A TWO-ENZYME CLEANING-IN-PLACE PROGRAMME FOR SOUTH AFRICAN DAIRIES

Report to the Water Research Commission
on the Project
"Isolation of Microbial Extracellular Enzymes
for Possible Use in Dairy
Cleaning-in-Place Applications"

by

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EXECUTIVE SUMMARY

Industrial Relevance of Project

Processing of dairy products is very complex with respect to production requirements. Unlike some other industries, a dairy plant is concerned with the processing of a highly perishable product for supply to the consumer who is becoming increasingly aware of nutritional requirements and has an increasing desire for high quality, value-for-money products. In order to produce such high quality, value-for-money products, the dairy industry has to concern itself with issues such as labour and its increasing costs, the cost of operation and maintenance of equipment, the cost of cleaning and maintaining hygienic conditions on the plant, pressures from regulatory agencies with regards to product specifications and the discharge of effluent and the rising cost of fuels. Dairies face a serious dilemma to improve their CIP systems cost efficiency whilst maintaining high sanitation standards and reducing effluent outputs. The hypothesis for this study is that, if it is possible to harness the enzymes which cause the currently unwanted degradation of milk constituents, these can be modified to be used as possible cleaning agents in the dairy industry. This approach would address the environmental concerns which have arisen through the use of chemicals for cleaning and the use of a single stage biological cleaning regime may decrease the total cost-of-clean in dairies. Both bacteria and fungi are capable of producing extracellular enzymes that are capable of breaking down milk constituents at low (Law, 1979; Muir et al, 1979; Cousin, 1982; Rowe et al, 1990) and high temperatures (Richardson et al, 1978; McKellar, 1981). This could be achieved by digesting the milk constituents on product surfaces by enzymatic action, rather than by removing these with high pressure and high temperature chemical solutions.

There is a dire need in South Africa to improve the quality of industrial effluents. A possible method to improve effluent by reducing its organic loading, not currently in use in South Africa, is to rinse all equipment prior to washing to collect the product residues from which dairy solids can be extracted for further processing or swine fodder. Furthermore, effluent treatment plants are being suggested as an attempt to improve the quality of effluent dumped. Effluent treatment plants are going to be expensive with respect to capital outlay and more costs will be incurred in the running of these plants. Therefore, a paradigm shift away from caustic and acid in dairy cleaning and sanitation is needed to avoid excessive costs which will inevitably rise as an environmentally conscious society continues to demand stricter legal limitations on effluent discharge. This can, however, only occur if the new technologies are equal to or greater in efficiency than the currently applied techniques.

Aims

1. The overall aim of this study is to produce an economically viable biological cleaning system for dairy plants. The system has to be as, if not more, effective than the current chemical systems used, both with respect to cleaning action and anti-microbial efficacy, while significantly improving the effluent released from the plant and reducing water consumption and energy expenditure. This would effectively reduce the total cost-of-clean in the dairy resulting in a decrease of energy, water, chemical and effluent discharge costs.
2. To screen microbes isolated on-site in a dairy for their extracellular enzymes which can degrade milk constituents, and to characterize the enzymes and optimize the conditions for

their activity. Furthermore, the enzyme activity is to be compared to that of commercial preparations of the enzymes as well as to the enzymes isolated from known secretors such as *Bacillus* (proteases) and *Pseudomonas* (lipases).

3. To build a laboratory-scale plant to test the efficacy of the screened enzymes on milk deposits and to compare these results to commercial preparations of the enzymes as well as to the currently used cleaning regimes.
4. To improve enzyme production and export from the selected microbes. This will be done by placing the micro-organisms under selective pressures upon which their efficacy to express the enzymes in question will be assessed on solid media as well as by biochemical means.

Accomplishment of Aims

1. The aims of this study were to develop a CIP system based on lipases and proteases, which would result in a more acceptable effluent being discharged while still providing effective cleaning for all surfaces in the dairy industry in South Africa. The twin enzyme lipase/protease CIP system proposed and investigated in this study produced an equivalent clean whilst producing a more acceptable effluent when compared to the current chemical CIP practices.
2. Novel biocatalysts were isolated from *Serratia liquefaciens* and *Acinetobacter lwoffi* and these were shown to be able to provide a far safer and cheaper alternative to the conventional chemical CIP systems.
3. A 'mini dairy' system was constructed of 9.6m of dairy grade stainless steel pipe with a diameter of 5cm with the help of Clover Dairies and Mr Albert Loubser of Ecolab SA. The milk and cleaning solutions were pumped through the system at 1.5m/s using a centrifugal pump. There are five dead-end sections with Pyrex ends that were designed to allow for access to the pipes for visual inspection of cleaning efficacy and for taking swabs. The ‘mini dairy’ allowed for the evaluation of the new technology as well as for the verification of the efficacy of current cleaning regimes.
4. The fourth aim was not attained as the current feeling towards GMOs is such that a natural rather than an engineered enzyme will be acceptable to both industry and the public.

Screening for Micro-organisms producing Potential Biocatalysts of Value

For the selection of extracellular enzymes it is realistic to evaluate their potential to degrade milk constituents in the considered environment. This includes the physico-chemical conditions such as temperature, pH and the presence of possible inhibitory compounds as well as the synergism or antagonism between the enzymes selected. The first step in selection consists of the screening of strains from a milk-processing site. The aim of this screening (usually done on solid media in the laboratory) is to assess the microbial performance in a wide range of situations related to the environment in which they have to operate. The selected cultures should then be carefully preserved as stock cultures. Once the cultures have been selected from the solid media, the extracellular enzymes should be evaluated with regards to temperature, pH and the presence of possible inhibitory compounds as well as interactions between the enzymes, in the laboratory using biochemical tests. After these evaluations the enzymes to be used for further study, should be characterized with regard to the kinetics and specificity. This can be achieved by stimulating extracellular enzyme production in liquid media by adding milk proteins or butter-fats as inducers (DeMartino, 1989). The presumptive enzyme solutions can

then be clarified by centrifugation and filtration and assayed for proteolytic or lipolytic activity in the solutions (Sutherland, 1993; Jaeger et al 1994). The enzyme solutions can then be purified according to Ray et al (1992), Dow et al (1990) or Debette (1991). After these evaluations, the enzymes should be evaluated in a small scale *in-situ* conditions to determine whether the enzymes are effective under the actual use conditions. A laboratory-scale milk transfer line and storage vessel made of stainless dairy steel with pump must be built to allow circulation of the milk through the system for a given period to allow for the deposit of milk constituents onto the stainless steel. Samples from the transfer line must be taken using Robin's devices for Scanning Electron Microscopy. The system must be flushed with water and then the enzyme solution. Samples must be taken from the line at different time intervals to determine the efficacy of the cleaning regime. The loss of activity of the enzymes over time must be assessed and the cleaning regime compared to the efficacy of current commercial chemical systems. Finally, if the use of microbial extracellular enzymes appears promising, the genes for the most active enzymes can be cloned into high expression systems for industrial applications.

Samples were taken from Bushy Park Farm Dairy on three different occasions and screened for organisms exhibiting lipolytic or proteolytic activity. Conventional media were modified to develop differential media that supported the growth of a wide range of micro-organisms and facilitated the identification of protease or lipase producing organisms (McComb and Graz, 1997).

The final proteolytic and lipolytic isolates satisfied the general criteria for industrial biocatalysts in that they: are the product of an intensive screening run; yield enzymes with high activity in a relatively short time; grow in media with minimal supplements; isolates produce extracellular enzymes which are readily isolated from the fermentation liquor; and are non-pathogenic and unrelated phylogenically to a pathogen.

The above criteria ensure the production of a safe active novel biocatalyst that can be used in dairy CIP for the cleaning of milk build-up. Enzymes have higher specificities than acids and bases when used in dairy CIP. Therefore, novel biocatalysts isolated from *Serratia liquefaciens* and *Acinetobacter lwoffi* could provide a far safer and cheaper alternative to the conventional chemical CIP systems.

Acinetobacter lwoffi, as isolated in our laboratory, failed to produce appreciable amounts of extracellular lipase under all nutritional conditions employed. A total number of 4.2 units per gram of cells was the highest extracellular lipase content obtained in a constant culture volume.

These results indicate that the culture of *A.lwoffi* and the isolation of its extracellular lipases would not be cost-effective to be applied in a cleaning system for South African dairies. A cheaper alternative is to purchase a mass produced but not as specific lipase from Novo-Nordisk™. The commercial lipase was shown to be most active at 50°C. At this temperature the activity of a 10mg/ml lipase solution was 70.3 U/g. The result was worked out in U/g because the lipase came in the form of a powder. This enabled convenient calculations of mass of lipase needed for further studies.

S.liquifaciens was cultured under varying conditions to determine those conditions under which the highest activity of free protease would be obtained. The highest protease activity was

obtained using 1l of starter culture cells incubated overnight at 37°C with a subsequent overnight incubation at 4°C. It was suggested that incubation at 4°C stimulated protease productions since the bacteria were under stress and were trying to survive.

Design and use of a Mini Dairy

The 'mini dairy' system used was constructed of 9.6m of dairy grade stainless steel pipe with a diameter of 5cm. The milk and cleaning solutions were pumped through the system at 1.5m/s using a centrifugal pump. There was no temperature control system in place. The solutions were drawn from the bottom of a holding tank (40 x 40 x 50cm) and returned through a pipe entering from the top of the vat. The vat was emptied from the bottom of the tank via a separate pipe. There are five dead-end sections with Pyrex ends that were designed to allow for access to the pipes for visual inspection of cleaning efficacy and for taking swabs. The length of the dead-ends is 4cm. A special insert was made to fit into the dead-ends to allow small disks of stainless steel to be inserted into the flow of liquid.

Raw milk was obtained from the Clover Rosedale Dairy in Grahamstown and run through the system for 1.75h before starting the cleaning procedure. The same cleaning regimes were used in the scale model for the chemical and commercial enzyme CIP systems. The lipase/protease system was used with 30U/l lipase activity being used as this was shown in preliminary studies to be most effective for cleaning and microbial control.

The cleanliness of the surfaces was determined by microbiological analyses and by visual and microscopical examination using scanning electron microscopy. Visually, all CIP procedures yielded effective cleaning. There was no visible protein or lipid residue on the stainless steel after cleaning and no milkstone residue was evident.

The composite samples of effluent taken during the cleaning experiments indicate that the enzyme-based CIPs exhibit a 'cleaner' more acceptable effluent than the chemical CIP systems. The results indicate that the new technology would ensure that dairy effluent would be within the legislative parameters and that dairies could save large amounts of money annually by reduced effluent costs.

Fortuitous Development of A Wastewater Management Programme

The investigation into effluent developed the following wastewater management ideas, which were later implemented at a major South African dairy. A product waste study should be conducted after completion of the water use survey as product waste has a direct influence on effluent quality. The water-waste supervisor and each production supervisor should observe operations closely and list all the waste that occurs. Photographs of leaks, spills, and other situations in which waste occurs should be taken to make slides for use in employee training. 500g of pollutants, in the form of Biological Oxygen Demand (BOD), is directly equivalent to 4.5ℓ of milk lost down the drain. If the BOD level in a plant's wastewater is known, an accurate idea of how much product is being lost into the drain can be obtained.

The waste load of a plant can be decreased substantially by controlling the volume of water used and reducing the amount of product lost into the sewerage/storm water system. Stopping pollution at its source is less expensive and more practical than end-of-pipe waste treatment. Wastewater from most dairy plants is discharged to publicly owned treatment works where the majority of the pollutants are removed before the water is discharged to the environment. Treating the water is costly, and most treatment works charge according to the volume of sewage treated. In addition, they commonly apply a surcharge if the waste load exceeds certain specified levels because it costs more to treat water that contains more pollutants.

A water-waste program can result in daily savings with a minimal amount of waste. The full co-operation of the company's employees in reducing waste and water must be procured in order to ensure an efficient operation - with money going into the bank rather than down the drain.

Total Cost-of-Clean

Further investigation into the Total Cost-of-Clean indicated that cost-effectiveness in cleaning requires CIP to be carried out with minimal waste of detergents and minimal output of effluent. Whilst CIP installations are a capital investment the chemical used is presently chosen specifically on product cost, product performance and supplier service levels. However, one cannot rely solely on the cost of the detergent to determine whether the CIP is cost-effective or not. The concept of “use-cost” or “cost-of-clean” must be applied when considering the cost implications of a CIP system. Any such calculation must take into account the cost per litre of product, the dilution rate, the performance, power and water consumption, cost of staff, down time of equipment for cleaning and effluent costs.

At present a major dairy will spend between R80 000 and R100 000 per month on cleaning chemicals alone. When one adds the costs of water, effluent and energy, the cost rises by over 25% to R100 000 to R125 000 per month.

However, penalties may reach levels of up to R100 000 per month with up to R160 000 having been reported. Using the data from the investigation into the use of an enzymatically-based CIP programme, the costs can be reduced quite dramatically. The chemical costs would decrease marginally by up to 7%, whilst the energy and effluent costs could be halved. Effluent penalties relating to CIP would be reduced by nearly 90%. Effluent costs due to product disposal would not be affected.

This cost does not include the penalties charged on effluent that does not conform to legislative requirements. If one includes the effluent penalties, the percentages change as indicated in Figure 1. It must be understood that for the protection of the plant the exact amounts are not mentioned.

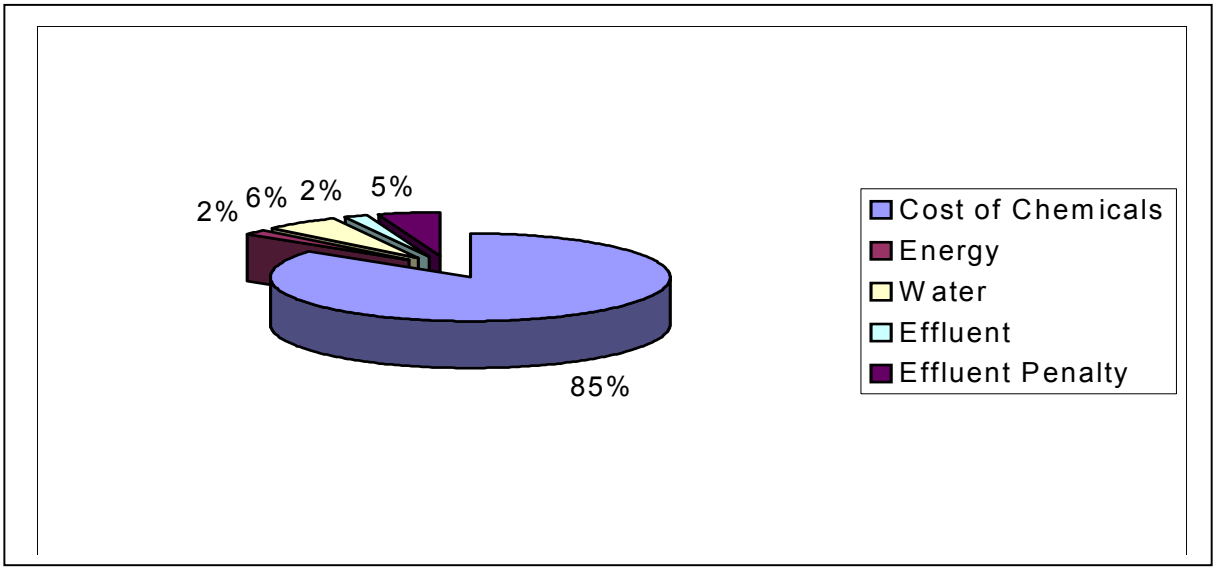

Figure 1 Distribution of the current CIP costs including the effluent penalties

Conclusions and Further Actions

The aim of this study was to develop a CIP system based on lipases and proteases, which would result in a more acceptable effluent being discharged while still providing effective cleaning for all surfaces in the dairy industry in South Africa. The twin enzyme lipase/protease CIP system proposed and investigated in this study produced an equivalent clean whilst producing a more acceptable effluent when compared to the current chemical CIP practices.

- The combination of enzymes and the application thereof needs to be patented as the concept has future viability when one considers the movement towards environmental management.

- Areas that still need to be addressed are 1) the purification of the protease to further improve the effluent obtained and 2) to increase the activity of protease used in the CIP procedure. By increasing the protease activity present, the system may become even more comparable to the chemical CIP in cleaning efficacy. Further research into these areas would benefit industry because if this system is shown to be effective it would save the companies money in both cleaning and effluent costs. This would be due to the decrease in or elimination of fines paid on effluent discharged into the sewer and a decrease in energy expenditure due to the lower cleaning temperatures used.

- The primary user group for the products envisaged from this project are the small and medium sized dairy processors in South Africa. The large multi-national dairy companies should rather be viewed as potential innovators of this process and potential financial supporters of the work. Whilst academic institutions may be interested in the project from a enzymological perspective, the research was meant to be application-based. Complete characterization of the proteases used in the study could, however, lead to further capacity building of the scientist base required in South Africa.

- Considering the efficacy of the two enzyme CIP in cleaning stainless steel in dairies, this research could now be expanded to be further developed for the **fruit juice**, **brewing** and **soft drinks** markets. The technology could potentially also be used in the **bottled water** industry, which is rapidly growing in South Africa. Finally, the technology should not be localized to cleaning-in-place applications. Large **open-area-cleaning** could also be attempted with multi-enzyme cleaners. In all cases the primary soils would have to be identified and a screening programme for biocatalysts, capable of degrading the soils, would have to be run. Enzymes capable of degrading these soils may be available commercially, thereby decreasing the time required to investigate the efficacy of the systems *in-situ.*

    - The technology developed and the information collected throughout the duration of this project should be made available to dairy processors in South Africa as soon as possible.

    - A suggestion would be to set up a series of technology transfer seminars with the aid of Ecolab South Africa, who have been involved in this project from its inception. The researchers involved in the project should then describe the benefits of water management and CIP management to all dairy producers. The potential of this work should also be highlighted, but should really be brought to the attention of the major role players in the industry.

    - By stimulating the interest of Clover-Danone and Parmalat, this work could be brought to completion and the technology implemented in South Africa in the foreseeable future. The seminars could be linked to one-day workshops in which dairy processors are taught about the different chemical types currently available to them, basic chemistry of their application, their use and abuse, development of water management programmes and the need for these programmes in light of the current South African legislation.

    - The seminars should be held in the major dairy centres in South Africa, namely Cape Town-Stellenbosch, Johannesburg, Bloemfontein, Durban and Port-Elizabeth/Tsitsikama.

Capacity Building

The research project, was run in part by the Department of Biochemistry and Microbiology at the University of Port Elizabeth. The project allowed the department to train postgraduate students in the newly established discipline of Microbiology beyond the level of Honours, which will contribute to building capacity of the student body which has, to date, been unable to study the discipline of Microbiology at the University of Port Elizabeth. The project involved the development of four post-graduate students, two of whom were from designated groups. Furthermore, the project involved unskilled and semi-skilled labour at three dairies in the Eastern Cape Province, allowing for skills development in CIP, water management and effluent management.

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The Steering Committee responsible for this project, consisted of the following persons:

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Dr J Theron	University of Pretoria
Mr R Jansen van Vuuren	Clover SA
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1.1 MOTIVATION

Processing of dairy products is very complex with respect to production requirements. Unlike some other industries, a dairy plant is concerned with the processing of a highly perishable product for supply to the consumer who is becoming increasingly aware of nutritional requirements and has an increasing desire for high quality, value-for-money products. In order to produce such high quality, value-for-money products, the dairy industry has to concern itself with issues such as labour and its increasing costs, the cost of operation and maintenance of equipment, the cost of cleaning and maintaining hygienic conditions on the plant, pressures from regulatory agencies with regard to product specifications and the discharge of effluent and the rising cost of fuels. The hypothesis for this study is that if it is possible to harness the enzymes which cause the currently unwanted degradation of milk constituents, these can be modified to be used as possible cleaning agents in the dairy industry. This approach would address the environmental concerns which have arisen through the use of chemicals for cleaning and the use of a single stage biological cleaning regime may decrease the total cost-of-clean in dairies. Both bacteria and fungi are capable of producing extracellular enzymes that are capable of breaking down milk constituents at low (Law, 1979; Muir et al, 1979; Cousin, 1982; Rowe et al, 1990) and high temperatures (Richardson et al, 1978; McKellar, 1981). This would be achieved by digesting the milk constituents on product surfaces by enzymatic action, rather than remove them with high pressure, high temperature chemical solutions.

There is a dire need in South Africa to improve the quality of industrial effluents (Reid, 1997). A possible method to improve effluent by reducing its organic loading, which is not currently in use in South Africa, is to rinse all equipment prior to washing to collect the product residues from which dairy solids can be extracted for further processing or for swine fodder. Furthermore, effluent treatment plants are being suggested as an attempt to improve the quality of effluent dumped. Effluent treatment plants are going to be expensive with respect to capital outlay and more costs will be incurred in the running of these plants (Van Vuuren, Personal Communication, 1997). Therefore, a paradigm shift away from caustic and acid in dairy cleaning and sanitation is needed to avoid excessive costs which will inevitably rise as an environmentally conscious society continues to demand stricter legal limitations on effluent discharge. This can, however, only occur if the new technologies are equal to or greater in efficiency than the currently applied techniques.

1.2 OBJECTIVES

Cleaning and sanitizing in the dairy industry is currently a three-step process based on the an initial rinse with water, cleaning using sodium hydroxide and nitric acid and sanitizing using peroxide-based sanitizer. The reason for this is that the cleaning step may involve the chemical and/or physical removal of soils. If this is not achieved before the application of the sanitizing agent, the sanitizer could be consumed in the sanitizing process before being able to act on the residual microbial population. Cleaning and sanitation techniques are

energy consuming, hazardous to operators, and to compound the problem, increasingly restricted by legislation. Dairies face a serious dilemma to improve their CIP systems cost-efficiency whilst maintaining high sanitation standards and reducing effluent outputs. One of the possible solutions for this is to use burst rinses, which use low volumes of water to flush product or chemicals out of the lines rather than extended rinses using large volumes of water. The effective use of chemicals and sanitizers requires the use of the correct materials, at the correct concentration and temperature applied in the correct sequence at the required time. Cost-effectiveness requires this to be carried out with minimal waste.

Partial reclaim CIP systems minimize waste by recovering up to 80% of sodium hydroxide and nitric acid after the cleaning process. This is achieved by the use of conductivity probes which measure chemical concentration and control valves which either allow the reclaim of the chemical or, once the solution drops below a set concentration, discharge the cleaning solution into the plant's effluent stream. Sanitizer is always fluxed to the drain. During the reclaim process the cleaning chemicals are diluted by the rinse water. The concentration of chemicals in the bulk balance tanks for CIP must, therefore, be maintained by conductivity probes mounted in the bulk tanks which control dosing equipment which maintain the bulk tank concentration at the supplier recommended concentration.

The process of enzymatic CIP harbours challenges which have to be overcome before true biological cleaning can be achieved. For dairy CIP two types of enzymes could be used. These enzymes exhibit proteolytic or lipolytic characteristics and would be able to remove the bulk of milk soils at biological temperatures (25°-42°C). Another method could be a combination of a proteolytic enzyme and a non-ionic surfactant, combining biocatalysis and chemical cleaning technologies.

Proteases are enzymes that catalyse the cleavage of peptide bonds resulting in the release of individual amino acids or peptide sub-units from peptide chains (Barrett, 1994). The largest current application for microbial enzymes is the use of proteases in detergents (Cheetham, 1987).

Enzymes exhibiting lipolytic activity or lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are produced by various micro-organisms (Eddy, 1965); either alone or together with esterases (carboxylic-ester hydrolases, EC 3.1.1.1). Lipases act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. A characteristic property of lipases is called interfacial activation, which is distinguished by a sharp increase in lipase activity when the substrate forms an emulsion. This results in the kinetics of the lipase reaction not conforming to the classical Michaelis-Menton model. Esterase activity is a function of substrate concentration as described by Michaelis-Menton kinetics with the maximal reaction rate being reached long before the solution becomes substrate saturated.

A major challenge that needs to be overcome with the use of enzymes in CIP is that the products used to clean the system could lead to the degradation of the product. Heat resistant proteases ideally should be avoided for chance of possible product spoilage (degradation) but this enzyme could be used effectively in a cleaning regime including high temperature enzyme mixtures which could be far more effective and specific than the chemicals in present use. Less than 30% of psychrotrophic bacteria (bacteria which grow at

low temperatures) in refrigerated raw milk produced lipolytic taints. However, the possible use of enzymes from these bacteria (in cleaning) and danger (in contaminating), lie in their heat-resistance. Certain extra-cellular lipases from *Pseudomonas*, *Achromobacter* and *Serratia* species are heat-resistant, while those of *Alcaligenes* and *Flavobacterium* species have been shown to be heat sensitive. Kishonti (1992) found that 40% of the psychrotrophic bacteria produced enzymes that retained 75% of their activity after heating for 2 minutes at 90°C. Considering that commercially sold milk is high-temperature-short-term (HTST) pasteurised at 72°C for 15 seconds, there is a danger of the enzyme solution used for cleaning, being a source of milk degradation during normal processing if the milk were contaminated. The problem of cleaning enzyme carryover must be considered as a minor problem in comparison to the presence of often heat-stable enzymes produced by spoilage organisms, which will reduce milk quality.

A further challenge, which must be addressed is the fact that not all enzyme-based cleaners are effective under use conditions and are unable to cleave proteins into smaller fragments which can be rinsed away from soiled surfaces. Hydrolysis of milk proteins by other enzyme-based cleaners is incomplete in the time currently considered adequate for cleaning with enzyme-based products. Thus there is still a need to develop a better understanding of the activity of enzymes destined for use as biologically-based CIP products.

Furthermore, if a two enzyme system is used rather than a product combining biocatalysis and chemical cleaning technologies, the order of enzyme application would have to be considered carefully. As enzymes are protein in nature, the lipases would always have to be applied first so that they can act on the lipid soils before they are degraded by the action of the proteases.

Enzyme-based cleaning has been shown to be ineffective in reducing microbial numbers from surfaces and thus sanitizing of surfaces after enzyme-based cleaning is essential.

The need for effective biological CIP cleaners can be met by the tremendous increase in screening for novel microbial enzymes for industrial use. The main advantages of the enzyme detergents would be energy and cost efficiency, safety and greater efficacy due to specificity. With current recombinant techniques available, the manufacture and isolation of crude, durable (heat and pH stable) enzyme solutions is not only feasible but relatively inexpensive considering the costs involved in current chemical CIP process.

Dairies are being pressured into stepping up effluent considerations with heavy penalties an unavoidable reality. Installing treatment plants is only a temporary alternative as inevitably the restrictions on acids, bases and heavy metal composition in water effluent will rise as the total potable water supply decreases in quality. Biological soil removal appears to be the best alternative to meet the cleaning needs of the dairy industry in the new Millennium.

1.3 AIMS

- The overall aim of this study is to produce an economically viable biological cleaning system for dairy plants. The system has to be as, if not more, effective than

the current chemical systems used, both with respect to cleaning action and anti-microbial efficacy, while significantly improving the effluent released form the plant and reducing water consumption and energy expenditure. This would effectively reduce the total cost-of-clean in the dairy resulting in a decrease energy, water, chemical and effluent discharge costs.

- To screen microbes isolated on site in a dairy for their extracellular enzymes which can degrade milk constituents, and to characterize the enzymes and optimize the conditions for their activity. Furthermore, the enzyme activity is to be compared to that of commercial preparations of the enzymes as well as to the enzymes isolated from known secretors such as *Bacillus* (proteases) and *Pseudomonas* (lipases).
- To build a laboratory-scale plant to test the efficacy of the screened enzymes on milk deposits and to compare these results to commercial preparations of the enzymes as well as to the currently used cleaning regimes.
- To improve enzyme production and export from the selected microbes. This will be done by placing the micro-organisms under selective pressures upon which their efficacy to express the enzymes in question will be assessed on solid media as well as by biochemical means.

1.4 LITERATURE SURVEY

1.4.1 GENERAL ASPECTS OF SOILING AND CHEMICAL CLEANING

A chemical cleaning reaction is a heterogenous reaction between a chemical solution and the fouling layer, soil or biofilm (Tragadh, 1989). According to Tragadh (1989), a cleaning reaction can be divided into six stages: 1) Bulk reaction of the chemical solution. 2) Transport of the chemical solution to the soiled surface. 3) Transport of the chemical into the soil. Penetration of the chemical into the soil allows chemical activity to occur at the soil-surface interface for optimum soil removal. 4) The phyico-chemical transformations and chemical reaction occurring due to the interaction between the chemical and the soil. 5) Transport of the reaction products to the surface of the soil. 6) Transport of the soils away from the product contact surface.

The most common dairy soil component, the carbohydrates, are the easiest to remove. These carbohydrates include polysaccharides, sugars, starches, and cellulose, all of which are at least partially water soluble as well as pectate, xylanate and some celluloses which are water insoluble (Lehninger *et al.*, 1993). Complex carbohydrates which are currently being used as “fat replacers” in fat free products also fall into this class. Heating will alter the structure of each, making them more difficult to remove, but still relatively easy compared to other soils. All of these soil types can easily be removed using a alkaline cleaner at temperatures between 40 and 80°C at a pH of 7-12.
Proteins are probably the most difficult dairy soils to remove from surfaces. Proteins consist of linear polypeptides, which may be folded into specific secondary and tertiary structures which make them functional (Ferscht, 1977). Changes in temperature and pH cause a change in the ionic characteristics of the amino acids making up the protein thereby changing the bonding which maintain the secondary and tertiary protein structures. This leads to the unfolding or denaturation of the molecule. Having lost their original structure

the proteins are linear, and this change in conformation together with the binding of the proteins to other molecules leads to irreversible coagulation of the proteins (Lehninger *et al.*, 1993).

Strongly alkaline or chlorinated alkaline chemicals (at pH 10-13) are usually used to clean areas soiled by proteinaceous materials. The drastic change in pH leads to a loss of protein structure and cleavage of peptide bonds resulting in smaller peptides with increased water solubility. Acidic chemicals denature proteins due to a decrease in pH but are not able to cleave peptide bonds as is evident with alkaline chemicals (Bodnar and Bradley, 1992).

Lipid soils are not water soluble, and are thus more difficult to clean than carbohydrates or water-soluble proteins (Lehninger *et al.*, 1993). Lipids are able to increase the adherance of other soils onto surfaces making extremely important to remove these soils (Jeurnink and Brinkman, 1994). Elevated temperatures are generally used for efficient cleaning of lipid containing soils as lipids are known to melt at higher temperatures. However, excessive temperatures may result in the polymerization of lipids increasing the size of globules making them more difficult to remove. Alkaline chemicals are able to saponify lipids creating soluble soaps which are easily washed away. Surfactants are added to help wet the surfaces and soils. In contradiction to the above statement, however, elevated temperatures are generally used for efficient cleaning .

Mineral salts account for the inorganic component of dairy soils. Hard water salts and inorganic components in the product being processed may precipitate onto equipment, especially heated surfaces. Scale formation is common in areas with hard water supplies. Thus the cleaning agent used must be able to chelate the hard water salts to facilitate effective cleaning. Minerals such as calcium or magnesium can come from the product or the processing water. Scale and other mineral deposits are removed with acid cleaners.

1.4.2 CHEMISTRY OF MILK

Milk is a complex food substance with over 100 000 different molecular species and can be described as an oil-in-water emulsion with the fat globules dispersed in the continuous serum phase. Serum (Whey) is milk plasma less casein micelles, while plasma is described as the raw milk with the fat removed and is also referred to as the skim milk. Other milk fractions include SNF (solids-non-fat) and TMS (total milk solids). The SNF fractions include proteins, lactose, minerals, acids, enzymes and vitamins. The SNF fraction together with the milk fat makes up the TMS (Goff, 1995). Table 1 shows the approximate composition of milk. The precise compositions of milk varies according to breed variations, cow to cow variations, herd to herd variations and geographic variations (Jacobs, 1958).

Milkfat is predominantly made up of triglycerides (98-99%), with a small component of phospholipids (0.2-1.0%) and cholesterol (0.25-0.40%). Phospholipids are mainly associated with the fat globule membrane, while cholesterol is mostly located in the fat globule core (Jennes and Patton, 1959). Triglycerides consist of a glycerol backbone binding up to three different fatty acids. Fatty acids are composed of hydrocarbon chains and a carboxyl group (Lehninger *et al.*, 1994).

Table 1: The Composition of Milk

Percentage	Component	Examples
85.5 to 88.7	Water	
2.4 to 5.5	Milkfat	
7.9 to 10.0	SNF	
3.25	Protein	3/4 Casein
4.6	Lactose	
0.65	Minerals	Calcium, phosphorous, citrate, magnesium, potassium, sodium, zinc, chlorine, iron, copper, sulfate, bicarbonate
0.18	Acids	Citrate, formate, acetate, lactate oxalate
	Enzymes	Peroxidase, catalase, phosphatase, lipase
	Gases	Oxygen, nitrogen
	Vitamins	A, C, D, thiamine, riboflavin, others

Table 2 shows common fatty acids that occur in milk triglycerides and their relative percentage presence, with long even carbon chain saturated fatty acids predominate. The one unsaturated fatty acid that is present in relatively large quantities is oleic acid with one double bond.

Table 2: Fatty acids commonly found in milk triglycerides.

Fatty Acids	Carbons	% in Milk
Long chain : myristic	C14	11%
Palmitic	C16	26%
Stearic	C18	10%
Oleic	C18:1	20%
Short chain : butyric	C4	11%
Caproic	C6	
Caprylic	C8	
Capric	C10	

The final melting point of milkfat is 33°C, just below the body temperature of the cow (Ling *et al.,* 1961). This is to be expected because the milk needs to be liquid at biological temperature. The actual melting points of the various triglycerides range from -75°C (tributyric glycerol) to 72°C (tristearin). The lipids with higher melting points dissolve in the liquid fats (Goff, 1995). The other physical factors of milkfat are summarized in Table 3 below.

95% of the total milk lipid is present in the form of globules that range in size from 0.1 to 10 μm in diameter, with a mean of 3μm (Ling *et al.*, 1961). These lipid globules are surrounded by a thin membrane that are 8 to 10 nm thick and are derived from the apical plasma membrane if the secretary cells (Goff, 1995). This membrane is called the native fat

globule membrane (FGM) in raw milk (Ling *et al.,* 1961). The FGM decreases the lipid-serum interface to very low values (1 to 2.5 $mN.m^{-1}$).

Table 3: Physical characteristics of milkfat.

Density (at 20°C)	$915kg.m^{-3}$
Refractive index (589nm)	1.458 to 1.464 at 15°C (decrease with increasing temperature)
Solubility of water in fat at 20°C	0.14% (w/w) (increases with an increasing temperature)
Thermal conductivity	$\pm 0.17J.m^{-1}.s^{-1}.K^{-1}$ at 20°C
Specific heat at 40°C	$\pm 2.1kJ\ kg^{-1}K^{-1}$
Electrical conductivity / Dielectric constant	± 3.1

The lowered interface prevents immediate flocculation and coalescence of globules as well as protecting them from enzymatic action (Ling *et al.,* 1961; Goff, 1995). The FGM changes during homogenization - the membrane is then called a "recombined" membrane. This is mainly because homogenization decreases the average globule size, thereby increasing total globular surface area. There is not enough original FGM to cover all the globular surfaces (Ling *et al.,* 1961). After homogenization the surface tension climbs to 15 $mN.m^{-1}$; this causes amphiphilic molecules in the plasma to absorb the lipid globule, and this effectively lowers the surface tension value. Therefore, recombined membranes consist mainly of serum proteins and casein micelles as well as FGM (Goff, 1995).

Caseins (78.5%), whey proteins (16.5%) and non-protein nitrogen (NPN) (5%) all contribute to the nitrogen content of milk (Ling *et al.,* 1961). Proteins carry most of the nitrogen conten
t of milk, casein and whey proteins together constituting 3.28% of raw milk (Fox *et al.*, 1961). Table 4 shows the distribution of protein as a fraction of total protein in the system.

Table 4: The concentration of proteins in milk

	Grams/litre	**% Total protein**
Total Protein	33	100
Total Casein	26	79.5
α S1	10	30.6
α S2	2.6	8.0
β	9.3	28.4
κ	3.3	10.1
Total Whey Protein	6.3	19.3
α - lactalbumin	1.2	3.7
β - lactoglobulin	3.2	9.8
BSA	0.4	1.2
Immunoglobins	0.7	2.1
Proteose peplane	0.8	2.4

Casein as mentioned before represents about 80% of milk proteins. Casein is a conjugate protein, present in raw milk as calcium-caseinate, with low solubility at pH 4.6 (Ling *et al.,* 1961). Most phosphate groups in casein molecules are esterified to serine residues and are important to the structure of the casein in proportion to the phosphate content.

Casein has a conformation similar to that of a denatured globular protein. The conformation is due to the high number of proline residues that cause particular bending and inhibits the formation of a closely packed, ordered secondary structure. This together with the lack of tertiary structure accounts for the heat stability of casein because there is very little structure to unfold. Casein is insoluble in water because of its lack of tertiary structure, leading to a considerable number of hydrophobic residues being exposed. This leads to string association reactions between the casein molecules, rendering them insoluble in water (Goff, 1995). This characteristics leads to the formation of casein micelles which are colloidal particles that function to carry insoluble calcium phosphate to mammalian young and to form a clot in the stomach for more efficient digestion (Mepham, 1976). Casein micelles also contain citrate, minor ions, lipase, plasmin enzymes and entrapped milk serum (Goff, 1995).

Whey proteins are globular in nature, and are more water soluble than casein and more heat sensitive. Upon denaturation, whey proteins increase their water holding capacity (Goff, 1995). The principal fractions of whey proteins are:

- β lactoglobulins
- α lactalbumins
- Bovine serum albumin (BSA)
- Immunoglobulins (Ling *et al.,* 1961; Mepham, 1976)

1.4.3 DAIRY SYSTEMS

Milk arrives at a Dairy processing plant in insulated tankers designed to keep the milk at 4°C when being transported from the dairy farms. The raw milk is pumped into insulated raw receiving tanks. From these raw receiving tanks the milk is then fed into the processing system that contains a number of steps: clarification, separation, standardization, homogenization, pasteurization, filling and packaging.
Clarification of milk is the removal of solid impurities from raw milk before it is separated (Jenness and Patton, 1959; Goff, 1995). Clarification is not, however, practiced by South African Dairies, instead the raw milk is separated directly (as per inspection). Separation entails the separating of raw milk into cream and skim milk components. The separated milk is then standardized by recombining the cream and skim milk to a specific fat content and is controlled by the Foodstuffs, Cosmetics and Disinfectants Act No 54 of 1972. The addition of the cream is controlled by adjusting the throttling valve of the cream outlet; with direct standardization the mixing occurs automatically at the separator to provide the desired fat content (Jennes and Patton, 1959; Goff, 1995).

Standardized milk is then homogenized (as per inspection). Homogenization of the standardized milk entails mechanical treatment of the fat globules by passing the milk under high pressure through a tiny orifice, resulting in a decrease in the average diameter of the fat globules. If the raw standardized milk is not homogenized fat would rise to the surface

resulting in a cream layer forming. This is because the fat globules are large and tend to aggregate; homogenization decreases the fat globule size and greatly reduces the tendency of creaming of fat globules (Corbin and Whittier, 1965; Goff, 1995).

Homogenization is followed by pasteurization using HTST (High Temperature Short Time) technique (as per inspection). HTST pasteurization is designed to kill the vegetative cells of the most heat resistant pathogen known, *Coxiella burnetti,* the rickettsial agent of Q-fever. The HTST technique consists of holding milk at a temperature of 71.7°C for 15 seconds and then rapidly cooling it again to 4°C. Vegetative cells of non-pathogenic organisms do survive pasteurization. These include *Streptococci, Lactobacillus* and some species of *Micrococcus*. These organisms will grow after pasteurization and eventually lead to milk spoilage if it is held beyond its shelf life (Boyd, 1988). Pasteurization can be done before homogenization, but this requires that standardization also takes place after pasteurization. This means that both the skim milk and the cream of the separated milk must be pasteurized separately. Standardizing and homogenizing the milk before pasteurization means that only one stream of milk needs to be pasteurized. After the milk is pasteurized it is then sent to pasteurized storage tanks, from where it is either further processed or is packaged into cartons, bottles or sachets. The packaged product is stored at 4°C until it is sold.

1.4.4 DAIRY SOILS AND THEIR REMOVAL

Dairy soils can vary greatly in composition depending on the product, processing conditions, and the geographical area from which the milk came. The major components in milk are water, sugar, lipids, protein and minerals.

Two different types (A and B) of deposits are found in a dairy environment (Burton, 1966). These depend largely on the temperature to which the milk in the heat exchanger is exposed. Type A consists mainly of whey proteins. The deposit is characterised by a protein-rich outer layer and a sub-layer, rich in calcium and phosphorous, directly attached to the heating surface. This type of soiling is commonly formed at temperatures between 70 and 90°C (Tissier and Lalande, 1997). Type B is composed mostly of casein entrapped in a calcium phosphate matrix. This type of soiling is found at temperatures ranging between 110 - 140°C. Milk soils can further be classified according to the part of the process in which they are found.

"Cold milk" soils contain a large amount of lactose and lipid, while hot soils contain only traces of these compounds. Since both sugars and lipids become more soluble with increasing temperature, they are found in higher proportions in cold milk soils as compared to pasteurized milk soils. Thus the cleaning regime used for cold milk soils must be able to cope with increased levels of carbohydrates and fats.

Since lipids melt at higher temperatures, they are less likely to de-emulsify onto surfaces, in hot milk soils. Proteins are a major component of both hot and cold milk soils because of their large size and ability to combine with minerals, sugars and lipids. There are two major proteins in milk. Whey proteins are water soluble, but begin to denature when heated above 65^{o}C. Casein is not water-soluble and is easily precipitated by minerals, enzymes or heat. Minerals are an important component of both hot and cold soils because of their relative

insolubility. Calcium is less soluble at elevated temperatures whilst magnesium is more soluble at elevated temperatures. It has been proposed that they are the first component to deposit onto surfaces, forming the foundation for other deposits (Stadhouders and Mulder, 1960).

"Hot milk" soils exhibit different characteristics depending on processing temperature and holding time at elevated temperatures. Soiling is generally greater in the hotter parts of processing equipment than in non-heated parts. As processing temperature increases, the concentration of minerals in the soil increases while the lipid content is reduced. Furthermore, precipitation of calcium occurs more readily at higher temperatures. The minerals can then combine with proteins (as in Type B soils), making them extremely difficult to remove. As previously indicated, proteins denature when the temperature is above 65°C, increasing the potential for deposit on product contact surfaces. This results in protein-based soils which are difficult to remove. Soils found in the pasteurizer are mainly mineral, with some protein, and are more difficult to remove than soils in other areas of the equipment.

A newer technology currently available includes the use of a phosphoric acid-based chemical-sanitizer, containing a blend of EPA-approved anionic surface active agents and a sequestrant. While this technology is allowed in South Africa, it may not be used in this way in the United States on food contact surfaces. This new technology in dairy CIP is being applied to minimise the cleaning chemical loading (especially sodium) in the plant effluent and to reduce cleaning time. This technology is only effective against "cold milk" soils as it contains minimal chemical activity and thus is able to solublilize soils only slightly. The product is effective in reducing both energy input, since it is used at 40°C, and washing time as it is used in a single pass situation based on a single rinse and subsequent wash to drain. The effect of the treatment on the coagulation of casein, which coagulates at pH 4.6 is, as yet, unknown. A major role player in the South African dairy market has indicated that use of this technology for raw milk silo and road tanker washing has reduced the total cost of clean by up to 40% for those sections of the plants where it is being applied (Van Vuuren, personal communication, 1997).

Whatever the soil composition, it appears that the mineral components and proteins are responsible for the degree of difficulty of removal of the soil. The change in the structure of proteins upon heating, and their increased ability to interact with the surface and with minerals are responsible for this. A major problem in dairy production systems is the deposit of milkstone (a precipitate of calcium and magnesium salts). This deposit imparts a roughness to the surface of the stainless steel vessels and tubing used in dairies. This roughness promotes adhesion/adsorption of microbes to the soil depending on the environmental factors associated with the solution flowing through the vessel (McCoy *et al*., 1981).

Milkstone can be found on all surfaces of milk processing equipment and is the result of inadequate removal of minerals during previous cleaning procedures. Acid chemicals must be used to remove milkstone, as the decrease in pH changes the ionic interaction between minerals, proteins and the processing surfaces resulting in an increase in solubility and a

release of the mineral into the washing solution. A chelating agent could also be used, not only to prevent milkstone formation but also to remove milkstone deposits.

The soils resulting from other dairy products are different from raw milk soils due to the additional processing procedures involved. Butter will require increased temperatures and higher concentrations of wetting agents in the chemical cleaners. In the production of cheese, as the protein structure is changed, these soils become more difficult to remove. Cultured dairy products like yoghurt contain micro-organisms and consequently microbial by-products which alter the composition of the soil. Additives such as fruits and nuts in frozen desserts and yoghurts may increase the particulate debris and may increase the complex sugar content.

Bacterial biofilms can develop on almost any surface in any environment in which viable micro-organisms are present (Allison and Gilbert, 1992; Costerton *et al*., 1973; 1987). The tendency of bacteria to adhere to, and colonise on inert dairy contact surfaces is a concern in the dairy processing industry. This is because of the significant health consequences that arise if even low numbers of potentially pathogenic micro-organisms remain on these surfaces after cleaning (Flint *et al*., 1997).

Biofilms play a more direct influence in the South African milk industry where equipment is often older and often not adequately maintained: a) Corrosion, particularly amongst the sulphate-reducing bacteria found in the anaerobic zone at the biofilm/metal interface. This results in irregularities such as roughness, crevices and pits which increase bacterial adherence by bacterial cell attachment and decreasing removal of attached cells by cleaning; and b) Post-pasteurization contamination. Costerton *et al.* (1973; 1987; 1995) suggests that the planktonic mode of growth is favoured for dissemination and persistence of the dormant form, while the biofilm mode is favoured for growth. Attached spoilage and pathogenic bacteria may increase in number, and detach on their own or by physical movement of product through a pipeline (Mostellor and Bishop, 1993). This process is called “sloughing” and is a major concern to product quality as well as a potential health hazard.

The microbial exopolysaccharide layer provides increased protection from phagocytosis and prophylactic agents such as sanitizers, antibiotics, preservatives and antibodies. Sanitizers are unable to penetrate the glycocalyx matrix and contact the bacterial cells in order to destroy them. Austin and Bergeron (1995) found that extensive bacterial biofilms may develop on gaskets and tubing in various areas of dairies even after regular CIP procedures. Contact surfaces of milk pipelines and processing equipment have been suggested to be a direct source of post-pasteurisation contamination. In severe cases of soiling where the soil is not removed completely by the CIP process, a dramatic decrease in sanitizer efficacy is observed. Classical methods to test the anti-microbial efficacy of commercial sanitizers rely mainly on kill tests using planktonic cultures. Subsequent evaluations have shown that sanitizers currently employed reduced planktonic cultures by the accepted South African standard (99.999% reduction/minute which is less stringent than the EPA requirement of 99.999% per half-minute), but did not affect the sessile organisms (Lindsay, 1997).

The daily cleaning of dairy production equipment by chemical CIP cannot be avoided as there will always be milk solids deposited on processing equipment during processing.

Processing costs as described by Sandu and Singh (1991) which may be influenced by cleaning and sanitation include: a) increased capital costs as a result of over-sizing of the heat transfer equipment; b) installation of parallel units as an alternative processing route during cleaning and early replacement of equipment because of the shortening of technical life-time; c) increased energy costs as a result of a decreasing heat transfer coefficient during operation; d) an increasing pressure drop during operation and cleaning at elevated temperatures to remove deposit; e) high maintenance costs due to the use of cleaning chemicals and water and treatment of cleaning effluent before disposal; f) costs for production losses as a result of product which remains on the wall of the heat exchanger; g) product losses during plant shut-down for cleaning and start-up procedures, and downtime for cleaning, during which no production can take place; h) costs for the recycling of product (not applicable to South Africa to date).

1.4.5 BIOCATALYSIS AS AN ALTERNATIVE FOR DAIRY CIP

The synthetic industrial chemicals available today have good cleaning power as a result of the numerous technical improvements since the advent of extensive use of cleaning chemicals in the dairy industry 50 years ago (Eveleigh, 1987; Hoshino and Ito, 1997). The cleaning power of chemicals seems to have peaked; most chemicals contain the same ingredients (at more or less similar concentrations) and are based on the same detergency mechanisms. In each case the soil that has adsorbed onto a surface is removed by surfactants and other builders, which lower the surface tension at the interface and enhance the repulsive forces between the surface and the soil which results in the removal of the soil. New detergents include the incorporation of enzyme mixtures into products for household and industrial use (May, 1992). The use of enzymes for industrial or domestic applications is part of the discipline called biocatalysis (Fogarty and Kelly, 1980).

Although enzymes are relatively expensive components of cleaning mixtures, in comparison to a chemically-based CIP product, an enzyme-based CIP product: a) would be more energy efficient as reactions could take place at biological temperatures (25°-42°C rather than 65°-80°C which is used with chemically based CIP); b) would be more cost-effective as there is currently no legislation on the discharge of biological effluent in South Africa; c) would be safer to work with; d) could be more effective in dealing with soiling as enzymes are the most efficient catalysts known, increasing rates of chemical reactions by factors of 10^9 to 10^{12}. as the majority of enzymes are distinguished by the specificity of their action. Enzyme function can be regulated which means that simple changes in the environment can alter enzyme function from their most active to their inactive forms; e) can continually be improved as an increased use of genetic and chemical engineering has proved to be a powerful influence in the production of industrially stable and novel enzymes (Nedleman, 1994).

2. ENZYMES USED IN STUDY

2.1. INTRODUCTION

A previous Honours study by Mr Darrin McComb of the Department of Biochemistry and Microbiology at the University of Port Elizabeth provided the enzymes utilized in the development of the two-enzyme component CIP programme investigated in this project. The following extract from the work illustrates the source of the enzymes used.

Samples were taken from Bushy Park Farm Dairy on three different occasions and screened for organisms exhibiting lipolytic or proteolytic activity. Conventional media were modified to develop differential media that supported the growth of a wide range of micro-organisms and facilitated the identification of protease or lipase producing organisms (McComb and Graz, 1997).

The final proteolytic and lipolytic have satisfied the general criteria specified by Fogarty and Kelley (1980) in that they:

a. are the product of an intensive screening run;
b. yield enzymes with high activity in a relatively short time;
c. grow in media with minimal supplements;
d. isolates produce extracellular enzymes which are readily isolated from the fermentation liquor; and
e. are non-pathogenic and unrelated phylogenically to a pathogen.

The above criteria ensure the production of a safe active novel biocatalyst that can be used in dairy CIP for the cleaning of milk build-up. Enzymes have higher specificities than acids and bases when used in dairy CIP. Therefore, novel biocatalysts isolated from the organisms listed in Tables 1 and 2 could provide a far safer and cheaper alternative to the conventional chemical CIP systems.

Table 1 A summary of the proteolytic organisms chosen to be used in further trials. The relative enzyme activities of the extracellular enzymes of the isolates were determined.

Isolate	Classification	Activity (units/ml)
C2	*Staphylococcus*	0.274
C15	*Aeromonas*	0.289
C18	*Serratia*	0.484
C19	*Serratia*	2.095
C21	*Aeromonas*	0.394

Table 2 A summary of the lipolytic organisms chosen to be used in further trials. The relative enzyme activities of the extracellular enzymes of the isolates were determined.

Isolate	Classification	Activity (units/ml)
MB24	*Acinetobacter*	68.34
MB25	*Dienobacter*	58.75
MB27	*Flavobacterium*	42.17

Further research must go into determining the rate of milk-soil degradation by the enzymes and bulk enzyme production, to allow the enzyme system to time economical and cheaper to purchase. A restrictive factor is that the enzyme system would have to use the conventional CIP systems to provide a feasible alternative for industry to convert to biological cleaning approaches.

2.2. LITERATURE REVIEW

2.2.1. LIPASES FROM *ACINETOBACTER LWOFFI*

The investigation of the secretion of an extracellular lipase of *Acinetobacter lwoffi* previously isolated in the Department of Biochemistry and Microbiology at the University of Port Elizabeth, was to determine whether the enzyme could be isolated cost-effectively in large concentrations.

The lipolytic activity of genus *Acinetobacter* has been studied since the late 1970's. This was due to the realization that one-eighth to one–half of the total microflora of soil, water and sediment consisted of micro-organisms that exhibited lipolytic activity, with the facultative psychrophiles *Pseudomonas* and *Acinetobacter* being very important in this regard in the dairy industry (Breuil & Kushner, 1975a).

Acinetobacter lwoffi and *Acinetobacter calcoaceticus* are the two best studied *Acinetobacter* species with regard to their lipolytic activity. Intensive research has been performed not only with regard to the physiological factors affecting the production of lipase by these two species, but also the partial purification and characterization of extracellular and cell–bound lipases as produced by these species. *A. lwoffi* has been indicated to produce an extracellular lipase when grown on a complex medium where most of the lipase was produced during the logarithmic phase of growth. Under the same conditions, *A.lwoffi* also produced cell-bound esterase activity, although significant amounts of the esterase activity appeared in the external medium upon cell death (Breuil & Kushner, 1975a).

Since *A. lwoffi* is psychrophilic, optimal growth occurs at lower temperatures, but optimal lipase production occurs at 15 or 20ºC. Nutrient conditions also greatly influence lipase production. Lowering either the yeast extract content or the peptone content lowered or totally abolished lipase production. The addition of synthetic amino acids or acid–hydrolyzed casein led to increased production of esterase activity, but no lipase activity. The addition of sodium acetate, oleic acid, olive oil or Tween 20 had no effect on lipase production, while the addition of Tween 80 or ethanol inhibits lipase production. The addition of taurocholate, however, led to a 3–fold increase in extracellular lipase production. It was also found that the best lipase production occurred in standing cultures (Breuil & Kushner, 1975a).

The production of extracellular lipase activity by *A. calcoaceticus*, on the other hand, can be stimulated by the presence of long–chain alkanes such as hexadecane as sole carbon source in the growth medium. The highest extracellular lipase production occurs during the stationary growth phase, but sharp decreases in the lipase content of the medium occur

shortly after the production peak due to the degradation of the enzyme/enzymes by proteases (Kok *et al.*, 1993).

A. lwoffi liberates two forms of lipase when grown in a complex medium as indicated by gel filtration, a low and a high molecular weight form where the high molecular weight form has been found to be associated with lipids or phospholipids. Thus, the low molecular weight form could be the high molecular weight form minus the lipids or phospholipids. The crude enzyme shows a pH optimum at 7.5 while the purified enzyme shows an optimum at 8.5. Both preparations show temperature optima at 35ºC. The crude enzyme preparation was stable at the indicated temperature optimum and relatively stable up to a temperature of 50ºC where 50% inactivation of lipase activity occurred after 40 minutes of incubation.

Calcium ions have been found to stimulate lipase activity with optimum activity occurring in the presence of 10–15 mM calcium ions. It should be noted, however, that attempts to fully characterize the lipase/lipases of *A.lwoffi* were hindered by the inability to obtain a pure preparation of the lipase/lipases (Breuil & Kushner, 1975b).

Enzymes that hydrolyse ester bonds in general are esterases, and in this regard lipases are specialized esterases in that they are activated by interfaces. Triacylglycerols, the normal substrates of lipases, form distinct lipid–water interfaces at high concentrations, leading to the activation of lipases. At low concentrations, the triacylglycerols have a higher solubility in aqueous solutions, leading to the formation of less interfaces and, thus, less activation (Nthangeni, 1997). This consideration had to be kept in mind during the development of various substrates to be used in the determination of lipase activity. The substrate had to contain an esterified long–chain fatty acid that showed low solubility in aqueous solutions and, therefore, a high probability to form distinct lipid–water interfaces. In this regard, β-naphtyl laurate, olive oil and *p*–nitrophenyl palmitate have found the most application. Here, *p*–nitrophenyl palmitate is most often used because the assay system is easy to use, short and sensitive. The principle of the assay involves lipase hydrolysis of the ester bond between palmitate and *p*–nitrophenol, liberating *p*–nitrophenol, the production of which is easily followed at 410 nm (Nthangeni, 1997).

In light of the aforementioned, the aim of this chapter was to study the production of extracellular lipase by an *A. lwoffi* strain isolated in our laboratory after sampling at a local dairy drain. In this regard, various nutritional conditions were tested to produce the extracellular lipase in sufficient quantity for commercial application as an enzymatic cleansing agent for dairies.

2.2.2. PROTEASES FROM *SERRATIA LIQUEFACIENS*

Enzymes are the reaction catalysts of biological systems. They possess extraordinary catalytic power, often far greater than that of synthetic catalysts. The distinguishing feature of an enzyme-catalyzed reaction is that it occurs within the confines of a pocket on the enzyme called the active site. The molecule bond by the active site and acted upon by the enzyme is called the **substrate**. They have a high degree of specificity for their substrates, accelerating the chemical reactions and they function in aqueous solutions under very mild

conditions of temperature and pH. With exception of a small group of catalytic RNA molecules, all enzymes are proteins. Their catalytic activity depends upon the integrity of their native protein conformation. Only a few non-biological catalysts show these properties (Lehninger *et al*., 1993).

Microbial growth is defined as an increase in cellular constituents and may result in an increase in cell size, population number, or both. When micro-organisms are grown in a closed system, after the initial inoculation, there is no immediate increase in cell numbers and is called the **lag phase**. The population growth then enters an exponential phase for a few generations depending on the concentration of the nutrients. The population finally reaches stationary phase due to factors such as nutrient limitation and waste accumulation. During each of these growth phases, the microbial metabolism may change due to varying factors. For this reason, the growth curve of the microbes in question must be monitored for protease activity to provide optimum enzyme yield.

Micro-organisms require about ten elements in large quantities used mainly to construct cell constituents. These elements make-up over 95% of cell dry weight. The requirements for carbon, hydrogen, and oxygen are often satisfied together (Prescott *et al*., 1993). Nutrient molecules frequently cannot cross the selectively permeable plasma membrane of the cells through passive diffusion. It is for this reason that exoenzymes are secreted by the bacterial cells with the function of cleaving large proteins into smaller, metabolically molecules that are more accessible.

The *de novo* biosynthesis of certain enzymes of a microbial cell involved in catabolism is subject to a switch-on mechanism elicited by a corresponding component of the growth medium. Such a component is by definition an inducer for the corresponding enzymes. The induction process effects a change in phenotype allowing further production of energy required for metabolism and/or growth (Parke, 1975). It is the triggering of existing genetic potentiality that is revealed by the inducer. The level of the inducible enzyme rises and in this way enzyme activity increases regulating metabolism. Many enzymes function in this manner or to some degree require induction for significant enzyme activity.

There are presently two theories of induction that is, the *abundance* and *starvation*. The abundance theory implies that induction proceeds due to the large variety and high concentrations of complex nutrients in the growth media thereby causing the micro-organisms - in an attempt to utilise these - to synthesis the enzymes required. Three culture media namely, nutrient broth (NB), tryptone soy broth (TSB) and a 1% peptone, 1% yeast extract and 0.01% NaCl were evaluated for use as media for the production of extracellular proteases. The second theory involves limiting the nutrients available to the micro-organisms in the growth media, and in this way forcing the microbes to biosynthesis enzymes in order to survive. In order to ensure the biosynthesis of the desired enzyme, normally an inducer is added to the culture medium. The inducer is in the form of a low concentration of the substrate acted upon by the enzyme desired. As in the case of the proteases isolated specifically from bacteria originating in a dairy drain, the milk protein, casein and its derivatives were used at low concentrations.

Minimal media functioned as the basic growth media and was substituted with various carbon sources in an attempt to achieve maximum protease activity. Of the bacteria previously isolated and identified from a dairy drain, two strains of *Serratia liquifaciens* were evaluated for their potential protease activity.

2.3 MATERIALS AND METHODS

2.3.1. LIPASES FROM *ACINETOBACTER LWOFFI*

2.3.1.1. Optimization of assay for the determination of lipase activity

The assay system used is based on that of Nthangeni (1997) with a few modifications. Two solutions were prepared, i.e., solution 1 (30 mg *p*–nitrophenyl palmitate made up to 10 ml with 2–propanol) and solution 2 (2g sodium deoxycholate and 0.5g gum arabic dissolved and made up to 450 ml with a 50 mM phosphate buffer, pH 8.5). Solution 2 was stored at 4°C, while solution 1 was stored at room temperature. The assay mixture was prepared by the dropwise addition of solution 1 to solution 2 in the ratio of 1:9 with continuous stirring. Just enough assay mixture was prepared to be used in a single triplicate determination. The stock commercial lipase (899 μg/ml) was diluted to correspond to 18; 9; 6 and 4.5 μg/ml, respectively. For each determination, 600 μl of buffered substrate was pipetted into a clean and dry plastic cuvette. The reaction was started by the addition of 25 μl of enzyme preparation. Following brief mixing, the production of *p*–nitrophenol was measured spectrophotometrically at 410 nm at room temperature over a period of 5 minutes, taking readings every 10s. Each assay was done in triplicate (for each enzyme concentration). After determining the slope of the linear portion of the progress curve, the curve of $\Delta A_{410\ nm}$ /min vs [enzyme] (μg/ml) was constructed. Protein determinations were done according to the BCA method (Stoscheck, 1990).

2.3.1.2. Cultivation of *a. Lwoffi*

Initially *A. lwoffi* was cultivated according to the method of Breuil and Kushner (1975a). To this end, 50 ml of a solution containing 2% peptone and 0.15% yeast extract was prepared (in 250 ml Erlenmeyer flasks) and autoclaved. Following inoculation of the medium with *A. lwoffi*, the bacterium was incubated at 24 °C. The growth of the bacterium was followed by taking readings at 600 nm spectrophotometrically 3–6 h apart. At each point the extracellular lipase activity was determined as described in section 2.2.1. At each point, serial dilutions of a sample taken from the culture were made and spread onto nutrient–agar plates. This was done firstly, to assess homogenicity of the culture and, secondly, to indicate that the increase of the reading at 600 nm was due to an increase in *A. lwoffi* cell number.

Very little lipase activity could be measured when *A lwoffi* was cultured under the aforementioned conditions. Therefore, experiments involving the induction of lipase production were carried out. To this end, 48 ml of the M9 minimal medium was prepared in 250 ml Erlenmeyer flasks. *A.lwoffi* was inoculated into 5 ml of a previously prepared complex medium and incubated at 24°C for 21.5–22 h. Following this incubation, 2ml of the bacterium containing solution was aseptically transferred to the previously prepared M9 medium and the growth of the bacterium as well as lipase production was followed as

previously described. 0.1% Glucose as growth enhancer as well as sodium taurocholate and Tween 80 as lipase inducers was added to the M9 minimal medium.

2.3.2. PROTEASES FROM *SERRATIA LIQUEFACIENS*

2.3.2.1. Culturing of *serratia liquifaciens*

The optimal growth conditions were determined for protease production using the information obtained from previous studies done at the University of Port Elizabeth. Starter cultures were obtained by growing up *S.liquifaciens* in tryptone soy broth at 37 °C overnight at 120rpm. The cells where pelletted by centrifuging at 9500 rpm for 6 minutes and inoculated into 3l of M9 supplemented with 0.1% glucose and 0.05% casein.

In the first case (flask 1) 100ml of starter cells were used to inoculate the M9 which was then incubated at 37°C for 20 to 24 hours at 120 rpm. The protease activity was determined as outlined above. The culture was left overnight at 4°C and the protease activity was determined again. Flask 2 was inoculated with 1l of starter cells and was treated in the same way as flask 1. Flask 3 was inoculated with 1l of starter cells but was incubated at 4°C overnight after which the protease activity was obtained. It was left at 4°C for another 24 hrs and the protease activity determined again. The M9 *S.liquifaciens* culture was centrifuged at 9500 rpm for 10 minutes to remove the cells. The supernatant was used to determine the protease activity.

2.3.2.2. Protease activity assay

Protease activity of each of the isolates was determined by a modification of Ray *et al*. (1992). Culture supernatant (600 µl) was added to 1 ml of 1% (w/v) casein and 400 µl of 50 mM sodium acetate buffer (pH 5.0) and incubated at 30 °C for 2 hrs. The reaction was stopped by the addition of 1 ml of 20% (w/v) tricloroacetic acid (TCA). The resulting precipitate was allowed to settle for 30 minutes on ice and then was pelleted by centrifugation (3000rpm/20°C/Beckman) for 15 minutes. Proteolytic activity was measured by determining the Abs_{280} of the final supernatant. Due to deviations between absorbance values it was necessary to stabilize the casein by modifying the pH of the buffer form 5 to 7. At a higher pH value the casein does not precipitate out. For this reason a 50mM Tris Acetate (pH7) was used instead of the 50mM Sodium Acetate buffer. All assay determinations were done in triplicate. The blank was prepared by adding TCA to the two-hour incubation mixture at zero time.

Alternate assays were performed according to Beydon and Bond (1989) outlined in Table 3. A standard curve was set up using *Aspergillus oryzea* protease (Sigma®). The standard curve ranged from 0 to 40 U/l.

The supernatant obtained from the *S.liquifaciens* was tested over a range of temperatures. The Beynon and Bond (1989) assay was used except that different temperatures were used for the 30 minute incubation period. The temperatures used were 30, 35, 40, 45, 50, 55, 60 and 65°C.

Table 3 Assay of proteinase activity with casein

1. Prepare a 1% (w/v) solution of casein in 0.2M glycine-NaOH buffer, pH 9.0, and a stopping solution of 0.11M trichloroacetic acid, 0.22M sodium acetate and 0.33M acetic acid.
2. Incubate 1ml casein solution with 1ml of proteinase solution at 37°C for 30min.
3. Stop the reaction by addition of 3ml of stopping solution.
4. Keep the solution at 4°C for 1 hr, then centrifuge at 1000g for 5 min. The A280 of the supernatant is measured relative to a blank. The blank was prepared by incubating the protease at 37°C for 30min after which stopping solution is added immediately after addition of proteinase.
5. An increase in absorbance of 0.1 corresponds to the hydrolysis of 0.7 mg of casein. The increase in absorbance should not exceed 0.5, which corresponds to the hydrolysis of about one-third of the casein initially present.

The supernatant was inactivated for 10 minute at temperatures from 45 to 80 °C before being assayed for protease activity. The incubation temperature used was that determined as the optimal temperature for *S.liquifaciens* protease activity in section 2.1.2.

Inactivation by industrial peroxide acid based sanitizer was done by diluting the sanitizer to 0.1% in the supernatant and then assaying for protease activity after 10 minutes.

2.3.2.3. Test for endotoxins in supernatant

The E-toxate® test (Sigma Chemical Company) was used for the detection of endotoxin in the supernatant. E-toxate® is prepared from a lysate of the circulating amebocyte of the horseshoe crab, *Limulus polyphemus*. When exposed to minute quantities of endotoxin (lipopolysaccharides from the walls of gram-negative bacteria), the lysate increases in opacity as well as viscosity and may gel depending on the concentration of endotoxin. While the mechanism for this reaction is not completely understood, it appears to be analogous to the clotting of mammalian blood involving two steps:

1. Endotoxin in the presence of calcium ions activates a trypsin-like, preclotting enzyme(s).

2. The activated enzyme(s) then modify a 'coagulogen' by limited proteolysis to produce a clottable protein.

This endotoxin-mediated effect is closely tied to the biologically active or 'pyrogenic' portion of the molecule since it has been shown that 'detoxified' endotoxin yields a negative *Limulus* lysate test (Sigma Chemical Company).

2.4. RESULTS

2.4.1. LIPASES FROM *ACINETOBACTER LWOFFI*

2.4.1.1. Optimization of assay for the determination of lipase activity

Figure 1 and Figure 2 indicate typical progress curves obtained with the highest and lowest enzyme concentrations.

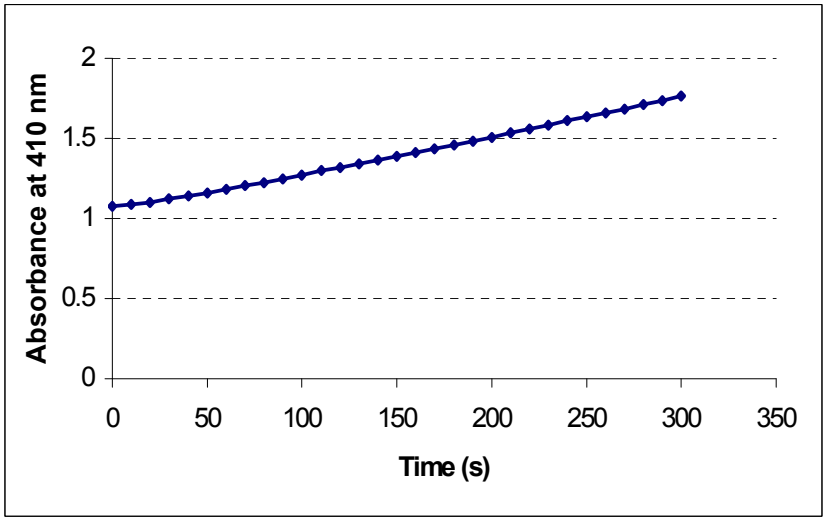


Figure 1 Progress curve obtained with 4.5 µg/ml enzyme

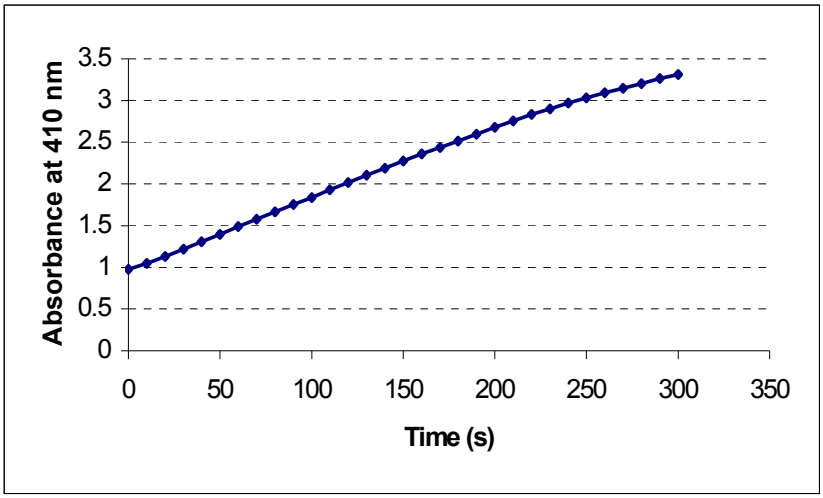
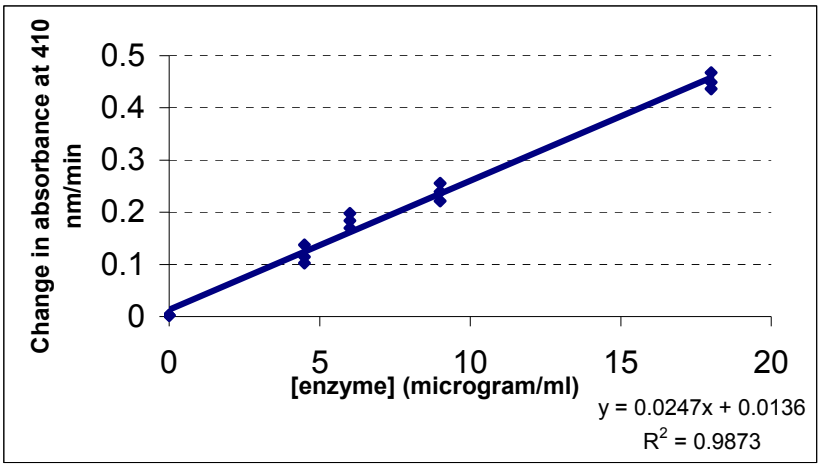


Figure 2 Progress curve obtained with 18 µg/ml enzyme

As can be seen from Figures 1 and 2, the progress curves obtained were linear over the full time period of this assay at these enzyme concentrations. Therefore, the slope of these curves was taken over 5 minutes and used to construct the curve of $\Delta A_{410\ nm}$/min vs [enzyme] (µg/ml). This plot can be seen in Figure 3.

Figure 3 Relationship between enzymatic activity and enzyme concentration



As can be seen from Figure 3, the relationship between enzymatic activity and enzyme concentration is linear over the whole range of enzyme concentrations used. At enzyme concentrations lower than that used in this optimization, an initial lag period could be observed (data not shown) where a decrease in the absorbance at 410 nm signifies an initial dilution of the enzyme. Therefore, the enzyme concentrations used are situated at the lower end of the sensitivity range of this assay system with this particular enzyme. We define one unit (U) of lipase activity as that amount of enzyme that releases one μmole of *p* – nitrophenol per minute in the aforementioned assay system.

The units/ml can be calculated as follow:

$$\mathbf{U/ml = \{V/v\varepsilon d\} \times \Delta A_{410\ nm}/min}$$
$$\mathbf{= 1.67 \times \Delta A_{410nm}/min}$$

where V = Total volume in cuvette (ml)
v = Enzyme volume (ml)
ε = Molar extinction coefficient of *p* – nitrophenol at 410 nm ($15\ ml.\mu mole^{-1}.cm^{-1}$)
d = Light path of cuvette (cm)

2.4.1.2. Culture of *a. Lwoffi*

A. lwoffi was initially cultured in a complex medium consisting of 2% peptone and 0.15% yeast extract, according to the procedure of Breuil & Kushner, 1975a. However, we also tested whether the addition of 0.8% NaCl would have an effect on the growth rate and cell density of the bacterium.

The bacterium grew faster in the absence of 0.8% NaCl, reaching higher cell densities in a shorter period of time. Therefore, subsequent experiments were carried out in the absence of the NaCl. What is also evident from these curves is that extracellular lipase production remained at a baseline level throughout the growth curve in either case. Therefore, we proceeded by adding 0.04% sodium taurocholate and 1% Tween 80 to two individual cultures.

A slight peak in lipase production occurred just at the onset of the logarithmic phase of growth, this peak tapering off quickly thereafter. An inhibitory effect of Tween 80 on lipase production is evident from the lowered baseline production of lipase.
As very little or no extracellular lipase could be measured by growing *A. lwoffi* in a complex medium even in the presence of known inducers of extracellular lipase, attempts where made to try and induce extracellular lipase production by culturing the bacterium in a minimal medium (M9) containing sodium taurocholate.

The extracellular lipase was produced during the whole of the logarithmic growth phase, with the extracellular lipase content of the medium reaching a maximum just at the onset of the stationary phase of growth. Immediately hereafter, lipase production decreases and the lipase content of the medium decreases, possibly due to the action of specific proteases.

2.4.2. PROTEASES FROM *SERRATIA LIQUEFACIENS*

S.liquifaciens was cultured under varying conditions to determine those conditions under which the highest activity of free protease would be obtained. Figure 4 reflects how flask 2 yields the highest protease activity. This flask was grown up using 1l of starter culture cells incubated overnight at 37°C with a subsequent overnight incubation at 4°C. Flask 1 had 100ml of starter culture with an initial incubation of 37°C followed by an incubation period at 4°C and yielded the second highest yield of free proteolytic activity. Flask 3 had 1l of starter culture with two consecutive incubations at 4°C, with minimal protease activity being achieved. This result was expected, since the incubation at 37°C, and large starter culture volumes, ensured that the culture quickly reached stationary phase.

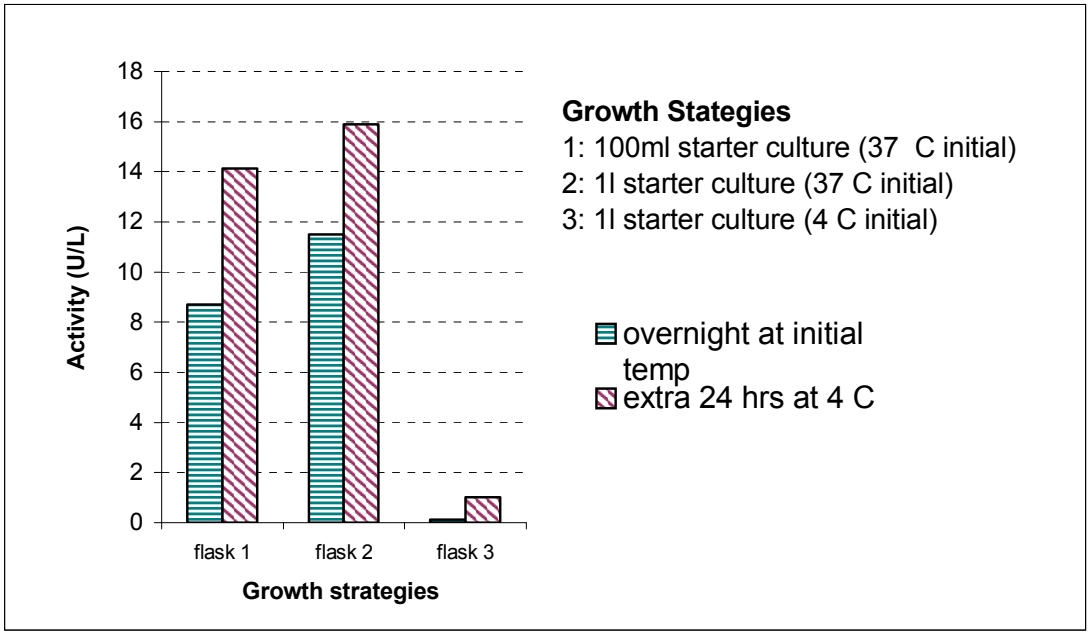


Figure 4 A comparison of the different growth conditions used for *S.liquifaciens*

Studies on *S.marcescens* showed that production of free proteolytic activity occurred during this stage (Hernándes-Delgadillo and Ruiz-Cruz, 1994). It was suggested that incubation at 4°C stimulated protease productions since the bacteria were under stress and trying to survive. The results cannot, however, substantiate this since the increase in protease activity is most probably due of normal cell activity and would have occurred anyway, especially since *S.liquifaciens* is known to grow comfortably at this temperature.

Once proteolytic activity was obtained and the *S.liquifaciens* cells centrifuged out of the culture medium, further studies on the protease were performed. These showed that its optimal activity was at 45°C, with a sharp increase in activity between 40 and 45°C. (Figure 5).

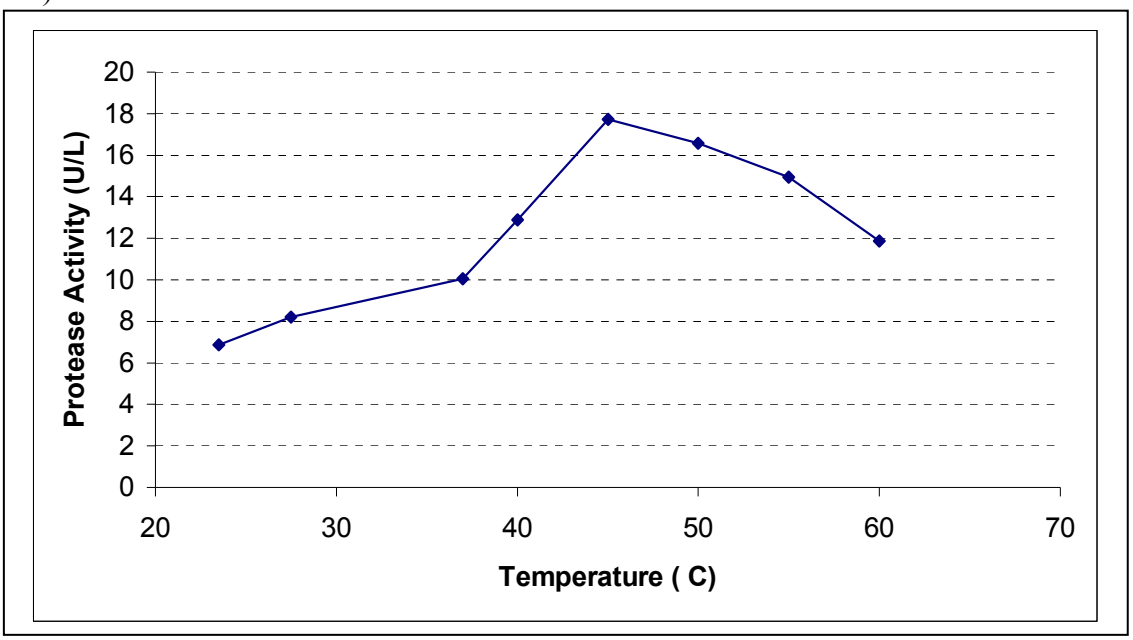


Figure 5 Effect of temperature on *S.liquifaciens* protease activity.

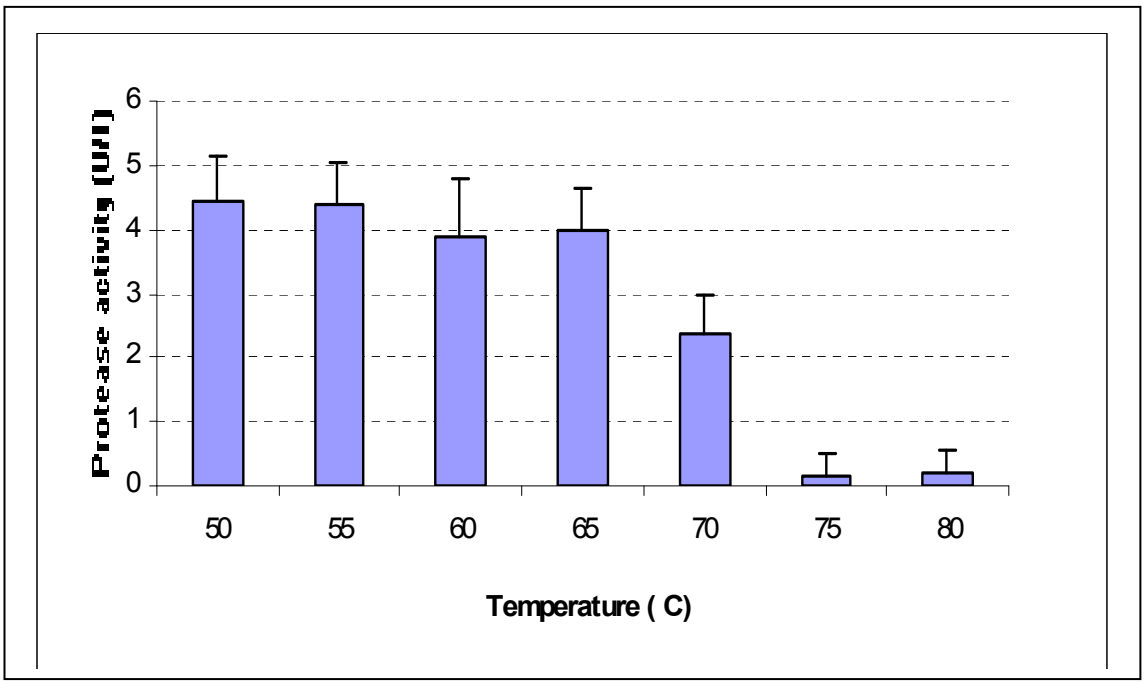


Figure 6 Temperature inactivation of *S.liquifaciens* protease

The protease was inactivated when incubated at 75°C for 10 min (Figure 6). Figure 6 indicates that incubation at 65°C for 10 min had less effect on the protease than when the protease is incubated at 60°C. This is seen by the higher activity of the protease exhibited after incubation at 65°C. If, however, the statistics are taken into account, and the error bars reflected in Figure 3 are assessed, the activity after incubation at 65°C could, in fact, be lower than that obtained after incubation at 60°C. The protease was also inactivated with 0.1% peroxide-based sanitizer.

The supernatant was tested for the presence of endotoxins but yielded a negative result, indicating the absence of endotoxins in the supernatant that was used in further studies.
The protease was inactivated when incubated at 75°C for 10 min (Figure 6). Figure 6 indicates that incubation at 65°C for 10 min had less effect on the protease than when the protease is incubated at 60°C. This is seen by the higher activity of the protease exhibited after incubation at 65°C. If, however, the statistics are taken into account, and the error bars reflected in Figure 3 are assessed, the activity after incubation at 65°C could, in fact, be lower than that obtained after incubation at 60°C. The protease was also inactivated with 0.1% peroxide-based sanitizer.

The supernatant was tested for the presence of endotoxins but yielded a negative result, indicating the absence of endotoxins in the supernatant that was used in further studies.

2.5. DISCUSSION

2.5.1. LIPASES FROM *ACINETOBACTER LWOFFI*

The assay system employing *p* – nitrophenyl palmitate was optimized and the relationship between enzymatic activity and enzyme concentration showed linearity throughout the range of enzyme concentrations used. As previously said, the enzyme concentrations used probably represent the lower limit of the sensitivity range of this assay system with this particular lipase. Since the assay was not optimized with a commercial lipase from an *A. lwoffi* strain, the relationship between enzymatic activity and enzyme concentration as well as the sensitivity range of this assay system for an *A. lwoffi* lipase are unknown. However, during the determination of extracellular lipase activity during the respective growth experiments, an initial lag period was observed where the absorbance at 410 nm decreased for a period of one minute, whereafter it increased linearly for the remaining four minutes. This indicates that the lipase/lipases are diluted upon addition to the assay mixture and, therefore, that the lipase concentration is close to the sensitivity limit of the assay system with this particular lipase. In the determination of lipase activity during the growth experiments, we used the linear portion of the progress curve and, therefore, the slope was calculated over a period of four minutes.

Extracellular lipase production was minimal throughout the various growth experiments. Because this production was so low, the lipase activity measured was expressed as ΔA_{410nm}/min without taking the cell number into consideration. If two different nutritional conditions yielded similar high extracellular lipase production, the cell number would have been taken into consideration. Nevertheless, the nutritional condition yielding the highest

extracellular lipase activity was the M9 minimal medium containing sodium taurocholate as sole carbon source. The peak observed represents 4.2 total lipase units. However, it is evident that the stationary phase cell density of this bacterium under this nutritional condition decreased dramatically as compared to the cell density obtained when culturing the bacterium in the complex medium. Attempts to increase the stationary phase cell density by the addition of 0.1% glucose and by increasing the nitrogen content of the medium relative to the carbon content in separate cultures not only failed to do so, but also decreased the production of extracellular lipase.

The results obtained from growing the bacterium in complex media clearly indicates that the production of extracellular lipase is not essential for the growth of the bacterium since the bacterium grew to high cell densities without the production of any appreciable amounts of lipase. Breuil and Kushner (1975a) indicated that the presence of amino acids in the medium resulted in no lipase production by an *A. lwoffi* strain in their laboratory. This probably indicates the ability of this bacterium to produce various proteases, these proteases not only maintaining growth of the bacterium, but also decreasing the lipase content of the medium. The fact that the highest extracellular lipase production was observed in a minimal medium containing a lipase inducer might just point to the absence of proteases increasing this lipase production rather than the lipase inducer itself having any marked effect on lipase production. Breuil and Kushner (1975a) did not present any conclusive results regarding this matter in that they cultured *A. lwoffi* in a minimal medium containing sodium taurocholate but also containing various amino acids that could lead to the production of various proteases. We, therefore, believe that a study regarding the protease production ability of *A. lwoffi* would also yield valuable information regarding its lipase production ability.

Acinetobacter lwoffi, as isolated in our laboratory, failed to produce appreciable amounts of extracellular lipase under all nutritional conditions employed. A total number of 4.2 units was the highest extracellular lipase content obtained in a constant culture volume.

These results indicate that the culture of *A.lwoffi* and the isolation of its extracellular lipases would not be cost-effective to be applied in a cleaning system for South African dairies. A cheaper alternative is to purchase a mass-produced, but not as specific, lipase from Novo-Nordisk™.

The optimal activity and optimal temperature of the commercial lipase was analysed in a temperature range of 25 to 60°C. The substrate was pre-heated to the test temperature before the assay was done. The assay was conducted in a Perkin-Elmer Lambda3 temperature-regulated spectrophotometer to keep the temperature of the assay solution constant during the assay period.

The commercial lipase was shown to be most active at 50°C. At this temperature the activity of a 10 mg/ml lipase solution was 70.3 U/g. The result was worked out in U/g because the lipase came in the form of a powder. This enabled convenient calculations of mass of lipase needed for further studies.

2.5.2. PROTEASES FROM *SERRATIA LIQUEFACIENS*

Although the growth curve for *S. liquifaciens* gave significantly higher OD_{600} readings, the modified protease assay of Ray *et al.* was not able to give reproducible results for the complex media due to the interference by compounds such as meat extract, peptone and yeast extract. Survey scans carried out for TSB show high absorbance readings from wavelength 200 to 300 nm. Due to the fact that nutrient broth and peptone solution contains meat extract, peptone or yeast extract, complex media could not be assessed for the production of protease.

Minimal media was used to form the basic component to the growth media for testing the starvation theory. Casein, casein hydrolosate or glucose was added as a carbon source and their action as an inducer monitored by the protease assay. The metabolism of glucose as a carbon source was above that of casein and casein hydrolosate since the growth curve was significantly steeper and obtained the highest OD_{600} reading than the others - a higher protease activity for the glucose media than the casein and casein hydrolosate. Whether or not this is a function of a higher microbial population density, remains unclear.

3. EVALUATION OF ENZYMES AS CLEANING AGENTS

The cleaning of dairy plants is regulated by the Foodstuffs, Cosmetics and Disinfectants Act No 54 of 1972. At the present time there are further regulations being passed in Parliament which will require the implementation of HACCP in most food processing plants. These regulations could be promulgated within the next two years (Hunt, 1999).

Correct, effective cleaning of dairy processing systems is essential in order to prevent milkstone and biofilm build-up. This is achieved by CIP systems that remove soils that accumulate during the normal running of the system. CIP processes are usually chemically-based with a heterogeneous reaction occurring between a chemical solution and the fouling layer, soil or biofilm (Tragadh, 1989).

Current CIP systems are based on a 3-chemical cleaning regime of:

1. Pre-rinse with ambient water to remove gross soil.
2. 0.5-2.0% Sodium hydroxide at 70-80°C for 15 mn.
3. Intermediate rinse with water (70-80°C).
4. 0.5 - 1.0% Nitric acid at 60-65°C for 15 min.
5. Intermediate rinse with water at ambient temperature.
6. Sanitizer (peroxide-based cleaner or steam injection) at ambient temperature for 5min.

The alkali is used to remove carbohydrates (pH 7-12), proteins (pH 10-13) and lipids. The carbohydrate soils are the easiest to remove and include polysaccharides, sugars, starches and cellulose. The use of strongly alkali or chlorinated alkaline components leads to the loss of protein structure and subsequent cleavage of peptides into smaller peptides, with increased water solubility (Bohner and Bradley, 1992). The removal of lipid soils from the dairy system is very important, as lipids are able to increase the adherence of other soils onto surfaces (Jeurnink and Brinkman, 1994). Lipids are more difficult to remove than carbohydrates or water-soluble proteins because they are not water-soluble. The alkali chemicals used for cleaning lead to the saponification of lipids, creating soluble soaps that are easily washed away (Lehninger *et al.*, 1993).

The acid step (HNO_3) removes milkstone and other mineral deposits, such as scale, by decreasing the pH. This leads to changes in the ionic interactions between minerals, proteins and the processing surfaces, resulting in an increased solubility and a release of the soil into the washing solution. The sanitizing step usually kills and removes any residual microbial presence in the system, rendering it sterile for the next milk load.

In all steps, surfactants are used to wet the surfaces and soils, allowing for more efficient cleaner-soil interaction. Elevated temperatures are used to increase the efficiency of cleaning although, in some cases, an increase in temperature may cause the formation of certain soil types.

Chemically-based CIP has many drawbacks. It is energy consuming, hazardous to the operators and is being increasingly restricted by the legislator. Another problem is that unless the first two chemical steps are effective in chemically and physically removing soils, the sanitizer may be consumed in the sanitizing process before being able to act on the residual microbial population.

The release of industrial effluent is subject to the control by Section 21(10(a) of the Water Act of 1956 (Act 54 of 1956) modified in the Water Amendment Act of 1984 (Act 96 of 1984). These Acts apply to discharging of both industrial effluent and domestic wastewater into the natural waterways. The untreated effluent, produced by the chemical CIP process, is mostly released into the sewers, although the effluent water should be treated before being disposed of. This practice is being increasingly restricted by legislation, with tariffs based on the effluent composition. Therefore, dairies are paying high tariffs because of their effluent, which has high COD and sodium levels and is at elevated temperatures and high pH (Personal Communication, Mr. J.C. Kritzinger). Dairies are now facing the problem of improving their effluent to cut down on production costs while still maintaining high sanitation standards.

The regulations set out in the Water Act are often transferred directly to industry, thereby ensuring that the wastewater management sites can cope with the influx of effluent in a manner that will ensure adequate treatment of the effluent. Individual municipalities may set regulations that are more stringent in order to maintain the standards of water released into natural waterways. The regulations as set out by the City Engineer's Department of Port Elizabeth are shown in Table 2 below (Personal Communication, Mr. J.C Kritzinger).

Table 1 Physical and chemical conditions required before effluent acceptance (Personal Comunication, Mr J.C. Kritzinger)

Standard:	**Value**:
Temperature	< 44°C
Electrical Conductivity	< 500 mS/m at 25°C
pH	< 12.0 or > 6.0
Permanganate	< 1 000 mg/ml
COD	< 10 000 mg/l
Fats, vegetable oil and like substances	400 mg/l
Mineral oils and grease	50 mg/l
Suspended solids	1 000 mg/l

Other parameters that are important when considering wastewater treatment and are sometimes tested for by a municipality are calcium, nitrates, potassium, sodium, TDS and water hardness. Testing for microbial numbers on equipment surfaces after cleaning is also important. Indicator organisms are tested for giving a general idea of the microbial quality of the system. Total coliform bacteria are used as an indicator to the general hygienic quality of the surfaces. Faecal coliform bacteria are indicators of faecal pollution and provide a more specific indicator of faecal contamination that originates from humans and other warm blooded animals (Prescott *et al.*, 1996).

If these limits are exceeded, an extra charge is imposed on the company. The current tariff formula for trade effluents discharged into the sewers in the Port Elizabeth Municipality is as follows (Personal Communication, Mr. J.C. Kritzinger).

Tariff = C x V x K x T

Where C = prescribed treatment charge of 74 cents per kilolitre (exclusive of VAT) for 1999.
V = metered volume of water entering premises in kilolitres
K = fraction of incoming water discharged into sewer
T = strength of effluent discharged into sewer

Strength (T):

$$T = \frac{1}{3}\frac{2\,PV - 160}{80} + \frac{S - 10}{10}$$

Where PV = permanganate value (mg/l)
S = volume in ml of solid matter that settles in 1 hour in an Imhoff cone from 1 litre of effluent.

The regulations pertaining to effluent may change slightly between different Municipalities. This includes the tariff formula for trade effluents discharged into the sewers.

Dairy effluent always exceeds the regulatory specification, with average values for dairies in Port Elizabeth and Johannesburg depicted in Table 4. The tariff charged on this effluent is charged on the average results obtained from the previous years samples. These values would remain for the following year unless it is thought that they are not representative of effluent strength. In the latter case re-sampling will occur, with 3 random samples being obtained from the drain at the company in question (Personal Communication, Mr. J.C. Kritzinger).

Table 2 Average values of effluent obtained from dairies in Port Elizabeth and Johannesburg

Standard:	Johannesburg Dairy:	Port Elizabeth Dairy:
pH	10.5	2.84 -10.27
COD	11 450 mg/ml	11 250 mg/ml
Sodium level	Increased by 300 mg/ml compared to water intake	Not tested in Port Elizabeth
Temperature	42°C	38°C

3.1 MATERIALS AND METHODS

The 'mini dairy' system used was constructed of 9.6 m of dairy grade stainless steel pipe with a diameter of 5 cm. The milk and cleaning solutions were pumped through the system at 1.5m/s using an electrical pump. Fig 1 shows the system and its features. There was no temperature control system in place. The solutions were drawn from the bottom of a holding tank (40 x 40 x 50cm) and returned through a pipe entering from the top of the vat. The vat was emptied from the bottom of the tank via a separate pipe. There are five dead-end sections with Pyrex ends that were designed to allow for access to the pipes for visual inspection of cleaning efficacy and for taking swabs. The length of the dead-ends is 4 cm. A special insert was made to fit into the dead-ends to allow small disks of stainless steel to be inserted into the flow of liquid. These disks were analyzed with a scanning electron microscope, in the Physics Department (UPE), to determine cleaning effectiveness. This insert is shown in Fig 2.

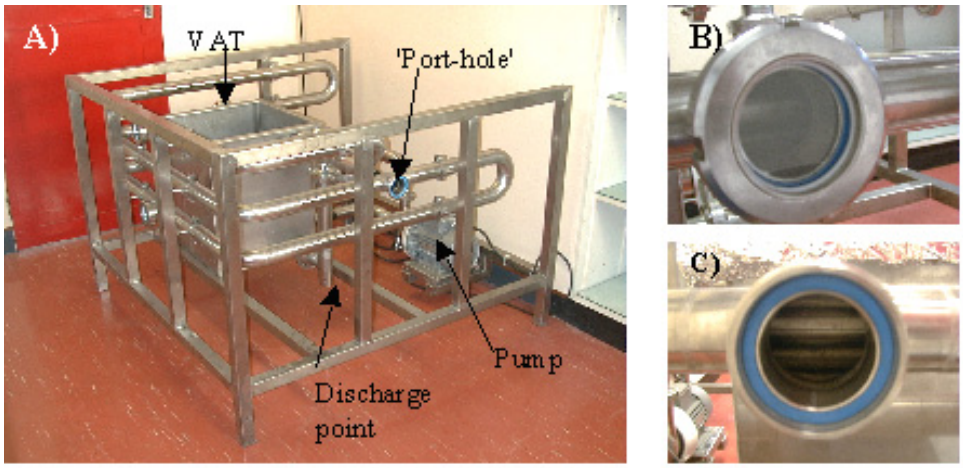


Figure 1 A) A picture of the 'mini dairy' system used for the cleaning and effleunt studies. B) A closer look at the port-hole through which visual inspections were made and microbial swabs were collected. C) The port-hole without the pyrex cover showing the Teflon O-ring that seals the end.

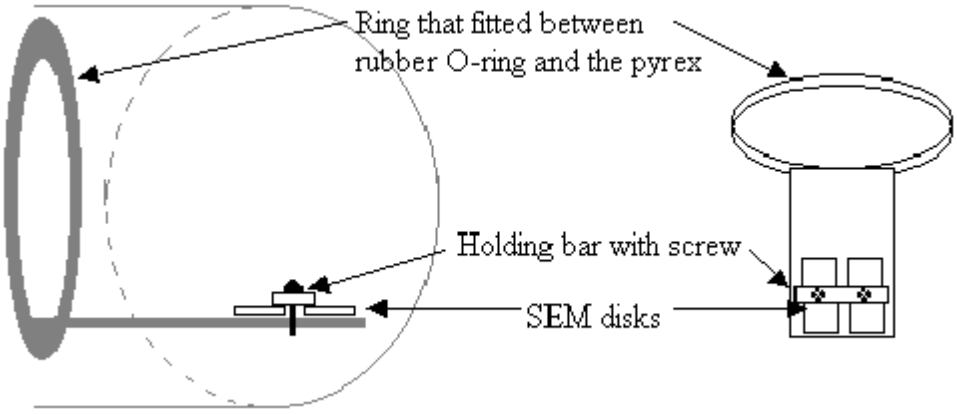


Figure 2 Diagram of insert used to insert disks that were used for analysis by SEM. This insert was fitted into a port-hole as shown in Fig 2 between the Teflon O-ring and the Pyrex.

3.1.1 CLEANING SYSTEMS USED

Raw milk was obtained from the Clover Rosedale Dairy in Grahamstown and run through the system for 1.75 h before starting the cleaning procedure. The same cleaning regimes were used in the scale model for the chemical and commercial enzyme CIP systems. The lipase/protease system was used with 30U/l lipase activity being used as this was shown in preliminary studies to be most effective for cleaning and microbial control.

Three main CIP systems where tested. Chemical CIP was used as a comparison for the lipase/protease CIP system, of what is currently being used in industry. The commercial enzyme-based CIP provides a comparison of the lipase/protease CIP system with an alternative enzyme-based CIP system that is currently available to the dairy industry. This commercial enzyme-based CIP is not, however, widely used as it has not been able to compete with the conventional chemical CIP system with respect to cleaning efficacy in industry.

3.1.1.1 Chemical CIP

The following procedure was used as a representative system for what is currently being used in the dairy industry.

a. Rinse with water at ambient temperature.
b. Wash with 1.0% industrial NaOH-based solution for 15 min at 70°C.
c. Rinse with water at 60°C.
d. Wash with 1.0% industrial HNO_3 -based solution for 15 min at 60°C.
e. Rinse with water at ambient temperature.
f. Rinse with 0.05% peroxide-based sanitizer for 1 min at ambient temperature.

3.1.1.2 Commercial enzyme-based CIP

The following procedure was used as a representative system for an alternative enzyme-based CIP system.

1. Rinse with water at ambient temperature.
2. Wash with 0.04% protease for 15 min at 50 °C.
3. Rinse with water at 50 °C.
4. Wash with 0.05% of an alkali solution for 15 min at 50°C.
5. Rinse with water at ambient temperature.
6. Rinse with 0.1% peroxide-based sanitizer for 1 min at ambient temperature.

3.1.1.3 Lipase and protease based CIP

Protease obtained from *S.liquifaciens* was used at 10 U/l while the commercial lipase was used at 30U/l.

1. Rinse with water at ambient temperature.
2. Wash with commercial lipase for 15 min at 50°C.
3. Rinse with water at ambient temperature.
4. Wash with *S.liquifaciens* protease at 30U/l for 30 min at 50°C.
5. Rinse with water at ambient temperature.
6. Rinse with 0.1% peroxide-based sanitizer for 1 min at ambient temperature.

3.1.2 MICROBIOLOGY OF SURFACES TESTED AFTER

Swabs were moistened in sterile water, and samples taken off approximately 3 cm^2 surfaces and placed in 2 ml of sterile water. A 3 cm^2 area was sampled instead of the prescribed 10 cm^2 area because of the limited surface available for swabbing.

The water was plated onto nutrient agar and MacConkey agar. The nutrient agar plates and one set of MacConkey agar plates were incubated at 37 °C overnight to obtain a total viable bacterial count and coliform count, respectively. Another set of MacConkey agar plates was incubated at 44 °C to determine *E.coli* counts. Counts were done in triplicate and incubated overnight (Marshall, 1992).

3.1.3 SEM FOR DETERMINATION OF CLEANING EFFECTIVITY

The experimental plates used were of a 1 mm thick stainless steel with the dimensions: 50 x 50mm. These plates were previously cleaned according to a modified protocol from Tissier and Lalande (1986) consisting of nine steps: (1) deionized water, ultrasonic bath (80°C, 10 min); (2) acid washing (1% HNO_3, 80°C, 20min); (3) deionized water, ultrasonic bath (80°C, 20 min); (4) alkaline washing (2% NaOH, 80°C, 20 min); (5) deionized water, ultrasonic bath (80°C, 10 min); (6) acid washing (1% HNO_3, 80°C, 10 min); (7) deionized water, ultrasonic bath (80°C, 10 min); (8) distilled water; and (9) water drainage and oven drying at 110°C overnight.

The disks were inserted at the beginning of a run, one disk was removed after each rinse step (as outlined in the cleaning procedures) and the final one was removed after peroxide-based sanitizing step. All the disks were fixed before coating with gold and analyzing with SEM. The fixing procedure was modified from Austin and Bergeron (1995), McCoy *et al.* (1981), and Tissier and Lalande (1986). The disks were fixed in 5% gluteraldehyde for one hour, followed by dehydration in a series of aqueous ethanol solutions (10 min each in 30, 60 and 90% followed by two 15min incubations in pure ethanol. The disks were air dried at 37°C overnight.

The disks were coated with gold in a Edwards spotter coater (40mA for 1.5min). All SEM was done on a Phillips XL30 Scanning electron microscope.

3.1.4 EFFLUENT TESTS

40 ml samples were collected in McCartney bottles during the cleaning procedures. The following tests were performed on the samples: temperature, pH, water hardness, COD, potassium, nitrates, TDS, calcium and sodium.

3.1.4.1 Chemical oxygen demand

The Nanocolor® COD 1500 test for determination of chemical oxygen demand was used (Macherey-Nagel, Düren). The test range is 100 to 1500 mg/l O_2. The chemical oxygen demand (COD) of a sample is determined by the silver-catalyzed oxidation with potassium dichromate/sulphuric acid at 148°C during a two hour period. The increase in green chromium(III) ions is measured at 620 nm.

The standard curve was set up using the Nanocontrol® COD standard with a concentration of 4.0 g/l O_2 (Macherey-Nagel, Düren). The standard was diluted to fall within the range of 0 to 1500 mg/l O_2.

3.1.4.2 Potassium

The Visocolor® Potassium test kit was used for the determination of potassium in the range 2 to 15 mg/l K^+. Potassium forms a precipitate with sodium tetraphenylborate, under controlled conditions. The resulting turbidity is used for concentration measurement. Turbidity interferes and turbid samples must to be filtered. Good reproducibility is obtained in drinking, surface, and ground water.

3.1.4.3 Nitrates

Method 1
Copper cadmium for the assay was prepared as follows: course cadmium powder was washed briefly with 5% HCL; rinsed with distilled water; washed with 0.5% $Cu(SO_4).5H_2O$ (turns from silver grey to dark grey); washed with 0.007N HCl / 0.005M EDTA by shaking, decanted and rewashed until supernatant is clear, silver grey copper cadmium results. The copper cadmium was stored in EDTA solution away from air.

A small amount of copper cadmium was added to 2 ml of sample or standard solution and mixed. The mixture was centrifuged at 5000 rpm for 5 min and the supernatant was used to make 1 ml serial dilutions. 0.25 ml Griess reagent was added and mixed. The solution was left to stand for 15 min and read A_{540nm}. A standard curve was set up using KNO_3 concentrations of 0 to 0.313 mM.

Method 2
Copper cadmium was prepared as in method 1. The assay solution was composed of a 1:1 mixture of solution A (0.165% sulpanilic acid in 5N acetic acid, dissolved by gentle heating) and B (0.25% 1-naphthylamine in 5N acetic acid, dissolved by gently heating). A small amount of copper cadmium was added to 2 ml of sample or standard, mixed and centrifuged at 5000 rpm for 5 min. Aliquots (200µl) were pipetted into a microtitre plate, 8 µl of the assay solution added and mixed. The mixture was left to stand for 30 min and the absorbance was read at 540 nm (modified from Barrow and Feltham, 1993).

A standard curve was set up using KNO_3 concentrations 0 to 0.15 mM.

3.1.4.4 Sodium

Flame photometry was used to determine the concentration of sodium in the effluent. The Zoology Department's (University of Port Elizabeth) Jenway Flame Photometer PFP7 was used. A flame standard of 140 mmol Na/l was used to calibrate the photometer.

3.2 RESULTS AND DISCUSSION

Visually, all CIP procedures yielded effective cleaning. There was no visible protein or lipid residue on the stainless steel after cleaning and no milkstone residue was evident. Figure 3(A) represents what the system looked like after the milk had been run through the system for 1.75 h. The surfaces within the system displayed a fatty texture. In the vat, substantial protein/lipid residue had accumulated where there was no continual flow of milk (Figure 3 (B)). The differences in the cleaning efficacy of the different CIP systems used

could be better illustrated by looking at the microbial counts obtained after cleaning (Tables 3 and 4) and the micrographs taken with the SEM.

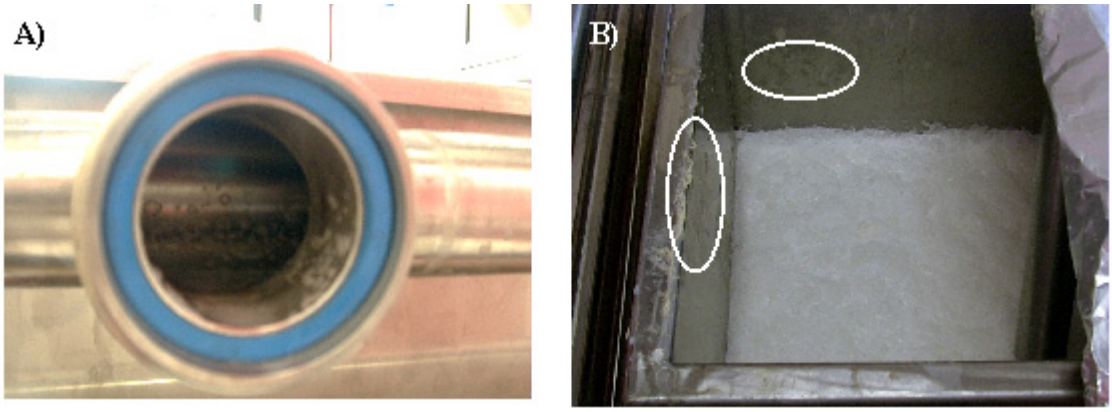


Figure 3 A) A view of a porthole after fouling of the system with raw milk for 1.75h. After the subsequent rinsing step the surfaces looked clean but still 'fatty'. Further washing steps removed this fattiness. B) Inside the vat large protein/lipid deposits were evident (circled).

Table 3 indicates that the enzyme-based CIP systems were less effective at removing bacteria from the system than the chemical CIP. The increased number of micro-organisms found on the Teflon surfaces is expected because of the differences in the surfaces of the Teflon, steel and Pyrex. The Teflon surfaces are porous when compared to the steel and Pyrex surfaces. Bacteria are known to colonize porous material and grow. This provides the micro-organisms with a sheltered environment that leads to an increased resistance of organisms on these surfaces to the peroxide-based sanitizer.

Table 3 A comparison of the microbial counts obtained off the different surfaces in the 'mini dairy' after the different CIP procedures as a measure of the CIP cleaning efficacy. All counts given in microorganisms/cm^2.

Incubation conditions	**Total Counts** **37°C overnight**	**Coliforms** **37°C overnight**	***E.coli*** **44°C overnight**
Chemical CIP			
Steel	<1	<1	<1
Teflon	<1	<1	<1
Glass	<1	<1	<1
Commercial enzyme CIP			
Steel	<1	<1	<1
Teflon	157	8	2
Glass	<1	<1	<1
Enzyme CIP (counts for one run)			
Steel	1	<1	<1
Teflon	2	<1	<1
Glass	4	<1	<1

Table 4 shows the counts obtained for four of the five lipase/protease washes. There was a definite increase in the microbial numbers, probably due to the formation of a biofilm. This was especially evident because the highest microbial numbers occurred on the Teflon surfaces that are more prone to biofilm formation because the surface provides a better substrate for attachment as mentioned previously. Another reason for the higher microbial numbers on Teflon is that the O-rings are not open to the flow of cleaning solutions and are, therefore, not cleaned as effectively. A suggestion here would be that the system is opened up periodically and the Teflon O-rings well cleaned.

Table 4 Microbial counts from successive lipase/protease CIP washes. Nutrient agar plates from total counts and the McConkey agar plates for the coliform counts were incubated at 37°C overnight and the McConkey agar plates for the *E.coli* counts were incubated at 44°C overnight.

Surface	Run			
	1	**2**	**3**	**5**
Total Counts				
Steel	<1	2	2	<1
Teflon	<1	4	7	283
Glass	<1	<1	2	40
Coliform counts				
Steel	<1	<1	<1	<1
Teflon	<1	<1	<1	80
Glass	<1	<1	<1	<1
***E.coli* counts**				
Steel	<1	<1	<1	<1
Teflon	<1	<1	<1	<1
Glass	<1	<1	<1	<1

Figure 4 exhibits a stainless steel disk which has just been cleaned and is representative of what all the SEM disks look like prior to fouling and cleaning in the system. The striations visible on the surface are due to the method of polishing employed.

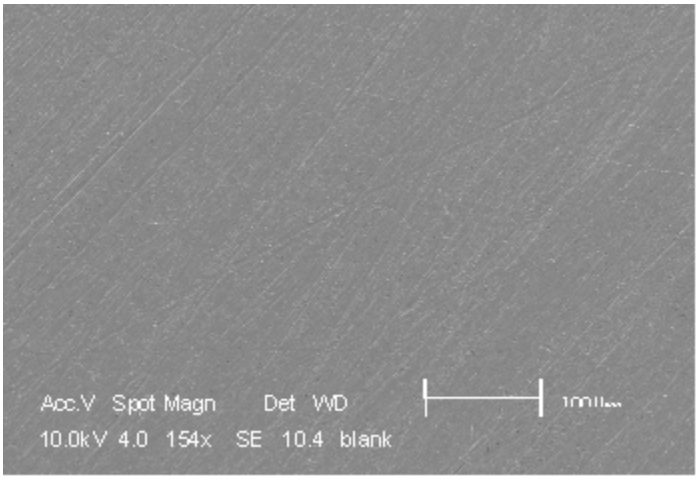


Figure 4 The blank disk that has just been cleaned according to section 2.4.2, used to determine amount of fouling and cleaning of disks.

Figure 5 exhibits the SEM micrographs of a chemical CIP run. A definite progression in cleaning can be seen from these. Figure 5(a) clearly shows the deposit left by the milk after the first rinse in the cleaning cycle. The deposit is seen as white flecks on the surface of the metal. A close-up of the disk removed after the first rinse (Figure 5(b)) gives an idea as to what the protein and lipid deposits look like. The cracks evident in the deposit are due to the fixing and dehydration process. Figure 5(c) depicts the disk removed after the NaOH step of the CIP process, where a decrease in deposit is noticeable. The disk shown in Figure 5(d) was obtained after the HNO_3 step. It looks very much like the disk obtained after the NaOH step. This is probably because the acid step is not very effective in removing protein and lipid deposits. Its primary function is to remove mineral deposits if present; therefore, any protein or lipid deposit remaining after the NaOH step will not be removed to any noticeable degree. The disk taken obtained after the sanitizing step (Figure 5(e)) looks clean, when compared to Figure 5(a) and Figure 4, with little deposit noticeable.

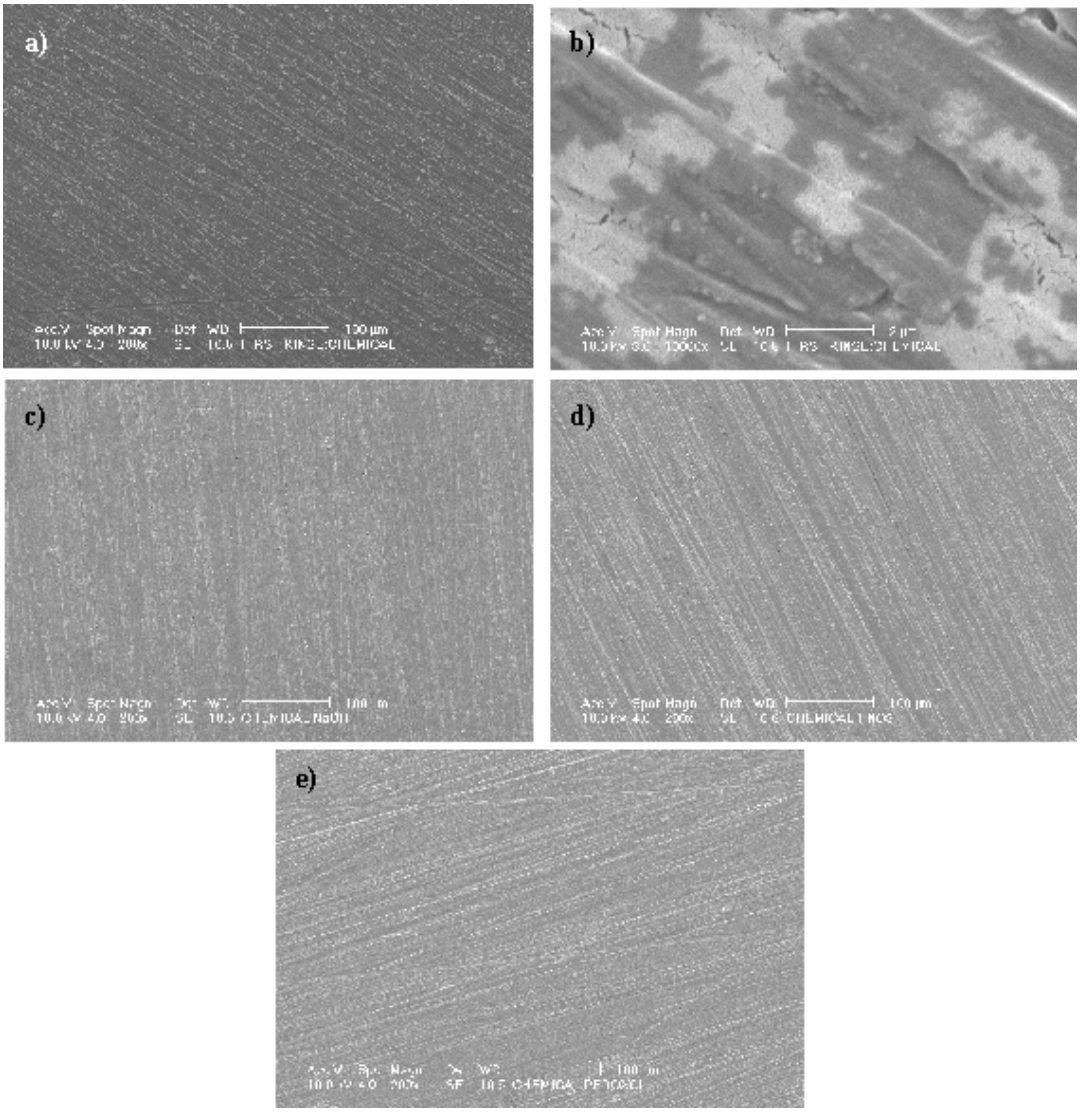


Figure 5 **Chemical CIP.** **a)** This micrograph represents a disk after the first rinse of the cleaning cycle. **b)** A close-up of the deposit seen in (a). **c)** Micrograph taken after the NaOH step. **d)** Micrograph taken after the HNO_3 step. **e)** Micrograph taken after the sanitizing step. The disk looks clean with little deposit noticeable.

Figure 5a represents the disk removed from the system after the first rinse. Clear fouling of the metal is evident by the white flecks on the steel. The striations visible on the steel surface are due to the polishing technique used in preparation of the disk. The lipase/protease CIP system reflects a clear progression of cleaning through the different steps. Figure 6(a) indicates a definite removal of deposit from the steel disks when compared to the disk

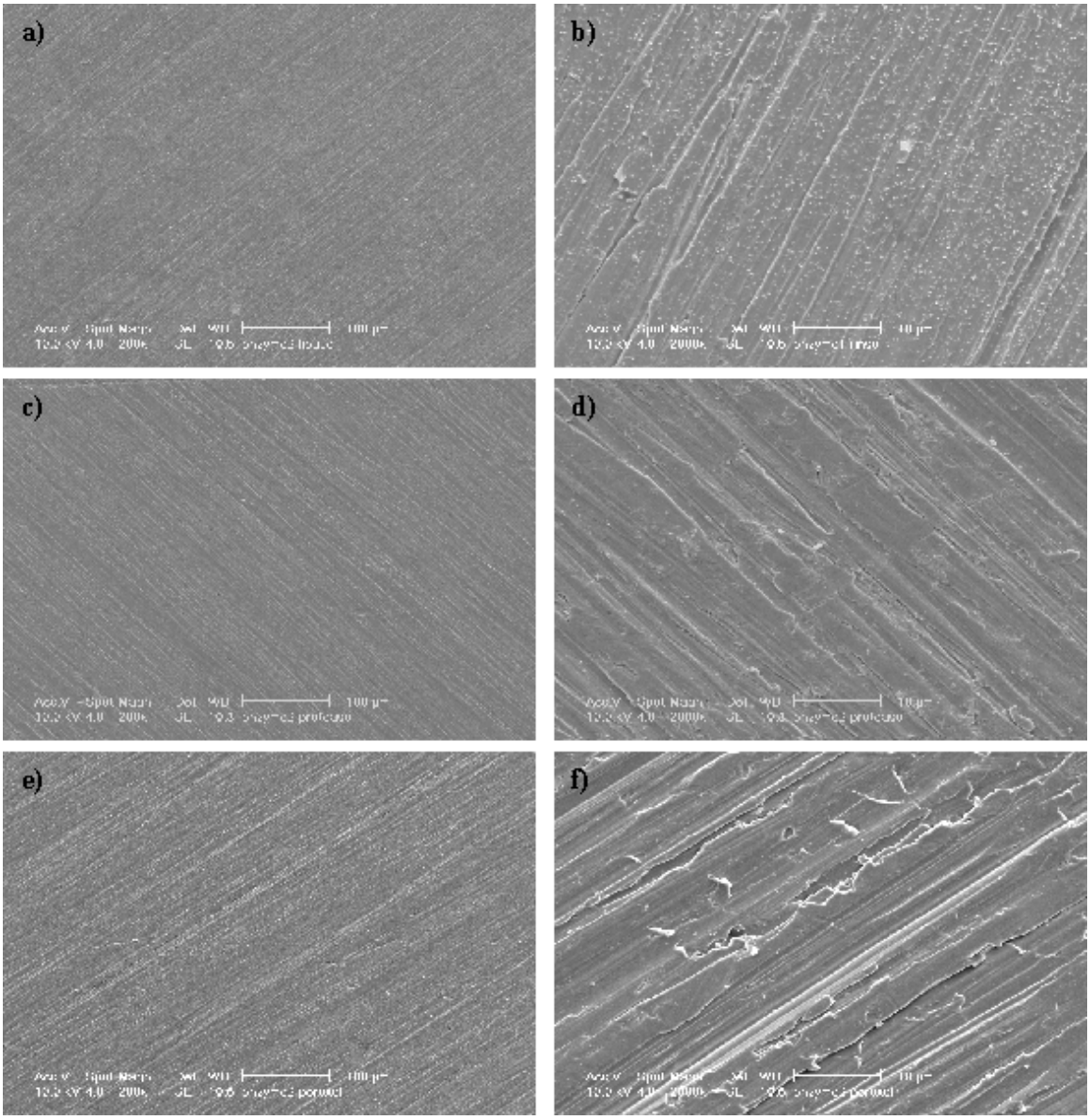


Figure 6 **Lipase/protease CIP.**
a + b) Micrographs of the disk removed after the lipase cleaning step. (b) is a 10x magnification of (a).
c + d) Micrograph of the disk removed after the protease cleaning step. (d) is a 10x magnification of (c).
e + f) Micrographs taken of a disk removed after the sanitizing step of the lipase/protease CIP. (f) is a 10x magnification of (d).

obtained after the first rinse (Figure 5a). The disks obtained from the lipase (Figure 6(a)) and protease (Figure 6(c)) cleaning steps were very similar. When studied under a higher magnification, however, it was observed that the protease step (Figure 6(e)) had removed a residual deposit left by the lipase step (Figure 6(b)).

3.2.1 EFFLUENT ANALYSIS

Analyses of the effluent produced after cleaning with the individual CIP systems indicated that the effluent from the biological CIP was of a better quality than that of the conventional CIP.

The pH comparisons (Figure 7) of the different systems show that the lipase/protease CIP system effluent stays roughly around neutral, except for the sanitizing step. This is because the sanitizing step contains a hydroxide-based sanitizing acid that lowers the pH. The hydroxide does, however, break down relatively quickly into water and hydrogen at the concentrations used. The pH's of the chemical and commercial enzyme-based CIP change from step to step. The end result of the effluent (composite sample) does, however, show that the chemical CIP (pH 11.65) is just below the upper limit accepted by Port Elizabeth Municipality (pH 12) while the commercial enzyme-based CIP effluent falls below the lower limit of pH 6. Both these pH values are legally unacceptable.

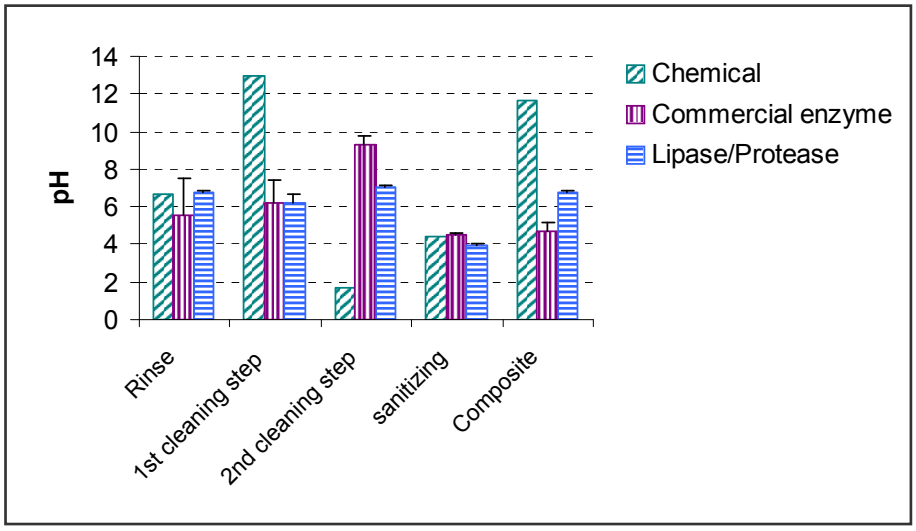


Figure 7 A comparison of the pH of the different CIP systems. Composite sample also included.

Figure 8 compares the COD levels of the different CIP systems. The COD levels seen in first rinse are dependent on the extent to which the milk was removed from the system prior to rinsing the first time. The protease step in the lipase/protease CIP system had a high COD when compared to the rest of the steps of the system. This could have been due to the unpurified state of the protease. This will be discussed later. The most important sample to note is the composite sample COD's from the different CIP systems. The COD's for the composite effluent samples were comparable and below the value set by the Port Elizabeth Municipality.

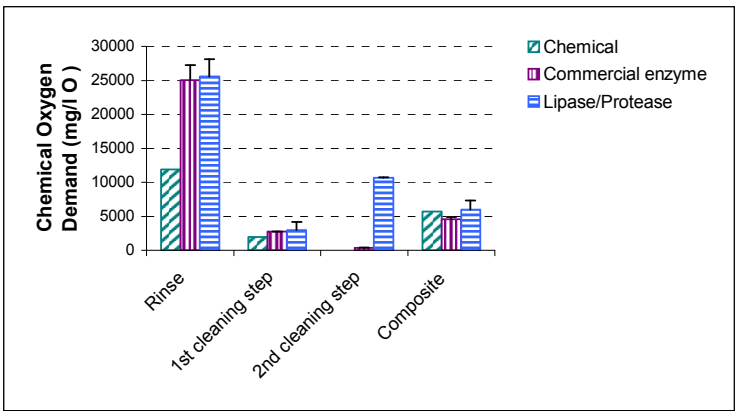


Figure 8 COD level comparison of the different CIP systems. The composite sample results are included.

The graph in Figure 9 shows a very high relative concentration of potassium in the lipase/protease CIP, especially from the protease step. This could be as a result of the M9 media used to grow the *S.liquifaciens* used to produce the protease. The commercial enzyme-based CIP system yielded a high potassium concentration derived from the alkali-cleaning step. Composite levels of potassium in the chemical and commercial enzyme-based CIP systems where both remarkably lower than the levels seen in the lipase/protease CIP system.

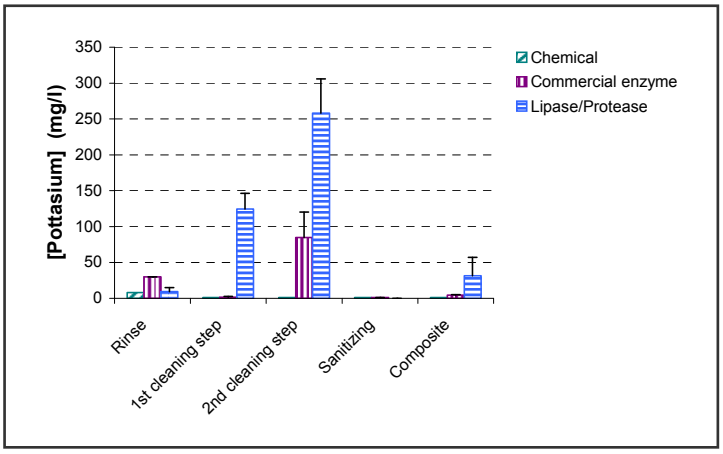


Figure 9 Comparison of the different potassium levels obtained after each of the main washing steps in the different CIP systems.

In Figure 10 the commercial enzyme-based CIP effluent is not shown. This is because this CIP system had no detectable levels of nitrates. The other two systems did, however, exhibit noticeable levels of nitrates, but originating from different steps. The nitrate levels in the chemical CIP was due to the acid wash (HNO_3), while in the lipase/protease system it originated in the lipase step. The composite nitrate levels of both the chemical and the lipase/protease CIP systems were roughly equal.

Figure 10 Comparison of the different nitrate levels obtained after each of the main washing steps in the different CIP systems.

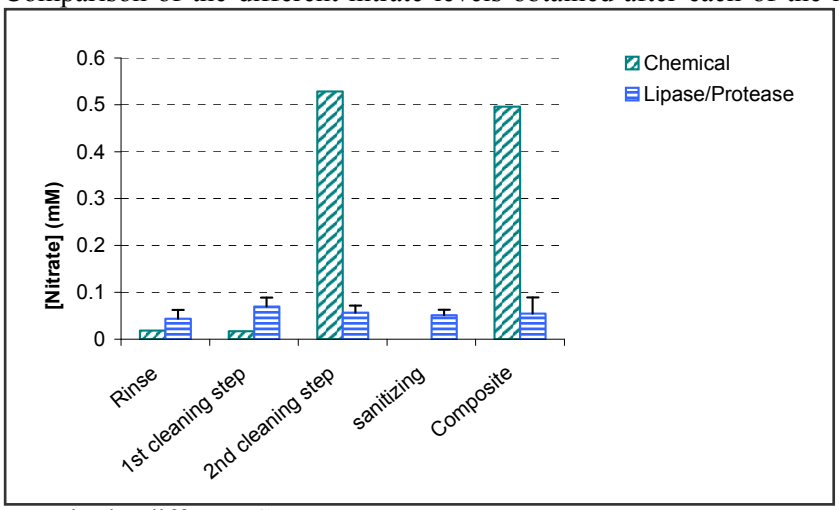


Figure 11 compares sodium levels of the different effluents. Here the chemical CIP exhibits the highest concentration of sodium because of the NaOH step in the CIP procedure. The high level of sodium present in the lipase/protease CIP effluent is because of the M9 media used to grow the *S.liquifaciens*. The commercial enzyme-based CIP exhibited a continually low level of sodium levels throughout all steps of cleaning.

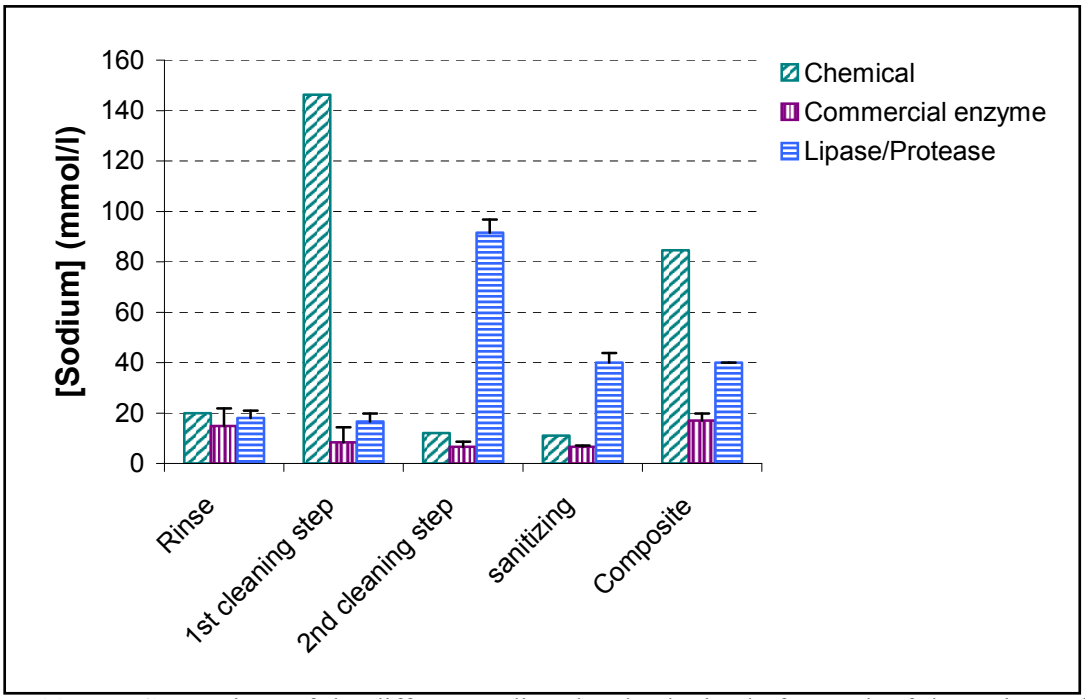


Figure 11 Comparison of the different sodium levels obtained after each of the main washing steps in the different CIP systems.

The composite results are summarised in Table 5. The overall results (based mainly on the composite samples) indicate that the commercial enzyme-based CIP exhibits a 'cleaner' more acceptable effluent than the other CIP systems. However, if it is considered that the protease obtained from *S.liquifaciens* is not purified, but used in the growth media minus the cells, the results can be looked at in a slightly different light

Table 5 A comparison of the composite effluent samples obtained from the different CIP systems tested.

Test	Chemical CIP	Commercial Enzyme CIP	Lipase/protease CIP
pH	11.65	4.665	6.782
Hardness (°d)	214	62	103.4
COD (mg/l O_2)	5700	4540	5980
Pottasium mg/l)	<2	<2	31.4
Nitrates (mmol)	0.496	0	0.542
TDS	3.77ppt	247ppm	731
Calcium (mmol)	0.44	0.75	0.916
Sodium mmol/l)	84.6	9.9	40

Table 6 gives the ingredients of M9 while Table 7 gives the levels of the different parameters tested on the effluent that fresh sterile M9 contains. These values give an indication as to why the TDS, calcium, COD and sodium levels of the lipase/protease CIP system are higher than those obtained with the commercial enzyme-based CIP.

Table 6 The components of the M9 used to grow up *S.liquifaciens*. The M9 was supplemented with casein hydrolysate and glucose.

Ingredient	Amount (g/l)	Parameter affected
$Na_2HPO_4.12H_20$	16.896	Sodium concentration
KH_2PO_4	3	Potassium concentration
NaCl	0.5	Sodium concentration
NH_4Cl	1	
$MgSO_4$	0.492	
$CaCl_2$	0.0147	Calcium concentration
Sodium succinate	0.2	Sodium concentration
Casein Hydrolysate	0.5	COD levels
Glucose	1	COD levels

Table 7 The values of different parameters tested for in fresh sterile M9. These are the same as tested for in the effluent.

Test	Level	Concentration in final composite effluent sample (12x dilution)
PH	7.17	
Water hardness	232 mg $CaCO_3$/l	19.33 mg $CaCO_3$/l
COD	15 020 mg O_2/l	1215.67 mg O_2/l
Potassium	415 mg/l	34.58 mg/l
Nitrates	0.118 mM	0.0098 mM
Calcium	1.5 mmol/l	0.125 mmol/l
Sodium	191 mmol/l	15.92 mmol/l

3.2.2 WASTEWATER MANAGEMENT

The investigation into effluent developed the following wastewater management ideas, which were later implemented at a major South African dairy (Table 8).

Table 8 Elements of a Successful Water and Wastewater Management Program

• Obtain full management commitment and understanding.
• Appoint a water-waste supervisor.
• Survey waste production in the plant.
• Survey water use in the plant.
• Conduct a management meeting to implement the programme.
• Train employees.
• Solicit ideas from employees.
• Monitor performance and maintain records.
• Implement the best ideas immediately; if suggestions will not be implemented right away or are rejected, let the employees know the reason.
• Ensure continued commitment of all employees.

A product waste study should be conducted after completion of the water use survey as product waste has a direct influence on effluent quality. The water-waste supervisor and each production supervisor should observe operations closely and list all the waste that occurs. Photographs of leaks, spills, and other situations in which waste occurs should be taken to make slides for use in employee training. 500g of pollutants, in the form of Biological Oxygen Demand (BOD), is directly equivalent to 4.5l of milk lost down the drain. If the BOD level in a plant's wastewater is known, an accurate idea of how much product is being lost into the drain can be obtained.

A plant's waste load can be decreased substantially by controlling the amount of water used and reducing the amount of product lost into the sewerage/storm water system. Stopping pollution at its source is less expensive and more practical than end-of-pipe waste treatment. Wastewater from most dairy plants is discharged to publicly owned treatment works where the majority of the pollutants are removed before the water is discharged to the environment. Treating the water is costly, and most treatment works charge according to the volume of sewage treated. In addition, they commonly apply a surcharge if the waste load exceeds certain specified levels because it costs more to treat water that contains more pollutants.

A water-waste program can result in daily savings and a minimal amount of waste. The full co-operation of the company's employees in reducing waste and water must be procured in order to ensure an efficient operation - and money going into the bank rather than down the drain.

3.2.3 COST IMPLICATIONS

The effective use of detergents requires the use of the correct materials, at the correct concentration, applied in the correct sequence at the required time. Cost-effectiveness

requires this to be carried out with minimal waste of detergents and minimal output of effluent. Whilst CIP installations are a capital investment the chemical used is presently chosen specifically on product cost, product performance and supplier service levels. However, one cannot rely solely on the cost of the detergent to determine whether the CIP is cost-effective or not. The concept of "use-cost" or "cost-of-clean" must be applied when considering the cost implications of a CIP system. Any such calculation must take into account the cost per litre of product, the dilution rate, the performance, power and water consumption, cost of staff, down time of equipment for cleaning and effluent costs.

At present a major dairy will spend between R80 000 and R100 000 per month on cleaning chemicals alone. When one adds the costs of water, effluent and energy the cost rises by over 25% to R100 000 to R125 000 per month. This cost does not include the penalties charged on effluent that does not conform to legislative requirements. These amounts can be broken down as percentages as follows (Figure 12).

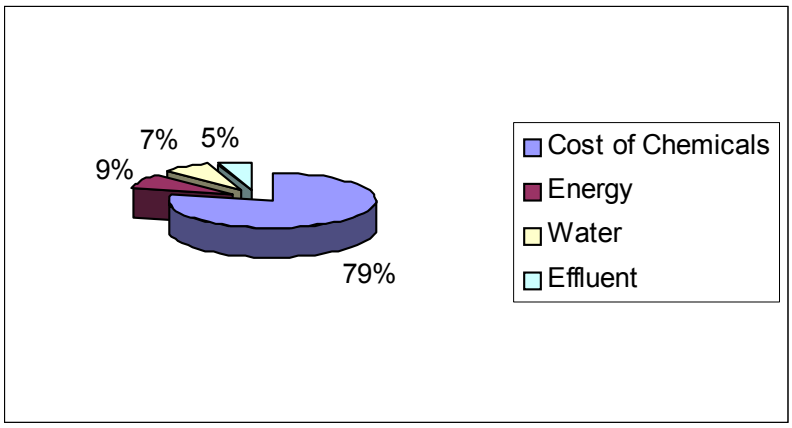


Figure 12 Distribution of CIP costs at a major South African Dairy.

If one includes the effluent penalties, the percentages change as indicated in Figure 13. It must be understood that for the protection of the plant the exact amount is not mentioned. However, penalties may reach levels of up to R100 000 per month with up to R160 000 having been reported. Using the data from the investigation into the use of an enzymatically-based CIP programme, the costs can be reduced quite dramatically.

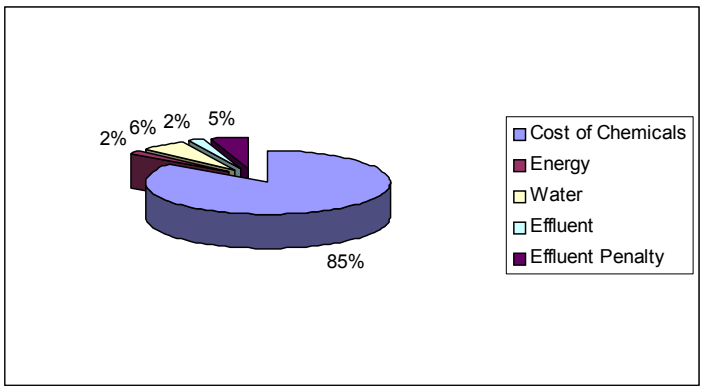


Figure 13 Distribution of the current CIP costs (Figure 12) including the effluent penalties.

The chemical costs would decrease marginally by up to 7%, whilst the energy and effluent costs could be halved. Effluent penalties related to CIP would be reduced by nearly 90%. Effluent costs due to product disposal would not be affected. The breakdown of these percentages is shown in Figure 14.

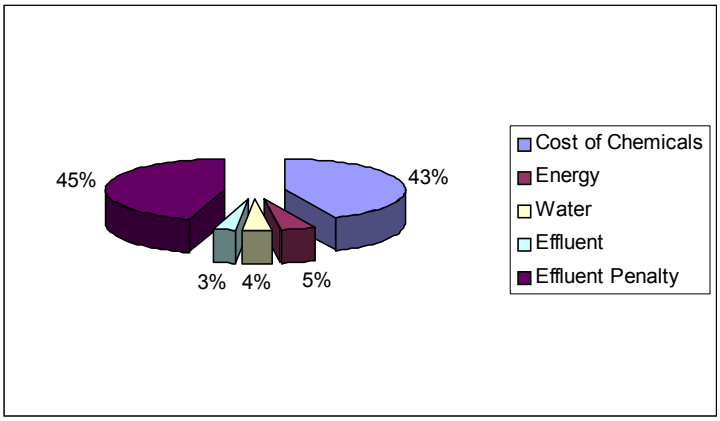


Figure 14 Distribution of the CIP costs including the effluent penalties when using an enzymatically-based CIP system.

The results indicate that an enzymatically-based CIP programme could potentially have marked impacts on the cost-of-clean of South African dairies.

4. CONCLUSION

The aim of this study was to develop a CIP system based on lipases and proteases, which would result in a more acceptable effluent being discharged while still providing effective cleaning for all surfaces in the dairy industry in South Africa. The two enzyme lipase/protease CIP systems proposed and investigated in this study produced an equivalent clean whilst producing a more acceptable effluent when compared to the current chemical CIP practices. Areas that still need to be addressed are 1) the purification of the protease to further improve the effluent obtained and 2) to increase the activity of protease used in the CIP procedure. By increasing the protease activity present, the system may become even more comparable to the chemical CIP in cleaning efficacy. Further research into these areas would benefit industry because if this system is shown to be effective it would save the companies money in both cleaning and effluent costs. This would be due to the decrease in or elimination of fines paid on effluent discharged into the sewer and a decrease in energy expenditure due to the lower cleaning temperatures used.

The primary user group for the products envisaged from this project are the small and medium sized dairy processors in South Africa. The large multi-national dairy companies should rather be viewed as potential innovators of this process and potential financial supporters of the work. Whilst academic institutions may be interested in the project from a enzymological perspective, the research was meant to be application-based. Complete characterization of the proteases used in the study could, however, lead to further capacity building of the scientist base required in South Africa.

The technology developed and the information collected throughout the duration of this project should be made available to dairy processors in South Africa as soon as possible. A suggestion would be to set up a series of technology transfer seminars with the aid of Ecolab South Africa who have been involved in this project from its inception. The researchers involved in the project should then describe the benefits of water management and CIP management to all dairy producers. The potential of this work should also be highlighted but should really be brought to the attention of the major role players in the industry. By stimulating the interest of Clover-Danone and Parmalat, this work could be brought to completion and the technology implemented in South Africa in the foreseeable future. The seminars could be linked to one-day workshops in which dairy processors are taught about the different chemical types currently available to them, basic chemistry of their application, their use and abuse, development of water management programmes and the need for these programmes in light of the current South African legislation.

The seminars should be held in the major dairy centres in South Africa, namely Cape Town-Stellenbosch, Johannesburg, Bloemfontein, Durban and Port-Elizabeth/Tsitsikama. The costs of such a series of seminars can be broken down into four aspects: 1) Travel and subsistence for the presenters; 2) consulting fees for the presenters; 3) course material to be developed and printed; and 4) the cost of the venues for the seminars. As the seminars are meant to be technology transfer especially for the small and medium sized dairy processors, attendees should not have to pay for their attendance. The expenses that would amount to R90 000 could potentially be reduced if venues for the seminars and workshops can be found for minimal costs. The funding, which was released for this project and has to date not been utilized, could be used in this technology transfer process, but would have to be supplemented. Further funding could be requested from Ecolab as these seminars could be used as advertising for the company.

The research project, which was run in part in the Department of Biochemistry and Microbiology at University of Port Elizabeth, was able to enhance the development of

Microbiology, a newly-established discipline in the department, which evolved due to the need for the discipline in the Eastern Cape Province. The department currently has more than 50% non-white students which is also reflected in its postgraduate students where the percentage of non-white students is also more than 50%. The department is closely associated with the PE Technikon where it is assisting in the capacity building of staff. The project allowed the department to train postgraduate students in the newly-established discipline of Microbiology beyond the level of Honours, which will contributed to building capacity of the student body which to date has not been able to study the discipline of Microbiology in Port Elizabeth. The project involved the development of four post-graduate students of which two were from designated groups. Furthermore, the project involved unskilled and semi-skilled labour at three dairies in the Eastern Cape Province who underwent skills development in CIP, water management and effluent management.

Considering the efficacy of the two enzyme CIP in cleaning stainless steel in dairies, this research could now be expanded to be further developed for the **fruit juice**, **brewing** and **soft drinks** markets. The technology could potentially also be used in the **bottled water** industry, which is growing rapidly in South Africa. Finally, the technology should not be localized to cleaning in place applications. Large **open-area-cleaning** could also be attempted with multi-enzyme cleaners. In all cases the primary soils would have to be identified and a screening programme for biocatalysts able to degrade the soils would have to be run. Enzymes capable of degrading these soils may be available commercially, thereby decreasing the time required to investigate the efficacy of the systems *in-situ.*

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