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HYDROLYTIC AND TRANSGALACTOSYLIC ACTIVITIES OF COMMERCIAL β-GALACTOSIDASE (LACTASE) IN FOOD PROCESSING

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I. INTRODUCTION

Food enzyme technology is the use of commercial sources of these biocatalytic compounds in the processing of food. The enzymes used for this

purpose may be derived from plant, animal, or microbial sources, but generally do not require much refining to reach food-grade status. Since the 1950s, most commercial enzymes have been derived from microbial sources, replacing many of the plant- and animal-derived enzymes first used in food processing. One of the more successful commercial microbial source enzymes in foods in recent years has been β-D-galactosidase (EC 3.2.1.23), commonly known as lactase, because its main action is the catalysis of the hydrolysis of lactose (milk sugar). Lactase hydrolyzes the β-D-galactoside bond between D-galactose and glucose. Since the only source of lactose is milk, the principal application of this enzyme has been as an additive (commercial food-grade lactase) to milk and milk products; the consequent incubation predigests the lactose and converts milk sugar into the component monosaccharides. One of the main reasons to consider producing this change in milk is the resulting increase in sweetness and solubility of the sugar. This approach takes advantage of two unique properties of enzymes:

1. The narrow specificity guarantees that only the desired reaction occurs and maintains the nutritional integrity of the food.
2. The catalytic function means that a very small amount of enzyme is required to produce a very large effect.

A. NUTRITION

Milk, nature's most nearly perfect food! This phrase has been used both to describe and to promote milk for years [Patton, 1969; National Dairy Council (NDC), 1977; Rosensweig, 1978]. The basis for this statement about a single food is the vital role milk plays in sustaining life, particularly for newborn mammals. Milk and other dairy foods have contributed significantly to human diets as sources of the essential nutrients protein, riboflavin, calcium, vitamin D, niacin, vitamin B_{12}, phosphorus, magnesium, vitamin A, zinc, and iodine (Phillips and Briggs, 1975; NDC, 1977; Savaiano and Kotz, 1988). The nutrients in milk have also found increasing roles as functional ingredients in an amazing variety of food products (Jonas, 1973; Jost, 1993; Allen, 1993).

1. Sugar Malabsorption

Problems with digestion and absorption of components in foods, such as the carbohydrates, is not a new issue (Townley, 1967). Early in this century, clinicians noted a correlation between dietary disaccharides and diarrhea. It has only been in the past 30 years, with the wide availability and dependence on milk and dairy foods in the food supply, that milk has created

serious problems for those individuals described as lactose intolerant (Solomons, 1986). Perhaps 70% of the world's population may develop gastrointestinal problems after ingesting milk products or foods containing dairy ingredients that incorporate sufficient amounts of the milk sugar lactose (Kretchmer, 1972; Rosensweig, 1978; Savaiano and Kotz, 1988). Similar to other mammals, after weaning, humans lose the majority of intestinal lactase (β-galactosidase) activity required for lactose digestion (Kretchmer, 1972). The majority of world population groups that have gastrointestinal problems with milk lose the most lactase and have genetic origins in zones of the world where milk has not been a component of the traditional diet (Kretchmer, 1972; Rosensweig, 1978; Simoons, 1981). The small proportion of the world's adults (about 30%) who have adapted to dairy foods by maintaining ample lactase levels genetically originated in areas with a long tradition of milk consumption (Simoons, 1981; Savaiano and Kotz, 1988). In the United States, the lactose-intolerant population varies widely, depending on genetic origin (Holsinger and Kligerman, 1991). It is estimated that on average about one-third of the U.S. population has lactose digestion difficulty (Houts, 1988; Holsinger and Kligerman, 1991).

Low intestinal lactase levels result in the gastrointestinal discomfort experienced by lactose-intolerant individuals. Intestinal lactase, immobilized in the brush border of the small intestine surface, accomplishes both digestion and absorption of dietary carbohydrate (Gray, 1971,1975; Rosensweig, 1978). Lactase deficiency is a reduction in the sites available to complex with dietary lactose and remove sugar from the intestinal fluid. The lactose bound to the enzyme at the brush border undergoes hydrolysis and absorption. The extent of lactose intolerance symptoms depends on the level of active lactase remaining after the postweaning decline and the ratio of lactase to ingested lactose. Following consumption of milk or dairy foods, lactose not bound and digested in the small intestine travels to the large intestine, where fermentation by the microorganisms converts the sugar into acids and gas (Wolin, 1981). The result is the rapid onset of flatulence, pain, and diarrhea, the classic symptoms of lactose intolerance. This problem is much more prevalent than the adverse reactions individuals can have to other components in milk, as in allergies that involve the body's immune system but do not impair the ability to digest lactose (Solomons, 1986; Matsuda and Nakamura, 1993).

The decrease in intestinal lactase levels with age, increase in symptom production, and resulting discomfort from ingesting lactose lead to the avoidance of dairy foods (Paige, 1981). Although these immediate effects of lactase deficiency represent natural consequences, the long-term impact has serious nutritional complications (Newcomer, 1981). A decline in consumption of dairy foods has been linked to poor nutritional status (Phillips

and Briggs, 1975). Phillips and Briggs found that adults who limit or omit milk from the diet run the risk of low intake of calcium and possibly other nutrients such as riboflavin, thiamine, phosphorus, and vitamin A. Dairy products contribute 42% of the riboflavin and up to 20% of the thiamine in the U.S. diet. Great concern has been focused on the loss of calcium in the diet since Birge *et al.* (1967) connected osteoporosis with intestinal lactase deficiency, which was affirmed in reviews of bone metabolism and biochemistry (Lutwak, 1975) and metabolic bone disease (Nutrition Reviews, 1979).

As dairy products account for just over half to perhaps three-quarters of the calcium intake by the U.S. population (Phillips and Briggs, 1975; Allen and Wood, 1994), there is cause for concern about adequate intake if people avoid milk. There has been increasing evidence that adequate dietary calcium not only helps prevent brittle bone disease (Chapuy *et al.*, 1992), but may help mitigate the risk from several other serious and chronic diseases (CRH, 1994; Heaney and Barger-Lux, 1994). Current recommendations for calcium intake are 1000 mg/day for all adults and 1500 mg/day for postmenopausal women (Heaney, 1993; CRH, 1994; Heaney and Barger-Lux, 1994). These levels can easily be met only if people consume milk-based foods. Calcium intake has also been linked to a role in blood pressure regulation and control of hypertension [National Academy of Sciences (NAS), 1989; Allen and Wood, 1994]. Other evidence suggests that increased calcium in the U.S. diet could be effective in lowering total and low density lipoprotein cholesterol (Bell *et al.*, 1992; Denke *et al.*, 1993). It has been estimated that the effects of elevated dietary calcium levels on cholesterol and hypertension could reduce the risk of heart attack by more than 20% (CRH, 1994). A high calcium intake in excess of the recommended daily allowance (RDA), 1000 mg, can also be protective against colon cancer (NAS, 1989; Allen and Wood, 1994; CRH, 1994). High intakes of calcium, vitamin D, and milk products could reduce the risk of colon cancer 20 to 30% (Bostick *et al.*, 1993). It thus seems clear that this extremely valuable nutrient must be made available to people at levels higher than those currently in existence. Most Americans consume too little of this valuable nutrient, and avoiding milk products prevents those individuals from obtaining the vast potential health benefits of calcium (Heaney and Barger-Lux, 1994).

2. *Lactase*

As most adults are lactose intolerant because they have insufficient quantities of lactase in their small intestine, these individuals tend to avoid milk because of the risk of serious discomfort. Development of commercial

lactase (β-galactosidase) has stimulated the production of lactose-reduced foods (Rand, 1981). This enzyme is able to hydrolyze lactose into the monosaccharides glucose and galactose, which are readily absorbed by the small intestine (Paige *et al.,* 1975; Turner *et al.,* 1976; Hourigan and Rand, 1977; Cheng *et al.,* 1979; Reasoner *et al.,* 1981). Short-term studies have established that lactose-intolerant individuals who consume low-lactose milk show improved absorption and reduced symptoms, and that the increase in osmolarity caused by the hydrolysis does not appear to have any side effects. The change in taste, resulting from the increased sweetness of the monosaccharides, has caused palatability problems. Cheng *et al.* (1979) demonstrated the acceptability of low-lactose milk over an extended period. Lactose-intolerant individuals are now able to benefit from the nutrients supplied by milk, thus reducing the danger of low intake of calcium, phosphorus, vitamin A, and riboflavin (Rand, 1981).

The impact of lactase on predigesting the lactose in milk and making the food available to more people raises some questions on the nutritional changes produced. It has been known since the 1930s that lactose has a stimulating effect on calcium absorption (NDC, 1977; Pansu *et al.,* 1979). In fact, lactose has profound effects on the absorption and retention of many minerals, including iron, zinc, magnesium, and manganese (Bushnell and DeLuca, 1981). What happens when lactose is converted to glucose and galactose by enzyme treatment? It appears that the presence of the monosaccharides does not alter the absorption of nutrients (Kobayashi *et al.,* 1975; Vega *et al.,* 1992).

B. FOOD PROCESSING

1. Commercial Enzyme Sources

Lactase (β-galactosidase) is widely distributed in nature and has been found in plants, animals, and microorganisms (Pomeranz, 1964a; Mahoney, 1985; Agrawal *et al.,* 1989). For use in food processing, however, the enzyme must be derived from sources that are classified "generally regarded as safe" (GRAS) by the Food and Drug Administration (FDA). Sources that have been regarded as food constituents have found easy acceptance as GRAS or in the Food Chemicals Codex (FCC) (Nelson, 1980). The approved sources of lactase all fall in the category of fermentation-derived enzymes, primarily from yeasts and molds (Mahoney, 1985; Agrawal *et al.,* 1989; Holsinger and Kligerman, 1991; Bigelis, 1993). Current production seems to be restricted to the molds *Aspergillus niger* and *Aspergillus oryzae* and the yeasts *Kluyveromyces lactis, Kluyveromyces fragilis,* and *Candida pseudotropicalis* (Kuntz, 1993). All enzymes from these sources and from

bacteria closely related to *Bacillus stearothermophilis* can be used to hydrolyze the lactose in milk and alleviate the problems with milk consumption by lactose-intolerant populations (Mahoney, 1985). These enzymes have varied properties that provide strengths and weaknesses for a variety of applications. A review of lactase products from commercial suppliers indicates that only sources derived from yeasts and molds are currently available for industrial application.

2. *Low-Lactose Milk*

The mold lactases can be used to produce low-lactose milk, but work best at higher temperatures, 50° to 60°C, and lower pH, 3 to 5. These enzymes can be used to hydrolyze lactose at temperatures close to that of pasteurization, which minimizes growth of more than 90% of common spoilage bacteria (Rand and Linklater, 1973; Nijpels, 1981; Rand, 1981). Figure 1 illustrates the possible use of *A. niger* lactase to process milk during pasteurization. This enzyme can be added to the milk while cold; it is then heat activated as the temperature is raised to 63°C. Hydrolysis of lactose occurs during the 30- to 60-minute holding period. The lactose

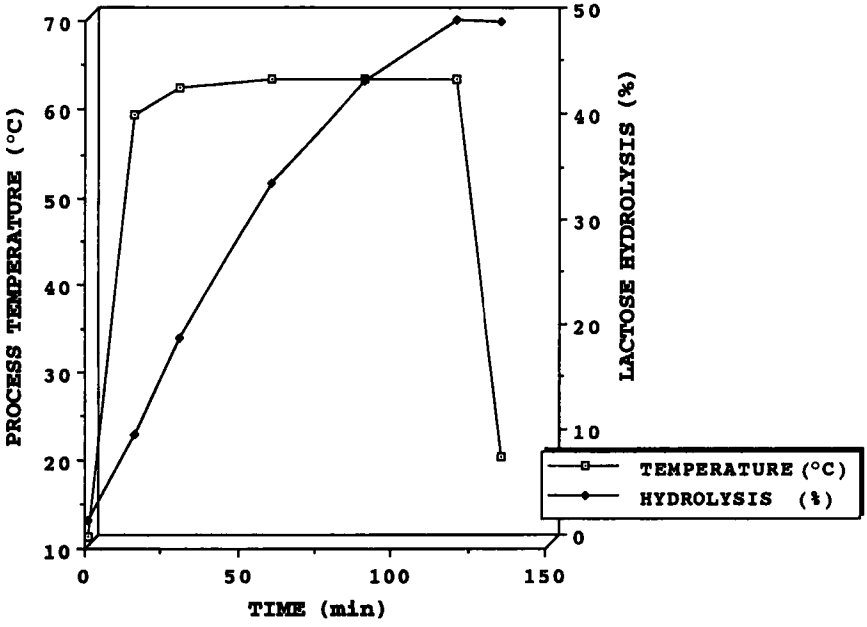


FIG. 1. Processing of lactose-hydrolyzed milk with fungal lactase (enzyme concentration = 0.33% w/v). Reprinted with permission from Rand and Linklater, 1973.

in milk is reduced while pathogenic microorganisms are eliminated and growth of spoilage bacteria is minimized. This illustrates how lactose reduction of milk products and pasteurization can be combined employing thermostable lactases. These enzymes might even survive conventional high temperature short time (HTST) pasteurization (Rand, 1981); however, the pH optima for these enzymes do not correspond well to that of milk, and they would be better suited for use in processing whey and acidified products (Nijpels, 1981; Holsinger and Kligerman, 1991; Bigelis, 1993).

The yeast lactases operate best near neutral pH and at milder temperatures, 30° to 40°C, conditions that prove very effective for production of low-lactose milk. This commercial enzyme source permits lactose hydrolysis in milk before or after pasteurization. The enzyme can be added to pasteurized milk, which is then incubated near the optimum temperature, about 30° to 35°C, for about 2 to 5 hours, hydrolyzing 70 to 90% of the lactose (Hourigan and Rand, 1977; Guy and Bingham, 1978; Mahoney, 1985). This, however, introduces an additional process step and additional expense. An approach has been proposed that avoids this problem: a very small amount of food-grade filter-sterilized yeast lactase is injected into ultrahigh-temperature (UHT)-sterilized and packaged milk (Dahlqvist *et al.,* 1977). This product can be stored at room temperature and undergoes nearly complete lactose hydrolysis in 7 to 10 days. The enzyme used for this process must be free of protease (and other hydrolases) to prevent deterioration of the milk during storage (Mahoney, 1985). In an alternative process, which has proved most successful in the United States, lactose hydrolysis is induced in milk before pasteurization by adding yeast lactase to cold milk (Hourigan and Rand, 1977; Guy and Bingham, 1978; Reasoner *et al.,* 1981). This treatment can accomplish up to 90% lactose hydrolysis while milk is held overnight at 4° to 6°C, a normal step before processing, which minimizes the growth of spoilage microorganisms.

3. Processing by the Consumer

The capability for low-temperature processing of low-lactose milk with yeast lactase led to the concept of low-lactose milk processing by the consumer (McCormick, 1976; Kligerman, 1981; Holsinger and Kligerman, 1991). Initially, lactase could be purchased in a powdered form, as packets, to treat a quart of milk overnight in the home refrigerator. This unique marketing concept, developed by Lactaid, Inc., made low-lactose milk available to many consumers long before commercial reduced-lactose fluid milk was in distribution in many areas of the United States. Now, the enzyme for home processing comes in a sterile liquid form, which is much more stable and easy to use.

Another alternative method for producing low-lactose milk at home supplies lactase to the consumer as a dose form (DeAngelis *et al.*, 1979). The initial product consisted of yeast lactase encapsulated in algin beads, partially dehydrated with sugar to yield a reduced water activity (aw), subcoated, and enteric-coated capsule as shown in Fig. 2. The final product was packaged in gelatin capsules. The enzyme is delivered to the site of lactose digestion and absorption, the small intestine. The efficacy of this lactase dose form was tested in a clinical trial, as shown in Fig. 3. The average symptoms of lactose intolerance per person were reduced by the administration of one or two capsules just prior to the consumption of

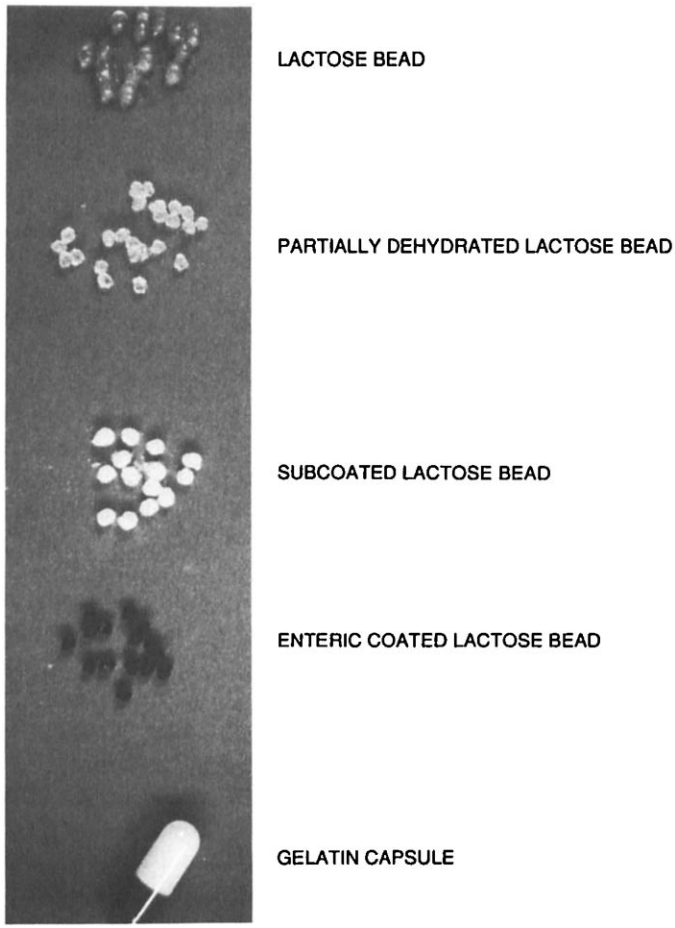


FIG. 2. Stages involved in lactase immobilization and development of a capsule dose form.

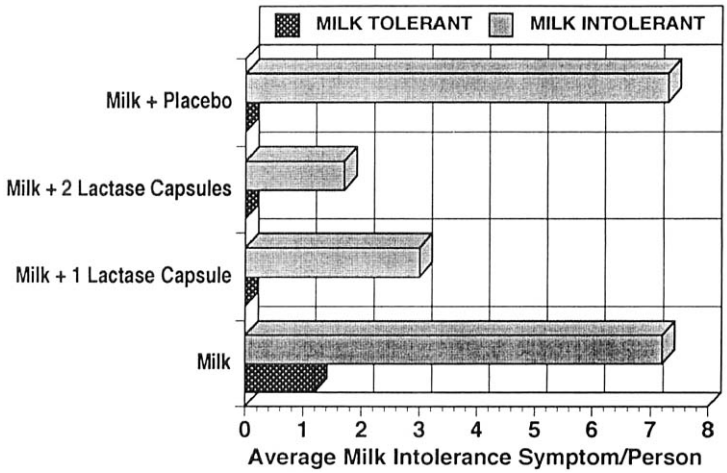


FIG. 3. Effect of lactase capsule dose form on milk intolerance symptoms with 240 ml of skim milk. Average symptom = response to 4 symptoms of bloating, cramps, flatulence, and diarrhea. Severity rated as 1 = mild, 2 = moderate, and 3 = severe.

240 ml of skim milk. Commercial forms of this product were introduced in the mid-1980s (Holsinger and Kligerman, 1991). Now tablets containing combinations of yeast and mold lactases are available for consumers to take with a meal. Thus, whenever lactose-intolerant individuals suspect they have ingested or intend to ingest foods containing lactose, they can mitigate the symptoms by taking one of these dose forms.

4. Lactase Product Inhibition

Utilization of low-lactose hydrolyzed milk has been hampered because of product inhibition, attributed to the formation of galactose (Rand and Linklater, 1973; Hourigan and Rand, 1977; Rand, 1981). Product inhibition has represented a major disadvantage to batch-type processing. Large quantities of expensive enzyme have been required to increase the degree of lactose conversion in the process, which has normally been conducted over a period of about 24 hours while the milk was refrigerated. Reduction of galactose during lactose hydrolysis would greatly increase enzyme efficiency. This concept can be visualized comparing the kinetic constants for galactose inhibition in the two major classes of food-grade lactases. Studies comparing the major data have been reviewed by Rand (1981) and Mahoney (1985) and are summarized in Fig. 4. The K_m values differ for mold and yeast lactases, depending on the medium used for evaluation. *A. niger* lactase had better affinity for lactose in milk than in buffer, whereas *K.*

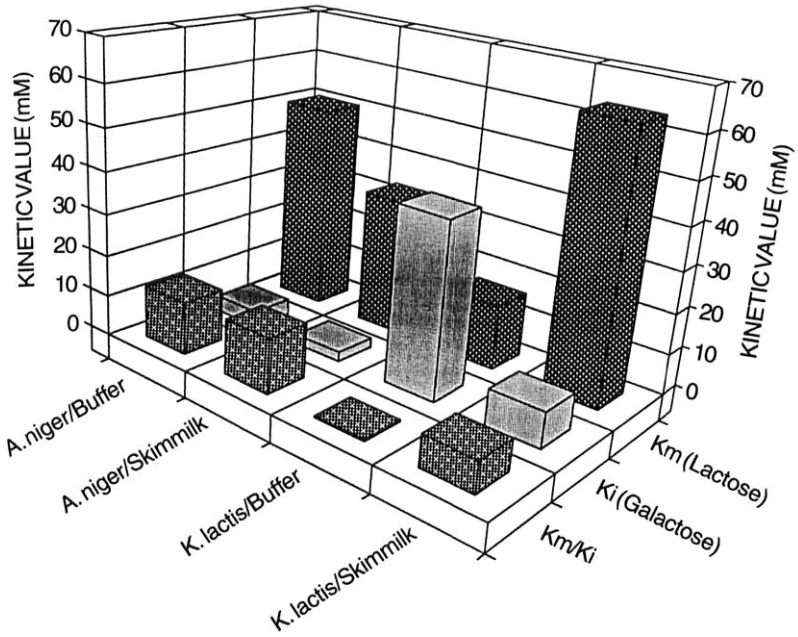


FIG. 4. Comparison of kinetic values and product inhibition for *A. niger* and *K. lactis* lactases in both buffer and skim milk.

lactis enzyme performed more efficiently in buffer than in milk; however, the affinity for galactose, as a competitive inhibitor, was always higher for the mold enzyme. Thus, the K_m/K_i ratios reveal that *K. lactis* lactase performs better than *A. niger* enzyme in both milk and buffer when lactose is the substrate. It was also clear that any method that could remove the galactose inhibitor would improve the performance of both enzymes, but particularly the yeast enzyme, which did not perform as well in the food system.

Enzymatic modification of galactose during lactose hydrolysis to reduce the inhibitor was first proposed by Finnie *et al.* (1979). β-Galactose dehydrogenase was coupled with lactase to specifically remove the inhibitor and convert the sugar to the corresponding lactone with an impact during reactions in buffer (Rand, 1981). This coupled enzyme approach was evaluated during the hydrolysis of lactose in milk at 4°C with three lactase enzymes, and the results are compared in Fig. 5. The most significant impact was observed with *K. fragilis* lactase (KFL) (Fig. 5A). Hydrolysis of milk proceeded over 24 hours and the effects of coupled enzyme modification of galactose improved as the reaction proceeded. The effect of coupled

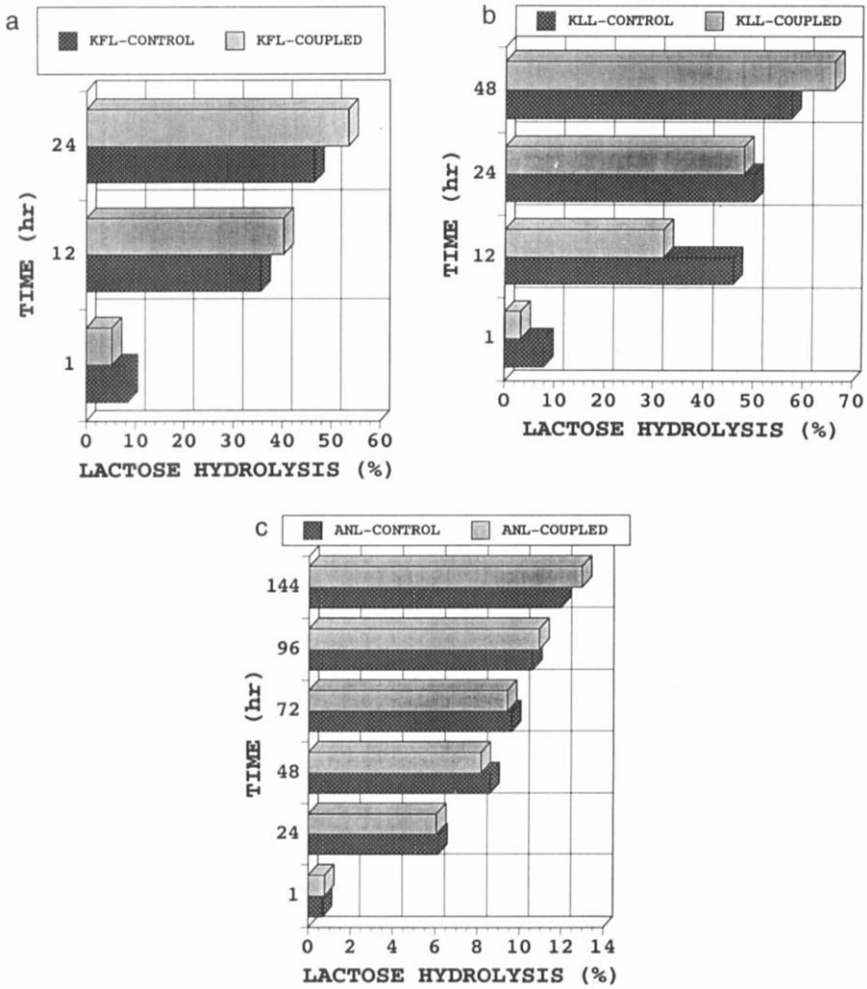


FIG. 5. (a) Hydrolysis of lactose in milk with *K. fragilis* lactase (KFL) at 4°C and coupled with β-galactose dehydrogenase (β-GDH). (KFL = 0.03 unit/ml, β-GDH = 0.04 unit/ml, 0.17 mg/ml NAD.) (b) Hydrolysis of lactose in milk with *K. lactis* lactase (KLL) at 4°C and coupled with β-galactose dehydrogenase. (KLL = 0.04 unit/ml, β-GDH = 0.04 unit/ml, 0.17 mg/ml NAD.) (c) Hydrolysis of lactose in milk with *A. niger* lactase (ANL) at 4°C and coupled with β-galactose dehydrogenase. (ANL = 0.09 unit/ml, β-GDH = 0.04 unit/ml, 0.17 mg/ml NAD.)

enzyme hydrolysis with *K. lactis* lactase (KLL) was less dramatic (Fig. 5B). Addition of β-galactose dehydrogenase did not improve the process until significant amounts of galactose had accumulated, but when conversion

exceeded 50%, the coupled system began to have a definite impact. The control hydrolysis reaction clearly began to slow down in efficiency when about half the lactose had been hydrolyzed to galactose; however, the coupled enzyme system continued to proceed at a relatively constant rate from 12 to 48 hours, showing minimum impact of galactose inhibition. Coupled enzyme hydrolysis with *A. niger* lactase (ANL) (Fig. 5C) progressed at a much slower rate, as expected at refrigeration temperatures, and did not reach the conversion levels of the yeast lactases; however, it seems clear that the coupled enzyme system was slowly improving the performance of the mold enzyme over the control process. This approach demonstrates the potential to improve significantly the efficiency of lactase enzymes to food systems by reducing the inhibitory galactose product.

The effect of galactose on lactase activity during milk processing can be described as typical competitive inhibition (Rand, 1981; Mahoney, 1985). This means that the effects of inhibition can be overcome by keeping the substrate concentration much higher in the vicinity of the enzyme. This might be the way the mammalian small intestine deals with galactose inhibition of lactase during digestion. Intestinal lactase, immobilized on the apical surface of the intestinal mucosa, permits rapid hydrolysis and absorption of the sugars in individuals who are not lactase deficient (Gray, 1975). Intestinal hydrolysis of lactose is much more efficient than that in milk, as the reaction is controlled by an absorptive process that maintains higher concentrations of substrate near the enzyme and rapidly removes products. This approach to minimizing product inhibition of lactase enzymes was evaluated in a membrane reactor that localized *K. lactis* lactase to the lumen side of a hollow fiber in a pressure-induced regime (Maculan *et al.*, 1978). The results from this study are presented in Fig. 6. It is clear that as the pressure increased from 2.5 to 10 psig, the flux or flow across the enzyme layer and the membrane also increased from 52 ml/h up to nearly 360 ml/h. The concentration of glucose in the permeate decreased as flux increased and contact time with the enzyme layer decreased, but the actual rate of glucose production increased from 180 mg/h at 2.5 psig to 244 mg/hr at 10 psig. Thus, the enzyme operated more efficiently when the substrate concentration was kept high in the vicinity of the enzyme, while displacing galactose and removing this competitive inhibitor through the membrane.

It is clear from these studies that any approach that reduces or removes galactose from the system during lactose hydrolysis has the potential to improve the efficiency of lactase enzymes. The implications for milk processing could result in the use of smaller amounts of enzyme, thereby reducing cost and saving time in processing.

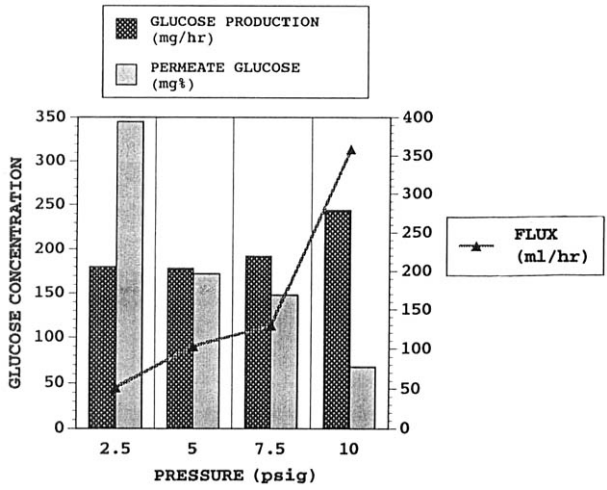


FIG. 6. Effect of lactose flow rate as pressure on flux and glucose production for a localized lactase UF bioreactor. (*K. lactis* lactase = 1000 $\mu g/cm^2$, temperature = 30°C.)

5. *Lactase in Ice Cream*

Some of the earliest applications of lactase in food processing occurred in frozen products like ice cream (Pomeranz, 1964b). The original goal was to eliminate crystallization of the lactose and sandiness, but these problems have been almost completely eliminated by the increased use of stabilizers. The reduction of lactose content could have another benefit: the ability to use increased amounts of whey and skim milk products in the formulation (Mahoney, 1985). It now appears that reduced-lactose ice cream mixes yield products with other improved properties and the potential for incorporation into reduced-calorie forms (El-Neshawy *et al.*, 1988; Alagialis *et al.*, 1990; Rand and Kleyn, 1991). Lactose hydrolysis has been shown to produce products of higher viscosity and whipping ability and products with higher overrun and improved organoleptic properties (El-Neshawy *et al.*, 1988). Alagialis *et al.* (1990) began with a premium-style ice cream of 16% fat and >40% total solids, which served as the control. The results, presented in Fig. 7, demonstrate that with lactose hydrolysis by lactase, advantage can be taken of the increased sweetness and the amount of sucrose in the formula can be reduced as one step toward calorie reduction. The control premium ice cream at 13.4% sucrose was evaluated on sensory scores for flavor, body/texture, melting, and color as 24.3 out of a maximum total of 25. When yeast lactase was added at 3.5% to the formula to achieve 96% hydrolysis of lactose, the sensory score decreased about 1 point, mainly

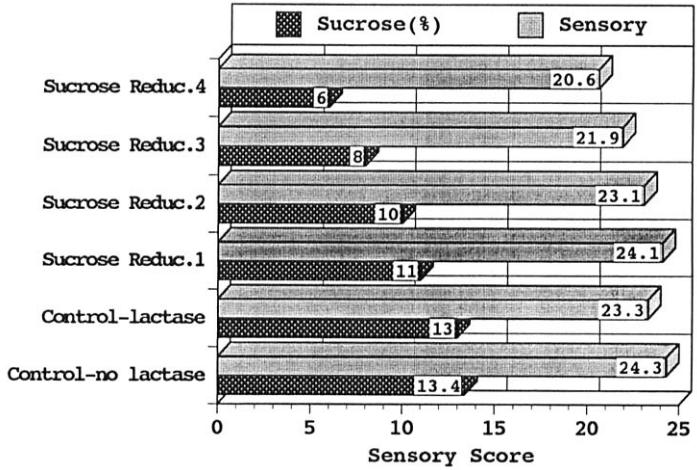


FIG. 7. Effect of lactose hydrolysis catalyzed by lactase on sucrose reduction as demonstrated by the sensory properties of premium ice cream. (Liquid lactase = 3.5%, Mix total solids = >40%, fat = 16%.)

because of a lower flavor score, which was attributed to increased sweetness. Reduction of the sucrose by 18% (11% sucrose) actually improved the sensory score to nearly the same as that of the control, demonstrating the benefits of improved bulking properties and the need to use less sucrose. Further reductions in sucrose to 10, 8, and 6% produced a steady decline in the sensory properties, which were reflected primarily in the flavor and body/texture scores as sweetness and bulking decreased. Rand and Kleyn (1991) evaluated the effects of lactase on the properties of nonfat ice milk of 31% total solids, as shown in Fig. 8. When formulated without lactase and frozen, this ice milk was evaluated as poor with respect to flavor, body/texture, and overall sensory perception. When yeast lactase was added to the mix, at 0.3%, and allowed to react overnight before freezing, the flavor and body/texture scores increased nearly 2.5 times and the overall sensory score substantially improved. The effects of lactase treatment on the properties of ice cream-type products was confirmed by a study from the NutraSweet Company (Keller *et al.*, 1991). It found that lactase had definite benefits in permitting the use of increased milk solids to replace sucrose. The enzyme treatment allowed the use of milk solids instead of alternative bulking agents for aspartame-flavored frozen dairy desserts.

6. *Lactase in Fermented Milk*

The lactose content of dairy mix intended for yogurt manufacture was shown by Goodenough and Kleyn (1976a) to average about 8.5%, which

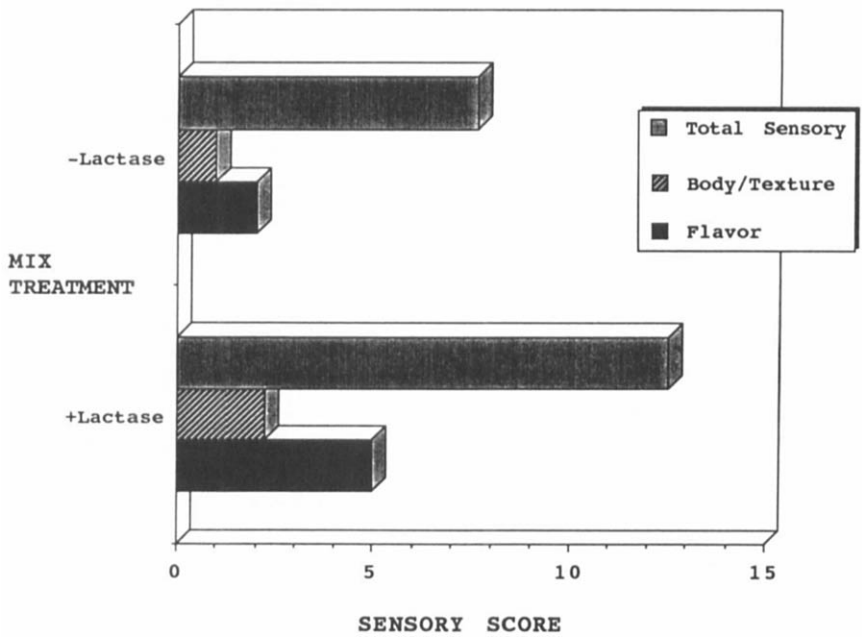


FIG. 8. Effect of lactase on the sensory properties of nonfat ice milk. (*K. lactis* lastase = 0.3%, Mix total solids = 31%.)

decreased 35% during fermentation to about 5.75%. This concentration was slightly greater than the normal lactose content of whole milk. Thus, it was somewhat surprising when consumption of lactose as yogurt, and other fermented dairy products, was found to cause minimal intolerance symptoms in lactase-deficient populations (Gallagher *et al.,* 1974; Kolars *et al.,* 1984; Gilliland, 1985). The beneficial effects of better digestion and utilization of lactose have been attributed in yogurt to the presence of active cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Kolars *et al.,* 1984; Kelley, 1984). An alternative approach to digestion problems caused by lactose was the use of sweet acidophilus milk, introduced in 1975 (Shearin, 1977). The beneficial effects on lactose digestion in humans of consuming nonfermented milk, following the addition of live *Lactobacillus acidophilus* cells, have been reported (Gilliland, 1985). Subsequently, these digestive benefits have been attributed more specifically to the presence of substantial amounts of active lactase from the cells of the microbial cultures, which contribute to lactose digestion after consumption of the food (Kilara and Shahani, 1974; Goodenough and Kleyn, 1976b; Onwulata *et al.,* 1989; De Vrese *et al.,* 1992). These studies attempted to measure

lactase activity either in the microbial cells in the food or in the intestinal tract after consumption. This led to the understanding that the lack of digestive problems after consuming these foods is due to the active cells and enzymes; this has discouraged a trend toward heat processing these products (Kelley, 1984; Gilliland, 1985).

Lactase has also found applications in the processing of fermented dairy foods. Although the microorganisms used to ferment dairy foods have been adapted to effectively ferment lactose, the process of hydrolysis before use appeared to be a rate-limiting step (Nijpels, 1981; Mahoney, 1985). Manufacture of yogurt from low-lactose mix has shown several benefits. Hydrolysis of lactose may reduce the gelation time for yogurt by 15 to 20% (Nijpels, 1981; Hilgendorf, 1981), body and texture properties may be improved (Dariani *et al.*, 1982), and the enhanced sweet taste can improve acceptability without increasing calories (Engel, 1973; Nijpels, 1981; Hilgendorf, 1981). The process of lactose hydrolysis may also permit the development of other approaches to manufacturing fermented dairy products, such as combining enzymatic and microbial acid development for the bioprocessing of yogurt (Tahajod and Rand, 1993). The effect of lactose hydrolysis by lactase on promoting enzymatic acidification of milk by glucose oxidase (GOX) for yogurt manufacture is shown in Fig. 9. This study demonstrated the impact of two yeast lactase concentrations on the production of glucose and the corresponding conversion to gluconic acid by GOX. Within 30 minutes, lactase at each concentration produced sufficient glucose for acidification of milk by GOX, which was added at that point, followed by hydrogen peroxide. The pH of milk declined to pH 5.6 to 5.7 within an additional 30 minutes, which was considered optimum for the addition of *Lactobacillus bulgaricus* to complete the conversion of yogurt. Lactose hydrolysis may also benefit the manufacture of fresh cheeses, like cottage cheese, resulting in a shorter gelation time, a firmer curd, and perhaps a 10% increase in yield (Nijpels, 1981). The process for making aged cheese, like cheddar, has also shown potential benefits from lactase treatment. The acidification step has been shown to occur at a slightly higher rate and may produce higher bacterial counts (Nijpels, 1981). The principal effect of lactase treatment has been shown to be a reduction in the expensive ripening time for these cheeses, due to acceleration of the aging process (Anonymous, 1977; Olson, 1979).

7. *Immobilized Lactase Systems*

Whether it is used to solve nutritional problems (lactose intolerance) or to improve the product (lactose solubility, sweetness, functionality), enzymatic hydrolysis must be economically feasible. The additional cost of

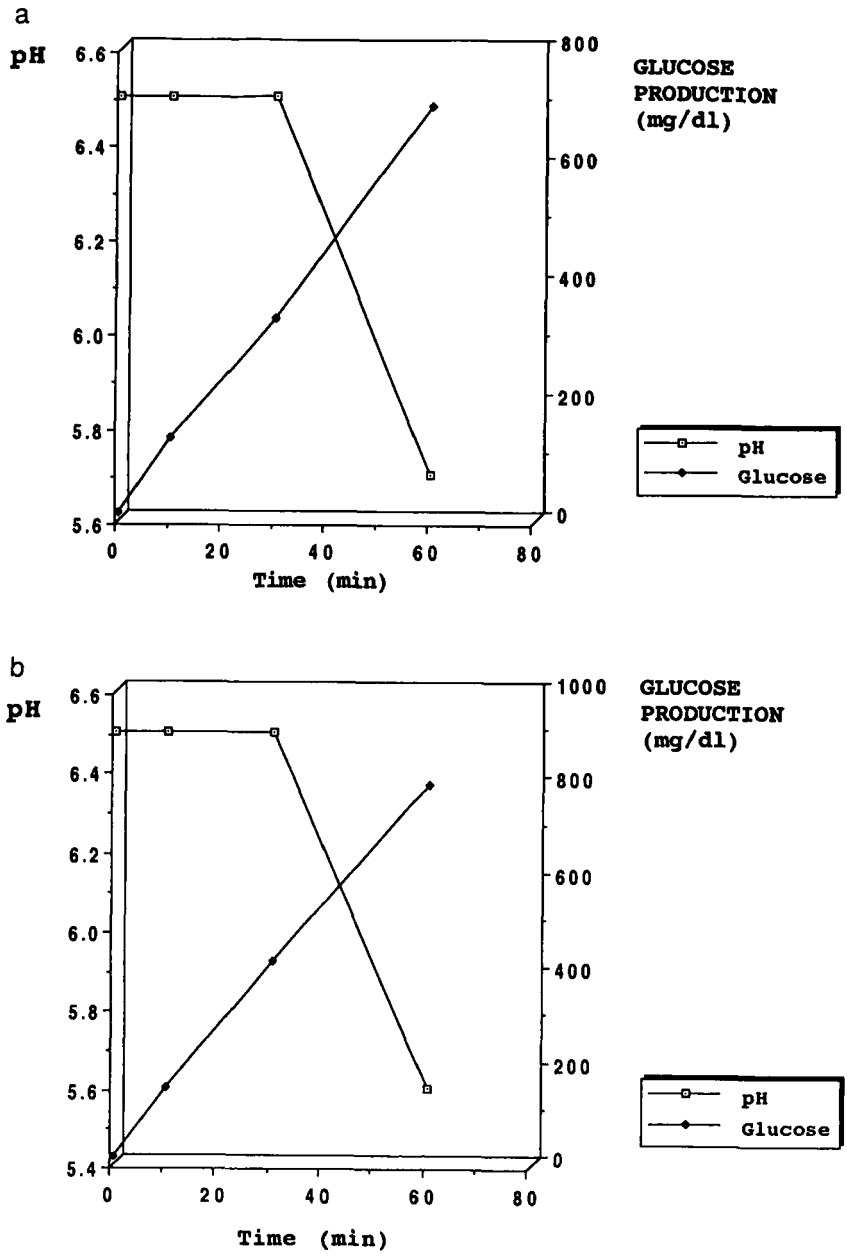


FIG. 9. Effect of lactase concentration on glucose production and acid formation by glucose oxidase in skim milk at 37°C: (a) 0.25 mg yeast lactase/ml milk; (b) 0.38 mg yeast lactase/ml milk. (Glucose oxidase = 2.5 units/ml, added at 30 min; 0.3 ml 50% H_2O_2 added in 0.1-ml increments at 10-minute intervals.)

adding soluble, food-grade lactase to milk has aroused interest in the use of immobilized systems for recovery and reuse of the expensive enzyme component. Immobilization of lactase has been reported employing virtually all of the major systems available: adsorption, covalent bonding, gel entrapment, and containment by semipermeable membranes (Agrawal *et al.,* 1989).

Adsorption of lactase to an immobilizing matrix has been reported using a variety of materials including phenol–formaldehyde resins (Olson and Stanley, 1973; Stanley and Palter, 1973; Okos *et al.,* 1978), alumina (Charles *et al.,* 1975; Finocchiaro *et al.,* 1980b), stainless steel (Charles *et al.,* 1975), and hydrophobic attachment to zeolites (Zentgraf and Gwenner, 1992). Covalent bonding of lactase to various supports has been the subject of studies with cellulose sheets (Sharp *et al.,* 1969), the most common matrix of porous glass beads (Woychick and Wondolowski, 1972; Wierzbicki *et al.,* 1973; Weetall *et al.,* 1974a,b Okos and Harper, 1974), gelatin (Sungur and Akbulut, 1994), solid-phase Ni(II) chelate (Irazoqui and Batista-Viera, 1994), and corn grits (Siso and Doval, 1994). The use of gel entrapment for lactase immobilization has been the subject of studies with polyacrylamide slices (Bunting and Laidler, 1972) and polyacrylamide beads (Nilsson *et al.,* 1972), which resulted in its application to production of low-lactose milk (Dahlqvist *et al.,* 1973). The gel entrapment process evolved to use of the more acceptable food-grade algin beads for lactase immobilization (DeAngelis *et al.,* 1979, Jacober-Pivarnik and Rand, 1984) and the development of fiber-entrapped lactase for milk processing (Dinelli, 1972; Morisi *et al.,* 1973). The entrapment process has also led to a unique approach to the use of immobilized enzymes for lactose hydrolysis by incorporating the lactase enzyme into the packaging material (J. A. Budny, personal communication, 1989). This approach has the potential to convert lactose in fluid milk products following processing and packaging.

The last method of lactase immobilization, which holds perhaps the most promise, is ultrafiltration (UF) and the containment of enzymes by semipermeable membranes. Membrane technology has become standard in the food industry, which means the equipment and technology have become widely available to take advantage of the size differences between enzyme and product (Cheryan, 1986; Cuperus and Nijhuis, 1993). The initial UF systems for immobilizing lactase employed continuous stirred tank reactors (CSTRS), in which the enzyme recirculated with the substrate, while the glucose and galactose products were removed through the membrane (Miller & Brand, 1980; Huffman-Reichenbach and Harper, 1982). The CSTR system was not very efficient for a product-inhibited enzyme process and has never achieved commercial success. Plug-flow reactor (PFR)-based systems consisting of a single pass showed much greater prom-

ise for lactase-catalyzed conversion of lactose, because at least part of the enzyme–matrix would always be exposed to fresh substrate to minimize product inhibition and maximize activity (Charles *et al.*, 1975; Finocchiaro *et al.*, 1980a). The use of a lactase tubular reactor was introduced by Ngo *et al.* (1976); the enzyme was placed on the inner surface of nylon tubes and shown to be potentially efficient. This type of enzyme reactor was applied to UF membranes by employing hollow-fiber technology (Breslau and Kilcullen, 1975; Huffman-Reichenbach and Harper, 1982; Jones *et al.*, 1988). Thus lactase UF bioreactors moved away from the CSTR mode and closer to the PFR type. The enzyme is added to the membrane on the sponge side (outside surface) of the hollow fiber, and lactase is kept in place essentially by adsorption and entrapment. If the enzyme is not permanently bound to the sponge layer, removal is achieved relatively easily by simply reversing flow during cleaning, and the membrane is immediately made ready for reloading. As already discussed, Maculan *et al.* (1978) demonstrated the potential of operating a UF reactor as an intestinal model, using pressure as the immobilizing force, as shown in Fig. 10. In this approach, the enzyme is layered on the lumen side of the hollow fiber and immobilized by pressure, allowing operation as a PFR with improved efficiency. This type of bioreactor was evaluated on skim milk UF permeate and found to operate efficiently at 5 psi and 30°C with a yeast lactase (Senecal and Rand, 1992a). The lactase UF bioreactor shows great potential for treatment of milk and whey permeates, and could be coupled into a system such as that

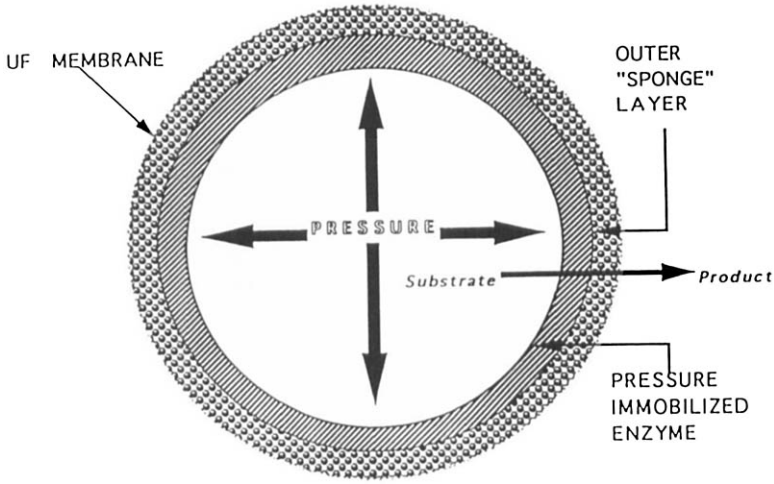


FIG. 10. Pressure-induced immobilization of lactase on the surface of a hollow-fiber ultrafiltration (UF) membrane.

proposed by Miller and Brand (1980). This would permit separation of milk into retentate and permeate fractions as shown in Fig. 11. The separation process would be coupled directly to hydrolysis of the lactose in the permeate stream by the UF bioreactor. Retentate and permeate streams could then be recombined to produce low-lactose milk products. The main advantage of this approach is that the bioreactor would be kept essentially free of protein fouling and microbial contamination, which would enhance its potential to operate continuously for extended periods.

C. WASTE UTILIZATION

1. Whey

The largest waste problem for the dairy industry is whey: sweet whey as the by-product of cheese or rennet casein with a pH greater than 5.5, and acid whey derived from the manufacture of cottage cheese or acid casein with a pH less than 5.0 (McDonough, 1977; Shahani *et al.*, 1978; Short, 1978; Newton, 1991; Siso and Doval, 1994). Only about half of the total world whey production has been used as human or animal food; the rest continues to be discarded, causing serious environmental problems (McDonough, 1977; Shahani *et al.*, 1978, Siso and Doval, 1994). The major constituuent of all types of whey is lactose, followed by high-quality protein as the second ingredient (Short, 1978). Whey proteins have usually been recovered by UF and used in foods as protein concentrates (Brinkman, 1976; Shahani *et al.*, 1978; Siso and Doval, 1994); however, protein recovery

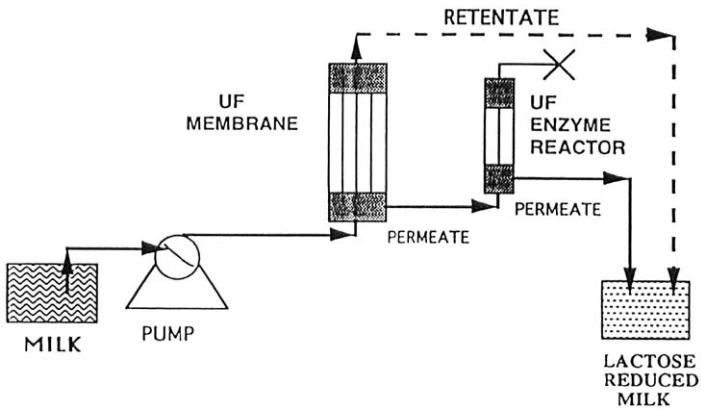


FIG. 11. Ultrafiltration process for the separation of milk and subsequent hydrolysis of lactose in the permeate stream with a hollow-fiber lactase bioreactor.

does not solve the environmental problems caused by whey, as most of the difficulties are due to lactose, which remains in the permeate (Siso and Doval, 1994). Direct utilization of lactose has been difficult, because in food applications the amount of lactose that can be added is limited by low solubility and sweetening power (Okos *et al.,* 1978; Short, 1978). Hydrolysis of lactose into glucose and galactose can solve many of these problems and potentially increase its value as a food ingredient. Hydrolysis produces a sugar mixture with improved solubility, sweetening power four times that of the original lactose, digestibility acceptable to lactose-intolerant people, and value as a microbial substrate.

The use of lactase to transform waste lactose into an improved form for food use has been a promising application for almost 20 years. Mold lactase has long been advocated for use in acid whey treatment, whereas yeast lactases would be the choice for sweet whey. As this treatment involves addition of enzyme to a waste product, the economics have not favored the use of free, soluble enzymes (Short, 1978). Immobilized enzyme reactors have the potential to lower the overall cost of processing by attaching enzymes to low-cost materials and reusing the catalyst (Short, 1978; Finocchiaro *et al.,* 1980b; Siso and Doval, 1994). The ability to hydrolyze the lactose in acid whey has been demonstrated using immobilized *A. niger* lactase bound to porous glass (Wierzbicki *et al.,* 1973) or phenol–formaldehyde resin, which retained nearly full activity over 120 days of continued processing at 40°C (Okos *et al.,* 1978). *K. lactis* lactase immobilized on alumina has been used to hydrolyze lactose in sweet whey for up to 48 hours at 40°C (Finocchiaro *et al.,* 1980b). *K. lactis* lactase immobilized on Ni(II)–iminodiacetate–agarose yielded 96% activity and reached 70% lactose conversion in whey permeate in 2 hours (Irazoqui and Batista-Viera, 1994). In a simpler approach, Siso and Doval (1994) immobilized lactase-rich *K. lactis* cells on corn grits, as an alternate low-cost support, and produced 80% hydrolysis of the lactose in sweet whey for up to 2 days at 37°C.

2. *Lactose Syrup Production*

Preparation of syrups from acid whey at 15% total solids using *A. niger* lactase has been reported (Wierzbicki *et al.,* 1974). Commercialization of lactose hydrolysis in acidified whey with immobilized lactase has been described (Moore, 1980). This process yields a glucose–galactose syrup, which is concentrated by evaporation to 60 to 68% solids (Mahoney, 1985). These glucose–galactose mixtures are about four times as sweet as lactose and about 60 to 75% as sweet as sucrose. The syrups can be made microbiologically stable with up to 75% total solids (Nijpels, 1981). Major potential

uses for these hydrolyzed lactose syrups have been described by Short (1978) and are summarized here.

a. Sweetener and Preservative. Syrups could be used in such foods as canned fruit, soft drinks, ice cream, and frozen yogurt. They could also find a role in the manufacture of toffees, fudge, and candy bars.

b. Enhancer. As a microbial substrate, these syrups could be used in beer, wine, and baked goods.

c. Browning Agent. The syrups could be used in various baked goods and confections.

3. Product Synthesis

Glycosidase enzymes, like lactase and invertase, are able to catalyze not only hydrolytic reactions, but also the reverse reaction for transferase activity (Zarate and Lopez-Leiva, 1990). This type of reverse reaction has been used for synthesis instead of hydrolysis with several enzymes, including chymotrypsin, subtilisin and urease (Butler *et al.*, 1976). Synthesis of sucrose from starch catalyzed by the enzyme invertase from yeast and molds (Butler *et al.*, 1976) and the enzyme from banana pulp (Glass and Rand, 1982) has been studied. The mechanism of lactase activity has demonstrated the capability to transfer galactose to any acceptor containing a hydroxyl group (Mahoney, 1985). This transferase activity results in the synthesis of oligosaccharides, which appear in highest concentration after 50 to 90% lactose hydrolysis has occurred. The initial lactose concentration and the product environment also seem to be important, as does the enzyme form: immobilized versus free (Zarate and López-Leiva, 1990). The potential of this reaction as a possible mechanism for conversion of lactose to useful products has received little attention. Considering the large amount of waste lactose available from whey protein recovery, the future application of immobilized lactase may lie in conversion of the waste sugar into a range of products, from vitamins like ascorbic acid to compounds with pharmaceutical potential.

II. HYDROLASE ACTIVITY

Reduction of lactose in food products can be accomplished either by physical separation or by hydrolysis; however, physical methods (such as gel filtration and ultrafiltration eliminate vitamins and minerals in addition

to lactose. Therefore, the logical approach, without complete reformulation of the milk product, has been lactose hydrolysis. Although hydrolysis can be achieved with acid- or cation-exchange resins, enzymatic conversion with β-galactosidase has been preferable because it is highly specific (Mahoney, 1985) and the other components of the substrate solution remain unchanged (Nijpels, 1981). Hydrolase activity would be the major route of catalysis for β-galactosidase. As stated in the introduction, lactases have been used commerically for the hydrolysis of lactose in dairy products. The catalytic properties of β-galactosidase, in both soluble and immobilized forms, have been extensively reviewed (Pomeranz, 1964b; Shukla, 1975; Richmond *et al.*, 1981; Gekas and López-Leiva, 1985; Mahoney, 1985).

β-Galactosidase has been purified from a vast number of microbes including yeast fungi, and bacteria (Shukla, 1975; Richmond *et al.*, 1981; Mahoney, 1985; Gekas and López-Leiva, 1985; Agrawal *et al.*, 1989). β-Galactosidase from *Escherichia coli* has specifically been examined and serves as a model for the study of the overall enzyme characteristics; however, *E. coli*-derived lactase has not been considered safe for commercial use and is used only as a valuable analytical tool for enzymatic determinations. Therefore, the following discussions pertain to those organisms used in the preparation of commercial lactases.

A. ENZYME MECHANISM FOR ACTIVITY

Glycoside hydrolases are named according to the type of bond hydrolyzed (Nilsson, 1991). β-Galactosidase, or lactase (EC 3.2.1.23), hydrolyzes the β1–4 glycosidic linkage between the galactose and glucose moieties of lactose by transferring galactose to water and simultaneously liberating glucose. Formation of an enzyme–galactosyl complex with simultaneous glucose liberation is the critical step in hydrolysis (Prenosil *et al.*, 1987a). The overall hydrolytic reaction is given in Fig. 12. Although the catalytic

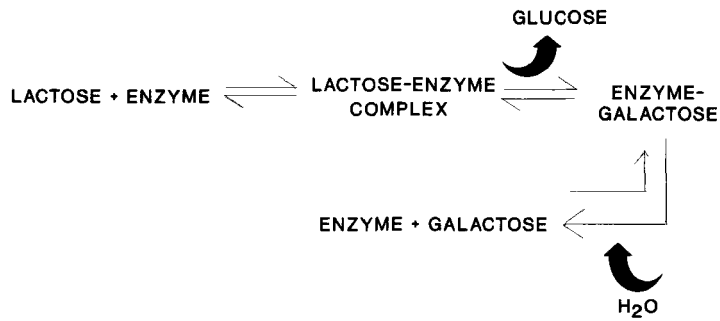
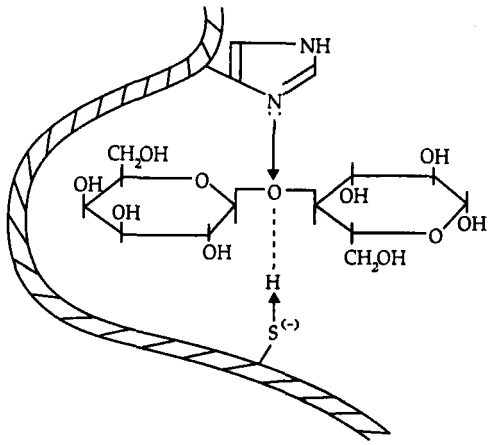
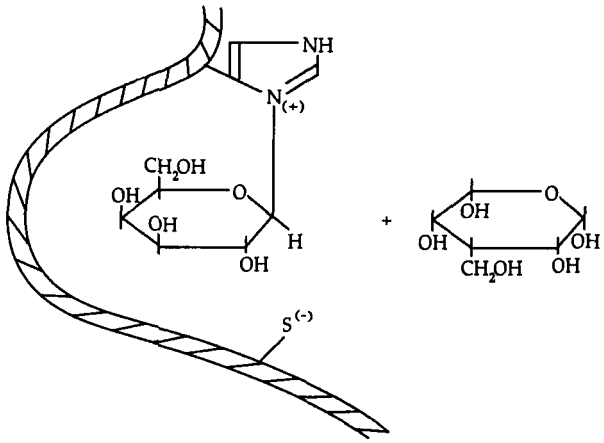


FIG. 12. General reaction for hydrolysis of lactose by β-galactosidase.

mechanisms of most glycosidases have not been elucidated (Nilsson, 1991), a specific mechanism of catalytic activity for *E. coli* β-galactosidase was originally proposed by Wallenfels and Malhotra (1961). This proposed mechanism for neutral-pH lactases, illustrated in Fig. 13, has been extensively reviewed and characterized (Shukla, 1975; Nijpels, 1981; Richmond



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



β-Galactosidase-galactose complex + glucose

FIG. 13. Proposed mechanism for lactose hydrolysis catalyzed by β-galactosidase. From Shukla (1975). Reprinted with permission of CRC Press, Inc.

et al., 1981; Mahoney, 1985; Prenosil *et al.*, 1987a; Zarate and López-Leiva, 1990). The hydrolysis reaction, as proposed by this model, corresponds to an S_N2-like displacement mechanism. The sulfhydryl group, at the active site of lactase, acts as a general acid to protonate the galactosidic oxygen atom, and an imidazole group acts as a nucleophile and attacks at C-1 of the glycone. This reaction led to the proposed formation of a covalent bond intermediate enzyme–galactosyl complex. Galactose is then removed from the enzyme through the abstraction of a proton from water by the sulfhydryl anion, which acts as a general base, and assists in the attack of OH^- at the C-1 position.

Wendorff and Admundson (1971) indicated that *K. fragilis* β-galactosidase appears to be a sulfhydryl enzyme, as it is inhibited by heavy metals, *para*-chloromercuribenzoate, and iodoacetate. Mahoney and Whitaker (1977) suggested that *K. fragilis* β-galactosidase, a neutral-pH enzyme, as is *E. coli* β-galactosidase, operated according to this same proposed mechanism based on inhibition experiments with sulfhydryl reagents and pH–activity studies. Some studies, however, have indicated the possibility that the sulfhydryl group, although important in maintaining the active site, does not participate in the actual catalytic mechanism (Mahoney, 1985). A second general mechanism postulated for glycosidases, has been implicated in the catalytic mechanisms of *E. coli* β-galactosidase, hen's egg white lysozyme, influenza virus neuraminidase, and other glycosidases as illustrated in Fig. 14 (Sinnott, 1987; Nilsson, 1991; Vulfson, 1993). Simply, this proposed catalytic mechanism involves an enzyme carboxylate group, which acts as a general acid that catalyzes cleavage of the glycosyl–oxygen bond. A covalent glycosyl–enzyme intermediate has been proposed that was reached from both directions, through oxocarbonium ion-like transition states. The final catalytic step for hydrolysis involves the deprotonation of water by A^- which increases the nucleophilicity of the acceptor, facilitating the formation of a new glycosidic bond forming free galactose. The active sites of fungal β-galactosidases have remained a mystery, and no direct evidence relating to their mechanisms is currently available.

B. ASSAY METHODS AND ENZYME ACTIVITY

Lactases, regardless of source, carry out the same hydrolysis and are inhibited by galactose, competitively, to different degrees; however, enzyme properties and, ultimately, catalytic efficiency and conditions for optimum activity differ. Enzyme activity is influenced not only by source, pH, and temperature but also by substrate. Catalysis is influenced by variation in the aglycone moiety (glucose, in the case of lactose), and various enzyme sources respond differently to substrate structure. Listings of various properties of

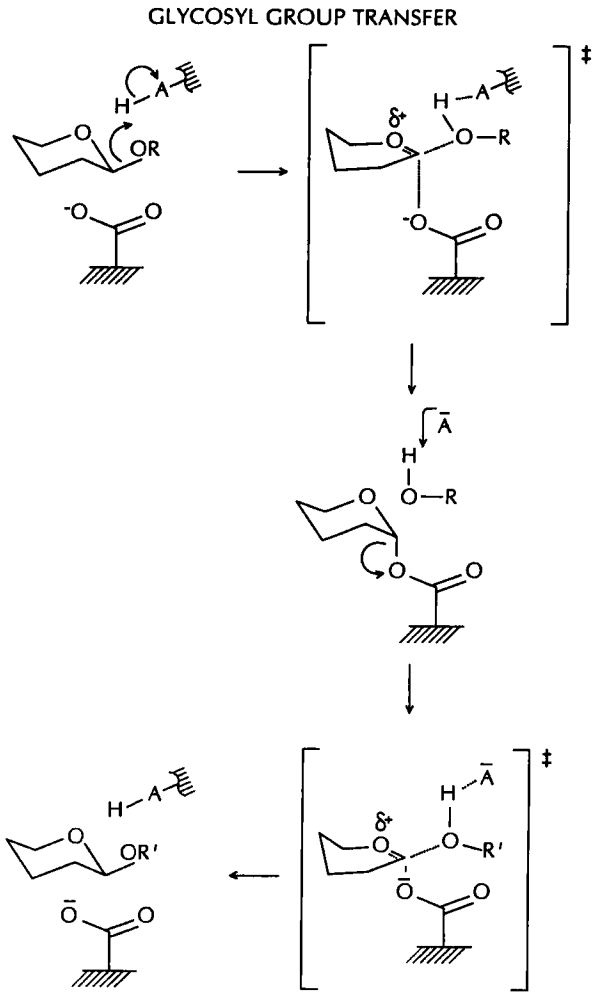


FIG. 14. Second general mechanism postulated for glycosidases. From Sinnott (1987). Reprinted with permission of Royal Society of Chemistry, London.

microbial lactases can be found in Shukla (1975), Greenberg and Mahoney (1981), Wasserman (1984), Gekas and López-Leiva (1985), and Agrawal *et al.*, (1989).

A number of methods have been developed to determine β-galactosidase activity using either lactose or other β-galactosides having an aglycone moiety different from glucose. Each procedure has its own built-in advantages and restrictions. The simplest methods use colorimetric analysis to

measure the quantity of monosaccharides in the presence of disaccharides (Bouvy, 1974; Nickerson *et al.,* 1976) or specific measurement of glucose with glucose oxidase (Dahlqvist, 1964). In addition to colorimetric determinations, glucose concentrations can be measured with biosensors that use glucose oxidase/catalase membranes. These methods illustrated are relatively rapid, specific, and highly sensitive.

Chemically modified substrates have been commonly applied for the determination of lactase activity because they are easy to use. Commercial enzyme manufacturers and researchers have characterized β-galactosidase from different commercial sources (Wendorff and Amundson, 1971; Uwajima *et al.,* 1972; Mahoney and Whitaker, 1977; Dickson *et al.,* 1979; Mahoney and Adamchuk, 1980; Hussein *et al.,* 1989) and have routinely defined lactase units using the artificial substrate *ortho*-nitrophenyl-β-D-galactoside (ONPG). The relatively simple and straightforward procedure involved has made ONPG the substrate of choice because of the high reaction velocities and lower K_m values (Richmond *et al.,* 1981). ONPG is hydrolyzed by the enzyme at the nonreducing end of the β-galactosidic bond, producing galactose and the yellow *o*-nitrophenol (Bouvy, 1974). One unit of lactase activity equals the production of 1 μmole of *o*-nitrophenol per minute under the conditions of the assay.

Wallenfels and Malhotra (1961) first reported that lactases from different sources exhibited optimum activity on different substrates because of variations in enzyme–substrate affinity. Pomeranz (1964a) noted that although hydrolysis rates have been routinely defined by ONPG activity, the affinity of the enzyme for different substrates varied; therefore, ONPG units did not directly reflect the activity lactase probably has on lactose in food. In addition, the analysis of enzyme activity in food substrates was complicated by the existence of both hydrolytic and transferase activities (Mahoney, 1985). This means that estimates of lactase activities on food lactose based on simple extrapolation from an ONPG assay may not be accurate. Buffering and artificial substrates may produce optimum activity, but these conditions do not properly indicate enzyme activity as it is reflected by real-life processing situations. Hourigan (1976) proposed that lactase activity should be studied with a milk system. K_m values for ONPG and lactose of lactases from commercially available yeast and fungal sources are listed in Table I. In all cases cited, the K_m value for lactose was higher than that for ONPG, thereby indicating lower affinity for the natural lactose substrate. Furthermore, investigators have shown that milk constituents also have an effect on lactose hydrolysis and the degree is source dependent. The *E. coli* enzyme, probably the most studied of all β-galactosidases, has been shown to have an overwhelming preference for ONPG substrate while losing 95% of its activity in milk (Morisi *et al.,* 1973). Yeast-derived lactases

TABLE I

COMPARISON OF K_m VALUES OF VARIOUS MICROBIAL β-GALACTOSIDASES ON ONPG AND LACTOSE SUBSTRATES

Enzyme source	K_m (*M*)		References[a]
	ONPG	Lactose	
A. niger	2.02	85	1,2
A. oryzae	0.77	50	2,3
K. fragilis	2.5, 2.72	24, 13.9	4,5
K. lactis	1.61, 1.25	16.8, 16.0, 24.3., 28.0	6–9

[a] (1) Greenberg and Mahoney, 1981a; (2) Greenberg and Mahoney, 1981b; (3) Park *et al.*, 1979; (4) Mahoney and Whitaker, 1977; (5) Wendorff and Amundson, 1971; (6) Dickson *et al.*, 1979; (7) Rand, 1981; (8) Forsman *et al.*, 1979; (9) Hussein *et al.*, 1989.

were affected to a lesser degree, with *K. fragilis* and *K. lactis* β-galactosidases losing 40% and 10 to 30% of their activity in milk when compared with ONPG (Morisi *et al.*, 1973; Mozaffar *et al.*, 1985). Finally, *A. niger* enzyme (Rand, 1981) activity has been shown to increase in milk. Therefore, proper choice of operating conditions, suitable to the β-galactosidase used, is important in establishing maximum enzyme activity.

Jacober-Pivarnik and Rand (1984) developed a milk assay to reflect the probable activity of lactase during dairy processing. They compared the lactase units of lactase powders derived from *K. lactis* and *K. fragilis* in a pH 6.5 imidazole-buffered lactose solution and reconstituted NFDM, both approximately 5% lactose. The milk system was found superior to the buffered enzyme–lactose system (Fig. 15). The assay method employing buffered lactose substrate did not accurately reflect the true enzyme activity in a milk system. Senecal and Rand (1992b) determined the lactase activity of five commercial yeast enzymes in a modified NFDM assay and compared the data with the manufacturer's data on ONPG activity (Table II). Although β-galactosidase from *K. lactis* achieved similar enzyme activity, the data revealed appreciable differences in the activity of lactases from *K. fragilis* and *Candida* species on NFDM and ONPG substrates. The data revealed that lactase activity, as reported by the manufacturer for an ONPG assay, did not reflect the activity that occurred in reconstituted NFDM. It appeared that the molecular complexity of milk may have interfered with the chemical attraction of *K. fragilis*- and *Candida*-derived lactases to lactose, resulting in decreased enzyme activity when compared with ONPG. This, however, was not observed with lactases derived from *K. lactis*. The reason for the difference in substrate affinities for various species of lactase remains unknown.

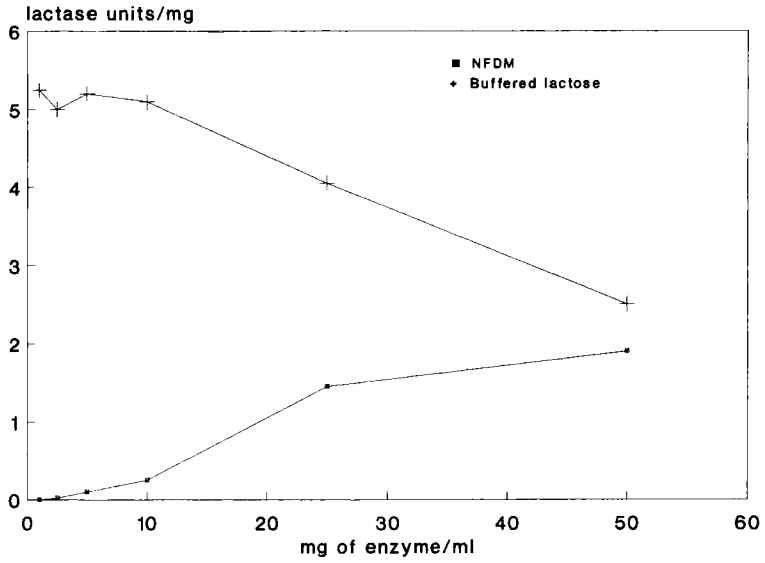


FIG. 15. Enzyme activity of Maxilact 40,000 lactase powder, isolated from *K. lactis*, in skim milk and pH 6.5 imidazole–HCl buffer at 37°C. From Jacober-Pivarnik and Rand (1984). Reprinted with permission of Institute of Food Technologists.

TABLE II

ACTIVITY UNITS OF COMMERCIAL YEAST LACTASES ON NFDM AND ONPG

	LAU[a]		
Lactase	NFDM	ONPG[b]	Activity (%) range
K. lactis	5300	5000	106
K. lactis	8400	8000	105
K. fragilis	1900	3000	63
C. pseudotropicalis	1400	2750	51
C. pseudotropicalis	1400	2500	56

[a] Lactase activity units as given are per gram of enzyme per milliliter.

[b] As reported by the manufacturer.

Presented at the 1992 annual Institute of Food Technologists meeting, New Orleans.

Mahoney (1985) indicated that because both hydrolytic and transferase activities are present, the degree of lactose hydrolysis cannot be established by evaluating a single monosaccharide unless the hydrolysis approaches 100%. It was perceived that the determination of glucose during hydrolysis could possibly overstate the actual amount of lactose remaining, as some of the glucose would be incorporated into oligosaccharides. Analyses using galactose could be inaccurate because of the even greater tendency to form compound sugars. To completely analyze the activity of the enzyme in a reaction mixture, separation of the components of the mixture is required. Chromatographic methods have been used for complete analysis of reaction mixtures; however, only high-performance liquid chromatography provided a simple and rapid method for the simultaneous determination of lactose, glucose, and galactose in reduced-lactose milk (Pirisino, 1983; Pivarnik, 1990).

A number of assay methods have been used to determine rates of hydrolysis of lactose-containing substrates by lactase (Table III). The majority of these are methods previously cited in reference to enzyme activity that were modified to satisfy the conditions. A rapid method that does not require expensive equipment and can be used routinely to determine the extent of lactose hydrolysis in dairy products is cryoscopic analysis. This method measures changes that occur in solutions as the result of production of monosaccharides during lactose hydrolysis. The method relies on the freezing point of the sample being depressed by an amount depending on the molal degree of hydrolysis of the lactose (Zadow, 1984). Baer *et al.* (1980) demonstrated the linear relationship between freezing point and lactose hydrolysis in neutralized acid whey and 5% lactose solution. Also, lactose hydrolysis of dairy products can be analyzed directly without deproteinization, which is required by the other methods of analysis. A disadvantage of the assay is the formation of oligosaccharides during hydrolysis and their impact on freezing point depression (FPD). Oligosaccharide production results in a smaller FPD than would be associated with the simple breakdown of lactose into glucose and galactose (Jeon and Saunders, 1986). Jeon and Saunders compared the percentages of hydrolysis of *C. pseudotropicalis* for 5% lactose solution in 0.025 *M* potassium phosphate buffer (pH 6.6), whey permeate, and milk, as measured by FPD and high-performance liquid chromatography. The percentage hydrolysis was substantially less for FPD and the degree of variability correlated to the concentration of oligosaccharide formed.

C. PROPERTIES OF COMMERCIAL ENZYMES

A variety of commercial lactase enzymes are available for use in food. These enzymes are derived from yeasts and molds and differ widely in

TABLE III

METHODS FOR ASSAYING β-GALACTOSIDASE ACTIVITY/LACTOSE HYDROLYSIS ON COMMERCIAL SUBSTRATES

Assay method	Substrate	Source of commercial enzyme	Reference
Colorimetric	Milk and whey	*K. lactis*	Guy and Bingham (1978)
	Milk	*K. fragilis*	Paul and Mathur (1989)
	Milk	*A. niger*	Rand and Linklater (1973)
	Milk	*K. lactis*	Hussein *et al.* (1989)
	Whey	*A. oryzae*	Ozbaas and Kutsal (1990)
	Milk and whey	*K. fragilis*	Mahoney and Adamchuk (1980)
	Milk	*K. marxianus*	Mahoney and Wilder (1988)
	Milk and whey	*K. lactis*	Dahlqvist *et al.* (1977)
	Milk	*K. lactis*	Forsman *et al.* (1979)
Chromatographic	Milk	*K. lactis*	Kosikowski and Wierzbicki (1973)
	Milk	*K. lactis*	Pirisino (1983)
Glucose analyzer	Whey	*A. oryzae*	Sheth *et al.* (1988)
	Whey	*A. oryzae/K. marxianus*	Jackson and Jelen (1989)
	Milk	*K. lactis/K. fragilis*	Jacober-Pivarnik and Rand (1984)
	Milk	*K. lactis*	Pivarnik and Rand (1992)
	Milk	*K. lactis/K. fragilis/ C. pseudotropicalis*	Senecal and Rand (1992a)
Cryoscopic	Milk	*K. lactis/A. oryzae*	Palumbo *et al.* (1995)
	Milk and whey	*A. oryzae/A. niger/ K. fragilis*	Shih-Ling *et al.* (1981)
	Whey	*K. lactis*	Baer *et al.* (1980)
		K. lactis	Nijpels *et al.* (1981)

their properties, particularly with respect to optimum pH and temperatures for enzyme activity. Shukla (1975) reported that β-galactosidase preparations from different sources are not identical with respect to structure, size, and optimum conditions for hydrolysis. Enzymes derived from different sources have demonstrated dissimilar properties as a result of variations in enzyme–substrate affinity (Jacober-Pivarnik and Rand, 1984; Agrawal *et al.*, 1989). Temperature and pH optima have been found to vary depending on the source and even the particular commercial preparation (Gekas and López-Leiva, 1985).

Table IV lists technical data on the commercial β-galactosidases enzymes available during 1993 in the United States. This comparison establishes that the two main sources of β-galactosidase being used commercially are

TABLE IV

MANUFACTURERS[a] 1993 TECHNICAL DATA FOR COMMERCIALLY AVAILABLE YEAST LACTASES

Lactase	Source	Substrate	Optimum pH	Optimum temperature (°C)	Activity units[a]
Enzeco fungal lactase	*A. oryzae*	ONPG[b]/lactose	4.5–5.0	55	100,000 FCC/g
Enzeco immobilized lactase	*A. orzyae*	—	4.0–5.0	50	~2,000 ILU/g
Yeast lactase L 50,000	*K. lactis*	Lactose	6.0	45	50,000 ONPG units/g
Fungal lactase 100,000	*A. oryzae*	ONPG	4.5–5.0	50–55	100,000 FCC/g
Lactozyme 3000L	*K. fragilis*	Lactose	6.5	37	3,000 LAU/mL
Lactase F	*A. oryzae*	ONPG	4.5	55	14,000 FCC/g
Neùtral lactase	*K. lactis*	Lactose	6.0	45	8,000 ONPG units/g
Biolactase	*A. oryzae*	—	4.5–5.0	55–60	30,000 FCC/g
Maxilact L2000	*K. lactis*	Whey powder	6.3–6.7	35–40	2,000 NLU/g
Maxilact LX5000	*K. lactis*	Whey powder	6.3–6.7	35–40	5,000 NLU/g
Neutral lactase	*K. lactis*	—	6.5	37	3,100 ONPG units/mL

[a] FCC, Federal Chemicals Codex Lactase Units; ILU, International Lactase Units; NLU, Neutral Lactase Units; LAU, lactase activity units.

K. lactis and *A. oryzae*. Previously, it had been reported that the sources of the two most widely used commerical lactases were *K. lactis* and *A. niger* (Nijpels, 1981). Mahoney (1985) indicated that *A. niger* lactase was preferred over *A. oryzae* enzyme for commercial applications because it was more stable and had a lower pH optimum, making it more applicable for immobilization. *A. oryzae* lactase (optimum pH 4.5–5.0), however, appeared to be more suitable for acid whey hydrolysis than *A. niger* enzyme (optimum pH 3.5–4.0). In addition, *A. oryzae* lactase was much less susceptible to product inhibition by galactose (Mahoney, 1985). The individual enzyme chosen for commercial utilization depends on a number of elements: substrate, pH, operational temperature, and stability.

1. pH

Operational pH has been the primary characteristic determining the application of a given enzyme (Mahoney, 1985). Not only does pH control enzyme activity, but it also is important in maintaining lactase stability. Fungal β-galactosidases have their pH optima in the range 3.0 to 5.0 and have an operational range of 2.5 to 6.0. This pH profile fits well for processing acid whey, acid whey permeate, or fermented dairy products. The optimum pH for purified *A. oryzae* lactase in ONPG and buffered lactose (0.1 *M* sodium acetate buffer) was found to be 5.0 (Park *et al.*, 1979). Ozbaas and Kutsal (1990) determined the optimum pH for purified *A. oryzae* lactase to be 4.5 using reconstituted whey powder as the substrate; however, fungal lactase preparations from *A. niger* have also been shown to effectively hydrolyze lactose in milk (Rand and Linklater, 1973). The yeast lactases demonstrate optimum activity in the neutral pH range. In addition, yeast lactases operate in a much more limited pH range (pH 6.0–8.5) than do fungal lactases. Experimental studies on purified *K. lactis* and *K. fragilis* lactases with different substrates have indicated the optimum pH range to be 6.5 to 7.0 (Wendorff and Admundson, 1971; Uwajima *et al.*, 1972; Dahlqvist *et al.*, 1977; Guy and Bingham, 1978; Hussein *et al.*, 1989). One study found that the optimum pH range for purified *K. lactis* enzyme was 7.0 to 7.5 and that lactase activity dropped off rapidly below pH 6.9 and above 7.5 (Dickson *et al.*, 1979). This deviation from other studies may have been due to the utilization of sodium phosphate buffer as the test medium. Both *K. lactis* and *K. fragilis* lactases have been shown to be slightly inhibited by sodium (Mahoney, 1985).

2. Temperature

Temperature is another important factor to consider when choosing a commercial source of β-galactosidase. Fungus-derived lactases have higher

optimum temperatures for both activity and enzyme stability, and are routinely used for hydrolysis of lactose at 50 to 55°C. Yeast lactases are rapidly denatured above 40°C. High concentrations of enzyme (although expensive) should be used when hydrolysis is conducted with yeast lactases in the optimum temperature range (30–40°C), to increase the rate of hydrolysis and, therefore, reduce microbial growth and oligosaccharide production, which are favored by these conditions (Mahoney, 1985). As a result, hydrolysis of milk products has been conducted at refrigeration temperatures (Guy and Bingham, 1978); however, the activity of the enzyme is severely impaired at these reduced temperatures, and a much longer period, generally overnight, is required to achieve lactose hydrolysis in excess of 70%.

Both the activity and the stability of β-galactosidase have been determined at various temperatures. Hourigan (1976) reported that *K. lactis* lactase in batch processes attained an 80 to 90% conversion rate in 5 hours at 35°C and in 18 hours at 6°C. Forsman *et al.* (1979) observed that incubation time and enzyme concentration have an effect on the temperature requirements for optimum lactose conversion with *K. lactis* lactase. They showed that the optimum temperature for hydrolysis increases when the enzyme concentration is decreased. In addition, the authors suggested the possibility of a saturation effect that becomes more pronounced with an increase in both temperature and incubation time. Guy and Bingham (1978) measured the effect of temperature on *K. lactis* lactase in cottage cheese whey substrate to determine the range in which the enzyme becomes inactivated. Activity was noticeably reduced when the enzyme was heated above 50°C for 1 minute, with complete inactivation at 70°C. Rand and Linklater (1973) showed no increase in the rate of hydrolysis catalyzed by *K. fragilis* lactase above 37°C and the enzyme was unstable. Lactose hydrolysis by *A. niger* lactase was about 15% lower than that by yeast lactase at 37°C for the same reaction time; however, increasing the temperature above 37°C had a pronounced effect on fungal lactase, resulting in an increase in the hydrolysis rate up to 63°C. The fungal enzyme was found to be stable up to 3 hours, indicating its potential utilization for batch-type pasteurization of milk. Studies on purified *A. oryzae* lactase in various substrates have found the optimum temperature range for maximum hydrolysis to be 50 to 55°C (Park *et al.*, 1979; Ozbaas and Kutsal, 1990). When the crude enzyme was tested in similar substrates to determine the effect of temperature on lactase activity (Park *et al.*, 1979), a higher optimum temperature range (55–60°C) was achieved (Fig. 16). This indicates that the crude enzyme is more heat resistant than the purified enzyme and may be a worthwhile subject of further research. This topic is discussed in later sections.

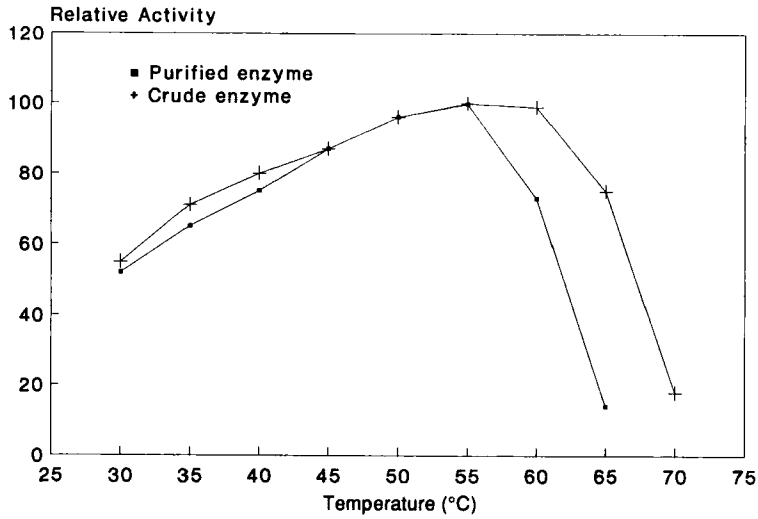


FIG. 16. Effect of temperature on the lactase activity of *A. oryzae* enzyme preparations. From Park *et al.* (1979). Reprinted with permission of Institute of Food Technologists.

3. *Stability*

The stability of β-galactosidase should be considered when choosing an enzyme for either batch or continuous processing. The thermal stability of an enzyme is especially important, as it can be used to establish optimum processing temperatures. Commercial preparations of liquid yeast lactase have been shown to be stable under refrigeration for 2 years (Dahlqvist *et al.*, 1977; Senecal, 1991). The yeast lactases, however, have proven to be extremely heat labile, particularly when used at temperatures needed to accelerate processing and inhibit microbial growth. Thus, considerable time and effort have been spent to identify a lactase source with greater thermostability at neutral pH, for use in processing milk products. Fungal lactases, although they display relatively good thermostability, have reduced activity at the pH of milk and, therefore, require increased amounts of expensive enzyme. Furthermore, larger amounts of enzyme could be a source of off-flavors in lactose-hydrolyzed milk because of the increased amount of insoluble residue present in food. Finnie (1980) studied the thermostability of *A. niger* lactase in the continuous pasteurization of milk. The fungal lactase did not display thermostability above 80°C, which would be necessary for UHT sterilization or pasteurization. For batch processing of milk at 63°C, the enzyme maintained 50% of its activity; however, for continuous

HTST pasteurization at 72°C, about 30% recovery of lactase activity was possible.

Very little data are available on the stability of soluble β-galactosidases in dairy products (Mahoney, 1985). Few studies have established the stabilizing effect of various milk components on lactase, other than those in simple buffer systems. Milk and whey have been found to increase the stability of *K. lactis* (Dahlqvist *et al.*, 1977) and *K. lactis* (*marxianus*) lactases at temperatures higher than those of simple buffer systems (Mahoney and Wilder, 1988). The increased stability is attributed to the stabilizing properties of the casein and whey proteins. It has been theorized that both proteins and polyols extend the stability of enzymes through the disruption of solvent–enzyme interactions, thus encouraging intramolecular hydrophobic bonding (Schmid, 1979; Klibanov, 1983). In the presence of lactose, glycerol was found to be less effective than caseinate, implying that the complexity of the substrate-dependent stabilization by protein was greater than the dehydration effect promoted by polyols (Mahoney and Wilder, 1988). Byeong-Seon and Mahoney (1994), in studies on the stabilization of *S. thermophilus* lactase by bovine serum albumin (BSA), proposed the formation of a dimer–BSA complex. They proposed that the native *S. thermophilus* enzyme exists mainly as a tetramer (N1) with some active dimer (D4) present. Heating at 60°C results in the formation of a new, kinetically less stable dimer (D3) from the D4 dimer and, ultimately, inactive aggregates. Addition of BSA with heating results in the production of a BSA–dimer complex more stable than the original dimer or tetramer. Formation of the BSA–dimer complex appears to improve enzyme dimer stabilization against unfolding and aggregation.

Mahoney and Wilder (1988) established that the stability of β-galactosidase in milk is also influenced by salts and sugars. Concentrations of milk solids up to 25% resulted in an increase in the factors important for lactase stability. Thompkinson *et al.* (1991) observed that increasing the concentration of milk solids to 35 to 40% allowed the optimum temperature for *K. lactis* lactase incubation to be increased from 37° to 50°C. The overall stability of lactase was noticeably reduced by the removal of divalent cations. Wendorff and Admundson (1971) determined that Mn^{2+} is a necessary cofactor and may increase stability by helping to maintain the conformation of the secondary and tertiary structure of the *K. fragilis* enzyme molecule. Enzyme stabilization was found to occur only with sugars capable of binding to lactase (substrate–products) (Mahoney and Wilder, 1988). It was determined that the stability afforded by sugars was not simply caused by complexing, but that other, as yet undetermined, phenomena must be involved. In addition, the reaction products appeared to be as effective as the substrate in enzyme stabilization. Mahoney and Wilder (1988) found that the

individual addition of lactose or caseinates resulted in a fivefold increase in enzyme stabilization; however, the synergistic effect of combined lactose and caseinate addition resulted in a 50-fold increase in the stability of *K. fragilis* lactase. When sugars and proteins were present together, the binding of lactose or galactose may result in an enzyme conformation that is more effectively stabilized by the proteins. Amino acids have also demonstrated stabilizing effects on *K. lactis* (*marxianus*) lactase at 45°C (Surve and Mahoney, 1994). All amino acids (except proline) were found to improve enzyme stability to some degree. Histidine was found to be the most effective amino acid, capable of stabilizing the lactase 9-fold and 40-fold in the presence of 5% lactose. It was determined that the α-amino group and N-1 ring nitrogen of histidine are required for enzyme stabilization.

Senecal (1991) investigated the stability of five commercial yeast lactases in skim milk and skim milk ultrafiltration permeate (SMUFP) at 20°, 30°, and 40°C. In general, half-life data indicated that the lactase enzymes were more stable in SMUFP at 30° and 40°C and in skim milk at 20°C (Table V). Lactases derived from *K. lactis* had the greatest overall thermostability of all the commercial yeast-derived products tested. In addition, the *K. lactis* lactases tested each had much higher units of activity per gram. Studies have been conducted to determine the effect of enzyme concentration on stability (Mahoney and Wilder, 1988; Senecal, 1991). Results demonstrate that enzyme concentration, even 30-fold, has no significant effect on lactase stability (Tables VI, VII).

Milk has been shown to have a greater thermostable effect on lactase than either salts or whey (Mahoney and Wilder, 1988). As in previous studies, increased stabilization was attributed to the combined presence of caseins and lactose. Dahlqvist *et al.* (1977) found *K. lactis* lactase to be more stable in UHT-processed milk than in UHT-processed whey at 24°C.

TABLE V

EFFECT OF MEDIA AND TEMPERATURE ON LACTASE HALF-LIFE IN SKIM MILK[a]

	Skim milk			SMUFP		
Lactase[b]	20°C	30°C	40°C	20°C	30°C	40°C
K. lactis	118	29	2.5	55	12	5
K. lactis	105	13	4.5	62	17	14
K. fragilis	84	6.5	<1	82	14	<1
C. pseudotropicalis	81	5	<1	67	11	<1
C. pseudotropicalis	108	7.5	<1	43	10	<1

[a] Half-life is expressed in hours.

[b] Enzyme concentration from all microbial sources is 11.9 mg/ml.

TABLE VI

EFFECT OF ENZYME CONCENTRATION ON *KLUYVEROMYCES LACTIS* LACTASE STABILITY IN MILK AT 45°C[a]

Enzyme concentration (ONPG units/ml)	Half-life (min)	
	Trial 1	Trial 2
0.66	85	—
1.0	86	65
2.0	85	63
4.0	82	74
10	90	66
20	94	74
30	—	66
Average	87 ± 4.3	68 ± 4.8
C.V. (%)[b]	4.9	7.0

[a] From Mahoney, R. R., and Wilder, T. Thermostability of yeast lactase (*Kluyveromyces marxianus*) in milk. *J. Dairy Res.* **55,** 423–433. Copyright (1988) by Cambridge University Press. Reprinted with the permission of Cambridge University Press.

[b] Coefficient of variation.

The compositions of the whey-derived substrates in these studies were different from those in the skim milk/SMUFP study (Senecal, 1991). SMUFP was processed through a 50,000-MW-cutoff hollow-fiber mem-

TABLE VII

EFFECT OF ENZYME CONCENTRATION ON STABILITY OF LACTASE IN SKIM MILK ULTRAFILTRATION PERMEATE

Lactase	Concentration (mg/ml)	Half-life (h)	
		20°C	30°C
K. fragilis	1.9	82	14
	32.1[a]	79	15
C. pseudotropicalis	11.9	67	11
	44.0[a]	53	12
C. pseudotropicalis	11.9	43	10
	44.0[a]	37	11

[a] Concentrations based on theoretical values for amounts to be equivalent to *K. lactis* lactase.

brane, similar to those commonly used in the dairy industry. The final product contained concentrations of proteins and salts greater than those used in the previous studies. The increased concentrations of these constituents may have been contributing factors to lactase stability at higher temperatures. In addition, soluble α-lactalbumin and nonprotein nitrogenous compounds may have had a greater thermostable effect than casein, because of their greater ability to react with the enzyme–substrate complex. Mahoney and Wilder (1988) found *K. lactis* (*marxianus*) lactase to have greater stability in synthetic milk salts with added caseinate than in milk. These investigators postulated that the increase in stability may have been due to solubility of protein as a stabilizer. In milk, casein would be primarily in the form of micelles so the concentration of soluble protein was reduced.

In addition to substrate composition, pH has played an important role in the overall stability of β-galactosidase. Senecal (1991) observed that changes in substrate pH, over time, for skim milk and SMUFP were different at 20° and 30°C. SMUFP was much more susceptible to changes in pH due to the onset of spoilage. These pH changes exceeded the optimum range for lactase and may have contributed to the reduction in stability of the enzymes in SMUFP at 20°C. At 30°C the enzymes might have attained greater half-life values if spoilage could have been delayed. Enzymatic activity was found to decrease rapidly for *K. lactis* lactase below pH 5.9 (Guy and Bingham, 1978) and for *K. fragilis* lactase below pH 5.5 (Wendorff and Amundson, 1971; Mahoney and Whitaker, 1977). Dahlqvist *et al.* (1977) observed that stability of *K. lactis* lactase declined rapidly if bacterial growth occurred in milk.

Palumbo *et al.* (1992) evaluated the storage stability of commercially available *K. lactis* and *A. oryzae* lactases dry blended into milk powders produced with 2% vegetable oils. The objectives were to determine lactase stability and to establish processing parameters to formulate a reduced-lactose shelf-stable milk powder for military applications. A 6-month storage trial of milk powders was conducted at different temperatures. *K. lactis* lactase proved to be relatively stable when stored under nitrogen at 20°C or below; however, when stored at 45°C in dry milk, the enzyme lost significant activity (25% within 1 month). β-Galactosidase derived from *A. oryzae* displayed much greater stability, retaining more than 90% of its activity even when stored at 45°C; furthermore, it demonstrated the potential for incorporation into milk powders. It appears that *K. lactis* lactase can only be added to milk powders that would be stored in cool places.

D. PURITY OF COMMERCIAL ENZYMES

Previous sections have alluded to the dissimilarities in the properties of lactase enzymes derived from different sources. An important property

that has received very little attention in the literature is the purity of the commercial preparation, especially with respect to the presence of other enzymes (i.e., proteases). Although proteases have not been a concern with oral lactase preparations, they could have a severe impact on the stability of lactase in continuous reactor systems. In addition, unless inactivated prior to packaging, protease could lead to undesirable changes in dairy products during storage.

The lactases used by researchers are either individually purified from cell-free extracts or obtained from commercial sources. Commercial lactases are obtained by the separation and purification of whole-cell extracts. Methods for the isolation and purification of β-galactosidase are summarized elsewhere (Shukla, 1975; Richmond *et al.*, 1981; Agrawal *et al.*, 1989). Early research was conducted on highly purified enzymes produced in the laboratory; however, references in the literature have implicated commercial sources of β-galactosidases as being far from pure and containing enzymes such as protease (Mahoney and Wilder, 1988, 1989). Protease activity present in commercial lactases may have influenced the results of thermostability studies (Mahoney and Wilder, 1989). Dickson *et al.* (1979) found that even under optimal storage conditions, *K. lactis* enzyme stability varies from preparation to preparation. It was assumed that *K. lactis* lactase stability is probably a function of protease contamination. This hypothesis was arrived at because of the loss of lactase activity with the decrease in molecular weight of the monomer and increase in lower-molecular-weight proteins. Results indicate that even a little protease activity can inactivate 25 to 50% of the *K. lactis* enzyme after 4 months at 4°C in glycerol phosphate buffer.

Protease present in lactase preparations may also cause undesirable flavor changes as a result of milk deterioration during storage (Mahoney, 1985). This is especially important if lactase is added to UHT-processed milk after processing. Dahlqvist *et al.* (1977) devised a method for adding β-galactosidase to UHT-processed milk. The lactase, which is readily inactivated by UHT processing, is added in much smaller amounts than normally required for batch processing, at the end of the UHT process. The commercial enzymes require further sterilization through a 0.22-μm filter to prevent bacterial contamination and a decrease in the stability of the UHT-processed milk.

Protease activity in commercial lactase preparations has also been implicated in the operational changes of a continuous membrane bioreactor (Senecal, 1991). Increasing the concentration of *K. lactis* lactase from 2000 to 3000 mg per 0.06 m^2 of hollow-fiber membrane resulted in an unexpected increase in flux. Protein analysis of the permeate established that 18.7% more nitrogen-containing material was penetrating the UF membrane. The

nitrogen-containing material was believed to originate from the protective protein bed added to increase enzyme stability by separating the enzyme from the polysulfone membrane material. Increasing the lactase concentration may have resulted in an increase in protease enzymes, which had a reducing effect on the concentration polarization layer over time.

Despite the evidence that commercial lactase preparations contain protease, only one supplier has actually listed protease activity in their product specifications for *K. fragilis* lactase. Others have indicated that their preparations contain very small amounts of protease. Therefore, depending on the intended application, commercial preparations should be assayed for protease activity by known analytical methods.

E. ACTIVATION AND INHIBITION

Table VIII lists the ionic activators of *K. lactis* and *K. fragilis* lactases. In general, heavy metals are inhibitory, whereas potassium, sodium, magnesium, and manganese stimulate the enzyme. It should be noted that some activators are more effective than others and some become inhibitory at high concentrations or in combination with other ions. Ammonium ion alone activates *K. fragilis* lactase, but when combined with adequate amounts of potassium, it inhibits the enzyme (Wendorff and Admundson, 1971). Therefore, the original literature should be consulted. In addition, the type of buffer used can also influence the effects of ions. For example, Mahoney and Whitaker (1977) found that although zinc stimulates *K. fragilis* β-galactosidase in phosphate buffer, it completely inhibits it in Tris buffer. The magnitude of the effect of these ions also differs depending on whether the enzyme is used in whey, milk, or buffered lactose. Finally, Dahlqvist *et al.* (1977) and Mahoney and Adamchuk (1980) found that milk proteins help activate and stabilize *K. lactis* and *K. fragilis* lactases, respectively. *A. niger* and *A. oryzae* β-galactosidases do not appear to be dependent on activating ions (Greenberg and Mahoney, 1981). As previously stated in the Introduction, product inhibition has been found to be a limiting disadvantage in obtaining the maximum effect of the β-galactosidase enzyme in commercial applications. Galactose has been credited as a competitive inhibitor. Rand and Linklater (1973) observed that galactose reduces the hydrolysis of *A. niger* lactase in milk by about 50% compared with glucose. Forsman *et al.* (1979) found that galactose inhibition occurs after the first 10 minutes of incubation and reaches a saturation level at 60 m*M* galactose. In their UHT system the saturation level was reached in about 2 hours, indicating the importance of product inhibition in reactions that are extended. β-Galactosidases from different organisms have displayed dissimilar effects of product inhibition. Wendorff and

TABLE VIII
EFFECTS OF IONS AS ACTIVATORS OR INHIBITORS OF β-GALACTOSIDASE FROM MICROBIAL SOURCES

Ion	Enzyme source	
	K. lactis	*K. fragilis*
Ag		–[a]
Ca^{2+}	–	+
Cl^-	ne	–
Cu^{2+}	–	
Co^{2+}	ne	+
Fe^{2+}	ne	
Hg^{2+}		–
K^+	+	+
Na^+	+	–
Mg^{2+}	+	+
Mn^{2+}	+	+
Ni^{2+}		
PO_4^{2-}	+	
Zn^{2+}	–	+
References[b]	1,2	3,4

[a] +, Activating effect; –, inhibiting effect; ne, no effect.

[b] (1) Dickson *et al.*, 1979; (2) Guy and Bingham, 1978; (3) Mahoney and Whitaker, 1977; (4) Wendorff and Amundson, 1971.

Amundson (1971) discerned that *K. fragilis* lactase is inhibited by high concentrations of both glucose and galactose. Furthermore, glucose was found to be more inhibitory than galactose in their experiments. It was concluded that glucose is a noncompetitive inhibitor, and galactose inhibition is competitive.

Recently, it has been reported that galactose had a noncompetitive inhibitory effect on fungal β-galactosidase from *A. oryzae* (Shukla and Chaplin, 1993). This study, conducted with a wide range of substrate and inhibitor concentrations, has been found to be contradictory to earlier investigations. The position taken by the authors was that the results of previous studies may not have been correctly interpreted and that a closer inspection of 1/v versus inhibition concentration demonstrated noncompetitive rather than competitive inhibition. They identified the importance of these findings (noncompetitive versus competitive inhibition) on the design of enzyme reactors. Reduction of competitive enzyme inhibition in a reactor can be

achieved through the addition of substrate. However, noncompetitive enzyme inhibition can only be reduced by raising the enzyme concentration or through the removal of inhibitor. Further studies under diversified reaction conditions and lactase sources may be required for complete understanding of the different mechanisms involved in lactase inhibition.

F. IMMOBILIZATION MECHANISMS AND REACTOR SYSTEMS

Many excellent articles have reviewed the immobilization methods available and the sources of lactase that have been investigated using a variety of techniques and support carriers (Shukla, 1975; Finocchiaro *et al.,* 1980b; Greenberg and Mahoney, 1981; Richmond *et al.,* 1981; Gekas and López-Leiva, 1985; Thompkinson, 1989; Bodalo *et al.,* 1991). In addition, Finocchiaro *et al.* (1980b) and Gekas and López-Leiva (1985) assembled extensive tables on lactase immobilization systems to include source, carrier, and reactor type. The immobilized reactor systems most frequently cited were batch, fluidized bed, and packed bed. Table IX lists methods that have been studied for β-galactosidase immobilization that use commercially available lactase sources. Methods that have been developed recently or have had limited review are discussed further.

Algin immobilization of lactases has received limited attention. Food-grade algin is considered a simple method for entrapment (Kierston, 1981), is inexpensive, and is available in both sodium and potassium forms (Na and K are activator ions for microbial sources of β-galactosidase). Algin encapsulation is accomplished by crosslinking with calcium chloride. Jacober-Pivarnik and Rand (1984) reported successful encapsulation of *K. lactis* lactase in both sodium and potassium forms and system performance for lactose hydrolysis. Lactase entrapped in potassium alginate resulted in twice as much lactose hydrolysis as lactase entrapped in sodium alginate. The alginate beads were easily recovered and washed to avoid microbial growth.

Immobilization of whole cells has been studied because it is less expensive and eliminates the need for enzyme purification (Champluvier *et al.,* 1988a,b). Champluvier *et al.* (1988a) studied the preparation and properties of β-galactosidase confined in cells from *Kluyveromyces* sp. to develop a treatment that could be combined with the various methods available for whole-cell immobilization. Treatment with glutaraldehyde was conducted to stabilize the confinement of the lactase to the cell. The combination of chloroform–ethanol treatment and immobilization with 0.4% glutaraldehyde resulted in a biocatalyst that could be stored for 14 months under

TABLE IX

IMMOBILIZED LACTASE SYSTEMS

Source	Reference[a]	Carrier	Method/immobilization agent	Substrate	Remarks
K. lactis	1	K/Na alginate	Ca-alginate beads	NFDM	Potassium showed activating effect in immobilized system
K. lactis/fragilis and *K. marxianus*	2	Whole cell	Chloroform/EtOH permeabilization confinement by glutaraldehyde	Buffered lactose	Biocatalyst used and recovered 7 times without loss of activity
K. lactis/fragilis	3	Whole cell	Polycations used to promote adhesion to solid supports	Buffered lactose	50% initial activity after 25 days of continuous operation
Aspergillus sp.	4	Microbial pellets	Immobilization of mycelium-associated β-galactosidase	Buffered lactose	50% of mycelium-associated lactase activity after 1300 h
B. circulans *A. oryzae*	5	PVC silica gel ribbed membrane	Covalently attached using PEI and glutaraldehyde	Skim milk	Determined kinetic parameters of rates of expressions
K. fragilis	6	Corn grits	Covalently attached using glutaraldehyde	Whey	50% hydrolysis with packed-bed reactor, reaction rate not limited by diffusion
K. lactis	7	Nylon-6 microbeads	PEI and gluteraldehyde	Skim milk	75% hydrolysis in a spin-basket reactor without plugging

[a] (1) Jacober-Pivarnik and Rand, 1984; (2) Champluvier *et al.*, 1988a; (3) Champluvier *et al.*, 1988b; (4) Réczey *et al.*, 1992; (5) Bakken *et al.*, 1990, 1991, 1992; (6) Siso *et al.*, 1994; (7) Ortega-Lopez *et al.*, 1993.

refrigeration with only 20% deactivation and could be used and recovered seven times without apparent activity loss.

Champluvier *et al.* (1988b) also studied the immobilization of whole yeast cells by adhesion of cells to a support. Cells were first treated with chloroform–ethanol and glutaraldehyde and immobilized to glass, polycarbonate, or polystyrene supports using chitosan to promote cell adhesion. After 25 days of continuous operation at 30°C, a microreactor of the immobilized lactase retained 50% of the initial activity. Réczey *et al.* (1992) investigated the *in situ* whole-cell immobilization of mycelium-associated β-galactosidase through mycelial pellet formation in 15 *Aspergillus* and *Penicillium* strains. The immobilized cells were produced through the growth of the fungal strains into pellets in shaker flasks, in an air-lift fermenter. The pellets were tested in repeated batch hydrolysis of lactose solutions (50 g/liter). Pellets produced from the fungal strain *A. pheonics* QM329 has the least amount of enzyme leakage during hydrolysis. It was estimated that 50% of the mycelium-associated lactase activity remained after 1300 hours of hydrolysis.

Batsalova *et al.* (1987) immobilized a fungal β-galactosidase in polyvinyl alcohol and studied the properties of the immobilized preparation in 4% lactose solution and whey substrate. The immobilized enzyme exhibited properties similar to those of the free enzyme and could be used repeatedly without any considerable loss of activity. The immobilized system initially suffered from enzyme leakage, which may have been due to gel pore size configuration. Treatment with 0.2% glutaraldehyde for 60 minutes or with 0.4% glutaraldehyde for 30 minutes was found to greatly reduce leakage without any adverse effect on hydrolysis. Combination of casein with the glutaraldehyde treatment resulted in greater retention of immobilized enzyme and an improved reusability. *K. fragilis* lactase has been covalently linked by glutaraldehyde to chemically modified corn grits for utilization in packed-bed bioreactors (Siso *et al.*, 1994). Corn grits were found to be very stable and to possess good mechanical properties as well as being an inexpensive enzyme support. Deproteinized whey from milk was hydrolyzed up to 50% within 3 hours of operation with negligible diffusional limitations in the packed-bed bioreactor. In addition, the immobilized system was recycled up to five times (4 hours each time) before significant reductions in enzyme activity occurred.

A novel chemical reactor for the hydrolysis of lactose has been tested with the immobilization of *A. oryzae* and *B. circulans* lactases onto a ribbed membrane of polyvinyl chloride (PVC) and silica (Bakken *et al.*, 1990, 1991, 1992). The fungal lactase is immobilized onto the PVC–silica membrane by adding polyethyleneimine (PEI) and glutaraldehyde. The immobilized membrane is modeled mathematically for lactose hydrolysis of skim milk

in an axial-annular flow reactor (Fig. 17). The reactor is found to avoid some of the problems associated with other reactor configurations, such as plugging, microbial contamination, and pressure drop (Bakken *et al.*, 1990). Ortega-Lopez *et al.* (1993) developed a novel spin-basket reactor using *K. lactis* lactase immobilized on nylon-6 microbeads. The reactor was capable of hydrolyzing 75% of the lactose in skim milk (28.6% total solids) within 7 minutes at 34°C and did not experience any of the plugging associated with packed-column reactors.

Despite all the studies on the development of immobilized lactose hydrolysis systems, very little information has been gathered on their commercial utilization. Why? The manufacturers may not disclose proprietary information, the systems may not be cost effective for wide-scale dairy applications, or the installation and operation of these systems may not be cost effective. Developers of commercial immobilized enzyme reactors for the continuous hydrolysis of milk have had to face many difficulties, such as the pH of milk, which encourages microbial growth; the presence of milk proteins, which can foul reactors; and neutral-pH yeast lactases, which are not very stable (Mahoney, 1985). Immobilized lactase reactors developed for whey hydrolysis have found more success because of the greater stability of the

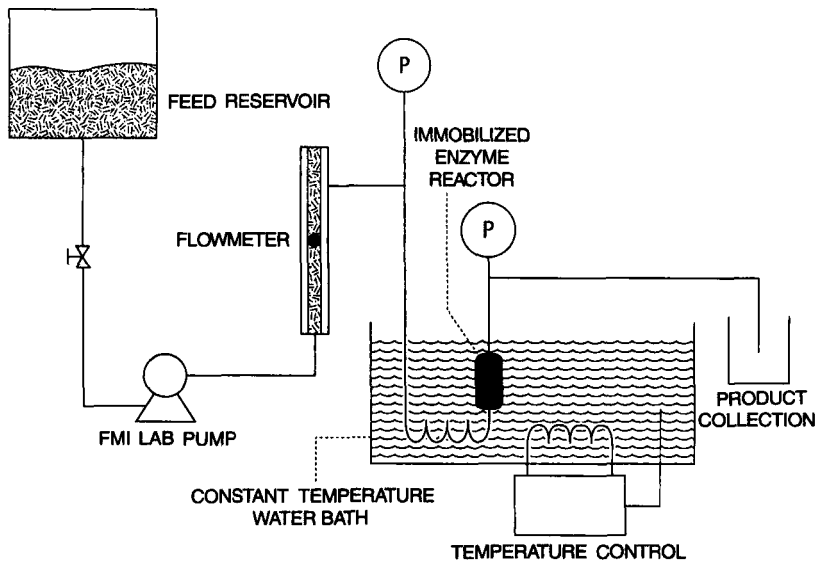


FIG. 17. Process schematic for the axial-annular flow reactor. From Bakken, A. P., Hill, C., Jr., and Amundson, C. H. Use of novel, immobilized β-galactosidase reactor to hydrolyze the lactose, constituent of skim milk. *Biotechnol. Bioeng.* **36**, 293–309. Copyright ©, 1990 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

fungal lactases and their activity at low pH, which inhibits microbial growth. Table X lists the lactase reactor systems that have been used in commercial applications. Until recently, Snamprogetti was the only immobilized lactase system known to hydrolyze lactose in milk on an industrial scale (Gekas and López-Leiva, 1985). The system has been operated in a recycle reactor with *K. lactis* lactase immobilized by entrapment in cellulose acetate fibers (Finocchiaro *et al.*, 1980a). The milk is first sterilized by UHT processing prior to lactose hydrolysis to reduce microbial contamination. At present, the Enzyme Development Corporation (New York) has made commercially available an immobilized fungal lactase for the hydrolysis of milk and whey. Lactase derived from *A. oryzae* is covalently bound to macroporous amphometric ion-exchange resin, with a multifunctional protein crosslinking agent. This system was initially developed for pilot plant application by Sumitomo of Japan. The immobilized *A. oryzae* system has been used in a column reactor for the continuous processing of milk and whey substrates (Fig. 18). The half-life data for the continuous column reactor were obtained at different concentrations of lactose for skim milk, whey, and lactose substrates (Table XI). The data demonstrate that the half-life for the column reactor increases with higher lactose concentrations, except when skim milk is used as the substrate. This improvement in reactor half-life may be due to the increase in the concentration of calcium in the skim milk substrate. Calcium ion concentrations have been found to have an inhibiting effect on remaining *A. oryzae* lactase activity (Enzyme Development Corp.).

Hydrolysis of lactose in whey has found greater commercial success, as demonstrated in Table X. The most successful systems to date have used immobilized lactase from *A. niger.* One of these is the system developed by the Corning Glass Company (Corning, NY) that immobilizes lactase on porous glass beads. The beads are packed into a cylindrical column for the continuous processing of whey (Fig. 19). The column is operated at 50°C with a residence time of 15 to 25 minutes, and the final product has an estimated 80% hydrolysis rate (Moore, 1980; Mahoney, 1985). The second commercial process using β-galactosidase from *A. niger* was developed by Valio Dairy in Finland. The enzyme is immobilized by absorption onto a phenol–formaldehyde resin and fixed in place by glutaraldehyde crosslinking (Olson and Stanley, 1973). For continuous processing, the immobilized lactase resin is packed into a fixed-bed columnar reactor and operated in a plug-flow mode (Mahoney, 1985).

G. ULTRAFILTRATION BIOPROCESS REACTORS

The third method of lactose hydrolysis is an alternative method of enzyme immobilization using ultrafiltration. As previously discussed in the Intro-

TABLE X

COMMERCIALLY AVAILABLE TECHNOLOGIES FOR IMMOBILIZING LACTASE

	System	Application
Snamprogetti	Fiber (cellulose triacetate)-entrapped yeast for lactose reduction in milk; batch process	Industrial; Centrale Lattern di Milano (Italy)
Corning Glass	*A. niger* lactase bound to silica beads to hydrolyze acid whey; UF permeate; fixed bed	Semi-industrial; ULN Condi (France), Dairy Crest (UK), Kroger (USA)
Connecticut/Lehigh Universities	*A. niger* lactase adsorbed to porous alumina carrier; fluidized bed; processing of whey UF permeate	Pilot plant (USA)
Valio Laboratory	*A. niger* lactase adsorbed to phenol–formaldehyde resin; Duolite ES-762; processing of whey UF permeate	Industrial Keymenlaakse Dairy (Finland)
Gist-Brocades	Immobilization of Maxilact enzyme; processing of milk	Pilot plant (Holland)
Rohm GmbH	*A. oryzae* lactase covalently bound to Plesxsym LA-1; processing of acid whey and milk	Pilot plant (Germany)
Sumitomo	*A. oryzae* lactase covalently bound to a macroporous amphoteric ion-exchange resin; processing of whey and milk	Pilot plant (Japan)
Amerace Corporation	*A. oryzae* lactase covalently bound to a microporous PVC–silica sheet; processing of whey	Pilot plant (USA)

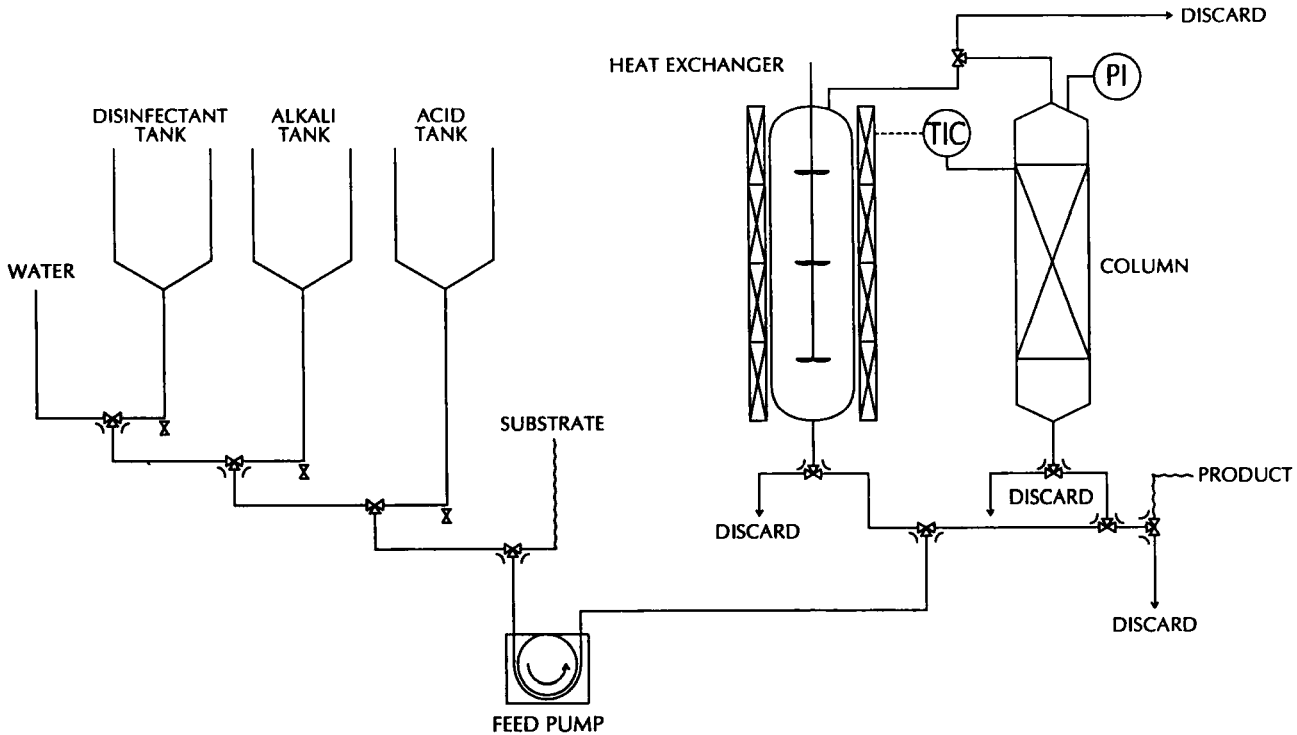


FIG. 18. Flow chart of column reactor using immobilized *A. oryzae* enzyme system. Manufactured by Sumitomo Chemical. For future information, please contact Enzyme Development Corporation.

TABLE XI

EVALUATION OF HALF-LIFE IN COLUMN REACTION[a]

Substrate	Concentrations	Temperature	Half-life
Whey (pH 4.5)	7%	50	250 (260)–680 h
		45	500 (520)–1780 h
		40	4880 h
		30	1000 ± 200 days
		4.5	3000 ± 1000 days
	12%	50	410 (435)–750 h
		45	820(870)–1950 h
		40	6090 h
		30	1600 ± 200 days
		4.5	2400 ± 1000 days
Skim milk (pH 6.65)	10%	50	870 (190)–1820 h
		45	2920 h
		40	9000 h
		30	760 ± 200 days
		4.5	2300 ± 500 (days)
	18%	50	1180 h
		45	1790 h
		40	8300 h
		30	1300 ± 300 (days)
		4.5	2000 ± 500 (days)
Lactose (pH 4.5)	5%	50	3330–3750 h
		40	1500 ± 100 (days)
	7%	50	3440–3880 h
		40	1600 ± 100 (days)
	12%	50	3760–4240 h
		40	1750 ± 150 (days)

[a] Lowering reaction temperature increases the half-life in column operation. Manufactured by Sumitomo. Chemical. For further information, please contact Enzyme Development Corporation.

duction, there are two ways of using UF membranes as membrane bioreactors: as a CSTR that continuously separates the biocatalyst from the reaction mixture, or as a hollow-fiber bioreactor that immobilizes the biocatalyst on one side of the membrane.

The second approach used for the development of UF bioreactors (hollow-fiber enzymatic reactors) allows the lactase to be immobilized under mild conditions without altering its kinetic behavior as might be experienced through chemical coupling onto solid supports (Jones *et al.*, 1988). Breslau and Kilcullen (1975) discussed alternative approaches to diffusion,

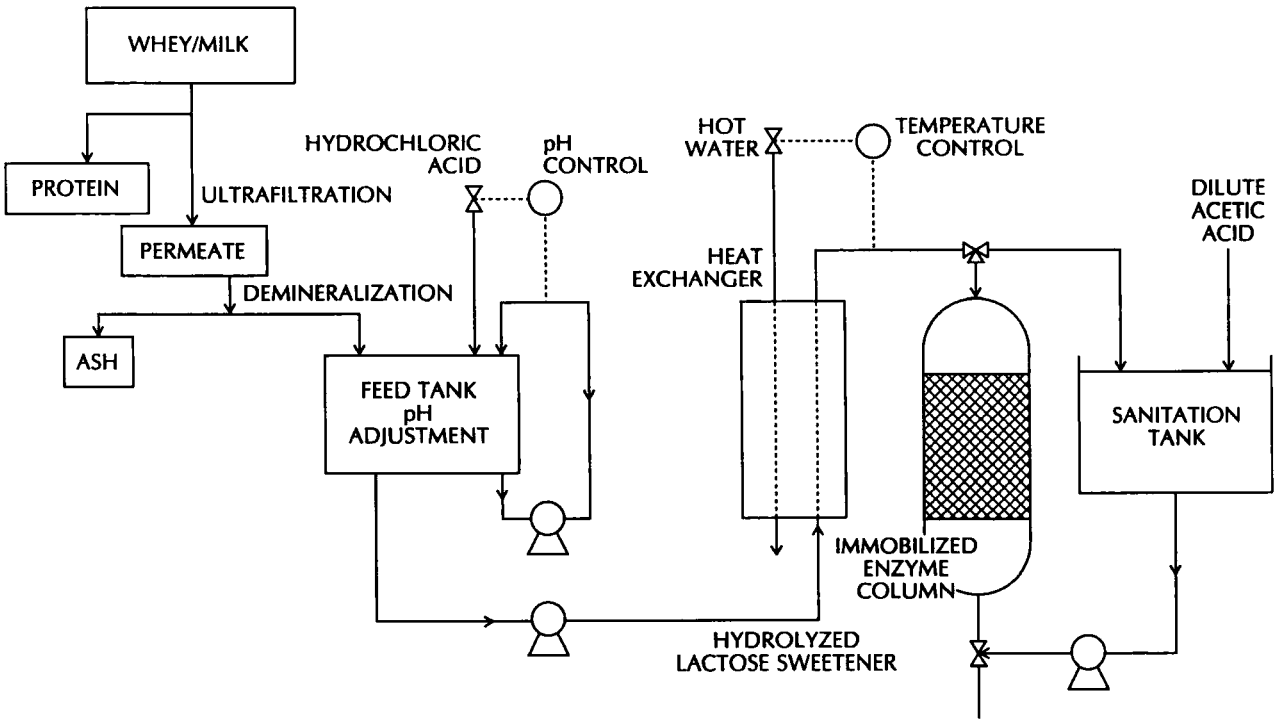


FIG. 19. Corning process for hydrolysis of lactose in ultrafiltered milk or whey. From Moore (1980). Reprinted with permission from Cahners Publishing Company.

the primary method that had been used for contacting substrate with enzyme in hollow-fiber reactors. These procedures took into account the unique properties of hollow fibers. Three modes of operation were proposed with the enzyme immobilized in the shell side of the hollow-fiber membrane reactor: UF, backflushing, and recycling. Huffman-Reichenbach and Harper (1982) studied the retention of *A. oryzae* lactase immobilized on the shell side of hollow-fiber membranes operated in the UF, recycle, backflush, and static modes; leakage averaged 5, 30, 40, and 7%, respectively. Loss in lactase activity was 50% after 2 hours in the UF mode and less than 10% for the other three modes of operation. Jones *et al.* (1988) conducted experimental and theoretical studies on a backflush hollow-fiber enzymatic reactor (HFER), using buffered lactose (Fig. 20). Four lactases derived from *A. niger, K. lactis, K. fragilis,* and *E. coli* were tested for stability and specific activity. The bacterial lactase was found to have the lowest specific activity and retained less than 10% of its initial activity after 3 days of storage at 37°C. The yeast-derived lactases demonstrated substantial losses in stability and had lower specific activity than the *A. niger* lactase. Only the fungal lactase retained 100% of its original activity under the conditions studied.

Maculan (1979) studied the application of a localized UF membrane lactase bioreactor modeled after mammalian intestinal mucosa. Lactases derived from *K. lactis, A. niger,* and a 50:50 combination of both were immobilized on the lumen side of a polysulfone UF membrane and tested for optimum conditions of hydrolysis and stability with 5% buffered lactose as substrate. *K. lactis* lactase was rather quickly denatured in the polysulfone hollow-fiber membrane. Addition of bovine serum albumin stabilized enzyme activity by shielding the lactase from the membrane material (Table XII). Studies involving hollow-fiber enzyme reactors reported in the literature have used buffered lactose solutions as substrate. These systems were important in establishing operating conditions, but have little correlation to actual industrial application.

Senecal and Rand (1993) tested a modified version of the UF lactase bioreactor for the hydrolysis of lactose in skim milk permeate (Fig. 21). Selection of hollow-fiber membrane porosity allows the use of the inherent larger-molecular-weight proteins, present in the skim milk permeate, to form a protective barrier against polysulfone membrane denaturation of the immobilized *K. lactis* lactase. Formation of the protein bed on the lumen side of a H1P30 hollow-fiber membrane is followed by loading with lactase derived from *K. lactis* to form a dynamic secondary membrane. The system is operated at a constant pressure, preserved by a pressure reservoir, and controlled with a pressure switch, to ensure laminar-flow conditions and reduce the possibility of disrupting the protein/lactase layer. Operating

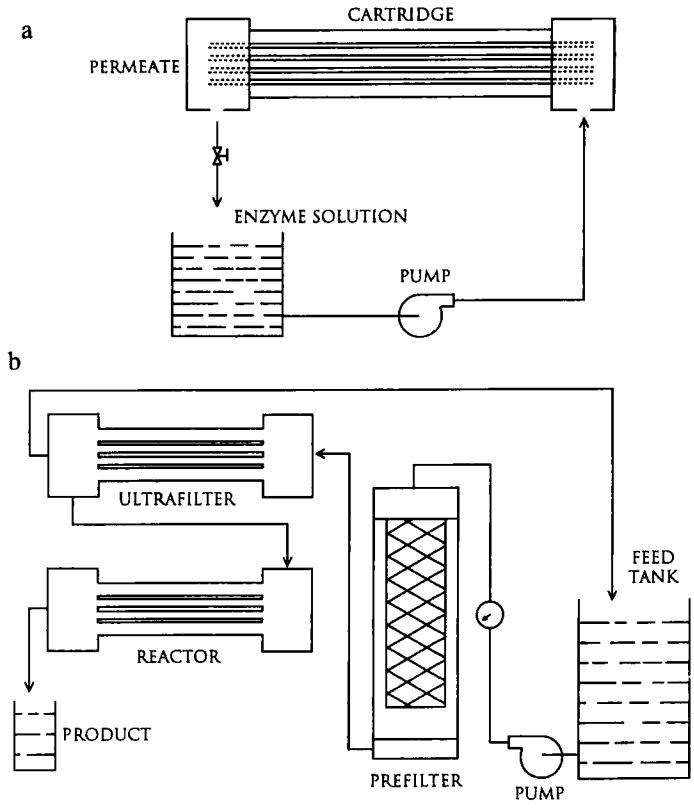


FIG. 20. (a) Experimental setup for enzyme loading. (b) Backflush hollow-fiber enzymatic reactor system. From Jones, C. K. S., Yang, R. Y. K., and White, E. T. (1988). A novel hollow-fiber reactor with reversible immobilization of lactose. *AIChE J.* **34**, 293–304. Reproduced by permission of the American Institute of Chemical Engineers. Copyright © 1988 AIChE. All rights reserved.

conditions that were tested for effects on flux and hydrolysis were temperature, pressure, and enzyme concentration. Results indicate that reactor conditions of 5 psi and 30°C with an enzyme load of 2000 mg of *K. lactis* lactase per 0.06 m^2 hollow-fiber membrane lead to the best performance of the bioreactors tested. The bioreactor has a half-life of approximately 19 hours and is capable of a greater than 40% rate of lactose hydrolysis (Table XIII). It appears that the protein layer, deposited on the lumen of the hollow-fiber membrane to protect the lactase from denaturation, eventually reduces the half-life of the bioreactor by introducing bacterial contamination over time. Permeate samples at 17 to 21 hours had pH readings around 5.8. *K. lactis* activity has been shown to drop rapidly below

TABLE XII

COMPARISON OF LACTASE HOLLOW-FIBER ENZYME BIOREACTORS LOADED ON THE LUMEN SIDE OF THE MEMBRANE

Reactor type	Permeate glucose (mg%)	Glucose production (mg/hr)	Half-life (h)
K. lactis	345	180	13.6
K. lactis technical grade	40	37	4.5
A. niger	277	234	57.0
A. niger/K. lactis	324	230	14.6
K. lactis/bovine albumin	500	240	26.0

pH 5.9 (Guy and Bingham, 1978). The enzyme load for maximum efficiency has been optimized at 3000 mg (16,000 lactase units) per 0.06 m^2, as shown in Fig. 22. This lactase membrane bioreactor demonstrated the potential to accomplish 84% hydrolysis of the lactose in skim milk UF permeate in one pass. It operated continuously for more than 40 hours with skim milk UF permeate, as shown in Fig. 23. The reactor operated at steady state for nearly 15 to 20 hours, with a flux that ranged between 9.5 and 13 liters/h/m^2 and glucose production that averaged nearly 70% hydrolysis of the lactose.

A major concern in studies of lactase immobilization with hollow-fiber reactors is their tendency to suffer enzyme leakage. This phenomenon occurs even when the molecular weight cutoff of the membrane used is much smaller than the enzyme molecular weight. The problems associated

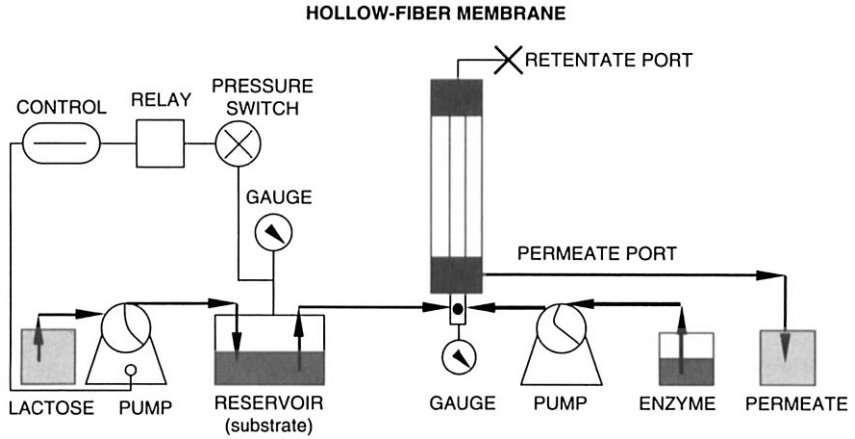


FIG. 21. Schematic diagram of the modified localized ultrafiltration enzyme bioreactor. Presented at the 1993 Institute of Food Technologists Annual Meeting, Chicago.

TABLE XIII

COMPARISON OF DIFFERENT UF LACTASE BIOREACTORS WITH RESPECT TO FLOW RATE AND GLUCOSE PRODUCTION[a]

Pressure (psi)	Temperature (°C)	Glucose (mg %)	Glucose (mg/h)	Flux (liters/m²/h)	% Hydrolysis	Half-life (h)
2.5	20	2342	411	0.30	41.5	47
2.5	30	2404	911	0.63	44.1	13
2.5	40	2087	1470	1.18	38.1	11
5.0	30	1716	2390	2.34	31.3	16
5.0	30[a]	2021	2264	1.77	39.2	19

[a] Bioreactors were operated with 1000 mg of lactase. Values are averages over 6 hours of operation for each bioreactor.

[b] Bioreactor operated with 2000 mg of lactase.

Presented at the 1993 annual Institute of Food Technologists meeting, Chicago.

with enzyme leakage are a result of the wide pore size distributions of commonly available UF membranes (Cheryan, 1986). Leakage has been controlled by choosing membrane pore sizes 5 to 10 times smaller than the

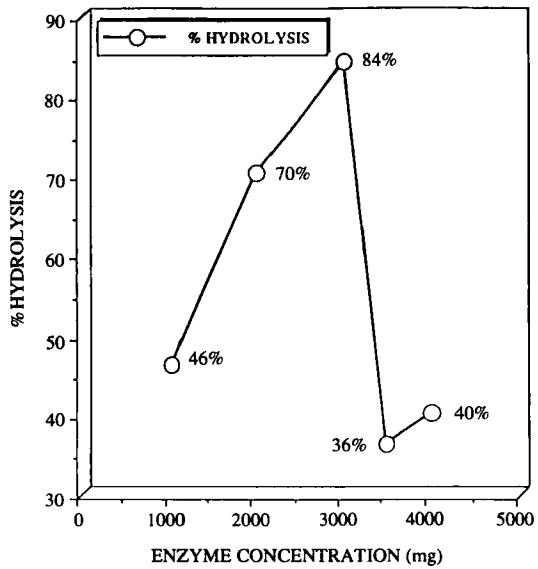


FIG. 22. Effect of *K. lactis* lactase concentration on lactase-catalyzed hydrolysis with a hollow-fiber UF membrane bioreactor operated in the intestinal mode at 30°C and 5 psi (substrate = 5% buffered lactose).

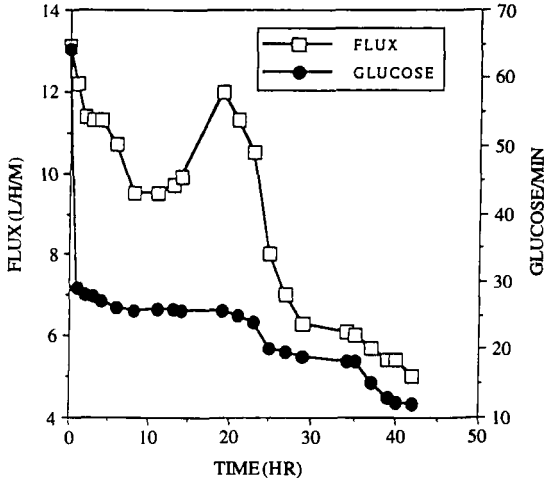


FIG. 23. Continuous operation of the localized membrane lactase bioreactor at 30°C and 5 psi, showing the flux and glucose production from skim milk UF permeate (lactase = 3000 mt/0.6 m^2).

molecular weight of the enzyme. No leakage was experienced in studies conducted with 10,000 MW permeable membranes (Maculan, 1979; Jones *et al.*, 1988); however, significant enzyme leakage occurred when XM50 membranes (Huffman-Reichenbach and Harper, 1982; Jones *et al.*, 1988) and H1P50 membranes (Maculan, 1979) were used. Enzyme leakage was not detected during operation with *K. lactis* lactase loaded on the lumen side of an H1P30 hollow-fiber membrane (Senecal, 1991). Reactor flow rates have also been found to have a significant effect on enzyme leakage. At 200 ml/min there was about 1% leakage from a PM30 membrane; however, at 6000 ml/min, there was an average of 29% enzyme leakage (Huffman-Reichenbach and Harper, 1982).

H. FUTURE POTENTIAL FOR COMMERICAL ENZYME SOURCES

Although development of the current commerical sources of β-galactosidase has allowed the consumption of dairy products by lactose-intolerant individuals, a great deal of work must still be performed to improve optimum enzyme performance, to reduce cost and increase the rate of hydrolysis. It has already been shown that commercial lactases perform differently under similar environmental conditions. In addition, industrial applications that use lactases differ in their operating temperature, pH, ionic environ-

ment, and substrate. The ideal enzyme would be β-galactosidase with the proper activity, specificity, and stability to satisfy the conditions of each individual application. Current research has been dedicated to the development of stable, lower-cost, and productive reactor systems. New lactase sources that perform in low- or high-temperature environments have been studied as means of improving existing continuous reactor systems.

Commerical lactases are produced by the extraction, separation, and partial purification of whole cells. This costly procedure has led to the study of immobilization of whole cells of suitable sources, such as *L. bulgaricus, K. lactis,* and *K. fragilis* (Mahoney, 1985). Utilization of the whole cell has demonstrated both advantages and disadvantages. The advantages are that the whole cell has been shown to have greater stability than the purified enzyme (Park *et al.,* 1979; Mahoney, 1985). The disadvantages are that diffusional restrictions can lead to lower enzyme activity and other active enzymes (i.e., lipase or protease) could affect end-product flavor (Mahoney, 1985). Chawan *et al.* (1993) studied the stability of liposome-encapsulated β-galactosidase in milk. Their objective was to determine the stability and feasibility of encapsulating β-galactosidase in liposomes to reduce the flavor changes that occur in milk during hydrolysis. These systems would allow lactase to be added to milk without changing the product composition or requiring oral supplements. Results indicate negligible hydrolysis of lactose in milk containing liposomes with *E. coli,* but significant (25%) hydrolysis in milk containing liposomes with *A. flavus.* Liposomes or other microencapsulation technologies can lead to enzyme systems that can be tailored to meet the requirements of the different available commercial lactases. In addition, the encapsulated enzymes can be adapted for utilization and release in either liquid or dry food systems. Important stabilizers and activators can be added with the lactase enzyme to improve both stability and functionality in different industrial applications. Research that incorporates encapsulated enzyme systems should include bioavailability studies, especially if the intended use is for activity within the intestine. Microencapsulation is a growing field that has tremendous potential for future applications in enzyme technology.

Enzyme modification is another potential area of research that could lead to improved commercial lactases. The diversity already exhibited by different sources of β-galactosidase could be expanded even further through enzyme modifications. These modifications could be effected through chemical or genetic methods that must be identified and require further extensive studies. Genetic methods have grown rapidly with the advent of increasingly sensitive analytical equipment. Genetic manipulation could lead to the development of a wide range of lactases, each engineered for a particular commercial application. The increase in the use of the thermostable en-

zymes α-amylase and neutral protease from thermophilic organisms is, in part, due to gene cloning of the thermophiles into mesophilic production strains (Zamost *et al.*, 1991).

1. Future Enzyme Sources

A great many sources of the enzyme β-galactosidase have been identified in the literature (Gekas and López-Leiva, 1985; Mahoney, 1985); however, only lactases derived from yeasts and molds and recognized as safe are currently used for industrial applications. Over the years, extensive studies have been conducted and technologies developed with the purpose of improving the stability and functionality of these commercial lactases. Still, these commercial enzymes lack important properties, such as thermostability at the pH of milk products, that are required to render them cost effective in a continuous commerical process. Fungal lactases have proven to be stable but costly in the commercial hydrolysis of whey. The industrial application of thermostable enzymes allows the process to operate at higher temperatures, resulting in shorter processing times and reduced chances of microbial growth. Therefore, studies have been continued on potential lactase sources in an attempt to discover new "safe" lactases that would function ideally in normal commercial processing.

Studies have been conducted on β-galactosidase derived from thermophilic lactic acid bacteria such as *Streptococcus thermophillus* (Greenberg *et al.*, 1985; Smart and Richardson, 1987; Chang and Mahoney, 1989). This lactase was attractive because it is active at temperatures above 50°C, it is a natural constituent in fermented milks, and competitive product inhibition by galactose is weak. Even though the enzyme is more stable in milk than in buffer, operation at temperatures above 50°C is limited (half-life of 146 minutes at 60°C) (Chang and Mahoney, 1989). Other thermophilic bacteria that have received attention were derived from *Bacillus. Bacillus sterarothermophilus* lactase, which has slightly greater thermostability than *S. thermophillus* lactase (Mahoney, 1985), was studied extensively by Griffiths and Muir (1978) in purified, whole-cell, and immobilized whole-cell forms. The enzyme in all forms was found to be very stable at 55° and 60°C. The whole cell was more stable than the purified extract above 65°C; however, all forms lost significant activity at higher temperatures. The only disadvantage to date has been a reported yield 10 times lower than what was currently attainable from yeast (Mahoney, 1985). There has been no evidence of an improvement of enzyme yield at this time. *Bacillus circulans* lactase has been investigated because of its pH optimum of 6.0 and its greater thermostability compared with yeast lactases. Applications for

GRAS status have been submitted to the Food and Drug Administration (Bakken *et al.*, 1992).

Psychotrophic microorganisms would be another potential source of β-galactosidase for dairy processing; however, little is known about their types, physiology, and safety. Loveland *et al.* (1994) isolated three possible members of the *Arthrobacter* genus with β-galactosidase activity. Lactase activity of whole cells was optimum at 20°C, and the cells were labile when incubated above 40°C. Lactases active at refrigeration temperatures would be beneficial in hydrolyzing lactose in dairy products at low temperature, thus reducing product spoilage from microbial contamination.

Fermented milk products have been identified as alternative dairy products for lactose-intolerant individuals who want to avoid the symptoms of lactose malabsorption (Marteau *et al.*, 1990; DeVrese *et al.*, 1992). Kefir, a cultured milk product produced by inoculation of cow's milk with kefir grains, contains β-galactosidase activity primarily as a result of the presence of lactobacilli. Itoh *et al.* (1992) isolated cell-free extracts of *Lactobacillus kefiranofaciens* from kefir grains. The enzymes had an optimal temperature of 50°C and a pH of 6.5 and were rapidly denatured above 55°C. In addition, the lactase was inhibited by both glucose and galactose; however, galactose inhibition was weaker than in most other lactase sources.

Finally, Gonzalez and Monsan (1991) purified β-galactosidase from the fungus *A. fonsecaeus* grown on wheat bran. They were able to obtain a yield of 81% without protease contamination. The *A. fonsecaeus* lactase was found to have greater thermostability than a commerical *A. oryzae* lactase. *A. fonsecaeus* lactase retained 100% activity for more than 1 week at 50°C and lost 50% of the initial activity in 36 hours at 65°C. In comparison, the enzyme activity for *A. oryzae* lactase decreased 23% after 8 hours at 50°C and 50% after 10 minutes at 65°C.

III. TRANSGALACTOSYLASE ACTIVITY

Of commercially available enzymes that command food industry interest, 90% are hydrolytic enzymes—proteases, carbohydrases, and lipases (Monsan *et al.*, 1989). Enzymatic hydrolysis of carbohydrates has found many applications in the food industry. The economic impact of glycosidases account for a third of the worldwide enzyme market (Vulfson, 1993). Like many hydrolases, however, the glycosidases can also catalyze the synthesis of simple and more complex saccharides, resulting in novel oligosaccharides and glycosides (Monsan *et al.*, 1989; Cote and Tao, 1990; Nilsson, 1991; Bucke, 1993; Vulfson, 1993).

Since the 1950s and early 1960s, investigators have been studying and reporting on the transgalactosylase activity of β-galactosidases derived from fungal, bacterial, and yeast sources using a variety of lactose-containing substrates (Aronson, 1952; Pazur, 1953, 1954; Roberts and McFarren, 1953; Roberts and Pettinati, 1957; Pazur *et al.*, 1958; Wallenfels and Malholtra, 1961). Subsequently, studies have shown that the number, concentration, and type of saccharides formed depend on the enzyme source, nature and concentration of substrate, pH, temperature, ion activators/inhibitors, type of process (free versus immobilized), and degree of lactose conversion (Shukla, 1975; Zarate and López-Leiva, 1990). Until recently, the primary reason for investigation of the transgalactosylation reaction of β-galactosidases has been nutritional concerns regarding the potential indigestibility of newly formed saccharides (Asp *et al.*, 1980; Kwak and Jeon, 1986; Burvall *et al.*, 1980; Richmond *et al.*, 1981; Mahoney, 1985; Zarate and López-Leiva, 1990) and the knowledge that allolactose, a major disaccharide formed during this enzymatic process, is involved in stimulation of the *lac* operon of *E. coli* (Jobe and Bourgeois, 1972; Huber *et al.*, 1976; Cote and Tao, 1990). In addition, formation of high levels of oligosaccharides in concentrated lactose solutions, such as whey, could lead to crystallization of these sugars during storage because of low solubility (Mahoney, 1985).

Interest in and recognition of the oligosaccharide moieties of glycoproteins and glycolipids in biological processes led to efforts to synthesize them (Smart, 1991; Thiem, 1993). Chemical synthesis has not been practical because of the laborious nature of conventional methods, the low yield of desired product, the multiple reaction steps, and the need to use expensive/hazardous chemicals (Hedbys *et al.*, 1984; Nilsson, 1990; Nilsson and Fernandez-Mayoralas, 1991; Vulfson and Law, 1993). Glycohydrolases, however, are inexpensive, are easily obtained, and tolerate handling and immobilization well (Thiem, 1993). Therefore, the development of alternative biosynthetic strategies employing enzymes has become attractive (Nilsson, 1988a; Vulfson and Law, 1993).

The characterization of galactosyl (and glycosyl) enzymatic transfer reactions only became of interest with the recognition of their potential applications in the synthesis of pharmaceutical, medical, and immunological products, the formation of biologically active compounds, and the synthesis of food ingredients (Nilsson, 1987, 1991; Vulfson, 1993; Vulfson and Law, 1993; Monsan *et al.*, 1989; Hedbys *et al.*, 1984, 1989; Cote and Tao, 1990; Zarate and López-Leiva, 1990; Thiem, 1993). The application of transgalactosylase activity to the production of a wide variety of products has enormous potential and can serve as an alternative to the more complex chemical syntheses of oligosaccharides.

A. MECHANISM

1. Transgalactosylation versus Reverse Hydrolysis

There are two options for the utilization of the stereospecific synthesis potential of β-galactosidase: reverse hydrolysis and transgalactosylation (Nilsson, 1987, 1991; Monsan *et al.,* 1989; Cote and Tao, 1990; Vulfson, 1993). Although not favored under physiological conditions, reverse hydrolysis reactions can be achieved by creating conditions, such as very concentrated sugar solutions, whereby the thermodymanic equilibrium is modified. β-Galactosidase can form saccharides by reverse hydrolysis via a condensation reaction and release of a water molecule (Monsan *et al.,* 1989; Cote and Tao, 1990; Nilsson, 1991). Although this has been done successfully by several authors (Ajisaka *et al.,* 1987a,b, 1988), there are drawbacks. First, synthesis is limited because of unfavorable equilibrium conditions that also require long reaction times (Monsan *et al.,* 1989). As reaction time is slow because of the limited activity in the concentrated sugar solutions needed to accomplish synthesis, higher enzyme concentrations are needed. Hahn-Hagerdahl *et al.,* (1987) showed that the very low water activity that accompanies concentrated sugar solutions is inhibitory to the synthetic (and hydrolytic) activity of β-galactosidase and maximum enzyme activity cannot be obtained. Furthermore, the enzyme is more specific to galactosides than to monosaccharides; therefore, not only are enzyme requirements higher, but pure enzyme preparations must be used and yields are lower (Nilsson, 1991; Vulfson, 1993).

Transgalactosylation or "transhydrolysis" is the synthesis mode addressed in this review. Because of the higher specificity, product formation using the transgalactosylic properties of β-galactosidase results in higher yields of compounds in shorter periods. Furthermore, crude enzyme preparations may be used (Nilsson, 1991) and, as will be discussed later, regioselectivity can be manipulated. The mechanism of β-galactosidase activity was outlined in the discussion of its hydrolytic characteristics. Figure 12 indicates that hydrolase activity is achieved by the transfer of galactose (glycone) from glucose to a water molecule. If the acceptor (aglycone) were other hydroxyl-containing compounds, synthesis would occur and galactosides would form (Shukla, 1975; Burvall *et al.,* 1979; Mahoney, 1985; Prenosil *et al.,* 1987a; Monsan *et al.,* 1989; Cote and Tao, 1990; Zarate and López-Leiva, 1990; Nilsson, 1991; Vulfson, 1993). Glycosidic bonds are formed via transfer of galactose to other acceptors, such as sugars, alcohols, amine-containing sugars, and nitrophenyl-containing sugars, at different positions, thus forming various di-, tri-, and other oligosaccharides. The enzyme exhibits preference for formation of a β(1–6) linkage when sugars

are used as the acceptors, but other glycosidic bonds have been formed depending on the microbial source of the enzyme (discussed later). In the presence of lactose, without addition of aglycone or acceptor molecules such as monosaccharides, the major disaccharide formed as a result of galactosyl transfer activity using β-galactosidase is allolactose, created by the β(1–6) bond between galactose and glucose (6-*O*-β-D-galactopyranosyl-D-galactose). Another commonly formed disaccharide is galactobiose [β-D-galactose-(1–6)-D-galactose]. The general principles for transgalactosylation were illustrated by Nilsson (1991) (Fig. 24). The donor glycoside (i.e., lactose) is hydrolyzed, an enzyme–galactosyl complex is formed, and glucose is liberated. The transgalactosylation product is formed via transfer of galactose to an acceptor molecule. The energy necessary for this synthesis comes from the splitting of the glycosidic bond stored as a covalent enzyme-derivative intermediate (Monsan *et al.*, 1989), in this case, an enzyme–galactosyl complex. The efficiency of this mechanism of galactosyl transfer can be enhanced by using increased concentrations of the desired donor and acceptor molecules, decreasing available water (without becoming inhibitory), and removing final product (Monsan *et al.*, 1989; Vulfson, 1993.

Huber *et al.* (1976) studied the transgalactosylase activities of β-galactosidase from *E. coli* on lactose and were the first to postulate a mechanism for activity. These researchers reported two types of transgalactosylase activities: direct and indirect. In the direct mode, the glucose moiety is not released from the active site of the enzyme after hydrolysis and the galactose is directly transferred to the glucose acceptor. Indirect activity involves the loss of glucose from the enzyme prior to galactose transfer and the binding of an acceptor in the second phase of the reaction. The authors postulated that the indirect mode of galactosyl transfer is the predominant route to the formation of higher-molecular-weight oligosaccharides and that, initially, direct transfer is the main mode of allolactose production. Free glucose

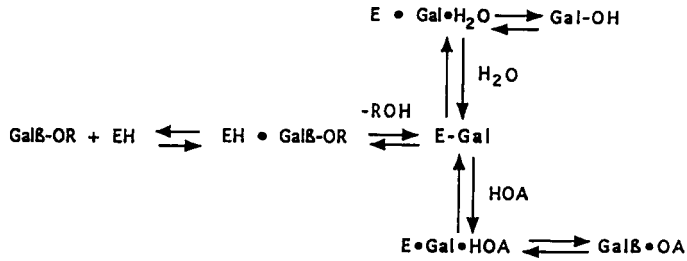


FIG. 24. General principles of transgalactosidation. Galβ-OR, donor (lactose); EH, enzyme; HOA, acceptor; Galβ-OA, transgalactosidation product. Reprinted from Nilsson (1991), pp. 135, by courtesy of Marcel Dekker, Inc.

becames a significant acceptor for formation of allolactose at concentrations greater than 0.01 *M*.

Kinetic models must incorporate both hydrolysis and transgalactosidation activities to accurately reflect enzymatic action (Zarate and López-Leiva, 1990). A simplified model (Fig. 25) describing both hydrolysis and transfer reactions was developed by Prenosil *et al.* (1987a). The model illustrates the numerous possibilities for β-galactosidase activity in the presence of lactose, glucose, galactose, and other oligosaccharides. Yang and Tang (1988) proposed a kinetic model for *A. niger*-derived lactase hydrolysis and transgalactosidation that fulfills a material balance for initial substrate and final product concentrations. These authors came to the conclusion that a kinetic model is plausible. Prenosil *et al.* (1987a), however, reviewed proposed models for β-galactosidase action on lactose and concluded that although models provide qualitative information on the reactions, the complexity of the equations and the number of kinetic constants involved make classic modeling extremely difficult.

Regardless of the mechanism proposed, it has been widely accepted that β-galactosidase, although specific for the glycone or galactose part of lactose, has less selectivity for the acceptor or aglycone portion. Griffith (1991) incubated *K. lactis* lactase with galactose and found poor disaccharide production, leading to the conclusion that transgalactosylation activity is not a viable alternative to chemical synthesis; however, this study actually measures reverse hydrolysis and not transgalactosylation. Research on

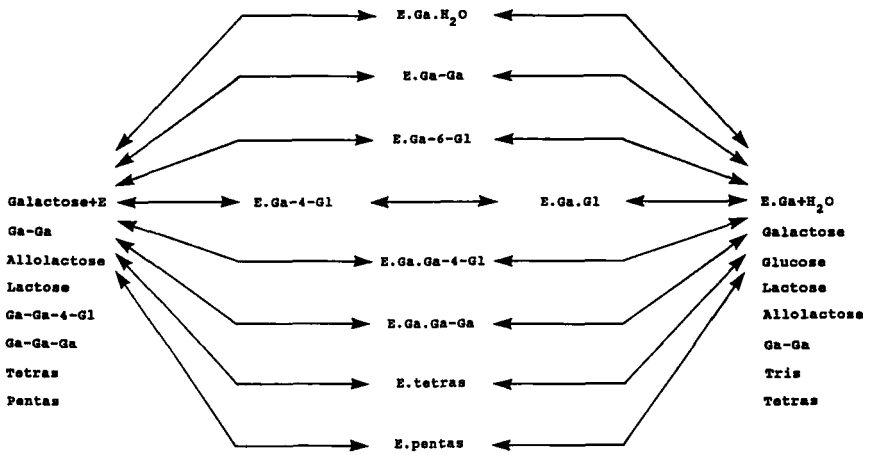


FIG. 25. Transgalactosylase and hydrolase activities of β-galactosidase. From Prenosil, J. E., Stuker, E., and Bourne, J. R. *Biotechnol. Bioeng.* **30,** 1019. Copyright © 1987 John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

transgalactosylase activity of yeast lactases has shown that saccharides are not formed when glucose or galactose is present, alone, in low concentrations, up to 10% (Tikhomirova *et al.*, 1980; Pivarnik, 1990). The efficiency of galactosyl transfer is maximized when lactose is present in the reaction mixture and is in the "hydrolytic" mode (Pivarnik, 1990; Pivarnik and Rand, 1991). Furthermore, the resulting products and their concentrations are critically impacted by the microbial source of the enzyme, the acceptor molecules available, and experimental conditions. Thus, it is impossible to make overall conclusions concerning the value of transgalactosylase activity. Two review articles (Prenosil *et al.*, 1987a, and Zarate; López-Leiva, 1990) discuss oligosaccharide formation during lactose hydrolysis by a variety of microbial lactases. Subsequent discussion in this review focuses on synthesis parameters with commerically available, food-grade β-galactosidases from *A. niger, A. oryzae, K. lactis, K. fragilis,* and *C. pseudotropicalis.*

B. ASSAY METHODS

The methodology to assay the transgalactosylic activity of β-galactosidase varies with the enzyme source and the products to be measured. One consequence associated with transgalactosylase activity that occurs during lactose hydrolysis concerns the erroneous estimation of lactase hydrolytic activity units due to the incorporation of monosaccharides into oligosaccharide production (Mahoney, 1985). Table XIV illustrates the numerous combinations of times, temperatures, pH, and substrates that have been used to study product formation due to transgalactosylase activity from commercially available β-galactosidases. The products formed, which are addressed later in this review, vary with the starting substrates and microbial source of the enzyme. The complete analysis of the oligosaccharides formed requires separation procedures, such as paper chromatography (PC), column chromatography, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas–liquid chromatography (GLC). Furthermore, identification of new products and the glycosidic linkages formed by the enzyme requires techniques such as mass spectroscopy (MS) and nuclear magnetic resonance (NMR). Generally, it has been shown that higher concentrations of lactose result in larger amounts of product; however, Vaheri and Kauppinen (1978) and Pivarnik and Rand (1991) both found that there are optimum lactose concentrations for the formation of products if acceptor molecules are present in the reaction mixtures. In addition, the presence of the aglycone molecule is one mechanism that leads to the more controlled synthesis of desired products. Although Table XIV shows the conditions under which product synthesis was followed and monitored, very little has been done to

delineate actual "units" of β-galactosidase activity, from any microbial source, devoted to synthesis of di-, tri-, and/or oligosaccharides under any conditions of activity.

Huber *et al.* (1976) reported kinetic data obtained from *E. coli* lactase, and their study was the first definitive research concerning velocity measurements. Information relating to the assay of units for lactase derived from commercial sources has, however, been lacking. Pivarnik (1990) and Pivarnik and Rand (1991) have shown that under controlled conditions of substrate and enzyme concentrations, transgalactosylase activity units for *K. lactis* β-galactosidase can be determined (Fig. 26). The linear relationship of allolactose formation over time means that units of transgalactosylase activity can be calculated as micromoles produced per minute under these conditions. As illustrated, the velocity is constant up to 10 and 15 minutes when 2.5 and 5.0% lactose substrate is used, respectively. Table XV shows that units can be calculated, uniformly, for the linear portion of the curves. When the units of transgalactosylase activity are defined as micromoles of allolactose produced per minute per milligram of enzyme, 0.42 unit of activity was calculated in this study versus 7.1 to 7.2 hydrolytic units (μmole glucose produced/min/mg). Therefore, under the conditions of this assay, it is possible to determine that the transgalactosylase activity constituted approximately 6% of the enzyme activity.

C. PROPERTIES

1. pH

The impact of pH on enzyme activity is, of course, critical to achieving optimal enzyme action on a substrate. Huber *et al.* (1976) showed that the pH profile for optimal production of oligosaccharides due to transgalactosylase activity in the presence of lactose is significantly different from that due to hydrolase activity. The curves representing transgalactosylase activity were clearly "skewed" toward higher pH values when compared with curves for glucose and galactose production. Other investigators working with food-grade lactases under various experimental conditions, however, found the pH effect to be variable.

Tikhomirova *et al.* (1980) investigated the effect of pH on transgalactosylase activity of lactase derived from *K. fragilis* and found that both the hydrolytic and transgalactosylic actions were optimal at pH 7.0. Figure 27 illustrates the effect of pH on the transgalactosylic and hydrolytic activities of *K. lactis* β-galactosidase. Although the optimum pH appears to be 7.0 for hydrolytic activity, the pH required for the transgalactosylic mechanism in the presence of lactose, glucose, and galactose is not as clearly delineated.

TABLE XIV

TRANSGALACTOSYLASE ASSAY CONDITIONS OF VARIOUS COMMERCIALLY AVAILABLE β-GALACTOSIDASES

Enzyme source	Substrate	pH	Activating ion	Temperature (°C)	Incubation time (h)	Analytical method[a]	Reference
A. niger	Acid whey @ 4–35% lactose	4.5	—	55	5	Carbon/celite TLC	Wierzbicki and Kosikowski (1973)
K. fragilis *K. lactis* *A. niger* *A. oryzae*	2–20% lactose 5–30% fructose	7.2	Mn^{2+}	37	1–5	GC	Vaheri and Kauppinen (1978)
K. fragilis	30% lactose	7.0	—	40	2–48	PC	Toba and Adachi (1978)
A. niger		4.0				GC	
K. lactis	5–20% lactose UHTST milk @ 5% lactose	6.8	—	24	0–24 0–14 days	PC SPEC	Burvall *et al.* (1979)
K. lactis	20% lactose	6.8	—	37	16	Sephadex G-15, PC, GC-MS	Asp *et al.* (1980)
K. fragilis	5, 10% lactose ± 10% glucose or galactose	7.0	Mg^{2+}	30	0.5–24	PC, GC, SPEC	Tikhomirova *et al.* (1980)
K. lactis	UF whey	6.8/7.0	—	4, 30	12–48	GC	Giec *et al.* (1981)
A. niger	5, 10, 25% lactose	4.5		30, 55	5–24		
K. lactis	4.56% lactose as skim milk	7.0		40	—	HPLC	Nakanishi *et al.* (1983)
A. oryzae	Phenyl-β-galactosides	5.0	—	10 20	720 min 20 min	Sephadex G-25 HPLC	Ooi *et al.* (1984)

A. oryzae	30% lactose	4.8	—	37	8	PC, GC, NMR	Toba *et al.* (1985)
C. pseudotropicalis	5,20% lactose	6.6	—	4	0–24	HPLC	Jeon and Mantha (1985)
K. lactis				37	0–4		
K. lactis	4.56% lactose 4.56,12% NFDM	6.6	—	25, 37	6, 10	PC SPEC	Mozaffar *et al.* (1985)
C. pseudotropicalis	HTST milk	—	—	1, 7, 37	0.5–14 days	HPLC	Kwak and Jeon (1986)
K. lactis					0.25–4		
C. pseudotropicalis	5% lactose, whey, whole milk	6.6		37	0–4	HPLC	Jeon and Saunders (1986)
A. niger	2–30% lactose	opt.	—	opt.	—	HPLC	Prenosil *et al.* (1987b)
A. oryzae							
K. lactis							
K. fragilis							
A. niger	2.5–25% lactose	opt.	—	50	0–8	HPLC	Yang and Tang (1988)
A. oryzae	Lactose +	6.0	—	40	1–15	TLC, HPLC	Kitahata *et al.* (1989)
K. lactis	Rubusoside					NMR	
K. lactis	2.5,5% lactose + 0–5% glucose + 0–5% galactose	6.5	Mg^{2+}	37	5 min 0–120 min	HPLC	Pivarnik (1990) Pivarnik and Rand (1991)
A. oryzae	ONPG + xylopyranoside derivatives	6.5	—	35	2	NMR,GC	Lopez *et al.* (1991)
A. oryzae	ONPG + L-serine	5.0	Mg^{2+}	20, 37	30 + min	Dowex, Sephadex G-10, NMR, HPLC	Sauerbrei and Thiem (1992)

(continues)

TABLE XIV
(*Continued*)

Enzyme source	Substrate	pH	Activating ion	Temperature (°C)	Incubation time (h)	Analytical method[a]	Reference
A. oryzae	Lactitol	5.0	—	50	4	TLC, HPLC, NMR, GC-MS	Yanahira *et al.* (1992)
A. oryzae *K. fragilis* *K. lactis*	20% lactose + 40% branched cyclodextrins	4.5 6.5 6.0	—	40	60 min	HPLC, FAB-MS	Kitahata *et al.* (1992)
C. pseudotropicalis	Chocolate/plain milk ± 3.95, 7.5% glucose	—	—	4, 7, 10	0–20	—	Huang and Rosenberg (1993)

[a] TLC, thin-layer chromatography; GC, gas chromatography; PC, paper chromatography; SPEC, spectrophotometry; MS, mass spectroscopy; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; FAB, flame absorption spectrometry; opt, optimum conditions.

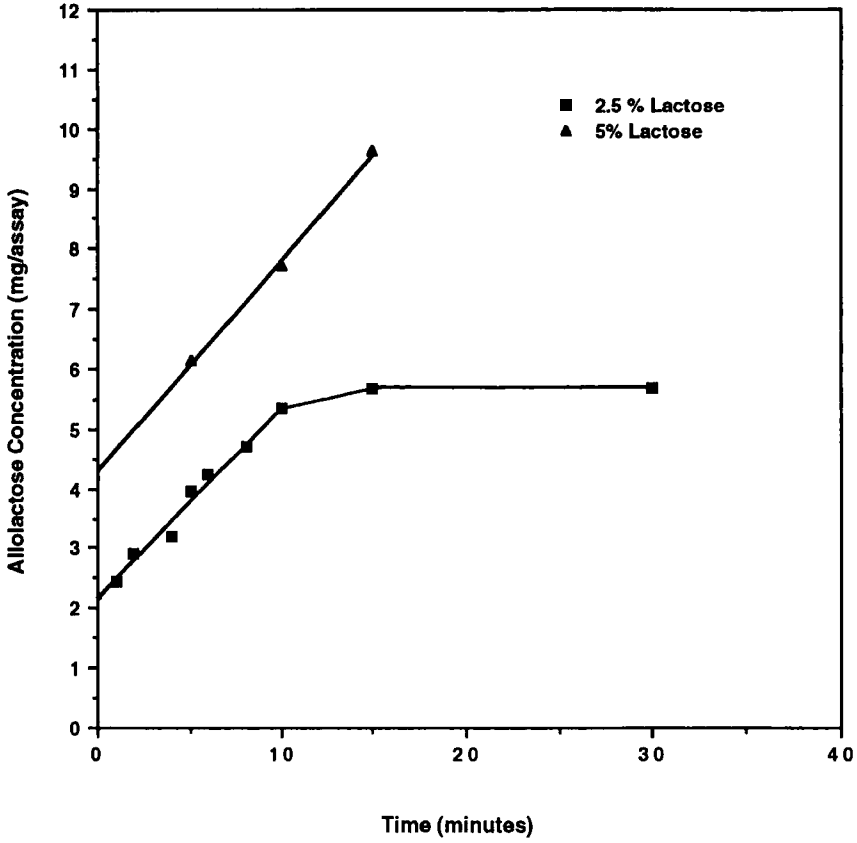


FIG. 26. Effect of lactose concentration on allolactose production catalyzed by *K. lactis* lactase. Experimental conditions: 2.5% glucose, 2.5% galactose, 0.001 *M* $MgCl_2$, and 17.5 hydrolytic units reacted at pH 6.5 and 37°C. Presented at the 1991 annual Institute of Food Technologists meeting, Dallas.

Allolactose production was not significantly different between pH 6.5 and 7.0. Vaheri and Kauppinen (1978) studied the effect of pH on the synthesis of lactulose from lactose (donor) and fructose (acceptor) using *K. fragilis* β-galactosidase. They reported that the optimum pH was 7.2 for both hydrolysis of lactose and formation of lactulose, and the ratio of lactulose formed to lactose hydrolyzed was unaffected by pH. Lopez *et al.* (1991), however, reported an increase in the optimal synthesis of β-galactopyranosyl-D-xylose disaccharides catalyzed by *A. oryzae* β-galactosidase when the pH was increased from 5.0 to 6.5. As illustrated in Tabel XIV, studies following the transgalactosylase activity of commerically available lactases

TABLE XV
KLUYVEROMYCES LACTIS β-GALACTOSIDASE VELOCITY FOR THE PRODUCTION OF ALLOLACTOSE

	Adjusted velocity (μmole/min)	
Time (min)	2.5% Lactose	5.0% Lactose
1	0.82	—
2	1.07	—
5	1.04	1.06
6	1.01	—
8	0.92	—
10	0.93	0.99
15	0.68	1.04
30	0.34	—
60	0.16	—
120	0.03	—

[a] Both systems contained 2.5% glucose, 2.5% galactose, and 17.5 hydrolytic units of β-galactosidase activity were reacted at pH 6.5 at 37°C.

have been conducted at or near the optimum pH for the microbial source. On the basis of limited information, it appears that the transgalactosylation may occur optimally at the same or similar pH, about pH 7.0, for yeast-derived lactases. This pH optimum may also correspond to the value for hydrolytic activity. The same conclusions may not be valid for fungus-derived lactases. More research is necessary to evaluate properly the effect of pH, and, more important, each experimental condition must be evaluated separately.

2. *Temperature and Reaction Time*

As with all enzymatic reactions, both temperature and reaction time have an effect on the pattern and/or concentration of di-, tri-, and oligosaccharides synthesized by β-galactosidases. Generally, as temperature is increased to an optimum, the transgalactosylase reaction rate increases. Although the resulting di-, tri-, and/or oligosaccharides reach their maximum concentrations sooner at higher temperatures, the tendency to form the same galactosyl transfer products remains, even at lower temperatures (Vaheri and Kauppinen, 1978; Giec *et al.*, 1981; Mozaffar *et al.*, 1985; Kwak and Jeon, 1986; Yang and Tang, 1988; Lopez *et al.*, 1991). The commercially available fungal lactases *A. oryzae* and *A. niger* produce saccharides differing little in concentraction when moderate or optimum temperatures are

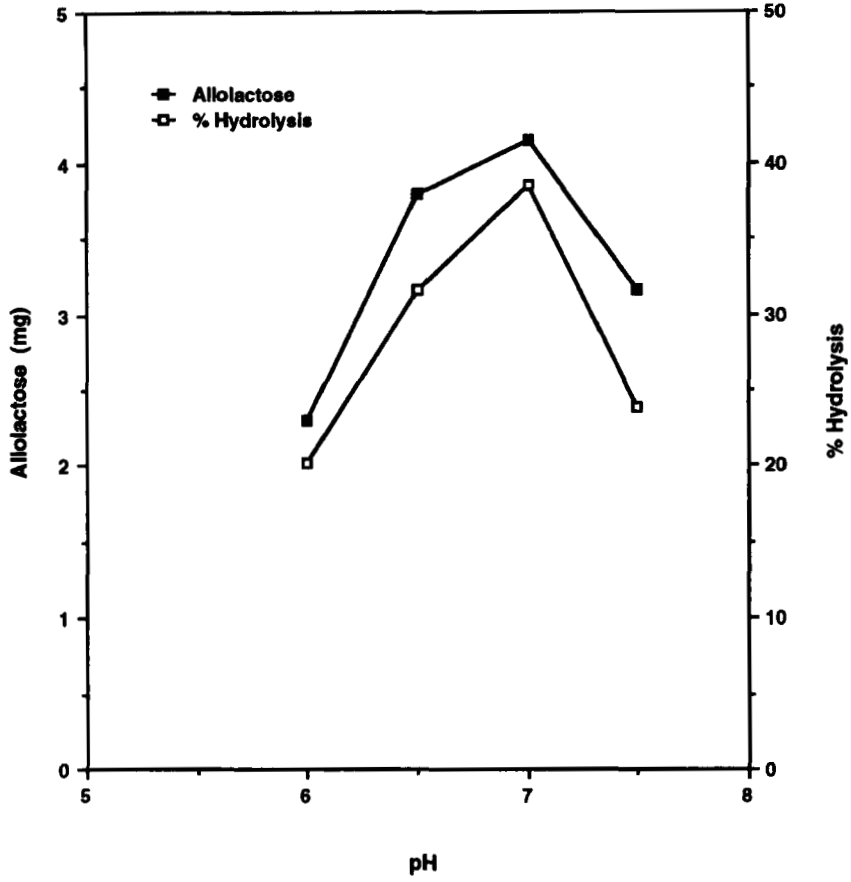


FIG. 27. Allolactose production and lactose hydrolysis by *K. lactis* β-galactosidase as a function of pH in a 5-minute assay at pH 6.5 and 37°C using 2.5% lactose, 2.5% glucose, and 2.5% lactose. Presented at the 1991 annual Institute of Food Technologists meeting, Dallas.

increased by 10° to 20°C, although some results indicate that the trisaccharides vary (Lopez *et al.*, 1991; Giec *et al.*, 1981; Prenosil *et al.*, 1987b). Yang and Tang (1988), using an immobilized system containing *A. niger* lactase, found that higher temperatures promote oligosaccharide formation. Research conducted with yeast β-galactosidase on various lactose-containing substrates showed that at very low temperatures (4°C), there is both a delay (as expected) and a reduction in peak product formation when compared with formation at higher temperatures (Kwak and Jeon, 1986; Giec *et al.*, 1981). Sugars formed under low-temperature conditions, however, have been shown to remain an extended period before being hydrolyzed by the enzyme (Kwak and Jeon, 1986). When product formation at lower

temperatures was evaluated over the longer time frame, the results appeared to show that *total* sugar formation is similar to that at the higher temperature peak. Vaheri and Kauppinen (1978) showed that the maximum temperature for transgalactosylase activity from *K. fragilis* shifts from the hydrolytic maximum of 36°C to 43°–53°C.

Reaction time has a significant effect on both the peak formation of transgalactosylase products and the maximum recovery of these products. Table XVI illustrates the relationship between lactose hydrolysis and maximum product synthesis. Transgalactosylase activity due to yeast-derived lactases results in maximum formation of oligosaccharides at higher degrees of lactose hydrolysis than that due to *Aspergillus*-derived lactases. All investigators have shown that the products formed appear to be related to the lactose hydrolyzed until lactose hydrolysis levels off and product hydrolysis begins. The decrease in available lactose in a reaction mixture will result in a shift in lactase activity, favoring the hydrolysis of the transgalactosylic products formed during transfer reactions (Monsan *et al.*, 1989). Figure 28 is a representative profile of the transgalactosidation products resulting from use of *K. lactis* β-galactosidase during lactose hydrolysis. Under assay conditions described by Pivarnik (1990) and Pivarnik and Rand (1991), of

TABLE XVI

PERCENTAGE LACTOSE HYDROLYSIS AT MAXIMUM FORMATION OF COMPOUNDS VIA TRANSGALACTOSYLASE ACTIVITY OF β-GALACTOSIDASES

Enzyme source	Lactose hydrolysis (%) at maximum synthesis	Reference
K. fragilis	80	Vaheri and Kauppinen (1978)
K. lactis	65–76	Burvall *et al.* (1979)
K. fragilis	60	Tikhomirova *et al.* (1980)
K. lactis	95–99	Giec *et al.* (1981)
A. niger	65–91	
K. lactis	60–85	Nakanishi *et al.* (1983)
K. lactis	80–90	Mozaffar *et al.* (1985)
K. lactis	82–85	Jeon and Mantha (1985)
A. niger	47[a]	Prenosil *et al.* (1987b)
A. oryzae	44–53	
K. fragilis	75	
K. lactis	68	
A. niger	40	Yang and Tang (1988)
K. lactis	70–85	Pivarnik (1990)
		Pivarnik and Rand (1991)

[a] All values estimated from graphic presentation.

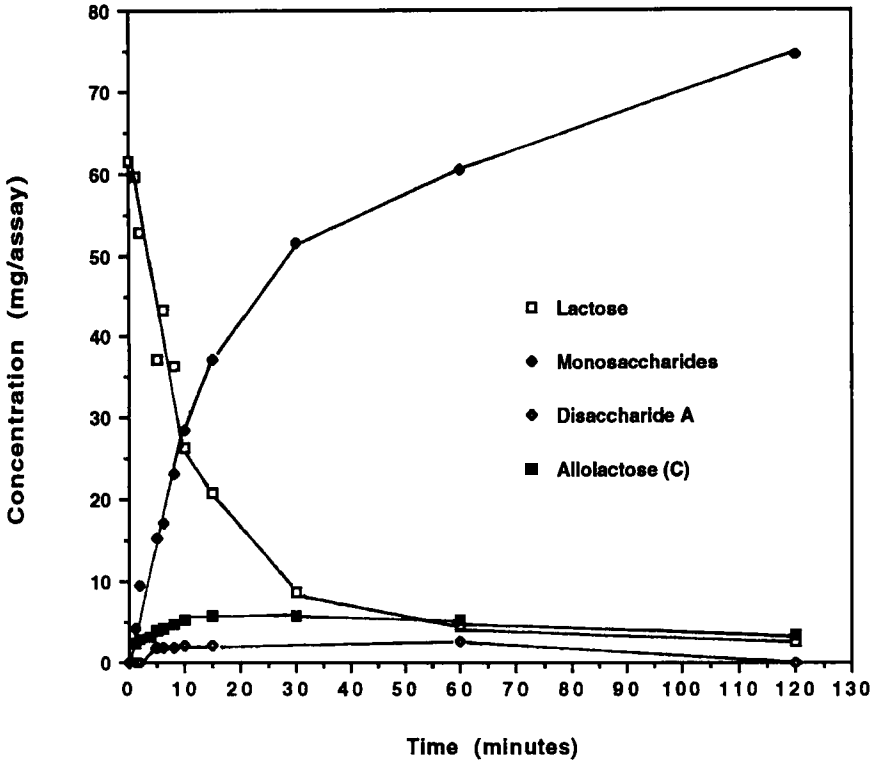


FIG. 28. Profile of substrate utilization and product formation during hydrolysis catalyzed by *K. lactis* β-galactosidase. Experimental conditions: 2.5% lactose, 2.5% glucose, 2.5% galactose, 17.5 hydrolytic units, 0.001 $MgCl_2$, and reacted at pH 6.5 and 37°C. Presented at the 1991 Annual Institute of Food Technologists Meeting, Dallas.

two disaccharides synthesized with yeast lactase transgalactosylase activity, one appears at 6.4% lactose hydrolysis, between 0 and 1 minute, and the other after 5 minutes, when lactose hydrolysis reaches 38.5%. Both compounds peak to a maximum concentration that remains constant and, ultimately, are hydrolyzed at different rates. A rapidly formed compound may be more susceptible to secondary hydrolysis, and the ratio of product isomers would depend on the time of reaction termination (Nilsson, 1991). Therefore, the impact reaction time has on product formation and, ultimately, hydrolysis depends on the specific product(s) formed, assay conditions, availability of acceptor molecules/substrate, and microbial lactase source.

There could be a nutritional implication for low-lactose milk products. If milk is hydrolyzed only 75 to 85% using yeast-derived lactases, as is

TABLE XVII

EFFECT OF LACTOSE CONCENTRATION ON THE PRODUCTION OF OLIGOSACCHARIDES BY TRANSGALACTSIDATION

Enzyme source	Lactose concentration (%)	Matrix	Oligosaccharide concentration (%)	Temperature (°C)	Reported as	Reference
A. niger	4 >4–35	Acid whey	1–2 inc. w/conc.	24	% Total sugars	Wierzbicki and Kosikowski (1973)
S. lactis	5 10 20	Buffer	5 8.5 12.5	55	% Total sugars	Burvall *et al.* (1979)
S. fragilis	5 10	Buffer	6 17.0	30	% Initial lactose	Tikhomirova *et al.* (1980)
S. lactis	5 15 25	Cheese Whey Permeate	ND, ND[a] 5.07, 6.88 9.26, 16.01	4,30	% Total sugars	Giec *et al.* (1981)
S. lactis	5 15	Deionized cheese whey permeate	3.21, 4.94, 6.24, 9.28	4,30	% Total sugars	Giec *et al.* (1981)
A. niger	5 15 25	Cheese whey permeate	2.11, ND 5.94, 4.02 10.44, 8.86	30,55	% Total sugars	Giec *et al* (1981)

A. niger	15	Deionized	4.45, 6.11	30,55	% Total sugars	Giec *et al.* (1981)
	25	cheese whey permeate	7.26, 7.74			
S. lactis	4.56	Skim milk	7.0[b]	40	% Initial lactose	Nakanishi *et al.* (1983)
S. lactis	4.56	Skim milk	14, 20, 23	25, 37, 25	% Initial lactose	Mozaffar *et al.* (1985)
	12					
K. lactis	5	HTST milk	8.87	7	% Lactose hydrolyzed	Kwak and Jeon (1986)
K. lactis	5	Buffer	11.3	37	% Initial lactose	Jeon and Mantha (1985)
	20		15.3–16.5			
A.	2	Buffer	5[b]	50	% Total sugar	Prenosil *et al.* (1987b)
oryzae	5		11			
	7.5		15			
	10		19			
	15		22			
	30		29			
A. niger	2.5	Buffer	2[b]	50	% Total sugar	Prenosil *et al.* (1987b)
	5		3			
	10		5			
	15		8			
	20		11			
	30		16			

[a] ND, not detected.
[b] Determined from data presented as graphs.

usually done commercially (Prenosil *et al.*, 1987b), research has shown that at least 5% of the total sugars could occur as oligosaccharides (Burvall *et al.*, 1979). The presence of the oligosaccharides may eliminate some of the beneficial effects of low-lactose milk for lactose-intolerant individuals. An increased reaction time could help alleviate any potential problems.

3. *Substrate Concentration*

a. Lactose. Transgalactosidation has been found, by many investigators, to be enhanced by increasing the concentration of lactose (Mozaffar *et al.*, 1985; Mahoney, 1985; Prensoil *et al.*, 1987a; Zarate and López-Leiva, 1990; Monsan *et al.*, 1989). Table XVII illustrates the effect of increasing initial lactose concentrations on the transgalactosylase activity of β-galactosidase from commercial sources. A similar trend was observed for immobilized β-galactosidase (Prenosil *et al.*, 1987b; Yang and Tang, 1988). Although it is evident that increasing the concentration of donor results in greater production of di-, tri-, and/or oliogosacchrides, direct comparisons between studies are difficult. The bases on which the results were calculated and reported were different and experimental parameters (time, temperature, enzyme concentration, microbial source, substrate matrix) also varied. Lactose concentration also has an impact on the types of transgalactosylase products formed: more tri- and tetrasaccharides are produced as lactose levels are increased. In addition, the values reported in Table XVII show a "relative" change, but may not reflect the maximum concentrations actually obtained. Investigators may have reported oligosaccharide concentrations that were determined at a point in the reaction where the products were also being hydrolyzed.

b. Donor plus Acceptor Molecules. Although the use of glycosidases is attractive for synthetic applications, the yield and regioselectivity of desired products have been low (Nilsson, 1987, 1988b, 1991; Lopez *et al.*, 1991; Lopez and Fernandez-Mayoralas, 1992). The variety of (1–6) linkages and, to a lesser extent, (1–2), (1–3), and (1–4) isomers has hampered the use of glucosidases and galactosidases for the synthesis of functional and biologically active carbohydrates (Nilsson, 1987, 1988b, 1991). Many investigators have used milk, whey, or buffered lactose as a medium to study the transgalactosylase activity of β-galactosidase and have reported a variety of results. The addition of monosaccharides to the system, in the presence of the donor (i.e., lactose), to maximize the formation of desired compounds is now receiving more attention. The presence and nature of the aglycone molecule can lead to more controlled synthesis and yield of desired products and the formation of oligosaccharides can be manipulated (Nilsson, 1987,

1988b, 1991; Monsan *et al.,* 1989; Nilsson and Fernandez-Mayoralas, 1991; Lopez and Fernandez-Mayoralas, 1992; Bucke, 1993).

Of the work cited above, delineation of the impact of acceptor molecules on activity of transgalactosylases derived from commercial sources has been limited. In 1952, Aronson reported the effects of adding sugars—glucose, galactose, xylose, glycerol—to a reaction mixture containing lactase from *K. fragilis* and 2% lactose. Although the results of the experiments were qualitative, the author concluded that addition of acceptor molecules would result in the formation of additional products. More recent work has quantitatively evaluated the formation of saccharides due to the action of commercially available lactases on substrate mixtures of donor and acceptor molecules. The formation of lactulose, cardiac glycosides, β-xylopyranosides, branched cyclodextrins, and lactitol-derived oligosaccharides has been reported (Vaheri and Kauppinen, 1978; Ooi *et al.,* 1984; Lopez *et al.,* 1991; Kitahata *et al.,* 1992; Yanahira *et al.,* 1992). All of these compounds have potential food ingredient, nutritional, or medical uses.

Optimizing the transgalactosylase reaction of commercial lactases in the presence of donor and acceptor molecules has received little attention. Vaheri and Kauppinen (1978) showed that although the absolute yield of lactulose increased with increasing concentrations of lactose, the ratio of lactulose to hydrolyzed lactose did not change when lactose concentrations increased above 10%. Pivarnik (1990) and Pivarnik and Rand (1991) studied *K. lactis* β-galactosidase to delineate the effects of adding glucose and/or galactose to lactose on the formation of allolactose. Table XVIII shows the relative impact of the monosaccharides on the production of allolactose. Increased concentrations of both lactose and glucose had an impact on product formation. The study showed that the presence of a desired acceptor molecule, in increasing concentrations, could result in a higher percentage of desired product while minimizing undesirable compounds. Table XIX illustrates the total di- and trisaccharide production as a percentage of the total sugars in the reaction mixture. It would appear that regardless of the lactose concentration used, once hydrolysis reaches 57 to 58%, the percentage of galactosyl transfer products formed is approximately the same, 9%. Combining lower concentrations of lactose with higher concentrations of acceptor molecules could maximize formation of desired end products and minimize formation of undesirable oligosaccharides.

4. *Effect of Commercial Source*

Many investigators have studied, both qualitatively and quantitatively, the structures of oligosaccharides formed during lactose hydrolysis catalyzed by transgalactosylase. The number and type of di-, tri-, and oligosac-

TABLE XVIII
RELATIVE TRANSGALACTOSYLASE ACTIVITIES OF *KLUYVEROMYCES LACTIS* β-GALACTOSIDASE WITH VARYING SUBSTRATE COMPONENT COMBINATIONS AFTER A 5-MINUTE REACTION TIME AT pH 6.5 AND 37°C

Initial substrate level (%)			Relative activity of allolactose production (%)[a]
Lactose	Glucose	Galactose	
0.0	2.5	2.5	0
2.5	2.5	2.5	100
2.5	0	0	25[b]
2.5	2.5	0	106
2.5	2.5	0.5	115
2.5	2.5	5.0	112
2.5	5.0	0	160
2.5	5.0	1.0	131
2.5	5.0	2.5	132
2.5	5.0	5.0	148
2.5	1.0	5.0	82
5.0	2.5	2.5	155
5.0	0	0	59
5.0	5.0	0	247

[a] Based on observed values and the standard assay (100%).
[b] Value obtained at 15 minutes; therefore, compared with 15-minute standard assay data to obtain relative activity.

charides produced varied with the yeast, fungal, and bacterial sources studied and the conditions of the experimental design and detection. The disaccharide allolactose [β-D-Gal(1–6)-D-Glu] has been reported to be the major product formed via transgalactosylase activity by many of the authors cited in this review. Galactobiose [β-D-Gal(1–6)-D-Gal] is another prominent disaccharide produced under conditions of lactose hydrolysis. The trisaccharides that have been reported in significant amounts are galactolactose, galactoallolactose and galactogalactobiose. Review articles by Prenosil *et al.* (1987a) and Zarate and López-Leiva (1990) present comparative data on structures delineated by a variety of researchers. Therefore, this review briefy describes the impact of the microbial source on oligosaccharide formation during lactose hydrolysis.

There is a lack of agreement in the literature concerning the identification and enumeration of di-, tri-, and oligosaccharides formed during galactosyl transfer for all commercially available lactases. Yeast-derived lactases have been studied extensively. Aronson (1952), Pazur (1954), and Pazur *et al.* 1958) reported the formation of 4 to 5 saccharides when *K. fragilis* β-

TABLE XIX

DISACCHARIDES AND OLIGOSACCHARIDES FORMED BY *KLUYVEROMYCES LACTIS* LACTASE AS A PERCENTAGE OF THE TOTAL SUGARS PRESENT IN THE ASSAY AT A GIVEN TIME INTERVAL[a]

	2.5% Lactose[b]				5.0% Lactose[b]			
Time (min)	Di (mg)	Tri (mg)	% Total	% Hydrolysis	Di (mg)	Tri (mg)	% Total	% Hydrolysis
2	0.73	ND[c]	1.2	15.2	—	—	—	—
5	3.50	ND	6.2	38.2	3.94	0.97	4.0	25.3
10	5.24	ND	8.7	57.2	4.81	1.89	5.6	49.0
15	5.56	ND	8.7	70.9	8.47	2.23	8.9	58.6
30	5.67	ND	8.6	86.2	—	—	—	—
60	5.76	ND	8.1	93.7	—	—	—	
120	1.17	ND	1.5	95.9	—	—	—	

[a] Lactose left + monosaccharides formed + di- and trisaccharides formed.

[b] Both systems contained 2.5% glucose, 2.5% galactose, and 17.5 hydrolytic units of β-galactosidase activity.

[c] ND, not detected.

galactosidase was used during lactose hydrolysis; however, Roberts and McFarren (1953) reported that the number of products changed with higher lactose concentrations and identified 10 distinctly different di- and higher oligosaccharides. Roberts and Pettinati (1957) and Toba and Adachi (1978) identified 11 and 12 oligosaccharides, respectively, due to galactosyl transfer for *K. fragilis* lactase. Tikhomirova *et al.* (1980) reported the production of only four: one trisaccharide and three disaccharides. Although many investigators have reported that production of oligosaccharides via *C. pseudotropicalis* and *K. lactis* activity is limited (only 5 or 6 oligosaccharides) (Asp *et al.*, 1980; Jeon and Mantha, 1985; Kwak and Jeon, 1986; Pivarnik, 1990; Pivarnik and Rand, 1991), Giec *et al.* (1981) reported 12 different saccharides produced by the action of *K. lactis* lactase (6 disaccharides and 6 trisaccharides).

Generally, mold-derived lactases, *A. niger* and *A. oryzae,* have been found to produce both a larger number of total transgalactosylase products and a higher proportion of trisaccharides when compared with yeast lactases (Giec *et al.*, 1981). Originally, Wierzbicki and Kosikowski (1973) described the formation of five oligosaccharides resulting from *A. niger* lactase activity on lactose in acid whey. As many as 10 to 16 different di-, tri-, and oligosaccharides have since been reported to have been formed during lactose hydrolysis in the presence of *A. niger* lactase (Toba and Adachi,

1978; Giec *et al.*, 1981); and as many as 20 different oligosaccharides appeared when *A. oryzae* β-galactosidase was used (Toba *et al.*, 1985). Glycosidic bonds consisting of β(1–6), β(1–3), and β(1–2) linkages and di-, tri-, tetra-, penta-, and hexasaccharides have been reported.

The impact of microbial source has also been evident when transgalactosylation has been used to form oligosaccharides from a variety of donor and acceptor molecules. Six trisaccharides with a variety of glycosidic bonds, including β(1–5) and β(1–1) to a small degree, were formed from lactitol by a transgalactosylation reaction catalyzed by *A. oryzae* lactase (Yanahira *et al.*, 1992). Transgalactosidation compounds produced from lactose and rubusoside by the action of *A. oryzae* lactase contained β(1–6) and β(1–4) linkages (Kitahata *et al.*, 1989). Vaheri and Kauppinen (1978) showed that *K. fragilis, K. lactis, A. niger,* and *A. oryzae* lactases all catalyze the formation of lactulose from lactose and fructose β(1–4), but formation proceeded at variable rates. Other glycosidic isomers were also formed. Although β-galactosidase derived from *A. oryzae* has been reported to successfully form β-xylopyranosides, the degree and velocity are not as great as with β-galactosidases from other sources (Lopez *et al.*, 1991).

Although all commercially available lactases studied are capable of forming transgalactosidation products during lactose hydrolysis, it has been shown that not all lactases perform galactosyl transfer reactions in the presence of all acceptors. Both *K. lactis* and *K. fragilis* lactases do not catalyze a transgalactosylation reaction to branched cyclodextrins (Kitahata *et al.*, 1992); however, β-galactosidase derived from *A. oryzae* successfully produces transgalactosylation derivatives, although not to the same degree as lactases from other microbial sources. In addition, *K. lactis* β-galactosidase does not form rubusoside derivatives, whereas *A. oryzae* lactase has some transgalactosylase activity under the same experimental conditions (Kitahata *et al.*, 1989). From the limited work, it would appear that yeast-derived lactases have a more restrictive transgalactosylase activity, and all food-grade lactases must be evaluated if optimum production of novel compounds is to be achieved.

The potential of the transgalactosylase-catalyzed reaction lies in the production of novel, functional products from a variety of donor and acceptor molecules. Research has shown that choice of the lactase source has a critical impact on final product type and concentration.

5. *Specificity*

As stated previously, transgalactosylase activity results in the formation of di- and trisaccharides which appear to be common to most of the micro-

bial lactases. Some sources of lactase, however, have been reported to be more restrictive than others with respect to the selectivity of saccharides formed. Although the β(1–6) linkage is generally regarded as the major route of galactosyl transfer, other glycosidic bonds have been formed. *E. coli*-derived β-galactosidase has been shown to produce galactosyl transfer products in a (1–6)>(1–4)>(1–3) order of preferred bond formation (Wallenfels and Maholtra, 1961; Nisizawa and Hashimota, 1970; Nilsson, 1991). Though a variety of glycosidic bonds are possible, some sources of lactase clearly are more restrictive with respect to the selectivity of bonds formed. *K. lactis*-derived β-galactosidase has transgalactosylation activity with a high specificity for the formation of β(1–6) galactosidic linkages (Asp *et al.*, 1980). Toba *et al.* (1985) elucidated the structures of oligosaccharides produced by *A. oryzae* lactase and found that both β(1–3) and β(1–4) linkages were formed, in addition to β(1–6) bonds. Both *A. niger* and *K. fragilis* lactases were found to form disaccharides that contained both β(1–2) and β(1–3) bonds; however, this was not considered the major route of galactosyl transfer and β(1–6)>β(1–3) was the predominant order of synthesis (Toba and Adachi, 1978).

Conducting the transgalactosidation reaction in low-water media may also help manipulate enzyme specificity and product recovery (Vulfson, 1993). Addition of organic solvents to the medium has been studied with successful results (Vulfson *et al.*, 1990; Sauerbrei and Thiem, 1992; Vulfson and Law, 1993).

The structure of acceptor molecules, or aglycones, can influence the specificity of the transgalactosylase reaction. The regioselectivity of glycosidase-catalyzed transglycosidations can be manipulated by changing the structure and/or anomeric configuration of the aglycone (Nilsson, 1987, 1990, 1991; Lopez and Fernandez-Mayoralas, 1992). Size and hydrophobicity of the acceptor aglycone may also have an impact on the specificity to form certain glycosidic linkages (Nilsson, 1991). The yield and regiospecificity of transglycosidation reactions have been controlled simply by modifying one or more of the hydroxyl groups of the acceptor molecule, other than the reducing end (Nilsson and Fernandez-Mayoralas, 1991). Lopez and co-workers (1991), using *A. oryzae*-derived β-galactosidase for the formation of β-xylopyranosides, showed that the aglycone acceptor molecules had a significant impact on the yield and types of products formed. Sauerbrei and Thiem (1992) studied products formed by *A. oryzae* β-galactosidase in a reaction system containing nitrophenyl galactosides as donor and acceptor. Under similar experimental conditions, *ortho*-nitrophenyl and *para*-nitrophenyl galactosides were converted to compounds containing different glycosidic bonds: β(1–6)/β(1–4) and β(1–2)/

$\beta(1-4)$, respectively. Most of the work on specificity manipulation, however, has not been done on commercially available, food-grade β-galactosidases.

6. *Activation and Inhibition*

Information on the activation and inhibition of the transgalactosylase activity of lactases has been limited. Huber *et al.* (1976) first showed the impact of Mg^{2+} ions on *E. coli* lactase-catalyzed production of allolactose in a buffered system. A dramatic 70% decrease in activity was observed when the ion was eliminated from the assay mixture. Potassium ions have also proven to be a powerful activator, in the presence of magnesium, for β-galactosidase derived from *S. thermophilus* (Smart, 1991). The effect of magnesium ions on *K. lactis* β-galactosidase (Table XX), however, has been shown to be less severe, with approximately 30% of activity loss in a buffered system when these ions were eliminated (Pivarnik, 1990; Pivarnik and Rand, 1991). This study also reported relatively little impact of potassium (K^+) ions on galactosyl transfer. Although potassium has an enormous impact on the hydrolytic activity of this enzyme (Jacober-Pivarnik and Rand, 1984), it would appear that it plays no role, either activation or inhibition, on the transgalactosidase activity of *K. lactis* lactase under the experimental conditions of this study. Vaheri and Kauppinen (1978) reported on the effects of ions on *K. fragilis* activity for the formation of lactulose. Incorporation of magnesium, potassium, or manganese ions increased lactulose formation more than lactose hydrolysis. Calcium had a

TABLE XX

EFFECTS OF POTASSIUM AND MAGNESIUM IONS ON THE TRANSGALACTOSYLASE-CATALYZED PRODUCTION OF ALLOLACTOSE AFTER A 5-MINUTE REACTION TIME

	Relative activity (%)	
Added KCl (*M*)	With Mg	Without Mg
0	100[a]	71.2[b]
0.05	94.1	63.7
0.106	100	70.5
0.502	106	63.5

[a] Assay–standard comparison using 2.5% lactose, 2.5% glucose, 2.5% galactose, and 17.5 hydrolytic units of lactase.

[b] Magnesium added as $MgCl_2$ at 0.001 *M*.

slight inhibitory effect, and cadmium profoundly inhibited the transfer reaction.

Ions are not the only possible source of lactase inhibition and activation. Pivarnik and Rand (1991) showed that galactose inhibition, common in hydrolytic reactions to varying degrees, does not seem to affect the transgalactosylase activity of *K. lactis* lactase for the production of allolactose, when a significant quantity of glucose is added during lactose hydrolysis (Fig. 29).

These studies illustrate that the transgalactosylase activities of all β-galactosidases must be evaluated separately under all unique conditions of desired product formation. Maximum transgalactosylase activity can be achieved only if the proper β-galactosidase is chosen for a specific purpose.

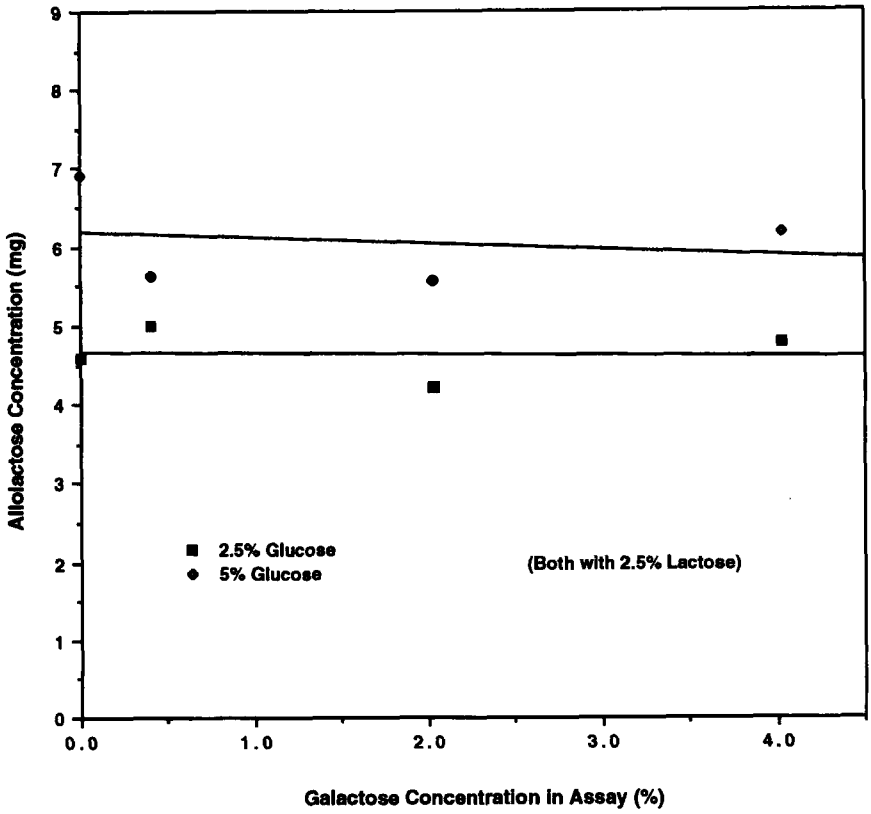


FIG. 29. Effect of changes in glucose and galactose concentrations on allolactose production catalyzed by *K. lactis* lactase. Presented at the 1991 Annual Institute of Food Technologists Meeting, Dallas.

D. ACTIVITY IN FOOD

Researchers have demonstrated that formation of high concentrations of oligosaccharides during lactose hydrolysis can cause intestinal discomfort in healthy humans. Unlike many microbial lactases, human small intestine β-galactosidase has strict substrate specificity and has been shown to have limited hydrolytic activity on galactosyl products with β(1–6) linkages (Burvall *et al.*, 1980). Therefore, most galactosyl transfer products, formed during lactose hydrolysis, would pass through the small intestine undigested and into the large bowel, where bacterial degradation would result in gastrointestinal discomfort (Zarate and López-Leiva, 1990). Although small amounts of transgalactosylation products would form in lactose-hydrolyzed milk, incomplete digestion by individuals, particularly those already compromised by lactase deficiency, could result in symptoms of lactose intolerance. Furthermore, lactose hydrolysis in concentrated milk or whey products would result in a much greater production of oligosaccharides and a greater possibility of intestinal distress (Burvall *et al.*, 1979). For these reasons, the presence of undesirable galactosyl transfer products in dairy products should be evaluated.

Commercially available, low-lactose milk or whey products have not been specifically studied with respect to the degree of oligosaccharides formed; however, other dairy products have been evaluated. Although microbial β-galactosidase activity in yogurt (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) has been shown to enhance lactose digestibility in lactose-intolerant individuals (Kolars *et al.*, 1984; Lin *et al.*, 1989; Onwulata *et al.*, 1989), these lactases have been demonstrated to form oligosaccharides (Toba *et al.*, 1981). Galactosyl transfer reactions of β-galactosidase from *S. thermophilus* have been shown to be significant (Greenberg and Mahoney, 1983; Smart, 1991), with both *S. thermophilus* and *L. bulgaricus* lactases producing the highest yield of galactooligosaccharides compared with other β-galactosidase-generating bacteria (Kobayasi *et al.*, 1990). Allolactose and galactobiose were first isolated from commercial yogurts by Toba *et al.* (1982). The concentrations were low, ranging from 0.03 to 0.09%. The investigators concluded that these compounds were formed by β-galactosidase from the lactic acid bacteria. The oligosaccharide content of yogurt has been shown to be affected by storage and fermentation time. The amount of transgalactosylic products formed in yogurts in a 4-hour incubation period was higher than the amount formed in a 6- or 10-hour incubation period (Toba *et al.*, 1983). When low-lactose yogurt was manufactured, the amounts of saccharides formed due to galactosyl transfer were 4 to 19 times higher than the amount obtained with a control yogurt (0.3–1.7%) (Toba *et al.*, 1986). Therefore, transgalactosylase activity can

be found in other fermented dairy products, particularly when additional lactase is added to the culture to form a low-lactose product.

E. FUTURE UTILIZATION

The importance of galactosides in biological processes, therapeutic applications, and food processes has dictated that simple, inexpensive, and rapid methods of synthesis be evaluated and optimized. Research has shown that utilization of β-galactosidase for synthesis of novel oligosaccharides and glycosides has enormous potential; however, the feasibility of synthesis via transgalactosidation requires more information and study. Characterization and control of galactosyl (and glycosyl) transfer reactions have received attention because of the potential applications of the oligosaccharides that can be produced. Glycoproteins, which contain sugar residues with unique characteristics, are involved in the determination of blood group type, play a role in bacterial and viral infections, are responsible for cell–cell recognition, and are involved in cell receptor sites (Cantacuzene *et al.*, 1991). Galactosides can be used as food additives for a variety of functional purposes, such as humectants, sweeteners, stabilizers, and solubilizers.

Of particular interest is the formation of galactosyl oligosaccharides for the production of *Bifidobacterium* growth promoters. Although the health benefits of *Bifidobacterium* spp. are still being questioned and studied (Hoover, 1993), transgalactosidation products of β-galactosidases are perceived as potential growth promoters for *Bifidobacterium* in the lower intestine of infants (Smart, 1991). Bifidogenic growth factors include the oligosaccharides that are the major components of human breast milk and have been known to accelerate the growth of *Bifidobacterium* (Garza *et al.*, 1987; Kobayashi *et al.*, 1990). Specifically, β-galactosyl (1–6) lactose, lactulose, and amine-containing galactosides have been shown to stimulate certain *Bifidobacterium* stains (Monsan *et al.*, 1989; Modler *et al.*, 1990). Other researchers have shown that many galactosyl oligosaccharides are used by bifidus flora (Zarate and López-Leiva, 1990). Dumortier *et al.* (1994) showed that β-galactosidase from *B. bifidium* exhibited transgalactosylase activity that resulted in 10 different galactooligosaccharides in the presence of lactose. The tri-, tetra-, and pentasaccharides had predominant linkages of β(1–3) and maximum transgalactosylase activity accompanied a 60% lactose conversion. Dumortier and co-workers determined the effects of metals and pH and found the location of transgalactosylase activity to be in the outer membrane region of the cells, indicating the importance of the presence of galactooligosaccharides to the growth of the organism. In addition, lactose derivatives formed through transgalactosylase catalysis not only have enormous future applications in the production of a variety of useful

products, but could take advantage of the large lactose waste problem that accompanies whey disposal.

1. Food and Other Applications

Future food, pharmaceutical, and medical applications of the transgalactosidation process lie in the synthesis of unique and functional compounds, in a controlled manner, through the addition of donor and acceptor molecules. Tables XXI and XXII illustrate the current status of production of novel galactooligosaccharides for potential practical applications. Patent requests have been associated primarily with the formation of galactosides for the promotion of *Bifidobacterium* growth, except for that of Nilsson (1989), who directs the use of galactooligosaccharide formation to therapeutic and diagnostic uses. Table XXII lists the wide variety of compounds synthesized by the transgalactosylase mechanism of β-galactosidases of different origins, not all commercially available and/or food grade. Although all the lactases illustrated are not acceptable for use, this review has shown that β-galactosidases from a variety of microbial sources can accomplish similar tasks given the correct experimental conditions. Therefore, all these products could have enormous potential applications if appropriate enzyme sources are studied.

2. Future Enzyme Sources

In addition to commercially available, food-grade lactases from yeasts and molds, β-galactosidases from other food-grade microbial sources could have potential uses. *S. thermophilus* and *L. bulgaricus* are both potential sources of lactase that have significant transgalactosylase activity but have not been extensively studied. *Bacillus subtilis* KL88, a psychotrophic, food-grade bacteria, has been shown to form galactooligosaccharides in dairy products (Rahim and Lee, 1991) and has potential as a future source of β-galactosidase for commercial applications of transgalactosidase activity. Of particular interest are the hydrolase and transgalactosylase activities associated with β-galactosidase derived from *Bacillus circulans*. *B. circulans* lactase has particularly strong transgalactosylase activity and wide acceptor specificity (Kitahata *et al.*, 1991). In instances where commercially available, food-grade β-galactosidases had limited or no transgalactosylase activity for the production of a novel compound (i.e., branched cyclodextrins, Kitahata *et al.*, 1992), *B. circulans* β-galactosidases catalyzed the synthesis of a significant amount of galactosyl transfer products. Mozaffar and co-workers (1984, 1985, 1986, 1987, and 1989) have been studying β-galactosidase-1 and β-galactosidase-2 derived from *B. circulans* in the free and immobi-

TABLE XXI

PATENTS USING β-GALACTOSIDASE ACTIVITY FOR PRODUCTION OF DESIRABLE GALACTOOLIGOSACCHARIDES FOR USE IN FOOD PRODUCTS

Inventor	Year	Title	Country	Patent No.
Kobayashi *et al.*	1988	Method for producing milk containing galactooligosaccharide	European	88312328.3
Okonogi *et al.*	1988	Enzymatic manufacture of β-D-galactopyranosyl-(1–6)-β-L-galactopyranosyl-(1–4)-D-fructose from lactulose	Japan	JP 6394, 987
Nilsson	1989	Enzymatic synthesis of complex oligosaccharides	International	WO 8909, 275
Kobayashi *et al.*	1990	Method for producing milk containing galactooligosaccharide	United States	4, 944, 952
Oki *et al.*	1990	Vinegar containing galactooligosaccharides	Japan	JP 04218, 362

TABLE XXII

COMPOUNDS FORMED BY TRANSGALACTOSYLASE ACTIVITY OF β-GALACTOSIDASES

Compound	Potential use	Enzyme source	Reference[a]
Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose)	Humectant *Bifidobacterium* growth promoter	*K. fragilis* *A. niger* *K. lactis* *A. oryzae*	Vaheri and Kauppinen (1978)
6-*O*-β-D-galactopyranosyl-2-acetoamido-2-deoxy-D-galactose	Cell surface receptor	*E. coli*	Hedbys *et al.* (1984)
Glycosides of gitoxigenin	Cardiac glycosides	*A. oryzae*	Ooi *et al.* (1984)
Isoraffinose (6-*O*-β-galactosyl sucrose)	*Bifidobacterium* growth promoter	*E. coli*	Suyama *et al.* (1986)
Monoacyl galactoglycerides	Surfactants	*E. coli*	Bjorkling and Gotfredson (1988)
Galβ1–3GlcNAc	*Bifidobacterium* growth promoter	Bovine testes	Hedbys *et al.* (1989)
Galβ1–3GlcNAcβ-Set	Blood group determinant	*E. coli* Bovine testes	Hedbys *et al.* (1989)
Rubusoside derivatives	Sweetner	*A. oryzae* *B. circulans* *P. multicolor* *E. coli*	Kitahata *et al.* (1989)
β-Galactosyl-serine	Glycoprotein component	*E. coli*	Cantacuzene *et al.* (1991)
β-Galactosyl-xylopyranosides	*In vivo* evaluation of intestinal lactase activity	*E. coli* *A. oryzae*	Lopez *et al.* (1991) Lopez and Fernandez-Mayoralas (1992)
4-*O*-β-galactosyl-maltopentose	*Bifidobacterium* growth promoter	*B. circulans*	Kitahata *et al.* (1991)
Galactosyl branched cyclodextrins	Emulsifier, stabilizer, masking odors, production of powders from viscous or oily compounds	*A. oryzae* *B. circulans* *P. multicolor*	Kitahata *et al.* (1992)
β-Galactosyl-serine	Glycoprotein component	*A. niger* *E. coli*	Sauerbrei and Thiem (1992)
Lactitol oligosaccharides	*Bifidobacterium* growth promoter	*A. oryzae*	Yanahira *et al.* (1992)

lized forms and have shown significant transgalactosylase activity even at low lactose concentrations. Experimental conditions optimized for β-galactosidase from one microbial source may not accommodate another. The potential future use of transgalactosidation processes must include evaluation of any feasible source of microbial β-galactosidase under a variety of experimental conditions.

IV. SUMMARY AND RESEARCH NEEDS

The functionality and stability of commercial and potential commercial sources of β-galactosidases for hydrolytic processes must be improved to use the enzymes under conditions that are safe from microbial contamination. To realize this goal, research is required to develop lactases that are resistant to normal processing temperatures, such as that of pasteurization. The continuing development of new microencapsulation technologies could result in a variety of heat-resistant enzymes and more research is needed. Encapsulated materials and geometries should be investigated for effects on lactase functionality as well as increased thermal stability. We may not be that far from realization of the goal. Finnie (1980) demonstrated that *A. niger* lactase has a potential application in HTST pasteurization of milk with 30% recovery. Studies investigating technologies for microencapsulation of lactases from thermostable microorganisms, such as *A. niger,* could result in increased enzyme stability during HTST processing of milk products. Research into genetic alterations or combinations of enzyme characteristics may also improve the functionality and stability of lactases. Genetic cloning of genes from molds into yeasts could result in a β-galactosidase that is stable at traditional processing temperatures yet functional at the pH characteristic of dairy products.

The transgalactosylase activity of lactases has enormous potential for conversion of lactose into useful products; however, because of the extreme diversity of microbial lactase activities, each commercially viable lactase must be evaluated individually, under a variety of experimental conditions, if optimum production of novel compounds is to be achieved. Other food-grade, microbial β-galactosidases, not usually used for their lactase capabilities, may have applications in this area and should be examined. Furthermore, if theses future procedures are to be reproducible, methods for assaying transgalactosylase activity must be optimized and used routinely. Research into methods to increase specificity through modification of substrate molecules should be expanded. Low-lactose milk and whey products should be analyzed to quantitate the extent of transgalactosidase reaction products present and to ascertain their effect on the digestion of these

commodities. Finally, the potential therapeutic, food product, and medical and pharmaceutical applications are almost unlimited; however, selectivity and isomeric control must be achieved to make transgalactosidation reactions an alternative to other mechanisms of synthesis.

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