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BETA-GALACTOSIDASE ACTIVITY OF FREE AND IMMOBILIZED CELLS OF KLUYVEROMYCES FRAGILIS

BY

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ABSTRACT

Kluyveromyces fragilis has been reported to be a potential source of beta-galactosidase for the hydrolysis of lactose in milk and sweet whey. The main objectives of this study were to characterize beta-galactosidase activity of immobilized cells of *K. fragilis* and to evaluate the potential of immobilized cells in continuous flow reactor operation, in view of application in the dairy industry. *K. fragilis* cells investigated were grown at suboptimal temperature (10°C) and optimal temperature (30°C). Cells were immobilized by covalent attachment to controlled pore glass with glutaraldehyde. Activity of immobilized cells was compared with activity of free cells and enzyme extracts in both soluble (free) and immobilized form employing oNPG as a substrate. Growth at suboptimal temperature seemed to facilitate increased extraction of beta-galactosidase preparation.

Stability of immobilized enzyme preparations in a continuous flow reactor were short (half life of 13.6 hr). Beta-galactosidase activity of free and immobilized cells was similar and, thus, not altered by immobilization. In terms of pH and temperature optima, identical values were found as for extracted enzyme preparation. However, immobilization of cells resulted in an increase of beta-galactosidase stability in continuous flow operations compared to the stability of immobilized enzyme extract. Half life found for beta-galactosidase activity was 86

and 76 hour for cells grown at 10^{o} and $30^{o}C$, respectively.

The covalent bonding of cells with glutaraldehyde seemed to inhibit hydrolysis of lactose since negligible hydrolysis of lactose was achieved in continuous flow reactors containing immobilized cells. This was not evident when oNPG was used as a substrate indicating that an active permease system was required for lactose but that oNPG relied on passive diffusion. In view of potential commercial application of immobilized K. fragilis cells for lactose hydrolysis, the immobilization technique has to be modified or changed to an alternative type (entrapment, adsorption, etc.), to preserve the permease system. It is in this way that the potential of beta-galactosidase of K. fragilis cells may be exploited.

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List of Abbreviations

CPG - controlled pore glass

ga-CPG - glutaraldehyde activated controlled pore glass

GAPTES - gama-aminopropyltriethoxysilane

oNPG - ortho-nitrophenol beta-D-galactopyranoside

p-CMB - p-chioromercuribenzoate

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CHAPTER 1

INTRODUCTION

Beta-galactosidase technology may potentially rectify numerous nutritional and technological problems related to lactose in the dairy industry. The hydrolysis of lactose in milk and whey to glucose and galactose results in several changes in the physical and chemical properties of treated products. These changes include: a prevention of lactose crystallization, increased carbohydrate solubility, increased sweetness, and more readily fermentable sugars. The controlled hydrolysis of lactose by beta-galactosidase, thus, enables the development of milk and whey products of modified nutritional, physical and functional properties.

It is the microbes (fungi, bacteria and yeast) that offer the highest beta-galactosidase productivity, hence, are the most suitable sources for industrial applications. Fungal beta-galactosidase (primarily Aspergillus niger) is relatively expensive to produce. Fungal beta-galactosidase being active at acid pH is well suited for the hydrolysis of lactose in acid whey. Bacterial beta-galactosidase is most active at neutral pH but is extremely expensive to produce, hence, is not a commercially feasible source. Yeast beta-galactosidase, however, is a relatively inexpensive commodity and has a pH optimum at neutral pH, thus, possesses good potential for the hydrolysis of lactose in milk and sweet whey. At present,

the commercial sources of beta-galactosidase are Aspergillus niger and Kluyveromyces lactis. The yeast Kluyveromyces fragilis has been reported as a good potential source of beta-galactosidase but at present is not being utilized.

Regardless of the source, beta-galactosidase preparations when added in the soluble form to dairy products can be used but once. The immobilization of beta-galactosidase is, therefore, a beneficial process to commercial operations. The advantages of immobilization compared with the soluble enzyme form may be summarized as follows: enables multiple or repetitive use of a single batch of enzyme; facilitates control of hydrolysis; may lead to enzyme stabilization; and results in a processed solution which is not contaminated by enzyme.

Previous work within this department focused on the characterization of beta-galactosidase from the yeast K. fragilis. The study was initiated to investigate the effect of growth at optimal and suboptimal temperatures upon the yield and properties of beta-galactosidase preparations extracted from K. fragilis cells. Thereafter, the immobilization of beta-galactosidase preparations and the effect of immobilization on enzymic properties was studied. Findings of these investigations indicated that yield and activity of the beta-galactosidase preparation increased when K. fragilis was grown at suboptimal temperature. However, enzymic

properties of the two beta-galactosidase preparations produced at optimal and suboptimal temperatures were similar, suggesting that the enzymes were identical. Comparison of the enzymic properties of these beta-galactosidase preparations with a commercial preparation derived from K. lactis indicated that K. fragilis indeed represented a feasible alternative source of beta-galactosidase to K. lactis.

The enzymic properties of immobilized beta-galactosidase preparations were similar to the properties of the soluble form. The immobilized K. fragilis beta-galactosidase preparations are, therefore, potentially applicable for the hydrolysis of lactose in milk and sweet whey. Less attractive in view of the potential application for continuous flow operation was the short half-life of the immobilized preparation. In this respect however, similar characteristics were obtained from the immobilized beta-galactosidase from K. lactis (commercial preparation).

The present study represents a continuation of the previous work performed in the department, on beta-galactosidase of K. fragilis. The objectives of the study were to investigate the potential hyperproduction of beta-galactosidase of K. fragilis when grown under suboptimal temperature and to evaluate the potential of lactose hydrolysis by immobilized K. fragilis cells.

The following criteria were investigated:

1) Beta-galactosidase activity of free and immobilized cells as affected by pH and temperature.
2) Side activities of intact K. fragilis cells.
3) Stability of beta-galactosidase activity of intact K. fragilis cells.
4) Effect of substrate flow rate on relative hydrolysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Lactose

2.1.1 Introduction

Lactose, poses a number of nutritional and technological problems within the realms of the dairy industry. These problems include: 1) Lactose intolerance, experienced by individuals deficient in intestinal beta-galactosidase.

2) High lactose levels in condensed milk products tend to crystallize causing grittiness upon prolonged storage (Shukla, 1975).

3) High lactose levels in cheese whey represent a major problem in respect to waste disposal (Jelen, 1977).

Beta-galactosidase technology offers solutions to these problems in health, dairy processing and pollution. The controlled hydrolysis of lactose into its constituent monosaccharides, glucose and galactose results in several desirable changes in the physical and chemical properties of milk and whey. These changes include: a reduced lactose content, prevention of lactose crystallization, increased sweetness and more readily fermentable sugars. Presented in the following section is a review of lactose and the importance of its hydolysis by beta-galactosidase in the dairy industry.

2.1.2 Lactose and the importance of its hydrolysis in dairy products.

2.1.2.1 Lactose intolerance.

Individuals low or deficient in the enzyme, beta-galactosidase may suffer from the condition known as lactose intolerance. In such cases where the ingested lactose exceeds the hydrolytic capacity of the available beta-galactosidase, unhydrolyzed lactose enters the large intestine. The osmotic pressure of the intestinal fluid increases with the presence of lactose in solution, causing an influx of water. Compounding the problem, the intestinal microflora metabolize the lactose. The overall results are the production of organic acids and carbon dioxide causing fermentative diarrhea, cramps, bloating, belching and watery explosive diarrhea (Shukla, 1975). In addition to the gastrointestinal discomfort brought about by milk ingestion, there existsa general impairment in the normal digestive process (Paige et al., 1972). This creates serious problems for nutrition rehabilitation programs based on milk products.

Bayless et al. (1971) estimate that lactose intolerance is prevalent in approximately 70% of the world's adult population. The reduction of lactose levels in dairy products is therefore of great nutritive value to mankind. Nijpels (1976) states that in most cases a 50 - 80% hydrolysis of

lactose is necessary to attain acceptiability by intolerant groups. A hydrolysis of 90% is required in extreme cases only.

2.1.2.2 Lactose crystallization.

Lactose crystallization may create a mealy, sandy or gritty texture in condensed dairy products when crystal length is 30 μm in length or greater (Shukla, 1975). The products most prone to lactose crystallization are: condensed milk (sweetened and unsweetened), ice cream, condensed and dried wheys, dry milk and frozen milk. Bouvy (1975) states 20% to 30% lactose hydrolyzation will result in complete inhibition of crystallization. Partial lactose hydrolyzation by beta-galactosidase, hence, represents a solution to lactose crystallization in nonfermented dairy products.

2.1.2.3 Use of beta-galactosidase in fermented dairy products.

The pre-hydrolysis of lactose to glucose and galactose results in an accelerated and more efficient fermentation for the production of cheese and yogurt. Curd formation, aging and stability is enhanced when utilizing lactose hydrolyzed milk in fermented dairy products (Nijpels, 1976). Production costs are subsequently lowered with the use of beta-galactosidase. An additional benefit is the production of low lactose whey that can be concentrated and used as a food ingredient (Thompson and Brower, 1974; Thompson and

Gyuricsek, 1974). This type of production scheme avoids the problems surrounding lactose intolerance, lactose crystallization and the utilization of whey.

2.1.2.4 Whey utilization.

Acid and sweet whey was often regarded as industrial waste and was subsequently dumped into sewage systems. This practise, not only resulted in the loss of protein and lactose which could have been recycled into product formulation but also increased the biological oxygen demand of the waste water (Shukla, 1975). The net result was a high cost of waste water treatment. Economics of the waste disposal problem selected for the development of the following corrective measures:

1) Strict pollution abatement programs.
2) The production of whey protein concentrates (Muller, 1976).
3) Yeast protein production using acid or sweet whey as a substrate (Bernstein et al., 1977).
4) Alcohol production using whey as a substrate; lactose hydrolyzation prior to the fermentation enhanced fermentation rate (O'Leary et al., 1977).
5) Ultrafiltration and reverse osmosis methods of producing protein and lactose concentrates; lactose may be subsequently hydrolyzed by beta-galactosidase to produce an industrial sweetener

(Weetal et al., 1974; Wierzbicki and Kosikowski 1973a).

6) The hydrolyzation of lactose in whey for the production of a sweet protein syrup; eliminates the costly ultrafiltration system (Morris, 1980).

Beta-galactosidase thus plays an important role in the production of various by-products from whey. As may be seen from the measures implemented in whey utilization, beta-galactosidase may be used alone or in conjunction with an alternative process such as alcohol production or ultrafiltration and reverse osmosis.

2.2 Beta galactosidase

2.2.1 Introduction

Beta-galactosidase (B-D-galactosidase galactohydrolase) is classified under the enzyme commission number EC 3.2.1.23. A wide distribution, throughout the plant, animal and microbial kingdoms, is reported for the enzyme (table #1). Wallenfels and Weil (1972) state that the widespread occurrence of beta-galactosidase is probably due to numerous physiological functions of the enzyme, including:

1) Hydrolysis of lactose in mammals and microorganisms.
2) Degradation of the galactosyl moieties of glycoproteins and glycolipids.
3) Degradation of galactolipids in plants.

TABLE #1

Potential sources of beta-galactosidase (Shukla, 1975)

PLANTS

- Peach
- Apricot
- Almond
- Kefit Grains
- Tips of Wild Roses
- Alfalfa Seed
- Coffee

ANIMAL ORGANS

- Intestine
- Brain and Skin Tissue

YEAST

- *Kluyveromyces lactis*
- *Kluyveromyces fragilis*
- *Candida pseudotropicalis*

FUNGI

- *Neurospora craussa*
- *Aspergillus foetidus*
- *Aspergillus niger*
- *Aspergillus flavus*
- *Aspergillus orizae*
- *Aspergillus phoenicis*
- *Mucor pucillus*
- *Mucor meihei*

BACTERIA

- *Escherichia coli*
- *Bacillus megaterium*
- *Thermus equaticus*
- *Streptococcus lactis*
- *Streptococcus thermophilus*
- *Lactobacillus bulgaricus*
- *Lactobacillus helareticus*

It is the microbes (bacteria, fungi and yeast), however, that offer the highest productivity, hence are the most suitable sources of beta-galactosidase for industrial applications.

Beta-galactosidase production in microorganisms has been described as an induced enzyme synthesis. The lac Operon of Escherichia coli is the most thoroughly investigated (Wallenfels and Weil, 1972). Hewitt and Groot Wassink (1980) working with Kluyveromyces fragilis, however, describe an induced beta-galactosidase synthesis in the presence of galactose and/or lactose.

Beta-galactosidase from different sources vary with respect to structure, size and optimal conditions for lactose hydrolysis (Shukla, 1975). This variability of enzymic properties is important to industrial processes in that it facilitates the selection of the beta-galactosidase source best suited to the nature of the substrate and subsequent product.

At present, the primary commercial sources of beta-galactosidase are Kluyveromyces lactis and Aspergillus niger. Beta-galactosidase from K. lactis possesses a pH optimum of 6.8 - 7.0, and a temperature optimum of 35^{o}C. K. lactis beta- galactosidase is, thus, ideal for the treatment of milk (pH 6.6) and sweet whey (pH 6.2). Beta-galactosidase from A. niger possesses a pH optimum of 4.0 - 4.5, and a temperature optimum of 55^{o}C. A. niger beta-galactosidase is, hence, suitable for the treatment of acid whey.

K. fragilis, though not a primary commercial source, is reported as being a good potential source of beta-galactosidase (Wendorff and Amundson, 1971). Wierzbicki and Kosikowski (1973b) studied the crude cell yields of beta-galactosidase from yeast and reported K. fragilis to be superior over K. lactis. Smith (1978) subsequently characterized and compared beta-galactosidase of K. fragilis and K. lactis and reported them as being similar. It is therefore evident, that beta-galactosidase from K. fragilis possesses good potential within the dairy industry for the hydrolyzation of lactose in milk and whey.

The purification and subsequent characterization of beta-galactosidase from K. fragilis has been reported by several authors. The following discussion focuses on the enzymic properties of beta-galactosidase from K. fragilis.

2.2.2 Beta-galactosidase from Kluyveromyces fragilis

2.2.2.1 Physical properties

The molecular weight of beta-galactosidase from K. fragilis was determined by Uwajima et al. (1972) to be 203 000. Mahoney and Whitaker (1978) report a molecular weight of 201 000, hence, support the findings of Uwajima et al. (1972).

Mahoney and Whitaker (1978) determined the amino acid composition of beta-galactosidase from K. fragilis (table #2).

TABLE #2 (Mahoney and Whitaker, 1978)

Amino acid composition of beta-galactosidase from K. fragilis

AMINO ACID	PERCENT
Aspartic Acid	12.50
Threonine	4.60
Serine	4.88
Glutamic Acid	10.80
Proline	4.74
Glycine	6.94
Alanine	5.16
Valine	6.44
Cystine	2.33
Methionine	0.56
Isoleucine	5.38
Leucine	7.86
Tyrosine	5.09
Phenylalanine	5.80
Lysine	7.64
Histidine	3.82
Arginine	3.32
Tryptophan	1.98

A high proportion (30.2%) of hydrophobic amino acid residues (i.e. proline, valine, leucine, isoleucine and phenylalanine) suggested a subunit structure of the molecule (Mahoney and Whitaker, 1978). This was in accordance to Van Holde (1966, 1977) who postulated a protein with more than 28 - 30% hydrophobic amino acid residues would be of subunit structure. The molecular structure, however, has yet to be determined, pending successful separation of the subunits.

Wendorff and Amundson (1971) report that the inactivation of K. fragilis beta-galactosidase indicates the presence of sulfhydryl groups on the molecule. The possible involvement of some of these sulfhydryl groups in the catalysis of lactose hydrolysis is indicated by the competitive inhibition of enzyme activity by p-chloromercuribenzoate (p-CMB) (Wendorff and Amundson, 1971). Mahoney and Whitaker (1978), subsequently, report that mercaptide formation with p-CMB suggests that five sites are present in K. fragilis beta-galactosidase.

Mahoney and Whitaker (1978), utilizing isoelectric focusing, found two active protein components; one major and one minor component, comprising 98% and 2% of the protein respectively. The isoelectric points were 5.1 $\pm$ 0.1 for the major component and 6.0 $\pm$ 0.1 for the minor component. Uwajima et al. (1972), however, reported the isoelectric point

of the major isozyme to be 4.4. It is most likely that the isoelectric point is approximately 5.0. Wendorff and Amundson, (1971), while studying the effect of pH on the stability of K. fragilis beta-galactosidase, report that at pH 5.0 precipitation of the enzyme occurred. Wendorff and Amundson, (1971), further, report a complete and irreversible loss of enzymic activity upon precipitation. Control and subsequent manipulation of the pH thus represents a method of enzyme inactivation and should be compensated for in the lactose hydrolyzation process. An example where pH may constitute a problem is during the fermentation of lactose hydrolyzed milk for the production of cheese and yogurt. Microbial lactic acid production would invaribly inactivate the beta-galactosidase upon lowering of the pH to 5.0. It is important, therefore, that sufficient time be given for optimal lactose hydrolysis.

2.2.2.2 Enzymic properties

The effect of temperature on the rate of lactose hydrolysis has been studied by several authors. A temperature optimum of 30 - 37^{o}C has been reported (Kovalenko et al., 1975; Mahoney and Whitaker, 1977; Woychik and Holsinger, 1977; Guy and Bingham, 1978; Smith, 1978). Additionally, Guy and Bingham (1978) reported a higher optimal temperature of hydrolysis was in effect when lactose in milk or whey was utilized as a

substrate. The heterogeneity of such substrates seemed to require higher temperatures of activation. Nonetheless, K. fragilis beta-galactosidase possesses a relatively low temperature optimum which is readily attainable, in view of commercial processes.

pH possesses a marked effect on the ionization of groups in the active site of the free enzyme (Whitaker, 1972) and the enzyme - substrate complex (Mahoney and Whitaker, 1977). The effect of pH on the rate of lactose hydrolysis shows a pH optimum in the range of 6.3 - 7.2 (Wendorff and Amundson, 1971; Kulikova et al., 1972; Kovalenko et al., 1975; Woychik et al., 1974; Kilara et al., 1977; Mahoney and Whitaker, 1977; Woychik and Holsinger, 1977; Smith, 1978). The utilization of K. fragilis beta-galactosidase for the hydrolyzation of lactose in milk (pH 6.6) and sweet whey (pH 6.2) is, thus, feasible.

Beta-galactosidase activity is affected by the presence of mono- and divalent cations. Wendorff and Amundson (1971) and Uwajima et al. (1972) report enhanced enzymic activities with increased potassium ion concentration. Mahoney and Whitaker (1977), however, report little effect of potassium ion concentration up to 10mM, above which, a slight inhibitory effect is evident. A major controversy surrounds the effect of potassium ions. Different experimental conditions maintained during these studies may be a factor. The divalent

metals, managanese, magnesium, colbalt, zinc and calcium are documented activators of the enzyme (Tikomirova et al., 1971; Wendorff and Amundson, 1971; Uwajima et al., 1972; Mahoney and Whitaker, 1977). The above mentioned mono-and divalent cations are all present in milk and whey, hence, the hydrolyzation of lactose should proceed at a near optimal rate (assuming all other conditions are optimal).

The effects of substrate concentration on initial rates of hydrolysis of ortho-nitrophenol-beta-D-galactopyranoside (oNPG) and lactose are well documented. Michaelis constants (Km) range from 1.2 to 5.6 mM for oNPG (Bierman and Glantz, 1968; Wendorff, 1969; Chan, 1971; Kulikova et al., 1972; Mahoney and Whitaker, 1977; Smith, 1978) and 13.9 to 30 mM for lactose (Wendorff, 1969; Chan, 1971; Uwajima et al., 1972; Ohmiya et al., 1977; Mahoney and Whitaker, 1977; Smith, 1978). These Km values are of particular importance when viewing immobilized enzymes in packed-bed reactor systems. Marazzo et al., (1975) state that for short columns if the substrate concentration within the column greatly exceeds the Km value of the immobilized enzyme, the phenomenon of substrate inhibition may be a factor. The Km value must therefore be taken into account in the designing of a continuous flow reactor system for a particular enzyme.

Wendorff (1969) stated that galactose behaves as a competitive inhibitor of beta-galactosidase (i.e. end product

inhibition). Mahoney and Whitaker (1977) are in support of this concept. The inhibition constant (Ki) for galactose has been reported from 19 to 46 mM (Kilara et al., 1977; Mahoney and Whitaker, 1977; Ohmiya et al., 1977; Smith, 1978). The Ki should be taken into account in the design of enzyme reactor systems as to avoid end product inhibition and in doing so maximize lactose hydrolysis.

2.2.2.3 Transgalactosidation

Transgalactosidation is a side reaction of beta-galactosidase which results in the production of oligo-saccharides of varying lengths and molecular weight. During the hydrolization of lactose, beta-galactosidase occassionally facilitates the transfer of the galactose moiety from lactose to various acceptors such as monosaccharides, polysaccharides and alcohols (Wierzbicki and Kosikowski, 1973c). Shukla (1975) states the number and type of oligosaccharide produced are affected by enzyme source, substrate concentration, pH, temperature and inorganic ions. An example of this is K. fragilis beta-galactosidase which is reported to produce 11 different oligosaccharides during the hydrolysis of lactose in skim milk (Roberts and Pettinatti, 1957). Beta-galactosidase from A. niger, on the other hand, is reported to produce 5 different oligosaccharided during the hydrolysis of lactose in acid whey (Wierzbicki and Kosikowski, 1973c).

Thus, it is important in the analysis of beta-galactosidase activity that the degree of hydrolysis is monitored through the liberation of glucose (if lactose is the substrate) or an aglycon (if a non-disaccharide is the substrate). Galactose is not a reliable indicator of hydrolysis due to potential transgalactosidation.

Additionally, transgalactosidation may constitute a nutritional problem. Intestinal beta-galactosidase is incapable of hydrolyzing the oligosaccharides produced during the hydrolysis of lactose due to a strict substrate specificity (Burvall et al., 1980). An analogous condition to lactose intolerance may arise assuming large quantities of oligosaccharides are produced. The net result would be intestinal discomfort, mainly flatulence.

2.2.2.4 Enzyme stability

Enzyme stability is of an essence when viewing commercial applicability of an enzyme. Enzyme preparations of K. fragilis beta-galactosidase offer good storage stability at a variety of temperatures. The beta-galactosidase activity of an acetone dried preparation was found to be relatively stable at room temperature; 50% activity remaining after 2 years (Davies, 1964). Wendorff and Amundson (1971) reported that beta-galactosidase in cell free extracts was stable for at least three months at -20^{o}C. Dried beta-galactosidase

preparations were also found to be stable for up to two years at 4°C (Dahlquist et al.,1977).

The effect of pH on enzyme stability is pronounced. Wendorff and Amundson (1971) report beta-galactosidase stability for 30 minutes at 25°C in the pH range 6 - 7. Similarly, Mahoney and Whitaker (1977) note enzyme stability from 6.5 to 7.5 for at least 10 minutes at 37°C. Rapid inactivation of the enzyme is in effect as the acidity is increased beyond this optimal range (Wendorff and Amundson, 1971; Mahoney and Whitaker, 1977). At pH 5.0, Wendorff and Amundson (1971) report complete and irreversible loss of activity due to precipitation of the enzyme. This is not surprising, considering Mahoney and Whitaker (1977) estimated the isoelectric point of the major isozyme to be 5.1 ± 0.1. Beta-galactosidase of K. fragilis, however, offers good functionality in the neutral pH range and,hence,is well suited for the hydrolysis of lactose in milk and sweet whey.

Temperature inactivation of beta-galactosidase follows a first-order rate kinetics (Mahoney and Whitaker, 1977). Uwajima et al. (1972) report stability of purified K. fragilis beta-galactosidase at pH 7.0 for 10 minutes at temperature up to 40°C. Above 40°C rapid inactivation of the enzyme is

documented (Uwajima et al., 1972; Mahoney and Whitaker, 1977). Thus, a mild heat treatment (over 40^{o}C) would invaribly terminate enzyme hydrolysis in industrial applications. This is important when considering the potential inactivation of beta-galactosidase in the processed end product. Such a process may be necessary as the result of a batch system of hydrolysis utilizing a soluble enzyme.

Dialysis of the enzyme against deionized water may result in irreversible inactivation (Wendorff and Amundson, 1971; Mahoney and Whitaker, 1977). Some protection against the dialysis effect is provided through the addition of mercaptoethanol and Mn^{++} (Wendorff and Amundson, 1971). Reactivation of the enzyme, however, is reported through the addition of potassium ion (Uwajima et al., 1972). Mahoney and Whitaker (1977) state that potassium ions are required for stability and cannot be replaced by sodium, maganese or magnesium. The dialysis effect, however, would not constitute a problem during the hydrolyzation of lactose in milk or whey due to the presence of stabilizing mono and divalent cations in these products.

2.2.2.5 The effect of growth temperature on microbial enzyme production

Growth of microorganisms at suboptimal temperature may have

various effects, including an increased production in proteolytic and lipolytic enzymes (Nashif and Nelson, 1953; Peterson and Gunderson, 1960). Additionally, properties of proteases produced at suboptimal temperature often differ from those of enzymes produced at optimal temperature (Scholefield, 1967; Fallon, 1972; Smith, 1976).

Smith (1978), investigated K. fragilis beta-galactosidase production at optimal and suboptimal temperatures and reported the enzymes produced at each temperature to be identical. Smith (1978), however, obtained increased yields and simultaneously, a more active beta-galactosidase preparation when grown at suboptimal temperature. Growth of K. fragilis at suboptimal temperature seemed to select for a hyperproduction of beta-galactosidase. Such a condition, would be advantageous to commercial production of beta-galactosidase from K. fragilis.

2.3 Immobilization

2.3.1 Introduction

Enzymes are defined as organic catalytic agents which perform the function of inducing and governing a specific reaction. Enzymes are generally soluble and unstable, thus, may be used but once in free solution.

The immobilization of enzymes is, therefore, a beneficial process to commercial operations. The advantages of

immobilizing enzymes may be summarized as follows:

1) Multiple or repetitive use of a single batch of enzymes.
2) Ability to stop reaction rapidly by removing the enzyme from the reaction solution.
3) In many cases, the enzyme is stabilized by immobilization (maintenance of tertiary structure).
4) The processed solution is not contaminated with the enzyme.

In recent years, increasing attention has been directed toward the utilization of immobilized microbial cells. A key example is in the industrial production of high fructose syrups where Streptomyces sp. cells are immobilized for their glucose isomerase activity (Takasaki and Kanbayashi, 1969). Immobilized cells offer several advantages over immobilized enzymes: the cost of extraction and subsequent purification is obviated; a wider scope of reactions is possible including multi-step reactions utilizing several enzymes; maintainence of the enzyme in its native state enhances its stability; and the presence of co-factors and continued biosynthesis within the cell contribute to longevity of enzymic activity (Jack and Zajic, 1977).

The process of immobilization, however, may inflict a number of detrimental effects upon the hydrolytic capacities of microbial cells. The difficultly to maintain the integrity of the cells frequently results in a loss of enzymic activity. Cell viability in some immobilized systems,on the other hand, may lead to contamination and subsequent microbial growth in the processed solution. The cell walls and membranes may offer permeability and diffusion barriers to the substrate and in thus doing so inhibit enzymic activity. The presence of numerous active enzymes in the cell may lead to potential unwanted side reactions. Careful study and control of these effects is required,therefore, when viewing the commercial feasibility of an immobilized cell system.

2.3.2 Immobilization of enzyme preparations

Enzyme immobilization is a well established and documented field. Immobilization techniques are numerous. Mosbach (1976) categorizes immobilization techniques as follows:

A Physical:
1) Adsorption on or in a carrier.
2) Encapsulation or entrapment.

B Chemical:
1) Covalent attachment to a soluble or insoluble carrier.
2) Cross-linking of the enzyme to enzyme with or without the benefit of a carrier.

Physical methods of immobilization involve the immobilization of the enzyme in any manner such that no covalent bond is formed. Physical immobilization methods involve:

1) Physical forces (electrostatic interaction, formation of ionic bonds, protein-protein interaction, etc.).
2) The entrapment within microcompartments.
3) The containment in special membrane dependant devices.

These methods often suffer leakage, desorption, and problems of diffusional resistences. Entrapment in cellulose acetate fibres, however, possesses good industrial potential (Morisi et al., 1973; Pastore et al., 1974). Beta-galactosidase entrapped in cellulose acetate fibres showed no loss of activity during spinning and very little leakage after fifteen days of continuous use (Pastore et al., 1974).

Chemical methods of immobilization involve the reactivity of functional groups on the enzyme. The involvement of these functional groups in covalent bond formation alters the structure and reactivity of the enzyme. Covalent methods which have been reported include immobilization of beta-galactosidase onto: alumina (Charles et al., 1976), sepharose (Kilara et al., 1977), porous glass (Woychik et al., 1974) and collagen (Woychik et al., 1974). Covalent bonding of enzymes to carriers

is often selected over other techniques because of the permanence of insolubilization.

2.3.3 Immobilized microbial cells as catalysts

Conditions surrounding the immobilization of microbial cells and techniques employed are analogous to those involved with the immobilization of enzyme preparations. Jack and Zajic (1977) categorize the methods of whole cell immobilization as follows:

1) Adsorption by an inert support.
2) Entrapment by an inert support.
3) Binding via immobilized biological macromolecules.
4) Covalent bonding of the cell to an inert support.

2.3.3.1 Adsorption of microbial cells

The physical adsorption of microbial cells to matrixes such as ion exchange materials is dependant upon:

1) Presence of metal ions or anions.
2) The balance of surface charges.
3) The configuration of charged sites.
4) The accessibility of these functional ion groups (Jack and Zajic, 1977).

Furthermore, Jack and Zajic (1977) state the affinity of a specific species for a given ion exchanger is not predictable.

Hattori and Furusaka (1960, 1961) successfully bound Escherichia coli and Azobacter agile to Dowex - 1 resin; the cells readily oxidized succinic acid. Johnson and Ciegler (1969) report that immobilized spores of Aspergillus sp. and Penicillium sp. bound to ion exchange cellulose derivatives exhibited good stability and were enzymically active catalysts, analogous to purified insoluble invertase.

Johnson and Ciegler (1969), however, report that column studies show spore germination after 8 - 10 hours continual use. In a subsequent study to Hattori and Furusaka (1960, 1961), Hattori et al., (1972) state that continuous operation of a column of adsorbed E. coli on Dowex - 1 shows periodic fluctuation of effluent cell density.

Cell viability is an important factor to be considered when viewing commercial applicability. The release of progeny into the column effluent and potential side activity of viable cells is often detrimental to the finished product. Jack and Zajic (1977), furthermore, state that the lability of the microorganisms adsorbed to ion exchange materials precludes their use in continuous industrial processes.

2.3.3.2 Entrapment of microbial cells

Entrapment techniques involve the restraint of cells in a water insoluble phase for the enzymatic conversion of water soluble substrate to product. In this method the following

matrixes may be employed:

1) Polyacrylamide gel (Chibata et al, 1974).
2) Collagen membranes (Vieth et al., 1973; Venkatasubramanian et al., 1974).
3) Agar pellets (Toda and Shoda, 1975).
4) Cellulose triacetate (Dinelli, 1972).

Collagen membrane inclusion, agar pelletization and entrapment in cellulose triacetate impose a diffusion barrier on the operation of cellular enzyme systems. The porosity of the polyacrylamide gel precludes diffusion problems, hence, is the most frequent employed procedure for the industrial application of immobilized microbial cells (Jack and Zajic, 1977).

The industrial application of polyacrylamide entrapment technique appeared in 1973. Chibata et al., (1974) describe the immobilization of E. coli in polyacrylamide gel for the continuous production of L-aspartic acid from ammonium fumarate.

2.3.3.3 Binding of cells by immobilized macromolecules

Lectins, naturally occuring proteins, possess the ability to agglutinate cells. The agglutinating activity arises from their ability to bind specific antigens on the cell surface. A naturally occuring example of such a process is the selective binding of Rhizobium trifolii to the surface of clover root

hairs via a species specific clover lectin complex which bridges the cross reactive antigen on the root hairs and the surface of the bacterial cells (Dazzo and Hubbel, 1975). Analogously, through the attatchment of macromolecules to an inert support, it is possible to selectively bind whole cells. The use of macromolecules, however, is thus far limited to the immobilization of cells of animal origin (Jack and Zajic, 1977).

2.3.3.4 Covalent binding of microbial cells

The immobilization of whole cells to a support via irreversible covalent bonds produces a system free of diffusion limitations present in an entrapment procedure and free of the release of progeny typical of adsorption systems (Jack and Zajic, 1977). In the immobilization process, a bifunctional reagent such as glutaraldehyde is utilized to covalently link the cell to either an organic or inorganic carrier.

The functionality of glutaraldehyde is reported to be the result of its high chemical affinity for epsilon amino groups (lysine residues), tyrosine, histidine, and sulfhydryl residues (Tomimatsu et al., 1971). Navarro and Monsan (1976) state that glutaraldehyde action on microorganisms is accompanied by the establishment of intercellular bonds that induce yeast or bacteria agglutination. Working upon this principle

Navarro and Durand (1977) immobilized Saccharomyces carlsbergensis cells on glutaraldehyde activated porous silica; imine bonds formed between support carbonyl groups and the amino groups of the cell walls.

The binding effect of glutaraldehyde, however, often alters the integrity of the cellular membrane. Subsequent transport of the substract across the membrane is hindered. Chibata et al., (1974) report that aspartase activity of E. coli cells treated with glutaraldehyde is 34% that of free cells and for the same cells entrapped in polyacrylamide, the yield is 73%. Navarro and Durand (1977) report that immobilization decreased cellular activity but increased ethanol production which is advantageous when viewing alcohol production in a continous process.

The use of glutaraldehyde, as a bifunctional reagent, in the covalent binding of K. fragilis cells, therefore, may reduce beta-galactosidase activity. The degree of potential inhibition or alteration of the enzymic process, however, is not predictable due to the specificity of the reaction i.e. reaction to the covalent bonding of cells to glutaraldehyde is species specific. The immobilization of K. fragilis to controlled pore glass utilizing glutaraldehyde as a bi-functional reagent has not previously been reported.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The strain of Kluyveromyces fragilis utilized, was number 8608 from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A.

The commercial beta-galactosidase preparation from Kluyveromyces lactis, maxilact 40 000, was donated by Gist-Brocades, Delft, Holland.

The chemicals used in this study, unless stated, were of reagent grade or better. The ortho-nitrophenol beta-D-galactopyranoside (oNPG) was lot number 17 C 5059 from Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, U.S.A. The standard for the oNPG assay, o-nitrophenol was crystallized once from ethanol and once from distilled water as a purification procedure. Gamma-aminopropyltriethoxysilane (A-1100) was donated by Union Carbide, NY 10017, U.S.A. The glutaraldehyde (50%) utilized in the study was of practical grade from the Eastman Kodak Company.

The Glucose/Fructose analysis test kit was obtained from Boehringer Mannheim GmbH. (cat. # 139106).

The control pore glass (product code number 44800) was obtained from Serva Feinbiochemica Heidelberg. The specifications were: particle size 80 - 120 mesh; pore diameter 2096 Å.

Unless, stated otherwise, the buffer utilized for enzyme assay, dilution, etc. was 0.02 M KH_2PO_4 containing 0.1mM $MnSO_4$, adjusted to pH 7.0 using 50% KOH.

3.2 Methods

3.2.1. Production of K. fragilis beta-galactosidase

Storage and propagation of the yeast was according to Mahoney et al., (1974), using 10 liters of medium in a 15 liter fermentation jar, equipped with magnetic stirrers and air spargers (aeration: 12.5ml/L/min). The culture was harvested at the onset of the stationary growth phase which was approximately 168 hours at 10°C and 30 hours at 30°C.

On completion of the incubation period, the cells were separated from the medium by centrifugation at 10 000 gn for 10 minutes using a Sorval superspeed RC2-B automatic refrigerated centrifuge. The cells were frozen at -40°C and subsequently freeze dried in a Virtis freeze dryer. The dried cells were stored at -40°C awaiting analysis or extraction.

3.2.2 Isolation and purification of beta-galactosidase

Extraction of the beta-galactosidase from the dried cells was according to a modified method of Mahoney et al., (1974). Dried cells (100mg/ml) were suspended in a solution of 2% toluene in phosphate buffer. This mixture was incubated for 24 hours at room temperature. The autolysate was centrifuged at 10 000 gn for 10 minutes to remove broken cell

walls and remaining whole cells. The beta-galactosidase contained in the supernatant was collected by adding one volume of acetone at 0°C and centrifuging at 6 000 gn for 10 minutes at 0°C. The precipitate was washed twice with acetone and dried under vacuum at room temperature. The dry beta-galactosidase preparation was stored at -40°C.

3.2.3 Assay methods

3.2.3.1 Assay of beta-galactosidase activity

The hydrolysis of beta-D-galactosides by beta-galactosidase results in the liberation of galactose and accompanying moiety. Galactose is not a reliable indicator of hydrolysis due to potential transgalactosidation (refer to section 2.2.2.3). Hence, beta-galactosidase activity is determined, utilizing concentrations of liberated glucose (if lactose is the substrate) or an aglycon (i.e. ortho-nitrophenol if oNPG is the substrate) in solution.

In this study, a modification of the Gist-Brocades method (Anonymous, 1972) was used to assay for enzymic activity of beta-galactosidase preparations of K. fragilis and intact K. fragilis cells. The assay procedure utilized was as follows. To 5.0 ml 1.67 mM oNPG in phosphate buffer was added, at timed intervals, 1.0 ml enzyme solution /or cell suspension. The reaction mixture was shaken at 35°C in a mangni whirl constant temperature shaker bath (88 cycles per minute), as according to Smith (1978). After 10 minutes,

the reaction was terminated with 2.0 ml of 1.0 M $Na_2 C0_3$. The absorbance was then measured at 410 nm in a Bausch and Lomb spectronic 710, to determine the concentration of o-nitrophenol.

A centrifugation of the terminated reaction mixture was necessary when assaying intact cells for beta-galactosidase activity. Suspended cells were removed by centrifugation at 10 000 gn for 10 minutes. The absorbance of the supernatant was read at 410 nm.

The procedure utilized to calculate enzymic activity was in accordance to Smith (1978). The unit of measurement was the nanokatal which represented the production of one nanomole of product (i.e. o-nitrophenol or glucose), per second, under the assay conditions specified.

When lactose was utilized as a substrate for determining beta-galactosidase activity of intact K. fragilis cells, the assay procedure was as follows. To 5.0 ml of 5% lactose in buffer was added 1.0 ml cell suspension in buffer. The reaction mixture was shaken at 35°C. After 20 minutes, the reaction mixture was heated at 80°C for 2 minutes, as to terminate the reaction. Glucose was determined employing a UV-method of analysis; Glucose/Fructose UV-test kit. The units of activity were expressed as nanokatals.

3.2.3.2 Lipase activity of K. fragilis cells

Lipase activity was determined according to Tietz and Fiereck (1966). The potential lipase activity was reflected in the ability of the enzyme preparations or K. fragilis cells to hydrolyze an olive oil emulsion. Hydrolysis of the oil would result in the production of fatty acids of which were detectable through titration with NaOH. The units of measurement were mls of 0.050 N NaOH required to titrate the reaction mixture to end point (pH10).

3.2.3.3 Proteolytic activity of K. fragilis cells

The proteolytic activity was determined according to a modified method of Anson (1939). The assay procedure was as follows. To 1.0 ml cell suspension/or enzyme preparation was added 5.0 ml of 2% hemoglobin in phosphate buffer. This reaction mixture was shaken at 35^{o}C. After 10 minutes, the reaction was terminated through the addition of 5.0 ml 0.5 M trichloroacetic acid. The trichloroacetic acid coagulated all the unhydrolyzed protein; hydrolyzed protein remained in solution. Centrifugation of the reaction mixture at 10 000 gn for 10 minutes removed all unhydrolyzed protein. The absorbance of the supernatant was read at 280 nm. The units of measurement were absorbance at 280 nm/mg enzyme preparation or yeast.

3.2.3.4 Protein determination

Protein was determined according to the method of Lowry et al., (1951), bovine serum albumin was used as the standard.

3.2.4 Characterization of beta-galactosidase

3.2.4.1 pH optima

The effect of pH upon enzymic activity was determined utilizing the following assay procedure. To 1.0 ml of enzyme solution/or cell suspension was added a mixture of 2.5 ml of 3.33 mM oNPG in 0.1 mM $MnSO_4$, which was adjusted to the appropriate pH, using 50% KOH. The reaction was terminated with 2.0 ml of 1.0 M Na_2CO_3. The absorbance was read at 410 nm. The percentage activity was calculated at each pH relative to the pH which displayed the greatest activity.

3.2.4.2 Temperature optima

The effect of temperature upon enzymic activity was determined using the standard beta-galactosidase assay procedure coupled with variable temperatures of incubation (i.e. 10^{o}, 15^{o}, 20^{o}, 25^{o}, 30^{o}, 35^{o}, 40^{o} and 45^{o}C). The percentage activity was calculated at each temperature, relative to the temperature which displayed the maximum activity.

3.2.4.3 Effect of temperature upon beta-galactosidase stability of intact K. fragilis cells

The effect of temperature upon beta-galactosidase stability was monitored at three temperatures: 47.5°, 54°, and 59°C. Cell suspensions were subjected to heat treatments of variable times (i.e. 0,2,4,8,12,16 and 20 min.) and then assayed for remaining beta-galactosidase activity. The standard beta-galactosidase assay procedure was utilized. The percentage activity was calculated at each time-temperature combination, relative to time zero for the given temperature. The results were reported as $\log_e$ remaining activity (%) vs time (min.).

3.2.5 Immobilization studies

3.2.5.1 Immobilization of beta-galactosidase and K. fragilis cells onto controlled pore glass (CPG)

Immobilization of beta-galactosidase and intact K. fragilis cells was according to a modified method of Monsan et al. (1975). The preparation of the control pore glass for immobilization studies involved the following four step procedure:

1) Preparation of the carrier.
2) Silane coupling.
3) Alkylamine coupling.
4) Immobilization of beta-galactosidase or K. fragilis cells.

Preparation of the carrier involved a 5% HNO_3 acid wash at 80 - 90°C followed by rinsing with distilled water. This cleaned and hydrated the carrier surface.

In the silane coupling step, alkyl amine groups from gama-aminopropyltriethoxysilane (GAPTES) were bound to the carrier surface. This was performed via an evaporative technique whereby 25 mls of a 1% solution of GAPTES in acetone was added to 1 gram of CPG and evaporated to dryness. Thereafter the CPG was heated at 115°C for 16 hours.

The silanized CPG was subsequently subjected to an alkyl amine coupling step. The bifunctional reagent glutaraldehyde was bound to the alkylamine groups and,thus,represented the active form of CPG (ga-CPG). The procedure for the preparation of the carbonyl derivative was as follows. To 1 gram silanized CPG was added 40 ml of 2% glutaraldehyde in 0.05 M pyrophosphate buffer (pH 8.5). This suspension was stirred at room temperature for 2 hours and then washed with distilled water.

The beta-galactosidase preparation was coupled to the activated carrier by adding 5.0 ml of a 1% enzyme solution in phosphate buffer to 1 gram of ga-CPG. This suspension mixture was shaken at room temperature for one hour and then washed repeatively in buffer (5X) to remove excess and weakly bound enzyme.

Cells of K. fragilis were immobilized in an analogous fashion to the beta-galactosidase preparation, with the exception that 5.0 ml of a 4% cell suspension in 0.05 M citrate phosphate buffer (pH 5.0) was added to 1 gram of ga-CPG.

3.2.5.2 Determination of the support retention capacity of K. fragilis cells

Support retention capacity was estimated utilizing a modified removal method of population estimation (Smith, 1974). The suspension mixture of cells and ga-CPG was washed five times in buffer. Two 1.0 ml aliquots were drawn from each wash and placed in, pre-dried and tared, aluminum dishes. The samples were dried in a forced air oven at 70^{o}C for 16 hours. The total weight of suspended yeast per wash was determined. The accumulated dry weight of yeast was plotted against the dry weight of yeast from each successive wash. The y-intercept represented the dry weight of yeast removed through washing. This value was subtracted from the dry weight estimate of the blank (all conditions identical except that untreated CPG was utilized); the difference being the estimate of the support retention capacity.

3.2.5.3 Assay of the immobilized beta-galactosidase or K. fragilis cells

The beta-galactosidase activity of immobilized enzyme or

cells was monitored utilizing a modified beta-galactosidase assay. Conditions of analysis were identical to those utilized in the standard assay procedure with the exception that a 1.0 ml suspension of immobilized enzyme or cells on CPG was used.

3.2.5.4 Effect of substrate flow rate upon the hydrolysis of oNPG in a fluidized bed reactor

Column studies were performed monitoring the effect of substrate flow rate upon the relative hydrolysis of oNPG by immobilized K. fragilis cells. The fluidized bed reactor utilized in the studies was prepared from a Pasteur pipette, in an analogous fashion to Smith (1978). The column dimensions were: inside diameter 0.5 cm; height 3.5 cm. The flow rate of oNPG (1.67 mM) was varied (6.5, 10.0, 15.0, 23.0, 26.0 and 40.0 $ml/cm^3/hr$) and the relative hydrolysis as a function of flow rate was monitored. The data was expressed as % relative activity; i.e. the percentage activity was calculated at each flow rate relative to the flow rate which displayed the greatest activity on a % hydrolysis/unit volume basis.

3.2.5.5 Beta-galactosidase stability of immobilized beta-galactosidase and K. fragilis cells

Column studies were performed monitoring the beta-galactosidase stability of immobilized beta-galactosidase preparations and K. fragilis cells as a function of time.

Columns were prepared from a Pasteur pipette according to the previously mentioned study on the effect of substrate flow rate. Substrate flow rate was 10 $ml/cm^3/hr$. The hydrolysis of oNPG was monitored by absorbancy reading at 410 nm.

A 5% lactose solution was also utilized as a substrate for immobilized K. fragilis cells produced at $10^{\circ}C$. Column conditions were identical to above except that a substrate flow rate of 6.5 $ml/cm^3/hr$ was utilized. The glucose concentration of the effluent was measured at 4 hour intervals as an indication of lactose hydrolysis.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Production and extraction of beta-galactosidase from K. fragilis

Cultures of K. fragilis, grown optimally at 30°C and suboptimally at 10°C, were harvested at the onset of the stationary growth phase. The maximal induced enzyme yield was found at this stage of growth, (figure #1) concurring with the report of Mahoney et al. (1974). The beta-galactosidase, produced at each temperature was isolated by a toluene autolysis buffer extraction followed by an acetone precipitation. The yield and catalytic activity of the beta-galactosidase preparations are reported in Table #3. For comparison, the catalytic activity of the commercial beta-galactosidase preparation, Maxilact 40 000 is also presented in Table #3. All data were based on assays employing oNPG as a substrate. Findings confirm observations reported by Smith (1978) that a greater quantity of enzyme preparation, with higher beta-galactosidase activity, was obtainable from K. fragilis cells grown at suboptimal temperature than of cells grown at optimal temperature. The data also showed that the activity of the preparation obtained from cells grown at 10°C was comparable to the commercial preparation, Maxilact 40 000. This supports the claim that K. fragilis is a good, commercially feasible source of beta-galactosidase.

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Figure 1: Effect of temperature on growth and beta-galactosidase production of K. fragilis (Edgar and Kupskay, 1979)

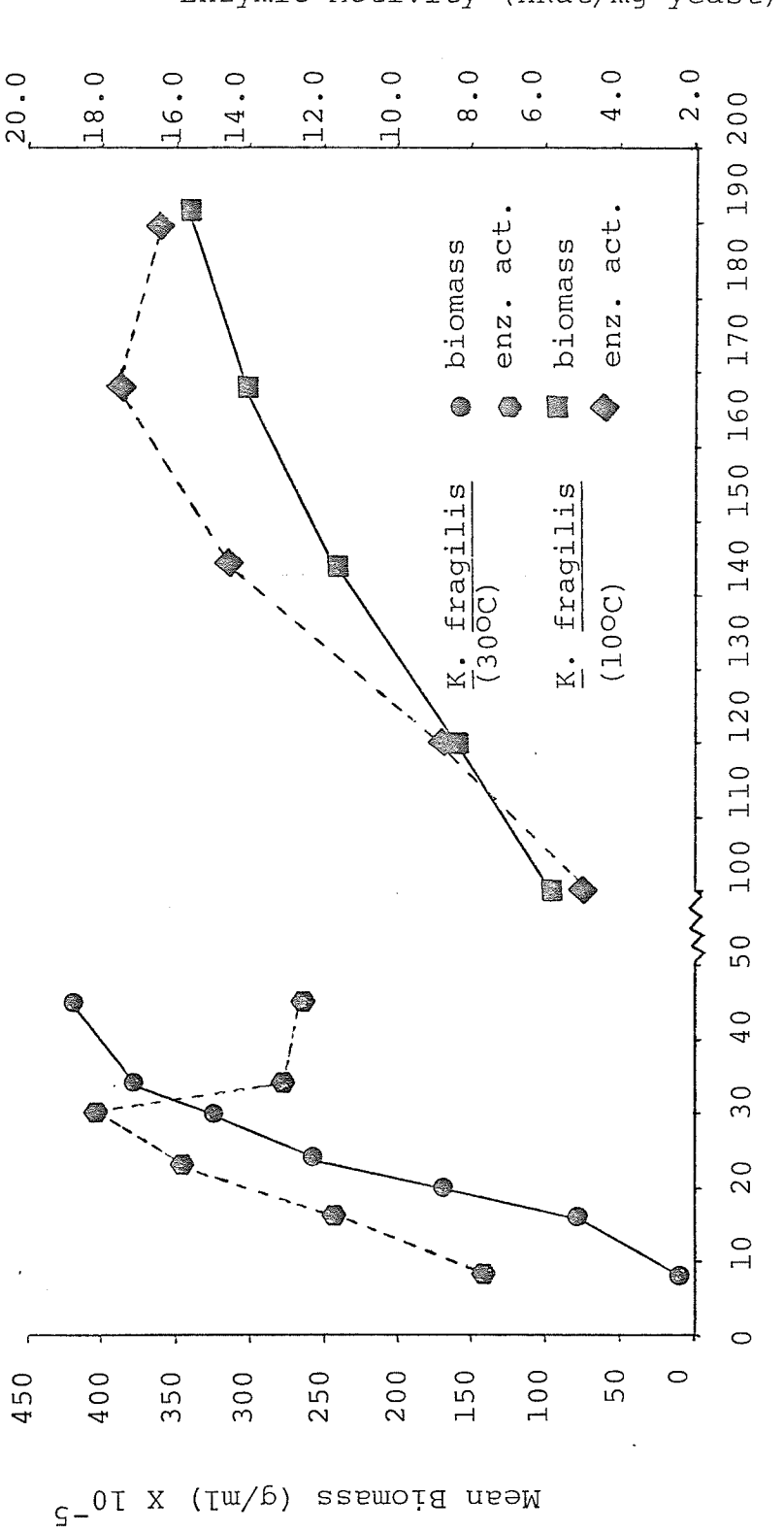


TABLE #3: Yield and catalytic activity of beta-galactosidase from K. fragilis grown at 10° and 30°C.

	Enzyme Yield mg preparation/g yeast	Relative Activity (nkat/mg preparation)	Specific Activity (nkat/mg protein)
K. fragilis beta-galactosidase (10°C)	186	9.81	47.84
K. fragilis beta-galactosidase (30°C)	39	3.73	18.53
Maxilact 40 000	-	14.38	48.27

K. fragilis grown suboptimally at 10°C yielded approximately five times more beta-galactosidase preparation of higher exzymic activity than K. fragilis grown optimally at 30°C. Growth of K. fragilis at suboptimal temperature, thus, seemed to induce a hyperproduction of beta-galactosidase. If hyperproduction was in occurrance, however, one would expect a proportionally higher beta-galactosidase activity per unit cell weight, with growth at suboptimal temperature. To test for hyperproduction, cells, grown at both temperatures, were harvested at the onset of the stationary growth phase and assayed for beta-galactosidase activity. The beta-galactosidase activities obtained were 17.82 nkat/mg yeast for K. fragilis grown at 10°C and 19.24 nkat/mg yeast for K. fragilis grown at 30°C. Based on these results, the concept of hyperproduction must be rejected. Thus, the increased enzyme yields obtained from K. fragilis grown at 10°C reflected easier extratability of beta-galactosidase. This may have been the result of altered cellular membranes of K. fragilis when grown at suboptimal temperature. From a practical point of view, however, this observation suggested that if the extraction procedure was modified, better yields of beta-galactosidase preparation with increased activity could be obtained from cells grown optimally at 30°C.

4.2 Characterization of beta-galactosidase

Smith (1978), found that the enzymic properties of beta-galactosidase preparations obtained from K. fragilis grown at 10° and 30°C were similar, and suggested they were identical. In addition, findings reported by several authors (Wendorff and Amundson, 1971; Kulikova et al., 1972; Kovalenko et al., 1975; Woychik et al., 1974; Kilara et al., 1977; Mahoney and Whitaker, 1977; Woychik and Holsinger, 1977) involving beta-galactosidase preparations from K. fragilis grown at optimal temperature (30°C) support Smith's results. Investigation of enzymic properties was, therefore, limited to beta-galactosidase preparations of K. fragilis grown at 10°C. The results were compared to published data reporting the enzymic properties of beta-galactosidase preparations of K. fragilis grown optimally.

The effect of pH on beta-galactosidase activity is shown in figure #2. Maximum enzyme activity was obtained at pH 7.0. A rapid loss of enzymic activity was evident below pH 6.5. At pH 5.5 the beta-galactosidase activity was negligible. Similar data has been reported by several authors (Wendorff and Amundson, 1971; Kulikova et al., 1972; Kovalenko et al., 1975; Woychik and Holsinger, 1977; Smith, 1978).

The effect of temperature on beta-galactosidase activity was examined over the range of 10 - 45°C (Figure #3). Maximum enzyme activity was obtained at 35°C. This lies within the

Figure 2: Effect of pH on enzymic activity of soluble beta-galactosidase from K. fragilis grown at 10°C.

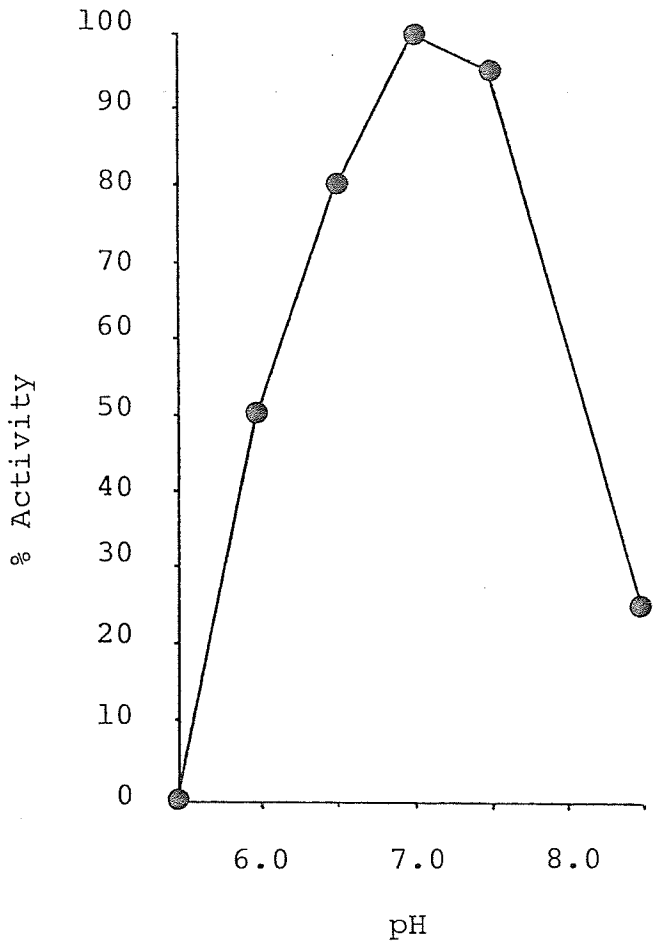
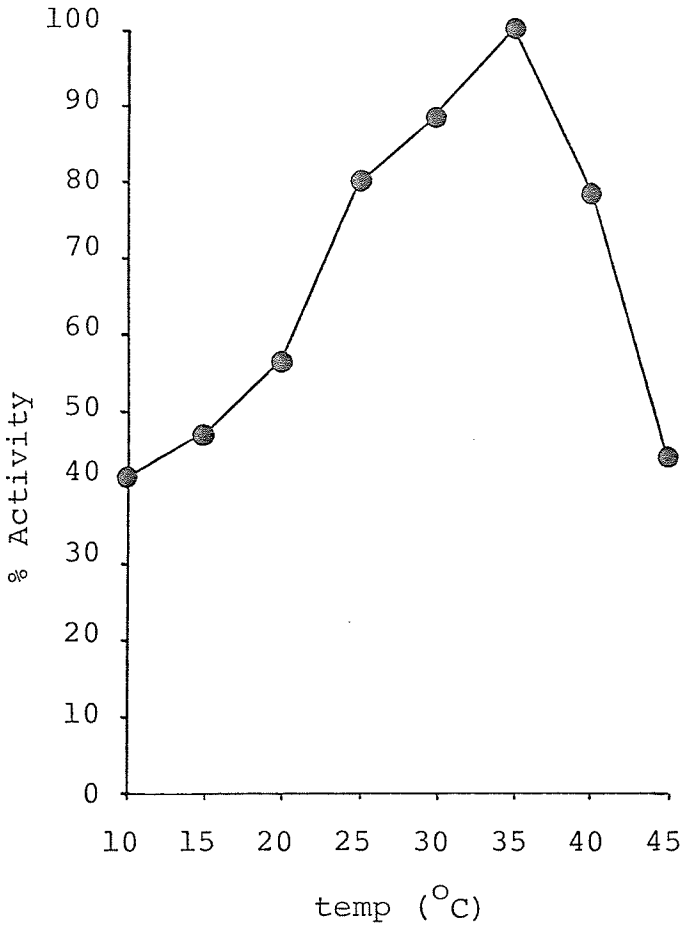


Figure 3: Effect of temperature on enzymic activity of soluble beta-galactosidase from K. fragilis grown at 10°C.



reported range of temperature optima of 30 - 37°C (Kovalenko et al., 1975; Mahoney and Whitaker, 1977; Woychik and Holsinger, 1977; Guy and Bingham, 1978; Smith 1978). Above the optimal temperature of 35°C enzymic activity decreased rapidly. At 45°C, the maximum temperature of growth, for K. fragilis approximately 40% of the maximum enzymic activity remained. This was in accordance to Uwajima et al., (1972) and Mahoney and Whitaker (1977), who report rapid inactivation of the enzyme above 40°C.

The findings of this study support Smith (1978). The results compared well to published data reporting the enzymic properties of beta-galactosidase preparations of K. fragilis grown at optimal temperature. The beta-galactosidase obtained from K. fragilis grown suboptimally at 10°C and optimally at 30°C appear to be identical.

4.3 Immobilization of beta-galactosidase from K. fragilis

Beta-galactosidase was immobilized to controlled pore glass, utilizing the bifunctional reagent glutaraldehyde. Tomimatsu et al., (1971) report the functionality of glutaraldehyde to be the result of its high affinity for epsilon amino groups (lysine residues), tyrosime, histidine and sulfhydryl residues. The involvement of these functional groups may potentially alter the structure and subsequent reactivity of the enzyme. Covalent bonding of the enzyme to

carriers by bifunctional reagents such as glutaraldehyde, however, is often selected over other techniques of immobilization because of its permanence of insolubilization.

The effect of pH on the activity of immobilized beta-galactosidase is shown in Figure #4. Immobilization seemed to cause a shift in the pH optimum from 7.0 to 6.5. This may be explained by a possible structural deformation of the enzyme upon immobilization which need not necessarily affect other characteristics such as temperature optimum. The pH optima of immobilized beta-galactosidase, however, remained within the range of pH optima reported for soluble K. fragilis beta-galactosidase (i.e., 6.3 - 7.2) (Wendorff and Amundson, 1971; Kulikova et al., 1972; Kovalenko et al., 1975; Woychik et al., 1974; Kilara et al., 1977; Mahoney and Whitaker, 1977; Woychik and Holsinger, 1977; Smith 1978).

The temperature activity profiles of free and immobilized beta-galactosidase were similar (Figure #5). Immobilization did not seem to affect the temperature optimum and temperature activity profile.

Immobilized beta-galactosidase from K. fragilis possessed similar enzymic properties to the soluble form. These findings concur with reports of Smith (1978), and confirm that immobilized beta-galactosidase may be potentially applicable for lactose hydrolysis in milk and sweet whey.

Figure 4: Effect of pH on enzymic activity of soluble and immobilized K. fragilis beta-galactosidase preparations (10°C).

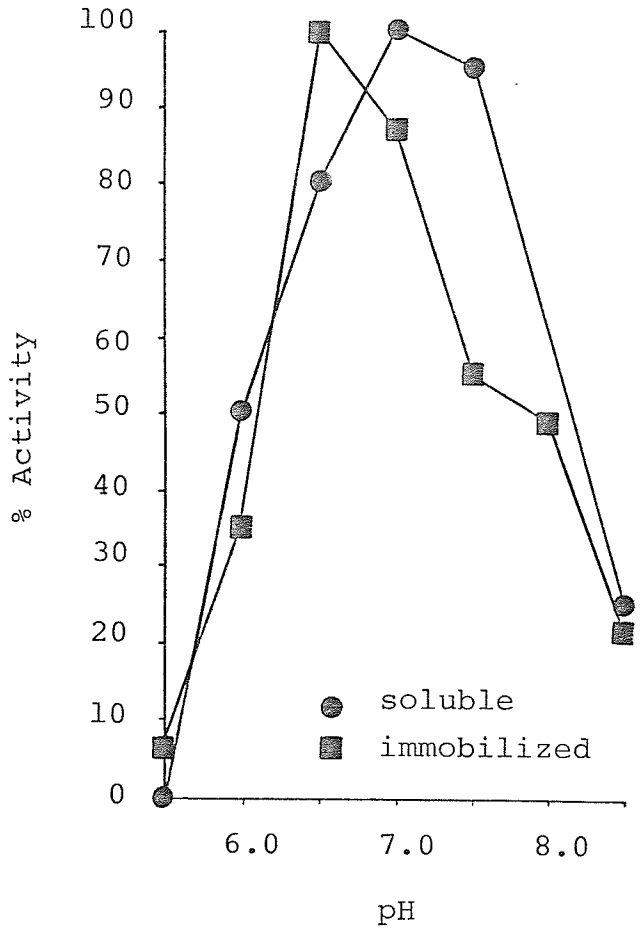
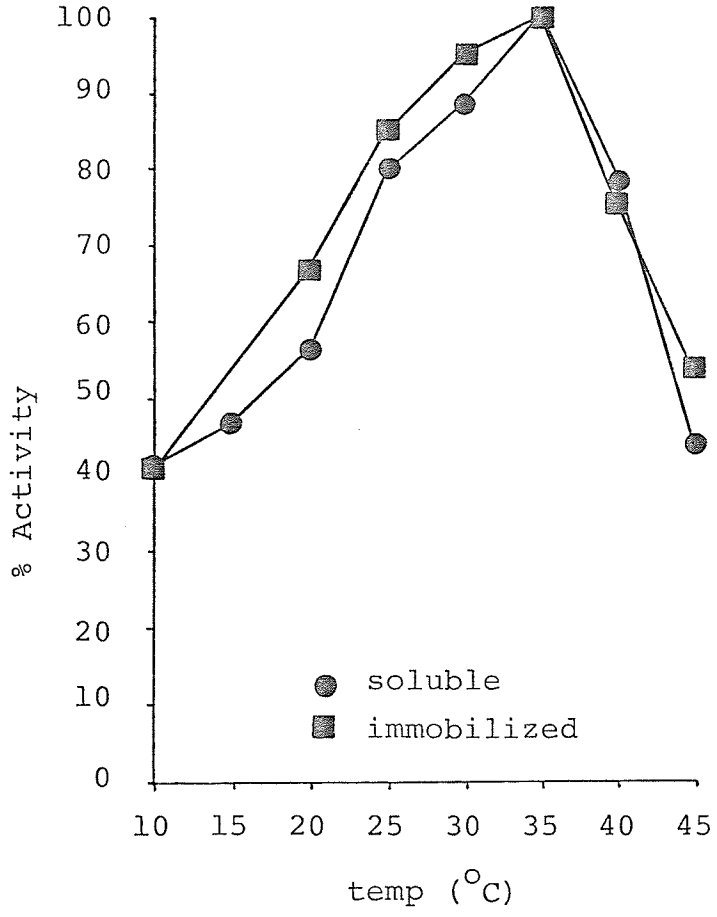


Figure 5: Effect of temperature on enzymic activity of soluble and immobilized K. fragilis beta-galactosidase preparations (10°C).



In view of a potential continuous flow operation, the stability of immobilized K. fragilis beta-galactosidase in a fluidized bed reactor, was investigated. Data showed that the half-life of the immobilized enzyme preparation was approximately 13.6 hours at room temperature. This was short in consideration of a continuous flow system. Hence, the stability of immobilized K. fragilis beta-galactosidase must be increased to make it more attractive for commercial applications.

4.4 Characterization of beta-galactosidase of intact K. fragilis cells

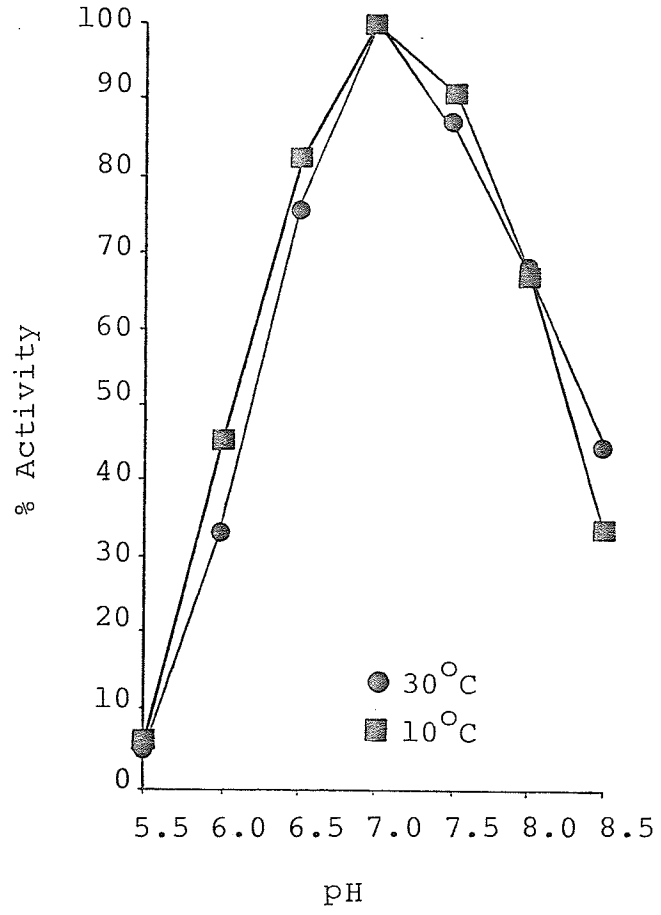
The relative instability of K. fragilis beta-galactosidase upon extraction may be potentially avoided by the fixation of intact K. fragilis cells to inert supports. Reports in literature (Jack and Zajic, 1977) stipulate that increased enzyme stability is often attained by the immobilization of intact cells. The following sections report the properties of beta-galactosidase activity of intact K. fragilis cells in the free and immobilized state.

K. fragilis cells were grown suboptimally at 10^{o}C and optimally at 30^{o}C. After harvest the cells were freeze dried and stored at -40^{o}C until subsequent analysis. The characterization studies were all based on assays employing oNPG as a substrate.

During the initial stages of the study, the enzymic activities of freeze dried K. fragilis cells seemed erratic. Investigation revealed that beta-galactosidase activity increased with time upon suspension of the cells in buffer. The cells reached a maximum level of enzymic activity after 3 hours and then stabilized. A possible explanation of this latency in activity may be that freeze drying of the yeast altered the integrity of the cellular membrane. This affected the permeability of the membrane, resulting in a hinderance of substrate penetration and hence reduced activity. Chayen et al., (1971), reported an analogous latency of napthylamidase activity in lysosomes of synovial lining cells. Findings showed that it was necessary to preincubate the cells prior to a full manisfestation of enzymic activity. In subsequent studies, therefore, K. fragilis cells were preincubated in buffer for 3 hours prior to assays or immobilization.

The pH optima and activity profile of intact K. fragilis cells was not affected by the temperature of growth (Figure #6). Additionally, it is evident upon comparison of Figures 2 and 6 that the pH opimum of 7.0 and corresponding activity profiles were similar to the soluble beta-galactosidase preparation. Thus, beta-galactosidase of K. fragilis, in vivo and in vitro seem to react analogously to changes in pH.

Figure 6: Effect of pH on beta-galactosidase activity of *K. fragilis* cells grown at 10°and 30° C.



The temperature activity profiles of intact K. fragilis cells, grown at 10° and 30°C, were similar (Figure #7). The temperature optimum of 35°C and corresponding activity profiles of intact K. fragilis cells were similar to the soluble beta-galactosidase preparation. K. fragilis cells, however, showed higher relative enzymic activities above 35°C. Assuming that beta-galactosidase activity above the optimum temperature of hydrolysis was a function of enzymic stability to heat inactivation, beta-galactosidase, in vivo, seemed to offer increased enzyme stability to heat.

The increased beta-galactosidase stability in intact K. fragilis cells, suggested by the temperature activity profile, prompted the investigation of the effect of temperature on beta-galactosidase stability. Three temperatures, above the maximum growth temperature of K. fragilis (45°C), were utilized in the study (47.5°, 54° and 59°C). Cell suspensions were subjected to heat treatments of different time-temperature combinations and then assayed for beta-galactosidase activity. Intact K. fragilis cells offered good beta-galactosidase stability, independant of the temperature of growth (Figures 8 and 9). A first order type of kinetics of enzyme inactivation was indicated by these figures. This was the same as reported for beta-galactosidase preparations (Mahoney and Whitaker, 1977).

Figure 7: Effect of temperature on beta-galactosidase activity of K. fragilis cells grown at 10° and 30° C.

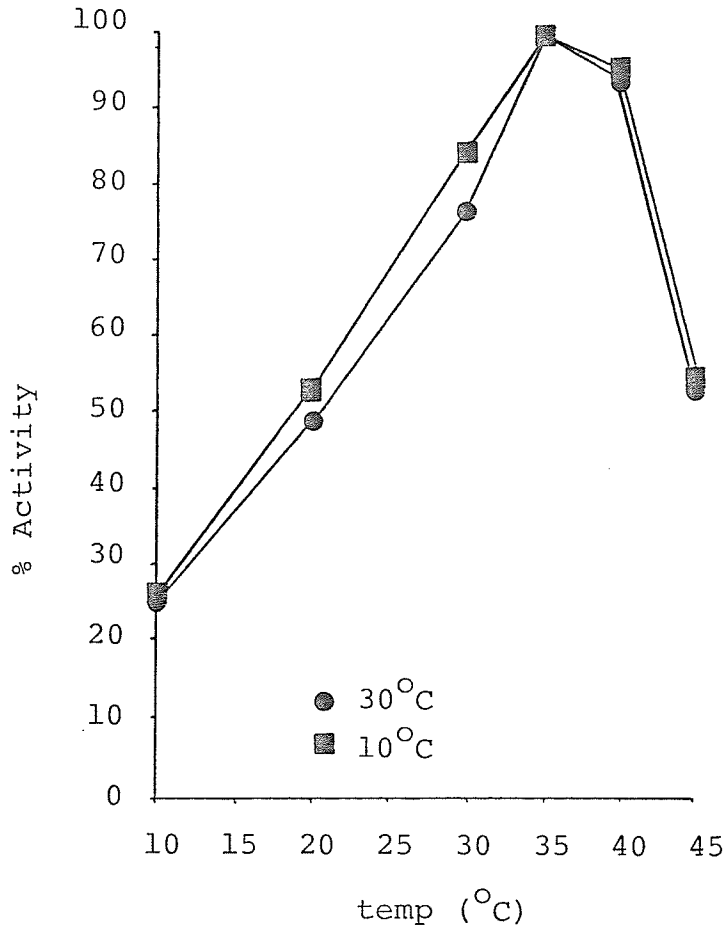
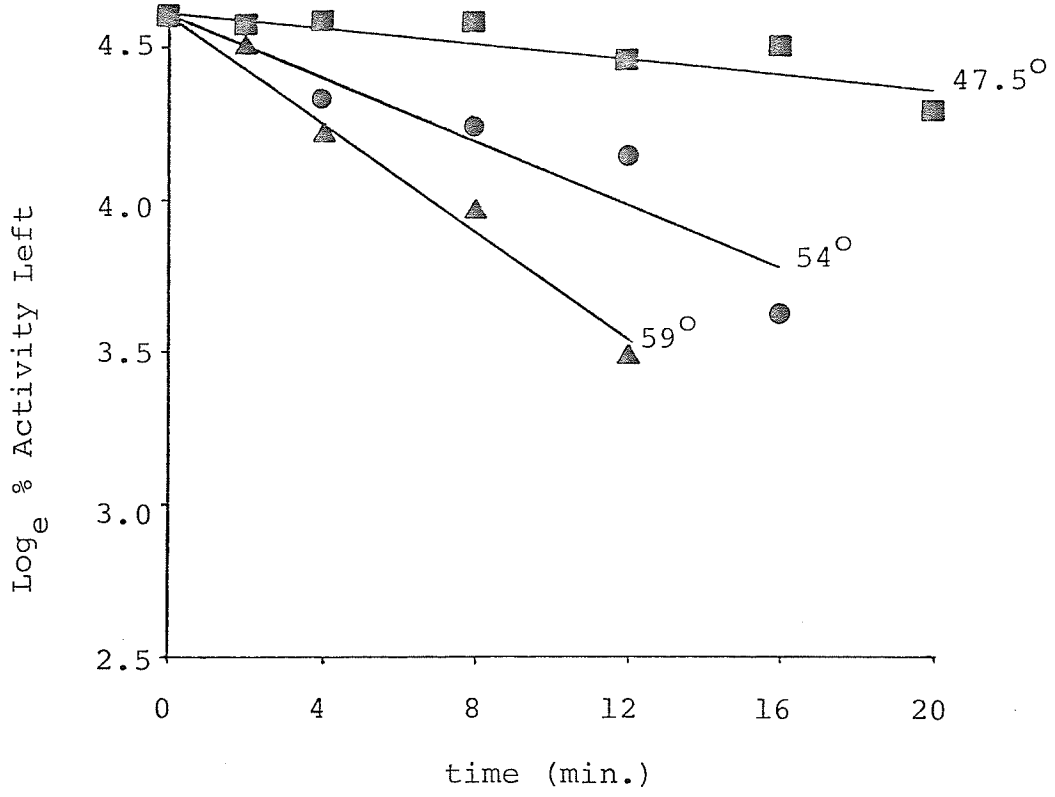


Figure 8: Effect of temperature on the stability of beta-galactosidase of K. fragilis cells grown at 10° C.

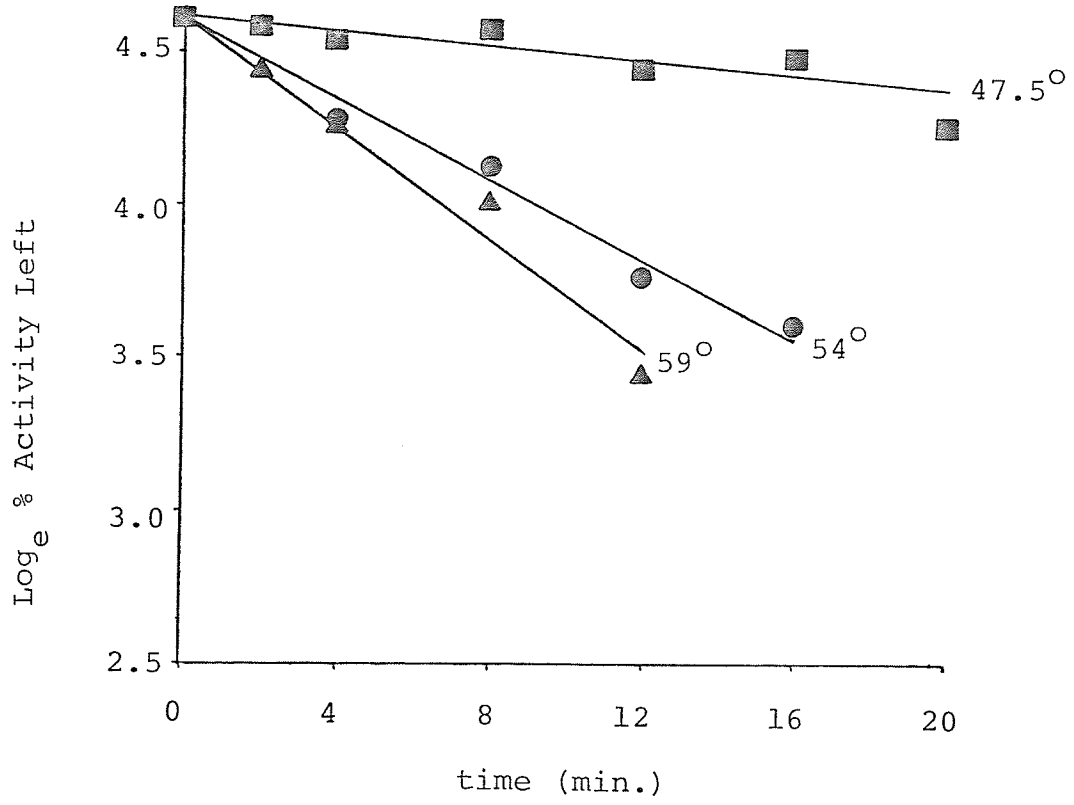


47.5°: y=-0.014x + 4.640 ; r=-0.885

54° : y=-0.055x + 4.647 ; r=-0.925

59° : y=-0.093x + 4.637 ; r=-0.992

Figure 9: Effect of temperature on the stability of beta-galactosidase of K. fragilis cells grown at 30° C.



47.5°: y=-0.013x + 4.626 ; r=-0.869

54° : y=-0.065x + 4.627 ; r=-0.986

59° : y=-0.093x + 4.646 ; r=-0.990

Table #4 compiled the calculated half-lives of beta-galactosidase activity of intact K. fragilis cells, as affected by temperature. For comparison, the calculated half-life of a beta-galactosidase preparation at 46°C, as reported by Mahoney and Whitaker, (1977), was included. Findings showed the stability of beta-galactosidase of intact cells exceeded that of the enzyme preparation.

The use of intact K. fragilis cells for the hydrolysis of lactose in milk and sweet whey, thus, seems feasible, since the pH and temperature optima of beta-galactosidase activity of intact cells were similar to that of enzyme preparations. Additionally, the beta-galactosidase activity of intact cells was more stable (at pH 7) than that of enzyme preparations. The presence of numerous active enzymes in the cell, however, may lead to potential unwanted side reactions. Contaminating lipolytic and proteolytic activity is detrimental to product quality, especially when working with a heterogenous material such as milk or whey. Subsequently, intact K. fragilis cells and beta-galactosidase preparation were compared to the commercial enzyme preparation, Maxilact 40 000, for lipolytic and proteolytic activity at pH 7 (Table #5). In all cases, lipase activity was negligible. The proteolytic activity, though evident in K. fragilis cells and beta-galactosidase

TABLE #4: Half-life of beta-galactosidase activity as a function of temperature.

	Half-life of beta-galactosidase activity (min.)			
	46°C	47.5°C	54°C	59°C
K. fragilis cells 10°	-	63.8	13.4	7.8
K. fragilis cells 30°	-	53.3	10.9	7.9
Beta-galactosidase preparation (Mahoney and Whitaker, 1977)	8	-	-	-

TABLE #5: Proteolytic and lipolytic activities of K. fragilis cells and beta-galactosidase preparations.

	Proteolytic * Activity	Lipolytic ** Activity
K. fragilis Cells (10°C)	1.40	< 0.02
K. fragilis Cells (30°C)	1.42	< 0.02
K. fragilis beta-galactosidase preparation (10°C)	1.05	< 0.02
Maxilact 40 000	1.35	< 0.02

* Units of activity: absorbance at 280 nm/mg yeast or beta-galactosidase preparation.

** Units of activity: ml of 0.050 N NaOH/mg yeast or beta-galactosidase preparation.

preparation were of about the same magnitude as for the commercial enzyme preparation. This may indicate that the K. fragilis preparation and cells at neutral pH would contain tolerable levels of side activity, in view of commercial application.

4.5 Immobilization of K. fragilis cells

Intact K. fragilis cells were immobilized to ga-CPG in an analogous fashion to enzyme preparations. Immobilization of the cells was accomplished by the establishment of imine bonds between support carbonyl groups (glutaraldehyde) and the amino groups of the cell walls (Navarro and Durand, 1977).

The support retention capacity of K. fragilis cells was estimated to be 2.6 $\pm$ 0.5 mg yeast/g ga-CPG and 2.3 $\pm$ 0.6 mg yeast/g ga-CPG for 10^{o} and 30^{o}C grown cells, respectively. Navarro and Durand (1977), reported a support retention capacity of Saccharomyces carlsbergensis on porous silica (spherosil: 34m^{2}/g area) to be approximately 3.0 mg yeast/g carrier. The efficiency of immobilization in this study was of about the same level as that reported by Navarro and Durand (1977).

The immobilized cells displayed total beta-galactosidase activities of approximately 55 nkat and 53 nkat/g carrier for 10^{o} and 30^{o} grown cells respectively. This would be equivalent to approximately 21.2 nkat/mg yeast and 23.0 nkat/mg yeast for 10^{o} and 30^{o}C grown cells, respectively, which was

similar to the activity found for free cells (page 45). Immobilization of K. fragilis cells appeared to have no effect on beta-galactosidase activity.

Immobilization of K. fragilis cells caused a shift in the temperature optima of beta-galactosidase from 35^{o} to 30^{o}C, as illustrated in Figures 10 and 11 which show enzymic activity as a function of temperature for free and immobilized cells. The temperature optimum of immobilized cells, however, remained within the reported range of temperature optima of 30^{o} - 37^{o}C (Kovalenko et al., 1975; Mahoney and Whitaker, 1977; Woychik and Holsinger, 1977; Guy and Bingham, 1978; Smith, 1978). Petre et al.,(1978), observed a similar shift in the optimum pH with the immobilization of E. coli when cross-linked with glutaraldehyde and bovine serum albumin. Elucidation of the mechanisms involved in such shifts in pH or temperature optima upon immobilization of intact microbial cells is complex and has yet to be determined.

The pH profiles of free and immobilized K. fragilis cells are presented in Figures 12 and 13. It is evident that pH optima and profiles were not altered by immobilzation of the cells. In addition, there appeared to be no difference between cells grown optimally at 30^{o} or suboptimally at 10^{o}C.

Figure 10: Effect of temperature on beta-galactosidase activity of free and immobilized K. fragilis cells grown at (10°C).

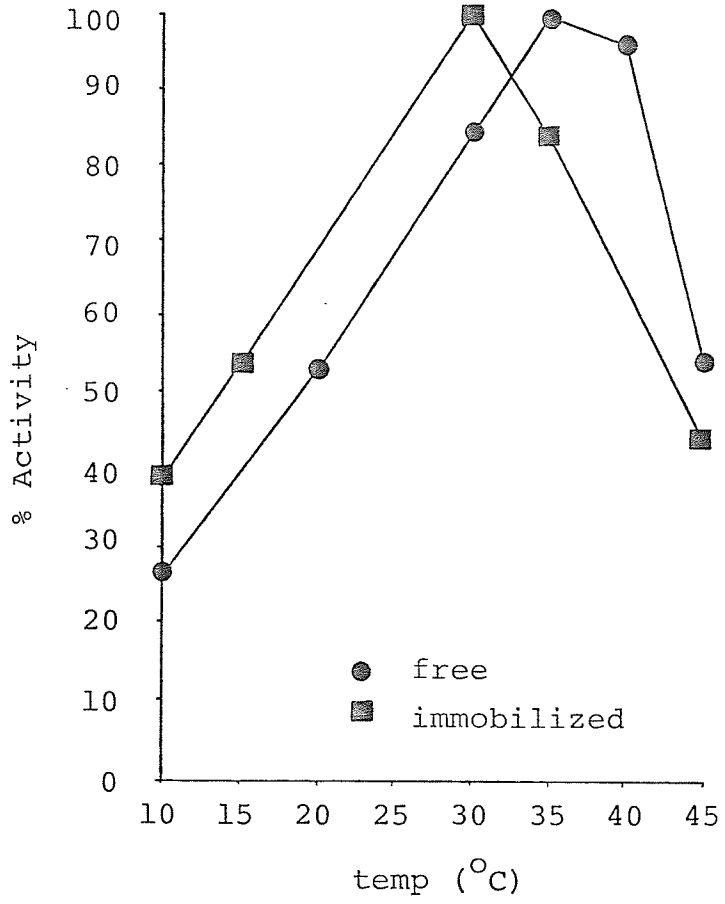


Figure 11: Effect of temperature on beta-galactosidase activity of free and immobilized K. fragilis cells grown at (30°C).

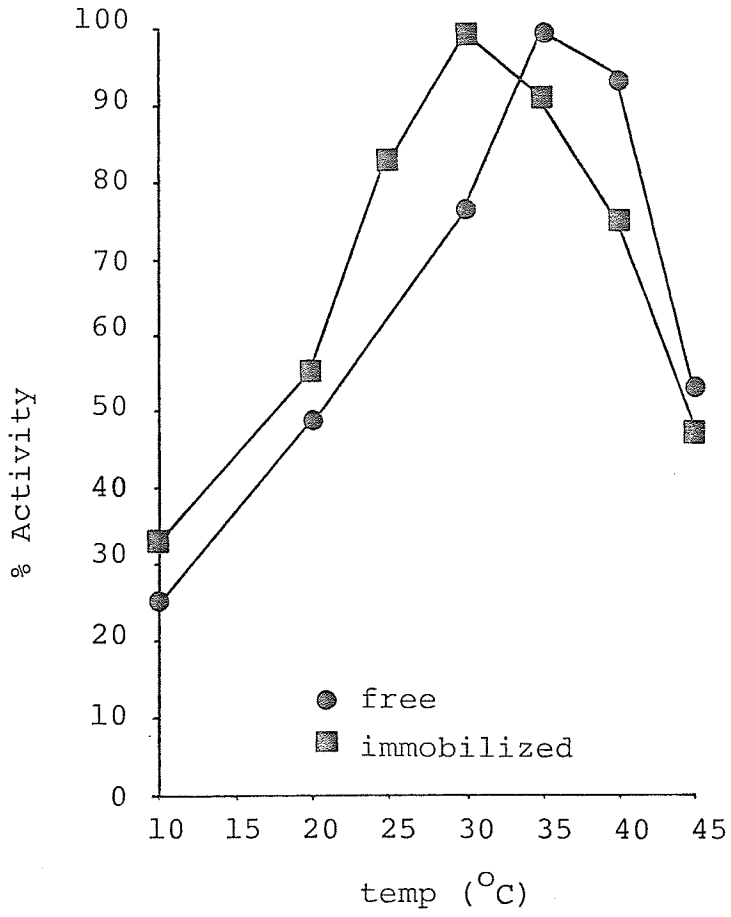


Figure 12: Effect of pH on beta-galactosidase activity of free and immobilized K. fragilis cells grown at (10°C).

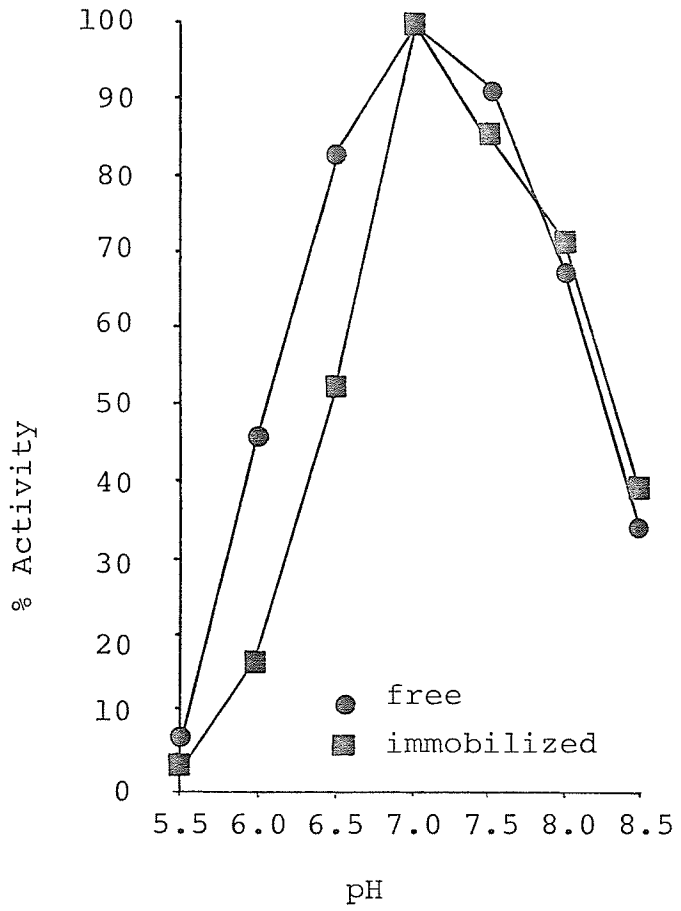
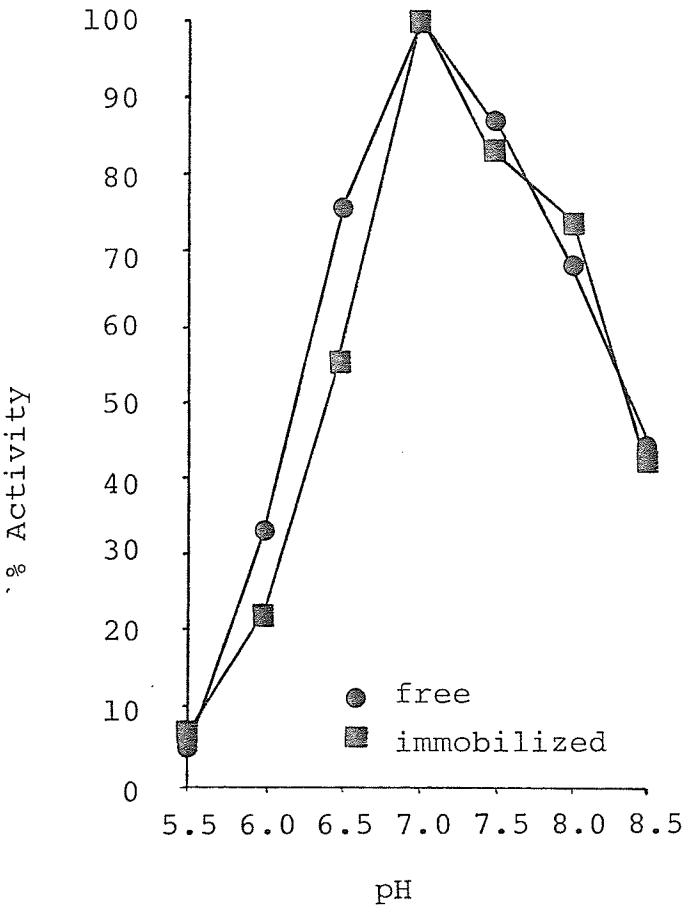


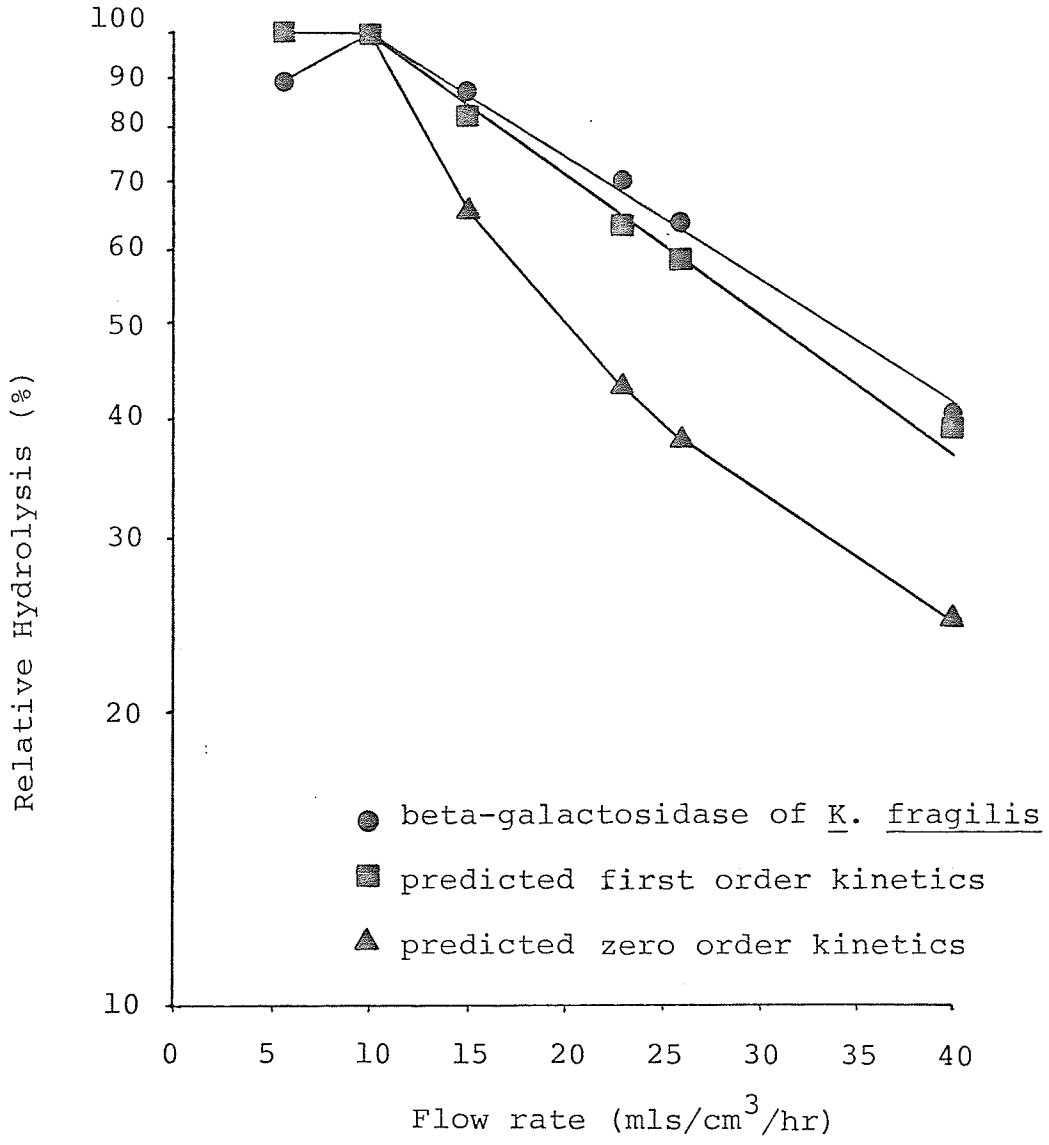
Figure 13: Effect of pH on beta-galactosidase activity of free and immobilized K. fragilis cells grown at (30°C).



4.6 Effect of substrate flow rate on the relative hydrolysis of oNPG in a fluidized bed reactor

In immobilized enzyme systems, the rate of substrate diffusion towards the active surface (site) of enzymes fixed on a matrix, plays an important role in determining the kinetics of a reaction (i.e. mass transfer effects). Thus, the relative hydrolysis of a specific substrate is often a function of substrate flow rate. Subsequently, the effect of substrate flow rate on the relative hydrolysis of oNPG by K. fragilis cells in a fluidized bed reactor was investigated (Figure #14). For comparison, the predicted conversions for a zero order and first order kinetics were also included in Figure #14. The hydrolysis of oNPG (1.67mM) by beta-galactosidase of K. fragilis seemed to follow first order kinetics. The optimal flow rate was 10.0 ml/cm^3/hr. Below this value a lower relative hydrolysis of oNPG was evident. A flow rate of 6.5 m/cm^3/hr showed approximately 90% of the maximum relative activity. End product inhibition may be the most likely cause of this reduction, but, in addition diffusional and partitioning effect may also cause a lower hydrolysis than expected. Above the optimal substrate flow rate of 10.0 ml/cm^3/hr was an exponential decrease in the relative hydrolysis. Assuming that the apparent Km for beta-galactosidase of intact

Figure 14: Effect of substrate flow rate on the hydrolysis of oNPG in a fluidized bed reactor.



K. fragilis cells was comparable to that of soluble preparations, these findings were expected in view of the concentration of oNPG being approximately one half of the Km for soluble beta-galactosidase (i.e. K. fragilis 10^{o} Km 2.72 $\pm$ 0.29 mM; K. fragilis 30^{o}C Km 3.30 $\pm$ 0.46 mM), as reported by Smith, (1978). The reaction was substrate dependant, hence, was expected to be near first order kinetics.

4.7 Stability of beta-galactosidase activity of immobilized K. fragilis cells in a continuous flow reactor

The use of immobilized K. fragilis cells as a technique of stabilizing beta-galactosidase activity was investigated. Studies monitored beta-galactosidase activity of immobilized K. fragilis cells in a fluidized bed reactor at ambient temperature, as a function of time (substrate: 1.67 mM oNPG). Findings showed the half-life of beta-galactosidase activity of immobilized K. fragilis cells to be 86.0 $\pm$ 1.5 hours and 76.8 $\pm$ 8.5 hours for 10^{o} and 30^{o}C grown cells, respectively. The half-life of beta-galactosidase of K. fragilis cells grown at suboptimal and optimal temperature were similar. The temperature of growth did not seem to affect beta-galactosidase stability or the degree of substrate hydrolysis. Nonetheless, the immobilization of intact K. fragilis cells

offered a six fold increase in beta-galactosidase stability compared to immobilized enzyme preparations (half-life: 13.6 hours). This is important, in view of a potential commercial continuous flow operation.

Beta-galactosidase stability studies were also performed using 5% lactose in phosphate buffer as a substrate. Experimental conditions were identical to previous column studies, involving oNPG as a substrate, except that only K. fragilis cells grown at $10^{o}C$ were immobilized and the substrate flow rate was reduced to 6.5 $ml/cm^{3}/hr$. The lower rate of substrate flow was to compensate for a higher Km value for lactose (Smith, 1978). However, negligible lactose hydrolysis by immobilized cells was evident. A check of beta-galactosidase activity with oNPG showed the appropriate levels of hydrolysis. Preliminary studies showed that the matrix (i.e. ga-CPG) was incapable of hydrolyzing oNPG. Immobilization of K. fragilis cells by the covalent bonding of glutaraldehyde, thus, seemed to inhibit the hydrolysis of lactose but not oNPG.

Subsequently, beta-galactosidase activity of free and immobilized K. fragilis cells were tested, using the standard assay procedure (5% lactose). A 20 minute assay time was utilized. This approximated the retention time of the substrate within the column. The free K. fragilis cells possessed a

relative beta-galactosidase activity of 6.8 nkat/mg yeast. Immobilized K. fragilis cells, on the other hand, possessed negligible activity. Immobilization of the K. fragilis cells seemed to inhibit the hydrolysis of lactose.

As a final test of beta-galactosidase inhibition, the time of the standard assay was lengthened to 14 hours and either 0.5% lactose or 5.0% lactose in buffer was used as the substrate. Upon analysis, the glucose in solution was estimated to be 0.70 $\pm$ 0.15 g/l and 0.17 $\pm$ 0.01 g/l for 0.5% and 5.0% lactose solutions, respectively. The hydrolysis of lactose by immobilized K. fragilis cells was very low, showing a maximum 14% hydrolysis of the 0.5% lactose solution. However, the relative lactose hydrolysis was approximately 4 X greater for the 0.5% lactose solution in comparison to the 5.0% solution. This indicated that substrate inhibition was in effect when 5% lactose was utilized as the substrate.

Sols et al., (1974), state that lactose is transported across the cellular membrane of yeast by an inducible specific system, to be subsequently hydrolyzed inside the cell by beta-galactosidase. The covalent bonding of glutaraldehyde seems to inhibit the lactose permease system, resulting in a lack of lactose hydrolysis. Hydrolysis of the two substrates, lactose and oNPG, was obviously different. The best explanation at this time would be that oNPG diffusion into the cell to the

enzyme is of passive nature, whereas, diffusion of lactose relies on an active transport system, most likely on a permease system. These observations would suggest that oNPG was not a perfect substrate to predict lactose hydrolysis by a reactor based on immobilized cells. In addition, the results of the reactor studies indicate that the permease system needs to be investigated as to the effect of immobilization on its activity.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

K. fragilis has long been considered to be a good potential source of beta-galactosidase for the hydrolysis of lactose in milk and sweet whey (Mahoney *et al.*, 1974). Previous studies (Smith, 1978), indicated that the employment of a relatively simple extraction technique resulted in an enzyme preparation which was comparable in activity and properties (pH, temperature profile) to that of the commercial preparation, Maxilact 40 000, derived from *K. lactis*. The yield and properties of beta-galactosidase derived from *K. fragilis* seem to confirm these claims.

Investigations by Smith (1978), suggested that when *K. fragilis* was grown at suboptimal temperature, hyperproduction of beta-galactosidase occured. During this study, however, findings lead to the rejection of hyperproduction. It was found that beta-galactosidase was more readily extracted from cells grown at 10^{o}C than at 30^{o}C. This indicated that possible changes or differences existed between the cellular membranes of cells grown at suboptimal and optimal temperature. In view of the planned immobilization of intact cells, this may have had some implications on the activity or subsequent performance of a continuous flow reactor, based on immobilized cells. Hence, *K. fragilis* cells grown at 10^{o}C and 30^{o}C were utilized in immobilization studies.

The purpose for immobilization of cells was to determine if the half-life of beta-galactosidase activity could be increased. Preliminary studies which involved the immobilization of extracted enzyme preparation showed a half-life of only 13.6 hours. It was obvious that the stability of beta-galactosidase activity had to be increased to make it more attractive for potential commercial use. Immobilization of intact cells proved to be successful in that half-life activity in continuous flow operations was increased six fold. Comparison of the beta-galactosidase activity of free and immobilized cells showed that the immobilization of the cells by the covalent bonding to controlled pore glass did not cause any major change in the hydrolytic properties of the enzyme (Figures 8 - 13). The hydrolytic properties of beta-galactosidase of intact cells, also, seemed to be independant of the temperature of growth, i.e. results obtained from $10^{o}C$ and $30^{o}C$ grown cells were similar. In addition, the side activities of cellular proteolytic enzymes, though present, appeared tolerable since they were comparable to the ones found in the commercial preparation, Maxilact 40 000 (Table #5). Future investigations may, however, persue this problem of side activity. The reduction of cellular proteolytic activity to a minimum would be advantageous to a commercial process.

The investigations to this point were all carried out employing oNPG as a substrate. oNPG is an analogue to lactose and is widely used to characterize beta-galactosidase activity. However, when lactose was used as a substrate in continuous flow operations, negligible hydrolysis was achieved by immobilized cells. The immobilized cells possessed beta-galactosidase activity as indicated by their capability to hydrolyze oNPG. An explanation of this difference was presumably related to the transport of lactose through the cell wall and membrane to the enzyme. It may be stipulated that lactose requires a permease system for active transport whereas oNPG relies on passive transport. The covalent bonding of glutaraldehyde seemed to inhibit the lactose permease system, resulting in a lack of lactose hydrolysis. The conclusion which may be drawn from this is that alternative immobilization techniques must be investigated. Techniques such as entrapment in polyacrylamide gel or the binding to macromolecules which potentially may not damage or inactivate the permease system should be considered. Cell viability, however, may constitute a problem in alternative immobilization procedures. Glutaraldehyde is a strong antimicrobial reagent and thus avoided the problem of cell viability in this study. A solution to the potential problem of cell viability is selective for a treatment developed to kill cells without

inactivating beta-galactosidase activity. Results in Table #4 or Figures 8 and 9 indicate that a potential heat treatment of the cells may be a solution. The increased beta-galactosidase stability offered by intact cells is essential in view of the retention of enzymic activity pending the potential need of heat inactivation of cell viability or accompanying cellular enzymes.

On a positive note, the half-life of beta-galactosidase was extended by the immobilization of intact K. fragilis cells. It should be possible, however, to increase the stability of beta-galactosidase even further. Findings of this study show a potential trend of increased enzyme stability when K. fragilis was grown at suboptimal temperature ($10^{o}C$) in comparison to growth at optimal temperature ($30^{o}C$). Control and manipulation of the growth enviroment, thus, represents a potential key to the increased stabilization of beta-galactosidase of intact K. fragilis cells.

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