EFFECT OF GLYCOSIDASES AND PROTEASES ON BIOFILMS FORMED ON BLACK BUNA-N RUBBER

by

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(ABSTRACT)

Proteolytic enzymes and a glycolytic enzyme used in dishwashing detergents and by the starch conversion industry were examined for their ability to remove bacteria attached to black buna-N rubber. Pure culture and mixed culture biofilms of Pseudomonas fluorescens and Listeria monocytogenes were treated with the proteolytic enzymes Purafect® (Genencor International), Durazym™, and Savinase® and the glycolytic enzyme Termamyl® (Novo Nordisk BioChem North America). Compared to controls, none of the enzyme treatments were able to significantly remove P. fluorescens cells adherent in pure culture ($p>0.05$). Durazym™, Purafect®, and Termamyl® did significantly reduce the number of adherent cells of L. monocytogenes grown in pure culture. Treatment with Purafect® reduced the number of attached cells of both P. fluorescens and L. monocytogenes

when grown in mixed culture. Material which absorbs at 280 nm was released from both pure and mixed culture biofilms when all three proteolytic enzymes were used. No survivors remained after planktonic (free floating) cells of both P. fluorescens and L. monocytogenes were incubated with Purafect®. Reduction in overall numbers of P. fluorescens and L. monocytogenes attached in mixed culture by Purafect® appeared to involve a combination of release of proteinaceous material followed by bactericidal effects on exposed cells.

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Introduction

Lappin-Scott et al (1992) defined a biofilm as a complex association of microorganisms and microbial products attached to a surface. In the food industry, the formation of a biofilm can occur when bacteria present in food adhere to surfaces of processing or storage equipment. Milk, for example, can pick up microorganisms at any juncture along the route to pasteurization and even after pasteurization. Contamination can occur during milking, directly from the cow or milking parlor, or through shipping and storage at the processing plant. All food product contact surfaces can be potential sites of inoculation and subsequent biofilm formation. Stainless steel piping, Teflon surfaces, or rubber gaskets between joints are all susceptible sites for potential biofilm formation.

The formation of biofilms on food processing equipment poses many problems for the manufacturer, and more importantly, for the consumer. Biofilm formation can cause reduction in the efficiency of operation by decreasing flow rates, reducing heat transfer and by stimulating the corrosion process. In addition to the health hazards and fouling of processing equipment, bacteria in adherent biofilms can block filters and injection interfaces, foul products, generate harmful metabolites (such as hydrogen

sulfide (H_2S)) and corrode metal piping. Biofilms also provide protection for bacteria by increasing their resistance to heat, sanitizers, and disinfectants (Criado et al., 1994). All these factors can lead to considerable economic loss due to product recall, unacceptability, and/or potential health problems for consumers.

Various bacterial species have been shown to have very different adhesive capabilities depending on the nature and configuration of the polymers present on the bacterial surface (Criado et al., 1994). Studies investigating the microflora present in refrigerated milk have demonstrated the almost exclusive dominance of gram-negative psychrotrophs, especially members of the genus Pseudomonas (Cousin, 1982). Pseudomonas species are capable of reproduction in raw milk maintained at or below 7°C, with generation times as short as 8 hr. Pseudomonas fluorescens is the primary spoilage organism in milk and other dairy products and produces off-flavors, or off-odors, and decreased shelf-life. P. fluorescens is also responsible for textural defects such as slimy curd in cottage cheese.

Another microorganism of more significance to consumers is Listeria monocytogenes. L. monocytogenes causes listeriosis in the immunocompromised including fetuses, the

elderly, cancer patients, and people with other diseases which lower body immunity. L. monocytogenes has been implicated in foodborne listeriosis linked to milk, ice cream and other dairy products (Farber and Peterkin, 1991).

Microbial populations present in food products are almost never entirely homogeneous. Biofilms of mixed cultures of L. monocytogenes and Pseudomonas aerugenosa have been shown to have increased strength of adherence to beef tissue and also produce an increase in the number of attached bacteria compared to pure single species biofilms (Chung et al., 1989). When L. monocytogenes was grown in the presence of P. fragi, adherence to a glass collecting surface was enhanced significantly (Sasahara and Zottola, 1993)

Several studies have used enzymes to characterize the various stages of biofilm attachment. Two of the most widely accepted stages are reversible and irreversible adhesion. The exact nature of the adhesive material involved in these stages is not entirely known. Reversibly attached cells are predominately anchored by proteinaceous material such as flagella and pili and can be removed through washing or product flow. Irreversibly attached cells are more strongly attached by a thick mat of fibers that are predominately polysaccharide in nature. Moreover, enzyme studies have

demonstrated the possibility that proteinaceous material may also be involved in adherence during the irreversible stage of biofilm formation. Trypsin was shown to be effective in removing P. fragi attached in a biofilm to stainless steel (Herald and Zottola, 1989).

The objectives of this research were to determine the ability of crude industrial enzyme preparations to remove biofilm of single organisms (P. fluorescens alone, and L. monocytogenes alone) and mixed organisms (P. fluorescens and L. monocytogenes together). Black buna-N rubber were the surface on which the biofilms were grown.

Literature Review

Chapter 1: Biofilm Formation

Bacteria benefit significantly when attached to surfaces compared to living in the surrounding fluids (Lappin-Scott and Costerton, 1992). Biofilms are therefore prevalent throughout natural, industrial, and medical environments. Microorganisms, primarily bacteria, adhere to a wide variety of surfaces ranging from the human tooth, throat or lung tissue to stainless steel, rubber, and Teflon surfaces found in food and paper processing facilities. Biofilms have been found to form on conduits, condensers and heat exchange tubes in water and wastewater treatment facilities, and on remote sensors, submarine periscopes, and sight glasses used for water quality data collection. Even rocks submerged in fresh and salt water under conditions of stress, due to water flow, contain bacteria present in biofilms.

Bacteria form biofilms by excreting extracellular polymer fibers made of both proteinaceous and polysaccharide material. Pili, flagella and fimbrea make up the proteinaceous aspect of the biofilm. Glycocalyx is the term used to describe the polysaccharide material. Costerton et al (1981) defined glycocalyx as those polysaccharide-

containing structures of bacterial origin, lying outside the integral elements of the outer membrane of gram-negative and the peptidoglycan of gram-positive cells.

A. Mechanism of Attachment

Many theories have been proposed to describe the mechanisms involved in biofilm attachment. Some mechanisms proposed include: bacterial attraction to charged surfaces, gravity, brownian motion, chemoattraction when the surface contains nutrients (Lappin-Scott et al, 1992), charge on bacterial cells, hydrophobicity of bacteria and the collecting surface (Hood and Zottola, 1995), and the distance between the bacterial cell and the collecting surface (Busscher and Weekamp, 1987). The most widely held hypothesis concerning the mechanism by which bacteria adhere to surfaces is a time dependent process incorporating some of the theories mentioned above into two consecutive stages called reversible and irreversible attachment (Marshall et al., 1971, Costerton et al., 1987).

In 1991 Notermans et al described bacterial adhesion as occurring in three stages and labeled them 1) adsorption 2) consolidation and 3) colonization. During adsorption, physical forces such as van der Waals forces, ionic bonds, entropic contributions and bridging interactions appear to play a role (Notermans et al., 1991). Van der Waals forces

are weak interactions between permanent dipoles, instantaneous dipoles, and induced dipoles. These weak interactions result from attraction of opposite charges produced by fluctuations in distribution of electrons within the molecule or molecules or changes in electronic distribution induced by external electronic charges or electric fields (Biokess, 1985). Weak interactions are only consequential when the interacting species are in very close proximity or when numerous weak interactions occur. In a dynamic environment such as a food processing plant, close proximity to surfaces is frequently due to random contact of product flowing under turbulent conditions. Adsorption is a reversible step, and at this stage, the bacteria can still be removed via washing or product flow.

The next stage described by Notermans et al (1991) is called consolidation, one of the two irreversible stages. Once bacteria are adsorped to the surface, Firstenberge-Eden et al (1979) observed the production of thin fibers and extracellular slime. Herald and Zottola (1988a) concluded that the extracellular material surrounding bacterial cells was comprised of acidic-mucopolysaccharide. Herald and Zottola reported that the time required for bacterial consolidation varies with the genus and species of the microorganism and the incubation environment (pH,

temperature, nutrient content, and collecting surface). L. monocytogenes has been shown to attach irreversibly to stainless steel after just a few minutes of contact time (Butler et al, 1979, Smoot, unpublished 1996).

Close contact between the cell and the collecting surface is facilitated by bacterial cell appendages such as flagella and pili, but these appendages are not necessary. Different species of the same genera lacking flagella and pili have also been shown to adhere to surfaces (Lillard, 1985). Once contact is established, whether by cell appendages or random contact, bacterial cells initiate the process of irreversible adhesion by binding to surfaces using exopolysaccharide glycocalyx polymers. Colonization begins when the biofilm grows by internal replication and by trapping bacterial cells that are transported in the fluid phase of the product. Thus, biofilms are highly hydrated, predominately anionic matrices of bacterial exopolymers, living bacterial cells, and trapped extraneous macromolecules creating an anchored population that will grow and multiply (Sutherland, 1977). These polymers help the organism to proliferate without being removed. Nutrients can be concentrated from the product biofilm interface and favor the growth of bacteria on the collecting surface. Another advantage of biofilm formation is the ability of the

biofilm matrix to form a barrier to heat, sanitizers and detergents (Frank and Koffi, 1990).

B. Composition of Adhesive Material

A variety of laboratory techniques have been used to characterize the material involved in anchoring and attaching biofilms to collecting surfaces. Scanning electron microscopy (SEM), various staining techniques using ruthenium red and alcian blue, and glycolytic and proteolytic enzymes have been used to view, visualize and hydrolyze the material responsible for biofilm initiation and formation (Hood and Zottola, 1995). Components of the cell wall such as pili, flagella, lipopolysaccharide O antigen, and excreted polysaccharide fibers have been shown to contribute to the anchoring and attachment of biofilms (Notermans et al, 1991).

Protruding linear structures such as lipopolysaccharide O antigen and exopolysaccharides are components on the surfaces of many species of bacteria involved in the initiation of bacterial adhesion (Costerton et al, 1987). Highly structured appendages such as pili, fimbriae, and flagella, which are mainly composed of proteinaceous material, are also involved (Isaacson, 1985). Studies have shown increased attachment with flagellated organisms (Notermans & Kampelmacher, 1974, Butler et al., 1983), but

both chemotaxis and flagellar activity appear to be involved in greater accumulation of motile organisms. Lipopolysaccharide O antigens are somatic antigens that are a constitutive part of the cell wall and also extend beyond this barrier. These antigens consist of a complex of lipopolysaccharide and protein (Banwart, 1989) and appear to be instrumental in the initial adhesion process. However, lipopolysaccharide O antigens are not sufficient to establish colonization if the adherent cells do not also produce glycocalyx, the sticky exopolysaccharide (Costerton et al, 1987).

Exopolysaccharide-mediated adhesion is strong and resistant to shear forces, while pili are relatively fragile (Costerton et al., 1987). Bacteria have been shown to attach themselves to surfaces by means of a mass of tangled fibers of polysaccharides that extend from the cell surface (Costerton et al., 1978). The sticky glycocalyx has been proposed to consist of polysaccharides complexed with proteins and or lipids (Herald and Zottola, 1988a, Jones et al., 1969).

Corpe (1970) demonstrated the involvement of acidic polysaccharides in bacterial adhesion; Fletcher and Floodgate (1973) verified this phenomenon using scanning electron microscopy. Corpe (1970) identified components of

the polysaccharide layer from Pseudomonas atlantica as containing pyruvate and monosaccharides identified as mannose, glucose, galactose, and galacturonic acid. Corpe also found a 1:1 ratio between uronic acid and hexose. Herald and Zottola (1988a) used ruthenium red and alcian blue to stain the extracellular substances involved in the attachment of Pseudomonas fragi to stainless steel chips. These two stains contain cation groups which bind to polyanions and are specific for acidic polysaccharides and acidic mucopolysaccharides.

Fletcher and Floodgate (1976) described two types of polysaccharides, termed primary and secondary polysaccharides, respectively, responsible for reversible and irreversible adhesion of marine bacteria. Primary polysaccharides form a thin layer around both attached and non-attached cells and are thought to be responsible for initial attachment. Secondary polysaccharides are excreted after initial attachment and produce a firmer adhesion to the collecting surface. After secondary polysaccharide excretion the culture was transferred to a cation-deficient media, which produced a rapid dissociation of the secondary polymer. Fletcher and Floodgate (1976) postulated that Ca++ and Mg++ are important in maintaining this adhesive polysaccharide structure.

Streptococcus mutans adheres to dental surfaces as well as other surfaces by means of an α-1,3 linked glucan (Johnson et al., 1977). Sutherland (1980) proposed that a high incidence of 1,3 or 1,4 linkages would confirm some degree of rigidity to the polymer; α-1,3 linkages would be relatively water insoluble. Rigidity and water insolubility of the polysaccharide material involved in the biofilm matrix would provide a strong protective barrier that would remain intact even in the presence of product flow and water-based cleaners and sanitizers.

Beech and Gaylarde (1989) found that a glycosidase and N-acetyl glucosaminidase reduced adhesion of P. fluorescens to mild steel when the biofilm was grown in the presence of these enzymes. Beech and Gaylarde also found that only N-acetyl glucosaminidase reduced the number of attached cells of Desulfovibrio desulfuricans. Based on these results, both glucose and N-acetyl glucosamine appear to be involved in adhesion of P. fluorescens. A di- or polymeric form of N-acetyl glucosamine is associated with D. desulfuricans adhesion.

C. Industrial Significance

Biofilms in food processing environments can collect on surfaces of processing equipment and food products before, during, and after processing. Some consequences of biofilm formation of industrial significance include: microbial corrosion, increased flow resistance, blockages and decrease in heat exchange efficiency, decreased efficiency of detergents and sanitizers, and attachment to product surfaces. Decreases in product quality and appearance are minor consequences of biofilms in food processing facilities compared to the increased health risks these industrial problems pose.

Corrosion of metals due to bacterial colonization is an economically important consequence of bacterial formation that illustrates several fascinating aspects of the structure and physiology of the adherent bacterial populations. Bacterial corrosion is an activity of structured bacterial biofilms in which physiochemical differences between adjacent loci on the metallic surface are created and maintained by differential metal binding and metabolic activity until deep corrosion pits have been produced. In the past, these corrosion pits were removed by regular scraping, also known as pigging, of the pipelines. Pigging is a labor-intensive, time-consuming process

designed to disturb the highly structured bacterial corrosion cells. After pigging of the pipelines, several days are required to reestablish the structure and activity of bacterial biofilms (Costerton et al., 1987). In addition, corrosion due to biofilm formation decreases the life of processing equipment which is a huge expenditure for the food manufacturer.

Biofilms which have formed on product contact surfaces cause a decrease in product flow rates as well as an increase in friction between the product and food contact surface. A decrease in product flow rates can produce an overprocessed product which can become organoleptically unacceptable to consumers. Bacteria have been known to colonize the water-cooled side of metal surfaces in heat exchangers and reduce heat exchange efficiency. The resultant biofilm insulates against heat exchange so effectively that exchange efficiency can be gradually reduced to as low as <10% of designed values (Costerton et al., 1987). Microorganisms which are able to survive the heat step can form biofilms on processing equipment further down the processing line. Biofilm can become dislodged and contaminate post processed food resulting in decreased product shelf-life and potential safety problems for consumers (Criado et al, 1994).

Once a biofilm becomes established on a food product contact surface, the thick mat of polysaccharide and proteinaceous fibers act as a barrier to cleaners and sanitizers. In the dairy industry, Clean-in-place (CIP) is the most common means of continuously cleaning and sanitizing product processing equipment. CIP systems have been shown to adequately clean and sanitize processing equipment. CIP systems must be operated for the designated times and at the designated temperatures recommended by manufacturers of detergents and sanitizers (Dunsmore et al., 1981a). If these strict guidelines are not followed a biofilm could develop and the CIP cleaning system would no longer have the ability to remove contaminating bacteria fouling processing lines. Resistance of bacteria in a biofilm to heat and sanitizers is covered more extensively later.

Notermans and Kampelmacher (1974) studied the attachment of E. coli, Lactobacillus brevis, a Klebsiella species and three Pseudomonas species to the skin of broiler chickens. As chicken meat is prepared for shipment to consumers, carcasses are dipped in a washing solution at poultry processing plants. This washing solution can be a source of cross contamination of microorganisms from carcass to carcass. Biofilms on the skins of the birds slough off

into the wash solution and contaminate the next bird to be rinsed. Dickenson and Koohmarie (1989) reported adherence of Bacillus subtilis, E. coli O157:H7, L. monocytogenes, Salmonella typhimurium, S. marcescens, S. aureas, and S. epidermidis to the surfaces of both lean and fat red meat tissue. Poultry skin and the surfaces of red meats containing E. coli O157:H7, Salmonella sp., S. aureus, L. monocytogenes or other pathogens present in a biofilm are a source of cross contamination in the home and illness to consumers.

Chapter 2: Pseudomonas fluorescens

Milk is rich in nutrients and has a pH close to neutrality; both factors make for a highly preferred media for growth by many microorganisms (Criado et al, 1994). Pseudomonas species are psychrotrophs and are the predominant organisms found in cold stored milk (Cousin, 1982). Present day collection and storage of raw milk in refrigerated tanks has lead to improvement in the bacteriological quality of milk. However, refrigeration temperatures allow the growth of psychrotrophic bacteria, many of which are able to produce thermostable extracellular proteases and lipases which decrease product acceptability and shelf-life. Lipolysis and proteolysis of milk products by Pseudomonas species have been associated with a bitter flavor and rancidity as well as a fruity odor. In cottage cheese, Pseudomonads produce a slimy curd defect on the surface and a fruity, putrid or rancid odor (Fairbairn and Law, 1986, Law et al., 1979).

A. Characteristics

P. fluorescens, biovar I, ATCC # 13525 is the type strain for this genus species. This organism can be isolated from soil and water, after enrichment in media containing various carbon sources and incubated aerobically (Palleroni,

1984). Since members of the genus Pseudomonas are aerobic, they possess a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. P. fluorescens is commonly associated with spoilage of foods such as eggs, cured meats, fish and milk, and is often isolated from clinical specimens (Palleroni, 1984). The optimum growth temperature is between 25 to 30 °C, but also has the ability to grow between 4 °C and 40 °C. Some distinguishing characteristics of P. fluorescens are motility by one or more polar flagella, production of oxidase, gelatin liquefaction and production of lechithinase which can be viewed on egg yolk agar by zones of clearing. No fimbriae have been observed in strains of P. fluorescens. (Palleroni, 1984).

Fluorescent pigments, pyocyanin and pyoverdin, are produced abundantly by fluorescent Pseudomonads in media of low iron content. Depending on the pigment, fluorescence varies from white to blue-green upon excitation with ultraviolet radiation. Pyocyanin, the pigment produced by Pseudomonas aeruginosa, can be visualized using wavelengths of 400 nm or lower. Pyoverdin, the fluorescent pigment produced by P. fluorescens, can be distinguished from other fluorescent pigments by examination of the cultures on solid

media under a source of ultraviolet light at 254 nm (Palleroni, 1984).

Most Pseudomonas strains can be maintained on slants of common bacteriological media such as nutrient agar. However, other standard complex media or various chemically defined media, with the addition of 0.5% yeast extract and lactate or glycerol can also be used. Transfers should be made every 1-2 months onto slants maintained at 4-8 °C (Palleroni, 1984).

B. Selective Media

King's medium B has been shown to enhance production of pyoverdin, the fluorescent greenish-yellow pigment produced by Pseudomonas fluorescens (King et al, 1954). King et al (1954) attributed the presence of phosphates in proteose peptone No. 3 with the ability to greatly enhance pyoverdin production and visualization. Proteose peptone No.3 contains more phosphorus (0.46%) than average peptones (0.079%). King et al (1954) also found the presence of sodium ions and phenylalanine to be detrimental to the production of pyocyanin, another fluorescent pigment produced by Pseudomonas species.

King's B can be used in conjunction with three antibiotics to select and differentiate fluorescent

Pseudomonads. Resistance to a broad spectra of antibiotics is a common feature of most fluorescent Pseudomonads. Sands and Rovira (1970) found that penicillin (75,000 units/L), novobiocin (45,000 ug/L), and cycloheximide (75,000 ug/L) limited the growth of nearly all fungi and bacteria except fluorescent Pseudomonads. The addition of these antibiotics to King's B medium, or other standard diagnostic media, produces a highly selective medium which is useful for detecting very low numbers of fluorescent Pseudomonads in soil samples (Sands & Rovira, 1970, King et al., 1954). Palleroni (1984), editor of Bergey's Manual of Systemic Bacteriology, endorse King's B containing the three antibiotics penicillin G, novobiocin, and cycloheximide as a selective medium for isolation of pyoverdin producing bacteria, such as P. fluorescens.

C. Role in Milk and Dairy Product Spoilage

In a study performed by Poffe and Mertens (1988), 143 species of psychrotrophic bacteria were isolated from 53 cooled raw bulk milk samples. The most frequently encountered isolates were P. fluorescens and P. fluorescens-like organisms. The main psychrotrophic microflora of milk were gram-negative rods with Pseudomonas species comprising 50% of the genera found in milk stored in cooling tanks (Stead, 1986). In another study of bacteria found in cold

milk storage tanks, Cousins & Bramley (1981) found that P. fluorescens predominated with the occurrence of other Pseudomonas species including P. putida, P. fragi, and P. aeruginosa less pronounced.

Growth of psychrotrophic bacteria in milk does not in itself cause spoilage (Law, 1979) and most gram-negative psychrotrophs are readily eliminated by pasteurization (Witter, 1961). However, many species produce heat resistant extracellular proteinases (Fairbairn & Law, 1986) and lipases (Cousin, 1982) that can withstand pasteurization and remain active in products made from heat-treated milk. Heat stable proteinases produced by Pseudomonas species have been associated with reduced yields of soft cheeses (Cousin, 1982) and gelation of UHT-sterilized milk (Law et al., 1977). Bacterial lipases produce flavor defects associated with fat breakdown and the release of free fatty acids in cream, butter, cheese and ultra-heat treated products (Cousin, 1982).

Chapter 3: Listeria monocytogenes

L. monocytogenes was first described in 1926 by Murry, who provided the name Bacterium monocytogenes because of a characteristic monocytosis found in infected laboratory rabbits and guinea pigs. Pirie in 1927 renamed the organism Listerella hepatolytica and in 1940 supplied the present name (Grey and Killinger, 1966). L. monocytogenes was first isolated in 1929 from sheep by Gill and from humans by Nyfeldt (Grey and Killinger, 1966); subsequent isolation from cows, (McCarthy, 1990), and other farm animals followed (Grey and Killinger, 1966). L. monocytogenes is widely distributed in nature and can be isolated from water, mud, sewage, vegetation, and the feces of animals and humans (Seeliger and Jones, 1986). Transmission from host animal or soil to food products is known to occur. Dairy products, as well as other farm fresh products, can be potential vehicles for transmission of L. monocytogenes which can cause listeriosis, a disease affecting immunocompromised individuals.

A. Characteristics

L. monocytogenes is a gram-positive, non-sporeforming, facultatively anaerobic rod which grows between -0.4 and 50 °C (Junttila et al, 1988); optimum growth temperature occurs

between 30 and 37 °C (Seelinger and Jones, 1986). Expression of β-hemolysin can be visualized on blood agar by the production of zones of clearing. L. monocytogenes produces catalase, does not produce oxidase, and possess peritrichous flagella, which give it a characteristic tumbling motility when grown between 20 and 25 °C. Growth in this temperature range produces flagella that are assembled at the cell surface (Seelinger and Jones, 1986). Growth at 37 °C greatly reduces the production of flagella (Peel et al, 1988).

Colonies of L. monocytogenes produce a characteristic blue-green sheen when viewed under obliquely transmitted light (Farber and Peterkin, 1991). L. monocytogenes is widely present in plant, soil, and surface water samples (Weis and Seeliger, 1975) and has also been found in silage, sewage, slaughter house waste, milk of normal and mastitic cows, and human and animal feces (McCarthy, 1990). Grey and Killinger, (1966) frequently isolated L. monocytogenes from cattle, sheep, goats, and poultry, but rarely from wild animals. L. monocytogenes is a normal resident of the human intestinal tract which may explain the reason antibodies to Listeria species are commonly found in healthy humans (Seelinger and Finger, 1976). At any one time, approximately 5 to 10% of the general population may be carriers of the

organism (Farber and Peterkin, 1991).

Carbohydrate fermentation by Listeria species was reported by Pine et al (1989). Under anaerobic conditions, only hexoses and pentoses support growth; under aerobic conditions, maltose and lactose, but not sucrose, support growth. L. monocytogenes and L. innocua utilize glucose, lactose, and rhamnose under aerobic conditions. Because of the need for these sugars growth is enhanced by the addition of glucose (0.2-1% w/v).

Listeria species grow well on usual bacteriological media such as Blood Agar Base No. or Tryptose Agar with 0.6% yeast extract (Seelinger and Jones, 1986). Yeast extract is added to provide biotin, riboflavin, thiamine, thioctic acid and several amino acids including cysteine, glutamine, isoleucine, leucine and valine required for growth. Cultures grown on solid media containing glucose have a characteristic sour, buttermilk-like odor. All strains grow best at neutral to slightly alkaline pH (Seelinger and Jones, 1986).

B. Listeriosis

Many pathogenic bacteria have the ability to invade host tissues by inducing their own endocytosis, with subsequent transport across the normally protective barrier of the cell wall. This phenomenon, called parasite-directed

endocytosis, seems to be the method used by L. monocytogenes to gain entry into intestinal cells and macrophages and cause listeriosis in immunocompromised hosts (McGee et al, 1988).

Listeriosis may well be the leading fatal food-borne infection in the United States (Gellin et al, 1991). The infective oral dose for 50% of mice studied ranges from 1.7e3 to 9.9e6 colony forming units (Golnazarian et al, 1989). The major factors influencing infective dose are bacterial strain differences and host factors. The majority of cases of listeriosis occur in individuals who have an underlying condition which leads to suppression of their T cell-mediated immunity. Some of the predisposing conditions which are often associated with listeriosis include neoplastic disease, immunosuppression, pregnancy, extremes of age, diabetes mellitus, alcoholism, cardiovascular and renal collagen diseases, and hemodialysis failure (Neiman and Lorber, 1980). However, apparently healthy individuals have occasionally become ill with listeriosis in both food-borne epidemics (Schlech et al., 1983) and sporadic cases (Azadian et al., 1989).

In 1989, a summary of listeriosis cases from 16 countries showed that 31% and 22% of the total cases occurred in patients older than 60 years and younger than 1

month, respectively (Azadian et al., 1989). The clinical symptoms associated with adult listeriosis include central nervous system infections and primary bacteremia, but can also include endocarditis (Farber and Peterkin, 1991). Meningitis occurs mainly in the elderly and immunocompromised adults. In 1988, 43% of the 782 reported cases of listeriosis from 20 countries were maternal and neonatal infections, 29% were septacemic infections, 24% were central nervous system infections, and 4% were atypical forms (Rocourt, 1989).

Two clinical forms of neonatal listeriosis are known to occur, early and late onset. The mean incubation time for occurrence of symptoms in early onset listeriosis is 1.5 days and presumably occurs in infants infected in utero. Manifestations of neonatal listeriosis include respiratory distress syndrome, rash, purulent conjunctivitis, pneumonia, hyperexcitability, vomiting, cramps, shortness of breath, shock, menatologic abnormalities, and either hyper- or hypothermia (Kessler and Dajani, 1990). In late-onset neonatal listeriosis, the mean onset of symptoms is 14.3 days, with meningitis as the predominate form of the disease.

C. Reported Cases

In 1983, 14 of the 49 people infected with listeriosis (7 were perinatal cases) died in Massachusetts after consuming pasteurized milk. Evidence of improper pasteurization at the plant could not be found. This report was the first to indicate a possible increased heat resistance of L. monocytogenes. Doyle et al (1987) suggested that an intracellular location of the organism due to endocytosis gives added protection against pasteurization. However, Bunning et al (1988) found that the intracellular position does not protect L. monocytogenes from pasteurization and that contamination post pasteurization was far more likely.

Doyle et al., (1987) used raw milk from listeric cows to demonstrate the increased heat resistance of L. monocytogenes during high temperature, short time pasteurization conditions. Listeric cows produce milk with L. monocytogenes contained in the leukocytes. Under these conditions, L. monocytogenes was able to survive a minimum heat treatment of 72.2 °F for 16.4 s. Bunning et al (1988) inoculated sterile milk with cells phagocytized under laboratory conditions to produce intracellular L. monocytogenes, and found that L. monocytogenes was not able

to survive high temperature short time pasteurization conditions. These findings lead Bunning et al (1988) to conclude that post pasteurization contamination was a more probable cause of the Massachusetts outbreak than inadequate thermal processing. Biofilms present on pasteurization heat exchangers or on processing equipment located after pasteurization could be responsible for post pasteurization contamination.

The Center for Disease Control (CDC) reported an outbreak occurring in California, lasting from January through August 1985, due to the consumption of contaminated Mexican style cheese. Of the 142 reported cases, 93 were perinatal and 49 were adult, with a total of 48 deaths (34% mortality rate) involving 30 fetuses and newborn infants and 18 nonpregnant adults. Among the 49 adult cases, 48 were immunosuppressed or elderly or had a severe chronic illness (Linnan et al, 1988). In August 1985, soft cheese produced in Ohio was found to contain L. monocytogenes and was recalled before any illness occurred. Ice cream (Berche et al, 1988), ice cream sandwiches, and ice cream bars (Matyunas, 1987) infected with L. monocytogenes have also been implicated in food-borne outbreaks and product recalls.

Overall worldwide incidence of L. monocytogenes in raw milk appears to be approximately 2.2% (Farber & Peterkin,

1991). Survival during manufacturing of dairy products such as cultured buttermilk, butter, and even yogurt, and the ripening of many different cheeses in the soft style varieties, such as Camembert and cottage cheese, has been shown (Farber & Peterkin, 1991). Enhanced growth of L. monocytogenes in milk has been shown in the presence of other psychrotrophic bacteria, including members of the genus Pseudomonas (Marshall and Schmidt, 1988).

D. Epidemiology

L. monocytogenes is an emerging pathogen. The incidence of listeriosis appears to be on the increase worldwide, with the number of cases rising especially in Europe (Bille 1990, Campbell 1990, McLauchlin 1989). The annual endemic disease rate varies from 2 to 15 cases per million population for 1986 (Farber and Peterkin, 1991). As the population ages and number of immunocompromised individuals increases, due to diseases such as AIDS and cancer, listeriosis becomes more of a threat to an increasing segment of the population.

All thirteen serovars of L. monocytogenes are capable of causing listeriosis in humans, however serovars 1/2a, 1/2b and 4b are involved in the most cases (Farber and Peterkin, 1991). Serovar 4b predominates in most of Europe, and an even distribution of serovars 1/2a, 1/2b, and 4b occur in Canada and the United States. No direct links have

been made between particular forms of listeriosis and certain serotypes, but recent work has shown an epidemiologic association between perinatal listeriosis and serovars 1/2a, 3b, and 4b (Gellin et al, 1991).

Chapter 4. Characteristics of Attached Microorganisms

Bacteria are found in every habitat on earth, even under the most extreme environmental conditions of temperature, pH, and dissolved minerals such as sulfur and sodium chlorine. The majority of bacteria in natural environments are found attached to available surfaces and not suspended in the liquid phase. Geesey et al (1978) showed that the organism surface count per cm^2 on submerged rocks in the Marmot Basin (Alberta, Canada) was 5 X 10^2 to 10^4 higher than in the surrounding flowing water. An increased presence of attached bacteria compared to planktonic bacteria has also been observed in medical, dental, industrial and agricultural environments (Lappin-Scott and Costerton, 1989).

Most environments that support bacterial survival are rarely able to supply the total nutrients necessary for uninhibited growth and therefore starvation conditions are not uncommon. Food plant cleaning procedures, for example, remove nutrients from surfaces providing starvation or low nutrient microenvironments. Conditions of nutrient limitation have been shown to increase the number of attached bacteria (Kjelleberg et al., 1983, Kjelleberg and Hermansson, 1984) and also increase resistance to biocides (Ren and Frank, 1993). Overall, attachment to surfaces,

whether starvation induced or not, provides many advantages including increased resistance to heat (Frank and Koffi, 1990, Hood and Zottola, 1995) and biocides such as sanitizers (Frank and Koffi, 1990, Mosteller and Bishop, 1993, Ronner and Wong, 1993, Stone and Zottola, 1985, Wirtanen and Mattila-Sandholm, 1992).

Bacterial attachment to a wide variety of surfaces by an equally diverse group of microorganisms has been the subject of many studies. Some examples of the microorganisms and surfaces involved in bacterial adherence studies include attachment of Pseudomonas aeruginosa to medical prosthetic devices (Anwar et al., 1990), E. coli, Lactobacillus brevis, a Klebsiella species and three Pseudomonas species to surfaces of poultry and red meat (Dickenson and Koohmarie, 1989, Notermans and Kampelmacher, 1974, Wirtanen and Mattila-Sandholm, 1992,). Marine Vibrio DW1, Spirillum species, Flavobacterium species, Pseudomonas species, Serratia marcescens and Acintobacter calcoaceticus have been documented to attach glass (Dawson et al., 1981, Kjelleberg and Hermansson, 1984,) and P. fluorescens, L. monocytogenes and Salmonella typhimurium, to stainless steel and rubber (Frank and Koffi, 1990), as well as L. monocytogenes to polypropylene (Mafu et al., 1990), and polyester (Krysinski et al., 1992). This list is by no means all inclusive.

A. Starvation

Adherent bacterial cells grown under conditions of limited nutrients have been observed to attach in greater numbers while decreasing in cell diameter. Production of exopolysaccharide material during starvation conditions seems to vary greatly depending on the organism and the nutrient limited. In the absence of nutrients, some bacterial species can achieve a minimum spherical volume more quickly than can cells in the liquid phase (Humphrey et al., 1983). The simultaneous decrease in cell size and production of exopolysaccharide material by some species allows the bacteria to penetrate deeper into crevices and pores of surfaces than full-sized vegetative cells (MacLeod et al., 1988). Concentrations of nutrients in liquid media are very low under starvation conditions, but available nutrients concentrate near surfaces. Therefore, adherence to surfaces during nutrient limitation may be a survival technique (Hood and Zottola, 1995).

Brown et al (1977) showed that, under conditions of glucose limitation, a mixture of bacteria isolated from a marine environment, including Vibrio species, Aeromonas species, Flavobacterium species, and Pseudomonas species, adhered to aluminum foil discs in greater numbers. Examination of the surface of the aluminum foil discs using

scanning electron microscopy did not detect the presence of an adhesive polymer. Klebsiella aerogens produced exopolysaccharide material from glucose under nitrogen, sulfate, and phosphate limitation but not under carbon limitation (Niejssel et al., 1975). Niejssel and colleagues (1975) suggested that exopolysaccharide production is the product of what they called overflow metabolism. Overflow metabolism occurs when nutrients are in excess, however, when nutrients are limited under starvation conditions some species can not muster the energy necessary to produce an exopolysaccharide adhesive material.

Starvation studies conducted on marine Vibrio DW1 for periods of time ranging from 5 hr to 5 days showed a decrease in cell volume, termed dwarfing, and a decline in total numbers and overall viability for all time periods investigated (Dawson et al., 1981). The outer surface of the dwarfed cell contained bridging polymers that were probably responsible for increased adhesiveness displayed by dwarfs. Dawson et al (1981) did not determine composition of the bridging polymers. Once these polymers were formed, the dwarfs were able to retain their stickiness despite the fall in viability as starvation time progressed. Dawson et al (1981) also noted an increase in the rate of attachment as the length of prior starvation increased and that the

starved dwarfs were more strongly attached than normal cells.

Kjelleberg and Hermansson (1984) also noted dwarfing of cells and surface changes of a marine Vibrio species, Flavobacterium species, Pseudomonas species, Serratia marcescens, and Acinetobacter calcoaceticus that lead to increased adhesion. The smaller cell volume experienced by starved cells was suggested to be a sequence of two processes, namely: fragmentation, which is division without growth (Novitsky and Morita, 1977, Kjelleberg and Hermansson, 1984), and continuous size reduction (Dawson et al., 1981, Kjelleberg et al., 1983 and 1984), which results in an increase in populations of smaller cells. Other changes in the outer cell topography were observed during starvation involving an increase in surface roughness (Kjelleberg and Hermansson, 1984) and the formation of fibrils that increased surface adhesion (Dawson et al., 1981.

B. Increased Heat Resistance

Bacteria present in biofilms have shown increased resistance to heat when compared to planktonic or free floating cells (Frank and Koffi, 1990). The outer polysaccharide layer surrounding the bacteria in a biofilm seems to be instrumental in providing protection against

temperatures used during cleaning, sanitizing and food processing. Once these bacteria are able to escape the heat step and cleaning temperatures they can cause contamination of post processed food and food contact surfaces (Hood and Zottola, 1995).

L. monocytogenes attached to glass slides in low nutrient medium containing excess glucose was the subject of a heat resistance study performed by Frank and Koffi (1990). Planktonic cells and microcolonies adherent after 14 days incubation were exposed to temperatures of 55 °C and 70 °C for 5 min. Planktonic cells at a level of 10^8 cfu/ml were decreased to a level of 10^1 cfu/ml after heating to 55 °C for 5 min. Adherent microcolonies were decreased from 10^6 to 10^5 cfu/ml after the same treatment. Planktonic cells were reduced to undetectable levels by the 70 °C treatment, a greater than 7 log decrease in number. Cells in adherent microcolonies were reduced by less than 5 log units by heating at 70 °C for 5 min. A population of over 10 cfu/ml survived this heat treatment. This study indicates that bacteria attached to a surface have a far greater resistance to heat than free floating cells.

Lee and Frank (1991) studied the effect of heat on L. monocytogenes adherent in a biofilm to stainless steel.

Single celled adherent populations were produced after 4 hr and microcolonies were produced after 8 days incubation. Both populations were present at a pre-treatment level of 10^5 cfu/cm^2. Cell numbers of both populations were reduced by 3.8 log units after treatment at 65 °C for 30 sec. Microcolony cells were still detected after 3 min, however, single adherent cells were not detected after the same treatment. After 30 sec of treatment at 72 °C, none of the single adherent cells were detected. Less than 1 log cfu/cm^2 of the microcolony cells remained after 30 sec and none after 1 min.

These two studies produced different degrees of heat resistance for L. monocytogenes on two different surfaces, stainless steel and glass. Cell numbers of adherent Listeriae on stainless steel were greatly decreased when heated to 65 °C and 72 °C (Lee and Frank, 1991). However, 5 min at 70 °C achieved only a 5 log unit decrease in numbers of cells attached to glass (Frank and Koffi 1990). Longer survival of L. monocytogenes attached to glass could be a result of the greater cell density on glass (10^6 cfu/cm2) verses stainless steel (10^5 cfu/ml). The greater density of cells may produce increased heat resistance because a higher population is associated with more and larger clumps. In

addition, glass is less heat conductive than stainless steel and may provide an insulation effect during heat treatment (Lee and Frank, 1991).

Butler et al (1980) documented an increase in heat resistance of Pseudomonas putrefaciens and Lactobacillus species attached to surfaces of pork skin. Experiments were performed on P. putrefaciens attached to pork skin at a level of 10^4 cfu/cm^2 and planktonic cells grown to a level of 10^6 cfu/ml. Lactobacillus species attached to pork skin to a level of 10^3 cfu/cm^2 and planktonic cells were grown to a level of 10^6 cfu/ml. When skin with attached P. putrefaciens was heated for 13 min at 43.5 °C, a 1.4 log reduction was shown. No survivors were detected after unattached P. putrefaciens cells were heated in buffer to 42°C for 13 min. Attached P. putrefaciens experienced a 1.6 log reduction when heated to 45 °C for 13 min, no survivors were recovered after planktonic cells were exposed to the same conditions. Lactobacillus species attached to pork skin experienced a 1.8 log reduction when exposed to 55 °C for 13 min, no survivors were recovered when planktonic cells underwent the same treatment.

Results of this study showed that P. putrefaciens and the Lactobacillus species attached to pork skin exhibit

greater heat resistance than unattached bacteria. Butler et al (1980) concluded that two conditions may be responsible for the greater heat resistance of attached bacteria: a) the protective effect of the skin when bacteria are trapped in capillary crevices and b) the protective effect of substances such as polysaccharides formed by bacteria in the attachment process. Existence of both of these phenomenon are supported by data obtained using scanning electron microscopy.

C Increased Resistance to Sanitizing Agents

Attachment of microorganisms to food contact surfaces poses many problems for the food industry. If adherent bacteria loosen and detach from a food contact surface, contamination of the food product could result. Industrial use of sanitizing agents is one method of preventing contamination of food product contact surfaces. Unfortunately, evidence exists to suggest that if cleaning and sanitizing procedures are not followed to specifications provided by the manufacturers, bacteria adherent in biofilms can continue to cause problems. Additional laboratory studies indicate that sanitation practices are less effective on biofilms than on free floating planktonic cells.

Frank and Koffi (1990) showed increased resistance of

L. monocytogenes cells present in a biofilm attached to glass slides when sanitizing agents benzalkonium chloride (n-alkyl dimethyl dichlorobenzyl ammonium chloride, BAC) and acid anionic sanitizer (dodecyl benzene sulfonic acid, DBSA) were applied (Frank and Koffi, 1990). The inactivation study included L. monocytogenes attached as single cells grown on glass slides for 4 hr, attached microcolonies grown on glass for 14 days, and planktonic cells. Attached single cells exhibited a 3 to 5 log decrease after 30 sec exposure to BAC and were unrecoverable after 12 to 16 min of treatment with DBSA. Adherent single cells could not be recovered 16 min following treatment with 100 ppm BAC and 12 min after exposure to 400 and 800 ppm BAC. Microcolonies experienced a 2 to 3 log decrease in cell numbers the first 30 sec of exposure. However, a resistant population remained after 20 min of exposure to 100, 400, and 800 ppm of both BAC and DBSA demonstrating resistance to both sanitizers at all concentrations. When planktonic cells were grown in broth, inactivation occurred in just 30 sec at all concentrations of both BAC and DBSA (Frank and Koffi, 1990).

Increased resistance to sanitizers was attributed to the lipophilic nature of the outer cell membrane of the gram-positive Listeria. Production of extracellular lipoteichoic acids and lipopolysaccharides may prevent

penetration of sanitizers. In addition, adherent cells used in this study were grown with excess glucose in the medium to stimulate production of protective glycocalyx. However, no attempt was made to affirm the presence of the glycocalyx.

Krysinski et al (1992) studied the effect of a variety of chemical cleaning and sanitizing compounds on the removal and/or inactivation of a multistrain cocktail of L. monocytogenes. A 24 hr biofilm was grown on stainless steel, polyester, and polyester/polyurethane. The chemical cleaners included a chlorinated alkaline detergent, an alkaline detergent, a mild, non-alkaline anionic detergent, a complex detergent containing chlorine dioxide, an alkaline quaternary ammonium peroxide and a broad spectrum enzymatic cleaner. The sanitizers included sodium hypochlorite, iodophor, a neutral quaternary ammonium compound, an acidic quaternary ammonium, a mixed halogen, an acidic anionic sanitizer, an acidic octenyl succinic anhydride, peracetic acid, chlorine dioxide, and a C_8-C_{10} fatty acid. Chemical cleaners and sanitizers were used at concentrations and temperatures recommended by the manufacturers except that exposure times were standardized to 10 min.

L. monocytogenes in the planktonic state experienced a 5 log reduction when exposed to 200 ppm chlorine, 200 ppm

neutral quaternary ammonium compound, and 25 ppm iodophor for only 30 sec (Lopes, 1986). The same sanitizers had little effect on attached cells even when exposure time was increased to 10 min (Krysinski et al., 1992). Efficacy of the cleaners and sanitizers was largely dependent on the surface. When attached to stainless steel, all sanitizers studied, with the exception of chlorine and iodophor, were effective in inactivating the adherent microorganisms. However, none of the sanitizers or cleaners were effective when Listeria species were attached to the polyester/polyurethane mix. In general, the chemical cleaners were much more effective in eliminating L. monocytogenes attached to stainless steel, particularly the chlorinated alkaline detergents and the alkaline QAC peroxide.

Stone and Zottola (1985b) used a specially constructed stainless steel piping system to study the effects of two different cleaning procedures on the removal of attached P. fragi. The first cleaning procedure (A) started with an alkaline detergent, followed by an acid detergent, and ended with a sodium hypochlorite sanitizer. The second cleaning procedure (B) started with an acid sanitizer, followed by an alkaline detergent, and ended with the same acid sanitizer. When proper cleaning procedures were followed as directed by

the manufacturers cells remaining in the system did not exhibit attachment fibrils and were not viable. Under suboptimum cleaning conditions of lowered detergent water temperature and detergent and sanitizer concentrations, cells present after CIP cleaning showed attachment fibrils and were viable.

Under suboptimum cleaning conditions, the acid detergent step of procedure A was more effective in removing the attached cells compared to the acid sanitizer of procedure B. The main purpose of an acid detergent is to remove mineral buildup. Stone and Zottola, (1985b) concluded that the acid detergent may also be altering the mechanism necessary for maintenance of the adhesive polymer. Fletcher and Floodgate (1976) found that when a fimbriated marine Pseudomonad was grown in the absence of Ca^{++} and Mg^{++}, production of the secondary polysaccharide material was greatly disrupted. Therefore, the acid detergent could be more effective than the acid sanitizer at removing Ca++ ions needed to maintain the adhesive structure.

Chapter 5: Attachment of Microorganisms to Dairy Processing Equipment

Milk is a complex media containing mineral and organic components which provide nutrients for bacterial growth. During processing many of these components are deposited on food contact surfaces. Residue buildup provides a source of nutrients as well as serves to protect the associated bacteria from cleaning products and disinfectants. The microtopography of the collecting surface also plays a role in increasing bacterial attachment, particularly if the surface has deep channels and crevices in which to trap bacteria. Speers et al (1984, 1985) reported a positive correlation between the degree of surface roughness and the number of bacteria attached. Scanning electron microscopy has been extensively used to examine the presence of bacteria trapped on the surfaces of Teflon, factory floor tile, aluminum, stainless steel, rubber, and glass.

Notermans et al (1991) used scanning electron microscopy to view Escherichia coli attached to five different surfaces (Teflon, floor tile, stainless steel, aluminum, and rubber) found in food processing facilities. Although, Teflon and tile surfaces were smooth and possessed few indentations and crevices, organisms appeared to be able

to attach. In contrast, the stainless steel surface contained channels and crevices of a width equivalent to that of the E. coli. The aluminum surface had even more channels and a sponge-like appearance. Magnification (50X) of the surface of a rubber plucker "finger" after several weeks of use showed deep pits and heavy bacterial contamination. Deep groves and crevices create areas where bacteria can become trapped and over time produce extracellular polysaccharide material and subsequent protection from cleaning and disinfecting agents.

Hydrophobicity and surface charge of both the organism and collecting surface are other factors that may influence attachment to surfaces found in processing plants. Several reports (Speers et al, 1984, 1985, Fletcher and Loeb 1979) have noted an increased level of attachment on positively charged or neutral surfaces such as stainless steel, compared with negatively charged surfaces such as glass. In part, these studies confirmed the hypothesis proposed by Rutter & Vincent (1980) that some hydrophilic organisms adhere more strongly to hydrophobic surfaces such as rubber than to hydrophilic surfaces such as glass.

Some microbial species isolated from substrates submerged in aquatic media exhibited higher levels of adhesion to hydrophobic than to hydrophilic surfaces

(Fletcher and Loeb, 1979, Pringle and Fletcher, 1983). Furthermore, the hydrophobic properties of some bacteria have been shown to correlate quantitatively with their tendency to form associations with various biological and synthetic surfaces. For example, van Loosdrecht et al (1987) examined a wide range of gram-positive and gram-negative microorganisms and found a relationship between the hydrophobicity of bacterial cells and their adhesion to hydrophobic polystyrene particles that were negatively charged as a result of SO_3^{2-} group fixation.

Additionally, inhibition of bacterial attachment by agents which interfere with hydrophobic interactions provide further evidence of their importance to the adhesion processes. Surface proteins in the form of fimbriae, fibrils and pili impart hydrophobic properties to the cell surface and contribute to adhesion. Paul and Jeffrey (1985) observed that the proteolytic enzymes pronase, trypsin, and chymotrypsin, in addition to the surfactant Triton X-100, inhibited attachment of Vibrio proteolytica to polystyrene, a hydrophobic surface. These treatments had no effect on attachment to hydrophilic surfaces such as glass or tissue culture dishes. Pronase treatment also produced a significant decrease in cell surface hydrophobicity when measured by phase partitioning in hexane or petroleum ether.

The results of this study showed that proteins located on the surface of some bacteria are involved with attachment to hydrophobic surfaces and that a separate mechanism is involved in attachment to hydrophilic surfaces.

While most surfaces involved in dairy processing are stainless steel, rubber is often employed as gasket material. Rubber is hydrophobic and very porous and would be expected to provide an excellent surface for bacterial proliferation through biofilm formation. For example, Noorlander and Heckman (1980) found cracks and pores on the inner surface of rubber teats ranging in size from 1 um in new teats to 15 ums after 1,000 milkings. When the surface of stainless steel and rubber gasket material was viewed by scanning electron microscopy, the rubber material was distinguished by the presence of an extensive amount of cracks and crevices. These cracks and crevices make removal of attached biofilms even more difficult and contribute significantly to final product contamination. Buna-n gasket material has been implicated as the source of bacterial contamination of dairy products such as milk, ice cream, and cheese (Czechowski, 1990).

Czechowski's (1990) research provides an example of how surface roughness can increase bacterial contamination and decrease product shelf-life under normal processing

conditions, including daily cleaning. Czechowski (1990) wished to determine if continual use and subsequent deterioration of the buna-N rubber gasket material could lead to greater bacterial adhesion. He found that most bacteria were present on the surface with only a few located in cracks or holes in the gaskets. Failure to detect bacteria deeply located in cracks and crevices may be attributed to the inability of the staining procedure to penetrate. In this study, new gaskets were placed into a properly cleaned dairy processing facility and examined over a six week period. After one week, 33% of the 18 gaskets distributed throughout the processing lines contained adherent bacteria; after three weeks 65% were contaminated and after six weeks all gaskets were observed to contain adherent microorganisms. He concluded that frequent changing of gaskets would help improve the quality of dairy products and allow dairy plants to extend shelf life by one day.

Chapter 6: Clean in Place (CIP) Sanitation and Cleaning System

Currently, most small scale operations use a manual process to clean processing equipment. However, most large scale operations, as well as plants where more complex equipment is used, more commonly employ cleaning in place (CIP) practices. CIP involves cleaning complete items of plant or pipeline circuits without dismantling or opening equipment and with little or no manual involvement. The process involves jetting or spraying of surfaces and circulation of cleaning solutions throughout the food processing facility under conditions of increased turbulence and flow velocity (Romney, 1990a).

As far as cleaning is concerned, components of food products can be broken down into two broad categories: organic and inorganic soils. Organic soil, mainly of plant or animal origin, is generally most susceptible to attack by alkaline detergents. Inorganic soil, mainly of mineral origin, is usually most effectively attacked by acidic detergents.

The CIP process consists of a series of three discrete cycles, generally composed of a pre-rinse cycle, a detergent cycle, and a final rinse. The pre-rinse cycle is designed to

remove any loosely adherent residual matter from food contact surfaces by means of highly turbulent, fast moving water. Cleaning is the main task of the detergent cycle, soil is lifted from product contact surfaces and held suspended or dissolved in the detergent solution. During final rinse all traces of detergent residues and entrained soil are removed by means of a clean water rinse (Romney, 1990b). Subsequent sterilization or disaffection of the cleaned facility is a distinct and separate process to the cleaning cycle and is ideally undertaken immediately prior to the next start up.

Chapter 7: Industrial Enzymes

Proteases account for approximately 48% of all sales of industrial enzymes. More than 80% of all industrial enzymes are hydrolytic and are used to depolymerize natural substrates. Major industrial uses include detergents, meat processing, cheese making, reagents, and diagnostic assays. Current commercial sources are derived from animals (stomach), plants (saps, juices), and microorganisms. Recombinant DNA technology is expected to have a dynamic impact on future sources of proteolytic enzymes by imparting the ability to express and amplify any enzyme to microorganisms (Loffler, 1986). Because of the need for government approval, most industrial microbial sources of enzymes used throughout the world are produced from no more than 11 fungi, 8 bacteria, and 4 yeasts (Godfrey and Reichelt, 1983).

Industrial glycosidases are used primarily in the enzymatic conversion of starch into various sugars, syrups and dextrins by the starch conversion industry. Controlled enzymatic degradation of starch can be used to generate products that contain various amounts of glucose, maltose and higher oligosaccharides, as well as a wide variety of desired physical properties. These hydrolysates have many uses in the food industry. Sugars are broken down and used

by yeast as carbon sources for conversion into alcohol in brewing and fermentation products such as beer and wine. Confectionery products utilize maltose syrup as a sweetener in soft drinks, jams, jellies, conserves, and ice cream. Dextrins can impart physical characteristics to food products by acting as thickeners, fillers, stabilizers, glues, and pastes (Bigelis, 1993).

A. Detergents

More than 80% of all industrial enzymes are hydrolytic and used for the depolymerization of natural substrates. The majority of these are proteolytic and are used in the detergent industry (Godfrey and Reichelt, 1983). Enzymes were first incorporated into detergents in 1913 when the German chemist, Otto Rohm, obtained a patent for a presoaking product which contained enzymes extracted from animal pancreas glands. The product consisted primarily of sodium carbonate containing added crude extract of pancreas (pancreatin). Enzyme contents were typically very low and activities also tended to be very low due to the high pH of the product. Despite the suboptimal conditions, this household enzyme detergent was still used heavily in Europe until the early 1950's (Barfoed, 1983).

The first enzyme detergents were used to clean work clothes for employees in the fish industry, slaughter

houses, bakeries and hospitals (Barfoed, 1983). Stains on these clothes consisted primarily of proteinaceous material, so proteolytic enzymes were effective during laundering. This industrial application was later introduced into household detergents to fight the same protein-based stains in ordinary home laundry (Barfoed, 1983). Even at low concentrations, proteins act as binding agents to fix other soil components to fabric and make complete removal of the stain difficult.

In 1959, the first detergent containing a bacterial proteinase was produced by the Swiss company Gebruder Schnyder for use in both industry and the home. The real breakthrough came when Novo Industri in Denmark (now called Novo Nordisk BioChem) developed the alkaline protease marketed as Alcalase®. This product became very successful and stimulated rapid growth in the area of enzyme-based detergents (Barfoed, 1983). In the 1970's, progress was slowed when workers in enzyme manufacturing plants developed severe allergy problems. Development of dust-free enzyme preparations overcame this problem and allowed sales of enzyme-based detergents to resume. Detergents containing heat and alkali stable amylases were developed for dishwashing applications (Barfoed, 1983). Foods which

contain starch, such as mashed potatoes, oatmeal or spaghetti, pose a major problem in machine washing of dishes.

In enzymatic machine dishwashing detergents, an alkali-resistant and moderately heat resistant (up to 60 °C) α-amylase produced by Bacillus licheniformis is most often used. Alkaline bacterial proteinases of the serine type, derived from the common soil bacteria Bacillus subtilis and B. licheniformis, are most prevalent proteinases used in laundry detergent formulations (Barfoed, 1983). General formulations for these two types of enzyme detergents are provided (Tables 1 & 2).

B. Proteolytic Enzymes or Proteases

Proteolytic enzymes hydrolyze proteins to produce small peptides and amino acids. In the past, proteases have been classified in a number of ways, such as on the basis of activity within a particular pH range (acid, neutral, or alkaline), ability to hydrolyze specific proteins (keratinase, collagenase, elastase), and similarities to well-characterized enzymes (trypsin, chymotrypsin, cathepsin, chymosin) (Loffler, 1986). Today, the most widely accepted classification system is based on active site mechanism (Hartley, 1960). This classification system

Table 1: General ingredients used in the formulation of industrial and household laundry detergent (Barfoed, 1983).

Ingredient	% of Total
Anionics	5-10%
Nonionics	10-40%
Solubilizer	5-15%
Sequestering agent	0-15%
Water	max. 45%
Optical brightener, perfume	0.1-0.5%
Enzyme preparation	0.4-0.8%
pH	7.0-9.5

Table 2: General ingredients used in the formulation of industrial and household dishwashing detergent. (Barfoed, 1983).

Ingredient	% of Total
Sodium tripolyphosphate	20-50%
Sodium metasilicate	10-30%
Sodium bicarbonate	40-60%
Surfactant	3-10%
Enzyme preparation	1-3%
pH	9.0-9.5

proposed by the Enzyme Commission (EC International Union of Biochemistry), consists of four groups: serine proteases, sulfhydryl (thiol proteases and cystein proteases), metal-containing proteases and aspartic proteases (carboxyl proteases and acidic proteases). These groups are distinguished from each other by the key catalytic groups involved in the mechanism of the active site (Loffler, 1986.)

Enzymes produced by microorganisms have many advantages over those extracted from animals and plants. Enzymes obtained from animals, such as chymosin from calf stomach, are relatively expensive and availability usually depends on current markets for those animals. Most commercial plant proteases such as papain, bromelain, and fican are easy to extract, but supply is governed by food demands for papaya, pineapple and figs. Environmental conditions under which microorganisms are grown can be monitored closely to provide consistency in purity, stability, and activity of enzymes produced (Loffler, 1986.). The Food and Drug Administration (FDA) has awarded GRAS status to proteases extracted from edible plant and animal tissue as well as those produced by Bacillus subtilis, Aspergillus oryzae, Aspergillus niger, Saccharomyces cerevisiae, or Kluyveromyces fragilis. Proteolytic enzymes derived from Penicillium roqueforti,

Penicillium caserilum, Mucor miehei, Mucor pusillus, Endothia parasitica, Bacillus liquiformis, and Bacillus cereus have also been granted GRAS status (Loffler, 1986). Enzymes produced from GRAS organisms that have been altered genetically cannot be considered GRAS unless analyses can demonstrate safety.

Calf chymosin is an example of an enzyme produced by recombinant technology with GRAS status. Cheese manufacturing utilizes two enzymes isolated from the stomach of suckling calves, chymosin and rennin, to initiate the clotting process. Chymosin is preferred by cheese manufacturers because of limited proteolysis leading to minimal bitterness in aged cheese. However, chymosin is produced only during the first two weeks of the calf's life (Wegstein and Heinsohn, 1993.) Through genetic engineering consistent production of chymosin with analogous properties to native chymosin is now possible. The proteins are identical, despite the use of three microorganisms for production: the bacterium Escherichia coli, the yeast Kluyveromyces marxianus variety lactis, and the fungus Aspergillus niger variety awamori. In order to gain GRAS status, companies producing the enzyme commercially by genetic engineering submitted data to the FDA. These firms were able to demonstrate that genetically engineered

chymosin is chemically and genetically identical to the native chymosin and that all safety and good manufacturing requirements have been met (Wegstein and Heinsohn, 1993).

Any compound that decreases the observed activity of an enzyme is an inhibitor (Whitaker, 1994a). Decreased enzyme activity can occur in a number of ways. At the cellular level, the inhibitor may function by (a) altering the permeability of a cell or depressing transport mechanisms; (b) interfering with the formation of high energy phosphate compounds (ATP); (c) interfering with synthetic reactions involved in the formation of cellular constituents; (d) interfering, at the level of DNA, RNA or ribosomes, with biosynthesis of the enzyme; or (e) inhibiting the enzyme directly. At the molecular level, an inhibitor can react with essential groups of the active site of the enzyme or with specific groups on the enzyme not involved with the active site per se but which are important in maintaining conformation of the active site or in holding multiunit enzymes together. A compound could also be inhibitory if reaction with a substrate, a cofactor, or an activator needed by the enzyme occurs (Whitaker, 1994a).

Enzymes which are characterized by mechanism of active site are inhibited by selective reagents. For example, chymotrypsin, trypsin, elastase, thrombin, subtilisin and

α-lytic protease from Sorangium species are all serine proteases. These enzymes are inhibited by diisopropyl-phosphofluoridate (DFP) and Phenylmethylsulfhydralfluoride (PMSF), which react with the hydroxyl group of a specific seryl residue in the active site of the enzyme. These enzymes are all endopeptidases and contain an imidizole group and an aspartyl carboxyl group involved in mechanisms of the active site (Whitaker, 1994b).

Although serine proteases share similarities in their mechanism of activity, individual members of this group can posses very different substrate specificities. In general, mechanisms of catalysis by members of groups such as serine proteases are identical; only specific groups involved in binding substrates are different, which results in different specificities. For example, the enzyme subtilisin, produced from Bacillus subtilis Carlsberg acts by cleaving -COOH-NH_2- of the peptide bond adjacent to the large hydrophobic side chains of the aromatic amino acids tyrosine, phenylalanine, and tryptophan. Bovine trypsin, another serine protease, attacks the basic side chains of the amino acids lysine and arginine in order for hydrolysis of the peptide bond to occur (Whitaker, 1994b).

Microbial proteases are classified together in sub-

group four of group three which classifies hydrolases. The enzymes are sub-divided based on their exo- or endo-nature of attack into peptidases and proteinases, respectively. The EC number for serine proteases is 3.4.21 and the entire EC number for the microbial serine protease called subtilisin is 3.4.21.14. The EC number for metalloproteinases is 3.4.24, Bacillus subtilis neutral proteinase is a metalloproteinase and has an EC number of 3.4.24.4.

Savinase® is a crude protease (Table 3) used in detergent formulations to remove protein-based stains such as grass, blood, mucus, feces, and various foods such as egg and gravy. These stains are almost insoluble and tend to adhere to the surface of textiles. Savinase® hydrolyzes protein in stains into peptides which are readily dissolved or dispersed in the washing liquid (Anonymous, 1992b). Savinase® is a subtilisin (E.C. 3.4.21.62) of the serine type produced by submerged fermentation of a genetically modified alkalophilic Bacillus species.

Also classified as a subtilisin (E.C. no. 3.4.21.14), Durazym™ (Table 3) is a generic engineered variant of Savinase® (Anonymous, 1992b). Durazym™ was developed to improve stability in the presence of bleaching agents and maintain performance in powder and liquid detergents.

Table 3: Characteristics and manufacturers of the enzymes Durazym™, Savinase®, Purafect®, and Termamyl®.
and amino acids which can then be easily removed from

Enzyme	Supplier	EC no.	Type	*pH range (opt)	*T range (opt)
[a]Durazym™[a]	Novo Nordisk BioChem	3.4.21.14	subtilisin	8-12	40-57°C (47°C)
[b]Purafect®[b]	Genencor	3.4.21	subtilisin	5-11 (10)	25-65°C (40°C)
[c]Savinase®[c]	Novo Nordisk BioChem	3.4.21.62	subtilisin	8-12	50-60°C (55°C)
[a]Termamyl®	Novo Nordisk BioChem	3.2.1.1	α-amylase	5-8	80-95°C (76°C)

[a] conditions: Dimethyl Casein, T=50°C, pH=8.3
[b] conditions: peptide substrate, T=25°C, pH=8.6
[c] conditions: Dimethyl Casein, T=50°C, pH=8.3
(Economic Microbiology, 1980)

Compared to Savinase®, Durazym™ possesses increased storage stability in detergents with activated bleach and performs better in some detergents at low-temperature wash conditions.

Purafect® is an alkaline protease (Table 3) for use in the formulation of laundry detergent products. Purafect® hydrolyzes insoluble protein stains into soluble peptides fabric. This alkaline protease is useful in removing common household stains such as blood, grass, milk and gravy. Purafect® is a high-efficiency, fully biodegradable enzyme derived from a selected strain of Bacillus lentus and expressed in another Bacillus species (Anonymous, 1994a). The protease works in conjunction with surfactants, builders, and other laundry chemicals to enhance removal of most stains from all types of cloth surfaces under a wide variety of laundering conditions.

B. Glycosidases

Glycosidases are enzymes involved in starch hydrolysis. Starch consists of mixtures of unbranched chains having α(1-4) linked α-D-glucopyranose molecules called amylose and chains having both α(1-4) linked and α(1-6) linked branches of α-D-glucopyranose molecules called amylopectin.

Glycogen occurs in animals and possess structure similar to amylopectin. The glucosyl hydrolases are classified into three groups designated by the heading EC 3.2.1. (glycosidases) in the official nomenclature for enzymes. The three groups are: enzymes specific for cleavage of α(1-4) bonds, enzymes specific for cleavage of α(1-6) bonds, and enzymes specific for cleavage of α(1-4), and α(1-6) bonds (Alais and Linden, 1991). Starch degrading enzymes are distributed widely throughout the plant, animal and microbial kingdoms. In the past 25 years, a number of starch degrading enzymes have been expressed and produced in large quantities in various bacterial sources.

α-Amylases, β-amylases, debranching enzymes specific for α(1-6) bonds, amyloglucosidases, and cyclodextrin glycosyl-transferases are examples of the types of enzymes that fit into the three groups used to classify glycosidases. These enzymes can be extracted from animal, plant, or microbial sources. Different segments of food and detergent industry utilize these enzymes.

α-Amylases, which can be from animal, plant, or microbial origin, randomly hydrolyze α(1-4) bonds of amylose and amylopectin. Also called endo-amylase and

liquefying or dextrinizing amylase, the main product formed upon hydrolysis of starch is an oligosaccharide of 6 or 7 glucose residues in addition to maltose (Alais and Linden, 1991). β-Amylases can be extracted from plants and from certain strains of microorganisms. This 'saccharifying enzyme' hydrolyzes amylose and amylopectin from their non-reducing ends and produces β-maltose. α(1-6) Bonds of amylopectin cannot be hydrolyzed, therefore, only cleavage of external parts of the amylopectin molecule occurs (Alais and Linden, 1991).

Debranching enzymes hydrolyze α(1-6) bonds of amylopectin chains and glycogen. These enzymes can be extracted from both plant and microbial sources. Two debranching enzymes that have been studied extensively are pullalanase and isoamylase, whose action depends essentially on the ease with which these enzymes are able to penetrate the complex web of branches that make up amylopectin and glycogen (Alais and Linden, 1991). Also known as glucoamylases, amyloglucosidase rapidly hydrolyzes α(1-4) and α(1-6) bonds of long chained amylose and amylopectin and produces D-glucose. Isolation is predominately from molds of the genus Rhizopus and Aspergillus (Alais and Linden, 1991). Cyclodextrin glycosyl-transferases are

synthesized by Bacillus macerans and Bacillus subtilis. Enzymatic degradation of starch produces large rings of 6 to 8 glucose units (Alais and Linden, 1991).

Products of starch hydrolysis have many industrial uses including brewing, bread making, and preparation of manufactured sugar products, in the form of 70% glucose syrup or sugars of varied molecular size (glucose, maltose, triose, tetrose) or in powdered form (92-94% reducing sugar). Low DE (dextrose equivalents) maltodextrins, maltose syrup, high maltose syrup, high DE syrups, glucose syrups and fructose syrups are examples of hydrolyzed starch products. Differences between these products depend on their per cent composition of glucose, maltose, and higher oligomers. For example, maltose syrups contain 16-20% glucose, 41-44% maltose, and 36-43% higher oligomers. Maltose syrups are added to confectionery products, soft drinks, jams, jellies, conserves, ice cream, sauces, and the brewing and fermentation industries to increase viscosity, reduce crystallization, and impart moderate sweetness (Bigelis, 1993). Low DE maltodextrins are composed of 1-12% glucose, 4-13% maltose, 6-20% maltotriose, 50-80% higher oligomers, and are used as thickeners, fillers, stabilizers, glues, pastes, and as raw materials for further enzymatic sacchrification (Bigelis, 1993).

Although proteolytic enzymes make up the bulk of enzymes used in the clothing detergent industry, glycosidases are used extensively in the manufacture of dishwashing detergents. Glycosidases contained in dishwashing detergent formulations act on starch present in foods such as mashed potatoes, oatmeal and spaghetti (Barfoed, 1983). Due to the adhesive nature of these starchy foods, glycosidases are necessary in order to cleave glucosidic bonds involved in adhesion of starch residues to dishware. Termamyl® is a liquid enzyme preparation (Table 3) containing a heat-stable α-amylase expressed in and produced by a genetically-modified strain of Bacillus licheniformis. The enzyme is an endoamylase which hydrolyzes 1,4-α-glucosidic linkages in amylose and amylopectin. Starch is rapidly hydrolyzed to soluble dextrins (Anonymous, 1994b).

Termamyl® has many industrial applications in addition to use in dishwashing detergent formulation. The starch industry utilizes Termamyl® for continuous liquefaction of starch in steam jet cookers or similar equipment operating at temperatures up to 105-110°C. In the alcohol industry, this glycolytic enzyme is used for thinning starch in

distilling mashes. The brewing industry uses Termamyl® for adjunct liquefaction. Termamyl® is also used to hydrolyze the starch present in cane sugar in the sugar industry.

C. Use of Enzymes in Biofilm Removal

Thus far, enzymatic treatment of adhered cells has been used primarily to characterize and more fully understand the nature of the attachment process. Christie et al (1970) found that proteolytic and amylolytic enzymes produced a consistent weakening action of "settled" zoospores of Enteromorpha intestinalis on a glass surface. The settled spores were allowed to adhere for 5 to 18 hr prior to enzyme treatment. A general pronase with broad specificity produced a pronounced loosening. Many of the cells exposed to the pronase were burst; treated cells could be easily removed using 50 mm Hg flow. Very high concentrations (5 mg enzyme/ml) of α-amylase also produced marked weakening of settled cells. The marked loosening effect produced by the pronase and the α-amylase reaffirm the belief that the glycocalyx is made of both polysaccharide and proteinaceous material. These results show that protein and polysaccharide cleaving enzymes can be used to produce breakdown of the adhesive layer produced in biofilm.

Danielsson et al (1977) treated marine Pseudomonas

species with protein and carbohydrate splitting enzymes and noted a weakened ability to adhere to glass. These investigators found that a general pronase produced a strong and rapid release. Treatment of attached Pseudomonas species for 5 min produced a 50% release which increased to 70% after 30 min. Two carbohydrate splitting enzymes were also used, a lysozyme and an α-amylase. Treatment with lysozyme produced a weak release of only 10% of adhered cells after 30 min. α-Amylase had no effect on attachment. The complete absence of cell release by α-amylase treatment indicates that polysaccharides with α-1,4 glucosidic bonds are not critical to the adhesion process. Similarly, the weak lysozyme effect indicates that β-1,4 glucosidic bonds do not play a major role in the attachment of settled cells. Alternatively, the glycosidases may not have been able to penetrate into the crevices of the glass surface and disrupt the slime layer protecting the cells.

Jones et al (1969) treated samples of biofilm formed from lyophilized microorganisms obtained from a polluted stream with deoxyribonuclease. The polysaccharide-like material surrounding the cells was not affected and maintained a thread-like appearance, indicating that deoxyribonucleic acid is not a major component of the

biofilm.

Beech and Gaylarde (1989) added glucosidase, N-acetyl glucosaminidase and crude pronase (Sigma) to cultures of P. fluorescens and Desulfovibrio desulfuricans 1-2 hr before introducing stainless steel stubs. Beech and Gaylarde found that glucosidase and N-acetyl glucosaminidase significantly ($p<0.05$) reduced adhesion of P. fluorescens. Bacterial adhesion was not affected by treatment with pronase. These results indicate that the presence of glucose and N-acetyl glucosamine on the cell surface may be associated with adhesion of P. fluorescens.

Herald and Zottola (1989) studied the effects chemicals known to react with polysaccharides (sodium periodate, cetyltrimethyl ammonium bromide, and Concanavalin A) and the two proteolytic enzymes trypsin and pepsin had on attachment of P. fragi to stainless steel. The ability of this organism to attach was monitored by three different assays; first, after pretreatment with the chemicals or proteases and second, after P. fragi was grown in the presence of the chemicals or enzymes. Finally, the organism was allowed to attach and a subsequent chemical or enzyme treatment was applied in an attempt to remove cells. After pretreatment, attachment of trypsin-treated cells was not significantly different from the phosphate buffer control. Prior pepsin

treatment had no effect upon attachment. Significantly lowered levels of attachment were observed with the Concanavalin A treated cells at 35.5% of the control. Sodium periodate and cetyltrimethyl ammonium bromide were the most effective in reducing attachment at 5.4% and 4.9% of the control, respectively.

When P. fragi was grown in the presence of trypsin and pepsin, no significant differences could be found in their ability to attach to stainless steel compared to controls. Sodium periodate and cetyltrimethyl ammonium bromide both reduced attachment, but only by 53.6% and 71.8%, respectively. Cell death was revealed due to sodium periodate, and cetyltrimethyl ammonium bromide when the number of cells remaining after the assay were recorded. Data provided from the removal assay revealed that treatment with trypsin was effective in decreasing the number of attached cells by more than half. Sodium periodate detached cells significantly when compared to the distilled water control. cetyltrimethyl ammonium bromide seemed to stabilize the level of attached cells and prevent their removal at significant levels when compared to the control. Pepsin was not used in the final part of the experiment.

Herald and Zottola (1989) drew many conclusions from the results provided by the polysaccharide reactants, sodium

periodate, Concanavalin A, and cetyltrimethyl ammonium bromide when their mechanisms of action were investigated. Sodium periodate, which cleaves carbon-carbon bonds between carbons with adjacent hydroxyl groups of carbohydrate moieties in an oxidation reaction, was effective not only in preventing attachment but also aided in the detachment of cells. Concanavalin A reacts with sugars or glycoproteins with branched terminal non-reducing α-D-glucopyranosyl, α-D-mannopyranosyl or β-D-fructofuranosyl residues. In the pretreatment assay, this binding may have occurred to block groups participating in the attachment process. The cationic detergent cetyltrimethyl ammonium bromide, a polysaccharide precipitant, may have lowered attachment levels by causing cells to clump on the stainless steel, resulting in the clump being counted as one rather than the sum of all the cells in the clump.

The objectives of this research will be to determine the ability of crude industrial enzyme preparations to remove P. fluorescens from black buna-n rubber, L. monocytogenes from black buna-n rubber, and a mixed biofilm containing both organisms attached to black buna-n rubber. The enzyme preparations selected are presently used in the clothing and dishwashing detergent industries. These enzymes

are inexpensive, easy to handle, and have broad specificities. Three of the enzyme preparations will contain predominately proteolytic activities and one will be predominately glycolytic. Black buna-N rubber will be the surface on which the biofilms are produced. Gasket material provides a junction between pipes in food processing plants and is made of black buna-N rubber which is very porous and provides an excellent microenvironment on which to study removal of bacteria adherent in biofilms.

Materials and Methods:

All experiments were performed using the equipment available at the Food Science and Technology building on the campus of Virginia Polytechnic Institute and State University, Blacksburg, Virginia. A pure live culture of Pseudomonas fluorescens ATCC #13525 was provided by the Biology Department at Virginia Polytechnic Institute and State University. A pure live culture of Listeria monocytogenes was obtained from the Food Science and Technology Department stock collection. Durazym™, Savinase®, and Termamyl® were provided by Novo Nordisk BioChem North America, Inc. located in Franklinton, North Carolina. Purafect® was a gift from Genencor International Inc., Rochester, New York. 2.0 cm X 5.0 cm strips of black buna-N rubber was purchased through Chicago-Wilcox, Manufacturing Co, South Holland, Illinois. Chlorinated solution used to sanitize black buna-N rubber was purchased from Klenzade a Division of Ecolab Inc., St Paul, Minnesota. 2% UHT milk was purchased from Wades Supermarket located in Blacksburg Virginia. All other materials and reagents were purchased from Fisher Scientific, Fair Lawn, New Jersey.

I: *Pseudomonas fluorescens* and *Listeria monocytogenes*

Daily culture transfers of *L. monocytogenes* and *P. fluorescens* were made into Tryptic Soy Broth (TSB) containing 0.6% yeast extract (YE) for *L. monocytogenes* and TSB alone for *P. fluorescens*. *L. monocytogenes* was maintained on a Tryptic Soy Agar (TSA) slant containing 0.6% YE and *P. fluorescens* was maintained on a TSA slant.

Broth cultures of *P. fluorescens* and *L. monocytogenes* were streaked weekly onto a non-selective media. Colony size, shape and odor were examined and their similarities to typical colonies were compared to ensure purity of cultures. Additionally, selective media was used to verify the use of the proper organism in this study. After 48 hr of growth on selective media, a single typical colony was picked to inoculate tubes of sterile TSB and TSBYE. For subsequent experiments, transfers into sterile TSB and TSBYE were made daily.

King's B medium with three antibiotics (Sands and Rovira, 1970) was used as selective media for *P. fluorescens*. King's B contains 2% Protease Peptone No. 3, 1.2% Agar Bacteriological (Agar No. 1), 0.8% Bacto glycerol, 0.009 M Potassium Sulfate crystals (K_2SO_4) and 0.006 M Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$). The pH was adjusted to 7.2

with 0.1 N NaOH before autoclaving for 25 min at 121 °C. The three antibiotics were penicillin-G (benzyl penicillin) sodium salt (75,000 units/L), novobiocin sodium salt (45 mg/L), and cycloheximide (75 mg/L). The antibiotics were mixed together with 0.3% of the total volume in 95% ethanol, diluted with 5% of the total volume in sterile distilled water, and added to the sterilized King's B medium at 45 °C.

The medium used to select and differentiate L. monocytogenes was Oxford Medium Base. After 48 hr at 37 °C, colonies of L. monocytogenes on Oxford agar are gray and cause the surrounding agar to turn dark brown.

II: Bacterial Enumeration

Initial and final bacteriological counts of pure culture biofilm growth media (TSB for P. fluorescens and TSBYE for L. monocytogenes) and mixed culture biofilm media (2% UHT milk) were made using the pour plate method (Busta et al., 1984) in duplicate with Tryptic Soy Agar (TSA) containing 0.6% YE. Plates containing P. fluorescens were incubated at 30 °C for 48 hr. Plates containing L. monocytogenes were incubated at 37 °C for 48 hr.

Prior to enumeration of pure culture biofilms, only one side of the black buna-N rubber surfaces were scrubbed, not the edges, with a sterile calcium alginate tipped swab for

each strip. Enumerations were performed using the pour plate method in duplicate on TSAYE. After swabbing, tips were broken off into a dilution blank and vortexed for 90 sec. Dilutions were made using 9.0 ml blanks of phosphate buffer solution (PBS) (AOAC, 1992). PBS contained 0.13 M Crystal Sodium Chloride (NaCl), 0.05 M Sodium Phosphate Anhydrous-Dibasic (Na_2HPO_4), and 0.02 M Potassium Phosphate Monobasic (KH_2PO_4). Prior to autoclaving, pH was adjusted to 7.4 with 0.5 N NaOH.

For mixed culture biofilm enumeration, surfaces were swabbed and two sets of duplicate TSAYE plates were poured. One set of plates was used to determine the number of both P. fluorescens and L. monocytogenes present and was incubated at 30 °C for 48 hr. A second set of plates was used to identify only the L. monocytogenes present. These plates were poured with a thin layer of TSAYE and allowed to set for 4 hr to recover injured cells. These plates were overlaid with Oxford medium base and incubated at 37 °C for 48 hr.

To confirm that swabbing was able to remove adherent bacteria, strips were removed from anaerobe tubes with sterile forceps and placed in petri plates. The strips were

then overlayed with TSAYE and incubated for 24 hr at 30 °C for P. fluorescens and at 37 °C for L. monocytogenes.

III: Biofilm Preparation

Biofilms were grown on 0.3 x 5.0 x 2.0 cm strips of black buna-N rubber cut from a 0.3 x 91.0 x 122.0 cm sheet. Prior to use, buna-N rubber strips were sanitized with 1.4% solution of HC10-Chlorinated Kleer-MOR in tap water for 24 hr at room temperature. Strips were rinsed initially with tap water followed by a thorough rinse in distilled water; strips were blotted dry with paper towels. A large surface area was needed in order to obtain sufficient release of hydrolyzed material for spectrophotomertic assays therefore, buna-N rubber strips were choose as the surface on which to grow the biofilm.

For formation of biofilm by L. monocytogenes or P. fluorescens, one strip was placed in a 100 ml Pyrex Media Bottle and autoclaved for 20 min at 121 °C. Sterile TSB (50 ml) with (L. monocytogenes) or without (P. fluorescens) 0.6% YE was added. Bottles were inoculated with 1 ml of a 24 hr culture (approximately 10^8 cfu/ml)of either L. monocytogenes or P. fluorescens (Figure 1). Inoculated media bottles were incubated at 30 °C for 24 hr in a reciprocating shaker bath (Precision Reciprocal Shaker bath Model 50) operated at 120

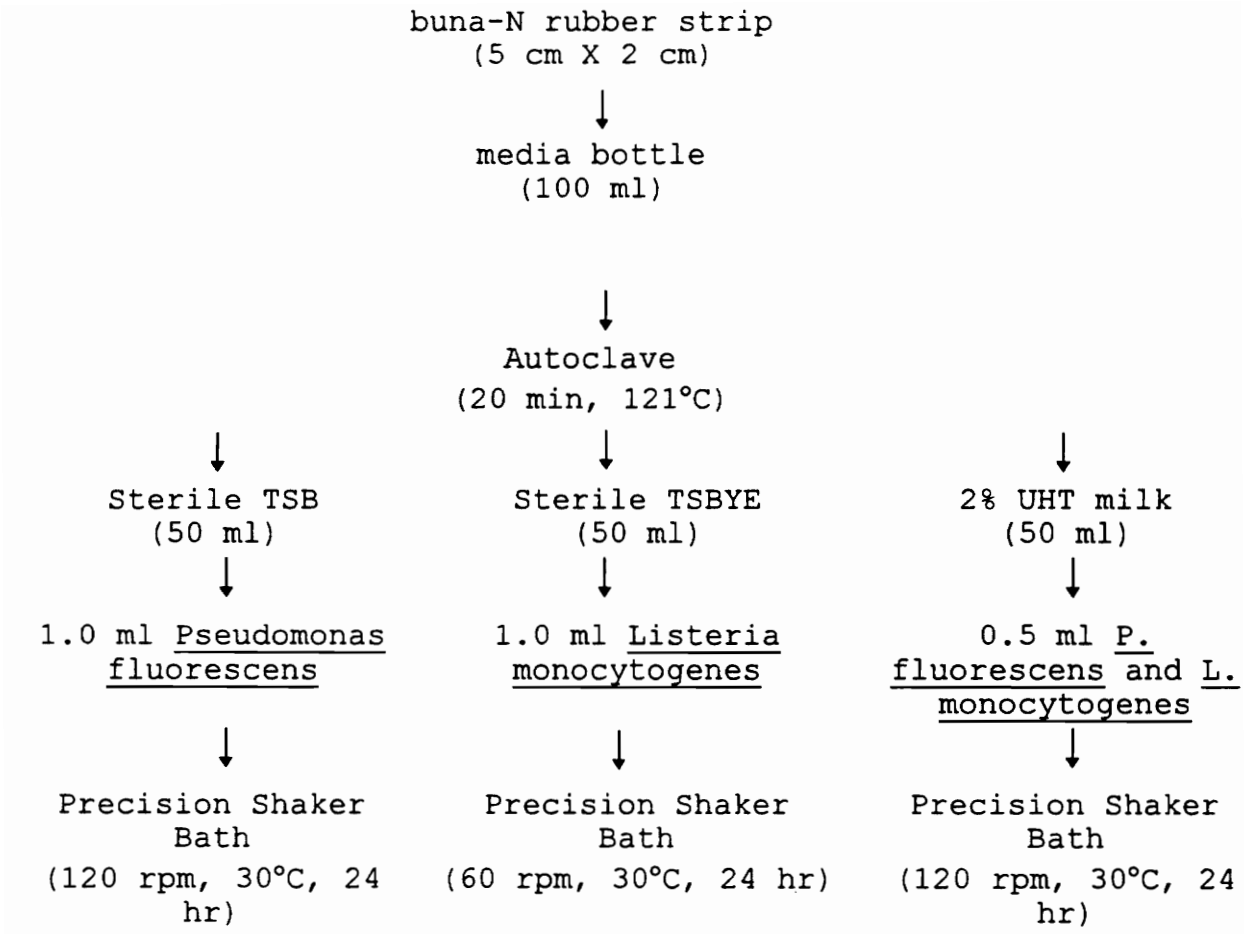

Figure 1: Flow diagram of the preparation and incubation of black buna-N rubber strips in pure and mixed cultures of P. fluorescens and L. monocytogenes.

rpm for P. fluorescens and 60 rpm for L. monocytogenes (Figure 1).

During mixed culture studies (P. fluorescens and L. monocytogenes) a black buna-N rubber strip was placed in a 100 ml Pyrex Media Bottle and autoclaved for 20 min at 121 °C (Figure 1). After addition of 50 ml 2% UHT pasteurized milk, media bottles were inoculated with 0.5 ml of a 24 hr culture of P. fluorescens and with 0.5 ml of a 24 hr culture of L. monocytogenes. Inoculated media bottles were incubated in a reciprocating shaker bath operated at 120 rpm for 24 hr at 30 °C.

After 24 hr incubation, strips were removed from media bottles with sterile forceps and placed into anaerobe tubes containing 20 ml sterile PBS (Figure 2). Strips which had been incubated with P. fluorescens or mixed culture were vortexed (Touch Mixer Model 232) for 30 sec at a setting of 7.0. Anaerobe tubes containing strips incubated with L. monocytogenes were inverted 5 times instead of being vortexed (Figure 2). Strips were removed with sterile forceps and placed into another sterile anaerobe tube containing 20 ml PBS. Strips were vortexed or inverted, as described above. Strips were washed two additional times to remove any bacterial cells which were not irreversibly

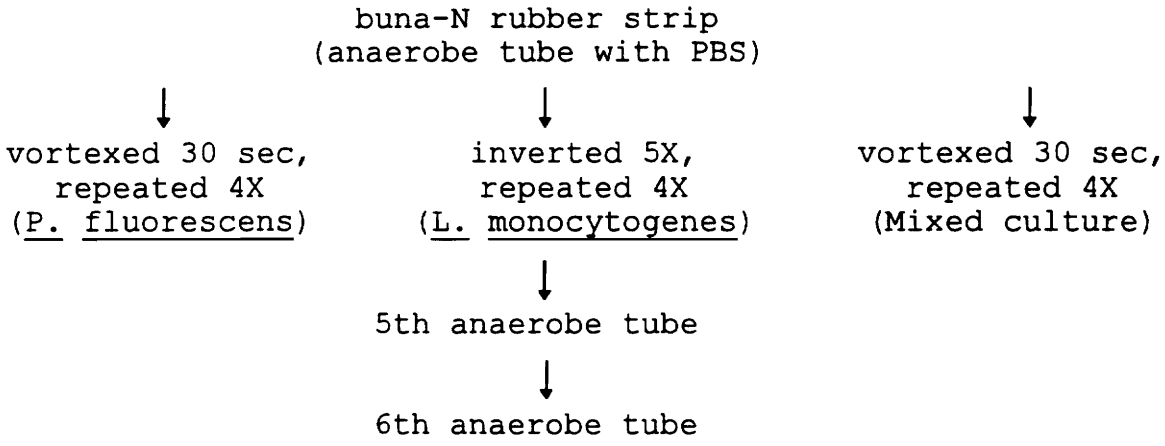

Figure 2: Rinse method used to remove reversibly attached cells of *P. fluorescens* and *L. monocytogenes* grown in pure and mixed culture.

attached. Strips were rinsed without vortexing in a fifth anaerobe tube and finally placed into a sixth anaerobe tube. Prior to enzyme treatment, PBS was removed by a vacuum pump. Solutions in anaerobe tubes used in rinses four and five were enumerated to determine the number of reversibly attached cells remaining (Appendix A, Table 10).

IV. Enzyme Assays

Due to the high background readings in enzyme assays, Savinase®, Termamyl®, Durazym™, and Purafect® were dialyzed prior to use. Enzymes were placed in Spectrapor membrane tubing (molecular weight cut off of 12,000-14,000) and dialyzed at 0-4°C against phosphate buffer solution (PBS), pH 7.4. Savinase®, Termamyl®, and Durazym™ were dialyzed with constant stirring for 48 hr with PBS changed every 9 hr. Due to break down of dialysis tubing, Purafect® was dialyzed for 6 hr. Prior to use in treatment of biofilm strips, dialyzed enzymes were passed through a 0.2 um filter (Acrodisc, Gelman Sciences) to remove any contaminating microorganisms.

Bovine casein was used as substrate in assays to detect proteolytic activity. Whole casein was prepared from milk obtained at the Virginia Tech Dairy Farm. Casein was prepared by direct acidification of skim milk to pH 4.6 with

0.5 M HCl. Skim milk was prepared by centrifugation (Elecrem, L'Electro-Ecremeuse, Boulogne-Billancourt, France) at 9,400 rpm at a flow rate of 125L/hr at 37 °C. Casein was washed three times with distilled water and filtered through double layered cheese cloth. Prior to use, storage was at -4 °C.

Proteolytic activities of dialyzed and undialyzed Savinase®, Termamyl®, and Durazym™ were determined by incubation with 3.0% casein in PBS, pH 7.4, at 40 °C for 5 hr with mild agitation (200 rpm) on a portable orbital platform shaker (Tekmar Electronic VXR). Periodically, aliquots (0.9 ml) were removed and non-hydrolyzed casein was precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 12% (w/v). After centrifugation (Allied Fisher Scientific Microcentrifuge, Model 235 C) at 15,000 rpm for 5 min (15.6 g), absorbancy of supernatants was determined in a spectrophotometer (Perkin-Elmer Lambda 3 System UV/Vis) at 280 nm (Appendix B, Figures 19-21).

Glycosidic activity present in dialyzed and undialyzed Termamyl® was determined using corn starch. Tubes containing 0.5% soluble starch were incubated with various levels (5% and 10%) of Termamyl® at 40 °C for 5 hr with mild

agitation (200 rpm) on a portable orbital platform shaker. Periodically, aliquots (0.3 ml) were removed and added to dinitrosalicylic acid reagent (0.9 ml) (Wood and Bhat, 1988). Reaction tubes were boiled for 15 min, cooled, centrifuged 2 times for 5 min each, and absorbancy determined at 625 nm (Appendix B, Figure 22).

V: Enzymatic Treatment of Biofilms

Biofilm strips were treated with 5% and 10% Savinase®, Durazym™, Purafect®, and Termamyl® in PBS (v/v). Controls contained PBS only. Treated and control strips were incubated for 5 hr at 30 °C in a reciprocating water bath (Precision) operated at 30 rpm. After treatment, biofilm strips were rinsed using the method described above (Figure 2). Rubber surfaces were swabbed and enumerated using the pour plate method.

Release of hydrolyzed products during incubation of buna-N rubber strips was monitored spectrophotometrically. When Savinase®, Durazym™, or Purafect® were used to treat biofilm strips, aliquots (0.9 ml) were obtained periodically during the 5 hr incubation. Activities were quenched by addition of 0.1 ml (0.1 M) Phenylmethyl Sulfonyl Fluoride (PMSF) to each aliquot. Samples were centrifuged (Allied Fisher Scientific Microcentrifuge) twice at 15,000 rpm for 4

min (15.6 g) at room temperature. Absorbancy was determined in a spectrophotometer at 280 nm. Material released from Termamyl® treated strips was assayed using the dinitrosalicylic acid assay described above.

VI: Bactericidal Effects of Dialyzed Enzymes

Pure broth cultures of P. fluorescens and L. monocytogenes incubated for 24 hr in TSBYE were used to inoculate 5% and 10% dialyzed enzyme solutions in PBS, pH 7.4, to an initial level of 2 to 3 mean log cells/ml. Initial levels were determined using the pour plate method with TSBYE. Test tubes containing 2 to 3 log cells/ml in PBS were used as controls. Control test tubes, and those containing 5% and 10% dialyzed enzyme were incubated in a reciprocating shaker bath for 5 hr, at 30 °C and a speed of 30 rpm. After treatment, the number of survivors was determined using the pour plate method.

VII. Statistical Methods

Analysis of variance (ANOVA) tests of the data were completed using SAS (SAS Institute, Inc. Cary, NC). Also an f-test with tukey HSD (Honestly Significant Difference) at the 0.05 level of significance was performed to determine were the difference occured among the four treatments.

Results and Discussion

A: Bacterial Attachment

Black buna-N rubber strips were placed in sterile TSB and sterile TSBYE and inoculated with either Pseudomonas fluorescens, or Listeria monocytogenes. Mixed culture studies utilized equivalent levels of both organisms inoculated into 2% UHT milk containing black buna-N rubber strips. Initial and final mean log cell counts of media were obtained prior to incubation with buna-N rubber strips (Table 4). In pure culture, the average initial inoculum level for P. fluorescens was 6.68 mean log cells/ml (n=24); after 24 hr incubation at 30 °C, levels increased to an average of 8.36 mean log cells/ml (n=24). L. monocytogenes had an average initial inoculum level of 7.13 mean log cells/ml (n=24) which increased to 8.73 mean log cells/ml (n=24) during incubation (Table 4).

Mixed culture studies involving P. fluorescens and L. monocytogenes obtained similar average initial and final mean log cell counts in 2% UHT milk (Table 4) (n=24). In mixed culture, levels of P. fluorescens increased from an average initial inoculum level of 6.27 mean log cells/ml (n=24) to 8.31 mean log cells/ml (n=24) after 24 hr at 30

TABLE 4: Initial and final mean log cells/ml of planktonic bacteria in media during 24 hr incubation at 30 °C for both pure and mixed culture studies. Additional data provides initial mean log cells/cm^2 of bacteria adherent to black buna-N rubber strips after 24 hr incubation and counts (mean log cells/cm^2) of adherent bacteria remaining on control strips after 5 hr incubation in a reciprocating shaker bath at 30 °C. (n=24)

Bacteria (media)	Initial counts in media (mean log cells/ml +/- std dev)	Final counts in media (mean log cells/ml +/- std dev)	Initial adherent counts (mean log cells/cm^2+/- std dev)	Control (PBS) in shaker bath (mean log cells/cm^2+/- std dev)
Pseudomonas fluorescens (TSB)	6.68 +/- 0.10	8.36 +/- 0.06	4.32 +/- 0.05	4.18 +/- 0.08
P. fluorescens in mixed culture (2% UHT Milk)	7.13 +/- 0.04	8.73 +/- 0.05	3.87 +/- 0.06	4.00 +/- 0.04
Listeria monocytogenes (TSBYE)	6.27 +/- 0.17	8.31 +/- 0.08	2.25 +/- 0.05	1.62 +/- 0.09
L. monocytogenes in mixed culture (2% UHT Milk)	6.75 +/-0.08	7.83 +/- 0.06	1.25 +/- 0.05	0.88 +/- 0.09

°C. L. monocytogenes, grown in mixed culture with P. fluorescens, increased from an average initial level of 6.75 mean log cells/ml (n=24) to a final level of 7.83 mean log cells/ml (Table 4) (n=24). Average values obtained for both initial and final mean log cell counts for organisms grown in pure and mixed culture were very similar for both P. fluorescens and L. monocytogenes. Little variation among these values indicates that the mixed culture environment did not inhibit or enhance growth of either organism in the liquid media. Similar growth and recovery data was obtained for both organisms in 2% UHT milk compared to the complete media TSB (P. fluorescens) and TSBYE (L. monocytogenes).

Biofilm was grown on 12 strips for each enzyme treatment. 3 strips were swabbed for initial counts, 3 strips were for control counts, and 3 strips were swabbed for each of the 5% and 10% enzyme treatments. Trials were performed in duplicate.

After incubation for 24 hr in pure and mixed culture solutions of P. fluorescens and L. monocytogenes, black buna-N rubber strips were rinsed in 5 successive anaerobe tubes to remove any reversibly attached cells. Rinse solutions of tubes 4 and 5 were enumerated to verify that only irreversibly attached cells remained (Appendix A, Table

9). Following rinsing 3 of the strips were swabbed and initial counts of attached bacteria were obtained. The remainder of the strips (9) were treated with proteolytic and glycolytic enzymes for 5 hr at 30 °C in a reciprocating shaker bath. Effects of the reciprocating shaker bath were distinguished from enzyme effects by including a control containing no enzyme. Control strips were incubated for 5 hr at 30 °C in PBS; remaining strips were treated with 5% and 10% enzyme and incubated under the same conditions.

P. fluorescens and L. monocytogenes were treated separately as well as in mixed culture. Buna-N strips incubated alone with P. fluorescens contained an average initial attachment level of 4.32 mean log cells/cm^2 (n=24). When incubated with L monocytogenes the average initial level of attached P. fluorescens was 3.88 mean log cells/cm^2 (Table 4) (n=24). The difference (0.44 log units) in attachment was not significant ($p>0.05$). Average initial attachment for L. monocytogenes alone was 2.30 mean log cells/cm^2 (n=24), almost 2 log units less than P. fluorescens. When grown in mixed culture, average initial attachment of L. monocytogenes (1.25 mean log cells/cm^2) (n=24), was 1.05 log units lower than when grown in pure culture (Table 4). Sasahara and Zottola (1993) reported

increased attachment of L. monocytogenes to glass when grown in mixed culture with P. fragi. Differences obtained in this study may be due to the bacteria used and/or variations in properties of the adhering surface.

Prior to enzyme treatment, effects of environmental conditions on cells attached to buna-N rubber strips during 5 hr treatment needed to be determined. During enzyme treatment, attached cells would undergo gentle agitation (30 rpm) in the reciprocating shaker bath. Therefore, a study was designed to account for mechanical stress experienced by attached cells during enzyme treatment. The number of cells adherent after incubation in the absence of enzymes was used as an indication of the effect of agitation due to mechanical action alone. The average number of P. fluorescens cells remaining after incubation in PBS was 4.18 mean log cells/cm^2 (n=24) and 4.00 mean log cells/cm^2, (n=24) whether adhering to buna-N rubber strips alone or with L. monocytogenes. Average values for initial attachment of P. fluorescens were not significantly different ($p>0.05$) whether present on strips in pure or mixed microflora (Table 4).

Mechanical sheer produced during treatment did not remove a significant number of attached P. fluorescens cells (Table 4). These results further verify that the rinse

method used following attachment of P. fluorescens to buna-N rubber strips was successful in removing only those cells that were reversibly attached. Although gentle mechanical agitation would be expected to enhance the effects of enzymes during treatment, any loosening experienced by attached P. fluorescens cells treated with enzyme would primarily be due to the action of the enzyme.

Since some additional removal occurred due to mechanical stress sustained during incubation, L. monocytogenes did not appear to attach as strongly as P. fluorescens. Average numbers of attached cells of L. monocytogenes remaining after 5 hr incubation in PBS were 1.62 mean log cells/cm^2 (n=24) and 0.88 mean log cells/cm^2 (n=24) when present as pure and mixed microflora, respectively (Table 4). Compared to initial attachment values of 2.30 mean log cells/cm^2 (n=24) and 1.25 mean log cells/cm^2 (n=24) for L. monocytogenes prepared from pure and mixed cultures, respectively, mechanical stress did appear to remove some of the cells (Table 4). Thus, the mechanism involved in the attachment of P. fluorescens appears to be very different and far more resistant to mechanical action than that of L. monocytogenes. P. fluorescens present on black buna-N rubber strips was able to resist both shear forces involved in vortexing during rinsing as well as the

mechanical action sustained during subsequent enzyme treatment.

B: Enzymatic Treatment

1. Pseudomonas fluorescens

In all enzyme treatments involving P. fluorescens present alone on black buna-N rubber strips, no significant differences could be detected in the initial number of attached bacteria and the number remaining after mechanical agitation (control). Thus, any reduction in adherent P. fluorescens could be attributed to enzyme activity. After treatment with 5% and 10% Durazym™ values of mean log counts were not statistically different from each other or the control (Table 5) ($p=0.16$). When Savinase® was used to treat attached cells of P. fluorescens (Table 5), no difference was found among treatments ($p=0.47$). Treatment with Termamyl® produced no statistical difference between initial numbers of attached P. fluorescens, control strips, and those remaining after 5% and 10% enzyme treatment ($p=0.51$). Finally, although treatment with 10% Purafect® did produce some decrease in the number of attached cells (Table 5), no statistically significant decrease could be detected ($p=0.64$).

Table 5: Mean log cells/cm^2 of Pseudomonas fluorescens grown in pure culture adherent to black buna-N rubber strips. After 24 hr incubation at 30 °C initial counts were taken; remaining strips were enumerated after incubation for 5 hrs at 30 °C in a reciprocating shaker bath with PBS (control), 5% or 10% enzyme preparation. (n=24)

Enzyme (treatment)	Adherent Cells (mean log cells/cm^2 +/- std dev)	% Recovered (initial=100%)	% Recovered (control=100%)
Durazym™			
initial	4.38 +/- 0.08	100.00	
control	3.82 +/- 0.03	87.26	100.00
5% enzyme	4.21 +/- 0.03	95.95	109.96
10% enzyme	4.65 +/- 0.10	106.10	121.60
Savinase®			
initial	4.84 +/- 0.05	100.00	
control	4.38 +/- 0.07	90.35	100.00
5% enzyme	4.49 +/- 0.04	92.76	102.66
10% enzyme	4.77 +/- 0.11	98.52	109.03
Purafect®			
initial	3.89 +/- 0.05	100.00	
control	4.30 +/- 0.14	110.54	100.00
5% enzyme	3.56 +/- 0.04	91.56	82.83
10% enzyme	3.00 +/- 0.08	77.09	69.74
Termamyl®			
initial	4.18 +/- 0.04	100.00	
control	4.22 +/- 0.06	100.94	100.00
5% enzyme	4.33 +/- 0.05	103.58	102.62
10% enzyme	4.84 +/- 0.11	115.77	114.70

2. Listeria monocytogenes

Durazym™, Purafect®, and Termamyl® produced a significant reduction in numbers of attached cells of L. monocytogenes grown in pure culture. Durazym™, a crude commercial enzyme containing primarily proteolytic enzyme, did reduce the number of attached cells at treatment levels of both 5% and 10%. Numbers of cells recovered after treatment with 5% and 10% Durazym™ were statistically different from counts obtained for both initial attachment and control (PBS) strips ($p=0.003$). Differences among all treatment levels were also obtained when Termamyl®, containing primarily glycolytic enzyme, was used to treat attached cells of L. monocytogenes ($p=0.007$) (Table 6). A paired comparison test showed differences among cells recovered after initial attachment, control strips, and strips incubated in 5% and 10% enzyme ($p<0.05$) (Table 6). After treatment with 5% and 10% Termamyl®, mean log values were reduced by 68% when compared to initial attachment levels ($p=0.007$). However, even though a reduction in attached cells by over 50% was recorded when compared to control strips this was not statistically significant ($p>0.05$). This may have been due to extensive removal due to mechanical action of the shaker bath. No differences were

Table 6: Mean log cells/cm^2 of Listeria monocytogenes grown in pure culture adherent to black buna-N rubber strips. After 24 hr incubation at 30 °C initial counts were taken; remaining strips were enumerated after incubation for 5 hrs at 30 °C in a reciprocating shaker bath with PBS (control), 5% or 10% enzyme preparation. (n=24)

Enzyme (treatment)	Adherent Cells (mean log cells/cm^2 +/- std dev)	% Recovered (initial=100%)	% Recovered (control=100%)
Durazym™			
initial	2.55 +/- 0.05	100.00	
control	2.00 +/- 0.05	78.67	100.00
5% enzyme	0.32 +/- 0.13	12.39[a]	15.75[b]
10% enzyme	0.42 +/- 0.02	16.64[a]	21.15[b]
Savinase®			
initial	2.33 +/- 0.04	100.00	
control	1.48 +/- 0.05	63.33	100.00
5% enzyme	0.28 +/- 0.16	11.87	18.74
10% enzyme	0.82 +/- 0.01	35.27	55.70
Purafect®			
initial	2.22 +/- 0.06	100.00	
control	1.63 +/- 0.04	73.43	100.00
5% enzyme	0.18 +/- 0.26	8.22[a]	11.19[b]
10% enzyme	0.00 +/- 0.00	0.00[a]	0.00[b]
Termamyl®			
initial	2.08 +/- 0.04	100.00	
control	1.35 +/- 0.19	64.92	100.00
5% enzyme	0.65 +/- 0.04	31.38[a]	48.33
10% enzyme	0.56 +/- 0.31	26.83[a]	41.32

[a] significantly different when compared to initial mean log cells/cm^2 ($p<0.05$)
[b] significantly different when compared to control mean log cells/cm^2 ($p<0.05$)

obtained between use levels of 5% and 10% Termamyl® ($p>0.05$). Savinase® (Table 6) produced no differences among the various treatments ($p=0.14$). Purafect® did produce a marked decrease in cells recovered after enzyme treatment compared to those initially present on control strips ($p=0.01$). No difference was found when initial and control counts were compared ($p>0.05$). However, statistically significant differences where found between both the number of bacteria remaining on control strips and those incubated in 5% Purafect® ($p<0.05$) and between the two treatment levels of 5% and 10% enzyme ($p<0.05$).

The ability of both proteolytic and glycolytic enzymes to remove significant numbers of attached L. monocytogenes cells provides some additional insight into the mechanism of attachment used by this organism. Durazym™ is a subtilisin produced by Bacillus amyloliquefaciens (EC 3.4.21.14). Durazym™ possesses broad specificity and can hydrolyze many different types of peptide bonds as well as some ester bonds (Aunstrup, 1980). Termamyl® is a heat stable α-amylase expressed in and produced by a genetically modified strain of Bacillus licheniformis. This enzyme is an endoamylase which hydrolyzes α-1,4-glucosidic linkages in amylose and

amylopectin; end products are soluble dextrins and oligosaccharides (Anonymous, 1994b).

Purafect® is a protease used in the preparation of liquid detergents. This enzyme, of the subtilisin type, is derived from a selected strain of Bacillus lentus and expressed in another Bacillus species. Purafect® hydrolyzes insoluble protein strands into soluble peptides and amino acids and is mainly used for the removal of common household stains such as blood, grass, milk, and gravy (Anonoymous, 1994a). The ability of Durazym™, Termamyl®, and Purafect® to remove L. monocytogenes from buna-N rubber confirms previous studies which have described the biofilm matrix as comprised of both proteinaceous and polysaccharide material (Herald and Zottola, 1989, Beech and Gaylarde, 1989, Herald and Zottola, 1988).

3. Mixed culture studies

Although studies performed with individual organisms are useful in providing information concerning attachment, mixed culture environments are more representative of those found throughout nature and, particularly, in food processing facilities. Therefore, additional experiments using mixed cultures of P. fluorescens and L. monocytogenes were conducted. The biofilm matrix produced by bacteria

grown in mixed culture compared to pure culture is very different. Genus and species as well as the dynamics between microorganisms can influence the materials and mechanisms involved in anchoring and protecting adherent mixed culture bacteria (Lillard, 1985, Johnson et al., 1977, Beech and Gaylarde, 1989).

No significant differences could be detected in numbers of P. fluorescens attached with L. monocytogenes to buna-N rubber strips when treated with Durazym™ ($p=0.80$), Savinase® ($p=0.15$), and Termamyl® ($p=0.11$) (Table 7). When P. fluorescens grown in mixed culture was treated with Purafect®, a significant difference was found among treatment levels (5% and 10%) and initial attachment ($p=0.001$) (Table 7). A pair-wise comparison revealed that initial attachment levels and control strips (PBS) were not significantly different at the 0.05 level. Treated cells also differed significantly from control strips ($p<0.05$). Similarities were found between 5% and 10% treated strips (Table 7).

Purafect® treatment of L. monocytogenes cells attached to strips with P. fluorescens also produced significant reduction in attached cells ($p=0.002$) compared to initial and control strips (Table 8). Statistically significant

Table 7: Mean log cells/cm^2 of Pseudomonas fluorescens grown in mixed culture adherent to black buna-N rubber strips. After 24 hr incubation at 30 °C initial counts were taken; remaining strips were enumerated after incubation for 5 hrs at 30 °C in a reciprocating shaker bath with PBS (control), 5% or 10% enzyme preparation. (n=24)

Enzyme (treatment)	Adherent Cells (mean log cells/cm^2 +/- std dev)	% Recovered (initial=100%)	% Recovered (control=100%)
Durazym™			
initial	3.93 +/- 0.06	100.00	
control	4.46 +/- 0.05	113.35	100.00
5% enzyme	3.84 +/- 0.05	97.69	86.18
10% enzyme	3.92 +/- 0.10	99.62	87.89
Savinase®			
initial	3.30 +/- 0.03	100.00	
control	3.93 +/- 0.03	119.23	100.00
5% enzyme	4.28 +/- 0.03	129.76	108.83
10% enzyme	4.20 +/- 0.05	127.52	106.96
Purafect®			
initial	4.19 +/- 0.09	100.00	
control	4.17 +/- 0.05	99.50	100.00
5% enzyme	1.08 +/- 0.03	25.75[a]	25.88[b]
10% enzyme	0.81 +/- 0.27	19.25[a]	19.34[b]
Termamyl®			
initial	4.08 +/- 0.05	100.00	
control	3.44 +/- 0.04	84.34	100.00
5% enzyme	4.42 +/- 0.19	108.34	128.46
10% enzyme	4.22 +/- 0.06	103.46	122.67

[a] significantly different when compared to initial mean log cells/cm^2 ($p<0.05$)
[b] significantly different when compared to control mean log cells/cm^2 ($p<0.05$)

Table 8: Mean log cells/cm^2 counts of Listeria monocytogenes grown in mixed culture adherent to black buna-N rubber strips. After 24 hr incubation at 30 °C initial counts were taken, remaining strips were enumerated after incubation for 5 hrs at 30 °C in a reciprocating shaker bath with PBS (control), 5% or 10% enzyme preparation. (n=24)

Enzyme (treatment)	Adherent Cells (mean log cells/cm^2 +/- std dev)	% Recovered (initial=100%)	% Recovered (control=100%)
Durazym™			
initial	1.07 +/- 0.09	100.00	
control	1.30 +/- 0.04	121.87	100.00
5% enzyme	0.89 +/- 0.03	83.91	68.85
10% enzyme	0.96 +/- 0.02	89.97	73.82
Savinase®			
initial	1.06 +/- 0.02	100.00	
control	0.81 +/- 0.19	76.47	100.00
5% enzyme	0.26 +/- 0.01	24.42	31.93
10% enzyme	0.31 +/- 0.02	28.86	37.74
Purafect®			
initial	1.59 +/- 0.04	100.00	
control	0.62 +/- 0.02	38.86[a]	100.00
5% enzyme	0.00 +/- 0.00	0.00[a]	0.00
10% enzyme	0.00 +/- 0.00	0.00[a]	0.00
Termamyl®			
initial	1.26 +/- 0.05	100.00	
control	0.77 +/- 0.13	61.26	100.00
5% enzyme	0.43 +/- 0.01	33.95	55.42
10% enzyme	0.20 +/- 0.02	16.14	26.34

[a] significantly different when compared to initial mean log cells/cm^2 ($p<0.05$)
[b] significantly different when compared to control mean log cells/cm^2 ($p<0.05$)

differences were detected between levels initially attached to strips and controls ($p<0.05$). Additionally, no differences were obtained between strips treated with 5% and 10% Purafect®. However, differences were found among the two groups; numbers of attached cells removed after incubation in 5% and 10% enzyme were statistically different than both initial counts and controls ($p<0.05$) (Table 8).

Treatment with the remaining three enzymes did remove additional cells compared to control and initial levels (Table 8). However, none of the differences were statistically significant, probably due to the low numbers of L. monocytogenes initially attached. In contrast to results obtained with L. monocytogenes attached alone (Table 6), 5% and 10% levels of Durazym™ did not produce a marked loosening effect on cells of L. monocytogenes attached with P. fluorescens ($p=0.98$). Although Savinase® appears to have some effect on attached cells (Table 8), no statistical difference existed among treatment levels ($p=0.208$). Similarly, attached cells remaining after treatment with 5% and 10% Termamyl® (Table 8) are not statistically different compared to initial and control levels ($p=0.073$).

Adherent bacteria grown in pure and mixed culture exhibit different attachment mechanisms. Treatment with

Durazym™, Savinase®, and Termamyl® produced no significant ability to remove adherent P. fluorescens whether prepared in pure or mixed culture (Tables 5 and 7) ($p>0.05$). However, results obtained with Purafect® did produce differences based upon whether pure or mixed cultures were used in preparation of buna-N rubber strips. Purafect® did have the ability to remove adherent bacteria when P. fluorescens was prepared in mixed culture ($p=0.001$) (Table 7), while strips prepared with P. fluorescens alone were not affected ($p=0.639$) (Table 5).

Overall, Savinase® had no effect on removal of P. fluorescens after treatment of both pure (Table 5) and mixed (Table 7) cultures adherent to black buna-N rubber. Differences in the ability of Durazym™, Termamyl®, and Purafect® to remove cells attached to buna-N rubber strips in pure and mixed culture were more evident during studies on L. monocytogenes. Durazym™ reduced the number of attached cells of L. monocytogenes only when grown in pure culture ($p=0.003$), however, it did not produce a marked reduction in attached mixed culture cells when grown with P. fluorescens ($p=0.977$). Termamyl® did produce a statistically significant reduction in the number of

attached L. monocytogenes cells grown in pure culture (Table 6) ($p=0.007$) but not when grown in the presence of P. fluorescens ($p=0.073$) (Table 5). The ability of Durazym™, and Termamyl®, to remove pure culture cells of adherent L. monocytogenes and not mixed culture adherent cells provides information about the role of P. fluorescens in a mixed culture biofilm. Differences in material involved in biofilm formation by L. monocytogenes and P. fluorescens together appear to offer some protection to L. monocytogenes toward susceptability to removal by these proteolytic and glycolytic enzymes.

Treatment with Purafect® did produce reduction of attached cells in all cases (Tables 6-8) except when Pseudomonas was present on buna-N rubber strips alone (Table 5). In fact,cell reduction provided by Purafect® on both attached cells of P. fluorescens and L. monocytogenes grown in mixed culture may provide the most substantial evidence concerning the nature of the attachment material. Purafect® was able to reduce numbers of attached L. monocytogenes alone (Table 6) and in mixed culture (Table 8) indicating that resistance displayed by P. fluorescens alone did not carry over to an ability to protect L. monocytogenes.

Moreover, in mixed culture, P. fluorescens did become susceptable to the hydrolytic action of Purafect®.

C. Products of Enzymatic Activity

Durazym™, Savinase®, and Purafect® are commercially available crude enzyme preparations containing primarily proteolytic activity. The ability of Purafect® to remove P. fluorescens in mixed culture with L. monocytogenes (Table 7) and L. monocytogenes alone and in mixed culture (Tables 6 and 8) attached to buna-N rubber strips would be expected to be related to the hydrolytic activity present in this enzyme preparation. Thus, measurement of material which absorbs at 280 nm was used to detect the release of proteolytic products formed during enzymatic treatment. Similarly, release of carbohydrate material by Termamyl® activity was monitored by the dinitrosalicylic acid method (Wood and Bhat, 1988) except that a wavelength of 625 nm was used. Wood and Bhat (1988) suggest using a wavelength of 490 nm to assay hydrolyzed material produced from carboxymethyl cellulose. However, glucose, galactose and mannose are reducing sugars expected to be produced during glycolysis of adhered P. fluorescens and L. monocytogenes. Thus, 625 nm was chosen based on spectra obtained with glucose,

galactose, mannose and the enzyme Termamyl® (Appendix B, Figures 15-18).

1. Pseudomonas fluorescens

Control strips containing attached P. fluorescens alone produced only a slight increase in absorbance indicating that some material was released during incubation (Figure 3). Rubber strips treated with 5% and 10% Durazym™ released only slightly more material with absorbance at 280 nm; final values were 0.18 and 0.35, respectively. When strips containing attached P. fluorescens were treated with Savinase®, slightly greater amounts of material which absorbed at 280 nm was released compared to control (A_{280}=0.17) (Figure 4). Absorbance values for strips treated with 5% and 10% Savinase® increased rapidly during the first 60 min and then only slightly throughout the remainder of the incubation period. Only strips treated with 5% Purafect® produced a slight increase of material which absorbed at 280 nm compared to control strips; 10% treated strips were similar to controls (Figure 5). No release of carbohydrate-containing material could be detected following treatment with Termamyl® (Figure 6).

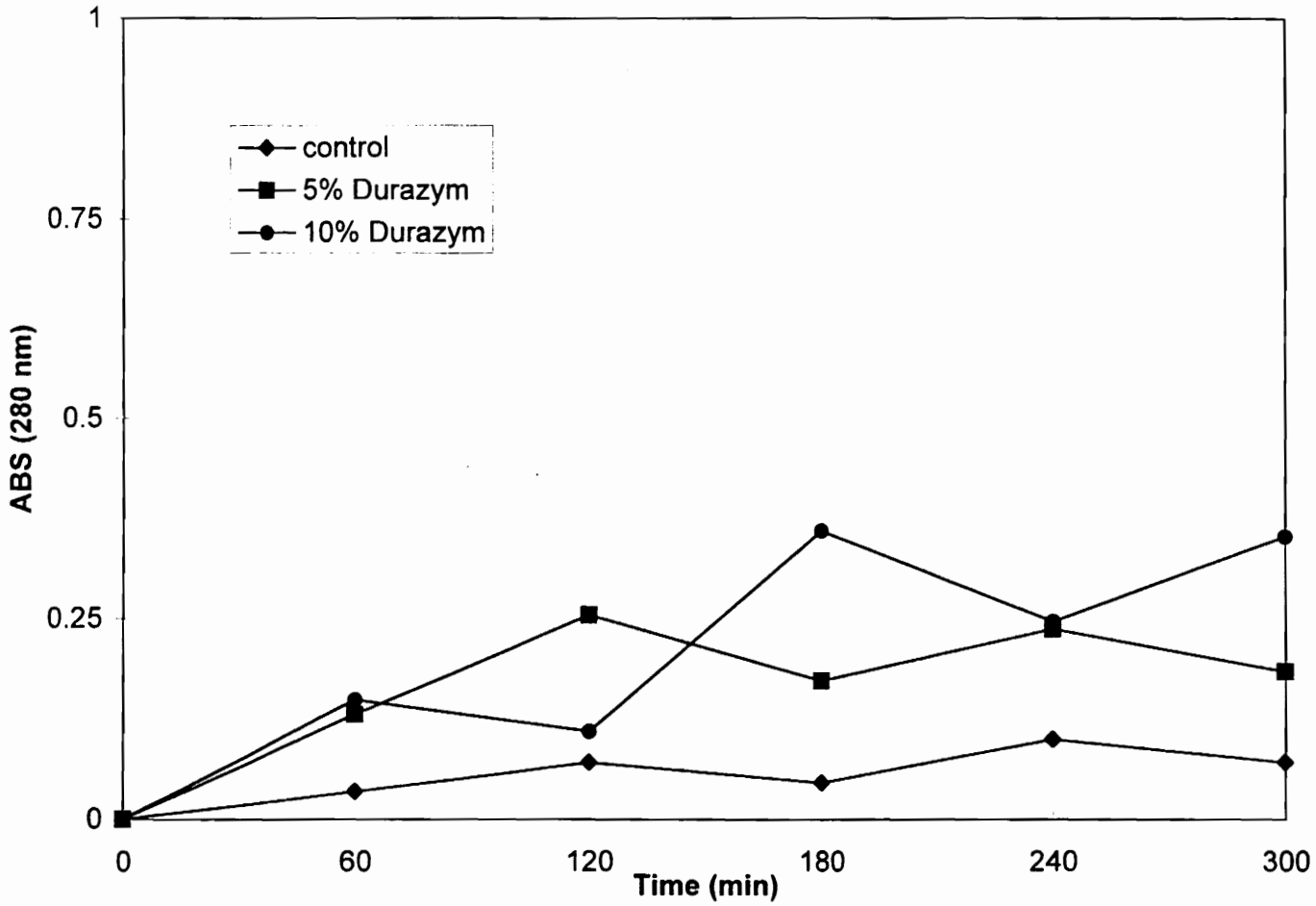

Figure 3: Results of assay for released material which absorbs at 280 nm after treatment of adherent pure culture cells of Pseudomonas fluorescens with Durazym™. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Durazym™ in PBS (v/v) are also shown.

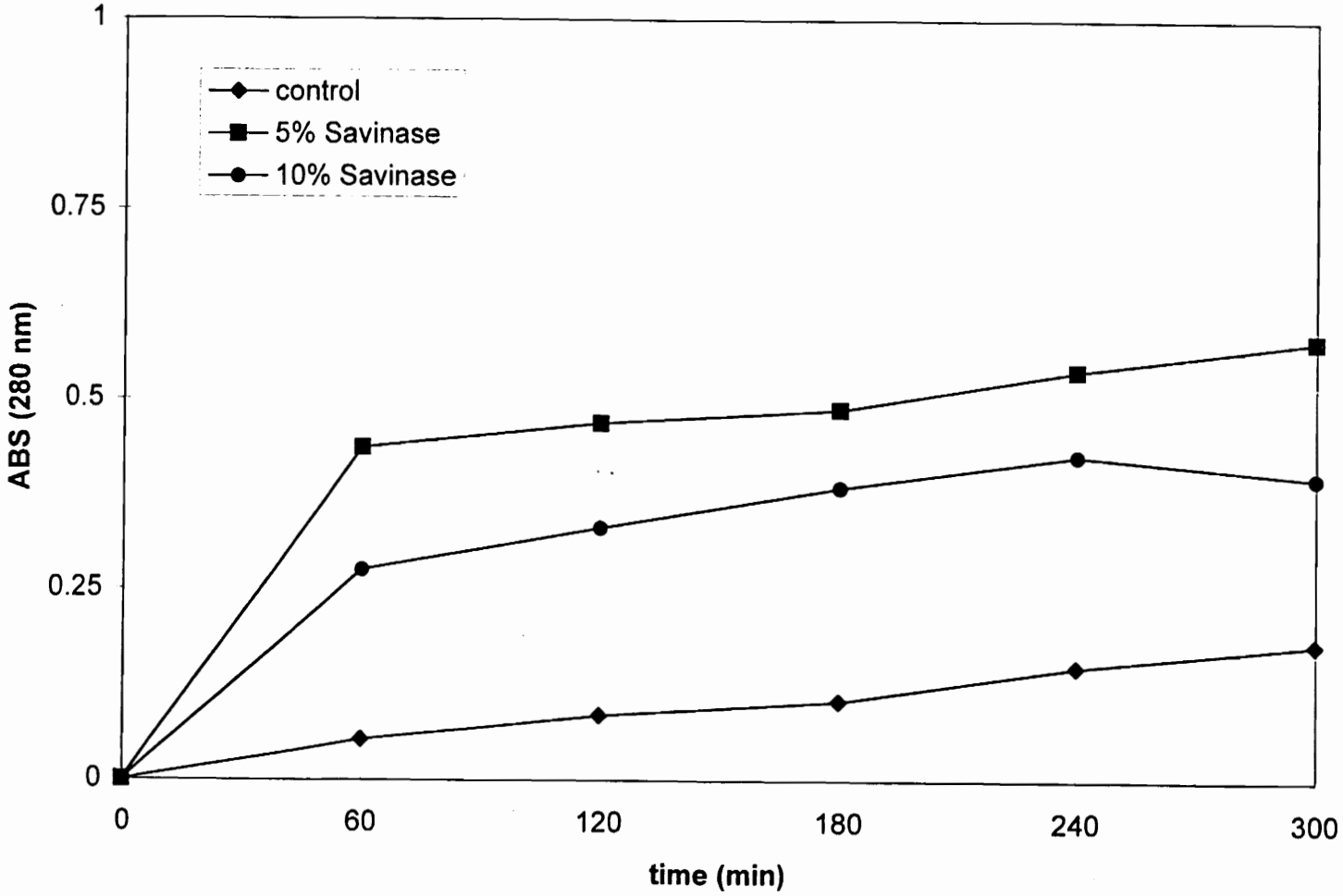

Figure 4: Results of assay for released material which absorbs at 280 nm after treatment of adherent pure culture cells of Pseudomonas fluorescens with Savinase®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Savinase® in PBS (v/v) are also shown.

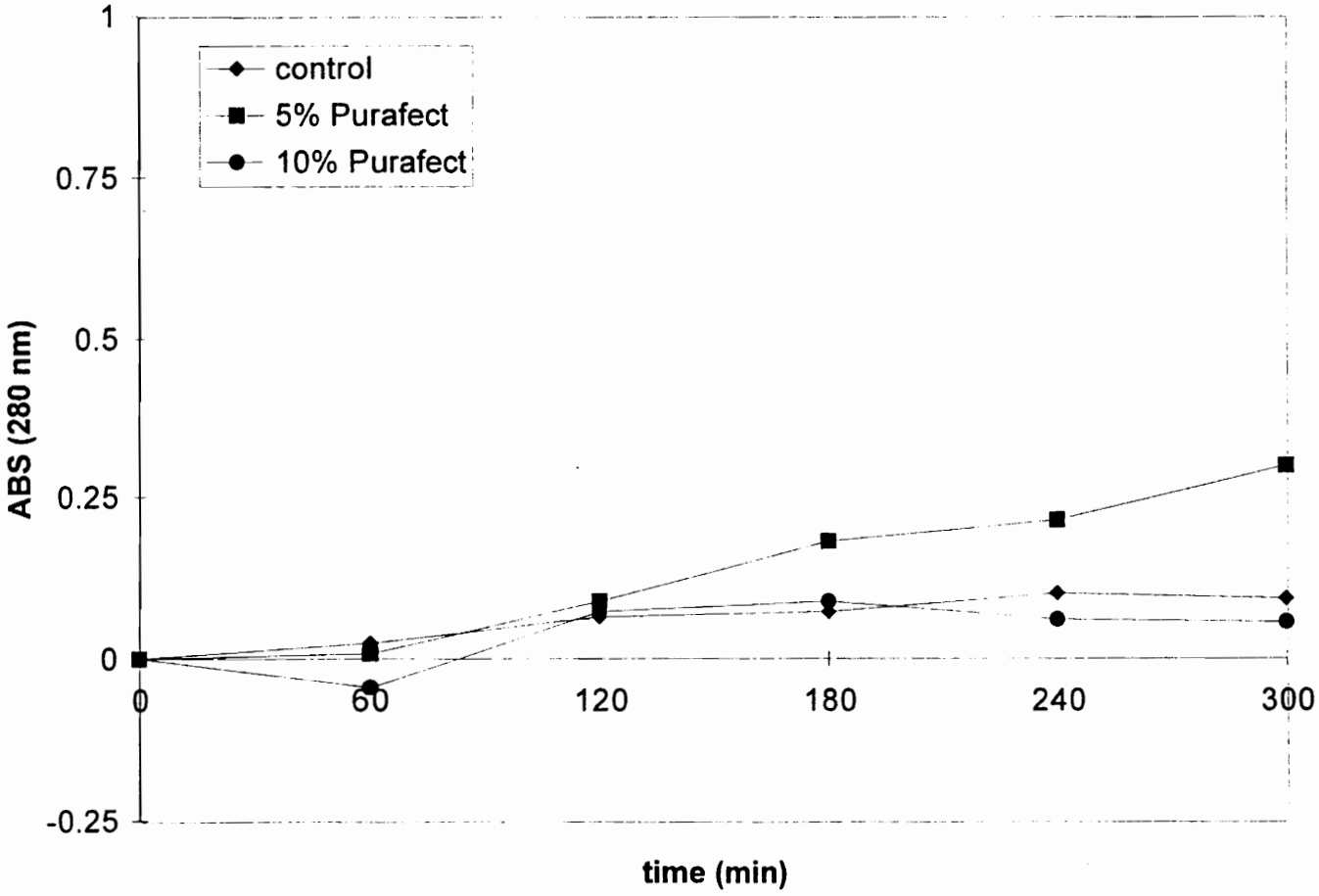

Figure 5: Results of assay for released material which absorbs at 280 nm after treatment of adherent pure culture cells of Pseudomonas fluorescens with Purafect®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Purafect® in PBS (v/v) are also shown.

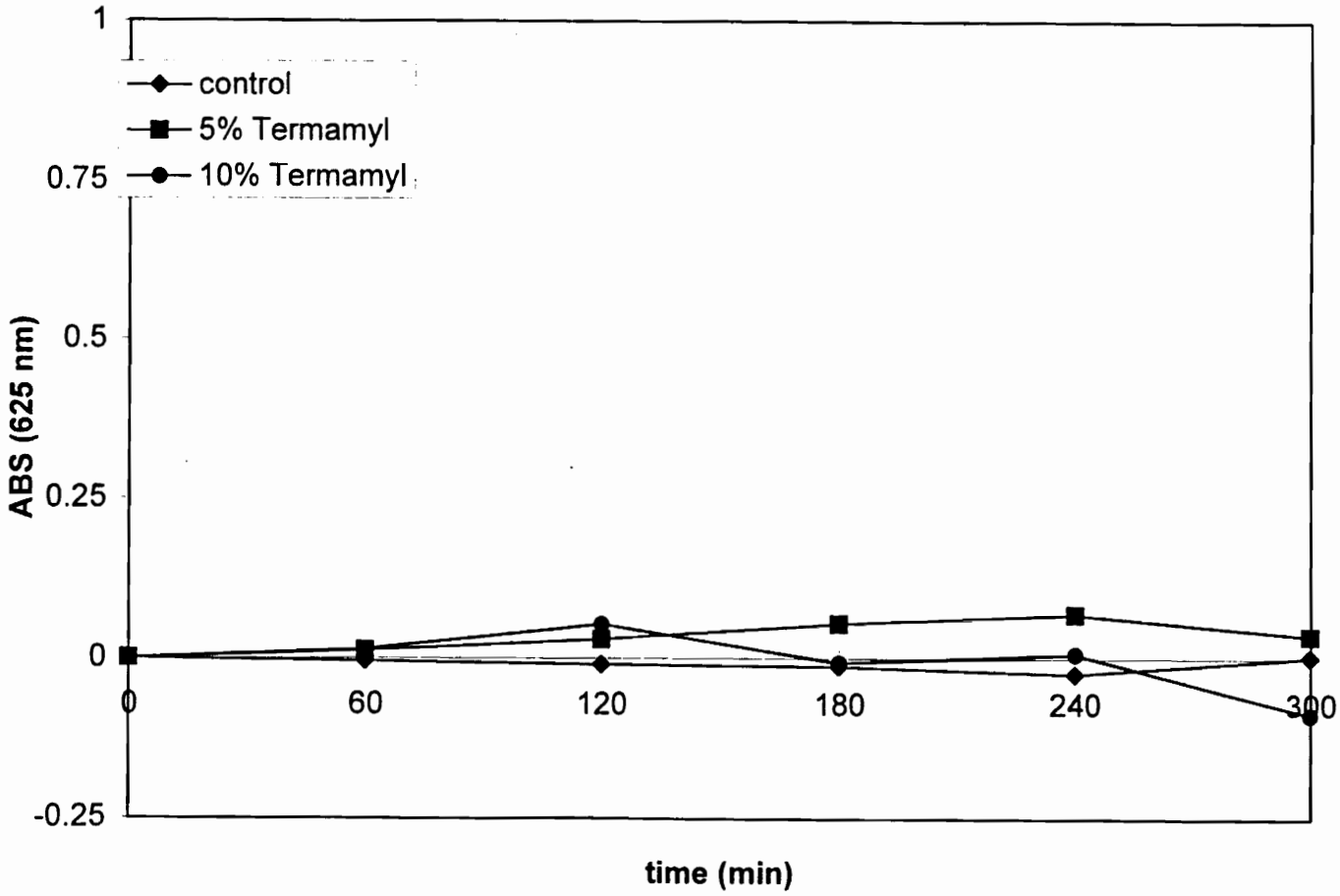

Figure 6: Results of assay for released carbohydrate containing material after treatment of adherent pure culture cells of Pseudomonas fluorescens with Termamyl®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Termamyl® in PBS (v/v) are also shown.

Although Durazym™, Savinase®, and Purafect® appeared to have no effect on P. fluorescens alone attached to rubber strips, (Table 5) some material which absorbed at 280 nm was released during treatment (Figures 3-5) These results would seem to indicate that although some proteolytic activity was occurring, any microbial activity associated with the biofilm was not affected. The glycosidase Termamyl® had no apparent effect on the biofilm matrix (Table 5, Figure 6).

2. L. monocytogenes

Durazym™ applied to pure culture cells of L. monocytogenes reduced the number of cells attached to buna-N rubber strips (Table 6) ($p=0.003$). Treatment with Durazym™ (Figure 7) also produced a corresponding increase in material which absorbed at 280 nm after the first 60 min of incubation. Final absorbance readings increased only slightly over the remainder of the incubation period (0.65 and 0.28 for 5% and 10% treatment levels, respectively). After the first 60 min, readings taken at 280 nm for attached cells treated with Savinase® (Figure 8) were 0.30 and 0.43, for levels of 5% and 10% enzyme, respectively. Final absorbance values rose slightly to 0.52 and 0.60.

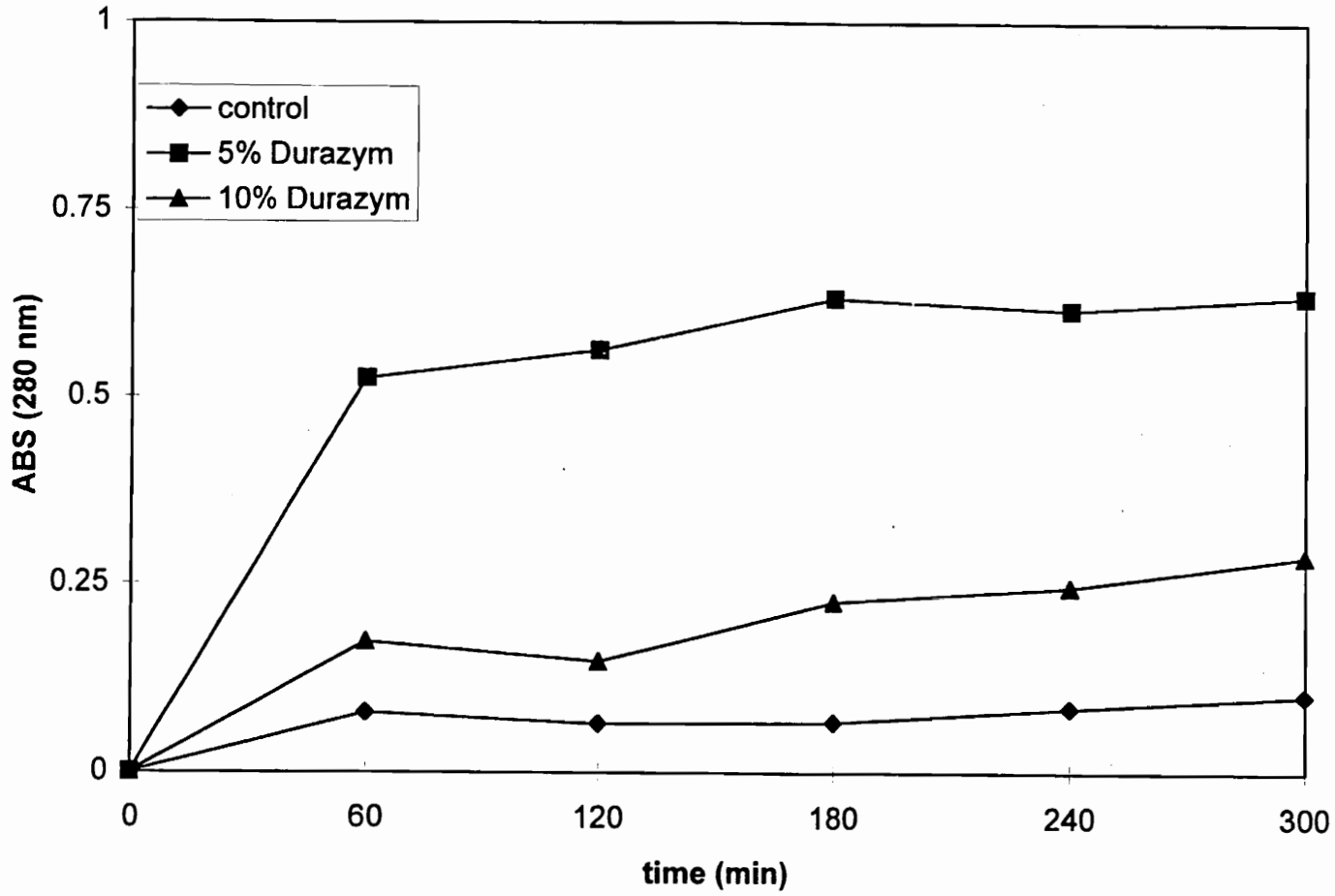

Figure 7: Results of assay for released material which absorbs at 280 nm after treatment of adherent pure culture cells of Listeria monocytogenes with Durazym™. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Durazym™ in PBS (v/v) are also shown.

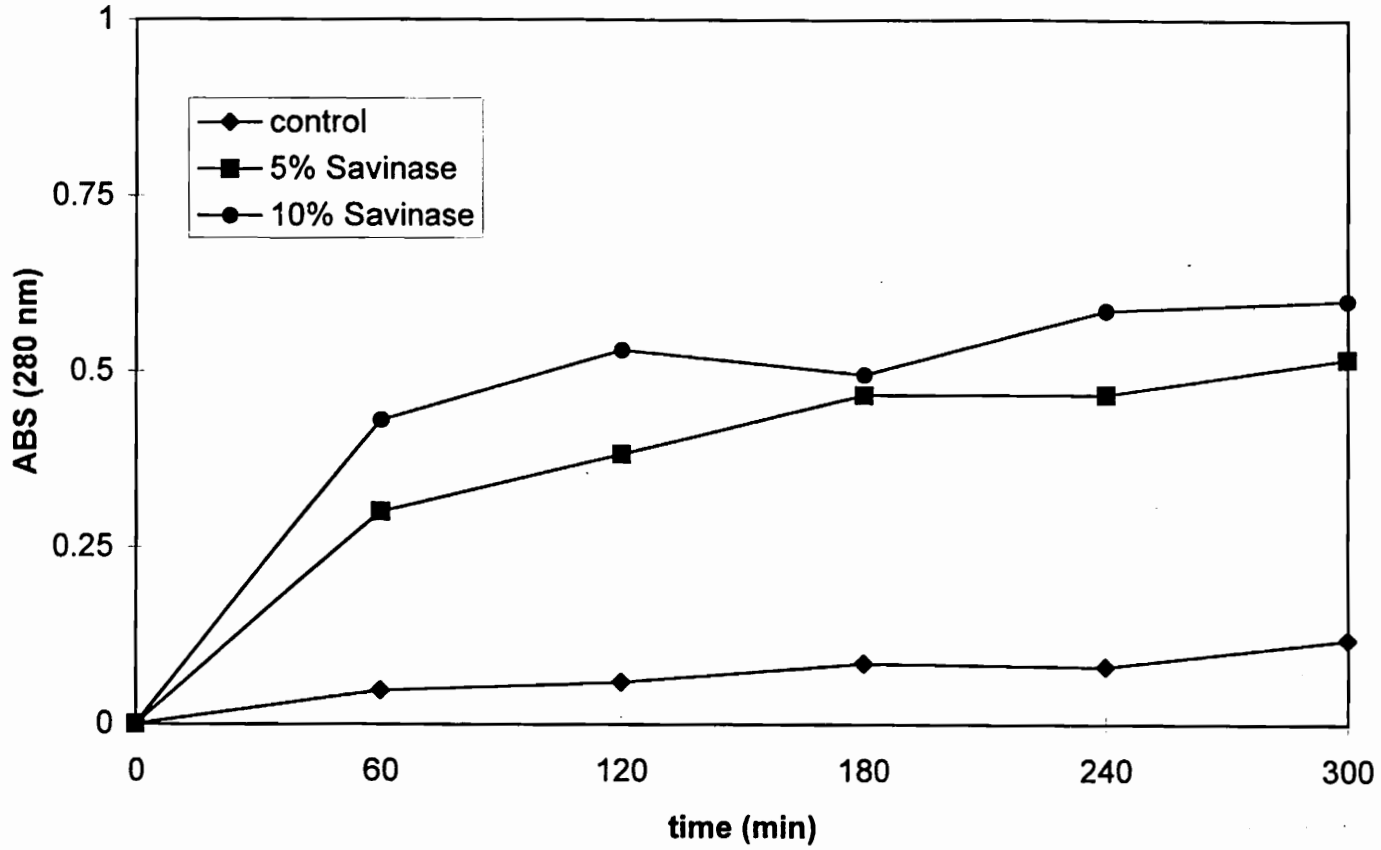

Figure 8: Results of assay for released material which absorbs at 280 nm after treatment of adherent pure culture cells of Listeria monocytogenes with Savinase®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Savinase® in PBS (v/v) are also shown.

Termamyl® applied to pure culture adherent cells of L. monocytogenes produced a marked reduction in attached cells (Table 6, p=0.007). However, no significant production of carbohydrate-containing material could be detected at either level (Figure 9). Material which absorbs at 280 nm released after treatment with Purafect® (Figure 10) produced values of 0.33 and 0.15 after the first 60 min. Absorbance values rose gradually to final values of 0.60 and 0.43 for treatment levels of 5% and 10% after 5 hr incubation. All control strips produced very minimal absorbance values at both 280 and 625 nm, indicating nominal release of proteinaceous and carbohydrate-containing material due to mechanical action.

Treatment of attached pure culture cells of L. monocytogenes with all three proteolytic enzymes resulted in release of proteinaceous material. However, in the case of Savinase®, hydrolysis did not appear to occur to the extent necessary to produce a marked reduction in attached cells (Table 6). The proteinaceous material released by Savinase® may not be involved in anchoring and/or sustaining the biofilm matrix. Durazym™ and Purafect® may release a larger portion of proteinaceous material involved in actually anchoring the cells to the buna-N rubber strips.

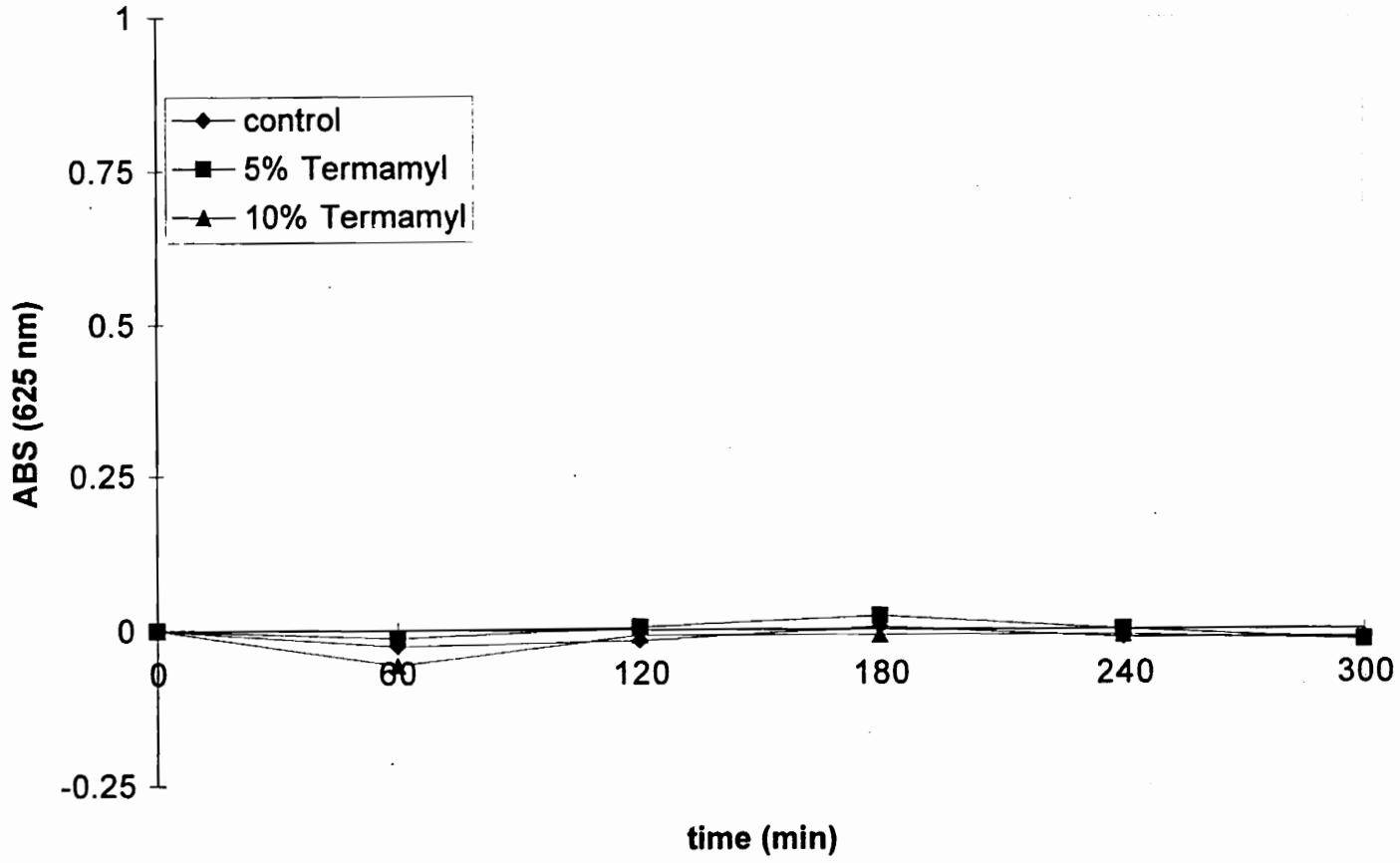

Figure 9: Results of assay for released carbohydrate containing material after treatment of adherent pure culture cells of Listeria monocytogenes with Termamyl®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Termamyl® in PBS (v/v) are also shown.

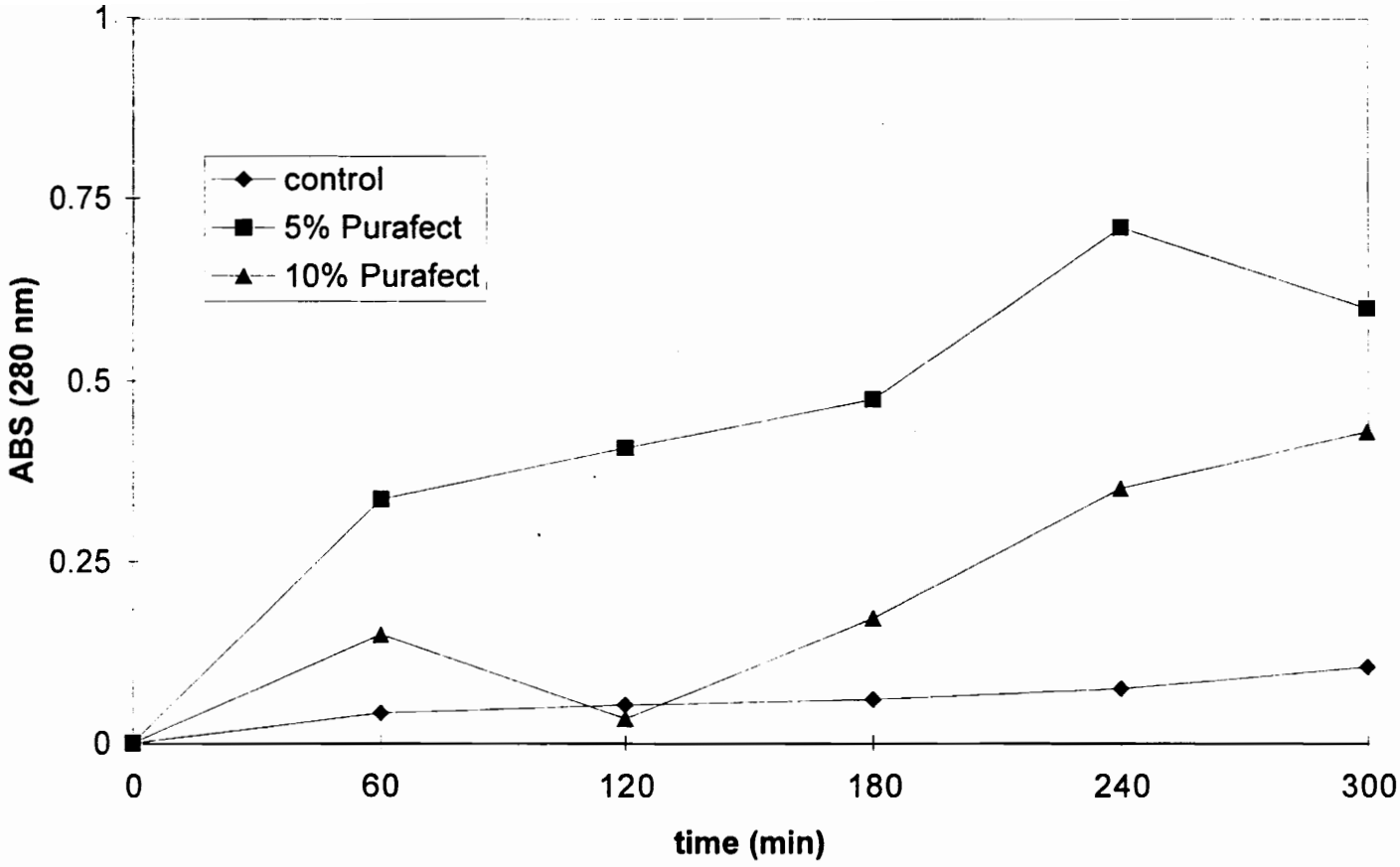

Figure 10: Results of assay for released material which absorbs at 280 nm after treatment of adherent pure culture cells of Listeria monocytogenes with Purafect®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Purafect® in PBS (v/v) are also shown.

Termamyl® was also able to produce a statistically significant reduction in the number of attached pure culture cells of L. monocytogenes (Table 6). However, no carbohydrate-containing material could be detected following treatment with Termamyl® (Figure 9). The sensitivity of the dinitrosalicylic acid assay may account for the inability to detect released reducing sugars. The dinitrosalicylic acid assay cannot detect levels of glucose below 100 ug/ml (Wood and Bhat, 1988). Since only microscopic amounts of biofilm material are necessary to cover and protect adherent bacteria, another more sensitive assay may be needed to determine whether carbohydrates are released following treatment with Termamyl®. Alternatively, enzymes used in this study are crude commercial preparations and are known to contain contaminating enzymes of varying activities. Many crude preparations of glycolytic enzymes are known to also contain proteolytic enzymes in small amounts with broad specificities (Godfrey and Reichelt, 1983). These proteolytic enzymes present in small amounts, may actually account for the ability of Termamyl® to reduce attached cells of L. monocytogenes.

3. Mixed culture

Treatment of attached mixed culture cells with 5% and 10% Durazym™ (Figure 11) produced absorbance values of 0.44 and 0.38 after 60 min incubation; values increased slightly to 0.66 and 0.57 after 5 hr in a reciprocating shaker bath. After 60 min mixed culture strips treated with Savinase® also produced a rapid increase in absorbance; again, final values rose only slightly to 0.55 and 0.70 (Figure 12). Once again, no carbohydrate containing material could be detected during Termamyl® treatment of strips (Figure 13)

Although Purafect® was able to produce a significant reduction in attached mixed culture cells of both P. fluorescens and L. monocytogenes (Tables 7 and 8), levels of released material which absorbs at 280 nm (Figure 14) were similar to the other proteolytic enzymes used in this study. After 60 min incubation, absorbance values for Purafect® treated adherent cells rose quickly to 0.44 and 0.38. Continued incubation produced a gradual increase in these values to 0.66 and 0.57 for 5% and 10% treatment levels (Figure 14). Since Purafect® was able to produce a statistically significant reduction in the number of attached mixed culture cells of both P. fluorescens (Table

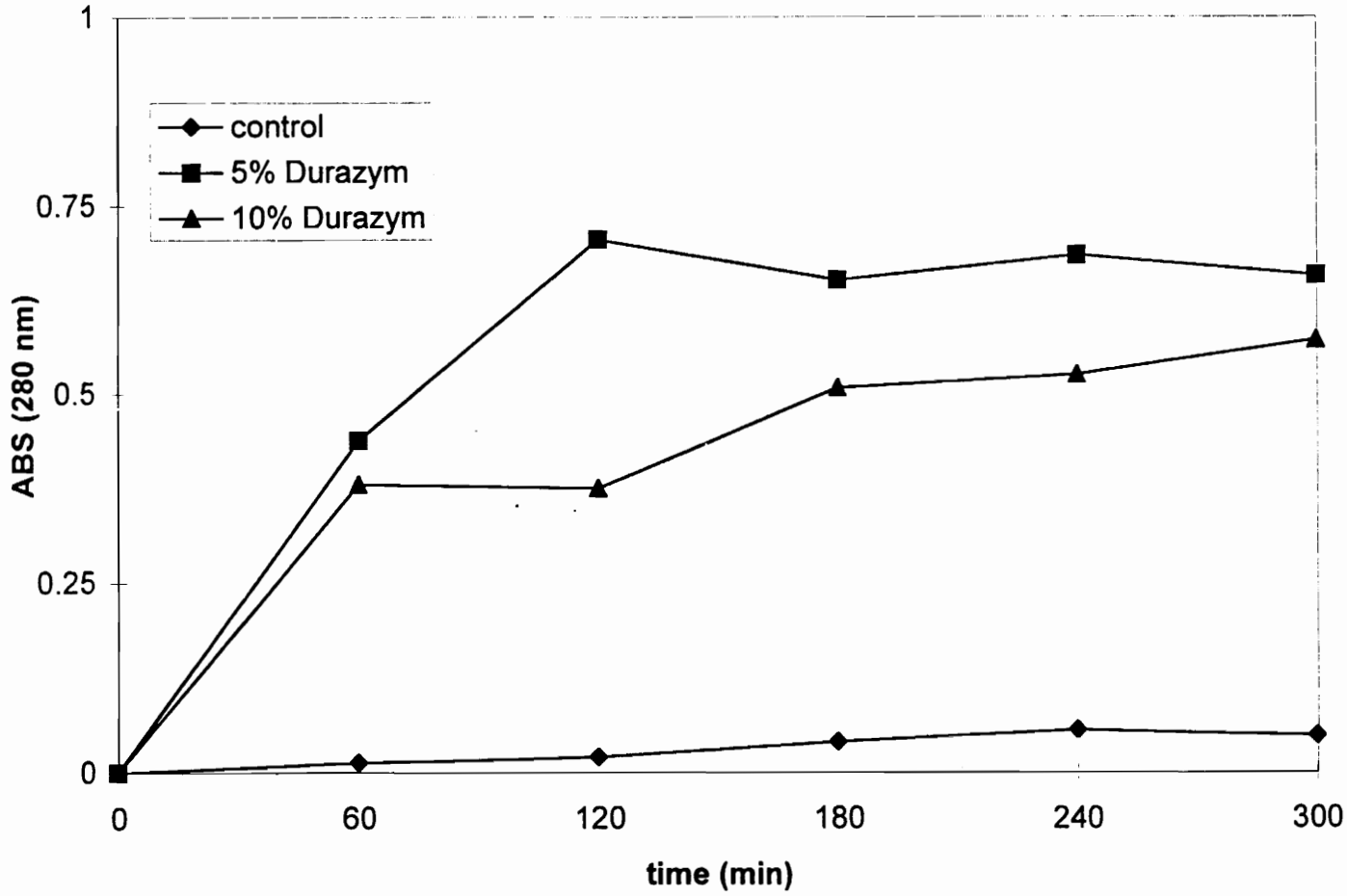

Figure 11: Results of assay for released material which absorbs at 280 nm after treatment of adherent mixed culture cells of Pseudomonas fluorescens and Listeria monocytogenes with Durazym™. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Durazym™ in PBS (v/v) are also shown.

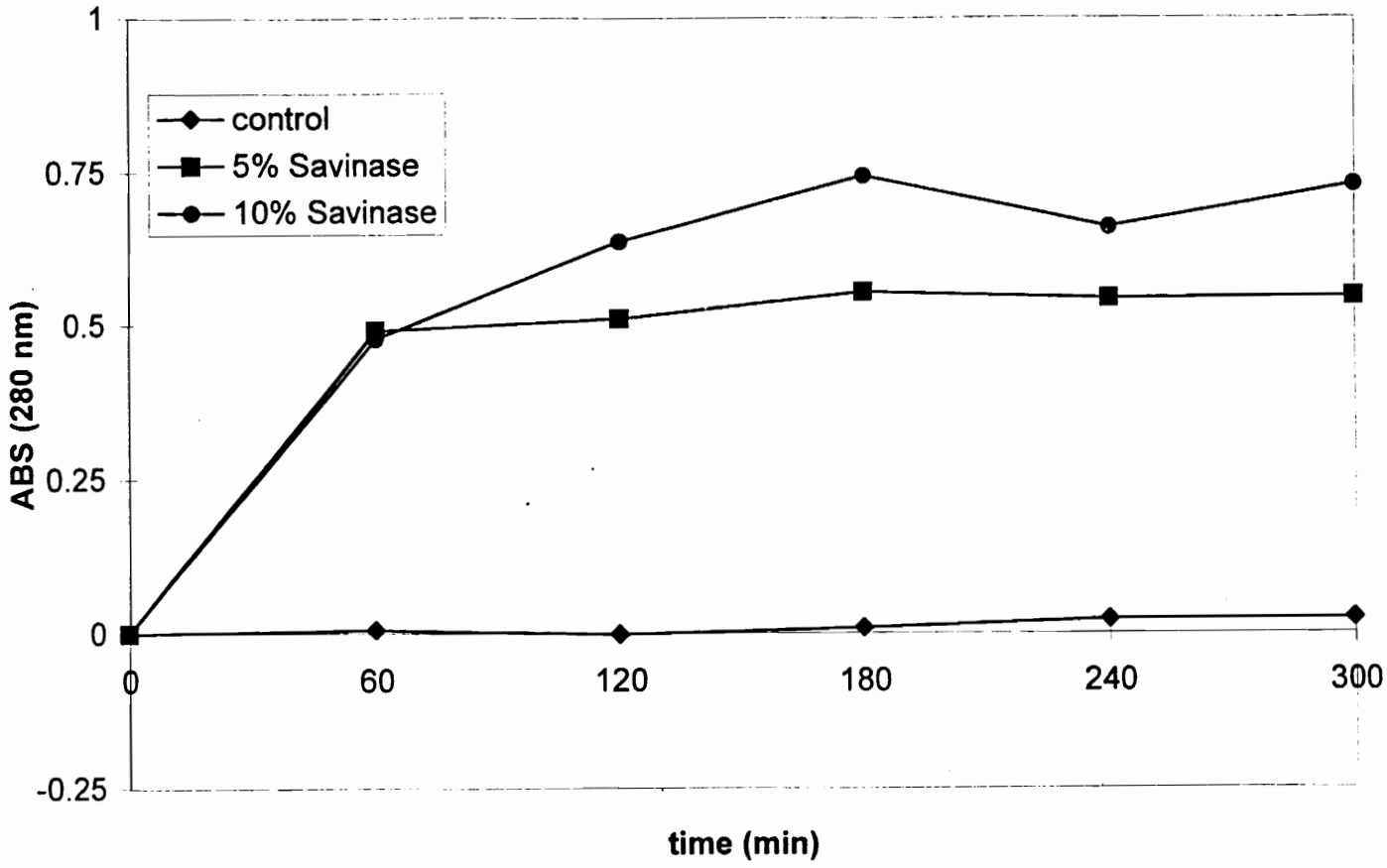

Figure 12: Results of assay for released material which absorbs at 280 nm after treatment of adherent mixed culture cells of Pseudomonas fluorescens and Listeria monocytogenes with Savinase®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Savinase® in PBS (v/v) are also shown.

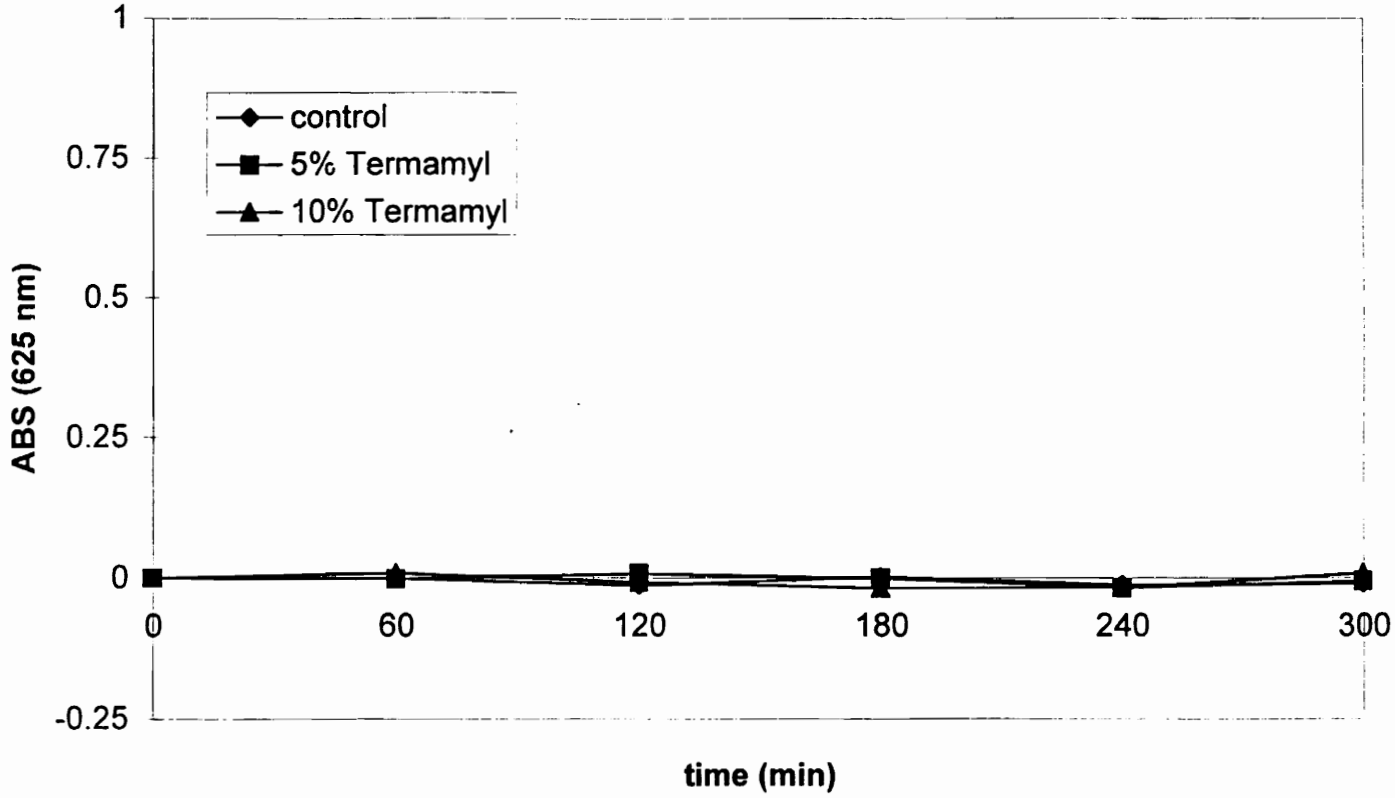

Figure 13: Results of assay for released carbohydrate containing material after treatment of adherent mixed culture cells of Pseudomonas fluorescens and Listeria monocytogenes with Termamyl®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Termamyl® in PBS (v/v) are also shown.

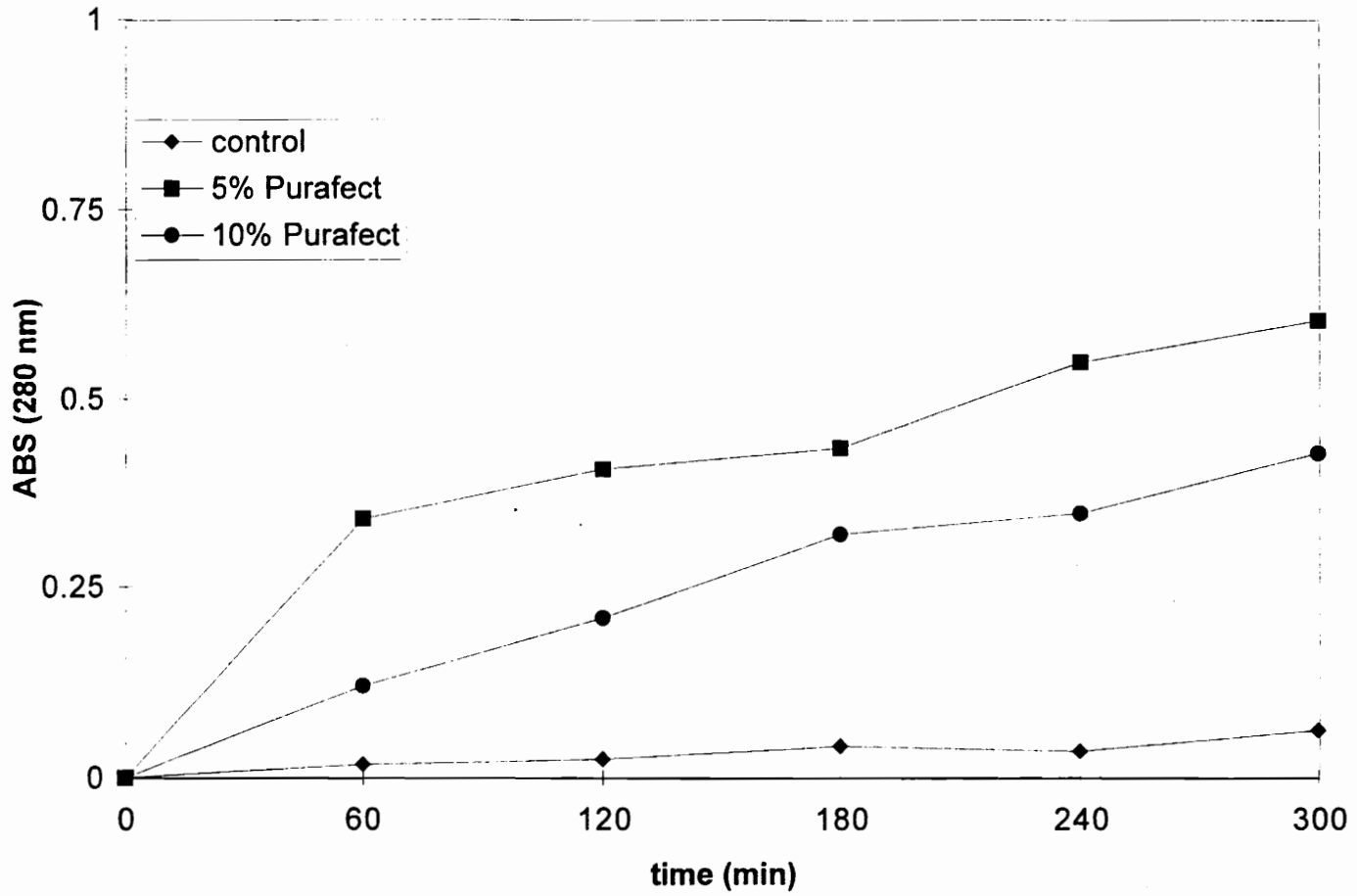

Figure 14: Results of assay for released material which absorbs at 280 nm after treatment of adherent pure culture cells of Pseudomonas fluorescens and Listeria monocytogenes with Purafect®. Strips were incubated for 24 hr at 30 °C and 30 rpm. Control data is for material released due to mechanical agitation of the shaker bath. Released material for treatment levels of 5% and 10% Purafect® in PBS (v/v) are also shown.

7, p=0.001) and L. monocytogenes (Table 8, p=0.002), these results would seem to indicate an effect in addition to hydrolysis and release of material anchoring cells in pure and mixed culture biofilms.

D. Bactericidal Effects of Dialyzed Enzymes

Studies were conducted to determine if the dialyzed enzyme preparations possessed bactericidal effects. Durazym™, Savinase®, and Termamyl® produced no differences between the number of live planktonic cells present at the start of the 5 hr incubation and the number of cells remaining after treatment for both P. fluorescens and L. monocytogenes (Table 9). However, Purafect® at concentrations of both 5% and 10%, had a considerable bactericidal effect on pure cultures of both P. fluorescens and L. monocytogenes in the planktonic state (Table 9). A decrease from initial values of 3 log units produced no survivors for either bacterium after treatment with Purafect®.

Planktonic cells and attached bacteria have very different behavioral characteristics. A bactericidal effect experienced by planktonic cells will not necessarily occur in cells adherent in a pure or mixed culture biofilm. Bacteria present in a biofilm have been known to exhibit

Table 9: Bactericidal effects treatment of 5% and 10% enzyme concentration had on planktonic cells of Pseudomonas fluorescens and Listeria monocytogenes incubated for 5 hrs at 30 °C in a reciprocating shaker bath at 30 rpm. Initial and Final data is also provided for untreated planktonic cells in PBS. (n=24)

Organism (PBS)	Initial (mean log cells/ml +/- std dev)	Final (mean log cells/ml +/- std dev)
Pseudomonas fluorescens	2.58 +/- 0.49	2.17 +/- 0.07
Listeria monocytogenes	2.91 +/- 0.04	2.79 +/- 0.11

Enzyme	Pseudomonas fluorescens 5% Enzyme (mean log cells/ml +/- std dev)	10% Enzyme (mean log cells/ml +/- std dev)	Listeria monocytogenes 5% Enzyme (mean log cells/ml +/- std dev)	10% Enzyme (mean log cells/ml +/- std dev)
Durazym™	2.58 +/- 0.08	2.62 +/- 0.07	2.91 +/- 0.03	3.07 +/- 0.06
Purafect®	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00
Savinase®	2.64 +/- 0.14	2.82 +/- 0.05	2.96 +/- 0.06	2.91 +/- 0.06
Termamyl®	2.64 +/- 0.01	2.50 +/- 0.08	2.73 +/- 0.03	2.65 +/- 0.12

increased heat resistance (Butler et al., 1980, Frank and Koffi, 1990) and increased resistance to sanitizers and detergents (Krysinski et al., 1992, Stone and Zottola, 1985b) when compared to planktonic cells. However, the increased ability of Purafect® to remove P. fluorescens and L. monocytogenes attached to black buna-N rubber strips compared to Durazym™, Savinase®, and Termamyl® would appear to potentially involve the bactericidal effect noted on planktonic cells.

The release of material which absorbs at 280 nm when the proteolytic enzymes Durazym™, Savinase®, and Purafect® were used to treat adherent pure or mixed culture cells indicates that hydrolysis of biofilm material occured. Removal of protective proteinaceous biofilm material followed by the bactericidal effects noted on planktonic cells may help explain the enhanced effectiveness of Purafect® in biofilm removal. Savinase®, and Durazym™ were also able to expose attached mixed culture bacteria but did not posses the ability to kill planktonic cells as efficiently as Purafect®.

Conclusions:

Purafect® was able to reduce the number of Pseudomonas fluorescens and Listeria monocytogenes attached in a mixed culture biofilm adherent to black buna-N rubber. None of the four enzymes examined were able to significantly reduce the number of P. fluorescens adherent in a pure culture biofilm. Pure culture cells of L. monocytogenes contained in a biofilm were removed when the proteolytic enzymes Durazym™ and Purafect® were used. Additionally, the glycolytic enzyme Termamyl® was effective in reducing the number of adherent cells.

Assays of the incubation media were used to determine if proteinaceous and polysaccharide material was released during mechanical action and treatment with proteolytic and glycolytic enzymes. For adherent cells formed from both pure and mixed cultures of P. fluorescens and L. monocytogenes, no release of carbohydrate-containing material was reported. However, all of the proteolytic enzymes (Durazym™, Savinase®, and Purafect®) did release material with the ability to absorb at 280 nm during treatment.

The ability of proteolytic enzymes to reduce the number of adherent cells and release proteinaceous material further verifies the involvement of protein in the biofilm matrix. However, this may only be true for P. fluorescens and L. monocytogenes grown in mixed culture on black buna-N rubber in a milk system. Bacteria are complex, diverse organisms surviving and secreting substances which allow them to attach to many surfaces in both natural (rocks, teeth, and the intestines of animals) and un-natural environments (internal prosthetic devices, and polyurethane). Therefore, the ability of industrial enzymes to reduce the numbers of adherent bacteria formed by other microorganisms pertinent to food processing environments needs further investigation.

Purafect® did reduce the number of attached cells of P. fluorescens, and L. monocytogenes adherent in mixed culture as well as attached cells of L. monocytogenes adherent in pure culture. Moreover, Purafect® did produce a bactericidal effect on planktonic cells of both P. fluorescens and L. monocytogenes. Hydrolysis of a protective proteinaceous layer may have exposed attached cells allowing the bactericidal components to reduce the number of recoverable cells.

Material involved in anchoring and sustaining a pure culture biofilm is very different from that secreted in mixed culture. Durazym™, Purafect®, and Termamyl® were able to produce a significant reduction in attached pure culture cells of L. monocytogenes adherent to black buna-N rubber. Durazym™ is a subtilisin (EC 3.4.21.14) produced by Bacillus amyloliquefaciens and has a broad specificity in hydrolyzing most types of peptide bonds. Termamyl® (EC 3.2.1.1) is an α-amylase expressed in and produced by a genetically modified strain of Bacillus licheniformis. This enzyme is a endoamylase which will hydrolyze 1,4-α-glucosidic linkages in amylose and amylopectin. Purafect® (EC 3.4.21) is a protease of the type subtilisin which hydrolyzes insoluble protein strains into soluble peptides and amino acids. The ability of both proteolytic and glycolytic enzymes to reduce the numbers of attached pure culture cells of L. monocytogenes grown in TSBYE seems to indicate the presence of proteinaceous and polysaccharide material in this particular biofilm matrix.

Overall, results of this research may be useful in the formulation of cleaning and sanitizing agents and also may provide information on characterization of the attachment

process. The crude enzymes in this study have a broad specificity and contain many types of enzymes which can hydrolyze both proteinaceous and polysaccharide material. In the future more highly purified preparations of the enzymes used in this study should be examined to determine their effect on P. fluorescens and L. monocytogenes attached to buna-N rubber. If these enzymes fail to produce the desired effect then components of the crude enzyme preparations should be isolated and characterized to identify fractions responsible for biofilm removal. Finally, several other enzymes utilized by the detergent and food manufacturing industry could have a potential use as a biofilm remover. Future studies should be performed to screen more potential biofilm removing detergent and food processing enzymes.

REFERENCES

Alais, C. and G. Linden. 1991. Polysaccharides. In *Food Biochemistry*, I. Morton (Ed.), p.38-52. Ellis Horwood, England.

Anonymous, 1992a. Durazym™. Product Sheet. Detergent Enzyme Division. Novo Nordisk A/S, Bagsvaerd, Denmark.

Anonymous, 1992b. Savinase®. Product Sheet. Detergent Enzyme Division. Novo Nordisk A/S, Bagsvaerd, Denmark.

Anonymous, 1994a. Purafect® 4000L. Protease enzyme for liquid detergents. Product Information. Genencor International, Inc., Rochester, New York.

Anonymous, 1994b. Termamyl®. Product Sheet. Enzyme Process Division. Novo Nordisk A/S, Bagsvaerd, Denmark.

Anwar, H., M.K. DasGupta, and W. Costerton. 1990. Testing the susceptibility of bacteria in biofilms to antibacterial agents. Antimicrob. Agents and Chemother. 34:2043-2046.

AOAC 1992. *Bacteriological Analytical Manual, 7th ed.* Association of Official Analytical Chemists International, Arligton, Virginia.

Aunstrup, K. 1980. Proteinases. In *Economic Microbiology, Vol 5*, A.H. Rose (Ed.), p.49-114. Academic Press, London.

Azadian, B.S., G.T. Finnerty, and A.D. Pearson. 1989. Cheese-borne *Listeria* meningitis in immunocompetent patient. Lancet. 1:322-323.

Banwart, G.J. 1989. Basic Food Microbiology, 2nd ed. Chapman & Hall, NY.

Barfoed, H.C. 1983. Detergents. In *Industrial Enzymology. The Application of Enzymes in Industry*, T. Godfrey and J. Reichelt (Ed.), p.284-293. Macmillan Publishers, Ltd., England.

Beech, I.B. and C.G. Gaylarde. 1989. Adhesion of *Disulfovibrio desulfuricans* and *Pseudomonas fluorescens* to mild steel surfaces. J. of Appl. Bacteriol. 67:201-207.

Berche, P., J.L. Gaillard, and S. Richard. 1988. Invasiveness and intracellular growth of *Listeria monocytogenes*. Infection. 16(suppl.2):S145-S148.

Bigelis, R. 1993. Carbohydrases. In *Enzymes in Food Processing, 3rd ed*, T. Nagodawithana and G. Reed (Ed.), p.121-158. Acedemic Press Inc., San Diego, California.

Bille, J. 1990. Epidemiology of human listeriosis in Europe, with special reference to the Swiss outbreak. In *Foodborne Listeriosis*, A.J. Miller, J.L. Smith, and G.A. Somkuti (Ed.), p.71-74. Society for Industrial Microbiology, Elsevier Science Publishing Inc., New York.

Boikess, R.S. and E. Edelson. 1985. *Chemical Principles. 3rd ed.* RD Publishing, Highland Park, NJ.

Brown, C.M., D.C. Ellwood and J.R. Hunter. 1977. Growth of bacteria at surfaces: Influence of nutrient limitation. FEMS Microbiol. Lett. 1:163-166.

Bunning, V.K., C.W. Donnelly, J.T. Peeler, E.H. Briggs, J.G. Bradshaw, R.G. Crawford, C.M. Beliveau, and J.T. Tierney. 1988. Thermal inactivation of *Listeria monocytogenes* within bovine milk phagocytes. Applied Environ. Microbiol. 54:364-370.

Busscher H.J. and A.H. Weekamp. 1987. Specific and non-specific interactions in bacterial adhesion to solid strata. FEMS Microbiol. Rev. 46:165-173.

Busta, F.F., E.H. Peterson, D.M. Adams, and M.G. Johnson. 1984. Chapter 4: Colony count methods. In *Compendium of Methods for the Microbiological Examination of Foods, 2nd ed*, M.L. Speck (Ed.), p.62-83. American Public Health Association, Washington, D.C.

Butler, J.L., J.C. Stewart, C. Vanderzant, Z.L. Carpenter, and G.C. Smith. 1979. Attachment of microorganisms to pork skin and surfaces of beef and lamb carcasses. J. Food Prot. 42:401-406.

Butler, J.L., C. Vanderzant, Z.L. Carpenter, G.C. Smith, R.E, Lweis, and T.R. Dutson. 1980. Influence of certain processing steps on attachment of microorganisms to pork skin. J. Food Prot. 43:699-705.

Campbell, D.M. 1990. Human Listeriosis in Scotland. 1967-1988. J. Infect. 20:241-250.

Christie, A.O., L.V. Evans, and M. Shaw. 1970. Studies on the ship fouling alga *Enteromorpha*. II. Effect of certain enzymes on the adhesion of zoospores. Ann. Bot. 34:467-482.

Chung, K.T., J.S. Dickenson, and J.D. Crouse. 1989. Attachment and proliferation of bacteria on meat. J. Food Prot. 52:173-177.
Corpe, W.A. 1970. An acid polysaccharide produced by a primary film-forming marine bacterium. Dev. Ind. Microbiol. 11:402-412.

Costerton, J.W., K.J. Cheng, G.G. Gheesey, T. Ladd, J.C. Nickel, M. Dasgupta, and T.J. Marrie. 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41:435-464.

Costerton, J.W., G.G. Geesey, & K.J. Cheng. 1978. How bacteria stick. Sci. Am. 238:86-95.

Costerton, J.W., R.T. Irwin, and K.J. Cheng. 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. 35:299-324.

Cousin, M.A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products: A review. J. Food Prot. 45:172-207.

Cousins, C.M. and A.J. Bramley. 1981. The microbiology of raw milk. In *Dairy Microbiology. Vol. 1. The Microbiology of Milk*, R.K. Robinson (Ed.), p.119-163. Applied Science Publishers Ltd., Reading, England.

Criado, M.T., B. Suarez, and C. Ferreiros. 1994. The importance of bacterial adhesion in the dairy industry. Food Technol. 48(2):123-126.

Czechowski, M. H. 1990. Bacterial attachment to buna-n gaskets in milk processing equipment. Aust. J. Dairy Technol. 45(2):113-114.

Danielsson, A, B. Norkrans, and A. Bjornsson. 1977. On bacterial adhesion-The effect of certain enzymes on adhered cells of a marine Pseudomonas species. Bot. Mar. XX:13-17.

Dawson, M.P., B.A. Humphrey, and K.C. Marshall. 1981. Adhesion: A tactic in the survival stategy of a marine *Vibrio* during starvation. Curr. Microbiol. 6:195-199.

Dickenson, J.S. and M. Koohmarie. 1989. Cell surface charge charecteristics and their relationship to bacterial attachment to meat surfaces. Applied Environ. Microbiol. 55:832-836.

Doyle, M.P., K.A. Glass, J.T. Beery, G.A. Garcia, D.J. Pollard, and R.D. Shultz. 1987. Survival of *Listeria monocytogenes* in milk during high temperature, short time pasteurization. Applied Environ. Microbiol. 53:1433-1438.

Dunsmore, D.G., A. Twomey, W.G. Wittlestone, and H.W. Morgan. 1981. Design and performance of systems for cleaning product-contact surfaces of food equipment: A review. J. Food Prot. 44:220-240.

Fairbairn, D.J. and B.A. Law. 1986. Review article proteinases of psychrotrophic bacteria: their production, properties, effects and control. J. Dairy Res. 53:139-177.

Farber, J.M. and P.I. Peterkin. 1991. *Listeria monocytogenes* a food-borne pathogen. Microbiol. Rev. 55:476-511.

Firstenburg-Eden, R., S. Notermans, F. Thiel, S. Henstra, and E.H. Kampelmacher. 1979. Scanning electron microscopic investigations into attachment of bacteria to teats of cows. J. Food Prot. 42:305-309.

Fletcher, M. and G.D. Floodgate. 1973. An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. J. Gen. Microbiol. 74:325-334.

Fletcher, M. and G.D. Floodgate. 1976. The adhesion of bacteria to solid surfaces. In *Microbial Ultrastructure*, R. Fuller and D.W. Lovelock (Ed.), p.101-107. Academic Press, London.

Fletcher, M. and G.I. Loeb. 1979. Influence of substratum charecteristics on the attchment of Pseudomonad to solid surfaces. Applied Environ. Microbiol. 37:67-72.

Frank, J.F., and R.A. Koffi. 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. J. Food Prot. 53:550-554.

Geesey, G.G. R. Mutch, J.W. Costerton, and R.B. Green. 1978. Sessile bacteria: An improtant component of the microbiol population in small mountain streams. Limnol. Oceanogr. 23:1214-1223.

Gellin, B.G., C.V. Broome, W.F. Bibb, R.E. Weaver, S. Gaventa, L. Mascola, and the Listeriosis Group. 1991. The epidemiology of listeriosis in the United States.-1986. Am. J. Epidemiol. 133:392-401.

Godfrey, T., and J.R. Reichelt. 1983. Introduction to industrial enzymology. In *Industrial Enzymology. The Application of Enzymes in Industry*, T. Godfrey and J.R. Reichelt (Ed.), p.1-7. Macmillan Publishers Ltd., England.

Golnazarian, C.A., C.W. Donnelly, S.J. Pintauro, and D.B. Howard. 1989. Comparison of infectious dose of *Listeria monocytogenes* F5817 as determined for normal versus compromised C57B1/6J mice. J. Food Prot. 52:696-701.

Grey, M.L. and A.H. Killinger. 1966. *Listeria monocytogenes* and listeric infections. Bacteriol. Rev. 30(2):309-382.

Hartley, B.S. 1960. Proteolytic enzymes. Annu. Rev. Biochem. 29:45-72.

Herold, P.J. and E. A. Zottoloa. 1988. Scanning electron microscopic examination of *Yersinia enterocolitica* attached to stainless steel at selected temperatures and pH values. J. Food Prot. 51:445-448.

Herold, P.J. and E. A. Zottoloa. 1988. The use of transmission electron microscopy to study the composition of *Pseudomonas fragi* attachment material. Food Microstruct. 7:53-57..

Hood, S.K. and E.A. Zottola. 1995. Review. Biofilms in food processing. Food Control. 6:9-18.

Humphrey, B., S. Kjelleberg, and K. C. Marshall. 1983. Responses of marine bacteria under starvation conditions at a solid-water interface. Applied Environ. Microbiol. 45:43-47.

Isaacson, R.E. 1985. Pilus Adhesions. In *Bacterial Adhesion*, D.C. Savage and M. Fletcher (Ed.), p.307-336. Plenum Publishing Corp. New York, New York.

Iwaida. M., Y. Kawaguchi, and T. Tsugo. 1967. Ultraviolet spectrophotometric determination of protein content in milk. J. Dairy Sci. 50:1322-1372.

Johnson, M.C., J.J. Bozzola, I.L. Schechmeister, and I.L. Shklair. 1977. Biochemical study on the relationship of extracellular glucan to adhere and carcinogenicity in *Streptococcus mutans* and an extracellular polysaccharide mutant. J. Bacteriol. 129:351-357.

Jones, H.C., I.L. Roth, and W.M. Sanders III. 1969. Electron microscopic study of a slime layer. J. Bacteriol. 99:316-325.

Junttila, J.R., S.I. Niemela, and J. Hirn. 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic listeria. J. Appl. Bacteriol. 65:321-327.

Kessler, S.L. and A.S. Dajani. 1990. *Listeria* meningitis in infants and children. Pediatr. Infect. Dis. J. 9:61-63.

King, E.O., M.K. Ward, and D.E. Raney. 1954. Two simple media for the demonstration of pyocyanin and flourescin. J. Lab. Clin. Med. 44:301-307.

Kirtley, S.A. and Mc Guirie. 1989. Dairy foods technology notes on differences in surface constitution of dairy product contact materials. J. Dairy Sci. 72: 1748-1753.

Kjelleberg, S. and M. Hermansson. 1984. Starvation-induced effects on bacterial surface charecteristics. Applied Environ. Microbiol. 48:497-503.

Kjelleberg, S., B. A. Humphrey, and K. C. Marshall. 1983. Initial phases of starvation and activity of bacteria at surfaces. Applied Environ. Microbiol. 46:978-984.

Krysinski, E.P., L.I. Brown, and T.J. Marchicello. 1992. Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. J. Food Prot. 55:246-251.

Lappin-Scott, H.M., J.W. Costerton, and T.J. Marrie. 1992. Biofilms and biofouling. Encycl. Microbiol. 1:277-284.

Lappin-Scott, H.M. and J.W. Costerton. 1989. Bacterial biofilms and surface fouling. Biofouling. 1:323-342.

Law, B.A. 1979. Reveiws of the process of dairy science: Enzymes of psychrotrophic bacteria and their effects on milk and milk products. J. Dairy Res. 46:573-588.

Law, B.A., A.T. Andrews, and M.E. Sharpe. 1977. Gelation of ultra-high-temperature-sterilized milk by proteases from a strain of *Pseudomonas flourescens* isolated from raw milk. J. Dairy Res. 44:145-148.

Law, B.A., C.M. Cousins, M.E. Sharpe, and F.L. Davies. 1979. Psychrotrophs and their effects on milk and dairy products. In *Cold tolerant microbes in spoilage and the environment*, A.D. Russell and R. Fuller (Ed.),. Acedemic Press, (Society for Applied Bacteriology Symposium Series), London.

Lee, S.H. and J.F. Frank. 1991. Inactivation of surface-adherent *Listeria monocytogenes*. Hypochlorite and heat. J. Food Prot. 54:4-6.

Lillard, H.S. 1985. Bacterial cell charecteristics and conditions influencing their adhesion to poultry skin. J. Food Prot. 48:803-807.

Linnan, M.J., L. Mascola, X.D. Lou, V. Goulet, S. May, C. Salminen, D.W. Hird, M.L. Yonekura, P.Hayes, R. Weaver, A. Audurier, B.D. Plikaytis, S.L. Fannin, A Kleks, and C.V. Broome. 1988. Epidemic Listeriosis associated with Mexican-style cheese. N. Engl. J. Med. 319:823-828.

Loffler, A. 1986. Proteolytic enzymes: Sources and applications. Food Technol. 40:63-70.

Lopes, J.A. 1986. Evaluation of dairy and food plant sanitizers against *Salmonella typhimurium* and *Listeria monocytogenes*. J. Dairy Sci. 69:2791-2796.

MacLeod, F.A., H.M. Lappin-Scott, and J.W. Costerton. 1988. Plugging of a model rock system by using starved bacteria. Applied Environ. Microbiol. 54:1365-1372.

Mafu, A.A., D. Roy, J. Goulet, and P. Magny. 1990. Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene, and rubber surfaces after short contact times. J. Food Prot. 53:742-746.

Marshall, K.C., and R.H. Schmidt. 1988. Growth of *Listeria monocytogenes* at 10 °C in milk preincubated with selected Pseudomonads. J. Food Prot. 51:277-282.

Marshall, K.C., R. Stout, and R. Mitchell. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. 68:337-348.

Matyunas, N.J. 1987. The Polar B'Ar ice cream recall-report or exposure in pregnant women. Vet. Hum. Toxicol. 29:469,ABS55.

McCarthy, S.A. 1990. *Listeria* in the environment. In *Food-borne Listeriosis*, A.J. Miller, J.L. Smith, and G.A. Somkuti (Ed.), p.25-29. Society for Industrial Microbiology, Elsevier Science Publishing, Inc., New York.

McGee, Z.A., G.L. Gorby, P.B. Wyrick, R. Hodinka and L.H. Hoffman. 1988. Parasite-directed endocytosis. Rev. Infect. Dis. 10:S311-S316.

McLauchlin, J. 1989. Rapid non-cultural methods for the detection of *Listeria* in food-a review. Microbiol. Aliments Nutr. 7:279-284.

Mosteller, T. and J.R. Bishop. 1993. Sanitizer efficacy towards attached bacteria in a milk biofilm. J. Food Prot. 56:34-41.

Neijssel, O.M. and D.W. Tempest. 1975. The regulation of carbohydrate metabolism in *Klebsiella aerogens* NCTC 418. Organisms growing in chemostat culture. Arch. Microbiol. 160:251-258.

Neiman, R.E. and B. Lorber. 1980. Listeriosis in adults: a changing pattern. Report of eight cases and review of the Literature, 1968-1978. Rev. Infect. Dis. 2:207-227.

Noorlander, D.O., and R. Heckman. 1980. Scanning electron microscopy and etiological studies of teat cup inflations for mastitis control. J. Food Prot. 43:205-207.

Notermans, S., J.A. Dormans, and G.C. Mead. 1991. Contribution of surface attachment to the establishment of microorganisms in food processing plants: A review. Biofouling 5:21-36.

Notermans, S. and E.H. Kampelmacher. 1974. Attachment of some bacterial strains to the skin of broiler chickens. Br. Poult. Sci. 15:573-585.

Novitsky, J.A. and R.Y. Morita. 1977. Survival of a psychrophilic marine *Vibrio* under long-term nutrient starvation. Applied Environ. Microbiol. 33:635-641.

Palleroni, N.J. 1984 Section 4. Gram-Negative Aerobic Rods and Cocci. Family 1. Pseudomonadaceae. In *Bergey's Manual of Systematic Bacteriology, Vol.1*, N.R. Krieg and J.G Holt (Ed.), p. 156-165. Williams & Wilkins, Baltimore.

Paul, J.H. and W.H. Jefferey. 1985. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces for *Vibrio proteolytica*. Applied Environ. Microbiol. 50:431.

Peel, M., W. Donachie, and A. Shaw. 1988. Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-Page, and Western Blotting. J. Gen. Microbiol. 134:2171-2178.

Pine, L., G.B. Malcolm, J.B. Brooks, and M.I. Daneshvar. 1989. Physilogical studies on the growth and utilization of sugars by *Listeria* species. Can. J. Microbiol. 35:245-254

Poffe, R. and W. Mertens. 1988. Heat stable proteases of psychrotrophic bacteria isolated from cooled raw milk. Appl. Microbiol. Biotechnol. 27:437-442.

Pringle, J.H. and M. Fletcher. 1983. Influence of substratum wettability on attachmnet if fresh water bacteria to solid surfaces. Applied Environ. Microbiol. 45:811-817.

Ren, T.J. and J.F. Frank. 1993. Susceptibility of starved planktonic and biofilm *Listeria monocytogenes* to quaternary ammonium sanitizer as determined by direct viable and agar plate counts. J. Food Prot. 56:573-576.

Rocourt, J. and B. Catimel. 1989. International phage typing center for *Listeria*: report for 1987. Acta Microbiol. Hung. 36:225-229.

Romney, A.J.D. 1990a. Chapter 1. Principles of Cleaning. In *CIP: Cleaning in Place*, A.J.D. Romney (Ed.), p.1-6. The Society of Dairy Technology, Cambridgeshire, England.

Romney, A.J.D. 1990b. Chapter 3. The Chemistry of Detergents. In *CIP: Cleaning in Place*, A.J.D. Romney (Ed.), p.17-29. The Society of Dairy Technology, Cambridgeshire, England.

Ronner, A.B. and A.C.L. Wong. 1993. Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and buna-n rubber. J. Food Prot. 56:750-758.

Rutter, P.R. and Vincent, B. 1980. The adhesion of micro-organisms to surfaces: physici-chemical aspects. In *Microbial Adhesion to Surfaces*, R.C.W. Berkeley, J.M. Lynch, J. Melling, P.R. Rutter, and B. Vincent (Ed.), p.79-92. Ellis Horwood Ltd., Chichester, England.

Sands, D.C., and A.D. Rovira. 1970. Isolation of flourescent *Pseudomonads* with a selective medium. Appl. Microbiol. 20:513-514.

Sasahara, K.C. and E.A. Zottola. 1993. Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. J. Food Sci. 56:1022-1028.

Schlech, W.F.III, P.M. Lavigne, R.A. Bortolussi, A.C. Allen, E.V. Haldane, A.J. Wort, A.W. Hightower, S.E. Johnson, S.H. King, E.S. Nicholis, and C.V. Broome. 1983. Epidemic listeriosis-evidence for transmission by food. N. Engl. J. Med. 308:203-206.

Seelinger, H.P.R. and H. Finger. 1976. Listeriosis. In *Infectious diseases of the fetus and new born infant*, J.S. Remington and J.O. Klein (Ed.), p.333-365. The W.B. Saunders Co., Philadelphia.

Seelinger, H.P.R., and D. Jones. 1986. Listeria. In *Bergey's Manual of Systemic Bacteriology, Vol 2*, P.H.A. Sneath, N.S. Mair, M.E. Sharpe, and J.G. Holt (Ed.),. Williams & Wilkins, Baltimore.

Speers, J.G.S., and A. Gilmour. 1985. The influence of milk and milk components on the atachment of bacteria to farm dairy equiptment surfaces. J. Appl. Bacteriol. 59:325-332.

Speers, J.G.S., A. Gilmour, T.W. Fraser, and R.D. McCall. 1984. Scanning electron microscopy of dairy equipment surfaces contaminated by two milk-borne micro-organisms. J. Appl. Bacteriol. 57:139-145.

Stead, D. 1986. Review Article. Microbiol lipases: their charecteristics, role in food spoilage and industrial uses. J. Dairy Res. 53:481-505.

Stone, L.S. and E.A. Zottola. 1985a. Relationship between the growth phase of *Pseudomonas fragi* and its attachment stainless steel. J. Food Sci. 50:957-960.

Stone, L.S. and E.A. Zottola. 1985b. Effect of cleaning and sanitizing on the attachment of *Pseudomonas fragi* to stainless steel. J. Food Sci. 50:951-956

Sutherland, I.W. 1980. Polysaccharides in the adhesion of marine and fresh water bacteria. In *Microbial Adhesion to Surfaces*, R.C.W. Berkeley, J.M. Lynch, J Melling, P.R. Rutter, and B. Vincent, (Ed), p.329-338. Horwood, Chichester, England.

Sutherland, I.W. 1977. Enzymes acting on bacterial surface carbohydrates. In *Surface Carbohydrates of the Prokaryotic Cell*, I.W. Sutherland (Ed.), p.209-245. Academic Press, London.

vanLoosdrecht, M.C.M., J. Lyklema, W. Norde, G. Schraa, and J.B. Zehnder. 1987. The role of bacterial cell hydrophobicity in adhesion. Applied Environ. Microbiol. 53:1893-1897.

Wegstein, J. and H. Heinsohn. 1993. Modern methods of enzyme expression and design. In *Enzymes in Food Processing*, T. Nagodawithana and G. Reed (Ed.), p.74-102. Acedemic Press Inc., San Diego, California.

Weiss, J. and P.R. Seeliger. 1975. Incidence of *Listeria monocytogenes* in nature. Appl. Microbiol. 30:29-32.

Whitaker, J.R. 1994a. Enzyme inhibitors. In *Principles of Enzymology for the Food Scinces, 2nd. ed*, O.R. Fennema (Ed.), p.241-270. Marcel Dekker Inc., New York.

Whitaker, J.R. 1994b. The proteolytic enzymes. In *Principles of Enzymology for the Food Sciences*, 2nd. ed, O.R. Fennema (Ed.), p.469-498. Marcel Dekker Inc., New York.

Wirtanen, G. and T. Mattila-Sandholm. 1992. Removal of food borne biofilms-comparison of surface and suspension tests. PartI. Lebensm. Wiss. Technol. 25:43-49.

Witter, L.D. 1961. Psychrophilic bacteria. A review. J. Dairy Sci. 44:983-1015.

Wood, T.M., and K.M. Bhat. 1988. Methods for measuring cellulose activity. In *Methods of Enzymology, Vol. 160, Biomass Part A Cellulose and Hemicellulose*, W.A. Wood and S.T. Kellogg (Ed.), p.87-112. Acedemic Press, Inc., London.

APPENDIX A

Table 10: Mean log cells/ml of planktonic bacteria in anaerobe tubes after the fourth and fifth rinse.

Bacteria	Rinse 4 (mean log cells/ml +/- std dev)	Rinse 5 (mean log cells/ml +/- std dev)
Pseudomonas fluorescens	3.53 +/- 0.09	2.66 +/- 0.09
Listeria monocytogenes	1.85 +/- 0.11	0.12 +/- 0.07
P. fluorescens in mixed culture	2.85 +/- 0.06	2.14 +/- 0.07
L. monocytogenes in mixed culture	1.22 +/- 0.16	0.00 +/- 0.00

APPENDIX B

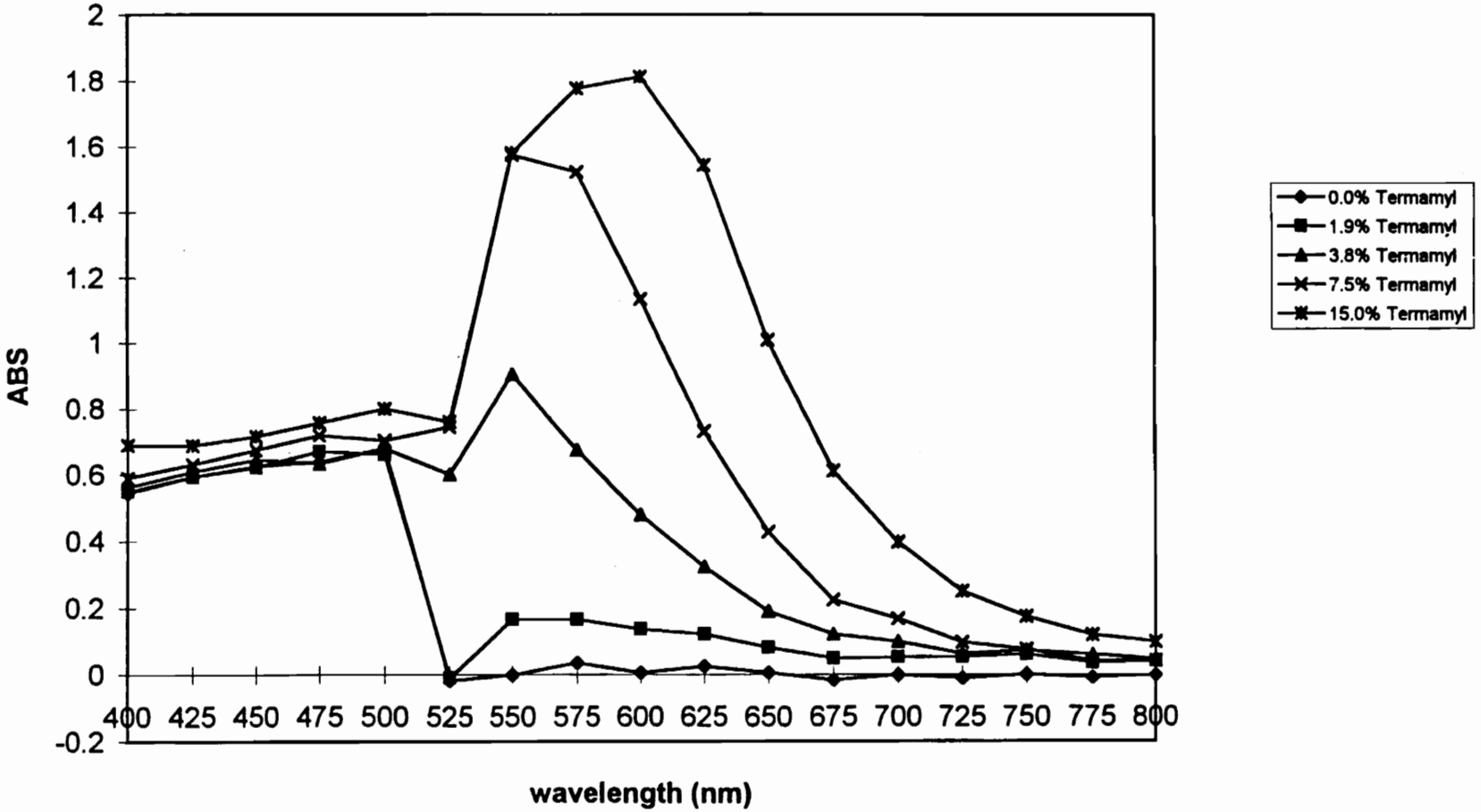

Figure 15: Spectra of dinitrisalicylic acid assay for the detection of reducing sugars using the enzyme Termamyl

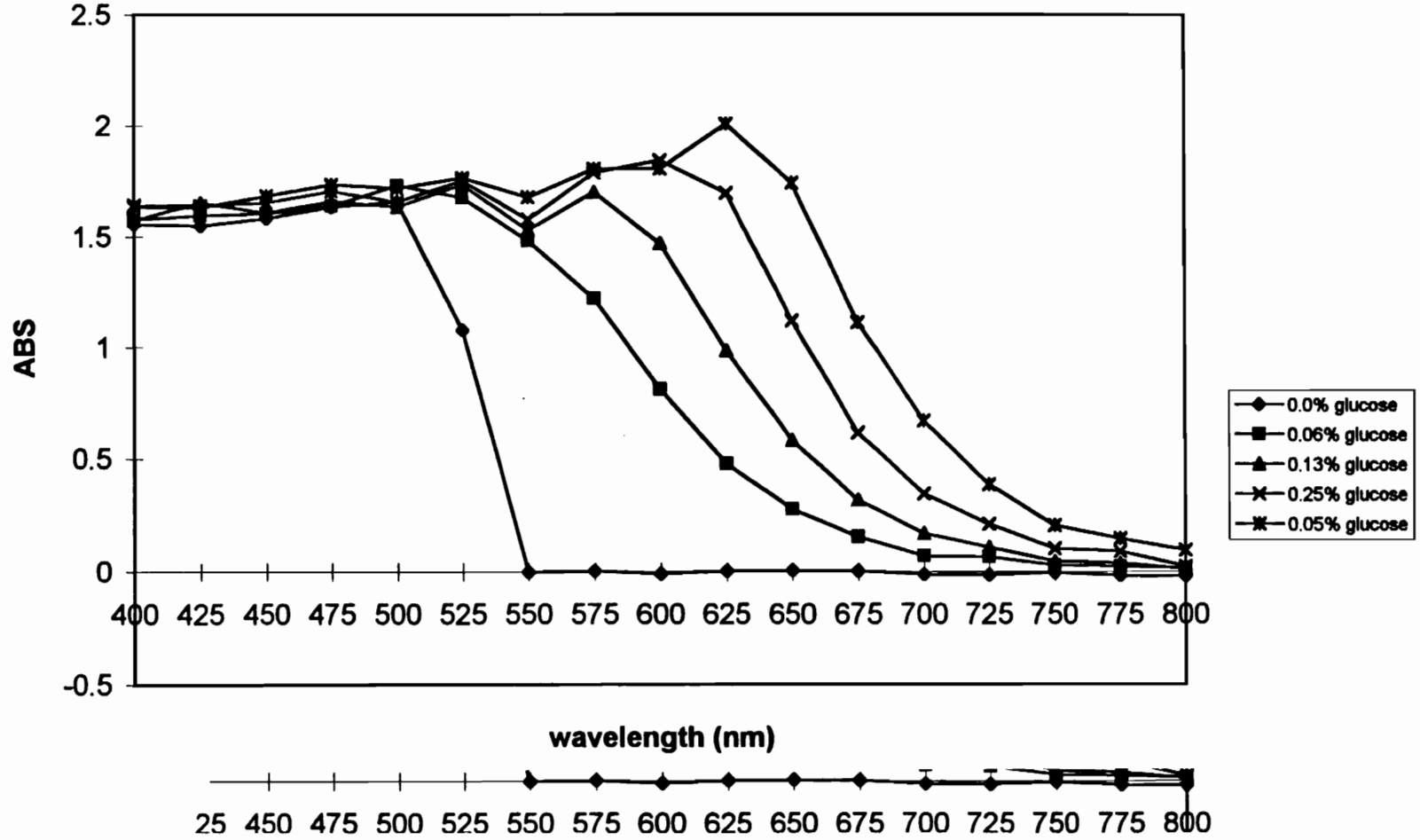

Figure 16: Spectra of dinitrosalycilic acid assay for the detection of reducing sugars using glucose

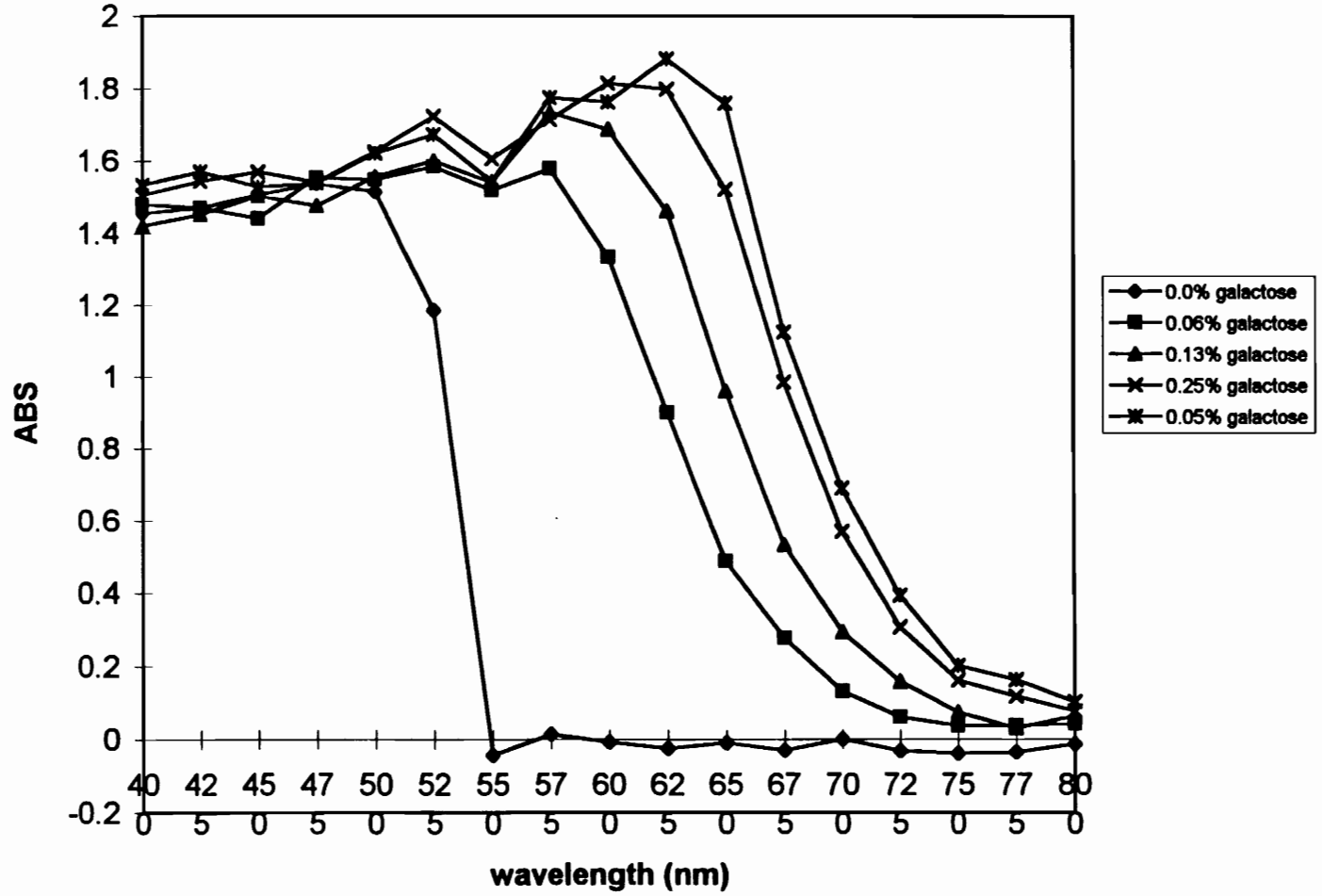

Figure 17: Spectra of dinitrosalicylic acid assay for the detection of reducing sugars using galactose

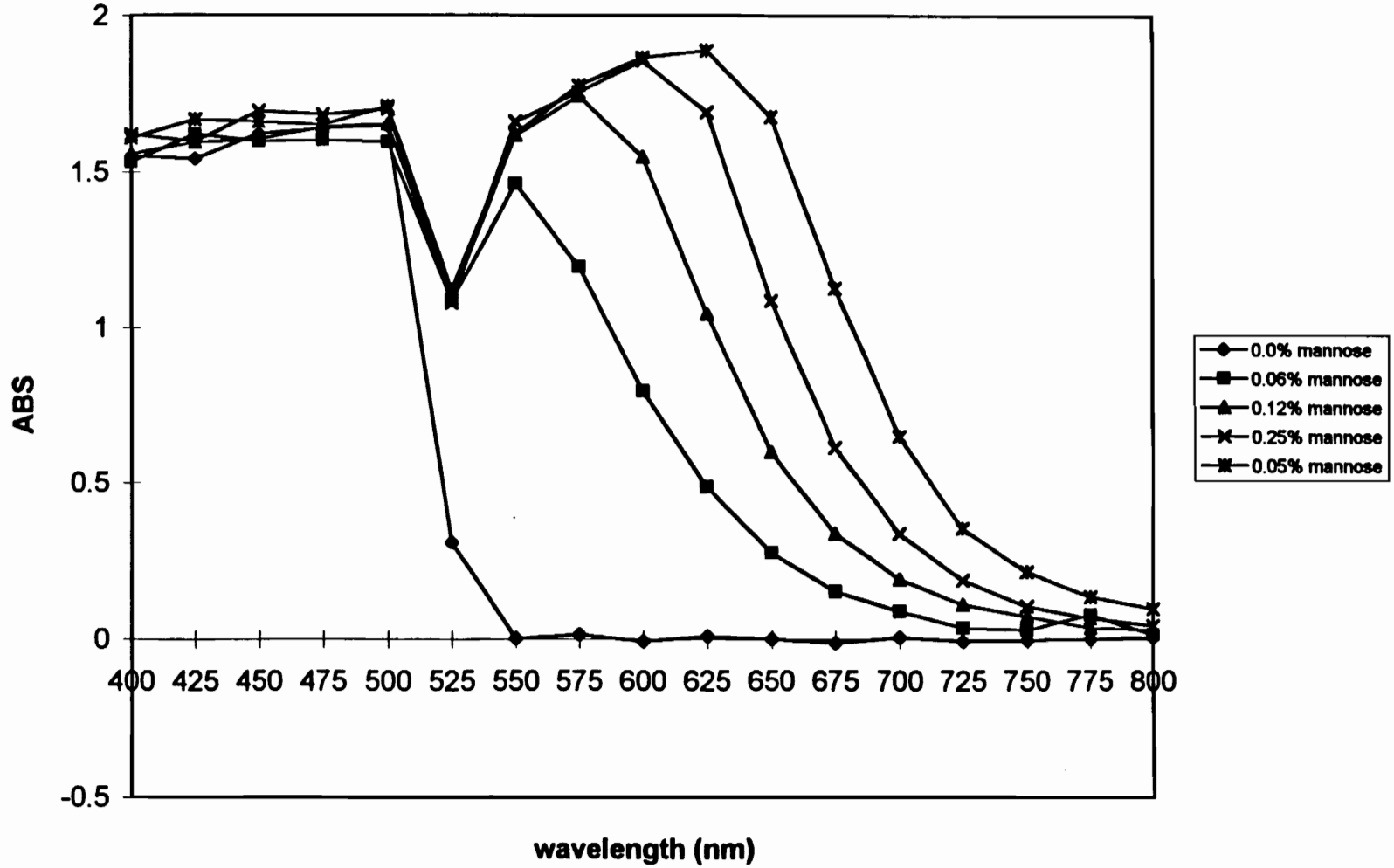

Figure 18: Spectra of dinitrosalicylic acid assay for the detection of reducing sugars using mannose

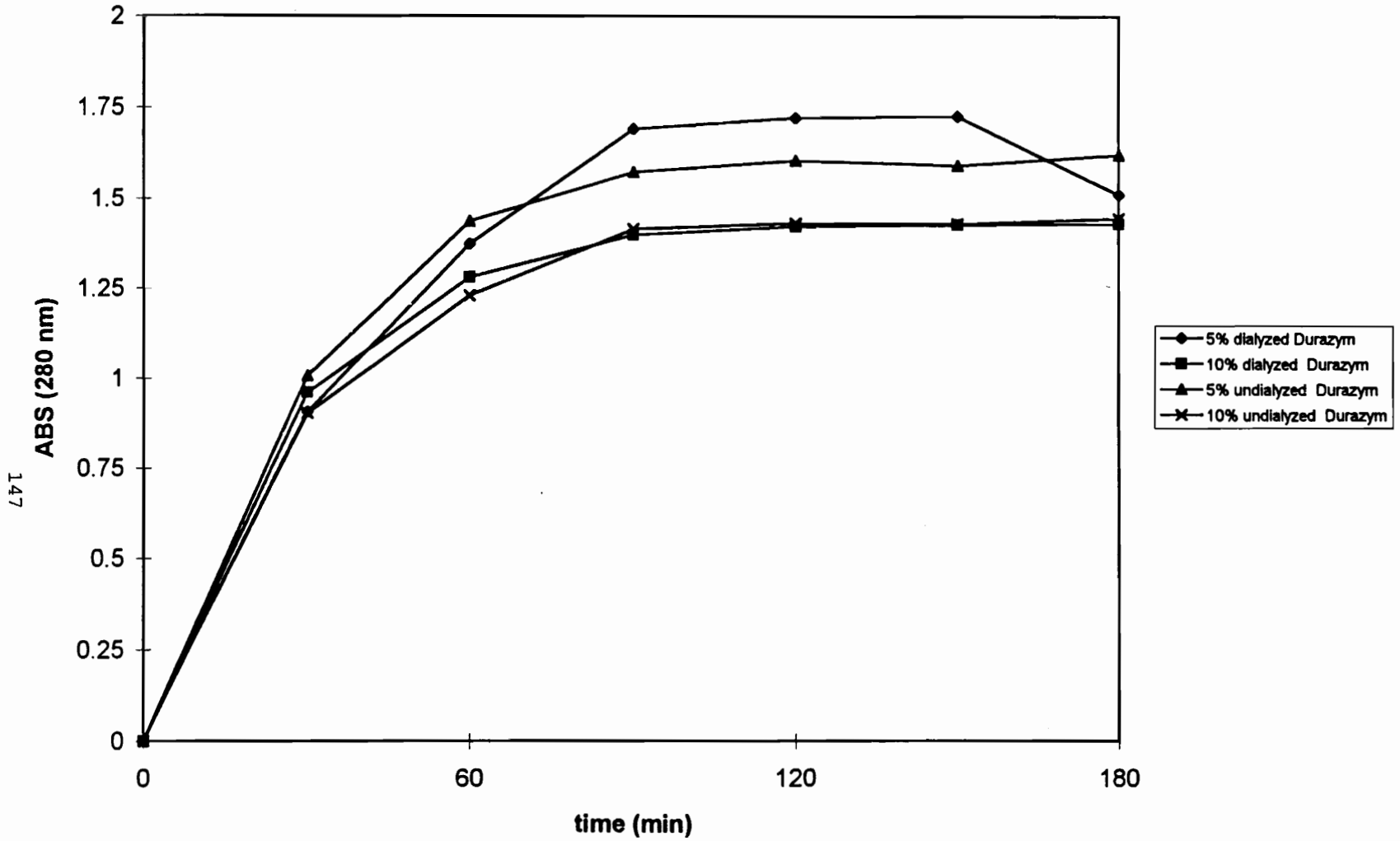

Figure 19: Comparison of the activities of 5% and 10% dialyzed and undialyzed Durazym using a 3.0% casein substrate

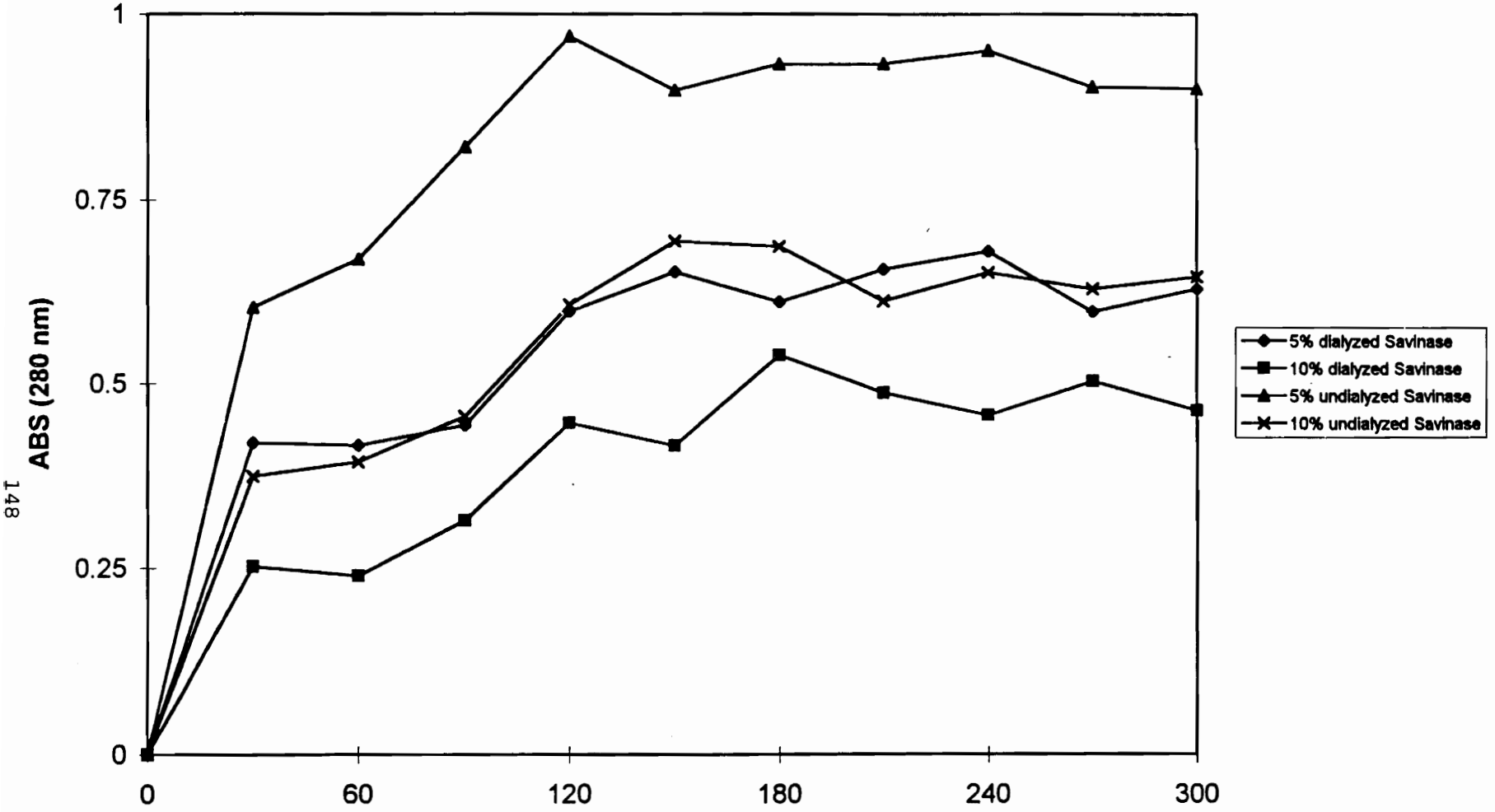

Figure 20: Comparison of activities of 5% and 10% dialyzed and undialyzed Savinase using a 3.0% casein substrate

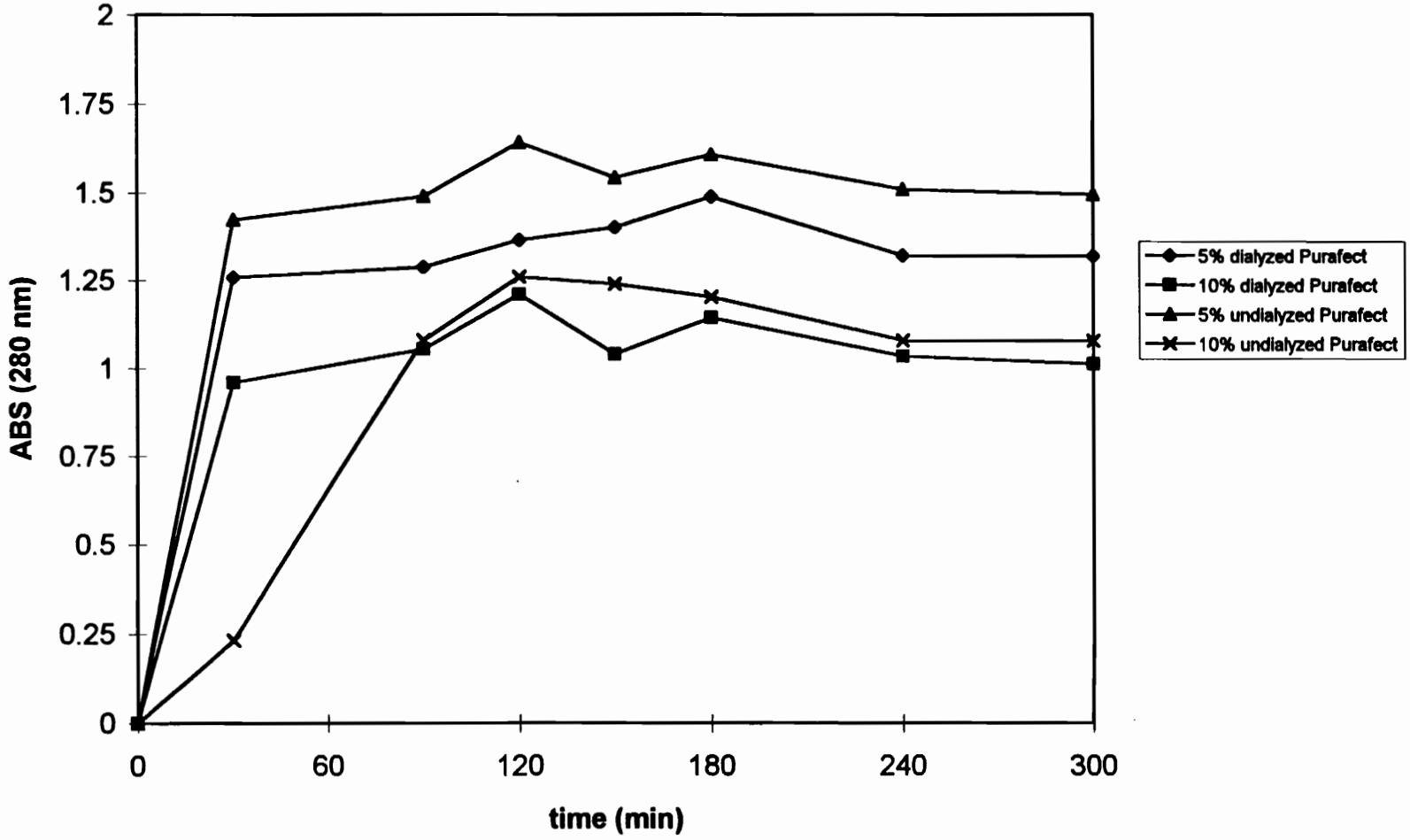

Figure 21: Comparison of activities of 5% and 10% dialyzed and undialyzed Purafect using a 3.0% casein substrate

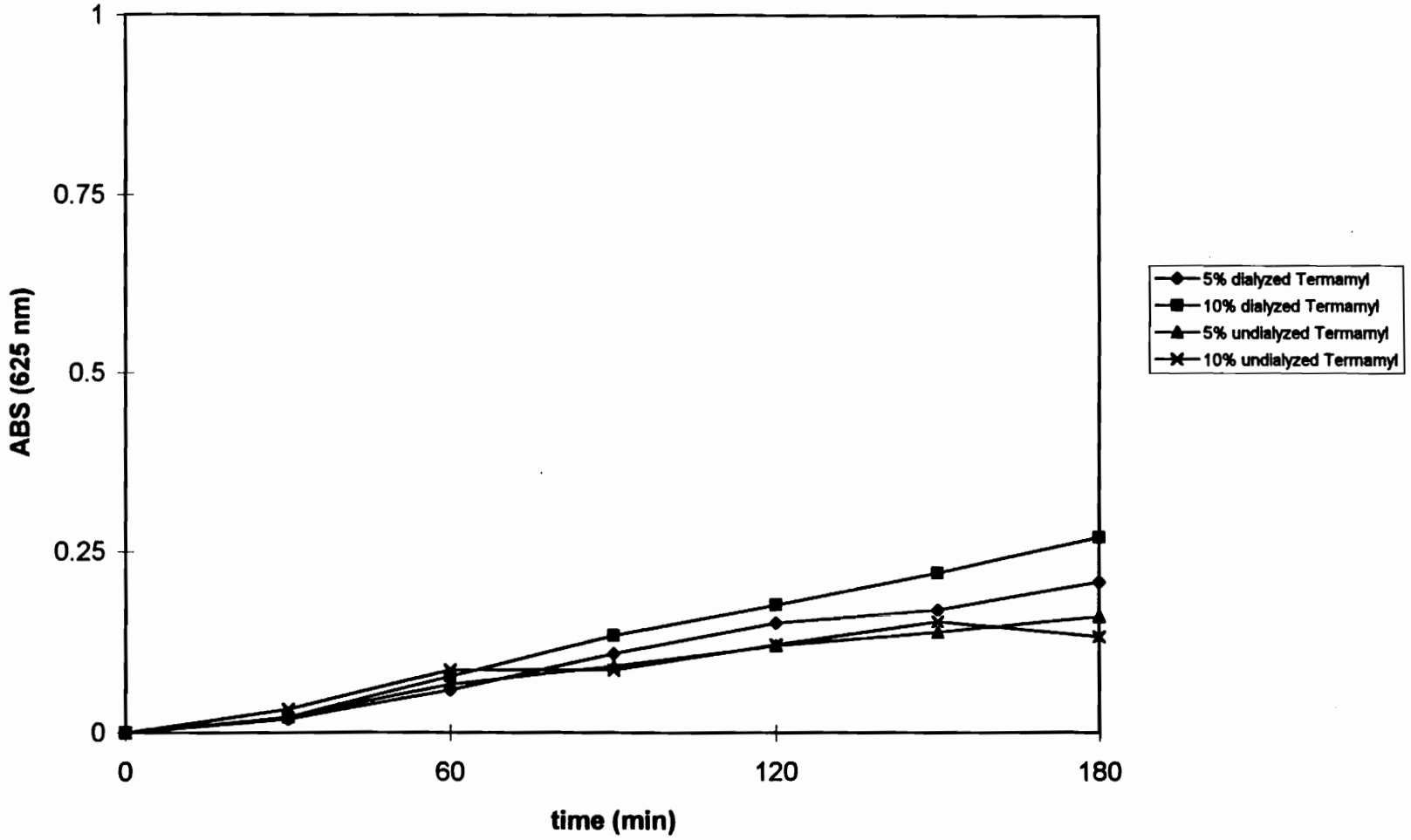

Figure 22: Comparison of the activities of 5% and 10% dialyzed and undialyzed Termamyl using a 0.5% corn starch substrate

VITA

The author, Patricia Maria Clark, was born April 6, 1972, in Somerdale, New Jersey. She is the second daughter and third child to Roselyn and James Clark. She grew up in Kirkwood, New Jersey and graduated from Pope John Paul IV High School in Hadonfield, New Jersey in June 1990.

In September 1990, she began attending Rutgers College, one of five colleges that make up Rutgers the State University of New Jersey in New Brunswick, working towards a degree in Chemistry. In May 1994, she graduated from Cook College, Rutgers University with a Bachelor of Science Degree in Food Science.

Patricia entered the Graduate School of Virginia Polytechnic Institute and State University in August 1994 and is currently a candidate for the degree of Master of Science in Food Science and Technology. She is a member of the Institute of Food Technology and the Co-editor of the Virginia Polytechnic Institute and State University Newsletter for the Food Science and Technology Department.