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I am submitting herewith a thesis written by Cleva Brock Claiborne entitled "Disposal of Cottage Cheese Whey by Microbial Degradation." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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DISPOSAL OF COTTAGE CHEESE WHEY BY MICROBIAL DEGRADATION

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

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March 1979

1091452

ACKNOWLEDGEMENTS

The author wishes to express deepest appreciation to Dr. W. W. Overcast and Dr. H. O. Jaynes, major professors, for their guidance and aid in the selection of courses and in the research presented herewith. Dr. M. J. Montgomery and Dr. C. C. Melton are also appreciated for their support and assistance while serving on the thesis committee.

Angela Cabrera and Cynthia Meeks are greatly appreciated for their laboratory assistance. Many thanks to Kathy Au for her friendship and help. Also, many sincere thanks to Mayfield Dairy Farms for their cooperation during this project.

The author wishes to express her deepest appreciation to her parents for their abiding love and guidance. To her husband Eddy, who continually encouraged and supported her in this study, the author wishes to express her deepest gratitude.

ABSTRACT

Whey disposal is a common problem among dairy plants. In recent years much research has been conducted to relieve this worrisome problem by using microorganisms. The purpose of this study was to further investigate the possibility of using microbes to reduce the Chemical Oxygen Demand (COD) of cottage cheese whey. The organisms considered in this study were *Kluyveromyces fragilis* (K) (formerly *Saccharomyces fragilis*) and *Candida lipolytica* (C).

The two starter cultures were added singly and in combination to make 3 different culture treatments. A 10 percent inoculum of the culture treatments was added to 1800 milliliters of whey. The cultures were grown for 48 hours in a New Brunswick Multigen fermenter at 32° C $\pm$ 2° C with constant agitation and aeration. Samples were removed at 3, 6, 12, 24, and 48 hour intervals, analyzed for pH and total solids, and then centrifuged. The supernatant was then used for COD, protein, lactose, precipitated solids and titratable acidity analysis. The effects of culture treatment and time and replications within cultures were studied. An analysis of variance was used to derive regression equations which demonstrated changes in each parameter.

There was no significant difference among culture treatments on the parameters COD and protein. There was a significant interaction between time and lactose utilization. Treatment three (K and C) was shown to respond best by reducing the organic content which resulted

in a reduction of the COD of whey. In one trial (K), up to 81 percent of the lactose was utilized which reduced the COD by 86 percent. In all trials the pH and titrable acidity were virtually unchanged due to the addition of K_2HPO_4.

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CHAPTER I

INTRODUCTION

In 1977 an estimated 1.1 billion pounds of cottage cheese was utilized (8, 9). Within the past two decades -- 1955 through 1974 -- per capita sales of cottage cheese in the United States increased by 50 percent, rising from 3.5 pounds per person in 1955 to 5.2 pounds per person in 1974 (4). During 1975 Tennessee manufactured ten million pounds of cottage cheese (6).

A by-product of cheese manufacturing is a greenish-yellow liquid called whey. One hundred pounds of milk yields about 16 pounds of cottage cheese and 84 pounds of whey (12). This fluid contains approximately one-half of the milk solids, most of the lactose, approximately one-fifth of the protein, most of the vitamins and minerals and has a pH range between 4 and 5 (43).

The organic matter of whey goes largely unused. Some is used for animal feed and various other feed and food products but a large percentage is waste. This waste becomes a costly burden on the sewage systems. Due to the high Chemical Oxygen Demand (COD), 60,000-70,000 ppm, disposal in streams aggravates pollution problems. Every 1,000 gallons per day of whey discharged to a sewage treatment plant imposes a load equal to that from 1,8000 people (2, 30, 36).

Different methods have been used to drain the whey and wash the curd. Each method has its own effect on the COD of wastewater (35).

In addition to the COD, the cottage cheese industry is concerned with pollution control of the following parameters: Five Day Biological Oxygen Demand (BOD_5), suspended solids, total solids and pH. Also important are the components that create the pollution problems such as nitrates, phosphates, protein, lactose, and lactic acid (35).

Keeping the pollution problem in mind, many methods have been used in an effort to utilize whey. These include spray drying which results in applying the solids to food products (26, 34). Other methods include ultrafiltration which yields a protein concentrate succeeded by reverse osmosis in order to retrieve the lactose from the concentrate (23, 33). Still others include electrodialysis and ion exchange to demineralize whey, a metaphosphate complex system and the sephandex system (17). Once the whey is purified it can then be added to food products such as ice cream, bakery products, processed meats, cheese spreads and beverages (17, 26, 34).

Since whey is not allowed in many municipal sewer systems, other means of disposal must be utilized. Disposal of cottage cheese whey is even more difficult, since this whey is acid and is produced in relatively small plants where the volume makes drying and other concentration methods unprofitable. Most of the methods mentioned above require at least 100,000 pounds of whey per day to be economical (32). Therefore, small dairies need an inexpensive process in order to utilize whey. With these economic restrictions in mind this study was conducted to further investigate the possibilities of using microorganisms to degrade cottage cheese whey. Upon degradation the effluent would have a much lower COD and the microbial products

could be used to supplement the nutritional requirements of several animals including man.

CHAPTER II

LITERATURE REVIEW

I. MICROBIAL UTILIZATION OF WHEY YIELDING BY-PRODUCTS

For years the utilization of whey has been studied. Methods such as drying have been employed to rid the cheese producer of this cumbersome by-product but due to the expense, drying is not always feasible. In the last few decades microbial degradation of whey has been studied and these studies have yielded many useful purposes for the microbial by-products. Whey is relatively inexpensive and is usually available locally; therefore, the microbial by-products are an extra for the cheese industry. The main by-products under investigation include supplements for food and feed products, lactase production and alcohol production.

A. Food and Feed Products

Much of the whey produced remains unused. This represents the loss of millions of pounds of protein. Adding yeast to whey is a means of recovering the protein that would otherwise become a pollution problem. The yeast can assimilate the organic matter of whey into a useful, protein rich product.

The protein content of whey varies according to the organism used. Dabbah (21) found the protein content of bacteria to vary from

47 percent to 87 percent depending on the species, while the protein content of fungi (40 percent) and yeast (50 percent) does not vary substantially. The amino acid content of yeast compares favorably with that of the Food and Agricultural Organization reference protein, except for a lower content of methionine (21). The amino acids deficient in protein from yeasts are also the same ones lacking in protein from bacteria (methionine, tryptophan, and cystine).

Porges et al. (38) found that yeast grown on dairy products are higher in protein and amino nitrogen than other yeasts. They are also higher in ascorbic acid, thiamine and riboflavin. *Saccharomyces fragilis* gave the highest yield of solids. Also used by Porges was *Candida lipolytica* and *Torula utilis*. These organisms produced small amounts of solids.

Using *Saccharomyces fragilis*, Wasserman (46) found the amino acids compare well with the average concentration of amino acids in yeast used for food and feed purposes. The concentration of lysine is considerably greater in *Saccharomyces fragilis* than in other yeasts and it can be grown in a whey medium to contain 50 percent protein. After analyzing various strains of yeasts it was found that the amino acid composition of the protein and vitamin content were the same.

Wasserman (47) also reported maximum yeast growth in 4 hours using raw whey and an inoculum of *Saccharomyces fragilis* equal on a dry weight basis to 25 percent of the whey sugar. High yields were dependent on the supply of sufficient oxygen to satisfy the demand of the yeast.

Becthle and Claydon (14) used *Torulopsis*, *Saccharomyces fragilis*, and *Candida* for yeast cultures and *Lactobacillus*, *Streptococcus*, and *Leuconostoc* for bacterial cultures to increase cell yields from whey. These cultures were added to a medium of single-strength whey and a few were added to 3 times single strength and 4 times single strength whey. Trace elements and essential nutrients were added for maximum microbial growth and these cultures were incubated at 32° C. They found no amino acid advantages in using a mixed inoculum of yeast and lactose-using bacteria. But it was found that lactose-using bacteria stimulate mixed food-yeast cultures by accelerating fermentations, indicating an advantage for the production of food yeast in whey media.

Bernstein and Tzeng (10, 13) found *Saccharomyces fragilis* grown in whey could produce a superior quality yeast, higher in protein (45 percent to 55 percent) and lower in ash (6 percent to 10 percent) content. The protein quality was good but was low in sulfur containing amino acids as are most single-cell proteins.

Once the whey has been processed using the above methods, the cell biomass can be added to food and feed products such as bakery goods. Added at a rate of 3 percent of the flour weight it can contribute flavor, texture and good nutrition. It can also be added to ice cream and processed cheese to improve the flavor and it can be added to beverages as a protein supplement. About 20 percent of the processed whey is used in food products with the remaining 80 percent used in animal feeds (17, 26, 28, 34, 53, 54).

A study performed by Mann (29) indicated that whey protein concentrates were a good source of B vitamins, but not of Vitamin A.

When added to bread the beneficial effects observed, besides those previously stated, included an increase in total and reducing sugars and the calcium content was increased by approximately 60 percent.

Not only can whey be used for human food but also for animal feed. At one time farmers were allowed to pick up whey from the dairy plants to feed their animals but it is no longer allowed. Protein concentrates derived from whey are now used in cattle and swine feed (1, 3, 17, 20, 27, 54).

At Michigan State University (7) it was found that when *Lactobacillus* was added to whey it would metabolize the lactose and convert it to lactic acid. Then when ammonia was added to neutralize the acid during and after the fermentation, ammonium lactate was formed. This would then increase the amount of total nitrogen which could be fed safely to cattle and other ruminants. This product was known as "Bactolac."

In various feeding trials it has been shown that whey added to feed has no harmful side effects. Rats and other experimental animals have been fed whey and no toxic levels were found (13, 19, 33, 48, 54).

It appears that whey added to bread, beverages, processed meats, and animal feed is an excellent means of utilization. Instead of allowing the whey to pollute streams or place a burden on municipal sewage systems, it can be used to supplement the diet of many animals and man.

B. Lactase Production

There are many microorganisms that ferment whey and during fermentation produce many metabolic products. One of the metabolic products is lactase enzyme. Microbial enzymes have shown value in the food industry and in genetic research.

Lactase enzymes are used to hydrolyze lactose to make it more soluble and also to increase its sweetness. The primary sources of commercial lactases are calf intestines and microorganisms (49).

Wendroff (49) using reconstituted dried cheddar cheese whey fortified with corn steep liquor, K_2HPO_4 and 5N NH_4OH, found the lactase activity of dried yeast increased with higher levels of lactose in the whey. Whey with additional nutrients exhibited a greater increase in lactase activity than did unfortified whey. The pH of the medium was held between 4.0 and 4.7 because as the pH increased, lactase activity decreased. The fermentation medium produced maximum lactase yields consisted of whey containing 10-15 percent lactose, 0.5 percent corn steep liquor, 0.3 percent K_2HPO_4 and 0.3 percent 5N NH_4OH.

Aeromonas hydrophila, a bacterium, has exhibited the ability to grow well on lactose. Butany and Ingledew (14) used *Aeromonas hydrophila* in a study of lactase production. At 30° C it is capable of complete oxidation of glucose, galactose and lactose with the theoretical oxidation ranging between 50 to 56 percent. Assays for B-galactosidase activity were carried out using the bacteria. It was found that *Aeromonas hydrophila* possessed 7 to 8 times more of the enzyme than *Saccharomyces fragilis*, a lactose fermenting yeast.

In experimental work organisms such as *Aeromonas hydrophila* are capable of utilizing whey with sufficient aeration and using whey with at least 1.5 percent carbohydrate content. Good cell yields will result using *Aeromonas hydrophila* with fewer yeast related problems such as more complex nutritional requirements and contamination which often occur in yeast propagation.

Due to the tendency for crystallization and low sweetness, Wiezbicki and Kosikowski (50) used B-galactosidase from *Aspergillus niger* to convert the lactose in acid whey to monosaccharides. Mixing the hydrolyzate with protein precipitants and minerals and subsequent concentration at low temperatures led to the production of food syrups of differing characteristics. The researchers used fresh fluid and spray dried cottage cheese whey. These were treated with an active B-galactosidase preparation from *Aspergillus niger* at pH 4.5 and 55° C. The resultant food syrups were blended individually with other basic food materials to yield a variety of colors and flavors.

In another study performed by Wiezbicki and Kosikowski (51) it was found that *Saccharomyces fragilis* as well as *Candida pseudotropicalis* and *Saccharomyces lactis* exhibited a high level of lactase potential and high crude cell yields. Lactose hydrolysis for crude yeast cells in 5 hours varied between 57.0 percent to 59.0 percent with an optimal pH for yeast lactase activity being between 6.0 and 7.0 and optimal temperature from 30° C to 50° C. Molds demonstrated a lesser ability to utilize lactose.

C. Alcohol Production

Before World War II ethyl alcohol was being produced from the fermentation of lactose in whey. In a report on lactose utilization by Rogosa et al. (39), it was found after 55 hours *Torula cremoris* had fermented all the lactose. Other organisms such as *Zygosaccharomyces lactis, Mycotorula lactis,* and *Torulopsis kefir* left 3.8 percent, 3.1 percent, and 2.9 percent residual lactose, respectively. So *Torula cremoris* was used for further investigation of lactose fermentation in whey. Adding yeast at the rate of 2 percent of the weight of the lactose initially present at a temperature of 33° C to 34° C, the fermentation was carried out at a pH range of 4.7 to 5.0. It was found desirable to conduct the fermentation at as low a pH value as possible in order to minimize growth of contamination microorganisms. Alcohol yields averaged 90 percent of theoretical and the quality of the alcohol was highly satisfactory. By-products of the fermentation, such as the whey protein, were of value as feed with favorable vitamin content.

In a more recent study presented by Bernstein and Tzeng (10, 13) it was found that by changing the fermentation conditions (operating under aerobic conditions, 0.1 to 0.3 volume of air per volume of medium) the amount of product formed was increased. After the initial period of aerobic operation to increase cell concentration, lactose in the form of condensed whey was added to a fermentation broth. Then it was operated under anaerobic conditions. The cell population remained at a constant level and by adding condensed whey incrementally, ethyl alcohol was produced in large amounts. Greater than

90 percent of the lactose was converted to ethyl alcohol; the rest was used for cell maintenance.

II. MICROBIAL REDUCTION OF CHEMICAL OXYGEN DEMAND OF WHEY

Microorganisms propagated in dairy wastes have been used in the past to produce protein enriched food and feed supplements, lactase enzymes and alcohol. Using whey as a medium, large cell yields can be obtained. This increased cell mass is the result of microorganisms converting the solids content of whey into their own body mass. By utilizing the solids in whey for their energy and growth, microorganisms reduce the objectionable COD of the particular dairy waste.

A. Reduction of Chemical Oxygen Demand of Whey by Yeasts

Mickle et al. (32) used *Kluyveromyces fragilis*, formerly called *Saccharomyces fragilis*, for further work concerning COD reduction of cottage cheese whey and *Rhodotorula gracilis* for fat production. The pH range of the media varied between 4.0 and 6.0. Optimum temperature for growth was found to be between 35° C and 40° C. Under these conditions 82 $\pm$ 2 percent of the COD was reduced by *Kluyveromyces fragilis* in 10-12 hours. *Kluyveromyces fragilis* also reached maximum cell counts of 4.5×10^8 in approximately 7 hours. Using *Rhodotorula gracilis* was found to be an impractical means of reducing the COD even though it may yield as much as 60 percent of its dry weight as lipid.

In 1959 Wasserman et al. (44) reported an 84 percent reduction

in the COD of whey using *Saccharomyces fragilis*. The whey was seeded to contain 5.0 x 10^8 cells of *Saccharomyces fragilis* per milliliter. In whey supplemented with 0.5 percent $(NH_4)_2SO_4$, 0.5 percent K_2HPO_4 and 0.1 percent Difco yeast extract, maximum growth and sugar utilization were obtained within 6 hours. The temperature was maintained at 32-33° C with best growth reported at pH 5.7. They also found that increasing the size of the inoculum decreased the time necessary to reach the maximum yield, but regardless of inoculum size, given enough time the final results were equivalent.

While studying the availability of whey nitrogen for growth of *Saccharomyces fragilis*, Wasserman (45) found that the organisms could utilize ammonia nitrogen much better (up to 50 percent was assimilated) than organic nitrogen of which only 25 percent could be used. The principal nitrogenous components were the heat and acid precipitable lactalbumin and lactoglobulin, whereas the non-protein nitrogen fraction consisted of proteose-peptones, amino acids and other substances. *Saccharomyces fragilis* was added to 500 milliliter quantities of deproteinated whey and untreated whey. Salts and yeast extracts were added to formulate the media. During the 3 hour growth period all of the ammonia nitrogen added to both media was assimilated by the yeast and in both media approximately the same quantity of nitrogen disappeared from the organic nitrogen. It was found the nitrogen used by the yeast in both media originated in the non-coagulable organic fraction.

Wasserman (46) analyzed various strains of yeast grown on different media and found the amino acid compositions of the proteins

and vitamin contents to be the same. The amino acids of *Saccharomyces fragilis* compared well with the average concentration of amino acids in yeast used for food and feed purposes.

The dissolved oxygen requirement for maximum growth of *Saccharomyces fragilis* was investigated by Wasserman et al. (57), Irvin (24), and others (40, 52), by studying various propagator systems. The efficiency of lactose assimilation depended upon the efficiency of the propagator's design for aeration and agitation. Since foaming was such a problem, conflicting results occurred. Once the bubbles were dispersed using agitation, an increase in the amount of dissolved oxygen occurred which was greater than the expected amount.

Knight (27) used *Saccharomyces fragilis* to define optimum conditions and also to learn how much the BOD of whey could be reduced and the time for maximum reduction. Optimum growth conditions were found to be whey plus 0.1 percent yeast extract and 2 percent peptone for a nitrogen source. These afforded maximum growth. Optimum growth rates were observed between pH values of 4.0 and 6.0 with the best temperature ranging from 35 to 40° C. An air flow rate of one volume per minute was maintained throughout the growth period. Also investigated was the COD reduction of whey. It was found that as cell counts increased the lactose and COD decreased. Using various inocula volumes of 5, 10, 20, 30, and 40 percent it was found that a 30 to 40 percent inocula caused maximum COD reduction of 70-80 percent while a 5 percent inoculum resulted in only a 55 percent reduction in COD within 14 hours.

B. Reduction of Chemical Oxygen Demand of Whey by Molds

Riboflavin, penicillin and fat are 3 end-products of whey fermentation using molds. Wix and Woodbine (54) found *Eremothecium ashbyii* would produce riboflavin when sugar and trace elements were added to the whey and the pH adjusted to 5.5. Penicillin production was also studied and yields were good; however, whey does not seem to have received any attention in penicillin production. Fat production seems to be the most widely studied area using molds. *Oospora lactis*, *Endomyces vermalis* and *Penicillium javanicum* are some of the molds capable of producing fat when grown in whey. *Aspergillius ustus* has been shown to produce good fat yields. The mycelium yielded 28 percent fat for a growth period of 12 days at 28 to 29° C. *Geotrichum candidum* was found to be impractical for fat production but it did demonstrate an ability to utilize lactose and lactic acid (54).

In studies conducted by Van Uden (41) and Carmichael (16), *Geotrichum candidum* was found to be a contaminant of dairy products. Carmichael defined the morphological properties of the species and found a great majority of *Geotrichum* strains which were isolated from dairy, human, and animal sources, belong to this species.

The growth of *Penicillum frequentans* was studied by Cook and Woodbine (18). The BOD of whey was reduced as the lactose was utilized. When the whey was supplemented with ammonium nitrate and potassium sulfate, the felt weight (organisms which have adhered to one another) and fat production increased. Using supplemented whey the BOD was reduced from 65,000 to 114 ppm in 40 days. Five-day BOD is generally accepted as being equivalent to 60 percent of

the COD (Chemical Oxygen Demand) of the whey.

C. Microbial Reduction of Chemical Oxygen Demand of Whey

Clostridum asetobutylicum has been called the most important organism used in bacterial fermentation of whey. This organism is capable of an acetone-butanol fermentation from which solvents and/or riboflavin may be extracted (54). According to Wix and Woodbine (53) a medium capable of giving riboflavin yields of 20 to 30 micrograms per milliliter and a useful solvents yield was prepared as follows: whey was adjusted to pH 6.0 to 7.0 and 1 to 3 pounds of calcium carbonate was added per 1,000 pounds of material. After sterilization at 250° F for 15-20 minutes, the iron content was adjusted with ferrous sulfate to 1.0 to 1.5 ppm. Fermentation took 12 to 48 hours at a temperature of 38° C.

Edmondson and Brazis (22) found Pseudomonas organisms to be very effective in reducing the BOD of dairy waste. A survey of small dairy plants in Missouri found the average BOD_5 of rinse waters and whey ranged from 270 to 6,200 ppm, with the majority in the upper range. *Pseudomonas cyanogenes, Pseudomonas puterfaciens* and *Pseudomonas viscosa* were found to effectively lower the BOD of dairy waste by acting on the proteins. Edmondson and Brazis concluded that certain *Pseudomonas* species can be used effectively to treat dairy wastes, provided alkalinity is maintained and aerobic conditions for fermentation are present. Also dairy waste can be treated with moderate results by means of continuous pH control. If buffered nutrient broth is added to dairy waste it too can cause desirable fermentations by microorganisms present in the waste, thereby producing a stable effluent.

CHAPTER III

MATERIALS AND METHODS

I. SOURCE OF WHEY AND STOCK CULTURES

Before each fermentation trial a fresh container of whey was obtained from Mayfield Dairy Farms, Athens, Tennessee. This was to insure freshness of the medium.

Kluyveromyces fragilis (NRRL No. Y1156) was obtained from the Northern Regional Research Laboratory of the U.S. Department of Agriculture Research Service for research concerning the reduction of the chemical oxygen demand of cottage cheese whey (42).

The *Candida lipolytica* had been isolated during routine laboratory analysis of milk. This too was used in research dealing with the reduction of the chemical oxygen demand of cottage cheese whey. It had been conditioned to utilize the lactose in whey (42).

II. PREPARATION OF INOCULUM

The stock cultures were stored at 4° C on 4 percent lactose agar slants. These organisms were transferred every 2 weeks in order to maintain viable cultures. The organisms were then transferred to 180 milliliters of 4 percent lactose broth and incubated at 32° C $\pm$ 1° C for 24 hours before each trial.

The 4 percent lactose broth with the growing culture was centrifuged at 1,000 x g. for 15 minutes in an International Number 2

Centrifuge. The supernatant was removed and the culture was added to 1,800 milliliters of whey plus 4 grams of K_2HPO_4. This was always a 10 percent inoculum (v/v). When a single culture was used, 180 milliliters of 4 percent lactose broth was centrifuged then resuspended in the whey. When a combination of 2 starter cultures were used, 90 milliliters of each organism was centrifuged then resuspended in 1,800 milliliters of whey.

A New Brunswick Multigen (2 liter capacity) fermenter was used. The cultures were chosen randomly and fermented at 32° C $\pm$ 1° C over a 48 hour period. Aeration was held constant at 0.5 standard cubic feet per hour and the media was agitated constantly. A dissolved oxygen meter was used to monitor the oxygen level. It remained at 100 percent dissolved oxygen throughout the trials.

III. SAMPLING

One hundred milliliters of sample were removed at 3, 6, 12, 24, and 48 hour intervals. At the beginning of each trial a sample of uninoculated whey was analyzed. This was done to assess the progress of the microbial degradation.

IV. ANALYSIS OF THE WHEY

A. Chemical Oxygen Demand

Chemical Oxygen Demand (COD) determinations were made on the whey supernatant according to the method outlined in the Agricultural Handbook of 176 (5). A 1 to 40 dilution of the sample was mixed with distilled water. A 5 milliliter aliquot of the diluted

sample was added to 50 milliliters of dichromate oxidizing solution. The mixture was then brought to 165° C within 6 minutes using a hot plate as the heat source. The samples were cooled to room temperature in ice water, 200 milliliters of distilled water was added, cooled again and 10 milliliters of potassium iodide solution was added. Titration of samples followed immediately using standard 0.05N sodium thiosulfate solution and a starch indicator. The endpoint was reached when the samples turned a pale blue-green color. A blank was run along with the samples. COD was calculated using the following equation:

$$\text{COD (ppm } O_2) = \frac{(\text{ml titrated}_{\text{blank}} - \text{ml titrated}_{\text{sample}}) \times (0.05 \text{ meq/ml}) (8 \text{ mg } O_2/\text{meq}) (1{,}000 \text{ ml/l}) (40)}{5 \text{ ml sample}}$$

$$\text{or COD (mg/l)} = (\text{Titr}_{\text{blank}} - \text{Titr}_{\text{sample}}) (80) (40)$$

B. Protein

Protein determinations were made using 5 milliliters of culture treatment supernatants by the Kjeldahl method according to the Association of Official Analytical Chemists (ACAC) (11). A factor of 6.38 x N was used (42).

C. Lactose

Lactose determinations were made on one-half milliliter of culture treatment supernatant using the picric acid colorimetric method of Perry and Doan (37). To one-half milliliter of the supernatant was added 49.5 milliliters of saturated picric acid. After thoroughly

mixing the two, the mixture was filtered through No. 1 Whatman filter paper to remove any protein precipitate. One milliliter of the filtrate was added to 2 milliliters of 4.7N Na_2CC_3 and placed in a hot water bath for 20 minutes to allow the color to develop. The samples were cooled and 20 milliliters of distilled water was added. A blank determination was also run using 1 milliliter of saturated picric acid and 2 milliliters of the Na_2CO_3 solution. A blank was used to zero the Bausch and Lomb Spectronic 20. A portion of each sample was used to record absorbance readings at 520 nm. Previous to this analysis, a standard curve had been constructed using standard lactose solutions ranging in concentration from 1 to 6 percent (w/v).

Absorbance = -0.0217497 + 0.0773285 (percent lactose)

D. pH

The pH of each sample was measured at the time of extraction using a Fisher Accumet Model 230A pH/Ion meter.

E. Total Solids

Total solids determinations were made using approximately 2 grams of each culture treatment. Each sample was heated in a tared aluminum pan to a slurry in duplicate on a hot plate, then placed in a vacuum drying oven at 380 torr for 24 hours at 60° C to insure a uniform weight. This method is listed by the AOAC (11).

F. Total Solids-Centrifuged Samples

The culture treatment samples were centrifuged at 1,000 x g for 15 minutes in 50 milliliter tubes in an International Number 2

Centrifuge. After centrifugation, a 2 gram sample was taken from the supernatant of each sample and determinations were made in duplicate as previously stated.

G. Titratable Acidity

Titratable acidity determinations were made using 5 milliliters of culture treatment supernatants (11). The sample was diluted with twice its volume of distilled water. A few drops of phenolthalein were added, then titrated with a standard sodium hydroxide solution. Titratable acidity was calculated as percent lactic acid.

$$\text{Percent Lactic Acid} = \frac{(\text{ml NaOH})\ (\text{N NaOH})\ (\text{Meq wt of acid})\ (100)}{\text{Wt of sample in grams}}$$

H. Statistical Analysis

Four replications were done for each of the 3 culture treatments. Each sample was run in duplicate. The experiment was a nested split plot design. The data was subjected to an analysis of variance to measure the time and treatment effects.

CHAPTER IV

RESULTS AND DISCUSSION

Due to the sanitary conditions which must be maintained in a dairy plant, it would be impractical to continue or preserve such conditions for microbes used in whey disposal. Therefore, the whey disposal organisms should be maintained in an area separate from those cultures used for lactic acid formation in the production of cottage cheese. The equipment used for each process should be kept separate also. Yeast cell numbers used in this study were such that contamination problems were at a minimum when cleanliness was routinely observed. Before each trial the fermentation jar was autoclaved to insure the yeasts used in the previous fermentation could not influence the succeeding fermentation. The incubation temperature used was 32° C $\pm$ 2° C because this supplies the optimum growing conditions and it is also the temperature at which the curd and whey are separated. The 1,8000 milliliters of uninoculated whey plus 4 grams of K_2HPO_4 were placed in a 2 liter capacity New Brunswick Multigen fermenter where agitation and aeration were maintained at constant rates throughout each trial. These conditions could be reproduced in an actual dairy plant situation. A silo designed for whey storage could be used for holding the liquid prior to inoculation and the clarifier could be used to remove the yeasts.

Each lot of cottage cheese whey was analyzed when it was received to determine the pertinent components. Composition of each batch of

whey is shown in Table I. The average of all replications for each culture treatment was taken to graphically illustrate 0 hour conditions when parameters were plotted against time. Protein, lactose, COD, and total solids vary somewhat from batch to batch. This variation can be explained by variations in batches of cottage cheese whey, the method of curd formation, temperature of cooking the curd, amount of fines of shattered curd, and the amount of acid produced in lactose fermentation.

At regular time intervals (3, 6, 12, 24, and 48 hours) samples from each treatment were removed to monitor the response of each batch of whey to the culture treatments. Those parameters which were determined on a percentage basis included lactose, total solids, precipitated solids (culture treatment supernatant), protein, and titratable acidity. The Chemical Oxygen Demand (COD) was calculated in ppm O_2 and the pH was measured with a pH meter. The data were subjected to analysis of variance (ANOVA) to measure treatment, time, and replication effects on each of the 7 parameters. A split-plot design with treatments completely randomized was used in order to increase the effectiveness of the statistical analysis. Each treatment was examined at designated time intervals to subjectively evaluate the microbial utilization of the various whey components. The "F" values from the ANOVA are listed in Table II. No significant difference was found among cultures or in the cultures x time interaction for all parameters except lactose and titratable acidity. Time and replications within cultures were shown to have a significant difference. The replication effect could be attributed to using a fresh batch of whey

TABLE I. Composition of Batches of Whey

Batch	pH	COD	Protein	Lactose	Total Solids*	Dissolved Solids*	Titratable Acidity
			% Solids				
1	4.40	68,640	.810	4.42	6.80	0.22	.646
2	4.50	67,840	.861	4.42	6.96	0.24	.611
3	4.55	70,880	.938	4.61	7.75	0.04	.611
4	4.51	51,520	.893	4.87	7.10	0.10	.663
5	4.55	56,800	.657	4.16	6.45	0.26	.650
6	4.45	56,800	.864	4.16	6.39	0.02	.590
7	4.80	72,960	.861	5.00	6.83	0.00	.908
8	4.40	61,760	.911	5.26	6.64	0.02	.523
9	4.40	60,000	.902	4.14	6.19	0.06	.698
10	4.60	65,120	.887	4.87	6.49	0.11	.559
11	4.40	60,640	.987	4.10	7.25	0.57	.590
12	4.40	62,080	.902	5.32	6.81	0.07	.560
Means	4.49	63,670	.873	4.61	6.81	0.14	.634
Std. Dev.	±.120	±6,331	±.141	±.444	±.433	±.162	±.099

*Means of duplicates

TABLE II. F Values from Statistical Analysis of Culture Treatment Variables

Source	COD	Protein	Lactose	pH	Total Solids	Precipitated Solids	Titratable Solids
Cultures (C)	0.73	0.14	0.42	2.00	2.77	1.36	7.48**
REPS/Culture	6.41**	6.08**	3.35**	5.85**	3.26**	3.71**	4.45**
TIME	135.84**	26.59**	89.25*	12.84**	25.33**	27.84**	3.05*
Cul x Time	1.16	0.47	2.52*	0.67	1.42	0.69	1.16
(Rep x Time)/Cul	0.11	0.02	0.13	0.34	0.85	0.37	1.68
r^2	.9433	.8106	.9178	.7391	.7361	.8019	.6450

*$p < 0.05$

**$p < 0.01$

for each fermentation. Therefore, each trial began from a different set of values. Another reason for such variation could be competition for growth and energy sources found in the whey. In mixed cultures there are often antagonistic and synergistic effects which affect the rate of growth, thereby affecting the rate at which fermentation progressed.

Time was found to have an unquestionably significant effect on the 7 parameters for all 3 culture treatments. Using mathematical equations, time can be represented more meaningfully when graphed. The course of protein digestion was depicted graphically using an exponential equation which was derived to fit the data. The COD data were an exponential function. The lactose determinations were calculated using linear and quadratic equations. The best fit line was found to be linear for culture treatment 1 (Table III) and quadratic for culture treatments 2 and 3. The rate of utilization can be expressed as a first-order reaction. The equations relating to lactose utilization are shown in Table IV.

Using these equations when the variable lactose is plotted against time, the concentration will decrease quickly at first. Lactose utilization seems to depend upon the concentration present in the whey. Theoretically each whey component can be completely assimilated by the microorganisms but experimentally this usually does not occur. Lactose is the limiting carbohydrate source for energy; therefore, once it is utilized to its fullest, the microbes are no longer able to reduce the total solids content which in turn

TABLE III. Microorganisms Involved in Culture Treatments

Treatment	Microorganisms	Symbols
1	*Kluyveromyces fragilis*	K
2	*Candida lipolytica*	C
3	*Klyveromyces fragilis*	K,C
	Candida lipolytica	

TABLE IV. Equations Giving Best Fit for the Variation Over Time of COD, Protein and Lactose for Culture Treatments[1]

Treatment 1, 2, and 3	
Protein (%)	$= 1.3101 - 0.4598e^{+0.0092t}$
Treatment 1, 2, and 3	
COD (ppm)	$= -0.1292 + 62.3875e^{-0.0262t}$
Treatment 1, 2, and 3	
Lactose 1 (%)	$= 4.67686 - 0.076379t$
Lactose 2 (%)	$= 3.99666 + 0.01021t - 0.00127t^2$
Lactose 3 (%)	$= 4.53495 - 0.02852t - .00096t^2$

[1] t = time

limits the COD reduction of the whey.

Data from COD determinations best fit an exponential equation with an adjusted asymptote. In order to fit an exponential equation to the degredation of a component which is not fully utilized or reduced, the exponential equation must be adjusted and an asymptote greater than 0 added. Such an equation is represented as follows:

$$COD\ (ppm) = -0.1292 + 62.3875e^{-0.0262t}$$

Table III lists the cultures used for each treatment. Table IV lists the equations which present the best fit lines for each culture treatment.

The pH and titratable acidity changed very little due to the addition of K_2HPO_4 which acts as a buffer plus the buffering action of the proteins. Each culture treatment doubled in cell mass in 24 hours and this parameter continued to increase steadily during the next 24 hour period of each trial. Mathematical functions for these parameters were not evolved.

There was no evidence of any difference in the 3 culture treatments in COD reduction as shown in Figure 1. At 12 hours the data from this study were very similar to data collected by Vigdorth (42) in a previous study on reducing the COD of cottage cheese whey. At 12 hours all culture treatments were around 46,000 ppm COD. But at 48 hours a difference was found. Vigdorth found culture treatment 3 (K and C) to reduce the COD by 68 percent in a 48 hour period whereas this study found the combination to reduce the COD by 82 percent during the same time period. Culture treatment 1 (K) was also found

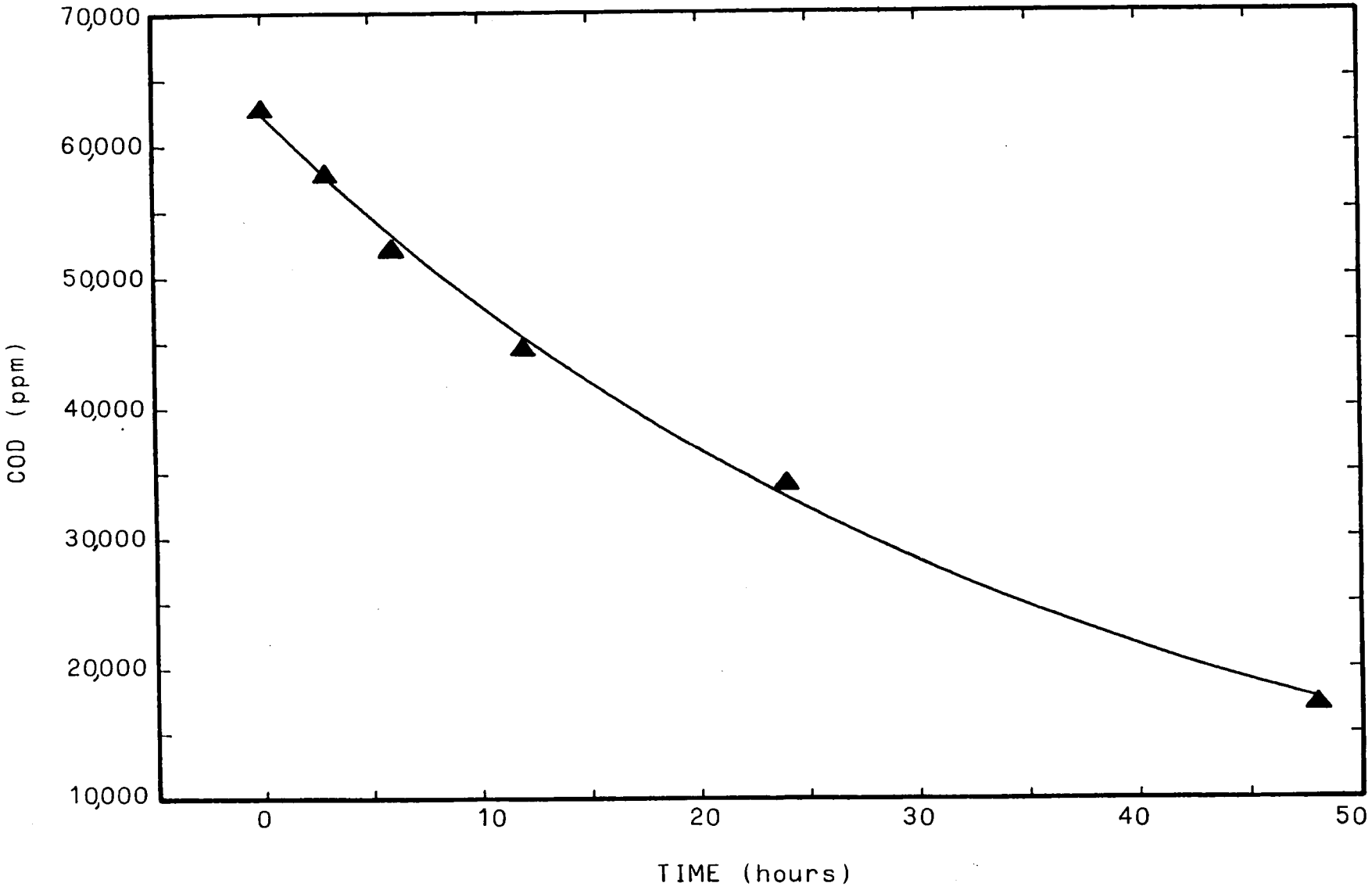


Figure 1. COD variation over time of culture treatments 1, 2, and 3.

to respond better in this study with an 86 percent reduction compared to a 69 percent reduction in the previous study. Considering all culture treatments in the present study the COD reduction was 72 percent. In the study conducted by Vigdorth the overall COD reduction with K and C was 75 percent; with K, 71 percent, and with C, 17 percent. The overall replication difference in this study could be attributed to the 4 percent lactose broth in which each culture treatment was grown as well as to variations in whey composition. The first 3 trials were conducted using a commercial lactose broth. The last 3 trials were conducted using broth made from scratch. The organisms responded much better to the "homemade broth" than the commercial broth. The commercial broth was old and had caked. This caused an increase in moisture content which in turn can cause protein denaturation and the sugars to undergo the browning reaction. The vitamin and mineral contents would also be reduced. But using fresh ingredients to make up the "homemade" broth made all the essential nutrients available to the organisms. The "homemade" broth was made to meet the requirements of the commercially-prepared broth.

The same response was true for the protein utilization as the COD reduction. Again, there was no indication of a significant effect from culture treatment on protein utilization as shown in Figure 2. There was an overall reduction of protein of 32 percent in 48 hours with all culture treatments. In 2 replications culture treatment 3 (K and C) utilized between 43 and 47 percent of the protein. Culture treatment 2 (C) also reduced the protein content by 43 percent in 1 replication. The overall protein content in this study was essentially

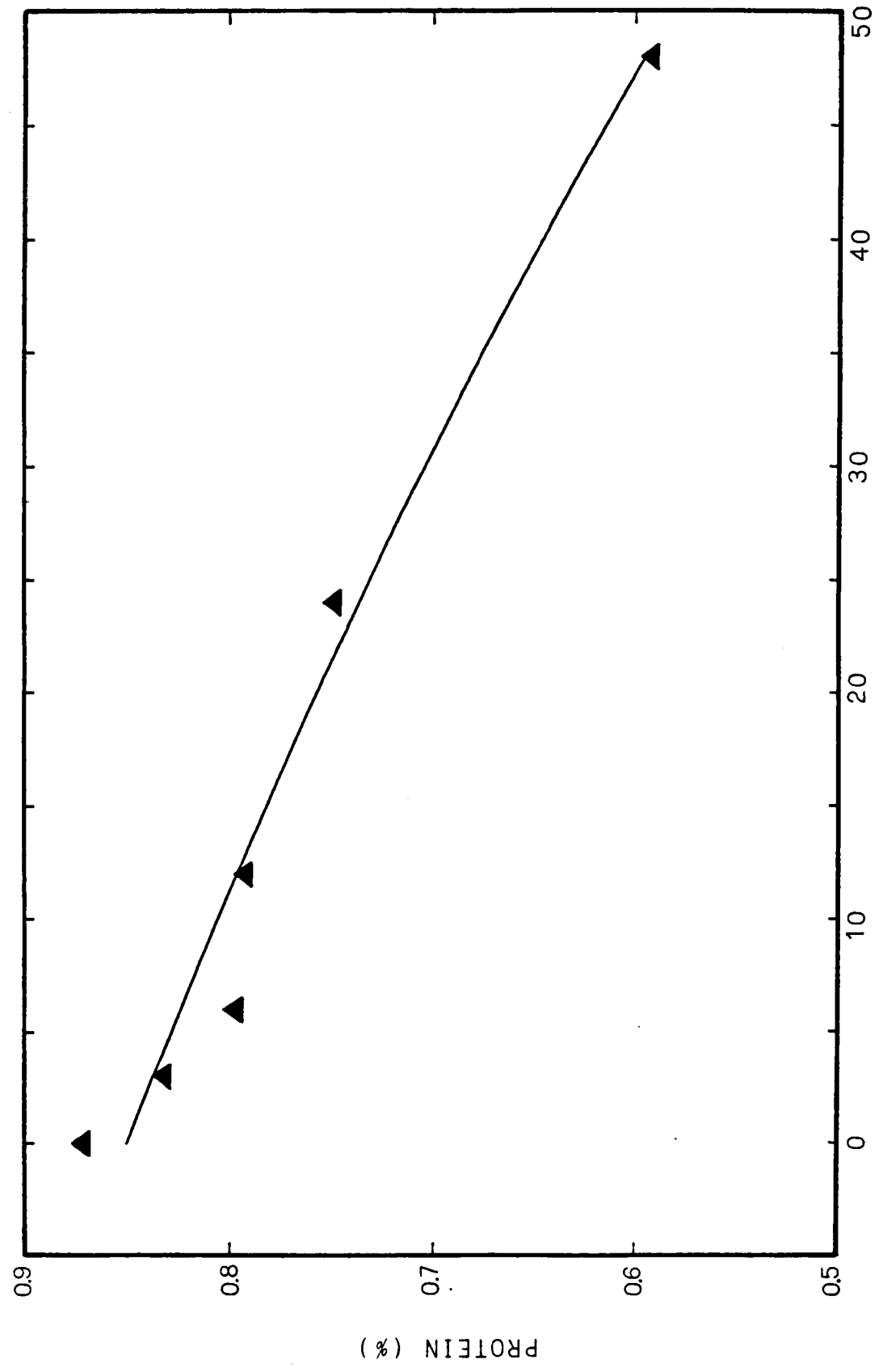


Figure 2. Protein variation over time of culture treatments 1, 2, and 3.

the same as in the study conducted by Vigdorth (42). Within 12 hours there was approximately a 10 percent decrease in protein content in both studies. At 48 hours both studies reported a decrease of approximately 30 percent in protein content. Again this overall difference among replications in this study can be attributed to the initial growth period in the broth and the initial protein concentration.

The rate of lactose utilization was approximately the same with each culture treatment (Figure 3) with culture treatment 3 (K and C) utilizing the most in a 48 hour period. Comparing the present study with that of Vigdorth (42) shows the lactose was reduced substantially more in the latter. At 12 and 48 hours Vigdorth had reduced the lactose content by 52 and 48 percent, respectively, for culture treatment K and C, whereas this study reduced it by 18 and 81 percent. Culture treatment K reduced it by 96 percent in Vigdorth's work and 77 percent in this study. In this study at 24 hours cultures K and C and C had utilized essentially equal amounts of lactose with the last 24 hours being the time period which demonstrates the greatest lactose utilization. The linear and quadratic slope coefficients (Table IV) for each culture treatment indicate the speed of reduction. Culture treatment 1 had a linear coefficient of -0.076379, culture treatment 2, 0.01021 (linear) and -0.00127 (quadratic), and culture treatment 3, -0.02852 (linear) and -0.00096 (quadratic). Culture treatments 2 and 3 generated quadratic functions. The difference in lactose utilization between the two studies could be attributed not only to the conditions under which each fermentation trial was conducted but also to a problem of foaming in this study. It was not unusual

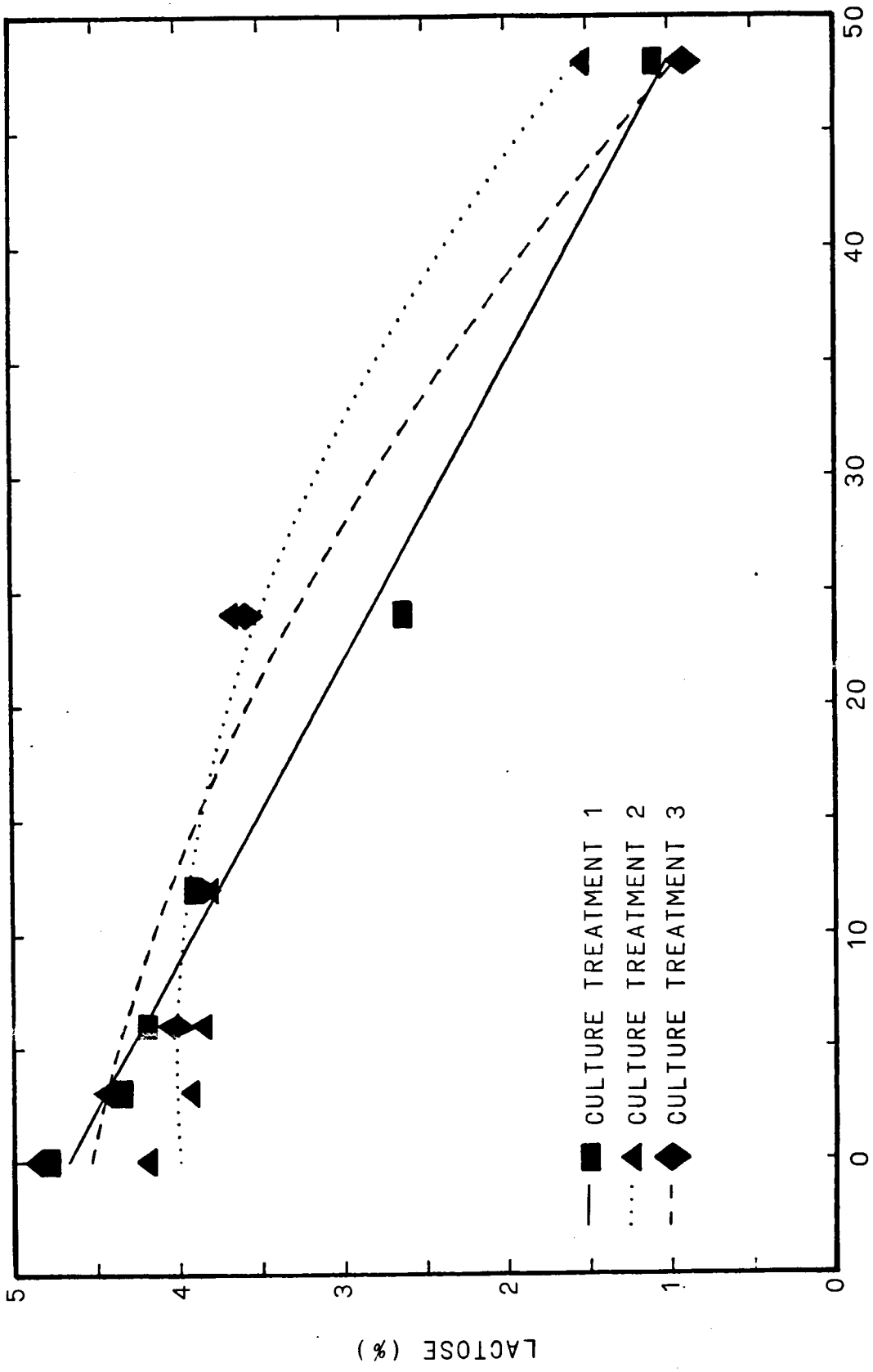


Figure 3. Lactose variation over time of culture treatments 1, 2, and 3.

for the foam to bubble out of the fermentation jar. This loss of whey due to foaming could decrease the microbial population plus decrease the available nutrients. No attempt was made to control the foaming problem because this in turn would change the composition of the media.

COD reduction and protein utilization in this study were similar to Vigdorth's (42) findings but lactose utilization was far greater in the latter work. Those cultures containing K indicated more ability to reduce the lactose and COD rapidly.

This study indicates the use of microbes as a means of cottage cheese whey disposal is possible. More control over the initial growth conditions and probably an increase in aeration and agitation could improve the process. Increasing the agitation and aeration would aid in the prevention of waste product build up which inhibits COD reduction.

Being able to run only 1 replication at a time complicated the usage of 1 batch of whey for 1 set of replications. Each treatment was chosen randomly and each trial lasted 48 hours with a minimum of 24 hours between each trial. Therefore, each trial lasted at least 3 days. If all trials had used the same batch of whey, the last replication would have started 9 days after receiving the whey. If the whey had been held 9 days its contents would have been drastically altered by indigenous microorganisms. Therefore it would not have been an accurate measure of the culture treatment.

Protein utilization remains a hurdle to be overcome in the problem of COD reduction of cottage cheese whey. But lactose and

lactic acid are of no real concern since they are readily utilized. With controlled conditions (temperature, aeration, and agitation) and nutrient supplementation, the fermentation time should not have to exceed 48 hours. The 2 yeast cultures used in this study and previous studies have proved to be reliable in the disposal of cottage cheese whey. The next step in this work would be at the dairy plant level. Most dairy plants already have the equipment needed to implement the use of microorganisms for whey disposal. A whey storage tank large enough to hold whey for 48 hours at a time and vats for growing the culture prior to inoculation would be the only equipment needed. A device to provide aeration would need to be installed in the whey fermentation tank. The mother culture could be stored and maintained in the laboratory.

The culture would be added to the whey and allowed to ferment for a 48 hour period. Then the whey could be passed through a desludging clarifier and the yeast organisms removed. This sludge could then be sold for animal feed and the residual liquid, with 10,000-15,000 ppm COD could be sent to a sewage plant.

CHAPTER V

SUMMARY

This study was undertaken to further investigate the possibilities of using microorganisms to reduce the Chemical Oxygen Demand (COD) and organic content of cottage cheese whey. Due to its ability to ferment lactose rapidly, *Kluveromyces fragilis* was chosen for this study. *Candida lipolytica* demonstrated proteolytic capabilities so it too was chosen for this study. These 2 cultures were then added singly and in combination to the whey making 3 culture treatments.

A 10 percent inoculum of each culture was added to 1,800 milliliters of whey. They were then incubated at 32° C $\pm$ 2° C with constant agitation and aeration for 48 hours in a New Brunswick Multigen fermenter. Samples from each treatment were taken at 3, 6, 12, 24, and 48 hour intervals and analyzed to follow the progress of each parameter studied which included pH, titratable acidity, total solids, precipitated solids, COD, lactose, and protein.

The change over time of each of the 7 parameters was studied. There was a significant culture x time interaction indicating that the 3 cultures utilized lactose according to different rate functions. Protein and COD showed no significant difference among cultures, but there was a significant difference among replications. Up to 87 percent of the lactose was utilized in the 48 hour period. The pH and titratable acidity remained virtually unchanged due to the addition of K_2HPO_4. With up to 47 percent protein utilization in some

cases, the COD was reduced up to 86 percent. Culture treatment 1 (K and C) and culture treatment 3 (K) reduced the COD to below 13,000 ppm during most of the 48 hour trial periods. Culture treatment 2 (C) did not reduce the COD as low as was needed. Regression equations which described the changes over time of COD, lactose, and protein were derived.

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LITERATURE CITED

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APPENDIX

TABLE V. Raw Data from Culture Treatments 1, 2, and 3 Replication 1

Culture	Time (Hours)	COD (ppm)	pH	Protein	Lactose	Total Solids	Precipated Solids	Titratable Acidity
				%				
1	0	68,640	4.40	0.810	4.42	6.80	6.78	0.65
	3	64,320	4.40	0.827	4.39	7.05	7.07	0.65
	6	60,960	4.45	0.780	4.16	6.97	7.00	0.77
	12	55,680	4.49	0.931	3.93	6.90	6.86	0.72
	24	28,640	4.59	0.849	1.83	7.02	6.95	0.72
	48	12,480	4.81	0.715	0.54	6.26	6.23	0.72
2	0	67,840	4.50	0.861	4.42	6.96	7.20	0.61
	3	58,160	4.50	0.850	4.15	7.07	7.14	0.66
	6	48,160	4.51	0.850	4.11	7.05	7.21	0.69
	12	49,440	4.57	0.850	4.06	7.04	7.21	0.68
	24	43,200	4.71	0.840	3.74	7.06	7.18	0.59
	48	28,000	5.10	0.800	3.48	6.79	6.96	0.52
3	0	70,880	4.55	0.938	4.61	7.75	7.79	0.35
	3	63,200	4.55	0.836	4.26	7.39	7.53	0.40
	6	53,120	4.55	0.827	3.87	7.39	7.46	0.45
	12	45,440	4.49	0.836	3.64	7.41	7.47	0.47
	24	41,440	4.50	0.846	3.45	7.40	7.46	0.45
	48	33,120	4.49	0.639	2.09	3.50	5.98	0.39

TABLE VI. Raw Data from Culture Treatments 1, 2, and 3 Replication 2

Culture	Time (Hours)	COD (ppm)	pH	Protein	Lactose	Total Solids	Precipated Solids	Titratable Acidity
				%				
1	0	51,520	4.51	0.893	4.87	7.10	7.00	0.66
	3	48,160	4.90	0.874	4.35	7.23	7.06	0.63
	6	39,520	4.90	0.761	4.42	7.29	7.15	0.62
	12	32,480	4.90	0.874	4.06	7.34	7.23	0.54
	24	28,480	4.98	0.752	3.71	7.38	7.25	0.61
	48	17,920	6.50	0.507	2.38	7.72	4.74	0.35
2	0	56,800	4.55	0.657	4.16	6.45	6.19	0.65
	3	49,760	4.95	0.676	3.87	5.96	6.30	0.60
	6	58,080	5.00	0.676	3.89	6.34	6.48	0.62
	12	42,080	5.00	0.676	3.90	6.38	6.42	0.57
	24	40,000	5.60	0.695	3.86	6.41	6.21	0.42
	48	20,800	5.20	0.529	1.06	3.89	3.07	0.52
3	0	56,800	4.45	0.864	4.16	6.39	6.41	0.59
	3	58,720	4.80	0.733	3.89	6.57	6.58	0.65
	6	52,000	4.80	0.785	3.64	6.15	6.16	0.63
	12	46,600	4.82	0.747	3.48	6.83	6.85	0.46
	24	38,240	5.20	0.799	3.09	6.47	6.43	0.43
	48	11,840	5.70	0.491	0.48	2.99	2.43	0.51

TABLE VII. Raw Data from Culture Treatments 1, 2, and 3 Replication 3

Culture	Time (Hours)	COD (ppm)	pH	Protein	Lactose	Total Solids	Precipated Solids	Titratable Acidity
				%				
1	0	72,960	4.80	0.861	5.00	6.83	6.83	0.91
	3	68,160	5.29	0.842	4.35	6.82	6.87	0.84
	6	56,480	5.29	0.817	4.10	6.75	6.74	0.66
	12	54,080	5.50	0.771	3.77	6.96	6.91	0.63
	24	45,120	5.50	0.761	3.16	6.92	6.94	0.58
	48	20,000	5.30	0.644	0.93	3.82	3.61	0.66
2	0	60,000	4.40	0.902	4.14	6.19	6.27	0.70
	3	54,720	4.87	0.799	3.85	6.54	6.48	0.54
	6	47,520	5.00	0.789	3.74	6.57	6.55	0.45
	12	35,520	5.47	0.780	3.66	6.51	6.40	0.52
	24	30,240	5.47	0.752	3.59	6.37	6.20	0.39
	48	11,040	5.20	0.583	0.83	2.83	2.11	0.62
3	0	61,760	4.40	0.911	5.26	6.64	6.66	0.52
	3	56,640	5.00	0.907	4,68	6.86	6.77	0.44
	6	52,640	5.00	0.888	4.35	6.65	6.43	0.93
	12	43,520	5.22	0.893	4.19	6.93	6.80	0.37
	24	32,160	5.31	0.871	3.93	6.61	6.31	0.49
	48	12,480	5.10	0.660	0.57	2.54	2.04	0.33

TABLE VIII. Raw Data from Culture Treatments 1, 2, and 3 Replication 4

Culture	Time (Hours)	COD (ppm)	pH	Protein	Lactose	Total Solids	Precipated Solids	Titratable Acidity
				%				
1	0	65,120	4.60	0.837	4.87	6.49	6.60	0.56
	3	61,120	5.10	0.861	4.29	6.59	6.88	0.23
	6	56,960	5.10	0.759	4.10	6.54	6.85	0.35
	12	51,360	5.11	0.689	3.87	6.63	6.84	0.30
	24	23,520	5.15	0.593	1.82	6.41	6.78	0.40
	48	9,120	5.20	0.504	0.51	2.62	2.48	0.61
2	0	60,640	4.40	0.987	4.10	7.25	6.68	0.59
	3	55,360	4.80	0.940	3.88	7.16	6.89	0.70
	6	49,280	4.99	0.864	3.71	7.17	6.88	0.56
	12	36,320	4.90	0.789	3.64	7.13	6.93	0.68
	24	31,040	5.70	0.733	3.56	6.91	8.59	0.31
	48	19,840	5.12	0.564	0.77	3.03	2.60	0.44
3	0	62,080	4.40	0.902	5.32	6.81	6.74	0.56
	3	57,280	4.72	0.855	4.81	6.85	6.77	0.51
	6	52,640	4.90	0.779	4.23	6.89	6.80	0.49
	12	44,000	4.90	0.686	4.10	6.87	6.78	0.44
	24	28,960	5.10	0.564	3.90	3.23	3.04	0.35
	48	11,360	5.30	0.479	0.48	2.93	2.68	0.40

VITA

The author was born November 11, 1954 in Clinton, Tennessee, to Mr. and Mrs. Willard C. Brock, Sr. In 1972 she graduated from Clinton Senior High School and in September of the same year entered the University of Tennessee at Knoxville. Completing her Bachelor of Science in Animal Science in August 1976, she graduated with honors. In September of the same year she married Donald Edwin Claiborne and also began a graduate program in the Department of Animal Science at the University of Tennessee, Knoxville. January 1978 she changed into the Department of Food Technology and Science at the University of Tennessee, Knoxville. She received a graduate research assistantship in May, 1978. Requirements for a Master of Science Degree were completed in March, 1979.