Optimization of Cultural Factors Influencing the Production of Extracellular Vesicles and Proteinase by *Pseudomonas fragi* ATCC 4973.

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ABSTRACT

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Pseudomonas fragi ATCC 4973 was grown in trypticase soy broth (TSB), on a trypticase soy broth + 1.5% agar (TSA) surface, and in a defined citrate broth. The citrate broth contained glutamine as the sole nitrogen source. *Pseudomonas fragi* grown in TSB started proteinase production at 24 h, during the late logarithmic early stationary growth phase. *Pseudomonas fragi* grown on TSA surfaces initiated proteinase production at 4 h, 20 hours earlier than in liquid medium. Electron micrographs of *P. fragi* grown on TSA revealed extracellular vesicles ca. 20 nm in diameter "blebbing" off the surface of the cells. These vesicles were absent from the surface of *P. fragi* cells grown in TSB, although vesicles could be isolated from the culture supernatant. Isolated extracellular vesicles were ca. 20 nm in diameter and contained a proteinase similar to that found in the supernatant. Electrophoretic analysis showed the vesicles and outer cell membrane of *P. fragi* to share similarities in their composition. Use of the centroid search technique of Aishima and Nakai, showed the optimum

cultural conditions for proteinase production by *P. fragi*, in defined citrate broth to be: incubation temperature, 12.5 C; incubation time, 38 h; initial pH, 6.8; organic nitrogen concentration, 314 mmole nitrogen/L (glutamine); a gas mixture containing 16.4% oxygen flowing over the medium (7.42 ppm dissolved oxygen). Oxygen was the major factor influencing proteinase production by *P. fragi*.

A comparison of optimization techniques suitable for microbiological experiments showed that the centroid search technique of Aishima and Nakai, the modified super simplex of Nakai and Kaneko and the simplex technique of Morgan and Deming all required similar time and experiment numbers to obtain the optimum point.

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INTRODUCTION

Psychrotrophic microorganisms are present in nearly all raw food products. They are common contaminants of processed food items such as pasteurized milk or post-slaughter meat surfaces. They are a major factor in determining the keeping quality of these foods. The growth and metabolism of these microorganisms has been selected by the development of systems leading to extended refrigerated storage of foods.

As a part of their metabolism, psychrotrophic bacteria produce extracellular enzymes, primarily lipases and proteinases, which are major contributors to advanced stages of microbial spoilage of refrigerated foods of animal origin. Many of the proteinases, especially those of the psychrotrophic pseudomonads, are extremely heat stable. The heat stable proteinases are able to withstand pasteurization and other heat treatments, and remain active in thermally processed foods. Consequently, they are of considerable commercial importance, since the production of shelf-stable food items depends, in part, on the destruction of deleterious enzymes.

The objective of this study was to determine how environmental and nutritional factors affected proteinase production by one of these psychrotrophic organisms, *P. fragi* ATCC 4973. In part, the study compared various techniques that could be used to examine the relationships

between the factors and proteinase production. Finally, this study attempted to understand and explain the function of extracellular vesicles produced by *P. fragi*. Specifically this study focussed on the relationship between extracellular vesicles, with bacteriocin or proteinase production.

LITERATURE REVIEW

SPOILAGE OF MEAT SURFACES

Definition

Meat, simply stated, is the flesh of a slaughtered animal which may be used as food. Immediately after slaughter, the meat undergoes degradation which may ultimately spoil the meat. Microbial spoilage is a process in which microorganisms produce proteinases capable of degrading functional and structural proteins. Some proteins may be degraded by cathepsins during the process of aging. It is important to be able to distinguish microbial enzymic breakdown of proteins from the protein decomposition by endogenous enzymes.

To delay spoilage, a dressed animal carcass is chilled below 25 C and stored at temperatures between -1 and +5 C.

Microbial flora

The establishment of a microbial flora on a meat carcass depends upon the environment in which the organisms are forced to grow. As such, the organisms involved are subject to changes in oxygen tension, water activity and pH. These factors are a consequence of the postmortem biochemical changes which take place in the muscle tissues. Besides those factors influenced by biochemical changes in the meat itself, the microbial flora may also be influenced by nutrient availability and microbial antagonism.

Temperature

At chill temperatures (temperatures between -1 and +5 C) the growing bacterial flora consists of cold tolerant organisms only. Gill and Newton (1980) found strains of *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Lactobacillus*, *Brochothrix thermosphacta* and some psychrotrophic *Enterobacteriaceae* to be the most common bacterial types found on spoiling meat.

Oxygen

Under normal meat storage conditions, where animal carcasses are hung in a cooler, spoilage is a surface phenomenon. If the humidity of the cooler air is high, a superficial layer of slime will develop. This slime layer is primarily produced by bacteria of the *Pseudomonas* genus (Ingram and Dainty, 1971). Gill and Newton (1980) found that *Pseudomonas* species grew faster than the other bacteria present on meat surfaces from 2 to 15 C, and that the difference in growth rates increased with a decrease in temperature. These investigators found that *Enterobacter* sp. and *B. thermosphacta* grew more slowly when competing with *Pseudomonas* sp. which had attained maximum cell densities on a meat surface. They suggested that the maximum cell density attainable by *Pseudomonas* sp. was determined by the rate at which oxygen became available to the cells. The strictly aerobic pseudomonads would have a greater requirement for oxygen than either *Enterobacter* sp. or *M. thermosphactum*. The latter two organisms must obtain

energy from the less efficient glycolytic pathway. Available nutrient utilization between the competing organisms was not as great a factor as oxygen availability.

Nutrient availability

Advanced microbial spoilage of meat involves the production of proteinases by the spoiling microorganisms. Meat spoiled at warm temperatures by clostridia clearly involved proteolytic breakdown of the tissues. It appeared, however, that initial spoilage at intermediate and chill temperatures involved the breakdown of low molecular weight water-soluble peptides and not larger structural or functional proteins.

Borton *et al.* (1970a) inoculated minced beef muscle with *P. fragi* and incubated the mixture at 2 C. These researchers found an initial decrease in soluble proteinaceous material. Sodium dodecyl sulfate polyacrylamide electrophoresis studies (Borton et al., 1970b) showed that, after eight days of incubation, no major destruction of salt soluble myofibrillar proteins could be detected. Those changes which did appear at this time were caused by endogenous enzymic activity. After 20 days incubation, however, considerable amounts of large molecular weight proteins disappeared. They concluded that the organism preferentially utilized those compounds most easily assimilated, and that proteolysis occurred only after these compounds were destroyed.

SPOILAGE OF DAIRY PRODUCTS

Processing

Fluid milk in Canada is almost exclusively stored at 4 to 6 C. Typically, milk producers store milk for pick-up every second day. The refrigerated bulk tank truck will collect and mix milk from several dairy farms. Milk is then usually delivered to the processing plant the same day. The delivered milk will be pumped to large bulk tanks, for refrigerated storage, prior to pasteurization. The time period between production and pasteurization may vary up to 120 h (6.5 days) depending upon storage capacity and fluid milk demand (Cousin, 1981).

Commercial raw milk in general is composed of about 90% moisture, 3% fat and 3.2% protein. The protein component is primarily casein.

Spoilage microorganisms

Constant agitation during storage is necessary to prevent milk and cream separation, and to maintain uniform temperature. Consequently, milk is well aerated during storage. As a result of this aeration, microorganisms responsible for milk spoilage tend to be aerobic or facultatively anaerobic. These microorganisms generally produce extracellular enzymes, which are capable of hydrolyzing the lipid and the proteinaceous components of the milk.

When raw milk is held at a constant refrigeration temperature, mesophilic aerobic bacterial spoilage can be

reduced considerably. These conditions, however, selectively favor the growth of aerobic psychrotrophic bacteria (Cousin and Marth, 1977). The most frequently found psychrotrophic bacteria in raw milk are gram negative rods, especially those of the *Pseudomonas* species (Kwan and Skura, 1985; Juffs, 1973). *Pseudomonas fluorescens* has been shown to be the species most often associated with milk spoilage (McKellar and Cholette, 1985; McKellar and Cholette, 1987; Patel *et al.*, 1983a; Jackman *et al.*, 1983; Fairbairn and Law, 1987; McKellar, 1982; Rowe and Gilmour, 1982). Kwan and Skura (1985) studying psychrotrophic spoilage pseudomonads from raw milk, found only half of the organisms belonged to the *fluorescens* species with *P. fragi* making up the bulk of the remaining species. This study found *P. fragi* and *P. fragi*-like organisms to reside in a closely related cluster when subjected to numerical taxonomy. Isolation of *P. fragi* from commercial raw milk samples has also been reported by Law *et al.* (1976), Samagh and Cunningham (1972), and Overcast (1968).

Extracellular proteinases

Growth of these pseudomonads in raw refrigerated milk is accompanied by the production of extracellular proteinases. These enzymes have been shown to produce bitter off flavors in milk due to peptide formation, and poor functionality of milk protein in cheese making, due to partial protein degradation (Speck and Adams, 1976; Law *et al.*, 1976; Law, 1979; Cousin, 1982; Hicks *et al.*, 1986).

These proteinases tend to be heat stable (Griffiths *et al.*, 1981), able to withstand commercial high temperature short time (HTST), and ultra high temperature (UHT) treatments. Subsequent to heat treatment, these enzymes have been responsible for breakdown of protein components in milk subject to long term storage. Typical defects have included bitter off flavors and milk gelation.

Since heat treatment results in milk unsuitable for manufacturing into products requiring formation of a curd, milk must be stored until needed in an unpasteurized form. Elimination of the microorganisms through other means would be difficult. Control of environmental factors, influencing the production of the proteinase by the microorganisms, may help to eliminate the problems associated with long term refrigerated raw milk storage.

PSEUDOMONADS

The term pseudomonad can be used to describe a group of bacteria belonging to the family *Pseudomonadaceae* (Bergey's Manual, 1984). This family is composed of straight or curved gram negative rods, motile by polar flagella, capable of growing at temperatures between 4 and 43 C. The organisms are strict aerobes (*i.e.* possess a respiratory metabolism) and are never fermentative. Organisms belonging to the genus *Pseudomonas* predominate in the family with over 265 species described. The *Pseudomonas* genus is ubiquitous. It has been associated with the decomposition of a wide

variety of substrates, reflecting the versatility of the genus.

Pseudomonas fluorescens belongs to a group of organisms including *P. aeruginosa, P. putida, P. chlororaphis,* and *P. aureofaciens* which produce a fluorescent pigment (Bergey's Manual, 1984). *Pseudomonas fragi* on the other hand is a non-fluorescent *Pseudomonas* species described as a species in Bergey's Manual (1984).

Using competitive DNA-rRNA hybridization studies Palleroni, *et al.* (1973), and De Vos and De Ley (1983) divided the genus *Pseudomonas* into five distinct RNA homology groups. Members of the first RNA group included *P. aeruginosa, P. fluorescens, P. putida, P. cichorii, P. syringae, P. savastanoi, P. phaseolicola, P. mori, P. glycinea, P. tomato, P. viridiflava, P. stutzeri, P. stanieri, P. mendocina* and *P. pseudoalcaligenes.* Recent DNA-DNA hybridization experiments conducted by Ursing (1986), Kiprianova, *et al.* (1987) and Kiprianova, *et al.* (1988) revealed that *P. fragi* (ATCC 4973) belonged to this first rRNA group which included *P. fluorescens.*

EXTRACELLULAR PROTEINASE

Extracellular proteinases produced by the genus *Pseudomonas* all appear to be closely related. The enzymes generally fall into the class of proteinases referred to as neutral metalloproteinases. As such, they are most active at neutral pH values and are inactivated by metal chelating

agents, since metalloproteinases contain a divalent metal such as Zn at their active site (Mihalyi, 1978). The enzymes are specific for peptide bonds whose imino group contains a hydrophobic or bulky residue. Moreover, the proteinases are active against large peptides only (≥4 residues) and thus do not display esterase activity (Morihara, 1968).

The proteinases have molecular weights between 35,000-36,000 daltons. They are most active at temperatures between 30 and 45 C, although many show extensive activity at 4 C. They display considerable thermal stability.

Jackman *et al.* (1983) examined proteinases produced by several *P. fluorescens* strains. All strains produced only one proteinase. These proteinases all had a molecular weight of 42,000 ± 1,500. The proteinases were inactivated by chelating agents. Rabbit antiserum was prepared from each of the proteinases isolated from the different strains. Inhibition of proteinases by the antisera, and gel precipitin bands of antisera and proteinases, showed that all the proteinases shared antigenic determinants. This suggested that the proteinases were similar in structure and amino acid content.

Patel *et al.* (1983a; 1983b) purified extracellular proteinases from *Pseudomonas* species isolated from raw milk. All isolates produced only one major proteinase. The proteinases ranged in molecular weight from 39,000 to 44,000. They were neutral metalloproteinases, being most

active at pH 7.2 to 7.4, and inactivated by EDTA. The enzymes were heat resistant, being able to retain partial activity after heat treatment of 120 C for 10 min.

Malik and Mathur (1984) purified a proteinase from *Pseudomonas* sp. B-25, an organism isolated from refrigerated butter samples. The enzyme, with a molecular weight of 41,200, was a neutral metalloproteinase since it was most active at pH 6-10, and was inactivated by EDTA. It was most active at a temperature of 65 C. The enzyme retained 26% of its activity after 10 minutes at 70 C.

Stepaniak and Fox (1985) and Yan *et al.* (1985) both purified proteinases from *Pseudomonas* species isolated from raw milk. The enzymes ranged in molecular weight from 44,000 to 46,000, were active at pH 7.0 to 7.5 and were inactivated by EDTA. The proteinase from *Pseudomonas* AFT 21 (Stepaniak and Fox, 1985) was most active at temperatures between 45 and 47 C, but retained 20% of its activity at 4 C. The proteinase examined by Yan *et al.* (1985) was heat resistant, retaining 50% of its activity after 30 min at 63 C.

Porzio and Pearson (1975) purified and characterized a proteinase produced by *P. fragi* ATCC 4973. The proteinase had a molecular weight of 40,000 to 50,000 and was most active at pH levels of 7.0 to 7.5. The enzyme was a metalloproteinase, since it was inactivated by EDTA. The enzyme had an isoelectric point of pH 5.2. Thompson *et al.* (1985a) also examined the proteinase produced by *P. fragi*.

They found the organism to produce a single proteinase with a molecular weight of 48,000 ± 1,200. Electron micrographs of *P. fragi* stained to localize the proteinase revealed high concentrations of the enzyme near the cell wall. No enzyme could be detected in the cytoplasm. They hypothesized that the enzyme would also appear in extracellular vesicles, which the organism may produce on occasion.

EXTRACELLULAR VESICLES

Introduction

Pseudomonads are the predominant psychrotrophic microflora of refrigerated raw milk (Stradhouder, 1975) and meat (Shaw and Latty, 1982). *Pseudomonas fragi* has been isolated from raw milk (Kwan and Skura, 1985) and spoiled meat (Shaw and Latty, 1982). *Pseudomonas fragi* is commonly involved in the psychrotrophic spoilage of meat and dairy products.

During the growth of *P. fragi* ATCC 4973, electron micrographs (Lee Wing *et al.*, 1983; Lee Wing 1984; Thompson *et al.*, 1985a) revealed the presence of extracellular vesicle-like structures. These vesicles appeared to emanate from the surface of the bacterium by a process of exocytosis and accumulated in the supernatant.

Bacteriocins

Some pseudomonads, spontaneously or under the influence of inducing agents, produce antimicrobial factors known as bacteriocins (Bradley, 1967). These bacteriocidal agents, which may emanate from the cell surface, are primarily

active against bacteria of the same genus and species, although antagonism towards different genera can occur.

Austin-Prather and Booth (1984) reported an active bacteriocin found in association with extracellular membrane vesicles released from whole *Bacteroides uniformis* cells. These membrane vesicles were similar in size and shape to those produced by *P. fragi* ATCC 4973.

Greer (1982) studied bacteriophage sensitivity of several meatborne pseudomonads and found that phage extended the lag phase of some bacteria under psychrotrophic conditions, resulting in the selection of phage resistant mutants. Patel and Jackman (1986) showed that pseudomonads isolated from milk were susceptible to bacteriophages active against meat derived pseudomonads. It is possible that *P. fragi* may produce membrane vesicle bound bacteriocins which could have effects similar to that of bacteriophage in limiting competition between bacteria of differing strains, species, or genera.

Bacteriocins are a group of entities produced by some "host" bacteria, under some conditions, which may be lethal to other "susceptible" bacteria of a different strain, species, genus or order. They are not clinically important in destroying pathogenic bacteria. Bacteriocins, however, may affect the pathogenicity of some bacteria, and have been studied in this context.

Of particular interest to this study, are the ecological ramifications of bacteriocins as they affect

competition between differing strains, species, or genera of bacteria contained in a dynamic mixed culture such as in spoiling foods, particularly fluid milk and muscle tissues. Both commodities, when stored under refrigeration conditions, soon develop a microflora composed of a small number of related organisms, mainly of the pseudomonad group. These organisms establish themselves at the expense of other organisms. The mechanisms of this competition is poorly understood and may involve antagonistic chemical compounds such as bacteriocins.

Historical perspectives

The study of bacteriocin has been for the most part centred upon colicin, the antibiotic agent produced by *Escherichia coli*. Several reasons for this restriction apply, such as the concomitant detailed study of this organism; but the main reason stems from the initial work of Gratia (1925). A strain of *E. coli* V (named V for virulent) produced a substance bacteriocidal towards *E. coli* ϕ. A filter sterilized culture supernatant of the virulent *E. coli* strain proved only lethal towards susceptible strains of *E. coli*. It differed from bacteriophage in that the principal agent did not reproduce itself in a fashion similar to bacteriophage. The antagonistic agent was stable after 1 h at 100 C and ½ h at 120 C and was active after exposure to chloroform vapors.

Jacob *et al.* (1953), in an attempt to define the term bacteriocin, suggested that these substances should be

protein in nature, produced by lethal biosynthesis, adsorbed onto specific receptor sites and active against organisms within the same species. Considerable complications have since confused this definition since many such substances; "classical" antibiotics, metabolic substances, enzymes and defective bacteriophage, have at one point or another been called bacteriocins. Hamon and Peron (1963) found many bacteriocins of gram positive bacteria did not fit the classical definition. In particular, they found a much wider spectrum of activity, not restricted to one species. The precise meaning of the term bacteriocin then, seems elusive. In general terms, the presence of a biologically active protein-like substance possessing a bacteriocidal property can be considered bacteriocin-like.

Detection of bacteriocins

For screening, the general test for antagonism involves growth on solid medium. Antagonism is displayed by a reduction or elimination in the growth of an indicator (passive) strain by the test (active) host strain.

There are two general methods used to detect antagonism: the direct and the deferred procedure.

The host and indicator are placed on the agar plate together. For example, the host may be stabbed into an agar plate seeded with the indicator organism. Both grow at the same time, and the inhibitor diffuses through the agar, inhibiting the indicator organism (Gratia, 1946).

In the deferred procedure, developed by Fredericq (1948), the host organism is grown on an agar plate for some period of time, following which the cells are killed with chloroform vapors. A second layer of sloppy agar medium (growth medium containing ca. 1.0% agar) seeded with the indicator organism is poured over the first agar layer. Zones of inhibition, observed around the dead host colonies, indicate bacteriocin activity.

The deferred procedure has been shown to be more sensitive at detecting antagonistic behavior than the direct method (Tagg *et al.*, 1976). With the deferred procedure, incubation time and conditions of the host or indicator organism can be independently varied, thus accommodating differences in growth rate.

Unrelated antagonists

When investigating the occurrence of a bacteriocin, it is important to eliminate other substances unrelated to but similar in action to bacteriocin. In many cases, the discovery of bacteriocins from specific species was the by-product of investigations into the lysogeny of the organism due to bacteriophage. Bacteriophage are viral particles which may infect specific bacterial cells, causing lysis. Unlike bacteriophage, bacteriocins cannot be propagated from one culture to another, due to the lack of necessary genetic material. Since bacteriocins cannot reproduce through bacterial lysis, bacteriocins may be diluted to the point where no zone of clearing occurs.

Bacteria can produce a variety of low-molecular weight inhibitors and bacteriolytic enzymes which may possess bacteriocin-like properties. An example of the former is the classical antibiotic nisin produced extracellularly by strains of *Streptococcus lactis* (Tagg *et al.*, 1976). Lysostaphin from *Staphylococcus* (Schendler and Schuhardt, 1965), possessing antibiotic-like activity, is an example of the latter. Classical methods of bacteriocin detection, whether direct or indirect, can only detect antagonistic substances produced by bacteria. Other methods must be used to determine the class of substances produced.

Factors affecting production of bacteriocins

In general terms, if one looks long enough at any given species, one may find a bacteriocin-like substance produced by the host organism antagonistic towards some indicator organism (Tagg *et al.*, 1976). The difficulty lies in finding the indicator. No special growth media or cultural conditions may be needed to produce bacteriocins, other than optimal growth conditions for the host (Bradley, 1967).

Maximum bacteriocin production, however, does depend upon some important factors. Kelstrup and Gibbons (1969) cultivated *Streptococcus* sp. of human and rodent oral cavity origin in trypticase medium (2%) plus 0.2% glucose. Supplementation of this growth medium with either agar (0.005% to 0.1%) or starch (0.05% to 1.0%) increased bacteriocin production. Similarly, Jetten *et al.* (1972) found that *S. epidermidis* produced ca. 20 times more

bacteriocin activity on a semisolid medium, than in liquid culture of the same volume. Vidaver *et al.* (1972) found that *P. phaseolicola* and *P. syringae* produced bacteriocin only on solid medium.

Growth phase does seem to affect bacteriocin production. *Streptococcus* sp. produced maximum streptococcin during the exponential phase, then declined sharply as the culture entered stationary phase. In contrast, *Streptococcus* A-FF22 started bacteriocin production in the late logarithmic phase and decreased slowly as the culture aged (Tagg *et al.* 1976). Vidaver *et al.* (1972) found that increasing incubation time well past the exponential phase (up to 72 h) resulted in an increase in bacteriocin production from *Pseudomonas sp.*

Bacteriocin induction

Bacteria may or may not produce bacteriocin spontaneously, depending upon the species. If the bacteriocin must be induced, its production usually is associated with cell lysis (Bradley, 1967). Both physical inducing agents such as ultraviolet (UV) radiation and chemical inducing agents such as mitomycin C tend to be metabolic disrupters.

Hamon *et al.* (1961) induced several bacteriocins from *Pseudomonas* sp. using ultraviolet radiation at 254 nm. Similarly, Crowley and DeBoer (1980) induced bacteriocin in *Erwinia* sp. with radiation of the same wavelength.

Mitomycin C was utilized as a chemical inducing agent by Takeya *et al.* (1969) and Haag and Vidaver (1974) to produce bacteriocin from *Pseudomonas* sp. The mitomycin C concentration varied from 0.2 to 2.0 µg/mL in these studies.

Physical properties of bacteriocins

Bacteriocin-like substances produced by bacteria have been classified by Bradley (1967) into two groups, designated as low (ca. 10,000 to 100,000 d) or high (1.0 x 10^6 to >1.0 x 10^7 d) molecular weight. The high molecular weight substances include particles resembling bacteriophage tails, or otherwise defective bacteriophage. These particles frequently are inducible upon exposure to U.V. radiation or mitomycin C, and are often associated with cell lysis.

Kelstrup and Gibbons (1969) described a low molecular weight bacteriocin produced by *Streptococcus* sp. It was dializable, suggesting a molecular weight of ca. 12,000. Jetten *et al.* (1972) described the production of a bacteriocin produced by *Staphylococcus epidermidis* with a molecular weight of 150,000 to 400,000, composed of 20,000 d subunits. In both of these cases, neither U.V. radiation nor mitomycin C increased bacteriocin production.

The literature contains many examples of bacteriocin-like particles produced by bacteria. These particles may or may not be closely associated with the cell outer layers. Austin-Prather and Booth (1984) found a bacteriocin of *B. uniformis* to be associated with membrane vesicles about 100

nm in diameter, which were released by bleb formation from the outer surface of the cells.

Hamon *et al.* (1961) induced bacteriocin-like particles with U.V. radiation from several *Pseudomonas* sp. including "fluocin" from a *P. fluorescens* strain. Takeya *et al.* (1969) described a rod shaped bacteriocin-like particle induced with mitomycin C from *P. aeruginosa* P28. This bacteriocin, labelled "pyocin" 28, varied in size from 9.0 to ca 400 nm in size when viewed under the electron microscope. Similar studies of such particles (Crowley and DeBoer, 1980; Haag and Vidaver, 1974) also indicated the need for either U.V. or mitomycin C induction.

Chemical composition

Various bacteriocins differ significantly in their chemical compositions, reflecting their diverse physical properties. Generally speaking, the low molecular weight bacteriocins are composed primarily of protein, consequently they are susceptible to attack by trypsin or other similar proteinases. The high molecular weight bacteriocins, reflecting a more diverse composition, are relatively immune to trypsin attack but are easily inactivated by high temperatures. Both bacteriocin types may or may not contain carbohydrate or lipid, but all must contain protein.

Kelstrup and Gibbons (1969) described an example of a low molecular weight bacteriocin produced from *Streptococcus* sp. with a molecular weight less than 12,000. Four proteinases, including trypsin, totally inactivated the

bacteriocin. The substance was, however, stable after heating at 80 C for 45 min. Gagliano and Hinsdill (1970) and Dandeu (1971) found staphylococcin and colicin respectively to be chemically similar to the cell wall of the originating bacterium. In particular, the latter study found the active protein component to be integral with the lipopolysaccharide on the surface of the producer cell.

In contrast to low molecular weight bacteriocin, Takeya *et al.* (1969) found high molecular weight rod shaped pyocin from *P. aeruginosa* to be resistant to treatment by trypsin and nagarase (a bacterial proteinase). It was, however, inactivated by exposure for 10 minutes at 60 C. Haag and Vidaver (1974) found similar results from a study of syringacin from *P. syringae*.

Mode of action of bacteriocins

Most of the original information regarding the mode of action of bacteriocins upon bacteria is based on studies of colicins. Research has tended to focus on two aspects of the interaction between bacteriocin and the susceptible bacteria. These are the kinetics of the physical interaction between the two, and the development of some fatal disruption in the biochemical process in an affected cell. The widely accepted hypothesis regarding the mode of action of bacteriocins is that it is a two step process. In the first step adsorption of the bacteriocin to the surface of the cell is facilitated by receptors on the surface of the cell. This first stage is thought to be a reversible

step. No damage is done to the cell since removal or destruction of the bacteriocin at this stage, with trypsin digestion, saved the cell from destruction and left no permanent damage. At a definite time period, following the first step, permanent damage begins to become evident through irreversible biochemical changes.

The adsorption of bacteriocin can be demonstrated by a drop in bacteriocin titre in a suspension with excess susceptible bacteria. Elution of bacteriocin from treated cells after elimination of free bacteriocin proved that the bacteriocin was, in fact, removed from solution or suspension and not enzymatically inactivated by the bacterial cells (Tagg *et al.*, 1976).

It is thought that the receptor sites on the bacterial cell surface specific for bacteriocins are the same sites to which bacteriophage also attach. This relationship between the two binding sites was strengthened when it was found that resistant mutants (to colicin) were resistant to both bacteriocin and bacteriophage attack. Sabet and Schnaitman (1973) isolated colicin E3-CA38 receptor from the cell surface of *E. coli* by treating the cell wall with Triton X-100 in the presence of ethylenediaminetetracetic acid (EDTA). It was found to be a glycoprotein with a molecular weight of 60,000. It is thought to be situated in the outer membrane. The purified receptor inactivated colicin. Significant binding of colicin to the isolated receptors was shown to prevent bacteriophage binding. The binding of

colicin and bacteriophages to common receptors suggests that both have similar ancestry.

Bacteriocin induced cell damage

The physiological state of the indicator cell seems to be important in bacteriocin activity. Actively growing cells are most susceptible to cell damage. Kinetic studies have shown that the lethal action is of a single hit. That is, one molecule will, with a certain probability, kill a bacterial cell. However, due to the presence of more than one active binding site, more than one bacteriocin molecule is likely to bind and kill one bacterial cell.

Specific targets of biochemical attack centre around energy production, macromolecule production and membrane transport and permeability. Colicins A, B, E1, D and S are similar in their action since energy dependent syntheses and transport processes are inhibited. Several energy dependent transport processes, such as the transport of amino acids, are inhibited, leading to loss of protein synthesis. The membranes are altered allowing release of K^+ and the freer movement of Mg^{2+} and Ca^{2+} through the membrane. Since the cell attempts to maintain a proper K^+ concentration across the membrane through active transport, ATP levels drop.

OPTIMIZATION

Introduction

Optimization techniques can be used to improve the efficiency of most processes when more than one factor is involved. In the case of chemical processes, optimization

may be required to improve the efficiency of an industrial or analytical method. Major improvements in the productivity of microbiological fermentation may also be achieved by controlling incubation conditions or culture medium compositions.

The objectives of any optimization strategy are two-fold: (1) to improve the overall efficiency of the process under study (frequently the process under study is referred to as the objective function); (2) to attain this gain in efficiency in as few experiments (or in the least time) as possible. Few "wasted" experiments (that is experiments which do not contribute to improvement of the objective function) should have to be evaluated (Hendrix, 1980).

The measure of the objective function can vary widely, depending upon the nature of the function. It may be a measure of some chemical, the result of a chemical process. In microbiological systems it may be a measure of enzymatic activity, or of some chemical metabolite. The factors involved in influencing the objective function can also vary greatly. In microbiological systems, the factors tend to be cultural conditions such as pH, incubation temperature, or oxygen level or culture medium composition differences (Greasham and Inamine, 1986). As such, certain constraints may have to be placed upon the factors. For example, pH values have to be constrained for microorganisms growing in a narrow pH range. As a result, the optimization technique used must be able to work satisfactorily within the boundaries set up by these constraints. Several

optimization techniques have been used which can, to varying extents, satisfy the above criterion. Two such techniques include linear programming and direct search methods.

Linear programming

Linear programming techniques can and have been used to optimize process control or product formulation (Harper and Wanninger, 1970). This technique entails solving a series of simultaneous equations, usually through zeroing of row functions. Linear programming techniques can handle boundaries well, since the technique depends upon constrained factors. Unfortunately, linear programming requires that each factor be represented by a linear equation. This situation is rarely, if ever, encountered in a chemical or microbiological system. Linear programming may therefore be of limited use.

Direct search

Linear programming techniques used to optimize processes rely upon solving an overall variation in a defined series of linear equations. When the behavior of each factor as applied to the objective function is unknown (i.e. when the linear functions are undefined), then a more direct approach must be taken. Direct search techniques (Saguy, 1983) rely upon the evaluation of the objective function at a sequence of trial points, points made up by a variation of factor levels within the constraint boundaries. Progress towards the optimum point is assured if the search is forced to move to the region of optimum response. Simply

stated, if the set of factor values results in an improvement of the objective function, then the technique will move in that "favored" direction. The term favored direction, as used here, refers to an improvement in the desirability of the objective. As such, optimization techniques may be used to maximize or minimize the objective function.

This technique of direct search and evolutionary operations (EVOP) was first formulated by Box (1957). Central to the concept was evaluation of trials, with an improvement of the objective function in mind.

Evolutionary operation techniques

Response surface methodology

The initial design of Box (1957) was centred around an established full-scale process. That is, the experimental levels of each factor were equally spaced and the experiments evaluated. When complete, the data were used to construct, by multilinear regression analysis, a response surface. Full scale factorial designs are impractical for large numbers of factors (Greasham and Inamine, 1986). As a result, Box and Behnken (1960) formulated a system of three level designs for reducing the total numbers of trials required. This combination of fractional factorial with multilinear regression analysis is commonly referred to as response surface methodology (McDaniel et al., 1976). Nakai (1982) found, however, that response surface methodology became inaccurate when constraint boundaries did not cover

the factor levels of the true function, or if initial boundaries were set too narrow.

Simplex methods

Shortly after Box (1957) formulated the concept of EVOP, Spendley *et al.* (1962) suggested a system of EVOP which was truly evolutionary. Rather than formulate a set number of trials, these researchers proposed an iterative procedure. The trials were formulated from a simplex, a geometric figure containing one more than the number of factors involved in the optimization. The simplex was derived from a Spendley matrix. By forcing the search to move to the region of optimum response, the simplex figure would work towards the optimum objective function value. This was achieved by a series of reflections, where the worst function point was abandoned and that point reflected towards a favored direction.

Morgan-Deming simplex

Flexible as the simplex technique appears, several problems remain. The simplex figure moved in a favorable direction, at a constant rate, regardless of the steepness of the ascent. Morgan and Deming (1974) proposed that expansion and contraction factors be used to accelerate movement in a more favorable direction, while moving away from a less desirable direction. This procedure tended to speed up the optimization rate. To further improve the efficiency of the simplex technique, Routh *et al.* (1977) introduced a quadratic curve fitting routine into the

simplex design. The quadratic curve fitted point replaced the worst point of the previous trial. This procedure tended to further improve the simplex technique. However, both the modified simplex of Morgan and Deming (1974) and the super modified simplex technique of Routh *et al.* (1977) occasionally stalled near to or at the constraint boundary.

Modified super simplex

In an attempt to improve the behavior of the simplex technique around constraint boundaries, Nakai (1982) proposed the modified super simplex. This technique was a basic super modified simplex (Routh *et al.*, 1977) with the addition of a "quadratic factorial regression analysis" step. The new procedure was shown to be more efficient, than the two above mentioned techniques, when the quadratic factorial regression equation fitted the experimental response surface. When the equation did not fit the response surface, however, no increased efficiency occurred.

To obtain a further improvement in efficiency, under all situations, Nakai *et al.* (1984) introduced a mapping procedure to the super modified simplex (Routh *et al.*, 1977). This technique shows, in a two dimensional plot, the relationships one separate factor has with all the other factors involved in the objective function. These researchers found a significant improvement in efficiency over the modified super simplex.

In all optimization techniques, a danger exists that the identified optimum point is in fact a local optimum and

that the global maximum has been missed. A solution to this problem, developed by Nakai *et al.* (1984), was to shift simultaneously all the factor levels away from the identified optimum point, towards new target values. Shifting is continued until a worse response value is obtained.

Centroid search

Simplex techniques are efficient at finding optimum points because they are evolutionary operations. As such, new trials are suggested based upon the results of the previous trial. By a succession of such trials, at smaller and smaller ranges, the optimum point is eventually found. This technique is efficient when individual experiments can be conducted quickly. In the case of lengthy experiments, such as in microbial fermentation where incubation times can take several days, the iterative nature of the simplex technique can be very time consuming. In an attempt to speed up the optimization, Aishima and Nakai (1986) proposed centroid search, an innovation of the classic simplex procedure. With the centroid search technique, a series of trials can be evaluated simultaneously. In this way, the time required for optimization may be substantially reduced. The centroid search technique includes evaluation of Spendley matrix, centroid and simultaneous search experiments.

MATERIALS & METHODS

BACTERIOCIN

Organisms

The host

Pseudomonas fragi ATCC 4973 was purchased from the American Type Culture Collection (Rockville, MD). All subsequent studies used this bacterium.

The indicators

Streptococcus faecalis ATCC 27286 and 12984; *P. fluorescens* ATCC 948; *P. aureofaciens* ATCC 13985; *P. fluorescens* biotype "A" ATCC 17397; *P. acidovorans* ATCC 15668; *P. lemmonieri* ATCC 12983; *P. pseudoalcaligenes* ATCC 17440; *P. putida* ATCC 12633; *P. stutzeri* ATCC 17588; *P. mendocina* ATCC 25411; and *E. coli* ATCC 25922 were purchased from the American Type Culture Collection. The following organisms, isolated by Kwan and Skura (1985) from raw milk and classified by numerical taxonomy, were also used as indicators: six different strains of *P. fragi* 4-1, 6-3, 8-4, 10-0, 10-7, A14; and six different strains of *P. fluorescens* 0-0, 6-0, 10-0, 10-6, B16, C10.

Storage

All organisms were suspended in trypticase soy broth (TSB) (BBL, Baltimore, MD) and stored frozen in liquid N_2. Cultures were thawed and incubated in TSB at 21 C for 18 h, and streaked onto trypticase soy agar (TSA, BBL) slants.

These stock cultures were stored at 4 C and replaced at 4 week intervals.

Growth of organism

The media

All cultures were grown in TSB. Bacteriocin activity was determined on TSA plates.

Washed cell suspension

A 24 h broth culture (TSB, 28 C) was centrifuged (10,400 x g, 10 min, 4 C) and the supernatant discarded. An equal volume of 0.1% peptone water was used to resuspend the pellet and the procedure repeated. The final suspension in 0.1% peptone contained 1.0×10^8 cfu/mL.

Detection plates

Plates for stab inoculation were prepared from 15 mL molten and cooled (45 C) TSA poured into a petri plate on a level surface and allowed to solidify (ca. 10 min), with the tops off, in a laminar flow hood. Lawns of *P. fragi* were also prepared by mixing 0.2 mL washed cell suspension with 15 mL of TSA (45 C) prior to pouring.

Detection of bacteriocinogenicity

Solid culture

The procedure of Crowley and DeBoer (1980) was used to detect antagonistic behavior. The host, *P. fragi* ATCC 4973, was stab inoculated onto TSA plates and incubated (28 C, 48 h). The incubated plates were inverted over chloroform soaked filter paper for 20 min, the colony material scraped off, and the plates treated an additional 20 min. Residual

chloroform vapors were allowed to dissipate by exposure to sterile air in a laminar flow hood (Model BM6-2A, Canadian Cabinets Inc., Nepean, ON) for 15 min. Indicator organisms were prepared by mixing 0.1 mL aliquots of washed cell suspension in 5.0 mL of 0.6% agar containing 1% peptone at 45 C. The resultant suspension was mixed and poured over the previously prepared host test plates and incubated (28 C, 24 h).

Induction

Radiation induction

Shake broth cultures of *P. fragi*, grown to the mid-logarithmic growth phase (28 C, 18 h), were poured into sterile glass pans (13 x 21 cm) to a depth of 2.0 mm. The pan was placed 15 cm below an ultraviolet light source (254 nm) with a dose rate, calculated by potassium ferrioxalate actinometry (Jagger, 1967), of 35.57 $\mu J/cm^2/s$. Total dose was 2000 $ergs/mm^2$.

Alternately, a mid-logarithmic broth culture was irradiated with 30, 90 and 160 Gy of gamma radiation in a Gammacell 220 irradiator (Atomic Energy of Canada, Kanata, ON). Both ionizing and non-ionizing dose levels reflected those used by Lwoff *et al.* (1950) to induce bacteriophage in *Bacillus megaterium*. Subsequent to irradiation, the cultures were incubated a further 4 h at 28 C.

After incubation, the bacterial cells were removed by centrifugation (10,400 x g, 10 min, 4 C) and the supernatant filter-sterilized by passage through a 0.45µm cellulose

acetate membrane filter (Millipore Corp., Malton, ON). Supernatant was left "as is", or was concentrated (x 20) in an ultrafiltration cell with a 10,000 MW cut-off membrane (Amicon, Danvers, MA).

The test for antagonistic activity involved spotting 0.02 mL drops of "as is" or concentrated supernatant onto the surface of TSA plates containing indicator organisms. A positive test would be revealed by a zone of clearing in the bacterial lawn where the supernatants were applied.

Alternately, indicator organisms were grown in 10.0 mL TSB for 18 h followed by the addition of 2.0 mL "as is" supernatant. The absorbance, at 600 nm, of the cultures was followed over a further 24 h period using a spectrophotometer (Shimadzu Scientific Instr. Inc., Columbia, MD, model UV-160).

Mitomycin C

A mid-logarithmic broth culture of *P. fragi* (450 mL) was centrifuged (10,400 x g, 10 min, 4 C) and the pellet resuspended in TSB. After incubation (21 C, 4 h), the chemical inducer mitomycin C (Sigma Chemical Co., St. Louis, MO) was added at a concentration of 1.0 to 20.0 µg/mL and the cell suspension incubated a further 6 h at 21 C. Subsequent to the second incubation period, the culture was again centrifuged, the supernatant collected and filter-sterilized. Some supernatant was used "as is" and some was dialyzed against distilled water. Twenty mL of retentate was collected. The retentate and "as is" supernatant were

spotted on indicator plates to test for antagonistic activity. The absorbance of indicator broth cultures treated with both supernatants, as described earlier, was determined.

Spiral plate detection

In addition to spot plating, the spiral plating technique was used to detect possible antagonism from *P. fragi* induced by ionizing radiation and mitomycin C. The technique involved the concomitant use of the uniform and variable deposition modes of a model DU spiral plater (Spiral Systems Inc., Cincinnati, OH). Initially, a diluted washed cell suspension of indicator organisms (1.0×10^5 cfu/mL) was deposited, using the uniform cam, onto a TSA plate (inoculum density 1.3 cfu/mm^2 of track) and the liquid allowed to diffuse into the agar. Radiation or mitomycin C induced *P. fragi* culture supernatants, in both regular or concentrated forms, were then deposited, using the variable cam. The supernatants were deposited directly on top of the bacteria, following the same track. A total of 0.05 mL of supernatant was deposited per plate. Regular or concentrated TSB was deposited on separate indicator plates which were used as controls.

GROWTH OF *P. fragi* ON SOLID AND LIQUID MEDIUM

Inoculum growth

Pseudomonas fragi was incubated, with agitation, at 21 C for 24 h in TSB.

Incubation on solid medium

A 0.01 mL aliquot of inoculum containing ca. 4.8×10^3 cfu was mixed with 100 mL 0.1% peptone water and the suspension drawn through a 47 mm polycarbonate membrane filter (0.45µm, Nucleopore Corp., Pleasanton, CA). The membrane, together with the collected cells, was placed onto the surface of 15 mL TSB + agar, contained in a 50 mm petri plate, and incubated at 21 C at 4 h time intervals up to 60 h. These experiments were repeated three times.

Incubation in liquid medium

Separate 250 mL flasks containing 150 mL TSB were inoculated with ca. 1.5×10^6 cfu washed cells and incubated, with agitation (150 RPM), at 21 C up to 95 h. These experiments were also repeated three times.

Enumeration of cells

After incubation, the membrane filter was removed from the agar surface and the *P. fragi* cells on the membrane filter were dispersed into 100 mL of 0.1% peptone water with a Stomacher 400 (Cooke Laboratory Products, Alexander, VA). Mixing time was 2 min.

Surface plating on TSA using the spiral plating technique (Spiral Systems Inc) was used for enumeration of *P. fragi* grown on solid and in liquid medium. Duplicates of all counts were conducted. Plates were incubated at 21 C for 24-48 h.

Proteinase activity

The substrate was prepared by adding 10.0 g Hammersten casein (Chemical Dynamics Corp., New Jersey, NJ) to 187.5 mL 0.4 M Na_2HPO_4 followed by boiling for 30 min to solubilize the casein. After cooling, 312.5 mL of 0.4 M NaH_2PO_4 was added and the mixture diluted with distilled water to 1 L.

For determination of proteinase activity in liquid medium, 0.2 mL of culture supernatant, clarified by centrifugation (10,400 x g, 10 min, 4 C) and filter sterilized, was added to 2.0 mL of substrate. The supernatant was added to the substrate within 30 min of cell/supernatant separation. Proteinase activity of whole cells grown in liquid medium was determined by adding 0.2 mL unclarified culture medium to 2.0 mL substrate. Proteinase activity in solid medium was determined by adding the agar medium contained within the 50 mm petri plate (15.0 mL agar), without the membrane filter, to 150.0 mL substrate, followed by blending for 2 min with a Stomacher 400. The agar was added to the substrate within 30 min. In all cases, 2.2 mL test mixture aliquots were incubated at 40 C for time periods ranging up to 2 h. For incubation times in excess of 1 h, 0.02% NaN_3 (Sigma) was added to the substrate. After incubation, the reaction was stopped with 2.0 mL 24% trichloroacetic acid (TCA), and the mixture centrifuged (2,000 x g, 10 min). Absorbance, at 280 nm, of the supernatants was determined using a spectrophotometer (Shimadzu). One enzyme unit of proteinase was defined as

the quantity of proteinase that liberated TCA soluble amino acids and peptides equivalent to 0.001 µMole of tyrosine per min. The absorbance of unincubated controls was deducted from incubated samples to compensate for nonproteinaceous U.V. absorbing material present in the substrate. In all cases duplicate proteinase determinations were performed.

Data presentation

At each time interval the mean of the six proteinase determinations, from the three repeated *P. fragi* cell growth experiments (both liquid and solid), was calculated. The means and their resultant standard deviations were plotted.

Electron microscopy

Sample fixation

Cells from liquid medium were centrifuged (2,000 x g, 10 min), washed in 0.05 M phosphate buffer (PB) pH 7.0, recentrifuged, and fixed with 2.5% glutaraldehyde (J.B. EM Services, Dorval, PQ) in PB at room temperature for 1 h. Fixed cells were washed in PB and post-fixed with 1% w/v osmium tetroxide (J.B. EM Services) in PB at room temperature for 1 h. Fixation of *P. fragi* cells grown on solid medium was achieved by placing the entire polycarbonate membrane filter (Nucleopore) into the fixatives.

Sample preparation

Scanning electron microscope samples were dehydrated on the surface of polycarbonate membrane filters (Nucleopore) through a graded series of aqueous ethanol solutions. The

filters were immersed in 30, 50, 70 and 80% ethanol, for 5 min for each concentration, followed by two changes of 90% ethanol for 10 min each, and ending with two changes of 100% ethanol for 20 min each. Samples were critical point dried in a Parr-bomb (Parr Instruments Co., Moline, IL) using CO_2 as the transition fluid. Dried membrane filters were mounted on aluminum stubs with silver paste (J.B. EM Services Inc.) and sputter coated with gold (SEMPREP II Sputter Coater, Nanotech Ltd., Preswich, England).

Fixed cells, to be examined by transmission electron microscopy, were suspended in a mixture of 1.5% agar in PB. After solidification, 1 mm^3 blocks were cut and immersed in a graded series of aqueous ethanol solutions as described for scanning electron microscopy. Samples were infiltrated with EPON 812 (J.B. EM Services Inc.), using propylene oxide as a transition solvent, and polymerized at 60 C for 36 h.

Embedded samples were sectioned with a Porter-Blum ultramicrotome (Ivan Sorvall Inc., Norwalk, CN). Sections were mounted on 3 mm copper grids and stained with 2% w/v uranyl acetate and Reynold's lead citrate (Reynold, 1963).

Microscopy

Scanning electron microscopic examination of cells was done with a Cambridge Stereoscan 250 operated at 40 Kv. Transmission electron microscopy was done with a Zeiss EM-10 at an accelerating voltage of 80 Kv.

Transmission electron micrographs of *P. fragi* cells grown in TSB + agar for 20, 32, 40 and 56 h were examined.

The total of the cell perimeters for each electron micrograph was calculated, in μm. In addition, the total number of extracellular vesicles appearing on these perimeters were counted. With this information the number of vesicles per μm of cell perimeter was calculated. At each time interval four electron micrographs were examined. Mean values and standard deviations were determined.

The media

Trypticase soy broth (TSB), trypticase soy agar (TSA) and Kosers citrate medium were from BBL. Nitrogen free citrate medium was composed of NaH_2PO_4 (1.0 g/L), Na_2HPO_4 (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L) and sodium citrate (2.94 g/L). All were obtained from Sigma. Nitrogen free citrate medium was supplemented with l-glutamine (Sigma), added on a mmole nitrogen/L basis.

Cell number enumeration

Pseudomonas fragi suspensions were enumerated on TSA plates using the Spiral plating technique. Plates were incubated at 21 C for 46 h. Colony forming units were counted with a Model 500A bacteria colony counter (Spiral Systems Inc.) interfaced with a Spiral Systems Computer Assisted Spiral Bioassay data system.

Cell suspension

Initial broth culture

An initial broth culture of *P. fragi* was prepared in 150 mL of TSB, incubated at 21 C for 18 h. After incubation, the cells were harvested by centrifugation

(10,400 x g, 10 min, 4 C) and resuspended in 150 mL nitrogen-free citrate medium.

Working inoculum

Kosers citrate medium (150 mL) was inoculated with 7.5 mL washed initial broth culture. The cultures were incubated at 21 C for 18 h in a gyratory shaking incubator (120 RPM). After incubation, the cells were harvested by centrifugation (10,400 x g, 10 min, 4 C) and resuspended in 150 mL nitrogen-free citrate medium. The resultant working inoculum contained 5.7×10^8 cfu/mL.

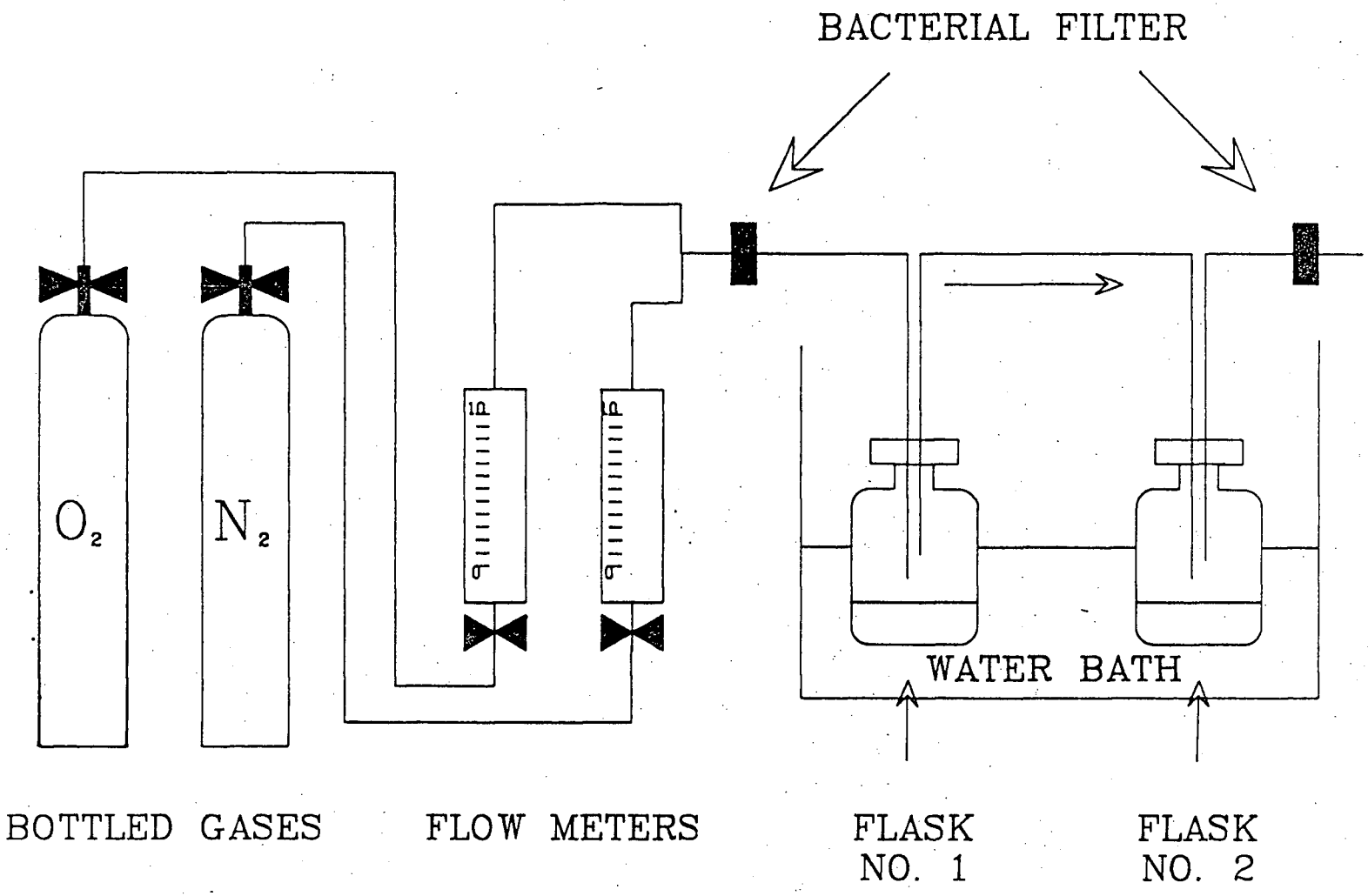
Experimental conditions

Growth of *P. fragi* during the experiments was carried out in 300 mL square-pak flasks (American Sterilizer Co., Erie, PN) (Figure 1). Each flask contained rubber septum type closures through which hypodermic needles carried gas mixtures and bacterial inoculum. Each experiment was repeated twice.

Gas mixtures

Gas mixtures consisted of U.S.P grade N_2 and O_2 (Union Carbide Corp., Toronto, ON). Gas pressures were controlled with oxygen and nitrogen two stage gas regulators (Linde, Union Carbide Corp.). Gas mixture flow was controlled by flow meters (Cole-Parmer Instrument Co., Chicago, IL). Passage of the gas mixture through presterilized 37 mm bacterial air vents (0.45 µm; Gelman Science Inc., Ann Arbor, MI) assured gas mixture sterility. Gas mixture flow

FIGURE 1. Diagram of gas mixture control and gyratory water bath setup.
Each Square Pak flask contained 50.0 mL nitrogen free citrate medium plus varying amounts of glutamine. The flasks were closed with rubber septums. Gases were supplied by two stage regulator valves, and the mixtures controlled by flow meters. The gas mixtures were sterilized by passage through bacterial filters (0.45 µm).


BACTERIAL FILTER
O_2
N_2
WATER BATH
BOTTLED GASES
FLOW METERS
FLASK NO. 1
FLASK NO. 2

rate was controlled at 400 mL/min. Dissolved oxygen in the experimental citrate medium was measured with a dissolved oxygen meter (Cole-Parmer). Experiments showed that this flow rate maintained a constant dissolved oxygen level during growth of *P. fragi*. Oxygen content of the gas mixture was expressed as percent O_2 present in the mixture. Dissolved oxygen present in the culture medium was expressed as ppm O_2. For experimental design purposes, percent oxygen was used as the factor level, although dissolved oxygen was dependant upon incubation temperature.

Each experimental unit consisted of two square-pak flasks, each containing 50 mL of experimental citrate medium (Figure 1). The gas mixture entered flask #1 which acted as a humidifier and experimental control. Humidified gas mixture then was passed by flexible tubing to the second flask. The second flask acted as the active experimental chamber. Gas mixture exited through a bacterial air vent.

The experimental unit was contained in a gyratory shaking water bath incubator (Lab-Line Instruments, Melrose Park, IL). Water bath temperature was monitored and recorded with a Microtemp model 811 temperature monitor/recorder (Intelicom Systems Inc., North Vancouver, BC). The experimental units were equilibrated at the indicated incubation temperature and gas mixture for 24 h prior to inoculation.

Subsequent to equilibriation, 2.5 mL of working inoculum was introduced into flask #2 of the experimental unit through the rubber septum (previously sterilized with a 70% ethanol swab) by hypodermic injection. Initial inoculum level was 2.8×10^7 cfu/mL. Inoculated flasks were shaken at 120 RPM during incubation.

After incubation, *P. fragi* cell numbers were enumerated, in duplicate, by spiral plating. The bacterial cells were then harvested by centrifugation (10,400 x g, 10 min, 4 C) and the supernatant filter sterilized by passage through a 0.45 µm membrane filter (Millipore).

Proteinase activity

Proteinase activity of the culture supernatants was determined, as previously described.

Electron microscopy

The harvested cells were examined by electron microscopy, as previously described.

Protein assay

Protein assay was performed using the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co., Rockford, IL). Briefly, 0.1 mL of sample was mixed with 2.0 mL of working BCA reagent and incubated at 37 C for 30 min. After cooling the absorbance was measured with a spectrophotometer (Shimadzu) at 562 nm. The absorbance of a blank containing BCA working reagent was subtracted from the sample absorbance. Bovine serum albumin (BSA, Sigma) was used as a standard.

Isoelectric focusing (IEF)

Filter sterilized supernatants were drop dialyzed on a 25 mm, 50 nm membrane filter (Millipore) as described by Marusyk and Sergeant (1980) and separated on IEF polyacrylamide gels pH 3.7 to 6.2 as described by Righetti (1987). Samples were applied onto the gel using filter paper squares. Duplicate gels were made and either overlayed with a casein-agar mixture or stained with silver stain.

Proteinase localization

Active proteinase was localized within IEF gels by overlaying a 1.5% agar suspension, containing (1%) casein substrate, over the gel. The casein substrate was the same preparation that was used for the proteinase assay. Briefly, the solubilized casein-agar mixture was heated to boiling, cooled to ca 45 C and poured over the IEF gel, still on its glass backing plate. The IEF gel and glass backing plate had previously been placed within an aluminum template to maintain a 2 mm thick casein-agar gel layer. The IEF gel-casein gel composite was incubated for periods up to 24 h at room temperature. Hydrolyzed areas in the casein gels were visualized by immersion, for 10 min, in a 4% sulfosalicylic acid, 12.5% trichloroacetic acid, 30% methanol solution.

Optimization procedures

The centroid search technique was used to optimize the production of proteinase from *P. fragi* under given cultural

conditions. The conditions and their ranges were: incubation temperature 1 to 40 C; incubation time 4 to 72 h; initial pH 5 to 10; glutamine concentration 200 to 650 mmole nitrogen/L; oxygen concentration 0 to 50%. The response value was enzyme units/mL.

Prior to optimization, a fractional factorial design experiment was conducted in order to evaluate the importance of each factor. The fractional factorial design used in this study was an L_8 (2^7) orthogonal array, as described by Taguchi (1957). It consisted of eight experiments at two factor levels. The five factors and their two levels were as follows: temperature 20 and 30 C; time 32 and 72 h; pH 7 and 9; glutamine concentration 7 and 125 mmole nitrogen/L; oxygen 10 and 30%. Each experiment was run twice.

A nested or hierarchial design (Snedecor and Cochran, 1971) analysis of variance of the data obtained from the Taguchi (1957) fractional factorial was performed on the individual experiments versus experiment replicates for both proteinase determinations and cell number enumerations (total of two analyses of variance). A nested design analysis of variance was also performed on the individual experiments versus experiment replicates for proteinase determinations from both the Spendley matrix and centroid experiments. Each experimental unit consisted of two replicates, each done in duplicate. These analyses were performed in order to determine whether replicates contributed significantly to the variance. Analysis of

variance was also performed on individual factors from the fractional factorial experiment. One analysis of variance was performed using enzyme units/mL and one used colony forming units/mL.

The mean of the proteinase determination for each experimental unit of the fractional factorial and centroid search (Spendley matrix, centroid and the simultaneous shift experiments) was determined. The standard deviation of each of the fractional factorial experimental unit means was calculated. The standard error of each of the Spendley matrix and centroid experiments experimental unit means was calculated. The mean of the means, and its associated standard error, of the individual experimental units for each of the fractional factorial, Spendley matrix and centroid experiments were determined.

Optimization models

The purpose of the following section is to provide the derivation of mathematical models used, in this study, to evaluate the efficiency of several popular optimization techniques.

It can generally be assumed that an organism will produce a specific enzyme, or group of similar enzymes, over a wide range of cultural conditions. If a specific enzyme can be identified, then there is most likely some unique set of cultural conditions where the maximum yield of enzyme would occur.

The rate at which the enzyme is produced at suboptimal culture conditions may change smoothly, rising at a constant rate of change far from the optimum point, and ending at the optimum point. This ideal situation in all probability does not occur. It is more likely that there is a variable rate of change in enzyme production.

If one could imagine a response surface on which microbial enzyme production varies over changing culture conditions, one would expect hills, valleys and plateaus to occur as the enzyme yield responds to changes in culture conditions. This picture would become increasingly complicated if a group of similar enzymes were being monitored, rather than one specific enzyme. Such situations may occur when one or more isoenzymes are produced. Under these circumstances, local maxima may populate the terrain.

A mathematical model designed to evaluate optimization techniques as they are applied to proteinase production by an organism, such as *P. fragi*, presents more of a challenge than simple continuous functions can provide. Several trigonometric functions exist which can produce discontinuous response surfaces. These functions are useful, but the response extremes are hard to predict and the computations can be complicated, especially with functions containing several factors.

Simple continuous functions can, however, be easily manipulated such that the point of maximum response can be

controlled both in position and value. In the following function:

$$f(X_1,X_2)=\{D-(X_1 \pm A)^2*C_1-(X_2 \pm B)^2*C_2\} \quad (I)$$

The maximum (or minimum) point will occur at the point X_1 = A, X_2 = B. The slope of the response surface can be controlled by C_1 and C_2. As an example:

$$Y_1=\{250-(X_1-3)^2*30-(X_2-7)^2*5\}/2.5 \quad (II)$$

The maximum response (100) will occur at X_1 = 3, X_2 = 7.

If another continuous function is superimposed on the original function by simple addition, then the resulting response surface will reflect this. If the maximum (or minimum) points of the two functions differ, then the resultant response surface becomes discontinuous. For example, if equation III with a maximum response value of 60 at X_1 = 8, X_2 = 2:

$$Y_2 = \{325-(X_1-8)^2*30-(X_2-2)^2*5\}/5.4 \quad (III)$$

were added to II such that:

$$Z=Y_1+Y_2 \quad (IV)$$

(values of Y_1 or Y_2 if <0 are changed to 0)

the response surface would have two maxima, one at $X_1 = 3$, $X_2 = 7$ with a value of 100, and one at $X_1 = 8$, $X_2 = 2$ with a value of 60. A three dimensional plot of factors X_1, X_2, and Z is shown in Figure 2. This same technique may be used to construct discontinuous response surfaces in dimensions greater than two. For example, two continuous three factor functions (V) and (VI) both designed to yield only one maximum response value are shown below:

$$Y_1=\{200+45X_1-50X_2+30X_3+140X_1X_2-10X_1X_3+61X_2X_3 \quad (V)$$
$$+45(X_1)^2-245(X_2)^2-11(X_3)^2\}/10$$

$$Y_2=\{200+45X_1-50X_2+30X_3+140X_1X_2-10X_1X_3+61X_2X_3 \quad (VI)$$
$$+45(X_1)^2-123(X_2)^2-43(X_3)^2\}/10$$

Functions (V) and (VI) can be combined in (VII).

$$Z=Y_1+Y_2 \quad (VII)$$
(same rules as IV)

Function (VII) would form a discontinuous four dimensional response surface. Equations V and VI were formulated according to the method of Bowman and Gerard (1967) and Saguy (1983) such that:

(1) for a minimum response value

$$D_i>0 \text{ for all } i=1,2,3,.....,n \quad (VIII)$$

(2) for a maximum response value

$$D_i<0 \text{ for all odd } i \ (i=1,3,5....)$$
$$D_i>0 \text{ for all even } i \ (i=2,4,6...)$$

where:

FIGURE 2. Three dimensional plot of model #1.
A three dimensional plot of the following equations:

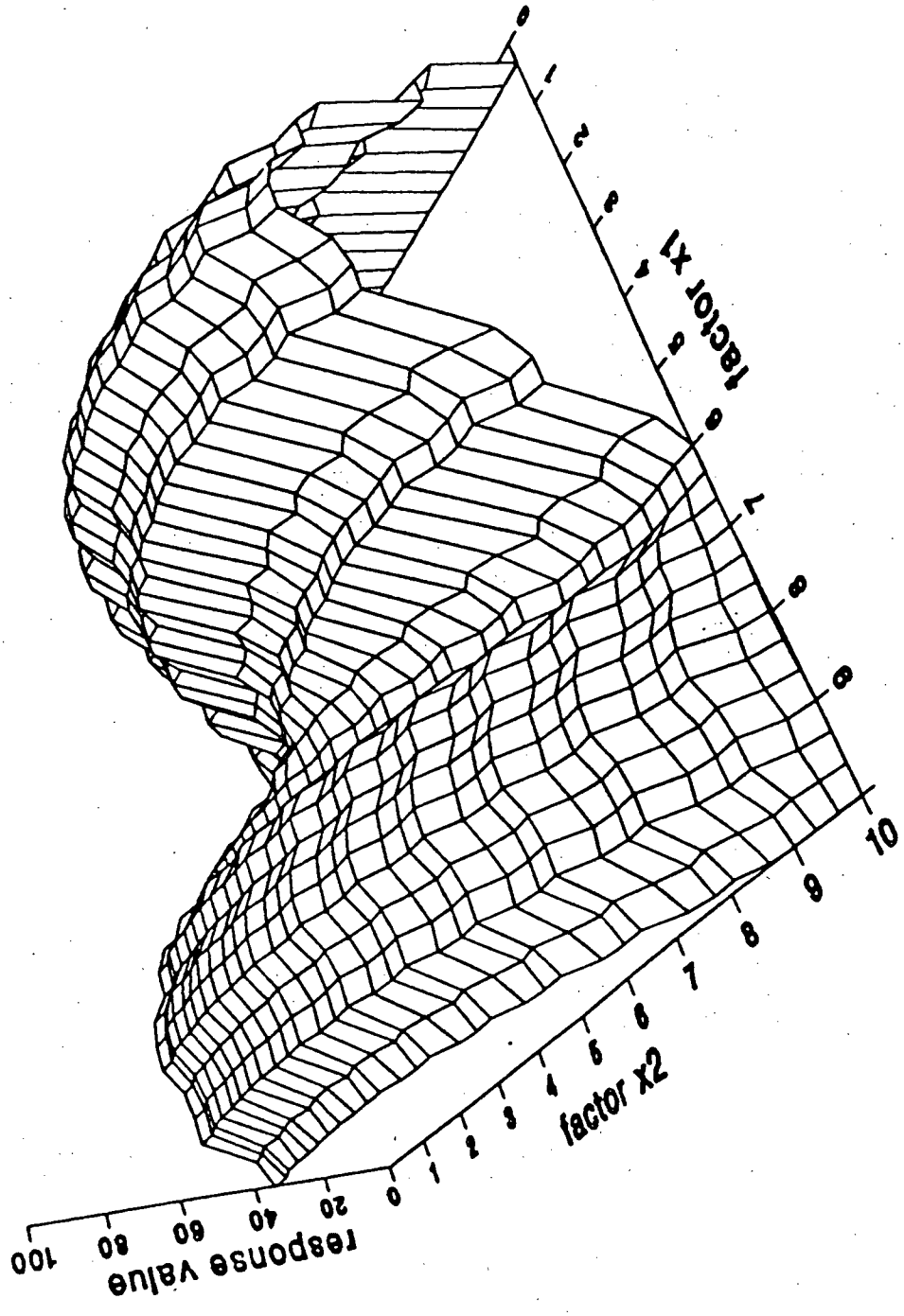
$$Y_1=\{250-(X_1-3)^2*30-(X_2-7)^2*5\}/2.5$$

and

$$Y_2 = \{325-(X_1-8)^2*30-(X_2-2)^2*5\}/5.4$$

$$Z=Y_1+Y_2$$

(values of Y_1 or Y_2 if <0 are changed to 0)
Factor X_1 is plotted on the X-axis and factor X_2 is plotted on the Y-axis. Factor Z is plotted on the Z-axis. Two maxima, one at $X_1 = 3$, $X_2 = 7$, and one at $X_1 = 8$, $X_2 = 2$ can be seen.


response value
0
20
40
60
80
100
factor x2
0
1
2
3
4
5
6
7
8
9
10
factor x1

$$D_1 = f_{11} \qquad D_2 = \begin{vmatrix} f_{11} & f_{12} \\ f_{21} & f_{22} \end{vmatrix} \qquad D_3 = \begin{vmatrix} f_{11} & f_{12} & f_{13} \\ f_{21} & f_{22} & f_{23} \\ f_{31} & f_{32} & f_{33} \end{vmatrix}$$

$$D_n = \begin{vmatrix} f_{11} & f_{12} & f_{13} & f_{1n} \\ f_{11} & f_{12} & f_{13} & f_{1n} \\ f_{11} & f_{12} & f_{13} & f_{1n} \\ \cdots & \cdots & \cdots & \cdots \\ \cdots & \cdots & \cdots & \cdots \\ \cdots & \cdots & \cdots & \cdots \\ f_{n1} & f_{n2} & f_{n3} & f_{nn} \end{vmatrix} \qquad \text{(IX)}$$

All the models constructed were used to evaluate the centroid search optimization technique (models are shown in Table I). The modified simplex technique (MDS) of Morgan and Deming (1974), the modified super simplex technique (MSS) with mapping (Nakai and Kaneko, 1985) and the response surface methodology (RS) derived from a Box and Behnken (1960) factorial design were compared against the centroid search technique (CS), using all models. Multiple regression analysis of the Box and Behnken (1960) factorial was performed on an IBM-XT compatible computer using the multiple regression procedure provided by the statistical graphing system "Statgraphics" (Statistical Graphics Corp., Baltimore, MD).

All models were created with a range of 10. All four optimization techniques were then applied to each model. Further runs of all the techniques were conducted using ranges of less than 10. These ranges were selected using a random number generator. A total of four runs were conducted. The stopping criterion was two successive

TABLE I. Mathematical models used to evaluate optimization techniques.

MODEL #1

$$Y_1=(250-(X_1-3)^2*30-(X_2-7)^2*5)/2.5$$

$$Y_2=(325-(X_1-8)^2*30-(X_2-2)^2*5)/5.4$$

MODEL #2

$$Y_1=(250-(X_1-3)^2*12-(X_2-7)^2*5)/2.5$$

$$Y_2=(325-(X_1-8)^2*12-(X_2-7)^2*5)/3.25$$

MODEL #3

$$Y_1=(200+45*X_1-50*X_2+30*X_3+140*X_1*X_2-10*X_1*X_3+61*X_2*X_3+45X_1^2-245*X_2^2-11*X_3^2)/10$$

$$Y_2=(200+45*X_1-50*X_2+30*X_3+140*X_1*X_2-10*X_1*X_3+61*X_2*X_3+45X_1^2-123*X_2^2-43*X_3^2)/10$$

response values ≥98% of the true response values. Measuring the amount of time required to complete the optimization was important in this study. Consequently, one independent factor was chosen to represent time (in hours).

An analysis of variance of the data obtained from the comparisons of three optimization techniques (Morgan Deming super simplex, modified super simplex and centroid search) versus the three models (model #1, model #2 and model #3) was performed (Snedecor and Cochran, 1971). The data consisted of means of experiment number and means of experiment time. A multiple range test (least significant difference) was used to compare differences between means.

Centroid optimization

The object of the following section is to demonstrate the centroid search and mapping procedures using function (IV), hereafter referred to as model #1.

Step 1 - Initial simplex

An initial simplex, a geometric figure defined by experimental points equal to one more than the number of independent variables (the number of independent variables being n), was created. The vertices of the simplex were obtained from a Spendley matrix once an origin and scale of dimension had been chosen. The origin is the lower limit (LL) and the origin + the scale is the upper limit (UL). In model #1 the origin is zero and the scale (S) is 10. The Spendley matrix suggests vertices (D_x) which have regularity

of spacing such that the values of the independent variables are of equal interest. The derivation is as follows:

$$
\begin{aligned}
D_1 &= (X_1, X_2, X_3, \ldots\ldots\ldots\ldots\ldots\ldots, X_n) \\
D_2 &= (p_n+X_1, q_n+X_2, q_n+X_3, \ldots\ldots\ldots\ldots, q_n+X_n) \\
D_3 &= (q_n+X_1, p_n+X_2, q_n+X_3, \ldots\ldots\ldots\ldots, q_n+X_n) \qquad \text{(X)} \\
&\vdots \\
D_{n+1} &= (q_n+X_1, q_n+X_2, q_n+X_3, \ldots\ldots\ldots\ldots, p_n+X_n)
\end{aligned}
$$

where: $$p = \frac{1}{n\sqrt{2}} [(n-1) + \sqrt{n+1}\,] * S \qquad \text{(XI)}$$

and: $$q = \frac{1}{n\sqrt{2}} [\sqrt{n+1} - 1\,] * S \qquad \text{(XII)}$$

S = scale factor
D_x = vertex
n = number of independent variables

For n=2, and S=10, p=9.659 and q=2.588. The resultant vertices of the simplex were:

$$D_1 = X_1, X_2$$

$$= 0, 0$$

$$D_2 = p_n+X_1, q_n+X_2$$

$$= 9.659, 2.588$$

$$D_3 = q_n X_1, p_n + X_2$$

$$= 2.588, 9.659$$

The Spendley matrix generated vertices, which are plotted in Figure 3, show the X_1 factor ranging from 0 to 10 on the X axis, with the X_2 factor ranging from 0 to 10 on the Y axis. The response values are shown as lines of contour, much like elevations on a topographical map.

Vertices are described as x,y pairs which have corresponding response values. The experiments and their responses are shown in Table II. These results show that vertex 3 produced the best result, with vertex 2 producing the next best result. Vertex 1 produced the worst response.

Step 2 - Evaluate the centroids

The centroids between the best and the next best vertices were searched. The centroid is the bisection point between the best (X_b) and the next best (X_n) vertex (point 4 in Figure 3). The optimum response is achieved by moving in the direction of improved response. If the centroid has a higher response than either the best or the next best vertex then the search is in fact moving in the direction of improved response. If this condition is met, then this point (point 4) becomes the next best vertex. If on the other hand, this condition is not met (i.e. if point 4 has a lower response value than either point 2 or point 3), then the search is

FIGURE 3. Topographical plot of model #1 with centroid generated experiments.
A topographical map of model 1. Factor X_1 is plotted on the X-axis and factor X_2 is plotted on the Y-axis. Lines shown correspond to equal values for the response value (Z). Vertices 1, 2 and 3 are points generated by the initial Spendley matrix. Vertex 3 produced the best result, with vertex 2 producing the next best result. Vertex 1 produced the worst result. The centroid (point 4) between the best and the next best results was evaluated and found to have a lower result than vertex 3. A new simplex with vertices 5, 6 and 7 was calculated. Centroids (points 8, 9 and 10) were evaluated. Point 10 came to within 99% of the true optimum value.

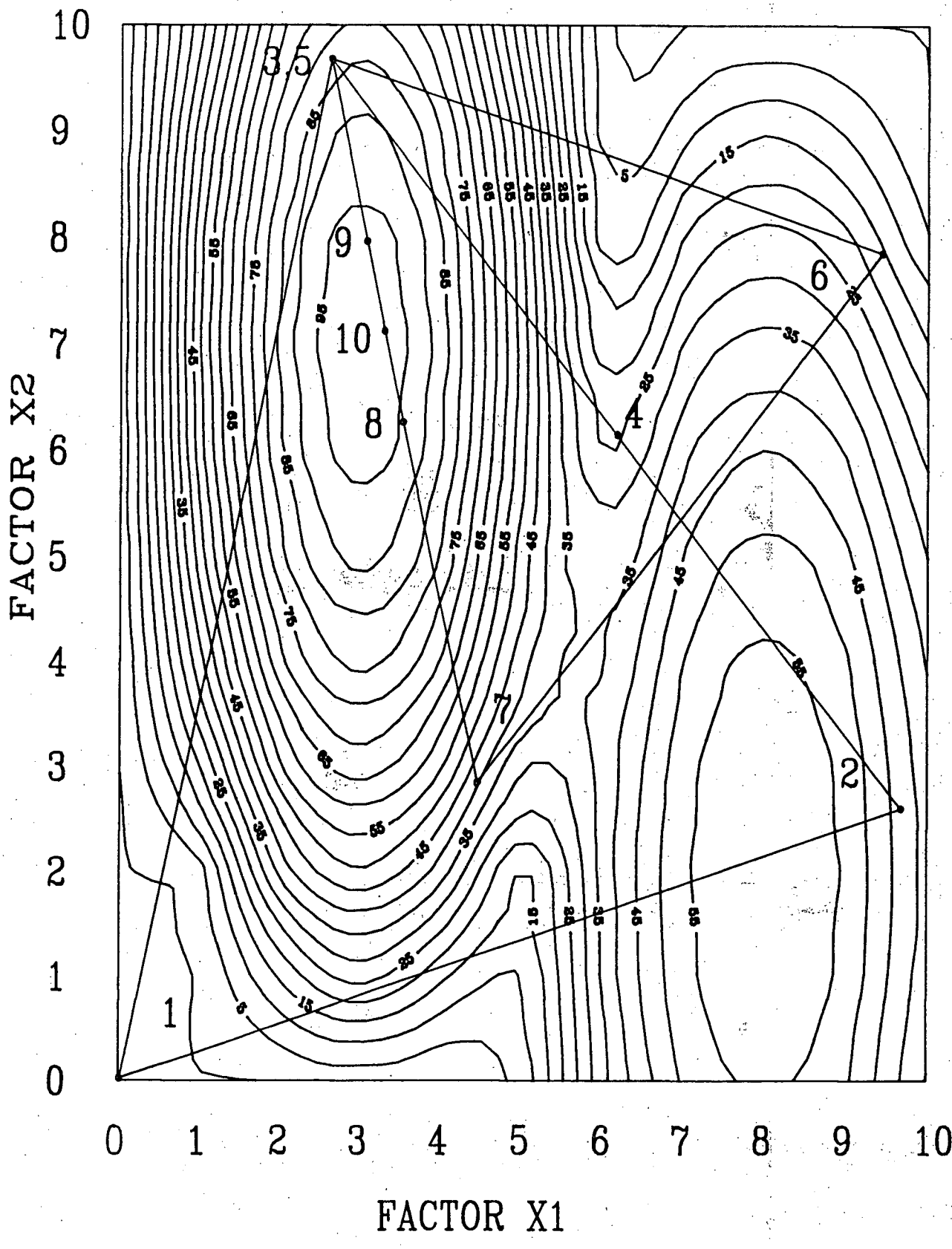

FACTOR X2
FACTOR X1
0
1
2
3
4
5
6
7
8
9
10
35
9
10
8
7
4
6
2
1

TABLE II. Experiments generated by centroid search and response values returned by model #1.

EXPERIMENTS		FACTOR		RESPONSE
		X_1	X_2	VALUE
initial	VERTEX 1	0.000	0.000	0.000
	VERTEX 2	9.659	2.588	44.570
	VERTEX 3	2.588	9.659	83.820
	CENTRD 4(1)	6.123	6.123	24.870
new	VERTEX 5	2.588	9.659	83.820
	VERTEX 6	9.418	7.829	17.550
	VERTEX 7	4.418	2.829	41.070
	CENTRD 8	3.503	6.244	95.820
	CENTRD 9	3.045	7.951	98.160
	CENTRD 10	3.274	7.097	99.080

(1) Centroid

not moving in the direction of improved response. This search line must be abandoned. Figure 3 and Table II show that this response was worse than the best from the initial simplex. This search line was abandoned and a new simplex with new ranges calculated.

Step 3 - Calculate new vertices and ranges

New vertices and ranges were calculated according to the rules set out by Aishima and Nakai (1986).

(1) When the responses of X_b and X_n (i.e. Y_b and Y_n) are close to one another:

$$|Y_b - Y_n| < |Y_b - Y_e|$$

Where: Y_b = best response value
Y_n = next best response value
Y_e = estimated best response value

then X_n is replaced by X_n+1 the third best vertex.

(2) When the difference between the best (X_b) and the next best (X_n) vertex is large:

$$|X_b - X_n| > |UL - LL| / 2$$

X_b and X_n are used as the new lower and upper limits.

(3) When the difference between the best (X_b) and the next best (X_n) vertex is small:

$$|X_b - X_n| < |UL - LL| / 20$$

$$\text{the new lower limit} = X_b - |UL - LL| / 20$$
$$\text{the new upper limit} = X_b + |UL - LL| / 20$$

(4) When the difference between the best (X_b) and the next best (X_n) vertex is small but X_b is near the boundary (UL or LL) then:

$$\text{the new lower limit} = X_b - q * |X_b - X_n|$$

$$\text{new upper limit} = X_b + |X_b - X_n| \; / \; 1 - q$$

A new Spendley matrix was produced at this point. The factor levels are shown in Table II (vertices 5,6,7).

Step 4 - Evaluate new simplex

Response values are shown in Table II. Vertex 5 (Figure 3) was the best vertex, while vertex 7 was the next best.

Step 5 - Evaluate new centroids

The new centroids are those points 8, 9 and 10 between the best and the next best vertices. The response values are shown in Table II. The value of the first centroid (point 8) was higher than the best from the new simplex (point 5), thus searching was continued. A new centroid (point 9), half way between the best and the new next best point, is evaluated. Centroids 9 and 10 yielded yet higher response values suggesting that this search line may be close to the optimum response value. At this point it is wise to stop further searches. Centroid searching involves a relatively small search area. As a result, local topography is evaluated, while global trends tend to be ignored. To remedy this situation a method must be used to map trends in the data and view this objectively. In this way it is possible to move to other areas where the true maximum may reside, and not get "bogged down" at a local maximum, the result of centroid search's "myopic" nature.

Step 6 - Mapping

Mapping (Nakai *et al.*, 1984) is an approximate visualization of the experimental response surface. The purpose of mapping is to demonstrate trends toward the true optimum. Each factor was plotted with the factor range on the X axis and the response values on the Y axis. For each factor not being compared, the range of values, as determined by the range of the response surface, was divided into four equal sections known as the large limit. Searches were made for two or more identical data points appearing in at least one section of each factor. Those data points found were then entitled to be joined in a map of the comparison factor. After all the factors were in turn compared using the large limit, a small limit was then selected and the process repeated. The small limit was determined from the plot of the comparison factor, including only those points suggested by the user as being of major importance. A medium limit was then used to evaluate all the comparison factors. The medium limit was calculated as the average of the values of the large and small limits. After all the matched data points from all three limits were plotted and linked, quadratic curve fitting was performed, if possible.

Figure 4a shows the mapping of factor X_1, while Figure 4b shows the mapping of factor X_2. The X values are shown on the abscissa while the response values are shown on the ordinate. Data points corresponding to factor values and

FIGURE 4. Map of factors from model #1.
The range for each factor is plotted against response value. The data points represent the vertices and centroids generated by centroid search. Data points are grouped into four groups. Those points entitled to be joined belong to the same group of all the other factors. The matched points are linked and quadratic curve fitting performed, if possible. Short line segments represent quadratic curves that have been fitted, but cannot be joined within the bounds of the map.

(a) Map of factor X_1. Optimum response is between 2.5 and 3.5.

(b) Map of factor X_2. Optimum response is between 6.5 and 9.0.

RESPONSE VALUE
0
20
40
60
80
100
(a)
0
2
4
6
8
10
FACTOR X1
RESPONSE VALUE
0
20
40
60
80
100
(b)
0
2
4
6
8
10
FACTOR X2

their respective response values are plotted. Figure 4a clearly shows an optimum response at X_1, values between 2.5 and 3.5, while Figure 4b shows an optimum response at X_2, values between 6.5 and 9.0.

New simplex

The centroid search procedure was repeated with either the new ranges suggested by the previous optimization procedure or by the mapping technique. The new vertices and their response values are shown in Table III and Figure 5. For brevity the vertices and their responses are given without comment.

UPPER AND LOWER LIMITS	
X_1	
LL	2.5
UL	3.5
X_2	
LL	6.5
UL	9.0

Mapping of the vertices for factor X_1 is shown in Figure 6a, while mapping of factor X_2 is shown in Figure 6b. Axes have been contracted to "zoom in" on the optimum points. Optimum points are close to vertex 4 (Figure 5), i.e. $X_1 = 2.982$, $X_2 = 6.823$.

TABLE III. Experiments generated by centroid search and evaluated by model #1 - new simplex.

EXPERIMENTS	FACTOR		RESPONSE
	X_1	X_2	VALUE
VERTEX 1	2.500	6.500	96.500
VERTEX 2	3.465	7.147	97.360
VERTEX 3	2.758	8.914	91.970
CENTRD 4	2.982	6.823	99.930
CENTRD 5	3.224	6.985	99.390
CENTRD 6	3.103	6.904	99.850

FIGURE 5. Topographical map of Model #1 and experiments generated by centroid search - new simplex.
Factor X_1 is plotted on the X-axis and factor X_2 is plotted on the Y-axis. Lines shown correspond to equal values for the response value (Z). Using ranges suggested from the initial mapping of model #1, a second centroid search optimization was performed. Points 1, 2 and 3 are vertices generated by the Spendley matrix. Point 2 is the best response and point 1 the next best. Centroid 4 came to within 99.93% of the true optimum.

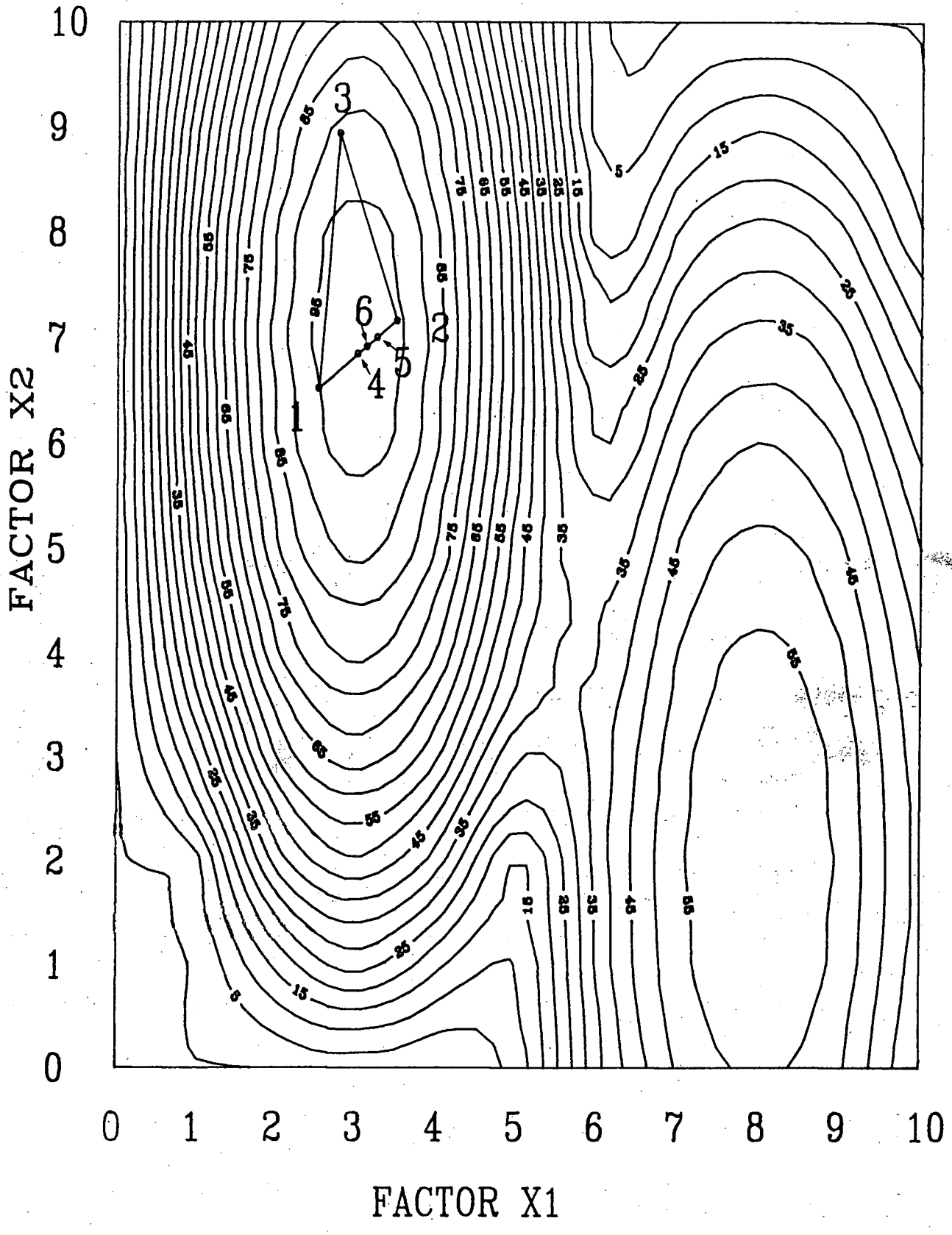

FACTOR X2
FACTOR X1
0
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
6

FIGURE 6. Map of factors from model #1 - new simplex. The range for each factor is plotted against response value. The data points represent the vertices and centroids generated by centroid search. The range for each factor has narrowed, converging towards the optimum point.

(a) Map of factor X_1. Optimum response is close to vertex 4 (2.982)

(b) Map of factor X_2. Optimum response is close to vertex 4 (6.823).

RESPONSE VALUE
99.0
99.2
99.4
99.6
99.8
100
2.75
2.85
2.95
3.05
3.15
3.25
FACTOR X1
(a)
RESPONSE VALUE
98.0
98.4
98.8
99.2
99.6
100.0
6.5
6.7
6.9
7.1
7.3
7.5
FACTOR X2
(b)

CELL AND GROWTH MEDIUM FRACTIONATION

Broth culture

Separate 250 mL flasks containing 150 mL TSB were inoculated with ca. 1.5 x 10^6 cfu *P. fragi* and incubated with agitation (150 RPM) at 21 C for 82 h. After incubation, flask contents were pooled and centrifuged (10,400 x g, 10 min, 4 C). Both cell pellet and supernatant were collected. Separate 150 mL culture units were used to maintain aerobic continuity.

Supernatant preparation

P. fragi was grown in 1 L TSB at 21 C for 82 h. Cells were removed by centrifugation (10,400 x g, 10 min, 4 C) and the supernatant filter sterilized. The supernatant was concentrated in ultrafiltration cells with 10,000 MW cut-off membranes (Amicon). The concentrated supernatant was then dialyzed against 4 L of water at 4 C and further concentrated by ultrafiltration. This procedure would eliminate salts which would interfere with electrophoretic separation of proteinaceous material present in the supernatant.

Extracellular vesicle preparation

Extracellular vesicles were precipitated from 1 L culture supernatant by slow addition (over a 1 h period) of 240 g $[NH_4]_2SO_4$ (40% saturation) at 4 C, with agitation.

The resultant suspension was centrifuged (20,000 x g, 40 min, 4 C) and the pellet resuspended in 150 mL of 50 mM

Tris buffer pH 6.8. The suspension was dialyzed for 24 h in 4 L of the same buffer.

After dialysis, the retentate was collected and centrifuged (27,000 x g, 40 min, 4 C). The pellet was suspended in 30 mL water and again centrifuged. The pellet was resuspended in 3.0 mL of water and the resultant preparation stored at -20 C for subsequent electron microscopy, or freeze-dried for chemical analysis.

Electron microscopy

A sample of extracellular vesicle suspension was deposited onto Formvar coated 3 mm copper electron microscopy grids and negative stained with 2% aqueous uranyl acetate. Negatively stained grids were examined by TEM, as previously described.

Buoyant density

The buoyant density of the extracellular vesicles was determined by high speed centrifugation in Percoll (Pharmacia) plus 0.25 M sucrose.

The Percoll working solution consisted of 15.0 mL 2.5 M sucrose, 127.5 mL Percoll (from the bottle) and water to make up to a total of 150 mL. The density of the resultant solution was 1.14 g/mL. Twenty mL of the Percoll working solution was placed in 30 mL high speed polycarbonate centrifuge tubes (Sorvall) and a 0.5 mL sample (suspended in 0.25 M sucrose) layered on top of the Percoll. The centrifuge tubes were spun at 60,000 x g in a T865 fixed-angle rotor head (Sorvall) for 45 min. Density marker beads

(Pharmacia), as well as refractometry, were used to measure the generation of the linear density gradient. The density ranged from 1.097 to 1.165 g/mL.

Carbohydrate assay

Total carbohydrate of the extracellular vesicles was determined by the Phenol-sulfuric acid reaction as described by Dubois, *et al.* (1956). Dextrose was used as a standard.

Outer cell membrane preparation

Outer cell membrane preparations were obtained by adaptations of the methods described by Filip *et al.* (1973), Hancock and Carey, (1979), and Anwar, *et al.* (1985). The cell pellet, collected by centrifugation (10,400 x g, 10 min, 4 C), was suspended in 20 mL 10 mM Tris buffer pH 8.0 containing 20% sucrose, and frozen. After thawing, 1000 µg DNase I (Sigma) was added to the mixture and incubated at room temperature for 15 min. After incubation, the cells were ultrasonically disrupted (Tekmar sonic disruptor Model TM500, Tekmar Inc., New York, NY) by 4 x 30 s pulses at 280 W. The mixture was maintained in an ice bath during treatment. Subsequent to disruption, the mixture was centrifuged (3,000 x g, 10 min, 4 C) and the pellet discarded. To the supernatant 0.4 g sodium lauryl sarcosinate (Sigma) was added and the mixture incubated 30 min at room temperature. After incubation, the mixture was ultracentrifuged (100,000 x g, 1 h, 4 C) (Sorvall model OTD-50 utilizing a T865 fixed-angle rotor head, (Ingram and Bell Scientific, Weston, ON) and the resultant pellet washed

twice with distilled water. The outer membrane was suspended in 3.0 mL water and stored at -20 C.

Soluble cell material preparation

Soluble cell material was prepared in a manner similar to that of outer cell membrane material. In the preparation of soluble cell material no sodium lauryl sarcosinate was used to solubilize membrane structures. Subsequent to the initial high speed centrifugation, the supernatant and the salmon colored pellet were reserved. The supernatant was the soluble cell material.

Proteinase activity

The proteinase activity of the supernatant, extracellular vesicles, outer cell membrane and soluble cell material was determined using casein substrate, as previously described. An aliquot of the extracellular vesicle suspension, maintained in an ice bath, was disrupted (solubilized) by a sonic disruptor (Tekmar) for 2 min at 280 W. The resultant disrupted mixture was centrifuged at 10,400 x g for 10 min at 4 C and the proteinase activity of the supernatant determined.

Protein assay

Protein assays were performed on the supernatant, extracellular vesicles, outer cell membrane and soluble cell material using the BCA protein assay reagent (Pierce), as previously described. Extracellular vesicles and outer cell membrane material were solubilized with 1.0% Triton X-100 (Pierce) prior to protein determination.

Electrophoretic techniques

Electrophoresis

The proteinaceous components of the extracellular vesicles and the outer cell membrane preparation were separated on horizontal slab sodium dodecyl sulfate containing polyacrylamide gels (SDS-PAG) using the buffer system of Laemmli (1970). The acrylamide concentration of the separating gel was 7.5% and that of the stacking gel was 4.0%. Samples were solubilized by heating at 100 C for 2.0 min in a sample buffer containing 2.0% SDS and 5.0% 2-mercaptoethanol (Pharmacia). Thirty-six µL of sample were added to the sample wells. Gels were run at 50 mA constant current until the bromophenol blue tracking dye reached the end of the gel. Gels were fixed in 11.4% trichloroacetic acid, 3.4% sulphosalicylic acid, 30.0% methanol, and stained with 0.25% Coomassie Blue R250 in 50.0% methanol.

The proteinase profiling technique described by Kelleher and Juliano (1984) was used in an attempt to profile the proteinases present in the vesicles. In this technique, casein is covalently bound to glutaraldehyde-activated linear polyacrylamide. The conjugated casein, subsequent to incorporation in SDS-Polyacrylamide gels, does not migrate during electrophoresis and remains susceptible to enzymic attack following regeneration of enzyme activity. Enzyme regeneration requires complete removal of SDS. The gel is initially washed with water, followed by 60 min incubation in 10 mM Tris buffer pH 6.6 containing 1.0%

Triton X-100 (Sigma). The washed gels are transferred to development buffer (50 mM Tris buffer pH 6.6) containing 10 mM Ca^{++} and incubated for 24 h. After incubation, gels are stained with Coomassie Blue.

Isoelectric focusing (IEF)

The supernatant and three preparations of the extracellular vesicles were separated using 2.0 mm IEF polyacrylamide gels pH 4.0 to 6.0. One vesicle preparation was unaltered, one was sonicated, as previously described and one was solubilized with 2.0% Triton X-100 (Pierce). Isoelectric focusing was performed on a LKB 2117 Multiphor II Electrophoresis System (Pharmacia Inc., Dorval, PQ). Samples were applied onto the gel using filter paper squares. Runs were conducted at 10 C for 2.5 h at 25 W. Power was supplied by a LKB MacroDrive 5 power supply (Pharmacia). Duplicate gels were made and either overlayed with previously poured 0.5 mm casein-agar gels, or stained with Coomassie Blue as described by Righetti (1987).

Proteinase localization

Active proteinase was localized within IEF gels by overlaying the gel with a 0.5 mm 1.5% agar (Noble, BBL) gel containing 1.0% casein substrate. The casein substrate was the same preparation that was used for the proteinase assay. Briefly, the solubilized casein-agar mixture was heated to boiling, cooled to ca. 70 C and poured into a preheated (70 C) gel mould and cooled. The cooled casein-agar gel supported by a LKB GelBond (Pharmacia) film was placed onto

the IEF gel and a weight applied in order to ensure contact between the two gels. The casein-agar gel was incubated for 24 h at 25 C. After incubation, hydrolyzed areas in the casein-agar gel were visualized by immersion, for 10 min, in a 3.5% sulfosalicylic acid, 11.5% trichloroacetic acid solution.

RESULTS

GROWTH OF *P. fragi* ON SOLID AND LIQUID MEDIUM

In liquid medium (Figure 7a), the *P. fragi* population increased from the initial 2.0 x 10^5 cfu/mL to 2.0 x 10^9 cfu/mL after 90 h. The bacteria entered an exponential growth rate at 8 h that continued up to 20 h, followed by a stationary growth phase. The population began to decline somewhat after 56 h, but began to increase again after 76 h.

On solid medium (Figure 8a), the *P. fragi* population increased from the initial 2.8 x 10^5 cfu/cm^2 to 8.2 x 10^9 cfu/cm^2 after 60 h. The growth rate became exponential at 4 h. From 16 to 32 h a much reduced rate of growth occurred. After 32 h, the culture entered a stationary growth phase. Cell colonies on the membrane surface became visible after 8 h. By 24 h a thick mat of cells could be seen growing on the membrane surface. At 60 h the mat was several millimeters thick.

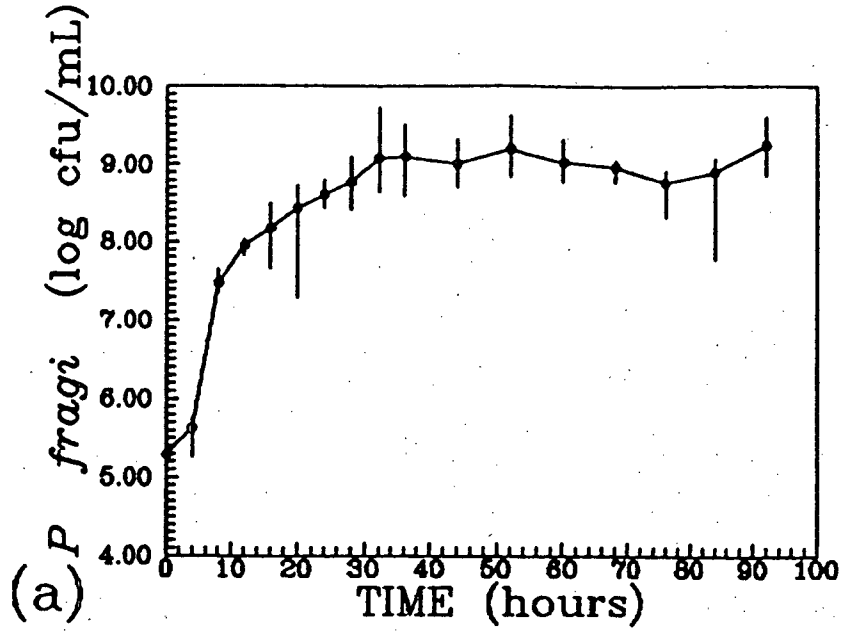
Production of proteinase by *P. fragi*

Proteinase production, by *P. fragi* cells grown in liquid medium (Figure 7b), was first detected in the culture supernatant at 26 h and increased steadily to a maximum at ca. 82 h. At this point proteinase production began to decline.

FIGURE 7. *P. fragi* growth and proteinase production in liquid medium.
P. fragi were grown in TSB at 21 C for time periods up to 95 h.

(a) Log *P. fragi* colony forming units/mL. The data points represent the geometric mean of six data points. Bars represent ranges.

(b) Proteinase enzyme units/mL. The data points represent the arithmetic mean of six data points. Bars represent standard deviation.


P fragi (log cfu/mL)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
0
10
20
30
40
50
60
70
80
90
100
TIME (hours)
(a)

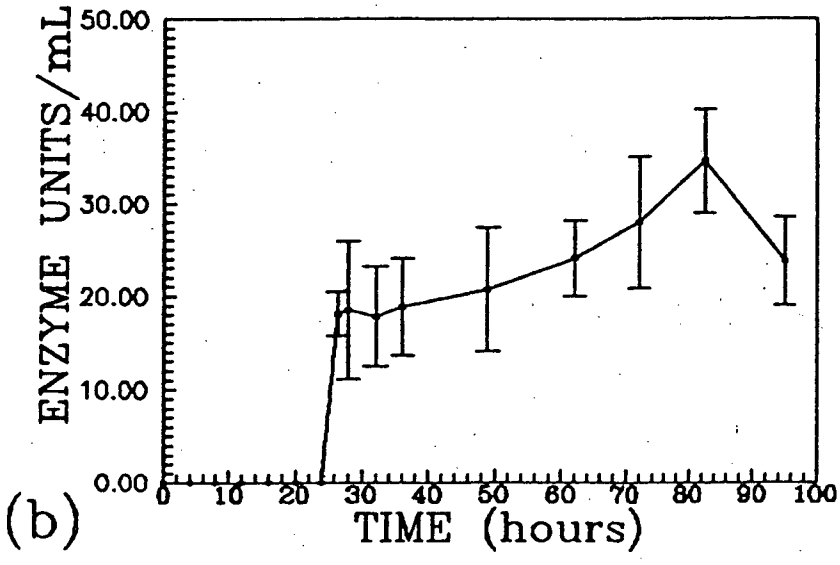
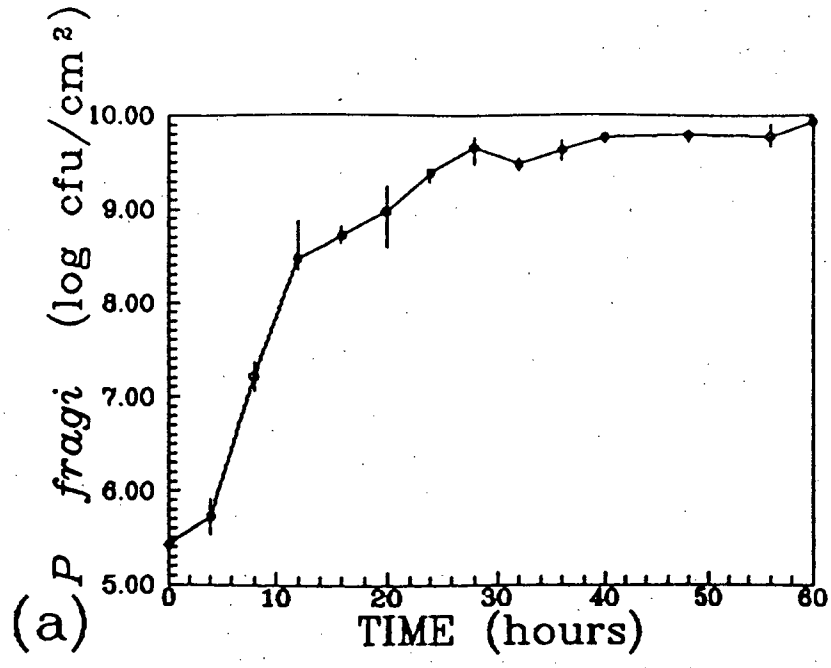

ENZYME UNITS/mL
50.00
40.00
30.00
20.00
10.00
0.00
0
10
20
30
40
50
60
70
80
90
100
TIME (hours)
(b)

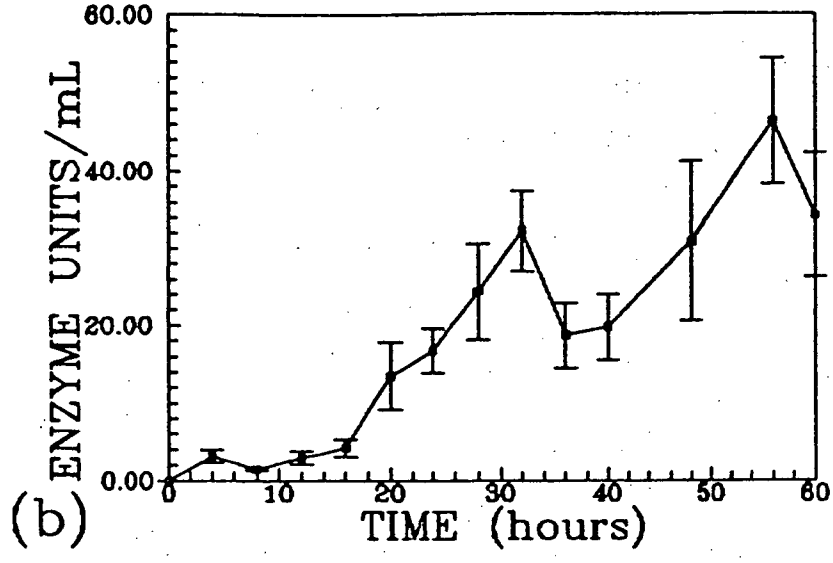
FIGURE 8. *P. fragi* growth and proteinase production on solid medium.
P. fragi were grown on TSB + agar surfaces at 21 C for time periods up to 60 h.

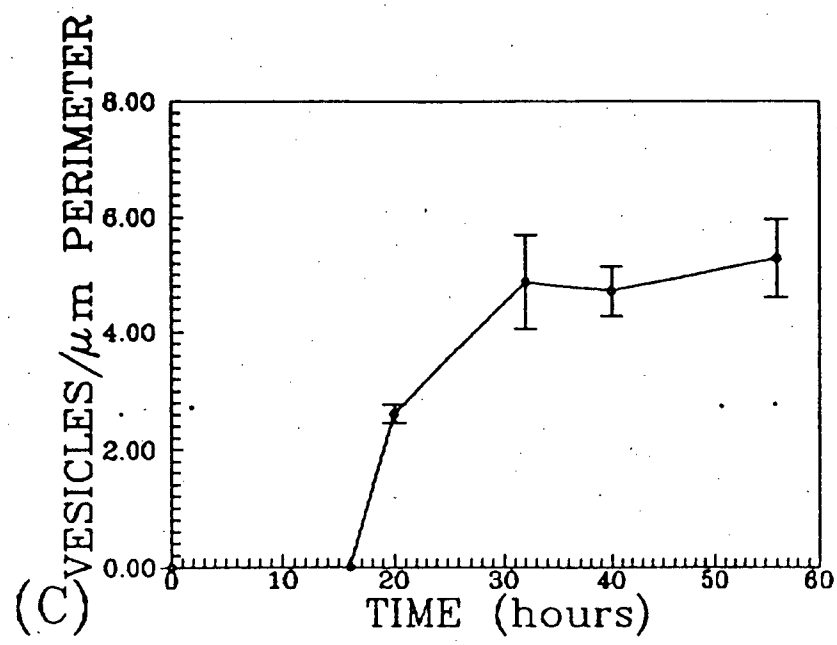
(a) Log *P. fragi* colony forming units/cm^2. The data points represent the geometric mean of six data points. Error bars represent ranges.

(b) Proteinase enzyme units/mL. The data points represent the arithmetic mean of six data points. Error bars represent standard deviation.

(c) Vesicles per unit perimeter. Transmission electron micrographs of *P.fragi* cells at 20, 32, 40 and 56 h were examined. Cell perimeters were calculated, and vesicles appearing on the perimeters were counted. Mean values of vesicles per µm were plotted versus time. Error bars represent sample mean standard deviation.


(a)
P fragi (log cfu/cm²)
10.00
9.00
8.00
7.00
6.00
5.00
0
10
20
30
40
50
60
TIME (hours)


(b)
ENZYME UNITS/mL
60.00
40.00
20.00
0.00
0
10
20
30
40
50
60
TIME (hours)


(C)
VESICLES/μm PERIMETER
8.00
6.00
4.00
2.00
0.00
0
10
20
30
40
50
60
TIME (hours)

Proteinase production of whole *P. fragi* cells grown in liquid medium could not be demonstrated at time periods before 26 h.

Proteinase production, by *P. fragi* cells grown on a solid surface of agar growth medium, first appeared at about 4 h (figure 8b), approximately 22 h sooner than in liquid culture. The production declined somewhat at 8 h but began to increase quickly after 16 h. At 20 h the increase in production slowed somewhat, but did increase until 32 h. At this point, production declined sharply and did not rise substantially for 8 h. At 40 h proteinase began to increase steadily for 16 h to a maximum at the 56 h mark. At 56 h production again declined to the end of the experiment.

Electron microscopy/extracellular vesicle production by *P. fragi*

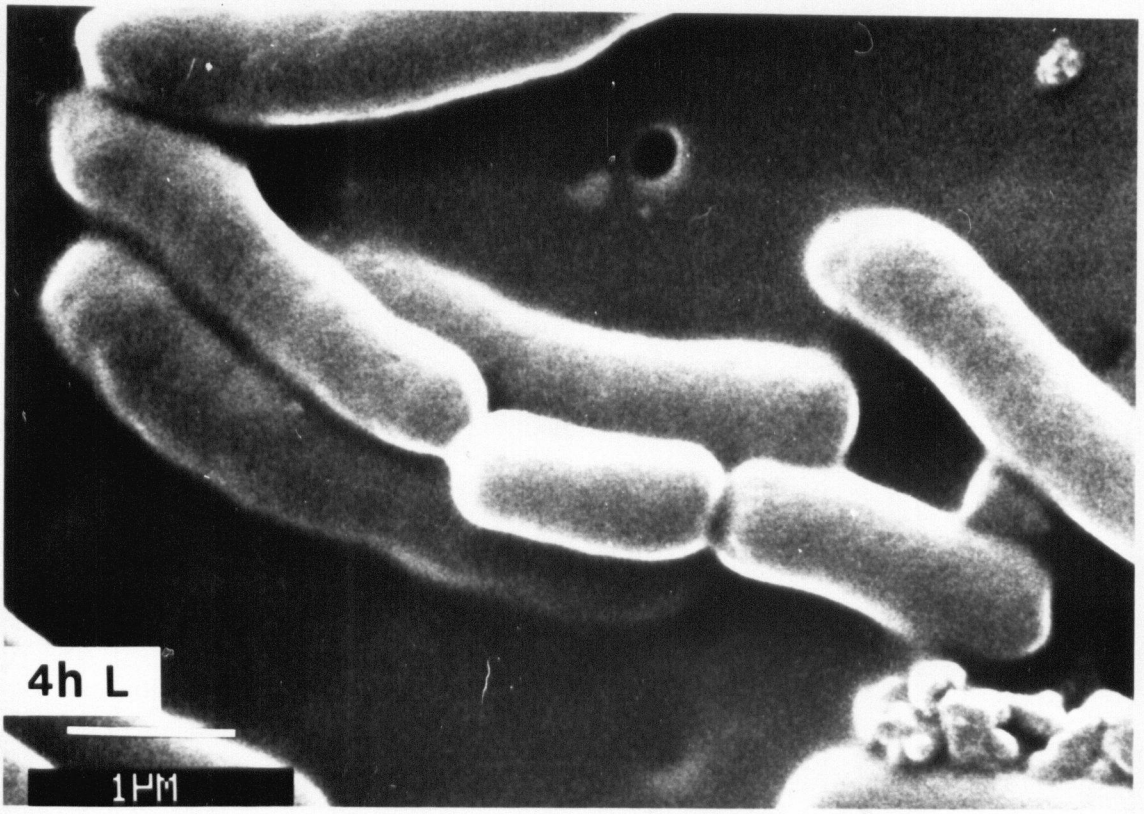
Scanning electron microscopy

At 4 and 27 h the surface of *P. fragi* cells grown in liquid medium appeared without globules (Figures 9 and 10). The cell surfaces did not change appreciably until the end of the logarithmic phase. At 76 h, during the stationary growth phase, globules, ca. 100-200 nm in diameter, were observed on the cell surfaces (Figure 11). This period coincided with the culture's maximum proteinase production.

On solid medium, at 4 h, globules were not observed on the *P. fragi* cell surfaces (Figure 12). At 24 h, during the late logarithmic early stationary phase, the cell surfaces

FIGURE 9. Scanning electron micrograph of *P. fragi* grown in liquid medium for 4 hours.
No globules appear on the cell surface.

FIGURE 10. Scanning electron micrograph of *P. fragi* grown in liquid medium for 27 hours.
No globules appear on the cell surface.


4h L
1PM

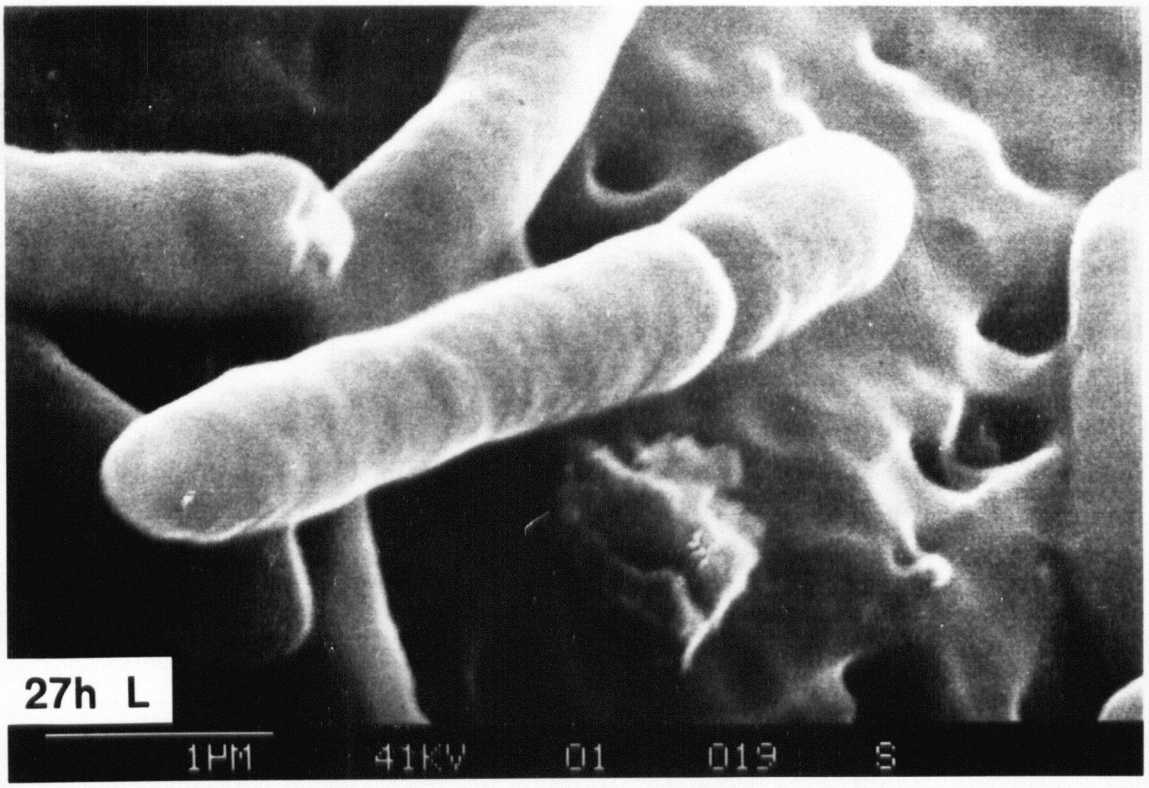
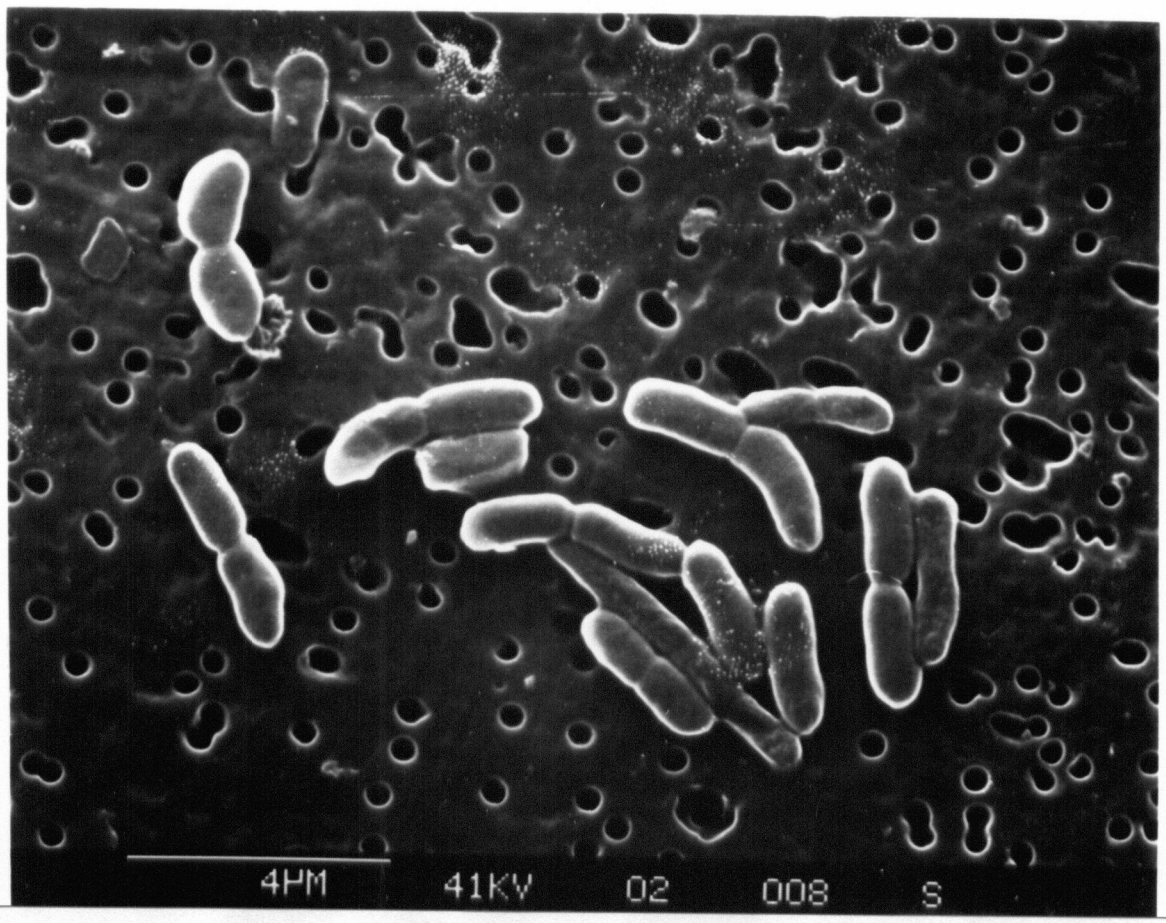

27h L
1PM
41KV
01
019
S

FIGURE 11. Scanning electron micrograph of *P. fragi* grown in liquid medium for 76 hours.
Globules approximately 100-200 nm in diameter were present on the cell surfaces.

FIGURE 12. Scanning electron micrograph of *P. fragi* grown on solid medium for 4 hours.
Globules were not observed on the cell surfaces.


1PM 41KV 05 029 S


4PM 41KV 02 008 S

had a ragged look with an accumulation of globules ca. 40-190 nm in diameter (Figure 13).

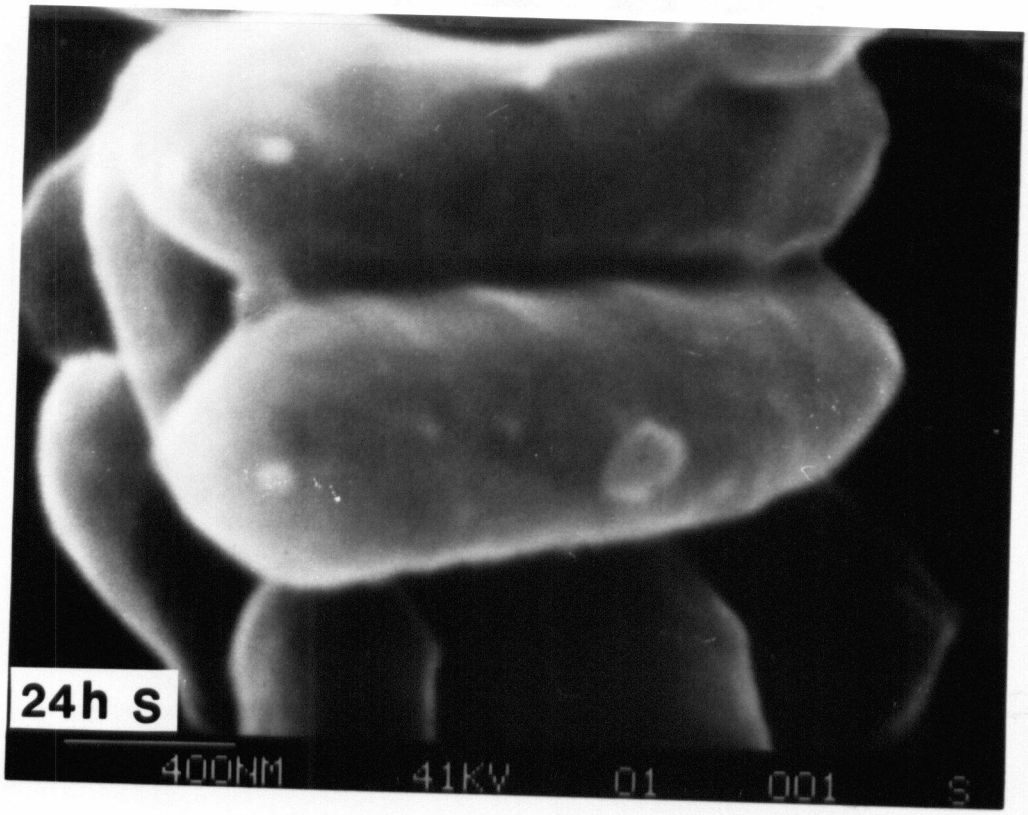
Thin section transmission electron microscopy

Transmission electron micrographs of *P. fragi* grown in liquid medium for 4 h showed the outer cell margin as a complex structure composed of several layers (Figure 14), the outer most portion being highly convoluted. The cytoplasmic portion (CP) of a *P. fragi* cell grown for 16 h (Figure 15) was surrounded by an inner cytoplasmic membrane (CM). This membrane was in turn contained by a cell wall (CW) structure. Together the cytoplasmic membrane and the cell wall contain the cytoplasm and define the overall shape of the cell. Beyond these structures the periplasmic gap (PG) separates the cell wall from the outer cell membrane (OM). It was this outer membrane, completely surrounding the cell, which gave the convoluted appearance to the cell outer margin. The outer cell membrane at times appeared to breach the gap between the outer cell membrane and the cell wall (Figure 15).

Young cells, such as those depicted in Figure 16 (16 h), contained a more or less homogeneous cytoplasm with no obvious inclusions or voids. The components which make up the cell margins were distinct and well defined. As the cells aged, such as that depicted in Figure 17 (44 h), the periplasmic gap widened and the outer cell membrane

FIGURE 13. Scanning electron micrograph of *P. fragi* grown on solid medium for 24 hours.
An accumulation of globules, approximately 40-190 nm in diameter, were observed on the cell surfaces.

FIGURE 14. Transmission electron micrograph of *P. fragi* grown in liquid medium for 4 hours.
Cells were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and suspended in agar. Agar blocks were dehydrated in ethanol and infiltrated with EPON 812. Embedded cells were sectioned, and stained with uranyl acetate and lead citrate.


24h S
400NM
41KV
01
001
S

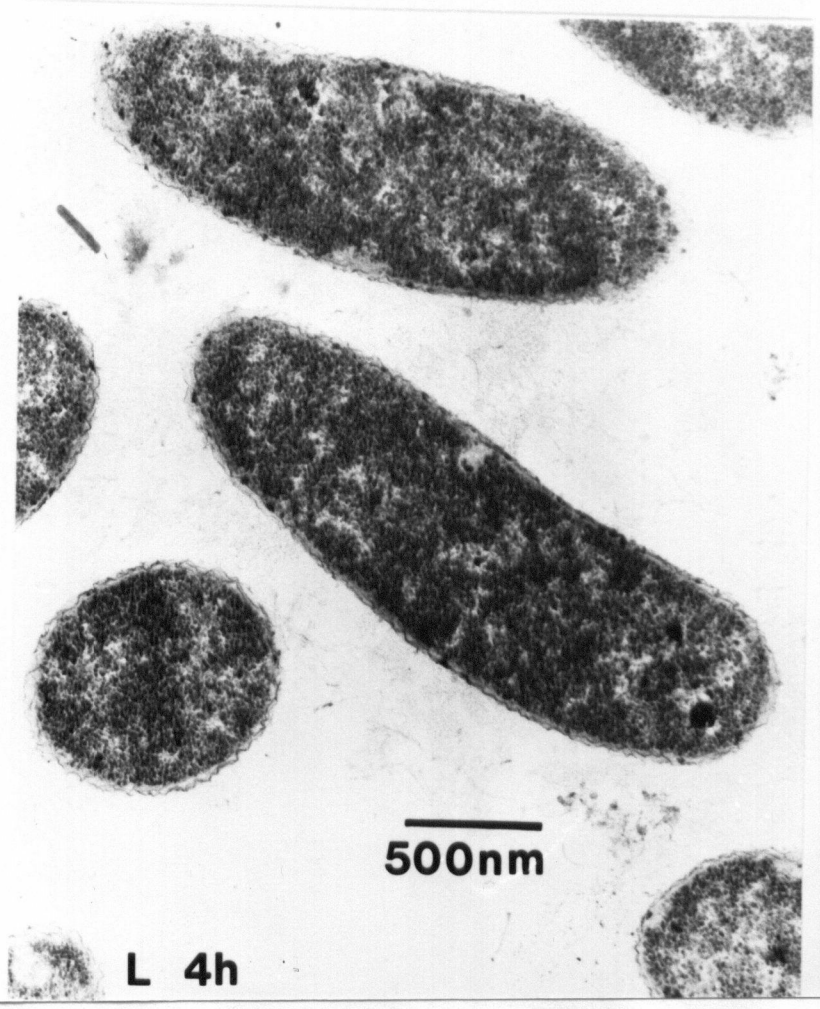

500nm
L 4h

FIGURE 15. Transmission electron micrograph of *P. fragi* grown in liquid medium for 16 hours.
(OM) outer membrane; (PG) periplasmic gap; (CP) cytoplasm; (CM) cytoplasmic membrane; (CW) cell wall.

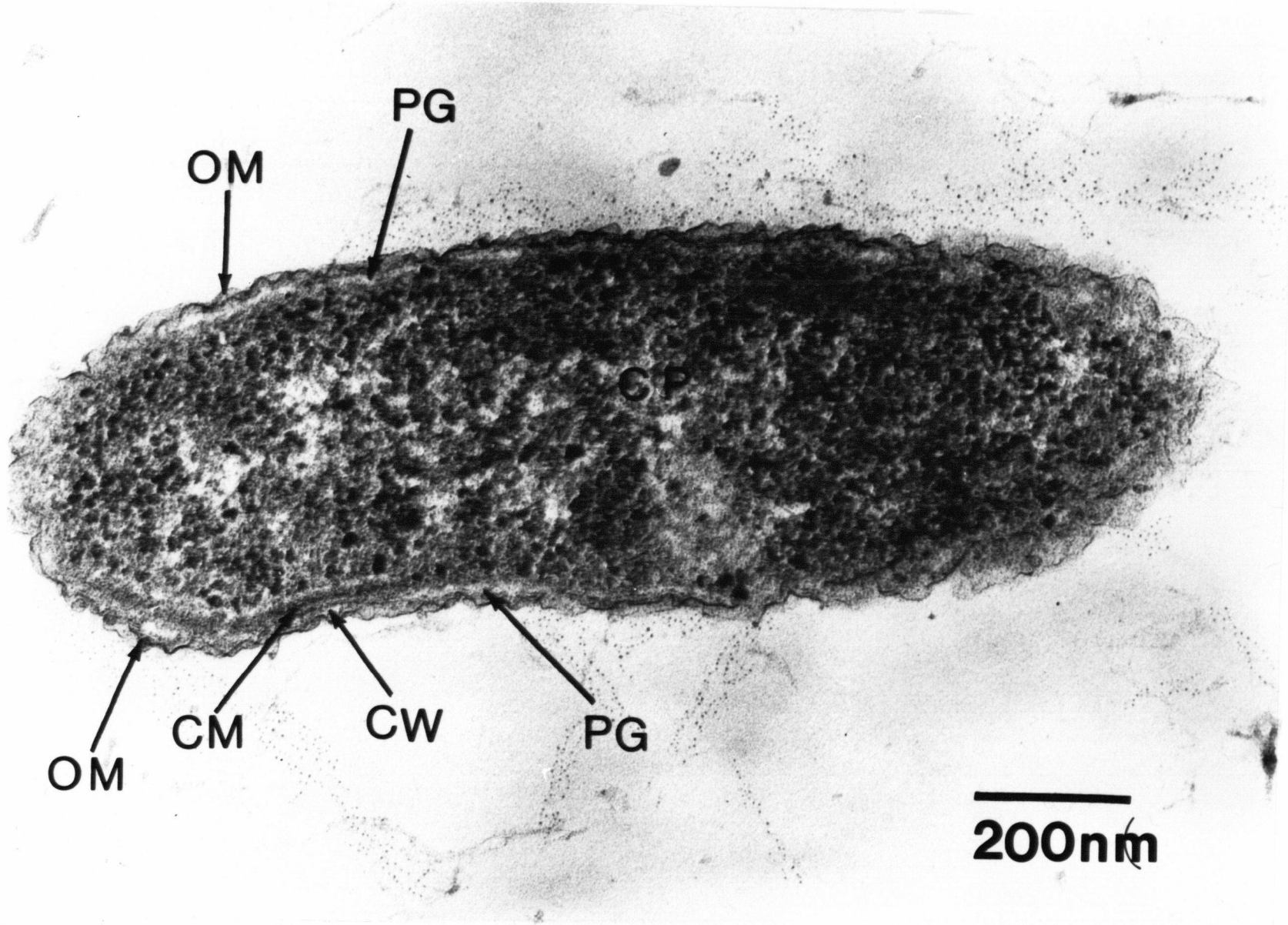
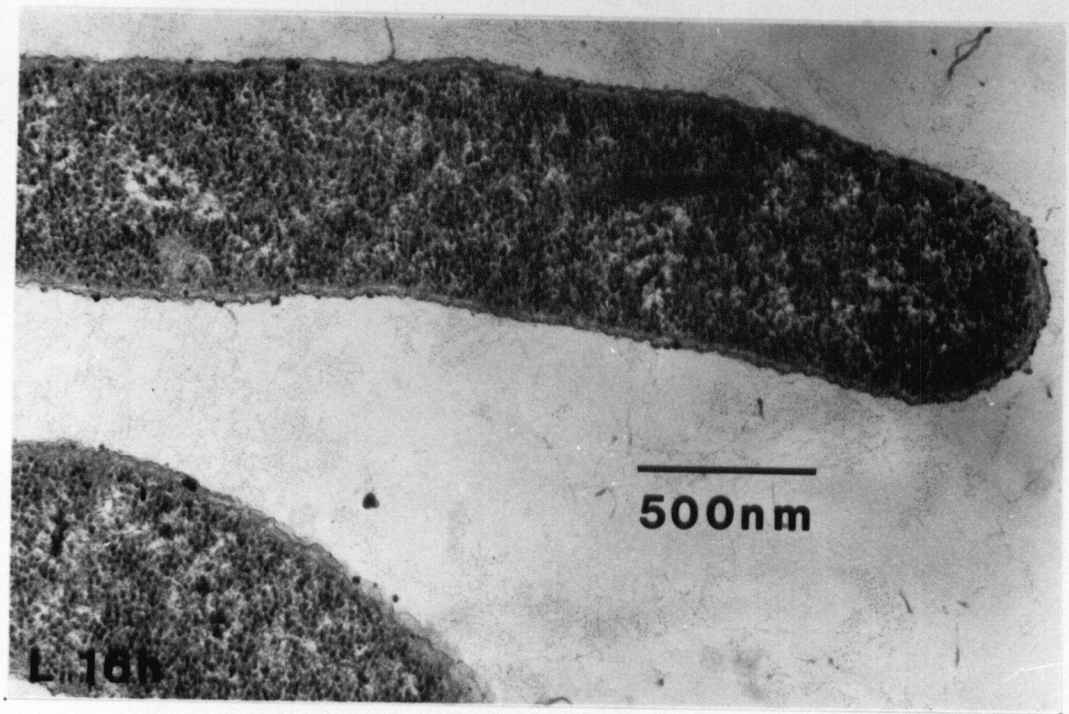
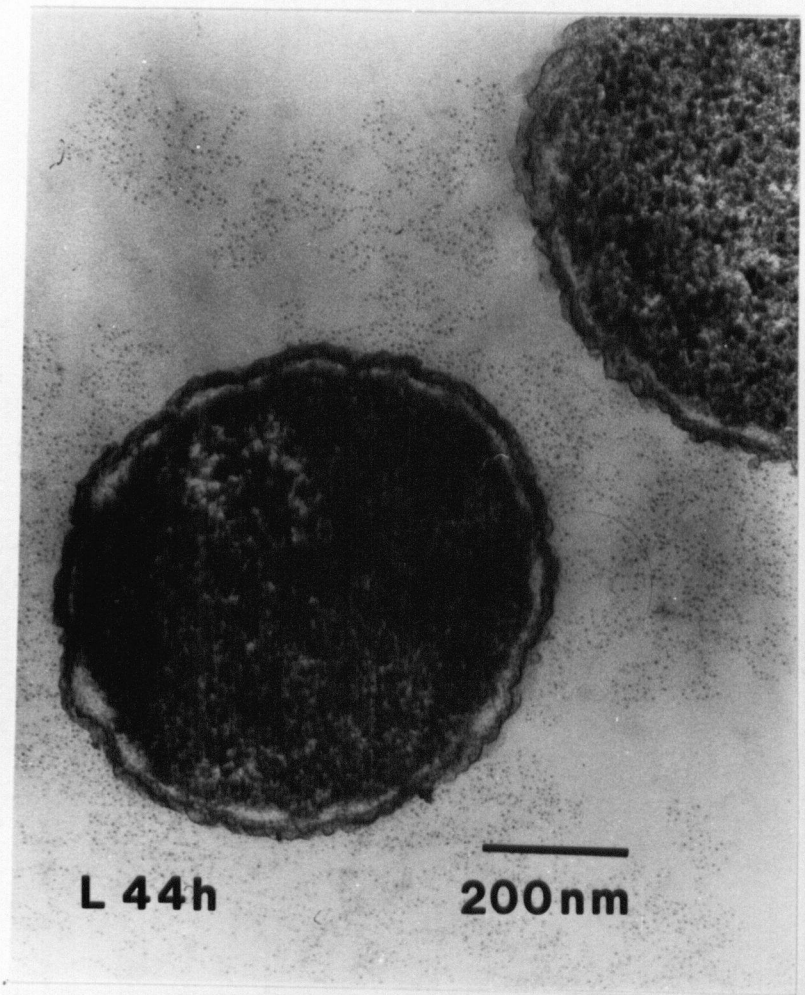

PG
OM
CP
OM
CM
CW
PG
200nm

FIGURE 16. Transmission electron micrograph of *P. fragi* grown in liquid medium for 16 hours.

FIGURE 17. Transmission electron micrograph of *P. fragi* grown in liquid medium for 44 hours.
Note thickening of the outer membrane and increase in periplasmic gap.


500nm
L 16h


L 44h
200nm

thickened. This gap widening and membrane thickening became extreme at 92 h (Figure 18).

Although the outer cell membrane was highly convoluted on cells grown in liquid medium, no evidence of extracellular vesicles or globule formation could be seen.

Transmission electron micrographs of *P. fragi* grown on a solid surface for 4 h (Figure 19), again showed the periplasmic gap separating the outer cell membrane from the cytoplasmic membrane-cell wall structures. At 20 h (Figure 20) most cells were surrounded by many round vesicles more or less bound to the cell surface. All the vesicles, between 30 and 40 nm in diameter, appeared to originate from the outer cell membrane. In some instances the vesicles appeared to be attached to the cell by a stalk. The individual vesicles, when examined in section, were surrounded with a membrane similar in appearance to that of the outer cell membrane surrounding intact cells. Those cells actively shedding particles appeared to have intact cell wall-cytoplasmic membrane structures. The cell wall did not appear to contribute to vesicle formation. The vesicles began appearing between 4 and 20 h. The vesicles continued to be present on the cell surfaces at 32 h (Figure 21) and at 56 h (Figure 22).

In a similar fashion to *P. fragi* grown in liquid medium, the outer cell membrane of cells grown on a solid surface thickened as the culture aged.

FIGURE 18. Transmission electron micrograph of *P. fragi* grown in liquid medium for 92 hours.
Note continued thickening of the outer membrane and increased periplasmic gap widening. The cytoplasm seems to contain voids or inclusions not seen in earlier cells.

250nm
L 92h

FIGURE 19. Transmission electron micrograph of *P. fragi* grown on solid medium for 4 hours.
Note the absence of extracellular vesicles.

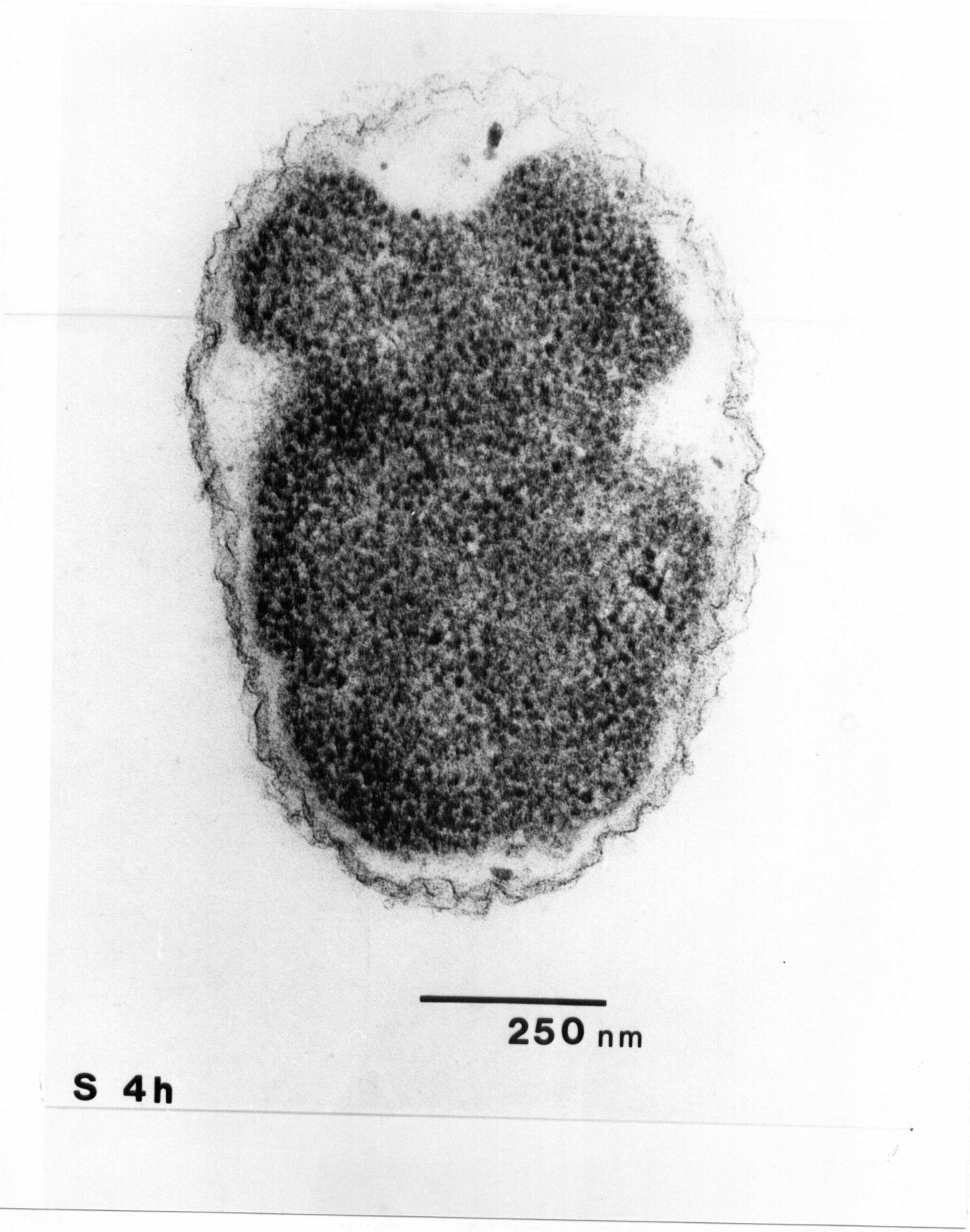

250 nm
S 4h

FIGURE 20. Transmission electron micrograph of *P. fragi* grown on solid medium for 20 hours showing presence of vesicles.
First observed occurrence of extracellular vesicles on cell surfaces.

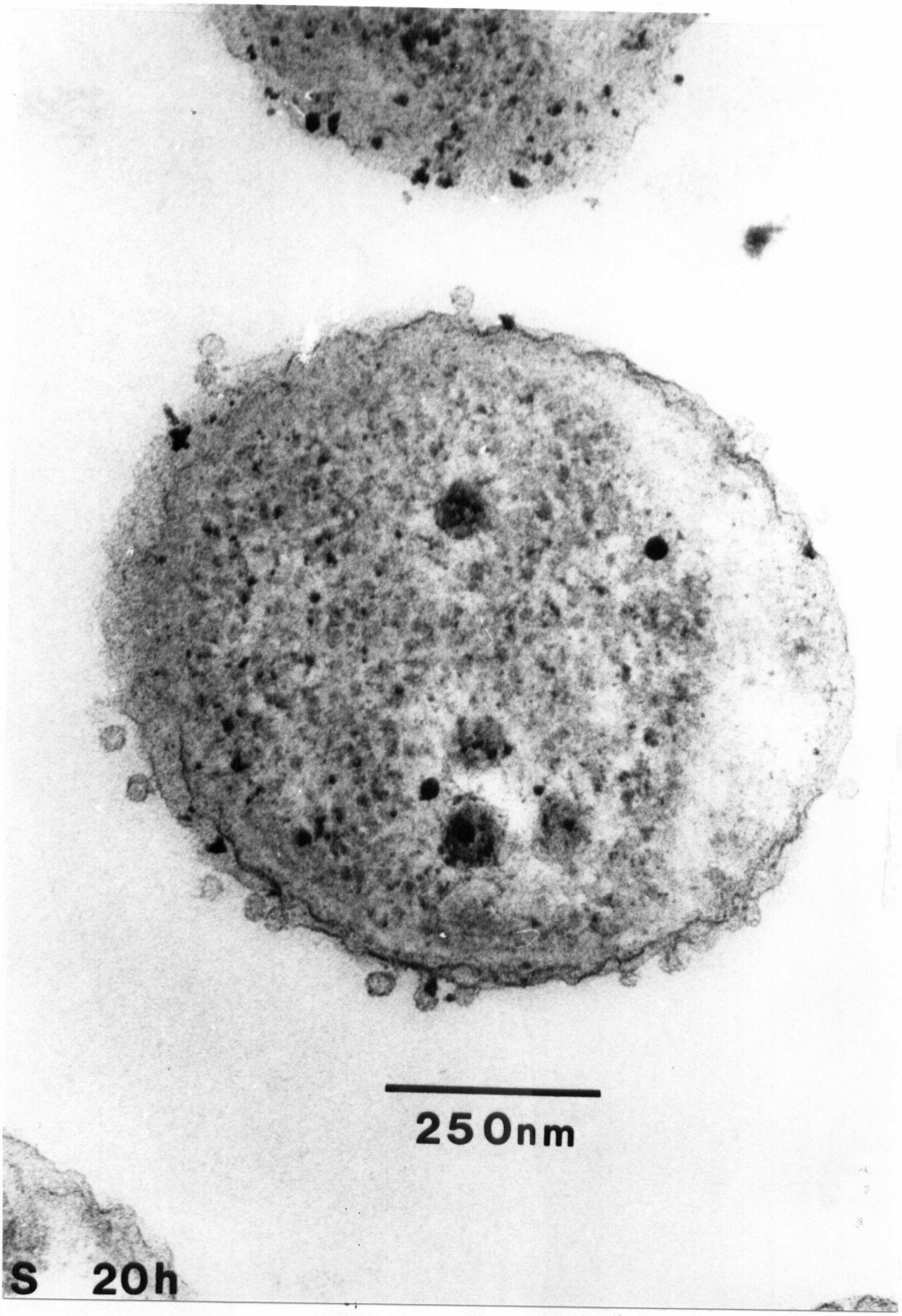

250nm
S 20h

FIGURE 21. Transmission electron micrograph of *P. fragi* grown on solid medium for 32 hours.
Vesicles continued to be present on the cell surfaces.

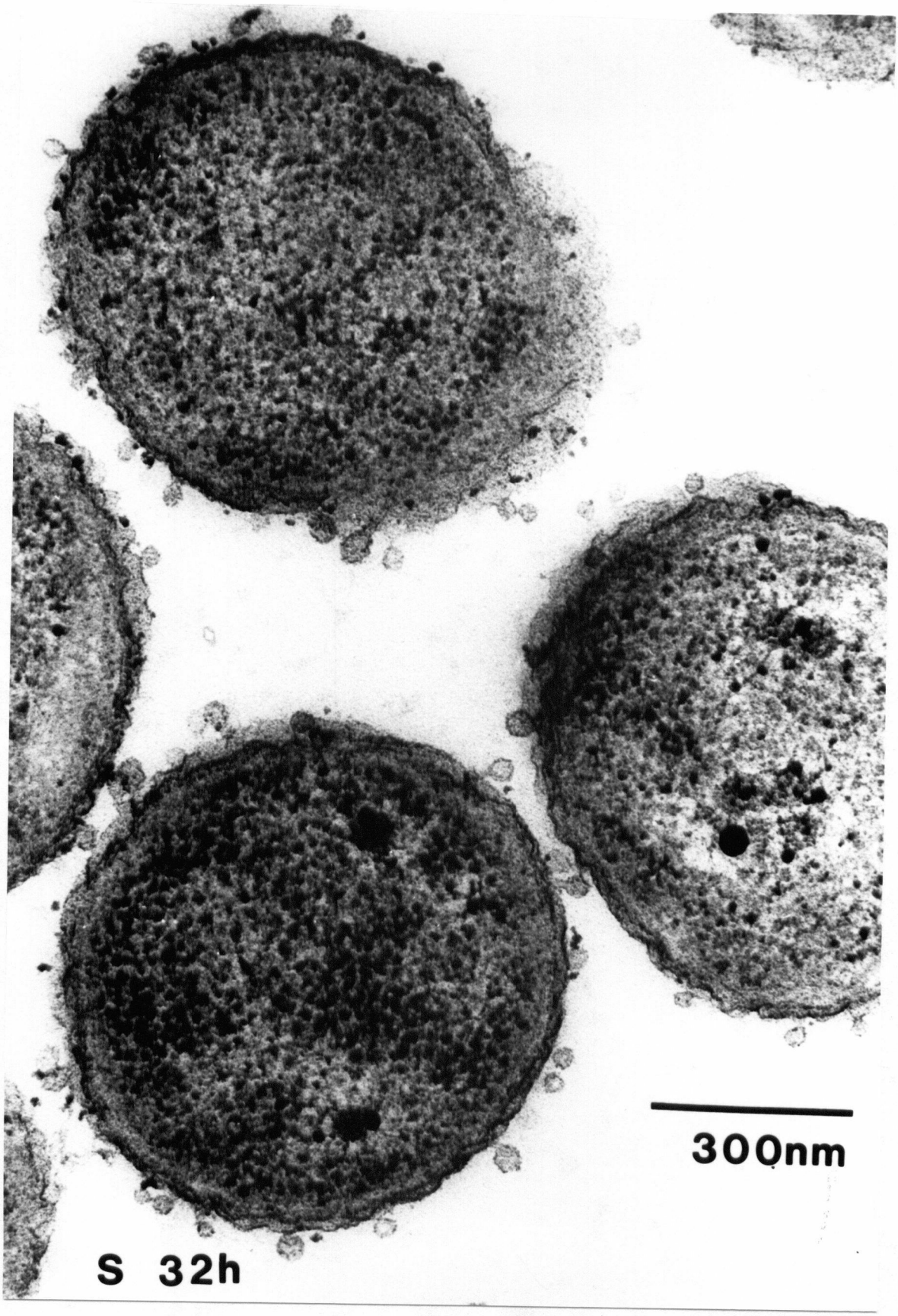
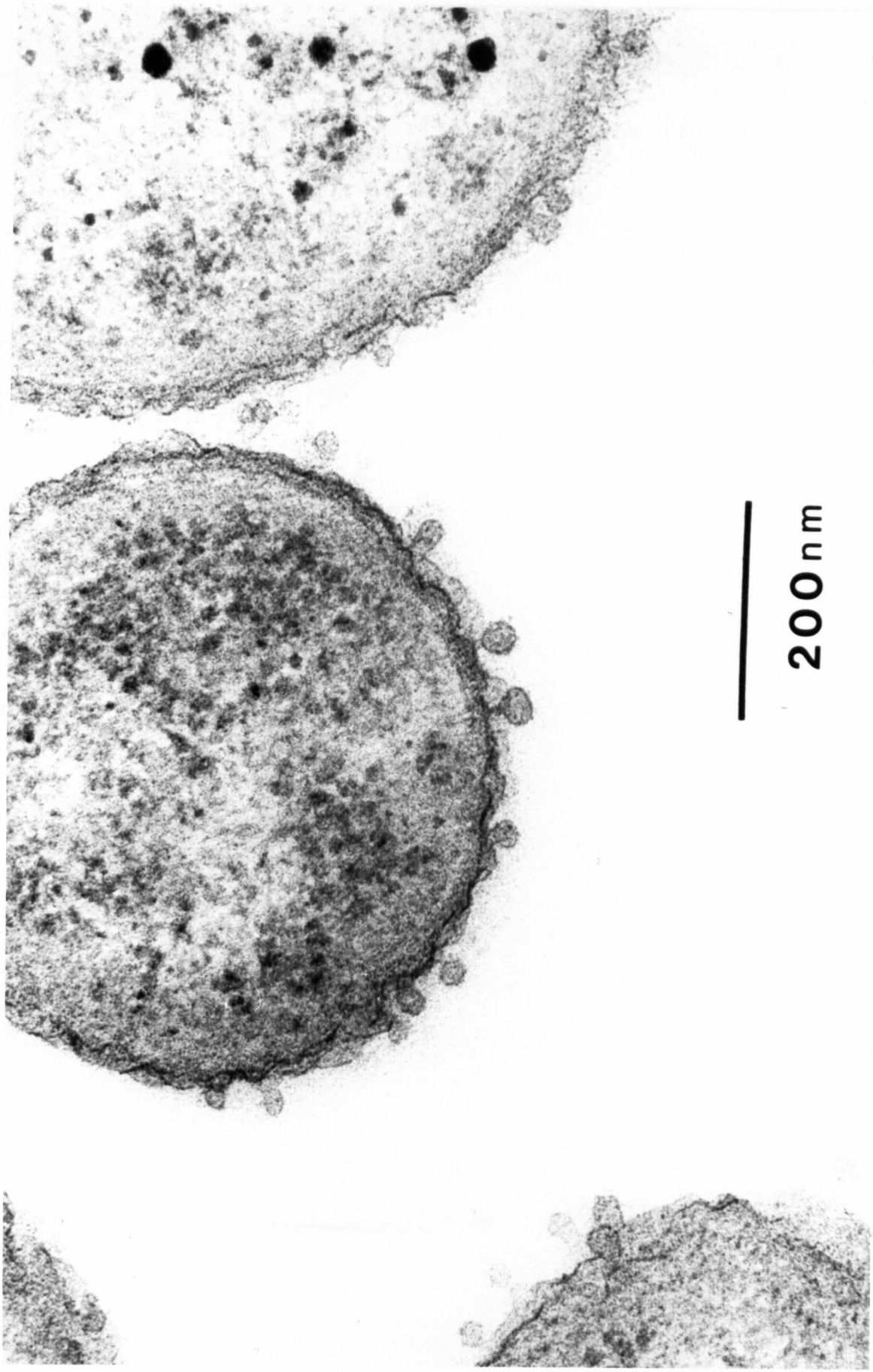

300nm
S 32h

FIGURE 22. Transmission electron micrograph of *P. fragi* grown on solid medium for 56 hours.
Maximum numbers of vesicles per unit perimeter of cells.


200nm

Vesicle shedding versus proteinase production by *P. fragi*

SEM of *P. fragi* cells grown in liquid medium revealed globules (Figure 11) 100 to 200 nm in diameter. These globules were visible only after 76 h, the approximate time period when proteinase production was at a maximum (Figure 7b).

Transmission electron micrographs of cells grown in liquid medium failed to display evidence of vesicle shedding at any of the time periods examined.

SEM revealed globules, approximately 40 to 190 nm in diameter, on *P. fragi* cells grown for 24 h on solid medium (Figure 13). This time period coincided with increased proteinase production by cells grown on solid medium (Figure 8b).

TEM revealed that extracellular vesicles appeared at 20 h (Figure 20). This point in time corresponded with a dramatic increase in total proteinase (Figure 8 b). A count of the extracellular vesicles per unit perimeter of cells grown on solid medium further supported the association of vesicle appearance and proteinase production (Figure 8c).

CELL AND GROWTH MEDIUM FRACTIONATION

Isolation of extracellular vesicles

Pseudomonas fragi was grown to the stationary growth phase in liquid culture (82 h at 21 C) and the culture supernatant collected. Ammonium sulfate precipitation and

subsequent dialysis of duplicates isolates yielded a mean of 11.55 ± 1.60 mg (mean of duplicate isolations ± one half the range) of particles per litre of culture supernatant. The particles were not artifacts, since uninoculated culture medium did not yield such particles.

After ammonium sulfate precipitation and subsequent dialysis, the particles readily associated together producing a loosely packed, black coloured precipitate which formed after less than one minute. This tendency to flocculate made particle separation by low speed centrifugation from the suspending menstrum quite easy. The black precipitate was easily resuspended by shaking.

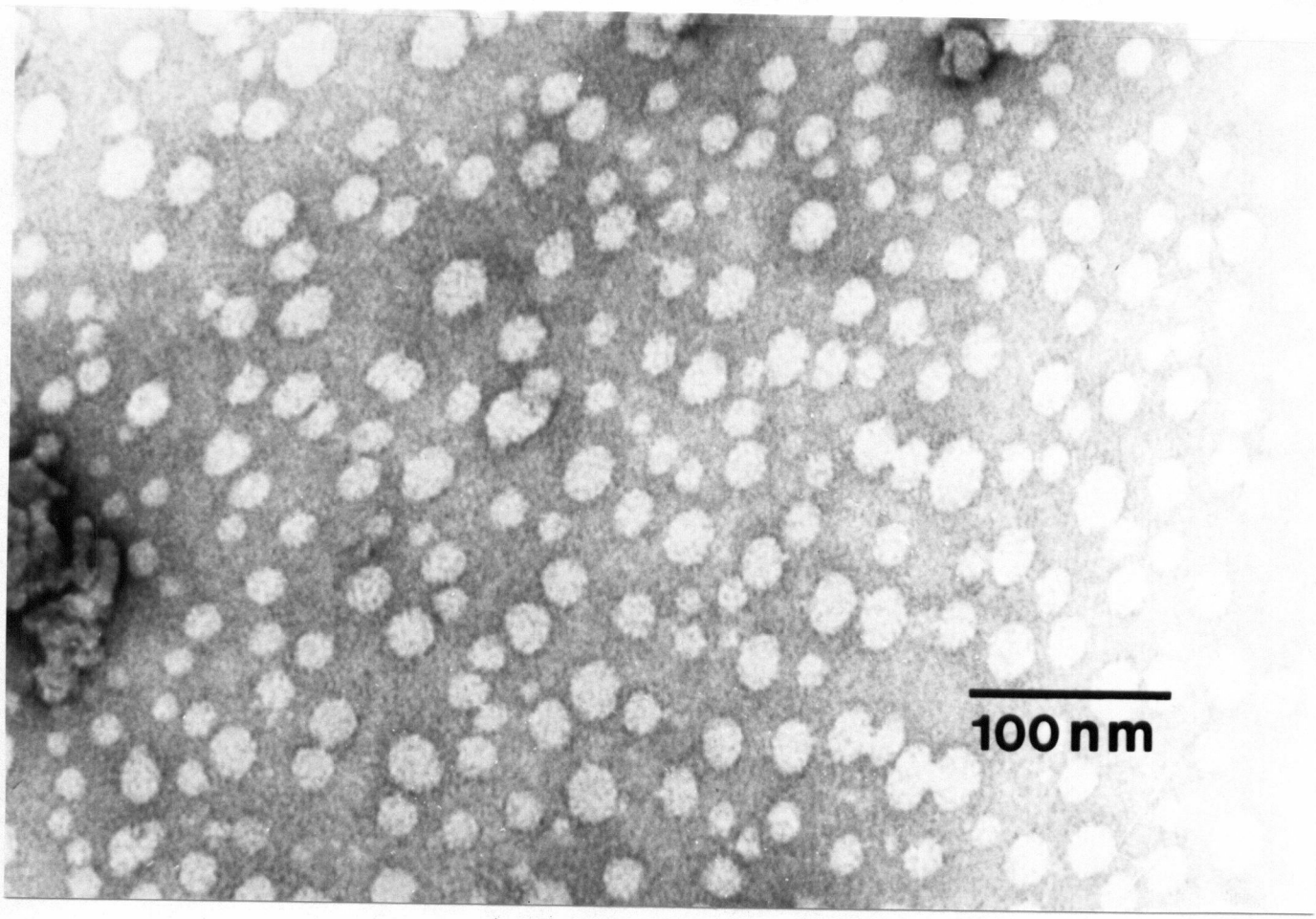
Electron microscopy

Examination of the vesicle preparation mounted and stained on Formvar coated grids (Figure 23) revealed the presence of a myriad of spherical vesicles ca. 20 nm in diameter. These vesicles were the same size as those particles observed on the surface of *P. fragi*, grown on solid medium, when the sectioned cells were examined by TEM (Figure 21). All of the vesicles appeared to be consistent in size. No evidence of pili could be seen.

Outer-cell membrane preparation

Outer-cell membrane material was isolated by selective solubilization of DNA and cytoplasmic membrane. High speed centrifugation yielded a jelly-like pellet. The outer cell membrane material contained approximately 5.91 ± 0.83 mg protein/mL.

FIGURE 23. Transmission electron micrograph of negatively stained isolated extracellular vesicles.
Extracellular vesicles were isolated by ammonium sulfate precipitation of culture supernatant. *P. fragi* was grown at 21 C. Washed vesicles were placed on the surface of Formvar coated E.M. grids and stained with uranyl acetate. Vesicles were approximately 20 nm in diameter.


100 nm

Buoyant density

The buoyant density of the extracellular vesicles was 1.153 g/mL.

Isoelectric focusing (IEF)

Extracellular vesicles and supernatant

Approximately 30 µL of supernatant, and 50 µL of untreated extracellular vesicles, extracellular vesicles solubilized by Triton X-100 and extracellular vesicles solubilized by sonication were separated by IEF. Several components with isoelectric points between pH 4.0 and 6.0 were resolved with Coomassie Blue stain (Figure 24). The supernatant produced the most bands in the gel, followed by the sonicated vesicles, the Triton X-100 treated vesicles and the untreated vesicles. A casein-agar gel was overlaid on a duplicate IEF gel. Approximately 60 µL of supernatant and 50 µL of each of the three extracellular vesicle preparations were applied. Visualization with TCA, sulphosalicylic acid fixative, revealed a region of hydrolysis at a point equivalent to pH 5.2 on the IEF gel, which corresponded to a major band on all four samples (Figure 25).

Outer-cell membrane/extracellular vesicle component profiles

Electrophoresis, on SDS-PAG, of the extracellular vesicles and the isolated *P. fragi* outer membrane (Figure 26) revealed several protein bands with molecular weights

FIGURE 24. IEF separation of culture supernatant and extracellular vesicle proteins.
Proteins were separated on 5.0% polyacrylamide gel containing pH 4-6 ampholyte. Bands were visualized with Coomassie Blue protein stain. (A) Culture supernatant. Approximately 448 µg of protein was applied to the gel; (B) Untreated extracellular vesicles. Approximately 225 µg of protein was applied to the gel; (C) Extracellular vesicles solubilized in 2.0% Triton X-100. Approximately 225 µg of protein was applied to the gel; (D) Extracellular vesicles solubilized by sonication. Approximately 225 µg of protein was applied to the gel; (E) Stained protein bands with the same pI and possessing proteinase activity.

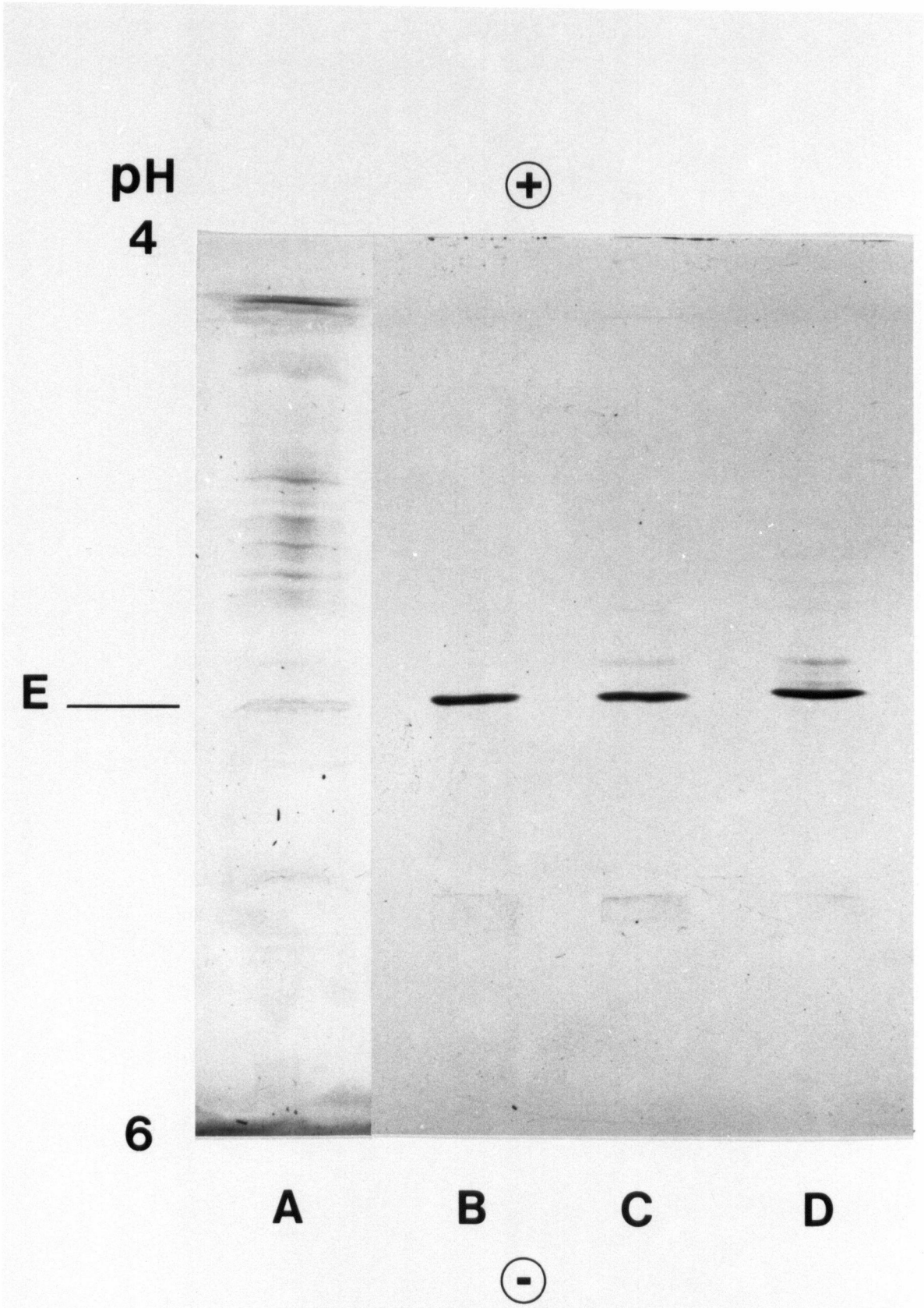

pH
4
⊕
E
6
A
B
C
D
⊖

FIGURE 25. Zymogram of IEF separated culture supernatant and extracellular vesicles.
Proteins were separated on 5.0% polyacrylamide gel containing pH 4-6 ampholyte. Areas containing active proteinase were visualized by overlaying the IEF gel with a casein-agar gel and incubating for 24 h at 25 C. Gel was developed with a TCA/sulphosalicylic acid fixative. The zymogram was backlit and photographed against a black background. Due to the fixative, the casein was opaque and appeared grey, while the translucent hydrolyzed areas around pH 5.2 (indicated by an E) revealed the black background. (A) Untreated extracellular vesicles. Approximately 225 µg of protein was applied to the gel; (B) Extracellular vesicles solubilized in 2.0% Triton X-100. Approximately 225 µg of protein was applied to the gel; (C) Extracellular vesicles solubilized by sonication. Approximately 225 µg of protein was applied to the gel; (D) Culture supernatant. Approximately 900 µg of protein was applied to the gel.

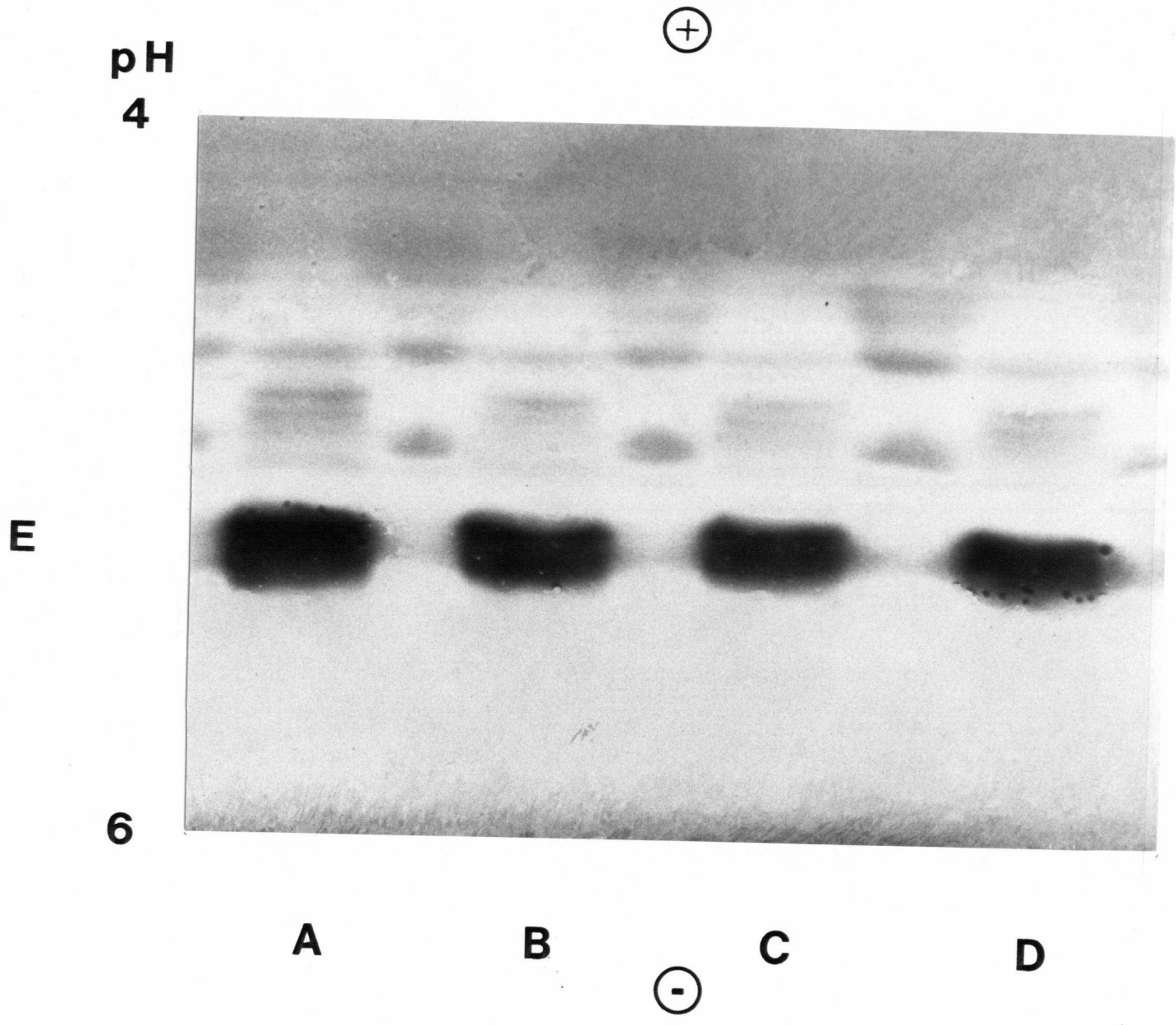
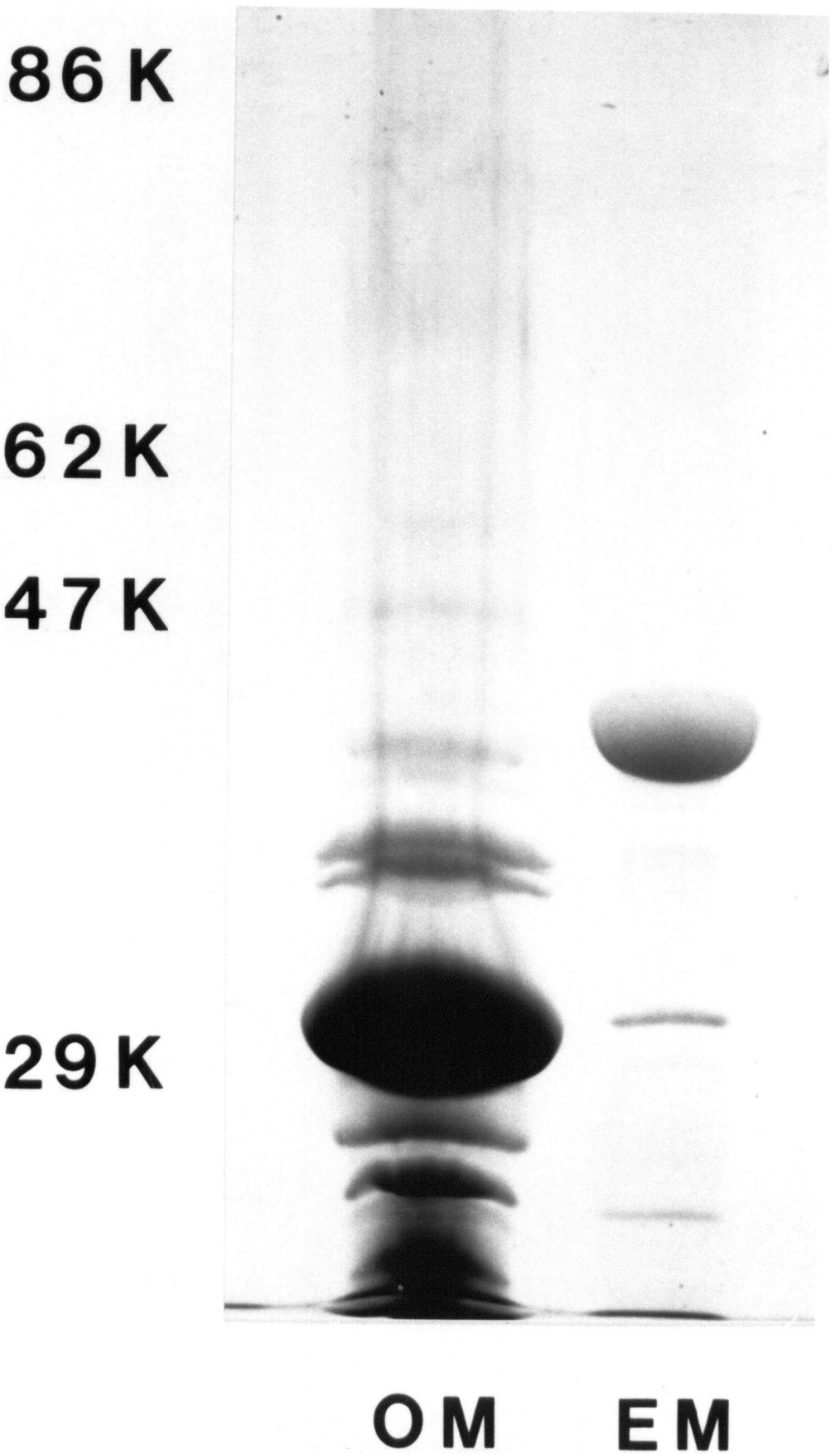

pH
4
6
E
+
-
A
B
C
D

FIGURE 26. SDS-PAGE separation of extracellular vesicle and outer cell membrane proteins.
Proteins were separated on a 7.5% acrylamide gel. Stacking gel consisted of 4.0% acrylamide. Samples were solubilized in a sample buffer containing 2.0% SDS and 5.0% 2-mercaptoethanol. (EM) extracellular vesicle material. Approximately 225 µg of protein was applied to the gel; (OM) *P. fragi* outer cell membrane, approximately 216 µg of protein was applied to the gel.


86K
62K
47K
29K
OM
EM

between 20,000 and 86,000 daltons. The outer membrane contained about 9 major bands and some minor bands.

The major bands occurred between 20,000 and 47,000 D. The extracellular vesicles showed four major bands and several minor bands. A comparison of the electrophoretic bands showed the small bands of the extracellular vesicles corresponded to major bands in the isolated outer cell membrane material. The extracellular vesicles may, at least in part, be made up of outer cell membrane material.

Extracellular vesicle/outer cell membrane enzyme localization.

Localization of the active proteinase was undertaken in normal SDS-polyacrylamide gel or SDS-polyacrylamide gel bound covalently by glutaraldehyde to casein. Subsequent to electrophoresis, regeneration of enzyme activity was attempted in the presence of Ca^{++} ion after removal of SDS with a non-ionic detergent wash. The regenerated enzyme was then free to hydrolyze the casein: in the case of normal SDS-polyacrylamide gel, present in a casein-agar gel overlay; in the case of the covalently bound gel, found in the casein-polyacrylamide gel itself. For electrophoresis, samples were prepared without mercaptoethanol or heat. In both instances, enzyme could not be localized despite several attempts to rejuvenate the enzyme, including extensive washings to eliminate SDS.

Composition of the components

The culture supernatant contained 34.71 enzyme units/mL (Table IV). The protein content was 0.31 mg/mL. The specific activity of the supernatant was 111.97 enzyme units/mg protein. The total amount of proteinase in one L was 34,710.00 enzyme units.

The extracellular vesicles contained 51.92 enzyme units/mL. The protein content was 0.45 mg/mL. The specific activity of the vesicles was 115.38 enzyme units/mg protein. The total amount of proteinase present in the vesicles associated with one L of *P. fragi* culture was 103.80 enzyme units.

The soluble cell material contained 16.32 enzyme units/mL. The protein content of the material was 11.71 mg/mL. The specific activity of the soluble material was 1.39 enzyme units/mg protein. The total amount of proteinase present in cells from one L was 244.80 enzyme units.

A freeze-dried extracellular vesicle preparation contained 11.7% (dry weight) protein. After sonic disruption of the original particle suspension, the soluble portion of the resultant mixture contained 1.7% (dry weight) protein.

The proteinase content of the sonicated vesicles was 58.52 enzyme units/mL. The protein content of the soluble material left after sonication was 0.09 mg/mL.

Table IV. Composition of vesicles, soluble cell material and supernatant.

ANALYSIS	VESICLES	VESICLES SONICATED	SOLUBLE CELL MATERIAL	SUPERNATANT
PROTEINASE[A]	51.92±2.40	58.52±1.12	16.32±1.80	34.71±2.82
PROTEIN[B]	0.45±0.05	0.09±0.00	11.71±0.46	0.31±0.03
SPECIFIC ACTIVITY[C]	115.38	680.50	1.39	111.97
TOTAL PROTEINASE	103.80	117.00	244.80	34710.00
DRY WEIGHT[D]	8.56±1.15			
CARBOHYDRATE[D]	1.04±0.04			
DRY WEIGHT[E]	11.55±1.60			

Determinations are the result of duplicate experiments, grown in one L quantities for 82h at 21 C.
Results are means of duplicate experiments ± one half the range.
[A] Enzyme units/mL.
[B] Milligrams/mL.
[C] Enzyme units/mg protein.
[D] Milligrams/milligram protein.
[E] Milligrams.

The specific activity of the crude vesicle preparation was 115.38 enzyme units/mg protein while that of the sonicated preparation was 680.50 enzyme units/mg protein.

Extracellular vesicles contained 1.04 mg carbohydrate/mg protein (Table IV).

BACTERIOCINS

Extracellular vesicles

The electron micrographs of *P. fragi* grown to the stationary growth phase (48 h) on TSB + agar revealed spherical membrane vesicles ca. 20 nm in diameter. These vesicles, bound to the cell surface, or free in the culture medium, bore some resemblance to bacteriocin particles produced by *B. uniformis* and described by Austin-Prather and Booth (1984). Supernatant, containing particles produced by *P. fragi* and described in this study, failed to elicit zones of inhibition when applied to TSA plates containing indicator organisms. In a like fashion, it did not alter the growth rate of indicator organisms as measured by absorbance over a 24 h period.

Bacteriocin induction.

Pseudomonas fragi ATCC 4973 also failed to show bacteriocidal activity towards the indicator organisms despite attempts to force bacteriocin production with U.V. or gamma ionizing radiation, or mitomycin C inducing agents.

Culture supernatant from *P. fragi* ATCC 4973 grown in TSB for 48 h, when treated with 20.0 µg/mL mitomycin C

displayed clear zones on TSA plates containing indicator organisms and altered the growth rate of indicator organisms, as measured by absorbance over time. This inhibitory effect was due entirely to mitomycin C, and not bacteriocin, since controls consisting of uninoculated culture medium containing 20 µg/mL mitomycin C displayed identical inhibitory properties. When *P. fragi* culture supernatants were dialyzed to remove mitomycin C they lost their ability to alter growth rates of the indicator organisms.

OPTIMIZATION

Optimization of proteinase production

Fractional factorial

Prior to optimization, a Taguchi (1957) fractional factorial design experiment was conducted to determine the importance of each factor. The experiments shown in Table V were evaluated. Analysis of the growth data in Table VIa revealed that all the factors significantly affected growth of *P. fragi* ($P<.01$). Time was the most significant factor, followed by oxygen, glutamine, temperature and pH. Replicates effects were not significant ($P>.05$) (Table VIb).

Analysis of proteinase response data (Table VIIa) revealed that oxygen level, glutamine concentration, incubation temperature and time were all significant factors ($P<.01$). Initial pH was significant at the 5% confidence level. Oxygen was the most significant factor. In general higher oxygen levels (>10%, or 4.2ppm) favored greater

TABLE V. Fractional factorial experiments

EXP #	TEMP (C)	TIME (h)	pH	GLUT[3] (mmole)	OXYGEN (%)	OXYGEN (ppm)	PROTEINASE (units/mL)	*P.fragi* (cfu/mL)
T1[2]	20	32	7	7	10	4.2[1]	5.2 (0.0)*	$6.9X10^8$
T2	20	32	9	125	30	12.9	6.2 (1.0)	$3.8X10^9$
T3	20	72	7	7	30	12.9	6.8 (0.8)	$1.1X10^8$
T4	20	72	9	125	10	4.2	3.0 (0.0)	$7.7X10^7$
T5	30	32	7	125	30	10.7	5.1 (1.9)	$3.2X10^9$
T6	30	32	9	7	10	3.6	2.6 (0.5)	$1.4X10^9$
T7	30	72	7	125	10	3.6	1.9 (0.3)	$2.7X10^7$
T8	30	72	9	7	30	10.7	6.9 (0.3)	$4.1X10^5$

(1) Parts per million of dissolved oxygen contained in the growth medium.
(2) "T" experiments refer to Taguchi experiments and are synonymous with fractional factorial experiments.
(3) GLUT = glutamine (mmole nitrogen / L).
* Standard deviation. Figures are the results of 4 experiments.

TABLE VIa. Analysis of fractional factorial growth data variance (Factors).

Source of variation	ss	df	ms	F	signif.
temp C.	1.26×10^{18}	1	1.26×10^{18}	163.85	**
time	2.80×10^{19}	1	2.80×10^{19}	3641.09	**
pH	2.82×10^{17}	1	2.82×10^{17}	36.67	**
glutamine	1.97×10^{19}	1	1.97×10^{19}	2561.77	**
oxygen %	1.98×10^{19}	1	1.98×10^{19}	2574.77	**
error	2.00×10^{17}	26	7.69×10^{15}		
total	6.92×10^{19}	31			

(**) P<.01

TABLE VIb. Analysis of fractional factorial growth data variance (Replicates).

Source of variation	ss	df	ms	F	signif.
Experiments	6.84×10^{19}	7	9.77×10^{18}	1570.74	**
Replicates	4.97×10^{16}	8	6.22×10^{15}	0.15	ns
error	6.86×10^{17}	16	4.29×10^{16}		
total	6.91×10^{19}	31			

(**) P<.01
(ns) not significant P>.05
F statistic calculation for experiments = $ms_{(experiments)}$ / $ms_{(replicates)}$

TABLE VIIa. Analysis of fractional factorial proteinase data variance (Factors).

Source of variation	ss	df	ms	F	signif.
temp C.	15.68	1	15.68	50.58	**
time h	3.25	1	3.25	10.48	**
pH	2.18	1	2.18	7.03	*
glutamine	17.19	1	17.19	55.45	**
oxygen %	77.30	1	77.30	249.35	**
error	7.94	26	0.31		
total	123.54	31			

(**) P<.01
(*) P<.05

TABLE VIIb. Analysis of fractional factorial proteinase data variance (Replicates).

Source of variation	ss	df	ms	F	signif.
Experiments	107.34	7	15.33	109.50	**
Replicates	1.15	8	0.14	0.15	ns
error	15.05	16	0.94		
total	123.54	31			

(**) P<.01
(ns) not significant P>.05
F statistic calculation for experiments = $ms_{(experiments)}$ / $ms_{(replicates)}$

proteinase production. Replicate effects were not significant (P>.05) (Tables VIIb).

Centroid search optimization

The same factors used in the fractional factorial experiments were used in the centroid search procedure. The response factor was again enzyme units/mL. The experiments (Table VIIIa) were evaluated for proteinase production and cell numbers. The best response (7.7 enzyme units/mL) occurred with experiment number S3. This experiment also supported the best cell growth. The next best response was experiment S4 (5.4 enzyme units/mL), while experiment S1 was the worst (0.0 enzyme units/mL).

From the results of the six Spendley experiments (Table VIIIa), six centroids were suggested. The experimental conditions for four centroids were evaluated and the response values are shown in Table IXa. Analysis of variance of the Spendley matrix experiments and centroid experiments (Table VIIIb and Table IXb), showed that both experimental units and replicates contributed significantly to the overall variance (P<.01).

Since the initial centroid response value (centroid C7) was greater than or equal to the best response value obtained from the starting Spendley matrix, evaluation of a majority of the remaining centroids was carried out. Centroid 11 with a response value of 8.4 enzyme units/mL was the highest result obtained from all experiments, including

TABLE VIIIa. Initial Spendley matrix experiments and response values.

EXP #	TEMP (C)	TIME (h)	pH	GLUT[B] (mmole)	OXYGEN (%)	OXYGEN (ppm)	PROTEINASE (units/mL)	*P.fragi* (cfu/mL)
S1	1.0	4	5.0	200	00.0	0.0	0.0 (0.0)*	$5.4X10^7$
S2	36.6	18	6.0	292	10.3	3.1	4.7 (1.1)	$2.0X10^7$
S3	9.0	66	6.0	292	10.3	5.4	7.7 (0.6)	$2.1X10^9$
S4	9.0	18	9.6	292	10.3	5.4	5.4 (0.0)	$3.6X10^7$
S5	9.0	18	6.0	610	10.3	5.4	4.0 (0.7)	$2.1X10^8$
S6	9.0	18	6.0	292	45.6	25.2	4.8 (0.4)	$3.5X10^8$
S7[A]	9.0	18	6.0	292	10.3	5.4	6.2 (0.4)	$3.4X10^8$

Mean value for the seven experiments 4.7. Standard error of the mean 2.2.

* Standard error of the mean. Figures are the result of 4 experiments.

[A] This experiment was an additional experiment not meant to be included in the optimization. It was included in the ANOVA only.

[B] GLUT = glutamine (mmole nitrogen / L).

TABLE VIIIb. Analysis of Spendley proteinase data variance (Replicates).

Source of variation	ss	df	ms	F	signif.
Experiments	138.00	6	23.00	16.79	**
Replicates	9.58	7	1.37	1957.14	**
error	0.01	14	$7.00x10^{-4}$		
total	147.59	27			

(**) P<.01

F statistic calculation for experiments = $ms_{(experiments)}$ / $ms_{(replicates)}$

TABLE IXa. Centroid experiments and their response values

EXP	TEMP	TIME	pH	GLUT[A]	OXYGEN		PROTEINASE	*P.fragi*
#	(C)	(h)		(mmole)	(%)	(ppm)	(units/mL)	(cfu/mL)
C7	14.5	27.6	6.7	356	17.3	8.1	7.7 (0.0)*	4.4X109
C8	15.6	29.5	6.9	305	18.7	8.8	5.0 (0.0)	4.4X109
C10	11.9	34.6	7.3	311	15.4	7.6	5.9 (0.5)	3.5X109
C11	12.5	37.9	6.8	314	16.4	7.4	8.4 (0.0)	3.2X109

Mean value for the four experiments 6.8. Standard error of the mean 1.4.
* Standard error of the mean. Figures are the result of 4 experiments.
A GLUT = glutamine (mmole nitrogen / L).

TABLE IXb. Analysis of centroid proteinase data variance (Replicates).

Source of variation	ss	df	ms	F	signif.
Experiments	30.39	3	10.13	42.21	**
Replicates	0.95	4	0.24	80.00	**
error	0.02	8	3.00x10-3		
total	31.36	15			

(**) P<.01
F statistic calculation for experiments = $ms_{(experiments)}$ / $ms_{(replicates)}$

those of the fractional factorial. The factor levels for centroid 11 are shown in Table X.

The fractional factorial experiments showed that oxygen concentration was the most significant factor. Response values at 30% (10.7-13.0 ppm) oxygen were higher than at 10% (3.1-5.4 ppm) (Table V). The Spendley experiments showed that at 45.6% (25.2 ppm) oxygen, response values were lower than those at 30% oxygen (Table VIIIa). The centroid experiments showed that 16.4% (7.4 ppm) oxygen had the highest response value overall (Table IXa). To test whether or not oxygen at this concentration was close to the optimum response value, oxygen levels were shifted to bracket this projected optimum point. These experimental conditions and the responses obtained are shown in Table XI. In general, raising or lowering the oxygen level away from 16.4% (7.4 ppm) oxygen resulted in lower proteinase production.

Mapping

Data points from the above experiments, mapped in Figures 27, 28 and 29, show the approximate values of the factors where the optimum response may exist. Broken lines represent curves manually added to estimate proposed directions determined by quadratic curve fitting. These curves could not be plotted because of limited map space. These maps indicate that the above values (i.e. centroid 11) are close to the maximum projected response in each factor.

TABLE X. Suggested optimum values for proteinase production by *P. fragi*

FACTORS	LEVELS
Temperature	12.5 C
Time	37.9 h
pH	6.8
Glutamine	314.0 mmole Nitrogen/L
Oxygen	16.4% (7.4 ppm)

TABLE XI. Simultaneous shift experiments and their response values.

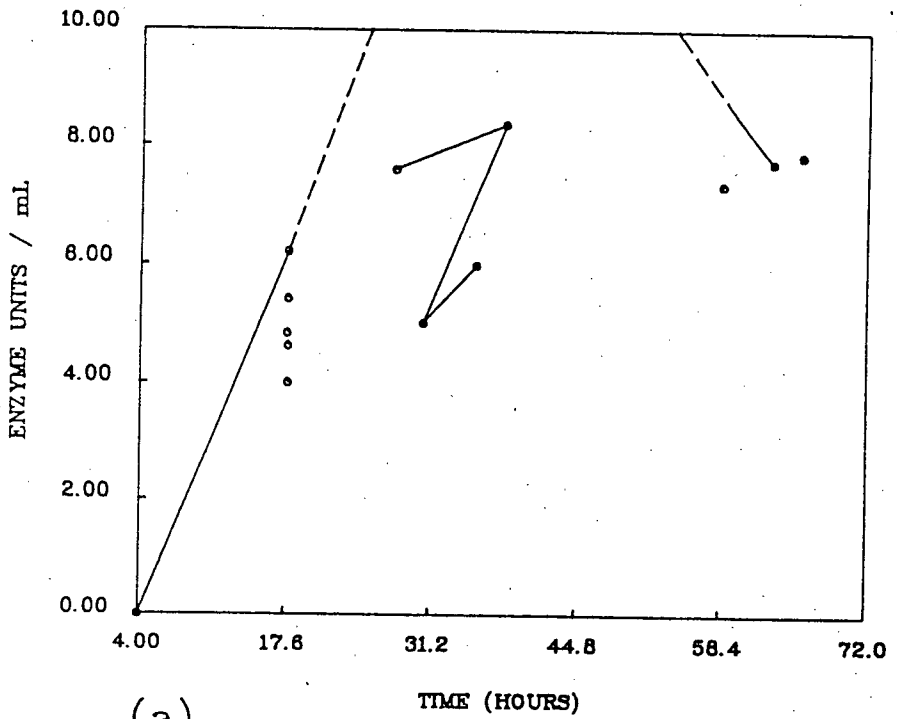
EXP #	TEMP (C)	TIME (h)	pH	GLUT[A] (mmole)	OXYGEN (%)	OXYGEN (ppm)	PROTEINASE (units/mL)	P.fragi (cfu/mL)
SS1	10.4	62	7	468	19.2	10.1	7.3 (0.5)*	$1.9X10^9$
SS2	8.0	68	7	468	10.2	5.5	7.9 (0.1)	$3.1X10^9$

[A] GLUT = glutamine (mmole nitrogen / L).
*Standard deviation. Figures are the result of 4 experiments.

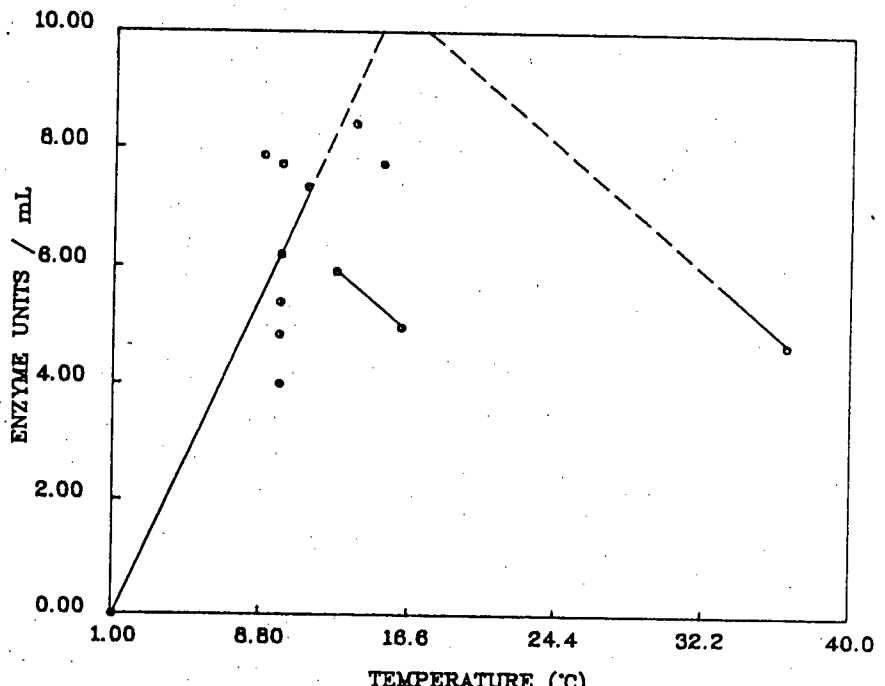
FIGURE 27. Optimization map of factors time and temperature.

(a) Optimum time appears ca. 45 h.

(b) Optimum temperature appears ca. 13 C.


10.00
8.00
6.00
4.00
2.00
0.00
ENZYME UNITS / mL
4.00
17.6
31.2
44.8
58.4
72.0
TIME (HOURS)

(a)

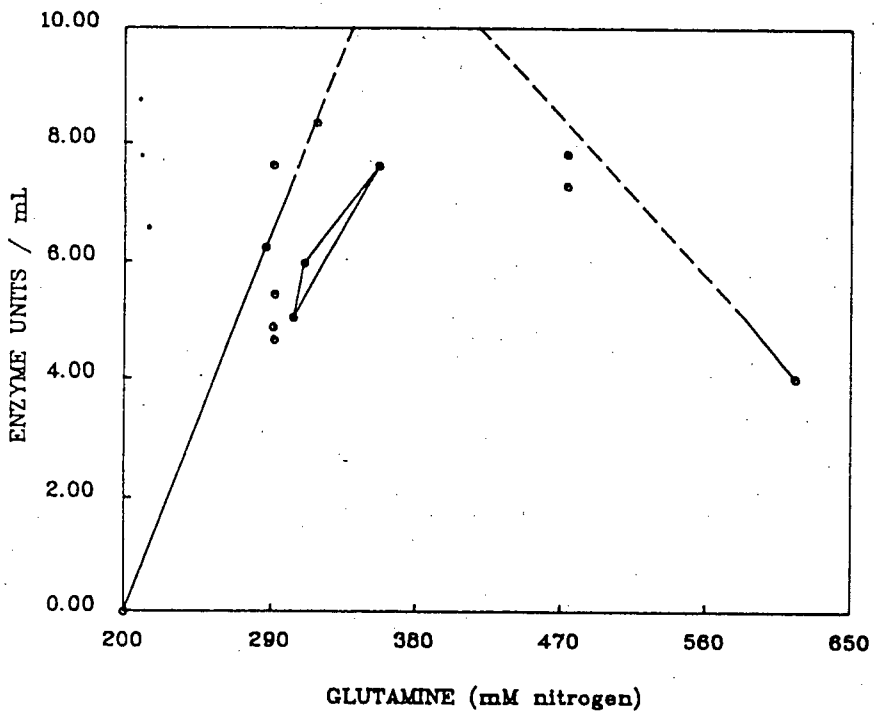

10.00
8.00
6.00
4.00
2.00
0.00
ENZYME UNITS / mL
1.00
8.80
16.6
24.4
32.2
40.0
TEMPERATURE (°C)

(b)

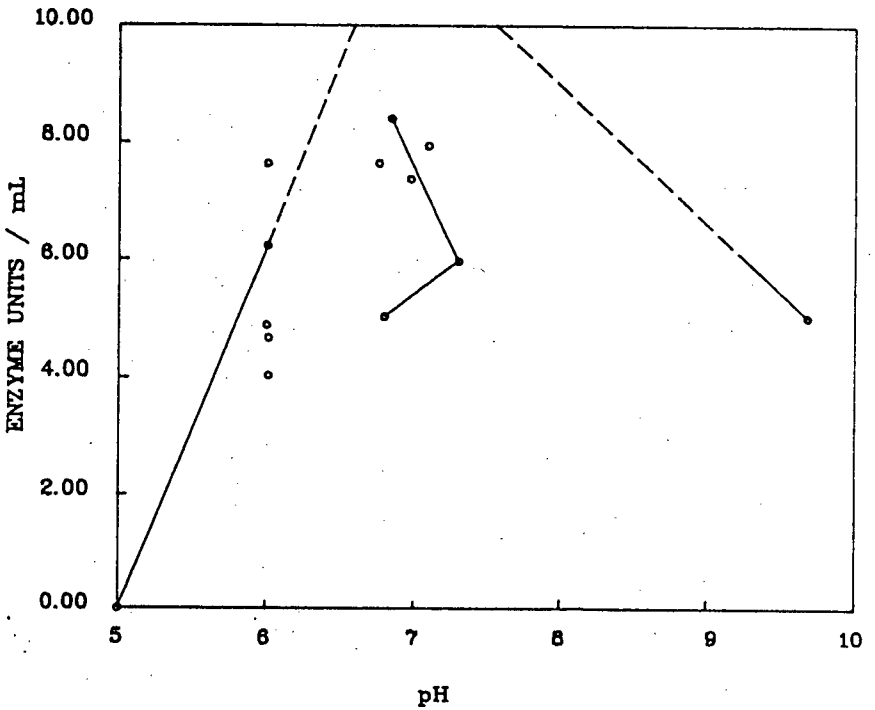
FIGURE 28. Optimization map of factors glutamine and pH.

(a) Optimum glutamine level appears ca. 300 mmole nitrogen/L.

(b) Optimum pH appears ca. pH 7.

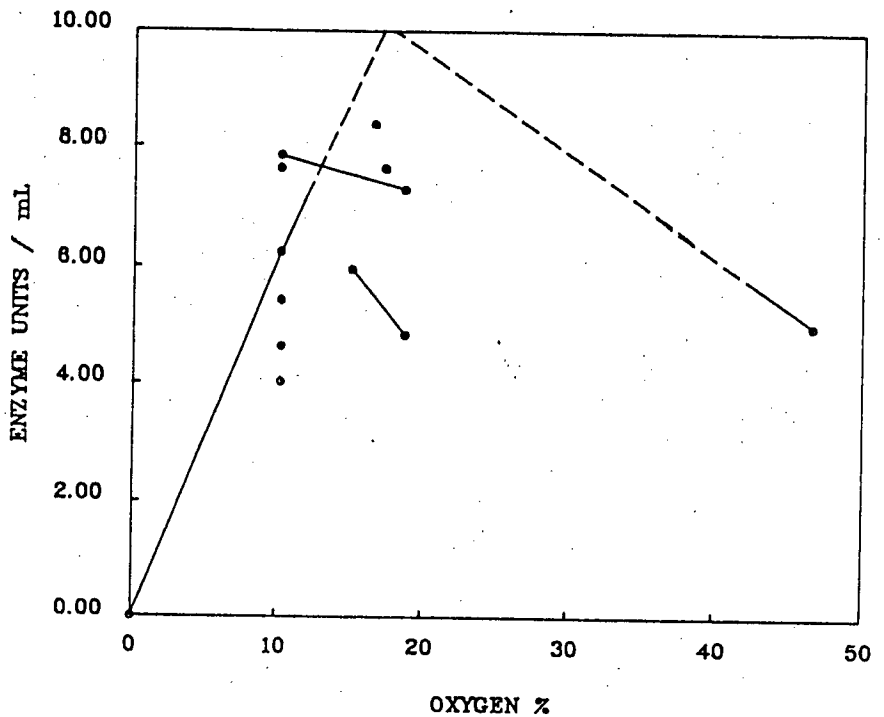

10.00
8.00
6.00
4.00
2.00
0.00
ENZYME UNITS / mL
200
290
380
470
560
650
GLUTAMINE (mM nitrogen)

(a)


10.00
8.00
6.00
4.00
2.00
0.00
ENZYME UNITS / mL
5
6
7
8
9
10
pH

(b)

FIGURE 29. Optimization map of factor oxygen.
Optimum oxygen level appears ca. 16.4% oxygen (7.4 ppm).


10.00
8.00
6.00
4.00
2.00
0.00
ENZYME UNITS / ml
0
10
20
30
40
50
OXYGEN %

As optimization continues, new factor ranges, as suggested by centroid search and mapping, will continue to decrease. The maps shown in Figures 27, 28 and 29 show data points clustering about a narrow range corresponding to the optimum point. Continuation of optimization would result in a further narrowing of the factor ranges. In particular, continuation of optimization would require a temperature factor range of less than one degree Celsius. This fine temperature control was not possible with the equipment available. As a result, a practical limit had been reached, and thus further optimization trials were not carried out.

Thin section transmission electron microscopy

A representative sample of *P. fragi* cells from the optimization trials, shown in Table XII, were selected for microscopic examination. Transmission electron micrographs (Figure 30a;b;c and Figure 31a;b) revealed the presence of extracellular vesicles on the surface of some cells grown in liquid culture.

At high temperatures cells were found both with (experiment T6, Figure 31a) and without (experiment T8, Figure 31b) extracellular vesicles on the cell surface. The same is true for *P. fragi* grown at low temperatures. Experiment S6 (Figure 30c) shows no vesicles attached to the cells, while experiments S5 and S3 (Figure 30b;a) show attached vesicles.

TABLE XII. Optimization trials selected for electron microscopy.

EXP	TEMP	TIME	pH	GLUT[A]	OXYGEN	
#	(C)	(h)		(mmole)	(%)	(ppm)
T8	30	72	9	7	30.0	10.7
T6	30	32	9	7	10.0	3.6
S5	9	18	6	610	10.3	5.4
S6	9	18	6	292	45.6	25.2
S3	9	66	6	292	10.3	5.4

[A] GLUT = glutamine (mmole nitrogen / L).

FIGURE 30. Transmission electron micrographs of *P. fragi* used in the optimization experiments.

(a) S3-Cells grown at 9 C; 66 h; pH 6; glutamine concentration 292 mmole nitrogen/L; 10.3% oxygen (5.4 ppm). Vesicles were present.

(b) S5-Cells grown at 9 C; 18 h; pH 6; glutamine concentration 610 mmole nitrogen/L; 10.3% oxygen levels (5.4 ppm). Vesicles were present.

(c) S6-Cells grown at 9 C; 18 h; pH 6; glutamine concentration 292 mmole nitrogen/L; 45.6% oxygen levels (25.2 ppm). Vesicles were not present.

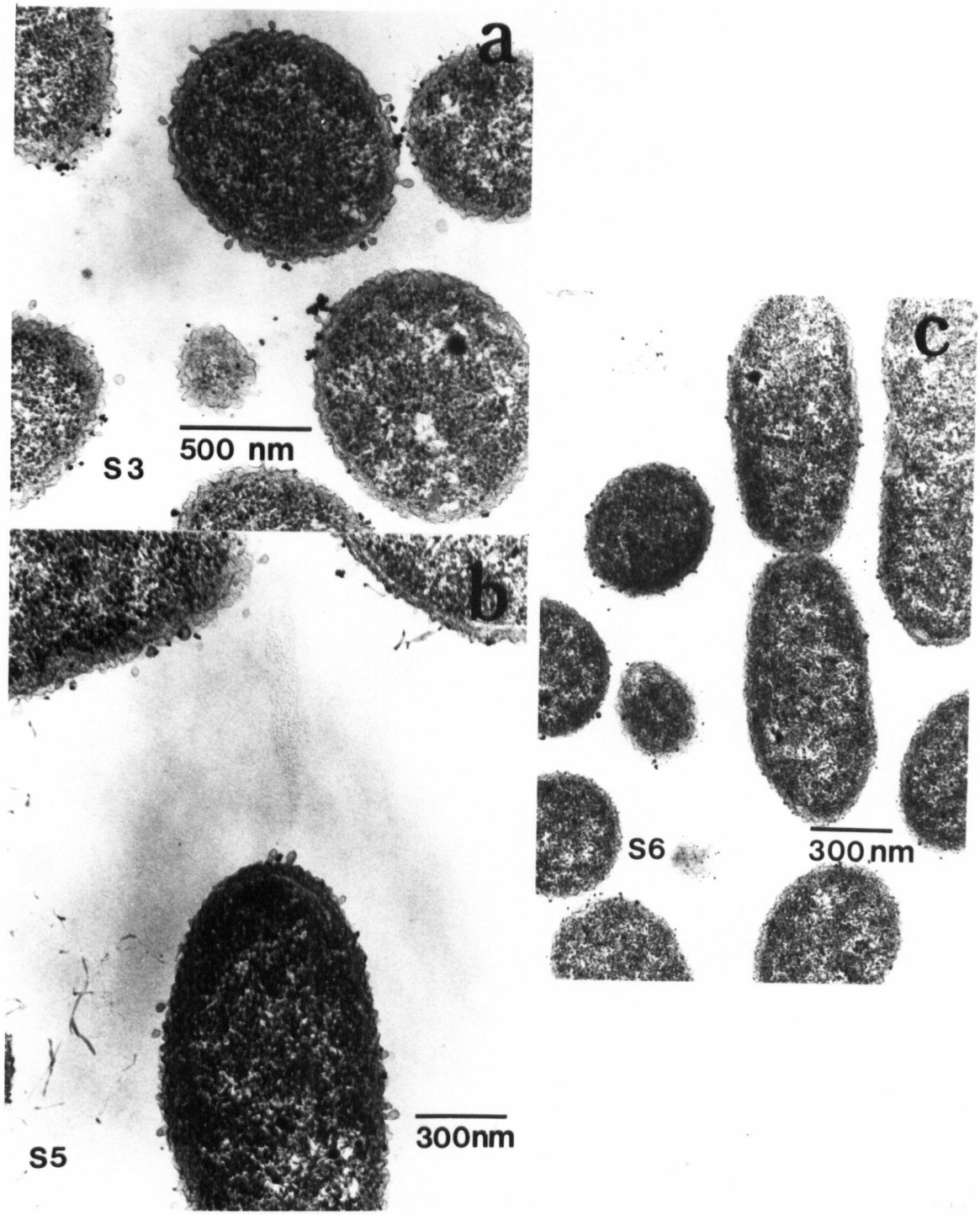
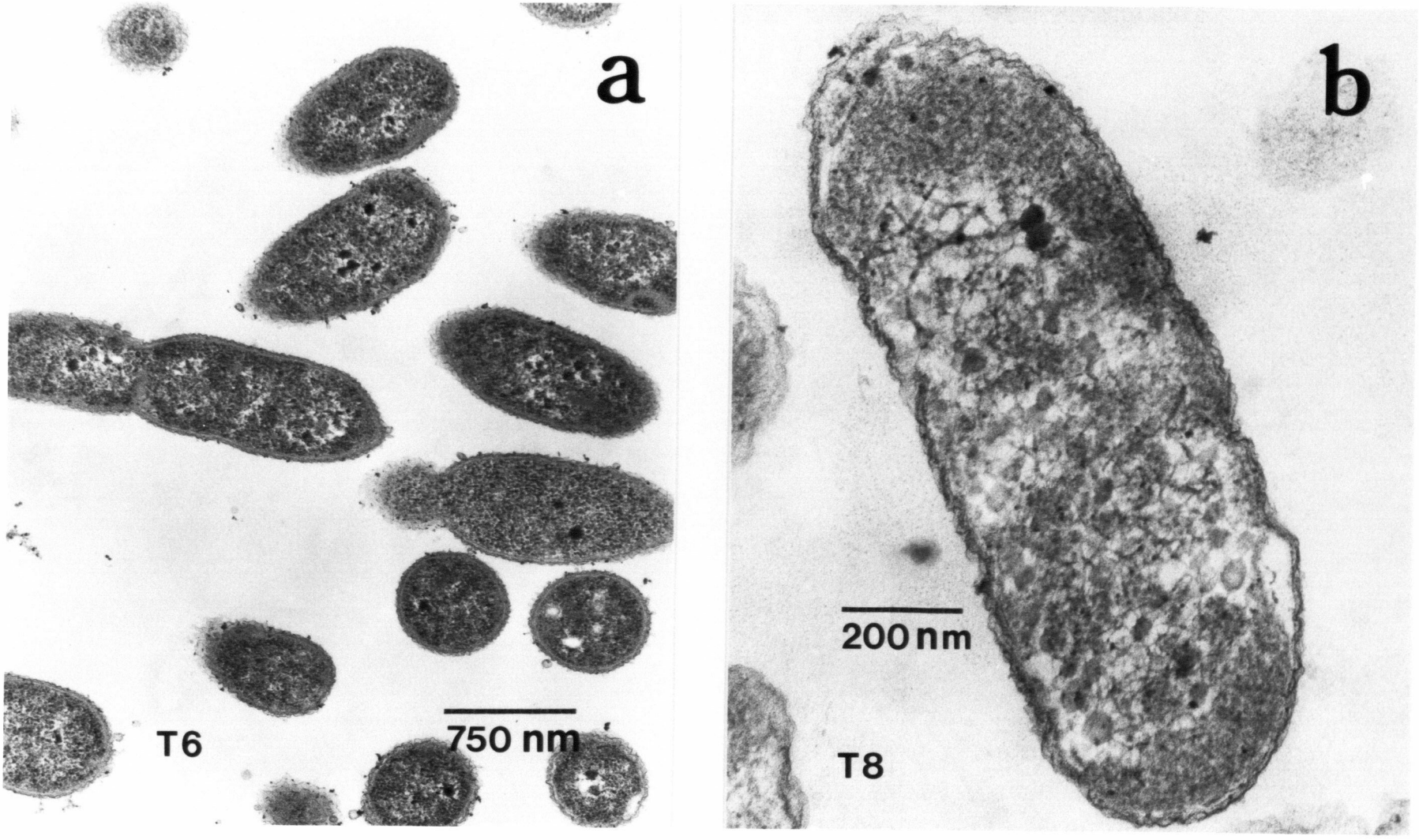

a
S3
500 nm
b
S5
300nm
c
S6
300nm

FIGURE 31. Transmission electron micrographs of *P. fragi* used in the optimization experiments.

(a) T6-Cells grown at 30 C; 32 h; pH 9; glutamine concentration 7 mmole nitrogen/L; 10.0% oxygen levels (3.6 ppm). Vesicles were present.

(b) T8-Cells grown at 30 C; 72 h; pH 9; glutamine concentration 7 mmole nitrogen/L; 30.0% oxygen levels (10.7 ppm). Vesicles were not present.


a
T6
750 nm
b
200 nm
T8

Those experiments with long incubation times were found both with (experiment S3) and without (experiment T8) vesicles attached to P. fragi cell surfaces. Experiments with a shorter incubation time also revealed cells with (experiment S5) and without (experiment S6) attached vesicles.

These trends continued with both high and low pH levels and glutamine concentrations (Table XII).

The oxygen level did affect the presence of vesicles on the cell surfaces. At high oxygen levels (experiments T8, S6) no extracellular vesicles were present on the cell surfaces. Those cells grown at lower oxygen levels clearly showed attached extracellular vesicles. This trend was evident regardless of the levels of the other growth factors studied.

Isoelectric focusing (IEF)

Isoelectric focusing of culture supernatants collected from optimization trials T4 and T3 and stained with silver stain (Figure 32) showed the presence of several protein bands. One of these bands, as revealed on the duplicate gel overlaid with casein-agar, possessed proteinase activity. The pI of this proteinase was pH 5.2.

Modeling

Description of centroid search

In model #1 (Figure 33), the first centroid (point 4) had a worse response than any of the originating vertices.

FIGURE 32. Supernatant from optimization experiments T3 and T4 separated on IEF gels.

(a) Zymogram of IEF separated culture supernatant. Proteins were separated on 5.0% polyacrylamide gel containing pH 3.7-6.4 ampholyte. Areas containing active proteinase were visualized by overlaying the IEF gel with a casein-agar gel and incubating for 24 h at 20 C. Gel was developed with a TCA/sulphosalicylic acid fixative. The zymogram was backlit and photographed against a white background. Due to the fixative, the casein was opaque and appeared dark, while the translucent hydrolyzed areas around pH 5.2 (arrow) revealed the white background.

(b) Culture supernatant from optimization experiments T3 and T4 separated on IEF gel pH 3.7-6.4. Arrow indicates protein bands around pH 5.2. The gel was stained with silver stain.

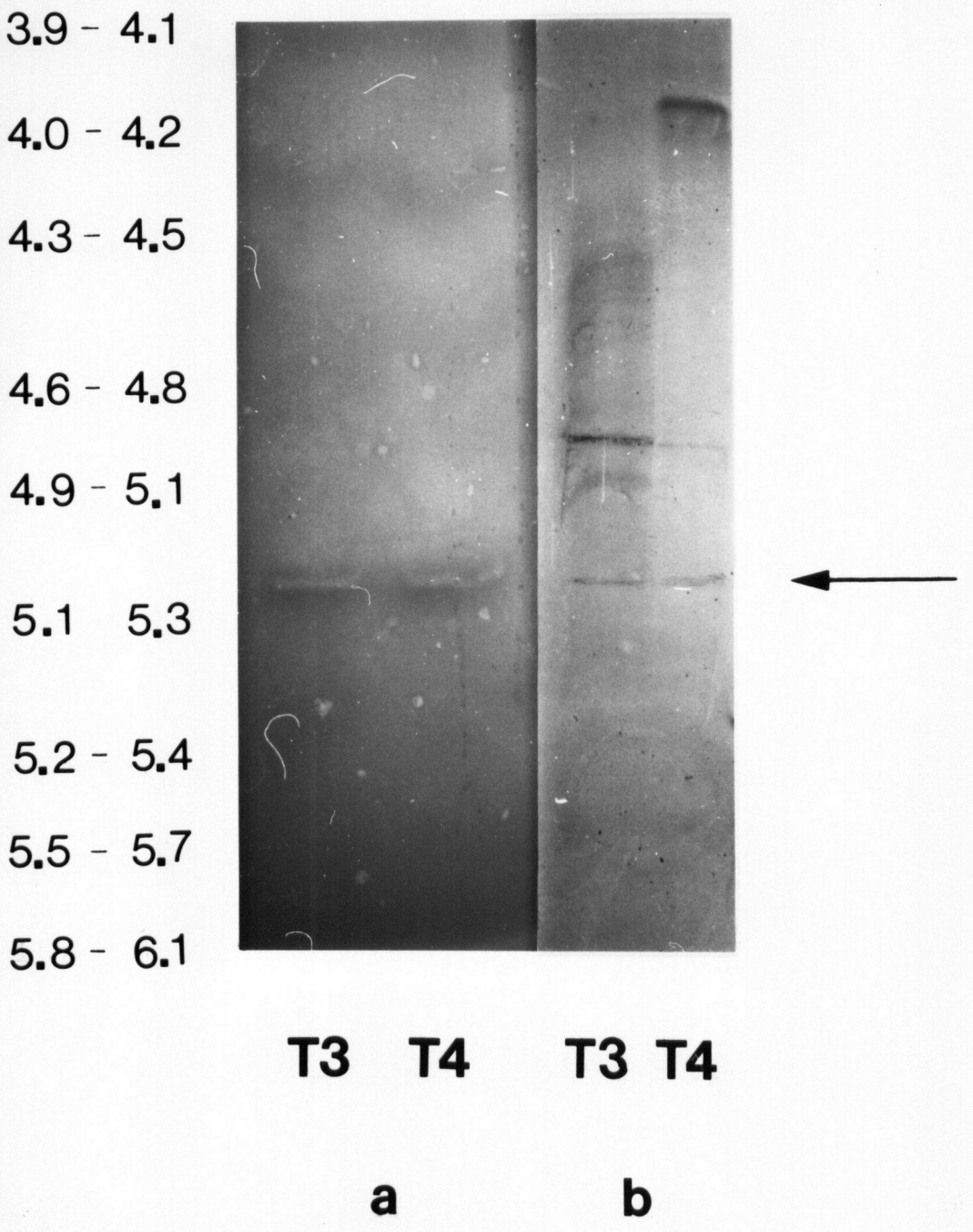
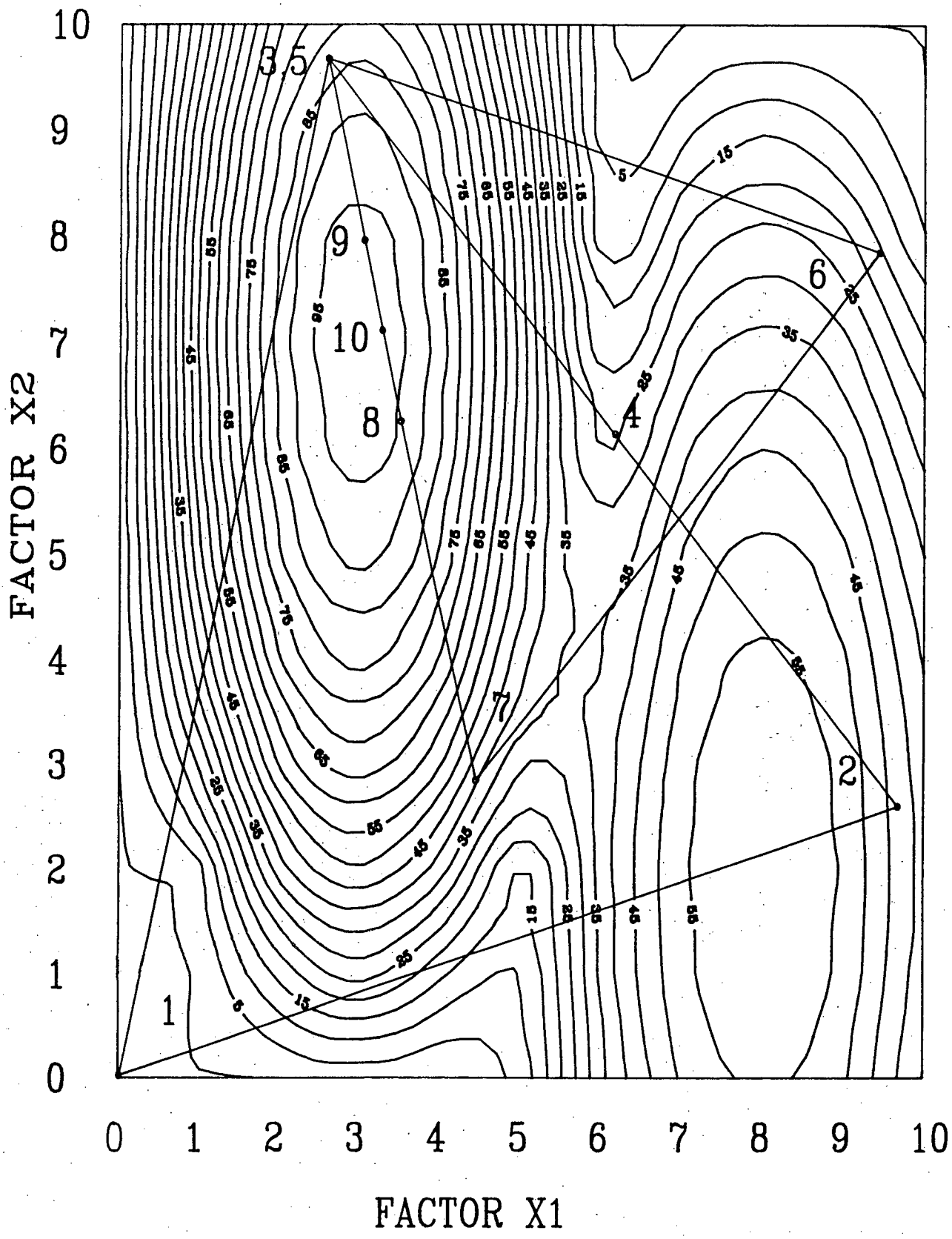

3.9 - 4.1
4.0 - 4.2
4.3 - 4.5
4.6 - 4.8
4.9 - 5.1
5.1 5.3
5.2 - 5.4
5.5 - 5.7
5.8 - 6.1
T3 T4 T3 T4
a
b

FIGURE 33. Topographical plot of model #1 with centroid generated experiments.
A topographical map of model 1. Factor X_1 is plotted on the X-axis and factor X_2 is plotted on the Y-axis. Lines shown correspond to equal values for the response value (Z). Vertices 1, 2 and 3 are points generated by the initial Spendley matrix. Vertex 3 produced the best result, with vertex 2 producing the next best result. Vertex 1 produced the worst result. The centroid (point 4) between the best and the next best result was evaluated and found to have a lower result than vertex 3. A new simplex with vertices 5, 6 and 7 was calculated. Centroids (points 8, 9 and 10) were evaluated. Point 10 came to within 99% of the true optimum value.


FACTOR X2
FACTOR X1
0
1
2
3
4
5
6
7
8
9
10
1
2
3,5
4
5
6
7
8
9
10

As a result, continuing searches along line 2,3 were abandoned and a new simplex was generated. The response values of all the resulting centroids of this simplex continued to improve. Mapping was carried out and new ranges derived. The first centroid of a new set of simplices (Figure 34) came within 99.93% of the theoretical optimum. Subsequent centroids had lower response values, and as such further searches were abandoned.

Centroid search was able to ignore the local maximum at X_1=8, X_2=2, and found the true maximum, which was centred on a simple ascending peak.

In model #2 (Figure 35), the response value of 112.7, for the first centroid (point 4), was better than all three of the originating simplices. Centroid two (point 5) had a lower response value (91.7). As a result, the search of line 2,3 was abandoned. By combining the results of the optimization with mapping of the data points, new ranges were obtained, resulting in the new simplex shown in Figure 36. The first centroid (point 4) came within 99.64% of the theoretical optimum point which was 126.13. Subsequent centroids (points 5 and 6) had lower response values and further searches were abandoned.

Comparison of optimization techniques

Three different response models were used to compare the optimization techniques. The three models were evaluated with an initial range of 10. Three repetitions,

FIGURE 34. Topographical map of Model #1 and experiments generated by centroid search - new simplex.
Factor X_1 is plotted on the X-axis and factor X_2 is plotted on the Y-axis. Lines shown correspond to equal values for the response value (Z). Using ranges suggested from the initial mapping of model #1, a second centroid search optimization was performed. Points 1, 2 and 3 are vertices generated by the Spendley matrix. Point 2 is the best response and point 1 the next best. Centroid 4 came to within 99.93% of the true optimum.

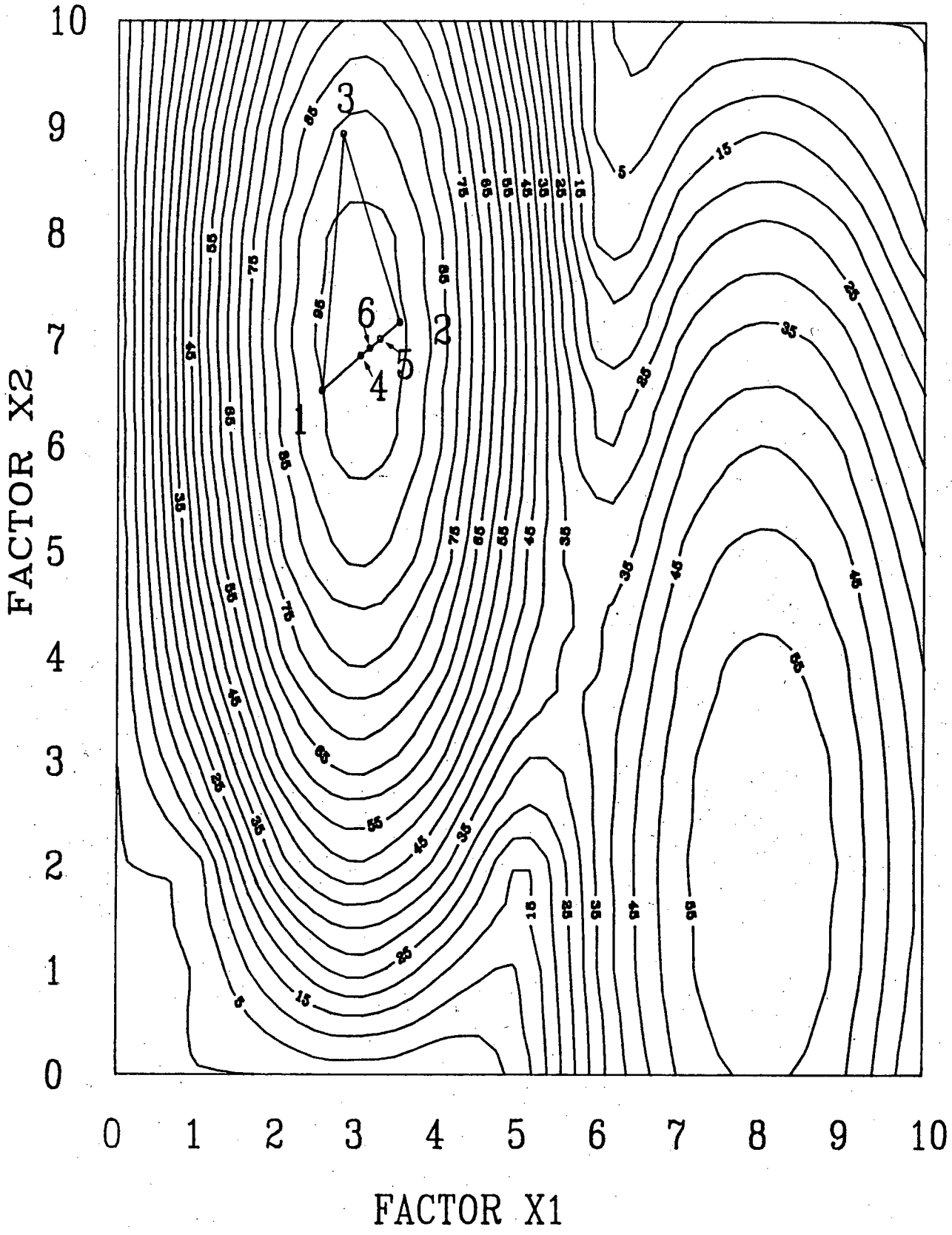

FACTOR X2
FACTOR X1
0
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
6

FIGURE 35. Topographical plot of model #2 centroid generated experiments.
A topographical map of model #2. Factor X_1 is plotted on the X-axis and factor X_2 is plotted on the Y-axis. Lines shown correspond to equal values for the response value (Z). The response value of the first centroid (point 4) was 112.7. The response value of the second centroid (point 5), at 91.7, was lower than point 4. As a result further searches were abandoned.

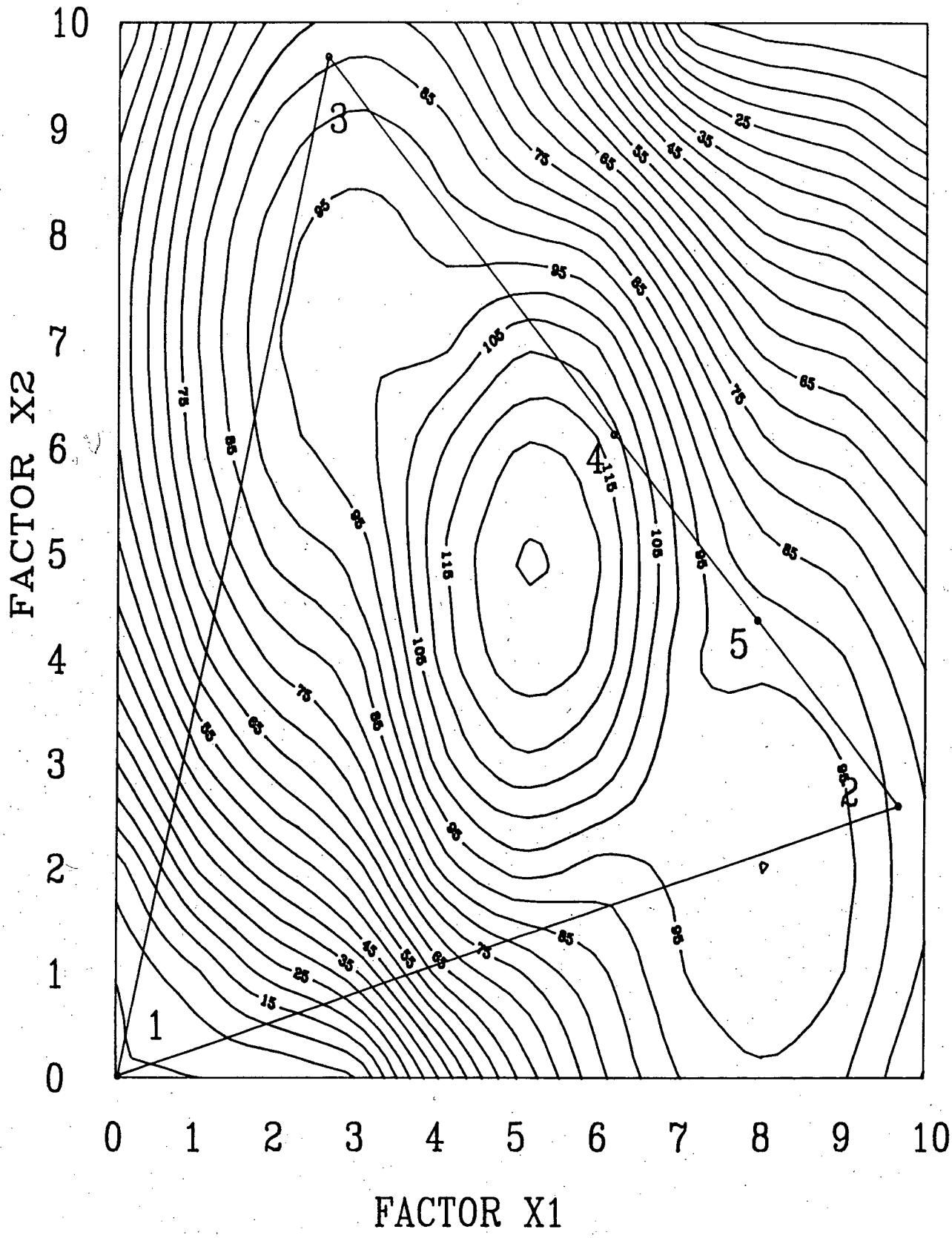
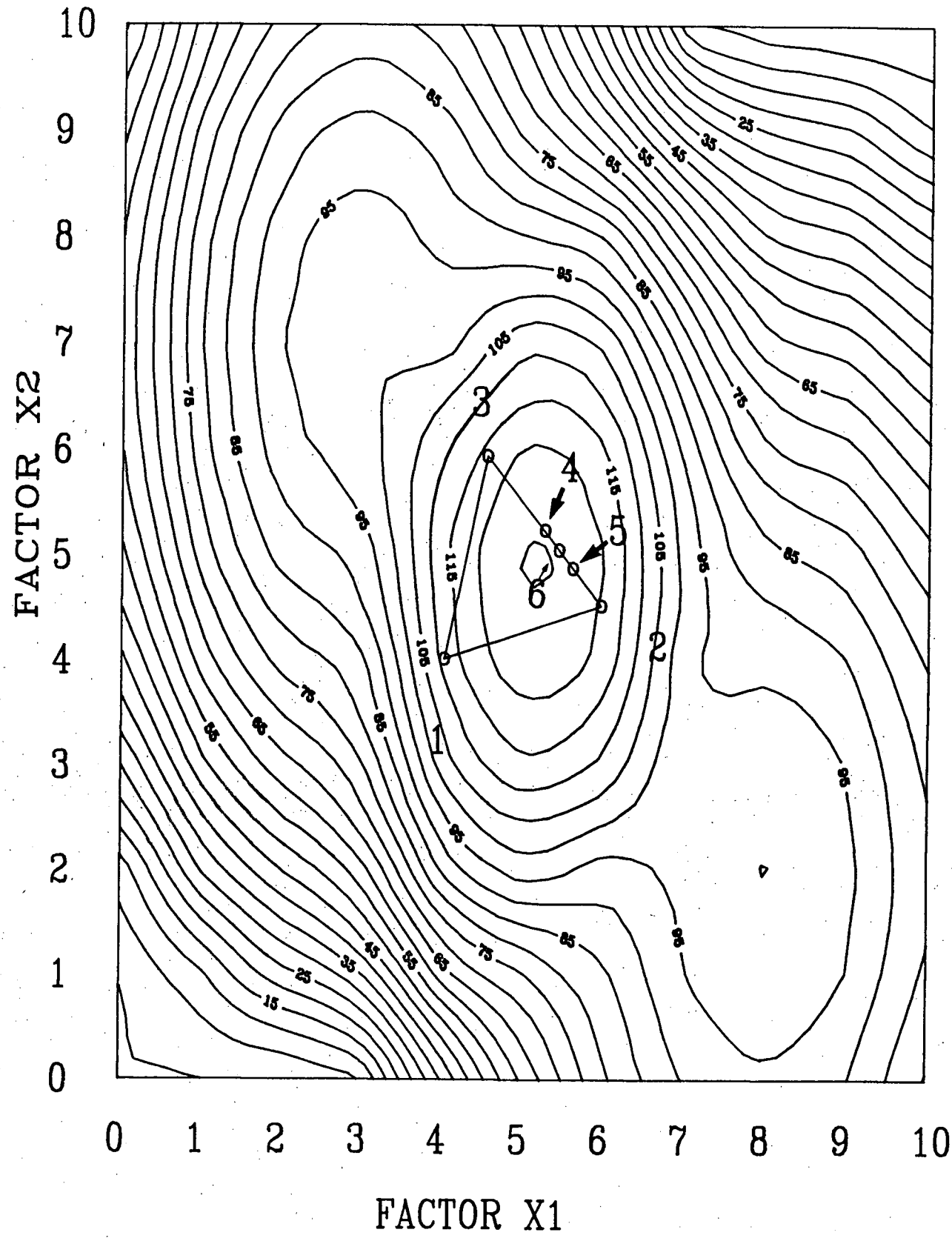

FACTOR X2
FACTOR X1
0
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
15
25
35
45
55
65
75
85
95
105
115

FIGURE 36. Topographical plot of model #2 centroid generated experiments-new simplex.
A topographical map of model #2. Factor X_1 is plotted on the X-axis and factor X_2 is plotted on the Y-axis. Lines shown correspond to equal values for the response value (Z). The first centroid (point 4), at 125.7, was within 99.64% of the theoretical optimum point.


FACTOR X2
FACTOR X1
0
1
2
3
4
5
6
7
8
9
10
15
25
35
45
55
65
75
85
95
105
115
1
2
3
4
5
6
7

using smaller ranges, were also evaluated (Table XIII). Analysis of the comparison data showed that, for experiment time (Table XIVa) and experiment number (Table XIVb) required to reach the optimum, there was no significant difference between the three optimization techniques.

Response surface methodology was unable to predict the optimum response value for model #1 and model #3. Only model #2 was correctly evaluated by RS, where the predicted response value came within 4.3% of the true optimum value.

TABLE XIII. Comparison of optimization techniques using mathematical models.

MODEL #1

$$Y_1=(250-(X_1-3)^2*30-(X_2-7)^2*5)/2.5$$

$$Y_2=(325-(X_1-8)^2*30-(X_2-2)^2*5)/5.4$$

MDS(1)		MSS(2)		CS(3)	
23 exp	143.6 h	13 exp	57.3 h	10 exp	33.4 h
17 exp	105.7 h	23 exp	62.5 h	15 exp	64.1 h
8 exp	52.8 h	13 exp	87.1 h	18 exp	90.5 h
18 exp	133.9 h	18 exp	137.7 h	15 exp	92.0 h

MODEL #2

$$Y_1=(250-(X_1-3)^2*12-(X_2-7)^2*5)/2.5$$

$$Y_2=(325-(X_1-8)^2*12-(X_2-7)^2*5)/3.25$$

MDS		MSS		CS	
21 exp	108.4 h	25 exp	88.0 h	11 exp	26.9 h
31 exp	114.1 h	28 exp	111.0 h	19 exp	73.2 h
10 exp	62.4 h	29 exp	162.9 h	13 exp	70.2 h
16 exp	84.6 h	14 exp	93.6 h	12 exp	73.0 h

MODEL #3

$$Y_1=(200+45*X_1-50*X_2+30*X_3+140*X_1*X_2-10*X_1*X_3+61*X_2*X_3+45X_1^2-245*X_2^2-11*X_3^2)/10$$

$$Y_2=(200+45*X_1-50*X_2+30*X_3+140*X_1*X_2-10*X_1*X_3+61*X_2*X_3+45X_1^2-123*X_2^2-43*X_3^2)/10$$

MDS		MSS		CS	
23 exp	191.6 h	23 exp	91.5 h	34 exp	118.4 h
16 exp	76.9 h	14 exp	52.6 h	25 exp	100.2 h
8 exp	33.7 h	14 exp	54.3 h	27 exp	90.0 h
23 exp	112.4 h	15 exp	73.5 h	17 exp	34.3 h

(1) MDS Morgan-Deming simplex
(2) MSS Modified super simplex
(3) CS Centroid search

TABLE XIVa. Comparison of optimization techniques with respect to time required.

Analysis of variance (time required)

Source of variation	ss	df	ms	F	signif.
technique	5266.44	2	2633.22	1.81	ns
model	70.51	2	35.26	0.02	ns
replicate	1679.90	3	559.97	0.39	ns
error	40767.18	28	1455.97		
total	47784.03	35			

(ns) not signigicant (P>.05)

Table of means (time required)

Techniques*	Mean (time)	$LSD_{.05}$ = 31.94**
MDS	101.67	a
MSS	89.33	a
CS	72.18	a

* MDS, Morgan and Deming simplex; MSS, modified super simplex; CS, centroid search.
** Means followed by the same letter do not differ significantly from one another.

TABLE XIVb. Comparison of optimization techniques with respect to experiment number required.

Analysis of variance (experiment number)

Source of variation	ss	df	ms	F	signif.
technique	11.06	2	5.53	0.13	ns
model	106.89	2	53.44	1.23	ns
replicate	196.31	3	65.44	1.51	ns
error	1215.39	28	43.41		
total	1529.64	35			

(ns) not signigicant (P>.05)

Table of means (experiment number)

Techniques*	Mean	$LSD_{.05}$ = 5.51**
MSS	19.08	a
CS	18.00	a
MDS	17.83	a

* MDS, Morgan and Deming simplex; MSS, modified super simplex; CS, centroid search.
** Means followed by the same letter do not differ significantly from one another.

DISCUSSION

GROWTH OF *P. fragi* ON SOLID AND LIQUID MEDIUM

Culture growth

Pseudomonas fragi, in general, grew equally well in liquid and on solid culture medium. The form of the culture medium had little effect on its rate of growth (Figure 7a & 8a). Following a short (4 h) lag phase, both cultures in liquid and on solid media grew exponentially until the beginning of the stationary growth phase (ca. 24 h). The cultures did continue to increase in cell numbers, however, to the end of the experiments.

Proteinase production

In liquid medium, proteinase production was first detected at 26 h during the late logarithmic, early stationary growth phase (Figure 7b). The cells were not producing measurable amounts of proteinase, since proteolytic activity could not be demonstrated in whole cells grown in liquid medium at 8 and 12 h. These observations were in agreement with previous research (McKellar, 1982; Thompson et al., 1985a). Several explanations regarding this behavior have been postulated including depletion of soluble nitrogenous nutrients (Lee Wing, 1984), or depletion of dissolved oxygen (Rowe and Gilmour, 1982). Priest (1983), however, suggested that nitrogen metabolism is related to extracellular proteinase

production. Basically, the enzymes are constitutive in nature and are produced in greater amounts only in the presence of some inducer. In the context of this study, it is possible that the proteinase was present, albeit in small quantities, but the enzyme assay lacked sufficient sensitivity to detect its presence.

On solid medium, proteinase first appeared at 4 h, approximately 22 h sooner than that of liquid culture (Figure 8b). Obviously the type of culture (solid versus liquid) influenced proteinase production. As with those cells grown in liquid culture, some sort of growth factor depletion may have been involved in stimulating proteinase production.

Electron microscopy

Scanning electron microscopy

Scanning electron micrographs of *P. fragi* cells in liquid medium, grown to the lag and beginning stationary growth phases, showed cell surfaces apparently devoid of globules (Figures 9 and 10). At 76 h, cells grown in liquid medium showed the presence of globules approximately 100-200 nm in diameter (Figure 11). Globules also appeared on cells grown on solid medium (Figure 13), but at 24 h rather than at 76 h. Lee Wing (1984) also described cell surface globule formation by *P. fragi* cells grown on solid culture medium. The globules observed in this study and those described by Lee Wing (1984), were much larger than the extracellular vesicles visible on the surface of *P. fragi*

cells and described as "blebs" by Lee Wing (1984) and Thompson, *et al.* (1985a). It is possible that globules found here and also reported by Lee Wing *et al.* (1983) were composed of coalesced extracellular vesicles. Further research is needed to determine the nature of these globules.

Thin section transmission electron microscopy

Transmission electron micrographs of *P. fragi* grown in liquid medium for 16 h (Figure 15), revealed structures typical of gram negative bacteria as described by Costerton et al. (1974) and Michaelis and Beckwith (1982). *Pseudomonas fragi* grown for 20 h on solid growth medium (Figure 20) displayed many extracellular particles attached to the cell surface. These particles, identical in size to those described by Lee Wing (1984) and Thompson *et al.* (1985a), were present while the cells were producing increased quantities of proteinase (Figures 8b and 8c). Larger numbers of vesicles appeared on the cell surfaces at 32 and 56h. This coincided with the time periods at which large amounts of proteinase production occurred. This would be expected if vesicles were a means of enzyme transport through the outer cell membrane.

CELL AND GROWTH MEDIUM FRACTIONATION

Isolation of extracellular vesicles, produced by *P. fragi* in liquid culture by $[NH_4]_2SO_4$ precipitation, revealed particles (Figure 23) similar in size and shape to those revealed by TEM on the surface of *P. fragi* cells (Figure

21). The particles bore a resemblance to particles produced by other gram negative bacteria, such as *B. gingivalis* (Grenier and Mayrand, 1987), *E. coli* (Hoekstra *et al.*, 1976) and *Aeromonas hydrophila* (MacIntyre *et al.*, 1980). Sodium dodecyl sulfate polyacrylamide electrophoretic separation (Figure 26) showed the vesicles to contain some small protein bands with mobilities similar to protein bands of the outer cell membrane of *P. fragi*. This indicated that the vesicles are associated with, and may have their origin in the outer cell membrane. Hoekstra *et al.* (1976) found membranes surrounding extracellular vesicles produced by *E. coli* to closely resemble the outer membrane of that organism. Markers identifying the cytoplasmic membrane or the accompanying cell wall were absent.

Only one proteinase with an isoelectric point of pH 5.2 could be detected in the supernatant from *P. fragi* cells grown in liquid culture (Figure 24 and 25). Isoelectric focusing showed that vesicles contained only one major proteinase. This proteinase, like that found in culture supernatant of *P. fragi* cells grown in liquid culture, had an isoelectric point of pH 5.2. The evidence strongly supports the theory that the same proteinase was present in both the supernatant and the vesicles.

Analysis of the liquid culture supernatant, extracellular vesicles and the soluble cell material, showed that the majority of the proteinase could be found in the supernatant (34,710.00 enzyme units). Smaller amounts were

associated with the extracellular vesicles, solubilized by sonication (117.00 enzyme units), and with the soluble cell material (244.80 enzyme units) (Table IV). Analysis of the specific activity of these components, however, showed that the proteinase was concentrated in the vesicles (680.50 enzyme units/mg protein) (Table IV). Jensen *et al.* (1980) found that the majority of the proteinase in a late logarithmic culture of *P. aeruginosa* was in the culture supernatant.

The total quantity of proteinase found in the supernatant (34,710.0 enzyme units) was the result of growth for 82 h (Table IV). The total enzyme associated with the extracellular vesicles (solubilized by sonication) was 117.0 enzyme units. If extracellular vesicles were the only means by which *P. fragi* could export proteinase outside the cell, it would have to produce 117.0 enzyme units once every 16.6 min., and produce (assuming 11.55 mg of vesicles in the supernatant at any one time) ca. 41.8 mg of vesicles/h.

Enzymes destined for the cell exterior may be synthesized in the cell cytoplasm by ribosomes attached to or free of the cytoplasmic membrane (Randall *et al.*, 1987; Priest, 1983). Transport through the cytoplasmic membrane may involve interaction between hydrophobic portions of the enzyme and hydrophobic portions of the cytoplasmic membrane (Wickner, 1979). The enzyme could appear in the cytoplasm as an active enzyme or as an inactive enzyme precursor (Priest, 1983). It could also appear in the periplasm as an

active or inactive enzyme. An inactive form of enzyme, associated with cytoplasmic membrane material was found in the periplasm of *P. aeruginosa* (Fecycz and Campbell, 1985). Active proteinase has been shown to be present in the interior of extracellular vesicles of *P. fragi* (Thompson et al., 1985a).

Whatever the state, proteinase residing in the periplasm must transverse the outer cell membrane. Fecycz and Campbell (1985) determined that transport through the outer membrane was not the same as that through the cytoplasmic membrane. Indeed, the small channels of pseudomonad porin molecules (Fairbairn and Law, 1986) would not allow a fully formed active enzyme with a molecular weight of ca. 45,000 through the outer cell membrane. It seems likely that some other form of transport would be required. Winkler and Stuckmann (1979) hypothesized a mechanism for the selective stimulation of exolipase formation from *Serratia marcescens* by a variety of exogenous polysaccharides. In that study, exolipase yield increased with an increase in either exogenous *Serratia* polysaccharide or glycogen concentration. The theory suggests hypothetical "sites", on or near the cell surface, where newly formed exolipase molecules accumulate. Exolipase enhancing polysaccharide detaches the exolipase from the cell surface by competing for these sites, or by changing the conformation of the enzyme. The glycogen probably mimics the exolipase enhancing properties of the bacterial

exopolysaccharide. An alternate hypothesis proposed by these authors suggested that the exogenous polysaccharides protect newly-formed enzymes from proteolysis during secretion. In either theory, the authors proposed that exoenzymes which are glycoproteins would preferentially bind to the cell surface and respond to enzyme-enhancing polysaccharides. In the case of *P. fragi*, proteinase is associated with outer cell membrane material. This material may complex with the proteinase, forming a vesicle. The vesicle then "blebs" off the cell surface. The resultant vesicle may contain polysaccharide material of outer cell membrane origin loosely bound with proteinase. The *P. fragi* vesicles did contain carbohydrate (1.04 mg carbohydrate/mg protein). MacIntyre *et al.* (1980) found that vesicles produced by *Aeromonas salmonicida* also contained carbohydrate (4.1 mg carbohydrate/mg protein). When the extracellular vesicles were disrupted by ultrasonic disruption, a 338% increase in specific proteinase activity occurred. If the proteinase had merely adsorbed to the vesicle surface, no such increase in specific proteinase activity would have been observed. The literature (Porzio and Pearson, 1975); (Noreau and Drapeau, 1979) does not indicate whether the proteinase produced by *P. fragi* is a glycoprotein. Fairbairn and Law (1986) described the proteinases produced by *Pseudomonas* 145-2 and *P. aeruginosa* strain 34362 to be glycoproteins. If the proteinase produced by *P. fragi* is a glycoprotein, then the theory of

Winkler and Stuckmann (1979) may apply. Further experimentation will be necessary to support these hypotheses.

The possible appearance of proteinase with outer cell membrane material may be related to transport of the enzyme through the membrane. The continued association of membrane material along with the proteinase in the form of a vesicle is, however, not readily apparent. Why would the cell allow a substantial portion of itself to be released into the supernatant along with the proteinase, when reclamation of the membrane material would appear to be more conservative? Winkler and Stuckmann (1979) proposed that the bacterial exopolysaccharide associated with the exoenzyme, is recycled back to the cell surface, liberating more exoenzyme.

Encapsulation of the proteinase may afford some advantage which the enzyme and ultimately the cell may benefit from. Lee Wing *et al.* (1983) postulated that *P. fragi* vesicles may serve to channel the proteinase produced by the cell, towards the nutrient source for which they were produced. Grenier and Mayrand (1987) echoed this hypothesis, suggesting that extracellular vesicles expelled by *B. gingivalis* may serve as a transport medium, conducting packages of highly concentrated enzymes, from the cell to the nutrient source. Indeed, since *P. fragi* is known to colonize meat surfaces, the vesicles may serve to transport these concentrated enzyme packages, between and around intercellular (i.e. meat) spaces just as the vesicles of *B.*

gingivalis may carry its proteinases to areas of tissue not normally accessible to them. This process which acts as a factor in *B. gingivalis*'s attack on the gingival cavity tissues, may be similar to the attack of *P. fragi* on a spoiling meat surface. The two situations are similar.

This discussion suggests that extracellular vesicles may be vehicles for the transport of proteinase only. A survey of the literature has revealed extracellular vesicles of other organisms to be complexed with many extracellular bound molecules including *Aeromonas hydrophila* acyltransferase (MacIntyre and Buckley, 1978; MacIntyre *et al.*, 1980), and *B. uniformis* bacteriocin (Austin-Prather and Booth, 1984). Apparently, extracellular vesicle formation takes place in several gram negative bacteria, acting to transport a wide variety of molecules through the outer cell membrane.

The association of enzymes with outer cell membrane material may have a stabilizing effect on the enzyme molecule. While the enzyme resides in the periplasm, it is in effect shielded from denaturing physical or chemical agents. While encapsulated by outer cell membrane material the enzyme may be afforded structural stability, perhaps through actual complex formation, or by concentration of stabilizing ions. The proteinase of *P. fragi* is a metalloproteinase and does require Zn^{++} for activity and Ca^{++} for stability (Porzio and Pearson, 1975).

This encapsulation creates a microenvironment which would differ from the surrounding menstrum. It would screen the enzyme and protect it from damage by physical and chemical means. The thermostability of proteinases produced by psychrotrophic pseudomonads has been well documented (Griffiths *et al.*, 1981; Malik and Mathur, 1984; Stepaniak and Fox, 1985). The mechanism of this stability is poorly understood. It is possible that at least part of this stability may be due to exopolysaccharide-proteinase complex formation. Porzio and Pearson (1975) found that *P. fragi* proteinase retained approximately 30% of its activity after 10 min at 60 C, in the presence of Ca^{++}. Pseudomonad proteinases tend to be further stabilized in the presence of milk and milk products (Patel *et al.*, 1983b).

BACTERIOCINS

Several researchers have found bacteriocinogenic pseudomonads, mainly of phytopathogenic (Cuppels *et al.*, 1978; Vidaver *et al.*, 1972) or phytopathogenic and clinical origin (Gonzalez and Vidaver, 1979). Few studies have detected bacteriocinogenicity in pseudomonads responsible for the spoilage of food products in cold storage. Hamon *et al.* (1961) described a strain of *P. fluorescens* to be bacteriocinogenic, active mainly against other *P. fluorescens* strains. *Pseudomonas fluorescens* has been implicated in spoilage of raw meat products (Greer, 1982), and raw and pasteurized milk (McKellar and Cholette, 1987). Smirnov *et al.* (1984) described a bacteriocinogenic *P.*

fragi, of unknown origin, which inhibited the growth of *P. lemonnieri*, *P. putida*, *P. stutzeri*, *P. mendocina*, *P. pseudoalcaligenes*, and *P. acidovorans*, but not other *fragi* strains. In this study, *P. fragi* ATCC 4973 did not inhibit the growth of the above species nor six different *P. fragi* strains, nine different *fluorescens* strains (including *P. aureofaciens* ATCC 13985), *E. coli*, and two *Streptococcus* strains.

P. fragi did produce extracellular membrane vesicles similar in size and shape to those bacteriocin containing particles of *B. uniformis* (Austin-Prather and Booth, 1984). The particles present in *P. fragi* culture supernatant did not have bacteriocin activity.

This lack of bacteriocinogenicity seen in *P. fragi* ATCC 4973 is in variance with the findings of Smirnov *et al.* (1984). Those researchers found that a *P. fragi* strain (not ATCC 4973) did produce bacteriocins inhibitory towards different species of *Pseudomonas*. The species was, however, limited in its range of antagonism. In this study, indicator organisms similar (*i.e.* same species, different strains) to those used by Smirnov *et al.* (1984) were not inhibited by *P. fragi* ATCC 4973. Some of the indicator organisms employed in this study, isolated by Kwan and Skura (1985), were implicated in milk spoilage. It appears that *P. fragi* ATCC 4973 may not gain a competitive advantage over other milk or meat spoilage bacteria due to bacteriocin

production. Such antibacterial properties could not be demonstrated under the conditions used in this study.

OPTIMIZATION

There have been several attempts by previous researchers (Fairbairn and Law, 1987; Griffiths and Phillips, 1984; Malik *et al.*, 1985; McKellar, 1982) to determine optimal culture conditions for the production of proteinase from pseudomonads (primarily *P. fluorescens*). All these studies, however, examined only one factor at a time. McKellar (1982) and Fairbairn and Law (1987) looked at nitrogen and carbon source, and their effect upon proteinase production. Griffiths and Phillips (1984) and Malik *et al.* (1985) looked at culture aeration, and oxygen's effect upon proteinase production. As well, Malik *et al.*, (1985) studied pH, time and nutrient source. By using optimization techniques, several cultural factors were examined simultaneously in this study to determine their effect upon the production of proteinase by *P. fragi*.

Since the optimization approach had not been previously used, decisions regarding appropriate factors, and their value ranges had to be made subjectively, using knowledge gained from previously published research. There are undoubtedly many cultural factors involved in the regulation of proteinase production from pseudomonads. Some of these factors seem to be of minor influence while others have been found to be of fundamental importance. The object of this study was, in part, to determine, from a selection of

cultural factors, which were of major and which were of minor importance for the production of proteinase by *P. fragi*. The factors chosen were ones that could be regulated by process control in an industrial food environment. In addition, it was the object of this study to determine what the optimum factor values were. The factors evaluated in this study were organic nitrogen level, initial culture pH, incubation time, incubation temperature, and culture oxygen levels.

Discussion of factors studied

Glutamine

McKellar (1982) found *P. fluorescens* capable of producing proteinase with glutamine as the sole nitrogen source at 7.0 mmole nitrogen/L. In this study, *P. fragi* was also found capable of producing proteinase when grown in the presence of glutamine as the sole nitrogen source. The glutamine levels used for optimization ranged from 7 to 610 mmole nitrogen/L. The upper glutamine level was close to the maximum solubility of the compound in culture medium (an equivalent of 650 mmole nitrogen/L). Glutamine was also used because it was shown to influence the system of nitrogen metabolism and enzyme regulation in pseudomonads (Fairbairn and Law, 1986).

Both proteins or amino acids may contribute carbon and nitrogen to the production of proteinase. The amount of nitrogen present may, however, control the production of proteinase by a process of feedback inhibition. Nitrogen

required for cellular biosynthesis may be acquired by *P. fragi* through N metabolism and ammonia assimilation. Two pathways of ammonia assimilation may be involved (Fairbairn and Law, 1986). In the first, 2-oxoglutarate is converted to glutamate, mediated by glutamate dehydrogenase (GDH). Ammonia contributes the nitrogen with NADPH as the terminal electron acceptor:

$$\text{2-oxoglutarate} + NH_3 + \text{NADPH} + H^+ \xrightarrow{\text{GDH}} \text{glutamate} + NADP^+ + H_2O$$

For the above reaction to occur, an association between GDH and NADPH must exist. This association can occur only during periods of high NH_3 levels.

Alternately, glutamate may be converted to glutamine, mediated by glutamine synthetase (GS). Ammonia again functions as the nitrogen source:

$$NH_3 + \text{glutamate} + \text{ATP} \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + P_i$$

The GS system produces a pool of glutamine, allowing the overall amination of 2-oxoglutarate to glutamate by the glutamine: 2-oxoglutarate aminotransferase (GOGAT) system:

$$\text{glutamine} + \text{2-oxoglutarate} + \text{NADPH} + H^+ \xrightarrow{\text{GOGAT}} \text{glutamate} + NADP^+ + H_2O$$

Comparing the two groups of reactions, the GS/GOGAT system has a much higher affinity for ammonia than the GDH

system. The GS/GOGAT does not require formation of a complex, such as GDH-NADPH, and thus is allowed to occur at low NH_3 levels. The GS/GOGAT system is irreversible and is repressed during growth on good nitrogen sources. Most of the nitrogen assimilated by the GS/GOGAT system is stored as glutamine. Glutamine not only acts as an intermediate in the amination of 2-oxoglutarate, but may contribute nitrogen to other biosynthetic processes. Thus glutamine is an important aspect of nitrogen metabolism.

The mechanism of nitrogen catabolite repression of GS was described by Shapiro and Stadtman (1970), and Tyler (1978). GS can be modified by addition of adenine to the molecule, such that the enzyme becomes less active. Adenylylation of GS is mediated by adenylyl transferase (AT). The activity of AT is itself controlled by a protein (P_{II}) which may exist in two forms. The unmodified P_{II} complexes with AT, catalyzing the transfer of adenine to GS. The protein may be modified by addition of uridine, mediated by uridylyl transferase (UT). Uridylyl transferase is stimulated by 2-oxoglutarate and ATP, while inhibited by glutamine, and inorganic phosphate:

$$GS \underset{[P_{II}UR]}{\overset{AT\ [P_{II}]}{\rightleftharpoons}} GS\text{-}AD$$

$$P_{II} \underset{[glutamine\ /\ P_i]}{\overset{UT\ [2\text{-}oxoglutarate\ /\ ATP]}{\rightleftharpoons}} P_{II}UR$$

The modified $P_{II}UR$ stimulates the deadenylylation of GS to a more active form. In summary, high levels of glutamine with low levels of 2-oxoglutarate, the result of a rich nitrogen substrate, renders the GS enzyme in a less active state. As a result less NH_3 will be assimilated. Low glutamine levels, with high 2-oxoglutarate levels, renders the GS enzyme more active, allowing more NH_3 assimilation.

Since proteins are sources of nitrogen, *P. fragi* produces extracellular proteinases, allowing the cell access to this nutrient. But since such proteinase production would contribute to the nitrogen pool, inhibition of the enzyme through nitrogen catabolic repression would seem logical.

It seems clear that glutamine would act as the perfect defined organic nitrogen source in order to study the effect of nutrient level on proteinase production. Being the only nitrogen source, glutamine must be deaminated, and the resultant NH_3 assimilated. Glutamine must stimulate proteinase production, since Kosers citrate medium (containing inorganic nitrogen) does not support proteinase production from *P. fragi* (Thompson *et al.*, 1985a). At some

level, however, glutamine must also act to limit proteinase production by N catabolic repression. At this point, maximum proteinase production would occur. This study indicates that glutamine at a level equivalent to approximately 300 mmole nitrogen/L culture medium, does act in this way.

Previous researchers (Lee Wing *et al.*, 1983, Borton *et al.*, 1970a; b) postulated that proteinase had been stimulated when psychrotrophic bacteria were subjected to limitation of nutrients. This effect would be more acute for cells grown on a solid medium, since the area immediately surrounding the cells would be quickly depleted of nutrients. Proteinase production, in this study, was clearly stimulated at lower nitrogen levels. This may explain the earlier occurrence of proteinase when *P. fragi* was grown on a TSB + agar surface (Figure 8b), as compared to those cells grown in liquid TSB medium (Figures 7b). A similar occurrence may have existed when Yada and Skura (1981) observed stimulation of proteinase from *P. fragi*, grown on sarcoplasmic depleted sterile beef tissue. As colony size on the beef surface increased, digested areas on the beef surface increased in size, particularly when *P. fragi* entered the late logarithmic, early stationary phase of growth (Yada and Skura, 1982).

pH

Malik *et al.* (1985) found maximum proteinase production from *P. fluorescens* at pH 7.0 ± 0.5. Fairbairn and Law

(1987) found little proteinase production below pH 6.7, but maximum proteinase at pH 6.8. In this study, pH values from 5.0 to 9.0 were used to determine the optimum proteinase production from *P. fragi*. Fairbairn and Law (1987) postulated that an increase in pH would result in a decrease in proteinase production. This study found pH 6.8 to be optimal for proteinase production. The effect of pH repression of proteinase production was weak, however, since large amounts of proteinase were produced by *P. fragi* at both pH 6.0 (experiment #S3) (Table VIIIa) and at pH 9.0 (experiment #T8) (Table V).

Time

Previous research had found proteinase production by *P. fluorescens* (McKellar, 1982) and *P. fragi* (Yada and Skura, 1981; Thompson *et al.*, 1985b; Stead, 1987) to commence at the late logarithmic, early stationary phase. For *P. fragi* in the present study, proteinase production in TSB (Figure 7b) commenced at approximately 30 h, which corresponded to the late logarithmic, early stationary phase. Production continued and extended to ca. 82 h at 21 C. The time range used for optimization was from 4 to 72 h. Extending the time range beyond 72 h made practical optimization experiments impossible. For *P. fragi*, the optimum time for proteinase production was 37.9 h.

Temperature

Previous research had shown that 20 C (McKellar, 1982), 22 C (Malik *et al.*, 1985), and 20 C (Fairbairn and Law,

1987) were the optimum temperatures for proteinase production by *P. fluorescens*. The temperature range chosen for optimization of proteinase production by *P. fragi* in this study was 1 C to 40 C. McKellar (1982) found maximum proteinase production occurred at 20 C. Fifty-five percent of maximum production was observed at 5 C. Additionally, McKellar and Cholette (1987) found that a shift from 20 to 30 C halted proteinase production by *P. fluorescens*. This study found 12.5 C to be the optimum temperature for proteinase production by *P. fragi*. *Pseudomonas fragi* did produce proteinase at high temperatures (incubating at 36.6 C produced 4.70 enzyme units/mL). Incubation at temperatures lower than the optimum, induced production of large amounts of proteinase, but only at longer incubation times. Proteinase production by *P. fragi* was favored at lower temperatures. As temperatures decline, however, metabolic activity in the cell also declines, to the point where the cell is unable to produce proteinase.

As was the case with *P. fluorescens* (McKellar and Cholette, 1987), temperatures higher than 30 C discouraged proteinase production by *P. fragi*. At these temperatures cell growth appeared to be greatly accelerated, and a true death phase had evidently occurred. Since proteinase production occurs primarily between the end of the logarithmic phase and the beginning of the death phase, any factor which tends to prolong this stationary phase, would promote increased total proteinase production. Decreasing

temperatures do favor a longer stationary growth phase, allowing more proteinase to accumulate.

Oxygen

Little information, regarding the optimum oxygen levels required for proteinase production from *P. fragi*, exists in the literature. Ambient oxygen levels centre around 18% (8.4 ppm maximum dissolved oxygen in water). The upper limit (50%) and the lower limit (0%) used for optimization of proteinase production, contain this ambient value.

Results of the first preliminary experiments showed oxygen levels to be the most important factor in determining proteinase production. Results of the fractional factorial experiment showed that oxygen levels above 10% oxygen (>3.6 ppm) strongly favored proteinase production. Malik *et al.* (1985) found liquid culture aeration stimulated the production of proteinase by *Pseudomonas* sp. B-25. Continuing on this theme, Murray *et al.* (1983) and Skura *et al.* (1986) observed that psychrotrophic microorganisms in raw milk did not produce proteinase when the milk was purged of oxygen by flushing with N_2. Rowe and Gilmour (1982), however, found proteinase production by *P. fluorescens* could be induced by forcibly decreasing the oxygen tension of the growth medium. In contrast, Griffiths and Phillips (1984) showed that accumulation of proteinases and lipases of psychrotrophic bacteria in raw milk could be suppressed by maintaining the milk in an aerated state. The results of this study indicated that optimum proteinase production

occurred when the gas mixture flowing over the growth medium contained 16.4% oxygen (7.4 ppm oxygen dissolved in the medium). This dissolved oxygen level was somewhat lower than that found when air was used as the gas (ca. 8.4 ppm). When Rowe and Gilmour (1982) reduced their oxygen level, the action initially may have resulted in a stimulation of proteinase production.

Thin section electron microscopy

Transmission electron micrographs of *P. fragi* cells, grown under various oxygen levels, revealed extracellular vesicles on the surfaces of cells grown under reduced oxygen levels only (Figure 30a, b; 31a). Cells grown under richer oxygen levels were devoid of vesicles (Figure 30c; 31b). Vesicles were found associated with cells grown close to the oxygen level optimal for proteinase production. These observations tend to support the hypothesis that vesicles are a means of transport for proteinase through the outer membrane.

Pseudomonas fragi cells grown in TSB liquid cultures (Figures 14,15,16,17,18) were devoid of accumulated extracellular vesicles, while the surfaces of cells grown on TSB + agar medium for more than 20 h (Figures 20,21,22) were studded with these particles. The particles were produced by cells grown in TSB liquid culture, since vesicle recovery from culture supernatant was possible. It appears likely that, of the factors examined in this study, dissolved oxygen concentrations mediate vesicle detachment from the

cell surface. Dissolved oxygen concentrations in liquid agitated cultures would be higher than those in the immediate vicinity of cells grown on solid medium. *Pseudomonas fragi* cells grown on solid medium attained a considerable mat thickness quickly, which probably cut off oxygen supplies to those cells nearest the nutrient source.

Oxygen level and the dairy industry

Presently, little attention is paid to the oxygen levels of raw milk, stored on the dairy farm, in the bulk tank truck, or in the processing plant silo. Optimum proteinase production occurs when *P. fragi* is grown at O_2 levels slightly below that of the atmosphere. It would seem advantageous to store milk containing *P. fragi* at oxygen levels either well below, or well above atmospheric levels. Dramatic flavor changes due to lipid oxidation would rule out storage of milk at very high oxygen levels. On the other hand, considerable reduction in proteinase production could be achieved by eliminating, possibly by N_2 flushing, oxygen from raw milk (Murray *et al.*, 1983; Skura *et al.*, 1986; Rowe and Gilmour, 1982). Rowe and Gilmour (1986) suggested that oxygen tension measurements may be a means of detecting proteinase and lipase mediated spoilage of raw milk by psychrotrophic organisms. Presently, raw milk in silos is often agitated by gentle release of air from the tank bottom. This practice may tend to stimulate proteinase production by continually supplying a small amount of oxygen to the psychrotrophic bacteria in the raw milk.

Optimization technique

Since optimization techniques had not previously been used to optimize proteinase production from *P. fragi*, the importance of each culture factor, and their influence on the optimization process were unknown. Consequently, an eight experiment fractional factorial design experiment using five factors was conducted to give some indication of their importance.

All factors were evaluated at two set factor levels (Table V). A fractional factorial experiment employing 3 factor levels (27 experiments) would have provided more information, but would have dramatically increased the number of experiments required.

Analysis of the variance of the data collected from the fraction factorial experiment (Table VIIa), showed that oxygen level was the most significant factor, of the factors studied, in regulating proteinase production from *P. fragi*. This fact may be of importance to the dairy industry in their efforts to control psychrotrophic spoilage of raw milk.

Centroid search

Evaluation of the progress of the optimization process could be checked by comparing the standard error of the mean for the Spendley matrix set of experiments with that of the centroid search experiments. It was assumed that as optimization continued, the variance between the experimental units would decrease. Theoretically, when the

true optimum value was reached the variance between experimental units would be zero. Practically, however, this point could not be reached because of random error. In this study, the standard error of the mean for enzyme units/mL declined from 2.2 for the Spendley matrix set of experiments to 1.4 for the centroid experiments mean. If optimization could have been continued, it would be expected that the standard error of the mean would continue to decline.

Subsequent to the initial Spendley matrix evaluation, six centroids were generated (Table IXa). Four of these, including centroid #1 (experiment C7) were evaluated. The strength of the centroid search technique lies, in part, in its ability to evaluate all centroids simultaneously, rather than one at a time as other iterative procedures do. This can result in a considerable saving of time, if the first centroid response value is better than that of the best response of the initial Spendley matrix. If on the other hand, the first centroid response value is less than the best response of the Spendley matrix, then the continued search of the remaining five experiments would be a waste of time. The optimum factor response would not lie on that search line. It was therefore decided that only a portion of the centroids should initially be searched. If this initial search showed continued improvement of response value, then the remaining centroids would be evaluated, otherwise the search line would be abandoned. It was hoped

that a search of sixty percent of the centroids would strike a balance between too few and too many searches. In the present situation four of six centroids were searched. Three centroids were selected randomly (only three were selected since the first centroid must always be evaluated). The selection proved fortuitous since the initial centroid response value (experiment C7, Table IXa) was not lower than the best response from the Spendley matrix (a tie was deemed an improvement). The second centroid (experiment C8) response value was lower than that of the first centroid, indicating that further searches along that line may not result in the optimum. At that point, a new Spendley matrix with a new (and narrower) range of factor levels was generated. Continued search of a new set of vertices with a narrower range of factor levels was not possible since the equipment used to maintain incubation temperature was not sufficiently precise to maintain the lower temperature differences suggested by the new Spendley matrix. As a result, further searching was stopped.

According to the centroid search procedure of Aishima and Nakai (1986) once the suspected area of optimal response value has been found, simultaneous factor shift could be used to move the factors quickly towards other areas on the response surface. The danger here is that centroid search has "homed in" on a local maximum and has missed the true maximum area. Subsequent to centroid search evaluation, factor levels were shifted to suspected areas of increased

response values. Continued shifting of factors were continued until the resultant response value became worse than the preceding one. In this study, simultaneous shift provided the set of factor levels given in Table XI. These experiments resulted in lower response values. Further simultaneous shifts were therefore not performed.

Mapping

Data from the Spendley matrix, centroid searches and simultaneous shift searches were used to construct initial data maps. The maps (Figures 27, 28, 29), of the factors time, temperature, glutamine concentration, pH and oxygen level, show a clustering of points, indicating a clear optimum response value. Mapping searches for data points present in groups, over all factors. The objective of the optimization technique used, be it centroid search, or some other technique, is to organize factor values together in such groups.

Optimization comparisons

The optimization technique in general can be used to alter two or more independent variables (factors), in an organized fashion, such that an improvement in the dependent variable (response value) can be achieved. Without such organization, improvement of the response value can only be gained by lucky or chance factor value changes.

Of the many optimization techniques available, only the simplex optimization techniques and the response surface methodology technique will be discussed. With the simplex

design, evolutionary operations (EVOP) are followed, constructing the experiments as it proceeds. Response surface methodology relies on a formal set factorial experiment designed beforehand. Both processes attempt to describe, in as few experiments as possible, enough of a response surface, to predict where the true optimum point may lie.

Morgan and Deming simplex

The Morgan and Deming (1974) simplex procedure is based, for the most part, upon the EVOP approach described by Spendley et al. (1962). The procedure is begun by defining ranges for the factors. After such scaling, an initial simplex containing n+1 vertices is generated. Subsequent to evaluation at each vertex, the vertex with the worst response value (lowest value for maximization, highest for minimization) is replaced by its reflection through the centroid residing on the line connecting the remaining n vertices, creating a new simplex. The new vertex is evaluated and the process continued. If the new vertex contains the worst response value, reflection of the next best vertex is done to avoid oscillations. This is the basic simplex design.

Modification of the above procedure, contained within the MDS (Morgan and Deming (1974) allows a more rapid convergence towards the optimum point. If the reflected vertex response value is better than that of the previous best vertex, movement in this direction is favorable. As a

result, an expansion factor is used to extend this new best point further in the indicated direction creating a new vertex for evaluation. If, on the other hand, the reflected vertex response value is worse than the previous best vertex, but better than the previous worst vertex, then this direction is not as desirable. A contraction factor is used to construct a new vertex away from the indicated direction. Reflection, expansion and contraction procedures are continued until some response value criterion is met. Such a stopping criterion may be the response value standard deviation, or a preset response value objective.

Modified super simplex

The modified super simplex procedure of Nakai and Kaneko (1985) relies upon the same general procedure as outlined above. In addition, a quadratic factorial regression analysis procedure was included in the process. The inclusion of this procedure results in the process generating a new reflected vertex, a centroid, and a curve fitted point generated by quadratic regression analysis. The modified simplex procedure, together with mapping (previously described in the Materials and Methods), constitutes the MSS.

Centroid search

Centroid search also relies upon an initial simplex generation. Subsequent centroid searches are undertaken as described in the Material and Methods section.

Response surface methodology

Response surface methodology as used in this study is not an EVOP, but rather a "batch type" operation. The RS technique begins with scaling of the independent factors. Subsequently, a symmetrical 3-level factorial design experiment is constructed as described by Box and Behnken (1960). Each experimental point is then evaluated, and the resultant data analyzed by multiple regression analysis. From such an analysis, the regression coefficient value for each factor is utilized to construct a response surface using the following response function:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_1X_2+b_4(X_1)^2+b_5(X_2)^2$$

where:

b_0 = constant
b_1, b_2 = linear coefficients
b_3 = cross product coefficient
b_4, b_5 = quadratic coefficients
X_1, X_2 = levels of the two variables

Response surfaces can be generated with a suitable graphing procedure.

Comparison of simplex methods

Both MDS and MSS are iterative procedures, with each new experimental point being generated one at a time. As a result, generation of the next experimental point must wait until the previous experiment is evaluated. Centroid search, however, generates n+1 experiments (centroids) simultaneously. All the centroids may be evaluated in one experimental run reducing the total time required to obtain the optimum point. This may be of considerable advantage to microbiological systems where incubation times may be long.

Analysis of variance of the comparison data showed, however, that none of the optimization techniques differed significantly in their ability to attain the optimum response value (Tables XIVa and b). This was true for both experiment time as well as for experiment number. The CS technique did require substantially, but not significantly less time to attain the optimum response value than the MDS technique (Table XIVa).

Theoretically, centroid search should have required less time to attain the optimum response value. Centroid search is, however, confined to searching centroids only. The search area tends to be much smaller than the search area available to MDS or MSS. This smaller search area tends to reduce the efficiency of the technique. The advantage of simultaneous centroid searching, then, is partly negated by a loss of efficiency due to a reduced search area.

Response surface methodology

Response surface methodology, in most cases, did not accurately predict the optimum response value. Since RS is a batch type process, experiments cannot be altered "mid stream" to take advantage of previously evaluated experimental points. Response surface methodology can, however, predict overall response surface shapes, thus allowing subsequent EVOP techniques to pinpoint the true optimum point. This philosophy was previously adopted (Greasham and Inamine, 1986) to optimize cephamycin C

fermentation by *Streptomycetes*. Modified super simplex or CS techniques in combination with the mapping procedure may be able to eliminate the RS step, however, allowing for a substantial increase in optimization efficiency.

Optimization in microbiological systems

In any system an increase in a response value, may be gained by altering one or more factor values. An increase in a response value is defined as an increase in the desired direction, either positive (maximization), or negative (minimization). The intuitive approach will most likely alter one factor, while keeping all the other factors constant. Not only is this technique inefficient, but it is unlikely to result in attainment of the optimum set of factor levels. A better approach would be to alter all the factors simultaneously, in a random fashion. It is possible using this technique to approach the true optimum. A random search is inefficient, however, because all possible factor level combinations would have to be evaluated, in order to ensure attainment of the optimum response value. A more efficient technique would be to evaluate factor level combinations, using a prescribed search strategy. One such search technique is simplex optimization. This technique forces the search to move to the region of optimum response, and is iterative in nature. Applying this to a microbial system could result in a lengthy search due to long incubation times. The centroid search system may be more effective under these conditions, since the vertices created

by the technique can be evaluated simultaneously. Whatever the optimization technique used (i.e. simplex or centroid) it will be more efficacious than either intuition, or random searches.

Optimization techniques such as simplex, or centroid search can initially appear to be complicated, and confusing. Although the mathematics involved with the generation of new experiments is not complex, it can be tedious. Utilization of computers can eliminate most of this tedium.

The performance of the search technique may suffer, if the choice of the starting factor levels are close to the actual optimum. In relative terms, the increase in the response values will be low. This is not a failing of the search technique, however, since attainment of the optimum response value is the purpose for employing the optimization method and what the system has accomplished is a quick and valid means of verifying this optimum.

In a microbial system the absolute response values of the individual experiments must be large enough for the technique to operate properly. In this study glutamine induced relatively small amounts of proteinase, when compared to other substrates such as TSB. At these low levels, optimization is difficult.

CONCLUSIONS

(1) When grown on solid culture medium *P. fragi* started proteinase production ca. 20 h earlier than when grown in liquid medium.

(2) Extracellular vesicles present on the surface of *P. fragi* cells grown on solid surfaces, were absent from those cells grown in liquid culture.

(3) Isolated extracellular vesicles were ca. 20 nm in diameter. The evidence is persuasive but not conclusive that the vesicles contained the same proteinase as that found in the culture supernatant. The vesicles may be similar in composition to the outer cell membrane of *P. fragi*, suggesting they are associated with the outer cell membrane.

(4) Counts done of the extracellular vesicles on the surface of *P. fragi* cells grown on solid medium revealed an association between numbers of vesicles and proteinase production.

(5) Optimization of cultural conditions promoting proteinase production by *P. fragi* showed the optimum conditions to be: (i) temperature, 13 C; (ii) time, 38h; (iii) pH, 6.8; (iv) organic nitrogen concentration, 314 mmole nitrogen/L; (v) oxygen, 16.4% oxygen in the gas mixture (7.4 ppm dissolved oxygen in the culture medium).

(6) Of the factors examined, oxygen was the largest contributing factor to proteinase production by *P. fragi* grown in a chemically defined medium.

(7) The centroid search optimization technique was successfully applied in optimizing proteinase production by *P. fragi* in a defined citrate medium.

(8) The MSS of Nakai and Kaneko (1985), the CS of Aishima and Nakai (1986) and the MDS of Morgan and Deming (1974) had similar efficiency in both experiment numbers and experimental time. The CS of Aishima and Nakai (1986) required substantially, but not significantly less time to attain the optimum response value than the MDS of Morgan and Deming (1974). Response surface methodology was unable, in most instances, to obtain the optimum point.

(9) Pseudomonas fragi, under the conditions tested, did not produce bacteriocins active against the indicator organisms used in this study. The extracellular vesicles did not appear to possess bacteriocin activity.

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