DEVELOPMENT OF ACID LACTASE MILK

by

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INTRODUCTION

Milk is often referred to as nature's nearly most perfect food because it is very nutritive. It contains a high quality protein (casein) and serves as our major source of calcium. The importance of including milk in our daily diet cannot be over-emphasized.

Although milk is consumed in large quantities in the United States and elsewhere, a majority (about 70%) of the world's population is unable to consume milk without experiencing some discomfort. This is because they cannot digest the lactose in milk. They are hence said to be lactose-intolerant. For a long time, food scientists have endeavored to solve this problem. The result of their efforts has been the appearance of low lactose milk products on the market. This product is obtained by the addition of neutral lactase to the milk before it is packaged. In this process, the lactose in the milk is hydrolyzed by the added enzyme to glucose and galactose resulting in increased sweetness. However, many consumers do not like this increased sweetness and consider the product to be artificial.

Thus, there is an urgent need to seek methods of reducing the increased sweetness accompanying low lactose milk, while it still remains "safe" for the lactose-intolerant consumer to drink. This is presently the challenge to food scientists.

Therefore, the objectives of this study were:

1) To study acid lactase enzymes from various sources for their stability and activity in the neutral and acidic conditions of milk;
2) To select and encapsulate one of these enzymes; and
3) To develop with the encapsulated enzyme, a market milk that is acceptable to the lactose intolerant consumer.

Fresh pasteurized milk was obtained from the dairy plant at Kansas State University (Manhattan, Ks), and used as the substrate for developing acid lactase milk. Lactase enzyme from Aspergillus oryzae was selected, encapsulated and added to the milk at a concentration of 0.2 g lactase per 100 ml milk. This milk was refrigerated for 10 days, accompanied by analysis of % lactose hydrolysis at specified intervals. Tests were made in duplicates.

LITERATURE REVIEW

I. Milk

A. Definitions

For a long time, there was no clear-cut definition for milk due to its varied sources. However, because of increasing adulteration of milk, it became necessary to define and set legal standards for milk. Hence, in 1962, FAO/WHO established that the term "milk" shall mean exclusively the normal mammary secretion obtained from one or more milkings without either addition thereto or extraction therefrom. Bovine milk is defined in the Milk Ordinance and Code recommended by the United States Public Health Service as: "the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, which contains not less than 8.25% of milk-solids-not-fat and not less than 3.25% of milkfat" (Webb et al., 1974).

B. Composition

Although bovine milk is a liquid and often considered a drink, it contains an average of 13% solids. This amount is comparable to the solids content of many other foods and more appropriately should be regarded as a food. For example, lettuce and tomato have a solids content of only 5% and 6%, respectively. The solids of milk contains protein, carbohydrate, fat, minerals and vitamins. Altogether, approximately 250 chemical components have been identified in milk, including about 140 individual fatty acids (Campbell and Marshall, 1975). These constituents vary in amount in different milk products according to the procedure used in their preparation.

It is interesting to note that the milks of all species contain the same nutrients, differing only in proportions. The approximate composition of milk from some mammals is

given in Table 1.

Table 1. Milk composition for selected mammals[1]

	Milk composition, in %				
Mammal	Protein	Lactose	Fat	Ash	Total Solids
Woman	1.6	7.0	3.7	0.2	12.5
Mare	2.2	5.9	1.3	0.4	9.8
Cow	3.3	5.0	4.0	0.7	13.0
Goat	3.7	4.2	4.1	0.8	12.8
Sow	4.9	5.3	5.3	0.9	16.4
Dog	7.1	3.7	8.3	1.3	20.4

[1] Campbell and Marshall (1975)

C. The Merits of Milk

Milk is nature's nearly most perfect food. It is the first food nature provides for man. Although he must learn to ingest other foods, man in common with other mammals, is born a milk drinker. Milk has evolved through the corridors of time specifically for the nutrition and well-being of mammalian infants to bridge the gap between the dependent intrauterine and the independent adult life (Campbell and Marshall, 1975).

The nutritional merits of milk are indicated by the fact that daily consumption of a quart of cow's milk furnishes an average person approximately all the fat, calcium, phosphorus, and riboflavin; one-half the protein; one-third of the vitamin A, ascorbic acid and thiamine; one-fourth the calories; and with the exception of iron, copper, magnesium and manganese, all the minerals needed daily (as shown in Table 2). Milk also provides considerable amounts of nicotinic acid and choline. However, milk is not suitable as an exclusive diet beyond the normal weaning age (Fitzgerald, 1976).

It is interesting to note that life expectancy is observed to be highest in countries where liberal amounts of milk and milk products are consumed: Sweden, 74.2 yr; the Netherlands, 73.7 yr; Norway, 73.0 yr; Denmark, 73.0 yr; Canada, 72.0 yr (Statistical Yearbook, 1972). However, other factors such as environmental and nutritional conditions would also affect life expectancy.

In the 1960's, milk was considered to offer to consumers important food nutrients at low costs (Anonymous, 1973), although this may not be true today. It has a mild sweet taste, pleasing palatability and few children dislike its flavor. Milk is essentially 100% digestible, a desirable characteristic of all foods. Very few approach this degree of digestibility of milk. Additionally, there is no waste associated with milk. All of milk is edible in contrast with the inedible bones, hides, feathers, etc. of animals from which we obtain meat. This is also true for vegetables, fruits, grains, tubers and other foods which have seeds, peelings and stems (Campbell and Marshall, 1975) which must however be accredited for providing us with indigestable dietary fiber.

It is believed that milk has a tranquilizing effect that is primarily due to its high content in calcium. Milk is valuable in the control of peptic ulcers. A study in Japan revealed that the stomach cancer death rate percentage was 28.2 for those who drank no milk, 22.5 for those drinking milk occasionally and 13.0 for persons drinking milk daily (Hirayama, 1968).

It is obviously an unfair solution to suggest that lactose intolerant consumers should abstain from consuming milk and other dairy products, as some have recommended (Anderson, 1976; Bayless et al., 1971; Paige et al., 1971).

Table 2. Recommended daily dietary intake for a 70 kg (154 lbs) man, compared with the nutrients supplied by one quart of milk.[1]

Nutrient	Av. daily requirement	Amount in one quart of milk	Approx. portion of daily requirement in one quart of milk(%)
Protein (g)	56	34	61
Calories	2,700 (46 for 58 kg woman)	665	25
Calcium (g)	0.8	1.2	150
Phosphorus (g)	0.8	0.9	112
Iron (mg)	10.0	2.0	20
Vit. A (IU)	5,000	1,500*	30*
Vit. D (IU)	400	25*	60*
Ascorbic acid (mg)	45	15	33**
Thiamine (mg)	1.4	0.4	29
Nicotinic acid (mg)	18	0.8	4=
Riboflavin (mg)	1.6	1.5	94

* Milk is commonly enriched with 4,000 to 5,000 IU of Vit. A and 400 IU of Vit. D per quart.

** Quantity in raw milk; pasteurization destroys about one-half of this ascorbic acid.

= Milk provides lactose which enables microorganisms of the intestine to synthesize nicotinic acid.

1 National Dairy Council (1985)

II. **Lactose**

Lactose is the principal sugar in milk (Alm, 1982), however, its percentage in milk varies from one mammal to the other. As shown in Table 1, the highest concentration is in milk produced by woman (7.2%).

Chemically, lactose (4-O-β-galactopyranosyl-D-glucopyranose) is a disaccharide made up of two monosaccharides, glucose and galactose (Fig. 1). Upon hydrolysis, it yields glucose and galactose in equal moiety. Lactose exits in two isomeric forms, alpha and beta. The isomers differ in the configuration of the hydroxyl group on the number one carbon atom of the glucose residue (Nickerson, 1974; Zadow,1984). These isomers have

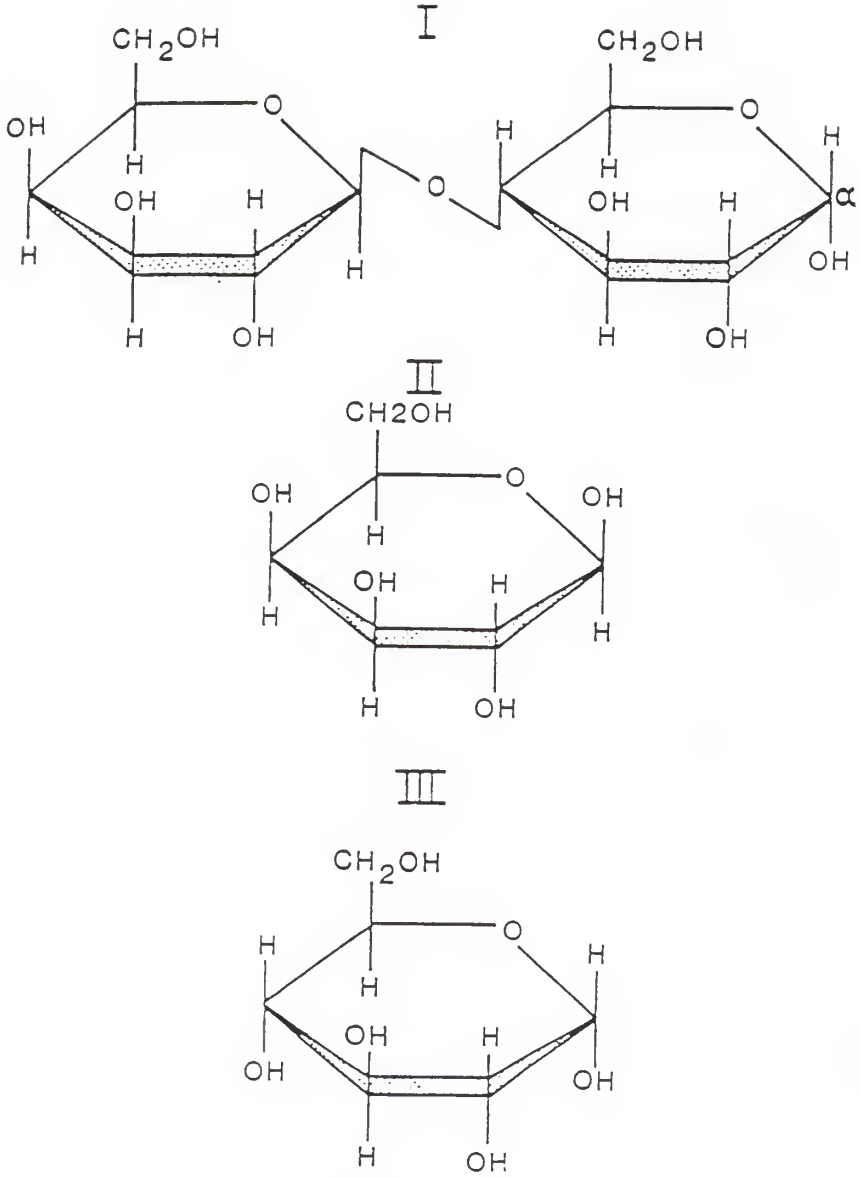


FIGURE 1 Structures of lactose (I), galactose (II), and glucose (III). The reducing residue is on the α-carbon atom of the glucose residue.

the same reducing power but show different solubility properties. The beta form is more soluble than the alpha form.

Man consumes lactose from milk and milk products only (Fitzgerald, 1976), but it has a multiple role in human nutrition. Banerjee (1972) identified three major roles for lactose:

i) it provides a significant amount of energy required by the body for proper functioning;

ii) it serves as source for the synthesis of galactoside of the brain and nerve tissues of the rapidly growing mammals;

iii) it functions for the better maintenance of intestinal conditions, as it is favorable for the production of certain types of bacteria, namely the Lactobacillus acidophilus.

A. Digestion of Lactose

Lactose is digested in the small intestine by the enzyme β-galactosidase (lactase) which is found in the microvilli of the epithelial cells lining the small intestines, principally the jejunum (which is the second of the three main segments of the small intestine). The glucose and galactose formed on hydrolysis are absorbed into the blood stream and are metabolized by the liver (Lehninger, 1982). If there is insufficient β-galactosidase to hydrolyze all of the lactose, it passes unaltered to the colon. This is called lactose malabsorption (Fitzgerald, 1976). The individual experiences some discomfort as a result of this, and is referred to as being lactose intolerant.

Except for protein, no nutrient is digested in the stomach due to the absence of other suitable enzymes. The gastric juice of the stomach has a pH range from 1.49 to 8.38, whereas that of the upper jejunum ranges from 5.07 to 7.07 (Altman, 1961). The pH

variation in the stomach is largely dependent upon its emptiness. Within the first thirty minutes of the start of a meal, the pH of the gastric contents rises rapidly from a pH of 2.0 or less, approaching the pH of the meal itself; but it soon begins to fall and within two hours or less, the pH of the contents of the stomach is again 2.0 or less (Weatherall et al., 1987).

B. Dietary significance of lactose

A high incidence of lactase deficiency has been observed among patients with osteoporosis (Birge et al., 1967). However, lactose is also recognized as having a positive effect on calcium absorption in humans (Ziegler and Forman, 1980). This effect of lactose is shared with other carbohydrates if they are present in the ileum with calcium. However, because of its low digestibility, lactose is the only carbohydrate that reaches the ileum intact. Here, it interacts with the brush border membrane, thereby increasing its permeability to calcium (Ambrecht and Wasserman, 1976).

C. Relative sweetness of sugars

Bouvy (1975) found the relative sweetness of some sugars in neutral water at a 10% concentration by weight to be: glucose - 75; galactose - 70; lactose - 40. Table 3 indicates the values for selected sugars. From this table, it is observed that alpha-D lactose has a relative sweetness in solution of 16-38%, while alpha-D glucose and alpha-D galactose have relative sweetness of 40-79% and 27%, respectively. This explains why milk tastes sweeter when β-galactosidase hydrolyzes its lactose to glucose and galactose. In unconcentrated milk or whey, the sweetness becomes pronounced when over 40 to 50% of the lactose is hydrolyzed. In fluid skim milk, 40% lactose hydrolysis gives a distinctively sweeter taste (Bouvy, 1975). Williams and Mcdonald (1982) found that hydrolyzing the lactose in milk to

30%, 60% and 90% has almost the same effect on sweetness as adding 0.3, 0.6, and 0.9% of sucrose, respectively, to the milk. In fact, when lactose is completely hydrolyzed, sweetness can be increased as much as four-fold (Anonymous, 1976). This increased sweetness is objectionable to a majority of consumers who may feel that the milk is too sweet or unnatural. Therefore, the hydrolysis of lactose in milk is certainly a solution for the lactose intolerance problem, but unfortunately creates a problem with consumer acceptability (Skala et al., 1971; Turner et al., 1976).

However, increased sweetness is advantageous in other fields of product development such as flavored milks which normally require extra sweetening, usually in the form of sucrose. The cost of sweetener addition is reduced, and the product is lower in calories (Fitzgerald, 1976).

Table 3. Relative sweetness (RS) of some sugars (w/w%).[1]

Sugar	Relative Sweetness (solution)	Relative Sweetness (crystalline)
β-D fructose	100 - 175	180
Sucrose*	100	100
α-D glucose	40 - 79	74
β-D glucose	< α-anomer	82
α-D galactose	27	32
β-D galactose	-	21
α-D lactose	16 - 38	16
β-D lactose	48	32

* Reference sugar, arbitrarily given a value of 100

1 Roy and James (1985).

D. Technical problems associated with lactose

One of the major problems associated with the use of lactose is the formation of large crystals. This is due to the fact that lactose has a very low solubility capacity compared to other sugars (Pazur, 1970; Shah and Nickerson, 1978). The solubility of some selected sugars is given in Table 4. The low solubility of lactose makes it quite unpopular in ice cream and other frozen desserts. The presence of lactose crystals in these products is considered a defect, often referred to as "sandiness". Because lactose is less sweet as compared to other sugars, it is uneconomical to use it as a sweetener.

Table 4. Solubility of some common saccharides.[1]

Sugar	Solubility (g/100g solution)		
	10°C	30°C	50°C
Sucrose	66	69	73
Lactose	13	20	30
D-galactose	28	36	47
D-glucose	40	54	70
D-fructose	--	82	87

[1] Shah and Nickerson, 1978.

III. Lactose Intolerance

A. Definition

Lactose intolerance is defined as a relatively flat blood sugar curve after the administration of relatively large amounts of lactose, 50 g in adults or about 50 g per meter

square of body surface in children (Anonymous, 1973). According to Houts (1988), 30 to 95 percent of the people who are lactose intolerant will experience some discomfort after drinking 8 ounces of milk containing 4.9% lactose

Terms often used in conjunction with lactose intolerance are:

1. Lactase deficiency is the inability for a normal person to produce lactase for lactose hydrolysis in the small intestine (Alpers, 1981; Newcomer and McGill, 1984). This could be at the primary or secondary level (Houts, 1988). Primary and secondary lactase deficiencies are described later.
2. Lactose malabsorption is the body's inability to metabolize some or all of the lactose as a result of the absence or deficiency of lactase (Simoons, 1980). Lactose intolerance is symptomatic lactose malabsorption which often causes the person to quit drinking milk and eating other dairy products.

B. Causes

In humans, lactose intolerance is seen in three situations:

1) This condition was first recognized as a congenital defect in infants in the late 1950s. It is referred to as congenital lactase deficiency. It is very rare, but is the most severe form of lactose intolerance. Virtually no lactase is present in the small intestine at all (Fitzgerald, 1976).
2) In children or adults, secondary lactase deficiency can be due to intestinal damage or diseases such as cystic fibrosis, ulcerative colitis, protein-calorie malnutrition, or following the ingestion of drugs after gastero-intestinal surgery. This is usually a temporary situation as normal tolerance levels return soon after the primary disease is relieved (Garza, 1981; Dahlqvist and Lindquist, 1971; NDC, 1985).

3) In the early 1960s, it was found that many healthy adults with no lesions of the intestinal mucosa and normal levels of other disaccharides were deficient in lactase. This is the most common reason for lactose intolerance throughout the world and is referred to as primary lactase deficiency or low lactase activity (Fitzgerald, 1976). In this case, the small intestine has nearly ceased production of lactase (Houts, 1988). This severe reduction of intestinal lactase is thought to be genetic in nature (Banerjee, 1972; McCracken, 1971), although others believe that it is also due to nutrition habits (Bolin et al., 1970).

C. Symptoms

Because of insufficient lactase in the small intestine to hydrolyse lactose, lactose passes unaltered to the colon where two processes ensue. The first of these is an osmotic effect due to the presence of lactose resulting in water being drawn from the surrounding tissues into the large intestine (Fitzgerald, 1976; Lehninger, 1982). The second is a biochemical effect. The bacteria in the colon ferment lactose to lactic acid as well as other organic acids and carbon dioxide, thereby lowering the pH to less than 6.0 (Fitzgerald, 1976).

Both processes combine to produce abdominal distention or bloating, cramping, gassiness and diarrhea (Anonymous, 1973; NewComer and McGill, 1984). These symptoms begin to be manifested as early as 30 minutes or as late as 12 hours after lactose ingestion (Fitzgerald, 1976). Some individuals who are diagnosed as malabsorbers develop these uncomfortable symptoms (Houts, 1988).

Lactose intolerance is very different from milk allergies. Allergies are usually associated with milk-protein hypersensitivity (Paige and Bayless, 1981). A milk allergy is the

body's response to an allergen-antibody reaction. An allergen is an ordinarily harmless substance present in the diet or environment which in sensitized persons is able to produce disorders such as asthma, vomiting, diarrhea and abdominal pain. Almost all proteins are antigenic and must be considered as potential allergens, although principal allergic activity among milk proteins has been ascribed to β-lactoglobulin and α-lactalbumin (Campbell and Marshall, 1975).

The statement that lactose malabsorption and intolerance are due to lactase deficiency is a fact, not a fad. However, Nnanyulego (1984) did not find any correlation between malabsorption and intolerance. This suggests that the symptoms usually associated with lactose intolerance may have other causes, and can affect absorbers as well as malabsorbers (Houts, 1988).

D. Diagnosis

Lactase deficiency is detected by administering a dose of lactose. For adults, this dose is 50 g which is equivalent to 1 liter milk, or 2 g/kg body weight for children. This is followed by one or more of the following tests:

1. Observation of clinical symptoms such as bloating, diarrhea, etc. (Banerjee, 1972).
2. A lactose intolerance test is done by measuring the increase of blood glucose level in response to the load. An increase of less than 20 mg/100 ml at 15 minutes after lactose ingestion is usually regarded as indicative of lactose deficiency (Fitzgerald, 1976). The person is said to have no increase in blood sugar levels (Banerjee, 1972).
3. A breath hydrogen test is done. Breath hydrogen level will be excessive if lactose is not hydrolyzed sufficiently (Anonymous, 1978; Flatz et al., 1984).

4. An intestinal biopsy is performed. A sample of mucosal tissue from the jejunum is obtained by a sampling device passed down the alimentary canal and the lactase activity of the tissue is measured (Fitzgerald, 1976; Norton and Rosenswing, 1969).

The last method is the most reliable for clinical purposes. However, since it is extremely traumatic for the subject, the second and third methods are preferred as a preliminary test (Fitzgerald, 1976). Clinical symptoms are variable and not really reliable.

E. Occurrence

The prevalence of lactose intolerance in human reportedly affects approximately 70% of the world's population (McCormick, 1976).

Lactose intolerance varies tremendously between ethnic groups as shown in Figure 2. It has been observed in 95% of African Bantus, more than 90% of Formosans, Phillipinos, Japanese, Thias and Columbian Indians, 90% in Chinese and 70% in African-Americans. About 40 to 60% of Mediterranean Europeans are intolerant (Fitzgerald, 1976).

The genetic theory, as explained by Harrison (1975), McCracken (1971), NewComer (1978) and Simoons (1980), is thought to offer the most potential for explaining the regional population distribution of lactose absorbers and malabsorbers.

Houts (1988) classified the world's population in three distinct groups: Those with a low percentage of lactose malabsorption (0 - 30%) includes people from north-west Europe, some pockets of the Mediterranean and Near East, Africa and Indian sub-continent. This is thought to be due to the fact that these people all share the longest

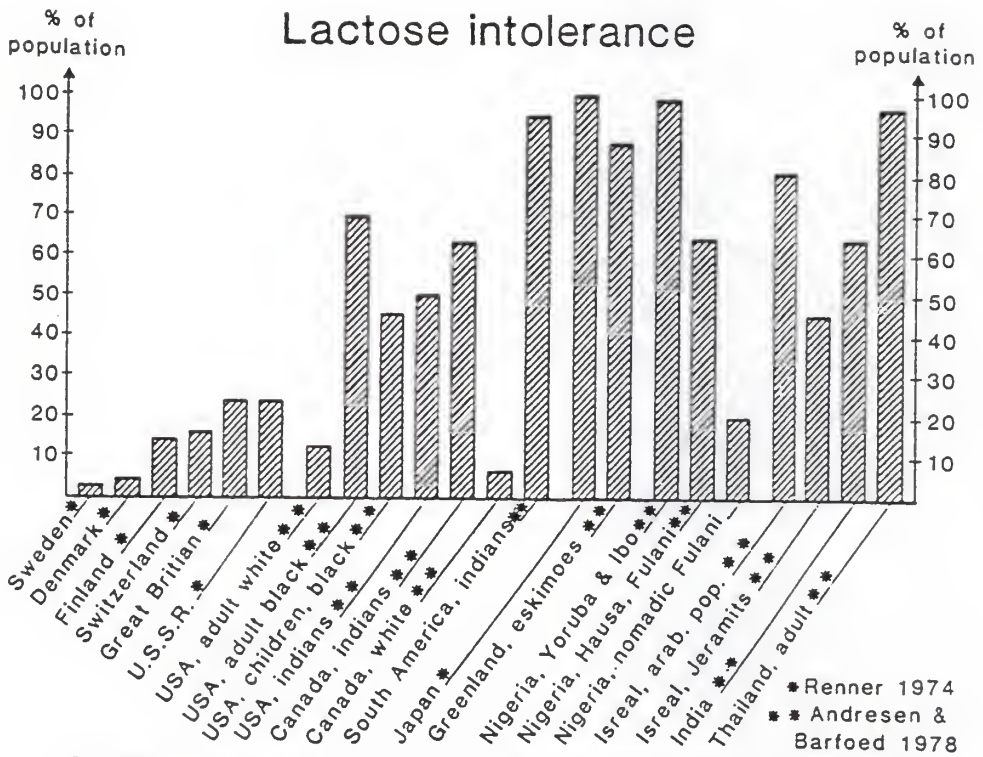


FIGURE 2. Frequency of lactose intolerance in percent of the population in some parts of the world. Adapted from Alm (1982).

known tradition of dairying. Those population that show very high proportions of intolerance (60 - 100%) include all American Indians and Eskimos, most Mediterranean and Near Eastern groups, all South-eastern and East Asians, The Pacific groups which have been studied - Fijians, New Guineans and Australian aborigines, and the remainder of African and Indian subcontinentsare also included in this group. These groups are found in geographic areas where dairying or milk has never, until perhaps recently been a part of their culture. Between these two extremes is a small group in the mid-range of lactose malabsorption (30 - 60%). These include some African-Americans, African-Arabs, Eskimo-Finnish people and Mexican-American among others (Simoons, 1980). These people originate from a mixture between the above mentioned extreme groups. It is also important to mention here that primary lactase deficiency is not prevalent in children who are four years old or younger, irrespective of their genetic and/or cultural background (Fitzgerald, 1976). Because a majority of adult humans have been shown to have low levels of lactase activity, it is probably a misnomer to term this a "deficiency" (Anonymous, 1976).

F. Nutritional Implications

The nutritional implications of lactose intolerance have a greater significance than just the inability to digest milk. The nutrients present in milk are not absorbed to the same degree by lactose intolerant subjects (McCormick, 1976). Thus, even if there are no signs of discomfort and gastro-intestinal reaction, nutrients supplied by certain foods might not be utilized effectively. The osmotic imbalance created by the higher dissolved solids level causes water to move into the intestine, increasing peristalsis and favoring the elimination of the food mass before it can be absorbed. There is also high acid production by the gut microflora attacking the lactose, which produces catharsis and tends to irritate the

gastrointestinal lining. The net effect is a waste of useful nutrients by the body (McCormick, 1976). Thus, asymptomatic lactose malabsorption conceivably can be a hidden source of malnourishment.

Of particular importance is the calcium in milk, since milk is its main food supplier to the body. Calcium deficiencies not only are now implicated in the occurrence of osteoporosis, but also may contribute to the occurrence of hypertension (Houts, 1988). All these implications underscore the importance of continued research in this area.

IV. Development of low lactose milk

Considerable research work has been done to solve the lactose problem in milk. Low lactose milk can be prepared entirely by the physical removal of the lactose by ultrafiltration or protein precipitation or by hydrolysis of lactose to its corresponding monosaccharides glucose and galactose.

Hydrolysis of lactose appears to be the most rational way of solving the lactose intolerance problem. Hydrolyzed lactose yields equal molar concentrations of glucose and galactose as discussed earlier. These saccharides are more functional than the unhydrolyzed lactose. The overall reaction for lactose hydrolysis is:

$$\underset{\text{(lactose)}}{C_{12}H_{22}O_{11}} + \underset{H_2O}{\xrightarrow{\beta\text{-galactosidase}}} C_6H_{12}O_6 + C_6H_{12}O_6$$

A. Enzymatic hydrolysis

The use of enzymes is the main method adopted today for lactose hydrolysis. The proposed mechanism for this reaction is presented in Figure 3 (Kilara and Shashani, 1979; Richmond et al., 1981; Shukla, 1975). The proposed groups involved in the catalytic mechanism are a sulfhydryl group acting as a general acid and an imidazole group providing

nucleophilic assistance for the breaking of the glycosidic linkage (Wallenfels and Weil, 1972). The sulfhydryl and imidazole groups of the enzyme attack lactose at the glycosidic linkage, resulting in the release of glucose as shown in Figure 3.

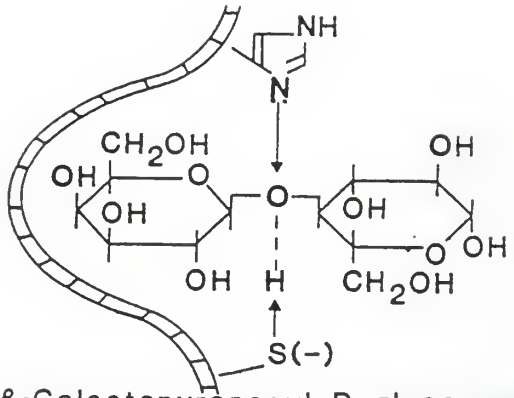
Enzyme activity is defined as one unit for the amount of enzyme causing the transformation of one micromole of substrate per minute at 25°C under optimal conditions of measurement. This varies considerably, depending on factors such as the source of the enzyme (animal, plant, microorganism, etc.), the kind of substrate, the ambient temperature, etc. (Lehninger, 1982).

The substrate used for determining lactase activity is o-nitrophenyl-β-D-galactopyranoside (ONPG). This compound is colorless, but opon reaction with the enzyme, it is split into two molecules, galactose and ortho-nitrophynol (ONP). ONP produces a yellow color whose intensity can be measured spectrophotometically (Dahlqvist et al., 1977; Mustranta et al., 1979). The overall reaction is:

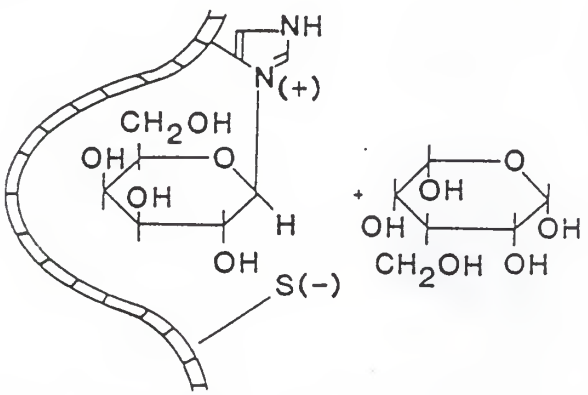
ONPG	**---------->**	**OPG**	**+**	**galactose**
(colorless)	**β-galactosidase**	**(yellow)**		

B. β-Galactosidase

Among the carbohydrolase of particular interest is β-galactosidase. It hydrolyzes the glycosidic bond of lactose between glucose and galactose (Kilara and Shashani, 1979). It is also referred to as β-D-galactoside galactohydrolase (Mahoney, 1985). Even though the term "lactase" is obsolete, many authors continue to use this nomenclature in preference to the scientific name (Richmond et al., 1981). β-Galactosidase induces the synthesis of certain oligosaccharides in a galactosyl transfer reaction (Richmond et al., 1981).



A 4-O-β-Galactopyranosyl-D-glucopyranose molecule at the active site of β-galactosidase enzyme



β-Galactosidase-galactose complex + glucose

FIGURE 3 Proposed mechanism of lactose hydrolysis by beta-galactosidases.

The proposed mechanism for the formation of oligosaccharides is given in Figure 4.

As discussed earlier in the hydrolysis reaction, β-galactosidase has one sulfhydryl (-SH) group and one imidazole group on its active site. It is involved in a reaction that corresponds to an S_N2-like displacement mechanism. The number of active sites in the enzyme depends on the temperature. Hence at low temperatures, not all the active sites are available (Shukla, 1975; Wallenfels and Weil, 1972). The enzyme is widely distributed in nature. It is found in plants, animal organs, yeasts, bacteria and fungi (Richmond et al., 1981; Shukla, 1975).

Only microbial enzymes can be considered for industrial use. Lactase producing bacteria include Escherichia coli, Streptococcus thermophilus, Bacillus stearothermophilus (Goodman and Pederson, 1976), Lactobacillus bulgaricus, and Lactobacillus helveticus (Pomeranz, 1964). Kluyveromyces fragilis, Kluyveromyces lactis and Candida kefir are the best known lactase producing yeasts. The most important molds that produce lactase belong to the genera Aspergillus, Neurospora and Mucor (Wierzbecki and Kosikowski, 1973). Aspergillus niger and Aspergillus oryzae are used for the commercial production of lactase. The lactase produced by yeasts is intracellular, but molds also produce extracellular lactase (Mustranta et al., 1979). For example, A. niger produces intracellular lactase, whereas A. oryzae enzyme is extracellular (Mahoney, 1985).

Lactases are similar in specificity to one another, but vary in physico-chemical, catalytic, and kinetic properties depending on the source of origin (Kilara and Shashani, 1979; Woychik and Wondolowski, 1973).

The pH optimum of yeasts and bacterial lactases is in the neutral area (pH 6.0 to 7.5). Lactic acid bacteria produce lactase with a somewhat lower pH optimum (pH 5.0 to

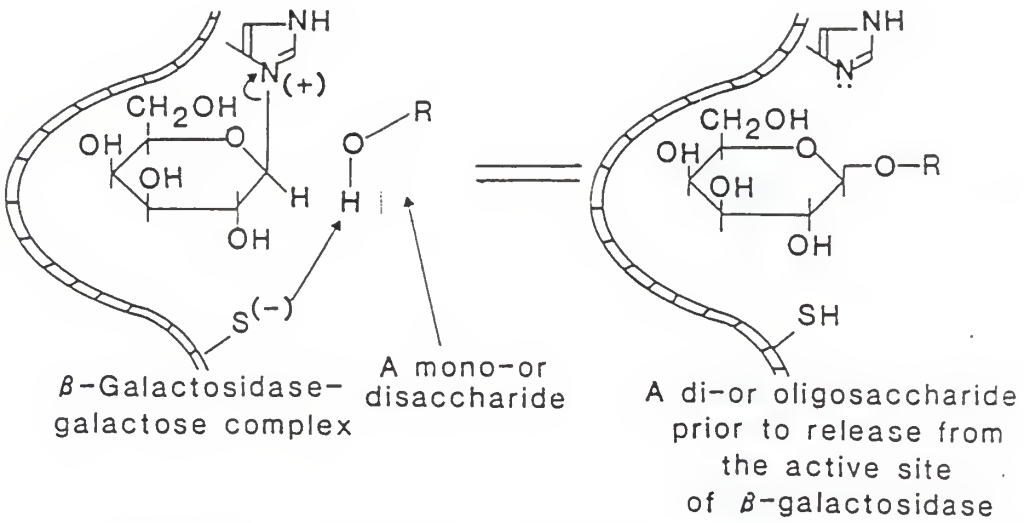


FIGURE 4 Proposed mechanism of galactosyl transfer reaction by beta-galactosidases.
Adapted from Shukla (1975)

6.5), while mold lactases have the lowest optima (pH 2.0 to 5.5). The optimum temperature for yeasts and bacterial lactases is reported to be between 30 to 50°C, while fungal lactases have an optimum action temperature of 40 to 65°C (Mustranta et al., 1979).

Olling (1972) found Streptococcus lactis to be the most promising source of lactase. Batch-wise incubation with this enzyme can almost completely hydrolyze the lactose in milk or whey. The optimum pH of this lactase coincides with that of milk. Below pH 5, lactase activity was completely and irreversibly lost. Its optimum temperature was found to be 35°C (Dahlqvist et al., 1977). In another study, Mustranta et al. (1979) found that the pH optimum of lactase produced by Aspergillus niger was 4.0 and the optimum temperature was 62°C. At a temperature of 45°C, the pH optimum was 3.0. Andrzej et al. (1985) studied lactase from Kluyveromyces lactis and reported that it was considerably more active than that from K. fragilis at lower temperatures. Brodsky and Grootwasink (1986) reported the optimum pH for a whole cell yeast lactase from K. fragilis to be 5.6 to 6.0. Mozaffer et al. (1984) reported the optimum pH of lactase from Bacillus circulans to be 4.5 while Chung et al. (1985) reported that lactase isolated from a strain of Aspergillus fumigtus to be at 5.5 to 6.0. Other factors that affect the rate of hydrolysis by this enzyme are time or duration of the reaction, and concentration of the substrate.

C. Formation of Oligosaccharides

As discussed earlier in the transfer reaction, β-galactosidase is a synthetase, catalyzing the formation of oligosaccharides. Researchers as early as the 1950's have reported the formation of oligosaccharides during lactose hydrolysis (Aronson, 1952; Roberts and McFarren, 1953). In this reaction, a sugar molecule becomes the acceptor of the β-D-galactoside moiety instead of water. This transfer principle, termed "transgalactosidation" is

responsible for small amounts of oligosaccharides present during and after lactose hydrolysis (Bouvy, 1975).

The number of oligosaccharides formed by transgalactosidation appeared to be five or six although three to eleven oligosaccharides were reported in earlier studies (Kwak and Jeon, 1988). The oligosaccharides reported were di-, tri-, tetra-, and pentasaccharides. Prenosil et al. (1987) reported that β-galactosidase from different microbial sources yielded different degrees of formation of oligosaccharides during hydrolysis of lactose in bovine milk systems. Weirzbicki and Kosikowski (1973) observed a 1 to 2 percent oligosaccharide formation when β-galactosidase from A. niger was used in acid whey that contained 4% lactose and a pH of 4.5. Burvall et al. (1979) reported that the maximum amount of oligosaccharides formed in a 5% lactose solution was about 5% of the total lactose content. However, a recent study by Jeon and Mantha (1985) indicated that maximum oligosaccharides formed in a 5% lactose solution were about 11.3% of the total lactose but decreased to 5.5% near completion of hydrolysis. Kwak and Jeon (1986) reported, however, that the amount of oligosaccharides formed in milk by the action of β-galactosidase were much smaller. They observed that the maximum concentration of oligosaccharides was 0.374 g/100 g milk which is equivalent to 6.5% for 4.9% lactose in milk, but was negligible after 7 days.

The higher the starting concentration of lactose, the more oligosaccharides are formed (Bouvy, 1975; Nickerson, 1974). Also, because a small amount of galactose is always transferred to another sugar molecule during hydrolysis, slightly more free glucose than galactose is present after the reaction (Bouvy, 1975; Dahqlvist et al., 1977). Kwak and Jeon (1986) found a higher level of enzyme produced a higher concentration of oligosaccharides.

In addition to substrate concentration, the amount of oligosaccharide formed is also affected by the source of the enzyme, pH and temperature (Shukla, 1975).

D. Methods for monitoring lactose in milk

Many options are available for the separation, detection and identification of oligosaccharides in milk or food systems. Some of the methods available include the Munson-Walker method that involves the formation of cuprous oxide, the Chloramine T method that involves back titration of liberated iodine, and the polarimetric method (AOAC, 1984; Kleyn, 1985; Zadow, 1984). There is also the enzymatic cryoscopic method (Zarb and Hourigan, 1979), and the high performance liquid chromatography method (Jeon et al., 1984).

The high performance liquid chromatography (HPLC) method is perhaps the most recent methodology developed for lactose analysis. It has been reported to be versatile, rapid, specific and accurate (Jeon et al., 1984; Tweenten and Euston, 1980). It permits both the qualitative and quantitative examination of mono- and oligosaccharides. Together with gas-liquid chromatography, these methods can distinguish very closely related compounds such as lactose, glucose and galactose, which is difficult to do with the traditional carbohydrate assay methods (Woollard, 1983).

According to Jeon and Kwak (1986), the success of this method heavily depends on sample preparation procedures. Several methods have been cited for the removal of the protein in milk. Euber and Brunner (1979) used trichloroacetic acid while West and Llorente (1981) used dilute perchloric acid, and still others used absolute ethanol (Warthesen and Kramer, 1979; Richmond et al., 1982). However, Kwak and Jeon (1986) reported that 2-propanol was a more effective than ethanol for precipitating milk proteins.

Buono (1988) found the 2-propanol method to be comparatively simple, more rapid, and more precise in eliminating proteins from soy milk or mixtures of soy and nonfat dry milk. Before sugar separation, it is necessary to remove other interfering compounds such as proteins with a simultaneous extraction of the sugar compounds into an aqueous phase. After mixing milk with 2-propanol as described by Kwak and Jeon, centrifugation is applied to obtain the sugars in the supernatant. The next step involves filtering the supernatant using Whatman paper, followed by elution through a C_{18} Sep-Pak cartridge. This serves to remove lipids, chromophores and residual proteins (Jeon et al., 1984). The eluant obtained is very clear and is ready for analysis.

Chromatography permits both a qualitative and quantitative examination of chemical components. It makes use of a heterogenous equilibrium established between a mobile phase and a stationary phase. The mobile phase can be a gas or a liquid, while the stationary phase can be a liquid or a solid. The four types of chromatographic techniques available are liquid-solid, liquid-liquid, gas-liquid, and gas-solid. The first technique is utilized in high performance liquid chromatography and is commonly called the high performance (or pressure) liquid chromatography (HPLC). The third technique is commonly known as the gas-liquid chromatography (GLC) or gas chromatography (GC). GLC is more complicated and time consuming than HPLC techniques in terms of sample preparation. The stationary phase for the HPLC uses a solid material for column packing which is either amine-based or resin-based.

Although a variety of non-polar solvents can be used as the mobile phase for HPLC analysis, Woolard (1983) recommended the use of a liquid mixture of acetonitrile and water for sugar separation with amine-based columns. High acetonitrile content results in longer

elution times, thus favoring resolution of the simpler pentoses and hexoses (Tweenton and Euston, 1980). The resin-based columns generally utilize water as the mobile phase and require operation at elevated column temperatures (60 to 80°C). In sugar separation, the amine-based matrix will elute carbohydrates in order of increasing molecular size, thereby allowing the rapid passage of small sugar molecules. Resin-based columns elute carbohydrates in order of decreasing molecular size (Richmond et al, 1982).

A detector is another critical component of the HPLC. The two common types used are the ultraviolet (UV) absorption detector and the refractive index (RI) detector. The RI is less sensitive than the UV detector, but is more applicable to carbohydrates than the UV because carbohydrates do not absorb strongly in the UV spectral region (except in the far UV region). Although carbohydrates absorb strongly in the far UV region, the use of a UV detector is discouraged due to possible spectral interferences with other compounds in the region. Therefore, unless carbohydrates are dissolved in pure water, the use of a UV detector should not be considered.

V. Applications of Hydrolysed Lactose

Hydrolysed lactose (HL) has numerous uses in food technology today. In the dairy industry, it is used for production of low-lactose fluid milk, acceleration of acid production to hasten the ripening of cheese and yoghurt, prevention of large crystals in ice cream and condensed milks, and the reduction of hygroscopicity in dehydrated dairy products. The modification of the functional properties of lactose such as increased solubility, increased sweetness, increased reducing power, higher osmotic pressure, lower viscosity, and greater fermentability has led to an increase in its range of uses in non-dairy products (Mahoney, 1985).

Some lactose-reduced products are already in the market. Lactose intolerant consumers can use freely or in limited quantities without discomfort. These products include fluid milk that has been modified in lactose concentration by the addition of lactase. Traditional fermented dairy products such as yogurt and cheeses can also be included.

A. Lactose-reduced Fluid Milk

A 75% conversion of the original lactose in milk to glucose and galactose is considered sufficient for a dairy product used by lactose intolerant consumers (Mosbach, 1976). The most common brand of hydrolyzed lactose fluid milk in the U.S. is Lactaid, produced by Lactaid Inc.(Pleasantville, N.J.). In this product, 70% of lactose is claimed to have been hydrolysed. This company has secured the exclusive rights to lactose hydrolysis of fluid milk from Enzyme Development Corporation (Keyport, N.J.) using the enzyme produced by Gist-Brocades NV of the Netherlands. Other brand names in the market are Lactolo, Sugarlo, etc. Lactase is also being marketed for home use in various forms such as lactaid in tablets and in liquid form. Lactase capsules are marketed by Witt Rover, Inc. (Washington, PA), while lactozyme capsules are marketed by Schiff Inc.(Monachie, N.J.). (Bouvy, 1975).

In Holland, a dairy product called Lactalac is sold by druggists and healthfood stores. It is a dried milk powder in which over 99% of the lactose has been hydrolysed by lactase treatment (Bouvy, 1975). A similar product called Kerulac is produced by Gist-Brocades NV and is sold in the Netherlands (Mahoney, 1985). In Italy, a product named Zymil is sold in pharmacies. It is a sterilized fluid milk with 80% of the lactose hydrolysed (Bouvy, 1975).

All these products taste much sweeter than regular milk. This is regarded by some consumers as a defect, while others do not even consider it to be real milk. Thus, the milk industry encounters difficulties convincing the lactose intolerant consumers, especially adults to accept their product as real milk (Skala et al., 1971; Turner et al., 1976). An alternate approach to this problem is to produce a similarly-treated milk which does not taste sweeter than untreated milk. This is the main objective of our study.

B. Other lactose-reduced dairy products

Successful attempts have been made to replace milk-solids-not-fat by using hydrolysed lactose in ice cream and other frozen deserts (Patel and Harper, 1977). Hydrolysed lactose is said to be a more functional ingredient that provides the ice cream manufacturer with a more economical choice. The resulting sugars, glucose and galactose are sweeter than lactose. Therefore, it is more likely that less sugar than normal will be needed in the ice cream mix (Rothwell, 1979).

Lactaid Inc. also markets cottage cheese and American cheese slices manufactured from lactase-treated milk (Bouvy, 1975). Lactose may be converted to lactobionic acid by lactose dehydrogenase and used in the acidification of cheeses, yoghurt and other fermented foods (Mahoney, 1985).

C. Lactose-reduced whey products

Whey contains small amounts of high quality protein nutritionally equivalent to that of egg, but fluid whey is essentially a crude solution of lactose. Whey from the manufacture of American cheese contains approximately 4.9% lactose, 0.9% protein, 0.6% ash, 0.3% fat and 0.2% lactic acid (Campbell and Marshall, 1975). Whey disposal has been a major concern of dairy industries for many years. Whey modification by lactase treatment offers

considerable potential in this regard. Pomeranz et al. (1962) showed that hydrolysed lactose makes acceptable bread when used even in the absence of other bakery sugars. The addition of hydrolyzed lactose to formulations under optimum conditions were found to have improved the aroma, appearance, flavor, volume and shelf life of baked goods (Holmes and Lopez, 1977). Hydrolysed lactose from whey can be used as a sweetener in the manufacture of beverages, ice cream, a variety of baked products, and other non-dairy products. For example, a shelf-stable athletic-type drink was made from direct-acid-set cottage cheese whey after the hydrolysis of lactose by β-galactosidase (Crippen and Jeon, 1984), and a white pan bread was made from hydrolyzed whey permeate syrups (Ogunrinola et al., 1988).

VI. Microencapsulation

The concept of microencapsulation began with the creation of a living cell. Most unicellular organisms are living wonders of microencapsulation. Even a chicken egg can be regarded as a form of encapsulation. Among the most important functions are protection of the interior or core matereial, and control of the flow of materials or permeation across the membrane (Vandegaer, 1974).

The earliest attempt to copy nature was by Barnett Green of Ohio in the 1930's. He was reported to have prepared the first gelatin microcapsules (Vandegaer, 1974). Today, microencapsulation is a constantly developing and rapidly expanding technology. It is pioneered by the National Cash Registrar Company (Dayton, Ohio), and is receiving considerable attention both industrially and academically (Bakan, 1973). The importance of microencapsulation techniques is well documented. This is reflected by the increasing number of articles in scientific literature and the increasing number of patents granted on

microencapsulation. Definitive literature in these areas is, however, still somewhat limited and will probably remain so for sometimes due to security measures on the part of those companies holding patent rights (Salib, 1977).

Microcapsules are composed of a polymeric skin or wall enclosing a core. The capsule wall should be inert to the substance it contains, be strong enough to permit normal handling without rupture, and be relatively thin so as to permit a high core to wall ratio. The contents of the capsule are contained within the shell until they are released by means that serve to break, crush, melt, dissolve or rupture the capsule wall, or until the internal phase is altered so it has the ability to diffuse through the capsule wall (Gutcho, 1979).

A. The core material

The core material is the specific substance to be coated. It can be liquid or solid of varying composition. The liquid core may be dispersed or dissolved. The solid core can be a mixture of active constituents, stabilizers, diluents, and release rate retardants or accelerators. Examples include vitamin A palmitate solution, aspirin, and meprobamate.

B. The coating material

The coating material is the substance that provides the coating for the creation of a wall. It must be capable of forming a film that is cohesive, chemically compatible and non-reactive with the core material. It should also provide the desired coating properties such as strength, flexibility, impermeability, optical properties and stability. The coating material may be subjected to some modification or chemical alteration such as cross linking. Therefore, the proper selection of coating material dictates to a major extent the resultant physical and chemical properties of the microcapsules. Examples of coating materials include water soluble polymers such as gelatin and gum arabic, water insoluble polymers such as

ethyl cellulose and cellulose acetate, waxes and lipids such as paraffin, beeswax, stearic, palmitic, myristic and lauric acids, lauryl, stearyl and myristyl alcohol glyceryl stearate, and enteric resins such as shellac, succinate, and cellulose acetate butyrate (Salib, 1977).

C. Microencapsulation Methods

A number of microencapsulation processes have been described in literature. The following are a summary of the examples (Salib, 1977):

1. Air suspension is a technique whereby the coating material is applied to the suspended particles as an atomized coating solution. The supporting air stream can be heated causing the evaporation of the volatile coating solvent, and depositing a thin layer of coating on the suspended core material. However, this method is useful for coating solids only.
2. Electrostatic deposition involves discharging a mist of the liquid coating material into a chamber, given an electrical charge, then deposited by electrostatic attraction upon the core material to be coated.
3. Multiorifice centrifugal process uses centrifugal forces to hurl a core material particle through an enveloping microencapsulation membrane, thereby effecting mechanical microencapsulation.
4. Spray drying and spray congealing both involve dissolving a core coating mixture into some environmental condition, whereby relatively rapid solidification of the coating is effected.
5. Vacuum deposition involves the vaporization of the coating material in a vacuum chamber, and its subsequent condensation onto a solid core material. Coating materials may be inorganic such as Al, Mg, Zn, etc.; or organic such

as waxes, paraffins, etc.

6. Polymerization techniques involve the dissolution of a monomer in the liquid to be microencapsulated, and dispersing the monomer into an immiscible continuous phase. Interfacial polymerization is then induced by activation.

7. Pan coating is carried out by applying the coating as a solution or as an atomized spray to the desired core material in the coating pan. Warm air is passed over the coating material as the coating is applied in the pan to remove the coating solvent.

8. Coacervation phase separation is the most widely and commonly used of the microencapsulation techniques. Coacervation is divided into simple and complex coacervation. Simple coacervation deals with systems containing only one colloidal solute, while complex coacervation deals with systems containing more than one colloid.

   a) Simple coacervation

      This process involves the addition of a strongly hydrophilic substance to a solution of a colloid. This added substance causes two phases to be formed. One phase is rich in colloidal droplets, and the other poor in such droplets. This process primarily depends on the degree of hydration produced. The addition of alcohol or sodium sulphate as a typical hydrophilic substance to an aqueous solution of gelatin could lead to phase formation. When suitable conditions including the presence of suitable nuclei are prevalent, microcapsules are formed.

   b) Complex coacervation

This depends primarily on pH. It has been reported that in gum arabic-gelatin systems, complex coacervation occurred and microcapsules formed at pH values below the isoelectric point (IEP) of gelatin, but would not occur above this pH. At pH values below the IEP of gelatin, it becomes positively charged, while acacia particles retained their negative charges regardless of pH. The same was found to be true of other systems containing two dispersed colloids, one of which was ampholytic.

Owing to the fact that core materials are microencapsulated while being dispersed in some liquid manufacturing vehicle, subsequent drying might be required. Typically, spray, freeze and tray drying.

D. Major steps in microencapsulation by coacervation techniques

This process consists of a series of three steps carried out under continuous agitation, namely (Bakan, 1973; Salib, 1977);

1) Formation of three immiscible chemical phases which includes a liquid manufacturing vehicle phase, a core material phase and a coating material phase.

2) Deposition of the liquid polymer around the core material is accomplished by controlled physical mixing of the coating material and the core material in the manufacturing vehicle. The adsorption phenomenon is a pre-requisite to effective coating. Continued deposition of the coating is promoted by a reduction in the total free interfacial energy of the system brought about by a decrease of the coating material surface area during coalescence of the

liquid polymer droplets.

3) Solidification or rigidization of the coating is done either by thermal, crosslinking or desolvation techniques, to form a self-sustaining entity - a microcapsule.

E. Application of microencapsulation technology

Microencapsulation is used advantageously in many fields. In fact, its application can benefit almost any field.

1. Food industry

The encapsulation of volatile flavors and aromas protects them from physical oxidation and from thermal decomposition (Gutcho, 1979). Other materials that have been encapsulated include acidulants, colorants, enzymes, edible oils, minerals, condiments, chewing gums, frosted coatings, leavening agents, animal feed supplements (Bakan, 1973; Gutcho, 1979). Milk fat capsules have been used by Magee and Olson (1981a, 1981b) to encapsulate cheese ripening systems.

2. Pharmaceutical industry

Microencapsulation has been used to mask unpleasant odor and taste, to protect against oxidation and spoilage, and also to control the release systems of drugs. It is also used to encapsulate aspirin, water insoluble medicaments, prepare antibiotics, chelating agents, etc. Vitamin A, C, E and riboflavin have been manufactured using this technique (Herbert, 1975; Webb et al., 1974). Also, beauty and health aids such as skin creams, lipsticks, makeup removers, perfumes, soaps, dentrifices and antiperspirants have been developed using

microencapsulation (Gutcho, 1979).

3. Additional uses

Laundry products such as bleaches, fabric conditioners, detergents have been microencapsulated. Agricultural chemicals such as pesticides, fertilizers, herbicides, and insecticides have been encapsulated.

Pigments, catalysts, adhesives, paper coatings, thermoplastics, pacifiers and a lot more have been manufactured using this technique.

The techniques of microencapsulation currently used successfully for a variety of commercial purposes are expected to find other new and exciting applications.

VII. Emulsion stability of milk fat globules

Milk is an oil-in-water emulsion with an average fat content of 3.5%. Fats or lipids are composed primarily of fatty acids esters and similar or derived compounds that are soluble in non-polar organic solvents, and insoluble or almost so in aqueous liquids. Fats have a high interfacial tension with water, and mostly occur in the form of small droplets. The lipids of milk, together forming the "milk fat" have a very complicated composition and structure, even more complicated than most other natural fats (Mulder and Walstra, 1974).

A. Size distribution

Nearly all of the fat in milk is in separate small globules 0.1 - 15.0 μm in size (Walstra and Jeness, 1984). According to Mulder and Walstra (1974), the size of the smallest globule is not exactly known, but it is certainly below 0.1 μm. The largest globule size cannot be established because it depends on the volume of milk. The larger the volume,

the higher the probability of finding extremely large globules. This has several consequences for the properties of milk and its products. It implies that milk is not homogenous and the fat emulsion is not entirely stable.The size distribution of milk fat globules can be split into three classes (Mulder and Walstra, 1974):

The first is the small globules, comprising about 80% of the number of globules, but only a small percentage of the fat. The second is the main comprising about 94% of the fat. The third is the large globule, a small tail of very large ones.

Small globule subdistribution is almost constant in size while the main subdistribution may vary in average and quantity. The size distribution can be greatly altered by treatment (Walstra and Jenness, 1984).

B. Milk fat crystallization

The melting and crystallization of natural fats are complicated phenomena, and this is particularly true for milk fat because of its very wide range of different triglycerides. Milk fat crystallization is of great practical importance, because it largely affects the susceptibility of globules to churning or clumping, the resistance of the globules to disruption, the consistency and mouthfeel of high-fat products and in some conditions, creaming rate (Mulder and Walstra, 1974).

Milk fat is a liquid above 40°C and usually completely solidified below -40°C. At intermediate temperatures, it is a mixture of crystals and oils. Crystallization is initiated by the presence of suitable nuclei, and in milk fat globules, considerable supercooling is needed. In fat, crystallization starts at the surface of extraneous particles called catalytic impurities. Once crystals have formed during cooling, they serve as catalytic impurities for the nucleation of other crystals of slightly different composition and crystallization proceeds

with little hindrance.

C. Emulsion stability

Emulsions are inherently unstable, and milk and milk products are no exception. There are several main types of instability in a fat emulsion: creaming, flocculation, coalescence and disruption.

Ceaming occurs because milk fat globules are lighter in density than plasma hence they rise under the influence of gravity.

Flocculation occurs when fat globules rise and aggregate. Different kinds of flocculation may occur, leading to different types of aggregates called floccules in which globules keep their identity, clusters in which globules share part of their interfacial layers), and granules formed from partly solid globules only. Granules always contain fat crystals (Mulder and Walstra, 1984).

Calescence and disruption occur when two globules are within 10 nm of one another. Globules start to coalesce when the thin film of liquid between adjoining globules suddenly ruptures. Coalescence is essentially the fusing together of smaller globules into larger ones. During coalescence, surface area decreases and proteins and polar lipids of the fat globule membrane (proteins and polar lipids) are released into the plasma. This is called disruption. With liquid fat, disruption and coalescence can occur simultaneously.

D. Emulsifiers

The stability of emulsions can be enhanced by adding emulsifiers or surfactants. Emulsifiers are substances which reduce the surface tension at the interface of two normally immiscible phases, allowing them to mix and form an emulsion. Emulsifiers assert their effects at the interface between oil, water or air dispersed in a second immiscible fluid

(Darling and Birkett, 1987). The key functions of emulsifiers may be summarized (Dziezak, 1988) as:

- to promote emulsion stability, stabilize aerated systems and control agglomeration of fat globules;
- to modify texture, shelf life and rheological properties by complexing with starch and protein components;
- to improve the texture of fat-based foods by controlling the polymorphism of fats.

Structurally, emulsifiers are amphiphilic molecules, possessing both hydrophilic and lipophilic moieties. The hydrophilic part may be any of a large variety of polar groups, while the lipophilic part is made up of hydrocarbons that are branched, straight-chained or cyclic.

Emulsifiers have been classified and selected by the use of two main methods:
-HLB (hydrophilic-lipophilic balance) system: this system provides an index (1 to 20) of the solubility of an emulsifier in oil or water systems and indicates the kind of emulsion for which the emulsifier is best suited. It is the most widely used method.
-PIT (phase inversion temperature) system: The PIT system takes into account the emulsion conditions.

Other methods based on physico-chemical properties are also used. These include the electrochemical charge of the emulsifier in aqueous systems and the functional chemical groups of the molecule (Friberg, 1976).

E. Examples of emulsifiers

Food emulsifiers are esters of edible fatty acids, derived from animal or vegetable sources and polyols such as glycerol, propylene glycol and sorbitol. They are usually formed

by either alcoholysis or direct esterification. Some commonly used food emulsifiers (Dziezak, 1988) include:

- Mono- and Diglycerides which are the most commonly used in foods. They are highly lipophilic compounds, and are used in cakes mixes, icings, and dairy products. Examples include glycerol monolaurate and polyglycerate 60.
- Stearoyl Lactylates are reaction products of stearic acid and lactic acid, converted to the calcium or sodium salts. They are highly hydrophilic, and are used in cake mixes, whipped toppings add breads. Examples are sodium stearoyl-2-lactylate, and calcium stearoyl-2-lactylate.
- Propylene Glycol Esters are formed from a reaction of propylene glycol and fatty acids. They are used in baked products.
- Sorbitan Esters are formed from the reaction of sorbitol and stearic acid and are also very hydrophilic. They are used in cake mixes, cocoa products, coffee whiteners, etc. An example is sorbitan monostearate.
- Polysorbates are polyethylene sorbitan esters and are formed from the reaction of sorbitan esters with ethylene oxide. They are used in ice cream, frozen custards, cakes, etc. Examples include polysorbate 60, polysorbate 65 and polysorbate 80.
- Polyglycerol Esters are formed by the reaction of fatty acids with polymerized glycerol consisting of 2 to 10 molecules, and are used in beverages, icings and margarine.
- Sucrose Esters are mono-, di-, tri-esters of sucrose with fatty acids. They are used in baked products and frozen dairy desserts.

- Lecithin is a mixture of phosphatides and other components. It is derived commercially from soybean and is used in baked goods, instant foods and margarine.

MATERIALS AND METHODS

To achieve the objectives of the research, the experiments were carried out in four major steps. As the first step, acid β-galactosidase was obtained from various sources and studied for their activities in milk under acidic conditions similar to those in the stomach and the small intestines as well as their activities in the natural pH of milk. In the second step, encapsulation efficiencies of various emulsifiers on enzymes were investigated with butterfat as a carrier. The third step involved the incorporation of various stabilizers to the best combination selected and testing their effectiveness on the encapsulation as well as keeping the encapsulated enzymes in the milk. Finally, the last step was intended to evaluate the overall effectiveness of the encapsulation of the enzyme with regard to lactose hydrolysis, enzyme stability and fat separation.

I. Activities of acid β-galactosidase in acidic conditions

A. The Substrate:

Fresh pasteurized whole milk was obtained from the dairy processing plant in the Department of Animal Sciences and Industries at Kansas State University. This milk was used without any further treatment.

B. The Enzyme:

Acid lactase (β-galactosidase) was obtained from various commercial sources as follows:

a. β-Galactosidase from Aspergillus niger, Grade V, Sigma Chemical Company (St Louis, MO). It was suspended in 3.5 M $_{\text{ammonium sulfate}}$ and 50 mM sodium acetate with a pH of approximately 5.2. It was indicated to have an activity of 10-15 units per mg protein (Biuret) at pH 4.0 (25 °C).

b. β-Galactosidase from Aspergillus oryzae, Grade IX, standardized with starch (Sigma Chemical Company). It was indicated to have an activity of 4 units per mg solid using lactose as substrate at pH 4.5 (30 °C).

c. β-Galactosidase from Bovine Testes (Sigma Chemical Company). It was suspended in 3.2 M $_{\text{ammonium sulfate}}$ with a pH of approximately 5.0 and its activity was labelled to be 1 to 3 units per mg protein using ONPG as the substrate at pH 4.4 (25 °C).

d. β-Galactosidase from Jack Beans, Grade VII (Sigma Chemical Company). It was suspended in 3.0 M $_{\text{ammonium sulfate}}$ and 25 mM sodium citrate at pH 5.5. Its activity was indicated to be 10-25 units per mg protein (Warburg-Christian) at pH 3.5 in citrate buffer at 25 °C.

e. Food grade Takamine Brand Fungal lactase (E.C.3.2.1.23 β-D-Galactoside galactohydrolase) from Asperillus oryzae (Miles Laboratories Inc., Elkhart, IN). It was indicated to have an activity of 30,000 units LU/g at pH 4.5 (37 °C).

f. Food grade Enzeco® Brand Fungal lactase from Aspergillus oryzae was purchased from Enzyme Development Corporation (EDC),

Keyport, NJ. It was said to have an activity of 10,000 units per gram using ONPG at pH 6.5 (30 °C).

C. Determination of enzyme activities

To estimate the amount of β-galactosidase needed to hydrolyze approximately 80% of the lactose in milk, 0.05, 0.10 and 0.25 g of enzyme from EDC and Miles Company and Sigma Chem. were weighed out, respectively, on a Mettler analytical balance (Mettler Instrument Corporation, Hightstown, NJ). Each enzyme was inoculated into 100 ml of milk and incubated at approximately 5 °C for 24 hrs. After incubation, the milk samples were analyzed for lactose content by HPLC procedures as described below. From the results obtained, the quantity of each enzyme needed for 80% lactose hydrolysis was estimated by use of a two-point graphic technique. The enzymes from Sigma Chemical Company except for A. oryzae were so small in quantity and expensive that their activities were not determined in milk. Estimated quantities were used from the activity units indicated on the label.

D. Acidification

Milk samples were acidified to the desired pHs utilizing a Beckman pH I 43 pH meter with a Beckman combination electrode and a thermo-compensator ATC/Temperature probe (Beckman Instruments Inc., Fullerton, CA). The pH meter was calibrated on a two-point standardization procedure using certified standard buffer solutions of pH 4.0 and 7.0 (Fisher Scientific Co.) prior to each set of measurement. Test solutions were continuously mixed using a Fisher magnetic bar and a Flexa-mix magnetic stirrer model 16 (Fisher Scientific Co.), while 10% hydrochloric acid solution was added until the pH was adjusted

to 4.0.

E. Selection of Ideal enzyme

After acidification, the samples were inoculated with the predetermined amount of enzymes as indicated in Table 5 and incubated in a water bath (Blue M Electric Company, Blue Islands, IL) at 37 °C for one hour. The samples were analyzed for lactose content using an HPLC procedure. The enzyme with the highest activity within the pH range of 2 and 4 was selected for further experimentation. The cost was also considered in the selection of the enzymes. It was not possible to use the same concentrations for all the six enzymes because of the limited quantities available.

Table 5. Acid β-galactosidases used from various commercial sources.

Type	Commercial Source	Concentration used
A. niger	Sigma Chem. Co.	1 ml/4 g milk
A. oryzae	Miles Labs.	0.2 g/100 g milk
A. oryzae	Enzyme Development Corp.	0.2 g/100 g milk
A. oryzae	Sigma Chem. Co.	0.2 g/100 g milk
Bovine Testes	Sigma Chem. Co.	1.8 ml/25 g milk
Jack Beans	Sigma Chem. Co.	0.5 ml/4 g milk

F. Stability of selected enzyme

The enzyme selected from the previous study was further evaluated for its stability in the neutral and acidic conditions of milk. Fungal lactase Enzeco (R) Brand (EDC), which appeared to be the most promising of all the six enzymes tested, was used to

inoculate pasteurized whole milk at a concentration of 0.2 g fungal lactase/ 100 ml milk. The milk was incubated at approximately 5 °C for 10 days. During incubation, samples were withdrawn at intervals of 1, 2, 3, 4, 5, 7 and 10 days and analyzed for lactose hydrolysis by the HPLC as described later. This would determine the activity of the enzyme at the neutral pH of milk under refrigerated conditions. To study its activity at acidic conditions, samples were withdrawn at the same intervals and acidified with 10% HCl to pH 3, which is the average acidity of the human small intestine. Then, all the acidified milk samples were incubated for one hour, the approximate length of time required to digest milk in the human alimentary canal and at 37 °C, the average human body temperature. A Blue M magic whirl constant temperature water bath (Blue M Electric Company, Blue Island, IL) was used to maintain the incubation temperature.

G. Sample preparation for high performance liquid chromatography (HPLC)

The procedure of Kwak and Jeon (1986) was used for the preparation of the milk samples for lactose analysis by the HPLC.

Milk samples (10.0 g each) were weighed into a 25 ml volumetric flask using an OHAUS model E400 electronic toploading balance (Ohaus Scale Corporation, Florham Park, NJ). These samples were gently mixed with about 10 ml of 2-propanol (Fisher Scientific) so that the denatured protein particles would not be trapped above the volume mark. Then, the samples were made to volume by adding more 2-propanol. After mixing thoroughly, the mixtures were left for 20 minutes at room temperature to ensure precipitation of proteins. The mixtures were then centrifuged at 5000 rpm for 10 minutes using a Beckman Centrifuge model J21 (Beckman Instruments, Inc.). The supernatant was collected and filtered through Whatman No. 42 paper (Fisher Scientific Co.). The filtrate

was then eluted through a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) following the procedure as recommended by the manufacturer. Using a 10 ml syringe the cartridge was first eluted with 2 ml methanol (Fisher Scientific Co.) and followed by 5 ml distilled, de-ionized water. Then, 2 ml of the supernatant was collected for HPLC analysis after discarding the first 2 ml eluent from the Sep-Pak.

H. Preparation of standard solutions

Pure solutions of lactose, glucose and galactose (5.00% w/v) were prepared from analytical grade reagents (Sigma Chemical Co.). Each of these sugars (5.00 g) was weighed on a Mettler analytical balance (Mettler Instruments Corp.) and made to 100 ml with distilled water. After thorough mixing to ensure complete dissolution, these solutions were kept frozen, thawed and used at the start of each injection into the HPLC. Their retention times were used to identify the sugars present in the milk samples.

I. HPLC analysis

The extent of lactose hydrolysis in the milk samples was determined by the high performance liquid chromatography (HPLC) procedure described by Jeon and Mantha (1985).

The HPLC system consisted of a Beckman model 100A pump, an Altex Scientific model 210 injection valve with a 20 microliter (μl) loop, a Model 156 refractive index detector, and a Fisher Recordall Series 5000 recorder. The Amino-Spheri-5 column (4.6 mm i.d. x 10 cm, Brownlee Labs., Santa Clara, CA) was operated at room temperature with a flow rate of 1.5 ml/min. A mixture of 75% acetonitrile (HPLC Grade, Fisher Scientific

Co.)/25% distilled, de-ionized water was used as the mobile phase after it had been de-aerated for 30 minutes in an ultrasonic machine. The guard column used was an Amino-Spheri-5 polar phase AS-GU cartridge (4.6 mm i.d. x 3.0 cm, Brownlee Labs., Santa Clara, CA). The chart speed was set at 0.5 cm per minute for all analyses. With the use of a 25 μl syringe (Fisher Scientific Co.), 20 μl of the prepared samples were injected into the HPLC.

The identification of the hydrolysates was done by comparing the retention time of the test solutions to that of the standard solution. Degree of hydrolysis was measured by taking the ratios of the peak heights of the treated milk samples and those of the untreated milk samples in each experiment. All samples were prepared and analyzed in duplicates. The formula used to calculate the percentage hydrolysis was:

$$\% \text{ hydrolysis} = 100 - [\ \{\ A/B\ \}\ x\ 100\ ]$$

where: **A** = peak height of lactose in treated milk sample; and

B = peak height of lactose in untreated milk sample.

II. Microencapsulation

Three main components were used: the core material, the coating material and the dispersion liquid.

A. The Core Material

The core material used was the enzyme lactase from EDC which had previously been selected as the most probable enzyme for our study. For each experiment, 2.0 g of the

enzyme was dissolved in 5 ml of distilled water and mixed thoroughly with the use of a Flexa-mix magnetic stirrer model 16 and a Fisher magnetic bar.

B. The Coating Material

The coating material used to encapsulate the enzyme was an emulsion of milk fat and emulsifiers.

Frozen unsalted butter obtained from the dairy processing plant at Kansas State University was melted at 70 °C, decanted and used without any further treatment as butterfat. Emulsifiers used were polyoxyethylene sorbitan monostearate (Tween 60) from Sigma Chemical Co., polyoxyethylene sorbitan monooleate (Tween 80), lecithin from Fisher Scientific Co., sorbitan monostearate (Span 60), and sorbitan monolaurate (Span 80) from Aldrich Chemical Co. These emulsifiers were used at various combinations as shown in Table 6, to determine which combination would give the best result for encapsulation efficiency.

C. The Dispersion Liquid

Fresh pasteurized skim milk obtained from the dairy processing plant at Kansas State University was used as the dispersion liquid without any further treatment.

D. Encapsulation Procedure

The method adopted to encapsulate β-galactosidase enzyme using milk fat globules was that developed by Magee and Olson (1981a).

Thirty five grams of butterfat was prepared from frozen unsalted butter, transferred to a 150 ml beaker, and tempered at 62 °C in a constant temperature water bath (Blue M, Blue Island, IL). The butterfat was continuously stirred at 300 rpm for approximately 15 min using a three-bladed multicraft stirring propeller attached to a stirring motor (Cole-Palmar Instruments and Equipment Co., Chicago, IL). To the butterfat, various emulsifiers were added at different combinations as indicated in Table 6. During the addition of the emulsifiers, the rate of stirring was increased to approximately 400 rpm and continued until the emulsifiers were completely dissolved. The temperature of the butterfat/emulsifier mixture was then reduced to 50 °C by adding cold water into the water bath. Stirring was further increased to approximately 500 rpm and the core material (dissolved enzyme) was slowly added to the mixture.

A Wagner airless paint sprayer series 220 with an orifice of 0.4 mm (Wagner Spray Technical Corporation, Minneapolis, MN) was used to disperse the emulsion/enzyme mixture into the milk. The sprayer was capable of generating a high pressure with a piston pump which could draw the mixture into a compression chamber and pump it out through an orifice. The sprayer was equipped with a nozzle extension, and a control screw was adjusted to produce a fine aerosol and maintained at this setting throughout the experiment. Before spraying into the milk, the mixture was sprayed into the tared volume of water at different time lengths to determine the length of time needed to spray 4.4 g of the emulsion into a 100 ml skim milk (to give 3.5% milk fat in the milk).

After 20 min of vigorous stirring, the emulsifier/enzyme mixture was quickly transferred into the sprayer. The orifice of the sprayer was submerged into the dispersion liquid (100 ml of skim milk at 5 °C) and sprayed for three seconds. After spraying, the milk

was stored at 5 °C for 10 days and analyzed as previously described at given intervals. Encapsulation efficiency (EE) was calculated as follows:

EE = 100 - % Lactose Hydrolysis.

Samples of the emulsion were observed under an OLYMPUS light microscope (Jacobs Instrument Co., Inc. Overland Park, KS.) in order to verify the presence of capsules. The hanging drop technique was used and the capsule size was estimated by a calibrated ocular grid.

Table 6. Emulsifier combinations used for microencapsulation.

Combination	Emulsifier concentration/35 g milk fat
I	2.5% Span 60 + 2.5% Tween 60
II	2.5% Span 60 + 2.5% Lecithin
III	2.5% Span 60 + 2.5% Tween 80
IV	2.5% Span 80 + 2.5% Tween 60
V	2.5% Span 80 + 2.5% Tween 80
VI	2.5% Span 80 + 2.5% Lecithin
VII	2.0% Span 60 + 2.0% Tween 60 + 1.0% Lecithin
VIII	2.0% Span 60 + 2.0% Tween 80 + 1.0% Lecithin
IX	2.0% Span 80 + 2.0% Tween 60 + 1.0% Lecithin
X	2.0% Span 80 + 2.0% Tween 80 + 1.0% Lecithin

III. Addition of stabilizers

Gum arabic from acacia tree and amylose from potato type III were obtained from Sigma Chemical Co. and used as dispersion agents (stabilizers). Also, carrageenan obtained from Star Blends Inc. (subsidiary of Grindsted Products Inc., St Joseph, MO) was used in this part of the experiment.

As a preliminary study, the stabilizers were incorporated at 1, 2 and 3% concentration levels to the 35 g emulsion mixtures to determine the concentration that gave the greatest reduction in cream separation. These stabilizers (1% each) were further used at various combinations as shown in Table 7. After stirring the enzyme into the milk for 20 min, the stabilizers were added and stirring continued for another 10 min, followed by spraying into the skim milk. Cream separation was measured after 24 hours of storage at 5 °C.

Stabilization efficiency (SE) was calculated as:

$$SE = 100 - (C/D \times 100)$$

where: C = volume of separated cream in milk sample

D = volume of separated cream in control

Table 7. Stabilizer combinations used in emulsifier/enzyme mixtures

Combination	Stabilizer concentration in emulsion mixtures
I	1.0% Gum arabic + 1.0% carrageenan
II	1.0% Gum arabic + 1.0% amylose
III	1.0% carageen + 1.0% amylose

IV. Efficiency of selected combinations

In this last step of the experiment, the most effiacious combination of emulsifiers from the previous steps were jointly used to create the final product. Two emulsion mixtures, one containing a combination of 1% gum arabic and 1% carrageenan and the other containing 1% carrageenan and 1% amylose were used.

The product was developed using procedures identical to the ones described above. The product was then incubated at 5 °C for 10 days and analyzed at intervals of 1, 3, 5, 7, and 10 days for the following parameters:

i) Stabilization efficiency was evaluated by preparing the two treatments mentioned above. These treatments were stored separately in 250 ml volumetric cylinders. Cream separation was observed at the above mentioned intervals and the stabilization efficiencies were calculated as described earlier.

ii) Encapsulation efficiency was evaluated by preparing another batch of the two treatments stored separately in 250 ml Erlenmeyer flasks and incubated as above. At the same intervals, samples were withdrawn after thorough mixing and analyzed immediately for enzyme stability under neutral conditions of milk as previously described. Samples were again withdrawn from these flasks, acidified to pH 3 and incubated at 37 °C for an hour followed by analysis for enzymatic activity under acidic conditions of milk as previously described.

RESULTS AND DISCUSSION

I. Activities of acid β-galactosidase

A. Determination of the amount of enzyme needed

In this first step of our study, it was necessary to know the approximate amount of the enzyme required to hydrolyze about 80% of the lactose in milk as recommended by Mosbach (1976).

The results in Table 8 show the activities of A. oryzae lactase preparations in milk at pH 4.0. The activities were similar among the three commercial enzymes although the lactase from Sigma Co. showed about 10% less at 0.25 g enzyme level. This is probably due to the possibility that the enzyme was from a different strain of the organism (Dahlqvist et al., 1977). A three-point graphical analysis of these data indicated that approximately 0.2 g of lactase per 100 g milk from E.D.C. and Miles Co. would suffice for the amount of hydrolysis desired. Since each company used a different enzyme activity unit, this was a good comparison for the activities of the enzymes as well as a good estimation for their activities in milk.

B. HPLC Analysis

Typical HPLC chromatograms obtained from the standard solutions of lactose, glucose and galactose are shown in Figure 5. Lactose was eluted at approximately 5 minutes after injection while glucose and galactose were eluted after about 3 minutes. The amino column used did not separate glucose and galactose under the conditions applied, but lactose was separated well from the monosaccharides. As mentioned earlier, the elution time of these standards were used to identify the sugars in the milk samples. It may be

Table 8. % Lactose hydrolysis by Aspergillus oryzae at various concentrations (pH = 4.0).

Concentration of Lactase per 100 g milk	Commercial Source		
	E.D.C. %	Miles Co. Lactose	Sigma Chem. Hydrolyzed
0.05 g	51.8	50.2	50.0
0.10 g	64.6	66.4	64.6
0.25 g	90.8	91.7	80.4

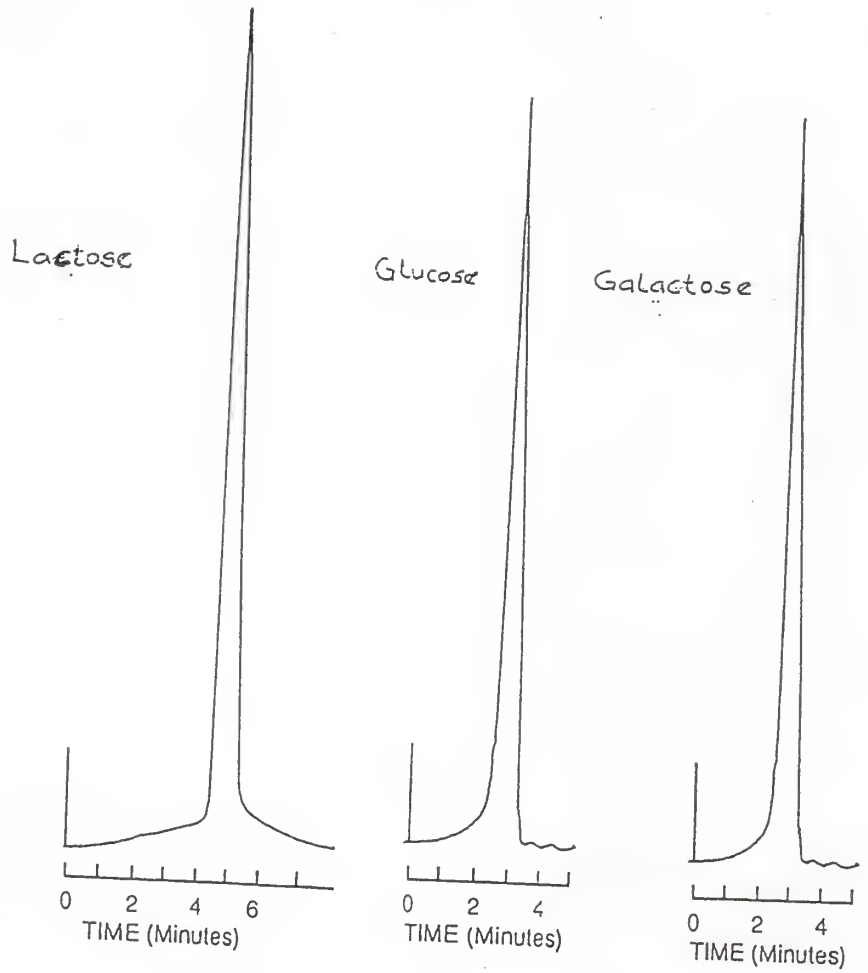


Figure 5. High performance liquid chromatography chromatograms of 5% standard solutions of lactose, glucose, and galactose (0.5 cm/min chart speed and 1.5 ml/min flow rate).

worthwhile to note that the retention time of the sugars decreased gradually as more injections were made. This is due to the fact that the amino column used is unstable, and loses some of the amino groups in the column solid phase. This was not a major set-back because the sequence of elution of the sugars remained the same at all times. However, the chart speed was increased from 0.5 cm/min to 1.0 cm/min during the last step of the experiment to facilitate the identification of the peak heights.

The lactose contents in skim milk and milk samples inoculated with lactase (after 24 hr refrigeration) are shown in Figure 6. The sugars were eluted in increasing order of their molecular weights with the amino-phase column used (Tweenton and Euston, 1980). Hence, peak 1 is glucose/galactose and peak 2 is lactose. Only one peak was observed in the skim milk sample as expected because lactose is the only measurable sugar present in milk (Alm, 1982). Two peaks were observed in the hydrolyzed milk samples instead of three. This is because glucose and galactose were not separated on the amino column as discussed above. Degree of hydrolysis was based on the ratios of the lactose peak height of the treated samples and that of untreated skim milk.

These chromatograms further demonstrate that the activity of the enzyme in neutral pH is high. The degree of increased sweetness in milk samples is solely dependent on the proportion of lactose, glucose and galactose present. As discussed earlier, the degree of hydrolysis will vary depending upon the quantity of the enzyme used, the pH conditions, the temperature of incubation, and the length of incubation (Bouvy, 1975; Mustranta et al., 1979). Since glucose and galactose are sweeter than lactose (Bouvy, 1975), it is to our interest in this experiment that lactose hydrolysis be minimized as much as possible during refrigeration, whereas the reverse is desirable after acidification and incubation at 37 ^{o}C.

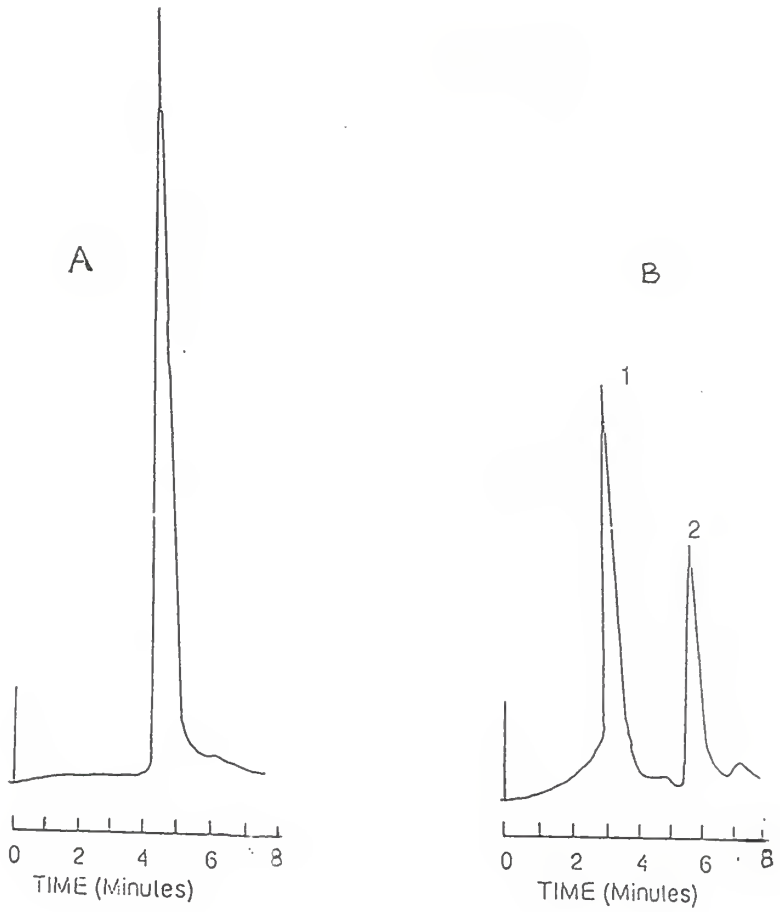


Figure 6. High performance liquid chromatography chromatograms of sugar extracts from skim milk at 1.5 ml/min flow rate and 0.5 cm/min chart speed.

A Lactose in skim milk.
B Sugars in skim milk containing lactose after 24 hours of refrigerated storage.

1 = glucose/galactose; 2 = lactose

C. Activities of acid β-galactosidase in acidic conditions

The results of enzymatic hydrolysis in acidic conditions of milk are presented in Table 9. At pH 2.0, the highest activity was observed with the lactase extracted from Jack Beans (63.8% hydrolysis), while the extract from A.oryzae (Miles Co.) gave the highest activity (90.8% hydrolysis) at pH 4.0. The same extract from Miles Co. was observed to be most sensitive to the change in pH. Despite its wide activity range, the extract from A.oryzae (E.D.C.) seemed to be more promising because its activity at pH 2.0 was nearly twice that from Miles Co. A very narrow activity range of 60.0% at pH 2.0 to 67.3% at pH 4.0 was observed with the extract from A. niger which was similar to the results reported by Mahoney (1984). Such a narrow range is probably due to the fact that this enzyme is strongly inhibited by galactose, a product of lactose hydrolysis (Mahoney, 1984). From our results, the optimum pH for this extract seemed to be somewhere between pH 2.0 and 4.0 inconsistence with Mahoney (1984). These researchers reported that the optimum pH of the enzyme was between 3.0 and 4.0, although Mustranta et al., (1979) reported that the optimum pH for the same enzyme dropped from 4.0 to 3.0 as the temperature was reduced from 62 °C to 45 °C. Lactase from Jack Beans and A. niger were not selected for further studies because they were very expensive. Considering both enzymatic activity in acidic conditions and economic feasibility, it seemed only logical to choose the enzyme extracted from A. oryzae (E.D.C.) for further experimentation.

D. Stability of the selected enzyme in neutral pH conditions

The data in Table 10 indicate that the selected enzyme was quite active both at neutral and acidic conditions. In fact, there was not much difference in activity observed

Table 9. Cost and percent lactose hydrolyzed by lactases from various sources.

Source	Cost	% Lactose Hydrolysis	
		pH 2	pH 4
A. niger (Sigma Chem.)	$30.00/2.5 ml	60.0	67.3
A. oryzae (Miles Co.)	$ 6.20/25 g	7.8	90.8
A. oryzae (E.D.C.)	$ 2.75/25 g	14.6	85.8
A. oryzae (Sigma Chem.)	$24.95/25 g	2.2	72.7
Bovine Testes (Sigma Chem.)	$75.00/1.5 ml	12.5	18.7
Jack Beans (Sigma Chem.)	$86.55/1.8 ml	63.8	39.6

Table 10. % Lactose hydrolyzed by lactase from EDC during 10 days of storage at 5°C.

	Duration of storage (days)					
Treatment	1	2	3	5	7	10
5°C	62.0	72.8	77.4	82.1	88.0	92.3
5°C, acidified to pH3, incubated at 37°C for 1 hr.	68.2	82.6	84.8	86.0	93.7	97.8

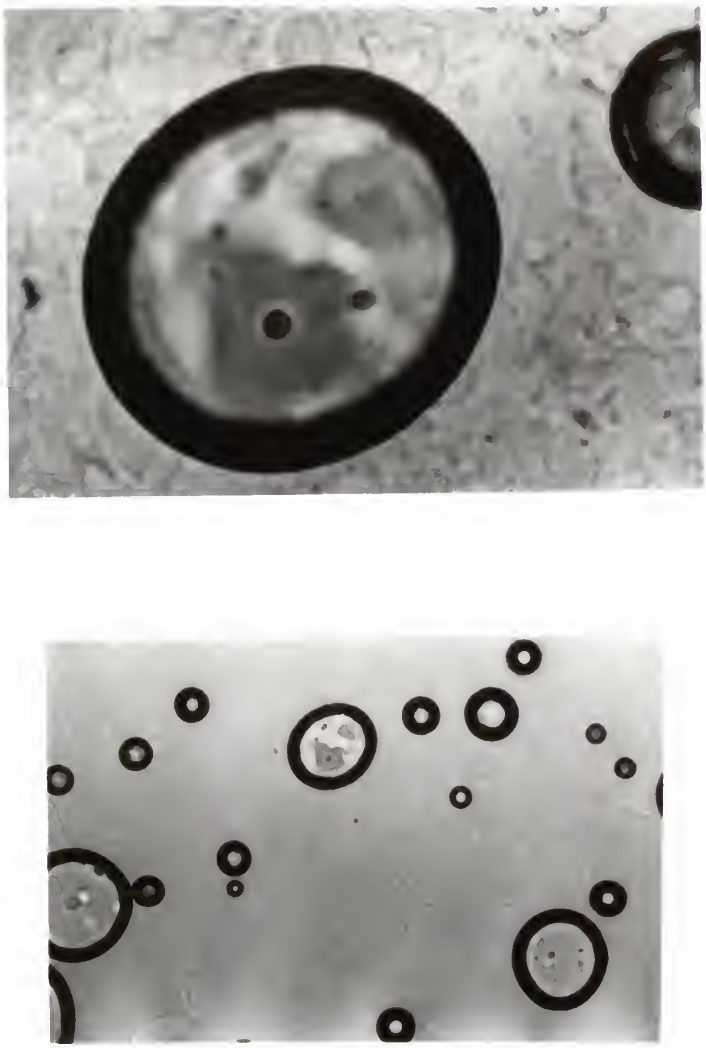


Figure 7. Photomicrographs of microcapsules, composed of a milk fat-emulsifier shell and encapsulated enzyme, dissolved in water. Plate A is an enlargement of a photomicrograph taken at 800 X; plate B is a 200 X magnification. The diameter of the largest microcapsule in each plate is 53 μm.

emulsion from the water bath to the sprayer resulted in a loss of heat and hence an increase in viscosity. This could have a negative effect with regards to encapsulation efficiency and dispersion of the capsules in milk.

B. Photomicrograph of capsules

A photomicrograph of the capsules is presented in Figure 7. This figure demonstrates that the capsule configuration was a multiple phase emulsion. The continuous phase is skim milk, and the apparent ring is a milk fat shell surrounding the encapsulated aqueous carrier vacuoles. This particular sample was taken from a mixture containing 35 g of milk fat, 0.88 g of Span 60, 0.88 g of Tween 60, and 2.0 g of lactase previously dissolved in 5 g of distilled water.

An estimate of the capsule size distribution ranged from a diameter of 12 μm to 60 μm. The average size calculated was 51 μm. Magee and Olson (1981a) also reported capsule sizes as large as 60 μm, although the average size was much smaller. Differences in the size of the capsules could be due to differences in stirring rate and time, the pressure applied to the sprayer during ejection, or to the size of the sprayer's orifice. It could also be attributed to the increase in viscosity of the emulsifier mixture as a result of heat loss during transfer to the sprayer as mentioned above.

C. Lactose hydrolysis with various emulsifier combinations

The results of lactose hydrolysis after enzyme encapsulation and refrigeration are indicated in Table 11. Percent hydrolysis was observed to be quite low compared to the results in Table 10. This indicates that some degree of encapsulation was achieved in milk.

between these two conditions. The indication that this enzyme was still active after acidification to pH 3.0 followed by incubation at 37 °C for an hour was very encouraging because it implied that the enzyme will remain active in the human small intestines. However, the fact that the enzyme was equally as active in neutral pH posed a major problem. The initial approach to this study was to select from a multitude of acid lactases, one that would be inactive or nearly at neutral pH, but very active in acidic pH conditions similar to that in the small intestines. The obtention of an enzyme with such ideal characteristics from a commercial source was not possible. Therefore, our next best alternative to achieving our objective was to retard the enzyme's activity during refrigeration by microencapsulation technique.

II. Microencapsulation

A. Encapsulation conditions

The emulsifiers available were used in various combinations as recommended by Magee and Olson (1981a, 1981b). They reported that blends of emulsifiers were superior for encapsulation as compared to the use individual emulsifiers. The combinations derived were based on their hydrophilic-lipophilic balance (HLB). Those with high HLB values (more hydrophilic) such as Tween 60, Tween 80 and lecithin were combined with those that have low HLB values (more lipophilic) such as Span 60 and Span 80.

Preliminary studies indicated that an increased rate of stirring as well as a longer stirring time directly reduced the size of the capsules. However, foaming and splashing was encountered as the stirring rate was increased above 500 rpm. Also, the transfer of the

Table 11. % Lactose hydrolysis with various emulsifier combinations during storage at 5°C for 10 days.

Combination (%/35g milk fat)	Days		
	1	5	10
	% Lactose Hydrolysis		
I 2.5 % Span 60 + 2.5% Tween 60	37.0	43.0	66.0
II 2.5% Span 60 + 2.5% lecithin	52.6	70.7	89.7
III 2.5% Span 60 + 2.5% Tween 80	44.4	69.8	83.0
IV 2.5% Span 80 + 2.5% Tween 60	48.3	64.7	70.7
V 2.5% Span 80 + 2.5% Tween 80	58.4	83.2	92.1
VI 2.5% Span 80 + 2.5% lecithin	44.3	72.2	84.5
VII 2.0% Span 60 + 2.0% Tween 60 + 1.0% lecithin	50.4	71.3	84.3
VIII 2.0% Span 60 + 2.0% Tween 80 + 1.0% lecithin	50.0	70.0	81.7
IX 2.0% Span 80 + 2.0% Tween 60 + 1.0% lecithin	49.2	77.7	80.0
X 2.0% Span 80 + 2.0% Tween 80 + 1.0% lecithin	52.3	72.3	88.5

The high melting point of the triglycerides in milk fat and possibly the added emulsifiers partially solidified when ejected into the dispersion liquid to form a rigid matrix that physically entrapped the carrier vacuoles within the milk fat shells (Magee and OLson, 1981a). However, a general trend observed was a constant increase in hydrolysis as refrigeration time increased. This implies that the capsules formed did not remain stable under refrigeration conditions as desired. Such destabilization during refrigeration was also encountered by Magee and Olson (1981b) during refrigerated storage. The cause of this destabilization has not been established, but the fact that hydrolysis increased gradually with time suggests that the capsules were not completely disintegrated initially. Probably, some of the capsules that were formed became cracked or broken (Magee et al., 1981), causing the enzyme to slowly seep out. It is also possible that some of the capsules formed only a partial barrier around the enzyme solution, resulting in exposure of the active site of the enzyme. Another possibility is that the capsules that were formed remained intact during the entire refrigeration period and the hydrolysis observed was as a result of that portion of the enzyme which was never really encapsulated at all.

As the results indicate, lactose hydrolysis was reduced in varied degrees with the use of different emulsifier combinations. A similar observation was made by Magee and Olson (1981a). The milk sample with the lowest percent hydrolysis (highest encapsulation) contained a combination of Span 60 and Tween 60. Samples that contained Span 60 were observed to give better encapsulation results, confirming a similar observation by Magee and Olson (1981a), and Minkov et al., (1974). The high efficiency of Span 60 may be attributed to its sorbitan moiety which forms a favorable association between the emulsifiers to enhance synergistically molecular orientation at interfaces and prevent the escape of the

carrier from the capsules (Magee and Olson, 1981a). In fact, Magee and Olson (1981a) reported that a combination of Span 60 and lecithin gave an encapsulation of 56.5% in a 16 °C dispersion liquid containing 0.01% Tween 60. Our results show that the same combination gave an encapsulation of 57.4% in a 5 °C dispersion liquid. These differences could be due to the differences in the composition and temperature of the dispersion liquid. The temperature of the dispersion liquid plays an important role in microencapsulation. Lower temperatures are favorable because this permits rapid formation of solid (rigid) capsules around the enzyme (Salib, 1977).

Phospholipids such as lecithin have an ionic moiety that increases the HLB oil/water to favor oil/water emulsions. The blending of phospholipids with Span 60 to give an emulsifier blend with an intermediate HLB was reported to give good encapsulation (Magee and Olson, 1981a). Although this was also observed in our study, encapsulation decreased sharply by the fifth day of refrigeration.

D. Lactose hydrolysis of encapsulated enzymes after acidification and incubation

The results in Table 12 reveal the percentage of lactose hydrolyzed after acidification to pH 3.0 followed by incubation at 37°C for an hour. Hydrolysis was a lot higher compared to the results in Table 11, and very close to those in Table 10. For example, percent hydrolysis in combination I increased from 37.0% (Table 11) to 67.0% (Table 12) after 24 hours of refrigeration. These results indicate that the various emulsifiers used did not inhibit the release of the enzyme. It is hence logical to assume that in the small intestines where similar pH and temperature conditions exist, the capsule wall will be destroyed releasing the enzyme for lactose hydrolysis as desired. The chromatographs shown in Figure 8 emphasize

the differences in the lactose content observed in the samples during refrigerated storage and after acidification and incubation.

Table 12. % Lactose hydrolysis with various emulsifier combinations during storage at 5°C for 10 days after acidification to pH 3 and incubation at 37°C for 1 hr.

Combination (%/35g milk fat)	Days		
	1	5	10
	% Lactose Hydrolysis		
I 2.5 % Span 60 + 2.5% Tween 60	67.0	77.0	87.0
II 2.5% Span 60 + 2.5% lecithin	62.7	77.4	86.8
III 2.5% Span 60 + 2.5% Tween 80	74.4	85.3	90.5
IV 2.5% Span 80 + 2.5% Tween 60	65.5	73.3	89.2
V 2.5% Span 80 + 2.5% Tween 80	67.3	89.1	93.6
VI 2.5% Span 80 + 2.5% lecithin	87.6	93.3	95.1
VII 2.0% Span 60 + 2.0% Tween 60 + 1.0% lecithin	73.9	83.5	87.8
VIII 2.0% Span 60 + 2.0% Tween 80 + 1.0% lecithin	71.7	80.0	97.5
IX 2.0% Span 80 + 2.0% Tween 60 + 1.0% lecithin	70.8	84.6	87.3
X 2.0% Span 80 + 2.0% Tween 80 + 1.0% lecithin	71.5	82.3	90.0

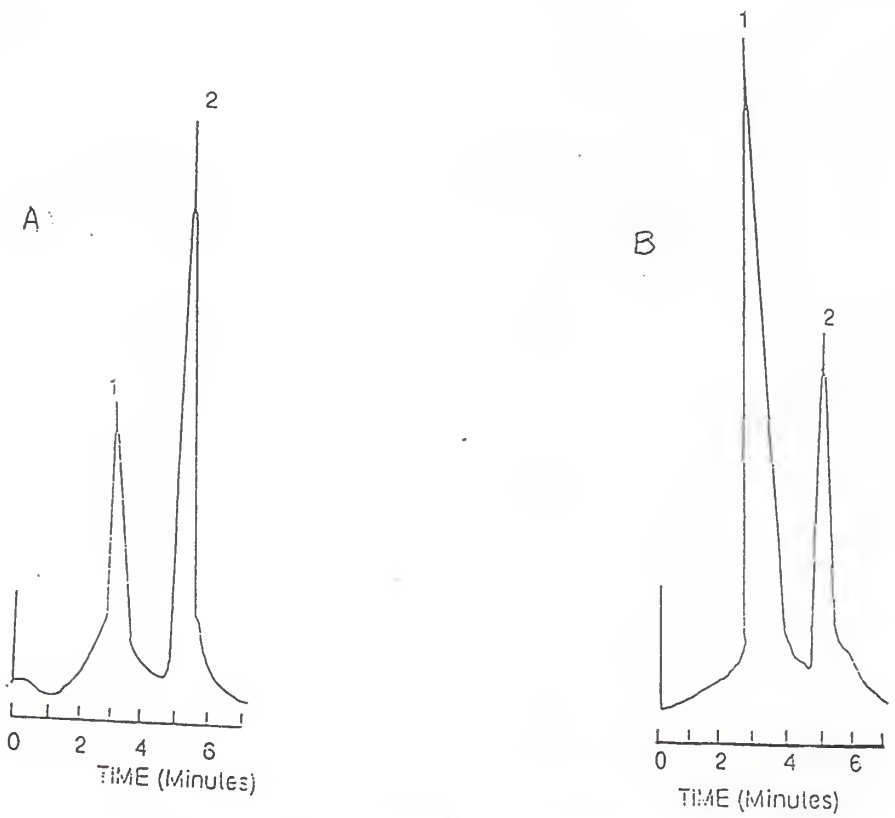


Figure 8. High performance liquid chromatography chromatograms of sugar extracts from skim milk.

A Skim milk with encapsulated enzyme after 24 hours of refrigerated storage. Chart speed is at 0.5 cm/min.

B Skim milk with encapsulated enzyme after 24 hours of refrigerated storage, acidification to pH 3 and incubation at 37°C for 1 hour. Chart speed is at 0.5 cm/min.

1 = glucose/galactose; 2 = lactose

E. Emulsion stability

Some creaming and/or clumping was observed during refrigeration in all the milk samples which contained the encapsulated enzyme, confirming the same report by Magee et al., (1981). They attributed this to insufficient pressure (energy) applied during ejection. In another study, Magee and Olson (1981a) reported that ejection into liquids below 16°C would produce large cylindrical capsules, suggesting that the carrier-milk fat emulsion emerged from the orifice as an elongated cylinder. This could also explain why creaming occurred.

Other factors may also be responsible for creaming. Shimizu et al., (1980) reported that the phospholipids and glycoproteins of the milk fat globule membrane plays an important role in stabilization of the milk fat emulsion. Mulder and Walstra (1974) explained the devastating effects on the milk fat globule proteins caused by agitation, foaming and heat shock. All these treatments were involved during encapsulation and could have contributed to cream separation. Oortwijn and Walstra (1982) reported that milk fat emulsified in skim milk did not churn without air but did so in the presence of air. This again suggests that the incorporation of air during encapsulation could also be partly responsible for the creaming observed. Magee and Olson (1981a) stated that the phase volume is important for emulsion stability, and that the volume encapsulated will influence the density and creaming rate of the capsules. This also could explain the creaming observed because the capsules formed were quite large as seen under the microscope. Emulsion instability may also be influenced by spoilage through microbial contamination.

III. Efficiency of stabilizers

Preliminary studies indicated that the volume of separated cream was observed decreased with an increase in the concentration of stabilizers used (Table 13). However, the difference in cream separation observed between incorporating 2% and 3% stabilizers appeared to be quite small. Hence, it seemed economically logical to use 2% stabilizers in the milk samples. Further investigation on the effect of using a combination of stabilizers indicated that this gave better results than when they were used individually (Table 14). The results in Table 14 further demonstrate that the sample containing 1% carrageenan and 1% amylose had the highest stabilization efficiency (76.7%).

According to Prasad and Balachandran (1987), carrageenan acts as a gelling against creaming by providing protection against calcium-induced clumping of globules. In another study, the stabilizing property of carrageenan was attributed to its capability of forming complexes with casein (Precht et al., 1988). A proposed mechanism for this interaction involves the interaction between the negatively charged carrageenan with casein and casein micelles which are also negatively charged by calcium ions acting as a cross-linking agent between α_s- and β-caseins with carrageenan (Douglas et al., 1988). The use of amylose as a stabilizer has not been extensively studied. However, the amylose molecule is considered to be a long, linear chain of anhydroglucose units. Because of its many hydroxyl groups, it readily interacts with positively charged ions present in a solution (Peterson, 1975). The interaction between the hydroxyl groups of amylose and the positively charged ions in skim milk such as casein, calcium, and phosphates could be responsible for its contribution to the stabilization of milk. Gum arabic on the other hand has been

extensively used as a stabilizer. Being a polysaccharide hydrocolloid, its stabilization action is attributed to its ability to increase viscosity in the continuous phase rather than by any action of the adsorbed polymeric material (Dickinson, 1986). Despite its wide usage, there is no clear understanding of its mode of action (Snowden et al., 1987).

The percent lactose hydrolyzed during refrigeration in the presence of stabilizers is given in Table 15. The lowest percent hydrolysis (35.1%) was observed in the sample containing 1% carrageenan and 1% gum arabic. The results of the other combinations however, indicate a slight undesirable increase in hydrolysis when compared to the control containing no stabilizers. This could imply that the stabilizers used were not compatible with the emulsifiers present, resulting in some disruption of the capsules.

IV. Efficiency of selected combinations

Two stabilizer combinations were selected from the last step. The combination containing 1% gum arabic and 1% carrageenan was selected because it had the lowest hydrolysis or highest encapsulation. The combination containing 1% carrageenan and 1% amylose was also selected because it had the highest stabilization efficiency.

Data on cream separation of these combinations during 10 days of refrigeration are given in Table 16. It is evident from this table that the control sample separated a lot faster, showing a constant volume as of the third day of refrigeration. The other two samples gave constant readings only after the fifth day. The combination containing carrageenan and amylose had the smallest volume of cream separation during the entire period of refrigeration. The same data is again represented graphically in Figure 9.

The percent lactose hydrolyzed during 10 days of refrigeration is given in Table 17. The combination containing gum arabic and carrageenan resulted in a lower extent of hydrolysis during the entire refrigeration period than that containing carrageenan and amylose. As earlier observed, the samples containing stabilizers showed an undesirable increase in hydrolysis compared to the control. Figure 10 shows lactose hydrolysis with various stabilizer combinations during 10 days of refrigerated storage.

Further information with regards to lactose hydrolysis after acidification and incubation is given in Table 18. Hydrolysis was higher in the sample containing amylose during the first three days of refrigeration. This is probably because amylose is more readily hydrolyzed than gum arabic in acidic conditions (Peterson, 1975). However, the sample containing gum arabic became more readily hydrolyzed after the fifth day of refrigeration. Figure 11 represents the chromatographs obtained for the sample containing gum arabic and carrageenan after 24 hours of refrigerated storage, followed by acidification and incubation. The greater peak height obtained for glucose/galactose after acidification and refrigeration again illustrates that these stabilizers did not obstruct the release of the enzyme under the given conditions. The results of hydrolysis obtained under these conditions during the 10 day refrigeration peroid are summarized in Figure 12.

The results of this entire study are summarized in Figures 13 through 15. Figure 13 is an illustration of the differences observed in cream separation in the various samples after 24 hours of refrigerated storage. From this, it is obvious that the combination of carrageenan and amylose served as an excellent stabilizer.

Figure 14 gives a summary of % lactose content in various milk samples after 24 hours of refrigerated storage. This concentration was found to significantly increase from

approximately 38% in the sample that contained the free enzyme to about 64% in the sample that contained the encapsulated enzyme. A small increased was observed in the encapsulated enzyme which contained an addition of carrageenan and gum arabic. Since our goal in the step is to maintain the lactose in our sample, it follows that this sample is preferred over the one containing carrageenan and amylose.

Figure 15 illustrates the % lactose hydrolyzed after 24 hr refrigeration, acidification and incubation. The percentages of hydrolyzed lactose are close to the sample that contained the encapsulated enzyme only, implying that the stabilizers did not affect hydrolysis under these conditions. It further implies that the same degree of hydrolysis could take place in the small intestines.

Table 13. Cream separation (ml) with various stabilizers after 24 hours storage at 5°C.

Stablizer	Concentration/35 g milk fat			
	0.0%	1.0%	2.0%	3.0%
	Cream Separation (ml)			
Gum arabic	30	25	21	20
Carrageenan	32	27	20	18
Amylose	30	24	13	10

Table 14. Cream separation with different combinations of stabilizers after 24 hours storage at 5°C.

Combination	Volume of Cream (ml)	% Stabilization Efficiency
1.0% Gum arabic and 1.0% Carrageenan	13	56.7
1.0% Gum arabic and 1.0% Amylose	9	70.0
1.0% Carrageenan and 1.0% Amylose	7	76.7
Control (No stabilizer)	30	--

Table 15. Lactose hydrolysis with different combinations of stabilizers after 24 hours storage at 5°C.

Combination	% Hydrolysis	% Encapsulation efficiency
1.0% Gum arabic and 1.0% Carrageenan	35.1	64.9
1.0% Gum arabic and 1.0% Amylose	38.8	61.2
1.0% Carrageenan and 1.0% Amylose	42.6	57.4
Control	37.9	62.1

Table 16. Cream separation (ml) with various stabilizer combinations during storage at 5°C for 10 days.

Combination	Days				
	1	3	5	7	10
	Cream Separation (ml)				
Control	30	32	32	32	32
1.0% Gum arabic and 1.0% carrageenan	15	19	21	21	21
1.0% Carrageenan and 1.0% amylose	8	16	19	19	19

Figure 9. Cream separation (ml) with various stabilizer combinations during storage at 5 C for 10 days.

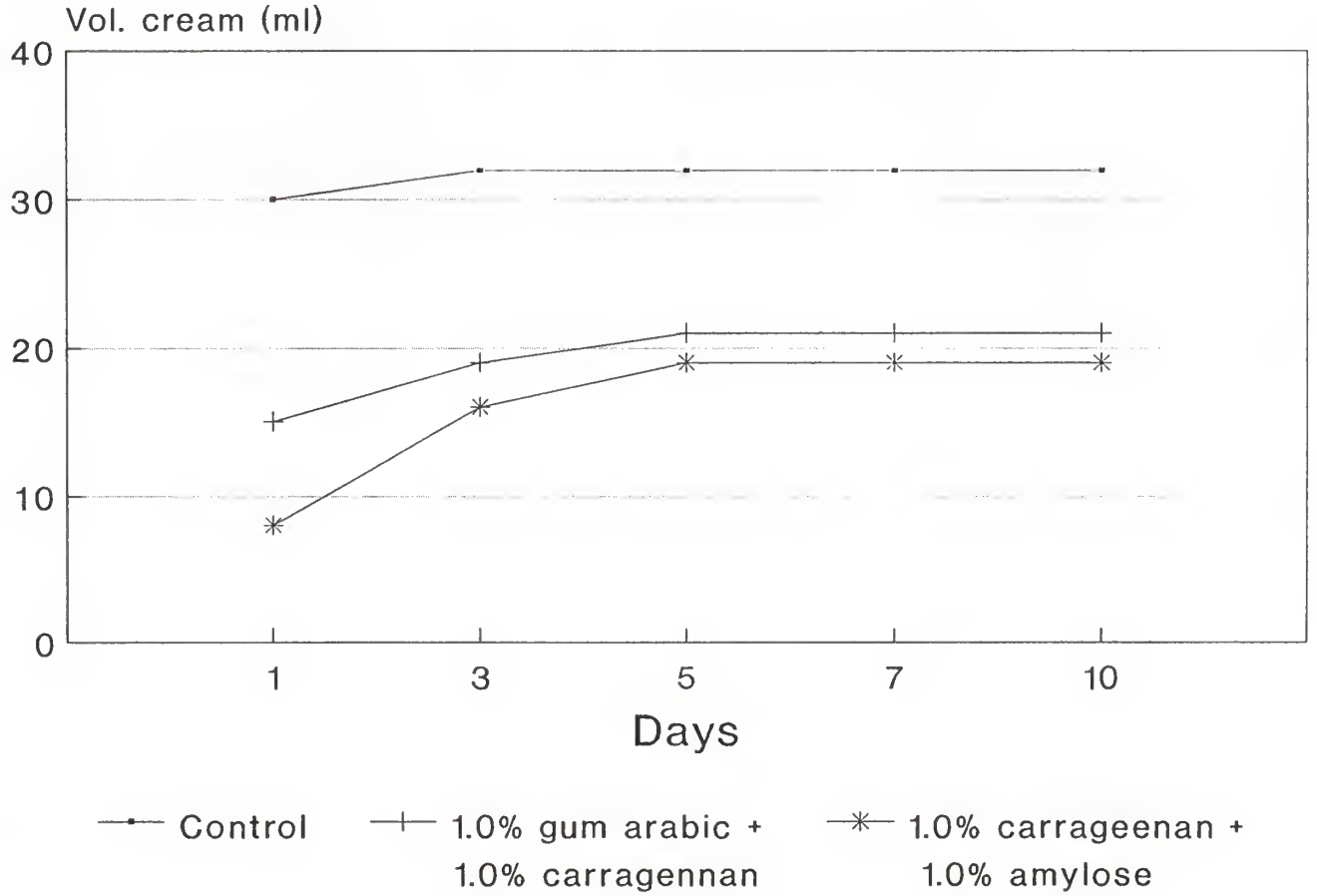


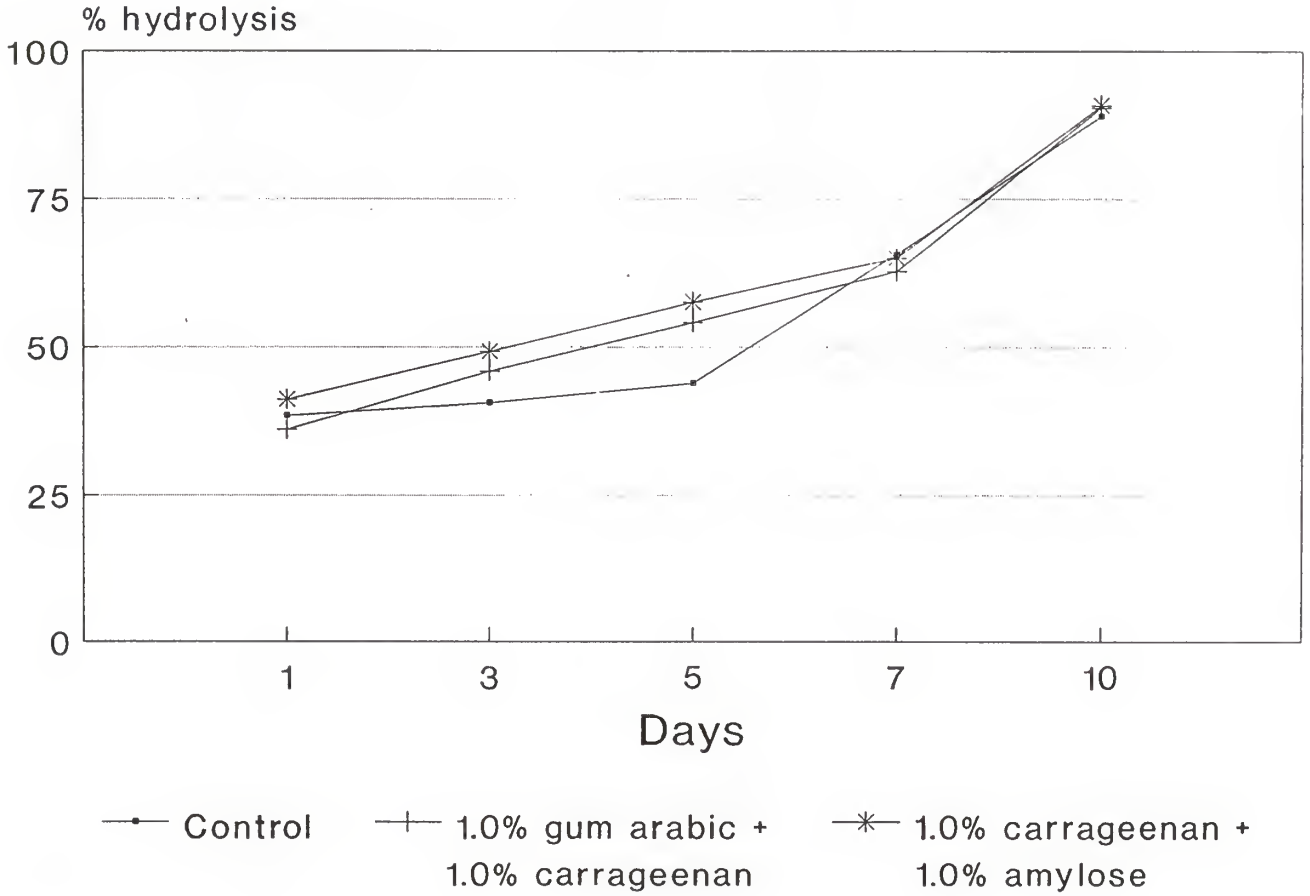
Table 17. % Lactose hydrolysis with various stabilizer combinations during 10 days of storage at 5°C.

Combination	Days				
	1	3	5	7	10
	% Lactose Hydrolysis				
Control	38.4	40.6	43.9	65.6	89.2
1.0% Gum arabic and 1.0% carrageenan	36.0	45.9	54.1	62.7	90.6
1.0% Carrageenan and 1.0% amylose	41.1	49.2	57.6	65.0	91.0

Table 18. % Lactose hydrolysis with various stabilizer combinations during 10 days storage, acidification to pH 3, and incubation at 37°C for 1 hr.

Combination	Days				
	1	3	5	7	10
	% Lactose Hydrolysis				
Control	66.2	69.6	73.0	77.8	93.5
1.0% Gum arabic and 1.0% carrageenan	56.6	62.4	71.2	76.1	94.4
1.0% Carrageenan and 1.0% amylose	62.3	64.0	68.8	74.3	93.6

Figure 10. % Lactose hydrolysis with various stabilizer combinations during 10 days of storage at 5 C.



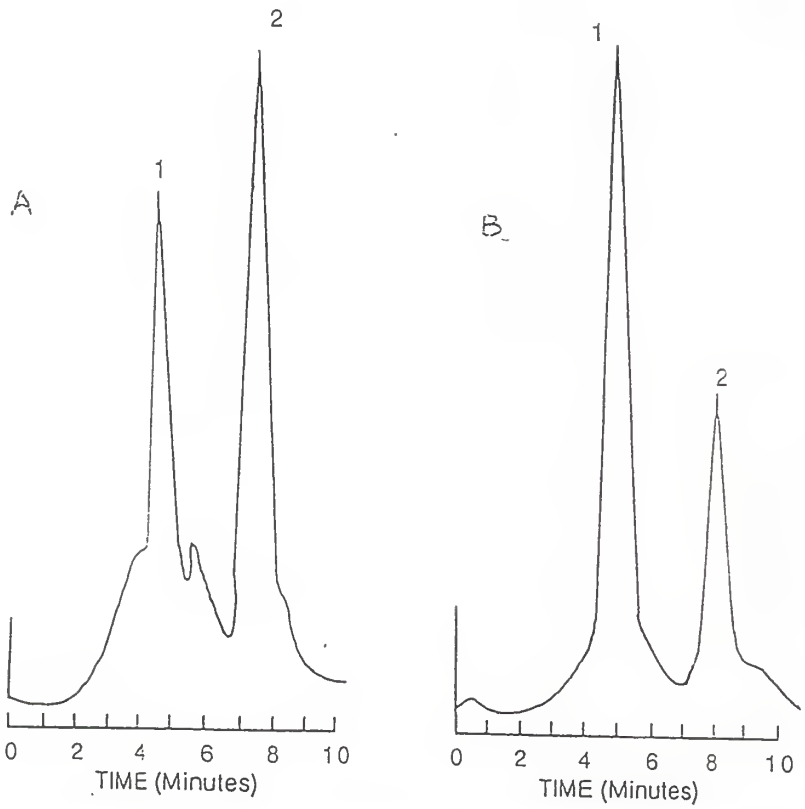


Figure 11. High performance liquid chromatography chromatograms of sugar extracts from skim milk containing the encapsulated enzyme and stabilizers (1.0 cm/min chart speed and 1.5 ml/min flow rate).

A Sample extract after 24 hours of refrigerated storage

B Sample extract after 24 hours of refrigerated storage, acifidication to pH 3, and incubation at 37°C for 1 hour.

1 = glucose/galactose; 2 = lactose

Figure 12. % Lactose hydrolysis with various stabilizer combinations during 10 days storage, acidification to pH3, and incubation at 37 C for 1 hr.

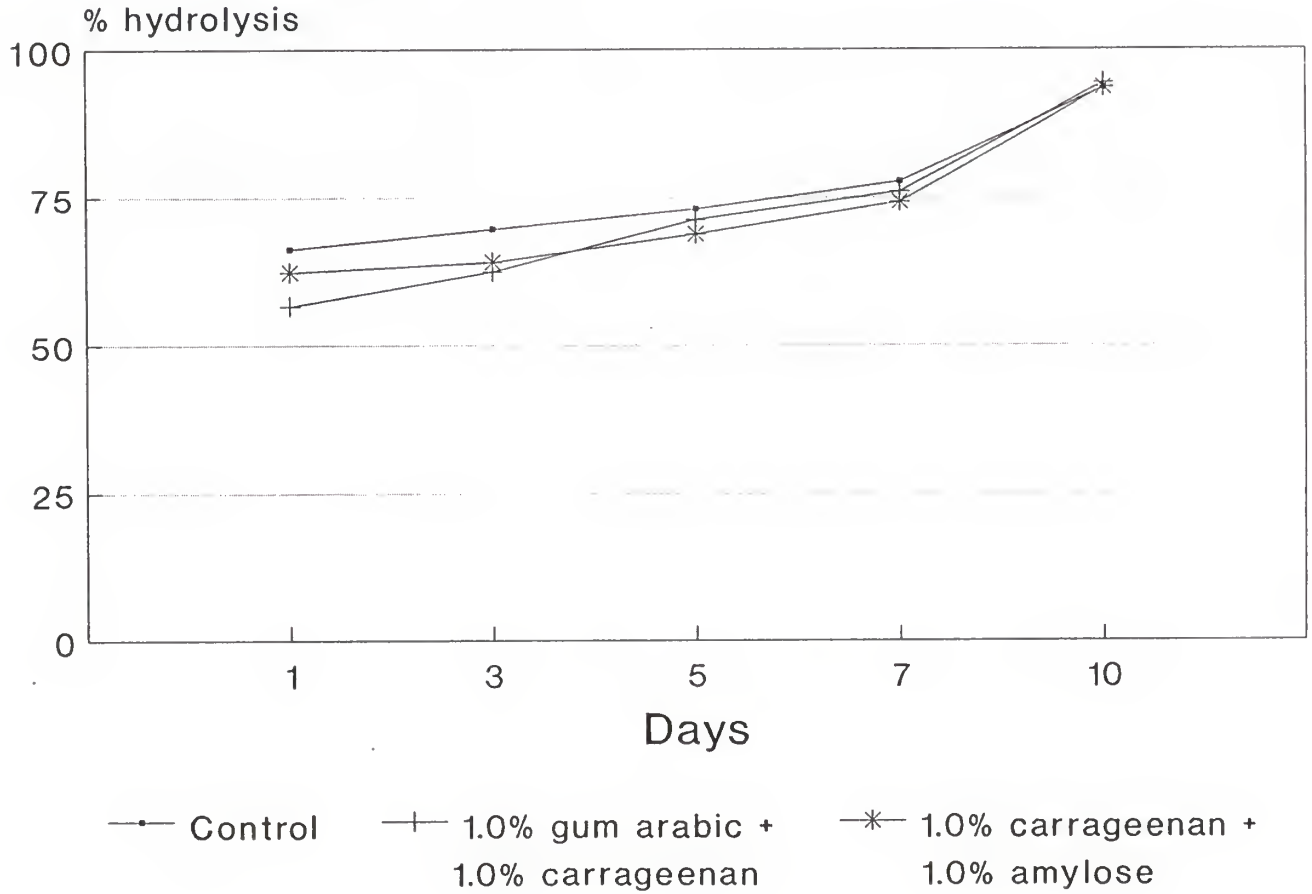


Figure 13. Cream separation in various milk samples containing encapsulated enzyme after 24 hours of storage at 5 C.

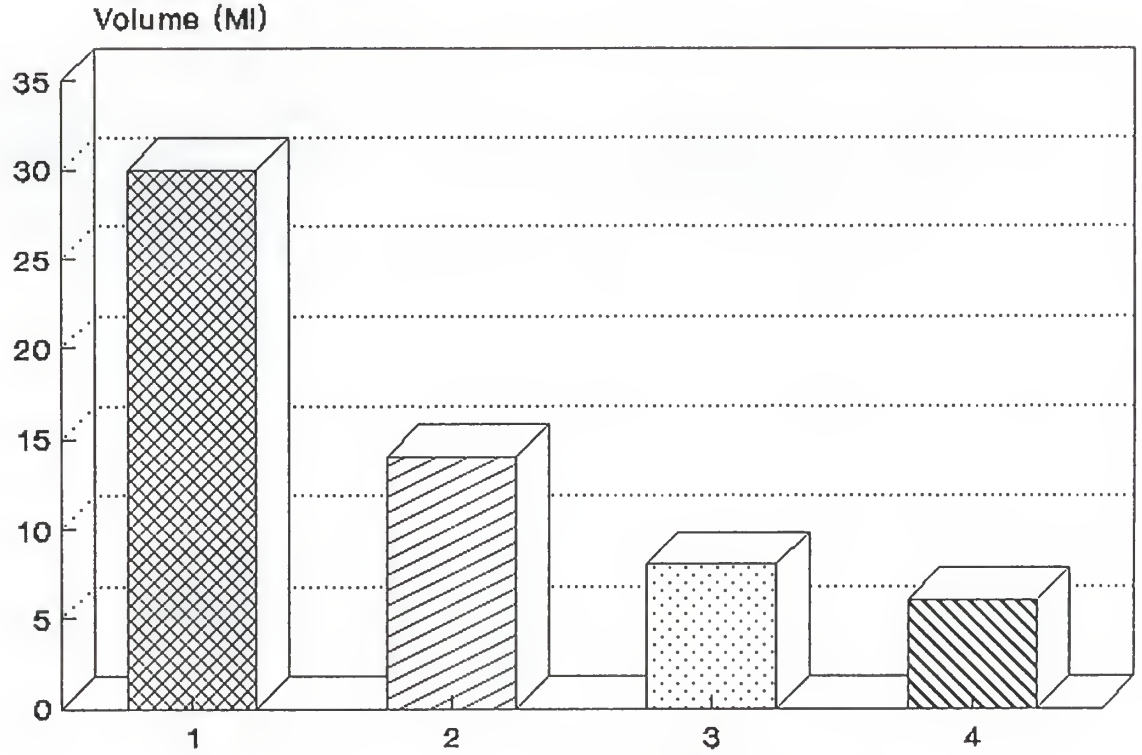
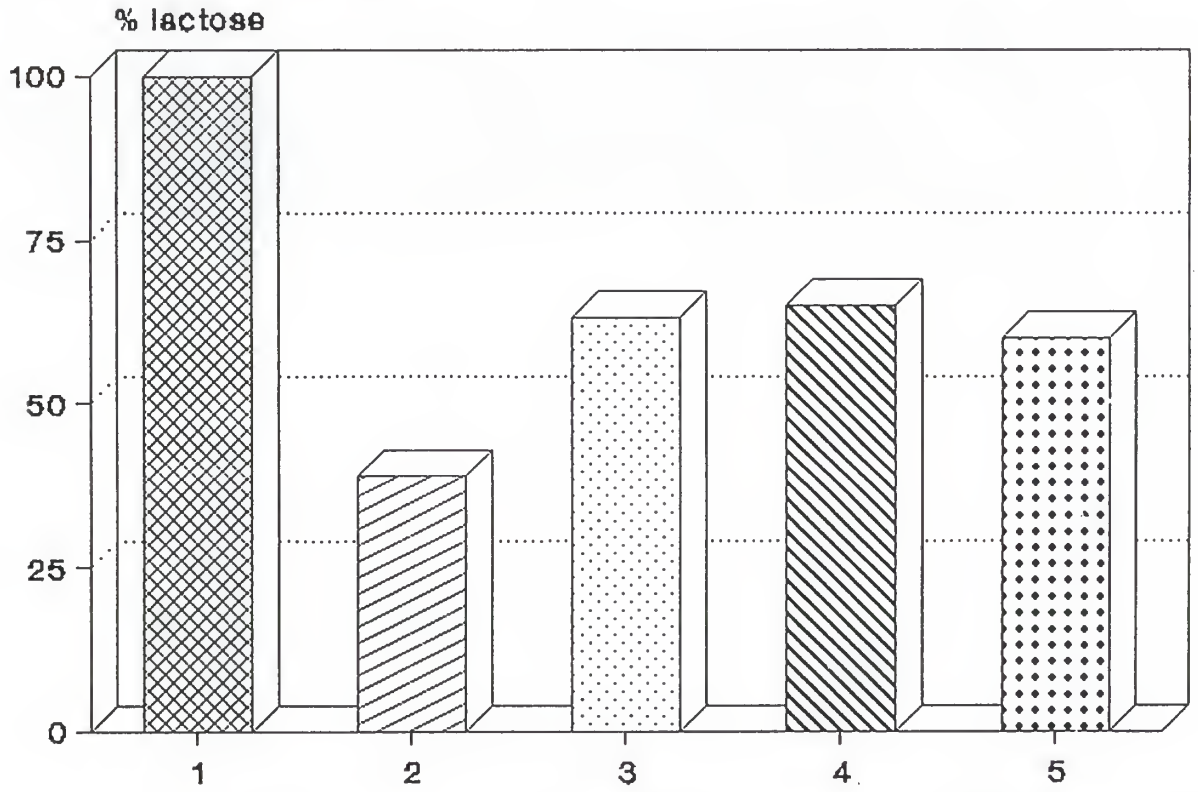


Figure 14 % Lactose content in various milk samples after 24 hours storage at 5 C.



1 = Skim milk

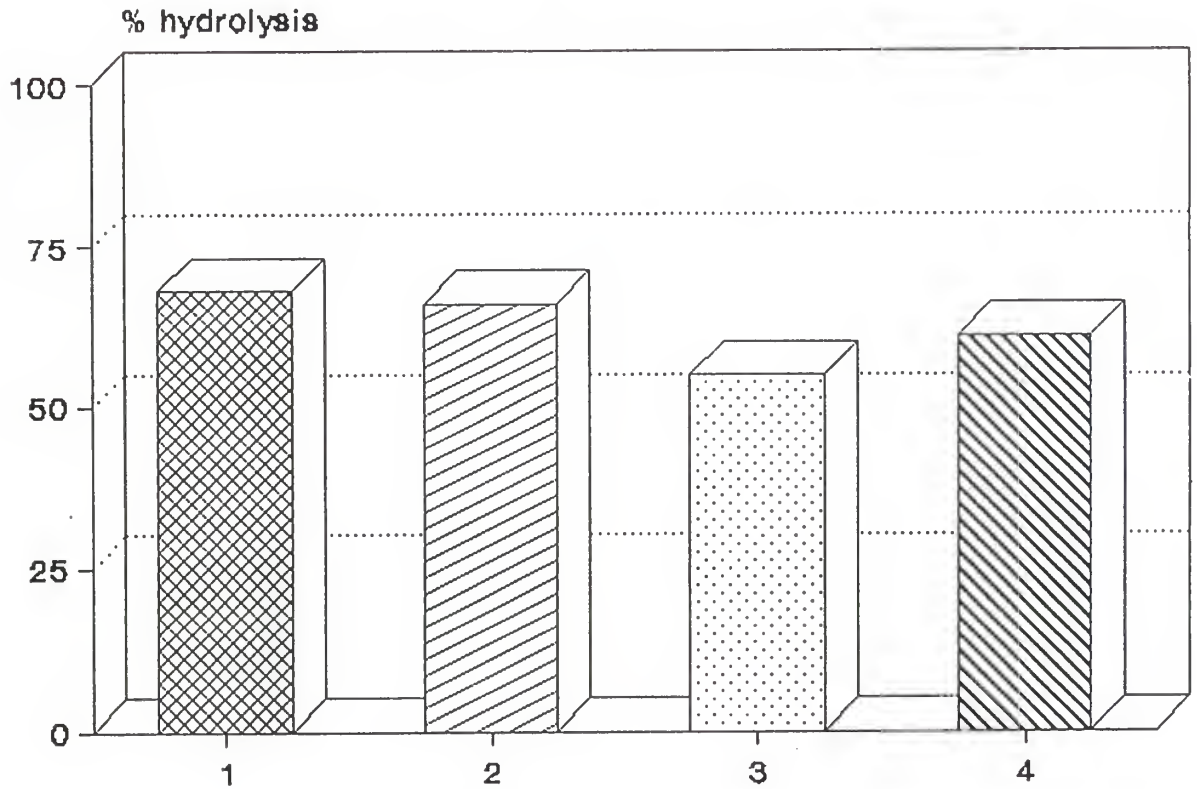
2 = Skim milk with free enzyme

3 = Skim milk with encapsulated enzyme

4 = Skim milk with encapsulated enzyme + gum arabic + carrageenan

5 = Skim milk with encapsulated enzyme + carrageenan + amylose

Figure 15. % Lactose hydrolyzed in various milk samples after 24 hours of storage at 5 C, acidification to pH3, and incubation at 37 C for 1 hour



CONCLUSIONS

The following conclusions can be drawn based on the results gained from this investigation.

1. Although β-galactosidase is classified as an acid enzyme, it is very active in both in the acidic and neutral conditions of milk. Further experimentation with a larger number of acid lactases is highly recommended. There still may exist an acid lactase that is active only in acidic conditions and also relatively cheap.
2. Enzyme encapsulation was successfully carried out using the Magee and Olson technique (1981a). Span 60 and Tween 60 were the most efficient emulsifier combination used in encapsulating the enzyme. Likewise, the list of emulsifiers used is not exhaustive. Other emulsifier combinations not studied in this investigation may give even better encapsulation results.
3. A stabilizer combination made of carrageenan and amylose gave the best results in reducing cream separation. Nevertheless, creaming continues to pose a problem during refrigerated storage of the product. More trials using a larger number of stabilizers is necessary. Cream separation can also be greatly reduced by avoiding some of the problems encountered during the encapsulation procedure as previously mentioned.
4. The emulsifiers and stabilizers used in this study did not hinder the release of the encapsulated enzyme after acidification to pH 3.0 and incubation at 37°C for one hour. Under similar conditions that exist in the human small

intestines, lactose hydrolysis will not be any problem. However, this is only an ideal situation and other factors present in the digestive tract should be considered. Therefore, actual consumption of this product by lactose-intolerant individuals is recommended in order to confirm this assumption.

5. Acid lactase milk was developed by enzyme encapsulation. Increased sweetness was effectively reduced for as long as five days of refrigerate ed storage as indicated by the amount of lactose hydrolyzed. A sensory evaluation by panelists is recommended for establishing the threshold of detectable increased sweetness of this product. The causes of increased hydrolysis during refrigeration need to be established and dealt with.

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DEVELOPMENT OF ACID LACTASE MILK

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ABSTRACT

Low lactose milk presently available on the market is considerably sweeter than regular milk, and the increased sweetness due to lactose hydrolysis is not acceptable to many lactose-intolerant consumers. It is desirable, therefore, to develop a lactase milk system that is not detectably sweeter than the regular milk. As a first step in the experiment, six analytical and food grade acid lactase samples were obtained from various commercial sources and evaluated for their activities in milk under acidic conditions. β-Galactosidase from Aspergillus oryzae was selected from this study because of its high activity in acidic pH as well as its relatively low cost. This enzyme was then evaluated for its activity in the natural pH of milk. It was found to be considerably active in an acidic environment, a property that is undesirable for the development of acid lactase milk. It was therefore necessary to restrict its activity in natural pH of milk by microencapsulation. To encapsulate the enzyme, the lactase was dissolved in distilled water (2.0 g/5 ml) and coated with a material that was composed of milk fat and various emulsifiers. The coating material used was prepared at 62 °C and cooled to 50 °C before addition of the dissolved enzyme. The encapsulated enzyme was then ejected into 100 ml fresh pasteurized skim milk by the use of a Wagner airless paint sprayer.

The milk was refrigerated at 5 °C for 10 days and a carbohydrate profile was obtained by HPLC analyses at specified intervals. The experimental and control milk samples were evaluated during refrigerated storage for cream separation, encapsulation efficiency, and enzymatic activity under similar temperatures and pH conditions found in the human stomach and small intestines. The most effective coating gave an encapsulation efficiency of 63.0%, and consisted of 35 g milk fat, 0.88 g Span 60, and 0.88 g Tween 60.

Cream separation was a problem encountered during refrigeration. To solve this problem, some stabilizers were incorporated at various concentrations into the emulsifiers. The combination that gave the highest stabilization efficiency (76.7%) included 0.35 g of carrageenan and 0.35 g amylose to the 35 g fat emulsion. However, the combination with the highest encapsulation efficiency (64.9%) was one containing an addition of 0.35 g gum arabic and 0.35 g carrageenan to the 35 g fat emulsion.