National Development Plan 2007 - 2013

Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

# STRIVE
## Report Series No.51

# Minimisation of Hazardous Waste Generated by Cleaning-in-Place Operations in the Dairy Processing Industry

STRIVE

Environmental Protection Agency Programme

2007-2013

SSTI
Strategy for Science, Technology and Innovation

Comhshaol, Oidhreacht agus Rialtas Áitiúil
**Environment, Heritage and Local Government**

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The Environmental Protection Agency (EPA) is a statutory body responsible for protecting the environment in Ireland. We regulate and police activities that might otherwise cause pollution. We ensure there is solid information on environmental trends so that necessary actions are taken. Our priorities are protecting the Irish environment and ensuring that development is sustainable.

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**EPA STRIVE Programme 2007–2013**

# Minimisation of Hazardous Waste Generated by Cleaning-in-Place Operations in the Dairy Processing Industry

## (2007-FS-ET-2-M5)

## STRIVE Report

Prepared for the Environmental Protection Agency

by

University of Limerick

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## DISCLAIMER



The EPA STRIVE Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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# Executive Summary

In dairy processing, effective cleaning of process equipment is essential for efficient plant operation and for the production of safe, quality dairy products. Such cleaning is generally achieved using sodium hydroxide (NaOH), caustic formulated detergents and acid at temperatures of up to 70–80°C. As such, cleaning has a negative impact on the environment. Cleaning has been reported to account for 30% of the energy use in dairy processing (Eide et al., 2003[1]) and is the largest contributor to overall wastewater volume in many plants (Danalewich et al., 1998[2]). Detergents represent the greatest proportion of chemicals used in dairy plants (Wildbrett, 2003[3]) and some of the chemicals used have adverse effects on human and ecosystem health and on the environment (Fryer et al., 2006[4]). Enzymes, which are biodegradable, have been exploited for cleaning in various other sectors, most notably in laundry and automatic dishwasher detergents. Reported benefits include reduced usage of chemicals, reduced energy consumption due to milder operating conditions and reduced rinsing volumes, resulting in reduced water consumption and reduced generation of waste. Similar benefits could potentially be achieved by using enzymes for cleaning-in-place (CIP) in dairy processing. However, with the exception of cleaning membranes and cold milk areas of dairy plants, little attention has been given to the widespread application of enzymes for this purpose, particularly for cleaning heated components where significant and problematic fouling occurs. The main objective of this study was to determine if enzymes display realistic potential to replace (either partially or entirely) currently used environmentally significant CIP chemicals and, hence, reduce the overall environmental impact of CIP in dairy processing.

Contact was established with several dairy co-ops and companies supplying cleaning chemicals to the dairy industry in order to establish details of how CIP is currently undertaken. Eight commercial protease products were evaluated on a laboratory scale by quantitatively assessing their ability to remove a milk fouling deposit, representative of that occurring on heated components, from stainless steel. Cleaning was carried out at 40°C, 50°C and 60°C for 1 h at the optimum pH determined for each enzyme under application-relevant conditions and compared with the cleaning performance of currently used CIP procedures. While satisfactory enzyme cleaning was observed at all three temperatures, cleaning at 40°C was most favourable in terms of enzyme stability and energy use. Following cleaning at 40°C, visibly clean surfaces were observed for six of the eight enzymes tested and the cleaning performance of four of the proteases (as judged by detection of residual organic matter and protein after cleaning) was comparable to that of 1% NaOH, the most commonly used CIP agent, at 60°C. The optimum product concentration for cleaning was determined for each enzyme and the three most suitable products for potential CIP application were identified, based on cleaning performance, enzyme stability and cost. Further assessments of these three products were undertaken, including confirmation of cleaning by confocal laser scanning microscopy. Initial cost analysis indicates that the cost of these enzyme cleaning solutions is unlikely to prohibit their use in CIP applications as the cost of two of these enzyme-based cleaning solutions is comparable to the average costs of cleaning solutions based on 1.5% NaOH and formulated detergents and, in addition, enzyme-based cleaning is likely to reduce energy costs. Laboratory-based studies undertaken confirmed that all enzyme activity is removed/inactivated by procedures normally

1. Eide, M.H., Homleid, J.P. and Mattsson, B., 2003. Life cycle assessment (LCA) of cleaning-in-place processes in dairies. *Lebensmittel-Wissenschaft Und-Technologie* (*Food Science and Technology*) **36:** 303–314.
2. Danalewich, J.R., Papagiannis, T.G., Belyea, R.L., Tumbleson, M.E. and Raskin, L., 1998. Characterization of dairy waste streams, current treatment practices, and potential for biological nutrient removal. *Water Research* **32:** 3555–3568.
3. Wildbrett, G., 2003. Dairy plant effluent. *In:* Roginski, H. (Ed.) *Encyclopedia of Dairy Sciences.* Academic Press, London, UK.
4. Fryer, P.J., Christian, G.K. and Liu, W., 2006. How hygiene happens: physics and chemistry of cleaning. *International Journal of Dairy Technology* **59:** 76–84.

undertaken after cleaning. The inclusion of lipase activity in the protease cleaning solution was also investigated with no improvement in cleaning performance observed under the conditions used. Screening of the in-house collection of micro-organisms was undertaken with the aim of identifying proteases of potential use in CIP operations. Of 35 micro-organisms screened, 14 were found to produce extracellular proteases with activity on denatured whey protein. Based on ability to remove milk fouling deposit from stainless steel, the crude activity produced by the fungus DSM 1024 *Schizophyllum commune* was found to be most suitable for CIP application.

Based on the results of this study it can be concluded that proteases, and in particular the three commercial products identified, are of potential use for CIP in dairy processing. While the laboratory-scale studies undertaken are a useful indicator of potential suitability, pilot- and industrial-scale studies are necessary to fully determine actual industrial applicability and confirm economic feasibility. If similar results are observed on these scales in terms of cleaning performance and cost, then enzyme-based cleaning offers several advantages over the currently used CIP procedures. These include reduced environmental impact as the enzymes operate at lower temperatures, resulting in reduced energy consumption, and are biodegradable in contrast to many of the products currently used for cleaning which have adverse effects on human and ecosystem health and on the environment. Enzyme-based cleaning is also more compatible with subsequent wastewater treatment and would create safer working conditions for plant operators. The three commercial proteases assessed in the study are already produced and sold in bulk by global enzyme manufacturers and could be readily incorporated in CIP operations if found to be suitable for this application.

# 1 Introduction

## 1.1 Overview of the Irish Dairy Industry

The dairy industry makes a substantial contribution to Ireland's food and drink industry. The export of dairy products and ingredients amounts to approximately €2.36 billion annually, with about 85% of Irish dairy products exported. This accounts for about 27% of Irish agri-food and drink exports. In addition, the Irish dairy industry provides employment for 22,000 farmers, 9,000 employees in the processing industry and a further 4,500 employees in support and ancillary services (Bord Bia, 2007; The National Dairy Council, 2008).

There are approximately 1.1 million dairy cows in Ireland (CSO, 2009a) and domestic milk supply is highly seasonal, with 78% of milk supplied from March to September inclusive (National Milk Agency, 2009). Milk intake in 2007 and 2008 amounted to 5,563 and 5,422 million litres, respectively (CSO, 2009b). In 2008, 91% of domestic milk supplies were used to manufacture dairy products, most of which were exported, while the remaining 9% were processed for liquid consumption (National Milk Agency, 2009). In 2008, 520.1 million litres of milk were sold for human consumption (357.3 million litres as whole milk and 162.8 million litres as skimmed and semi-skimmed milk) (CSO, 2009b). In addition to liquid milk, other major product streams include butter, cheese and milk powders. The quantities of various dairy products produced in Ireland in 2007 and 2008 are shown in Table 1.1.

There are 36 dairy co-ops in Ireland (IDB, 2007). These range in size from large global organisations (e.g. Glanbia and Kerry) to very small co-ops that collect milk and sell it to larger co-ops for processing. A strategic development plan for the Irish dairy processing sector, carried out in 2003, reported that eight companies are responsible for processing 90% of the milk pool (Promar International, 2003). The three largest dairy co-ops, Kerry, Glanbia and Dairygold, and other major players are shown in Table 1.2. In 2008,

**Table 1.1. Production of Irish dairy products in 2007 and 2008.[1]**

| Product | Quantity produced ('000 tonnes) | |
|---|---|---|
| | 2007 | 2008 |
| **Cheese** | 127.2 | 163.0 |
| **Butter** | 148.6 | 129.0 |
| **Whole milk powder** | 34.0 | 33.0 |
| **Chocolate crumb** | 44.2 | 40.5 |
| **SMP (incl. BMP)** | 105.1 | 70.0 |
| **Proteins** | 44.0 | 38.0 |

[1]IDB (2009).
SMP, skimmed milk powder; BMP, buttermilk powder.

**Table 1.2. Milk pools of major Irish co-ops.[1]**

| Processor | Estimated milk pool (billion kg) |
|---|---|
| **Glanbia** | 1.5 |
| **Kerry** | 0.93 |
| **Dairygold** | 0.88 |
| **Lakeland** | 0.45 |
| **Arrabawn** | 0.24 |
| **Connacht Gold** | 0.22 |
| **Drinagh** | 0.13 |
| **Tipperary** | 0.10 |
| **Wexford** | 0.10 |
| **Thurles Centenary** | 0.09 |
| **Bandon** | 0.07 |
| **Town of Monaghan** | 0.07 |
| **Lisavaird** | 0.07 |
| **Barrryroe** | 0.063 |
| **Donegal** | 0.054 |
| **North Cork** | 0.036 |
| **Newmarket** | 0.036 |
| **Lee Strand** | 0.022 |
| **Boherbue** | 0.018 |

[1]Kennedy and O'Neill (2009).

there were 19 heat treatment establishments licensed to process milk for liquid consumption, with two processors accounting for 75% of the milk supplies for this purpose (National Milk Agency, 2009). There were an estimated 59 dairy sites in Ireland in 2006 of which 9 were butter plants, 10 were powder plants and 8 were cheese plants (Lascurettes, 2008).

Ireland has a long tradition in dairying and over the years the industry has developed into a challenging environment where quality, innovation and new product development are key deliverables. In 2007, the Department of Agriculture, Food and Fisheries awarded €114 million in grant assistance towards capital projects which aimed to enhance the future competitiveness of the industry through innovation, increased productivity and new product development.

## 1.2 Milk

### *1.2.1 Composition of milk*

Milk is secreted by the female of all mammalian species and its primary natural function is the nutrition of the young of the species (Walstra et al., 2006). Interspecies differences are observed in the composition of milk and within a particular species the exact composition varies depending on the breed, stage of lactation, health and nutritional status of the animal (Fox and McSweeney, 1998; Walstra et al., 2006). The average composition of cows' milk, which is of relevance to the current study, is shown in Table 1.3.

From a physico–chemical perspective, milk is a very complex fluid with its constituents occurring in three phases. Lactose, organic and inorganic salts and vitamins occur in aqueous solution. Proteins are dispersed in this aqueous solution – whey proteins at the molecular level and caseins as large colloidal aggregates of diameter 50–600 nm. Lipids occur in an emulsified state, in the form of globules with diameters of 0.1–20 µm (Fox and McSweeney, 1998). Milk has a pH of around 6.7 at room temperature (Walstra et al., 2006).

### *1.2.2 Major constituents of milk*

The major components of milk are protein, fat and lactose. The protein in milk can be divided into two groups – casein and whey protein. Caseins precipitate out of solution when milk is acidified to pH 4.6 while the whey proteins remain soluble. Casein and whey proteins account for about 80% and 20% of the protein in bovine milk, respectively. Whey protein is also commonly referred to as serum protein or non-casein nitrogen. Casein is heat stable at pH 6.7 but becomes insoluble at temperatures in excess of 120°C due to chemical changes. In contrast, whey proteins are considered to be relatively heat labile (Fox and McSweeney, 1998; Walstra et al., 2006).

Casein does not occur as a globular protein but rather individual casein molecules associate with each other to form roughly spherical casein micelles which also contain calcium phosphate. There are different types

**Table 1.3. Composition of bovine milk.[1]**

| Component | Average content in milk (% w/w) | Range (% w/w) | Average content in dry matter (% w/w) |
|---|---|---|---|
| **Water** | 87.1 | 85.3–88.7 | – |
| **Solids – not fat** | 8.9 | 7.9–10 | – |
| **Fats in dry matter** | 31 | 22–38 | – |
| **Lactose** | 4.6 | 3.8–5.3 | 36 |
| **Fat** | 4.0 | 2.5–5.5 | 31 |
| **Protein** | 3.3 | 2.3–4.4 | 25 |
| **Mineral substances** | 0.7 | 0.57–0.83 | 5.4 |
| **Organic acids** | 0.17 | 0.12–0.21 | 1.3 |
| **Miscellaneous** | 0.15 | – | 1.2 |

[1]Walstra et al. (2006).

of casein – $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein and these occur in a number of variants. β-Casein may be cleaved into γ-casein and proteose peptone by proteolytic enzymes. Some properties of caseins are shown in Table 1.4.

The major whey proteins are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins (Igs) (Table 1.4). β-Lactoglobulin accounts for about 50% of total whey protein and occurs in three main genetic variants, A, B and C. The structure of β-Lg has been well studied. It contains two disulphide bonds and one free sulfhydryl group, which in the native state is buried within the protein structure. Its quaternary structure is pH and temperature dependent. At pH values below 3.5 and greater than 7.5, it exists as a monomer. At pH 3.5–5.5, it associates to form an octomer, while, at 5.5–7.5, it is present as a dimer. If milk is heated at high temperature, the dimer will dissociate. β-Lactoglobulin is reported to bind some apolar molecules such as retinol and fatty acids (Fox and McSweeney, 1998; Walstra et al., 2006).

α-Lactalbumin is a compact globular protein which acts as a coenzyme in the synthesis of lactose. It is a metallo-protein and binds a calcium ion in a pocket containing four Asp residues. This stabilises the protein conformation and removal of the calcium ion or its loosening (which occurs at pH 4) causes the protein to partially unfold into a molten globule state in which it is susceptible to irreversible heat denaturation at relatively low temperatures. In its native state, α-La appears to be more stable than β-Lg and can renature after heat treatment provided that no other proteins were present during heating (Fox and McSweeney, 1998; Walstra et al., 2006).

Bovine serum albumin is found in low levels in bovine milk and its presence is assumed to be due to leakage from blood serum. It is a large molecule composed of three domains and contains 17 disulfide bonds and one cysteine with a free thiol (–SH) group (Walsh, 2002). Immunoglobulins are antibodies synthesised in response to specific antigens and their function is to provide immunological protection to the calf; IgG, IgM and IgA are found in milk. Immunoglobulins are large glycoprotein molecules and are thermolabile in the presence of other whey proteins (Walstra et al., 2006).

Miscellaneous proteins include lactoferrin and various enzymes. Lactoferrin inhibits some bacteria due to the removal of the ferric ion ($Fe^{3+}$) from the serum. Milk contains several enzymes, many of which are

**Table 1.4. Characteristics of major bovine milk proteins.[1]**

| Protein | g/kg milk | Mol. mass | pI | Amino acid residues/molecule | Cysteine residues/molecule | Disulfide linkages/molecule | Glycosylated |
|---|---|---|---|---|---|---|---|
| **Casein** | 26 | | | | | | |
| **$\alpha_{s1}$-Casein** | 10.7 | 23.6 | 4.5 | 199 | 0 | 0 | No |
| **$\alpha_{s2}$-Casein** | 2.8 | 25.2 | 5.0 | 207 | 2 | 1 | No |
| **β-Casein** | 8.6 | 23.9 | 4.8 | 209 | 0 | 0 | No |
| **κ-Casein** | 3.1 | 19.0 | 5.6 | 169 | 2 | | Yes |
| **γ-Casein** | 0.8 | 20.5 | | | | | |
| **Serum proteins** | 6.3 | | | | | | |
| **β-Lactoglobulin** | 3.2 | 18.2 | 5.2 | 162 | 5 | 2 | No |
| **α-Lactalbumin** | 1.2 | 14.2 | 4.3 | 123 | 8 | 4 | No |
| **BSA** | 0.4 | 66.3 | 4.8 | 582 | 35 | 17 | No |
| **Immunoglobulins** | 0.8 | | | | | | Yes |

[1]Walsh (2002); Walstra et al. (2006).
pI, isoelectric point; BSA, bovine serum albumin.

associated with the fat globule membrane. Examples of enzymes found in milk include lactoperoxidase, lysozyme, oxidoreductases, phosphatases, lipolytic enzymes and proteinases (Walstra et al., 2006).

Triacylglycerols, also known as triglycerides, account for 97–98% of the total lipids in bovine milk, as shown in Table 1.5, and as such greatly influence the properties of milk fat. Other lipid components include di- and monoacylglycerols, free fatty acids, cholesterol, phospholipids and trace amounts of cerebrosides and gangliosides. The main phospholipids are phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, and these represent 17–30% and 10% of the total lipid content of skim milk and buttermilk, respectively (Fox and McSweeney, 1998).

**Table 1.5. Lipid composition of bovine milk.[1]**

| Lipid | % Weight of total lipids |
|---|---|
| **Triacylglycerols** | 97.5 |
| **Diacylglycerols** | 0.36 |
| **Monoacylglycerols** | 0.027 |
| **Cholesterol** | 0.31 |
| **Free fatty acids** | 0.027 |
| **Phospholipids** | 0.6 |

[1]Fox and McSweeney (1998).

Milk fat contains some 250 different fatty acid residues, including some with uneven numbers of carbon atoms, branched carbon chains and with keto or hydroxy groups. Milk fat contains a relatively high proportion of short-chain fatty acid residues (4–10 carbon atoms) and a high proportion of saturated fatty acid residues. Oleic acid accounts for about 70% of the unsaturated fatty acid residues present (Walstra et al., 2006). Fat in milk occurs in the form of fat globules which range in diameter from 0.1 to 20 µm, with a mean of about 3.5 µm. Such globules are surrounded by the milk fat globule membrane, which functions to prevent coalescence caused by interfacial tension between the water and lipid phases. The milk fat globule membrane contains proteins (including many enzymes), lipids and trace metals (Fox and McSweeney, 1998).

Lactose is the major carbohydrate found in milk. It is a disaccharide composed of glucose and galactose and is a reducing sugar (Walsh, 2002).

## 1.3 Fouling in Dairy Processing

### *1.3.1 Composition of fouling deposits*

Fouling may be defined as the unwanted build-up of deposits on a surface. While a film of milk will adhere to cold surfaces such as the walls of tanks and pipelines, etc., real or problematic fouling is associated with areas where milk comes in contact with heated surfaces, such as in heat exchangers used for pasteurisation. Such fouling may be classified as Type A or Type B, depending on the temperature at which the deposit is formed. Type B deposits are also commonly referred to as milkstone or scale. Type A and Type B deposits differ in their composition and appearance, as shown in Table 1.6.

**Table 1.6. Composition and appearance of Type A and Type B milk deposits.[1]**

| | Type A | Type B |
|---|---|---|
| **Temperature (°C)** | 75–105 | >100 |
| **Processing condition** | Pasteurisation | Ultra heat treated (UHT) |
| **Composition** | 50–60% protein<br>30–40% mineral<br>4–8% fat | 15–20% protein<br>70–80% mineral<br>4–8% fat |
| **Appearance** | Soft, voluminous, spongy, curd-like material, white or cream in colour | Compact, hard and granular, grey in colour |
| **Layering** | Two layers: a protein-rich outer layer and a thin layer rich in minerals near the heated surface | No distinct layers but protein concentrated near the outside of the deposit |

[1]Jeurnink et al. (1996); Changani et al. (1997); Visser and Jeurnink (1997); Fryer et al. (2006).

The protein in Type A deposits is mainly β-Lg. It has been reported that at least 50% of the protein in a deposit formed at 70–80°C is β-Lg (Fryer et al., 2006). However, at the higher end of the Type A temperature range, more casein may be present (Changani et al., 1997). A Type A deposit may be considered as a spongy protein matrix to which minerals are bound and in which fat globules are embedded (Jeurnink et al., 1996). The proteins found in Type B deposits are mainly β-casein (50%) and $\alpha_{s1}$-casein (27%) (Visser and Jeurnink, 1997).

It is evident upon comparison of Tables 1.3 and 1.6 that the composition of both types of fouling deposit differ significantly from that of milk in terms of casein and whey protein content, mineral content and fat and lactose levels (Jeurnink et al., 1996). Calcium and phosphate account for only 30% of the mineral content of raw milk but represent 90% of the mineral content of milk fouling deposit. Lactose, being water soluble, does not contribute to the fouling deposit except at temperatures greater than 100°C, under which conditions caramelisation or Maillard reactions can occur (Jeurnink et al., 1996; Bansal and Chen, 2006).

Biofilms can also form on the surfaces of dairy processing equipment. They may be considered as aggregations of bacterial cells that grow on a surface to which they are attached (Kulozik, 2003; Bremer et al., 2006). Cell-associated proteins are reported to play an important role in the adhesion of such biofilms (Flint et al., 1999). Micro-organisms may deposit directly onto the surface or may become entrapped in the deposits formed from other biological material, as described above. In the latter case, the fouling deposit may accelerate biofilm formation by providing favourable conditions for the attachment of normally non-adhesive micro-organisms and by providing nutrients for microbial growth (Kulozik, 2003; Bansal and Chen, 2006). Bacteria known to form biofilms in dairy plants include *Bacillus* sp. and thermo-resistant *Streptococci* (Flint et al., 1999; Bremer et al., 2006).

### *1.3.2 Mechanism of fouling*

Fouling in milk processing may be attributed to the denaturation of proteins and the decreasing solubility of milk salts with increasing temperature. The first step in the fouling of dairy processing equipment is the adsorption of a monolayer of proteins. Such adsorption will occur immediately at room temperature, with further fouling only observed at temperatures greater than about 72°C and in the presence of calcium ions. This significant fouling is the result of the heat-induced formation of whey protein aggregates and calcium phosphate particles in the bulk, which are subsequently transported to the heating surface by diffusion where they are deposited (Visser and Jeurnink, 1997). In addition to the physical and chemical changes occurring in the dairy fluid, mass transfer between the fluid and the heated surface is therefore an important step in fouling (Bansal and Chen, 2006).

Milk and milk fluids are generally processed at pH 6–7. In this pH range, β-Lg exists as a dimer. Upon heating to about 50°C, the dimer dissociates into a monomer. Both the monomer and dimer forms of β-Lg adsorb onto stainless steel as a monolayer. This modifies the surface and is likely to provide the basis for further attachment of particles. At temperatures greater than 60–65°C, the β-Lg monomer unfolds exposing the free sulfydryl (–SH) group which was previously buried inside the protein (see Section 1.2.2). This free sulfydryl group enables the molecule to react with other β-Lg molecules, resulting in the formation of aggregates in the bulk of the liquid. These aggregates, which are insoluble in water, may be deposited on the heated surface. Aggregate formation likely proceeds via an intermolecular exchange reaction between the exposed reactive –SH groups and intramolecular disulfide bonds (Visser and Jeurnink, 1997). The formation of larger aggregates is observed in the presence of calcium ions due to reduced electrostatic repulsion (Jeurnink et al., 1996). Calcium ions further promote aggregation of β-Lg molecules by decreasing the denaturation temperature of β-Lg, binding to β-Lg molecules and stabilising aggregates. Calcium ions also enhance deposition by forming bridges between proteins already adsorbed on the surface and aggregates formed in the bulk, and are considered essential for the formation of a protein deposit layer (Changani et al., 1997; Bansal and Chen, 2006).

In addition to forming aggregates as described above, the exposed –SH groups of heat-denatured β-Lg molecules may also bind to (a) protein molecules

already adsorbed on the surface of equipment, (b) κ-casein at the surface of the casein micelle via an S–S bridge, or (c) other milk components, e.g. fat globules and α-La. These particles may subsequently bind to the surface via the attached whey protein molecules, thus incorporating casein and fat, etc., into the fouling deposit.

Mineral deposition may be attributed to the decreased solubility of calcium phosphate salts at higher temperatures. During heating of milk, a calcium phosphate precipitate forms which may associate with the calcium phosphate already present in the casein micelle and/or with the β-Lg aggregates. These calcium phosphate particles may deposit directly onto the heating surface or indirectly via binding to whey protein aggregates (Jeurnink et al., 1996; Visser and Jeurnink, 1997).

Casein micelles may be incorporated into a deposit via interaction with β-Lg as described above. The optimum temperature of the heat-induced interaction between casein and β-Lg is about 90°C (Visser and Jeurnink, 1997). Alternatively, if their colloidal stability is decreased, the incorporation of casein micelles may be attributed to aggregation/coagulation due to high temperature at the heated surface. Colloidal stability of the micelles may be decreased for example by proteolytic action, decreased pH or a change in calcium ion activity (Jeurnink et al., 1996). The amount of casein found in deposits increases with increasing temperature and deposits rich in casein are formed at temperatures greater than 100°C (Visser and Jeurnink, 1997).

Fat accounts for 4–8% of both types of deposits as shown in Table 1.6. Fat globules are most likely entrapped in such deposits by interaction of the fat globule membrane constituents with whey proteins. In the case of homogenised milk, the surface layers of fat globules are altered and are composed mainly of micellar casein and some native and denatured whey proteins. Such globules are incorporated more readily into the deposit, resulting in a fat content of up to 40%.

Analysis of fouling deposits formed after a period of time reveals the presence of a mineral layer near the surface, as described in Section 1.3.1. Although there is some debate in the literature, it is generally considered that mainly proteins form the first deposit layer and that over time minerals diffuse through the deposit to the surface (Bansal and Chen, 2006).

The influence of protein denaturation on fouling is underlined by the fact that very little deposit (and one of a predominantly mineral composition) is obtained when milk is preheated at 80–85°C for 5–10 min. This is attributed to the fact that the whey proteins are already denatured (Visser and Jeurnink, 1997). The thermal properties of β-Lg are affected by various factors, including pH and the presence of other components such as calcium ions (as discussed above), lactose and casein. Thermal stability is enhanced in the presence of lactose and the thermal properties of β-Lg are also affected by interaction of the protein with small molecules, such as retinol and fatty acids, at a hydrophobic pocket on the protein (Visser and Jeurnink, 1997). Heating at temperatures in excess of 85°C results in the formation of β-Lg aggregates which are so large that they are entrained in the product flow and are no longer deposited on the surface of equipment. The deposition of calcium phosphate occurs faster at higher temperatures, explaining the formation of milkstone (Type B deposit) under such conditions.

### *1.3.3 Factors affecting fouling*

As evident from the previous section, fouling is strongly dependent on the composition of the milk/dairy fluid being processed. As a result, natural seasonal and lactational variations in milk composition can affect fouling (Changani et al., 1997). It has been reported that holding milk at 4°C for up to 24 h before processing results in less fouling while prolonged storage is associated with enhanced fouling. Reconstituted milk results in less fouling due to its decreased calcium concentration and also about 25% of the β-Lg is already denatured during production of the milk powder (Changani et al., 1997; Bansal and Chen, 2006). However, recombined milk containing emulsified milk fat is associated with severe (fat-rich) fouling due to the altered fat globule membrane (Jeurnink et al., 1996). Sweet whey (obtained by rennet-mediated coagulation of casein) results in more fouling than milk. This is attributed to the absence of casein which results in increased transport of whey protein aggregates to the surface and increased

association of calcium ions with the whey proteins. Acid whey (obtained after isoelectric precipitation of casein) results in more fouling than sweet whey as its pH is near the isoelectric point of the whey proteins, resulting in reduced electrostatic repulsion and enhanced 'sticking' onto the deposit (Jeurnink et al., 1996). Fouling due to unhomogenised cream is similar to that of whole milk.

Temperature (the absolute temperature, the surface temperature and the temperature difference between the heating surface and the fluid being heated) is a critical factor in fouling. Velocity/Turbulence is also important with increased velocity associated with reduced fouling. This may be due to reduced deposition of foulant and/or deposit re-entrainment (Bansal and Chen, 2006). The characteristics of the heating surface, for example the surface coating/ finishing, surface charge, surface energy and surface microstructure, may also affect fouling (Visser and Jeurnink, 1997).

Enhanced fouling has been reported in the presence of air bubbles (Changani et al., 1997). The presence of dissolved air results in air bubbles when milk is heated. Fouling is significantly increased if such air bubbles are formed at the heated surface as they act as nuclei for deposit formation. These bubbles are stabilised by casein micelles at their gas–liquid interface and may cause localised overheating of the surface. Such overheating causes the adhering bubbles to collapse and hence additional deposition of the stabilising casein micelles onto the heated surface (Visser and Jeurnink, 1997).

### *1.3.4 Impact of fouling*

Fouling causes reduced flow rates and reduced fluxes (in membrane processes). Fouling of heat exchangers decreases heat transfer efficiency and increases pressure drop. Overall, this results in reduced production capability and efficiency within the processing plant. Product quality may be adversely affected if, due to fouling, the milk/dairy fluid cannot be heated to the temperature required for pasteurisation/ sterilisation. In addition fouling deposits may become dislodged during processing causing product contamination (Bansal and Chen, 2006). Fouling deposits also provide sites for the attachment and multiplication of bacteria, as described in Section 1.3.1. Biofilm formation is of major concern to dairy manufacturers, as established biofilms are difficult to eliminate and can contaminate other surfaces as well as product, thereby affecting the quality, functionality and safety of the products produced (Kulozik, 2003; Bremer et al., 2006). In addition, biofilms may contribute to reduced heat transfer and flow rates and deterioration of process equipment (Kulozik, 2003).

In dairy processing, costs associated with fouling include additional energy and equipment, lost productivity, manpower, chemicals and environmental impact. It is reported that about 80% of the total production costs in the dairy industry can be attributed to fouling and cleaning of processing equipment (Bansal and Chen, 2006).

## 1.4 Cleaning-in-Place (CIP) in Dairy Processing

### *1.4.1 Overview of CIP*

Cleaning-in-place may be defined as the circulation of cleaning (and sanitising) liquids through the milk-handling equipment under conditions of increased turbulence and flow velocity. In the case of components with a large cross-section such as tanks, CIP involves spraying or jetting of the internal surfaces with cleaning solution, using specially designed spray devices. In this way, cleaning of pipes, storage tanks, heat exchangers, etc., is achieved without dismantling or opening the equipment and with little or no manual labour. CIP is economical relative to manual cleaning and reduces downtime, hence increasing the time available for productive use of the equipment (Bylund, 1995; Reinemann, 2003; Bremer et al., 2006).

The exact CIP procedure employed depends on a number of factors, as described in Section 1.4.3. The CIP system used must be validated to ensure consistent and acceptable cleaning. This involves demonstrating that cleaning, according to a specified standard operating procedure, will bring about the desired level of cleanliness, including removal of cleaning agents, in a reproducible manner (Chisti, 2000). Equipment design is very important for effective cleaning. Equipment should be installed such that it can be efficiently drained and that all product contact surfaces are freely accessible to the cleaning solutions

with no dead ends or pockets, etc. Surfaces should be smooth and free of crevices and pits. Materials used in the process equipment must be able to withstand the conditions and cleaning agents used. Stainless steel (Grade 304 or 316) is the preferred construction material in dairy processing (Bylund, 1995; Chisti, 2000).

In CIP operations, cleaning is achieved through a combination of chemical, thermal and mechanical actions (Reinemann, 2003). Most of the cleaning action is due to the chemical agents used. Thermal energy is provided by heating the cleaning solution prior to circulation and in some cases heat is also added by heat exchangers in the cleaning circuit. Mechanical action is provided by the turbulent flow conditions employed or by spraying solutions onto surfaces (Chisti, 2000; Reinemann, 2003). Chemical concentrations are often increased if mechanical or thermal action is limited (Reinemann, 2003). Although the activity of cleaning agents generally increases with increasing temperature, high temperatures are also associated with increased protein denaturation and mineral deposition and may also cause volatilisation of some chemical constituents, reducing their cleaning effect. Time is also an important parameter in CIP.

### *1.4.2 Design of CIP system*

A CIP system consists of all the equipment required for the storage, distribution and monitoring of cleaning solutions, including piping, tanks and reservoirs, heat exchangers, spray heads, various flow management devices and a programmable control unit (Chisti, 2000; Eide et al., 2003). Dairy plants are often divided for the purposes of cleaning into a number of circuits which can be cleaned at different times. The components of each circuit must be available for cleaning at the same time and contain the same type of fouling deposit such that the same cleaning agents and conditions can be used.

Cleaning may be centralised or decentralised. In centralised cleaning, water and cleaning agents are circulated from one central CIP station to all CIP circuits in the plant. Such a system is generally used in small plants. In larger plants, such a system would involve excessively long pipe systems and therefore the large CIP station is replaced by a number of smaller units located near different circuits/processes. Such decentralised cleaning involves small batches of cleaning solutions and offers advantages in terms of water and steam consumption as well as facilitating optimisation of cleaning for particular circuits (Bylund, 1995).

CIP may be performed using single-use or reuse systems. The system employed is dictated mainly by the severity of fouling, with single-use systems favoured if fouling is heavy. In single-use systems, cleaning solutions are discarded after one use whereas in reuse systems the cleaning solution is recovered and stored for use in the next cleaning. Fresh cleaning solution may be added prior to further use if necessary. Reuse systems are advantageous in terms of reduced water and cleaning product usage and reduced waste generation. However, there is a risk of recontamination and such a system is often only feasible where fouling levels are low and cleaning is frequent. Alternatively, the amount of fouling material in the used cleaning solution may be reduced by decantation, filtration or mechanical means, thereby reducing its recontamination potential and extending its period of use.

### *1.4.3 Cleaning procedure*

CIP in dairy processing entails several general steps – recovery of product residues, pre-rinsing with water, cleaning with detergent, rinsing with clean water and sanitation. The exact cleaning regime employed depends on the product produced and the type and intensity of fouling and varies from plant to plant. Pre-rinsing with water is carried out as soon as possible following the recovery of all residual product from the production line. The water used for pre-rinsing is often heated to aid the removal of milk fat residues but temperatures greater than about 55°C are avoided to prevent further protein denaturation. The function of this step is to remove loosely bound material and reduce the organic soil load. This decreases detergent consumption in subsequent cleaning steps, with efficient pre-rinsing contributing significantly to overall cleaning. Pre-rinsing is continued until the return water is clear (Bylund, 1995; Reinemann, 2003).

Cleaning with detergent may involve one or more steps and the procedure used depends on whether the

circuit being cleaned contains heated surfaces or not. Circuits containing cold components only (e.g. pipes and tanks) are cleaned by circulation of alkaline detergent at a minimum temperature of about 70°C (Bylund, 1995). Alkaline detergents dissolve fat and proteins (Eide et al., 2003). Such cold surfaces are cleaned only occasionally with acidic detergents to remove mineral deposits. Cleaning with acid detergents is generally performed at 68–70°C. In contrast, circulation of acidic detergents is always carried out when cleaning circuits contain heated surfaces (for example pasteurisers) that are susceptible to mineral deposition. In this case, alkaline detergent is generally circulated initially, followed by a water rinse and then circulation of acidic detergent. Such a regime is described as two-stage cleaning. For some processes, where the deposit is rich in minerals, it is beneficial to use acidic detergent initially followed by alkaline detergent (D'Souza and Mawson, 2005; Fryer and Christian, 2005). The alkaline and acid detergents used for cleaning are described in more detail in Section 1.4.4.

Important parameters when cleaning with detergents include detergent concentration, temperature and duration of cleaning. Detergent solutions must be circulated for a sufficient length of time to dissolve deposits, which in turn depends on the severity of fouling and the temperature of the detergent solution. In addition to optimum cleaning, the usage and cost of electricity, heating, water and labour as well as associated downtime must be considered in deciding on the duration of cleaning.

After cleaning, the circuit is flushed with water to carry away suspended soils and to remove all traces of cleaning chemicals (Bylund, 1995; Reinemann, 2003). This final rinse water is often collected and used in the pre-rinse step of the subsequent cleaning cycle. The final step of cleaning involves disinfection by thermal or chemical means with the aim of reducing the number of micro-organisms to an acceptable level (Eide et al., 2003). Thermal disinfection may be achieved by the circulation of hot water (outlet temperature >80°C; 5 min) or steam (condensate temperature >80°C; 15 min). Commonly used chemicals include chlorine, acids, iodophors and quaternary ammonium sanitisers (Reinemann, 2003).

In dairy plants, cleaning is normally carried out once per day, corresponding with breaks in the normal processing schedule (Reinemann, 2003). Fouling in heat exchangers and other heated components is so rapid that daily cleaning is essential (Bansal and Chen, 2006). Other cold components that are used continuously may be cleaned less frequently (Reinemann, 2003). A typical CIP programme for hot components may consist of a 10-min warm water wash, circulation of alkaline detergent at 75°C for 30 min, a warm water rinse for 5 min, circulation of acid solution at 70°C for 20 min, followed by a cold water rinse and disinfection. In the case of cold components, a CIP programme may involve a warm water rinse for 3 min, circulation of alkaline detergent at 75°C for 10 min, followed by a warm water rinse for 3 min and subsequent disinfection (Bylund, 1995).

### *1.4.4 Chemicals used in CIP*

Protein and fat found in milk fouling deposits are insoluble in water. The proteinaceous material is soluble in alkali and slightly soluble in acid while the fat is soluble in alkali. The solubility of minerals in water is variable, while most are acid soluble (Bylund, 1995; Fryer and Christian, 2005). Cleaners are therefore generally based on acidic or alkali compounds.

Sodium hydroxide (caustic soda, NaOH) is the most commonly used alkali. It promotes rapid hydrolysis of proteins and saponification of fats and to an extent is bactericidal (Purnell, 1993; D'Souza and Mawson, 2005). Upon contact of the alkali solution with the deposit layer, the cleaning solution penetrates into the deposit causing it to swell, forming a protein matrix of high void volume. This swelling has been attributed to repulsion between charged amino acid side chains in the deposit at high pH (Jeurnink et al., 1996; Fryer et al., 2006). When the pH is increased above the isoelectric point of the protein (5.1 for β-Lg), the net surface charge increases with a big increase in charge observed near pH 11 for whey protein solution due to conversion of the ammonium groups of the lysine residues to amine groups (Mercadé-Prieto and Chen, 2005). This swelling leads to cracks in the deposit structure which results in further penetration of the cleaning solution. The swollen deposit is then removed by shear with pieces loosened and carried away by the turbulence of the cleaning solution (Jeurnink et al.,

1996; Fryer et al., 2006). The cleaning and protein removal processes are complex and not fully understood and are reported to be affected by conditions at the deposit–solution interface, reaction and diffusion of protein in the deposit, transport of cleaning solution in the deposit and flow rate (Jeurnink et al., 1996; Gillham et al., 1999; Fryer et al., 2006). A concentration of 0.3–0.7 wt% sodium hydroxide is recommended for the most efficient deposit removal. Higher concentrations are reported to modify the deposit surface such that it is more difficult to remove (Visser and Jeurnink, 1997). Other alkalis used in cleaning include potassium hydroxide, sodium carbonate, alkaline silicates and phosphates (Purnell, 1993).

While single-component cleaners (e.g. sodium hydroxide) can be used for cleaning, products have been developed which contain additional chemicals/additives to enhance cleaning. Such products are referred to as built or formulated detergents and generally consist of a mixture of alkalis, phosphates, sequestering agents and wetting agents (surfactants), as shown in Table 1.7 (Purnell, 1993; D'Souza and Mawson, 2005).

Nitric acid and phosphoric acid are commonly used in detergent applications. Nitric acid is cheaper than phosphoric acid and has a passivating effect on stainless steel. Caution must be exercised with the use of sulfuric acid and hydrochloric acid due to their corrosive effect (Purnell, 1993). Organic acids such as acetic acid, hydroxyacetic acid, lactic acid, gluconic acid and citric acid may also be used (Reinemann, 2003), as well as blends of acids (Bremer et al., 2006). Acid cleaners generally contain about 0.5% (w/w) acid (Chisti, 2000) and other constituents may also be added to amplify their cleaning effect, as described above. The main role of acid in cleaning applications is to dissolve the mineral scale remaining on surfaces after alkaline cleaning. Acid also improves draining and drying and provides bacteriostatic conditions in which microbial growth is reduced (Bremer et al., 2006).

Single-stage cleaners are now also available. Such products usually contain sodium hydroxide and a complex blend of chemicals such as chelating compounds, wetting and other surface agents (Changani et al., 1997; Fryer and Christian, 2005). These products contain additives, such as sequestrants, which target inorganic material,

**Table 1.7. Major components of alkaline detergents and their function.[1]**

| Component | Function |
|---|---|
| **Alkalis**<br>(e.g. sodium hydroxide, sodium silicates, trisodium phosphate) | • Soil dissolution<br>• Protein hydrolysis and solubilisation<br>• Fat saponification |
| **Sequestering agents**<br>(e.g. sodium polyphosphate, EDTA and NTA) | • Formation of complexes with calcium and magnesium ions to avoid precipitation of salts |
| **Surfactants**<br>(e.g. alkyl aryl sulfonates (anionic), alkyl sulfonates (anionic), alkylphenol ethoxylates (non-ionic) | • Wetting<br>• Soil removal<br>• Solubilisation<br>• Emulsification |
| **Oxidisers**<br>(e.g. hydrogen peroxide, sodium hypochlorite) | • For intensifying the cleaning effect |
| **Minor components**<br>(e.g. dispersing agents, antifoaming agents, anticorrosion agents, stabilisers) | • Promoting dispersion<br>• Preventing foam formation<br>• Preventing alkaline erosion<br>• Prolonging storage |

[1]Fryer and Christian (2005).
EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetic acid.

therefore eliminating the need for separate acid cleaning (Gillham et al., 1999). Only three steps are required when these products are employed for cleaning, i.e. rinse, clean, rinse, resulting in reduced cleaning times but such products are generally more expensive (Changani et al., 1997; Fryer and Christian, 2005).

### *1.4.5 Environmental impact of cleaning*

Cleaning has an impact on the environment due to the chemical agents described above and also because it is a water- and energy-demanding process. It is reported that cleaning processes account for 80% of the total eutrophication potential from dairy processing. It is noteworthy, however, that while detergent nitrogen and phosphorus influence the eutrophication potential, the emission of milk product residues during rinsing has a much greater influence (Eide et al., 2003). Cleaning processes reportedly account for 30% of energy use in dairy processing (Eide et al., 2003). In many dairy plants, cleaning is the largest contributor to the overall wastewater volume (Danalewich et al., 1998).

According to Wildbrett (2003), detergents represent the greatest proportion of chemicals used in dairy plants. The chemicals employed in cleaning are generally biologically non-degradable substances. The use of alkaline detergents and acids causes extreme fluctuations in wastewater pH (3–13) which interferes with biological treatments (Bylund, 1995; Danalewich et al., 1998). Chemicals such as sodium hydroxide and potassium hydroxide and nitric acid and phosphoric acid are often neutralised to pH 6.5–10, prior to being discharged to wastewater, resulting in a high salt concentration (Fryer and Christian, 2005; Grasshoff, 2005). In Germany, the resultant non-biodegradable salt load amounts to 2,000–6,000 t/year (Grasshoff, 2002).

Some components of cleaning detergents have adverse effects on human and ecosystem health and on the environment. For example, phosphates found in cleaning products contribute to eutrophication and complexing agents may remobilise heavy metals (Fryer and Christian, 2005). Some surfactants affect fish reproduction and may form a foam on surface waters, reducing oxygen uptake into water or activated sludge systems (Wildbrett, 2003). Peracetic acid and some non-ion active tensides are considered harmful to water-living organisms (Eide et al., 2003). In a qualitative impact assessment of the chemical compounds in detergents, non-ion active tenside and potassium carbonate were considered irritating to human health while nitric acid, acetic acid, phosphoric acid, sulfuric acid, hydrogen peroxide and potassium hydroxide were described as cauterising (Eide et al., 2003).

## 1.5 Enzymes

### *1.5.1 Overview of the use of enzymes in industry*

Enzymes are biological catalysts that drive chemical reactions in living cells. Enzymes are highly specific in terms of substrate binding and the type of reaction catalysed. As they originate from living cells, most enzymes function under mild conditions. These two properties make enzymes particularly attractive for industrial applications.

Historically, enzymes have been used, in some cases unknowingly, in processes such as brewing and cheese making. Currently a range of enzymes finds application in different industrial sectors, such as the detergent, textile, leather, pulp and paper, animal feed, dairy, brewing, juice and wine industries. Enzymes are also used in biocatalysis and waste hydrolysis (Walsh, 2002; Olsen, 2004). The global market for industrial enzymes was estimated at $2 billion in 2004 and is expected to reach $2.4 billion in 2009 (Hasan et al., 2006). Advances in genetic and protein engineering continue to impact positively upon the industrial enzyme sector. Industrial enzymes can now be economically produced in large quantities by recombinant means, with a relatively higher degree of purity. In recent years, protein engineering has been successfully used to alter the properties of several enzymes, rendering them more suitable for specific applications (Walsh, 2002; Hasan et al., 2006). It has been reported that only about 2% of the world's micro-organisms have been tested as enzyme sources (Hasan et al., 2006) and therefore, in addition to improving existing enzymes, current research efforts are also focussed on isolating enzymes from novel sources.

Enzymes for use in industry are produced by fermentation of micro-organisms using primarily renewable resources and hence contribute to sustainable development. The use of enzymes in industrial processes is associated with reduced energy and chemical consumption and reduced generation of waste (Olsen, 2004). Enzymes are biodegradable and, as they are used in relatively low concentrations, their contribution to the waste stream biochemical oxygen demand (BOD) is negligible (Hasan et al., 2006) and they occupy very little storage space (Olsen, 2004).

Industrial enzymes are produced by growing the selected micro-organism in a fermentor containing the appropriate medium. Most industrial enzymes are extracellular and therefore are secreted into the medium during fermentation. The enzyme is harvested, most commonly by centrifugation or filtration, and concentrated by evaporation, membrane filtration or crystallisation, depending on the intended application. The insoluble material or biomass collected during harvesting may be used as a fertiliser following treatment with lime to inactivate the micro-organisms present. For most industrial applications, further purification is not necessary and the crude enzyme product is formulated as a liquid or dry (granulated) product. The exact formulation will depend on the intended application and requirements for storage stability will also be taken into account (Olsen, 2004).

The current project focusses on proteases and lipases and these enzymes are discussed in detail in Sections 1.5.2 and 1.5.3, respectively.

### *1.5.2 Proteases*

Proteases (peptidases or proteolytic enzymes) are protein-degrading enzymes that cleave peptide bonds within proteins. Proteases play essential roles in biology and are essential constituents of prokaryotes, fungi, plants and animals (Dunn, 2002; Gupta et al., 2002b). It is estimated that approximately 2% of all genes code for proteases (Dunn, 2002). Proteases may be classified according to the type of proteolytic reaction catalysed, i.e. the positioning of the peptide bond hydrolysed, or according to the type of catalytic mechanism by which such hydrolysis is achieved. On the basis of the position of the peptide bond hydrolysed, proteases may be described as endopeptidases or exopeptidases. Endopeptidases act internally in the polypeptide chain, usually some distance from the carboxyl or amino termini and their action results in the production of large fragments. Exopeptidases act near the end of the polypeptide chain, resulting in the liberation of a single amino acid, a dipeptide or tripeptide. Exopeptidases are further classified according to whether they act at the amino or carboxyl termini and on the number of amino acid residues liberated (Barrett, 2000; Walsh, 2002).

Proteases are divided into five groups based on the chemical nature of the catalytic site and associated mechanism of action – serine-, aspartic-, cysteine-, metallo-, and threonine-type proteases (Barrett, 2000). Serine proteases have a nucleophilic serine residue in their active site as well as essential aspartate and histidine residues, which together form the catalytic triad. The serine proteases are further divided into subclasses on the basis of structural homology to well-known proteases. These subclasses include subtilisin-like proteases or subtilases, chymotrypsin-like proteases and wheat serine carboxypeptidase II-like proteases (Gupta et al., 2002b). Aspartic proteases, for example pepsin, contain an essential aspartic acid residue at the active site and operate under acidic conditions (Neurath, 1989). In cysteine proteases, a catalytic triad is formed by cysteine, histidine and asparagine (Bott, 1997), while metalloproteases require divalent metal ions for activity (Walsh, 2002).

Proteases are used in a range of industrial applications, as shown in Table 1.8 and are one of the most important groups of industrial enzymes. Such proteases are obtained from various sources depending on application requirements. The most prominent use of proteases is in the detergent industry and, for reasons discussed in Section 1.5.4.1, the subtilisin class of serine proteases is favoured for this application. Subtilisins used in the detergent industry generally originate from *Bacillus* species, mainly *B. amyloliquefaciens*, *B. licheniformis*, *B. clausii* and *B. lentus*. These strains are capable of producing extracellular proteases over a short period of time by submerged fermentation (SbF) (Maurer, 2004). Protease production generally occurs during the stationary phase and is affected by carbon, nitrogen

**Table 1.8. Industrial applications of proteases.[1]**

| Industry | Application |
|---|---|
| **Detergent** | • Removal of proteinaceous stains |
| **Food** | • Preparation of protein hydrolysates of high nutritional value |
| **Dairy** | • Flavour development<br>• Cheese ripening |
| **Meat** | • Meat tenderisation |
| **Baking** | • Modification of dough texture and gluten elasticity |
| **Leather** | • De-hairing of animal hides and skin |
| **Photographic** | • Bioprocessing of used photographic films for silver recovery |
| **Brewing** | • Solubilisation of grain proteins<br>• Stabilisation of beer |
| **Textile** | • Wool treatment<br>• De-gumming of raw silk |
| **Organic synthesis** | • Ester and amide hydrolysis<br>• Peptide synthesis<br>• Synthesis and resolution of D,L-amino acids |
| **Other** | • Conversion of waste (e.g. horn, feather, nail and hair) into useful biomass, protein concentrate or amino acids |

[1]Anwar and Saleemuddin (1998); Gupta et al. (2002b); Walsh (2002); Olsen (2004).

and divalent cations as well as by physiological conditions such as pH, temperature and agitation (Gupta et al., 2002a).

### *1.5.3 Lipases*

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) catalyse the hydrolysis of lipids (triacylglycerols) to glycerol and free fatty acids by acting on the carboxyl ester bonds present in triacylglycerol, as shown in Fig. 1.1. Under micro-aqueous conditions, lipases are also capable of catalysing the reverse reaction (Gupta et al., 2004).

Lipases are found widely in nature and are produced by a range of plants, animals and micro-organisms, including bacteria, fungi and yeast. Some examples of micro-organisms capable of producing lipases are shown in Table 1.9. Lipases play a role in lipid

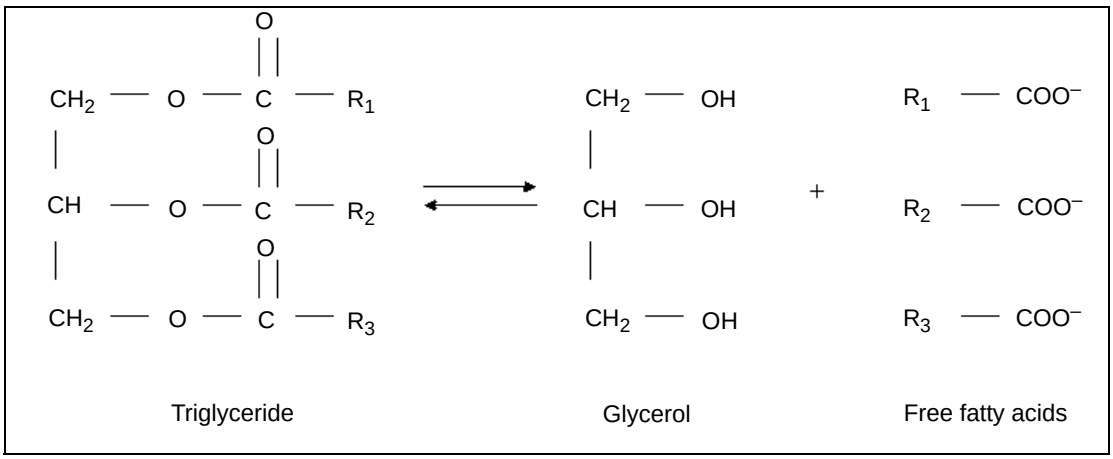


**Figure 1.1. Reaction catalysed by lipases (Walsh, 2002).**

**Table 1.9. Examples of lipase-producing micro-organisms.[1]**

| Source | Genus |
|---|---|
| **Bacteria** | • *Bacillus*<br>• *Staphylococcus*<br>• *Lactobacillus*<br>• *Streptococcus*<br>• *Micrococcus*<br>• *Propionibacterium*<br>• *Burkholderia*<br>• *Pseudomonas*<br>• *Chromobacterium*<br>• *Acinetobacter*<br>• *Aeromonas*<br>• *Achromobacter*<br>• *Alcaligenes*<br>• *Arthrobacter*<br>• *Archaeoglobus*<br>• *Corynebacterium*<br>• *Cryptococcus*<br>• *Enterococcus*<br>• *Microthrix*<br>• *Mycobacterium*<br>• *Pasteurella*<br>• *Proteus* |
| **Fungi** | • *Rhizopus*<br>• *Aspergillus*<br>• *Penicillium*<br>• *Mucor*<br>• *Geotrichum*<br>• *Humicola*<br>• *Rhizomucor*<br>• *Fusarium*<br>• *Acremonium* |
| **Yeast** | • *Candida* |

[1]Sharma et al. (2001); Gupta et al. (2004).

metabolism in eukaryotes and are found in energy reserve tissues in plants (Sharma et al., 2001). Lipases used commercially are generally of microbial origin.

Microbial lipases may be divided into three groups on the basis of their substrate specificity – non-specific lipases, regio-specific lipases and fatty acid-specific lipases. Non-specific lipases, for example those produced by *Staphylococcus aureus* and *Chromobacterium viscosum*, act randomly on triacylglycerol bringing about complete breakdown to fatty acids and glycerol. Regio-specific lipases or 1,3-specific lipases hydrolyse the ester bonds at atoms C1 and C3 of glycerol only resulting in the generation of free fatty acids, 1,2(2,3)-diacylglyceride and 2-monoacylglyceride. Extracellular bacterial lipases such as those produced by various *Bacillus* species belong to this class. Fatty acid-specific lipases exhibit a fatty acid preference. The only known bacterial lipase showing fatty acid specificity is that from *Achromobacter lipolyticum*. Several lipases also show specificity for triacylglycerols with long-chain, medium-chain or short-chain fatty acids (Gupta et al., 2004; Houde et al., 2004).

Lipases display little activity against soluble substrates in aqueous solution but rather the lipase-mediated reaction occurs at the lipid–water interface (Sharma et al., 2001). This enhanced lipase activity at the lipid–water interface is termed 'interfacial activation'. Upon determination of their 3-D structures it became apparent that the lipase active site was covered by a surface loop which formed a lid or flap. This lid moves away upon binding of the enzyme to the substrate–water interface, making the active site accessible to substrate molecules. At the same time, a large hydrophobic surface is exposed which likely facilitates binding of the enzyme to the interface (Jaeger and Reetz, 1998; Houde et al., 2004).

Lipases are serine hydrolases with a catalytic triad composed of Ser–Asp/Glu–His and usually also contain a consensus sequence (Gly–x–Ser–x–Gly) around the active site serine (Gupta et al., 2004). The 3-D structures of several lipases have been resolved and reveal the characteristic $\alpha/\beta$-hydrolase fold with the lipase core composed of a central $\beta$ sheet consisting of up to eight different $\beta$ strands connected by up to six $\alpha$ helices (Jaeger and Reetz, 1998; Gupta et al., 2004).

Several microbial lipases have been characterised extensively and on the basis of their properties have found numerous applications in various industries, as shown in Table 1.10. The use of lipases in the detergent industry is discussed further in Section 1.5.4.1. Currently available commercial lipase products are derived mainly from the genera *Aspergillus*, *Mucor*, *Rhizopus*, *Candida*, *Penicillium*, *Rhizomucor* and *Pseudomonas*. Some commercial lipase products are targeted for a specific application while many find use in various different fields.

Microbial lipases are generally produced by SbF and to a lesser extent by solid-state fermentation (SSF) (Sharma et al., 2001). Lipases are inducible enzymes

**Table 1.10. Industrial applications of microbial lipases.[1]**

| Industry | Application |
|---|---|
| **Detergent** | • Removal of fat-containing stains from clothing |
| **Food** | • Acceleration of cheese ripening<br>• Production of enzyme-modified cheeses (EMCs)<br>• Flavour development/enhancement<br>• Improvement of dough texture and colour and as an emulsifier in baking<br>• Fat modification in infant formula<br>• Production of structured lipids and reduced-calorie fats<br>• Production of cocoa butter substitutes |
| **Pharmaceutical** | • Synthesis of enantiopure molecules such as alcohols, amides, carboxylic acids and esters<br>• Resolution of racemic mixtures |
| **Oleochemical** | • Alcoholysis<br>• Acidolysis<br>• Hydrolysis<br>• Glycerolysis |
| **Cosmetic** | • Production of cosmetic components, e.g. isopropyl myristate and wax esters |
| **Leather** | • Liming and fat dispersion |
| **Pulp and paper** | • Removal of pitch |

[1]Houde et al. (2004); Hasan et al. (2006).

and are generally produced in the presence of a lipidic carbon source. Lipase production is also influenced by the type and concentration of nitrogen source and physical parameters such as pH, temperature, agitation and aeration. Bacterial lipases are generally extracellular with peak production, which varies from hours to days, observed by the late log phase (Gupta et al., 2004). Current lipase research focusses on the identification of novel and improved lipases in order to further enhance the industrial usefulness of this class of enzyme. This is being achieved by natural selection, protein engineering studies and directed evolution (Sharma et al., 2001).

### *1.5.4 Use of enzymes in cleaning applications*

#### *1.5.4.1 Laundry/Automatic dishwasher detergents*

The detergent industry is the largest application area for enzymes. Proteases, lipases, amylases and cellulases are used in laundry detergents while mainly proteases, lipases and amylases are used in automatic dishwasher detergents. The popularity of enzymes in this area can be attributed to their effective cleaning performance at moderate temperatures and pH values which results in reduced energy and water consumption and reduced environmental impact (Olsen, 2004). Proteases degrade protein-based stains (e.g. blood, egg, gravy) into fragments that are generally considered to be more soluble than the intact protein stain (Bott, 1997; Egmond, 1997). Lipases act on fat-based stains (e.g. butter and fat, mayonnaise, sebum, sweat, lipstick) and the resultant products are more water soluble than the original triglyceride. This facilitates the removal of fatty stains even in cool water, at a temperature below the fat's melting point (Wolff and Showell, 1997). Enzyme-based detergents also generally contain other ingredients such as surfactants, sequestering agents, bleach and components to provide buffering capacity (Walsh, 2002).

In terms of laundry detergent applications, endopeptidases are considered to be more useful than exopeptidases as it is assumed that a reduction in substrate molecular size is favourable for stain removal (Egmond, 1997). Of the various classes of proteases described in Section 1.5.2, serine proteases (in particular subtilisins) are considered the most suitable detergent additives. This is mainly due to their broad substrate specificity, stability in the presence of other ingredients and high activity under wash

conditions. In contrast, aspartic proteases exhibit little or no activity under the alkaline conditions typical of wash liquors while metalloproteases and cysteine proteases will be inactivated by sequestering agents and bleach, respectively (Bott, 1997; Egmond, 1997).

For optimum effect, enzymes used in detergents should be stable during storage and in the presence of other detergent components and should exhibit high activity under the wash conditions. Many detergent enzymes have been modified by protein engineering with the aim of improving their performance and enhancing their stability (Egmond, 1997). There is also continued interest in screening for novel enzymes with enhanced properties in nature. Recent trends in Europe towards lower washing temperatures has led to the development of new enzyme products capable of achieving satisfactory washing performance at temperatures as low as 30°C.

#### *1.5.4.2 Membranes*

There are several reports in the literature on the use of enzymes to clean membranes fouled with biological fluids. Arguello et al. (2002, 2003, 2005) described the use of proteolytic enzymes to clean inorganic ultrafiltration membranes fouled by whey proteins and reported high cleaning efficiencies. Effective cleaning was also achieved when proteases and lipases were used to clean ultrafiltration membranes fouled with abattoir effluent, the major constituents of which are lipids and proteins (Maartens et al., 1996; Allie et al., 2003). Enzyme cleaning offers several advantages in this area. In addition to being biodegradable and reducing chemical usage, rinsing volumes are also decreased, resulting in a reduced volume of wastewater and one which does not require neutralisation (D'Souza and Mawson, 2005). The ability of enzymes to operate under mild conditions reduces energy costs and increases the lifetime of membranes, which are generally sensitive to chemicals, pH and/or temperature (Maartens et al., 1996; Arguello et al., 2003).

#### *1.5.4.3 Contact lenses and surgical instruments*

Deposits that form on contact lenses and necessitate their frequent cleaning are derived from tears and contain proteins (lysozyme and antibodies) as well as a lipid component. Proteases and combinations of proteases and lipases have been shown to be effective in removing such deposits (Walsh, 2002). Begley et al. (1990) compared the performance of four marketed enzyme cleaners for this purpose and reported that all were effective in removing deposits from the surfaces of contact lenses. Stern and Zam (1987) reported that cleaning of contact lenses with enzymatic cleaners reduced the adherence of *Pseudomonas* which may reduce the incidence of *Pseudomonas* corneal ulceration in wearers. Enzymatic medical instrument cleaners are also available for the routine cleaning of surgical instruments. Lawson et al. (2007) demonstrated the ability of such cleaners to reduce the infectivity associated with a human-derived prion strain. Jackson *et al.* (2005) also reported on the use of an enzyme detergent for effective prion decontamination of surgical steel.

#### *1.5.4.4 CIP in dairy processing*

With the exception of membrane cleaning, little attention has been given to the widespread application of enzymes for CIP in dairy processing, where fouling deposits are predominantly biological in nature. In the 1990s, Ecolab developed an enzyme-based cleaning product P3-paradigm® for cleaning dairy plant equipment. This product contains proteolytic enzyme activity, surfactants, a buffer and complexing agents and is used at pH 8.5–9.5 and 50–55°C. Cleaning performance was confirmed in plant trials and field tests and benefits reported included reduced water and energy consumption, reduced costs and decreased risks associated with handling and storing hazardous chemical products (Pottchoff and Serve, 1997). However, this product is only suitable for use on non-heat treated equipment surfaces, where (as discussed in Section 1.3.1) substantial fouling is not observed and cleaning is relatively easily achieved. In contrast, hot components such as heat exchangers, etc., present a much greater cleaning challenge and significant environmental benefit could potentially be achieved by the use of enzymes in CIP operations in this area. Grasshoff (2002, 2005) investigated the use of proteases to remove milk soils generated in a laboratory heat exchanger and reported that deposit-free surfaces were obtained with three of the enzymes tested. A field trial was subsequently carried out in which an enzyme cleaning solution containing one of

these enzymes, surface active agents, a little sodium hydroxide and buffering substrates (pH 9–9.5) was used to clean a milk pasteuriser at 55–60°C, with visually clean surfaces reported after cleaning. While these results indicate the ability of enzymes to remove milk fouling deposits from heated components such as pasteurisers, cleaning was assessed only by visual inspection.

The use of enzymes for CIP in dairy processing would reduce the overall environmental impact of cleaning. The potential benefits are similar to those outlined above for laundry detergents and membranes – reduced chemical usage, reduced energy consumption due to milder operating conditions, reduced rinsing volumes resulting in reduced water consumption and lower wastewater volumes as well as reduced waste generation as enzymes are biodegradable. In a study comparing conventional cleaning, one-phase cleaning and enzyme-based cleaning in the cold milk area of a German dairy, the enzyme-based procedure was found to have the lowest overall environmental impact, consuming the least energy and water (Eide et al., 2003). Eide et al. (2003) carried out a life-cycle assessment of four CIP methods using the cleaning of an average Norwegian dairy as the functional unit. The methods investigated were conventional alkaline/acidic cleaning by nitric acid and sodium hydroxide followed by hot water disinfection, one-phase alkaline cleaning with acid chemical disinfection, enzyme-based cleaning with acid chemical disinfection and conventional alkaline/acidic cleaning with disinfection by cold nitric acid at pH 2. It was concluded that the CIP methods with low volumes and temperatures (enzyme-based cleaning and one-phase alkaline cleaning) were the best options for the impact categories energy use, global warming, acidification, eutrophication and photo-oxidant formation. Energy use for these two methods was 20–40% lower than the other methods and heating of large volumes of cleaning solution was reported to be a major contributor to the impact categories. The enzyme-based cleaning method was identified as the most toxic as the enzyme product used contained non-ionic tensides classified as harmful to the environment and related estimations undertaken in the study were worst case. It is noteworthy that the environmental effect of non-ion active tensides varies and can be taken into account in formulating enzyme cleaning products.

In addition to reducing the environmental impact of cleaning, enzymes could potentially be more effective in removing biofilms from processing equipment. The effectiveness of conventional cleaning methods in biofilm removal is variable and often unsatisfactory (Flint et al., 1999; Bremer et al., 2006). Given the important role of proteins in cell adhesion, it is possible that enzyme-containing products may give better results. Flint et al. (1999) reported that proteolytic enzymes removed more biofilm cells of thermo-resistant streptococci than cleaning chemicals commonly used in dairy processing. Improved and more reliable methods for biofilm removal are of interest to dairy manufacturers owing to increased concerns regarding biofilms and their potential deleterious effects on product safety and quality.

Various issues need to be considered if enzymes are to be successfully applied as cleaning agents in dairy processing. For optimum effect, enzymes used should be highly active on the target substrate, i.e. the protein and lipid components of the fouling deposit. Such enzymes should exhibit good activity at the temperature and pH used for cleaning and should be stable during storage and during the conditions encountered in the washing process. It is essential that enzyme activity is not adversely affected by other components that may be present in the cleaning fluid. From an economic standpoint, acceptable cleaning performance should be achieved in a time comparable to or less than that for conventional cleaning and at low enzyme levels. For acceptance of enzyme-based cleaning in industry, confirmation of the removal of all traces of enzyme from the processing equipment by subsequent rinsing/sanitation steps will be essential as contamination of product with enzyme in subsequent production runs could have deleterious effects on the product.

## 1.6 Aims of Study

The main objective of this study was to determine if enzymes display realistic potential to replace (either partially or entirely) currently used environmentally significant CIP chemicals and hence reduce the overall environmental impact of CIP in dairy processing.

Due to the composition of the fouling deposit encountered in dairy processing, the aim was to look at both proteases and lipases for this purpose and cleaning performance was evaluated in a quantitative manner using a fouling deposit representative of that occurring on heated components.

The first part of the study focussed on commercially available protease and lipase products which are already produced and sold in bulk quantities by global enzyme manufacturers and could be readily incorporated into CIP operations if found to be suitable. The aim of the second part of the study was to screen the University of Limerick's in-house collection of micro-organisms for proteases of potential use in CIP. A further aim was to establish details of typical CIP procedures currently undertaken in the Irish dairy industry via communication with dairy co-ops and suppliers of cleaning chemicals as well as a review of relevant literature. Other aims included confirmation of the removal/inactivation of all enzyme activity post enzyme-based cleaning and analysis of the cost implications of enzyme-based CIP. These issues are important for acceptance of enzyme-based cleaning in industry.

# 2 Laboratory-Scale Assessment of the Suitability of Currently Available Commercial Enzyme Products for Use in CIP Operations in the Dairy Processing Industry

## 2.1 Background and Aims

Minimisation of the environmental impact of cleaning operations is receiving increasing attention and becoming more important (Wilson, 2005; Fryer and Asteriadou, 2009). The environmental benefits of using enzymes for cleaning in other sectors have been well established and it is frequently reported that similar benefits could potentially be achieved by using enzymes for CIP in dairy processing (Section 1.5.4). However, with the exception of cleaning membranes and the cold milk areas of plants (Section 1.5.4.4), little attention has been given to the widespread application of enzymes for CIP operations in dairy processing. Reasons given for this include low cleaning efficiency, unsuitability for cleaning heated surfaces, poor sensitivity of enzymes to the conditions encountered during cleaning, high costs, and concerns that enzyme activity remaining after cleaning could have a deleterious effect on product subsequently processed (D'Souza and Mawson, 2005; Fryer et al., 2006). The aim of this part of the study was to determine if currently available commercial enzymes (proteases and lipases) could satisfy these challenges and, if so, to determine if they display realistic potential to replace (either partially or entirely) currently used environmentally significant CIP chemicals.

Assessment of the commercial enzyme products was based mainly on their ability to remove milk fouling (representative of that occurring on industrial heat exchangers) from the surface of stainless steel panels, as judged by quantification of residual organic matter and protein after cleaning and analysis of the cleaned panels by confocal laser scanning microscopy (CLSM). In order to assess enzyme cleaning performance under application-relevant conditions and facilitate comparison with currently used CIP procedures, details of how CIP is currently undertaken in industry were obtained by contacting the major Irish dairy co-ops and companies supplying cleaning agents to the dairy industry. The commercial enzyme products selected for assessment are all produced and sold in bulk quantities by major enzyme manufacturers with global supply chains and could therefore be readily used for CIP application if found to be suitable for this purpose. In addition to cleaning performance, other issues of importance in the context of industrial CIP application were also investigated, including enzyme stability, enzyme removal/inactivation after cleaning and the economic implications of enzyme-based cleaning.

## 2.2 Materials and Methods

### *2.2.1 Materials*

All reagents were purchased from Sigma–Aldrich Ireland Ltd, Wicklow, and Melford Laboratories Ltd, Suffolk, UK, unless otherwise stated. Commercial enzyme products were obtained from Novozymes, Bagsvaerd, Denmark (Liquanase® and Lipex® 100L), Genencor International Ltd, UK (Protex 6L, Protex 30L, Protex 40L and Protex 89L), Sigma–Aldrich Ireland Ltd, Wicklow (Esperase®) and National Centre for Biotechnology Education, Reading, UK (Alcalase®, Savinase® and Lipolase® 100T). Whey protein isolate (Power Health Products Ltd), produced by crossflow microfiltration and containing 90 g protein per 100 g, was obtained locally. Azocasein was obtained from Megazyme International Ireland Ltd, Bray, Co. Wicklow. Citifluor was obtained from UKC Chemical Laboratory, UK. Hypochlorite (Superbrand) was from O'Connor Group, Newmarket, Co. Cork. Stainless steel panels (20 × 70 × 1.5 mm) were obtained from a company supplying process systems to the dairy industry.

### *2.2.2 Methods*

#### *2.2.2.1 Determination of protease activity*

##### *2.2.2.1.1 Thermally induced whey protein gel substrate*

Protease activity was measured by quantification of the free amino groups generated during hydrolysis of the substrate (thermally induced whey protein gel) using the trinitrobenzenesulfonic acid (TNBS) method of Adler-Nissen (1979). The substrate was prepared by incubating 300-µl aliquots of 25% (w/w) whey protein isolate solution (prepared in distilled water) at 80°C for 60 min, following gradual heating from 50°C to 80°C over a 30-min period. After cooling, the gels were washed with distilled water and 500 µl of 0.1 M potassium phosphate buffer, pH 8 and 100 µl of enzyme sample, suitably diluted in the same buffer, were added to the gel substrate and incubated at 50°C for 30 min. After this time, 200 µl of the reaction mixture were added to an equal volume of 2% (w/v) sodium dodecyl sulfate (SDS) and heated at 80°C for 15 min. Blanks were prepared by adding the enzyme to the gel and buffer immediately before removing 200 µl and heating with SDS. To quantify the free amino groups generated during the reaction, 100 µl from each sample were added to a test tube containing 1 ml of sodium phosphate ($NaPO_4$) buffer, pH 8.2 and 1 ml of freshly prepared 0.1% (w/v) TNBS was added. Following mixing, tubes were incubated at 50°C for 60 min in a covered water bath. The reaction was stopped by addition of 2 ml of 0.1 M hydrochloric acid (HCl) and, after cooling to room temperature for 30 min, absorbance was measured at 340 nm. A standard curve was prepared using 0–5.0 mM L-leucine in 1% (w/v) SDS. One unit of enzyme activity is defined as the amount of activity that released the equivalent of 1 µmol of L-leucine from whey protein gel per minute under the assay conditions. All determinations were performed in triplicate.

##### *2.2.2.1.2 Azocasein substrate*

Determination of protease activity using azocasein as substrate was carried out as described by Sarath et al. (1989). A 150-µl enzyme sample and 250 µl substrate (2% (w/v) azocasein in 0.1 M potassium phosphate buffer, pH 8) were pre-equilibrated separately for 3 min prior to 30 min co-incubation at 50°C. The reaction was terminated by the addition of 1.2 ml of 10% (w/v) trichloroacetic acid (TCA). After standing at room temperature for 15 min, samples were centrifuged at 8000 × *g* for 5 min and 600 µl of the resulting supernatant were added to 700 µl of 1 M sodium hydroxide. The absorbance of this solution was measured at 440 nm. One unit of protease activity is defined as the amount of enzyme required to produce an absorbance change of 1.0 in a 1-cm cuvette, under the assay conditions.

#### *2.2.2.2 Effect of temperature on protease activity*

The effect of temperature on protease activity was determined by measuring protease activity using whey protein gel as substrate, as described in Section 2.2.2.1.1, at 22°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C.

#### *2.2.2.3 Effect of pH on protease activity*

The effect of pH on enzyme activity was determined by measuring protease activity at pH 6–11.3, using the following buffers: 0.1 M $Na_2HPO_4$–$NaH_2PO_4$ (pH 6–8), 0.1 M $Na_2B_4O_7$–HCl (pH 9), 0.1 M $Na_2CO_3$–$NaHCO_3$ (pH 10–10.5) and 0.1 M $Na_2HPO_4$–NaOH (pH 11.3). Determination of protease activity was carried out as described in Section 2.2.2.1.1, with the following modifications: the buffers listed were used in place of 0.1 M potassium phosphate buffer, pH 8, and enzyme dilutions were prepared in distilled water immediately prior to assay commencement.

#### *2.2.2.4 Generation of milk fouling deposit on stainless steel panels*

Before fouling, stainless steel panels were cleaned based on a typical dairy CIP procedure using 1.0% (w/w) sodium hydroxide at 75°C for 45 min and 1.0% nitric acid at 70°C for 20 min, with intermediate and final water rinses. After drying, the panels were placed on the base of a stainless steel container and heated for 2 min at setting 4 on a Yellowline MSH Basic magnetic stirrer with a stainless steel heating plate. Five hundred millilitres of raw, untreated milk which had been preheated to 50°C with stirring over a 12-min period were added and maintained at a temperature of 84–89°C for 90 min. After this time, the milk was removed and the fouled panels were rinsed extensively with water at 50°C.

#### 2.2.2.5 *Analysis of milk fouling deposit generated on stainless steel panels*

##### 2.2.2.5.1 *Determination of amount of organic matter on fouled panels*

The amount of organic material on the fouled panels was quantified by complete removal of all fouling material from the panel by incubation with 1.0% (w/w) sodium hydroxide at 70°C for 1 h and subsequent determination of the chemical oxygen demand (COD) of the wash solution. The COD was determined by the closed reflux, colorimetric method (APHA, 1995) using a HACH COD reactor and a HACH DR/2000 Direct Reading Spectrophotometer.

##### 2.2.2.5.2 *Determination of amount of protein on fouled panels*

The amount of protein on the fouled panels was quantified by complete removal of all fouling material from the panel by incubation with 1% (w/w) sodium hydroxide at 70°C for 1 h and subsequent determination of protein using a modified Bradford assay (Gotham et al., 1988). A 123-µl sample was added to 323 µl resolubilisation solution (300 µl 12 M urea in 0.2 M phosphate buffer, pH 6.0, and 23 µl 2-mercaptoethanol). The resulting mixture was vortexed and incubated in a boiling water bath for 1.5 min. After cooling to room temperature, protein concentration was determined using the Bradford method (Bradford, 1976). A standard curve was prepared in the concentration range 0–0.35 mg/ml protein using heat-denatured milk (95°C; 60 min) containing 1% sodium hydroxide and subjected to the resolubilisation procedure outlined above.

##### 2.2.2.5.3 *Analysis of fouling by scanning electron microscopy (SEM) and CLSM*

**Scanning electron microscopy:** Samples were placed in 0.3% paraformaldehyde:1.0% formamide in 0.1 M cacodylate buffer and 0.1 M PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] buffer, pH 7.4, before fixing with 4% (w/v) aqueous osmium tetroxide in 0.1 M cacodylate buffer. Samples were dehydrated stepwise in ethanol, with eight changes (10 min) in 0%, 20%, 30%, 40%, 50%, 60%, 80%, 95%, and 100% ethanol. Samples were subsequently air-dried, placed onto copper stubs, and gold coated to a depth of 80 nm before analysis using a Hitachi SU-70 SEM.

**Confocal laser scanning microscopy:** Samples were fixed with 2.0% osmium oxide in 0.1% cacodylate buffer and Mixture A (0.3% paraformaldehyde:1.0% formamide in 0.1 M cacodylate buffer). Lipids, proteins and polysaccharides were stained using Nile Red (Nile Blue A oxazone), fluorescein isothiocyanate (FITC) and calcofluor white, respectively. After staining, samples were washed three times by immersion in 0.l M cacodylate buffer with gentle agitation for 15 min. Stained samples were air-dried and stored in the dark at 4°C before image acquisition. Samples were mounted using Citifluor fluorescent mounting media. Images were obtained using a Carl Zeiss Confocal Laser Scanning Microscope Model 710 (Carl Zeiss, Germany), based on the inverted optical microscope Axio Observer II, and the spectral detection system Meta 710 (Carl Zeiss). Digital image creation, 3-D rendering and image analysis were performed using Software Zen 2008 (Carl Zeiss). Optical sectioning was also undertaken (Brooker, 1991, 1995).

#### 2.2.2.6 *Assessment of the cleaning performance of the commercial protease products*

Cleaning was initially performed at an enzyme concentration of 0.05 units/ml (determined using whey protein gel as substrate as outlined in Section 2.2.2.1.1) and at the enzyme optimum pH (determined in Section 2.2.2.3), using the following buffers: 0.1 M $Na_2CO_3$–$NaHCO_3$ (pH 10 and pH 10.5) and 0.1 M $NaPO_4$–NaOH (pH 11.3). Enzyme cleaning solution was prepared by adding the enzyme product to the appropriate buffer (preheated to the cleaning temperature) immediately before cleaning. The cleaning solution was subsequently added to a 250-ml conical flask containing a fouled panel (obtained as outlined in Section 2.2.2.4) on its base and incubated at 40°C, 50°C or 60°C, at 110 rpm for 60 min. After this time the cleaning solution was transferred to a 100-ml volumetric flask. Two sequential rinse steps were performed in which 20 ml of distilled water at the cleaning temperature were added to the conical flask containing the panel and incubation was continued under similar conditions for 10 min. The rinse solutions were added to the original cleaning solution and diluted to a final volume of 100 ml with distilled water (Wash 1). A second wash of the panel was carried out using 1.0% (w/w) sodium hydroxide at 70°C for 1 h to

remove any remaining fouling. This solution is referred to as Wash 2. Wash 1 and Wash 2 were analysed for COD and total protein, as outlined in Sections 2.2.2.5.1 and 2.2.2.5.2, respectively. The COD of fresh cleaning solution was also measured and used to calculate the COD from fouling material in Wash 1. In order to assess enzyme stability during cleaning, the protease activity of the cleaning solution was determined as outlined in Section 2.2.2.1.2, immediately before and after the 60-min cleaning period. For comparative purposes, parallel cleaning studies were also performed using buffer only, distilled water only and 1.0% (w/w) sodium hydroxide in place of the enzymes.

#### *2.2.2.7 Long-term protease stability studies*

Studies on long-term enzyme stability in used cleaning solution were carried out by performing cleaning with the enzyme at 40°C, as described in Section 2.2.2.6. After the 60-min cleaning period, ampicillin was added to the cleaning solution to give a final concentration of 100 µg/ml cleaning solution, and incubation was continued at 40°C at 110 rpm. Samples were withdrawn at various time intervals over a period of 172 h and residual enzyme activity was measured, as described in Section 2.2.2.1.2. A parallel study was carried out in which the enzyme was incubated in buffer instead of cleaning solution in order to assess long-term enzyme stability in buffer. The buffer and enzyme concentration used were similar to those used to prepare the cleaning solution.

#### *2.2.2.8 Determination of lipase activity*

Lipase activity was determined using a modification of the methods described by Kwon and Rhee (1986) and Mateos *et al.* (2007), based on copper soap colorimetry. A 50-µl quantity of the enzyme sample was added to 3 ml of pasteurised, homogenised whole milk, pre-equilibrated to 40°C. Following vortexing, the reaction mixture was incubated at 40°C for 15 min. Then 1 ml of 6 M hydrochloric acid and 5 ml isooctane were added and the resulting mixture was vortexed for 2 min and placed in a boiling water bath for 5 min. The mixture was centrifuged at 4,000 rpm for 5 min and 2.5 ml of the upper isooctane layer containing the free fatty acids were removed and added to 0.5 ml of cupric acetate–pyridine reagent (5% (w/v) aqueous solution of cupric acetate adjusted to pH 6.1 with pyridine). The mixture was vortexed vigorously for 90 s and allowed to stand still until the aqueous phase had settled from the solution of isooctane and fatty acids. The absorbance of the latter was measured at 715 nm. A standard curve was constructed by adding 0.5 ml cupric acetate–pyridine reagent to 2.5-ml samples containing 0–25 µmol oleic acid in isooctane and measuring absorbance at 715 nm after vortexing and standing as described above. One unit of lipase activity was defined as the amount of enzyme that will release 1 µmol fatty acid/min under the assay conditions.

#### *2.2.2.9 Assessment of the cleaning performance of commercial lipase products*

Assessment of the cleaning performance of the commercial lipase products was undertaken as described in Section 2.2.2.6, with the following modifications. Cleaning was carried out at 40°C, with a final concentration of 1.0% (w/v) lipase product in the cleaning solution and with the addition of protease activity where indicated. Lipase stability during cleaning was assessed by measuring the lipase activity of the cleaning solution (Section 2.2.2.8) before and after the cleaning period. The fatty acids produced during cleaning were quantified by adding 1 ml of 6 M hydrochloric acid and 5 ml isooctane to a 10-ml sample of the cleaning solution with subsequent boiling and reaction with cupric acetate–pyridine reagent, as outlined for the lipase assay in Section 2.2.2.8.

#### *2.2.2.10 Confirmation of the removal/inactivation of all residual enzyme activity post enzyme-based cleaning*

The effect of rinsing on enzyme activity was determined by conducting cleaning as described in Section 2.2.2.6, with the following modifications. After the 60-min cleaning period, the used cleaning solution was removed and the cleaned panel was rinsed with five 20-ml aliquots of water. All rinsing solutions were analysed for protease activity (Section 2.2.2.1.2) and the panels were analysed by CLSM (Section 2.2.2.5.3) following two 20-ml rinses. Enzyme thermal stability was assessed by measuring residual protease activity after heating protease solutions at 80°C or 90°C for defined periods using a thermal cycler. Enzyme stability at low pH was determined by exposure to 0.5% or 1.0% nitric acid for defined periods, with subsequent readjustment of pH before determination of residual protease activity. The effect of sanitation chemicals on

enzyme activity was determined by separate exposure to hypochlorite and an iodine-based disinfectant for 1 and 5 min at the concentration recommended by the suppliers, followed by determination of residual protease activity.

## 2.3 Results and Discussion

### *2.3.1 Survey of Irish dairy co-ops and suppliers of cleaning products*

Contact was established with nine Irish dairy co-ops and two companies that provide CIP chemicals to the dairy processing industry. Details of CIP procedures currently employed were obtained from five of the dairy co-ops surveyed. The four other co-ops agreed to provide information but failed to do so despite follow-up contact. General information on CIP in the dairy industry was provided by the two suppliers of CIP products.

From the information obtained, it can be concluded that the CIP procedures undertaken within the Irish dairy processing industry are similar to those described in the literature and outlined in Section 1.4. Cleaning is achieved by circulation of sodium hydroxide or caustic formulated detergents, which also contain surfactants, sequesterants and other additives to improve cleaning. Exact details of the CIP undertaken in terms of concentration, temperature and time depend on the area of the plant being cleaned and the level of fouling. Cleaning of road tankers, raw milk lines and silos is generally carried out using 0.25–0.35% sodium hydroxide at 60–70°C for 5–15 min. Milk process tanks, pasteurised lines and silos are cleaned at 70–75°C for 12–20 min using 0.35–0.6% sodium hydroxide. The concentration of sodium hydroxide used to clean pasteurisers, which are associated with high levels of fouling, ranged from 0.6% to 2%, with cleaning times and temperatures ranging from 40 to 90 min and from 70°C to 80°C, respectively.

Conductivity-controlled detergent dosing is frequently employed. In the case of low fouling levels, where the caustic-based cleaning solution is relatively clean after use and above a preset conductivity value, the cleaning solution may be recovered to a CIP tank and reused. This practice is generally not carried out for washes of the pasteuriser and other components associated with high levels of fouling, in which case the cleaning solution is discarded after a single use.

Prevention of scale build-up is achieved by acid circulation, with all of the co-ops surveyed using nitric acid for this purpose, generally at 65–70°C and at a concentration of 0.8–1.0%. As outlined in Section 1.4, acid washing may be undertaken only periodically in the case of tanks, etc., but after every wash for heated components such as pasteurisers. In some cases, cold components are sterilised by a peracetic acid flush, in which case no acid washing is required.

This survey of co-ops and cleaning product suppliers provided useful information on the practical aspects of currently used CIP protocols from a user perspective. This information was used during subsequent experimental design and execution to ensure that the study proceeded in a user-relevant manner and that enzyme cleaning performance was assessed under application-relevant conditions. Typical CIP procedures, based on the information above, were compared with enzyme-based cleaning (Section 2.2.2.6) and the information obtained was also used to simulate industrial conditions in studies to confirm removal/inactivation of enzyme activity after cleaning (Section 2.2.2.10). Data received in relation to the costs associated with CIP were used in the cost analysis of enzyme-based cleaning (Section 2.3.5).

### *2.3.2 Suitability of currently available commercial protease products for CIP operations in the dairy processing industry*

#### *2.3.2.1 Selection of commercial proteases suitable for use in the study*

The range of protease products available commercially from the major industrial enzyme manufacturers was identified and the associated enzyme technical data were analysed to select the products most suitable for potential application in CIP. Various application-relevant parameters were considered, including the type of enzymatic activity, optimum conditions for enzyme activity, product storage stability, ease of enzyme inactivation after use, and cost. Details of the commercial protease products selected are shown in Table 2.1.

**Table 2.1. Details of commercial protease products assessed in the study. Data generated from company literature unless otherwise indicated.**

| Product name (company) | Enzyme type & IUB No.[1] | Source | Typical application | pH and temperature ranges and optima | Molecular weight and isoelectric point (pI) |
|---|---|---|---|---|---|
| **Alcalase 3.0 T (Novozymes)** | Subtilisin 3.4.21.62 | Produced by submerged fermentation of a selected strain of *Bacillus licheniformis* | • Detergent formulations (laundry and automatic dishwasher) to remove protein-based stains<br>• Meat processing<br>• Textiles | >80% activity pH 6–11.5 (opt. 7.5–9.0)[2]<br>>80% activity 50–65°C[2] | Mol. wt: 27.5 kDa[3] |
| **Liquanase 2.5 L (Novozymes)** | Subtilisin 3.4.21.62 | | • Liquid laundry detergents | Effective at 30°C | |
| **Savinase 6.0 T (Novozymes)** | Subtilisin 3.4.21.62 | Produced by submerged fermentation of a genetically modified *Bacillus* micro-organism | • Detergent formulations (laundry and automatic dishwasher) to remove protein-based stains<br>• Textiles | >80% activity pH 7.5–12.0 (opt. 9.0–10.0)[2]<br>>80% activity 40–55°C[2] | Wild-type subtilisin<br>Mol. wt: 28 kDa<br>pI 10[4] |
| **Esperase 8.0 L (Novozymes)** | Subtilisin 3.4.21.62 | Origin: *Bacillus clausii* (wild type);<br>Production strain: *Bacillus clausii*[6] | • Detergent formulations (laundry and automatic dishwasher) to remove protein-based stains | >80% activity pH 7.0–12.0 (opt. 8.5–9.5)[2,5]<br>>80% activity 55–65°C (opt. 60°C)[2,5] | |
| **Protex 6L (Genencor)** | Alkaline serine endopeptidase 3.4.21.62 | Derived from a selected strain of *Bacillus licheniformis* | • Baking<br>• Protein processing<br>• Silver recovery from film material<br>• Pet food production | pH 7.0–10.0 (opt. pH 9.5)[7]<br>25–70°C (opt. 60°C)[7] | Mol. wt: 22.5 kDa<br>pI 6.8 |
| **Protex 30L (Genencor)** | Alkaline serine endopeptidase 3.4.21.62 | Derived from a genetically modified strain of *Bacillus subtilis* | • Protein processing<br>• Hydrolysing proteins used in pet foods, meat or vegetable protein hydrolysis | pH 7.5–10.0 (opt. pH 8.5)[7]<br>20–65°C (opt. 55°C)[7] | |
| **Protex 40L (Genencor)** | High alkaline protease (subtilisin) 3.4.21.62 | Derived from a genetically modified strain of *Bacillus subtilis* | • Protein processing<br>• Heparin and chondroitin sulfate production<br>• Waste treatment<br>• Membrane cleaning | pH 6.0–10.0 (opt. pH 8.6)[7]<br>40–65°C (opt 65°C)[7] | Mol. wt: 28 kDa<br>pI 9.4 |
| **Protex 89L (Genencor)** | Serine endopeptidase 3.4.21.62 | Derived from a genetically modified strain of *Bacillus subtilis* | • Protein processing<br>• Waste treatment<br>• Silver recovery from film material | pH 5.5–10.0 (opt. pH 8.0)[7]<br>25–60°C (opt. 50°C)[7] | Mol wt: 28 kDa<br>pI 9.4 |

[1]Enzyme Classification number (International Union of Biochemistry, IUB).
[2]Substrate-denatured haemoglobin; deviations from these values are to be expected for other substrates.
[3]Walsh (2002).
[4]Maste et al. (1997).
[5]Eriksen (1996).
[6]Maurer (2004).
[7]Exact pH and temperature optima will depend on process variables including temperature (in the case of pH optima), pH (in the case of temperature optima), time, substrate nature and concentration.

The proteases selected are all classified as subtilisins (EC 3.4.21.62) belonging to the class of serine proteases. This class of enzymes catalyse the hydrolysis of proteins with broad specificity for peptide bonds and exhibit high stability (Maurer, 2004), which is desirable in the context of potential CIP application. Subtilisins are endoproteases (act internally in the protein chain) that result in a rapid decrease in protein molecular weight which is assumed to be beneficial in terms of the removal of adsorbed protein (Egmond, 1997). The products selected are used in a range of applications, as shown in Table 2.1. Several of the products selected were developed for use in laundry and automatic dishwasher detergents and have been shown to be effective in removing protein-based stains by hydrolysing the protein in stains into peptides which are easily dissolved or dispersed in the washing solution. Protex 6L is reported to be more effective if the protein to be hydrolysed is partially unfolded or denatured, which is the case in the current study.

The optimum pH and temperature conditions reported by the manufacturers were considered in selecting suitable enzymes for the study, with emphasis on enzymes displaying activity over broad temperature and pH ranges, thereby allowing flexibility of cleaning conditions. It is noteworthy that the optimum conditions reported by the manufacturers were determined using different substrates and reaction conditions than those which would be encountered during CIP application and therefore were only used as an indication of optimal conditions until application-relevant determinations were undertaken at a later stage (Sections 2.3.2.2 and 2.3.2.3).

Enzymes displaying optimum activity in neutral to alkaline pH conditions were selected in order to facilitate the possibility of using the enzymes with reduced quantities of sodium hydroxide (relative to that currently used in CIP) if satisfactory cleaning could not be achieved by the enzymes alone. Enzymes with pH optima greater than 10 were avoided as neutralisation of the resulting cleaning solution, if required prior to discharge to wastewater (Section 1.4.5), would require a high concentration of non-biodegradable salt (Grasshoff, 2005).

In order to minimise energy consumption during cleaning, enzymes displaying high activity at relatively low temperatures were selected. The product Liquanase is relatively new to the market (launched in 2006) and was developed specifically to achieve high cleaning performance in liquid laundry detergents at low temperatures. According to the manufacturer, Liquanase can remove dried blood and other difficult protein stains at 30°C and is effective across a range of water hardness levels and against both normal and high soil loads. The other enzymes selected also display high activity at temperatures significantly less than those currently used in CIP operations, providing potential for reduced energy consumption.

Enzyme stability during use and storage was also considered in the selection process. Based on a review of the product technical data, none of the products selected are likely to be significantly inactivated by conditions encountered during CIP within the recommended pH and temperature conditions. The stability of Alcalase and Savinase is reported to be enhanced in the presence of protein and peptides in laundry stains. Calcium ions, which would almost certainly be present in the CIP washing liquor, are listed as an activator/cofactor for Protex 40L. The enzyme products are formulated to maximise storage stability, with enhanced stability observed at lower storage temperatures. Protex 40L is reported to lose less than 13% of its activity per year at 35°C and maintains its activity for a minimum of 24 months under refrigerated conditions. An activity loss of less than 14% and 20% per year is reported for Protex 30L and Protex 89L, respectively, when stored at 25°C.

Due to the importance of enzyme inactivation/removal after cleaning (Section 2.3.4), ease of enzyme inactivation was also considered in selecting enzymes for use in the study. Several of the enzymes selected are thermolabile, indicating that they would almost certainly be inactivated by the high temperatures encountered during heat sanitation frequently carried out following cleaning in dairy processing. Based on the product technical data, some of the selected enzymes are also likely to be inactivated by the low pH conditions experienced upon circulation of acid for removal of mineral deposits and/or for sanitation purposes after cleaning.

All the enzymes selected are produced and sold in bulk industrial quantities by Novozymes and Genencor, two major players in the industrial enzyme market with global supply chains, and could therefore be readily obtained if found to be suitable for CIP application. Although the cost of enzyme cleaning solutions ultimately depends on the required use concentration and was determined at a later stage in the study (Section 2.3.5), cost per tonne enzyme product was considered during initial product selection. As emphasis was on developing an economically viable enzyme-based cleaning procedure, high-value speciality proteases, such as those developed for the food industry, were avoided.

There are reports in the literature on the use of two of the enzyme products selected for removal of milk-based fouling. Investigation of the use of Alcalase and Savinase to remove milk soil deposits has been previously described by Grasshoff (2002, 2005) but with assessment of cleaning by visual inspection only. Under the conditions tested, a clean surface was obtained with Savinase but not with Alcalase. The enzyme-based cleaning method P3-paradigm used to clean the cold milk zone in some German dairies, as described in Section 1.5.4.4, contains both Savinase and Alcalase. In addition, Arguello et al. (2002) investigated the use of Alcalase to clean inorganic membranes fouled with whey protein solutions and reported high cleaning efficiencies although residual matter was observed on the membrane surface after cleaning.

Due to commercial sensitivity, the eight protease products selected for use in the study were randomly labelled P1 to P8 and are referred to using these designations in all subsequent sections.

#### *2.3.2.2 Effect of temperature on enzyme activity*

In order to determine the most suitable temperatures for subsequent enzyme cleaning studies, the effect of temperature on the activity of each commercial product was determined using application-relevant assay conditions. In CIP application, the enzyme is not acting on a soluble substrate in solution but on a predominantly water-insoluble substrate bound to a solid surface. In addition, the protein in the fouling layer is denatured which could potentially render it less accessible to enzymatic hydrolysis than the undenatured form. In order to simulate the conditions encountered during CIP application in industry, the effect of temperature on enzyme activity was determined using a substrate produced by thermal gelation of concentrated whey protein solution. Whey protein gels have been used in several cleaning studies to simulate milk fouling and their morphology and cleaning properties are reported to be similar to those of industrial milk deposits (Xin et al., 2002a,b; Hooper et al., 2006; Yoo et al., 2007). The ability of the commercial enzymes to degrade the whey protein gel was assessed at different temperatures by determination of the free amino groups produced during substrate hydrolysis using the TNBS method (Adler-Nissen, 1979). This method is based on the spectrophotometric detection of the chromophore produced upon reaction of TNBS with primary amines (Adler-Nissen, 1979) and is more suitable for analysis of whey protein hydrolysis than other available methods (Samples et al., 1984; Spellman et al., 2003). The temperature versus activity profiles of the commercial protease products are shown in Fig. 2.1.

Of the products assessed, five displayed maximum activity at 60°C and three displayed maximum activity at 70°C. Although decreased activity was observed at the lower temperatures tested, the products assessed displayed 17–36% and 35–66% of their maximum activity at 40°C and 50°C, respectively. This indicates the possibility of enzyme-based cleaning at temperatures significantly less than those currently employed (Section 1.4), which would be beneficial in terms of energy consumption. The temperature profiles generated differ from those reported by the enzyme manufacturers (Table 2.1), which may be attributed to different substrate and assay conditions. It is noteworthy that significant differences were also observed when temperature profiles were generated using 6% (w/v) whey protein isolate solution as substrate instead of the whey protein gel (data not shown). This highlights the importance of assessing the enzymes under application-relevant conditions and a similar whey protein gel was used in subsequent studies to assess the effect of pH on enzyme activity.

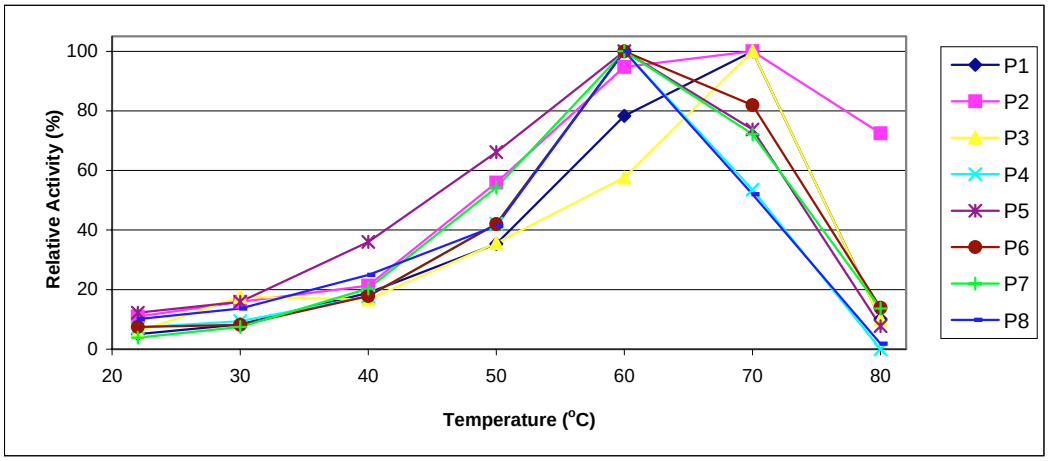


**Figure 2.1. Temperature versus activity profiles of commercial proteases, generated using whey protein gel as substrate, as described in Section 2.2.2.2. Data are expressed as a percentage of the maximum value. Each value represents the mean (n = 3) but error bars have been omitted for clarity.**

*2.3.2.3 Effect of pH on enzyme activity*

Figure 2.2 illustrates the effect of pH on the activity of the commercial protease products. Six of the products assessed displayed maximum activity at pH 10.5, with the other two products displaying maximum activity at pH 10 and pH 11.3. Decreased activity was observed at the lower pH values tested. Some of the enzymes assessed (for example P8) displayed a high proportion of their maximum activity over a broad pH range, indicating potential flexibility in terms of cleaning solution pH. The pH optima observed were generally higher than those reported by the manufacturer (Table 2.1), which may be attributed to the different substrate and assay conditions used. In the case of laundry detergent proteases, improved enzyme performance has been reported at pH values close to the isoelectric point of the protease due to improved enzyme adsorption to the protein stain (Eriksen, 1996; Egmond, 1997). Of the four commercial products assessed with reported isoelectric points (Table 2.1), three were found to display maximum activity at pH values close to their isoelectric points.

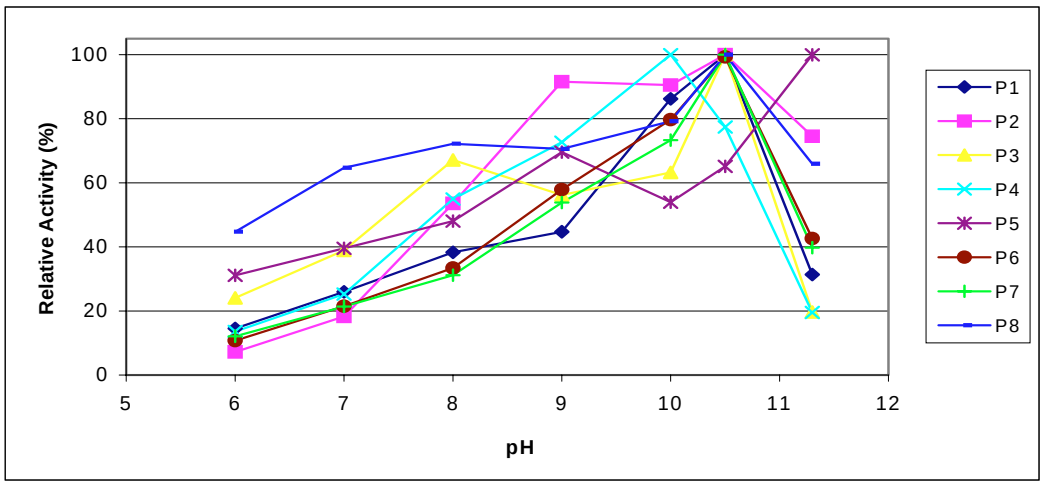


**Figure 2.2. pH versus activity profiles of commercial proteases, generated using whey protein gel as substrate, as described in Section 2.2.2.3. Data are expressed as a percentage of maximum value. Each value represents the mean (n = 3) but error bars have been omitted for clarity.**

#### 2.3.2.4 *Generation and analysis of milk fouling deposit on stainless steel panels*

A procedure for generating a fouling deposit on the surface of stainless steel panels was developed. As this fouling deposit would be used in subsequent experiments to assess the cleaning efficiency of enzymes/current CIP agents, it was essential that it would be reproducible and representative of the fouling occurring in industry. Based on a review of the literature published to date on the mechanism of fouling in dairy processing (Jeurnink et al., 1996; Visser and Jeurnink, 1997; Bansal and Chen, 2006), it was concluded that the generation of an industrial-like fouling deposit would require the use of raw, untreated whole milk, as pasteurised milk would produce an altered and predominantly mineral deposit due to pre-denaturation of the whey proteins.

Various experimental set-ups were tested in which such milk was heated (at industrially relevant temperatures) in the presence of the stainless steel panels. In some cases, this resulted in poor fouling which was easily removed by rinsing with water. Severe fouling (which could not be removed with water) was achieved when the stainless steel panels were in almost direct contact with the heat source and, based on this finding, an optimised procedure, outlined in Section 2.2.2.4 was developed. A stainless steel panel fouled by this method is shown in Fig. 2.3.

The amount of fouling on each panel was quantified by complete removal of all fouling material from the panel with subsequent analysis of the COD and protein concentration of the wash solution. COD is a measure of the oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant (APHA, 1995) and was used to quantify the amount of organic material on the fouled panels. As the protein removed from the panel is thermally denatured, the resolubilisation procedure described in Section 2.2.2.5.2 was necessary to facilitate accurate quantification of protein concentration by the Bradford assay. Using these approaches, the total amount of protein and organic material associated with panels fouled using the procedure outlined in Section 2.2.2.4 was 50 ± 10 mg/panel and 400 ± 85 mg/panel, respectively.

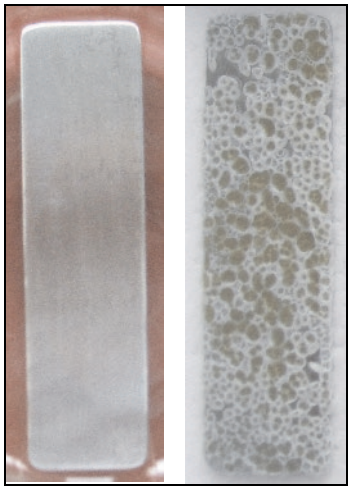


**Figure 2.3. Stainless steel panel (a) before and (b) after fouling, using the procedure outlined in Section 2.2.2.4.**

An image of the fouling deposit from SEM is shown in Fig. 2.4.

In SEM, a spatial image is generated due to the reflection of secondary electrons from the sample (Lee et al., 2005). SEM provides information on the structure and surface morphology of the fouling deposit and is frequently used to analyse fouling (Chan and Chen, 2004; Zator et al., 2007).

The milk fouling deposit generated was also analysed by CLSM (Fig. 2.5) to obtain information on its composition. This technique has previously been used

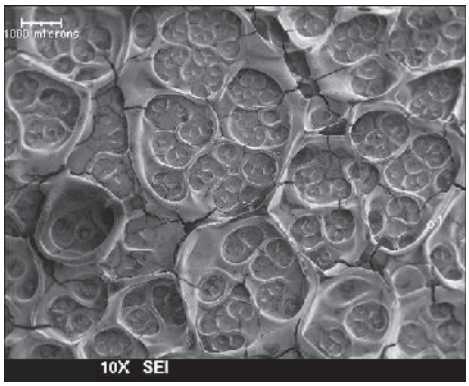


**Figure 2.4. Image from scanning electron microscopy showing surface morphology of milk fouling deposit generated on stainless steel panel.**

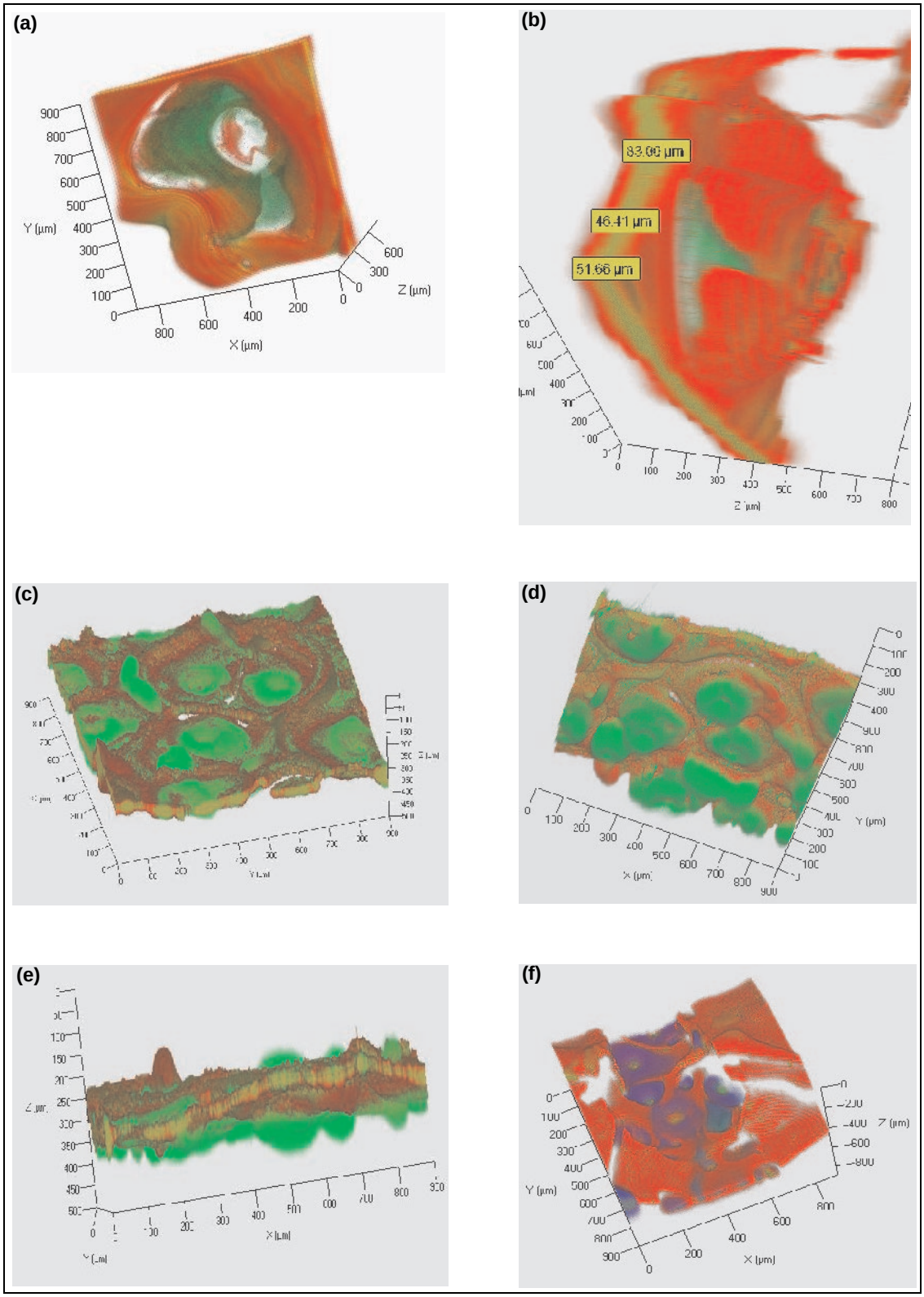


**Figure 2.5. Confocal laser scanning microscopy images of milk fouling deposit (refer to text for interpretation of colour). Images (a) and (b): staining for protein and lipid. Image (a) shows the view from the top of the fouling deposit. Image (b) shows the view from the side of the deposit, with the top of the deposit on the left. Images (c)–(f): Image of fouling deposit from different angles after staining for protein, lipid and polysaccharide.**

to study fouling and to examine the effectiveness of cleaning procedures (Siu et al., 2006; Le-Clech et al., 2007; Yang et al., 2007).

CLSM is a powerful technique that can be used to visualise and identify different constituents in a fouling deposit based on the emission of fluorescent light following treatment with specific stains or fluorochromes (Chan and Chen, 2004; Ferrando et al., 2005). Optical sectioning of the sample can also be carried out to obtain information about the pattern and composition of the fouling layer at different depths without physical slicing of the sample. This information can subsequently be combined to construct a 3-D image of the fouling deposit (Ferrando et al., 2005; Zator et al., 2007).

In the present study, the fouling deposit was stained for protein, lipids and polysaccharides and in the resulting images, red represents collected emission of the fluorescent dye with binding affinity to the hydrophobic domains of the lipids and proteins, green represents detected emission of the fluorescent dye with binding affinity to the amino group of proteins and blue is due to emission from fluorescent dye which binds to carbohydrate with slight input from autofluorescent material.

The CLSM images shown in Fig. 2.5 illustrate the composition and structure of the milk fouling deposit generated on the stainless steel panels. The deposit contains a large amount of protein and lipid with polysaccharide also evident in some areas. Distinct layering patterns were not observed but rather the fouling deposit appeared as a matrix of protein and fat with some embedded polysaccharide. Similar observations were also made upon analysis of the internal structure of the fouling deposit at different depths, with some small amounts of fat observed dispersed within the protein material.

#### *2.3.2.5 Assessment of the cleaning performance of the commercial proteases and selection of the most suitable commercial proteases for potential use in CIP*

A series of cleaning studies was carried out at 40°C, 50°C and 60°C, using the eight commercial protease products, 1% sodium hydroxide solution and distilled water or buffers, as outlined in Section 2.2.2.6. For six of the eight commercial protease products assessed (P2, P3, P4, P6, P7 and P8), visibly clean surfaces were observed after cleaning at all three temperatures (Fig. 2.6) and very little or no protein and less than 0.5% residual organic matter were detected on the panels after cleaning (Table 2.1).

When the enzymes were omitted from the cleaning solution and the panels were cleaned under similar conditions using buffer at pH 10 or pH 10.5 only, substantial fouling was observed on the panels, with subsequent quantification indicating the presence of up to 59% of the original organic matter and 27 mg of protein. These results confirm that the proteases are capable of removing milk fouling deposit from the surface of the stainless steel panels under the conditions tested.

Significant fouling (16.59%, 14.71% and 6.08% of original organic matter at 60°C, 50°C and 40°C, respectively) was observed after cleaning with P5. The poor performance of this product can be attributed, at least in part, to its poor stability at higher temperatures, with the enzyme retaining only 21% and 1% of its original activity after cleaning for 1 h at 50°C and 60°C, respectively (Fig. 2.7). As this enzyme displayed only 3% of its activity after 30 min at 60°C, much of the cleaning observed at this temperature is likely due to the sodium hydroxide component of the pH 11.3 buffer used with this enzyme, which removed a significant amount of fouling when used without added enzyme (Table 2.2). At 40°C, the contribution of the buffer sodium hydroxide to cleaning is reduced due to low temperature and the improved cleaning performance of P5 may be attributed to improved enzyme stability. On the basis of its poor stability and cleaning performance, P5 was considered to be unsuitable for potential CIP application and was eliminated from further studies. Cleaning with P1 was also not entirely satisfactory due to the observation of traces of fouling on the panels after cleaning and the detection of a small amount of organic matter. P1, however, has greater potential for CIP application than P5 on the basis if its cleaning performance, stability and cost and therefore assessment of this product was continued.

Figure 2.7 shows the amount of enzyme activity detected in the cleaning solution after 30 and 60 min of

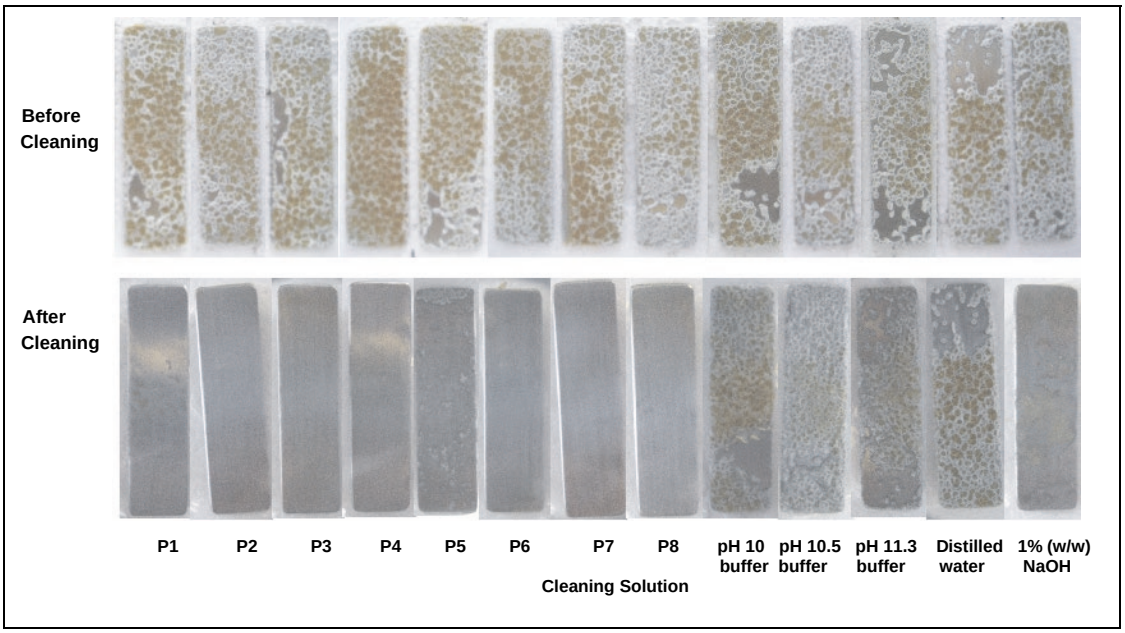


**Figure 2.6. Stainless steel panels before and after cleaning at 40°C with commercial protease products (P1–P8), buffers, distilled water and 1% (w/w) sodium hydroxide.**

**Table 2.2. Residual organic matter and protein not removed by cleaning with 0.05 units/ml commercial proteases (P1–P8), buffers, distilled water and 1% (w/w) sodium hydroxide (NaOH) at 40°C, 50°C and 60°C using the procedure outlined in Section 2.2.2.6.**

| Cleaning solution | Organic matter (COD) not removed (% of total before cleaning) Mean ± SD (n = 2) | | | Protein not removed (mg) Mean ± SD (n = 2) | | |
|---|---|---|---|---|---|---|
| | 60°C | 50°C | 40°C | 60°C | 50°C | 40°C |
| **P1 (pH 10.5)** | 0.11 ± 0.00 | 0.18 ± 0.07 | 0.42 ± 0.00 | 0.10 ± 0.02 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **P2 (pH 10.5)** | 0.11 ± 0.15 | 0.12 ± 0.04 | 0.10 ± 0.00 | 0.23 ± 0.09 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **P3 (pH 10.5)** | 0.12 ± 0.00 | 0.47 ± 0.55 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **P4 (pH 10.0)** | 0.17 ± 0.06 | 0.10 ± 0.02 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **P5 (pH 11.3)** | 16.59 ± 0.35 | 14.71 ± 0.72 | 6.08 ± 0.18 | 5.38 ± 0.26 | 0.37 ± 0.06 | 0.00 ± 0.00 |
| **P6 (pH 10.5)** | 0.00 ± 0.00 | 0.01 ± 0.01 | 0.23 ± 0.21 | 0.21 ± 0.20 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **P7 (pH 10.5)** | 0.08 ± 0.12 | 0.09 ± 0.08 | 0.04 ± 0.03 | 0.25 ± 0.08 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **P8 (pH 10.5)** | 0.00 ± 0.00 | 0.09 ± 0.05 | 0.20 ± 0.01 | 1.12 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| **pH 10.0 buffer** | 48.02 ± 0.17 | 54.17 ± 0.11 | 48.15 ± 0.50 | 14.75 ± 1.24 | 27.04 ± 0.48 | 19.39 ± 1.15 |
| **pH 10.5 buffer** | 45.96 ± 0.25 | 46.21 ± 0.23 | 58.82 ± 0.88 | 12.98 ± 0.96 | 21.32 ± 1.58 | 9.86 ± 2.06 |
| **pH 11.3 buffer** | 27.06 ± 0.41 | 46.50 ± 2.68 | 44.96 ± 2.10 | 7.44 ± 0.62 | 20.97 ± 0.96 | 8.41 ± 0.46 |
| **Distilled water** | 92.01 ± 0.23 | 83.28 ± 0.34 | 95.63 ± 0.08 | 40.11 ± 5.12 | 52.44 ± 2.22 | 37.45 ± 0.12 |
| **1% (w/w) NaOH** | 0.10 ± 0.02 | 0.27 ± 0.01 | 1.54 ± 0.08 | 0.00 ± 0.00 | 0.21 ± 0.04 | 1.02 ± 0.01 |

COD, chemical oxygen demand.

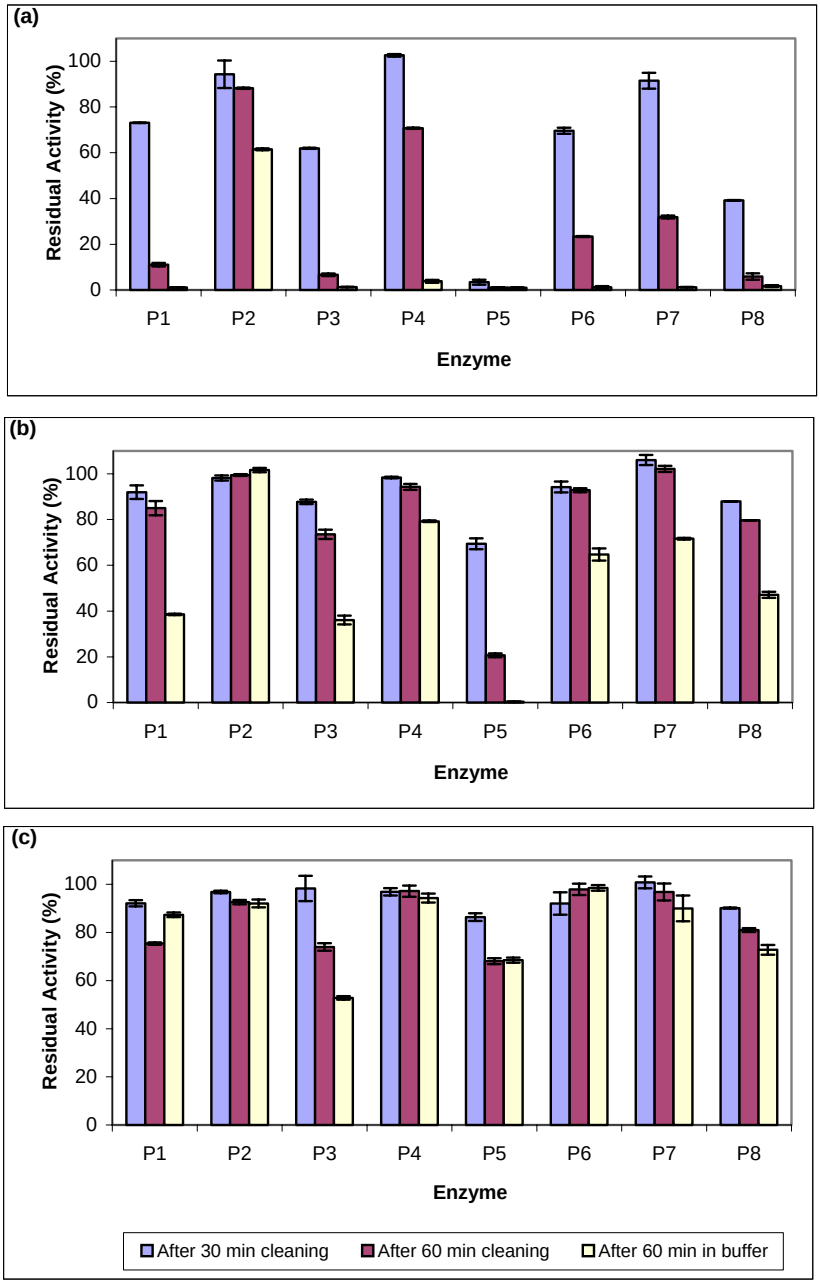


**Figure 2.7. Enzyme stability during cleaning at (a) 60°C, (b) 50°C and (c) 40°C. Residual enzyme activity determined after 30 and 60 min cleaning and after 60 min in buffer at each temperature. Residual activity expressed as a percentage of original activity before cleaning. Each value represents the mean ± SD (n = 2).**

cleaning at each temperature and the amount of activity detected after incubating each enzyme for 60 min in the buffer used for cleaning. Cleaning temperature was found to have a significant effect on enzyme stability, with reduced stability observed at higher temperatures. With the exception of P5, the proteases assessed retained 6–88%, 73–100% and 74–100% of their original activity after the 60-min cleaning period at 60°C, 50°C and 40°C, respectively.

Improved stability was observed in the presence of cleaning solution relative to incubation in buffer only. This may be attributed to the accumulation of protein and peptides in the cleaning solution during the cleaning process which may bind to the native conformation of the enzyme exerting a thermo-

protective effect. As discussed in Section 2.3.2.1, improved stability in the presence of protein and peptide laundry stains has been reported for some of the products assessed. Improved thermal stability of non-protease enzyme activity in the presence of increased protein concentration has also been previously reported (Hrmova and Fincher, 1993). Upon comparison of the residual enzyme activity detected in the cleaning solution relative to that in buffer, it can also be concluded that the fouling material removed during cleaning does not appear to exert an inhibitive effect on enzyme activity under the conditions used in the present study.

For comparative purposes, cleaning was also carried out using water only and 1% (w/w) sodium hydroxide, the most commonly used cleaning agent in the dairy processing industry (Section 1.4). Very little fouling was removed when cleaning was performed using water only, with 83–96% of original organic matter detected on the panels after cleaning. A visibly clean panel was observed and approximately 0.1% of original organic matter was detected after cleaning with 1% (w/w) sodium hydroxide at 60°C. At the lower temperatures tested, the amount of organic matter and protein detected on the panels after cleaning with sodium hydroxide increased, indicating reduced cleaning performance and, after cleaning at 40°C for 60 min, fouling was observed on the panel. At 40°C, the cleaning performance of all of the proteases tested, with the exception of P5, was better than that of 1% sodium hydroxide, as judged by the amount of organic matter and protein detected after cleaning. In addition, the cleaning performance of at least four of the proteases at 40°C was comparable to that of sodium hydroxide at 60°C, indicating that some of the protease products assessed display realistic potential to not only replace sodium hydroxide but also to achieve satisfactory cleaning at 40°C, hence reducing the energy consumption and overall environmental impact of the cleaning process.

While satisfactory enzyme-based cleaning was observed at all three temperatures, cleaning at 40°C and 50°C was most favourable in terms of enzyme stability. The enzymes assessed are more active at 50°C than at 40°C (Fig. 2.1) and therefore less enzyme product is required to achieve satisfactory cleaning at 50°C. However, cleaning at 40°C requires less energy and was therefore used as the cleaning temperature for all subsequent cleaning studies. The minimum amount of each enzyme product required for satisfactory cleaning at 40°C was determined by assessing the cleaning performance of each enzyme, as described in Section 2.2.2.6, using decreasing concentrations of the enzyme. This is important from an economic standpoint as using less than the minimum amount required could result in poor cleaning performance while using excess amounts of the enzyme will result in increased product costs. The minimum amount of each enzyme required for satisfactory cleaning and the corresponding cost per litre cleaning solution are shown in Table 2.3. The cost per litre cleaning solution was calculated using prices

**Table 2.3. Minimum concentration of each enzyme product required for satisfactory cleaning and cost of the resulting cleaning solution.**

| Enzyme product | Enzyme activity (units/ml cleaning solution)[1] | Cost (€/l cleaning solution) |
|---|---|---|
| **P1** | 0.125 | 0.330 |
| **P2** | 0.05 | 0.074 |
| **P3** | 0.025 | 0.294 |
| **P4** | 0.025 | 0.015 |
| **P5** | Not determined | |
| **P6** | 0.05 | 0.022 |
| **P7** | 0.025 | 0.015 |
| **P8** | 0.05 | 0.117 |

[1]Determined as described in Section 2.2.2.1.1.

obtained from the relevant enzyme suppliers for bulk quantities of each enzyme.

Based on cleaning performance, enzyme stability and cost per litre cleaning solution, the three most suitable products for potential CIP application were identified to be P4, P6 and P7. All further studies focussed on these three products.

#### *2.3.2.6 Further analysis of the three most suitable proteases*

##### *2.3.2.6.1 Analysis of cleaning performance by CLSM*

CLSM was undertaken to confirm the cleaning performance of P4, P6 and P7. Cleaning was carried out for 60 min at 40°C, as described in Section 2.2.2.6, at the optimum pH and enzyme concentration determined for each product. After the 60-min cleaning period, the used cleaning solution was removed and the enzymatically cleaned panels were rinsed with two 20-ml aliquots of water at 40°C, prior to analysis by CLSM as outlined in Section 2.2.2.5.3. A fluorescent signal due to the presence of protein was not detected from any of the panels examined, confirming that the three proteases tested were capable of removing all proteinaceous fouling material from the panels during cleaning. Upon analysis of the panel cleaned using P4, no fluorescent signal due to the presence of lipid or polysaccharide material was detected, confirming satisfactory cleaning by this product. In the case of P6 and P7, satisfactory cleaning was also confirmed with the exception of a very small region in the centre of the panels where a trace amount of residual fouling was detected, consisting of only lipid in the case of P7 and mostly polysaccharide in the case of P6. It is noteworthy that the area where this residual fouling was detected is at an identical location at the centre of both panels where there would have been less exposure to the enzyme cleaning solution and at a reduced turbulence relative to the rest of the panel, due to the orbital motion of the shaking incubator in which the cleaning experiments were performed. On this basis, it can be concluded that the rest (and indeed majority) of the panel, where no residual fouling was detected, is a more reliable indication of enzyme cleaning performance. The type of residual fouling detected (lipid and polysaccharide) indicates that the proteases, as expected, are more efficient at removing proteinaceous fouling material.

##### *2.3.2.6.2 Effect of pH on cleaning performance*

Cleaning studies were carried out at pH 6 to pH 11.3, as described in Section 2.2.2.6, using P4, P6 and P7, in order to assess the effect of pH on cleaning performance and to confirm that the optimum pH for the removal of milk fouling from stainless steel was similar to the enzyme pH optimum determined by enzyme assay, using denatured whey protein gel as substrate, as outlined in Section 2.2.2.3. For these experiments, the enzyme concentration used was half of the optimum concentration determined in Section 2.2.2.6 in order to facilitate comparison of cleaning performance at different pH values by direct visual observation and quantification of residual fouling after cleaning. Enzyme stability during cleaning at each pH was also assessed. The results generated are shown in Fig. 2.8.

P4, P6 and P7 exhibited optimal cleaning at pH 10–10.5, pH 10 and pH 10.5, respectively. These results are similar to the optimum pH values determined earlier using the whey protein gel enzyme assay, with the exception of P6 which displayed maximum activity at pH 10.5 in the enzyme assay but slightly better cleaning performance at pH 10. These results confirm the suitability of the whey protein gel assay for assessment of proteases for this application. The three enzymes were relatively stable during cleaning at pH 6 to pH 10.5, displaying in excess of 85% of their original activity after the 60-min cleaning period. Reduced stability was observed at pH 11.3 with P4, P6 and P7 retaining 88%, 72% and 73%, respectively, original activity after cleaning. For all three enzymes, poor cleaning performance was observed at pH 6, which is most likely attributable to the low activity of these enzymes at this pH, as shown in Fig. 2.2. The pH of the cleaning solution had a greater effect on the cleaning performance of P6 than on that of P4 and P7. The cleaning performance of P6 decreased significantly at pH values outside pH 10–10.5, whereas P4 and P7 maintained their cleaning performance over a broader pH range, as shown in Fig. 2.8. This indicates that P4 and P7 are likely to offer more flexibility in terms of cleaning solution pH, which would be advantageous in the context of industrial application as cleaning agents.

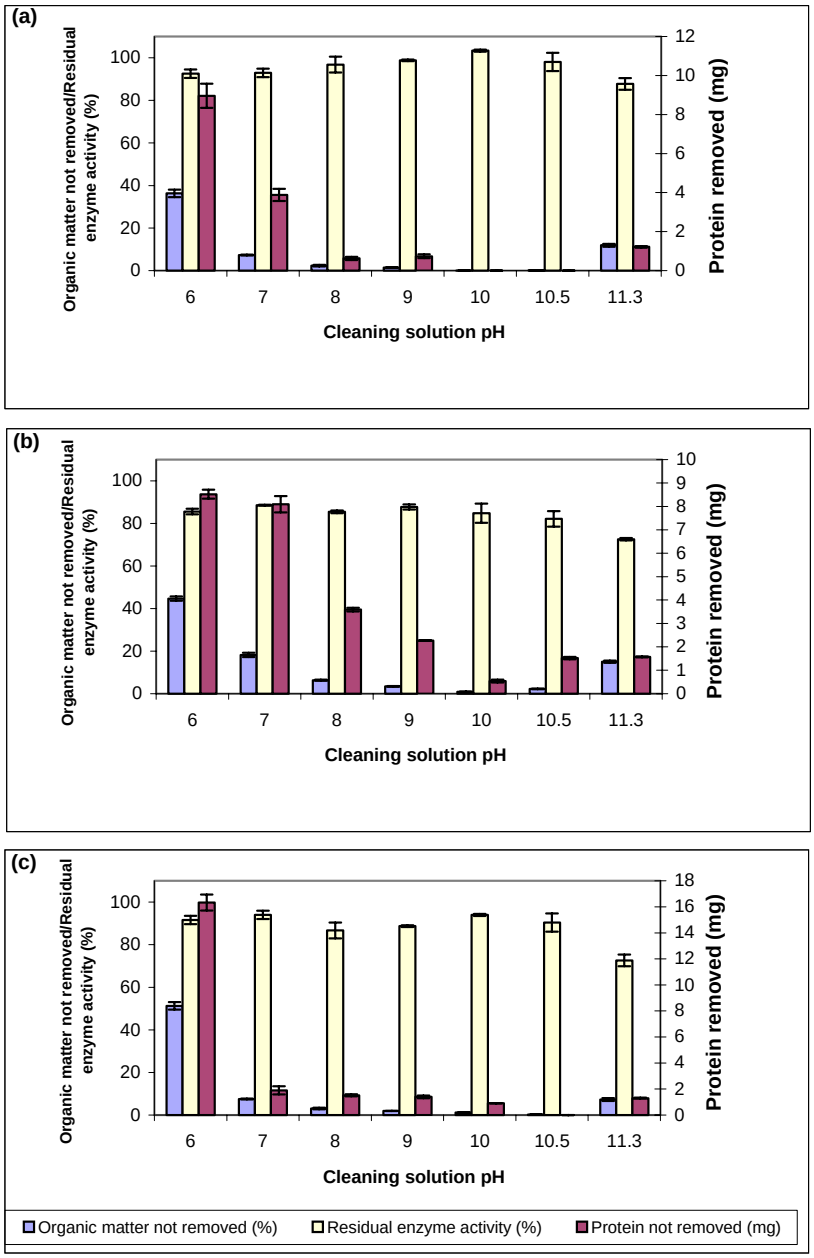


**Figure 2.8. Results of cleaning studies undertaken at different pH values using (a) P4, (b) P6 and (c) P7. Each graph shows residual organic matter and protein not removed after 60 min cleaning at 40°C at each pH and residual enzyme activity detected after cleaning. Residual organic matter is expressed as a percentage of the total amount of organic matter on the panel before cleaning. Residual enzyme activity is expressed as a percentage of the enzyme activity of the cleaning solution before cleaning. Each value represents the mean ± SD (n = 2).**

*2.3.2.6.3 Long-term protease stability studies*

Long-term enzyme stability studies were undertaken as described in Section 2.2.2.7 to ascertain the stability of P4, P6 and P7 when incubated separately in buffer and used cleaning solution at 40°C for periods longer than those assessed during the initial stability studies undertaken during assessment of cleaning performance. Long-term stability in buffer at 40°C is desirable in the context of CIP application as it would facilitate the preparation of the cleaning solution well in advance of commencing CIP if necessary for practical or operational reasons and is also an indication of the

long-term storage stability of the enzyme product. The stability of the enzyme in used cleaning solution (i.e. containing fouling material already removed from process equipment) was assessed to determine if the enzyme cleaning solution could potentially be reused, as is carried out in some cases with caustic cleaning solutions. In addition, long-term enzyme stability in the presence of cleaning solution would facilitate its use over longer cleaning periods, if necessary, for example where severe fouling is present.

Upon incubation in buffer at 40°C, the three enzymes retained in excess of 92%, 84% and 82% of their original activity after 1, 2.5 and 5 h, respectively. While the stability of the three enzymes was similar during the initial period, P7 was found be less stable over longer periods retaining 45% of its activity after 24 h in comparison with over 64% in the case of P4 and P6. After 100 h at 40°C, P4, P6 and P7 retained 35%, 18% and 34% of their original activity, respectively. Stability during the shorter incubation periods is more relevant to industrial CIP application and indicates that immediate use of the enzyme cleaning solution after preparation/heating would not be strictly necessary, providing some scope for flexibility in the timing of the CIP procedure, if required.

The results of long-term stability studies in the presence of used cleaning solution are shown in Fig. 2.9. Enhanced stability was observed in the presence of the used cleaning solution relative to buffer, with all three enzymes retaining over 80% of their original activity after 24 h. The observed losses of activity may be attributed to self-hydrolysis of the enzymes, denaturation of the enzymes caused by shear stress during shaking for extended periods, and/or adsorption of the enzyme onto the glass and stainless steel surfaces (Arguello et al., 2003). While these results are promising in the context of potential reuse of the enzyme cleaning solutions, actual reusability will depend on the cleaning conditions used, the level of fouling encountered and storage conditions between cleanings and can only be fully determined by pilot- and industrial-scale studies.

### *2.3.3 Suitability of currently available commercial lipase products for CIP operations in the dairy processing industry*

#### *2.3.3.1 Selection of commercial lipase products suitable for use in the study*

Two commercial lipase products (details shown in Table 2.4) were selected based on the same criteria outlined for the commercial protease products in Section 2.3.2.1.

Lipolase was the first commercial recombinant lipase product launched and its use in detergents for the removal of greasy food, cosmetic and body soil stains expanded rapidly in the late 1980s (Wolff and Showell,

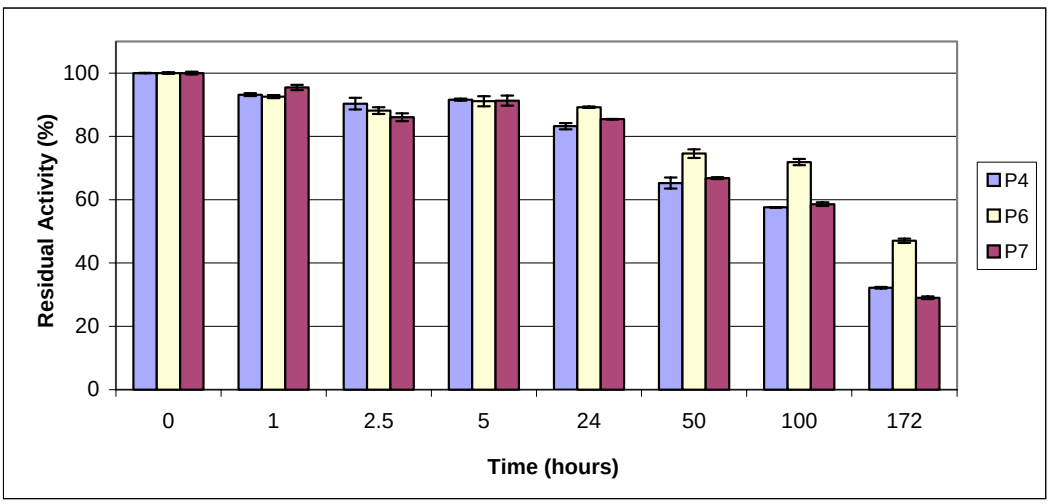


**Figure 2.9. Stability of P4, P6 and P7 when incubated at 40°C in used cleaning solution (i.e. containing fouling material). Each value represents the mean ± SD (n = 2).**

**Table 2.4. Details of commercial protease products assessed in the study. Data generated from company literature.**

| Product name (company) | Enzyme type and IUB No.[1] | Source | Typical application | pH and temperature ranges and optima | Molecular weight and isoelectric point |
|---|---|---|---|---|---|
| **Lipolase 100 T (Novozymes)** | 1,3-specific lipase 3.1.1.3 | *Humicola lanuginosa* lipase expressed in *Aspergillus oryzae* | • Used in detergent formulations to remove fat-containing stains | >80% activity at pH 10–11 (opt. pH 11)[2] >80% activity 18–48°C (opt. ~30°C)[2] | Mol. wt: 27 kDa pI 4.3 |
| **Lipex 100 L (Novozymes)** | 3.1.1.3 | Third variant of Lipolase obtained by directed evolution technology | • Used in detergent formulations to remove fat-containing stains | Effective under neutral to alkaline conditions and works well at temperatures as low as 20°C | |

[1]Enzyme classification number (International Union of Biochemistry, IUB).
[2]Determined using a pH-stat method with tributyrin as substrate, deviations are to be expected with different substrates and reaction conditions.

1997; Sharma et al., 2001). Lipolase has a broad substrate specificity and hydrolyses a range of triglycerides into mono- and diglycerides, glycerol and free fatty acids, which are more soluble in water than the original fats at a temperature below the fat's melting point (Wolff and Showell, 1997). The enzyme is a 1,3-specific lipase and therefore cleaves the ester bonds at positions 1 and 3 of the triglyceride. Lipolase is associated with the 'multicyle effect', whereby four to five washing cycles with intermediate drying are required for complete removal of fat stains from fabrics. This has been attributed to increased enzyme activity during the reduced water content of the drying phase (Misset, 1997). Lipex, a variant of Lipolase launched in 2002, has been developed to achieve effective removal of fatty stains in the first wash as well as deep cleaning inside the cloth fibres (Houde et al., 2004).

The pH and temperature profiles reported for both products are compatible with those of the commercial proteases used in the study, although deviations from the optimal conditions reported may be observed under CIP conditions, as discussed for the commercial proteases. Both lipases are active at low temperatures with Lipex reported to be capable of removing fat stains at 20°C, which is favourable in terms of reduced energy consumption during CIP operations.

The use of Lipolase to clean polysulfone ultrafiltration membranes fouled in abattoir effluent, containing proteinaceous substances and lipids, has been previously reported (Allie et al., 2003). A 46% reduction of the lipid content on the membrane was observed following cleaning with 1.15% (w/v) Lipolase and 0.1% (v/v) Triton X-100. Due to commercial sensitivity, the two lipase products were randomly labelled L1 and L2 and are referred to using these designations in all subsequent discussion.

#### *2.3.3.2 Assessment of the cleaning performance of the commercial lipases*

The cleaning performance of the commercial lipases was initially assessed in cleaning solutions containing 1.0% (w/v) lipase (L1 or L2) and P4, P6 or P7 at 25% of the concentration determined for satisfactory cleaning in Section 2.3.2.5 (0.00625, 0.0125 and 0.00625 units/ml, respectively). Cleaning was carried out for 60 min at 40°C, as described in Section 2.2.2.9, at the optimum pH of the protease employed. The lipase activity of a 1% (w/v) solution of L1 and L2 was determined to be 39.09 ± 5.53 units/ml and 37.58 ± 1.21 units/ml, respectively, using milk as substrate as outlined in Section 2.2.2.8.

The total amount of organic matter removed from the panels during cleaning with 1.0% (w/v) L1 or L2 alone (140 ± 44 and 144 ± 19 mg $O_2$, respectively) was comparable to the amount removed during cleaning

with buffer only (148 ± 8 to 178 ± 16 mg $O_2$). When cleaning was carried using the proteases at the reduced concentrations described above, residual organic matter (up to 3% of the original amount) and protein (up to 1.8 mg) were detected on the panels after cleaning. When cleaning was carried out with inclusion of 1.0% (w/v) L1 or L2 in the protease cleaning solutions, improved cleaning was not observed with similar levels of residual fouling detected on the panels after cleaning. These results indicate that, under the conditions used in the present study, the incorporation of L1 or L2 in the protease cleaning solutions does not result in a significant improvement in cleaning relative to cleaning with the proteases alone.

To determine if the lipases were active under the cleaning conditions used, further cleaning experiments were performed using 1.0% (w/v) L1 or L2 together with P4, P6 or P7 (at the concentrations determined for satisfactory cleaning in Section 2.3.2.5), with subsequent determination of residual lipase activity after cleaning (Section 2.2.2.8) and quantification of the fatty acid content of the cleaning solution (Section 2.2.2.9). Residual lipase activity following the 60-min cleaning period in the presence of the commercial proteases and following 60 min incubation in buffer only at 40°C is shown in Fig. 2.10.

After incubation at 40°C for 60 min without commercial proteases, the lipases exhibited over 90% of their original activity. Both lipases retained in excess of 85% of their activity after the 60-min cleaning period in the presence of the commercial proteases, indicating that they are relatively stable under the cleaning conditions employed and are not susceptible to excessive proteolytic degradation by the commercial proteases. In contrast, both lipases were significantly less stable when incubated with P7 in buffer only, with the activity of L2 almost completely abolished. The enhanced stability observed in the cleaning solution is most likely due to the accumulation of protein and peptides in the solution during cleaning, which would provide an alternative substrate for the protease and enhance lipase stability due to the accumulation of its substrate (fat) in the solution.

The free fatty acid content of the cleaning solution was determined after the 60-min cleaning period to determine if L1 and L2 exhibited activity on the fat component of the milk fouling deposit. When buffer, P4, P6 or P7 was used as cleaning solution, the amount of free fatty acids detected ranged from 0 to 1.6 µmol/50 ml cleaning solution, indicating little or no fat breakdown. Similarly, very low levels ranging from 0.11 to 1.6 µmol/50 ml cleaning solution were detected using L1 or L2 alone. When the proteases were included with L1 and L2, the amount of free fatty acids

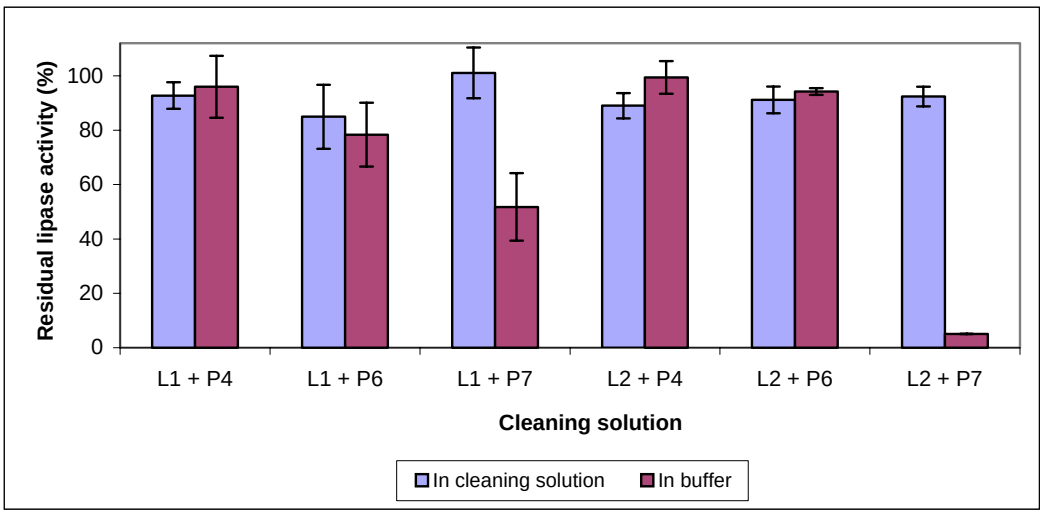


**Figure 2.10. Lipase stability during cleaning. Residual lipase activity determined in cleaning solution and in buffer after 60 min at 40°C. Residual activity is expressed as a percentage of original activity before cleaning. Each value represents the mean ± SD (n = 3).**

detected ranged from 30 to 200 µmol/50 ml cleaning solution, indicating a significant increase in fat breakdown. When taken in conjunction with the results of the cleaning experiments described above and the enzyme stability data shown in Fig. 2.10, these results indicate that the two commercial lipase products tested, while stable under the cleaning conditions used, are able to catalyse significant hydrolysis of the fat component of the fouling deposit only when used in conjunction with protease activity. As both lipases are capable of hydrolysing the target substrate, this may be due to poor access of the lipases to the fat component of the fouling deposit, which is improved upon degradation of the associated protein by the action of the proteases.

The commercial proteases do not exhibit lipase activity (based on their inability to release free fatty acids during cleaning) but are capable of removing lipid material from the stainless steel when used alone. A similar observation was reported by Maartens et al. (1996) during the enzymatic cleaning of ultrafiltration membranes and was attributed to the disturbance of lipid–protein interactions in the fouling layer upon degradation of protein 'anchors' by protease activity. Given the structure of the milk fouling deposit visualised by CLSM, the removal of lipid material by the proteases in the current study is probably also due to the degradation and removal of the associated protein in the fouling layer.

While inclusion of lipase was not beneficial in the present study it is noteworthy that lipases could potentially be of use in the removal of milk fouling deposits with higher levels of fat. Such deposits have been observed during processing of recombined milk, where fouling deposits of up to 60% fat have been reported (Jeurnink et al., 1996). High fat deposits are also possible under conditions of decreased pH (Bansal and Chen, 2006) and upon pasteurisation of previously homogenised milk (Jeurnink et al., 1996). The breakdown of fat achieved by incorporation of lipase activity in cleaning solutions could also be of potential benefit in subsequent wastewater treatment processes. There are several reports in the literature on the use of lipases to reduce the operational problems associated with lipid-rich wastewater (including wastewater from the dairy industry), with reported benefits including increased organic matter removal efficiency, increased biogas production and improved colour removal (Cammarota and Freire, 2006; Leal et al., 2006; Mendes et al., 2006).

### *2.3.4 Confirmation of the removal/inactivation of all residual enzyme activity post enzyme-based cleaning*

In the dairy industry, circulation of cleaning solution is generally followed by rinsing with water, acid circulation and sanitation. In order to confirm the removal/inactivation of all enzyme activity following enzyme-based cleaning, P3, P4 and P7 (at the concentrations determined for optimum cleaning) were subjected to conditions simulating those that would be encountered during such practices with subsequent detection of residual protease activity.

Enzyme activity was easily removed (as judged by detection of residual enzyme activity in the rinse solution and analysis of the stainless steel by CLSM) by rinsing the enzyme-cleaned stainless steel panels with water following removal of the enzyme cleaning solution, as described in Section 2.2.2.10. No protein was detected on the stainless steel panels by CLSM following two 20-ml rinses with water, indicating complete removal of enzyme activity from the stainless steel surface. Less than 2.5% of original protease activity was detected in the first 20-ml rinse solution collected and by rinses three to four no activity was detected.

As discussed in Section 1.4, circulation of 0.5–1.0% nitric acid at 60°C to remove mineral deposits is always undertaken after cleaning heated components and is carried out occasionally after cleaning cold components. Exposure to 0.5% and 1% nitric acid at room temperature resulted in complete enzyme inactivation after 1 min, indicating that any residual activity remaining on the processing equipment after cleaning is likely to be rapidly inactivated during any subsequent acid circulation step.

Sanitation may be achieved by the use of steam, hot water or chemicals. Steam sanitation is typically achieved using a condensate temperature greater than 80°C for a contact time of 15 min (Reinemann, 2003). Reported conditions for sanitation using hot water

include circulation of hot water for 5–15 min at a temperature of 90–95°C (Bylund, 1995) or with a minimum outlet temperature of 80°C (Reinemann, 2003). As the proteases used in the present study are thermolabile, retaining no activity after 3 min at 80°C or 1 min at 90°C, they would almost certainly be inactivated by any heat-based sanitation steps employed.

The most widely used sanitation chemicals are chlorine compounds (hypochlorites of sodium and calcium), iodophors (at a concentration of 12–25 mg/l available iodine) and acid sanitisers (organic acids) (Reinemann, 2003). In the Irish dairy industry, peracetic acid is now becoming the sanitiser of choice over sodium hypochlorite in an effort to minimise the content of trichloromethane in dairy products, in line with legal and exporting requirements (O'Brien, 2008). Protease activity was completely abolished upon incubation with industrially relevant concentrations of hypochlorite for 1 min, while 58–100% of original protease activity was detected following incubation with an iodine disinfectant at a concentration of 25 mg/l available iodine for 5 min. While complete enzyme inactivation was not observed upon incubation with the iodine disinfectant, it is noteworthy that enzyme removal would likely be achieved upon subsequent rinsing with potable water which is recommended after iodophor circulation to prevent product contamination with iodine (Reinemann, 2003; O'Brien, 2008). The poor enzyme stability observed at low pH in the presence of nitric acid indicates that exposure to acid sanitisers is also likely to result in enzyme inactivation.

The complete removal/inactivation of all enzyme activity after cleaning is essential for acceptance of enzyme-based cleaning in industry as any residual activity could potentially have a deleterious effect on product subsequently processed in the same equipment. From these results it can be concluded that an additional enzyme inactivation step is not necessary as any enzyme activity remaining on the equipment after removing the enzyme cleaning solution is likely to be removed by the subsequent water rinse, and, in the unlikely event that complete removal is not achieved, any residual activity would almost certainly be inactivated by acid circulation and/or sanitation and would not pose a threat to product quality.

### *2.3.5 Analysis of the cost implications and economic viability of enzyme-based CIP*

Information on the cost of purchasing bulk industrial quantities of the commercial enzymes assessed was obtained from the relevant enzyme manufacturers and used to calculate the cost of the corresponding cleaning solutions at the optimum concentrations determined for satisfactory cleaning (Section 2.3.2.5). The costs of cleaning solutions prepared using the three commercial enzymes selected as most suitable for CIP application were €0.015/l for P4 and P7 and €0.022/l for P6.

To facilitate cost comparisons with currently used CIP protocols, data relating to the costs of bulk sodium hydroxide and formulated detergent products were obtained from relevant Irish suppliers and also from some dairy co-ops. While the price of bulk sodium hydroxide fluctuates over time, the costs of sodium hydroxide cleaning solutions at the commonly used concentrations of 1.0% and 1.5% were calculated using prices quoted in July 2009 and are shown in Fig. 2.11. The cost of formulated detergent products depends on the actual product, the use concentration and the quantity purchased and the cleaning solution costs calculated, based on the information obtained in the present study, are also presented in Fig. 2.11.

Based on the calculations undertaken in the present study, the cost of the P4 and P7 enzyme cleaning solutions is comparable to the average cost of both the 1.5% sodium hydroxide cleaning solution and the caustic formulated detergent cleaning solution and is approximately 1.6 times that of the 1.0% sodium hydroxide cleaning solution. It is noteworthy that the costs calculated for the enzyme cleaning solutions are based on optimum enzyme concentrations determined for satisfactory cleaning on a laboratory scale and may change when cleaning is carried out on an industrial scale. However, the approximate cost analysis undertaken suggests that the cost of the enzyme-based cleaning solutions is unlikely to prohibit their use in CIP applications. Also, the cost of the enzyme cleaning solutions could be reduced by increasing the cleaning temperature from 40°C (basis of data shown)

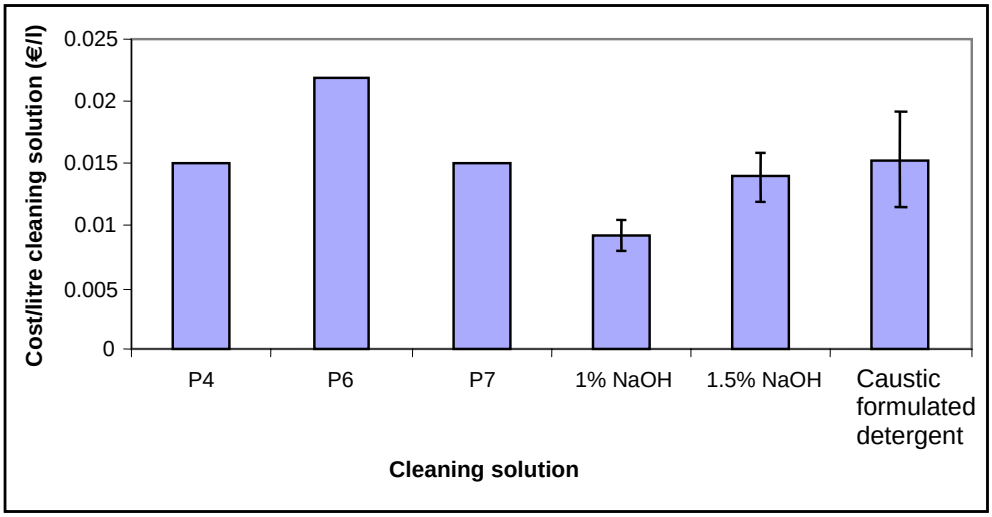


**Figure 2.11. Cost, expressed in €/l, of cleaning solutions prepared using commercial protease products (P4, P5 and P6), sodium hydroxide (NaOH) and caustic formulated detergents. For NaOH and caustic formulated detergent, the error bars shown represent the range in cost that is possible based on the information used.**

to 50°C, as lower enzyme concentrations would be required to achieve satisfactory cleaning due to the higher activity of these products at 50°C (Fig. 2.1).

In the food and drink sector, energy is generally the largest input cost when raw materials are excluded (FDII, 2009). Decreasing normal household laundry washing temperatures from 60°C and 40°C to 30°C and 20°C, respectively, by inclusion of low-temperature enzymes resulted in a reduction in electricity consumption of approximately 60% (Novozymes, 2007). Similarly, conducting CIP at 40–50°C using enzymes would result in reduced energy consumption relative to cleaning with sodium hydroxide or caustic formulated detergents at 70–80°C. With industrial electricity prices in Ireland (>€11/100 kWh in 2007) the second highest in the EU25 and almost 19% above the average (FDII, 2009; IAE, 2009), this reduction in energy consumption is likely to result in significant savings in energy costs.

## 2.4 Conclusions

For acceptance of enzyme-based cleaning in industry, satisfactory cleaning performance, comparable to that of currently used CIP procedures, must be achieved without incurring excessive costs or posing a threat to product quality. The three commercial protease products identified as being most suitable for CIP application satisfy these criteria on the basis of their observed cleaning performance, ease of removal/ inactivation after use and initial cost analysis. While the laboratory-scale cleaning studies undertaken are a useful indicator of the potential suitability of these proteases for CIP application, pilot- and industrial-scale studies are necessary to fully determine actual industrial applicability and confirm economic feasibility. The performance and cost of enzyme-based cleaning were found to be comparable to those of the currently used CIP procedures when investigated on a laboratory scale. If similar results are observed on an industrial scale, then enzyme-based cleaning offers several advantages over the currently used CIP procedures. These include reduced environmental impact as the enzymes operate at lower temperatures, resulting in reduced energy consumption, and also enzymes are biodegradable in contrast to many of the products currently used for cleaning which have adverse effects on human and ecosystem health and on the environment. Enzyme-based cleaning is also more compatible with subsequent wastewater treatment and would create safer working conditions for plant operators.

# 3 Screening Studies to Identify Microbial Proteases of Potential Use in CIP Operations in the Dairy Processing Industry

## 3.1 Background and Aims

While satisfactory removal of milk fouling deposit from stainless steel was achieved using currently available commercial protease products (Chapter 2), these commercial proteases were originally developed for use in laundry detergents and other non-CIP applications and are derived mainly from *Bacillus* species. Although the criteria required for optimum performance of an enzyme in detergent applications are broadly similar to those desirable for CIP application, some differences exist between both application types and therefore enzymes which were previously developed for detergent applications are not necessarily the most suitable for CIP application. In comparison with detergent applications, the substrate encountered during CIP in dairy processing is well defined and the choice of enzyme is not strictly limited by its oxidative stability, stability in the presence of builders and other detergent components and activity and stability in the alkaline pH range of detergent formulations. On this basis, it is possible that proteases from other microbial sources could potentially be more suitable for CIP application. As described in Section 1.5.2, a range of micro-organisms is capable of producing proteases, most of which have never been assessed in terms of their suitability for CIP application in dairy processing. In this part of the study a range of micro-organisms was screened for ability to produce proteases displaying high activity on denatured whey protein, with the ultimate objective of identifying a protease of potential use in CIP operations in the dairy processing industry.

## 3.2 Materials and Methods

### *3.2.1 Materials*

Reagents were obtained as described in Section 2.2, unless otherwise stated. The microbial strains (with DSM numbers) were obtained from DSMZ, Mascheroder Weg 1b, 38124 Braunschweig, Germany. Environmental isolates had previously been isolated from soil, maize silage and animal feed. Potato dextrose agar, malt extract and soya peptone were obtained from Oxoid, Hampshire, UK. Granulated agar and casamino acid (casein acid hydrolysate) were from Melford Laboratories Ltd., Suffolk, UK. Wheat bran was obtained locally. BioLabs Protein Marker Broad Range (P7702S) was purchased from ISIS, Bray, Co. Wicklow, Ireland.

### *3.2.2 Methods*

#### *3.2.2.1 Growth of microbial strains*

Microbial strains were initially grown under the conditions shown in Table 3.1. *Alicyclobacillus* medium contained per litre: 500 ml Solution A, 1 ml Solution B and 500 ml Solution C. Solutions A, B and C were autoclaved separately (121°C, 15 min, 15 psi) to avoid acid hydrolysis of the agar and cooled to about 50°C before mixing. Solution A contained (per 500 ml): 0.25 g $CaCl_2.2H_2O$, 0.50 g $MgSO_4.7H_2O$, 0.2 g $(NH_4)_2SO_4$, 2 g yeast extract, 5 g glucose and 3 g $KH_2PO_4$, pH adjusted to pH 3–4 or pH 4.5 as appropriate. Solution B contained (per 1,000 ml): 0.10 g $ZnSO_4.7H_2O$, 0.03 g $MnCl_2.4H_2O$, 0.30 g $H_3BO_3$, 0.20 g $CoCl_2.6H_2O$, 0.01 g $CuCl_2.2H_2O$, 0.02 g $NiCl_2.6H_2O$ and 0.03 g $Na_2MoO_4.2H_2O$. Solution C contained (per 500 ml): 15 g agar. Medium 674 contained per litre: 500 ml Solution D and 500 ml Solution E, which were autoclaved separately and cooled to about 50°C before mixing. Solution D contained (per 500 ml): 0.2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.25 g $CaCl_2.2H_2O$, 3.0 g $KH_2PO_4$, 1.0 g yeast extract, 1.0 g tryptone, 1.0 g glucose and 1.0 g starch, pH adjusted to pH 4.3. Solution E contained (per 500 ml): 20 g agar. Potato dextrose agar was prepared according to the manufacturer's instructions. Malt extract peptone agar contained (per litre): 30 g malt extract, 3 g soya peptone and 15 g agar, pH adjusted to 5.6. Universal medium for yeasts (YM) contained (per litre): 3 g yeast extract, 3 g malt extract, 5 g peptone from soybeans,

**Table 3.1. Growth conditions of microbial strains used in study.**

| Strain number | Microbial strain | Growth medium | Growth temp. (°C) |
|---|---|---|---|
| **Bacteria** | | | |
| **DSM 446** | *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* | *Alicyclobacillus* medium (pH 3–4) | 60 |
| **DSM 448** | *Alicyclobacillus acidocaldarius* | *Alicyclobacillus* medium (pH 3–4) | |
| **DSM 449** | *Alicyclobacillus acidocaldarius* | *Alicyclobacillus* medium (pH 3–4) | 60 |
| **DSM 17614** | *Alicyclobacillus sendaiensis* | *Alicyclobacillus* medium (pH 5) | 50 |
| **DSM 3922** | *Alicyclobacillus acidoterrestris* | *Alicyclobacillus* medium (pH 4.5) | 45 |
| **DSM 3923** | *Alicyclobacillus acidoterrestris* | *Alicyclobacillus* medium (pH 4.5) | 45 |
| **DSM 14558** | *Alicyclobacillus acidiphilus* | *Alicyclobacillus* medium (pH 4) | 45 |
| **DSM 9199** | *Bacillus* sp. | Medium 674 (pH 4.3) | 55 |
| **DSM 5552** | *Bacillus subtilis* | Nutrient agar (pH 7) | 30 |
| **DSM 7264** | *Bacillus atrophaeus* | Nutrient agar (pH 7) | 30 |
| **Fungi** | | | |
| **DSM 1330** | *Rhizomucor miehei* | Potato dextrose agar | 35 |
| **DSM 1331** | *Rhizomucor pusillus* | Potato dextrose agar | 35 |
| **DSM 915** | *Tolypocladium inflatum* | Malt extract peptone agar | 26 |
| **DSM 1963** | *Trichoderma virens* | Potato dextrose agar | 24 |
| **DSM 871** | *Aspergillus asperescens* | Potato dextrose agar | 24 |
| **DSM 1831** | *Thermoascus aurantiacus* | Potato dextrose agar | 40 |
| **DSM 1547** | *Phanerochaete chrysosporium* | Malt extract peptone agar | 35 |
| **DSM 3515** | *Ganoderma lucidum* | Potato dextrose agar | 30 |
| **DSM 9621** | *Ganoderma lucidum* | Potato dextrose agar | 30 |
| **DSM 1024** | *Schizophyllum commune* | Malt extract peptone agar | 30 |
| **AB001** | *Mucor hiemalis* Wehmer | Potato dextrose agar | 30 |
| **DL002** | *Aspergillus niger* van Tiegh | Potato dextrose agar | 30 |
| **G2B** | Environmental isolate | Potato dextrose agar | 30 |
| **23A** | Environmental isolate | Potato dextrose agar | 30 |
| **MW1A** | Environmental isolate | Potato dextrose agar | 30 |
| **Yeast** | | | |
| **DSM 5418** | *Kluyveromyces marxianus* | YM medium | 25 |
| **DSM 5419** | *Kluyveromyces marxianus* | YM medium | 25 |
| **DSM 5420** | *Kluyveromyces marxianus* | YM medium | 25 |
| **DSM 7238** | *Kluyveromyces marxianus* | YM medium | 25 |
| **DSM 7239** | *Kluyveromyces marxianus* | YM medium | 25 |
| **DSM 70292** | *Kluyveromyces marxianus* | YPD medium | 25 |
| **DSM 3434** | *Kluyveromyces thermotolerans* | YM medium | 26 |
| **DSM 3795** | *Kluyveromyces lactis* | YPD medium | 30 |
| **DSM 70290** | *Kluyveromyces africanus* | YPD medium | 25 |
| **DSM 70294** | *Kluyveromyces polysporus* | YPD medium | 25 |

YM, universal medium for yeasts; YPD, yeast peptone glucose medium.

10 g glucose and 15 g agar. Yeast peptone glucose medium (YPD) contained (per litre) 10 g yeast extract, 20 g peptone and 20 g glucose, pH adjusted to 6.5.

#### *3.2.2.2 Screening for extracellular protease production*

##### *3.2.2.2.1 Screening on solid screening media*

Initial screening for extracellular protease activity was carried out on milk agar plates according to the method described by Saran et al. (2007). The medium contained (per litre): 20 g skim milk powder in 200 ml distilled water, 15 g agar in 200 ml distilled water and 600 ml 0.2 M phosphate buffer (pH 7). The three media components were autoclaved separately and cooled to about 50°C before mixing. The microbial strains were subcultured onto the milk agar plates and incubated at the recommended temperatures shown in Table 3.1. For detection of extracellular protease activity after growth, plates were flooded with 10% tannic acid and examined for zones of clearance due to hydrolysis of the milk protein. For screening of *Alicyclobacillus* strains, a modified version of these screening plates were prepared containing the components of *Alicyclobacillus* medium Solution A listed above in addition to skim milk powder and agar at a pH of 4.

##### *3.2.2.2.2 Screening in SbF and SSF*

The *Alicyclobacillus* strains were screened for extracellular protease production on the following media – A1 medium described by Catara et al. (2006), containing (per litre): 1.3 g $(NH_4)_2SO_4$, 0.3 g $KH_2PO_4$, 0.6 g $MgSO_4.7H_2O$, 0.13 g $CaCl_2$, 1 g yeast extract, 1 g casamino acid and 2 g sucrose (pH adjusted to 4); A2: Medium A1 in which casamino acid was replaced with 10 g casein and final pH adjusted to 5; A3: Medium A1 in which casamino acid was replaced with 5.55 g whey protein isolate and final pH adjusted to 4; A4 medium described by Tsuruoka et al. (2003), consisting of: potato dextrose broth with 0.2% $KH_2PO_4$ at a final pH of 4; A5: modified *Alicyclobacillus* medium, prepared as outlined in Section 3.2.2.1 but with omission of agar and $(NH_4)_2SO_4$; Medium A5 with inclusion of 10 g casein (A6), 10 g whey protein isolate (A7), 10 g skim milk powder (A8), 10 g tryptone (A9) and 20 g wheat bran (A10).

Screening of *Bacillus* strains was carried out using the following media – B1 medium, starch casein broth, described by Doddapaneni et al. (2009) containing (per litre): 10 g starch, 3 g casein, 2 g $KNO_3$, 2 g NaCl, 2 g $K_2HPO_4$, 0.05 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2$, 0.01 g $FeSO_4$; B2 medium described by Oskouie et al. (2008) containing (per litre): 5.2 g $KNO_3$, 4 g $K_2HPO_4$, 0.5 g $MgSO_4.7H_2O$, 0.002 g $CaCl_2$, 11 g sucrose, 5 g yeast extract, 10 g $Na_2CO_3$ and 4 g tri-sodium citrate; B3 medium described by Hadj-Ali et al. (2007) containing (per litre): 5 g casein, 0.5 g $K_2HPO_4$, 4 g yeast extract, 0.5 g $Na_2CO_3$ and 1.5 g KCl; B4 medium described by Joo et al. (2002) containing per litre: 30 g tryptone soya broth and 10 g casein. The pH values of Media B1–B4 were adjusted to pH 7 for screening of DSM 5552 and DSM 7264 and to pH 5 for screening of DSM 9199.

The fungal strains were screened for extracellular protease activity by SbF and SSF. The media used for SbF was based on those described previously for the production of extracellular proteolytic enzymes (Sandhya et al., 2005; Dunaevsky et al., 2006) and contained (per litre): 1 g $K_2HPO_4$, 1 g $MgSO_4.7H_20$, 0.5 g KCl, 0.05 g $FeSO_4.7H_2O$, 0.5 g $CaCl_2$ and 10 g wheat bran at a final pH of 5.5. The media for SSF contained 10 g wheat bran and 10 ml salt solution (0.1% $K_2HPO_4$, 0.5% $MgSO_4.7H_20$, 0.5% NaCl and 0.004% $FeSO_4$ in distilled water) (Sandhya et al., 2005).

Screening of the yeast strains was undertaken using the following media – Y1 containing (per litre): 10 g casein, 3 g $K_2HPO_4$, 5 g yeast extract and 3 ml 5 N $NH_4OH$ at a final pH of 7–8; Y2 modified Y1 with omission of $NH_4OH$; Y3 modified Y1 with casein replaced with 10 g whey protein isolate; Y4: 10% (w/v) skim milk; Y5: 0.5% (w/v) whey protein isolate; Y6: 1% (w/v) casein.

Flasks were incubated at 200 rpm for SbF or in a static incubator for SSF and at a temperature appropriate to the micro-organism being screened (Table 3.1). During SbF, samples were removed daily and assayed for protease activity using the plate assay method (Section 3.2.2.3.1) and/or quantitatively with azocasein or whey protein gel as substrate (Sections 3.2.2.3.2 and 3.2.2.3.3, respectively). Extraction of the enzymes produced during SSF was undertaken on Day 5, with the exception of DSM 3515 which was harvested on Day 16. For enzyme extraction from SSF media, 50 ml

of distilled water were added and the resulting mixture was incubated at 200 rpm for 60 min at 30°C. Wheat bran was removed by filtration through a milk filter and the supernatant obtained after centrifugation of the filtrate at 12,000 rpm for 20 min at 4°C was used as crude enzyme extract and assayed as outlined above.

#### *3.2.2.3 Detection and quantification of protease activity produced*

##### *3.2.2.3.1 Plate assay by radial enzyme diffusion*

Protease activity was detected and quantified based on radial diffusion of the enzyme through a substrate-containing gel, based on a modified method of those described (Walsh et al., 1995, 2005). The substrates used were skim milk and denatured whey protein isolate. Skim milk gel plates were prepared as outlined for the skim milk screening plates in Section 3.2.2.2.1, with each Petri dish (diameter 8.5 cm) containing 20 ml of media. Denatured whey protein isolate gel plates were prepared by combining 100 ml of 5% (w/v) whey protein isolate solution, previously denatured by heating at 80°C for 90 min, with 400 ml of autoclaved 1.9% (w/v) agar and pouring 20-ml aliquots of the resulting solution into Petri dishes. Circular wells (0.7 cm diameter) were punched and removed from the gel. For detection of protease activity during screening, 100-µl aliquots of each sample were added to the wells and plates were incubated at 45°C for 15 h. Plates were examined for zones of hydrolysis by direct visual inspection and contrast was improved by flooding the plates with 10% tannic acid. Protease activity was determined in a semi-quantitative manner by measuring the diameters of the zones of hydrolysis observed. The linear relationship between the diameter of the zone of hydrolysis and the log of protease activity was confirmed using 100-µl aliquots of the commercial protease product P7, containing 0.6–350 units/ml determined as outlined in Section 3.2.2.3.3.

##### *3.2.2.3.2 Determination of protease activity using azocasein as substrate*

Determination of protease activity using azocasein as substrate was as outlined in Section 2.2.2.1.2, with the following modifications: azocasein was prepared at pH 4 for screening of *Alicyclobacillus* strains and DSM 9199 and at pH 8 for all other samples and assay time was increased to up to 90 min.

##### *3.2.2.3.3 Determination of protease activity using thermally induced whey protein gel as substrate*

Protease activity was measured by reaction of Folin–Ciocalteu's reagent with the tyrosine and tryptophan residues of the TCA-soluble peptides generated during hydrolysis of the substrate (thermally induced whey protein gel), using a method modified from that of Sandhya et al. (2005). The substrate was prepared by incubating 500-µl aliquots of 6% (w/v) whey protein isolate solution (prepared in distilled water) in 1.5-ml tubes at 80°C for 60 min. A 500-µl quantity of the sample was added and tubes were closed and vortexed to mix the sample and gel before incubating at 50°C for 150 min. The reaction was stopped by adding 500 µl 20% TCA. After standing at room temperature for 20 min, samples were centrifuged at 12,000 rpm for 5 min. Then 1 ml 0.44 M sodium carbonate and 100 µl Folin–Ciocalteu's phenol reagent (Fluka 47641) were added to 200 µl of the supernatant and the resulting solution was incubated at 30°C for 30 min before measuring absorbance at 660 nm. Blanks were prepared by adding enzyme sample to the substrate after the addition of TCA. A standard curve was prepared by adding 1 ml 0.44 M sodium carbonate and 100 µl Folin–Ciocalteu's phenol reagent to 200-µl samples containing 0–40 µg tyrosine. One unit of activity was defined as the amount of enzyme that liberated 1 µg tyrosine per min under the conditions of the assay. Prior to assay by this method, crude enzymes extracted from SSF media were diafiltered with distilled water.

#### *3.2.2.4 Assessment of crude proteases*

##### *3.2.2.4.1 Effect of temperature on activity*

The effect of temperature on protease activity was determined by measuring protease activity using denatured whey protein gel as substrate, as described in Section 3.2.2.3.3, at the following temperatures: 20°C, 30°C, 40°C, 50°C and 60°C.

##### *3.2.2.4.2 Effect of pH on activity*

The effect of pH on enzyme activity was determined by measuring protease activity at pH 3–9, using the following buffers: citric acid–$Na_2HPO_4$ buffer (pH 3–4), sodium acetate–acetic acid (pH 5), $Na_2HPO_4$–$NaH_2PO_4$ (pH 6–8) and $Na_2B_4O_7$–HCl (pH 9). Determination of protease activity was as described in Section 3.2.2.3.3 with the following modifications: the

500-µl enzyme sample added to the whey protein gel was prepared immediately before assay commencement and consisted of 250 µl 1 M buffer listed above, 200 µl distilled water and 50 µl of crude enzyme concentrated by ultrafiltration.

*3.2.2.4.3 SDS-PAGE analysis*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970), as described by Sambrook et al. (1989), using a 10% polyacrylamide gel and a vertical electrophoresis system. The 10% resolving gel contained 4.0 ml distilled water, 3.3 ml 30% acrylamide mix (29% (w/v) acrylamide and 1% (w/v) *N,N'*-methylene-bis-acrylamide), 2.5 ml 1.5 M tris(hydroxymethyl) aminomethane (Tris) (pH 8.8), 0.1 ml 10% SDS pH 7.0, 0.1 ml 10% ammonium sulfate and 0.004 ml *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel contained 2.1 ml distilled water, 0.5 ml 30% acrylamide mix, 0.39 ml 1.0 M Tris (pH 6.8), 0.03 ml 10% SDS pH 7.0, 0.03 ml 10% ammonium persulfate and 0.003 ml TEMED. The loading buffer contained 50 mM Tris–Cl (pH 6.8), 2% (w/v) SDS, 0.1% bromophenol blue and 10% (v/v) glycerol. Tris–glycine electrophoresis buffer contained 25 mM Tris, 250 mM glycine and 0.1% SDS. The protein molecular weight marker used (BioLabs Protein Marker Broad Range P7702S) contained myosin (212,000 Da), maltose binding protein (MBP)-β-galactosidase (158,194 Da), β-galactosidase (116,351 Da), phosphorylase b (97,184 Da), serum albumin (66,409 Da), glutamic dehydrogenase (55,561 Da), MBP2 (42,710 Da), thioredoxin reductase (34,622 Da), triosephosphate isomerase (26,972 Da), trypsin inhibitor (20,040–20,167 Da), lysozyme (14,313 Da), aprotinin (6,517 Da), insulin A (3,400 Da) and B chain (2,340 Da).

Glass plates were assembled according to the manufacturer's instructions and the resolving and stacking gels were allowed to polymerise separately. A 15-µl aliquot of protein sample and 5 µl loading buffer were mixed and centrifuged at 12,000 rpm for 10 s immediately before loading. A 5-µl aliquot of protein marker and 20 µl of each sample were added to separate wells. Electrophoresis was carried out at 120 V until the bromophenol blue reached the bottom of the resolving gel. Gels were removed from the electrophoresis system for subsequent staining. Protein staining was carried out using EZBlue Gel Staining Reagent (Sigma G1041) according to the manufacturer's instructions. Activity staining was carried by modifying the method described by Morcelle et al. (2004). The gel was washed in 2.5% (v/v) Triton X-100 for 30 min at room temperature before washing three times for 10 min in distilled water. For activity staining using casein as substrate, the gel was incubated in 1% (w/v) casein in 0.1 M $Na_2H_2PO_4$–$NaH_2PO_4$ buffer, pH 7 at 45°C for 90 min, before rinsing briefly in distilled water and staining for protein as described above. For activity staining using denatured β-Lg as substrate, the washed gel was overlaid onto a 10% polyacrylamide gel containing denatured β-Lg and incubated at 45°C for 120 min. The substrate-containing gel was stained for protein. The denatured β-Lg-containing gel contained 3.3 ml 30% acrylamide mix, 0.1 ml 10% ammonium sulfate, 0.004 ml TEMED, 1 ml 1 M sodium acetate–acetic acid buffer (pH 5) and 1.5 ml 1% denatured β-Lg (70°C, 5 min) diluted to a final volume of 10 ml with distilled water.

*3.2.2.4.4 Assessment of cleaning performance*

Assessment of the cleaning performance of the crude enzymes from screening was undertaken as described in Section 2.2.2.6 with the following modifications: cleaning studies were carried out by incubating 9 ml of cleaning solution with a fouled panel in a 2 × 7.5 cm container so that the cleaning solution covered the surface of the fouled panel. The pH of the cleaning solution was adjusted to the enzyme optimum pH determined in Section 3.2.2.4.2 by inclusion of the following buffers at a final concentration of 0.1 M: sodium acetate–acetic acid (pH 4–5), $Na_2HPO_4$–$NaH_2PO_4$ (pH 6–8) and sodium carbonate–sodium bicarbonate (pH 10). The enzyme activity of the cleaning solution was determined using denatured whey protein as substrate (Section 3.2.2.3.3) and protease activity was determined before and after cleaning using azocasein as substrate (Section 3.2.2.3.2) to assess enzyme stability during cleaning. Cleaning was performed at 40°C and 100 rpm for various times as indicated. After enzyme cleaning, residual fouling was removed by incubation with 30 ml 1% sodium hydroxide or with 10 ml 1% sodium hydroxide to increase sensitivity of detection if very

little fouling was evident. Where Triton X-100 and propylene glycol were included in the cleaning solutions, 1% (v/v) and 1% (w/v) solutions, respectively, were prepared in distilled water and added to the cleaning solutions immediately before cleaning to give a final concentration of 0.1% in the cleaning solution.

*3.2.2.4.5 Effect of protease inhibitors on enzyme activity*

The effects of the protease inhibitors ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) on enzyme activity were determined by incubating the enzyme with each inhibitor at a final concentration of 10 mM for 15 min at room temperature. Protease activity was determined after this time, as described in Section 3.2.2.3.2 and expressed as a percentage of original activity.

## 3.3 Results and Discussion

### *3.3.1 Screening of micro-organisms for extracellular protease activity*

Screening focussed on identifying extracellular proteases as extracellular enzymes require less downstream processing, facilitating more cost-effective commercial production. Micro-organisms screened were therefore predominantly fungal as well as some strains of *Alicyclobacillus* and *Bacillus* sp., which have been previously shown to produce extracellular enzymes. There are reports in the literature of the production of serine-carboxyl proteinases by *Alicyclobacillus acidocaldarius* (Catara et al., 2006) and *Alicyclobacillus sendaiensis* (Tsuruoka et al., 2003) and several proteases produced by *Bacillus* sp. have been characterised and exploited commercially, including in cleaning applications (Gupta et al., 2002a). Some of the bacteria are acidophilic, with recommended growth at pH 3–4.5, and were screened as potential sources of acid-stable proteases which could be used for cleaning in conjunction with the acid circulation step to achieve removal of organic and mineral fouling in a single step. Fungi that have been exploited in the past for the production of various industrial enzymes, for example *Trichoderma*, *Aspergillus* and *Rhizomucor* were also selected for screening. Proteases have already been identified and in some cases characterised from some of the fungal strains and genera screened, for example *Aspergilli* (Gupta et al., 2002a; Sandhya et al., 2005), *Thermoascus aurantiacus* (Merheb et al., 2007), *Rhizomucor miehei* (Trujillo et al., 2000), *Trichoderma virens* (Pozo et al., 2004), *Phanerochaete chrysosporium* (Cruz-Córdova et al., 1999), *Schizophyllum commune* (Johnston et al., 2000) and *Mucor* sp. (Belyauskaite et al., 1980), but there are no known reports of their activity on milk fouling deposits. Several strains of the yeast *Kluyveromyces* were also screened, most of which were isolated from dairy products and a creamery environment and could therefore potentially produce proteases with activity on milk protein. There are reports on the purification and characterisation of an aminopeptidase and a serine carboxypeptidase from *Kluyveromyces marxianus* (Ramírez-Zavala et al., 2004a,b). In order to target proteases with high activity at low temperatures (for low-temperature cleaning) micro-organisms screened were mostly mesophilic.

Screening was initially carried out on solid milk agar plates. Micro-organisms producing extracellular protease activity generate zones of clearance in this media due to disappearance of the milk protein. Visualisation of this hydrolysed zone was improved by flooding with tannic acid which forms insoluble complexes with protein, thereby increasing the contrast between the hydrolysed zones and intact protein (Saran et al., 2007). Figure 3.1 shows a zone of hydrolysis observed on the milk agar medium and

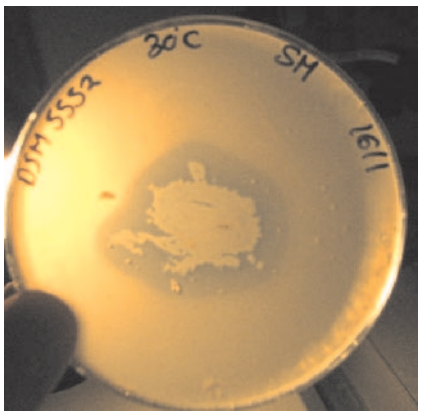


**Figure 3.1. Zone of substrate hydrolysis produced on a milk agar plate by DSM 5552 *Bacillus subtilis*, indicating the production of extracellular protease activity.**

the results from the screening studies undertaken using this approach are shown in Table 3.2. Of the 35 micro-organisms screened, 22 produced zones of substrate hydrolysis in the milk agar.

Micro-organisms were grown by SbF and/or SSF, using the media described in Section 3.2.2.2.2, to confirm the production of extracellular protease activity and to facilitate quantification and characterisation of the enzymes produced. Microbial proteases are generally constitutive and at times partially inducible (Gupta et al., 2002a). Extracellular protease production is affected by media composition, in particular by C/N ratio and the presence of metabolisable sugars and metal ions and by physical factors such as pH, temperature, inoculum size and aeration (Anwar and Saleemuddin, 1998; Gupta et al., 2002b). Screening was carried out using media described in the literature for protease production as well as various modified media supplemented with different proteins. Screening of fungi was also undertaken by SSF using a wheat-bran-based medium. In contrast to SbF where the micro-organisms and nutrients are submerged in water, water is present only in the solid substrate in SSF (Graminha et al., 2008). In a comparative evaluation of protease production by *Aspergillus oryzae* in SbF and SSF, Sandhya et al. (2005) reported 3.5-fold more enzyme production in SSF. In addition to high enzyme yields, other advantages of SSF include low water input, low wastewater output, shorter enzyme production times, lower production costs and the possibility of using agricultural and agro-industrial wastes and by-products as media which are plentiful and inexpensive (Sandhya et al., 2005; Cammarota and Freire, 2006; Graminha et al., 2008).

Protease production was initially determined by assay using azocasein as substrate (Section 3.2.2.3.2) or by plate assay (Section 3.2.2.3.1). Although activity on azocasein is not indicative of ability to degrade the proteinaceous component of milk fouling deposits, this general endopeptidase assay is convenient and less time-consuming for initial screening than using whey protein gel as substrate. The plate assay method employed is also useful for screening applications and facilitates detection of activity on substrates directly relevant to the present study, i.e. denatured skim milk and denatured whey protein. A linear relationship was observed between the diameter of the zone of hydrolysed substrate and the log of enzyme activity when samples of the commercial protease product P7 containing different levels of activity were assayed (Fig. 3.2), confirming that this method can be used to determine protease activity in a semi-quantitative manner. The results from the screening studies undertaken in SbF and SSF are shown in Table 3.2.

All *Alicyclobacillus* strains were screened for protease production using normal *Alicyclobacillus* medium and media A1–A4. Of the strains tested, only DSM 3922 and DSM 3923 were found to produce extracellular proteases. Further screening using these two strains was undertaken on Media A5–A10. Activity was detected only when azocasein was used as substrate with maximum activity observed on Days 2–3. No activity was detected by the plate assay method or on whey protein isolate gel, indicating that the extracellular proteases produced by these strains are not active on the substrate of interest in the present study. Upon screening of the *Bacillus* strains in nutrient broth and Media B1–B4, no protease production by DSM 9199 was detected while DSM 5552 and DSM 7264 were found to produce extracellular proteases in all media tested with activity detected on both azocasein and skim milk plates. Of the media tested, DSM 5552 produced maximum activity on nutrient broth and B3 while maximum DSM 7264 activity was observed using nutrient broth and B1.

Solid-state fermentation was found to be more suitable for the production of fungal extracellular protease activity than the SbF media tested. While four of the fungal strains tested gave positive results using the skim milk plate assay, appreciable levels of activity on azocasein were observed for DSM 915 only during screening by SbF. In contrast, 12 of the 14 fungal strains screened produced extracellular proteases during SSF with activity on both skim milk plates and azocasein. Differential expression of enzymes in SbF and SSF has previously been reported (Graminha et al., 2008). The three yeast strains that produced good zones of hydrolysis on solid screening media (DSM 7238, DSM 7239 and DSM 70292) were screened for production of extracellular proteases on YM, YPD and Y1–Y6 media. While growth was observed in all media

**Table 3.2. Results from screening studies on solid screening media and in submerged (SbF) and solid-state fermentation (SSF).**

| Strain number | Microbial strain | Zone of hydrolysis on milk agar plates | Screening in SbF and SSF: media in which extracellular protease activity detected |
|---|---|---|---|
| **Bacteria** | | | |
| **DSM 446** | *Alicyclobacillus acidocaldarius* | No | |
| **DSM 448** | *Alicyclobacillus acidocaldarius* | No | |
| **DSM 449** | *Alicyclobacillus acidocaldarius* | No | |
| **DSM 17614** | *Alicyclobacillus sendaiensis* | No | |
| **DSM 3922** | *Alicyclobacillus acidoterrestris* | No | *Alicyclobacillus* medium, A1, A2, A3, A4, A5, A6, A7, A8, A9, A10[1] |
| **DSM 3923** | *Alicyclobacillus acidoterrestris* | No | *Alicyclobacillus* medium, A1, A2, A3, A4, A5, A6, A7, A8, A9, A10[1] |
| **DSM 14558** | *Alicyclobacillus acidiphilus* | No | |
| **DSM 9199** | *Bacillus* sp. | No | |
| **DSM 5552** | *Bacillus subtilis* | Yes | Nutrient broth, B1, B2, B3, B4[1,2] |
| **DSM 7264** | *Bacillus atrophaeus* | Yes | Nutrient broth, B1, B2, B3, B4[1,2] |
| **Fungi** | | | |
| **DSM 1330** | *Rhizomucor miehei* | Yes | SSF[1,2] |
| **DSM 1331** | *Rhizomucor pusillus* | Yes | SSF[1,2] |
| **DSM 915** | *Tolypocladium inflatum* | Yes | SbF[1,2], SSF[1,2] |
| **DSM 1963** | *Trichoderma virens* | Yes | SbF[2], SSF[1,2] |
| **DSM 871** | *Aspergillus asperescens* | Yes | |
| **DSM 1831** | *Thermoascus aurantiacus* | Yes | SSF[1,2] |
| **DSM 1547** | *Phanerochaete chrysosporium* | Yes | SSF[1,2] |
| **DSM 3515** | *Ganoderma lucidum* | Yes | SSF[1,2] |
| **DSM 9621** | *Ganoderma lucidum* | Yes | SbF[2], SFF[1,2] |
| **DSM 1024** | *Schizophyllum commune* | Yes | SSF[1,2] |
| **AB001** | *Mucor hiemalis* Wehmer | Yes | SSF[1,2] |
| **DL002** | *Aspergillus niger* van Tiegh | Yes | |
| **G2B** | Environmental isolate | Yes | SbF[2], SFF[1,2] |
| **23A** | Environmental isolate | Yes | SSF[1,2] |
| **MW1A** | Environmental isolate | Yes | SSF[1,2] |
| **Yeast** | | | |
| **DSM 5418** | *Kluyveromyces marxianus* | Very small zone | |
| **DSM 5419** | *Kluyveromyces marxianus* | Very small zone | |
| **DSM 5420** | *Kluyveromyces marxianus* | | |
| **DSM 7238** | *Kluyveromyces marxianus* | Yes | |
| **DSM 7239** | *Kluyveromyces marxianus* | Yes | |
| **DSM 70292** | *Kluyveromyces marxianus* | Yes | |
| **DSM 3434** | *Kluyveromyces thermotolerans* | | |
| **DSM 3795** | *Kluyveromyces lactis* | | |
| **DSM 70290** | *Kluyveromyces africanus* | | |
| **DSM 70294** | *Kluyveromyces polysporus* | | |

[1]Activity detected using azocasein as substrate.
[2]Activity detected using plate assay method with skim milk as substrate.

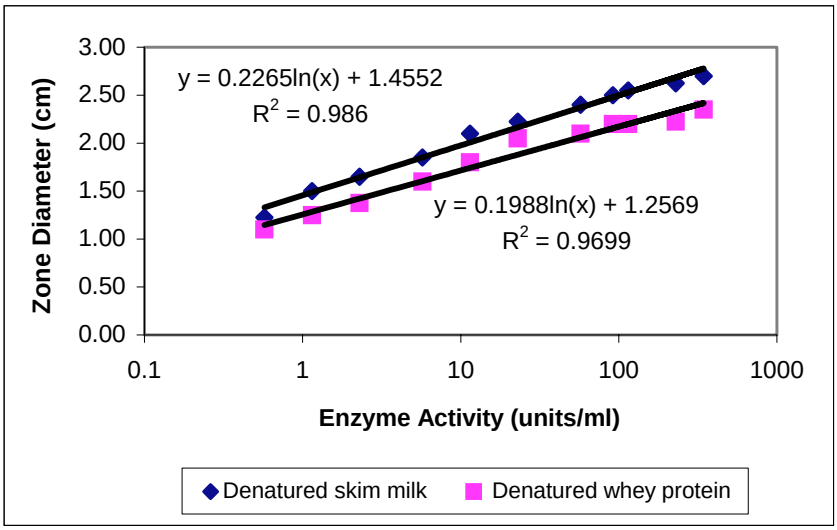


**Figure 3.2. Plot of the relationship between the diameter of the zone of substrate hydrolysed and enzyme concentration of the commercial protease product P7 with denatured skim milk and denatured whey protein as substrate, using the plate assay method described in Section 3.2.2.3.1.**

tested, no protease activity was detected in the culture media.

In order to identify proteases potentially capable of degrading the proteinaceous component of milk fouling deposits, the 14 crude proteases showing activity on denatured skim milk in the plate assay method were re-assayed by this method using denatured whey protein as substrate (Section 3.2.2.3.1) and quantitatively assayed using whey protein gel as substrate (Section 3.2.2.3.3). Images of the zones of hydrolysed substrate observed and measured zones of diameter are shown in Fig. 3.3 and Table 3.3, respectively. For the quantitative assay, the whey protein gel was prepared at a lower concentration and is less rigid than that used for assessment of the commercial proteases in order to facilitate detection of lower levels of enzyme activity. Activity was quantified by reaction of Folin–Ciocalteu's reagent with the tyrosine and tryptophan residues of the TCA-soluble peptides generated during hydrolysis of this substrate. This approach, while less accurate than the TNBS method employed in Section 2.2.2.1.1, is less time-consuming and laborious and more convenient for assaying the large number of samples from screening. Crude enzyme samples from SSF gave high blank values when assayed by this method, most likely due to the co-extraction of wheat-bran media components and/or compounds produced during fermentation. This was overcome by diafiltration of the enzyme samples before assay as described in Section 3.2.2.3.3. For the 14 micro-organisms found to produce extracellular protease activity of potential interest the amount of activity produced during fermentation is shown in Table 3.3.

Of the fungal strains tested, the crude enzyme from DSM 1024 *Schizophyllum commune* showed the highest amount of activity as measured by both methods and was the only fungal enzyme that showed appreciable activity when denatured whey protein was used as substrate in the plate assay method. This substrate is more relevant to the present study than denatured skim milk due to its high β-Lg content. The crude enzyme produced by DSM 5552 *Bacillus subtilis* also showed activity on this substrate and, upon quantitative comparison, the amount of activity produced by DSM 5552 was significantly greater than that produced by the DSM 7264 *Bacillus atrophaeus*. Due to the low amount of activity produced, the latter was excluded from further studies.

### *3.3.2 Assessment of crude proteases*

#### *3.3.2.1 Effect of temperature and pH on activity*

Figure 3.4 shows the effect of temperature on the activity of the crude proteases, with the three commercial protease products, P4, P6 and P7,

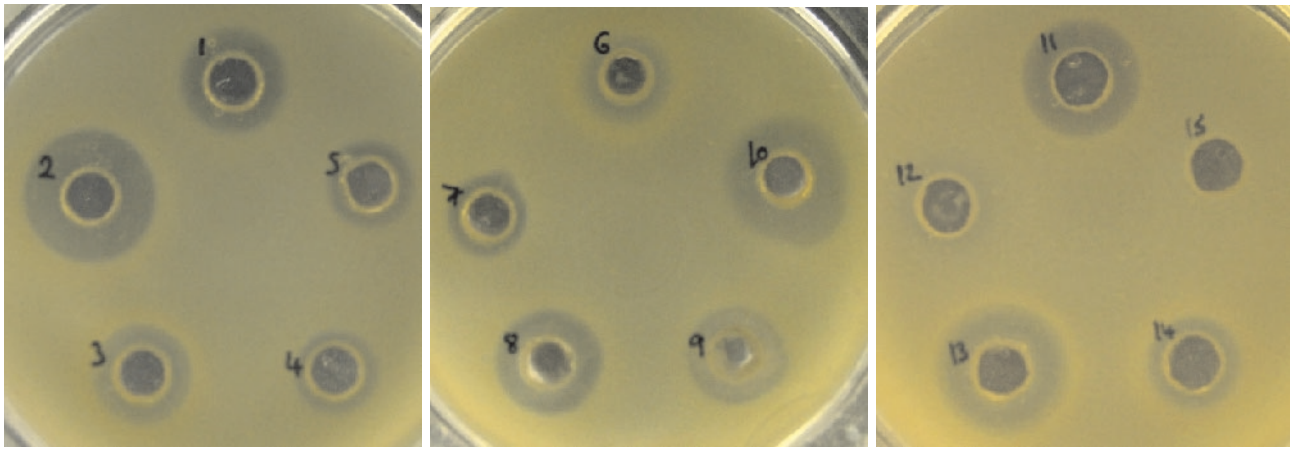


**Figure 3.3. Zones of hydrolysis produced by crude enzymes on skim milk plates when plate assay was carried out as described in Section 3.2.2.3.1. 1, DSM 1963; 2, DSM 1024; 3, G2B; 4, 23A; 5, MW1A; 6, DSM 915; 7, *Mucor hiemalis* Wehmer; 8, DSM 1330; 9, DSM 1331; 10, DSM 1547; 11, DSM 1831; 12, Control (SSF media not inoculated); 13, DSM 5552; 14, DSM 7264; 15, Control (nutrient broth not inoculated).**

**Table 3.3. Extracellular activity produced during fermentation, determined by the semi-quantitative plate assay method and by quantitative assay using denatured whey protein as substrate (mean ± SD).**

| | Plate assay zone diameter (cm) | | Activity produced (units/g wheat bran) |
|---|---|---|---|
| | Skim milk | Whey protein | |
| **Fungi** | | | |
| **DSM 1963 *Trichoderma virens*** | 1.75 ± 0.00 | 0 | 4.81 ± 0.13 |
| **DSM 1024 *Schizophyllum commune*** | 2.17 ± 0.03 | 1.15 | 5.05 ± 0.14 |
| **G2B (environmental isolate)** | 1.70 ± 0.00 | 0 | 1.16 ± 0.17 |
| **23A (environmental isolate)** | 1.38 ± 0.03 | 0 | 1.30 ± 0.13 |
| **MW1A (environmental isolate)** | 1.40 ± 0.00 | 0 | 1.43 ± 0.02 |
| **DSM 3515 *Ganoderma lucidum*** | 1.30 ± 0.00 | 0 | 1.41 ± 0.07 |
| **DSM 915 *Tolypocladium inflatum*** | 1.70 ± 0.00 | 0 | 2.42 ± 0.02 |
| ***Mucor hiemalis* Wehmer** | 1.40 ± 0.00 | 0 | 1.63 ± 0.07 |
| **DSM 1330 *Rhizomucor miehei*** | 1.78 ± 0.03 | 0 | 2.82 ± 0.21 |
| **DSM 1331 *Rhizomucor pusillus*** | 1.65 ± 0.09 | 0 | 2.56 ± 0.06 |
| **DSM 1547 *Phanerochaete chrysosporium*** | 2.12 ± 0.03 | 0 | 2.63 ± 0.33 |
| **DSM 1831 *Thermoascus aurantiacus*** | 1.92 ± 0.03 | Very small zone | 2.12 ± 0.25 |
| **Bacteria** | **Plate assay zone diameter (cm)** | | **Activity produced (units/ml media)** |
| | **Skim milk** | **Whey protein** | |
| **DSM 5552 *Bacillus subtilis*** | 2.12 ± 0.03 | 1.30 | 2.32 ± 0.16 |
| **DSM 7264 *Bacillus atrophaeus*** | 1.57 ± 0.03 | 0 | 0.08 ± 0.00 |

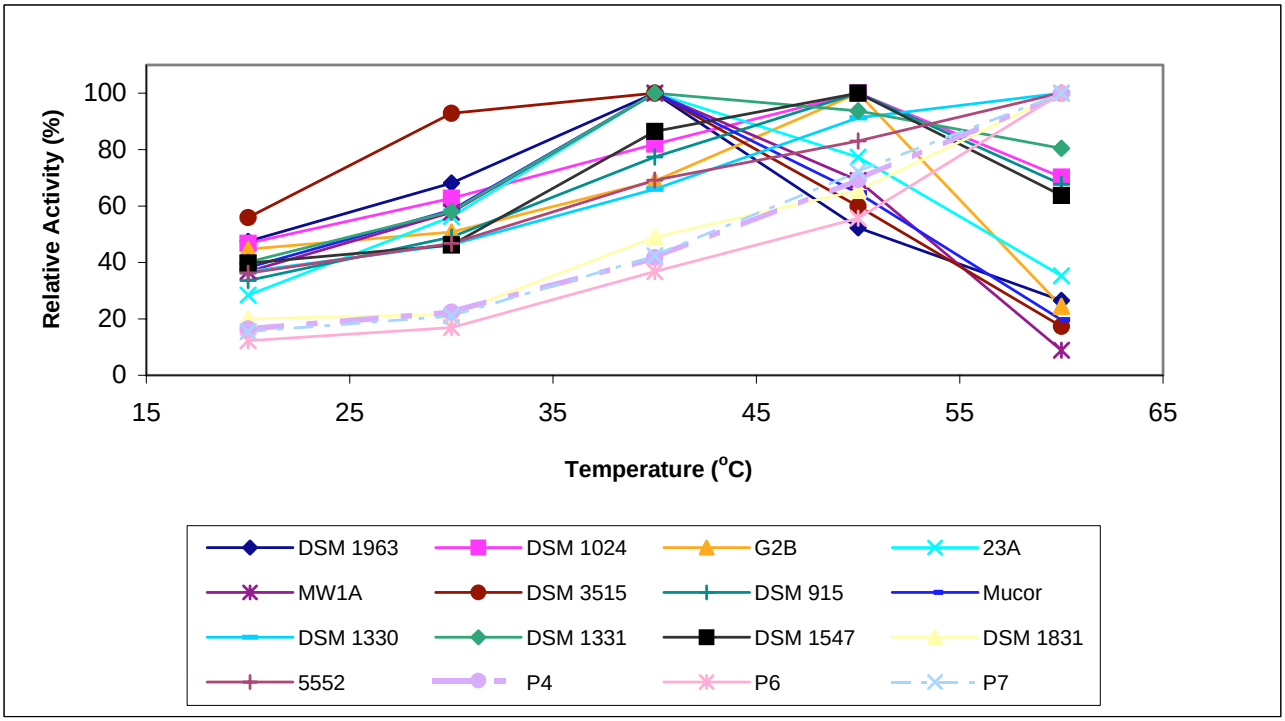


**Figure 3.4. Temperature versus activity profiles of crude proteases from screening and commercial enzymes P4, P6 and P7, generated using whey protein gel as substrate, as described in Section 3.2.2.4.1. Data are expressed as a percentage of maximum value. Each value represents the mean (n = 3) but error bars have been omitted for clarity.**

included for comparison. Six of the crude proteases from screening displayed optimum activity at 40°C and four had an optimum temperature of 50°C. Eight of the crude enzymes displayed over 50% of their maximum activity at 30°C and the crude enzyme produced by DSM 3515 *Ganoderma lucidum* displayed 56% of its optimum activity at 20°C. In comparison with the commercial protease products, the crude enzymes produced during screening exhibited a greater proportion of their maximum activity at lower temperatures, which is favourable in terms of low-temperature cleaning.

The effect of pH on enzyme activity is shown in Fig. 3.5. The crude enzymes generally displayed maximum activity at pH 5–6, with the exception of DSM 3515 (pH 4), DSM 915 (pH 8) and DSM 5552 (pH 10). The pH range of the fungal enzymes produced during screening is lower than that of the commercial products tested, which all displayed maximum activity at pH 10–10.5. In the context of CIP application, cleaning at neutral pH could potentially be beneficial in terms of subsequent wastewater treatment and eliminate the need for neutralisation prior to discharge to wastewater, as described in Section 1.4.5. It is noteworthy that the crude enzyme from DSM 5552 *Bacillus subtilis* has an optimum pH similar to the *Bacillus*-derived commercial proteases assessed (pH 10–10.5) but displays a greater proportion of its maximum activity at lower pH (63–73% at pH 6–7).

### *3.3.2.2 SDS-PAGE analysis*

Upon analysis by SDS-PAGE, all the crude samples from screening were found to contain at least one band with activity on casein as shown in Fig. 3.6b.

In some samples, more than one band with activity on casein was observed. Such bands may be due to genetically distinct enzymes or may result from partial proteolysis or differential glycosylation of a single enzyme. For the fungal samples, multiple protein bands (in addition to those displaying protease activity) were observed (Fig. 3.6a), indicating the production of multiple extracellular proteins during SSF on wheat bran. In contrast, little or no protein bands were

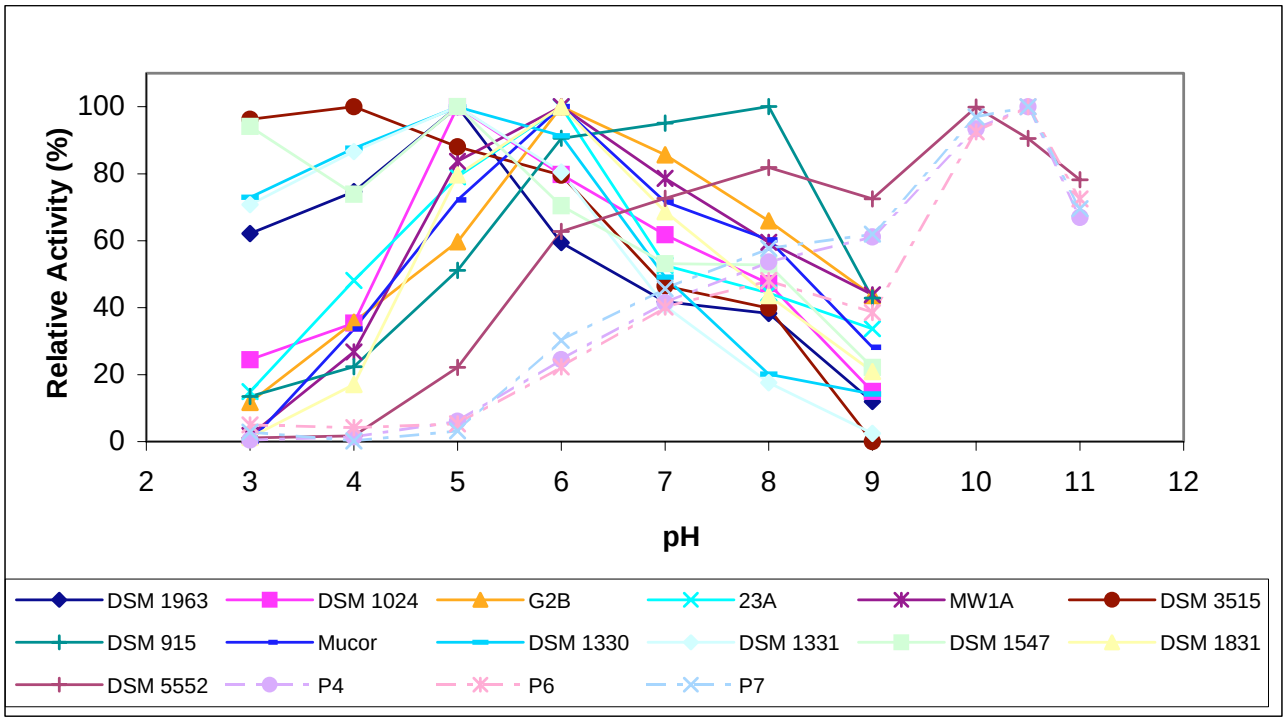


**Figure 3.5. pH versus activity profiles of crude proteases from screening and commercial enzymes P4, P6 and P7, generated using whey protein gel as substrate, as described in Section 3.2.2.4.2. Data are expressed as a percentage of maximum value. Each value represents the mean (n = 3) but error bars have been omitted for clarity.**

observed upon SDS-PAGE analysis of the crude enzyme harvested from DSM 5552 *Bacillus subtilis* although multiple bands with protease activity were observed upon activity staining.

### *3.3.2.3 Assessment of cleaning performance*

Initial assessment of the cleaning performance of the crude proteases was undertaken as outlined in Section 3.2.2.4.4 by incubation of each crude enzyme (1.7–6.1 units/ml) with a milk-fouled stainless steel panel (265 ± 60 mg $O_2$ per panel) at 40°C for 14 h. Cleaning was carried out at the optimum pH of each enzyme determined in Section 3.3.2.1: pH 4 (DSM 3515), pH 5 (DSM 1963, DSM 1331, DSM 1024, DSM 1330, DSM 1547), pH 6 (23A, MW1A, *Mucor hiemalis* Wehmer, G2B, DSM 1831), pH 8 (DSM 915) and pH 10 (DSM 5552). The results of the quantitative analysis of cleaning are shown in Fig. 3.7. When cleaning was carried out under similar conditions using buffer only, the percentage of organic matter not removed during cleaning was approximately 90% (pH 4), 80–87% (pH 5–6), 67% (pH 8) and 50% (pH 10), respectively. The increased removal of organic matter with increasing pH can most likely be attributed to increased swelling of the protein deposit at high pH, due to an increase in the net surface charge of the protein above its isoelectric point (approx. 5.1 for β-Lg) and hence increased repulsion between charged amino acid side chains (Mercadé-Prieto and Chen, 2005; Gunasekaran et al., 2007). Such improved swelling aids deposit removal as outlined in Section 1.4.4.

Based on comparison of the amount of organic matter not removed during cleaning with the crude enzymes with that not removed using the corresponding buffers, it is evident that all the crude enzymes tested are capable of removing milk fouling deposit from stainless steel. This confirms the suitability of the screening approach undertaken to identity proteases with the required activity for potential application in CIP operations in dairy processing. Significant differences in cleaning performance were observed however with less than 4% of organic matter detected after cleaning with the crude enzymes from DSM 1963, DSM 1024, DSM 5552, DSM 1547 and DSM 1831 compared with

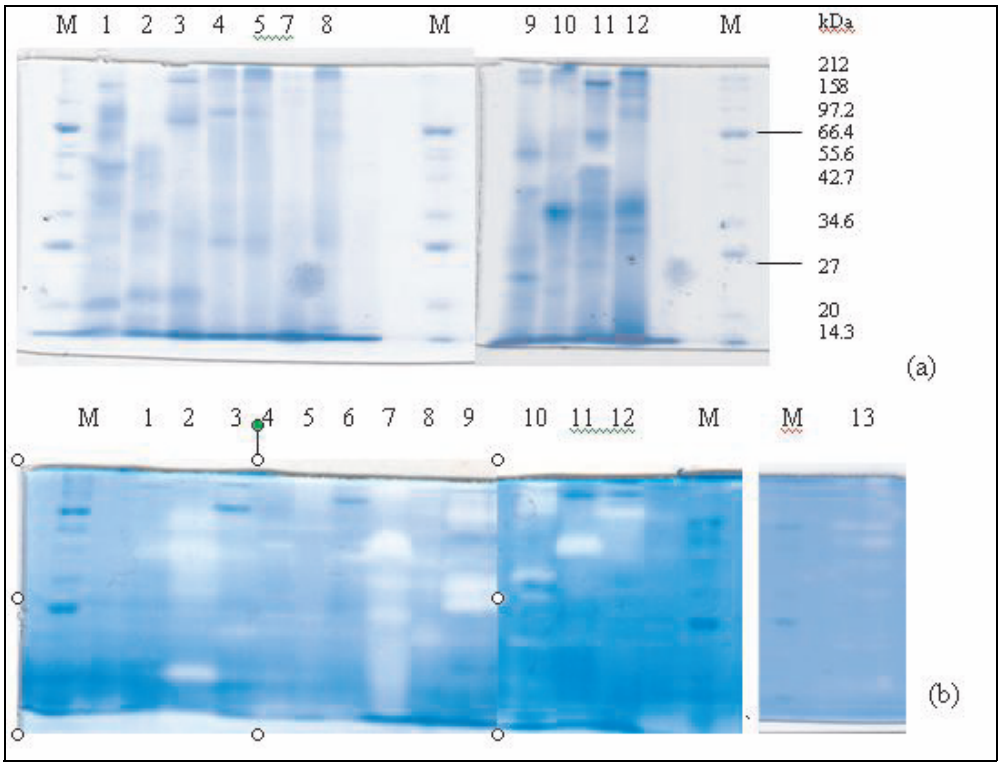


**Figure 3.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of crude enzymes with staining for (a) protein and (b) activity on casein. M, protein marker; 1, DSM 1963; 2, DSM 1024; 3, G2B; 4, 23A; 5, MW1A; 6, DSM 3515; 7, DSM 915; 8, *Mucor hiemalis* Wehmer; 9, DSM 1330; 10, DSM 1331; 11, DSM 1547; 12, DSM 1831; 13, DSM 5552.**

55–60% for 23A, MW1A and *Mucor hiemalis* Wehmer. The poorer cleaning performance of the latter three enzymes was also evident upon quantification of the amount of protein not removed during cleaning (Fig. 3.7b). The crude enzymes were found to be relatively stable during cleaning, with five of the enzymes assessed retaining over 80% of their original activity and a further five displaying 70–80% of their original activity after the 14-h cleaning period (Fig. 3.7c). The crude enzymes produced by DSM 1331, 23A and MW1A were the least stable of the enzymes tested. While the crude enzymes produced by DSM 1963, DSM 1024, DSM 5552, DSM 1547 and DSM 1831 are of potential interest for CIP application based on their cleaning performance and stability, the enzymes produced by the DSM 1024 *Schizophyllum commune* and DSM 5552 *Bacillus subtilis* were selected for further analysis in the current study. Of the fungal strains assessed, DSM 1024 produced the greatest amount of protease activity during SSF (Table 3.3) and was the only crude enzyme that displayed substantial activity on denatured whey protein in the plate assay method. In addition, the temperature profile of the crude enzyme indicates potential suitability for low-temperature cleaning. DSM 5552 *Bacillus subtilis* also produced large quantities of protease activity under conditions used in the present study and within a short time. Although, there are several *Bacillus*-derived proteases available commercially, the temperature and pH profiles of the crude enzyme produced by DSM 5552 are more favourable for CIP application than those of the commercial proteases assessed in the present study, with increased activity observed at lower temperatures (Fig. 3.4) and over a broader pH range (Fig. 3.5). All further cleaning studies focussed on these two enzymes.

### *3.3.3 Further studies on DSM 1024* Schizophyllum commune *crude protease*

#### *3.3.3.1 Optimisation of cleaning performance*

The cleaning performance of the crude enzyme was optimised in relation to pH and the inclusion of

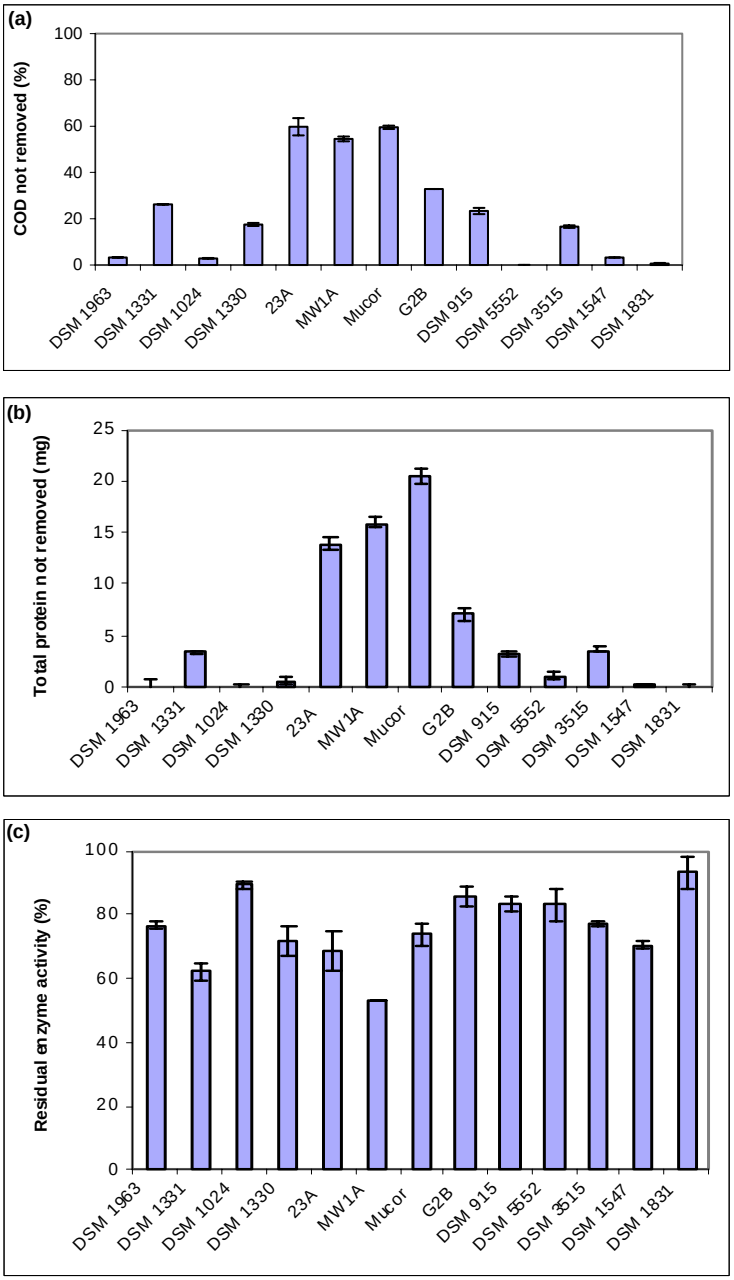


**Figure 3.7. Assessment of the cleaning performance of the crude enzymes from screening studies. Assessment undertaken as described in Section 3.2.2.4.4, at 40°C and at the enzyme optimum pH for 14 h. (a) Organic matter not removed during cleaning, expressed as a percentage of the amount of organic matter on each panel before cleaning; (b) amount of protein (mg) not removed during cleaning; (c) enzyme stability during cleaning expressed as percentage of original activity determined in cleaning solution after 14-h cleaning period. Each value represents the mean ± SD (n = 2).**

additives. To determine the effect of pH on cleaning performance, cleaning was undertaken at 40°C for 2 h, with the pH of the cleaning solution left unadjusted or adjusted to pH 5–7.5 by the addition of buffers. The enzyme concentration of each cleaning solution was circa 5 units/ml. Optimum cleaning performance, as judged by the amount of organic matter removed from the panels, was observed when the crude enzyme was used without pH adjustment or addition of buffer components. Under these conditions, the cleaning solution had a pH of 5.8 and the amount of organic matter removed was approximately 1.7 times that

removed by the same concentration of crude enzyme, with pH adjusted to pH 5 or pH 6 by addition of buffer (Fig. 3.8a). Within the pH range tested, optimum enzyme stability was observed at pH 5–6, with reduced stability observed with increasing pH (Fig. 3.8b). Poor removal of organic matter was observed when cleaning was carried out at pH 7 and pH 7.5. The poor cleaning performance observed at these pH values may be attributed to the reduced stability (Fig. 3.8b) and activity (Fig. 3.5) of the enzyme under these conditions.

A series of cleaning experiments was carried out in which 0.1% Triton X-100 and 0.1% propylene glycol were included in the enzyme cleaning solution. Triton X-100 is a non-ionic detergent that is frequently used in biological applications to solubilise proteins. It is reported to improve enzyme accessibility to fouling material, weaken lipid–protein interactions and reduce readsorption of proteins after removal during cleaning (Allie et al., 2003). Propylene glycol is commonly included in commercial enzyme preparations as a preservative and enzyme stabiliser (Becker et al., 1997; Herrmann et al., 1997).

Table 3.4 shows the effect of 0.1% (v/v) Triton X-100 and 0.1% (w/v) propylene glycol on the cleaning performance of the crude DSM 1024 enzyme when cleaning was carried out for 1 h at 40°C. Improved cleaning was observed upon inclusion of the additives,

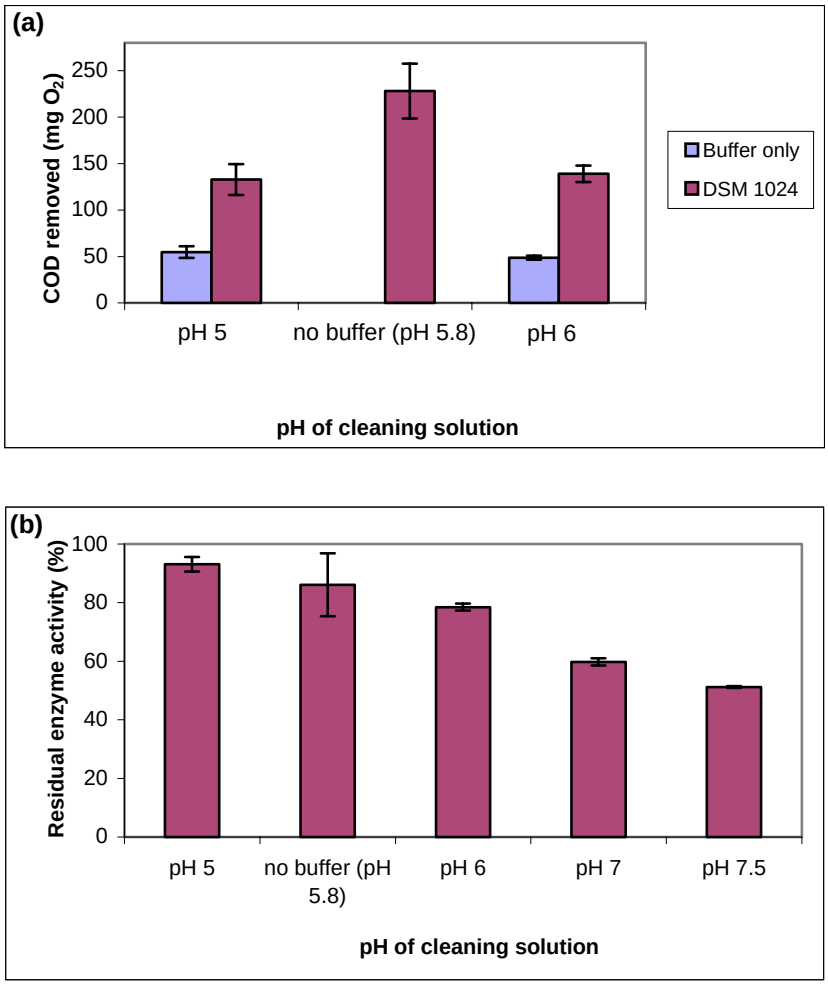


**Figure 3.8. Effect of pH on the cleaning performance of the crude enzyme from DSM 1024 *Schizophyllum commune*. (a) Amount of organic matter removed during a 2-h cleaning period at different pH values; (b) enzyme stability at different pH values, expressed as percentage of original activity detected in the cleaning solution after the 2-h cleaning period. Each value represents the mean ± SD (n=2).**

**Table 3.4. Effect of additives on the cleaning performance of DSM 1024 *Schizophyllum commune* crude protease. Cleaning was carried out at 40°C for 1 h.**

| Cleaning solution | pH | Enzyme activity mean ± SD | COD removed Mean ± SD (mg $O_2$) | Residual enzyme activity (%) |
|---|---|---|---|---|
| **DSM 1024 crude enzyme only** | 5.93 | 58.15 ± 0.90 | 185.49 ± 2.76 | 96.54 ± 1.22 |
| **DSM 1024 crude enzyme + 0.1% (v/v) Triton X-100 + 0.1% (w/v) propylene glycol** | 5.93 | 56.76 ± 1.83 | 247.97 ± 13.81 | 93.93 ± 2.21 |
| **Buffer only** | 6.15 | 0 | 33.59 ± 3.31 | – |
| **Buffer + 0.1% Triton X-100 + 0.1% propylene glycol** | 6.13 | 0 | 29.69 ± 4.42 | – |

COD, chemical oxygen demand.

with the amount of organic matter removed approximately 130% of that removed using the enzyme only, even though these additives were incapable of removing fouling from the stainless steel panels when used without the enzyme. Inclusion of the additives at the concentrations indicated had no effect on enzyme stability during cleaning. Similar improvements in the removal of organic matter from the panels were observed when the additives were used with different concentrations of the enzyme and for different cleaning times.

### *3.3.3.2 Biochemical analysis of crude enzyme*

The crude enzyme produced by DSM 1024 was analysed further by SDS-PAGE with activity staining using casein and denatured β-Lg as substrates (Fig. 3.9).

Activity on denatured β-Lg is of more relevance for CIP application as it is the most abundant protein in milk fouling deposits, accounting for at least 50% of the protein in a deposit formed at 70–80°C (Fryer et al., 2006). Upon analysis by SDS-PAGE, the crude DSM 1024 enzyme was found to contain several bands with activity on casein (Fig. 3.9a) and at least two distinct bands with activity on β-Lg of approximately 20 and 42 kDa (Fig. 3.9b).

Exposure of the crude enzyme from DSM 1024 to the protease inhibitors EDTA and DTT resulted in a significant loss of protease activity, with 33.64 ± 1.55% and 56.30 ± 2.03% of original activity remaining after incubation with 10 mM EDTA and 10 mM DTT, respectively. This indicates that the crude preparation from DSM 1024 contains both cysteine and metalloprotease activities. *Schizophyllum commune* is known to produce a variety of proteolytic enzymes, many of which are believed to be involved in mycelial autolysis during growth in nitrogen-limiting conditions (Johnston et al., 2000).

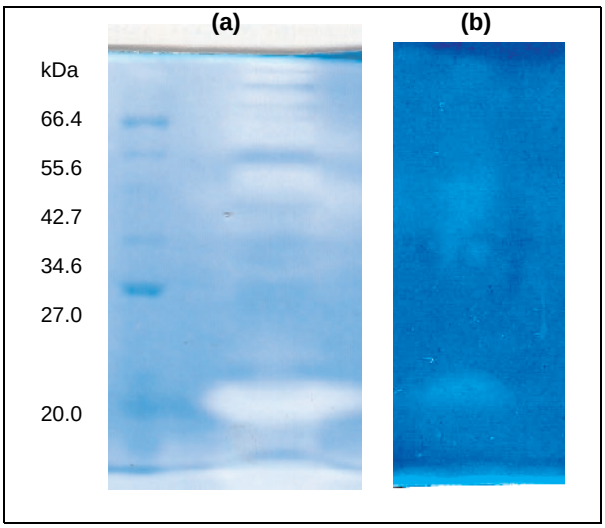


**Figure 3.9. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of DSM 1024 crude enzyme with activity staining using (a) casein and (b) denatured β-lactoglobulin as substrate.**

**Table 3.5. Results from cleaning studies using the crude enzyme produced by DSM 5552 *Bacillus subtilis.***

| Cleaning solution and cleaning time | Enzyme activity (units/ml) | COD removed (mg $O_2$) (% of total) | Protein not removed (mg) | Residual activity (%) |
|---|---|---|---|---|
| **DSM 5552, pH 10, 14 h** | 1.91 ± 0.11 | 336.72 ± 3.31 (100 ± 0.98%) | 0.96 ± 0.40 | 83.46 ± 5.12 |
| **DSM 5552, pH 10, 2 h** | 5.72 ± 0.24 | 255.00 ± 10.10 (85.66 ± 3.39%) | 5.14 ± 0.50 | 87.39 ± 0.34 |
| **DSM 5552, pH 10, 2 h** | 12.96 ± 0.38 | 423.75 ± 17.06 (90.63 ± 3.65%) | 5.39 ± 0.36 | 89.70 ± 1.72 |
| **DSM 5552, pH 10, 1 h** | 66.23 ± 6.27 | 252.68 ± 6.69 (88.43 ± 2.34%) | 3.44 ± 0.47 | 94.94 ± 3.88 |
| **pH 10 buffer** | 0 | 137.50 ± 3.54 (44.21 ± 1.14%) | 16.79 ± 0.71 | – |

COD, chemical oxygen demand.

### *3.3.4 Further studies on DSM 5552* Bacillus subtilis *crude protease*

The results of further cleaning studies undertaken using the crude enzyme produced by DSM 5552 *Bacillus subtilis* are shown in Table 3.5. No effect on protease activity was observed when 0.1% (v/v) Triton X-100 and up to 2% (w/v) propylene glycol were included in the azocasein enzyme assay. Improved cleaning performance could potentially be achieved by inclusion of these additives in the enzyme cleaning solution.

## 3.4 Conclusions

The crude activity from the fungus DSM 1024 *Schizophyllum commune* was found to be most suitable for potential use in CIP operations in dairy processing on the basis of its cleaning performance, stability during cleaning and high activity on denatured whey protein. Optimum cleaning performance using this crude enzyme was observed at approximately pH 5.8. Cleaning at this pH would have minimal impact on the pH of biological wastewater treatment systems and would eliminate the need for neutralisation of cleaning solutions prior to release, as described in Section 1.4.5.

While the crude enzyme is capable of removing milk fouling deposit from stainless steel at pH 5.8, cleaning at this pH is more difficult than cleaning at higher pH values due to the pH dependence of protein swelling which occurs during cleaning (Section 1.4.4). Minimum protein swelling occurs during cleaning at pH 5.8, which is close to the isoelectric point of β-Lg, the most abundant protein in the deposit, which is likely to render subsequent removal of the deposit more difficult. Enhanced cleaning was observed when Triton X-100 and propylene glycol were included in the cleaning solution. In order to achieve satisfactory cleaning at pH 5.8 using low concentrations of DSM 1024 activity, further optimisation of cleaning needs to be undertaken. This could potentially be achieved by the use of other enzyme-compatible additives. For example, Onaizi et al. (2009) reported improved cleaning performance when the surfactant sodium dodecyl benzyl sulfonate (SDOBS) (2 ppm) was included in a protease cleaning solution. This was attributed to enhanced weakening of the deposit by the surfactant via breakdown of intermolecular physical bonds between the protein molecules which, in turn, improved penetration of the enzyme and the surfactant. Biosurfactants, produced from renewable sources, are also of potential interest (Becker et al., 1997; Rosenberg and Ron, 1999). In the Onaizi et al. study, enhanced cleaning was also observed when the protease was used with a biosurfactant. Improved cleaning could also possibly be achieved by performing an acid circulation step prior to the enzyme cleaning step which has been reported by Grasshoff (2002, 2005) to result in more intense enzymatic deposit removal.

While the laboratory-scale cleaning performance of the crude activities from DSM 1024 and several other micro-organisms was found to be promising, the actual suitability of these enzymes for CIP operations in dairy processing can only be fully determined by pilot-scale studies.

# 4 Overall Conclusions

Based on the results of the laboratory-scale studies undertaken, three commercial protease products were found to be of potential use for CIP operations in the dairy processing industry on the basis of their cleaning performance, ease of removal/inactivation after use and initial cost analysis. It is noteworthy, however, that while these laboratory-scale studies are a useful indicator of potential suitability, pilot- and industrial-scale studies are necessary to confirm actual industrial suitability as well as economic feasibility. The three commercial protease products are currently produced and sold in bulk industrial quantities by global enzyme manufacturers and therefore could be readily obtained in sufficient quantities for such studies. The performance and cost of enzyme-based cleaning were found to be comparable to those of the currently used CIP procedures when investigated on a laboratory scale. If similar results are observed on an industrial scale then enzyme-based cleaning offers several advantages over the currently used CIP procedures. These include reduced environmental impact as the enzymes operate at lower temperatures, resulting in reduced energy consumption, and also enzymes are biodegradable in contrast to many of the products currently used for cleaning which have adverse effects on human and ecosystem health and on the environment. Enzyme-based cleaning is also more compatible with subsequent wastewater treatment and would create safer working conditions for plant operators.

Screening of selected micro-organisms resulted in the identification of 13 strains that produced extracellular protease activity capable of removing milk-based fouling deposit from stainless steel. The crude activity produced by the fungus DSM 1024 *Schizophyllum commune* was found to be the most suitable for potential CIP application and, while some optimisation of its cleaning performance was undertaken, further work is required to achieve satisfactory cleaning at low enzyme levels. The majority of microbial proteases have never been assessed in terms of their potential suitability for CIP operations in dairy processing and the application-relevant screening approach developed and optimised in the current study will be of use in future screening studies in this area.

While future work on this topic should focus primarily on pilot-scale cleaning studies as outlined above, an additional avenue for further investigation is the ability of the three selected commercial proteases to remove biofilms from processing equipment. Biofilms, which are aggregations of bacterial cells that grow on a surface to which they are attached, are of concern to dairy manufacturers, as discussed in Sections 1.3.1 and 1.5.4.4, due to their potential effects on product safety and quality. A review of the relevant literature indicates that enzymes could potentially be more effective in removing biofilms than conventional cleaning methods.

# References


Adler-Nissen, J., 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry* **27:** 1256–1262.

Allie, Z., Jacobs, E.P., Maartens, A. and Swart, P., 2003. Enzymatic cleaning of ultrafiltration membranes fouled by abattoir effluent. *Journal of Membrane Science* **218:** 107–116.

Anwar, A. and Saleemuddin, M., 1998. Alkaline proteases: A review. *Bioresource Technology* **64:** 175–183.

APHA, 1995. *Standard Methods for the Examination of Water and Wastewater.* American Public Health Association, American Water Works Association, Water Environment Federation, Washington DC, USA.

Arguello, M.A., Alvarez, S., Riera, F.A. and Alvarez, R., 2002. Enzymatic cleaning of inorganic ultrafiltration membranes fouled by whey proteins. *Journal of Agricultural and Food Chemistry* **50:** 1951–1958.

Arguello, M.A., Alvarez, S., Riera, F.A. and Alvarez, R., 2003. Enzymatic cleaning of inorganic ultrafiltration membranes used for whey protein fractionation. *Journal of Membrane Science* **216:** 121–134.

Arguello, M.A., Alvarez, S., Riera, F.A. and Alvarez, R., 2005. Utilization of enzymatic detergents to clean inorganic membranes fouled by whey proteins. *Separation and Purification Technology* **41:** 147–154.

Bansal, B. and Chen, X.D., 2006. A critical review of milk fouling in heat exchangers. *Comprehensive Reviews in Food Science and Food Safety* **5:** 27–33.

Barrett, A., 2000. Proteases. *In:* Coligan, J., Dunn, B., Ploegh, H., Speicher, D. and Wingfield, P. (Eds) *Current Protocols in Protein Science.* John Wiley and Sons, Inc.

Becker, T., Park, G. and Gaertner, A., 1997. Formulation of detergent enzymes. *In:* Ee, J.H.V., Misset, O. and Baas, E. (Eds) *Enzymes in Detergency.* Marcel Dekker, Inc., New York, USA. pp. 299–325.

Begley, C., Paragina, S. and Sporn, A., 1990. An analysis of contact lens enzyme cleaners. *Journal of the American Optometric Association* **61:** 190–194.

Belyauskaite, I.P., Palubinskas, V.J., Anchenko, O.E., Vesa, V.S. and Glemzha, A.A., 1980. Purification and some properties of the extracellular acid proteases from *Mucor renninus*. *Enzyme and Microbial Technology* **2:** 37–44.

Bord Bia, 2007. *Factsheet on the Irish Agriculture and Food & Drink Sector.* Available http://www.bordbia.ie [accessed May 2008].

Bott, R., 1997. Development of new proteases for detergents. *In:* Ee, J.H.V., Misset, O. and Baas, E. (Eds) *Enzymes in Detergency.* Marcel Dekker, Inc., New York, USA. pp. 75–92.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein dye-binding. *Analytical Biochemistry* **72:** 248–254.

Bremer, P.J., Fillery, S. and Mcquillan, A.J., 2006. Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *International Journal of Food Microbiology* **106:** 254–262.

Brooker, B., 1991. The study of food systems using confocal laser scanning microscopy. *Microscopy and Analysis* **27:** 13–15.

Brooker, B., 1995. Imaging food systems by confocal scanning laser microscopy. *In:* Dickenson, E. (Ed.) *New Physico-Chemical Techniques for the Characterization of Complex Food Systems.* Blackie Academic and Professional, London, UK. pp. 53–68.

Bylund, G., 1995. *Dairy Processing Handbook.* Tetra Pak Processing Systems AB, Lund, Sweden.

Cammarota, M.C. and Freire, D.M.G., 2006. A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. *Bioresource Technology* **97:** 2195–2210.

Catara, G., Fiume, I., Iuliano, F., Maria, G., Ruggiero, G., Palmieri, G., Capasso, A. and Rossi, M., 2006. A new kumamolisin-like protease from *Alicyclobacillus acidocaldarius*: An enzyme active under extreme acidic conditions. *Biocatalysis and Biotransformation* **24:** 358–370.

Chan, R. and Chen, V., 2004. Characterization of protein fouling on membranes: Opportunities and challenges. *Journal of Membrane Science* **242:** 169–188.

Changani, S.D., Belmarbeiny, M.T. and Fryer, P.J., 1997. Engineering and chemical factors associated with fouling and cleaning in milk processing. *Experimental Thermal and Fluid Science* **14:** 392–406.

Chisti, Y., 2000. Modern systems of plant cleaning. *In:* Robinson, R. (Ed.) *Encyclopedia of Food Microbiology.* Academic Press, London, UK.

Cruz-Córdova, T., Roldán-Carrillo, T.G., Díaz-Cervantes, D., Ortega-López, J., Saucedo-Castañeda, G., Tomasini-Campocosio, A. and Rodríguez-Vázquez, R., 1999. $CO_2$ evolution and ligninolytic and proteolytic activities of *Phanerochaete chrysosporium*

grown in solid state fermentation. *Resources, Conservation and Recycling* **27:** 3–7.

CSO, 2009a. *Lifestock Survey December 2008*. Central Statistics Office, Ireland.

CSO, 2009b. *Statistical Product – Agriculture Milk Production*. Central Statistics Office, Ireland.

Danalewich, J.R., Papagiannis, T.G., Belyea, R.L., Tumbleson, M.E. and Raskin, L., 1998. Characterization of dairy waste streams, current treatment practices, and potential for biological nutrient removal. *Water Research* **32:** 3555–3568.

Doddapaneni, K.K., Tatineni, R., Vellanki, R.N., Rachcha, S., Anabrolu, N., Narakuti, V. and Mangamoori, L.N., 2009. Purification and characterization of a solvent and detergent-stable novel protease from *Bacillus cereus*. *Microbiological Research* **164:** 383–390.

D'Souza, N.M. and Mawson, A.J., 2005. Membrane cleaning in the dairy industry: A review. *Critical Reviews in Food Science and Nutrition* **45:** 125–134.

Dunaevsky, Y.E., Zhang, D., Matveeva, A.R., Belyakova, G.A. and Belozersky, M.A., 2006. Degradation of proteinaceous substrates by xylotrophic basidiomycetes. *Microbiology* **75:** 35–39.

Dunn, B., 2002. Peptidases. *In:* Coligan, J., Dunn, B., Ploegh, H., Speicher, D. and Wingfield, P. (Eds) *Current Protocols in Protein Science.* John Wiley and Sons, Inc.

Egmond, M.R., 1997. Application of proteases in detergents. *In:* Ee, J.H.V., Misset, O. and Baas, E.J. (Eds) *Enzymes in Detergency.* Marcel Dekker, Inc., New York, USA. pp. 61–91.

Eide, M.H., Homleid, J.P. and Mattsson, B., 2003. Life cycle assessment (LCA) of cleaning-in-place processes in dairies. *Lebensmittel-Wissenschaft Und-Technologie – Food Science and Technology* **36:** 303–314.

Eriksen, N., 1996. Detergents. *In:* Godfrey, T. and West, S. (Eds) *Industrial Enzymology.* 2nd ed. Macmillan Press Ltd, London, UK. pp. 188–200.

FDII, 2009. *The Food and Drink Industry in Ireland. Closing the Gap. Competitive Indicators 2009*. Food and Drink Industry Ireland, IBEC, Dublin, Ireland.

Ferrando, M., Rozek, A., Zator, M., Lopez, F. and Guell, C., 2005. An approach to membrane fouling characterization by confocal scanning laser microscopy. *Journal of Membrane Science* **250:** 283–293.

Flint, S.H., Van Den Elzen, H., Brooks, J.D. and Bremer, P.J., 1999. Removal and inactivation of thermo-resistant *Streptococci* colonising stainless steel. *International Dairy Journal* **9:** 429–436.

Fox, P. and McSweeney, P., 1998. *Dairy Chemistry and Biochemistry*. Blackie Academic and Professional, London, UK.

Fryer, P. and Christian, G., 2005. Improving the cleaning of heat exchangers. *In:* Lelieveld, H., Mostert, M. and Holah, J. (Eds) *Handbook of Hygiene Control in the Food Industry.* Woodhead Publishing Ltd, Cambridge, UK. pp. 468–491.

Fryer, P.J. and Asteriadou, K., 2009. A prototype cleaning map: A classification of industrial cleaning processes. *Trends in Food Science and Technology* **20:** 255–262.

Fryer, P.J., Christian, G.K. and Liu, W., 2006. How hygiene happens: Physics and chemistry of cleaning. *International Journal of Dairy Technology* **59:** 76–84.

Gillham, C.R., Fryer, P.J., Hasting, A.P.M. and Wilson, D.I., 1999. Cleaning-in-place of whey protein fouling deposits: Mechanisms controlling cleaning. *Food and Bioproducts Processing* **77:** 127–136.

Gotham, S.M., Fryer, P.J. and Paterson, W.R., 1988. The measurement of insoluble proteins using a modified bradford assay. *Analytical Biochemistry* **173:** 353–358.

Graminha, E.B.N., Gonçalves, A.Z.L., Pirota, R.D.P.B., Balsalobre, M.A.A., Da Silva, R. and Gomes, E., 2008. Enzyme production by solid-state fermentation: Application to animal nutrition. *Animal Feed Science and Technology* **144:** 1–22.

Grasshoff, A., 2002. Enzymatic cleaning of milk pasteurizers. *Food and Bioproducts Processing* **80:** 247–252.

Grasshoff, A., 2005. Enzymatic cleaning in food processing. *Handbook of Hygiene Control in the Food Industry*. Woodhead Food Series No. 116. Woodhead Publishing Limited, Cambridge, UK. pp. 516–538.

Gunasekaran, S., Ko, S. and Xiao, L., 2007. Use of whey proteins for encapsulation and controlled delivery applications. *Journal of Food Engineering* **83:** 31–40.

Gupta, R., Beg, Q.K. and Lorenz, P., 2002a. Bacterial alkaline proteases: Molecular approaches and industrial applications. *Applied Microbiology and Biotechnology* **59:** 15–32.

Gupta, R., Beg, Q.K., Khan, S. and Chauhan, B., 2002b. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbiology and Biotechnology* **60:** 381–395.

Gupta, R., Gupta, N. and Rathi, P., 2004. Bacterial lipases: An overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology* **64:** 763–781.

Hadj-Ali, N.E., Agrebi, R., Ghorbel-Frikha, B., Sellami-Kamoun, A., Kanoun, S. and Nasri, M., 2007. Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. *Enzyme*

*and Microbial Technology* **40:** 515–523.

Hasan, F., Shah, A.A. and Hameed, A., 2006. Industrial applications of microbial lipases. *Enzyme and Microbial Technology* **39:** 235–251.

Herrmann, H., Good, I. and Laufer, A., 1997. Manufacturing and downstream processing of detergent enzymes. *In:* Ee, J.H.V., Misset, O. and Baas, E. (Eds) *Enzymes in Detergency.* Marcel Dekker, Inc., New York, USA. pp. 251–297.

Hooper, R.J., Paterson, W.R. and Wilson, D.I., 2006. Comparison of whey protein model foulants for studying cleaning of milk fouling deposits. *Food and Bioproducts Processing* **84:** 329–337.

Houde, A., Kademi, A. and Leblanc, D., 2004. Lipases and their industrial applications – an overview. *Applied Biochemistry and Biotechnology* **118:** 155–170.

Hrmova, M. and Fincher, G.B., 1993. Purification and properties of 3 (1-3)-beta-d-glucanase isoenzymes from young leaves of barley (*Hordeum vulgare*). *Biochemical Journal* **289:** 453–461.

IAE, 2009. *Review of Ireland's Energy Policy in the Context of the Changing Economy*. The Irish Academy of Engineering, Dublin, Ireland.

IDB, 2007. *Irish Dairy Industry Profile*. Irish Dairy Board, available http://www.idb.ie [accessed May 2008]

IDB, 2009. *Annual Report 2008*. Irish Dairy Board, Dublin, Ireland.

Jackson, G.S., Mckintosh, E., Flechsig, E., Prodromidou, K., Hirsch, P., Linehan, J., Brandner, S., Clarke, A.R., Weissmann, C. and Collinge, J., 2005. An enzyme-detergent method for effective prion decontamination of surgical steel. *Journal of General Virology* **86:** 869–878.

Jaeger, K.E. and Reetz, M.T., 1998. Microbial lipases form versatile tools for biotechnology. *Trends in Biotechnology* **16:** 396–403.

Jeurnink, T.J.M., Walstra, P. and Dekruif, C.G., 1996. Mechanisms of fouling in dairy processing. *Netherlands Milk and Dairy Journal* **50:** 407–426.

Johnston, J.M., Ramos, E.R., Bilbrey, R.E., Gathman, A.C. and Lilly, W.W., 2000. Characterization of scpri, a small serine protease, from mycelia of *Schizophyllum commune*. *Mycological Research* **104:** 726–731.

Joo, H.-S., Kumar, C.G., Park, G.-C., Kim, K.T., Paik, S.R. and Chang, C.-S., 2002. Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. *Process Biochemistry* **38:** 155–159.

Kennedy, J. and O'Neill, L., 2009. Dairy industry – the issues. *Irish Farmers Journal* (18 April 2009). Dublin, Ireland.

Kulozik, U., 2003. Biofilm formation. *In:* Roginski, H. (Ed.) *Encyclopedia of Dairy Sciences.* Academic Press, London, UK.

Kwon, D.Y. and Rhee, J.S., 1986. A simple and rapid colorimetric method for determination of free fatty-acids for lipase assay. *Journal of the American Oil Chemists Society* **63:** 89–92.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of head of bacteriophage-t4. *Nature* **227:** 680–685.

Lascurettes, C., 2008. Processing revamp required. *Irish Farmers Journal* (15 November 2008). Dublin, Ireland.

Lawson, V.A., Stewart, J.D. and Masters, C.L., 2007. Enzymatic detergent treatment protocol that reduces protease-resistant prion protein load and infectivity from surgical-steel monofilaments contaminated with a human-derived prion strain. *Journal of General Virology* **88:** 2905–2914.

Leal, M., Freire, D.M.G., Carnmarota, M.C. and Sant'anna, G.L., 2006. Effect of enzymatic hydrolysis on anaerobic treatment of dairy wastewater. *Process Biochemistry* **41:** 1173–1178.

Le-Clech, P., Marselina, Y., Ye, Y., Stuetz, R.A. and Chen, V., 2007. Visualisation of polysaccharide fouling on microporous membrane using different characterisation techniques. *Journal of Membrane Science* **290:** 36–45.

Lee, N., Amy, G., Croue, J.P. and Buisson, H., 2005. Morphological analyses of natural organic matter (NOM) fouling of low-pressure membranes (MF/UF). *Journal of Membrane Science* **261:** 7–16.

Maartens, A., Swart, P. and Jacobs, E.P., 1996. An enzymatic approach to the cleaning of ultrafiltration membranes fouled in abattoir effluent. *Journal of Membrane Science* **119:** 9–16.

Maste, M.C.L., Norde, W. and Visser, A., 1997. Adsorption-induced conformational changes in the serine proteinase savinase: A tryptophan fluorescence and circular dichroism study. *Journal of Colloid and Interface Science* **196:** 224–230.

Mateos, J.C., Ruiz, K., Rodriguez, J.A., Cordova, J. and Baratti, J., 2007. Mapping substrate selectivity of lipases from thermophilic fungi. *Journal of Molecular Catalysis B-Enzymatic* **49:** 104–112.

Maurer, K.H., 2004. Detergent proteases. *Current Opinion in Biotechnology* **15:** 330–334.

Mendes, A.A., Pereira, E.B. and De Castro, H.F., 2006. Effect of the enzymatic hydrolysis pretreatment of lipids-rich wastewater on the anaerobic biodigestion. *Biochemical Engineering Journal* **32:** 185–190.

Mercadé-Prieto, R. and Chen, X.D., 2005. Caustic-induced gelation of whey deposits in the alkali cleaning of membranes. *Journal of Membrane*

*Science* **254:** 157–167.

Merheb, C.W., Cabral, H., Gomes, E. and Da-Silva, R., 2007. Partial characterization of protease from a thermophilic fungus, *Thermoascus aurantiacus*, and its hydrolytic activity on bovine casein. *Food Chemistry* **104:** 127–131.

Misset, O., 1997. Development of new lipases. *In:* Ee, J.H.V., Misset, O. and Baas, E. J. (Eds) *Enzymes in Detergency.* Marcel Dekker, Inc., New York, USA. pp. 107–131.

Morcelle, S.R., Caffini, N.O. and Priolo, N., 2004. Proteolytic properties of *Funastrum clausum* latex. *Fitoterapia* **75:** 480–493.

National Milk Agency, 2009. *National Milk Agency Annual Report and Accounts 2008*. National Milk Agency, Dublin, Ireland.

Neurath, H., 1989. The diversity of proteolytic enzymes. *In:* Beynon, R. and Bond, J. (Eds) *Proteolytic enzymes – A Practical Approach.* IRL Press, Oxford, UK. pp. 1–12.

Novozymes, 2007. The detergent that lets consumers save as they wash. *Biotimes* 02/07.

O'Brien, B. (Ed.), 2008. *Milk Quality Handbook – Practical Steps to Improve Milk Quality.* Moorepark Dairy Production Research Centre, Teagasc, Fermoy, Ireland.

Olsen, H., 2004. *Enzymes at Work*. Novozymes A/S, Bagsvaerd, Denmark.

Onaizi, S.A., He, L.Z. and Middelberg, A.P.J., 2009. Rapid screening of surfactant and biosurfactant surface cleaning performance. *Colloids and Surfaces B-Biointerfaces* **72:** 68–74.

Oskouie, S.F.G., Tabandeh, F., Yakhchali, B. and Eftekhar, F., 2008. Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. *Biochemical Engineering Journal* **39:** 37–42.

Pottchoff, A. and Serve, W., 1997. The cleaning revolution. *Dairy Industries International* **62:** 25.

Pozo, M.J., Baek, J.-M., Garcia, J.M. and Kenerley, C.M., 2004. Functional analysis of tvsp1, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. *Fungal Genetics and Biology* **41:** 336–348.

Promar International, 2003. *Strategic Development Plan for the Irish Dairy Processing Sector*. Promar International, Cheshire, UK.

Purnell, P., 1993. Cleaning procedures in the factory. *In:* Macrae, R., Robinson, R. and Sadler, M. (Eds) *Encyclopedia of Food Science, Food Technology and Food Nutrition.* Academic Press, London, UK.

Ramírez-Zavala, B., Mercado-Flores, Y., Hernández-Rodríguez, C. and Villa-Tanaca, L., 2004a. Purification and characterization of a lysine aminopeptidase from *Kluyveromyces marxianus*. *FEMS Microbiology Letters* **235:** 369–375.

Ramírez-Zavala, B., Mercado-Flores, Y., Hernández-Rodríguez, C. and Villa-Tanaca, L., 2004b. Purification and characterization of a serine carboxypeptidase from *Kluyveromyces marxianus*. *International Journal of Food Microbiology* **91:** 245–252.

Reinemann, D., 2003. Hygiene in dairy production and processing. *In:* Roginski, H. (Ed.) *Encyclopedia of Dairy Sciences.* Academic Press, London, UK.

Rosenberg, E. and Ron, E.Z., 1999. High- and low-molecular-mass microbial surfactants. *Applied Microbiology and Biotechnology* **52:** 154–162.

Sambrook, J., Fritsch, E. and Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, New York, USA.

Samples, D.R., Richter, R.L. and Dill, C.W., 1984. Measuring proteolysis in cheddar cheese slurries – comparison of Hull and trinitrobenzene sulfonic-acid procedures. *Journal of Dairy Science* **67:** 60–63.

Sandhya, C., Sumantha, A., Szakacs, G. and Pandey, A., 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. *Process Biochemistry* **40:** 2689–2694.

Saran, S., Isar, J. and Saxena, R.K., 2007. A modified method for the detection of microbial proteases on agar plates using tannic acid. *Journal of Biochemical and Biophysical Methods* **70:** 697–699.

Sarath, G., Motte, R.D.L. and Wagner, F., 1989. Protease assay methods. *In:* Beynon, R. and Bond, J. (Eds) *Proteolytic Enzymes – a Practical Approach.* IRL Press, Oxford, UK. pp. 25–54.

Sharma, R., Chisti, Y. and Banerjee, U.C., 2001. Production, purification, characterization, and applications of lipases. *Biotechnology Advances* **19:** 627–662.

Siu, S.C., Boushaba, R., Topoyassakul, V., Graham, A., Choudhury, S., Moss, G. and Titchener-Hooker, N.J., 2006. Visualising fouling of a chromatographic matrix using confocal scanning laser microscopy. *Biotechnology and Bioengineering* **95:** 714–723.

Spellman, D., McEvoy, E., O'Cuinn, G. and Fitzgerald, R.J., 2003. Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal* **13:** 447–453.

Stern, G. and Zam, Z., 1987. The effect of enzymatic contact lens cleaning on adherence of *Pseudomonas aeruginosa* to soft contact lenses. *Ophthalmology* **94:** 115–119.

The National Dairy Council, 2008. *Dairy: Food for Life, A Strategy to Promote Irish Milk and Dairy Products*

*2008–2012*.

Trujillo, A.J., Guamis, B., Laencina, J. and López, M.B., 2000. Proteolytic activities of some milk clotting enzymes on ovine casein. *Food Chemistry* **71:** 449–457.

Tsuruoka, N., Nakayama, T., Ashida, M., Hemmi, H., Nakao, M., Minakata, H., Oyama, H., Oda, K. and Nishino, T., 2003. Collagenolytic serine-carboxyl proteinase from *Alicyclobacillus sendaiensis* strain ntap-1: Purification, characterization, gene cloning, and heterologous expression. *Applied and Environmental Microbiology* **69:** 162–169.

Visser, J. and Jeurnink, T.J.M., 1997. Fouling of heat exchangers in the dairy industry. *Experimental Thermal and Fluid Science* **14:** 407–424.

Walsh, G., 2002. *Proteins, Biochemistry and Biotechnology.* Wiley, Chichester, UK.

Walsh, G.A., Murphy, R.A., Killeen, G.F., Headon, D.R. and Power, R.F., 1995. Technical note – detection and quantification of supplemental fungal beta-glucanase activity in animal feed. *Journal of Animal Science* **73:** 1074–1076.

Walsh, G., Murphy, R.A., Killeen, G.F. and Power, R.F., 2005. Quantification of supplemental enzymes in animal feedingstuffs by radial enzyme diffusion. *Applied Microbiology and Biotechnology* **67:** 70–74.

Walstra, P., Wouters, J. and Geurts, T., 2006. *Dairy Science and Technology.* Taylor and Francis, Boca Raton, FL, USA.

Wildbrett, G., 2003. Dairy plant effluent. *In:* Roginski, H. (Ed.) *Encyclopedia of Dairy Sciences.* Academic Press, London, UK.

Wilson, D.I., 2005. Challenges in cleaning: Recent developments and future prospects. *Heat Transfer Engineering* **26:** 51–59.

Wolff, A. and Showell, M., 1997. Application of lipases in detergents. *In:* Ee, J.H.V., Misset, O. and Baas, E.J. (Eds) *Enzymes in Detergency.* Marcel Dekker, Inc., New York, USA. pp. 93–106.

Xin, H., Chen, X.D. and Ozkan, N., 2002a. Cleaning rate in the uniform cleaning stage for whey protein gel deposits. *Trans IChemE* **80:** 240–246.

Xin, H., Chen, X.D. and Ozkan, N., 2002b. Whey protein-based gel as a model material for studying initial cleaning mechanisms of milk fouling. *Journal of Food Science* **67:** 2702–2711.

Yang, Z., Peng, X.F., Chen, M.Y., Lee, D.J. and Lai, J.Y., 2007. Intra-layer flow in fouling layer on membranes. *Journal of Membrane Science* **287:** 280–286.

Yoo, J.Y., Chen, X.D., Mercade-Prieto, R. and Wilson, D. I., 2007. Dissolving heat-induced protein gel cubes in alkaline solutions under natural and forced convection conditions. *Journal of Food Engineering* **79:** 1315–1321.

Zator, M., Ferrando, M., Lopez, F. and Guell, C., 2007. Membrane fouling characterization by confocal microscopy during filtration of BSA/dextran mixtures. *Journal of Membrane Science* **301:** 57–66.

Zator, M., Warczok, J., Ferrando, M., Lopez, F. and Guell, C., 2009. Chemical cleaning of polycarbonate membranes fouled by BSA/dextran mixtures. *Journal of Membrane Science* **327:** 59–68.

# Acronyms and Annotations

| | |
|---|---|
| **α-La** | α-Lactalbumin |
| **β-Lg** | β-Lactoglobulin |
| **BMP** | Buttermilk powder |
| **BOD** | Biochemical oxygen demand |
| **BSA** | Bovine serum albumin |
| **CIP** | Cleaning in place |
| **CLSM** | Confocal laser scanning microscopy |
| **COD** | Chemical oxygen demand |
| **DTT** | Dithiothreitol |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EMC** | Enzyme-modified cheese |
| **FITC** | Fluorescein isothiocyanate |
| **HCl** | Hydrochloric acid |
| **Ig** | Immunoglobulin |
| **IUB No.** | Enzyme classification number (International Union of Biochemistry) |
| **MBP** | Maltose binding protein |
| **NaOH** | Sodium hydroxide |
| **$NaPO_4$** | Sodium phosphate |
| **NTA** | Nitrilotriacetic acid |
| **PDA** | Potato dextrose agar |
| **pI** | Isoelectric point |
| **PIPES** | Piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) |
| **SbF** | Submerged fermentation |
| **SDOBS** | Sodium dodecyl benzyl sulfonate |
| **SDS** | Sodium dodecyl sulfate |
| **SDS-PAGE** | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| **SEM** | Scanning electron microscopy |
| **SMP** | Skimmed milk powder |
| **SSF** | Solid-state fermentation |
| **TCA** | Trichloroacetic acid |
| **TEMED** | *N*,*N*,*N'*,*N'*-tetramethylethylenediamine |
| **TNBS** | Trinitrobenzenesulfonic acid |
| **Tris** | Tris(hydroxymethyl) aminomethane |
| **YM** | Universal medium for yeasts |
| **YPD** | Yeast peptone glucose medium |

# An Ghníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomh-nithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Ghníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil agus Rialtais Áitiúil a dhéanann urraíocht uirthi.

## ÁR bhFREAGRACHTAÍ

### CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreail.
- Scardadh dramhuisce

### FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain.
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce.
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí comhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

### MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOL

- Monatóireacht ar chaighdeán aeir agus caighdeáin aibhneacha, locha, uiscí taoide agus uiscí talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntí a dhéanamh.

### RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

### TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdéan aeir agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

### MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

### PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

### BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

### STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Ghníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstiúrthóir agus ceithre Stiúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

NDP
National Development Plan 2007 - 2013

**Science, Technology, Research and Innovation for the Environment (STRIVE) 2007-2013**

The Science, Technology, Research and Innovation for the Environment (STRIVE) programme covers the period 2007 to 2013.

The programme comprises three key measures: Sustainable Development, Cleaner Production and Environmental Technologies, and A Healthy Environment; together with two supporting measures: EPA Environmental Research Centre (ERC) and Capacity & Capability Building. The seven principal thematic areas for the programme are Climate Change; Waste, Resource Management and Chemicals; Water Quality and the Aquatic Environment; Air Quality, Atmospheric Deposition and Noise; Impacts on Biodiversity; Soils and Land-use; and Socio-economic Considerations. In addition, other emerging issues will be addressed as the need arises.

The funding for the programme (approximately €100 million) comes from the Environmental Research Sub-Programme of the National Development Plan (NDP), the Inter-Departmental Committee for the Strategy for Science, Technology and Innovation (IDC-SSTI); and EPA core funding and co-funding by economic sectors.

The EPA has a statutory role to co-ordinate environmental research in Ireland and is organising and administering the STRIVE programme on behalf of the Department of the Environment, Heritage and Local Government.

epa
Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

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SSTI
Strategy for Science, Technology and Innovation

Comhshaol, Oidhreacht agus Rialtas Áitiúil
**Environment, Heritage and Local Government**