Journal of Applied Microbiology 2004, **96**, 110–116 doi:10.1046/j.1365-2672.2003.02136.x

Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel

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2003/0371: received 5 May 2003, revised 15 June 2003 and accepted 26 August 2003

ABSTRACT

S.G. PARKAR, S.H. FLINT AND J.D. BROOKS. 2003.

Aims: To determine the mechanism for both the removal and inactivation of 18-h biofilms of a thermophilic *Bacillus* species that optimally grows at 55°C on stainless steel.

Methods and Results: The cleaning strategies tested were based on biofilm biochemistry and physiology, and focused on the chemistry of the cleaners, the duration and temperature of the cleaning process and a combination of various cleaners. The success of the cleaning regimes was determined based on the removal of cells and organic debris and the elimination of viable cells. The results confirmed that a caustic (75°C for 30 min) and acid (75°C for 30 min) wash, relied upon heavily in most food processing industries for cleaning-in-place systems, was successful in removing these biofilms. However, any changes in the concentrations of these cleaners or the temperature of cleaning drastically affected the overall outcome. Alternative cleaning agents based on enzymatic or nonenzymatic breakdown of cellular proteins or polysaccharides, surfactant action, use of oxidative attack and free radicals varied in degrees of their success. Combining proteolytic action with surfactants increased wetability and therefore enhanced the cleaning efficiency.

Conclusions: Several procedures, including caustic/acid and enzyme based cleaners, will be satisfactory, provided that the correct process parameters are observed i.e. concentration, time, temperature and kinetic energy (flow). Confirmation of these results should be carried out in a pilot plant through several use/clean cycles.

Significance and Impact of the Study: Confidence in standard and alternative cleaning procedures for food manufacturing plant to prevent contamination with thermophilic bacilli that threaten product quality.

Keywords: biofilm, clean in place, cleaning, milk, stainless steel, thermobacilli.

INTRODUCTION

Bacteria form biofilms on the surfaces of stainless steel equipment in food processing industries, releasing bacteria that compromise the safety and quality of the final product. Other unfavourable conditions associated with biofilms that affect food manufacturers include reduced flow through blocked tubes, reduced plant run times, corrosion of stainless steel, and reduced heat transfer through plate heat exchangers. Cleaning and disinfection have been incorporated into the cleaning-in-place (CIP) regimes in food manufacturing industries (Romney 1990; Zottola and Sasahara 1994). However, bacterial contamination and product spoilage because of biofilm formation are recurring problems (Carpentier *et al.* 1998). Cleaning and disinfection studies have focused on eliminating food-borne pathogens (e.g. *Listeria*) and have neglected other contaminating organisms (e.g. thermophilic bacilli) that threaten food quality.

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Bacterial biofilms may be defined as cells adhering to, and growing on, artificial substrates and are often associated with an extracellular polysaccharide matrix. The very nature of their structure and formation provides greater resistance to cleaning and disinfection, compared with planktonic cells (Joseph *et al.* 2001). Many agents have traditionally been used for CIP (Romney 1990) to allow cleaning to within acceptable levels.

It is difficult to find references in the literature about effective cleaning systems for sporeforming bacteria in food manufacturing plant. Studies on biofilms in dairy manufacturing plant have focused on *Pseudomonas* species and food borne pathogens (Dunsmore 1980; Frank and Koffi 1990; Zottola and Sasahara 1994). There are a few publications dealing with sporeforming bacteria (Husmark and Rönner 1992) and only limited information on thermophilic sporeforming bacilli as biofilms in dairy manufacturing plants (Parkar *et al.* 2001).

We have chosen to use the vegetative form of a thermophilic bacillus for our study because spores have not been observed within the biofilms of these organisms (Parkar *et al.* 2001). Any residual bacteria left after a cleaning cycle can potentially sporulate and therefore continue to proliferate and cause problems within the food manufacturing system. Here, we have compared for the first time, various cleaning strategies to control the formation of biofilms of sporeforming thermophilic bacilli (e.g. *Bacillus flavothermus)*.

Cleaners were selected for this study on the basis of their mechanisms of action as follows:

- i Alkaline cleaning, causing dissolution of cell material and removal of calcium deposits e.g. standard caustic soda and acid, and Eliminator.
- ii Enzyme based cleaning:
  - a Protease cleaners such as Paradigm which is an enzyme based cleaner combining proteolytic activity with surfactant action, and Purafect®, a serine protease.
  - b Polysaccharidase based agents such as Purastar™, an α-amylase, CellulaseL, a polysaccharidase and mutanolysin, a cell wall peptidoglycan hydrolase.
- iii Oxidizing chemicals such as sodium hypochlorite, Halamid (a chloramine), Oxine® (chlorine dioxide) and Perform® (combination of peracetic acid and hydrogen peroxide). These are all general pro-oxidants generating reactive oxygen and/or chlorine species. They initiate a cascade of oxidative attack on bacterial -SH group containing moieties such as enzymes, and peroxidative attack on membrane unsaturated fatty acids (Estrela *et al.* 2002).
- iv A quaternary ammonium chloride, such as Bactosolve®, that causes breakdown of cell membrane followed by intracellular potassium efflux and finally cellular protein/nucleic acid damage (Romney 1990).
- v Detergents, such as Tween-80 (a synthetic polysorbate) and dobanic acid, have surfactant effects; they enhance access of other cleaners to the biofilms by increasing their wetability.

On the basis of these treatments this paper aims to: (i) validate and establish the correct process parameters to achieve good cleaning practices in standard commercial procedures within a laboratory scale system, (ii) estimate the effect and efficiency of other agents that target cleaning in a defined biochemical mode of action within a laboratory scale system and (iii) examine the efficiency of some of the most effective cleaning procedures in a pilot industrial scale system.

MATERIALS AND METHODS

Sources of bacterial strains and cleaning chemicals

Bacillus flavothermus (B12-C^{m}), originally isolated from a milk powder manufacturing plant, was grown in trypticase soya broth (TSB) (BBL, Becton Dickinson, Cockeysville, MD, USA) at 55°C for 18 h, from stocks stored on Microbank cryobeads (Pro-Lab Diagnostics, Austin, TX, USA) at −20°C at the Biofilm Research Unit at the Institute of Food, Nutrition and Human Health, Massey University.

Paradigm (proteolytic enzyme cleaner) was obtained from Ecolab, Hamilton, New Zealand. Eliminator (caustic based cleaner), Perform® (hydrogen peroxide/peracetic acid) and Bactosolve® (a quarternary ammonium compound) were obtained from Orica Chemnet, Mt Maunganui, New Zealand.

Purafect® (a subtilisin), Purastar™ HPAmL (an α-amylase), and CellulaseL (Genencor International, Rochester, NY, USA) were supplied by Enzyme Services, Auckland, New Zealand. Oxine® was a gift from Biocide Pacific Ltd, Auckland, New Zealand. Mutanolysin was obtained from Sigma-Aldrich, Auckland, New Zealand.

Dobanic acid (a linear alkylbenzene sulphonic acid), was obtained from Chemcolour Industries NZ Ltd, Auckland, New Zealand. Tween-80 and lecithin were obtained from BDH, Palmerston North, New Zealand. All other chemicals were of analytical grade and obtained locally, unless otherwise mentioned.

Biofilm substrate

Cold rolled stainless steel, grade 316 with 2B surface finish as supplied for dairy manufacturing plant construction, was cut into coupons (1 cm × 1 cm × 1 mm) (Parkar *et al.* 2001). The coupons were then inserted into the laboratory biofilm reactor (Flint *et al.* 2001) and these were used to grow biofilms for all laboratory studies. An alternative

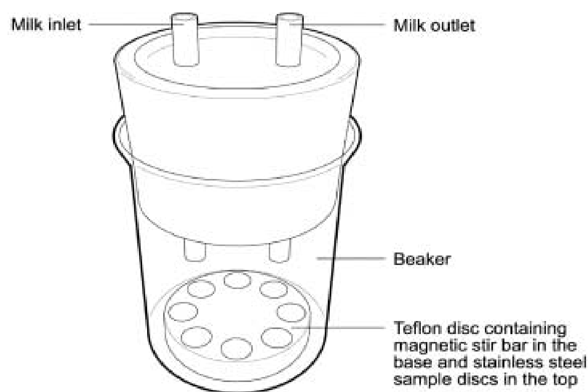

Fig. 1 Diagram of the annular reactor used for biofilm growth on stainless steel coupons (Reservoir is a 500-ml glass beaker operating with a volume of 200 ml. The sample discs are represented by the small circles in the large Teflon disc. This system was placed on a heated magnetic stirrer controlled at a temperature of 55°C)

'annular' reactor (Fig. 1) was used to grow biofilms on stainless steel discs of 1 cm diameter and 1 mm thick (designed to fit in the pilot plant), of the same grade and finish and this was used to prepare surfaces for pilot plant trials.

Biofilm generation

For the first phase of work, i.e. screening of a variety of cleaning regimes, a laboratory coupon reactor was used in the same setup as described previously (Flint *et al.* 2001). Cleaned and sterile stainless steel coupons, inserted into clean silicone tubing, were the substrates for biofilm formation. The entire system was cleaned and autoclaved before use. The planktonic cultures used to inoculate biofilm reactors were obtained from a 6-h culture of the cells in TSB grown, with agitation, at 55°C. To inoculate the substrates in the biofilm reactors, a 6-h culture, containing *ca* 8 $\log_{10}$ cells ml^{-1} was centrifuged at 3000 *g* for 10 min, and the cell pellet was washed and resuspended in sterile distilled water. These cells were used to initiate biofilm formation in the reactors by incubating them at ambient temperature with sterile stainless steel coupons for 30 min followed by five rinses with sterile distilled water.

There were two milk flows in the reactors. Pasteurized skim milk at 55°C was recirculated past the inoculated coupons in the reactor at 140 ml min^{-1} for 18 h to generate biofilms. Fresh milk was continuously added to the system (working volume 225 ml) at 4·7 ml min^{-1} and the surplus overflowed to drain. This dilution rate exceeded the growth rate of the bacteria, and avoided an increase in numbers of planktonic cells. The coupons with biofilms attached to them were aseptically cut from the tubing and rinsed five times with sterile distilled water prior to examination for biofilms.

For the second phase of work, an annular rotating disc reactor was used. In the annular reactor, eight stainless steel discs were fitted into a Teflon holder containing a bar magnet in the base. This assembly was placed in a 400 ml beaker on a magnetic stirrer. The assembly was fitted with a rubber bung that could take in two silicone tubes. One tube allowed the milk to flow in and the other tube, which had a baffle dipping into the milk to cause turbulence, passed the milk to the waste sink from the reactor (Fig. 1). The setup was autoclaved and the stainless steel discs were inoculated with washed *B. flavothermus* (B12-C^{m}) cells as already described. The milk flow rate was 4·7 ± 1 ml min^{-1}, determined from the doubling rate of the bacteria to avoid a net increase in the numbers of bacteria in the planktonic culture.

Cleaning regimes – laboratory trials

Once the 18-h mature biofilms were generated on the stainless steel coupons in the biofilm reactor, sterile distilled water was passed through the reactor tubing to rinse off residual milk or loosely attached cells. The coupons were aseptically removed by cutting the silicone tubing and were transferred to sterile 15-ml tubes. The coupons were subjected to cleaning by adding 2 ml of the different cleaning agents under specified conditions (Table 1) and agitated on a vortex mixer for 5 s and then

Table 1 Cleaning procedures used

1. 2% NaOH at 75°C, 30 min followed by 15 min distilled water rinse at ambient temperature and 1·8% HNO_3 at 75°C, 30 min
2. Eliminator (sodium hydroxide + sodium metasilicate) 2% at 75°C, 20 min
3. Paradigm (0·08% P2010 and 0·09% P2030) at 60°C, 30 min
4. Purafect®, 0·12 subtilisin U ml^{-1} at 37°C for 30 min
5. Purastar^{TM} α-amylase, 0·05 amylase U kg^{-1} at 37°C for 30 min
6. Cellulase^{L}, 1·5 cellulase U ml^{-1} at 37°C for 30 min
7. Mutanolysin, 1 U ml^{-1} at 37°C for 1 h
8. Sodium hypochlorite 500 ppm 5 min, ambient temperature
9. Halamid (chloramine) 0·3% 5 min, ambient temperature
10. Oxine® (chlorine dioxide) 500 ppm 5 min, ambient temperature
11. Perform® (Peracetic acid/hydrogen peroxide) 0·2%, 5 min, ambient temperature
12. Quarternary ammonium chloride (Bactosolve®) 25 ppm, 30 min, ambient temperature
13. Tween-80, 6% w/v followed by wash with lecithin–Tween-80 (3%) (Eginton *et al.* 1998)
14. Dobanic acid HFP (Hydrogen Fluoride Process) 0·1%, 5 min, ambient temperature

The cleaning regime for these cleaners was based on the literature/manual from the respective companies, or chosen by us. The control coupons were rinsed five times with sterile distilled water at 22°C and used for total count or viable count.

rinsed five times with sterile distilled water at 22°C. In the control treatment, coupons were rinsed five times with sterile distilled water at 22°C and used for total count or viable count. The cleaning efficiency was assessed in terms of numbers of viable cells remaining by impedance measurement using the Bactrac 4100™ (Sy–Lab Geräte GmbH, Purkersdorf, Austria) micro-organism growth analyser (Flint and Brooks 2001) and in terms of the total cell count by using epifluorescence microscopy (Parkar *et al.* 2001). The presence of polysaccharide matrix was noted microscopically as fluorescent material of the acridine-orange stained coupons. It was expressed as percentage of total field by arbitrarily dividing the field into a grid format, and counting the squares with debris vs total number of squares.

After the initial stage, when a number of cleaners were tested individually as mentioned in Table 2, different combinations of agents, temperatures and cleaning treatments were tried as given in Table 3. Again, the cleaning efficiency was estimated in terms of viability and the total number of cells left behind.

Control preparations

Biofilms on stainless steel surfaces all reached levels of 6–7 $\log_{10}$ cells cm^{-2} and were totally covered with biofilm.

Cleaning regimes – pilot scale trials

The chemical cleaning combinations that gave the most promising results in the laboratory experiments were run on a pilot plant. Duplicate discs containing biofilm were used for each cleaning treatment viz. full strength caustic and acid cleaning at 75°C (see details later), Paradigm treatment and Perform® with 0·1% dobanic acid at 60°C. The remaining two discs served as controls. For the pilot scale trials, the biofilms were grown in the annular reactor and then transferred to a modified Robbins device (MRD). This MRD, containing eight sample ports, was designed at the Fonterra Research Centre and built at a local engineering company from grade 316 stainless steel. Biofilms were not grown in the MRD in the pilot scale cleaning rig as a large quantity of milk would have been required to grow the

Table 2 Effect of different cleaning agents on the viability of biofilms of the thermophilic bacillus *Bacillus flavothermus* (B12-C^m) (control coupon $7{\cdot}0 \times 10^7$ cells cm^{-2}) in the laboratory scale reactor

Treatment*	Total cell reduction ($\log_{10}$ reduction)†	Viable cell kill ($\log_{10}$ reduction)‡	Residue§ remaining after cleaning (0 = 0%; 1 = 1–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%
1. Alkali/acid 75°C, 30 min	7	7	0
2. Alkali/acid 60°C, 30 min	5	7	0
3. Alkali/acid 50°C, 30 min	5	7	0
4. Half strength alkali/acid 60°C, 30 min	5	7	0
5. Half strength alkali/acid 50°C, 30 min	5	5·5	0
6. Eliminator, 75°C, 20 min	2	6	1
7. Paradigm, 2010 + 2030, 60°C, 30 min	7	7	0
8. Paradigm, 2030 then 2010, 60°C, 30 min each	7	7	0
9. Paradigm, 2010 then 2030, 60°C, 30 min each	7	7	1
10. Purafect®, 0·5%, 37°C, 30 min	5	3·6	1
11. Purastar™ 0·5%, 37°C, 30 min	5	6·6	1
12. Cellulase^L, 0·3%, 37°C, 30 min	5	5·8	1
13. Mutanolysin,1 U ml^{-1}, 37°C, 1 h	4	4·3	1
14. Sodium hypochlorite, 22°C, 5 min	1	5·5	2
15. Halamid, 22°C, 5 min	2	6·8	1
16. Oxine® 22°C, 5 min	1	7	2
17. Perform® 22°C, 5 min	7	7	0
18. Bactosolve®, 22°C, 30 min	2	7	2
19. Tween-80, 6% w/v, 22°C, 5 min	0	1	4
20. Dobanic acid, 22°C, 5 min	1	4	2

*Details in materials and methods and Table 1.
†Based on epifluorescence microscopy.
‡Based on impedance microbiology.
§Polysaccharide material observed using epifluorescence microscopy.
Results based on duplicate test; mean ± SD (SD about 0·2 $\log_{10}$ cells cm^{-2}).

Table 3 The effect of alternative cleaning conditions on the inactivation of biofilms of the thermophilic bacillus *Bacillus flavothermus* (B12-C^m) in the laboratory scale reactor, based on viable cell count, using Bactrac analyser

Treatment	Time (min)	Temperature (°C)	Viable cell kill ($\log_{10}$ reduction)
1. 100 ppm Oxine®	15	22	6
2. 100 ppm Oxine®	60	22	7
3. 250 ppm Oxine®	15	60	7·8
4. 250 ppm Oxine® + 1% Dobanic acid	15	22	6·8
5. 100 ppm Oxine® + 1% Dobanic acid	15	22	6·7
6. 100 ppm Oxine® + 1% Dobanic acid	15	60	7·5
7. 0·1% Dobanic acid/100 ppm Oxine®	15	22	7
8. 100 ppm Oxine® + 0·1% Dobanic acid	15	37	8
9. 100 ppm Oxine® + 0·1% Dobanic acid	15	45	8
10. 0·2% Perform®	15	22	8
11. 0·4% Perform®	15	22	8
12. 0·1% Dobanic acid/0·2% Perform®	15	22	8
13. 0·2% Perform® + 0·1% Dobanic acid	15	22	8
14. 0·4% Perform® + 0·1% Dobanic acid	15	37	8
15. 0·2% Perform® + 0·1% Dobanic acid	15	45	8
16. 0·2% Perform® + 0·1% Dobanic acid	15	60	8

biofilms. The pilot-scale cleaning rig allowed the effect of the cleaning chemicals to be determined under turbulent flow conditions (maximum pump rate of 200 kg h^{-1}) (*Re* > 2000), similar to those in a dairy manufacturing plant (Romney 1990).

Statistical analysis

All the studies were performed at least in duplicates. Viable counts obtained from the Bactrac 4100™ cell analyser were averages of duplicate counts; and total counts obtained by cell epiflurescent microscopy were averages of five counts. Results have been expressed as mean values, the standard deviation given as required.

RESULTS

Caustic and acid cleaning involved treating the biofilm coupons with 2% NaOH at 75°C for 30 min, followed by a sterile distilled water rinse and then treating with 1·8% HNO_3 at 75°C for 30 min followed by another rinse with distilled water. This was the most effective of all the caustic and acid treatments used to kill and remove biofilms. This is referred to as full strength caustic acid at 75°C (fsca 75) in our further studies. This killed all the cells in the biofilm (Table 2) and removed practically all the cells and polysaccharide matrix from the stainless steel. Using the same concentrations, or half strengths, of alkali and acid for the same time but at lower temperatures of 60 and 50°C, the cleaning efficacy was reduced. For full strength caustic acid at both 50 and 60°C (fsca 50, fsca 60) and half strength caustic acid treatment at 60°C (hsca 60) there was a 6–7 $\log_{10}$ cells cm^{-2} reduction (100% loss) in viability but only a 2 $\log_{10}$ cells cm^{-2} reduction in total cells detected by epifluorescence microscopy. The half strength caustic acid treatment at 50°C (hsca 50) resulted in only a 5·5 $\log_{10}$ cells cm^{-2} decrease in viable cells with a total cell reduction of 2 $\log_{10}$ cells cm^{-2}. Polysaccharide matrix was not detected when the cleaning cycles were carried out at 75 and 60°C, but small amounts (about 1·5%) were detected with fsca 50, and larger amounts (about 8%) were detected with hsca 50. The commercial caustic cleaner Eliminator (2%, 75°C, 20 min) reduced cell viability in the B12-C^m biofilm by 6 $\log_{10}$ cells cm^{-2} and the total cell count by *ca* 2 $\log_{10}$ cells cm^{-2} (Table 1). Fluorescent polysaccharide remnants were also observed on the coupons.

Paradigm, a commercial proteolytic enzyme cleaner consisting of two components, was used at 0·08% P2010 (enzyme/surfactant) combined with 0·09% P2030 (alkali/chelants) at 60°C for 30 min and was successful in cleaning biofilm from the test coupons in the laboratory trials. Paradigm was also used in the following combinations (0·08% P2010 followed by 0·09% P2030, and 0·09% P2030 followed by 0·08% P2010) at the same temperature with 30 min treatments for each component, resulting in a total 1 h cleaning time. When P2030 and P2010 were used simultaneously, there was a 7 $\log_{10}$ cells cm^{-2} reduction in total and viable cells and no visible polysaccharide. When P2030 was followed by P2010, there was again a 7 $\log_{10}$ cells cm^{-2} reduction in viable biofilm cells and no visible cells or polysaccharide. However, when P2010 was followed by P2030, there was a 7 $\log_{10}$ cells cm^{-2} reduction in viable biofilm cells, but residual cell material (about 11%) was seen under epifluorescence microscopy.

Under the given conditions, Purafect®, Purastar™, Cellulase^L and mutanolysin did not achieve 100% killing

of the biofilms (Table 1). The viability was decreased by 3·6, 6·6, 5·8 and 4·3 $\log_{10}$ cells cm^{-2}, respectively compared with the original biofilm. The total cell count was decreased by 5, 5, 5, and 4 $\log_{10}$ cells cm^{-2}, respectively, and residual polysaccharide was seen as well.

Of the oxygen based agents, Oxine® and Perform® caused a 7 $\log_{10}$ cells cm^{-2} loss of viability of the biofilms, followed by Halamid (6·8 $\log_{10}$ cells cm^{-2} reduction) and sodium hypochlorite (5·5 $\log_{10}$ cells cm^{-2} reduction) (Table 2). However, in terms of removing cells from the surface, Perform® was the most effective causing 100% removal of cells and attached polysaccharides. Halamid caused a 2 $\log_{10}$ cells cm^{-2} total cell reduction followed by Oxine® and sodium hypochlorite, each causing a 1 $\log_{10}$ cells cm^{-2} reduction of total cells. Halamid also removed more of the polysaccharide (0·5% polysaccharide seen) than did Oxine® and sodium hypochlorite. As the latter two had a high number of cells still attached to surface, it was difficult to estimate the polysaccharide content but one can state that the cells were obviously still attached by the polysaccharide glue.

Bactosolve® produced a 7 $\log_{10}$ cells cm^{-2} reduction in viability although only a 2 $\log_{10}$ cells cm^{-2} reduction in total cells on the stainless steel. Tween-80 was least effective of all treatments tried resulting in only a 1 $\log_{10}$ cells cm^{-2} reduction in viable cells and negligible detachment of total cells, while dobanic acid caused more cell kill (4 $\log_{10}$ cells cm^{-2} reduction in viability) and cell removal (1 $\log_{10}$ cells cm^{-2} reduction) and at least 25% of the polysaccharides were removed.

All the Oxine® combinations caused a >6 $\log_{10}$ cells cm^{-2} decrease in viability (Table 3). However, microscopic examination of the coupons showed no significant removal of the cells or breakdown of the polysaccharide matrix. The Perform® combinations were 100% successful in killing the biofilms (Table 2), although microscopic observations showed that the best clean was obtained with a combination of 0·2% Perform® with 0·1% dobanic acid at 60°C.

Three different treatments (fsca 75, Paradigm® and 0·2% Perform® with 0·1% dobanic acid) selected from successful laboratory trials were tested in the pilot plant cleaning rig. The treatments resulted in no viable cells remaining on the substrate and the complete removal of the biofilm. However, fluorescent material (about 20%) was seen along the striations (part of the surface topography) of the stainless steel disc in all three trials.

DISCUSSION

Cleaning with caustic (2% NaOH) and acid (1·8% HNO_3) at 75°C for 30 min was confirmed as a reliable cleaning protocol for stainless steel coupons with confluent 18-h biofilms of *B. flavothermus* (B12-C^{m}). Caustic and acid cleaning has been the standard method used in many food processing industries (Chisti 1999). However, this is the first report detailing the effect of a standard cleaning system on sporeforming thermophilic bacteria, and the effect of changes in concentrations or temperature on biofilm viability, detachment of cells and polysaccharide removal.

Our cleaning trials prove that it is very important to use the right concentrations of agents and the recommended temperatures. A decrease in the strengths of the agents killed the cells but failed to remove all the cells from the surface. Marshall (1994) suggests that this residue may serve as a locus for attachment of more organic remnants or bacteria, resulting in faster biofilm formation and product spoilage in subsequent runs. Polysaccharide dissolution and removal using alkali in our trials needed temperatures higher than 60°C, as some exopolysaccharide was observed after cleaning with full strengths of alkali and acid at 50°C. Temperature is a critical factor in cleaning, with many effects increasing linearly with temperature (Gibson *et al.* 1999). Eliminator, even at 75°C, was not successful in completely removing cell matter. A longer cleaning or a higher concentration of the agent may be required.

When Paradigm was used according to the manufacturers' instructions at 60°C, no viable cells or cell debris were left behind on the stainless steel. Using the alkali component to dissolve proteins and polysaccharides before proteinase action was more effective than using the proteinase before the alkali. The enzyme preparations, Purafect®, Purastar™ and Cellulase^{L} and mutanolysin (a polysaccharidase that breaks the glycosidic bonds in the bacterial peptidoglycan network) were not completely effective. Possible explanations may be low wetability, and the fact that these enzyme cleaners only target one part of the biofilm. The addition of surfactants or a combination of these agents may result in better cleaning.

Although kinetic energy (flow) was not specifically examined as a factor in cleaning for these trials the pilot plant cleaning rig, that operates under turbulent flow, ensured that the most promising treatments were tested under conditions of flow reflecting that seen in a manufacturing plant.

In cleaning industrial plants, even a large decrease in the percentage of cells killed and removed may be insufficient for optimum running of the manufacturing plant, as the remaining cell debris may act as an organic 'conditioning film' aiding the attachment of micro-organisms in subsequent runs (Marshall 1994).

Many factors need to be considered when judging the cleaning efficacy of various agents. Irreversible attachment of cells to a cleaned surface occurs within 30 min after exposure to a fresh suspension of cells of *B. flavothermus* (B12-C^{m}). Hence, the aim of the CIP procedure should be to achieve total cleaning in the shortest possible time, to

maximize the availability of the manufacturing plant for production. The final outcome of a cleaning procedure should be judged both by the number of cells remaining viable after a treatment and by checking for the presence of any cell residue on the cleaned surface.

The work in this trial examined the effect of different cleaners on the vegetative form of *B. flavothermus*. Although spores of this organism are a concern, the vegetative forms predominate in the manufacturing plant and are the source of spores. The origin of spores in the final product is under study.

Several procedures, including caustic/acid and enzyme based cleaners, will be satisfactory, provided that the correct process parameters are observed i.e. concentration, time, temperature and kinetic energy. Confirmation of these results should be carried out in a pilot plant through several use/clean cycles.

ACKNOWLEDGEMENTS

Tuan Truong, Fonterra Research Centre, for help with the pilot scale rig. Michael Dunne for the generous gifts of Purastar^{TM}, Purafect$^{®}$ and Cellulase^{L}. Graeme Ogg, from Biocide International, for the generous gifts of Oxine and dobanic acid. Dave Lowry, from Ecolab, for the supply of Paradigm. Jeff Old, from Orica Chemnet, for the supply of the other cleaning chemicals. Tim Coolbear and Kylie Walker, from the Fonterra Research Centre for proof reading this script.

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