OPTIMISATION OF PROPIONIBACTERIAL ECP PRODUCTION AND THE INFLUENCE OF PROPIONIBACTERIA ON THE UASB GRANULATION PROCESS

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously, in its entity or in part, been submitted at any university for a degree.

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ABSTRACT

The "classical" propionibacteria are used in a variety of natural dairy fermentations where they produce natural preservatives (propionic and acetic acids and bacteriocins) and large amounts of vitamin B_{12}. The extracellular polysaccharide (ECP) producing ability of these bacteria also make them of special interest to the food and waste water management industries as the ECP has been illustrated to play a role in the initial granule formation in upflow anaerobic bioreactor systems.

There is little known on the ECP production by propionibacteria and in this study different environmental conditions that influence ECP production were studied. Nineteen different *Propionibacterium* strains were examined in terms of ECP production and *Propionibacterium* strain 278 was identified as the best ECP producer. Further studies were only done on this strain because of its high ECP production and because it was originally isolated from an anaerobic digester. The influence of temperature, pH and sucrose concentration was determined through the measurement of ECP production and medium viscosity. It was found that more ECP was produced at temperatures lower than the optimum for growth with the optimum being between 22° and 25°C. Lower initial pH conditions of the growth medium (below pH 7.0) were found to inhibit ECP production and the influence when the initial pH values were between 7.0 and 8.5, was not significant. A higher carbon: nitrogen ratio, when 8% sucrose was added, was also found to enhance the ECP production.

The upflow anaerobic sludge bed (UASB) bioreactor process depends on the upward movement of soluble matter through a blanket of active methanogenic granular sludge. The long start-up times as a result of the slow granulation process, as well as the need for a speedy replacement of granules once they have been washed out of the system, are limitations that restrict the general application of this excellent waste water treatment technology. Full exploitation of this biomass immobilisation technique can thus not be realised until the granule formation conditions are defined and optimised. The precise nature of the mechanisms involved in the formation of granules and the reason for their stability, is still not fully understood. It was hypothised by Britz *et al.* in 1999 that, through the implementation of environmental 'stress' conditions, a shift in the population

dynamics of the anaerobic community can be obtained. This results in a concurrent increase in ECP formation that appears to enhance aggregate formation.

In the second study it was found that, when 'stress' conditions were applied to already formed granules, the Gram-positive lactate-utilising acidogenic population gained an advantage and more propionic acid producing bacteria were present. The propionic and acetic acid concentrations were also found to increase, and concurrently, a decrease in the growth medium pH occurred. This confirms part of the granulation hypothesis that, when granules are 'stressed', the acidogenic population dynamics change and the lactate-utilising population responds to the gradual decrease in pH and the more acid-tolerant propionic acid producing bacteria gain a competitive advantage resulting in the increase in the propionic acid concentration.

When propionibacteria were added to raw sludge during the granule production process, the granules were found to be more active than when no propionibacteria had been added. This was probably due to the ECP formation by the propionibacteria that enhances the aggregation of the granules. Enhanced granulation was thus found in the batch systems with the fatty acids formed in correlation with the model for granulation. A good correlation was evident between the hypothesis and the experimental data and the hypothesis was partially verified in this study.

UITTREKSEL

Die "klassieke" propionibakterieë word in 'n verskeidenheid van natuurlike suiwel fermantasies gebruik waarin hulle verantwoordelik in vir die produksie van natuurlike voedsel preserveermiddels (propioonsuur, asynsuur en bakteriosiene) en groot hoeveelhede vitamiene B_{12}. Die Ekstra Sellulêre Pollisakkaried (ESP) produserende eienskap van hierdie groep bakterieë maak hulle ook van belang in die voedsel en afvoerwater beheer industrieë, aangesien gevind is dat ESP 'n rol speel in die aanvanklike granule formasie in anaerobiese bioreaktor sisteme.

Daar is nog baie min bekend oor die ESP produksie van propionibakterieë en in hierdie studie is verskeie omgewings faktore wat die ESP produksie beïnvloed, bestudeer. Negentien verskillende *Propionibakterium* stamme was bestudeer in terme van ESP produksie en *Propionibakterium* stam 278 was geïdentifiseer as die stam wat die meeste ESP produseer. Verdere studies was op hierdie stam gedoen na aanleiding van sy hoë ESP produksie en omdat dit oorspronklik uit 'n anaerobiese verteerder geisoleer is. Die invloed van termperatuur, pH en sukrose konsentrasie was bepaal deur die meting van die ESP produksie en die medium viskositeit. Die was gevind dat meer ESP geproduseer was by temperature laer as die optimum vir groei, met die optimum temperatuur tussen 22° en 25°C. Dit is ook gevind dat laer aanvangs groei-medium pH (laer as pH 7.0) , ESP produksie inhibeer. Die invloed van die aanvangs groei-medium pH tussen 7.0 en 8.5 was egter nie betekenisvol nie. Dit is ook gevind dat 'n hoër koolstof tot stikstof verhouding, verkry deur die byvoeging van 8% sukrose, die ESP produksie verhoog.

Die "upflow anaerobic sludge blanket" (UASB) proses vind plaas as gevolg van die opwaarste beweging van opgeloste organiese materiaal deur 'n granule bed van aktiewe metanogeniese granulêre slyk. Die lang 'start-up' tye as gevolg van die stadige granulasie proses, en die nodigheid om 'n vinnige verplasing van granules te hê nadat dit uit die sisteem gewas is, is beperkings wat die algemense toepassing van hierdie fantastiese afvoerwater tegnologie, strem. Volle implementering van hierdie biomassa immobilisereings tegniek kan dus nie plaasvind voordat die granule formasie gedefinieer en geoptimiseer is nie. Die presiese eienskappe van die meganismes betrokke en die formasie van die granules en die rede vir hul stabiliteit word egter nog nie ten volle verstaan nie. Volgens 'n hipotese deur Britz *et al.*

(1999), vind 'n verskuiwing in die populasie dinamika van die anaerobiese gemeensakp plaas tydens die implementasie van omgewings 'stress' toestande. Die resultaat is 'n verhoging in ESP produksie en 'n gevolglike verbetering in die granulasie proses.

In die tweede studie was dit gevind dat, wanneer 'stress' toestande op die reeds gevormde granulasie toegepas word, die Gram-positiewe laktaat-benuttende asetogeniese populasie voordeel geniet en meer propioonsuur produserende bakterieë was teenwoordig. Die propioonsuur en asynsuur konsentrasies het ook verhoog en met 'n gevolglike daling in die groei-medium se pH. Dit bevestig 'n gedeelte van die hipotese dat, wanneer die granules onder 'stress' geplaas word, die asetogeniese populasie dinamika verander en die laktaat-benuttende populasie reageer tot die gedeeltelike afname in pH. Die meer suur-tolerante propioonsuur produserende bakterieë verkry 'n kompeterende voordeel en gevolglik is daar 'n verhoging in propioonsuur konsentrasie.

Propionibakterieë was gevoeg by die onbehandelde slyk gedurende die granule produksie proses, en daar is gevind dat meer aktiewe granules gevorm word as andersins. Dit is moontlik as gevolg van die die ESP produksie van propionibakterieë wat die granulasie versnel het. Verbeterde granulasie was dus verkry in die sisteme waar propionibakterieë bygevoeg is. Vetsuur analises het gedui dat die gevormde vetsure ook in korrelasie was met die model van granulasie. Goeie korrelasie was dus verkry tussen die hipotese en die eksperimentele data en en die hipotese is gedeeltelik bewys in hierdie studie.

Dedicated to my parents

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CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

The genus *Propionibacterium* can be divided into two principal groups, the 'classical' or dairy propionibacteria and 'cutaneous' or clinical group. The 'cutaneous' propionibacteria are predominant members of the bacterial population on human skin, where their role as a possible cause of disease is still unresolved (Benno & Mitsuoka, 1982). The 'classical' strains, originally isolated from dairy sources, are used extensively in both the dairy and food industry as starter cultures, as well as in the production of organic acids, especially propionic acid, bacteriocins (Grinstead & Barefoot, 1992; Lyon & Glatz, 1993), vitamin B_{12} and other specific metabolites (Marcoux *et al.*, 1992). In addition to the metabolic uniqueness of the propionibacteria, the antimutagenic properties (Vorobjeva *et al.*, 1995; 1996), and the fact that propionibacteria rank among the most potent immunomodulatory stimulating cell populations involved in non-specific resistance (Roszkowski *et al.*, 1990), have made them objects of great scientific interest. One of the characteristics of the genus *Propionibacterium* that has received very little attention in the past, is their extracellular polysaccharide (ECP) formation.

Some *Propionibacterium* strains are capable of synthesising ECP under certain cultural conditions (Skogen, 1970; Racine *et al.*, 1991; Reddy *et al.*, 1973), which these are released to the environment during fermentation. Depending on their structural relationship to the bacterial cell, these polymers have previously been recorded as slime, capsular or microcapsular polysaccharides. The quantities of ECP produced vary considerably and are mainly influenced by the strain and cultural conditions under which the bacteria are grown. Exopolysaccharide production has often been found to be greater at lower temperatures (Cerning, 1995; Mozzi *et al.*, 1995; Skogen *et al.*, 1974) and is also influenced by the pH (Racine *et al.*, 1991; Skogen *et al.*, 1974), carbon source and the carbon to nitrogen ratio (Cerning, 1995; Sutherland, 1982) of the growth medium. Polysaccharides are of interest to the food industry, since they can be used at low concentrations to thicken, stabilise or even solidify solutions (Maury & Roque, 1986). An example is the use of ECP producing

strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, which are mainly used as starter cultures for yoghurt manufacture, particularly in the Netherlands and France where the addition of texture-promoting additives are not allowed (Cerning, 1995).

The ECP producing trait in thermophilic and mesophilic lactic acid bacteria is known to be unstable and this instability has been attributed to loss of plasmids (Cerning *et al.*, 1994; Vedamuthu & Neville, 1986). Propionibacteria are known to contain plasmids (Rehberger & Glatz, 1990), but their functions have not been clearly established. Little is known on the biosynthesis of ECP produced by propionibacteria. It is, however, probable that the mechanism proposed for ECP production by Gram-negative bacteria can also be accepted for ECP production by Gram-positive bacteria, since the structure of the latter is based, as for the former, on the polymerisation of repeating sugar units (Cerning, 1995; Gruter *et al.*, 1993; Marshall *et al.*, 1995). There are, however, still major gaps in our knowledge about what governs the amount, and the structural organisations of ECP in a given organism and what the instability of the ECP producing may be.

Propionibacteria have also previously been isolated from anaerobic digestion processes (Quatibi *et al.*, 1990; Riedel & Britz, 1993) and Dubourgier *et al.* (1988) argued that *Propionibacterium* could be one of the main genera of the microbial community present in granular sludge. However, even though their presence has been shown in anaerobic digestion systems, the species diversity, numbers and ecological importance are not yet known (Riedel & Britz, 1993).

The implementation of the UASB (upflow anaerobic sludge blanket) reactor has been very successful world wide where it has been applied for the anaerobic biological treatment of a wide range of industrial and municipal waste waters (Lettinga, 1995; Lettinga *et al.*, 1997). The advantage of the UASB reactor compared to traditional anaerobic treatments, is its ability to retain high biomass concentrations despite the high upflow velocity of the waste water and the production of biogas. Consequently, this reactor design can be operated at short hydraulic retention times (HRT), since the sludge retention time is almost independent of the HRT. Successful operation under these conditions requires a highly active biomass with good settling abilities. In UASB reactors, the biomass is retained as aggregates (granules) formed by the natural self-immobilisation of the bacteria. The formation and stability of the granules are essential for the successful operation (Schmidt & Ahring, 1996).

Several researchers have shown, using microscopic observations, that the bacteria in granules are surrounded by ECP, and it is generally accepted that the formation of granules is correlated with the production of ECP (Grotenhuis *et al.*, 1991; Schmidt *et al.*, 1992). It is not known whether specific species produce ECP or whether several or even all the species are able to do so. However, the population balance within the granules appears to be influenced mainly by the ECP production by the acidogenic, rather than the methanogenic population (Forster & Quarmby, 1995). Riedel & Britz (1993) speculated on the role of propionibacteria as ECP producers in the UASB granulation process and developed the following hypothesis: when sudden 'stress' conditions are applied to batch and laboratory-scale UASB systems containing fresh anaerobic sludge under controlled conditions, an enhancement of the granulation process will take place.

In 1999, Britz and co-workers reported that, under balanced operational conditions, no lactate and very little propionate can be detected in the UASB bioreactors. However, when 'stress' conditions are put on granules in a bioreactor treating carbohydrate rich waste waters, the first detectable metabolite that appears is propionate (Myburg & Britz, 1993), while simultaneously, hydrogen can be detected in the gas phase and lactate starts to accumulate (Eng *et al.*, 1986). These metabolic changes result in a shift of the population dynamics of the anaerobic community (Riedel & Britz, 1993). Subsequently, ECP producing and aggregate forming *Propionibacterium* strains were isolated under the 'stress' conditions where granule formation was stimulated (Britz *et al.*, 1999). The increase in the lactate concentration, as result of the unbalanced conditions, resulted in an orderly shift of the predominant lactate-utilising bacteria in response to the gradual decrease in the pH and increase in H_2 partial pressure. Britz *et al.* (1999) also postulated that *Propionibacterium* strains could then gain a competitive advantage, as they obtain a maximum of ATP per mol of lactate fermented. Once they have the advantage at the lower pH, they start producing extracellular compounds, with the subsequent formation of aggregates (Riedel & Britz, 1993; Vanderhaegen *et al.*, 1992). There is, however, still research that needs to be done to validate this hypothesis (Britz *et al.*, 1999; Riedel & Britz, 1993)which may have far reaching implications for industries using the UASB bioreactor concept to treat industrial waste waters.

The objectives of this study were firstly to determine the influence of different environmental conditions on propionibacterial ECP production and to identify cultural

conditions that will lead to an enhancement of ECP production by propionibacteria. The second objective was to determine if the lactate-utilising population, and specifically propionibacterial numbers, change after 'stress' conditions have been applied during the mass culturing of granules. Thirdly, the influence of different *Propionibacterium* inoculum concentrations on the granulation process, was also determined.

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CHAPTER 2

LITERATURE REVIEW

A. Background

The first *Propionibacterium* strains were isolated from Swiss-type cheese by Von Freudenreich and Orla-Jensen in 1906 (Van Niel, 1928). The members of this genus are characterised as Gram-positive, non-motile and non-sporing pleomorphic rods. The cells are generally 0.5 - 0.8 μm in length, often club shaped with one end rounded and the other tapered. They may be coccoid, bifid or branched and occur singly, in pairs or short chains, in V or Y configurations, or in clumps with a "Chinese Character" morphological arrangement. Colonies are small, elevated and range in colour from cream to reddish (Britz & Riedel, 1994; Holt *et al.*, 1994).

Propionibacteria are facultative, anaerobic mesophiles with a variable aerotolerance. They are chemoorganotrophic, with complex nutritional requirements and a fermentative metabolism and prefer lactose to glucose (Lee *et al.,* 1974) and L(+)-lactate over D(-)-lactate (Crow, 1986; Piveteau, 1998). Characteristically, propionibacteria produce high concentrations of propionic and acetic acids and often smaller amounts of gas and are usually catalase positive (Babuchowski *et al.*, 1993; Holt *et al.*, 1994; Riedel & Britz, 1993). The optimum growth temperature is 30° - 37°C and the G + C content of the DNA ranges from 53 - 67 mol % (Tm) (Cummins & Johnson, 1986).

On the basis of habitat and G+C content, the genus *Propionibacterium* can be divided into two groups. The "classical" or dairy propionibacteria (64 - 68% GC), originally isolated from cheese and dairy products and subsequent isolations have also been reported from soil (Hayashi & Furuska, 1979; Van Niel, 1928) and as part of the microbial community of anaerobic digesters (Dubourgier *et al.,* 1988; Riedel & Britz, 1993). The other group is the clinical or "cutaneous" propionibacteria (57 - 63% GC) which are frequently isolated from the human skin, anterior nose conjunctiva, mouth and upper respiratory tract. Although generally isolated as a

common contaminant in cultures obtained precutaneously and considered as being opportunistic pathogens, recent reports indicated that species of the cutanous group could be primary pathogens (Brook & Frazier, 1991; Ramos *et al.*, 1995).

A few plasmids have been identified in propionibacteria (Miescher *et al.*, 1998). The only phenotypic characteristics that have so far been linked to these plasmids are lactose fermentation, associated with plasmid pRG03 in a strain of *P. freudenreichii*, and a clumping phenomenon associated with plasmid pRG05 in a strain of *P. jensenii* (Glatz, 1992; Rehberger & Glatz, 1990).

The classical strains are used in the fermentation and dairy industries and the group is divided into four species: *P. acidipropionici*, *P. freudenreichii*, *P. jensenii* and *P. thoenii* (Cummins & Johnson, 1986; Riedel & Britz, 1993 and 1996). These species can be distinguished from each other on the basis of sugar fermentation patterns, β-haemolysis, nitrate reduction, pigmentation and the diaminopimelic acids in their cell walls (Cummins & Johnson, 1986; Glatz, 1992; Riedel & Britz, 1993). The classical propionibacteria, especially *P. acidipropionici* and *P. freudenreichii*, are used in the dairy industry as starter cultures for the manufacture of Swiss-type cheese. Acid production by propionibacteria (propionic and acetic acid, diacetyl and various amino acids, especially leucine and proline) contributes to the characteristic sweet, nutty flavour of the Swiss-type cheese (Chaia *et al.,* 1990; Langsrud & Reinbold, 1973; Sherman, 1921) and carbon dioxide is responsible for the characteristic "eye" formation (Langsrud & Reinbold, 1973; Rymaszewski *et al.*, 1998; Sherman, 1921). Propionibacteria also contribute to the proteolysis and lipolysis of the curd by releasing intracellular enzymes after their autolysis (Dupuis *et al*, 1993; Dupuis, 1994; Perreard & Chamba, 1998). The utilisation of the products of proteolysis is important in the ripening of Swiss-type cheese and could account for the low propionate: acetate ratios observed in Swiss-type cheese (Piveteau, 1998). Proline specific peptidase that exhibits esterase activity against the short chain fatty acids in cheese was recently detected by Kakariari *et al.* (1998) in propionibacteria.

The classical propionibacteria have previously been used for the commercial production of vitamin B_{12} and are still the main cultures used in Russia (Prof. L. Vorobjeva, 1998, Personal Communication). A variety of materials of natural origin were used as substrates, including cheese whey (Bullerman & Berry, 1966),

distiller's waste (Kato & Shimizu, 1963), spent potato wash (Majchrzak & Czarnockza-Roczniakowa, 1966), casein hydrolosates (Neujahr *et al.*, 1960) and corn starch (Shaposhnikov *et al.*, 1969). Vitamin B_{12} is an important co-factor for the metabolism of carbohydrates, lipids, amino acids and nucleic acids, and can be used in chemotherapy (Quesda-Chanto *et al.*, 1994).

The addition of propionibacteria to lactic acid bacteria (LAB) used in the fermentation processes, has been reported to lead to an increase in the folacin, vitamin B_{12} and the propionic and acetic acid content. Propionibacteria also produce CO_2, bacteriocins, vitamins, other minor organic acids that, together with the antimutagenic properties displayed by propionibacteria (Grinstead & Barefoot, 1992; Vorobjeva *et al.*, 1995 and 1996), may be important in the application of propionibacteria as probiotic agents in foods (Chaia *et al.*, 1998). It led to the inhibition of harmful and pathogenic micro-organisms and to an extension of the shelf-life of the obtained products. The final products usually had better organoleptic characteristics than the products made with lactic acid bacteria only. The amount of metabolites produced was influenced by the starter cultures and the medium, as well as by the interactions between the strains. It was found that during long fermentation times, like that used with sauerkraut, pickles and potatoes, substantial amounts of metabolites were produced during processing and storage. On the other hand, in yoghurt and kefir which were made with short fermentation times, the concentration of metabolites in the final product depended predominantly on the concentration of the inoculum used (Babuchowski *et al.*, 1998). Combinations of propionibacteria and lactic acid bacteria have also been used to inhibit yeasts and moulds in fermented milk products, bakery products, silage and spent grains (Suomalainen & Mäyrä-Mäkinen, 1998).

Riedel & Britz (1993) obtained the first pure isolates from anaerobic digestion processes and according to Dubourgier *et al.* (1988), *Propionibacterium* could be one of the main genera of the microbial community in granular sludge. Riedel & Britz (1993) also speculated on the importance of this group during granulation in upflow anaerobic sludge blanket (UASB) bioreactors.

B. Extracellular Polysaccharides (ECP)

Bacterial polysaccharides (also known as exopolysaccharides, extracellular polysaccharides, heteropolysaccharides, extracellular slime and slime) can be found intracellularly as reserve material (granules of starch or glycogen), associated with the cell layers, or more or less free outside the cell as slime or free extracellular polysaccharides. Depending on their structural relationship to the bacterial cell, these polymers have been named slime, capsular or microcapsular polysaccharides. The name exopolysaccharides as proposed by Sutherland (1972), provides a general term for all these forms of bacterial polysaccharides found outside the cell wall. In some cases, the same microbe produces both capsular and unattached polysaccharides (Cerning, 1990).

Exopolysaccharides associated directly with the cell layers include: lipopolysaccharides present in the cell wall of Gram-negative bacteria; peptidoglycans of most bacterial cell walls in which polysaccharide chains are cross-linked via short peptide chains; teichoic acids that are present in cell-walls and membranes of Gram-positive bacteria; and capsular polysaccharides which are bound to the cell wall (Graber *et al.,* 1988). Electron micrographs showed that there are interactions between the extracellular polysaccharides, cell surface and proteins (Shellhaass & Morris, 1985; Teggatz & Morris, 1990).

Despite the knowledge and importance of the exopolysaccharides, there is still little understanding of the relationship between the chemical structure and the conformation and of the inter- or intramolecular associations of these polymers, and how these are responsible for their functional properties. This understanding is complicated by the complex structure of exopolysaccharides. Fortunately, many of the polysaccharides consist of complex but defined chemical repeat units. Some of these polymers can be grouped into families of structures, within which individual members of the family show small naturally arising modifications of the chemical structure. Such systems provide a basis for identifying critical structural features, which determine polymer functionality. The similarities of such structures suggest a similar biosynthetic pathway. Manipulation of the pathways in individual bacteria offers an opportunity for selectively manipulating the polysaccharide structure by deletion or inactivation of genes (Griffin *et al.*, 1996).

B1. Production

The synthesis of bacterial exopolysaccharides involves a larger number of enzymes. Sugar nucleotides (nucleotide diphosphate sugars) play an important role in exopolysaccharide synthesis: they are the activated form of the monosaccharides and provide the microbial cell with a means of interconversion of various monosaccharides through epimerisation, dehydrogenation and decarboxylation reactions. Furthermore, isoprenoid-glycosyl carrier lipids are involved in the polymerisation of exopolysaccharides (Sutherland, 1982; Sutherland & Tait, 1992; Whitfield & Valvano, 1993). The lipid involved is undecaprenol phosphate, identical to the carrier lipid involved in the synthesis of cell wall polymers such as lipopolysaccharides, enterobacterial common antigen (Sutherland, 1977), peptidoglycan and teichoic acids. This explains why exopolysaccharide production is reduced under conditions that stimulate increased lipopolysaccharide or teichoic acid production due to the competition for the same lipid carrier (Cerning, 1995).

Two distinct mechanisms for exopolysaccharide synthesis are known. Homopolysaccharides such as dextrans and levans are synthesised by an extracellular process involving a donor and acceptor molecule and enzymes that are secreted from bacteria or loosely associated with the cell surface. The donor is a specific substrate, usually sucrose, reflecting the involvement of high specific enzymes acting on oligosaccharide carbon sources (Cerning, 1990). Heteropolysaccharides are synthesised by a more complex system in that they are produced at the cytoplasmic membrane utilising precursors formed intracellularly (Cerning, 1995). In contrast to exopolysaccharides and capsular polysaccharides from Gram-negative bacteria which have been extensively investigated (Sutherland, 1982; Whitfield & Valvano, 1993), little is known of the biosynthesis of exopolysaccharides produced by lactic acid bacteria and propionibacteria. It is however, probable that the mechanism proposed for exopolysaccharide and capsular polysaccharide formation by Gram-negative bacteria can also be accepted for the exopolysaccharide production by propionibacteria. This is because the structure of the latter are based, as are the former, on the polymerisation of repeating sugar units (Doco *et al.*, 1990; Marshall *et al.*, 1995).

In some bacteria, exopolysaccharide synthesis appears to share common precursors and co-factors with cell wall and protein synthesis, hence competition for these intermediates would take place and the rate of polysaccharide synthesis would be influenced by the growth rate. The use of antibiotics such as tetracycline, streptomycin and chloramphenicol to uncouple the production of a polysaccharide from other synthesis could permit the selection of high ECP producing strains. Bacteriophage resistance has also been used as selective agents of mucoid strains (Sutherland, 1977).

The isolation of exopolysaccharide producing micro-organisms can be performed on rich complex media or a selective media using culture conditions known to promote the production of polysaccharides. Carbohydrate rich environments such as effluents from the sugar, paper and food industries, breweries and waste water plants are sources susceptible to contain micro-organisms that produce polysaccharides (Lawson & Sutherland, 1978). The colonies of these micro-organisms can be detected macroscopically since some of them have a mucoid or watery aspect.

Polysaccharide production is normally highest under aerobic conditions. Therefore, more polymers are usually excreted during growth on solid media than obtained from comparable amounts of cells grown in liquid media. Synthesis and secretion of exopolysaccharides occur during different growth phases and the type of polymer is influenced by growth conditions. Factors influencing the production of polysaccharides are the source and concentration of carbon, the carbon: nitrogen ratio, type and concentration of ions, dilution rate, aeration rate, incubation temperature and time, pH, age of the culture and nutrient limitation (Williams & Wimpenny, 1977). Deficiency of nitrogen, phosphorous, or sulphur sources, for example, in the presence of carbohydrates, leads to increased exopolysaccharide production in *Escherichia coli* (Cerning, 1990; Duguid & Wilkinson, 1953). Culture conditions promoting the production of microbial polysaccharides vary from one organism to another. The only enhancing parameter that is constant in most media, is the cultivation of the polysaccharide producing micro-organism in a medium with a high carbon to nitrogen ratio (Cerning, 1990; Graber *et al.*, 1988).

Mozzi *et al.* (1995) demonstrated an increased exopolysaccharide production from *Streptococcus sulvaricus* subsp. *thermophilus* and *Lactobacillus*

bulgaricus at incubation temperatures of 32°C or 37°C instead of 42°C. Exopolysaccharide production from mesophilic lactic acid bacteria is almost 50% higher when the organisms are grown at 25°C instead of 30°C. Mineral requirements also effect the exopolysaccharide production (Cerning *et al.*, 1992; Clarke-Sturman *et al.*, 1986). It has been reported that $MnSO_4$ exerted a s timulating effect on the exopolysaccharide production of *Lactobacillus casei* CRL 87 (Mozzi *et al.*, 1995). The growth of certain strains of *Lactococcus* increased the viscosity of whey, but the culture gradually lost this property when frequently transferred. Also, this organism lost the property of slime production more quickly at high temperatures (Cerning, 1990). Sometimes a decrease in the amount of polysaccharides is observed at the end of the fermentation (Racine *et al.,* 1991). Macura & Townsley (1984) found a decrease of viscosity in lactic streptococci after 24 hours of growth, but the decrease varied with the strains of ropy bacteria. These observations suggested that a degrading agent, possibly a glucohydrolase, is destroying slime progressively by hydrolysing it to oligomers and monomers (Cerning *et al.,* 1988). Similar observations have been made with strains of *S. salivarius* subsp. *thermophilus* (Cerning *et al.*, 1988) and with *Lb. hilgardii* (Cerning, 1990).

There is a significant correlation between biomass obtained and polysaccharides produced. Racine *et al.* (1991) showed that when the medium composition is modified to increase polysaccharide yield, it generally resulted in a parallel increase in biomass produced, but this was not the case when pH and temperature were varied. Results obtained by Mozzi *et al.* (1996) showed that at a constant pH of 6.0, the exopolysaccharide production of *Lb. casei* CRL 87 was linked to biomass, while at lower pH values (4.0) a higher cell efficiency in the polymer synthesis was observed. The maximal amount of polysaccharide containing fractions are normally reached at the end of the fermentation when the nitrogen source may become a limiting factor (Duguid & Wilkinson, 1953).

B2. Composition

Bacterial polysaccharides can be classified into two groups based on chemical and physical criteria. In general, group one polysaccharides contain

uronic acid as the acidic component, have a high molecular mass and are co-expressed with specific O-polysaccharides. In contrast, group two polysaccharides contain a large variety of acidic components and have a relative low molecular mass (Jann & Jann, 1990).

Microbial exopolysaccharides are either homopolymers composed of a single sugar (dextrans, α-glucans and fructans), or heteropolysaccharides in which the structure contains several different monosaccharides, commonly two to four in number (Cerning, 1995; Sutherland, 1994), like that produced by dairy lactic acid bacteria. Glycosidic linkages join these monosaccharides. Polysaccharides are an incredibly diverse range of molecules by virtue of not only containing different possible monosaccharide units but also as to how these units are joined together. The presence of a number of hydroxyl groups that may be involved in the formation of a glycosidic bond means that any two monosaccharides may be joined in a number of ways. Additional structural complexity may be achieved by the introduction of branches into the polysaccharide chain and the substitution with both organic and inorganic molecules. Therefore, polysaccharides represent a rich source of structural diverse molecules, properties which have been exploited by man in a number of industrial, biochemical and food processes (Roberts, 1995).

Neutral hexoses such as D-glucose, D-galactose and D-mannose are of widespread occurrence, as are the methylpentoses (6-deoxyhexoses), L-fucose and L-rhamnose. Polyols, ribitol and glycerol, are essential constituents of several exopolysaccharides, which closely resemble the teichoic acids of Gram-positive cell walls and membranes. Both amino sugars and uronic acids have been widely recognised; the former as neutral N-acetylamino-sugars, most frequently N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, but the more complex N-acetyl neuraminic acid is also found. D-Glucuronic acid is present in a large number of bacterial exopolysaccharides, D-galactoronic acid and D-mannuronic acid have also been reported, while L-guluronic acid is a component of bacterial alginates (Carlson & Matthews, 1966; Linker & Jones, 1966; Sutherland, 1977 and 1994).

Many exopolysaccharides, as well as monosaccharides, contain non-sugar residues as integral components of the macromolecule. Phosphate is present in those polymers that resemble teichoic acids. Acyl groups are of widespread occurrence, especially the O-acetyl groups, but formate (Sutherland, 1977) and

succinate (Harada, 1965) have also been identified. These three components are similar in that they all appear to be present in the form of ester linkages, which are readily removed by treating the polysaccharides with a weak alkali or acid. A further type of substitute, that resists mild alkali treatment, is a ketal-linked pyruvate (Garegg *et al.*, 1971; Sloneker & Orentas, 1962). Unlike the ester-linked residues, ketals normally confer a charge on the exopolysaccharides, rendering them acidic in the same way as those containing uronic acids (Sutherland, 1977 and 1994).

The exopolysaccharides of lactobacillus are essentially composed of glucose and galactose, and mannose and arabinose are present in smaller proportions. The proportions vary during the different stages of the fermentation cycle. The structure of the dairy starter *Streptococcus thermophilus* CNRZ 404 contains neutral sugars (*i.e.*, galactose, glucose and glucosamine) along with low levels of pentose sugars and mannose (Cerning *et al.*, 1988; Doco *et al.*, 1990). Despite earlier indications that all strains within some bacterial genera tended to produce exopolysaccharides of similar composition, it is now apparent that wide variations occur even within a single bacterial species (Sutherland, 1977).

Approximately 1 – 4% of the total solids of granular sludge are ECPs (Morgan *et al.*, 1991) and protein and carbohydrates are the dominant components of the ECPs (Jorand *et al.*, 1998; Shen *et al.*, 1993). The carbohydrate component is composed of a range of sugars including galactose, glucose, mannose and glucuronic acid. Although rhamnose and ribose have rarely been reported in bacterial ECPs in pure cultures (Sutherland, 1977), they appeared to be primary components of sugars in sludge ECP (Dignac *et al.*, 1998; Morgan *et al.*, 1991). The protein component of the sludge ECP includes the amino acids asparagine, glutamine and alanine. The remaining components of ECP include lipopolysaccharides, RNA, DNA and inorganic molecules. The consortium found in a granule ensures that no extracted ECP will be homogenous, thus explaining the range of constituents found in the biopolymers (Shen *et al.*, 1993).

B3. Techniques associated with ECP production

Media used for polysaccharide production in the laboratory or industry are based on high ratio's of carbon substrate limiting nutrient, where nitrogen is usually the favoured component to induce growth limitation and stimulate

exopolysaccharide production. Glucose at concentrations of 2 - 5% (w/v) is usually the preferred carbon substrate as it is utilised by a very wide range of microbial species and is also widely available, the cost depending on the degree of purity employed (Cerning, 1995). The usual source is starch hydrolysates. Sucrose provides an acceptable alternative for many species and is better than glucose as carbon source for polysaccharide production. However, at sucrose concentrations of more than 4%, polysaccharide yields were reduced; this effect was not apparent when glucose was used. The presence of pyruvate, succinate or α-ketoglutarate induced a stimulatory effect on the yield from sucrose-based media, as did L-glutamic acid (Sutherland, 1994). Polysaccharide yield and the rate of production are both effected by the oxidation state of the carbon source; both decrease when the substrate is at a higher or lower oxidation state than hexose (Linton *et al.*, 1987).

Under industrial conditions only the cheapest available substrates that will provide consistent yields and quality of the product, are used. Thus, corn-steep liquor, distillers soluble, acid or enzymatic hydrolysates of starch or other substrates have been used to form the bases for large-scale culture media (Sutherland, 1996). In the industry, microbial polysaccharides are usually produced by batch fermentation, which are difficult to control. Variations in the biopolymer properties, e.g. rheological parameters, are frequently observed (Sutherland, 1990). Most polysaccharide-producing micro-organisms are incubated at or near 30°C, although incubation at sub-optimal temperatures frequently favours polysaccharide production.

The isolation and purification of exopolysaccharides is difficult and tedious, because the exopolysaccharides are always found together with the surrounding micro-organisms that produced it, and from which they can hardly be entirely separated. Furthermore, ECP is often mixed with different carbohydrates and proteins or peptides from the medium itself (Cerning, 1995). Centrifugation of the cells and then the precipitation of the exopolysaccharides with a lower alcohol are usually used for the isolation of the exopolysaccharides.

Complex media for the cultivation of lactic acid bacteria usually contain a high concentration of ethanol insoluble materials and, with a few exceptions (Van Den Berg *et al.*, 1995), lactic acid bacteria produce only low amounts of

polysaccharide (<500 mg.l^{-1}) (Cerning, 1995). Therefore, the direct measurement of the ECP concentration in fermentation broth by precipitation with ethanol followed by drying, is impractical. Spectrophotometric (Phenol/sulphuric acid or anthrone) methods for total carbohydrate concentration are generally used, but this approach requires the separation of the polysaccharide into simple sugars. A variety of procedures have been used to this end: dialysis (Petit *et al.*, 1991; Van Den Berg *et al.*, 1995), enzymatic treatments followed by dialysis (Gancel & Novel, 1994) and ethanol precipitation, with or without enzymatic treatments, followed by dialysis (Cerning *et al.*, 1992; Mozzi *et al.*, 1995, 1996). These procedures are very slow and have a relatively high error rate (5 – 10%) (Cerning *et al.*, 1992). Size exclusion chromatography with gels with a nominal exclusion limit of 6 000 daltons is a rapid and convenient alternative to dialysis and has been used for preparative purposes (desalting and buffer exchange) (Ricciardi *et al.*, 1998).

Visual observations or viscosity measurements can also be used to estimate the amount of exopolysaccharides formed in liquid media. It remains however, difficult to relate viscosity and exopolysaccharides produced, because viscosity depends not only on structure, but also on the type of polymer, the apparent molecular mass of the polymer, the physical state of the proteins and the effect of other metabolic products which are excreted in the medium (Cerning, 1990; Shellhaas & Morris, 1985). The quantities of exopolysaccharides produced in milk by different species and strains vary considerably; the amount of exopolysaccharides reported range from 50 to 350 mg.l^{-1} for *Streptococcus thermophilus* (Cerning *et al.*, 1988; Garcia-Garibay & Marshall, 1991), and from 80 to 600 mg.l^{-1} for *Lactococcus lactis* subsp. *cremoris* (Cerning *et al.*, 1992). Exopolysaccharide production by *Lactobacillus casei* is relatively low in skim milk (50 to 60 mg.l^{-1}), but is stimulated by the addition of glucose or sucrose with the values increasing to about 200 mg.l^{-1}.

The characterisation of the exopolysaccharide composition is usually done with thin-layer chromatography, HPLC and gel filtration chromatography. Purification of exopolysaccharides from dairy lactic acid bacteria has been successfully achieved by DEAE cellulose chromatography (Doco *et al.*, 1990; Marshall *et al.*, 1995). Contaminating proteins or peptides can also be removed by

gel filtration chromatography, but the occasionally high viscosity of the exopolysaccharide solutions may be a problem (Cerning, 1995).

Although several methods for the extraction of ECP from anaerobic sludge have been reported, there is not yet a standard method universally accepted by researchers studying ECP. Fang & Jia (1996) showed that the amount of ECP extracted from anaerobic sludge is strictly dependent upon the extractant and the extraction procedure. According to Urbain *et al.* (1993), the dispersion of the floc matrix by sonication gave interesting results. Extraction was achieved in a crushed ice bath, thus avoiding heating of the sludge samples, and no chemical products were used. Therefore, the sonication did not appear to influence the chemical composition of the extract. Moreover, the disruption of bacterial cells of pure cultures by sonication has shown to be limited (Jorand *et al.*, 1994). The removal of multivalent cations by cation exchange resin (CER) (Rudd *et al.*, 1983), is another way of dispersing natural aggregates. The use of cation exchange with CER should be more preferable than the use of a chemical compound like EDTA (ethylenediaminetetraacetic acid) (Brown & Lester, 1980), since the risk of contamination of the samples by organic compounds is limited and the resin is easily removed from the cells by settling. The Dowex resin proposed by Rudd *et al.* (1983) has a strong affinity with calcium ions. This method preserves the integrity of bacterial cells and extracts large quantities of ECP (Frolund *et al.*, 1996).

According to Fang & Jia (1996), EDTA was more effective than formaldehyde as an extractant. The increase of temperature and the addition of caustic also enhanced the extraction. Regardless of the extraction methods, the ratio of the ECP carbohydrate content to the ECP protein content for both acetate and benzoate-degrading sludge was consistently 0.16 - 0.18. Under the same extraction conditions, the amount of ECP extracted from the acetate-degrading sludge consistently amounted to 40 - 45% of that extracted from the benzoate-degrading sludge. This may likely be the reason that the latter sludge formed superior quality of granular sludge in UASB (upflow anaerobic sludge blanket) reactors (Fang & Jia, 1996).

C. Propionibacteria as ECP producers

The genus *Propionibacterium* also belongs to the group of micro-organisms that produce slime (Sherman & Shaw, 1921). The yield of exopolysaccharides produced by propionibacteria is not a direct function of growth (Cerning, 1995) and in comparison with other micro-organisms such as leuconostocs, dairy propionibacteria are very low exopolysaccharide producers (Cerning, 1995; Sherman & Shaw, 1921). The exopolysaccharide yields produced by propionibacteria are in the range of milligrams rather than grams. This makes the analytical approach and the isolation of exopolysaccharides from complex fermentation media particularly difficult (Cerning, 1995).

Dairy propionibacteria produce heteropolymers composed of repeating units varying in size from disaccharides to heptasaccharides (Cerning, 1995). The exopolysaccharides isolated from a fermentation medium of *P. freudenreichii* subsp. *shermanii*, contained three fractions with a molecular mass of 200 - 350, 350 - 450 and 5 x 10^3 (Crow, 1988), while that of *P. acidipropionici* had a molecular mass of 5.8 x 10^3 (Racine *et al.*, 1991). The exopolysaccharides of the latter bacteria, were composed of a water-soluble and a water-insoluble fraction. The soluble fraction was composed of 22% rhamnose, 10% mannose and 34% of galactose and glucose. The water-insoluble fraction of the exopolysaccharides was composed of 7% fucose, 22% rhamnose, 40% galactose and 31% glucose. It was not certain whether two distinct exopolysaccharides were produced (Racine *et al.*, 1991).

The exopolysaccharide produced by *P. freudenreichii* subsp. *shermanii* contained high proportions of methylpentoses along with large amounts of mannose and lesser amounts of glucose and galactose (Skogen *et al.*, 1974). Similarly, "*P. zeae*" P74 produced exopolysaccharides composed of large amounts of mannose and lesser quantities of glucose and galactose (□erná & Vàña, 1982; Skogen *et al.*, 1974).

Skogen (1970) found that within limits of strain variability, the pH of the medium, incubation temperature and nitrogen source were the main factors effecting the slime formation of propionibacteria. These bacteria can produce exopolysaccharides on a glucose-based medium (Reddy *et al.*, 1973) and also on

whey supplemented with yeast extract (Crow, 1988). However, when *P. acidipropionici* was grown in a whey-based medium, a decline in viscosity and exopolysaccharide yield was observed (Racine *et al.*, 1991). The influence of different carbon sources on slime production are summarised in Table 1. Sucrose appeared to be a better carbon source than sodium lactate for slime production. This is probably because the precursors (sugar units) must first be synthesised before being used as building blocks for extracellular slime. Skogen (1970) found that the slime production of "*P. zeae*" P74 was aided by heat treatment of sucrose. The heat treatment probably caused the breakdown of sucrose to glucose and fructose that may be more readily usable in the production of slime (Skogen, 1970).

Exopolysaccharide production by propionibacteria has been found to be greater at lower growth temperatures (Cerning *et al.*, 1992; Racine *et al.*, 1991). Among the species tested for exopolysaccharide production, some produced more polysaccharides at 25°C, a temperature slightly lower than their optimal growth temperature (Racine *et al.*, 1991). "*P. zeae*", for example, showed an increase in viscosity of over 100% when grown at 15°C rather than at 21°C and also "*P. arabinosum*" when it was grown at 21°C instead of 32°C (Skogen *et al.*, 1974). At lower temperatures the cells were growing more slowly, and thereby the cell wall polymer formation was slower and more isoprenoid phosphate was made available for exopolysaccharide synthesis (Sutherland, 1972 and 1982). It can also be because the enzymes involved in the synthesis of slime, may have a lower optimum temperature for activity (Skogen, 1970).

Skogen (1970) found that when "*P. zeae*" P74 was incubated at 15°C, it reached maximum viscosity in 21 days, the viscosity then decreased until the 180th day. This indicated that some degradation of the exopolysaccharides had taken place after the maximum viscosity had been reached. It is probable that the breakdown of slime exceeded the production after the 21st day and that the result was a decrease in viscosity. This breakdown could have either been caused by the incubation temperature, pH of the broth, autolysis of cells releasing degradative enzymes into the broth, or a combination of these factors. Skogen (1970) also showed that the cell numbers of "*P. zeae*" P74 and "*P. shermanii*" P19 had reached the stationary phase of growth before the 21st day when it was incubated at 15°C.

Table 1. Effect of different carbon sources on slime production in selected strains of *Propionibacterium* (Skogen, 1970) (data given as an increase in the relative viscosity of a sterile broth).

	Species			
Carbon Source	***"P. arabinosum"* P42**	***"P. arabinosum"* P9**	***"P. zeae"* P74**	***P. freudenreichii* P6**
Sucrose	0.01	0.07	0.78	0.00
Galactose	0.01	0.57	2.37	0.08
Arabinose	0.01	0.09	0.16	0.03
Mannose	0.00	0.05	0.71	0.06
Mannitol	0.02	0.06	0.61	0.00
Maltose	0.02	0.19	3.89	0.02
Lactose	0.05	0.08	0.04	0.00
Sorbitol	0.04	0.06	0.06	0.00
Starch	0.00	0.13	0.40	0.00
Na-lactate	0.05	0.08	0.11	0.05
Rhamnose	0.02	0.03	0.05	0.00
Glucose	0.03	0.08	1.01	0.44
Fructose	0.02	0.06	0.69	0.12
Raffinose	0.03	0.15	8.05	0.03
Xylose	0.02	0.00	0.04	0.00
Yeast extract	0.01	0.01	0.04	0.00

Slime production is not favoured by acid conditions (Skogen, 1970). "*P. shermanii*" formed bulky biomass even in extreme alkaline media, especially in media with aseptically added glucose. This could be caused by morphological changes in the media. At a pH value of 8.5 and higher, the cells began to form slime, probably as protection against intoxication by excess free ammonia (□erná & Vàña, 1982). At a lower initial pH, self limiting acid production was reached more quickly, resulting in less slime formation and less growth.

Skogen (1970) found considerable strain variation in response to NaCl concentrations. The greatest slime production occurred when the medium was devoid of NaCl for "*P. zeae*" P74, whereas *P. freudenreichii* P56 produced the maximum amount of slime at a concentration of 3% NaCl.

At present the function of exopolysaccharides produced by propionibacteria has not been investigated in depth. Exopolysaccharides produced by three species of propionibacteria (Reddy *et al.*, 1973) did not induce detectable precipitating antibody formation in rabbits and it was concluded that the exopolysaccharides did not have immunological properties. Capsules may, however, be advantageous for propionibacteria to protect against possible attack from bacteriophage by covering the receptor sites, to protect against moisture loss because of the hygroscopic nature of capsular material, and as an aid to dispersal (Skogen, 1970; Wilkinson, 1958).

Because dairy propionibacteria have the potential to ferment low-value substrates such as whey, they can possibly be used to upgrade this dairy by-product by lactose fermentation, with concurrent production of a useful polymer for food and non-food uses (Racine *et al.*, 1991).

D. Importance of ECP

A feature of many bacteria is the production of ECP. These polysaccharides may be organised into distinct structures like capsules, or may be excreted as extracellular slime. However, this distinction is arbitrary and in practise may be of no functional significance (Boulnois & Roberts, 1990).

Whilst much of the market is still currently taken up by plant or animal derived gelling agents, the susceptibility of the former to climatic influents and the unsuitability of the latter in non-meat products, suggests a long term future for microbial derived polysaccharides with the appropriate physico-chemical properties (Roberts, 1995). Disadvantages of the use of polysaccharides as gelling agents, are the high costs involved in screening programs, the production costs and also the difficulties involved in obtaining food usage clearance which is not only costly, but requires justifying the need for a new polymer as well as proving that it is safe (Morris, 1992).

Bacterial fermentation's provide a source of new polysaccharides often with unique functional properties, with the additional prospects of stable cost and supply together with a reproducible structure and function (Griffin *et al.*, 1996; Morris, 1990). The commercial usefulness of most industrial polysaccharides is based on their ability to alter the basic properties of water. They can be used as stabilisers, suspending agents, film-forming agents, water-retention agents, coagulants, colloids and also as gelling agents and lubricant or friction reducers. Other uses include micro-encapsulation, blood plasma substituents and also uses in laundry products, textiles, adhesives and the paper and paint industries (Griffin *et al.*, 1996; Roberts, 1995; Smith & Pace, 1982; Sutherland, 1994). Hassen *et al.* (1996) showed that the use of ropy cultures produced a gel structure with more bonds than the non-ropy strains, possibly because of interactions among polysaccharides or between protein and polysaccharides. However, the use of ropy strains resulted in yoghurt with a lower value for yield stress than that of yoghurt made with uncapsulated non-ropy strains. The ropy strains were also encapsulated, and the capsule, in addition to the slime, could reduce interactions between proteins. These interactions seem to be the strongest bonds within the gel structure, and resulted in the high value for yield stress of the gels made with uncapsulated cultures (Hassen *et al.*, 1996).

As stabilisers, polysaccharides are widely used in dried products that are reconstituted by the addition of water (Roberts, 1995). Microbial polysaccharides such as xanthan and alginate are used as gel formers to change the texture of food (Morris, 1990). The presence of these polysaccharides prevents the subsequent phase separation.

The presence of exopolysaccharides in fermented milk improves the consistency and viscosity of the final product and decreases the susceptibility of wheying off (Cerning, 1990; Teggatz & Morris, 1990). One of the main problems in this field is the transitory nature of the thickening trait. This instability is not yet completely understood. Capsular and ropy slime both influence the rheological properties of yoghurt, each produces different effects, but unlike capsular polysaccharides, slime polysaccharides produce a stretchable structure (Hassen *et al.*, 1996). It is obvious the exopolysaccharides are necessary to increase viscosity and improve the texture of fermented milks, but the magnitude increase varies because of differences in culture strains, incubation conditions, total solid content and viscosity measurements. Moreover, viscosity may not only be effected by the amount of exopolysaccharides released, but also by an exopolysaccharide with a slightly different structure, resulting in different rheological characteristics of the medium (Shellhaass & Morris, 1985; Teggatz & Morris, 1990).

Most microbial exopolysaccharides are relatively hygroscopic compounds and will retain some water when subjected to freeze-drying and similar techniques. However, they can be used to form tough resilient films on evaporation of water and they also have the capacity to bind and retain water, a property of commercial value in the cosmetic industry. One of the most efficient moisture-retaining agents is hyaluronic acid, which is capable of retaining up to 6 000 times its own weight, i.e. one polymer can retain six litres of water (Sutherland, 1994).

ECP plays a key role in waste water treatment where they are important for the removal of pollution from waste water (Dignac *et al.*, 1998) and for the flocculation of bacterial cells (Fang & Jia, 1996; Tenney & Stumm, 1968; Urbain *et al.*, 1993). It also has a large influence on the activated sludge floc structure and may also have an impact on sludge treatment (Dignac *et al.*, 1998).

The ECP is critical for the granulation of anaerobic sludge (Riedel & Britz, 1993), which is the key element for the success in the UASB (upflow anaerobic sludge blanket) waste water treatment technology (Lettinga *et al.*, 1980; Li *et al.*, 1995). The main function of ECP within the granule is thought to be linked to the formation of bacterial aggregates and as the mediator of cell to cell adhesion (Shen *et al.*, 1993). This is a similar role to that played by ECP in the aerobic flocs of activated sludge. In both cases, the high molecular weight polymer is able to

bridge between different microbial surfaces to form a three-dimensional floc matrix and, therefore, the amount of ECP is one of the factors which can influence the stability of granules (Forster & Quarmby, 1995). Results obtained by Forster & Quarmby (1995) also suggested that the stronger granules could be associated with higher ECP yields and a greater percentage of carbohydrate in the ECP. As the amount of ECP present in the granule increases, flocs became more stable and resistant to break-up. However, too much ECP may cause a deterioration in floc formation as ECP has a negative charge and therefore, repulsion can occur. The strength of flocs can also be influenced by the waste water composition as well as by physical, biological and biochemical factors (Forster & Quarmby, 1995).

A variety of natural roles have been suggested for bacterial polysaccharides, many of which involve protection of the host in one way or another. It serves as protection against unfavourable effects, such as desiccation (Ophir & Gutnick, 1994) or may act as receptor sites for bacteriophage (Lin *et al.*, 1994) and conversely, differentiation of the polysaccharide structure may block or mask bacteriophage attachment (Lin *et al.*, 1994). Similarly, exopolysaccharides together with the lipopolysaccharide O-antigens, constitute the principal immunogens and antigens of bacteria (Bishop & Jennings, 1982). The secreted slimes and capsules are also important virulence factors whose role is to protect the organism from phagocytosis (Dudman, 1977). Exopolysaccharides have been implicated in the selective and non-selective adhesion of bacteria to surfaces, including inert surfaces, cultured human mucoid epithelial layers and plant cell walls (Costerton *et al.*, 1974; Costerton *et al.*, 1987; Zottola, 1994). Exopolysaccharides may also trap soluble nutrients (Forster & Quarmby, 1995) and accumulate moisture and thereby preventing cell dehydration. In the case of soil micro-organisms, the exopolysaccharides help to retain water in soils, promote soil adhesion and inhibit soil erosion (Lynch & Bragg, 1985).

Spoilage lactic acid bacteria of the genus *Pediococcus* found during wine and beer manufacture, can produce small amounts of β-glucan, which is very undesirable and makes the product unsuitable for consumption. The quantity produced varies from 50 - 100 $mg.l^{-1}$ when the medium contains glucose, and 5 to 10 $mg.l^{-1}$ in the presence of various sugars (Llauberes *et al.*, 1987). It was shown by Korkeala *et al.* (1988) that two homofermentative lactobacilli and a *Leuconostoc*

strain, produced slime on vacuum-packed cooked sausages, even in the absence of added sugar. Again the end-product becomes unsuitable for consumption.

Studies of such 'natural' polysaccharides offer the possibility of improving yields, genetically modifying polysaccharide structure to improve functions, and transferring the production to other starter bacteria to optimise the properties of the fermented food product by 'building-in' textural characteristics. Such polysaccharides could be extracted for use as more 'natural additives', partially extracted for use as novel functional ingredients, or produced *in situ* to texturise given food products (Morris, 1993).

D. Regulation and Genetic Control of ECP

The synthesis of the bacterium capsular polysaccharides varies from species to species, strain to strain, growth medium, growth temperature, carbohydrate source, pH and genetic background. In general, heteropolysaccharide-producing micro-organisms require an intracellular substrate for polymer production, therefore any system that regulates substrate uptake is likely to influence exopolysaccharide synthesis. Ideally, as much substrate as possible should be taken up into the cell and be converted to polysaccharides rather than be converted into cell material or catalysed to products excreted from the cell. In fact, very high conversions of substrate into polysaccharides can be achieved, so nutrient uptake may not always be a limiting factor (Cerning, 1990).

The presence of an outer membrane protein associated only with capsulated strains of *Escherichia coli* suggested that this protein may be responsible for some aspects of capsule synthesis (Paakenen *et al.*, 1979). Since exopolysaccharide synthesis primarily involves processes within the cytoplasmic membrane or the cytoplasm, it is difficult to rationalise a role for outer membrane proteins in polymer production (Sutherland, 1985). It is more likely that such proteins would have a role in either excretion or attachment of exopolysaccharides and that there is some regulatory control of the manufacture and availability of intermediates. Two cell surface proteins were found to be present in a slime-forming mucoid variant of *Lactococcus lactis* subsp. *cremoris*, but they were absent from the non-mucoid

variants. These two proteins may have some role in slime secretion as enzymes or as attachment site or they may likewise be part of the slime material itself (Cerning, 1990).

Dextransucrases of *Leuconostoc mesenteroides* are inducible enzymes, whereas the corresponding enzymes from *Streptococcus sanguis* and *Streptococcus mutans* are constitutive and are produced in the absence of sucrose. In species that produce the constitutive enzyme, its formation is associated with the logarithmic phase of bacterial culture growth. Addition of inhibitors of protein synthesis caused cessation of dextransucrase production (Sutherland, 1985). A possible plasmid involvement in the control of dextransucrase by *S. mutans* has been postulated (Montville *et al.*, 1978), however strains lacking plasmids, are still capable of glucan synthesis and very few strains from human carcinogenic isolates, contain plasmid DNA (Cerning, 1990).

The spontaneous loss of slime-producing ability by lactic acid bacteria has also been related to the involvement of plasmid-encoded genes (Cerning *et al.*, 1994; Vescovo *et al.*, 1989). This is true for mesophilic lactic acid bacteria (Neve *et al.*, 1988; Vescovo *et al.*, 1989), but does not seem to be true for exopolysaccharide-producing thermophilic lactic acid bacteria, which often do not contain plasmids (Vescovo *et al.*, 1989). Propionibacteria are known to contain plasmids (Rehberger & Glatz, 1990), but their functions have not clearly been established. Therefore, the question whether the exopolysaccharide-producing ability of propionibacteria is plasmid-encoded cannot be answered yet (Cerning, 1995).

Induced or increased slime and capsule synthesis was found in *Lc. lactis* subsp. *cremoris,* when it was grown in the presence of ρ-fluorophenylanaline (Forsèn & Häivä, 1981). This effect has been interpreted in *E. coli* to be mediated through the production of an inactive repressor, thus enabling depressed synthesis of several enzymes (Markovitz, 1977).

The structural genes involved in polysaccharide biosynthesis are clustered in a large number of Gram-negative and Gram-positive organisms (Arrecubieta *et al.*, 1994), for example *E. coli* (Trisler & Gottesman, 1984), *Pseudomonas solancearum* (Coplin & Majerczak, 1990) and *Acetobacter xylinum* (Standal *et al.*, 1994). Regulation of the synthesis of capsules occurs at the level of transcription of the

genes necessary for capsular synthesis. In *E. coli* it is the *cps* genes (Gottesman, 1995). The regulatory strategies used for colanic acid biosynthesis, an extracellular exopolysaccharide produced by *E. coli* K12 under appropriate growth conditions, usually serves as the model for the regulation of capsule synthesis. The *cps* gene cluster's products function in polymerisation, transport and modification of the polysaccharides. Transcriptional regulation of these genes is dependent on at least four positive regulators, RcsA, RcsB, RcsC and RcsF and two negative regulators, RscA and Lon. None of these regulators appears to effect the synthesis or the other regulators, indicating that any interactions among the regulators probably occur at protein level (Stout & Gottesman, 1990).

According to the model of Gottesman & Stout (1991) for the synthesis of colanic acid in *E. coli*, there are two pathways that regulate the synthesis of colanic capsular polysaccharides (Fig. 1). Colanic acid, or M antigen, causes cells to become extremely mucoid (Goebel, 1963). RscA and Lon compose one regulatory pathway and are conserved in a number of organisms. RcsA functions as an unstable, positive regulator of the *cps* genes of *E. coli*, and as a substrate for Lon. RcsA is rapidly degraded by the Lon protease and is normally limiting in cells (Torres-Cabassa & Gottesman, 1987). The stability of RcsA is enhanced in cells lacking Lon protease activity. One result of a *lon* mutant is that the cells are very mucoid (Dierksen *et al.*, 1997). Gupte *et al.* (1997) speculated that RcsA may have two roles: first it interacts with the *cps* promoter itself and/or interacts with phosphorilated RcsB and helps RcsB bind the promoter region to highly stimulate capsule expression. RcsA may also effect RcsC-RcsB interaction, but not RcsB-RcsC interaction.

Recent studies showed that RcsA and RcsB are heterodimers (Lopez Torrez, 1995). This suggests that RcsA and RcsB may bind the *cps* promoter region together and activate transcription. If Lon is not present, enough RcsA accumulates to stimulate *cps* gene expression either directly or indirectly by functioning in the RcsB-RcsC interaction to allow RcsB to stimulate capsule expression (Fig. 1). Stout *et al.* (1991) showed that both RcsA and RcsB, contain a possible helix-turn-helix motif. This information favours a model in which RcsA and RcsB both bind DNA and RcsA increases the activity of RcsB, either by the binding of RcsB or by promoting an active conformation of RcsB. RcsC and RcsB share

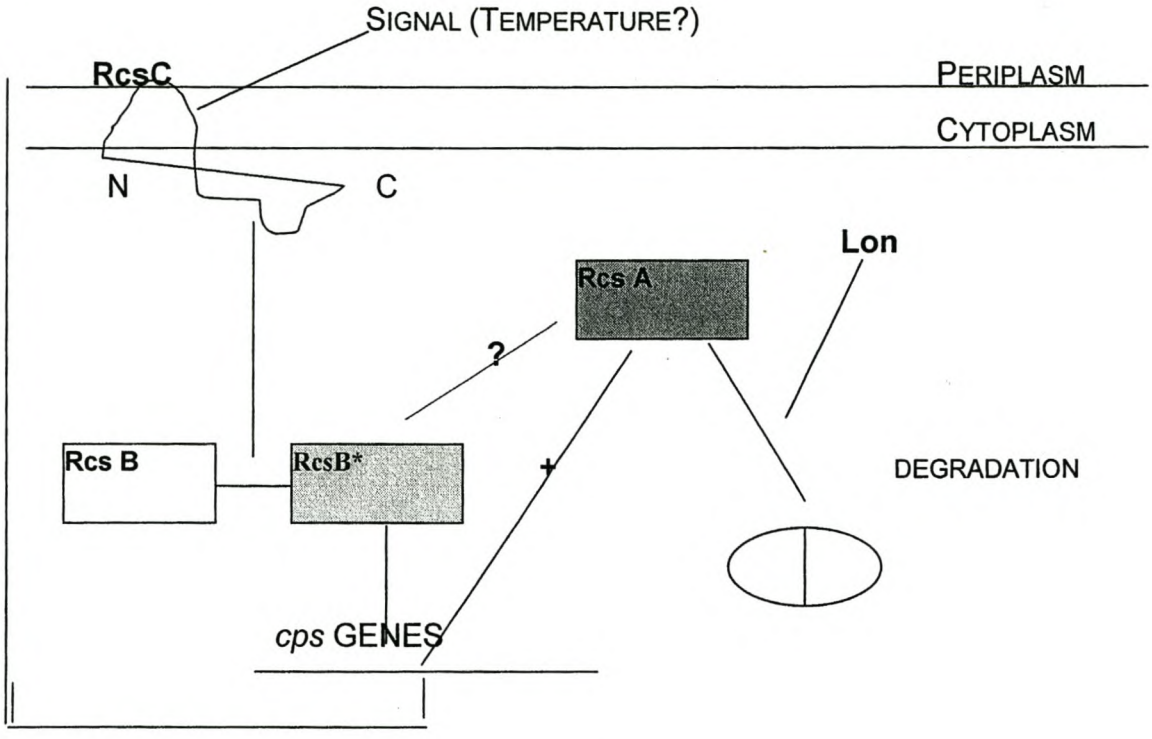

Figure 1. Model of capsule regulation in *E.coli* (Stout & Gottesman, 1990).

[Lon: intracellular, ATP dependent serine protease and negative regulator of *cps* genes.

RcsA: unstable, positive regulator of *cps* genes and substrate for Lon.

RcsB: bind promotor region of cps genes en activates genes to transcript for capsule expression (only maximum level when RcsA is present.

RcsC: Membrane kinase that, when it receives a signal (eg. A shift to a lower temperature), transmit signal to rcsB (perhaps phosphorilation or dephosphorilation).**]**

sequence homology with other two-component regulator pairs in the appropriate domains.

Synthesis of Vi antigen, a capsular polysaccharide expressed by *Salmonella typhi*, is controlled by the *viaA* and *viaB* chromosomal loci (Virlogeux *et al.*, 1996). It is known that Vi antigen expression is regulated by a system similar to the *rcs* regulatory system involved in colanic acid synthesis in *E. coli* (Houng *et al.*, 1992). The RcsA protein was not involved in Vi antigen synthesis. In contrast, the RcsB protein acted as a positive regulator of Vi polysaccharide expression. RcsB or TviA did not influence the transcriptional start point of *tviA* mRNA.

In the absence of RcsB or TviA proteins, transcription of *tviA* gave rise to only a monosistronic *tviA*-spesific mRNA. The presence of RcsB and TviA not only increased the amount of monosistronic *tviA*-spesific mRNA, but also resulted in co-transcription of *tivA* and *tviB*, which is located immediately downstream of *tviA* on the *viaB* locus. In addition, the TviA protein did not appear to be subjected to degradation by the Lon protease. These results strongly suggested that TviA might act in concert with RcsB at the *tviA* promoter to activate transcription of the genes involved in Vi polymer synthesis in *S. typhi* in a Lon-dependent manner (Virlogeux *et al.*, 1996).

Structural evidence suggested that Lon protease is present in a number of Gram-negative organisms (Dierksen *et al.*, 1994; Nobuki *et al.*, 1993), Gram-positive organisms (Dierksen *et al.*, 1994; Rietdorf *et al.*, 1994), yeasts (Suzuki *et al.*, 1994), and all human tissue types tested (Adams *et al.*, 1992; Wang *et al.*, 1993). This data strongly suggested that it has an ubiquitous nature. Mutations, or a deletion of the *lon* gene of *E. coli* (Gottesman, 1995) and *Erwinia* species. (Eastgate *et al.*, 1995) increased expression of exopolysaccharides. A similar role of Lon-protease has been found for *E. amylovora* (Eastgate *et al.*, 1995). RcsB has been found as an important activator of amylovoran synthesis (Bereswill & Geider, 1997).

RscA has been identified in a few Gram-negative bacteria (Allen *et al.*, 1987; Coleman *et al.*, 1990; Dierksen *et al.*, 1994), but in only one Gram-positive batch (Dierksen *et al.*, 1994). Complementation of an *E. coli* or *S. amylovora* restores

exopolysaccharide expression (Torres-Cabassa *et al.*, 1987). The *rcsA* gene was characterised in detail for its role in the synthesis of colanic acid of *E. coli*, amylovoran of *E. amylovora* (Bernhard *et al.*, 1990) and stewartan of *E. stewartii* (Painbeni *et al.,* 1993). These observations suggested that a common regulatory pathway for polysaccharide expression exists in these organisms. Conservation of Lon, RcsA or both in lactococci suggests that this common regulatory pathway exist in Gram-positive organisms as well as in Gram-negative organisms (Dierksen *et al.*, 1994).

On the basis of a number of biochemical and genetic criteria, *E. coli* capsules were divided in at least three groups of gene clusters (I, II and III) (Pearce & Roberts, 1995; Stevenson *et al.*, 1996). Studies revealed that that there is a common genetic organisation consisting of three functional regions (Boulnois & Roberts, 1990), as can be seen in Fig. 2. Two of these regions, 1 and 3, are common to all the group II capsule gene clusters so far analysed and flank a central serotype-specific region 2. Regions 1 and 3 encode functions that are needed for the cell-surface expression of group II capsules albeit that these polysaccharide molecules are chemically distinct (Roberts, 1995).

Region 3 was found to contain two genes, *kpsM* and *kpsT*, organised in a single transcriptional unit (Smith *et al.*, 1990). Analysis of the predicted amino acid sequences of KpsM and KpsT indicated that they are members of the family of ATP-binding cassette (AMC)-type transporters and may comprise an inner-membrane polysaccharide-export system (Pavelka *et al.*, 1991 and 1994).

Analysis of region 1 showed the presence of six genes, *kps* FEDUCS, organised in a single transcriptional unit (Pazzani *et al.*, 1993a and b). The precise function of all the proteins encoded within region 1 is not yet fully elucidated although computer aided database searches have revealed homologies to other proteins involved in the expression of polysaccharide capsules in other bacteria. This indicated that there must be functional conservation in the export of capsular polysaccharides in these Gram-negative bacteria. The periplasmic location of polysaccharide in *kpsE* and *D* mutants suggests a role for these two proteins on the export of polysaccharide onto the cell surface (Bronner *et al.*, 1993).

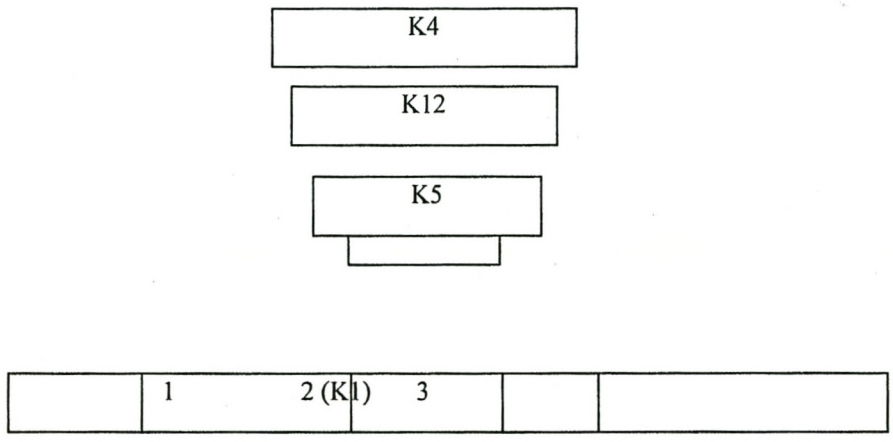

Figure 2. Schematic representation of the organisation of group II capsule gene clusters. The K1 capsule gene cluster is shown with the three functional regions. Boxes labelled K92, K5, K12 and K4 represent the serotype-specific region 2s that are inserted between conserved regions 1 and 3 (Roberts, 1995).

The periplasmic location of the KpsD protein that is anchored to the inner membrane with a large periplasmic domain (Rosenow *et al.*, 1995), are in keeping with this notion. The kpsC and S proteins are located in the cytoplasm associated with the inner face of the cytoplasmic membrane (Roberts, 1995). Roberts (1995) also speculated that the KpsC and S proteins may be involved in the attachment of 2-keto-3-deoxy-octonate (KDO) to phosphatidic acid and the subsequent ligation of the phosphatidyl-KDO to the reducing terminus of the polysaccharide prior to export across the cytoplasmic membrane by KpsM and T. If this is the case, this would infer that the presence of phosphatidyl-KDO at the reducing terminus of group II *E. coli* polysaccharides motif recognised by the proteins involved in polysaccharide export. This is an appealing notion since it might explain how a conserved set of proteins could export chemically different polysaccharide molecules independent of the repeat structure of the polysaccharide.

The *kpsU* gene within region 1 encodes a functional CMP-KDO transferase enzyme (Pazzani *et al.*, 1993a and b). This enzyme acts as a capsule-specific CMP-KDO synthetase enzyme to provide the CMP-KDO for the attachment of KDO to phosphatidic acid. The presence of this gene within region 1 of group II capsule gene clusters explains why strains expressing group II capsules have elevated levels of CMP-KDO synthetase activity (Finke *et al.*, 1990).

In contrast to the conservation of the regions 1 and 3 between different group II capsule gene clusters, the specific region 2 appears unique for a particular K antigen (Boulnois & Roberts, 1990; Petit *et al.*, 1995). It contains four genes encoding proteins for the biosynthesis of the K5 polysaccharide. The KfiC protein would appear to be the K5 transferase enzyme which adds the alternating sugar residues to the growing polysaccharide chain, whilst the KfiD protein is an UDP-glucose dehydrogenase enzyme which catalyses the production of UDP-glucuronic acid, a component of the K5 polysaccharide (Petit *et al.*, 1995). The functions of the KfiA and B proteins are so far unknown, but it may be that they participate in the initial stages of polysaccharide biosynthesis (Roberts, 1995). By bringing together all these data, it has been possible to propose a model to explain K5 polysaccharide biosynthesis and how group II polysaccharides may be exported onto the cell surface in *E. coli*. There are still a lot of unanswered questions on the

production and genetic control of ECP production and much research needed still to be done before it will be fully understood.

E. Role of Propionibacteria in the UASB Granulation Process

The direct treatment of waste waters was greatly stimulated by the development of the upflow anaerobic sludge blanket (UASB) process (Lettinga *et al.*, 1980; Weiland & Rozzi, 1991) and its successful full-scale application (Rinzema *et al.*, 1993). The UASB design containing granular sludge (Chynoweth, 1987), has permitted high space loading rates (30 $kgCOD.m^{-3}$) to be achieved at low hydraulic retention times (6 - 24 h) and this implies that smaller digester volumes are required (Iza *et al.*, 1991). However, one of the main problems still remaining in the application of the UASB process, is the extensively long start-up periods (Goodwin *et al.*, 1992). With non-carbohydrate wastes, it can take a few months before a highly effective granular sludge blanket can be cultivated. Since the operational efficiency and performance of these systems are mainly dictated by the formation, amount and specific activity of the granules, the potential use of the UASB is limited by the extended start-up periods (TJ Britz, 1999, Personal communication).

The mechanism of granule formation is still poorly understood (Slobodkin & Verstraete, 1993) and it would appear that granule sludges can only be formed with certain types of carbohydrate/protein containing waste waters generated in the agricultural and food processing industries. With other types of waste waters, granulation has not been possible (Sam-Soon *et al.*, 1991). In many cases, even after seeding the systems with granules it has been found that in time these have disintegrated or washed out (Sorensen *et al.*, 1991). This clearly restricts the general application, unless the granulation reaction can be induced. Full exploitation of this cell immobilisation method can thus not be realised until the granule formation conditions are defined. The granulation process appears to be a unique type of bioflocculation, with is similar to an agglutination reaction as induced by polymers. Moosbrugger *et al.* (1992) concluded that the granules are formed by the generation of an extracellular polypeptide produced by a hydrogenotrophic methanogen of the genus *Methanobacterium*. In contrast, Vanderhaegen *et al.*

(1992) found the granules rather to contain equal amounts of extracellular proteins and carbohydrates. According to Riedel & Britz (1993) and Slobodkin & Verstraete (1993), these compounds are produced by the propionate forming acidogens that are effective slime and aggregate formers.

During stable state, the anaerobic digestion process requires the concerted action of various microbial metabolic groups. Under these balanced operational conditions no lactate and very little propionate can be detected in the UASB digester. However, when 'stress' conditions are put on a digester treating carbohydrate rich waste waters, the first metabolite that appears is propionate (Myburg & Britz, 1993), while simultaneously, hydrogen can be detected in the gas phase and lactate starts to accumulate (Eng *et al.*, 1986). These metabolic changes result in a shift of the population dynamics of the anaerobic community. This was confirmed by Riedel & Britz (1993). Subsequently, slime producing and aggregate forming *Propionibacterium* strains can be isolated under these 'stress' conditions (TJ Britz, 1999, Personal communication). We have found that under these unbalanced 'stress' conditions granule formation is stimulated. The increase in the lactate concentration, as result of the unbalanced conditions, results in an orderly shift between the predominant lactate-utilising species. This possibly begins with the acid sensitive *Veillonella* and *Selenomonas* genera and then being superseded by the more acid tolerant *Propionibacterium*. According to Riedel & Britz (1993) these *Propionibacterium* strains gain a competitive advantage during the 'stress' condition, as they obtain a maximum of ATP per mol of lactate fermented. Once they have the advantage at the lower pH, they start producing extracellular compounds, with the subsequent formation of aggregates (Vanderhaegen *et al.*, 1992). The production of the extracellular polymers by the propionate producing bacteria (Slobodkin & Verstraete, 1993) could contribute directly to the formation of the highly settleable granules found in efficient operating UASB digesters.

In order to shorten the start-up period of the UASB process, the need exists to stimulate the aggregation of microbes into granules. A faster UASB digester start-up period as well as the exploitation of the UASB technology in the treatment of difficult degradable waste waters will be of great value in conservation of our water resources.

F. Conclusions

ECP's are an incredibly diverse range of biologically important molecules and they play vital roles in mediating a broad range of biological processes. Clearly, a greater understanding of how polysaccharides are synthesised and exported in bacteria will be important in allowing the synthesis of polysaccharides of industrial and pharmaceutical importance. New areas for research include altering and controlling the composition and molecular weight of polymers, for example, by manipulating culture conditions or mutation and selection techniques.

ECP production by diary propionibacteria has not been investigated in depth. However, Sherman already noted in 1921 that: "In suitable nutrient broth a heavy slimy growth occurs at the bottom and the whole broth becomes turbid, with the exception of a narrow zone at the surface". Environmental factors such as pH and incubation temperature play a definite role in the amount of ECP produced, but more work needs to be done on the influence of other environmental factors, growth conditions and strain differences. There are also still major gaps in our knowledge in what governs the structural organisation of ECP in a given organism and how can the instability of the ECP-producing trait be explained? It can be speculated that the natural function of ECP production by propionibacteria is protection against unfavourable growth conditions and possible attack by bacteriofage, but more research are needed to confirm this. Propionibacteria have also been shown to play an important role in the granulation process of UASB systems. However, more research is still needed to determine the influence of ECP and the presence of propionibacteria on the granulation process of anaerobic digesters.

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CHAPTER 3

INFLUENCE OF ENVIRONMENTAL FACTORS ON EXTRACELLULAR POLYSACCHARIDE PRODUCTION BY PROPIONIBACTERIA

Summary

Certain strains of propionibacteria have the ability to produce extracellular polysaccharides (ECP) under specific environmental conditions. It is known that the fermentation conditions (temperature and incubation time) and the medium composition (carbon and nitrogen sources) effect the yield of ECP produced. In this study the influence of incubation temperature (18°C, 22°C, 25°C and 30°C), initial pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) and incubation period on the ECP producing ability of different strains of *Propionibacterium* were studied. It was found that *Propionibacterium jensenii* strain 278 produced the most ECP. This strain was originally isolated from an anaerobic digester, and thus had the potential to be used to improve the granulation process in UASB reactors. The ECP production was the highest under temperature conditions that were not the optimum for growth (22°C = 0.72 $mg.ml^{-1}$ compared to 30°C = 0.35 $mg.ml^{-1}$) and higher sucrose concentrations (22°C, pH 7.0, 4% sucrose = 2.3 $mg.ml^{-1}$ and 22°C, pH 7.0, 8% sucrose = 2.9 $mg.ml^{-1}$). Further analyses, using a 4 x 2 x 4 factorial design, identified specific positive trends in the ECP production at the different environmental conditions. A positive trend was observed when the main effect of sugar concentration was studied. The influence of the initial pH on the main effect was not very prominent, but the trends obtained indicated that an initial pH near 7.0 would be more positive for ECP production. The main effect from 22°C to 30°C was the most negative, indicating that the temperature range around 22°C need further investigation to optimise the ECP production. The two-factor interactions again indicated a positive trend in terms of higher pH and sucrose concentration and it was found that a pH of higher than 7.5, but lower than 8.5 would be more in the optimised range. It could also again be seen that a temperature range between 22°C and 25°C leads to the optimisation of the ECP production. The three-factor interactions confirmed that the optimum conditions for ECP production is between 22° and 25°C and also pH 8.0

and 8.5 and a sucrose concentration of 8%. No proteins could be found in the ECP, indicating that the ECP probably only consisted of carbohydrates. ECP analysis and further analyses of the complex ECP polysaccharide isolated from the *P. jensenii* strain 278 indicated that the ECP consists out of in mannose, glucose and galactose.

Introduction

The genus *Propionibacterium* is an important bacterial group, widely distributed not only in dairy products where they influence the characteristic flavour and eye production of Swiss-type cheese (Rehberger & Glatz, 1990), but also as predominant members of the microbial population of the human skin (Brook & Frazier, 1991). The "classical" (dairy) propionibacteria are used as starter cultures in the production of Swiss-type cheese and for several other industrial fermentations (Glatz, 1992). During fermentation, these bacteria can produce large amounts of vitamin B_{12}, bacteriocins and organic acids (Glatz, 1992) that can also serve as natural antibacterial and antifungal agents (Marcoux *et al.*, 1992).

One of the characteristics of the dairy propionibacteria is their ability to produce extracellular polysaccharides (ECP) (Crow, 1988; Reddy *et al.*, 1973; Riedel & Britz, 1993) under specific cultural conditions (Skogen, 1970). The function of the ECP in propionibacteria has not been investigated in depth (Cerning, 1990), but natural roles ascribed to the ECP are the ability to trap soluble nutrients, increase pathogenicity or decrease the susceptibility to phagocytosis and the mediation of adhesion of bacteria in natural ecosystems (Costerton *et al.*, 1987). The amount of ECP produced is dependent on the strain and the cultural conditions. In previous studies, it was shown that ECP formation was generally favoured by excess nutrient carbohydrates, low concentrations of nitrogen, lower growth temperatures and more neutral pH values (Sutherland, 1977; Wilkinson, 1958). Wilkinson (1958) also reported that mineral requirements strongly influenced the ECP production of different bacterial strains.

Riedel & Britz (1993) postulated that ECP producing propionibacteria probably play a role in the initial granule formation in upflow anaerobic sludge blanket (UASB) reactors, used in the treatment of industrial food effluents. The bacteria in the granules are surrounded by ECP and it is generally accepted that the formation of granules is correlated with the production of ECP (Forster, 1992; Li *et al.*, 1995; Morgan *et al.*, 1991). Nevertheless, the understanding of the precise role of ECP in the granulation of anaerobic sludge is still very limited (Jia *et al.*, 1996).

The aim of this study was to examine the influence of different environmental conditions (initial pH, incubation period, carbohydrate concentration and incubation temperature) on propionibacterial ECP production and to determine the optimum growth conditions for ECP production.

Materials and methods

Propionibacterial strains and culture preservation

The 19 *Propionibacterium* strains evaluated in this study are listed in Table 1 and were obtained from the University of Stellenbosch Food Science Culture Collection. These strains were cultivated in Yeast Extract Lactate medium (YEL-medium) which, in certain studies was supplemented with different sucrose concentrations. The YEL-medium consisted of ($g.l^{-1}$): yeast extract (Biolab) 5.0; lactate (Saarchem) (60% v/v) 20.0; peptone (Biolab) 2.0; KH_2PO_4 (Saarchem) 10.0; and Tween 80 (Merck) 1.0 ml. The pH was adjusted as required using a 1 M NaOH solution (Riedel & Britz, 1993) and the medium autoclaved for 15 min at 121°C and 100 kPa.

Microscopical examination of Gram-stained slides, as well as a study of the colony pigment and cell morphology of the strains after growth for 5 d on YEL-plates at 30°C, was done to ensure the purity of the strains.

Viscosity and final pH measurement

The viscosity of the different growth media inoculated with propionibacteria, was measured using a Brookfield viscometer (Model RTN 55878). This was done according to a modification of the method of Racine *et al.* (1991). The viscosity was measured at 30°C with the use of a number 1 spindle at a rotation rate of 100 r.p.m.

Table 1. The different *Propionibacterium* strains examined.

Culture	Strain	Isolated from
64	*P. jensenii*	Anaerobic digester
71	*P. jensenii*	Dairy products
F87	*P. jensenii*	Cheese
262	*P. jensenii*	Anaerobic digester
271	*P. jensenii*	Anaerobic digester
273	*P. acidipropionici*	Anaerobic digester
276	*P. thoenii*	Anaerobic digester
278	*P. jensenii*	Anaerobic digester
283	*P. jensenii*	Anaerobic digester
309	*P. freudenreichii* subsp. *shermanii*	Anaerobic digester
312	*P. thoenii*	Anaerobic digester
346	*P. acidipropionici*	Leerdammer cheese
347	*P. freudenreichii* subsp. *shermanii*	Leerdammer cheese
354	*P. jensenii*	Leerdammer cheese
356	*P. jensenii*	Leerdammer cheese
423	*P. freudenreichii* subsp. *freudenreichii*	Swiss cheese
447	*P. thoenii*	Dairy products
456	*P. acidipropionici*	Dairy products
1032	*P. freudenreichii* subsp. *shermanii*	Anaerobic digester

The results were obtained after a stabilisation time of 30 sec and were expressed in centipoise per sec. The change in pH that resulted from the production of propionic and acetic acid by the strains at the different incubation conditions, was measured with a Portamess 751 pH-meter.

Isolation of the ECP

The ECP was isolated from the bacterial cells using a modification of the method of Ludbrook *et al.* (1997). After the incubation period, cultures were centrifuged (Beckman J-21B) at 51 500 x g for 45 min at 4°C and the supernatant containing the ECP, was collected. The supernatant was centrifuged for a second time under the same conditions. One volume of cold 99.8% ethanol (Merck) was gently added to two volumes of the supernatant and a stringy white mass formed as the ethanol and supernatant mixed. This ethanol mixture was left overnight at 4°C. The precipitate was collected by centrifugation (12 900 x g for 60 min) in a MSE GF-6 centrifuge and then dissolved in deionised water. The ECP was dialysed against distilled water for 24 h and the water was changed five times during that period. The water content was then reduced under vacuum with a Büchi Rotavapor R-114 and the ECP material freeze-dried (Heta FD. 1.0) to remove any water that was still present. The final mass of the ECP was then determined.

Protein determination

The presence of proteins in the ECP was determined using the Pierce Bicinchonic acid (BCA) protein assay kit (Pierce, USA) according to the manufacturer's instructions. The method is based on the biuret reaction by which proteins react with Cu^{2+} in alkaline medium to yield Cu^{1+}. BCA is a sensitive, stable and specific reagent that reacts with the cuprous ion (Cu^{1+}) to produce a purple reaction product that is water-soluble and exhibits a strong absorbance at 562 nm. A set of protein standards of known concentration was prepared using a 2 $mg.ml^{-1}$ stock solution of bovine serum albumin (BSA) for use with the Standard Microtiter Plate Protocol (37°C for 30 min). Absorbance was read at 562 nm with a Titertek Multiscan PLUS microtiter plate reader (Labsystems, Finland).

Carbohydrate analysis

A modification of the method of Cato *et al.* (1970) was used for the analysis of the ECP sugars. A 0.3 g sample of ECP was hydrolysed in 1 ml of 2 N H_2SO_4 for 1 h in a boiling waterbath. When cooled, the acid was neutralised with solid barium hydroxide to a pH of 7.0. The precipitate was removed by centrifugation. The supernatant was evaporated in a boiling waterbath. One dimensional ascending thin-layer chromatography was performed on silica gel G plates (Merck) (Bolliger *et al.*, 1965). The hydrolysate (1 µl) or known standard (1 µl) was applied to the base line of the chromatogram plates and a solvent system consisting of ethyl acetate (65 ml) and a 35 ml isopropanol-water mixture (2:1), was used (Akhrem & Kuznetsova, 1965). The chromatograms were run for 3 h at 4°C. Spots were visualised by spraying with acetonic-aniline-phthalate (aniline 2.0 ml; phthalic acid 3.0 g; acetone 95 ml and 5 ml water) followed by heating at 105°C for 5 min.

Experimental design

In the first Experimental Study (I), the optimum incubation period for ECP production was determined (Table 2). Strain 1032 (*P. freudenreichii* subsp. *shermanii*) was used, as it visually appeared to produce a large amount of ECP. This strain was incubated in YEL-broth with 4% (m/v) added sucrose at 30°C for different time periods of up to 24 d. In the literature it was reported that strains of "*P. shermanii*" were found to optimally produce ECP at pH values > 8.5 (□ernà & Vàña, 1982), thus an initial pH of 9.0 was used.

In the second Experimental Study (II), the ECP production of different strains of propionibacteria that were visually identified as high ECP producers, was studied (Table 2). Strains 276, 278, 283, 346, 423 and 1032 were incubated for 20 d at 30°C and an initial pH of 8.5. Sucrose (4% m/v) was reported to enhance propionibacterial ECP production (Skogen, 1970; Sutherland, 1994) and was thus added to the YEL-medium.

Table 2. Experimental studies performed to determine the influence of different environmental factors on ECP production by *Propionibacterium* strains in YEL-medium.

	Study	Culture	Incubation time (d)	Initial pH	Incubation temperature (°C)	Sucrose concentration (%)
I	Incubation time	1032	4; 8; 12; 16; 20; 24	9.0	30	4
II	Different strains	276; 283; 423; 278; 346, 1032	20	8.5	30	4
III	Initial pH	278	20	5.5; 6.0; 6.5; 7.0; 7.5; 8.0; 8.5; 9.0	30	4
		283	20	5.5; 6.0; 6.5; 7.0; 7.5; 8.0; 8.5; 9.0	30	4
IV	Temperature	278	20	8.5	22; 25; 30	
V	Combinations	278	20	7.0	18; 22; 25;30	4; 8
				7.5	18; 22; 25; 30	4; 8
				8.0	18; 22; 25; 30	4; 8
				8.5	18; 22; 25; 30	4; 8

Based on the data obtained during Studies I and II, Experimental Study III (Table 2) was designed to determine the influence of the initial pH on ECP production of strains 278 and 283. The strains were again grown for 20 d at 30°C, with the addition of 4% (m/v) sucrose to the YEL-medium.

Skogen (1970) and Cerning (1990) both reported that ECP production was favoured by lower incubation temperatures and thus in Experimental Study IV (Table 2), the influence of different temperatures (22°, 25° and 30°C) on ECP production by strain 278, was studied.

In Experimental Study V, the influence of different combinations of environmental factors (pH and temperature) on ECP production by strain 278, was determined. The culture was incubated for 20 d at either 18°, 22°, 25° or 30°C in YEL-media supplemented with either 4% or 8% (m/v) sucrose at different initial pH values (7.0, 7.5, 8.0 or 8.5).

Statistical design

A 4 x 2 x 4 factorial design was used to evaluate the quantitative effects of the variables, individually and in combination, on the different environmental combinations used in Experimental Study V. The Yate's algorithm (Box *et al.*, 1978) was used to calculate the response of the effects and interactions of temperature (T), sucrose concentration (S) and pH (P) on the responses. The main effect, either T, S or P, is defined as the influence of a specific variable on a response, averaged over the span of the other variables. The detection of positive two or three factorial interaction values, imply an enhancement of the final response when the two or three variables are increased simultaneously.

Results and discussion

Cultures

The strains that were used in this study (Table 1), were originally isolated from two totally different ecosystems - from Swiss-type cheese (Britz & Riedel, 1994) and anaerobic digesters (Riedel & Britz, 1993). The presence of ECP produced by the different *Propionibacterium* strains (Table 1) in YEL-medium was, in the first part of

the study, visually determined. Six strains (276, 278, 283, 346, 423 and 1032) that produced a reasonable amount of ECP, were identified and used in the subsequent studies.

Experimental studies

The isolation and purification of ECP is difficult and tedious as ECP is always found together with the surrounding microbial cells from which it can only, with difficulty, be separated. Furthermore, it is known that ECP can be mixed with different carbohydrates and proteins or peptides present in the growth medium itself (Cerning, 1995).

In this study, when using the BCA protein assay kit, no proteins could be found in the ECP and it was concluded that the ECP probably only consisted of carbohydrates. This could be an indication that the ECP could possibly be utilised as a carbon source under less favourable conditions.

Thin layer chromatography was done to identify the carbohydrates present in the ECP. It was found that the extracted ECP from *P. jensenii* strain 278 contained a complex polysaccharide and which, with further hydrolysis, resulted in major amounts of mannose and lesser amounts of glucose and galactose. No further extractions or purifications were undertaken due to a lack of specific facilities.

According to reports in the literature, viscosity determinations give a good indication of the amount of ECP produced (Cerning, 1990; Shellhaass & Morris, 1985). The polysaccharides are usually linear molecules, although side chains of variable length may also be present in some structures. These linear, high molecular weight polysaccharides, have the tendency to form helical structures which may occasionally be triple stranded or, more commonly, double-stranded. The molecules may vary considerably in their flexibility and some have proved to be very rigid. A consequence of these properties is the high viscosity of many aqueous solutions (Sutherland, 1994). However, viscosity, as an indication of ECP production, not only depends on the amount and linearity of ECP produced, but also on the structure, the polymer molecular mass and type, the presence and physical state of the carbohydrates, and the presence of proteins and other metabolites (Cerning, 1990; Shellhaass & Morris, 1985).

Study I - Incubation time ECP production by *Propionibacterium* strain 1032 was examined at 30°C at incubation-time intervals (4, 8, 12, 16, 20 and 24 days) (Table 2). The strain was grown in YEL-medium at an initial pH value of 9.0. The relative high amount of ECP isolated after 20 d (Fig. 1A) indicated that the most ECP was present in the medium after that period. In contrast to the ECP production, which had reached a maximum after 20 d, the viscosity (Fig. 1B), was still slowly increasing by day 24. The pH was found to decrease from 9.0 to 5.6 (Fig. 1C) by the end of the 24 d incubation period. This decrease in pH was probably due to the continuous production of volatile fatty acids, especially propionic and acetic acids.

The decrease in ECP production (Fig. 1A) after day 20 can possibly be an indication of the start of degradation of the extracellular material. It is also possible that the catabolism of the ECP had exceeded the production after day 20. Similar decreases in propionibacterial ECP production were previously reported by Skogen (1970) and were ascribed to either breakdown because of unfavourable incubation temperatures, unfavourable pH, autolysis of cells releasing degradative enzymes into the media, or a combination of these factors (Skogen, 1970). Based on the data from this study, it was concluded that an incubation time of at least 20 days at 30°C was necessary for optimum ECP production for this strain in YEL-medium and at an initial pH value of 9.0. Since the initial pH of the growth medium dropped to below 8.5 just after incubation, it was decided to rather set the initial pH at 8.5 in the follow-up studies.

Study II - ECP production by different strains In this study only the strains of *Propionibacterium* that were visually identified as strong ECP producers were (276, 278, 283, 346, 423 and 1032) (Table 2). The strains were grown in YEL-medium supplemented with 4% sucrose and the medium set at an initial pH of 8.5.

Strain 278 was found to produce the most ECP and the highest medium viscosity (Fig. 2A and 2B) under the conditions used in this study. Although the second highest mass of ECP was produced by strain 1032, ECP production by strain 423 resulted in a higher viscosity value. This could probably be because the two strains produced ECP that consisted of different carbohydrates or combinations (Sutherland, 1994), but this was not determined. Photos of the growth medium and ECP isolated from strain 278 are shown in Fig. 3.

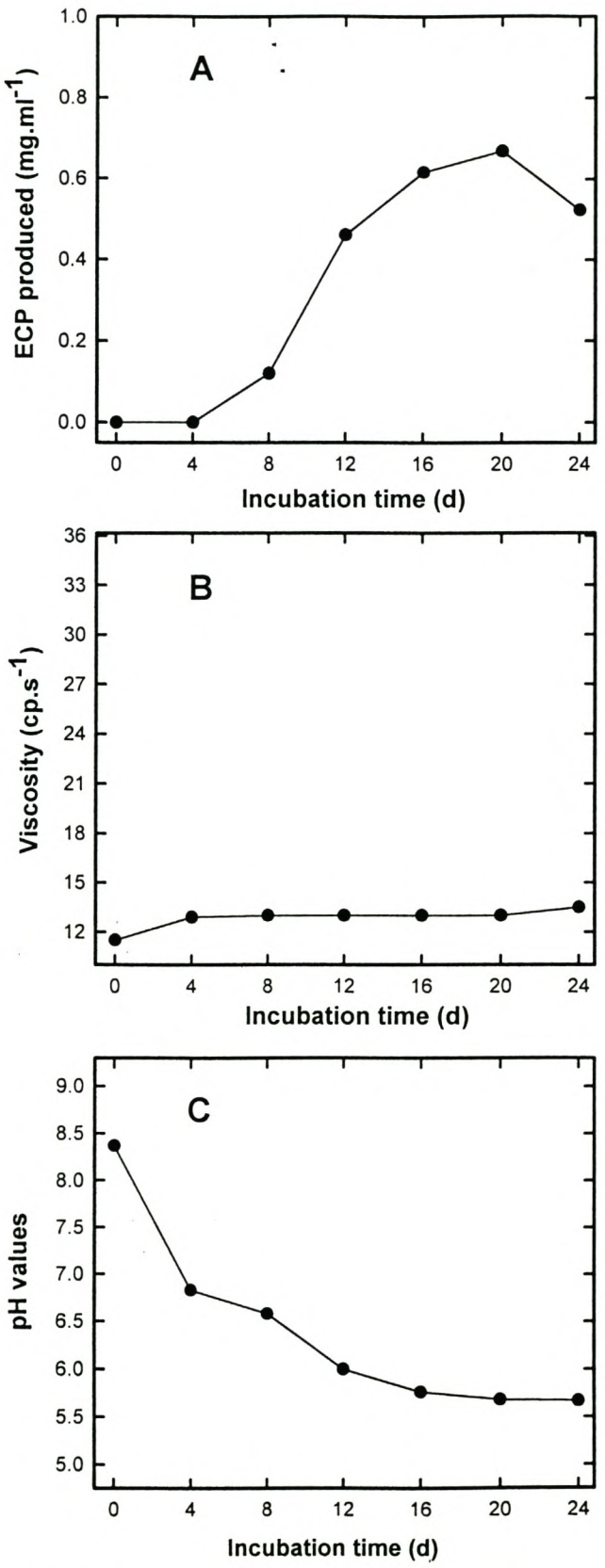

Figure 1. Influence of the incubation time at 30°C on ECP production (A), on the medium viscosity (B), and on the final pH (C) of medium inoculated with *P. freudenreichii* subsp. *shermanii* strain 1032.

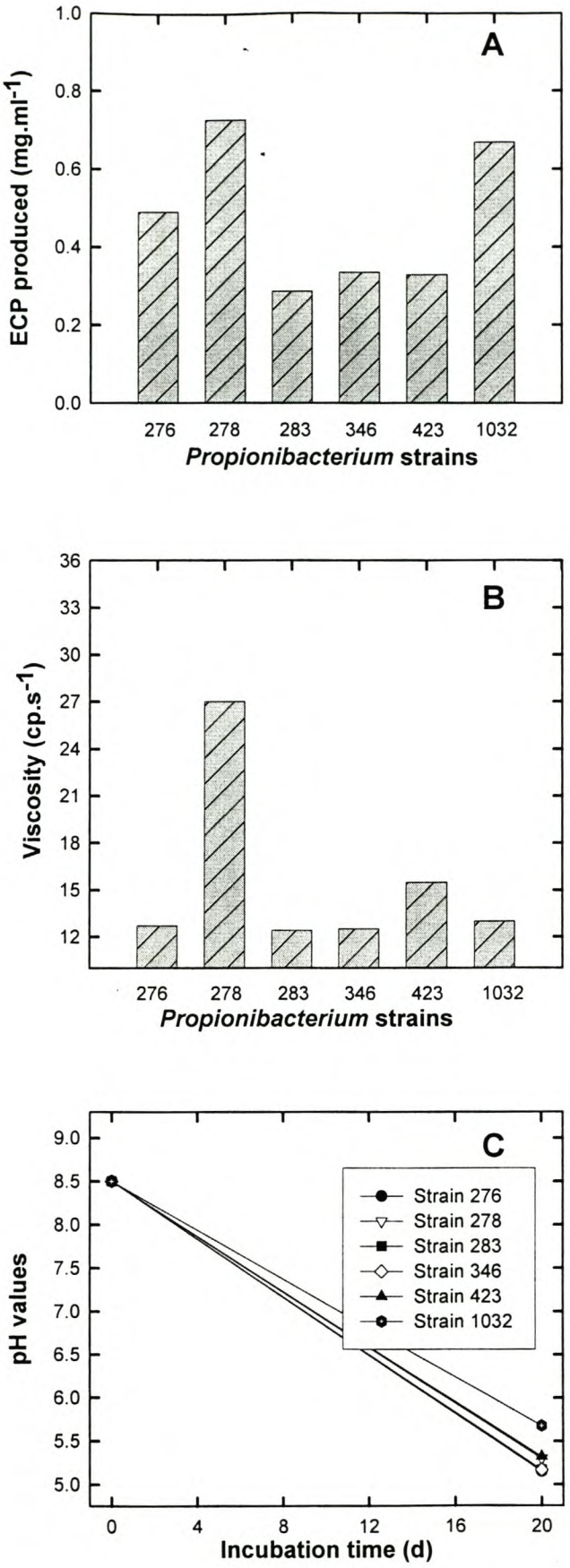

Figure 2. ECP production (A), viscosity (B) and pH (C) changes in YEL-medium (4% sucrose and pH 8.5 at 30°C) inoculated with the different propionibacterial strains.

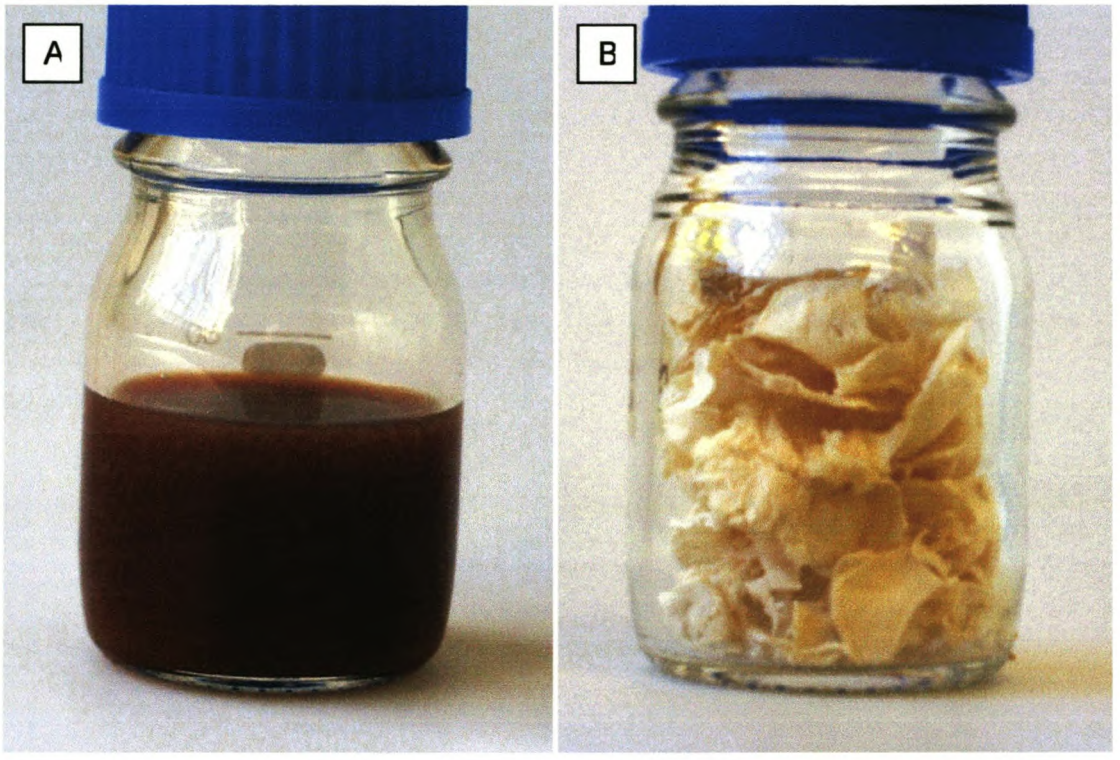

Figure 3. Growth medium (A) and the freeze-dried polysaccharide (B) for *Propionibacterium strain* 278.

Final pH values attained after the 20 d period are given in Fig. 2C. The data showed that strains 278 and 1032 not only had higher ECP production, but also showed higher final pH values. It can be hypothised that this is because ECP formation can serve as an alternative hydrogen sink reaction during fermentation (Vanderhaegen *et al.*, 1991). Thus, less hydrogen ions are formed and the final pH is not so low (Vanderhaegen *et al.*, 1991). During the production of ECP, less hydrogen ions are thus available that can lower the pH. Based on the fact that *P. jensenii* strain 278 produced the most ECP and that it was initially isolated from an anaerobic digester, it was used in the follow-up studies.

Study III - Initial pH It has been reported in the literature (Skogen, 1970; Skogen *et al.*, 1974) that the initial pH usually has a major influence on propionibacterial ECP production. Two strains isolated from anaerobic digesters (278 and 283), were taken as representatives of high and low ECP producers (Fig. 2) and used to determine what the influence of the initial pH would be on the ECP production by these propionibacteria (Fig. 4A and 5A). These strains were grown in YEL-medium supplemented with 4% sucrose at 30°C and at initial pH values that varied from 5.5 to 9.0 (Table 2).

Strain 278 was found, as already observed in Study II, to produce more ECP (data not shown) and a higher medium viscosity than strain 283 (Fig. 4A). The maximum ECP production by strain 278 (0.53 $mg.ml^{-1}$) was obtained at pH 8.5 with a viscosity of 23.5 $cp.s^{-1}$ (Fig. 5A). Strain 283 produced the most ECP (0.41 $mg.ml^{-1}$) at pH 7.5 with a viscosity of 17.2 $cp.s^{-1}$ (Fig. 4A). The results obtained in this study confirm the results of other researchers (Černà & Vàña, 1982; Skogen, 1970) who observed that ECP production by propionibacteria is not favoured by pH conditions below neutrality (Fig. 4A and 5A). At a lower initial pH value, an acidity that is unfavourable for growth is reached more quickly, resulting in less ECP formation as well as less growth (Skogen, 1970).

In Fig. 4B and 5B, although the final pH was only measured after the 20 d incubation period, slightly higher final pH values for the higher initial pH media, were observed. Thus, based on the data obtained in this study, the media used in further studies with strain 278, was set at an initial pH value of 8.5.

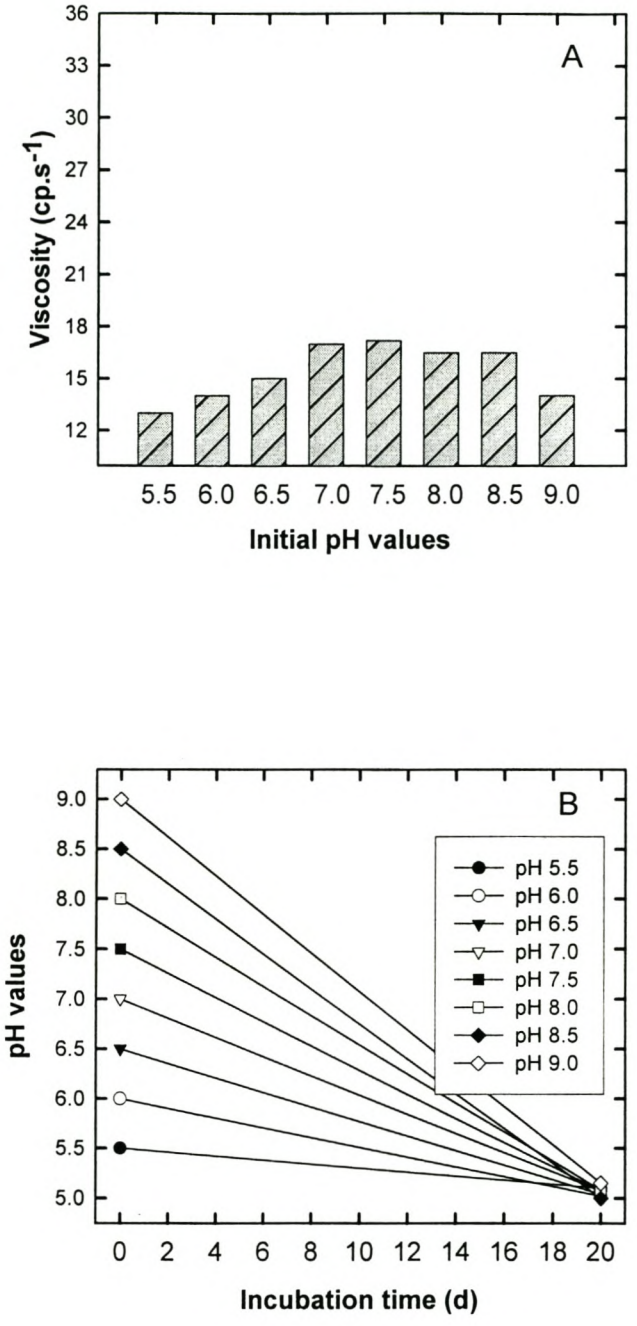

Figure 4. Influence of initial pH on the viscosity (A) and pH (B) of YEL-medium (4% sucrose and 30°C) inoculated with *Propionibacterium* strain 283.

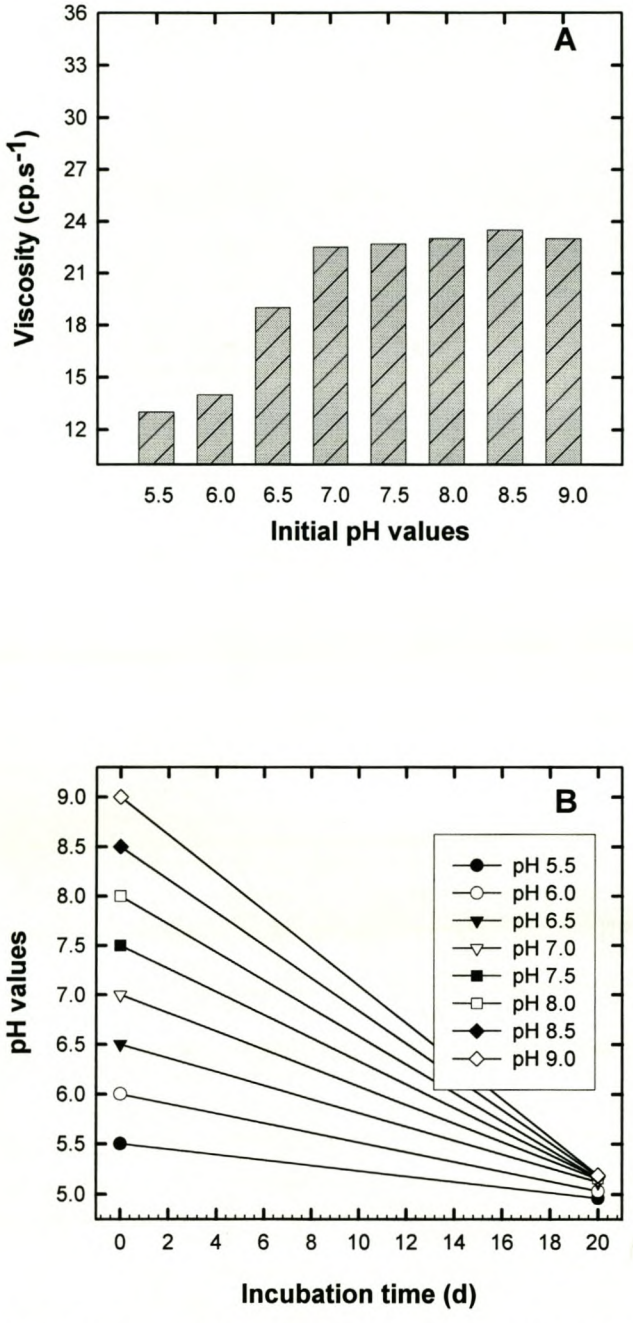

Figure 5. Influence of initial pH on the viscosity (A) and pH (B) of YEL-medium (4% sucrose and 30°C) inoculated with *Propionibacterium* strain 278.

Study IV – Temperature variation In this study, the influence of incubation temperature on ECP production by *Propionibacterium* strain 278, was examined. The strain was inoculated in YEL-sucrose media set at an initial pH of 8.5 and incubated at either 22°, 25° or 30°C (Table 2) (Fig. 6A and 6B).

A better cell efficiency in the conversion of carbon sources into ECP at lower incubation temperatures was observed as the highest ECP production and viscosity were obtained when strain 278 was incubated at 22°C (Fig. 6A and 6B). This can probably be due to the large number of enzymes (Cerning, 1995) that are involved in the synthesis of bacterial ECP. Sugar nucleotides are the activated form of monosaccharides and provide the microbial cell with a means of interconversion of various monosaccharides through epimerisation, dehydrogenation and decarboxylation reactions. Furthermore, isoprenoid-glycosyl carrier lipids are involved in the polymerisation of exopolysaccharides (Sutherland & Tait, 1992; Whitfield & Valvano, 1993). When grown at lower temperatures, growth and thus cell wall formation is slower because more enzymes and lipid carriers are available for ECP production (Cerning, 1995). Thus, cultural conditions that stimulate increased lipopolysaccharide or teichoic acid production inhibit exopolysaccharide formation due to the competition for the same lipid carrier (Cerning, 1995). The enzymes involved in the synthesis of propionibacterial ECP may also have a lower optimum temperature for activity and thus, when the culture was incubated at a lower temperature (22°C), more ECP was produced. The lower production of ECP at higher temperatures might also be due to the activation of certain hydrolysing agents, such as glucohydrolyses, which are capable of degrading the polysaccharides (Racine *et al.*, 1991). The production of slightly more ECP at 30°C than at 25°C (Fig. 6A and 6B) is probably because ECP production is correlated with the biomass obtained (Racine *et al.*, 1991). The results obtained in this study correlate with data from the literature (Cerning *et al.*, 1992; Racine *et al.*, 1991; Skogen, 1970) that indicated that more ECP was produced by strains incubated at lower temperatures.

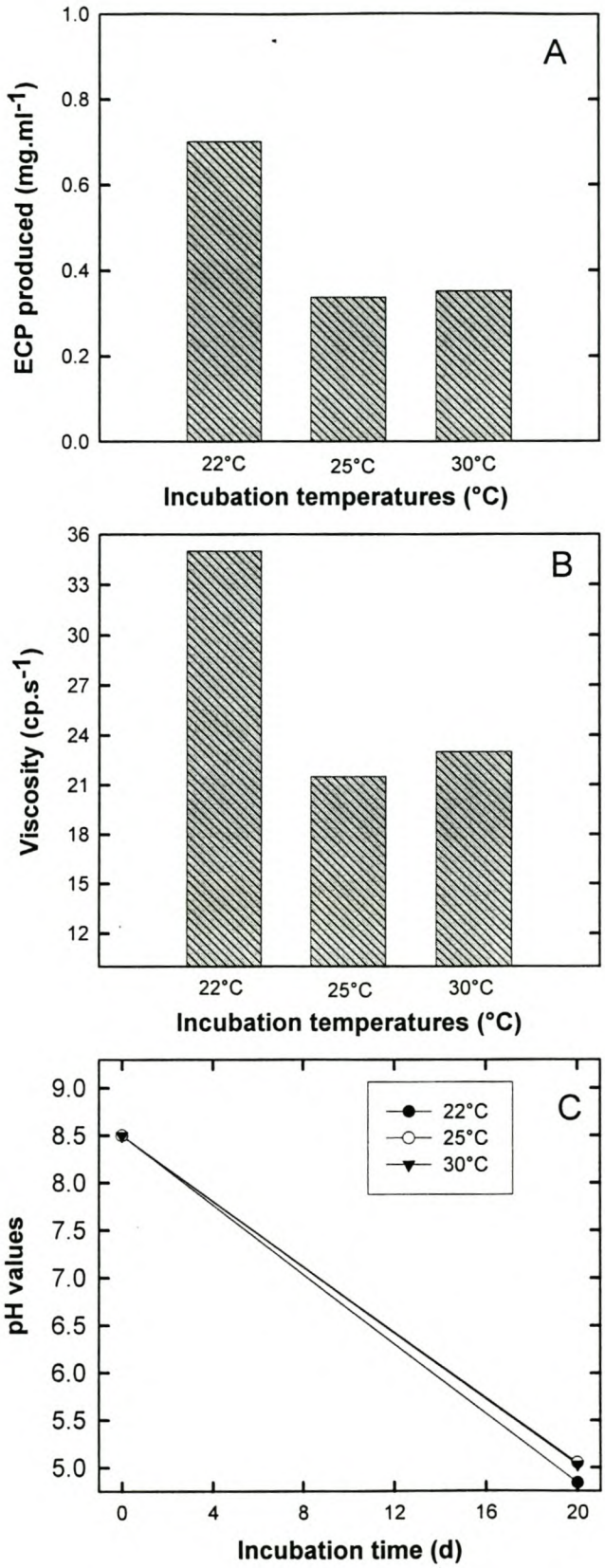

Figure 6. The influence of incubation temperature on the ECP production (A), mediun viscosity (B) and final pH of *Propionibacterium* strain 278, inoculated in YEL-media (4% sucrose and pH 8.5) for 20 days.

Study V - Influence of environmental parameter combinations In this study the influence of varying combinations of incubation temperature, initial pH and sucrose concentration on ECP production and medium viscosity, was determined (Table 2). Strain 278 was firstly inoculated into YEL-sucrose (4 or 8% sucrose) media with different initial pH values (5.5, 6.0, 7.0 and 8.0) and incubated at either 18°, 25°, 30° or 35°C. In this first section, because the isolation of ECP is very difficult and time consuming, only viscosity measurements were done so as to give an indication of the influence of the different environmental combinations. The data (not shown) showed that the viscosity was very low at conditions below pH 7.0 (5.5 and 6.0) and at 35°C. These results correlate with the data illustrated in Fig. 4, 5 and 6 where it was found that pH values below 6.5 and above 8.5 and also higher temperatures (35°C) inhibited ECP production. Based on these results, it was decided only to further investigate the influence of the interaction of pH values between 7.0 and 8.5 and temperatures below 35°C on ECP production by strain 278 with both 4 and 8% sucrose added to the medium.

Strain 278, at an inoculum concentration of 1×10^7 cfu. ml^{-1}, was then again inoculated into YEL-sucrose (4 or 8% sucrose) media set at different initial pH values (7.0, 7.5, 8.0 and 8.5) and incubated at either 18°, 22°, 25° or 30°C. In this section, however, both ECP (Fig. 7) and viscosity (Fig. 8), was measured.

When 4% sucrose was added to the medium (Fig. 7A), the highest ECP concentration was produced at a combination of 22°C and pH 8.5. In total the best temperature for ECP production at 4% sucrose appeared to be 22°C with the exception at a pH of 8.0, where it was found that more ECP was produced at 25°C. The lowest ECP production was found at 30°C combined with pH values of 7.0, 8.0 and 8.5.

The ECP production, when 8% sucrose was added to the medium, was again the highest at 22°C and an initial pH of 7.0 (Fig. 7B). In total the best temperature for ECP production under these conditions was 22°C and the lowest in all the cases, 30°C.

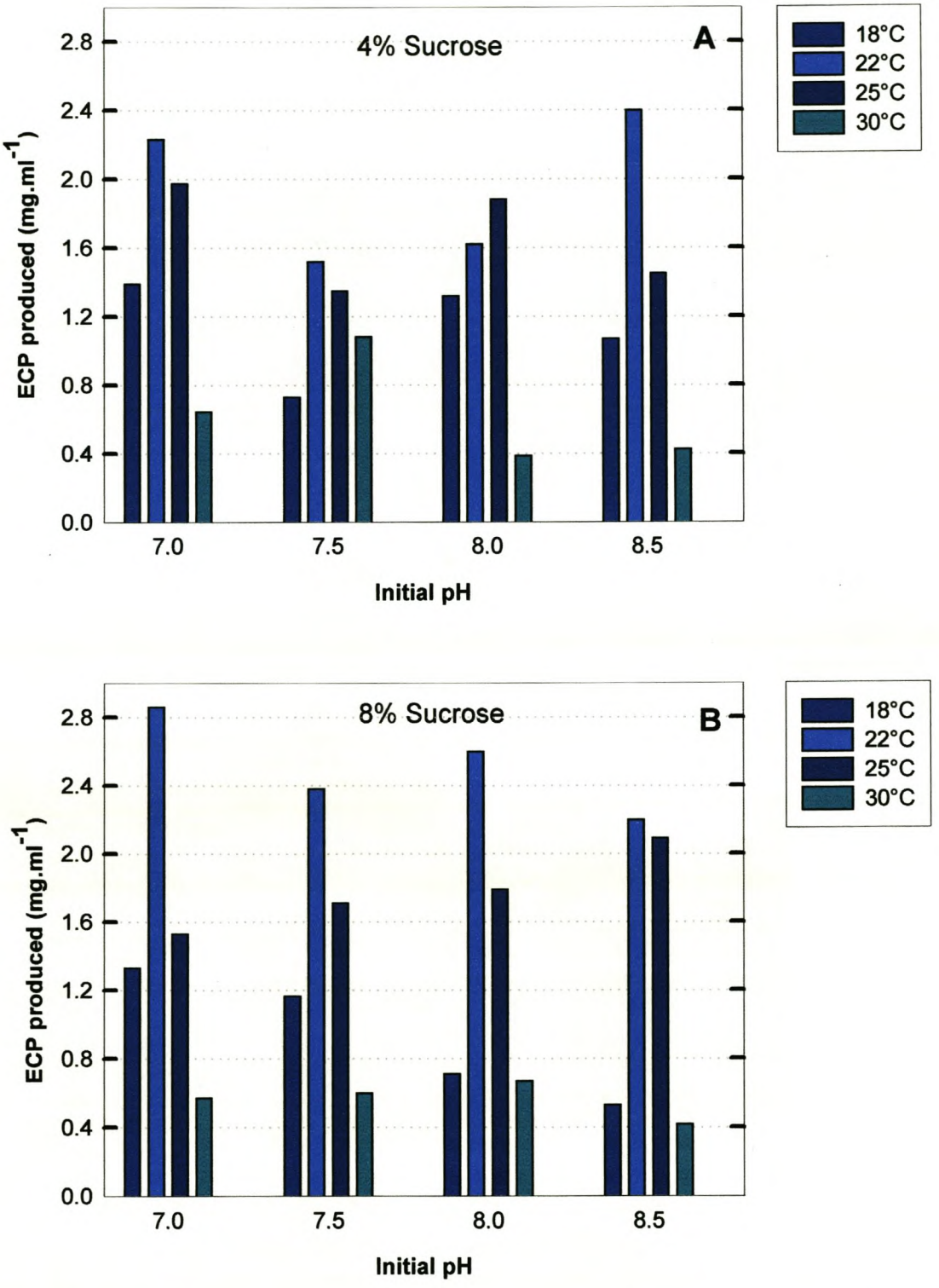

Figure 7. The ECP production by *Propionibacterium* strain 278 in YEL-medium with either 4% (A) or 8% (B) sucrose, at different temperatures and initial pH values.

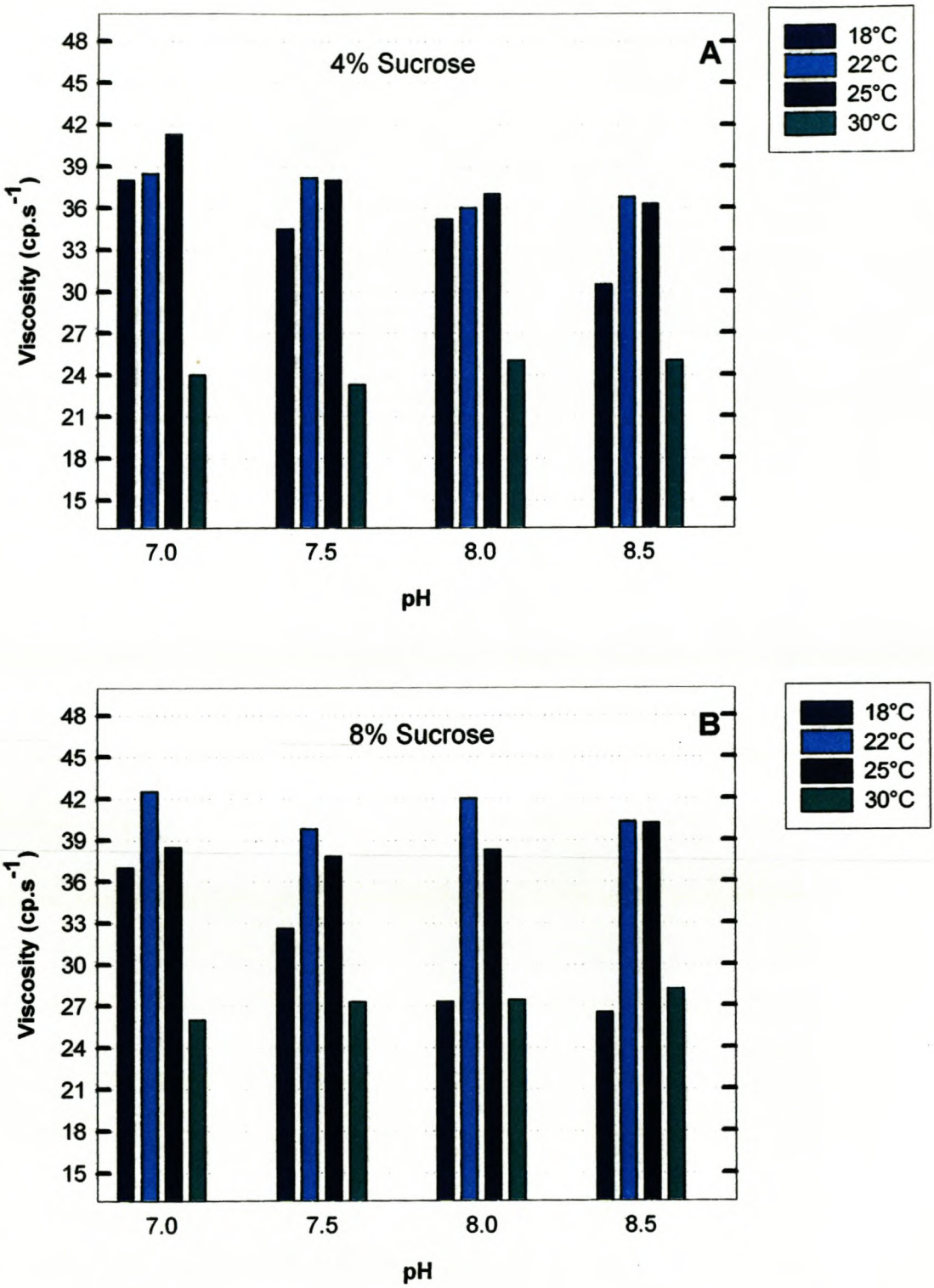

Figure 8. Viscosity of YEL-medium with either 4% (A) or 8% (B) sucrose, inoculated with *Propionibacterium* strain 278 and incubated at different temperatures and initial pH values.

The viscosity of the medium at the different environmental parameter combinations is given in Fig. 8A and B. In contrast to the ECP production it was found that the viscosity at 4% sucrose (Fig. 8A) was the best at 25°C and a pH of 7.0, however the difference in viscosity was found to vary very little between 22° and 25°C for all the pH values. The worst temperature for ECP production at all the pH values was 30°C. An initial pH value of 7.0 appeared to be the best for viscosity.

The best parameter for viscosity at 8% sucrose was 22°C and a pH of 7.0. The best temperature for viscosity at 8% sucrose for all the pH values was 22° with 25°C for all the pH's being the second best (Fig. 8B). The worst temperature was again found to be 30°C, although at pH 8.0 and 8.5, the viscosity was almost as low as at 18°C.

From the above data it was clear that that ECP production by propionibacteria was favoured by a lower incubation temperature. The best temperature for ECP production for both sucrose concentrations was at 22°C, while 25°C was better than 18°C and 18°C better than 30°C. At 18°C, the growth was probably too slow or the temperature too low for the ECP synthesising enzymes, and less ECP was formed after 20 d. The lowest yield of ECP was produced at the more optimal growth temperature for propionibacteria, 30°C (Holt *et al.*, 1994). The amount of ECP produced by the propionibacteria evaluated in this study was found to be very similar to ECP production by lactic acid bacteria (Ludbrook *et al.*, 1997) where variations were reported between 0.114 and 0.600 $mg.ml^{-1}$.

Optimisation of production parameters using a 4 x 2 x 4 factorial design

The data used in the above study was further analysed to identify specific trends in the ECP production at the different environmental conditions. The factorial design (Box *et al.*, 1978), as used in this study, is of practical importance as it requires relative few runs per factor studied. Although the specific design is unable to fully explore a wide region in the factor space, it can indicate major trends and thus directions for further experimentation. In this study the variables studied were sucrose concentration (S), temperature (T) and initial pH (P), giving a 4 x 2 x 4 factorial design which is illustrated in Fig. 9. The results of the ECP production obtained with the different parameter combinations are given in Table 3 and the trends in ECP production obtained from the factorial design are given in Table 4.

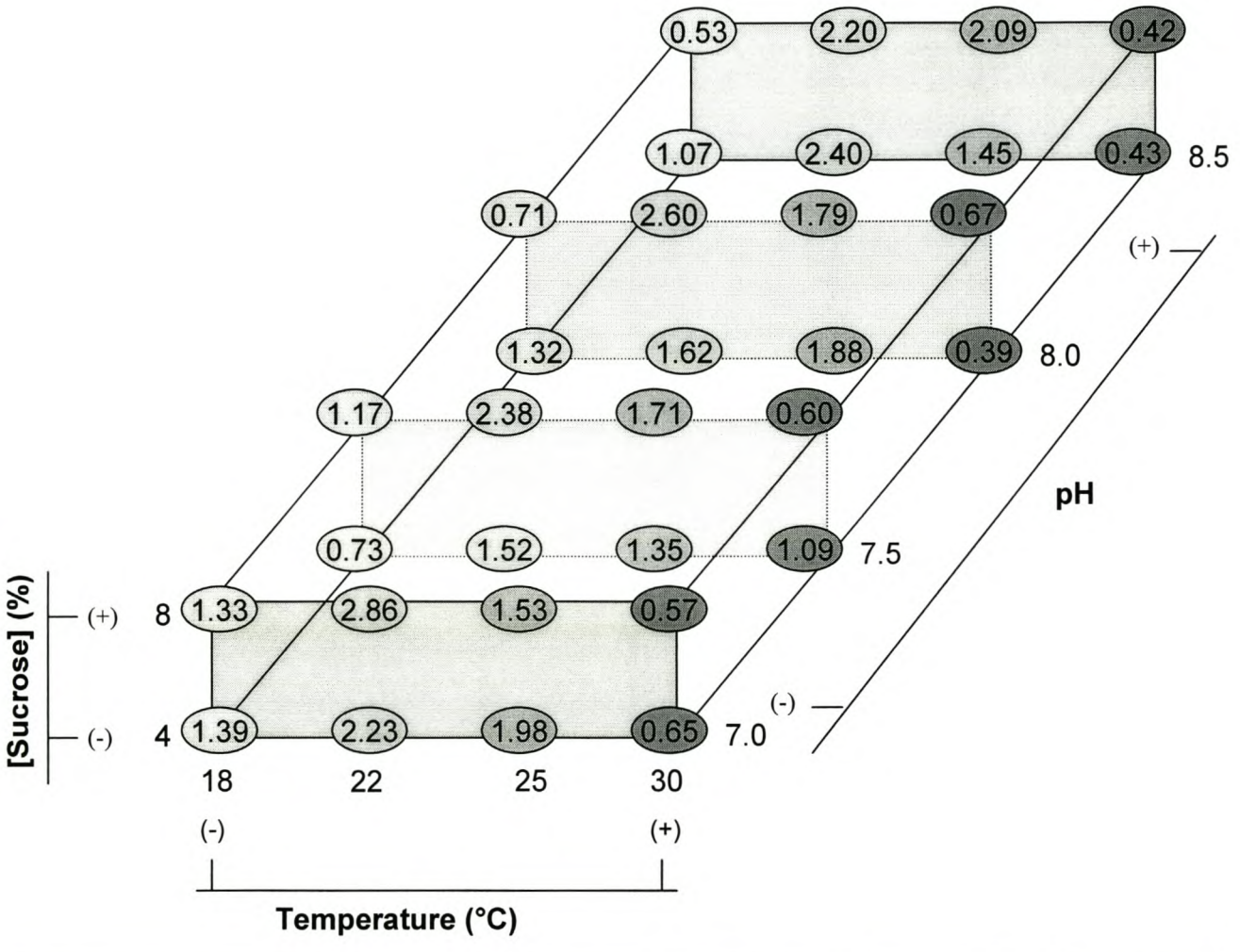

Figure 9. The factorial design used in this study showing the ECP production of strain 278 for the various test conditions.

Table 3. ECP yield by *Propionibacterium* strain 278 at different environmental parameter conditions.

Environmental Conditions			ECP Yield ($mg.ml^{-1}$)
Temperature (°C)	**pH**	**[Sucrose] (%) (w/w)**	
18	7.0	4	1.39
22	7.0	4	2.23
25	7.0	4	1.98
30	7.0	4	0.65
18	7.5	4	0.73
22	7.5	4	1.52
25	7.5	4	1.35
30	7.5	4	1.08
18	8.0	4	1.32
22	8.0	4	1.62
25	8.0	4	1.88
30	8.0	4	0.39
18	8.5	4	1.07
22	8.5	4	2.40
25	8.5	4	1.45
30	8.5	4	0.43
18	7.0	8	1.33
22	7.0	8	2.86
25	7.0	8	1.53
30	7.0	8	0.57
18	7.5	8	1.17
22	7.5	8	2.38
25	7.5	8	1.71
30	7.5	8	0.60
18	8.0	8	0.71
22	8.0	8	2.60
25	8.0	8	1.79
30	8.0	8	0.67
18	8.5	8	0.53
22	8.5	8	2.20
25	8.5	8	2.09
30	8.5	8	0.42

Table 4. Statistical evaluation of the effects and interactions of sucrose concentration and pH at the different temperatures on the ECP production by strain 278.

Effects	Interactions	Estimates
<u>Main effects</u>		
Sucrose concentration (S)	4% - 8% (S)	0.11
pH (P)	18 - 22°C (T_1)	1.20
	18 - 25°C (T_2)	0.70
	18 - 30°C (T_3)	-0.43
	22 - 25°C (T_4)	-0.51
	22 - 30°C (T_5)	-1.63
	25 - 30°C (T_6)	-1.13
Temperature (T)	7.0 - 7.5 (P_1)	-0.23
	7.0 - 8.0 (P_2)	-0.20
	7.0 - 8.5 (P_3)	-0.24
	7.5 - 8.0 (P_4)	0.06
	7.5 - 8.5 (P_5)	0.01
	8.0 - 8.5 (P_6)	-0.05
<u>Two-factorial interactions</u>		
[Sucrose] x pH (S x P)	S x P_1	0.08
	S x P_2	0.10
	S x P_3	0.01
	S x P_4	-0.08
	S x P_5	-0.17
	S x P_6	-0.09
Temperature x pH (T x P)	T_1 x P_1	0.18
	T_1 x P_2	-0.05
	T_1 x P_3	-0.33
	T_1 x P_4	-0.23
	T_1 x P_5	-0.51
	T_1 x P_6	-0.28
	T_2 x P_1	0.33
	T_2 x P_2	-0.05
	T_2 x P_3	0.40
	T_2 x P_4	-0.37
	T_2 x P_5	-0.27
	T_2 x P_6	-0.04
	T_3 x P_1	0.02
	T_3 x P_2	-0.08
	T_3 x P_3	-0.06
	T_3 x P_4	-0.23
	T_3 x P_5	-0.12
	T_3 x P_6	-0.28
	T_4 x P_1	0.26
	T_4 x P_2	0.29
	T_4 x P_3	0.06
	T_4 x P_4	-0.08
	T_4 x P_5	-0.20
	T_4 x P_6	-0.11
	T_5 x P_1	-0.05
	T_5 x P_2	0.18
	T_5 x P_3	-0.19
	T_5 x P_4	0.22
	T_5 x P_5	0.26
	T_5 x P_6	-0.37
	T_6 x P_1	0.10
	T_6 x P_2	0.29
	T_6 x P_3	0.29
	T_6 x P_4	0.08
	T_6 x P_5	0.19
	T_6 x P_6	0.11

Table 4. (Continued)

Effects	Interactions	Estimates
Two-factorial interactions		
Temperature x [Sucrose] (T x S)	T_1 x S	0.42
	T_2 x S	0.14
	T_3 x S	0.04
	T_4 x S	-0.28
	T_5 x S	-0.38
	T_6 x S	-0.10
Three-factorial interactions		
[Sucrose] x pH x Temperature (S x P x T)	S x P_1 x T_1	-0.07
	S x P_1 x T_2	-0.06
	S x P_1 x T_3	-0.23
	S x P_1 x T_4	-0.02
	S x P_1 x T_5	-0.23
	S x P_1 x T_6	-0.31
	S x P_2 x T_1	0.23
	S x P_2 x T_2	0.25
	S x P_2 x T_3	0.23
	S x P_2 x T_4	-0.20
	S x P_2 x T_5	0.00
	S x P_2 x T_6	0.07
	S x P_3 x T_1	-0.09
	S x P_3 x T_2	0.39
	S x P_3 x T_3	0.14
	S x P_3 x T_4	0.00
	S x P_3 x T_5	0.23
	S x P_3 x T_6	-0.26
	S x P_4 x T_1	0.29
	S x P_4 x T_2	0.29
	S x P_4 x T_3	0.15
	S x P_4 x T_4	-0.14
	S x P_4 x T_5	0.16
	S x P_4 x T_6	0.31
	S x P_5 x T_1	-0.05
	S x P_5 x T_2	0.32
	S x P_5 x T_3	0.37
	S x P_5 x T_4	0.34
	S x P_5 x T_5	0.39
	S x P_5 x T_6	0.05
	S x P_6 x T_1	-0.31
	S x P_6 x T_2	0.17
	S x P_6 x T_3	-0.09
	S x P_6 x T_4	0.48
	S x P_6 x T_5	0.22
	S x P_6 x T_6	-0.26

Schematical illustrations of the individual and combined effects are given in Fig. 10 – 12, while the responses are summarised in Table 5.

Main effects

A graphic illustration of the main effects is given in Fig. 10. Although the influence of sucrose concentrations was found not to be very prominent, a positive trend (+0.11), which indicated that a higher sucrose concentration (as a main effect) would promote ECP production, was obtained. The influence of initial pH on the ECP production was also not very prominent and in many cases more negative. The trends obtained indicate clearly that pH values higher than 7.5 would be more positive for ECP production.

The influence of certain temperatures were found to be the most positive of the three main parameters investigated with the positive and negative effects varying between +1.2 (T_1) and –1.6 (T_5) (Fig. 10). The best temperature trend for ECP production was found to be between 18° and 22°C (T_1) and 18° and 25°C (T_2). The main effect of T_5 (22° – 30°C) was the most negative, and the trends suggested that it would be of value to investigate a temperature range around 22°C so as to further optimise ECP production.

Two-factor interactions

The two-factor interactions are graphically illustrated in Fig. 11. From the bar diagrams it can be seen that the influence of the two factors, pH and sucrose, was again not very prominent. The interaction between P_2 and S (+0.10) was the highest of this group (P x S) and again, as with the main factors, indicated a positive trend in terms of a higher pH and an increase in sucrose concentration.

The interactions between incubation temperature and sucrose (T x S) varied between -0.38 and +0.42 with the most positive trend at T_1 x S (+0.42). The lower, but still positive, effect of T_2 x S (+0.14) suggests that an evaluation of a temperature range lower than 25°C, but above 22°C, could possibly lead to a more positive optimisation of the ECP production.

Table 5. Summary of the optimal temperature, sucrose and pH conditions as indicated by the statistical and quantitative evaluation data.

	ECP production
Main effects	
4% Sucrose	S x T_1 x P_1/P_3
8% Sucrose	S x T_1 x P_2
Two-factor interactions	S x P_2
	T_2 x P_3
	T_1 x S
Three-factor interactions	S x P_6x T_4
Optimal interactions	S x P_6 x T_4
Estimates at three-factor level	+0.48
ECP yield	2.86

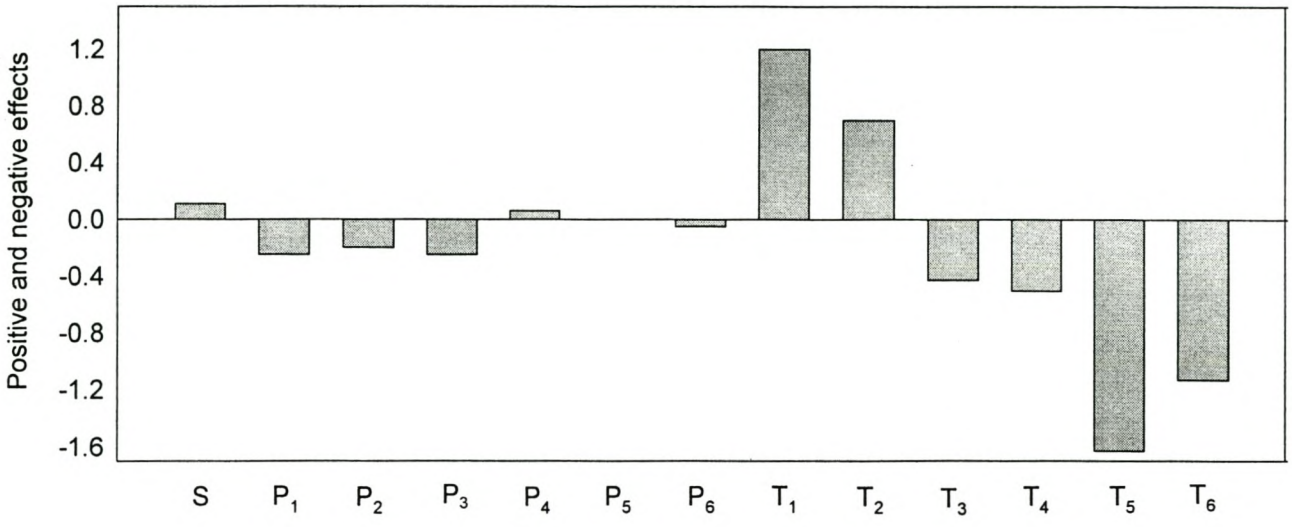

Figure 10. Histograms illustrating the main effects of temperature, initial pH and sucrose concentration on the ECP production by *Propionibacterium* strain 278.

Sucrose	pH	Temperature
4% - 8% (S)	7.0 - 7.5 (P_1)	18 - 22°C (T_1)
	7.0 - 8.0 (P_2)	18 - 25°C (T_2)
	7.0 - 8.5 (P_3)	18 - 30°C (T_3)
	7.5 - 8.0 (P_4)	22 - 25°C (T_4)
	7.5 - 8.5 (P_5)	22 - 30°C (T_5)
	8.0 - 8.5 (P_6)	25 - 30°C (T_6)

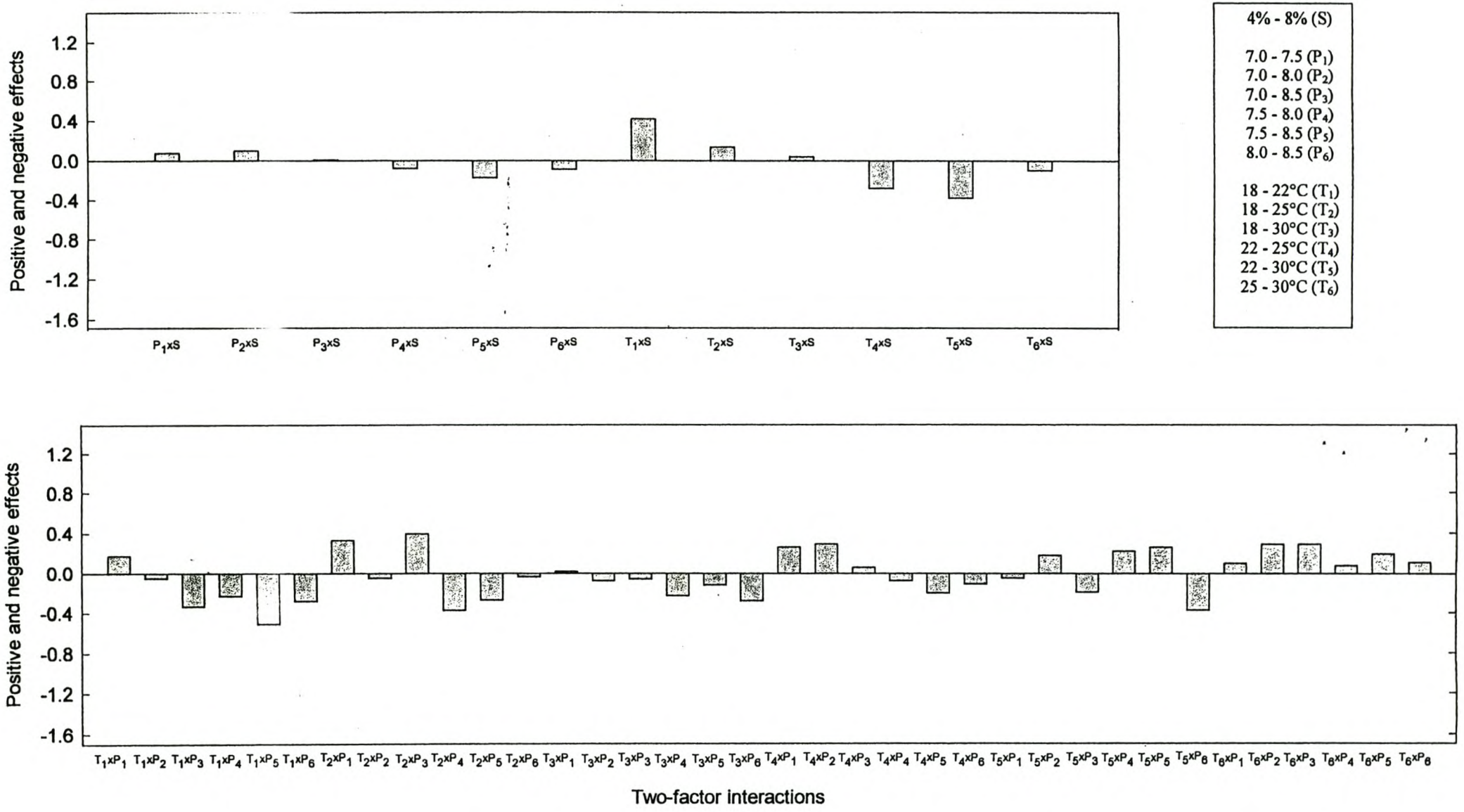

Figure 11. Histograms illustrating the <u>two-factor</u> interactions of the temperature, initial pH and sucrose concentration on the ECP production by *Propionibacterium* strain 278.

The combination T_2 x P_3 gave a positive effect of +0.40, once again indicating a positive trend for the lower temperature range of above 22° but lower than 25°C. This combination (T_2 x P_3) also indicates a trend to a higher initial pH of above 7.0, but below 8.5. Since the effect is higher than found for the T_2 x P_1 (+0.33), it could be possible that a pH of higher than 7.5 but lower than 8.5 would be more in the optimised range. This is confirmed by the positive values of the T_4 x P_1 (+0.26) and T_4 x P_2 (+0.29) combinations and the +0.29 values of the T_6 x P_3 and T_6 x P_2 combinations. These trends from the two-factor interactions of temperature and pH clearly show that this is still an area that can be examined before final optimisation may be achieved.

Three-factor interactions

The highest positive three-factorial interaction was found for the S x P_6 x T_4 combination. This indicates a trend in terms of an increase in sucrose concentration from 4 to 8%, a pH range from 8.0 - 8.5, and an incubation temperature of above 22°C but below 25°C. When the effects are studied carefully it is clear that sucrose concentration together with temperature (S x T_1) influences the trends, but when pH is taken into consideration as a third parameter, the impact of a change in sucrose concentration becomes of less importance. Thus, with three-factorial interactions, pH and temperature strongly impacts the trends, but it is impossible to predict from this data what the precise parameters are. It would in future, therefore, be advisable that a new range be evaluated based on the S x P_6 x T_4 combination, other small simultaneous changes (S x P_5 x T_5; S x P_3 x T_2) also appear, but at smaller levels, to impact the positive trends (Table 4) and these must always be taken into consideration.

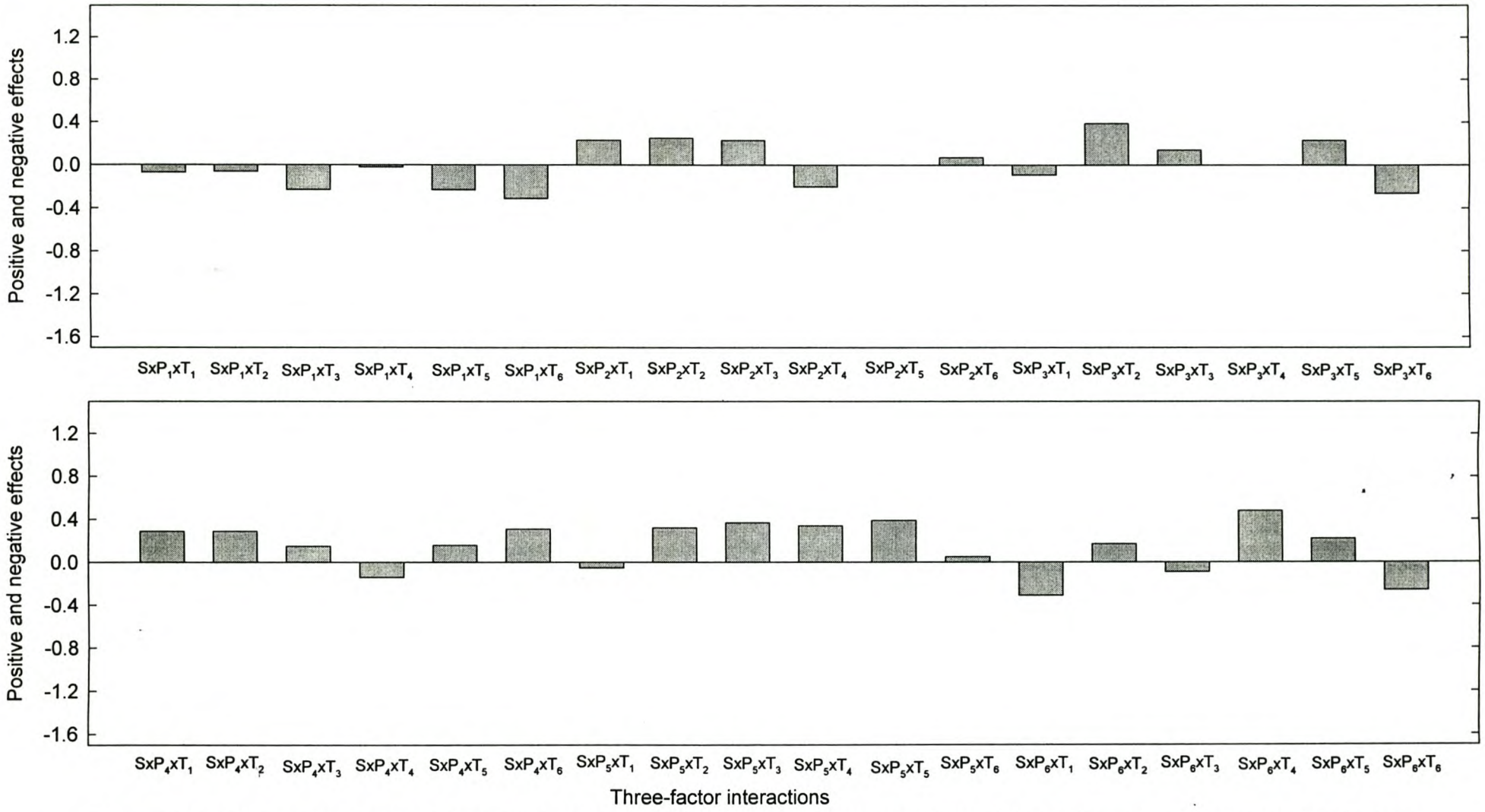

Figure 12. Histograms illustrating the <u>three-factor</u> interactions of the temperature, initial pH and sucrose concentration on ECP production by *Propionibacterium* strain 278.

4% - 8% (S)	7.0 - 7.5 (P_1)	18 - 22°C (T_1)
	7.0 - 8.0 (P_2)	18 - 25°C (T_2)
	7.0 - 8.5 (P_3)	18 - 30°C (T_3)
	7.5 - 8.0 (P_4)	22 - 25°C (T_4)
	7.5 - 8.5 (P_5)	22 - 30°C (T_5)
	8.0 - 8.5 (P_6)	25 - 30°C (T_6)

Conclusions

ECP is thought to effect the physical properties of the granular sludge used in UASB reactors (Roos, 1998) and is also believed to be important in bioflocculation (Forster, 1971). ECP producing propionibacteria have been found in the granules of anaerobic digesters and it was speculated by Riedel & Britz (1993) that the ECP production by these organisms may assist in the formation of granules in UASB reactors, especially during 'stress' conditions (Britz *et al.*, 1999).

It is known that ECP production and capsule formation are widespread in the genus *Propionibacterium* (Skogen, 1970). This suggests that, although the formation of ECP is not essential to the survival of the "classical" propionibacteria in their natural environments, it probably serves as a protection mechanism against unfavourable conditions such as lower incubation temperatures, low pH conditions and as protection against possible attack from bacteriophage by covering receptor sites or even to protect against moisture loss. The production of ECP by propionibacteria can thus give the bacteria a competitive advantage in the presence of other bacteria, for example in granules, where they could survive in more acidic conditions (Britz *et al.*, 1999).

In this study 19 different propionibacterial strains that had originally been isolated from two totally different ecosystems, were found to produced ECP (although the different strains produced different concentrations of ECP). Strain 278 was found to produce the most ECP of the strains examined and was used in further studies. It was found that the best ECP production was between 22° and 25°C. The data showed that the optimum temperature for ECP production was well below the optimum of 30°C for growth. The amount of ECP production varied from 0.3 – 0.7 $mg.ml^{-1}$, which is very similar to the reported production (Cerning *et al.*, 1992) of ECP by *Lactococcus lactis* subsp. *cremoris* strains (0.08 – 0.6 $ml.ml^{-1}$) and by *Streptococcus thermophilus* strains (0.05 - 0.35 $mg.ml^{-1}$) (Cerning *et al.*, 1988; Doco *et al.*, 1990). The influence of pH on the ECP production was not really significant, although it appeared that the best ECP production by strain 278 was near neutrality (Fig. 7A and B).

Experimental studies done by Skogen (1970) and visual determinations done during this study, indicated that sucrose appears to be a better carbon source for

ECP production than lactate alone. This is probably because ECP precursors (sugar units) must first be synthesised before being utilised as building blocks (Skogen, 1970). According to Sutherland (1994), at sucrose concentrations of more than 4%, polysaccharide yields by many species, was reduced. This was not true for strain 278, as the average ECP yield was higher at 8% sucrose than at 4% sucrose (Fig. 7A and B and 8A and B). However, at 30°C, the ECP production was reduced when 8% sucrose was added to the YEL-medium. The higher yield of ECP produced at the higher sucrose concentration, may be due to the higher carbon to nitrogen ratio of the YEL-media (Cerning, 1990; Graber *et al.*, 1988).

From the data obtained during the five experimental studies (I - V) it was clear that the parameters used showed variable ECP production and thus use was made of an factorial design to indicate positive trends that could, in future, be favourably exploited. The main effect of T_5 (22°C - 30°C) showed the most negative trend, indicating that a temperature range around 22°C needs further investigation to optimise the ECP production. The two-factor interactions again indicated a positive trend in terms of higher initial pH and sucrose concentration and it was found that a pH of higher than 7.5, but lower than 8.5 would be more in the optimised range. It was also again seen that a temperature range between 22° and 25°C would be a positive trend in optimisation of the ECP production. The three-factor interaction again suggested that the temperature range between 22° and 25°C and a pH range between 8.0 and 8.5 needed further investigation to optimise the ECP production by *P. jensenii* strain 278.

The enhancement of the ECP production at 22° - 25°C and between pH 8.0 and 8.5 by this specific propionibacterial strain, may be explained as a possible protection mechanism against unfavourable growth conditions. The production of ECP functions as an alternative hydrogen pathway (Riedel & Britz, 1993) and may therefore also protect the cells against acid conditions.

ECP producing strains of *Propionibacterium* may, in the future, play a more important role in the formation of highly settleable aggregates in UASB reactors. However, more research is still also required to determine the carbohydtrate composition of the ECP and the 'stress' conditions that promote the production of ECP. Different trends in the ECP production of *Propionibacterium* strain 278 were obtained in this study when the interactions of temperature, pH and sucrose was

studied. These trends must also be further investigated to determine the precise parameters for the optimum conditions for ECP production.

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CHAPTER 4

INFLUENCE OF PROPIONIBACTERIA ON THE UASB GRANULATION PROCESS

Summary

The formation and stability of granules are essential for the successful operation of the UASB (upflow anaerobic sludge blanket) bioreactor. It has been shown that the granules in these bioreactors are surrounded by extracellular polymers (ECP), and it is generally accepted that the formation of granules is correlated with the production of ECP. In this study, a hypothesis for the enhancement of the granulation process was partially validated against experimental observations. Granules were 'stressed' with YEL-media (containing 5.5 $g.l^{-1}$ glucose) on day 6 of an 8 day incubation period and the changes in pH, volatile fatty acid profiles and concentration of the lactate-utilising bacteria, were studied. Twenty four hours after the application of 'stress', the propionic acid concentration was found to increase above that of the acetic acid concentration and concurrently, the pH dropped from 6.6 to 6.5. The viable lactate-utilising population was 1.36×10^7 and 8.25×10^7 cfu per ml, respectively for the YEL and YELN-media 48 h after these granule units had been 'stressed'. The average counts on the Pal Propiobac medium for the 'unstressed' units were 1.37×10^5 $cfu.ml^{-1}$ and 2.72×10^5 $cfu.ml^{-1}$ for the 'stressed' units.

The influence of different propionibacteria and sludge concentrations on the granulation process were also examined. The number and size of the granules formed, metabolic and pH profiles, and the activity of the granules were examined. Granules were made in a batch replacement mode for 20 days and the volatile fatty acids and pH monitored daily. As postulated in the hypothesis, the granulation process was facilitated by a drop in pH at the start and an increase in the propionic and acetic acid concentrations was found. This was followed by an increase and stabilisation in pH, with a steady decrease in propionic and acetic acid concentrations until stabilisation. It was also seen that, when more propionibacteria were added to the sludge, more granules formed. The granules were also more active and more methane and biogas was produced when more propionibacteria and

sludge were added. When no propionibacteria was added, the biogas and methane production was almost half of that produced when 60 ml propionicbacteria were added. It was thus concluded that the addition of propionibacteria had a definite influence on the granulation process as well as the biogas and methane activity of anaerobic sludge.

Introduction

Upflow Anaerobic Sludge Bed (UASB) reactors are normally used for the high-rate anaerobic treatment of waste waters (Lettinga *et al.*, 1980 and 1997). This process depends on the upward movement of waste water through a blanket of granules and the success of this design is related to the capacity for biomass accumulation by settling without the need of a carrier (Laguna *et al.*, 1999). Thus, the operational efficiency and performance of the UASB system is mainly dictated by the formation, amount and specific activity of these granules (Schmidt & Ahring, 1996). The formation of granules has the advantage that a higher active biomass concentration can be retained inside the UASB reactor with simple and low cost equipment (Lettinga *et al.*, 1997).

It is known that anaerobic granular sludge is formed by self-immobilisation of bacteria that results in a bacterial consortium where cells live in close proximity (Schmidt & Ahring, 1995), but the precise nature of the mechanisms involved in the formation of granules and their continued stability is still not fully understood. According to Schmidt & Ahring (1996), granulation consists of four steps: transport of the cells to the surface of an inert material; adsorption to the substrate; the adhesion to the substrate; and the multiplication of the cells. Extracellular polymers (ECP), which form a matrix for the cells to divide in, is then produced by acidogens and a macro-colony of identical cells is formed. Sanin & Vesilind (1996) confirmed this theory and postulated that the building blocks of a granule are colonies, which are formed when two or more micro-organisms become attached to each other. Once the colonies are formed, they react with other colonies to form larger structures.

In 1999, it was reported by Britz and co-workers that under 'balanced' operational conditions, no lactate and very little propionate can be detected in well operated UASB bioreactors. However, when 'stress' conditions are put on granules in a bioreactor treating carbohydrate rich waste waters, the first detectable

metabolite that appears is propionate (Myburg & Britz, 1993), while simultaneously, hydrogen can be detected in the gas phase and lactate starts to accumulate (Eng *et al.*, 1986). These metabolic changes probably result from a shift of the population dynamics of the anaerobic community (Riedel & Britz, 1993). The increase in the lactate concentration, as result of the 'unbalanced' conditions, leads to an orderly shift between the predominant lactate-utilising bacteria in response to the gradual decrease in the pH and increase in H_2 partial pressure. ECP producing *Propionibacterium* strains were isolated under these 'stress' conditions (Britz *et al.*, 1999; Riedel & Britz, 1993) and it was postulated that *Propionibacterium* strains could gain a competitive advantage, as they obtain a maximum ATP per mol of lactate fermented. Once they have the advantage at the lower pH, they start producing extracellular compounds, with the subsequent formation of aggregates (Riedel & Britz., 1993; Vanderhaegen *et al.*, 1992).

It is known that propionibacteria are present in anaerobic digesters and granules (Britz *et al.*, 1999; Riedel & Britz, 1993), but the question arises whether their numbers do change once a 'stress' condition is applied. Thus, the aim of this study was to determine if the lactate-utilising population, and specifically propionibacterial numbers, change after 'stress' conditions have been applied during the mass batch culturing of granules. The influence of different *Propionibacterium* inoculum concentrations on the granulation process of anaerobic sludge was also studied.

Materials and methods

Experimental Study I - Influence of 'stress' conditions on the lactate utilising population in granules

Two triplicate sample sets of granules (20 g each) from a brewery effluent treating UASB digester, were inoculated into 500 ml containers with 400 ml Yeast Extract Lactate medium (YEL) (Table 1). The units were incubated in a linear shake waterbath (95 r.p.m.) (Scientific Manufacturing, Cape Town) at 35°C.

Daily, for a period of 8 days, 100 ml from each test unit was removed and replaced with 100 ml fresh sterile YEL-medium. On day 6, one triplicate sample set was 'stressed' by replacing the YEL-medium with 100 ml of a glucose rich 'stress'-

Table 1. Composition of the Yeast Extract Lactate-medium.

Component	**Concentration ($g.l^{-1}$)**
Sodium lactate (60% v/v) (Saarchem)	20.0
Yeast Extract (Biolab)	5.0
Peptone (Biolab)	2.0
KH_2PO_4 (Saarchem)	10.0
Tween 80 (Merck)	1.0 ml
pH	7.0

medium. The 'stress'-medium consisted of the YEL-medium to which 5.5 $g.l^{-1}$ glucose and 1.1 $g.l^{-1}$ urea were added. Volatile fatty acid analysis and pH measurements were done daily. On day 8, a sample of the granules was aseptically withdrawn and aseptically mashed in 9 ml sterile saline solution with the use of a Stomacher (Interscience, France). Triplicate samples were serially diluted and then plated on the respective media to determinate the lactate-utilising population.

Experimental Study II – Influence of different propionibacterial inoculum concentrations on the granulation process

Non-granulated sludge was obtained from the local Athlone Sewage Plant. A shake table (135 r.p.m.) was used in the cultivation process at 35°C in a batch system. Containers (500 ml) containing 300 ml of YEL-medium (Table 1) were inoculated in triplicate, with different combinations of sludge and *Propionibacterium jensenii* strain 278 (Table 2) at a concentration of 10^6 $cfu.ml^{-1}$. Distilled water was used to make the volumes of the test units up to 400 ml.

Every 24 h for 20 days, 100 ml of each test unit was removed and replaced with a 100 ml of fresh sterile YEL-medium to simulate UASB operational parameters and organic overloading (Britz *et al.*, 1999).

With the use of enumeration and isolation procedures (described below), strains that morphologically resemble propionibacteria were selected on day 20 with the use of a *Propionibacterium* selective medium, Pal Propiobac (Standa Industrie, France). Brownish colonies surrounded by a yellow zone, were selected after 5 days of incubation at 35°C and purified Gram-positive isolates producing major amounts of propionic and lesser amounts of acetic acid as the major metabolic end-products, were considered as members of the genus *Propionibacterium* (Riedel & Britz, 1993).

The granular sludge was also sieved on day 20. The pore sizes of the sieves (Endecotts) that were used were 2.00, 1.00, 0.71, 0.50 and 0.212 mm. The granules in the different size fractions were then counted and activity tests were done on these granules.

Enumeration and isolation

Enumeration of the lactate-utilising population was performed using the YEL-medium (Table 1). The Gram-positive, lactate-utilising population was enumerated using Yeast Extract Lactate Naladixic acid (YELN) medium that was prepared by

Table 2. Combinations of sludge and propionibacteria added to the YEL-medium batch systems to monitor granulation. The propionibacterial concentration was 10^6 cfu.ml^{-1}.

Sludge (ml)	Propionibacteria (ml)	YEL-medium (ml)	Distilled water (ml)
15 (Control)	0	300	85
15	10	300	75
15	20	300	65
15	30	300	55
30 (Control)	0	300	70
30	10	300	60
30	20	300	50
30	30	300	40
50 (Control)	0	300	50
50	10	300	40
50	20	300	30
50	30	300	20

adding filter-sterilised naladixic acid (Sigma) (0.02% w/v) (Riedel & Britz, 1993) to sterilised YEL-medium. Propionibacteria were isolated using a *Propionibacterium* selective medium, Pal Propiobac (Thierry & Madec, 1995), and the medium was prepared as described in the manufacturer's instructions (Standa Industrie, France). All the plates were incubated at 30°C for 10 days. The colony forming units from the highest dilution yielding a count of over 30 but less than 300, were then counted. Colonies from the Pal Propiobac-medium were selected and streaked out on YEL-medium until pure colonies were obtained. Ten colonies from the YELN-medium were also selected with the use of the Harrison Disk method (Harrigan & McCance, 1976) from the plates with the highest dilution. All the colonies suspected of being members of the genus *Propionibacterium* were then inoculated into sterile YEL-broth and incubated for 10 days before volatile fatty acid analysis was done. The following identification tests were also done: catalase, oxidase, Gram and endospore staining (Harley & Prescott, 1993).

Gas chromatography

The volatile fatty acids (VFA) were determined using a Varian (Model 3700) gas chromatograph, equipped with a flame ionisation detector and a Nukol (30 m x 0.53 mm, ID 0.5 μm) fused silica capillary column (Supelco, USA). The column temperature was initially held at 105°C for two min, then increased, at a rate of 8°C.min^{-1} to 190°C where it was held for 10 min. The detector and inlet temperatures were set at 300°C and 130°C, respectively, and nitrogen gas was used as carrier gas at a flow rate of 6.1 ml.min^{-1}.

Samples were prepared for volatile fatty acid (VFA) analysis by combining 3 ml of the test sample with 1 ml of 35% (v/v) formic acid and 2 μl hexanol. The mixture was centrifuged at 10 000 x g for 10 min and the supernatant (1 ml) drawn off. One μl was then used for the gas chromatographic determinations. A standard solution was prepared by pipetting 1 ml each of propionic (Merck), acetic (Merck), iso-butyric (Sigma), n-butyric (Merck), n-valeric (Aldrich), iso-valeric (Hopkin & Williams Ltd.) and 0.5 ml n-hexanol (BDH) into a 1 000 ml volumetric flask. A solution of 250 ml of 35% formic acid and 750 ml distilled water was prepared by adding to a volumetric flask and then gently agitating. Of this standard solution, 1 μl was injected into the gas chromatograph. Borwin™ computer software (JMBS

Developments, France) was then used to quantitatively determine the presence of volatile fatty acids by integrating the peak areas, using the internal standard calibration method. Identification of unknown compounds was achieved by comparing their retention times to those of analytical grade standards. Hexanol, as internal standard, was chosen because its polarity and boiling point allowed it to be closest to the fatty acid peaks (Britz *et al.*, 1999).

Activity testing

Activity tests were done by measuring of the volume of biogas and methane produced. A 3 gram sample of the granular sludge was inoculated (in triplicate) into 20 ml vials together with 13 ml of the test substrate (Table 3) and the vials sealed. An additional loading of glucose (4 $g.l^{-1}$) was added to certain samples to specifically investigate the acidogenic population activity. The samples were then incubated at 35°C and the amount of biogas produced was determined after 5, 10 and 25 h (O.D. O'Kennedy, 2000, Department of Food Science, University of Stellenbosch, Personal communication).

Biogas-volume sampling was done using a free moving gas-tight 10 ml syringe equipped with a 21 gauge needle. Measurement determinations were made by holding the syringe horizontally and allowing the plunger to move freely (gentle turning of plunger). This was done until equilibrium was reached between the container and atmospheric pressure. Readings were verified by withdrawing the plunger past the equilibrium and allowing it to return to the original position.

The methane and carbon dioxide content was determined by injecting the biogas sample into a Fisons GC 8000 series gas chromatograph (Fisons Instruments SpA, Milan, USA). The GC was equipped with a thermal conductivity detector and a 2.0 x 2.0 mm i.d. column packed with Hayesep Q (Supelco, Bellefonte, PA), 80/100 mesh. The oven temperature was set at 45°C and helium was used as carrier gas at a flow rate of 40 $ml.min^{-1}$.

Image analysis

The granule numbers were enumerated on days 0, 10 and 20 with the use of a Nikon stereo microscope and online camera (Panasonic CP/410). One ml of each sample was placed in a round glass container (with a diameter of 140 mm) with the addition of enough melted gelatine (30 $g.l^{-1}$) to cover the sample (Jeison & Chamy,

Table 3. Composition of the nutrient solution used for activity testing (Valcke & Verstraete, 1993).

Component	Concentration ($g.l^{-1}$)
Glucose (Merck)	2.0
KH_2PO_4 (Saarchem)	2.6
K_2HPO_4 (Merck)	1.0
$MgCl_2$ (Merck)	0.1
NH_4Cl (Merck)	1.0
$(Na)_2SO_4(7\text{-}9)H_2O$ (Merck)	0.1
Yeast extract (Biolab)	0.2
Urea (Merck)	1.1
pH	7.1

1998). The gelatine was allowed to set and a graded grid was placed underneath the container. Ten fields, each with a diameter of 6 x 10 mm were scanned into the computer using an Intelcam (Matrox Electronic Systems Ltd.) frame-grabber program (Matrox Intellicam Interactive 2.0). The granules were then counted with the use of the Scion Image (Release Beta 3b) computer software (Scion Corporation, Maryland, USA).

The granules formed were generally very small, and in combination with a cloudy and very viscous solution, it was fairly difficult to always detect the black nuclei visually. Even when using the computer software, the amount of granules was difficult to determine and the method can, therefore, only be used to monitor the granulation tendency.

Total solid and volatile suspended solids determination

The total solids were determined at days 0 and 20 using standard methods (Standard Methods, 1992). This was done to measure the mass accumulation of the granules.

Results and discussion

Experimental Study I - Influence of 'stress' conditions on the lactate utilising population in granules

It has often been reported that, when unfavourable conditions (overloading) are applied to an anaerobic digester treating carbohydrate rich waste water, lactate starts to accumulate (Eng *et al.,* 1986). Major amounts of propionic and lower concentrations of acetic acid are then detected in the digester effluent and, simultaneously, hydrogen can be found in the gas phase (Hickey & Switzenbaum, 1991). It has been postulated that these changes take place due to the unfavourable conditions resulting from a shift in the metabolic pathway of the dominant acidogens. This 'unbalanced' situation leads to a decrease in the pH. It is then postulated that the increase in the lactate concentration subsequently results in an orderly shift in the acidogenic population (Riedel & Britz, 1993) to a more predominant lactate-utilising population in response to the gradual decrease in the pH. Once lactate-utilising microbes gain a competitive advantage (Thauer *et al.,* 1977), the propionic

acid concentration increases. These lactate-utilising propionic acid producers can then produce ECP and the system will then display clumping characteristics that may lead to the formation of bacterial aggregates (Britz *et al.*, 1999).

In this experimental study, granules in a batch system at 35°C (Britz *et al.*, 1999) were 'stressed' with a glucose rich YEL-medium and the influence of these 'stress' conditions on the lactate utilizing-propionic acid producing organisms in the granules, was determined.

pH profiles The pH changes in the granule batch system were determined since it was shown by Britz *et al.* (1999) that the first change after an anaerobic digester had been 'stressed', was a sudden decrease in the pH. It was also argued that pH changes should give an indication of metabolic productivity as a result of the production of volatile fatty acids.

In this study it was found that in the batch units, the pH dropped within the first 48 h (Fig. 1A and 1B) from 7 to below 6.5 by day 3, confirming the fact that when an excess of an easy degradable carbohydrate is added to an anaerobic system, a sharp drop in pH can be expected. From day 3 onwards the pH started to recover and then stabilised at around 6.6. This stabilisation persisted when no further 'stress' was placed on the granules. However, when the glucose rich 'stress'-medium was added to one of the granule sets on day 6, the pH dropped within 24 h from 6.6 to 6.5. After a further 24 h, the pH recovered again to around 6.6. The control unit (Fig. 1A) did not show this drop in pH. The drop in pH after the application of the 'stress'-medium was probably as a result of the production of more organic acids. This sudden drop in pH confirms part of the granulation hypothesis of Britz *et al.* (1999).

Metabolite production As part of the hypothesis (Britz *et al.*, 1999), it was also postulated that after a sudden change in environmental conditions a large increase in the concentration of propionic and acetic acids would be found. To confirm this part of the hypothesis, the volatile fatty acids (VFA) were measured daily to monitor the metabolite production and the profiles obtained are shown in Fig. 2.

At the start of this study (Fig. 2) it was found that the acetic and propionic concentrations in both the control (unstressed) (Fig. 2A) and 'stress' (Fig. 2B) batch units started to increase in conjunction with the decrease in pH (Fig. 1). For the first

Chap 4-Fig. 1

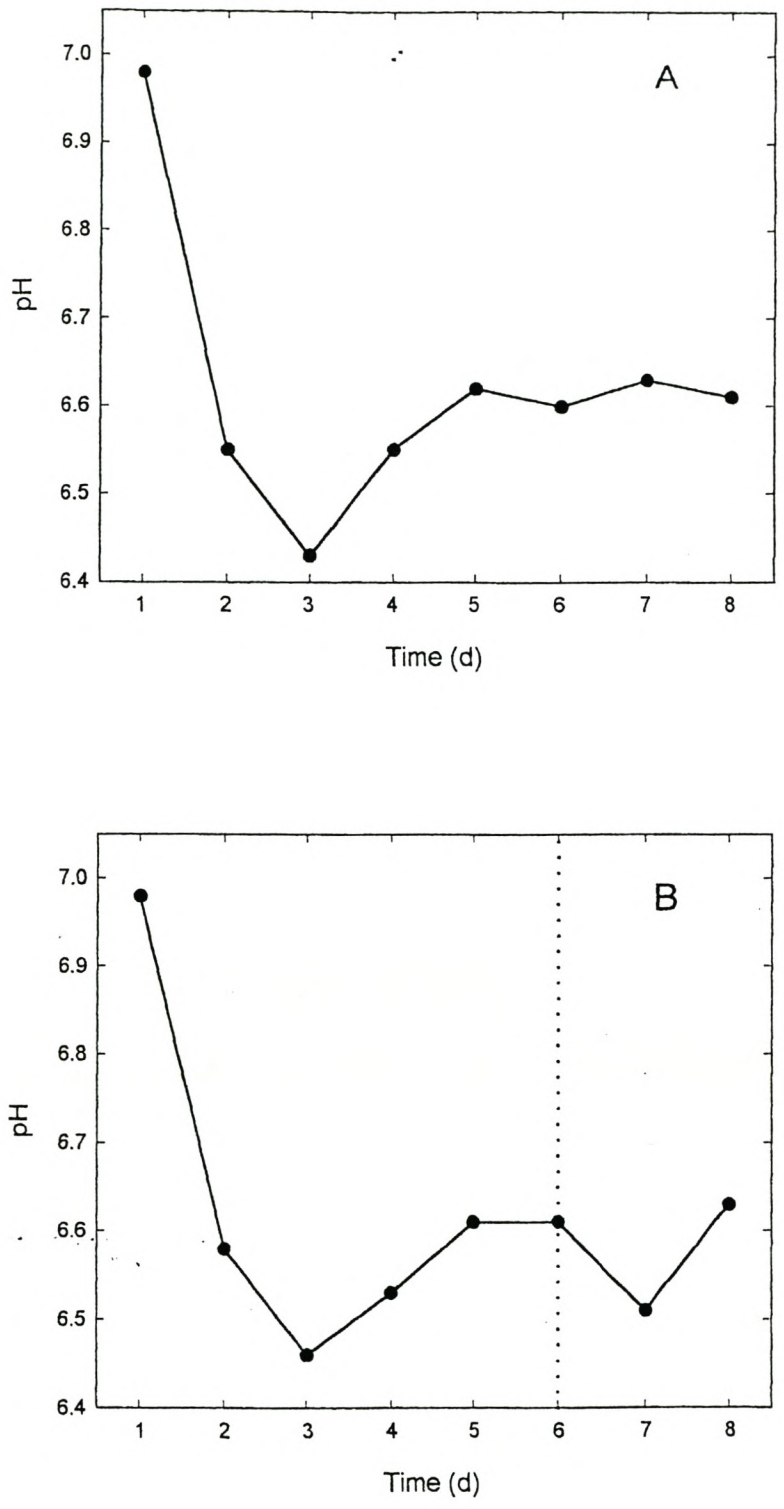

Figure 1. The pH profiles of granules incubated in a batch system (A = without the 'stress' treatment; B = with the 'stress' treatment on day 6). The dotted line represents the application of the 'stress' treatment.

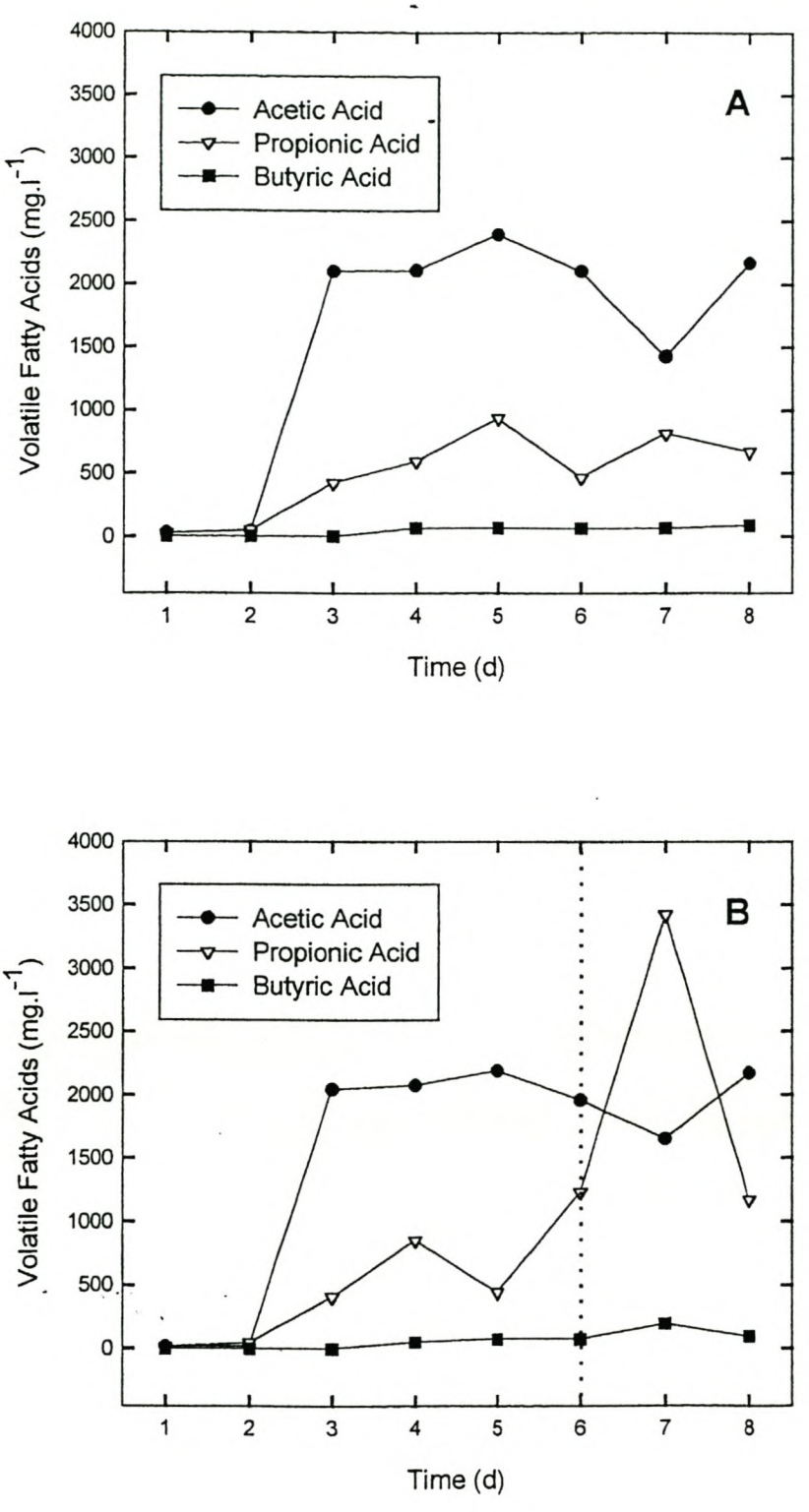

Figure 2. Volatile fatty acid profiles in 'unstressed' (A) and 'stressed' (B) batch granule units (average of triplicates). The dotted line represents the application of the ' stress' treatment.

6 days acetic acid was the major metabolite detected in both batch units. On day 7 however, the propionic acid concentration of the batch units that were 'stressed' on day 6, was found to be higher than the acetic acid concentration. On day 8, the propionic acid concentration in these units decreased again to below the acetic acid concentration (Fig. 2B). A slight increase in the butyric acid concentration (Fig. 2B) was also observed in the 'stressed' granule units. The units where no 'stress' conditions had been applied, showed no significant increase in the propionic acid concentration on day 7 and the acetic acid concentration remained higher than the propionic acid concentration (Fig. 2A). These changes in the volatile fatty acid profile when granules were 'stressed' are similar to the changes found when the lab-scale UASB reactors were subjected to sudden shock changes (Britz *et al.*, 1999) indicating that a microbial population shift had possibly taken place.

Bacterial enumeration and identification In this experiment it was determined whether the lactate-utilising population does change after sudden changes in the environment. The data on the changes in the lactate-utilising population when granules, grown in YEL-medium, were subjected to a sudden 'stress' loading of glucose, are presented in Fig. 3.

The average viable count of the lactate-utilising bacteria on the YEL-medium, when the granules were not 'stressed' was 8.2×10^6 cfu.ml^{-1} and the average Gram-positive lactate-utilising counts on the YELN-medium, were 3.85×10^7 cfu.ml^{-1}. However, 48 h after these granule units had been subjected to a sudden increase in 'stress' conditions, the viable lactate-utilising population was 1.36×10^7 and 8.25×10^7 cfu.ml^{-1}, respectively, for the YEL and YELN-media (Fig. 3A and 3B). These increases in the number of the lactate-utilising population (Fig. 3) after the granules had been subjected to a sudden 'stress' situation, supports the hypothesis (Britz *et al.*, 1999) in that the dynamics of the acidogenic group do change and specifically in the lactate-utilising population.

The *Propionibacterium* selective medium, Pal Propiobac, was used to enumerate the propionibacteria present in the granules. The average counts for the 'unstressed' units were 1.37×10^5 and 2.72×10^5 cfu.ml^{-1} on the 'stressed' units, respectively. Propionibacteria represent, on average, 60% of the total micro-organisms able to grow on Pal Propiobac and can usually be identified as brownish colonies, larger than 0.5 mm in diameter and surrounded by a yellow zone (Fig. 4).

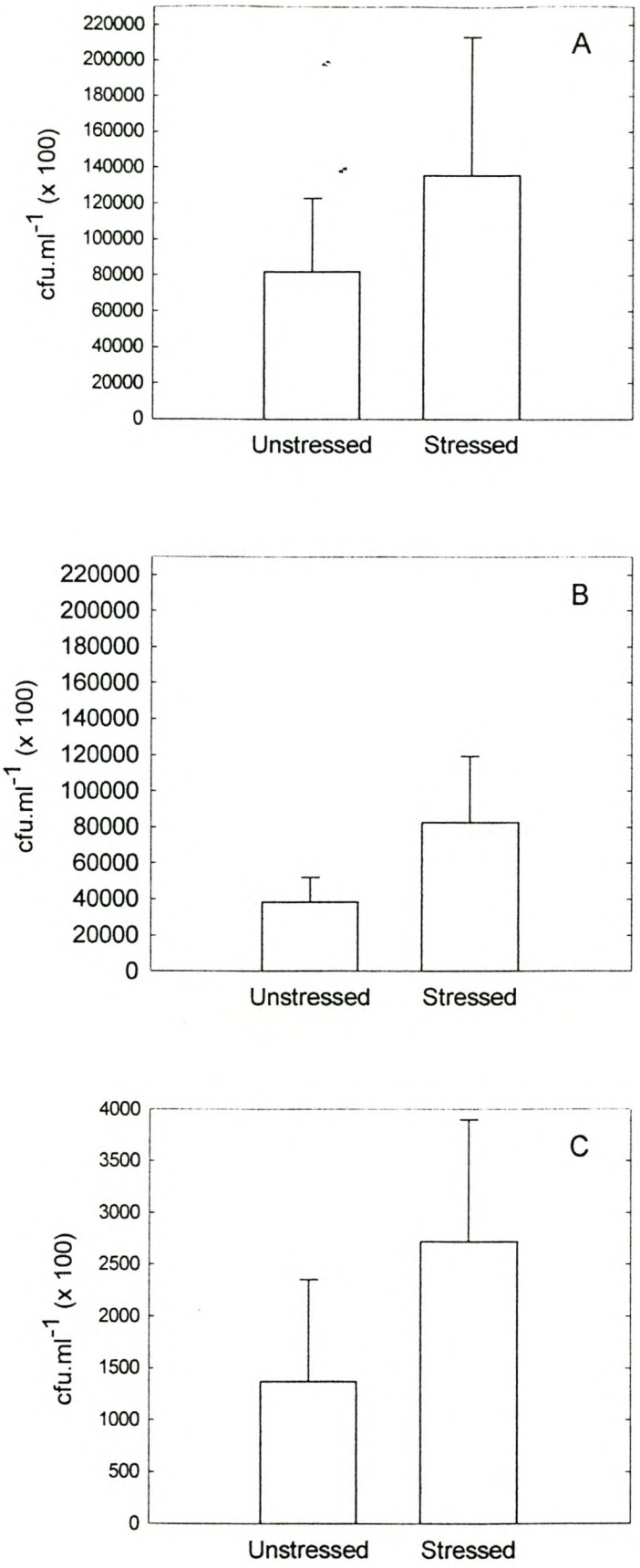

Figure 3. Changes in the lactate-utilising population on YEL (A), YELN(B) and Pal Propiobac-(C) media in granules cultured in YEL-medium before and after being subjected to a sudden 'stress' loading of 5.5 g per l glucose. (The standard deviation was used as the error bar length.)

The yellow zone (Fig. 4) is caused by a decrease in the pH that results from glycerol fermentation in the medium by the propionic acid bacteria (Thierry & Madec, 1995). In this study is was found that the colonies on the Pal Propiobac-medium could only be counted if there were less than 100 propionic acid producing colonies per plate. When the numbers were higher, the complete medium turned yellow and it was impossible to differentiate between the different acid producers. Therefore, although colonies on the plates with the highest dilution were usually less than 30, only those plates were used to select colonies for further studies.

As was already observed by other researchers (Riedel & Britz, 1993; Dubourgier *et al.*, 1988; Quatibi *et al.*, 1990) that propionic acid producing bacteria could be isolated from granules, irrespectively if they were 'stressed' or not and this was confirmed in this study (Fig. 3C). It was found that there was *ca* 1.35×10^5 more cfu of propionic acid producers per ml present after the granules had been 'stressed' (Fig. 3C).

Volatile fatty acid analysis done on the isolated colonies with the use of the Harrison Disk method (Harrigan & McCance, 1976), showed that the isolated bacteria produced more propionic than acetic acid and this was thus taken as proof that they were definitely members of the genus *Propionibacterium*. The members of propionibacteria isolated from the different YELN-plates, are given in Table 4.

This data, together with the VFA and pH profiles, confirms the part of the hypothesis postulated by Riedel & Britz (1993) that a sudden increase in a readily degradable substrate like glucose leads to a shift in the acidogenic population dynamics. It was also found that the propionic acid producers then gain a competitive advantage resulting in an increase in the propionic acid concentration with a concurrent decrease in pH, and the formation of ECP (Chapter 3 of this thesis).

Experimental Study II – Influence of different propionibacterial inoculum concentrations on the granulation process

The formation of granules in an UASB reactor allows the active methanogenic biomass to be retained independent of the flow rate, maintaining a good conversion efficiency at relatively high flow rates (Schmidt & Ahring, 1995). Results obtained by Britz *et al.* (1999) showed that separate culturing of granules holds a lot of promise for the application of the UASB technology in South Africa as a stable, high rate

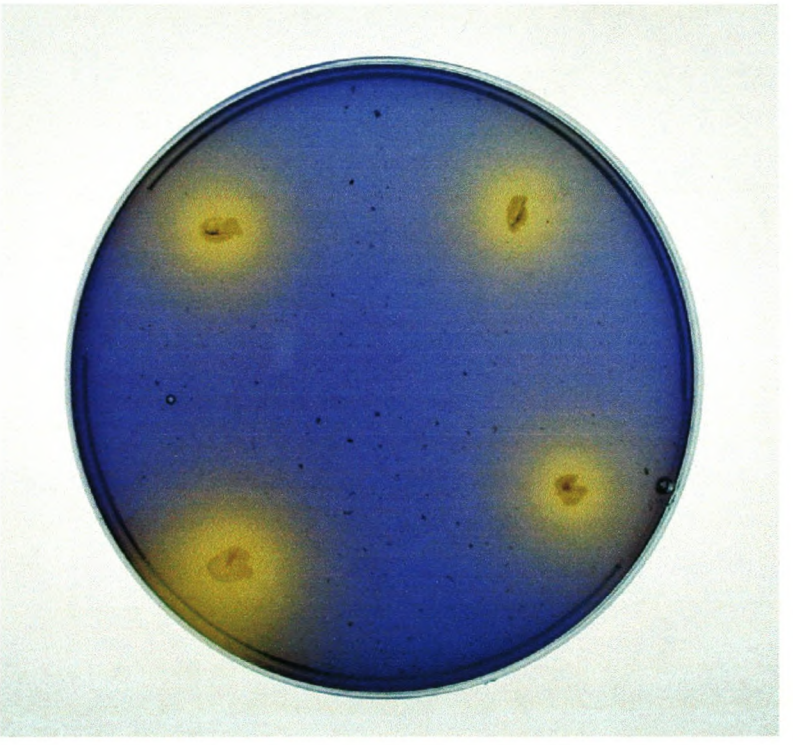

Figure 4. Photo of propionic acid producing colonies isolated from the granules and grown on the Pal Propiobac-medium.

Table 4. Numbers (%) of propionic acid producing bacteria selected from the YELN plates with the highest dilution using the Harrison Disc method.

	'Unstressed' granules		'Stressed' granules	
	Colonies on YELN plates	**% cfu of Propionic Acid producing Bacteria**	**Colonies on YELN plates**	**% cfu of Propionic Acid Producing Bacteria**
Sample 1	29	0	121	70
Sample 2	48	70	60	40
Sample 3	35	10	65	30

biological waste water treatment option. In order to shorten the start-up period of the UASB process, the need exists to stimulate the aggregation of microbes into granules. ECP is thought to play an important role in the granulation process (Schmidt & Ahring, 1995) and according to the hypothesis of Britz *et al.* (1999) and Riedel & Britz, (1993), the propionic acid producing bacteria that gain a competitive advantage during 'stress' conditions could produce ECP that enhances the granulation process. In this study, the influence of different propionibacterial inoculum concentrations (Table 2) on the batch granulation process, was determined.

In this study, the influence of different inoculum concentrations of the *P. jensenii* strain 278 on granule formation, was determined. Strain 278 was used because it was identified in Chapter 3 of this thesis, as an excellent ECP producing strain that had originally been isolated from an anaerobic digester. Different propionibacterial inoculation volumes (concentration of 10^6 cfu.ml^{-1}) were added (0, 10, 20 and 30 ml) (triplicate studies) to each of three different sludge volumes (15, 30 and 50 ml sludge) and inoculated in YEL-medium (Table 2). The samples were incubated at 35°C on a shake table in a batch replacement mode for 20 days and the pH and VFA's were measured daily. The change in pH was used as an indicator of metabolic productivity at the start and during the study.

pH profiles The typical pH profile was found for all the different sludge and *Propionibacterium* inoculum concentrations (Fig. 5). The pH dropped from 7.0 to 6.40 within the first 2 days in the samples to which the propionibacteria had been added. The controls (no added propionibacteria) showed a smaller drop in pH, from 7.0 to about 6.80 for the same period. This was expected as a result of the high volatile fatty acid formation from the lactate in spite of a high K_2HPO_4 buffering capacity (Roos, 1998). However, in all the batch systems, from day 2 onwards the pH increased and stabilised by day 7 - 9. This stabilisation persisted with the extended incubation of up to 20 days (Fig. 5). In most cases it was found that the initial pH drop was lower when a larger propionibacteria inoculum was used.

Metabolite production The profiles of the volatile fatty acids (VFA) produced by day 1, 2, 3, 4, 6, 11 and 20 are shown in Fig. 6 - 8. The metabolic profiles were

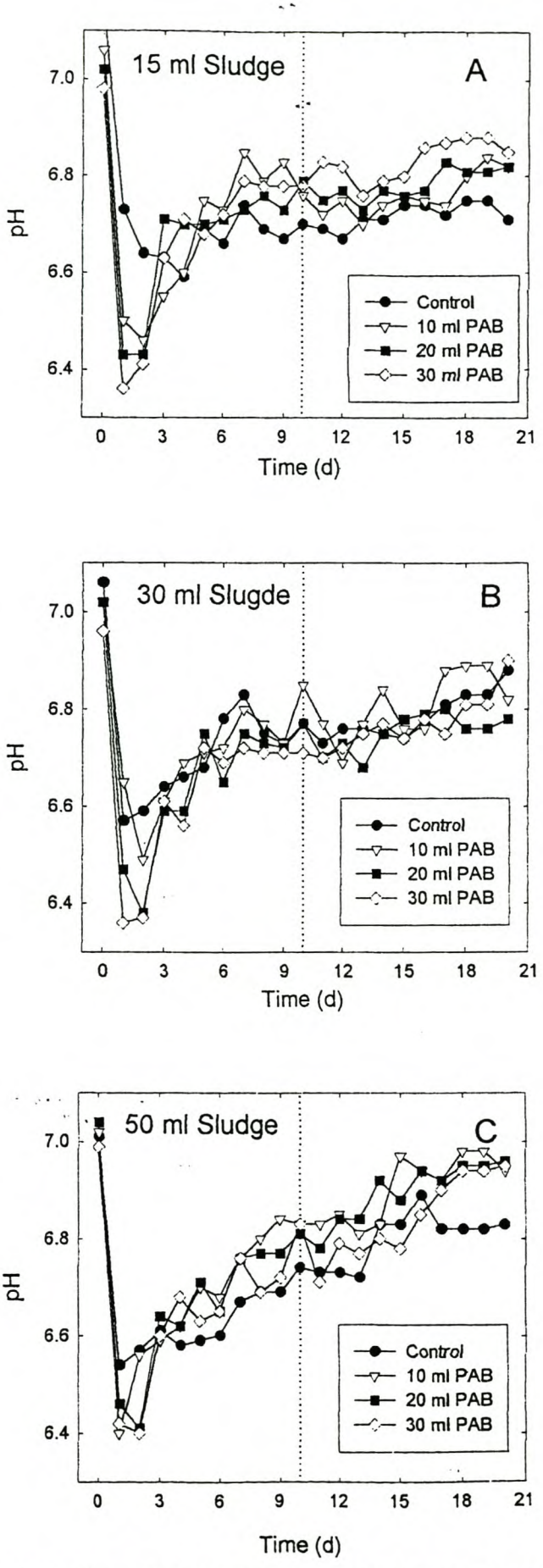

Figure 5. The impact of different sludge and propionibacteria concentrations on the pH values of the batch systems operated over a period of 20 days at 35°C (PAB = propionibacteria inoculum concentration).

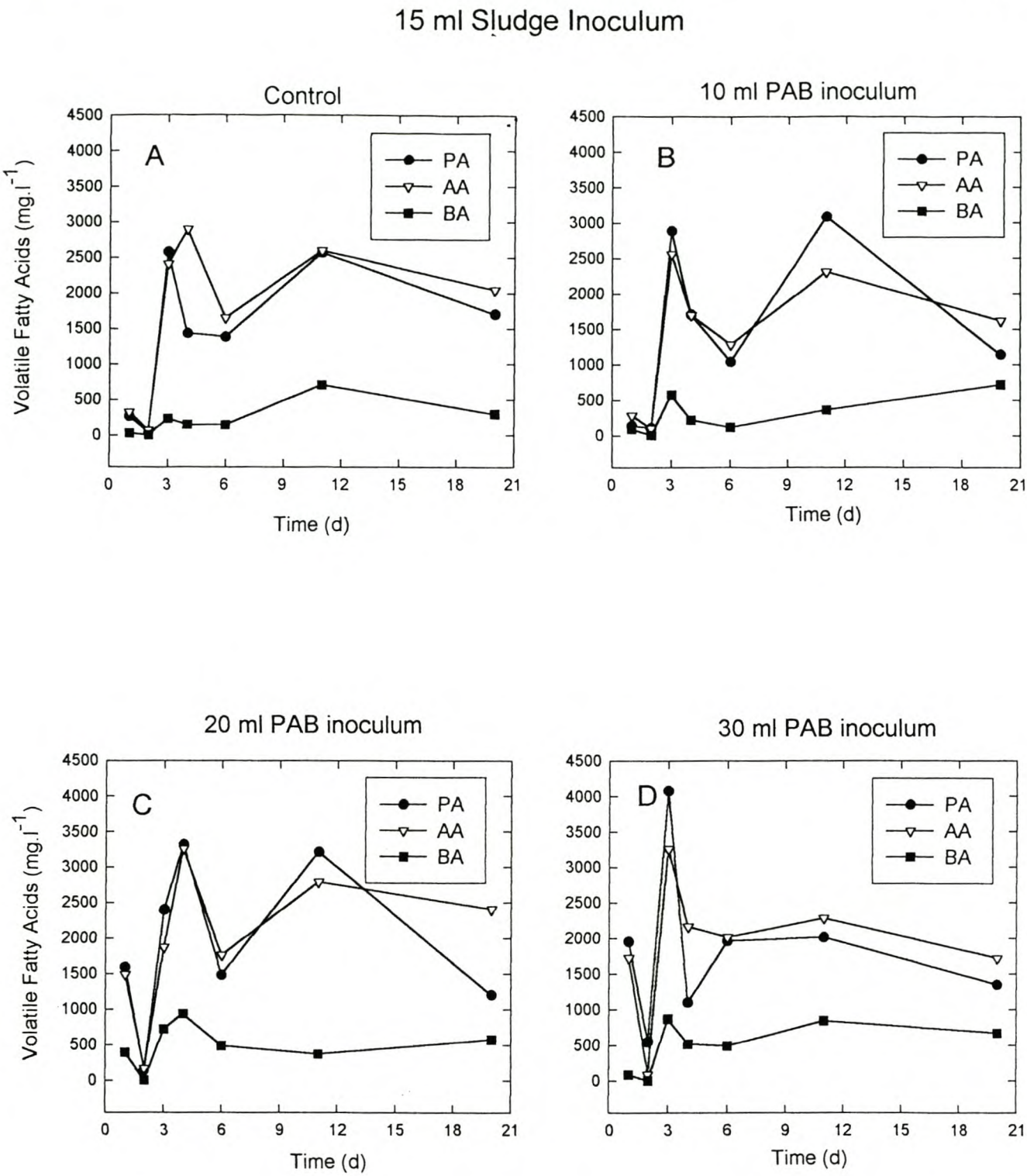

Figure 6. Influence of different concentrations of propionibacteria (A = control, no PAB added; B = 10 ml PAB; C = 20 ml PAB and D = 30 ml PAB) and 15 ml sludge inoculum on the volatile fatty acid production of the batch systems operated over a period of 20 days at 35°C (PA = propionic acid, AA = acetic acid and BA = butyric acid).

30 ml Sludge Inoculum

Fig7-chap4

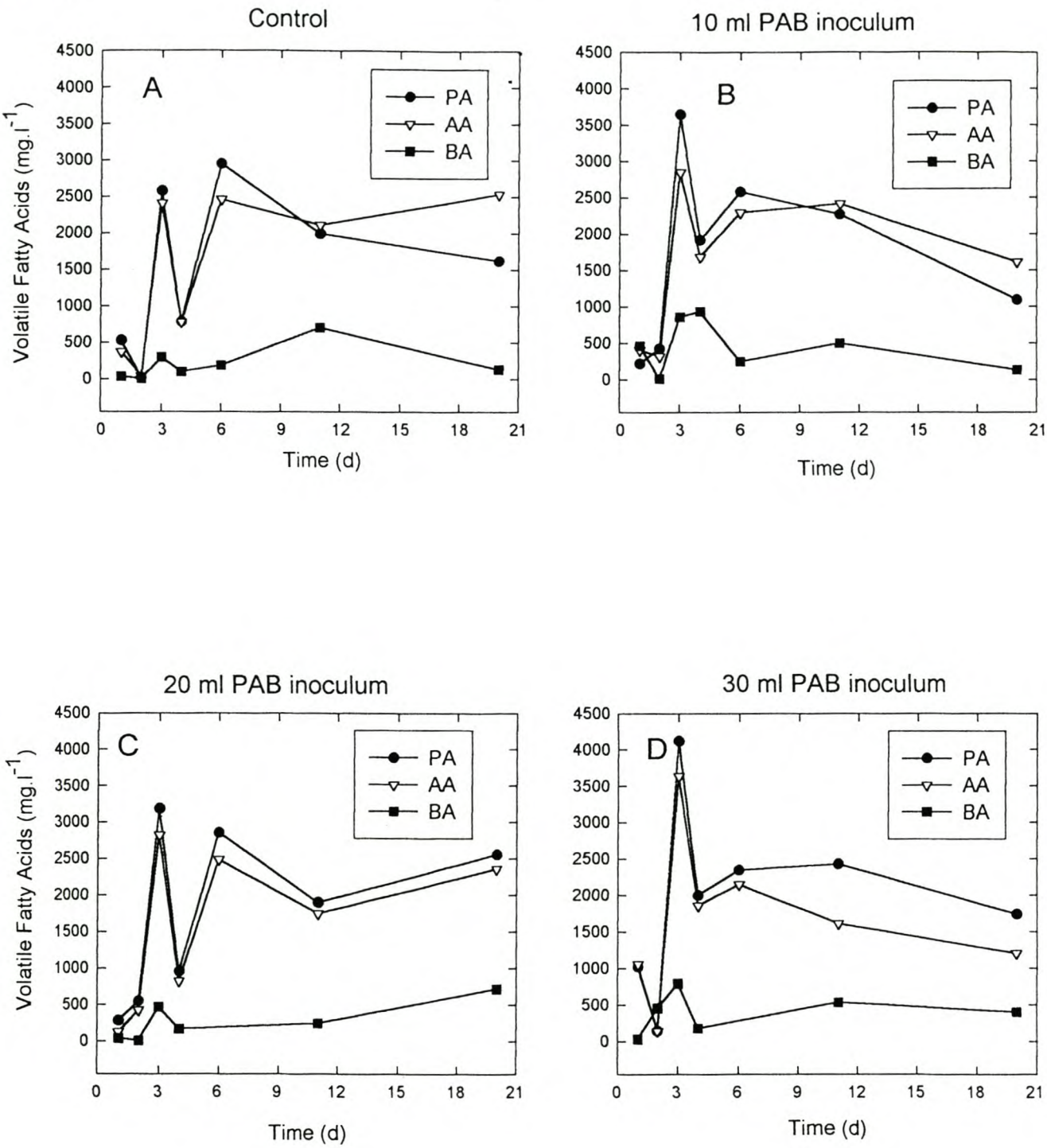

Figure 7. Influence of different concentrations of propionibacteria (A = control, no PAB added; B = 10 ml PAB; C = 20 ml PAB and D = 30 ml PAB) and a 30 ml sludge inculum on the volatile fatty acid production of the batch systems operated over a period of 20 days at 35°C (PA = propionic acid, AA = acetic acid and BA = butyric acid).

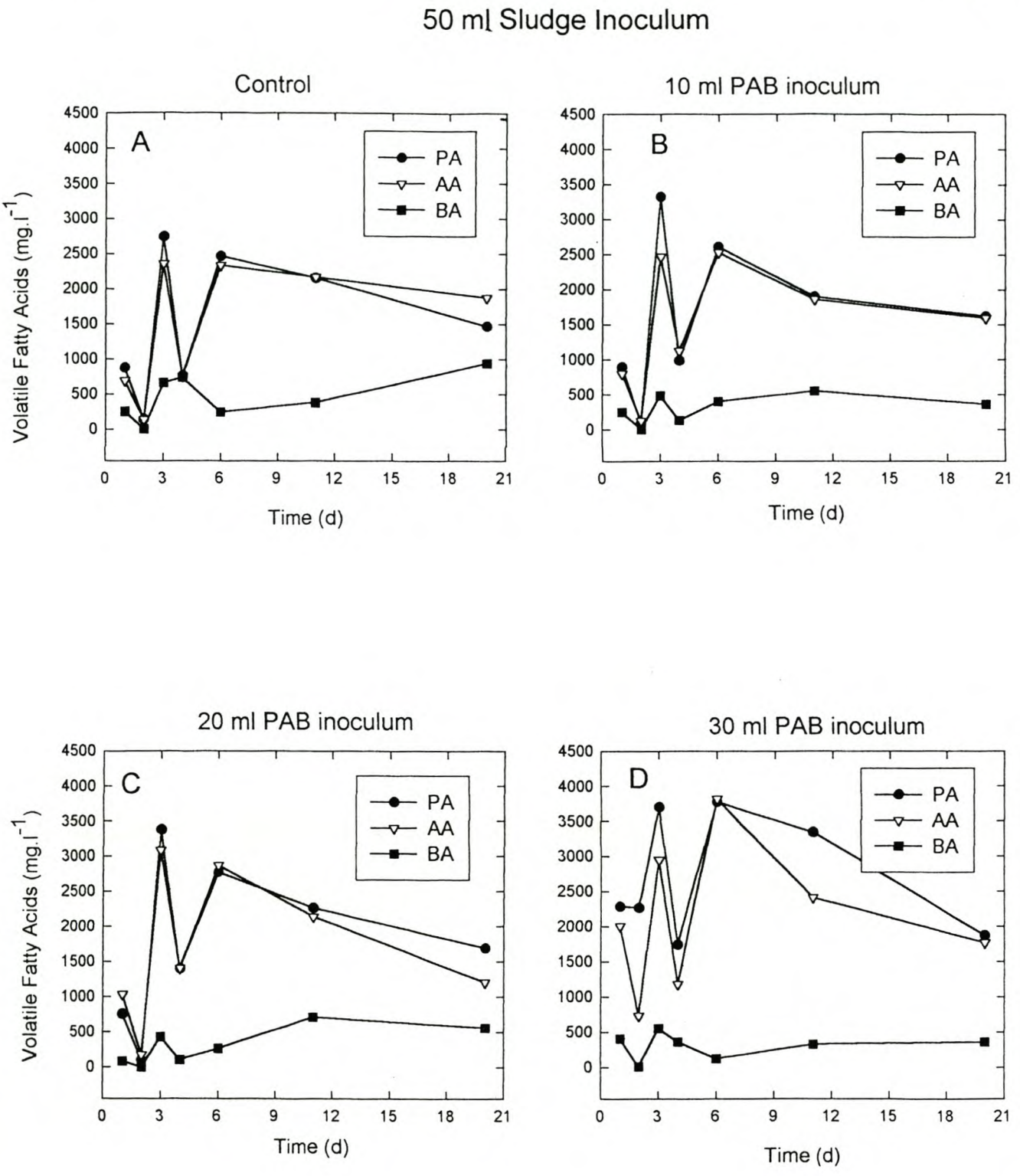

Figure 8. Influence of different concentrations of propionibacteria (A = control, no PAB added; B = 10 ml PAB; C = 20 ml PAB and D = 30 ml PAB)and 60 ml sludge inoculum on the volatile fatty acid production of the batch systems operated over a period of 20 days at 35°C (PA = propionic acid, AA = acetic acid and BA = butyric acid).

concurrent drop by day 4, an increase by day 7 and a drop by the end of the incubation period. Even though all the metabolite profiles were similar, the tempo of the second increase in acetic and propionic acid concentrations, increased as the sludge concentration was increased. The slightly higher concentration of propionic to acetic acid at the start is typical of the growth and metabolic profiles of members of the genus *Propionibacterium* (Riedel & Britz, 1993) and may be an indication of the lactate-utilising propionic acid producing organisms gaining an advantage.

Granule formation The number of granules of each set was determined on day 0, 10 and 20. In all the studies, the granules formed were very small, and in combination with a very cloudy solution, it was fairly difficult to always accurately detect the black nuclea (Fig. 9).

The influence of different sludge and propionibacterial concentrations on the amount of granules formed is given in Fig. 10A – 10C. The data points are the average values of three replica batches. When comparing the data of the three controls, it can be seen that there was a definite increase in the amount of granules formed as the sludge concentration was increased (at day 20 the control for 15 ml sludge = *ca* 6 330 per 10 ml and 50 ml sludge = *ca* 22 300 per 10 ml). An increase in the amount of granules formed by day 10 was observed in all the systems and can probably be ascribed to the high activity in the cultures. This fact is confirmed by the pH (Fig. 5) and fatty acid profiles (Fig. 6 - 8) of the batch cultures during the first 10 – 15 days. However, it is difficult to ascribe the increase in granules in the samples where propionibacteria had been added to either the propionibacteria or to the increased sludge concentration, and this is something that must be further investigated.

The granule samples were also sieved (Fig. 11) using a set of 5 sieves (2.0 mm, 1.0 mm, 0.71 mm, 0.5 mm and 0.121 mm) at day 20 and then again counted (Fig. 12). This was done to determine the size distribution in the granular sludge from the different batches. From this data (Fig. 12) it can be seen that more, small granules were formed when both the sludge and propionibacterial concentrations were higher with most in the 0.5 mm range. Only a few granules were larger that 1.0 mm.

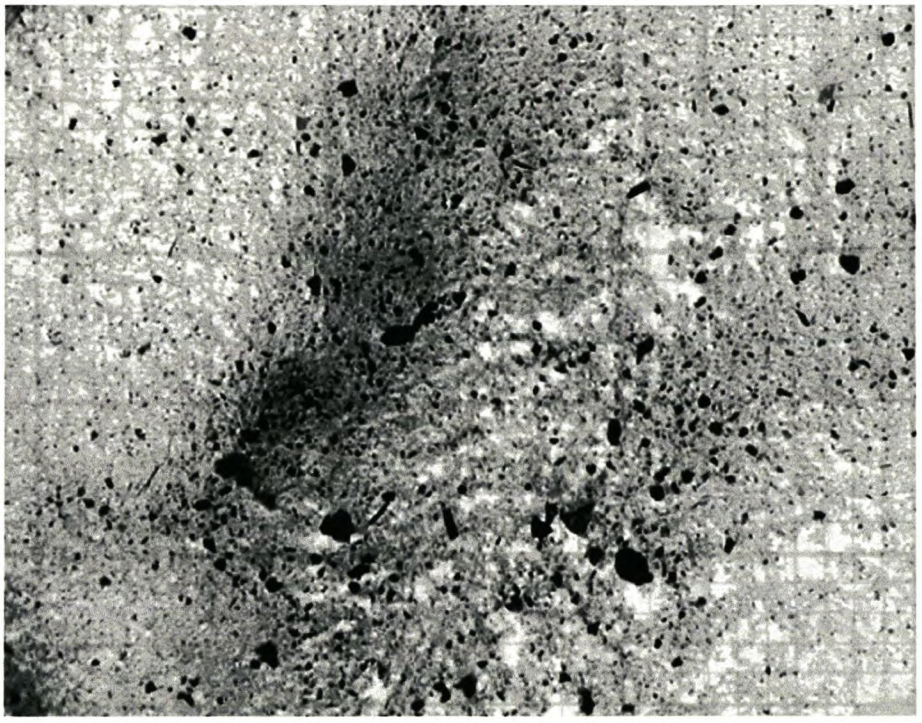

Figure 9. Granules made in the batch replacement mode for 20 d in YEL-medium with 50 ml sludge and the addition of 30 ml propionibacteria.

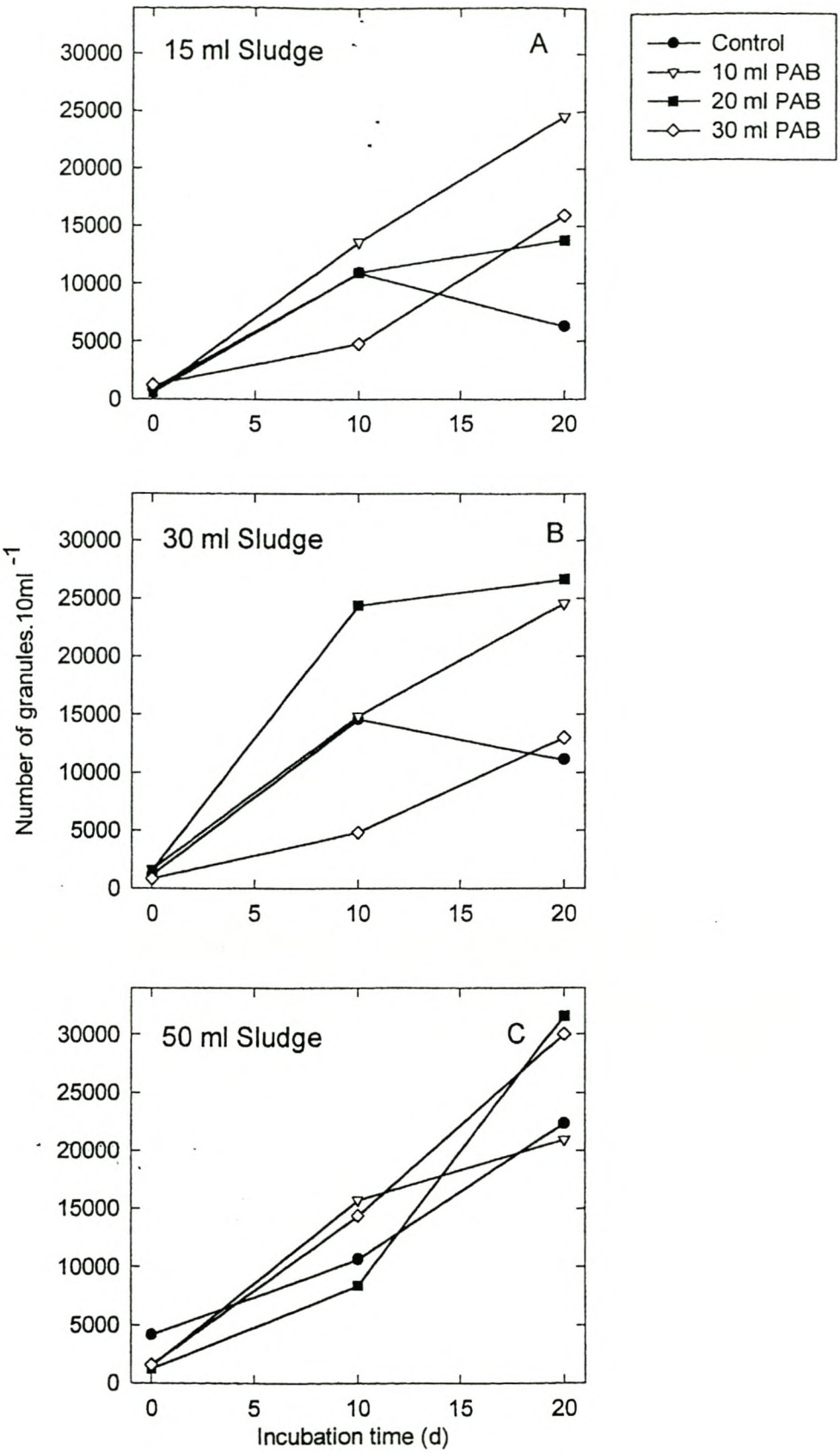

Figure 10. The impact of different *Propionibacterium* inoculum and sludge concentrations on the granule formation in the batch systems operated over a period of 20 days at 35°C (PAB = propionibacterial inoculum concentration).

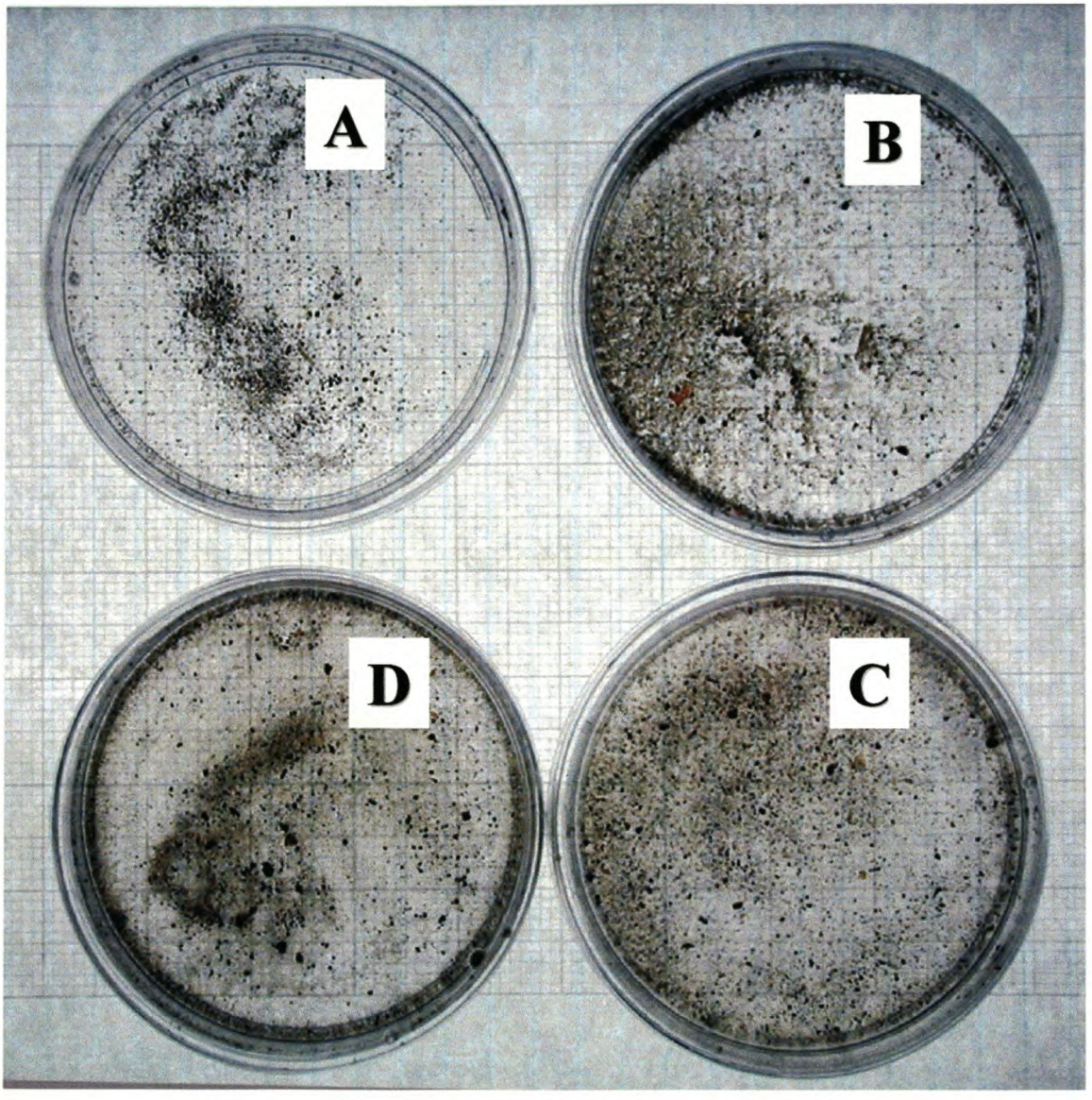

Figure 11. Photo of the granules produced in the batch systems with 50 ml sludge. A = control, B = 10 ml propionibacteria, C = 20 ml Propionibacteria and D = 30 ml propionibacteria.

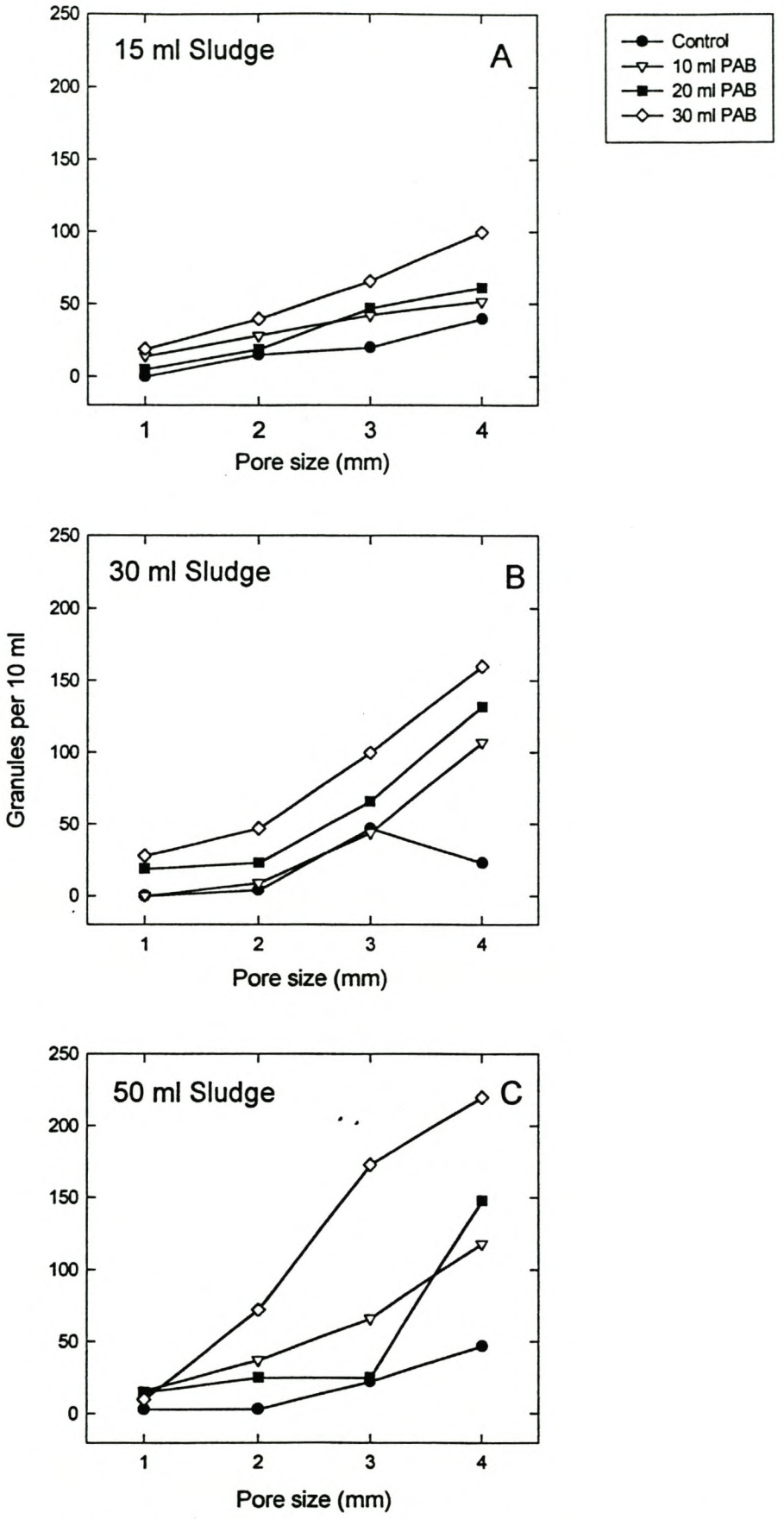

Figure 12. Influence of different *Propionibacterium* and sludge concentrations on the number and size of granules produced in a batch system and sieved after 20 days of incubation at 35°C (PAB = propionibacteria) (1 = 2.0 mm, 2 = 1.0 mm, 3 = 0.71 mm, 4 = 0.50 mm).

Propionibacterium isolation from the batch grown granules The propionic acid producing bacteria present in the granules were isolated on day 20 to determine if the same strain that was initially added to the sludge, could be re-isolated after the granular batch cultivation period. The isolation data from the batch cultures using the Pal Propiobac-medium, are given in Table 5. All the brown colonies surrounded with yellow zones on the Pal Propiobac-medium were isolated and further identified to determine if they were members of the genus *Propionibacterium*. From this it was found that all these bacteria were Gram-positive rods or cocci, with no endospore formers present. The isolates all produced propionic acid, but acetic acid was found to be the major metabolite.

The data showed no direct correlation between the number of bacteria isolated from the Pal Propiobac-medium and the concentration of propionic acid bacteria added to the batch cultures. This was probably because the Pal Propiobac-medium was not as selective as was expected and it was found that several other bacterial strains were also capable of growing and producing the characteristic yellow zone on the Pal Propiobac-medium. This was also found in Experimental Study I.

Activity tests (methane and biogas production) According to Schmidt & Ahring (1995), activity tests are necessary for the determination of the methanogenic potential of granules. In this study activity tests were done on the granular sludge on day 20 before and after the granular sludge was sieved to remove all non-granular material. The activity data are presented as the cumulative biogas and methane production in Fig. 13.

The activity data showed that the biogas increased as the sludge inoculum volume was increased (control = 1.40 ml for the 15 ml sludge unit; 2.30 ml for the 30 ml sludge unit and 2.60 ml for the 50 ml sludge unit) (Fig. 13A - 13C). This was to be expected as the granule counts were also higher when more sludge was added (Fig. 10). This was also true for the methane production (Fig. 13D - 13 F)

The influence of different concentrations of propionibacteria on the activity was also determined, and from the data given in Fig. 13, it is clear that the size of this propionibacterial inoculum has a definite enhancing effect on the activity. The highest biogas and methane activities were obtained when 50 ml sludge was used. In all cases when 30 ml propionibacteria was added, the highest biogas and

Table 5. Colonies isolated from the different granular sludge samples using the Pal Propiobac-medium.

Sludge concentration (ml)	***Propionibacterium* inoculum concentration (ml) (1×10^6 cfu.ml^{-1})**	**Colonies on Pal Propiobac-plates (cfu. ml^{-1})**
15	0	15 000
15	10	40 000
15	20	23 000
15	30	9 000
30	0	19 000
30	10	23 000
30	20	37 000
30	30	16 000
50	0	13 000
50	10	8 000
50	20	13 000
50	30	100 000

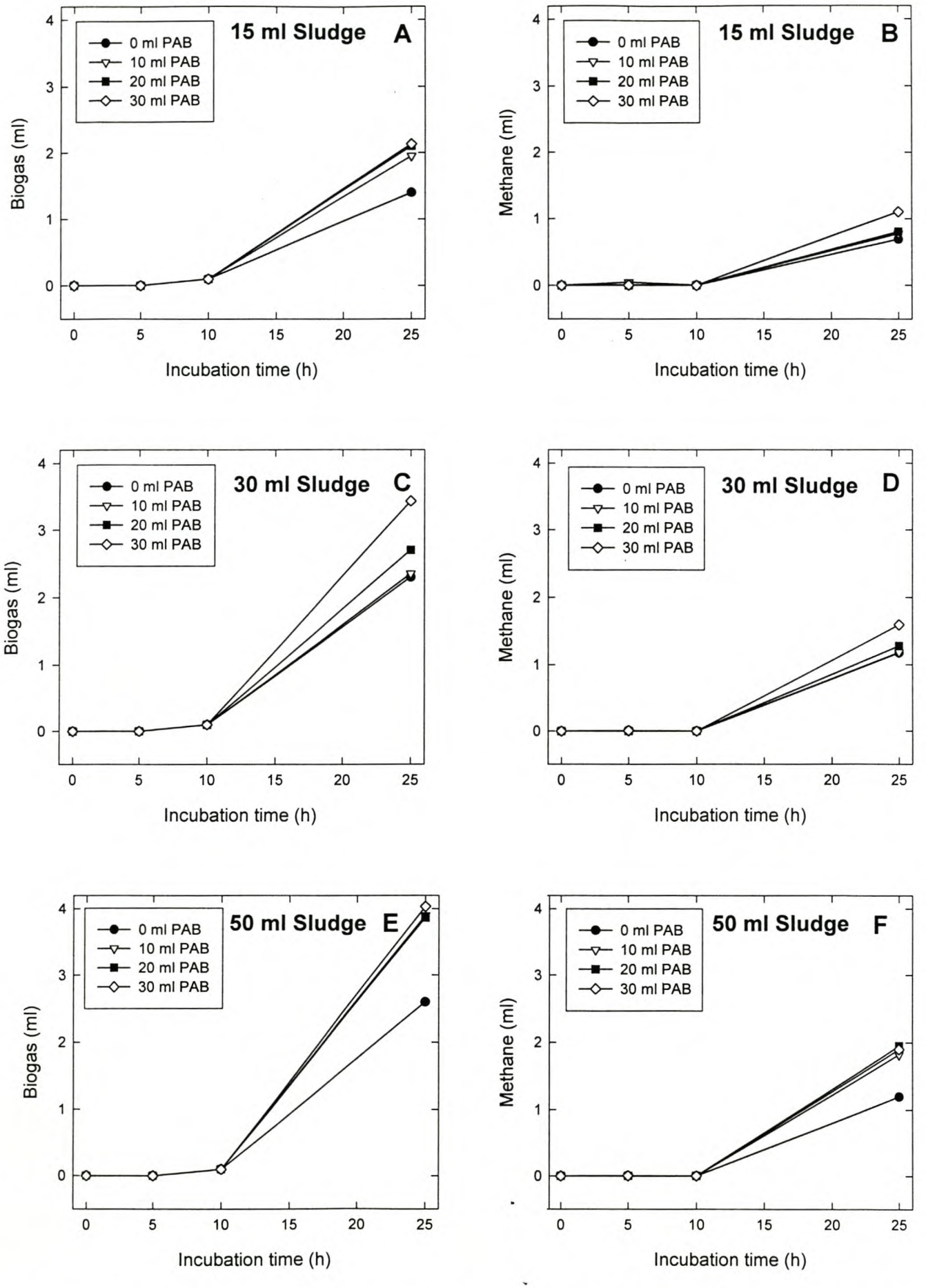

Figure 13. Influence of different *Propionibacterium* inculum and sludge concentrations on the activity of granular sludge produced in the batch systems operated over a period of 20 days at 35°C (PAB = 10^6 cfu ml^{-1} propionibacteria)

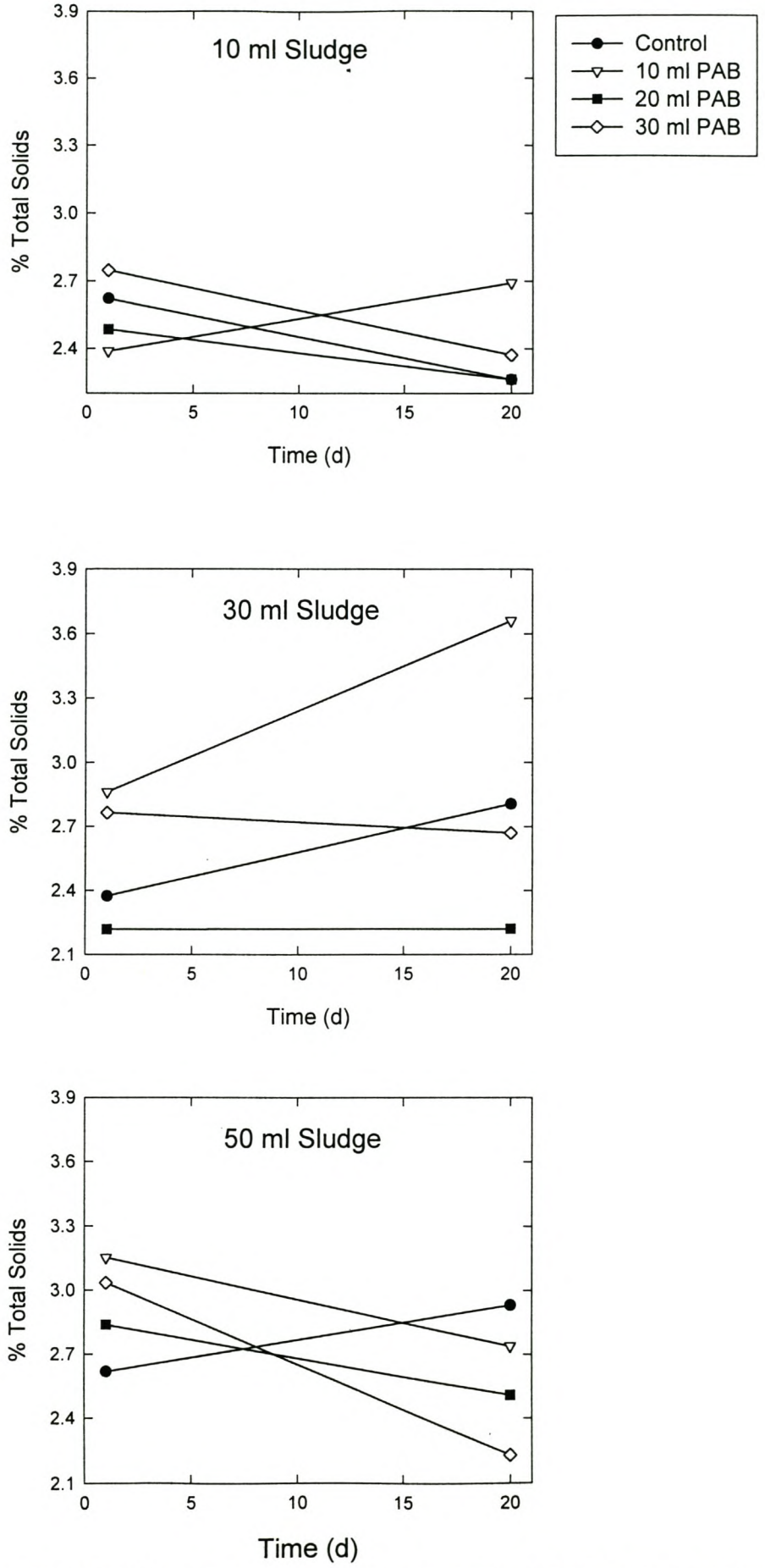

Figure 14. Influence of the different concentrations of propionibacteria and sludge on the total solid values (%) of the granular sludge (PAB = propionibacteria).

As postulated in the hypothesis, the granulation process was facilitated by a drop in pH at the start resulting from a sudden accumulation of organic acids. Subsequently, an increase in the reduced metabolites, like propionic acid, is found. This is followed by an increase and stabilisation in the pH with a steady decrease in propionic and acetic acid concentrations until stabilisation. According to the hypothesis (Britz *et al.*, 1999; Riedel & Britz, 1993), excessive production of ECP would then occur as an alternative hydrogen sink mechanism, with the concurrent aggregation of the granular sludge. From the data obtained in this study it was found that, when higher concentrations of the propionibacteria were added to the sludge, more granules formed. The granules were also more active in terms of biogas and methane production when propionibacteria were added. It was thus found that, when more propionibacteria are added, better granulation takes place. This is probably because the propionibacteria produce ECP that is important for the structure and maintenance of the granules (Forster & Quarmby, 1995), but this fact must still be confirmed in future research.

It can be concluded from the data that the environmental conditions in batch cultures can be changed by applying 'stress' to give the lactate-utilising propionic acid producers a competitive advantage, which then leads to an enhancement of the granulation process. It was also found that the presence of *Propionibacterium* strain 278 has a definite enhancing effect on the granulation of the sludge.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Background

The "classical" propionibacteria have previously mostly been isolated from dairy products, but there have also been reports of their presence in anaerobic digestion systems (Riedel & Britz, 1993). It has also been argued that members of the genus *Propionibacterium* could be one of the main genera of the microbial community present in granular sludge (Dubourgier *et al.*, 1988). The extracellular polysaccharide (ECP) producing ability (Cerning, 1995; Sherman & Shaw, 1921; Skogen, 1970) of propionibacteria makes them of special interest for the food and also waste water management industries. It has been hypothised by Riedel & Britz (1993) that propionibacteria gain a competitive advantage during 'stress' conditions in the anaerobic upflow sludge blanket (UASB) reactor and that their ECP producing trait might contribute to the enhancement of the granulation process. There is, however, little knowledge on the ECP production by propionibacteria and in the first section of this study, different strains and environmental conditions that influence ECP production, were studied.

ECP production

In the first study (Chapter 3), 19 different propionibacterial strains, originally isolated from two totally different ecosystems, were found to visually produce ECP, with *P. jensenii* strain 278 producing the most ECP under the study conditions. Fermentation conditions (temperature, pH and incubation time) and the cultivation medium composition (carbon and nitrogen sources) are known to influence the ECP composition and the yield of the polymers produced by several different microbes (Skogen, 1970: Sutherland & Tait, 1992). The ECP producing potential of strain 278 was further optimised, not only because of its higher ECP production ability, but also because it was originally isolated from an anaerobic digester and thus has potential to improve granulation.

It was reported in the literature that more ECP is produced at lower temperatures (Cerning, 1995; Mozzi *et al.*, 1995). In the first study it was found that

the optimum temperature for ECP production by strain 278 was around 22°C. The data also showed that lower pH conditions clearly resulted in a lower ECP production. When a higher concentration of sucrose (8%) was added to the growth medium, and thus the carbon to nitrogen ratio changed, the viscosity and the ECP production were enhanced. This enhancement was the best at 22°C. It was also observed that incubation at 18°C led to a negative ECP enhancement. Although it is reported in the literature (Racine *et al.*, 1991; Williams & Wimpenny, 1977) that the initial medium pH influences ECP production, very little variation in the ECP production occurred because of the change in initial pH under the conditions used in this study. However, it was found that the best ECP production by strain 278 was nearer to neutrality, but not below pH 7.0. This was ascribed to the fact that, at such a low initial pH, the inhibitory pH for the growth of the strain was probably reached more quickly and less cells were thus available for ECP production.

When the results were evaluated using a 4 x 2 x 4 factorial design, the best ECP producing trend when evaluating the main effects, was once again found to be between 22° and 25°C and with the use of 8% sucrose in the growth medium. Higher incubation temperatures of above 25°C showed a very negative trend, indicating that a temperature range around 20° - 25°C needs further investigation to optimise the ECP production. The two- and three-factor interactions again indicated a positive trend in terms of higher initial pH and sucrose concentration and it was found that a pH of higher than 7.5, but lower than 8.5 would be more in the optimal range. It was also again seen that a temperature range between 22° and 25°C was a positive trend in the optimisation of ECP production. The optimum conditions for the parameters evaluated in this study for ECP production were 8% sucrose, 22°C and pH nearer to 8.0 and 8.5.

The optimal ECP production at lower temperatures may be because of the slower growth of the bacteria at these temperatures and thus the availability of the enzymes needed for ECP production. It may also be that the transcription of the enzymes are activated by lower temperatures. The higher ECP production at 22°C and between pH 8.0 and 8.5 by this specific propionibacterial strain, may also be explained as a possible protection mechanism against unfavourable growth conditions. It has been reported that the production of ECP can also function as an alternative hydrogen pathway (Vanderhaegen *et al.*, 1992) and may, therefore, also

protect the cells against acid conditions. Although proteins are dominant in the ECP of granular sludge, none was found in the ECP from propionibacteria.

The parameters used for the production of ECP must, however, be further investigated to determine the best optimum conditions for ECP production. Further research is also needed on the biosynthesis of ECP in propionibacteria and the cause of the instability of the ECP producing trait as it appeared that the ECP production by strain 278 was enhanced by sub-cultivation. It can be speculated that this is because the genes for ECP are transcribed by plasmids, as a plasmid associated with a clumping phenomenon, was identified in *P. jensenii*.

Influence of propionibacteria in the UASB granulation process

During the last 20 years, the development of the upflow anaerobic sludge blanket (UASB) reactor has played an important role in the treatment of waste waters. In the UASB, degradation kinetics are enhanced by biomass immobilisation in the form of granules (Schmidt & Ahring, 1996). However, the formation, amount and specific activity of the granules dictate the operational efficiency and performance of the UASB reactors. Furthermore, the application of the UASB system is restricted by the long start-up times resulting from the slow process of granulation, as well as the need for a speedy replacement of granules once they have been washed out of the system. Thus, unless the granulation formation conditions can be defined and optimised, full exploitation of this rather unique biomass immobilisation technique cannot be realised (Schmidt & Ahring, 1996).

The precise nature of the mechanisms involved in granule formation is still not fully understood and many researchers have suggested theories for granulation, but community composition, structure and microbial growth of granules still needs to be investigated in more detail. It is however, generally accepted that the formation of granules is associated with the production of ECP (Schmidt *et al.*, 1992). It was postulated by Britz *et al.* (1999) that, through the implementation of environmental 'stress' conditions, a shift in the population dynamics of the anaerobic community can be obtained. This results in a concurrent increase in ECP formation that appears to enhance aggregate formation. The aim of the second part of this study (Chapter 4) was to partially verify this hypothesis.

It was firstly found that, when a fermentable carbon source (glucose-rich YEL-medium), and thus 'stress' conditions, w~s applied to granules incubated in a batch replacement mode, the Gram-positive lactate-utilising population gain an advantage. The propionic and acetic acid concentrations increased 24 h after the granules were 'stressed' with a concurrent sharp decrease in the pH. The propionic acid concentration was higher than the acetic acid concentration, which is characteristic of the metabolic profiles of members of the genus Propionibacterium and thus an indication that propionibacteria were present. Isolation and enumeration of the Gram-positive lactate-utilising (YELN-medium) and propionic acid bacteria (Pal Propiobac-medium) also indicated that an increase in members of these bacteria were stimulated. It was found that there was ca 1.35 x 105 more cfu's of propionibacteria per ml present after the granules had been 'stressed' indicating a definite influence of 'stress' conditions on the population dynamics of the granules.

These findings verify part of the hypothesis of Britz et al. (1999) and Riedel & Britz (1993) that, when granules are 'stressed', the acidogenic population dynamics of the anaerobic community change. As a result of the 'unbalanced' conditions, an orderly shift between the predominant lactate-utilising bacteria took place in response to the gradual decrease in the pH. In response to the decrease in pH, the more acid-tolerant propionic acid producing bacteria clearly gained a competitive advantage and the propionic and acetic acid concentration increased.

The influence of different concentrations of propionibacteria added to batch systems in the production of granules (Roos, 1998), was also studied. Although the optimum temperature for ECP production by Propionibacterium strain 278 was identified in Chapter 3 of this thesis, to be between 22° and 25°C UASB reactors function at 35°C, and therefore, the anaerobic sludge was incubated in batch replacement mode at 35°C. The granules that were formed were very small and it was difficult to determine the numbers accurately, but with the use of image analysis, trends could be observed. In this study, the drop in pH and the concurrent increase in propionic and acetic acids at the start of the process led to a.stimulation of granule formation. According to the hypothesis (Britz et al. , 1999; Riedel & Britz, 1993), in response to the decrease in pH, an orderly shift of the population dynamics in the anaerobic consortium takes place with the more acid-tolerant propionic acid producing bacteria gaining a competitive advantage. Subsequently, an increase in more reducedmetabolites like propionic acid is found and this can then also lead to the production of more ECP as an alternative hydrogen sink mechanism, and the

system then displays clumping characteristics. Thi.s was especially true with the samples containing 30 ml propionibacteria and 50 ml sludge as inoculums. The activity (biogas and methane production) of the granules was also dependent on the amount
of sludge and propionibacteria added to the batch samples. The activity was higher when the concentration of both the sludge and propionibacterial concentration was higher.

Propionibacterium strain 278 could not be isolated at the end of the incubation period and this needs to be done before the hypothesis can be fully verified. A possible reason for the inability to isolate the specific strain from the granular sludge is that the Pal Propiobac-medium that was used, was not selective enough. Another reason for this difficulty to isolate propionibacteria, may be the long growth period required by propionibacteria and thus that other, faster growing bacteria, over grew the slower growing propionibacteria. This is one aspect that must be researched in the future and the use of molecular enumeration techniques might be of great value in enumerating and confirming the presence of the propionibacteria in the granules.

The data obtained in this study clearly shows that part of the granulation be verified, although research is still needed to verify the complete hypothesis. In the future, with the addition of propionibacteria and the application of 'stress' to the anaerobic sludge in UASB reactors, it may be possible to optimise the stimulation of the granulation process and thereby enhance the efficiency and potential of UASB technology.

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