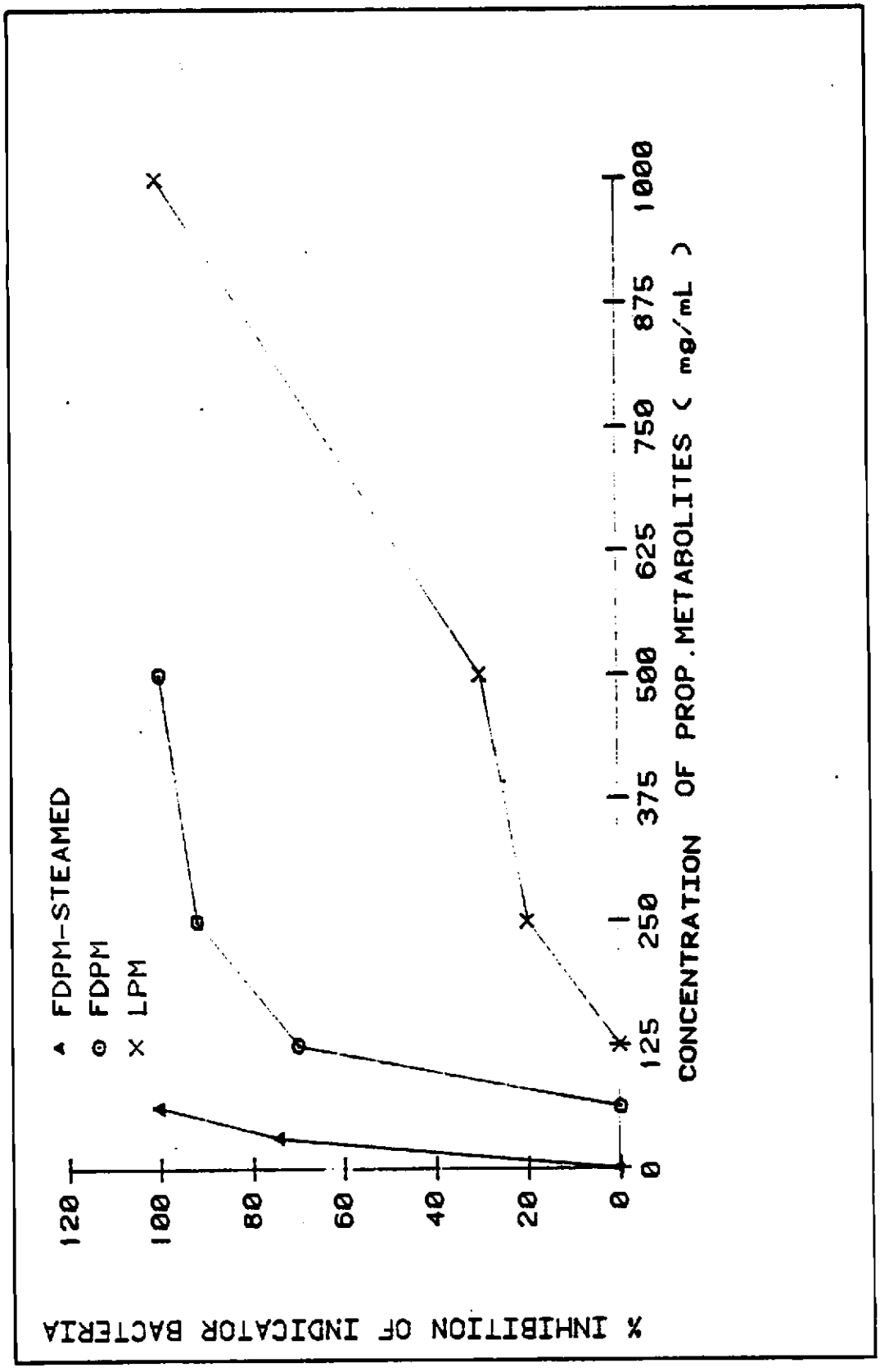

% INHIBITION OF INDICATOR BACTERIA
120
100
80
60
40
20
0
0
125
250
375
500
625
750
875
1000
CONCENTRATION OF PROP.METABOLITES (mg/mL)
▲ FDPM-STEAMED
⊙ FDPM
X LPM

Figure 1.3

Figure 1.4 Effect of PGM produced in whey (static) against the indicator bacterium (Pseudomonas). A density of 1.0 was assumed in the liquid metabolites when assaying them for activity as shown in the figure.

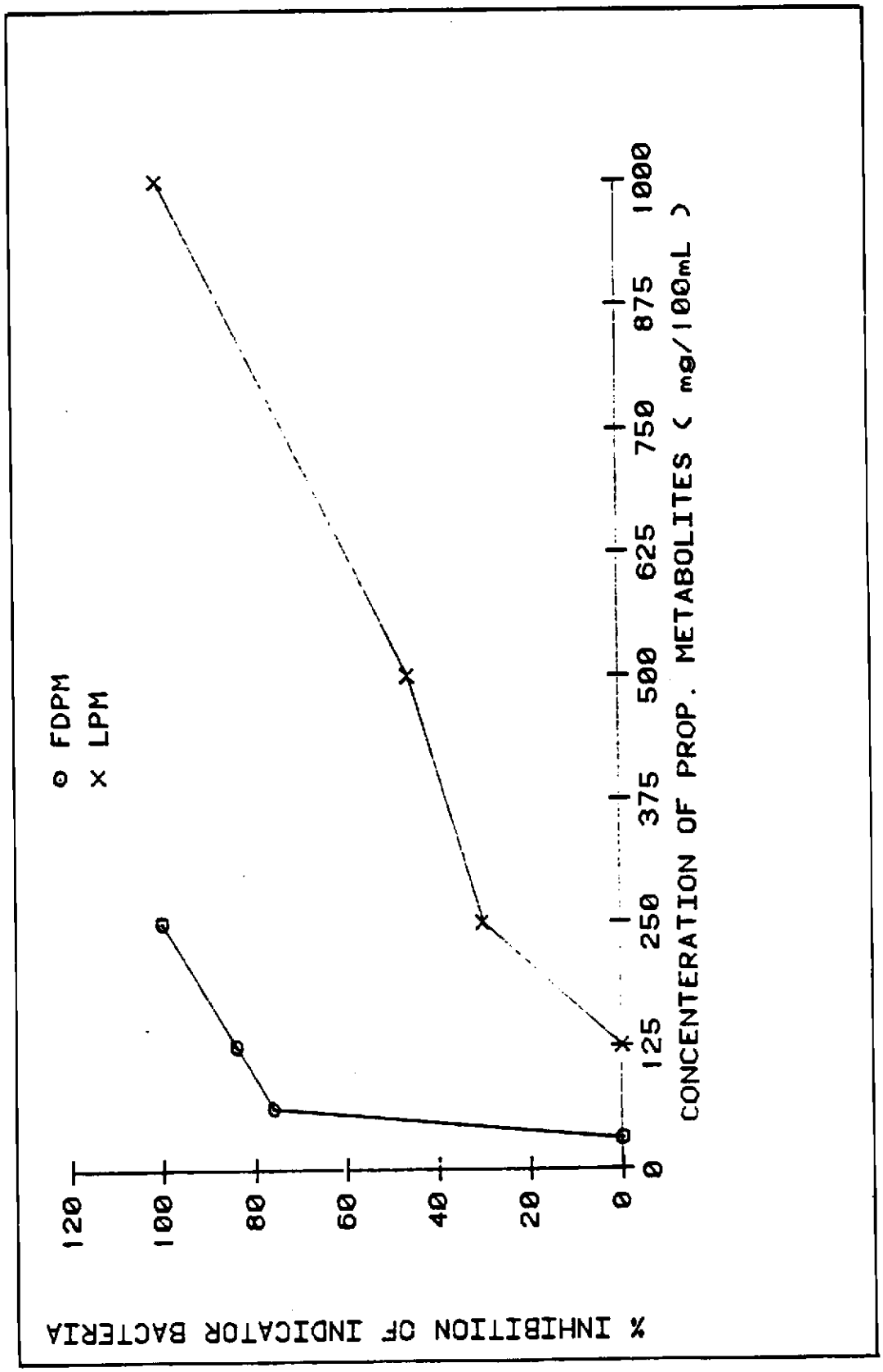

% INHIBITION OF INDICATOR BACTERIA
120
100
80
60
40
20
0
0
125
250
375
500
625
750
875
1000
CONCENTRATION OF PROP. METABOLITES (mg/100mL)
o FDPM
x LPM

Figure 1.4

Figure 1.5 Effect of PGM produced in whey (neutralized) against the indicator bacterium (Pseudomonas). A density of 1.0 was assumed in the liquid metabolites when assaying them for activity as shown in the figure.

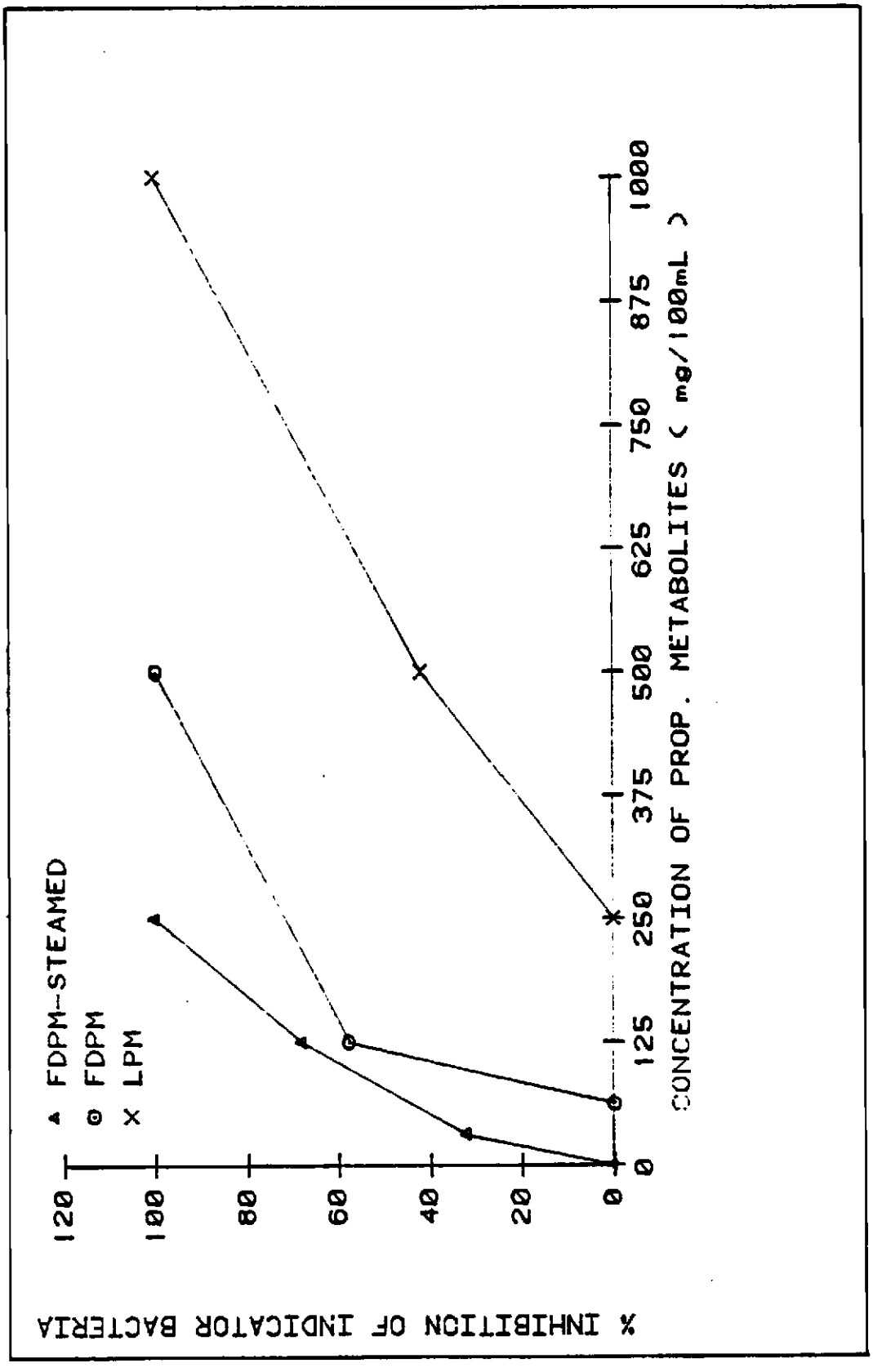

% INHIBITION OF INDICATOR BACTERIA
120
100
80
60
40
20
0
▲ FDPM-STEAMED
⊙ FDPM
X LPM
0
125
250
375
500
625
750
875
1000
CONCENTRATION OF PROP. METABOLITES (mg/100mL)

Figure 1.5

Figure 1.6 Effect of heat treatment on the activity of PGM as assayed against the indicator bacterium (Pseudomonas). A density of 1.0 was assumed in the liquid metabolites when assaying them for activity as shown in the figure.

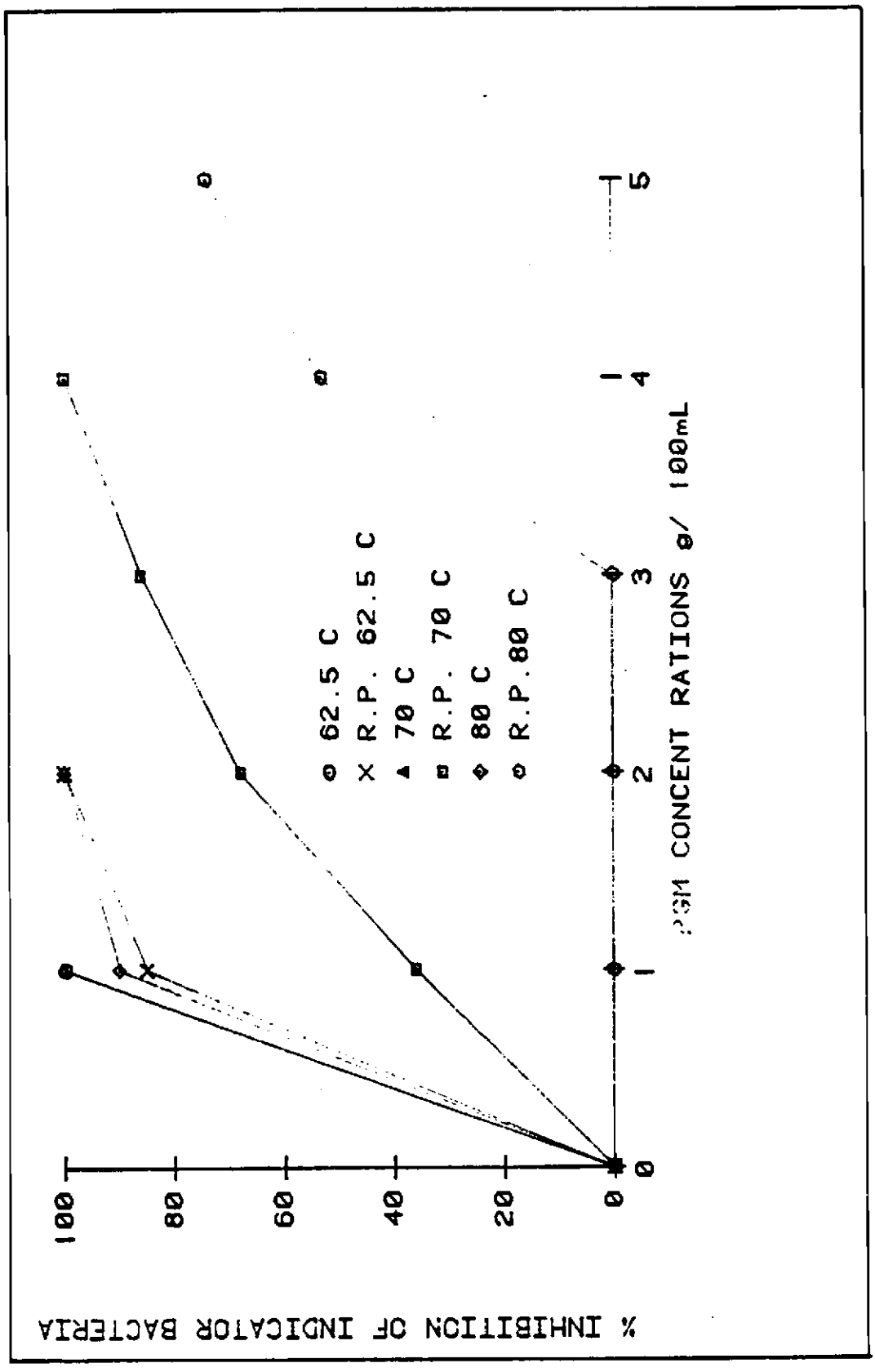

% INHIBITION OF INDICATOR BACTERIA
100
80
60
40
20
0
0
1
2
3
4
5
PPM CONCENTRATIONS g/ 100mL
62.5 C
R.P. 62.5 C
70 C
R.P. 70 C
80 C
R.P.80 C

Figure 1.6

Table 1.4 Mold assay[a] (*A. niger* the indicator organism) using continuously-neutralized and statically-grown *Propionibacterium* cultures in a whey medium, 5 days old. The assay was done on freeze dried and freeze dried, steamed material.

Concentration (g/100 ml)	Control	Freeze Dried				Freeze dried[b] and steamed (1 1/2 hrs in sealed aluminum cans)
		Static		Neutralized		
		Non-pasteurized	Pasteurized	Non-pasteurized	Pasteurized	
1	4	4	4	4	4	2
2	4	4	4	4	4	1
3	4	3	4	3	4	1
4	4	2	3	2	3	0
5	4	1	2	1	2	0

[a] see footnote a Table 1.1
[b] was done only with the continuously neutralized culture.

Table 1.5 Propionibacterial growth metabolite (from the static and the continuously-neutralized milk medium) inhibitory effects against Kluyveromyces fragilis.

% PGM Concentration (w/v)	Static (freeze dried) % Inhibition[a]			Neutralized (freeze dried) % Inhibition			
	Non-pasteurized	Pasteurized	Control	Non-pasteurized	Pasteurized	Steamed	Control
1	0	0	0	0	0	0	0
2	0	0	0	0	0	12	0
3	0	0	0	34	0	36	0
4	29	0	0	42	38	49	0
5	31	0	0	52	44	65	0

[a] % inhibition = control count – sample count/control count x 100

Table 1.6 Fungistatic action of sodium propionate, calcium propionate and propionic acid against Aspergillus niger[a] grown in a mineral medium at pH 5.0 containing different concentrations of propionates.

Time (hours)	Control	Sodium Propionate M					Calcium Propionate M					Propionic Acid M				
		2.08	1.04	0.52	0.26	0.13	1.1	0.54	0.27	0.13	0.067	2.7	1.35	0.68	0.34	0.17
2	4	2	4	4	4	4	2	3	4	4	4	0	0	0	0	1[b]
4	4	2	3	4	4	4	1	3	4	4	4	0	0	0	0	0
6	4	1	2	3	4	4	1	2	3	4	4	0	0	0	0	0
8	4	0	1	2	4	4	0	0	1	2	4	0	0	0	0	0
20	4	0	0	0	0	3	0	0	0	0	2	0	0	0	0	0

[a] see footnote a Table 1.1

[b] 3.1 was the higest pH that allowed growth of the mold for all tested propionic acid concentrations.

Table 1.7 Fungistatic actions of propionibacterial growth metabolites (PGM) against Aspergillus niger.[a] Propionibacterim shermanii (ATCC 9616) was grown for 5 days under continuous neutralization at pH 4.8 in a medium containing (g/L: whey, 96; yeast extract, 2; cobaltous nitrate 0.01). The medium was pasteurized at 85°C for 45 minutes and acidified with lactic acid (85% syrup) to pH 5.3. The grown cultures were then freeze dried and different concentrations were assayed against the indicator A. niger over a period of 72 hours.

Time (hours)	% of Freeze dried PGM (w/v, g/100 ml)						
	30	20	10	5	2.5	1.25	Control
2	4	4	4	4	4	4	4
6	3	4	4	4	4	4	4
8	2	2	3	3	4	4	4
20	1	1	1	2	3	3	4
72	0	0	0	0	0	0	4

[a] see footnote a Table 1.1

Table 1.8 Effects of propionic and acetic acids on growth of *Aspergillus niger* [a] in milk.

	Milk[b] with propionic and acetic acids					The freeze-dried milk containing propionic and acetic acids		
% of total acid used	pH of the acidified milk	propionic acetic ratio	total acid concentration x 10^{-4} M	Assay against *A. niger*	Control	Total[c] acid concentration x 10^{-4} M	Assay against *A. niger*	Control
0.3	5.77	1.86	3.22	4	4	2.83	4	4
			6.44	4	4	5.66	4	4
			9.66	4	4	8.49	4	4
			12.88	4	4	11.32	4	4
			16.10	4	4	14.15	4	4
0.6	4.76	1.86	8.76	4	4	6.24	4	4
			17.52	4	4	12.48	4	4
			26.28	4	4	18.72	4	4
			35.04	4	4	24.96	3	4
			43.80	4	4	31.20	2	4
0.9	4.46	1.86	13.10	4	4	9.77	4	4
			26.20	4	4	19.54	4	4
			39.30	4	4	29.31	3	4
			52.40	4	4	39.10	2	4
			65.50	4	4	48.85	1	4

[a] see footnote a Table 1.1
[b] Milk was made of 10% NFDM + 0.2% yeast extract steamed and fortified with propionic and acetic acids.
[c] Calculated after recovery of 9% solids from each % total acid concentration group.

Discussion

The nutritional requirements of propionibacteria have long been regarded as complex. Various vitamins, minerals, carbon and nitrogen sources have been found to influence the growth and metabolism of these organisms (4, 9, 11, 41). Similar complexities would be expected among the diverse metabolites produced by P. shermanii, despite the fact that acetate, propionate, diacetyl and propionin are the only antimicrobial metabolites recognized by research workers (8, 25, 26, 32). The insignificant effect of additional carbohydrates and nitrogenous sources in the milk or whey based media (acidified or not) was indicative of the nutritional adequacy provided by lactate and/or lactose and casein for inhibitory metaboliite production by P. shermanii. In contrast, Ingram (22) reported that the presence of additional carbohydrate and nitrogenous sources increased the production of extracellular proteinases by P. acnes. The addition of glycerol was intended to induce acyl coenzyme A carboxylase and fatty acid synthase which are required in the de novo synthesis of fatty acids and other hydrocarbons (1, 18, 39). Therefore the addition of glycerol was expected to increase production of

propionibacterial inhibitors by enhancing fatty acid production.

Microbial growth media which offer metabolic precursors, are becoming commonly used to encourage the overproduction of microbial products (42). Thus, it was not suprising that the addition of fumarate improved the effeciency of P. shermanii for over producing antimicrobial metabolites active against A. niger (Table 1.2 and 1.3). However, the inhibitory metabolites were never produced in sufficient quantity to completly eliminate mold growth.

Various elements such as Fe^{++}, Mg^{++}, Mn^{++}, Co^{++} and K^{+} have been demonstrated to have stimulatory effects on the production of propionic acid by P. shermanii (34, 36, 37). Some metals in the trace element mixture solution exerted positive effects on the ability of P. shermanii to produce the inhibitory metabolites. Recently, Leadlay (24) demonstrated the adverse effect of Cu^{++} on the activity of methylmalonyl CoA epimerase of P. shermanii which intercoverts methyl malonyl CoA epimers, an essential step in propionate production. Furthermore, the inverse relationship between the proteolytic activity and the effect of the inhibitory metabolites of P. shermanii was reversed when cobalt was the only supplemented

element. Jordan et al. (23) observed that the addition of $CoCl_2$ to P. shermanii growth medium, enhanced the production of vitamin B_{12} and adversely affected cell growth and protein synthesis but, remarkably increased cellular DNA contents. The oxygen tolerance of P. shermanii grown in a chemostat with lactate as energy source, was studied by Pritchard et al. (3, 35). Below measurable dissolved oxygen levels, the organism was suggested to act as a facultative anaerobe, producing more acetate and CO_2 and less propionic acid. The similarity between the effect of the inhibitory metabolites produced by P. shermanii cells grown under slow agitation and no agitation (static) suggested a possible contribution of some inhibitory metabolites other than the known organic acids. The continuous neutralization process also did not render a more active product. Suprisingly, most of the laboratory trials gave products of less inhibitory activity compared to large scale fermentations carried out under the same conditions (data not shown).

End product inhibition of P. shermanii growth by its organic acids and their salts has been documented by some workers (2, 17, 33). Nevertheless, the inhibitory amounts cited for these organic acids (33) were far less than the amounts we encountered in this study. The simulation of

the inhibitory effects of these organic acids in milk showed the need for higher total acid concentration (< 450 mM) to eliminate the molds. The undissociated form of propionic acid displayed similar inhibitory actions to the propionate salts except when the low pH came into effect. Steaming PGM likely resulted in possible browning reaction products which showed strong inhibitory action. The brown color and the hard texture of the steamed PGM were problems associated with this product. The production of votalite organic acids by P. shermanii appeared to mask the inhibitory action of other metabolites. Future positive identification of the different metabolites produced by the organism will provide better opportunity to optimize the growth conditions needed for maximum production of these materials.

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Chapter II

Inhibitory Effects of *Propionibacterium Shermanii* Growth Metabolites on Yogurt and Cottage Cheese Spoilage Organisms

Abstract

Three different concentrations of freeze-dried *Propionibacterium shermanii* metabolites (FDPM) from milk grown cells were investigated for their inhibitory action against spoilage organisms in two commercial brands of strawberry flavored yogurt. Evaluation of the FDPM activity in the commercial yogurt samples focused on the acid tolerant yeasts and psychrotrophic bacteria. Yeasts were found to be the principal spoilage organisms contaminating commercial yogurts. FDPM (10% concentration) inhibited viable yeasts and preserved one commercial yogurt brand for over 82 days at 5°C. In the other yogurt brand, yeasts were not eliminated, but their early growth was restricted by 10% FDPM. Mixtures of short chain fatty acids were suggested as synergistic inhibitors of the yeasts. The inhibitory action of FDPM against yeasts was found to be concentration dependent. FDPM-supplemented yogurts were also protected from spoilage by gram negative psychrotrophs.

In the case of commercially produced cottage cheese, a significant reduction in spoilage due to inhibition of gram negative psychrotrophs by liquid *P. shermanii* metabolites (LPM) was observed. In 24 days, 90% (105/114 cartons) of cheese that contained LPM had less than 800 gram negatives bacteria per g of cheese. In 30 days, 68% (78/114 cartons) of the cheese had undetectable levels of spoilage organisms (less than 100/g) when the cheese was kept at 7°C. Less than 1% of the cheese that contained LPM showed any surface growth (mold) and thirty-three percent of the cheese that did not contain LPM underwent surface spoilage in 21 days.

Introduction

Over the years, yogurt has developed different identities and appeals as a fermented milk product. The recent increase in yogurt popularity has been attributed to the introduction of fruits, stabilizers, flavors, coloring agents and unique starter culture combinations into the manufacture of the product (7, 9, 15, 30). However, these additives and techniques have also increased processing control demands, thus requiring more stringent manufacturing practices. The low pH of yogurt offers a selective environment for the growth of acid tolerant yeasts and molds (3, 27). Therefore, it is not surprising that various investigators have found that yeasts are the primary spoilage microorganisms for yogurt and that fruits, flavors and coloring agents are frequent contamination sources (1, 10, 18, 33, 34). The spoilage of yogurt by yeasts has been generally characterized by yeasty off-flavors, loss of textural quality due to gas production, swelling and occassional rupturing of the product containers (9). Benzoate and sorbate, through their permitted utilization in fruits to inhibit yeasts and molds, may be found in yogurt (22, 25, 29). Also benzoate, as a metabolic product of yogurt starter

bacteria in fruit and non-fruit yogurts, has been noted (2, 24). Furthermore, some manufactures add benzoate or sorbate to yogurt, thereby forfeiting their right to label the product "natural." This is done despite the fact that yeast cells develop resistance to these preservatives (28, 30, 35). As a result, there is an apparent need for an effective preservation method to control acid tolerant spoilage yeasts and molds in yogurt. Therefore one objective of the present study was to investigate the inhibitory action of the freeze-dried propionibacterial metabolites (FDPM) on the spoilage microorganisms in commercial yogurt.

Cottage cheese is a highly perishable food and a good growth medium for many spoilage organisms. Emmons and Tuckey (11) mentioned numerous factors that contribute to the microbial load of such a product. Gram negative psychrotrophic bacteria are the most important spoilage organisms encountered in milk and on equipment used in the manufacture of the cheese (19, 20, 26). Cooper (6) cited psychrotrophic yeasts and molds as other important contaminating spoilage organisms of the cheese. Also, meseophiles and gram negative coliform bacteria were present in cheeses produced under unsanitary manufactuing practices, esspecially when storage temperatures were

abused (11). Becuase of this, numerous approaches have been made to extend the shelf-life of the product and recently Branch (4) patented some cottage cheese preservative compounds claimed to be effective against pathogens and spoilage microorganisms.

Another objective of the present study therefore was intended to investigate the use of liquid Propionibacterim shermanii metabolites to preserve cottage cheese, especially to inhibit gram negative psychrotrophs.

Materials and Methods

Sources

Two different brands of strawberry flavored yogurt were purchased from local retail outlets. The FDPM preparations were obtained from Wessman Foods, Inc., Portland, Oregon. Brain heart infusion (BHI), Eosin methylene blue (EMB), violet red bile agar (VRBA) and yeast malt extract agar (YMA) were obtained from Difco Laboratories, Detroit, MI, 48232; they were prepared according to manufacurer's directors.

Procedures

Yogurt Brand A: Twelve yogurt containers each containing product were divided into four groups.

Aliquots (150 g) from each of three control yogurt containers were weighed into sterile blender jars (total weight of 450 g) and blended for 1-2 minutes. The mixed yogurt was returned to their respective precleaned containers. One-ml samples from each container were serially diluted into 9 ml of 0.1% peptone water and 0.1 ml from each dilution spread over the surface of four different agar meda. For the other three yogurt groups, 1%, 5% and 10% concentrations of FDPM were introduced by blending with the 450 g of yogurt which was then redistributed to their original containers as done with the control. One ml samples were taken from each container at zero time and distributed on the 4 agar media as done with the control. All sample containers were stored at $5^{o}C$ and sampled every two weeks over a period of 82 days. Microbial counts for the three yogurt samples within each group were geometrically averaged.

Yogurt Brand B: Twenty-four containers of another commercial brand of strawberry flavored yogurt were divided into two groups (12 each) and treated as follows: Yogurt Brand B-1: Twelve yogurt containers were grouped and received the same treatment as yogurt brand (A). Yogurt Brand B-2: The contents of the other 12 yogurt containers were combined in a sterile beaker and mixed

well. Aliquots (150 g) of the pooled yogurt were weighed into sterile blenders and blended for 1-2 minutes. Then the contents of the blenders were returned to their respective precleaned containers. Three such treated aliquots were designated controls. For the other three groups, 1%, 5% and 10% FDPM concentrations were achieved by blending with yogurt as appropriate to achieve a total net weight of 150 g. Homogeniety was insured for each cup by blending for 1-2 minutes and the yogurts were returned to their respective precleaned paper cups. After zero time sampling, all the yogurt samples were stored at $5^{o}C$ and sampling continued every week for a period of 28 days.

Cottage Cheese:

Twenty-five sets of commercially produced cottage cheese (150 cartons) were employed in the study. In nineteen of the cheese sets, 1% LPM (Microgard) was added to the cheese dressing. The other 6 sets received regular cheese dressing that contained no LPM (control). All cheese cartons were stored at $7^{o}C$ and sampled every 5 days over a period of a month. The cheese samples were analyzed for physical appearance, taste and smell, pH, total baterial counts, gram negative bacteria and sometimes coliforms.

Plating media and conditions:

Eleven grams of cottage cheese were added to a 99 ml sterile dilution blank (0.11% peptone water) which was blended and then serially diluted. The different dilutions were spread plated on BHI, CVT and EMB agar media. EMB was employed when necessary to differentiate between coliforms and gram negatives present in the sample. Incubation was at 30^{o}C for 48 hours.

All yogurt samples were assayed for different microorganisms by the surface spread-plate technique using four different agar media. BHI agar was used for total viable microbial counts. Crystal violet tetrazolium agar (CVT) was employed for enumerating gram negative psychrotrophic bacteria. Both these media were incubated at 30^{o}C for two days. VRB agar was utilized for coliform counts and plates were incubated at 35^{o}C and observed every 24 hours over a period of 5 days. YMA was made selective by the addition of 100 g/ml of filter sterilized oxytetracycline and designated by OYMA. OYMA plates were used for yeast and mold detection after an incubation period of 4-5 days at 25^{o}C.

Results:

Strawberry flavored yogurt was utilized to investigate the inhibitory effects of FDPM because it is the most widely sold type of fruit flavored product. Yeasts were observed to be the dominant spoilage microorganism. None of the tested yogurts exhibited any coliform contamination. Mold infection was limited to control samples which contained no FDPM.

Yogurt Brand A: The inhibitory effect of FDPM was dependent on concentration. A significant gradual reduction in spoilage occurred at 1% and 5% FDPM concentrations. Yeasts were completely inhibited by 10% FDPM over a storage period of almost 3 months at $5^{o}C$. Late in the storage time (67 days), gram negative psychrotrophic bacteria contaminated the control yogurt but none appeared in the yogurt supplemented with FDPM (Figure 2.1).

Yogurt Brand B-1: A significant reduction in the yeast population was observed for yogurt containing FDPM relative to the untreated controls. Despite the late insignificant differences between the different FDPM concentration effects, the initial yeast population was slowed with the 10% FDPM concentration (Figure $2.2\text{-}A_1$).

Complete elimination of the yeasts was not observed for any of the FDPM concentrations used. All the yogurt containers of the control and 1% FDPM group exhibited blown lids due to gas production by the yeasts.

Yogurt Brand B-2: No differences in yeast inhibition results were observed or compared to yogurt brand B-1 (Figure 2.2-A_2). Figure 3 is a photograph showing mold spoilage of control (no FDPM) yogurt in contrast to yogurt containing 10% FDPM. Also shown in Figure 3 is the effect of FDPM at 5% concentration when incorporated into potatoe dectore agar at pH 3.5. The control plates (top) show luxuriant growth of Penicillium roqueforti (left) and Penicillium camamberti (middle) and a mixture of the two (right). The FDPM containing plates showed no growth of the mold even though the plates were held for several months. The control group of yogurt brand B-2 also exhibited gross contamination with psychrotrophic gram negative microorganisms.

Cottage Cheese: Out of the six control cheese sets examined for psychrotrophs, only 2 sets contained cheese that had a low number of these organisms at 24 days. However, these 2 sets of cheese were spoiled (5×10^3 - 4

x 10^4 cells/g) by yeasts, resulting in either yeasty or sour off flavors (Table 2.5, A-G). The other 4 sets of the cheese had an excess of 10^7 cells/g of spoilage organisms (gram negatives) in 21-24 days. With one exception, all control cheese spoiled in 24 days. In contrast to the controls, cheese suplemented with LPM exhibited a dramatic reduction in the number of gram negative spoilage organisms over a testing period of 30 days (Figure 2.5). Seventeen individual sets of cheese (90%), representing 14 different production days, had less than 800 spoilage organisims per gram of cheese in 24 days. Thirteen sets of cheese (68%) had undetectable gram negatives in 30 days. Only two out of 19 groups examined showed spoilage organisms greater than 10^6/g, one of which contained substantial coliforms. Similar reductions in yeast counts were observed in cheese with LPM. Eight out of 36 cartons (22%) of control cheese showed yeast whereas in only 1 out of 114 LPM containing cartons (0.9%) was yeast present.

Table 2.1. Geometrical averages of the total viable microbiol populations in the commercial strawberry flavored yogurt brand A treated with FDPM. (BHI plate counts CFU/G)

	FDPM Concentrations			
Days	0	1%	5%	10%
0	$1x10^3$	$1x10^3$	$5x10^3$	$1x10^3$
4	$1x10^3$	$6x10^3$	$6x10^3$	$2x10^2$
20	$2x10^4$	$3x10^5$	$1x10^2$	$2x10^1$
35	$2x10^6$	$6x10^5$	$1x10^2$	$2x10^1$
52	$2x10^6$	$3x10^4$	$1x10^2$	$2x10^1$
67	$2x10^6$	$5x10^4$	$2x10^1$	$1x10^1$
82	$3x10^6$	$6x10^4$	$3x10^5$	$6x10^6$

Table 2.2. Geometrical averages of the total viable microbial populations in yogurt brand B-1[(a)] treated with FDPM. (BHI plate counts CFU/G)

	FDPM Concentrations			
Days	0	1%	5%	10%
0	0_3	0	$1x10^1$	$2x10^1$
8	$1x10^3$	$2x10^1$	$2x10^3$	$2x10^3$
16	$1x10^3$	$2x10^2$	$3x10^2$	$2x10^3$
24	$2x10^3$	$2x10^2$	$3x10^3$	$1x10^2$
32	$2x10^4$	$2x10^2$	$2x10^2$	$2x10^3$

* No gram negative bacteria were seen to contaminate this yogurt.

Table 2.3. Total viable microbial populations in yogurt brand B-2 (initially pooled yogurt) treated with FDPM (BHI plate counts CFU/G).

	FDPM Concentrations			
Days	0	1%	5%	10%
0	$2x10^{6}$	$2.3x10^{6}$	$2x10^{6}$	$2.3x10^{6}$
7	$3x10^{8}$	$2.3x10^{8}$	$5x10^{7}$	$6x10^{6}$
14	$3.5x10^{9}$	$4.3x10^{8}$	$5x10^{7}$	$3.9x10^{8}$
21	$4.9x10^{9}$	$3.8x10^{8}$	$4.3x10^{8}$	$2.2x10^{8}$
28	$7x10^{9}$	$2x10^{8}$	$2.5x10^{8}$	$1.5x10^{8}$

Table 2.4. Gram negative psychrotrophic bacterial populations in yogurt brand B-2 (initially pooled yogurt containers) treated with FDPM. (CVT viable counts CFU/G)

	FDPM Concentrations			
Days	0	1%	5%	10%
0	0	0	0	0
7	$1x10^{2}$	0	0	0
14	$3x10^{4}$	0	0	0
21	$3x10^{4}$	0	0	0
28	$5x10^{4}$	0	0	0

Figure 2.1 The effect of different inhibitory concentrations (1%, 5% and 10%) of FDPM against spoilage yeasts of commercially produced yogurt brand A. a) Counts of gram negative psychrotrophic bacteria observed in control samples (after 67 days of storage). b) Mold growth in control samples (last dilutions that showed growth against time).

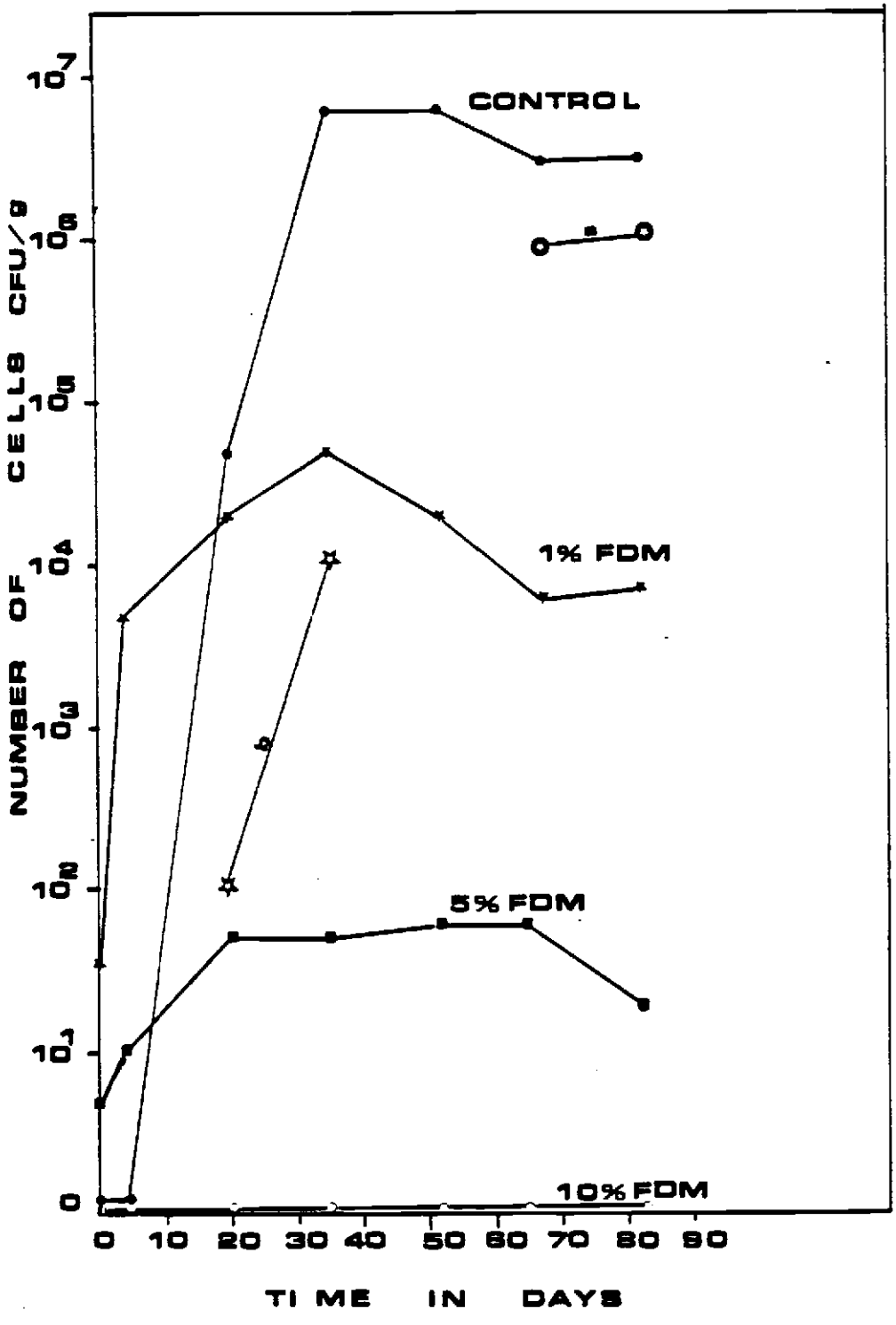
CONTROL
1% FDM
5% FDM
10% FDM
NUMBER OF CELLS CFU/g
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
0 10 20 30 40 50 60 70 80 90
TIME IN DAYS

Figure 2.1

Figure 2.2-A_1 The inhibitory effects of FDPM on yeast populations of the commercial yogurt brand B-1 (heterogenous yogurt samples). The counts were geometrically averaged.

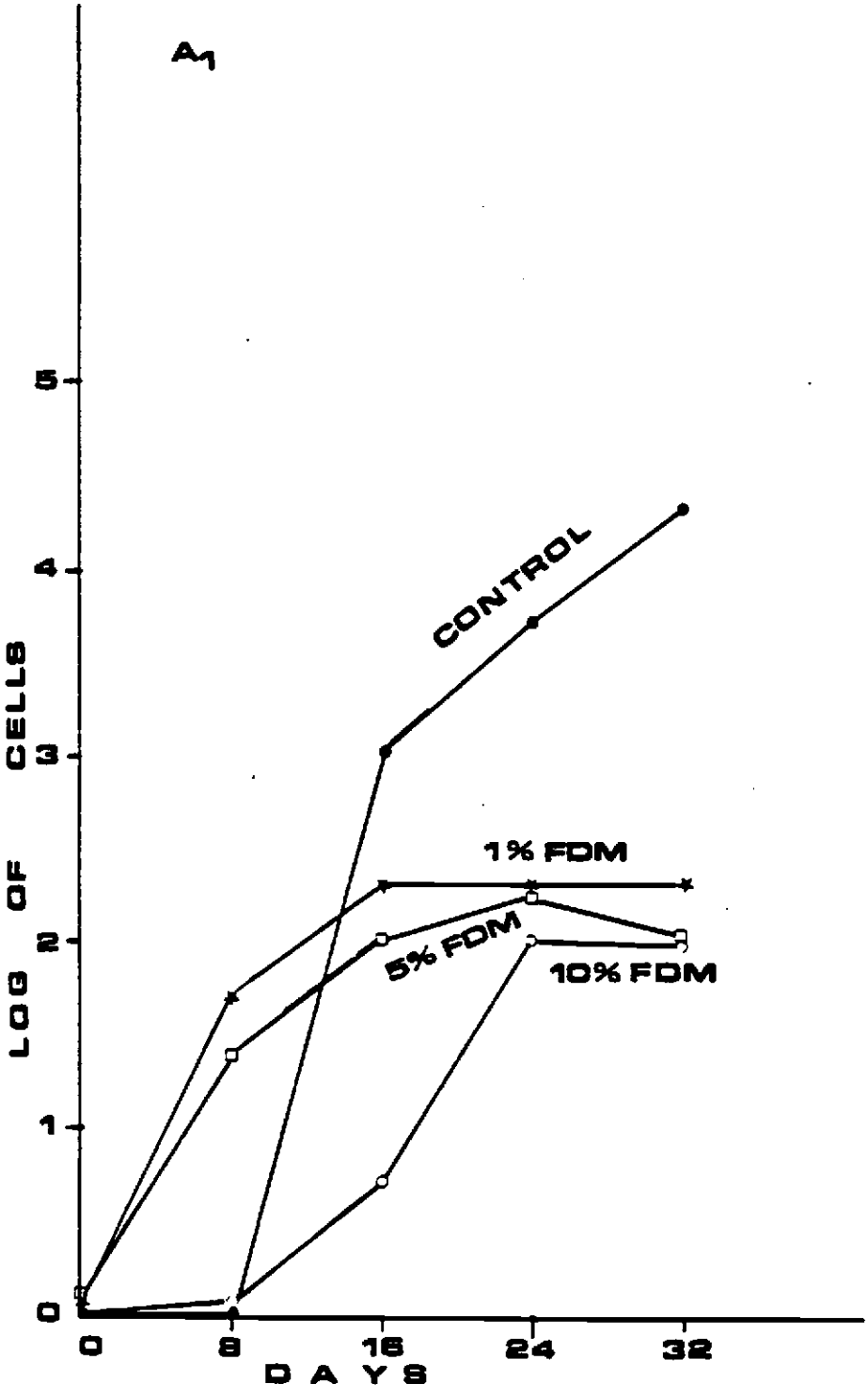
A1
LOG OF CELLS
0
1
2
3
4
5
CONTROL
1% FDM
5% FDM
10% FDM
0
8
16
24
32
DAYS

Figure 2.2-A
1

Figure 2.2-A$_2$ The inhibitory effect of FDPM effect against spoilage yeast populations of yogurt brand b-2. The initial yogurt was pooled (homogenous initial pool).

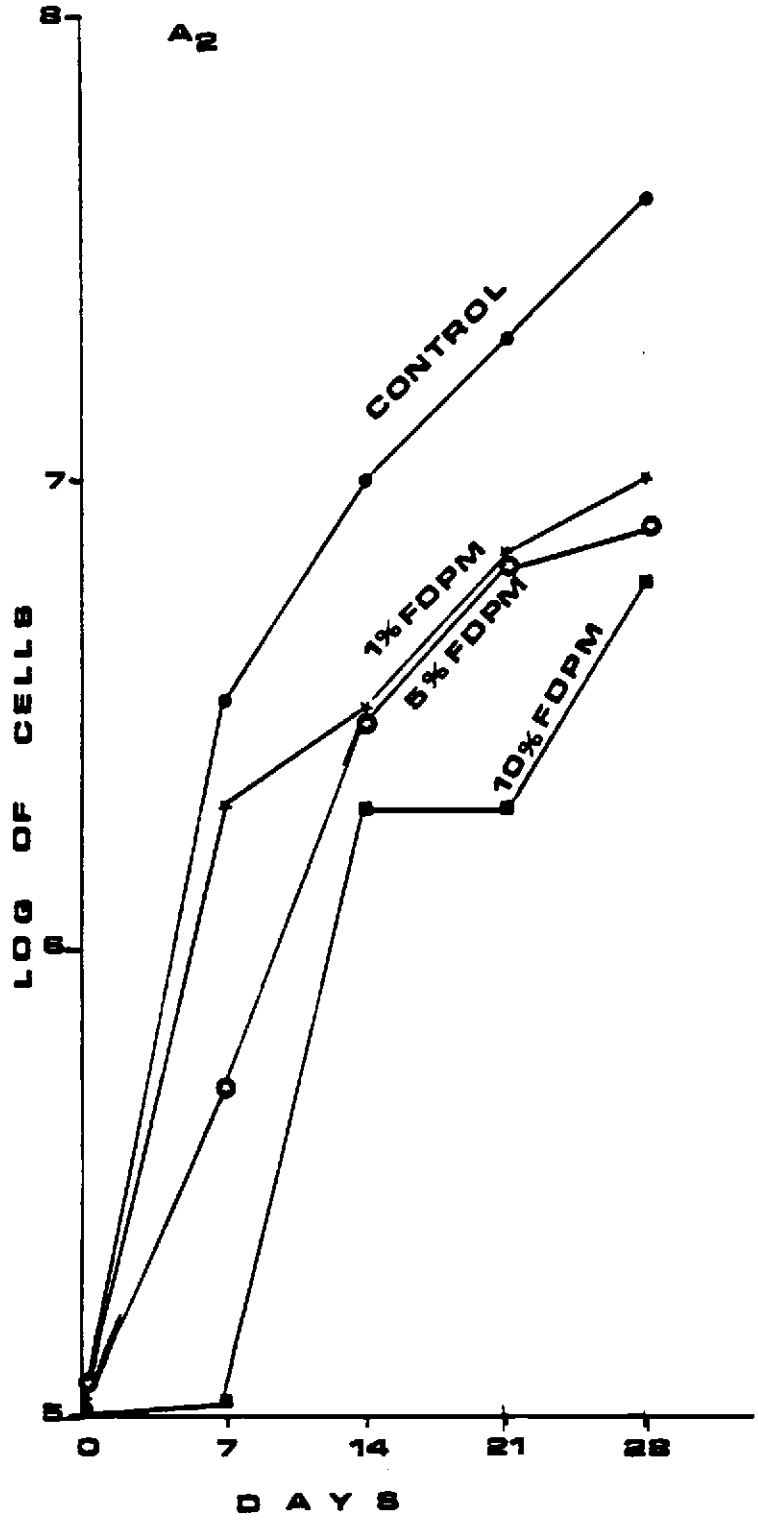
A2
8
7
6
5
LOG OF CELLS
CONTROL
1% FDPM
5% FDPM
10% FDPM
0
7
14
21
28
DAYS

Figure 2.2-A
2

Figure 2.3 Photograph (top) showing mold spoilage of control yogurt (above) in contrast to yogurt containing 10% FDPM (below).

Figure 2.4 Photograph (bottom) showing the effect of FDPM at 5% concentration when incorporated into potato dextrose agar at pH 3.5. The control plates (above-left to right) showed luxuriant growth of P. roqueforti, P. camemberti and a mixture of two respectively. FDPM containing agar (below) plates showed no growth of these molds.

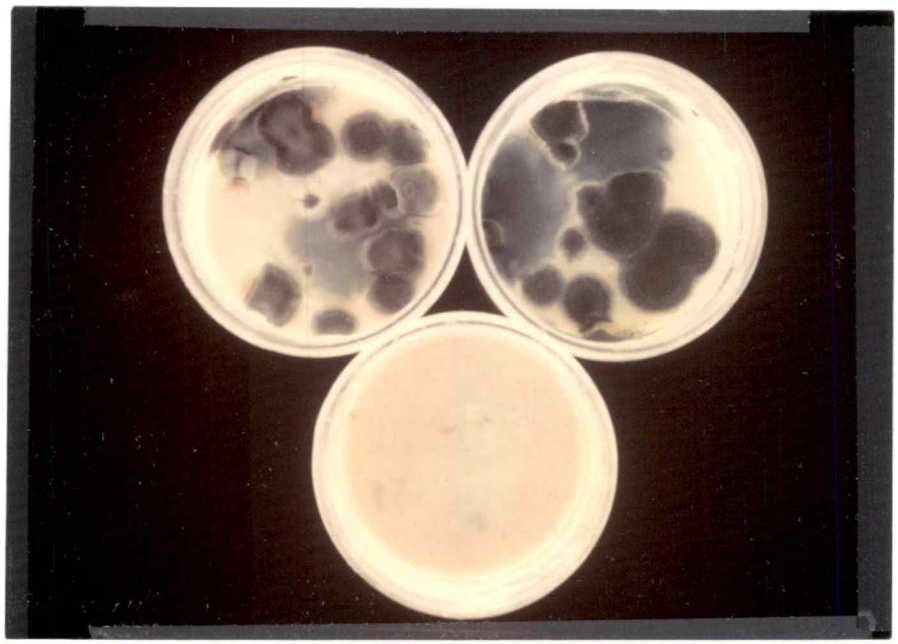

Figure 2.3

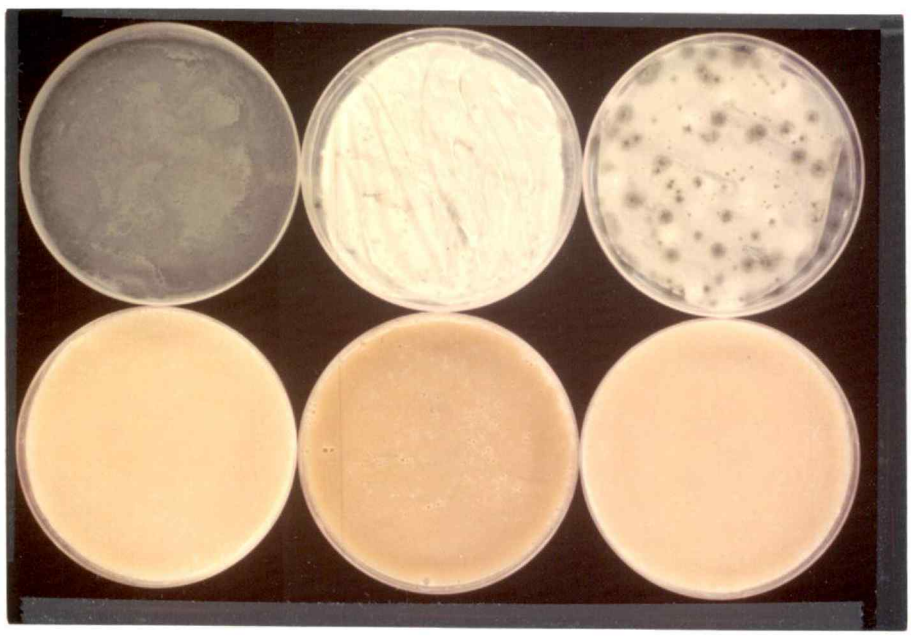

Figure 2.4

Figure 2.5 Summary of the inhibitory effects of liquid propionibacterial metabolites (LPM) against spoilage gram negative psychrotrophic bacteria of commercially produced cottage cheese (1% of LPM added to the cheese dressings). Microgard is the trade name for LPM.

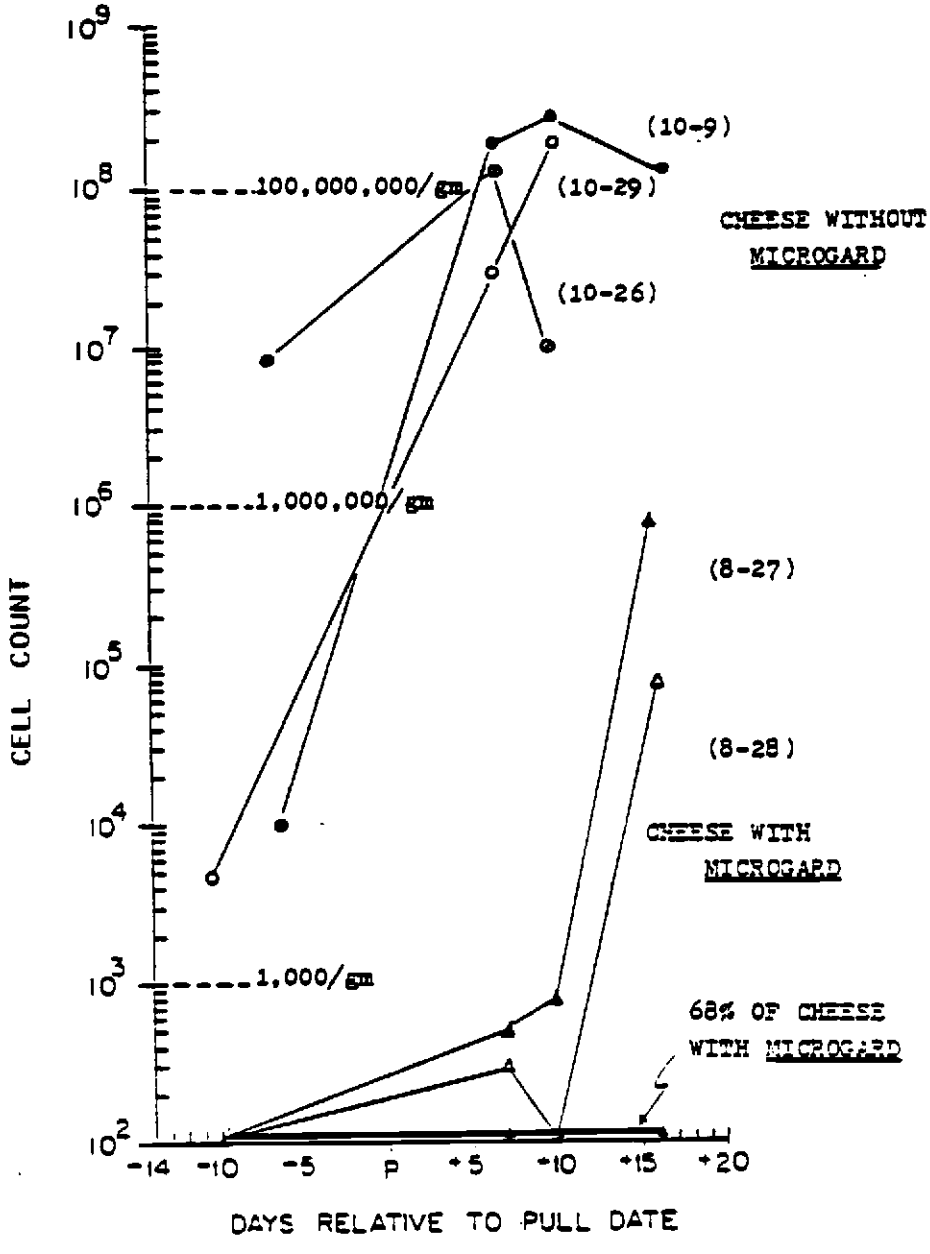
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
100,000,000/gm
1,000,000/gm
1,000/gm
CELL COUNT
(10-9)
(10-29)
(10-26)
CHEESE WITHOUT MICROGARD
(8-27)
(8-28)
CHEESE WITH MICROGARD
68% OF CHEESE WITH MICROGARD
-14
-10
-5
P
+5
+10
+15
+20
DAYS RELATIVE TO PULL DATE

Figure 2.5

Table 2.5 (A-G) Results of total viable bacterial counts (BHI), gram negative psychrotrophic bacteria (CVT), coliforms (EMB), pH measurements and taste and smell of all commercial cottage cheese lots used in studying the effect of LPM (Microgard).

ALL CARTONS: 16oz. SMALL CURD, WITH HIGHOARD

5-A	SAMPLE DATE 8-17-84		21 DAY		24 DAYS		30 DAYS	
	BHI	CVT	BHI	CVT	BHI	CVT	BHI	CVT
CODE DATE 8-27 4% FAT	$7.3x10^5$	$< 10^2$	(D) $3.2x10^7$ $2.8x10^7$	$5.0x10^2$ $2.0x10^4$	(D) $3.3x10^7$ $7.3x10^7$	$8.0x10^2$ $8.0x10^2$	(D) $2.5x10^7$	$8.0x10^5$
8-28 2% FAT	$1.1x10^6$	$< 10^2$	(B) $6.2x10^7$ $4.9x10^7$	$3.0x10^2$ $4.0x10^2$	(B) $2.8x10^7$ $2.2x10^7$	$< 10^2$ $< 10^2$	(B) $4.3x10^7$	pH=4.64 $8.5x10^4$
8-28 4% FAT	(A) $8.1x10^5$	$< 10^2$	(C) $7.0x10^7$ $8.4x10^7$	$2.1x10^7$ $4.9x10^7$	(E) $2.7x10^7$ $4.4x10^7$	$3.7x10^6$ $1.9x10^6$ EMB: $2.9x10^5$ $7.0x10^2$	(F) $1.1x10^8$	$1.0x10^7$

(A) Very flat taste

(B) Whey, slight sour taste

(C) Very dry, bland

(D) Taste and smells like new

(E) Whey, compact curd, slight fruity smell

(F) Cracked carton, whey, fruity smell

Table 2.5-A

ALL CARTONS: 16oz, WITH MICROGARD

5-B	SAMPLE DATE 8-23-84		21 DAY		24 DAY		30 DAY	
	BHI	CVT	BHI	CVT	BHI	CVT	BHI	CVT
CODE 8-29 4% FAT	8.7×10^5	$< 10^2$	(B) 3.2×10^6 3.8×10^6	1.0×10^2 1.0×10^2	(D) 9.1×10^6 1.6×10^7	$< 10^2$ $< 10^2$	(E) 2.2×10^7	pH=5.31 5.7×10^5
8-30 2% FAT	(A) 1.4×10^6	$< 10^2$	(C) 5.0×10^7 3.4×10^7	$< 10^2$ $< 10^2$	(C) 2.9×10^7 3.7×10^7	3.0×10^2 4.0×10^2	(C) 8.8×10^7	$< 10^2$
9-3 4% FAT	1.0×10^6	$< 10^2$	(E) 2.0×10^8 2.1×10^8	$< 10^2$ 2.0×10^2	(F) 2.8×10^8 2.4×10^8	pH=5.18 $< 10^2$ $< 10^2$	4.0×10^8	$< 10^2$
9-4 4% FAT	5.9×10^5	$< 10^2$	(D) 2.9×10^8 3.0×10^8	$< 10^2$ $< 10^2$	(D) 3.7×10^8 2.5×10^8	pH=5.40 $< 10^2$ $< 10^2$	(G) 2.3×10^8	pH=5.11 $< 10^2$

(A) Very "soupy"

(B) Tastes like new

(C) Whey, compact curd, Slight sour

(D) Tastes O.K.

(E) Smell good, slight sour taste

(F) Smell O.K., slight moldy taste

(G) Surface mold, slight sour smell

Table 2.5-B

ALL CARTONS: 16oz, 4% FAT WITH MICROGARD

S-C	SAMPLE DATE 8-30-84		21 DAY		24 DAY		30 DAY	
	BHI	CVT	BHI	CVT	BHI	CVT	BHI	CVT
CODE 9-6	5.0×10^5	$< 10^2$	3.6×10^8 2.8×10^8	pH=5.34 $< 10^2$ $< 10^2$	(E) 3.6×10^8 3.4×10^8	$< 10^2$ $< 10^2$	(E) 3.2×10^8	pH=5.10 $< 10^2$
9-7	2.5×10^6	$< 10^2$	(B) 6.3×10^7 4.4×10^7	pH=4.91 $< 10^2$ $< 10^2$	(C) 9.0×10^7 9.3×10^7	$< 10^2$ $< 10^2$	(F) 6.8×10^7	pH=4.59 $< 10^2$
9-10	3.4×10^5	$< 10^2$	(D) 1.0×10^8 1.2×10^8	2.6×10^3 $< 10^2$	(B) 3.8×10^7 4.1×10^7	pH=4.72 $< 10^2$ $< 10^2$	(B) 2.4×10^8	pH=4.51 $< 10^2$
9-11	(A) 4.7×10^5	$< 10^2$	(E) 2.7×10^7 5.0×10^7	1.0×10^2 $< 10^2$	(E) 2.1×10^8 3.2×10^8	pH=5.37 $< 10^2$ $< 10^2$	(E) 3.2×10^8	pH=5.31 $< 10^2$

(A) Foamy

(B) Sl. whey, good smell sl. sour taste

(C) Sour smell, taste

(D) Dry curd, sl. sour taste

(E) Good smell and taste

(F) Whey, sour taste

Table 2.5-C

ALL CARTONS: 16oz, WITH MICROGARD

S-D	SAMPLE DATE 9-7-84		21 DAY		28 DAY		30 DAY	
	BHI	CVT	BHI	CVT	BHI	CVT	BHI	CVT
CODE 9-13 2% FAT	(A) 1.4×10^6	$< 10^2$	(D) 1.6×10^8 1.8×10^8	pH=5.14 $< 10^2$ $< 10^2$	(F) 1.2×10^8 1.6×10^8	pH=4.94 $< 10^2$ $< 10^2$	1.3×10^9 yeast.	pH=5.01 $< 10^2$ 1.1×10^6
9-14 4% FAT	(B) 5.3×10^5	$< 10^2$	(D) 2.1×10^7 4.4×10^7	pH=5.20 $< 10^2$ $< 10^2$	(B) 4.7×10^7 4.1×10^7	pH=5.18 $< 10^2$ $< 10^2$	1.6×10^8	pH=5.14 $< 10^2$
9-17 4% FAT	6.3×10^6	$< 10^2$	(G) 3.8×10^7 5.0×10^7	pH=4.70 $< 10^2$ 1.0×10^2	(H) 3.7×10^7 5.2×10^7	pH=4.68 $< 10^2$ $< 10^2$	(H) 1.9×10^7	pH=4.68 $< 10^2$
9-18 2% FAT	(C) 1.0×10^6	$< 10^2$	(E) 1.1×10^8 4.4×10^7	pH=4.99 4.0×10^7 1.0×10^7	1.0×10^8 2.5×10^9	pH=4.99 3.9×10^7 1.0×10^8	(H) 6.8×10^8	6.6×10^7

(A) Bland
(B) Dry and bland
(C) Soupy, sl. acid
(D) Good taste and smell
(E) Sl. whey, bland taste
(F) Looks and smells good
(G) Whey, sour smell
(H) Whey, sour smell, taste

Table 2.5-D

ALL CARTONS: 16oz, WITH HICROGARD

S-E	SAMPLE DATE 9-20-84		21 DAY		24 DAY		30 DAY	
	BHI	CVT	BHI	CVT	BHI	CVT	BHI	CVT
CODE	(A)	pH=5.24	(A)	pH=5.24	(H)	pH=4.94	(D)	pH=4.83
9-28	$2.5x10^6$	$< 10^2$	$1.4x10^8$	$2.8x10^4$	$2.3x10^8$	$< 10^2$	$6.6x10^7$	$< 10^2$
4% FAT			$1.1x10^8$	$< 10^2$	$1.5x10^8$	$< 10^2$		
	(A)	pH=5.25	(A)	pH=5.31	(B)	pH=4.99	(D)	pH=4.84
9-28	$3.5x10^6$	$< 10^2$	$2.5x10^8$	$< 10^2$	$1.5x10^8$	$< 10^2$	$6.7x10^7$	$< 10^2$
4% FAT			$7.4x10^7$	$< 10^2$	$2.0x10^8$	$< 10^2$		
	(B)	pH=5.20	(D)	pH=5.12	(B)	pH=4.82	(A)	pH=4.66
9-28	$1.0x10^6$	$< 10^2$	$5.1x10^8$	$< 10^2$	$4.5x10^8$	$< 10^2$	$4.0x10^7$	$4.0x10^5$
2% FAT			$4.8x10^7$	$< 10^2$	$2.8x10^8$	$< 10^2$		
	(B)	pH=5.20	(B)	pH=5.16	(C)	pH=4.73	(E)	pH=4.62
9-28	$6.0x10^5$	$< 10^2$	$5.5x10^8$	$1.0x10^2$	$4.5x10^8$	$< 10^2$	$5.3x10^7$	$< 10^2$
2% FAT			$3.4x10^8$	$< 10^2$	$3.2x10^8$	$< 10^2$		

(A) Bland (C) Sl. sour (E) Sl. bitter taste

(B) Dry (D) Good smell, taste

Table 2.5-E

ALL CARTONS: 16oz, CONTROL CHEESE: NO MICROGARD

S-F	SAMPLE DATE 10-3-84		21 DAY		24 DAY		30 DAY	
	BHI	CVT	BHI	CVT	BHI	CVT	BHI	CVT
CODE	(A)	pH=5.20	(C)	pH=4.90	(D)	pH=5.00	(D)	pH=4.85
10-9	$1.6x10^6$	$9.5x10^3$	$2.2x10^8$	$2.0x10^8$	$4.4x10^8$	$3.0x10^8$	$2.5x10^8$	$1.5x10^8$
2% FAT			$3.7x10^8$	$3.7x10^8$	$3.9x10^8$	$3.0x10^8$		
	(B)	pH=5.19	(B)	pH=5.00	(E)	pH=5.16	(F)	pH=5.07
10-9	$9.3x10^5$	$< 10^2$	$2.4x10^8$	$< 10^2$	$4.3x10^8$ $2.5x10^8$	$1.0x10^2$ $4.0x10^2$	$6.5x10^7$	$2.0x10^2$
4% FAT			$1.6x10^8$	$< 10^2$	yeast:	$5.3x10^3$	yeast:	$2.2x10^3$
	(A)	pH=5.31	(B)	pH=5.01	(D)	pH=5.24	(B)	pH=5.60
10-11	$4.7x10^6$	$< 10^2$	$7.2x10^7$	$< 10^2$	$7.9x10^7$ $1.8x10^8$	$8.0x10^2$ 10^4	$1.1x10^8$	$1.0x10^2$
2% FAT			$6.3x10^7$	$< 10^2$	yeast:	$4.0x10^4$	yeast:	$1.5x10^4$

(A) Good taste and smell

(B) Bland taste

(C) Surface slime, sour smell

(D) Surface growth, sour smell, whey

(E) Sour smell, taste

(F) Sour smell, sour and bitter taste

Table 2.5-F

ALL CARTONS: 16oz, 4% FAT, CONTROL CHEESE: NO MICROGARD

5-G	SAMPLE DATE 10-19-84		21 DAY		24 DAY		30 DAY	
	BHI	CVT	BHI	CVT	BHI	CVT	BHI	CVT
CODE	(A)	pH=5.35	(B)	pH=4.93	(B)	pH=4.48		
10-26	$1.1x10^7$	$8.7x10^6$	$2.2x10^9$	$2.0x10^8$	$3.9x10^8$	$9.8x10^6$		
	coliform	$7.0x10^2$	$1.6x10^6$	$1.1x10^8$	$2.8x10^8$	$1.2x10^7$		
	(A)	pH=5.35	(B)	pH=5.01	(B&C)	pH=5.04		
10-29	$1.5x10^6$	$4.9x10^3$	$1.7x10^8$	$4.4x10^7$	$2.2x10^8$	$1.1x10^8$		
			$1.8x10^8$	$2.5x10^7$	$1.7x10^8$	$3.8x10^8$		
	(A)	pH=5.14	(D)		(E)	pH=4.79		
10-30	$1.8x10^6$	$< 10^2$	$4.3x10^8$	$7.4x10^3$	$1.1x10^8$	$< 10^2$		
					$1.4x10^8$	$< 10^2$		
					yeast:	$1.4x10^3$		
						$5.0x10^3$		

(A) Looks, smells and tastes good

(B) Surface slime, sour smell

(C) Whey, compact curd

(D) Whey, sour smell

(E) Surface mold, sour smell

Table 2.5-G

Discussion

Propionic acid and acetic acid are the two principal metabolites produced by Propionibacterim shermanii in milk. Propionic acid and its salts were previously reported to restrict the surface growth of molds on butter (12). Lennox and McElroy (16) demonstrated the detrimental effects of potassium sorbate and sodium propionate on the growth of Penicillium expansum. They also concluded that propionate blocked the synthesis of patulin, the toxic and carcinogenic unsaturated lactone produced by the mold. Nevertheless, the lack of a persistent mold contamination in the tested yogurt could be related to the possible levels of sorbate or benzoate that found their way into the product through their permitted utilization in fruits (2, 8). Two monphologically distinct yeast colonies were observed from samples of the two commercial yogurt brands. Thus, the inhibitory action of the FDPM was likely against two different types of yeasts in the two yogurt brands. Moon (21) investigated the mechanism and the inhibitory actions of acetate, lactate and propionate on 4 different genera of acid tolerant yeasts. She concluded that propionate was much more inhibitory than acetate or lactate but the synergistic mixture of the three acids was even more

potent. Therefore, a possible synergistic effect between propionibaterial metabolites and sorbate or benzoate in yogurt brand A may have restricted the growth and multiplication of the yeast cells. Certainly, the yeast populations in yogurt brand B reflected a limited response to the action of FDPM. The resistance of yeast cells to the action of food preservatives of the short chain monocarboxylic acid types has been documented by various investigators (5, 13, 17, 23). Warth (35) postulated an inducible transport system active in the presence of acid inhibitors as a mechanism of resistance rather than the ability of microorganisms to degrade the inhibitors. Furthermore, Ueda et al. (31) demonstrated the ability of Candida tropicalis as a propionate degrader by unravelling the enzymic system involved in the metabolism of propionate as a sole carbon source. However, it seems that yogurt brand B had the least amount of synergistic inhibitors and the noticeable yeast inhibition was mostly due to the effect of FDPM. Suriyarachchi and Fleet (24) observed that some proteolytic yeast species were able to raise the pH of yogurt through their excessive casein hydrolysis capabilities. Such a casein degradation process probably created favorable growth conditions signified by the gross psychrotrophic bacterial

contamination of yogurt brand B-2. Generally, the possible contamination of yogurt by gram negative psychrotrophic bacteria has been recognized by several workers (3, 14, 32). The complete control of the psychrotrophic bacterial contamination in all tested yogurt brands was evident. The relative similarity between the total viable cell populations and the yeast counts was mostly due to the death of the yogurt starter cultures as Yarabioff observed (32).

The preservation effect of LPM against gram negative psychrotrophic bacterial spoilage of cottage cheese, was evident. Despite the short shelf-life extension of cottage cheese by LPM (6-9 days), the significant economic impact is obvious.

Future research directed towards the possible utilization of different synergistic short chain fatty acids against different yeast genera might be needed for a potent yeast inhibitor in acid foods.

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Chapter III

Preliminary Characterization of Propionibacterium Shermanii Metabolites Active Against Gram Negative Organisms

Abstract

Propionibacterium shermanii metabolities produced in milk were separated by gel filliration, ultrafilteration and a cationic exchange. Activity of the fractions was determined by a plate assay, using a gram negative psychrotroph (Pseudomonas bacterium) as the indicator organism. Fatty acid content of the fractions was also scanned in the UV-range. No protein molecules were found to be involved in the inhibitory action. Phenyl substituted fatty acids were detected on mass spectrometry chromatograms but were believed to be transient occurrences because of their likely inevitable metabolism by the organism; also no confirmatory proton magnetic resonance spectra could be obtained to prove the existance of aromatic rings in the samples. Physical evidence supporting the existance of open-structured tetrapyrroles (bactobitin), or their degraded forms, was concluded from: 1. the presence of significantly higher amounts of fatty acids in the early chromatographic fractions relative to the unfractionated sample, mimicing the chemical bonding

nature of the fatty acids with other molecules; 2. the molecular weight profile of the active fractions reflecting the possible degradation of a parent compound; 3. the UV-peaks of the yellow colored chromatographic fractions in the range of 250-300 nm was diagnostic for dipyrrole units; 4. the addition of water and exposure to oxygen resulting in the decomposition and washability of the membrane filter retentates.

Pyrrole units were thought to function as ionophores in the inhibitory action of the propionibacterial metabolites and as natural buffering agents protecting the organism against its own toxic metabolities. Other molecules such as diacetyl, ∂ , B- diaminopropionic acid and possibly B-nitropropionic acids were also sought and considered to have a possible additive effect on the inhibitory action.

Introduction

Propionibacterium Shermanii is the principal organism used in Swiss-cheese manufacture and for the industrial production of vitamin B_{12}. Antimicrobial activities also are attributed to metabolities produced by propionibacteria; for example antifungal activity of short chain fatty acids is documented in the literature (13, 18, 28, 40) and the antibacterial activity exhibited by these acids has been extensively studied (33). Kabara et al. (23) screened over 40 natural and synthetic lipophilic compounds for their antimicrobial activities against gram positive and gram negative bacteria as well as yeasts. Shibaski et al. (37) studied the combined effects of fatty acids and their esters against gram negative organisms and concluded that short chain fatty acids were the most inhibitory. Evidence for antiviral activity of propionibacterial metabolites have come from studies done with P. freudenreichii extracts (10). A peptide (propionin) recognized in these extracts, exhibited antiviral activity against Columbia SK virus both in vivo and in vitro and also against vaccinia virus in vitro. Ramanathan et al. (29, 30) succeeded in purification and identification of three peptides participating in

different antiviral activities. The peptides were named propionin A, B and C. A lethal active agent (not antiviral) against lymphocytic Choriomeningitis virus infection in mice was also purified and characterized in the same laboratory (31).

Propionates and acetates, major metabolites of propionibacteria, may be derivatized by living cells into various antimicrobial compounds. In this regard, several plant species have been reported to contain ∂ , β - diaminopropionic acid and various other ∂ or β - substituted forms of the acid (4, 32). Actinomycetes have been shown to incorporate ∂ , β -diaminopropionic acids into the antibiotic molecule, viomycin (8). Roncari et al. (33), in their study on the chemical nature of ediene, found ∂, β diaminopropionic acid to be an essential part of the antibiotic molecule as synthesized by Bacillus brevis. Recently, Cane et al (7) confirmed that all the oxygen atoms and most of the carbon skeleton in erythromycin A and B molecules originated from propionates when produced during fermentation by Streptomyces erytherus. Also, a Japanese patent (35) reports on the associative usefulness of propionibacterial and Streptomyces metabolites in the production of erythromycins and the anthelmintic and miticidal

antibiotics B-41D, B-41E and B-41G. Hideo et al. (16) synthesized a pentapeptide containing a 1, 2 diaminopropionic acid residue and found the compound to exhibit strong antimicrobiol activity against gram negative organisims. β- nitropropionic acid (ON_2-CH_2-CH_2-COOH- Bovinicidin) is widely distributed among microorganisms, indicating a possible functional biosynthetic mechanism in propionate-producing organisms (5).

The remarkable diversity in propionibacterial metabolities was remarkably shown in the cutaneous propionibateria, which were characterized by their numerous extracellular enzymes (15, 19, 20, 21). Abrahamsson et al (1, 2, 3) spent seven years on the purification and identification of a lipid component from Propionibacterium acnes. Their effort was rewarded by the discovery of the first known prostaglandin-like substance (PLS), produced by microorganisms. PLS was found to be a potential inflammatory mediator involved in the physiology of acne vulgaris. Cummins et al. (9) isolated from P. acnes cell wall a strange polysaccharide, diamino hexuronic acid, which was characterized as 2, 3 diamino-2, 3 dideoxy glucuronic acid. The physiological role of the compound is not yet known. Brumm et al. (6)

successfully isolated, identified and synthesized a bile pigment possessing four acetic and four propionic side chains from extracts of the anaerobes Clostudium tetanomorphum and P. shermanii. The bile pigment was named Bactobilin. Bactobilin was related to the bilatrienes group by possessing an open chain tetrapyrrole structure. It had a molecular weight of 962 and was characterized by decomposition on exposure to oxygen and light. Bactobilin was not demonstrated in vivo. It was synthesized by the incubation of ∂-aminolevulinic acid and bacterial enzyme extracts.

The present work was undertaken to study the metabolities produced by P. shermanii in milk with activity produced against gram negative organisms. The possible physiological role of these metabolities is considered.

Materials and Methods

Source of Materials: Freeze-dried propionibacterial metabolite(s) (FDPM) was a neutralized (pH 7.0) pasteurized Propionibacterium culture grown commercially in skim milk. The unneutralized and unpasteurized liquid material (UNPM) was Propionibacterium culture grown commercially at 25°C in skim milk for 4 days (final pH 4.5). Both samples were obtained from Wesman Foods Inc., Portland, Oregon, under the trade name of Microgard. Membrane filters (YC05, YM2 and YM5) and the ultra filtration stirring cell (model 52) were purchased from Amicon Inc. Glass columns with top and bottom fittings were Pharmacia products. Sephadex G-25 gel (mesh size 40-120 mm) and Sephadex CM-50 (mesh 40-120 mm) were Sigma grades. Molecular weight reference molecules also were Sigma grades. Whatman filters #40 (W & R, Balston Ltd., England) and 0.45 µm and 0.2 µm filters (Gelman Service Inc.) were used in the filtration of samples.

Chromatographic Separations:

A. Gas Chromatography (GC): 5710A GC series (Hewlett Packard) instrument with flame ionzation detector (FID), was connected to a reporting integrator (Model No. 3390,

Hewlett Packard). The GC was fitted with a glass column (GP-Carbopack, Supelco, Inc.) 30" x 1/4" O.D. x 4 mm I.D.. Conditions used were: column temperature 120°C; inlet and Det., 200°C; gas flow rate, 60 ml/min; sample size, 1 1. A mixture solution of volatile fatty acids (C_2-C_5) in water, containing 50 ppm of each (Supelco Inc.) was used as an external standard.

B. Gel Filtrations:

Column I: thirty grams of FDPM was dissolved in 170 ml of distilled water (15% solution W/V) and allowed to equilibrate by stirring for 30 minutes. The pH of the milk solution was adjusted to 4.6 with 10% HCL (V/V). Centrifugation of the milk solution was carried out at 9880 x g for 20 min at 4°C (Beckman Model J-21 Centrifuge). The collected supernatant was filtered with Whatman Filters to remove any gross particles. The filtrate was refiltered with 0.45 m and 0.2 m filters respectively. Retentate on each filter was washed with 2 µml of 0.1 M sodium phosphate buffer (pH 5.0). Two ml of the original supernatant, 2 ml of the last filtrate (0.2 µm) and the washed retentates were all assayed biologically by the gram negative plate assay to determine if there was a loss in activity.

Twenty ml of the last filtrate (0.2 μm) was loaded on the Sephadex G-25 column which was preequilibrated with sterile 0.2 M Nacl solution at 4°C. The bed volume was 30 ml and the flow rate maintained at 7.5 ml/hr using the same equilibrating buffer solution. Five ml fractions were collected. All collected fractions were filter-sterilized (0.45 μ mm) separately to eliminate possible contaminants that would have interfered with the biological assay. Active fractions in the biological assay were injected into the GC to determine their fatty acid contents.

C. Column II:

Another G-25 Column (1.5 cm I.D. X 50 cm) had been settled with a bed volume of 56 ml and was equilirated with sterile 0.2 M NaCl Solution (at 4°C). Elution of the column was maintained with the same buffer (0.2 NaCl solution). Molecular weight calibration of the column was constructed using these molecules as molecuar weight reference molecules: Cyt-C (13,000), Bactracin (1,400), Vitamin B_{12} (1,165), Erythromycin Glucono-heptonoate (960), phenol red (354) and reduced glutathione (300). In 2 ml of deionized water, a mixture of these molecules was prepared in the following concentrations: Cyt C (2.5

mg/ml), Bactracin (5 mg/ml), Vitamin B_{12} (2.5 mg/ml), erythromycin glucono-heptonoate (5 mg/ml), phenol red (2 mg/ml) and reduced-glutathione (GSH 200 mg/ml). The mixture solution was loaded on the previously preequilibrated sephadex G-25 Column (Column II). The flow rate was maintained at 7.5 ml/hr and 5 ml fractions were collected (60 fractions). The fractions were analyzed by measuring optical densities at 280 nm using a Varian VHS - spectrophotometer. The log of the molecular weight of the reference molecules was plotted against their relative elution volumes (Velvo).

The UNPLM sample (pH 4.5) was centrifuged and filtered with Whatman filters #40 as before. Twenty ml of the filterate were loaded on the same column (after flushing the column with the elution buffer for a day). The sample was run under the same conditions as before. Fractions collected (75 fractions) were filter sterilized (0.45 μ mm) separately to make them ready for the biological assay. Active fractions from the biological assay were spectrophotmeterically scanned in the range of 200-400 nm using the programable DU-8 Spectrophotometer (Beckman). The fatty acids content of the fractions was also determined using the gas chromatograph.

Ultrafiltration procedures: A 15% solution of FDPM was prepared. The sample was centrifuged and coupled with consecutive filterations using Whatman #40, (0.45 μm) and 0.2 μm filters as before. Sixty-five ml of the last filtrate (0.2 μm) were loaded into an Amicon ultrafiltration cell, which was subsequently fitted with YM5, YM2 and YC05 membrane filters (having Molecular weights cut-off valves at 5,000, 1,000 and 500 respectively). Ultrafiltration with YM5 and YM2 was carried out to the extinction point. Retentates from these filters were washed with 2 ml of distilled water and then biological activity was assessed. Ultrafiltration with YC05 membrane filter was terminated after 2 days, when only 50% reduction in volume occurred. The retentate was very viscous, oily and slightly yellowish in color. A five ml sample from each the retentate and the filterate were assayed for their biological activity and their fatty acid content was determined by GC. Three ml of retentate were diluted with 3 volumes of distilled water and refiltered again, using the same filter (YC05)), to the original volume (3 ml). Fatty acids in the resulting retentate and filtrate were assessed again to check on the stability of the material. All the ultrafiltration

processes were done under nitrogen gas pressure (45-50 psi) and slow stirring at 4°C.

Cationic Exchanger: Sephadex CM-50 cellulose derivative was precycled and equilibrated with 0.1 M sodium phosphate buffer (pH 4.44) at 25°C room temperature. Twenty ml gel bed volume was maintained in a glass column (15 cm I.D. X 30 cm) at 4°C. Fifteen ml of the YC05 membrane filter retentate (from previous ultrafiltration) was loaded on the column. Elution to waste was done with 500 ml of 0.1 M sodium phosphate buffer (pH 4.44). Subsequently collected fractions were eluted with a salt gradient built into the same phosphate buffer as follows:

1. 0.1 M Nacl (150 ml)
2. 0.5 M Nacl (250 ml)
3. 1.0 M Nacl (410 ml)

The flow rate was maintained at 10 ml/hr and 5-ml fractions were collected. Fractions were analyzed by measuring optical densities at 280 nm. Observed peaks (above 0.01 O.D.) were filter sterilized (0.45 μmm), assayed biologically, scanned and their fatty acid content determined by gas chromatography.

Contaminants: possible contaminants from the commercial products were isolated on BHI (Brain Heart

Infusion) and grown in milk for 4 days under the same commercial conditions. They were then treated the same way as FDPM and UNPM and assayed against the indicator organism.

The Biological Assay (Plate Assay):

Organism: A gram negative psychrotrophic slime-producing spoilage bacterium isolated from cottage cheese supplied by H.P. Hood, Inc., Boston, MA. was used as the indicator organism. The organism was identified in our laboratory as a Pseudomonus species from transmission electron microscope pictures which showed distinct monopolar flagella. The organism was maintained by weekly transfere into sterile lactose broth (pH 7.3) and kept at $5^{o}C$. Propogation of the organism was conducted by 1% inoculum of the organism into the sterilized lactose broth and incubation at $30^{o}C$ overnight.

Selective Medium (acidified CVT agar):

To each one liter of plate count agar (Tryptone 5; yeast extract, 2.5; glucose, 1; and agar, 15 gm/l), 1 ml of 0.1% aqueous crystal violet solution was added. The pH was adjusted to 7.1 with 1 M NaOH solution. The medium was dispensed, 100 ml each, into presterilized bottles and

autoclaved for 15 minutes. The agar was cooled to 50°C and 0.5 ml of 2, 3, 5 triphenyl tetrazolum chloride solution (filter sterilized) was added. The pH was then readjusted to 5.3 with sterile 10% tartaric acid solution (0.4 ml added).

Assay procedure:

The overnight culture of the indicator organism was serially diluted to provide 10^{-4}, 10^{-5} and 10^{-6} dilutions. One ml of each dilution was added to sterile peteri plates (in duplicate). Plates were poured with 12-15 ml of sterile CVT agar containing 1% of the test material. Control plates had received the same treatment except that either only acidified CVT agar was poured or 1% of the eluant buffer (in chromatographic fractions) was added to the agar and poured. All plates were incubated at 30°C for 2 days and only red colonies were counted.

GC-MS Systems:

Fractions in peak A (Column II) were pooled, methylated with diazol-methane and injected into the GC which was connected to a Funigan mass spectrometry interfaced to an acquisition computer data system.

NMR - Spectrometry:

Sixty ml from the retentate of the ultrafiltration (YC05, cut off 500) were freeze dried (4.5 gms), suspended in D_{20} and freeze dried again to exclude water. The NMR spectra were run with this sample, with pooled fractions in peak A and other chromatographic fractions. An FT-80A (Varian) NMR spectrometer was used. Acetonitrite was used as an internal standard.

Results

The numbers of actively inhibitory peaks from both samples (FDPM and UNPLM) were almost the same, except for the noticable low resolution in column 2 resulting from the prolonged use of the column (Fig. 3.1, top and bottom). Concentration of the antimicrobiol material was a function of the indicator organism cell concentration. Surprisingly, significant amounts of organic acids appeared in the early fractions collected from both columns (Table 3.1 and 3.2). No free acids were detected, though pronounced inhibition peaks against the indicator organism were observed in the absence of any significant levels of the organic fatty acids. Surprisingly, the total amounts of organic fatty acids associated with the chromatographic fractions were higher than the amounts in the original unfractionated sample (despite its dilution 1/10, 1/100).

CM-50, cationic exchanger, analyzed fractions indicated the non-availability of accessable protein molecules, despite the obvious trapping of some positively charged molecules (Figure 3.2 and Table 3.5). This confirmed our previous findings, when we precipitated the protein fractions by organic solvents, collected the pellets by centrifugation, freeze-dried and assayed them

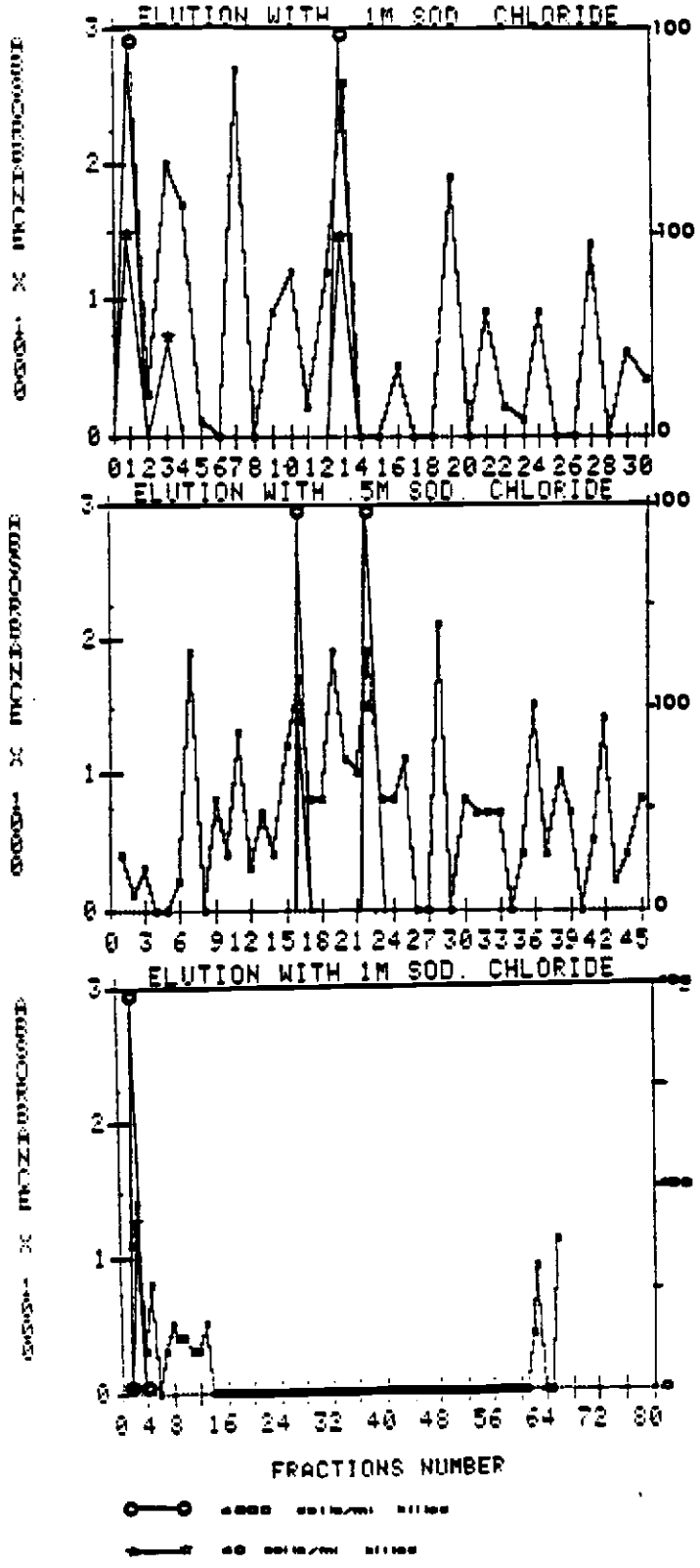
ELUTION WITH 1M SOD. CHLORIDE
ELUTION WITH .5M SOD. CHLORIDE
ELUTION WITH 1M SOD. CHLORIDE
FRACTIONS NUMBER

Figure 3.2

Figure 3.3 Standard molecular weight curve constructed by the elution of the following reference molecules on Column II; Cytochrome C (13,000), bacitracin (1,200), vitamin B_{12} (1,165), erythromycin gluconoheptonate (960), phenol red (354) and reduced glutathione (300). The following concentrations were used respectively, mg/mL^{-1}: cytodone C, 2.5; bacitracin, 5; vitamin B_{12}, 2.5; erythromycin, 5; phenol red, 2.5 and GSH 200.

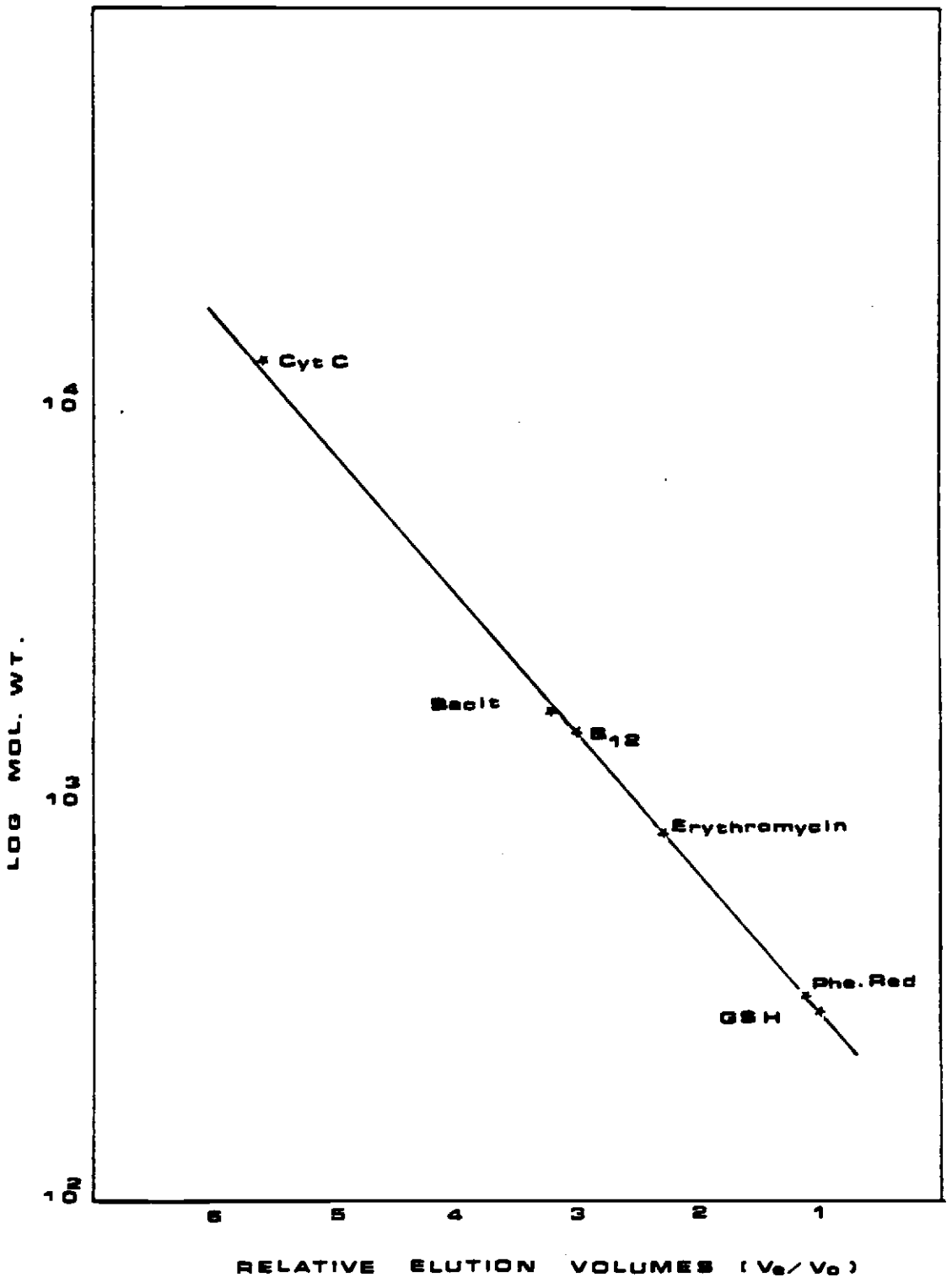
Cyt C
Bacit
B_{12}
Erythromycin
Phe. Red
GSH
10^4
10^3
10^2
LOG MOL. WT.
6
5
4
3
2
1
RELATIVE ELUTION VOLUMES (V_e/V_o)

Figure 3.3

Table 3.3 Distribution of the organic volatile fatty acids in the ultrafiltration fractions (µg/g).

action	Propionic	Acetic	Isobutyric	n butyric	Propionic/ acetic ratio	Remarks
ɔle whey ɔn- ltrafiltered) 5% FDM lution)	712.04	444.94	9.244	158.64	1.72	
p portion 500 Cut f. etentate)	675.74	411.135	8.62	260.52	1.64	viscous and yellow- ish
0 Retentate ter washing th 3 volume water	783.385	439.780	9.287	93.17	1.78	clear solution
loss by shing	59	60	46.40	80		

Table 3.4 Estimated Molecular Weights of the fractions collected from Column II.

Peak #	M. Wt.
A	1010
B	800
C	460
D	400
E	350
F	300
G	240
H	230

Table 3.5 Determination of fatty acids content in CM-50 fractions (µg/g).

Peak #	Acetic	Propionic	N-butyric
1	2.188	0	0
2	4.989	0	0
3	3.139	4.71	0
4	6.000	0	0
5	4.050	0	40.00

Figure 3.4 Programable DU-8 spectophotemeter scanning of FDPM biologically active fractions from Column I (Figure 1). Functions were scanned spectrophotometrically in the range of 200-400 nm using the programable DU-8 spectorphotometer (Beckman). The appearance of distinct absorption peaks in fractions 18, 19, 24 and 28 was thought to be diagnostic for dipyrrole units (in the range of 250-300 nm). The inhibitory action of FDPM fractions was also observed in the absence of any noticable absorption peaks as in fraction 33, 34, 37, 45, 62 and 72. Propionic plus acetic acid was a control fatty acid sample containing 5,000 ppm of each (1:1). GLU was a glucose sample (0.5g/4.5ml) as a carbohydrate control.

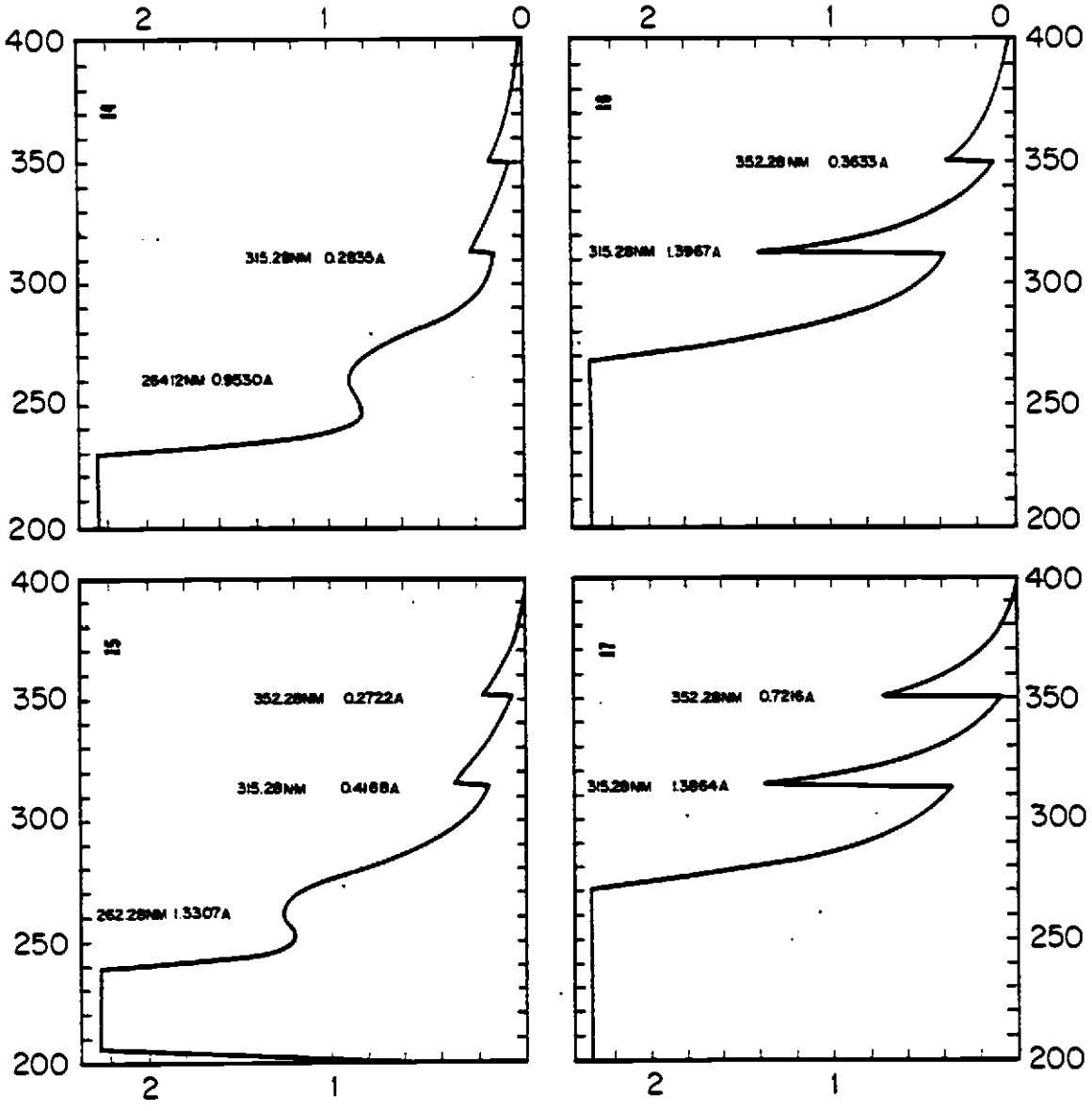
400
350
300
250
200
2
1
0
315.28NM 0.2835A
264.12NM 0.9530A
352.28NM 0.3633A
315.28NM 1.3967A
352.28NM 0.2722A
315.28NM 0.4168A
262.28NM 1.3307A
352.28NM 0.7216A
315.28NM 1.3864A

Figure 3.4

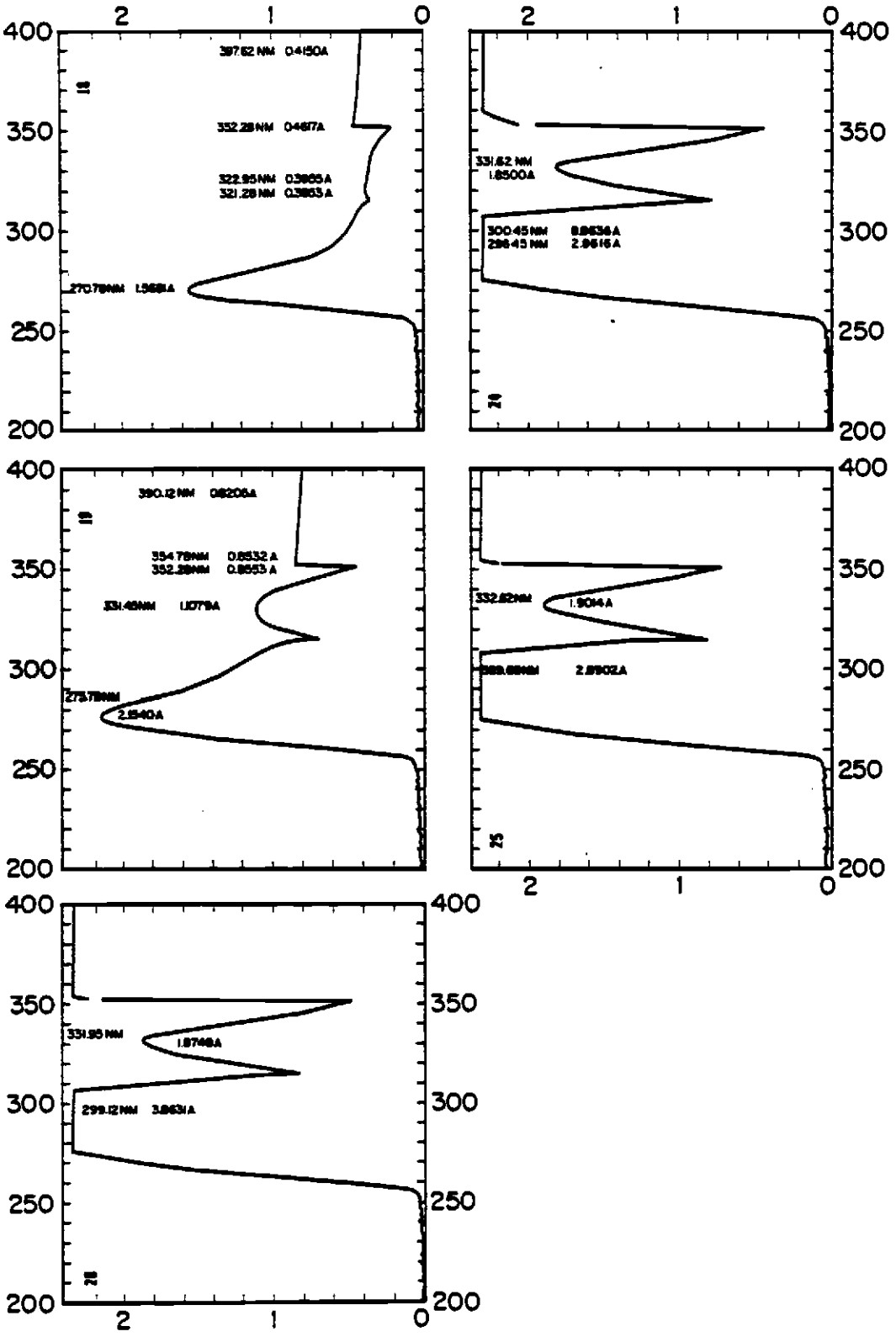

Figure 3.4 (continued)

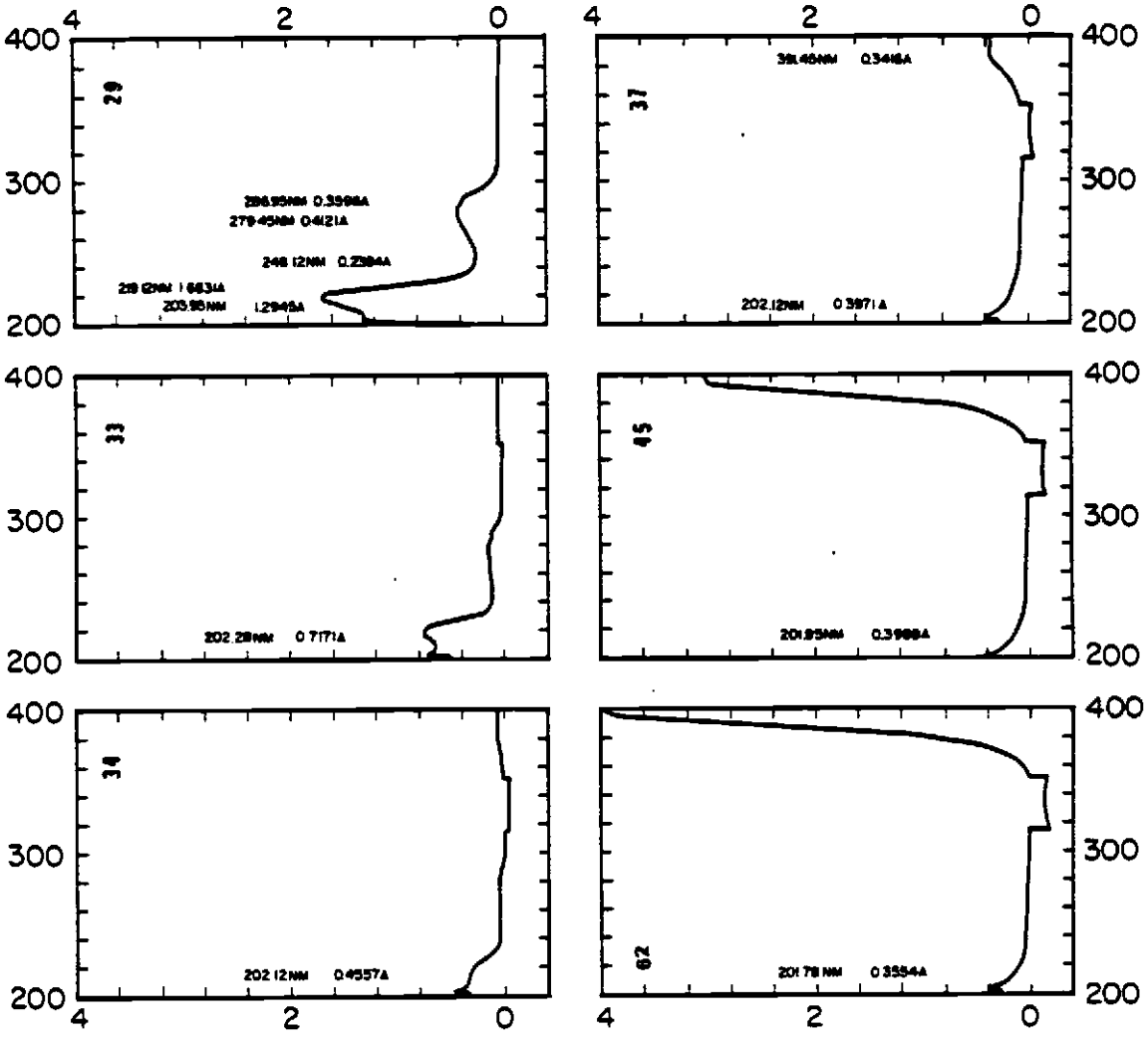

Figure 3.4 (continued)

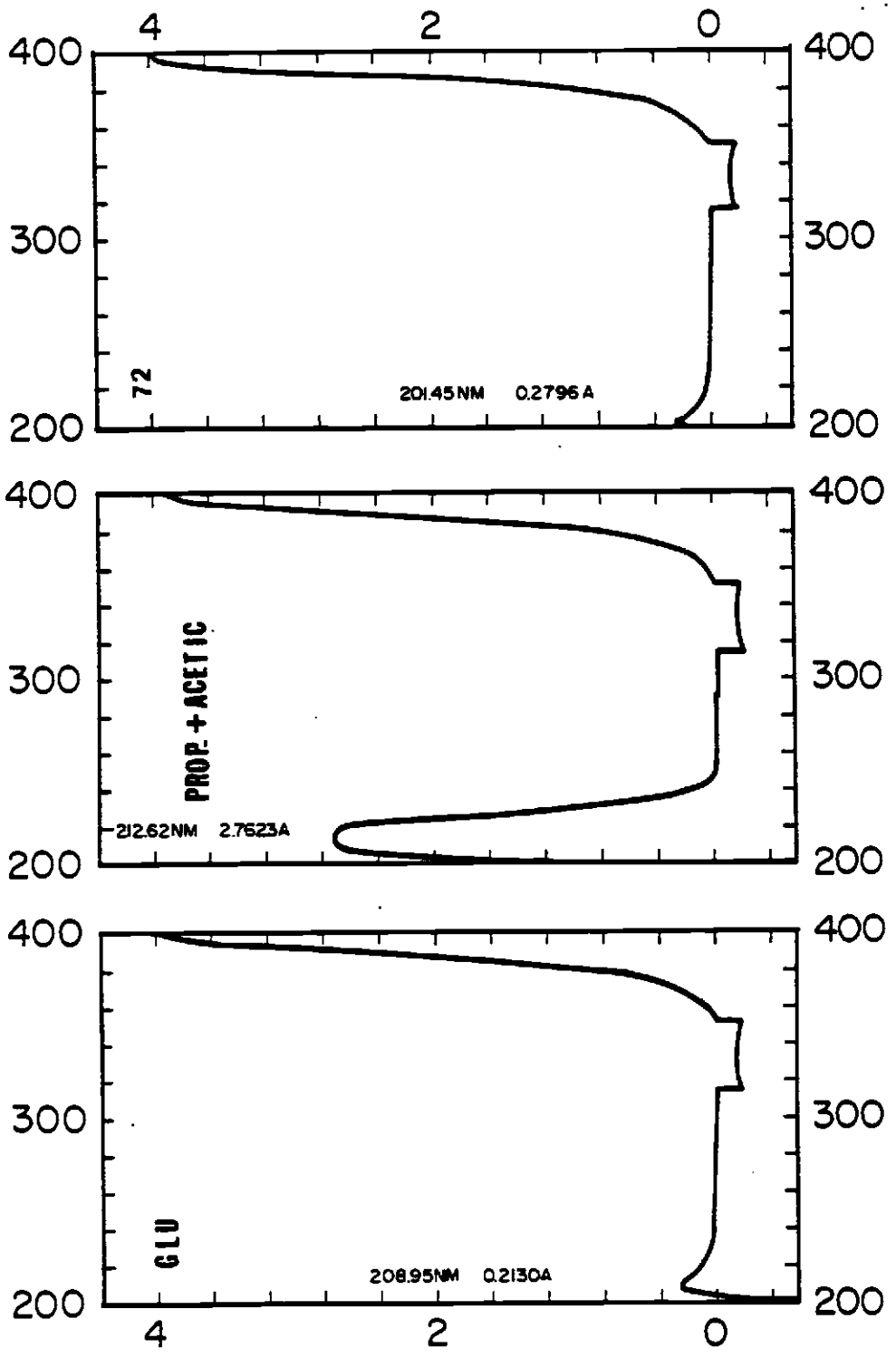

Figure 3.4 (continued)

Figure 3.5 Bilogically active fractions collected from the cationic exchanger and scanned in the UV range (200-400 nm) using the programable DU-8. No absorption peaks were apparent, though the fractions were very inhibitory to the indicator bacterium.

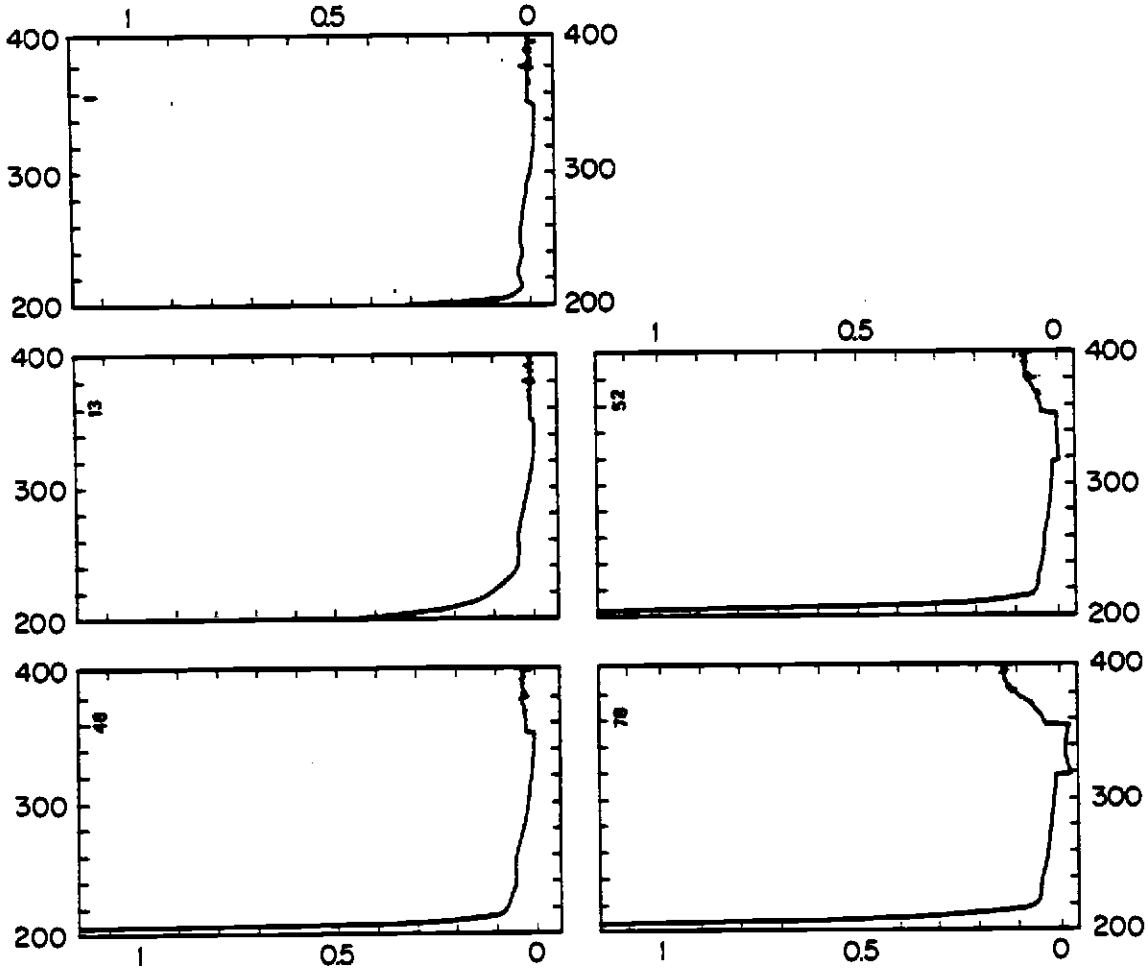

Figure 3.5

Figure 3.6 GS-MS- chromatogram: fractions in peak A (Column II) were pooled, methylated with diazol-methane and injected into the GC connected to a funigan mass spectrometer interfaced to an aquisition computer data system. M/C at 91 indicated the presence of an aromatic ring and a phenyl substituted lactic acid was identified. The occurrance of the compound was thought to be transient.

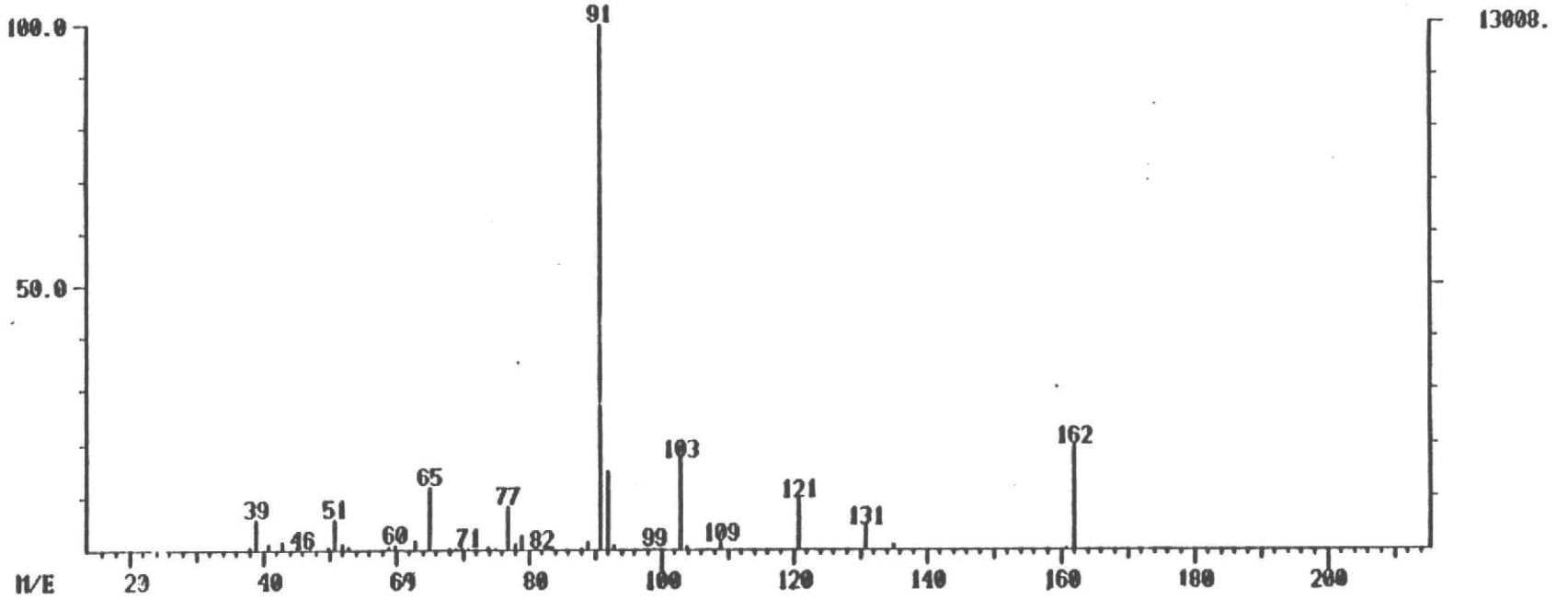
100.0
50.0
M/E
13008.
91
162
103
65
77
121
131
39
51
46
60
71
82
99
109
20
40
60
80
100
120
140
160
180
200

Figure 3.6

Discussion

Propionibacterium shermanii is a rumen microorganism that has the ability to deaminate aromatic amino acids to produce phenyl substituted fatty acids (36). The phenyl substituted fatty acid, hydrocinnamic acid (3-phenyl propionic acid) was demonstrated to competitively inhibit Eschevchra coli phenyl-alanyl tRNA-synthetase (26) and yeast phenylalanine ammonia lyase (17). B-phenyl propionic acid was also found to inhibit reversibly carboxy peptidase (24). In view of this, the occurrence in our samples of a phenyl-substituted fatty acid was no suprise, however, it apparently is a transient compound. Support for this view stemmed from the failure of the NMR spectra to provide supporting evidence for the existence of aromatic rings in the same or other samples.

Concentration dependent inhibition of microorganisms by lipophylic acids was demonstrated by Freese et al. (12). Nevertheless, some of these metabolities which are almost devoid of fatty acids exhibited the same concentration dependency. The early appearance of significant amounts of fatty acids in the gel filtration fractions suggested the physical or chemical association of these fatty acids with some other compounds. Physical

association appears unlikely for the following reasons: 1, the stability of this physical association is questionable under nitrogen pressure and continuous stirring during the ultrafiltration process; 2, the retentates in the ultrafiltration (with YC05) were found to pass with ease through the membrane filters after addition of water and exposure to air; 3, the tendency of the chromatographic fractions to lose activity at room temperature (kept mostly at -20^{o}C). Obviously, the chromatographic separation not only exposed volatile compounds but accessability of the GC to chemically-associated fatty acids (especially with open structure compounds) was facilitated by this exposure. The result of this expossure was recovery of higher total amounts of fatty acids in the fractionated compared to the unfractionated original sample. Discrepencies in the ratios of propionic to acetic acid as reported by Van Neil (38) (1.6-1.8) and Wood and Werkman (41) (2.1-14.7), might be attributed to these chemically-associated fatty acids. The estimated molecular weights of the active peaks indicated a profile of a possibly degraded parent compound. Bactobilin is a candidate compound exhibiting this synonymity. Bile pigments are ampholites containing pyrrole nitrogen and carboxyl groups. They are extremely

reactive in forming esters with various alcohols and acids and metal complexes with ions such as Cu++, Zn++, Ni++ and Fe++. It is well established that the open structure of bile pigments can easily decompose to monopyrroles, dipyrroles and tripyrroles in the presence of oxidizing agents (39). The occurance of dipyrrole units originating from tetrapyrroles has been documented in the urochrome of urine (mesobilifuscin) and in stool (14).

The yellow colored material seen in peaks I and A in both Sephadex columns and in the ultrafiltrates exhibiting a UV-peak between 250-300 nm (fractions 18,19, 24, 25, 28) indicated the presence of dipyrrole units. (39) It seems likely that the tetrapyrroles or their degraded pyrrole units elicit by themselves or in combination with other molecules, the major inhibitory action against the indicator organism. Dieter et al. (11) studied the carrier-mediated function of the pyrrole pigments, using them as ionophores. They found that ions such as Cr++, Fe++, Cu++ and Zn++ were rapidly and selectively transported by these pigments. Therefore, the dissipation of energy by the electron translocation process in the presence of these pyrrole units, is the possible inhibitory mechanism against the indicator organism.

The antimicrobiol effect observed with the fractions lacking fatty acids suggested the possible inhibitory participation by compounds such as diacetyl (21, 24) and positively-charged ions such as , diaminopropionic acid or neutral ions such as B- nitropropionic acid. Nanba et al. (27) studied the kinetics of the inhibitory effects of propionic and acetic acids on P. shermanii in a turbidostat culturing apparatus. The non-competitive inhibition was found to be severe under acidic conditions and the organism barely sustained growth at pH 5.2 in the presence of 0.1 M propionate. Their turbidostat culture apparatus had a light source constantly transmitted through the culture-vessel. Therefore, destruction of the bile pigment (bactobilin) was inevitable. However, in the present study the amounts of propionates and acetates the organism produced in milk at a low pH (5.2-5.3) were higher than the inhibitory levels cited by Nanba (Table 2). Hence, it is appropriate to suggest that in the presence of the bile pigment (bactobilin), natural buffering occurred, protecting the organism against its own metabolites.

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Chapter IV

Selenium Utilization and Accumulation by *Propionibacterium Shermannii*

Abstract

The ability of Swiss cheese starter organisms to accummulate elemental selenium was investigated using a differential milk agar medium. Five *Propionibacterium shermanii* strains were found to utilize and accumulate (in the presence of 300-400 µg/L selenite) elemental selenium. Some cells within each propionibacterial strain (resistant cells) failed to accummulate elemental selenium.

Generally, the uptake of elemental selenium by *P. shermanii* strain ATCC 9616 retarded its growth in a broth medium. Growing cells developed a muddy pink color within 24 hours in a broth medium containing 5 to 50 µg/L selenite. At 100 µg/L selenite and above, the pink color developed by 48 hours. Additional sugars (D-dulcitol & α-lactose) in Tryptic Soy Broth enhanced the selenium toxicity to the organisms. *Lactobacillus bulgaricus* R-5 and *S. thermophilus* C-5 accummulated selenium when the element was present in the growth medium at a concentration of 50 µg/L. *Streptococcus cremoris* SK11G accumulated the least amount of selenium relative to the

other Swiss cheese starter bacteria studied. Depending on selenite concentration, all organisms tested were considered potential participants in the pink discoloration defect of Swiss cheese. An extra cellular rennet like protease produced by propionibacterial strains in the presence of selenite, appeared to be an oxygen sentitive selenoprotein.

Introduction

Organisms utilizing selenium as a micronutrient must have the enzymic systems to release elemental selenium from inorganic compounds. In this regard, crude bacterial extracts have been reported to reduce selenite to elemental selenium and selenide (20, 46, 47). Dilworth et al. (8) were able to demonstrate an ATP - dependent sulfurylase in Saccharomyces cerevisiae, which catalyzed the reduction of selenate to elemental selenium and selenide in the presence of Mg++, inorganic pyrophosphatase and glutathione (GSH - reduced form). They proposed a reduction pathway involving oxygen. In addition, Burnell (4) established a dual role for ATP - dependent sulfurylase in the catalysis of both sulfate and selenate compounds in plants (Neptunia amplexicaulis). The absolute requirement for selenium as a micronutrient by anaerobic organisms was recognized in Methanococcus vannielii (17) and Clostridium purinolyticum (10). Recently, an aerobic, selenium utilizing Bacillus (strain SS) was isolated from Astragulus crotalariae seeds (21). The Bacillus was observed to grow under certain nutritional conditions, only in the presence of selenite.

The incorporation of selenium into macromolecules is well documented for several enzymes, some proteins of unknown bilogical function and certain amino acyl transfer nucleic acids (tRNAS) (42, 43). Depending on concentration, selenium is thought to substitute extensively for sulfur in macromolecules (28, 41). Ng and Anderson (29) studied the light-dependent incorporation of selenium and sulfur into selenocysteine and cysteine, respectively, by isolated pea chloroplasts. Recently, Wittwer (45) presented more evidence on the specific incorporation of selenium into E. coli tRNAS by processes markedly different from the mechanism of sulfur incorporation. Furthermore, the specific incorporation of selenium into protein molecules was substantiated and shown to involve a specific enzyme induction process in anerobic organisms (19). Today, selenium is viewed as an arduous antioxidant with terminal inhibitory effects on the metabolic progression of certain compounds to mutagens and carcinogens (9, 23, 24, 44).

The present study was undertaken to investigate the effect of selenite on Swiss cheese starter organisims. Special consideration was given to propionibacteria due to their anaerobic nature. Preliminary attempts to isolate

and concentrate an extracellular protease produced by these organisms also are reported.

Materials and Methods

Organisms:

Propionibacterim shermanii strains 9615, 9616, 9617 and 13673 were obtained from the American Type Culture Collection in Rockville, Maryland. Organisms were grown on ASLA selective medium described by (34) and incubated anaerobically (BBL GasPak, Baltimore, Maryland) for 11 days. Selected colonies were transfered into sterile Difco Tryptic Soy Broth (TSB) and incubated at 30°C for 2 days. Under aspetic conditions, 40% sterile glycerol solution was added to the stock culture which was then stored at -40°C. Inoculum for each experiement was prepared by transfering 2% thawed stock cultures into freshly sterilized TSB medium. Propogation of cultures was carried out under static conditions at 30°C for 2 days. Lactobacillus bulgaricus R-5, Streptococcus thermophilus C-5 and Streptococcus cremoris SK11G, were from the Oregon State University stock culture collection maintained in the Department of Microbiology. Lactobacillus bulgaricus and S. thermophilus were grown in M17 broth at 45°C for 6 hours to obtain higher cell counts. Streptococcus cremoris SK11G was propogated in sterile milk at 21°C for 16 hours.

Differential milk agar medium with selenite (Na_2SeO_3):

Sodium selenite was a donation from Dr. J. Oldfield, Animal Science Department, Oregon State University, Corvallis. The medium was prepared in 2 separate flasks as follows: (A) 100 g nonfat dry milk (NDM) + 2 g yeast extract were dissolved in 450 ml of distilled water and the pH of the milk solution was adjusted to 6.8 with 1M NaOH. (B) Fifteen grams of agar were dissolved into 550 ml of distilled water and steamed for 15 minutes and then 0.002% bromocresol purple was added to the flask. Both flasks A and B were autoclaved separately at 121°C for 12-13 minutes and cooled to 50-55°C in a water bath. Different selenite concentrations, from a 1% filter sterilized stock solution, were then added to the mixture of A and B. Fifteen to twenty ml of the medium then were poured into sterile petri plates. The plates were dried at room temperature (25°C) overnight. The propogated organisms were blended, diluted (10^{-1}-10^{-7}) and 0.1 ml of each dilution was spread over the agar surface. Inoculated plates were further dried at 30°C for 2 to 3 hours before anaerobic incubation. Propionibacterial cultures were incubated at 30°C for 5 to 6 days. Lactobacillus bulgaricus, and S. thermophilus were incubated at 37°C and S. cremoris at 30°C for 2 days. All the anaerobic

incubations were carried out in GasPak anaerobic jars (10% CO_2 and 90% N_2).

Screening trails for suitable growth conditions to maximize extracellular protease production in batch cultures were conducted as follows:

(A) Tryptic Soy Broth (TSB): Difco TSB with a low glucose concentration (0.25%) was investigated for support of protease production, because it allowed good growth. One liter of TSB was prepared, dispensed equally into 2 one-L flasks and autoclaved at 121°C for 15 minutes. To one flask 10 g of filter sterilized selenite were added. Two percent of the propogated P. shermanii strain was transfered into each flask. Cultures were incubated statically at 30°C. Samples (20 ml) were withdrawn every 24 hours and transfered aseptically to sterile centrifuge tubes (25 ml). Centrifugation was carried out at 5,000 X g at 4°C for 20 minutes. Supernatant solutions were collected for the determination of the proteolytic activity using the azocasein assay of Ingram et al. (16). The cellular pellets were washed twice with sterile phosphate buffer (pH 6.5) and resuspended in 5 ml of the same buffer. The cell suspensions were then transfered into sterile preweighed vials and freeze dried to determine cell dry weight.

(B) Effect of sugar supplementation:

1. D-sorbitol (Dulcitol): one liter of TSB was equally divided into 2 one-L flasks and autoclaved for 15 minutes at 121°C. Filter sterilized Dulcitol (Sigma) was added to each flask to a final concentration of 0.4% (TSB-D). From a 1% filter sterilized stock solution, 100 g were added to one of the flasks. Both flasks were then inoculated with 2% P. shermanii. Cultures were incubated statically at 30°C. Twenty-ml samples were withdrawn aseptically every 12 hours. Five ml of the sample were used to moniter the growth of the organism spectrophotometerically at 600 nm (Perkin-Elmer model 35 Oak Brook, Ill.). The remaining 15 ml were centrifuged at 9880 X g for 15 minutes (Beckman model J) at 4°C. The collected supernatant solutions were assayed for their proteolytic activities by the azocasein assay (16).

2. D-Fructose: Four flasks each containing 300 ml of TSB were autoclaved for 15 minutes at 121°C. A final concentration of 0.4% filter sterilized D-fructose (Sigma Chemical Co., St. Louis, MO) was achieved in each of the 4 flasks (TSB-F). To three flasks 25, 50 and 100 g selenite concentrations were made in the TSB-F media. Growth and proteolytic activity were monitored as above.

3. α -Lactose (TSB-L): Four flasks each containing

300 ml received the same treatment as for the TSB-F medium, except that 1, 5, and 10 g selenite and 0.4% α-lactose concentrations were used.

(C) Calcium caseinate medium:

One liter of the medium formulated as follows was made: Calcium caseinate (Gallaway West Co., Fond du Lac, WI), 10 g; yeast extract, 2 g; α-Lactose, 5 g, distilled water, 1 liter. The pH of the medium was adjusted to 6.8 with 1M NaOH, dispensed equally into 2 one-L flasks and autoclaved at 121°C for 15 minutes. To one flask, 10 g filter sterilized selenite was added. Both flasks were inoculated with 5% P. shermanii cultures. Growth was monitered by the Kanasaki et al. method (18). Proteolytic activity was measured by the procedure of Samples et al. (36) using trinitrobenzene sulfonic acid (TNBS). Results were reported in μM glycine equivalents determined from a standard glycine curve.

Milk coagulation assay for the protease:

To concentrate the protease, 500 ml of each formulation in sections A, B and C above, were prepared. Five percent inoculum of P. shermanii cultures (ATCC 9616) was transfered into each flask. Incubation was at 30°C for 2 days. Supernatants, collected by centrifugation

(9880 X g at 4°C for 15 minutes) of the cultures, were freeze dried. Freeze dried powders (13 to 14 gms) were suspended in 30 ml of distilled water and 70% $(NH_4)_2SO_4$ saturation was achieved in each collection. The suspension was stirred for 8 hours at 4°C and then transferred into dialysis tubes (spectrapor-membrane tubing, molecular weight restriction at 6,000-8,000). Dialysis was performed over 48 hours and contents then were transfered into Amicon stirred ultrafiltration cells fitted with YM5 membrane filters (molecular weight restriction 5,000). Desalting was ensured by washing retentates twice with distiled water. The concentrated protease was then suspended in 10 ml of phosphate buffer, pH 6.5. The milk coagulation assay of Roth et al. (36) was then used to measure milk coagulation at different temperatures and pH values.

Results

Growth on the differential milk agar medium (DMA):

Large red colonies with or without casein hydrolysis zones were observed. Pinpoint colorless colonies were also seen as an indication of their inability to accummulate selenium. In all tested *P. shermanii* strains, the frequency of occurrence of elemental selenium accumulators and non-accummulators did not follow specific distribution patterns (Table 4.2). All selected *P. shermanii* strains were relatively resistant to toxicity of high levels of selenite (300 - 400 µg) on the solid medium (DMA) (Table 4.1). *Lactobacillus bulgaricus* and *S. thermophilus* strains, grown on DMA, gave red colonies with distinct casein hydrolysis zones in the presence of 50 µ g/L selenite. *Streptococcus cremoris* strain SK11G gave few tiny red colonies (at the same selenite concentrations) with evident growth stimulation.

Response of *P. shermanii* strain 9616 to selenite in broth:

1. Pink coloration: A pink color developed within 24 hours in TSB medium at 5 to 50 µg selenite concentrations. At high selenite concentrations (100-150 µg), the pink coloration developed in 48 hours. The pink

coloration was found to be associated with the cellular pellets, evident after centrifugation of cultures grown in TSB selenite medium.

2. Effect of additional carbohydrates: Additional sugars in TSB (with 0.25% glucose) medium retarded growth of the organism to various degrees in the presence of different selenite concentrations. With D-sorbitol, the organism ceased to grow in the presence of 100 µg of selenite (Figure 4.1). The relative resistance of the organism to selenite was reduced in the presence of lactose as an additional sugar (Figure 4.2). Moderate selenite tolerance was observed with D-fructose as an additional sugar (Figure 4.3). Propionibacterium shermanii strain ATCC 9616 tolerated up to 150 µg selenite in broth before being completely inhibited.

3. Production of an extracellular protease: Cultures grown in TSB medium with selenite were tested for the production of a protease in cellular pellets and supernatant solutions (Figure 4.4). The cellular pellets were found to contain no proteolytic activity even after sonication. Proteolytic activity from all media formulations was very low despite using different assay systems. However, when the organism was grown in milk or calcium caseinate medium, protein curdling occured within

24 to 48 hours in the presence of selenite (Figure 4.5). In the absence of selenite, the organism coagulated the casein in 72 to 96 hours. Attempts to concentrate the protease were unsuccessful. No attempt was made to detect or concentrate the protease under reduction conditions (e.g. using reducing agents).

Figure 4.1 Growth of P. shermanii in TSB media amended with 0.4% Dulcitol with and without selenite.

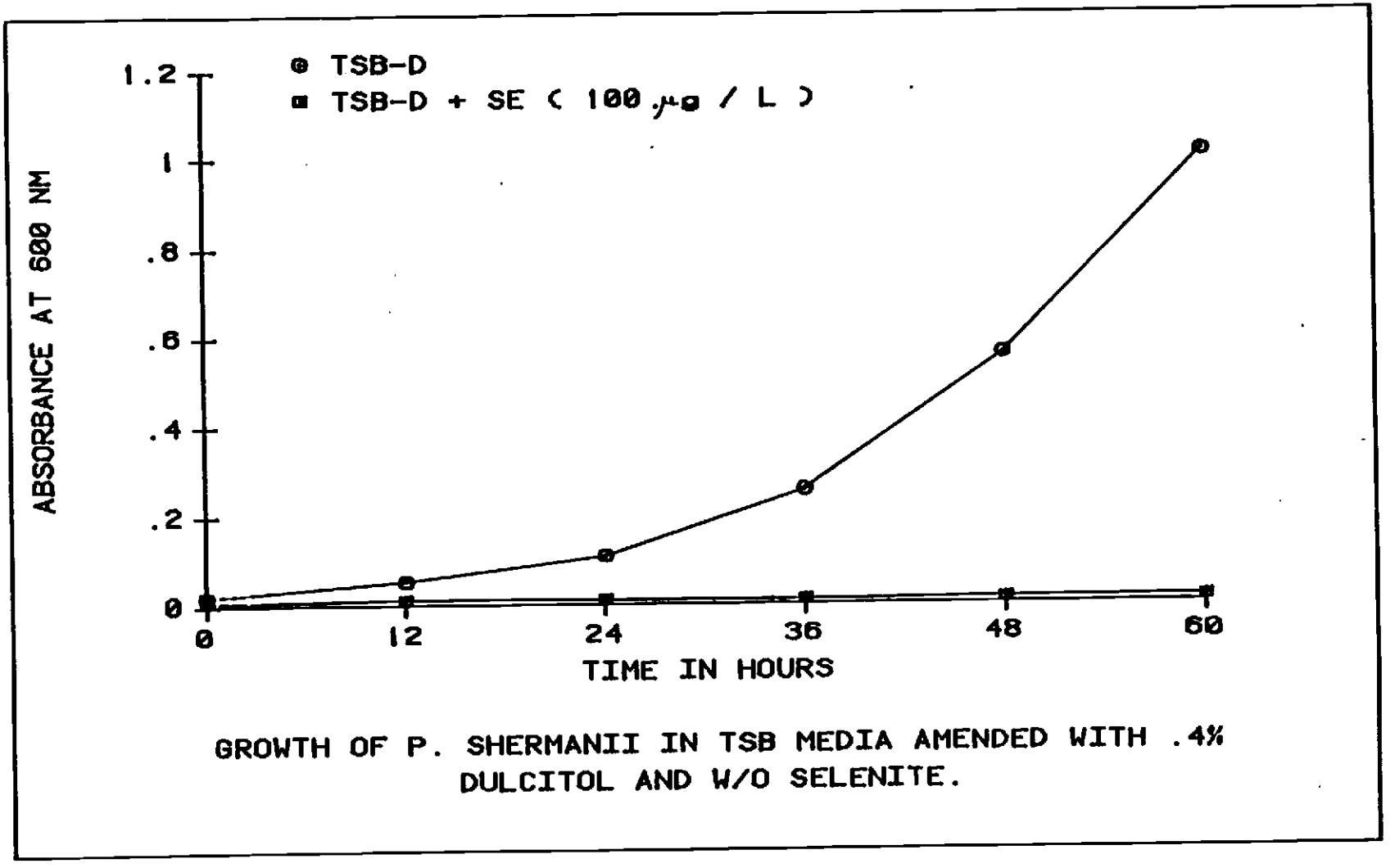
TSB-D
TSB-D + SE (100 µg / L)
ABSORBANCE AT 600 NM
1.2
1
.8
.6
.4
.2
0
0
12
24
36
48
60
TIME IN HOURS
GROWTH OF P. SHERMANII IN TSB MEDIA AMENDED WITH .4% DULCITOL AND W/O SELENITE.

Figure 4.1

Figure 4.2 Growth of P. shermanii in TSB media amended with 0.4% lactose with and without selenite.

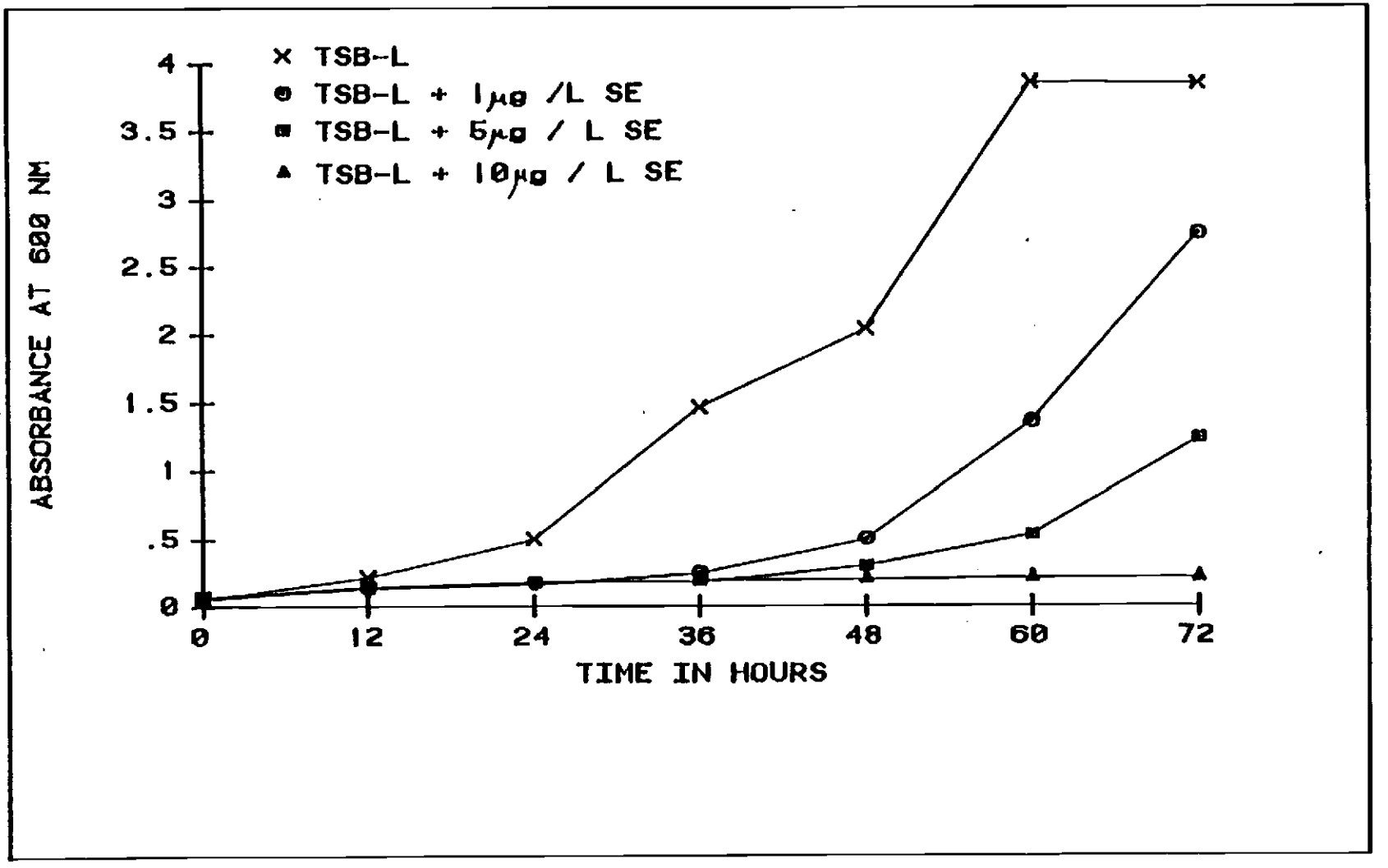
TSB-L
TSB-L + 1µg /L SE
TSB-L + 5µg / L SE
TSB-L + 10µg / L SE
ABSORBANCE AT 600 NM
4
3.5
3
2.5
2
1.5
1
.5
0
0
12
24
36
48
60
72
TIME IN HOURS

Figure 4.2

Figure 4.3 Growth of *P. shermanii* in TSB media amended with 0.4% fructose, with and without selenite.

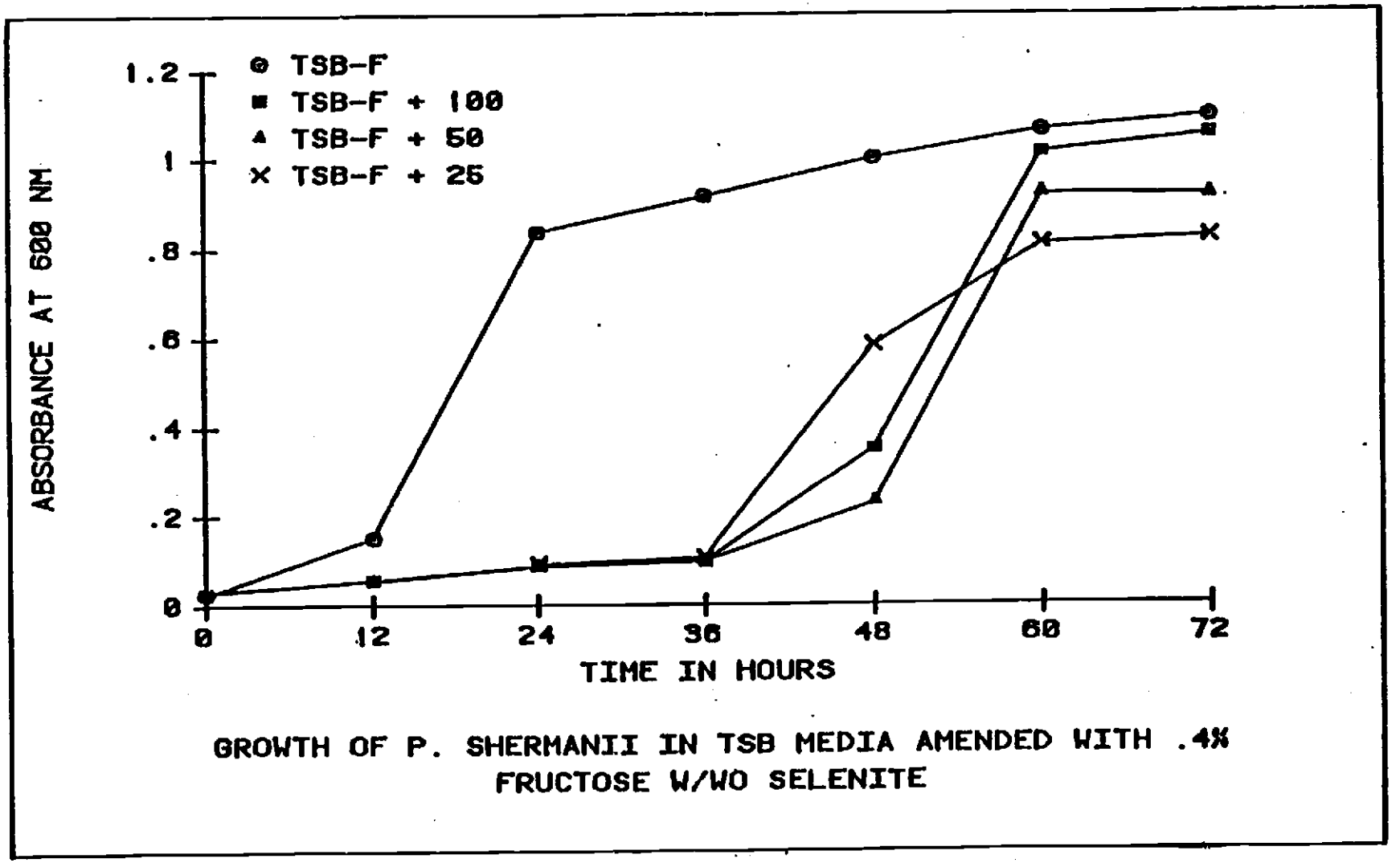
TSB-F
TSB-F + 100
TSB-F + 50
TSB-F + 25
ABSORBANCE AT 600 NM
1.2
1
.8
.6
.4
.2
0
0
12
24
36
48
60
72
TIME IN HOURS
GROWTH OF P. SHERMANII IN TSB MEDIA AMENDED WITH .4% FRUCTOSE W/WO SELENITE

Figure 4.3

Figure 4.4 Proteolytic activity of P. shermanii in TSB media, with and without selenite.

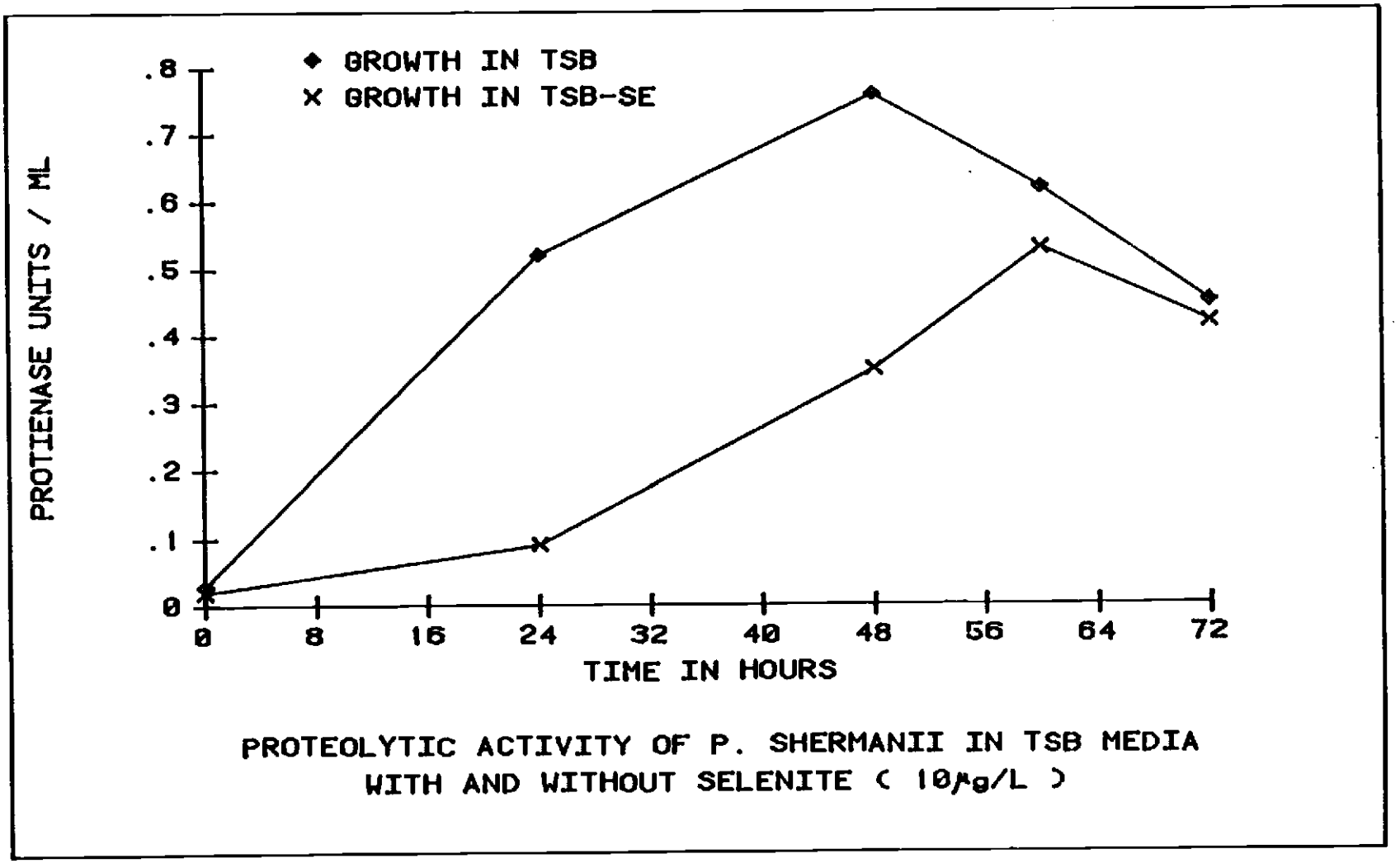
◆ GROWTH IN TSB
× GROWTH IN TSB-SE
PROTIENASE UNITS / ML
.8
.7
.6
.5
.4
.3
.2
.1
0
0
8
16
24
32
40
48
56
64
72
TIME IN HOURS
PROTEOLYTIC ACTIVITY OF P. SHERMANII IN TSB MEDIA
WITH AND WITHOUT SELENITE (10µg/L)

Figure 4.4

Figure 4.5 Proteolytic activity of P. shermanii in a medium (w/v calcium caseinate, 10; yeast extract, 2; lactose, 5; g/L- and pH 6.8), with and without selenite (10 µg/L).

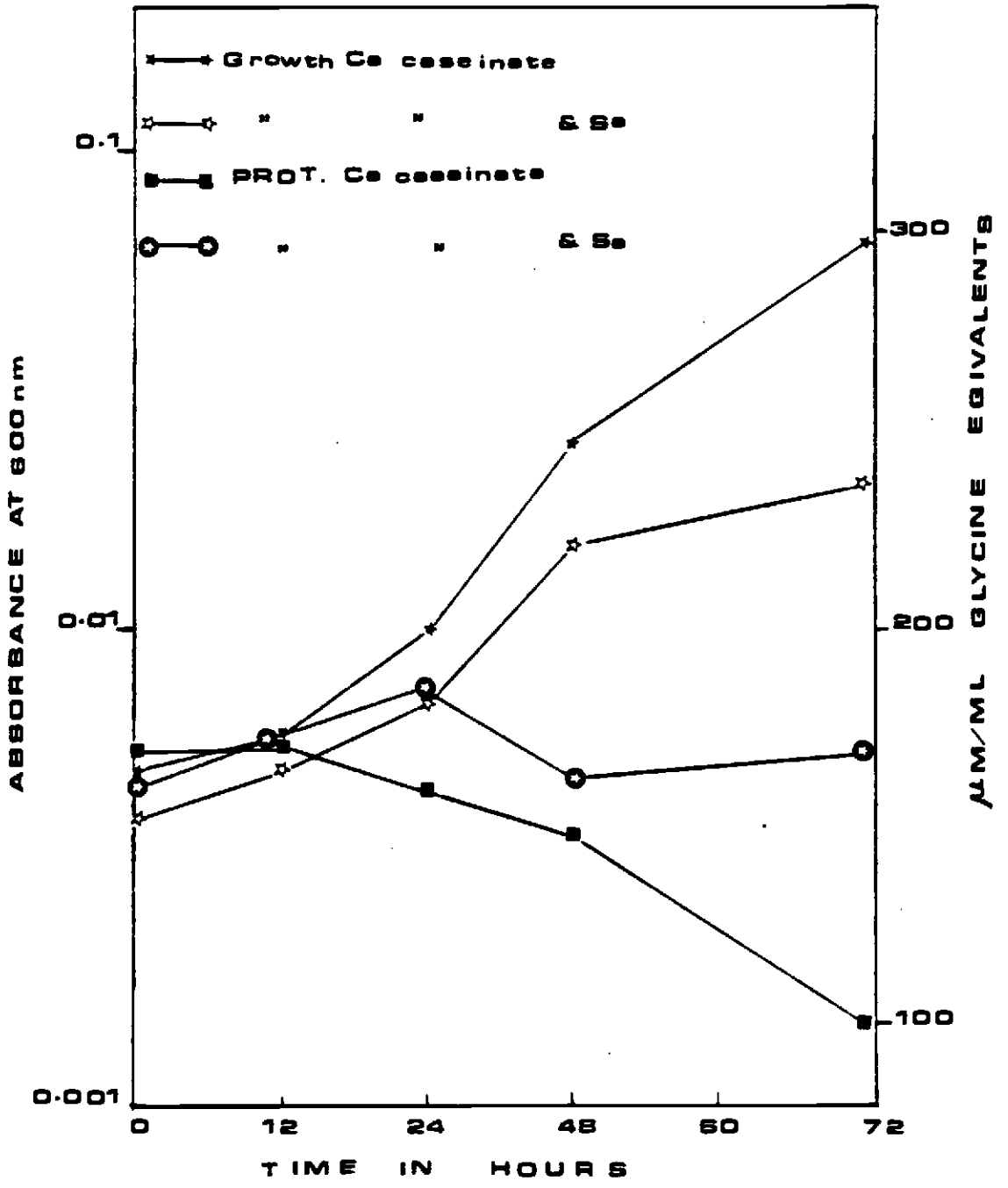
Growth Ce caseinate
" " & Se
PROT. Ce caseinate
" " & Se
0.1
0.01
0.001
ABSORBANCE AT 600 nm
300
200
100
µM/ML GLYCINE EQUIVALENTS
0
12
24
48
60
72
TIME IN HOURS

Figure 4.5

Table 4.1 Determination of selenite critical concentrations for ATCC propionibacterium strains:

Amount of Selenite μg/L	Propionibacteria Plate Counts on DMA (CFU/mL)				
	8262	9616	9615	9617	13673
5	$1.8x10^{7}$	$5.6x10^{6}$	$1.6x10^{7}$	$3.4x10^{6}$	$4.6x10^{6}$
100	$1.3x10^{6}$	$2.7x10^{6}$	$2x10^{6}$	$7x10^{4}$	$3x10^{3}$
200	$1x10^{4}$	$1.9x10^{4}$	$3x10^{2}$	$4x10^{2}$	$5x10^{2}$
300	$7x10^{2}$	$3x10^{2}$	$1x10^{2}$	$1x10^{2}$	$1.5x10^{2}$
400	$3x10^{2}$	$1x10^{2}$	0	0	0
500	0	0	0	0	0
1000	0	0	0	0	0
1500	0	0	0	0	0

Table 4.2 Description of colony distribution on Differential Milk Agar plates.

P. shermanii Strains	Colonies that grew out on 1st plating	Colonies that grew out on 2nd plating	Plate counts on 2nd plating (CFU/ml)
ATCC 13673	red	all red	3.1×10^8
	colorless	red	6×10^5
		colorless	2.5×10^5
ATCC 9615	red	red	2.3×10^7
		colorless	1×10^7
	colorless (tiny)	red	1.1×10^7
		colorless	1.2×10^7
	colorless (large)	red	3.4×10^4
ATCC 9616	red (not proteolytic)	tiny colorless (non-proteolytic)	3.1×10^8
	red (proteolytic)	red-proteolytic	2.4×10^6
		colorless - non-proteolytic	3×10^3
ATCC 8262	red non-proteolytic	red mostly proteolytic	3.2×10^6
	red (proteolytic)	red proteolytic	1.3×10^8

Discussion

The accumulation of elemental selenium by P. shermanii strains was variable. When selected single red or colorless colonies were transfered into a broth medium (TSB with no selenite) and replated again on the differential milk agar medium (DMA) containing selenite, a mixture of white and red colonies were observed. It was an indication of the inability of some cells within the population to accummulate elemental selenium. Inorganic ion resistance has been demonstrated in S. lactis C_2 and recognized as a plasmid mediated function (11). We were unable to detect any plasmids in P. shermanii strain 9616, using Orberg and Sandine's microscale procedure (32) for plasmid isolation (data not shown).

Elemental selenium could be inserted into either cysteine or methionine to form selenocysteine and selenomethionine respectively (7,14). Consequently, selenocysteine and selenomethionine could either be integral parts of bacterial protein moieties (7, 14) or be converted into non-protein amino acids such as Se-methyl selenocysteine or selenocystathionine, that serve as accummulation sites in seleniferous plants (4). Stadtman (42) noticed the rapid accumulation and instability of

selenocysteine, in preparations made from Clostridium sticklandii. Saki et al. (12) isolated, purified and identified a novel selenocysteine lyase enzyme from pig liver that catalyzed the conversion of selenocysteine into alanine and hydrogen selenide (H_2Se). The H_2Se was reported to be detoxified and removed by methylaltion processes in animal tissues (22). Hence, the inability of some cells (resistant) to accumulate elemental selenium could be attributed to: 1. The inability of cells to release elemental selenium from selenite as was reported by Arst (1). 2. Development of some detoxification mechanisms that continually purged cells of H_2Se and elemental selenium.

A pink discoloration defect had been observed in Italian varieties of cheese, Cheddar and Swiss cheese (2, 33, 35, 38). The discoloration defect was described as a uniform pink band near the cheese surface or as a uniform discoloration through out the entire cheese. The defect was controversially attributed to chromogenic bacteria and higher oxidation reduction potential in the cheese (25, 26, 27, 40). Shannon et al. (39) associated the pink discoloration to a non-dialyzable fat free fraction that could not be extracted by a wide variety of chemical treatments.

The ability of these cheese starter organisms to accumulate elemental selenium, suggests their possible contribution in the observed pink discoloration defects. The avarage selenium content in milk (61±12 µg/l) and Swiss cheese (0.34 µg/g) as reported recently (31), supports the possible involvement of these microorganisms in the pink discoloration defects.

Additional sugar sources were utilized to accelarate the growth of propionibacteria (16). In contrast, a growth retardation marked by a prolonged lag phase in the presence of selenite, was observed. A defective translational process resulting from the significant participation of selenomethionyl $tRNA^{met}$ that ultimately reduced the overall rate of protein synthesis, was cited as the mechanism of selenium toxicity (13). Additional sugars (D-Dulcitol and α-Lactose) seemed to enhance selenium toxicity by significantly retarding growth. Selenium was reported to provide protective functions in rat kidney lysosomes *in vitro* (30) and other cell membranes (3, 5). Therefore, probably the selenium protective function of the membrane phospholipid layers was interferred with in the presence of these additional sugars.

Excessive proteolytic activities by certain propionibacterial strains was thought to be involved in Swiss cheese split defects (15). As it was evident from the selenite DMA plates, a protease production was indicated by clear zones of casein hydrolysis. In addition, by growing the organism in milk and calcium caseinate media, casein coagulation occured in 24-48 hours at pH 6-6.5. Our unsuccessful attempts to isolate and concentrate the protease make us inclined to view this protease as an oxygen sensitive rennet-like enzyme. All seleno proteins so far recognized are extremly oxygen sensitive. Therefore, the extracellular proteolytic enzyme(s) produced by P. shermanii strain 9616 in the presence of selenite may have been a selenoprotein(s).

The strong ability of these cheese microorganisms to concentrate elemental selenium intracellularly could pose a potential health hazard for humans. Indeed more research work is needed to investigate this possibility.

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Chapter V

A Selective Differential Medium for Enumeration of *Lactobacilus bulgaricus* and *Streptococcus thermophilus*

Abstract

Differences in selenite and tellurite tolerance by *Streptococcus thermophilus* and *Lactobacilus bulgaricus* were investigated in broth and on a differential milk agar (DMA) medium. Selenite concentrations above 160 µg/L completely inhibited the growth of *S. thermophilus*. In contrast, *L. bulgaricus* exhibited a stimulatory response at the same selenite concentrations. A milk agar medium containing selenite (160 & 320 µg/L) provided a selective differential environment for the growth of *L. bulgaricus* alone. At concentrations above 80 µg/L potassium tellurite in broth medium, *S. thermophilus* was completely inhibited. *Lactobacillus bulgaricus* growth was favored by the same tellurite concentrations. Conversely, the survival of the two organisms was reversed on DMA with tellurite under anaerobic incubation at 37°C for 48 hours. Potassium tellurite (100 µg/L) provided a selective differential atmosphere for only the growth of *S. thermophilus*. *Lactobacillus bulgaricus* failed to grow under the same conditions. Potassium tellurite (10-20

µg/L) in the DMA medium was reduced to black metallic tellurium which was precipitated by both organisms resulting in morphologically distinct colonies. Development of differentially distinct red and black colonies by the two organisms on the same plate, was considered an attainable objective using this approach.

Introduction

The associative and symbiotic growth of Lactobacilus bulgaricus (rods) and S. thermophilus (cocci), has promoted their continual utilization in the manufacture of yogurt, Swiss cheese and various Italian cheeses (19). One of the problems associated with this symbiotic relationship is how to achieve desirable rod-coccus ratios in the bulk starter and the milk product during manufacture. There is agreement among various investigators that the rod-coccus ratio should be approximately 1:1 in yogurt starters and in the milk during the manufacturing processes. This is necessary to obtain an acceptable flavor and texture in the finished product (16, 17, 21).

A number of differential enumeration procedures for rods and cocci are known but none of them so far provided the convenience and the adequate selectivity needed for the differential enumeration of L. bulgaricus and S. thermophilus (2, 4, 5, 18, 20). Recently, Hamann and Marth (8) compared the ability of six media to recover L. bulgaricus and S. thermophilus from yogurt and pure cultures. They concluded that the Modified Hansen's Yogurt Agar medium was slightly better than others in differential ability and for obtaining total cell counts

of the two organisms. However, it still is apparent that available media are neither selective against contaminating microorganisms nor sufficiently differential for the two microorganisms in question. The purpose of the present study was to investigate selenite and tellurite reduction and accumulation by L. bulgaricus and S. thermophilus as a new approach for a selective differential enumeration of these important bacteria.

Materials and Methods

Organisms

Lactobacilus bulgaricus R-5 and Streptococcus thermophilus C-5 strains were from the Oregon State University stock culture collection maintained in the Department of Microbiology. Originally they were isolated from rod-coccis culture CR-5 obtained on a frozen concentrate from the Morechall Division of Miles Laboratories, Inc., Madison, WI. The organisms were routinely propagated in MRS broth (12) at 37°C using a 1% inoculum with incubation for 15 to 18 hours.

Proceedures

Growth in broth containing sodium selenite (Na_2SeO_3)

(A) MRS broth (800 ml) was dispensed in 100 ml aliquots into presterilized bottles and autoclaved at 121°C for 15 minutes.

(B) ST broth (4) (800 ml) was also dispensed in 100 ml aliquots into presterilized bottles and autoclaved at 121°C for 15 minutes. From a 1% filter-sterilized sodium selenite stock solution (Na_2SeO_3); 10, 20, 40, 80, 160, and 320 µg/L selenite concentrations were achieved in each of the two broth media. Each medium was individually inoculated with 2% cultures of either L. bulgaricus (set

A) or S. thermophilus (set B) and a control (no selenite) bottle was included for each culture. One bottle from each medium was used as a blank. Both sets were incubated in a water bath pre-adjusted to 42°C. Five-ml samples were withdrawn every 2 hr for the first 12 hours to moniter growth by measuring optical density (O.D.) at 600 nm (Elmer - Perkin spectrophotometer model 35) and acid production by following the drop in pH (corning 125 pH meter). Twenty four hour fermentation samples were also included. The same protocol was used to follow growth in broth containing potassium tellurite, except that M_{17} broth was used to grow S. thermophilus instead of ST broth.

Growth on agar medium containing selenite and tellurite.

To prepare differential milk agar (DMA) medium, four flasks containing the following media were prepared:

(A) One hundred grams of non-fat dry milk (NFDM) + 2 g yeast extract were dissolved in 450 ml of distilled water and the pH of the milk solution was brought to 6.8 using 1 M NaOH.

(B) Fifteen grams of Difco agar were added to 550 ml of distilled water, steamed for 15 minutes and bromocresol purple then was added to provide a final concentration of

.002% for one liter of medium. Both flasks (A & B) were autoclaved separately at 121°C for 12-13 minutes and cooled to 50-55°C and combined. To the mixture, 50, 80 and 320 µg/L final selenite concentrations were made in three separate flasks (250 ml volume) each from a filter-sterilized 1% sodium selenite stock solution. The fourth flask was inoculated with 1% potassium tellurite solution to provide a final concentration of 10 µg/L. Fifteen to twenty ml of each medium were poured into sterile petri plates and dried overnight (25°C). Lactobacilus bulgaricus and S. thermophilus pure cultures were serially diluted and 0.1 ml of each dilution was spread on the surface of each agar medium. A mixture from both cultures (1:1) was made and treated the same way as the individual cultures. For growth on DMA medium containing tellurite, the same experimental proceedure was followed except that 1% filter-sterilized potassium tellurite was used instead of sodium selenite. All plates were incubated anaerobically in GasPak anaerobic jars (10% CO_2, 90% N_2) at 37°C for 48 hr. Emerging colonies were observed for the reduction and accumulation of elemental tellerium or selenium.

Results

Response of the organisms to selenite in broth medium

Initial growth by L. bulgaricus was retarded at all selenite concentrations tested in MRS broth. Some stimulatory effects were more evident (15 to 25% increased OD over controls) by 24 hr incubation at selenite concentrations of 160 & 320 µg/L (Figure 5.1). A prolonged lag by S. thermophilus in the control experiment apparently was due to an enzymic induction period for utilization of sucrose as a sole carbon source in the ST broth. Streptococcus thermophilus growth inhibition was evident at 160 & 320 µg/L selenite (Figure 5.2). Acid production for the two organisms showed a typical reciprocal relationship with respect to the growth curves (Table 5.1).

Response of the organisms to potassium tellurite in broth medium:

Gennerally, all potassium tellurite concentrations retarded the growth of both organisms, though L. bulgaricus exhibited an increased tolerance to potassium tellurite in broth media (Figure 5.3). Above 80 µg/L, potassium tellurite was found very inhibitory to the

growth of S. thermophilus in broth media (Table 5.2).

Growth on differential milk agar medium containing selenite and tellurite

Lactobacilus bulgaricus pure cultures developed large red colonies surrounded by casein hydrolysis zones at all chosen selenite concentrations (80, 160 and 320 µg/L) on the DMA plates. Also, plates inoculated with a mixture of the two organisms developed similar colonies at the same selenite concentrations. Microscopic examination, however, showed only L. bulgaricus colonies at 160 and 320 µg/L selenite concentrations. Streptococcus thermophilus pure cultures completely failed to grow at these selenite concentrations (Figure 5.4, top - upper).

Under anaerobic incubation at 37°C for 48 hours, 100 µg/L potassium tellurite was reduced and tellerium was accummulated by S. thermophilus colonies (microscopic confirmation). Lactobacillus bulgaricus failed to grow under these conditions (Figure 5.4, bottom)

Potassium tellurite was reduced by both organisms at the level of 10-20 µg/L. Pale black colonies developed for both organisms as indicated by the metallic precipitations. From the mixture of the two organisms, S.

thermophilus differentially developed depressed colonies while L. bulgaricus had large moist raised colonies

Table 5.1 The pH of Lactobacillus bulgaricus (LB) and Streptococcus thermophilus (ST)cultures grown in media that contained different concentrations of sodium selenite.

Time in hours	LB[a] Sodium Selenite concentration							ST[b] Sodium Selenite concentration						
	0	10	20	40	80	160	320	0	10	20	40	80	160	320
0	6.45	6.36	6.31	6.40	6.40	6.42	6.47	6.84	6.86	6.87	6.88	6.89	6.95	7.01
2	5.30	5.46	5.37	5.66	5.96	6.01	6.13	6.75	6.75	6.79	6.80	6.83	6.88	6.96
4	4.70	4.84	5.05	5.33	5.74	5.75	5.95	6.48	6.58	6..67	6.71	6.78	6.86	6.95
6	4.37	4.48	4.98	5.15	5.64	5.61	5.83	6.09	6.42	6.59	6.67	6.77	6.84	6.93
8	4.18	4.24	4.89	4.99	5.53	5.48	5.69	5.32	6.26	6.52	6.66	6.76	6.84	6.94
10	4.07	4.11	4.81	4.82	5.32	5.27	5.42	4.79	6.04	6.41	6.62	6.76	6.86	6.94
12	3.99	4.02	4.70	4.62	5.10	5.15	5.10	4.57	6.04	6.41	6.62	6.76	6.86	6.94
24	3.86	3.88	4.17	4.03	4.12	4.27	4.17	4.44	4.31	4.42	4.70	5.83	6.88	6.98

[a] LB was grown in MRS broth supplemented with different concentrations of sodium selenite.

[b] ST was grown in ST broth (4) supplemented with different concentrations of sodium selenite.

Figure 5.1 Effect of different selenite concentrations on the growth of L. bulgaricus (strain R-5) in MRS broth medium. Selenite concentrations of 40, 80, 160 and 320 µg/L caused stimulatory effect at 24 hours of incubation. The initial growth of the organism was retarded at all selenite concentrations tested.

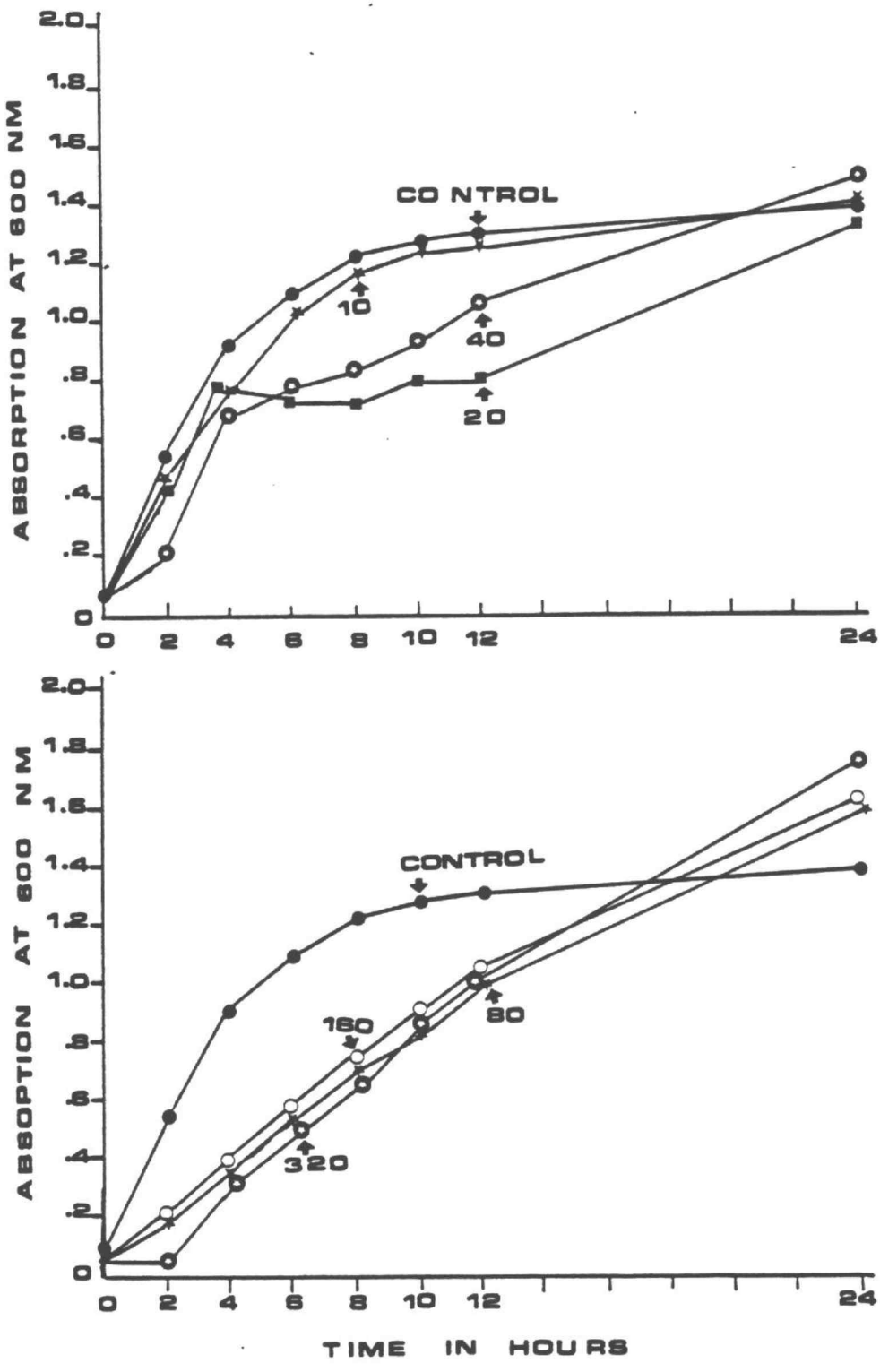
ABSORPTION AT 600 NM
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
CO NTROL
10
40
20
0 2 4 6 8 10 12 24
ABSOPTION AT 600 NM
CONTROL
160
80
320
TIME IN HOURS

Figure 5.1

Figure 5.2 Effect of different selenite concentrations on the growth of S. thermophilus (strain C-5) in ST broth medium. Low selenite concentrations (10 to 20 µg/L) showed slight stimulatory effects relative to control. Higher selenite concentrations above 160 µg/L were very toxic to the organism.

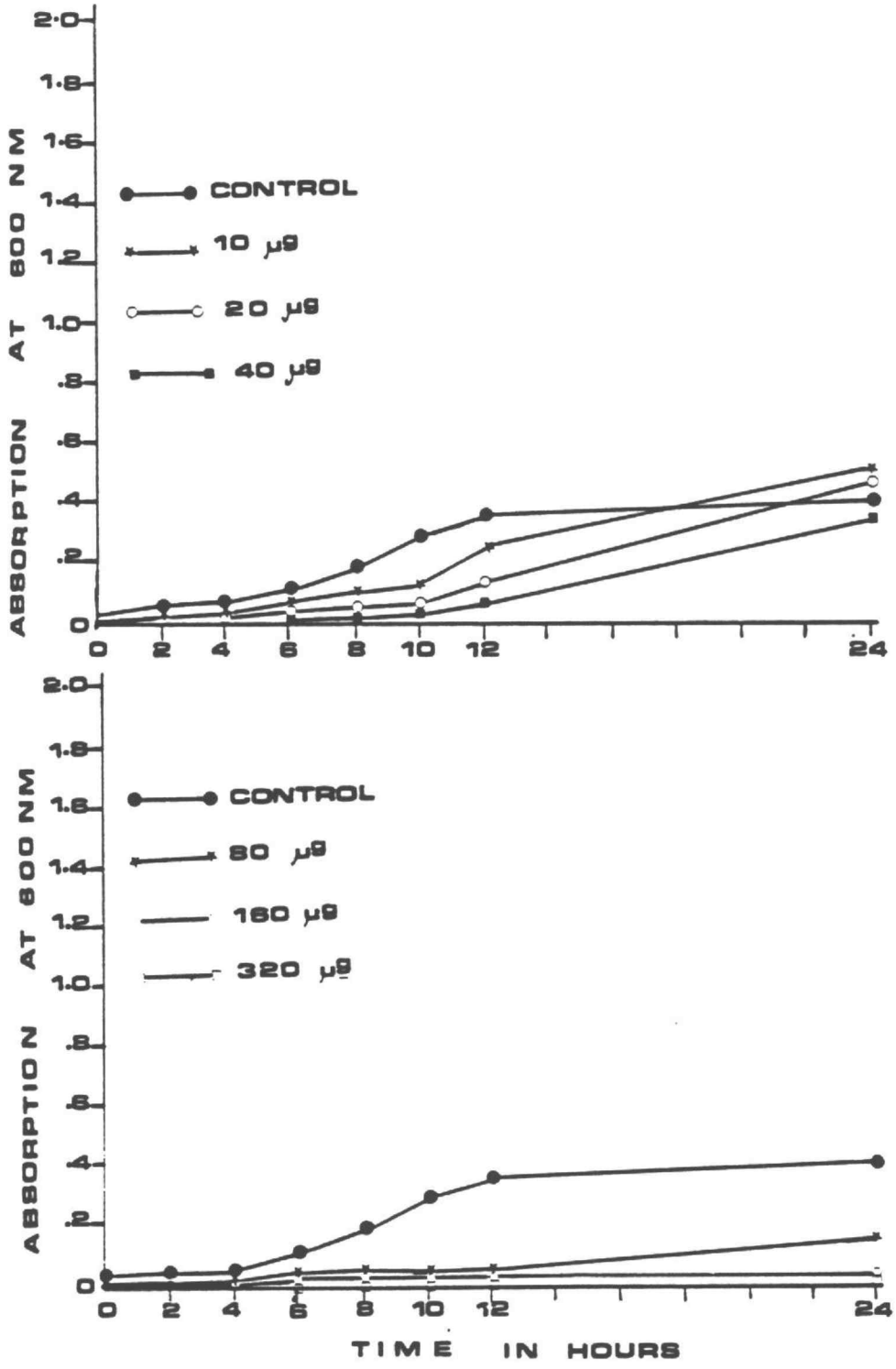
ABSORPTION AT 800 NM
2·0
1·8
1·6
1·4
1·2
1·0
·8
·6
·4
·2
0
CONTROL
10 µg
20 µg
40 µg
0 2 4 6 8 10 12 24
ABSORPTION AT 800 NM
CONTROL
80 µg
160 µg
320 µg
TIME IN HOURS

Figure 5.2

Figure 5.3 Effect of potassium tellurite concentrations on the growth of S. thermophilus (ST) and L. bulgaricus in broth media. At high tellurite concentrations (above 80 µg/L), the growth of S. thermophilus was completely inhibited. The growth of L. bulgaricus was retarded by all tellurite concentrations tested but never completely inhibited.

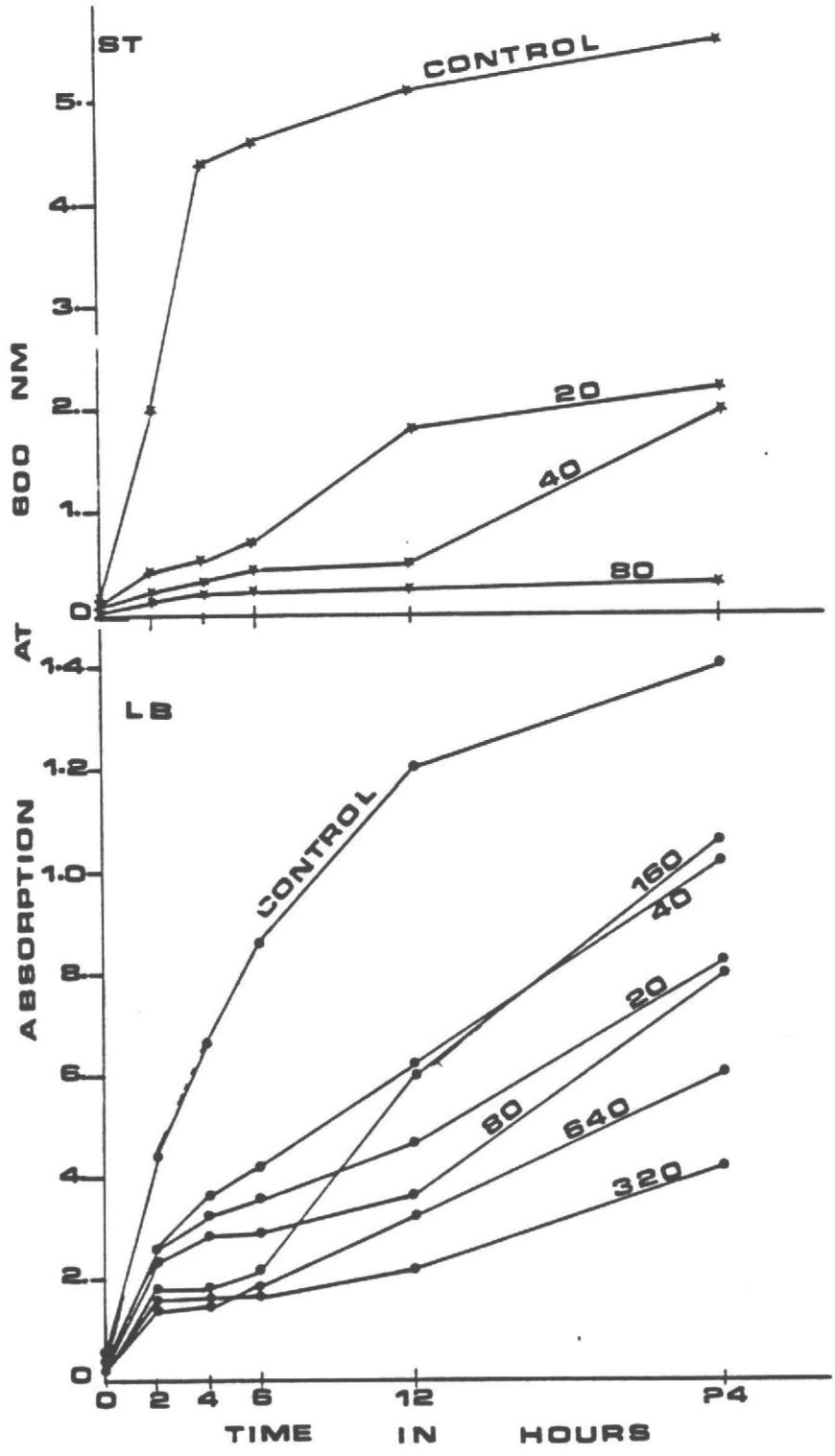
ST
CONTROL
5
4
3
2
1
0
800 NM
20
40
80
AT
LB
1.4
1.2
1.0
8
6
4
2
0
ABSORPTION
CONTROL
160
40
20
80
640
320
0 2 4 6 12 P4
TIME IN HOURS

Figure 5.3

Figure 5.4 Top photograph - upper picture: the selective growth of L. bulgaricus on DMA plates with high selenite concentrations. A mixture of L. bulgariucs and S. thermophilus (1:1) was grown on DMA plates containing 320 µg/L (upper right) and 160 g/L (upper left) of sodium selenite. 25-30 colonies, when gram stained, showed the growth of only rod shaped cells. Casein hydrolysis was evident by the halos around the red colones.

Lower picture: a similar mixture of the two organisms spread on DMA plates contianing 10 g/L of potassium tellurite. Lactobacilus bulgaricus developed moist raised colonies (pale) in contrast to the S. thermophilus depressed and flat colonies (dark).

Bottom photograph: the selective growth of S. thermophilus on DMA plates containing 100 µg/L potassium tellurite. Lactobacillus bulgaricus was unable to grow at this tellurite concentration.

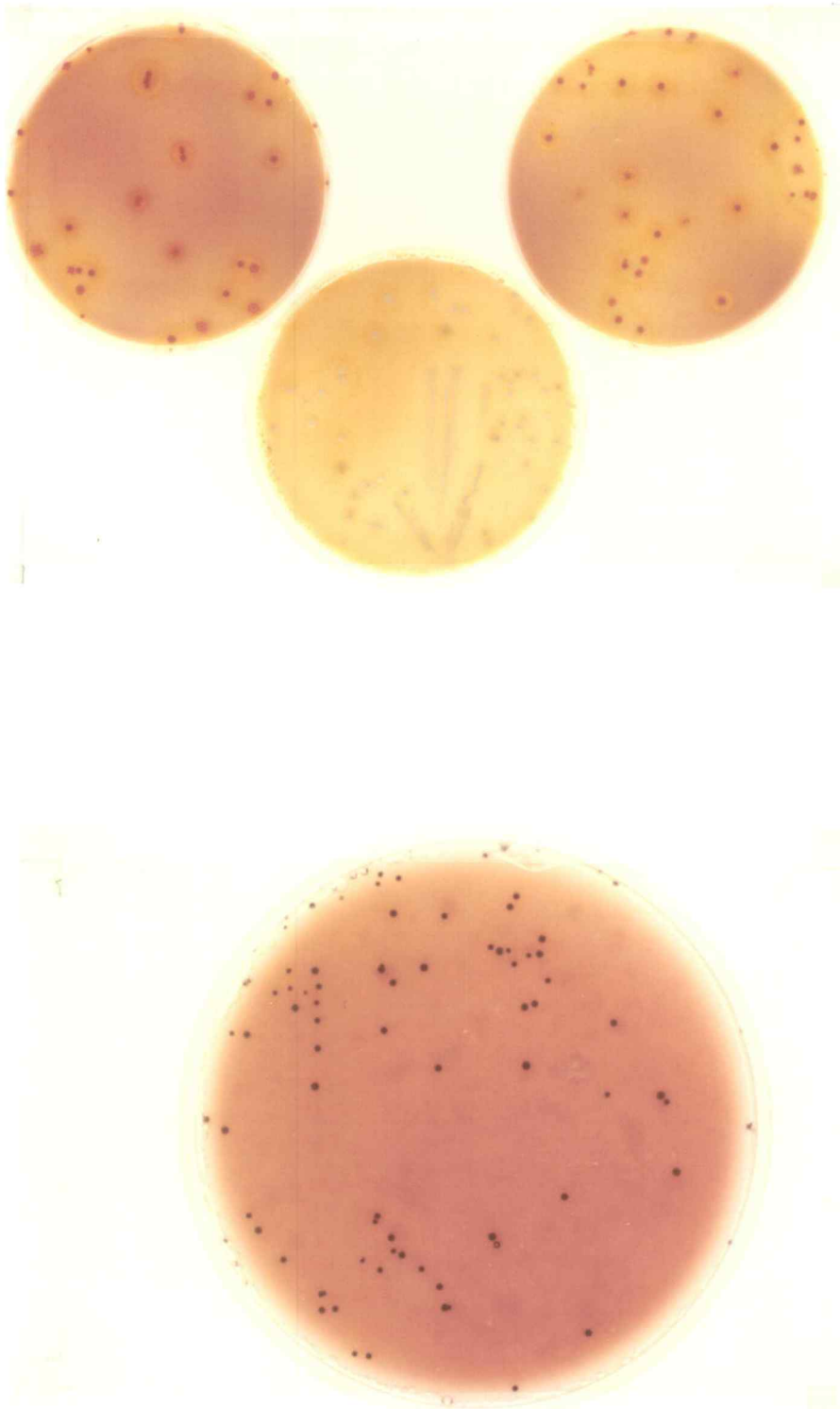

Figure 5.4

Table 5.2 The pH of Lactobacillus bulgaricus (LB) and Streptococcus thermophilus (ST) cultures grown in media that contained different concentrations of potassium tellurite.

Time in in hours	LB[a] Potassium tellurite concentration							ST[b] Potassium tellurite concentration						
	0	20	40	80	160	320	640	0	20	40	80	160	320	640
0	6.47	6.49	6.51	6.56	6.58	6.66	6.75	6.41	6.40	6.42	6.47	6.54	6.66	6.87
2	5.46	5.80	5.84	5.91	6.13	6.32	6.50	5.03	6.19	6.30	6.40	6.49	6.63	6.83
4	4.86	5.42	5.42	5.62	6.02	6.32	6.49	4.30	5.99	6.24	6.39	6.49	6.61	6.82
6	4.46	5.30	5.29	5.53	5.92	6.31	6.48	4.18	5.79	6.19	6.38	6.48	6.61	6.82
12	3.90	5.20	5.14	5.38	5.24	6.31	6.49	4.13	5.03	6.10	6.40	6.52	6.64	6.85
24	3.64	4.68	4.64	4.79	4.53	5.98	6.48	4.05	4.75	4.88	6.38	6.47	6.59	6.77

[a] LB was grown in MRS broth supplemented with different concentrations of potassium tellurite.

[b] ST was grown in M_{17} broth supplemented with different concentrations of potassium tellurite.

(Figure 5.4, top - lower). The same colonial features were also seen with the individual pure cultures.

Discussion

The two microorganisms exhibited different abilities to accumulate and survive relatively high selenite concentrations as was evident from the growth curves and DMA plates (Figure 5.4).

Streptococcus thermophilus growth was dramatically inhibited by higher selenite concentrations (160 and 320 g/L). In contrast, the same selenite concentrations exerted a noticeable stimulatory effecct on the growth of L. bulgaricus relative to the control. Selenite tolerance variability among microorganisms was observed by Levine (11) some time ago. Later, Leifson (10) applied these observations to formulate a commercial medium for the isolation of Salmonella species. The mechanism of selenite toxicity in S. thermophilus as well as the stimulatory effects on L. bulgaricus, could be attributed to different functioning systems. Microorganisms are known to metabolize and transform selenium compounds into either available micronutrients or highly toxic compounds. Hydrogen selenide (H_2SE), resulting from selenium transformation by soil microorganisms' (3), has been considered the most toxic form of the element (13).

Recently, Eustice (6) found that selenomethionine, as a competitive substrate for amino acid activation, caused a reduced rate of protein synthesis. This, he suggested, was the mechanism for selenium toxicity. Whatever the mechanism, the selective elimination of S. thermophilus at relatively high concentrations served as a basis for selective isolation and enumeration of L. bulgaricus. The same bacterial counts observed with L. bulgaricus pure cultures were obtained for mixed cultures (5×10^5 cells/ml). Thus the selectivity had no depressing effect on L. bulgaricus.

Conversely, the selective inhibiton of L. bulgaricus by potassium tellurite (100 µg/L), provided a selective proceedure in the isolation and enumeration of S. thermophilus. Furthermore at lower tellurite concentrations (10-20 µg/L), each organism exhibited specific colonial characteristics, even though the compound was reduced by both organisms to elemental tellurium as indicated by pale black precipitation in colonies. Tucker et al. (23) using x-ray diffraction analysis, reported on the complete reduction of potassium tellurite to metallic tellurium by Streptococcus faecalis and Corynebacterium diphtheriae. Cooper and Ramadan (1) using a heat treatment followed by incubation in a medium

containing tellurite, were able to differentiate between faecal streptococci from different sources. Unheated S. faecalis, but not Streptococcus faecium, was found to resist 0.04% potassium tellurite. Tellurite reductase activity of S. faecalis was viewed as a detoxification mechanism (22). Payne et al. (7, 14, 15) probed the tellurite resistant function of S. faecalis in studying the mechanism of heat injury.

Development of a selective differential medium distinctly showing black and red colonies on the same plate for the enumeration of rods and cocci, seemed like an attainable objective based on the differential ability of the two organisms to reduce and accumulate tellurite and selenite.

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