SIMULTANEOUS PRODUCTION OF INULASE AND LACTASE IN BATCH AND CONTINUOUS CULTURE BY THE YEAST *KLUYVEROMYCES FRAGILIS*

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Gary Maxwell Hewitt

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ABSTRACT

Hewitt, Gary Maxwell. M.Sc., The University of Manitoba, June, 1981. Simultaneous Production of Inulase and Lactase in Batch and Continuous Culture by the Yeast Kluyveromyces fragilis.

With the aim of optimizing simultaneous production of inulase and lactase in a single-step fermentation, carbon limited batch and continuous cultures of K. fragilis were examined. Initially, separate production of each enzyme was studied. In batch culture, inulin supported the highest inulase yields per unit biomass; however, these levels were less than half those obtained in continuous cultures grown on sucrose or fructose. Lactose and galactose limited continuous cultures produced five times more lactase than similar batch cultures. Both enzymes were repressed by rapidly metabolizable carbon substrates. This finding, plus the observed relationship between dilution rate and the production of enzymes, suggested that each enzyme is regulated by catabolite repression. Continuous cultivations on various monosaccharides indicated that inulase and lactase biosynthesis also require induction by fructose and galactose, or their metabolic derivatives, respectively.

Simultaneous enzyme production was investigated using numerous carbon substrate combinations. Good dual enzyme yields were obtained in a continuous culture grown on an equal mixture of fructose plus galactose. Attempts to determine the optimal fructose to galactose

feed ratio were unsuccessful. In one such experiment, the K. fragilis stock culture was displaced by a mutant which produced elevated inulase levels in batch culture on 11 carbon substrates. A second approach to optimizing simultaneous enzyme yields of K. fragilis involved addition of non-metabolizable galactose analogs to sucrose limited continuous cultures. By this method, lactase was not induced and inulase was repressed.

Important criteria used to assess a fermentation process for industrial application were evaluated. On this basis, continuous culture proved superior to batch culture for production of inulase and lactase, separately and simultaneously, by K. fragilis. The inulase hyperproducing mutants may further enhance the value of this fermentation technique.

1. INTRODUCTION

Increased competition for markets from the chemical industry, in addition to increasing labour, energy, and raw material costs, are forcing the fermentation industry to continually improve processes in order to minimize manufacturing costs. Presently, the majority of fermentations are oriented to a single end product such as an antibiotic, amino acid, organic acid, or an enzyme. Improvements in production are generally obtained through strain improvement geared to higher product yield or more efficient substrate utilization (Demain, 1971a and 1971b; Solomons, 1977). However, production could be improved by other means such as diversification of the products recovered from one culture. Nyiri (1977) states that microbial cells have the capability of producing more than one product of commercial value. For example, Saccharomyces cerevisiae cultured under certain environmental conditions is capable of simultaneously producing ethanol, L-lysine, flavour enhancing nucleotides, and edible protein. Very few other reports on multiple product fermentations have been published leaving this potentially rewarding area wide open to further investigation.

To pursue the study of multiple product fermentations, the biosynthesis of inulase and lactase in Kluyveromyces fragilis was investigated.

Inulase has potential commercial application in the production of high-fructose syrups from Jerusalem artichoke. The tubers of this plant contain large amounts of storage carbohydrates which can be hydrolysed enzymatically or with acid to yield a high proportion of fructose. Fleming and GrootWassink (1979), in a recent review of this topic, and Byun and Nahm (1978) concluded that enzymatic hydrolysis is preferred because of reduced purification procedures and a more palatable product. However, commercial application of an enzymatic process would only be feasible if large quantities of enzyme were available at reasonable prices. GrootWassink and Fleming (1980) have further suggested that certain characteristics of K. fragilis inulase make it a more versatile enzyme for industrial application than invertase. Both enzymes catalyze the hydrolysis of the same glycosidic bond in saccharides but have different substrate specificities. Invertase is currently used in production of soft-centered candies and jams that are based on sucrose (Wiseman, 1975) and in the formulation of certain bread doughs (Negoro and Kito, 1973b).

Lactase has many current and potential applications to the nutritional, technical, and pollution problems created by the presence of lactose in the food products and effluents of the dairy industry (for a review see Shukla, 1975). Although there are several commercial producers of lactase (Shukla, 1975), the enzyme preparations are still relatively expensive and the full range of possible applications is not yet realisable.

The purpose of the present study was to optimize simultaneous inulase and lactase production by K. fragilis. The effects on enzyme yields of a variety of carbon substrates and substrate combinations

were investigated using batch and continuous culture techniques. In addition, certain non-metabolizable galactose analogs known to induce lactase in the bacterium Escherichia coli were tested for enhancement of lactase production in K. fragilis. Certain inferences as to the regulation of each enzyme were made at various points during the study.

2. LITERATURE REVIEW

2.1 Inulase

2.1.1 Sources of Inulase

Inulases have been isolated from a variety of sources (Fleming and GrootWassink, 1979). Although the enzymes derived from plants (Jerusalem artichoke, chicory, and dandelion) and filamentous fungi have had some attention, the most common origin of inulase for biochemical studies has been yeasts.

Three inulase producing yeasts have been reclassified since publication of the earlier literature: Saccharomyces marxianus, Sacch. lactis, and Sacch. fragilis were transferred by Van der Walt (1965) to the emended genus Kluyveromyces. In this review, these yeasts are always referred to as Kluyveromyces. If the obsolete name was used in a cited publication, the new genus name will be followed by Sacch. in parentheses (e.g. K. (Sacch.) fragilis).

The ability of K. (Sacch.) marxianus and other unspecified yeasts to actively ferment inulin was first documented by Lindner (1900). Much later, K. (Sacch.) fragilis (Sachetti, 1933) and several baker's and brewer's yeasts (Adams et al., 1943) were accredited with this same ability. More recently, Lodder (1970), in an impressive treatise on yeast, identified 25 species capable of inulin fermentation plus another 50 species capable only of inulin assimilation. In many of

these species, inulin utilization is very slow and is limited to a small number of strains. The inulase from three separate inulin fermenting yeast species has been examined, but only K. fragilis regularly serves as the enzyme source for biochemical or applied studies. Different investigators have independently concluded that this species produces the highest levels of inulase (Kovaleva and Yurkevich, 1973; Negoro, 1978; GrootWassink and Fleming, 1980).

2.1.2 Specifity of Inulase

Inulases are named for their ability to hydrolyse inulin, a linear polymer of fructose units all linked by β-2,1-fructofuranosidic bonds and terminated by a single glucose residue. The glucosyl moiety is also linked with its adjacent fructose by a β-2,1-fructofuranosidic bond. Inulin polymers are about 35 units long, but a whole series of similarly constructed fructans of 2 (sucrose) to 35 units occurs naturally in plants.

Several enzymes capable of hydrolysing β-2,1-fructofuranosidic bonds have been described (for a review see Avigad and Bauer, 1966). True inulases, β-2,1-fructan fructanohydrolases [EC 3.2.1.7], are active only on oligo- and polyfructans of the inulin series and have decreasing activity towards increasingly shorter chains and virtually no activity on sucrose. Two such enzymes have been isolated from Jerusalem artichoke. A closely related class of glycolytic enzymes are the invertases or β-D-fructofuranoside fructohydrolases [EC 3.2.1.26]. Sucrose is the primary substrate and as chain length increases activity decreases drastically so that inulin or oligofructans of the inulin series are resistant to hydrolysis. A third type of enzyme does not

fit either classification because of its high activity on both inulin and sucrose plus activity on β-2,6-fructofuranosidic linkages in levans. These non-specific enzymes, also called inulases [EC 3.2.1.7], are the type elaborated by certain yeasts including K. fragilis.

The ratio of hydrolysis rates of sucrose to inulin (S/I ratio) can be used to determine whether a yeast produces an invertase or an inulase. This ratio will be low for inulase producers because the rate of inulin hydrolysis approaches that of sucrose. From Table 1 it can be seen that strains of K. fragilis, K. lactis, and C. kefyr produce inulases. In addition to varying among yeast species, the S/I ratio is affected by the assay conditions and the purity of the inulin used as the enzyme substrate.

Classification of the non-specific K. fragilis enzyme as an inulase probably results from early studies which used crude enzyme preparations. In these studies, inulin and sucrose hydrolysis were attributed to a true inulase and a contaminating invertase, respectively (Adams et al., 1943). In this case, even small amounts of contaminating invertase would increase the S/I ratio of crude enzyme preparations and as the inulase was purified the ratio would drop. However, during purification of K. fragilis inulase the S/I ratio remains constant indicating that no invertase is produced (Snyder and Phaff, 1960; Negoro and Kito, 1973a; Nahm and Byun, 1977; Negoro, 1978; GrootWassink and Fleming, 1980). Furthermore, Kovaleva and Yurkevich (1973) demonstrated that a single active site of inulase participates in hydrolysis of sucrose and oligofructans. If all strains of K. (Sacch.) fragilis produce only an inulase, then some reports which refer to the sucrose hydrolysing enzyme as an invertase

TABLE 1. S/I ratios for inulases and invertases obtained from various yeasts

Yeast source	S/I ratio	Reference
Saccharomyces cerevisiae	14,000	Snyder and Phaff (1960)
Kluyveromyces (*Sacch.*) *fragilis* 351	25	
Sacch. cerevisiae	16,666	Negoro and Kito (1973b)
Candida utilis	11,111	
C. kefyr 0616	41.6	
Sacch. cerevisiae	180	Kovaleva and Yurkevich (1973)
Sacch. paradoxus	520	
K. fragilis	6.0	
K. lactis	3.5	
K. fragilis 351	3.5	Nahm and Byun (1977)
K. fragilis 0541	28	Negoro (1978)
Sacch. cerevisiae	1,600	GrootWassink and Fleming (1980)
K. fragilis ATCC 12424	10.5	

(R. Davies, 1953; Davies, 1956a; R. Davies and Elvin, 1964; Kidby and Davies, 1970a and 1970b) may, in retrospect, be considered as studies on inulase.

Inulase attacks inulin polymers at the fructose terminal and removes single fructose residues until the last bond is broken to yield one D-glucose molecule (Snyder and Phaff, 1962; Nahm and Byun, 1977). A more random multichain attack results at a pH of less than 3.0 (Snyder and Phaff, 1962). Although principally a hydrolytic enzyme, some transferase activity with sucrose as the substrate has also been attributed to K. fragilis inulase (GrootWassink and Fleming, 1980).

For K. fragilis inulase, the reported pH optima for sucrose hydrolysis are generally lower than those for inulin hydrolysis. The values reported for sucrose and inulin hydrolysis respectively are: 4.2 and 5.1 (Snyder and Phaff, 1960), 3.3 and 4.1 (Kovaleva and Yurkevich, 1973), 3.0 and 5.5 (Nahm and Byun, 1977), and 4.5 and 4.5 (Negoro, 1978). These authors, plus GrootWassink and Fleming (1980), agreed that the enzyme activity on both substrates is stable to at least 55^{o} C.

2.1.3 Regulation of Inulase Biosynthesis

Microbial metabolic regulation has been reviewed by Vogel (1971) and Copeland and Marzluf (1977). Clarke (1971) has reviewed the techniques used to study microbial regulation and Demain (1971a and 1971b) has discussed the approaches available to cause overproduction of enzymes by microbes. The vast majority of research in this area has concentrated on bacteria (Escherichia coli in particular) so that several bacterial regulatory systems are well defined at the molecular

level. Although many techniques and perhaps general regulatory phenomena are also applicable to simple eucaryotes like yeast, it cannot be assumed that the molecular mechanics of regulation are identical. A general survey of the more limited knowledge of enzyme regulation in yeast has been collected by Rose and Harrison (1971).

Yeast inulases are glycoproteins that remain associated with the cell or are secreted into the medium. Characterization of the cellular and extracellular enzymes has revealed a high degree of similarity suggesting that they may be identical (Snyder and Phaff, 1980; Nahm and Byun, 1977; Negoro, 1978). The cellular inulase of K. (Sacch.) fragilis is primarily associated with the cell wall and is rapidly released during protoplast formation (Davies and Elvin, 1964). The cellular inulase is not physically bonded to the cell wall, but is prevented from solubilizing by the intact cell wall which serves as a permeability barrier (Kidby and Davies, 1970b).

The extracellular location of inulase is significant if the utilization of sugars by yeast, as reviewed by Barnett (1976), is considered. Since sugars cannot penetrate the yeast plasma membrane, they must be carried across by specific transport mechanisms. Constitutive or inducible carriers exist for monosaccharides and some disaccharides but no such mechanism exists for sucrose or inulin. Instead, these substrates must be hydrolysed extracellularly to monosaccharides which can be transported. Sucrose does not occur inside K. (Sacch.) fragilis (De La Fuente and Sols (1962) or Sacch. cerevisiae cells (Sutton and Lampen, 1962).

The amount of inulase produced by K. fragilis is significantly influenced by the nature and concentration of the carbon source in the

medium. Inulin, although not essential for inulase production, supports the highest enzyme yields in aerated batch culture (Snyder and Phaff, 1960; Negoro and Kito, 1973a). K. fragilis ATCC 12424 grown on inulin in batch culture grows most rapidly at a temperature of 33 to 35° C, but maximum inulase accumulation occurs at 26 to 30° C (GrootWassink and Fleming, 1980). Total accumulated inulase is much less in cultures grown above 33° C. R. Davies (1953) and A. Davies (1956a) demonstrated that certain sugars (glucose, fructose, and sucrose) are normally very repressive but effectively stimulate enzyme production if present in low concentrations. Raffinose was less repressive at normal concentrations. Continuous culture was used in the 1956 study to show that glucose is repressive at levels over 0.001% (w/v) and that galactose and lactose were repressive at all concentrations. It was also demonstrated that ammonium ion concentration, growth factors, or pH (up to 6.0) can vary relative to the carbon substrate concentration, with little effect on the inulase yields. GrootWassink and Fleming (1980) also used continuous culture and found that K. fragilis can be grown on sucrose with double the enzyme yields achieved in batch culture on inulin. These authors also demonstrated that variation of pH from 3.5 to 6.0 had no effect on the amount of enzyme produced and that the critical oxygen tension for enzyme formation is less than 2.5%.

Repression of enzyme synthesis by readily metabolized carbon substrates, such as glucose, is not a trait peculiar to inulase regulation but is a general regulatory phenomenon termed carbon catabolite repression (Magasanik, 1961). How the external sugar concentration affects production of yeast enzymes is not clear, but it

may involve a process similar to that reported for various procaryotes (Magasanik, 1970). The external sugar concentration may affect the level of an intracellular molecule such as cyclic-3',5'-adenosine monophosphate (cAMP) which has the ability to interact with various proteins and DNA to promote the synthesis of repressible enzymes. In effect, low sugar concentrations might cause high cAMP levels and therefore promote enzyme biosynthesis.

The production of inulase in K. fragilis possibly mimics that of the extensively studied enzyme, invertase, in Sacch. cerevisiae. Invertase shares several traits with inulase: both are glycoproteins that occur extracellularly and in association with the yeast cell wall, they hydrolyse the same glycosidic bond, and both are catabolite repressible (for invertase reviews see Lampen, 1968 and 1971; Hackel, 1977). With respect to invertase regulation, Gascon and Ottolenghi (1972), using continuous culture, demonstrated an inverse relationship between glucose concentration and enzyme yield. In addition, Dodyk and Rothstein (1964) found that various intermediates of glucose metabolism (acetaldehyde, ethanol, acetate, fumarate, succinate, and malate) are also repressive.

Some information concerning the mechanism of glucose catabolite repression of yeast invertase, that is perhaps applicable to inulase, has accumulated. Glucose interferes with transcription and translation of nucleic acids coding for invertase and also increases the rate of mRNA degradation, but it has no effect on enzyme secretion and does not interfere with the catalytic activity of the enzyme (Elorza et al, 1977). Some workers have found an inverse relationship between glucose and cAMP levels suggesting that this nucleotide may be involved

(Sy and Richter, 1972; Schlanderer and Dellweg, 1974; Wheeler et al, 1974). However, Montenecourt et al (1973) found no correlation between cAMP levels and the sensitivity of invertase to catabolite repression. Fang and Butow (1970) suggested possible involvement of other nucleotides. Haussmann and Zimmermann (1976) postulated that the mitochondria produce a factor which is translated in the cytoplasm and then causes catabolite repression. As a result, mutants without functional mitochondria produce invertase at derepressed levels. In fact, mutants resistant to catabolite repression have been isolated from several invertase producing yeasts (Montenecourt et al, 1973; Toda, 1976a; Zimmerman and Scheel, 1977; Hackel and Kahn, 1978). These organisms produce high levels of enzyme even in the presence of ordinarily repressive substrate concentrations.

Since invertase yields can be dramatically increased in wild type yeasts by reducing the concentration of repressive substrates, and because no invertase inducer has actually been identified, Hackel (1975) has stated that invertase, particularly in Sacch. cerevisiae, is a constitutive enzyme regulated solely by catabolite repression. However, according to other workers, the possibility of induction cannot be eliminated. For example, Zimmermann and Scheel (1977) found no catabolite repression resistant mutants that were not simultaneously derepressed for invertase and maltase synthesis thus demonstrating a link between the two enzyme systems. Maltase is under dual control by induction and catabolite repression - maltase activity can be induced to higher levels in a derepressed yeast culture by addition of maltose or a maltose analog (Van Wijk et al, 1969; Zimmermann and Eaton, 1974). Citing maltase regulation as a model,

Zimmermann and Scheel (1977) suggested that carbon catabolite repression may not be the only regulatory mechanism controlling invertase synthesis. Furthermore, Toda (1976b) has suggested that induction, possibly by a fructose-related compound common to both sucrose and glucose metabolism, may be involved in invertase regulation in Sacch. carlsbergensis.

Despite similarities between the regulation of invertase in Sacch. cerevisiae and inulase in K. fragilis, caution must be exercised in extrapolation of information from one system to the other. There are significant differences between invertase and inulase concerning substrate affinities, the proportion of enzyme associated with the cell wall, and the metabolic processes of the yeast species. This latter point is well illustrated by considering a biochemical feature that could result in significant differences in the mechanism of catabolite repression. Sacch. cerevisiae and K. fragilis differ with respect to regulation of mitochondrial function: Sacch. cerevisiae exhibits a strong Crabtree effect while K. fragilis does not (De Deken, 1966). In yeast demonstrating the Crabtree effect, rapid growth on glucose causes repression of mitochondrial biogenesis which results in repression of respiration. When feeding on glucose the response in predictable; respiration is repressed while the available glucose is metabolized via aerobic fermentation to ethanol. Once the glucose is exhausted, respiration is derepressed and the ethanol is further oxidized through the respiratory chain. Diauxic growth is often observed. If the mitochondria are involved in regulation of sugar-catabolizing enzymes, as suggested by Haussman and Zimmermann (1976), Puglisi and Algeri (1971); Furst and Michels (1977), and Ferrero et al (1978), then

it is possible that yeasts which have no Crabtree effect might differ from those which do in their mechanism of catabolite repression.

In summary, compared to some other yeast enzymes, the current knowledge of inulase regulation in K. fragilis is brief. Firstly, carbon catabolite repression is involved although enzyme yields are dependent upon the nature of the carbon substrate as well as the concentration. The mechanism of catabolite repression in yeast is undetermined. Secondly, in batch cultures, inulin stimulates higher enzyme yields than do simple sugars. However, induction has been neither proven nor eliminated as a possibility.

2.2 Lactase

2.2.1 Sources of Lactase

The variety of plant, animal, and microbial sources of lactase have been reviewed by Wallenfels and Weil (1972) and Shukla (1975). Microbes offer the highest enzyme yields and Aspergillus niger, Escherichia coli, and Kluyveromyces lactis are currently used for commercial lactase production. Each enzyme preparation possesses unique characteristics that make it suited to particular applications. The low pH optimum of the fungal enzyme makes it suitable for lactose hydrolysis in acid whey, while the bacterial enzyme has a higher optimum temperature that facilitates lactose hydrolysis when immobilized in a bioreactor. Because the yeast is an acceptable food additive, the enzyme for this source is preferred for use in neutral milk products intended for human consumption (Peppler, 1978).

Sixty-two strains of molds, bacteria, and yeasts were screened for lactase producing ability by Ramana Rao and Dutta (1978). Molds had the poorest enzyme yields while three bacterial strains and

K. fragilis were considered the best producers. Mahoney et al (1974) screened 41 strains of K. fragilis and found a 60-fold variance in their ability to produce lactase. Candida pseudotropicalis, also a yeast, has been identified as another good source of lactase (Shukla, 1975). (Lodder (1970) identified only three species that appear to have both good inulin and lactose fermenting ability: K. fragilis, C. pseudotropicalis, and C. kefyr.)

2.2.2 Specificity of Lactase

The purification and characterization of lactases have been reviewed by Wallenfels and Weil (1972) and Shukla (1975). Lactase and β-galactosidase are trivial names for β-D-galactoside galactohydrolases [EC 3.2.1.23], a group of enzymes that hydrolyse the disaccharide lactose (4-(β-D-galactopyranosyl)-D- glucopyranose) into its component monomers galactose and glucose. Lactases are also capable of transgalactosylation reactions in which a molecule other than water is the receptor of the galactosyl moiety. In a special case using E. coli lactase, there is an intramolecular galactosyl transfer from the C-4 to the C-6 position of the glucosyl residue to produce a disaccharide called allolactose (Jobe and Bourgeois, 1972). Transfer products have been reported for K. (Sacch.) fragilis lactase by Aronson (1952) and Pazur et al (1958).

Lactases are very specific for the galactosyl moiety and the β-configuration at the anomeric carbon but the glucosyl portion of lactose can be replaced by another sugar or an aglycan moiety. Some substitutions, such as replacement of the glycosidic oxygen with sulphur to produce a thiogalactoside, result in resistance to enzymatic

hydrolysis. However, the affinity for some substrates, ortho-nitrophenylgalactopyranoside (ONPG) for instance, is greater than for lactose (Uwajima et al, 1972; Mahoney and Whitaker, 1977). The hydrolysis of ONPG quantitatively liberates the chromogen ONP and provides a means for a rapid spectrophotometric assay of β-galactosidase activity.

K. fragilis lactase is activated by K^+ and Mg^{++} and appears to require Mn^{++} as a cofactor (Caputto et al, 1948; Bierman and Glantz, 1968; Wendorff and Amundson, 1971; Uwajima et al, 1972; Mahoney and Whitaker, 1977). Wendorff and Amundson (1971) reported competitive inhibition of enzyme activity by heavy metals, cysteine, and galactose, while glucose and some amines were non-competitive inhibitors. Mahoney and Whitaker (1977) found that D-galactono-1,4-lactone is also a good inhibitor. The K. fragilis enzyme displays optimum activity between 30 and 35° C and in the pH range of 6.7 and 7.1. In addition, it is stable at less than 40° C and between the pH values of 6.0 and 7.5 (Uwajima et al, 1972; Kulikova et al, 1972).

2.2.3 Regulation of Lactase Biosynthesis

The lactase of K. fragilis is strictly intracellular and has access to lactose only after this disaccharide has been transported across the plasma membrane by a stereospecific permease (Barnett, 1976). The lactase and the β-galactoside permease are both inducible (De la Fuente and Sols, 1962) and an absence of either enzyme results in an inability to assimilate lactose (Fiol, 1972). Lactose fermenting ability is inducible by lactose and galactose (R. Davies, 1964). These two sugars were the best inducers of

15 compounds, including mono-, di-, and trisaccharides plus substituted galactopyranosides, that were tested by Tikhomirova *et al* (1972).

Dickson and Markin (1978 and 1980) have further studied lactase induction in *K. lactis*. They found that lactase activity began to increase within 10 to 15 minutes after inducer addition and continued to increase linearly until a maximum lactase level 125 to 150 times the basal activity was reached. Maximum enzyme production was maintained as long as the culture remained in log growth but dropped to 5 to 10 times basal levels if stationary phase occurred. Apparently, lactase induction required *de novo* enzyme synthesis, a constant energy supply, and constant inducer presence. Maximum rates of induction were found to result with lactose concentrations greater than 1 to 2 mM. The presence of glucose caused transient catabolite repression, did not prevent lactose from entering the cells, and did not prevent lactose utilization.

Although lactose and galactose seem to be the best inducers of lactase activity in yeast, these sugars may not be the actual inducing compounds. In *E. coli*, lactase and β-galactoside permease biosynthesis are regulated by a block of regulatory and structural genes called the lac operon (for a review see Barkley and Bourgeois, 1977). Neither lactose nor galactose interact with the lac operon; rather, during growth on lactose, allolactose is formed and acts as the inducer (Jobe and Bourgeois, 1972). Allolactose is also formed by *K. lactis* lactase but no regulatory function has been demonstrated and the natural inducer in yeast remains unknown (Dickson *et al*, 1979).

Gratuitous inducers are non-metabolizable compounds that induce specific enzyme biosynthesis but are not substrates for the induced

enzymes (Clarke, 1971). These compounds usually strongly resemble the natural inducer. Isopropylthiogalactoside (IPTG) and methylthiogalactoside (TMG) are resistant to hydrolysis by β-galactosidase but stimulate higher enzyme yields in E. coli than does lactose (Jacob and Monod, 1961). IPTG, a very poor inducer of lactase in K. lactis (Dickson and Markin, 1978 and 1980), causes some induction in K. fragilis but is still inferior to lactose (Mahoney et al, 1974) and galactose (Algeri et al, 1978). Szabo and Davies (1964) demonstrated that TMG can be transported across the K. (Sacch.) fragilis plasma membrane but is not fermented. The effectiveness of this compound as a gratuitous inducer was not reported. Tingle and Halvorson (1972) claimed that TMG is as effective as lactose in inducing lactase in K. (Sacch.) lactis. However, more recently Dickson and Markin (1980) showed that TMG uptake by K. lactis does not result in lactase induction.

Lactose fermenting ability in K. fragilis is also subject to carbon catabolite repression. A. Davies (1956b) used continuous culture to demonstrate that K. (Sacch.) fragilis lactase levels are repressed by sugar concentrations greater than 0.001% (w/v). However, the magnitude and range of repression depends upon the nature of the sugar. Even lactose and galactose, which cause enzyme levels ten times higher than other sugars under derepressed conditions, reduce lactase production at concentrations greater than 0.001% (w/v).

Dual control by induction and catabolite repression also regulates maltose (as previously mentioned) and galactose utilization and β-glucosidase production in yeasts. The galactose system is genetically well defined and a model for regulation of enzyme activity

has been proposed (Douglas and Hawthorne, 1966). Galactose in the medium causes the induction of a galactose transport system and several enzymes which channel galactose into the glycolytic pathway (Mortimer and Hawthorn, 1971; Matern and Holzer, 1977). Glucose represses biosynthesis of the galactose system (Adams, 1972) even in the presence of appropriate inducers. Addition of glucose to cells growing on galactose also causes inhibition of the galactose uptake system which accounts for the immediate repression of galactose fermentation (Matern and Holzer, 1977). β-glucosidase biosynthesis by a hybrid yeast (MacQuillan et al, 1960) and by K. lactis (Herman and Halvorson, 1963) is induced by 1 mM glucose but repressed by higher concentrations. This is an interesting system since glucose acts as both inducer and repressor.

2.3 Continuous Culture

The basic theory of continuous culture was first forwarded by Monod (1950). Additional models have been proposed to account for deviations from this theory during application, but the basic model still provides a satisfactory basis for most studies. A rigorous treatise on the theoretical and methodological basis of continuous culture was published by Malek and Fencl (1966), but more recent articles by Tempest (1970) and Wang et al (1979) provide sufficient information for a basic understanding of the technique. Evans et al (1970) describe practical considerations while Harrison (1972) and Dawson (1977) have reviewed developments and applications of the technique.

2.3.1 Basic Theory of Continuous Culture

In batch culture, growth and product formation are processes that always terminate after a finite period of time. Growth, as measured by increasing cell mass, can be divided into three distinct phases: lag phase, growth phase, and stationary phase. During the lag phase there is very little growth because the inoculum has to adapt to the new culture conditions. Once adapted, the cells enter the growth phase which is usually characterized by an exponential (log) increase in cell mass. The specific growth rate of the culture can be calculated from:

$$\frac{dX}{dt} = \mu X$$

where X is the cell concentration in mg/ml, t is time in hours (h), and μ is equal to the maximum specific growth rate (μmax), a value that is determined by genetic and environmental factors. Eventually, due to substrate depletion or accumulation of inhibitory products, μ diminishes until net growth is zero. Thus the stationary phase begins when all cells stop dividing or the growth of viable cells is off-set by an equal death rate. The stationary phase may show a decline in biomass due to cell lysis.

During batch culture, the environment is always changing so that the effect of individual environmental parameters is difficult to evaluate. A steady state in which cell concentration, specific growth rate, and culture environment do not change with time can be achieved by using continuous culture. The chemostat, so named for the constant chemical environment resulting at steady state, is the most common apparatus used for continuous culture. Simply, it consists of a culture vessel to which fresh sterile medium can be continuously supplied while an equal volume of culture is simultaneously removed.

The medium is prepared so that all but one nutrient required for growth are present in excess. Additional environmental control is possible by inclusion of devices to regulate pH, temperature, foaming, and the dissolved oxygen tension.

The specific growth rate in a chemostat is determined by the rate of medium addition:

$$\mu = \frac{F}{V}$$

where F is the medium flow rate (liters/h) and V is the culture volume. This ratio, called the dilution rate (D), is equal to μ under steady state conditions.

The density of a steady state cell population is determined by the concentration of the growth limiting nutrient in the medium reservoir. The concentration of the limiting nutrient in the culture is independent of the reservoir concentration but depends upon the specific growth (dilution) rate as follows:

$$\mu = \mu max \frac{S}{K_s + S}$$

where S is the culture concentration of the limiting nutrient and K_s is the half-rate saturation constant that equals S when μ equals 0.5 μmax. At low dilution rate steady states, S is very low because the limiting nutrient is utilized rapidly after addition. As μ(D) is increased, S also increases until μ equals μmax. If D is further increased, the cells will be incapable of utilizing all of the limiting nutrient before they are washed out of the culture vessel along with the unused medium. Eventually the entire culture will be washed out of the vessel. At μmax, D equals the critical dilution rate, D_c. The

critical dilution rate, which usually corresponds to the maximum growth rate in batch culture, represents the maximum dilution rate at which the chemostat can be operated.

Continuous culture theory predicts that the biomass concentration in carbon limited culture will be the same at all values of D less than D_c. In practice, the cell concentration drops at low dilution rates. This normally occurs because the carbon source provides energy for growth and maintenance of essential processes and structural components. At lower growth rates, a larger proportion of the available energy is required for maintenance and, as a consequence, the cell mass decreases.

2.3.2 Enzyme Production in Continuous Culture

"Physiological state" refers to the total metabolic activity and physiological make-up of a microbial population at a given time (Malek, 1976). Culture history and current environmental conditions working within the genetic framework of an organism determine this state. Since microorganisms posess a tremendous capacity to adapt to alterations in their environment, the physiological state must be always changing during adaptation. Growth limiting conditions in the chemostat force cells to modify their behavior to ensure balanced growth under suboptimal conditions. To this end, modulation of the rates of enzyme synthesis is of key importance (Tempest and Neijssel, 1976).

Enzyme production in continuous culture is governed by the nature of the growth limiting substance and the dilution rate (for a review

see Dean, 1972). Steady state enzyme levels, as a function of dilution rate, will usually fit one of the following four patterns:

(1) Steady state enzyme activity (units of enzyme per unit of biomass) is the same at all dilution rates. Constitutive enzymes that are not catabolite repressible will fit into this category.

(2) Enzyme activity increases with increasing dilution rates. As growth rate increases, there may be a greater demand for these enzymes. The increased enzyme levels may result from derepression or induction of enzyme biosynthesis.

(3) Enzyme activity is reduced as the dilution rate increases. This response is attributed to increased catabolite repression due to increased concentrations of the growth limiting nutrient in the culture at higher dilution rates.

(4) Enzyme activity passes through a maximum value at a given dilution rate. Clarke et al (1968), in studies on amidase production by Pseudomonas aeruginosa, have proposed that the peak in enzyme synthesis is due to a balance between induction and catabolite repression. Toda (1976b) states that a similar regulatory trade-off is also responsible for the peak in invertase production by Sacch. carlsbergensis.

More complex patterns of enzyme dependence upon dilution rate also occur and require additional investigation to determine the nature of the regulation (Dean, 1972).

2.3.3 Mixed Substrate Utilization

Microorganisms presented in batch culture with a mixture of two utilizable carbon sources will normally use only one source at a time. A lag period will result if the culture must adapt to the second substrate. The resulting tiered growth pattern is termed "diauxic". Carbon catabolite repression by the preferred compound is responsible for preventing utilization of the second substrate.

In carbon limited continuous culture, the microbial response to two carbon substrates is considerably different (for reviews see Dean, 1972; Harder and Dijkhuizen, 1976). At low dilution rates total carbon substrate concentration is very low, resulting in reduced catabolite repression and simultaneous utilization of both substrates. However, at higher dilution rates the concentration of the preferred carbon source may be sufficiently high to impair utilization of the second substrate.

The presence of two carbon sources, or of a single carbon source plus an inducer, in a culture at a low dilution rate causes biosynthesis of enzyme systems in response to each compound. Sikyta and Fencl (1976) demonstrated that *E. coli* K 12 in carbon limited culture containing lactose and tryptophan produces good levels of β-galactosidase and tryptophanase. Sikyta and Fencl (1976) and Pavlasova *et al* (1980) also examined a β-galactosidase hyperproducing mutant of *E. coli* K 12 in lactose limited continuous cultures. Short term feeding of tryptophan to induce tryptophanase partially repressed the β-galactosidase. However, addition of the gene regulator cAMP allowed for maximum production of both enzymes (Pavlasova *et al*, 1980).

Therefore, it seems that simultaneous induction may place an excessive drain on endogenous cAMP pools.

2.3.4 Selection in Continuous Culture

Selection in a continuous culture is discussed by Jannasch and Mateles (1974) and Wang et al (1979). If two organisms compete for the same growth-limiting nutrient in a chemostat, the organism capable of the higher growth rate at a given substrate concentration will outgrow and displace its competitor. Displacement results from the indiscriminate removal of cells by dilution. Only after elimination of the slower growing organism can the resulting pure culture attain a steady state.

Culture conditions can be designed to isolate and propagate a single, desired phenotype from a mixed population. Alternatively, a pure culture can be displaced by faster growing contaminants or mutants. Contaminants enter the culture from outside if less than sterile conditions exist, but mutants arise from within the population. Heineken and O'Connor (1972) reported that spontaneous mutation of Bacillus subtilis led to cultures that produced much lower levels of alkaline protease, neutral protease, and α-amylase. Conversely, mutants may be selected that are constitutive or hyperproducers of certain enzymes (Horiuchi et al, 1962; Hegeman, 1966; Hope and Dean, 1974). A mixture of two substrates can lead to mutants that will constitutively use both substrates simultaneously (Silver and Mateles, 1969).

3. MATERIALS AND METHODS

3.1 Cultivation Methods

3.1.1 Preservation of Stock Culture

The yeast Kluyveromyces fragilis ATCC 12424 was used throughout this study. A lyophilized stock culture was maintained in silica gel and stored at 4° C in a tightly capped vial.

3.1.2 Agar Slant Cultures

To prepare agar for the slants, 20 gm glucose, 5 gm Difco yeast extract, and 20 gm Difco Bacto-agar were dissolved in 1 liter of distilled water and the mixture was brought to a boil. Aliquots of 10 ml each were dispensed into 8 dram screw-cap vials and autoclaved at 121° C for 15 minutes; subsequently, the agar was allowed to solidify with the vials positioned at an angle. Agar slants were stored at 4° C until used.

Slants were inoculated by streaking their surface with a loopful of silica gel stock culture. Small individual colonies formed after 48 hours incubation at 28° C. By spreading these colonies across the agar surface and incubating for an additional 24 hours, confluent growth was obtained. Cultures were stored at 4° C.

The cultures from these slants were used to inoculate liquid media. Since the viability of the slant cultures was reduced upon

extended storage, cultures older than three weeks were not used as inoculum.

3.1.3 Agar Plates

Agar for pour plates was prepared using 5 gm inulin (dahlia tuber, Sigma Chemical Co.), 2.5 gm Difco Yeast Nitrogen Base, and 10 gm Difco Bacto-agar in 500 ml distilled water. The agar was dissolved by bringing this mixture to a boil and the solution was autoclaved for 20 minutes. While still hot, the medium was poured into sterile petri dishes and allowed to solidify. Agar plates were stored at 4^{o} C.

3.1.4 Liquid Growth Media

The liquid growth media used in this study can be divided into two categories depending upon the nitrogen source. "Complex" media were prepared with yeast extract which contains organic nitrogen as a complex mixture of amino acids. "Defined" media contained Yeast Nitrogen Base (Difco) which provides nitrogen in the form of ammonium sulfate. Both nitrogen sources also contain the essential vitamins required for yeast growth.

The concentrations of carbon and nitrogen sources in all media are expressed on a percent weight by volume basis.

(1) Media containing yeast extract:

Carbon source	1.0%
Yeast extract	0.5%

Yeast extract was of technical grade (Anheuser-Busch) while all carbon sources were of reagent grade or better with the exception of the sucrose (Alberta Sugar Co., technical grade) used in media for the 3 liter continuous cultures. Carbon sources included: inulin (dahlia

tuber, Sigma Chemical Co.), sucrose, fructose, glucose, galactose, lactose, mannose, glycerol, ethanol, and succinic acid. All carbon sources were added to media prior to autoclaving except for ethanol which was aseptically added afterwards.

Distilled water was used in the preparation of all media for shake flask cultures and for the 225 ml continuous cultures. Tap water was used for all cultures in the 7.5 liter fermentors.

(2) Media containing yeast nitrogen base (YNB):

Carbon source	0.5 or 0.6%
YNB (Difco)	0.25 or 0.3%, respectively
KH_2PO_4	13.6 gm/l (0.1 M)

All carbon sources were of reagent grade or better and all media were prepared with distilled water. The carbon source and KH_2PO_4 were autoclaved together. Prior to autoclaving the carbon source solution, the pH was adjusted using NaOH. The pH varied according to the carbon source: succinate, 4.0; inulin and galactose, 5.0; glycerol, 5.5; and fructose, 6.0. A 10% YNB (w/v) solution was filter sterilized using cellulose acetate membrane filters with 0.22 µm diameter pores (Millipore). The required volume of YNB solution was aseptically added to the autoclaved mixture.

3.1.4.1 Media Sterilization. Most media to be used in 3 liter continuous cultures were prepared in a medium sterilizer (120 liter, Nutrient Vessel, New Brunswick Scientific). The temperature of a medium was raised to 118 to 121° C and was maintained for 40 minutes. All other media were sterilized in an autoclave at 121° C. Holding times varied from 15 minutes for flasks containing small volumes to 60 minutes for 54 liter carboys containing up to 32 liters of medium.

3.1.5 Culture Inoculation

Inoculum for liquid growth media was prepared by suspending the cells of an agar slant culture in 10 ml of sterile distilled water. The volumetric ratio of inoculum to growth medium was 1:100.

To ensure uniformity of inoculum within a series of shake flask cultures, the total volume of medium was inoculated prior to being dispensed into individual flasks.

3.1.6 Batch Cultures

3.1.6.1 Shake Flask Cultures. These cultures were grown in 25 or 50 ml of medium contained in 250 or 500 ml Erlenmyer flasks, respectively. Inoculated flasks were placed on a rotary shaker at 280 rpm and 28° C. For analysis, whole cultures were harvested.

3.1.6.2 Fermentor Cultures. These cultures were grown in a 7.5 liter jar fermentor (New Brunswick Scientific, Microferm) containing 5 liters of medium. At the time of inoculation, the aeration rate was 0.5 lpm and the impeller speed was 300 rpm. A dissolved oxygen controller (New Brunswick Scientific, Model DO 81-14) was employed to maintain the dissolved oxygen tension (DOT) at 20% of saturation by automatic adjustment of impeller speed (300-500 rpm) and aeration rate (0.5-3.0 lpm). DOT was measured using a galvanic probe and was expressed as a percentage of dissolved oxygen saturation of the uninoculated medium. The DOT controller was activated only when vigorous culture growth had reduced the DOT to less than 20%.

Cultures were grown at 30° C and the pH was continuously monitored using a pH controller (New Brunswick Scientific).

Cultures were sampled by withdrawing 40-50 ml through a submerged sampling tube after the initial tube contents had been discarded.

3.1.6.3 Carbon Limitation in Batch Cultures. In order to check that the biomass yields in batch cultures were limited by the amount of carbon substrate, shake flask cultures were grown using a series of media containing 0.5% yeast extract and various concentrations (0.5-1.6%) of a carbon source (sucrose, lactose, inulin, glycerol, or ethanol). Cultures were harvested in the stationary phase. The biomass yields of duplicate samples were plotted against percent carbon source.

3.1.7 Continuous Cultures

3.1.7.1 3-Liter Continuous Cultures. To start an experiment, an assembled fermentor jar (7.5 liter) containing 2 liters of tap water was autoclaved and set into position on a fermentor (New Brunswick Scientific, Microferm). Next, sterilized medium (2 liters) from the nutrient vessel or a carboy was added to the fermentor using a peristaltic pump (Cole Parmer Instrument Co., Masterflex Model No. 7564-10). Batch cultivation using the same procedure for inoculation and monitoring as described before (Section 3.1.5.2) preceded the initiation of continuous cultivation. Growth in the batch culture was allowed to proceed until there was a rapid increase in the DOT indicating that the stationary phase had been reached. At this time, the air exit line was clamped and the culture overflow line was opened to reduce the culture volume to 3 liters, the pH controller was activated to maintain a pH of 4.2 by addition of 4N HCl, and the peristaltic pump was turned on to continuously meter fresh medium into

the culture vessel. The culture volume was kept constant by carefully positioning an overflow tube at the 3 liter mark. The overflow was collected and measured volumetrically in order to calculate the dilution rate. Samples were also obtained through this tube by allowing 50 to 60 ml of culture to run into a graduate cylinder packed in ice.

Sampling of a continuous culture was initiated after at least six replacement volumes (18 liters) had passed through the fermentor. To ensure that steady state had been reached, at least two samples were obtained at each dilution rate with at least one replacement volume (3 liters) passed between samples. Subsequent to sampling one steady state, the dilution rate was fixed at a new value and, after at least 3.5 replacement volumes (10.5 liters), samples were taken to test for a new steady state. Unless specifically stated otherwise, all continuous cultures were initiated at a low dilution rate and run through a series of successively higher rates.

During a fermentation, the temperature and DOT were held constant at 30^{o} C and 20%, respectively. An automatic foam controller (New Brunswick Scientific, Model PA-6 or Model AFP-101) eliminated severe foaming by addition of the antifoam agent polypropylene glycol P2000 (Matheson, Coleman, and Bell Manufacturing Chemists).

3.1.7.2 250 Milliliter Continuous Cultures. Continuous culture experiments that necessitated smaller total medium volumes were executed in a system assembled around a fermentor jar with a 350 ml maximum culture volume (Bellco Biological Glassware). Agitation was provided by a bar magnet rotated by a magnetic stirrer (Fisher Scientific Co., Model: Versamix 115). Air was supplied by an aquarium

pump (The Oscar Co., Model: Oscar's 55) and passed through a gas flow meter (Matheson Gas Products, Model No. 7631T), a sterile glass wool filter, and finally a submerged glass sintered disc sparger. The fermentor jar was placed in a shallow glass container with water that was kept at 30° C using a circulating water bath (Haake, Model No. T31). A peristaltic pump fitted with two channels (LKB-Produkter AB, Model No. LKB 4912A) was used for adding media. An open-ended 5 ml pipette was placed in one nutrient line between the medium carboy and the pump to facilitate flow rate determinations.

An overflow tube was positioned for a culture volume of approximately 225 ml. Samples of 25 to 30 ml were collected via the overflow line into a graduate cylinder packed in ice.

High biomass concentrations equivalent to those in the large fermentor could not be supported in this small fermentor presumably due to oxygen limitation. This limitation was overcome by reducing nutrient concentrations in the media to 0.2 or 0.25% carbon source and 0.1 or 0.125% nitrogen source.

To start an experiment using this system, the fermentor jar containing about 200 ml of distilled water was autoclaved along with the media carboys and tubing. After sterilization, all auxilliary equipment was attached and approximately 400 ml of medium was passed through the culture vessel. The batch culture phase was initiated by addition of 2 ml of inoculum. After overnight growth, fresh medium addition was initiated at an estimated dilution rate. Steady states and sampling criteria, in volumes, were the same as for the 3 liter cultures. Culture pH was uncontrolled and ranged from 3.5 to 5.0.

3.1.7.3 Carbon Limitation in Continuous Cultures. Media containing 0.5% yeast extract but with varied concentrations (0.2 to 1.6%) of a carbon source (sucrose or fructose) were sequentially fed at the same dilution rate to a 3 liter continuous culture. Two duplicate samples for biomass and inulase determination were obtained at the steady state established at each concentration of substrate.

3.2 Assay Procedures

3.2.1 Dry Weight

Culture samples were suction filtered through cellulose acetate membrane filters with 0.45 μm pore diameters. If needed for analysis, the filtrates were stored at -20° C. The yeast mats were washed twice with 10 to 20 ml of distilled water, placed with the filter membrane in a vial, frozen at -20° C, freeze dried, and weighed to 1 mg accuracy.

3.2.2 Total Reducing Sugars (TRS)

The neocuproine method of Dygert et al (1965) was used for TRS determination. Two solutions were separately prepared.

Solution A:	Na_2CO_3 (anhydrous)	40	gm/liter
	glycine	16	gm/liter
	$CuSO_4 \cdot 5\ H_2O$	0.45	gm/liter
Solution B:	neocuproine hydrochloride (Sigma Chemical Co.)	1.2	gm/liter

Distilled, deionized water was used in each solution. Solution B was stored in a brown bottle to protect it from light.

Just prior to testing for TRS, equal volumes of solutions A and B were thoroughly mixed. Aliquots of 10 ml were pipetted into large graduated test tubes. Solutions of samples to be tested were added (100 µl of a given sample). The tubes were then placed in a boiling water bath for 10 minutes, cooled in cold water to room temperature, filled to 25 ml with distilled water, and shaken. The absorbance at 450 nm was measured using a Beckman Model 35 spectrophotometer with a clinical sipper system. Standard solutions of fructose (20-60 µg/tube) were included in every TRS test.

3.2.3 Inulase Assay

Total inulase content (cell-bound plus soluble) of cultures was determined using duplicate dilutions of fresh culture samples in acetate buffer (0.1 M, pH 5.0). The samples were diluted 10-100 fold and stored at -20° C until analysed. Freeze-dried cells were used to assay cell-bound inulase, by suspending 15 or 25 mg in 100 ml of acetate buffer. Intact cells could be used in assays involving the substrate sucrose since the inulase associated with the cell wall is completely accessible to sucrose. However, if inulin was used the enzyme had to be solubilized for full expression of activity (GrootWassink and Fleming, 1980). To solubilize inulase, cells were disrupted as follows: 50 mg of freeze-dried cells were placed in a disruption bottle with 20 gm of glass beads (Glasperlen, B. Braun Melsungen; 0.45-0.50 mm) and 5 ml of acetate buffer. After one minute shaking in a Braun homogenizer (Bronwill Scientific, Type 2876), the mixture was suction filtered through Whatman No. 4 filter paper, washed, and made to 100 ml with acetate buffer.

Inulase was usually measured with sucrose as the substrate and its ability to hydrolyse inulin was occasionally verified. Substrate solutions were prepared by dissolving 4.4 gm of sucrose (Sigma Chemical Co., Grade 1) or inulin (dahlia tuber, Sigma Chemical Co.) in 100 ml of acetate buffer. To perform an assay, 0.9 ml of substrate solution was preincubated for two minutes at 50° C and 0.1 ml of cell suspension was added. Over a period of 20 minutes, at 5 minute intervals, 0.1 ml aliquots were withdrawn from the reaction mixture and added to 10 ml of TRS assay solution (Section 3.2.2). The high pH of this solution (>10) immediately inactivated the inulase. Samples were processed according to the TRS test. The resulting optical densities were plotted against time of incubation and the slope of the best straight line was calculated (ΔOD/min). Enzyme activity was expressed in terms of the rate of hydrolysis of either sucrose or inulin as determined by the increase in total reducing sugars. Volumetric enzyme activity on sucrose was calculated as follows:

$$\frac{\Delta\ OD}{minute} \times \frac{1}{m} \times \frac{1}{360\ \mu g} \times 100 \times D = I$$

where m is the slope of the standard curve in ΔOD per μg fructose, 360 μg represents the weight resulting from hydrolysis of 1 μmole of sucrose, 100 represents volume factors, D is the number of times the culture was diluted for the assay, and I is the enzyme activity expressed in μmoles sucrose hydrolysed per minute per ml of original culture (μmoles sucrose/min/ml). Dividing this volumetric activity by the dry weight of cells gave the specific activity (μmoles sucrose/min/mg). One unit of inulase activity was defined as that

amount of enzyme which hydrolyses 1.0 µmole sucrose per minute at 50° C and pH 5.0.

Inulase activity on inulin was calculated as follows:

$$\frac{\Delta \text{ OD}}{\text{minute}} \times \frac{1}{m} \times \frac{1}{180\ \mu g} \times 100 \times D = I$$

where 180 µg represents the weight in reducing sugars from inulin hydrolysis equivalent to hydrolysis of 1 µmole of sucrose. Here, I equals the µmoles of reducing sugars per minute per ml.

3.2.4 Lactase Assay

The assay of lactase activity was modeled on the method of Mahoney and Whitaker (1977) with ortho-nitrophenylgalactopyranoside (ONPG) as the enzyme substrate.

Cell extracts for enzyme assays were prepared by glass bead homogenization as follows: 25 mg of lyophilized cells were placed in a 50 ml disruption bottle along with 20 gm of glass beads and 5 ml of cold phosphate buffer (0.02 M, pH 7.0) containing $MnCl_2$ (5×10^{-5} M). The mixture was shaken for 1 minute in the Braun cell homogenizer and kept cold with periodic blasts of liquid carbon dioxide. Following disruption, the mixture was suction filtered throught Whatman No. 4 filter paper and the filtrate was centrifuged for 10 minutes at 4,000 x g.

For the assay, 0.2 ml of the cell extract was added to 2.8 ml of prewarmed phosphate buffer containing 1.66 mM ONPG (Sigma Chemical Co.) in a cuvette of a temperature controlled (30° C) recording spectrophotometer (Beckman, Model 35). After rapid mixing with a cuvette stirrer, the increase in OD at 420 nm was monitored.

Enzyme activity was calculated using the following formula:

$$\frac{\Delta \text{ OD}}{\text{minute}} \times \frac{1}{\Sigma} \times \frac{3 \text{ ml}}{0.2 \text{ ml}} \times 1000 \times 5 \text{ ml} = L$$

where Σ is the molar extinction coefficient (1668 liters/mole) of ONP at pH 7.0, 3 ml is the assay volume, 0.2 ml is the volume of the cell extract added, 1000 is a conversion factor (mmoles to μmoles), 5 ml is the volume of buffer added for cell disruption, and L is the enzyme activity expressed in μmoles ONPG hydrolyzed per minute per ml of originial culture (μmoles ONPG/min/ml). Dividing this volumetric activity by the dry weight of cells gave the specific activity (μmoles ONPG/min/mg). One unit of lactase activity was defined as that amount of enzyme which hydrolyses 1.0 μmole ONPG per minute at 30^{o} C and pH 7.0.

3.3 Repression and Induction Studies

3.3.1 Repression of Enzyme Production in Batch Culture

YNB media (pH 5.0) containing 0.5% inulin were used to study repression of inulase production. During early growth phase, 0.4% of a fermentable or a non-fermentable carbon source was added to each of a series of cultures. The timing of effector addition was based upon a predictable drop in pH associated with culture growth (effectors were added at pH 3.75). At regular intervals, cultures were removed for dry weight and inulase determinations. The inulase activity per ml of culture for each sample was plotted against dry weight and the slopes of the resulting lines, which represent the differential rate of enzyme

production, were compared. Inulase production in an inulin series with no additions was used to estimate non-repressed enzyme production.

The procedure was the same for determination of repression of lactase production except that the media contained 0.5% galactose and the carbon compounds were added at a pH of 3.9 to 4.0.

The pH values of organic acid solutions were adjusted to 4.0 with NaOH prior to addition.

3.3.2 Induction of Lactase in Batch Culture

Two defined media were used to study lactase induction: 0.6% succinic acid (pH 4.0) and 0.6% glycerol (pH 5.5). Yeast growth caused a pH increase in succinate media and a pH decrease in glycerol media. Compounds to be tested for induction were added early in the growth phase (to succinate cultures at pH 4.2 and to glycerol cultures at pH 4.9). Samples, procured at timed intervals, were analysed for dry weight and lactase content. For each sample the lactase activity per ml of culture was plotted against the dry weight. Enzyme activity of a series of cultures with no compounds added was taken as the basal activity.

Galactose, methyl-β-thiogalactoside (TMG), and isopropyl-β-D-thiogalactopyranoside (all from Sigma Chemical Co.) were tested at a concentration of 10 mM.

3.3.3 Induction of Lactase by Galactose Analogs in Continuous Culture

The 350 ml fermentor was used to study the effect of isopropyl-thiogalactoside (IPTG), thiogalactoside (TDG), and 6-deoxygalactose (fucose) addition to *K. fragilis* cultures growing at steady state in a

sucrose-yeast extract medium. The fermentor was assembled so that one nutrient reservoir contained medium at double concentration while the second contained only distilled water. Once a steady state was established, the water reservoir was replaced by a carboy containing a galactose analog in distilled water. After 3.5 to 7.0 replacement volumes, the analog was diluted out by reinstalling the distilled water reservoir. Samples were procured prior to addition of a galactose analog, during the period of addition, and during removal of an analog.

The analog solutions were prepared at the following concentrations: IPTG, 14.0 mM; TDG, 11.2 mM; and fucose, 12.6 mM.

4. RESULTS

In order to evaluate the relative advantages of a dual enzyme production process, one criterion is to compare yields with those from systems used to produce each enzyme individually. Therefore, prior to investigation of simultaneous production of inulase and lactase, fermentation conditions were studied for each enzyme separately. A better understanding of the regulation of separate enzyme production was sought to use as a basis for the study of combined production.

4.1 Inulase Production

4.1.1 Inulase Production in Batch Culture

Previous studies by GrootWassink and Fleming (1980) on inulase production showed that batch cultures of K. fragilis gave good enzyme yields when grown on the natural substrate of the enzyme, inulin. The question arose whether alternate carbon sources could support inulase production under similar cultivation conditions. Thus, seven additional carbon substrates were tested in shake flask cultures with yeast extract as the nitrogen, vitamin, and inorganic ion source (Table 2). Growth of the cultures was carbon limited as verified later in Section 4.1.1.1. The listed substrates were tested for the following reasons. Sucrose, like inulin, is a natural substrate of K. fragilis inulase. Lactose is the natural substrate of lactase; the

TABLE 2. Biomass and inulase yields from shake flask cultures of *K. fragilis* grown on 8 different carbon substrates. Duplicate cultures were monitored until the stationary phase; 19 h of incubation for inulin and sugar containing media and 36 h for glycerol and ethanol containing media.

Carbon substrate	Dry weight (mg/ml)	Inulase (units/mg)
Inulin	5.3	10
Sucrose	4.6	2
Glucose	4.1	2
Fructose	4.2	2
Lactose	4.4	1
Galactose	4.5	1
Glycerol	3.6	3
Ethanol	4.8	2

second enzyme of interest in this study. Glucose, fructose, and galactose are the monosaccharides that comprise the two disaccharides as well as inulin. Glycerol and ethanol were tested because, unlike the other substrates, they are non-fermentable (i.e. they are not metabolized through the glycolytic pathway) and are therefore often found to be non-repressive.

Inulin caused the production of over three times more inulase than did any other carbon source.

Time course analysis of shake flask cultures showed that maximum inulase yields occurred during the stationary growth phase on all substrates. On the mono- and disaccharides, the bulk of the enzyme was produced during a short period towards the end of the active growth phase, as shown in Figure 1 for a sucrose grown culture. On the contrary, cultures grown on inulin, glycerol, and ethanol exhibited a more growth associated increase in inulase content. The growth rates of the resulting cultures varied depending upon the carbon source. Most rapid growth occurred on the sugars followed in decreasing order by inulin, ethanol, and glycerol.

During the stationary phase of cultures grown on sucrose, an inexplicable but reproducible dip in inulase content was observed (see Figure 1).

To exclude the inherent low oxygen transfer rates in shake flasks as the possible cause of some of the low inulase yields, two batch fermentations were carried out in a 7.5 liter fermentor with the dissolved oxygen tension maintained above 20% of saturation. The inulin grown culture gave inulase and biomass yields of 10.8 units/mg and 5.4 mg/ml, respectively. A similar culture on sucrose yielded

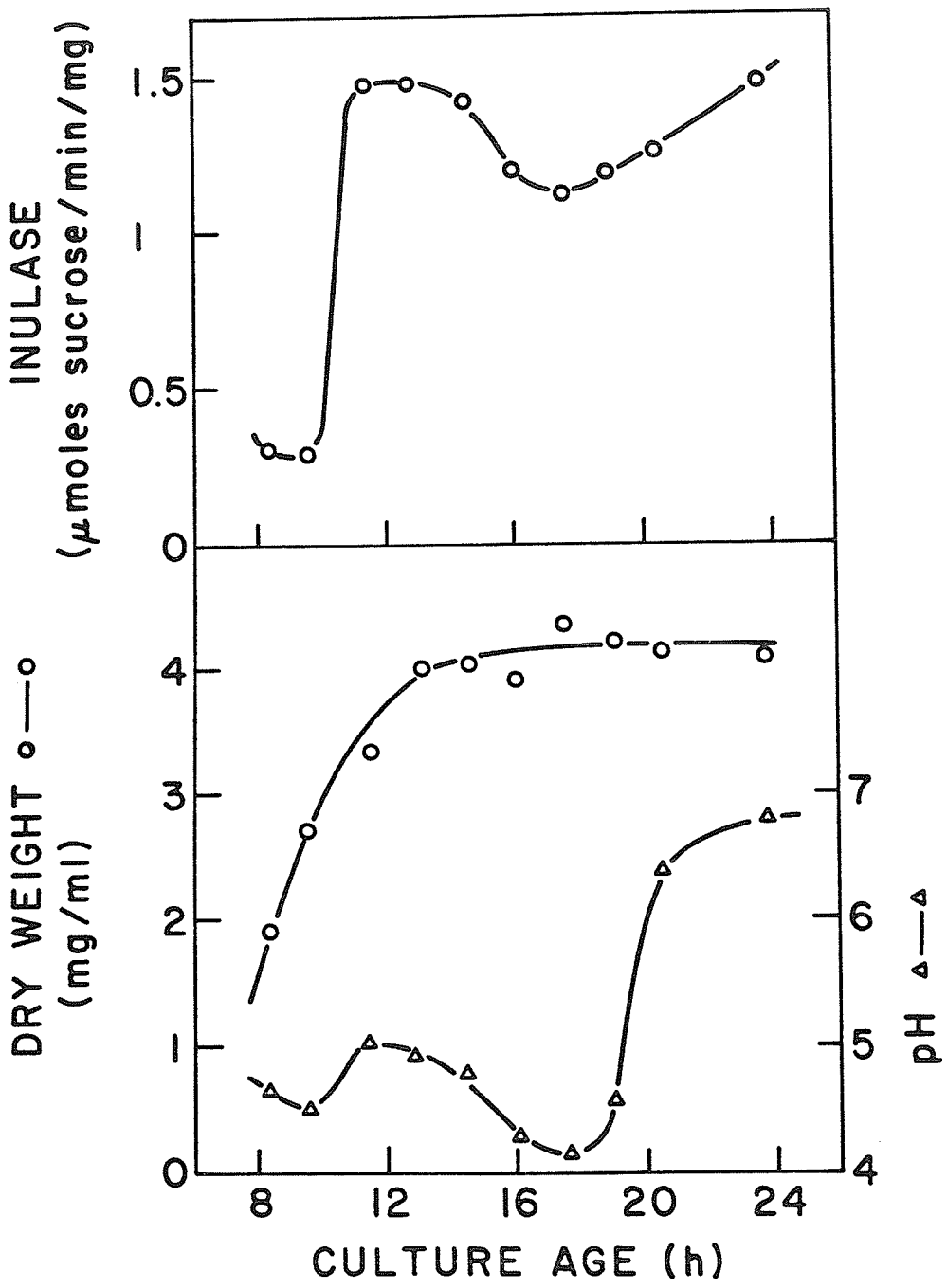

Figure 1. Shake flask batch culture of K. fragilis grown on 1% sucrose and 0.5% yeast extract.

1.0 units/mg of inulase and 4.7 mg/ml of dry biomass. With the exception of the slightly lower inulase yield on sucrose, these results are very close to those obtained from shake flask cultures on the same two carbon substrates. The enzyme production profiles were also similar to those of shake flask cultures. These findings indicate that the shake flask cultures were good approximations of those grown in the fermentor.

The results from the above set of experiments suggested that inulase formation in batch cultures was repressed during the active growth phase by rapidly metabolizable carbon substrates such as the mono- and disaccharides. This seemed concentration dependent since at the end of the growth phase as the substrate concentrations became low, a sudden surge in inulase formation occurred. In this case, only cultures whose total growth was carbon limited would have experienced temporal substrate concentrations that were low enough to cause derepression of inulase synthesis.

4.1.1.1 Carbon Limitation of Growth in Batch Culture. Results from two experiments to determine the carbon limiting concentration in sucrose/yeast extract media are presented in Figure 2. In one test (Figure 2A) cultures were grown on 0.5% yeast extract plus various concentrations of sucrose. Biomass yields increased linearly up to 1.4% sucrose, thus demonstrating a direct dependence of biomass yield upon sugar concentration. In a second experiment (Figure 2B) the yeast extract concentration was varied against 1.0% sucrose. Yeast extract did not become growth limiting until less than 0.4% was supplied. These tests both indicate that *K. fragilis* batch cultures were carbon limited when grown in the standard medium of 1.0% sucrose and 0.5% yeast extract.

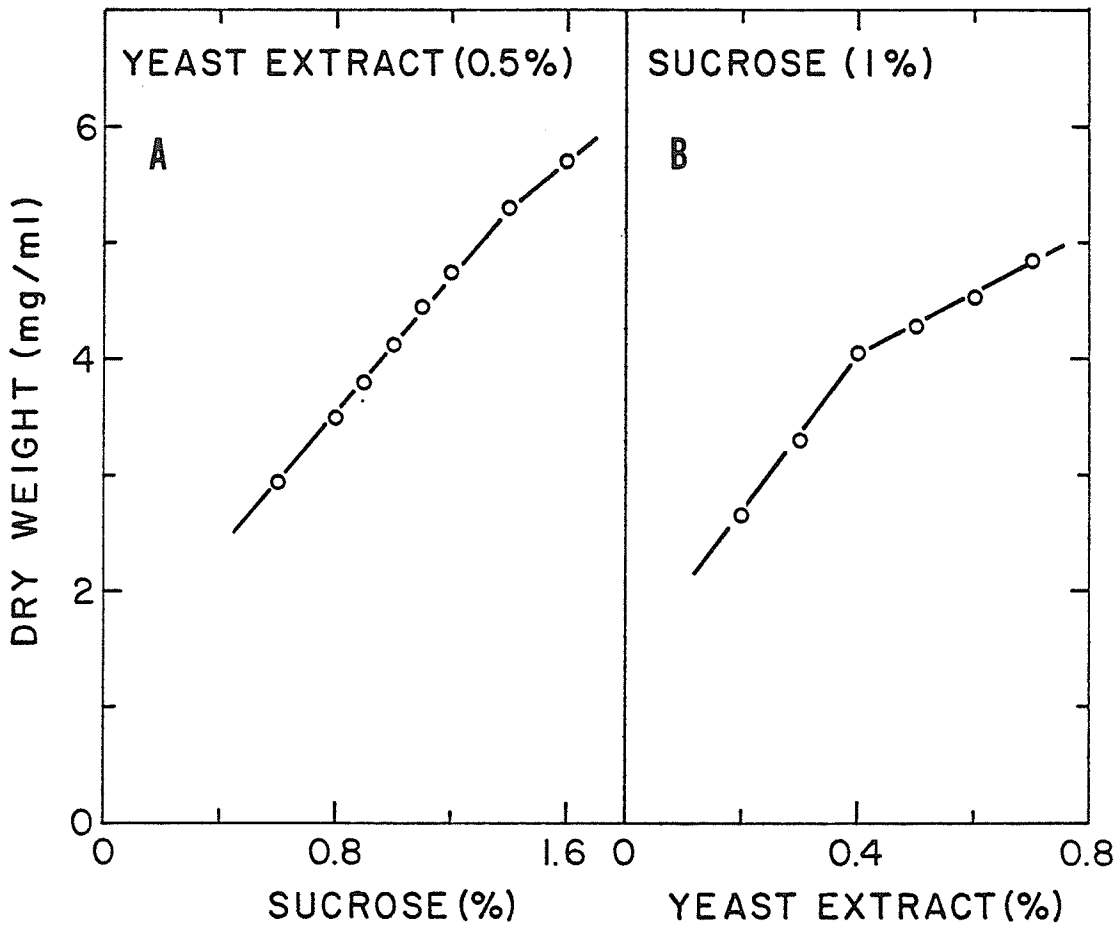

Figure 2. Carbon limitation of growth in batch cultures of K. fragilis grown in sucrose/yeast extract media. A: standard shake flask cultures were grown in liquid media containing 0.5% yeast extract with various concentrations of sucrose (0.6 to 1.6%). B: standard shake flask cultures were grown in liquid media containing 1% sucrose with various concentrations of yeast extract (0.2 to 0.7%). All cultures were harvested after 24 hours of incubation.

Lactose, inulin, glycerol, and ethanol were also tested for carbon limitation against 0.5% yeast extract. The results indicated that cultures grown on these substrates at a concentration of 1.0% were also carbon limited.

Since the cultures grown on 1% sucrose and lactose were carbon limited, the monosaccharides glucose, fructose, and galactose were also considered to be growth limiting at this concentration. This assumption was made because monosaccharides provide 5% less utilizable carbohydrate than an equal weight of a disaccharide.

Thus it appeared that the total growth of each of the batch cultures providing the data in Section 4.1.1 was carbon limited.

4.1.2 Inulase Repression in Batch Culture

To obtain more information about inulase production in response to different substrates, fermentable and non-fermentable carbon sources were tested for their ability to suppress inulase production. The carbon substrates were added to batch cultures during growth on inulin (Figure 3). Inulin was used in the test medium because it promoted the highest differential rate of inulase production during the growth phase. An added carbon source was considered to be repressive if it decreased the differential rate of inulase production. To avoid artifacts caused by variations in the nitrogen supply, media were used that contained the defined nutrients of YNB instead of the complex nitrogen source, yeast extract.

On addition, all the sugars caused increased specific growth rates and severe repression of inulase production (Figure 3A). These findings are consistent with the theory of catabolite repression which

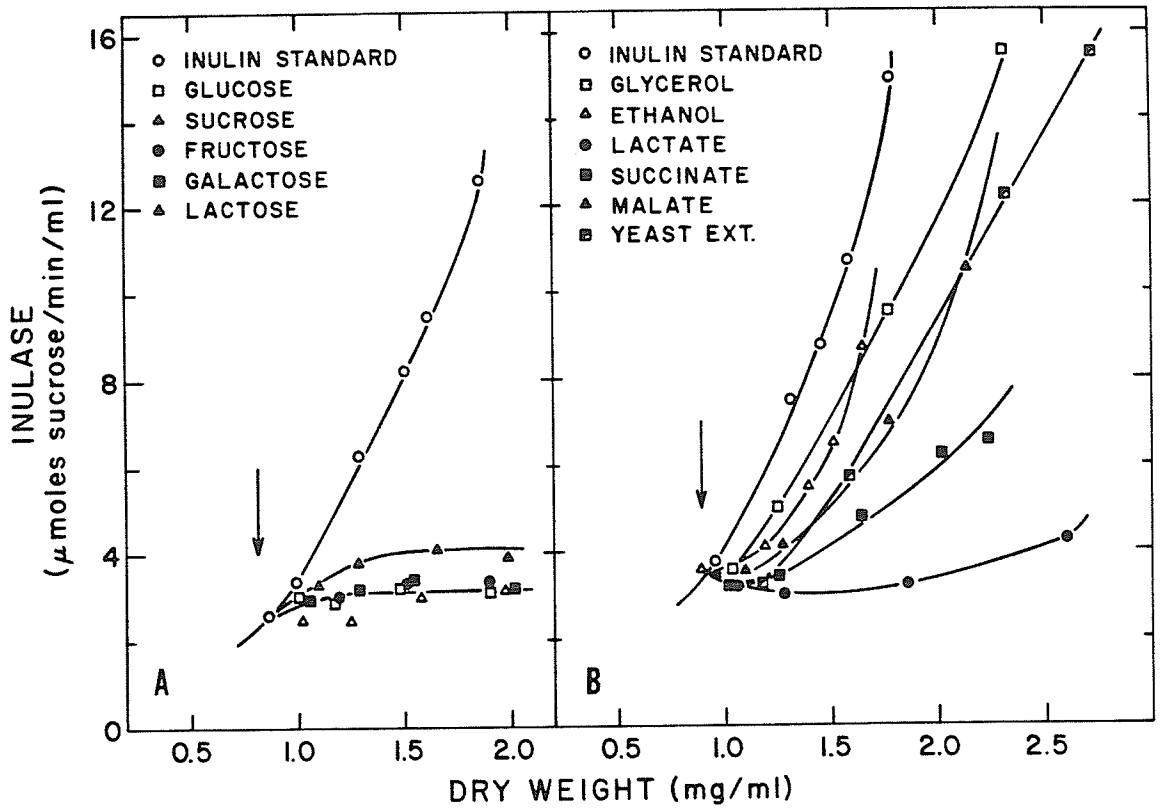

Figure 3. Repression of inulase production in K. fragilis by fermentable (A) and non-fermentable (B) carbon substrates. Shake flask cultures were grown in a medium containing 0.5% inulin and 0.25% YNB. Effectors were added for a concentration of 0.4% at the points indicated by the arrows.

states that an organism given a more easily metabolized substrate will immediately use it and at the same time, repress the biosynthesis of enzymes that were required for the metabolism of the old substrate but are no longer needed. Of the sugars, only lactose did not cause immediate cessation of enzyme accumulation. Lactose, unlike the other sugars tested, requires the induction of a specific permease before it can be rapidly utilized (Barnett, 1976). Therefore, the delay in action of this sugar may reflect the time required for permease induction. Alternatively, sucrose, which is not taken up in any amount by yeast cells (Barnett, 1976), caused immediate repression because of rapid hydrolysis to fructose and glucose by inulase already present in the cultures at the time of addition (2.5 units/ml).

The non-fermentable carbon sources suppressed inulase production to varying degrees (Figure 3B). Lactate, which is rapidly metabolized by K. fragilis (A. Davies, 1956a), was a strong repressor with immediate effect. Succinate caused some continuous repression but glycerol, ethanol, and malate caused only a transient reduction in the rate of inulase production. The transient repression was probably a result of the cultures' adaptation to the presence of new carbon sources. Once adapted, the cultures produced new inulase at rates similar to those in the control culture. The resumption of high rates of inulase production was not due to utilization and exhaustion of the added substrates because each compound was added at a concentration (0.4%(w/v)) that would sustain the growth of each culture to well over 2 mg/ml, even if it served as the sole carbon source.

Yeast extract was also tested for repression of inulase production. The transient repression that resulted was likely a

consequence of an adaptation to the new nitrogen supply. Two observations indicated that a switch of nitrogen sources occurred: the specific growth rate increased and the culture pH began to rise instead of continuing to gradually decrease as was normal for growth on inulin plus YNB. The shift from YNB to yeast extract would have involved a reorganization of metabolic activity to eliminate the biosynthetic pathways for those amino acids that were suddenly available in the medium. During reorganization, inulase biosynthesis might have been neglected, but after adaptation it was recommenced as demonstrated by the resumed high differential rate of inulase production. Thus it appeared that yeast extract was only slightly repressive, if repressive at all. This conclusion was supported by the observation that batch cultures on inulin produced more inulase when the nitrogen was supplied by yeast extract (10 units/mg) than by YNB (8 units/mg).

In summary: the results of the inulase repression studies indicate that inulase is repressed by rapidly metabolizable carbon sources. Substrates like ethanol and glycerol that support slow growth rates cause little repression.

4.1.3 Inulase Production in Continuous Culture

Continuous culture of *K. fragilis* using a variety of carbon sources was studied with two main objectives in mind. The first was to improve inulase yields over those in batch culture. It was believed that the continuous culture technique, by reducing the substrate concentration in the culture, could lead to high inulase yields thus eliminating the need for inulin in the medium. GrootWassink and Fleming (1980) had already demonstrated effective stimulation of

inulase production by growing K. fragilis under sucrose limitation in continuous culture. The second objective was to gain an increased understanding of the regulatory system governing inulase biosynthesis. Information in this regard would be obtained by examining the patterns of inulase yields as a function of dilution rate during growth on a variety of carbon sources.

4.1.3.1 Carbon Limitation of Growth in Continuous Culture. In continuous culture the steady-state concentration of the limiting nutrient is most sensitive to changes in dilution rate. Consequently, if the limiting nutrient is the carbon substrate, then enzyme production that is regulated in response to carbon substrate concentration should be significantly affected by changes in dilution rate.

For a given medium, a concentration of the carbon substrate that limits growth in batch culture may not do so in continuous culture. This discrepancy arises since differences in the efficiency of substrate utilization exist between batch and continuous cultures. As a demonstration of the difference in efficiency, compare the biomass yields resulting from growth of K. fragilis in media containing 1.0% sucrose and 0.5% yeast extract: in batch culture the dry weight is less than 5 mg/ml (Table 2) but continuous culture supported 6.0 mg/ml (Figure 5). Therefore, despite prior determination of carbon limitation for batch cultures it was necessary to establish carbon-limiting conditions for growth in continuous cultures.

Sucrose and fructose media containing 0.5% yeast extract were tested for carbon limitation in the chemostat (Figure 4). At relatively low medium concentrations of both sugars, biomass yields

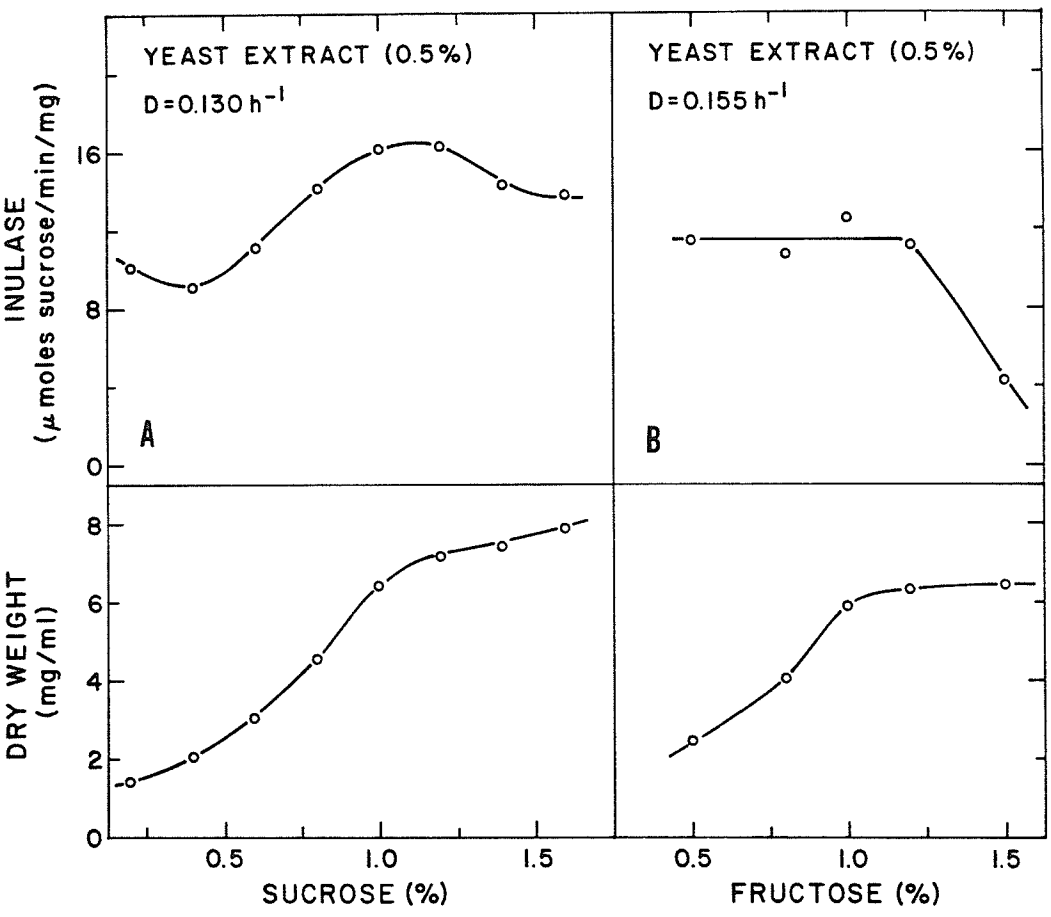

Figure 4. Carbon limitation of growth in 3-liter continuous cultures of K. fragilis. Inulase and biomass yields were determined for two series of media containing 0.5% yeast extract and various concentrations of sucrose (A) and fructose (B).

were dependent upon the amount of carbon source supplied. Both cultures became non-carbon limited between 1.0 and 1.2% sugar, as indicated by plateaus in biomass yields. As a further measure of carbon limitation, the inulase content was measured for each sugar concentration. Neither culture had a significant drop in activity until 1.2% sugar was exceeded. In the fructose culture the inulase level dropped sharply above 1.2% fructose. This drop reflects the repression of inulase biosynthesis caused by excess fructose. In contrast, inulase formation in the culture containing excess sucrose was not as strongly repressed.

The above results indicate that continuous cultures of K. fragilis grown on media containing 0.5% yeast extract plus sucrose or fructose at a concentration of 1.0% were carbon limited. They also suggest that up to 1.2% of either sugar could be used without adversely affecting inulase levels. Based on these findings, chemostat cultures grown on other mono- and disaccharides were assumed to be carbon limited at 1% carbon source and 0.5% yeast extract.

4.1.3.2 Inulase Yields and Regulation in Continuous Culture. Sucrose was tested as a carbon source in continuous culture (Figure 5A) since K. fragilis must produce inulase to utilize this disaccharide. The maximum inulase yield in this culture, approximately 27 units/mg, was more than ten times greater than that obtained in sucrose grown batch cultures. More importantly, the activity was more than double the amount produced from batch cultures on inulin. The higher specific enzyme activity in continuous culture did not result at the expense of the biomass yield. In fact, the dry weights were greater than those

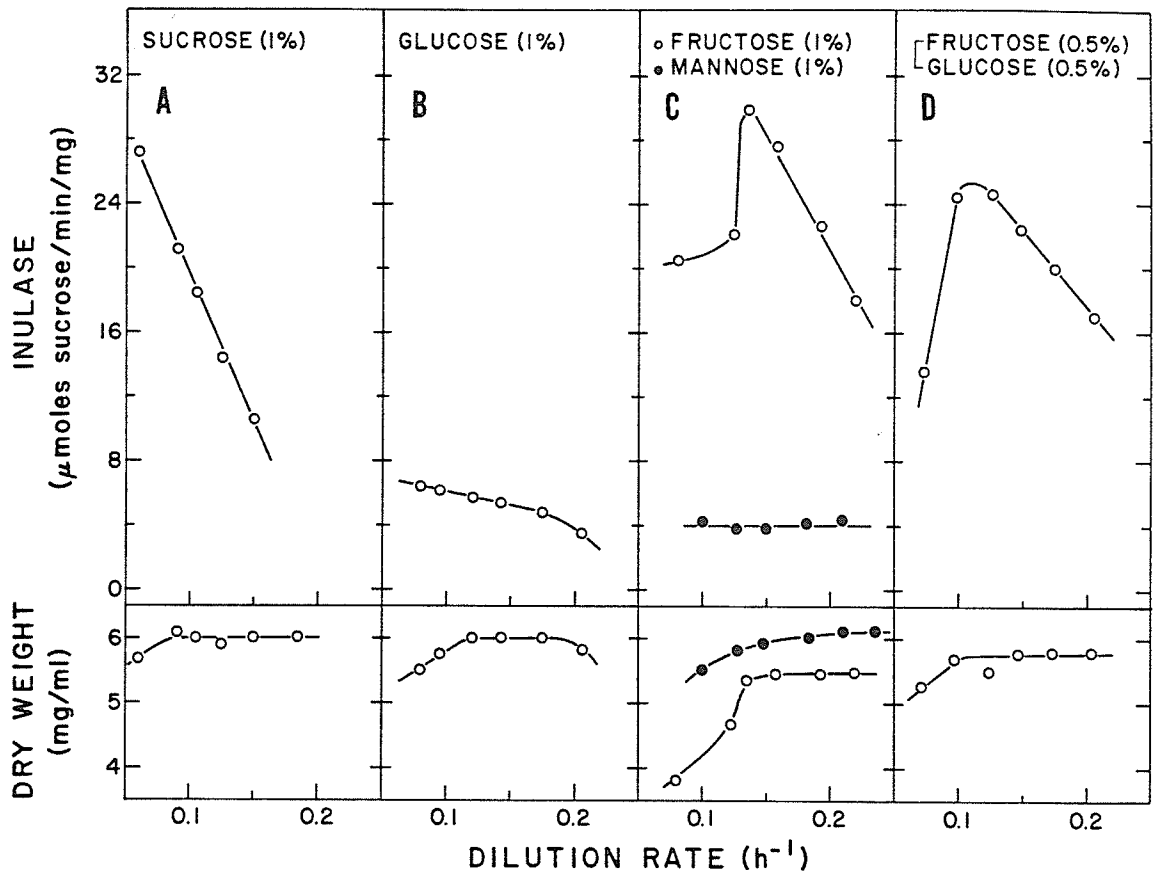

Figure 5. Inulase and biomass yields as a function of dilution rate in 3-liter continuous cultures of K. fragilis grown on sucrose (A), glucose (B), fructose (C), mannose (C), and fructose plus glucose (D).

obtained in batch culture on equal concentrations of sucrose or inulin.

In the chemostat culture on sucrose, the inulase yield was highest at the lowest dilution rate and rapidly decreased as the dilution rate increased. Based on the interpretations of enzyme production patterns in continuous culture proposed by Dean (1972) and Toda (1976b), these results indicate that inulase was constitutively produced but was subject to carbon catabolite repression. A similar enzyme activity pattern for K. fragilis grown on sucrose was described by GrootWassink and Fleming (1980).

Inulase repression at high dilution rates could not have been directly caused by sucrose since this disaccharide cannot enter yeast cells (Barnett, 1976). Instead, repression must have been caused by the glucose and/or fructose that was liberated by extracellular sucrose hydrolysis. To determine if these monosaccharides equally repress inulase, glucose and fructose were separately tested as carbon sources in continuous culture.

In the glucose culture (Figure 5B) the highest inulase level was approximately 6 units/mg and the dry weight leveled off at 6.0 mg/ml above a dilution rate of 0.12 h^{-1}. Despite a three-fold increase in inulase production over glucose based batch cultures, the enzyme level was still substantially lower than those obtained using sucrose in the chemostat. However, like the sucrose continuous culture, the pattern of inulase production suggested increased repression at higher dilution rates.

Continuous culture of K. fragilis on fructose (Figure 5C) resulted in a very prominent peak in inulase activity of about 30 units/mg at a

dilution rate of 0.14 h^{-1}. Biomass yields, 5.5 mg/ml at dilution rates over 0.13 h^{-1}, were slightly less than for continuous cultures grown on sucrose or glucose. The peak in inulase yield suggests that both induction and repression are involved in inulase regulation during growth on fructose. The apex might have occurred when enzyme induction was just balanced by repression.

The above results indicated that there is a radical difference between the effects of glucose and fructose on inulase production. Considering this difference, it was hypothesized that the inulase production profile that resulted from the sucrose culture may not have been a simple case of an increasing substrate concentration which caused increased repression. Rather, it may have resulted from a more complex interaction involving a mixture of an inducing and a non-inducing carbon substrate. The extracellular sugar mixture would have involved sucrose, glucose, and fructose in a ratio that depended upon inulase activity and the relative rates of glucose and fructose uptake at a given dilution rate. To determine if sucrose hydrolysis influences inulase production during continuous culture on sucrose, a continuous culture of K. fragilis was grown on a medium containing equal concentrations of glucose and fructose (Figure 5D). The highest inulase activity obtained in this culture was about 24 units/mg and the dry weight leveled at 5.8 mg/ml at a dilution rate of 0.1 h^{-1}. The peaking inulase profile, suggesting induction and repression, is different from the pattern that resulted from the sucrose culture. Therefore, the hydrolytic step during growth of K. fragilis on sucrose in the chemostat influences the level of inulase produced.

The final sugar to be examined in this series of continuous culture experiments was mannose. Apart from an initial isomerization step, mannose metabolism in yeast is very closely related to that of fructose (Barnett, 1976). Figure 5C shows inulase at about 4 units/mg at all dilution rates and biomass yields of about 6.0 mg/ml at dilution rates above 0.13 h^{-1}. The featureless enzyme profile gave no indication that mannose plays a similar role to fructose in the regulation of inulase formation.

Inulase production was also measured in continuous cultures grown on lactose and galactose (Figure 9B). At no dilution rate did the inulase production rise above 0.5 units/mg. These enzyme levels were not greater than those obtained from batch culture.

In summary, the foregoing experiments demonstrated that continuous culture can be used to substantially increase the inulase activity of K. fragilis cultures that are grown on sucrose, glucose, or fructose. In addition, it appears that inulase is not necessarily a constitutively produced enzyme regulated solely by catabolite repression, but may be inducible.

4.1.3.3 Effect of the Sequence of Dilution Rates on Inulase Yields. Towards the end of this study it became evident that the relationship between inulase yield and the dilution rate of a continuous culture is not fixed and reproducible under all conditions. Previous environmental conditions also appeared to influence the productivity at any particular dilution rate. Since inulase profiles were used to make inferences about the regulation of inulase formation, a close examination was made of the effect on inulase levels of the sequence in which steady states were established.

The standard operating technique adopted in this study for continuous cultures was that they were initiated at a low dilution rate and subsequent steady states were established at sequentially increased dilution rates (shift-up experiment). The results from such a continuous culture grown on fructose were compared with those of a culture that was passed through dilution rates in the reverse direction (shift-down experiment) (Figure 6). The patterns of inulase production were very different. Instead of peaking at an intermediate dilution rate, the inulase level during the shift-down experiment slowly increased until a dilution rate of 0.11 h^{-1} was reached. The activity then jumped to approximately 43 units/mg on further reduction of the dilution rate to 0.09 h^{-1}. This phenomenon, in which the trajectory of ascent differs from that of descent, is termed "hysteresis" and has been previously found to occur in continuous culture (Imanaka, 1972; Tanner, 1978).

At the lowest dilution rate of the shift-down experiment, the steady state was not established as quickly as usual. Instead, more than five culture replacement volumes passed before the inulase levels began to stabilize. Instability and very high inulase yield may be indications that mutation and selection for a high inulase producing yeast occurred. This speculation was supported when such mutants were isolated from another continuous culture that behaved abnormally (see Section 4.3.4).

In contrast to the shift-up culture, the profile of inulase production of the shift-down culture suggested regulation solely by catabolite repression; there was no implication of induction.

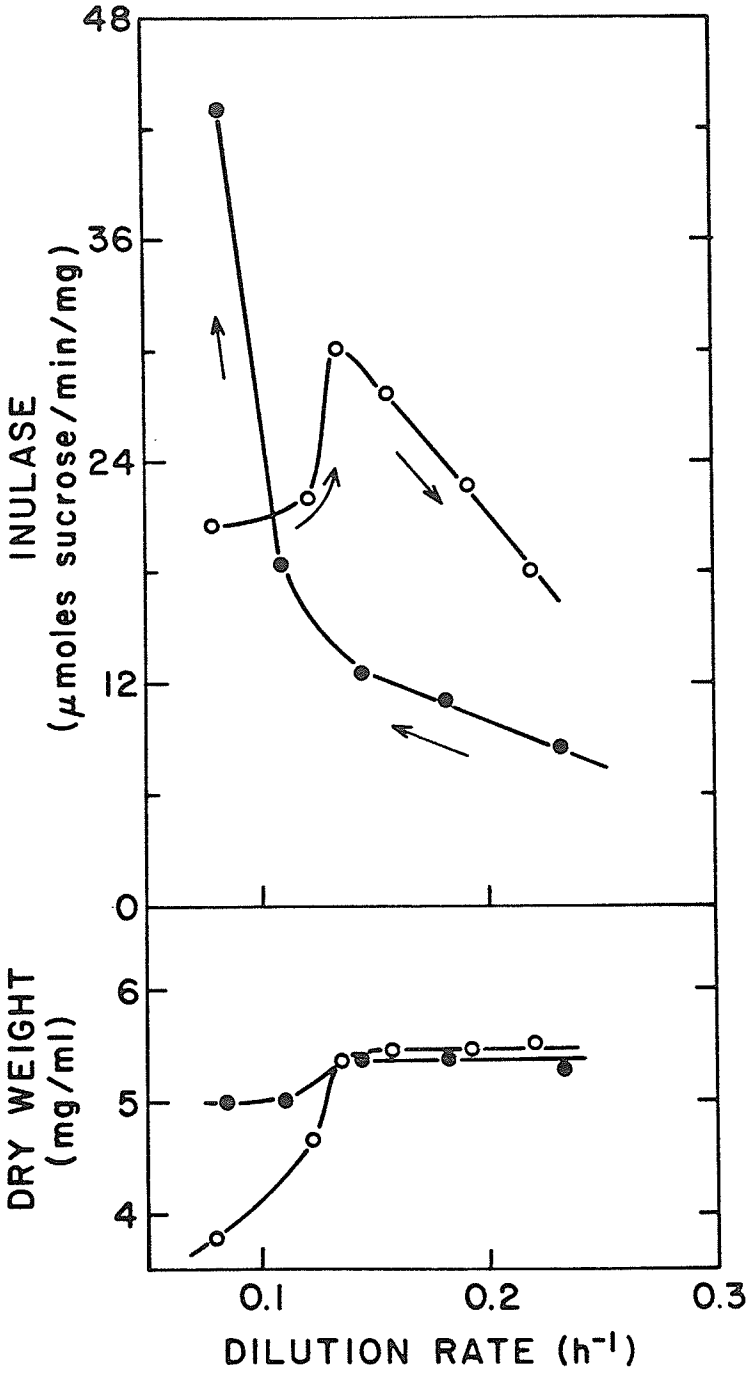

Figure 6. The effect of the sequence of dilution rates on inulase yields. Two separate 3-liter continuous cultures of K. fragilis were grown on 1% fructose and 0.5% yeast extract liquid media. The arrows indicate the order in which the steady states were established ("shift-up" o and "shift-down" ●).

The above results indicate that culture history, in particular the sequence of steady states, strongly influences the pattern of inulase production during growth of K. fragilis in continuous culture. Selection of mutant strains would of course seriously complicate the interpretation of these patterns.

4.2 Lactase Production

4.2.1 Lactase Production in Batch Culture

Some reports on lactase production by strains of K. (Sacch.) fragilis grown on various carbon sources in aerobic batch culture have been published (A. Davies, 1956b; Tikhomirova et al, 1972). The lactase of these strains was induced during growth on galactose or lactose but remained low on other sugars. In these reports the yeast strains were not clearly defined and no results were presented concerning lactase yields obtained on non-fermentable carbon sources. Therefore, for the purposes of this study, the investigation of lactase production was initiated by growing K. fragilis under the batch cultivation conditions described in Materials and Methods. The lactase and dry weight yields from carbon limited batch cultures grown on six different carbon sources are presented in Table 3. Lactose and galactose supported the highest lactase production. Growth on ethanol resulted in an intermediate lactase content but sucrose, fructose, and glycerol supported very poor enzyme yields.

Specific lactase activity (units/mg) increased rapidly and peaked towards the end of the active growth phase during growth on lactose (see Figure 7) and galactose. On sucrose and fructose most enzyme was

Table 3. Biomass and lactase yields from shake flask cultures of *K. fragilis* grown on 6 different carbon substrates. Cultures were monitored until early stationary phase.

Carbon substrate	Dry weight (mg/ml)	Lactase (units/mg)
Sucrose	4.6	0.04
Fructose	4.2	0.02
Lactose	4.4	0.31
Galactose	4.5	0.31
Glycerol	3.6	0.04
Ethanol	4.8	0.09

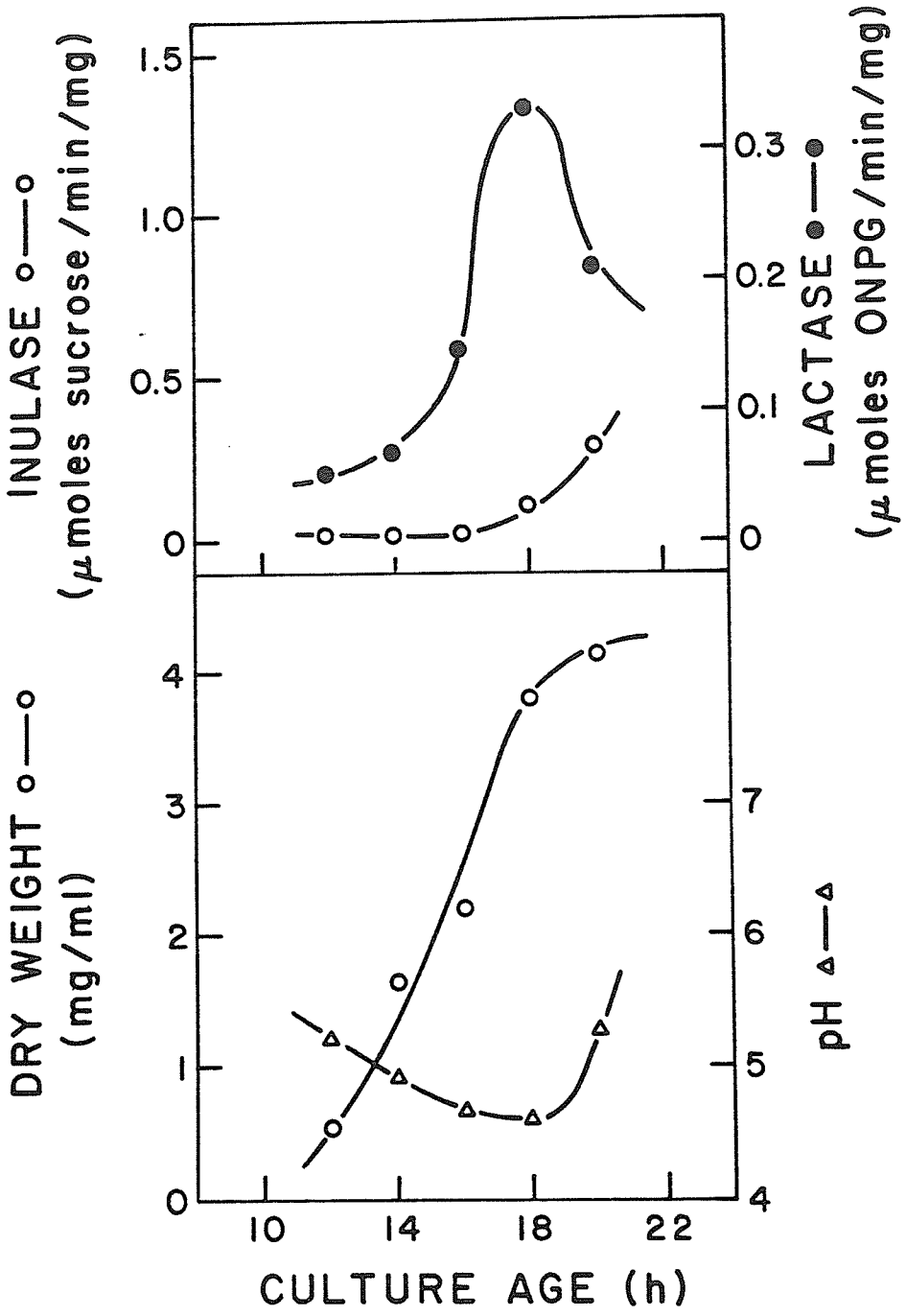

Figure 7. Shake flask batch culture of K. fragilis grown on 1% lactose and 0.5% yeast extract.

produced towards the end of the growth phase but there was no peak in activity. Ethanol supported a growth associated increase in specific activity but on glycerol there was no increase in lactase activity at any point during the cultivation.

The preceding results are consistent with the belief that lactose and galactose, in addition to being readily metabolized carbon sources for K. fragilis, are effective lactase inducers. It also appeared that lactase formation was repressed during the active growth phase by rapidly utilizable substrates but was derepressed as these carbon substrates neared exhaustion.

4.2.2 Lactase Repression in Batch Culture

The low lactase levels in K. fragilis cultures grown on substrates other than lactose or galactose could be due to repression, lack of inducer, or both. In order to gain more information regarding the repression of lactase production, six fermentable and five non-fermentable carbon sources were tested for repression of lactase production in batch cultures of K. fragilis growing on galactose (Figure 8). The addition of fermentable carbon sources, with the exception of galactose, caused repression. The severity of repression, as indicated by the decrease in the slope of the line representing the differential rate of lactase production, varied among the carbon sources with glucose being the most repressive. Glucose, fructose, and mannose, for which yeast have constitutive transport mechanisms (Barnett, 1976), represses immediately upon addition but sucrose and lactose repressed only after an initial lag, suggesting that the cultures had to adapt to the presence of these two disaccharides.

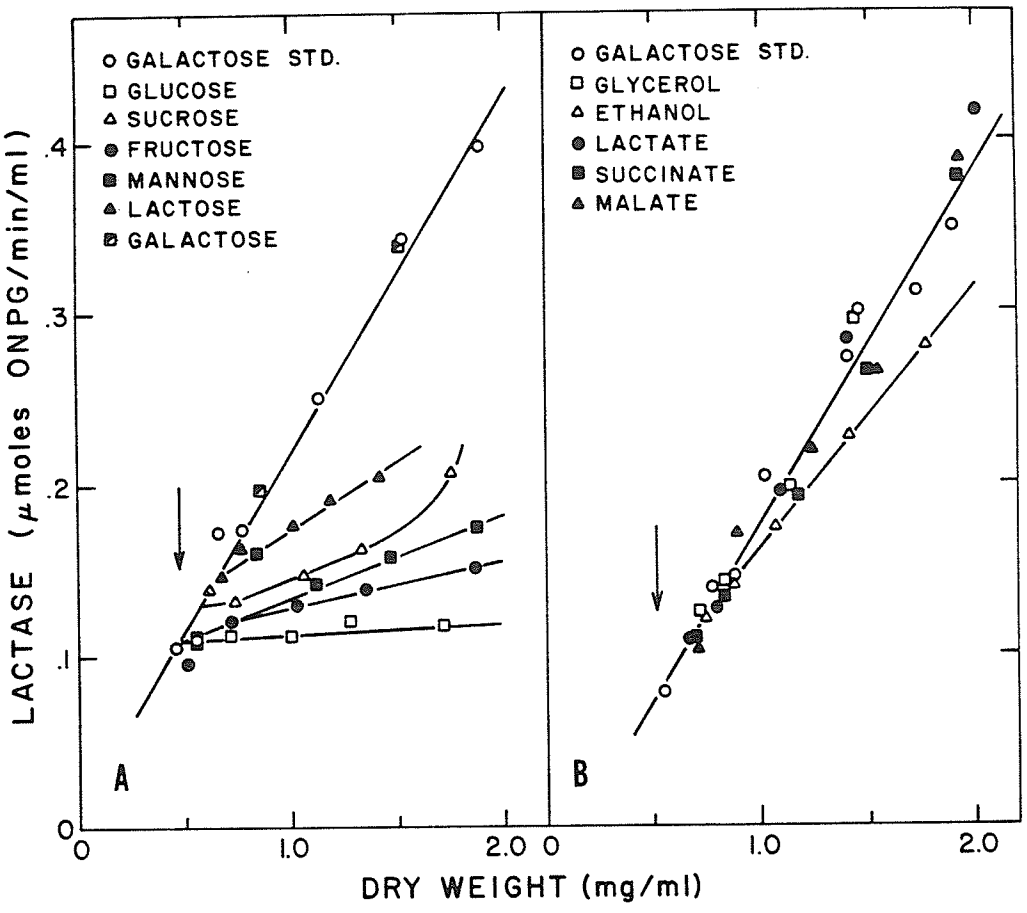

Figure 8. Repression of lactase production in K. fragilis by fermentable (A) and non-fermentable (B) carbon substrates. Shake flask cultures were grown in a medium containing 0.5% galactose and 0.25% YNB. Effectors were added for a concentration of 0.4% at the points indicated by the arrows.

Addition of galactose caused no repression, thereby ensuring that the repression caused by the other substrates was not simply due to increased concentration of the carbon substrate.

All effectors were added at a concentration sufficient to support biomass increases to the end of the sampling period, even if galactose was completely displaced as the carbon source. Of the added sugars, none, except lactose, caused the culture growth rate to differ from that of the control culture. Lactose caused a decrease in growth rate.

The mechanism of repression by glucose, fructose, mannose, and sucrose may have involved inducer exclusion at the level of sugar transport across the plasma membrane. Matern and Holzer (1977) demonstrated that, in Sacch. cerevisiae, glucose causes a decrease in the affinity for galactose of the galactose uptake system. If glucose and the other sugars have a similar effect in K. fragilis, then the degree of lactase repression observed in this experiment may reflect the effectiveness of each sugar in inhibiting inducer (galactose) uptake.

Interestingly, lactose, a lactase inducing carbon source (Tikhomirova et al, 1972), was repressive in this experiment. Perhaps the repression was caused by new intracellular glucose resulting from lactose hydrolysis or maybe it reflects an extended adaptation period during which the culture attempted to convert from galactose to lactose utilization. It is improbable that the repression resulted from more rapid metabolism of lactose than galactose since the specific growth rate decreased after lactose addition.

The addition of the non-fermentable substrates, with the possible exception of ethanol, caused no suppression of the differential rate of lactase synthesis. Subsequent to their introduction, none of these compounds significantly increased or decreased the specific growth rate of the cultures. These results suggest that the non-fermentable carbon sources are non-repressive. However, since there is no assurance that these compounds were absorbed or assimilated, the results do not unequivocally support this suggestion.

Overall, it appears that the fermentable carbon sources, except galactose, repress lactase production while the non-fermentable substrates may be non-repressive.

4.2.3 Lactase Production in Continuous Culture

With the object of producing higher lactase yields than obtained in batch culture, the production of lactase by *K. fragilis* was examined in carbon limited continuous cultures. This was considered to be a promising approach since repression caused by high substrate concentrations could be relieved allowing for increased enzyme production. Repression of lactase in *K.* (*Sacch.*) *fragilis* by sugars including lactose and galactose has been reported (A. Davies, 1956b). However, since lactase is also inducible (De la Fuente and Sols, 1962) and galactose and lactose appear to be the only two inducing carbon sources (Tikhomirova, 1972), it was of primary interest to determine the lactase yields using these two sugars in continuous culture (Figure 9).

The highest lactase yields that resulted on each sugar (about 1.5 to 1.7 units/mg) were five times greater than the highest yields that

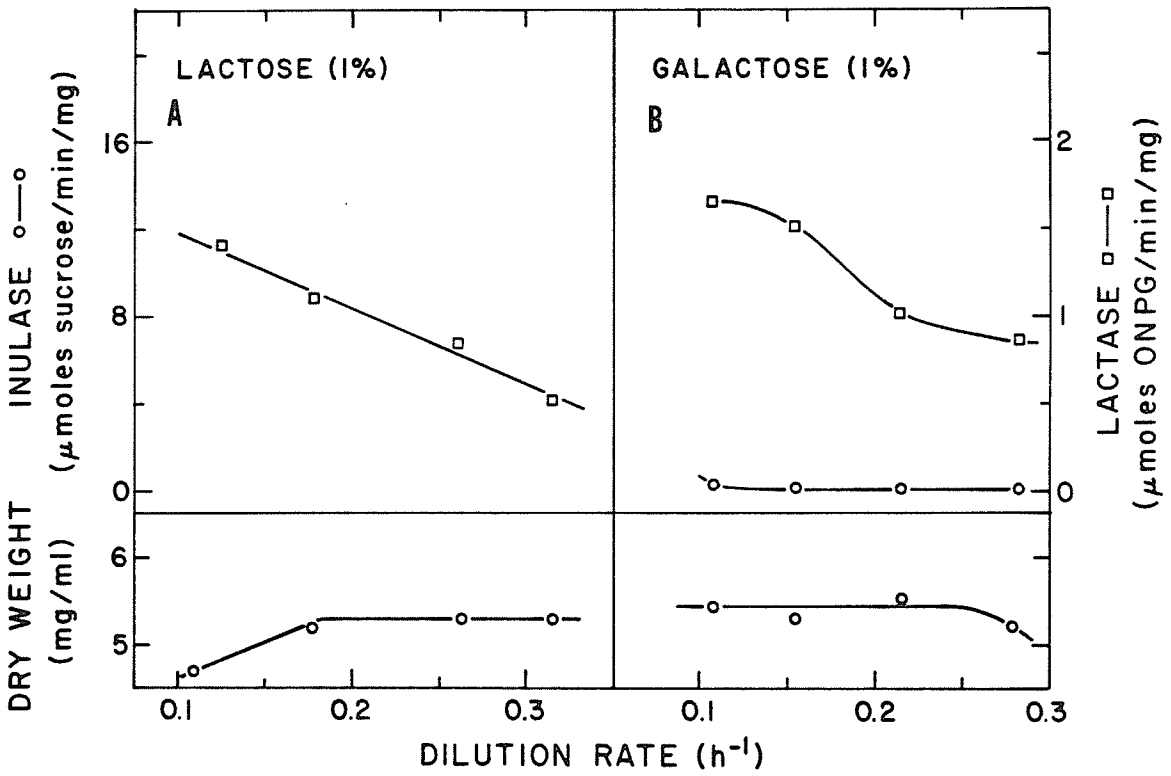

Figure 9. Lactase and biomass yields as a function of dilution rate in 3-liter continuous cultures of K. fragilis grown on lactose (A) and galactose (B). Inulase yields are shown for the galactose culture.

occurred in batch cultivations. The dry weights that resulted on each sugar were also higher than those in batch culture, indicating that the higher specific activities were not gained at the expense of biomass yield.

In the continuous cultures using lactose and galactose, steady state lactase activities decreased with increasing dilution rate implying that lactase biosynthesis is subject to greater repression at higher concentrations of lactose or galactose. In addition to this repressive effect, lactose and galactose must have a specific inductive function since continuous cultures grown on other substrates, including sugars and non-fermentable carbon sources, at all dilution rates gave low lactase yields that never exceeded 0.18 units/mg.

The above results demonstrate that much higher lactase production can be obtained in continuous cultures than in batch cultures of K. fragilis if lactose or galactose is the carbon source.

4.3 Simultaneous Inulase and Lactase Production

4.3.1 Inulase and Lactase Production in Batch Cultures Grown on a Mixture of Two Carbon Sources

No single carbon source supported high inulase and lactase yields in batch cultures of K. fragilis. In fact, substrates which gave good production of one enzyme resulted in very poor yields of the other. With the object of obtaining good yields of both enzymes, batch cultures of K. fragilis were grown on pairs of complementary substrates (i.e. one to stimulate lactase and one to stimulate inulase production) (Table 4).

TABLE 4. Biomass, inulase, and lactase yields from shake flask cultures of K. fragilis grown on 5 pairs of carbon substrates. Cultures were monitored until early stationary phase. The yields reported were the highest attained during each cultivation.

Carbon substrates A	Carbon substrates B	Dry weight (mg/ml)	Inulase (units/mg)	Lactase (units/mg)
Sucrose	Lactose	4.9	1	0.23
Sucrose	Galactose	4.4	1	0.30
Sucrose	Fructose	4.4	2	----
Fructose	Galactose	4.6	1	0.32
Fructose	Glucose	4.4	2	----

Galactose in combination with sucrose or fructose resulted in lactase activities equivalent to those obtained in cultures grown on galactose alone (see Table 2) whereas lactose paired with sucrose was not as effective. The three cultures containing galactose or lactose together with sucrose or fructose produced half as much inulase as those grown on sucrose or fructose alone.

Two other substrate combinations, neither one containing a lactase inducer (sucrose plus fructose and fructose plus glucose), were also tested, but only for inulase. The inulase levels were equivalent to to those obtained in cultures grown on sucrose, fructose, or glucose alone. Therefore, the lower inulase yields in the cultures grown on substrate pairs containing galactose or lactose were caused by the lactase inducing compound and not simply by the presence of two distinct carbon sources.

The above results demonstrate that inulase and lactase can be simultaneously produced in a single batch fermentation using complementary substrates. Unfortunately, inclusion of a lactase inducing carbon source results in reduced inulase yields.

4.3.2 Inulase and Lactase Production in Continuous Cultures Grown on a Mixture of Two Carbon Sources

Since overall yields of inulase and lactase in batch cultures were low on mixed substrates, the continuous culture technique appeared to be the method of choice for yield improvements. In continuous culture a mixture of two sugars will be utilized simultaneously, providing that the critical dilution rate is not approached (Dean, 1972; Harder and Dijkhuizen, 1976). Confronted with two carbon sources the culture must

produce the enzymes required to metabolize each one. Therefore, to determine if continuous culture of K. fragilis on a mixture of complementary substrates results in simultaneous production of inulase and lactase, the enzyme levels in continuous cultures grown on three different pairs of sugars were measured (Figure 10). The sugar pairs chosen to be tested were those that caused dual enzyme production in batch culture: sucrose plus lactose, sucrose plus galactose, and fructose plus galactose.

The two cultures grown on sucrose containing media gave similar results (Figure 10A; 10B). The highest activities of both enzymes were considerably greater than those obtained in batch culture on the same sugar mixtures. However, neither culture produced inulase activities comparable to those on sucrose alone nor lactase levels as high as those obtained on lactose or galactose alone. The shape of the enzyme activity curves suggests that there is increased repression of both enzymes at higher dilution rates.

On the third mixed substrate, fructose plus galactose (Figure 10C), a dramatic peak in inulase activity occurred at a dilution rate of 0.14 h^{-1}. Peak inulase activity was of the same magnitude as that obtained in the fructose and fructose plus glucose grown continuous cultures (see Figure 5C; 5D). The highest lactase yield (about 1.2 units/mg) exceeded that obtained on the first two substrate pairs (about 1.0 units/mg) but was still slightly inferior to the highest activity in the continuous culture on galactose alone (see Figure 9).

Unfortunately, the inulase and lactase activity maxima in the fructose plus galactose culture did not occur at the same dilution

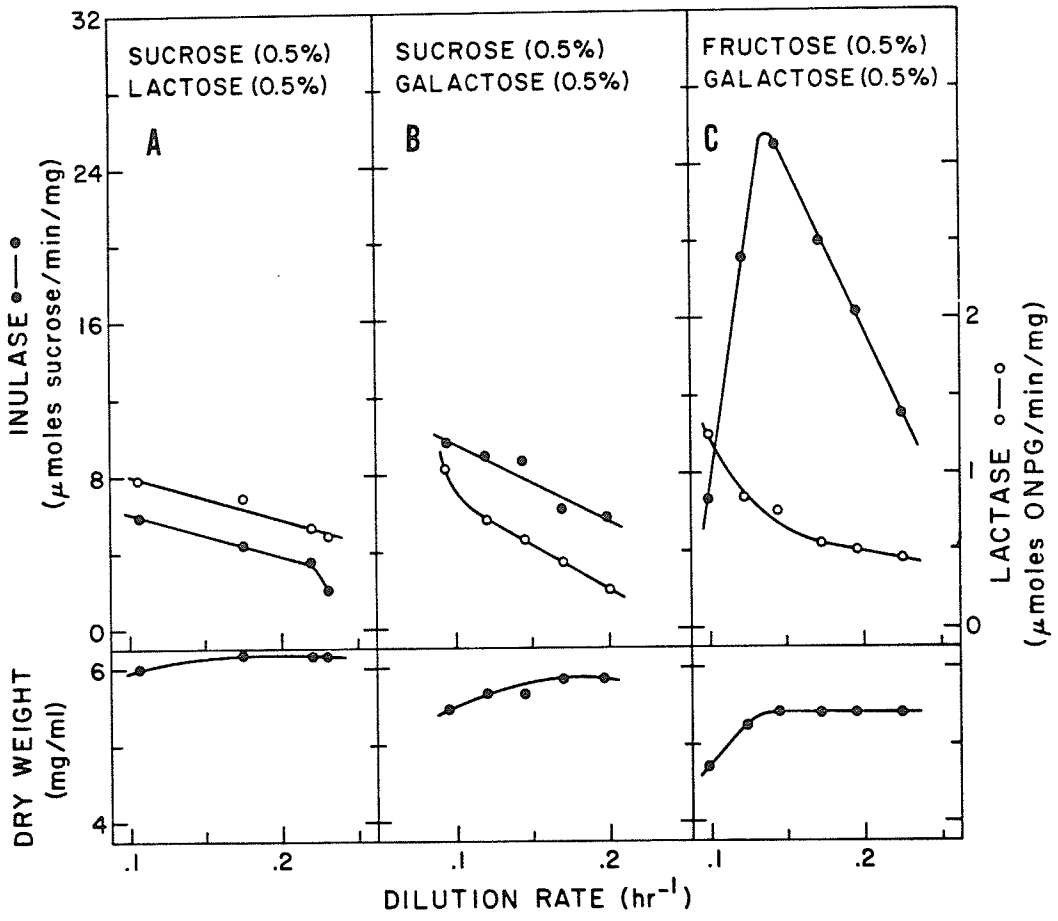

Figure 10. Inulase, lactase, and biomass yields as a function of dilution rate in 3-liter continuous cultures of K. fragilis grown on sucrose plus lactose (A), sucrose plus galactose (B), and fructose plus galactose (C).

rate. In fact, the lactase levels rapidly decreased over the range of dilution rates that the inulase levels increased. This may indicate a trade-off in which the ability to produce one enzyme is sacrificed in order to produce the other.

The high biomass yields obtained in each culture indicate simultaneous substrate utilization.

The results of the mixed substrate cultures indicate that K. fragilis can simultaneously produce inulase and lactase, perhaps with high yields of both. Two approaches to optimization of simultaneous dual enzyme production are described in Sections 4.3.3 and 4.3.4.

4.3.3 Gratuitous Induction of Lactase

Combinations of compounds other than carbon sources can be used to stimulate the simultaneous production of diverse enzymes in carbon limited continuous culture. For example, Sikyta and Fencl (1976) grew E. coli K 12 under growth limitation by glycerol and continuously added methylthiogalactoside (TMG), a non-metabolizable β-galactosidase inducer, to the culture with the result that high levels of β-galactosidase were produced. In this example, a non-repressive carbon source was used to support growth; however, it may have made no difference if a repressive carbon source was used because low steady state substrate concentrations might also have alleviated the repression. On this premise, an approach similar to that of Sikyta and Fencl (1976) was proposed. In this plan, a continuous culture growing at a low dilution rate on sucrose and producing high levels of inulase

would be fed a gratuitous lactase inducer to stimulate lactase production.

This proposal required first that a gratuitous inducer be identified and then tested in continuous culture.

4.3.3.1 Gratuitous Induction of Lactase in Batch Culture. In order to screen for gratuitous lactase inducers it was necessary to grow K. fragilis in batch culture on a non-repressive carbon source. The previously described lactase repression experiments (Section 4.2.2) indicated that glycerol, lactate, malate, and succinate are carbon sources that might not repress lactase production. Since Algeri et al (1978) used a succinate medium to demonstrate lactase induction by isopropylthiogalactoside (IPTG) in K. lactis, succinate was the first substrate tried.

Methyl-β-D-thiogalactoside (TMG), isopropyl-β-D-thiogalacto-pyranoside (IPTG), and galactose were all tested for lactase induction in K. fragilis cultures growing in a defined succinate medium. The increase in lactase content following addition of either galactose analog was so minute compared to the induction subsequent to galactose addition that it was questionable if any induction actually did occur.

The same experiment was attempted using a defined glycerol medium (Figure 11). Both TMG and IPTG caused increases in lactase content above the basal levels obtained on glycerol; however, both analogs were inferior to galactose as lactase inducers. Only galactose caused an increase in the specific growth rate of the culture indicating that the new carbon source was being utilized.

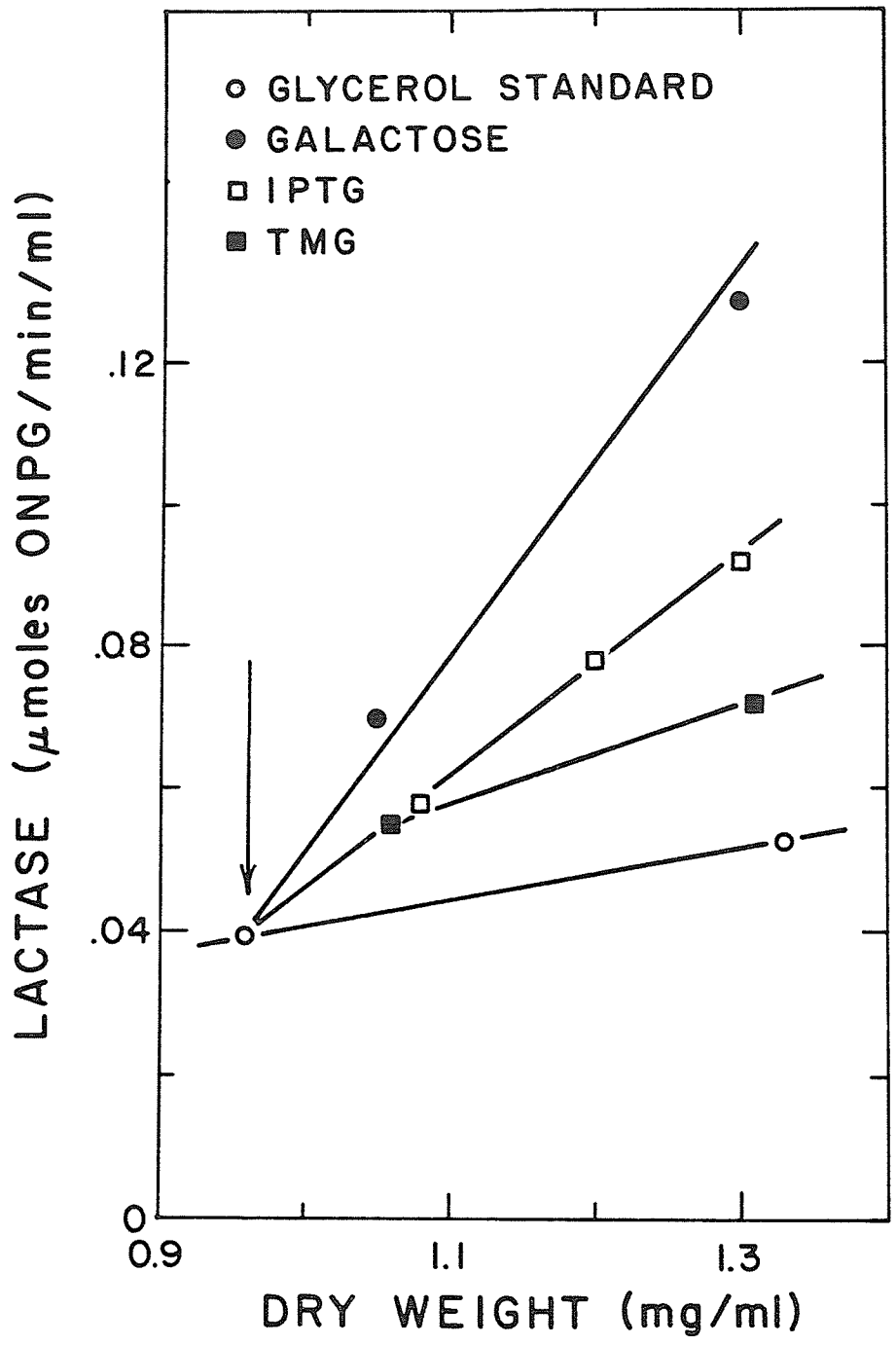

Figure 11. Lactase induction by galactose, isopropylthiogalactoside (IPTG), and methylthiogalactoside (TMG) in shake flask cultures of K. fragilis grown on glycerol. Effectors were added at the point indicated by the arrow.

The above results indicate that TMG and IPTG are lactase inducers in K. fragilis but do not induce as effectively as the metabolizable inducer, galactose.

4.3.3.2 Gratuitous Induction of Lactase in Continuous Culture. IPTG was tested as a lactase inducer in a continuous culture of K. fragilis under growth limitation by sucrose (Figure 12). Although a smaller culture volume of 225 ml was used, the high inulase activity that occurred before and after IPTG addition was consistent with the enzyme yields obtained in the 3-liter fermentation on sucrose at the same dilution rate (see Figure 5A). During IPTG addition, inulase levels were repressed with the degree of repression increasing with the concentration of the effector. The initial steady state lactase level was low, about 0.1 units/mg, and did not significantly change during the period of IPTG addition. Biomass yields remained constant throughout the experiment, while the pH underwent minor fluctuations from 3.5 to 3.65.

Very similar results were obtained when two other galactose analogs, fucose and thiodigalactoside, were similarly tested. Gas-liquid chromatography of hot water extracts of cells harvested at steady state revealed that 15% of the supplied thiogalactoside was cell associated. Fucose uptake was not measured, while IPTG uptake was assumed from batch culture induction experiments where IPTG uptake most probably preceded lactase induction.

The above results indicate that the presence of a non-metabolizable galactose analog in a sucrose limited continuous culture of K. fragilis represses the production of inulase. At the

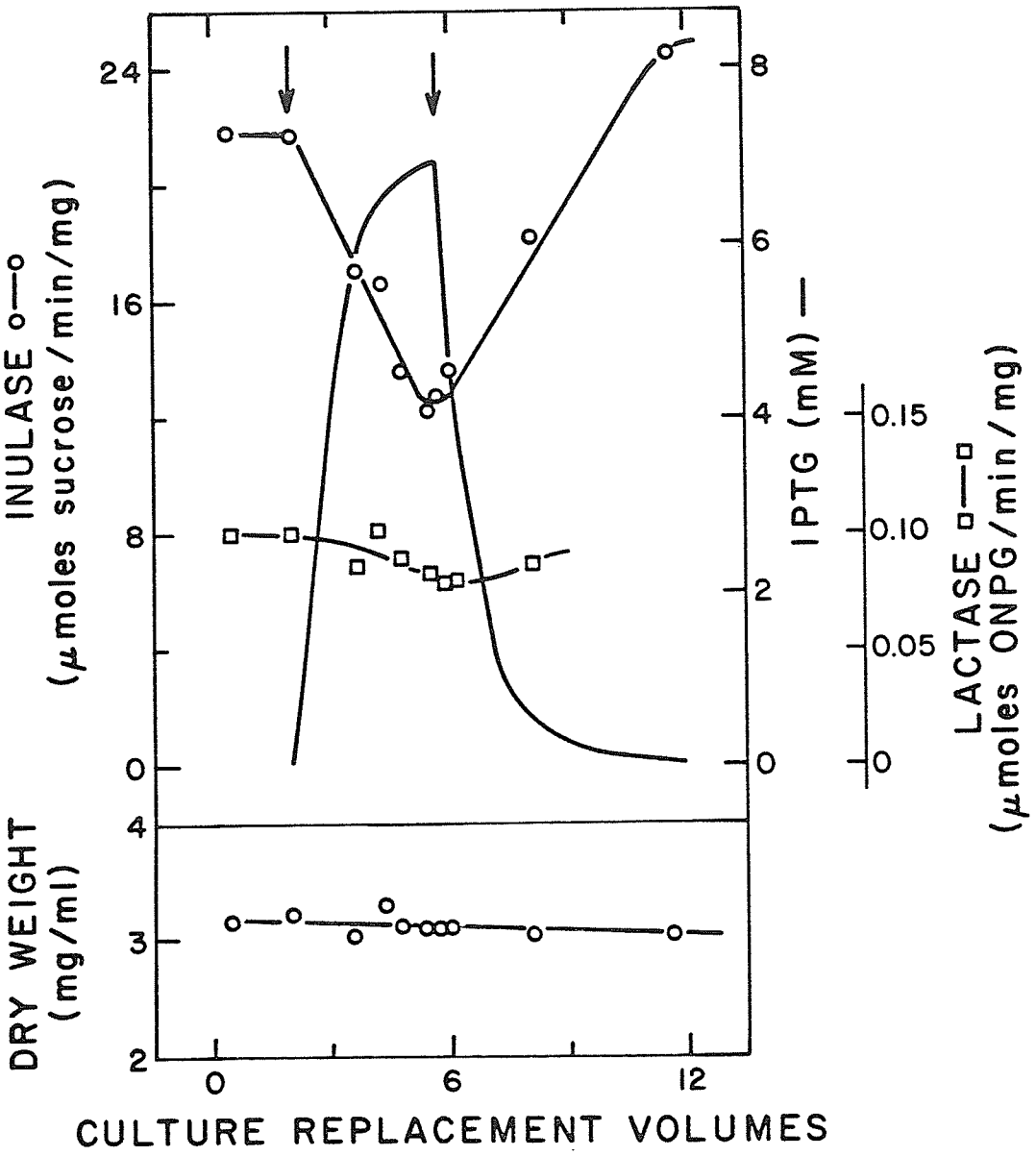

Figure 12. The effect of isopropylthiogalactoside (IPTG) on inulase, lactase, and biomass yields in a sucrose limited 225 ml continuous culture of K. fragilis. The culture was grown on 0.5% sucrose and 0.25% yeast extract. The dilution rate was 0.10 h^{-1} for the duration of the experiment and 14 mM IPTG was metered into the culture during the period delimited by the arrows.

same time, there is no induction of lactase, not even by IPTG which is a lactase inducer under derepressed conditions in batch culture.

4.3.4 Inulase and Lactase Production in Continuous Cultures Grown on Different Ratios of Fructose to Galactose

Continuous culture of K. fragilis in a medium containing equal concentrations of fructose and galactose caused high-level production of inulase and lactase (see Figure 10C). Unfortunately, the enzyme maxima occurred at different dilution rates. To determine if a different ratio of fructose to galactose might cause coincident high enzyme yields, an experiment was proposed in which a K. fragilis culture would pass through a series of steady states established on different ratios of these two monosaccharides. Initially, high inulase production would be encouraged by establishing the steady state at a dilution rate of 0.14 h^{-1} in a 1% fructose (1:0) and 0.5% yeast extract medium. Under these conditions inulase yields of approximately 28 units/mg had been previously obtained (see Figure 5C). Next, the medium reservoir would be exchanged for one containing 0.75% fructose, 0.25% galactose, (0.75:0.25), and a new steady state would be established. Media substitution would continue until only 1% galactose (0:1) was supplied as the carbon substrate. By starting the culture under conditions favouring inulase production and then slowly increasing the galactose content of the medium to induce lactase production, a balance was sought where the yields of both enzymes would be optimal. It is important to note that a dilution rate of 0.14 h^{-1} would not restrict lactase production, since K. fragilis

had previously produced more than 1.5 units/mg at this dilution rate on a 1% galactose medium (see Figure 9).

In the first attempt of this experiment the initial steady state yielded a meagre 12 units/mg of inulase. The first medium exchange resulted in even lower inulase levels. A second attempt of the procedure gave similar results. Why were the inulase yields so low compared to those previously obtained during growth on a fructose/yeast extract medium at a dilution rate of 0.14 h^{-1}? Previously, the initial steady state was not fixed at D=0.14 h^{-1}. Instead, cultures were started at dilution rates below 0.10 h^{-1} and two dilution rates were examined before steady states were established at dilution rates of 0.14 h^{-1} or higher. By this method, inulase production peaked at D=0.14 h^{-1}. Perhaps the initial low dilution rates are a prerequisite of high inulase production. Following this conjecture, the experiment concerning different ratios of fructose to galactose was redesigned to include a preparatory phase in which a K. fragilis culture growing on a 1% fructose medium would be passed through a series of six dilution rates ranging from 0.08 to 0.20 h^{-1}. The culture would then be returned to a dilution rate of 0.14 h^{-1} for initiation of the fructose to galactose procedure.

A peak in inulase production of about 28 units/mg at D=0.14 h^{-1} occurred during the preparatory phase. Reduction of the dilution rate from 0.20 to 0.14 h^{-1} resulted in a steady state inulase yield of about 30 units/mg. This high inulase producing culture was now ready to be tested on the different fructose to galactose ratios. Results from this phase of the experiment are presented in Figure 13. Biomass yields decreased almost linearly from

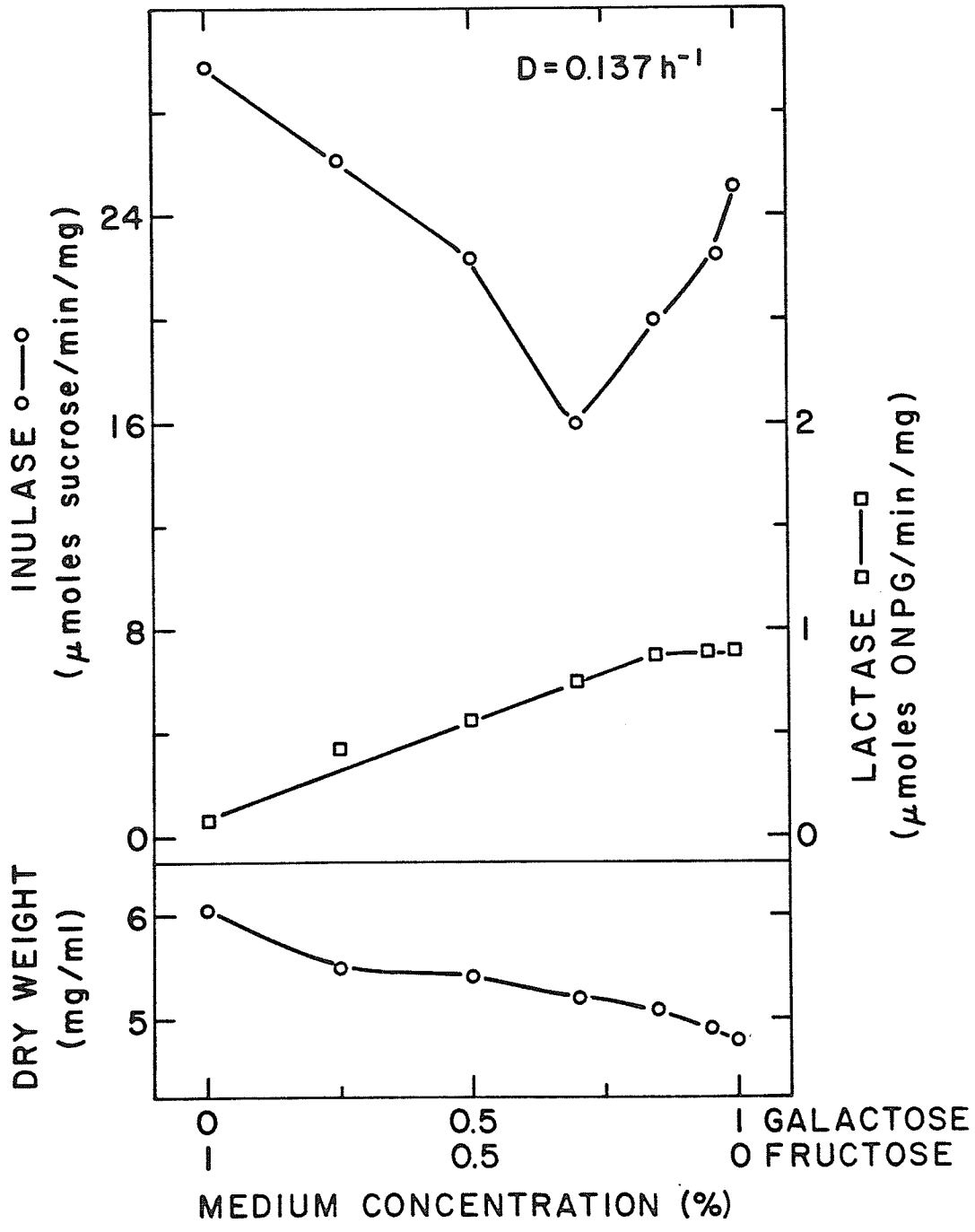

Figure 13. The effect of the relative medium concentrations of fructose to galactose on inulase, lactase, and biomass yields in a 3-liter continuous culture of K. fragilis.

6.0 to 4.9 mg/ml as the galactose content increased. The reason for this drop is not apparent. Lactase production increased from 0.08 to 0.90 units/mg as the proportion of galactose in the medium increased to 0.85. Further increases in galactose content caused no greater lactase yields. Inulase yields decreased from 30 to 16 units/mg as the galactose proportion was raised to 0.70. Then, unexpectedly, inulase production began an upturn until 25 units/mg were produced when galactose became the sole carbon substrate. The previous continuous culture of K. fragilis with galactose as the only carbon source (see Figure 9) at D=0.14 h^{-1} produced more lactase (1.5 units/mg) but had no detectable inulase.

It was odd that two K. fragilis continuous cultures growing at the same dilution rate on galactose media (Figure 9 versus Figure 13) could differ so vastly in inulase production. Microscopic examination of a culture sample from the galactose (0:1) medium steady state (Figure 13) revealed no bacterial contamination or detectable changes from normal K. fragilis morphology. Culture samples from the steady states at four sugar ratios (75:25, 15:85, 05:95, and 0:1) were tested for their ratios of hydrolysis rates of sucrose to inulin (S/I ratios). The resulting values were 12.5, 14.4, 11.2, and 12.7, respectively, indicating that the culture was continuously producing an inulase. These observations suggest that a pure culture of K. fragilis existed throughout the above experiment. However, the extreme difference in inulase production indicates a significant departure from the normal behavior of the stock culture; perhaps mutation to and selection for a new genotype occurred. This possibility is further examined in Section 4.3.4.

A clear-cut conclusion is difficult to draw from the above experiments. Uncritical examination of the results might suggest that a 1% galactose (0:1) medium is the medium of choice for optimal dual enzyme yields. To the contrary, previous results have demonstrated that this is a very poor medium for inulase production. What the results do imply is the involvement of complicating factors. For instance, certain culture manipulations appear to be required to promote high inulase yields. In addition, the stock culture seems to be unstable upon prolonged continuous culture and/or when the carbon substrate is gradually changed from fructose to galactose during continuous culture.

4.3.5 Inulase Production by the K. fragilis Isolates

The odd behavior of the culture towards the end of the continuous culture on media containing different ratios of fructose to galactose indicated a possible genotypic change in the culture and not just a phenotypic change caused by the various alternations in culture conditions. To test for such a change, culture samples from the galactose medium (0:1) of the preceding experiment were plated on inulin/YNB agar medium. Ten isolated colonies were picked and transferred to glucose/yeast extract agar slants. Four of these isolates were tested against the K. fragilis stock culture for inulase production in shake flask cultures using eleven different carbon substrates (Table 5). The isolates were superior inulase producers in every instance. On inulin, sucrose, glucose, and fructose the isolates produced from 4 to 7 units/mg more inulase activity than did the stock culture. Greater increases of 7 to 11 units/mg were obtained when

TABLE 5. Comparison of inulase yields from shake flask cultures of *K. fragilis* wild type and from four isolates grown on eleven different carbon substrates. Most media contained 1% carbon substrate and 0.5% yeast extract, however, those prepared with lactate, succinate, and malate contained 0.5% carbon substrate and 0.25% yeast extract.

Carbon substrate	Inulase (units/mg)				
	Isolate #1	Isolate #2	Isolate #3	Isolate #4	ATCC 12424
Inulin	19.1	18.9	16.2	19.1	12.0
Sucrose	9.1	8.5	7.8	8.6	2.2
Glucose	7.1	5.9	7.9	7.3	2.0
Fructose	6.2	7.3	7.0	6.4	1.9
Lactose	10.8	12.4	10.4	11.3	1.4
Galactose	9.9	9.4	7.7	9.1	0.7
Glycerol	28.7	29.2	32.7	32.0	2.5
Ethanol	19.0	18.3	15.3	17.4	1.8
Lactate	7.8	6.1	9.6	4.6	0.3
Succinate	16.5	11.9	19.0	10.4	0.3
Malate	18.3	14.1	19.9	14.1	0.4

lactose or galactose served as carbon substrate. These two sugars caused higher inulase levels in the isolates than did the other fermentable carbon substrates - a response opposite to that of the stock culture.

Of the non-fermentable carbon substrates, by far the most impressive increases in inulase production were obtained when the isolates were grown on glycerol. The inulase levels (28.7 to 32.7 units/mg) were of a magnitude previously obtained only in continuous culture and represented a 12-fold increase over the yields obtained with the stock culture grown on glycerol. Ethanol, succinate, and malate also supported large increases in inulase yields of 10 to 19 units/mg. Isolates grown on lactate, however, produced inulase at similar level to those obtained on fermentable carbon sources.

The observed variation in inulase levels among the isolates when grown on different carbon sources may not be due to genetic differences, but simply due to variability in the tests. It should not be surprising that the isolates are the same since they were selected from a culture that appeared to be at steady state.

The above results demonstrate that the isolates differ significantly from the stock culture of K. fragilis. An extension of this finding is that, during the continuous culture using different fructose to galactose media ratios, a mutant with altered inulase regulation arose and displaced the stock culture. The isolates may be constitutive for inulase since they produce it at elevated levels, even on substrates that give very low yields in the stock culture of K. fragilis. Catabolite repression is still involved, however, since growth on the more rapidly metabolized substrates results in lower enzyme yields.

5. DISCUSSION

The objective of this study was to optimize simultaneous inulase and lactase production in K. fragilis. Although an unequivocal optimum for dual production of these enzymes was not determined, considerable improvements in individual and dual yields were obtained. Most significant increases in enzyme yields resulted from the implementation of continuous culture techniques, although utilization of specific carbon sources also had a dramatic effect.

Before discussing the improved enzyme yields, the regulation of inulase and lactase will be discussed in light of the information contained in the literature and of findings of the current study.

There was an accumulation of evidence supporting the role of catabolite repression in inulase regulation in K. fragilis. Firstly, batch cultures grown on simple sugars (substrates that allowed rapid growth) only elaborated inulase in a sudden burst towards the end of the growth phase. Presumably, this would coincide with carbon source depletion as the cultures were carbon limited. Thus the diminishing substrate concentration resulted in decreasing repression allowing for some inulase synthesis. Secondly, rapidly metabolizable carbon substrates caused severe inulase repression upon addition to batch cultures growing on inulin. Thirdly, compared to batch cultures, glucose, fructose, and sucrose limited continuous cultures produced superior inulase levels, presumably due to minimized steady-state

substrate concentrations. Furthermore, in continuous cultures growing at a fixed dilution rate on sucrose or fructose, inulase levels dropped when growth was no longer limited by the carbon substrate. Fourthly, continuous culture profiles of inulase activity versus dilution rate implicated catabolite repression.

Hackel (1975) suggests that sucrose hydrolysing enzymes in yeasts are regulated solely through catabolite repression since no inducer has been identified. For K. fragilis, however, the possibility that inulase is inducible cannot be eliminated. Induction of K. (Sacch.) fragilis inulase was suggested by Snyder and Phaff (1960), who measured relative inulase levels among aerobic batch cultures grown on different carbon sources. Their data are paralleled by those obtained in the present study. Cursory examination of either set of results reveals that the highest inulase levels occur in inulin grown batch cultures, suggesting that inulin is an inducer. This is not unreasonable because catabolic enzymes are frequently inducible by their natural substrates or by direct metabolites of their natural substrates. True inducers, however, operate at the gene level - a qualification that precludes inulin as an inducer as it cannot cross the plasma membrane (Barnett, 1976). Induction by sucrose, another natural inulase substrate, must also be eliminated since it cannot traverse the plasma membrane (Barnett, 1976), nor does it appear inside K. fragilis cells (De La Fuente and Sols, 1962).

Although inulin and sucrose are improbable inulase inducers, induction was implicated in the present study. Initially, the influence of specific carbon substrates was demonstrated in batch culture experiments where growth on glucose, sucrose, and fructose

caused consistently higher inulase levels than did growth on lactose or galactose. Subsequent continuous cultures limited by sucrose, glucose, fructose, or substrate pairs containing at least one of these sugars produced inulase at levels far exceeding those of batch cultures on the same substrates. Conversely, it made no difference if lactose or galactose limited cultures were grown in the batch or continuous mode; even at low dilution rates, where catabolite repression is minimized, almost no inulase was produced.

Chemostat studies further indicated that the nature of the carbon substrate is critical. Despite an intimate relationship among glucose, mannose, and fructose catabolisms, continuous cultures on these sugars resulted in significant differences in inulase levels and in the change in inulase levels with increasing dilution rate. Only fructose promoted truly high enzyme levels and did so at an intermediate dilution rate which suggests regulation by repression and induction (Clarke et al, 1968; Dean, 1972; Toda, 1976b).

The inducer may be fructose, or an immediate metabolic derivative of fructose, as suggested by the following evidence:

(1) Only chemostat cultures containing fructose, supplied as the free sugar or as the fructosyl moiety within sucrose, produced truly high inulase levels.

(2) Continuous cultures fed free fructose, either alone or in combination with other carbon sources, exhibited maximum enzyme levels at the same intermediate dilution rate.

(3) Continuous culture on mannose, a sugar absorbed and catabolized like fructose except for a single isomerization reaction (Barnett, 1976), did not appear to induce inulase nor did it cause high enzyme levels.

Continuous cultures fed either 1% fructose or 0.5% fructose plus 0.5% glucose or galactose produced inulase maxima of similar magnitudes at the same intermediate dilution rate (0.14 h^{-1}). These coincidences are not unexpected if fructose is a unique substrate. Continuous culture theory predicts that the fructose culture concentration should be the same in each of the above chemostats at a given dilution rate. This occurs because the steady state sugar concentration depends upon the dilution rate but not upon the sugar concentration of the nutrient feed. A different steady-state fructose concentration will result in cultures fed sucrose despite extracellular hydrolysis to an equimolar ratio of fructose to glucose. The required hydrolytic reaction might cause a shift in the fructose to glucose ratio. As a result, the sucrose limited K. fragilis continuous culture did not pass through an inulase maximum at $D=0.14h^{-1}$; instead, it produced high enzyme levels at lower dilution rates. A. Davies (1956a), who measured only cell-bound enzyme, reported a peak in invertase (inulase) production for a sucrose limited K. (Sacch.) fragilis culture at a dilution rate of $0.09h^{-1}$. If lower dilution rates had been examined in the present study, perhaps a peak in total inulase may have been detected.

If inulase regulation involves both induction and repression it is not unique. Several yeast enzymes involved in sugar catabolism are regulated in this manner. Regulation of β-glucosidase in K. (Sacch.) lactis may be the most similar to that of inulase in K. fragilis. The β-glucosidase is induced by glucose at concentrations less than 1 mM but is severely repressed at concentrations greater than 10 mM (Herman

and Halvorson, 1963). Fructose may play an analogous role in *K. fragilis* inulase regulation.

Inulase production in response to fructose availability is particularly well suited to efficient fructan utilization by *K. fragilis*. In the presence of abundant free fructose or other rapidly metabolizable substrates such as glucose or lactate, there would be no need to produce inulase. Upon depletion of the good carbon substrate the yeast would be forced to seek other substrates. Some enzyme production in response to derepression would be necessary. The discovery of sucrose or inulin would initially result in small amounts of free fructose to induce more inulase. The level of inulase production would be mediated by the nature of the fructan. Since each hydrolysis of sucrose liberates two readily utilized monosaccharides, the demand for more enzyme would be minimal. However, as the length of available oligofructans approaches that of inulin, the frequency at which two sugars are liberated in a single hydrolytic reaction decreases. This would cause reduction in the apparent substrate concentration and a reduction in catabolite repression. In addition, longer polyfructans are not as accessible (Snyder and Phaff, 1960; GrootWassink and Fleming, 1980) and the yeast would send more inulase into solution.

The ability to simulate increasing polyfructan length to the extreme is the reason that application of fructose limited continuous culture to *K. fragilis* inulase production was successful. Repression by fructose could be minimized, induction maximized, and interference by glucose eliminated.

The regulation of lactase in K. fragilis resembles that of inulase. Catabolite repression was implicated by the sudden, late log phase appearance of lactase in batch cultures, by the suppression of lactase production in galactose growing cultures upon addition of other readily metabolizable substrates, and by the changes in lactase production with increasing dilution rate in galactose and lactose limited continuous cultures. The special inducing role of galactose and lactose was also demonstrated since only cultures grown on these sugars, whether batch or continuous, produced high levels of lactase. These findings, which implicated dual control by induction and repression, concur with the relevant literature (De La Fuente and Sols, 1962; R. Davies, 1964; Mortimer and Hawthorn, 1971; Barnett, 1976).

Although less effective than galactose or lactose, two non-metabolizable galactose analogs, TMG and IPTG, also induced lactase. This suggests that the true lactase inducer might be galactose or a galactose derived metabolite.

In studies of lactase induction in K. lactis, Dickson and Markin (1980) also found the natural sugars to be the best inducers. These workers further demonstrated that maximum lactase production requires log growth, a constant energy source, and constant presence of an inducer. Batch cultures of K. lactis require seven to nine cell doublings (11 to 14 h of log growth) to attain maximum lactase levels. Onset of stationary phase results in a rapid decrease in lactase activity . If the same requirements apply to K. fragilis, then the galactose and lactose batch cultures examined in the current study (which experienced log growth for only 6 to 8 h) had insufficient time

for full lactase production. This may also be the reason that Wendorff et al (1970) and Mahoney et al (1974) found that maximum lactase levels occurred in K. fragilis batch cultures supplied with abundant lactose (15%). The high sugar concentration could allow for a greater number of cell doublings.

If lactase regulation in K. fragilis parallels that in K. lactis, then the inherent advantages of continuous culture for producing lactase become apparent. In lactose or galactose limited continuous culture there would be continuous growth, a constant energy source, and a constant supply of inducer. Since galactose and lactose also repress lactase, catabolite repression could also be minimized.

Simultaneous enzyme production occurred in cultures fed an inulase inducing plus a lactase inducing sugar. Under the conditions of reduced catabolite repression in continuous culture, the monosaccharide pair of fructose plus galactose supported the highest dual enzyme yields. Considering the regulation of each enzyme this is not unexpected, but is intriguing, since neither enzyme appears to be required to metabolize either monosaccharide. Such wasteful metabolite production heightens the value of a commercial fermentation process (Demain, 1971a).

The controls governing inulase and lactase biosynthesis in K. fragilis are not wholly independent. This became evident from the examination of cultures grown on two complementary carbon substrates. In batch cultures, inulase levels suffered in the presence of lactose or galactose, whereas lactase levels were affected to a lesser degree by the inclusion of sucrose or fructose. This may result from preferential utilization of the inulase inducing over the lactase

inducing substrates. In this situation, subsequent to sucrose or fructose depletion, the less favoured substrate will have persisted and repressed further inulase production. Therefore, the reinforcing stimula of reduced repression and low inulase inducer concentration could not coincide.

Although two-substrate continuous cultures produced more of both enzymes, simultaneous maximal production was not observed. Instead, a trade-off occurred in which production of one enzyme was partially sacrificed to allow for increased production of the other. For example, in the equimolar fructose plus galactose continuous culture, lactase levels fell as inulase levels rose with increasing dilution rate. Additionally, as the galactose proportion increased during the early part of the fructose to galactose feed ratio experiment, falling inulase levels were countered by increased amounts of lactase. Regardless of the substrate pair, lactase levels never equalled those attained by cultures grown on lactose or galactose alone. Similarly, inulase levels were low except when free fructose was supplied.

Perhaps the trade-off during simultaneous induction resulted from competition for a common intermediate required for the biosynthesis of each enzyme. For example, supplemental cAMP allowed for simultaneous maximum β-galactosidase and tryptophanase production in continuous cultures of an E. coli K 12 mutant in which the β-galactosidase was ordinarily repressed (Pavlasova et al, 1980). Alternatively, a more direct interaction between the complemetary sugars may be involved. Some sort of competitive inhibition may explain the severe inulase repression upon addition of non-metabolizable galactose analogs to sucrose limited continuous cultures.

Industrial enzyme production is conventionally performed using batch cultivation. However, as alternative fermentation techniques improve their application must be considered. The important criteria to be evaluated in assessing the merits of an enzyme production method are volumetric productivity, conversion yield of product from the most expensive substrate in the medium (usually the carbon substrate), and final product concentration (Wang et al, 1979). Continuous culture has been criticized because final product concentrations are usually lower than those achieved in batch culture with the result that recovery costs are inflated. In the present study, maximal inulase and lactase levels of batch cultures were only 33% and 18%, respectively, as high as those attained in continuous cultures. Consequently, volumetric productivities (enzyme units/ml-hour) were much higher for the continuous system. Values for the volumetric productivities of the batch and continuous cultivations that produced the highest levels of inulase and lactase are presented in Table 6. The calculated volumetric productivities ignore down time between production runs and the obligatory batch growth prior to initiation of nutrient feed for continuous cultures. The first consideration reduces batch culture productivity because of more frequent down periods, while the second decreases the efficiency of the continuous process. Further optimizations of either fermentation system are also not considered. For example, inoculation with starter culture in the log growth phase would eliminate the initial lag phase of batch cultures and perhaps double their volumetric productivity. Likewise, a chemostat run at a higher dilution rate may have increased enzyme output.

TABLE 6. Volumetric productivities and efficiencies of substrate conversion for batch and continuous cultures of *K. fragilis* wild type and *K. fragilis* isolates.

Yeast strain	Enzyme	Cultivation technique	Carbon substrate(s)	Substrate concentration (mg/ml)	Volumetric enzyme activity (units/ml)	Hours to gain max. activity or dilution rate (h^{-1})	Volumetric productivity (units/ml-h)	Efficiency of substrate conversion (units/mg)
K. fragilis ATCC 12424	inulase	batch	inulin	10	53	20	2.7	5.3
			inulin*	20	120	18	6.7	6.0
			fructose	10	8.4	16	0.5	0.8
			fructose + galactose	10	4.6	17	0.3	0.5
		continuous	fructose	10	159	0.14	22.3	15.9
			fructose + galactose	10	135	0.14	18.9	13.5
	lactase	batch	lactose	10	1.4	18	0.1	0.14
			galactose	10	1.4	18	0.1	0.14
			fructose + galactose	10	1.5	17	0.1	0.15
		continuous	lactose	10	7.6	0.14	1.1	0.76
			galactose	10	8.0	0.14	1.1	0.80
			fructose + galactose	10	3.8	0.14	0.5	0.38
isolate #4	inulase	batch	inulin	10	97	24	4.1	9.7
#1			sucrose	10	40	12	3.3	4.0
#2			lactose	10	61	18	3.4	6.1
#3			glycerol	10	82	35	2.3	8.2
combined isolates**	inulase	continuous	galactose	10	123	0.14	17.2	12.3
	lactase		galactose	10	4.4	0.14	0.6	0.44

* Values taken from GrootWassink and Fleming (1980).

** Values obtained from the final medium of the fructose to galactose feed ratio experiment.

The volumetric productivities might increase if higher carbon substrate concentrations are used. Evidence in this regard comes from batch cultures of K. fragilis ATCC 12424 grown on 2% inulin and 1% yeast extract by GrootWassink and Fleming (1980). The volumetric productivity of these cultures was more than double that of cultures grown on 1% inulin. As discussed previously, higher initial substrate concentrations are also likely to augment lactase yields in batch cultures. Continuous cultures should show similar improvements since the growth limiting substrate concentration in the culture vessel is independent of its concentration in the nutrient feed. This means that greater substrate supplies should not cause more repression of specific enzyme activity at a given dilution rate.

Although neither the batch nor continuous cultures were optimized, the improvements in volumetric productivities obtained in the present study through use of continuous culture are impressive. A fructose limited chemostat had a volumetric productivity 25 times greater than a similarly limited batch culture and 8 times greater than an inulin grown batch culture. Lactase productivity was 11 times greater in lactose and galactose limited continuous cultures. Furthermore, the values for dual enzyme production in fructose plus galactose grown cultures are significantly better in the continuous system. Even the inulase hyperproducing isolates grown batchwise on inulin are only one-fifth as productive as a fructose limited continuous culture of the stock K. fragilis strain.

Finally, the two fermentation processes should be evaluated based upon their efficiencies of substrate conversion into inulase and lactase. In all instances continuous culture was superior (see

Table 6). Of particular note are the high conversion efficiencies of the fructose plus galactose limited chemostat; 13.5 inulase units and 0.38 lactase units resulted from each mg of sugar. Batch cultures did not produce either enzyme at equal efficiencies.

Optimism concerning the potential industrial application of continuous culture to simultaneous inulase and lactase production must be tempered despite the above mentioned merits. In the present study all carbon substrates were highly pure and unquestionably too expensive for commercial use. Perhaps the individual production of inulase or lactase using continuous culture is viable since cheap sources of sucrose (molasses) and lactose (whey) are available. Economical whey based media have already been shown to encourage good lactase yields in batch cultures of K. fragilis (Wendorff et al, 1970; Mahoney et al, 1974; DeBales and Castillo, 1979). Unfortunately simultaneous enzyme production faces greater financial barriers since a mixture of two expensive sugars, fructose and galactose, appears necessary. Had the addition of gratuitous lactase inducers to sucrose limited continuous cultures caused high level production of both enzymes, this procedure could have been a reasonable alternative. Despite the cost of the galactose analogs, their use could be justified if they could be readily recovered and reintroduced into the culture vessel.

Greater industrial application of continuous culture has been limited by several other drawbacks (Wang et al, 1979). Suitable equipment is lacking and owners of existing fermentation facilities are reluctant to incur the expense of retooling. As new facilities are constructed, however, companies are more likely to provide for continuous fermentation processes. Secondly, since it is necessary to

maintain sterile conditions for prolonged periods, there is a fear of regular production losses to contamination. Improvements in equipment design and methods are helping to overcome this problem. Additional problems include culture unpredictability and mutation of stock cultures; both were encountered in the present study. The unpredictability was manifested in the phenotypic differences among continuous cultures under identical conditions but which were developed in different manners. For example, in fructose limited chemostats, hysteresis with respect to inulase activity was observed between dilution rate shift-up and shift-down experiments. In shift-up fructose limited continuous cultures, a development phase using low dilution rates was required before maximum inulase levels were attained. Special developmental phases diminish the efficiency of the continuous fermentations since extra time elapses before commencement of maximum enzyme production.

Mutation of the K. fragilis stock culture and selection for the mutants in a chemostat were confirmed in one instance and were likely, but unconfirmed, in another. Selection for mutants in the fructose to galactose feed ratio experiment prevented determination of a sugar mixture for optimum simultaneous enzyme levels. However, from this experiment isolates were obtained which seem to produce inulase constitutively. Selection for such isolates was fortunate since degeneration to less productive strains is common (Jannasch and Mateles, 1974).

Although mutation is generally considered to be a problem in continuous culture, the constitutive isolates obtained in the present study may prove more useful than the stock culture. For instance,

lactose or galactose might serve as the sole carbon substrate in continuous cultures to promote high dual levels of inulase and lactase. The mutant culture in a galactose limited chemostat (i.e. at the time the isolate were obtained) had volumetric productivities of 17.2 units/ml-h and 0.62 lactase units/ml-h while the efficiencies of galactose conversion were 12.3 inulase units/mg and 0.44 lactase units/mg. These values are equivalent to those obtained in the fructose plus galactose (50:50) limited chemostat of the stock culture K. fragilis. As a further advantage, constitutive enzyme production may allow for use of higher dilution rates to increase volumetric productivities.

The importance of mutants with altered mechanisms of induction and catabolite repression has been stressed by Demain (1971a). Improved strains derived from the K. fragilis mutants of the present study could be developed by further random mutation and selection or through genetic engineering. The fact that K. fragilis is of commercial value in the production of single cell protein (Kulp, 1975; Laskin, 1977) and also produces a pectinase suggests that a diverse organism capable of forming several products in a single fermentation might be feasible.

CONCLUSION

K. fragilis is capable of simultaneously producing inulase and lactase in a single-step fermentation. Both enzymes are carbon catabolite repressible and appear to be inducible by specific carbon substrates. As a result, cultures grown on a mixture of fructose (inulase inducing) and galactose (lactase inducing), and experiencing reduced catabolite repression in continuous culture, produce good levels of both enzymes. The continuous culture technique is superior to batch culture for the production of inulase and lactase, either separately or simultaneously, by *K. fragilis*. Mutant strains of *K. fragilis* are more likely to find industrial application, and those isolated in the present study may be used in further studies to optimize a multi-product fermentation.

Suggestions for future investigations include: determination of the molecular basis of inulase and lactase regulation, including identification of the true inducers; determination of the nature and stability of the mutants which appear to be inulase constitutive; examination of the mutants in continuous culture; and, diversification of the fermentation to include other products. Of a more applied nature are the following suggestions: optimization of substrate concentrations for maximum volumetric productivity in continuous cultures; extraction, separation, and purification of inulase and

lactase from K. fragilis cultures; utilization of more economical carbon substrates; and, investigation of multi-stage continuous culture.

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