IOWA STATE UNIVERSITY
Digital Repository

1972

Beta-Galactosidase activity of Propionibacterium shermanii

James Charles Hartley
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Food Microbiology Commons, and the Microbiology Commons

Recommended Citation

Hartley, James Charles, "Beta-Galactosidase activity of Propionibacterium shermanii" (1972). *Retrospective Theses and Dissertations*. 5209.
https://lib.dr.iastate.edu/rtd/5209

www.manaraa.com

INFORMATION TO USERS

β-Galactosidase activity

of Propionibacterium shermanii

by

James Charles Hartley

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Food Technology

Approved:

In Charge of Major Work

For the Major Department

For the Graduate College

Iowa State University
Ames, Iowa

1972

PLEASE NOTE:

Some pages may have

indistinct print.

Filmed as received.

University Microfilms, A Xerox Education Company

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Propionibacteria are taxonomically interesting and economically important bacteria. Their natural habitat is the digestive tract of ruminants and, possibly, other mammals. They are frequently found in such fermenting natural products as silage or whey. Because of their common occurrence in milk and their relatively unique metabolism, it is not surprising that they should be responsible for both beneficial and undesirable results in cheese curing. Although of much less commercial significance, propionibacteria may be used to synthesize vitamin B_{12} and, also, propionic acid which is used in perfumes and solvents.

Propionibacteria play an important part in ruminant nutrition. Occurring in large numbers in the rumen, they ferment carbohydrates and lactic acid formed by other rumen microorganisms to carbon dioxide and acetic and propionic acids. These fatty acids plus butyric acid serve as the principal energy source for ruminants (171, p. 550).

The major commercial use of propionibacteria is in the manufacture of Swiss cheese, which is an important cheese variety in the United States (178). In the earlier prototypes of what we now call Swiss cheese, and, indeed, even today in Swiss cheese manufactured in other countries of the world, the necessary propionibacteria were either present in the raw milk or were added as contaminants in the rennet prepared by steeping calf stomachs in whey. The common practice in the United States now is to inoculate pure cultures of propionibacteria into the milk.

The homofermentative starter organisms (lactic streptococci and Streptococcus thermophilus) and lactobacilli, also added during the

manufacture of Swiss cheese, produce lactic acid from the lactose of the vat milk. The propionibacteria then utilize the lactic acid during "hot-room" curing to form acetic and propionic acids, carbon dioxide, and water according to the following formula:

$$3CH_3\overset{\overset{\displaystyle OH}{|}}{C}HCOOH \longrightarrow CH_3COOH + 2CH_3CH_2COOH + CO_2 + H_2O$$
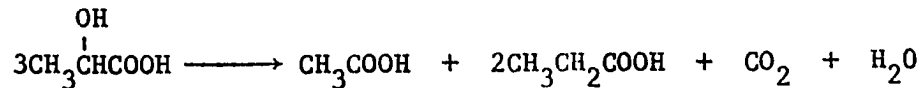
Lactate is oxidized to pyruvate by lactate dehydrogenase in the presence of the oxidized coenzyme nicotinamide adenine dinucleotide (NAD^+). The pyruvate then enters the propionic-acid fermentation pathway of Allen et al. (2) shown in Fig. 1. Acetic and propionic acids contribute to the characteristic flavor of Swiss cheese and the carbon dioxide collects in weak areas of the curd to form spherical openings called "eyes".

All species of Propionibacterium, except the pigmented species, could be used for Swiss cheese manufacture, provided they do not induce off-flavors. The pigmented species frequently form visible colonies which appear as undesirable pink, brown, or red spots. Some strains, able to grow at low temperatures, contribute to the "split" defect of Swiss cheese by producing too much carbon dioxide during the later stages of curing.

The Dairy Microbiology section of the Food Technology Department, Iowa State University, has been active in research on members of the genus Propionibacterium. Some of these investigations have been related to their previously unrecognized ability to grow at low temperature (140), their relationship to the split defect of Swiss cheese (83, 139), their inhibition by the globulin fraction of milk and whey (183), their production of capsules and loose slime (169), their production of diacetyl (107), and the taxonomic interrelationships of the various species within the

Acetyl-P $\longrightarrow$ acetate + ATP

$\uparrow$ +P$_i$

Acetyl-CoA + DPNH + CO$_2$

$\uparrow$ DPN
CoA

Pyruvate $\longrightarrow$ "C$_1$" $\longleftarrow$

$\uparrow$

P-enol   +CO$_2$ Me-malonyl-CoA (a)
pyruvate $\longrightarrow$ Oxaloacetate
P$_i$ PP$_i$ DPNH

(glycolysis)   Malate   Me-malonyl-CoA (b)   Propionyl-CoA

Glucose

Fumarate

ADP + P$_i$ FPH$_2$ Succinyl-CoA

ATP   FP

Succinate $\longrightarrow$ "CoA" $\longleftarrow$ Propionate

3
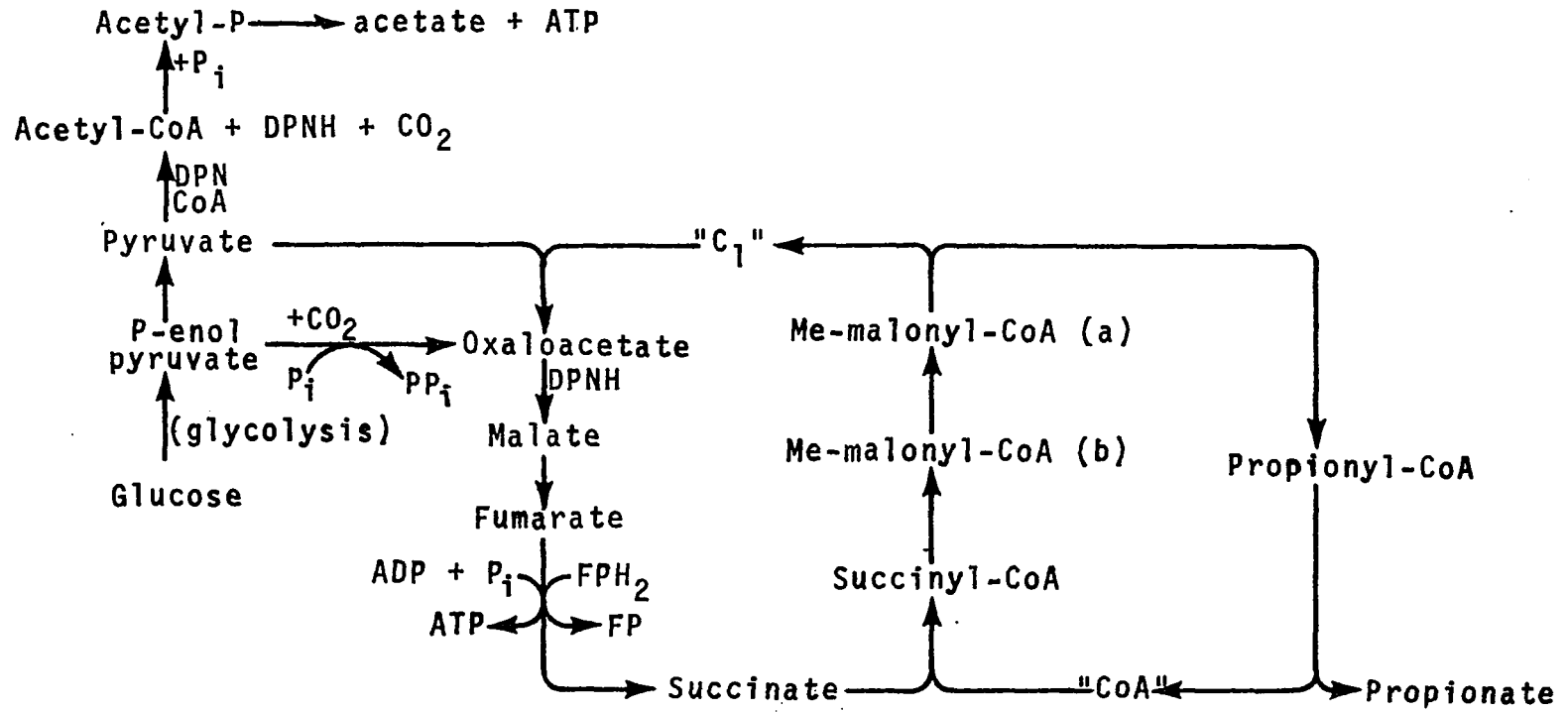
Fig. 1. Reactions of the propionic-acid fermentation and the formation of acetate, propionate, carbon dioxide, and ATP

genus (122). The taxonomic study, conducted by Malik et al. (122), sub-
stantiated the earlier observation (16) that the only difference between
Propionibacterium shermanii and Propionibacterium freudenreichii is in
lactose-utilizing ability. Propionibacterium shermanii can ferment lac-
tose, while P. freudenreichii cannot. Furthermore, all other species of
propionibacteria are known to utilize lactose (16). In a current investi-
gation in our laboratory, DNA base ratios of Propionibacterium species
are being determined to supplement earlier taxonomic investigations.

The question immediately arose, "Is P. freudenriechii deficient in β-
galactosidase or in the specific permease responsible for transporting
the substrate lactose into the cell?" The enzyme β-galactosidase (E.C.
3.2.1.23, β-D-galactoside galactohydrolase) hydrolyzes lactose into glu-
cose and galactose. Although considerable work has been done on the nu-
trition and metabolism of propionibacteria (82), very few investigations
have been made on the β-galactosidase of this important genus.

Lactose utilization by these bacteria has usually been determined by
using lactose broth containing bromthymol blue indicator to detect pH
change. This procedure is not as sensitive as the enzymatic method of
Lederberg (106) which uses the chromogenic substrate o-nitrophenyl-β-D-
galactopyranoside (ONPG). The enzyme β-galactosidase hydrolyzes the β-
linkage of ONPG to yield galactose and o-nitrophenol (ONP), a colored com-
pound with maximum absorbance at 420 nm. It is possible that P. freuden-
reichii might possess a low level of enzyme activity that results in insuf-
ficient acid to change the color of the indicator in lactose broth but
which could probably be detected by the sensitive ONPG hydrolysis technique.

Knowledge of the exact reason for the failure of P. freudenreichii to

actively utilize lactose would provide important taxonomic information on these two closely related _Propionibacterium_ species. Because the β-galactosidase activity of P. _shermanii_ and P. _freudenreichii_ has not been studied previously, and because such a study would supplement the previous accomplishments in our laboratory, this study was undertaken. Results of this investigation should be of taxonomic, academic, and economic importance.

LITERATURE REVIEW

Because β-galactosidase is a very stable enzyme, it has been a favorite for the enzymologist. Consequently, there has been a vast amount of work done using this enzyme system. Escherichia coli has a high level of β-galactosidase activity, is easy to cultivate, and undergoes genetic recombination. Therefore, it has been used for many of the investigations with β-galactosidase. The literature, however, contains few references to the study of Propionibacterium in this context.

To orient the reader who may not be fully familiar with this enzyme system, a brief discussion of the significance and distribution of β-galactosidase is given.

β-Galactosidase

β-Galactosidase (E.C. 3.2.1.23, β-D-galactoside galactohydrolase) (67) hydrolyzes lactose (4-0-β-D-galactopyranosyl-D-glucopyranose) (28, p. 161) into glucose and galactose. β-Galactosidase was called lactase in earlier literature (12, 28, 126, 184). Knopfmacher and Salle (97) credit Beyerinck (12) with naming this enzyme lactase. Many authors continue to refer to β-galactosidase activity on lactose as lactase activity (46, 160, 179, 199, 200).

In addition to hydrolytic activity, β-galactosidase possesses transfer activity. That is, the galactose moiety of the galactoside molecule may be transferred to water (the hydrolytic reaction), or to some other hydroxylic acceptor (such as another sugar or an alcohol (9, 19, 52, 141, 142, 143, 156, 173, 174, 184, 186, 190, 191, 194).

Specificity of β-Galactosidase

Pigman (144) reported that β-galactosidase hydrolyzes β-D-galactosides, α-L-arabinosides, lactose, and β-D- and L-glycero-D-galacto-aldo-heptosides. He was not certain if β-D-fucosides and α-D-galactouronides would be hydrolyzed. β-Galactosidase is inactive on sugars not possessing the β-galactosidic configuration (36) but exhibits strict specificity requirements for the structure of the glycon part of the substrate molecule (189, 190, 195). Only changes in substituents on C-5 of the D-galactose (glycon) residue are compatible with hydrolyzability. β-Galactosidase shows great tolerance for changes of the aglycon part of the molecule, which may be another sugar residue, an alkyl group, or an aryl group (190). The hydrolysis rate, however, is affected by the aglycon (100, 144, 184).

Distribution in Nature

β-Galactosidase is widely distributed in nature. Veibel (184) and Wallenfels and Malhotra (189, 190) reviewed its distribution in plants, animals, birds, insects, and microorganisms.

β-Galactosidase in plants

Wehmer and Hadders (196) have listed the families of the plant kingdom in which the enzyme is known to occur. The function of β-galactosidase in plants is believed to be to hydrolyze glycosides which have the β-linkage (181). It also catalyses synthesis (transferase) reactions in plants (173).

β-Galactosidase in animals

The primary site of β-galactosidase activity in animals is the intestine (1, 3, 21, 34, 46, 58, 76, 121) where it hydrolyzes lactose from the diet. The enzyme also is present in the pancreas, kidney, adrenal, thyroid, spleen, liver, testis, epididymis, vas deferens, and male accessory secretions (20, 34, 40, 41, 42, 55, 76, 162). Cohen et al. (34) reported that enzymatic activity, when present, was in the cytoplasm of epithelial cells and was absent from nuclei, connective tissue, and muscle. β-Galactosidase activity is much higher in the intestine of the fetus and suckling animal (3, 47, 55, 59, 76, 121, 187). This presence of high activity coincides with the time that milk forms the major or entire nutrient source for the animal.

β-Galactosidase in humans

β-Galactosidase is present in adult human saliva (24) and intestines (14, 58, 76, 88, 125). Some humans have low β-galactosidase activity in their intestines which results in lactose intolerance (160). The incidence of low β-galactosidase activity among humans is much higher in Negroes, American Indians, Greek Cyriots, and Asians (160).

β-Galactosidase in microorganisms

Because of the extensive work with β-galactosidase, it will not be possible or practical to cite all studies conducted or even the microorganisms investigated. Consequently, only some of the more pertinent and recent investigations on some of the more common microorganisms of interest in the dairy industry will be considered. Characteristics of the

β-galactosidase systems of these microorganisms will be compared with results obtained in this investigation on β-galactosidase of P. shermanii. Investigations on the following microorganisms are of particular interest: E. coli (35, 36, 44, 54, 75, 97, 100, 106, 109, 124, 188, 194); Aerobacter aerogenes (150); Klebsiella aerogenes (138); Streptococcus lactis (26, 27, 136, 179, 180); S. thermophilus (117); Lactobacillus (84, 117, 128); Staphylococcus aureus [phospho-β-galactohydrolase] (45, 93, 112, 132); Bacillus (7, 103, 159); Aeromonas formicans (158); Streptococcus faecium (17); Pseudomonas aeruginosa (157); Paracolobactrum aerogenoides (5); Shigella (29, 152, 163); Salmonella (165, 166); Alcaligenes faecalis (73); Corynebacterium simplex (11); Diplococcus pneumoniae (87); Pneumococcus (66); Neisseria (43); Aspergillus (72, 134, 190); Saccharomyces (13, 22, 48, 49, 70, 181, 199); Fabospora fragilis (65); and Neurospora (102, 104, 108). Feniksova et al. (65) and Estienne et al. (64) investigated many yeast and mold cultures for β-galactosidase activity. McKay et al. (118) recently reviewed lactose utilization by lactic acid bacteria.

β-Galactosidase in Propionibacterium   Wiśniewski (202) separated β-galactosidase of P. shermanii and Propionibacterium arabinosum on a diethylaminoethyl-cellulose column in the Cl⁻ form using stepwise ionic strength elution at constant pH 7.0. He did not characterize the enzyme.

Characterization of β-Galactosidase

β-Galactosidase from different microbial species is not identical, but possesses unique chemical, physical, and immunological properties (5, 29, 49, 70, 100, 103, 128, 144, 157, 158, 159).

Purification of β-galactosidase

β-Galactosidase has been purified and characterized from the following microbial sources: E. coli (35, 36, 44, 86, 100, 106, 124, 177, 194); K. aerogenes (138); S. lactis (113, 115, 116); Bacillus (7, 103, 159); Alc. faecalis (73); S. faecium (17); Pseudomonas aeruginosa (157); D. pneumoniae (87); Saccharomyces (13, 22, 49, 181); Aer. formicans (158); and Neurospora (104, 108).

The initial step in purification is usually fractionation with ammonium sulfate (7, 13, 36, 44, 86, 100, 106, 115, 194). β-Galactosidase also has been precipitated with acetone (7, 49), alcohol (100, 104, 181, 190, 194), and trichloroacetic acid (104). The enzyme is further separated on Sephadex (8, 13, 44, 115, 116, 157, 158) and diethylaminoethyl cellulose columns (8, 86, 158, 202). As the purification progresses, the enzyme becomes increasingly labile (13, 115). Adding 3 to 11% ammonium sulfate partially stabilizes the purified enzyme.

Permease Systems

Because β-galactosidase is an intracellular enzyme, it is necessary for the substrate to enter the cell before it can be metabolized. Transportation into the cell is accomplished by functional systems called permeases. Diffusion-like entry of galactosides into cells is 100 to 1,000 times slower than uptake by a permease (81).

Many workers (11, 17, 26, 29, 48, 81, 99, 103, 106, 117, 153) have observed much greater β-galactosidase activity in bacterial cells after the cells have had their cell walls and membranes ruptured or removed by

solvents or mechanical treatments. Their observations showed that the cell wall and membrane offers a barrier to entry of certain substances into the cell.

Deere (53), 1939, was one of the first workers to recognize that a permeability barrier might be the reason a specific substrate is not utilized by a microbial cell. He observed that E. coli-mutabile, which did not ferment lactose, contained normal amounts of β-galactosidase. After cell injury, this strain was capable of fermenting lactose. One of his interpretations was that failure to utilize lactose was due to impermeability of the cell to the sugar. In 1949, Doudoroff et al. (60) observed that a strain of E. coli did not utilize glucose, but did utilize maltose. Because glucose was released intracellularly and subsequently metabolized, they realized that permeation of glucose was blocked. Because maltose is a larger molecule than glucose, they suspected the permeation system was stereospecific. The fact that some cells accumulated certain nitriles against a concentration gradient demonstrated that there was not free passage of substances through the cell membrane (33, 110). Doudoroff et al. (60), 1949, and Davis (50), 1956, were among the first to emphasize the occurrence of selective permeability.

Cohen and Rickenberg (32), 1955, and Rickenberg et al. (154), 1956, described the galactoside permease system of E. coli. In 1957, Cohen and Monod (33) reviewed the literature on permeases for the transportation of organic compounds into bacterial cells. They recommended that these selective stereospecific systems be called permease systems and proposed a simple model describing the essential features of these systems. At that time, eight different permeases had been identified in E. coli. They

predicted that as many as 50 different permeases for organic substrates might exist in this microorganism.

Further elucidation of permease systems and components was accomplished by Kepes (94, 95), Koch (98), Fox and Kennedy (68), Scarborough et al. (164), and Egan and Morse (61, 62, 63). For additional information on permease systems, other articles may be consulted (25, 96).

The phosphotransferase systems

Kundig et al. (101) discovered a unique permease system in E. coli K235. The system, which they called the phosphotransferase system, utilizes phosphoenolpyruvate (PEP) to phosphorylate a heat-stable, histidine-containing protein, which they called HPr. The phosphate is transferred to specific carbohydrates while they are being transported through the cell membrane. This phosphotransferase system was capable of transporting N-acetylmannosamine, glucose, mannose, glucosamine, mannosamine, N-acetylglucosamine, and N-glycolylmannosamine. Carbohydrates, if phosphorylated in a different reaction, are not transported by this permease but must be phosphorylated during passage through the cell membrane (78, 93). In initial studies, the authors also detected the phosphotransferase system in A. aerogenes, Aerobacter cloacae, and Lactobacillus arabinosus.

Similar, but apparently not identical, phosphotransferase systems have been detected and investigated in Bacillus subtilis, L. arabinosus, Salmonella typhimurium, A. aerogenes, Staphylococcus aureus, and S. lactis (6, 92).

Carbohydrates are accumulated intracellularly as phosphorylated derivatives by microorganisms possessing the phosphotransferase system (78, 79,

101, 105, 119). Pleiotropic car⁻ mutants, which simultaneously lose the ability to utilize several carbohydrates, are a result of mutations resulting in loss of function of phosphotransferase system components (18, 61, 62, 63, 80, 132, 167, 168, 175, 176). Carbohydrates which depend on this permease system are not transported into the car⁻ mutant cell.

Hydrolysis of galactosides by S. aureus   Several workers (45, 61, 93, 112) observed that while untreated whole cells of Staphylococcus aureus possessed "β-galactosidase" activity, activity was not observed in solvent-treated, physically disrupted, or lyophilized cells. Kennedy and Scarborough (93) observed that acetone-treated Staphylococcus aureus cells possessed "β-galactosidase" activity when PEP was added to the assay system. They postulated that carbohydrates were transported into the cell by a phosphotransferase system similar to the system in E. coli K235, described by Kundig et al. (101). Staphylococcus aureus transports at least 10 carbohydrates (lactose, maltose, sucrose, galactose, mannitol, fructose, trehalose, mannose, melizitose, and ribose) by the phosphotransferase system (78).

Egan and Morse (61) concluded that the car⁻ mutation in Staphylococcus aureus NTCC 8511 was the result of a single gene mutation affecting the phosphotransferase system. Use of radioactive carbohydrates demonstrated the inability of the car⁻ phenotype to transport the substrate into the cell, while the car⁺ phenotype did accumulate radioactive carbohydrate. Egan and Morse (63) demonstrated that carbohydrates were accumulated inside the cells as derivatives. Hengstenberg et al. (78) determined that the derivatives accumulated by Staphylococcus aureus were phosphorylated

carbohydrates. Hengstenberg et al. (78) concluded that <u>Staphylococcus</u>

<u>aureus</u> cannot hydrolyze lactose or ONPG, but possesses an enzyme which

hydrolyzes the phosphorylated derivatives of these compounds that are

formed during passage through the cell membrane. Subsequent work also

demonstrated the accumulation of phosphorylated derivatives by

<u>Staphylococcus aureus</u> (79, 105). Hengstenberg et al. (79) verified that

car⁻ mutants did not make phosphorylated derivatives. Hengstenberg and

Morse (77) demonstrated that crude staphylococcal β-galactosidase hy-

drolyzed o-nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-6PO$_4$).

Before the phosphotransferase system was recognized in <u>Staphylococcus</u>

<u>aureus</u>, workers were measuring phospho-β-galactohydrolase activity when

they thought they were measuring β-galactosidase activity. The substrate

was phosphorylated during passage through the cell membrane, and hy-

drolyzed by phospho-β-galactohydrolase. No "β-galactosidase" activity was

evident with solvent-treated or mechanically disrupted cells because the

source of PEP was eliminated when the membrane was disrupted. When ex-

ternal PEP was added (93), "β-galactosidase" activity was present.

While the phosphotransferase system is utilized by <u>Staphylococcus</u>

<u>aureus</u> (78, 93) and <u>S</u>. <u>lactis</u> (119) to transport lactose, and by <u>E</u>. <u>coli</u>

to transport monosaccharides (101), <u>E</u>. <u>coli</u> does not use this system to

transport lactose (93).

Constitutive and Adaptive Systems

β-Galactosidase enzyme may be either constitutive or adaptive. In a

constitutive system, the enzyme is always produced whether an inducer is

present in the growth medium or not. Most adaptive enzymes are produced

in small amounts in the absence of an inducer (37). When an inducer is present, however, up to a 10,000-fold increase in enzyme activity is observed (5, 90).

Inducers of β-galactosidase

To be an inducer, a substance must possess an intact galactoside ring (131). Rotman (161), however, observed that borate induced an E. coli culture. He suspected that the borate affected transcription. Borate did not induce β-galactosidase synthesis by S. lactis (26).

Lactose, the natural substrate for β-galactosidase, will induce most adaptive β-galactosidase systems (17, 19, 26, 29, 49, 99, 102, 103, 109, 138, 179, 199, 202). With Staphylococcus aureus, however, Creaser (45) found that galactose was a much better inducer than lactose. McClatchy and Rosenblum (112) observed that galactose and lactose were efficient inducers of β-galactosidase by Staphylococcus aureus but that the thio-galactosides were not inducers. Morse et al. (132) reported that galactose-6-phosphate was a better inducer for Staphylococcus aureus than galactose was. Because Staphylococcus aureus has a phosphotransferase system and a phospho-β-galactohydrolase instead of a β-galactosidase, it is not surprising that the induction pattern is different than with microorganisms possessing β-galactosidase. Galactose also was a better inducer than lactose for S. lactis C2F, which possesses a phosphotransferase system (119).

Galactose also was a good inducer for Bacillus megaterium (103), S. faecium (17), E. coli (99, 109), Saccharomyces fragilis (49), and Neurospora (102). Galactose did not induce strains of Shigella sonnei (29), E. coli

(29), S. lactis (26, 27), and Sac. fragilis (198).

Compounds do not have to be utilized by the microorganism to induce enzyme synthesis (109, 127, 131). Thiogalactosides, which are not utilizable, induce many strains (26, 29, 37, 56, 81, 103, 127, 133, 149). Thiogalactosides did not induce Staphylococcus aureus (112) or S. faecium (17).

Inhibitors of β-galactosidase

Although lactose is an inducer, it also may be inhibitory. Inhibition has been attributed to accumulation of metabolites (56, 85). Feniksova et al. (65) reported that lactose inhibited β-galactosidase production by Sac. fragilis. Hofsten (85) found that the growth of E. coli strains possessing high β-galactosidase activity was temporarily inhibited by the addition of lactose to the growth medium. A strain of E. coli which produced very large amounts of β-galactosidase (hyper strain) was remarkably sensitive to lactose (135). Dénes (56) observed that lactose inhibited synthesis of β-galactosidase by E. coli cells which had previously been induced by a thiogalactoside. Citti et al. (26) found slight repression of β-galactosidase by galactose and marked repression by glucose.

Glucose inhibits β-galactosidase synthesis and activity (6, 26, 37, 38, 39, 120, 123, 133, 185, 201).

o-Nitrophenyl-β-D-Galactopyranoside Assay Method

Lederberg (106) used the chromogenic substrate, ONPG, to measure β-galactosidase activity. The β-linkage of this galactoside is hydrolyzed

by β-galactosidase. The hydrolysis products are galactose and ONP, a
colored compound which has maximum absorbance at 420 nm. By measuring
absorbance at 420 nm, the μmoles of ONP liberated can be calculated. This
method is much simpler and faster than other procedures (22, 31, 36, 42,
49, 54, 75, 91, 136, 145, 148, 199). Cohn and Monod (36) demonstrated that
lactose and ONPG were hydrolyzed by the same enzyme. The affinity of the
enzyme, however, for the substrate and the rate of the hydrolysis reaction
is different for the two substrates (36, 45, 102, 104, 106, 179, 180, 188,
192). These workers observed that microbial β-galactosidase was more
active toward ONPG than toward lactose (36, 102, 104, 106, 179, 180, 188,
192).

Optimum assay temperature

The assay temperature for optimum β-galactosidase activity is often
considerably above the optimum growth temperature of the microorganism.
McKay (117) found 50 C to be the optimum assay temperature for untreated
cells of Lactobacillus helveticus, Lactobacillus lactis, and Lactobacillus
acidophilus, using ONPG. Untreated cells of L. arabinosus, Lactobacillus
casei, and S. lactis had greater β-galactosidase activity at 37 than at 50
C. Citti et al. (26) reported that S. lactis 7962 untreated cells had
optimum activity with ONPG at 50 C, and toluene-acetone treated cells had
optimum activity at 40 C. Enzyme from B. subtilis also had optimum activ-
ity with ONPG at 50 C (7).

Escherichia coli possessed greater lactose hydrolytic activity at 46
C than at 36 C. There was no hydrolytic activity at 56 C (97). Wierzbicki
and Kosikowski (200) found that the optimum assay temperature for lactose

hydrolysis by mold cell-free extracts was 50 C, and the optimum for yeast and bacteria was 40 to 50 C. β-Galactosidase from Sac. fragilis cell-free extract had optimum activity on lactose at 33 C (181). Studies by Pomeranz et al. (145) revealed an optimum assay temperature of 50 C for a fungal and a bacterial β-galactosidase, and 37 C for a yeast and a bacterial (E. coli) β-galactosidase for hydrolyzing lactose.

Optimum pH

The optimum pH is dependent upon the ionic environment, and is usually near neutrality for bacterial β-galactosidase. Reithel and Kim (148) observed that purified β-galactosidase from E. coli ML 308 and K-12 had maximum hydrolyzing ability on ONPG at pH 6.8 when the assay system contained 4 mM Na^+ and 2 mM Mg^{++} ions. When only Na^+ ions were added (4 mM), the optimum pH was 7.4. Lederberg (106) reported a pH optimum of 7.3 for an extract of E. coli K-12 in sodium phosphate buffer. The pH optimum for intact cells was slightly lower. Kuby and Lardy (100) observed that, with 0.14 M Na^+ ions in the assay system, maximum enzyme activity was obtained between pH 7.2 and 7.3 with purified enzyme from E. coli K-12. Wallenfels et al. (194) reported an optimum pH of 7.3 with purified β-galactosidase of E. coli ML 309. Knopfmacher and Salle (97) obtained optimal lactose hydrolysis between pH 7.0 and 7.5 with untreated E. coli cells.

Wallenfels and Malhotra (190) summarized the results of studies on the effect of pH on β-galactosidase activity of E. coli. Buffers, activating ions, and assay temperatures were reported. Optimum activity occurred from pH 6.6 to pH 7.5.

The optimum pH for cell-free extracts of S. lactis (26), B. subtilis

(7), and Paracolobactrum aerogenoides (5) was 7.0. McKay (117) observed
that L. helveticus cell-free extract had maximum activity at pH 6.6. Op-
timum β-galactosidase activity with enzyme from D. pneumoniae occurred in
the pH range 6.3 to 6.5 (87). Landman (103) reported that cell-free ex-
tract of B. megaterium had a pH optimum of 7.7. He used the continuous
method to measure ONPG hydrolysis, however. He was measuring the optimum
pH of the colored tautomer of o-nitrophenol and not the optimum pH for
enzyme activity (7).

The pH for optimum β-galactosidase activity with toluene-acetone
treated cells and cell-free extract may be different from the optimum for
untreated cells, because pH may affect the rate of transport of substrate
into the cell, which is often the rate-limiting step (25, 33, 49, 50, 62,
98). Desai and Goldner (57) observed that maximum uptake of thiomethyl-β-
D-galactoside by S. lactis occurred at pH 3.6 to 4.0, while the maximum β-
galactosidase activity occurred at pH 7.0.

Yeasts and molds, which grow at low pH, have a lower pH for optimum
activity. The optimum pH for many mold β-galactosidases lies between 3.0
and 5.5, while the optimum pH for most yeast β-galactosidase is between 5.0
and 7.0 (65, 104, 181, 198, 200).

Effect of buffer

Because the enzyme from different sources is different, one buffer
would not be expected to give optimum activity with enzyme from all sources.
Sodium phosphate gave higher β-galactosidase activity than potassium phos-
phate with toluene-acetone treated cells of S. lactis 7962 (26) and with
cell-free extracts from L. helveticus and Paracolobactrum aerogenoides (5).

Anema (7) observed that the two buffers were equivalent with β-galacto-
sidase in cell-free extract from B. subtilis.

Kuby and Lardy (100) found that β-galactosidase from E. coli K-12 was
almost completely inactivated by tris-(hydroxymethyl)-aminomethane (tris) as
the phosphate unless Na^+ ions were present. Citti et al. (26) reported
that tris was a poor buffer for S. lactis 7962 toluene-acetone treated
cells, and that sodium chloride and, particularly, sodium phosphate buffer
added to tris partially restored β-galactosidase activity. Stárka (172)
observed that the inhibitory effect of tris-HCl on E. coli β-galactosidase
was reversed by phosphate ions. Purified B. megaterium β-galactosidase
possessed high activity in tris-sodium chloride buffer (103). Rickenberg
(151) reported that tris-sodium chloride buffer was equivalent to sodium
phosphate buffer with E. coli cell-free extract. Landman (103) stated
that the high activity of purified dialyzed B. megaterium enzyme in tris-
sodium chloride buffer strongly suggested that β-galactosidase did not
require phosphate for its activity. Since phosphate ions reverse the in-
hibitory effect of tris buffer (172), it is possible that Na^+ ions might
do the same. This would explain the acceptability of tris-sodium chloride
buffer. Caputto et al. (22) attributed activation by phosphate during
assay of Sac. fragilis β-galactosidase to removal of inhibitory Zn^{++} ions.

Rohlfing and Crawford (158) observed that 0.5 M sodium or potassium
phosphate buffer increased the thermal stability of β-galactosidase for
Aer. formicans and E. coli when suspended in 20 mM tris-thioglycolate
buffer (pH 7.7) which contained 20 mM $MgCl_2$.

Effect of ions

Manganous ions increase the activity of β-galactosidase from many sources (22, 100, 103, 148, 194, 198). Buecher and Brock (17) found that β-galactosidase of S. faecium became inactivated in cell-free extracts unless Mn^{++} ions were present. Cohn (35) reported that the β-galactosidase of E. coli does not show Mn^{++} ion activation unless it is first treated with a complexing agent, such as versene. Cohn and Monod (36) found that 0.02 M MnCl$_2$ caused a 62% inhibition of β-galactosidase from E. coli ML. This anomaly is explained by work of Rickenberg (151) who found that Mn^{++} ions increased β-galactosidase activity at low concentrations, but was inhibitory at 0.01 M and above. Manganese chloride stabilizes β-galactosidase above 50 C (5, 151, 197).

Rickenberg (151) observed that β-galactosidase in E. coli cell-free extract lost activity when diluted to protein concentrations below 100 µg/ml unless protected by Mn^{++}, Mg^{++}, or Na^+ ions. He did not rule out the possibility that the phosphate or chloride anion might be responsible for stabilizing β-galactosidase. A variety of extraneous proteins also protected the enzyme against loss of activity. The enzyme was completely inactivated by prolonged dialysis against water. The presence during dialysis of Mn^{++}, Mg^{++}, or Na^+ ions, as the phosphate or chloride salt, protected the enzyme. Corbett and Catlin (43) also observed that β-galactosidase of Neisseria was unstable to dialysis against water and to protein concentrations below 100 µg/ml. Two hundred micrograms albumin per milliliter stabilized the enzyme. Manganese chloride also stabilized, but not as effectively as albumin. Lederberg (106) did not detect an effect by divalent cations or by anions (chloride, sulfate, nitrate, acetate, and

phosphate) with β-galactosidase from E. coli.

Reithel and Kim (148) observed that both Na^+ and Mg^{++} ions must be present in the ONPG assay to obtain maximum activity with E. coli β-galactosidase. Sodium ions resulted in greater stimulation of β-galactosidase activity than K^+ ions when added to ONPG assay systems containing β-galactosidase from E. coli (36, 100, 106), and Aer. formicans (158). Potassium ions gave greater stimulation than Na^+ ions when lactose was being hydrolyzed (36, 49, 91, 148). Activity of D. pneumoniae β-galactosidase was not affected by adding Na^+, K^+, or Mn^{++} ions to the ONPG assay system (87).

Effect of storage

The β-galactosidase enzyme is very stable to several storage methods. Clausen and Nakamura (29) observed that β-galactosidase activity of Shigella sonnei cells decreased only slightly when stored 7 days at 40 C. Escherichia coli cell suspensions decreased only slightly in β-galactosidase activity during several months storage at 5 C (97). Kuby and Lardy (100) stored dried E. coli K-12 cells, in a vacuum desiccator over P_2O_5, in the cold room for several months with only a small loss in β-galactosidase activity.

Lyophilized Neurospora mycelial mats could be stored in a vacuum desiccator for several weeks without loss of β-galactosidase activity (104).

β-Galactosidase activity in cell-free extracts also is very stable to storage. Lederberg (106) reported that E. coli enzyme activity was retained during several months of refrigerated storage.

β-Galactosidase also is very stable to frozen storage (87). Puri-
fied enzyme is much more stable than crude extract. Landman (103) reported
that crude lysozyme lysates of B. megaterium lost 49% of their activity
during 12-day storage at -20 C. He attributed the loss of enzyme activity
to proteinase activity. On the other hand, a partially purified prepara-
tion did not lose activity when stored 60 days at -20 C. Anema (7) also
found that purification increased the storage stability of B. subtilis
β-galactosidase. Crude extract was stable for only 3 weeks at -20 C,
while purified extract could be stored 3 months without loss of activity.

Presence of Sulfhydryl Groups

β-Galactosidase contains free sulfhydryl groups (33, 44, 87, 189,
190, 198) which are important for enzyme activity. Some of the compounds
which react with sulfhydryl groups are N-ethylmaleimide (155), iodoacetate
(44, 106, 198), ρ-mercuribenzoate (49, 155), ρ-chloromercuribenzoate (33,
190), iodoacetamide (190), iodine (97), and heavy metals (87, 190). When
a sulfhydryl group reacts with one of these compounds, or other reactive
compounds, that active site no longer possesses enzymatic activity. Com-
pounds containing sulfhydryl groups, as reduced glutathione and cysteine
often prevent or reduce inhibition by the sulfhydryl group blocking
reagents (33, 155). Partial protection against inhibition by ρ-chloro-
mercuribenzoate was provided to S. lactis 7962 β-galactosidase by 0.85 M
ammonium sulfate (114) and to E. coli ML 309 β-galactosidase by 0.05 M
sodium chloride (190). For an extensive discussion of sulfhydryl groups
in enzymes, Boyer (15) should be consulted.

Propionibacteria

For a comprehensive discussion of the significance and usage of propionibacteria in the manufacture of Swiss cheese, <u>Swiss Cheese Varieties</u> (147) should be consulted. Their growth and metabolism, as has been mentioned, is covered in the review by Hettinga and Reinbold (82).

MATERIALS AND METHODS

Cultures

Strains of propionibacteria were obtained from the culture collection of the Department of Food Technology, Iowa State University. Original sources were listed by Malik et al. (122). _Streptococcus lactis_ C2F was received from the Department of Food Science and Industries, University of Minnesota, St. Paul. The other _S. lactis_, the _E. coli_ B, and _Proteus vulgaris_ cultures were obtained from the Department of Microbiology, Oregon State University, Corvallis. The lac⁻ mutanta, _S. lactis_ F22 and G2, were obtained by treating _S. lactis_ C2F with N-methyl-N-nitro-N-nitrosoguanidine (117). _Escherichia coli_ 11775 was obtained from the American Type Culture Collection, Washington, D.C.

Media

Propionibacteria were propagated in sodium lactate broth, the composition of which is given in the Appendix. When lactose, glucose, and galactose media were used for growing propionibacteria, these sugars were substituted for sodium lactate in sodium lactate broth. Purified sodium lactate was used in the medium for propagation of stock cultures and for growing cells for whole cell studies. Technical grade sodium lactate was used in the medium for growing cells used to prepare cell-free extract. Purified sodium lactate was produced by crystallizing and hydrolyzing lactide. The procedure is described in the Appendix.

Streptococcus lactis and _E. coli_ cultures were grown in the lactose

broth used by Citti et al. (26). Composition of this medium is given in

the Appendix. Sufficient filter sterilized 15% lactose solution was added

to the basal medium to bring the lactose concentration to 1%.

Unless stated otherwise, the following procedure was used for growing

all propionibacteria to produce cells for enzyme assay: 500 ml of medium

were placed in a 1,000-ml Erlenmeyer flask closed by four layers of alu-

minum foil. A 1.4% inoculation was used.

Source of Reagents

All reagents were A grade except N-ethylmaleimide which was B grade.

The ONPG, dithiothreitol, α-iodoacetamide, N-ethyl-maleimide, and ρ-

chloromercuribenzoate were obtained from Calbiochem, Los Angeles, Califor-

nia. Reduced glutathione was acquired from Mann Research Laboratories,

Inc., New York, New York, The ONPG-6PO$_4$ was obtained from Research Plus

Laboratories, Inc., Denville, New Jersey, and the Sephadex G-100 and Blue

Dextran 2000 were purchased from Pharamacia Fine Chemicals, Inc.,

Piscataway, New Jersey.

Propagation of Cultures

All cultures were transferred daily on at least three consecutive

days before use. Propionibacteria were inoculated at the rate of 7.1%

and incubated for 24 to 28 hr at 32 C. Lactic cultures were transferred

in reconstituted 11% nonfat dry milk (Matrix medium, Galloway-West, Fond

Du Lac, Wisconsin) using a 1% inoculum, and were incubated at 32 C until

coagulation. They were then transferred twice in lactose broth before

final propagation in lactose broth to produce a cell crop. <u>Streptococcus</u>
<u>lactis</u> C2F was streaked on lactose agar. Individual colonies were picked
and inoculated into tubes of sterile reconstituted nonfat dry milk, which
were then incubated at 32 C. The milk which coagulated first was then
inoculated into broth. This culture was transferred in broth once before
being used to inoculate lactose broth to produce a cell crop. The purpose
of transferring in lactose broth was to dilute out milk solids which would
interfere with determining the mg dry cells/ml. Trypticase soy broth
(Baltimore Biological Laboratory, Baltimore, Maryland) was used for routine
propagation of lac⁻ mutants (<u>S</u>. <u>lactis</u> F22 and G2) and for <u>Proteus</u>
<u>vulgaris</u>. These strains were inoculated into lactose broth to produce a
cell crop. <u>Escherichia</u> <u>coli</u> cultures were routinely grown in lactose
broth; the same medium was used to propagate cells for harvesting.

Buffer Preparation

Sodium and potassium phosphate buffers were prepared by mixing 0.2 $\underline{M}$
stock solutions of the monobasic and dibasic salts (71). Tris buffer was
prepared by adding HCl to tris until the desired pH was obtained. The
final concentration of all buffers was 0.05 $\underline{M}$.

Whole Cell Studies

Harvesting of cells

After the desired incubation period, the broth culture was cooled in
an ice-water bath. The culture was dispensed into three 250-ml polycar-
bonate centrifuge bottles and was centrifuged at 6870 $\underline{g}$ for 20 min in a

Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk,

Connecticut, GSA rotor) at 4±1 C. This centrifuge was used exclusively

in this study. The supernatant liquid was decanted, and the compacted

cell mass in each bottle was dispersed in 15 ml of sodium phosphate buffer

by shaking on a Kahn shaker (Burton Manufacturing Company, Los Angeles,

California, Model 1430). After the cells were resuspended, the contents

of all bottles were pooled. The first rinse was decanted after centrifu-

gation. Approximately 45 ml of buffer were added to the pellet and the

cells were again dispersed. After resuspension, an additional 120 ml of

buffer were added to the bottle. Following centrifugation, the superna-

tant (second rinsing) was decanted, and the cells were resuspended in 5 ml

of buffer to yield a 100X cell suspension.

Determination of dry cell weight

The milligrams of dry cells used in a β-galactosidase assay were cal-

culated from the weight of dry cells per milliliter of the 100X cell sus-

pension and the specific dilution used. The dry cell weight per milli-

liter of the 100X cell suspension was determined in duplicate by adding 1

ml of 100X cell suspension to an aluminum planchet (17 X 60 mm). The

planchets were previously heated at 100 C until constant weight was

attained on two successive weighings made after 24 and 48 hr. The planchets

were provided with covers fabricated from aluminum foil which extended

over the side of the dish by approximately 5 mm. After the sample was

added, the lip of the cover was crumpled over the top of the aluminum

planchet to hold the cover in place. The samples were air dried at 100 C,

cooled for 30 min in a glass desiccator containing anhydrous $CaCl_2$, and

weighed every day until two constant values were obtained. Weighings were performed on a Mettler balance (Mettler Instrument Corp., Hightstown, New Jersey, Model B5H26). One milliliter of the suspension buffer was dried to determine the buffer solids weight. This value was used for correcting the weight of the cells per milliliter of the 100X suspension.

β-Galactosidase assay procedure using whole cells

Lederberg's assay procedure (106), as described by Citti et al. (26), was used to determine β-galactosidase activity. One milliliter each of cell suspension was added to a series of 20 X 125 mm screw-capped test tubes placed in an ice water bath. Four milliliters of 5 mM ONPG were added to each tube; the tubes were then transferred to a water bath held at the desired temperature. After the required incubation period, the enzyme reaction was stopped by placing the tubes in an ice water bath, and adding 5 ml cold (3.3 C) 0.5 M sodium carbonate to each tube. Whole cells and debris were removed by centrifuging in 50-ml polycarbonate centrifuge tubes at 9,750 g for 12 min using a SS-34 rotor. Absorbance of the supernatant was determined at 420 nm using a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, California) with a tungsten lamp. Pyrex cuvettes with a 1-cm light path were used. All absorbance measurements were made with this equipment. A β-galactosidase assay control and a boiled-cell control also were included. The β-galactosidase assay control contained 1 ml of sodium phosphate buffer instead of 1 ml of cell suspension. One milliliter of cell suspension, held in boiling water for 3 min, was used for the boiled control. The spectrophotometer was adjusted to zero absorbance with either the β-galactosidase assay control

or with distilled water. The absorbance of the boiled control was subtracted from sample absorbance to obtain the corrected value. Duplicate assays were performed unless stated otherwise.

Toluene-acetone treatment   Whole cells were subjected to toluene-acetone treatment by adding 4 ml of cell suspension and 0.2 ml of a 1:9 toluene-acetone mixture to a 20 X 125 mm screw-capped test tube and mixing on a Vortex Genie Laboratory mixer (Fisher Scientific by Scientific Industries, Springfield, Massachusetts) at room temperature for 5 min. One milliliter of this treated cell suspension was then used for the sample in an enzyme assay.

Preparation of o-nitrophenol standard curve

A stock solution of ONP containing 40 µg/ml was prepared by dissolving 20 mg ONP in 10 ml ethyl alcohol and then diluting to 500 ml with 0.05 $\underline{M}$ sodium carbonate buffer (pH 10.0). Absorbance of dilutions of this stock solution was determined at 420 nm. All points fell on or very near a straight line which went through the origin. The concentration of ONP (µmoles/ml) necessary to increase the absorbance by 0.001 was close to the value of 0.238 determined by McFeters et al. (115).

Calculation of β-galactosidase activity

Since the final volume of solution in the assay system was 10 ml, the total number of micromoles of ONP in the supernatant for each 0.001 absorbance unit was 2.38. Multiplying absorbance by 2.38 gives the number of micromoles of ONP liberated during the assay. One unit of enzyme activity is equivalent to 1 µ mole of ONP liberated per mg dry cells per

minute incubation time (26; 67, p. 6). Since there was usually less than 1 unit of activity with the propionibacteria used in this investigation, results are presented as the milliunits activity which are calculated as follows:

$$\text{Milliunits enzyme activity} = \frac{\text{absorbance X 2.38 X 1,000}}{\text{mg dry cells X min incubation}}$$

β-Galactosidase activity of cultures

Ten strains of P. shermanii and 10 strains of P. freudenreichii were purified by streaking on sodium lactate agar. Plates were incubated 4 days at 32 C in a candle oats jar (182). Cells from an individual colony of each strain were inoculated into sodium lactate broth. β-Galactosidase activity of these strains was determined using the procedures as described. The dilution of cells for an enzyme assay depended upon amount of growth, activity of the cells, and assay conditions. For assay of these 20 propionibacteria strains, the cell crop was diluted to a 10X concentration. That is, the cells were 10 times more concentrated in the buffer than they were in the growth medium. The assay systems contained from 0.58 to 6.05 mg dry cells/ml.

The same procedure was used for determining the β-galactosidase activity of the S. lactis, E. coli, and Proteus vulgaris species.

Effect of carbon source in growth medium on β-galactosidase activity

Five P. shermanii strains were grown for 24 hr in seven combinations of carbon sources and then harvested and assayed for β-galactosidase activity. The carbon sources were 1% sodium lactate, 0.5% lactose, 0.5%

lactose + 0.5% sodium lactate, 0.5% glucose, 0.5% glucose + 0.5% sodium
lactate, 0.5% galactose, and 0.5% galactose + 0.5% sodium lactate. These
carbon sources were substituted for sodium lactate in sodium lactate broth.
Cultures were transferred on three consecutive days in medium containing
the same carbon source to be used for growing the cell crop. The sugars
were filter-sterilized and were added to the medium immediately before
inoculation.

Effect of lactose analogs and related sugars on β-galactosidase activity

Propionibacterium shermanii P7 and P22 were inoculated into 1% sodium
lactate broth and incubated 24 hr at 32 C. Cells were centrifuged from
the medium in sterile 250-ml polycarbonate centrifuge bottles, and rinsed
once with sterile sodium phosphate buffer (pH 7.0). Five milliliters of
sterile sodium phosphate buffer were added to the cells, which were held
on a Kahn shaker until complete cellular suspension was achieved. One-
milliliter portions were removed for determination of mg dry cells/ml.
One milliliter was added to a sterile bottle containing 9 ml of the desired
lactose analog (0.01 $\underline{M}$) or sugar (0.5%). A second 1-ml aliquot was added
to another sterile centrifuge bottle, which contained 9 ml of buffer, to
serve as the control. The samples were incubated 6 hr at 32 C. After the
incubation period, the cells were centrifuged, rinsed twice with sodium
phosphate buffer, and then resuspended to 10X concentration in sodium
phosphate buffer. β-Galactosidase activity was then determined. The
milligrams dry cells present in the assay were assumed to be 1/10th the
mg dry cells/ml in the 100X cell suspension after the cells were harvested.

Growth curves

As a further test to determine if β-galactosidase was inducible, sodium lactate- and lactose-grown cells were separately inoculated (1%) into two broths. Both broths contained the ingredients of sodium lactate broth except for these changes: 1) one broth contained a high sodium lactate concentration (0.0145 M) and a low lactose concentration (0.0029 M); 2) the other medium contained a low sodium lactate concentration (0.0029 M) and a high lactose concentration (0.0145 M). Growth was measured by determining the absorbance of cells in the growth medium at 600 nm. A well-mixed aliquot was removed from each flask of broth every hour for 48 hr. Aliquots were stored at 3.3 C, and the absorbance was determined after every fourth sampling period.

Effect of temperature and pH on β-galactosidase activity of whole cells

β-Galactosidase activity of untreated and toluene-acetone treated cells of P. shermanii P7 and P22 was determined at specific incubation temperatures between 32 and 65 C. Also, activity was determined at specific pH values between pH 6.0 and 8.9.

Effect of age of cells on β-galactosidase activity

Three-hundred-milliliter quantities of 1% sodium lactate broth in 500-ml Erlenmeyer flasks, were inoculated at the rate of 2.3% with P. shermanii P7. Cells from two flasks were harvested after 12 hr, and cells from two more flasks were harvested after 16 hr of growth. Because higher numbers of cells were present after 20, 24, 28, 36, 48, and 72 hr, one flask of medium provided sufficient cells for assays representing these

incubation times. Harvested cells were stored 30-36 hr at 3.3 C before being assayed for β-galactosidase activity.

Effect of incubation time on rate of ONPG hydrolysis by untreated cells

Suspensions of untreated cells of P. shermanii P7 and P22 were incubated for time intervals ranging from 2 to 30 min during the β-galactosidase assay, to determine the length of time that the hydrolysis reaction was linear. Single assays were performed.

Effect of buffers on β-galactosidase activity of whole cells

The effect of buffers on β-galactosidase activity was determined using 0.05 M sodium phosphate, potassium phosphate, and tris buffer. Tris buffer (0.05 M) was mixed with an equal volume of sodium phosphate (0.05 M) and sodium chloride (0.05 M) to provide buffering systems that were 0.025 M in each component of the mixture. Sodium phosphate, potassium phosphate, and tris buffer were used to wash portions of the cell crop. An aliquot of each portion of cells was diluted with the respective buffer, to proper cell concentration. A portion of the cells washed with sodium phosphate was diluted with a mixture of sodium phosphate and tris. A portion of the cells washed with tris buffer was diluted with a mixture of tris and sodium chloride. One-milliliter samples of untreated and toluene-acetone treated cell suspensions were assayed with ONPG solution, which contained the respective buffer or buffers.

Effect of manganese chloride on β-galactosidase activity of whole cells

Suspensions of P. shermanii P7 and P22 cells were assayed at 32 and 52 C in the presence of 0.4 mM $MnCl_2$ to determine the effect of $MnCl_2$ on

β-galactosidase activity. Control samples did not contain $MnCl_2$.

Statistical evaluation of effect of manganese chloride
on β-galactosidase activity of untreated cells

The t test was performed using the mean difference between β-galacto-sidase activity of both P7 and P22 untreated cells receiving 0.4 $m\underline{M}$ $MnCl_2$ in the enzyme assay and control samples which did not receive $MnCl_2$. Statistical procedures from Statistical Methods (170) were used.

Cell-Free Extract Studies

Preparation of cell-free extract

Five liters of sodium lactate broth, in a 6-liter Erlenmeyer flask, were used for growing cells for preparation of the cell-free extract. Cells were harvested by centrifuging the growth medium at 27,000 $\underline{g}$ in the continuous-flow system of the Sorvall RC2-B refrigerated centrifuge (4±1 C). Cells were rinsed twice with sodium phosphate buffer and then diluted to a 100X cell concentration.

Cells were disrupted with a French pressure cell in a power laboratory press (American Instrument Co., Inc., Silver Springs, Maryland). The pressure cell was chilled to 3.3 C before use. Pressure within the cell was a minimum of 16,000 pounds per square inch. The cell suspension was passed through the pressure cell two times. Unbroken cells and debris were removed by centrifuging at 27,000 $\underline{g}$ for 15 min at 4±1 C. The cell-free extract contained from 7 to 21 mg protein/ml.

Protein determination of cell-free extract

Protein concentration of the cell-free extract was determined by

Lowry's procedure (111). Sodium citrate, instead of sodium or potassium

tartrate, was used to dissolve the $CuSO_4 \cdot 5H_2O$ (B2). The procedure is

given in the Appendix.

β-Galactosidase assay procedure using cell-free extract

The β-galactosidase assay with cell-free extract was conducted

similarly to the procedure described for whole cells. One milliliter of

cell-free extract was added to the assay system instead of 1 ml of cell

suspension. Cell-free extract samples also were centrifuged. The milli-

units enzyme activity are expressed per milligram protein.

Lyophilization of cell-free extract

Cell-free extract was lyophilized in a Virtis Freeze Dryer (The

Virtis Company, Inc., Gardiner, New York, Model 10-147MR-BA). Lyophilized

cell-free extract was stored in a desiccator, with anhydrous $CaCl_2$ as the

desiccant, at 3.3 C.

Reconstitution of lyophilized cell-free extract

Propionibacterium shermanii P7 lyophilized cell-free extract was

reconstituted by adding 2 g lyophilized cell-free extract per 100 ml

distilled water; this amount of cell-free extract contained approxi-

mately 10 mg protein/ml. Propionibacterium shermanii P22 lyophilized

cell-free extract was reconstituted by adding 0.5 g lyophilized cell-free

extract per 100 ml distilled water; this amount of cell-free extract con-

tained approximately 1.5 mg protein/ml. These quantities of cell-free

extracts were selected because they usually yielded absorbance readings between 0.100 and 0.400.

Effect of temperature and pH on β-galactosidase activity of cell-free extract

β-Galactosidase activity of cell-free extracts of P. shermanii P7 and P22 was determined at specific incubation temperatures between 18 and 65 C. Also, activity was determined at specific pH values between 6.0 and 8.0.

Linearity of product formation with time during ONPG hydrolysis by cell-free extract

Cell-free extracts of P. shermanii P7 and P22 were incubated for time intervals ranging from 2 to at least 20 min during the β-galactosidase assay to determine the length of time that the hydrolysis reaction was linear.

Effect of buffers on β-galactosidase activity of cell-free extract

Buffers were prepared the same as in the whole cell study. Cells used to prepare cell-free extract were suspended in sodium phosphate buffer. Consequently, some Na^+ and phosphate ions were present in all assay systems, except when the cell-free extract was dialyzed.

Effect of manganese chloride on β-galactosidase activity of cell-free extract

Cell-free extracts of P. shermanii P7 and P22 were assayed at 18, 28, 32, 37, 45, and 65 C in the presence and absence of 0.4 mM $MnCl_2$. Manganese chloride was included in the β-galactosidase control which did not

contain any cell-free extract.

Statistical evaluation of effect of manganese chloride
on β-galactosidase activity of cell-free extract

The t test was performed using the mean difference between β-galacto-sidase activity of both P7 and P22 cell-free extracts receiving 0.4 m$\underline{M}$ MnCl$_2$ in the enzyme assay and control samples which did not receive MnCl$_2$. Statistical procedures from Statistical Methods (170) were used.

Dialysis of cell-free extract

Fifty milliliters of $\underline{P}$. shermanii P22 cell-free extract (reconstituted by dissolving 0.5 g lyophilized cell-free extract in 50 ml redistilled water) were dialyzed against 1,000 ml of redistilled water in a 1,000-ml glass cylinder. The dialysate was mixed with a Teflon-coated stirring bar driven by a magnetic stirrer. The dialysate was changed every 12 hr for five changes. When indicated, 0.1 m$\underline{M}$ dithiothreitol was added to the dialysate. The dialyzed cell-free extract was diluted 1:2 with redistilled water. The sample dialyzed against redistilled water containing 0.1 m$\underline{M}$ dithiothreitol was diluted (1:2) with redistilled water containing 0.1 m$\underline{M}$ dithiothreitol.

Effect of ions on β-galactosidase activity of dialyzed cell-free extract

Dialyzed cell-free extract of $\underline{P}$. shermanii P22 was used to determine the effect of ions on β-galactosidase activity. To simplify the experiment, ONPG was made double strength (0.01 $\underline{M}$) in distilled water. Sodium phosphate, potassium phosphate, ammonium phosphate, sodium chloride, potassium chloride, and tris were made double strength (0.10 $\underline{M}$) also.

Each 1 ml of cell-free extract received 2 ml of 0.01 $\underline{M}$ ONPG solution and 2 ml of 0.10 $\underline{M}$ buffer or salt solution. Results reported are of single assays.

Effect of sulfhydryl group blocking reagents on β-galactosidase activity of cell-free extract

Cell-free extract of $\underline{P}$. shermanii P22 was assayed for β-galactosidase activity in the presence of the sulfhydryl group blocking reagents α-iodoacetamide, N-ethylmaleimide, and ρ-chloromercuribenzoate. The sulfhydryl group protector, dithiothreitol (30) also was added to samples receiving the sulfhydryl group reagents to see if it would reverse the blocking effect. Reduced glutathione, another sulfhydryl group protector, also was used.

Effect of storage at 3.3 and 25 C on β-galactosidase activity of cell-free extract

Because β-galactosidase enzyme from other sources is reported to be a very stable enzyme, the stability of $\underline{P}$. shermanii P22 β-galactosidase was investigated. A sample of cell-free extract was divided into two portions and placed in 50-ml Erlenmeyer flasks. One portion was stored at 3.3 C, and the other at room temperature (25 C). β-Galactosidase activity of both portions was determined initially, and after 9, 24.5, 49.5, and 74 hr.

Effect of preservation method on β-galactosidase activity of cell-free extract

To determine the effect of preservation methods on β-galactosidase

activity, cell-free extracts of P. shermanii P7 and P22 were frozen at -20 C. Portions of cell-free extracts were lyophilized and stored at -20 C. β-Galactosidase activity of the fresh cell-free extracts was compared against preserved cell-free extracts after 32, 73, and 75 days of storage.

<div style="text-align:center">

Phospho-β-Galactohydrolase Activity of

P. shermanii and S. lactis Strains

</div>

The presence or absence of a phospho-β-galactohydrolase in P. shermanii P7 and P22 was tested by adding phosphoenolpyruvate (PEP) and sodium fluoride (NaF) to an assay system containing ONPG. A phosphorylated substrate, ONPG-6PO$_4$, also was used. Streptococcus lactis C2F, known to possess a phospho-β-galactohydrolase (119), was assayed for comparison, and as a control on the assay procedure.

The P. shermanii strains were examined for the presence of an alkaline phosphatase by methods outlined in Standard Methods for the Examination of Dairy Products (4).

<div style="text-align:center">

Preparative Isolation of β-Galactosidase by Ammonium

Sulfate Precipitation from Cell-Free Extract

</div>

Preparative isolation of β-galactosidase from P. shermanii P22 cell-free extract was accomplished by fractionating with ammonium sulfate. One gram of lyophilized cell-free extract was dissolved in 100 ml of redistilled water. A β-galactosidase assay of this sample was performed. Ten grams of ammonium sulfate were added to the remaining cell-free extract sample. After 12-hr storage at 3.3 C, the sample was centrifuged 20 min

at 6870 g in a 250-ml polycarbonate centrifuge bottle. The precipitate
was suspended in 0.05 M sodium phosphate buffer, and assayed for β-galacto-
sidase activity and protein concentration. Ammonium sulfate was added to
the supernatant in 10-g amounts, using the procedures just described,
until 60 g had been added.

<p style="text-align:center;">Sephadex Separation of Cell-Free Extract</p>

A Sephadex column was prepared using the procedure outlined by
Garcia (69). Twelve and one-half grams of Sephadex G-100 were slowly
added to 500 ml of 0.05 M sodium phosphate buffer containing 0.1 mM
dithiothreitol in a 1,000-ml Berzelius beaker. The buffer was gently
stirred with a Teflon-coated stirring bar while the Sephadex was being
added. One liter of Sephadex solution was prepared. Five milliliters of
toluene were added to each liter to retard bacterial growth. The Sephadex
was swollen in the eluant for 5 days with daily removal of "fines" by
decanting, followed by gentle stirring with the magnetic stirrer.

A non-jacketed glass column (Pharmacia Fine Chemicals, Inc.,
Piscataway, New Jersey) measuring 2.5 X 45 cm was equilibrated at room
temperature for 48 hr with the eluant. A protein fraction, obtained by
adding an additional 20 g ammonium sulfate to the supernatant from 100 ml
of 1% (W/V) P22 cell-free extract which had been previously precipitated
with 10 g ammonium sulfate, was suspended with 30 ml sodium phosphate
buffer and then condensed to 12.5 ml by surface evaporation in dialysis
tubing (Union Carbide Corporation, Food Products Division, 6733 West 65th
Street, Chicago, Illinois. 60638) in front of a stream of air from a fan.
β-Galactosidase activity of this concentrate was determined. Five milli-

liters of concentrate, which contained 8.0 mg protein/ml, were added to the column. The flow rate was 0.82 ml/min.

Five-milliliter aliquots were collected by a LKB Radi Rac Fraction Collector Controller (Type 3403B), Distributor (Type 3402B), and Rotator (Type 3401B). The eluted fractions were continuously monitored at 253.7 nm through a LKB Uvicord (Type 4701A) Optical and Control unit, and the per cent transmittance was transcribed on a LKB Recorder (Type 6520A). These instruments are manufactured by LKB-Produkter AB, Stockholm-Bromma, Sweden.

The void volume of the column was determined using Blue Dextran 2000. Fifty milliliters of Blue Dextran, containing 4 mg/ml (69) were prepared by adding 200 mg of Blue Dextran to 50 ml of 0.05 $\underline{M}$ sodium phosphate buffer containing 0.1 m$\underline{M}$ dithiothreitol. The solution was stored for 24 hr at 3.3 C to permit the beads to swell. A 1-ml sample of Blue Dextran solution was added to the column when determining the void volume.

RESULTS AND DISCUSSION

Whole Cell Studies

β-Galactosidase activity of propionibacteria strains

β-Galactosidase activity of untreated and toluene-acetone treated cells of 10 strains of P. shermanii is presented in Table 1. For these initial assays, 32 C was selected as the assay temperature because it is near the optimum growth temperature of propionibacteria (16).

All P. shermanii strains studied showed detectable activity in both untreated and toluene-acetone treated cell assays. Strain P12 had the greatest activity (15.7 milliunits) using untreated cells. Toluene-acetone treated cells of this strain exhibited 26.6 milliunits activity.

Toluene-acetone treatment resulted in increased activity with P. shermanii strains P8, P12, P22, P51, and P59. Propionibacterium shermanii P22 showed the greatest response to the solvent treatment; treated cells yielded greater than five times the enzyme activity found in untreated cells. There are many reports in the literature confirming that toluene-acetone (or other solvent) treatment, which destroys the permeability barrier of the cell membrane, results in increased enzyme activity in microbial cell systems (11, 26, 29, 81, 99, 106, 117). Several authors (29, 33, 49, 50, 62, 98) have emphasized that permeability is often the limiting factor in substrate utilization. Koppel et al. (99), working with E. coli B cells, found a 10-fold increase in β-galactosidase activity in toluene-acetone treated cells over that obtained with untreated cell suspensions. Citti et al. (26) observed a fivefold increase in the enzyme activity of S. lactis 7962 when treated with toluene-acetone.

Table 1. β-Galactosidase activity of 10 P. shermanii strains grown 24 hr at 32 C in 1% sodium lactate broth

	Milliunits enzyme activity[a] (nMoles ONP/mg dry cells/min)	
	Untreated cells	Toluene-acetone treated cells
P. shermanii		
P7	8.2	4.4
P8	15.0	23.6
P12	15.7	26.6
P18	10.7	4.5
P22	12.8	64.6
P32	9.4	7.8
P48	7.7	4.4
P51	11.6	29.9
P55	4.9	2.8
P59	5.6	8.4

[a] Assay conditions: 15-min incubation at 32 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0).

In contrast to the aforementioned propionibacterial strains, P. shermanii P7, P18, P32, P48, and P55 responded negatively to solvent treatment. Toluene-acetone treated cells yielded about 50% of the enzyme activity found in untreated cell suspensions. Other workers also have observed lowered activity or complete destruction of activity when microbial cells were treated with toluene-acetone (5, 26, 29, 45, 112, 117). With

organisms possessing the phosphotransferase system, toluene interferes with the production of PEP (93, 119). With other microorganisms, the exact reason for such anomalous phenomenon is still unknown. It is probable that there are strain differences in lability of enzymes to solvents like toluene and acetone. Another possibility could be the differences in the manner in which the specific enzyme is bound to the cell membrane or mesosomes, the organelles most affected by the solvent treatment.

None of 10 P. freudenreichii strains (P1, P6, P16, P23, P30, P39, P40, P49, P56, P57) possessed detectable enzyme activity. These results, obtained by using ONPG as the substrate, correlated entirely with previous observations made with lactose as the substrate in broth systems. Recent taxonomic work by Malik et al. (122) confirmed that inability to utilize lactose by P. freudenreichii was the only physiological difference between P. shermanii and P. freudenreichii (16). The sensitive enzymatic assay results of this study support the conclusion that P. freudenreichii fails to produce acid from lactose because of either absence or complete inactivity of enzymes to hydrolyze this disaccharide. It was considered that these microorganisms might possess a low level of enzyme activity that could be detected by the sensitive enzymatic assay, but which would not produce sufficient acid to be detected by the indicator in lactose broth.

It is concluded that a cryptic system is not present because none of the 10 P. freudenreichii strains responded positively to toluene-acetone treatment. Three strains, P1, P30, and P40, received prolonged incubation in broths containing lactose concentrations up to 6.3%. None of these strains produced any more acid in lactose broth than in control tubes which contained only basal medium. To test the stability of propioni-

bacteria β-galactosidase to toluene-acetone, aliquots of P. shermanii P7 and P22 cell-free extracts which were assayed at the different incubation temperatures (Fig. 11 and 12) were also assayed under the same conditions except that they received the same toluene-acetone treatment, including agitation, that whole cells received during solvent treatment (unpublished results). The greatest inhibition of β-galactosidase was 54% when P7 cell-free extract was incubated at 65 C. There was less than 30% inhibition of this strain when assayed at 32, 37, 45, and 52 C. The greatest inhibition of P22 by toluene-acetone, 41%, also occurred at 65 C. There was less than 10% inhibition by toluene-acetone treatment at 28, 32, 37, and 45 C incubation. There was only 19% inhibition when the assays were incubated at 52 C. These observations show that strain P7 β-galactosidase is more labile to solvent treatment than enzyme from strain P22. The solvent has a detrimental effect on the enzyme itself and the inhibition is more pronounced at higher assay temperatures.

Propionibacterium freudenreichii P30 did not possess detectable activity when toluene-acetone treated cells were assayed with ONPG-6PO$_4$ as substrate. This observation indicated that this strain does not contain a phospho-β-galactohydrolase.

In the assay system containing toluene-acetone treated cells of P. freudenreichii P30, an absorbance reading >0.100 at 420 nm was consistently obtained. The yellow color that would normally be associated with the liberation of o-nitrophenol, however, was absent. A control sample, which received buffer instead of ONPG, did not have absorbance. Apparently, the solvent was releasing some substance from the cells which, in the presence of ONPG, caused absorbance at 420 nm.

Two strains of P. shermanii, P7 and P22, were selected for further

intensive study. Strain P7, which retained only 54% of the untreated

cell enzyme activity in the solvent-treated system, was chosen at random

to represent the group of cultures which yielded reduced enzymatic activity

after toluene-acetone treatment. Strain P22, which exhibited the maximum

stimulation of enzyme activity with solvent treatment, was selected to

represent the other group.

β-Galactosidase activity of Propionibacterium, Streptococcus,

Escherichia, and Proteus species

To evaluate the relative β-galactosidase activity of P. shermanii,

cultures of two other bacterial species, E. coli and S. lactis, which

have been extensively investigated with regard to this enzyme system, were

assayed along with the propionibacteria. Additionally, Proteus vulgaris,

which does not utilize lactose (16), also was included in this experiment.

These assays were conducted at 37 C. The results are summarized in Table

2.

Compared to the β-galactosidase activities observed with the E. coli

strains, and S. lactis 7962, the response of the two propionibacterial

strains was low.

Untreated cell suspensions of the P. shermanii strains exhibited in-

creased activity at the higher assay temperature used in this experiment.

The enzyme activity of untreated P7 cells at 37 C was almost three times

the value obtained at 32 C; and, untreated cells of P22 exhibited greater

than a 100% increase over the activity obtained at 32 C (Table 1).

Increase in assay temperature was, however, detrimental to the enzyme

Table 2. β-Galactosidase activity of P. shermanii, S. lactis, and E. coli strains

Strain	Incubation time at 32 C	Milliunits enzyme activity[a] (nMoles ONP/mg dry cells/min)	
		Untreated cells	Toluene-acetone treated cells
P. shermanii	24 hr in sodium lactate broth		
P7		23.0	6.0
P22		26.0	50.5
S. lactis	7 hr in lactose broth		
7962		42.0	130
C2F		10.2[b]	0.3[b]
	24 hr in lactose broth		
F22		NDA[c]	0.4
G2		NDA	NDA
E. coli	7 hr in lactose broth		
B		358	1,870
11775		1,140	1,350

[a] Assay conditions: 15-min incubation at 37 C, 0.05 M sodium phosphate buffer (pH 7.0), 0.4 mM MnCl$_2$ except with S. lactis F22 and G2.

[b] β-Galactohydrolase activity.

[c] No detectable activity.

activity of solvent-treated cells of strain P22. Toluene-acetone treated cells of P. shermanii P22 contained 64.6 milliunits activity when assayed at 32 C (Table 1), but only 50.5 milliunits activity when assayed at 37 C (Table 2). Activity of untreated cells assayed at 37 C was more than double the activity obtained at 32 C incubation. The favorable effect of disrupting the permeability barrier of the bacterial cells is a function of temperature. This was borne out in a later experiment conducted to determine the optimum temperature of enzyme activity in untreated and toluene-acetone treated cell systems (Fig. 4). Citti et al. (26) found toluene-acetone treated S. lactis 7962 cells had a lower temperature for optimum activity, 40 C, than untreated cells, 50 C. The loss of activity in solvent-treated cells with increasing temperature is probably a synergistic effect leading to denaturation of the enzyme per se or the alteration of the cell membrane systems to which the enzyme may be attached.

The enzyme activity of 42.0 milliunits observed for untreated cells of S. lactis 7962 agrees well with the value of 50.0 milliunits reported by Citti et al. (26) using the same assay conditions. They, however, observed a much greater stimulation of activity by solvent treatment of the S. lactis cell suspension. Their value of 770 milliunits is nearly six times greater than the value (namely 130 milliunits) obtained in this experiment.

Although untreated cells of P. shermanii P7 and P22 possessed, respectively, as much as 55 and 62% of the β-galactosidase activity of S. lactis 7962, these strains fail to coagulate milk over a 7-day incubation period at 32 C. On the other hand, S. lactis 7962, a homofermentative microorganism, coagulates milk after as little as 12-hr incubation at 32 C.

The lactic streptococci convert some lactose into lactic acid, a relatively dissociable acid, while the heterofermentative propionibacteria form very little lactic acid, but convert the common metabolic intermediate, pyruvate, to poorly dissociable acetic and propionic acids (Fig. 1), which fail to depress the pH of the milk near or past the isoelectric point of casein.

Untreated cells of S. lactis C2F had a "β-galactosidase" activity of 10.2 milliunits. Citti et al. (26) using an untreated cell suspension of S. lactis C2, the parent strain of S. lactis C2F, found a "β-galactosidase" activity of 15.0 milliunits under identical assay conditions. Although S. lactis C2 is relatively a much faster acid producer in milk as compared with S. lactis 7962 (146), Citti et al. (26) found that this strain exhibited only one-third the "β-galactosidase" activity found in the latter strain when measured with ONPG. Table 2 shows that S. lactis C2F possessed less than one-fourth as much activity as strain 7962. A possible explanation for this anomaly was recently furnished by McKay et al. (119) who found that while S. lactis 7962 possesses a β-galactosidase system, S. lactis C2F in reality has a phospho-β-galactohydrolase system for the utilization of lactose. McKay et al. (119) concluded that S. lactis C2F lacked β-galactosidase but possessed a different enzyme as a part of a PEP-dependent system. The presence of a phospho-β-galactohydrolase in S. lactis C2F was verified in a later experiment in this investigation (Table 15).

Toluene-acetone treatment of S. lactis C2F cells reduced the response in the β-galactosidase assay considerably (10.2 milliunits for untreated cells versus 0.3 milliunits for solvent-treated cells). Citti et al. (26)

failed to elicit any activity from solvent-treated cells of S. lactis C2.

McKay et al. (119) recently observed a highly enhanced enzyme activity

with solvent-treated S. lactis C2F cells when PEP was added to the assay

system. The system as described by McKay et al. (119) when applied in

this investigation gave only a slight enhancement of enzyme activity with

solvent-treated cells of this strain (Table 15). However, the phosphory-

lated substrate, ONPG-6PO$_4$, gave very high enzyme activity, which substan-

tiates the previous observation that S. lactis C2F possesses a phospho-β-

galactohydrolase (119).

Although the lac⁻ mutants, S. lactis F22 and G2, which were isolated

from S. lactis C2 (117), would not be expected to utilize lactose, they

were inoculated into and incubated in lactose broth so that an inducer was

present. Because the lactose broth contained relatively high concentra-

tions of other carbon sources [Tryptone (1%), yeast extract (0.5%), gelatin

(0.25%), sodium acetate (0.15%), ascorbic acid (0.05%), and some residual

carbon sources from the Trypticase soy broth of the inoculum], there were

sufficient carbon sources present to permit good growth.

The lac⁻ mutants did not possess any detectable β-galactosidase ac-

tivity in untreated cell systems; after toluene-acetone treatment, strain

F22 exhibited a very low level of activity. McKay (117) reported that

neither of these mutants could hydrolyze lactose. McKay et al. (119)

observed that S. lactis C2F cell-free extract possessed phospho-β-galacto-

hydrolase activity when a highly concentrated extract (5.7 mg protein/ml)

was employed, but he did not obtain detectable activity with toluene-

acetone treated cells unless PEP was added to the assay system. Neverthe-

less, the most logical explanation for the low level of activity with S.

lactis F22 is that there was sufficient PEP contributed from the high concentration of solvent-treated cells used in this assay (17.7 mg dry cells/ml) to elicit phospho-β-galactohydrolase activity. Also, because the concentration of dry cells was greater, the toluene-acetone treatment might have been less detrimental to PEP production.

The two E. coli strains possessed very high enzyme activity. The value of 358 milliunits obtained for untreated E. coli B cells is in agreement with the value of 400 milliunits reported by Citti et al. (26). They, however, reported a value of 6,600 milliunits for solvent-treated cells, which is more than three times the level observed in this study. Escherichia coli 11775 failed to show a very dramatic increase in enzyme activity with toluene-acetone treatment. The increase, however, amounted to 210 milliunits, which is four times the entire enzymatic activity detected in the toluene-acetone treated cell suspension of P. shermanii P22. Kennedy and Scarborough (93) reported that E. coli does not depend on the phosphotransferase system for utilization of lactose.

Proteus vulgaris did not exhibit detectable activity. This was expected, because Proteus species do not utilize lactose (16). The comments regarding the carbon sources for the S. lactis lac⁻ mutants also apply for the growth of Proteus vulgaris.

Effect of carbon source in growth medium on β-galactosidase activity

To determine the carbon source that resulted in maximum β-galactosidase activity, P. shermanii P7, P8, P12, P22, and P51 were grown separately in basal medium containing four different carbon sources added

singly or in combinations. Cells from each of these media were separately harvested and examined for the relative amounts of enzyme activity. The carbon sources chosen were sodium lactate, lactose, glucose, and galactose. The results are shown in Table 3.

Although some differences in enzyme levels in cells harvested from media containing different carbon sources were observed, the variations were insignificant. From these data, it appears that the β-galactosidase system of P. shermanii is probably constitutive rather than inducible. Unfortunately, it cannot be definitely stated that the enzyme is not inducible because the high concentration of yeast extract (1%) and Trypticase (1%) in the basal broth may have yielded metabolic products that repressed enzyme synthesis.

The β-galactosidase system in many bacterial species is inducible. There are several reports relating to the inducibility of β-galactosidase in E. coli (19, 33, 35, 81, 89, 90, 99, 109, 128, 129, 131, 137). The enzyme also is inducible in S. faecium (17), Staphylococcus aureus (45, 112), and Sac. fragilis (199). Wiśniewski (202) found that this enzyme is inducible in P. shermanii NCDO 839 and P. arabinosum ATCC 4965.

Lactose is an effective inducer of β-galactosidase in many microorganisms (17, 26, 27, 45, 108, 109, 199, 202). Jacob and Monod (90) reported an E. coli strain increased the rate of synthesis of β-galactosidase 10,000-fold in response to the addition of the galactoside to the growth medium. Lactose repressed β-galactosidase in several systems (56, 65, 85, 135). In some of these instances, inhibition by lactose was attributed to the accumulation of metabolites (56, 85).

Table 3. Effect of carbon source in growth medium on β-galactosidase activity of five P. shermanii strains grown 24 hr at 32 C

| P. shermanii strain | Milliunits enzyme activity[a] (nMoles ONP/mg dry cells/min) | | | |
| | P7 | | P8 | |
Carbon source	U.C.[b]	T.A.[c]	U.C.	T.A.
Sodium lactate (1.0%)	8.2	4.4	15.0	23.6
Lactose (0.5%)	6.9	3.6	9.0	11.6
Lactose (0.5%) + sodium lactate (0.5%)	9.2	4.5	7.2	9.7
Glucose (0.5%)	10.4	5.7	12.0	13.6
Glucose (0.5%) + sodium lactate (0.5%)	8.8	4.8	13.2	23.9
Galactose (0.5%)	10.0	3.8	----[d]	----
Galactose (0.5%) + sodium lactate (0.5%)	12.1	6.5	13.1	19.5

[a] Assay conditions: 15-min incubation at 32 C, 0.05 M sodium phosphate buffer (pH 7.0).

[b] Untreated cells.

[c] Toluene-acetone treated cells.

[d] Not determined.

Milliunits enzyme activity (nMoles ONP/mg dry cells/min)							
P12			P22			P51	
U.C.	T.A.		U.C.	T.A.		U.C.	T.A.
15.7	26.6		12.8	64.6		11.6	29.9
11.6	14.3		9.0	25.7		9.9	6.4
11.4	16.6		5.6	20.3		9.0	14.4
12.7	15.6		23.0	60.2		6.9	6.2
13.3	27.9		20.8	71.1		10.2	20.8
----	----		14.8	54.2		----	----
14.5	22.8		13.5	60.8		11.8	15.9

Effect of lactose analogs and related sugars on β-galactosidase activity

To further investigate the nature of the cellular control mechanism governing the β-galactosidase system in P. shermanii, the relative ability of various synthetic non-utilizable lactose analogs and other sugars to elicit enzyme response in whole cell assay systems of strains P7 and P22 were examined. These assays were made on eight different days. To eliminate variations inherent in such a procedure so that direct comparisons could be made, the results are expressed as per cent of control activity for each assay (Table 4).

With the possible exception of isopropyl-β-D-thiogalactopyranoside (IPTG), no significant differences were observed in the relative enzyme levels in whole cell suspensions of P7 and P22 exposed to various lactose analogs and other sugars. With IPTG, a relatively lower response was encountered with both strains. This was in direct contrast to known inducible β-galactosidase systems as in E. coli, for which IPTG and methyl-β-D-thiogalactopyranoside (TMG) were found to be good inducers (37) with S. lactis 7962, Citti et al. (26) observed that lactose, TMG, and IPTG were good inducers; and, in S. faecium TMG again proved to be a good inducer (17). One of the few systems where IPTG failed to induce β-galactosidase was encountered in Staphylococcus aureus (112). This is understandable, because this microorganism possesses a phospho-β-galactohydrolase instead of a β-galactosidase (78, 93).

The response to various sugars in this experiment agreed with the results obtained in the first experiment using different carbon sources to grow the cells for enzyme assay (Table 3). These results also suggest

Table 4. Effect of lactose analogs and sugars on β-galactosidase activity
of P. shermanii P7 and P22 untreated cells[a]

P. shermanii strain	Lactose analog or sugar[c]	Milliunits enzyme activity[b] (nMoles ONP/mg dry cells/min)		
		Control	Lactose analog or sugar	Per cent activity of control
P7	IPTG	8.7	7.0	80
	TMG	5.0	5.2	104
	PNPG	3.0	3.2	107
	ONPG	2.6	2.9	112
	Lactose	3.6	3.3	92
	Glucose	2.7	3.0	111
	Galactose	3.4	4.4	129
	Maltose	7.7	7.6	99
P22	IPTG	12.8	7.7	60
	TMG	13.5	9.6	71
	PNPG	6.4	4.4	69
	ONPG	6.7	5.2	78
	Lactose	5.2	5.0	96
	Glucose	4.9	5.7	116
	Galactose	10.2	10.1	99
	Maltose	12.0	11.0	92

[a] Cells, centrifuged from 1% sodium lactate broth after 24-hr growth,
were washed once with sodium phosphate buffer. Lactose analogs (0.01 $\underline{M}$)
and sugars (0.5%) were added to aliquots of cells which were then incubated for 6 hr at 32 C. After incubation, cells were centrifuged from the
lactose analog or sugar solution and rinsed twice with sodium phosphate
buffer. The β-galactosidase assay was then performed. Control samples,
which received buffer instead of lactose analogs or sugars, received the
same incubation and cell rinsings before being assayed.

[b] Assay conditions: 15-min incubation at 32 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0).

[c] IPTG - Isopropyl-β-D-thiogalactopyranoside.
TMG - Methyl-β-D-thiogalactopyranoside.
PNPG - p-Nitrophenyl-β-D-galactopyranoside.
ONPG - o-Nitrophenyl-β-D-galactopyranoside.

that the β-galactosidase system of P. shermanii P7 and P22 is probably

constitutive rather than inducible. It is assumed that there were suffi-

cient endogenous metabolites in the cells for enzyme synthesis if the

strains were inducible.

Growth curves

To confirm the observations made in the previous two experiments,

another experiment was designed to determine if a differential growth rate

and/or diauxie growth occurred in two parallel cultures in a basal medium

containing combinations of lactose and sodium lactate; one with limiting

lactose and the other with limiting sodium lactate. The results are shown

in Fig. 2. No difference in growth rate was seen; the lag phases of the

growth curves were similar for all combinations. Furthermore, no diauxie

growth pattern was observed, which again indicated that the β-galactosidase

system of P. shermanii P7 is constitutive. Within the same overall incu-

bation period, an elevated total growth was observed in the medium con-

taining the high level of lactose. Because lactose is a disaccharide

consisting of a glucose and a galactose moiety, each mole of lactose would

yield 4 moles of lactate. Such an elevated overall growth could be ex-

pected in the medium containing the higher level of lactose. The fact

that higher numbers of microorganisms were present in the media containing

the high concentration of lactose indicates that the cells were utilizing

the lactose and so should have been induced in β-galactosidase is an induc-

ible enzyme in P. shermanii P7.

Fig. 2. Growth curves of P. shermanii P7 in the four combinations
of high and low concentrations of sodium lactate and lactose

A - Culture grown in sodium lactate broth was inoculated
into broth containing 0.0145 M (0.16%) sodium lactate +
0.0029 M (0.10%) lactose.

B - Culture grown in sodium lactate broth was inoculated
into broth containing 0.0145 M (0.50%) lactose + 0.0029
M (0.03%) sodium lactate.

C - Culture grown in lactose broth was inoculated into broth
containing 0.0145 M (0.16%) sodium lactate + 0.0029 M
(0.10%) lactose.

D - Culture grown in lactose broth was inoculated into broth
containing 0.0145 M (0.50%) lactose + 0.0029 M (0.03%)
sodium lactate.
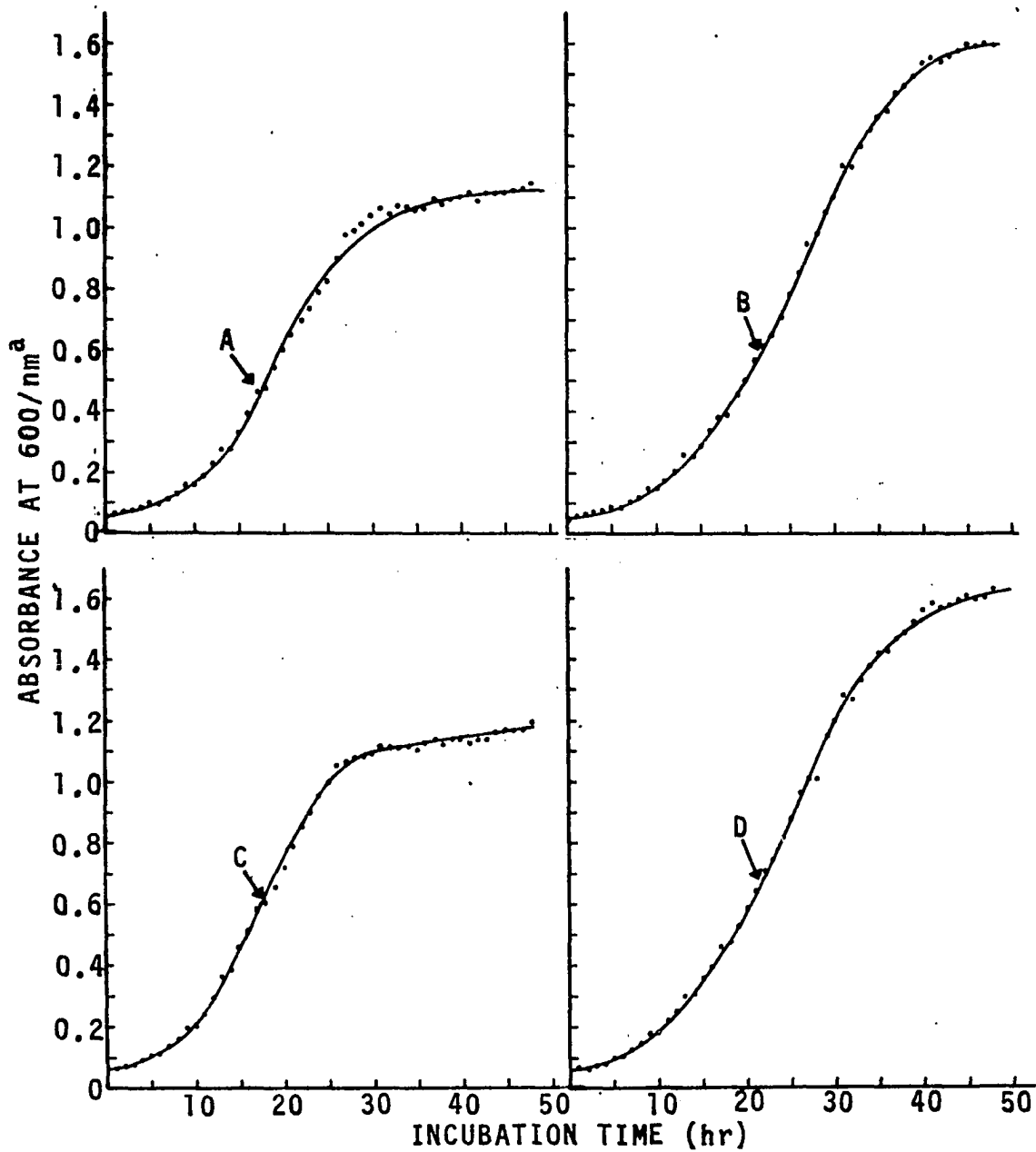
a Absorbance of cells was determined in growth medium.

Effect of incubation temperature on β-galactosidase activity

To establish the optimum conditions for β-galactosidase activity in untreated and toluene-acetone treated cell suspensions of P. shermanii P7 and P22, the effect of temperature was first investigated. The results are graphically represented in Fig. 3 and 4. With P. shermanii P7, the optimum temperature for both the untreated and toluene-acetone treated cell systems was 52 C. The effect of temperature on the enzyme activity in the solvent-treated cell system was, however, not as pronounced as in the untreated cell system. The enzyme activity at 52 C in the untreated cell system was nearly 17 times the level observed at 32 C.

The optimum temperature for β-galactosidase activity in untreated cell suspensions of P. shermanii P22 was 58 C. The increase in activity at 58 C (the optimum temperature) over the level observed at 32 C, however, was not as pronounced as that found in the other strain. With strain P22, the increase was about sevenfold. In the toluene-acetone treated cell system, an inverse relationship between enzyme activity and temperature was observed. Maximum activity in this system was observed at 32 C; activity declined gradually up to 45 C beyond which a more rapid decrease was observed. The probable reason for this phenomenon was discussed in an earlier section.

Although the optimum growth temperature of propionibacteria is around 32 C (16), the optimum temperature for β-galactosidase activity in untreated cell suspensions of these microorganisms is above 50 C. Similar observations have been made with other bacterial systems. McKay (117) found that β-galactosidase from L. helveticus, L. lactis, and L. acidophilus
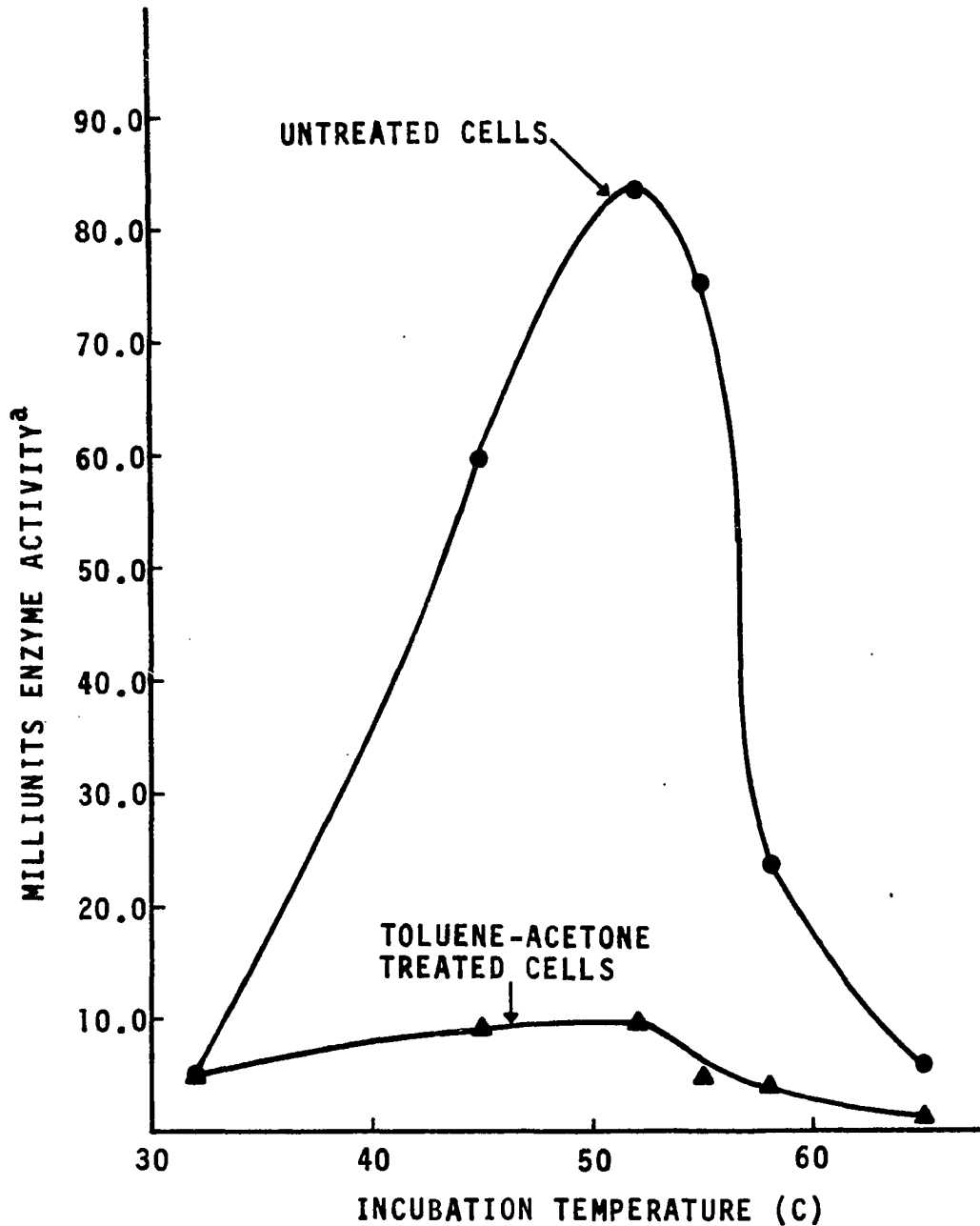
[a] nMoles ONP liberated from ONPG/mg dry cells/min incubation. Assay conditions: 15-min incubation, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0).

Fig. 3. Effect of incubation temperature on β-galactosidase activity of P. shermanii P7 untreated and toluene-acetone treated cells
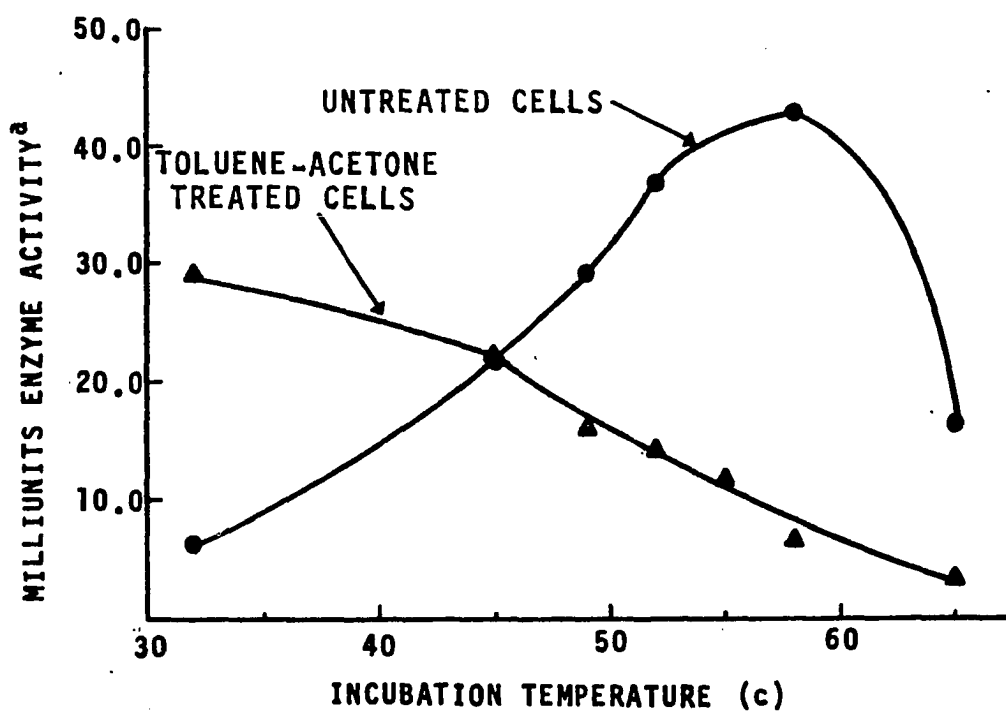
^{a} nMoles ONP liberated from ONPG/mg dry cells/min incubation. Assay conditions: 15-min incubation, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0).

Fig. 4. Effect of incubation temperature on β-galactosidase activity of $\underline{P}$. shermanii P22 untreated and toluene-acetone treated cells

exhibited greater activity as measured by ONPG hydrolysis at 50 C than at 37 C. Citti et al. (26) found maximum β-galactosidase activity in S. lactis 7962 at 50 C. Knopfmacher and Salle (97) observed that greater lactose hydrolysis by E. coli occurred at 46 C than at 36 C. Some authors consider that such microbial enzyme systems exhibiting anomalous optimum temperature for activity in relation to the optimum growth temperature of the microorganisms themselves, are probably not directly related to the vital functions of these microbial cells (23).

Citti et al. (26) found that toluene-acetone treated cells of S. lactis 7962 showed maximum β-galactosidase activity at 40 C, although the optimum temperature for the enzyme in untreated cell suspensions was 50 C. The responses obtained in their study (26) were very similar to the P. shermanii P22 system in this investigation. McKay (117), on the other hand, found that β-galactosidase activity of toluene-acetone treated cells of L. helveticus, L. lactis, and L. acidophilus, as in untreated cell sus-pensions, was greater at 50 C than at 37 C. It is probable that the cell-membrane associated enzyme systems in the lactobacilli are more stable to the combined action of the solvent treatment and elevated temperature.

Effect of pH on β-galactosidase activity

The effect of pH on β-galactosidase activity in untreated and solvent-treated cell suspensions of P. shermanii P7 and P22 is shown in Fig. 5 and 6. Optimum activity occurred at pH 7.5 in both P7 and P22 untreated and toluene-acetone treated cell suspensions. In several other microbial systems also, the pH optima for β-galactosidase have been reported to occur
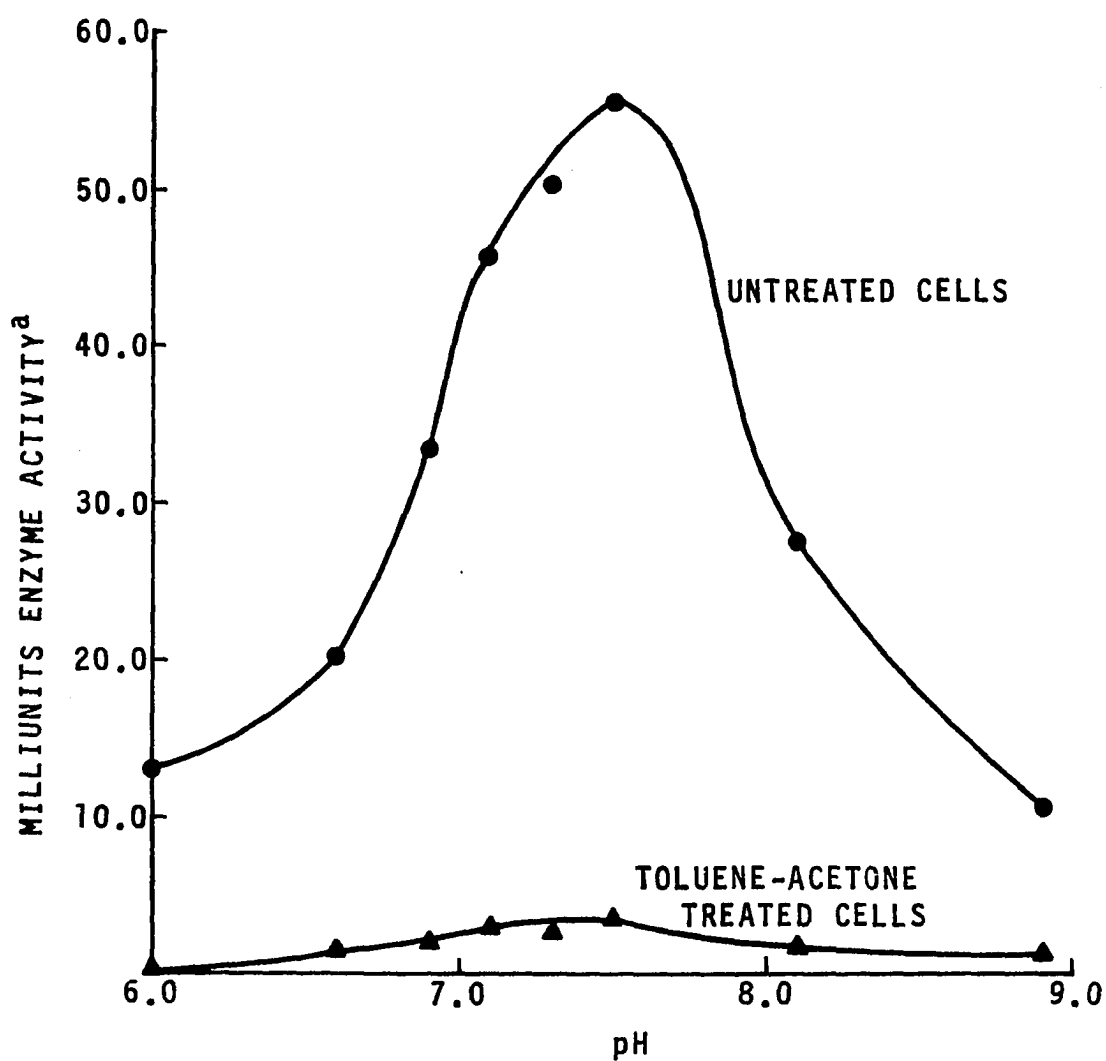
[a] nMoles ONP liberated from ONPG/mg dry cells/min incubation.
Assay conditions: 15-min incubation at 52 C, 0.05 $\underline{M}$
sodium phosphate buffer.

Fig. 5. Effect of pH on β-galactosidase activity of $\underline{P}$. shermanii
P7 untreated and toluene-acetone treated cells
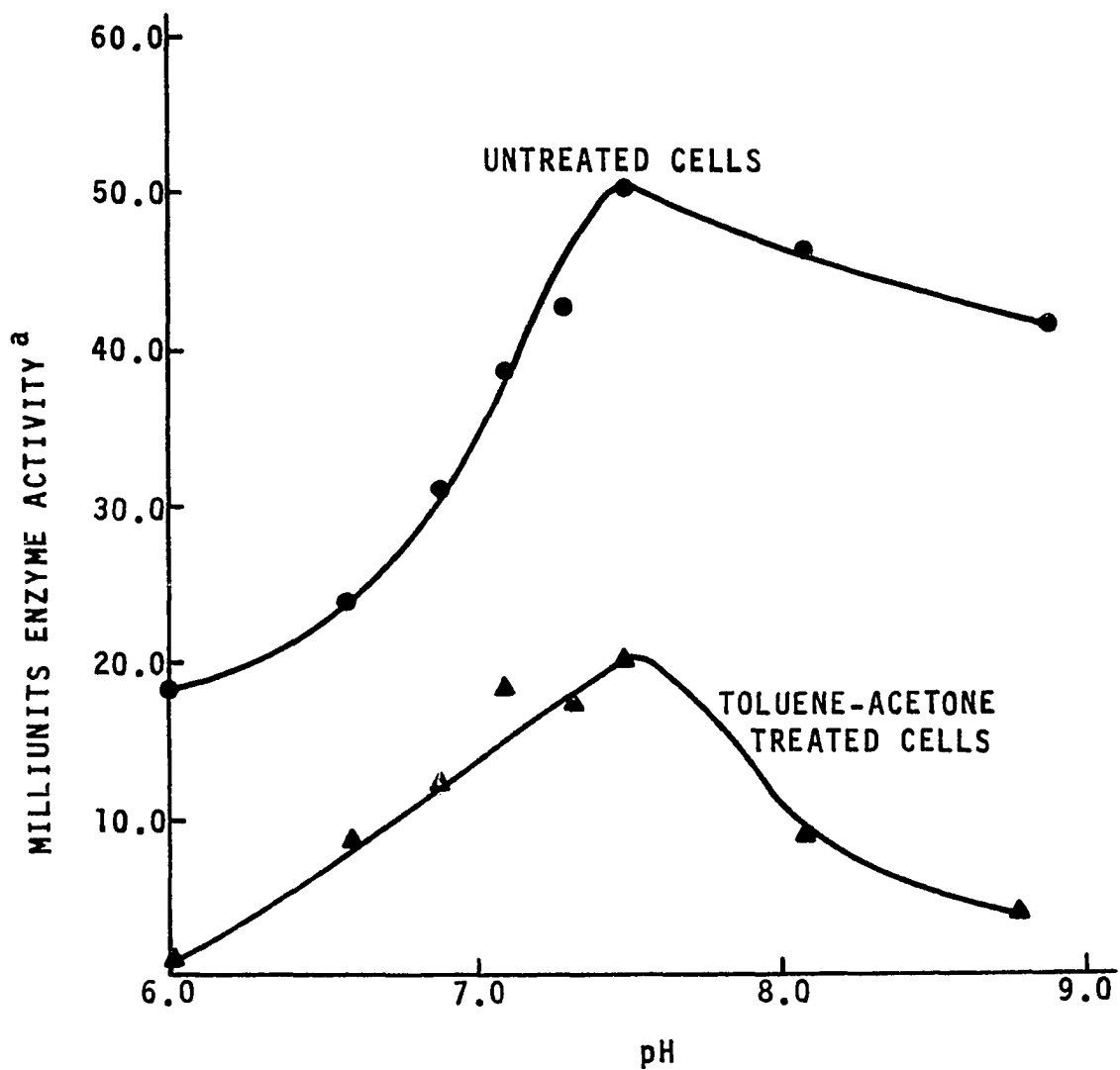
[a] nMoles ONP liberated from ONPG/mg dry cells/min incubation. Assay conditions: 15-min incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer.

Fig. 6. Effect of pH on β-galactosidase activity of P. shermanii P22 untreated and toluene-acetone treated cells

in close proximity to neutral conditions (26, 36, 97, 100, 106, 117, 148, 193, 198).

The pH effect in the P. shermanii P7 system was more prominent in untreated cell suspensions than in the toluene-acetone treated cells. Enzyme activity in untreated cells of strain P22 was not greatly affected by pH change beyond 7.5 up to a maximum of 8.9 which was similar to the response obtained with Staphylococcus aureus whole cells by Creaser (45). The pH curve for β-galactosidase activity in the untreated cell system of P. shermanii P7, on the other hand, was more typically bell-shaped with a sharp peak at pH 7.5 and a rapid decline below or above this value. In direct contrast to the toluene-acetone treated cell system of P. shermanii P7, the enzyme activity in the solvent-treated cell suspensions of strain P22 exhibited greater dependence on pH for optimum activity. The variability in the behavior of the enzyme systems in response to pH changes in the assay systems suggests that there could be basic differences in the structure of the enzyme proteins from these two strains.

Effect of age of cells on β-galactosidase activity

Enzyme activities in several P. shermanii P7 whole cell suspensions, made from cells harvested at different stages of growth in sodium lactate broth at 32 C over a 72-hr period, are represented in the histogram shown in Fig. 7. The maximum enzyme activity was observed in cells harvested after 28-hr growth. During the early stages of growth, viz., up to 16 hr, the cells had very low enzyme levels. From Fig. 2, which depicts the growth response of this strain in sodium lactate-containing medium at 32 C,
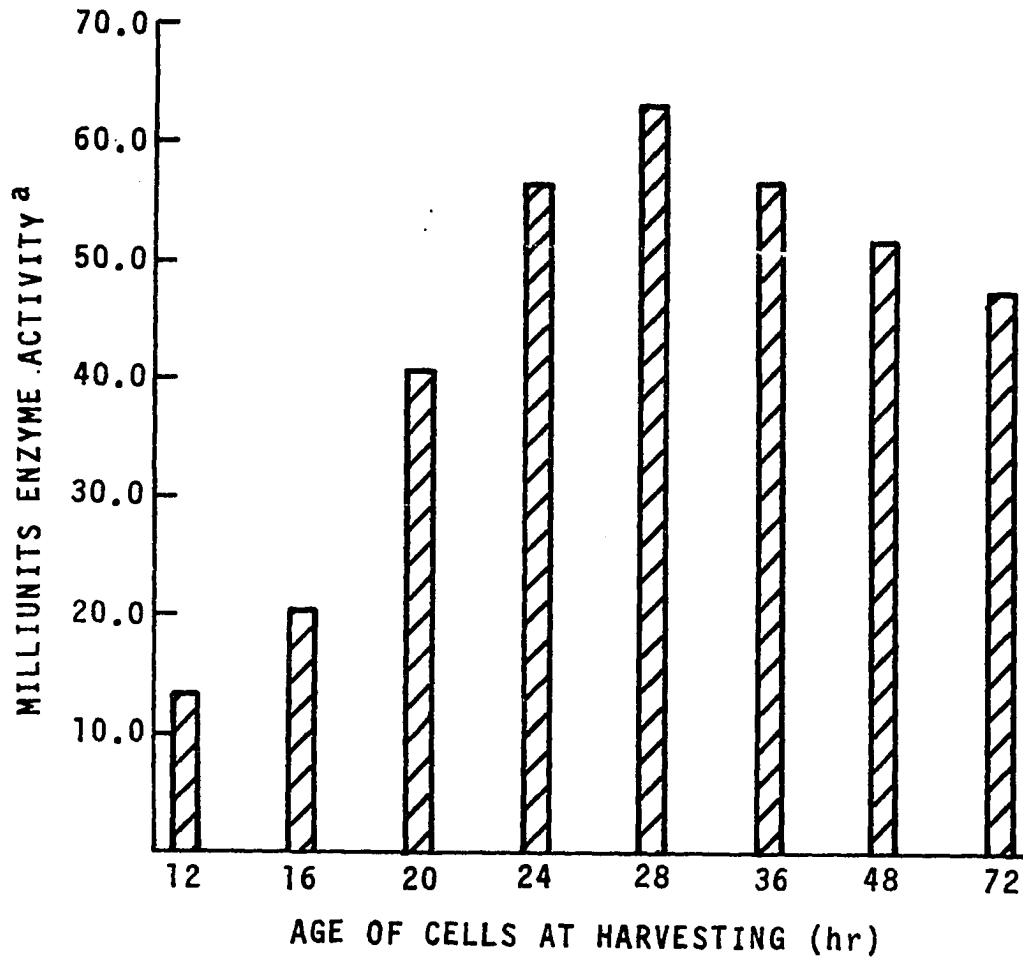
[a] nMoles ONP liberated from ONPG/mg dry cells/min incubation. Assay conditions: 15-min incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.5).

Fig. 7. Effect of age of cells at harvesting on β-galactosidase activity of $\underline{P}$. shermanii P7 untreated cells

it is evident that the culture attains the early logarithmic phase by about 16 hr. The 28-hr culture, according to the same growth curve, would represent the late logarithmic phase of growth. Similar observations have been made with other bacterial systems. Richards and Hinshelwood (150) observed that the β-galactosidase activity of A. aerogenes, growing in aerated lactose broth, was constant during the greater part of the logarithmic phase but registered a rapid increase towards the onset of the stationary phase of growth. The decrease in activity in the older cultures agrees with the observations of McFeters et al. (116) who observed that β-galactosidase concentration in S. lactis 7962 decreased when growth of the culture slowed down. Based on the observations made in this experiment, all further enzyme assays were conducted on cultures grown 28 hr at 32 C.

Effect of incubation time on the rate of ONPG hydrolysis

Hydrolysis of ONPG by untreated cells of P. shermanii P7 and P22 was linear for at least a 15-min assay incubation period (Fig. 8). Propionibacterium shermanii P22, however, exhibited a zero order reaction over a longer incubation period. This experiment showed that all assays conducted in this investigation fell within the period where the catalytic reaction exhibited zero order kinetics.

Effect of buffers on β-galactosidase activity

To determine the ionic requirement for optimum β-galactosidase activity in untreated and toluene-acetone treated cell systems of P. shermanii P7 and P22, several different buffer combinations made up of different ionic groups were used in the assay system (Fig. 9). Presence of potassium
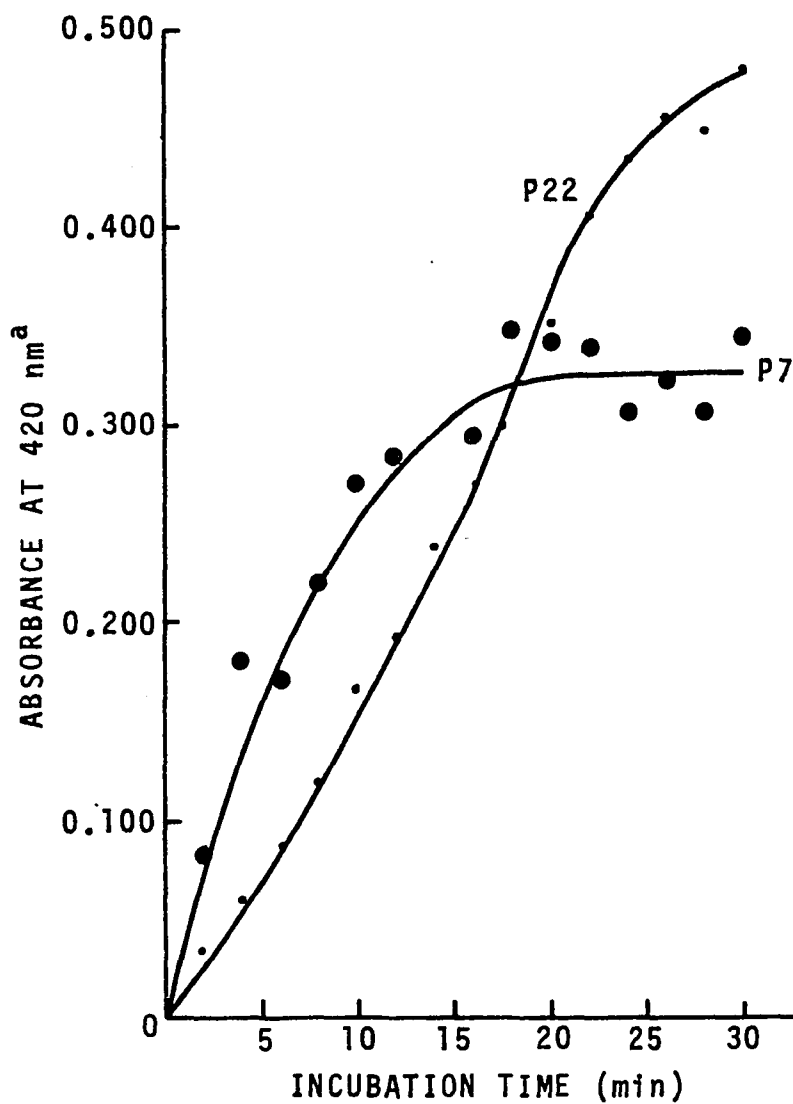
[a] Assay conditions: incubation at 52 C, 0.05 M sodium phosphate buffer (pH 7.5). Weight of cells: P7 = 0.76 mg dry cells/ml, P22 = 0.78 mg dry cells/ml.

Fig. 8. Effect of incubation time on rate of ONPG hydrolysis by P. shermanii P7 and P22 untreated cells

BUFFERS

0.05 M SODIUM PHOSPHATE
0.05 M POTASSIUM PHOSPHATE
0.05 M TRIS
0.025 M TRIS + 0.025 M SODIUM PHOSPHATE
0.025 M TRIS + 0.025 M SODIUM CHLORIDE

MILLIUNITS ENZYME ACTIVITY[a]

50.0
40.0
30.0
20.0
10.0

UNTREATED CELLS TOLUENE-ACETONE TREATED CELLS UNTREATED CELLS TOLUENE-ACETONE TREATED CELLS

P7   P22
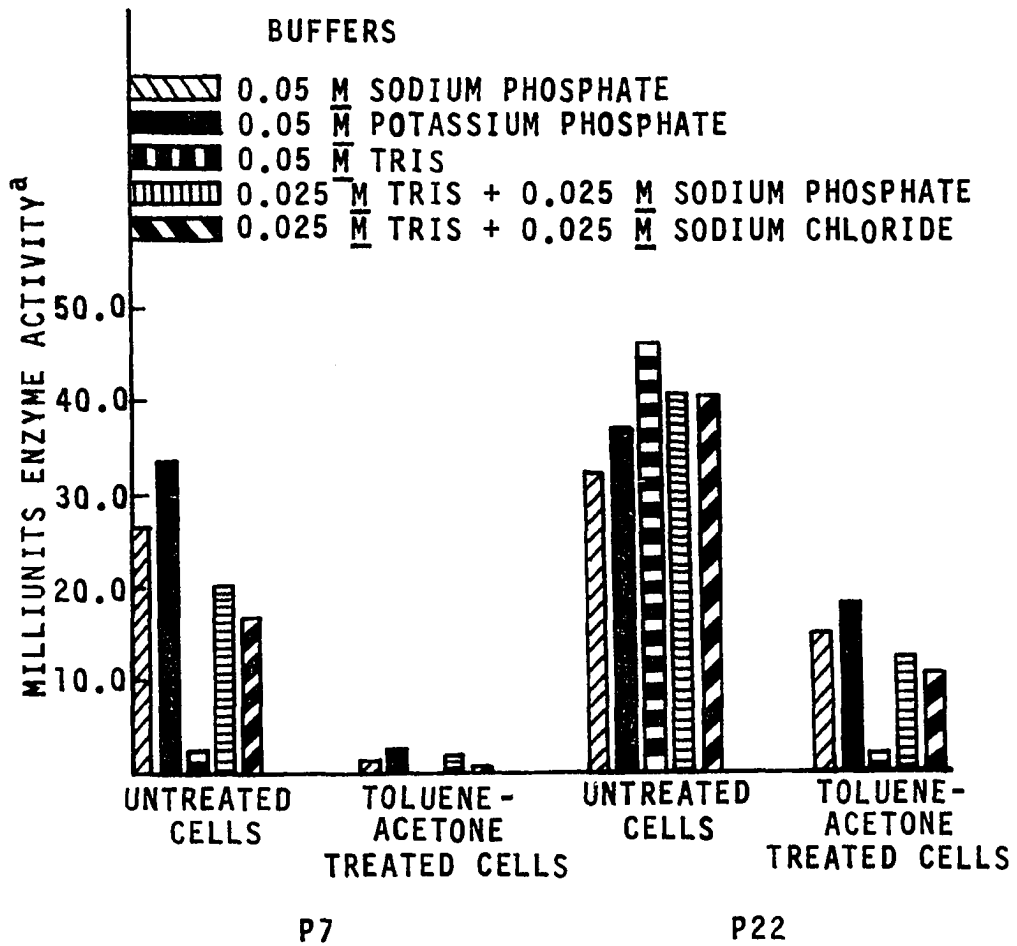
[a] nMoles ONP liberated from ONPG/mg dry cells/min incubation.
Assay conditions: 15-min incubation at 52 C, pH 7.5.

Fig. 9. Effect of buffer on β-galactosidase activity of P. shermanii
P7 and P22 untreated and toluene-acetone treated cells

phosphate in the system yielded higher enzyme activity with untreated cell suspensions of both strains. Sodium ions appeared to be fairly comparable to K^+ ions. The near equal response obtained with sodium phosphate and sodium chloride added to tris buffer indicated that Na^+ was the essential ion. Sodium and K^+ ions also have been reported to stimulate β-galactosidase in other bacterial systems (26, 117).

Tris buffer by itself appreciably inhibited enzyme activity in P. shermanii P7, but in the P22 system, maximum response with untreated cells was obtained with tris buffer. This observation also indicates that the β-galactosidase systems in the two strains are quite different. With S. lactis 7962, a tris assay system failed to elicit good β-galactosidase activity (26). Sodium chloride, and especially sodium phosphate partially reversed the tris inhibition. Stárka (172) observed that phosphate ions reversed the inhibitory effect of tris-HCl buffer on E. coli β-galactosidase. Tris-sodium chloride buffer gave good enzyme activity in other investigations (103, 151).

In toluene-acetone treated cell suspensions, the enzyme activity was relatively poor because of the high assay temperature (52 C). The most noteworthy observation was that the enzyme activity was greatly affected by the presence of tris buffer without any other accompanying buffer salts in the toluene-acetone treated assay system of P. shermanii P22. The reason for this observation is not known.

It is puzzling that tris buffer should give the greatest activity with P22 untreated cells, but markedly lower activity in the other assay systems, including toluene-acetone treated P22 cells. The protective

effect of Na^+ and phosphate ions could result from the ions binding to the enzyme and altering its stereochemistry. Tris also could affect the enzyme activity by altering the stereochemistry of the molecule. It is possible that the amino group of tris could react with a carboxyl group of the enzyme. With P22 untreated cells, the molecular configuration would be altered so the enzyme could react with the substrate more rapidly. With toluene-acetone treated cells, the combined effect of the tris, solvent, and high incubation temperature could change the molecular configuration so that it reacted very slowly with the substrate.

Effect of manganese chloride on β-galactosidase activity

Manganous ions have been reported to stimulate β-galactosidase enzyme obtained from many different sources (22, 100, 103, 148, 194, 198). To investigate if Mn^{++} ions also would cause an increase in the enzyme activity of the two P. shermanii strains, assays were conducted in the presence and absence of 0.4 $\underline{m}\underline{M}$ $MnCl_2$ in the two systems.

Marked and statistically significant stimulation of enzyme activity by Mn^{++} ions was observed only with P. shermanii P7 cells (Table 5). The difference in response to the presence of Mn^{++} ions in the assay systems of P7 and P22 again indicates that the β-galactosidase systems in the two strains are quite different.

Untreated cells of P. shermanii P7, the strain showing greater β-galactosidase activity in the presence of $MnCl_2$ (Table 5), gave much higher activity in the presence of $MnCl_2$ when a 52 C assay incubation temperature was used than when the assay was conducted at 32 C (Fig. 10). In a later experiment, $MnCl_2$ stabilized β-galactosidase from cell-free extracts of

Table 5. Statistical evaluation of effect of manganese chloride on β-galactosidase activity of P. shermanii P7 and P22 untreated cells

Strain	Milliunits enzyme activity[a] (nMoles ONP/mg dry cells/min)		Difference
	$MnCl_2$ (none)	$MnCl_2$ (0.4 m$\underline{M}$)	
P7	84.0	115	31.0
	56.4	108	51.6
	72.5	110	37.5
			$\bar{x}_d$= 40.03
			s_d= 6.08
			t= 6.58*
P22	65.5	66.2	0.7
	62.4	64.7	2.3
	55.7	54.5	-1.2
			$\bar{x}_d$= 0.60
			s_d= 1.01
			t= 0.59[b]

[a] Assay conditions: 15-min incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.5).

[b] Not significant at P<0.05.

* Significant at P<0.05.

P22 at high temperatures also (Table 10, Fig. 16). Other workers have observed that $MnCl_2$ stabilizes β-galactosidase in cell-free extracts at temperatures above 50 C (5, 151, 197).
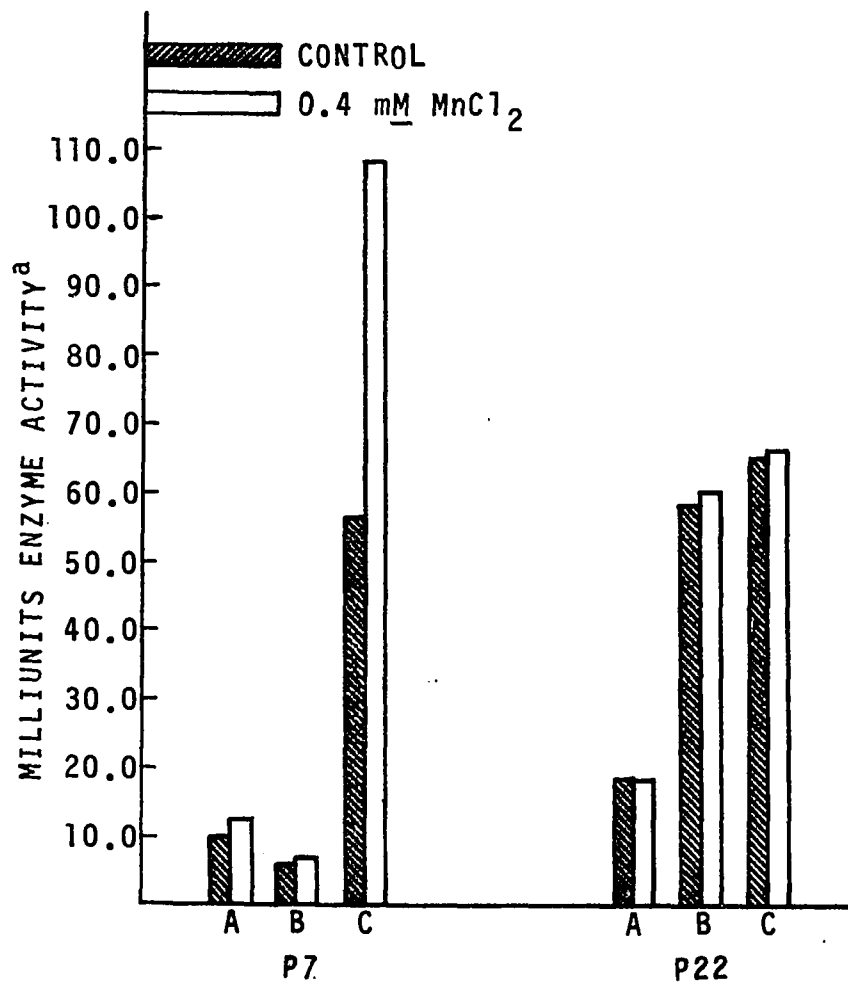
^{a} nMoles ONP liberated from ONPG/mg dry cells/min incubation.

Assay conditions

0.05 M sodium phosphate buffer.
A - 15-min incubation at 32 C, pH 7.0.
B - 15-min incubation at 32 C, pH 7.0, cells
      received toluene-acetone treatment.
C - 15-min incubation at 52 C, pH 7.5.

Fig. 10. Effect of manganese chloride on β-galactosidase activity of
          P. shermanii P7 and P22 untreated and toluene-acetone treated
          cells

Stability of β-galactosidase enzyme in resting cell suspensions

The β-galactosidase activity in resting cell suspensions of Shigella

sonnei and E. coli is quite stable over several days storage (29, 97).

To determine the relative stability of the enzyme in the propionibacterial

systems, whole cell suspensions of P. shermanii P7 and P22 in sodium phos-

phate buffer (pH 7.0) were stored at 3.3 C for 16 days and were then

assayed for the enzyme. The activity after storage was compared with

determinations made before storing the cell suspensions. From Table 6 it

is evident that the enzyme system in strain P22 is more stable than that

in strain P7. This again further substantiates differences in the enzyme

systems between the two strains.

Table 6. Effect of storage at 3.3 C on β-galactosidase activity of P.
shermanii P7 and P22 untreated cells grown 24 hr at 32 C in
0.5% lactose broth

Strain	Days storage[a]	Milliunits enzyme activity[b]	Per cent of initial activity
P7	0	62.1	
	16	38.6	62
P22	0	26.9	
	16	29.8	111

[a] Cells were stored in 0.05 M sodium phosphate buffer.

[b] nMoles ONP liberated from ONPG/mg dry cells/min incubation. Assay
conditions: 10-min incubation at 52 C, 0.05 M sodium phosphate buffer
(pH 7.5).

Cell-Free Extract Studies

β-Galactosidase activity of cell-free extracts

Cell-free extract of P. shermanii P22 possessed over six times more

β-galactosidase activity per milligram protein than extract from P. sher-

manii P7 (Table 7). This observation shows that enzyme from the two

strains also exhibits differences when assayed in cell-free extracts. With

untreated cells, strain P7 had only slightly lower activity than strain P22

(Table 1). Enzymes from the two strains differed in several other proper-

ties. Strain P7 did not show increased activity when cells were treated

with solvent (Table 1), was less stable to storage (Table 6), and had a

slightly lower optimum assay temperature (Fig. 3 and 4). Strain P7 un-

treated cells, however, did possess much higher enzyme activity at 52 C

than strain P22 untreated cells (Fig. 3 and 4), and gave much greater

response to $MnCl_2$ in the assay system (Table 5, Fig. 10). The low activity

Table 7. β-Galactosidase activity of P. shermanii P7 and P22 cell-free
extracts

Strain	Milliunits enzyme activity[a] (nMoles ONP/mg protein/min)
P7	19.5
P22	128

[a] Assay conditions: 10-min incubation at 52 C, 0.05 M sodium phos-
phate buffer (pH 7.0), 0.4 mM $MnCl_2$.

of strain P7 in cell-free extract relative to P22, based on their relative activities in untreated cells, indicates that β-galactosidase in P7 cells is labile to cell disruption. The loss in enzyme activity with strain P7 could be due to the release of a protease, oxidase, or ion or substance that reacted with sulfhydryl groups or other sites important for enzyme activity. Also, the sudden decrease in pressure when the cells leave the French press pressure cell could have an adverse effect on the enzyme configuration.

The French press was the sole method used for cell disruption. Wierzbicki and Kosikowski (200), however, obtained equivalent results from ultrasonic disintegration, press crushing, solvent treatment, and air and freeze drying. Strain P7 (Table 7), which was adversely affected by disrupting the cells with the French press, also showed reduced activity when the cell walls and membranes were disrupted with solvent (Table 1 and 2).

Effect of incubation temperature on β-galactosidase activity

Maximum β-galactosidase activity with cell-free extracts of both strains occurs near 52 C, the same optimum observed with untreated cells (Fig. 11 and 12). Activity was much lower at assay temperatures above 52 C. Although P. shermanii P7 contained approximately only one-tenth as much β-galactosidase activity as cell-free extract from strain P22, both strains showed the same general response to change in assay incubation temperature. Because of the lower β-galactosidase activity of P7 cell-free extract, it was necessary to reconstitute lyophilized cell-free extract to 6.53 mg protein/ml, while P22 cell-free extract was reconstituted to only 1.02 mg protein/ml.
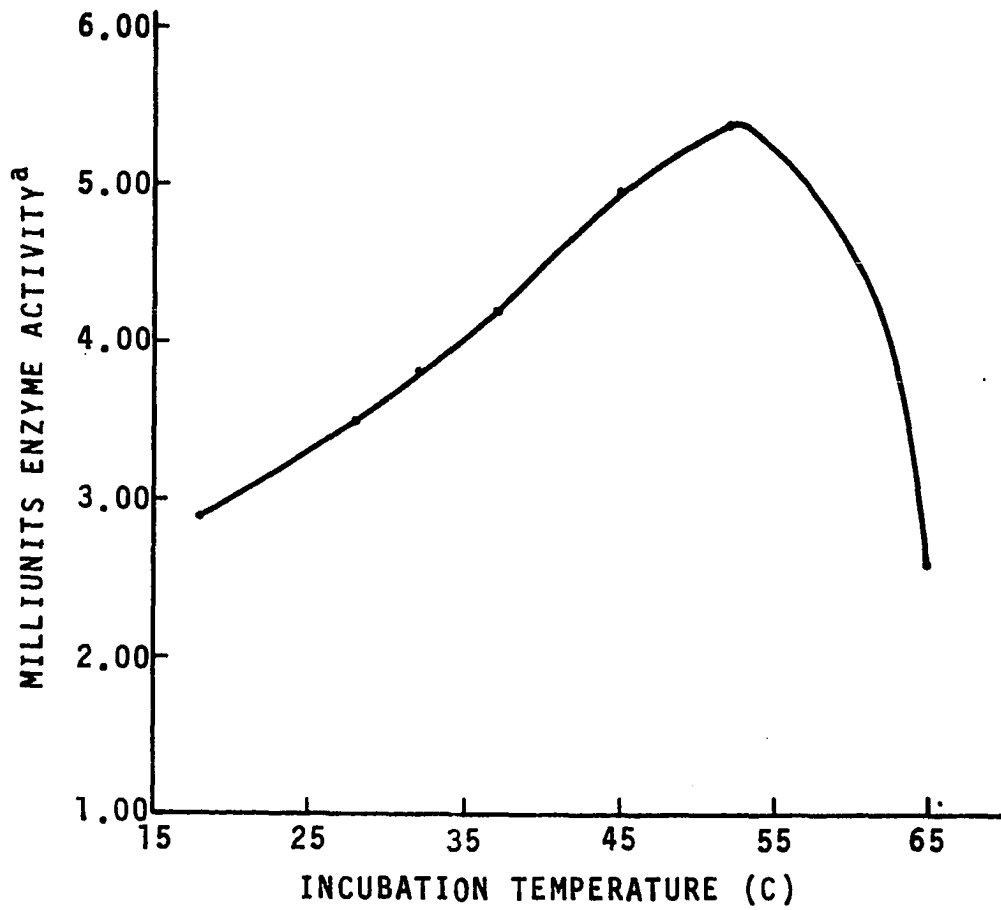
Fig. 11. Effect of incubation temperature on β-galactosidase activity of P. shermanii P7 cell-free extract
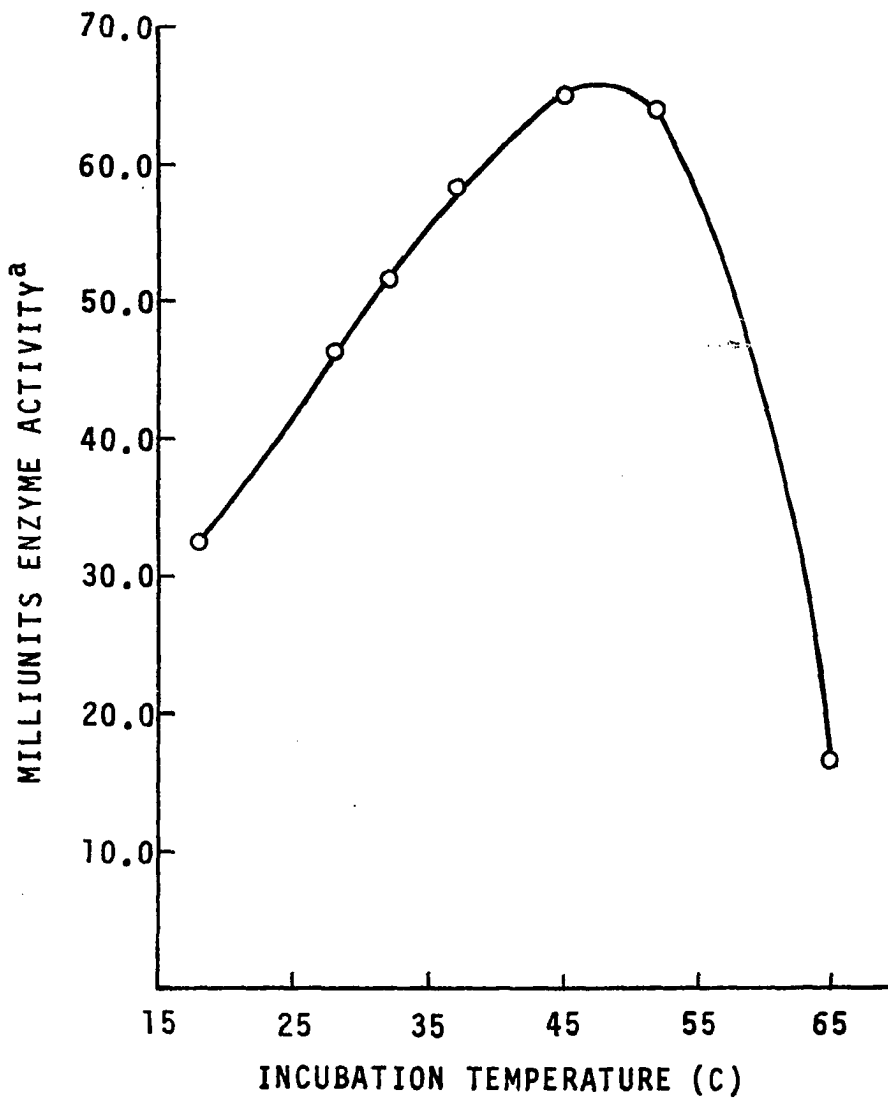
[a] nMoles ONP liberated from ONPG/mg protein/min incubation. Assay conditions: 15-min incubation, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0), 0.4 m$\underline{M}$ $MnCl_2$.

[a] nMoles ONP liberated from ONPG/mg protein/min incubation. Assay conditions: 15-min incubation, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0), 0.4 $\underline{mM}$ $MnCl_2$.

Fig. 12. Effect of incubation temperature on β-galactosidase activity of $\underline{P}$. shermanii P22 cell-free extract

It is surprising that the optimum assay temperature for cell-free extract is so near the optimum for whole cell systems. One would expect the cell to offer some protection to the enzyme. The high temperature for optimum β-galactosidase activity has been observed with other microorganisms as well as with propionibacteria. The optimum assay temperature for B. subtilis cell-free extract was 50 C (7). Wierzbicki and Kosikowski (200) found that the optimum assay temperature for hydrolysis of lactose by β-galactosidase in cell-free extracts from microbial species used in the dairy industry was between 40 and 50 C. The significance of optimum assay temperature was discussed in the whole cell system.

Experiments were not conducted in this study to determine if β-galactosidase was inactivated at the optimum assay temperature. Other workers report that β-galactosidase was very stable to incubation at 40-50 C. β-Galactosidase from E. coli ML 309 lost very little activity when incubated 30 min at 40 C (194). Cohn and Monod (36) observed less than a 5% loss of activity when a concentrated preparation of purified E. coli β-galactosidase was incubated 10 min at 47 C. Other workers report even greater stability. Purified B. subtilis β-galactosidase was not inactivated by storing 1 hr at 50 C (7) and B. megaterium cell-free extract did not lose β-galactosidase activity when held 5 min at 55 C in 1.5 M potassium phosphate buffer (103).

Effect of pH on β-galactosidase activity

Optimum β-galactosidase activity of P22 cell-free extract was obtained at pH 7.0 (Fig. 13). Activity decreased more rapidly at higher pH than was observed with untreated cells (Fig. 6). Cell-free extract of strain

[a] nMoles ONP liberated from ONPG/mg protein/min incubation. Assay conditions: 15-min incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer, 0.4 m$\underline{M}$ $MnCl_2$.

Fig. 13. Effect of pH on β-galactosidase activity of $\underline{P}$. shermanii P7 and P22 cell-free extracts

P7 was insensitive to pH changes. There also was very little response to pH change by toluene-acetone treated cells (Fig. 6). The factor which is responsible for reducing enzyme activity when the cell wall and membrane is disrupted may alter the tertiary and even the secondary structure of the 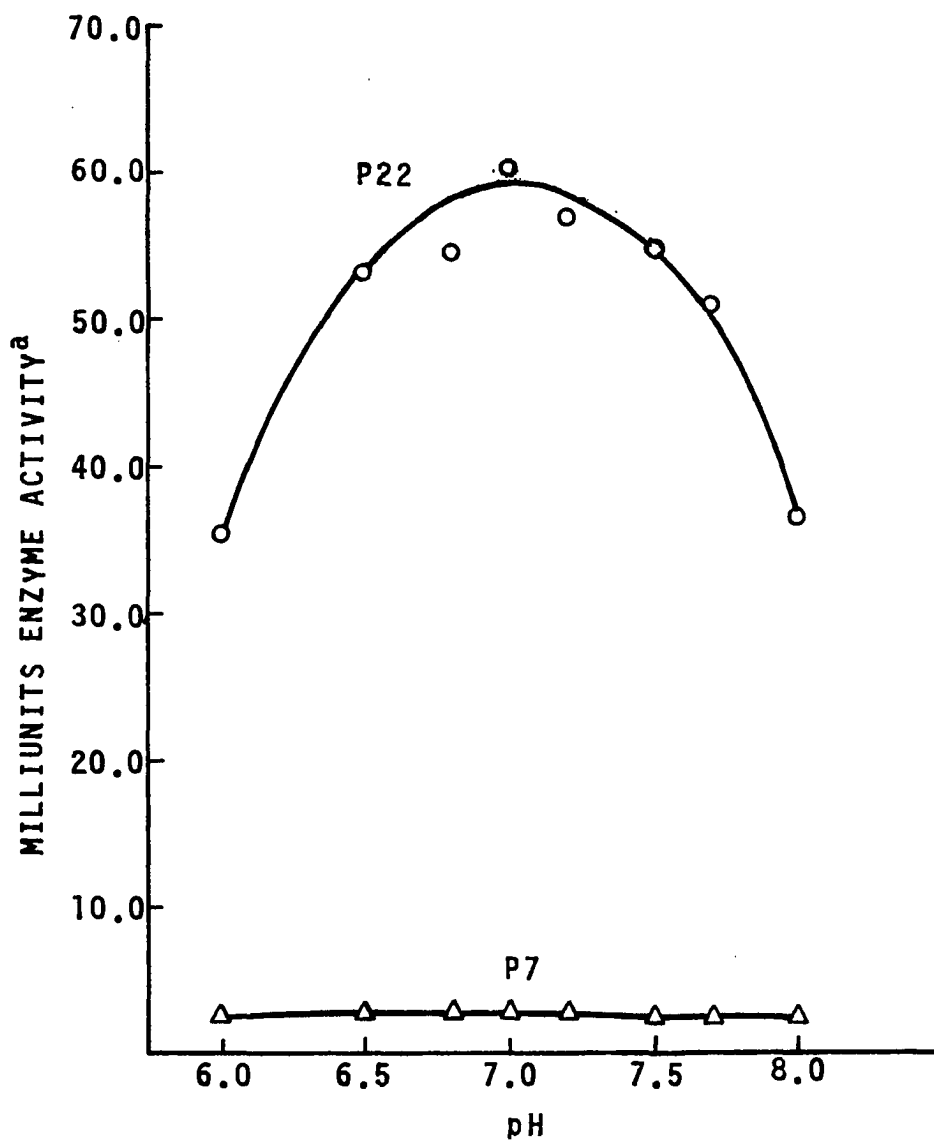enzyme molecule so that a majority of the enzyme activity is lost. That configuration which remains, and accounts for the remaining enzyme activity, may not be affected by dissociation and association of carboxyl and amino groups. Enzyme activity with P7 whole cells was sensitive to pH (Fig. 5). Lederberg (106) found a pH optimum of 7.3 for E. coli K-12 cell-free extract which was slightly higher than the pH optimum for E. coli whole cells. He observed that enzyme in intact cells was less responsive to pH changes than enzyme in cell-free extracts. He assumed that the intact cells were regulating the pH environment of the enzyme. Cell-free extract of B. subtilis had maximum activity at pH 6.5 (7). It is possible that this lower pH gave optimum activity because the protease may have been less active at this hydrogen ion concentration.

Linearity of ONPG hydrolysis rate with time

Hydrolysis of ONPG by P7 and P22 cell-free extracts was linear for at least a 15-min assay incubation period (Fig. 14). Because of the relatively lower activity of P7 cell-free extract compared to P22 cell-free extract activity, a much higher protein concentration was required with this strain to get absorbance readings in the proper range. To be certain that the reaction was being measured in the linear region of ONPG hydrolysis, that is, that the same amount of product was formed per minute at the end of the incubation period as at the beginning, 10-min incubation was
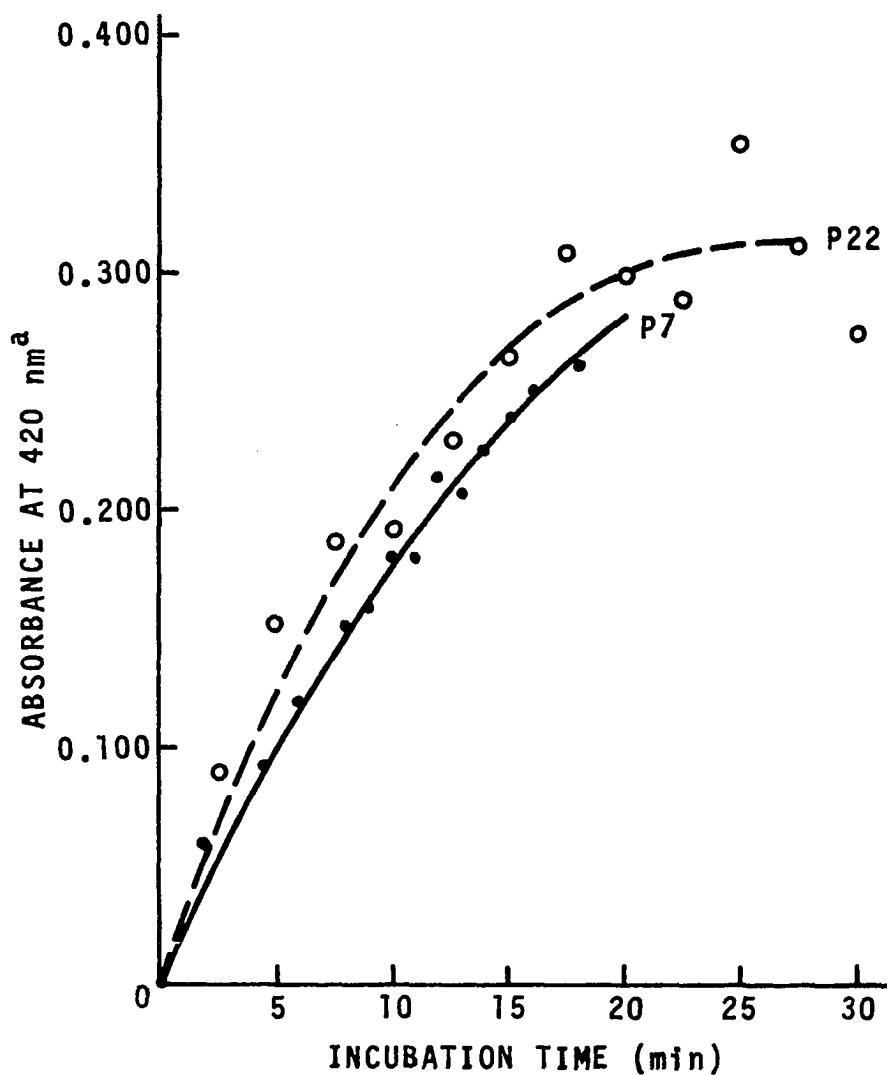
[a] Assay conditions: incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0). Amount of protein: P7 = 10.84 mg protein/ml, P22 = 0.95 mg protein/ml.

Fig. 14. Linearity of product formation with time during ONPG hydrolysis by $\underline{P}$. shermanii P7 and P22 cell-free extracts

adopted in later assays.

Effect of buffers on β-galactosidase activity

To determine the buffer system for optimum β-galactosidase activity with cell-free extracts, crude extracts of strain P7 and P22 were assayed using the buffers listed in Fig. 15. There was very little difference in β-galactosidase activity of P7 cell-free extracts with the different buffers. As discussed previously with pH effect, the reason for this is probably that, as a result of cell disruption, the structure of the enzyme was changed, resulting in greatly reduced enzyme activity. The resulting enzyme structure was equally active in all buffers used in this experiment.

Although tris buffer gave the greatest activity with P22 untreated cells (Fig. 9), it gave the poorest response with P22 cell-free extract. The tris-containing buffers (tris + sodium phosphate and tris + sodium chloride) gave a poor response compared to the phosphate buffers. As observed with whole cells (Fig. 9), potassium phosphate gave slightly greater activity than sodium phosphate. The reversal of tris inhibition by sodium phosphate and sodium chloride also was observed with whole cells and was discussed in that section.

Effect of dialysis on β-galactosidase activity

Dialysis is inhibitory to enzyme activity because it removes stimulatory ions and also may inactivate the enzyme. Dialyzed P22 cell-free extract contained only 21% as much activity as cell-free extract which was not dialyzed (Table 8). Dithiothreitol, a sulfhydryl group protector, stabilized the enzyme during dialysis. Cohn and Monod (36) observed that β-galactosidase activity in a purified E. coli preparation was irreversibly
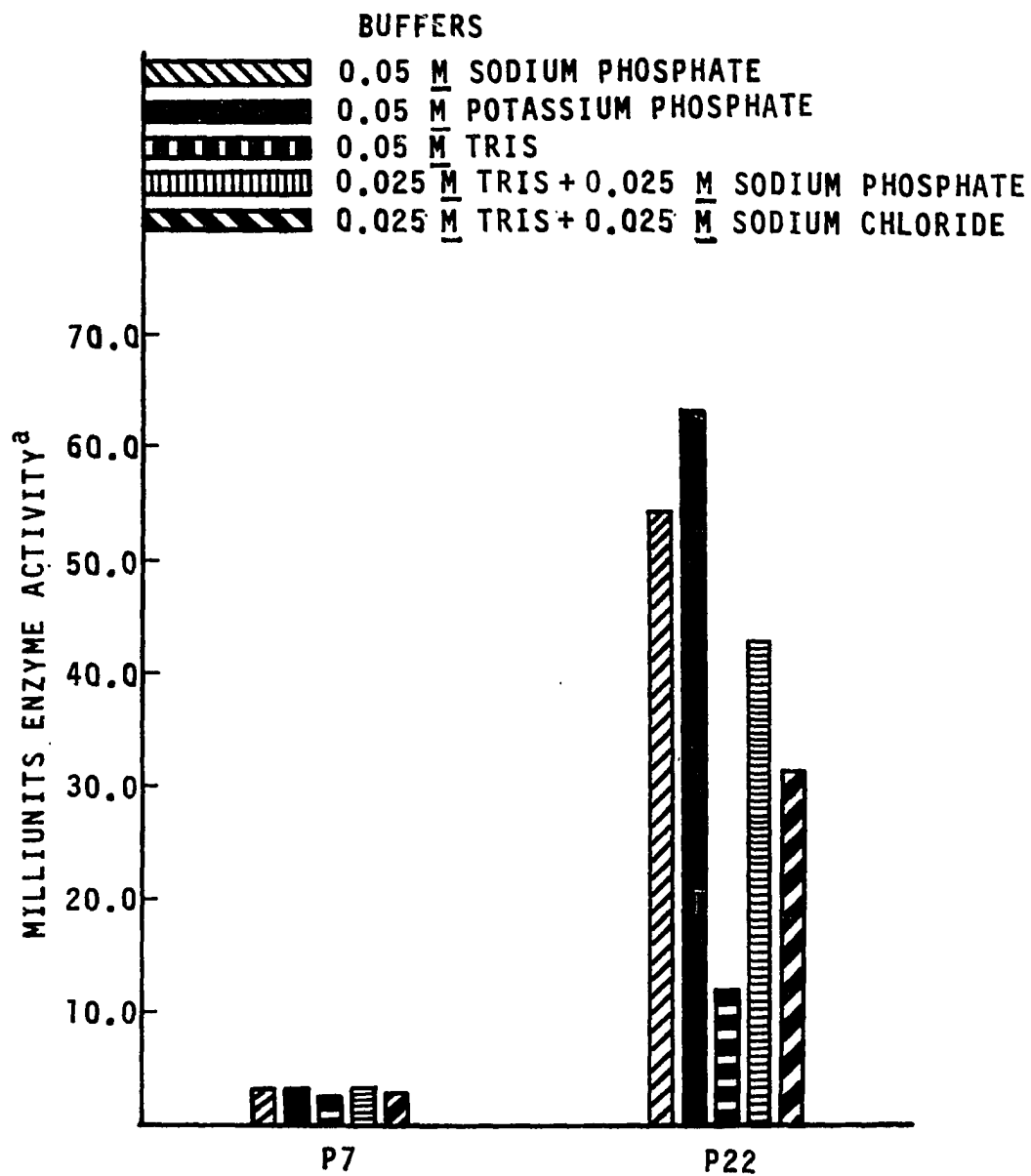
BUFFERS

0.05 M SODIUM PHOSPHATE
0.05 M POTASSIUM PHOSPHATE
0.05 M TRIS
0.025 M TRIS + 0.025 M SODIUM PHOSPHATE
0.025 M TRIS + 0.025 M SODIUM CHLORIDE

[a] nMoles ONP liberated from ONPG/mg protein/min incubation.
Assay conditions: 15-min incubation at 52 C, pH 7.0.

Fig. 15.   Effect of buffer on β-galactosidase activity of P. shermanii
P7 and P22 cell-free extracts

Table 8. Effect of dialyzing[a] P. shermanii P22 cell-free extract against
redistilled water and redistilled water containing 0.1 m$\underline{M}$
dithiothreitol, on β-galactosidase activity

Dialysate	Milliunits enzyme activity[b] (nMoles ONP/mg protein/min)
None (control)	88.1
Water	18.5
Water + dithiothreitol	33.0

[a] Fifty milliliters of cell-free extract were dialyzed against 1,000
ml redistilled water at 3.3 C. Dialysate was changed every 12 hr for five
changes.

[b] Assay conditions: 10-min incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0), 0.4 m$\underline{M}$ MnCl$_2$.

inactivated by prolonged dialysis against water. Cell-free extract of $\underline{B}$.

megaterium, dialyzed for 8 hr against two changes of 0.6% versene + 0.1 $\underline{M}$

methionine and for 6 hr against three changes of triple distilled water,

retained only one-fourth of its original activity (103).

Effect of ions on β-galactosidase activity

Sodium phosphate, potassium phosphate, ammonium phosphate, sodium

chloride, potassium chloride, and tris were added to dialyzed P22 cell-

free extract to determine the effect of the various ions on β-galacto-

sidase activity (Table 9). Results of this experiment substantiate the

observations in the buffer study (Fig. 15). Much higher activity was

obtained in the presence of MnCl$_2$. It appears that either Na$^+$ or K$^+$ ions

will give good stimulation. The lower activity with ammonium phosphate

suggests that the Na$^+$ and K$^+$ ions, rather than the phosphate ions are

Table 9. Effect of ions on β-galactosidase activity of P. shermanii P22 cell-free extract dialyzed against redistilled water containing 0.1 mM dithiothreitol

	Milliunits enzyme activity (nMoles ONP/mg protein/min)	
Ions added (0.05 $\underline{M}$)	$MnCl_2$ (none)[a]	$\underline{M}$ $MnCl_2$ (0.4 m$\underline{M}$)[b]
Control (not dialyzed)[c]	49.8[b]	71.4
Sodium phosphate	5.6	27.0
Potassium phosphate	5.2	30.5
Ammonium phosphate	2.5	20.4
Sodium chloride	2.8	40.6
Potassium chloride	6.0	53.6[d]
Tris	0.4	Int.[d]

[a] Assay conditions: 30-min incubation at 52 C.

[b] Assay conditions: 10-min incubation at 52 C.

[c] Assay conditions: 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0). Enzyme activities of the controls are averages of several determinations made on the same source of lyophilized cell-free extract. The assays were performed in separate experiments.

[d] Interference. A red-colored compound which absorbed at 420 nm formed in the sample when sodium carbonate was added.

responsible for the increased activity. Potassium ions gave slightly more activity than Na^+ ions in the assay system containing $MnCl_2$.

β-Galactosidase activity is not presented for cell-free extract in the presence of $MnCl_2$ with tris buffer because a red-colored complex formed which had absorbance at 420 nm. The red color also formed in the samples receiving sodium chloride and potassium chloride, approximately 1 hr after sodium carbonate was added. Results in Table 9 are acceptable, however, because absorbance of controls [(buffer + ONPG + sodium carbonate) and

(boiled cell-free extract + ONPG + sodium carbonate)] which received $MnCl_2$ was in the same range as absorbance of controls which did not receive $MnCl_2$. Also, no visible red color was observed in the controls or assay samples. The red color did not form in samples which received phosphate buffers. A reddish brown color formed when $MnCl_2$ was added to sodium carbonate. However, the color was obviously different than the color which developed in assay samples. The color could result from a combination of Mn^{++} ions in an alkaline environment, $MnCo_3$, and complexes of Mn^{++} ions with proteins of the cell-free extract.

Both Na^+ and K^+ ions are reported to stimulate β-galactosidase activity with other microorganisms. Potassium ions caused maximum stimulation of ONPG hydrolysis by β-galactosidase from E. coli (100) and Sac. fragilis (49). With some microorganisms (26, 36, 103, 148), Na^+ ions caused greater ONPG hydrolysis than K^+ ions.

Effect of manganese chloride on β-galactosidase activity

Cell-free extract of strain P7 did not show statistically significant increased β-galactosidase activity when $MnCl_2$ was added to the assay (Table 10). Only three trials were performed, however. When the data presented in Fig. 16 were statistically analyzed, a significant increase in β-galactosidase activity of P7 cell-free extract was observed in response to $MnCl_2$. Table 10 shows that there was increased activity in one of the trials, but decreased activity in two trials. It could be that the response of P7 cell-free extract to $MnCl_2$ is dependent upon the state of denaturation of the enzyme. Because of the low enzymatic activity of β-galactosidase from P7 cell-free extract, this phenomenon was not investigated further.

Table 10. Statistical evaluation of effect of manganese chloride on β-galactosidase activity of P. shermanii P7 and P22 cell-free extracts

| Strain | Milliunits enzyme activity[a] (nMoles ONP/mg protein/min) | | Difference |
	MnCl$_2$(none)	MnCl$_2$ (0.4 m$\underline{M}$)	
P7	4.64	6.02	1.38
	2.99	2.94	-0.05
	3.33	2.86	-0.47
			$\bar{x}_d$ = 0.287
			s_d = 0.56
			t = 0.54[b]
P22	31.2	64.2	33.0
	39.7	60.4	20.7
	59.9	82.0	22.1
			$\bar{x}_d$ =25.27
			s_d = 3.89
			t = 6.50*

[a] Assay conditions: 15-min incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0).

[b] Not significant at P<0.05.

* Significant at P<0.05.

Manganese chloride caused statistically significant increased β-galactosidase activity with P. shermanii P22 cell-free extract (Table 10). The increased activity may have partially resulted from stabilization of β-galactosidase by MnCl$_2$ at the 52 C incubation temperature. Results in Fig. 10 and 16 and observations of other workers suggests that MnCl$_2$

stabilizes β-galactosidase above 50 C (5, 151, 197).

Table 11 presents a statistical analysis of the effect of $MnCl_2$ on β-galactosidase activity of P7 and P22 cell-free extract when assays were incubated at temperatures from 18 to 65 C. As mentioned previously, $MnCl_2$ caused a significant increase in enzyme activity of strain P7 cell-free extract. The increase with cell-free extract was approximately the same at all incubation temperatures used. With strain P22, $MnCl_2$ increased β-galactosidase activity at all temperatures used, but because the stimulation was much greater at 52 C than at other temperatures, the over-all effect of $MnCl_2$ was not statistically significant. It should be emphasized that $MnCl_2$ does cause statistically significant increased β-galactosidase activity when assays are incubated at 52 C (Table 10).

The effect of $MnCl_2$ on β-galactosidase activity of P7 and P22 cell-free extracts at assay incubation temperatures from 18 to 65 C is presented graphically in Fig. 16. With both strains, $MnCl_2$ caused a greater increase in activity as the incubation temperature was increased up to 52 C, but the effect is much more pronounced with strain P22. At 52 C, P22 cell-free extract possessed over twice as many milliunits activity when $MnCl_2$ was added to the assay system. The stimulatory effect of $MnCl_2$ with enzyme from other microorganisms has been discussed previously.

Effect of sulfhydryl group blocking reagents on β-galactosidase activity

The stabilization of P22 β-galactosidase by dithiothreitol (Table 8) suggests that sulfhydryl groups are important for enzyme activity with this strain. As shown in Table 12, sulfhydryl group blocking reagents and sulfhydryl group protectors were added to the ONPG assay system with P22
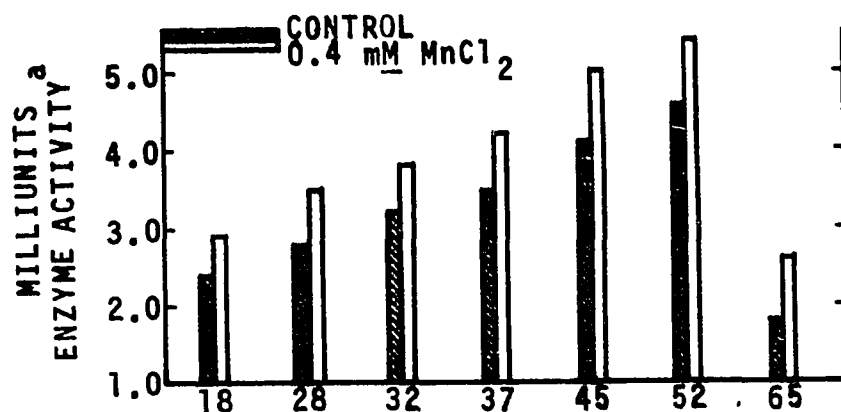
P7
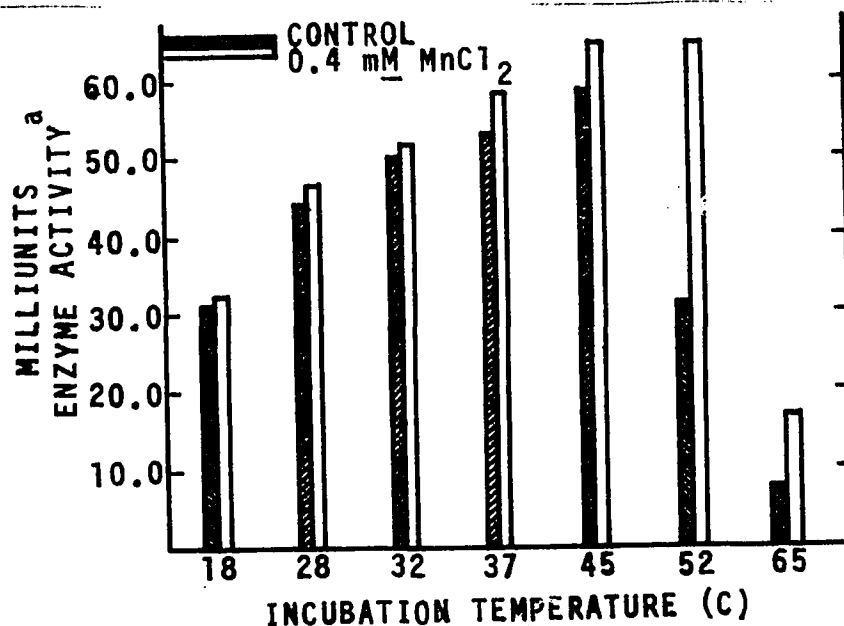
P22

[a] nMoles ONP liberated from ONPG/mg protein/min incubation.
Assay conditions: 15-min incubation, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0).

Fig. 16. Effect of manganese chloride on β-galactosidase activity of $\underline{P}$. shermanii P7 and P22 cell-free extracts when assays were incubated at 18, 28, 32, 37, 45, 52, and 65 C

93

Table 11. Statistical evaluation of effect of manganese chloride on
β-galactosidase activity of P. shermanii P7 and P22 cell-free
extracts when assays were incubated at 18, 28, 32, 37, 45, 52,
and 65 C

| Strain | Assay temp (c) | Milliunits enzyme activity[a] (nMoles ONP/mg protein/min) | | |
		$MnCl_2$ (none)	$MnCl_2$ (0.4 mM)	Difference
P7	18	2.37	2.88	0.51
	28	2.80	3.48	0.68
	32	3.18	3.79	0.61
	37	3.46	4.20	0.74
	45	4.06	4.96	0.90
	52	4.64	5.38	0.74
	65	1.84	2.60	0.76

$$\bar{x}_d = 0.706$$

$$s_d = 0.0466$$

$$t = 15.1**$$

P22	18	32.2	32.4	0.2
	28	44.5	46.4	1.9
	32	50.3	51.6	1.3
	37	53.5	58.4	4.9
	45	59.0	65.2	6.2
	52	31.2	64.2	33.0
	65	7.6	16.9	9.3

$$\bar{x}_d = 8.11$$

$$s_d = 4.32$$

$$t = 1.88^b$$

[a] Assay conditions: 15-min incubation, 0.05 M sodium phosphate buffer (pH 7.0).

[b] Not significant at $P<0.05$.

** Significant at $P<0.01$.

Table 12. Effect of sulfhydryl group reagents on β-galactosidase activity
of P. shermanii P22 cell-free extract

Treatment[a]	Milliunits enzyme activity[b]	Per cent enzyme activity
Control	35.6	100
DTT (1 mM)	34.3	96
IA (1 mM)	37.4	105
IA (1 mM) + DTT (1 mM)	42.3	119
IA (20 mM)	25.3	71
IA (20 mM) + DTT (3 mM)	28.2	79
NEM (1 mM)	19.2	54
NEM (1 mM) + DTT (1 mM)	34.9	98
NEM (10 mM)	12.2	34
PCMB (0.5 mM)	1.0	3
PCMB (0.5 mM) + DTT (3 mM)	31.5	88
PCMB (1 mM)	0.7	2
PCMB (1 mM) + DTT (1 mM)	12.7	36
PCMB (1 mM) + DTT (5 mM)	31.3	88
GTH (2 mM)	1.1	3

[a] DTT - dithiothreitol, IA - α-iodoacetamide,
NEM = N-ethylmaleimide, PCMB - ρ-chloromercuribenzoate,
GTH - glutathione (reduced).

[b] nMoles ONP liberated from ONPG/mg protein/min incubation. Assay
conditions: 10-min incubation at 52 C, 0.05 M sodium phosphate buffer
(pH 7.0), 0.4 mM $MnCl_2$.

cell-free extract to verify the importance of sulfhydryl groups in β-

galactosidase of this strain.

The least inhibitory sulfhydryl group reagent was α-iodoacetamide.

An assay mixture with 20 mM α-iodoacetamide exhibited 71% of the activity

in the unpoisoned system. Wallenfels and Malhotra (190) reported virtually

no inhibition of E. coli ML 309 β-galactosidase by 0.9 mM iodoacetamide.

Iodoacetate also has little inhibitory action on β-galactosidase (44, 49, 106).

A 1 mM concentration of N-ethylmaleimide reduced the enzyme activity to 54% of the initial activity. Wallenfels and Malhotra (190) found that 1 mM N-ethylmaleimide in 2-amino-2-(hydroxymethyl)-1,3-propanediol acetate buffer (pH 7.5), did not inhibit β-galactosidase. By increasing the concentration to 3 mM and increasing the pH to 8.6, N-ethylmaleimide became inhibitory. Table 12 shows that a sample of cell-free extract which received 10 mM N-ethylmaleimide retained only one-third of the original enzyme activity.

The most inhibitory sulfhydryl group reactant was ρ-chloromercuribenzoate. A 5 mM concentration of this reactant reduced the enzyme activity to only 3% of the original activity. Davies (49) reported that a 0.63 mM concentration of ρ-chloromercuribenzoate completely inhibited lactose hydrolysis by β-galactosidase from Sac. fragilis. Sulfhydryl groups are important for β-galactosidase activity in other microorganisms also (44, 97, 190, 198).

Inhibition by all sulfhydryl group reagents was partially reversed by dithiothreitol (30), a sulfhydryl group protector. Increasing the concentration of dithiothreitol resulted in increased β-galactosidase activity.

A 2 mM concentration of glutathione resulted in almost complete inhibition of β-galactosidase activity. Because the reduced glutathione adds additional sulfhydryl groups to the system, it should protect sulfhydryl groups of β-galactosidase by competing with them for the inhibitor. The reason for the inhibition by glutathione is unknown. Wallenfels and

Malhotra (190) also observed that β-galactosidase (E. coli ML 309) was inhibited by reduced glutathione. They did not offer an explanation for this observation. Glutathione may alter the configuration of the enzyme molecule, resulting in reduced activity. It is possible that the amino group of glutathione is responsible for the inhibitory effect, since tris, which also reduces β-galactosidase activity, also contains an amino group.

Effect of storage on β-galactosidase activity

β-Galactosidase activity in P22 cell-free extract was stable to storage (Table 13). Even after storing over 3 days at 25 C, there was still more than 50% of the initial activity remaining in the extract. Enzyme activity of both strains was very stable to freezing (Table 14). β-Galactosidase activity of strain P7 was more labile to lyophilization than β-galactosidase in strain P22. Strain P7 retained only 42% of the original enzyme activity after being lyophilized and stored at -20 C for 73 days, while strain P22 retained 69% of the original activity after being lyophilized and stored at -20 C for 75 days. β-Galactosidase from these propionibacteria, however, is not as stable as the enzyme from other microorganisms. Landman and Bonner (104) observed that lyophilized mycelial pads of Neurospora could be stored in a vacuum desiccator for weeks without loss of β-galactosidase activity. Other workers have found β-galactosidase enzyme in cell-free extracts is stable to refrigerated (106) and frozen storage (7, 87, 103). Purified extract was more stable to storage than crude extract (7, 103).

Table 13. Effect of storage up to 74 hr at 3.3 and 25 C on β-galactosidase activity of P. shermanii P22 cell-free extract

Storage temp	hr	Milliunits enzyme activity[a] (nMoles ONP/mg protein/min)	Per cent of initial activity
3.3 C	0	98.6	
	9.0	98.8	100
	24.5	97.4	99
	49.5	94.6	96
25 C	0	98.6	
	9.0	92.0	93
	24.5	81.6	83
	49.5	76.0	77
	74.0	53.9	55

[a] Assay conditions: 10-min incubation at 52 C, 0.05 M sodium phosphate buffer (pH 7.0), 0.4 mM $MnCl_2$.

Table 14. Effect of preservation method on β-galactosidase activity of P. shermanii P7 and P22 cell-free extracts stored at -20 C

Strain	Milliunits enzyme activity[a] Fresh	Storage (days)	Per cent activity of fresh cell-free extract Frozen	Lyophilized
P7	19.5	32	99	45
		73	99	42
P22	128	75	97	69

[a] nMoles ONP liberated from ONPG/mg protein/min incubation. Assay conditions: 10-min incubation at 52 C, 0.05 M sodium phosphate buffer (pH 7.0), 0.4 mM $MnCl_2$.

Phospho-β-Galactohydrolase Activity

Phosphoenolpyruvate (PEP) did not appreciably increase and NaF did not inhibit β-galactosidase activity of P. shermanii P7 or P22 untreated or toluene-acetone treated cells (Table 15). Activity with ONPG-6PO$_4$ was generally less than when ONPG was the substrate. Strains P7 and P22 gave negative results in the alkaline phosphatase test which demonstrates that an alkaline phosphatase was not present. A phosphatase could dephosphorylate the substrate before it was hydrolyzed. These observations indicate that neither P. shermanii P7 nor P22 rely on a PEP-dependent enzyme for hydrolyzing ONPG. β-Galactoside hydrolysis is dependent upon a β-galactosidase, not a phospho-β-galactohydrolase. It was suspected that P. shermanii P7 might possess the PEP-dependent enzyme because of the decreased enzyme activity with this strain after toluene-acetone treatment.

McKay et al. (119) demonstrated that S. lactis C2F contained the PEP-dependent system for β-galactoside utilization. They attributed substrate hydrolysis to an enzyme different from β-galactosidase. Streptococcus lactis C2F was assayed in this experiment to demonstrate that the assay system in this investigation was functioning properly. Toluene-acetone treated C2F cells had over a 400-fold increase in activity with ONPG-6PO$_4$ as substrate compared to ONPG. Manganese chloride was necessary to obtain optimum activity in this assay system. Streptococcus lactis C2F did not show high activity with ONPG-6PO$_4$, unless the cells received toluene-acetone treatment. This resulted because the phosphotransferase system does not transport phosphorylated substrates, but phosphorylates the substrate during passage through the cell membrane (78, 93). When the cell

Table 15. Phospho-β-galactohydrolase activity of <u>P. shermanii</u> P7 and P22
grown 28 hr in 1% sodium lactate broth and of <u>S. lactis</u> C2F
grown 7 hr in 1% lactose broth

| | | | Assay system | |
| | | Incubation | $MnCl_2$ | $MgCl_2$ |
Strain	Cell treatment	temp (C)	(0.4 m$\underline{M}$)	(0.4 m$\underline{M}$)
P7	Untreated[b]	32	−	−
	Untreated	32	+	−
	Toluene-acetone[b]	32	−	−
	Toluene-acetone	32	+	−
	Cell-free extract[d]	52	+	−
P22	Untreated[b]	32	−	−
	Untreated	32	+	−
	Toluene-acetone[b]	32	−	−
	Toluene-acetone	32	+	−
	Cell-free extract	52	+	−
C2F	Untreated[f]	37	+	−
	Untreated	37	+	+
	Untreated	37	−	−
	Toluene-acetone[f]	37	+	−
	Toluene-acetone	37	+	+
	Toluene-acetone	37	−	−

[a] nMoles ONP liberated from ONPG/mg dry cells/min incubation. With
cell-free extract, the activity is expressed per mg protein.

[b] Assay conditions: 15-min incubation, 0.05 $\underline{M}$ sodium phosphate
buffer (pH 7.5).

[c] Not determined.

[d] Assay conditions: 10-min incubation, 0.05 $\underline{M}$ sodium phosphate
buffer (pH 7.0).

[e] No detectable activity.

[f] Assay conditions: 15-min incubation, 0.05 $\underline{M}$ sodium phosphate
buffer (pH 7.0).

| Milliunits enzyme activity[a] | | | |
ONPG	ONPG + PEP(8 m$\underline{M}$)	ONPG + NaF(8 m$\underline{M}$)	ONPG-6PO$_4$
11.2	12.7 c	10.5	0.4
8.0	--	--	5.0
5.8	6.4	6.2	1.4
7.2	7.0	--	7.4
3.4	3.6	--	2.8
5.8	5.6	6.0	NDA[e]
10.0	--	--	5.8
48.8	46.2	42.6	2.1
53.9	38.0	--	17.0
94.0	83.8	--	84.0
10.2	--	--	3.0
9.6	--	--	2.8
9.1	--	--	2.9
0.3	--	--	121
1.0	4.3	--	126
0.8	--	--	50.1

membrane is disrupted by the solvent, the phosphorylated substrate can come in contact with the phospho-β-galactohydrolase. Cell-free extract of S. lactis C2F was not investigated in this study, but McKay et al. (119) observed β-galactohydrolase activity in cell-free extract of this strain.

Much lower activity of S. lactis C2F was obtained when PEP was added to an ONPG assay system than when the phosphorylated substrate was used (4.3 milliunits activity for ONPG + PEP vs. 126 milliunits activity with ONPG-6PO$_4$). McKay et al. (119) observed 107 milliunits activity when 2 mM PEP was added to an ONPG assay system containing 3 ml of 5 mM ONPG (this activity value has been corrected for the difference in volume of the two assay systems). The same assay conditions were employed in both experiments. The activity of C2F with the phosphorylated substrate (121 and 126 milliunits activity, Table 15) agrees fairly well with the activity observed by McKay et al. (119) with ONPG + PEP (107 milliunits activity).

Low enzyme activity was observed when toluene-acetone treated cells were assayed with ONPG. McKay et al. (119) did not observe detectable activity when toluene-acetone treated C2F cells were assayed with ONPG. They concluded that S. lactis C2F did not contain a β-galactosidase but possessed a different enzyme which hydrolyzed the phosphorylated substrate. McKay et al. (119) observed that highly concentrated cell-free extract (5.7 mg protein/ml) did hydrolyze ONPG. Apparently, there was sufficient PEP present in the concentrated extract to transport some of the ONPG substrate. The low level of activity in this experiment (Table 15) could be due to PEP present with the cells or perhaps this strain actually possessed a β-galactosidase as well as a phospho-β-galactohydrolase.

Concentration of β-Galactosidase

with Ammonium Sulfate Precipitation

An attempt was made to precipitate the nucleic acids from P. shermanii

P22 cell-free extract by adding 1 mg protamine sulfate/mg protein (115),

but this also precipitated 88% of the β-galactosidase activity, so this

step was not used. Protein was precipitated from cell-free extract by

adding ammonium sulfate in 10% (W/V) increments to determine the amount of

ammonium sulfate to add for maximum concentration of β-galactosidase.

Results in Table 16 show that a majority of the β-galactosidase precipi-

tates when 20 and 30% (W/V) ammonium sulfate are added. Consequent ,

β-galactosidase was concentrated by discarding the protein which preci-

pitated at 10% (W/V) ammonium sulfate and then collecting the fraction

which precipitated after an additional 20% (W/V) ammonium sulfate was

added. This fraction contained only a 1.9-fold increase in β-galactosidase

activity, as shown in Table 17. Craven et al. (44) and Cohn and Monod

(36) reported a 3-fold and a 3.1-fold concentration of β-galactosidase

from E. coli by ammonium sulfate precipitation at 40 and 50% of saturation.

McFeters et al. (113, 115) observed a fourfold and a fivefold concen-

tration of β-galactosidase activity with ammonium sulfate precipitation of

enzyme from S. lactis 7962.

Sephadex Separation

An attempt was made to separate β-galactosidase from a P22 cell-free

extract on a Sephadex G-100 column. The extract was precipitated with 30%

(W/V) ammonium sulfate as previously described. β-Galactosidase was almost

Table 16. Ammonium sulfate fractionation of P. shermanii P22 cell-free extract[a] for the partial isolation of β-galactosidase

Per cent $(NH_4)_2SO_4$	Volume of resuspended fraction	Mg protein/ml	Specific activity[b]
10	2	1.08	61.5
20	10	3.60	110
30	20	3.72	129
40	5	5.97	65.0
50	5	14.3	10.6
60	5	3.92	1.4

[a] A 1% solution (W/V) of lyophilized cell-free extract was made. The protein concentration of this solution was 3.48 mg/ml and the specific activity was 99.2 milliunits activity.

[b] nMoles ONP liberated from ONPG/mg protein/min incubation. Assay conditions: 10-min incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0), 0.4 m$\underline{M}$ $MnCl_2$.

Table 17. Relative purification of β-galactosidase from P. shermanii P22 cell-free extract, by ammonium sulfate precipitation

Sample	Mg protein per milliliter	Milliunits specific activity[a]	Extent of concentration
Cell-free extract	1.70	98.6	
Reconstituted precipitate obtained at >10 to 30% $(NH_4)_2SO_4$ (W/V)	1.40	185	1.9

[a] nMoles ONP liberated from ONPG/mg protein/min incubation. Assay conditions: 10-min incubation at 52 C, 0.05 $\underline{M}$ sodium phosphate buffer (pH 7.0), 0.4 m$\underline{M}$ $MnCl_2$.

completely inactivated by the column. Because of the low enzyme activity, a qualitative micro-assay was performed instead of the conventional β-galactosidase assay. One drop of the fraction and 4 drops of ONPG were added to a 10 X 75 mm culture tube and the mixture was incubated at 37 C until distinguishable yellow color was present. Because of the low activity, it was necessary to incubate the assay samples 1 hr or longer to detect activity. When samples were incubated at 52 C, activity was not detected. The enzyme was apparently inactivated before sufficient hydrolysis of ONPG occurred to yield detectable product. The smaller quantity of sample and substrate were used because of the many fractions that were assayed.

The highest β-galactosidase activity was present in fraction 17 (80-85 ml eluant). This fraction hydrolyzed ONPG to produce visually detectable yellow color (absorbance of approximately 0.100) after 1-hr incubation at 37 C. After 3-hr incubation, activity also was detected in fractions 18 and 19 (85-95 ml eluant). After 9-hr incubation, activity was detected in fractions 15 and 16 (70-80 ml eluant), fractions 35 to 40 (170-200 ml eluant), fraction 42 (205-210 ml eluant), and fraction 46 (225-230 ml eluant). Separation of β-galactosidase from other sources on G-200 columns has shown that the β-galactosidase activity peak occurs just after the main protein peak (44, 74, 115), as was observed in this experiment. Blue Dextran 2000 was collected in fractions 9-25 (40-125 ml). The main protein peak occurred in fraction 15 (70-75 ml eluant).

Fractions were pooled, condensed in dialysis tubing, and assayed for β-galactosidase activity. No detectable activity was observed using the conventional β-galactosidase assay. With the micro-assay procedure, lower

activities were present in the pooled fractions than in the individual fractions. Biermann and Glantz (13) concentrated pooled fractions from Sac. lactis by adding solid ammonium sulfate to 65% of saturation. This may be less detrimental to enzyme activity than concentrating in dialysis tubing. Perhaps $MnCl_2$, added to the eluant, also would have helped stabilize the enzyme.

Wiśniewski (202) successfully separated β-galactosidase from P. shermanii and P. arabinosum. He obtained a 30- to 40-fold purification on a diethylaminoethyl-cellulose solumn in the Cl^- form using stepwise ionic strength elution at pH 7.0. β-Galactosidase was eluted at ionic strength 0.5 μ [$\mu = \frac{1}{2} (\sum_{i=1}^{n} M_i \times Charge_i^2$) of the n ionic species in solution].

β-Galactosidase from E. coli has been successfully concentrated and separated by many workers (8, 36, 44, 86, 100, 106, 124, 130, 177, 194). β-Galactosidase from Sac. lactis (13), S. faecium (17), Sac. fragilis (49), B. megaterium (103), Neurospora (104), and S. lactis (113, 115) also has been purified. Purified enzyme is very labile (13, 113, 115, 202). If the procedures which were used successfully by other workers were used with P. shermanii P22 cell-free extract, it is probable that the enzyme could be successfully purified.

This investigation was an outgrowth of earlier studies on propionibacteria in this department. Although β-galactosidase has been studied extensively in many other microorganisms, it has not been investigated in detail in propionibacteria. Results of this investigation show that P. freudenreichii does not possess β-galactosidase activity. This was consistent with the previous observations made in a taxonomic study in this department. Examination of the β-galactosidase of P. shermanii revealed

that it was similar in many respects to β-galactosidase from other microorganisms. Its optimum assay conditions; incubation at 52 C, pH 7.5 for whole cells and 7.0 for cell-free extracts, stimulation by $MnCl_2$, Na^+ ions, and K^+ ions; were similar to the optima observed with other microorganisms. β-Galactosidase activity of the propionibacteria strains studied was stable to storage, but it was not as stable as with other microorganisms. The P. shermanii strains investigated, P7 and P22, did not possess β-galactohydrolase activity.

SUMMARY

β-Galactosidase activity of P. shermanii was investigated as a continuation of the extensive work with propionibacteria in our laboratory. Enzyme activity was measured with ONPG. Because of taxonomic significance, P. freudenreichii, which differs from P. shermanii only by its inability to ferment lactose, also was assayed for β-galactosidase activity.

Two strains of P. shermanii, P7 and P22, were selected from 10 strains of this species, based on their response to toluene-acetone treatment when assayed at 32 C, for extensive investigation. Toluene-acetone treatment of whole cells reduced the activity of strain P7 by 46%, while strain P22 showed a fivefold increase in activity in response to the solvent treatment. None of 10 strains of P. freudenreichii possessed detectable activity with untreated or toluene-acetone treated cells.

Growth and assay conditions for maximum β-galactosidase activity were determined. Cells grown in sodium lactate medium possessed as high or higher activity than cells grown in media containing lactose, glucose, or galactose. Enzyme activity was not appreciably affected by incubating cells 6 hr at 32 C with lactose, glucose, galactose, and maltose or with the lactose analogs IPTG, TMG, ONPG, and PNPG. This lack of response suggests that β-galactosidase is not inducible in these strains. As an additional test for an inducible system, strain P7 cells were inoculated into media containing the two combinations of high and low (0.0145 and 0.0029 M) concentrations of sodium lactate and lactose. Growth was measured spectrophotometrically every hour for 48 hr. The lag period was the same whether lactose- or sodium lactate-grown cells were used as the

inoculum. There was no evidence of diauxie growth. These observations also suggest that β-galactosidase is not induced in this strain.

Cells from strain P7 were harvested after 12, 16, 20, 24, 28, 36, 48, and 72 hr of growth and assayed for β-galactosidase activity to determine the optimum growth time. Highest activity was present in 28-hr cells.

Cell-free extracts of both strains possessed β-galactosidase activity. Extract from strain P22 was much more stable to cell disruption (128 milli-units activity) than extract from strain P7 (19.5 milliunits activity). With cell-free extracts, activity is expressed per milligram protein.

Standard β-galactosidase assay procedures were adopted which most nearly matched the optimum assay conditions for both strains. Un-treated cells were incubated 10 min at 52 C using sodium phosphate buffer (pH 7.5). There was little difference in activity of solvent-treated P7 cells when the assays were incubated at different temperatures, while un-treated cells showed a 17-fold increase in activity between 32 and 52 C incubation. Activity of P22 toluene-acetone treated cells decreased as the assay incubation temperature was increased. Untreated P22 cells had greater activity than solvent-treated cells when assayed at 45 C and above. Optimum activity with cell-free extracts also was obtained at 52 C, but optimum pH was 7.0.

Potassium phosphate gave slightly greater activity than sodium phos-phate buffer but tris gave decidedly lower activity in all assay systems except with P22 untreated cells. In the systems where tris gave low activity, sodium phosphate and sodium chloride increased activity.

Manganese chloride significantly increased β-galactosidase activity with P7 untreated cells and cell-free extracts from strains P7 and P22.

The stimulatory effect of $MnCl_2$ was more pronounced at 52 C than at lower incubation temperatures, which suggests that it stabilized the enzyme against heat inactivation.

Stability to storage was investigated. β-Galactosidase activity of strain P22 was more stable to storage than enzyme in strain P7. Untreated cells of P7, stored 16 days at 3.3 C, retained 62% of the original enzyme activity, while strain P22 possessed 111% of the original enzyme activity. Cell-free extract from strain P22 still retained 96% of the original activity after being stored 2 days at 3.3 C, and 77% of the original activity after being stored 2 days at 25 C. Enzyme activity in both strains was very stable to freezing. Freezing at -20 C was less detrimental to enzyme activity than lyophilization. Strain P22 retained 97% of the original enzyme activity after freezing and storing at -20 C for 75 days. Lyophilized cell-free extract, stored under the same conditions, retained only 69% of the original activity.

β-Galactosidase activity in cell-free extracts was labile to dialysis, but was partially protected by dithiothreitol, a sulfhydryl group protector. Activity was partially restored to dialyzed cell-free extract by addition of potassium chloride, sodium chloride, potassium phosphate, sodium phosphate, and ammonium phosphate. β-Galactosidase activity of cell-free extract was reduced by the sulfhydryl group reagents ρ-chloromercuribenzoate, N-ethylmaleimide, and α-iodoacetaminde, but was partially protected by dithiothreitol, which indicates that sulfhydryl groups are important in β-galactosidase activity.

Propionibacterium shermanii P7 and P22 were assayed for a phospho-β-galactohydrolase by employing both ONPG + PEP and ONPG-6PO$_4$. Neither

strain possesses the PEP-dependent enzyme.

β-Galactosidase activity was concentrated 1.9 times by fractionating P22 cell-free extract with ammonium sulfate. Attempts to purify the enzyme with a Sephadex G-100 column resulted in loss of most of the β-galactosidase activity.

LITERATURE CITED

1. Alexander, F., and A. K. Chowdhury. 1958. Enzymes in the ileal juice of the horse. Nature, 181: 190.

2. Allen, S. H. G., R. W. Kellermeyer, R. L. Stjernholm, and H. G. Wood. 1964. Purification and properties of enzymes involved in the propionic acid fermentation. J. Bacteriol., 87: 171-187.

3. Alvarez, A., and J. Sas. 1961. β-Galactosidase changes in the developing intestinal tract of the rat. Nature, 190: 826-827.

4. American Public Health Association. 1967. Standard methods for the examination of dairy products. 12th ed. American Public Health Association, Inc., New York. 304 p.

5. Anderson, J. M., and H. V. Rickenberg. 1960. β-Galactosidase of Paracolobactrum aerogenoides. J. Bacteriol., 80: 297-304.

6. Anderson, R. L., and W. A. Wood. 1969. Carbohydrate metabolism in microorganisms. Ann. Rev. Microbiol., 23: 539-578.

7. Anema, P. J. 1964. Purification and some properties of β-galactosidase of Bacillus subtilis. Biochim. Biophys. Acta, 89: 495-502.

8. Appel, S. H., D. H. Alpers, and G. M. Tomkins. 1965. Multiple molecular forms of β-galactosidase. J. Mol. Biol., 11: 12-22.

9. Aronson, M. 1952. Transgalactosidation during lactose hydrolysis. Arch. Biochem. Biophys., 39: 370-378.

10. Bailey, J. L. 1962. Techniques in protein chemistry. Elsevier Publishing Company, New York. 310 p.

11. Bernaerts, M. J., and J. De Ley. 1957. The catabolism of lactose and lactobionate by Corynebacterium simplex. Antonie van Leeuwenhoek, 23: 333-344.

12. Beyerinck, M. W. 1889. Die Lactase, ein neues Enzym. Centralblatt für Bakteriologie und Parasitenkunde, 6: 44-48.

13. Biermann, L., and M. D. Glantz. 1968. Isolation and characterization of β-galactosidase from Saccharomyces lactis. Biochim. Biophys. Acta, 167: 373-377.

14. Borgström, B., A. Dahlqvist, G. Lundh, and J. Sjövall. 1957. Studies of intestinal digestion and absorption in the human. J. Clin. Invest., 36: 1521-1536.

15. Boyer, P. D. 1959. Sulfhydryl and disulfide groups of enzymes, Vol. 1, p. 511-588. In P. D. Boyer, H. Lardy, and K. Myrbäck [ed.] The enzymes. 2nd ed. Academic Press, New York.

16. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology. 7th ed. Williams and Wilkins Co., Baltimore. 1094 p.

17. Buecher, E. J., and T. D. Brock. 1962. The β-galactosidase in Streptococcus faecium. Bacteriol. Proc., 1962: 105.

18. Burd, G. I., I. V. Andreeva, V. P. Shabolenko, and V. N. Gershanovitch. 1968. Absence of phosphotransferase-system components in mutant Escherichia coli K-12 with a disrupted carbohydrate-transfer system. (Russian, translated to English). Mol. Biol., 2: 70-83.

19. Burstein, C., M. Cohn, A. Kepes, et. J. Monod. 1965. Rôle du lactose et de ses produits métaboliques dans l'induction de l'opéron lactose chez Escherichia coli. Biochim. Biophys. Acta. 95: 634-639.

20. Cajori, F. A. 1934. Some characteristics of intestinal lactase. Amer. J. Med. Sci., 187: 295-296.

21. Cajori, F. A. 1935. The lactase activity of the intestinal mucosa of the dog and some characteristics of intestinal lactase. J. Biol. Chem., 109: 159-168.

22. Caputto, R., L. F. Leloir, and R. E. Trucco. 1948. Lactase and lactose fermentation in Saccharomyces fragilis. Enzymologia, 12: 350-355.

23. Carpenter, P. L. 1967. Microbiology. 2nd ed. W. B. Saunders Company, Philadelphia. 476 p.

24. Chauncey, H. H., F. Lionetti, R. A. Winer, and V. F. Lisanti. 1954. Enzymes of human saliva. I. The determination, distribution, and origin of whole saliva enzymes. J. Dental Res., 33: 321-334.

25. Cirillo, V. P. 1961. Sugar transport in microorganisms. Ann. Rev. Microbiol., 15: 197-218.

26. Citti, J. E., W. E. Sandine, and P. R. Elliker. 1965. β-Galactosidase of Streptococcus lactis. J. Bacteriol., 89: 937-942.

27. Citti, J. E., W. E. Sandine, and P. R. Elliker. 1967. Lactose and maltose uptake by Streptococcus lactis. J. Dairy Sci., 50: 485-487.

28. Clamp, J. R., L. Hough, J. L. Hickson, and R. L. Whistler. 1961. Lactose. Advances Carbohydrate Chem., 16: 159-206.

29. Clausen, C. R., and M. Nakamura. 1963. Beta-galactosidase of
    Shigella sonnei. Nature, 197: 570-573.

30. Cleland, W. W. 1964. Dithiothreitol, A new protective reagent for
    SH groups. Biochemistry, 3: 480-482.

31. Coffey, R. G., and F. J. Reithel. 1969. An enzymic determination of
    lactose. Anal. Biochem., 32: 229-232.

32. Cohen, G. N., et H. V. Rickenberg. 1955. Étude directe de la
    fixation d'un inducteur de la β-galactosidase par les cellules d'
    Escherichia coli. Comptes Rendus Acad. Sci., 240: 466-468.

33. Cohen, G. N., and J. Monod. 1957. Bacterial permeases. Bacteriol.
    Rev., 21: 169-194.

34. Cohen, R. B., K. -C. Tsou, S. H. Rutenburg, and A. M. Seligman. 1952.
    The colorimetric estimation and histochemical demonstration of β-D-
    galactosidase. J. Biol. Chem., 195: 239-249.

35. Cohn, M. 1957. Contributions of studies on the β-galactosidase of
    Escherichia coli to our understanding of enzyme synthesis. Bacteriol.
    Rev., 21: 140-168.

36. Cohn, M., et J. Monod. 1951. Purification et propriétés de la β-
    galactosidase (lactase) d'*Escherichia coli.* Biochim. Biophys. Acta,
    7: 153-173.

37. Cohn, M., and J. Monod. 1953. Specific inhibition and induction of
    enzyme biosynthesis, p. 132-149. In R. Davies and E. F. Gale [ed.]
    Adaptation in micro-organisms. Cambridge University Press, Cambridge,
    England.

38. Cohn, M., and K. Horibata. 1959. Inhibition by glucose of the
    induced synthesis of the β-galactoside-enzyme system of *Escherichia
    coli.* Analysis of maintenance. J. Bacteriol., 78: 601-612.

39. Cohn, M., and K. Horibata. 1959. Physiology of the inhibition by
    glucose of the induced synthesis of the β-galactoside-enzyme system
    of *Escherichia coli.* J. Bacteriol., 78: 624-635.

40. Conchie, J., and A. J. Hay. 1959. Mammalian glycosidases. 2.
    Properties of α-mannosidase and β-galactosidase from rat epididymis.
    Biochem. J., 73: 327-334.

41. Conchie, J., and T. Mann. 1957. Glycosidases in mammalian sperm and
    seminal plasma. Nature, 179: 1190-1191.

42. Conchie, J., J. Findlay, and G. A. Levvy. 1959. Mammalian glyco-
    sidases. Distribution in the body. Biochem. J., 71: 318-325.

43. Corbett, W. P., and B. W. Catlin. 1968. Galactosidase activity of lactose-positive Neisseria. J. Bacteriol., 95: 52-57.

44. Craven, G. R., E. Steers, Jr., and C. B. Anfinsen. 1965. Purification, composition, and molecular weight of the β-galactosidase of Escherichia coli K12. J. Biol. Chem., 240: 2468-2477.

45. Creaser, E. H. 1955. The induced (adaptive) biosynthesis of β-galactosidase in Staphylococcus aureus. J. Gen. Microbiol., 12: 288-297.

46. Dahlqvist, A. 1961. The location of carbohydrases in the digestive tract of the pig. Biochem. J., 78: 282-288.

47. Dahlqvist, D. 1961. Intestinal carbohydrases of a new-born pig. Nature, 190: 31-32.

48. Davies, A. 1956. Some factors affecting lactase formation and activity in Saccharomyces fragilis. J. Gen. Microbiol., 14: 425-439.

49. Davies, R. 1964. Lactose utilization and hydrolysis in Saccharomyces fragilis. J. Gen. Microbiol., 37: 81-98.

50. Davis, B. D. 1956. Relations between enzymes and permeability (membrane transport) in bacteria, p. 509-522. In O. H. Gaebler [ed.] Enzymes: units of biological structure and function. Academic Press, Inc., New York.

51. Deane, D. D., and E. G. Hammond. 1960. Coagulation of milk for cheese making by ester hydrolysis. J. Dairy Sci., 43: 1421-1429.

52. Dedonder, R. A. 1961. Glycosidases and transglycosidation. Ann. Rev. Biochem., 30: 347-382.

53. Deere, C. J. 1939. On the "activation" of the lactase of Escherichia coli-mutabile. J. Bacteriol., 37: 473-483.

54. Deere, C. J., A. D. Dulaney, and I. D. Michelson. 1939. The lactase activity of Escherichia coli-mutabile. J. Bacteriol., 37: 355-363.

55. De Groot, A. P., and P. Hoogendoorn. 1957. The detrimental effect of lactose. II. Quantitative lactase determinations in various mammals. Neth. Milk Dairy J., 11: 290-303.

56. Dénes, G. 1960. Enzyme repression as the control mechanism in the synthesis of induced β-galactosidase. Nature, 188: 852-853.

57. Desai, P. D., and M. Goldner. 1969. Effect of low pH on thiomethyl-β-D-galactoside uptake by Streptococcus lactis. J. Bacteriol., 100: 1415-1416.

58. Doell, R. G., and N. Kretchmer. 1962. Studies of small intestine during development. I. Distribution and activity of β-galactosidase. Biochim. Biophys. Acta, 62: 353-362.

59. Dollar, A. M., and J. W. G. Porter. 1957. Utilization of carbohydrates by the young calf. Nature, 179: 1299-1300.

60. Doudoroff, M., W. Z. Hassid, E. W. Putnam, A. L. Potter, and J. Lederberg. 1949. Direct utilization of maltose by Escherichia coli. J. Biol. Chem., 179: 921-934.

61. Egan, J. B., and M. L. Morse. 1965. Carbohydrate transport in Staphylococcus aureus. I. Genetic and biochemical analysis of a pleiotropic transport mutant. Biochim. Biophys. Acta, 97: 310-319.

62. Egan, J. B., and M. L. Morse. 1965. Carbohydrate transport in Staphylococcus aureus. II. Characterization of the defect of a pleiotropic transport mutant. Biochim. Biophys. Acta, 109: 172-183.

63. Egan, J. B., and M. L. Morse. 1966. Carbohydrate transport in Staphylococcus aureus. III. Studies of the transport process. Biochim. Biophys. Acta, 112: 63-73.

64. Estienne, V., E. Castagne, and E. Bertrand. 1947. Lactase of fungi. Bull. Soc. Chim. Biol., 29: 620-627. (Chem. Abstr., 42: 5078. 1948).

65. Feniksova, R. V., A. S. Tikhomirova, A. N. Kulikova, and V. D. Kuznetsov. 1968. Production of β-galactosidase by various microorganisms. Microbiology, 37: 836-840.

66. Fleming, W. L., and J. M. Neill. 1927. Studies on bacterial enzymes. III. Pneumococcus maltase and lactase. J. Exp. Med., 45: 169-174.

67. Florkin, M., and E. H. Stotz [ed.] 1964. Comprehensive biochemistry. Rev. ed. Elsevier Publishing Company, New York. Vol. 13. 164 p.

68. Fox, C. F., and E. P. Kennedy. 1965. Specific labeling and partial purification of the M protein, A component of the β-galactoside transport system of Escherichia coli. Nat. Acad. Sci., Proc., 54: 891-899.

69. Garcia, V. V. 1969. Practical methods for packing reproducible gel filtration columns; their application to protein separations. Unpublished M.S. thesis. Libr., Iowa State Univ., Ames, Iowa. 124 p.

70. Gilmour, M. H. N. 1957. The induced synthesis and properties of beta-galactosidase of Saccharomyces fragilis. Diss. Abstr., 17: 946.

71. Gomori, G. 1955. Preparation of buffers for use in enzyme studies, vol. 1, p. 138-146. In S. P. Colowick and N. O. Kaplan [ed.] Methods in enzymology. Academic Press, Inc., New York.

72. Guagnini, O., and D. Jacovkis. 1947. Utility of enzymes of *Aspergillus oryzae*. I. Amylolytic enzymes. Ind. y quim (Buenos Aires) 9: 207-212. (Chem. Abstr., 42: 6881. 1948).

73. Han, Y. W., and V. R. Srinivasan. 1969. Purification and characterization of β-glucosidase of *Alcaligenes faecalis*. J. Bacteriol., 100: 1355-1363.

74. Harrap, G. J., and W. M. Watkins. 1970. Enzymes of *Trichomonas foetus*. Separation and properties of two β-galactosidases. Biochem. J., 117: 667-675.

75. Heilskov, N. S. C. 1951. Studies on animal lactase. I. Lactase activity determination. Acta Physiol. Scand., 22: 267-276.

76. Heilskov, N. S. C. 1951. Studies on animal lactase. II. Distribution in some of the glands of the digestive tract. Acta Physiol. Scand., 24: 84-89.

77. Hengstenberg, W., and M. L. Morse. 1968. Synthesis of o-nitrophenyl β-D-galactoside 6-phosphate, a substrate of the staphylococcal β-D-galactosidase. Carbohydrate Res., 7: 180-183.

78. Hengstenberg, W., J. B. Egan, and M. L. Morse. 1967. Carbohydrate transport in *Staphylococcus aureus*. V. The accumulation of phosphorylated carbohydrate derivatives, and evidence for a new enzyme-splitting lactose phosphate. Nat. Acad. Sci., Proc., 58: 274-279.

79. Hengstenberg, W., J. B. Egan, and M. L. Morse. 1968. Carbohydrate transport in *Staphylococcus aureus*. VI. The nature of the derivatives accumulated. J. Biol. Chem., 243: 1881-1885.

80. Hengstenberg, W., W. K. Penberthy, and M. L. Morse. 1968. The phosphotransferase system of *Staphylococcus aureus*. Fed. Proc., 27: 643.

81. Herzenberg, L. A. 1959. Studies on the induction of β-galactosidase in a cryptic strain of *Escherichia coli*. Biochim. Biophys. Acta, 31: 525-538.

82. Hettinga, D. H., and G. W. Reinbold. 1971, 1972. The propionic-acid bacteria- a review. I. Growth: II. Metabolism: III. Miscellaneous metabolic activities. J. Milk Food Technol., in press.

83. Hettings, D. H., E. R. Vedamuthu, G. W. Reinbold, and E. G. Hammond. 1969. Split defect in Swiss cheese. J. Dairy Sci., 52: 880.

84. Hofmann, E. 1934. Über das Vorkommen von Glucosidasen bzw. Galaktosidasen und Disaccharide spaltenden Enzymen in Bakterien. Biochem. Zeitschrift, 272: 133-143.

85. Hofsten, B. von. 1961. The inhibitory effect of galactosides on the growth of Escherichia coli. Biochim. Biophys. Acta, 48: 164-171.

86. Hu, A. S. L., R. G. Wolfe, and F. J. Reithel. 1959. The preparation and purification of β-galactosidase from Escherichia coli, ML 308. Arch. Biochem. Biophys., 81: 500-507.

87. Hughes, R. C., and R. W. Jeanloz. 1964. The extracellular glycosidases of Diplococcus pneumoniae. I. Purification and properties of a neuraminidase and a β-galactosidase. Action on the α1-acid glycoprotein of human plasma. Biochemistry, 3: 1535-1543.

88. Jackson, W. P. U. 1952. Observations on an isolated loop of human jejunum. Clin. Sci., 11: 209-216.

89. Jacob, F., and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol., 3: 318-356.

90. Jacob, F., and J. Monod. 1961. On the regulation of gene activity. Cold Spring Harbor Symp. Quant. Biol., 26: 193-211.

91. Jasewicz, L., and A. E. Wasserman. 1961. Quantitative determination of lactase. J. Dairy Sci., 44: 393-400.

92. Kaback, H. R. 1970. Transport. Ann. Rev. Biochem., 39: 561-593.

93. Kennedy, E. P., and G. A. Scarborough. 1967. Mechanism of hydrolysis of o-nitrophenyl-β-galactoside in Staphylococcus aureus and its significance for theories of sugar transport. Nat. Acad. Sci., Proc., 58: 225-228.

94. Kepes, A. 1960. Études cinétiques sur la galactoside-perméase d' Escherichia coli. Biochim Biophys. Acta, 40: 70-84.

95. Kepes, A. 1962. Permeases: identification and mechanism, p. 38-48. In N. E. Gibbons [ed.] 8th Int. Congr. Microbiol., Montreal, University of Toronto Press. 1963.

96. Kepes, A., and G. N. Cohen. 1962. Permeation, vol. IV, p. 179-221. In I. C. Gunsalus and R. Y. Stanier [ed.] The bacteria. Academic Press, Inc., New York.

97. Knopfmacher, H. P., and A. J. Salle. 1941. Studies on the lactase of Escherichia coli. J. Gen. Physiol., 24: 377-397.

98. Koch, A. L. 1964. The role of permease in transport. Biochim. Biophys. Acta, 79: 177-200.

99. Koppel, J. L., C. J. Porter, and B. F. Crocker. 1953. The mechanism of the synthesis of enzymes. 1. Development of a system suitable for studying this phenomenon. J. Gen. Physiol., 36: 703-722.

100. Kuby, S. A., and H. A. Lardy. 1953. Purification and kinetics of β-D-galactosidase from Escherichia coli, strain K-12. J. Amer. Chem. Soc., 75: 890-896.

101. Kundig, W., S. Ghosh, and S. Roseman. 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. Nat. Acad. Sci., Proc., 52: 1067-1074.

102. Landman, O. E. 1954. Neurospora lactase. II. Enzyme formation in the standard strain. Arch. Biochem. Biophys., 52: 93-109.

103. Landman, O. E. 1957. Properties and induction of β-galactosidase in Bacillus megaterium. Biochim. Biophys. Acta, 23: 558-569.

104. Landman, O. E., and D. M. Bonner. 1952. Neurospora lactase. I. Properties of lactase preparations from a lactose utilizing and a lactose non-utilizing strain. Arch. Biochem. Biophys., 41: 253-265.

105. Laue, P., and R. E. MacDonald. 1968. Studies on the relation of thiomethyl-β-D-galactoside accumulation to thiomethyl-β-D-galactoside phosphorylation in Staphylococcus aureus HS1159. Biochim. Biophys. Acta, 165: 410-418.

106. Lederberg, J. 1950. The beta-D-galactosidase of Escherichia coli, strain K-12. J. Bacteriol., 60: 381-392.

107. Lee, S. Y., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold. 1970. Diacetyl production by Propionibacterium shermanii in milk cultures. Can. J. Microbiol., 16: 1231-1242.

108. Lester, G., and A. Byers. 1965. Properties of two β-galactosidases of Neurospora crassa. Biochem. Biophys. Res. Commun., 18: 725-734.

109. Lester, G., and D. M. Bonner. 1952. The occurrence of β-galactosidase in Escherichia coli. J. Bacteriol., 63: 759-769.

110. Lester, G., D. Azzena, and O. Hechter. 1962. Permeability and metabolism of lactose in Neurospora crassa. J. Bacteriol., 84: 217-227.

111. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem., 193: 265-275.

112. McClatchy, J. K., and E. E. Rosenblum. 1963. Induction of lactose utilization in Staphylococcus aureus. J. Bacteriol., 86: 1211-1215.

113. McFeters, G. A., W. E. Sandine, and P. R. Elliker. 1966. Purification and properties of Streptococcus lactis β-galactosidase. Bacteriol. Proc., 1966: 91.

114. McFeters, G. A., W. E. Sandine, and P. R. Elliker. 1967. Further studies on β-galactosidase from Streptococcus lactis. Bacteriol. Proc., 1967: 53-54.

115. McFeters, G. A., W. E. Sandine, and P. R. Elliker. 1967. Purification and properties of Streptococcus lactis β-galactosidase. J. Bacteriol., 93: 914-919.

116. McFeters, G. A., W. E. Sandine, R. R. Becker, and P. R. Elliker. 1969. Some factors affecting association-dissociation of β-galactosidase from Streptococcus lactis 7962. Can. J. Microbiol., 15: 105-110.

117. McKay, L. L. 1970. Mechanisms of lactose utilization by lactic acid bacteria: enzymatic and genetic studies. Ph.D. Thesis. Oregon State Univ. (Libr. Congr. Card No. Mic. 70-5178) 130 p. Univ. Microfilms. Ann Arbor, Mich. (Diss. Abstr. Int. 30:4725-B).

118. McKay, L. L., W. E. Sandine, and P. R. Elliker. 1971. Lactose utilization by lactic acid bacteria: a review. Dairy Sci. Abstr., 33: 493-499.

119. McKay, L. L., L. A. Walter, W. E. Sandine, and P. R. Elliker. 1969. Involvement of phosphoenolpyruvate in lactose utilization by group N streptococci. J. Bacteriol., 99: 603-610.

120. Magasanik, B. 1961. Catabolite repression. Cold Spring Harbor Symp. Quant. Biol., 26: 249-256.

121. Maio, J. J., and H. V. Rickenberg. 1960. The β-galactosidase of mouse strain L-cells and mouse organs. Biochim. Biophys. Acta, 37: 101-106.

122. Malik, A. C., G. W. Reinbold, and E. R. Vedamuthu. 1968. An evaluation of the taxonomy of Propionibacterium. Can. J. Microbiol., 14: 1185-1191.

123. Mandelstam, J. 1962. The repression of constitutive β-galactosidase in Escherichia coli by glucose and other carbon sources. Biochem J., 82: 489-493.

124. Marchesi, S. L., E. Steers, Jr., and S. Shifrin. 1969. Purification and characterization of the multiple forms of β-galactosidase of Escherichia coli. Biochim. Biophys. Acta, 181: 20-34.

125. Marx, A. B. 1924. Lactase in the adult intestine. Arch. Verdauungs-Krankh., 33: 77-86. (Chem. Abstr., 18: 2745. 1924).

126. Mehler, A. H. 1957. Introduction to enzymology. Academic Press, Inc., New York. 425 p.

127. Monod, J. 1956. Remarks on the mechanism of enzyme induction, p. 7-28. In O. H. Gaebler [ed.] Enzymes: units of biological structure and function. Academic Press, Inc., New York.

128. Monod, J., et M. Cohn. 1952. La biosynthèse induite des enzymes. (adaptation enzymatique). Advances Enzymol., 13: 67-119.

129. Monod, J., A. M. Pappenheimer, Jr., et G. Cohen-Bazire. 1952. La cinétique de la biosynthèse de la β-galactosidase chez E. coli consideré comme fonction de la croissance. Biochim. Biophys. Acta, 9: 648-660.

130. Monod, J., A.-M. Torriani, et J. Gribetz. 1948. Sur une lactase extraite d'une souche d'Escherichia coli mutabile. Comptes Rendus Acad. Sci., 227: 315-316.

131. Monod, J., G. Cohen-Bazire, et M. Cohn. 1951. Sur la biosynthese de la β-galactosidase (lactase) chez Escherichia coli. La specificite de l'induction. Biochim. Biophys. Acta, 7: 585-599.

132. Morse, M. L., K. L. Hill, J. B. Egan, and W. Hengstenberg. 1968. Metabolism of lactose by Staphylococcus aureus and its genetic basis. J. Bacteriol., 95: 2270-2274.

133. Nakada, D., and B. Magasanik. 1964. The roles of inducer and catabolite repressor in the synthesis of β-galactosidase by Escherichia coli. J. Mol. Biol., 8: 105-127.

134. Nishizawa, K. 1951. The enzymic cleavage of galactosides. III. (in German). J. Fac. Textiles Sericult Shinshu Univ., Ser. C, 1: 1-73. (Chem. Abstr., 47: 11272. 1953).

135. Novick, A., and T. Horiuchi. 1961. Hyper-production of β-galactosidase by Escherichia coli. Cold Spring Harbor Symp. Quant. Biol., 26: 239-245.

136. Novikova, S. I. 1956. Lactase activity of Streptococcus lactis (Russian Summary, 44). Mikrobiol. Zhur., Akad. Nauk Ukr. S. S. R., Inst. Mikrobiol. im. D.K. Zabolotnogo, 18(3): 40-43. (Chem. Abstr., 51: 2936. 1957).

137. Pardee, A. B., F. Jacob, et J. Monod. 1958. Sur l'expression et le rôle des alléles "inductible" et "constitutif" dans la synthèse de la β-galactosidase chez des zygotes d'Escherichia coli. Comptes Rendus Acad. Sci., 246: 3125-3128.

138. Pardoe, G. I. 1970. The inducible β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) of Klebsiella aerogenes NCIB 9479. Pathologia et Microbiologia, 36: 108-122.

139. Park, H. S., G. W. Reinbold, and E. G. Hammond. 1967. Role of propionibacteria in split defect of Swiss cheese. J. Dairy Sci., 50: 820-823.

140. Park, H. S., G. W. Reinbold, E. G. Hammond, and W. S. Clark, Jr. 1967. Growth of propionibacteria at low temperatures. J. Dairy Sci., 50: 589-591.

141. Pazur, J. H. 1954. The mechanism of enzymatic synthesis of galactosyl oligosaccharides. J. Biol. Chem., 208: 439-444.

142. Pazur, J. H., J. M. Marsh, and C. L. Tipton. 1958. Reversible transgalactosylation. J. Amer. Chem. Soc., 80: 1433-1435.

143. Pazur, J. H., M. Shadaksharaswamy, and A. Cepure. 1961. The use of o-nitrophenyl β-D-galactoside for the synthesis of galactosyl oligosaccharides. Arch. Biochem. Biophys., 94: 142-147.

144. Pigman, W. W. 1943. Classification of carbohydrases. J. Res. Nat. Bur. Standards, 30: 257-265.

145. Pomeranz, Y., R. J. Robinson, and J. A. Shellenberger. 1962. Evaluation of β-galactosidase (lactase) activity by paper chromatography. Enzymologia, 25: 157-166.

146. Reddy, M. S. 1971. Development of cultural techniques for the study of starter streptococci. Unpublished M.S. thesis. Libr., Iowa State Univ., Ames, Iowa. 158 p.

147. Reinbold, G. W. 1971. Swiss cheese varieties. Pfizer Cheese Monographs, vol. VI. Chas. Pfizer and Co., New York. In press.

148. Reithel, F. J., and J. C. Kim. 1960. Studies on the β-galactosidase isolated from Escherichia coli ML 308. I. The effect of some ions on enzymic activity. Arch. Biochem. Biophys., 90: 271-277.

149. Richards, N. 1969. Regulation of gratuitous β-galactosidase in Aerobacter aerogenes during an adaptive process. J. Gen. Microbiol., 55: 361-370.

150. Richards, N., and C. Hinshelwood. 1961. Observations on the β-galactosidase activity of Bact. lactis aerogenes (Aerobacter aerogenes). Royal Soc. London, Proc., Ser. B, 154: 463-477.

151. Rickenberg, H. V. 1959. The effect of metal ions and proteins on the stability of the β-galactosidase of Escherichia coli. Biochim. Biophys. Acta, 35: 122-129.

152. Rickenberg, H. V. 1960. Occurrence of β-galactosidase in the genus Shigella. J. Bacteriol., 80: 421-422.

153. Rickenberg, H. V., C. Yanofsky, and D. M. Bonner. 1953. Enzymatic deadaptation. J. Bacteriol., 66: 683-687.

154. Rickenberg, H. V., G. N. Cohen, G. Buttin, et J. Monod. 1956. La galactoside-perméase d'Escherichia coli. Ann. Inst. Pasteur, 91: 829-857.

155. Riordan, J. F., and B. L. Vallee. 1967. Reactions with N-ethyl-maleimide and p-mercuribenzoate, vol. XI, p. 541-548. In C. H. W. Hirs [ed.] Methods in enzymology. Academic Press, Inc., New York.

156. Roberts, H. R., and E. F. McFarren. 1953. The chromatographic observation of oligosaccharides formed during the lactase hydrolysis of lactose. J. Dairy Sci., 36: 620-632.

157. Rohlfing, S. R. 1968. The estimated size of Pseudomonas aeruginosa β-galactosidase. Soc. Exp. Biol. Med., Proc., 127: 1185-1187.

158. Rohlfing, S. R., and I. P. Crawford. 1966. Purification and characterization of the β-galactosidase of Aeromonas formicans. J. Bacteriol., 91: 1085-1097.

159. Rohlfing, S. R., and I. P. Crawford. 1966. Partial purification and physical properties of Bacillus megaterium β-galactosidase. J. Bacteriol., 92: 1258-1259.

160. Rosensweig, N. S. 1969. Adult human milk intolerance and intestinal lactase deficiency. A review. J. Dairy Sci., 52: 585-587.

161. Rotman, B. 1964. Specificity of induction of enzymes in the lactose operon of Escherichia coli and the effect of boric acid. Bacteriol. Proc., 1964: 106-107.

162. Rutenburg, A. M., S. H. Rutenburg, B. Monis, R. Teague, and A. M. Seligman. 1958. Histochemical demonstration of β-D-galactosidase in the rat. J. Histochem. Cytochem., 6: 122-129.

163. Sarkar, S., and S. E. Luria. 1962. A heat-sensitive β-galactosidase from Shigella dysenteriae. Fed. Proc., 21: 255.

164. Scarborough, G. A., M. K. Rumley, and E. P. Kennedy. 1968. The function of adenosine 5'-triphosphate in the lactose transport system of Escherichia coli. Nat. Acad. Sci., Proc., 60: 951-958.

165. Schäfler, S., and L. Mintzer. 1959. Acquisition of lactose-fermenting properties by salmonellae. I. Interrelationship between the fermentation of cellobiose and lactose. J. Bacteriol., 78: 159-163.

166. Schäfler, S., L. Mintzer, and C. Schäfler. 1960. Acquisition of lactose fermenting properties by salmonellae. II. Role of the medium. J. Bacteriol., 79: 203-212.

167. Simoni, R. D., M. F. Smith, and S. Roseman. 1968. Resolution of a staphylococcal phosphotransferase system into four protein components and its relation to sugar transport. Biochem. Biophys. Res. Commun., 31: 804-811.

168. Simoni, R. D., M. Levinthal, F. D. Kundig, W. Kundig, B. Anderson, P. E. Hartman, and S. Roseman. 1967. Genetic evidence for the role of a bacterial phosphotransferase system in sugar transport. Nat. Acad. Sci., Proc., 58: 1963-1970.

169. Skogen, L. O. 1970. Capsulation of Propionibacterium. Unpublished M.S. Thesis. Libr., Iowa State Univ., Ames, Iowa. 72 p.

170. Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods. 6th ed. Iowa State University Press, Ames. 593 p.

171. Stanier, R. Y., M. Doudoroff, and E. A. Adelberg. 1963. The microbial world. 2nd ed. Prentice-Hall, Inc., Englewood Cliffs, New Jersey. 753 p.

172. Stárka, J. 1964. The effect of some ions on the activity of β-galactosidase and the inhibitory effect of tris (hydroxymethyl) aminomethane. Folia Microbiologica, 9: 24-27.

173. Takano, K. 1956. Acceptor relations in the enzymatic transfer of β-glucosyl and β-galactosyl groups. J. Biochem., Tokyo, 43: 205-216.

174. Takano, K., and T. Miwa. 1953. Enzymatic transfer of β-D-galactose. J. Biochem., Tokyo, 40: 471-476.

175. Tanaka, S., and E. C. C. Lin. 1967. Two classes of pleiotropic mutants of Aerobacter aerogenes lacking components of a phospho-enolpyruvate-dependent phosphotransferase system. Nat. Acad. Sci., Proc., 57: 913-919.

176. Tanaka, S., D. G. Fraenkel, and E. C. C. Lin. 1967. The enzymatic lesion of strain MM-6, a pleiotropic carbohydrate-negative mutant of Escherichia coli. Biochem. Biophys. Res. Commun., 27: 63-67.

177. Ullmann, A., F. Jacob, and J. Monod. 1968. On the subunit structure of wild-type versus complemented β-galactosidase of Escherichia coli. J. Mol. Biol., 32: 1-13.

178. United States Department of Agriculture. Statistical Reporting Service. Crop Reporting Board. 1971. Production of manufactured dairy products, 1970. Da 2-1 (71). July. 62 p.

179. Vakil, J. R., and K. M. Shahani. 1969. Carbohydrate metabolism of lactic acid cultures. II. Different pathways of lactose metabolism of Streptococcus lactis and their sensitivity to antibiotics. J. Dairy Sci., 52: 162-168.

180. Vakil, J. R., and K. M. Shahani. 1969. Carbohydrate metabolism of lactic acid cultures. V. Lactobionate and gluconate metabolism of Streptococcus lactis UN. J. Dairy Sci., 52: 1928-1934.

181. Van Dam, B., J. G. Revallier-Warffemius, and L. C. Van Dam-Schermerhorn. 1950. Preparation of lactase from Saccharomyces fragilis. Neth. Milk Dairy J., 4: 96-114.

182. Vedamuthu, E. R., and G. W. Reinbold. 1967. The use of candle oats jar incubation for the enumeration, characterization and taxonomic study of propionibacteria. Milchwissenschaft, 22: 428-431.

183. Vedamuthu, E. R., G. J. Washam, and G. W. Reinbold. 1971. Isolation of inhibitory factor in raw milk whey active against propionibacteria. Appl. Microbiol., 22: 552-556.

184. Veibel, S. 1950. Hydrolysis of galactosides, mannosides, and thioglycosides, vol. 1, part 1, p. 621-634. In J. B. Sumner and K. Myrbäck [ed.] The enzymes. Academic Press, Inc., New York.

185. Vogel, H. J. 1961. Aspects of repression in the regulation of enzyme synthesis: pathway-wide control and enzyme-specific response. Cold Spring Harbor Symp. Quant. Biol., 26: 163-172.

186. Wallenfels, K. 1951. Enzymatische Synthese von Oligosacchariden aus Disacchariden. Die Naturwissenschaften, 38: 306-307.

187. Wallenfels, K., und J. Fischer. 1960. Untersuchungen über milchzuckerspaltende Enzyme. X. Die Lactase des Kälberdarms. Hoppe-Seyler's Zeitschrift Physiol. Chem., 321: 223-245.

188. Wallenfels, K., und M. L. Zarnitz. 1957. Kristallisation der β-Galaktosidase aus Escherichia coli. Angewandte Chemie, 69: 482.

189. Wallenfels, K., and O. P. Malhotra. 1960. β-Galactosidase, vol. 4, p. 409-430. In P. D. Boyer, H. Lardy, and K. Myrbäck [ed.] The enzymes. 2nd ed. Academic Press, Inc., New York.

190. Wallenfels, K., and O. P. Malhotra. 1961. Galactosidases. Advances Carbohydrate Chem., 16: 239-298.

191. Wallenfels, K., E. Bernt und G. Limberg. 1953. Über den Verlauf der enzymatischen Spaltung von Lactose mit β-Galaktosidase von Schimmelpilzen, Helix pomatia, Escherichia coli und Kälberdarm. Justus Liebigs Annalen der Chemie, 584: 63-85.

192. Wallenfels, K., J. Lehmann, und O. P. Malhotra. 1960. Unter-
suchungen über milchzuckerspaltende Enzyme. VII. Die Spezifität
der β-galactosidases von E. coli ML 309. Biochem. Zeitschrift, 333:
209-225.

193. Wallenfels, K., O. P. Malhotra, und D. Dabich. 1960. Untersuchungen
über milchzuckerspaltende Enzyme VIII. Der Einfluss des Kationen-
Milieus auf die Aktivität der β-Galaktosidase von E. coli ML 309.
Biochem. Zeitschrift, 333: 377-394.

194. Wallenfels, K., M. L. Zarnitz, G. Laule, H. Bender, und M. Keser.
1959. Untersuchungen über milchzuckerspaltende Enzyme. III.
Reinigung, Kristallisation und Eigenschaften der β-galactosidase von
Escherichia coli ML 309 (English summary). Biochem. Zeitschrift,
331: 459-485.

195. Webb, E. C. 1960. Steric factors in enzyme action: hydrolytic
enzymes. Symp. Biochem. Soc., 19: 90-107.

196. Wehmer, C. V., und M. Hadders. 1933. Systematische Verbreitung und
Vorkommen der Enzyme, vol. IV, part 2, p. 861-907. In G. Klein [ed.]
Handbuch der Pflanzenanalyse. Julius Springer, Vienna.

197. Wendorff, W. L., C. H. Amundson, and N. F. Olson. 1969. Characteri-
zation of β-galactosidase from Saccharomyces fragilis. Presented at
the 64th Annual Meeting of the American Dairy Science Association,
University of Minnesota, Minneapolis, June 1969. University of
Wisconsin, Madison.

198. Wendorff, W. L., C. H. Amundson, and N. F. Olson. 1969. Characteri-
zation of β-galactosidase from Saccharomyces fragilis. J. Dairy Sci.,
52: 890.

199. Wendorff, W. L., C. H. Amundson, and N. F. Olson. 1970. Nutrient
requirements and growth conditions for production of lactase enzyme
by Saccharomyces fragilis. J. Milk Food Technol., 33: 451-454.

200. Wierzbicki, L. E., and F. V. Kosikowski. 1971. Preparation and
characterization of lactase (βD galactosidase) from microbial sources.
J. Dairy Sci., 54: 763.

201. Winkler, H. H., and T. H. Wilson. 1967. Inhibition of β-galactoside
transport by substrates of the glucose transport system in Escherichia
coli. Biochim. Biophys. Acta, 135: 1030-1051.

202. Wiśniewski, W. 1965. Separation of proteins from propionibacteria
on cellulose ion exchangers. Acta Biochim. Polonica, 12: 115-122.

APPENDIX

Sodium Lactate Broth

Trypticase (Baltimore Biological Laboratory)	10 g
Yeast extract (Difco)	10 g
Potassium phosphate, dibasic	0.25 g
Sodium lactate	10 g
Distilled water	1 liter

The hydrogen ion concentration was adjusted so that, after autoclaving for 20 min at 121 C, the pH was 7.0 ± 0.1. When solid medium was desired, 15 g agar (Difco) were added.

Preparation of Purified Sodium Lactate

Sodium lactate was purified by crystallizing lactide (3,6-dimethyl-2,5 ρ-dioxanedione. Clinton Corn Processing Company, Clinton, Iowa) according to the procedure of Deane and Hammond (51). One hundred and sixty-six milliliters of chloroform were added to 315.7 g lactide in a 1,000-ml Erlenmeyer flask which was then heated on a hot plate. The flask, which contained a boiling stick, was covered with a watch glass during the heating. Since the lactide did not readily go into solution, an additional 190 ml of chloroform were added. After the lactide dissolved, it was decanted into a 1,000-ml beaker to remove most of the sediment. After cooling to room temperature, the solution was placed at 3.3 C for 24 hr to permit crystallization. Crystals were rinsed on a Buchner funnel with 200 ml cold (3.3 C) chloroform. After evaporation of the chloroform at room temperature, the crystals were weighed to determine the number of moles of lactide. Twice this number of moles of NaOH (in the form of a 10% NaOH solution) were slowly added to hydrolyze the lactide to sodium

lactate. After the calculated amount of NaOH was added, the solution was
heated on a hot plate. The pH was measured to make sure it was above 7.0,
which would indicate that all the lactide was hydrolyzed. The sodium lac-
tate was cooled, diluted to 500 ml, and the per cent sodium lactate calcu-
lated.

Lactose Broth

Lactose	10 g
Tryptone (Difco)	10 g
Yeast extract (Difco)	5 g
Gelatin	2.5 g
Sodium chloride	4 g
Sodium acetate	1.5 g
Ascorbic acid	0.5 g
Distilled water	1 liter

The hydrogen ion concentration of the medium was adjusted so that
after autoclaving 20 min at 121 C, the pH was 7.0 ± 0.1. The lactose was
incorporated into the medium by adding a 15% lactose solution (filter-
sterilized) immediately before inoculation. When a solid medium was de-
sired, 15 g agar (Difco) were added.

Lowry's Procedure for Determining Protein

Alkaline copper reagent was prepared on the day it was to be used by
mixing 50 ml of 2% Na_2CO_3 in 0.1 $\underline{N}$ NaOH with 1 ml 0.5% $CuSO_4 \cdot 5H_2O$ in 1%
sodium citrate. Folin reagent was prepared by diluting phenol reagent 2 $\underline{N}$
solution (Fisher Scientific Company, Fair Lawn, New Jersey) 1:1 with dis-
tilled water. One milliliter of sample was mixed with 5 ml alkaline copper
reagent and held in a 37 C water bath for 10 min. Then, 0.5 ml of diluted
phenol reagent was added and it was immediately mixed 30 sec on a Vortex

Genie laboratory mixer. After mixing, the sample was incubated 30 min at 37 C. Absorbance was read at 500 nm. Bovine serum albumin (Pentex Incorporated, Kankakee, Illinois) in concentrations of 100 to 700 µg/ml was used to make a standard curve.