Natural (Unhydrolyzed) Milk Versus Lactose-Hydrolyzed Milk for Cultured Dairy Products: Physiological and Practical Implications for the Starter Industry

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ABSTRACT

As a microbiological medium, lactose-hydrolyzed milk is quite different from natural milk in that the restrictive role of milk lactose (the major energy source for microorganisms) is eliminated. To emphasize the restrictive role of lactose, the enzymatic and genetic mechanisms involved in the utilization of this beta-galactoside are discussed. Elimination of this selectivity leads to certain manufacturing and storage difficulties with cultured dairy products. This important change in the raw material (milk) should be recognized in selection of starter strains for use in conversion of lactose-hydrolyzed milk to cultured dairy products.

In considering the implications of lactose-hydrolyzed milk for the starter industry, emphasis should be directed at the difference(s) between natural (untreated) and enzyme-treated milk as microbiological media. Milk is indeed an excellent medium for microbial growth and metabolic activity. The moisture content and the pH of milk are ideal for most microorganisms to thrive, and the solid components of milk, i.e. milk-fat, casein, whey proteins, lactose, ash and minor components, can more than adequately provide the carbon, nitrogen, mineral and vitamin (accessory growth-factor) requirements of microorganisms. The only difference between natural milk and lactose-hydrolyzed milk lies in the relative concentrations of the disaccharide, lactose, and its monosaccharide components. It is important to examine how this change influences the course and rate of microbial growth in the enzyme-treated milk.

MILK SUGAR-LACTOSE

Lactose is the major carbohydrate in milk and it accounts for about 4.9% of the weight of this biological fluid. From the viewpoint of chemical structure, lactose is designated as 4-0-{\beta-D-galactopyranosyl-D-glucose} pyranose. It is a disaccharide made up of D-glucose and D-galactose, linked together by a 1,4, \(\beta\)-linkage. The first moiety in the linkage is galactose and the second, glucose. The other free sugars in milk—glucose, galactose and certain phosphate esters of hexoses—occur in relatively trace amounts (26, 47) that lactose is for all practical purposes the major energy and carbon source for microorganisms (32).

LACTOSE UTILIZATION AMONG MICROORGANISMS

In examining the role of lactose as the sole carbon source for microorganisms in milk, one has to consider the mechanism(s) of utilization of lactose by microorganisms. The major metabolic pathways involved in energy trapping and in producing short chain carbon intermediates (for synthesis), accommodate simple sugars or their derivatives, primarily the aldohexose, glucose (30). So in the utilization of lactose, microorganisms have to first convert the disaccharide into its hexose components before they are fed into the relevant energy-yielding pathways. From an operational standpoint, use of any substrate involves two phases—viz: the uptake or transport of the substrate into the cell; and, the actual breakdown of substrate. The uptake of the substrate may involve specific permeases or reactions involving expenditure of bond energy—energy dependent active transport systems (57). The breakdown of disaccharide substrates usually involves enzyme systems capable of cleaving the bond between the monosaccharide components. In the case of utilization, the mechanisms needed are the specific permease or other transport systems and the specific enzyme system to cleave the 1,4 \(\beta\)-linkage between the galactose and glucose moieties. Conversely, only those microorganisms that possess the specific transport system(s) and the enzyme capable of cleaving the \(\beta\)-linkage can derive their carbon requirements from lactose. This in effect is the selective or restrictive role of lactose as far as microbial growth in milk is concerned (32).

Four enzymatic mechanisms are recognized for use of lactose by microorganisms:

1. \(\beta\)-Galactosidase (\(\beta\)-gal), formerly known as lactase, catalyzes the hydrolysis of lactose to glucose and...
galactose. This system is linked either to an inducible galactoside permease or an energy-requiring active transport system. This enzyme is widely distributed among microorganisms and has been described in Aeromonas formicanca, Shigella sonai, Escherichi coli, Bacillus megaterium, Bacillus subtilis, Kluyveromycess fragilis, Neurospora crassa, Aspergillus niger (38), lactobacilli (50), Streptococcus thermophilus (35), and Propionibacterium shermanianii (18).

2. Lactose dehydrogenase system (38) found among pseudomonads which converts lactose to lactobionic acid through two steps with the intermediate formation of lactobionic-\(\xi\)-lactone. The intermediate is then cleaved to gluconate and galactose.

3. In the linked PEP-phosphotransferase-\(\beta\)-D- phosphogalactose galactohydrolase system (PEP-\(\beta\)-D-gal) lactose is first phosphorylated at carbon 6 of the galactose moiety during its passage through the transferase system, and the phosphorylated disaccharide is cleaved to galactose-6-PO₄ and glucose by \(\beta\)-D-phosphogalactose galactohydrolase (\(\beta\)-P-gal). This integrated system is found among lactic streptococci (36,37), lactobacilli (50), and Staphylococcus aureus (27).

4. The fourth is a plausible system, but for which no experimental evidence is now available. It is a scheme similar to that involved in maltose utilization, where there is direct phosphorylation of the disaccharide (\(\alpha\).46). In the case of lactose, this would involve the transfer of a phosphate group to the first carbon in the galactose moiety.

The known lactose utilization mechanisms among dairy starter bacteria are summarized in Table 1. For detailed discussion of these systems, the reader should consult excellent reviews by McKay et al. (38) and Lawrence et al. (31). An excellent treatment of the transport systems involving carbohydrates is given in a recent article by Kornberg (28).

### GENETIC ASPECTS OF LACTOSE UTILIZATION AMONG STARTER BACTERIA

Phenotypic characteristics such as lactose utilization reflect the genetic make-up of the microorganisms, and the genetic control mechanisms that govern their expression. For a more complete understanding of lactose utilization among starter bacteria, the genetic aspects of this function needs to be surveyed here. Currently, only something of the genetics of this function among lactic streptococci is known. This system will be examined here to understand the complexities involved in lactose utilization by starter bacteria.

### INSTABILITY OF LACTOSE UTILIZATION

One of the very first observations made about the lactose fermentative characteristics (as manifested by lactic acid accumulation) of lactic streptococci was that it is relatively unstable. As early as 1931, Harriman and Hammer (17) reported on the emergence of slow acid-producing variants within pure cultures of lactic streptococci. Later Hunter (21) reported on occurrence of slow variants within pure cultures of Streptococcus cremoris. Recently McKay et al. (39) and McDonald (34) observed the spontaneous and dominant emergence of slow lactose-fermenting or lactose-negative variants when fast acid producing single strains of lactic streptococci were propagated in a continuous fermentation system. The numbers of such variants were very high as the fermentation progressed beyond 120 h.

These observations plus the data showing the dramatic increase of lactose-negative variants within a pure lactic Streptococcus culture treated with acriflavin (a mutagen that selectively induces the loss of extrachromosomal elements within cells), prompted McKay et al. (39) to propose that the genetic information for lactose utilization in these bacteria resides in extrachromosomal elements.

### LACTOSE UTILIZATION - CODED ON TRANSIENT GENETIC ELEMENTS

The first evidence for a transient genetic element-linked lactose fermentation function among lactic streptococci was reported by McKay et al. (40). They found that certain UV-induced prophages from Streptococcus lactis C₇ can transduce the ability for active lactose fermentation to spontaneous and acriflavin-induced lactose-negative mutants of the same strain. Transduction is the process by which genetic transfer among bacteria is mediated by bacteriophages. This was confirmed by induction of high frequency transducing particles from lactose-positive transductants. Transductants are bacteria that have been genetically altered by acquiring genetic markers transferred via bacteriophages. Later, Cords et al. (6) presented the first evidence for a plasmid-linked lactose utilization function in S. cremoris B₃. Plasmids are characteristically small, covalently closed, circular duplex DNA molecules which may confer upon the host known genetic functions (6).
RELATIONSHIP BETWEEN PROTEOLYTIC FUNCTION AND LACTOSE UTILIZATION IN MILK

Another interesting sidelight to the loss of $\beta$-galactosidase utilization by lactic streptococci is the observation that this phenotypic expression is accompanied by the relative loss of efficient proteolytic function. In milk, slow acid production from lactose is invariably accompanied by considerable weakening or complete loss of caseolytic ability (4,44). The relatedness of these functions was clearly demonstrated in the analysis of transductants derived from lactose and proteinase-negative S. lactis $C_2$ mutants by treating them with prophages induced from lactose and proteinase-positive wild-type S. lactis $C_2$ culture (41). In publications that followed (9,43), further experimental proof was presented to show that both lactose utilization and proteinase functions are genetically coded on plasmids carried by lactic streptococci.

COMPLEXITY OF GENETICS OF LACTOSE UTILIZATION AMONG LACTIC STREPTOCOCCI

In a recent paper Anderson and McKay (1) demonstrated the complexities of the genetics of lactose utilization in S. cremoris $B_1$. They used wild-type S. cremoris $B_1$, its mutants and revertants in complementation studies with site-specific mutants of S. aureus and showed the following:

1. The location of genetic determinants for lactose-specific transport enzymes of PEP-phosphotransferase system appears to be on a plasmid with a molecular weight of $36 \times 10^6$ daltons.
2. The site for the induction of $\beta$-D-phosphogalactosidase galactohydrolase by galactose-6-PO$_4$ also appears to reside in the same plasmid.
3. There appears to be an alternative site for the genetic information for the elaboration of $\beta$-P-gal enzyme on the bacterial chromosome which is expressed under special conditions. Figure 1 depicts the complex genetic system governing use of lactose by lactic streptococci.

So far, the mechanism(s) and genetics of lactose utilization among starter bacteria have been surveyed at length, because in natural milk, lactose presents the major hurdle to rapid and uniform acid producing function by lactic streptococci, none of the lactose-negative mutants lacked the ability to utilize glucose. So glucose utilization appears to be a stable phenotypic characteristic. This observation assumes great significance in dealing with lactose-hydrolyzed milk.

Lactic streptococci preferentially utilize glucose (4,44) and when this hexose is available, rapid lactic acid accumulation is observed. Galactose, on the other hand, is slowly metabolized. Gilliland et al. (13), using glucose, galactose and lactose individually in a broth system, showed that the fastest growth of lactic streptococci is obtained with glucose; the growth rate was the lowest with galactose. The same workers also found that in milk supplemented with E. coli $\beta$-galactosidase, and inoculated with a lactic starter, there was rapid use of glucose, but galactose accumulated gradually. Other authors (15,19,31) have also reported on the lingering presence of galactose in milk and cheese during and after active fermentation by lactic bacteria. Recently O'Leary and Woychick (48) made similar observations with yogurt bacteria in natural and lactose-hydrolyzed milk.

IMPLICATIONS OF LACTOSE-HYDROLYZED MILK TO STARTER INDUSTRY

What implications do these observations have in selection of starters for lactose-hydrolyzed milk? It may be safely assumed that removal of the restrictive role of lactose in the hydrolyzed milk makes it easier to select and formulate starters. In all the published work relating to the manufacture of cultured products from lactose-hydrolyzed milk (16,48,58,61) with one exception (8), the major observation was that the rate of acid production in this substrate was much faster than in untreated milk. This is definitely because of the ready availability of glucose in free form in the enzyme-treated milk. Slow use of galactose is not of much significance because in most fermentations in dairy manufacturing only a very small portion, about 16-18% of the available sugar, is utilized (53,54). In a recent article, Kosikowski (29) has proposed the possible application of a suitable galactose isomerase to stimulate the rate of dissipation of this persis tant hexose.

Based on the foregoing discussion, several important implications can be projected for the starter industry:

1. Lactose-hydrolyzed milk affords a wider selection of conventional starter strains because many of the so called "slow strains" can be included in mixed-strain starters.
2. Variation in rate of acid production either due to inherent "slowness" or genetic changes may not be as critical as in untreated milk.
3. Wider selection of strains would offer a greater number of strain combinations to provide multiplicity of and flexibility in culture rotation to protect against phage failures.
4. Faster rate of acid production in itself (as found in hydrolyzed milk in comparison with untreated milk) may provide greater phage protection, because the faster
FIGURE 1. Diagrammatic representation of genetic recombination of Lac and Prt markers in S. lactis C2. A-Lactose; Prt - Proteinase; C-Bacterial chromosome; HB-Hybridphage containing phage DNA, small pieces of Lac and Prt plasmids caused by copy error during proliferation of prophage (Spontaneous or induced).

HBP-Hybrid plasmid containing portions of Lac and Prt plasmids plus a small piece of phage DNA. This plasmid is produced in large numbers because it contains phage DNA. As phage DNA is replicated, enzymes are furnished which allow increased replication of the hybrid plasmid.

HFT-High frequency transductants produced as a result of replication on HBP. These HFT phages transfer Lac and Prt markers at high frequency.

1-Induction-sporontaneous or UV; L-Lactose plasmid; LP-Phage containing small piece of lac plasmid plus the phage DNA; LY-Lysis of cell liberating mature phages; P-Proliferation of phage; PL-Loss of plasmid(s)-spontaneous or induced by agents like acridine; PP-Phage DNA.

PH-Proteinase plasmid; RC-Phage-Lac!Prt plasmid recombination; T-Transduction.

A. Lactose fermenting and proteinase producing S. lactis C2 cell containing phage DNA (PP) which inserted into the bacterial chromosome and the five identified plasmids. Three of the plasmids of unknown function have been designated as open circles, the proteinase plasmid as (PR), and the lactose plasmid as (L).

B. Exposing the cell to UV irradiation or other inducing agents will release the phage DNA from the chromosome. The phage DNA then begins self-replication to form mature phage particles.

C. S. lactis C2 cell containing plasmid DNA and mature phage particles. Most of the phage particles will contain phage DNA but a few, due to errors in recombination events, will contain phage DNA plus lactose plasmid DNA (LP) or phage DNA plus lactose plasmid DNA and proteinase plasmid DNA (HBP). These particles serve as transducing phage.

D. The bacterial cell has lysed releasing the phage particles.

E. An S. lactis C2 cell is unable to ferment lactose or produce the proteinase enzyme system because it has lost the lactose and proteinase plasmids.

This cell is infected with the phage lysate from D.

F. If the Lac Prt cell becomes infected with LP phage only lactose fermenting ability is added back and the cell is now able to ferment lactose but does not produce the proteinase enzyme system.

G. If the Lac Prt cell becomes infected with HBP phage both lactose fermenting ability and proteinase activity are added back and the cell now regains ability to ferment lactose and produce the proteinase enzyme system.

H. Steps B and C are repeated upon release of the phage from the chromosome.
fermentation attains pH values lower than 5.0, the smaller is the chance for massive phage infection (49).

5. Use of lactose-hydrolyzed milk for cultured products would probably offer wider application of "protective cultures." The term "protective cultures" refers to specific lactic Streptococcus and Leuconostoc cremoris cultures that are used to extend the shelf-life of products like cottage cheese (33,45,59). Some of these cultures also have inhibitory properties against certain foodborne pathogens (2,7,51,56). With removal of the restrictive lactose barrier, and the easy availability of glucose, a sugar that is readily fermentable by a wide variety of flora, spoilage of lactose-hydrolyzed fluid milk will be a problem. Currently, a highly concentrated culture of S. lactis subspecies diacetylactis is available for direct application to cottage cheese curd to lengthen shelf-life (55). There is further need to develop procedures and technology for application of such protective cultures to lengthen the shelf-life of fluid lactose-hydrolyzed milk and products made from it (14).

6. Production of concentrated culture for application in lactose-hydrolyzed milk will be simpler and cheaper. For use in natural milk, it is recommended that milk or milk derivatives be used in fermentation media to retain the caseolytic function in starter bacteria, which would ensure production of good acid-producing cells (60). The need to retain specific proteolytic function in milk necessary for lactose metabolism is not critical when glucose is available. So it would be possible to substitute milk or milk derivatives with more soluble, non-opaque and more heat-stable components. Also a wider variety of ingredients and stimulants can be used in the fermentation media to obtain high cell count without the loss of acid-producing activity (60). Removal of milk or milk derivatives from the fermentation media would make separation of bacterial cells (centrifugation or filtration) easier and more efficient. However, care should be taken to include strains with good caseolytic function in combining mixtures for the production of ripened natural cheeses.

7. The availability of free glucose in sufficient amounts in lactose-hydrolyzed milk would allow the use of safe, clean lactic acid producing bacteria used in non-dairy fermentations. This will provide additional safeguards against phage failures. For example, selected strains of Pedicoccus cerevisiae, which are widely used in sausage fermentations (10), can be employed. The procedure for applying such cultures in lactose-hydrolyzed milk has been patented (20). Certain selected low temperature lactobacilli like Lactobacillus planta-rum, which is used in sausage, pickle, sauerkraut and silage fermentations (12), could also be considered.

Another possibility is the application of carefully screened strains of enterococci, which are capable of fast acid production. Recently a series of papers was published on production of safe, excellent quality cheese using selected strains of enterococci as supplemental starters (23-25). Although the relationship between enterococci and food poisoning outbreaks is considered tenuous (9), extensive testing of strains for the absence of any trace of enteropathogenicity should be done before large scale application.

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