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A Lactic Culture Stimulant Blend from *Kluyveromyces fragilis* and Whey

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A LACTIC CULTURE STIMULANT BLEND FROM *Kluyveromyces fragilis* AND WHEY

by

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Steven L. Wright

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ABSTRACT

A Lactic Culture Stimulant Blend from *Kluyveromyces fragilis* and Whey

by

Steven L. Wright, Doctor of Philosophy

Utah State University, 1984

Major Professor: Dr. Gary H. Richardson
Department: Nutrition and Food Sciences

A buffered growth medium was developed that sustained a significantly greater concentration of various strains of *Streptococcus lactis* and *Streptococcus cremoris* than did milk. The active buffering ingredients of this medium were magnesium hydroxide, ammonium and sodium salts of phosphate and citrate. This medium was entitled Lactic Culture Buffered Growth medium (LABGRO) and possessed about 8 times the buffering strength between pH 6.6 and 5.1.

Significant differences in growth and acid production rates were noted among Prt+ and Prt- lactic strains evaluated. Also noted were significant differences in cellular acid production rates. The two Prt- strains having the slowest growth rate in milk were selected as test strains to evaluate stimulants.

Three strains of Kluyveromyces fragilis were aerobically propagated in whey medium and afterwards subjected to autolysis conditions. Whey protein and casein were added to some yeast samples prior to initiation of autolysis. To some of these latter samples, pepsin was added. After autolysis the yeast and yeast-protein samples were dried and their stimulatory properties for lactic culture propagation were evaluated with a test strain and LABGRO medium. No product was found to be as stimulatory as a control commercial yeast autolysate. Yeast-protein samples with added pepsin were markedly more stimulatory than the other samples. The whey-derived yeast extract significantly improved the lactic culture growth stimulating properties of protein hydrolysates.

(219 pages)

INTRODUCTION

The use of pH controlled whey-based bulk medium for cheese making is increasing as more cheese plants modify their culture facilities to accommodate pH controlled fermentation (Personal communication, Reed Ernstrom, Biolac Inc., 1983). Even though the cost of pH controlled whey-based medium is markedly less expensive than milk-based phage inhibitory media, a typical cheese plant using pH controlled bulk medium and processing 1,000,000 lbs of milk a day pays approximately $45,000 a year for a stimulant and phosphate blend to add to whey. Yeast extract, yeast autolysate and casein hydrolysate are common stimulants added to bulk media and range in price from $1.80/lb for yeast autolysate to $4.50/lb for casein hydrolysate, exclusive of shipping costs (Personal communication, Blake Butterfield, Biolac Inc., 1983).

The cheese maker often discards the raw materials from which stimulants for propagation of his cheese cultures could be obtained. Properly processed whey could yield yeast autolysates and whey protein hydrolysates that would serve as stimulants for a lactic culture bulk medium. A regionally located plant could produce a yeast autolysate-whey protein hydrolysate product at minimal cost for several cheese plants using pH controlled whey-based medium for bulk fermentations. Production of

less expensive stimulants would make pH controlled whey-based bulk medium more appealing to the cheese maker.

Less expensive stimulants for pH controlled whey-based medium would also encourage use of protease negative lactic cultures (lactic cultures lacking some protease enzymes and which grow to lower concentrations in cheese milk than cultures with protease enzymes) for cheese making. Protease negative lactic cultures can be propagated in pH controlled whey-based medium to concentrations similar to those reached with regular lactic cultures (133). However, 2 to 3 times the inoculum is required with protease negative lactic cultures than is required to make cheese with regular cultures. Benefits of using protease negative lactic cultures for cheesemaking include: less chance of bitter cheese developing (102), greater protection against bacteriophage and antibiotics (45), greater cheese yield (115), and greater control of acid production during high temperature cheese production (141).

The goals of this research project were (A) to develop methods to evaluate whey-derived nutrients for incorporation into lactic culture bulk media, (B) to develop a procedure to monitor whey-derived nutrients during production and (C) to establish production parameters for whey-derived products.

The term lactic cultures refers to *Streptococcus lactis* and *Streptococcus cremoris*, unless otherwise noted.

REVIEW OF LITERATURE

Nutrition of Lactic Cultures

Pont and Holloway (125) found pH controlled whey to be unsatisfactory in propagating lactic cultures unless stimulants such as tryptone or yeast extract are added. Law et al. (83) observed similar results. Wright and Richardson (178) found that a pair of lactic strains propagated in pH controlled whey-based medium with 0.4% yeast extract and 0.1% casein hydrolysate are 220% more active than they are when propagated in pH controlled whey-based medium with no added stimulants. McDonald (97) reported that lactic cultures grow poorly in medium where casein is the sole source of protein and that growth increased significantly when the sodium caseinate is treated with papain. Wright and Richardson (178) found that a pair of lactic strains propagated in pH controlled nonfat dry milk-based medium with 0.4% yeast extract and 0.1% casein hydrolysate are 150% more active than they are when propagated in pH controlled milk-based medium with no added stimulants. Other researchers have also observed the stimulatory properties of yeast extracts and protein hydrolysates on lactic cultures (6, 7, 26, 29, 36, 46, 73, 75, 91, 145, 146, 149, 154, 177).

Growth Factors

Amino acids. Essential nutrients of lactic cultures have been extensively investigated. Reiter and Oram (129) found amino acids GLU, VAL, MET, LEU, ILE and HIS to be essential amino acids for the S. lactis strains tested. In addition to these, PRO and PHE were essential for S. cremoris strains tested. Niven (109) reported that a minimum of 14 amino acids were required to produce prompt growth of the S. lactis strains tested and he found GLU and ASP to be essential for all cultures. Anderson and Elliker (5) found that of 35 strains of S. lactis and S. cremoris tested, all required PRO, ILE, VAL, LEU, HIS, GLU, and MET; and all but one strain required ARG. Husain and McDonald (70) found LEU, ILE, MET, VAL, ARG and HIS to be required for lactic culture growth. Ramasamy and Natarajan (128) found ALA, ARG, GLU, LEU to be required by their S. lactis test strain and ARG, GLU, HIS, ILE and LEU to be required for the S. cremoris test strain.

Vitamins and Minerals. Reiter and Oram (129) found niacin, pantothenic acid and biotin to be essential vitamins for lactic cultures. Niven (109) and Anderson and Elliker (5) observed similar results. Anderson and Elliker (5) also observed that thiamin and pyridoxine are required for maximum growth. Koburger et al. (78) found inosine, hypoxanthine and adenine to be stimulatory. The

stimulatory properties of purine bases was confirmed by other researchers (30, 100, 146). Pyrimidines were found to have little affect on lactic culture growth (30). Olson and Qutub (116) found iron, magnesium, molybdenum and selenium to be stimulatory to 2 strains of lactic cultures.

Protein Utilization

Preliminary Observations. Several researchers have observed lactic streptococcal strains that are unable to coagulate milk (23, 47, 86, 98). A lactic strain designated a slow starter meant that it could not coagulate milk in 18 h at 1% inoculum at 22 C (89). About 2% of the total cell population of the lactic strains in one study were slow (23, 59). Slow lactic strains propagated in skim milk were reported only to reach 20-30% the final cell concentration of fast strain (23, 120). Citti et.al. (23) found the growth rate of the slow lactic strain equal to that of the fast strain in their respective exponential growth phases. Pearce et.al. (120) and Thomas and Mills (156) reported similar results. Limsowtin et.al. (90) observed that the lag phase of slow strains is shorter than that of the fast strains.

Citti et al. (23) found the fast lactic strain to be four times as proteolytic as the slow strain. Exterkate (41) and Pearce et al. (120) found the slow variant to

have no cell wall associated proteolytic enzymes that were found in parent strains. Exterkate (41) also reported that a slow lactic strain of S. cremoris HP had no intracellular proteolytic enzyme. However, Exterkate (41) and Law (84) confirmed slow lactic strains to possess peptidase activity.

As genetic research of lactic cultures progressed, proteolyzing capability was found to be plasmid-linked (31, 39, 98). Hence, slow lactic strains were termed protease negative (Prt-). Limsowtin and Terzaghi (89) introduced a selective plating method involving milk and β-glycerol phosphate for isolation of protease positive (Prt+) and Prt- lactic strains. The Prt+ colonies utilize casein and therefore grow larger than Prt- colonies which cannot utilize casein. Mills and Thomas (102) defined those colony isolates requiring more than 2 days to coagulate autoclaved milk at 22 C as Prt-. Yu et al. (179) found multiple and differentiable plasmids in various lactic culture strains. Davies et al. (32) suggested the use of plasmid profiles to characterize lactic strains.

Proteolytic capabilites of various degrees have been observed in lactic cultures. Exterkate (42) discovered the presence of three separable proteolytic enzymes in S. cremoris HP and found that a group of 14 strains of S. cremoris varied as to the sets of enzymes possessed.

Limsowtin et al. (90) noticed that different plate isolates from the same "pure culture" required from 18 h to 10 days to coagulate milk incubated at 22 C.

Proteolytic System of Lactic Cultures. Lawrence and Thomas (87) state that in order for lactic cultures to reach a density in milk of 0.5 mg dry weight/ml (corresponding to 10^9 colony forming units/ml), 0.25 mg protein substrates/ml milk, or 0.025% amino acids are required. Milk contains only 0.004-0.010% total free amino acids and small peptides. Lactic cultures therefore must breakdown milk proteins in order to grow to concentrations they normally attain in cheese milk (i.e. 10^9 colony forming units/ml).

The metabolic role played by lactic culture proteolytic enzymes was investigated by Mills and Thomas (103). They observed that lactic cultures first deplete the amino acids and peptides in the milk and then start proteolyzing and assimilating the milk caseins as the amino acid and peptide levels become too low to sustain growth. As cell density levels increase there is a concommitant increase in proteinase and peptidase levels. Speck and Williamson (148) observed an inverse relationship between the amount of proteolysis and the concentration of pancreas extract added to milk which signifies that the presence of peptides inhibits the

lactic cultures from hydrolyzing milk proteins. Exterkate (42) observed similar results.

Proteinases (9, 27, 28, 40, 41, 80, 114, 153, 172) and peptidases (40, 93, 161) have been isolated from lactic cultures. In an extensive study of the proteolytic system of S. cremoris HP, Exterkate (40) identified an acid proteinase (P1), a neutral proteinase (P2), an intracellular proteinase (Pi), two endopeptidases (P37, P50), an aminopeptidase (AP) and a proline iminopeptidase (IP).

Sussman and Gilvarg (151) stated that most peptide-utilizing microorganisms bring peptides into the cell where they are cleaved. Peptides are transported as dipeptides or oligopeptides by permeases and not by diffusion. Amino acids are usually transported into the cell by permeases which are group specific. The bacterial cell wall acts as a selective sieve. Eschericia coli can accept peptides up to 6 amino acid residues long (118).

Law (84, 85) confirmed that lactic cultures do use peptides as an amino acid source. He found that amino acid transport is different than peptide transport. He also found that not all peptides are utilized as efficiently and suggested that this may be due to the particular transport system of the strain and that the degree of nitrogen assimilation by the lactic strain is related both to the presence of proteolytic enzymes as

well as to the transport systems of the strain. Rice et al. (130) confirmed a Prt- lactic strain to contain a peptide transporting system. Exterkate (41) confirmed a Prt- strain of S. cremoris HP to contain peptidase activity comparable to the Prt+ strain. Desmazeaud and Hermier (36) found peptides with molecular weights between 1000-2500 daltons to be stimulatory to Streptococcus thermophilus. When the most stimulatory fraction was analyzed, it was found to contain 14 pure peptides of various molecular weights. The average molecular weight was 1500 daltons (about 12 amino acid residues).

Lawrence et al. (86) observed that some step of lactic culture metabolism in milk with no added protein hydrolysates is growth limiting since cell growth rate was increased when protein hydrolysates were added. Law et al. (83) observed that greater lactic culture growth occurs in whey supplemented with soy peptone than that which occurs in whey supplemented with papain-digested milk peptides. The soy peptone was found to contain smaller peptides (average number of amino acid residues was between 3 and 4 residues) than the papain milk digest (7 residues was average).

Yeast and Whey Protein as Nutrient Sources

Yeast Products

Preliminary Observations. Yeast products have been shown to be growth stimulants for lactic cultures (29, 73, 91, 145, 146). They have been incorporated into pH controlled milk-based media for the propagation of concentrated cultures (92) and have been included in non-pH controlled phage inhibitory media (101).

Cox and MacBean (29) have found yeast products to be more stimulatory than corn steep liquor. Smith et al. (146) found 1% yeast extract in milk to give about the same amount of stimulation as did 1% tryptic digest of casein. Keen (73) observed a 40% increase in specific growth rate of lactic cultures when 1% yeast extract was added to skimmilk. In another article, Keen (72) observed that a yeast product eliminated the inhibitory effect of hydrogen peroxide produced when the growth medium containing the lactic culture was stirred. Lloyd and Pont (91) found that a yeast product was stimulatory in milk-based medium up to 1% level.

Definition and Composition of Yeast Products. The term yeast products refers to autolysed yeast or autolysed yeast extract. Autolysed yeast contains products from hydrolytic activity of enzymes of edible yeast. These

products primarily include amino acids, peptides, polypeptides, vitamins and nucleic acids (122). Autolysed yeast contains the yeast cell walls whereas autolysed yeast extract does not (122). Table 1 lists the composition of a commercial autolysed yeast extract product (Amberex 1003, Amber Laboratories, Juneau, WI). Amberex 1003 is an extract from Saccharomyces uvarum (Personal communication, Gerald Reed, Amber Laboratories, 1983).

Composition analysis of Kluyveromyces fragilis has also been conducted. Bednarski et al. (13) gave the following analysis: dry matter 94.8%; total nitrogen, 5.9%; total protein, 37.1%; ash, 12.1%. Martini et al. (95) reported a total nitrogen of 9.9%; crude protein, 56.2%; and true protein 47%. They also reported the following amino acid ccmposition (g/100g): ILE, 5.2; LEU, 3.8, LYS, 1.8; PHE, 2.4; TYR, 1.7; THR, 2.5; TRP, 0.7; VAL, 3.0; CYS, 0.9; MET, 0.5; ASP, 4.4; SER, 2.2; GLU, 4.9; GLY, 2.3; ALA, 3.1; PRO, 1.5; HIS, 0.9; ARG, 2.4.

Wasserman (168) reported the vitamin content of K. fragilis (ug/g) as follows: thiamine, 24.1; pyridoxine, 13.6; riboflavine, 36.0; niacin, 280.0; folic acid, 5.8; pantothenate 67.2; biotin, 2.0; choline, 6,760.

Nutrients of Yeast Products. The stimulatory properties of yeast products on lactic cultures have been

Table 1. Composition of a commercial autolysed yeast extract product, Amberex 1003 (Amber Laboratories, Juneau, WI). Amberex 1003 is a yeast extract from Saccharomyces uvarum. (Personal communication, Gerald Reed, Amber Laboratories, 1983).

TYPICAL ANALYSIS:

% Total Nitrogen	8.0	% Ash	9.7
Protein (N*6.25)	50.0	% Cl as NaCl	2.5
% Moisture	3.0		

VITAMINS:

Thiamine	50 ug/g	Pantothenate	100 ug/g
Riboflavine	35	Choline	2000
Niacin	550	Biotin	2
Pyridoxine	25	Inositol	3000

AMINO ACIDS: (Protein Base)

Arginine	3.9 %	Phenylalanine	4.2 %
Lysine	6.3	Threonine	3.4
Tryptophan	1.0	Leucine	6.3
Methionine	2.1	Isoleucine	4.4
Cystine	0.8	Valine	4.2
Histidine	2.4	Glutamic Acid	8.8
Tyrosine	4.0	Glycine	5.3

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investigated. Smith (145) concluded that the stimulatory component(s) is not a vitamin and that it can not be replaced with a combination of 23 amino acids. He found that the stimulatory property of the yeast product is destroyed by heating it at 210 C under vacuum. Smith et al. (146) fractionated a yeast product and found the most stimulatory fraction to contain over 70% of the total amino nitrogen present in the yeast product and was found to contain a wide variety of free amino acids and small peptides. Purines and pyrimidine bases were also found to be stimulatory. These researchers concluded that the major stimulatory component of the yeast product are free amino acids while bases and inorganic constituents exert a smaller affect. Westhoff et al. (170) confirmed the superior stimulation of peptides and amino acids over organic bases when they reported that casein hydrolysate is much more stimulatory to slow lactic cultures than purines, pyrimidines or minerals. Cowman and Speck (26) observed that pancreas extract, comprised mainly of peptides and amino acids, is stimulatory to lactic cultures. Grant and Pramer (53) have shown a yeast product to contain substantial amounts of magnesium and iron which are stimulatory to lactic cultures. Smith et al. (146) isolated a yeast product fraction that decomposes hydrogen peroxide.

Whey Protein Hydrolysate

Preliminary Observations. Protein hydrolysates have been shown to be stimulatory to lactic cultures (6, 26, 36, 46, 75, 147, 148, 154, 177). Anderson et al. (7) found that as the peptide content of cow's milk increases so does the acid production of cultures inoculated into the milk. Anderson and Elliker (6) found that greater lactic culture stimulation results when cultures are grown in peptonized and tryptonized milk than results from any single amino acid or vitamin. Wright and Skeggs (177) also observed the stimulatory nature of trypsinized milk proteins. Lactalbumin hydrolysate is commercially available as a nutrient source in microbiological media (Difco Laboratories, Detroit, MI and Sheffield Products, Norwich, NY).

Amino Acid Content of Whey Protein. Glass and Hedrick (51) reported the amino acid content (g/100 g protein) of whey protein to be as follows: LYS, 8.8; HIS, 2.0; ARG, 2.6; TRP, 2.4; ASP, 10.2; THR, 6.8; SER, 5.3; GLU, 18.0; PRO, 6.9; GLY, 1.9; ALA, 4.6; CYS, 2.3; VAL, 5.9; LEU, 10.3; TYR, 2.7; PHE, 3.5.

Yeast and Whey Protein Products for Lactic Culture Nutrition

Autolysed Yeast Products

Choice of Lactose-fermenting Yeast. Much interest has been shown in reducing whey biochemical oxygen demand while concurrently producing a utilizable product (4, 12, 18, 44, 111, 127). Biochemical oxygen demand measures the amount of organic material present that could be oxidized by microorganisms. Porges et al. (126) found K. fragilis to be more effective than Torula cremoris, Candida lipolitica, and Torulopsis utilis in converting lactose to biomass. In their survey, the K. fragilis strain took the whey biological oxygen demand from 1,050 ppm to 86 ppm after 48 hour incubation. Giec and Kosikowski (48) evaluated K. fragilis, Kluyveromyces bulgaricus, Kluyveromyces lactis Bretanomyces anomalus and Candida blankii strains for biomass production from whey. They found K. fragilis ATTC 8582 to produce the most biomass (8.4 g dry weight/l). Friend et al. (44) evaluated 5 strains of K. fragilis and found all 5 to take whey lactose from 5.1% to 0.1-0.2% in 48 hours. Orberg et al. (117) found that of seven strains of K. fragilis, K. fragilis NRRL Y-610 produces an autolysate most stimulatory to lactic cultures. K. fragilis is an approved food yeast (24).

Propagation Parameters for Kluyveromyces fragilis. Whey has been shown to be an acceptable medium for propagation of K. fragilis (12, 112, 160, 169). Harvesting of K. fragilis is facilitated by the flocculating properties of the yeast. Hang and Woodhams (58) reported that 99% of the yeast population sediments in sedentary medium after 1 hour. Wasserman et al. (169) used a separator to harvest yeast in a pilot plant. Orberg et al. (117) obtained a yeast concentration of about 15% by allowing K. fragilis NRRl Y-610 to sediment for 1 h.

Significant yield differences with K. fragilis have been observed through varying the growth parameters: lactose concentration, dissolved oxygen concentration, temperature, pH, and added growth factors. Propagation time is affected by inoculum size.

Lactose concentration. Moresi et al. (107) found that lactose concentration has a marked affect on biomass yield. Giec and Kosikowski (48) evaluated lactose utilization rates in whey permeates and found that lactose utilization is most efficient at an initial lactose concentration of 4% and dramatically decreases at initial concentrations above 15%. With aeration, significantly higher yields of biomass is achieved in 12% lactose whey permeate than in 5% lactose whey permeate. The higher

lactose concentration yielded 27.5 g dry yeast/mL. Castillo et al. (20) propagated K. fragilis in 2% whey permeate and obtained a yield of 3.7 g dry yeast/1. Amundson (4) reported a yield of 16 g dry yeast/1 when he propagated K. fragilis in whey of 5% lactose concentration. Nour El-Dien et al. (111) obtained a yield of 7 g dry yeast/1 in 2% lactose whey. De Sanchez and Costillo (35) reported a yield of 5 g dry yeast/1 from 2% lactose whey.

In continuous propagation of K. fragilis, Vananuvat and Kinsella (159) found a whey lactose concentration of 2% to be optimal. Nour El-Dien et al. (111) found that a 2% whey lactose concentration effects the fastest growth rate from K. fragilis. Moresi and Sebastiani (108) found 2.5% whey lactose to achieve optimal biomass yield of K. fragilis propagated in a reciprocating Erlenmeyer flask.

Yeast yield has also been reported as g dry yeast/g lactose. Reported yields include 62% (160), 35% (4), 23% (48), 48% (127), 38% (35) and 40% (20). Wasserman et al. (169) reported a yield of 55% in the laboratory and 42% in a pilot plant.

Dissolved oxygen. Vananuvat and Kinsella (160) reported that yeast yields increase as medium agitation increase. Wasserman (166) found that peak oxygen demand of actively growing K. fragilis to be 5 mM/1/min. Knight

et al. (76) reported no significant increase in yeast levels when air volume increased above 1 volume/min. However, Giec and Kosikowski (48) found that yeast concentrations increase about 150% when air sparging goes from 1 volume/min to 4 volume/min.

Temperature. Jones et al. (54) reported that optimum growth temperatures for mesophilic strains of the Saccharomyces genus (closely related to K. fragilis) to be between 28 C and 35 C. Knight et al. (76) observed highest yeast growth at 40 C. Penman and Duffus (121) observed the shortest generation time (90 min) in K. fragilis to occur at 30 C. Moresi and Sebastiani (108) found 36 C to be optimal for K. fragilis propagation in whey.

pH. De Sanchez and Castillo (35) reported that K. fragilis can grow over a wide pH range but that significantly superior yeast production and lactose utilization was achieved at an initial pH of 5.0. Whey with an initial pH of 5.0 produces 155%, 125% and 120% more yeast than that produced in whey with initial pH of 3.5, 4.0, and 4.5, respectively. When whey pH is maintained throughout the propagation, whey at pH 5.0 produces 316%, 37% and 2% more yeast than that produced in whey with maintained pHs of 3.5, 4.0, and 4.5, respectively. De Sanchez and Castillo (35) noted that

whey pH would usually increase and then fall to lower than initial pH by the end of the propagation period. These authors reported that initial utilization of lactic acid followed by ammonia assimilation (from added ammonium sulphate) is the reason for these pH changes. They observed no advantage for maintenance of whey pH at 5.0. Amundson (4) decreased the initial whey pH to 3.5 in his propagation procedure for K. fragilis. Knight et al. (76) found there is little difference in final optical density in samples of K. fragilis propagated at pH 4.0, 4.5, 5.0, or 6.0. Moresi and Sebastiani (108) found pH 5.1 to be optimal for propagation of K. fragilis in whey.

Added growth factors. Whey was shown to be a better medium for K. fragilis propagation than a mineral medium consisting of 0.01% magnesium sulfate, 0.014% monopotassium phosphate, 0.08% disodium phosphate, 0.1% sodium chloride, 0.4% diammonium sulfate and 2% lactose (112). When yeast extract was added to the above mineral medium, K. fragilis growth is better than that in whey. Wasserman et al. (164) found a 45% increase in K. fragilis biomass yield when 0.1% yeast extract is added to the whey. Nour El-Dien and Halasz (113) compared growth of K. fragilis in whey (2% lactose) to growth in whey (2% lactose) plus 0.5% yeast extract and found a 32% decrease

in generation time and a 55% increase in net dry weight in the whey supplemented with yeast extract.

Jones et al. (54) reported that S. cereviseae takes up peptides, amino acids, purines and pyrimidines which have been shown to increase yeast yields. Inositol and biotin were also cited as being required by many yeasts for growth.

K. fragilis apparently does not utilize whey protein as a nitrogen source. Wasserman (167) found that K. fragilis utilizes 25% of the whey nitrogen when propagated in whey but that none of this was whey protein nitrogen. K. fragilis can utilize ammonium compounds as a nitrogen source. Nour El-Dien et al. (111) observed an increase in specific growth rate (0.346/h vs 0.325/h) and in yeast dry weight (7g/l vs 5g/l) when K. fragilis is propagated in whey (2% lactose) plus 0.5% diammonium sulfate and 0.5% dipotassium phosphate than when propagated in whey alone. Wasserman (165) observed a 65% increase in net yield when K. fragilis is propagated in whey supplemented with 0.5% diammonium sulphate as opposed to propagation in whey alone. Castillo et al. (20) found a 47% yield increase when K. fragilis is propagated in whey (2% lactose), 0.1% yeast extract, and 0.1% inorganic ammonium salts than when it is propagated in the same medium without ammonium salts. These researchers found ammonium sulphate to be the best nitrogen salt to use. Moresi and Sebastiani

(108) found the optimal concentration of yeast extract and nitrogen salts (1:1 ammonium suplate, dipotassium phosphate) for K. fragilis propagation in whey to be 0.11% and 0.47%, respectively. Amber Laboratories, a principal U. S. yeast manufacturer, adds phosphoric acid, yeast extract and ammonia gas to whey for yeast propagation (34).

Inoculum size. Wasserman et al. (164) found that final yeast concentration is essentially the same but that time for lactose utilization (4% lactose) decreases from 6 to 3 h when initial yeast concentration goes from $3x10^8$ to $2x10^9$/ml. Moresi et al. (107) observed that with initial cell concentration between $0.2x10^6$ and $3.3x10^8$/ml, whey stationary phase is achieved between 24 and 5 h respectively. They confirmed the observation of Wasserman et al. (169) that biomass is independent of inoculum size.

General Anatomy and Physiology of a Yeast Cell. The processes involved in yeast autolysis are better understood when yeast cell anatomy is considered. Yeast hydrolytic enzymes are the primary vehicles responsible for yeast autolysis (3, 61, 96, 142). Shetty and Kinsella (142) reported that around 40% of yeast proteins are hydrolyzed after 5 h incubation at 55 C. Extract of autolysed yeast was found to contain twice the amount of protein and seven times the amount of amino acids than

extract of fresh yeast (10). Ahearn et al. (3) found that K. fragilis has caseolytic activity. Chang et al. (21) reported no proteolytic activity on casein in supernatant of milk where K. fragilis was grown but that autolysed yeasts have caseolytic activity. Matite and Wienken (96) determined yeast vacuoles to be where yeast lytic enzymes are located. They were able to distinguish those enzymes secreted for nutrient metabolization from those enzymes released for cell digestion. These researchers also observed these autolyzing enzymes are released only as the cell approaches death phase. Holzer et al. (65) reported that yeast intracellular proteolytic enzymes are required for protein turnover which adapts the microorganism to new environmental conditions by providing essential amino acids by degrading pre-existing ones. They confirmed that these enzymes are contained in membrane-bound compartments or vacuoles.

Proteolytic enzymes have been found in yeasts (1, 61, 69, 88, 94, 174, 176). Lenney (88) isolated two distinct proteolytic enzymes in S. cerevisiae which are liberated during autolysis. One of these enzymes, proteinase A, had an optimum pH of 3.7; the other enzyme, proteinase B, had an optimum pH of 6.2. Maddox and Hough (69, 94) isolated 4 proteolytic enzymes, A, B, C, and D, from S. carlsbergenesis all with a maximum pH stability of 6.0-6.5. Optimum pH for activity was 7.5, 6.2, 3.5 and

optimum temperature for activity was 35, 50, 50, and 60 C for the proteolytic enzymes A, B, C, and D, respectively. Enzyme A was found to be the most abundant in this group. The products of proteolytic enzyme A are amino acids and low molecular weight peptides. Enzymes B, C, and D produce peptides with molecular weight greater than 5,000. Hough and Maddox (69) speculate that enzyme A acts on the products of enzymes B, C, and D. Wolf (174) reported 7 proteolytic enzymes in S. cerevisiae. Of these, 2 were endoproteinases, 2 were carboxypeptidases, 3 were amino peptidases and one was a dipeptidase. Woods and Kinsella (176) investigated S. carlsbergensis as a potential source of proteolytic enzymes. They isolated a protease which had a pH optima for casein of 3.0 and 6.8. Proteolytic activity was substantially higher at pH 3 than pH 6.8, however. The enzyme was more stable at 45 C than 55 C. Kominami et al. (79) determined proteinase B of S. cerevisiae to have no aminopeptide activity but to have proteolytic activity on trypsin substrates.

Kinsella and Shetty (74) reported that yeast cell walls contain 30-40% mannans, 30-60% glucans, 5-10% proteins and 1% chitin. The cell wall remains intact during autolysis (162).

Production Parameters for Autolysed Yeast Products. Peppler (122, 123) reported detailed procedures for

obtaining autolysed yeast products. Autolysis is induced by heat alone or by heat with added sodium chloride or ethyl acetate. Endogenous yeast lytic enzymes are subsequently activated and digest the cell thereby freeing amino acids, peptides, vitamins, and nucleic acids into the supernatant. Yeast autolysis is induced by slowly heating yeasts to 50 C and then allowing them to remain around 45 C for 12 to 24 h until the desirable concentration of soluble nitrogen is achieved. Peppler (122) reported that around 48% of the yeast cell components are recovered as autolysed yeast extract.

Mechanical (105, 143, 158) and enzymic (77) disruption of yeast cell walls yields more protein than autolysis alone. A yeast protein product can also be produced with acid hydrolysis wherein yeast is heated at 100 C in hydrochloric acid. More yeast protein is recovered in an acid hydrolysate than in an autolysate (10). Amino acid content, however, is lower with acid hydrolysis than with autolysis.

Vosti and Joslyn have done extensive research on conditions for yeast autolysis (55, 56, 162, 163). The major points of their articles are now given.

1. More soluble nitrogen was observed in the supernatant when the incubation temperature is 45 C than when it is 55 C.

2. Cell density has no affect on degree of autolysis.

3. Aerobically grown yeast were found to autolyze more easily than anaerobically grown yeast.

4. Autolysis is markedly greater when the yeast is propagated in a medium deviating from optimum growth pH.

5. Observed phases of autolysis include an 8 h latent period, a 16 h product release period followed by a period of little change in product level up to 24 h.

6. Proteases, peptidases, amino acids, polypeptides, peptides, purines, pyrimidines and vitamins are among yeast autolysis products.

7. No observable yeast cell wall fractionation occurs during autolysis.

8. Toluene, chloroform, and ethyl acetate appear to retard protein hydrolysis during autolysis.

9. Amount of extracellular nitrogen is significantly influenced by the autolysate medium pH. pH optima for a 24 h autolysis in 0.5 M citrate at 45 C is 4.0 for C. lipolytica and a Spanish sherry yeast, 5.0 for S. carlsbergensis, and 4.5 for S. cerevisiae.

10. There is no difference in degree of autolysis in S. cerevisiae autolysed in 0.5 M citrate or 0.5 M phosphate. However, there is more autolyzate products produced in 0.1 M citrate than 0.5 M citrate.

11. Autolysis yields are greater with S. carlsbergensis than with S. cerevisiae, a Spanish sherry yeast or C. lipolytica.

12. Permeability of yeast cell wall largely determines the degree of protein and enzyme extractability.

Hough and Maddox (69) observed that proteolytic activity of yeast grown in absence of growth medium protein in substantially less than the proteolytic activity of yeast propagated in media containing even small amounts of protein such as in Brewer's wort.

Orberg et al. (117) found optimum autolysis conditions of K. fragilis NRRL Y-610 after 12 h incubation without agitation to be 55 C and initial pH of 5.5. They obtained a yeast autolysate comparable in lactic culture stimulation to a commercial yeast extract.

Skupin et al. (144) found that spray drying doesn't affect biological value of vitamins and proteins of bacterial-yeast biomass.

Quantitative Measurement of Autolysate Product. Formol titration has been used to determine protein levels (7, 135), as well as degree of autolysis (12, 110). Vosti and Joslyn (162) did not use formol titration to measure protein content because of the buffering capacity of the autolysate medium; but rather used the napththaquinone

method of Frame et al. (43). Since extracellular nucleic acid concentration increases during autolysis (56, 69) and A260 is linear with nucleic acid content (37), ultra-violet spectrophotometry at 260 nm can be employed to monitor nucleic acid concentration.

Whey Protein Hydrolysate

Isolation of Whey Protein.

Whey protein can be concentrated on an industrial scale by either precipitation and collection (usually by decantation or centrifugation) or by ultra-filtration (52, 134). Modler and Harwalkar (104) reported whey protein ultra-filtration procedures. Typical ingredient composition of ultra-filtered, dried whey (whey protein concentrate) includes 30-70% protein, 20-55% lactose, 3-5% minerals and 4-5% fat (33).

Hydrolysis of Whey Protein.

Whey proteins are cleavable by pepsin, trypsin, papain (8), *Bacillus subtilis* protease (106), and Pronase^{tm} (protease from *Streptomyces griseus*, Cal Biochem) (81). Undoubtedly many other proteolytic enzymes would also be effective. Pronase^{tm} was shown to be about 2.5 times as proteolytic as pepsin (81). Trypsin was shown to cleave only 8% of whey protein peptide bonds (57). Through proteases and peptidases lactalbumin is hydrolyzed to 40% amino acids

and 60% di, tripeptides (Personal communication, technical representative, Sheffield Products, Kraft Inc., 1983).

Hough and Maddox (69) found that proteolysis occurs outside the yeast cell during hydrolysis.

Nutritional Evaluation of Yeast and Whey Protein Products

Lactic culture concentration after propagation in a growth medium is the basis for evaluation of the growth medium. Specifically, the nutrient characteristics of a product added to a base medium is evaluated by the increased (or decreased) final bacterial concentration.

Measurement of Cell Populations in Growth Medium

Milk has long been the most popular medium for lactic culture propagation. Milk, however, is poorly buffered compared to buffered media and cannot produce the cell concentration that buffered medium can (82, 173). Methods to estimate the growth of lactic cultures in milk and other growth media include cell dry weight measurements (25, 63, 71), plate counts (124, 140, 150), optical density measurements (25, 71, 124, 155, 157), and activity tests (6, 14, 62, 68, 157). Activity tests do not directly measure growth medium final cell concentration unless growth and acid production are coupled; but rather give an indication of concentration by the amount of

lactic acid produced by inoculum in milk under spcified temperature and time incubation conditions.

Buffered Lactic Culture Growth Medium. The growth-inhibitory properties of accumulating lactic acid during lactic culture growth in unbuffered media prevents accurate appraisal of an added stimulant since low pH stops culture growth before stimulant depletion occurs. The deleterious effect of reduced medium pH on lactic culture growth is well attested. Harvey (60) found that lactic culture growth is inhibited in medium below pH 5 and that there is a direct relationship between length of storage of lactic cultures in medium below pH 5 and the length of their lag phase when incubated in medium of pH 6.0. Harvey (60) also noticed a reduction in some enzyme activities at pHs below 5.0. Wilkowski and Fouts (171) found that in continuous cultivation of lactic cultures, the growth rate is reduced if the pH falls below 5.3. Wright and Richardson (178) found that lactic cultures propagated in pH controlled nonfat dry milk (7% solids) have 125% greater activity than they have with no pH control in nonfat dry milk. Pettersson (124) found that pH controlled milk produces about 3 times the population produced by unneutralized milk. Other researchers have observed similar results (11, 14, 15, 25, 38, 49, 137, 150).

Various bases have been added as buffering compounds to lactic culture growth medium. Ammonium hydroxide has generally been more effective as a neutralizing agent than has sodium hydroxide (38, 49). Stadhouders et al. (150) found calcium hydroxide better than ammonium hydroxide in neutralizing viable cultures for preservation.

Several internal pH controlled media have been shown to be successful in propagating greater densities of lactic cultures than are possible in unbuffered media. Thomas and Turner (155) were able to achieve 50% greater cell population in milk with .0075 M β-glycerol phosphate than in milk alone. Heap and Lawrence (62) reported similar results. Lamprech and Foster (82) attained 10 times the lactic culture concentration in a medium containing 2.0% magnesium phosphate than that attained in unbuffered milk. Magnesium phosphate is insoluble in the medium initially but solubulizes as acid is produced. These researchers further reported that the amounts of soluble phosphates necessary in the growth medium to maintain the pH above 5.0 during incubation were inhibitory. Sandine et al. (138) reported an internal pH controlled medium that produced cell densities comparable to external pH controlled whey-based medium. This medium is commercialized as PHASE IV. Birlison and Stanley (17) in a field report said PHASE IV performs better than milk for culture propagation and that only a small portion

of lactic cultures did not grow better in PHASE IV medium than in milk.

The contents of PHASE IV was reported by Mermelstein (99) to be sweet whey, autolysed yeast, phosphate and citrate buffers. The patent for PHASE IV lists the suggested contents of the formulation reported by Sandine and Ayres (139) to include 1.5% magnesium phosphate (tribasic), 1.5% diammonium phosphate, 1.5% trisodium citrate dihydrate, 3.5% sweet whey powder and 0.5% yeast extract. Apparently a 1.5% diammonim phosphate concentration in a lactic culture growth medium is not too high to be inhibitory. Zottola and Marth (180) were able to propagate all the lactic cultures they inoculated into a medium containing 2% 1:1 mono-diammonium phosphate.

Phosphate salts have successfully been incorporated into agar plates for buffered enumeration of lactic cultures. Barach (11) improved the enumeration of lactic cultures on Elliker agar plates when he replaced disodium phosphate with diammonium phosphate. Terzaghi and Sandine (152) developed an effective agar medium for the enumeration of lactic cultures by incorporating β-glycerol phosphate in a medium termed M17. Limsowtin and Terzaghi (89) stated that β-glycerol phosphate is a better buffering compound than inorganic phosphates because it does not induce precipitate formation of

calcium and growth inhibition at higher concentrations as the inorganic phosphates do.

Direct Measurement of Medium Cell Concentration. Milk has long been a difficult medium to estimate lactic culture populations by optical density measurements. Hogg and Jago (64) stated that optical density measurements can not be taken in milk because of its opacity and presence of acid coagulum. Kanasaki et al. (71) , however, developed a method using 0.2% EDTA, pH-adjusted to 12.2, to solubulize casein so that optical density measurements could be taken. Hong (66) found precipitates occuring in whey medium which interfered with accurate optical density measurements of lactic culture concentrations in pH controlled whey-based medium. Modifications on the Kanasaki method for optical density measurement as suggested by Thomas was reported by Richardson et al. (133).

Milk Activity Tests. Activity tests have been extensively used to evaluate acid producing capability of lactic cultures (14, 62, 68). Pearce (119) developed an activity test that closely resembles cheese making conditions. Heap and Lawrence (62) emphasized the importance of strict temperature control and the use of nonfat dry milk in order to maintain uniform results. They concluded that any one starter does not produce the same activity test results from day to day even when the

procedure is well regulated. Wolk and Tittsler (175) also emphasized the importance of temperature control in cheese making. Heap and Lawrence (62) used pasteurized reconstituted nonfat dry milk (in boiling water for 10 min) for activity tests after they observed amino acids being released due to autoclaving the milk. Citti et al. (22) observed similar results.

MATERIALS AND METHODS

Cultures

Lactic Cultures

Strains of Streptococcus lactis and Streptococcus cremoris were obtained from the Utah State University culture bank. These cultures were isolated in 1980 at Utah State University by Howard Heap and Gene Hong (131).

Lactic cultures were maintained in frozen storage (-40 C) in sterilized reconstituted nonfat dry milk until use. Prior to frozen storage, 0.1 mL of milk coagulated with the lactic strain was inoculated into 10 mL of sterilized nonfat dry milk and incubated at 27 C for 3 h. At this time the strain had achieved log phase and was put into a freezer at -40 C. A frozen culture was re-activated by removing the test tube of culture from the freezer and incubating it in a 27 C water bath until coagulation (usually 7 h). A 0.1 mL aliquot of this milk was then inoculated into 10 mL of sterilized reconstituted nonfat dry milk and incubated at 27 C until coagulation. Other lactic culture media were then inoculated from this medium.

The Prt- lactic strains were isolated from parent strains using the buffered milk agar plate method of Limsowtin and Terzahgi (89). A lactic strain was plated onto milk agar plates and incubated at 30 C until large

small colonies developed. Small colonies were picked off and inoculated into 10 mL sterilized RNDM and incubated at 22 C. Those isolates requiring more than 2 days to coagulate the milk were identified as Prt- (102). Isolates from the milk agar plate that were able to coagulate the RNDM within 2 days were identified as Prt+.

The Prt- lactic strains used in this research project were: S. lactis UL21, UL7, UL21A, UL18, UL33, UL8; S. cremoris UC91, UC63, UC85, UC169, UC320, UC310, UC161, UC318, UC171, UC73, UC77, UC45, and UC97. Prt+ strains used were S. lactis UL7 and S. cremoris UC171. Prt+ lactic strains were maintained in 10% reconstituted nonfat dry milk and Prt- lactic strains were maintained in 10% reconstituted nonfat dry milk with 0.5 (wt/vol) yeast autolyzate (Amberex 1003, Amber Laboratories, Juneau, WI).

Yeast Cultures

One strain of Kluyveromyces fragilis was obtained from Utah State University microbiology department. K. fragilis strains ATCC-8582 and ATCC-12424 were obtained from the American Type Culture Collection (Professional Services Department, 12301 Parklawn Drive, Rockville, MD).

K. fragilis strains were maintained on refrigerated lactose agar slants.

Enzymes

Pepsin; 1: 1200-2000 units/mg protein (Sigma Chemical Co., St. Louis, MO); and HT-Proteolytic^{tm} (a fungal protease), (Enzyme Products Division, Miles Laboratories, Inc., Elkhart, IN) were used to hydrolyze whey protein.

Media

Reconstituted Nonfat Dry Milk Medium

Reconstituted nonfat dry milk medium (RNDM) consisted of low heat, spray processed pasteurized nonfat dry milk (Western General Dairies, Inc., Ogden, UT) reconstituted to 10% solids (10 g/90 mL deionized water), automatically dispensed (Digital Dispensing Pump, Dynatech Laboratories, Inc., Alexandria, VA) into 10 mL aliquots into 16x150 mm test tubes and autoclaved at 15 psi for 15 min.

RNDM was also used for activity tests. The RNDM for activity tests, however, was not autoclaved but was pasteurized by putting the RNDM-filled test tubes into boiling water until RNDM reached 75 C.

pH Controlled Whey-Based Medium

pH controlled whey-based medium (WB) was composed of 5.0% (wt/vol) spray process pasteurized dry whey (Mulligan Sales, Inc., City Industry, CA), 0.375% monosodium phosphate, 0.375% disodium phosphate (Stauffer Chem Co.),

0.4% yeast extract (Amberex 1003, Amber Laboratories, Juneau, WI) and 0.1% casein hydrolysate (N-Z Amine Type E, Sheffield Products, 1983). N-Z Amine Type E gel electrophoretic analysis showed 23% of the product had molecular weight between 100-200 daltons; 55% was between 200-500 daltons and 22% was above 500 daltons (Personal communication, Technical Salesman, Sheffield Products, 1983). WB was reconstituted with deionized water and heat-treated 90 C for 40 min by immersing the container of WB into boiling water for 45 min.

Lactic Culture Buffered Growth Medium

Lactic culture buffered growth (LABGRO) medium was developed in order to better evaluate yeast and whey protein stimulant products. LABGRO base medium consisted of 0.7% (wt/vol) monosodium phosphate (A.C.S. certified, Fisher Scientific Col, Fair Lawn, NJ), 0.85% monoammonium phosphate (A.C.S. reagent, Matheson, Coleman, and Bell, Norwood, OH), 0.3% trisodium citrate (Pfizer, Chas. Pfizer and Co., Inc., New York, NY), 0.55% diammonium citrate (reagent grade, J. T. Baker Chemical Co., Phillipsburg, NJ), 1.0% magnesium hydroxide (laboratory grade, Fisher Scientific Co., Fair Lawn, NJ), 3.6% whey permeate solids (Hi-Land Dairy, Murray, UT). Deionized water was used to reconstitute the medium. Yeast and/or whey protein products were added to this base medium at 0.5%.

M17 Agar

M17 agar (152) was prepared in 2 L amounts by adding 10 g lactose (analytical reagent grade, Mallinckrodt Inc., St. Louis, MO) and 100 mL deionzed water to a 150 mL Erlenmeyer flask. Ten grams of Bacto-Peptone (Difco Laboratories, Detroit, MI), 10 g Phytone Peptone (BBL, Becton, Dickinson and Co., Cockeysville, MD), 8.4 g beef extract (Difco Laboratories, Detroit, MI), 5 g yeast autolyzate (AYS, Busch Industrial Prod. Corp., St. Louis, MO), 1 g ascorbic acid (Eastman Kodak Co., St. Louis, MO), 38 g disodium beta-glycerol phosphate (grade II, Sigma Chemical Co., St. Louis, MO), and 20 g agar (100 mesh, Sargent-Welch Scientific Co., Skokie IL). Nineteen hundred milliters of deionized water was added and the contents stirred. In another 150 mL Erlenmeyer flask, 1 M calcium chloride was made up. The contents of these three flasks were autoclaved at 121 C for 15 min. Twenty milliliters of 1 M calcium chloride was then added to the lactose solution. The lactose solution was then poured into the agar medium. Fifteen milliter aliquots of liquid M17 agar were dispensed via sterile pump tubing with a dispensing pump (Digital Dispensing Pump, Dynatech Laboratories Inc., Alexandria, VA) into sterile disposable petri dishes (10x1.5 cm, Fisher Scientific Company, Pittsburg, PA).

Buffered Milk Agar

Milk agar (89) consisted of autoclaved 8.9% (% wt/vol) nonfat dry milk, 1.7% disodium β-glycerophosphate and 0.7% agar.

Lactose Agar

Lactose agar slants (19) contained 2.5% (wt/vol) agar, 2.0% lactose, 1.0% peptone, 0.1% yeast extract. Lactose agar medium was autoclaved 15 min at 15 psi.

Yeast Subculture Medium

Yeast subculture medium (19) consisted of 2.0% lactose, 1.0% peptone, and 0.1% yeast extract. Yeast subculture medium was autoclaved 15 min at 15 psi.

Yeast Whey Permeate Medium

Whey permeate medium (18, 48) consisted of 15% whey permeate, 0.5% dipotassium phosphate, 0.5% diammonium sulfate and 0.1% yeast extract. pH adjustments were made with phosphoric acid and ammonium hydroxide. Five percent whey solids whey permeate medium was also used.

Culture Propagation

Lactic culture propagation was initiated by incubating at 27 C both Prt- and Prt+ strains in sterilized RNDM until coagulation occurred. From this

fermented RNDM, pH controlled whey-based medium and lactic culture buffered growth medium were inoculated.

Yeast propagation was initiated by incubating the strains in yeast subculture medium with agitation for 16 h at 27 C. From this medium, yeast whey permeate medium was inoculated.

In pH Controlled Whey-Based Medium

A batch laboratory pH controlling fermentor similar to the one used by Richardson and Pearce (132) was used for pH controlled whey-based fermentations. A recording pH converter (Great Lakes Instruments, Milwaukee, WI) was used. An Ingold pH electrode (cat. no. 465, 120 mm combination electrode, steam sterilizable, Ingold Electrodes Inc., Andover, MA) was steam sterilized in place along with the lid and medium container holder. The container with 1 L heat-treated (40 min 90 C) whey-based medium was put into the holder and covered with the metal lid holding the electrode. The lactic strain was inoculated, generally 1% inoculum, when the medium temperature had reached 27 C. Water was maintained at the appropriate temperature by a water bath (Magni Whirl constant temperature bath, Blue M Electric Company, Blue Island, IL) and pumped into copper tubing, which encircled the whey-based medium container, by a water pump (model 9M818, Dayton Electric Manufacturing Co., Chicago, IL).

WB medium temperature was maintained at 27 C. During the course of fermentation, ammonium hydroxide of known normality was pumped into the WB medium by a pump (Buchler Duostatic Pump, Buchler Instruments, Ft. Lee, NJ) to maintain the pH between 6.1 and 6.3. Weight of injected ammonium hydroxide was recorded.

In Lactic Culture Buffered Growth Medium

The buffering system of lactic culture buffered growth medium contained an insoluble compound at inoculating pH. It was therfore necessary to dispense LABGRO medium with the use of an automatic dispenser (Digital Dispensing Pump, Dynatech Laboratories Inc., Alexandria, VA) while the medium was continuously stirred. Fifteen mL aliquots were thereby dispensed into 25 mL Erlenmeyer flasks. Each flask contained a 3.2 by 12.7 mm stirring rod. The medium was heat-treated by immersing the Erlenmeyer flasks into boiling water for 45 min or by immersing the flasks into 90 C water bath for 45 min. This latter procedure provided a more uniform heat treatment for all the flasks. After heat treatment, the insoluble salts had become caked and were broken up by agitation with the stirring rod. Lactic cultures were inoculated into cooled (22 C) LABGRO medium in Erlenmeyer flasks. These flasks were inserted onto a shaker platform (Lab-line Junior Orbit Shaker, Lab-line Instruments Inc.,

Melrose Park, IL). The shaker was set at 150 rpm. The shaker was located in an air incubator (Fisher Low Temperature Incubator, GM Corp., Dayton, OH) set at 27 C. Cultures were incubated for 19 h.

In Yeast Subculture Medium

Two inoculating loopfuls of culture growing on lactose agar slants were inoculated into a 150 mL Erlenmeyer flask containing 70 mL lactose medium. The flask was inserted on a shaker (Labline Junior Shaker, Labline Instruments Inc., Melrose Park, IL), shaken at 350 rpm while it was incubating at 27 C for 16 h.

In Yeast Whey Permeate Medium

Seventy milliliters of yeast-grown subculture medium was added to 4 L of heat-treated (95 C for 30 min) (48) whey permeate medium which was either adjusted to pH 4.5 (117) with phosphoric acid or hydrochloric acid; or left unadjusted (pH 5.5). Yeast propagation was carried out in 4 l quantities in a Microferm bench top fermentor (model MF-107, New Brunswick Scientific Co. Inc., New Brunswick, NJ). Incubation temperature was 30 C (35, 48, 121). Aeration rate was initially maintained at 2 volumes filtered air/volume medium/min but was later increased to 4 volumes air/volume medium/min. Agitating impeller rate was set at 800 rpm. Dissolved oxygen (ppm) was monitored with an oxygen electrode (see Dissolved Oxygen in Yeast

Whey Permeate Medium section). Propagation conditions were maintained until stationary phase was achieved. Anti-foam spray (Antifoam A Spray, Dow Corning Corp., Midland, MI) or Marschall defoamer (silicone-based emulsion, Marschall Products, division Miles Laboratories, Madison, WI) was added to medium as needed.

K. fragilis was also propagated simulataneously in six 250 mL Erlenmyer flasks containing yeast whey permeate medium. One hundred seventy-five milliliters of yeast whey permeate medium was added to flasks and heat treated to 90 C for 40 min. Filtered, humid air was delivered to each of the flasks. Media was agitated by stirring rods placed in each flask and rotated by a multiple stirring plate (LABLINE Multi-Magnestir, LABLINE Instruments Inc., Melrose Park, ILL.)

Yeast Autolysis

Yeast was concentrated to various levels by either natural sedimentation or by centrifugation (Lourdes, Beta-Fuge, Model A-2, Lourdes Instrument Corp., Brooklyn, NY). Natural sedimentation of yeasts was carried out by allowing whey medium to remain undisturbed until sedimentation occurred and then siphoning off the supernatant. Sedimented yeast was stored in an ice bath until autolysis was begun. The method of autolysis as prescribed by Peppler (123) and modified by Orberg et al.

(117) was used to induce autolysis in yeasts. Yeast dry weight (see Yeast Dry Weight section) was determined on the yeast concentrate. Yeast concentrate was pH-adjusted with 15 M phosphoric acid (reagent A. C. S., Baker and Adamson, General Chemical Division, Morristown, NJ) or 28% ammonia, reagent ammonium hydroxide (reagent A. C. S., Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey) and then slowly warmed to 50 C. The yeast concentrate was moderately agitated until autolysis was complete. Later a temperature of 55 C was selected and no agitation was used during yeast autolysis. Formol titration measurements were the basis for determining when autolysis was complete (110). The effect of yeast autolyzing enzymes on whey protein breakdown was studied by adding amounts of freeze-dried isolated whey protein to autolyzing yeast.

Quantitative Measurements

Acid in Fermented pH Controlled Whey-Based Medium

The initial and final weight of the ammonium hydroxide container for WB medium fermentation was taken. The concentration of lactic acid (lactate) in 1 liter of WB medium during the course of fermentation was calculated according to the formula: Y = AxBx90.08; where Y = grams lactic acid/L WB medium, A = normality of ammonium

hydroxide base, B = L of added base (converted from weight of base). The molecular weight of the acid used was 90.08.

Lactic Culture Plate Counts

Plate counts were made on lactic cultures using M17 medium. Appropriate dilutions were made with 0.1% peptone and plated onto 10 cm disposable petri dishes (Fisher Scientific Products) by the Spiral Plater (Spiral System Inc., Cincinnati, OH). Inoculated plates were incubated 24 h at 30 C before counting. In order to obtain more accurate colony forming unit counts, sufficient dilutions were made so as to be able to count colonies over the total plate area. Usually this required a $1x10^{-6}$ dilution on WB medium and LABGRO medium.

Lactic Culture Optical Density

A spectrophotometer (Beckman DB-G, Beckman Instruments Inc., Fullerton, CA) was used for all optical density readings. All optical density readings were taken at 480 nm with disposable plastic cuvettes (Fisherbrand styrene cuvets, 10x10x45 mm volume, Fisher Scientific Company, Pittsburgh, PA).

In Reconstituted Nonfat Dry Milk Medium. The method of Kanasaki et al. (71) reported by Richardson et al. (133) was used to obtain optical density measurements in RNDM. Optical density was expressed as OD(480nm) where

OD(480 nm) = ((Absorbance at 480 nm (medium + culture)) - ((Absorbance at 480 nm (medium)) x Dilution Factor.

For RNDM optical density readings it was not necessary to dilute the milk sample. A solution of 0.2% ethylene-diaminetetra-acetate in deionized water (EDTA) solution) was adjusted to pH 12.2 with 10 M sodium hydroxide. Aliquots of 4.5 mL were dispensed into 13x100 mm test tubes with an automatic dispensor (Digital Dispensing Pump, Dynatech Laboratories Inc., Alexandria, VA). An optical density measurement on RNDM was performed by pipetting exactly 0.5 mL RNDM sample into the test tube containing 4.5 mL EDTA solution, gently drawing the mixture into the pipet three times to assure thorough mixing with minimal mixing in of air bubbles, pouring the mixed solution into a cuvette, inserting the cuvette into the spectrophotometer and taking the reading. The time for preparing and reading each sample was uniform.

In pH Controlled Whey-Based Medium. Higher cell concentrations in pH controlled whey-based medium required that a 1/10 dilution be made. Optical density readings on whey-based medium was performed by adding exactly 0.5 mL of WB to 4.5 mL EDTA solution, mixing thoroughly with a pipette, adding 0.5 mL of this solution to another tube of 4.5 mL EDTA solution, again mixing thoroughly with a pipet and then pouring a sample into a cuvette and inserting the

cuvette into the spectrophotometer to take the reading. Sample preparation time for spectrophotometry was uniform. Figure 1 confirms OD(480 nm) WB and cell concentration in WB to have an increasing relationship.

In Lactic Culture Buffered Growth Medium. There were no milk proteins in LABGRO medium due to the use of whey permeate solids rather than whey solids. The insoluble salts of LABGRO medium which were remaining after fermentation were dissolved by adding sufficient phosphoric acid to bring the LABGRO medium pH to 4.0. The medium was continually being mixed by the stirring rod during this acid addition. All salts were solubulized when the medium pH went to 4.0. Sample preparation time for spectrophotometry was uniform.
Figure 2 confirms OD(480 nm) in LABGRO and cell concentration in LABGRO to have an increasing relationship.

Conversion of RNDM Optical Density into Estimated Colony Forming Units/mL RNDM

Estimated colony forming units of lactic cultures propagated in 10% RNDM medium was obtained from medium optical density by the equation: 100 Million CFU/ml 10% RNDM = 1.168 + 6.066(OD(480nm) 10% RNDM). The analysis of variance and regression analysis from which this equation

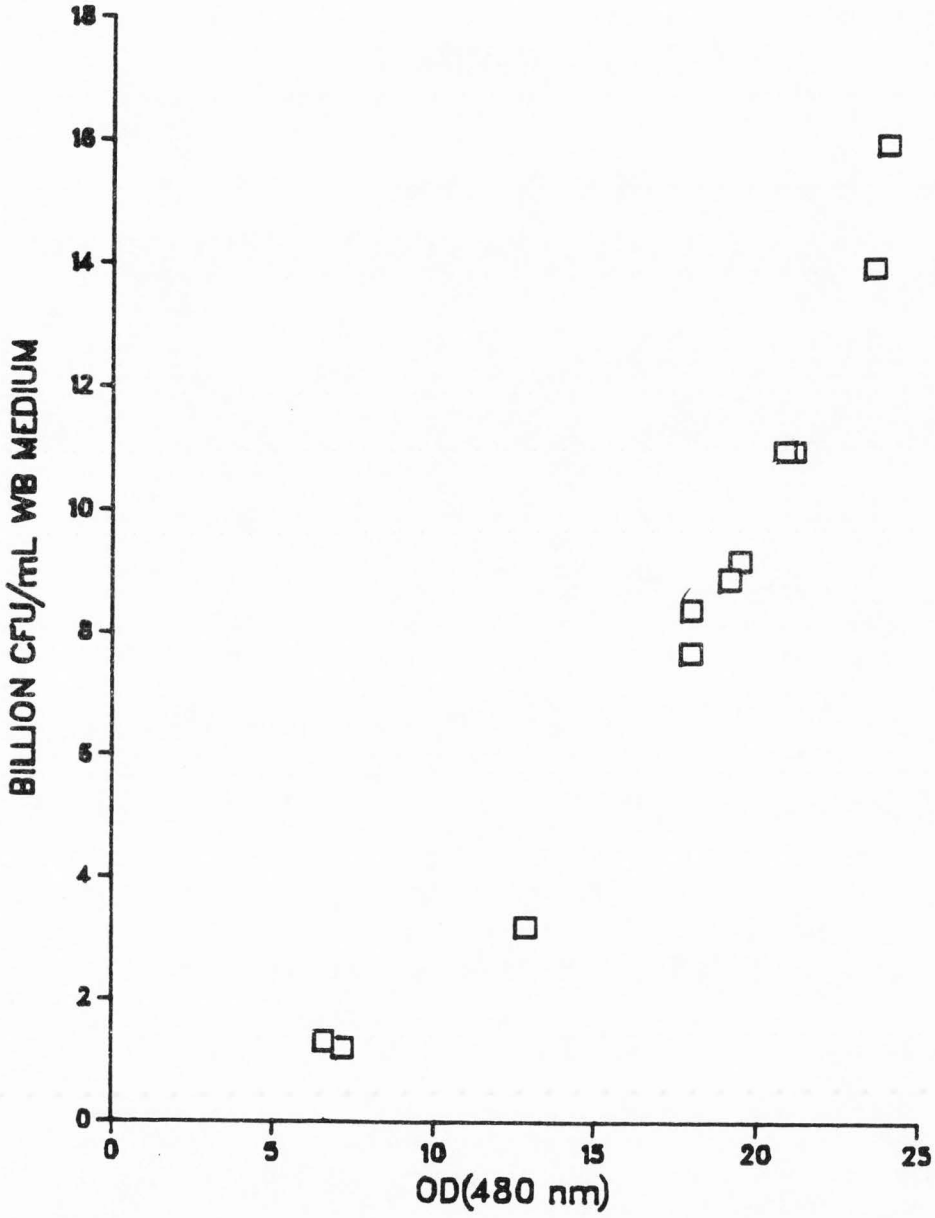

Figure 1. Relationship of cell concentration (billion CFU/mL pH controlled whey-based medium) to OD(480 nm) pH controlled whey-based medium.

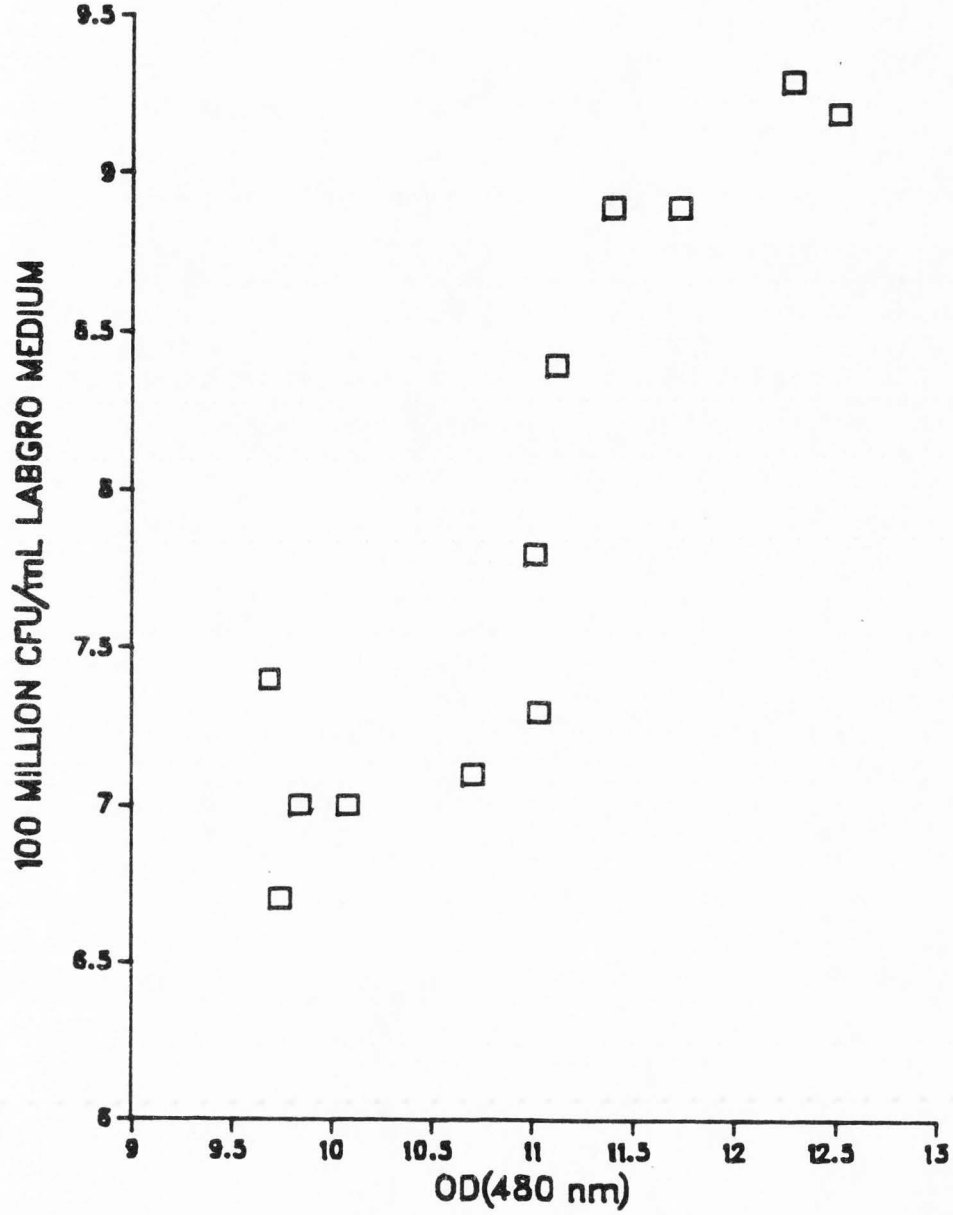

Figure 2. Relationship of cell concentration (100 million CFU/mL LABGRO medium) to OD(480 nm) LABGRO medium.

Table 2. Analysis of variance for estimated cell concentration (100 million CFU/mL 10% RNDM *) from OD(480 nm) 10% RNDM.

Source	DF	Mean Square	F Value	Pr > F
Model	1	1480.80	659.77	.0001
Error	17	2.24		

R^2 = .975

Table 3. Regression analysis of cell concentration (100 million CFU/mL 10% RNDM) from OD(480 nm) 10% RNDM.

Parameter	Estimate	Std Error of Estimate	T for HO: Parameter=0	Pr > |T|
Intercept	1.168	.455	2.57	.0200
OD(480 nm)	6.066	.236	25.69	.0001

* Reconstituted nonfat dry milk.

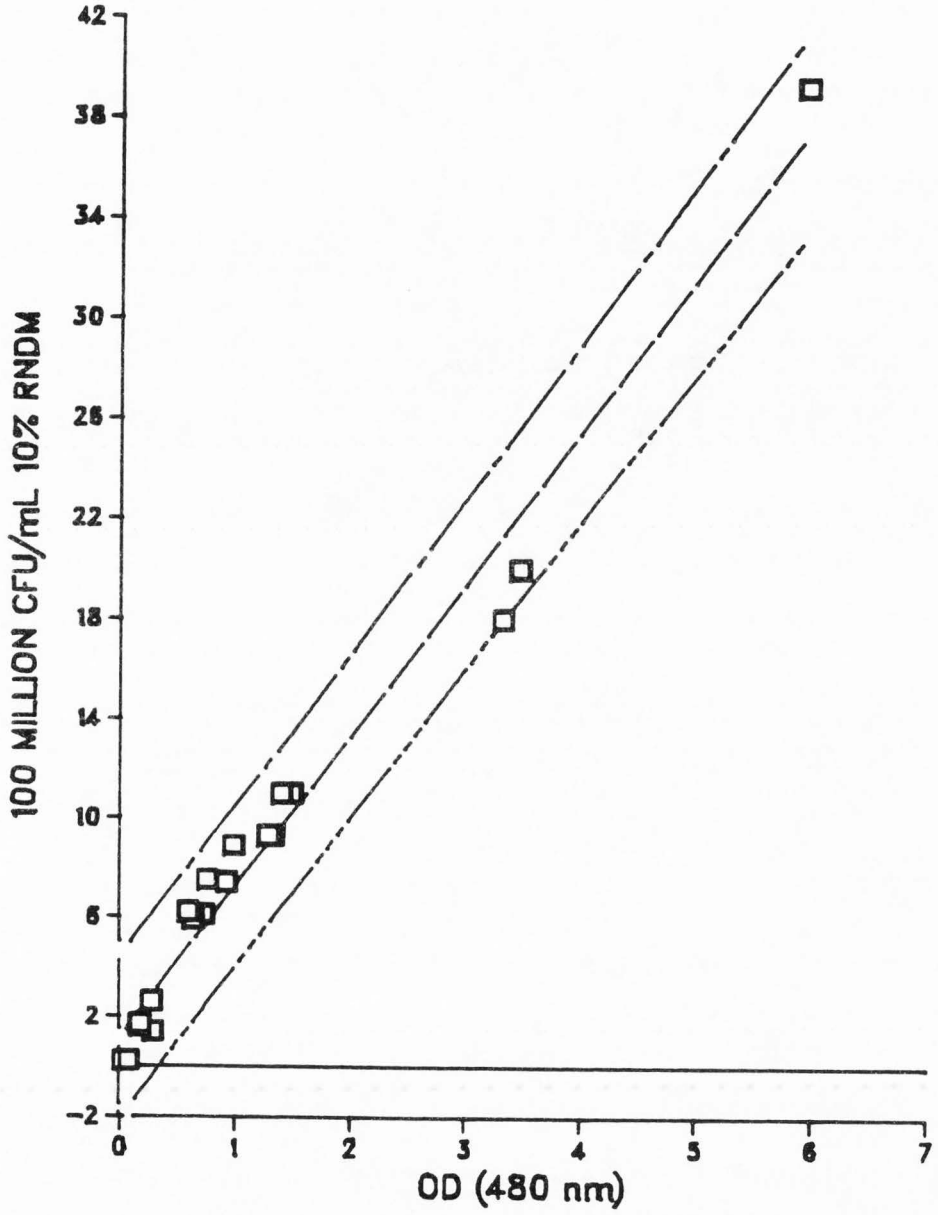

Figure 3. Linear regression of cell concentration (100 million CFU/mL 10% reconstituted nonfat dry milk) and OD(480 nm) 10% RNDM. (See tables 2 and 3). 100 million CFU/mL 10% RNDM = 1.168 + 6.066(OD(480 nm) 10% RNDM). 95% confidence curves are drawn.

was obtained are given in tables 2 and 3. The plot of this relationship is given in Figure 3.

Buffering Strength of Medium Formulations

Buffering strength of medium formulations was evaluated by inserting a 50 mL syringe filled with 10% lactic acid solution (A. C. S. specified, J. T. Baker Chemical Col, Phillipsburg, NJ) into an automatic syringe pump (Sage Model 341, Sage Instruments, Division Orion Research inc., Cambridge, MA). The automatic syringe delivered 10% lactic acid at 1.1 ml/min. A combination pH electrode (Sensorex, Westminister, CA) attached to a pH recorder (Sargent-Welch Scientific Co., 7300 N Linden Ave, Skokie, IL) monitored pH. Fifty milliliters of formulated medium was tested at a time. Buffering strength between pH 6.8 (initial pH varied between pH 7.0 and pH 6.6) and pH 5.2 of each formulation was evaluated by amount of 10% lactic acid solution required to bring buffer pH to 5.2.

pH Measurements

pH measurements were performed with an Altex 060 pH meter (Beckman Instruments Inc., Fullerton, CA) and a Ross combination pH electrode(model 81-02, Orion Research Inc., Cambridge, MA). An Altex Thermo Compensator (531550, Beckman Instruments, Inc., Fullerton, CA) was used.

Acid in Fermented Reconstituted Nonfat Dry Milk Medium

Lactic acid of known normality was added by a buret in increments to pasteurized 10% RNDM used for activity tests. The pH reading of the RNDM after each acid increment addition was recorded. Since the amount of and normality of the added lactic acid was known, lactic acid concentration in the RNDM was calculated as follows. mg lactic acid /mL 10% RNDM = (mL lactic acid solution added)(Normality lactic acid solution/1000)(MW lactic acid) / total 10% RNDM volume. Variation in buffering capacity among bags of nonfat dry milk necessitated that the same bag of nonfat dry milk be used throughout these experiments.

Conversion of RNDM pH into Estimated Lactic Acid Concentration

Estimated lactic acid produced in 10% RNDM by lactic cultures was obtained from the equation: mg Lactic Acid/ml 10% RNDM = $43.304 - 12.086(pH) + .840(pH^2)$. The analysis of variance and regression analysis from which this equation was derived are given in tables 4 and 5. The plot of this relationship is given in Figure 4. This prediction for lactic acid is accurate only for the bag of RNDM used for this research due to variation of buffering capacity among lots of RNDM.

Table 4. Analysis of variance for lactic acid concentration (mg/mL 10% RNDM) and 10% RNDM pH.

Source	DF	Mean Square	F Value	Pr > F
Model	2	149.790	32125.51	.0001
Error	115	.005		

R^2 = .998

Table 5. Regression analysis of lactic acid concentration (mg/mL 10% RNDM) from 10% RNDM pH.

Parameter	Estimate	Std Error of Estimate	T for HO: Parameter=0	Pr > |T|
Intercept	43.304	.583	74.22	.0001
pH	-12.086	.211	-57.19	.0001
pH^2	.840	.019	44.28	.0001

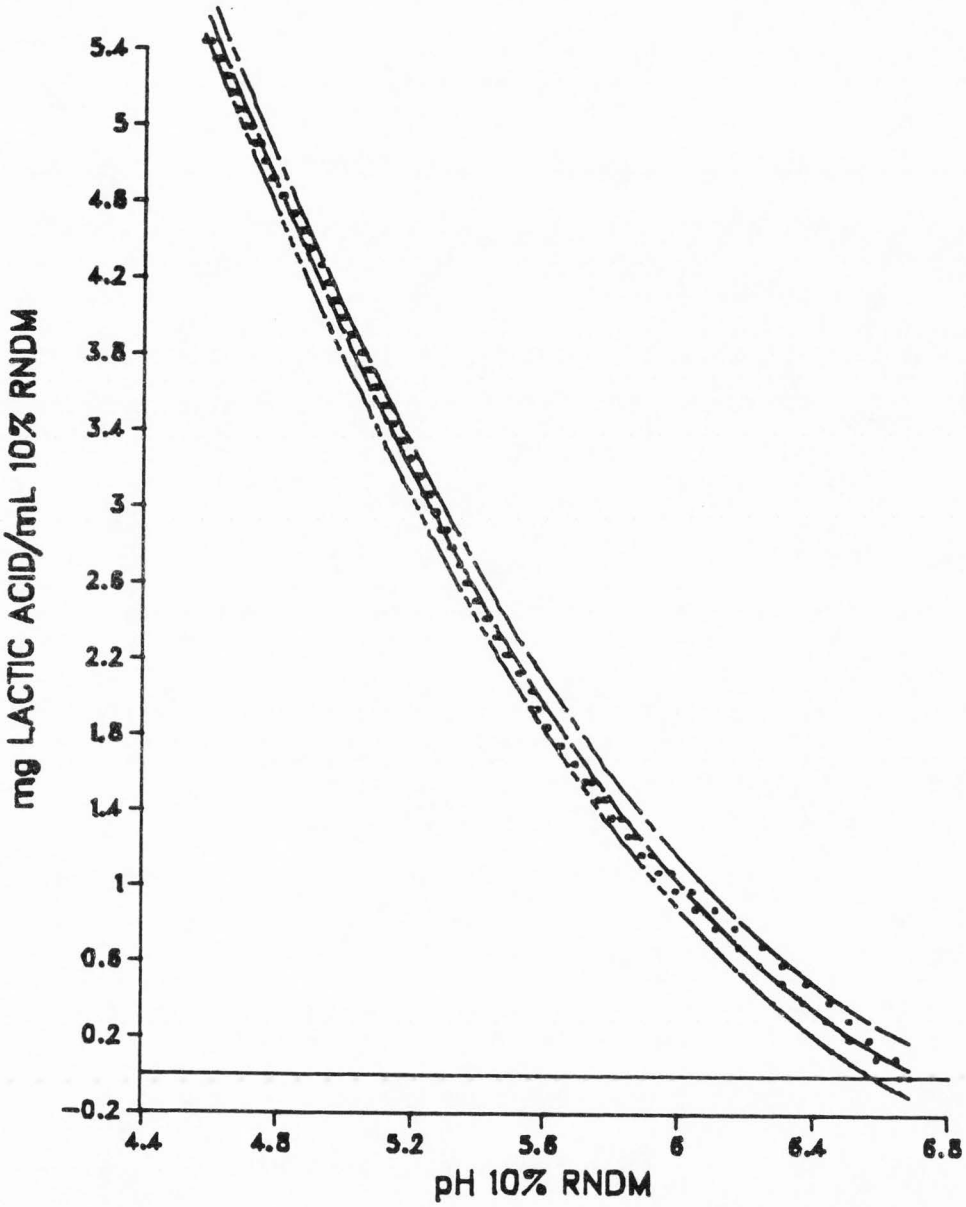

Figure 4. Quadratic regression of lactic acid concentration (mg/mL 10% reconstituted nonfat dry milk) and 10% RNDM pH. (See tables 4 and 5). mg lactic acid/mL 10% RNDM = 43.304 - 12.086(pH 10% RNDM) + .840(pH 10% RNDM)^2. 95% confidence curves are drawn.

Lactic Culture Activity Tests

All activity tests were performed with the same bag of spray process dry pasteurized nonfat dry milk (Western General Dairies, Ogden, UT). Activity tests were performed with RNDM heated to 75 C and immediately cooled to 4 C.

Pearce Activity Test. The Pearce activity test was modified to exclude the use of rennet. It was performed by inoculating 10 mL pasteurized, cold (4 C) RNDM with 0.2 mL of fermented WB or LABGRO medium of the particular culture, mixing, taking the initial pH and incubating duplicate test tubes according to the schedule illustrated in Table 6. After incubation, the test tubes were immersed into an ice bath and final pH readings were taken on the RNDM. Activity measurements were expressed either as Δ pH units (initial RNDM pH - final RNDM pH) or as estimated acid concentration (see Acid in Fermented Reconstituted Nonfat Dry Milk Medium section).

High Temperature Activity Test. A high temperature activity test (133) was performed by inoculating into separate test tubes containing 10 mL of cold (4 C) RNDM, 0.05, 0.1, 0.2, 0.5, 0.9 mL of fermented WB or fermented LABGRO medium. The initial pH of the tubes were taken. Duplicate samples of the milk along with a control sample with no inoculum was then incubated 5 h at 38 C. All

Table 6. Temperature, time schedule for the Pearce activity test[1].

Minute Interval	Interval Temperature (C)	Interval Time Fraction
0-70	32	.23
70-75	33	.02
75-80	34	.02
80-85	35	.02
85-90	36	.02
90-95	37	.02
95-100	38	.02
100-115	38	.05
115-260	37	.48
260-268	36	.03
268-276	35	.03
276-284	34	.03
284-292	33	.03
292-300	32	.03

[1] Pearce, L. E. 1969. Tests for Cheese Starter Cultures. New Zealand J. Dairy Technol. 4:246.

tubes were then immersed in ice water for 10 min and final pH readings were taken. Results were expressed the same as in Pearce activity test. Some activity tests were done as described but 30 C was the incubating temperature.

Growth Potential in Reconstituted Nonfat Dry Milk Medium of Individual Lactic Strains

Lactic strains were characterized according to their acid production and growth in 10% RNDM after 5 h incubation at 38 C or 30 C. The lactic strains to be characterized were initially propagated in WB or LABGRO medium. The fermented medium was added in 0.05, 0.1, 0.2, 0.5, and 0.9 mL aliquots into test tubes containing 10 ml aliquots of 10% RNDM. After the cultures were mixed into the RNDM, optical density measurements were taken of the RNDM samples. The test tubes were then incubated at 38 C or 30 C for 5 h and then placed in an ice bath for 10 min before pH and optical density measurements were taken. RNDM pH after 5 h culture incubation was converted into mg lactic acid/mL 10% RNDM according to the prediction equation in Figure 4. When 10% RNDM acid concentration was plotted against initial optical density of the inoculated 10% RNDM, a hyperbola-like curve similar to the Michaelis-Menton enzyme kinetics curve was observed. The equation describing the Michaelis-Menton curve, i.e., $Y = (AX)/(B+X)$, was selected to describe the relationship

observed between initial cell concentration in RNDM and final acid concentration in RNDM as the initial cell concentrations increased. Y was set equal to the final acid concentration in 10% RNDM. X was set equal to the initial cell concentration (OD(480nm) in 10% RNDM. The A and B coefficients were estimated by the nonlinear least squares approximation curve fitting program of Ruckdeschel (136) with the aid of an Apple Computer (Apple II Plus, Apple Computer Inc., Cupertino, CA). When the A and B coefficients were determined by the computer an equation was achieved that closely predicted the initial cell concentration vs final acid concentration of the inoculated RNDM.

Each strain was characterized according to what predicted initial cell concentration (OD(480nm)) in 10% RNDM was required in order to produce an acid concentration of 2.5 mg lactic acid/ml 10% RNDM or to produce a final cell concentration of 1.46 OD(480nm) after a 5 h incubation at a set temperature. These values were chosen because they approximate a 10% RNDM pH of 5.4 and a cell concentration in 10% RNDM of 1×10^9 CFU/ml, respectively. Predicted initial cell concentration (OD(480 nm)) was derived for each strain by rearranging the above equation and solving for X : $X = (BxY)/(A-Y)$. Y was either 2.5 or 1.46 depending on which criteria was desired to characterize the strain.

Lactic strains were further characterized according to their number of generations in 10% RNDM at 38 C during a 5 h incubation period. OD(480nm) of inoculated 10% RNDM was converted to estimated 100 million CFU/1 10% RNDM by the prediction equation in Figure 1. Number of generations during the 5 h period was calculated according to : $k = \log_{10}(Xt) - \log_{10}(X_0)/0.301$

where k is the number of doublings in 5 h.

Lactic strains were also characterized according to cellular acid rate expressed as pg lactic acid produced / cell. The average cell number during the 5 hour incubation period was determined for each level of initial cell concentration by adding the logs of the initial and final cell concentration, dividing by 2 and taking the antilog. Microbial cellular expressions of this kind have been reported by other authors (2).

Lactose in Yeast Whey Permeate Medium

Lactose in yeast whey permeate medium was measured enzymically (Lactose/Galactose spectrophotometric test, Boehringer Mannheim Biochemicals, Indianapolis, IN). The enzyme kit included citrate buffer plus NAD, pH 6.6; about 100 units of β-galacosidase suspension, potassium diphosphate buffer, pH 8.6; and about 35 units of galactose dehydrogenase suspension. All solutions were added to the 1 cm disposable cuvette used for

spectrophotometric readings. The whey permeate medium was diluted as required with distilled water. The blank was prepared by mixing 0.20 mL of citrate buffer plus NAD and 0.05 mL of β-galactosidase suspension. After 10 min 1.00 mL of potassium phosphate buffer and 2.00 mL of distilled water were added. These were mixed and absorption at 365 nm was read after 2 min (A1b). Then 0.05 mL of galactose dehydrogenase suspension was mixed and absorbance at 365 (A2b) was read after 15 min. Then 0.10 mL of diluted whey sample was mixed with 0.20 of citrate buffer plus NAD and allowed to sit for 10 min. Then 1.00 mL of potassium diphosphate buffer and 1.95 mL of distilled water was mixed in and absorption at 365nm was read after 2 min (A1s). To this, 0.05 mL of galactose dehydrogenase suspension was mixed in and absorbance at 365nm (A2s) after 15 min. Absorbance (ΔA) = As - Ab. (As = As2 - As1; Ab = Ab2 - Ab1). The concentration of lactose in the original whey permeate medium sample (g/liter) was determined by the equation:

$$c = (VxMW/Exvxdx1000)xFxA;$$

where V = final volume, v = sample volume, MW = lactose molecular weight, d = cm of cuvette light path, E = absorption coefficient of NADH at 365 nm and F is the dilution factor.

Dissolved Oxygen

Dissolved oxygen (ppm) was monitored by an oxygen electrode (mode 97-08 Orion Research Inc., Cambridge, MA) connected to a pH meter (Corning pH meter, model 7, Corning Glassworks, Corning, NY).

Yeast Optical Density in Yeast Whey Permeate Medium

Optical density readings were measured with the spectophotometer at 560 nm (16). Samples were prepared by transferring exactly 10 mL of incubating yeast whey permeate into a 12 mL centrifuge tube (Kimax). This sample was then spun in a bench centrifuge (International Clinical centrifuge, International Equipment Co., Needham Heights, MA) at setting 5 for 10 min. The supernatant was discarded and replaced with 10 mL of 0.2 M sodium acetate, pH 5.8 (technical grade, Baker and Adamson, General Chemical Division, New York, NY). This sample was mixed with a vortex mixer (model K-550-G, Scientific Industries Inc., Bohemia, NY) and centrifuged in the same manner. Again the supernatant was discarded and 10 mL of new 0.2 M sodium acetate buffer was added to the sample and mixed. One tenth milliliter of this mixture was added to 10 mL 0.2 M sodium acetate, pH 5.8 and vortexed. Optical density was read at 560 nm and optical density (OD(560nm) was determined according to the formula:

OD(560 nm) = ((Absorbance at 560 nm (sample) - (Absorbance at 560 nm (bank)) x Dilution Factor.

Yeast Dry Weight

Yeast dry weight was determined according to the A O A C method for total solids determination of yeast (67). Ten milliliters of yeast-grown whey permeate medium was added to a 15 mL centrifuge tube (Kimax). The sample was centrifuged in a bench top centrifuge and the supernatant was discarded and replaced with 10 mL of deionized water and then mixed with the yeast sediment with a mixer. This procedure was then repeated. The washed resuspended yeast cells were then added to a metal weighing dish along with about 5 g of sea sand (washed, ignited, Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, NJ) and mixed together with a small glass rod. The weight of these contents (including the glass rod and weighing pan lid) was then determined to the nearest mg. Five milliliters of ethanol was then added and thoroughly mixed in with the glass rod. The entire contents of the weighing pan were dried for 3 h at 105 C in an air oven (Thelco, Model 18, GCA/Precision Scientific, Chicagao, IL), cooled in a dessicator for 20 min and weighed. Yeast dry weight was expressed as g dry yeast/L yeast whey permeate medium.

Table 7. Analysis of variance for yeast dry weight (g/L whey permeate medium) and OD(560 nm) whey permeate medium.

Source	DF	Mean Square	F Value	Pr > F
Model	2	225.195	208.99	.0001
Error	18	1.078		

$R^2 = .959$

Table 8. Regression analysis for yeast dry weight (g/mL whey permeate medium) and OD(560 nm) whey permeate medium.

Parameter	Estimate	Std Error of Estimate	T for HO: Parameter=0	Pr > |T|
Intercept	8.610	3.067	2.81	.0116
OD(560 nm)	-.295	.135	-2.19	.0424
OD(560 nm)^2	.007	.001	4.97	.0001

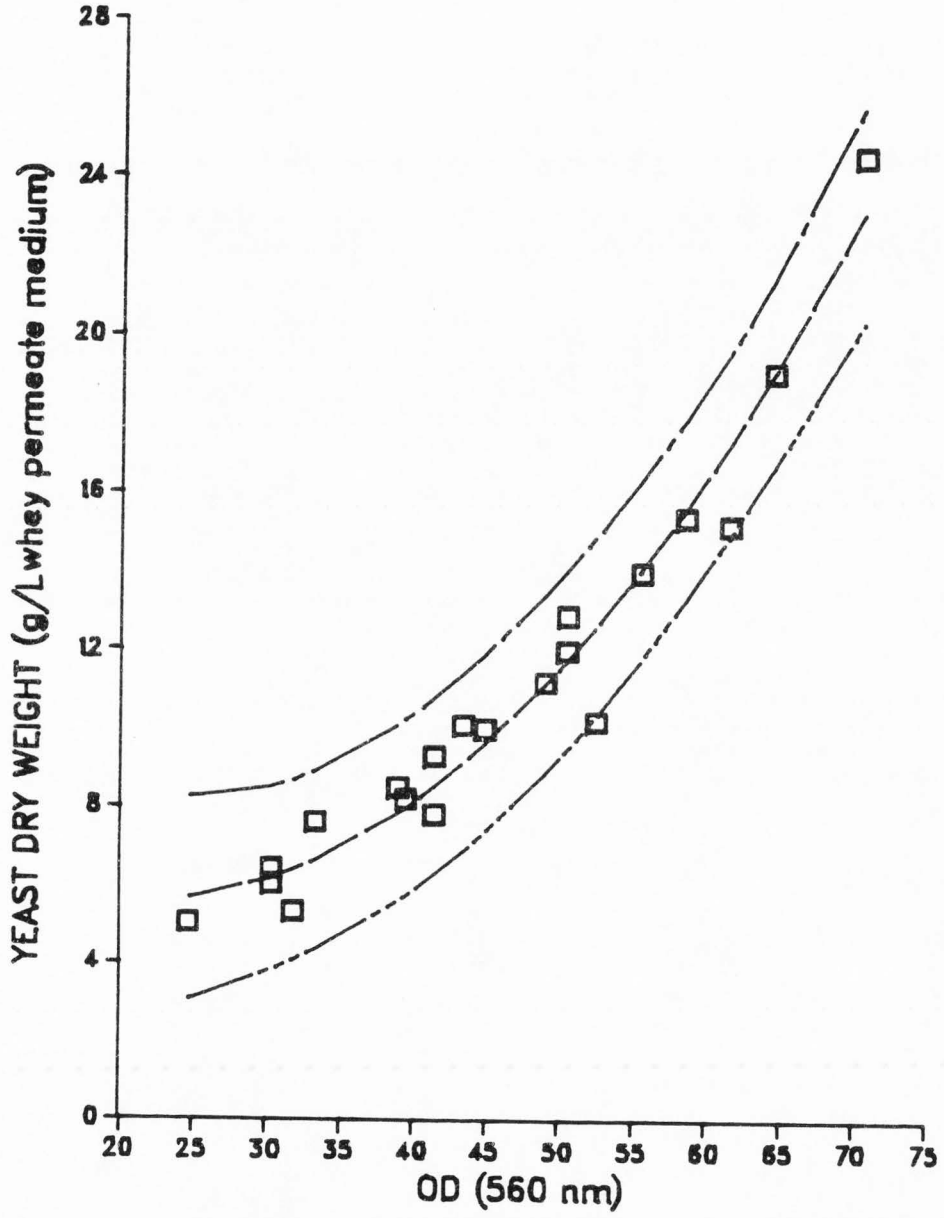

Figure 5. Quadratic regression of yeast dry weight (g/L whey permeate medium) and OD(560) whey permeate medium. (See tables 7 and 8). g dried yeast/L whey permeate medium = 8.610 - .296(OD(560 nm)) .007(OD(560 nm))^2. 95% confidence curves are drawn

Conversion of Medium Optical Density into Estimated Yeast Dry Weight

Estimated yeast dry weight (g/L) in whey permeate medium was predicted from OD(560 nm) of whey permeate medium by the equation: yeast dry weight (g/mL whey permeate medium) = 8.160 - 0.295(OD(560 nm) + .007(OD(560 nm))^2. The analysis of variance and regression analysis from which this equation was obtained are in tables 7 and 8. The plot of this relationship is given in Figure 5.

Autolysate Products

Autolysate products were monitored by formol titration and ultra-violet absorption (absorbance index).

Commercial Yeast Products. Various commercial yeast products were nutritionally evaluated for lactic culture growth. Yeast products of the following companies were evaluated: Busch Industrial Products Corp., St. Louis, MO: Product 2313 (yeast extract); AYE 2312 (yeast extract), AYS (yeast autolysate).

Amber Laboratories, Juneau, WI: Amberex 1003 (yeast extract), Amberex 1400 (yeast autolyzate), Prymex 212 (yeast extract), EP 440 (yeast autolysate).

BBL Microbiological Systems, Becton Dickinson and Col, Cockeysville, MD: BBL yeast extract.

Pure Culture Products, Inc., Chicago IL: ZYEST-70H (yeast autolysate).

Ardamine "Yes" Yeast Products Co., Patterson, NJ: Ardamine "Yes" yeast extract.

Formol Titration. Amount of protein hydrolysis in supernatant of yeast autolyzate was determined by formol titration (110). Formol titration was carried out by adding 20 mL of concentrated yeast of known solids content to a Karl Fisher automatic titration instrument cup (Multi-Dosimat E415, Metrohm Herisau, Switzerland). A combination pH electrode (Sensorex, Westminister, CA) attached to a pH meter (Corning pH meter model 10, Corning Glassworks, Corning, NY) was inserted into the cup to monitor pH of the yeast mixture. Drops of 1 M NaOH were added initially to get the yeast mixture pH close to 8. Thereafter sodium hydroxide of known normality was added until the yeast mixture pH reached 8.3 (phenolthalein endpoint). Four and a half milliliters of 37% w/w formaldehyde (certified A. C. S., Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, NJ) was then added via the automatic titrimeter into this cup. The yeast mixture was then titrated to pH 8.3 with 0.1 M NaOH. The automatic titrimeter recorded the amount of base added. From this amount of base was subtracted the amont of 0.1 M NaOH required to bring 4.5 mL of the formaldehyde to pH 8.3. The amount of protein and extent of protein hydrolysis was measured by formol titration

units expressed as mmoles NaOH/g yeast solids and added milk protein solids. Yeast solids were estimated by turbidity according to the prediction equation in Table 12.

Absorbance Index. Protein and protein hydrolyzate concentration was monitored indirectly by ultraviolet absorption measurements. Spectrophotometric measurements (Beckman DB-G, Beckman Instruments Inc., Fullerton, CA) were taken at 260 nm where yeast nucleic acids absorb optimally (37). Yeast autolysate was diluted with deionized water to give a concentration of approximately 0.2 to 0.5 mg yeast solids/mL prior to taking the absorption reading. A quartz cuvette (Precision Cells Inc., Hicksville, NY) was used for all ultra-violet absorption measurements. The amount of protein and extent of protein hydrolysis as measured by absorption index was expressed as (Absorbance 260 nm/mg yeast solids/ml)x100.

Whey Protein Isolation

Whey protein was isolated (52) by reconstituting spray process pasteurized dry whey (Mulligan Sales, Inc., City Industry, CA), adjusting pH between 6.3 and 6.5 and heating the whey to 90 C with constant stirring. One hundred milliliters of 33% acetic acid/45.4 kg of whey was then added with stirring until the acid was thoroughly

mixed in. When whey protein had coagulated it was collected by filtration and freeze dried at 35 C (Dura-Dry, FTS Systems Inc., Stone Ridge, NY). Freeze dried whey protein was stored at 4 C until used.

Statistical Analysis

All statistical analysis was performed with an IBM 4341 using the SAS (Statistical Analysis System, SAS, Cary, NC).

RESULTS AND DISCUSSION

Development of an Internal pH Controlled Medium for Evaluation of Yeast, Protein Hydrolysate Products

An internal pH control buffering system was preferred to an external system to evaluate bulk medium nitrogen sources in the laboratory because more nutrient sources could be evaluated concurrently. The inclusion of buffering compounds in culture media that largely neutralizes lactic acid produced by metabolizing cultures is what constitutes a medium being termed internal pH control medium. An internal pH control medium was considered to give better repeatability and to be much simpler than the method we used previously wherein drops of ammonium hydroxide were manually added (178).

A buffering system was desired to be equal or better than those available in commercial internal pH control media and to be void of significant nitrogenous compounds.

First, soluble salts were incorporated into medium formulations and compared against PHASE IV^{tm}, a commercial internal pH control bacteriophage inhibitory medium. Table 9 shows that none of the soluble salt formulations were comparable to PHASE IV^{tm} in buffering strength. Since experitmental results shown in tables 9, 10, 11 and 12 were exploratory in nature no duplicates were run.

Table 9. Buffering of media and salt formulations. The milliliters of 10% lactic acid solution to drop the pH from 6.6-7.0 to pH 5.3 were determined.

Formulation	10% Lactic Acid (mL)
3.0[1] BGP[2] 1.8 monoSP 0.2 diSP	7.7
5.0 BGP	12.1
3.0 BGP 1.8 monoSP 0.2 diSP 0.4 triSC	11.1
2.5 monoAP 2.5 diAP	14.3
5.0 W 0.4 YA 0.1 CH 3. BGP 1.8 monoSP 0.2 diSP	13.2
8.5 PHASE IV^{tm}	22.0

[1] %weight/volume

[2] BGP, disodium β-glycerophosphate; P, phosphate; S, sodium; C, citrate; A, ammonium; W, whey solids; YA, yeast autolysate; CH, casein hydrolysate.

Approximately 4% salts were considered present in the reconstituted PHASE IV^{tm} since 8.5% reconstituted solids was recommended by the PHASE IV^{tm} manufacturer. Formulations with approximately 5% reconstituted soluble salts buffered half that provided by 8.5% PHASE IV^{tm}. Disodium β-glycerophosphate (BGP) buffered less than the same amount of 1:1 mono, disodium phosphate. Duplicated measurements were made on experiments once a successful formula was achieved.

Varied soluble salts were compared via activity tests with a 1:1 inoculum blend of S. cremoris UC73 and S. lactis UL8 (Table 10). One formulation sustained good culture growth. It contained 1.0% monoammonium phosphate, 1.0% diammonium phosphate and 3% trisodium citrate and it sustained culture activity similar to that in PHASE IV^{tm}. The 19 h medium pH, however, was 5.2 whereas the 19 h PHASE IV^{tm} medium pH was only 5.8. Superior buffering existed in PHASE IV^{tm} allowing a superior growth environment which would have been apparent given a longer incubation time.

Varied initially insoluble salts including calcium hydroxide, magnesium phosphate and calcium phosphate were then evaluated unsuccessfully. An antacid tablet (MAALOX^{tm}) was used in a formulation along with ammonium phosphate. One formulation containing 1% monoammonium phosphate, 1% diammonium phosphate and 2% pulverized

Table 10. Comparison of various buffered media for suitability in propagating S. cremoris UC73 and S. lactis UL8, 1:1 blend. All media were heated to 90 C for 40 min, incubated at 27 C for 19 h. Activity was measured with the Pearce activity test.

Buffered Medium	19 h pH[1]	Activity (pH)
8.5[2] PHASE IV[tm]	5.8	1.04
3.5 W, 0.4 YA 0.1 CH, 3.0 BGP 1.8 monoSP, 0.2 diSP	6.4	0.40
5.0 N, 0.4 YA 0.1 CH, 3.0 BGP 1.8 MonoSP, 0.2 diSP	6.3	0.34
3.5 W, 0.4 YA 0.1 CH, 5.0 BGP	6.7	0.33
5.0 N, 0.4 YA 0.1 CH, 2.5 monoAP 2.5 diAP	6.4	0.26
5.0 N, 0.4 YA 0.1 CH, 2.5 monoSP 2.5 diSP	6.3	0.34
5.0 N, 0.4 YA 0.1 CH, 2.0 monoAP 2.0 diAP, 2.0 triSC	6.3	0.49
5.0 N, 0.4 YA 0.1 CH, 1.0 monoAP 1.0 diAP, 3.0 triSC	5.2	1.00

[1] pH of medium after 19 h incubation at 27 C.

[2] % weight/volume

[3] W, whey solids; YA, yeast autolysate; CH, casein hydrolysate; BGP, β-glycerophosphate; S, sodium; P, phosphate; N, nonfat dry milk, A, ammonium; C, citrate.

antacid had similar buffering capacity to PHASE IV^{tm} (Table 11). Low buffering strength was observed in the absence of ammonium phosphates. Magnesium hydroxide and aluminum hydroxide were the main antacid ingredients. Aluminum hydroxide was shown to possess little buffering power with the ammonium phosphates in the desired pH range, i.e., pH 7.0-5.2. The successful formulation apparently existed as a complex buffering system involving ammonium, phosphate and magnesium salts.

Other salts were combined with the pulverized antacid tablet. Ammonium and sodium carbonate were effective in buffering (Table 12). Both 3% ammonium phosphates/carbonates and sodium phosphates/ carbonates provided equal buffering to PHASE IV^{tm} when combined with 1.5% pulverized antacid tablet. The sodium salts had greater buffering strength. Sodium phosphates as the only phosphate medium ingredient were not evaluated further because of greater inhibition of lactic culture growth (11) and a more insoluble precipitate developed during the 40 min/90 C heat treatment used. A combination of ammonium and sodium phosphates was therefore desired in the formulation. Carbonates lost buffer capacity during heat treatment and were not further considered for incorporation into a medium..

Three different internal pH controlled media were developed and evaluated for propagation of Prt- lactic

Table 11. The effect of antacid (Maalox^{tm}) addition on buffering capacity of a 1% monoammonium phosphate, 1% diammonium phosphate solution. Buffering strength was evaluated by how many mLs of 10% lactic acid solution was required to bring initial pH (6.6-7.0) to pH 5.2. Pulverized antacid tablets contained 50% magnesium hydroxide and 50% aluminum hydroxide.

Buffer	Antacid Addition[1]	10% Lactic Acid (mL)
1% monoammonium phosphate 1% diammonium phosphate	0.0	6.5
	0.5	9.7
	1.0	13.5
	1.5	17.2
	2.0	22.5
(1.0% aluminum hydroxide)		6.3
0% monoammonium phosphate 0% diammonium phosphate	1.0	0.2
8.5% PHASE IV	----	24.1

[1] % weight/volume

Table 12. Buffering strength of formulations containing various concentrations of pulverized antacid (Maalox^{tm}) tablets, phosphates and carbonates. Buffering capacity was evaluated by how many milliliters of 10% lactic acid solution was required to bring initial pH (from 6.6 to 7.0) to pH 5.2. Pulverized antacid tablets contained 50% magnesium hydroxide and 50% aluminum hydroxide.

Buffer	Antacid Addition[1]	10% Lactic Acid (mL)
1[1] monoS[2]P, 1 diSP	0.0	6.2
.75 monoSP, .75 diSP	1.0	11.7
1 monoSP, 1 diSP	1.0	11.2
1.25 monoSP, 1.25 DiSP	1.0	12.7
1.5 monoSP, 1.5 diSP	1.0	14.0
1.5 monoSP, 1.5 diSP	1.5	16.2
1 monoAP, 1 diAP, 1 ACO	1.5	24.6
1 monoSP, 1 diSP 1 SCO	1.5	32.3
8.5 PHASE IV^{tm}	----	23.6

[1] % weight/volume

[2] S, sodium; P, phosphate; A, ammonium; CO, carbonate.

cultures. Composition of these media is given in Table 13.

Table 13. Composition of internal pH controlled media for lactic culture propagation. MSP = monosodium phosphate, MAP = monoammonium phosphate, TSC = trisodium citrate, DAC = diammonium citrate, DAP = diammonium phosphate MH = magnesium hydroxide, CA = citric acid, YE = yeast extract, CH = casein hydrolyzate, WS = whey solids. Quantities are expressed at % weight/volume. Media were heated 90 C for 40 min prior to inoculation.

Ingredient	Medium A	B	C
MSP	0.8	---	---
MAP	1.0	1.5	---
TSC	0.3	1.5	---
DAC	0.7	---	1.2
DAP	---	---	1.5
MH	1.2	1.0	1.2
CA	---	0.3	0.8
YE	0.4	0.4	0.4
CH	0.1	0.1	0.1
WS	5.0	5.0	5.0

These 3 media were evaluated for Prt- lactic culture propagation by inoculating the Prt- cultures: *S. cremoris* UC320, UC73, *S. lactis* UL7, and UL8 separately into each of the media. Inoculated media were incubated at 27 C for 14 h. Activity of the cultures in the respective media was then evaluated with the Pearce activity test. Analysis of variance was performed on the results (Table 14). Both the LSD and Duncan's multiple range test showed that medium A was superior to media B

and C and that there was no significant difference between media B and C at alpha = .05.

Table 14. Analysis of variance on various internal pH controlled media.

Source	DF	Mean Square	F Value	Pr > F
Block	1	.213	3.69	.0628
Strain	3	2.794	48.37	.0001
Medium	2	.711	12.31	.0001
Strain*Medium	6	.033	.57	.7496
Error	35	.058		

$R^2 = .835$

Whey solids were replaced by whey permeate to reduce coaguable protein content which interferred with turbidity readings (66). Modified medium A was then entitled LActic culture Buffered GROwth (LABGRO) medium (Table 15). Reconstituted LABGRO medium contained 7.5% solids.

The buffering capacity of LABGRO medium before 15% reduction of its buffering salts (medium A in Table 13) is illustrated in Figure 6. Here LABGRO medium was found to have about 8 times the buffering capacity between pH 6.6 and pH 5.2 than 10% RNDM.

The effect of agitation and addition of a reducing agent (ascorbic acid (.1%)) on cell propagation in LABGRO medium was studied (Tables 16, 17). LABGRO medium

Table 15. Ingredient composition of lactic culture buffered growth medium (LABGRO Medium). Quantities given are reconstituted values (% wt/vol).

LActic culture Buffered GROwth medium

LABGRO Medium

INGREDIENT	CONCENTRATION
Monosodium phosphate	.70
Monoammonium phosphate	.85
Trisodium citrate	.30
Diammonium citrate	.55
Magnesium hydroxide	1.00
Whey permeate solids	3.60
Nutrient source	.50
Water	92.50
	100.00

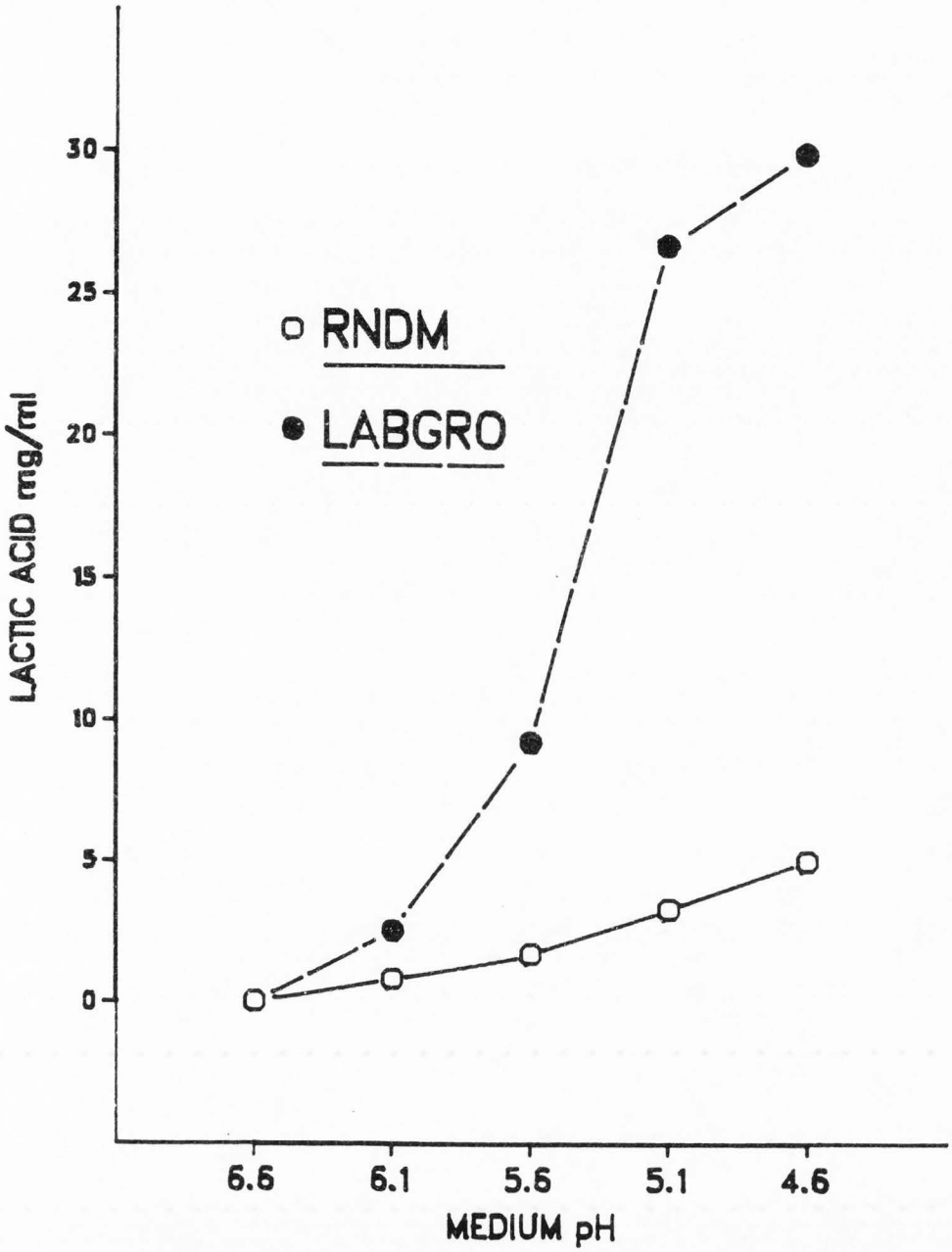

Figure 6. Buffering capacity of 10% reconstitued nonfat dry milk and LABGRO medium.

sustained greater concentrations of two Prt- lactic cultures when mildly agitated during culture incubation (Table 17). The analysis of variance of this comparison is given in Table 16.

Table 16. Analysis of variance on various incubating conditions on LABGRO medium.

Source	DF	Mean Square	F Value	Pr > F
Strain	1	49.88	43.51	.0001
Agitation	2	40.03	34.91	.0001
Ascorbic Acid(AC)	1	.01	.01	.9403
Strain*Agitation	2	2.76	2.40	.2841
Strain*AC	1	1.58	1.38	.1234
Agitation*AC	2	1.63	1.42	.2740
Error	14	1.15		

R^2=.897

A reducing agent, .1% ascorbic acid, gave no beneficial affect. Increased lactic culture growth in agitated medium was probably due to better solubulization and mixing of buffering salts. Growth-inhibiting hydrogen peroxide levels resulting from agitation were probably minimized by the yeast extract which has been shown to prevent cell growth inhibition due to hydrogen peroxide build up (146).

Table 17. Effect of continuous medium agitation and added reducing agent (ascorbic acid) on growth of S. cremoris UC171 Prt- and S. lactis UL7 Prt- in 7.5% LABGRO medium. Culture was propagated for 19 h at 27 C. Means are duplicate averages.

Incubation Condition	OD(480 nm)[1]		
Agitation[2]	14.5	a[3]	a[4]
Agitation + AC[5]	14.0	a	a
Initial agitation[6] + AC	11.0	b	b
No agitation[7] + AC	10.5	b	b
No agitation	10.0	b	b
Initial agitation	9.9	b	b

[1] Optical Density =((Absorbance at 480 nm (medium+culture) - (Absorbance at 480 nm(medium)) x Dilution factor

[2] Agitation at 100 rpm on flask shaker throughout incubation.

[3] LSD test.

[4] Duncan's multiple range test. When two means in a column share the same letter, they are not significantly different at alpha=.05.

[5] 0.1% ascorbic acid.

[6] Agitated initially to break up salts caked on bottom of flask.

[7] No agitation at all. Culture was initially mixed into medium by gently swirling.

Growth of a Prt- lactic variant in LABGRO medium was compared to the culture concentration sustained by pH controlled whey-based medium and two commercial internal pH controlled bacteriophage inhibitory media; PHASE IV^{tm} and INSURE^{tm}. The analysis of variance and results are shown in tables 18, 19, 20 and 21. The performance of LABGRO medium was approaching that of pH controlled whey-based, equal to that of INSURE^{tm} medium and superior to PHASE IV^{tm}. LABGRO medium was selected as a laboratory medium to evaluate the nitrogenous sources used in conditions simulating commercial pH controlled bulk media fermentation. It is not suggested as a commercial internal pH control bacteriophage inhibitory medium since calcium chelating properties were inferior to PHASE IV^{tm} and INSURE^{tm} (personal communication, dairy products laboratory technician, Stauffer Chemical Co., 1983). Also it probably would violate the patent rights of PHASE IV^{tm} (139)

Selection of a Test Strain for Evaluation of Yeast, Protein Hydrolysate Products

A Prt- variant was desired as a test strain for evaluating yeast and protein products. Such a strain should be more sensitive to utilizable nitrogenous matter than Prt+ strains.

Table 18. Analysis of variance on culture growth in WB, PHASE IV^{tm} and LABGRO media.

Source	DF	Mean Square	F Value	Pr > F
Medium	2	1.2×10^{19}	36.77	.0004
Error	3	3.2×10^{17}		
		R^2=.925		

Table 19. Growth of S. lactis UL7 Prt- in WB, PHASE IV^{tm}, and LABGRO media. S. lactis UL7 Prt- was inoculated (1%) into heat treated (90 C for 40 min), cooled media. Incubation was for 19 h at 27 C. PHASE IV^{tm} and LABGRO media were moderately agitated during incubation to assure dispersion of salts. M17 media was used for plate counts.

Medium	CFU/mL Medium	
pH control Whey-Based	11.0×10^9	a[1]
LABGRO	9.4×10^9	b
PHASE IV^{tm}	7.3×10^9	c

[1] When two means in a column share the same letter, they are not significantly different at alpha=0.05, Duncan's multiple range test.

Table 20. Analysis of variance on culture growth stimulation in INSURE^{tm}, PHASE IV^{tm} and LABGRO media.

Source	DF	Mean Square	F Value	Pr > F
Medium	2	18.77	26.50	.0124
Error	3	.71		

R^2=.946

Table 21. Growth of S. lactis UL7 Prt- in INSURE^{tm}, PHASE IV^{tm} and LABGRO media. S. lactis UL7 Prt- was inoculated, 1%, into heat treated (90 C,40min), cooled media. Incubation was for 19 h at 27 C. PHASE IV^{tm} and LABGRO media were moderately agitated during incubation to assure dispersion of salts.

Medium	OD(480 nm)[1]
INSURE^{tm}	13.5 a[2]
LABGRO	13.2 a
PHASE IV^{tm}	8.1 c

[1] Optical Density = ((Absorbance at 480 nm (medium + culture) - (Absorbance (medium)) x Dilution factor.

[2] When two means in a column share the same letter, they are not significantly different at alpha=0.05, Duncan's multiple range test.

During the course of obtaining data plotted in Figure 1, S. cremoris UC171 and S. lactis UL7 and a Prt- variant of each were individually propagated in pH controlled whey-based medium.

Prt- strains grew in pH controlled whey-based medium (WB) to within 95% of the growth sustained by Prt+ strains (Figure 7). Each Prt- strain also produced about the same amount of lactate in pH controlled whey-based fermentation (Figure 8). No significant difference among the Prt+ and Prt- samples were shown (Table 22).

Table 22. Analysis of variance on final lactate concentration in WB medium fermented by S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt-. Each strain was propagated individually in WB medium.

Source	DF	Mean Square	F Value	Pr > F
Strain	1	27.556	79.18	.0030
Protease Type	1	.900	2.58	.2066
Strain*Type	1	2.500	7.18	.0751
Error	3	.348		

R^2=.739

Effect of Initial Concentration and Strain Type on Cell Growth Rate

After propagation in WB medium each strain was then inoculated at .5, 1, 2, 5, and 9% (%vol/vol) into

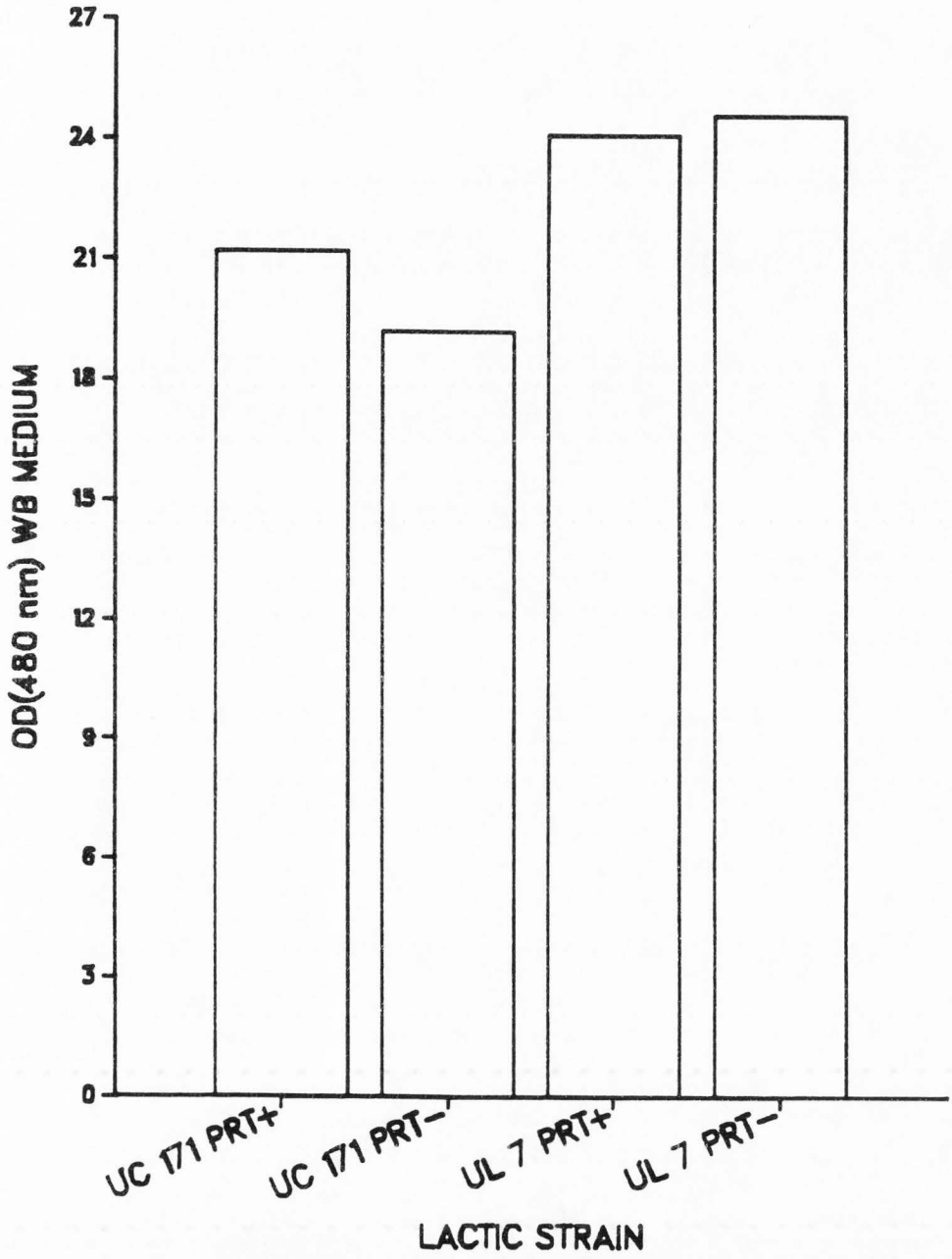

Figure 7. Final optical density (480 nm) in pH controlled whey-based medium after growth of S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt-.

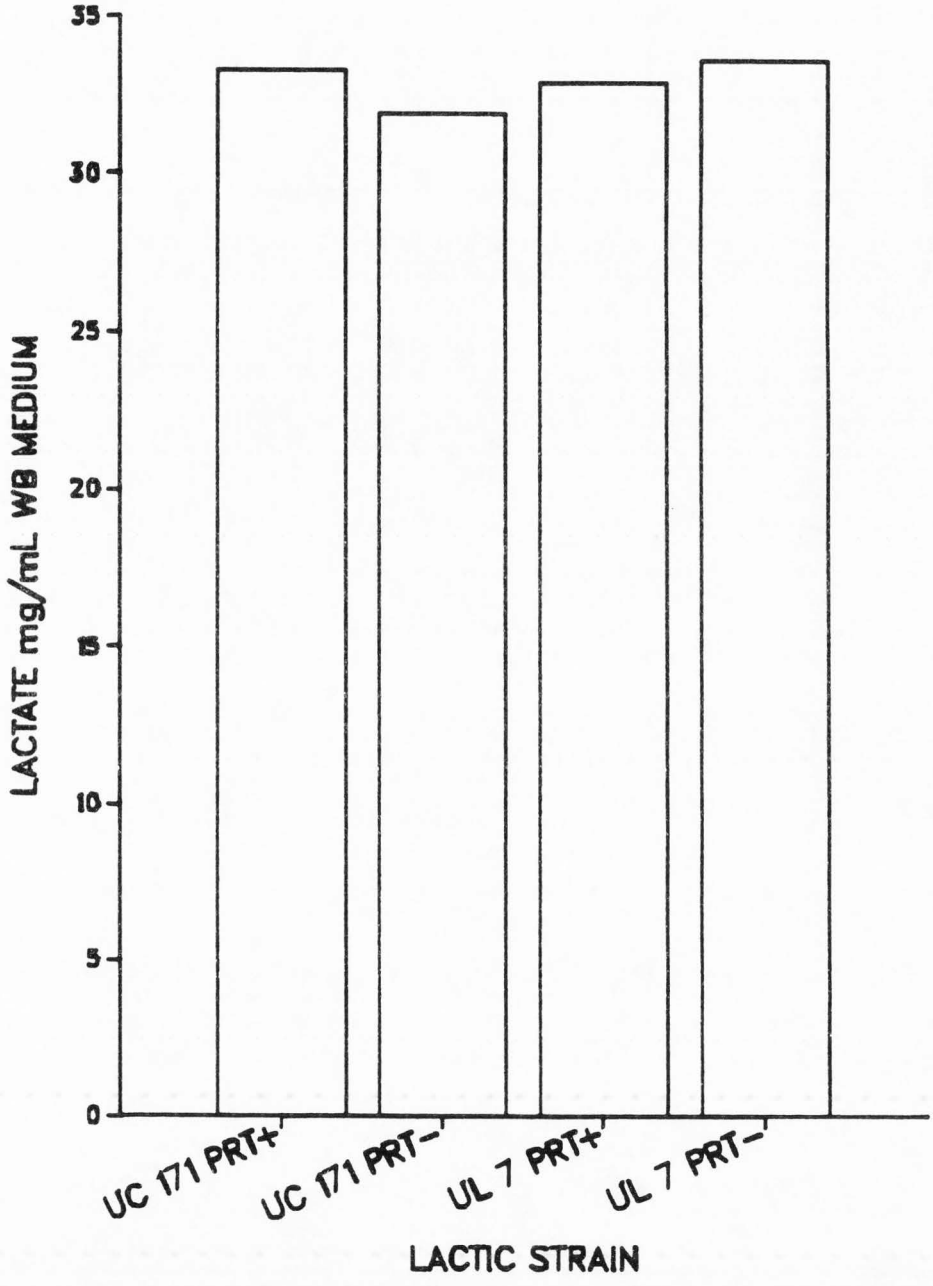

Figure 8. Final lactate concentration in pH controlled whey-based medium after growth of S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt-. (See Table 22).

pasteurized 10% RNDM and incubated for 5 h. Initial, final cell concentration and total acid produced in 10% RNDM were estimated from optical density (OD(480 nm)) and pH measurments using prediction equations (figures 3 and 4).

The relationship between initial and final cell concentration (OD(480 nm)) of the strains after 5 h incubation at 38 C is shown in Figure 9. Though initial cell concentrations in milk were nearly equal, there were marked differences in growth between the Prt+ and Prt- cultures. The growth rate (number of generations during 5 h incubation) was calculated for each strain for each initial 10% RNDM concentration level (Figure 10). When initial cell numbers were approximately $2x10^8$/mL 10% RNDM (the linear prediction equation in Figure 3 possibly overestimates cell concentration at the low end of the plot) the Prt+ strains doubled more rapidly during 5 h at 38 C. These rates were reduced as initial cell concentrations approached $2x10^9$ cells/ml. Prt+ strains approached the slow growth patterns of Prt- strains as initial cell concentration increased.

Analysis of variance shows the effects of strain type, protease type and cell concentration to be significant on growth rate (Table 23).

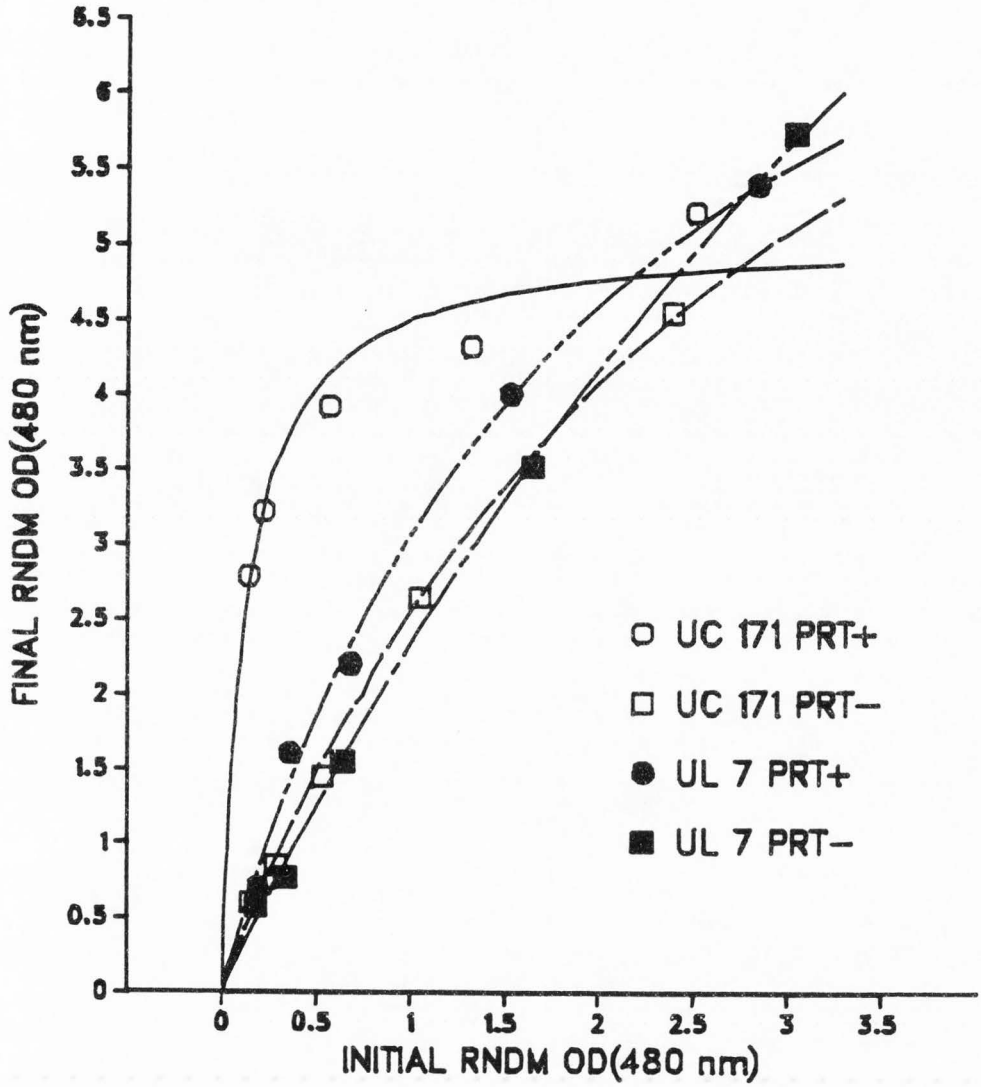

Figure 9. Final cell concentrations (OD(480 nm)) in 10% RNDM of S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+, UL7 Prt- inoculated into 10% RNDM at different initial concentrations and incubated for 5 h at 38 C. Final 10% RNDM OD(480 nm) were approximated by the equation: Final 10% RNDM OD(480 nm) = (A x initial 10% RNDM OD(480 nm))/(B + initial 10% RNDM OD(480 nm)). A and B coefficients were determined by aid of a computer (Table 25). Curves represent this approximation.

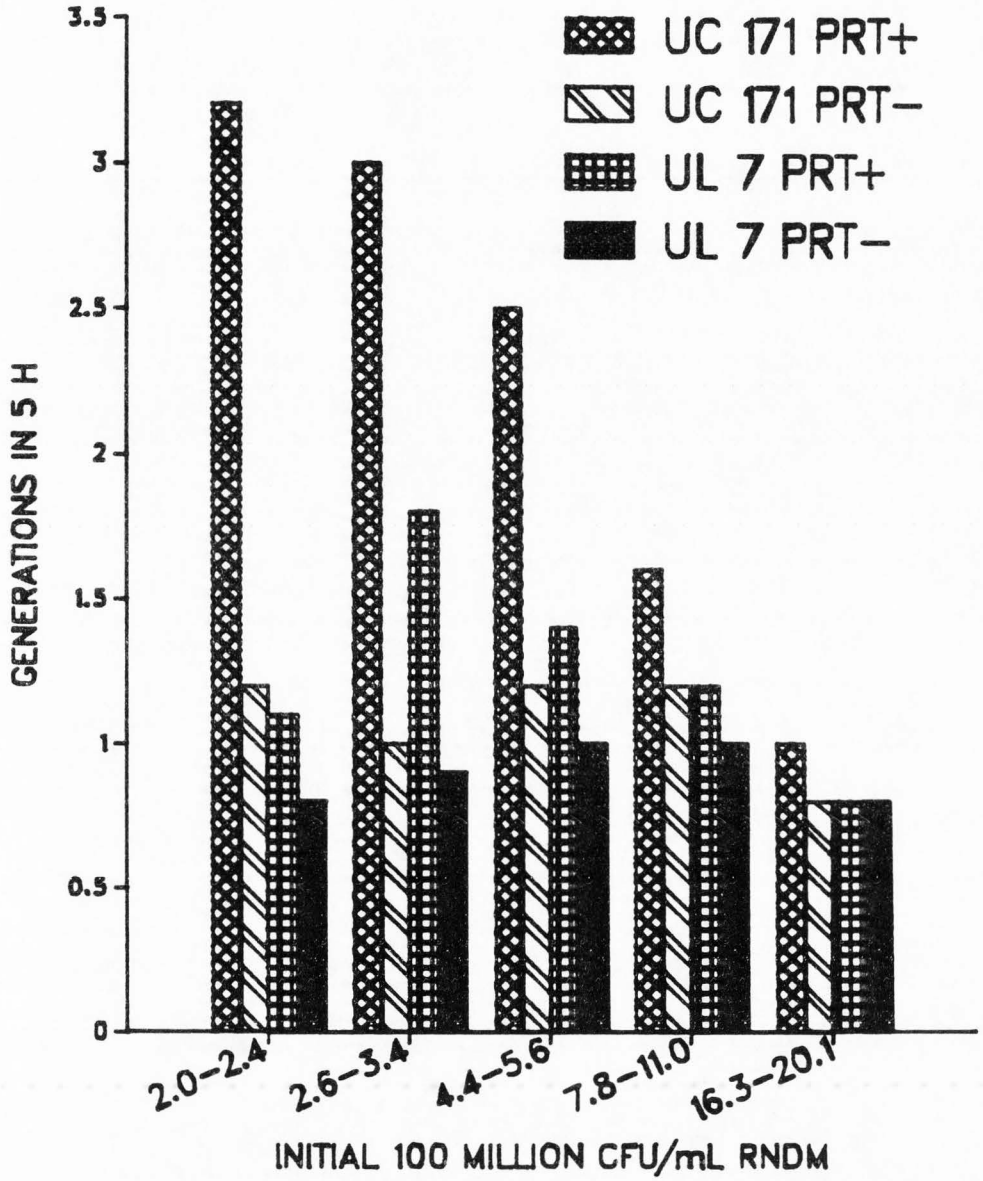

Figure 10. Number of generations during incubation (5 h, 38 C) of S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt- inoculated at different initial concentrations into 10% RNDM. CFU/mL 10% RNDM was estimated by 10% RNDM OD(480 nm) from a reference curve (Figure 3)

Table 23. Analysis of variance on growth rate of S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt- at various initial concentrations in 10% RNDM.

Source	DF	Mean Square	F Value	Pr > F
Strain	1	3.136	250.88	.0001
Protease Type	1	5.929	474.32	.0001
Concentration	4	.908	72.67	.0001
Strain*Protease Type	1	1.681	134.48	.0001
Strain*Concentration	4	.355	28.43	.0001
Protease Type*Conc.	4	.656	52.47	.0001
Strain*Prt Type*Conc.	4	.230	18.43	.0001
Error	20	.0125		
		R^2=.987		

Also shown to be significant were the interactions shown in Table 23. Statistical analysis on the main effects of these four strains shows S. cremoris UC171 and Prt+ cells to have a significantly higher (alpha=.05) growth rate than S. lactis UL7 and Prt- cells, respectively (Table 24). Cell concentration seemed to have an inverse relationship with cell growth rate (Table 24).

Growth rate differences due to protease type were expected (23, 133) and reflects impaired ability of Prt- cells to utilize milk protein (23, 41, 120). The inverse relationship between cell concentration and growth rate is probably due to higher cell numbers collectively producing sufficient lactic acid to inhibit cell growth (60) earlier

Table 24. Effect of strain, protease type and initial cell concentration on growth rate.

Parameter	N[1]	Mean[2]	
Strain			
UC	20	1.67	a[3]
UL	20	1.11	b
Protease Type			
Prt+	20	1.78	a
Prt-	20	1.01	b
Concentration[4]			
2.6-3.4	8	1.70	a
2.0-2.4	8	1.58	b
4.4-5.6	8	1.54	b
7.8-11.0	8	1.29	c
16.3-20.1	8	.85	d

[1] Number of measurements for the mean.

[2] Number of generations during a 5 h incubation at 38 C.

[3] LSD test. When two columns share the same letter they are not significantly different at alpha=.05.

[4] 100 miilion CFU/mL 10%RNDM.

than occurs in RNDM samples with lower initial cell concentrations. The higher growth rate of S. cremoris UC171 over S. lactis UL7 suggests possible growth rate differences among Prt+ strains and among Prt- strains themselves.

The interaction effects are most easily seen in graphs (figures 11, 12 and 13). A greater difference in growth rate appears to exist between the Prt+ and Prt- strain of S. cremoris UC171 than exists between the Prt+ and Prt- strain of S. lactis UL7 (Figure 11). This observation again suggests possible variations in growth rate among the strains themselves. This topic will be discussed shortly.

When the initial cell concentration vs lactic strain type interaction was plotted, steadily decreasing growth rates seemed to follow increasing initial cell concentration (Figure 12). The lowest initial cell concentration of S. lactis UL7 was an exception. Growth rate of S. lactis UL7 never reached that of S. cremoris UC171 but also did not decrease as rapidly.

The plot of initial cell concentration vs protease type interaction on growth rate indicates a more uniform growth rate among Prt- cells than among Prt+ cells (Figure 13). The dramatic decline in Prt+ cell growth rate is probably due to high acid concentrations. The Prt- cell initial concentration does not become sufficient to

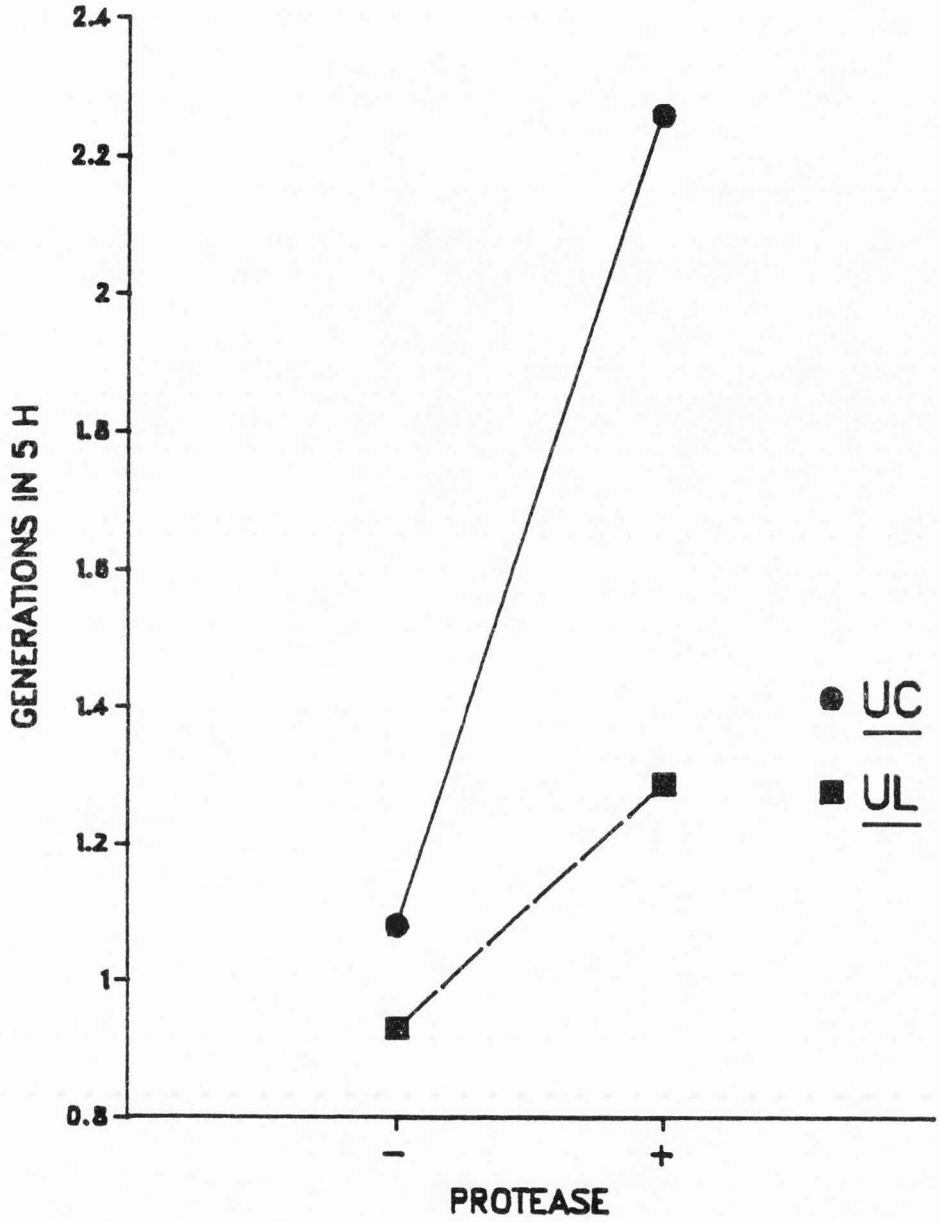

Figure 11. Effect of lactic strain type vs protease type on growth rate. (See Figure 10).

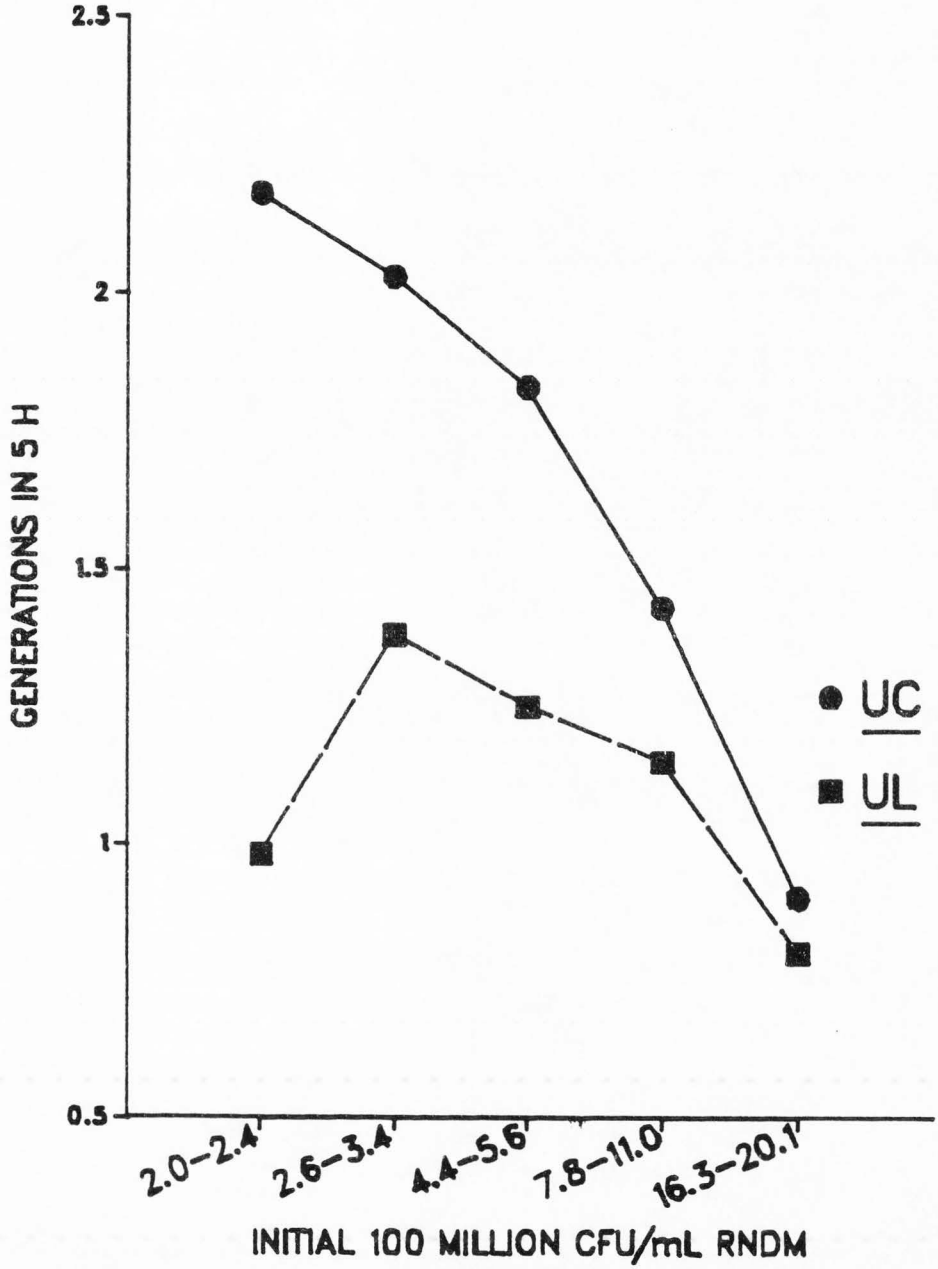

Figure 12. Effect of lactic strain type vs initial cell concentration on growth rate. (See Figure 10)

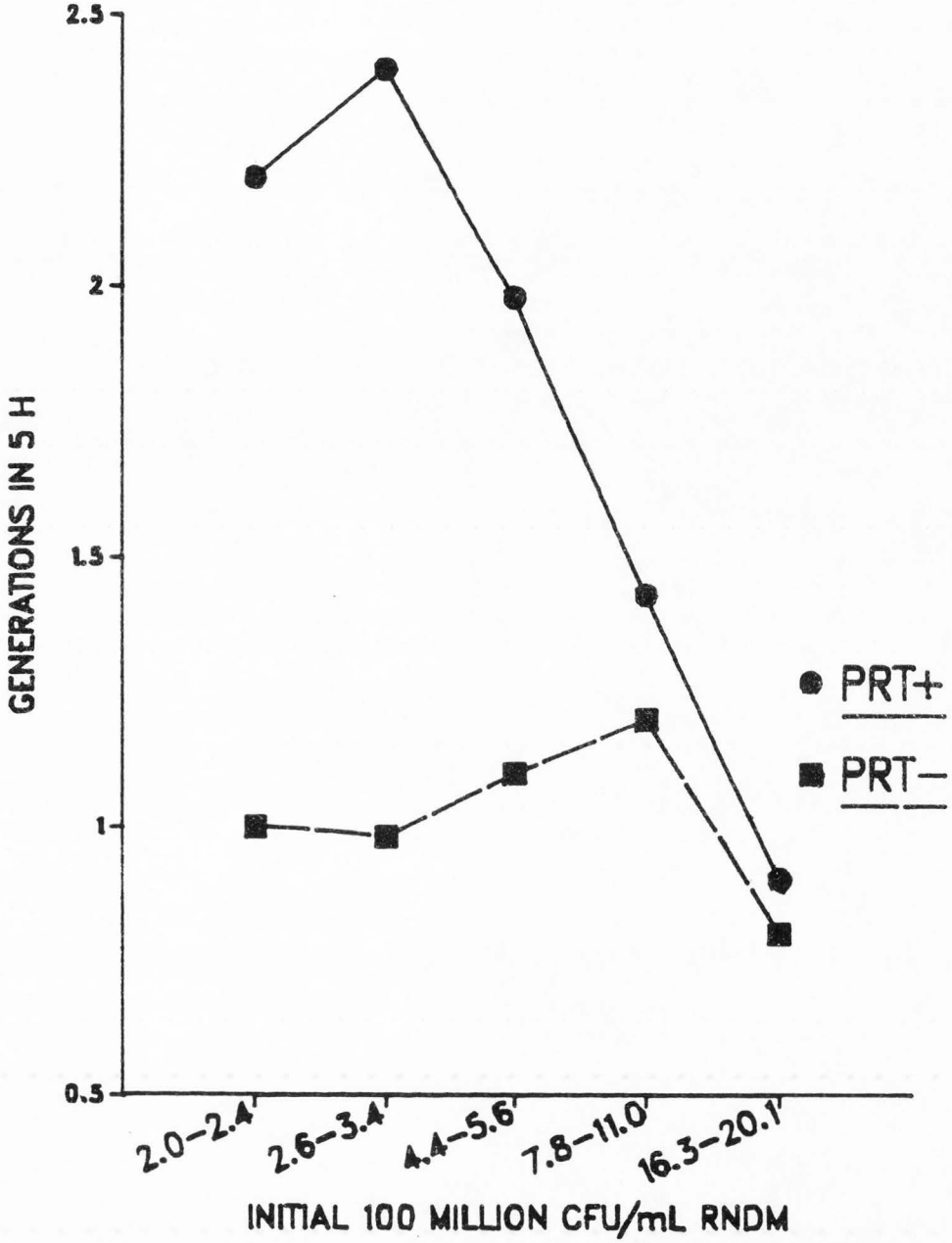

Figure 13. Effect of protease type vs initial cell concentration on growth rate. (See Figure 10).

produce inhibiting amounts of acid until initial cell concentration of 1.6×10^9 CFU/mL 10% RNDM is reached.

Foregoing results suggest possible differences in growth rate among the individual strains tested. A preferred evaluation method for growth rate would be based on actual initial cell concentration in the 10% RNDM rather than on estimation of cell concentration from percentage of added inoculum. The problem of equalizing initial concentrations in 10% RNDM among the strains could be solved by fitting a mathematical equation relating final cell concentration to intial cell concentration. Concern for tediously adjusting initial cell concentrations to achieve equality would thereby be eliminated.

A hyperbolic regression equation was chosen to predict final cell concentration (OD(480 nm)) from initial cell concentration (OD(480 nm)). The equation $Y = AxX/(B+X)$ was evaluated to see how well it would predict final cell concentration (Y) from initial cell concentration (X) in 10% RNDM. The A and B coefficients were estimated using the parametric curve fitting basic computer program of Ruckdeschel (136). Final cell concentration predicted from this equation is represented by the curves in Figure 9 and correlated well with actual measurements shown by the symbols (Figure 9). The estimated A and B coefficient values are listed (Table

25). Also listed are correlation values for degree of fit. Standard deviation between predicted and actual final cell concentrations are given (Table 25).

It was possible to estimate initial individual strain cell concentration required to reach a target final cell concentration by setting Y (final cell concentration) at the target value (1.46 OD(480 nm)) and then solve for X (initial cell concentration (OD(480 nm)). One billion CFU/mL 10% RNDM is estimated by the target value 1.46 OD(480 nm) (Figure 3). These predicted X values to reach the target level were derived for each strain (Table 25) and were the basis for strain growth rate comparison. Significant differences in growth rate were found not only between protease types but among the four individual strains themselves (tables 26 and 27).

Table 25. Coefficient values and standard deviation of fit between actual and predicted final optical density (OD(480 nm)) values. Strains compared were S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+, UL7 Prt-. Strains originally were propagated in WB medium. Data was fitted to a curve described by the equation: final cell concentration in milk (OD(480 nm)) = (A x Initial cell concentration) / (B + Initial cell concentration). Initial concentration was also OD(480 nm). Refer to Figure 9 for a visual comparison of actual and predicted values.

Strain	Coefficient Values A	B	SD	Predicted Initial OD[1]
UC171 Prt+	5.312	.151	.548	.06
	*[2] 5.075	.128	.325	.05
UC171 Prt-	* 10.104	2.965	.096	.50
	8.561	2.443	.177	.50
UL7 Prt+	7.911	1.600	.350	.36
	* 9.192	2.004	.149	.38
UL7 Prt-	14.376	5.127	.181	.58
	* 19.274	7.259	.090	.59

[1] Initial cell concentration in 10% RNDM (OD(480 nm)) when final cell concentration in milk (OD(480 nm)) is set equal to 1.46 according to the equation : initial cell concentration (OD) = (Bx1.46) / (A-1.46). An OD of 1.46 in the 10% RNDM used in these experiments approximated a cell concentration of 1 x 10^9 CFU/ml RNDM.

[2] A and B coefficients used for prediction curves in Figure 9.

Table 26. Analysis of variance for required initial concentration in 10% RNDM of lactic strains originally propagated in WB medium to achieve a final cell concentration of 1.46 OD(480 nm) after 5 h incubation at 38 C.

Source	DF	Mean Square	F Value	Pr > F
Strain	1	.0800	1066.67	.0001
Protease Type	1	.2178	2904.00	.0001
Strain*Prt Type	1	.0265	352.67	.0001
Error	4	.000075		
		R^2=.999		

Other lactic strains were evaluated for differences in growth rate using the curve-fitting method explained previously. Six Prt- strains were propagated concurrently and in duplicate in LABGRO medium. Coefficient estimates were derived and corresponding correlation values as measured by standard deviation are given in Table 28. Also given in Table 28 are the predicted initial cell concentrations to achieve the target final cell concentration of 1.46 OD(480 nm). Significant differences in growth rate were once again found among individual Prt- lactic strains (tables 29 and 30).

Table 27. Initial concentrations (OD(480 nm)) of lactic strains in 10% RNDM required to achieve a final cell concentration of 1.46 OD(480 nm) after 5 h incubation at 38 C. Strains were originally propagated in WB medium. Strains compared were S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt-. All strains were run in duplicate.

Strain	Required Initial RNDM Cell Concentration[1]		
UL7 Prt-	.59	a[2]	a[3]
UC171 Prt-	.50	b	b
UL7 Prt+	.37	c	c
UC171 Prt+	.06	d	d

[1] Optical Density =((Absorbance at 480 nm (RNDM + culture)) - (Absorbance(RNDM)) x Dilution Factor.

[2] LSD test. When two means in a column share the same letter, they are not significantly different at alpha = .05.

[3] Duncan's multiple range test. When two means in a column share the same letter, they are not significantly different at alpha = .05.

Table 28. Coefficient values and standard deviation of fit between actual and predicted final optical density (OD(480 nm)) values. Strains compared were S. cremoris UC171, UC310, UC73, S. lactis UL7A, UL33 and UL21A. All strains were Prt-. Strains originally were propagated in LABGRO medium. Data was fitted to a curve described by the equation: final cell concentration in milk (OD(480)) = (A x Initial cell concentration (OD(480 nm)) / (B + Initial cell concentration (OD(480 nm)).

Strain	Coefficient Values A	B	SD	Predicted Initial OD[1]
UL7A	3.382	.965	.128	.73
	2.949	.723	.065	.71
UL33	5.835	2.536	.113	.85
	7.645	3.685	.050	.87
UC171	3.496	1.125	.118	.81
	2.882	.853	.120	.88
UC310	4.027	1.548	.110	.88
	5.075	2.308	.059	.93
UC73	6.218	2.781	.099	.85
	5.700	1.893	.127	.65
UL21A	4.970	1.101	.113	.46
	4.858	1.437	.039	.62

[1] Initial cell concentration in 10% RNDM (OD(480 nm)) when final cell concentration in milk (OD(480 nm)) is set equal to 1.46 according to the equation : initial cell concentration (OD(480 nm)) = (Bx1.46) / (A-1.46). An OD(480 nm) of 1.46 in the 10% RNDM used in these experiments approximated a cell concentration of 1×10^9 CFU/mL RNDM.

Table 29. Analysis of variance for required initial concentration in 10% RNDM of lactic strains originally propagated in LABGRO medium to achieve a final cell concentration of 1.46 OD(480 nm) after 5 g incubation at 30 C.

Source	DF	Mean Square	F value	Pr > F
Strain	5	.0351	5.71	.0279
Error	6	.0062		
		R^2=.826		

The analysis of variance F value on these six strains (Table 29) was markedly lower than the F value on the 4 strains propagated in pH controlled whey-based medium (Table 26) which suggests possible greater sensitivity to detecting culture growth rate differences when pH controlled whey-based medium is used to propagate cultures rather than LABGRO medium.

Differences among strain growth rate (number of generations in 5 h) for 4 lactic strains were noted when the strains were inoculated at various initial concentrations (tables 23 and 24). Also differences among strain growth rates (initial RNDM concentration required to reach 1.46 OD(480 nm) in 5 h) were also noted when the 4 strains were inoculated at various initial concentrations into 10% RNDM (tables 26 and 27). The

Table 30. Initial concentrations (OD(480 nm)) in 10% RNDM required to achieve a final cell concentration of 1.46 (optical density units) after 5 h incubation at 30 C. Strains were originally propagated in LABGRO medium. Strains compared were S. cremoris UC171, UC310, UC73, and S. lactis UL7A, UL21A, and UL33. All strains were Prt-. All strains were run in duplicate.

Strain	Required Initial RNDM Cell Concentration[1]		
UC310	.91	a[2]	a[3]
UC33	.86	a	a
UC171	.85	a	a
UC73	.75	a	a
UL7A	.72	a b	a b
UL21A	.54	b	b

[1] Optical Density = ((Absorbance at 480 nm (RNDM + culture)) - ((Absorbance(RNDM)) x Dilution Factor.

[2] LSD test. When two means in a column share the same letter, they are not significantly different at alpha = .05.

[3] Duncan's multiple range test. When two means in a column share the same letter, they are not significantly different at alpha = .05.

latter method is the more realistic for strain evaluation for cheese making since approximately $1x10^9$ cells/g curd exist at packing. These apparent differences among strains in growth rate may be due to differing proteolytic capabilities. Different proteolytic capabilities have been observed by Exterkate in lactic cultures (42). Observations that proteolytic capabilities are plasmid-linked (31, 39, 98) and the observation of Davies et al. (32) and Yu et al. (179) on the presence of multiple and differentiable plasmids in lactic strains suggest a relationship between proteolytic enzyme encoding plasmid genes and cell growth rate. This possibility should be investigated by running a plasmid profile on each of these strains tested to see how plasmid content and growth rate correlate. A high correlation would suggest the possibility of stabilizing growth rate of the particular strain through incorporation of the plasmid(s)' genes of interest into the bacterial chromosome.

Effect of Initial Concentration and Strain Type on Cellular Acid Production

Figure 14 shows the relationship between initial cell concentration and the amount of lactic acid produced by each strain during 5 h incubation at 38 C. At approximately equal initial cell concentrations, there were marked differences in acid production capability

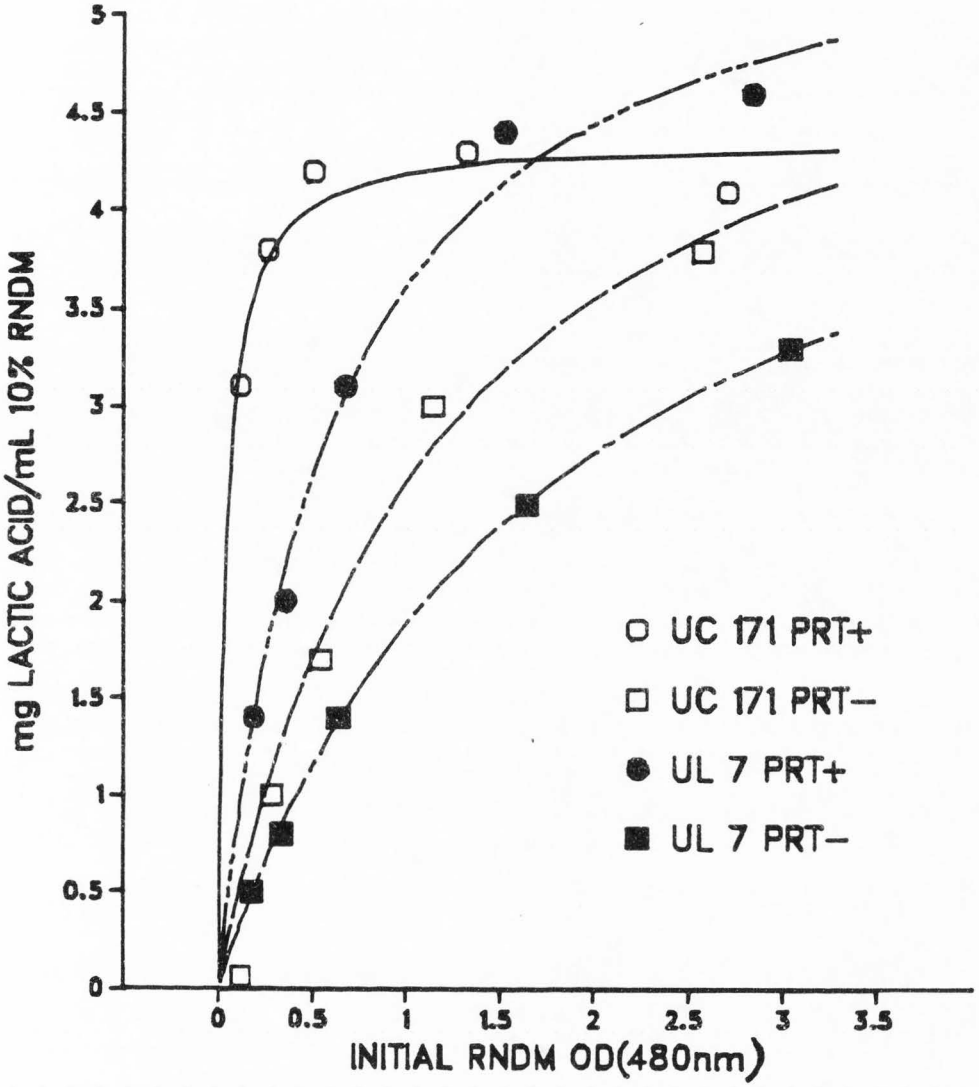

Figure 14. Lactic acid produced in 10% RNDM by S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+, UL7 Prt- inoculated into 10% RNDM at different concentrations and incubated for 5 h at 38 C. Final lactic acid concentration (mg/mL 10% RNDM) was approximated by the equation: mg lactic acid/mL 10% RNDM = (A x initial 10% RNDM OD (480 nm))/(B + initial 10% RNDM OD(480 nm)). A and B coefficients were determined by aid of a computer (Table 33). Curves represent this approximation.

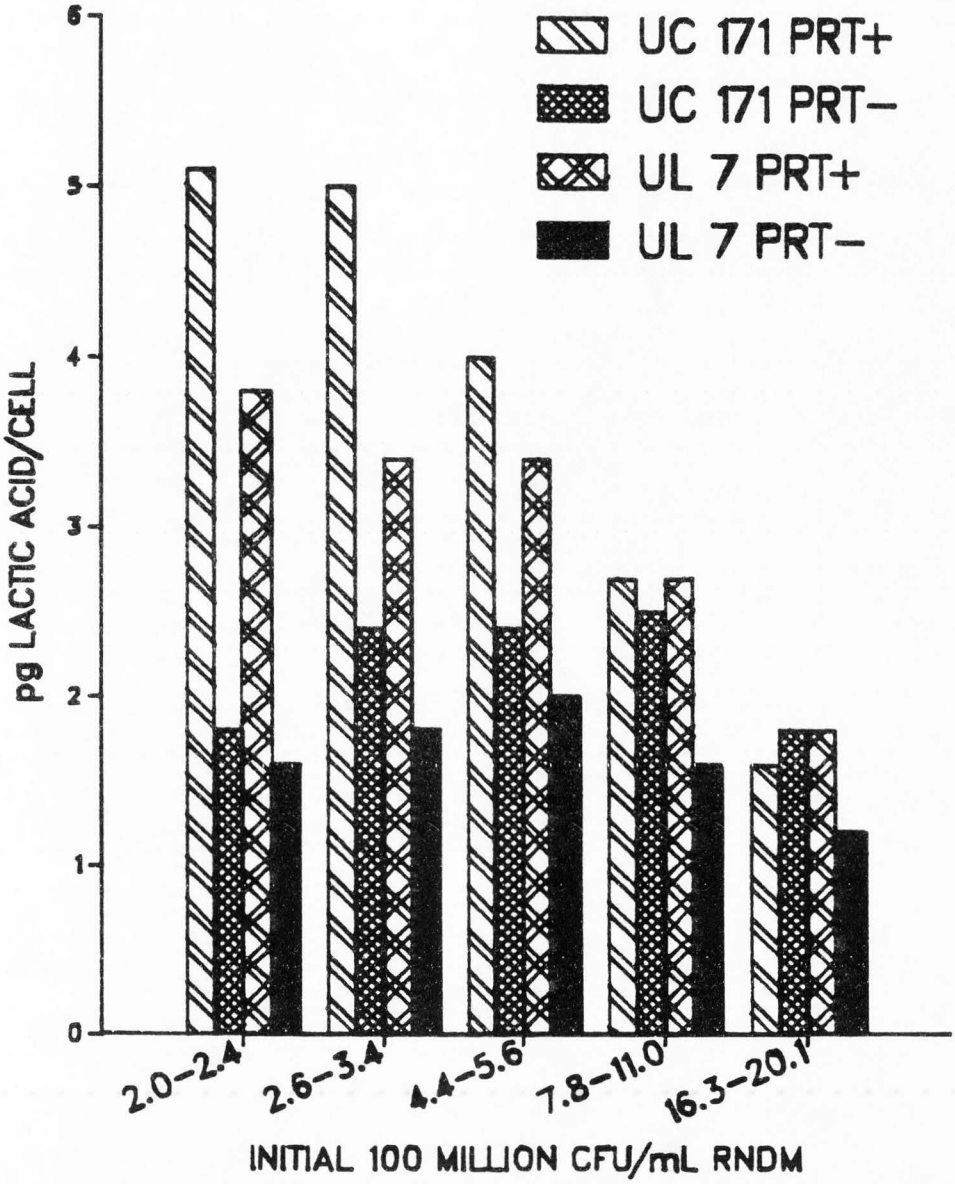

Figure 15. Cellular acid production during incubation (5 h, 38 C) of S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt- inoculated at different initial concentrations into 10% RNDM. pg lactic acid/cell was estimated by 10% RNDM pH and 10% RNDM OD 480 nm) from reference curves (figures 3 and 4).

between the Prt+ and Prt- cultures (Figure 14). Cellular acid production (pg lactic acid produced per cell during 5 h incubation at 38 C per cell) relative to initial cell concentration was calculated (Figure 15). When initial cell concentration in the RNDM was approximately $2x10^8$ cells/mL 10% RNDM (the linear prediction equation in Figure 3 possibly overestimates cell concentration at the lower end of the plot), the Prt+ cells produced significantly higher quantities of lactic acid per cell. However, these differences diminished as initial cell concentrations were increased to $2x10^9$ cells/ml RNDM. Greater uniformity of cellular acid production rate of Prt- strains over the Prt+ strains was evident.

Analysis of variance shows the effects of strain, protease type and cell concentration to be significant on cellular acid production (Table 31).

Table 31. Analysis of variance on cellular acid production of S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt- at various initial concentrations in 10% RNDM.

Strain	DF	Mean Square	F Value	Pr > F
Strain	1	3.969	122.12	.0001
Protease Type	1	20.736	638.03	.0001
Concentration	4	3.394	104.42	.0001
Strain*Protease Type	1	.049	1.51	.2337
Strain*Concentration	4	.207	6.35	.0018
Protease Type*Conc.	4	2.104	64.72	.0001
Strain*Prt Type*Conc.	4	.432	13.28	.0001
Error	20	.0325		
		R^2=.987		

Also shown to be significant were all interactions except strain*protease type.

Statistical analysis of these four strains shows S. cremoris UC171 and Prt+ cells to have significantly higher (alpha=.05) cellular acid production rates than S. lactis UL7 and Prt- cells, respectively (tables 31 and 32). Cell concentration seemed to have an inverse relationship with cellular acid production rate. The effects of lactic strain type, protease type and initial cell concentration on cellular acid production rate and growth rate follow similar patterns. This is probably due to acid production being generally coupled to growth rate. The higher cellular acid production rate of S. cremoris UC171 over S.

Table 32. Effect of strain, protease type and initial cell concentration on cellular acid rate.

Parameter	N[1]	Mean[2]	
Strain			
UC	20	2.94	a[3]
UL	20	2.32	b
Protease Type			
Prt+	20	3.35	a
Prt-	20	1.91	b
Concentration[4]			
2.6-3.4	8	3.15	a
2.0-2.4	8	3.08	b
4.4-5.6	8	2.95	b
7.8-11.0	8	2.38	c
16.3-20.1	8	1.60	d

[1] Number of measurements for the mean.

[2] pg lactic acid produced per cell during 5 h incubation at 38 C.

[3] LSD test. When two columns share the same letter they are not significantly different at alpha=.05.

[4] 100 million CFU/mL 10%RNDM.

lactis UL7 suggests possible differences in cellular acid production rate among individual strains.

The interaction effects on cellular acid production rate are portrayed (figures 16, 17 and 18). A uniform pattern of cellular acid production rate is evident between protease and lactic strain types (Figure 16). Possible variations in cellular acid production rates exist among the individual strains. This topic will be further discussed shortly.

When the initial cell concentration *vs* lactic strain type interaction was plotted, steadily decreasing growth rates generally followed increasing cell concentrations (Figure 17). Evidences of differing cellular acid production rates were seen among lactic strain types.

The plot of initial cell concentration *vs* protease type interaction on cellular acid production rate indicates a more uniform rate among Prt- cells than among Prt+ cells (Figure 18). The dramatic decline in Prt+ cellular acid production rate as initial cell concentration increases is probably due to growth-inhibiting acid concentrations occurring sooner in the 5 hour incubation period. A condition of more cells producing less acid therefore develops. Up to an initial concentration of about 1 billion cells/mL 10% RNDM, Prt- cells produce almost one half as much acid as do Prt+ cells on a cellular basis.

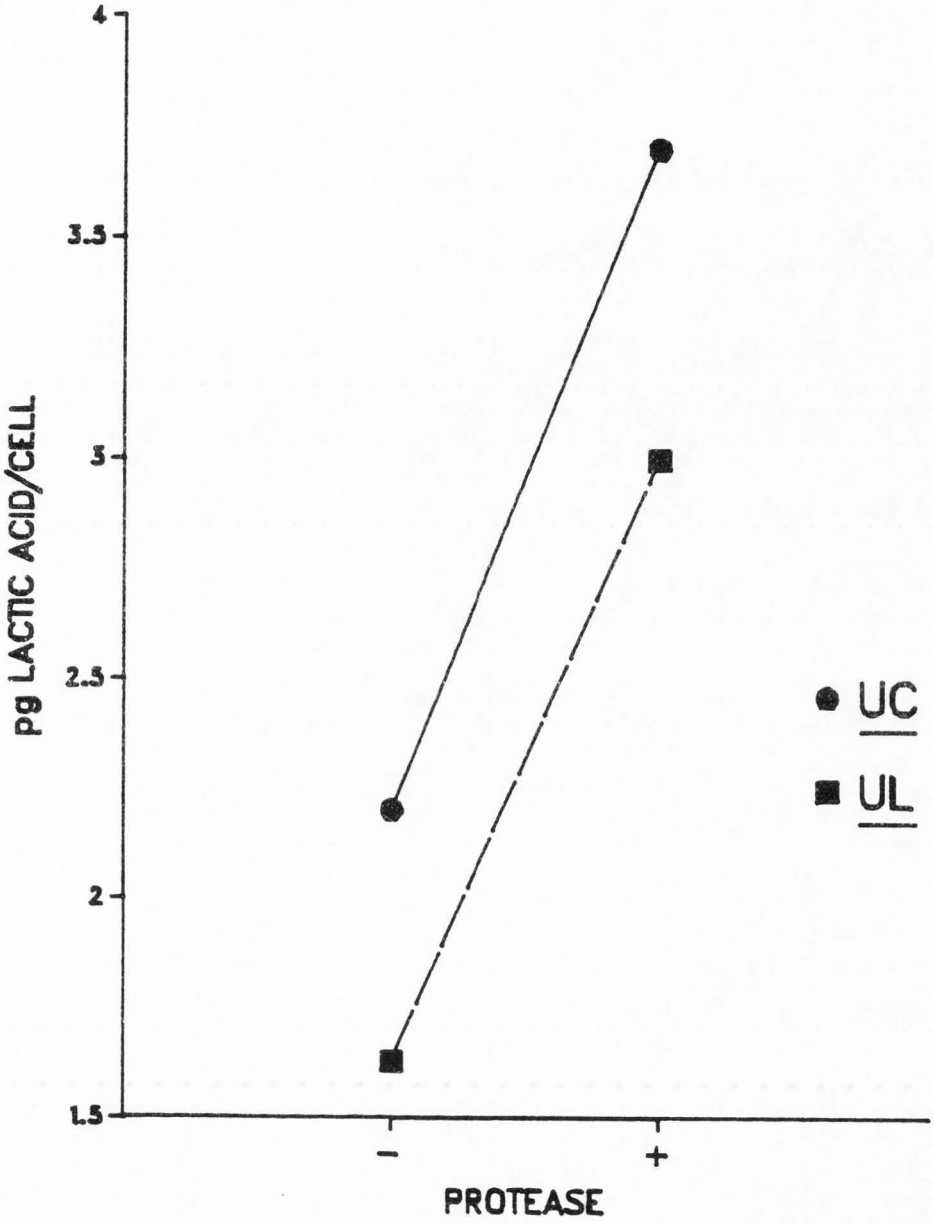

Figure 16. Effect of lactic strain type vs protease type on cellular acid production. (See Figure 15).

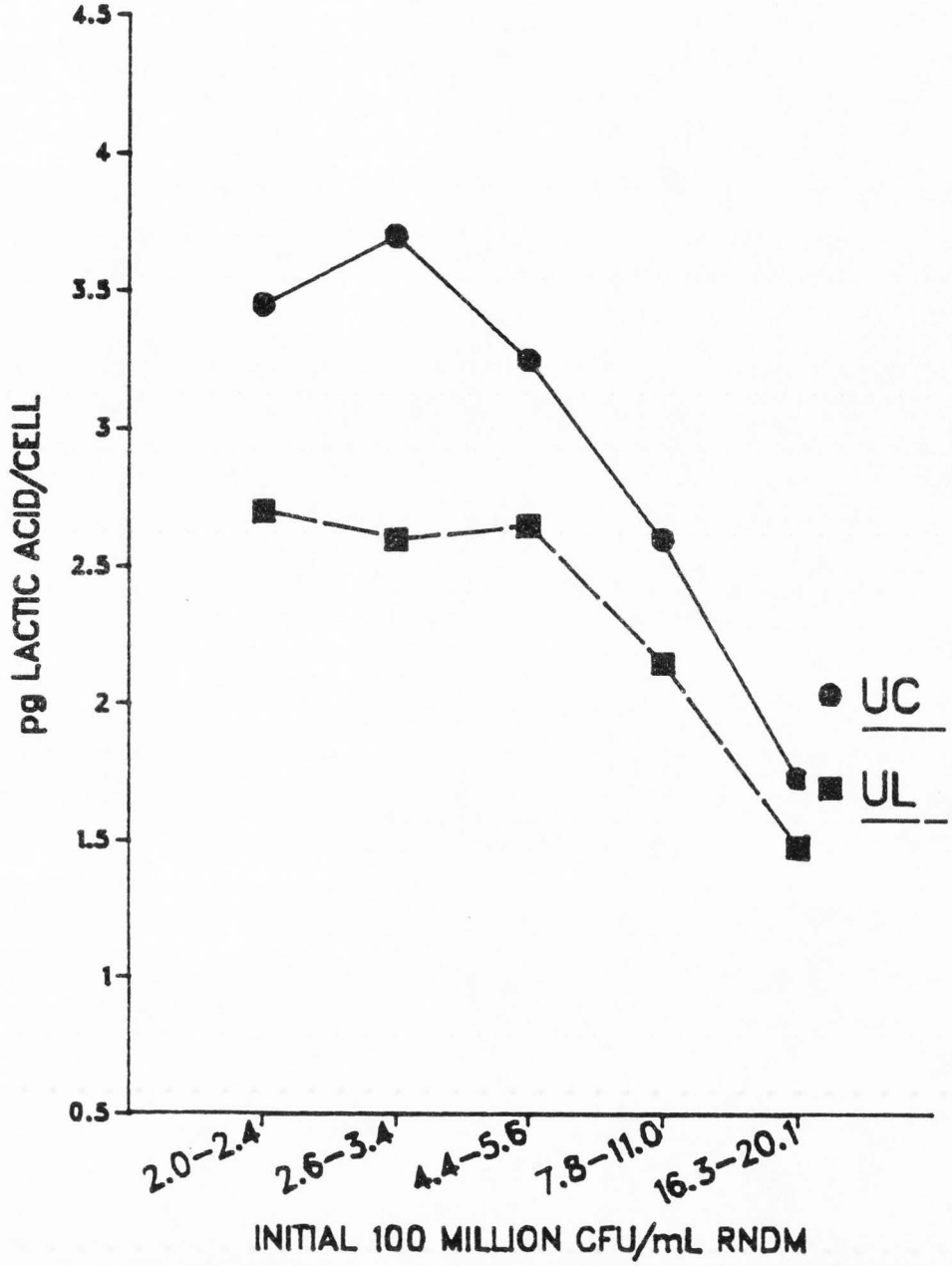

Figure 17. Effect of lactic strain type vs initial cell concentration on cellular acid production. (See Figure 15).

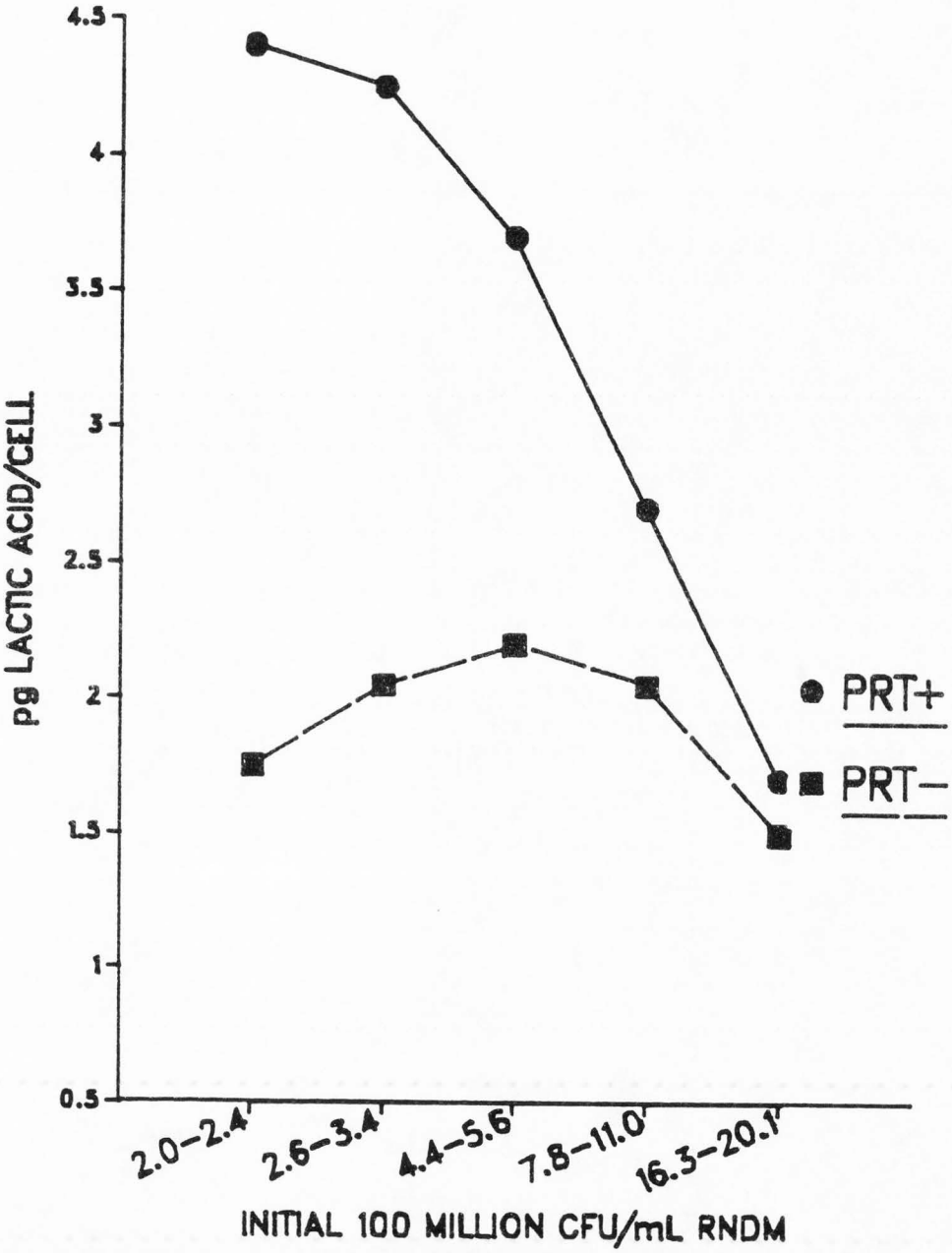

Figure 18. Effect of protease type vs initial cell concentration on cellular acid production. (See Figure 15).

Above results suggest possible differences in cellular acid production rates among individual strains tested. The curve-fitting method previously used to evaluate differences in growth rate among lactic strains was modified to evaluate differences of acid production rate.

The equation, $Y = AxX/(B+X)$ was evaluated to see how well it would predict final acid concentration (mg lactic acid/mL 10% RNDM) (Y) from initial cell concentration (OD(480 nm)) (X). Lactic acid concentration in 10% RNDM was predicted from pH by a prediction equation (Figure 4). The A and B coefficients were estimated by Ruckdeshel's parametric curve-fitting basic program (136) as previously explained. Final lactic acid concentrations in 10% RNDM predicted from these equations are represented by the curves in Figure 14 and correlated well with actual measurements shown by the symbols (Figure 14). The estimated A and B coefficient values are listed along with their standard deviations (Table 33).

It was possible to estimate initial strain cell concentration required to reach a target final acid level in 10% RNDM by setting Y (mg lactic acid/mL 10% RNDM) equal to the target level (2.5 mg lactic acid/mL 10% RNDM) and then solve for X (initial cell concentration as measured by OD(480 nm). 2.5 mg lactic acid/mL 10% RNDM corresponded to a 10% RNDM pH of 5.4 (Figure 4). These

Table 33. Coefficient values and standard deviation of fit between actual and predicted final acidity values (mg lactic acid/mL 10% RNDM). Strains compared were S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt-. Strains originally were propagated in WB medium. Data was fitted to a curve described by the equation: final cell concentration in milk (OD(480 nm)) = (A x Initial cell concentration (OD(480 nm))) / (B + Initial cell concentration (OD(480 nm))). Refer to Figure 14 for a visual comparison of actual and predicted values.

Strain		Coefficient Values A	B	SD	Predicted Initial OD[1]
UC171 Prt+	*[2]	4.375	.0447	.171	.06
		4.380	.056	.278	.07
UC171 Prt-		6.361	1.421	.214	.92
	*	5.611	1.161	.163	.93
UL7 Prt+		5.629	.569	.289	.45
	*	5.782	.603	.201	.46
UL7 Prt-	*	5.230	1.789	.028	1.64
		5.264	1.843	.098	1.67

[1] Initial cell concentration in 10% RNDM (OD(480 nm)) when final acidity in 10% RNDM is set equal to 2.5 according to the equation : initial cell concentration (OD(480 nm)) = (Bx2.5) / (A-2.5). An acidity of 2.5 mg lactic acid/mL 10% RNDM used in these experiments approximated a pH of 5.4.

[2] A and B coefficients used for prediction curves in Figure 14.

predicted X values were determined for each strain (Table 33) and were the basis for strain acid production rate comparison.

Significant differences in acid production rates were found not only between protease types but also among individual strains themselves (tables 34 and 35).

Table 34. Analysis of variance for required initial concentration in 10% RNDM of lactic strains originally propagated in WB medium to achieve a final acidity of 2.5 mg lactic acid/mL 10% RNDM after 5 h incubation at 38 C.

Source	DF	Mean Square	F Value	Pr > F
Strain	1	.6272	4181.33	.0001
Protease Type	1	2.1218	14145.33	.0001
Strain*Prt Type	1	.0578	385.33	.0001
Error	4	.00015		
		R^2=.999		

Of the four strains tested, S. cremoris UC171 Prt+ required the least initial cell concentration in 10% RNDM and S. lactis UL7 Prt- required the greatest initial cell concentration to produce the same amount of acid (Table 35). These results were confirmed by a separate experiment wherein these same strains were individually propagated in LABGRO medium, inoculated into 10% RNDM and incubated at 30 C. The same strain order for acid

Table 35. Initial concentrations (OD(480 nm)) of lactic strains in 10% RNDM required to achieve a final acidity of 2.5 mg lactic acid/mL 10%RNDM after 5 h incubation at 38 C. Strains were originally propagated in WB medium. Strains compared were S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt-. All strains were run in duplicate.

Strain	Required Initial RNDM Cell Concentration[1]		
UL7 Prt-	1.66	a[2]	a[3]
UC171 Prt-	.93	b	b
UL7 Prt+	.46	c	c
UC171 Prt+	.07	d	d

[1] Optical Density = ((Absorbance at 480 nm (RNDM + culture)) - ((Absorbance(RNDM)) x Dilution Factor.

[2] LSD test. When two means in a column share the same letter, they are not significantly different at alpha = .05.

[3] Duncan's multiple range test. When two means in a column share the same letter, they are not significantly different at alpha = .05.

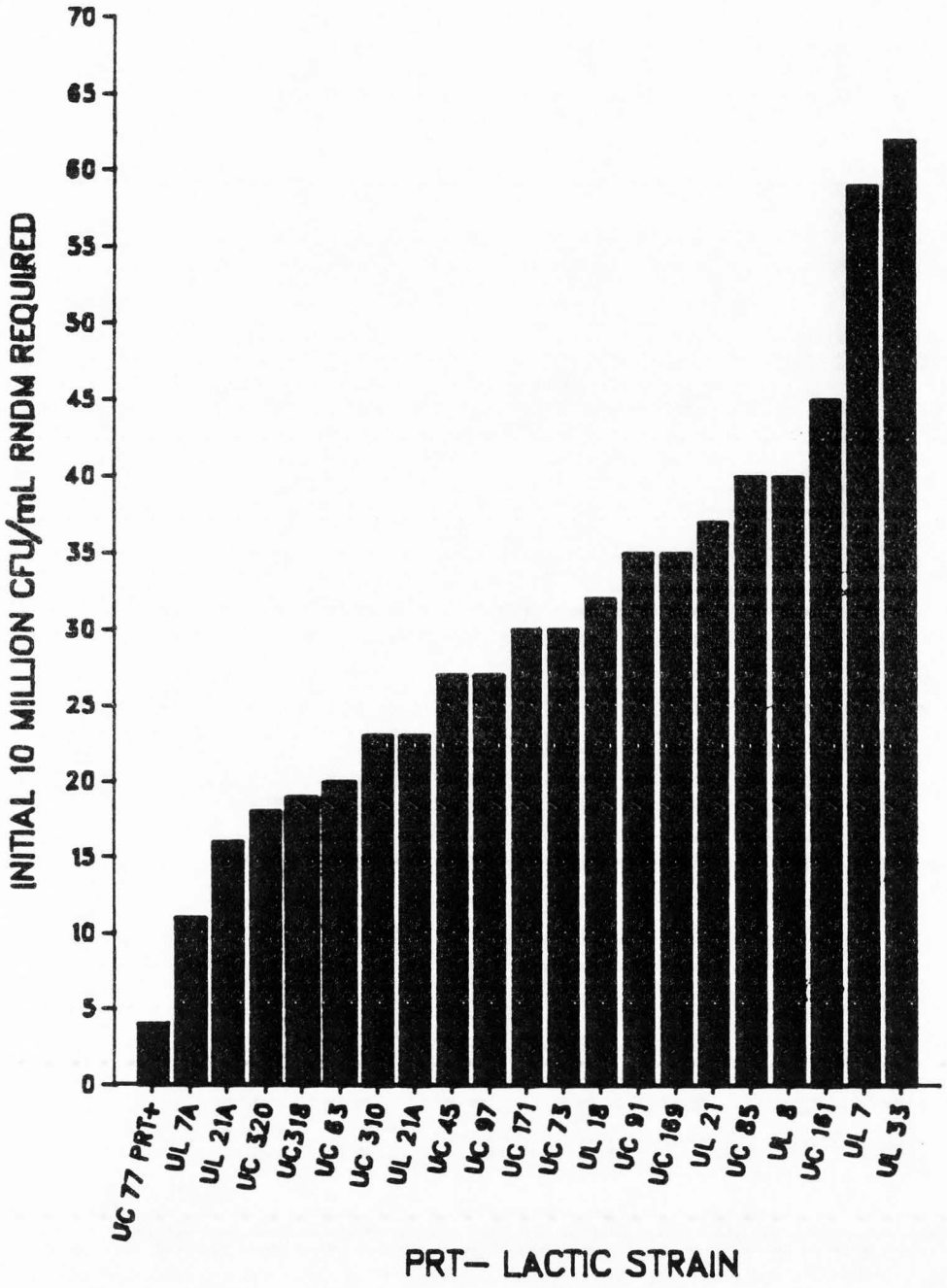

Figure 19. A preliminary study of required initial cell concentrations of Prt- lactic streptococci required in 10% RNDM to produce 1.1 mg lactic acid/mL 10% RNDM after 5 h incubation at 38 C. UL strains are S. lactis and UC strains are S. cremoris. Required initial concentrations were predicted as were predictions in Table 33. Because predicted initial concentrations listed in this table were not replicated, no statistical comparisons can be made.

production and growth rate was observed in Table 35 (Table 36).

Other lactic strains were evaluated for differences in acid production rate using the curve-fitting method of Ruckdeschel (136). A preliminary evaluation of acid production rate among collected Prt- strains was desired. Since this was a preliminary investigation replicates were not performed. Apparent differences in acid production rates among strains tested was observed (Figure 19) but not statistical inference can be drawn.

Six strains were selected for further evaluation. They were chosen from both the low and high ends of the spectrum (Figure 19). They were propagated concurrently and in duplicate in LABGRO medium. The A and B coefficient values were estimated and correlation values as measured by standard deviation are given in Table 37. Also given are the predicted initial cell concentrations to achieve the target final acid concentration (2.5 mg lactic acid/mL 10% RNDM). Significant differences in acid production rates were once again found among individual Prt- lactic strains (tables 38 and 39).

Table 36. Growth and acid production in 10% RNDM of *S. cremoris* UC171 Prt+, UC171 Prt-, *S. lactis* UL7 Prt+ and UL7 Prt-. Lactic strains were initially grown in LABGRO medium prior to 10% inoculation into 10% RNDM. Lactic strains were incubated at 30 C until 10% RNDM pH reached 5.4. Incubation of *S. lactis* UL7 Prt+ was not terminated until RNDM pH had reached 5.2.

Strain	Initial RNDM OD(480 nm)[1]	Time to Reach pH 5.4 (min)	Final RNDM OD(480 nm)	Generation Time (h)
UC171 Prt+	1.0	130	2.7	1.4
UC171 Prt-	1.0	234	2.0	3.6
UL7 Prt+	1.1	165	3.6	1.5
UL7	1.5	258	1.8	15.1

[1] OD(480 nm) 10% RNDM = ((Absorbance at 480 nm (medium+culture))-((Absorbance at 480 nm (medium)) x Dilution Factor.

Table 37. Coefficient values and standard deviation of fit between actual and predicted final acidity values. Strains compared were S. cremoris UC171, UC310, UC73, S. lactis UL7A, UL33 and UL21A. All strains were Prt-. Strains originally were propagated in LABGRO medium. Data was fitted to a curve described by the equation: final cell concentration in milk (OD(480 nm)) = (A x Initial cell concentration (OD(480 nm))) / (B + Initial cell concentration (OD(480 nm)).

Strain	Coefficient Values A	B	SD	Predicted Initial OD[1]
UL7A	12.323	2.665	.176	.68
	7.440	1.311	.051	.66
UL33	9.254	3.555	.182	1.32
	9.724	4.569	.084	1.58
UC171	11.913	3.013	.137	.80
	11.073	2.579	.179	.75
UC310	22.668	7.051	.177	.87
	24.730	7.082	.257	.80
UC73	29.077	9.005	.224	.85
	25.967	5.932	.227	.63
UL21A	11.698	2.040	.157	.55
	28.437	8.101	.167	.78

[1] Initial cell concentration in 10% RNDM (OD(480 nm)) when final acidity in 10% RNDM is set equal to 2.5 mg lactic acid/mL 10% RNDM according to the equation : initial cell concentration (OD(480 nm)) = (Bx2.5) / (A-2.5). An acidity of 2.5 mg lactic acid/mL 10% RNDM used in these experiments approximated a pH of 5.4.

Table 38. Analysis of variance for required initial concentration in 10% RNDM of lactic strains originally propagated in LABGRO medium to achieve a final acidity of 2.5 mg lactic acid/mL 10% RNDM after 5 h incubation at 30 C.

Source	DF	Mean Square	F Value	Pr > F
Strain	5	.1778	12.07	.0044
Error	6	.0147		
		R^2=.910		

The majority of strains were found not to be significantly (alpha=.05) different in acid production rate. The same ranking among these 6 strains for significant (alpha=.05) acid production rates was observed (Figure 43) as observed in the preliminary investigation (Figure 19). The analysis of variance F value on these six strains (Table 38) was markedly lower than the F value on the 4 strains propagated in pH controlled whey-based medium (Table 34) again suggesting the apparent greater sensitivity of WB medium over LABGRO medium for this type of analysis.

Variability in lactic acid production rates among lactic strains may be plasmid linked. Evidence suggests lactose metabolism in some lactic cultures is plasmid linked (39). Degrees of acid production rates among

Table 39. Initial concentrations (OD(480 nm)) in 10% RNDM required to achieve a final acidity of 2.5 mg lactic acid/mL 10%RNDM after 5 h incubation at 30 C. Strains were originally propagated in LABGRO medium. Strains compared were S. cremoris UC171, UC310, UC73, and S. lactis UL7A, UL21A, and UL33. All strains were Prt-. All strains were run in duplicate.

Strain	Required Initial RNDM Cell Concentration[1]		
UL33	1.45	a[2]	a[3]
UC310	.84	b	b
UC171	.78	b	b
UC73	.74	b	b
UL7A	.67	b	b
UL21A	.67	b	b

[1] Optical Density = ((Absorbance at 480 nm (RNDM + culture)) - ((Absorbance(RNDM)) x Dilution Factor.

[2] LSD test. When two means in a column share the same letter, they are not significantly different at alpha = .05.

[3] Duncan's multiple range test. When two means in a column share the same letter, they are not significantly different at alpha = .05.

different plate isolates from the same "pure culture" have been observed (90). Are these apparent differences in acid production rates explained by apparent differences in growth rate which have been previously noted ? Would the same number of cells of different cultures produce the same amount of acid given uniform incubation conditions ?

To answer the above questions significant differences in the activity/growth rate ratio were investigated. Activity/growth ratio is the initial cell concentration required for the particular strain to produce 2.5 mg lactic acid/ mL 10% RNDM in 5 h over the initial cell concentration required to achieve an OD(480 nm) of 1.46 in the same 10% RNDM in 5 h. This ratio should remain uniform from strain to strain if the same number of cells of different cultures do indeed produce the same amount of acid given uniform incubation conditions. Significant differences in the activity/growth rate ratio were found among WB propagaed strains (S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt-) (tables 40 and 41). Individual cells of S. lactis UL7 Prt- produce acid at a statistically slower rate than do cells of S. cremoris UC171 Prt- (tables 40 and 41).

Table 40. Analysis of variance for activity/growth rate ratio (initial cell concentration required for the particular strain to produce 2.5 mg lactic acid/mL 10% RNDM in 5 h over the initial cell concentration required to achieve an OD(480 nm) of 1.46 in the same 10% RNDM in 5 h). S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt- propagated in WB medium were evaluated.

Source	DF	Mean Square	F Value	Pr > F
Strain	1	2.531	126.55	.0004
Protease type	1	.510	25.50	.0070
Strain*Type	1	.450	22.50	.0090
Error	4	.020		

R^2=.977

Differences in the activity/growth ratio among the two Prt+ strains tested are not statistically confirmed but appear to exist (tables 27 and 35).

Significant differences were also found in activity/growth rate ratio among the 6 LABGRO propagated Prt- strains (S. cremoris UC171, UC73, Uc310, S. lactis UL33, UL7A and UL21A). Individual cells S. lactis UL33 Prt- produce acid at a statistically slower rate (alpha=.05) than do all other Prt- strains tested (tables 42 and 43). Individual cells of S. lactis UL21A produces acid at a statistically slower rate than other compared strains except for S. lactis UL33 (tables 42 and 43).

Table 41. Activity/ growth rate (ratio (initial cell concentration required for the particular strain to produce 2.5 mg lactic acid/mL 10% RNDM in 5 h over the initial cell concentration required to achieve an OD(480 nm) of 1.46 in the same 10% RNDM in 5 h). S. cremoris UC171 Prt+, UC171 Prt-, S. lactis UL7 Prt+ and UL7 Prt- propagated in WB medium were evaluated. Means are duplicate averages.

Strain	Activity/Growth Rate Ratio	
UL7 Prt-	2.83	a[1]
UC171 Prt-	1.85	b
UL7 Prt+	1.23	c
UC171 Prt+	1.20	c

[1] When two means in a column share the same letter, they are not significantly different at alpha=.05, Duncan's multiple range test.

Table 42. Analysis of variance for activity/growth rate ratio (initial cell concentration required for the particular strain to produce 2.5 mg lactic acid/mL 10% RNDM in 5 h over the initial cell concentration required to achieve an OD(480 nm) of 1.46 in the same 10% RNDM in 5 h). All evaluated strains were Prt-. S. cremoris UC171, UC73, UC310, S. lactis UL33, UL7A and UL21A propagated in LABGRO medium were evaluated.

Source	DF	Mean Square	F Value	Pr > F
Model	5	.1853	19.53	.0012
Error	6	.0095		
		R^2=.942		

These results suggest that the same number of cells of different cultures do not necessarily produce the same amount of acid during the same time period given uniform incubation conditions. These observations may find application in the cheese industry. Cheese plants interested in the exclusive use of Prt- cultures for cheese production (133) may use Prt- strains with a higher cellular acid production rate to cut down on bulk media costs. Bacteriophage and antibiotic milk problems would diminish (45) and improvements in yield (115) and flavor (102) might come about. Cheese ripening time might be shortened by using S. lactis UL33 Prt- or UL7 Prt- which have much lower cellular acid production rates. Higher

Table 43. Activity/ growth rate ratio (initial cell concentration required for the particular strain to produce 2.5 mg lactic acid/mL 10% RNDM in 5 h over the initial cell concentration required to achieve an OD(480 nm) of 1.46 in the same 10% RNDM in 5 h). All strains evaluated were Prt-. S. cremoris UC171, UC310, UC73, S. lactis UL33, UL7A and UL21A were LABGRO propagated and evaluated. Means are duplicate averages.

Strain	Activity/Growth Rate Ratio	
UL33	1.69	a[1]
UL21A	1.23	b
UC73	0.99	c
UL7A	0.93	c
UC310	0.93	c
UC171	0.92	c

[1] When two means in a column share the same letter, they are not significantly different at alpha=.05, Duncan's multiple range test.

cell concentrations in milk could exist while acid control can still be maintained. The presence of higher cell concentration might speed the ripening process.

Due to their apparent stringent requirement for amino acids and peptides, S. lactis UL7 Prt- and UL33 Prt- were chosen as test strains for evaluation of yeast autolysates, yeast extracts and protein hydrolysates.

Evaluation of Yeast Products for Incorporation into Lactic Culture Bulk Media

Stimulatory properties of commercial yeast autolysates and yeast extracts were evaluated using LABGRO medium. There were significant differences among commercial yeast products in stimulating growth of the test strain, S. lactis UL 7, Prt- (tables 44 and 45).

Table 44. Analysis of variance on growth of S. lactis UL7 Prt- in LABGRO medium with different brands of commercial yeast extracts or yeast autolysates.

Source	DF	Mean Square	F Value	P > F
Brand	11	25.59	59.23	.0001
Error	12	.43		

R^2=.982

Table 45. Formol titration, absorbance index, and lactic streptococcal growth measurements on various commercial yeast extracts and yeast autolysates. S. lactis UL7 Prt- was inoculated, 1% into LABGRO medium plus 0.5% of respective yeast products (YA=yeast autolysate, YE=yeast extract) and incubated 19 h at 27 C.

Product	Type	Formol Titration	Absorbance Index	OD(480 nm)[1]
Product 2313	YE	---	6.2	14.85 a[2]
BBL	YE	---	5.2	14.15 a
AYE 2312	YE	1.7	6.4	13.40 ab
Ardamine Yes	YE	1.6	5.0	12.15 bc
Amberex 1003 lot B 8144	YE	1.3	4.8	11.10 cd
AYS	YA	2.5	7.1	10.80 cd
ZYest 70H	YA	1.4	5.6	10.10 d
Prymex 212	YE	1.5	5.2	9.90 d
Amberex 1003 lot B 1020	YE	---	4.8	8.30 e
Amberex 1003 lot B 1073	YE	1.3	5.0	7.70 ef
Amberex 1400	YA	---	4.6	6.50 f
EP 440	YA	0.8	3.8	2.20 g

[1] 19 h optical density(480 nm)of lactic strain in LABGRO medium. OD(480 nm) = ((Absorbance at 480 nm (medium + culture)) - ((Absorbance at 480 nm (medium)) x Dilution Factor.

There were also significant differences among different lots of the same brand (Amberex 1003). Yeast extracts contain no yeast cell wall materials whereas yeast autolyzates do. Yeast extracts are generally more stimulatory than yeast autolysate (tables 44 and 45). Beer yeast extracts and autolysates (Product 2313, AYE 2312, Amberex 1003, Amberex 1400, Prymex 212 (half brewer's yeast extract and half baker's yeast extract) were all statistically more stimulatory than the autolysate of K. fragilis (EP 440) (tables 44 and 45).

Formol titration and absorbance index of yeast products were evaluated to assess culture stimulatory properties of yeast products. The formol titration method quantitates free α-amino groups and thereby indicates extent of protein hydrolysis (110). Stimulatory yeast autolysates contain more peptides and amino acids (due to yeast autolyzing enzymes hydrolyzing yeast protein) which stimulate greater lactic culture growth. Ultraviolet absorbance at 260 nm (absorbance index) measures extracellular nucleic acid levels and also indicates the presence of peptides and amino acids since nucleic acids are released along with peptides and amino acids from the yeast cell during autolysis (55, 56).

Use of absorbance index to estimate lactic culture growth stimulating properties of yeast extracts and yeast autolysates was investigated (Table 45). A low R^2 value

was shown between absorbance index and lactic culture growth stimulation given by yeast extracts and yeast autolysates grouped together (tables 46, 47 and Figure 20). R^2 did not improve when absorbance index was correlated to yeast extracts alone (tables 48, 49 and Figure 21). A much higher R^2 was achieved when absorbance index was correlated to yeast autolysates (tables 50, 51 and Figure 22).

Use of formol titration to estimate lactic culture growth stimulating properties of yeast extracts and yeast autolysates was investigated (Table 45). A low R^2 value was shown between formol titration and lactic culture growth stimulated by yeast extracts and yeast autolysates grouped together (tables 52, 53 and Figure 23). A markedly improved R^2 was achieved when formol titration was correlated to yeast extracts alone (tables 54, 55 and Figure 24). Similarly, a high R^2 was shown when formol titration was correlated to yeast autolysates (tables 56, 57 and Figure 25).

Absorbance index could evaluate yeast autolysates to be incorporated into lactic culture bulk media. Relative growth stimulating properties of different lots could quickly be screened. Formol titration could evaluate yeast extracts and yeast autolysates to be incorporated into lactic culture bulk media. More efficient yeast product evaluation could be achieved with the absorbance

Table 46. Analysis of variance for absorbance index and stimulatory properties of yeast extracts and yeast autolysates.

Source	DF	Mean Square	F Value	Pr > F
Model	1	64.00	8.34	.0162
Error	10	7.68		
		R^2=.455		

Table 47. Regression analysis of absorbance index and stimulatory properties of yeast extract and yeast autolysate.

Parameter	Estimate	Std Error of Estimate	T for HO Parameter = 0	Pr > |T|
Intercept	-4.236	5.025	-0.843	.4189
Absorbance index	2.698	.935	2.887	.0162

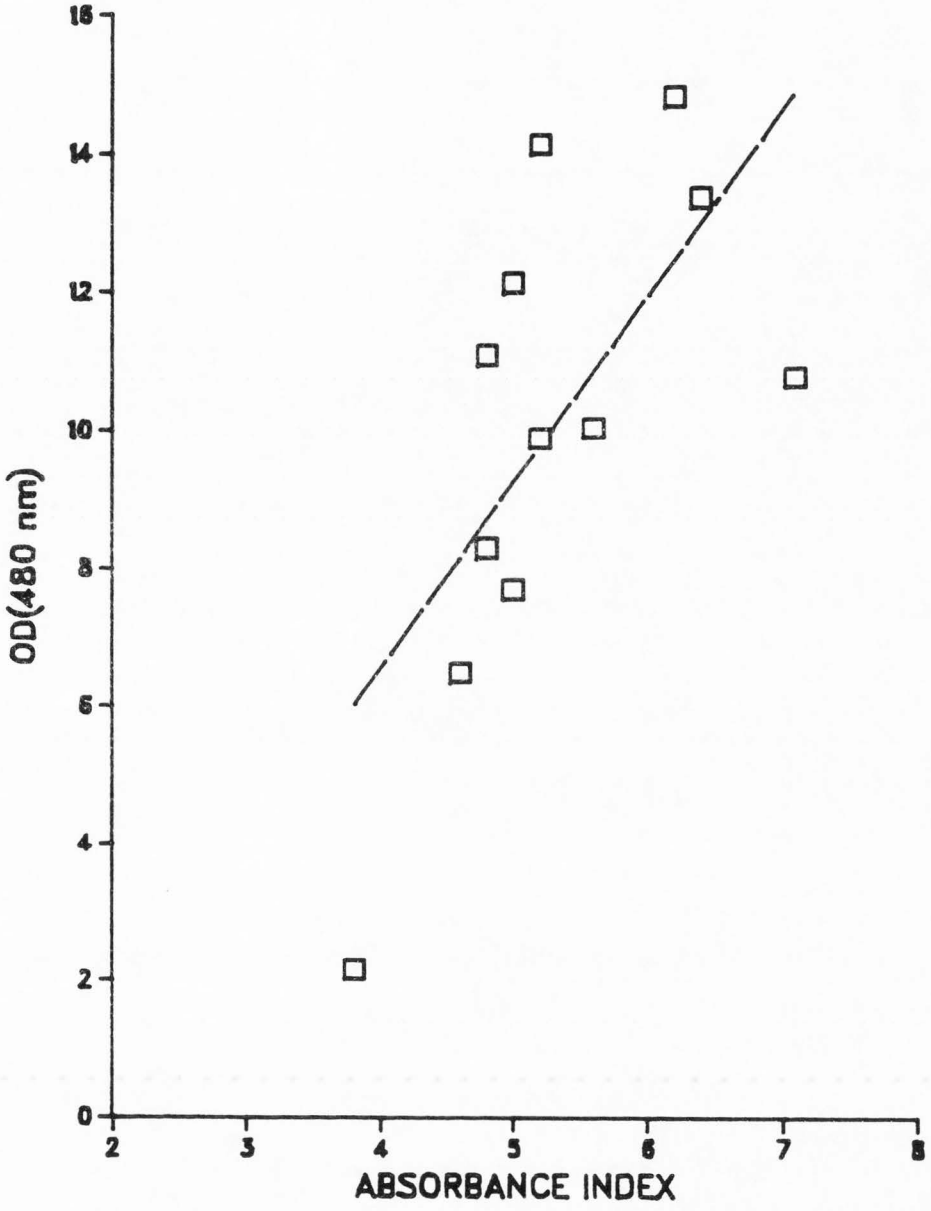

Figure 20. Linear regression of absorbance index of different brands (or different lots of the same brand) of yeast extracts and yeast autolysates incorporated in LABGRO medium and growth of S. lactis UL7 Prt- in LABGRO medium. (See tables 46 and 47).

Table 48. Analysis of variance for absorbance index and stimulatory properties of yeast extracts.

Source	DF	Mean Square	F Value	Pr > F
Model	1	22.16	4.84	.0702
Error	6	4.58		
		R^2=.446		

Table 49. Regression analysis of absorbance index and stimulatory properties of yeast extract.

Parameter	Estimate	Std Error of Estimate	T for HO Parameter = 0	Pr > |T|
Intercept	-3.769	6.959	-0.542	.6076
Absorbance index	2.857	1.299	2.199	.0702

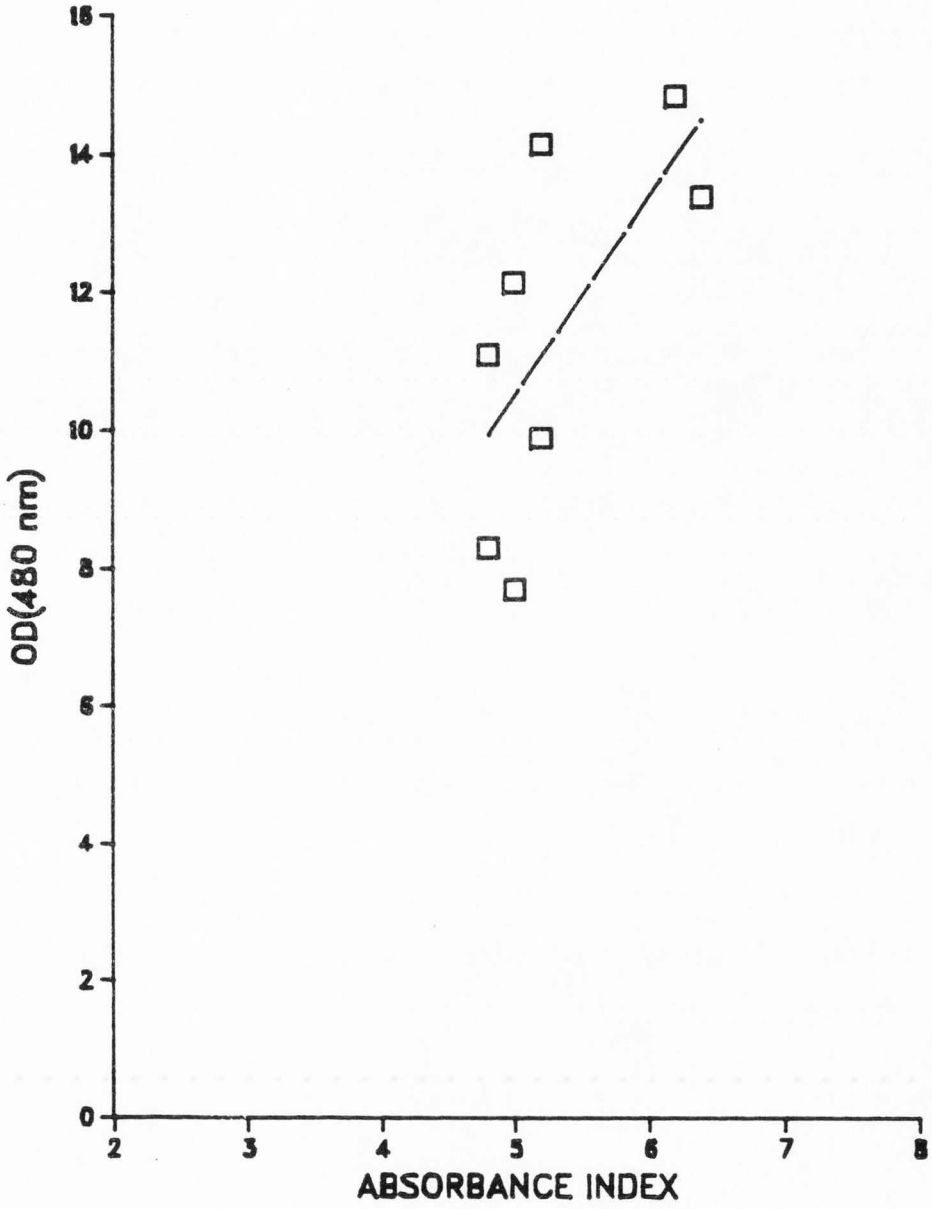

Figure 21. Linear regression of absorbance index of different brands (or different lots of the same brand) of yeast extracts incorporated in LABGRO medium and growth of S. lactis UL7 Prt- in LABGRO medium. (See tables 48 and 49)

Table 50. Analysis of variance for absorbance index and stimulatory properties of yeast autolysates.

Source	DF	Mean Square	F Value	Pr > F
Model	1	39.18	10.08	.0866
Error	2	3.89		
		R^2=.834		

Table 51. Regression analysis of absorbance index and stimulatory properties of yeast autolysates.

Parameter	Estimate	Std Error of Estimate	T for HO Parameter = 0	Pr > |T|
Intercept	-6.029	4.336	-1.390	.2990
Absorbance index	2.541	0.801	3.174	.0866

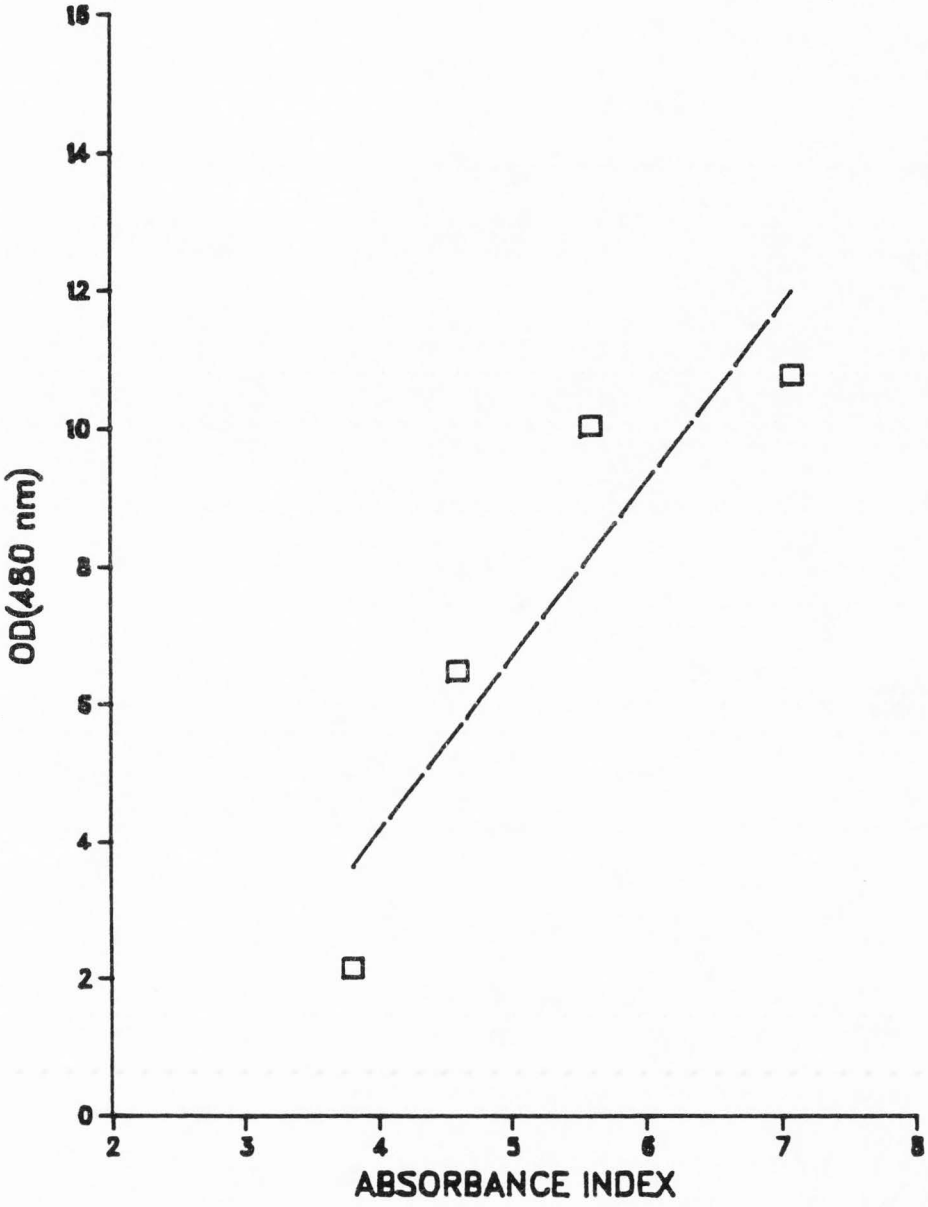

Figure 22. Linear regression of absorbance index of different brands of yeast autolysates incorporated in LABGRO medium and growth of S. lactis UL7 Prt- in LABGRO medium. (See tables 50 and 51).

Table 52. Analysis of variance for formol titration and stimulatory properties of yeast extracts and yeast autolysates.

Source	DF	Mean Square	F Value	Pr > F
Model	1	34.16	4.11	.0889
Error	6	8.31		
		R^2=.407		

Table 53. Regression analysis of formol titration and stimulatory properties of yeast extracts and yeast autolysates.

Parameter	Estimate	Std Error of Estimate	T for HO Parameter = 0	Pr > |T|
Intercept	2.729	3.565	.766	.4729
Formol Titration	4.580	2.259	2.028	.0899

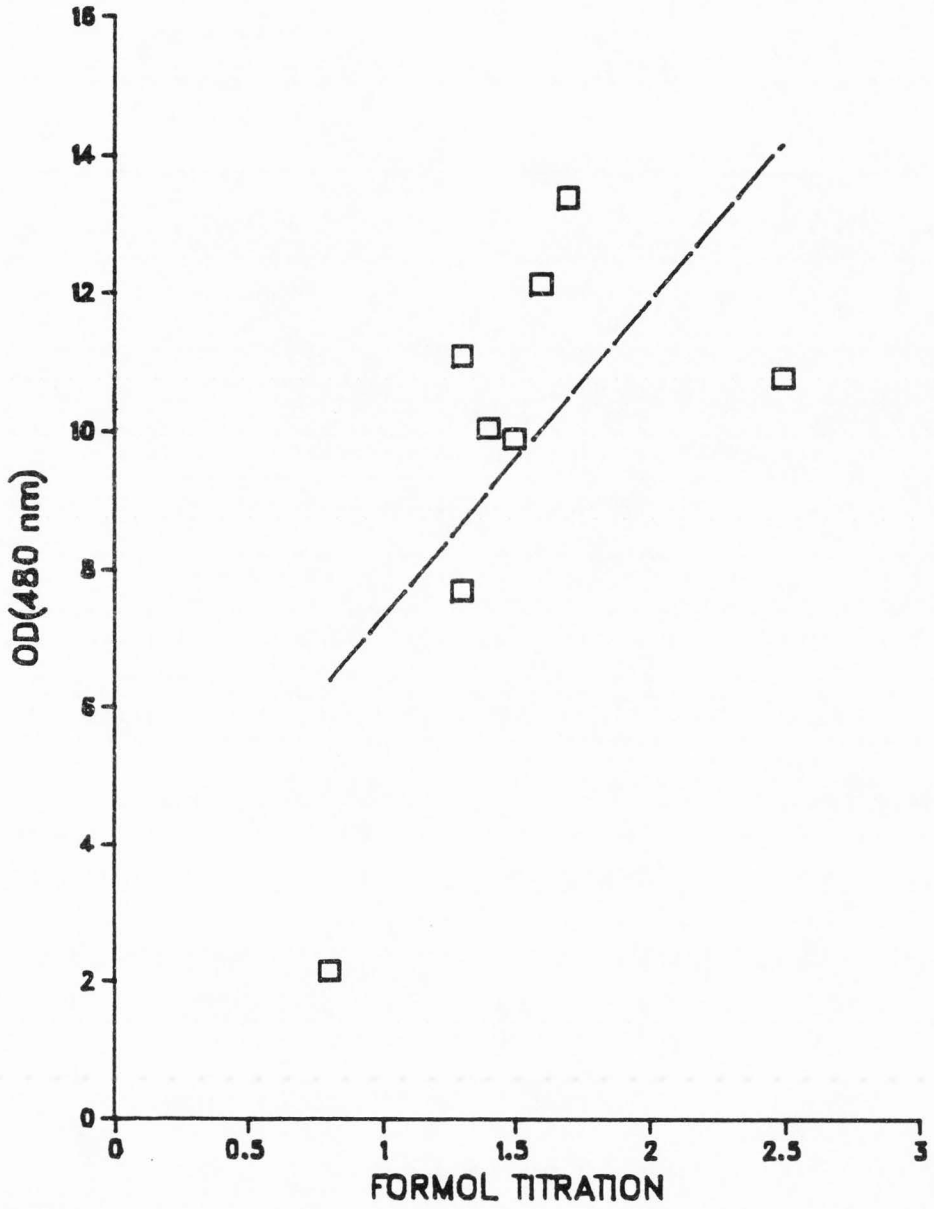

Figure 23. Linear regression of formol titration of different brands (or different lots of the same brand) of yeast extracts and yeast autolysates incorporated in LABGRO medium and growth of S. lactis Prt- in LABGRO medium. (See tables 52 and 53).

Table 54. Analysis of variance for formol titration and stimulatory properties of yeast extracts.

Source	DF	Mean Square	F Value	Pr > F
Model	1	73.68	34.78	.0041
Error	4	2.12		
		R^2=.897		

Table 55. Regression analysis of formol titration and stimulatory properties of yeast extracts.

Parameter	Estimate	Std Error of Estimate	T for HO Parameter = 0	Pr > |T|
Intercept	-6.973	2.839	-2.456	.0700
Formol Titration	11.981	2.032	5.897	.0041

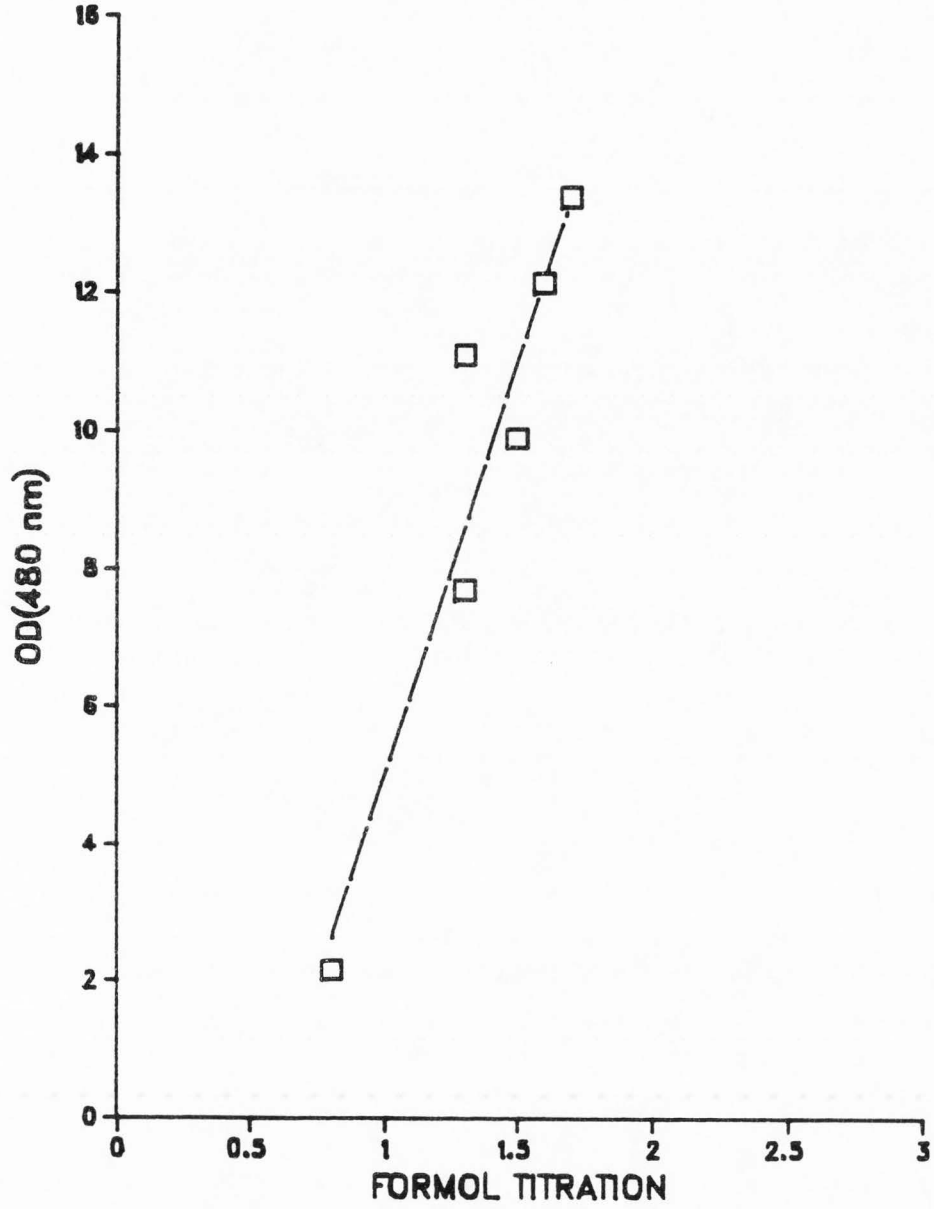

Figure 24. Linear regression of formol titration of different brands (or different lots of the same brand) of yeast extracts incorporated in LABGRO medium and growth of S. lactis UL7 Prt- in LABGRO medium. (See tables 54 and 55).

Table 56. Analysis of variance for formol titration and stimulatory properties of yeast autolysates.

Source	DF	Mean Square	F Value	Pr > F
Model	1	.439	16.92	.1518
Error	1	.026		

R^2=.944

Table 57. Regression analysis of formol titration and stimulatory properties of yeast autolysates.

Parameter	Estimate	Std Error of Estimate	T for HO Parameter = 0	Pr > |T|
Intercept	8.864	.350	25.350	.0251
Formol Titration	.770	.187	4.114	.1518

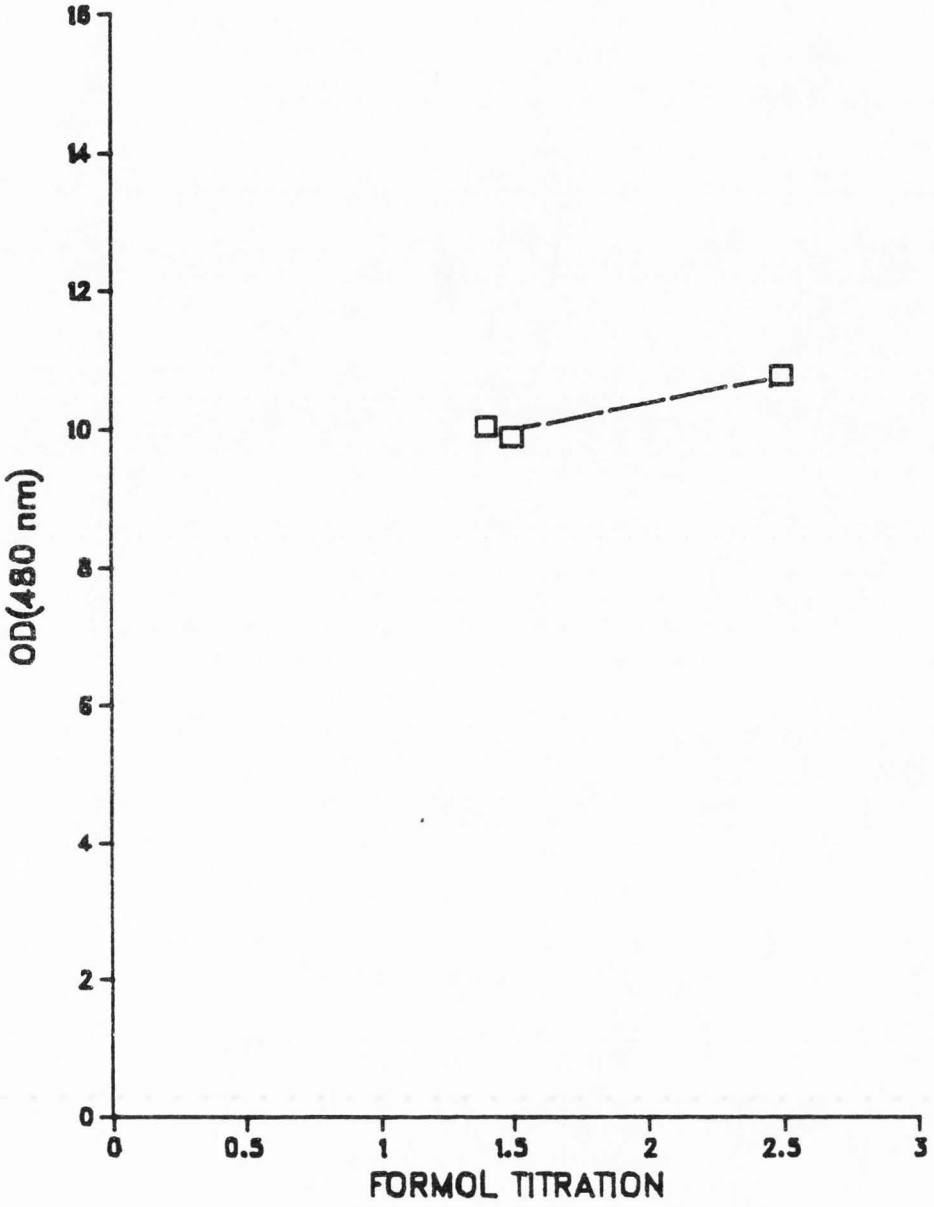

Figure 25. Linear regression of formol titration of different brands of yeast autolysate incorporated in LABGRO medium and growth of S. lactis Prt- in LABGRO medium. (See tables 56 and 57).

index and formol titration methods over the microbiological growth measurements in LABGRO medium. These measurements take minutes to run whereas at least 19 hours is required for microbiological growth measurements. Activity tests require a total of about 24 hours and plate counts require about 43 hours. Respective coefficients of variation (C.V) for 4 of these five methods are given in Table 58.

Better repeatability for sample measurement was shown for absorbance index and formol titration as evidenced by lower coefficients of variation (Table 58). Note the lower coefficient of variation for optical density measurements in LABGRO medium compared to the plate counting method (Table 58).

Conditions for autolysis were imposed on an 18% (dried active) baker's yeast mixture. Autolysis conditions were also imposed on mixtures of baker's yeast and whey protein or casein. Extract solids percentage (% total solids in supernatant after centrifugation of mixture) increased with time (Figure 26). A significant increase in extract solids percentage with time was shown (Table 59).

Table 58. Evaluation of various yeast products for suitability as nutrient sources for propagation of S. lactis UL 7 Prt- in LABGRO medium. Medium was incubated at 27 C for 14 h and for 19 h. The lactic strain was propagated in duplicate. Plate counts were taken on each medium sample in triplicate. All other measurements were taken on each medium sample in duplicate. A = Product # 2312, Busch Industrial Products Corp.; B = AYS, Busch Industrial Products Corp.; C is the same as B; D = ZYEST-70H, Pure Culture Products Inc..

Yeast Product	FT[1]	Absorbance Index	CFU[2]	Microbiological Measurement 14 h OD(480 nm)	19 h CFU	19 h OD(480 nm)
A	1.98	5.8	9.1	11.84	9.1	12.13
B	2.52	7.2	7.4	9.88	7.8	10.91
C	2.54	7.4	6.8	9.79	7.6	11.02
D	2.20	5.9	3.2	8.48	----	6.03
S.D.[3]	.01	.11	.80	.38	.98	.09
C.V.[4]	.22	1.69	12.09	3.83	12.44	3.06

[1] Formol titration.

[2] Plate count, CFU/mL LABGRO medium.

[3] Standard deviation of parameter being measured.

[4] Coefficient of variation of parameter being measured.

Table 59. Analysis of variance on prediction of extract solids percentage (% total solids in supernatant after centrifugation of mixture) by time and yeast mixture type.

Source	DF	Mean Square	F Value	Pr > F
Type	2	73.57	542.33	.0001
Time	2	156.88	1118.13	.0001
Type*Time	4	5.24	37.35	.0001
Error	21	.14		

R^2=.994

Possibly some casein was hydrolysed (and solubulized) by the yeast proteolytic enzymes (3, 61, 96, 142) since a greater rate of extract solids percentage was observed for the yeast and casein mixture (Figure 26).

Methods to monitor production of yeast extract (and protein hydrolysate) during autolysis were then investigated. Absorbance index measurements were taken on the same yeast and yeast and protein mixtures subject to autolysis conditions in Figure 26. Absorbance index increased with time (Figure 27). A significant increase in absorbance index with time was shown (Table 60).

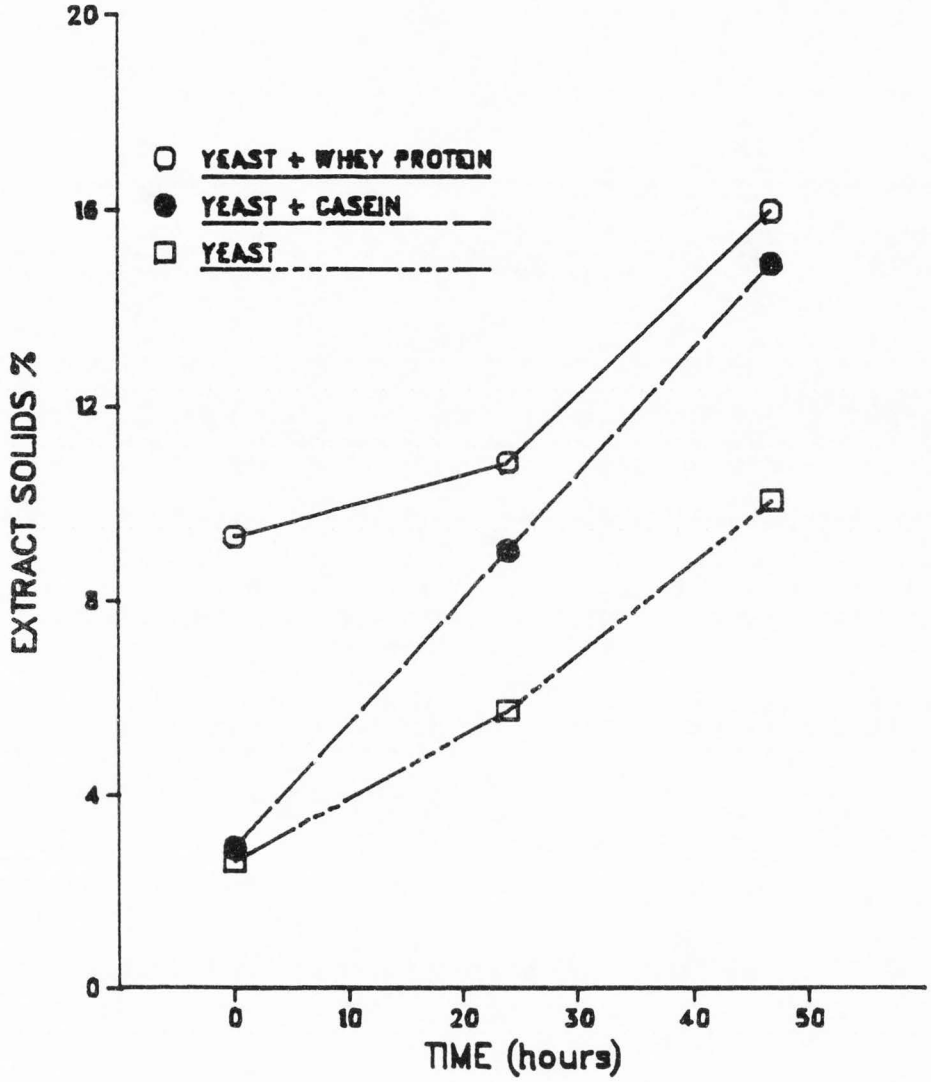

Figure 26. Extract solids during yeast autolysis. Dried active baker's yeast was reconstituted to 18% solids, heated to 50 C for 1 h and thereafter maintained at 45 C. Three groups of yeast or yeast and protein evaluated were: 18% dried yeast + 6% dried whey protein, 18% dried yeast + 6% dried casein, 18% dried yeast. (See Table 59). Extract solids % is the percent total solids in supernatant after centrifugation of mixture.

Table 60. Analysis of variance for prediction of absorbance index by time and yeast mixture type.

Source	DF	Mean Square	F Value	Pr > F
Type	2	.0675	14.97	.0001
Time	6	.3257	72.23	.0001
Type*Time	12	.0127	2.81	.0186
Error	21	.0045		

R^2=.960

Formol titration measurments were also taken on the same yeast and yeast and protein mixtures subject to autolysis conditions in Figure 26. Formol titration increased with time (Figure 28). A significant increase in formol titration with time was shown (Table 61).

Table 61. Analysis of variance for prediction of formol titration by time and yeast mixture type.

Source	DF	Mean Square	F Value	Pr > F
Type	2	.0874	790.64	.0001
Time	6	.3411	3085.71	.0001
Type*Time	10	.0024	21.98	.0001
Error	19	.0001		

R^2=.999

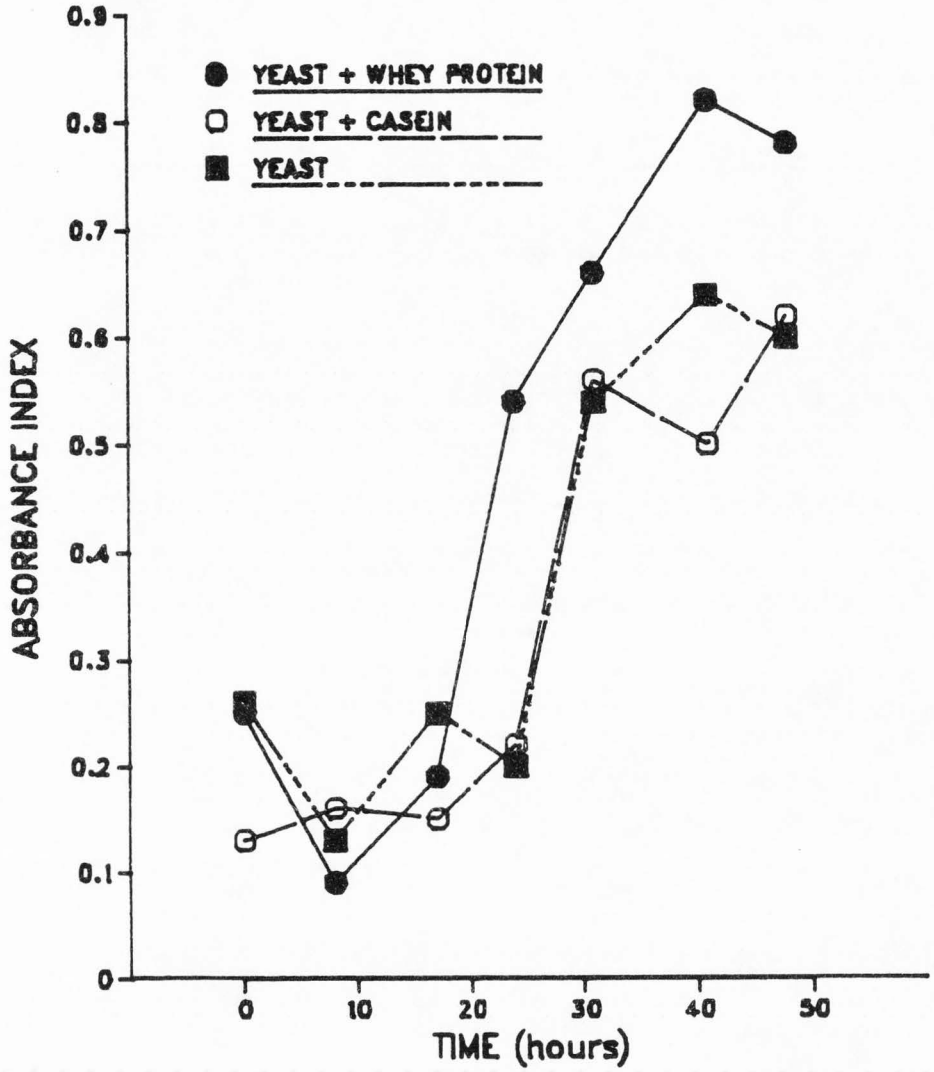

Figure 27. Absorbance index during yeast autolysis. Dried active baker's yeast was reconstituted to 18% solids, heated to 50 C for 1 h and thereafter maintained at 45 C. Three groups of yeast or yeast and protein evaluated were: 18% dried yeast + 6% dried whey protein, 18% dried yeast + 6% dried casein, 18% dried yeast. (See Table 60).

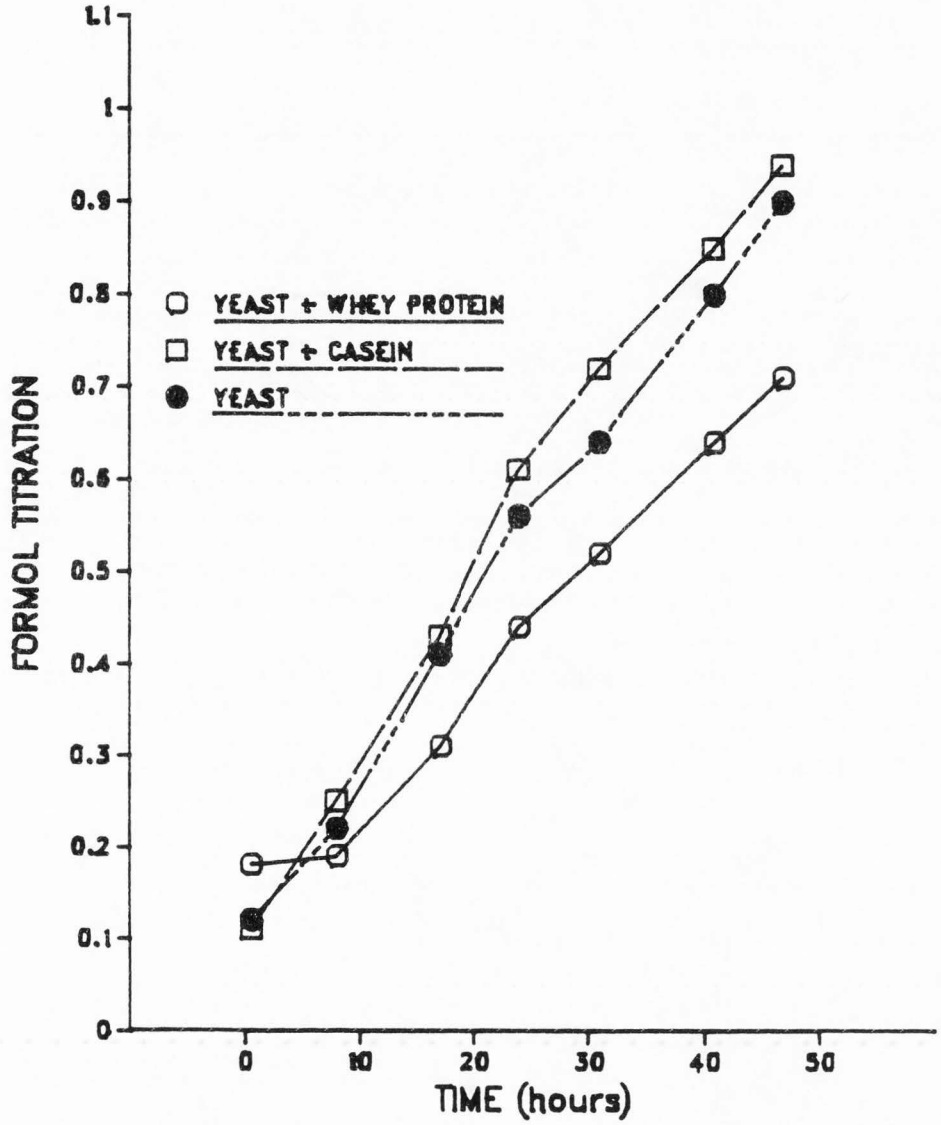

Figure 28. Formol titration during yeast autolysis. Dried active baker's yeast was reconstituted to 18% solids, heated to 50 C for 1 h and thereafter maintained at 45 C. Three groups of yeast or yeast and protein evaluated were: 18% dried yeast + 6% dried whey protein, 18% dried yeast + 6% dried casein, 18% dried yeast. (See Table 61).

Apparently casein was being hydrolysed by yeast enzymes to a greater extent than was whey protein (Figure 28). This was also indicated in Figure 26. Formol titration seems to be superior to absorbance index for monitoring yeast extract/protein hydrolysate production since more uniform readings with time were achieved (figures 27 and 28).

Effect of Yeast Autolysate and Casein Hydrolysate on Lactic Culture Activity

The stimulatory properties of yeast autolysate and casein hydrolysate on lactic cultures were evaluated. S. cremoris UC73 Prt- and UC171 Prt- were blended (1:1) and propagated in LABGRO medium (Table 62). The brand of yeast autolysate used became inhibitory at concentrations above 1.0%. Casein hydrolysate, however, was stimulatory even at 2.5%. Casein hydrolysate appeared to be a better stimulant than the particular yeast autolysate used.

A cost effective lactic culture bulk medium formulation for yeast autolysate and casein hydrolysate evaluated (Table 62) was investigated. A multi-regression analysis was run on results shown in Table 62. The multi-regression model (tables 63 and 64) was utilized in a basic program which determined what yeast autolysate, casein hydrolysate blend would predict an activity test pH change (Pearce activity test) of 1.1 for the least cost.

Table 62. Growth of S. cremoris UC73 Prt- and S. cremoris UC171 Prt- in LABGRO medium with various amounts of added commercial yeast autolysate and casein hydrolysate. Cultures were incubated for 14 h at 27 C. Media were evaluated with the Pearce activity test.

%YP[1]	%CP[2]	Medium 14 h pH	Activity Test[3] (Δ pH)	YP,CP cost/pH unit[4] ($)
0.1	0.1	5.2	.53	.22
0.1	1.0	5.1	1.08	1.18
0.1	2.5	5.1	1.57	2.78
1.0	0.1	5.1	.83	.59
1.0	1.0	5.1	1.14	1.56
1.0	2.5	5.2	1.42	3.16
2.5	0.1	5.6	.26	1.21
2.5	1.0	6.1	.07	2.17
2.5	2.5	6.1	.29	3.77
0.0	0.0	----	.16	----

[1]Yeast product, AYS (yeast autolysate), Busch Industrial Products Corp., St. Louis, MO.

[2]Casein product, N-Z Amine E, Sheffield Products, Norwich, NY.

[3] Δ pH = initial RNDM pH - final RNDM pH.

[4]Cost of yeast product and casein product per 1 Δ pH unit.

The most cost effective blend determined contained 0.4% of the yeast autolysate brand and 0.8% of the casein hydrolysate brand. This blend is much higher than the optimized yeast extract, casein hydrolysate blend for pH controlled whey-based media (.4% yeast extract, .1% casein hydrolysate) reported by Wright and Richardson (178). Differences probably are attributed to brands of stimulants and to media differences. Wright and Richardson used pH controlled whey-based medium rather than LABGRO medium for culture propagation. The observation that pH controlled whey-based medium produced greater concentrations of lactic cultures than LABGRO medium given the same stimulants and stimulant ingrediant levels (Table 19) indicates greater utilization of stimulant concentrations may be achieved in external pH controlled media over internal pH controlled media. This computer-assisted bulk medium optimization method might find application in industry for developing more cost effective media blends.

The yeast autolysate and casein hydrolysate blend (0.4% and 0.8%, respectively) effected a 1.1 pH change in the Pearce activity test. A stimulant composition of 1.2% yeast autolysate with no casein hydrolysate contained the same total amount of stimulants but the predicted activity (tables 63 and 64) was only a 0.7 pH change in the activity test. This suggests a significant positive

Table 63. Analysis of variance for effect yeast autolysate and casein hydrolysate concentration on activity of lactic cultures.

Source	DF	Mean Square	F Value	Pr > F
Yeast autolysate	2	1.398	3699.84	.0001
Casein hydrolysate	2	.290	768.71	.0001
YA*CH	4	.169	447.33	.0001
Error	9	.0003		
		R^2=.999		

Table 64. Multiple regression analysis on effects of yeast autolysate and casein hydrolysate on lactic culture activity.

Parameter	Estimate	Std Error of Estimate	T for HO: Parameter	Pr > |T|
Intercept	0.391	.017	23.67	.0001
YA	0.690	.036	19.31	.0001
CH	.770	.036	21.56	.0001
YA*CH	-.298	.077	-3.85	.0039
YA^2	-.291	.013	-22.53	.0001
$CH*YA^2$	-.065	.028	-2.34	.0438
CH^2	-.120	.013	-9.28	.0001
YA^2*CH^2	.034	.01	3.35	.0086

interaction between the brands of yeast autolysate and casein hydrolysates used. A significant interaction between yeast autolysate and casein hydrolysate was indeed shown (Table 63). There appears to be a concentration range wherein superior stimulation is given by a blend of casein hydrolysate (whey protein hydrolysate also?) and yeast autolysate over the same total concentration of yeast autolysate. This will be confirmed in following results (Table 74).

Results previously cited (figures 26 and 28) suggest that during autolysis, proteolytic enzymes of active dry baker's yeast hydrolyse casein. Would this apparent casein hydrolysis also occur with a lactose-fermenting yeast (K. fragilis)? Would the casein hydrolysate be sufficiently cleaved to be stimulatory? Also could K. fragilis sufficiently hydrolyze whey protein? These topics were investigated.

Extent of whey protein hydrolysis on culture stimulation was evaluated (Table 65). Formol titration indicated extent of whey protein hydrolysis (110). Greater apparent hydrolysis was observed in whey protein treated initially with pepsin followed with a neutral fungal protease than with the fungal protease alone or with acid hydrolysis alone (Table 65). Formol titration of lactalbumen hydrolysate (Difco) was not recorded but should be close to that of casein hydrolysate. A

Table 65. Whey protein hydrolysis from pepsin[1], HT Proteolytic[2] and acid. Freeze-dried whey protein was reconstituted to 7% solids. Twenty ml aliquots of whey protein concentrate was used for formol titration. Whey protein mixture was acidified with phosphoric acid to pH 2.0 and 1 g of powdered pepsin was added to 100 ml of whey protein mixture. Pepsin was mixed in and the whey protein mixture was incubated for 5 hours. The whey protein mixture pH was then adjusted to 7.3 with NaOH and 0.6 HT-Proteolytic^{tm} (Marschall Products) enzyme was added to 60 ml of whey protein mixture and incubated at 55 C for 1 h. Acid hydrolysis was carried out by adding 5.6 g phosphoric acid to 100 ml of whey protein mixture whereupon the mixture was heated at 100 C for 6 hour.

Incubation Time (h)	Mixture pH	Enzyme Added	Formol Titration	Lactic Culture Growth[1]
0	7.0	HT-Proteol.	0.27	
4.0			0.26	
5.0			0.33	
0	2.0	pepsin	0.24	
1.5			0.51	
2.0			0.54	
5.0	7.0	HT-Proteol.	0.64	
6.0			0.73	5.99 / 6.66
0	Acid Hydrolysis		(ca 0.20)	
6			0.52	6.74 / 6.83
	casein hydrolysate[2]		1.42	6.05 / 6.83
	lactalbumen hydrolysate[3]		----	5.85 / ----

[1] pH in LABGRO medium after 19 h incubation at 27 C / pH of uninoculated LABGRO medium after 19 h incubation at 27 C. 0.5% (% weight/volume) hydrolysate, whole whey protein added to LABGRO medium.

[2] N-Z Amine E, Sheffield Products, Norwich, NY 13815.

[3] Difco Laboratories, Detroit, MI.

preliminary investigation indicated lactalbumen hydrolysate, casein hydrolysate and the enyzme-hydrolysed whey protein to contain similar lactic culture growth stimulating properties (Table 65). Acid-hydrolysed whey protein appeared markedly less stimulatory.

These hydrolysates and the yeast autolysate used in the factorial experiment (Table 62) were compared for growth stimulating properties. Significant differences were found (tables 66 and 67).

Table 66. Analysis of variance for effect of whey protein hydrolysates, yeast autolysate and casein hydrolysate on growth of *S. lactis* UL33 Prt- in LABGRO medium (19 h at 27 C).

Source	DF	Mean Square	F Value	Pr > F
Stimulant	4	.4400	188.6	.0001
Error	5	.0023		
		R^2=.993		

The yeast autolysate was significantly more stimulatory than the protein hydrolysates. This was surprising in view previous results (Table 62) wherein the casein hydrolysate appeared more stimulatory. Possibly there is some growth factor other than amino acids or

Table 67. Effect on lactic culture growth of whey protein hydrolysates, yeast autolysate and casein hydrolysate. S. lactis UL33 Prt- was propagated in LABGRO medium for 19 h at 27 C. Stimulants were added at 0.5%.

Stimulant	Culture Growth 19 h LABGRO pH	/	19 h LABGRO pH (Control)
WPH-acid[1]	6.75 a[2]	/	6.83
Casein Hydrolysate[3]	6.05 b	/	6.76
WPH-enzyme[4]	5.99 b	/	6.66
LH[5]	5.84 c	/	6.80
AYS[6]	5.46 d	/	6.85

[1] Acid-hydrolysed whey protein.

[2] When two means in a column share the same letter, they are not significantly different at alpha = .05, Duncan's multiple range Test.

[3] NZ Amine type E.

[4] Enzyme-hydrolysed whey protein. Enzymes used were pepsin and HT Proteolytic, a neutral fungal enzyme.

[5] Lactalbumin hydrolysate. Difco Laboratories.

[6] Yeast autolysate. Annheuser-Busch.

small peptides present in yeast autolysate but lacking in protein hydrolysate (145).

Unlike results in Figure 28 where formol titration measurements of the yeast and protein mixture during hydrolysis was higher than for the yeast itself, results in Table 71 show otherwise. This suggests little protein hydrolysis by K. fragilis during autolysis. When pepsin was initially added to yeast and protein samples greater protein hydrolysis was achieved (Table 71). The yeast autolysate and casein mixture with pepsin appeared to be more stimulatory than yeast autolysate and whey protein mixture with pepsin (Table 71). Little apparent difference between the two was shown by formol titration, however.

Growth of Kluyveromyces fragilis in Yeast Whey Permeate Medium

A major research objective was to see if a stimulatory yeast autolysate or extract for incorporation into lactic culture bulk medium could be produced from a lactose-fermenting yeast, i.e., K. fragilis.

A strain of K. fragilis was obtained at the Utah State University culture bank and propagated in whey containing 5.0% whey solids and growth factors. One volume of air/volume medium-min was delivered during the propagation period. By 18 h 11.5 g dried yeast/L whey

Table 68. Growth of *K. fragilis* in whey medium (5.0% whey solids, 0.1% yeast extract, 0.85% diammonium sulphate, 0.23% diammonium phosphate. Incubation temperature was 35 C. Yeast dry weight was measured in duplicate.

Incubation Time (h)	Dissolved O_2 (ppm)	Medium pH	Yeast Dry Weight (g/l)
0	------	5.7	-------
8	0.0	4.6	7.0
18	5.8	4.5	11.7
22	6.1	4.6	11.4

Dry Yeast Weight : 11.6 g/l
Estimated Initial Lactose : 35 g/l
% Yield (g dried yeast/g lactose) : 33%

Dry Yeast Weight (centrifuged) : 98.8 g/l
Yeast Extract Solids : 41.6 g/l
% Yield (g yeast extract solids/g dry yeast): 42%

medium was present (Table 68). Conversion of lactose into yeast (g dried yeast/g lactose) was 33%. These values compare with those reported in literature which range from 23 to 62% (4, 20, 35, 48, 50, 127, 160). Recovery of yeast extract (42%) (Table 68) was comparable to the level generally harvested in industry (122).

Giec and Kosikowski (48) found K. fragilis ATTC 8582 to yield the highest biomass in a field of 10 lactose-fermenting yeasts. K. fragilis ATTC 8582 was obtained from the American Type Culture Collection and propagated in whey permeate medium adjusted to pH 5.0 with phosphoric acid. Whey permeate medium contained 0.1% yeast extract. Incubating temperature was 30 C. Two volumes of air/volume medium/min was delivered for 19 h when it was increased to 4 volume air/volume medium/min. Over 25 g dry yeast/l medium was obtained after a 42 h incubation period (Figure 29). Giec and Kosikowski (48) reported a yield of 27.5 g/l with this strain and under the same general (they did not add 0.1% yeast extract to their whey medium) conditions. They, however, sparged 4 volume air/volume medium/min into the whey medium throughout the 48 h propagation period. When these researchers delivered 2 volumes air/volume medium/min under the same conditions, they obtained a yield of 10.8 g/l. A 155% increase in yield is achieved by the increase of air sparging rate from 2 volume/volume medium/min to 4 volume/volume

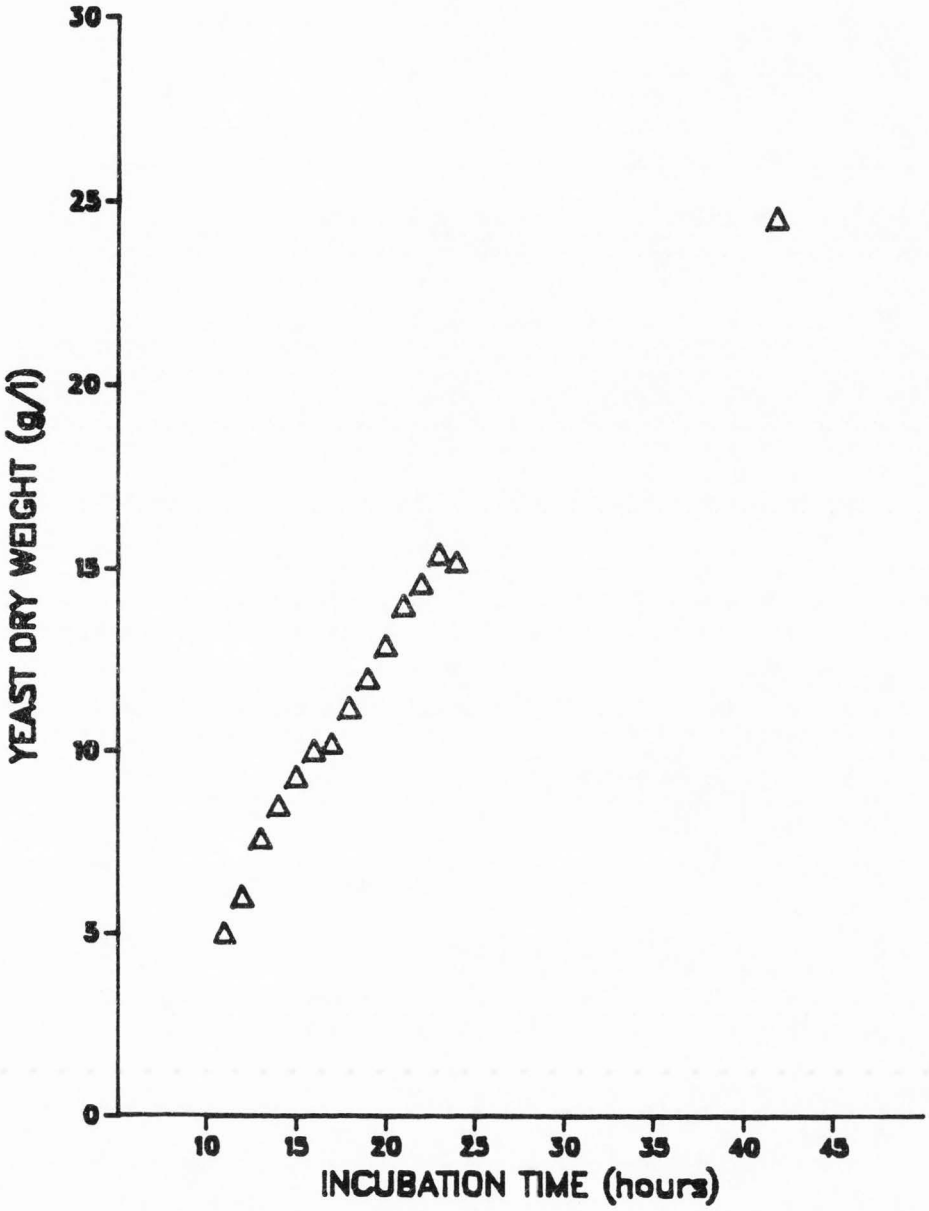

Figure 29. Growth of Kluyveromyces fragilis ATCC 8582 in yeast whey permeate medium. Initial pH was 5.0. Incubation temperature was 30 C. Filtered air sparging rate was 2 volumes air/volume medium-min until 19 h whereupon the air rate was increased to 4 volumes air/volume medium-min.

medium/min. When K. fragilis ATTC 12424 was propagated 42 h in yeast whey permeate medium with 4 volume air/volume medium/min being delivered, 31.5 g dry yeast/l medium was harvested (Table 71). When the amount of air delivered was increased from 2 volumes to 4 volumes/volume medium-min a yield increase of 296% was achieved.

Autolysis of Whey-Grown Kluyveromyce fragilis and Evaluation of Autolysate Products

Giec and Kosikowski (48) reported K. fragilis ATTC 8582 to be produce the greatest biomass in whey. This strain was therefore propagated in yeast whey permeate medium and autolysis conditions were imposed. The 24 h autolysate product was then evaluated with the LABGRO medium method. K. fragilis ATTC 8582 autolysate, was markedly inferior in stimulating lactic culture growth when compared to a commercial yeast autolysate--even when the concentration was doubled (Table 69). The K. fragilis autolysate became more stimulatory when it was added to LABGRO medium in a 1:1 blend with the commercial yeast autolysate.

The optimum pH for autolysis was investigated. K. fragilis was propagated in yeast whey permeate medium and the pre-autolysate mixture was pH adjusted (Table 70). Formol titration measurements indicated an initial pH of 5.5 optimal for producing the most hydrolyzed yeast

Table 69. Autolysis of *K. fragilis* ATCC 8582 propagated in yeast whey permeate medium. Autolysis period was 24 h. The sample was heated from 30 C to 50 C during a 6 h period. Initial autolysis pH was adjusted to 5.0 with HCL or NaOH. Degree of stimulation from the yeast autolysate was evaluated by incorporating it into LABGRO medium. *S. lactis* UL33 Prt- was inoculated into LABGRO medium and incubated at 27 C for 19 h. pH LABGRO control is the 19 h uninoculated LABGRO pH.

Yeast Autolysate	Amount added (%)	Culture Growth 19 h LABGRO pH / 19 h LABGRO control pH
ATCC 8582	0.5	6.73 / 6.82
	1.0	6.58 / 6.70
ATCC 8582 + AYS[1]	0.25 0.25	6.44 / 6.90
AYS	0.5	5.59 / 6.99

[1] Yeast autolysate, Busch Industrial Products Corp., St. Louis, MO.

Table 70. Autolysis of K. fragilis ATCC 8582 propagated in yeast whey permeate medium. Autolysis period was 24 h. The sample was heated from 30 C to 50 C druing a 6 h period. Initial autolysis pH was adjusted with HCL or NaOH. Yeast concentration was estimated from OD(560 nm) to be 15 g/L (Table 12). The yeast autolysate was incorporated into LABGRO medium (0.5%) and S. lactis UL33 was inoculated and incubated for 19 h at 27 C.

pH treatment	Formol titration	Culture Growth 19 h LABGRO pH / pH LABGRO blank
4.0 (0 h)[1]	----	6.50 / 6.60
4.0 (12 h)	----	6.44 / 6.60
4.0 (24 h)	3.10	6.50 / 6.61
4.0 (43 h)	3.70	6.49 / 6.70
5.0	3.99	6.48 / ----
5.5	4.65	---- / ----
6.0	3.90	---- / ----

[1] Hours of autolysis.

proteins. These results agree with the work of Orberg et al. (117). However, no autolyzate of K. fragilis ATTC 8582 was comparable in lactic culture stimulating properties to the commercial yeast autolyzate (tables 69 and 70).

Orberg et al. (117) reported that K. fragilis NRRl Y-610 (same as K. fragilis ATCC 12424) was superior to a group of various K. fragilis strains in producing an autolyzate stimulatory to lactic cultures. K. fragilis ATTC 12424 was obtained and propagated in yeast whey permeate medium and subjected to autolysis conditions. No autolysate of K. fragilis ATCC 12424 stimulated lactic culture growth to the extent of a commercial yeast autolysate (Table 71). The autolysate of K. fragilis ATCC 12424 was hydrolysed about one half the extent of the commercial yeast autolysate (Table 71). Significant less hydrolysed proteins (or total proteins) in the K. fragilis 12424 autolysate may account for inferior stimulatory properties.

An extract of K. fragilis ATCC 12424 was then produced and tested for lactic culture stimulation. The yeast extract of K. fragilis ATTC 12424 was found to contain little stimulatory value (tables 72 and 73).

Table 71. Autolysis of K. fragilis ATCC 12424 propagated in yeast whey permeate medium for 48 h to a concentration of 31.5 g dried yeast/L medium. Air was sparged into medium at 4 volumes air/volume medium-min. Autolysis period was 24 h. The sample was heated from 30 C to 55 C during a 6 h period. Initial autolysis pH was adjusted to 5.5 with HCL or NaOH. Heat treated (90 C for 40 min) whey protein and acid casein were added to the yeast to a final concentration of 6% prior to autolysis. Degree of stimulation from the yeast autolysate was evaluated by incorporating it into LABGRO medium (0..5%) and uncubating S. lactis UL33 Prt- for 19 h at 27 C. PC = 20 mg pepsin added to yeast+casein mixture at beginning of autolysis; PWP = 20 mg pepsin added to yeast+whey protein mixture at beginning of autolysis. pH LABGRO control is the 19 h uninoculated LABGRO pH.

Treatment	Formol Titration	Culture Growth 19 h LABGRO pH / pH LABGRO control
yeast (0 h)[1]	.78	6.68 / 6.83
(24 h)	1.46	6.72 / 6.81
(36 h)	1.56	6.76 / 6.78
yeast+casein	.57	
0.5%[2]		6.58 / 6.81
1.0%		6.54 / 6.69
yeast+WP	.49	6.66 / 6.75
yeast+PC	.98	
0.5%		6.24 / 6.88
1.0%		5.88 / 6.65
yeast+PWP	.95	
0.5%		6.30 / 6.83
1.0%		6.28 / 6.68
AYS[3]	2.78	5.47 / 6.92

[1] Hours of autolysis.

[2] Amount of product added to LABGRO medium (%wt/vol).

[3] Yeast autolysate, Busch Industrial Products Corp., St. Louis, MO

Table 72. Analysis of variance on growth stimulation of S. lactis UL33 Prt- by a yeast extract and yeast autolysate of K. fragilis, a commercial yeast extract and yeast autolysate. S. lactis was propagated in LABGRO medium.

Source	DF	Mean Square	F Value	Pr > F
Stimulant	3	1.124	890.1	.0001
Error	4	.001		
		R^2=.999		

Both the extract and the autolysate were found not very stimulatory. Moreover the extract was not more stimulatory than the autolysate (Table 73). The commercial yeast products were once again proven more stimulatory to the growth of lactic cultures.

An extract from K. fragilis ATCC 12424 propagated under the same conditions as before except 1 volume of air rather the 4 volumes/volume medium-min was given. This treatment more closely simulated the yeast propagation procedures of Orberg et al. (117). The K. fragilis ATCC 12424 extract and the previously enzyme-hydrolysed whey protein (Table 65) were evaluated for lactic culture growth stimulation and compared with a commercial yeast extract, yeast autolysate and lactalbumen hydrolysate. Results again confirm the previous observations that

Table 73. Stimulatory properties of an extract (YE) and autolysate (YA) of Kluyveromyces fragilis ATCC 12424 and whey protein hydrolysate on the growth of S. lactis UL33 Prt- in LABGRO medium. Twenty-one percent of the weight of the yeast was recovered as yeast extract. Measurements are duplicate averages. Stimulants were added to LABGRO medium at 0.5%. Media were incubated 19 h at 27 C.

Product	19 h LABGRO pH	/	19 h LABGRO control pH
K. fragilis ATCC 12424 (YE)	6.55 a[1]	/	c.a. 6.8
K. fragilis ATCC 12424 (YA)	6.61 a	/	c.a. 6.8
AYE[2]	5.30 b	/	6.66
AYS[3]	5.27 b	/	6.73

[1] Duncan's multiple range test. When two means in a column share the same letter, they are not significantly different at alpha=.05

[2] Yeast extract, Busch Industrial Products Corp., St. Louis, MO.

[3] Yeast autolysate, Busch Industrial Products Corp., St. Louis, MO.

extracts of K. fragilis heretofor produced were not very stimulating to lactic culture growth (Tables 74 and 75).

Table 74. Analysis of variance for effect of an enzyme-hydrolysed whey protein, a commercial yeast extract and yeast autolysate, a commercial casein hydrolysate and a commercial lactalbumen hydrolysate on growth of S. lactis UL33 Prt- in LABGRO medium (19 h at 27 C).

Source	DF	Mean Square	F Value	Pr > F
Stimulant	8	50.50	114.78	.0001
Error	9	.44		
		R^2=.990		

Very little growth was stimulated by the yeast extract or whey protein hydrolysate themselves. When the two were added in equal proportions at the same final concentration (0.5%) significant growth occurred. Moreover, when the yeast extract of K. fragilis ATCC 12424 was added in equal proportions with lactalbumen hydrolysate (Difco), significant growth again occurred (Table 75). This might be due to a peroxidase or an essential growth factor not associated with protein hydrolysate. The superior growth properties of LABGRO medium containing yeast autolysate but no casein or lactalbumen hydrolysate over medium containing casein or

Table 75. Stimulatory properties of yeast extract of *K. fragilis* ATCC 12424 on *S. lactis* UL33 Prt-. Incubation was for 19 h at 27 C.

LABGRO Basal Medium plus	OD(480 nm)[1]
.25 (%w/v) product 2313[2] .25 lactalbumin hydrolysate[4]	14.45 a[3]
.25 product 2313 .25 WPH[5]	11.35 b
.5 Product 2313	11.05 b
.5 AYS[6]	10.50 b
.25 YE[7] .25 lactalbumin hydrolysate	10.10 b
.5 lactalbumin hydrolysate	6.35 c
.25 YE .25 WPH	5.70 c
.5 WPH	0.30 d
.5 YE	0.10 d

[1] Optical density(480 nm) = ((Absorbance at 480 nm(medium+culture))-((Absorbance at 480 nm(medium)) x Dilution Factor.

[2] Yeast extract, Annheuaser-Busch Company,

[3] When two means in a column share the same letter, they are not significantly different at alpha = .05, Duncan's multiple range test.

[4] Difco

[5] pepsin, neutral fungal protease-hydrolysed whey protein (Table 69), [6] yeast autolysate, Annheuser-Busch Company, [7] yeast extract of *K. fragilis* ATCC 12424.

lactalbumen hydrolysate but no yeast autolysate has previously been observed (Table 67).

Growth stimulating properties of a blend of commercial yeast extract and whey protein hydrolysate (Table 65) were equally stimulatory (alpha=.05) to that of only commercial yeast extract at equal total concentrations (tables 74 and 75). This suggests that whey protein less hydrolysed than commercial protein hydrolysate (Table 65) can replace half the commercial yeast extract and still effect the same growth. If this whey protein hydrolysate could be produced for $2.45/lb (cost of the commercial yeast extract) a more cost effective bulk medium could be developed. Lactalbumen hydrolysate was clearly more stimulatory than the same amount of the whey protein hydrolsate. This was probably due to the commercial lactalbumen hydrolysate being more hydrolysed than the pepsin, neutral fungal enzyme-hydrolsed whey protein (Table 65).

No lactic culture growth stimulating autolysates or extracts of two strains of K. fragilis comparable to commercial yeast autolysates and extracts were found. Also, no significant hydrolysis of milk proteins by K. fragilis proteolytic enzymes was evident. Methods other than simple autolysis should be investigated. Knorr et al. suggest mechanical disruption of the yeast cell wall would markedly stimulate cell hydrolytic enzymes with a

concommitant increase in released hydrolysed protein (77). Reed advises homogenization pressures of at least 10,000 psi would be required to break yeast cell walls (Personal communication, Gerald Reed, Universal Foods Inc., 1984). Knorr et al. also suggest that an excellent yeast autolysate could be produced by adding lytic and proteolyzing enzymes to the yeast (77). Reed suggests acid-hydrolysing the yeast may produce a more stimulatory product (Personal communication, Gerald Reed, Universal Foods, Inc., 1984). Autolysates from lactose-fermenting yeasts other than K. fragilis should be also be evaluated.

Given the lactose eliminating ability of K. fragilis (one strain eliminated 130 g lactose/liter medium in 42 h) and their ability to produce protein from ammonium compounds; significant benefits to the cheese industry may exist. As ultrafiltration technology moves into the cheese industry, problems with milk permeate and whey permeate disposal may become even more significant than whey disposal problems are currently. A recycling program for this waste material utilizing a lactose-fermenting yeast would produce a plentiful supply of yeast extract and animal feed from the cell debris. Even if this yeast extract could not be made as stimulatory as commercial extracts from beer yeast or baker's yeast; as long as a minimal amount of capability to stimulate lactic culture growth is achieved, this product may have an important

role in the cheese industry. This product could be used in bulk media, even if three to four times the amount of this than commercial yeast extract is required. Furthermore, this extract added to cheese milk could significantly enhance culture growth thereby reducing amounts of bulk media added. Also the cheese cultures would use the amino acids and peptides supplied by the yeast extract for their growth instead of hydrolysing casein in the milk. Yield would then probably increase. This yeast extract could be added to milk retentate to encourage lactic culture activity in milk retentate which has heretofor been shown to be quite low (Personal communication, Ron Raynes, Utah State University, 1984).

Minimally hydrolysed whey proteins when added with yeast extract of K. fragilis has been shown to be more stimulatory than the yeast extract or the hydrolysed whey protein alone (Table 75). Both these products should be used together. Plants producing whey protein concentrate powder may realize a greater profit (whey protein concentrate sells for about $0.50/1b) if they were to hydrolyse the whey protein and sell the hydrolysate/lactose/phosphate dried product resulting from the enzyme treatment to growth media blending companies or use it themselves for their own culture propagation.

The goals of this research project were (A) to develop methods to evaluate whey-derived nutrients for

incorporation into lactic culture bulk media, (B) to develop a procedure to monitor whey-derived nutrients during production and (C) to establish production parameters for whey-derived products.

Microbiological growth assays using LABGRO medium was proven to be an effective method to evaluate stimulatory properties of yeast extracts, autolysates and protein hydrolysates. Formol titration was shown to be a good indicator of lactic growth stimulating properties of individual yeast extract and yeast autolysate samples. Absorbance index was a good evaluation assay for yeast autolysate but not for yeast extracts. Objective A was accomplished.

Formol titration was shown to be a good assay for monitoring production of yeast extract during yeast autolysis. Formol titration was shown to be effective for monitoring protein hydrolysis. Objective B was also accomplished.

The fundamental aim of this research, however, was to produce products from whey that are stimulatory to lactic culture growth in bulk media. This objective was not fully realized. Whey derived yeast autolysates and yeast extracts were not as stimulatory as commercial yeast autolysates and yeast extracts which were generally made from beer yeast. Also, hydrolysed whey proteins were not as stimulatory as commercial casein hydrolysates and

lactalbumen hydrolysate. However, these whey-derived protein hydrolysates did possess significant stimulatory properties when coupled with a commercial yeast extract (tables 74 and 75). Also these whey-derived yeast extracts did possess significant stimulatory properties when coupled with a commercial protein hydrolysate (tables 74 and 75). Such application may result in savings for bulk media formulation and should be investigated. Homogenation and lytic/proteolytic enzymes treatment of K. fragilis should be investigated for improved yeast autolysate production. Why should the dairy industry pay to help the beer industry get rid of their waste products (yeast) while neglecting their own waste disposal?

CONCLUSIONS

1. Optical density measurements could be used on phosphate-containing lactic culture media to estimate cell concentration.
2. pH measurements could be used on reconstituted nonfat dry milk where lactic cultures are fermenting to estimate the amount of lactic acid present.
3. Lactic culture buffered growth (LABGRO) medium was an effective internal pH controlled medium for the propagation of a variety of S. lactis and S. cremoris strains to higher concentrations not achievable in milk. This medium could be used to evaluate the stimulatory properties of yeast autolysates, extracts or protein hydrolysates.
4. There were significant differences in growth rate among Prt- and Prt+ strains of S. cremoris and S. lactis.
5. There were significant differences in acid production rates among Prt- and Prt+ strains of S. cremoris and S. lactis.
6. There were significant differences cellular acid production rates among Prt- (and probably among Prt+) strains of S. cremoris and S. lactis.

7. There were significant differences in lactic culture stimulatory properties among commercial yeast autolysates and yeast extracts.
8. Formol titration could be used to efficiently evaluate lactic culture growth stimulating properties of both yeast extracts and yeast autolysates (not grouped together).
9. Absorbance index could be used to efficiently evaluate lactic culture growth stimulating properties of yeast autolysates.
10. Absorbance index and formol titration measurement could be used to monitor the extent of autolysis and protein hydrolsysis occurring during yeast autolysis. Such methods could be used by manufacturers of yeast autolysate/ protein hydrolysate products to monitor process completion.
11. K. fragilis reduced an initial whey permeate lactose content from 130 g/l whey permeate to below 0.1 g/l whey within a 42 h period when supplied with the proper nutrients and sufficient oxygen.
12. K. fragilis strains did not produce an autolysate or an extract comparable in lactic culture stimulatory properties to a commercial yeast autolysate under the autolysis conditions imposed.

13. Lactic culture stimulating properties of a *K. fragilis* autolysate appeared to be enhanced by the pre-autolysis addition of casein and pepsin. The addition of whey protein and pepsin to the pre-autolyzed yeast also enhanced the stimulatory properties, but less than when casein was used.
14. Casein appeared to be more readily hydrolyzed by autolyzing yeast enzymes and pepsin than was whey protein.
15. Commercial whey protein hydrolysate and casein hydrolysate were not very stimulatory to lactic culture growth in LABGRO medium. However, when yeast extract was added a marked increase in lactic culture stimulation occurred.

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