FROZEN CONCENTRATED CULTURES OF *KLUYVEROMYCES FRAGILIS*

By

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KLUYVEROMYCES FRAGILIS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Whey, produced as a by-product of the cottage cheese industry, can be cultured with the yeast Kluyveromyces fragilis and the biological oxygen demand (BOD) of the liquid reduced significantly (Knight, et al. 1972). In return, a yeast-whey protein material can be recovered which may have some value as a nutritious food supplement for humans and animals alike. Storage of the yeast culture required for this fermentation poses several problems. In the past, large volumes of the yeast-whey mixture have been held back and used as a starter for the next fermentation (Smith, et al., 1977). This procedure is troublesome and requires considerable time and storage space. Furthermore, such a procedure can result in reduced viability and activity of the yeast culture.

The objective of this study was to find a means of storing concentrated yeast cultures in a frozen state, at either -19°C or -196°C, in such a manner that the culture would retain its viability and activity. Storage of such concentrated cultures would require less space and simplify utilization of the culture for fermenting whey. Results from this study also could have important applications for other areas of food processing involving the use of yeast cultures.

CHAPTER II

REVIEW OF LITERATURE

A limited amount of work has been reported on the freezing and frozen storage of yeast cultures, particularly in liquid nitrogen. The generalization has been made that both yeasts and molds appear to be more resistant to the effects of freezing than do vegetative cells of bacteria (Rose, 1967). Those factors which have been shown to be most important in survival of yeast cells during freezing are the rate of cooling, the minimum temperature to which the cells are cooled, the rate of thawing, and the growth conditions employed in preparing the culture for freezing (Mazur, 1961a).

The rate at which yeast cells are cooled to the storage temperature has received considerable attention. When Saccharomyces cerevisiae was cooled rapidly to -30°C or below less than 0.01 percent of the cells survived (Mazur, 1961a). However, when these cells were slowly cooled to the same temperature, as many as 50 percent survived. Hwang (1966) found that he could preserve pathogenic strains of yeast in liquid nitrogen from 44 - 57 months by slowly freezing the cultures to -35°C (1°C/minute) and then using an accelerated rate to bring them to -196°C. The highest survivals of brewing yeasts frozen in liquid nitrogen by Wellman and Stewart (1973) were obtained with a freezing rate of 1°C/minute. Souzu (1973) reports that the baker's yeast he worked with yielded the highest survivals following a slow freezing process to

liquid nitrogen temperature. Bank and Mazur (1973) have shown a 60 percent survival of S. cerevisiae when cooled at the rate of 7°C/minute.

Mazur and Schmidt (1968) and Bank and Mazur (1973) have explained the mechanisms by which yeast cells are damaged due to their cooling rates. In any freezing situation, the external medium begins to freeze first. The water present within the cell becomes supercooled and begins to move out of the cell because of its elevated vapor pressure. When yeast cells are frozen slowly, they are permitted to equilibrate with the external medium and are less likely to freeze internally, due to partial removal of intracellular water. With a rapid cooling rate, the yeast cells have less time to equilibrate with the environment. Therefore, the cells lose less water, and the probability of internal ice formation increases. These internal ice crystals are the principal cause of damage to rapidly frozen cells. It was also pointed out by Bank and Mazur (1973) that if the rate of freezing were suboptimal, or too slow, "solution effects" could damage the yeast cells. These effects are the result of increased cellular dehydration, pH changes, and a concentration and precipitation of solutes. The effect of concentrated solutes, both extra cellularly and intracellularly, is probably not immediate, but occurs over the period of frozen storage (Meryman, 1966).

The thawing of frozen yeast cells is another factor to consider. Mazur (1961b) found that in cells frozen at -10°C, survival was not dependent on thawing temperature. However, with cells frozen from -30°C to -75°C, the survival rate was very sensitive to cooling and thawing rates. Slow warming yielded viable cell numbers 1/3 to 1/140 of those with rapid warming. Rapid cooling and slow warming proved

to be the worst combination for yeast cells, resulting in 1/2000 to 1/4000 as many viable cells as the optimum conditions. Mazur and Schmidt (1968) report that rapid warming is the least harmful to frozen yeast cells. The greatest danger in the thawing of yeast cell suspensions is recrystallization, or grain growth, of the ice crystals already present in the cell. A rapid rate of warming reduces the amount of recrystallization and the exposure of the thawing cells to concentrated solutes. They have found that if warming is extremely fast (48,000°C/minute) for super-cooled cells (-196°C), there should be no additional damage to the cells due to warming.

While freezing and thawing rates have received considerable attention, the temperature and time of yeast culture storage has also been studied. Wood (1957) has shown there is little inactivation of yeast cultures above -10°C, and that exposures of yeasts to even very low temperatures will not totally destroy the population. Mazur and Schmidt (1968) also believe -10°C to be the breaking point between high and low survival of yeast during freezing. In addition, they found that survival was not due to the time a culture was held at a particular temperature level. Meryman (1966), however, states that survival of cells frozen at temperatures above -70°C is affected by storage time, and he feels the extent to which the number of viable cells decreases is dependent on the suspending medium. In another study, Mazur (1961b) reported results showing that damage to cells exposed to low temperatures occurred fairly rapidly. With cells rapidly cooled to -30°C, survival after a one minute exposure was 1 percent. With extended exposure of up to 90 minutes, there was little additional damage.

The process of freeze-thawing yeast cells causes damage to the cell membrane. Mazur (1963) has found that the cell membrane of rapidly frozen cells remains sufficiently intact after freezing to maintain the solutes within the cell. However, during thawing, when the temperature of the cell reached 4°C, the solutes of the cell moved out quickly, due to the breakdown of membrane barriers. The membrane damage may be due to phospholipid degradation, which in turn leads to decreased cell survival (Souzu, 1973). A disturbance of the hydrophobic bonds maintaining the membrane yielded a greater access to the phospholipids, increasing the extractable lipid material from freeze-thawed cells.

Hossack and Rose (1976) have reported that sterols function in maintaining the stability of the phospholipid molecules in the cell membrane. The chemical composition of the growth medium can be used to alter the lipid composition of microbial cells. Through the use of high saline concentrations, the lipid content of cells of S. cerevisiae and Endomyces vernalis has been increased. While inhibiting the growth of Candida albicans, sodium chloride also increased the lipid content of this yeast. The sodium ions seem to function in increasing the rate of glucose fermentation by the yeasts (Combs, et al., 1968). Hossack (1977) has found that the two principal phospholipids synthesized by most strains of S. cerevisiae are phosphatidylcholine and phosphatidylethanolamine. The rate of synthesis of these compounds can be increased when either 1mM chlorine or 1mM ethanolamine is supplied in the growth medium. With an increase of ethanolamine, S. cerevisiae becomes more resistant to osmotic lysis. White and Werkman (1948) have found that acetate can be converted into fat and sterols by yeasts. According to Dawson and Craig (1966), the lipid content of Candida utilis, when grown in a batch

culture, reached a maximum in the stationary phase and began to decline when the glucose in the medium was depleted.

The use of various growth media and freezing menstrua in storing Saccharomyces carlsbergensis in liquid nitrogen has been studied by Tsuji (1966a). He found that when grown and frozen in Sabouraud Liquid Medium, 0.2 percent of the yeast cells in the logarithmic growth phase survived, while 17 percent of those in the maximum stationary phase survived. When Trypticase Soy Broth was used, seven percent of the cells frozen in the log phase recovered, but 81 percent of those from the maximum stationary phase survived. He believed that cells in the log phase showed greater sensitivity to freezing due to an unfavorable electrolyte concentration or unfavorable cell wall permeability. Additives to the freezing menstrua which have been shown to decrease storage death rates for yeast cells include milk protein, gelatin, mucin, meat extract, serum, glycerol, sucrose, glucose, and lactose (Meryman, 1966).

Tsuji (1966b), preserved cultures of S. carlsbergensis at -196°C in ten percent glycerol. Hwang (1966) also used a ten percent w/v glycerol-water menstruum for freezing his pathogenic yeast cultures in liquid nitrogen.

CHAPTER III

EXPERIMENTAL PROCEDURE

Source and Maintenance of Cultures

K. fragilis Y-1156, Y-1171, Y-108, and Y-1196 were obtained from the Northern Regional Research Laboratory (Peoria, Illinois), and *K. fragilis* 50-16, 50-29, 55-1, 72-297, C-21, and C-106 were obtained from the Department of Food Science and Technology, University of California at Davis.

Stock cultures were maintained by inoculating lactose agar slants (2.5 percent agar, 2.0 percent lactose, 1.0 percent peptone, and 0.1 percent yeast extract) and incubating them at 35°C for 18 hours. The cultures were held at refrigeration temperature (6°C) between subcultures. The yeasts were subcultured before experimental use by inoculating 10 ml of the appropriate growth medium with a loopful of the culture from a lactose agar slant and incubating it for 16-18 hours at 35°C. A one percent inoculum of this broth culture was then used to prepare a second broth subculture in a similar manner.

Identification of Yeasts

The identity of the yeasts were confirmed using procedures described by Lodder (1970). The procedures are repeated here for convenience. The basal medium for the fermentation tests contained 4.6 g of yeast

extract and 7.6 g of peptone per liter of distilled water, plus adequate one percent aqueous Bromothymol Blue to produce a dense green color. The broth was dispensed in 8 ml quantities, into screw cap test tubes, Durham tubes were inserted, and the medium was autoclaved at 121°C for 15 minutes.

In preparing the basal medium for the carbon assimilation tests, 6.6 g of Yeast Nitrogen Base broth (Difco, Detroit, Michigan) per liter of distilled water was dissolved by stirring and heating slightly. The solution was "filter sterilized" through a sterile 0.45 u membrane filter, and 4.5 ml aliquots were aseptically dispensed into sterile screw cap test tubes.

The substrates for the tests were prepared in the following manner. Forty ml of 10 percent aqueous solutions of glucose, galactose, sucrose, maltose, lactose, cellobiose, trehalose, melezitose, and α-methyl-D-glucoside and 40 ml of 20 percent raffinose were "filter sterilized" by passage through sterile 0.45 u membrane filters and aseptically dispensed into sterile screw cap test tubes (approximately 20 ml per tube). All substrates were stored in the refrigerator. The sugars were used at the prepared concentrations for the fermentation tests. For the carbon assimilation tests, each was diluted one-to-one with sterile distilled water, yielding five percent solutions for all except raffinose (10 percent). An additional substrate included in the carbon assimilation tests was ethanol, prepared as a five percent aqueous solution.

Each yeast culture was streaked onto a lactose agar plate and incubated overnight at 35°C. The plates were observed for culture purity and colonial morphology. Using a sterile cotton swab a portion

of the surface growth was collected and placed in nine ml of sterile peptone (0.1 percent) dilution water. This suspension served as the inoculum for each culture.

To carry out the fermentation test, sufficient basal broth tubes were labeled--one for each substrate for each culture, plus one extra to serve as an uninoculated control. Two ml of each substrate was added to the appropriate tubes. One tube of each substrate was inoculated with 0.1 ml of inoculum for each culture. All tubes were incubated at 35°C and observed at 48 and 72 hours for gas and acid production. A distinct yellow color was considered positive for acid production. The presence of collected gas in the Durham tube was positive for gas production.

For the carbon assimilation tests, one Yeast Nitrogen Base broth tube was labeled for each substrate for each culture, plus one for an uninoculated control. One-half ml of each substrate was aseptically added to the appropriate tubes. One tube of each substrate was inoculated with 0.1 ml of inoculum for each culture. All tubes, including the uninoculated controls, were incubated at 35°C and checked for growth at 7 and 21 days. To check for growth, each tube was mixed thoroughly on a Vortex Mixer (Scientific Industries, Inc., Bohemia, N.Y.). A white card with black lines 3/4 mm wide was held behind the tube. If the black lines could not be seen or appeared as diffuse broad bands, the test was considered positive for assimilation.

Determination of Yeast Viability and Activity

Colony Counts

Colony counts of yeasts were determined using a pour plate method. Dilutions were prepared using peptone dilution blanks containing 99 ml of 0.1 percent peptone and 0.001 percent Anti-foam A Emulsion (Sigma

Chemical Company, St. Louis, Missouri). All plating media and dilution blanks were autoclaved at 121°C for 15 minutes. The initial dilution for the concentrated cultures was made by weighing one gram of the yeast culture concentrate into an empty sterile dilution bottle and aseptically pouring 99 ml of sterile dilution water into the bottle. The dilution was shaken and additional dilutions made as described in Compendium of Methods for the Microbiological Examination of Foods (Speck, 1976). Duplicate plates were poured with melted Sabouraud Dextrose Agar tempered to 45°C for all dilutions. After solidification, the plates were inverted and incubated for 48 hours at 35°C. All visible colonies were enumerated with the aid of a Quebec Colony Counter.

Measurement of Ability to Grow in Whey and Utilize Lactose

To compare the abilities of the nine strains of yeasts to utilize the lactose in whey, the following activity test was employed. The inoculum for the test was prepared in clarified cottage cheese whey. It was clarified by heating the required quantity of the whey in a boiling water bath for 30 minutes. The whey was then cooled and centrifuged at 3000 x g for 10 minutes in sterile 50-ml centrifuge tubes. Fifty ml of the supernatant was aseptically transferred to a sterile 250 ml Erlenmeyer flask. The yeast culture was subcultured in 10 ml of lactose broth (containing 4.0 percent lactose, 2.0 percent peptone, and 0.1 percent yeast extract; autoclaved 15 minutes at 121°C) and incubated statically at 35°C for 18 hours. Fifty ml of the clarified cottage

cheese whey was inoculated with 0.5 ml of the lactose broth culture. Following inoculation, the flask was incubated in a 35°C reciprocating shake water bath (82 strokes of 40 mm per minute) for 16-18 hours (Precision Scientific Company, Chicago, Illinois).

For each culture, 60 ml of fresh unheated cottage cheese whey, obtained from the Oklahoma State University dairy plant, was dispensed into a sterile 250-ml Erlenmeyer flask. A one percent inoculum from the clarified whey culture was added, and the flask was incubated in the 35°C shake water bath (82 strokes of 40 mm per minute). Five ml samples were removed at 0, 8, 9 1/2, 11, 12 1/2, 14, and 15 1/2 hours, placed in sterile screw cap test tubes, and submerged in an ice bath until analyzed. The yeasts were enumerated by colony counts. The samples were also assayed for lactose.

Lactose Assay

An anthrone method similar to that described by Morris (1948) was used to measure the sugar content of the whey. The reagent was prepared by dissolving one gram of anthrone in 500 ml of 95 percent H_2SO_4. Two ml of the sample, diluted sample, or distilled water (for the blank) were placed in a test tube. Four ml of the anthrone reagent was added to each tube and the contents mixed thoroughly. All tubes were placed in a boiling water bath for three minutes, cooled, and the percent transmittance determined at 620 nm on a Klett Summerson colorimeter (Klett Manufacturing Company, New York, N.Y.). To determine the sugar content, the sample readings were compared to a standard curve prepared using lactose.

Preparation of Concentrated Cultures for Freezing

Growth Media

For producing cells of *K. fragilis*, it was desirable to find a growth medium containing ingredients similar to fresh whey, but which could be easily stored between experiments. Krafen dried, sweet whey (Kraft Industrial Foods Division, Chicago, Illinois) was selected for use. The reconstituted whey was pepsinized, supplemented with various nutrients, and evaluated for its ability to support the growth of *K. fragilis*. The dried whey was reconstituted as a five percent aqueous solution and adjusted to pH 3.0 with 30 percent citric acid. Pepsin (Sigma Chemical Company, St. Louis, Missouri) was added to the mixture (25 mg/100 ml whey), and the medium was incubated in a 37°C water bath for 30 minutes. The digested whey was adjusted to pH 6.5 with 20 percent NaOH. It was then dispensed into containers and autoclaved for 15 minutes at 121°C.

Several media supplements, which have been reported to alter the lipid composition of microbial cells, were evaluated to determine if they would have any effect(s) on stability of the yeast cells to freezing and frozen storage. These supplements, which were added to pepsinized whey, included 0.1 percent Tween 80 (Hossack and Rose, 1976), 1mM ethanolamine (Hossack, 1977), and 0.5 percent NaCl (Combs, et al., 1968). Sabouraud Liquid Medium (composed of 2.0 percent dextrose, 0.5 percent trypticase, and 0.5 percent thiotone) was also compared to the pepsinized whey medium (Tsuji, 1966a). The media were prepared, dispensed in 50-ml quantities in 250-ml Erlenmeyer flasks, and autoclaved 15 minutes at 121°C.

Growth Conditions

Erlenmeyer flasks (250 ml), containing the prescribed amount of growth media, were inoculated with a fresh pepsinized whey culture of the yeast at a level of one percent. They were placed in the 35°C reciprocating shake water bath, agitating the cultures 82 strokes of 40 mm per minute, and incubated the prescribed length of time.

Recovery and Resuspension of Yeast Cells

The cells were harvested by centrifugation at 12,000 x g (50-ml tubes) or 15,000 x g (250-ml bottles) for 10 minutes at 10°C in a Sorvall model RC-5 Superspeed Refrigerated Centrifuge (Dupont Company, Newtown, Connecticut). The pellets were resuspended in cold sterile 10 percent nonfat milk solids (NFMS). Resuspension was accomplished by gently swirling the milk in the centrifuge container. The resulting concentrated cell suspension was dispensed into sterile 2-ml plastic freezing vials (Cooke Pro-vial, Dynatech Laboratories Inc., Alexandria, Virginia). The vials were placed in a freezer (-19°C) or submerged in liquid nitrogen (-196°C) for storage.

Effect of Cellular Age and Freezing Temperature on Survival of Yeasts

Experiments were conducted to determine the effect of cellular age on survival of yeast cells during freezing and frozen storage. For each experiment, four 250-ml Erlenmeyer flasks containing 50-ml of pepsinized whey were used for growing the yeast cells for concentrate preparation. One flask was removed at 8, 11, 14, and 17 hours. Concentrated cultures

were prepared from the cells in each flask. Duplicate vials were prepared for each. One was frozen and stored at -19°C and the other at -196°C. The concentrated cultures were plated to determine colony counts before freezing and after a three-day storage period.

Effect of Added Glucose on Survival of Frozen Concentrated Cultures

In order to test the effect of various glucose concentrations on the survival of yeasts over prolonged storage periods, the following experiment was conducted. Eight hundred ml of a pepsinized whey culture of K. fragilis Y-1156 was prepared, using ten flasks containing 80-ml each of the growth medium. The cells were harvested and resuspended in 20-ml of cold sterile 10 percent NFMS. Five gram aliquots of this concentrated culture were placed into each of four test tubes, containing 15-ml of 10 percent NFMS and glucose in the amounts of 0, 10, 20, and 30 percent. The milk and glucose were autoclaved separately, aseptically combined, and heated in a boiling water bath to dissolve the sugar. The tubes of suspending media were held at refrigeration temperature until needed. Four 2-ml vials of each concentrated culture were frozen at -19°C and four at -196°C. Each sample was plated before freezing and after 1, 7, 14, and 21 days of frozen storage.

To determine the effect of a freezing at -19°C followed by frozen storage at -196°C, additional experiments were conducted. The protocol was the same as in the previous paragraph except that additional concentrates were prepared so some samples could be frozen at -19°C for 24 hours and then transferred to liquid nitrogen for storage. From each glucose preparation, eight 2-ml and three 5-ml vials were prepared.

Five of the small vials and two of the large ones were frozen at -19°C; the others were placed in liquid nitrogen. Each sample was plated before freezing. After 24 hours, a sample from -19°C and one from -196°C were plated. At the same time, two 2-ml vials and one 5-ml vial from each glucose preparation were transferred from -19°C to liquid nitrogen. Samples from each of the three freezing and storing processes were plated to determine colony counts after 7, 14, and 21 days of storage. The larger vials were used for the 21 day sample. This provided sufficient cell concentrate to be used for determining the ability of the yeasts to grow in cottage cheese whey following three weeks of frozen storage. In this phase of the experiment each vial was thawed in two liters of tap water at 20°C.

In evaluating the ability of the yeast to grow in cottage cheese whey after frozen storage, the inoculum for the test was prepared by diluting one gram of the concentrated culture in 99 ml of cold sterile ten percent NFMS. Sixty ml of fresh cottage cheese whey was placed in sterile 250-ml Erlenmeyer flasks. One and a half ml of inoculum was added to each flask, and the flasks were incubated in the 35°C reciprocating shake water bath. Five ml samples were taken at 0 and 10 hours and colony counts determined.

Comparison of Procedures for Resuspending Yeast Cells

To determine whether the use of glass beads in resuspending the yeast cell pellet after centrifugation would result in a more uniform cell suspension than a simple swirling of milk in the centrifuge bottles, the following experiment was done. Eight hundred ml of a pepsinized

whey culture of K. fragilis Y-1156 was prepared. The cells were harvested in four 250 ml centrifuge bottles. Five ml of 10 percent NFMS was added to each bottle; approximately 30 sterile glass beads (1/8 inch in diameter) were added to two of the bottles. After a thorough suspension of the pellets, by swirling the bottles with and without glass beads, the cell concentrates for each procedure were combined into two bottles and mixed. Two ml quantities of each preparation were placed in five sterile screw cap test tubes. All samples were immediately plated to determine colony counts.

Statistical Analyses

An analysis of variance procedure was used to determine if significant differences occurred among glucose concentrations for cell concentrates frozen at -19°C, and to determine if there was significant variation among replicates of these same experiments. Duncan's new multiple range test was used to compare the differences between mean cell survivals of yeasts frozen in 0, 10, 20, and 30 percent glucose at -19°C. The least significant difference procedure was used to compare mean cell survivals of yeasts frozen over an extended storage period with the 0-time count for each glucose preparation. The methods for these analyses are outlined in Principles and Procedures of Statistics (Steel and Torrie, 1960).

CHAPTER IV

RESULTS

Identification of Yeasts

To ensure the identity of the yeasts as strains of Kluyveromyces fragilis, a series of fermentation and carbon assimilation tests were conducted. The identities of the yeasts were confirmed using these procedures. The results appear in Table I. The characteristics of all strains except C-21 matched those of K. fragilis as presented by Lodder (1970). Thus, strain C-21 was not included in further studies.

Growth and Utilization of Lactose in Cottage Cheese Whey

The abilities of the nine strains of K. fragilis to grow in cottage cheese whey and utilize lactose were compared in an activity test in which the yeasts were grown in fresh cottage cheese whey. Samples were taken periodically for determining colony counts and lactose content. The data in Table II reveals that the amount of growth based on colony counts was similar for all strains after 14 hours. When the yeasts were compared for their ability to utilize the lactose in whey, numerous differences resulted (Figure 1). The data is presented graphically as percent lactose remaining after 8, 11, and 14 hours. K. fragilis 50-29, 50-16, and 55-1 used less than 30 percent of the lactose in a 14-hour period, whereas approximately 90 percent or more of the lactose was used in the whey cultured with K. fragilis 72-297, Y-1156, Y-108, Y-1171,

TABLE I

IDENTITY CHARACTERISTICS OF KLUYVEROMYCES FRAGILIS

Test	Substrate	Strains of Kluyveromyces fragilis										
		Y-1156	Y-1171	Y-1196	Y-108	50-16	50-29	55-1	72-297	C-106	C-21	Lodder*
FERMENTATION	Glucose	+	+	+	+	+	+	+	+	+	+	+
	Galactose	+	+	+	+	+	+	+	+	+	+	+
	Sucrose	+	+	+	+	+	+	+	+	+	+	+
	Maltose	-	-	-	-	-	-	-	-	-	-	-
	Lactose	+	+	+	+	+	+	+	+	+	+	+
	Cellobiose	-	-	-	-	-	-	-	-	-	-	-
	Trehalose	-	-	-	-	-	-	-	-	-	-	-
	Melezitose	-	-	-	-	-	-	-	-	-	-	-
	α -Methyl-D-Glucoside	-	-	-	-	-	-	-	-	-	-	-
	Raffinose	+	-	+	+	+	+	+	+	+	-	+
ASSIMILATION	Glucose	+	+	+	+	+	+	+	+	+	-	+
	Galactose	+	+	+	+	+	+	+	+	+	-	+
	Sucrose	+	+	+	+	+	+	+	+	+	-	+
	Maltose	-	-	-	-	-	-	-	-	-	-	-
	Lactose	+	+	+	+	+	+	+	+	+	+	+
	Cellobiose	+	-	-	+	+	+	+	-	+	-	+
	Trehalose	+	+	+	-	+	-	-	-	+	-	-
	Melezitose	-	-	-	-	-	-	-	-	-	-	-
	α -Methyl-D-Glucoside	-	-	-	-	-	-	-	-	-	-	-
	Raffinose	+	+	+	+	+	+	+	+	+	-	+
	Ethanol	+	+	+	+	+	+	+	+	+	-	+

*Reactions for K. fragilis as presented by Lodder (1970).

TABLE II

GROWTH OF KLUYVEROMYCES FRAGILIS IN COTTAGE CHEESE WHEY

Strain	Hr.	Count/ml	Strain	Hr.	Count/m.
Y-1156	0	2.6×10^6	50-29	0	2.9×10^6
	8	1.4×10^8		8	7.6×10^7
	11	2.4×10^8		11	1.7×10^8
	14	3.2×10^8		14	2.6×10^8
Y-1171	0	5.9×10^5	55-1	0	1.1×10^6
	8	2.1×10^8		8	4.4×10^7
	11	3.2×10^8		11	9.1×10^7
	14	3.5×10^8		14	2.0×10^8
Y-1196	0	3.2×10^6	72-297	0	2.9×10^6
	8	1.8×10^8		8	1.4×10^8
	11	3.2×10^8		11	2.6×10^8
	14	2.9×10^8		14	3.1×10^8
Y-108	0	3.6×10^6	C-106	0	1.4×10^6
	8	1.6×10^8		8	4.1×10^7
	11	3.6×10^8		11	1.2×10^8
	14	3.9×10^8		14	2.0×10^8
50-16	0	-			
	8	9.2×10^7			
	11	1.8×10^8			
	14	2.6×10^8			

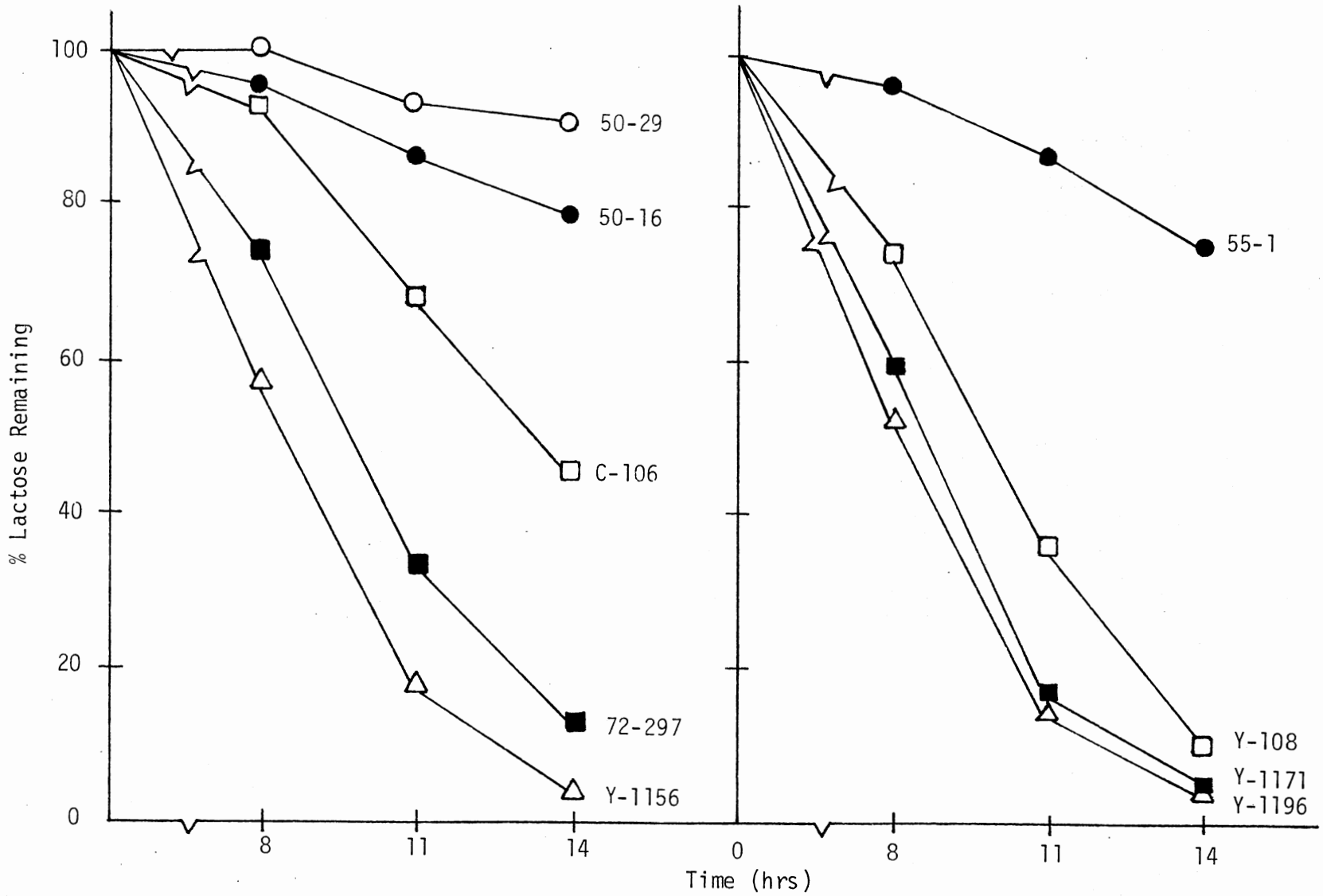

Figure 1. Lactose Utilization by Kluyveromyces fragilis in Cottage Cheese Whey

and Y-1196 in 14 hours. K. fragilis C-106 was intermediate in lactose utilization, with 45 percent remaining at the end of the test period.

From the data collected in this phase of the study, K. fragilis Y-1156 was chosen for further experimentation in culture concentrate preparation and freezing trials. Its ability to grow in whey and to readily utilize most of the lactose were the determining factors.

Effect of Cellular Age on Survival After Freezing

The survival of K. fragilis Y-1156 in concentrated cultures during frozen storage varied with physiological age and storage temperature (Table III). The viability of concentrated cultures prepared after 8, 11, 14, and 17 hours of growth in a pepsinized whey medium was determined before and after three days of frozen storage. In both trials, the cultures frozen at -19°C survived much better than did those frozen at -196°C. Greatest survival of cultures frozen at -19°C was observed for those frozen after eight hours growth. The number of cells surviving freezing after 11 hours growth was somewhat lower than that observed for the eight-hour culture. The percentages of survival of the yeast cultures frozen at the 14 and 17-hour growth periods were slightly higher than at 11 hours but not as high as for the eight-hour cultures. There was one exception to this trend, however. The percentage survival of the concentrate prepared at 14 hours in Trial II was quite a bit lower than all other values for this phase of the study.

The yeast cultures frozen at -196°C responded in a contrary manner to those frozen at -19°C. There was a negligible amount of survival from cells frozen after eight hours of growth. The survival level

TABLE III

EFFECT OF CELLULAR AGE AND FREEZING TEMPERATURE ON SURVIVAL OF KLUYVEROMYCES FRAGILIS Y-1156

Trial	Age(hr)	Days Storage	-19°C		-196°C	
			Count/g	% Survival	Count/g	% Survival
I	8	0	2.0×10^8	-	2.0×10^8	-
		3	1.6×10^8	80	2.1×10^6	1
	11	0	8.7×10^8	-	8.7×10^8	-
		3	5.0×10^8	57	1.0×10^8	11
	14	0	2.0×10^9	-	2.0×10^9	-
		3	1.3×10^9	65	2.5×10^8	13
	17	0	2.6×10^9	-	2.6×10^9	-
		3	1.7×10^9	65	2.3×10^8	9
II	8	0	2.0×10^8	-	2.0×10^8	-
		3	1.6×10^8	80	4.1×10^5	<1
	11	0	6.2×10^8	-	6.2×10^8	-
		3	3.8×10^8	61	8.3×10^7	13
	14	0	2.0×10^9	-	2.0×10^9	-
		3	5.5×10^8	28	4.1×10^8	21
	17	0	1.9×10^9	-	1.9×10^9	-
		3	1.4×10^9	74	5.3×10^7	3

seemed to peak between growth times of 11 and 14 hours and decrease again at the 17-hour point.

Effect of Glucose Concentration on Survival After Freezing

Various concentrations of glucose were added to concentrated cultures of K. fragilis Y-1156 to determine their effect on survival of yeast cells. The concentrated cultures were prepared by resuspending cells from a 15-hour pepsinized whey culture in 10 percent non-fat milk solids (NFMS) plus either 0, 10, 20, or 30 percent glucose. The concentrated cultures were frozen at -19°C and -196°C, and samples were plated after 0, 1, 7, 14, and 21 days of frozen storage. Results from four trials, presented as percent survival, are shown in Table IV. The addition of glucose to the suspending medium had little or no apparent beneficial effect on survival after one day storage at -19°C. However, during extended storage periods of 7 to 21 days, the number of yeast cells surviving increased as the amount of glucose in the medium increased. The concentrated cultures frozen at -196°C again exhibited much lower survival than those frozen at -19°C (Table V). The addition of glucose to the medium, even at a level of 30 percent, provided no apparent protection to the cells.

Effect of a Dual Freezing Treatment on Survival After Freezing

From the previous experiments, it was observed that damage to the concentrated cultures frozen at -19°C occurred over the total storage period at a slow rate. However, the damage to yeast cells frozen at

TABLE IV

EFFECT OF GLUCOSE CONCENTRATION AND STORAGE TIME ON THE SURVIVAL OF KLUYVEROMYCES FRAGILIS Y-1156 AT -19°C

Glucose Concentration	Days Storage	% Survival				
		1	2	3	4	AVG.
0%	1	90	85	83	71	82
	7	19	48	36	23	32
	14	49	39	41	37	42
	21	5	32	43	11	23
10%	1	74	92	69	81	79
	7	60	68	63	59	63
	14	79	63	58	56	64
	21	37	55	53	48	48
20%	1	79	100	78	93	88
	7	66	85	69	60	70
	14	80	85	56	73	74
	21	54	81	51	68	64
30%	1	84	83	83	100	88
	7	69	97	64	78	77
	14	82	83	72	90	82
	21	83	79	53	85	75

TABLE V

EFFECT OF GLUCOSE CONCENTRATION AND STORAGE TIME ON THE SURVIVAL OF KLUYVEROMYCES FRAGILIS Y-1156 AT -196°C

Glucose Concentration	Days Storage	% Survival				
		1	2	3	4	AVG.
0%	1	7.5	12	7	6	8
	7	5	12	7	4	7
	14	6	10	7	5	7
	21	1	10	5	6	6
10%	1	4	4	2	2	3
	7	5	6	3	1	4
	14	5	4	2	2	3
	21	2.5	4	3	2	3
20%	1	8	6	5	5	6
	7	10	6	6	4	7
	14	9	4	6	5	6
	21	8	6	8	5	7
30%	1	-	7	7	10	8
	7	-	8	6	6	7
	14	-	5	6	8	6
	21	-	10	10	1	7

-196°C seemed to occur almost immediately, and the viability of these cells changed little during additional storage. Therefore, the previous experiment was repeated along with a third freezing treatment. Additional cell concentrates were prepared with the various concentrations of glucose and were frozen for 24 hours at -19°C. At this time, they were transferred to frozen storage in liquid nitrogen. The results from two trials are summarized in Table VI. Cultures frozen and stored at -19°C exhibited the highest survivals. Increasing the amount of glucose in the suspending medium again increased the number of cells surviving freezing and storage at -19°C over extended storage periods. Concentrated cultures frozen and stored at -196°C again failed to benefit from the added glucose in the medium. For those cultures exposed to the dual freezing treatment, higher survivals were observed as compared to cultures frozen and stored at -196°C. However, survival levels were not as high as for those frozen and stored at -19°C. The addition of glucose to the suspending medium was not as effective in preventing death of the cultures exposed to the dual freezing treatment as was observed for the cultures frozen and stored at -19°C.

Ability to Grow in Whey After Frozen Storage

Concentrated cultures of K. fragilis Y-1156 frozen 21 days at -19°C (from the previous study) were used to determine if freezing had any effect on the cell's ability to grow in cottage cheese whey. Fresh cottage cheese whey, inoculated with the culture concentrates and incubated at 35°C, were sampled at zero and 10 hours for determining colony counts. The results from two trials appear in Figure 2. The variations seen among the zero-time counts among all glucose

TABLE VI

EFFECT OF FREEZING TREATMENT, GLUCOSE CONCENTRATION AND STORAGE TIME ON % SURVIVAL OF KLUYVEROMYCES FRAGILIS Y-1156

Glucose Concentration	Days Storage	-19°C			-196°C			-19°C/-196°C		
		Trail 1	Trial 2	AVG	Trail 1	Trial 2	AVG	Trial 1	Trail 2	AVG
0%	1	73	78	76	23	12.5	18	-	-	-
	7	56	31	44	13	15	14	27	16	22
	14	50	22	36	22	15	19	42	19	31
	21	38	6	22	19	11	15	27	9	18
10%	1	100	100	100	22	14	14	-	-	-
	7	57	77	67	17	11	11	45	18	32
	14	69	61	65	20	14	14	60	25	43
	21	78	47	63	19	13	13	45	10	28
20%	1	79	83	81	31	17	17	-	-	-
	7	58	74	66	33	17	17	18	8	13
	14	65	70	68	23	13	13	41	14	28
	21	59	54	57	23	13	13	53	7	30
30%	1	100	100	100	19	10	10	-	-	-
	7	100	100	100	21	11	11	39	11	25
	14	96	83	90	19	10	10	47	14	31
	21	69	80	75	20	11	11	88	8	48

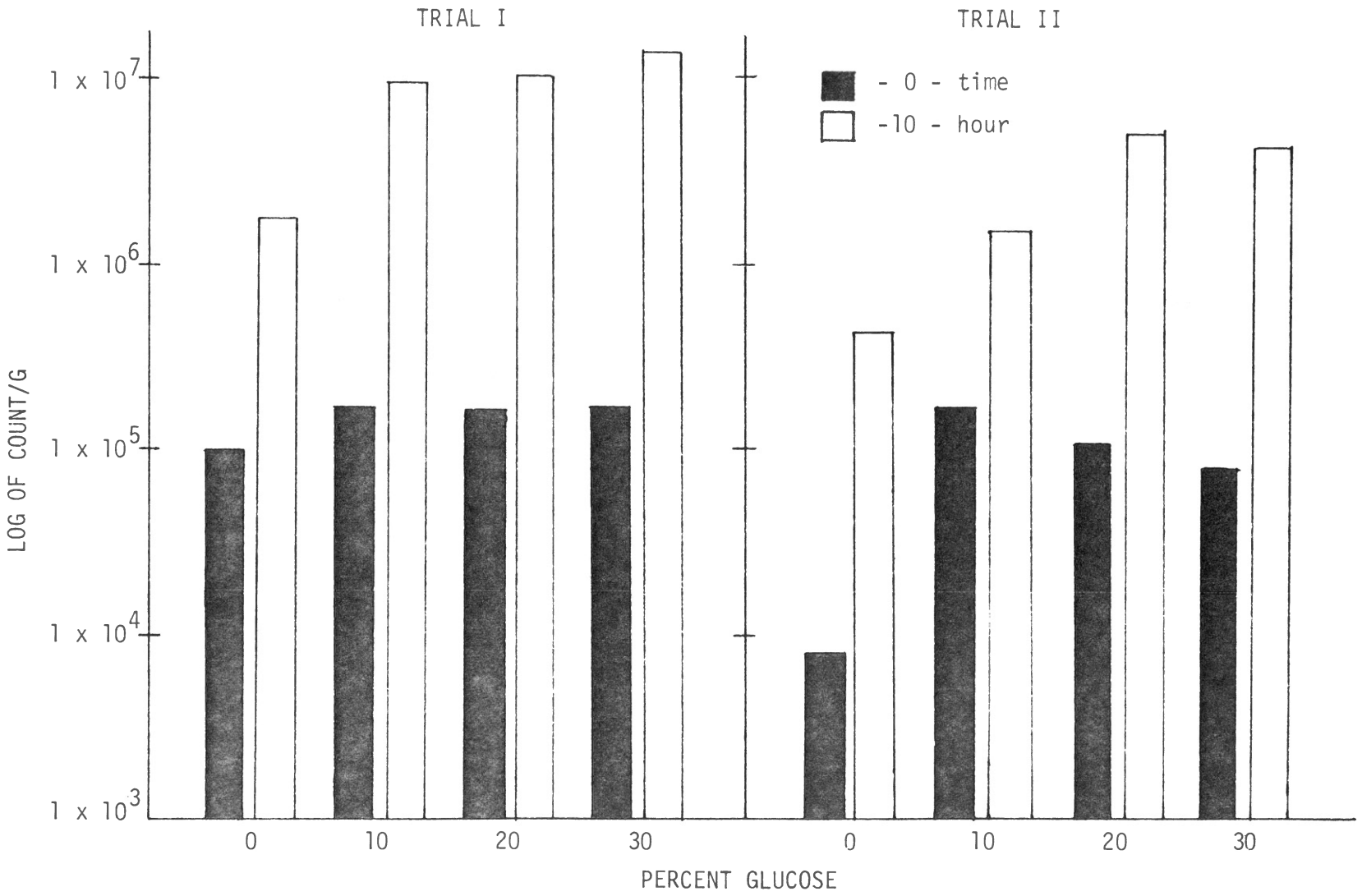

Figure 2. Growth of Kluyveromyces fragilis Y-1156 in Cottage Cheese Whey After 21 Days Storage At -19°C.

concentrations were due to the differing percentages of survival of the cultures after freezing. In both trials, the cells frozen in zero percent glucose did not achieve as high a count at 10 hours as the other cultures. The cultures frozen in 10, 20, and 30 percent glucose in Trial I all appeared to grow equally well in the cottage cheese whey. There was some variation in Trial II, with those frozen in 20 and 30 percent glucose achieving the highest levels of growth. Glucose added to the freezing medium seemed to have a beneficial effect on the cell's ability to grow in whey after freezing. This is probably related to increased survival of the cultures during freezing and storage.

Effect of Composition of Growth Media on Stability of Frozen Cells

In attempts to find a growth medium which might produce yeast cells which were more resistant to freezing damage and prove more suitable than pepsinized whey for the growth of K. fragilis Y-1156 in preparing frozen culture concentrates, four media were examined. They included pepsinized whey supplemented with Tween 80, ethanolamine, and 0.5 percent sodium chloride. Sabouraud Liquid Medium was also included for comparison. Concentrated cultures were prepared from K. fragilis cells from each medium and frozen at -19°C and -196°C. Viability was determined by plate counts, both before freezing and after three days of frozen storage. The media and percentages of survival from two trials are shown in Table VII. For the cultures frozen at -19°C, survival levels from all media appeared to be fairly comparable. The pepsinized whey and the pepsinized whey plus sodium chloride yielded slightly higher survival levels than the other three media. The cells frozen at -196°C

TABLE VII

EFFECT OF GROWTH MEDIA AND STORAGE TEMPERATURE ON % SURVIVAL OF KLUYVEROMYCES FRAGILIS Y-1156

Growth Medium	-19°C			-196°C		
	Trial 1	Trial 2	AVG	Trial 1	Trial 2	AVG
Pepsinized Whey (PW)	63	75	69	12	9	11
PW + 0.1 % Tween 80	57	59	58	6	3	5
PW + 1mM ETHANOLAMINE	57	56	57	11	10	11
PW + 0.5% NaCl	48	80	64	12	12	12
Sabouraud Liquid Medium	47	68	58	18	12	15

did not survive as well as those at -19°C for any growth medium. The addition of Tween 80 to the growth medium seemed to have a detrimental effect on the survival of the yeast in liquid nitrogen. The Sabouraud Liquid Medium yielded cells with a slightly higher percentage of survival for those cultures frozen at -196°C than for those from the other media.

Effect of Procedure Used for Resuspension of Yeast Cells

Because of some inconsistencies in survival levels from one week to the next within a particular storage study, an experiment was done to determine if the yeasts were being uniformly resuspended after centrifugation. Samples from five vials of single concentrated cultures, resuspended with and without the aid of glass beads, were plated immediately after preparation. Results from analyses of these samples appear in Table VIII. The counts of those cultures resuspended with the glass beads appear to be most uniform in both trials. There was more diversity among the cultures resuspended without the aid of glass beads.

Statistical Analyses

Comparison of Glucose Concentrations

To test for any significant differences among the percentages of survivals of *K. fragilis* in concentrated cultures containing various glucose concentrations, the data for cultures frozen at -19°C for 21 days was subjected to statistical analyses. A summary table of results obtained from seven trials is shown in Table XX of the Appendix. The

TABLE VIII

EFFECT OF RESUSPENDING TECHNIQUE ON UNIFORMITY OF COUNTS OF KLUYVEROMYCES FRAGILIS Y-1156

TRIAL	SAMPLE	Count/G	
		BEADS	WITHOUT BEADS
I	1	4.8×10^9	4.6×10^9
	2	4.6×10^9	3.1×10^9
	3	3.2×10^9	4.6×10^9
	4	4.5×10^9	3.6×10^9
	5	4.1×10^9	3.7×10^9
II	1	4.8×10^9	5.2×10^9
	2	4.7×10^9	5.4×10^9
	3	4.6×10^9	4.8×10^9
	4	4.8×10^9	4.7×10^9
	5	5.7×10^9	5.2×10^9

analysis of variance of these results appears in Table IX. These results indicate that effects of the glucose concentrations (treatments) were highly significant ($P < 0.005$), but that no significant variation occurred among trials for this experiment.

To compare the effects of individual glucose concentrations, the differences between mean percentages of survivors for the four glucose concentrations were also analyzed for significant differences using Duncan's new multiple-range test. A summary of these results is presented in Table X. The survival levels for all concentrated cultures containing added glucose were significantly higher ($P < 0.01$) than for the cultures containing zero percent added glucose. The survival in concentrated cultures containing 20 percent glucose was not significantly better ($P > 0.05$) than that observed in cultures containing 10 percent glucose, but the 30 percent glucose treatment was significantly better than 10 percent glucose ($P < 0.01$). The difference in survival between the samples containing 20 and 30 percent glucose was significant at the five percent level, but not at the one percent level.

Significance of Storage Time on Cell Survival

In order to determine at what point in the storage of the concentrated cultures (at -19°C) a significant drop in survival occurred, data for each glucose concentration was analyzed separately with percentages of survival being compared to day zero (100 percent). Table XX, in the Appendix, summarizes the data used in these analyses. Analysis of variance tests were done first for each glucose concentration to determine if significant variation occurred among days of storage and among replicate trials. These results appear in Tables XI-XIV. At all glucose

TABLE IX

TEST FOR SIGNIFICANT DIFFERENCES AMONG GLUCOSE CONCENTRATIONS AT -19°C

Source	df	SS	MS	F*
Total	27	15700.43	-	-
Treatments	3	11457.57	3819.19	23.21
Trials	6	1281.43	213.57	1.30
Error	18	2961.43	164.52	-

*F value required for significance of treatments = 6.03 at the 0.5% level

*F value required for significance of trials = 2.13 at the 10% level

TABLE X

SURVIVALS OF YEAST CULTURES SUSPENDED IN VARIOUS GLUCOSE CONCENTRATIONS AT -19°C

Glucose Concentrations	Mean Survivals(%)	Differences Between Means	LSR*	P .05	LSR	P .01
0%	20	10% - 0% = 32	19.74	+	14.40	+
10%	52	20% - 0% = 40	20.71	+	15.13	+
20%	60	30% - 0% = 55	21.24	+	15.57	+
30%	75	20% -10% = 12	19.74	-	14.40	-
		30% -10% = 23	20.71	+	15.13	+
		30% -20% = 15	19.74	+	14.40	-

*LSR = Least significant range = difference required for significance

TABLE XI

TEST FOR SIGNIFICANCE OF SAMPLE DAYS ON CELL SURVIVAL AT 0% GLUCOSE LEVEL

Source	df	SS	MS	F*
Total	34	35210.97	-	-
Days	4	31234.11	7808.53	68.60
Trials	6	1244.97	207.50	1.82
Error	24	2731.89	113.83	-

*F value required for significance of days = 4.89 at 0.5% level

*F value required for significance of trials = 2.04 at 10% level

TABLE XII

TEST FOR SIGNIFICANCE OF SAMPLE DAYS ON CELL SURVIVAL AT 10% GLUCOSE LEVEL

Source	df	SS	MS	F*
Total	34	13690.74	-	-
Days	4	10841.60	2710.4	30.06
Trials	6	684.74	114.12	1.27
Error	24	2164.4	90.18	-

*F value required for significance of days = 4.89 at 0.5% level

*F value required for significance of trials = 2.04 at 10% level

TABLE XIII

TEST FOR SIGNIFICANCE OF SAMPLE DAYS ON CELL SURVIVAL AT 20% GLUCOSE LEVEL

Source	df	SS	MS	F*
Total	34	9741.54	-	-
Days	4	7305.54	1826.39	38.25
Trials	6	1289.94	214.99	4.50
Error	24	1146.06	47.75	-

*F value required for significance of days = 4.89 at 0.5% level

*F value required for significance of trials = 4.20 at 0.5% level

TABLE XIV

TEST FOR SIGNIFICANCE OF SAMPLE DAYS ON CELL SURVIVAL AT 30% GLUCOSE LEVEL

Source	df	SS	MS	F*
Total	34	5822.97	-	-
Days	4	2470.11	617.53	7.10
Trials	6	1266.17	211.03	2.43
Error	24	2086.69	86.95	-

*F value required for significance of days = 4.89 at 0.5% level

*F value required for significance of trials = 2.04 at 10% level

concentrations, a significant difference occurred among storage days ($p < 0.005$). In comparing replicate trials of the same experiment, no significant differences resulted for the zero and 10 percent glucose ($P > 0.1$); however, there was a significant variation among trials for the 20 percent glucose treatment ($P < 0.005$) and for 30 percent glucose ($P < 0.1$).

In determining the point at which a significant drop occurred in cell survival over a three-week storage period for the cultures frozen in 0, 10, 20, and 30 percent glucose at -19°C, a least significant difference test was applied to the data for each glucose concentration. The results of this analysis appear in Table XV. For those cultures frozen in 0, 10, or 20 percent glucose, a significant drop in survival occurred after one day frozen storage ($P < 0.01$ for 0 and 20 percent glucose; $P < 0.05$ for 10 percent glucose). However, cultures frozen in 30 percent glucose did not show a significant drop in cell survival until they were stored for 14 days ($p < 0.01$). The additional glucose appears to add some protection to the yeast cells over extended storage periods.

Data obtained from freezing cultures at -196°C or from freezing them at -19°C followed by storage at -196°C were not evaluated statistically. The percentages of survival obtained from such treatments were too low to suggest the use of such methods for storing cultures of K. fragilis.

TABLE XV

SURVIVALS OF YEAST CULTURES STORED AT -19°C
FOR VARIOUS TREATMENT TIMES

Glucose Concentration	Days Stored	Mean Survivals %	Differences Between Means	Significance			
				LSD*	P .05	LSD	P .01
0%	0	100	- -	-	-	-	-
	1	82	0 - 1 = 18	11.77	+	15.95	+
	7	38	0 - 7 = 62	11.77	+	15.95	+
	14	40	0 -14 = 60	11.77	+	15.95	+
	21	20	0 -21 = 80	11.77	+	15.95	+
10%	0	100	-	-	-	-	-
	1	88	0 - 1 = 12	10.48	+	14.20	-
	7	65	0 - 7 = 35	10.48	+	14.20	+
	14	65	0 -14 = 35	10.48	+	14.20	+
	21	51	0 -21 = 49	10.48	+	14.20	+
20%	0	100	-	-	-	-	-
	1	86	0 - 1 = 14	7.62	+	10.33	+
	7	68	0 - 7 = 32	7.62	+	10.33	+
	14	69	0 -14 =31	7.62	+	10.33	+
	21	60	0 -21 = 40	7.62	+	10.33	+
30%	0	100	-	-	-	-	-
	1	91	0 - 1 = 9	10.29	+	13.94	-
	7	87	0 - 7 = 13	10.29	+	13.94	-
	14	82	0 -14 = 18	10.29	+	13.94	+
	21	75	0 -21 = 25	10.29	+	13.94	+

*LSD = least significant difference = difference required for significance

CHAPTER V

DISCUSSION

Whey disposal is a major concern of dairy processing plants. The waste load is sufficiently high so that dumping it in municipal sewer systems can overtax them. One possible alternative to dairy processors in treating cottage cheese whey is to culture it with the yeast Kluyveromyces fragilis. Such a fermentation has been reported to reduce the biochemical oxygen demand of the liquid through the utilization of the lactose by the yeast culture (Knight, et al., 1972). A high protein material can be recovered from this process which could be used as a valuable and nutritious food supplement. A convenient means of handling the yeast culture in the dairy plant, however, is needed for this process. Frozen concentrated cultures of K. fragilis could help eliminate the problems involved in a continuous transfer of the culture and save time, labor, and storage space.

In choosing a culture suitable for this process, it was desirable to find one which would grow well in fresh cottage cheese whey and utilize the lactose it contained fairly rapidly and completely. Nine strains of K. fragilis were evaluated. The abilities to grow in the whey and utilize the lactose varied considerably among the nine cultures. Those yeasts which grew best in the whey did not necessarily reduce the lactose content sufficiently during the test period. Thus it is important to be selective in choosing a strain of K. fragilis for culturing cheese whey. K. fragilis Y-1156 was chosen for further study in the

preparation of culture concentrates and for freezing trials because it grew well in the whey and utilized the lactose quite readily.

It was then necessary to find a growth medium for K. fragilis that would produce a high yield of cells which would function well when the resulting concentrated culture was inoculated into cottage cheese whey. It was assumed that a growth medium similar in composition to cottage cheese whey might be best suited for this purpose. A reconstituted dried, sweet whey was evaluated. It was digested with pepsin to make the nitrogen contained in the whey proteins more readily available to the yeasts.

A definite difference in cell survival was found in experiments where the physiological ages of the cultures were varied. Cells frozen at -19°C survived much better than those frozen at -196°C. However, there appeared to be an optimum age for maximum cell survival at each temperature. At -19°C, concentrated cultures prepared from cells in the exponential growth phase yielded higher survivals than those from the stationary phase. Cells frozen in liquid nitrogen exhibited the opposite behavior. Survival for these cultures peaked when the yeasts were harvested in the mid-stationary growth phase. Cells from the exponential phase and late stationary phase were more susceptible to freezing damage. While cells of K. fragilis Y-1156 harvested after eight hours of growth survived freezing at -19°C better than did cells harvested after 14 and 17 hours of growth, the difference in populations at 14 and 17 hours versus that at eight hours would more than compensate for the lower survivor level. This must be considered when evaluating the overall efficiency of producing the concentrated cultures.

Survival of the concentrated yeast cultures suspended in 10 percent NFMS did not appear to be sufficiently high for use in culturing cottage cheese whey, particularly for those stored at -196°C. The use of cryoprotective agents was then examined in hopes of increasing cell survival. Additives to the freezing menstrua which have been shown to decrease storage death for yeast cells include milk protein, sucrose, glucose, and lactose (Meryman, 1966). Glucose, in amounts of 0, 10, 20, and 30 percent, was added to the NFMS, and these suspending media tested for the possibility of increasing the survival of yeast cells. The addition of glucose appeared to have no beneficial effect on the survival of the concentrated cultures at -196°C. Those cells frozen at -19°C did benefit from the sugar added to the freezing menstruum. Little or no difference was noted after one day of frozen storage, but after extended storage periods of one to three weeks, it was found that cell survival increased significantly with additional glucose in the suspending medium.

In comparing the effect of the various glucose concentrations on the survival of K. fragilis Y-1156 cultures at -19°C, a significant difference was found among these treatment effects ($P < 0.005$). The addition of any amount of glucose to the suspending medium resulted in significant improvements in yeast survival ($P < 0.01$). A 30 percent glucose concentration was found to be significantly better than all other glucose levels ($P < 0.05$), especially for extended storage periods. For those cultures frozen in 0, 10, or 20 percent glucose, a significant drop in survival occurred after one day of frozen storage ($P < 0.05$). However, for those cultures frozen in 30 percent glucose, a significant drop in survival did not occur until the cultures were stored 14 days

($P < 0.01$). While freezing the concentrated cultures in 30 percent glucose at -19°C yields the highest survival levels, a storage period longer than 14 days is needed for this process to be practical for use by dairy processors. The decrease in survival for these cultures was significant at 14 days. Other methods need to be investigated to increase the storage period for the cultures.

The key to successfully preserving yeast cells in liquid nitrogen has still not been found. Mazur (1961a) found that when yeasts were frozen rapidly to temperatures below -30°C, less than one percent survived, but that as many as 50 percent survived when the cells were cooled slowly to this same temperature. Wellman and Stewart (1973) reported that a freezing rate of 1°C/minute resulted in the highest survivals of brewing yeasts stored in liquid nitrogen. In the present study, it was observed that the survivals of the concentrated cultures frozen at -19°C appeared to decrease gradually over the study period as a whole. However, damage to those cultures frozen in liquid nitrogen was evident within one day, and there was little or no additional loss of viability for the rest of the storage period. These results seemed to point to a possible advantage of freezing the yeast cells slowly at first, where they would undergo minimal damage, and then transferring them to liquid nitrogen for storage. Hopefully, at this stage, the cultures would receive no additional damage. A new experiment was conducted at this point. In addition to freezing trials at -19°C and -196°C with various concentrations of glucose in the freezing medium, a dual-temperature freezing treatment was tested. Concentrated cultures were frozen for 24 hours at -19°C and then transferred to liquid nitrogen for storage up to three weeks. Those yeasts undergoing

the dual-temperature treatment yielded survival levels better than the liquid nitrogen but not as high as those frozen and stored at -19°C. This method of freezing appeared to be superior to freezing and frozen storage in liquid nitrogen, but temperatures of -19°C still seemed optimal.

In determining the viability of frozen cultures during the three-week storage studies, some inconsistencies resulted which required some additional investigation. The survivals of some of the cultures fluctuated up and down within one study period. Two factors were considered as possible cause(s) of such occurrences--the thawing temperature, and the method used to resuspend the yeast cells in the freezing medium. The thawing temperature was considered first. Mazur and Schmidt (1968) have reported that rapid warming is least harmful to frozen yeast cells. In the present study, the frozen concentrated cultures were thawed in hot tap water (40-45°C). This would be similar to rapid thawing as described by Mazur and Schmidt (1968). The method of resuspending the yeast cells in the freezing menstrua following centrifugation may have been partially responsible for variation in cell numbers in the concentrated cultures. The use of glass beads to aid in suspending the cells appeared to result in more uniform cell suspensions. It is suggested that this be used in further experiments. The diversity of the counts for concentrated cultures prepared without the aid of glass beads could have been responsible for some of the inconsistencies observed in the storage experiments in this study.

Additional work is needed to find a satisfactory means of storing frozen concentrated cultures of K. fragilis. One area which should be further evaluated is the alteration of the growth medium to produce a

culture which may be more stable to freezing. The chemical composition of the growth medium can be used to alter the lipid composition of microbial cells. Smittle, et al. (1974) showed that supplementation of the growth medium for Lactobacillus bulgaricus with sodium oleate resulted in cells that survived freezing in liquid nitrogen. Cells grown without the oleate did not survive freezing. The improved survival was correlated to alterations in composition of the cellular lipids. It has been found that the freezing and thawing of yeast cells causes damage to the cell membrane (Souzu, 1973). He suggested that this was due to phospholipid degradation. Alterations of the lipid material in the cells may provide more stability during freezing. High saline concentrations in the growth medium have been found by Combs, et al. (1968) to increase the lipid content of various species of yeast. Hossack (1977) has found that the synthesis of phospholipids can be increased in the yeast cell when ethanolamine or choline is supplied to the growth medium. Sabouraud Liquid Medium was used by Tsuji (1966) in preparing concentrated yeast cultures for storage in liquid nitrogen. In the present study, several modifications of the growth media were used to grow cells of K. fragilis for freezing. Sodium chloride, ethanolamine, and Tween 80 were added to pepsinized whey. The Sabouraud Liquid Medium was also tested in this study. However, none of these appeared to have any beneficial effect on the culture with respect to stability during freezing. It is possible that other alterations in the growth medium besides those mentioned here may produce yeast cells more able to withstand freezing.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Nine strains of Kluyveromyces fragilis were compared for their ability to grow in cottage cheese whey and utilize lactose. K. fragilis Y-1156 was chosen for use in concentrated culture preparation and freezing storage studies. The yeast culture was grown in a five percent aqueous solution of dried, sweet whey, in which the protein had been digested with pepsin. Concentrated cultures were prepared by resuspending the yeast cells in 10 percent non-fat milk solids (NFMS) and freezing at -19°C and -196°C. Viability was determined before and after freezing with the plate count method.

Survival of the K. fragilis Y-1156 in concentrated cultures at both -19°C and -196°C was influenced by the physiological age of the culture. The concentrated cultures survived better at -19°C than at -196°C. Cells from the mid-exponential growth phase survived better at -19°C than those from the stationary phase. The opposite was observed for the yeasts frozen at -196°C. They exhibited highest levels of survival at the mid-stationary phase.

Glucose was added in various concentrations to the concentrated cultures of K. fragilis Y-1156 to determine its effect on cell survival after frozen storage. The glucose had no apparent beneficial effect on cell survival after a one day storage period at -19°C. However, over extended storage periods of 21 days, the number of yeast cells surviving increased significantly as the amount of glucose in the freezing menstrua

increased. The concentrated cultures frozen at -196°C again exhibited much lower survival than those frozen at -19°C. The addition of glucose to the suspending medium prior to freezing at -196°C provided no protection to the cells at any concentration.

The death of cells in concentrated yeast cultures frozen at -19°C occurred gradually over the total storage period, while damage to those cells frozen in liquid nitrogen resulted immediately. The viability of the cultures frozen at -196°C remained relatively constant throughout the entire storage study. A dual-freezing treatment, with the yeast cells frozen at -19°C and stored at -196°C, was tested for its effect on yeast cell survival. The concentrated cultures undergoing this treatment resulted in survivals intermediate between those stored at -19°C and those in liquid nitrogen. The addition of glucose to the suspending medium in this freezing and storage procedure also increased cell survival over the total storage period, with the highest glucose levels yielding the highest survival levels.

The means of suspending yeast cells for concentrate preparation should be handled with strict controls to insure that the cells are uniformly dispersed in the culture concentrates. The use of glass beads in suspending yeast cells in the freezing menstrua did not appear to damage the cells and helped increase the consistency of population among all the concentrated cultures that were prepared.

The ability of the concentrated cultures to grow in fresh cottage cheese whey after frozen storage is a good indication of the suitability of such storage conditions for these yeast cultures. Those cultures frozen at -19°C for 21 days varied in their ability to grow in the fresh

whey. Those frozen in the highest concentration of glucose appeared to grow best.

The culture concentrates were stored at -19°C in a 10 percent NFMS-30 percent glucose medium for up to 14 days without a significant loss in cell viability. Studies need to be conducted to extend the length of this storage period to one more feasible for use by dairy processors. Adequate cell survival of yeast cultures frozen at -196°C was not attained. Additional study in this area is still needed.

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APPENDIX

TABLE XVI

EFFECT OF GLUCOSE CONCENTRATION AND STORAGE TIME ON THE GROWTH OF KLUYVEROMYCES FRAGILIS Y-1156 AFTER FREEZING AT -19°C

Glucose Concentration	Days Storage	COUNT/G				
		1	2	3	4	AVG.
0%	0	1.1×10^9	1.3×10^9	1.2×10^9	1.3×10^9	1.2×10^9
	1	9.9×10^8	1.1×10^9	1.0×10^9	9.2×10^8	1.0×10^9
	7	2.1×10^8	6.2×10^8	4.3×10^8	3.0×10^8	3.9×10^8
	14	5.4×10^8	5.1×10^8	4.9×10^8	4.8×10^8	5.1×10^8
	21	5.4×10^7	4.1×10^8	5.1×10^8	1.4×10^8	2.8×10^8
10%	0	1.0×10^9	1.2×10^9	1.2×10^9	1.2×10^9	1.2×10^9
	1	7.4×10^8	1.1×10^9	8.3×10^8	9.7×10^8	9.1×10^9
	7	6.0×10^8	8.1×10^8	7.5×10^8	7.1×10^8	7.2×10^8
	14	7.9×10^8	7.6×10^8	6.9×10^8	6.7×10^8	7.3×10^8
	21	3.7×10^8	6.6×10^8	6.3×10^8	5.8×10^8	5.6×10^8
20%	0	9.5×10^8	1.0×10^9	1.2×10^9	1.0×10^9	1.0×10^9
	1	7.5×10^8	1.0×10^9	9.3×10^8	9.3×10^8	9.0×10^8
	7	6.3×10^8	8.5×10^8	8.3×10^8	6.0×10^8	7.3×10^8
	14	7.6×10^8	8.5×10^8	6.7×10^8	7.3×10^8	7.5×10^8
	21	5.1×10^8	8.1×10^8	6.1×10^8	6.8×10^8	6.5×10^8
30%	0	8.3×10^8	8.9×10^8	1.1×10^9	7.9×10^8	9.0×10^8
	1	7.0×10^8	7.4×10^8	9.1×10^8	8.8×10^8	8.1×10^8
	7	5.7×10^8	8.6×10^8	7.0×10^8	6.2×10^8	6.9×10^8
	14	6.8×10^8	7.4×10^8	7.9×10^8	7.1×10^8	7.3×10^8
	21	6.9×10^8	7.0×10^8	5.8×10^8	6.7×10^8	6.6×10^8

TABLE XVII

EFFECT OF GLUCOSE CONCENTRATION AND STORAGE TIME ON THE GROWTH OF KLUYVEROMYCES FRAGILIS Y-1156 AFTER FREEZING AT -196°C

Glucose Concentration	Days Storage	COUNT/G				
		1	2	3	4	AVG
0%	0	1.1×10^9	1.3×10^9	1.2×10^9	1.3×10^9	1.2×10^9
	1	8.3×10^7	1.5×10^8	8.4×10^7	8.0×10^7	9.9×10^7
	7	5.3×10^7	1.5×10^8	8.2×10^7	4.7×10^7	8.3×10^7
	14	6.1×10^7	1.3×10^8	8.0×10^7	7.1×10^7	8.6×10^7
	21	1.4×10^7	1.3×10^8	6.3×10^7	7.7×10^7	7.1×10^7
10%	0	1.0×10^9	1.2×10^9	1.2×10^9	1.2×10^9	1.2×10^9
	1	3.9×10^7	5.1×10^7	2.0×10^7	2.1×10^7	3.3×10^7
	7	4.6×10^7	6.7×10^7	3.0×10^7	1.3×10^7	3.9×10^7
	14	4.9×10^7	5.2×10^7	2.6×10^7	1.9×10^7	3.7×10^7
	21	2.5×10^7	5.3×10^7	3.8×10^7	2.2×10^7	3.5×10^7
20%	0	9.5×10^8	1.0×10^9	1.2×10^9	1.0×10^9	1.0×10^9
	1	7.6×10^7	6.1×10^7	6.4×10^7	4.9×10^7	6.3×10^7
	7	9.1×10^7	5.8×10^7	7.4×10^7	4.1×10^7	6.6×10^7
	14	8.3×10^7	4.3×10^7	7.4×10^7	5.2×10^7	6.3×10^7
	21	8.0×10^7	6.0×10^7	9.1×10^7	4.6×10^7	6.9×10^7
30%	0	8.3×10^8	8.9×10^8	1.1×10^9	7.9×10^8	9.0×10^8
	1	-	6.6×10^7	7.4×10^7	7.7×10^7	7.2×10^7
	7	-	6.7×10^7	6.6×10^7	5.0×10^7	6.1×10^7
	14	-	4.7×10^7	6.6×10^7	6.5×10^7	5.9×10^7
	21	-	8.9×10^7	1.1×10^8	1.0×10^7	6.9×10^7

TABLE XVIII

EFFECT OF FREEZING TREATMENT, GLUCOSE CONCENTRATION, AND STORAGE TIME ON THE GROWTH OF KLUYVEROMYCES FRAGILIS Y-1156

Glucose Concentration	Days Storage	-19°C		-196°C		-19°C/-196°C	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
0%	0	1.2×10^9	8.8×10^8	1.2×10^9	8.8×10^8	1.2×10^9	8.8×10^8
	1	8.8×10^8	6.9×10^8	2.8×10^8	1.1×10^8	-	-
	7	6.7×10^8	2.7×10^8	1.5×10^8	1.3×10^8	3.2×10^8	1.4×10^8
	14	6.0×10^8	1.9×10^8	2.6×10^8	1.3×10^8	5.0×10^8	1.7×10^8
	21	4.6×10^8	5.5×10^7	2.3×10^8	1.6×10^8	3.2×10^8	7.8×10^7
10%	0	1.1×10^9	7.7×10^8	1.1×10^9	$7.7.10^8$	1.1×10^9	7.7×10^8
	1	1.1×10^9	9.2×10^8	2.4×10^8	4.6×10^7	-	-
	7	5.7×10^8	5.9×10^8	1.9×10^8	4.1×10^7	5.0×10^8	1.4×10^8
	14	7.6×10^8	4.7×10^8	2.2×10^8	5.1×10^7	6.6×10^8	1.9×10^8
	21	8.6×10^8	3.6×10^8	2.1×10^8	5.7×10^7	4.9×10^8	7.8×10^7
20%	0	1.2×10^9	9.2×10^8	1.2×10^9	9.2×10^8	1.2×10^9	9.2×10^8
	1	9.5×10^8	7.6×10^8	3.7×10^8	1.5×10^7	-	-
	7	7.0×10^8	6.8×10^8	3.9×10^8	1.1×10^7	2.2×10^8	7.4×10^7
	14	7.8×10^8	6.4×10^8	2.8×10^8	1.5×10^7	4.9×10^8	1.3×10^8
	21	7.1×10^8	$5.0.10^8$	2.8×10^8	2.0×10^7	6.4×10^8	6.6×10^7
30%	0	9.4×10^8	7.0×10^8	9.4×10^8	7.0×10^8	9.4×10^8	7.0×10^8
	1	1.0×10^9	7.7×10^8	1.8×10^8	3.1×10^6	-	-
	7	9.6×10^8	7.1×10^8	2.2×10^8	3.1×10^6	3.7×10^8	7.9×10^7
	14	9.0×10^8	5.8×10^8	1.8×10^8	2.8×10^6	4.4×10^8	9.9×10^7
	21	6.5×10^8	5.6×10^8	1.9×10^8	4.0×10^6	8.3×10^8	5.9×10^7

TABLE XIX

EFFECT OF GROWTH MEDIA AND STORAGE TEMPERATURE ON GROWTH OF KLUYVEROMYCES FRAGILIS Y-1156

Growth Medium	Days Storage	-19°C			-196°C		
		Trial 1	Trial 2	AVG	Trial 1	Trial 2	AVG
Pepsinized Whey (PW)	0	1.9×10^9	2.4×10^9	2.2×10^9	1.9×10^9	2.4×10^9	2.2×10^9
	3	1.2×10^9	1.8×10^9	1.5×10^9	2.2×10^8	2.2×10^8	2.2×10^8
PW + 0.1% TWEEN 80	0	2.1×10^9	2.7×10^9	2.4×10^9	2.1×10^9	2.7×10^9	2.4×10^9
	3	1.2×10^9	1.6×10^9	1.4×10^9	1.2×10^8	8.0×10^7	1.0×10^8
PW + ImM ethanolamine	0	2.1×10^9	2.5×10^9	2.3×10^9	2.1×10^9	2.5×10^9	2.3×10^9
	3	1.2×10^9	1.4×10^9	1.3×10^9	2.3×10^8	2.6×10^8	2.5×10^8
PW + 0.5% NaCl	0	2.0×10^9	2.0×10^9	2.0×10^9	2.0×10^9	2.0×10^9	2.0×10^9
	3	9.6×10^8	1.6×10^9	1.3×10^9	2.3×10^8	2.4×10^8	2.4×10^8
Sabouraud Liquid Medium	0	1.5×10^9	9.8×10^8	1.2×10^9	1.5×10^9	9.8×10^8	1.2×10^9
	3	7.1×10^8	6.7×10^8	6.9×10^8	2.8×10^8	1.2×10^8	2.0×10^8

TABLE XX

EFFECT OF GLUCOSE CONCENTRATION AND STORAGE TIME ON THE SURVIVAL OF KLUYVEROMYCES FRAGILIS Y-1156 AT -19°C

Glucose Concentration	Days Storage	% Survival						
		1	2	3	4	5	6	7
0%	1	90	85	83	71	91	73	78
	7	19	48	36	23	54	56	31
	14	49	39	41	37	44	50	22
	21	5	32	43	11	4	38	6
10%	1	74	92	69	81	100	100	100
	7	60	68	63	59	72	57	77
	14	79	63	58	56	71	69	61
	21	37	55	53	48	43	78	47
20%	1	79	100	78	93	91	79	83
	7	66	85	69	60	66	58	74
	14	80	85	56	73	55	65	70
	21	54	81	51	68	52	59	54
30%	1	84	83	83	100	88	100	100
	7	69	97	64	78	100	100	100
	14	82	83	72	90	65	96	83
	21	83	79	53	85	78	69	80

VITA[2]

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