THE UNIVERSITY OF MANITOBA

RAPID ENUMERATION OF FAECAL COLIFORMS IN MILK USING A COLOURIMETRIC β-D-GALACTOSIDASE ASSAY

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF FOOD SCIENCE

WINNIPEG, MANITOBA

SEPTEMBER 1980

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"TO MY HUSBAND ANDREWS

and

MY DAUGHTER OTEMA"

A C K N O W L E D G E M E N T S

I wish to express deep gratitude to my advisor, Dr. G. Blank, for his advice and guidance throughout this study.

The invaluable advice and assistance of Dr. E.D. Murray, Dr. E. Hoehn and Dr. H.B. Lejohn are truly appreciated. Sincere thanks are also due to Dr. K. Mount and P. Stephen, for their assistance regarding statistical analysis.

I also wish to express thanks to my dear friend, Miss E. Gordon, and other staff and fellow graduate students of the Food Science Department, who contributed in one way or the other to make this thesis a success.

Finally, my sincere gratitude goes to my husband, Dr. A.A. Adade, for his unfailing support, patience and encouragement.

A B S T R A C T

A new, rapid method for the enumeration of faecal coliforms recently devised for water has been extended for use in milk. The assay is based on the ability of coliforms to synthesize β-D-galactosidase which is then used to hydrolyse O-nitrophenyl-β-D-galactoside (ONPG). The amount of liberated ONP resulting from the enzymatic hydrolysis of ONPG was then related to the initial concentration of faecal coliforms. Selectivity for faecal coliforms was achieved by incubation at 44.5°C in EC medium. In this investigation, mid-log grown Escherichia coli K-12 were added to samples of 2% pasteurized milk at different inoculum levels and the resulting ONP analyzed spectrophotometrically. The actual hydrolysis time was dependent on the inoculum size. This technique was found to be fast, simple and selective for faecal coliforms, and provided an estimate of faecal coliform densities of 10^2 cells/ml and higher, in less than 24 hours.

In this study, the ability of faecal coliforms to synthesize β-D-galactosidase and hydrolyze ONPG was also applied to an agar diffusion plate method. The extent of hydrolysis, as well as the time taken for hydrolysis was

dependent on the inoculum size. Cell concentrations of 10^3/ml represented the lower limit of detection using this procedure. Although agar concentrations of 1.5 to 2.5% had negligible effects on the extent of hydrolysis, adjustment of the pH of the agar medium to 8.0 improved the colour of the zone of hydrolysis and allowed for good contrast with the agar background.

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1. INTRODUCTION

One of the principal concerns of regulatory officials both in the government and industry, is the assurance that the food eaten by the consumer should be both safe and wholesome from a microbiological standpoint. This major concern emphasizes the need for fast and accurate methods for the detection and enumeration of microorganisms in food. Since it is both impractical and time consuming to examine each food product for the possible presence of hazardous or potentially hazardous microorganisms, it has become necessary to monitor these food products using various index or indicator organisms.

The faecal coliform (FC) group of indicator organisms have been successfully used to monitor food products for many years. The approved bacteriological test procedures - most probable number (MPN) and membrane filter (MF) techniques, although satisfactory and straight forward in implementation, lack precision and are also time consuming. Recommendation of a single standard method for FC detection is exceedingly difficult owing to a lack of comparative data.

There is thus an urgent need for vastly improved methods for the quick, bacteriological evaluation of water and foods, such as milk. This is especially true in cases of natural disasters, malfunctions in potable water distribution, or contamination of foods by faecal matter. During such outbreaks, the usual 48 - 96 hours normally required to complete faecal coliform analysis is too long for the selection of the most reasonable solution to the problem.

In addition, most bacteriological tests for total counts, coliforms, enterococci, Staphylococci and Salmonellae have been designed for clinical tests and water analysis. Investigators often apply these test procedures to foods in the same manner even though their original application was not intended nor designed for food analysis. Thus, the presence of various components such as spices and preservatives peculiar to food, may influence the behaviour of the test medium, the test procedure, per se, and the microorganisms under analysis.

To improve the detection and enumeration of FC, efforts have been directed towards developing more sensitive and accurate procedures that are economical, simple and fast, and which could make available some comparative data, so that

with the added economic advantages they could be used alone and convincingly. No single rapid method has so far been devised which fulfils these criteria. A new, rapid enumeration method for FC in water (Warren et al., 1978) has created several interesting possibilities towards the continuing search of improved techniques for this group of indicator microorganisms. This rapid assay is based upon the ability of coliforms to elaborate β-D-galactosidase which is then used to hydrolyse the chromogenic substrate, ortho-nitrophenyl β-D-galactoside (ONPG). The test procedure also makes use of the 44.5°C temperature requirement for faecal coliform tests.

Theoretically, the time required to achieve detectable ONPG hydrolysis in an inoculated medium is directly proportional to the quantity of faecal coliforms in that medium.

In this investigation, the feasibility of applying a colorimetric β-D-galactosidase assay to milk for FC enumeration will be investigated. Before adopting the new technique, it was necessary to study some aspects of the assay in greater detail.

The objectives of this study were:

1. To determine and select the optimum conditions of the β-D-galactosidase assay for faecal coliforms with respect to pH, temperature and growth phase.
2. To evaluate the rapid β-D-galactosidase assay and show how time of color production from ONPG hydrolysis is related to inoculum size.
3. To apply the rapid assay to the enumeration of faecal coliforms in a natural food sample - milk.
4. To compare the new assay technique to a conventional method of coliform enumeration - the (MPN) technique.

2. L I T E R A T U R E R E V I E W

2.1 Incidence and pathological significance of sanitary indicator organisms

Foods, per se, in addition to providing the body with essential nutrients, can also serve as vehicles or vectors for the transmission of a wide variety of microorganisms. Many of these microorganisms represent part of the natural microflora of the various plant and animal materials used in the production of food. Although such transmissions occur via the natural microflora, foods can also become contaminated during the handling and processing of the final product (Frazier, 1958).

The safety and sanitary quality of foods is of particular importance to the food manufacturer (de Figueiredo, 1970). For food to be of good sanitary quality, it must be shown to be free of hazardous microorganisms, or those present should be at a safe, low level (Chordash and Insalata, 1978). In general, it is not feasible to examine each food product directly for the presence of pathogens since it is a time-consuming and costly effort. To minimize these factors, it has been the practice for many

years to determine the sanitary quality of foods by their content of certain "indicator organisms" (de Figueiredo, 1970), the presence of these organisms can also reflect conditions that could lead to the entry and proliferation of pathogens.

Indicator organisms have thus been used with success to monitor and improve the quality of certain foods, such as milk (Milk Industry Foundation, 1964).

The safety of a food product from food-poisoning and other pathogenic microorganisms is normally ascertained by the examination of that food for the presence of faecal and/or other indicator organisms. To be useful, these indicator microorganisms must delineate real or potential effects of hazard-posing conditions in the food, and should do so using relatively simple and quick microbiological tests. In some cases, these microorganisms should indicate the spoilage potential of the food product (de Figueiredo, 1970).

The types and/or groups of indicator microorganisms used in the food industry are varied, and their use is largely dependent upon the food product to be analyzed, and the type of information to be gained.

The most useful indicators used in the milk industry include total aerobic plate counts (TAPC), coliform count,

Escherichia coli (*E. coli*) count, yeast and moulds and faecal streptococci or enterococci counts (Miskimin *et al.*, 1976).

It is generally believed that high TAPC in food products are indicative of a high risk of pathogens being present, while presence of the coliform group in high numbers signals possible unsanitary processing conditions, and therefore, the presence of potential pathogens. The absence of coliform microorganisms is assumed to be an indication of safe, sanitary conditions under which the food product was handled and processed (Buttiaux and Mossel, 1961; Thatcher and Clark, 1968). *E. coli* has been the traditional indicator of faecal contamination, and has therefore, been used to monitor the possible presence of food-poisoning organisms, such as *Salmonella* (Buttiaux and Mossel, 1961). Yeasts and moulds are used as indicator microorganisms in acid foods, dairy products, carbonated beverages, etc. There is the additional potential hazard from production of mycotoxins by mycotoxigenic moulds (Saito and Tatsuno, 1971). Yeast spoilage, however, is not a health hazard (ICMSF, 1978). Faecal streptococci are also used as indicators of faecal contamination, but their identity as causative agents of

food borne gastroenteritis has not been proven with certainty (Deibel, 1964).

2.1.1 Total aerobic plate count (TAPC)

The TAPC as an indicator group has been reviewed by Silliker (1963). Total counts performed on foods reflect the handling history, state of decomposition or degree of freshness, and sanitary quality of foods. Its use, however, has been shown to have some limitations. For example, in frozen food products, no direct relationship has been reported between the TAPC and the spoilage potential of that product (Silliker, 1963). TAPC do not indicate the safety of a food product, or the possibility of a public health risk. A low TAPC does not always represent safety upon consumption (Montford and Thatcher, 1961). The presence of small numbers of pathogens such as Salmonellae cannot be predicted especially from low TAPC in a food product. Despite low TAPC, toxins have been shown to remain stable to conditions which may not favour the continued survival of vegatative cells (de Figueiredo, 1970).

The sanitary quality of many cultured and naturally fermented foods such as sauerkraut, pickles, as well as yoghurt and other related dairy products cannot be ascertained by

TAPC, since many of the microorganisms pertaining to the TAPC have been produced by their multiplication during the processing of that food product. In such products, a high count has practically no significance because objectionable organisms cannot be differentiated from those starter or normal microflora used in the fermentation process (Barber and Deibel, 1972).

A more recent study of many ready-to-eat foods however, has suggested that the TAPC is the most suitable indicator of their microbial quality (Miskimin et al., 1976). Fluid milk, both raw and pasteurized, as well as spray dried milk are routinely analyzed by TAPC. In addition, the milk industry has used direct microscopic counts (DMC) to check the sanitary quality of raw and pasteurized milk (APHA, 1967). In such perishable foods, low counts have been associated with a longer shelf life (de Figueiredo, 1970).

2.1.2 Enterococci

Another group of microorganisms which has been extensively employed as sanitary indicators of food products are the Group D streptococci, also referred to as faecal streptococci or enterococci. The enterococci are members of the genus Streptococcus, which consists of Gram-positive

cocci, producing long or short chains, and differing from most other Gram-positive cocci in being catalase negative. Although there is some confusion as to which species of streptococci should be included under the term enterococci, it is now generally agreed that the enterococci are all members of Lancefield's serologic Group D streptococci. These organisms classically include Streptococcus faecalis and its three subspecies - faecalis, liquefaciens and zymogenes; S. faecium; and S. durans. Less heat-resistant streptococci such as S. bovis and S. equinus are also included (Jay, 1978). Proposals have also been made to include S. mitis and S. salivarius as faecal streptococci (Smith and Shattock, 1962; Niven, 1963).

The presence or absence of faecal streptococci has been used to provide information regarding the sanitary history of many food products including milk (Bartley and Slanety, 1960). Their significance as indicators of faecal contamination is severely limited because of their widespread occurrence both in faeces and in the environment (Martin and Mundt, 1972). Despite this limitation, the occurrence of large numbers of enterococci in foods has implied either inadequate sanitary practices or exposure to conditions that have permitted

extensive multiplication of contaminants. Faecal streptococci, have been shown to be more resistant than coliforms, to refrigeration, freezing, heating, dessication, and therefore, provide a more sensitive indication of faecal pollution in foods processed and stored under these conditions (Burton, 1949; Kereluk and Gunderson, 1959; Niven, 1963; Deibel, 1964). They are also extremely resistant to detergents and disinfectants. Raw milk, dairy products and other foods may contain faecal streptococci as concomitant microflora, therefore, their presence alone may not indicate the sanitary history of that food product. Because of their relatively high heat resistance, the enterococci may survive thermal treatments that would allow survival of viruses in some pasteurized or dried foods, thus making these cocci unreliable as general indicators of faecal contamination. In addition, they have also been shown to have a greater potential for survival in areas remote from the source of pollution (Niven, 1963). Their complete elimination is extremely difficult even under good sanitary conditions. They may survive adverse conditions so well that their occurrence bears little relation to the hazard from less durable pathogens such as

Salmonella and *Shigella* (Niven, 1963; Deibel, 1964). Microbiological analyses for these organisms should therefore, be performed concurrently with those for coliforms, faecal coliforms (FC), and/or *E. coli*, until their significance as sanitary indices has been established for a specific product or process (Chordash and Insalata, 1978). Buttiaux and Mossel (1961) noted the value of the association between Group D streptococci and coliforms in certain raw foods in giving indications of faecal contamination.

2.1.3 Yeasts and moulds

Yeasts and moulds represent a diverse group of microflora whose use as indicator organisms has increased in popularity (ICMSF, 1978). Yeasts and moulds grow more slowly in non-acid foods and therefore, seldom cause problems in such foods. However, in acid foods of low water activity, they outgrow bacteria and cause spoilage losses in fresh fruits, vegetables, cheeses, cereal products, salted and pickled foods and frozen or dried foods which are improperly stored (Onishi, 1963; Koburger, 1971; Corry, 1973).

"Machinery mould" is used to describe the occurrence of *Geotrichum candidum*, as a regulatory problem in food

products. The term refers to the build up of the organism on factory equipment in processing plants (Eisenberg et al., 1977). Another term associated with the occurrence of this organism in milk products is "dairy mold". The association of this fungus with Geotrichosis in man has also been noted (Conant et al., 1968).

2.2 Coliform organisms as sanitary indicators

Many bacterial contaminants found in foods are members of the family Enterobacteriaceae. The family includes the following genera: Escherichia, Klebsiella, Paracolobactrum, Erwinia, Serratia, Hafnia, Citrobacter, Proteus, Salmonella, Shigella, Edwardsiella, Enterobacter and Yersinia. They are Gram-negative, asporogenous, rod-shaped bacteria that grow well on and in artificial media. Most of these organisms grow as saprophytes in foods, and others, such as Salmonella and Shigella consist chiefly of pathogens (Chordash and Insalata, 1978). The possibility of including all organisms belonging to the Enterobacteriaceae as indicators of poor sanitary processing conditions or potential health hazards in foods has been reported (Mossel et al., 1962; 1963; 1970).

Members of the Enterobacteriaceae, in the genera Escherichia and Aerobacter (Klebsiella and Enterobacter) and Paracolobactrum, are included in the coliform or coli-aerogenes group of microorganisms. This group consists of "all the aerobic and facultative anaerobic, Gram-negative, non-spore-forming bacteria which ferment lactose with gas formation within 48 hours at 35°C" (A.P.H.A., 1974). The primary coliform species include E. coli and Aerobacter aerogenes (Klebsiella aerogenes - non-motile form, and Enterobacter aerogenes - motile form of A. aerogenes), and are further classified according to their origin or natural habitat of growth. The faecal coliforms primarily inhabit the intestinal tract of man and other warm-blooded mammals, while the non-faecal coliforms are normally associated with soil and vegetation, and occasionally with the intestinal tract of warm-blooded mammals (Jay, 1978).

2.2.1 Growth and distribution of Coliforms

Coliforms grow well on a wide variety of media and are found ubiquitously in many foods, that provide the proper nutrients, pH and temperature conditions (Jay, 1978). Unlike most bacteria, they are capable of fermenting

lactose and are also capable of growth in the presence of bile salts which inhibit the growth of many Gram-positive bacteria (Warren et al., 1978). Advantage is taken of this fact in their isolation and enumeration. By incorporating lactose and bile salts into culture media, (such as MacConkey agar), it has become possible to differentiate between the coliform organisms and most other contaminants that may be present in foods and water. The general ease of cultivating coliforms and differentiating them from other microorganisms makes this group ideal as indicators of sanitary quality (Jay, 1978).

With respect to pH, these organisms have been reported to grow well over a rather wide range. Growth of coliform organisms from pH 4.4 to 9.0 has been reported by Griffin and Stuart (1940). They have also been reported to grow as low as $-2^{o}C$ and as high as $50^{o}C$ with an optimum growth temperature of $35^{o}C$ for non-faecal coliforms, and $44.5^{o}C$ for FC organisms (Geldreich, 1966).

Coliforms of faecal origin, such as E. coli, primarily inhabit the mammalian intestinal tract, whereas Enterobacter aerogenes, a non-faecal coliform, is normally associated with vegetation and other extra-external

environments (Geldreich et al., 1962). Klebsiella, on the other hand, has been found in human faeces (Montogerie et al., 1970; Thom, 1970), industrial wastes (Bauer, 1972) and soil deposits (Duncan and Razzell, 1972). As early as 1940, Griffin and Stuart came to the conclusion that the occurrence of E. coli outside the intestinal tract and E. aerogenes and intermediates in places other than non-faecel materials were adventitious.

Because of their ubiquitous nature, non-faecal and faecal coliforms are present in a variety of raw and commercially processed foods (Jones et al., 1967; Taylor et al., 1967; Duitschaever et al., 1973; Miskimin et al., 1976). Most market vegetables can be shown to harbour small numbers of lactose-fermenting, Gram-negative rods of the coliform type. With proper harvesting and handling, the numbers tend to be quite low and consequently, of no real significance from the standpoint of public health (Jay, 1978).

It was thought that these FC organisms exclusively inhabited the intestinal tracts of warm-blooded animals, suggesting that their presence in water resulted from direct faecal contamination. Coliforms were thus well established as faecal indicators for water. Their use as indicators of

food sanitary quality has been derived from their successful use in water quality control (ICMSF, 1974).

The coliform index as it is applied to foods, was originally proposed for use in the dairy industry to indicate the quality of both raw and pasteurized fluid milk (Jones et al., 1967; A.P.H.A., 1974). Their presence in milk especially after pasteurization, indicated inadequate heat treatment or mishandling.

Coliforms were instituted as indicators of sanitation in the United States by the Public Health Service as early as 1914 and have been shown (Burton, 1949) to be very efficient sanitary indicators.

In processed foods, the presence of coliforms has been shown to indicate improper processing or sanitation, or post-processing contamination, most probably from the workers or their dirty equipment or from bacteriologically poor raw materials. McCoy (1961) concluded that the presence of coliforms during the examination of foods should be indicative of a lack of cleanliness, not safety.

2.2.2 Faecal Coliforms

Since coliforms have been isolated from numerous sources other than faecal material, their presence in some foods does

not necessarily imply faecal contamination or unsanitary production facilities. Some species of Erwinia which closely resemble the criteria used for defining faecal coliforms, are plant pathogens and do not indicate faecal contamination (ICMSF, 1974). As a result, specific testing for faecal coliforms has been widely adopted as a more precise method to indicate the presence of faecal pollution and possibly the presence of pathogens such as Salmonellae (Chordash and Insalata, 1978).

"Faecal coliforms", defined according to criteria listed in Standard Methods for the Examination of Water and Wastewater (1966), are those members of the Enterobacteriaceae which ferment lactose with the production of gas within 24 - 48 hours at elevated incubation temperatures, such as 42, 44, 44.5 or 45.5°C in selective medium. The term "faecal coliform" has arisen from several attempts to find some rapid and dependable analytical methods to establish the presence of E. coli, or closely related variants in food products, without the need of specifically purifying or identifying this microorganism (Chordash and Insalata, 1978). Faecal coliform populations usually contain a high proportion of E. coli types I and II, and thus are useful to indicate

a probable faecal source (Fishbein and Surkiewicz, 1964; Geldreich, 1966), without the actual proportion of these types I and II being established, positively.

2.2.3 Escherichia coli as faecal indicators

The practice of using coliforms and faecal coliforms as indicators of water contamination has been questioned recently, particularly with regard to their use in establishing the possible presence of a water health hazard (Dutka, 1973). The fact that some coliform biotypes are widely distributed in nature and may represent only transient inhabitants of the gastro-intestinal tract of warm-blooded animals has caused microbiologists to re-examine the significance of FC in water. Although faecal coliforms are a more restricted portion of the total coliform population, they too, are a heterogenous group of bacteria composed primarily of E. coli and Klebsiella biotypes. More important, about half of the Klebsiella isolates obtained from certain industrial effluents, free of faecal contamination, would fit the definition of faecal coliforms (Bauer, 1972). There also remains inadequate information on the ability of some coliforms to reflect the microbial pathogen population in an aquatic environment (Gray, 1964;

Thom, 1970; Dufour and Cabelli, 1975). These shortcomings of using FC as sanitary indicators has prompted the specific use of E. coli as a more direct and specific indicator of faecal pollution (Fishbein, 1976).

The use of E. coli as an indicator for the presence of water-borne pathogens was first suggested as early as 1892 by Shardinger. He suggested that E. coli should be more readily detected and recovered in water than Salmonellae due to their higher numbers and adaptability. Consequently, E. coli was employed as an indicator of faecal pollution in water and foods.

Prior to the use of E. coli as an indicator of faecal pollution, Escherich (1887), demonstrated the ubiquity of E. coli from human faeces. This organism was first proposed as an indicator organism because it was primarily found in the intestinal contents of man and mammals, hence, "coli" for colon. It was noted to be so uniformly present in the intestinal tract that its presence outside this environment was regarded as a faecal contaminant. From his studies on the flora of the intestinal tract of man, Haenel (1961), placed the percentage of coliforms at less than one. This investigator found 10^8 to 10^9/g of these organisms to be common in adult faeces.

In general, approximately 90% of the faecal coliforms from the human intestinal tract are E. coli strains. Hence, E. coli is the classical indicator for the presence of enteric pathogens. However, it should be emphasized that the presence of this organism has no direct relationship to the presence of pathogens, only a risk is implied (Buttiaux and Mossel, 1961; Lewis and Angellotti, 1964; APHA, 1966; Geldreich, 1966). In foods, E. coli is the generally preferred indicator of contamination of relatively recent faecal origin. In appraising the safety of a food product, the presence of E. coli usually warrants a more positive assumption of a health hazard than that of other coliforms (Ewing et al., 1963). Contamination of a food product implies a risk in which faecal matter containing one or more types of enteric pathogens may have gained access, and hence have introduced a potential health hazard (Hall et al., 1967; Miskimin et al., 1976).

The presence of E. coli is unavoidable in food products which have been in direct or indirect contact with the soil. Significant numbers of E. coli in heat processed products have indicated post processing contamination. Buttiaux and Mossel (1961) concluded that various pathogens may persist

after the death of E. coli, especially in frozen foods or foods stored under prolonged refrigerated conditions and in radiated foods as well as in treated waters. These authors have concluded that only in acid foods, does E. coli have particular value as an indicator organism due to its relative resistance to low pH. Growth of E. coli on food processing equipment and subsequent contamination of the finished product indicates not faecal contamination, but poor manufacturing practice. When E. coli is found, it is important to determine the source, and only then is its significance judged. The absence of E. coli in food does not indicate the absence of pathogens (Elliott, 1963).

With respect to the overall choice of indicator organisms, Buttiaux and Mossel (1961) stated that the organism or group of indicator organisms should exist in faecal matter in high numbers; possess properties of survival outside the intestinal tract close to that of the intestinal pathogens; possess a certain degree of specificity for faecal matter; and be detectable by simple and rapid means. The enteric origin of E. coli is well established (Shardinger, 1892; Geldreich et al., 1962; Mossel, 1962) as is the ease of its recovery (Fishbein, 1976). In addition, E. coli conforms

to most biochemical and morphological criteria used to describe coliform and faecal coliform indicators (Chordash and Insalata, 1978).

Since E. coli is more indicative of faecal pollution than E. aerogenes, it has become an effective indicator of faecal pollution and, or poor sanitation in water, milk, shell-fish and other raw and commercially processed foods (Fishbein and Surkiewicz, 1964; Geldreich, 1966). However, detection of E. coli does not necessarily indicate the presence of other index organisms, nor their relative numbers.

The partial confirmation of E. coli identification is accomplished by IMViC (Indole, (I); Methyl red, (MR); Voges-Proskaeur, (VP); and Citrate (C) tests) patterns of either (++--) or (-+--) for E. coli types I and II, respectively (Chordash and Insalata, 1978). This typing was an approach towards increasing the specificity of coliforms as sanitary indicators (Wolfe and Amsterdam, 1968). IMViC typing, however, presents some problems. Closs and Digranes (1971) showed that IMViC patterns are non-specific relative to the genera of the coliform group. This is evident with Klebsiella and Enterobacter, both of which are (--++).

Klebsiella isolates have been shown to be highly variable with respect to their IMViC reactions. All strains of Citrobacter are MR positive and VP negative. In addition, while most species are citrate positive, their indole production varies. Although E. coli isolates tend to be rather consistent in their IMViC reactions, the IMViC typing requires the isolation and identification of individual pure colonies, which is a time-consuming and laborious procedure. To enhance confirmation of E. coli identification, Geldreich (1966) recommended an increased incubation temperature of 44.5°C (± 0.5°C) as offering the best compromise between acceptable sensitivity and specificity for the faecal coliforms.

2.2.4 Enteropathogenic - Enteroinvasive and Enterotoxigenic E. coli

Numerous strains of Enteropathogenic - Enteroinvasive (EEC) and Enterotoxigenic (ETEC) E. coli capable of causing human gastroentenitis have been identified. Delepine (1903), and Tanner and Tanner (1944) implicated E. coli in such outbreaks, and gave a complete assessment of their public health significance.

The EEC whose pathogenecity has been reported by

many investigators (Gitter, 1967; Cramblett et al., 1971; Howard and Glynn, 1971; Jones and Rutter, 1972; Sakazaki et al., 1974), attach to the epithelium of the colon and multiply intracellularly, producing a syndrome resembling dysentery. Symptoms may include ulceration and lesion formation, resulting in blood, mucus or inflammatory cells in the excreta.

Enterotoxigenic E. coli cause infantile and childhood diarrhoeas (Guerrant et al., 1975) travellers diarrhoea (Merson et al., 1976) and illness resembling cholera (Sack et al., 1971) as well as certain adult enteric disorders (Ewing et al., 1963; Sack et al., 1971).

2.3 Incidence and public health aspect of the coliform group

2.3.1 Public health aspects

Contamination of foods by disease-producing micro-organisms has been known and studied since 1880 (Escherich 1887; Shardinger, 1892). Since that time, numerous instances of food-borne diseases have been recorded in addition to those commonly referred to as food poisoning.

The Group D streptococci are not believed to be the cause of food borne disease (Moore, 1955; Hartman et al., 1965) and may be considered to possess little overt public

health significance. While generally regarded as being non-pathogenic, they have been incriminated in mild food poisoning outbreaks where large number of cells were recorded.

Federal courts in the U.S. recognize the presence of E. coli in foods as a public health hazard which originates from its use as an indicator of faecal pollution (Chordash and Insalata, 1978). Available incidence data from the U.S. indicate a low level of occurrence of food and water-borne gastro-enteritis resulting from E. coli. Feig (1950) reported that from 1945 to 1947, 10 out of 926 outbreaks of such nature were associated with E. coli and that 5 out of this 10 were transmitted by food and one by water.

Edwards and Ewing (1972) summarized several of the classical E. coli serogroups causing clinical cases of infantile diarrhoea. Other investigators incriminated serogroups in food and water-borne outbreaks of gastroenteritis (Hobbs et al., 1949; Costin et al., 1964).

Several investigators have demonstrated that EEC and ETEC could be isolated from foods and water. It has therefore, been concluded that these groups of organisms are of public health significance (Chordash and Insalata, 1978). They

have been isolated from numerous foods and water sources and have been incriminated in outbreaks of food and water borne gastroenteritis in both U.S., Canada and other parts of the world. The 1971 outbreak involving 387 persons in the U.S. was traced to imported French cheese and was the first documented occurrence of foodborne gastroenteritis in the U.S. resulting from EEC (Barnard and Callahan, 1971).

Food and waterborne outbreaks of gastroenteritis resulting from EEC/ETEC appear to be more prevalent in other parts of the world than in the U.S., as indicated in the literature (Agenjo, 1945; Costin et al., 1964; Barnard and Callahan, 1971; Ryder et al., 1976). In Japan, for example, EEC has been established as an important foodborne pathogen, accounting for approximately 5% of the reported outbreaks and 9% of the cases, ranking after Vibrio parahaemolyticus, Staphylococcus, and Salmonella (Sakazaki et al., 1974).

2.4 Methods for the Enumeration of Faecal Coliforms

The utilization of the coliform group as an indication of fecal pollution is historically based on the development of sanitary public water supplies and the problems inherently associated with such supplies (Fishbein and Surkiewicz, 1964). As such, systems of bacterial methodologies have been devised, elaborated, and refined through the years in order to seek out this group of indicator organisms in water supplies (A.P.H.A., 1966).

The application of these bacteriological methodologies to food also involves the use of the coliform group as indicator organisms, and more specifically, E. coli. The problem of bacterial isolation and identification, however, is more complicated when analyzing food products. Many food products are not inert, but instead may affect the physicochemical environment in which the organism - food medium reaction occurs; the associated bacterial flora may be of a nature to compete more successfully with the indicator organism; and finally, the handling and storage of the food may debilitate the indicator organism (Fishbein and Surkiewicz, 1964).

Methods for the enumeration of faecal coliforms originated

when MacConkey (1901) and Eijkman (1904) first utilized elevated incubation temperatures of 43° and 45°C, respectively in order to differentiate the coliform types inhabiting the intestinal tracts of warm-blooded animals from those of non enteric sources. Eijkman (1904) showed that coliform bacteria from the intestines of warm-blooded animals would grow and produce gas in glucose broth at 46°C, whereas coliforms from the intestines of cold-blooded animals would not.

Modifications of the Eijkman test, with regard to the nature of the medium and the temperature, have been introduced from time to time. Wilson *et al.* (1935), considerably modified the test and demonstrated the value of this technique in detecting the presence of the coliform group, and subsequently recommended its use for routine detection of *E. coli*. The modified Eijkman test consisted of the ability of coliforms to ferment MacConkey's broth at 44.0°C. This method was shown to have advantages over the routine agar plating methods in terms of saving considerable time, labour and cost.

Geldreich *et al.* (1959), Tennant *et al.* (1959), Fishbein and Surkiewicz (1964) and Fishbein (1962), have

since evaluated the use of selective, elevated incubation temperatures ranging from 35.5 to 45.5°C to detect and enumerate faecal coliforms. The experimental data, however, varied as to both the temperatures and the medium employed (Fishbein and Surkiewicz, 1964). Numerous media have also been used in the isolation of faecal coliforms. The application of EC medium, which contains bile salts, appears to have become stabilized in regard to its use as a selective agent for the isolation of coliforms (Geldreich et al., 1959; Tennant et al., 1959; Fishbein, 1962). Nevertheless, proper culturing conditions and temperature of incubation of EC mediumare still subject to wide variation, depending on the nature of the food product involved. Incubation of coliforms in EC medium has been carried out at temperatures of 44.5, 45.0 or 45.5°C. Substances examined have been water (Geldreich et al., 1959); seawater (Tennant et al., 1959); precooked frozen foods (Shelton et al., 1962); and seafoods (Raj and Liston, 1961).

2.4.1 Standard methods for faecal coliform enumeration

The methods used to enumerate the coliform group are, in recommended practice (A.P.H.A., 1974; W.H.O., 1974) restricted to the use of membrane filtration (MF), and

multiple tube or the most probable number technique (MPN), although previously, A.P.H.A.,(1955) stated that depending upon the type of selective media used, imperfect degrees of coliform recovery are realized, thus the pour plate methods have not achieved the status of a standard procedure. Despite this failing, the pour plate methods possess inherent advantages of precision and simplicity as to merit consideration as a standard procedure. Mara (1973) found it curious that this procedure was no longer recommended, for it would appear to be most suitable for enumerating coliforms and E. coli densities in polluted waters. The pour plate method is a simple technique familiar to water bacteriologists. It is less time-consuming than the MPN, and cheaper than, but as accurate as MF counts. Pour plate methods using VRB agar have been recommended for use in milk analyses (A.P.H.A., 1978).

The enumeration of coliforms is performed using three main methods involving either solid, liquid, or MF procedures.

2.4.1.1 The most probable number technique

The standard MPN technique accepted by the United States Food and Drug Administration (F.D.A.) and American

Public Health Association (A.P.H.A.) for coliform enumeration in water (Hackney et al., 1979), requires a maximum of 48 hours of incubation in Lauryl Sulphate Tryptose broth (LST) at 35°C, which represents an enrichment period needed to obtain optimum recovery of faecal coliforms (Andrews and Presnell, 1972). Aliquots from positive tubes showing gas formation are then transferred to test tubes containing EC medium and incubated for an additional 24 - 48 hours at either 44.5 or 45.5°C (A.P.H.A., 1976; F.D.A., 1976) in order to determine the MPN of faecal coliforms. Gas production within 24 - 48 hours in the fermentation vial of a tube of EC medium is considered a positive reaction indicating an organism of faecal origin.

Similarly, the Association of Official Analytical Chemists (A.O.A.C.) multiple-tube dilution method for E. coli, (A.O.A.C., 1975) has remained the accepted method for analyzing the quality of many foods. Thus faecal coliforms in foods are commonly enumerated by the MPN fermentation technique or by the EC confirmation procedure (Buttiaux and Mossel, 1961; I.C.M.S.F., 1974). The MPN procedure also employs a presumptive test which requies an LST

enrichment broth, followed by confirmation of gas positive tubes in Brilliant Green Bile Broth (BGLB) at 35°C, and confirmation in EC broth at 44.5°C. This latter method yields adequate information as to the source of the coliform groups (faecal or non-faecal), when used as a confirmatory test procedure.

Geldreich (1966) however, has stated that the coliform count is presently the best available method to determine the bacteriological quality of water, and although this method is statistically valid, its major disadvantage is that it requires from 48 to 96 hours to complete, as outlined in the standard methods (A.P.H.A., 1976).

The MPN technique consists of a series of three or more decimal dilutions with 3, 5 or 10 tubes in each dilution. The result of each test may be characterized by the most probable number (Eisenhart and Wilson, 1943). The mean coliform density cannot be obtained by a simple arithmetic averaging of the MPN values. Estimates based on such computations may be grossly underestimated as to the true coliform density (Thomas, 1955). The reasons for these possible coliform underestimations include: (i) some of the MPN values are apt to be indeterminate, as is the

case when all tubes are positive or negative, and (ii) the MPN is a biased estimator of the true density (Eisenhart and Wilson, 1943), and the amount of bias depends upon the number of tubes tested in each dilution. Consequently, a factor must be applied to correct for this bias (Thomas, 1955). The correction factor, C, for an estimation of the true density is $C = e^{-0.805/n}$, where n is the number of tubes used. The following are values of C for common values of n:

n	3	5	10
C	0.764	0.851	0.922

Incorporating the correct MPN bias factor as described by Thomas (1955) reduces the tendency for coliform underestimation. Also, since the MPN is an indirect enumeration procedure, it inherently is less accurate than the direct plating methods unless the population densities are very low (A.P.H.A., 1976).

Additional drawbacks of the MPN method include the relative expensive and cumbersomeness of the test; LST broth being a selective medium, could interfere with the recovery of stressed and injured coliform cells (Ray and Speck, 1973).

Several attempts which have been made to improve this laborious and time-consuming procedure include: (i) reduction in the time for the test (Andrews and Presnell, 1972; Abshire and Guthrie, 1973; Francis et al., 1974). Andrews and Presnell (1972), simultaneously evaluated Fishbein's elevated temperature test (Fishbein, 1976); an experimental 24-hour temperature test with a newly formulated medium; and the 72-hour standard methods procedure. Their data indicated that E. coli in raw seawater could be determined in 24 hours without significant loss of accuracy. (ii) Modification that will allow the test to detect injured and stressed organisms: Mossel and Ratto (1970) used a pre-enrichment treatment for the resuscitation of sublethally impaired cells by overnight incubation in lactose broth. This restoration treatment appeared to be significantly more productive than the use of LST because it allowed any injured coliform cells present the opportunity to repair in a non-selective medium at room temperature before being exposed to a specific selective growth. This method was also shown to be more effective than the standard MPN for FC determination since it enumerated injured coliform cells which might otherwise

have remained undetected in the selective medium used in MPN determinations. (iii) The third improvement involved the detection of faecal coliforms, nominally, E. coli and not just coliforms. Raj and Liston (1961) observed that the EC confirmation test had low specificity for E. coli in several frozen seafoods. Powell et al. (1979) used two variations of the multiple-tube technique to enumerate FC in commercially processed frozen crab meat after they also showed that the specificity of the EC confirmation test for E. coli was not as high for such a food product. They therefore assessed a rapid method requiring Medium A-1 (Andrews et al., 1975) as an alternative to EC test. The newly formulated Medium A-1 included: lactose, tryptone, NaCl, Triton X-100 - a surfactant, and salicin - a carbohydrate readily utilized by Escherichia species. This method also required the use of an elevated temperature of incubation, 44.5± 0.2°C, but did not require a pre-enrichment step, and as such a time period of 24 hours was used as opposed to the 48 to 72 hours required for the EC confirmation test. Andrews and Presnell (1972), showed that this medium was more satisfactory than EC broth for estimating E. coli in oyster. E. coli was isolated from

84% of the positive medium A-1 tubes, whereas it was isolated in only 64% of the positive EC broth cultures. Also, fewer species of non faecal coliforms were isolated from medium A-1, thus making the method a better alternative for FC in crab meat.

The major limitations of the MPN method are low precision and the excessive time necessary for analysis. Despite these limitations, it was advocated that the presumptive enrichment step of the MPN may increase recovery of coliforms at elevated temperatures by allowing for repair and minimum stress (Rose et al., 1975).

2.4.1.2 Direct plate-count methods

To overcome the shortcomings of the MPN method, attempts have been made to use plating procedures for the enumeration of faecal coliforms (Hefferman and Caballi, 1967; Klein and Fung, 1976). These procedures have involved the use of VRB agar and the agar overlay technique which has prevented the formation of atypical colonies. The disadvantage of this presumptive plate method occurs when the coliform densities in the food are low, since their presence cannot always be revealed, particularly if the initial food product is of a non-liquid nature

(ICMSF, 1974).

Warseck et al. (1973) have shown that VRB agar does not recover injured coliforms from food when incubated at 35°C, but the use of 45.5°C, as suggested by Klein and Fung (1976), would probably result in exclusion of injured coliforms.

Plating techniques that enumerate both injured and uninjured coliforms in a food sample, and still selectively enumerate only the desired microorganisms, have been developed by Speck et al. (1975) and others (Hartman et al., 1975; Hackney et al., 1979). Their "repair-detection" procedure involved surface plating on Trypticase soy agar (TSA) and subsequent overlay with VRB, for the repair and enumeration of coliforms injured by freezing. However, their method was found to be relatively less effective for enumeration in semi-preserved foods as compared with dairy products, but more effective than the MPN method.

Elliot and Millard (1976) showed that VRB plates incubated at 32°C gave a reliable count of total coliforms. In the light of this development, they recommended that the VRB plate count be performed in duplicate with one set incubated at 44.5°C and the other set incubated at 32°C for

24 hours. The 44.5°C incubation temperature estimated the counts of FC, and the latter incubation temperature indicated the total coliform count.

Elevated temperature tests for the separation of coliform organisms into those of faecal and non-faecal origin have been used in many parts of the world with various modifications (W.H.O. 1974). Varga et al. (1977) found that some of these methods underestimated the FC population by 20%. They assessed the Elevated Temperature plate count (ETPC) of Hefferman and Caballi (1967) to measure sanitary quality of soft shell clams. The ETPC of FC were obtained in a modified single strength MacConkey agar after 24 hour incubation at 44.5 ± 0.5°C. Their results indicated that the heat shock and the ensuing selective growth environment employed by the ETPC procedure lead to a 10 - 20% underestimation of FC numbers. This underestimation could be rectified by multiplying the ETPC value by a factor of 1.2. They therefore, indicated the need for modification of the method when applying it as a standard faecal coliform test. This ETPC technique, either by MPN estimate or MF procedure, was developed to alleviate the shortcomings of the MPN method (Elliot and Millard,

1976). However, considering the speed of ETPC technique, 24 hour incubation period, this option of using the correction factor of 1.2 would be quite attractive. The MPN procedure using LST or EC broth at 44.5°C incubation without pre-enrichment, however, gave a better estimate of FC density as compared to ETPC method.

Mara (1973) indicated that the pour plate technique using Lactose Teepol Agar (LTA) was also a reliable alternative to both the MF and the MPN techniques. This method was found to be suitable only for coliform densities over 3,000 per 100 ml.

2.4.1.3 Membrane filter technique

In 1965, Geldreich et al. proposed an MF method for the detection of faecal coliforms using a lactose broth base to which an indicator system of aniline blue and rosolic acid was added. This method was an attempt to develop a precise MF test which would overcome the inherent limitations of the MPN ,which included low precision and excessive time necessary for analysis. Its advantages over the plate count methods were speed, economy of media, and incubator space. This membrane faecal coliform (M-FC) medium employed an incubation temperature of 44.5°C ± 0.2°C for 24 hours, and is currently accepted for FC recovery

using the MF procedure (Geldreich et al., 1965; A.P.H.A., 1971).

Several other investigators have modified this technique bringing about a significant decrease in the incubation time required for a confirmation count (Guthrie and Reeder, 1969).

This procedure was accepted in 1971 as an official method for determining FC densities in water (A.P.H.A., 1974). Its distinct advantages over the plate count method included speed of performance of the test, reproducibility, economy of media utilized and incubator space (Sharpe and Michaud, 1975).

Several investigators, however, have reported that E. coli injured during physical or chemical treatment failed to: (i) form colonies on MF incubated on m-FC broth; (ii) grow and produce gas in lactose broth; or (iii) grow on selected media (Maxey, 1970; Braswell and Hoadley, 1974). Recently, Hufham (1974) reported that relatively large errors in coliform recovery resulted with the use of this method. These errors were influenced by the nature of membrane filter used, the medium and the temperature of incubation.

Subsequent methods have been proposed to improve the recovery of nonlethally injured cells by the MF procedure. These include: pre-incubation (Lin, 1973); temperature acclimation (Burman et al., 1969), and alternate media usage (Braswell and Hoadley, 1974), or a combination of these (Rose et al., 1975). Lin (1976) found that the one-step m-FC broth MF procedure was much less effective than the MPN technique. He, therefore, developed a two-step MF method, using a pre-enrichment step with phenol red lactose broth and incubation at 35°C for 4 hours, followed by plating on m-FC agar, and further incubation at 44.5°C for 18 ± 2 hours. This enhanced coliform recovery from chlorinated effluents. The results reported by Lin (1976) suggest that a combination of these methods was comparable to the MPN procedure in FC recovery efficiency. LST used as an enrichment medium did not improve FC recovery. Pre-enrichment at 44.5°C for 2 and 4 hours, however, significantly increased FC counts when phenol red lactose broth (PRLB) was used (Braswell and Hoadley, 1974). Dutka (1973) compared Gelman and Millipore autoclaved membrane filters for toxicity against E. coli and found that at 35°C both were able to recover 92% of the test organisms. At 44.5°C,

neither filter was able to recover more than 40% of the test organisms. Temperature acclimation was therefore, a very important consideration in culturing chlorine-injured cells. Hufham (1974) and Lin (1976) demonstrated the adverse, detrimental effects of using a 44.5°C incubation temperature. A pre-enrichment incubation temperature of 35°C was therefore considered the temperature of choice for FC recovery using MF techniques (Lin, 1973).

2.4.1.3.1 Application of membrane filters to milk and other foods

An MF technique was shown to be useful in enumerating and isolating sublethally, heat injured bacteria (Goff et al., 1972). The procedure used broth cultures of a mixed flora obtained from milk incubated at 32°C. Goff et al. (1972) and Claydon (1975) reported that there were several problems associated with milk filtration; the fat content of homogenized whole milk was shown to be the biggest problem since it limited the quantity of milk that could be filtered through bacterial membrane filters (pore size 0.45 μm).

Little data on membrane filtration of foods, at levels equivalent to those used in plate count analyses exist. The most relevant published uses of MF are restricted to easily

filterable beverages such as wines, beers, sugar liquids (Moroz, 1957; Halden, 1957) and to a few dairy products such as milk, butter and ice-cream (Fifield et al., 1957; Busta and Speck, 1965; Graves et al., 1966; Goff et al., 1972; Claydon, 1975). The membrane filtration of wines, beers and sugar liquids for microbiological analysis would appear to present little problem. The American Public Health Association Compendium (Speck, 1976) recommends microbiological analysis by filtration for foods which "can be dissolved and passed through a bacteriological filter, without, however, offering any examples of suitable subjects."

The m-FC procedure for coliforms is authenticated in standard methods for the examination of dairy products (A.P.H.A., 1978), and by a membrane filter apparatus manufacturer (Application Data Manual - 10, Millipore Filter Corporation, 1964).

Modifications of MF technique for use with milk (Busta and Speck, 1965) include the use of Triton as a surfactant. Another modification whereby the milk sample to be filtered was added to warm (45 to 50^{o}C) surfactant solution already in the filter, was described by Fifield et al. in 1957. The use of surfactant filtering aids, however,

was generally shown to be unsatisfactory since many of these acids inhibited the growth of heat-injured bacteria (Maxey, 1970). Claydon (1975) applied vacuum and further dilution of the milk (1:100 dilution) with sterile distilled water, but observed that the milk sample was still difficult to filter. Problems associated with membrane porosity and the use of higher decimal dilutions ($>10^3$) did not favour the technique.

Improvements in filterability resulting from the dilution of the milk sample with phosphate buffer have been claimed (Graves and Schipper, 1966; Goff et al., 1972) and supported by Sharpe et al. (1979). Changes in degree of disperal, dissolution and conformation of tissue components etc., are important factors that contributed to improvement. It is also possible that changes in ionic strength, pH, etc., might also be significant, and should be investigated if difficulties in filtering are encountered (Geldreich, 1978).

Factors affecting the MF of food suspensionswere studied (Sharpe et al., 1979). Lot number within a brand, pore size (0.45 or 0.8 μm), and time elapsed before filtration had little effect on filterability. Types of

MF, flow direction, pressure differential, age (microbiological quality) of food had significant and often predictable effects. Preparation of suspensions by stomacher (relative to rotary blender) addition of surfactant at elevated temperatures, and prior incubation with proteases enhanced food filterability.

It is apparent that combinations of the use of elevated temperature, suitable surfactants and incubation with proteases are capable of improving filterability of foods, according to whether fat, protein or starch particles are the principle cause of filter pore blocking. Thus, if filtration difficulties are encountered, and if the organisms are relatively tolerant, an array of procedural modifications is available to provide the desired level of filterability (Geldreich, 1970).

Apart from the cited examples, the microbiological applications of MF appear to be limited to the analysis of waters and sterilization of fluids (Sharpe et al., 1979). The absence of publications pertinent to MF of foods may be due in part to: (i) The numerical operating range of the ubiquitous 47 μM diameter MF pad which is so small (eg., lower and upper counting limits of 20 and 80 colonies, respectively) that many discs would be required per analysis

to accommodate the range of counts found in most foods; (ii) Most microbiologists tend to dismiss food suspensions as being generally unfilterable (Sharpe et al., 1979).

2.4.1.3.2 The hydrophobic grid membrane filter (HGMF)

At high microbial concentrations, the apparent discreteness of countable colonies using the MF and plate count methods decreases largely due to the increased probability of colony growth overlap. With spreading organisms, the limit might occur below 300 colonies, and confluence might make an estimate of the number of cells impossible (Sharpe and Michaud, 1975). The HGMF procedure of Sharpe and Michaud (1975) had the effect of separating colonies and allowing growth in an orderly array, thus making counting easier and more accurate, and in addition, reduced the need of diluting the samples to be analyzed.

The HGMF has great attractions in the automation of enumerative microbiology because of its potential ability to simplify the engineering requirements for diluting and counting, and to eliminate the likelihood of false,low counts at high levels of contamination (Sharpe and Michaud, 1975). The HGMF has been shown to have a numerical operating range far greater than any conventional membrane filter.

Very high colony-packing densities were achieved ca. $1.61 \times 10^5/cm^2$, making it possible to enumerate up to 9×10^4 organisms per filter.

Of all the enumeration methods thus far evaluated, HGMF has the ability to provide linear colony recoveries at levels as high as 9×10^4 colony forming units (CFU) per filter (Sharpe et al., 1979).

2.4.1.3.3 MPN - MF comparisons

No satisfactory coliform differential pour plate medium is available which can be adapted to water samples and used in a comparative study of MF media. Thus, MPN - MF comparisons are generally used even though the MPN method is not entirely satisfactory for such quantitative comparisons with the high bias inherent in the mathematics of MPNs (Thomas et al., 1956). There is a need to recognize the limitations of the MPN tube method, and to be aware of the possible error in use, particularly in MPN - MF comparisons. An example is seen in the paper of Morgan et al. (1965) in which confirmed coliforms not completed MPN data were used as the base line for evaluation of the Endo-MF one step and the two-step MF procedures. It has been observed by Geldreich et al. (1976) that when any evaluation of MPN or MF data were to be made, and the MPN

tube results were to be used as a base line for comparison, the confirmed MPN test would not suffice unless previous tests had indicated close correlation between the confirmed and completed coliform tests. Comparison of results of confirmed and completed coliform tests have demonstrated that coliform numbers can differ significantly, and that the difference is related to the bacterial flora and age of the sample, and effective suppression by the brilliant green dye and bile salts in the confirmatory medium. Geldreich et al. (1976) therefore concluded that any evaluation of MF data based on these improbable MPN values is questionable.

Also, failure to be aware of the broad confidence limits inherent in the MPN calculations had at times, led to conclusions from MPN - MF comparisons that the MF did not detect all of the coliform organisms present in a sample. The MF count is based on an "actual count" of cells, whereas the MPN procedure yields a "statistical estimate" of the most probable number of organisms in a given amount of water or food (Geldreich et al., 1976). The 95% confidence limits of the five-tube three-dilution MPN test ranges between 31 and 289% of the true bacterial density of a sample.

2.4.1.4 Rapid methods of faecal coliform determination

The need for rapid and reliable methods in the determination of coliform and FC densities is an important aspect of the continuing search for methods of improvement of bacterial quality of water and foods (Andrews and Presnell, 1972). Various methods that have accelerated bacteriological assessment of sanitary quality of foods have been described (Donnelly et al., 1960). For example, Mackenzie et al. (1948) suggested a rapid test for water analysis which consisted of a MacConkey presumptive test at 37°C followed by a confirmatory BGLB and peptone broth (for indole determinations) both at 44°C. E. coli density could be estimated within 48 hours using this method.

Mossel (1962), in the examination of human milk and ice cream, utilized BGLB at 44°C for both primary and secondary tests supplemented with a test in tryptone-water at 44°C. Other rapid procedures used to determine FC densities were described by Fishbein et al. (1967); Hefferman and Caballi, (1967); Andrews and Presnell, (1972); and Francis et al. (1974). In the rapid 7 hour plate count method of FC enumeration in food, developed by Francis et al. (1974), an agar pour-plate medium was used. These

researchers found that after 7 hours of incubation at 41.5 $\pm$ 0.05^{o}C the formulated medium used effectively allowed the growth of only FC organisims.

A single instance of a rapid E. coli technique directly related to milk was recorded (Moran and Witter, 1976). Their method was based on the use of a Technicon Auto Analyzer II system which assayed for the presence of glutamate decarboxylase, an enzyme which is found in E. coli and a few other bacterial species that are highly unlikely to be found in milk. The procedure yielded results in 8 hours, but required 10 hours of incubation for the analysis to be completed. The MPN technique was combined with the automated system to quantitate E. coli in milk.

Despite the numerous attempts that have been made to improve the time-consuming MPN procedure, no single, really rapid method has yet been devised.

2.5 The β-D-Galactosidase Assay

Recently, Warren et al. (1978) reported a rapid colorimetric β-D-galactosidase assay method for the enumeration of faecal coliforms in water. The assay was based upon the enzymatic hydrolysis of the synthetic substrate, o-nitrophenyl-β-D-galactoside (ONPG) by FC. The technique, as reported by these authors, provided an estimate of FC density in water within 8 - 20 hours and represented a departure from most recent, rapid, FC enumeration methods, because of the simplicity, low cost, accuracy and relative specificity of the test.

The method, as reported by these authors, makes use of the chromogenic substrate, ONPG, in conjunction with an incubation temperature of 44.5°C, which is a requirement of the FC test. The test also makes use of the selective EC medium and takes advantage of the active biosynthesis of β-D-galactosidase by E. coli (Feniksova et al., 1968). This assay was based partially on a modification of a procedure first reported by Eijkman (1904). The author observed that coliform bacteria of faecal origin could produce gas, via their formic hydrogenlyase system, in a glucose-based medium if incubated at 46°C, whereas

coliforms of non-faecal origin were rarely able to do so under the same conditions.

Warren et al., (1978) found that the time required between the start of incubation of the test and the half-maximum absorbance of ONP was proportional to the concentration of FC present in the water sample. High cell densities produced an immediate response, whereas low cell densities, 1 cell/ml, produced a response in less than 20 hours.

2.5.1 General characteristics of β-D-galactosidase

β-D-Galactosidase, or β-D-galactoside-galactohydrolase, commonly referred to as lactase has been classified under the Enzyme Commission number 3.2.1.23 (Enzyme Nomenclature Recommendations, 1973). The enzyme, β-D-galactosidase, catalyses the hydrolysis of β-D-galactosides including lactose and its derivatives in the following manner:

β-D-galactoside + H_2O -----> galactose + alcohol

2.5.2 Occurrence of β-D-Galactosidase

β-D-Galactosidase, obtainable from various plant and animal sources, has been studied widely both in its soluble (Lederberg, 1950; Rickenberg, 1959; Anderson and Rickenberg, 1960) and in immobilized forms (Dahlqvist et al., 1973;

Okos and Stanley, 1973; Giacin et al., 1973). Microbial sources of the enzyme include: Mycobacteria spp, Aspergillus oryzae, Kluyveromyces fragilis, Coliforms, Fabospora fragilis and other yeast, fungal and bacterial sources (Feniksova et al., 1968; Tikhomivora et al., 1974).

Since biochemical tests for the fermentation of lactose play an important role in the diagnostic bacteriology of the Enterobacteriaceae, (Bulow, 1964), the occurrence of β-D-galactosidase in Gram-negative rods has been investigated extensively (LeMinor and Hamida, 1962; Wilson et al., 1971). Enzymatic activity has been found in several hundred strains of Enterobacteriaceae, Pseudomonadaceae, Parvobacteriaceae and Neisseriaceae (Bulow, 1964). Among the Gram-positive bacteria, Diplococcus pneumoniae, Streptococcus lactis, Bacillus megaterium, B. subtilis and several Propionibacteria spp have been shown to synthesize β-D-galactosidase.

The enzyme has also been shown to occur in the intestine of animals, particularly those which suckle their young (Reed, 1975).

Milk, however, has not been shown to contain lactase,

although the presence of a lactase-synthesizing enzyme in milk has been reported (Babad and Hassid, 1964).

2.5.3 General properties of β-D-galactosidase

β-D-Galactosidase enzymes differ in several characteristics, such as pH and temperature optima, stability and activity, depending on the source of their isolation (Reed, 1975). For example, yeast lactose from Kluyveromyces fragilis with a pH optimum of 6.5 is suitable for applications involving milk products. Fungal lactase from Aspergillus spp. with pH optimum of 4.5 - 5.5 is close to the pH of bread dough and is useful in this industry (Morisi et al., 1972; Reed, 1975).

The in vitro pH optima for the β-D-galactosidase of several strains of Citrobacter are slightly lower than 7.0 (Pickettand Goodman, 1966); and that of E. coli is pH 7.2 to 7.7 (Lederberg, 1950). In some cases, the temperature of growth and assay incubation temperature may be a critical factor in the synthesis and activity of β-D-galactosidase. For example, Anderson and Rickenberg (1960), found progressively increasing enzyme activity in two strains of Paracolobactrum aerogenoides grown on lactose at 37, 30 and 19°C. Bulow (1964) mentioned three strains of Hafnia which

produced β-D-galactosidase when grown at 22°C, but not at 35°C. Thus, β-D-galactosidase and its formation, may be temperature-sensitive. Pickett and Goodman (1966) confirmed these possibilities by growing Citrobacter both at 26°C and 35°C. Marr et al. (1964) also studied the effect of temperature on the growth and formation of β-D-galactosidase in E. coli.

Cohn and Monod (1951) showed that the β-D-galactosidase of E. coli, A. aerogenes and Shigella sonnei were indistinguishable from one another both kinetically and serologically, but the lactase from K. fragilis and Lactobacillus bulgaricus differed on the basis of these criteria from the E. coli enzyme. Landman (1957) showed that the β-D-galactosidase produced by B. megaterium was similar to the E. coli enzyme in its catalytic properties, but differed from B. megaterium with respect to its stability and serology.

2.5.4 Characteristics of β-D-galactosidase from E. coli, K - 12

Research performed by Cohn and Monod (1951) has established that most of the β-D-galactosidase preparations from various bacteria belonging to the Enterobacteriaceae

have identical kinetic properties, although their serological properties may vary. Warren et al. (1978) showed from results of preliminary studies, that ONPG hydrolysis by mixed faecal coliform cultures proceeded at rates similar to those of E. coli K - 12 using standardized inocula.

The β-D-galactosidase from E. coli, K - 12, isolated and characterized by Craven et al. (1965) and Steers et al. (1965), after an extensive study by Lederberg (1950), was shown to have a molecular weight of 518,000 at pH 7.6 (Weber et al., 1964); 540,000 at pH 7.5 (Craven et al., 1965) and 595,000 (Goldberg, 1969). Erickson (1970), however, demonstrated discrepancies in previous studies concerning the enzyme's molecular weight. His work suggested a monomer weight of 91,000.

Inhibitors and activators of the enzyme have been studied by Shifrin et al. (1970), and Moses and Sharp (1970), respectively. The enzyme is protected against heat-inactivation by 5-phosphorylribose 1-phophosphate in the presence of β-mercaptoethanol. In the absence of mg^{+2} ions, β-mercaptoethanol causes dissociation (Shifrin et al., 1970). Rickenberg (1959) evaluated the effects of metal ions and whey proteins on the activity of

β-D-galactosidase. The enzyme was found to be activated by mono and divalent cations (manganese, magnesium, potassium, and cobalt); low whey protein concentrations of 100 μg/ml lead to extremely rapid inactivation. The pH optimum for E. coli β-D-galactosidase was shown to be between 7.2 and 7.7, with an Isoelectric point (IEP) at 4.61 (Lederberg, 1950; Wallenfels and Weil, 1972).

Wondolowski and Woychik (1974) observed no loss in activity of the enzyme after 90 days storage in buffer at 4°C. The β-D-galactosidase enzyme was shown to retain full activity after 30 minutes incubation at temperatures up to 50°C. A 10% loss in enzyme activity was observed at 55°C, while complete enzyme inactivation resulted from incubation at temperatures above 60°C.

A review of substrate requirements was given by Wallenfels and Malhorta (1960). The β-D-galactosidase specifically hydrolyses terminal β-D-galactosidic bonds of several β-D-galactosides, such as lactose and other oligosaccharides, glycoproteins and glycolipids. The specificity of β-D-galactosidase is shown by the strict requirement of β-D-galactoside for the glycon part of the molecule (Johnston and Pivnick, 1970). The rate of

hydrolysis of this enzyme, however, is strongly influenced by the aglycon part of the substrate molecule. Minor structural changes of either part of the galactoside molecule can drastically alter its activity as an inducer or inhibitor of induction, or even make it inert with respect to induction (Johnston and Thatcher, 1967). It has been established that the nature of the substrate composition used to culture this organism, specifically the carbon source, could influence the amounts of β-D-galactosidase contained within the cellular protein fraction (Tikhomirova et al., 1972). Normal E. coli strains, for example, have been shown to contain about 6% β-D-galactosidase in their total protein fraction, however, certain hyper strains of E. coli have been reported to contain as much as 24% (Horiuchi et al., 1961).

2.5.5 Constitutive and adaptive enzymes

Ever since attention was drawn to the difference between "constitutive" and "adaptive" or "inducible" enzymes, it has become more evident that substrate induction of enzymes plays a very important and prominent role in bacteria. It has been shown (Deere et al., 1939; Lowe, 1960; Rickenberg, 1960) that β-D-galactosidase could

be present in bacteria even if they were unable to ferment lactose or were late lactose fermentors, and that non-lactose fermentors were often more or less impermeable for lactose. Induction of the enzyme was not shown to occur unless the substrate was transferred to the interior of the cell (Johnston and Pivnick, 1970).

Many workers have observed that the lactase activity of E. coli cells was dependent on their prior exposure to lactose, preferably under conditions of growth, that is, the lactase of E. coli is an adaptive enzyme. Adaptation to lactose has been regarded as a substrate-induced synthesis of enzyme protein, although small amounts of the enzyme might persist in unadapted cells (Monod, 1947; Lederberg, 1950).

2.5.6 Effect of lysis on β-D-galactosidase activity

Deere (1939), observed that lytic treatments of unadapted E. coli cells activated their lactase activity to a marked extent. He concluded that adaptation might be comparable to lytic activation viz., as some sort of permeability effect. Studies by Rickenberg et al. (1956) and Rotman (1964) have revealed the presence of a galactoside-permease system in E. coli. This enzyme system, which is

specifically induced, is responsible for the transportation of β-D-galactosides through the cell membrane. The possibility of liberating the enzyme from the cell by disintegration, instead of establishing direct contact between the β-D-galactosidase enzyme and the substrate has been investigated (Lederberg, 1950). This author found that the β-D-galactosidase activity of E. coli cells was greatly influenced by toluene treatment.

During his studies on the β-D-galactosidase of E. coli strain K-12 using a chromogenic substrate, Lederberg (1950) found discrepancies of 10 to 47-fold between the activity of intact and disrupted cells or cell extracts. In addition, the author realized that the activity of the intact cells was protected from pH changes and inhibitory cations. Such large differences in enzymatic activity were also found by Rotman (1964). Lacking a better explanantion, it has been assumed frequently that the penetration of the substrate molecule is the rate-limiting step in intact cells.

Similarly, Rickenberg (1960) was able to demonstrate in several members of the genus Shigella, a higher enzymatic activity after toluene treatment than before. On the other hand, it is known that toluene labile β-D-galactosidase

exists (Anderson and Rickenberg, 1960).

Addition of toluene to a suspension of intact cells has been shown to both periodically promote the hydrolysis of ONPG (Lederberg, 1950; Rickenberg, 1960), and inactivate β-D-galactosidase (Anderson and Rickenberg, 1960). These authors found that the yellow colour (representing a positive ONPG reaction) was often more intense if toluene had been used in their enzyme recovery processes, and indicated that the cell membranes of the bacteria did not always permit unrestricted passage of ONPG from the environment to the interior of the cell.

These findings have helped to explain the necessity of performing the ONPG test as a routine bacteriological test to include an enzyme-inducing substrate in the culture medium, and why attempts have been made to enhance autolysis by addition of toluene to cell suspensions (Bulow, 1964).

2.5.7 Applications of β-D-galactosidase

Lederberg (1950) noted that the glycolysis of lactose by E. coli was of interest in the study of biochemical problems associated with disaccharide utilization, and was also a point of attack for studies investigating the genetic basis of enzyme constitution. In these studies, E. coli,

especially K-12 strains, were used because of their suitability for genetic recombination analysis. One of the greatest achievements of molecular genetics, the general concept of operon structure, expression and regulation, is based largely on the work concerning the lac-operon of E. coli (Lederberg, 1947; Wallenfels and Weil, 1972). In the food industry, a variety of β-D-galactosidases have been investigated as to their potential application in the utilization of cheese whey lactose (Woychik and Wondolowski, 1973; Olson and Stanley, 1973; Shukla, 1975). The enzymes primarily used and investigated were of fungal (Woychik and Wondolowski, 1973; Olson et al., 1973) and yeast (Wendroff et al., 1971) sources, although some efforts have been made with bacterial galactosidases (Sharp et al., 1969) such as selected E. coli strains (Wallenfels and Weil, 1972).

Other interests of the enzyme are centered on analytical uses in food chemistry, clinical biochemistry, microbiology and pharmaceutical industries. Lactose has a calorific value and aids in calcium assimilation. These advantages are, however, offset by three main nutritional and technological problems it creates, viz: - lactose

intolerance, lactose crystallization and whey utilization (Hood, 1971). These problems have determined the application of β-D-galactosidase in various industries, especially in branches of the food industry which use processed milk products (Okos et al., 1978; Holsinger, 1978). The use of β-D-galactosidase in dairy products yield these desired chemical and physical properties: - reduced lactose content, prevention of lactose crystallization, increased solubility, increased sweetness, more universally fermentable sugars, increased level of reducing sugars. The enzyme may therefore, be used for digestive upsets due to indigestibility of milk products (Holsinger, 1978).

2.5.8 Assay methods for β-D-galactosidase

The enzyme, β-D-galactosidase hydrolyses the β-D-galactoside bond in both the natural substrate lactose, and the synthetic substrate, ONPG (Cohn and Monod, 1951). The rate of hydrolysis of β-D-galactosides such as lactose, by β-D-galactosidase can be followed by measuring the liberation of either the glycon or aglycon portion. Thus, glucose formed from hydrolysis of lactose is determined specifically with the help of glucose oxidase (notatin), in a gasometric method (Keilin and Hartree, 1948). The

liberation of galactose as a basis of hydrolase assay is permissible only under conditions where no transfer to suitable acceptors occurs (Cohn and Monod, 1951). Most β-D-galactosidase assay procedures are based on the determination of the aglycon portion (Lederberg, 1950).

2.5.8.1 Quantitative β-D-galactosidase assay using ONPG as substrate

Synthetic substrates are used for a series of hydrolases because the natural substrates are often not precisely definable or because the products obtained by the action of the enzymes on synthetic substrates are easier to measure. The choice of substrate is of decisive importance, since synthetic substrates are hydrolyzed at different rates (Bergmeyer, 1978). Lederberg (1950), reported that a variety of physical, chemical and biochemical methods have been used to measure and monitor β-D-galactosidase activity, but that none of the assay methods was totally satisfactory, particularly for intact cells that glycolyse the hydrolyzed products. The most common substrates for assaying β-D-galactosidase today, are synthetic chromogenic galactosides, of which the first to be used was p-nitrophenyl β-D-galactoside (Huggins and

Smith, 1947). The successful application of chromogenic substrates for enzyme assay suggested that a chromogenic galactoside might be used for assaying galactosidases. Accordingly, orthronitrophenyl β-D-galactoside (ONPG) was prepared and was found to be suitable, sensitive and convenient, and therefore, generally accepted (Seidman and Link, 1950). The intact glycoside, ONPG, has negligible optical density at visible wavelengths. On the other hand, free o-nitrophenol (ONP) is capable of a tautomeric change that produces a yellow colour in alkaline solutions with an absorption peak at 420 nm. ONP is a weak acid, with a pK value of 7.3, and the acid, benzoic tautomer is practically colourless. The assay must therefore, be determined in well-buffered solutions, in which a fixed portion of the ONP is dissociated (and coloured) or in alkaline solutions, pH 10 or higher, in which a negligible fraction remains undissociated (and colourless). At pH 7.25 in the presence of 0.14 M Na^+, the hydrolysis proceeds with maximum velocity (Rickenberg, 1959; Hill and Huber, 1971). The low dilution at which ONP can be accurately measured permits the assay of extremely dilute extracts equivalent to 10 μg or fewer cells in 10 ml. The advantage of using ONPG as a substrate for lactase

hydrolysis is based on the yellow breakdown product, o-nitrophenol (ONP) which is liberated by the action of β-D-galactosidase on the β-D-galactoside bond. Thus, by using ONPG to demonstrate β-D-galactosidase activity, it is possible to see and measure directly the activity of the enzyme (Bulow, 1964).

ONPG stock solutions have been shown to be somewhat unstable as evidenced by weak yellow colourations developing after storage for some time at 4°C (Bulow, 1964). It has been observed that various agents, such as traces of zinc, or contamination with bacteria containing β-D-galactosidase can promote this initial hydrolysis.

2.5.8.2 Qualitative β-D-galactosidase assay using ONPG as substrate

Ortho-nitrophenyl-β-D-galactoside (ONPG) is a colourless, synthetic chromogenic substrate composed of one molecule of ortho-nitrophenol and one molecule of galactose, which are joined together by a β-D-galactoside bond (Bulow, 1964). The chromogen was first prepared by Seidman and Link (1950) and used by Lederberg (1950). Since its discovery, ONPG has been used in a wide variety of biochemical and bacteriological investigations by many

researchers. ONPG, for example, was used by Le Minor and Hamida (1962) in the diagnostic bacteriology of Enterobacteriaceae. Mollaret and Le Minor (1962) used the chromogen as an aid in the differentiation of the genus Pasteurella. Other workers, Lowe (1962), Szturm-Rubinsten (1962), Leelere (1962), used ONPG in similar bacteriological investigations involving the Enterobacteriaceae. These authors found that all the lactose fermentors in this family were ONPG positive, while the non-lactose fermentors were ONPG negative. The qualitative ONPG sensitivity test is therefore, used in diagnostic bacteriology (Bulow, 1964).

3. MATERIALS AND METHODS

3.1 Bacterial Cultures

Escherichia coli K-12 (209), Klebsiella pneumoniae (111) Citrobacter freundii (320) and Enterobacter cloacae (121) were obtained from the Culture Collection, Department of Microbiology, University of Manitoba, for use in this study.

3.2 Culture Maintenance and Purity

E. coli K-12 was routinely maintained on nutrient agar (Difco, NA) slants stored at 4°C. The slant cultures were periodically streaked on Eosin Methylene Blue Agar (Difco, EMB) plates incubated for 18 hours at 37°C in order to check their purity, then restreaked on NA slants and incubated at 37°C for 18 hours. This same procedure was followed for the remaining cultures used throughout this investigation. In addition, the purity of K. pneumoniae, C. freundii and Enterobacter cloacae was appraised using violet red bile agar (Difco, VRB), gram staining (BBL Manual, 1973) and IMViC typing (A.P.H.A., 1966). All bacteriological media used throughout this investigation

were formularized and reconstituted according to the manufacturer's directions, unless otherwise specified, and sterilized for 15 minutes at 121°C using a Barnstead autoclave.

3.3 Cell Propagation

Escherichia coli K-12 cultures were routinely propagated in 1-litre Erlenmeyer flasks containing 500 ml EC medium using a New Brunswick shaker incubator (175 rpm) at 35°C to 37°C, for 10 - 12 hours unless otherwise stated. The pH of the medium was adjusted to 6.8 - 6.9 with 1N HCL. prior to sterilization. Cell propagation was initiated by transferring a loopful of NA grown culture to the EC medium.

3.4 Cell Harvest

Unless otherwise stated, cells of E. coli K-12 were harvested in their logarithmic phase of growth after approximately 10 - 12 hours of incubation at 37°C. Samples were centrifuged at 7,500 x g in a Sorval RC2B centrifuge at 1°C for 20 minutes. The supernatant was discarded and the resultant bacterial pellet was washed twice with sterile 0.85% saline and finally resuspended in 200 ml

sterile 0.85% saline. This suspension consituted the stock of E. coli K-12 cells. The viable cell concentration of this stock solution ranged between 10^7 to 10^9 cells/ml. In those preliminary studies which investigated the effects of pH, temperature and growth phase as a function of enzyme activity, the harvested cells were washed twice in 200 ml of cold Sorensen's phosphate buffer, pH 7.0. Centrifigation was carried out at 14,000 x g for 10 minutes at $1^{\circ}C$. The washed cells were finally resuspended in 25 ml of Sorensen's phosphate buffer pH 7.0.

3.5 Preparation of Toluene-Treated Cells

Toluene treated E. coli K-12 cells were prepared according to the method of Citti et al. (1964). Four ml volumes of stock cell suspension, contained within a 25 ml Erlenmeyer flask were treated with 0.2 ml toluene-acetone (1:9) solution, and incubated for 5 minutes at $25^{\circ}C$ with vigourous agitation. After incubation, 1.0 ml aliquots of the toluene-treated E. coli K-12 cells were analyzed for β-D-galactosidase activity according to the method outlined by Citti et al. (1964).

3.6 Growth and β-D-galactosidase Activity of E. coli K-12 Grown in EC Medium

Two percent (v/v; ca 4×10^6 / ml) inocula of saline-suspended, mid log stock E. coli K-12 cells were inoculated into 1-litre Erlenmeyer flasks containing 500 ml EC medium, pH 6.8. The flasks were incubated at 37°C on a shaker operating at 175 rpm for 28 hours. At 2 hour regular intervals, 0.1 ml volumes of the resultant growth were removed and analyzed for total plate counts (VRB) and β-D-galactosidase activity (Ikura and Horikoshi, 1979).

3.7 Assay of β-D-galactosidase Activity

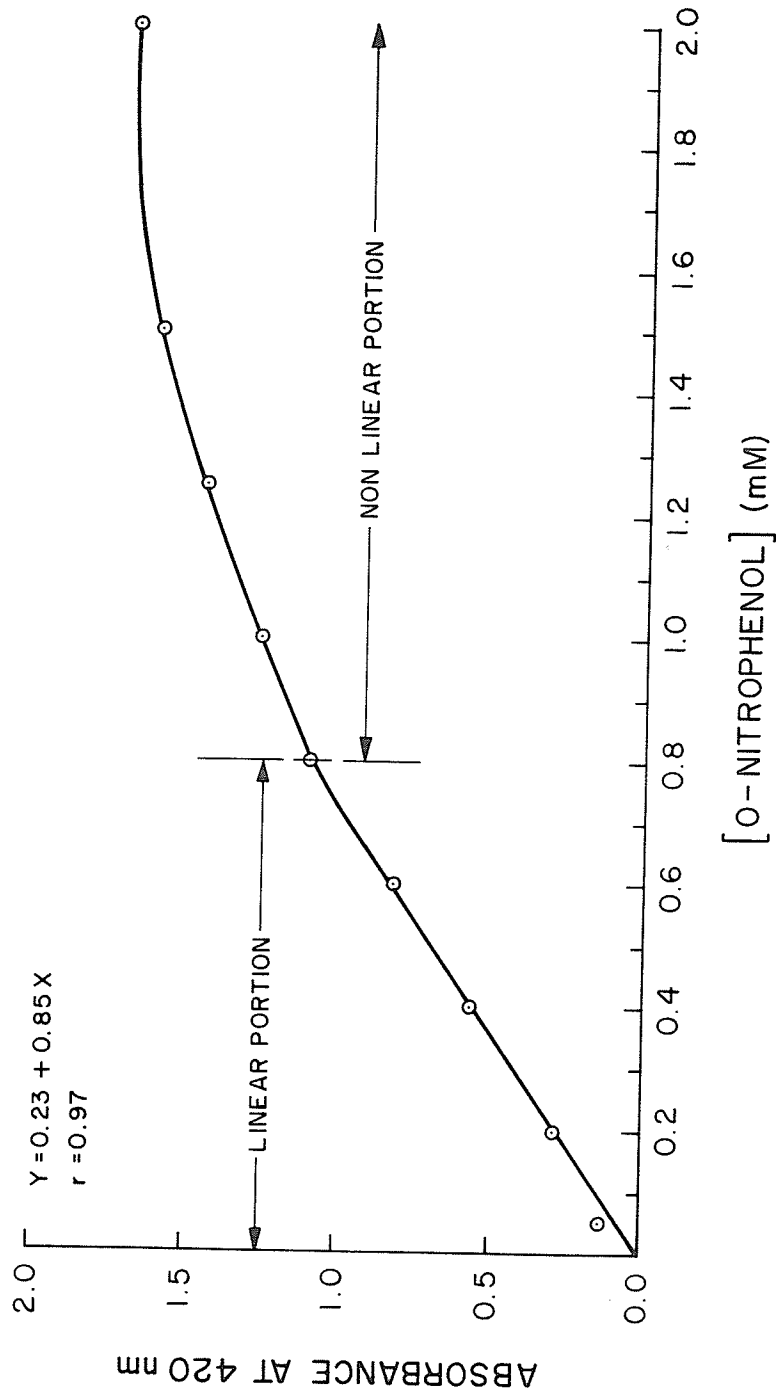
The method of Citti et al. (1964) was followed for the assay of β-D-galactosidase activity. One ml volumes of whole cell suspensions or toluene-treated cell suspensions were incubated with 4.0 ml of 0.005M ONPG solution for 5 minutes. The 15 minute time period used by Citti et al. (1964) was not used in these investigations, since earlier studies showed complete hydrolysis of ONPG after 10 minutes of assay. Unless otherwise stated, an assay incubation temperature of 37°C was used. The colour development was terminated by the addition of 5.0 ml of 0.5M Na_2CO_3. Cells

were centrifuged at 9,000 x g for 12 minutes at 1°C. Absorbancy of the resulting supernatant was measured at 420 nM. The corresponding amount of o-nitrophenol liberated were determined from a standard curve prepared by measuring the change in absorbancy produced by varying concentrations of o-nitrophenol (Sigma, ONP) at 420 nM (Figure 1).

A second method outlined by Ikura and Horikoshi (1979) was also used to determine β-D-galactosidase activity. This procedure had the advantage that microquantities (0.1 ml) of cell suspension could be withdrawn from the reaction mixture with negligible effect on the remaining reaction mixture. Smaller assay volumes, 0.4 ml were used in the standard reaction mixture, containing 0.1 ml, 10m M ONPG; 0.2 ml, 0.1 M Sorensen's buffer pH 6.5; 0.1 ml stock cell suspension. Incubation was carried out at 37°C for 5 minutes. The reaction was terminated by the addition of 0.2 ml 1M Na_2CO_3 solution, and subsequently diluted with 3.0 ml of distilled water. Absorbance was measured at 420 nM. Enzyme activity in both procedures was expressed as mkatal; where 1 katal (kat) = moles of ONP liberated/second. Therefore, mkatal (mkat) = mmoles/sec.

Figure 1

Standard Curve for β-D-Galactosidase Activity Using ONPG as Substrate



3.3 Coliform Determination

3.8.1 Coliform enumeration

FC enumeration was performed using a three-tube MPN technique (A.P.H.A., 1974). Presumptive coliforms, confirmed coliforms and faecal coliforms were determined using LST, BGLB and EC medium, respectively. Presumptive and confirmatory coliform tests were incubated at 37°C for 24 - 48 hours. Confirmed coliforms were incubated at 44.5°C (± 0.2°C) for 24 - 48 hours. Test tubes showing gas production in EC medium were recorded as positive for FC and their densities were computed using standard MPN tables (A.P.H.A., 1974).

3.8.2 E. coli confirmation

Inocula from gas positive cultures of EC broth were streaked on Levine EMB plates and incubated at 37°C for 18 - 24 hours. After incubation, typical, discrete E. coli colonies exhibiting a green metallic sheen and showing a nucleated center were picked from the EMB plates and subcultured in lactose broth (Difco, LB) tubes for 18 - 24 hours at 37°C.

If a plate showed no typical E. coli colonies, a representative atypical colony was chosen and subcultured

under the same conditions in LB. After incubation, the LB tubes were examined for gas production and the partial identification of the E. coli K-12 cells and other isolates was made on the basis of IMViC patterns. Further identification of both typical and atypical colonies was performed by ONPG sensitivity tests.

3.8.3 ONPG sensitivity tests

ONPG sensitivity tests as outlined by Bulow (1964) were used to further identify coliform isolates by testing for their β-D-galactosidase activity, qualitatively.

Loopfuls of the culture to be tested were suspended in 0.25 ml sterile distilled water to which 0.25 ml of 0.25 M ONPG reagent was added. The test tubes were then incubated at 37°C and analyzed every 30 minute, 60 minute and 240 minute intervals. A final reading was taken after 24 hours of incubation. The intensity of the resultant yellow colourations was visually ranked, viz, 1+ (weak ONPG hydrolysis) to 4+ (strong ONPG hydrolysis).

3.9 β-D-Galactosidase Activity of E. Coli K-12 as a Function of Growth Medium pH

Cultures of E. coli K-12 were initially grown in EC

media adjusted to final pH values of 6.0, 6.4, 6.8, 7.2, 7.6, 8.0, 8.4 and 9.2 prior to sterilization by the addition of 1N HCL or 1N NaOH.

Aliquots of the resultant growth from each pH growth media were adjusted with Sorensen's phosphate buffer pH 7.0. One ml aliquots of the standardized stock cell suspensions were then each assayed for their β-D-galactosidase activity by the method of Ikura and Horikoshi (1979).

3.10 Effect of Assay Incubation Temperature on the β-D-galactosidase Activity of E. coli K-12

One ml aliquots of washed E. coli cell suspensions containing approximately 3.0×10^8 cells/ml were assayed for β-D-galactosidase activity using the procedure of Citti et al. (1964). Assay incubation temperatures of 37, 40, 45, 50, 55, 60, 70 and 80°C were used. The influence of the assay incubation temperature on the β-D-galactosidase activity was determined after 10 minute exposures to the respective temperatures (ibid).

3.11 The Enumeration of E. coli K-12 Using the Colorimetric β-D-galactosidase Assay

3.11.1 Preparation of EC-ONPG reagent

O-nitrophenol β-D-galactopyranoside (ONPG) Sigma Chemical Corp., St. Louis, was prepared using Sorensen's phosphate buffer, pH 7.0, as the diluent. Unless otherwise stated, the stock reagent had a molarity of 0.022 M and was routinely sterilized by Millipore filtration (0.22 μm) (Millipore Corp., Bedford, Mass., U.S.A.). For the assay of β-D-galactosidase activity, the ONPG reagent was combined aseptically with rehydrated EC medium (24.67 g/ L) in a 1:1 volume ratio. The resultant pH of the reaction substrate was approximately 6.8 - 7.0. The reaction substrate was then aseptically distributed into previously sterilized 150 x 15 mm test tubes, to contain a final volume of 9.0 mls, unless otherwise specified.

3.11.2 Assay procedure for saline-suspended E. coli K-12 cells

The enumeration of E. coli K-12 using the colorimetric β-D-galactosidase assay was followed as outlined by Warren et al. (1978). One ml aliquots of saline stock E. coli K-12 cells were used as the inocula. The initial cell density of the inocula was determined by the MPN technique. The inoculated reaction substrate was incubated at 44.5°C

($\pm$ 1.0°C) for varying lengths of time. Rates of colour production as a result of ONPG hydrolysis were determined in duplicate by periodically removing culture tubes from the incubator. The enzyme reactions were terminated with 3.0 ml of 1M Na_2CO_3. The absorbance was read at 420 nM in a Bausch and Lomb Spectromic 710 spectrophotometer. An uninoculated test tube containing EC-ONPG medium incubated at 44.5°C for a period equal to that of the inoculated medium was used as the control. Any spontaneous ONPG hydrolysis occurring in the control was similarly terminated with 3.0 ml of 1M Na_2CO_3. This same procedure was repeated using varying cell concentrations, viz 10^2 - 10^9.

3.11.3 Assay procedures for milk-suspended E. coli

Twenty ml samples of fresh, 2% pasteurized milk obtained from the Commercial Dairy Section, Food Science Department, were distributed into sterile 1-litre Erlenmeyer flasks. Varying initial inocula of E. coli K-12 (5% v/v) were then inoculated into the milk samples. The final concentrations of the inoculated milk samples were determined by the MPN technique (A.P.H.A., 1974). The final inoculum levels in the milk samples ranged

from 10^1 to 10^9 cells/ml. An uninoculated sample of 2% pasteurized milk, boiled for 5 minutes, served as the control.

A series of EC-ONPG tubes prepared for each known concentration of milk-suspended E. coli cells were then inoculated in triplicate, with 1.0 ml of the respective milk samples. The fourth tube in each series was inoculated with 1.0 ml of the boiled, uninoculated milk sample. All EC-ONPG tubes, including the control were subsequently incubated at 44.5°C and observed for ONPG hydrolysis. The incubation was terminated with 3.0 ml of Na_2CO_3 and the length of incubation recorded when the absorbance reading was visually judged to be approximately at the half maximum absorbance of 0.7.

The reaction mixture was then filtered through a coarse Whatman No. 4 filter in order to remove any resulting precipitate. The filtrate was then membrane filtered using a Millipore 0.22μM filter. The resulting filtrate was analyzed for actual ONPG hydrolysis (Warren et al., 1978). The absorbance readings obtained from the various cell concentration filtrates were located on the standard curve (Appendix 1), and the difference between them and the half-

maximum absorbance was noted. This difference could be translated on the X axis to a time correction factor such that all values could be compared to that time required to reach half-maximum absorbance. The corrected time required to reach half-maximum absorbance was then used to compute the FC density from another standard curve relating FC density to time required to achieve half-maximum absorbancy. The actual FC densities of the milk samples enumerated via the MPN method, were then compared to the FC densities obtained through half-maximum absorbancy computations.

3.12 The Effects of Na_2CO_3 and Trichloroacetic Acid on the Termination of ONPG Reaction in Milk Samples

Nine ml volumes of 2% pasteurized milk, contained in sterilized 50-ml centrifuge tubes were each inoculated with a 1.0 ml stock suspension of mid-log grown *E. coli* K-12 cells. The centrifuge tubes were vigorously mixed and the concentration of the *E. coli* was determined by plate counting on VRB at 37°C for 24 hours (A.P.H.A., 1974). Ten-ml volumes of ONPG were then added to each tube to give a final concentration of 0.022 M ONPG. The tubes were

incubated at 44.5°C for 4 hours. After incubation the reactions were terminated by use of various combinations of either 1M Na_2CO_3 and/or 5% trichloracetic acid (TCA) 1.0, 2.0, 3.0. 4,0. 5.0 and 6.0 mls of 1M Na_2CO_3 in one series, and 3:1, 2:2, 1:3, 0:4 ml combinations of 1M Na_2CO_3 and 5% TCA, respectively, in a second series. After the reaction mixtures were terminated, the pH of each sample was recorded and the contents centrifuged at 20,000 x g for 20 minutes at 1°C. The resultant supernatants were then analyzed for ONPG hydrolysis (Warren et al., 1978). Control tubes consisting of 9 mls of uninoculated 5 minute boiled milk samples were set up in the same fashion as the inoculated tubes, and similarly analyzed. All tests were performed in triplicate.

3.13 Application of the Colorimetric β-D-galactosidase Assay to an Agar Diffusion Plate Method

3.13.1 Media composition

The colorimetric β-D-galactosidase assay method for coliforms was extended for use in an agar diffusion plate technique by the incorporation of ONPG into an EC basal

agar medium composed of (g/l):24.67, dehydrated EC medium; 15.0 agar (Bacto). ONPG reagent was prepared as outlined (Section 3.11.1). The final pH of the medium was 6.8 and is henceforth referred to as EC-ONPG agar medium.

3.13.2 Preparation of assay plates

The agar diffusion plates were prepared by dispensing 10 ml aliquots of the EC basal agar medium into standard pyrex test tubes followed by sterilization. The sterilized basal medium was tempered (50°C) and 10 ml of filter sterilized ONPG reagent was then added. The final concentration of ONPG in each test tube was 0.022M. The test tubes were then gently mixed and each was aseptically poured into 150 x 25 mm petri plates. The depth of the agar bed was approximately 5 mm. The medium was allowed to set at room temperature on levelled bench tops.

Wells of 12 x 5 mm diameter were then prepared by means of a sterile, stainless steel 12 mm cork borers centrally located on the assay plate.

3.13.3 Assay plate inoculum

Prepared wells were inoculated with 0.5 ml aliquots of 12 hour old stock *E. coli* K-12 cells at 37°C, unless otherwise specified.

3.14 Effect of pH of EC-ONPG on the Agar Plate Assay

EC-ONPG assay plates were prepared as outlined (Section 3.13.2). The assay media, however, was adjusted to pH values of 6.8 (unadjusted) 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 with 1N NaOH prior to sterilization and also to 3.0, 4.0 and 5.0 with 1N HCL.

Following a 4 hour incubation period, the plates were removed and visually observed for zones of hydrolysis.

3.15 Effect of Incubation Temperature on the Agar Plate Assay

Prepared EC-ONPG agar plates (pH 8.0) were incubated at room temperature (ca. 22°C), 37°C and 44.5°C for 6 hours. At one hour intervals, zones of ONPG hydrolysis were measured to the nearest perceptible mm, through the center of both axis by means of precision calipers.

3.16 Effect of Agar Concentration on the Agar Plate Assay

Petri plates of EC-ONPG agar (pH 8.0) were prepared with agar concentrations of: (%) 1.25, 1.5, 1.75, 2.0 and 2.25.

The inoculated assay plates were monitored for increase in zone size after one hour regular intervals, up to six hours of incubation.

3.17 Statistical Analysis

The results obtained in these studies were analyzed by conducting the analysis of variance, and comparing the treatment means using Tukey's test (Snedecor and Cochran, 1967). Standard deviations, linear regressions and correlations were calculated where applicable according to Snedecor and Cochran (1967).

4. RESULTS AND DISCUSSION

4.1 Growth Curve of E. coli K-12 and Related β-D-galactosidase Activity

The growth curve and related β-D-galactosidase activity of E. coli K-12 when grown in an EC based medium, at 37°C for 28 hours, are shown in Figure 2.

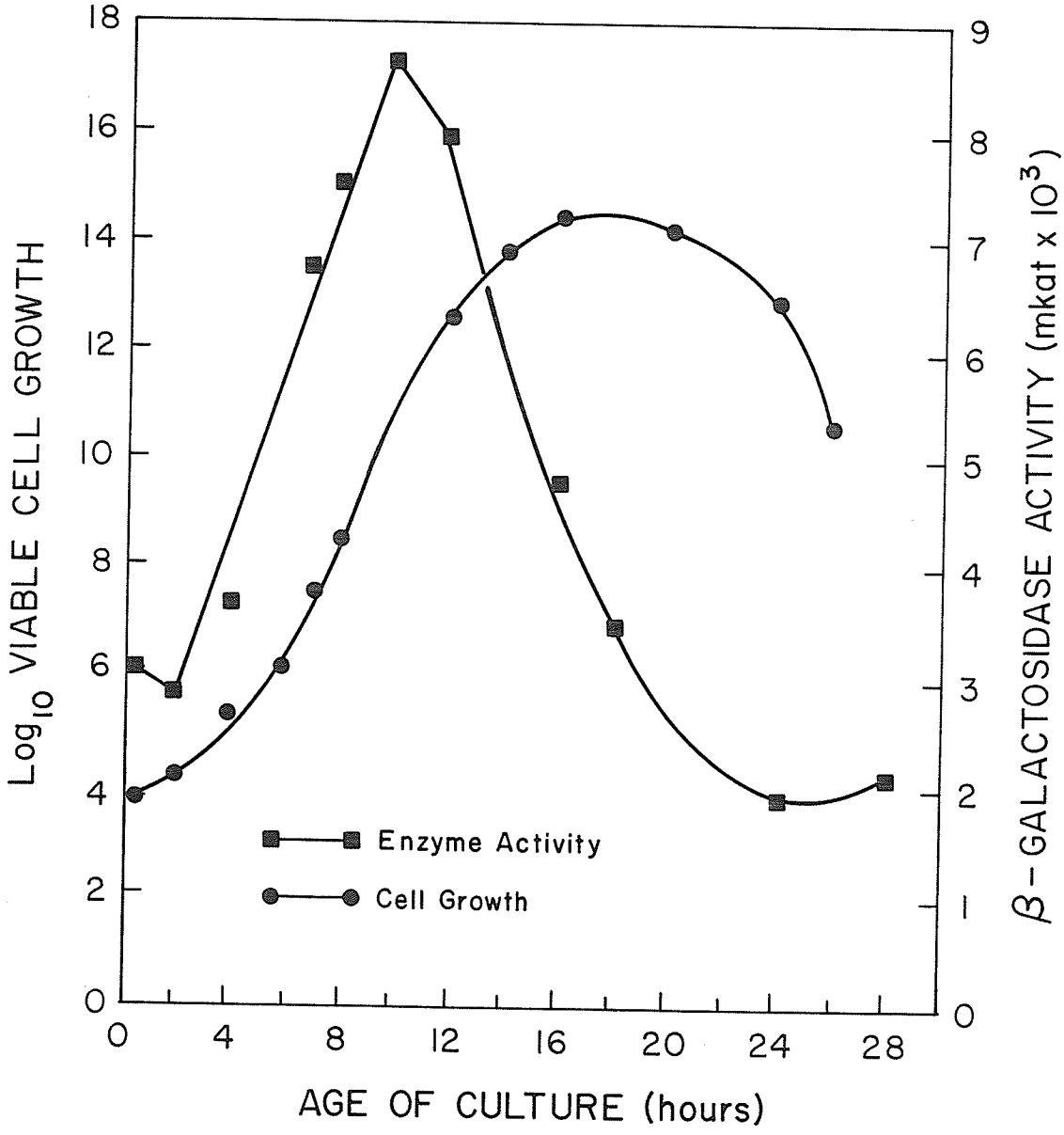
Maximum enzyme activity was shown to occur at approximately 10 hours of incubation (8750 mkat). The enzyme activity initially showed a decrease, then it progressively increased after the first two hours of growth.

Rotman (1955) showed that β-D-galactosidase activities were influenced by the age of the E. coli culture, since the duration of the growth period seemed to be of some importance for the synthesis of β-D-galactosidase, and as such, may relate to the fact that the process of enzyme induction does require some time under the conditions of growth. This explanation may account for the initial decrease in activity. Since the initial inoculum was transferred from nutrient agar slants, devoid of lactose, into a more selective lactose based medium, it is quite probable that the decrease in enzyme activity is attributable to a time factor required for β-D-galactosidase induction by the carbon source in the substrate.

Figure 2

Growth Curve of E. Coli K-12 and Related

β-D-Galactosidase Activity



Although the growth of the organism maintained a fairly logarithmic trend when maximum β-D-galactosidase activity occurred, it is not clearly understood why the enzyme activity decreased so sharply after 10 hours of cellular growth even though log growth was maintained up to 14 - 16 hours. Since lactose utilization is most rapid during the exponential phase of growth, the decreased β-D-galactosidase activity exhibited after 10 hours may reflect the de-accelerating portion of the log growth phase. In such an instance, enzyme levels would be expected to fall. Maximum growth (3.5×10^{14} cells/ml) occurred at approximately 16 hours, thereafter decreasing. The mid log growth phase of E. coli K-12 was evidenced to be between 10 - 12 hours. No well defined lag phase was observed. Although Warren et al. (1978) had reported the mid log growth phase of E. coli K-12 to be 12 hours when grown in the same medium, there was no indication by these authors of the corresponding enzyme activity of the cells.

Many workers (Anderson and Rickenberg, 1960; Warren et al., 1978), have recommended the use of E. coli cultures in their exponential growth phase for enzyme studies. Since the optimum enzyme activity of E. coli in these studies was

established to be approximately in the mid log phase, ca. 10 - 12 hours, all further studies were conducted with similarly grown cultures.

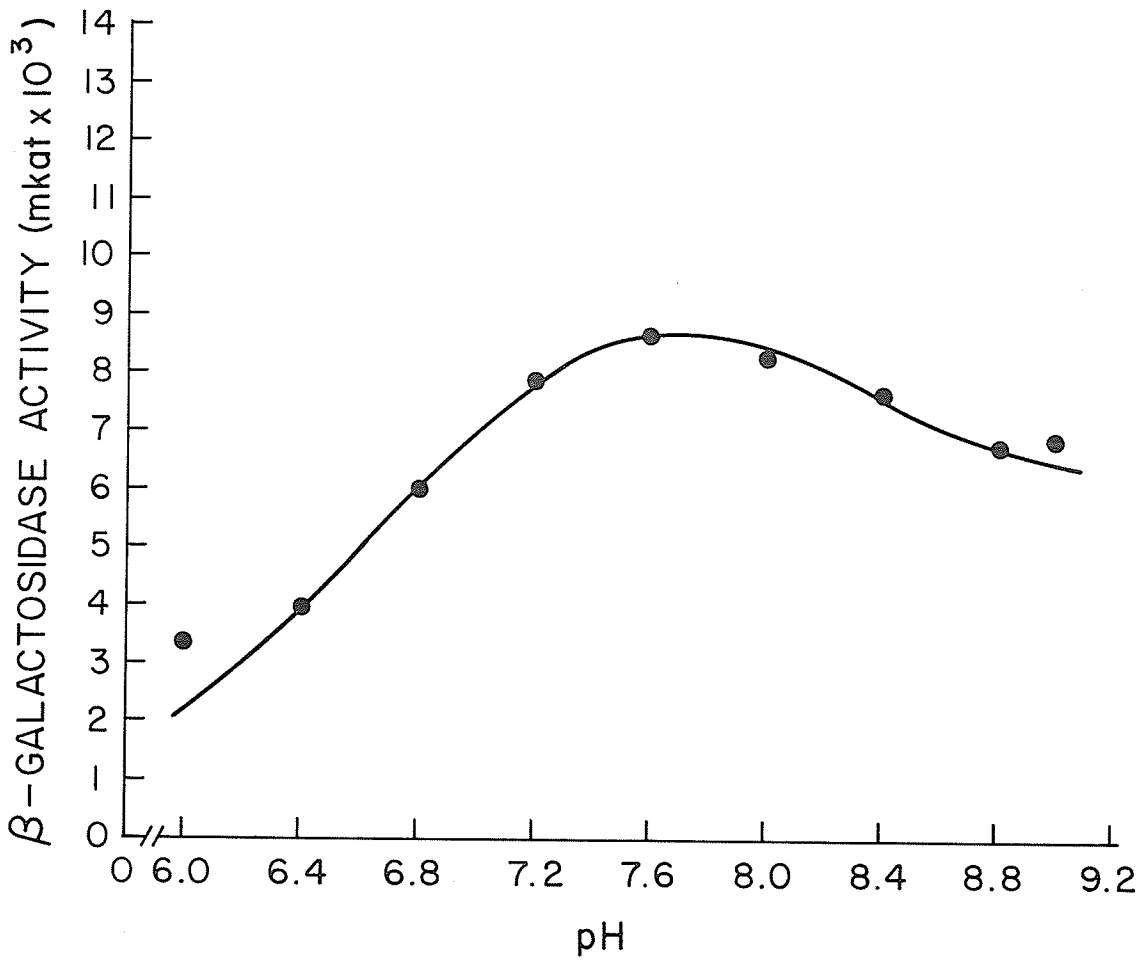
4.2 β-D-Galactosidase Activity of E. coli K-12 as a Function of Growth Media pH

Since the pH of EC medium, rehydrated according to the manufacturer's directions was 6.8, it was necessary to evaluate this pH value and others in order to determine a pH optimum for β-D-galactosidase activity. As such, a pH activity curve in the range of 6.0 to 9.2 was determined for the β-D-galactosidase assay of E. coli K-12 in EC medium at 37°C using ONPG as substrate (Figure 3). The pH optima for β-D-galactosidase activity of various E. coli strains has been reported at 7.2 to 7.7 (Lederberg, 1950; Wallenfels and Weil, 1972). Results from this study have confirmed this observation, however, a much broader range of 7.2 to 8.4 was observed. An optimum pH of 7.6 was established in these studies. In agreement with Lederberg's finding (1950), the flatness of the plateau around the optimum pH range was characteristic of intact cells whose response to the pH of the medium was usually much less abrupt as compared to enzyme extract, thus making an accurate

Figure 3

Effect of pH on β-D-Galactosidase Activity of

E. coli K-12



determination of the optimum pH rather difficult.

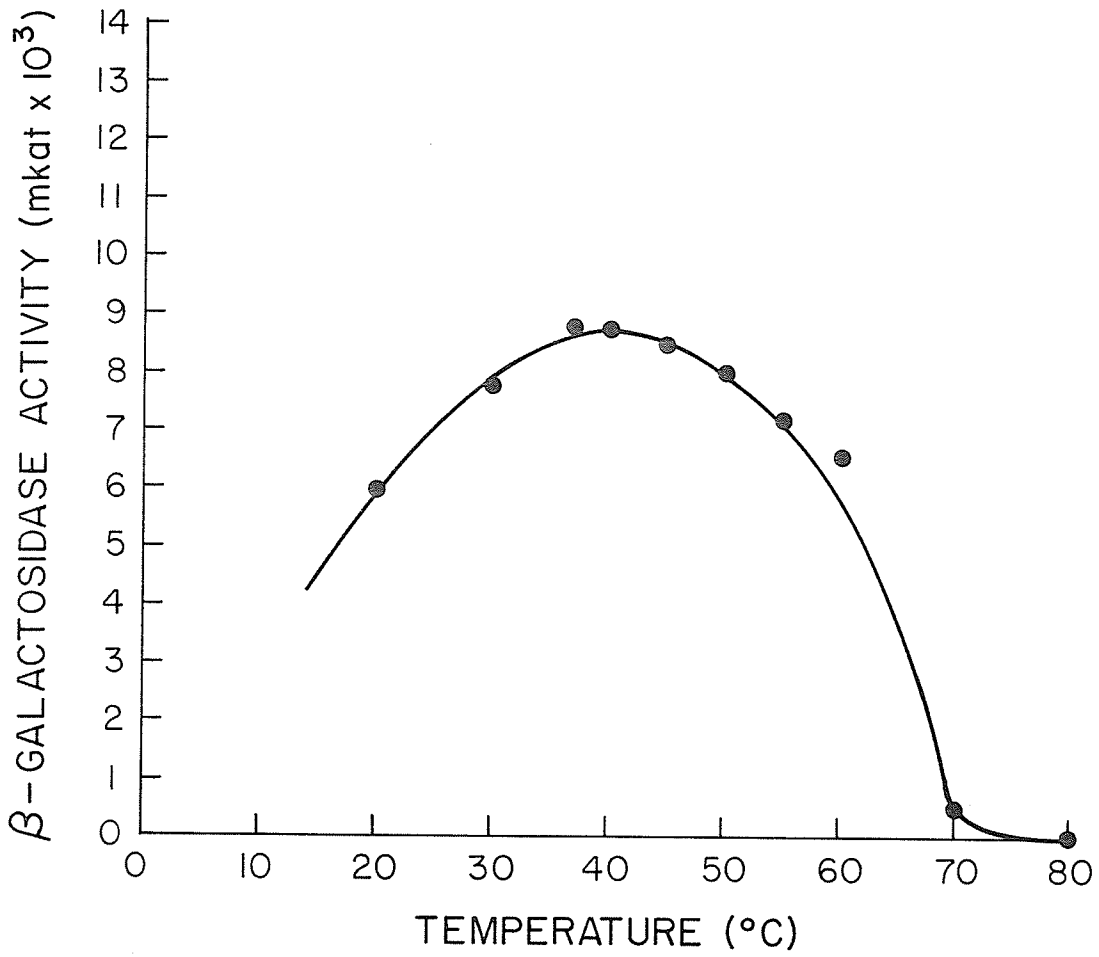
4.3 Effect of Assay Incubation Temperature on the β-D-galactosidase Activity of E. coli K-12

The effect of assay incubation temperature on enzyme activity is shown in (Figure 4). The enzyme activity of the culture was observed to increase with temperature increase up to a maximum of 40°C (8700 mkat). Further increase in temperature up to 60°C lead to a gradual decrease in activity. Exposure of the cells to temperature above 60°C decreased the activity sharply. Incubation at 70°C appreciably inactivated the enzyme, and enzyme activity at 80°C was negligible.

The synthesis and activity of β-D-galactosidase has been shown to be temperature-sensitive (Anderson and Rickenberg, 1960; Bulow, 1964). This temperature sensitivity suggested that some bacterial strains might yield more rapid ONPG hydrolysis if grown and assayed at lower temperatures. However, since it has been established that FC determinations at such low temperatures, 40°C, are relatively impractical (Eijkman, 1904; Wilson et al., 1935; Fishbein, 1962) it cannot be suggested that the ONPG test

Figure 4

The Effect of Assay Incubation Temperature on β-D-Galactosidase Activity of E. coli K-12



be performed below 40°C, if selectivity is to be maintained for FC organisms. Warren et al. (1978), observed significantly faster rates of ONPG hydrolysis at 43.5°C and proposed among other recommendations that it may be possible to gain a 10 to 15% decrease in the incubation time of the test procedure by lowering the temperature (1 to 2°C). Although optimum enzyme activity was obtained between 37.5 - 42.5°C, subsequent assays were performed at 44.5°C. This incubation temperature was preferred in this faecal coliform enumeration studies, since it gave fairly high activity as well as affording an opportunity to retain species specificity. However, a lower temperature within the range of 37.5° and 42.5°C is clearly more desirable if specificity is not reduced. This is also in agreement with the proposal of Warren et al. (1978).

4.4 Assay Procedure for Saline-Suspended E. coli K-12 Cells

Although numerous references on the qualitative uses of β-D-galactosidase for diagnostic bacteriology and the differentiation of the Enterobacteriaceae members exist (Bulow, 1964; Pickett and Goodman, 1966), there have been

no reports on the quantitative applications of the β-D-galactosidase assay relating enzyme activity with bacterial numbers. LeMinor and Hamida (1962) found a definite correlation between the density of the coliform cell suspension and the time lapse for a positive ONPG reaction. Until 1978, little attention was given to the relation between bacterial density and β-D-galactosidase activity. Warren et al. (1978) described a method for the enumeration of FC in water by a quantitative β-D-galactosidase assay.

To evaluate how the time of colour production was related to inoculum size, the response time for different cell concentrations of pure cultures of E. coli K-12 cells were studied. Theoretically, it has been hypothesized that the time required to achieve detectable ONPG hydrolysis is directly proportional to the quantity of FC present in the inoculum (Warren et al., 1978). The reaction mixture of EC-ONPG was that formulated by Warren et al. (1978). It consisted of EC medium with ONPG reagent. It is a well buffered medium that meets the requirement of β-D-galactosidase synthesis. In addition, the medium shows little colour interference with ONP, and is a good growth medium for E. coli (Warren et al., 1978).

The rates of ONPG hydrolysis of the different cell concentrations are shown in Figures 5 and 6. High concentrations of 10^7 to 10^9 cells/ml produced a direct linear relationship between the absorbance and time. At these high cell concentrations, spontaneous ONPG hydrolysis was evident, presumably because the inoculum was sufficient to permit immediate observable enzymatic hydrolysis. The reaction was completed within 20 minutes, 1.25 hours and 2.25 hours for cell concentrations of 10^9, 10^8 and 10^7 cells/ml, respectively. Relatively lower concentrations of 10^2 - 10^6 cells/ml gave a slightly different response. These curves became logarithmic and not sigmoidal as observed by Warren et al. (1978). However, an attempt was made to obtain a linear relationship between absorbance and time in order to accommodate the observed variations at these low cell concentrations. This relationship was first accomplished by plotting log absorbance 420 vs. time and comparing the results with those proposed by Warren et al. (1978) (Figure 6). The general trend (Figures 5 and 6) indicated that the higher the cell concentration, the more spontaneous the reaction. There was a progressive reduction

Figure 5

Rate of ONPG Hydrolysis by Different Inoculum Sizes of *E. coli* K-12 in EC-ONPG Medium at 44.5°C

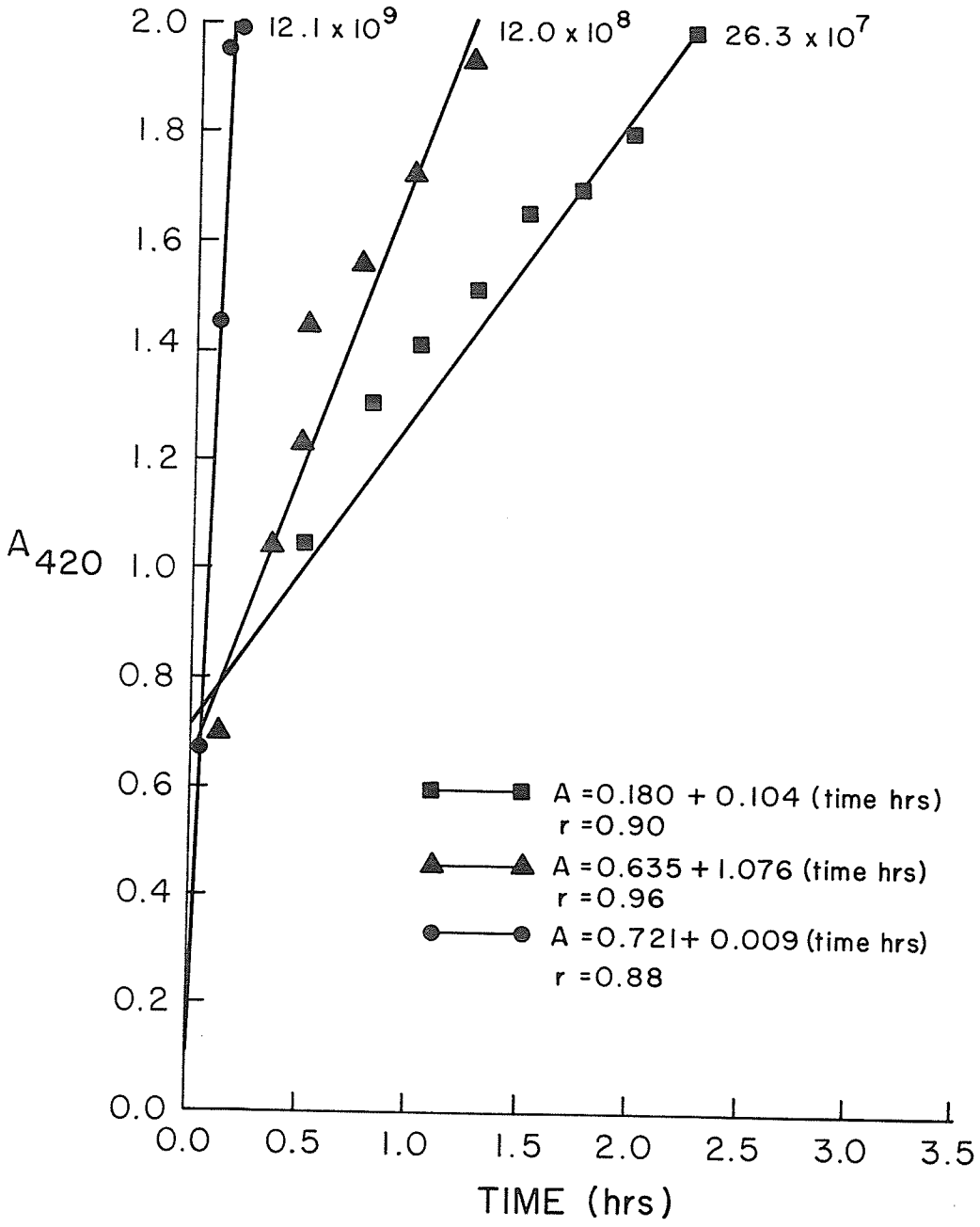
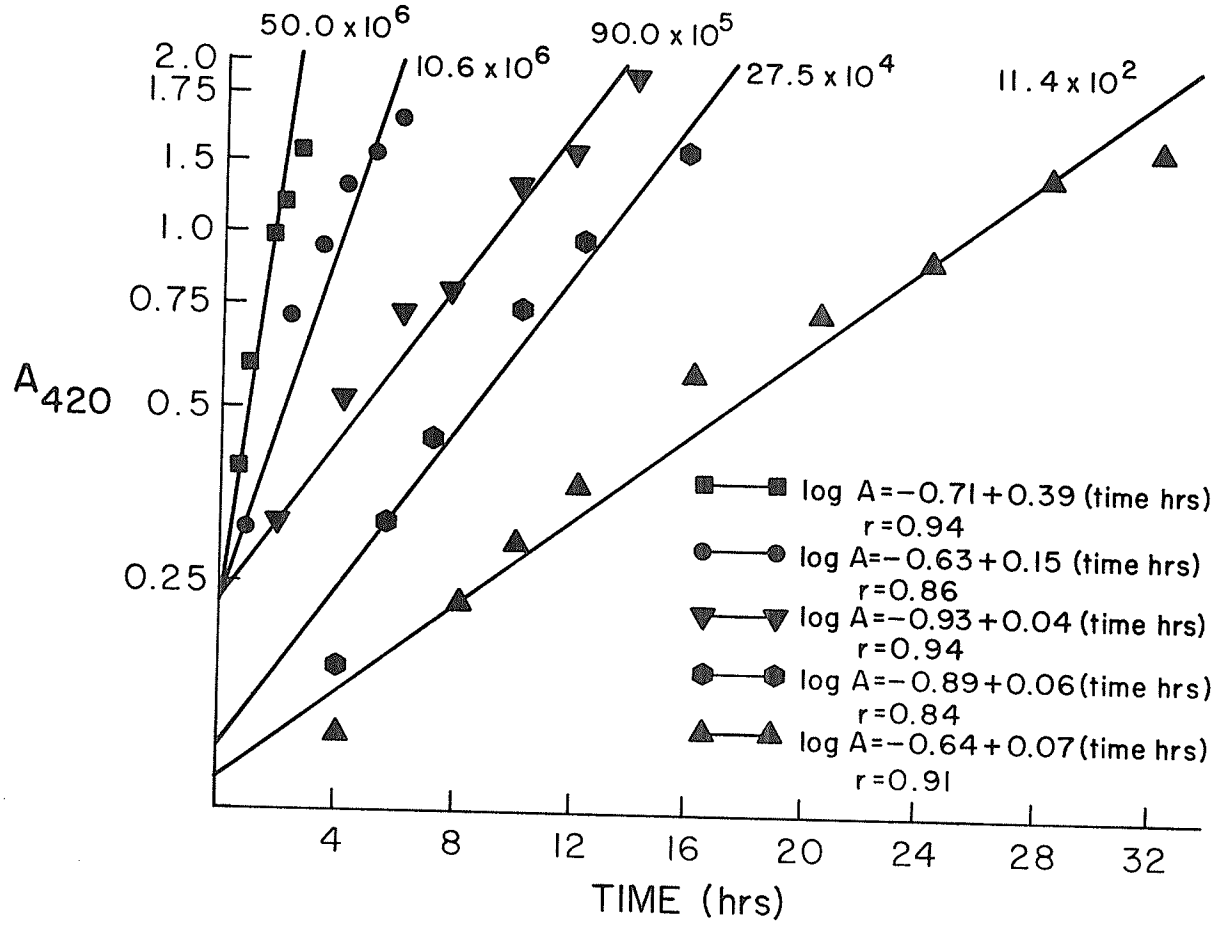


Figure 6

Rate of ONPG Hydrolysis by Different Inoculum Sizes of E. coli K-12 in EC-ONPG Medium at $44.5^{0}C$



in slope with decreasing cell densities. R-square values at 0.05 probability levels ranged from 0.84 to 0.96, indicating that log of absorbance reading significantly correlated with time. Results thus obtained in this study were similar to those of Warren *et al.* (1978) and it was found appropriate to use his linear equation, viz.

$$\text{Absorbance} = \frac{k}{1 + e^{a-bx}} = \frac{1.3312}{1 + e^{(2.24397 \; -0.03216 \text{ time (h)})}} \quad 1$$

Where $k = 1.3312$

$a = 2.24397$

$b = -0.03216$

x = time (hrs)

The above equation was rearranged to the form presented:

$$\ln \left(\frac{k}{A} - 1\right) = a - bx \quad 2$$

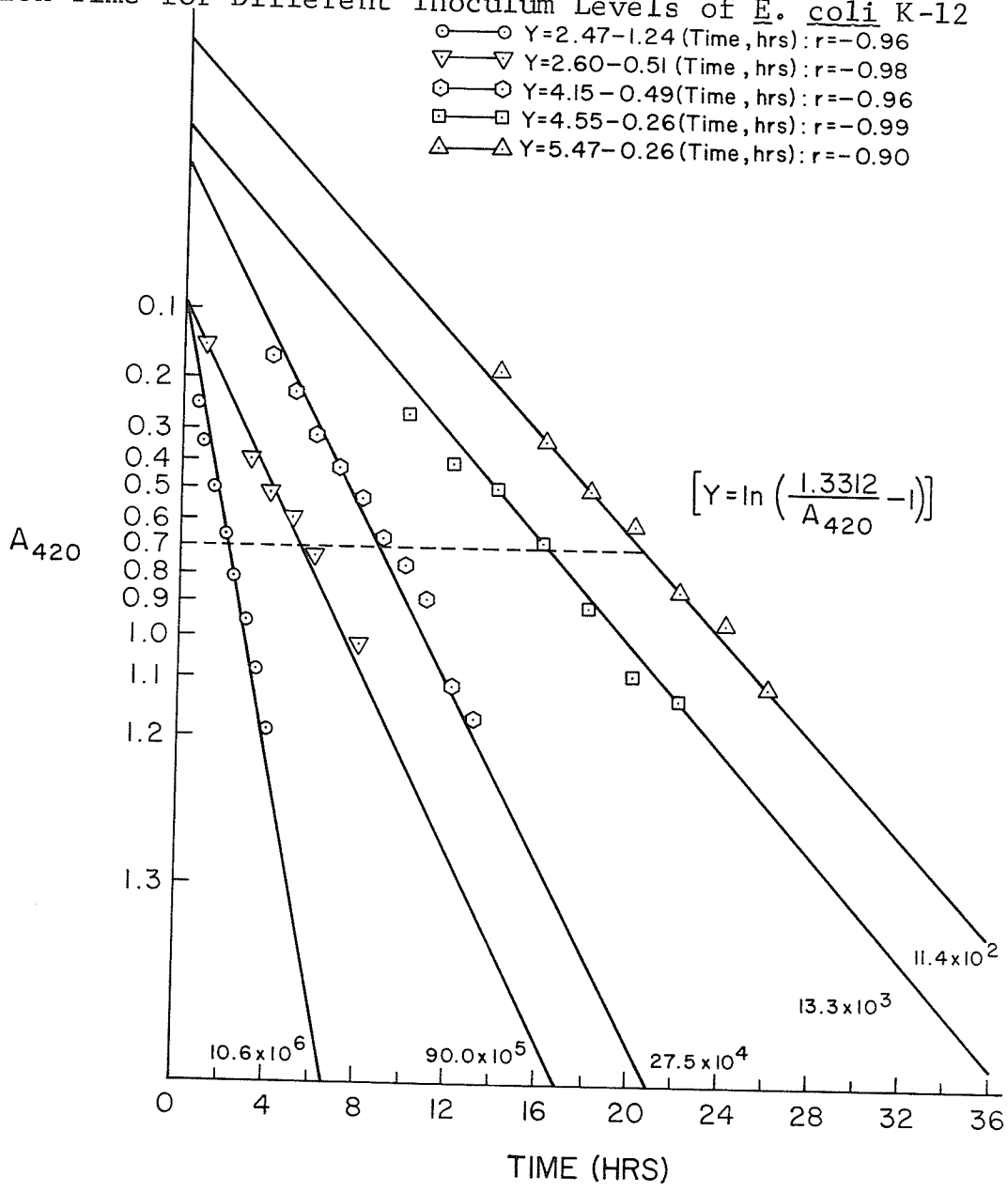
Where A = absorbance

x = time

Equation 2, which represents a linear relationship between absorbance and time was derived for use in this investigation, and as such, was applied to data obtained from saline-suspended cells at the inoculum levels of 10^2 - 10^6 cells/ml. The curves, shown in Figure 7, were generated using equation 2 and linear regression analysis (Snedecor and Cochran, 1967). For the range of inoculum

Figure 7

Linear Regressions Analysis Between Absorbance and Incubation Time for Different Inoculum Levels of E. coli K-12



levels tested, this latter method was found to be more appropriate in establishing a linear relationship, as indicated by the relatively higher R-square values of 0.96 to 0.99 (Figure 7), and was therefore accepted as the method for the analysis and interpretation of the results of all the studies relating FC numbers to time for half-maximum absorbance. Warren et al. (1978) selected a standard absorbance of 0.7, that is, using the fixed concentration (variable time) method (Guilbault,1973). Thus, the time required to reach this present absorbance would be inversely proportional to the concentration, and a plot of $\ln (\frac{k}{A} - 1)$ vs. time was linear (Figure 7). The general trend again indicated a reduction in slope with decreasing cell densities. However, the mean slope (-0.5536) was calculated from the curves in Figure 7 and used to obtain one linear calibration curve for time correction (Appendix 1).

The use of fixed concentration (variable time) method (Guilbault, 1973), indicated that this method can lend itself to automization. The time correction can also be computed using the mean slope (-0.5536) as follows:

Since $b = \frac{y_1 - y_2}{X_1 \; X_2}$ 3

Therefore rearranging: $\Delta X = \frac{y_1 - y_2}{b}$ 4

where $\Delta X = X_1 - X_2$

$$y_1 = \ln \left(\frac{k}{0.7} - 1\right) = -0.1035 = C \qquad 5$$

$$y_2 = \ln \left(\frac{k}{A} - 1\right) \quad (A = \text{measured absorbance})$$

Time corrected (i.e. time for 0.7 abs.)

$$= \text{time measured} + \frac{C - y_2}{b} \qquad 6$$

This equation (6) was preferred for use in calculating the time correction because it was found to be more accurate when estimating the cell density of E. coli inoculated, pasteurized milk samples. A half-maximum absorbance of 0.7 was selected as the standard to which all readings on the milk samples were corrected. However, since it was almost impossible to terminate the reaction at exactly 0.7 absorbance reading, during a manual operation of the assay, it was necessary to use the time correction factor based on the mean slope of -0.5536 (equation 6). This correction factor was used with the full awareness of the errors it could introduce, especially when testing samples with extreme cell densities (Table 1). This data shows the effect of the range of absorbancy reading on the accuracy of the method using two extreme inoculum levels, and for comparison, one intermediate

Table 1

Over - and Underestimation of *E. coli* Densities Inherent in the use of Time Correction for Given Absorbancies and Inoculum Levels (% Error)

A_{420} \ Inoculum Level/ml	10.6×10^6	27.5×10^4	11.4×10^2
0.5	+ 2.01	- 0.85	- 6.25
0.6	+ 1.3	- 0.85	- 4.7
0.8	- 2.68	+ 0.85	+ 6.25
0.9	- 2.74	+ 0.85	+10.77

Obtained corrected readings = 100%

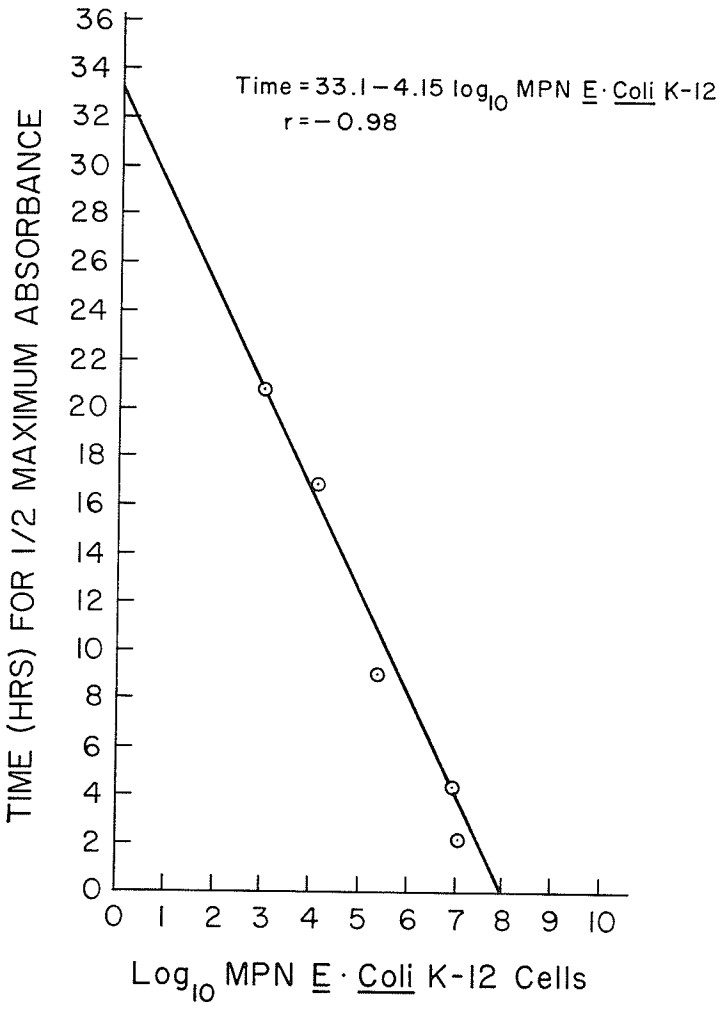
\+ Corrected values obtained for FC densities indicate underestimation, therefore, add the % error.

\- Corrected values obtained for FC densities indicate overestimation, therefore, substract the % error.

level. These values (Table 1) represent estimated error (that is, error introduced solely by using the mean slope in the time correction factor) regardless of inherent variability in a sample. For inoculum levels of approximately 10^4 cells/ml, a greater amount of variation was not introduced by varying the absorbance values within 0.7 ± 0.2. The error term, 0.85% was constant. The results in Table 1 indicated that high cell densities, ca. 10^6, would tend to overestimate at absorbance readings greater than 0.7, and vice versa for A420 less than 0.7. For lower cell densities, ca. 10^2 cells/ml, absorbancy readings greater than 0.7 would underestimate the faecal coliform numbers and vice versa for absorbance readings less than 0.7. However, if the validity of this method is limited to the inoculum levels of 10^2 to 10^6, and readings taken between 0.7 ± 0.2, one should expect to incur the errors illustrated in Table 1, up to a maximum of 10.77% at extremely low concentrations. These errors should be acceptable in the sensethat any methodology would contain an inherent weakness or fallacies which will over-or underestimate the specific organisms the test desired to enumerate. A curve relating the time required to achieve half-maximum absorbance (0.7) to log of

Figure 8

Regression Analysis of E. coli K-12 Density and Time Required to Achieve Half-maximum Absorbance



the cell densities is shown in Figure 8. As expected, a negative Pearson's r value of -0.98 was obtained which indicated that as cell numbers increased, time needed to reach half maximum ONPG hydrolysis decreased. This high correlation coefficient indicates a linear relationship between the two variables.

4.5 Assay Procedure for Milk-Suspended E. coli K-12 Cells

To evaluate the ONPG method in water, Warren et al. (1978) concentrated the FC cells by filtration, then preincubated the filters in EC medium at 37°C for 1 hour, before the addition of ONPG and subsequent incubation at 44.5°C. A modified medium and procedure were formulated after preliminary studies using pure cultures of E. coli K-12 inoculated into 2% pasteurized milk samples. Media composition and recoveries were adjusted to give maximum screening for faecal strains. As a result of these experiments, a suitable method for the performance of the ONPG test was devised for FC enumeration in milk.

To estimate the faecal coliform density of a given milk sample, the colour development of the inoculated EC-ONPG medium was stopped with Na_2CO_3, when the hydrolysis

was visually judged to be approximately 0.7. The method adapted was similar to that described for the pure culture studies. The only modification introduced was that after the reaction, the mixture was passed through a coarse Whatman #4 filter and then through a 0.22 μm Millipore filter in order to clarify the filtrate. The length of incubation was recorded, and the absorbance was measured and located on the standard curve (Appendix 1) and the difference between it and 0.7 absorbance was noted. This difference was translated on the axis to a time correction factor as recommended by Warren et al. (1978). Alternatively, the time correction was calculated using equation 6, such that all values could be compared to the time required to reach 0.7 absorbance. This correction would thus permit the investigator to check samples at set intervals, such as every hour, and still obtain consistent results.

4.5.1 Concentration of milk-suspended E. coli K-12 cells by membrane filtration

Attempts were made to concentrate the milk-suspended E. coli K-12 cells by membrane filtration using a 0.45 μM membrane filter. The method used was the same in principle as described by Goff et al. (1972). The original sample

was diluted (1:10) with warm sterile distilled water during filtration. This method was not satisfactory. Filtration was difficult because of blockage of the membrane pores by fat molecules which limited the quantity that could be filtered as observed by these authors. The use of higher decimal dilutions of 1:100 or 1:1000 eased the filtration process. The use of such high dilutions, however, was still not favourable since the limits of sensitivity of this assay technique would be grossly reduced, and a correction factor would have to be introduced to compensate for the dilution effect.

4.5.2 Concentration of milk-suspended E. coli K-12 cells by centrifugation

Another modification tried was centrifugation. Twenty ml portions of 2% pasteurized milk samples were pipetted into sterile centrifuge tubes. 1.0 ml of E. coli stock suspension were added to the milk. E. coli densities of these samples were determined on VRB. The inoculated milk samples were centrifuged at 20,000 x g for 20 minutes at 0°C. E. coli counts were then performed on the resulting supernatant and sediment in order to evaluate the distribution of cells and the effectiveness of the

process. The results of the distribution of E. coli after centrifugation (Appendix 2) indicated that more than 99% of the initial inoculum was sedimented along with a large protein fraction. To eliminate the protein precipitate, the cells were washed 2 times with 0.85% saline and recentrifuged. However, the time lapse between initial inoculation of the milk samples and the end of the centrifugation process (ca. 1.5 hours) allowed more growth as evidenced by higher plate counts after centrifugation (Appendix 2). Good recoveries were observed in the sediment. The resultant cell density in the supernatant was negligible, 0.06%, but the increased growth during centrifugation could not be ignored, and as such, concentration of the E. coli cells by centrifugation, prior to the assay, was not found to be desirable.

4.5.3 The effect of Na_2CO_3 and TCA on the termination of the ONPG reaction

Warren et al. (1978) used Na_2CO_3 to develop the full colour of the liberated o-nitrophenol and eliminate any turbidity caused by the bacterial cells in the medium. Although the sole use of Na_2CO_3 was found to be effective as a reaction terminator in experiments with saline-suspended

E. coli K-12 cells, the additional turbidity caused by the milk protein could not be eliminated by using Na_2CO_3 alone. It would have been ideal to terminate the enzyme reaction and remove the protein precipitate in one step, but this was not feasible in this study, thus subsequent coarse filtration and membrane filtration were used to further clarify the reaction mixture.

The data (Table 2) showed that termination of the hydrolysis reaction with 3 or 4 ml of Na_2CO_3 was more effective in developing the colour of ONP than any other combinations tried. The use of more than 5.0 ml Na_2CO_3 caused a slight dilution effect while volumes below 2.0 ml were not great enough to develop the full colour of ONPG. This was in accordance with the findings of Lederberg (1950). In the standard assay conditions specified by this author, the colour density determination was to be made at pH 10, that is, a range within which the phenolic group of ONP remains completely dissociated (pK = 7.3), and the colour density is maximum. It was therefore, recommended that the assay must be determined in well-buffered solutions, or in an alkaline pH, 10 or higher, in which a negligible fraction of the substrate remains undissociated, and colourless. The

Table 2

Effect of Na_2CO_3 and Trichloroacetic Acid on the Termination of ONPG Reaction

Volume of Terminator (ml) 1M Na_2CO_3	5% TCA	Total Volume (ml)	Final pH	Mean Absorbance at 420 nm
6	0	6	10.4	0.610
5	0	5	10.3	0.626
4	0	4	10.2	0.665*
3	0	3	10.1	0.638*
2	0	2	10.0	0.429
1	0	1	9.6	0.332
3	1	4	10.0	0.593
2	2	4	9.5	0.408
1	3	4	7.8	0.299
0	4	4	7.0	0.332

* Maximum values

use of 3.0 ml Na_2CO_3 for the termination of the reaction is therefore, justifiable since the final pH of the assay mixture was above 10.0, with a concomitant, effective development of the full colour of ONP. The use of TCA alone precipitated the protein but did not develop the full colour of ONP, presumably because of the reduction in pH.

4.5.4 Comparison of faecal coliform densities of milk estimated by the colourimetric assay and MPN techniques

Estimated times to reach half maximum absorbance and corresponding cell densities obtained on the basis of the standard curve (Figure 8) in this colourimetric assay and MPN techniques are compared in Table 3. As expected, estimated faecal coliform density using the rapid colourimetric assay and MPN techniques were not identical.

Figure 9 correlates faecal coliform densities obtained by the colourimetric assay and the MPN techniques. Each data point represents the average of triplicate readings. An R-square value of 0.93 was computed.

Warren et al. (1978) obtained an R-square value of 0.602 for water samples. The values that fell outside the

Table 3

Faecal Coliform Densities of Milk Estimated by the Colourimetric Assay and MPN Techniques

Milk Sample	A_{420}	Time Measured (hrs)	Time Corrected (for 0.7 Abs) (hrs)*	Estimated Faecal Coliform Density per ml	
				Colourimetric Assay	MPN Method
1	0.538	23.50	24.39	12.60×10^1	12.50×10^1
2	0.715	12.70	12.60	89.10×10^3	17.00×10^2
3	0.609	17.75	18.24	39.81×10^2	70.00×10^2
4	0.699	16.00	16.01	19.95×10^3	85.00×10^2
5	0.644	14.50	14.80	25.10×10^3	13.60×10^3
6	0.700	14.25	14.25	44.67×10^3	58.00×10^3
7	0.622	9.60	10.00	39.80×10^4	12.60×10^4
8	0.687	10.40	10.45	31.60×10^4	20.00×10^4
9	0.640	8.50	8.83	70.79×10^4	48.00×10^4
10	0.575	5.75	6.38	28.20×10^5	50.00×10^4
11	0.591	8.00	8.57	60.26×10^4	80.00×10^4
12	0.695	5.00	5.02	56.20×10^5	15.70×10^5
13	0.606	5.40	5.94	39.90×10^5	35.00×10^5
14	0.632	6.70	7.04	20.00×10^5	11.00×10^6
15	0.766	3.15	3.50	14.10×10^6	20.00×10^6
16	0.734	4.50	4.31	79.40×10^5	29.00×10^6
17	0.711	2.20	2.13	28.20×10^6	63.00×10^6
18	0.621	2.75	2.32	25.10×10^6	79.00×10^6
19	0.677	0.10	0.23	70.80×10^6	15.80×10^7
20	0.703	1.50	1.48	56.20×10^5	15.70×10^5

* Time corrected (time for 0.7 Abs) = Time measured + $\frac{C-y_2}{b}$

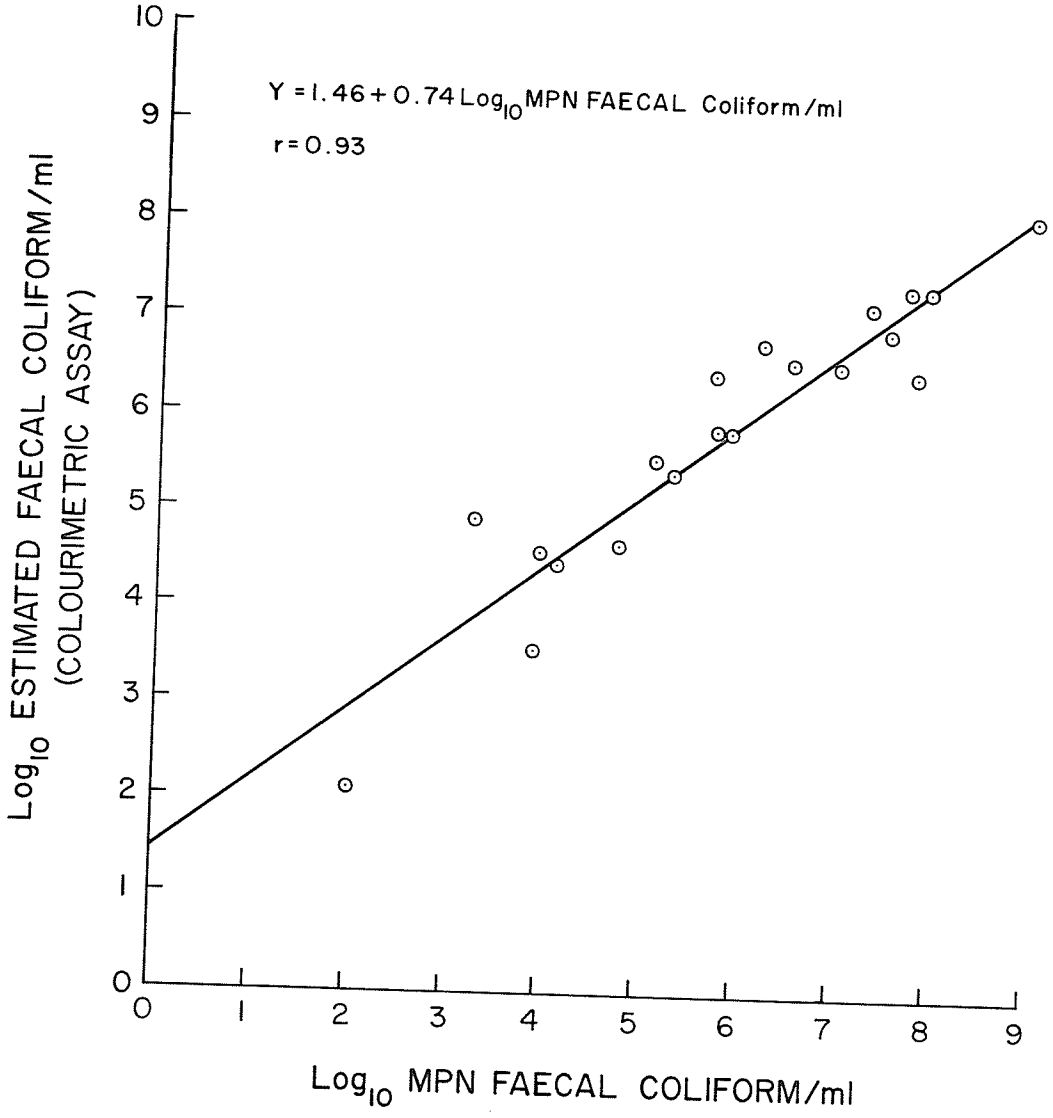
Where $C = \ln\left(\frac{1.3312}{0.7} - 1\right) = -0.1035$

$y_2 = \ln\left(\frac{1.3312}{A420} - 1\right)$

b = mean slope = -0.5536

Figure 9

Correlation Between the Faecal Coliform Density Estimated by the Colourimetric Assay and MPN Technique



95% confidence limits of regression line in their studies, reflected some of the differences in physiological conditions and genetic diversity within the group of bacteria that qualify as faecal coliforms. Results from the current investigation showed a marked improvement over the results of Warren et al. (1978). The improved correlation coefficient is primarily due to the use of standardized E. coli K-12 stocks, which do not exhibit much genetic variability as would be expected from more natural field samples. Despite this high correlation (Figure 9) extreme cell densities, particularly in the lower range, were less statistically reliable due to the increased variability. The results at lower cell densities (10^2 cells/ml) exhibited larger deviations from the regression line ($p < 0.05$) (Figure 9). These deviations are a measure of the failure of cell density to account for all the variation in time for half maximum absorbance. Thus, the findings in Table 1, which showed that the use of this colourimetric assay technique yielded more accurate results with cell densities of 10^4 to 10^6/ml was further substantiated. This is an important observation with respect to interpreting results of concentrations in excess of 10^7 and below 10^2 cells/ml during

the quantitative application of this assay. The safest range of densities within which one can operate without incurring excessive error (10.77%), seemed to be 10^2 - 10^6 cells/ml, bearing in mind that linear relations are a good approximation provided the values of the independent variable do not cover too wide a range.

4.6 Comparative Studies on Selected Members of Enterobacteriaceae

4.6.1 Temperature Sensitivity Tests for E. coli and Other Selected Members of Enterobacteriaceae

A salient goal in these studies was to develop an ONPG assay for detecting and enumerating faecal coliforms. The temperature sensitivity study was prompted by the proposal made by Warren et al. (1978), that the usefulness of the rapid colourimetric assay of FC enumeration was based on the premise that the test was relatively specific for E. coli and yet did not exclude bacteria that fit the accepted FC definitions. This specificity was ascertained by these authors when they isolated these members of Enterobacteriaceae in these proportions from water samples.

E. coli	96.69%
Enterobacter cloacae	2.32%
Citrobacter freundii	0.66%
Klebsiella pneumoniae	0.33%

To test this hypothesis, pure cultures of these selected strains were assayed for β-D-galactosidase activity at 37°C and 44.5°C, using the method of Citti et al. (1964). These results are presented in Table 4.

It was apparent that E. coli K-12 exhibited the highest enzyme activity at both temperatures (Table 4), however, the magnitude of the activity was not the same, it was higher at 37°C than 44.5°C. This marked decrease in activity, confirmed Warren et al. (1978) suggestion that lower temperatures were clearly more desirable if specificity was not reduced. Hydrolysis of ONPG by Citrobacter freundii was negligible at both temperatures. Data presented in Table 4 was in agreement with the findings of Geldreich (1967) and that of Warren et al. (1978) and therefore, confirmed the fact that the ONPG assay is specific for E. coli, yet does not exclude a significant portion of the FC population. Warren et al. (1978) also observed that other members of the Enterobacteriaceae such as Klebsiella spp. demonstrated luxuriant, rapid growth patterns similar to E. coli K-12, and also produced ONPG hydrolysis at temperatures up to 43°C but not at 44.5°C. This was also confirmed by the data in Table 4. The enzyme activity of

Table 4

Temperature Sensitivity of Selected Strains of Enterobacteriaceae

Organism	β-D-Galactosidase Activity (mkat) 37°C	44.5°C
E. coli K-12	2100.0	1060.0
Enterobacter cloacae	1500	40.0
Klebsiella pneumoniae	30.0	20.0
Citrobacter freundii	0.0	0.0

Klebsiella pneumoniae at 44.5°C was almost negligible (20.0 mkat) as compared to *E. coli* K-12 (1060 mkat). Preliminary studies conducted by Warren *et al.* (1978) at 43.5°C showed significantly faster rates of ONPG hydrolysis. Geldreich *et al.* (1962) had also shown that 96.4% of the human FC strains they examined were detected by EC broth when incubated at 44.5°C. Van Donsel *et al.* (1969) observed the optimum temperature for growth of FC to be between 40 and 44.5°C. Based on these observations, and results presented in Table 4, the selective 44.5°C incubation temperature was used in this study primarily to enhance specificity.

4.6.2 ONPG Sensitivity Test

Although the principal concern in this study was to define optimal conditions for E. coli K-12, selected as the model FC, IMViC typing and the ONPG sensitivity test were performed on these members of Enterobacteriaceae: - Klebsiella pneumoniae, Enterobacter cloacae and Citrobacter freundii, as a partial identification process.

Several investigators have used the chromogen, ONPG as an aid in the differentiation of members of Enterobacteriaceae in diagnostic bacteriology (Lowe, 1962; Szturm-Rubinsten, 1962; Leelere, 1962; Bulow, 1964). These authors found that all the lactose fermentors in this family were ONPG positive, while the non-lactose fermentors were ONPG negative. In agreement with these other investigators, the strains that were able to ferment the lactose in EC broth at the elevated temperature of 44.5°C, were able to hydrolyze ONPG (Table 5). E. coli K-12 showed immediate moderate hydrolysis, whereas Citrobacter freundii was ONPG negative. This also confirms the results of several other investigators who have reported that some strains of Citrobacter yielded negative ONPG results (Bulow, 1964; LaPage et al., 1964).

Table 5

IMViC Typing and ONPG Sensitivity Tests of Some Members of Enterobacteriaceae

Organism	I	M	Vi	C	Growth in EC$_o$ at 44.5°C	ONPG Reaction after Specified Time (hrs) 0.5	1	4	24
E. coli K-12	+	+	-	-	+	2+	3+	4+	4+
Enterobacter cloacae	-	-	+	+	+ (Slow)	-	1+	2+	2+
Klebsiella pneumoniae	-	-	+	+	+	-	1+	2+	2+
Citrobacter freundii	-	+	-	+	-	-	-	-	-

4.7. Effect of Toluene-Treatment on β-D-Galactosidase Activity

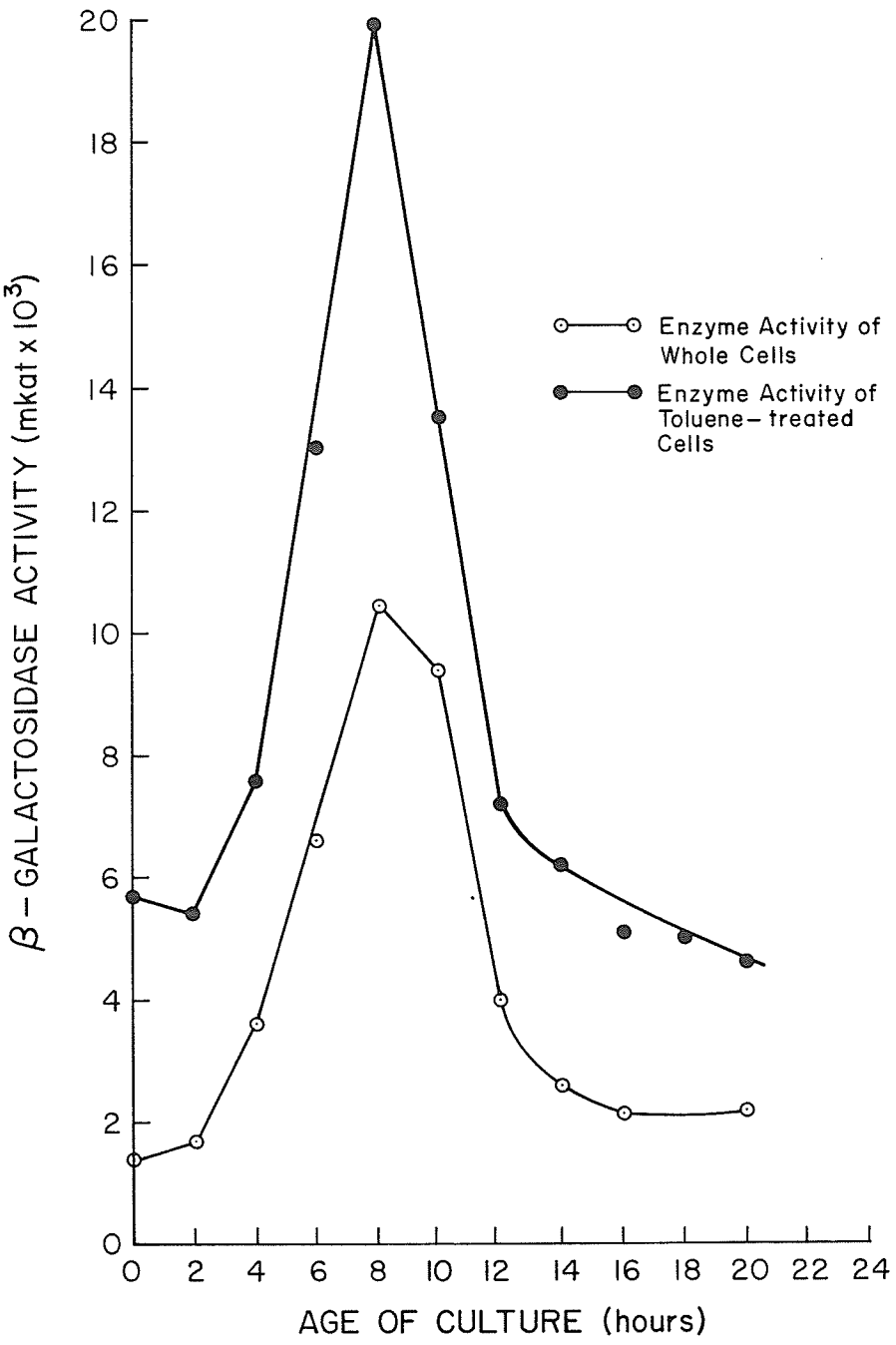
An attempt was made to find the effect of toluene-treatment on the β-D-galactosidase activity of E. coli K-12 cells, in view of the fact that toluene treatment had been shown to promote the hydrolysis of ONPG in intact cells (Lederberg, 1950; Rickenberg et al., 1956), and sometimes inactivate the enzyme (Anderson and Rickenberg, 1960). Results in Figure 10, showed that the β-D-galactosidase activity of whole cells had relatively lower activity values as compared to the lysed cells.

The difference in sensitivity of the two cell preparations was probably due to a difference in the principles behind the method, that is, permeability of the cell membranes for ONPG, and the amount of permease and β-D-galactosidase.

Studies by Rickenberg et al. (1959) and Rotman (1964), had revealed the presence of a galactoside-permease system in E. coli, which was responsible for the transportation of β-D-galactoside across the cell membrane. Lederberg (1950) had earlier investigated the possibility of liberating the enzyme from the cell by disintergration,

Figure 10

β-D-Galactosidase Activity of Whole and Toluene Treated Cells



instead of establishing direct contact between the β-D-galactosidase enzyme and the substrate. The data in this study was compatible with an inducible penetration mechanism, which has been reported necessary for β-D-galactosidase (Rotman, 1955). Toluene treatment of whole cells permitted the bypass of the penetration mechanism and thus hastened the reaction, presumably by destroying the integrity of the cell membrane. Lederberg (1950), and Jolly and Kosikowsi (1976) observed increased β-D-galactosidase activity in E. coli cells. Some workers (Bulow, 1964; LaPage and Jayaraman, 1964), therefore, enhanced autolysis by the addition of toluene to cell suspensions, when performing the qualitative ONPG sensitivity test.

Cell lysis of any form, is not recommended in these studies because of the high activities realized after toluene-treatment. This could yield false high results when computing the density of faecal coliforms using the β-D-galactosidase assay.

4.8 Application of the Colourimetric Assay to an Agar Diffusion Plate Method

The agar diffusion plate method was devised to observe the response of different inoculum sizes to ONPG hydrolysis in an EC based agar medium at pH 6.8. The resulting yellow zones of hydrolysis were observed to be indiscrete and fast-spreading, especially after 6 hours of assay. In addition, the colour intensity was not satisfactory, thus making any quantitative measurements of zone diameters very difficult. Initial attempts were thus directed at improving the technique. Variables tested in these attempts included: pH of the medium, temperature of incubation, and agar concentration.

4.8.1 Effect of pH on the agar plate assay

Varying the pH of the EC-ONPG agar medium from 6.8 through 10.0, showed a distinct improvement on the agar plate assay technique. The colour intensity was especially enhanced on plates adjusted to pH values above neutral (Illustration 1). It was therefore, evident that pH of 7.0 or higher, was optimal for the demonstration of the enzyme activity. At pH below 6.8, there was a decrease in contrast between the agar background and the zone of hydrolysis. At

Illustration 1

Effect of pH on the Agar Plate Assay

A	Control
B	pH 3
C	pH 4
D	pH 5
E	pH 6.8
F	pH 7.5
G	pH 8.0
H	pH 8.5
I	pH 9.0
J	pH 9.5

pH values at and below 4.0, the enzyme was precipitated as a white flocculent material, and enzyme activity was completely lost, presumably because at this pH, it completely dissociated into inactive monomers (Marchesi et al., 1969). Above pH 10.0, some non-enzymatic hydrolysis of ONPG was observed in the periphery of the medium surrounding the actual zone of hydrolysis (Illustration 2).

The improved zonation observed by shifting the pH of the EC-ONPG media to values above neutral, may in part be due to the nature of the alkaline used - NaOH.

The requirement for sodium ions to maximally activate the β-D-galactosidase enzyme when ONPG was the substrate, was evident in this study. Similar results were obtained with E. coli cells by Cohn and Monod (1951) and P. aerogenoides by Anderson and Rickenberg (1960), using broth assay techniques. According to Rickenberg (1959), at pH 7.25, in the presence of Na^+ ions, the hydrolysis of ONPG proceeds with maximum velocity (Vmax. = 178). This fact was verified in this study since the synergistic effect of Na^+ ions and increase in pH towards alkalinity enhanced the colour intensity.

In the assay conditions specified by Lederberg (1950),

Illustration 2

Non-enzymatic Hydrolysis of ONPG at pH 10.0

A control
B actual zone of ONPG hydrolysis
C faint non-enzymatic ONPG hydrolysis

the colour density determination was made at pH above 10.0, that is, a range in which the phenolic group of o-nitrophenol was completely dissociated (pK = 7.3) and the colour density was at a maximum. Thus β-D-galactosidase reactions are usually terminated with Na_2CO_3 to develop the full colour of ONP. The use of optimal alkaline pHs in the plate assay would therefore, eliminate the need to terminate reactions with Na_2CO_3 as in broth assays.

Based on the visual observations made in Illustration 1, a narrower pH range of 7.5 - 8.5 was selected for further studies. This pH range was compared with pH of the unadjusted EC medium, viz., 6.8. The statistical analysis, (AOV), and Tukey's test on this pH range are presented in Appendix 3a, and Table 6, respectively.

The rate of reaction and ease of reading the qualitative plate test did not differ significantly with pH values of 7.5 and above. Within this range, pH was not critical for the demonstration of β-D-galactosidase activity. Only pH 6.8 differed significantly from the others with respect to rate of zone spread (Table 6). Accordingly, a pH adjustment to 8.0 was selected and adopted for use in

Table 6

Effect of pH on Agar Plate Assay

pH	Mean Zone Diameter*
6.8	2.05[a]
7.5	3.03[b]
8.5	3.03[b]
8.0	3.04[b]

* Mean values not followed by the same superscript are significantly different at $p < 0.05$.

subsequent plate assays.

4.8.2 Effect of agar concentration on the agar plate assay

A series of EC based media with agar concentrations ranging from 1.25 to 2.20% were tested, to study the sensitivity of the assay procedure. Agar concentrations of 1.2% were too soft and did not yield clearly visible zones of hydrolysis. The spread of the zones was very fast and appeared uneven. Therefore, plates prepared with 1.5 to 2.5% agar were selected for further quantitative measurements. Results of the effect of different agar concentrations, 1.5, 2.0, 2.5%, on the plate assay, are shown in Illustration 3. There was no apparent relationship between the agar concentration and the rate of spread of the yellow zones. Thus, the rate of increase of zone diameters did not seem to be a function of the agar concentration, and the trials failed to improve the technique. The analysis of variance, (AOV), on zone diameters are presented in Appendix Table (3b). Comparison of mean diameters of the 4 agar concentrations was performed by Tukey's test (Snedecor and Cochran, 1967), and are presented in Table 7.

Although there were significant differences among the

Illustration 3

Effect of Agar Concentration on the Agar Plate Assay

A 1.5% Agar
B 2.0% Agar
C 2.5% Agar

Table 7

Effect of Agar Concentration on the Agar Plate Assay

Agar Concentration	Mean Zone Diameter*
2.50	2.52^{a}
2.00	2.94^{b}
1.75	3.02^{c}
1.50	3.36^{c}

* Mean values not followed by the same superscript are significantly different at $p < 0.05$.

different agar concentrations, no apparent trend was obtained to show that changing this variable would enhance the agar plate assay. Therefore, a standard agar concentration of 1.5% was found to be adequate.

4.8.3 Effect of incubation temperature on the agar plate assay

Plates of EC-ONPG agar were prepared after the pH was adjusted to 8.0. Incubation was carried out at room temperature, 37°C and 44.5°C. Results in Illustration 4 revealed some differences in the rate of spread of zone diameters. Generally the rate of zonation increased with increasing temperature. This fact was substantiated in the trends evidenced in Appendix Table 3c, which showed highly significant differences between the three temperatures and time ($p < 0.05$). Comparison of mean diameters also confirmed these observed differences (Table 8). The results presented indicated that for the temperature range tested, incubation temperatures were not critical for enhancing the agar plate technique, as intensity of colour did not vary much. However, one encouraging observation, as evidenced from Illustration 4, was that the selective incubation temperature of 44.5°C recommended by Warren

Illustration 4

Effect of Incubation Temperature on the Agar Plate Assay

A Room temperature
B 37°C
C 44.5°C

Table 8

Effect of Temperature of Incubation on Agar Plate Assay

Temperature	Mean Zone Diameter*
Room Temperature	3.03[a]
37°C	3.41[ab]
44.5°C	3.87[b]

* Mean values not followed by the same superscript are significantly different at $p < 0.05$.

et al. (1978), was favourable to the plate assay since it also gave a fairly distinct zonation.

4.8.4 Effect of cell concentration on the agar plate assay

To study the response of different cell concentrations on the agar plate assay, plates with different inoculum levels were observed over a 24 hour period. A colour change in the medium from clear to yellow was indicative of a positive reaction, which signified ONPG hydrolysis. Illustrations 5a and 5b show the reactions after 4 and 24 hours, respectively. The colour intensity and the size of yellow zone increased with increases in cell density. However, after 24 hours of incubation, the yellow colour had spread over the surface of the whole plate for cell densities of 10^3 - 10^6 (Illustration 5b). These results demonstrate that there is a definite relation between cell density and resulting intensity of the yellow colour; and on the other hand, between density and time lapse for observable hydrolysis. Concentrations of 10^4 - 10^6 cells/ml gave an immediate response, whereas, 10^3 cells/ml showed a slower response. Concentration of 10^2 cells/ml did not show a positive reaction even after 24 hours incubation. Thus, the cell concentration of 10^3 cells/ml represented the limit

Illustration 5a

Effect of Cell Concentration on the Agar Plate Assay

(After 4 hours Incubation)

A 10^1 cells/ml
B 10^2 cells/ml
C 10^3 cells/ml
D 10^4 cells/ml
E 10^5 cells/ml
F 10^6 cells/ml

Illustration 5b

Effect of Cell Concentration on the Agar Plate Assay

(After 24 hours of Incubation)

A Control
B 10^1 cells/ml
C 10^2 cells/ml
D 10^3 cells/ml
E 10^4 cells/ml
F 10^5 cells/ml
G 10^6 cells/ml

of detection on the agar plates. Several workers have recommended the use of a heavy inoculum for any rapid routine qualitative ONPG test (LeMinor and Ben Hamida, 1962; Bulow, 1964; Wilson et al., 1971). They found that the density of the suspension and time lapse before a positive reaction was observed, showed a definite correlation. Furthermore, these authors reported that with very dense suspensions, results could be reproduced with sufficient accuracy. These results (Illustrations 5a and b) compare well with these findings and show the advantage of a dense suspension over a dilute one for the purposes of routine tests.

5. CONCLUSION

The present investigation was prompted by a study (Warren et al., 1978) in which a technique of using a colourimetric β-D-galactosidase assay to enumerate faecal coliforms in water was described.

In this investigation, mid-log grown cultures of E. coli K-12 were used to investigate some of the parameters of the assay and extend its use as a possible rapid method for faecal coliform enumeration in milk. The findings in this study include:

1. The age of culture that was desirable for optimum β-D-galactosidase activity was established at 10 - 12 hours. pH and temperature optima were 7.6 and 40°C, respectively.
2. The actual time to reach half-maximum absorbance reading (0.7) at 420 nm was proportional to the cell density. High cell densities gave an immediate response, whereas 10^2 to 10^3 cells/ml responded well in less than 24 hours. Thus, the hypothesis that a short time faecal coliform test could be developed based on the rate of ONPG hydrolysis was established. This relationship between ONPG hydrolysis time and

actual FC density was found to be linear within certain limits - 10^2 to 10^6 cells/ml.

3. It was also established that for cell densities 10^2 to 10^6/ml, the relationship holds true for absorbancy readings taken at 0.7 ± 0.2, without incurring errors over 10.77%.

4. Based on the comparison between FC densities estimated by MPN and the colourimetric assay, it is reasonable to conclude that there is a fairly good correlation between the two techniques ($r^2 = 0.93$).

5. It has value as a rapid screening procedure for the detection and enumeration of organisms indicative of faecal contamination of milk, and it possibly has potential application to other foods. Thus, continuing studies could be directed towards evaluating a wider applicability of the rapid enumeration method. However, a baseline should be established for half-maximum absorbance of each particular processing condition.

6. The economic advantage of using the ONPG assay over other rapid bacteriological assays of water, such as radiometric methods using labelled substrates

(Bachrach and Bachrach, 1974), or the gas chromatographic presumptive test for coliforms (Newman and O'Brien, 1975), lies in the fact that its cost may be equal to or lower than that of conventional methods.

7. The colourimetric assay was found to be specific for E. coli and yet did not exclude a significant portion of the FC population.

8. The superiority of the test over the MPN technique lies in its speed, selectivity and simplicity, and portrays a significant improvement in the practical enumeration of bacteria and is therefore, recommended for routine qualitative and quantitative use. However, the purpose was less in determining the status of the new test than in the establishment of the validity of the test for FC enumeration.

9. In the course of the qualitative agar diffusion plate assay, no improvement in response was observed which might be ascribed to the variables investigated, temperature of incubation and agar concentration. The pH of the medium was found to be critical for the improvement of the colour contrast. Generally,

the plate technique lacked precision with respect to quantification. It was therefore, decided that the agar plate assay be used qualitatively, not quantitatively.

10. The plate assay shows promise as a rapid, specific qualitative test. It is especially promising for use where a specific faecal coliform limit has been established in a food product. For example, if the limit is to be 10^3 cells/ml, detectable enzymatic hydrolysis should be observed in less than 24 hours, using the protocol of this investigation. This application would be valid only if the growth pattern and/or the state of the organism had not been altered by any processing steps. If, for example, there has been thermal injury, one would expect a lag period which would affect time required to obtain observable hydrolysis.

B I B L I O G R A P H Y

Abshire, R.L., and R.K. Guthrie, 1973. Fluorescent antibody as a method for the detection of faecal pollution: E. coli as indicator organism. Can. J. Microbiol. 19:201-206.

A.O.A.C. "Official Methods of Analysis", 1975. 12th Edition, Washington, D.C.

A.P.H.A. (American Public Health Association), 1955. Standard Methods for the Examination of Water, Sewage and Industrial Wastes. 10th Edition. A.P.H.A. Inc., New York.

A.P.H.A., 1966. Subcommittee on Methods for the Microbiological Examination of Foods, 2nd Edition, A.P.H.A. Inc., New York.

A.P.H.A., 1974. Standard Methods for the Examination of Water and Wastewater. 14th Edition., A.P.H.A. Inc., New York.

A.P.H.A., 1976. Compendium of Methods for Microbiological Examination of Foods, 1st Edition, A.P.H.A. Inc., Washington, D.C.

A.P.H.A., 1978. Standard Methods for the Examination of Dairy Products, 14th Edition, A.P.H.A. Inc., New York.

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

Andrews, W.H., and M.W. Presnell, 1972. Rapid enumeration of E. coli from estuary water. Appl. Microbiol. 23:521-523.

Andrews, C., D. Diggs and C.R. Wilson, 1975. Evaluation of a medium for the rapid recovery of E. coli from shellfish. Appl. Microbiol. 29:130-131.

Babad, H., and W.Z. Hassid, 1964. J. Biol. Chem. 239 PC 946.

Bachrach, V., and Z. Bachrach, 1974. Radiometric method for the detection of coliform organisms in water. Appl. Microbiol. 28:169-171.

Barber, L.E., and R.H. Deibel, 1972. Effect of pH and oxygen tension on staphylococcal growth and enterotoxin formation in fermented sausage. Appl. Microbiol. 24:891.

Barnard, R., and W. Callahan, 1971. Follow up on gastroenteritis attributed to French cheese. Morbility and Mortality 25:445.

Bartley, C.H., and L.W. Slanety, 1960. Types and sanitary significance of faecal streptococci isolated from feces, sewage and water. Am. J. Public Health 50:1545.

Bauer, R.R., 1972. History and background on occurrence of fecal coliforms in industrial wastes. In "Proceeding of the Seminar on the Significance of Fecal Coliform in Industrial Wastes". R.H. Bordner and B.J. Carroll, Eds., U.S. Environmental Protection Agency, Denver, Colorado.

Bergmeyer, H.U., Eds., 1978. In "Principles of Enzymatic Analysis". pp. 56-60. Verlag Chemie Weinheim, New York.

Braswell, J.R., and A.W. Hoadley, 1974. Recovery of E. coli from chlorinated secondary sewage. Appl. Microbiol. 28:328-329.

Bulow, P., 1964. The ONPG test in diagnostic bacteriology. (i) Methodological Investigations. Acta Pathol. Microbiol. Scand. 60(3):387-402.

Burnam, N.P., E.W. Oliver, and J.K. Stevens, 1969. Membrane filtration technique for the isolation of coli-aerogenes, E. coli, fecal streptococci, Clostridium perfringens, actinomycetes and microfungi. In "Isolation Methods for Microbiologists." pp. 127-135. D.A. Shapton and G.W. Gould, Eds., Technical Series No. 3. Academic Press Inc., New York.

Burton, M.O., 1949. Comparison of coliform and Enterococci organisms as indices of pollution in frozen foods. Food Res. 14:434.

Buttiaux, R., and D.D.A. Mossel, 1961. The significance of various organisms of fecal origin in foods and drinking water. J. Appl. Bacteriol. 24:353-364.

Busta, F.F., and M.L. Speck, 1965. Enumeration of Bacillus stearothermophilus by use of membrane filter techniques to eliminate inhibitors present in milk. Appl. Microbiol. 13(6):1043-1044.

Chordash, R.A., and N.F. Insalata, 1978. Incidence and pathological significance of Escherichia coli and other sanitary indicator organisms in food and water. Food Technol. 32(10):54-62.

Citti, J.E., W.E. Sandine, and R.R. Ellikan, 1964. β-Galactosidase of Streptococcus lactis. J. Bact. 89(4):937-942.

Claydon, T.J., 1975. A membrane-filter technique to test the significance of sublethally injured bacteria in retail pasteurized milk. J. Milk Food Technol. 38(2):87-88.

Closs, O., and A. Digranes, 1971. Rapid identification of prompt lactose-fermenting genera within the family Enterobacteriaceae. Acta Pathol. Microbiol. Scand. Sect. 79:673-678.

Cohn, M., and J.P. Monod, 1951. Purification et propietes de la β-galactosidase d'Escherichia coli. Biochim et Biophys. Acta. 7:1471-1478.

Connant, N.F., D.T. Smith, R.D. Baker, T.T. Calloway, and D.S. Martin, 1968. "Manual of Clinical Mycology". 2nd Ed. W.B. Saunders and Co., Philadelphia.

Corry, J.E.L., 1973. The water relations and heat resistance of microorganisms. Progress and Microbiol. 12:73.

Costin, I.D., D. Voiculesen,and V. Gorcea, 1964. An outbreak of food poisoning in adults associated with serotype 086:B7:H34. J. Path. Microbiol. 27:68.

Cramblett, H.G., P. Azim, and R.E. Haynes, 1971. The etiology of infectious diarrhea in infancy with special reference to Enteropathogenic E. coli. Ann. New York Acad. Sciences. 176:80.

Craven, G.R., E. Steers Jr., and C.B. Aufinsen, 1965. Purification, composition and molecular weight of the β-galactosidase of Escherichia coli K-12. J. Biol. Chem. 240:2468-2477.

Dahlqvist, A., B. Mattiason, and K. Mosbach, 1973. Hydrolysis of β-galactosidase using polymer entrapped lactase. A study towards producing lactose-free milk. Biotechnol. Bioeng. 15:395.

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

deFigueiredo, M.P., 1970. Microbial indices for quality assurance. Food Technol. 24:157-159.

Deibel, R.H., 1964. The group D streptococci. Bacteriol. Rev. 28:330.

Delepine, S., 1903. Food poisoning and epidemic diarrhea. J. Amer. Med. Assn. 40:657.

Donnelly, C.B., L.A. Black and K.H. Lewis, 1960. An evaluation of simplified viable methods for determining viable counts of raw milk. J. Milk Food Technol. 23:275-277.

Dufour, A.P., and V.J. Cabelli, 1975. Membrane filter procedure for enumerating the component genera of the coliform group in seawater. Appl. Microbiol. 29(6): 826-833.

Duitschaever, C.L., D.R. Arnott, and D.H. Bullock, 1973. Bacteriological quality of refrigerated ground beef. J. Milk Food Technol. 36:375.

Duncan, D.W., and W.E. Razzell, 1972. Klebsiella biotypes among coliform isolates from forest environments and farm produce. Appl. Microbiol. 24:933-938.

Dutka, B.J., 1973. Coliforms are an inadequate index of water quality. J. Environ. Health 36:39-46.

Edwards, P.R., and W.H. Ewing, 1972. In "Identification of Enterobacteriaceae". 3rd Ed., Burgess Pub. Co., Minneapolis, Minn.

Eijkman, C., 1904. Die garungsprobe bei 46° alb hilfstmittel beider trinkwasseruntersuchung. Zentralbl. Bakteriol. Parasitenkal. Infektionskr. Hyg. Abt. 1 Orig. 34:742-748.

Eisberg, W.V., and M. Cichowicz, 1977. Machinery mold - indicator organism in food. Food Technol. 31:52-56.

Eisenhart, C., and P.W. Wilson, 1943. Statistical method and control in bacteriology. Bact. Rev. 7:57.

Elliott, J.A.,and G.E. Millard, 1976. A comparison of two methods for counting coliforms in cheese. Can. Inst. Fd. Sci. Tech. J. 9(2):95-97.

Enzyme Nomenclature Recommendations, 1973. The International Union of Pure and Applied Chemistry and the International Union of Biochemistry. p. 17-22. Elsevier Scientific Publishing Co., Amsterdam.

Erickson, R.P., 1970. Molecular weight of E. coli β-galactosidase in concentrated solutions of guanidine hydrochloride. Biochem. J. 120:255-261.

Escherich, T., 1887. Central Bacteriol. Parasiteuk 1:705.

Ewing, W.H., B.R. Davis, and T.S. Montague, 1963. Studies on the occurrence of Escherichia coli serotypes associated with diarrheal disease. Pub. Health Lab. 15:118.

Feig, M., 1950. Diarrhea, dysentery, food poisoning and gastroenteritis. Am. J. Public Health 40:1372.

Feniksova, R.A., A.S. Tikhomirova, A.N. Kulikova, and V.S. Kuznetsov, 1968. Production of β-galactosidase by various microorganisms. Mikrobiologiya 37(6):988-992.

Fifield, C.W., J.E. Hoff, and B.E. Proctor, 1957. The millipore filter for enumerating coliform organisms in milk. J. Dairy Sci. 40:588-589.

Fishbein, M., 1962. The aerogenic response of E. coli strains of Aerobacter in EC broth and selected sugar broth at elevated temperatures. Appl. Microbiol. 10:79-85.

Fishbein, M., 1976. Coliforms, fecal coliforms, E. coli, human enteropathogenic E. coli. In "Compendium of Methods for the Microbiological Examination of Foods". Am. Pub. Health Assn. Inc., Washington, D.C.

Fishbein, M., and B.F. Surkiewicz, 1964. Comparison of the recovery of Escherichia coli from frozen foods and nut meats by confirmatory incubation in EC medium at 44.5°C and 45.5°C. Appl. Microbiol. 12:127.

Fishbein, M., B.E. Surkiewicz, E.F. Brown, H. Oxley, A.P. Pardon, and R.J. Groomes, 1967. Coliform behaviour in frozen foods. I. Rapid test for recovery of E. coli from frozen foods. Appl. Microbiol. 15:233-238.

Food and Drug Administration (FDA), 1976. Bacteriological Analytical Manual. 4th Ed. A.O.A.C., Washington, D.C.

Francis, D.W., J.T. Peeler, and R.M. Twedt, 1974. Rapid method for detection and enumeration of fecal coliforms in fresh chicken. Appl. Microbiol. 27(6):1127-1130.

Frazier, W.C., 1958. In "Food Microbiology". 2nd Ed. McGraw Hill Book Co. Inc., New York.

Geldreich, E.E., 1966. Sanitary Significance of Fecal Coliforms in the Environment. U.S. Dept. of the Interior. Federal Water Pollution Control Administration Publication WP 20-3. U.S. Govt. Printing Office, Washington, D.C.

Geldreich, E.E., R.H. Bordner, C.B. Huff, H.F. Clark and P.W. Kabler, 1962. Type distribution of coliform bacteria in the faeces of warm-blooded animals. J. Water Pollut. Control Fed. 34:295-301.

Geldreich, E.E., H.F. Clark, C.B. Huff, and L.C. Best, 1965. Fecal coliform organism medium for the membrane filter technique. J. Am. Water Works Assoc. 56:208-214.

Geldreich, E.E., H.L. Jeter, and J.A. Winter, 1976. Technical Considerations in Applying the Membrane Filter Procedure. H.L.S. 4(2):113-125.

Gitter, M., 1967. The enteropathogenic effect of E. coli in rabbits and pigs. Brit. Vet. F. 123:403.

Goff, J.H., J.J. Claydon, and J.J. Iandolo, 1972. Revival and subsequent isolation of heat-injured bacteria by a membrane filter technique. Appl. Microbiol. 23:857-862.

Goldberg, M.E., 1969. Tertiary structure of Escherichia coli β-galactosidase. J. Molecular Biol. 46:441-446.

Graves, D.C., and I.A. Schipper, 1966. Membrane filtration technique for isolating organisms from raw milk of normal udders. Appl. Microbiol. 14(4):535-539.

Gray, R.D., 1964. An improved formate lactose glutamate medium for the detection of Escherichia coli and other coliform organisms in water. J. Hyg. 62:495-508.

Griffin, A.M., and C.A. Stuart, 1940. An ecological study of the coliform bacteria. J. Bacteriol. 40:83-100.

Guerrant, R.L., R.A. Moore, P.M. Kirschenfeld and M.A. Sande, 1975. Role of toxigenic and invasive bacteria in acute diarrhea of childhood. New England J. Med. 293:567.

Guilbault, G.G., 1973. In "Enzymatic Method of Analysis". p. 265-293. Pergamon Press Inc., Oxford, New York. Toronto, Australia, Braunschwerg.

Guthrie, R.K., and D.J. Reeder, 1969. Membrane filter-fluorescent antibody method for detection and enumeration of bacteria in water. Appl. Microbiol. 17:399-401.

Hachney, C.R., B. Ray, and M.L. Speck, 1979. Repair detection procedure for enumeration of faecal coliforms and enterococci from seafoods and marine environments. Appl. and Environ. Microbiol. 37(5):947-953.

Haenel, H., 1961. Some rules in the ecology of the intestinal microflora of man. J. Appl. Bacteriol. 24:242-251.

Halden, H.E., 1957. Methods for counts of microorganisms in liquid sugar. J. Am. Soc. Sugar Beet Technol. 9:393-399.

Hall, H.E., D.F. Brown and K.H. Lewis, 1967. Examination of market foods for coliform organisms. Appl. Microbiol. 15:1062.

Hartman, P.A., G.W. Reinbold, and D.C. Saraswat, 1965. Indicator organisms, a review: Role of enterococci in food poisoning. J. Milk Food Technol. 28:344.

Hartman, P.A., P.S. Hartman and W.W. Lanz, 1975. Violet red bile 2 agar for stressed coliforms. Appl. Microbiol. 29:537-539.

Hefferman, W., and V. Cabballi, 1967. Modified McConkey Agar Plate Technique for Faecal Coliform Determination. Shellfish Sanitation Research Center, U.S. Public Health Service, Department of Health, Education and Welfare, Washington, D.C.

Hill, J.A. and R. Huber, 1971. Effect of different concentrations of Na^{+} and Mg^{2+} on E. coli β-galactosidase activity. Biochem. Biophys. Acta. 25:630.

Hobbs, B.C., M.E.M. Thomas and J. Taylor, 1949. School outbreaks of gastroenteritis associated with a pathogenic paracolon bacillus. Lancet, ii:530.

Holsinger, V.H., 1978. Applications of lactose-modified milk and whey. Food Technol. 32(3):35-40.

Hood, L.F., 1971. Enzymatic hydrolysis of lactose in dairy foods. J. Dairy Sci. 54:761.

Horiuchi, T., J. Tomizawa, and A. Novick, 1961. Isolation and properties of bacteria capable of high rates of β-galactosidase synthesis. Biochim. Biophys. Acta. 25:114.

Howard, C.J., and A.A. Glynn, 1971. Some physical properties of K. antigens of Escherichia coli related to their biological activity. Infec. Immun. 4:6.

Hufham, J.B., 1974. Evaluating the membrane faecal coliform test by using Escherichia coli as an indicator organism. Appl. Microbiol. 27:771-776.

Huggins, C., and D.R. Smith, 1947. Chromogenic substrates. III. P-nitrophenyl sulfate as a substrate for the estimation of phenolsulfatase activity. J. Biol. Chem. 176:391-398.

Ikura, Y., and K. Horikoshi, 1979. Isolation and some properties of β-galactosidase producing bacteria. Agric. Biol. Chem. 43(1):85-88.

International Commission on Microbiological Specifications for Foods (ICMSF) of the International Association of Microbiological Societies, 1974. In "Microorganisms in Food", M. Ingram et al. (ed). Vol. 2 University of Toronto Press, Toronto.

I.C.M.S.F., 1978. Their Significance and Methods of Enumeration. In R.P. Elliott et al. (ed). Microorganisms in Foods, Vol. 1, 2nd Ed., University of Toronto Press, Toronto, p. 103-136.

Jay, J.M., 1978. Indices of Food Sanitary Quality and Microbiological Standards and Criteria. In "Modern Food Microbiology". 2nd Ed. p. 291-320. D. Van Nostrand Co., New York, Cincinatti, Toronto, London, Melbourne.

Johnston, M.A., and H. Pivnick, 1970. Use of autocytotoxic β-galactosidases for selective growth of Salmonella typhimurium in the presence of coliforms. Can. J. Microbiol. 16:83-89.

Johnston, M.A., and F.S. Thatcher, 1967. Selective inhibition of E. coli in the presence of enterobacteria including Salmonellae. J. Clin. Pathol. 17:117-121.

Jolly, R.C., and F.V. Kosikowski, 1976. β-galactosidase activity by the enterococci. Milkwissenschaft 31(6):355-358.

Jones, G.A., D.L. Gibson, and K.J. Cheng, 1967. Coliform bacteria in Canadian pasteurized dairy products. Can. J. Public Health 58:527.

Jones, G.W., and J.M. Rutter, 1972. Role of the K88 antigen in the pathogenesis of neonatal diarrhea caused by Escherichia coli in piglets. Infect. Immun. 6:918.

Keilin, D., and E.F. Hartree, 1948. Biochem. J. 42:230.

Kereluk, K., and M.F. Gunderson, 1959. Studies on the bacteriological quality of frozen meat pies. IV. Longevity studies on coliform bacteria and enterococci at low temperatures. J. Appl. Microbiol. 7:327.

Klein, H., and D. Fung, 1976. Identification of fecal coliforms using violet red bile agar at elevated temperatures. J. Milk Food Technol. 39:768-770.

Koburger, J.A., 1971. Fungi in foods. II. Some observations on acidulants used to adjust media pH for yeasts and mould count. J. Milk Food Technol. 34:475.

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

LaPage, S.P., and M.S. Jayaraman, 1964. β-galactosidase and lactose fermentation in the identification of enterobacteriaceae including Salmonella. J. Clin. Path. 17:117-121.

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

Leelere, H., 1962. Etude biochimique d'Enterobacteriaceae pigmentées. Ann. Inst. Pasteur. 102:726.

leMinor, L., and F. Ben Hamida, 1962. Avantages de la recherche de la β-galactosidase sur celle de la fermentation du lactose en milien complexe dans le diagnostic bactériologique, en particulier des Enterobacteriaceae. Ann. Inst. Pasteur. 102:267.

Lin, S.D., 1973. Evaluation of coliform tests for chlorinated secondary effluents. J. Water Pollution Control Fed. 45:498-506.

Lin, S.D., 1976. Membrane filter method for recovery of fecal coliforms in chlorinated sewage effluents. Appl. and Environmental Microbiol. 32:547-552.

Lowe, G.H., 1960. A study of the factors controlling lactose-fermentation in the coliform and paracolon groups. J. Gen. Microbiol. 23:127.

Mackenzie, E.F.W., E.W. Taylor, and W.E. Gilbert, 1948. Recent experiences in the rapid identification of Bacterium coli Type 1. J. Gen. Microbiol. 2:197-204.

Mara, D.D., 1973. Coliform Counts of Polluted Waters: A Comparison of Media and Methods. In "Water Research". Pergamon Press, Vol. 7, p. 1899-1903. Gr. Britain.

Marchesi, S.L., E. Steers, and S. Shifrin, 1969. Biochim. Biophys. Acta. 181:20.

Marr, A.G., J.L. Inghaham, and C.L. Squires, 1964. Effect of the temperature of growth of E. coli on the fermentation of β-galactosidase. J. Bacteriol. 87(2):356-362.

Martin, J.D., and J.O. Mundt, 1972. Enterococci in insects. Appl. Microbiol. 24:575.

Maxey, R.B., 1970. Non-lethal injury and limitations of recovery of coliform organisms on selective media. J. Milk Food Technol. 33:445-448.

McCoy, J.H., 1961. The safety and cleanliness of waters and foods. J. Appl. Bacteriol. 24:365-367.

Merson, M.H., G.K. Morris, D.A. Sack, J.G. Wells, W. Greech, J.C. Feeley, R.B. Sack, A.Z. Kapikian, and E.J. Gangarosa, 1976. Traveler's diarrhea in Mexico: A prospective study. New England J. Med. 294:1299.

Milk Industry Foundation, 1964. Methods of Analysis of Milk and Its Products. 3rd Ed. Milk Ind. Fndn., Washington, D.C.

Miskimin, D.K., K.A. Berkowitz, M. Solberg, W.E. Riha Jr., W.C. Franke, R.L. Buchanan and V. O'Leary, 1976. Relationships between indicator organisms and specific pathogens in potentially hazardous foods. J. Food Sci. 41:1001.

Monod, 1947. The phenomenon of enzymatic adaptation. Growth. 11:223-289.

Moore, B., 1955. Streptococci and food poisoning. J. Appl. Bacteriol. 18:606.

Montford, J., and F.S. Thatcher, 1961. Comparison of 4 methods of isolating Salmonellae from foods, and elaboration of a preferred procedure. J. Food Sci. 26:510-517.

Montogerie, J.Z., P.B. Doak, D.E.M. Taylor, J.D.K. North, and W.J. Martin, 1970. Klebsiella in fecal flora of renal-transplant patients. Lancet 2:787-792.

Moran, J.O. and L.D. Witter, 1976. An automated rapid test for Escherichia coli in milk. J. Fd. Sci. 41:165-167.

Morgan, G.B., and P. Gubbins, and V.A. Morgan, 1965. A critical appraisal of the membrane filter technique. H.L.S. 2:227-237.

Moroz, R., 1957. Determination of yeast in sugar liquids using membrane filters. Int. Sugar J. 59:70-71.

Moses, V., and P.B. Sharp, 1970. Interactions between metabolic intermediates and β-galactosidase in skin. Clin. Chim. Acta. 20:1.

Mossel, D.A.A., 1962. An ecological investigation of the usefulness of the 2 specific modifications of Eijkman's test as one of the methods used for the detection of faecal coliform contamination of foods. J. Appl. Bacteriol. 25:20-29.

Mossel, D.A.A., W.H.J. Mengerink and H.H. Scholts, 1962. Use of a modified MacConkey agar medium for the selective growth and enumeration of Enterobacteriaceae. J. Bacteriol. 84:381.

Mossel, D.A.A., M. Visser, and A.M.R. Correlissen, 1963. The examination of foods for Enterobacteriaceae using a test of the type generally adopted for the detection of Salmonellae. J. Appl. Bacteriol. 26:444.

Mossel, D.A.A., and S. Ratto, 1970. Enterobacteriaceae in dried foods. Appl. Bacteriol. 20:273.

Newman, J.S., and R.T. O'Brien, 1975. Gas chromatographic presumptive test for coliform bacteria in water. Appl. Microbiol. 32:547.

Niven, C.F.Jr., 1963. Microbial indexes of food quality: faecal streptococci. In "Microbiological Quality of Foods". Proceedings Conference on Microbiological Quality of Foods, Franconia, N.H. August 27-29, 1962, Ed. L.W. Slanetz, C.O. Chichester, A.R. Ganfin and Z.J. Ordal. p. 119. N. York, Academic Press.

Okos, E.S., E.A. Grulke, and A. Syverton, 1978. Hydrolysis of lactase in acid whey using β-galactosidase absorbed to a phenol formaldehyde resin. J. Food Sci. 43:567-571.

Olson, A.C., and W.L. Stanley, 1973. J. Agr. Food Chem. 21:440.

Onishi, H., 1963. Osmophilic yeasts. Adv. Food Res. 12:53.

Pickett, M.J., and R.E. Goodman, 1966. β-galactosidase for distinguishing between Citrobacter and Salmonella. Appl. Microbiol. 14(2):178-182.

Powell, J.C., A.R. Moore and J.A. Gow, 1979. Comparison of EC broth and medium A-1 for the recovery of Escherichia coli from frozen shucked snow crab. Appl. and Environ. Microbiol. 33(5):836-840.

Raj, H., and Liston, J., 1961. Detection and enumeration of fecal indicator organisms in frozen sea foods. I. Escherichia coli. Appl. Microbiol. 9:171-174.

Raj, H., J. Wiebe and J. Liston, 1961. Detection and enumeration of fecal indicator organisms in frozen sea foods. II. Enterococci. Appl. Microbiol. 9:295.

Ray, B., and M.L. Speck, 1973. Enumeration of E. coli in frozen samples after recovery from injury. Appl. Microbiol. 25:499-503.

Reed, G.,(Editor), 1975. In "Enzymes in Food Processing." 2nd Ed. Academic Press, New York, San Francisco, London.

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

Rickenberg, H.V., 1960. The role of the inducer in the induced synthesis of β-galactosidase in Escherichia coli. Nature 185:240.

Rickenberg, H.V., G.N. Cohen, Buttin, G., and J. Monod, 1956. Galactosidepermease d'Escherichia coli. Ann. Inst. Pasteur, 91:829-857.

Rose, R.E., E.E. Geldreich, and W. Litsky, 1975. Improved membrane filter method for faecal coliform analysis. Appl. Microbiol. 29:532-536.

Rotman, B., 1955. Factors controlling the enzymatic activity of intact cells of Escherichia coli. Bacteriol. Proc. p. 133.

Rotman, B., 1964. Specificity of induction of enzymes in the lactose operon of Escherichia coli. J. Mol. Biol. 8:105-127.

Ryder, R.W., D.A. Sack, A.Z. Kapikian, A.S.M.M. Rahman, M.H. Merson and J.G. Wells, 1976. Enterotoxigenic Escherichia coli using Y1 adrenal cells in miniculture. Infec. Immun. 11:334.

Sack, R.B., S.L. Golback, J.G. Branwell, B. Jacobs, R.D. Chatterjee and R.C. Mitra, 1971. Enterotoxigenic Escherichia coli isolated from patients with severe cholera-like diseases. J. Infect. Dis. 123:378.

Saito, M., and T. Tatsuno, 1971. Toxins of Fusarium nivale. In "Microbial Toxins, Vol. VII: Algae and Fungal Toxins". Ed. S. Kadis, A. Ciegler and S.J. Ajl. p. 293. New York Academic Press.

Sakazaki, R., K. Tamura and A. Makamura, 1974. Further studies on enteropathogenic E. coli associated with diarrhoeal diseases in children and adults. Japan J. Med. Sci. Biol. 27:7.

Seidman, M., and K.P. Link, 1950. O-nitrophenyl β-galactopyranoside and its tetra acetate. J. Am. Chem. Soc. 72:21-24.

Shardinger, F., 1892. "Ueber das Vorkommen Gahrung errengender Spaltzillze in Trink wasser und ihre Bedeuting fur die Hygiensche". Wien. Klin. Wochschr. 5:403.

Sharpe, M.P., and G.L. Michaud, 1975. Enumeration of high numbers of bacteria using hydrophobic grid-membrane filters. Appl. Microbiol. 30:519-524.

Sharpe, A.N., P.I. Peterkin and I. Dudas, 1979. Membrane filtration of food suspension. Appl. and Environ. Microbiol. 37(1):21-35.

Shelton, L.R., H.V. Leininger, B.F. Surkiewicz, E.F. Baer, R.R. Elliott, J.B. Hyndman, and N. Kramer, 1962. "A Bacteriological Survey of the Frozen Precooked Food Industry". Washington, D.C. U.S. Department of Health, Education and Welfare, Food and Drug Administration.

Shifrin, S., B.J. Grochowski, and S.W. Luborsky, 1970. Dissociation of β-galactosidase by thiols. Nature 227:608.

Shukla, T.P., 1975. β-galactosidase technology: a solution to the lactose problem. CRC Critical Reviews in Food Tech. 5(3):325-356.

Silliker, J.H., 1963. Total Counts as Indexes of Food Quality. In "Microbiological Quality of Foods". L.W. Slanetz et al. Editors. Academic Press, N.Y., p. 102-112.

Smith, D.G., and P.M.F. Shattock, 1962. The serological grouping and Streptococcus equinus. J. Gen. Microbiol. 29:731.

Snedecor, G.W., and W.G. Cochran, 1967. Statistical Methods, 6th Ed. Iowa State University Press, Ames, Iowa.

Speck, M.L., B. Ray, and R.B. Read Jr., 1975. Repair and enumeration of injured coliforms by a plating procedure. Appl. Microbiol. 29:549-550.

Steers, E, Jr., G.R.Craven, C.B. Anfinsen, and J.L. Bethune, 1965. Evidence for non-identical chains in the β-galactosidase of E. coli K-12. J. Biol.Chem. 240:2478.

Szturm-Rubinstein, S., and D. Piechaud, 1963. Observations sur la recherche de la β-galactosidase cans le genre Shigella. Ann. Inst. Pasteur (Ibid.) 104:284-291.

Tanner, F.W., and L.P. Tanner, 1944. "Foodborne Infections and Intoxications". 2nd Ed. The Garrand Press, Champaign, IU.

Taylor, J. Jr., R.L. Maston, N.J. Schneider, and A.W. Morrison Jr., 1967. Sanitary Quality of Salad Type Foods. Presented at Ann. meeting, Am. Public Health Assn., Inc., Miami Beach, Fla. Oct. 22.

Tennant, A.D., J.E. Reid, and L.J.Rockwell, 1959. An Evaluation of the E.C. (44.5°C) Confirmation of E. coli Densities in Sea Water and Shellfish. Manuscript Rept. 59-6, p. 1-37. Laboratory of Hygiene, Dept. of National Health and Welfare.

Thom, B.T., 1970. Klebsiella in faeces. Lancet 2:1033.

Thomas, S.B., 1955. Coli-aerogenes bacteria in raw milk. J. Appl. Bacteriol. 18:331.

Thomas H.A.Jr., R.L. Woodward, and P.W. Kabler, 1956. Use of molecular filter membranes for water potability control. J. Amer. Water Works Assoc. 48:1391-1402.

Tikhomirova, A.S., A.K. Kulikova, and R.V. Feniksova, 1972. Conditions for biosynthesis of β-galactosidase in Sacch fragilis. Mikrobiologiya 41(3):236-241.

Varga, S., R. Dobson, and R. Earle, 1977. Comparison of the ETPC and MPN procedure to estimate the densities of faecal coliforms and E. coli in soft shell clams. J. Food Protec. 40:763-764.

Wallenfels, K., and R. Weil, 1972. In "The Enzymes", Vol. 7, 3rd Ed. P.O. Boyer, Ed., Academic Press, New York, 1971, p. 618-663.

Warren, L.S., R.E. Benoit, and J.A. Jessee, 1978. Rapid enumeration of faecal coliforms in water by a colourimetric β-galactosidase assay. Appl. and Environ. Microbiol. 35(1):136-141.

Warseck, M., B. Ray, and M.L. Speck, 1973. Repair and enumeration of injured coliforms in frozen foods. Appl. Microbiol. 26:919-924.

Weber, K., H. Sand, and K. Wallenfels, 1964. Biochem. Z. 339:498.

Wendroff, W.L., C.H. Amundson, N.F. Olson, and J.C. Garver, 1971. J. Milk Food Technol. 34:294.

World Health Organization, 1974. "International Standards for Drinking Water". 3rd Ed. W.H.O. Geneva.

Wilson, C.R., O. Adrian, P. Pardon and W.B. Dockstader, 1971. Salmonella screening procedure with tests for β-galactosidase and Flagellar antigens. Appl. Microbiol. 21(2):346-349.

Wilson, G.S., R.S. Twigg, R.C. Wright, C.B. Hendry, M.P. Cowell, and I. Maier, 1935. The bacteriological grading of milk. Med. Res. Council Spec. Rept. Ser. 206.

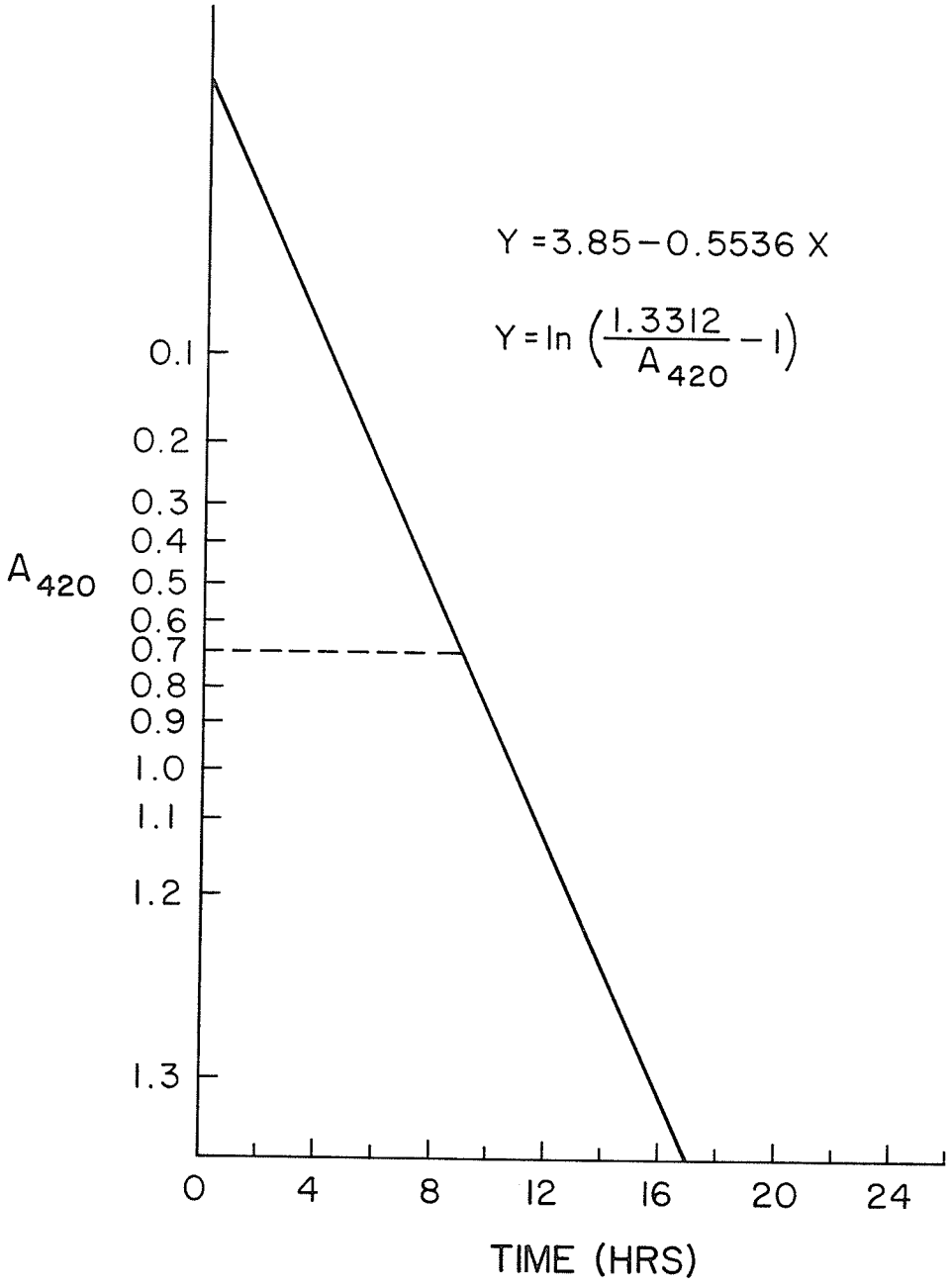
Wolfe, M.W., and E. Amsterdam, 1968. New diagnostic system for the identification of lactose-fermenting gram-negative rods. Appl. Microbiol. 16:1528-1531.

Wondolowski, M.V., and Woychik, J.H., 1974. Characterization and immobilization of E. coli (ATCC-26) β-galactosidase. Biotechnol. Bioeng. 16:1633-1644.

Woychik, J.H., and M.V. Wondolowski, 1973. Covalent bonding of fungal β-galactosidase to glass. J. Milk Food Technol. 36:31.

Appendix 1

Standard Curve for the Time Correction Factor



Appendix 2

Distribution of *E. coli* K-12 Cells in Milk upon Centrifugation

Trial	Treatment of Milk Sample		*E. coli* Density/ml	% Total Recovery
I	Before Centrifugation		3.0×10^4	
	After Centrifugation	Supernatant	4.9×10^2	0.004
		Sediment	1.31×10^7	99.996
II	Before Centrifugation		5.25×10^5	
	After Centrifugation	Supernatant	4.3×10^3	0.001
		Sediment	3.69×10^8	99.999
III	Before Centrifugation		1.35×10^5	
	After Centrifugation	Supernatant	9.0×10^1	0.001
		Sediment	3.02×10^7	99.9997
IV	Before Centrifugation		4.25×10^3	
	After Centrifugation	Supernatant	6.94×10^4	0.168
		Sediment	4.11×10^7	99.831

Appendix 3a

Statistical Analysis of Agar Plate Assay Data

Analysis of Variance (AOV)

3a

Effect of pH

SV	df	SS	MS	F
pH	3	8.6050	2.8683	87.4481**
Time	5	13.0317	2.6063	79.4606**
Error	39	1.2800	0.0328	
Total	47			

** $p < 0.05$

Appendix 3b

Statistical Analysis of Agar Plate Assay Data

Analysis of Variance (AOV)

Effect of Agar Concentration

SV	df	SS	MS	F
Agar Concentration	3	4.5973	1.5324	62.0405**
Time	5	4.6024	0.9205	37.2672**
Error	39	0.9624	0.0247	
Total	47	10.1621		

** $p \leq 0.05$

Appendix 3c

Statistical Analysis of Agar Plate Assay Data

Analysis of Variance (AOV)

Effect of Temperature of Incubation

SV	df	SS	MS	F
Temperature	2	4.2593	2.1297	57.5595**
Time	5	24.4087	4.8817	131.9378**
Error	28	1.0361	0.0370	
Total	35			

** $p < 0.05$