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Modification of Milk Using Immobilized Enzymes

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Abstract

The preparation and use of immobilized enzyme systems for the modification of the principle components of milk—casein, lactose, and butterfat—are discussed.

Design of a pilot-scale bioreactor is described. Also the use of tea polyphenols as a crosslinking agent is reported.

A review of methods for hydrolyzing lactose and characteristics of a concentrated liquid sweetener derived from dairy byproducts is presented. Further, the application of immobilized esterases to modify milk, cream, and butterfat is reported.

Index Entries: Immobilized enzymes, in milk modification; continuous reactor, for modified milk; tea polyphenol crosslinker; milk-clotting; liquid sweetener; butterfat-fatty acids.

Introduction

The reasons why food manufacturers are concerned with processes utilizing immobilized enzymes have been the subject of earlier reviews (*1–4*). Among these is the recognition that immobilized bioreactors will allow manufacturers a level of process control previously unavailable. In some instances, by simply altering flowrate of feedstock, the desired degree of modification can be attained. In addition, there

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is the added economic benefit of enzyme reuse over a period of weeks or months. Finally, since no enzyme appears as an additive in the final product, an additional processing step to inactivate the enzyme is unnecessary.

The advantages of this new enzyme technology from a corporate point of view are summarized in Fig. 1. It can be observed that reduced plant size and enhanced automation at the plant level are desirable production goals. Reductions in processing costs and improvements in process time are continuing goals for plant engineers. Additionally, the ability to provide food products with enhanced flavor, consistent quality, and increased shelf life are of great interest to food processors. Finally, the ability to develop new foods, along with new food analogs, by enzyme technology offer added incentives to establish these new products in the market place.

World cheese production has more than doubled in the past 10 yr, reflecting growing populations and per capita consumption in the developed nations. One of the first steps in the manufacture of cheese involves gelation of the casein proteins present in milk. This coagulation of milk is accomplished by the addition of animal or fungal proteases that have the ability to induce coagulation. Thus, as production has increased, so has the need for enzymes with this unique characteristic. Estimates of the commercial value of the enzyme market for animal and fungal milk-clotting enzymes indicate a combined 20 million dollar expenditure per annum for enzymes with this functionality.

The concept of attaching chymosin or pepsin to water-insoluble polymers for milk-clotting has been of interest to researchers for many years (*5–9*). Although active enzyme systems were developed, the loss in activity in milk systems was a common defect.

This report summarizes results of studies on milk-clotting using an immobilized pepsin–resin polymer. A schematic illustration of the attachment process is presented in Fig. 2. In addition, the development and use of an immobilized lactase system employing tea polyphenols for crosslinking is described. Finally, data on the use of two immobilized esterase systems to modify butterfat is presented.

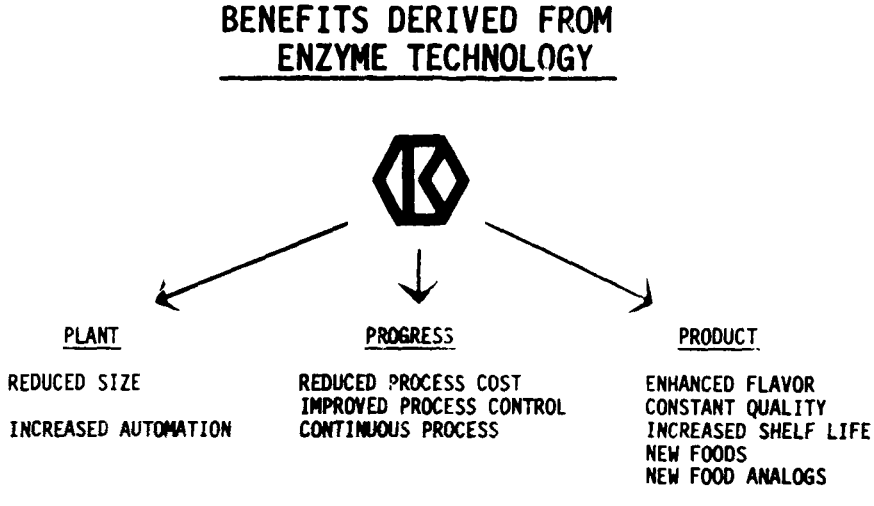

Fig. 1. Advantages of enzyme technology—corporate viewpoint.

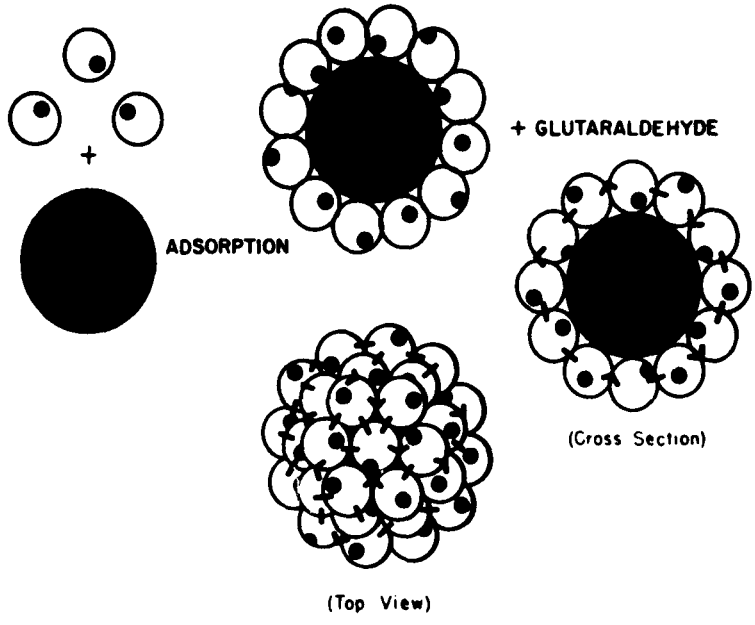

Fig. 2. Schematic illustration of enzyme attachment process.

Materials and Methods

Immobilized Pepsin

3× Crystalline pepsin (Worthington) was used as the protease for milk-clotting studies. The protease was attached to a Duolite amine resin, 16 × 15 mesh (Diamond Shamrock), at the isoelectric point of pepsin (pH 1.2). After 18 h at 10°C, the bound enzyme was washed with 0.5% sodium acetate buffer pH 3.5, followed by 0.5% K_2HPO_4 buffer, pH 5.0, until no traces of proteases were detectable in the washing. The bound enzyme was then crosslinked with 3.0% glutaraldehyde and again buffer washed as before.

Milk-clotting activity was estimated by passing acidified milk, adjusted to pH 6.2, through a column of immobilized pepsin–resin particles at 15°C. The procedure was previously described by Ferrier et al. (*6*) in their studies on milk-clotting. The treated milk was then warmed to 37°C and allowed to clot (in less than 5 min in the presence of 0.1% calcium chloride). In order to characterize the functionality of the immobilized system in continuous operations, the use of space velocity units of activity were established using the defined formula similar to that previously described by Tosa et al. (*10*).

$$\text{Space velocity units} = [\text{Milk flow rate (mL h}^{-1})/\text{Volume of wet resin (mL)}][1/\text{Set time (min)}]$$

The specific activity of the attached enzyme was determined using hemoglobin for substrate as described by Herriot (*11*). Additionally, the phenyl-sulfite procedure of Stein et al. (*12*) was employed.

Immobilized Lactase

Asp. niger lactase (GB Fermentation Ind., Inc.) was adsorbed on pretreated carboxymethyl cellulose (CMC) particles (Vistec). Combined enzyme and CMC loadings were carried out and enzyme protein loadings of 7–12 mg g^{-1} cellulose media were attained. Crosslinking was achieved using tea polyphenols derived from water extracts of green or black teas. The bound enzyme was soaked for several hours in tea extract, followed by cold acetate buffer (pH 4.5) washings to remove excess tea derivatives.

Activity of the bound enzyme was determined using lactose buffer solutions or whey permeate (*13*).

Immobilized Lipase

Nylon 6 particles, 16–20 mesh F 8202 (Allied Chemical), were pretreated with formic acid (50%) for 30 min, washed and soaked in 30% $FeCl_2$ for 72 h. After washing, the particles were treated with succinic anhydride at pH 8.0 for 2 h. Again the particles were washed and placed in 30% $FeCl_2$ for 18 h. The twice complexed nylon–iron complex was water-washed and used for attachment of the enzyme.

A solution of *Mucor miehei* lipase (5.0 mg mL^{-1}) or pregastric esterase in 0.05*M* sodium acetate buffer pH 5.5 was mixed with the nylon–iron support for 3 h at 2°C. Crosslinking of the enzyme–iron–nylon complex was achieved using tea polyphenols or 25% glutaraldehyde which was added to the complex and slowly stirred for 18 h at 6°C. Subsequently, the enzyme complex was thoroughly washed with cold water and 0.05 sodium acetate buffer. Assays of the washed product (*M. miehei*) showed 27.4 $Lu.g^{-1}$ wet resin and 3.5 $Lu.g^{-1}$ wet resin for the pregastric esterase product. Assay of free fatty acids was conducted using gas chromatography.

Results and Discussion

Immobilized Pepsin

In Figs. 3 and 4 are presented results of a study to determine optimal enzyme loading conditions for the pepsin–resin system. In this experiment quantities of resin were exposed to increasing levels of 3× crystalline pepsin at pH 1.0 and equilibrated for several hours. The buffer-washed resins were then crosslinked with glutaraldehyde, again washed, placed in columns, and used in milk-clotting studies. It can be observed that loading conditions relating to a concentration of 60 mg pepsin/g wet resin resulted in a minimal binding ratio and showed maximal milk-clotting activity relative to the control resin polymer #32931.

Using these established conditions, scale-up of the pepsin–resin system to the 4 kilo level was carried out. Milk-clotting studies were conducted on a semi-continuous (8–10 h daily) basis using a specially designed borosilicate glass column. Cooled, acidified milk was passed down-flow through the unit at flow rates varying from 1 to 4 L/min. After an operational day, the unit was washed with cold

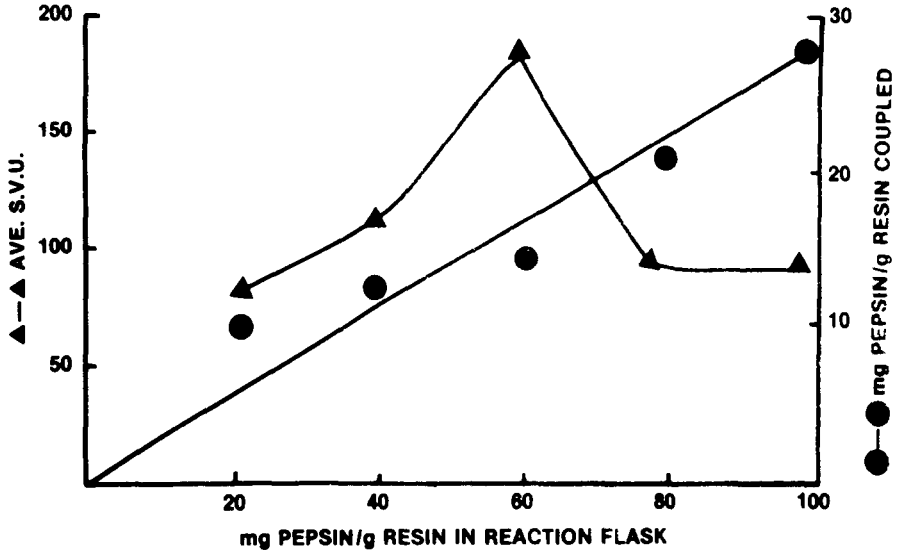

Fig. 3. Graphic illustration of optimal enzyme–resin loading conditions.

acetate and phosphate buffers and then sanitized with 100 ppm chlorine solution prior to storage overnight at 5–10°C.

A schematic of the design of a 2 ft^3 bioreactor that was used for larger pilot plant scale trials is presented in Fig. 5.

The durability of the pepsin–amine–resin system was established over several weeks time and was found to be very stable. Data illustrating the durability of the system showing full milk-clotting activity being retained for the 25-d duration of the experiment is presented in Fig. 6. In contrast, when pepsin was attached to a phenolic–formaldehyde resin containing only functional hydroxyl groups and crosslinked with glutaraldehyde, milk-clotting activity declined very rapidly and fell to nonfunctional levels within 5 d. Results of another long-term study indicating that milk-clotting activity could be retained over a period of 25 wk are presented in Fig. 7. Therefore, it can be seen that a durable system for the continuous coagulation of milk has been achieved.

RESIN	PEPSIN (mg/g)	BOUND (%)	ACTIVITY RESIN / ACTIVITY 32931 (%)
32931	20	48.5	100
32932	40	30.3	139
32933	60	23.0	247
32934	80	27.6	117
32935	100	28.1	113

Fig. 4. Tabulated data of enzyme–resin milk-clotting activities.

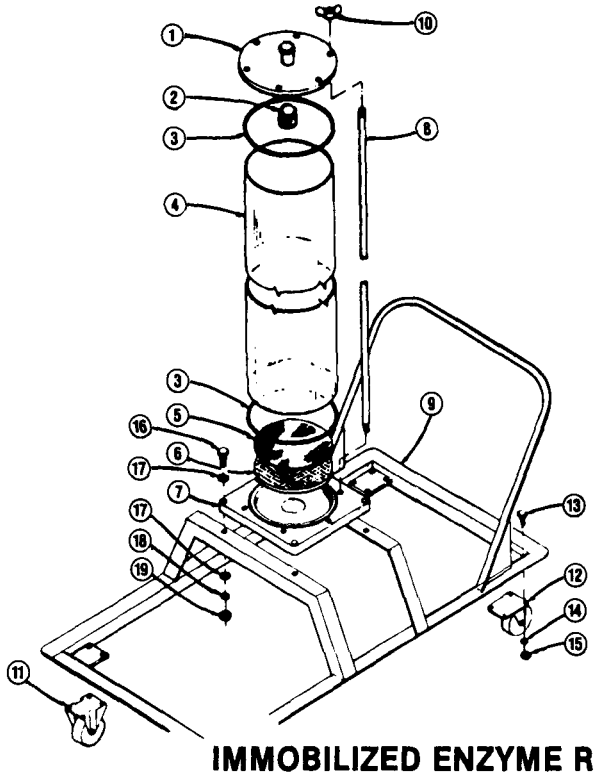

Fig. 5. Schematic of 2-ft^3 immobilized-enzyme pilot plant reactor.

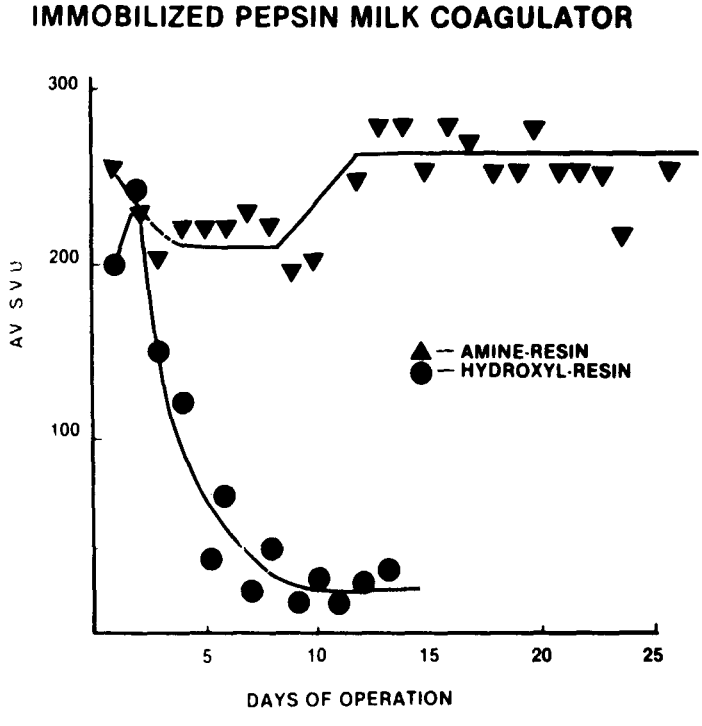

Fig. 6. Stability trial of immobilized-pepsin milk coagulator.

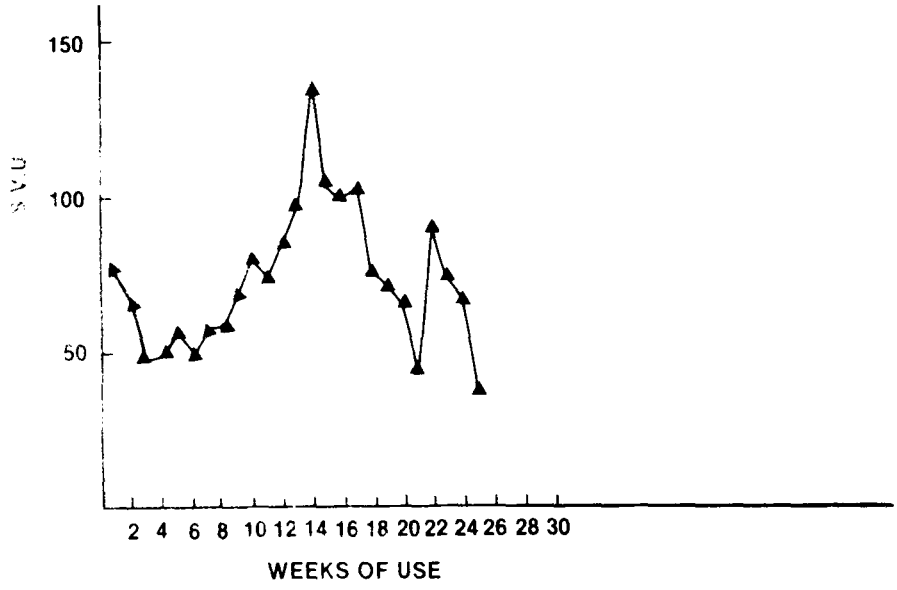

Fig. 7. Long-term stability trial of immobilized-pepsin milk coagulator.

Immobilized Lactase

There are a variety of ways to hydrolyze lactose. In Fig. 8. are listed some of the processes that have been studied over the past few years. First, there are the batch processes using the free enzyme as an additive. Then there are the semicontinuous lactase membrane processes where the free enzyme is contained in protein-retaining membrane and lactose is percolated through the membrane.

The immobilized lactase system developed by the Italian group at SNAM PROGETTI involved entrapment of yeast lactase in cellulose fibers. The system has been brought to the industrial scale for the production of low-lactose milk.

Likewise, the highly efficient Corning immobilized lactase system is now operating at semi-works and plant scale. Finally, there are those systems that rely on acid-catalysis for the hydrolytic degradation of lactose.

PROCESSES FOR HYDROLYZING LACTOSE

1. **BATCH PROCESS USING SOLUBLE YEAST OR MOLD ENZYME (LACTASE)**
2. **SEMI-CONTINUOUS HYDROLYSIS USING SOLUBLE LACTASE-MEMBRANE PROCESS**
3. **IMMOBILIZED YEAST LACTASE (SNAM PROGETTI)**
4. **IMMOBILIZED MOLD LACTASE (CORNING)**
5. **MINERAL ACID HYDROLYSIS**
6. **HYDROLYSIS BY ION EXCHANGE RESIN CATALYSIS**

Fig. 8. Various processes proposed for modifying milk and dairy byproducts.

The results of the studies using an immobilized enzyme system in which *Asp. niger* lactase was adsorbed to carboxymethyl cellulose and subsequently crosslinked with tea polyphenols are also reported. The adsorbed lactase was tested under operational conditions and found to have a half-life of about 30–40 d. In other trials the adsorbed enzyme was allowed to soak in water extracts of either green or black teas for several hours. The crosslinked enzyme system was then washed with cold buffer to remove excess tea derivatives. It was possible to recover an immobilized lactase system with an enhanced half-life functionality of 120 d. This level of stability was comparable to other systems using glutaraldehyde as the crosslinking agent.

Tea polyphenols are chemically and functionally distinct from vegetable tannins. The chemical structures of some of these complex polyphenols are presented in Fig. 9. In order to establish which chemical compounds are active participants in the enzyme crosslinking reactions, a solvent phase partition fractionation (*14*) was carried out. Isolation of purified components of the tea flavanols was achieved. The epi-gallo catechin, epi-catechin gallate, epi-gallocatechin gallate, epicatechin were found to be active as crosslinking agents.

The immobilized lactase system of Corning was used to prepare pilot plant quantities of the liquid sweetener syrup prepared from hydrolyzed whey permeate. The analytical composition of the liquid sweetener is presented in Fig. 10. It was found that hydrolysis of lactose to about 80% was efficient from an enzyme functionality point of view and also provided an acceptable level of sweetness. One very interesting aspect of the condensed syrup is its low viscosity. Unlike high solids corn syrups, the condensed syrup (65% TS) is very fluid, with a poise reading of 3.1. This presents an important advantage over corn syrups, which require heavy-duty pumps to transport the syrups into industrial process areas.

The sweetener syrup was successfully utilized in a variety of food products. An excellent grape jelly was prepared utilizing the hydrolyzed whey syrup. Flavor evaluations over several months by an expert taste panel indicated the product prepared with the dairy byproduct sweetener was equivalent in flavor, or slightly preferred, to the corn syrup-sweetened jelly.

HYDROLYZED WHEY SYRUP

TENTATIVE SPECIFICATIONS, LOT -2258 - 106

TOTAL SOLIDS, %	68.0			
ASH, %	0.15	MICROBIOLOGY		
TOTAL NITROGEN, %	0.04	TOTAL COUNT, gm		900
pH	5.1	YEAST, gm		20
COLOR	Amber	MOLD, gm		<10
TOTAL SUGARS, %	67.4	COLIFORM		<10
GLUCOSE	21.6			
GALACTOSE	21.6			
LACTOSE	22.4			
OLIGOSACCHARIDES	1.8	DEGREE BAUME		35.5
VISCOSITY, POISE	25°C	50°C		
SYRUP SOLIDS, 82.5%	17.0	12	HAAKE	
68.0%	3.1	0.7	BROOKFIELD	

Fig. 9. Compositional analysis of concentrated hydrolyzed whey syrup.

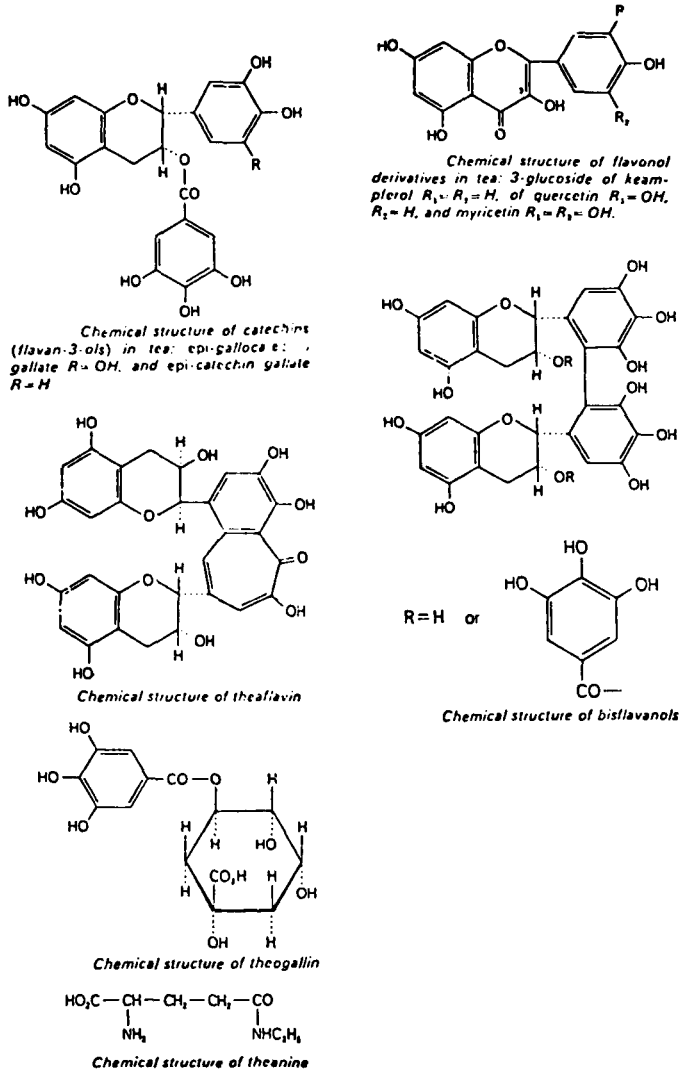

Fig. 10. Chemical structures of various tea components.

Utilization of the concentrated syrup as a fermentation carbon energy source was also investigated (*15*). The hybrid grapes grown in New York State and Ohio, although full of flavor, are low in fermentable sugar. Therefore, it is conventional wine-making technique to chaptalize the extracted juice with fermentable sugar in order to produce wines containing 10–12% alcohol. In Fig. 11 is illustrated a typical fermentation pattern in which dairy byproduct syrup, fully hydrolyzed, was chaptalized into the fermenting "must" over a period of several days in gradually decreasing amounts. It can be observed that unlike many *Saccharomyces cerevisiae* strains, the *ellipsoides* variant used in these trials can utilize both glucose and galactose for growth and ethanol formation.

Wines prepared in this manner were found to conform to the standards regulating wine composition and were acceptable to wine-tasting panelists. It

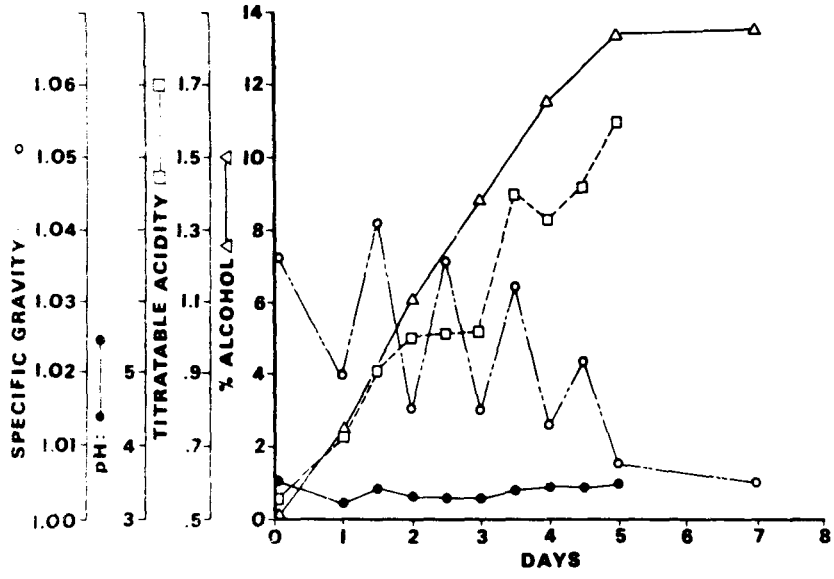

Fig. 11. Wine fermentation using chianti red grape concentrate and hydrolyzed whey syrup.

would therefore appear that a fully hydrolyzed whey syrup could be utilized as a carbon source in a variety of industrial fermentations.

Immobilized Esterase

Application of immobilized enzyme systems to modify the third component of milk—butterfat—has not previously been described. The bulk of milk fat exists in milk as small globules with an average diameter of 3–6 μm. The surface of these globules is coated with an adsorbed layer, usually referred to as the milk fat membrane. This membrane, composed of complex lipoproteins and other phospholipids, helps the fat globule to maintain its integrity and reduces the tendency toward coalescence (*16*).

Various esterases and lipases from microbial, fungal, or animal sources can be used to liberate the acylglycerol esters of butterfat. Because of the action of the esterase, desirable flavor changes in the lipolyzed fat are induced. These preparations can then be used in a wide variety of bakery products, cheese products, margarines, confectionary products, sauces, and soups.

At present, the only FDA-approved enzyme that may be employed in butterfat modifications is pregastric esterase, a complex of esterases derived from the oral tissues of young calves, kid goats, and lambs. The biochemistry and use of pregastric esterase have recently been extensively reviewed (*17*). Another esterase, derived from the fermentation broth of *Mucor miehei,* has recently received FDA approval for use in a limited number of cheese processes (*18*).

In Fig. 12 is illustrated the pooled results of stirred batch trials using immobilized pregastric esterase with an activity of 3.5 $Lu.g^{-1}$. The immobilized system was used to lipolyze the different milk fat substrates—butter–oil emulsion, whole milk, and 10% cream. It can be observed the immobilized esterase can liberate

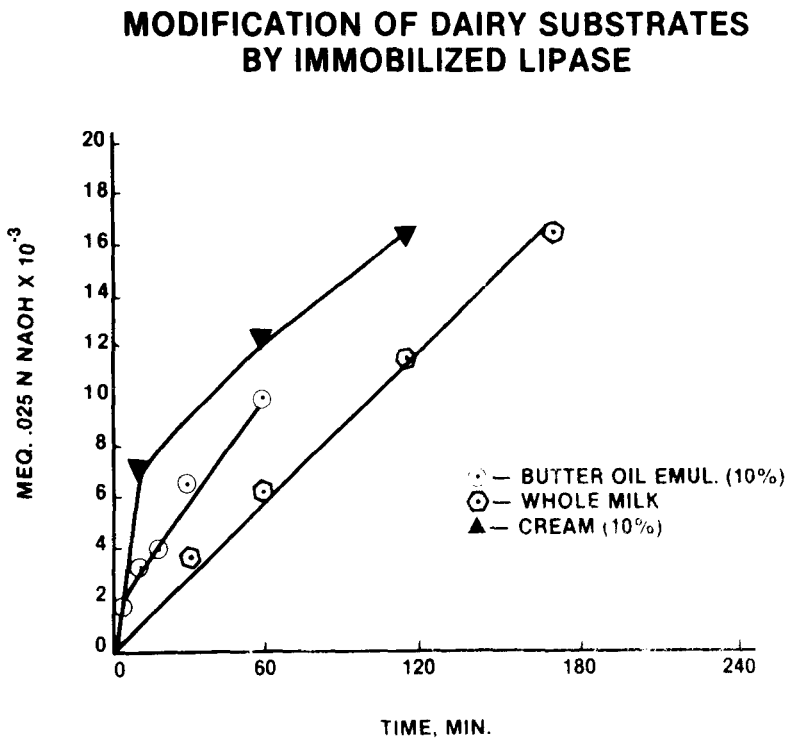

Fig. 12. Liberation of short-chain fatty acids from dairy products using immobilized lipase.

fatty acids from all three butterfat substrates. It should be noted that pregastric esterase has a unique specificity for acylglycerols of C 4:0 to C :10 and preferentially hydrolyzes the 3-position of triglycerol esters, which is predominantly C : 4 butyric acid in butterfat.

Results of modifying cream using an immobilized esterase (*M. miehei*) system in a column bioreactor are presented in Fig. 13. The enzyme complex had an activity of 24.5 $Lu.g^{-1}$. The bound enzyme (43 g) was stabilized in the reactor bed (1.5 × 90 cm) by pumping 0.05*N* sodium acetate through the column for 1 h. Then 10% cream was pumped through the reactor in plug flow at $Q = 2.2$ mL min^{-1}.

LIBERATED SHORT CHAIN FATTY ACIDS, CHAIN LENGTH
%

TREATMENT	C2:0	C4:0	C6:0	C8:0	C10:0
NONE	.0053	.0007	.0005	.0005	.0011
IMMOBILIZED LIPASE	.0038	.0038	.0023	.0041	.0070
SOLUBLE LIPASE	.0033	.0256	.0223	.0228	.0365

Fig. 13. Comparison of free and immobilized lipase activity on cream.

After 1 h of flowing fresh cream through the reactor, a 300 mL sample was then collected, cooled, and assayed for free short-chain fatty acids by gas chromatography. As a control, a 300 mL aliquot of the fresh cream was treated with soluble *M. miehei* esterase (0.5 mg/mL) for 1 h at 40°C. The lipolyzed product was cooled and assayed in conjunction with the sample collected from the immobilized esterase column. In Fig. 13 it can be seen that lipolysis occurred in both enzyme processes and that the immobilized esterase bioreactor liberated about 10–15% of the fatty acids compared to the free enzyme.

Summary

Immobilized enzyme systems have been described that have functional applications in the future development of continuous processes for modifying milk systems. It should be noted there are major constraints that tend to restrict the development and industrial-scale application of these and other immobilized enzyme systems. Among these are the limitations of supply of purified enzymes derived from animal tissues, along with their current high costs. In addition, the rising expense of regulatory compliance, coupled with prolonged affirmation time, will permit the development of only those enzyme systems where cost/benefit ratios are highly favorable.

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