HYDROLYSIS AND UTILIZATION OF LACTOSE IN WHEY
BY SELECTED MICROORGANISMS FOR POTENTIAL
USE IN BREADMAKING

by

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INTRODUCTION

Whey, a by-product of the cheese industry, constitutes approximately 89 per cent of the weight of milk used in cheese production. The chemical composition of fluid whey is about 93 per cent water and seven per cent solids, of which approximately five per cent is lactose, one per cent is protein and one per cent is mineral (1, 2).

On a world basis, about 3.5 million tons of whey solids are produced annually; in the United States, about 875 thousand tons (1). A little over one half of that produced in the United States is utilized in the production of lactose, as a medium base for fermentation, as dried whey for food and feed, and in numerous non-food uses. The remainder creates an enormous disposal problem for the dairy industry due to a BOD (biological oxygen demand) of twelve to eighteen times that of normal sewage. Thus, plants and animals in whey-adulterated waters can be killed by a lack of oxygen. As a result, many municipalities assess a gallonage tax on all whey entering public sewers. Other agencies have prohibited further dumping of whey into natural waters.

With the per capita consumption of cheese increasing annually (1), more whey will be produced in the years ahead. This increase in supply, the growing emphasis on pollution control, and the high cost of suitable methods of whey disposal seem to indicate that new uses for whey or whey products, with reduced disposal costs and pollution, would be welcomed by the dairy industry and the public as well.

The baking industry normally adds malt (0.6%) and sugar (4-6%) to dough formulations to provide a fermentable carbohydrate required by Saccharomyces cerevisiae (baker's yeast) during the proof period. Nonfat dry milk (NDM) is also used in breadmaking due to its favorable effects on the final product, namely improved nutritional value, improved crumb tenderness and texture, enhanced flavor, improved crumb and crust color, high buffering capacity, and increased water absorption and retention (3). Whey products could contribute some of these favorable effects. Besides containing nutritionally good protein and being more economical, whey products contain large amounts of lactose. Since S. cerevisiae will not ferment lactose, the lactose present could serve as a source of fermentable sugar for the yeast if it could be hydrolyzed into glucose and galactose. The glucose could then be utilized by the yeast for fermentation. Not only would the loaf depressing lactose concentration be reduced, but also new uses for whey proteins would be established as replacements for malt, sugar, and the costly NDM.

The objectives of this research were (a) to use selected lactic acid microorganisms to either hydrolyze lactose in whey and whey products to glucose, galactose or lactic acid, or to utilize the lactose and thus reduce its level, and (b) to utilize this altered whey product as an energy source for Saccharomyces cerevisiae. The altered whey product was analyzed for lactose, glucose, and galactose content; proteolytic activity; and the amount of acid produced.

LITERATURE REVIEW

As the world's population increases, a lack of dietary protein may become highly critical in densely populated areas. Even today there is a protein shortage in some areas of the world. Cereal products form a large portion of the caloric intake among the people of these areas and are therefore a prime target for protein enrichment, especially so because of the limited quantity of certain essential amino acids in cereal grains (4).

Whey provides one of the highest known protein efficiency ratios (PER) among commonly available protein materials, as well as being a rich source of lactose (1). The lactalbumin fraction of whey protein is exceptionally high in lysine and methionine, thus enhancing its utility as a supplement to cereal proteins.

The baking industry has long been a major domestic buyer of nonfat dry milk (NDM). In 1969, Mertens (3) published the results of a survey of bakers which gave the following as advantages of using dairy products in bread: nutritional value, improved crumb tenderness and texture, improved crumb and crust color, enhanced flavor, high buffering capacity, and increased water absorption and retention. On a flour weight basis, 3 per cent NDM is commonly used in the straight and sponge-dough methods, although it is possible to use 4-6 per cent and still get an acceptable loaf of bread. In the continuous mix system, 1-2 per cent NDM is used, and acceptable bread can be produced using 3-4 per cent. Kirk (5), in

a review of milk products used by bakers, found the main reason for using the lesser amounts of NDM was the high cost.

Whey Proteins in Baking

Numerous studies have been made on the factors that influence the baking quality of milk solids. The necessity of heat treatment was first discovered by the pioneer work of Greenbank et al. (6) in 1927. Skimmilk was scalded before being used in bread and compared to low heat-treated milk. High heat treatment imparted a greater water holding capacity to the dough, thus giving increased loaf volume and better grain and texture. Grewe and Holme (7) heat-treated skimmilk, before drying, at temperatures ranging from 50-100°C for 30 minutes. Baking experiments showed improved loaf volume as the heat treatment was increased. The cause of low loaf volume, produced by low heat-treatment, was given the name "loaf depressant factor".

Through the use of farinograph studies, Stamberg and Bailey (8) showed that the loaf depressant factor was present in the serum or whey protein fraction. They also found that there were sufficient sulfhydryl (-SH) groups present in raw milk to cause dough slackening, and suggested that heat treatment oxidized the -SH groups to more stable S-S bonds, thereby reducing loaf depressant action. In 1943, Harland et al. (9) studied the effect of different milk fractions on loaf volume and found that the non-dialyzable fraction of acid and rennet wheys contained a loaf depressant factor, and that their baking properties could be improved by heating. However, the depressant factor in casein could not be overcome by heating.

Both Larsen et al. (10) and Morton and Coppock (11) studied the three
major components of NDM (casein, lactose, and milk serum proteins) and
demonstrated that all unheated fractions depressed loaf volume. Heat
treatments improved the baking quality of the milk serum proteins only.
Larsen et al. (12) demonstrated that there is a decrease in titratable
-SH groups with increased heating. Since Coulter et al. (13) demonstrated
that sulfhydryl-containing compounds such as glutathione and cysteine
slacken dough and depress loaf volume, it was believed that this decrease
in -SH groups explained the improved baking quality of dry milk solids.
However, in 1952, Larson et al. (14) showed that β-lactoglobulin, which
accounts for almost all the -SH groups in milk, was not detrimental to loaf
volume or dough consistency. Gordon et al. (15) tested several fractions
of serum protein and found that neither β-lactoglobulin nor insoluble
protein exhibited loaf volume depression. The lactalbumin fraction did
not contain high concentrations of the loaf depressant factor either.
They suggested it might be present in the lactoglobulin fraction of the
serum protein.

In later work, Jenness (16) showed evidence that a "component 5",
present in both acid and rennet whey, exerts pronounced depression of
loaf volume.

Work by McGugan et al. (17), Trautman and Swanson (18), Zittle et al.
(19), Sawyer et al. (20), and Swanson et al. (21) has established an inter-
action between the κ-casein, β-lactoglobulin, and a component of α-casein.
This interaction between casein and whey protein, on heating, appears to
form a new protein complex which is responsible for improving the baking
properties of NDM, whey, and whey products.

Use of hydrogen peroxide-oxidized NDM samples at the six per cent level in continuous mix and sponge dough by Patel et al. (22), Kopp (23), and Guy et al. (24), again indicates that -SH groups may be involved in loaf volume depression. Grinrod and Nickerson (25) studied the changes produced in milk proteins upon treatment with hydrogen peroxide. They found that migration rates of individual proteins on polyacrylamide gel electrophoresis were reduced for α_s-casein and β-lactoglobulin but were increased for β-casein and bovine serum albumen. Fish and Mickelsen (26), in a study to determine which whey proteins were modified by hydrogen peroxide, suggested it may modify β-lactoglobulin so that it does not form the κ-casein complex mentioned previously.

Lactose in Baking

Swanson and Sanderson (27) report that the major whey components (α-lactalbumin, β-lactoglobulin, blood serum albumin, and lactose) seemed not to be detrimental to loaf volume or bread quality. This contrasts with the work of Larsen et al. (10) and Morton and Coppock (11) that was cited previously. These workers found that lactose depressed loaf volume more than any other whey component after heating. Proper heat treatment and the use of bromate as an oxidizing agent overcame the depressant effect of the serum proteins. Casein depressed loaf volume somewhat but lactose depressed it the most. In 1971, Guy et al. (28) demonstrated that lactose does definitely depress loaf volume as well as carbon dioxide production. They suggest that lactose, as well as extra sucrose, inhibits carbon dioxide production due to osmotic effects of the sugar on yeast. They also suggest that lowered lactose contents in whey products should be beneficial in overcoming volume depressant effects of

high levels of whey products in baking. Pomeranz et al. (29) found that an acceptable loaf of bread could be obtained using lactose and lactase without adding additional sugars. However, this process is probably uneconomical due to the slow hydrolysis of lactose by lactase to produce glucose for yeast fermentation, thus requiring increased proof times to obtain a good loaf of bread.

At the proper concentrations, lactose serves several functions in baking. Since it is not fermented by baker's yeast, it is available during baking to react with free amino groups of proteins or amino acids to form the golden brown crust of bread (30). This reaction, known as the Maillard reaction, combines the free aldehyde group of sugar and amino groups of proteins to form melanoidens (31). Lactose also produces a marked tenderizing effect on crumb structure and improves bread flavor (4).

Nutritional Aspect of Whey and Whey Products

Approximately 54 per cent of the nutrients from whole milk are found in the sweet fluid whey of cheddar cheese while about 73 per cent of the nutrients in NDM are found in cottage cheese whey (32). Guy (33) of the United States Department of Agriculture and the Foremost (34) and Borden (35) Companies give some typical compositions of whey products as shown in Table 1. The protein content ranges from 12 per cent for cottage cheese whey to 35 per cent for Foretein (Foremost) and 55-61 per cent for Protolac (Borden), the latter two being commercial whey protein-concentrate products. The lactose content of these products is 66.4%, 50-60%, and 18-22%, respectively. The increased protein and lowered lactose concentrations of the whey protein-concentrate products make them ideal for partial or complete

replacement of NDM in baking. The lowered lactose content could help

alleviate the osmotic effects of this carbohydrate mentioned earlier.

Table 1. Typical analysis of some whey products.

Product	% Lactose	% Protein	% Ash	% H_2O
Cottage Cheese Whey	66.4	12.0	8.6	3.5
Sweet Whey	73.0	12.5	8.4	2.7
Foretein[a]	50-60	35.0	3.0	5.0
Protolac[b]	18-22	55-61	7-18	3-5

a. a product of Foremost
b. a product of Borden

Complete proteins supply all the essential amino acids in sufficient

quantity so that none is a limiting factor in the utilization of the whole

protein. Lactalbumen, which comprises about 77 per cent of whey proteins

(36), is a complete protein and has long been recognized as highly superior

to most other proteins in animal nutrition (37, 38, 39). Although whey

does not have as much total protein as NDM, whey proteins have greater

nutritional value than equal amounts of casein, as well as being richer in

lysine and tryptophan, the first and second limiting amino acids of wheat

flour (40). Thus whey proteins can increase the nutritional value of wheat

protein, more than does casein, by improving the amino acid balance.

Winegerd et al. (41) in a study of the nutritional value of whey protein

concentrates showed that the protein and minerals of the soluble whey

protein concentrate are completely available to both animals and humans.

Nutritionally, lactose is unique. Like other carbohydrates it can serve as an energy source. It is, however, hard to hydrolyze and absorb. Therefore it is not fully utilized for energy purposes as are most other carbohydrates. Atkinson et al. (42) and Boutwell (43) have listed some of the valuable effects of lactose as pertains to nutrition. They found that lactose favors an acid type fermentation which helps in the absorption, retention, and utilization of calcium, phosphorous, and magnesium. This fermentation aids in digestion and also discourages the growth of putre-factive organisms in infants. Thus lactose is used in infant formulas. Lactose supplies galactose, a structural sugar necessary for the synthesis of cerebrosides in the brain and medullary sheaths of the nerves. Lactose is used in weight reduction diets, due to its inefficient utilization, and as a sugar for diabetics.

Summary

Dried dairy products are used in the baking industry because of their improving effects on nutrition, crumb and crust color, tenderness, flavor, moisture absorption and retention, and buffering capacity. These improving qualities are due to protein and lactose present in these products. The levels of these two ingredients determine the effects they have on the baked product.

NDM has traditionally been the major dairy product used by the baking industry. However, the high cost of NDM and new developments in whey protein concentrates have started a shift of importance toward these whey products.

Whey protein does contain a loaf depressant factor. However, this factor can be overcome by proper heat treatment and the use of dough oxidants. Lactose also exhibits loaf volume depression, due to osmotic effects of the sugar. Thus it appears that whey and whey products could be used as an economical replacement for NDM if the lactose content can be lowered enough so as not to cause loaf volume depression.

EXPERIMENTAL PROCEDURES

Selection of Microorganisms

Food acceptable lactose-fermenting bacteria were selected as a means of hydrolyzing the lactose in whey. Streptococcus lactis, which has been shown to have various metabolic pathways for hydrolyzing lactose, was selected (44). Lactobacillus casei and Lactobacillus delbrueckii, which have been reported advantageous in fermenting lactose (45), also were selected. Lactobacillus bulgaricus has a stimulatory effect on other lactose-utilizing bacteria in mixed culture fermentation, as well as hydrolyzing lactose itself (2, 46), and it, therefore, was selected. Lactobacillus acidopholus, which uses lactose as a food source (47), also was selected. Stock cultures were carried on slants of Elliker's broth with 1.5% agar added (48). The sources of the five cultures used were:

L. acidopholus	KSU
L. bulgaricus	KSU
L. casei	KSU
L. delbrueckii	NRRL B-445
S. lactis	KSU

Those designated KSU were from the collection of the Department of Dairy and Poultry Science, Kansas State University. The culture with an NRRL number was from the Northern Utilization Research and Development Division of the United States Department of Agriculture (USDA), Peoria, Ill. 61604. All cultures were maintained in pure culture.

The <u>Saccharomyces</u> <u>cerevisiae</u> stock culture was carried on Sabouraud dextrose agar (48) and was obtained by plating a sample of a Red Star yeast cake on Sabouraud dextrose agar and removing a pure yeast colony for the stock culture.

Whey Sources and Preparation

Three samples of whey products were obtained. The first was a sweet spray-dried whey obtained from Milk Specialties Inc., Dundee, Illinois. The second and third samples were two partially delactosed and demineralized whey products (Protolac); calcium whey protein concentrate (Ca-WPC) and sodium whey protein concentrate (Na-WPC). They were obtained from Borden, Inc., New Ulm, Minnesota.

One-liter solutions of 6, 9, and 12 per cent concentrations were prepared from each of the whey products. "Cool sterilization" was used to sterilize the wheys and to protect the whey proteins from heat damage (49). This procedure, as reported by Bechtle (50), involves addition of one per cent of hydrogen peroxide (30 ml of 30-35 per cent stabilized hydrogen peroxide per liter) followed by heating at $65^{o}C$ for one hour. The solutions were cooled to $20^{o}C$ or lower and 50 Keil units of beef catalase L (Marschall Division of Miles Laboratories, Inc., Elkhart, Indiana) were added to destroy residual hydrogen peroxide. This amount of catalase is five times the amount theoretically required as suggested by Roundy (51). This excess is required to destroy any hydrogen peroxide that may be produced by Lactobaccilli (52) that could inhibit bacterial growth. Very low concentrations of hydrogen peroxide have been found to be highly toxic to yeast (50). After two hours the Roundy test (51) for

residual hydrogen peroxide was carried out. Negative results were obtained in all tests.

<center>Incubation, Growth, Inoculation, and Sampling</center>

Procedure for each organism. Preliminary trial experiments were performed to determine which bacterial culture or combination of cultures produced the greatest amount of lactose hydrolysis. Six sterile test tubes containing 10 ml each of sterilized Elliker's broth were inoculated, using a standard bacterial inoculation loop, with L. acidopholus. The six tubes were then incubated for 18 hours at 32^{0}C. After 18 hours, the contents of each tube were aseptically transferred to 500-ml Erlenmeyer flasks, each containing an additional 100 ml of sterile broth. The flasks were connected as shown in Fig. 1, and placed in a 32^{0}C water bath. Cotton plugs placed in the end of each glass tube which carried air into each flask were used to prevent contamination between flasks and to filter incoming air which was bubbled through the system by using a water aspirator at 650 ml/minute. The entire apparatus was sterilized before each use at 248^{0}F with 15 pounds of pressure for 15 minutes using an American Sterilizer Company autoclave (Erie, Penn.). After 24 hours, an additional 140 ml of sterile Elliker's broth was aseptically added to the system and growth continued for an additional 24-hour period. The bacterial solutions were then transferred to sterile 250-ml screw-cap centrifuge bottles and centrifuged for 20 minutes at 1,500 xG (International centrifuge, size 2, model V). The supernatant solution was decanted, the cell packs washed from the centrifuge bottles into 40-ml sterile capped centrifuge tubes with 10 ml of sterile 0.5 per cent physiological saline solution, and then centrifuged at 23,500 xG for 20 minutes (Sorvall SS-3 automatic super-

Fig. 1.   Bacterial incubation and growth apparatus.
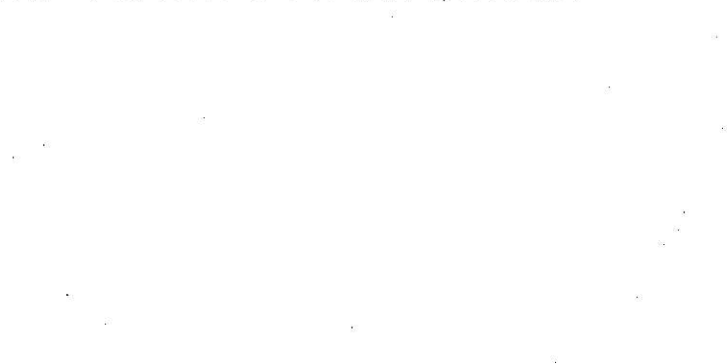
WATER VACUUM
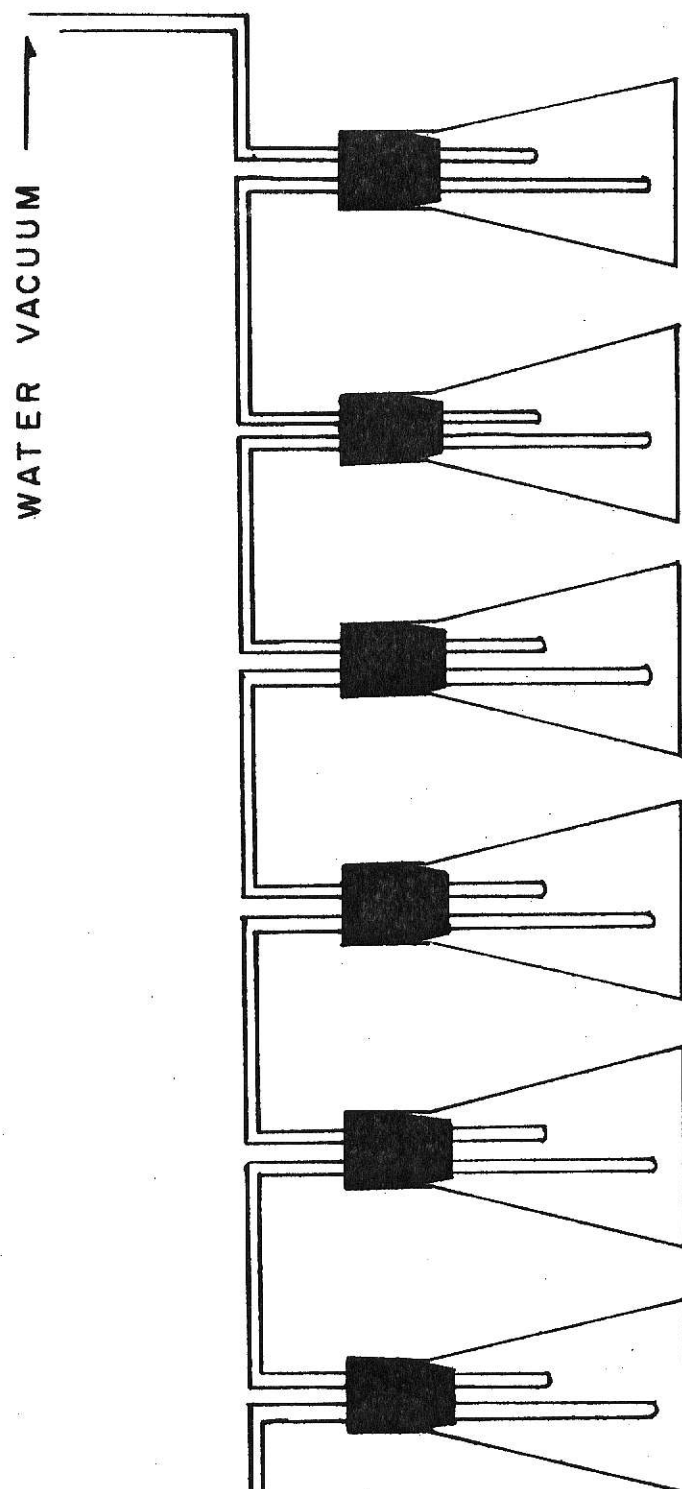
speed centrifuge). The supernatant solution was then decanted leaving approximately 0.75 cc of cells in each tube. Triplicate 25-ml portions of 6 or 12 per cent Ca-WPC were used to wash the cells from each tube into sterile 6-ounce prescription bottles which were then incubated at 32^0C. Samples (5 ml) were removed after 4 and 8 hours of incubation. The pH of all samples was determined immediately upon collection, after which 14 ml of 95 per cent ethanol (gives a 70 per cent solution) were added to each sample to kill the bacteria and precipitate the protein. The samples were then centrifuged at 23,500 xG for 20 minutes to remove the precipitated protein. The supernatant solutions were decanted for analysis.

This same procedure was used for the remaining four bacteria, L. bulgaricus, L. casei, L. delbrueckii, and S. lactis.

Procedure for combined organisms. Six sterile test tubes containing 10 ml each of sterile Elliker's broth were inoculated with all five bacteria. The same procedure previously described for bacterial growth, inoculation, and sampling was used. Each cell pack contained approximately 3.5 cc of cells.

Selecting the bacterial concentration. L. bulgaricus was selected to determine the optimum concentration of cells with which to inoculate each sample. The procedure previously described was used with the following alterations. Various amounts (0.38, 0.75, 3.0, and 7.5 cc of cells) of bacterial cells were inoculated into the Ca-WPC sample. Samples (5 ml) were removed after 4, 8, 12, and 24 hours for analysis.

Addition of buffer. Since the combined bacterial system lowered the pH (see Results and Discussion) below the isoelectric point of the Ca-WPC

causing precipitation and coagulation of the protein, a 0.6 M phosphate buffer (pH 6.5) was prepared for use in the combined bacterial growth. The procedure originally described was used with the following changes. After washing with the saline solution and centrifuging, the bacterial cell packs were each suspended in 5 ml of the buffer solution and added to the Ca-WPC, giving a 0.1 M concentration of buffer and a pH of 6.5. Twenty-five ml of 6, 9, and 12 per cent Ca-WPC were used. Five-ml aliquots were removed as before after 4, 8, 12, and 24 hours.

Addition of yeast. Saccharomyces cerevisiae was added to the system to determine if yeast could grow in the bacterial system. Two sterile test tubes containing 10 ml of Sabouraud's dextrose broth (made by leaving the agar out of Sabouraud's dextrose agar) (48) were inoculated with yeast. The same propagation system was used as before, with the addition of two more flasks to the system for each yeast culture. Each yeast culture produced approximately 1.5 cc of cells. Inoculation of the bacteria into the whey samples was the same except for using 4 ml of buffer instead of five. Each yeast cell pack was suspended in 3.2 ml of buffer, with 1 ml portions then being transferred to each triplicate whey sample. The final amount of buffer added was the same as before. The whey samples were incubated in 40-ml test tubes with inverted gas tubes to determine if the yeast cells were fermenting glucose. The test tubes were placed in a dessicator and the air removed from the gas tubes by means of a water vacuum. The whey samples then were incubated at $32^{o}C$ with 5-ml samples being removed after 4, 8, 12, and 24 hours.

Methods of Analysis

pH. The pH of each sample was determined immediately after collection, before addition of the alcohol to stop the reaction and precipitate the protein, using a Corning model 10 pH meter.

Determination of sugars. Paper chromatography was used for qualitative determination and identification of the sugars present in each sample. Standards of glucose, galactose, and lactose were used for identification. The solvent system was ethyl acetate: pyridine: water (10:4:3 v/v/v). Chromatograms were developed in a descending system for 24 hours. After removing and drying, carbohydrates were located on the chromatograms with an alkaline silver nitrate dip reagent (53).

Lactose determination. Lactose concentration in all samples was determined by the phenol-sulfuric acid method of Dubois et al. (54). One-ml aliquots of properly diluted samples were added to 1 ml of 5 per cent phenol in water. Five ml of concentrated sulfuric acid were rapidly added with a Mini-pet automatic pipette (VWR Scientific, Denver, Colorado). The tubes were thoroughly mixed, allowed to stand for 30 minutes at room temperature, and the absorbance of the solution was measured at 490 nm (Beckman model DU quartz spectrophotometer). A standard curve was prepared using 10-70 μg of reagent grade lactose in 70 per cent ethanol. Standards of each whey solution were analyzed for lactose contents before inoculation with bacteria and yeast. The per cent of lactose disappearance from the samples was determined with the standards being set at 100 per cent.

Proteolysis. The amount of proteolytic activity was determined by a modified Lowry procedure (55), in which sodium citrate was substituted for sodium tartrate in reagent B below, as difficulty was encountered when trying to solubilize the sodium tartrate.

The following reagents were used:

A. 2% Na_2CO_3 in 0.1 N NaOH.

B. 0.5% $CuSO_4 \cdot H_2O$ in 1% sodium citrate.

C. 1 ml of reagent B mixed with 50 ml of reagent A.

D. Folin-Ciocalteau reagent (obtained from Fisher Scientific Co., Fairlawn, N.J.) diluted with an equal volume of distilled water to give a 1.0 N solution.

One ml of properly diluted sample was mixed with 5 ml of reagent C and the solution allowed to stand for 10 minutes at room temperature. Then 0.5 ml of reagent D was rapidly added with thorough mixing. After standing for 30 minutes at room temperature, the absorbance was determined at 750 ηm. Standards of all whey solutions were analyzed to determine the amount of free amino groups present before bacterial activity. The results were compared as absorbance values.

RESULTS AND DISCUSSION

Analysis of Whey Products

The chemical composition of the three whey products used in this study was determined by proximate analysis. The results are given in Table 2. Lactose per cent was determined by difference since lactose was the only carbohydrate present as determined by paper chromatography.

Preliminary Investigations

Individual and combined bacteria. Since one of the objectives of this work was to utilize lactic acid organisms for hydrolysis of lactose, the selection of appropriate organisms was of paramount importance. Preliminary tests were performed to determine which organism(s) hydrolyzed the greatest amount of lactose in a given period of time. Figure 2 shows the results of analyses for lactose in six and twelve per cent Ca-WPC solutions after incubation for four and eight hours. Lactose concentration was measured by the phenol-sulfuric acid method (54) for total carbohydrate since paper chromatograms of all samples showed lactose to be the only carbohydrate present. The results are shown as per cent lactose disappearance during the incubation. The greatest percentage of lactose disappearance occurred in the combined bacterial samples after eight hours incubation using both the six and twelve per cent solutions. S. lactis appears to produce some type of carbohydrate during incubation since the per cent of carbohydrate present increases in both 6 and 12 per cent Ca-WPC solutions as the incubation time increases from

Table 2. Proximate analysis of whey products used.

Sample	% Protein	% Moisture	% Ash	% Fat	% Fiber	% Lactose
Spray-Dried Whey	13.5	4.9	8.3	0.2	0.4	72.7
Calcium Whey Protein Concentrate (Ca-WPC)	54.5	4.9	15.2	0.4	0.4	24.6
Sodium Whey Protein Concentrate (Na-WPC)	55.6	4.4	16.1	0.4	0.4	23.1

Fig. 2.   Lactose analysis of individual and combined bacteria in 6 and 12 per cent Ca-WPC. A=L. acidopholus, B=L. bulgaricus, C=L. casei, D=L. delbrueckii, S=S. lactis, ALL=combined bacteria, Org=organism, Time=hours of incubation. All values plotted are an average of triplicate analysis of three samples.
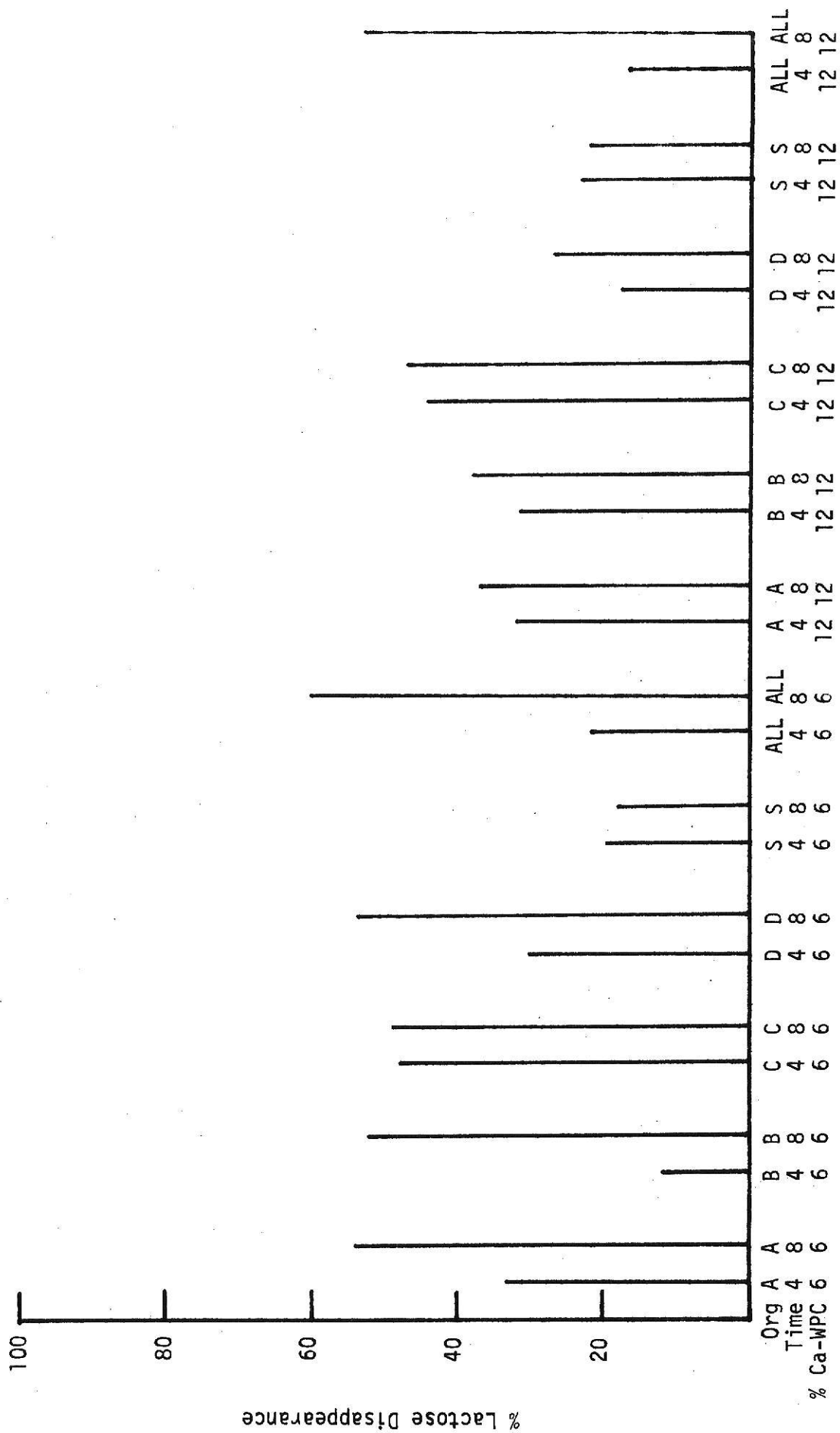
4 to 8 hours. However, samples spotted on paper chromatograms showed only one sugar present, which cochromatographed with lactose. This does not eliminate the possibility of some type of polysaccharride formation which is not detected on the paper chromatograms.

Bacterial concentration. Concentrations of L. bulgaricus, varied from 0.38 to 7.5 cc of cells, were added to 25 ml of six per cent Ca-WPC solutions. Increases in the per cent of lactose hydrolyzed were observed although the results were inconsistent as seen in Fig. 3. For a given incubation time, there was less than sixteen per cent variation in the lactose used at all bacterial concentrations. There seems to be no clear explanation for this apparent inconsistency in results obtained. A concentration of 0.75 cc of cell inoculum per 25 ml of whey product was chosen for use in the remainder of the investigation.

Effect of buffering. Measurement of the pH of mixtures containing the individual and combined organisms incubated for 4 and 8 hours is given in Table 3. All the samples incubated 8 hours with the combined bacterial cells showed increased viscosity or precipitation. The low pH values could slow or stop bacterial growth, thus lessening the amount of lactose hydrolyzed by the bacteria. From these data it is apparent that the whey product solutions have to be buffered.

Figure 4 shows the results obtained for the amount of lactose disappearance after incubation of buffered 6, 9, and 12 per cent Ca-WPC with the combined bacteria. Nine per cent Ca-WPC shows the greatest amount of lactose disappearance at each time interval except at 24 hours where all the lactose present was degraded at all three Ca-WPC concentrations.

Fig. 3.   Lactose analysis of variations in _L. bulgaricus_ concentration.
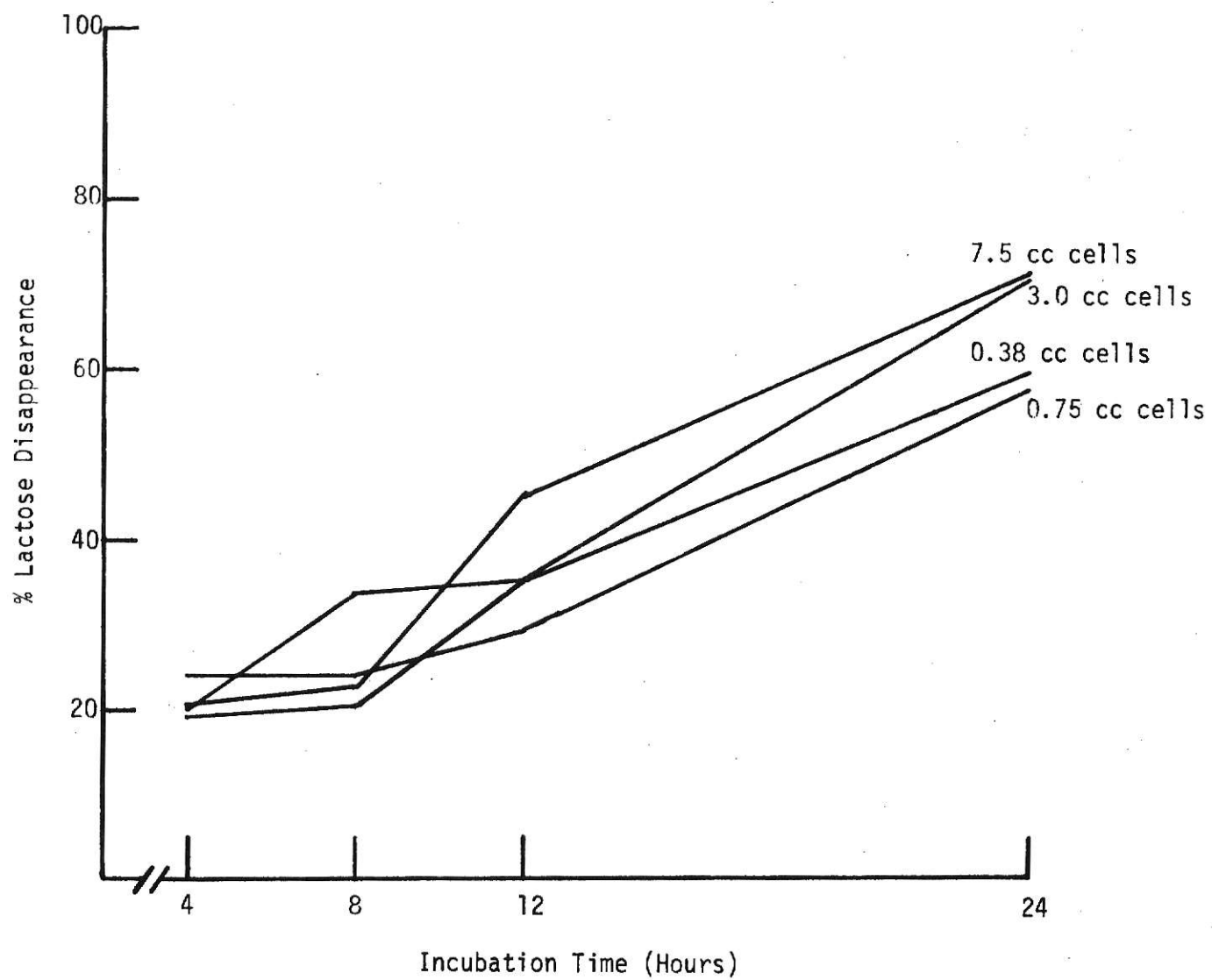
Table 3. pH of samples after incubation with individual and combined bacteria in 6 and 12 per cent Ca-WPC.

Bacteria	Whey Conc.	pH*	
		4 Hour Ferm.	8 Hour Ferm.
L. acidopholus	6%	6.0	5.8
	12%	6.0	5.7
L. bulgaricus	6%	6.1	5.9
	12%	6.4	6.3
L. casei	6%	6.5	6.3
	12%	6.4	6.1
L. delbrueckii	6%	6.5	6.1
	12%	6.5	6.2
S. lactis	6%	6.3	6.1
	12%	6.3	6.2
Combined bacteria	6%	5.6	4.5
	12%	6.0	4.5

*The pH of the Ca-WPC solution was 6.8 at the beginning of the incubation period. Values are an average of pH readings from triplicate samples.

Table 4. pH of Ca-WPC + combined bacteria + buffer.

Whey Conc.	Ferm. Time	pH*	Whey Conc.	Ferm. Time	pH*
6%	4 hrs	6.5	6%	12 hrs	6.5
9%	4	6.5	9%	12	6.5
12%	4	6.5	12%	12	6.5
6%	8	6.5	6%	24	5.8
9%	8	6.5	9%	24	5.6
12%	8	6.5	12%	24	5.5

*The pH of the Ca-WPC solution was 6.8 at the beginning of the incubation period. Values are an average of pH readings from triplicate samples.

Fig. 4.   Lactose analysis of combined bacteria + buffer in 6, 9, and
           12 per cent Ca-WPC. Time=hours of incubation. All values are
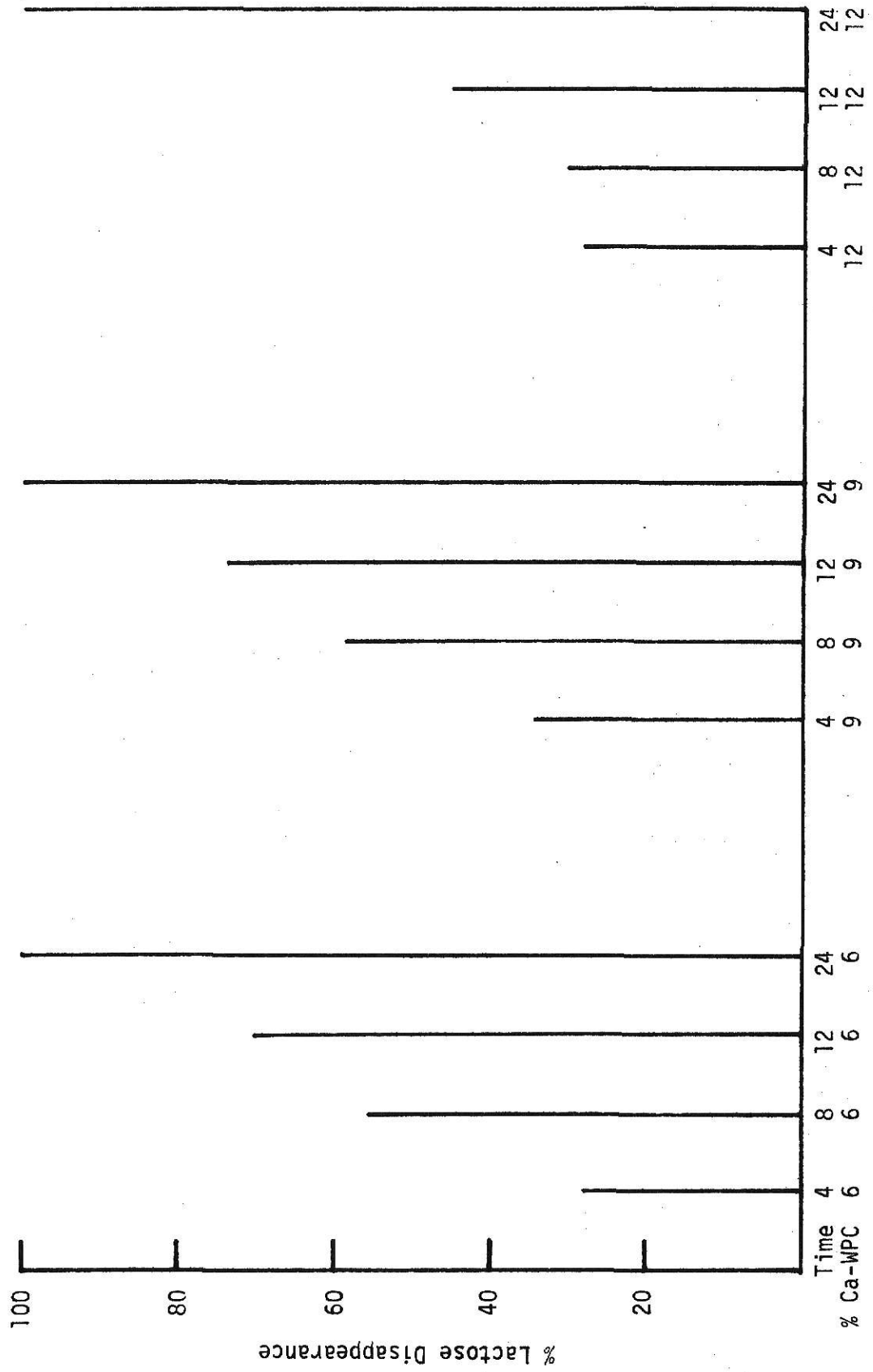   an average of triplicate analysis of three samples.

Table 4 shows the effect on the final pH of buffering the original Ca-WPC. The pH was 5.5 or above after 24 hours incubation as compared to a pH of 4.4-4.8 after 8 hours incubation without buffer. No precipitation occurred, and buffering the solutions thus kept the pH high enough that bacterial growth could continue for the entire 24 hours of incubation.

Analysis of proteolysis. Figures 5 and 6 show the results of analyses for the amount of proteolysis of the individual and combined bacterial samples in 6 and 12 per cent Ca-WPC, respectively. L. bulgaricus, L. casei, L. delbrueckii, S. lactis, and the combined bacteria sample gave decreasing absorbances after 4 and 8 hours of incubation as compared to the standard, indicating the bacteria utilized the free amino groups of the protein as a nitrogen source. L. acidopholus, however, showed a decrease in absorbance values after 4 hours but increased to a higher value after 8 hours, exceeding even the standard in the 6 per cent Ca-WPC sample. This may be due to an induced enzyme system present in the L. acidopholus which started to attack protein sometime after 4 hours thus increasing the amount of amino groups in solution. In both the 6 and 12 per cent Ca-WPC solutions, the greatest absorbance decrease was observed in the combined bacteria sample after 8 hours incubation.

Analysis of the buffered Ca-WPC solutions for proteolysis (Fig. 7) gave the same trend of results as those obtained from the combined bacteria samples (Fig. 5, 6) without buffer in that increases in incubation time decreased the absorbance readings. The range of values obtained increased as the Ca-WPC concentration increased, but this increase was proportional to the increase in the absorbance values of the 6, 9, and 12 per cent standards as shown in Fig. 8. It should be noted, however,

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Fig. 5.   Proteolysis of individual and combined bacterial samples in 6
           per cent Ca-WPC. A=L. acidopholus, B=L. bulgaricus, C=L. casei,
           D=L. delbrueckii, S=S. lactis, ALL=combined bacteria, Org=
           organism, Time=hours of incubation. All values are an average
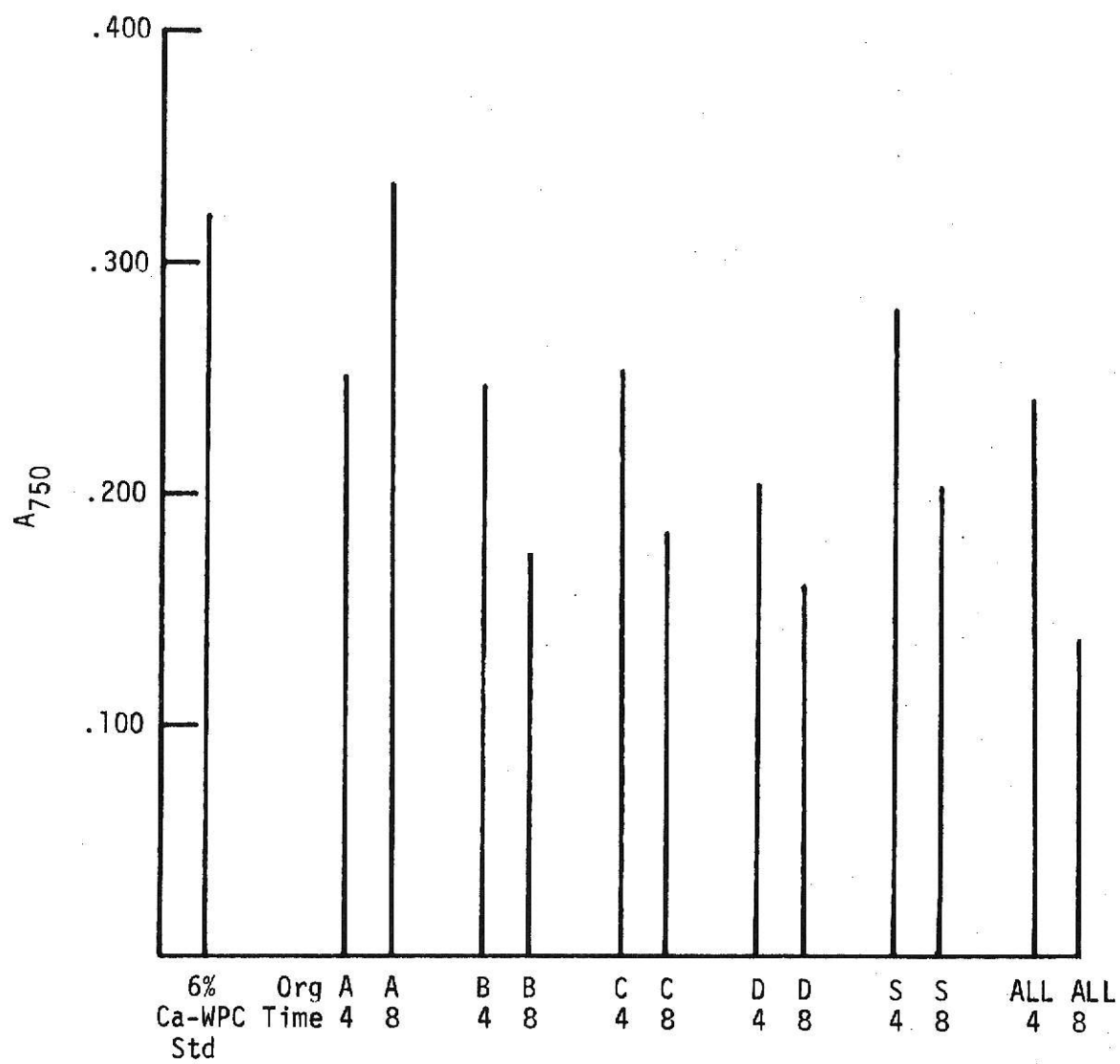   of triplicate analysis of three samples.

Fig. 6. Proteolysis of individual and combined bacterial samples in 12 per cent Ca-WPC. A=L. acidopholus, B=L. bulgaricus, C=L. casei, D=L. delbrueckii, S=S. lactis, ALL=combined bacteria, Time=hours of incubation. All values are an average of triplicate analysis of three samples.

A_{750}

.400

.300

.200

.100

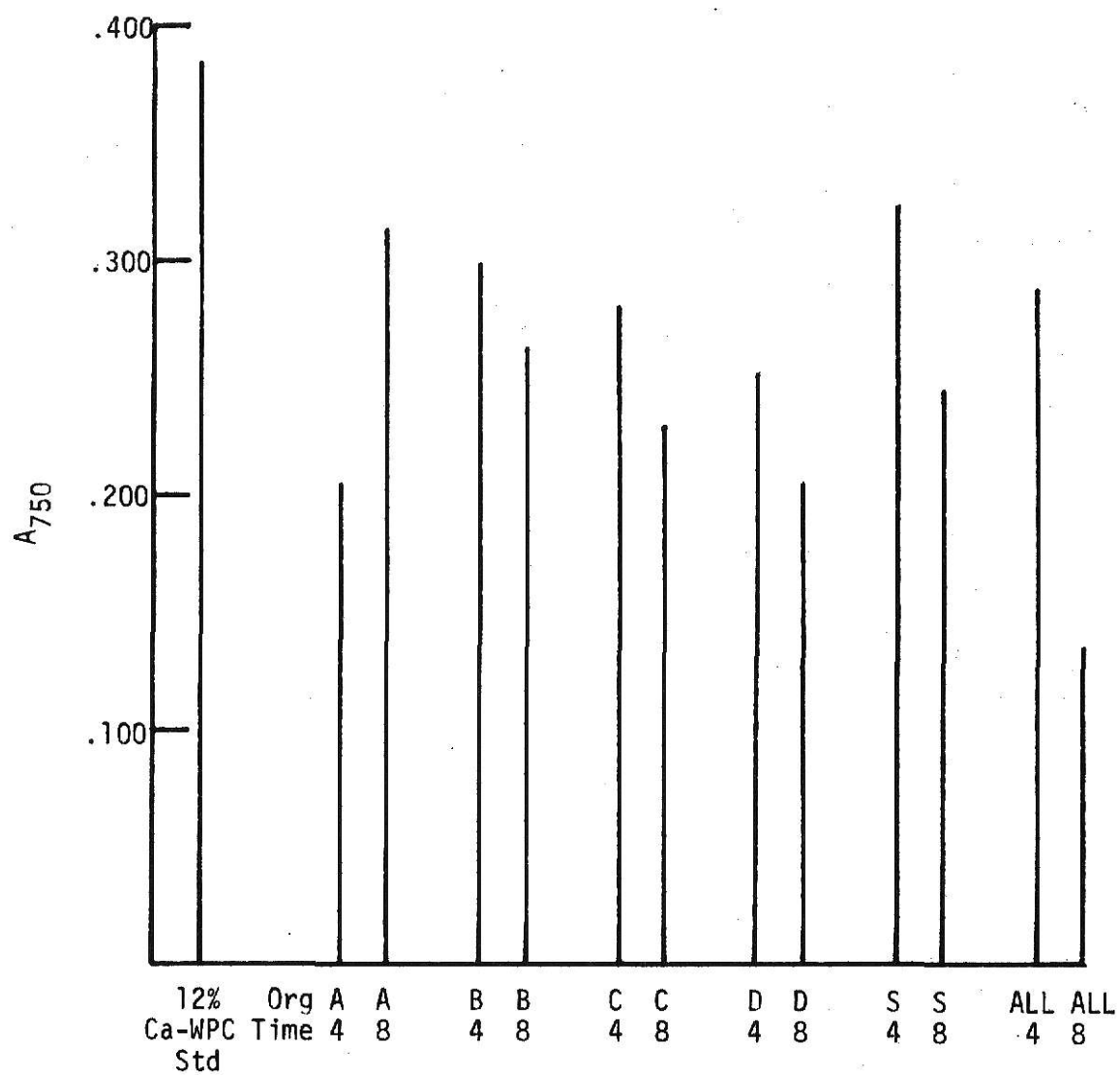| 12%
Ca-WPC
Std | Org
Time | A
4 | A
8 | B
4 | B
8 | C
4 | C
8 | D
4 | D
8 | S
4 | S
8 | ALL
4 | ALL
8 |

Fig. 7.   Proteolysis of combined bacteria in buffered 6, 9, and 12 per
            cent Ca-WPC. Time=hours of incubation. All values are an
            average of triplicate analysis of three samples.
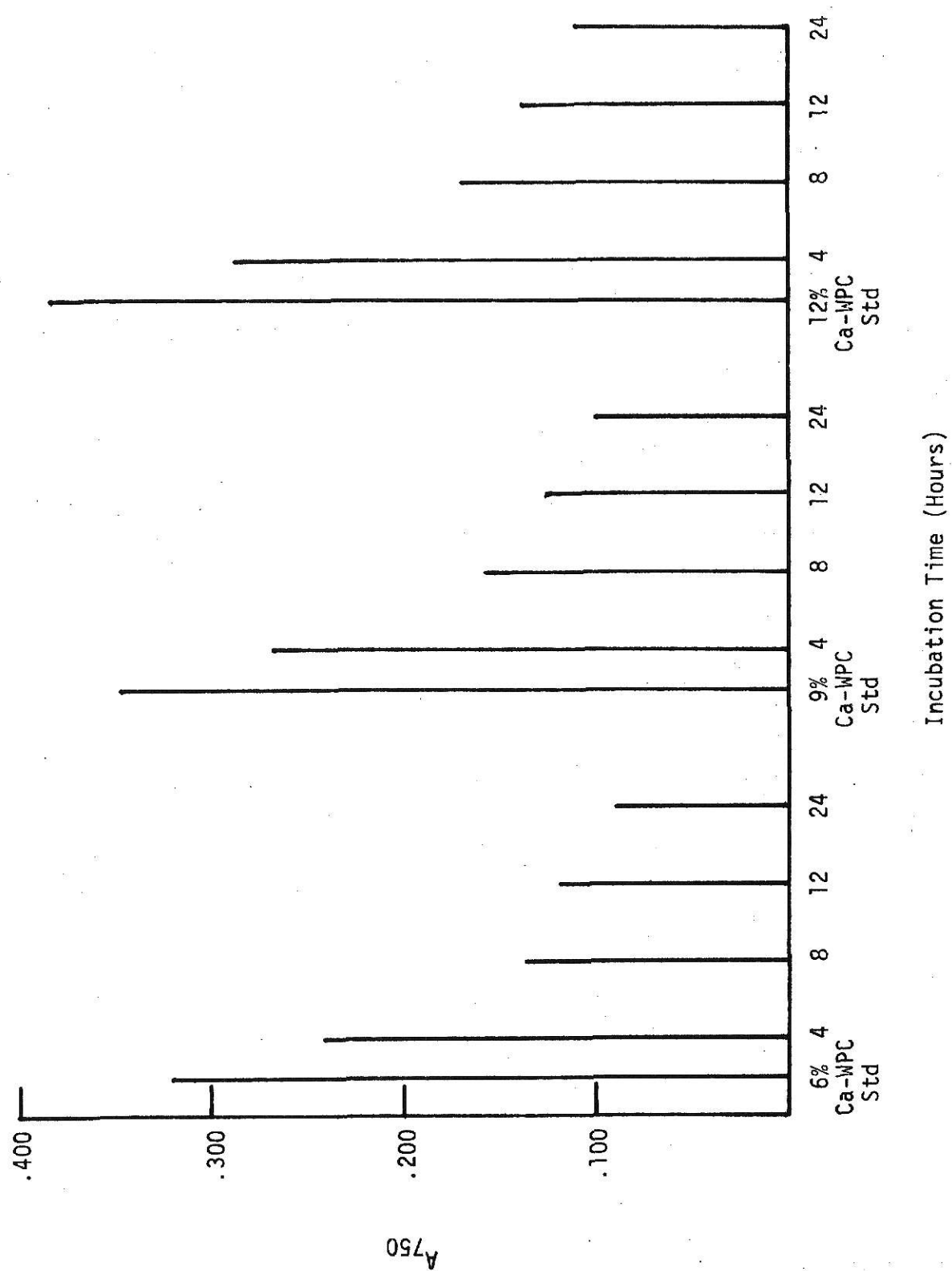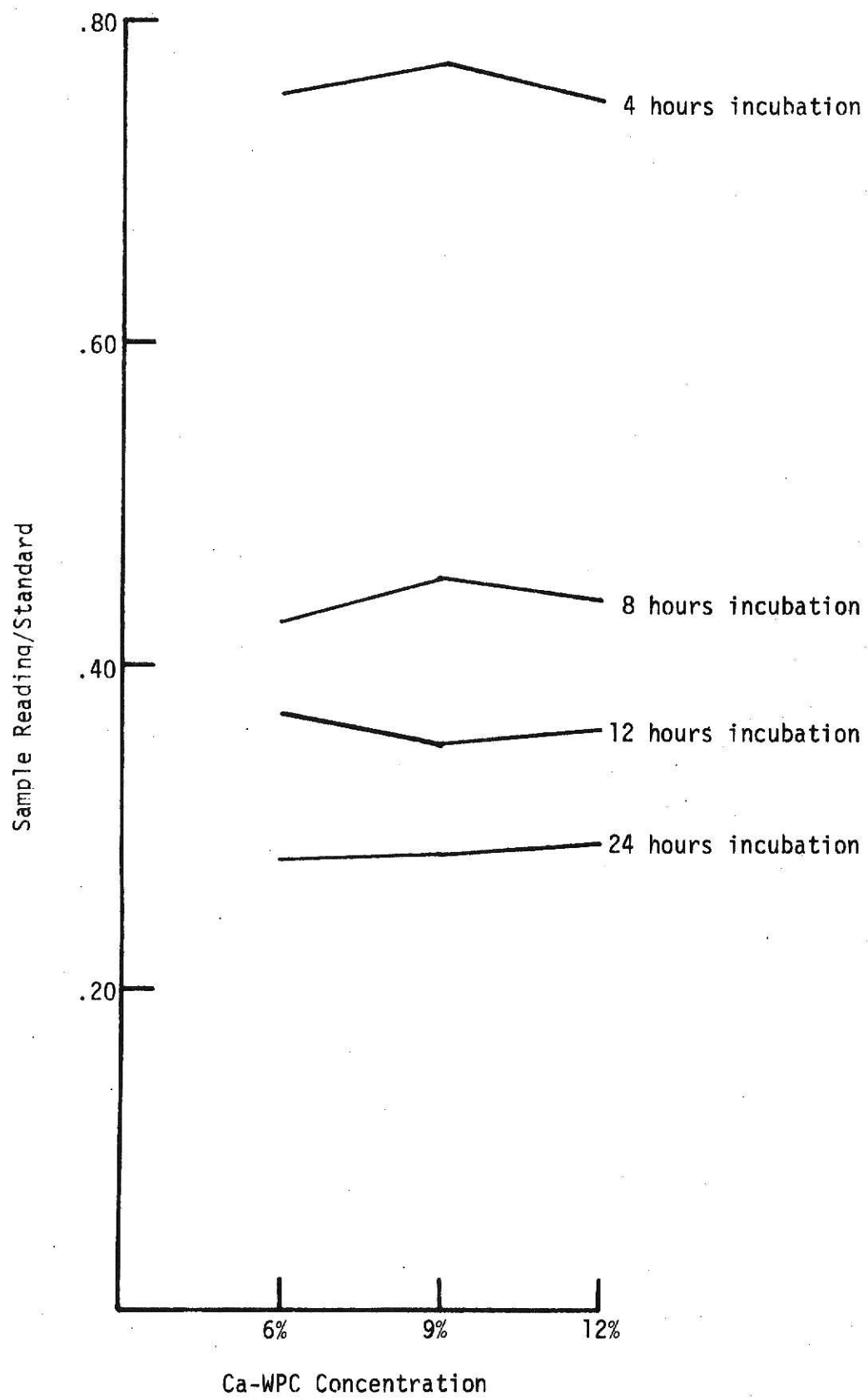
37

Fig. 8.   Comparison of increases in standard absorbance values to
             increases in sample absorbance values shown in Fig. 7.

that although the standards for Ca-WPC, Na-WPC, and spray-dried whey rose approximately the same number of absorbance units for each 3 per cent increase in sample concentration, this increase is not proportional to the actual increase in sample concentration as doubling the concentration does not double the absorbance reading. This may be due to the increased protein present in the more concentrated solutions which, upon precipitation, carried some of the shorter peptide chains out of solution. In the lower concentration solutions these short peptide chains would have stayed in solution.

Results from Whey Samples

From the data obtained in the preliminary investigations, it was apparent that best results would be obtained by using combined bacteria and buffering the whey sample being used. Yeast cells were added to each sample as described previously to determine if the yeast could reproduce in the bacterial system. By means of inverted gas tubes, small amounts of gas production were observed in all samples containing yeast. The slow yeast growth was probably due to a lack of available carbohydrate for energy. The glucose and galactose produced by bacterial degradation of lactose were the only available carbohydrates that the S. cerevisiae could utilize (56), and, even then, the yeast had to compete with the bacteria for these carbohydrates. Thus, although glucose and galactose are being formed, they are immediately utilized by bacteria and yeast and are therefore not detected in the subsequent paper chromatographic examinations.

Lactose hydrolysis. Results from analyses of the supernatant solutions from Ca-WPC, Na-WPC, and spray-dried whey after incubation with yeast, buffer, and the combined bacteria are shown in Fig. 9, 10, and 11, respectively. The per cent of lactose disappearance increased with increases in incubation time for all samples, as would be expected. Comparison between 6, 9, and 12 per cent Ca-WPC and Na-WPC shows very little difference in the results obtained, except for the slightly higher per cent of lactose disappearance in the Na-WPC samples at all three concentrations after 24 hours incubation. However, when converted to mg/ml of lactose disappearance (see footnotes in Fig. 9, 10, and 11), the difference between the two samples was not statistically significant at the .05 level. The spray-dried whey gave much smaller percentage increases in the amount of lactose disappearance over the 24 hour incubation period. This is probably due to the 48-50 per cent increase in lactose concentration of the spray-dried whey samples as compared to the same concentrations of Ca-WPC and Na-WPC (Table 2).

All three whey-product samples showed decreases in the per cent of lactose disappearance for given incubation times as the per cent of whey-product concentration increased. However, calculation of the actual number of mg/ml of lactose disappearance showed more lactose was degraded as the per cent concentration of the samples increased. The most lactose disappearance occurred in the 12 per cent spray-dried whey sample after 24 hours incubation.

Addition of yeast. Addition of yeast to the incubation system seems to have an adverse affect on the rate of lactose hydrolysis when

Fig. 9.   Lactose analysis of combined bacteria + buffer + yeast in 6, 9, and 12 per cent Ca-WPC. Time=hours of incubation. All values are an average of triplicate analysis of three samples. 6% Ca-WPC=11.6 mg lactose/ml. 9% Ca-WPC=17.2 mg lactose/ml. 12% Ca-WPC=22.5 mg lactose/ml.
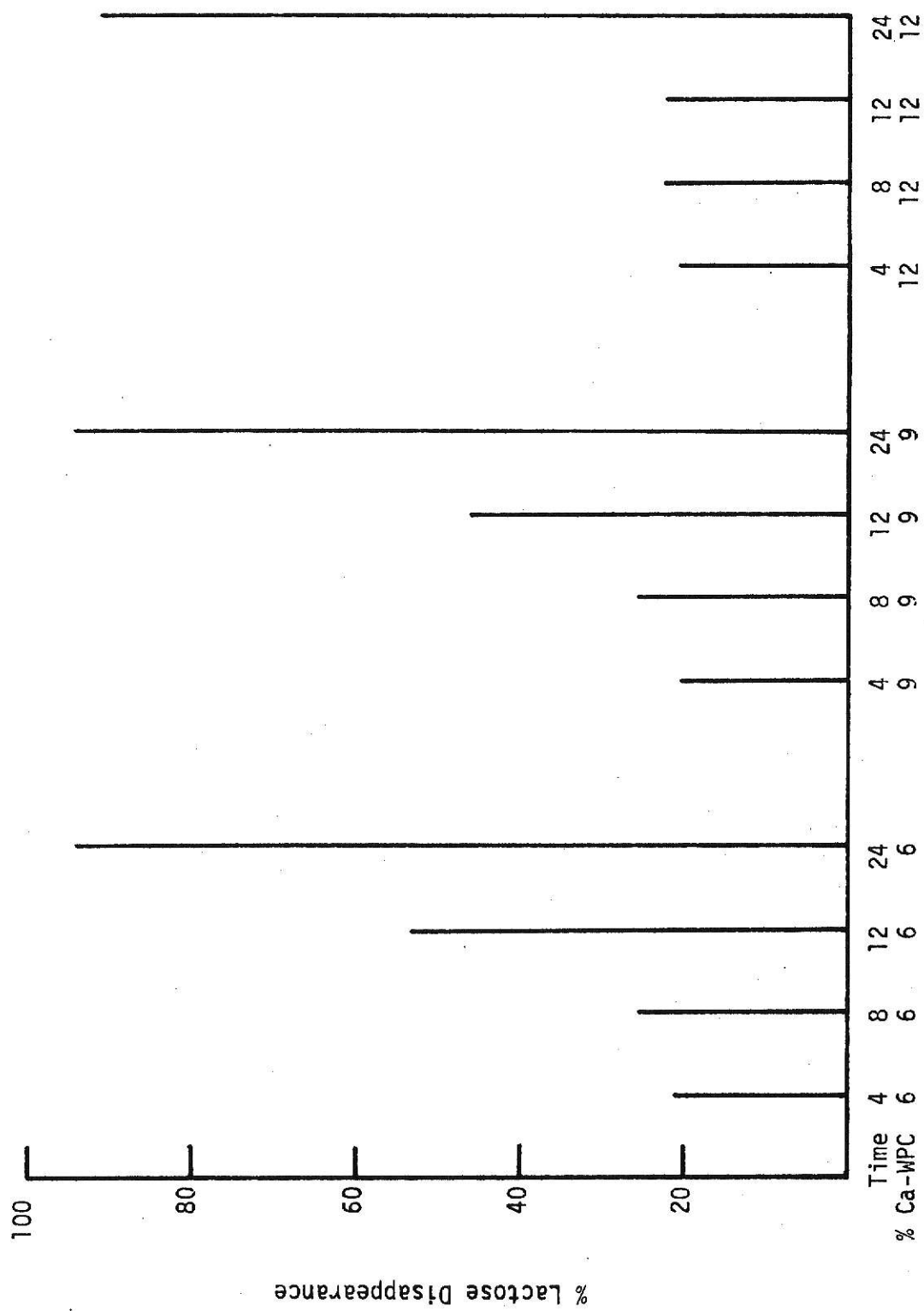
Fig. 10.   Lactose analysis of combined bacteria + buffer + yeast in
6, 9, and 12 per cent Na-WPC. Time=hours of incubation.
All values plotted are an average of triplicate analysis
of three samples. 6% Na-WPC=10.9 mg lactose/ml. 9% Na-WPC=
16.2 mg lactose/ml. 12% Na-WPC=21.1 mg lactose/ml.

44

% Lactose Disapperance

Time
% Na-WPC

100   80   60   40   20

4   8   12   24   4   8   12   24   4   8   12   24
6   6   6   6   9   9   9   9   12   12   12   12
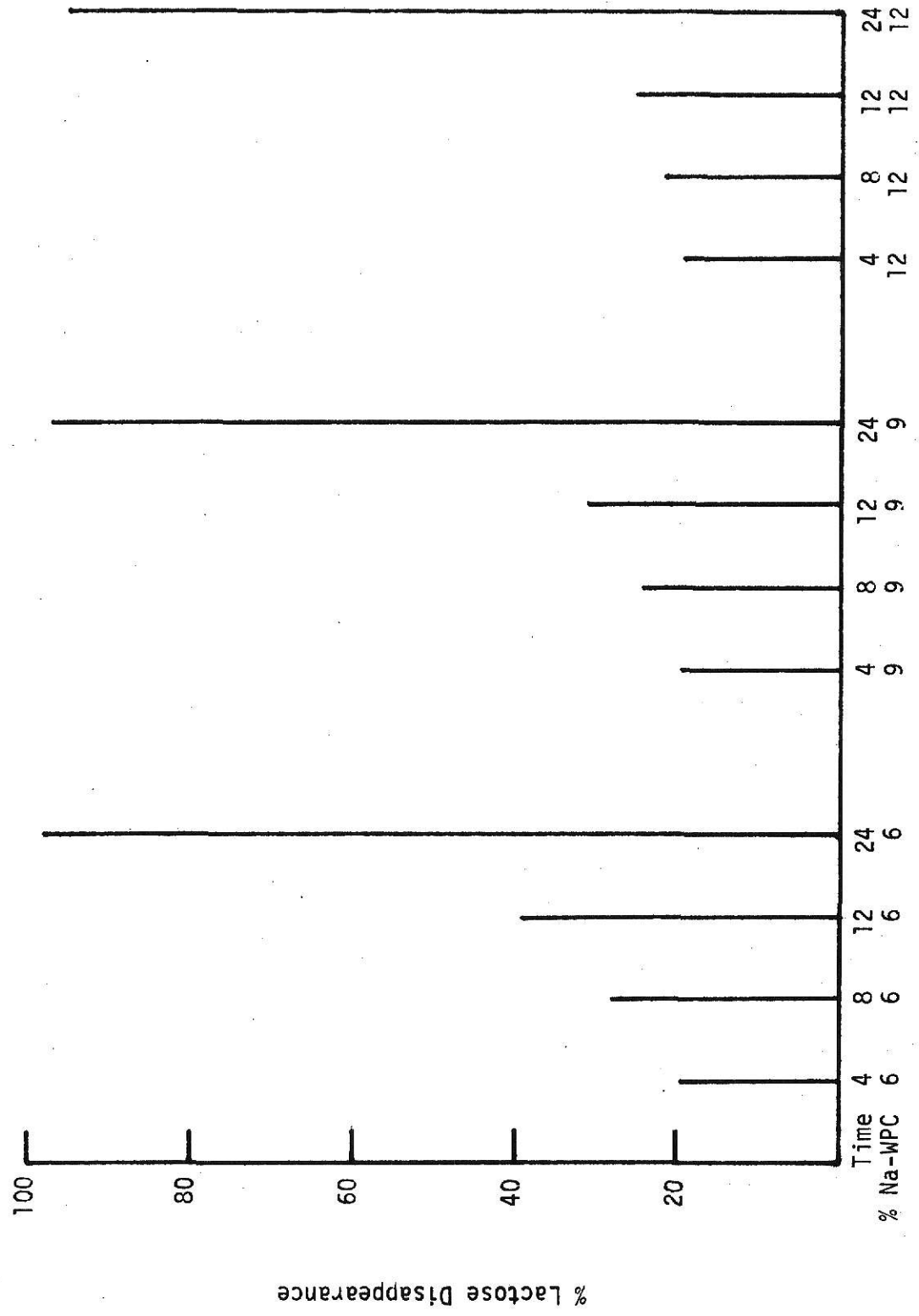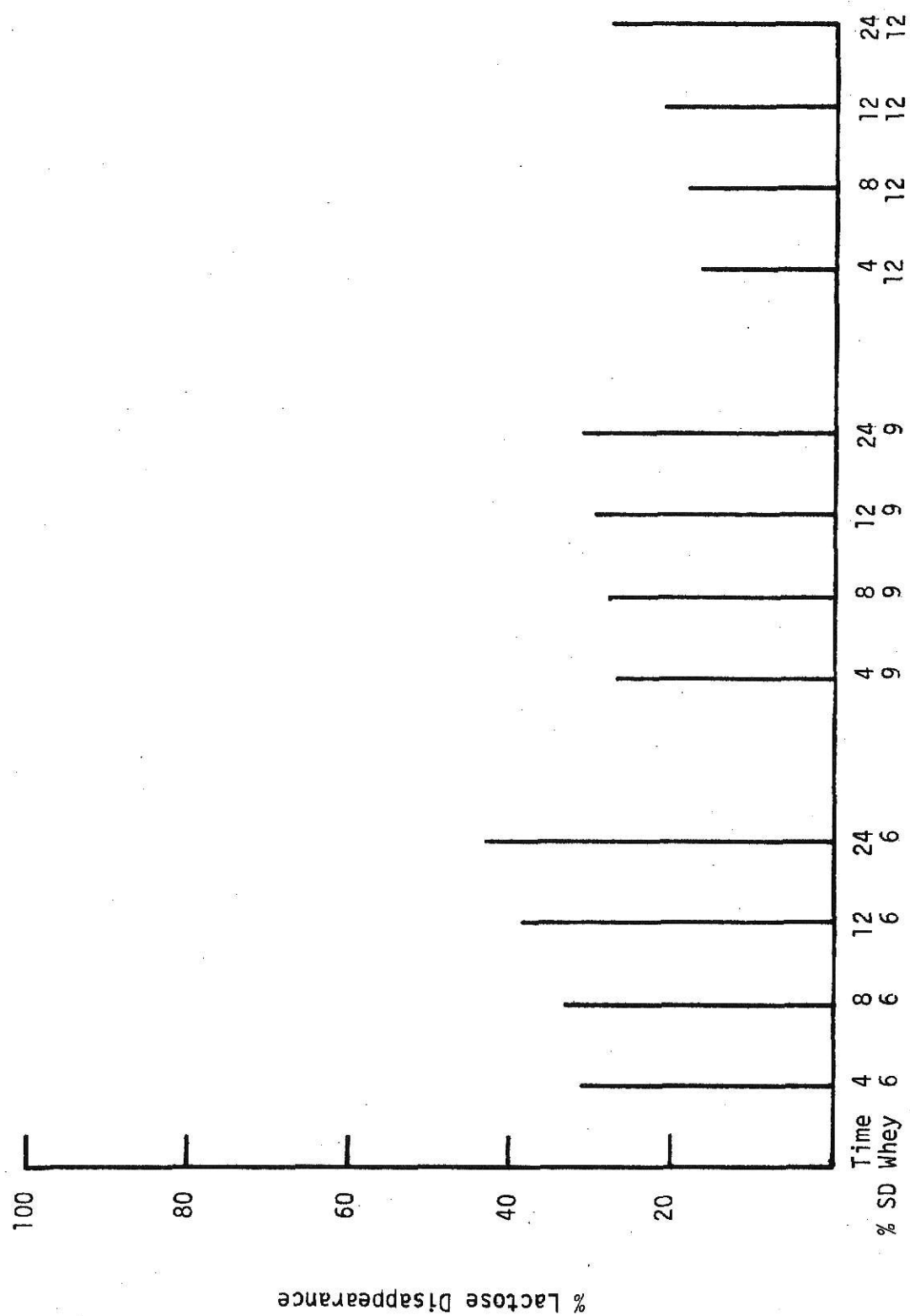
45

Fig. 11.   Lactose analysis of combined bacteria + buffer + yeast in
            6, 9, and 12 per cent spray-dried whey. Time=hours of
            incubation. All values plotted are an average of triplicate
            analysis of two samples. 6% spray-dried whey=41.6 mg lactose/
            ml. 9% spray-dried whey=60.4 mg lactose/ml. 12% spray-dried
            whey=73.7 mg lactose/ml.

% Lactose Disappearance

| Time | 4 | 8 | 12 | 24 | 4 | 8 | 12 | 24 | 4 | 8 | 12 | 24 |
| % SD Whey | 6 | 6 | 6 | 6 | 9 | 9 | 9 | 9 | 12 | 12 | 12 | 12 |

comparing the rates obtained for Ca-WPC with and without yeast (Fig. 9
and 4, respectively). In all samples of comparable concentration and
incubation time, the amount of lactose disappearance ranged from 6-33
per cent higher in the samples without yeast. This slower rate of
hydrolysis may be due to competition between the bacteria and yeast for
the glucose and galactose produced as mentioned previously. The mono-
saccharides utilized by the yeast are not available for use by the bacteria.
Therefore, the bacteria must break down more lactose to get the energy
required for growth and this could slow the growth rate.

pH. The pH readings obtained after incubation of Ca-WPC, Na-WPC,
and spray-dried whey are given in Table 5. In Ca-WPC and Na-WPC samples,
buffer maintained the pH above 5.0, the isoelectric point of the protein
in these samples (35). No precipitation was observed. The pH of the
spray-dried whey reached as low as 4.5 in one sample after 24 hours, but
no precipitation was observed. The lower pH in the spray-dried whey
sample could be due in part to the greater amount of lactose disappearance
in these samples as stated earlier. One possible degradation path of
lactose yields lactic acid, thus helping to lower the pH values.

Proteolysis. Figures 12, 13, and 14 show the results of the Lowry
test for proteolysis of the Ca-WPC, Na-WPC, and spray-dried whey,
respectively. As expected, increases in incubation time brought about
decreased absorbance values in all three samples, indicating as before
that the bacteria utilized the available nitrogen that was present in
the soluble amino groups. The greatest decrease in absorbance value as
compared to each standard for Ca-WPC, Na-WPC, and spray-dried whey
occurred at the 12 per cent sample concentration after 24 hours.

Table 5. pH of Ca-WPC, Na-WPC, and spray-dried whey samples after incubation with combined bacteria + buffer + yeast.

Hrs	%	pH*			Hrs	%	pH*		
		Ca-WPC	Na-WPC	SD Whey			Ca-WPC	Na-WPC	SD Whey
4	6	6.5	6.5	6.5	12	6	6.4	6.4	5.4
4	9	6.5	6.5	6.5	12	9	6.4	6.4	5.3
4	12	6.5	6.5	6.5	12	12	6.4	6.4	5.2
8	6	6.5	6.5	5.8	24	6	5.5	5.5	4.8
8	9	6.5	6.5	5.7	24	9	5.2	5.2	4.7
8	12	6.5	6.5	5.7	24	12	5.0	5.1	4.6

*Each pH value reported is an average of the pH of three samples.

Fig. 12.   Proteolysis of combined bacteria + buffer + yeast in 6, 9, and 12 per cent Ca-WPC. Dilution factor=38. All values plotted are an average of triplicate analysis of three samples.
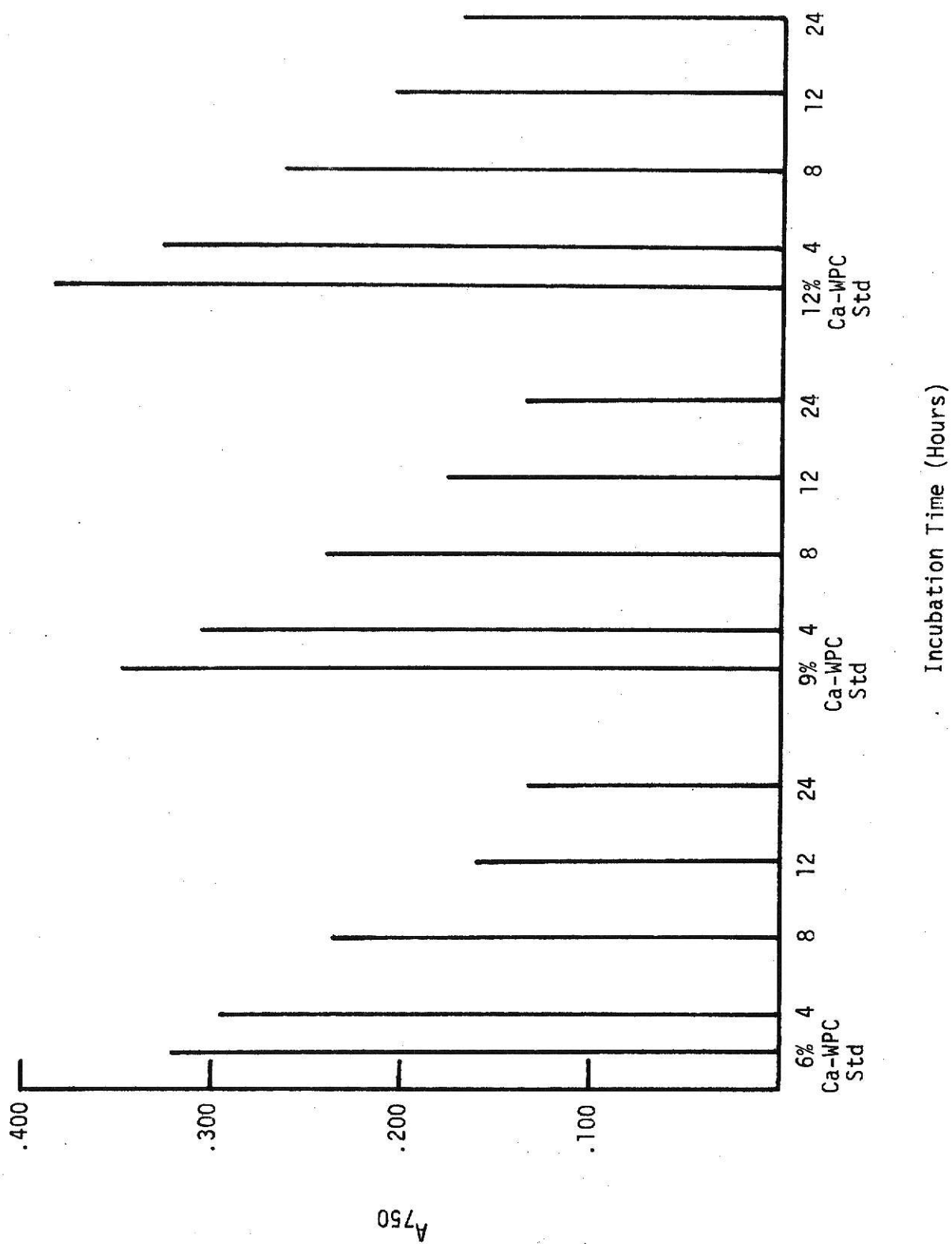
Fig. 13.   Proteolysis of combined bacteria + buffer + yeast in 6, 9, and 12 per cent Na-WPC. Dilution factor=38. All values plotted are an average of triplicate analysis of three samples.
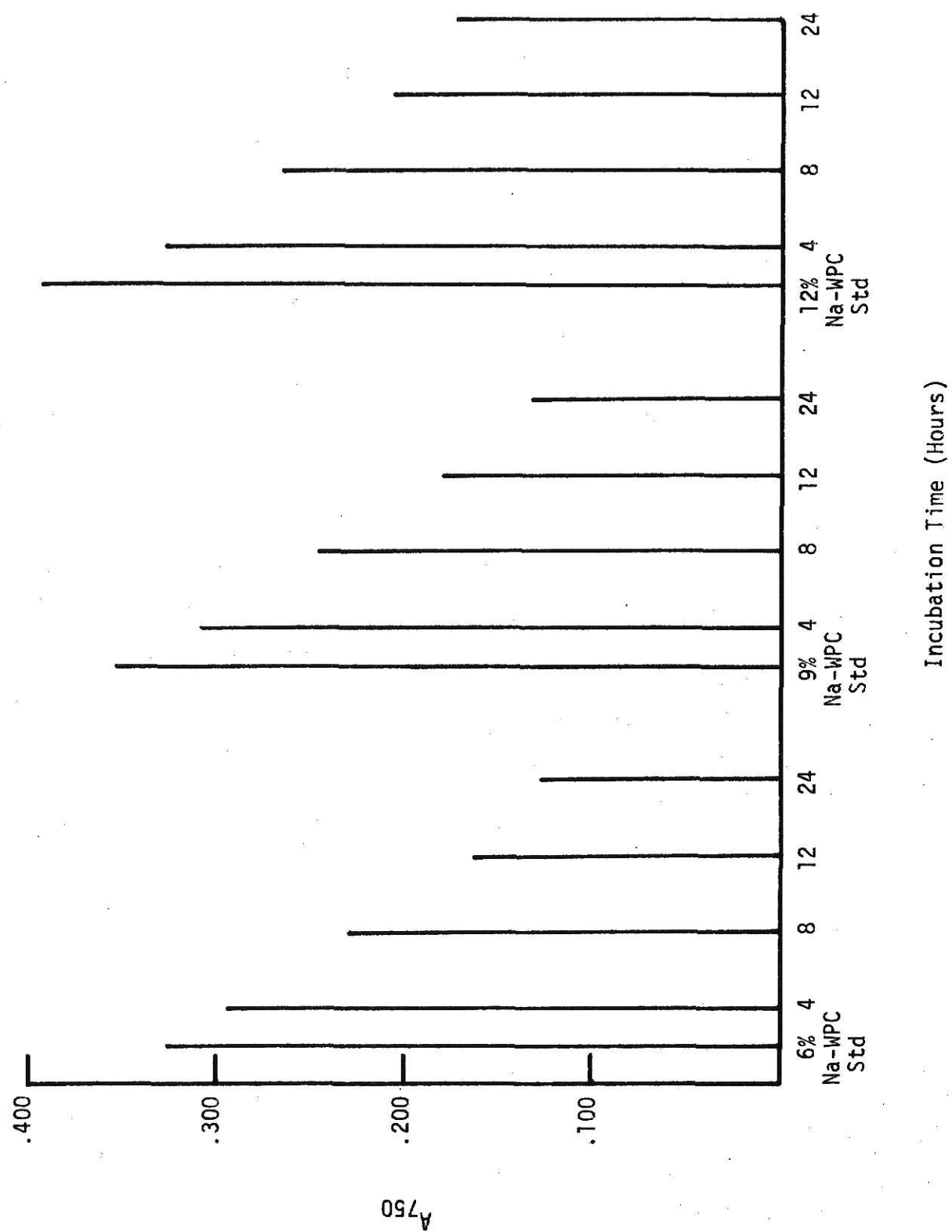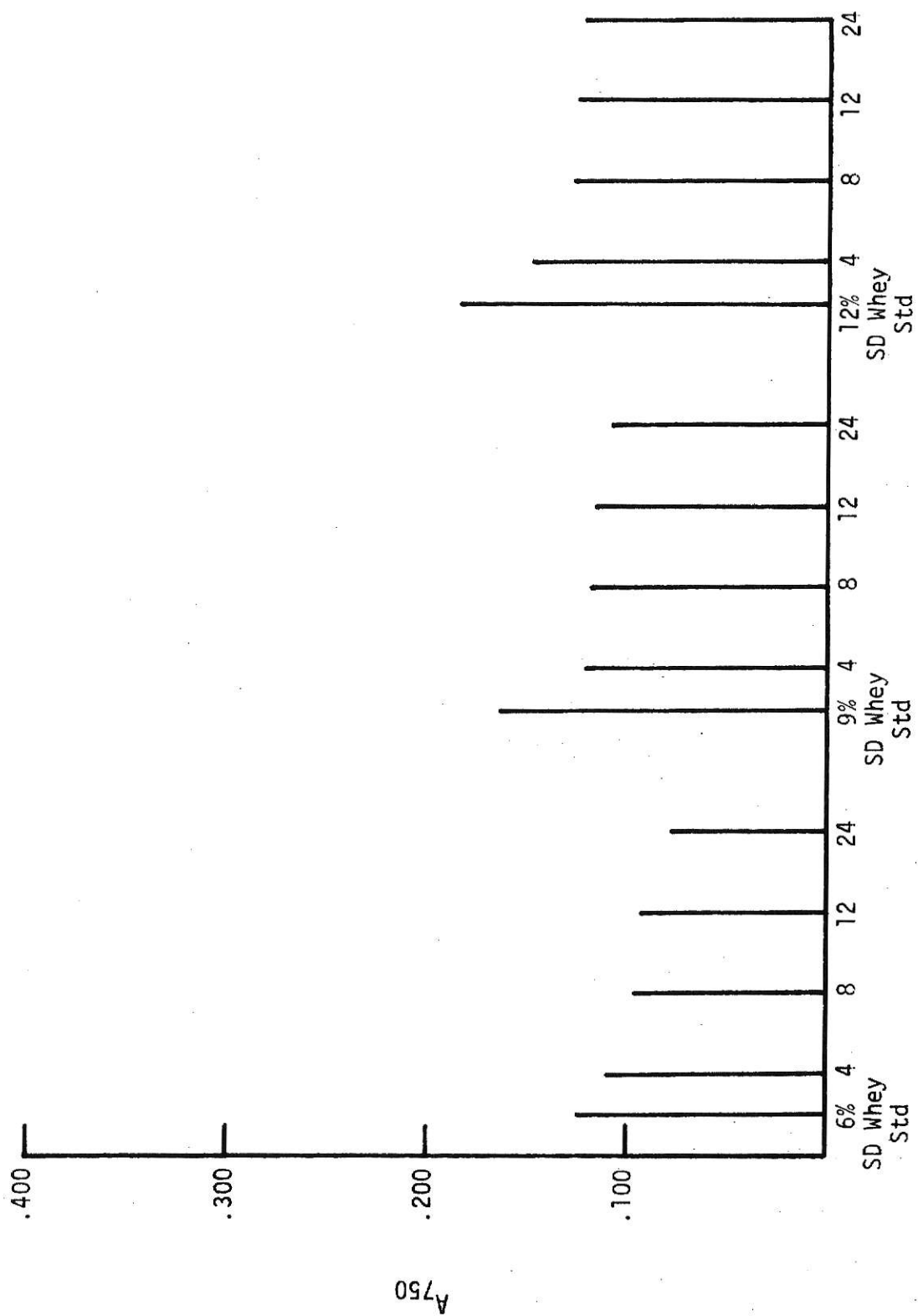
53

Fig. 14.   Proteolysis of combined bacteria + buffer + yeast in 6,
            9, and 12 per cent spray-dried whey. Dilution factor=57.
            All values plotted are an average of triplicate analysis of
            two samples.

However, values obtained for the spray-dried whey samples are in the less sensitive range of the spectrophotometer, thus results could contain a machine error.

SUMMARY

Investigations were conducted to determine the feasibility of using selected microorganisms as a means of reducing the lactose level in whey and whey products, and to utilize these altered whey products as an energy source for S. cerevisiae.

Three whey products were used for these studies. They were sweet spray-dried whey, Ca-WPC, and Na-WPC, the latter two being commercial partially delactosed and demineralized whey protein concentrate products.

Preliminary investigations using the Ca-WPC sample were conducted on five selected lactose-utilizing bacteria. A combination of all five were found to grow compatibly and to cause the most lactose disappearance. Lactose disappearance was increased slightly by buffering the whey product with a 0.1 M phosphate buffer at pH 6.5, thus keeping the pH high enough to allow bacterial growth for incubation periods up to 24 hours. Proteolytic activity also was highest in the combined bacteria sample.

Varied results were obtained after incubation of the combined bacteria with S. cerevisiae (baker's yeast) in each of the three whey samples. The Ca-WPC and Na-WPC samples gave similar results when analyzed for lactose disappearance. Values ranged from 91 to 98 per cent of the lactose utilized after 24 hours incubation. For the spray-dried whey, lactose disappearance after 24 hours incubation varied from 43 per cent utilized in the 6 per cent solution to 23 per cent in the 12 per cent solution. When converted to mg of lactose disappearance, however, the greatest

amount of lactose utilized after 24 hours incubation was in the 12 per cent spray-dried whey sample.

Proteolytic activity in all three samples increased with increased incubation time, indicating that the bacteria utilized the nitrogen present in the soluble amino groups.

CONCLUSIONS

Results of this investigation have shown that the lactose content of whey and whey products can be reduced through the use of lactose-utilizing microorganisms. Ninety-one to ninety-eight per cent of the lactose present in 6, 9, and 12 per cent Ca-WPC and Na-WPC can be removed after 24 hours of incubation. Twenty-three to forty-three per cent of the lactose in 6, 9, and 12 per cent spray-dried whey can be removed after 24 hours incubation. It would appear that this reduction in lactose, especially in the Na-WPC and Ca-WPC, would be high enough to enable these altered whey products to be used in bread at higher than normal levels as the loaf depressing effect of the lactose would be greatly reduced.

Results also indicate that this system could not be used as an energy source for yeast due to competition for avialable mono-saccharides between the yeast and bacteria.

The cost of putting such a system into operation might possibly be prohibitive, however, as special continuous-growth bacterial fermenters would probably be required to produce the quantity of bacteria necessary for any large scale operation. More research as to the feasibility of such an operation is needed.

ACKNOWLEDGEMENTS

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HYDROLYSIS AND UTILIZATION OF LACTOSE IN WHEY
BY SELECTED MICROORGANISMS FOR POTENTIAL
USE IN BREADMAKING

by

NORVAL KEITH SPIELMAN

B.S., Kansas State University, Manhattan, Kansas, 1970

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

This investigation was undertaken (a) to determine the feasibility of using selected lactic acid microorganisms to reduce the level of lactose present in whey and whey products, and (b) to utilize this altered whey product as an energy source for Saccharomyces cerevisiae.

Preliminary investigations indicated that the greatest amount of lactose disappearance occurred when a combined bacterial inocula was used which contained five lactose-utilizing bacteria (L. acidopholus, L. bulgaricus, L. casei, L. delbrueckii, and S. lactis).

Two commercial protein concentrate products, Calcium-Whey Protein Concentrate and Sodium-Whey Protein Concentrate, and a sweet spray-dried whey were used at 6, 9, and 12 per cent concentrations for incubation times up to 24 hours. Results showed 91-98 per cent lactose disappearance in the Ca-WPC and Na-WPC samples. The spray-dried whey sample gave 23-43 per cent lactose disappearance after 24 hours incubation. Conversion to mg of lactose disappearance, however, showed that the greatest amount of lactose utilized was in the spray-dried whey sample.

Addition of yeast (S. cerecisiae) to the system seemed to have an adverse affect on the rate of lactose hydrolysis, and yeast growth was minimal. This slow yeast growth was probably due to a lack of available carbohydrate for energy due to competition between the yeast and bacteria for the glucose and galactose being produced by lactose degradation.

Measurement of proteolytic activity of the bacteria during incubation showed increases of activity in all samples with increased incubation time, indicating the bacteria were utilizing the amino groups of the whey protein as a source of nitrogen.

The reduction in lactose concentration, especially in the Ca-WPC and Na-WPC, would appear to be high enough to enable these altered whey products to be used in bread or baked products at higher than normal levels since the loaf-depressing effect of the lactose would be greatly reduced.