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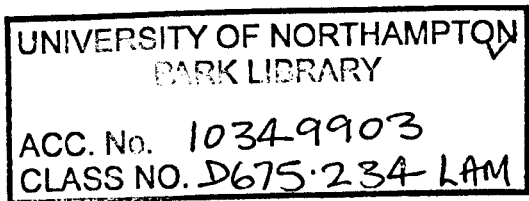
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The Impact of the Leather Manufacturing Process on Bacterial Growth

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Acknowledgements

Abstract

Hides and skins used as a raw material for leather manufacture may be contaminated with various microbial species including potential pathogens. Many bacterial species such as *Bacillus*, *Staphylococcus*, *Micrococcus* and *Pseudomonas* were isolated from raw hides/skins, and hides/skins at different stages of leather making process. The extreme environmental conditions present during a conventional tanning process due to hazardous chemicals may prevent the growth of bacteria present on hides/skins. On the other hand, partial or total replacement of the hazardous chemicals with non-hazardous chemicals, during a best available technologies (BAT) process, may provide suitable conditions for microbial growth in tannery effluent and hides/skins. Therefore, the aim of the project was to determine the survival and growth of the various bacterial species during the conventional and BAT leather-making processes. The beamhouse and tanning stages were studied, as the majority of the environmental pollution occurs during the early stages of the leather making process.

Both the pre-soaking and soaking stages during the conventional and BAT-leather making processes provided suitable conditions for bacterial (*Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus* spp.) growth and proliferation. The results showed a significant reduction in the number of *B. cereus* found during the conventional and BAT unhairing processes. Limited *B. cereus* growth was observed during the subsequent reliming process. *Bacillus cereus* growth was also occurred during the deliming and bating processes (conventional and BAT), followed by a decrease during the conventional and BAT pickling processes. No *B. cereus* colonies were isolated during the chrome tanning process.

Growth of *P. aeruginosa* was inhibited during both the unhairing and reliming stages of the conventional and BAT leather making processes. A reappearance and recovery of *P. aeruginosa* in the subsequent deliming and bating (conventional and BAT) processes, indicated that deliming and bating processes may provide suitable growth conditions for *P. aeruginosa*. On the other hand, both the conventional and BAT pickling processes, and the chrome tanning processes hindered *P. aeruginosa* growth.

Staphylococcus spp. were present throughout the conventional and BAT leather-making processes. A large reduction in the number of *Staphylococcus* spp. was observed during the unhairing and reliming processes (conventional and BAT). Growth of *Staphylococcus* spp. occurred during the subsequent deliming, bating, pickling and chrome tanning stages for both the conventional and BAT leather-making processes.

The biochemical assays for bacterial identification confirmed the presence of *B. cereus*, *P. aeruginosa* and *Staphylococcus* spp. during the leather processing. The pulsed-field gel electrophoresis (PFGE) method of DNA fingerprinting confirmed that the bacterial species isolated during the leather

manufacturing processes were the inoculated *B. cereus* and *P. aeruginosa*, and no alteration of the DNA of above-mentioned bacteria occurred during the processing.

Overall, the research showed that bacterial species are capable of surviving during both the conventional and BAT leather-manufacturing processes. The bacterial species prefer the environmental conditions during the pre-soaking and soaking processes, while the unhairing and reliming processes did not favour the growth of bacterial species. Bacterial colonies were enumerated during the deliming and bating processes indicating that the unhairing and reliming processes did not cause total destruction of the bacterial cells. Alternatively pickling and chrome tanning processes were found to have suppressed the growth of bacterial colonies.

List of Abbreviations

BAT: Best available technology

BBCA: Brilliance *Bacillus cereus* agar

BOD: Biological Oxygen Demand

Cfu: Colony forming units

COD: Chemical Oxygen Demand

Conv: Conventional

cm: Centimetre

g: Gram

Hrs: Hours

lb: pound

IPPC: Integrated Pollution Prevention and Control

in: Inches

kb: Kilobase

ml: Millilitre

μ: Micro

Mins: Minutes

MP: Megapixel

n: Number of replicates

NA: Nutrient agar

nm: Nanometre

PBS: Phosphate buffered saline

PCA: Plate count agar

PEMBA: Mannitol phenol egg yolk polymixin agar

PFGE: Pulsed-field gel electrophoresis

RPM: Revolutions per minute

Spp.: Species

TSA: Tryptone soya agar

TSB: Tryptone soya broth

V: Volt

v: Volume

W: Weight

WPCA: Water plate count agar

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Chapter 1: Literature Review

1.1. Microorganisms

Microorganisms are microscopic organisms, generally single-celled, but may also be multi-cellular (Madigan *et al.*, 1997; Pelczar *et al.*, 1993; Wilson, 2005; Wilson *et al.*, 2002). Primarily microorganisms are classified into two groups: eukaryotic and prokaryotic. The major difference between a eukaryotic and prokaryotic cell is; a eukaryotic cell consists of a nucleus, which is surrounded by a nuclear membrane and contains several DNA molecules, whereas a prokaryotic cell contains a nuclear region (known as nucleoid), which is not surrounded by a nuclear membrane and contains a single DNA molecule. Eukaryotic cells include fungi, algae and protozoa, and prokaryotic cells include bacteria and archaea (Madigan *et al.*, 1997; Maier *et al.*, 2000; Todar, 2008; Wilson *et al.*, 2002).

1.1.1. Bacteria

Bacteria (singular: bacterium) are single-celled organisms and have the simplest cell structure. A typical bacterial cell consists of a cytoplasm (protoplasm) surrounding by a cell envelope. The cytoplasm of a bacterial cell contains DNA chromosomes, mRNA, ribosomes, proteins and metabolites. The cell envelope is composed of several layers, such as a capsule, cell wall and cytoplasmic membrane. Bacteria may also have other organelles such as flagella (singular: flagellum) and pili (singular: pilus) (Murray *et al.*, 2005; Todar, 2008; Wilson, 2005; Wilson *et al.*, 2002).

Based on the Gram-reaction, bacteria are classified into two groups: Gram-positive and Gram-negative (Barrow and Feltham, 2003; Murray *et al.*, 2005; Wilson, 2005; Wilson *et al.*, 2002). The Gram-staining technique generally involves application of crystal violet to a bacterial smear, subsequent addition of a mordent, iodine, followed by a wash with alcohol/acetone and application of a red dye safranin. The cell wall of Gram-positive bacteria is largely (as much as 90%) consists of a component known as peptidoglycan. The thick cross-linked, mesh-like structures of peptidoglycan retain the violet-iodine complex, which is not removed by the alcohol or acetone wash, and appears as purple when observed under a light microscope. On the other hand, the cells wall of Gram-negative bacteria consists of a comparatively thinner pepdidoglycan layer (10%) than Gram-positive bacteria and therefore does not retain crystal-violet-iodine complex. In addition, the outer layer of Gram-negative bacteria consists of lipid, which is generally removed during the alcohol/acetone wash along

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with the violet-iodine complex. Gram-negative bacteria therefore stains red due to the addition of safranin (Madigan *et al.*, 1997; Murray *et al.*, 2005; Pelczar *et al.*, 1993; Wilson, 2005). The schematic structure of a typical bacterial cell (Gram-positive and Gram-negative) is shown in Figure 1.1.
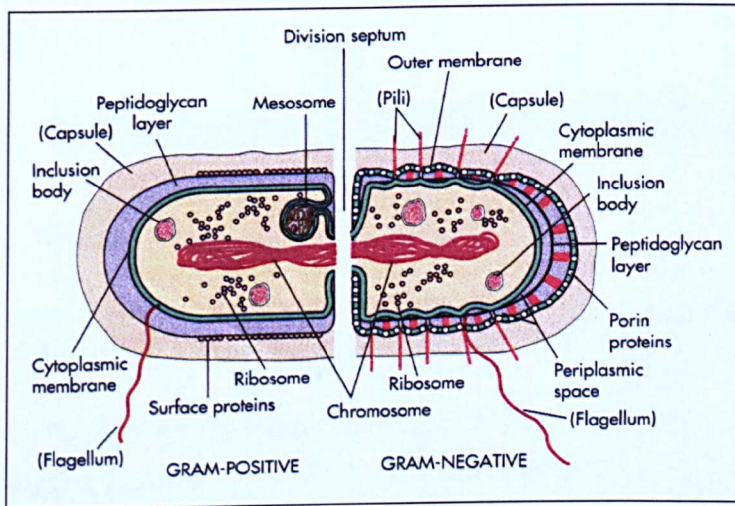
Figure 1.1: Schematic diagram of a typical bacterial cell, showing various major features such as a cell envelop, cytoplasm and other important organelles (Murray *et al.*, 2005).

Bacteria cells are generally 0.5-1 µm in diameter (Maier *et al.*, 2000; Todar, 2006) and are the most abundantly found organisms on earth, due to their ability to survive almost every environmental condition and ability to use any substrate as a nutritional source (Wilson, 2005). Most of the bacterial population are harmless; however, some of those may cause disease in humans and are known as pathogens (Madigan *et. al.*, 1997; Wilson, 2005).

1.1.2. Fungi

Fungi (singular: fungus) are unicellular or multicellular. Fungi may exhibit two morphological structures: yeast and moulds (mycelia fungi). Yeasts are generally unicellular and reproduce by budding or by fission. Moulds are multicelluar, which consist of a threadlike structure called hyphae (singular: hypha). Hyphae are generally two types: coenocytic and septate. Hollow and multinucleate hyphae are known as coenocytic, whilst, hyphae, which are divided by partitions, are known as septate (Figure 1.2). Hyphae intertwined together to form a mycelium (plural: mycelia) (Murray *et al.*, 2005; Wilson, 2005). Moulds reproduce by forming spores, either sexual or asexual. The schematic structure of a mycelial fungus (*Aspergillus* spp.) is shown in Figure 1.2.
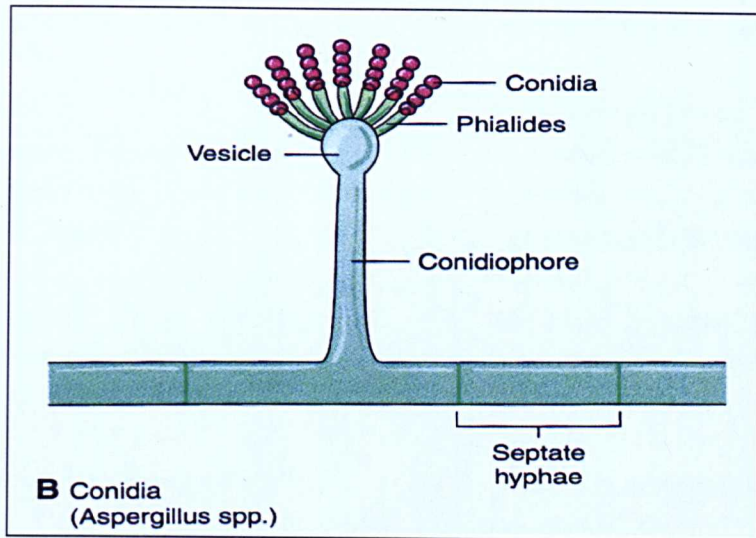
Figure 1.2: Schematic diagram of a mycelial fungus (*Aspergillus* spp.) showing various major features (Murray *et al.*, 2005).

Fungi are also found in abundance in soils although these are less numerous than bacteria. Fungi are heterotrophic organisms; most of them are aerobic or micro-aerophilic in nature (Maier *et al.*, 2000). Fungi lack chlorophyll and absorb nutrients by degrading complex organic materials into simple organic and inorganic compounds, such as carbon, nitrogen and phosphorous (Prescott *et al.*, 1993).

1.1.3.   Influencing Factors for Microbial Growth

The following factors have been found to influence the growth of microorganisms

- **Nutrients**: Microbes require nutrients for their survival and growth. The sources and functions of various major essential elements for microbial metabolisms are given in Table 1.1. Microorganisms may require other elements in trace such as zinc, copper, manganese, molybdenum and cobalt (Maier *et al*, 2000; Murray *et al.*, 2005; Wilson, 2005). Although it has not been proved as yet, chromium (Cr) may be an essential element for growth of some microorganisms (Maier *et al.*, 2000).

Table 1.1: Sources and functions of the major essential elements for microbial metabolisms (Murray *et al.*, 2005; Todar, 2008).

Major Element	Source	Function in Metabolism
Carbon	Organic compounds, carbon dioxide (CO_2)	Major components of cellular materials
Oxygen	Oxygen (O_2), water (H_2O) and organic compounds	Constituent of cell material and cell water, oxygen act as an electron acceptor in aerobic respiration
Nitrogen	Ammonia (NH_3), trinitrate (NO_3^-) and organic compounds, nitrogen (N_2)	Constituent of amino acids, nucleic acids, nucleotides and coenzymes
Hydrogen	H_2O, organic compounds, hydrogen (H_2),	Major components of organic compounds and cell water
Phosphorus	Inorganic phosphates (PO_4^{-3})	Constituents of nucleic acids, nucleotides and phospholipids
Sulfur	Sulfate (SO_4^{-2}), hydrogen sulfide (H_2S), sulfur, organic sulfur compounds	Constituent of sulfur -containing amino acids, cystine, methionine, glutathione, thiamine pyrophosphate and several coenzymes
Potassium	Potassium salt	Inorganic cellular cation, cofactor for certain enzymatic reactions
Calcium	Calcium salts	Inorganic cellular cation, cofactor for certain enzymes, component of cell wall and endospores
Iron	Iron salts	Component of cytochromes, ferredoxins and other iron-sulfur proteins, cofactor of some enzymatic reactions
Sodium	Sodium salts	Transport
Chlorine	Chloride	Inorganic anion

- **Oxygen**: Air contains 21% oxygen. Microorganisms those are able to tolerate free oxygen in air or higher are known as aerobic organisms. Those which can only grow in the absence of free oxygen are classified as anaerobic micro-organisms, whereas facultative microorganisms are able to grow with or without oxygen. Micro-aerophilic organisms require a small amount of free oxygen for their growth (Clarke, 2004; Madigan *et al.*, 1997; Rushing *et al.*, 2006).

- **Water**: Though water is an important factor for microbial growth, pure water does not provide suitable growth conditions. The availability of water for microbes is measured as water activity or A_w. The A_w of pure water is 1 (100%) (Rushing *et al.*, 2006, Todar, 2008). Most of the bacteria related to food spoilage require a minimum of 0.90 A_w. Yeasts and moulds require an A_w of 0.75 (Rushing *et al.*, 2006).

- **Temperature**: The temperature at which a particular organism grows best is known as its optimum temperature. Every microorganism has a minimum and maximum temperature, beyond which the organism is incapable of growing. Microorganisms require an optimum temperature below 20°C for growth are known as psychrophiles, microbes which grow within the temperature range of 20-40°C are termed as mesophiles, thermophiles can grow at 40-80°C, while extreme thermophiles have the ability to grow at a temperature above 80°C (Figure 1.3) (Madigan *et al.*, 1997; Rushing *et al.*, 2006). The optimum temperature of the normal flora (indigenous microbial population in a specific environment) and pathogens in humans is 37°C (Wilson, 2005).
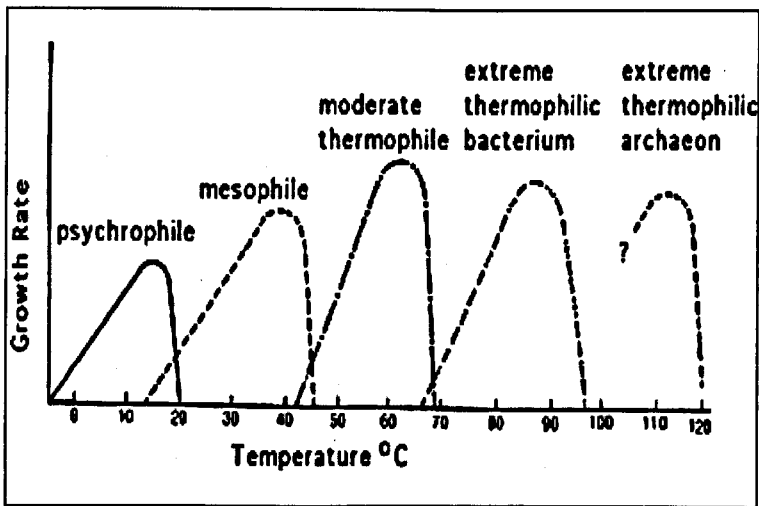
Figure 1.3: Bacterial classification based on temperature (Todar, 2008).

- **Acidity and pH**: Microorganisms also require an optimum pH for their growth. Neutrophiles describe the microorganisms which grow best at neutral pH range, whereas acidophiles grow best at a pH range below the neutral pH. Generally, fungi are acidophiles, therefore able to grow at a pH 5 or below. Microorganisms those grow above the neutral pH range are known as alkaliphiles (Pelczar *et al.*, 1993; Madigan *et al.*, 1997; Todar, 2008). Most of the bacteria prefer a neutral or slightly alkaline pH (6-8). The optimum pH for most of fungi is 5-6 (Madigan *et al.*, 1997; Wilson, 2005). Figure 1.4 shows microbial classification based on the optimum pH range.
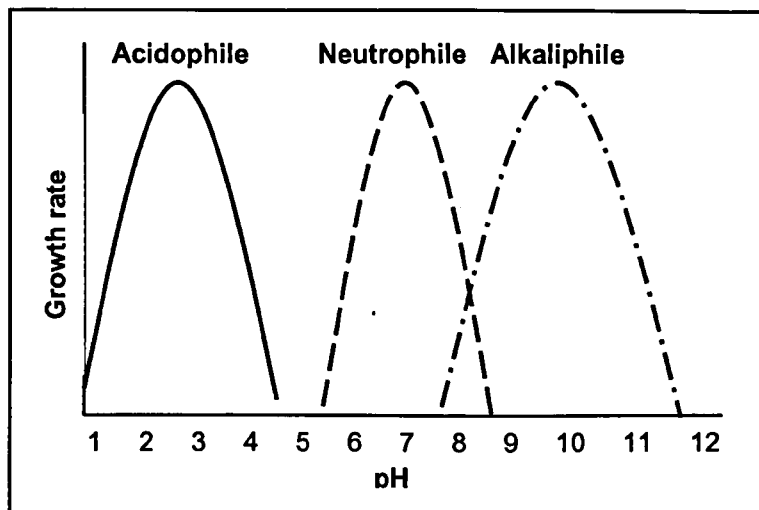
Figure 1.4: Optimum pH range for the different types of bacteria (Todar, 2008).

- **Light:** Microorganisms are sensitive towards high-energy radiation such as x-rays, gamma rays and ultraviolet light. Gamma rays and x-rays have the ability to ionise molecules, disrupting the cells through the penetration into the microorganisms (Pelczar *et al.*, 1993; Wilson, 2005). Ultraviolet light can cause alteration to, or inhibition of DNA replication, which can eventually cause cell death. Unlike gamma rays and x-rays, ultraviolet light does not have the ability to penetrate matter, therefore, is only effective for surface decontamination (Madigan *et al.*, 1997; Pelczar *et al.*, 1993).

- **Chemicals:** The presence of certain chemicals, as for example formaldehyde, glutaraldehyde, chlorine, iodine, peroxygen compounds, ethylene oxide gas and phenol can reduce or prevent microbial growth. Therefore, these chemicals are often used as disinfectants. Hypochlorites and other chlorine based compounds are active against a wide range of microorganisms including fungi, viruses, bacteria and bacterial spores (Madigan *et al.*, 1997; Murray *et al.*, 2005; Wilson, 2005). Although 70% (w/v) alcohol is also active against a wide range of bacteria and fungi, but it is inactive against bacterial spores (Wilson, 2005).

1.1.4. Bacterial Growth Patterns

Bacteria undergo growth phases when cultured in a closed media containing a liquid nutrient media. A typical bacterial growth pattern shows four different phases (Figure 1.5), which are, as follows (Prescott *et al.*, 2000; Todar, 2008):

- **Lag phase:** When bacterial colonies are first introduced to a fresh media, cell division may not occur. Bacterial cells in the lag phase may be adapting to the new environment

or repairing any damages through synthesising enzymes. During the period, bacterial cells may increase in mass and volume (Pelczar *et al.*, 1993; Prescott *et al.*, 2000; Todar, 2008).
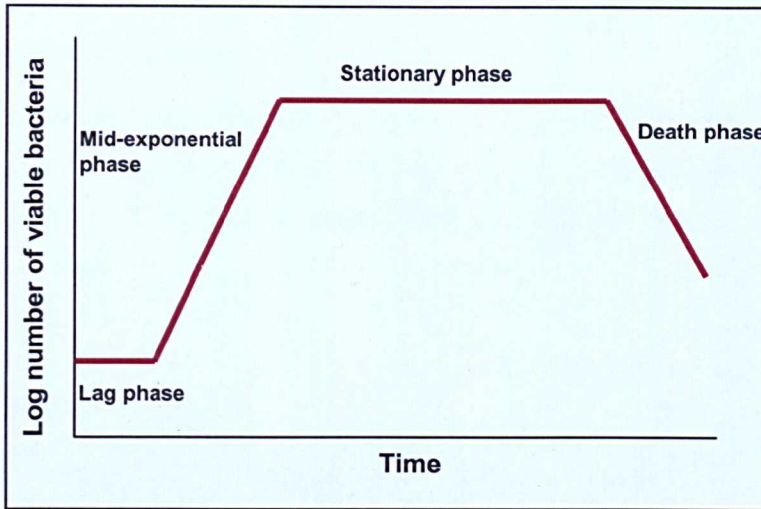
Figure 1.5: Bacterial growth curve (Pelczar *et al.*, 1993).

- **Exponential phase**: Bacterial cells are most active in the exponential phase. During the exponential phase, bacterial cells divide in binary fission at a constant rate. The growth rate may vary based on the growth media and incubation conditions (Prescott *et al.*, 2000; Todar, 2008). The physical and chemical properties of the bacterial cells are most consistent in the exponential phase and therefore are generally used in biomedical and physiological studies (Pelczar *et al.*, 1993; Prescott *et al.*, 2000).

- **Stationary phase**: In the stationary phase, the rate of bacterial growth is reduced; however, the cell numbers remain constant. The reduction of growth rate occurs due to a variety of reasons such as nutrient depletion or production of toxic waste as a result of metabolic activity (Pelczar *et al.*, 1993; Prescott *et al.*, 2000). Oxygen depreciation also effects the growth of aerobic micro-organisms (Prescott *et al.*, 2000).

- **Death phase**: The death phase follows the stationary phase if further incubation is carried out, without the addition of fresh media. In the death phase, the bacterial population is reduced due to the accumulation of toxic waste or further exhaustion of nutrients (Pelczar *et al.*, 1993; Prescott *et al.*, 2000; Todar, 2008).

Bacteria have been found on hides/skins and to understand the survival and growth of bacteria within tannery effluents or hides/skin during the processing, understanding of the leather manufacturing processes is essential.

1.2. Leather Manufacture

Hides and skins of vertebrates, particularly cattle, sheep, pig, buffalo and goat, are generally used to manufacture leather. According to the Food and Agriculture Organisation (2001), the total global production of leather is approximately 1.67×10^9 square metres (cited in Thanikaivelan et al., 2005).

1.2.1. Structure of Skins
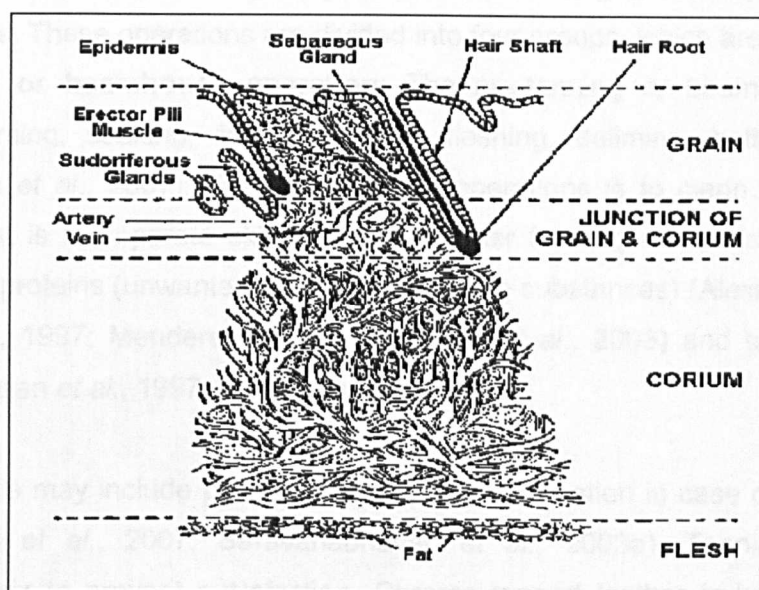
Figure 1.6: Schematic diagram of the cross section of hides (Sharphouse, 1971).

The structure of skin is divided into three distinct layers (Figure 1.6), the grain (epidermis) layer, the corium or dermis and the flesh (hypodermis) layers (Daniels, 2003). The grain layer of the skin gives the aesthetic value (Leafe, 1999) and determines the final appearance of the leather (Bailey and Paul, 1998). The outermost layer of the grain layer is epidermis, which is removed during the pre-tanning process (Daniels, 2003).

The corium is relatively thick (Daniels, 2003) and composed of white interlacing collagen fibres cemented together by a substance called dermatan sulfate, in a net-like structure (Bailey, 1992; Reich, 1999). The corium is responsible for the mechanical strength (Bailey, 1992; Leafe, 1999; Reich, 1999) and resiliency (Leafe, 1999). Collagen type I is one of the

major collagen types in animal bodies (Heidemann, 1993). Due to the formation of internal cross-links within the fine fibrils, the collagen is highly stable (Daniels, 2003).

The flesh layer composed of subcutaneous tissue mainly consists of adipose tissues (Gustavson, 1956). The flesh layer is also removed during the beamhouse processes (see Section 1.2.2).

1.2.2.   Different Stages of the Leather Manufacturing Process

Leather manufacturing processes involves the conversion of putrescible biodegradable hides/skins, into non-putrescible leather (Covington, 2001). A series of physical and chemical operations are required to convert raw hides and skins into leather. The different mechanical and chemical processes may be varied according to the required properties in the final products. These operations are divided into four groups, which are as follows:

- **Pre-tanning or beamhouse operation**: The pre-tanning or beamhouse operations include trimming, soaking, liming/unhairing, fleshing, deliming, bating and scudding (Aravubdhan et al., 2007). The aim of these operations is to clean or purify the skin collagen, that is to separate skin collagen (leather forming materials) from other non-collagenous proteins (unwanted non-leather making substances) (Alexander et al., 1992; Buljan et al., 1997; Menderes et al., 1999; Rao et al., 2003) and to split the fibrous structure (Buljan et al., 1997; Menderes et al., 1999).

- **Tanning**: This may include pickling, tanning and basification in case of chrome tanning (Aravubdhan et al., 2007; Saravanabhavab et al., 2003a). Tanning stabilises the collagen-matrix to prevent putrefaction. Chrome tanned leather is known as wet-blue (Covington, 2001; Heidemann, 1993; Rao et al., 2003).

- **Post-tanning**: Post-tanning consists of sammying, splitting, shaving, re-chroming, neutralisation, re-tanning, fatliquoring, dyeing, setting and drying (Aravubdhan et al., 2007). Post-tanning operations are carried out to confer additional properties. The products after post tanning processes are known as crust (Integrated Pollution Prevention and Control (IPPC), 2001; Rao et al., 2003; Saravanabhavab et al., 2003a).

- **Finishing**: Finishing includes conditioning, staking, toggling, trimming, buffing, spraying/ roller coating or plating/polishing. Finishing increases the aesthetic value of the final product (Aravubdhan et al., 2007; Rao et al., 2003; Saravanabhavab et al., 2003a).

In this study, the primary stages of the leather manufacturing processes that are beamhouse and tanning processes were focused and the details of the beamhouse and tanning processes are given below.

1.2.3. Beamhouse and Tanning Processes

1.2.3.1. Soaking

Soaking is the first stage in the leather manufacturing processes. There are two main purposes for the soaking process (Daniels, 2003; Thanikaivelan et al., 2005):

- Re-hydration of hides and skins to bring them back to the state of fresh hides or skins (just after flaying) as far as possible, and

- Removal of adhered blood, urine, soil, dung, salt or other extraneous materials from hides or skins.

A soaking process also assists removal of non-collageneous proteins, water-soluble albumins and salt-soluble globulins, constituents of the interfibrilary fluid and fibroblasts (Heidemann, 1993).

Generally, a soaking process is carried out in two steps, the first step is known as dirt-soaking or pre-soaking, which is done for a short period of time, usually an hour. The second step is known as main-soaking (IPPC, 2001). Strong mechanical action may also be required for proper cleaning (Heidemann, 1993). The quantity of water required for soaking per tonne dried hides can be as high as 20 m^3 water (Ludvik and Buljan, 1998). Normally it requires 5 to 24 hours to complete the soaking process depending on the condition of preserved hides (Heidemann, 1993).

Alkalis such as sodium hydroxide or sodium carbonate, surface-active agents (surfactants)/wetting agents, bactericides and proteolytic enzymes are usually used as additives during soaking, and are based on the type of the raw material (Heidemann, 1993; Thanikaivelan et al., 2005). Water absorption by hides/skins increases at higher pHs. The pH of the soaking bath is generally 9.5-10.5. The pH is increased by using mild alkalis such as sodium hydroxide or sodium carbonate. The temperature of soaking is usually between 25-28°C. A higher temperature accelerates the soaking procedure (Heidemann, 1993).

1.2.3.2. Unhairing

Unhairing processes are carried out to remove hair and some non-structural proteins such as proteoglycans from the hides. The main structural protein of hair is keratin. Keratin mainly consists of an amino acid called cystine. Reduction or oxidation can break down the disulfide bonds (S-S) of cystine causing keratin degradation, without affecting the collagen. The most commonly used reducing agents are sulfur or thio-compounds. The removal of proteoglycan splits up the fibre structure into fibril levels and is known as "opening up". Swelling of hides and opening up of the fibre structure takes place during liming. A hair-burning method for removal of hair is caused by the total destruction of keratin. Usually in a conventional hair burning method, unhairing and liming is carried out at the same time in a drum or paddle. This process requires 14-24 hours at room temperature for complete hair removal and opening up. Mechanical movements increase the reaction rate as well as assisting in the removal of hair roots from the hair follicle (Frendrup, 2000; Heidemann, 1993).

A hair-saving method of unhairing is the removal of intact hair and considered as an environmentally friendly method of unhairing. A conventional hair-saving method, known as "painting" is mainly carried out to remove hair from sheep or goatskins. In this process of hair removal, a thick paste of lime and sulfide is applied on the flesh side of skins, folded flesh side to flesh side, and left for 4-6 hours. Sulfides penetrate through the skins and attack the hair roots and epidermis causing removal of intact hair (Daniels, 2003; Heidemann, 1993; Frendrup, 2000). Subsequent liming is required for swelling of unhaired skins (Heidemann, 1993).

1.2.3.3. Fleshing

Fleshing is carried out to remove excessive organic materials (fats and tissues) and to aid chemical penetration into the hides. Fleshing may be carried out at various stages such as prior to soaking, after soaking, after unhairing or after pickling. The fleshing process, if undertaken prior to unhairing, is known as green fleshing, and lime fleshing if carried out after liming (IPPC, 2001). Green fleshing reduces the chemical uptake during liming and assists in achieving a uniform liming effect (Thanikaivelan et al., 2005) to enhance leather quality (Ludvik and Buljan, 1998). This also produces fleshings at neutral pH that are free from chemical contamination. By-products such as protein and fat may be extracted from green fleshings (Ludvik and Buljan, 1998).

1.2.3.4.   Deliming

Deliming processes are carried out to neutralise lime and other alkalis from hides and skins, which were absorbed during the liming process. The deliming process also prepares the pelts (unhaired hides/skins) for the subsequent bating process. Weakly bound alkalis can easily be removed by washing the pelts with water. Acid salts such as ammonium salts (ammonium sulfate or chloride), or sodium bisulfite are used to neutralise chemically bound alkalis. Ammonium salts are the most common deliming agents since the salts buffer the pH to 8-9, at which the commercial proteolytic enzymes (added in the subsequent process bating) operate at optimum levels. Organic acids such as lactic acid, acetic acid or boric acid, and strong mineral acids such as hydrochloric acid may also be used. Careful controls are required while using strong mineral acids as they may reduce the pH of the deliming liquor to a very low pH. A low pH cause the precipitation of proteins onto the grain and acid swelling, as a result, the grain may be damaged (Leafe, 1999).

1.2.3.5.   Bating

The main purpose of the bating process is to clean the grain by removing scuds (e.g., hair roots, pigments). Proteolytic enzymes with a mild cleaning action are used in bating. The commercial bating enzymes are mainly based on pancreatic trypsin or bacterial proteases. These enzymes require an optimal pH range of 8-9 (Daniels, 2003). The enzymes may also break down other non-structural and structural proteins which relax the skin and soften the leather (Daniels, 2003; Leafe, 1999).

1.2.3.6.   Pickling

Chromium is the most commonly used tanning agent (Saravanabhvan et al., 2004). It is sparingly soluble in water and requires low pH for higher solubility. A high pH in the tanning liquor will cause chromium to precipitate onto the pelt surface, so reducing penetration into the pelts. The pickling process is therefore carried out before tanning to reduce the pH of the liquor, in order to allow the penetration of the chromium salt into the pelts. The pH is generally reduced by adding acids, such as sulfuric and formic acids. The pH of the pickle liquor for chrome tanning is approximately 2.5. The low pHs cause the swelling of the skins, known as "acid swelling". Sodium chloride (NaCl) (6-8% based on the pelt weight) is therefore added during a conventional pickling process to control acid swelling (Leafe, 1999; Thanikaivelan et al., 2005).

1.2.3.7. Tanning

Tanning of hides and skins is defined as the conversion of putrescible biodegradable materials (hide/skins) into a non-putrescible material that is leather, which can resist microbial attack. Tanning causes changes in physical appearance and properties, such as opacity and increase in hydrothermal stability. The newly acquired properties due to tanning should be permanent so the leather should have resistance to prevent it reverting back to the properties of the raw pelts (Covington, 2001; Heidemann, 1993).

Tanning is generally carried out by effective crosslinking or stabilising the animal hide-collagen, using tanning agents known as tannins. Although there are various types of vegetable, synthetic, organic and mineral tannins, chrome tanning is still the most common mineral tanning agent used to convert hides and skins into leather (Qi and Brown, 2002). Approximately 90% of the leather produced globally is chrome-tanned. Chrome-tanned leather shows high shrinkage temperature or high hydrothermal stability, good flexibility, excellent fastness properties, lightweight with good comfort characteristics, good moulding and shape retention properties (Dasgupta, 2004).

1.2.4. Emissions from the Beamhouse and Tanning Processes

1.2.4.1. Soaking

Soak liquor is characterised by the presence of a high amount of total dissolved solid (TDS) and chloride content. Due to the presence of soluble proteins and other organic materials, soak liquors contribute to the total biological oxygen demand (BOD) and chemical oxygen demand (COD) loads of tannery effluents to some extent (Thanikaivelan et al., 2005). Soaking of hides and skins generates 7 m^3-9 m^3 effluent per tonne of raw hides (based on the weight of the preserved hides and skins). The pollution loads from a conventional soaking process is given in Table 1.2 (Ludvik and Buljan, 1998).

Table 1.2: Discharge of pollution load from a conventional soaking process (Ludvik and Buljan, 1998).

Pollution loads	Load kg/tonne raw hides
Suspended solids (SS)	11- 17 kg
Chemical oxygen demand (COD)	22 – 23 kg
Biochemical oxygen demand (BOD)	7 – 11 kg
Ammoniacal nitrogen (NH$_3$-N)	0.1 – 0.2 kg
Total Kjeldahl nitrogen (TKN)	1 – 2 kg
Chlorides (Cl$^-$)	85 – 113 kg
Sulfate (SO$_4$$^{2-}$)	1– 2 Kg

Alkyl phenol ethoxylates (APEs), particularly nonylphenol ethoxylates (NPEs) are the most commonly used surfactant in tanneries. The latter compound has a toxic effect on the environment. Nonylphenol is known to cause disturbances in the endocrine system (IPPC, 2001).

The salinity in soaking effluent is due to the use of NaCl during the curing process (curing: hide preservation method to protect raw hides/skins from microbial putrefaction) (Confederation of National Associations of Tanners and Dressers of the European Country (COTANCE), 2002). The presence of NaCl at high levels in the environment can adversely affect aquatic and plant life particularly fresh water species. Mineral nutrients of soil may be replaced by NaCl (IPPC, 2001).

1.2.4.2.   Unhairing

The conventional lime-sulfide hair burning unhairing process is considered the most polluting process step in leather manufacture. The pollution load is mainly due to the degradation of keratin (Paul *et al.*, 2001) that generates an effluent, contributing 55% COD, 55% suspended solids (SS), 70% BOD and 40% nitrogen. Nitrogen has a high oxygen demand and therefore stimulates eutrophication (Bosnic *et al.*, 2000; IPPC, 2001). Lime or calcium hydroxide and sodium sulfide (Na_2S) is harmful to aquatic species even at low concentrations (Weiss, 1980). Pollution loads from a conventional hair-burning unhairing process is shown in Table 1.3.

Table 1.3: Discharge of pollution load from a conventional hair burning unhairing process (Ludvik and Buljan, 1998).

Pollution load	Load kg/tonne raw hide
Suspended solids (SS)	53-97 kg
Chemical Oxygen demand (COD)	79-122 kg
Biological oxygen demand (BOD)	28-45 kg
Sulfide (S^{2-})	3.9-8.7 kg
Ammoniacal nitrogen (NH_3-N)	0.4-0.5 kg
Total Kjeldahl nitrogen (TKN)	6-8 kg
Chlorides (Cl^-)	5-15 kg
Sulfate (SO_4^{2-})	1-2 kg

1.2.4.3.   Deliming

The added ammonium salts during a conventional deliming process reacts with the alkaline liquor. As a result, the formation of ammonia gas occurs. This also produces large amount of

ammoniacal nitrogen (NH_3-N). The presence of nitrogen at high concentrations is toxic for aquatic species as it reduces the oxygen level in water, stimulating eutrophication (Bosnic *et al.*, 2000; IPPC, 2001).

1.2.4.4. Bating

Bating liquor consists of small amount of proteinacious matter and other debris, which may cause an increase in BOD and COD loads (Thanikaivelan *et al.*, 2005).

1.2.4.5. Pickling and Chrome Tanning

The pickling process is a major source of salt in effluents (IPPC, 2001; Leafe, 1999) due to the use of NaCl to prevent acid swelling (see Section 1.2.3.6). Generally, pickling and chrome tanning is carried out in the same drum and effluent is discharged after chrome tanning. Pollution from such a process is given in Table 1.4. An analysis of mass balance done by Buljan *et al.* (1997) showed that in a conventional chrome tanning process, less than 50% of the chromic oxide added during tanning remains in the chrome tanning effluent.

Table 1.4: Effluent from a conventional pickling and chrome tanning process (Ludvik and Buljan, 1998).

Pollution load	Load kg/t raw hide
Suspended solids (SS)	5-10 kg
Chemical oxygen demand (COD)	7-11 kg
Biological oxygen demand (BOD)	2-4 kg
Chrome(III)	2-5 kg
Ammoniacal nitrogen (NH_3-N)	0.6-0.9 kg
Total Kjeldahl nitrogen (TKN)	0.6-0.9 kg
Chlorides (Cl^-)	40-60 kg
Sulfate (SO_4^{2-})	30-35Kg

Due to the emission of a high amount of pollution load during the conventional leather-making process, various alternative cleaner processes have been developed in order to reduce the pollution load. The alternative cleaner processes are known as best available technologies or BAT (Alexander *et al.*, 1992; IPPC, 2001; Rao *et al.*, 2003).

1.2.5. *Best Available Technologies (BAT)*

1.2.5.1. Soaking

The effluent or liquid from the conventional soaking process may be reused or recycled, instead of discarding. This may reduce the discharge of pollution load from the soaking process. The physical, chemical and biological treatment of wastewater cannot remove the salt (NaCl) from the effluents (Ludvik and Buljan, 1998). Physical means such as shaking off the salt in special drums before soaking may decrease the amount of salts in soaking effluent. Salt may be recovered from the soaking effluent (Thanikaivelan *et al.*, 2005; Ludvik and Buljan, 1998) however; the recovered salt is usually too dirty and contaminated for further direct use (Ludvik and Buljan, 1998).

Pollution problems due to the presence of salt in the discharged soaking effluent may be lessened by avoiding the use of salt during processing of raw hides. Hides may be preserved for a short period of time at 2°C (COTANCE, 2002). A small amount of salt is required to dissolve some of the globular proteins during soaking of the non-salted hides. The quantity of salt used in the curing process may also be reduced by using some environmentally friendly antiseptics and biocides (IPPC, 2001). A variety of antiseptics and biocides have been recommended by IPPC (2001) to use during leather processing.

1.2.5.2. Unhairing

By following a conventional "hair-save" unhairing process (see Section 1.2.3.2), it is possible to reduce the volume of water generally used during a conventional "hair-burning" process. This "hair-save" unhairing technique reduces the volume of water usage and the disposal of effluents (Paul *et al.*, 2001).

Since 1980, many processes have been developed to make the unhairing process cleaner and more environmentally friendly. For example, in 1981, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) developed a "hair-saving" unhairing system known as *Sirolime*, which is practiced in many tanneries in Australia and New Zealand. In 1985, Rohm and Hass Co. developed the *Blair* method of unhairing in cooperation with Eagle Ottawa Leather Co. The principle of both *Sirolime* and *Blair* methods were based on immunisation and removal of the intact hair. The resistance of the disulfide bonds (S-S) of keratin (main structural protein of the hair) to biological and chemical degradation may be increased by alkaline treatment in absence of Na_2S. This assists the removal of intact hair from the hair roots (Frendrup, 2000; Heidemann, 1993).

Various organic sulfur compounds such as mercaptoethanol, salts of mercaptoacetic acid (thioglycolic acid) and formamidinesulfinic acid may be used instead of Na_2S during unhairing to reduce the sulfide pollution load. A lesser amount of organic sulfur may be required to remove the hair than Na_2S; therefore, the amount of discharged chemicals is reduced in the effluent. However, sulfur compounds are more expensive than sulfide, increasing production costs (Frendrup, 2000).

Lyotropic agents (hydrogen bond breakers) can cause the loosening of the hair and the epidermis by attacking the basement membrane. Usually this method of unhairing takes several days but the method may be accelerated when applied in combination with enzymes (Frendrup, 2000; Hawkins and Wassenberg, 1997). Amines can increase the unhairing rate when used with lime. Dimethylamine may produce carcinogenic nitrosamines. Amine compounds which do not produce nitrosamine, such as hydroxylamines or some alkanolamines, are used as liming auxiliaries in combination with sulfide, organic sulfur compounds or enzymes (Frendrup, 2000).

1.2.5.3. Deliming

An ammonium-free deliming process can reduce ammoniacal nitrogen generation by 97% during the deliming process. Organic acids may be used instead of ammonium salts, to reduce the pollution load due to the presence of ammonia in the deliming effluent. Organic acids are expensive, thus increasing the production costs. Carbon dioxide (CO_2) may be used as an alternative to ammonium salts during the deliming of the pelts (Ludvik and Buljan, 1998; Leafe, 1999; IPPC, 2001). Frendrup (2000) recommended the use of carbon dioxide deliming in combination with "hair-save" unhairing for a larger elimination of nitrogen from tannery wastewater.

Carbon dioxide neutralises the lime and solubilises the calcium chloride. In addition, carbon dioxide deliming stabilises the pH at 6.5-7.0, therefore, acid swelling does not occur. However, production of the hydrogen sulfide gas (H_2S) may occur due to the low pH and may be avoided by the addition of hydrogen peroxide before the addition of carbon dioxide (Ludvik and Buljan, 1998).

1.2.5.4. Pickling

The use of organic acids can reduce or eliminate the use of salt (NaCl) (Environmental Technology Best Practice Programme (ETBPP), 2000). A salt-free pickling process using

phenol sulfonic acid-based compounds has been developed (Pojer and Huynh, 2006). Weak organic acids such as lactic acids and acetic acid can also be used in the pickle bath (IPPC, 2001).

1.2.6.   Use of Enzymes During the Beamhouse Processes

The use of enzymes in the leather industry is not a new technology. The earliest recorded enzymatic unhairing technique is known as sweating. In this process, hides/skins were placed in a hot and humid room, which encouraged the bacteria present on the hides/skins to proliferate. Hair loosening occurs due to the enzymatic action of the bacteria, and the loose hair can be easily removed by mechanical action (Frendrup, 2000; Ockerman and Hansen, 2000).

Use of proteolytic enzymes for unhairing was reported in 1913 (Heidemann, 1993). An alkaline pre-treatment and 12 hours incubation time may required for effective hair removal. The alkaline pre-treatment is also required to cause swelling or the decomposition of the epidermis in order to allow enzymes to penetrate into the papillary layer and into the hair follicle of the epidermis. The outermost layer of the skin, works as a protective layer of the grain, and therefore prevents penetration of enzymes into the grain or papillary layer (Heidemann, 1993). Use of proteolytic enzymes and carbohydratase was stated in a patent, by Otto Grimm in 1966 (Taylor *et. al.*, 1987a). Cattle hides contain 4-8 proteoglycans, which are susceptible to a wide range of proteolytic enzymes (Daniels, 2003; Heidemann, 1993). Use of alkaline proteases along with Na_2S reduces the percentage of sulfide loads, biological oxygen demand (BOD) and suspended solids (SS) in the tannery effluent (ETBPP, 2000). A number of proteolytic enzymes, mainly alkaline proteases extracted from a variety of sources (e.g. bacterial or fungal proteases) have been investigated for unhairing (Dayanandan *et al.*, 2003; Gehring *et al.*, 2002; Macedo *et al.*, 2005; Najafi *et al.*, 2005; Thangam *et al.*, 2001). There are both advantages and disadvantages of using enzymes during leather processing, which are as follows:

Advantages:
- Reduction of the quantity of Na_2S in effluent (Ludvik and Buljan, 1998; Frendrup, 2000).
- Recovery of the quality hair (ETBPP, 2000).

Disadvantages:
- High control is required while using enzymes for unhairing of hides (Frendrup, 2000).

- Enzymatic unhairing processes are more expensive than the conventional unhairing operations (Frendrup, 2000; Taylor *et al.*, 1987a), thus increases the production cost.

While using enzymes for unhairing, it is important that the enzymes should not affect or damage the collagen fibre structure (Cantera *et al.*, 2003). Proteolytic enzymes may influence collagen causing damage to the grain. In addition, enzymes may enhance bacterial proliferation, which may ruin the quality of leather. Most of the enzymatic unhairing processes may require a reducing agent to achieve complete hair removal (Frendrup, 2000; IPPC, 2001). Proteolytic enzymes are commonly used during the bating process (see Section 1.2.3.5) Enzymes may also be used in the soaking process to increase the water absorption by the hides and skins (see Section 1.2.3.1). The pH of the soaking liquor may be increased by the addition of mild alkalis such as sodium hydroxide or sodium carbonate in order to achieve the optimum enzyme activity (Heidemann, 1993; IPPC, 2001; Thanikaivelan *et al.*, 2005).

Best available technology processes may involve the reduction of the amount of hazardous chemicals used as well as recycling of the used liquor and chemicals, with a less extreme processing condition. The environmental pollution primarily occurs due to the presence of certain hazardous chemicals and their consequent presence in the tannery effluent. This research project concerns a BAT process reducing hazardous chemicals by partial or total replacement with non-hazardous chemicals.

Animal skins naturally contain various types of microorganisms, which either are natural inhabitants or acquired from the environment. The microorganisms present on the hides and skins may multiply rapidly due to the following factors: nutritional source, high moisture content, suitable pH and temperatures in the tannery environment (Birbir and Ilgaz, 1996; Kayalvizhi *et al.*, 2008; Oppong *et al.*, 2006; Rangarajan and Didato, 2003). Control of the microorganisms is essential during the leather manufacturing processes since microorganisms can cause damage to the collagen structure of the hides/skins and such damaged hides/skins produce inferior quality leather (Kayalvizhi *et al.*, 2008; Rangarajan and Didato, 2003). Various research, as described below, was therefore carried out in order to isolate and identify microorganisms that may cause damage to the hides/skins and leather.

1.3. Microorganisms Isolated from Raw Hides/Skins and Hides/Skins at Various Stages of the Leather Manufacturing Process

McLaughlin and Rockwell (1922) observed the presence of a wide range of microorganisms on fresh steer hides. The microorganisms varied in their morphology (e.g., Bacilli, Cocci, Spirilla, Spirochaeta), biological characteristics (e.g. Gram-positive and Gram-negative), and their action on hides (e.g., proteolytic or non-proteolytic). Presence of yeasts, moulds and protozoa was also observed on the fresh hides by the authors. Bacteria responsible for hide putrefaction were found to be mainly proteolytic types and require an aerobic environment for their growth. The presence of a high concentration of carbon dioxide, a lack of oxygen, acidity and absence of proteins were thought to prevent bacterial proliferation and so bacterial deterioration (McLaughlin and Rockwell, 1922).

Anderson (1945) found that the various bacterial species isolated from fresh calf skins had the ability to withstand a high level of salt (NaCl) concentrations (1.5-9% w/v). The isolated bacterial species included *Bacillus coli*, *Bacillus proteus*, *Bacillus megatherium*, *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus albus*, *Staphylococcus aureus*, *Sarcina lutea* and *Micrococcus roseus*. *Bacillus subtilis* and *Bacillus mycoides* was found to have survived in a dormant state at a high salt concentration (20% w/v) (Anderson, 1945).

Rawlings and Cooper (1975) carried out a study in order to determine the most suitable 'routine technique' of bacterial enumeration during the early stages of the leather manufacturing processes (soaking-pickling). It was concluded by the authors that the most appropriate method of counting viable bacterial colonies in the tannery effluent was inoculation of the diluted effluents on a nutrient agar media with at pH either 7 or 9, and at 30°C for 5-7 days (Rawlings and Cooper, 1975).

Hanlin *et al.* (1995) isolated various Gram-positives (such as *Staphylococcus* spp. and *Corynebacterium* spp.) and Gram-negatives (such as *Micrococcus* spp., *Proteus* spp., *Agrobacterium* spp. and *Shewanella* spp.) from cattle hides. Bacterial enumeration was carried out on tryptic soy agar at 25°C for 4 days. The research showed that a mixture of the two bacteriocins, nisin and pediocin AcH from *Lactococcus lactis* and *Pediococcus acidilactici* respectively, are effective against the Gram-postive bacteria, while ineffective on Gram-negative bacteria. Addition of 0.25% w/v sodium dodecyl sulfate to a mixture of the two bacteriocins increased the effectiveness of the bacteriocin mixture toward the Gram-negative bacteria (Hanlin *et al.*, 1995).

Birbir and Ilgaz (1996) isolated various bacterial species from fresh hides, salted hides, hides after soaking and liming, chromed, retanned, dried and finished leather. Samples were enumerated on nutrient agar, nutrient gelatine and beef extract media containing 0.85% w/v, 7% w/v and 30% w/v NaCl at 25°C, 37°C, and 41°C for over 2 days. The isolated microbial species are shown in Tables 1.5-1.7. The research showed that the bacterial species in the genus the *Bacillus* may have survived an unhairing process, which was carried out using 2% w/w lime, 3% w/w Na_2S and 3.5% w/w calcium hydroxide (Birbir and Ilgaz, 1996).

Table 1.5: Microorganisms isolated from chrome tanned, re-tanned, dried and finished leather (Birbir and Ilgaz, 1996).

Microorganisms	CT	RH	DR	FH*	L¹
Bacillus brevis		✓			
Bacillus cereus	✓	✓	✓	✓	
Bacillus licheniformis	✓		✓	✓	
Bacillus pumilus	✓	✓		✓	✓
Bacillus sphaericus				✓	
Bacillus subtilis	✓	✓	✓		✓
Micrococcus roseus			✓		
Staphylococcus aureus		✓			

(Note: CT; chrome tanned hides, RH; retanned hides, DR; dried hides, FH*; finished hides, L¹; 1 day old leather: referred by Birbir and Ilgaz, 1996).

Table 1.6: Microorganisms isolated in fresh, wet salted and limed hides (Birbir and Ilgaz, 1996).

Microorganisms	FH	SH¹	SH²	SH	LH
Bacillus brevis		✓			
Bacillus cereus	✓	✓	✓	✓	✓
Bacillus firmus			✓		
Bacillus laterosporus				✓	
Bacillus licheniformis		✓	✓	✓	✓
Bacillus megaterium	✓	✓	✓	✓	✓
Bacillus pumilus		✓	✓		✓
Bacillus sphaericus	✓	✓	✓		
Bacillus subtilis	✓	✓	✓	✓	✓

(Note: FH; fresh hides, SH¹; 1 week old salted hides, SH²; 2 months salted hides, SH; soaked hides, LH; limed hides; referred by Birbir and Ilgaz, 1996).

Table1.7: Microorganisms isolated in fresh, wet salted and limed hides (Birbir and Ilgaz, 1996).

Microorganisms	FH	SH[1]	SH[2]	SH	LH
Kurthia variabilis	✓	✓	✓	✓	
Micrococcus candidus			✓		
Micrococcus luteus			✓		
Micrococcus roseus	✓	✓	✓		
Micrococcus rubens		✓	✓	✓	
Pseudomonas aeruginosa				✓	
Staphylococcus aureus	✓	✓	✓	✓	
Staphylococcus epidermidis		✓	✓	✓	

(Note: FH; fresh hides, SH[1]; 1 week old salted hide, SH[2]; 2 months salted hides, SH; soaked hides, LH; limed hides: referred by Birbir and Ilgaz, 1996).

An environmental mycological survey was carried out by Nigam (1997) at the liming section of the Tannery and Footwear Corporation (TAFCO) at Kanpur, India, in 1985. Nigam (1997) isolated and identified 33 fungal species. *Aspergillus* spp. and *Penicillium* spp. were the two predominantly isolated fungal species. The other isolated species were *Alternaria* spp., *Cephalosporium* spp., *Chaetomium* spp., *Cladosporium* spp., *Cunninghamella* spp., *Curvularia* spp., *Drechslera* spp., *Fusarium* spp., *Mucor* spp., *Phoma* spp., *Rhizopus* spp. and *Trichoderma* spp. The allergic reaction of 7 potential allergens, which were *A. flavus, A. oryzae, A. sulphureus, A. sydowii, A. terreus, Mucor geophila* and *R. stolonifer* were tested on 50 patients and 25 healthy humans. However, the study generated no significant correlation between allergens produced by *A. flavus, A. oryzae, A. sulphureus, A. terreus, Monilla geophila and Rhizopus stolonifer* and allergic reactions among human patients, who were suffering from various diseases (Nigam, 1997).

Özdilli *et al.* (2007) isolated various fungal species such as *Penicillium* spp., *Aspergillus* spp., *Alternaria* spp., *Scopulariopsis* spp. and *Cladosporium* spp. from 14 tanneries in Istanbul, Turkey. *Penicillium* spp. was found to be the most commonly isolated fungal species followed by *Aspergillus* spp. Özdilli *et al.* (2007) concluded the allergen from the isolated fungal species may be the reason for the development of respiratory infections in tannery workers.

In the tannery, the growth of fungi normally occurs on pickled skins, since fungi are capable of growing at a lower pH (Maier *et al.*, 2000; Rangarajan and Didato, 2003). Growth of fungi also occurred on vegetable tanned, chrome tanned and finished leather. *Aspergillus* spp., *Penicillium* spp. and *Paecilomyces* spp. were the most common type of fungi isolated from leather and may be responsible for the discolouration on the skins or leathers. *Aspergillus terreus, Aspergillus niger, Aspergillus fumigatus, Penicillium restrictum, Penicillium citrinum,*

Alternia spp. and *Cladosporium* spp. were isolated from salted sheep skins by Bitlisli *et al.* (2004). In the tannery environment, fungal spores and vegetative cells are spread through the air or by physical contact during the leather manufacturing process. These spores may germinate in suitable environmental conditions, such as presence of nutrition, suitable pH and temperature. Similar to bacteria, fungi are also natural inhabitants of hides/skins. Fungi species can also tolerate high NaCl concentrations (20-30% w/v) (Bitlisli *et al.*, 2004).

Recently Oppong *et al.* (2006) used molecular-based (16s ribosomal RNA) methods to identify bacteria in fresh hides. Various bacterial species including proteolytic and non-protolytic bacteria were isolated from fresh hides before and after a soaking process. Proteolytic bacteria are thought to be responsible for the putrification of hides/skins. Bacterial enumeration was carried out on plate count agar containing either 7% w/v or 25% w/v NaCl and was incubated for 2-7 days at 30°C. Bacterial growth was observed at 7% NaCl. The bacterial species isolated from the hides before and after soaking are shown in Table 1.8. No bacterial growth was observed when the NaCl concentration was 25% w/v. The total number of bacterial colonies isolated from the hides was approximately 10^6 colony forming units (cfu)/gram (g). According to the authors, the isolated non-proteolytic (e.g., *Acinetobacter* spp. and *Pseudomonas* spp.) bacteria may utilise non-proteinous matters, fat and other extraneous organic matter as nutritional sources.

Table 1.8: Microorganisms identified using PCR based genotype identification (Oppong *et al.*, 2006)

Isolated form	Isolated bacterial species
Bacteria isolated from the fresh hides before soaking	*Aeromonas hydrophila, Shewanella putrefaciens, Pseudomonas fulgida, Staphylococcus homonis, Aeromonas veronii, Proteus vulgaris, Brevibacterium lutescens, Dietzia maris, Janibacter terrae, Pseudomonas cannabina, Pseudomonas* spp., *Arthrobacter protophorniae, Pseudomonas putida*
Bacteria isolated from the fresh hides after soaking	*Aeromonas hydrophila, Aeromonas johnsonii, Aeromonas veronii, Comamonas acquatica, Klebsiella* spp., *Shewanella putrifaciens, Citrobacter freundii, Proteus vulgaris, Pseudomonas* spp., *Acinetobacter radioresistens, Acinetobacter calcoaceticus, Pseudomonas stutzeri,* Aeromonas media, Brevibacterium *lutescens, Jeotgalicoccus psychrophilus, Nocardiopsis* spp.

Kayalvizhi *et al.* (2008) isolated various Gram-positive and Gram-negative bacteria from goat and sheep skins. Most of the identified bacteria (78.7%) were Gram-positive. The isolated bacteria were identified as *Bacillus cereus, Bacillus subtilis, Bacillus megaterium, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus fermentum, Micrococcus luteus, Neisseria flavescens, Neisseria sicca, Proteus mirabilis, Proteus* spp. *Pseudomonas* spp. *Staphylococcus luteus, Staphylococcus aureus, Staphylococcus epidermis* and

Streptococcus faecalis. However, Kayalvizhi *et al.* (2008) found that the Gram-positive Bacilli and Cocci with proteolytic activity are responsible for degradation of goat and sheep skins.

Shede *et al.* (2008) isolated various bacterial species with proteolytic and/or lypolytic activity, such as *Acinetobacter caviae*, *Bacillus cereus*, *Bacillus sphaericus*, *Brevibacterium luteolum*, *Brevibacterium otitidis*, *Escherichia coli*, *Proteus mirabilis*, *Proteus penneri*, *Proteus vulgaris*, *Myroides odoratimimus*, *Staphylococcus sciuri*, *Vagococcus* species and *Weeksella virosa*. In addition, the genus *Bacillus*, Acinetobacter, *Proteus*, *Aeromonas*, *Brevibacterium*, and *Weeksella* showed collagenase activity. Bacterial cells were isolated using nutrient agar consisting of 1% w/v skimmed milk powder and 1% w/v tributyrine and incubated for 48 hours at 37°C. Bacterial identification was carried out based on 16S ribosomal DNA gene sequencing method using the gradient gel electrophoresis (DGGE) system and was stated as an effective molecular-based method of DNA profiling by the authors (Shede *et al.*, 2008). However, the bacterial species that were isolated by the above mentioned authors exhibited proteolytic and/or lypolytic activity and were found to be the most likely to be responsible for degradation of hides.

Current and past research has been concerned with microbial growth and putrefaction of hides/skins. Although the majority of the isolated microbial species are non-harmful and do not cause infections to humans (Hanlin *et al.*, 1995; Wilson, 2005), studies also show that some species in the genera *Bacillus*, *Staphylococcus*, *Pseudomonas*, *Klebsiella*, *Aspergillus*, and *Candida* are considered to be pathogens or potential pathogens (Wilson, 2005). The presence of pathogens in tannery effluents and the pathogen related health issues requires investigation. During conventional leather-making processing, due to the extreme environmental conditions, the probability of bacteria surviving on hides is reduced. Total or partial replacement of the hazardous chemicals with non-hazardous chemicals during the BAT leather-making processes may provide suitable conditions for bacterial growth. Since the leather industry in moving toward more environmentally friendly methods of leather manufacturing, therefore, the survival and growth of pathogens/potential pathogens during the BAT leather-making processes also requires investigation.

1.4.   Aim and Objectives

1.4.1.   Overall Aim

The aim of the project is to determine the survival and growth of bacteria, particularly pathogens, present on hides/skins during a conventional and BAT leather-making processes (beamhouse and tanning).

1.4.2.   Objectives

- Carry out preliminary experiments to identify the bacteria present during the BAT leather-making process (beamhouse and tanning).

- Optimisation of the experimental methods, such as the leather-making processes (conventional and BAT), procedures for bacterial isolation, growth and identification.

- Inoculation of known bacterial species (*Bacillus cereus* ATCC11778, *Staphylococcus aureus* NCTC12981/ATCC25923 *and Pseudomonas aeruginosa* ATCC10145) on calf skins and to monitor the survival and growth of the above-mentioned bacterial species at various stages (beamhouse and tanning) of the conventional and BAT leather-making processes.

- Enumeration and isolation of the inoculated bacterial species from the effluent and skin samples, during various stages (beamhouse and tanning) of the conventional and BAT leather-making processes.

- Confirmatory tests to ensure the presence of the inoculated bacterial species using biochemical assays (standard biochemical test and the Biolog identification method).

- Use of the pulsed-field gel electrophoresis method of DNA fingerprinting to ensure that the isolated bacterial species (*Bacillus cereus* and *Pseudomonas aeruginosa*) are the same as the inoculated bacterial species based on their DNA profiles.

2.1.   Preparation of Microbial Media

Media for microbial enumeration were prepared according to the instructions provide by the suppliers (Oxoid, UK and Sigma-Aldrich, UK). The prepared media was stored in a refrigerator at 4°C. The prepared selective solid media for *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* was used within two weeks.

2.2.   Sample Plating

Samples (50 μl) for analysis were spread on solid media using a Whitley automated spiral plater (WASP), (model WASP2, Don Whitley Scientific Ltd., UK). The inoculated petri-dishes (plates) were left on the worktop for 15 minutes to allow the samples to be absorbed by the solid media (Health Protection Agency (HPA), 2005a). The inoculated plates were inverted and incubated at required temperature for 24 hours.

2.3.   Bacterial Species for Inoculation

The following bacterial species were selected for inoculation:

Bacillus cereus (ATCC11778)
Pseudomonas aeruginosa (ATCC10145)
Staphylococcus aureus (NCTC12981/ATCC25923)

The above-mentioned bacterial species were selected, as *Bacillus*, *Staphylococcus* and *Pseudomonas* are a few of the most isolated species from raw hides and skins (Anderson, 1945; Birbir and Ilgaz, 1996; Hanlin *et al.*, 1995; Kayalvizhi *et al.*, 2008; McLaughlin and Rockwell, 1922; Oppong *et al.*, 2006; Özdilli *et al.*, 2007).

2.4.   Growth Curve

Growth patterns of the bacterial species were observed using a UV spectrophotometer (UV-250 1PC, Shimadzu, Japan). A pure culture of the bacterial species (*B. cereus* ATCC11778, *P. aeruginosa* ATCC10145 and *S. aureus* NCTC12981/ATCC25923) was incubated overnight in tryptone soya broth (TSB). An aliquot (1ml) was collected from the overnight

incubated culture and added to 9 ml TSB to prepare a 10^{-1} dilution. Tryptone soya broth, without any bacterial inoculation, was used as a reference sample. A temperature controller (TCC-controller, TC-240A, Shimadzu, Japan) was connected to the spectrophotometer to maintain the incubation temperatures as required (see Sections 2.5.1, 2.5.2 and 2.5.3). The spectrophotometer was set at the wavelength of 600 nm (Alksne *et al.*, 2000; Auger *et al.*, 2006; Borges *et al.*, 2008; Cotroneo *et al.*, 2008; Cao *et al.*, 2001). The experimental and reference samples were placed in the spectrophotometer, and absorbance of the previously prepared 10^{-1} bacterial cell suspensions read at every 30 minutes for up to 24 hours. A graph was plotted using the absorbance readings against time (minutes). The plotted graph was used to calculate the mid-exponential phase. Figure 2.1 shows a schematic diagram of a typical bacterial growth curve indicating the mid-exponential phase.
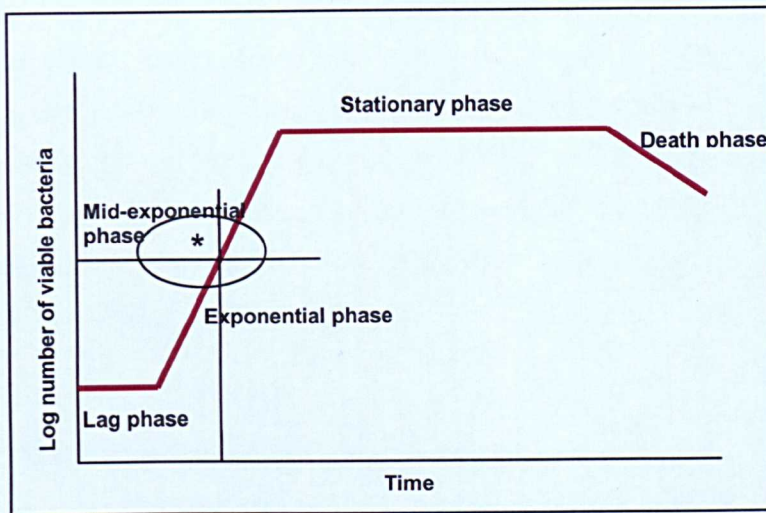
Figure 2.1: Bacterial growth curve, (*) showing mid-exponential phase (Pelczar *et al.*, 1993).

2.5.   Bacterial Enumeration

For inoculation and incubation of the bacterial species, the national standard methods (HPA, 2007, 2005a, b, c and d) were followed. A few adjustments were made for this study. As for example, a phosphate buffered saline (PBS; pH 7.4±0.2) solution was used for dilution instead of maximum recovery diluents or peptone buffered water, in order to maintain consistency, since PBS was used during the preliminary experiments (Chapter 3), such as, for the optimisation of solid media (Section 3.3.1) and dilution of the pre-soaking and soaking effluents (Section 3.3.5). In addition, the recommendation of using maximum recovery diluents or peptone buffered water, by HPA (2007, 2005c and d) is optional.

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2.5.1. *Bacillus cereus*

Bacillus cereus was enumerated on Polymyxin pyruvate egg yolk mannitol agar (PEMBA) (CM0617, SR0099, SR0047, Oxoid UK) media (for details of the chemical composition see Appendix 1). Samples for microbial analysis were inoculated on PEMBA media using a spiral-plater, as described in Section 2.2. Incubation was carried out at 30°C for 24 hours. Further incubation was carried out if the growth of bacterial colonies were not observed on the inoculated plates following 24 hours incubation. The plates were examined and any presumptive *B. cereus* colonies that appeared (see Section 2.6.1) on the inoculated plates were counted and recorded.

2.5.2. *Staphylococcus aureus*

For enumeration of *S. aureus*, Baird-Parker media with egg yolk emulsion and tellurite (CM0275, SR0054, Oxoid, UK) (chemical composition in Appendix 1) was used. Samples for microbial analysis were inoculated on the media using a spiral-plater (see Section 2.2). Incubation was carried out at 37°C for 24 hours. The plates were incubated further if the appearance of bacterial species were not observed on the inoculated plates after 24 hours incubation. *Staphylococcus aureus* colonies on the Baird-Parker media (see Section 2.6.2) were counted and recorded.

2.5.3. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was enumerated on *Pseudomonas* CN media containing glycerol, cetrimide and nalidixic (CM0559, SR0102, Oxoid, UK). See Appendix 1, for details of the chemical composition of the *Pseudomonas* CN media. Samples for microbial analysis were inoculated on *Pseudomonas* CN media using a spiral-plater (see Section 2.2). The inoculated plates were incubated at 37°C for 24 hours. The plates were examined and incubated further if the absence of the bacterial colonies was observed on the inoculated plates after 24 hours incubation. *Pseudomonas aeruginosa* colonies on the *Pseudomonas* CN media (see Section 2.6.3) were counted and recorded.

2.6. Primary Identification of Inoculated Bacterial Colonies

2.6.1. *Bacillus cereus*

The PEMBA media contains mannitol (sugar alcohol) and egg yolk. The PEMBA media also contain a pH indicator bromothymol blue exhibiting yellow and blue colour in acidic and

alkaline pH respectively. *Bacillus cereus* does not ferment mannitol (mannitol-negative); as a result, *B. cereus* colonies on PEMBA media appear as a turquoise colour or peacock blue colour, whereas mannitol-positive bacterial colonies appear as a straw colour (acid production). Generally, *B. cereus* produces an enzyme lecithinase, which degrades lecithin in egg yolk causing precipitation of the hydrolysed lecithin (HPA, 2005c). A photo of typical blue *B. cereus* colonies (on PEMBA media) surrounded by blue precipitated zone of hydrolysed lecithin is shown in Figure 2.2. However, some *B. cereus* strains may produce little or no lecithinase (HPA, 2005c). The turquoise coloured or peacock blue coloured colonies, which may or may not be surrounded by an egg yolk precipitated zone, were counted as presumptive *B. cereus*.
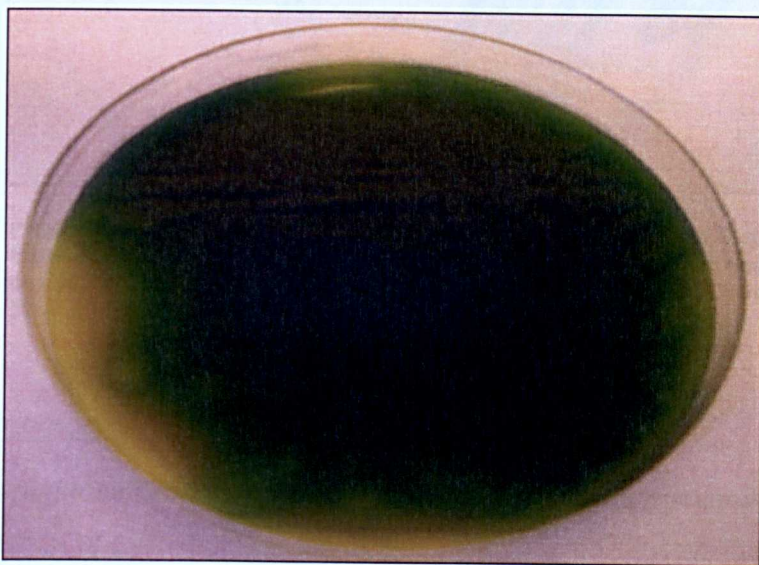
Figure 2.2: *Bacillus cereus* ATCC11778 on PEMBA media. The plate was incubated at 30°C for 24 hours. Photo was taken using a Pentax Optio 57, 7.0 Megapixel (MP).

2.6.2.   *Staphylococcus aureus*

Staphylococcus aureus can reduce tellurite; therefore, appears as shiny, grey-black-coloured colonies on Baird-Parker media. *Staphylococcus aureus* produces convex colonies. Similar to *B. cereus*, *S. aureus* produces the enzyme lecithinase. As a result, precipitation of hydrolysed lecithin occurs and appears as an opaque zone surrounded by a clearing zone around the *S. aureus* colonies on Baird-Parker media (HPA, 2005d). Figure 2.3 shows typical black, convex, shiny *S. aureus* colonies on Baird-Parker media. It was observed that the black *S. aureus* NCTC12981/ATCC25923 colonies were surrounded by an opaque zone and the opaque zone was surrounded by a narrow zone of clearing. *Staphylococcus aureus*

of bovine origin may not show opaque halos or clear zone (HPA, 2005d). The shiny-black, convex colonies with and without opacity or clearing were counted as *S. aureus* colonies.
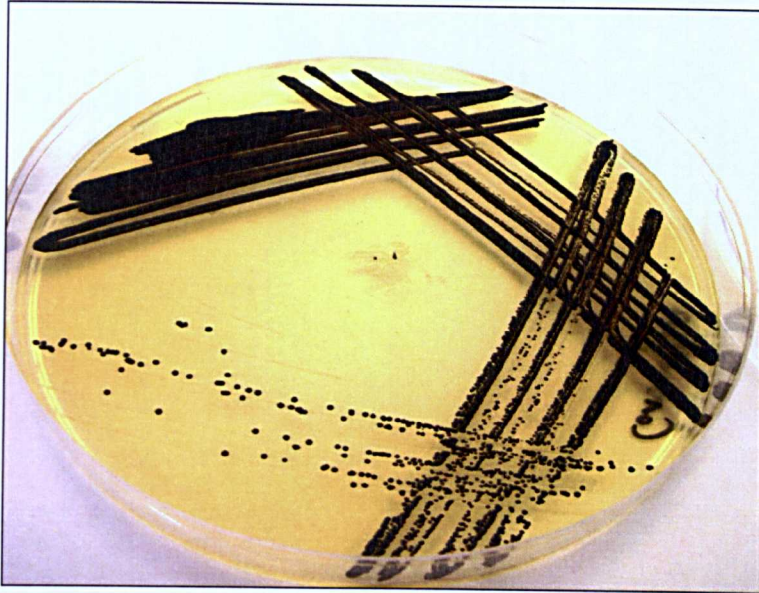
Figure 2.3: ***Staphylococcus aureus*** **NCTC12981/ATCC®25923 on Baird-Parker egg yolk tellurite agar media. Incubation was carried out at 37°C for 24 hours. Picture was taken using a Pentax Optio 57 (7.0 MP).**

2.6.3.   *Pseudomonas aeruginosa*

Pseudomonas aeruginosa produces two water soluble pigments pyoverdin and pyocyanin. Due to the production of pyocyanin, *P. aeruginosa* produces distinctive blue/green colonies (Barrow and Feltham, 2003; HPA, 2007; Madigan *et al.*, 1997; Todar, 2008). Figure 2.4 shows green *P. aeruginosa* colonies on *Pseudomonas* CN media. Green colonies on *Pseudomonas* CN agar media were counted as *P. aeruginosa*.

Figure 2.4: *Pseudomonas aeruginosa* ATCC10145 on *Pseudomonas* CN agar media. Incubation was carried out at 37°C for 24 hours. Picture was taken using a Pentax Optio 57 (7.0 MP).

2.7.   Gram-Staining

Bacterial smears were prepared on microscopic slides, air-dried and heat-fixed. The slides were flooded with crystal violet (0.5% w/v) for 1 minute, rinsed with water and an iodine solution (0.375% w/v) of potassium iodide was applied for 1 minute. The crystal violet forms a complex with iodine. The slides were then rinsed with water and washed with ethanol (95% w/v) for 10 seconds, followed by a wash with water. Safranin (0.4% w/v) was applied as the counterstain for 1 minute, rinsed with water and then pat-dried (Murray *et al.*, 2005).

As mentioned in Section 1.1.1, due to the presence of a thick peptidoglycan layer, the cell wall of a Gram-positive bacterium retains the violet-iodine complex even after the alcohol wash, and appears purple when observed under a light microscope. On the other hand, due to a comparatively thinner pepdidoglycan layer the cells wall of a Gram-negative bacterium does not retain the violet-iodine complex. In addition, the outer layer of Gram-negative bacteria consists of lipid, which is removed during the alcohol wash along with the violet-iodine complex. Gram-negative bacteria therefore stain red after the addition of safranin (Madigan *et al.*, 1997; Murray *et al.*, 2005; Pelczar *et al.*, 1993; Wilson, 2005).

2.8.   Spore-Staining

Malachite green dye (0.4% w/v) was used to stain spores and safrinin (0.1% w/v) was used as the counterstain to stain the vegetative cells. Bacterial smears were prepared on

microscopic slides, air-dried and heat-fixed. The prepared microscopic slides were placed on a slide rack and flooded with malachite green (0.4% w/v). A small piece of paper towel was placed on the stain, in order to prevent the quick drying of malachite green. The slide racks, containing the microscopic slides, were then placed on the steaming water bath for 5-10 minutes, using a 85-90°C water bath whilst making sure that the paper towels or the smears did not dry on the slides. The slides were then washed thoroughly with water. A safranin (0.1% w/v) counterstain was applied for one minute, and rinsed with water. Washing with water before applying safranin removes the malachite green dye bound to the vegetative cells, but not to the spores. The spores therefore retain the green stain, whereas vegetative cells do not and stain red when safranin is applied, hence, the spores appear green against the red vegetative cells when observed under the microscope (Barrow and Feltham, 2003; Todar, 2008).

2.9.   Decontamination of Calf Skins

2.9.1.   Raw Materials

Calf skins, supplied by Latco Ltd., UK, were used as raw materials for the experiments conducted in this study. Calf skins were pre-soaked and soaked as shown in Table 2.1, and fleshed. The fleshed calf skins were salted using sodium chloride (NaCl) and stored until used. The salted calf skins were stored no longer than 6 months. The fleshed and salted calf skins were used for the trials (Section 2.11) as well as for the conventional (section 2.12.1) and BAT (Section 2.12.2) leather-making processes. Each calf skin was cut into 5 pieces, weighing 100±10 g and, used for the conventional and BAT leather-making processes. These calf skin pieces used for the leather-making processes will be referred as the 'experimental samples', in this instance. Calf skin pieces, weighing approximately 25 g, were used for the trials and will be referred as the 'trial samples' (Section 2.11).

Table 2.1: Pre-soaking and soaking of the calf skins used for the experiments in this study.

Process	Chemicals	Amount (% w/v)*	Time (minutes)
Pre-soaking	Water	300	60
	Truposept BA	0.2	
Soaking	Water	300	120
	Corilene®W385	0.2	
	Truposept BA	0.2	

*Note: Percentage of the added chemicals was based on the weight of the salted calfskin pieces.

2.9.2. *Decontamination of Calf Skins using Sodium Hypochlorite*

Experimental and trial samples were decontaminated using a chlorine-based disinfectant, sodium hypochlorite. A 0.125 g/ml sodium hypochlorite (NaClO) solution was prepared by diluting 125 g/l stock solution (Fisher Scientific, UK) using deionised water. The decontamination procedure was carried out in a category II cabinet, which provides a sterilised environment and limiting the risk of microbial contamination through air. Calfskin pieces were placed in sterilised beakers and washed twice with sterilised tap water to remove salt and extraneous matters. The experimental calfskin pieces were immersed in sterilised water and stored overnight in a refrigerator at 4°C.

Sodium hypochlorite (0.125 g/ml) was added to each of the experimental samples and manually agitated for 3 minutes. The procedure was repeated twice. Presence of NaClO at a high concentration may hinder the growth of inoculated bacterial species. The sodium hypochlorite-treated calfskin pieces were therefore washed until the total chlorine concentrations in the water after the washings were below 0.2 mg/ml. A chlorine test kit (Chlor-test Reflectroquant®Plus 00109888/462, Merck, France) was used to measure the total chlorine concentrations. It was observed that the total chlorine concentration was reduced below 0.2 mg/ml following 15-20 washes with sterilised water. Water from the final washing cycle was collected and enumerated on respective selective solid media (see Section 2.5.1, 2.5.2 and 2.5.3) using a spiral-plater (see Section 2.2).

The flesh side of the decontaminated calf skin pieces was swabbed after the final washing cycle, in order to determine the presence of viable microbial colonies. The swabs were placed in a sterilised 5 ml bottle containing 2.5 ml PBS solution (Oxoid, U.K), and agitated using a vortex at a high speed for 20 seconds before plating (see Section 2.14.1). The last washed water and swabs were plated within 24 hours of collection, using a spiral-plater (see Section 2.2.). The decontaminated skin samples were re-immersed in sterilised tap water stored overnight in a refrigerator at 4°C. This was carried out to assist further reduction of the NaClO concentration absorbed by the calf skins and to aid effective time management. The following day the samples were rinsed with sterilised tap water before inoculating with the selected bacterial species on the flesh side of the calf skins (see Section 2.10).

2.10. Bacterial Inoculation

2.10.1. Bacterial Inoculation on Calf Skin Pieces for Trials and the Leather-Making Process Experiments

Samples of the bacterial culture for inoculation were collected from the mid-exponential phase. The time required to reach the mid-exponential phase was determined by plotting growth curves for each of the bacterial species (see Sections 2.4). A pure culture of a particular bacterial species was incubated overnight in TSB (Oxoid, UK) at a required temperature (see Section 2.5.1, 2.5.2 and 2.5.3). The microbial culture was mixed using a vortex at a high speed for 10 seconds and an aliquot was transferred to a 12 ml bottle containing TSB, to prepare a 10^{-1} dilution. The diluted sample was then incubated at a required temperature (see Sections 2.5.1, 2.5.2 and 2.5.3) until the mid-exponential phase (Section 2.4). The bacterial culture in the mid-exponential phase was agitated using a vortex at a high speed for 10 seconds and 4 ml aliquot was inoculated on the flesh sides of the calfskin pieces. The trial samples were inoculated with 1 ml aliquot of the bacterial culture in the mid-exponential phase. The fibre structure of the grain side of a hide or skin is compact and dense while the fibre structure of the flesh side is less compact than the grain side (Leafe, 1999). The bacterial culture was therefore inoculated onto the flesh side of calf skin pieces as the compact fibre structure of the grain side may hinder bacterial penetration into the skin samples. The total number of bacterial colonies inoculated on the calf skin pieces were estimated as described in Section 2.10.2. The inoculated calf skin pieces were incubated overnight at a required temperature (see Sections 2.5.1, 2.5.2 and 2.5.3) in order to allow the inoculated bacterial species to adjust to the new environment.

2.10.2. Total Number of Inoculated Bacterial Colonies

Enumeration of the inoculated bacterial colonies was carried out in order to determine the number of total bacterial colonies inoculated on the calf skin pieces used for the conventional and BAT leather-making processes as well as for the trial experiments. An aliquot was collected from the bacterial culture that was used for the inoculation (see Section 2.10.1) and added to PBS to prepare 10^{-2} or 10^{-4} dilution. The diluted samples were inoculated on the corresponding selective media using a spiral-plater as described in Section 2.2. The inoculated plates were incubated for 24 hours at the required temperatures (see Sections 2.5.1, 2.5.2 and 2.5.3) and the number of the bacterial colonies appeared on the plates were counted and expressed as colony forming units (cfu)/ml.

2.11. Trials for Measuring Decontamination of Calf Skins and the Growth of Bacterial Species on the Decontaminate Calf Skins

The trials were carried out before conducting the conventional and BAT leather-making process experiments in order to observe the survival of the bacterial species (*B. cereus, S. aureus* and *P. aeruginosa*) on the sodium hypochlorite-treated calf skin pieces. Trial experiments were undertaken in triplicate for each of the above-mentioned bacterial species. Fleshed and salted calf skin pieces (see Table 2.1), approximately 25 g, were decontaminated using NaClO as described in Section 2.10.1. The trial samples were washed 5 times with the sterilised tap water. Water from the final washing cycle was collected and the flesh side of the treated calf skin pieces were swabbed. Water and swabs were stored in a refrigerator at 4°C and were analysed within 24 hours of collection to determine the presence of the microbial colonies. The swabs were placed in 2.5 ml sterilised PBS and vortexed at a high speed for 20 seconds before analysis (see Section 2.14.1). Water and swabs after final washing cycles were inoculated on the respective selective media (see Sections 2.5.1, 2.5.2 and 2.5.3) in triplicate using a spiral-plater (see Section 2.2).

The decontaminated and washed calf skin pieces were inoculated with 1 ml bacterial culture from the mid-exponential phase (see Section 2.4). The total number of the inoculated bacterial colonies was determined as described in Section 2.10.2. The inoculated calf skin pieces were incubated overnight at required temperatures (see Sections 2.5.1, 2.5.2 and 2.5.3) to allow the bacterial species to adjust to the new environment. Sterilised water, 300% w/v was added to the overnight-incubated calf skin pieces and incubated further in a 25°C water bath at a speed of 100 rpm for an hour. The water samples were collected after the incubation and the flesh side of the calfskin pieces were swabbed. Water and swab samples were stored in a refrigerator at 4°C and analysed within 24 hours. Water samples were diluted (see Section 2.14.1) as required and based on the initial concentrations of bacterial colonies. Swabs (1 from each calfskin piece) were placed in 2.5 ml PBS, vortexed at a high speed for 20 seconds before analysis (also see Section 2.14.1). The samples were inoculated on the respective selective media (see Section 2.5.1, 2.5.2 and 2.5.3) in triplicate using a spiral-plater (see Section 2.2). The inoculated plates were incubated for 24 hours at the required temperature (see Section 2.5.1, 2.5.2 and 2.5.3). Colonies that appeared on the plates were counted and recorded.

A trial was conducted using calf skin pieces (5), weighing approximately 100 g, in order to determine whether the decontamination procedure is effective for comparatively larger samples. Although the decontamination procedure is found to be effective, however, it was

found that 5 water washes, following the NaClO decontamination, may not have reduced the concentration of absorbed NaClO by the calf skins, to a minimal level in order to allow survival of the bacterial cells. The growth and proliferation of the inoculated bacterial colonies (*B. cereus*) was found to be inhibited in these instances.

However, growth and proliferation of the inoculated bacterial species occurred when the total chlorine concentration in the water after final washing cycle was lower than 0.2 mg/ml. A chlorine test kit (Chlor-test Reflectroquant®Plus 00109888/462, Merck, France) was used to measure the total chlorine concentrations. Therefore, decontaminated calf skin pieces were washed until the total chlorine concentration was below 0.2 mg/ml.

2.12. Conventional and BAT Leather-Making Processes

The conventional and BAT leather-making processes were carried out in a closed cabinet containing 6 removable trial drums. The drums were removed before the processing, washed thoroughly with tap water and sterilised at 121°C for 15 minutes using an autoclave to inactivate the microorganisms present in the drums and therefore to control microbial contaminations. Each of the decontaminated experimental calf skins were placed in an individual drum. Sterilised tap water was used during the leather-making processes in this study, in order to limit microbial contaminations through water. The pH of the effluent and room temperature was monitored. A control experiment was carried out during the conventional and BAT processes without the addition of calf skin pieces, whilst water and all the chemicals used for the tanning processes, were added. The experimental and the control samples were carried out following the same method at the same time.

The conventional and BAT leather-making processes were established based on the recipes supplied by Together For Leather (TFL) (Germany), Southern Petrochemicals Industries Corporation Limited (SPIC) (India) and through personal communication with Chris Barnard, tannery technician, and Jeffry Guthrie-Strachan, senior lecturer at the British School of Leather Technology (BSLT), the University of Northampton, UK. Quantities of the used chemicals such as ammonium chloride, sulfuric acid and Sellatan®P were adjusted, if required, to obtain the required pH during the processing. The following stages of the conventional and BAT leather-making processes were carried out in this study: pre-soaking, soaking, unhairing, reliming, deliming, bating, pickling and chrome tanning.

2.12.1. Conventional Leather Manufacturing Process

A conventional leather-making process, to manufacture wet-blue (chrome-tanned leather) is shown in Table 2.2. Chemicals available at the BSLT, The University of Northampton, UK, were used for processing. A bactericide Truposept BA (sodium dimethyldithiocarbamate, Trumpler, Germany) was used during the pre-soaking and soaking process. Addition of a bactericide to control the bacterial population during the pre-soaking and soaking processes is a common practice. An anionic surfactant Corilene®W385 (sodium salt of alkyl ether phosphate) (STAHL Europe, The Netherlands), was used during the conventional soaking process. Surfactants are usually added during the soaking process to assist rehydration of the preserved hides and skins. Soaking processes are generally carried out for 5-24 hours depending on the condition of the preserved hides/skins (Heidemann, 1993). In this instance, an overnight soaking process was conducted. A conventional hair-burning unhairing process was carried out using sodium sulfide (Na_2S) and lime (calcium hydroxide-$Ca(OH)_2$) to degrade hair, followed by a reliming process with water and lime for 48 hours. The reliming process is generally carried out in tanneries particularly during the manufacture of soft leather, such as leather for garments and clothing accessories.

A deliming process was conducted using ammonium chloride (NH_4Cl), and the pH was adjusted to 8.5±0.5. A bating process was carried out using a pancreatic-based enzyme Oropon ON 2 (TFL, Germany) and was added to the deliming bath. A conventional pickling process was carried out using sulfuric acid (H_2SO_4), formic acid (HCOOH) and sodium chloride (NaCl). The pH during the pickling process was reduced to 2-3 to assist chrome penetration into the skins. The chrome tanning process was conducted using chrome(III) sulfate salt. Chrome(III) sulfate was added to the pickle liquid, and the tanning process was conducted for 90 minutes to achieve chrome penetration into the pickled pelts (unhaired skins). The pH was adjusted using a magnesium oxide-based compound Feliderm®MGO powder (previous name: Tanbase, Clariant, Switzerland).

Table 2.2: The conventional leather-making process that was followed in this study.

Process	Chemicals	Amount (%, w/w)*	Time	pH	Temperature
Pre-soaking	Water	300	60 mins	7.6 – 8.0	21.1 - 25.8°C
	Truposept BA	0.2			
Soaking	Water	300	120 mins and	7.6 – 8.1	21.5 – 25.6°C
	CorileneW385	0.2	left overnight		
	Truposept BA	0.2			
Unhairing	Water	100	20 - 24 hrs	11.1 - 12.7	21.2 – 27.8°C
	Sodium sulfide	2			
	Lime	1			
	Water	100			
	Sodium sulfide	1			
	Lime	1			
Washing	Water	300	10 mins		
Reliming	Water	200	72 hrs	12.5 - 12.6	20.6 – 25.8°C
	Lime	2			
Washing	Water	300	10 mins		
Washing	Water	300	10 mins		
Washing	Water	300	10 mins		
Deliming	Zero float			8.2 – 8.8	24.5 – 26.8°C
	Ammonium chloride	2	15 mins		
	Water	100	30-45 mins		
Bating	Oropon ON 2	0.1	30 mins	8.0 – 8.7	24.9 – 26.8° C
Washing	Water	300	10 mins		
Washing	Water	300	10 mins		
Pickling	Water	100	3-4 hrs and left overnight	1.5 – 3.1	20.4 – 26.6°C
	Sodium chloride	8			
	Sulfuric acid	1.2			
	Formic acid	1.0			
Chrome-tanning and Basification	Chrome(III) sulfate	8	90 mins	4.1 - 4.9	22.8 – 27.7° C
	Feliderm MGO Powder	0.4	4 hrs		

*Note: Percentage of the added chemicals was based on the weight of the salted calfskin pieces; mins=minutes and hrs=hours.

2.12.2. *Best Available Technology (BAT) Process of Leather Manufacturing*

The used chemicals were supplied by TFL, Germany, SPIC, India and BSLT, UK. Similar to the conventional pre-soaking process, the BAT pre-soaking was carried out using water and bactericide Truposept, BA. In addition, during the BAT soaking process a proteolytic enzyme-based product Pellvit C (TFL, Germany) was added to the soaking liquid, instead of adding a surfactant. Addition of a proteolytic enzyme to the soaking liquid assists the

rehydration of the hides/skins with a minimal environmental impact. Pellvit C is most active within the pH range of 8.5-11.0, therefore, sodium carbonate was added to the soaking bath in order obtained the required pH.

An enzyme-based hair-save unhairing method was followed (Aravubdhan *et al.*, 2007; Saravanabhavan *et al.*, 2005a, b and 2003a, b; Thanikaivelan *et al.*, 2004 and 2002) during BAT unhairing, to reduce the environmental pollution. A protease, unhairing enzyme (SPIC, India) with an active pH range of 6-11, was used in combination with a small amount of Na_2S and lime for unhairing (for the details of the recipe see Table 2.3). A soaked piece of skin was placed in a sterilised beaker containing water, enzyme, Na_2S and lime, mixed well for 3 minutes, covered with aluminium foil to avoid airborne contamination and incubated at 25°C for 18-24 hours.

Similar to the conventional process, the unhaired calf skin pieces were relimed using water and lime. The BAT deliming process was carried out using carbon dioxide (CO_2) instead of NH_4Cl to reduce nitrogen pollution load (see Sections 1.2.3.4, 1.2.4.3 and 1.2.5.3). Alpha amylase (SPIC, India), active pH range 6-8, was used during the BAT bating process. Use of NaCl was avoided during the BAT pickling process in order to reduce the salt pollution load (see Sections 1.2.3.6, 1.2.4.5 and 1.2.5.4). Formic acid and a non-swelling acid (aromatic polysulfonic acid-based chemical), Sellatan®P (TFL, Germany) were added to the pickle liquor. Similar to the conventional process the calfskin pieces were tanned using a chrome(III) sulfate and the pH adjusted using Feliderm®MGO Powder. The details of a BAT leather-making process is shown in Table 2.3.

Table 2.3: A BAT leather-making process that was followed in this study.

Process	Chemicals	Amount (% w/w)*	Time	pH	Temperature
Pre-soaking	Water	300	60 mins	7.8 – 8.2	23.1 – 28.7°C
	Truposept BA	0.2			
Soaking	Water	300	120 mins and left overnight	9.2 – 9.6	21.2 – 27.8°C
	Sodium carbonate	0.5			
	Pelvit C	0.5			
	Truposept BA	0.2			
Unhairing	Water	20	20 - 24 hrs	10.3 – 11.5	25°C
	unhairing enzyme	1.5			
	Lime	1.0			
	Sodium sulfide	0.5			
Manual unhairing					
Washing	Water	300	10 mins		
Reliming	Water	200	72 hrs	12.0 – 12.7	20.6 – 25.8°C
	Lime	2.0			
Washing	Water	300	10 mins		
Washing	Water	300	10 mins		
Washing	Water	300	10 mins		
Deliming	Water	50	30 mins	6.3 – 7.5	23.3 – 25.8°C
	Carbon dioxide gas	2.0			
Bating	Water	200	120 mins	6.9 – 7.5	25.5 – 25.8°C
	α -Amylase	1.25			
Washing	Water	300	10 mins		
Washing	Water	300	10 mins		
Pickling	Water	80	3-4 hrs and left overnight	2.3 – 3.1	29.5 – 26.6°C
	Sellatan P	2.0			
	Sodium formate	0.35			
	Formic acid	0.5			
Chrome-tanning and Basification	Chrome(III) sulphate	8	90 mins	4.2 – 4.7	24.0 – 27.7°C
	Feliderm MGO Powder	0.40	4 hrs		

*Note: Percentage of the added chemicals was based on the weight of the salted calfskin pieces; mins.=minutes and hrs.=hours

2.13. Sample Collection

Effluent samples (approximately 15 ml) were collected from the removable trial drums aseptically, and promptly, at the end of each stage of the beamhouse and tanning processes: pre-soaking, soaking, liming, reliming, deliming, bating, pickling and tanning process. The effluent was collected in 15 ml sterilised centrifuge tubes (Fisher Scientific, UK). Centrifuge tubes were completely filled with the effluents; this was carried out to limit air contact with the collected samples and so bacterial contamination. Effluent was collected

from each of the above-mentioned stages in duplicate. Hair samples were collected in duplicate from the unhairing effluents.

The flesh side of calf skin pieces were swabbed (in duplicate) at the end of each stage of the conventional and BAT leather-making processes after collecting the effluent. This will only provide with qualitative data, since the total number of bacterial colonies per unit area was not determined due to the potential for contamination and due to the impractical nature of the task. However, this was carried out in order to observe the presence of the inoculated bacterial species on the flesh side during the leather manufacturing processes. As no calfskin pieces were added during the control experiment, therefore, swab samples were not collected from the control experiments.

To collect swab samples, transport swabs (SWA3018, Scientific Laboratory Supplies Ltd., UK) were used, which consists of a charcoal-based microbial media to maintain bacterial samples during transport and storage. The microbial media of the transport swabs preserve the bacterial samples without damaging or assisting regeneration of the bacterial cells. Swabs were collected aseptically by rubbing a sterilised swab on the flesh side of the skin samples 50 times to ensure consistency throughout the experiments. Effluent samples and swabs were stored immediately in a refrigerator at 4°C and analysed within 24 hours of collection. Bacterial enumeration in effluent, swabs and hair was carried out as described in Section 2.14.

2.14. Bacterial Enumeration

2.14.1. Effluent and Swab Dilution

Effluent samples were collected from the pre-soaking and soaking processes and diluted using sterilised PBS solution. The samples were agitated by inverting the centrifuge tubes, containing effluent, five times. An aliquot was then transferred to a PBS solution, to prepare a 10^{-2} or 10^{-4} dilution (HPA, 2005b), the dilution ratio was based on the initial bacterial concentrations in the effluent.

Swabs were placed in a sterilised 5 ml bottle containing 2.5 ml PBS solution (Oxoid, U.K) and agitated using a "vortex mixture" at high speed for 20 seconds. This agitation will allow the bacterial cells in swabs to transfer to the PBS solutions, the PBS solutions were then analysed to determine the presence of microbial species (see Section 2.14.2).

2.14.2. Bacterial Enumeration in Effluent and Swabs

Bacterial enumeration in effluent was carried out promptly and aseptically in order to limit microbial contamination. The pre-soaking and soaking effluents, collected from both the conventional and BAT leather manufacturing processes, were diluted as described in Section 2.14.1. The diluted samples were mixed well using a "vortex mixture" at a high speed for 10 seconds and inoculated on the respective selective media (see Sections 2.5.1, 2.5.2 and 2.5.3) using a spiral-plater in duplicate (see Section 2.2).

The conventional and BAT unhairing effluents (10 ml) were centrifuged at 4°C, at 5000 rpm for 5 minutes. The centrifuged supernatant was inoculated without dilution on the respective selective media as mentioned above. The effluents collected from the conventional and BAT reliming, deliming, bating, pickling as well as chrome tanning processes were inoculated on the respective selective media without dilution using a spiral-plater as mentioned above.

The swabs from the flesh side of the calfskin pieces at the end of each of the conventional and BAT processes were diluted as described in Section 2.14.1. The PBS solutions (50 µl) were inoculated on the respective selective media (see Sections 2.5.1, 2.5.2 and 2.5.3) using a spiral-plater (see Section 2.2) in duplicate.

2.14.3. Bacterial Enumeration on Hair

Bacterial enumeration on hair was carried out in order to determine the presence of inoculated bacterial species in hair after the unhairing processes. The conventional and BAT unhairing effluents (10 ml) were dispensed in sterilised centrifuge tubes, 15 ml (Fisher Scientific, UK), and was centrifuged at 8000 rpm for 15 minutes to separate solid hair waste from the effluent. The supernatant was decanted carefully and TSB (10 ml) was added to the sediment (solid hair waste) and incubated overnight. An overnight incubation is thought to assist the recovery of damaged or stressed bacterial cells. However, this may also allow bacterial cells to multiply increasing the initial number of bacterial cells in solid hair waste. Therefore, determination of the initial or original number of the bacterial cells in hair may not be feasible following this method. These experiments were therefore used for qualitative analysis in order to determine the presence of the inoculated bacterial species in hair after the conventional and BAT unhairing processes. After incubation, the samples were mixed using a vortex for 10 seconds. The samples were maintained stationary for few minutes to allow settling of the hair. Liquid broths from the hair samples were decanted carefully and plated without further dilution. Each of the samples (50 µl) were inoculated in duplicate on

the respective selective media (see Sections 2.5 and 2.6) using a spiral-plater as described in Section 2.2.

2.15. Confirmatory Tests for *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Typical and atypical bacterial colonies were collected at various stages of the leather manufacturing processes. The collected mixed cultures were then purified to a single colony by streaking. The purified single colony was re-streaked in order to produce more colonies and to obtain sufficient bacterial colonies to perform the confirmatory tests. Presumptive *B. cereus*, *S. aureus* and *P. aeruginosa* were then subjected to the confirmatory tests.

2.15.1. Biochemical Tests

2.15.1.1. *Bacillus cereus*

A national standard method (HPA, 2005c) was followed to undertake the biochemical tests for confirmation of *B. cereus*. Typical *B. cereus* shows anaerobic growth, reduces nitrate to nitrite, ferments glucose but does not ferment arabinose, mannitol and xylose, and shows beta-haemolysis. *Bacillus cereus* ATCC11778 was used as a positive control for the biochemical tests.

- **Anaerobic Growth:** The anaerobic growth test was carried out in an oxygen-depleted environment. Bacterial colonies were streaked on Columbia Blood agar-based media (CM0331, Oxoid, UK) with 5% v/v horse or sheep blood (SR0050, Oxoid, UK) and placed in an airtight, approximately 2.5 l, anaerobic jar. A controlled anaerobic environment in the airtight jar was generated using Anaerocult®A, (cat: 1.13829, Merck, Germany) and incubated at 30°C for 18-20 hours. The following day the plates were examined to observe growth of the inoculated colonies.

- **Carbohydrate Fermentation Ability Test:** A sugar-free media Phenol-Red broth (P8976, Sigma-Aldrich, UK), was used for the fermentation test. Phenol-Red broth contains a pH indicator that changes colour from red to yellow when acid production takes place due to the fermentation of sugars by the inoculated microorganisms. An overnight incubated culture of *B. cereus* was collected using a 10 µl sterilised loop and transferred to 4 test tubes containing 5 ml of phenol red broth. Four separate discs of arabinose (80372, Sigma-Aldrich, Uk), dextrose (63367 Sigma-Aldrich, UK), mannitol (94438, Sigma-Aldrich, Uk) and xylose (07411, Sigma-Aldrich, Uk), were placed in each

of the test tubes and incubated at 30°C for 18-20 hours. The following day the test tubes were examined for colour transformation.

- **Nitrate reduction test:** Using a 10 µl sterilised loop, presumptive *B. cereus* colonies were inoculated in nitrate broth (B2676, Sigma-Aldrich, Uk) and incubated for 18-24 hours at 30°C. The following day the tubes were agitated, and 0.5 ml nitrate reagent A, 1-aminonaphthalene (5 g/1000 ml in 5N acetic acid) solution (38497, Sigma-Aldrich, UK) and 0.5 ml nitrate reagent B, p-aminobenzenesulfonic (8 g/1000 ml in 30% w/v acetic acid) solution (39441, Sigma-Aldrich, UK) was added to each tube. *Bacillus cereus* produces an enzyme called nitrate reductase, are capable of reducing nitrate to nitrite, which then reacts with sulfanilic acid to form a diazonium salt. The diazodium salt reacts with 1-aminonaphthalene and produces a red azo dye (Maria and Csaba, 1999).

- **Beta-haemolytic Test:** For beta (β)-haemolytic tests, an overnight incubated *B. cereus* (on PEMBA media) colony was streaked on Columbia Blood agar-based media with 5% v/v horse/sheep blood. The streaked plates were inverted and incubated for 18-24 hours at 30°C. *Bacillus cereus* produces haemolysins, which destroys the red blood cell (cell envelopes) and appears as a clear, colourless zone (see Figure 2.5) of haemolysis on blood agar (Barrow and Feltham, 2003). Figure 2.5 shows β-haemolysis of *B. cereus* on blood agar.

Figure 2.5: Beta haemolysis by *Bacillus cereus* colonies on blood agar (Todar, 2008).

2.15.1.2.   *Staphylococcus aureus*

Confirmatory tests of presumptive *S. aureus*, isolated at various stages of the leather manufacturing processes, were undertaken according to the national standard methods (HPA, 2005d). Typical *S. aureus* appears as cream or golden coloured colonies (up to 3 mm in diameter) on blood agar media and, produces enzymes DNase and coagulase. *Staphylococcus aureus* NCTC12981/ ATCC25923 was used as a positive control for the biochemical tests.

- **Growth on Blood Agar:** A colony of *S. aureus* was streaked on Columbia-Blood agar-based media with 5% v/v horse or sheep blood and incubated at 37°C for 24 hours. The following day the colour and colony size was compared with *S. aureus* NCTC12981/ ATCC25923 colonies.

- **DNase Production:** *Staphylococcus aureus* produce a DNA hydrolysing enzyme DNase. colonies were inoculated on DNase agar (CM0321, Oxoid, UK) and incubated for 24 hours at 37°C. The following day the inoculated plates were flooded with 1M hydrochloric acid and discarded after 30 seconds. Hydrochloric acid precipitates DNA and if the bacterial colonies produce enough DNase, the DNase will therefore hydrolyse the precipitated DNA and will be visible as a clear zone surrounding the bacterial colonies (HPA, 2005c). Figure 2.6 shows the hydrolysis effect of DNase on the precipitated DNA that is visible as a clear zone surrounding the bacterial culture.
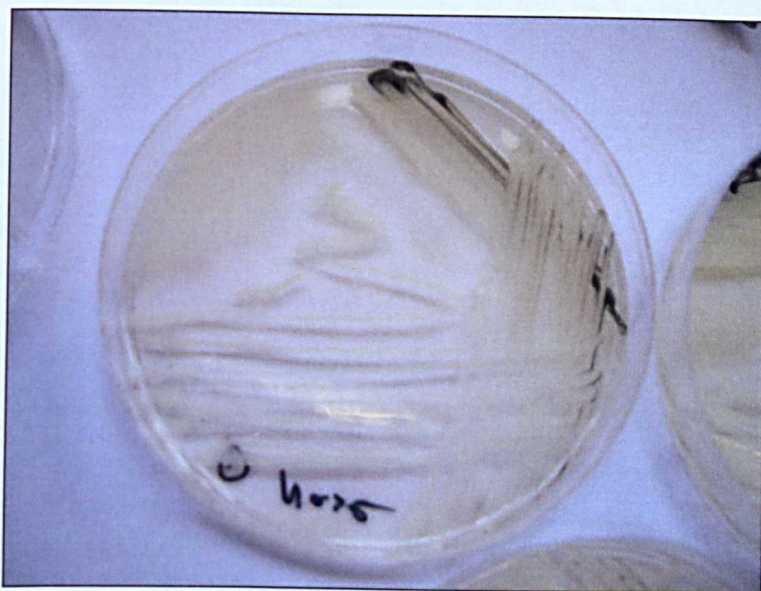
Figure 2.6: Precipitation of DNA (clear zone) due to the production of an enzyme DNase by *S. aureus*. Picture was taken using a Pentax Optio 57 (7.0 MP).

- **Coagulase Production:** A coagulase test was carried out using lyophilised rabbit plasma (74226 Sigma-Aldrich, UK). *Staphylococcus. aureus* colonies were collected from Baird-Parker media or Columbia-Blood agar-based media and mixed with 0.3 ml of hydrated rabbit plasma in 5 ml McCartney bottles. The bottles were incubated at 37°C and examined for coagulation after 2 hours, 6 hours and 24 hours by carefully tilting the bottles. *Staphylococcus aureus* produce an enzyme coagulase, which binds with a serum factor of plasma and convert fibrinogen to fibrin causing coagulation or clotting of the blood plasma (Madigan *et al.*, 1997; Murray *et al.*, 2005). *Staphylococcus aureus* NCTC12981/ATCC25923 showed the coagulation within 4 hours of incubation.

2.15.1.3. *Pseudomonas aeruginosa*

According to the national standard method, published by the HPA (2007), no further confirmation tests will be required if *P. aeruginosa* produces blue or green coloured colonies on the *Pseudomonas* CN agar media (see Sections 2.5.3 and 2.6.4). Alternatively, *P. aeruginosa* colonies that produce red or brown pigments (red or brown coloured colonies) require further confirmation. *Pseudomonas aeruginosa* isolated from the conventional and BAT leather-manufacturing processes, appeared as green coloured colonies on *Pseudomonas* CN media. Therefore, no further biochemical tests were carried out with the *P. aeruginosa* colonies, however, the Biolog Identification system (Section 2.15.2) was applied this instance.

2.15.2. *The Biolog System for Bacterial Identification*

The Biolog system was developed by Biolog Inc. (California, USA) and is generally used as an easier option of phenotypic identification (Klingler *et al.*, 1992). The Biolog technique of microbial identification is based on carbohydrate utilisation by microorganisms (Klingler *et al*, 1992; Sutton and Cundell, 2004). The tiny wells of a Biolog microplate consist of various media of specific carbohydrates and a redox indicator. The redox dye, tetrazolium, turns into purple colour if microbial growth occurs in a particular well representing catabolism of the substrate (Sutton and Cundell, 2004). The colour transformation of the dye is considered as a positive reaction. The Biolog microplates consist of 96 wells that contain 95 carbon sources and water. The well containing water is used as a control for the biolog tests. Different microorganisms use different carbon sources depending on their nutritional requirement; therefore, based on the positive and negative reaction, a species-specific signature can be produced (Solit, 2001). Samples for the Biolog identification method were prepared according to the manual guide provided by Biolog Inc., USA (Solit, 2001). A

Microlog™ 4.2 database was used to interpret the data obtained on the microplates. Figure 2.7 shows an image of a Biolog microplate indicating the positive results by the colour transformation.
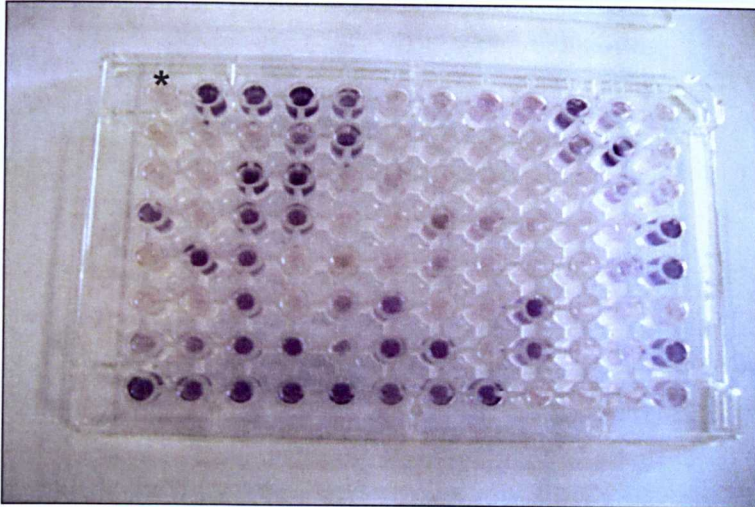
Figure 2.7: Photograph of a Biolog microplate. The microplate was inoculated with *B. cereus* and incubated at 30°C for 18 hours. Utilisation of a particular carbohydrate is indicated by the transformation of the indicator dye to purple. Colour transformation did not occur (colourless wells) when the bacterial species did not use a carbohydrate. Well number 1 (*) contains water and used as a control. Photograph was taken using Pentax Optio 57 (7 MP).

2.16. Preservation of the Bacterial Species

Bacterial samples were preserved in a cryopreservation tubes, Microbank™, (Porlab Diagnostics, UK) at -80°C.

2.17. Pulsed-Field Gel Electrophoresis

A pulsed-field gel electrophoresis (PFGE) was carried to analyse *B. cereus* and *P. aeruginosa* DNA, isolated at various stages of the conventional and BAT leather-making processes. The DNA band patterns obtained were then compared with *B. cereus* ATCC11778 and *P. aeruginosa* ATCC10145 DNA band patterns. For more information regarding PFGE see Chapter 4.

2.17.1. Restoration of the Lambda Ladder

A lambda ladder (48.5-970 kb) (Bio-Rad, UK), was used as a marker to standardise the size of the DNA fragments, when an electrophoresis was carried out. It was observed that the

ladder was not migrating through the agarose gel during the electrophoresis. Advice was sought by the suppliers (Bio-Rad, UK) to apply a restoration method before loading the lambda ladder into the agarose gel. The restoration method is described in the following paragraph.

Before electrophoresis a 1 ml thick slice of Lambda Ladder was cut, which was further cut into half. A disinfected scalpel was used to cut the Lambda Ladder. Lambda ladder pieces were then placed in a microcentrifuge tube and submerged completely in 0.5 ml of 0.5x (concentration) TBE buffer. The microcentrifuge tube was then placed in a heating block and heated at 45°C for ten minutes. The restored plug was either immediately loaded or placed on ice until loaded into an agarose gel.

2.17.2. *Bacillus* cereus

The PFGE protocol for *B. cereus* DNA sequencing was provided by Dr. Babetta L. Marrone and Yulin Shou, Los Alamos National Laboratory, Los Alamos (Ferris *et al.*, 2004; Zhong *et al.*, 2007). However, the protocol was modified for this study. *Bacillus cereus* colonies for the experiment were grown overnight on nutrient or PEMBA media. A single colony was added to 5 ml TSB and incubated for 14-16 hours in a 37°C water bath with an agitation speed of 200 rpm. The following day 5 µl of the overnight grown bacterial culture, was added to 5 ml fresh TSB and incubated for a further 4 hours in a water bath with an agitation speed of 200 rpm. The grown bacterial culture was centrifuged (centrifuge 5804R Eppendorf, Germany) at 3200 g and washed in 1 ml PET-IV buffer (10 mM tris-HCl (pH 7.5) 1 M LiCl at) at 8000 rpm. The centrifuged cells were suspended in 0.15 ml lysis buffer (6 mM tris-HCl (pH 7.6), 1 M LiCl, 100 mM EDTA, 0.2% w/v deoxycholate, 0.5% w/v sodium lauryl sarcosine). A 0.7% w/v low melting point agarose (Bio-Rad, USA) was prepared in PET-IV buffer and dissolved by using a microwave. The melted, or dissolved, low melting-point agarose was stored and re-melted as required. A bacterial suspension, in lysis buffer, was agitated with 0.15 ml of the prepared molten agarose (approximately 50°C), dispensed in a plug mould (Bio-Rad, UK) and solidified on ice.

The plugs were placed in 2 ml microcentrifuge tubes (Fisher Scientific, UK). The microcentrifuge tubes were incubated overnight in 1 ml lysis buffer with lysozyme (2 mg/ml) in a 37°C water bath with an agitation speed of 200 rpm. The following day proteinase K (1 mg/ml) was added to each of the test tubes and incubated in a 55°C water bath for 2 hours without agitation. The test tubes were inverted occasionally (approximately every 30

minutes). To inactivate proteinase K, the plugs were washed 4 times for 30 minutes in 1 ml tris-EDTA buffer [10 mM tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] at 4°C.

Before digestion, approximately a 1 mm thick slice was cut from each of the plugs and rinsed in 200 μl of 1x (concentration) digestion buffer SH (33 mM tris acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol at pH 7.9), supplied by the corresponding company (Sigma-Aldrich, UK). Digestion was carried out using 20 units (10000 units/ml) of *SmaI* restriction enzyme (Sigma-aldrich, UK) in 200 μl 1x (concentration) SH buffer for 2 hours in a 25°C water bath with an agitation speed of 200 rpm.

A lambda ladder (48.5 - 970 kb) (Bio-Rad, USA) was used as a marker, which was restored in 0.5x (concentration) tris borate EDTA (TBE) buffer (Sigma-Aldrich, UK) as described in Section 2.22. A 1% w/v electrophoresis gel was prepared by melting 1 g certified megabase agarose (Bio-Rad, USA) in 100 ml of 0.5x (concentration) TBE buffer (pH 8.3), using a microwave. To stain bacterial DNA, 100 μl ethidium bromide (500 μg/ml) (Sigma-Aldrich UK) was added to the molten agarose in order to achieve a final concentration of 0.5 μg/ml. Digested plugs and Lambda Ladder were loaded into the 1% w/v agarose (Bio-Rad, USA) gel and sealed with a small amount of 1% w/v molten agarose in TBE. A CHEF-DR II system (Bio-Rad, USA) was used for the electrophoresis. The electrophoresis was carried out at 14°C using a 0.5x (concentration) TBE buffer at 6 V/cm for 20 hours with an initial switch time 2.2 seconds and a finial switch time 54.2 seconds. The gel was visualised under UV (336 nm) using a Gel-Doc system (Bio-Rad, USA) and photographed.

2.17.3.   *Pseudomonas aeruginosa*

Pseudomonas aeruginosa colonies were incubated overnight on nutrient agar. Bacterial cells were suspended in 5 ml PET-IV buffer (10 mM Tris-HCl (pH 7.5), 1 M NaCl). A spectrophotometer (CE 1011, Cecil Instrument Ltd., UK) was set at a wavelength of 600 nm and the bacterial cell density was adjusted to 1.5 using the PET-IV buffer. The bacterial suspension in PET-IV buffer (2 ml) was dispensed in a sterilised centrifuge tube (Fisher Scientific, UK) and centrifuged (centrifuge 5804R Eppendorf, Germany) at 8500 rpm for 5 minutes. The supernatant was removed carefully using a pipette and re-suspended in 2 ml of PET-IV buffer, this procedure was repeated once more. A 2% w/v low melting point agarose (Bio-Rad, USA) was prepared in water and dissolved completely using a hot plate with constant stirring. The prepared low melting point agarose was placed in a heating block at 50°C.

The bacterial cell suspension in PET-IV (0.5 ml) was mixed with 0.5 ml of the prepared 2% w/v low melting-point agarose (approximately 50°C). The mixed suspension was then transferred to a plug mould (Bio-Rad, UK) and solidified on ice. The plugs were incubated in 2.5 ml lysis buffer (6 mM tris, 0.1 M EDTA, 1 M NaCl, 0.5 % w/v Brij 58 (polyethylene glycol hexadecyl ether), 0.4% w/v sodium deoxycholate, 0.5% w/v sodium lauryl sarcosine, pH was adjusted at 6.4 using HCl) with lysozyme (1 mg/ml) for 24 hours at 37°C. The plugs were rinsed with sterilised distilled water and incubated with 2.5 ml proteolysis buffer (0.5 M EDTA (pH 8.2), 1% w/v sodium lauryl sarcosine) with proteinase K (50 µg /ml) for overnight at 50°C. The plugs were washed 4 times for 30 minutes with 2.5 ml of tris-EDTA (TE) buffer [10 mM tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. DNA plugs were stored in 5 ml TE buffer at 4°C and used within 5 weeks (Campana et al., 2004; Corona-Nakamura et al., 2001; Patzer and Dzierżanowska, 2007; Römling et al., 1994; Stępińska and Trafny, 2008).

Before digestion approximately 1 mm thick slices were cut from each of the plugs using a disinfected scalpel, placed in 100 µl 1x (concentration) digestion buffer SA, (50 mM Tris-HCl (pH 7.5), 100 mM sodium chloride, 10 mm magnesium chloride, 1 mM dithiothreitol) supplied by the corresponding company (Sigma-Aldrich, UK) and placed in a refrigerator for one hour at 4°C. Digestion was carried out using 15 units (10000 units/ml) of SpeI restriction enzyme (Sigma-Aldrich, UK) in 100 µl of 1x (concentration) SA buffer overnight at 37°C (Campana et al., 2004; Corona-Nakamura et al., 2001; Patzer and Dzierżanowska, 2007; Römling et al., 1994; Stępińska and Trafny, 2008).

A 1% w/v certified megabase agarose was prepared in TBE (0.5x concentration) buffer and 100 µl ethidium bromide (500 µg/ml) was added to the molten agarose. Digested plugs and lambda ladder (48.5-970 kb) were loaded into the gel and sealed with 1% w/v molten agarose. The lambda ladder (48.5-970 kb) was restored using 0.5% w/v TBE buffer as described in Section 2.22 before loading it in the gel. The gel was then placed in a clamped homogenous electric field (CHEF-DR II) system (Bio-Rad, USA). The electrophoresis was carried out at 14°C using 0.5 x (concentration) TBE buffer at 6 V/cm. The running time was 20 hours (block 1) with switch time ramped from 5 seconds (initial switch time) to 45 seconds (final switch time) and for a further 4 hours (block 2) with an initial switch time 45 seconds to a final switch time 90 seconds. The gel was visualised under UV (336 nm) using a Gel Doc system (Bio-Rad, USA) and photographed (Barekzi et al., 1999; Campana et al., 2004; Corona-Nakamura et al., 2001; Patzer and Dzierżanowska 2007; Römling et al., 1994; Stępińska and Trafny, 2008).

2.18.   Statistical Analysis

Total number of bacterial colonies were expressed as colony forming units per millilitre (cfu/ml) of the samples and transformed in to Log_{10} value. SPSS was used for data analysis One sample Kolmogorov-Smirnov test was used to determine whether the data distribution is normal. Parametric tests such as independent T-test and ANOVA (with Post Hoc tests) were carried out to compare means between groups if the data were found to have normal distribution following one sample Kolmogorov-Smirnov test. On the other hand non-parametric tests such as Mann-Whitney test and Kruskal-Wallis test were carried out if the data distribution was skewed. Values of $p \leq 0.05$ were accepted as statistically significant. Microsoft Excel was used for creating graphs and charts.

Chapter 3: Preliminary Investigation and Optimisation of the Analysis of Bacterial Proliferation in Tannery Effluent

3.1. Introduction

Hides and skins may be contaminated with a variety of microorganisms. Various types of bacterial and fungal species have previously been isolated from hides/skins and leather at various stages of the leather manufacturing processes (Anderson, 1945; Birbir and Ilgaz, 1996; Hanlin et al., 1995; Kayalvizhi et al., 2008; Nigam, 1997; McLaughlin and Rockwell, 1922; Bitlisli et al., 2004; Özdilli et al., 2007; Oppong et al., 2006; Rawlings and Cooper, 1975). Microorganisms, present on the raw hides and skins are able to degrade protein and consequently the degraded raw materials may produce inferior quality leather (Heidemann, 1993; Kayalvizhi et al., 2008). Therefore, on most occasions the concern related to microorganisms in the leather industry is the quality of leather production.

Although, the majority of the isolated microorganisms from hides/skins and leather are non-pathogenic, pathogenic and opportunistic pathogenic microorganisms have also been found (Anderson, 1945; Birbir and Ilgaz, 1996; Bitlisli et al., 2004; Hanlin et al., 1995; Kayalvizhi et al., 2008; McLaughlin and Rockwell, 1922; Nigam, 1997; Oppong et al., 2006; Özdilli et al., 2007; Rawlings and Cooper, 1975;). Pathogens are present in the hides and skins and consequently their presence in the tannery effluent may infect tannery personnel, especially those with compromised immune systems (Brock and Madigan, 1991). Research by Özdilli et al. (2007) and Verma et al. (2001) showed that the microorganisms in the tannery environment may cause various health hazards among tannery workers. Microbial transmissions to humans occur through intact or compromised skin, inhalation, ingestion and direct contact with mucous membrane (Clesceri et al., 1998). Additionally microbial contamination of a water body may occur through the discharge of contaminated tannery effluents, and so be responsible for the spread of pathogens.

A conventional leather manufacturing process involves various chemical and mechanical operations. Approximately 500 kg chemicals may be required to process 1000 kg raw hides/skins (Integrated Pollution Prevention and Control (IPPC), 2001). The chemicals added during leather processing, particularly during the pre-tanning or beamhouse processes, may not remain in the hides/skins and therefore transfer to the effluent increasing the pollution load (Buljan et al., 1997). Moreover, many of the commonly used chemicals such as salts (ammonium salts or sodium chloride), sodium sulfide, tanning agents, biocides,

organic solvents, surfactants and dye are considered as hazardous chemicals and cause environmental pollution. Tannery effluents may exert a toxic effect on a variety of organisms such as bacteria, algae, fish and in some cases the human population (Taleb-Ahmed et al., 2005). The extreme environmental conditions during conventional leather-making processes may not assist the growth and survival of the microorganisms.

Various studies (Aravubdhan et al., 2007; Bass, 2007; Crispim and Mota, 2003; Gousterova et al., 2005; Hana, 2003; Marsal et al., 2002; Nazer et al., 2006, Paul et al., 2001; Saravanabhvan et al., 2005a, b, 2004 and 2003a, b; Thanikaivelan et al., 2001-2004) have been carried out in order to develop clean or cleaner technologies to reduce the pollution load during leather manufacturing. These clean and cleaner technologies are also known as best available technologies (BAT). Environmental pollution occurs due to the addition of certain hazardous chemicals and their consequent presence in the tannery effluent. The replacement of the hazardous chemicals with non-hazardous chemicals may reduce pollution levels. For example, replacing sodium sulfide with enzymes reduces sulfide pollution (He et al., 2005, Frendrup, 2000; IPPC, 2001; Thanikaivelan et al., 2004). Replacement of hazardous chemicals with non-hazardous chemicals may also provide suitable conditions for microbial growth.

Growth and survival of microorganisms, particularly pathogenic/opportunistic pathogenic bacteria related to health issues, at various stages of the leather manufacturing processes have not been investigated. Since it is assumed and accepted that the conventional unhairing process with sodium sulfide and lime destroys most of the microorganisms/pathogens on hides/skins, therefore unhaired/limed hides/skins and unhairing/liming effluents are considered low risk where pathogens are concerned (Biosecurity, New Zealand, 2008; Department of Agriculture, Fisheries and Forestry (DAFF), Australia, 2001). Birbir and Ilgaz (1996) showed that a conventional lime-sulfide unhairing process causes a considerable reduction in the number of bacterial colonies present on the hides. In addition, pickling and chrome tanning processes, during the leather-making process may also effectively inhibit microbial growth (Biosecurity, New Zealand, 2008; DAFF, Australia, 2001). Chrome-tanned hides and skins are considered free from pathogens and carry no risk of microbial hazard (Biosecurity, New Zealand, 2008; DAFF, Australia, 2001). On the other hand, the BAT processes of leather-making are still under development, issues regarding pathogens, if hazardous chemicals are replaced by non-hazardous chemicals, requires investigation.

Therefore, in this study, preliminary experiments were carried out in order to investigate the presence of certain microbial species such as *Bacillus* spp., *Coliforms* and *Salmonella* spp. in the effluent during the BAT leather manufacturing process. *Coliform* is a group of Gram-negatives, non-spore forming rod-shaped bacteria, which includes *Citrobacter* spp., *Enterobacter* spp., *Escherichia* spp., *Hafnia* spp., *Klebsiella* spp., *Serratia* spp. and *Yersinia* spp. Coliform is mainly non-pathogenic and used as indicator bacteria to assess water quality (Clesceri *et al.*, 1998; Environment Agency, 2002a and b). The presence of *Salmonella* spp. is also determined to assess water quality. As the *Bacillus* species are the most common species isolated from hides/skins, therefore, presence of *Bacillus* spp. In effluents collected from a BAT leather-making processes was investigated. Total bacterial count in effluents was also carried out.

3.2.   Aims and Objectives

- To study the effect of the BAT leather-making processes on the bacterial growth:
  - Analysis of the effluent that was collected from the various stages of the BAT leather-making processes in order to determine the presence of total *Coliform*, *Bacillus* spp. and *Salmonella* spp.
  - To measure the number of the total bacterial colonies in the effluent at various stages of the BAT leather-making process.

- Modification and optimisation of the experimental methods, such as the beamhouse processes and procedures for bacterial growth, enumeration and identification based on the results obtained during the preliminary investigation.

3.3.   Materials and Methods

3.3.1.   Optimisation of Agar for Total Bacterial Count

An experiment was conducted in order to determine the most suitable growth media for total bacterial count. Four different solid media: nutrient agar (NA), water plate count agar (WPCA), plate count agar (PCA) and tryptone soya agar (TSA) was selected (Clesceri *et al.*, 1998; Hanlin *et al.*, 1995; Rawlings and Cooper, 1975). The details of the chemical formula of the above-mentioned media are given in Appendix 1. The experiment was carried out using 2 cm^2 calf skin pieces in triplicate. These pieces were obtained from the same area (butt) of the same calf skin in order to ensure equitable results.

Each calf skin piece was placed in a sterilised 50 ml centrifuge tube (Fisher Scientific, UK) containing 20 ml sterilised phosphate buffered saline (PBS) solution, pH 7.4±0.2 (Oxoid, UK). Sterile PBS, 20 ml in a 50 ml centrifuge tube was used as a control. Calf skin pieces and the control were incubated for 24 hours at room temperature using an orbital shaker with an agitation speed of 100 rpm. The following day, the samples were agitated using a vortex at high speed for 10 seconds and an aliquot was added to the sterilised PBS solutions to a prepare 10^{-4} dilution. The control sample was not diluted.

The diluted samples and the control sample were vortexed at high speed for 10 seconds and inoculated on 5 petri-dishes containing each of the four media (NA, WPCA, TSA and PCA) using a spiral-plater (see Section 2.2). The petri-dishes were incubated at 37°C for 24 hours. The following day, the bacterial colonies that appeared on the petri-dishes were counted and recorded. The results obtained are given in Section 3.4.1.

3.3.2.   *Microbial Enumeration in Sterilised Water and PBS*

An experiment was undertaken in order to examine the presence of microorganisms in the sterilised water and PBS. Deionised water, tap water and PBS were sterilised by autoclaving at 121°C for 15 minutes. Non-selective media promote the growth of most of the microorganisms. Therefore, sterilised water and PBS, were inoculated on a non-selective nutrient media, TSA, in triplicate using a spiral-plater (see Section 2.2). The inoculated plates were incubated at 37°C for 24 hours; a further 24 hours incubation was carried out if no microbial growth occurred. The results obtained are given in Section 3.4.2.

3.3.3.   *BAT Leather-Making Processes*

Trials were carried out in order to enumerate *Bacillus* spp., *Coliform*, *Salmonella* spp. as well as total bacterial colonies in the effluent at various stages of the BAT process. Fleshed and salted calf skins (see Section 2.9.1) were cut into 5 pieces. Each of the calf skin pieces were placed in separate trial drums. The trial drums were washed with commercial bleach and rinsed with water for 3 times before placing the calf skin pieces in the drums. The recipe for the BAT leather-making process was established based on the recipes supplied by Together For Leather (TFL) (Germany), Southern Petrochemicals Industries Corporation Limited (SPIC) (India) and through personal communication with Chris Barnard, tannery technician, and Jeffry Guthrie-Strachan, senior lecturer at the British School of Leather Technology (BSLT), the University of Northampton, UK. The BAT process was carried out in the tannery situated in BSLT, The University of Northampton, Northampton, UK.

The BAT process was carried out using tap water. A bactericide, Truposept BA, (sodium dimethyldithiocarbamate, Trumpler, Germany) was added to control the bacterial growth during the pre-soaking and the soaking process, which is common practice in the tannery. A proteolytic enzyme-based compound Pelvit C (TFL, Germany) was used during the soaking process to assist rehydration of the preserved skins. Sodium carbonate (Na_2CO_3) was added to the soaking liquor to adjust the pH required for the enzyme (Pelvit C) activity, which is 8.5-11. A hair-save unhairing method was carried out using a protease-based unhairing enzyme with an active pH range of 6-11 (SPIC, India). Sodium sulfide and lime were also added during the unhairing process. The deliming process was carried out using carbon dioxide (CO_2) and the bating process was carried out using an enzyme α-amylase (active pH range 6-8) (SPIC, India). The pickling process was conducted using a non-swelling acid (modified aromatic amine-based compound) Picaltal®Flakes (BASF, Germany). The chrome tanning process was carried out using chrome(III) sulfate and the pH was adjusted using Feliderm®MGO Powder (previous name: Tanbase, Clariant, Switzerland). The details of the BAT process are given in Table 3.1. A control experiment was also conducted in this instance in the 6th trial drum, without addition of a calf skin piece, while water and all the chemical used for the tanning processes were added. The experimental and control samples were carried out following the same methods at the same time.

Table 3.1: Best available technology (BAT) leather-making process.

Process	Chemicals	Amount (%, w/v)*	Time	pH
Pre-soaking	Water	300	60 mins	7.8 – 7.9
	Truposept BA	0.2		
Soaking	Water	300	120 mins, left over	11.2 – 11.4
	Sodium carbonate	0.5	night	
	Pelvit C	0.5		
	Truposept BA	0.2		
Unhairing	Water	20	20 to 24 hrs	
	unhairing enzyme	1.5		
	Lime	1.0		
	Sodium sulfide	0.5		
Manual unhairing				
Washing	Water	300	10 mins	
Washing	Water	300	10 mins	
Washing	Water	300	10 mins	
Deliming	Water	50	30 mins	6.0 – 7.8
	Carbon dioxide	2.0		
Bating	Water	200	120 hrs	6.8 – 7.0
	α -amylase	1.25		
Washing	Water	300	10 mins	
Washing	Water	300	10 mins	
Pickling	Water	80	3-4 hrs, left over	2.8 – 2.9
	Picaltal Flakes	2.5	night	
	Sodium formate	1.0		
	Formic acid	0.25		
Chrome-tanning and	Chromium(III) sulfate	8.0	90 mins	3.9 – 4.1
Basification	Feliderm MGO Powder	0.40	180 – 240 mins	

*Note: Percentage of the added chemicals was based on the weight of the salted calf skin pieces, mins= minutes and hrs=hours

3.3.4.   *Sample Collection*

Effluent samples were collected at the end of the BAT pre-soaking, soaking, unhairing, deliming, bating, pickling and tanning processes using a sterilised 15 ml centrifuge tube. The centrifuge tubes were filled completely with effluent as to avoid air contact with the effluent and so limit bacterial contamination. The collected samples were placed in a cooling unit containing ice blocks, transferred to the microbiology lab as soon as possible and were analysed within 24 hours of collection.

3.3.5. Enumeration of Bacterial Species in Effluents Obtained from the BAT Leather Manufacturing Process

Effluents collected from the BAT leather-making process was inoculated on the required chromogenic selective media (Sections 3.3.5.2-3.3.5.4), and non-selective media (Section 3.3.5.1) in triplicate using a spiral-plater (see Section 2.2). Details of the chemical combination of the media used are given in Appendix 1. Selective media for a particular microbial species inhibits the growth of unwanted microorganisms promoting the growth of the particular microbial species (Pelczar *et al.*, 1993).

3.3.5.1. Total Bacterial Count

Effluent samples were enumerated on TSA media (CM0131, Oxoid, UK) media in order to measure the total bacterial colonies in effluents. The inoculated plates were incubated at 37°C for 24 hours, followed by a further 24 hours incubation if no microbial growth was observed on the inoculated plates. The number of colonies that appeared on the plates were counted and recorded. The results obtained are given in Section 3.4.3.1.

3.3.5.2. *Bacillus* Species

Bacillus species were enumerated on chromogenic brilliance *Bacillus cereus* agar (BBCA) (CM1036, Oxoid, UK) media, in combination with a brilliance *Bacillus cereus* selective supplement (SR0230, Oxoid, UK). The inoculated BBCA plates were incubated at 30°C for 24 hours and a further 24 hours incubation was carried out if no bacterial growth was observed on the inoculated plates. Bacterial colonies that appeared as of blue or blue-green were counted as *Bacillus* spp. (see Section 3.4.3.2 for the obtained results).

3.3.5.3. *Salmonella* Species

Salmonella species were enumerated on *Salmonella* chromogenic agar base (CM1007, Oxoid, UK) with supplements cefsulodin and novobiocin (SR0194, Oxoid, UK). The inoculated plates were incubated at 37°C for 24 hours and a further 24 hours incubation was carried out if no bacterial growth occurred on the inoculated plates. The colonies that appeared as magenta were counted as *Salmonella* spp. (see Section 3.4.3.3 for the results).

3.3.5.4. Total *Coliform*

Effluent samples were inoculated on *E. coli/Coliform* chromogenic agar base media (CM0956, Oxoid, UK) to enumerate total *Coliform*. The inoculated plates were incubated at 37°C for 24 hours and a further 24 hours incubation was carried out if no bacterial growth occurred on the inoculated plates. The *E. coli/Coliform* chromogenic agar base medium is a selective as well as a differential medium for *E. coli* and *Klebsiella* spp. *Klebsiella* spp. appears as pink-or maroon-coloured colonies whilst *E. coli* appears as blue coloured colonies on the inoculated media. The number of *E. coli* and *Klebsiella* colonies that appeared on the media were counted and recorded (see Section 3.4.3.4 for the results).

3.3.6. Optimisation of Solid Media for <u>Bacillus cereus</u>

During the preliminary experiments *Bacillus* spp. was enumerated on the BBCA media (see Section 3.3.5.2), as all the selective media selected to enumerate microorganisms during the preliminary experiments were chromogenic agar-based media. However according to the national standard method, Polymixin pyruvate egg yolk mannitol agar (PEMBA) is the recommended media for *Bacillus cereus* (Health Protection Agency (HPA), 2005c). A trial was therefore carried out in order to determine the most suitable media between BBCA and PEMBA for *B. cereus* enumeration.

Bacillus cereus ATCC11778 (Oxoid, UK) was incubated overnight in tryptone soya broth (TSB) (CM0129, Oxoid, UK) and, 10^{-2} and 10^{-4} dilutions were prepared in PBS using the overnight-incubated culture. Sample dilution was carried out in triplicate. Each diluted sample was inoculated in duplicate on PEMBA and BBCA media, using a spiral-plater (see Section 2.2), and incubated overnight for 24 hours at 30°C (HPA, 2005c). The following day the number of colonies that appeared on the media were counted and recorded. No significant difference was found (p>0.05) between the number of enumerated colonies on the BBCA and PEMBA media. For details of the results, see Section 3.4.4. PEMBA media was used, since it was recommended by HPA (2005c) for the enumeration of *B. cereus*.

3.3.7. Optimisation of Disinfectants

Based on the results obtained during the preliminary experiments, it was decided to inoculate calf skins with known microbial species and carry out a conventional as well as a BAT leather-making processes using the inoculated calf skins to determine the effect of the leather-making processes on bacterial growth. Microorganisms already present on the skin

(skin microbial flora) may interfere with the inoculated microbial species. Therefore, decontamination of calf skins before inoculation of the known microbial species is required to remove skin microbial flora.

Fleshed and salted calf skin pieces (see Section 2.9.1), approximately 25 g each, were used for this trials. Calf skin pieces were washed with tap water to remove salt and other adhered dirt, before treatment with disinfectants. The decontamination trials were carried out in a Category II cabinet in order to avoid airborne contamination.

3.3.7.1.   Ethanol

A trial was carried out using a calf skin piece in order to observe the effect of decontamination with 70% w/v ethanol. A calf skin piece was washed with 70% w/v ethanol 3 times for 3 minutes each time. The treated calf skin piece was washed with sterilised tap water five times, 3 minutes each time. Water after the final washing cycle was collected and inoculated in triplicate on PEMBA media using a spiral-plater (see Section 2.2). The flesh side of the calf skin piece was swabbed and the swabs were placed in a sterilised bottle containing 2.5 ml PBS, vortexed at high speed for 20 seconds before inoculating on PEMBA media in triplicate using a spiral-plater (see Section 2.2). The inoculated plates were incubated at 30°C for 24 hours and incubated for a further 24 hours if no bacterial growth occurred on the inoculated plates. Due to satisfactory decontamination with 70% w/v ethanol, the experiment was repeated in triplicate to ensure the reliability of the results obtained. Bacterial growths in water and swabs after the final washing cycle were examined.

A trial was undertaken in order to determine the growth and survival of the bacteria on the ethanol-treated calf skin pieces, when inoculated with a known bacterial species. *Bacillus cereus* ATCC11778 was incubated overnight in TSB. The ethanol-treated calf skin pieces were inoculated with the overnight-incubated culture (1 ml) of *B. cereus* and incubated overnight at 30°C to allow the bacterial species to adjust to the new environment. The number of total inoculated colonies was determined as described in Section 2.10.2.

The following day sterilised water, 300% w/v, was added to each of the calf skin pieces. A further incubation was carried out for an hour in a 25°C water bath with an agitation speed of 100 rpm. This was carried out in order to imitate the pre-soaking process, since the pre-soaking process in this study was carried out for 1 hour using 300% w/v water. After incubation, water samples and swabs (from the flesh side of the calf skin pieces) were obtained. The collected samples (water and swabs) were diluted as described in Section

2.14.1, and inoculated on PEMBA media in triplicate using a spiral-palter (see Section 2.2). The inoculated plates were incubated at 30°C for 24 hours. The number of blue-coloured *B. cereus* colonies (see Section 2.6.1) that appeared on the plates were counted and recorded. The results obtained are given in Section 3.4.5.1.

3.3.7.2. Sodium Hypochlorite

Trials were carried out in order to determine the most effective sodium hypochlorite (NaClO) concentration to decontaminate calf skins. A stock NaClO solution, 1.25 g/ml (Fisher Scientific, UK) was diluted using deionised water to prepare diluted NaClO solutions. Calf skin pieces (approximately 25 g) were placed in separate 250 ml conical flasks (sterilised) and treated with 0.125 g/ml, 0.25 g/ml, 0.375 g/ml, 0.5 g/ml, 0.625 g/ml, 0.75 g/ml, 0.875 g/ml, 1 g/ml and 1.25 g/ml concentrated sodium hypochlorite solutions. Calf skin pieces were washed with NaClO at various concentrations 3 times for 3 minutes each time. The treated calf skin pieces were washed with sterilised tap water five times for 3 minutes each time. The water and swabs, after the final washing cycle, was incubated on PEMBA media in triplicate using a spiral-plater (see Section 2.2). Before inoculation, the swabs were diluted by placing in a 5 ml sterilised bottle containing 2.5 ml PBS and vortexed at high speed for 20 seconds. The inoculated media was incubated for 24 hours at 30°C and incubated for a further 24 hours if no bacterial growth occurred on the inoculated media. Bacterial colonies appeared on the inoculated media were counted and recorded.

Bacillus cereus ATCC11778 colonies were incubated overnight in TSB at 30°C. The flesh side of the NaClO-treated and washed calf skin pieces were inoculated with 1 ml of the overnight-incubated *B. cereus*. The inoculated calf skin pieces were incubated overnight at 30°C to allow *B. cereus* colonies to adapt to the new environment. The following day the calf skin pieces were agitated and liquid samples was collected by pipetting. The flesh side of the calf skin pieces was swabbed as the bacterial species was inoculated on the flesh side. Collected sample (liquid samples and swabs) were diluted based on the initial concentration of the *B. cereus* cells (see Section 2.14.1) and inoculated on PEMBA media in triplicate using a spiral-plater (see Section 2.2). The inoculated plates were incubated at 30°C for 24 hours. The number of blue-coloured *B. cereus* colonies (see Section 2.6.1) that appeared on the inoculated plates were counted and recorded. An appropriate NaClO concentration to decontaminate calf skins was selected based on the following two criteria:

1. Lowest bacterial growth in the water and swabs after the final washing cycle following a NaClO treatment and wash, and
2. Highest recovery of the inoculated bacteria.

Based on the results obtained from the above trials, it was decided that a 0.125 g/ml concentrated NaClO solution is most suitable in this regard (see Section 3.4.6.2 for details). A trial was therefore carried out in triplicate to verify the effectiveness of decontamination with 0.125 g/ml NaClO.

Calf skin pieces (3 and each weighing approximately 25 g) were placed in three separate 250 ml sterilised conical flasks. Skin samples were treated with NaClO (0.125 g/ml) 3 times for 3 minutes each time. The NaClO-treated calf skin pieces were washed 5 times, 3 minutes each time, with sterilised tap water. The water and swabs, after the final washing cycle, were inoculated on PEMBA in triplicates using a spiral-plater (see Section 2.2). Before inoculation, the swabs were placed in a 5 ml bottle containing 2.5 ml PBS and vortexted at high speed for 20 seconds. The inoculated plates were incubated at 37°C for 24 hours and a further 24 hours incubation was carried out if no microbial growth occurred.

Decontaminated and washed calf skin pieces were then inoculated with the overnight-incubated culture of *B. cereus* (1 ml) in TSB. The number of total inoculated *B. cereus* colonies was determined (see section 2.10.2). The inoculated calf skin pieces were incubated overnight at 30°C. The following day 300% w/v sterilised deionised water was added to each conical flask and incubated in a 25°C water bath with an agitation speed of 100 rpm for an hour. Water and swab samples were collected after incubation followed by a dilution as described in section 2.14.1 and inoculated on PEMBA media in triplicate using a spiral-plater (see Section 2.2). The inoculated plates were incubated for 24 hours at 30°C. Blue-coloured colonies that appeared on PEMBA were counted as *B. cereus*. For the details of the results see Section 3.4.5.2.

3.3.8. *Microbial Enumeration in Sodium Hypochlorite (0.125 g/ml)*

A NaClO solution (0.125 g/ml) was prepared by adding non-sterilised deionised water to a stock NaClO solution (1.25 g/ml, Fisher Scientific). A trial was carried out to ensure the diluted NaClO (1.25 g/ml) solution was free from microbial contamination.

A diluted hypochlorite solution was inoculated in triplicate on TSA media using a spiral-plater (see Section 2.2), since TSA is a non-selective medium and therefore will allow the growth of majority of the culturable microorganisms. The inoculated plates were incubated at 37°C for 24 hours and incubated further 24 hours if no microbial growth occurred on the inoculated plates. The number of microbial colonies that appeared on the inoculated media (if any) was counted and recorded (see Section 3.4.6 for the results obtained).

3.3.9. Optimisation of Bacterial Enumeration on Hair

Hair collected from the conventional and BAT unhairing effluents (see Section 2.14.3 and Chapters 5, 6 and 7) was analysed in order to determine the presence of the inoculated microbial species. A trial was therefore undertaken to optimise the bacterial enumeration on hair. Bovine hair was used for this trial. Hair (0.10 g) was placed in two separate bottles (approximately 12 ml) containing 10 ml TSB. The samples were vortexted at high speed for 20 seconds, and incubated for 6 and 14 hours at 30°C. After incubation, the samples were vortexed for 10 seconds at high speed and inoculated on PEMBA media in triplicate using a spiral-plater (see Section 2.2) followed by a 24 hours incubation at 30°C. Blue-coloured bacterial colonies that appeared on the agar media were counted as *Bacillus* spp. The results obtained are given in Section 3.4.7.

3.3.10. Microbial Enumeration in Chemicals used for the Leather Manufacturing processes

This study was carried out to enumerate microorganisms in chemicals used for the conventional and BAT leather-making process. The chemicals analysed were as follows: Troposept BA (Trumpler, Germany), Corilene®W385 (STAHL Europe, The Netherlands), sodium carbonate, Pelvit C (TFL, Germany), lime, sodium sulfide, unhairing enzyme (SPIC, India), Oropon ON 2 (TFL, Germany), α-amylase (SPIC, India), sodium chloride, sodium formate, formic acid, sulfuric acid, Sellatan®P (TFL, Germany), chromium(III) sulfate and Feliderm®MGO Powder (Clariant, Switzerland). Each chemical (1 g) was weighed in sterilised bottles and dissolved in 1-5 ml sterilised PBS as required. If the prepared solution is too dense, it may congest the intake tube of the spiral-plater during inoculating on the agar media, therefore the prepared solution was diluted further ($10^{-1}/10^{-2}/10^{-4}$ dilutions). The solutions were mixed and vortexed at high speed for 20 seconds, inoculated on TSA in triplicate using a spiral-plater (see Section 2.2) and incubated at 37°C for 24 hours. The plates were examined for the appearance of the microbial colonies. A further 24 hours incubation was carried out if no bacterial growth occurred (see Section 3.4.8 for the results).

3.3.11. Microbial Enumeration in Unhairing Enzyme and α-Amylase

Unhairing enzyme and α-amylase (SPIC, India), which were used during the BAT unhairing and bating process respectively, were found to be contaminated with bacteria, during the previous trials (see Section 3.4.10). The total number of bacterial colonies/g enzymes (unhairing enzyme and α-amylase) was measure as described in Sections 3.3.11.1 and

3.3.11.2. Experiments were also carried out to identify the isolated bacterial colonies (see Section 3.3.11.3). The results obtained are given in Section 3.4.9.

3.3.11.1. Total Bacterial Count in Unhairing Enzyme

Samples were prepared in triplicate by adding 0.1 g unhairing enzyme to 10 ml sterilised PBS and agitated. The solutions were vortexed at high speed for 10 seconds, and an aliquot was added to prepare 10^{-2} dilutions in PBS. The dilutions were prepared in duplicate for each sample. The dilutions were vortexed at high speed for 10 seconds and inoculated on TSA media in duplicate using a spiral-plater (see Section 2.2). The inoculated plates were incubated for 24 hours at 37°C and bacterial colonies that appeared on TSA media were counted and recorded.

3.3.11.2. Total Bacterial Count in α-Amylase

Samples were prepared in triplicate by dissolving 1 g α-amylase in 10 ml sterilised PBS. Each enzyme solution was vortexed at high speed for 10 seconds and an aliquot was added to PBS to prepare a 10^{-2} dilution. Sample dilution was carried out in duplicate and dilutions were vortexed at high speed for 10 seconds followed by the inoculation on TSA media in duplicate using a spiral-plater (see Section 2.2). The inoculated plates were incubated at 37°C for 24 hours. The following day, bacterial colonies that appeared on the TSA media were counted and recorded.

3.3.11.3. Identification of the Bacterial Species Isolated from Unhairing Enzyme and α-Amylase

It was observed that the bacterial colonies that were isolated from unhairing enzyme and α-amylase have similar colony morphology. This suggest that both of Unhairing enzyme and α-amylase were manufactured using the same bacterial source. Bacterial colonies (5) enumerated from each of the enzymes (Unhairing enzyme and α-amylase) were purified by streaking on TSA media. The purified colonies were re-streaked on TSA in order to produce sufficient amount of the purified colonies for this experiment. Gram-staining and spore-staining method were followed as described in Sections 2.7 and 2.8 to stain the isolated bacterial cells and spores respectively. The stained bacterial cells were observed under a light microscope (Nikon Eclipse 600, Nikon, Japan) to examine the microscopic structure. The Biolog identification system (Biolog Inc., USA) (see Section 2.15.2) was applied to identify the isolated microbial colonies.

3.3.12. Removal of Bacterial Contamination from Enzymes through Filtration

Membrane filters are often used as a sterilisation technique to separate or to remove microorganisms from a solution (Pelczar *et al.,* 1993). A quick trial was carried out to examine the effectiveness of the membrane filtration to remove microorganisms from the enzyme solutions in this regard. Unhairing enzyme (1.25 g) was dissolved in deionised water (20 ml) and filtered through a 0.2 μm sterilised disposable membrane filter unit (Fisher Scientific, UK), using a vacuum pump. The enzyme solution before and after filtration was inoculated on TSA media in duplicate using a spiral-plater (see Section 2.2) to determine total bacterial colonies in enzyme before and after filtration. The inoculated plates were incubated at 37°C for 24 hours. The following day, bacterial colonies that appeared on the TSA media were counted and recorded (see Section 3.4.10 for results).

3.4. Results and Discussion

3.4.1. Optimisation of Agar for Bacterial Count

Figure 3.1 shows the average number of bacterial colonies, when calf skin pieces (2 cm^2) were incubated in sterilised PBS for 24 hours at a room temperature (see Section 3.3.1 for the detailed method). Figure 3.1 also shows the average number of bacterial colonies isolated from the control. The total number of replicates will be referred to as n in this study.
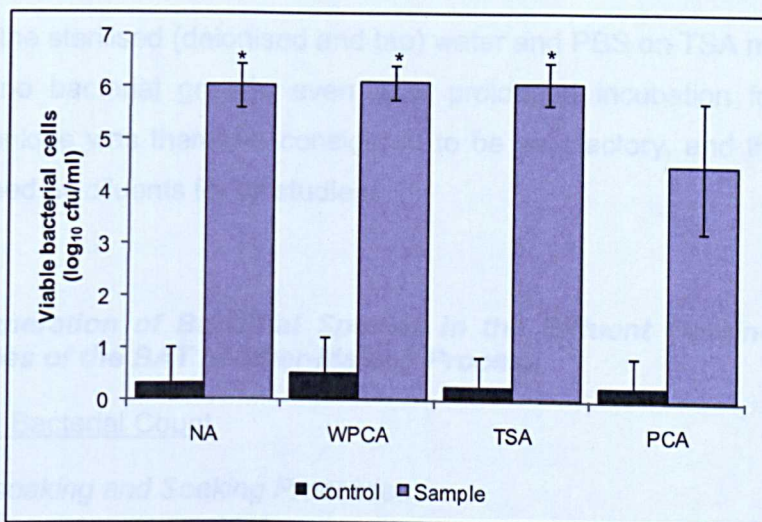
Figure 3.1: Total bacterial count on NA (nutrient agar), WPCA (water plate count agar), TSA (tryptone soya agar) and PCA (plate count agar), n=3. The error bars represent standard deviations, * indicates a significant difference when compared to PCA (F=18.126, p<<0.001).

Bacterial enumeration was carried out on nutrient agar (NA), water plate count agar (WPCA), tryptone soya agar (TSA) and plate count agar (PCA). Minimal bacterial growth

was observed in the control samples when compared with the experimental samples. Bacterial growth for the experimental samples were significantly higher ($p \ll 0.001$) when compared to the corresponding control samples (Table 3.2). The average number of bacterial colonies enumerated on PCA media was significantly lower ($F=18.126$, $p \ll 0.001$) than TSA, NA and WPCA media. On the other hand, no significant difference ($p > 0.05$) was obtained between the total number of bacterial colonies enumerated on NA, WPCA and TSA media. Therefore, any of the media studied (TSA, NA and WPCA) was considered to be suitable for total bacterial enumeration. However, TSA was available within the school (The School of Health, The University of Northampton, UK) as well as inexpensive and therefore it was decided to use TSA media for the enumeration of total bacterial colonies for various experiments conducted in this study.

Table 3.2: t, df and p values when the mean value of the bacterial count in the experimental samples were compared with the mean value of the corresponding control samples for the media studied.

Name of the Media	t	df	p value
Nutrient Agar (NA)	21.956	17	$\ll 0.001$
Water Plate Count Agar (WPCA)	16.986	4.550	$\ll 0.001$
Tryptone Soya Agar (TSA)	25.223	18	$\ll 0.001$
Plate Count Agar (PCA)	9.786	15.045	$\ll 0.001$

3.4.2. Microbial Enumeration in Sterilised Water and PBS

Enumeration of the sterilised (deionised and tap) water and PBS on TSA media (see Section 3.3.2) showed no bacterial growth, even after prolonged incubation for 48 hours. The sterilisation technique was therefore considered to be satisfactory, and the sterilised water and PBS was used as diluents for all studies.

3.4.3. Enumeration of Bacterial Species in the Effluent Obtained from Various Stages of the BAT Leather-Making Process

3.4.3.1. Total Bacterial Count

3.4.3.1.1. Pre-soaking and Soaking Processes

Figure 3.2 shows that the BAT pre-soaking process may provide suitable conditions for bacterial growth. An average of log 4.72 (± 0.53 (standard deviation), n=5) cfu/ml was isolated from the pre-soaking effluent during a BAT leather-making process. A significant reduction in the number of bacterial colonies from log 4.72 (± 0.53, n=5) to 3.45 (± 0.57, n=5) cfu/ml was observed during the BAT soaking process (t=6.338, df=28, $p \ll 0.001$). Bacterial growth was observed in the control samples during both of the pre-soaking and soaking

processes. Statistical analysis showed that bacterial growth in the pre-soaking effluent was significantly higher that its corresponding control sample (t=7.397, df=16, p<<0.001). On the other hand, although bacterial count in the soaking effluent was higher than its corresponding control sample, however, the difference was not statistically significant (t=2.933, df=2.118, p=0.093).
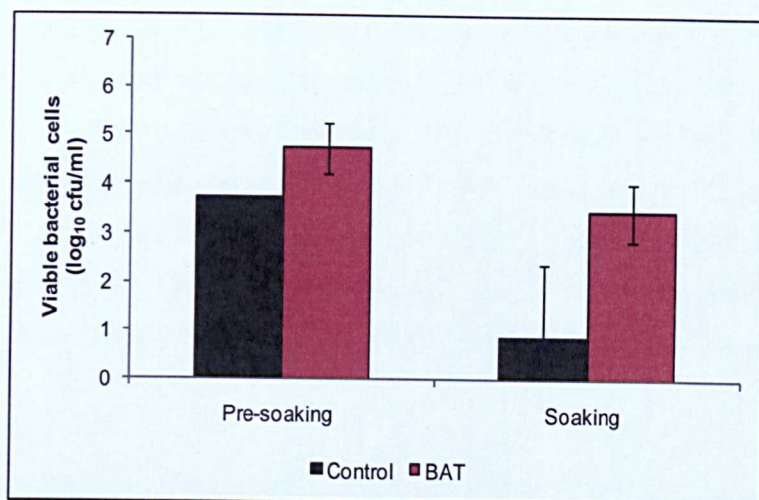
Figure 3.2: Total bacterial count in the BAT pre-soaking and soaking effluents, (n=5, t=6.338, d.f.=28, p<<0.001) the error bars represent standard deviation.

A 0.2% w/w of a bactericide Truposept BA (sodium dimethyldithiocarbamate) was added during the pre-soaking and soaking process. Bactericides are generally added during the pre-soaking and soaking process mainly to control the growth of microorganisms, which does not cause total inactivaton of the bacterial cells. Hides and skins are mainly composed of protein (33%), moisture and fat. Microorganisms are able to degrade the protein by proteolysis (Kayalvizhi et al., 2007). In addition, microorganisms, such as the species in genera *Bacillus* and *Pseudomonas* have lipolytic activity and are able to degrade fat or lipid (Loperena et al., 2009). Therefore, control of microbial growth during the leather-making process is important for the quality of leather production (Kayalvizhi et al., 2007). Bacterial concentration above 10^5 cfu/ml (=log 5.0), in the soaking effluent may affect adversely the quality of the leather produced (Rangarajan and Didato, 2003).

A proteolytic enzyme and Na_2CO_3 was also added during the soaking process. Addition of proteolytic enzymes during the soaking process promotes the rehydration of the preserved hides/skins. As biological catalysts, enzymes are considered to have less of an environmental impact (Thanikaivelan et al., 2004), and therefore may not inhibit bacterial growth. Sodium carbonate increases the pH of the soaking liquor, which not only aids in achieving the optimum pH range for enzyme activity, but also increases the water absorption

by the skins (Heidemann, 1993). Very little is known about the antimicrobial activity of Na_2CO_3. The toxicity of Na_2CO_3 towards microorganisms may depend on the pH (Lakhanisky, 2002) since the pH of an environment is an important factor for microbial growth and the majority of the bacteria prefer a neutral to slightly alkaline pH (6-8) for their growth and proliferation.

Moisture content is one of the vital factors for microbial growth. Use of salts (sodium chloride) during preservation reduces microbial numbers by reducing the moisture content in the raw hides/skins. Salt preservation may not completely eliminate microorganisms, particularly spore-forming microorganisms. The microorganisms may remain in the dormant state during salt preservation. During the pre-soaking and soaking process, the salt-preserved raw hides/skins gain moisture, which may encourage microbial growth and recovery (Biosecurity, New Zealand, 2008; DAFF, Australia, 2001; Rangarajan and Didato, 2003).

During processing, due to the mechanical agitation, microorganisms on the hides/skins may transfer to the effluent reducing the number of microbial cells on the hides/skins (Birbir and Ilgaz, 1996). This may be the reason why a lower number of bacterial colonies are found in the soaking effluent than the pre-soaking effluent.

As mentioned previously, the pH is an important factor for microbial growth. The pH of the pre-soaking effluent during the BAT process was 7.8-7.9, which is within the pH range required for the bacterial growth and therefore may aid the growth of the bacteria. On the other hand, the pH of the BAT soaking process was higher (10-11) due to addition of Na_2CO_3. A higher pH range during the BAT soaking process may have an adverse effect on the bacterial growth.

3.4.3.1.2. Unhairing, Deliming and Bating

The BAT unhairing process appears to reduce the number of bacterial colonies significantly from log 3.45 (±0.57, n=5) to 0.13 (±0.49, n=5) cfu/ml (Mann-Whitney Test: $U_{15, 15}$=0.00, p<<0.001) (see Figure 3.3). Due to the presence of lime and sodium sulfide (Na_2S) the pH of the BAT unhairing effluent was above 12.5, which was higher than the required range for bacterial growth. Moreover, both lime and Na_2S has antimicrobial properties (Al-Nazhan, 2002; Estrela *et al.*, 2003; Taleb-Ahmed *et al.*, 2005), and therefore, effectively reduce the number of bacterial colonies in the effluent. No bacterial growth was observed in the control sample during the unhairing process, but the difference between the experimental samples

and the corresponding control sample in this instance was insignificant (Mann-Whitney Test: $U_{15,3}=21.00$, p=0.655).

Bacterial growth was observed in control samples during both the deliming and bating processes. A lower bacterial count was observed in the deliming effluent that the corresponding control sample, however the difference was not statistically significant (t=-0.746, d.f.=8.299, p=0.476). A significantly higher bacterial growth was observed in the bating effluent than the corresponding control sample (Mann-Whitney Test: $U_{15,\ 3}=1.5$, p=0.005).
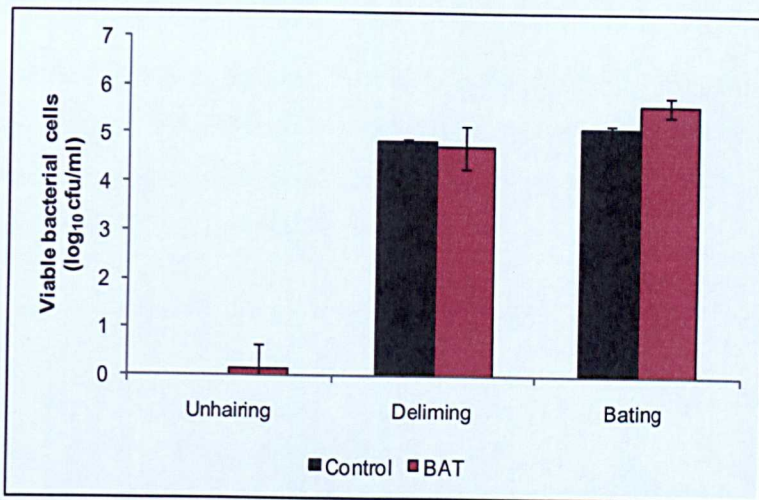
Figure 3.3: Total bacterial count in the BAT unhairing, deliming and bating effluents, n=5 for unhairing and bating processes, and n=4 for deliming process. The error bars represent standard deviation.

A significantly increased number of bacterial colonies from log 0.13 (±0.49, n=5) to 4.74 (±0.44, n=4) cfu/ml was observed during the subsequent BAT deliming process (Mann-Whitney Test: $U_{15,\ 9}=0.000$, p<<0.001) (see Figure 3.3). The results obtained from one of the samples were not included while calculated the mean as the bacterial colonies were overspread and therefore, count of the separate colonies were not possible. The BAT deliming process was carried out using CO_2. Many bacteria are able to utilise CO_2 as a carbon source, which is one of the essential elements for microbial growth and metabolism (Madigan *et al.*, 1997; Murray *et al.*, 2005). Moreover, CO_2 reduces the pH of the BAT deliming effluent (Ludvik and Buljan, 1998) providing a suitable pH range for bacterial growth (Cono and Colomé, 1986). In this instance, the pH of the BAT deliming effluent was 6.0-7.8.

A further significant increase from log 4.74 (±0.44, n=4) to 5.60 (±0.19, n=5) cfu/ml in the number of total bacterial colonies was observed during the BAT bating process (Mann-

Whitney Test: $U_{9,\,15}$=3.0, p<<0.001) (see Figure 3.3). Due to growth of numerous bacterial colonies on the inoculated media, the average number of enumerated colonies was estimated. Calf skin samples, after deliming were left in the drum overnight without any bactericide. Bacterial growth and multiplication may have occurred overnight. The pH of the BAT bating was 6.7-7.0, which was within the optimum pH range required for the growth of bacteria. An enzyme α-amylase was added to the deliming liquid. The presence of an available protein source and presence of a lower amount of hazardous chemicals may have promoted bacterial growth.

3.4.3.1.3. Pickling and Chrome Tanning Processes

Analysis of the control sample showed bacterial growth during the pickling and chrome-tanning processes (Figure 3.4). Bacterial count was found to be significantly lower in the control sample than the experimental sample during pickling (t=42.581, d.f.=16, p<<0.001) and chrome tanning (t=5.330, d.f.=14.542, p<<0.001).
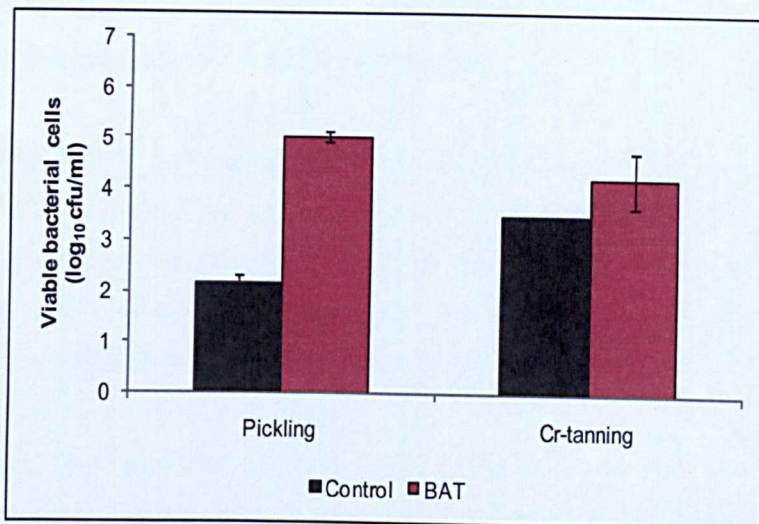
Figure 3.4: Total bacterial count in the BAT pickling and chrome (Cr) tanning effluent (n=5, t=5.713, d.f.=14.830, p<<0.001), the error bars represent standard deviation.

A significant reduction was observed in pickling (Mann-Whitney Test: $U_{15,\,15}$ = 2.0, p<<0.001) and chrome tanning (t=5.713, d.f.=14.830, p<<0.001) effluent. The number of bacterial colonies reduced from log 5.60 (±0.19, n=5) to 5.03 (±0.09, n=5) cfu/ml during the pickling process followed by a further reduction from log 5.03 (±0.09, n=5) to 4.22 (±0.54, n=5) cfu/ml during the chrome tanning process (see Figure 3.4).The pH of the pickling effluent was 2.8-2.9. It is less likely for the bacteria to survive at such a low pH, however in this instance it appears that the low pH did not prevent bacterial growth. Aromatic amines are toxic towards

microorganisms (Lu *et al.*, 2009; Martins *et al.*, 2009). In addition, microorganisms are sensitive towards chromium (Cr), particularly Cr(VI) (Chandra *et al.*, 2004). However, it appears that the presence of Cr did not affect bacterial growth.

3.4.3.2. *Bacillus* Species

3.4.3.2.1. Pre-soaking, Soaking and Unhairing Processes

Growth of *Bacillus* spp. did not occur in the control samples during the pre-soaking, soaking, unhairing processes. A low number of *Bacillus* spp., log 0.37 (±0.63, n=5) cfu/ml, were isolated from the pre-soaking effluent. No significant difference was obtained between the bacterial count in the pre-soaking effluent and the corresponding control sample (Mann-Whitney Test: $U_{15, 3}$=16.5, p=0.327). The lower *Bacillus* count in the pre-soaking effluent is probably due to the initial lower viable *Bacillus* colonies on the raw materials (calf skin pieces) used in this instance. The lower *Bacillus* count in the pre-soaking effluent may also be due to the presence of viable but non-culturable bacterial cells. As no bacterial growth was observed in the control as well as experimental samples except pre-soaking, therefore no statistical analysis was carried out in this instance.

Absence of *Bacillus* spp. in the soaking effluent was observed, which may also be due to the initial lower bacterial count on calf skins. The mechanical action during the processing may cause the microorganisms on the hides/skins to transfer to the effluent (Birbir and Ilgaz, 1996). Discharge of the pre-soaking effluent may therefore cause a reduction in the number of bacterial colonies isolated from the subsequent soaking process.

Absence of *Bacillus* spp. was also observed during the unhairing process. The antimicrobial activity due to the alkaline properties of lime and Na_2S may prevent bacterial growth during the unhairing process (Al-Nazhan, 2002; Estrela *et al.*, 2003; Taleb-Ahmed *et al.*, 2005). The toxicity of Na_2S is due to the strong reducing properties (Bajza and Vrcek, 2001; Tišler, 2004). The low initial *Bacillus* count on the calf skin pieces may also influenced the number of bacterial colonies in the unhairing effluent.

3.4.3.2.2. Deliming and Bating Processes

No bacterial growth was observed in the control sample during the deliming processes, while an average of log 0.83 (±1.12, n=5) cfu/ml was isolated from the deliming effluent (see Figure 3.5). However, the difference between the bacterial count in the control and

experimental samples was not significant (Mann-Whitney Test: $U_{15, 3}=13.5$, p=0.203). On the other hand, a significantly higher *Bacillus* growth was observed in the control sample than the experimental sample during the bating process (t=-4034, d.f.=15.883, p=0.001).

A significant increase in the number of *Bacillus* colonies, from log 0.83 (±1.12, n=5) to 3.08 (±1.06, n=5) cfu/ml was isolated from the bating effluent (Mann-Whitney Test: $U_{15, 15}=20.0$, p<<0.001) (Figure 3.5). The high standard deviation is due to the inconsistent growth of *Bacillus* spp. on the plates that were inoculated with the effluents. Reasons for the increased number of *Bacillus* colonies in the bating effluent are likely to be as follows: a suitable pH range, available nutritional source, a reduced amount of hazardous chemicals (Na_2S and lime), and an adequate time for the recovery of the stressed cells.
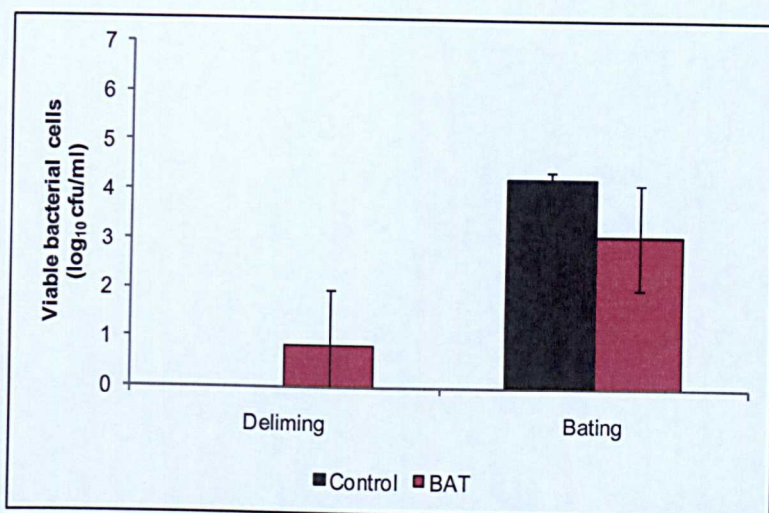
Figure 3.5: *Bacillus* **spp. in the BAT deliming and bating effluents (n=5, Mann-Whitney Test: $U_{15, 15}=20.0$, p<<0.001) the error bars represent standard deviation.**

3.4.3.2.3. Pickling and Chrome Tanning processes

Absence of bacteria was observed in the control sample during the pickling process, whilst *Bacillus* spp. growth was observed in the control samples during the chrome tanning process (Figure 3.6). Statistical analysis showed that the difference between the number of bacterial colonies obtained from experimental and respective control sample during the pickling process was not significant (Mann-Whitney Test: $U_{15, 3}=18.0$, p=0.412). Bacterial count in the chrome tanning effluent was significantly lower than the corresponding control sample (t=3.462, df=14.557, p=0.004).

A significant decrease in the number of *Bacillus* spp. from log 3.08 (±1.06, n=5) to 0.31 (±0.65, n=5) cfu/ml was observed in the pickling process (Mann-Whitney Test; $U_{15, 15}=2.5$,

p<<0.001) (Figure 3.6). A low pH (2.8-2.9), due to the presence of a non-swelling acid (modified aromatic amine-based compound) Picaltal®Flakes and formic acid may reduce the number of bacterial colonies in the pickling effluent. Microorganisms are sensitive towards aromatic amines (Lu *et al.*, 2009; Martins *et al.*, 2009), since the aromatic amines may have genotoxic (toxic to the genetic material) and cytotoxic (toxic to the cells) effects on microorganisms (Martins *et al.*, 2009). Therefore, the presence of an aromatic amine-based compound in the pickling process may have hindered *Bacillus* growth.
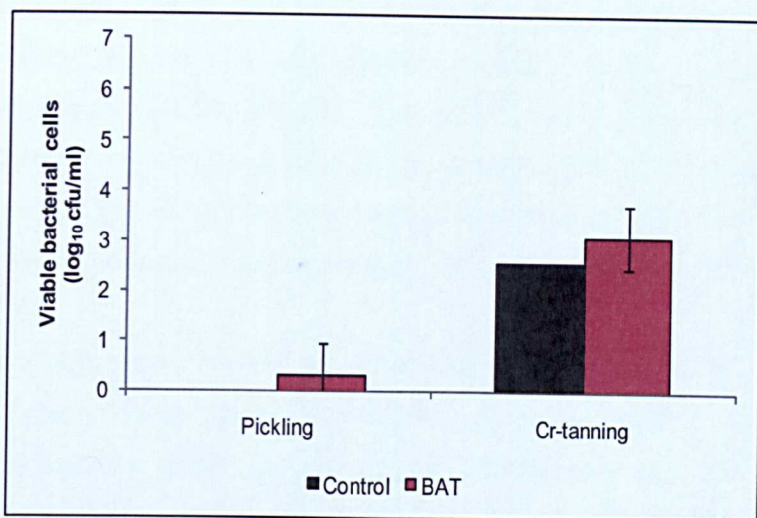
Figure 3.6: *Bacillus* spp. in the BAT pickling and chrome (Cr)-tanning effluents (n=5, Mann-Whitney Test: $U_{15,\ 15}$=1.5, p<<0.001) error bars represent standard deviation.

A significant increase in the number of *Bacillus spp.* from log 0.31 (±0.65, n=5) to 2.56 (±0.62, n=5) cfu/ml occurred during the chrome tanning process (Mann-Whitney Test: $U_{15,\ 15}$=1.5, p<<0.001), even though chromium may exert toxic effects toward microorganisms. The solubility of Cr(VI) is higher than Cr(III) (Cervantes *et al.*, 2001; Verma *et al.*, 2001), which may assist the diffusion of Cr(VI) through the cell membrane. Chromium(VI) is a strong oxidant and able to interrupt the microbial activity by oxidation (Bosnic *et al.*, 2000; Jun *et al.*, 2008; Megharaj *et al.*, 2003). Therefore, Cr(III) is generally considered less toxic than Cr(VI) (Cervantes *et al.*, 2001; Verma *et al.*, 2001). Various *Bacillus* species such as *Bacillus sphaericus, Bacillus marisflavi* and *Bacillus aquimaris* are found to withstand Cr(VI) (Liu *et al.*, 2006; Megharaj *et al.*, 2003; Mishra, and Doble, 2008).

3.4.3.3. <u>*Salmonella* Species</u>

3.4.3.3.1. Pre-soaking, Soaking and Unhairing Processes

Similar to *Bacillus* spp., a low number of *Salmonella* spp. log 0.26 (±0.54, n=5) cfu/ml was isolated from the pre-soaking process. As mentioned previously (Section 3.4.3.2) the high standard deviation is due to the inconsistent growth of the bacterial species, on the petri-dishes that were inoculated with the effluent, and was observed throughout the preliminary experiments. The inconsistent growth was may be due to the presence of variable number of bacterial colonies in the effluent. Growth of *Salmonella* spp. occurred in the control sample during the pre-soaking process and was significantly higher than the experimental samples (Mann-Whitney Test: $U_{15, 3}=0.00$, p=0.01). Analysis of the soaking and unhairing effluent (control and experimental samples) showed no growth of the *Salmonella* spp. The low bacterial growth and no growth are probably due to the following reasons:

1. An initial lower count of *Salmonella* spp. on calf skin pieces that were used in this experiment.
2. A pH that is higher than the pH range required for the bacterial growth.
3. Bacterial cells present on the skins may be transferred to the effluent due to mechanical agitation during the processing, and removal of the effluent at the end of each process reduces the number of the bacterial cells on skins.
4. Presence of antimicrobial agents particularly Na_2S and lime during the unhairing process may prevent microbial growth.

3.4.3.3.2. Deliming and Bating Processes

Figure 3.7 shows the average number of isolated *Salmonella* spp. from the BAT deliming and bating effluents. *Salmonella* growth did not occur in the control sample during the deliming process, whilst growth of *Salmonella* was observed in the control sample during the bating process. Bacterial growth in the deliming and bating effluent was significantly higher than the corresponding control sample (Mann-Whitney test: $U_{30, 6}=0.00$, p=0.012 [deliming]; t=5.370, df=7, p=0.001 [bating]).

Although, effluent was collected from 5 experimental samples, however, some of the inoculated plates were dehydrated. Therefore counting those plates was impossible and were not taken into account when the average bacterial count was calculated. The average number of isolated *Salmonella* colonies from the deliming and bating effluents was log 3.31 (±0.73, n=2) cfu/ml and log 4.91 (±0.16, n=3) cfu/ml respectively. Statistical analysis showed bacterial count in the bating effluent was significantly higher than the bacterial count in the

deliming effluent (t=-6.346, d.f.=9.054, p<<0.001). Factors such as, suitable pH range, an available nutritional source and the presence of a reduced amount of hazardous chemicals (Na_2S and lime), may promote the proliferation of *Salmonella* spp. during the BAT deliming and bating processes.
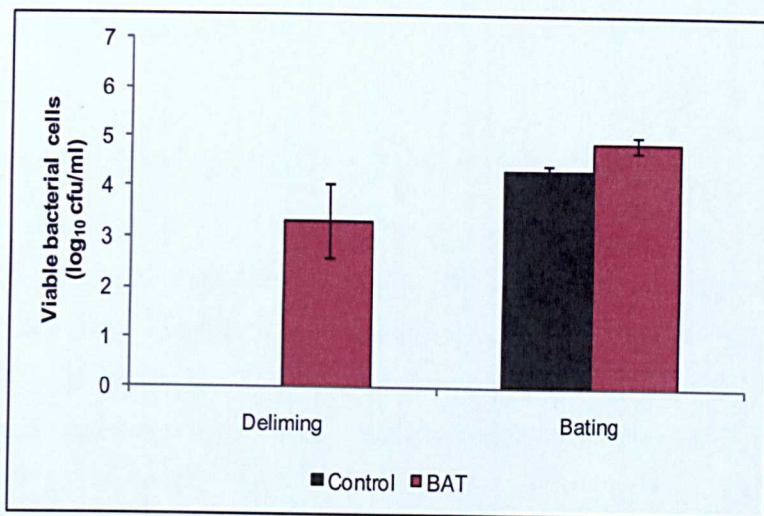
Figure 3.7: *Salmonella* **spp. in the BAT deliming (n=2) and bating effluents (n=3, t=-6.346, d.f.=9.054, p<<0.001), the error bars represent standard deviation.**

3.4.3.3.3. Pickling and Chrome Tanning Processes

Analysis of the pickling and chrome tanning effluents showed absence of *Salmonella* spp. No bacterial growth was occurred in the control samples during the pickling and chrome tanning processes. The addition of an aromatic amine based compound (Picaltal®Flakes, BASF, Germany) (Lu et *al.*, 2009; Martins *et al.*, 2009) to the pickling liquid may hinder the growth of *Salmonella* spp. In addition, the pH of the pickling effluent was 2.8-2.9, which is lower than the required pH range for bacterial growth and therefore did not provide a suitable pH range for *Salmonella* spp. Most of the microorganisms are found to be sensitive towards chrome (Chandra *et al.*, 2004); the presence of a high level of chrome may therefore also prevent the growth of *Salmonella* in the chrome-tanning effluent.

3.4.3.4. Total *Coliform* (*Klebsiella* Species)

Coliform is a group of bacteria that includes *Citrobacter* spp., *Enterobacter* spp., *Escherichia* spp., *Hafnia* spp., *Klebsiella* spp., *Serratia* spp., and *Yersinia* spp., generally found in natural water bodies. Although *Coliform* is mainly harmless and are often used as an indicator bacteria to monitor water quality, but some species of *Coliform* such as *E. coli*, *K. pneumoniae* and *K. oxytoca* are considered as pathogens (Department for Environment

Food and Rural Affairs (DEFRA), 2009; Environment Agency, 2002a and b; HPA, 2009a and b). According to the bathing water directive (DEFRA, 2009) the maximum limit of total *Coliform* in bathing water is 100 cfu/ml. Therefore, effluent from the BAT process was analysed to determine the level of *Coliform*. Based on the colony morphology the isolated *Coliform* colonies from the various stages of the BAT process were identified as *Klebsiella* spp. Oppong *et. al.* (2006) found the presence of *Klebsiella* spp. on the soaked hides.

3.4.3.4.1. Pre-soaking, Soaking and Unhairing Processes

A limited number of *Klebsiella* spp., log 0.9 (±0.34, n=5) cfu/ml was enumerated in the pre-soaking effluent followed by no bacterial enumeration in the soaking and unhairing effluents. The analysis of the control samples show *Klebsiella* growth during the pre-soaking process, however, there was no significant difference between the bacterial count in the pre-soaking effluent and the corresponding control sample (Mann-Whitney Test: $U_{15, 3}$=16, p=0.159).

3.4.3.4.2. Deliming and Bating Process

The total number of isolated *Klebsiella* spp. in the deliming and bating effluents was log 3.87 (±0.62, n=5) cfu/ml and log 5.57 (±0.20, n=5) cfu/ml respectively (Figure 3.8). The results shows that a significantly higher number of bacterial colonies were enumerated form the bating effluent than deliming effluent (Mann-Whitney Test: $U_{12, 15}$ = 0.000, p<<0.001).
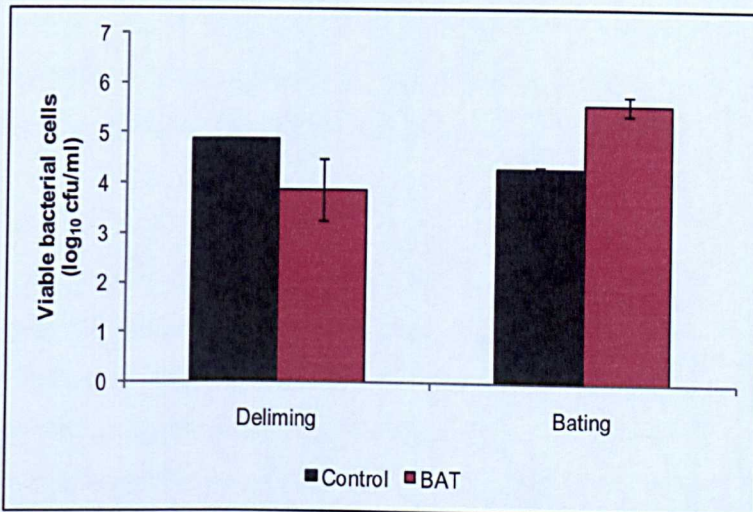
Figure 3.8: *Klebsiella* spp. in the BAT deliming and bating effluents (n=5, Mann-Whitney Test: $U_{12, 15}$=0.00, p<<0.001). The error bars represents standard deviation.

Growth of *Klebsiella* spp. was observed in the control samples during both of the deliming and bating processes (Figures 3.8). Bacterial count in the control sample was considerably

higher than the experimental samples during the deliming process (t=-2.729, df=13, p=0.017). On the other hand, bacterial count in the control sample was significantly lower than the experimental samples during the bating process (Mann-Whitney Test: $U_{15,\,3}$=0.00, p=0.003). However, the experiment shows the deliming and bating processes may favour bacterial growth.

3.4.3.4.3. Pickling and Chrome Tanning Processes

Similar to the *Salmonella* spp. absence of *Klebsiella* spp. was observed in the pickling and chrome tanning effluents, indicating that the pickling and chrome tanning processes did not provide suitable environmental conditions for the growth of *Klebsiella* spp.

Factors such as initial low bacterial cells on calf skin pieces, pH, discharging the effluents, available nutritional source, presence or absence of the chemicals that promote or hinder bacterial growth and processing time may have influenced the growth of *Coliform* at various stages of the BAT leather-making process.

The above study shows variable growth of bacterial species during various stages of the BAT leather-making processes. In addition, a high bacterial growth was observed in the control sample during the processing. As no calf skin pieces were added during the control experiments, the presence of bacterial colonies in the control samples indicates that the calf skin pieces may not be the sole source of bacterial species. Bacterial contamination may have occurred through water and chemicals added during the leather-making process. However, the preliminary investigation to determine effect of the BAT leather-making process on the bacterial growth shows that the used experimental methods were not suitable for this study.

Alternative methods such as decontamination of the calf skin pieces (see Section 2.9) and inoculation of known bacterial species (see Sections 2.3 and 2.10) were adopted. These methods were followed to standardise the raw materials by eliminating the possibility of having variable bacterial species on the skin samples. In addition, inoculation of known species will also allow ease in monitoring the presence of bacterial species throughout the leather manufacturing process enabling the identification of the principle colonies and differentiation from contamination, in order to obtain data that is more reliable. In order to avoid microbial contamination during processing as well as during sample collection, the following precautions were taken:

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1. Use of removable drums for the experiments, which were sterilised by autoclaving at 121°C for 15 minutes before conducting the leather manufacturing processes.
2. Use of sterilised tap water for the leather processing.
3. Addition of water and chemicals, as well as collection of samples aseptically and promptly.

Bacterial species that were already isolated from raw hides/skins or leather were reconsidered. Modes of transmission were also taken into account. Bacterial species, which were already isolated from hides/skins/leather and are capable of transmission via open wounds or breakage of the protective barrier (skin), were selected for the next experiments. The selected bacterial species were *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (also see Section 2.3).

Samples for bacterial analysis are required to be analysed within 24 hours of collection (Clesceri *et al.*, 1998), therefore, difficulties arose with time management of sample collection, in order to complete both of the tannery procedures as well as undertaking microbial analysis efficiently. In order to manage time, workload and to carry out experiments proficiently, it was determined to split the tannery procedures in 2 weeks instead of a 1 week time limit. An additional procedure (reliming) was added (for details see Section 2.12). Further trials were carried out to optimise the conditions and to ensure the reliability of the adopted methods. The results obtained are given in Sections 3.4.4-3.4.10.

3.4.4. Optimisation of Solid Media for <u>Bacillus cereus</u>

During the preliminary experiments, BBCA was used for the enumeration of *Bacillus* species (see Section 3.3.5.2). According to the national standard method PEMBA is the recommended media for the enumeration of *B. cereus* (HPA, 2005c). A trial was therefore carried out in order to determine the optimal growth media for *B. cereus* enumeration among BBCA and PEMBA as described in Section 3.3.6.

The average number of enumerated colonies on PEMBA and BBCA media was log 7.28 (±0.19) cfu/ml and log 7.33 (±0.34) cfu/ml respectively (Figure 3.9). Although a slightly higher number of *B. cereus* colonies were enumerated on BBCA than on PEMBA, but no significant difference was found between the number of *B. cereus* colonies enumerated on PEMBA and BBCA media (t=-0.440, df=22, p=0.666).
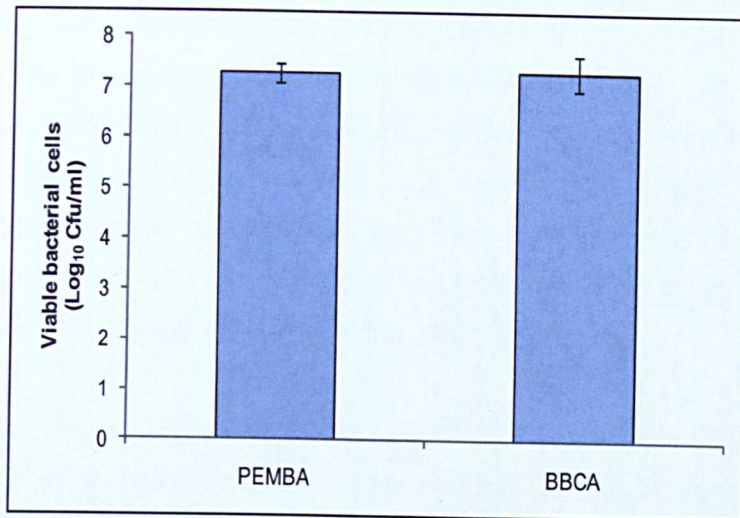
Figure 3.9: *Bacillus cereus* growth on polymixin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) and brilliant *Bacillus cereus* agar (BBCA), n=3, the error bars represent standard deviation. There was no significant difference between PEMBA and BBCA media (t=-0.440, df=17.293, p=0.666).

3.4.5. Optimisation of Disinfectants for Decontamination of Calf Skins

This experiment was carried out in order to find a suitable disinfectant to decontaminate calf skins by eliminating the microorganisms that are present on the skins. Various chemical disinfectants such as alcohol, halogen based compound, formalin, phenol based compound and glutaraldehyde inactivate microorganisms. Among the chemical agents, ethyl alcohol or ethanol (70-90% w/v) and NaClO (a halogen-based antimicrobial agent) are the most widely applied disinfectants (Pelczar *et al.*, 1993; Madigan *et al.*, 1997; Cords, 2005; Wilson, 2005). Additionally, both ethanol and NaClO are the most cost effective and easily accessible. Ethanol (70% w/v) and NaClO were therefore selected for decontamination purposes. Trials were carried out to determine an appropriate antimicrobial agent among ethanol and NaClO for this study. The trials were primarily carried out with one calf skin piece and if a satisfactory result was obtained, the experiments were repeated in triplicate. Calf skin pieces, approximately 25g, were used for the trials. Bacterial enumeration was carried out on PEMBA media as the decontaminated calf skin pieces were inoculated with *B. cereus*, ATCC11778.

3.4.5.1. Ethanol

No bacterial growth was observed in the water and swabs after the final washing cycle, when a calf skin piece was treated with 70% w/v ethanol. Therefore, this experiment was repeated in triplicate. Microbial analysis of the water (obtained after the final washing cycle)

following an ethanol-decontamination of the calf skin pieces showed no microbial growth. The average number of isolated bacterial colonies from swabs after the final washing cycle was log 0.29 (±0.57, n=3) cfu/ml. Use of a disinfectant may not cause total elimination of microorganisms. Microorganisms, which are more resistance towards extreme environmental condition such as bacterial and fungal spores, may survive the ethanol-treatment (Murray *et al.*, 2005). However, due to limited bacterial growth, the antimicrobial activity of ethanol in this respect was considered to be satisfactory.

An average of log 7.54 (±0.03) cfu *B. cereus* ATCC11778, colonies was inoculated on the ethanol-treated calf skin pieces (for details see Section 3.3.7.1). It was found that log 1.74 (±1.17, n=3) cfu/ml and log 1.03 (±1.54, n=3) cfu/ml of the inoculated *B. cereus* colonies were recovered from the water samples and swabs respectively. Ethanol may have a persistent antimicrobial activity when applied to skin (Ascenzi, 1995). This may prevent the growth of the inoculated bacterial colonies on the ethanol-decontaminated calf skin pieces. Due to the limited growth of the inoculated bacterial species on ethanol treated calf skin pieces, ethanol was considered unsuitable for this study.

3.4.5.2. Sodium Hypochlorite

Sodium hypochlorite is effective towards a wide range of microorganisms including bacteria, fungi, viruses and bacterial spores, ensuring a high level of decontamination (Cords, 2005; Murray *et al.*, 2005; Rutala and Weber, 1997; Wilson, 2005). The antimicrobial property of diluted NaClO solutions may reduce rapidly (Wilson, 2005). This property may allow growth of inoculated bacterial species on the NaClO-treated calf skin pieces and therefore, may be beneficial for this study. Microbial analysis of the water and swabs after the final washing cycle showed no growth of microbial species, when calf skin pieces were treated with NaClO w/v at various concentrations (Figures 3.10 and 3.11)

Figures 3.10 and 3.11 also show the recovery of the inoculated *B. cereus* on the calf skin pieces treated with NaClO (w/v) at various concentrations. The highest recovery of the inoculated bacteria was observed on the calf skin piece that was treated with a 0.125 g/ml NaClO solution. A lower number of bacterial colonies were recovered at higher NaClO concentrations. Due to a satisfactory decontamination effect and a higher bacterial recovery, 0.125 g/ml NaClO was selected, and a trial was therefore carried out in triplicate with 0.125 g/ml NaClO to reduce the probability of error before conducting the main experiments.
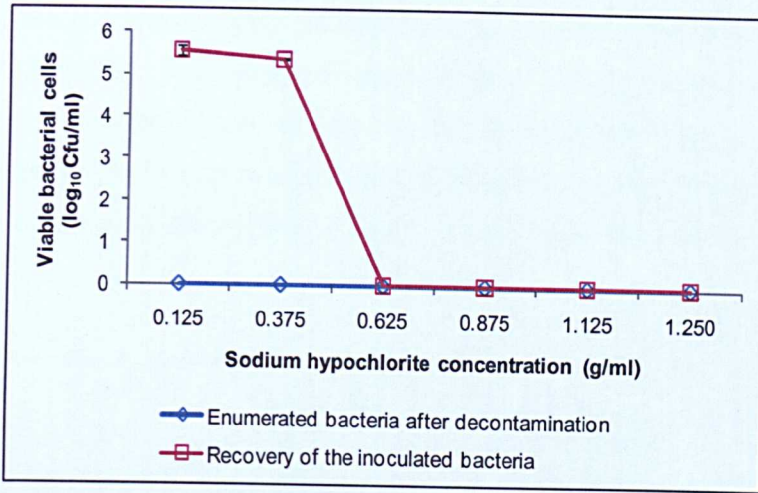
Figure 3.10: The number of bacterial colonies isolated from the water after the final washing cycle following sodium hypochlorite-decontamination and recovery of the inoculate bacteria (*B. cereus*) from water samples after overnight incubation at 30°C, n=1, the error bars represent standard deviation.
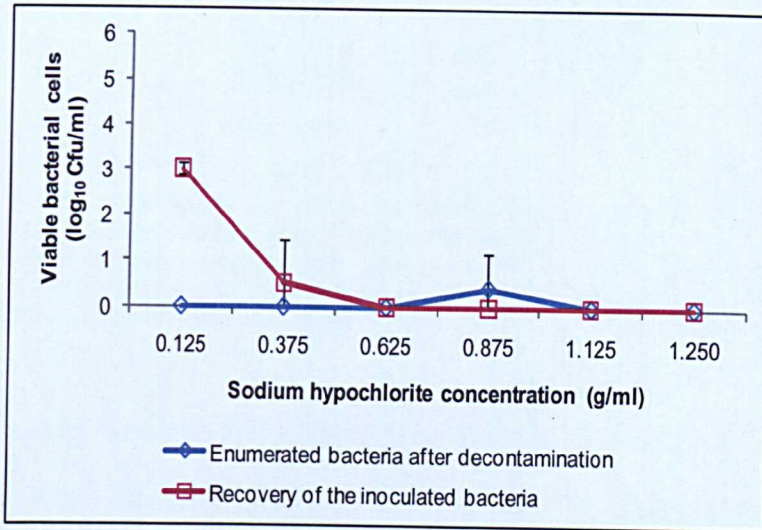
Figure 3.11: The number of bacterial colonies isolated from swabs after decontamination with sodium hypochlorite and recovery of the inoculate bacteria (*B. cereus*) after overnight incubation at 30°C, n=1, the error bars represent standard deviation.

An average of log 0.29 (±0.57, n=3) cfu/ml and log 0.72 (±0.69, n=3) cfu/ml bacterial colonies was enumerated from the water and swabs, after the final washing cycle, following decontamination of calf skin pieces with 0.125 g/ml NaClO. Sodium hypochlorite may not cause total elimination of microorganisms, but often found to reduce the viable microbial cells to log 1 or below (Allenda *et al.*, 2009; Koide *et al.*, 2009). Due to the enumeration of limited microbial colonies, the decontamination activity of 0.125 g/ml NaClO was considered to be satisfactory in this instance.

The flesh side of the decontaminated calf skin pieces were inoculated with log 7.54 (±0.03) cfu *B. cereus* ATCC11778. An average of log 5.38 (±1.16, n=3) cfu/ml and log 4.59 (±0.89, n=3) cfu/ml of the inoculated *B. cereus* was recovered from the water samples and swabs respectively (Figure 3.12). Due to the recovery of a high number of inoculated *B. cereus* on the NaClO-treated calf skin pieces, NaClO (0.125 g/ml) was considered to be suitable for this study.
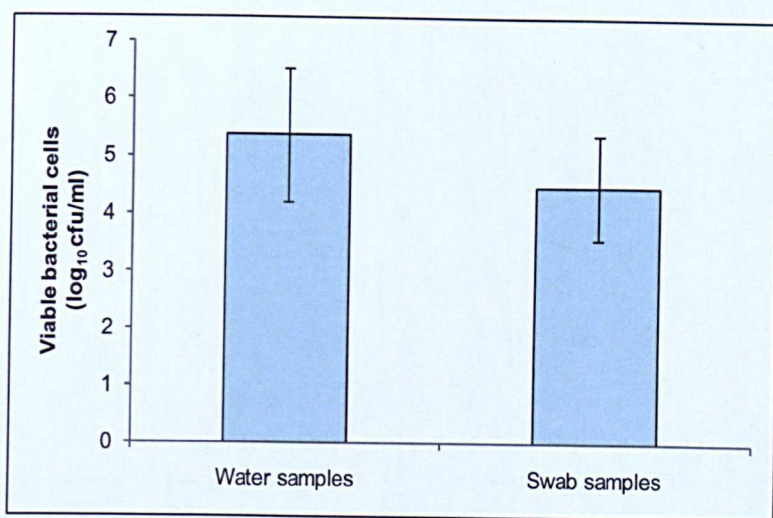
Figure 3.12: Recovery of the inoculated bacteria (*B. cereus*) on the sodium hypochlorite (0.125 g/ml) treated calf skin pieces, when water samples and swabs were analysed. Bacterial enumeration was carried out on PEMBA media, n=3 and the error bars represent standard deviation.

3.4.6. *Microbial Enumeration in Sodium Hypochlorite (0.125 g/ml)*

Microbial analysis of a 0.125 g/ml NaClO solution showed the absence of bacterial colonies and was considered to be satisfactory for decontamination purposes.

3.4.7. *Optimisation of Bacterial Enumeration on Hair*

In Chapters 5, 6 and 7, hair from the conventional and BAT unhairing effluents was analysed to determine the presence of the inoculated bacterial colonies. A preliminary trial was carried out to reduce experimental errors before conducting the main experiments. Bovine hair was used for this trial. For details of the method, see Section 3.3.9. The results obtained are given in Figure 3.13.

Bacillus spp. was selected in this instance, as it is one of the most common bacterial species isolated from raw hides and skins, therefore, have high possibility of isolating those from bovine hair. Bacterial enumeration was carried out on PEMBA media. The average number

of recovered *Bacillus* species from hair after 6 and 14 hours incubation was log 4.0 (±0.16, n=2) cfu/ml and log 3.98 (±0.07, n=2) cfu/ml respectively. The difference between bacterial count obtained after 6 hours and 14 hours incubation was found to be statistically insignificant (t=-0.332, df=10, p=0.747). Prolonged incubation did not increase the number of bacterial colonies; this may be due to entering of *Bacillus* spp. into the stationary phase.
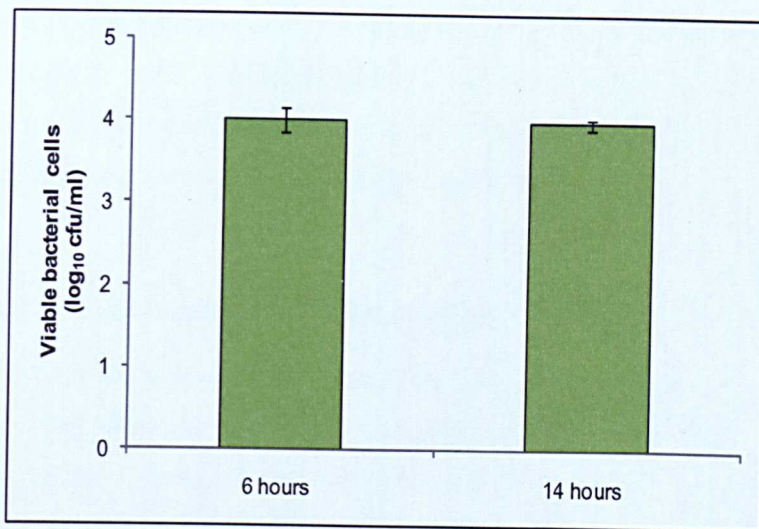
Figure 3.13: Enumeration of *Bacillus* species from bovine hair. Colonies were enumerated on PEMBA media, (n=2, t=-0.332, df=10, p=0.747). The error bars represent standard deviation.

Enumeration of the damaged bacterial cells may not be possible if inoculated directly on a selective media. Incubation of the damage bacterial colonies in TSB, before inoculating on a solid media, may allow recovery of the damaged bacterial cells and consequently enumeration of the recovered bacterial cells may be achievable. However, this is only a qualitative analysis and the initial number of bacterial colonies cannot be obtained, as 6-14 hours incubation would provide sufficient time for the bacteria not only to repair the damaged cells, but also for the cell multiplication.

3.4.8. Microbial Enumeration in Chemicals used for the Leather Manufacturing Processes

It was thought that the chemicals used for the leather manufacturing processes in this study may be contaminated with microorganisms. An experiment was therefore carried out to examine the presence of microorganisms in the chemicals used during the processing (conventional and BAT). Microbial analysis of the following chemicals showed the absence of microorganisms:

1. Truposept BA (Trumpler, Germany),
2. Corilene®W385 (STAHL Europe, The Netherlands);

3. Sodium sulfide, lime (calcium hydroxide), sodium chloride, formic acid, sulfuric acid, sodium formate, Na_2CO_3 and chrome(III) sulfate (supplied by BSLT, The University of Northampton, UK),

4. Sellatan®P and Pelvit C (TFL, Germany)

5. Feliderm®MGO powder (Clariant, Switzerland).

The presence of bacterial colonies was observed in unhairing enzyme and α-amylase, which were supplied by SPIC, India. Bacterial growth was also observed in Oropon ON2 (TFL, Germany). However, a limited number of bacterial colonies, log 1.02±1.27 cfu/g was isolated from Oropon ON2 and therefore, may be considered to be negligible.

3.4.9. *Microbial Enumeration in α- Amylase and Unhairing Enzyme*

An average of log 8.61 cfu/g (±0.05, n=3) and log 6.42 cfu/g (±0.03, n=3) bacterial colonies were enumerated from α-amylase and the unhairing enzyme respectively. It was observed that the colony morphology and microscopic structure of the isolated bacterial colonies from α-amylase and unhairing enzyme was the same. The bacterial cells appeared as Gram-positive, spore-forming rod-shaped (Figures 3.14-3.16), when observed under a light microscope (Nikon Eclipse 600, Nikon, Japan).
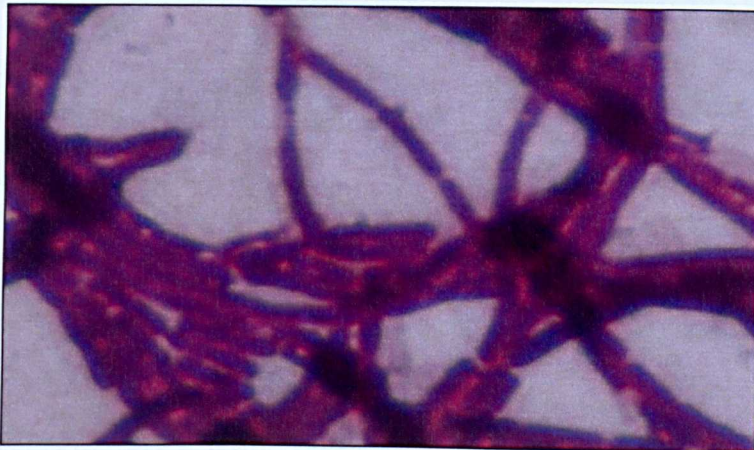
Figure 3.14: Microscopic structure of the isolated bacteria from α-amylase (1 k magnification). The bacterial cells appeared as Gram-positive rods and occurred in chains, when observed under a light microscope (Nikon Eclipse 600, Nikon, Japan). Image was taken using a Nikon digital camera (4 MP)
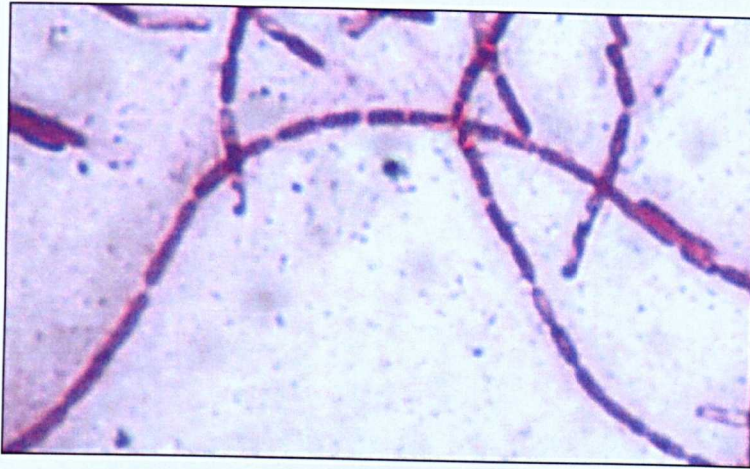
Figure 3.15: Microscopic structure of the isolated bacteria from the unhairing enzyme (1 k magnification). The bacterial cells appeared as Gram-positive rods and occurred in chains, when observed under a light microscope (Nikon Eclipse 600, Nikon, Japan). Image was taken using a Nikon digital camera (4 MP).

Figure 3.16: Bacterial spores of the bacteria isolated from α-amylase (1 k magnification). The spores were stained green using malachite green, while the vegetative cells were stained red using safranin. Cells were observed under a light microscope (Nikon Eclipse 600, Nikon, Japan) and the image was taken using a Nikon digital camera (4 MP).

The Biolog identification technique (Biolog Inc., USA) (see Section 2.15.2) was used to identify isolated bacterial species. The majority of the bacterial colonies isolated from α-amylase and unhairing enzyme were identified as *Bacillus amyloliquefaciens* A (Table 3.2). It was advised by the supplier (SPIC, India) that a *Bacillus* sp. was used for the production of protease (unhairing enzyme) and α-amylase. The spores produced by the *Bacillus* spp. may have survived and recovered when suitable environmental conditions were given.

Table 3.3: The Biolog Identification of the isolated microorganisms from α-amylase and unhairing enzymes.

Enzyme	Biolog identification	Probability (%)
Alpha amylase	*B. amyloliquefaciens*	100
	B. amyloliquefaciens	100
	B. amyloliquefaciens	99
	B. amyloliquefaciens	91
	B. amyloliquefaciens	87
Unhairing enzyme	*B. amyloliquefaciens*	94
	B. amyloliquefaciens	100
	B. amyloliquefaciens	98
	B. amyloliquefaciens	99
	B. licheniformis	-

3.4.10.   Filtration to Remove Micro-organisms in Enzymes

It appears filtration of the enzyme solution through a 0.2 µm membrane filter effectively removed microorganisms. Before filtering the unhairing enzyme solution, the total number of enumerated colonies was log 4.46 cfu/ml, while no bacterial growth was observed in the filtered enzyme solutions.

However, it was found during the experiments that the unhairing enzyme was too concentrated to filter through a 0.2 µm membrane filter. Therefore, the unhairing enzyme was not filtered before adding to the unhairing liquid, during the BAT unhairing processes, for the studies that were carried out in Chapters 5, 6 and 7. Based on the preliminary experiments (see Section 3.4.3), the BAT unhairing process was expected not to provide suitable environmental conditions for the bacteria or bacterial spores present in the unhairing enzyme to recover or germinate. Moreover, use of selective media for bacterial enumeration will prevent the growth of unwanted bacterial species assisting the isolation of the particular bacterial species. Additionally, confirmation tests (see Section 2.15) were carried out to ensure the presence of the inoculated bacterial colonies during the leather manufacturing processes.

On the other hand, it was found during the preliminary experiment that the bating process may provide suitable growth conditions for the bacteria to recover. Therefore, the enzyme α-amylase was filtered through a 0.2 µm membrane filter before addition during the BAT bating process for the studies that were carried out in Chapter 5, 6 and 7.

3.5.   Summary

During the preliminary experiments, variable results were obtained when bacterial enumeration (total bacterial count, total *Coliform*, *Bacillus* spp. and *Salmonella* spp.) in effluent, collected from the BAT leather-making process, was carried out. In some instances, the number of enumerated bacterial colonies was found to be larger in the control samples than the experimental samples. This indicated that bacterial contamination may have occurred during the processing through water or chemicals. Bacterial proliferation occurred in all stages from the pre-soaking to chrome tanning effluents, when total bacterial colonies in the effluent were measured. Growth of *Bacillus* spp. occurred in the pre-soaking effluent and in effluent collected from the deliming-chrome tanning processes. Growth of *Salmonella* spp. and total *Coliform* was observed in the pre-soaking, deliming and bating effluents. Due to the inefficacy of the preliminary methods to study the effect of the BAT leather manufacturing processes on bacterial growth, it was decided to modify the experimental methods as follows. Decontamination of calf skin pieces using a suitable disinfectant, inoculation of the decontaminated calf skin pieces with known bacterial species, carry out a conventional and BAT leather-making processes with the inoculated calf skin pieces, and use of sterilised drums and water during processing.

Further trials were therefore conducted to optimise the decontamination methods and the optimal results were obtained when used 0.125 g/ml NaClO for the decontamination purposes. The presence of bacterial contaminants in the chemicals used during the leather manufacturing processes was investigated. The results obtained showed that a majority of the chemicals were free from bacterial contamination. A limited quantity of bacterial colonies was isolated from Oropon ON 2, and therefore considered to be negligible. On the other hand, a high bacterial growth and proliferation was observed in unhairing enzyme and α-amylase. The bacterial species isolated from the unhairing enzyme and α-amylase were identified as *B. amyloliquefaciens* and was found to be effectively removed after filtering an unhairing enzyme solution through a 0.2 μm membrane filter.

3.6.   Conclusion

In this study, although bacterial growth was observed in the effluent during various stages of the BAT leather-making process, but it was not possible to conclude the effect of the BAT leather-making process on the bacterial growth due to the potential contamination. In order to obtain a reliable data and to ease identification of bacterial species, it was decided to decontaminate calf skin pieces using 0.125 g/ml NaClO followed by the inoculation of known

bacterial species, such as *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (see Chapter 2). Additionally, in order to avoid potential contamination, sterilised trial drums and sterilised water were used during leather processing. Unhairing enzyme and α-amylase was found to be contaminated with *B. amyloliquefaciens* and may be removed using a 0.2 μm membrane filter. Moreover, an additional reliming stage was added to manage time and workload. The optimised methods were described in Chapter 2 and were applied to investigate the growth of *B. cereus* (Chapter 5), *P. aeruginosa* (Chapter 6) and *S. aureus* (Chapter 7) during the conventional and BAT leather-making processes.

Chapter 4: Optimisation of Pulsed-Field Gel Electrophoresis to Determine the DNA Profiles of *Pseudomonas aeruginosa* and *Bacillus cereus*

4.1.　　Introduction

Phenotypic characteristics of bacteria may vary depending on the environmental conditions during incubation, and therefore possibly hinder accurate identification of the bacterial species. Additionally a phenotypic identification method does not provide information to identify microorganisms to the strain level. Alternatively, a genotypic method can provide accurate identification of bacterial species, up to strain level.　A pulsed-field gel electrophoresis (PFGE) method was used to determine DNA profiles, leading to the isolated bacteria being matched with the inoculated bacteria. This also assists to differentiate the contaminants from the inoculates, strengthening the validity of the obtained results.

A DNA molecule is composed of nucleotides and each nucleotide consists of a five–carbon sugar molecule, a phosphate molecule and a nitrogen base. The sugar-phosphate molecules remain the same, the variable component is the nitrogen base (Madigan *et al.*, 1997; Pelczar *et al.*, 1993). DNA contains four different nitrogen bases, which are classified into two groups (Dale, 1994; Madigan *et al.*, 1997; Pelczar *et al.*, 1993):

1. Purine base: adenine (A) and guanine (G)
2. Pyrimidine bases: cytosine (C) and thymine (T).

In the double helical structure of DNA, the nitrogen bases, A to G and C to T, of single-stranded DNA molecules are linked together via hydrogen bonds. The nitrogen bases, which are linked by a hydrogen bond, are known as base pairs (Dale, 1994; Madigan *et al.*, 1997; Pelczar *et al.*, 1993).

Appropriate restriction endonucleases (also known as restriction enzymes) recognise and cleave the nitrogen base sequence at a specific site (Pelczar *et al.*, 1993; Madigan *et al.*, 1997). Restriction enzymes are classified into three groups: type I, II and III. The most commonly used restriction enzymes are type II, which generally recognise 4-8 base sequences in DNA molecules. These enzymes either cut the DNA within the recognised sequence, or the sites, which are immediately adjacent to the recognised sequence (Dale, 1994). For example, a restriction enzyme, *Eco*RI, from *E. coli* recognises a 6-base sequence GAATTC, where the GA is the cleavage site (Dale, 1994, Madigan *et al.*, 1997). In a DNA molecule, if the distributions of the base pairs are considered random, the probability of the

occurrence of 6-base sequence is one in every $4^6 = 4096$ bases, therefore, the restriction enzyme would cleave a DNA molecules into fragments with a size of approximately 4 kb (=4000 b). Restriction enzymes are therefore able to cleave a large DNA molecule into smaller fragments (Dale 1994; Madigan *et al.*, 1997), which therefore can be separated using an electrophoresis system.

Gel electrophoresis systems are the most commonly used electrophoresis method for microbial DNA profiling. In general, the molecules may migrate towards anodes (positive electrode) or cathodes (negative electrodes) depending on the net charge of the molecules, when an electric field is applied. During gel electrophoresis, the pH of the electrophoresis buffers (usually tris-borate EDTA) is approximately 8.3, at which the DNA molecules are negatively charged (Burmeister and Ulanovsky, 1992; Dale, 1994), and therefore, migrates towards the anodes. The migration rates of the DNA fragments depend on the size and shape of the molecules. During electrophoresis, DNA fragments with a comparatively smaller size and compact shape may migrate faster than the larger DNA fragments through the agarose or acrylamide gel pores (Dale, 1994) and as a result, separation of the fragments occurs. The DNA fragments may be visualised as luminous bands if stained with a fluorescent dye, ethidium bromide, and when observed under UV-lights. Electrophoresis of a mixture of DNA fragments with various sizes and shapes for a limited period of time would therefore provide a specific band patterns depending on the distance travelled by the fragments. Based on the band pattern, obtained after the electrophoresis, a genetic fingerprinting of a particular microbial DNA may be obtained (Gautom, 1997; Madigan *et al.*, 1997).

In a conventional gel electrophoresis system, the current is generally applied in a single direction. Linear DNA fragments larger than 20 kb may migrate together regardless of the size, when a conventional electrophoresis system is used. This may prohibit the separation of the large DNA fragments. Alternatively, in a PFGE system, the direction of current is changed periodically forcing the DNA fragments to change directions. The larger DNA fragments may require a longer time to re-orient in the new direction with the change of the current directions than the smaller DNA fragments, which may assists separation of the large DNA (>20 kb) (Basim and Basim, 2001; Dale, 1994; Gautom, 1997; Madigan *et al.*, 1997).

Various types of PFGE systems are available, such as field inversion gel electrophoresis, counter-clamped homogenous electric field (CHEF), transverse-alternating field electrophoresis, rotating gel electrophoresis, orthogonal-field alternation gel electrophoresis, programmable autonomously-controlled electrodes and pulsed-homogeneous orthogonal

field gel electrophoresis (Dale, 1992; Joppa *et al.*, 1992; Basim and Basim, 2001). In this study, a CHEF (CHEF-DR®II, Biorad, UK) system was used. Counter-clamped homogenous electric field systems of PFGE generally consist of 24 electrodes. The hexagonal array of electrodes generates two different alternating electric fields at 120° orientation. The periodic alternation between the two electric fields is defined as switch time (Herschleb et al., 2007). In addition, due to the generation of a homogenous electric field in the CHEF system, the DNA fragments migrate in straight lines (Burmeister and Ulanovsky, 1992; Dale, 1992; Herschleb *et al.*, 2007). Figure 4.1 shows a schematic diagram of the CHEF system.
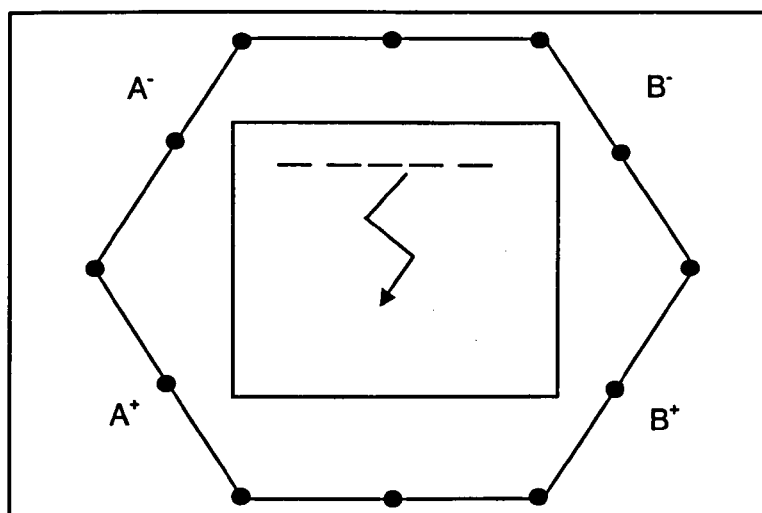
Figure 4.1: Schematic diagram of a counter clamped homogenous electric field system, the hexagonal electrodes, represented by the black spheres, create two alternative electric field oriented at 120° (A and B). The direction of the electric field changes periodically, forcing the DNA molecule migrating through the gel pores in a zigzag fashion (Burmeister and Ulanovsky, 1992).

The microbial cells for PFGE analysis are generally harvested by centrifugation and an appropriate buffer is added to prepare a cell suspension. Plugs are prepared by mixing molten agarose with the bacterial suspension and transferring it to a plug mould followed by the lysis of the embedded bacterial cells to extract DNA. Proteolysis may be required in order to remove remaining protein debris with the extracted DNA, followed by the digestion of the DNA with an appropriate restriction enzyme that cleaves the DNA into fragments with specific sizes and shapes. Electrophoresis of such DNA fragments for several hours may result in the separation of the DNA fragments producing a specific band pattern for the experimental bacterial DNA (Gautom, 1997; Madigan *et al.*, 1997). The various PFGE protocols that were trialled to obtain quality DNA from *P. aeruginosa* and *B. cereus* and so to obtain clear DNA band separation are included in this chapter.

4.2. Aim and Objective

- Optimisation of the PGFE protocols in order to determine *B. cereus* and *P. aeruginosa* DNA profiles.

- Analysing the DNA extracted from *B. cereus* and *P. aeruginosa*, isolated during various stages of the conventional and BAT processes, to ensure that the strain of the isolated bacteria are same as the inoculated bacteria.

4.3. Materials and Methods

4.3.1. Pulsed-Field Gel Electrophoresis (PFGE)

The chemicals and the instruments that were used in this chapter are given in Appendix 2. The chemical compositions of various buffers are shown in Table 4.1. The pH of the buffers was adjusted using either hydrochloric acid (HCl) or 30% w/v sodium hydroxide (NaOH). The concentration of the buffer solutions will be expressed as 'x' in this instance.

Table 4.1: Chemical composition of various buffers used in this chapter for electrophoresis.

Buffer	Chemical compositions
PET-IV buffer-1	10 mM tris-HCl, (pH 7.5) 1 M NaCl
PET-IV buffer-2	10 mM tris, (pH 7.5) 1 M LiCl
Lysis Buffer	6 mM tris-HCl (pH 7.5), 0.1 M EDTA, 1 M NaCl, 0.5% (w/v) Brij 58 (polyethylene glycol hexadecyl ether), 0.4% (w/v) sodium deoxycholate, 0.5% (w/v) sodium lauryl sarcosine
EC lysis buffer	6 mM tris-HCl (pH 7.5) 1 M LiCl, 100 mM EDTA, 0.2% (w/v) deoxycholate, 0.5% (w/v) sodium lauryl sarcosine
Proteolysis buffer	0.5 M EDTA at pH 8.0 – 8.5, 1% (w/v) sodium lauryl sarcosine
TE buffer-1	50 mM EDTA (pH 8.0), 20 mM tris-HCl (pH 8.0)
TE buffer -2	100 mM EDTA (pH 8.0), 100 mM tris-HCl (pH 8.0)
TE buffer-3	1 mM EDTA (8.0), 10 mM tris-HCl (pH 8.0)
SE buffer	75 mM NaCl, 25 mM EDTA (pH 7.5)
Digestion Buffer SA 10x	50 mM tris-HCl, 100 mM NaCl, 10 mm $MgCl_2$, 1 mM dithioerythritol (pH 7.6)
Digestion Buffer SH 10x	33 mM tris acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol (pH 7.9)

Note: the pH of the buffers except SA and SH were adjusted using HCl and NaOH.

In this instance, plugs were prepared by mixing a bacteria cell suspension with a low melting point agarose (Bio-Rad, USA). Lysis and proteolysis of the bacterial cells were carried out using enzymes lysozyme (~70000 units/mg) (Sigma-Aldrich, UK) and proteinase K (≥30 units/mg protein) (Sigma-Aldrich, UK) respectively. The detailed procedures of

harvesting bacterial cells, DNA extraction and purification methods are described in Sections 4.3.1.1-4.3.1.7. The plugs were subsequently cut into 1-2 mm pieces and digested using appropriate restriction enzymes. The plugs were loaded into a 1% w/v agarose gel, which was prepared by adding 1 g certified megabase agarose (Bio-Rad, USA) to 100 ml 0.5x tris-borate EDTA (TBE) buffer (pH 8.3) (Sigma-Aldrich, UK) and dissolved completely using a microwave. Ethidium bromide, a fluorescent compound, was used to stain bacterial DNA. Due to the presence of ethidium bromide, the DNA fluorescence, when observed under UV radiations (336 nm) and the DNA fragments appear as luminous bands. The molten agarose was mixed with 100 µl ethidium bromide (500 µg/ml) to achieve a final concentration of 0.5 µg/ml. The agarose gel was placed in a counter-clamped homogenous electric field system (CHEF-DR II, Bio-Rad, USA). Electrophoresis was carried out at 14°C using a 0.5x TBE buffer (Sigma-Aldrich, UK) at 6 V/cm. A Lambda Ladder, 48.5-970 kb, (Bio-Rad, USA) was used as a DNA marker. A Gel-Documentation (Gel-Doc) system (Bio-Rad, USA) was used to visualise and photograph the gel.

4.3.1.1.   Method 1 (PFGE)

Pseudomonas aeruginosa were grown overnight on tryptone soya agar (TSA) media at 37°C and suspended in PET-IV buffer-1. Bacterial suspensions were washed three times by centrifugation at 8000 rpm and re-suspended in PET-IV buffer-1. A spectrophotometer (Cecil Instrument Ltd., UK) was set at a wavelength of 600 nm and the bacterial cells density adjusted to 1.5 using PET-IV buffer-1. A 2% w/v low melting-point agarose was dissolved completely in water using a hot plate with constant stirring and transferred to a 1.5 ml centrifuge tube, which was then placed in a 50°C heating block until used. Bacterial suspensions (0.5 ml) were mixed with 0.5 ml of the 2% w/v molten agarose, transferred immediately to a plug mould and solidified at room temperature for 10-15 minutes. The plugs were placed in 15 ml centrifuge tubes containing lysis buffer with lysozyme (1 mg/ml) and incubated for 20 hours at 37°C followed by a wash using TE buffer-1. The plugs were washed twice more using TE buffer-1. A proteolysis buffer containing proteinase K (1 mg/ml) was added to the plugs and incubated overnight at 50°C. The following day, the plugs were washed twice (2 hours each time), in TE buffer-3. The prepared plugs were stored in 5 ml TE buffer-3 at 4°C and used within 4 weeks.

The plugs (1-2 mm) were placed in 100 µl digestion buffer SA (1x), and the DNA was digested at 37°C with 15 units *Spe*I (10000 units/ml; Sigma-Aldrich, UK) restriction enzyme overnight. The digested plugs and Lambda Ladder, were placed into a 1% w/v agarose gel

containing ethidium bromide (0.5 μ/ml), sealed with 1% w/v molten agarose and the electrophoresis was carried out for 20 hours using two time blocks at 14°C. The first block was set with an initial to final switch time of 0.5 to 60 seconds respectively. The second block was set for 4 hours with a ramping time from 60 to 120 seconds. The agarose gel was then visualised and photographed using a Gel-Doc system.

4.3.1.2. Method 2 (PFGE)

A rapid PFGE protocol according to Gautom (1997) was followed in this experiment. A *P. aeruginosa* culture was incubated overnight on TSA media and suspended in TE buffer-2. The bacterial suspensions were adjusted to 20% transmittance using TE buffer-2. A 0.5 ml aliquot was collected from the bacterial suspension in TE buffer and, 0.5 mg lysozyme and 0.5 mg proteinase K was added followed by a 15 minutes incubation at 37°C. A 1.2% w/v low melting-point agarose was prepared in water by using a hot plate, transferred to a 1.5 ml centrifuge tube and placed in a heating block at 50°C. Sodium lauryl sulfate (20% w/v), 25 μl, was added to 0.5 ml of the 1.2% w/v low melting-point agarose. The low melting-point agarose was then added to the incubated bacterial suspensions, transferred immediately to a plug mould and allowed to solidify at room temperature for 10-15 minutes. The plugs were placed in a 2.5 ml proteolysis buffer containing 2.5 mg proteinase K (1 mg/ml) and incubated for 2 hours in a 55°C water bath. The proteolysis buffer was removed and the plugs incubated with 8 ml sterilised deionised water (pre-heated to 50°C), in a 50°C water bath for 10 minutes with an agitation speed of 100 rpm. The plugs were placed in TE buffer-3 (preheated to 50°C) and incubated further for 15 minutes in a 50°C water bath. The washing procedure was carried out three more times. The plugs were stored in a cold TE buffer at 4°C and used within 4 weeks.

DNA was digested using 15 units of *Spe*I (10000 units/ml) (Sigma-Aldrich, UK) restriction enzyme in 100 μl digestion buffer SA (1x) at 37°C for 18 hours. Electrophoresis of the digested DNA and Lambda Ladders were carried out using 1% w/v agarose gel containing ethidium bromide (0.5 μ/ml) for 14-18 hours with an initial switch time 2.1 seconds to a final switch time 35.1 seconds at 14°C. The gel was visualised and photographed using a Gel-Doc system.

No clear band patterns were obtained after electrophoresis of the DNA extracted from *P. aeruginosa* following the above-mentioned method. A trial was undertaken to investigate whether the incubation periods influence the separation of the DNA. The extracted DNA was

placed in 100 µl of 1x SA buffer and, incubated with 15 units of *SpeI* for 3, 6 and 18 hours. The DNA was incubated overnight in 100 µl of 1x SA buffer without the enzyme, and used as a control.

4.3.1.3. Method 3 (PFGE)

A method described by Ouoba *et al.* (2004) was followed in order to extract *B. cereus* DNA. *Bacillus cereus* cells were collected from an overnight-incubated culture on TSA media and suspended in SE buffer. Bacterial suspensions were adjusted to 1.5 absorbance value at a wavelength of 600 nm using SE buffer. Bacterial suspensions, 2 ml, were placed in a centrifuge tube and was centrifuged at 8500 rpm for 5 minutes and re-suspended in 2 ml SE buffer. A 2% w/v low melting point agarose was prepared in SE buffer. Prepared molten agarose (approximately 50°C), 0.5 ml, was mixed with 0.5 ml bacterial suspensions, transferred immediately to a plug mould and solidified on ice. The prepared bacterial plugs were placed in a 2.5 ml lysis buffer containing 5 mg lysozyme (to obtain a final concentration of 2 mg/ml) and incubated overnight at 37°C. The following day, lysis buffer was removed followed by an addition of 2.5 ml proteolysis buffer containing 5 mg proteinase K (2 mg/ml), and incubated overnight at 53°C. The plugs were washed 5 times with EDTA (50 mM EDTA at pH 8.5) for 30 minutes at room temperature and stored in TE buffer-3 until required (no longer than 4 weeks).

Overnight digestions of the DNA were carried out using 15 units of *SmaI* (10000 units/ml) (Sigma-Aldrich, UK) restriction enzyme in 100 µl of 1x digestion buffer SH at 25°C. Lambda Ladder and digested DNA were loaded into a 1% w/v agarose gel containing ethidium bromide (0.5 µ/ml) and sealed using 1% w/v molten agarose. Electrophoresis was carried out for 18 hours, with a switch time ramped from 1 to 25 seconds at 14°C. The gel was visualised and photographed using a Gel-Doc system.

4.3.1.4. Method 4 (PFGE)

Bacillus cereus cells were collected from an overnight-incubated culture on nutrient agar media and suspended in a 5 ml PET-IV buffer-1. The density of the bacterial cells was adjusted to 1.5 at a wavelength of 600 nm using PET-IV buffer-1 and washed twice by centrifugation at 8500 rpm for 5 minutes using PET-IV buffer-1. Lysozyme was added to the bacterial suspensions to achieve a final concentration of 2 mg/ml. A 2% w/v low melting-point agarose was prepared in water. A bacterial cell suspension (0.5 ml) was added to 0.5 ml of the 2% w/v low melting-point agarose (approximately 50°C), transferred immediately to

a plug mould and solidified on ice. The bacteria were lysed using lysozyme (0.5 mg/ml) as mentioned in Section 4.3.1.3 and rinsed with sterilised distilled water followed by incubation in 2.5 ml proteolysis buffer with proteinase K (2.0 mg/ml) for 48 hours at 50°C. After incubation, the DNA plugs were washed 5 times, for 30 minutes each time, using 2.5 ml of 50 mM EDTA at pH 8.7, stored in 5 ml TE buffer-3 at 4°C and used within 4 weeks.

Plugs were placed in 100 μl of 1x SH buffer and digestion of DNA was carried out overnight using 15 units of SmaI (10000 units/ml) (Sigma-Aldrich, UK) at 25°C. Digested DNA and Lambda Ladder were placed into a 1% w/v agarose gel containing ethidium bromide (0.5 μ/ml), sealed with a small amount of 1% w/v molten agarose and electrophoresis was carried out for 18 hours with an initial switch time of 1 second to a final switch time of 25 seconds at 14°C. The gel was visualised and photographed using a Gel-Doc system.

4.3.1.5.   Optimising PFGE Trials

To assess the difficulty in obtaining well-defined DNA profiles from B. cereus, the above-mentioned experiment (Section 4.3.1.4) was then repeated using different parameters, which are shown in Table 4.2. Digested DNA and Lambda Ladder were placed into a 1% w/v agarose gel containing ethidium bromide (0.5 μg/ml), sealed using a 1% w/v molten agarose and the electrophoresis was carried out as described in Section 4.3.1.4. The gel was visualised and photographed using a Gel-Doc system (see Table 4.2).

Table 4.2: Extraction, purification and digestion of B. cereus DNA using various parameters for PFGE analysis.

Parameters	Methods
Preparation of plugs using bacterial cells suspensions at various cell concentrations	The bacterial cells density was adjusted to 0.2, 0.5, 0.8, 1.0 and 1.2 at a wavelength of 600 nm using PET-IV-1 buffer. Lysis and proteolysis of the bacterial cells were undertaken using lysozyme (0.5 mg/ml) and proteinase K (2 mg/ml) followed by washing in TE buffer as described in Section 4.1.3.4. DNA digestion was carried out using 15 units of SmaI as mentioned in section 4.3.1.4.
Proteolysis using proteinase K at various concentrations	The plugs were prepared and lysis of the bacterial cells was carried out as mentioned in Section 4.3.1.4. The plugs were subsequently placed in proteolysis buffer containing either 0.5 mg/ml or 1 mg/ml proteinase K followed by a washing in TE buffer (see Section 4.3.1.4). DNA was digested as described in Section 4.3.1.4.
Digestion of DNA using various restriction enzyme concentrations	The plugs were prepared and lysis of the bacteria cells was carried out using the protocol described in section 4.3.1.4, followed by a proteolysis in 1 mg/ml proteinase K buffer and washed 5 times in TE buffer (see Section) 4.3.1.4. The plugs were placed in 100 1x SH buffer and the DNA was digested overnight in using 15, 20, 25, 30 and 35 units of SmaI at 25°C.

4.3.1.6.   Method 5 (PFGE)

A DNA extraction kit (CHEF genomic DNA plug kits, 170-3591, Bio-Rad, UK) was used to extract DNA and to prepare the plugs for electrophoresis. The corresponding company, Bio-Rad (USA) supplied the DNA extraction and preparation protocol. The DNA was digested using 15 units of *Sma*I as described in Section 4.3.1.4. Digested DNA and Lambda Ladder were placed into a 1% w/v agarose gel containing ethidium bromide (0.5 μg/ml); sealed using 1% w/v molten agarose and the electrophoresis was carried out as mentioned in Section 4.3.1.4. The gel was visualised and photographed using a Gel-Doc system.

4.3.1.7.   Method 6 (PFGE)

This PFGE protocol for *B. cereus* DNA sequencing was provided by Dr. Babetta L. Marrone and Yulin Shou, Los Alamos National Laboratory, Los Alamos (Ferris *et al.*, 2004; Zhong *et al.*, 2007). *Bacillus cereus* colonies were incubated overnight on a nutrient or PEMBA agar media. A single colony was added to 5 ml tryptone soya broth (TSB) and incubated in a 37°C water bath with an agitation speed of 200 rpm for 14-16 hours. The following day 5 μl of the overnight-incubated bacterial culture was added to 5 ml fresh TSB and incubated for a further 4 hours in a 37°C water bath with an agitation speed of 200 rpm. After incubation, the bacterial culture was centrifuged at 3200 *g* followed by a washing in 1 ml PET-IV buffer-2 at 8000 rpm using a centrifuge and suspended in 0.15 ml EC lysis buffer. A 0.7% w/v low melting-point agarose was prepared in PET-IV buffer-2 and dissolved using a microwave. The melted or dissolved low melting-point agarose was stored at 4°C and re-melted as required. Bacterial suspensions in EC lysis buffer was mixed with 0.15 ml of the prepared molten agarose, dispensed in a plug mould and solidified on ice.

Plugs were placed in 2 ml centrifuge tubes and incubated overnight in 1 ml EC lysis buffer, containing 2 mg lysozyme, in a 37°C water bath with an agitation speed of 200 rpm. The following day 1 mg proteinase K was added to each of the centrifuge tubes, followed by a further 2 hours incubation in a 55°C water bath without agitation. The test tubes were inverted occasionally (approximately every 30 minutes). The plugs were then incubated at 70°C for 30 minutes and the EC lysis buffer removed, followed by a washing in water (pre-heated to70°C) at 70°C for 30 minutes. Plugs were placed in 1 ml cold sterilised water, stored at 4°C and electrophoresis was carried out the following day.

Approximately 1 mm thick slice was cut from each of the DNA plugs and rinsed in 200 μl digestion buffer SH (1x). Digestion was carried out using 20 units of *Sma*I (10000 units/ml)

(Sigma-Aldrich, UK) in 200 μl SH buffer (1x) for 2 hours in a 25°C water bath with an agitation speed of 200 rpm. Digested DNA and Lambda Ladders were loaded onto a 1% w/v agarose gel containing ethidium bromide (0.5 μg/ml) and sealed using 1% w/v molten agarose. The electrophoresis was carried out for 20 hours with an initial switch time 2.2 seconds to a finial switch time 54.2 seconds at 14°C. The gel was visualised and photographed using a Gel-Doc system.

4.3.2. Electrophoresis Trials Using a Mini Electrophoresis Unit

A marker, 1 kb DNA Ladder, (New England Biolabs, UK) and super-coiled DNA pBluescript (Stratagene, UK), which were supplied by Dr. Stuart Allen (School of Health, The University of Northampton) was used to validate the electrophoresis methods. The pBluescript DNA was digested using 1 μl Smal (10000units/ml) for 30 minutes at 25°C. Liquid DNA samples for electrophoresis were prepared as follows:

1. Preparation 1: 1 μl DNA with 2 μl 6x loading buffer and 9 μl sterilised deionised water
2. Preparation 2: see Table 4.3

Table 4.3: Liquid DNA samples (DNA marker (1 kb), super-coiled DNA pBluescript) preparation-2.

Component	Super-coiled DNA pBluescript (digested)	Super-coiled DNA pBluescript (un-digested)	DNA marker (1 kb)
DNA samples	1 μl	1 μl	0.5 μl
Digestion buffer SH (1x)	2 μl	2 μl	0
Smal (μl)	1 μl	0	0
Sterilised deionised water (μl)	16 μl	17 μl	8 μl
Loading buffer 5 x	5 μl	5 μl	1.5 μl

The pBluscript DNA (digested and undigested), Lambda Ladder and DNA marker were placed into a 1% w/v agarose gel. The wells were sealed using 1% (w/v) molten agarose after placing the Lambda Ladders into 1% agarose gel. Electrophoresis was conducted using a smaller electrophoresis tank (Mini Horizontal Electrophoresis Unit, Scic-plus Gel Units, Scientific Laboratory Supply, UK) at 100 V for 30 minutes at room temperature. The gel was visualised and photographed using a Gel-Doc system. Electrophoresis was undertaken for a further 30 minutes, 2 hours and 24 hours if migration of the DNA samples had not occurred.

4.3.3.   Electrophoresis of Lambda Ladder

Difficulties arose with the separation of the bands during the electrophoresis of Lambda Ladder (48.4-970 kb). The Lambda Ladder was subsequently treated with 0.5x TBE buffer as described in Section 2.17.1, to achieve clear separations. Electrophoresis was carried out using a 1% w/v agarose gel (prepared in 0.5x TBE buffer) containing ethidium bromide (0.5 μg/ml) for 24 hours with an initial switch time of 45 seconds to a final switch time of 90 seconds at 14°C and at 6 V/cm.

4.3.4.   Preparation of Loading Buffer

A 6 x DNA loading buffer was prepared using 25 mg bromophenol blue, 5 ml glycerol (≥99% w/v), 5 ml deionised water and few drops of tris-EDTA buffer at pH 8.0. A 5x loading buffer was prepared using 30% v/v glycerol, 10 mM tris-EDTA (pH 7.5) and 0.05% w/v bromophenol blue.

4.4.   Results and Discussion

4.4.1.   Pulsed-Field Gel Electrophoresis (PFGE) of the *Pseudomonas aeruginosa* DNA

Figure 4.2 shows the image of the agarose gel that was obtained by following a PFGE protocol described in Section 4.3.1.1. Presence of the bacterial DNA and Lambda Ladders in the wells is evident by the bright fluorescence due to the ethidium bromide staining (Figure 4.2). It appears that bacterial DNA as well as Lambda Ladder remained in the wells, instead of travelling towards the bottom of the gel or anodes through the agarose gel pores. This suggests that there may have been experimental errors during the electrophoresis. Electrophoresis of the Lambda Ladder was therefore undertaken in order to evaluate the electrophoresis method to minimise the possibility of experimental errors. The results obtained are shown in Figure 4.3.
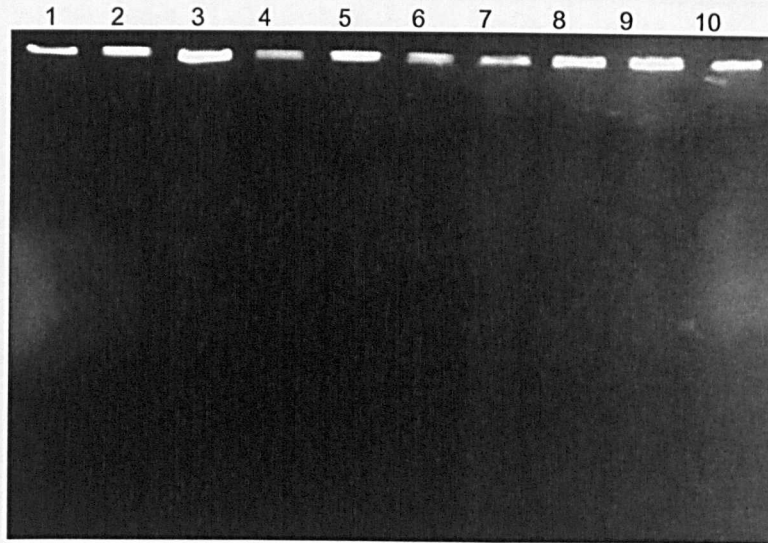
Figure 4.2: Pulsed-field gel electrophoresis using the protocol described in Section 4.3.1.1. Lanes 1 and 10: Lambda Ladder (45.8-970 kb).

Figure 4.3 shows, although the Lambda Ladder (lanes 1-3) migrated towards the bottom of the gel; the migration through the gel even after 20 hours electrophoresis was limited. A number of factors, such as agarose gel concentrations and electrophoresis buffer are may be responsible for the phenomenon. Electrophoresis of the Lambda Ladder and a 1 kb DNA marker was trialled using a smaller electrophoresis unit (see Section 4.3.2) in order evaluate the electrophoresis gel and buffer concentrations used during the PFGE analysis. The evaluation was undertaken using a smaller electrophoresis unit, as the trials with the smaller unit can be accomplished within a short period of time using a reduced quantity of the chemicals and buffers. The results obtained are shown in Figure 4.4.
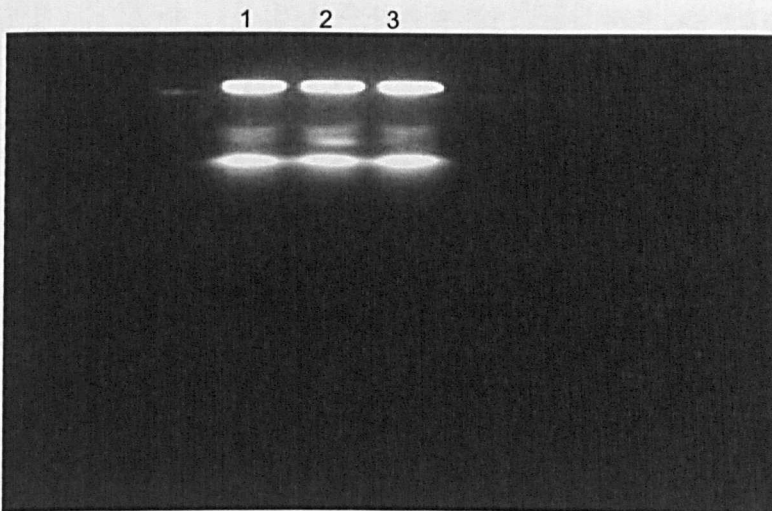
Figure 4.3: Pulsed-field gel electrophoresis of the Lambda Ladders, 45.8-970 kb, (lanes 1-3).

It appears that 1 kb DNA marker (lane 1) transferred through the gel to anodes with a visible clear band patterns, whereas migration of the Lambda Ladder (lanes 2-3) was limited (Figure 4.4). This experiment established that the electrophoresis gel concentration and buffer used for the PFGE analysis were appropriate. The limited migration is probably due to the use of damaged Lambda Ladder. The Lambda Ladder that was used in these experiments (Sections 4.3.1.1 and 4.3.2) may have been stored for a long period of time, which may have influenced the band separations. However, a restoration technique was followed as described in Section 2.17.1 to restore the Lambda Ladder.
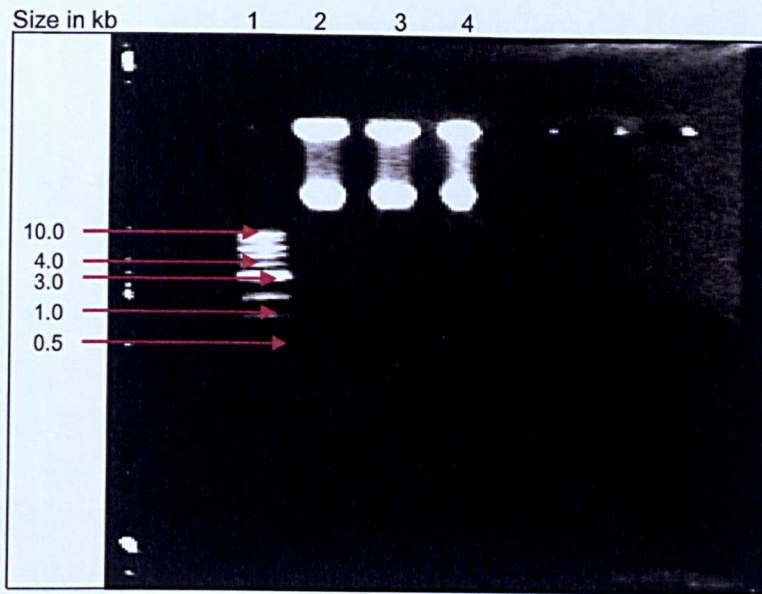
Figure 4.4: Electrophoresis of the 1 kb DNA marker (lane 1) and Lambda Ladder, 45.8-970 kb, (lanes 2-4), using a mini electrophoresis unit.

Figure 4.5 shows an image of the agarose gel when PFGE was carried out after following a restoration technique (see Section 2.17.1) to achieve band separation of the Lambda Ladder. The restored Lambda Ladder (lane 1 and 2) migrated through the agarose gel with clear visible band patterns, this shows that the restoration method was successful.
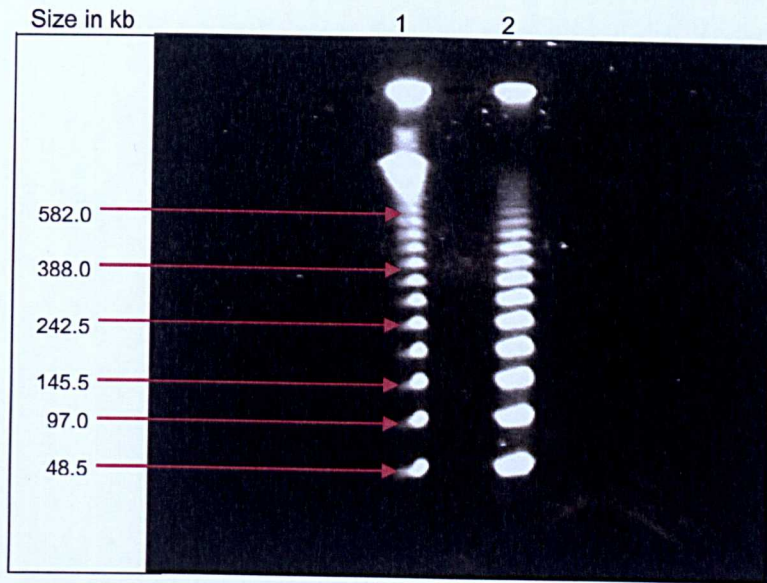
Figure 4.5: Pulsed-field gel electrophoresis of the Lambda Ladder, 45.8-970 kb, (lanes 1 and 2), following a restoration technique (see Section 2.17.1).

It has already been established (see Figure 4.4) that the agarose gel concentration and electrophoresis buffer used for the PFGE analysis were appropriate for the experiments. The reason for the DNA (Figure 4.2) to remain in the well may not be therefore due to the use of an inappropriate buffer or gel concentration. It was thought that the followed protocol (Section 4.3.1.1) may be unsuitable for this study. Therefore, a new method as described in Section 4.3.1.2 was trialled to extract DNA from *P. aeruginosa*.

Figures 4.6 shows the results, when the PFGE method was carried out with the DNA that was extracted from *P. aeruginosa* following the method described in Section 4.3.1.2. No visible band patterns was occurred when electrophoresis of the *Spe*I digested *P. aeruginosa* DNA (lanes 4, 5, 8, 9, 12 and 13) was carried out.
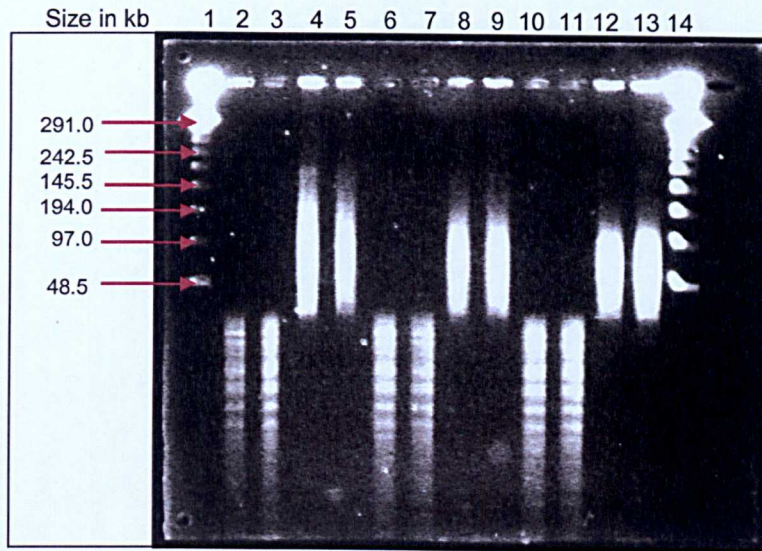
Figure 4.6: Pulsed-field gel electrophoresis of the DNA extracted from *P. aeruginosa* (Section 4.3.1.2). Lanes 1 and 14: Lambda Ladders (45.8-970 kb); lanes 4, 5, 8, 9, 12 and 13: *Spe*I digested *P. aeruginosa* DNA.

A trial was undertaken to investigate the effect of the incubation periods on the DNA separations. The extracted DNA was incubated with 15 units of *Spe*I for 3, 6 and 18 hours. The DNA was also incubated overnight in SA buffer (1x) without the enzyme and used as a control. The results obtained are shown in Figure 4.7.

It appears that the incubation period had no influence in this regard as all the trialled DNA samples appeared as unclear streaks (lanes 2-6, 8, 9, 11-13) regardless the incubation period with *Spe*I. Moreover, PFGE of *P. aeruginosa* DNA, which was only incubated in buffer showed the same results as mentioned above (lanes 3, 7 and 10). This suggests that the buffer itself may have an impact on *P. aeruginosa* DNA and therefore, electrophoresis was carried out using *P. aeruginosa* DNA without a prior incubation to determine whether the unclear streaks were due to the digestion buffer (SA). The results obtained are shown in Figure 4.8.
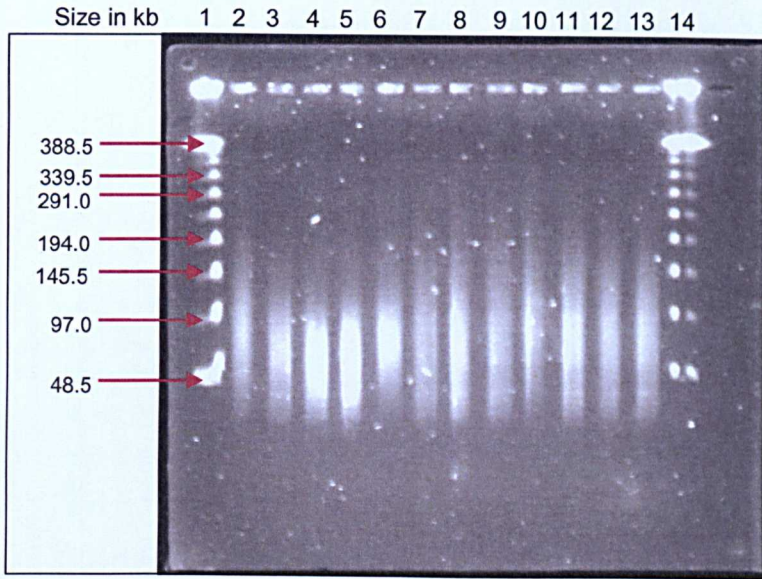
Figure 4.7: Pulsed-field gel electrophoresis of the *P. aeruginosa* DNA that was incubated with *Sma*l for various incubation periods. Lanes 1 and 14: Lambda Ladder (45.8-970 kb), lanes 2, 6 and 10: *P. aeruginosa* DNA without enzymatic digestion, lanes 3, 7 and 11: *P. aeruginosa* DNA incubated with *Spe*l for 3 hours, lanes 4, 8 and 12: *P. aeruginosa* DNA incubated with *Spe*l for 6 hours, lanes 5, 9 and 13: *P. aeruginosa* DNA incubated with *Spe*l for18 hours.

Similar to Figure 4.7, electrophoresis of the *P. aeruginosa* DNA (lanes 2 and 3) showed no clear band patterns (Figure 4.8), indicating the inferior quality of the extracted DNA. The unclear streaks (lanes 2 and 3) also suggest that the trialled DNA may have been degraded (Corkill *et al.*, 2000; Fawley and Wiocox, 2002). Degradation of the DNA may have occurred during the storage or during extraction. Lysis of the bacterial cells may release enzyme nucleases, which may cause DNA degradation (Corkill *et al.*, 2000; Herschleb *et al.*, 2007) resulting in unclear streaks. Degradation of DNA may also cause by the presence of excess protein debris after cell lysis. Proteinase K removes the excess protein or nucleases through digestion purifying the DNA molecules (Pelczer *et al.*, 1993; Corkill *et al.*, 2000; Schwalbe *et al.*, 2007). Presence of a large amount of protein due to improper proteolysis may therefore be responsible for the unclear streaks (Schwalbe *et al.*, 2007) during electrophoresis.
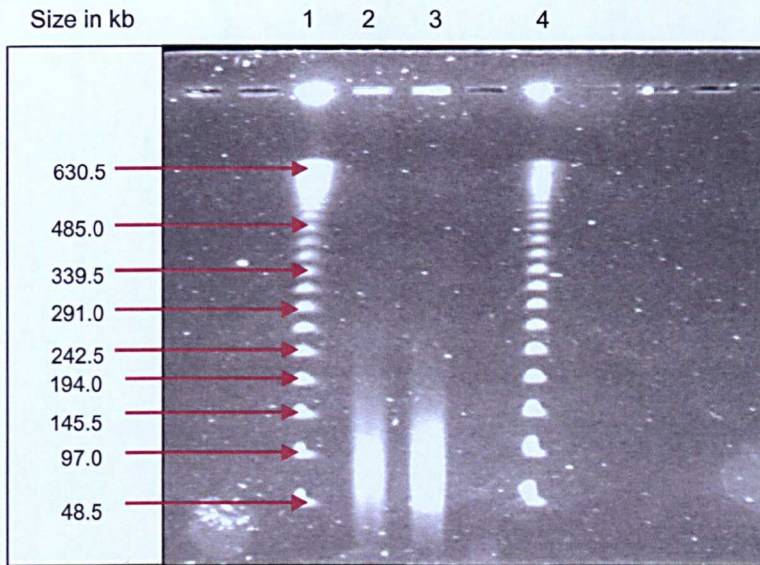
Figure 4.8: Pulsed-field gel electrophoresis of undigested *P. aeruginosa* DNA. Lanes 1 and 4: Lambda Ladder (45.8-970 kb), Lanes 2 and 3: *P. aeruginosa* DNA without prior incubation or digestion.

Due to failure of the above-mentioned protocol, a new protocol as described in Section 2.17.3 was therefore trialled to obtain quality DNA from *P. aeruginosa*. The results obtained are shown in Figures 4.9 and 4.10.

Figure 4.9 shows that *P. aeruginosa* DNA remained in the wells, when electrophoresis was carried out with undigested DNA, indicating presence of un-degraded DNA. Alternatively, electrophoresis of the *Spe*I digested *P. aeruginosa* DNA (lanes 1 and 2) showed distinctive band patterns (Figure 4.10). The DNA extracted from *P. aeruginosa* was digested using *Spe*I restriction endonuclease. *Spe*I recognises the 6-base sequence, ACTAGT, and the cleave site is AC as shown below;

$$5'\cdots A \mathbin{|} C-T-A-G-T-\cdots 3'$$
$$3'\cdots T-G-A-T-C \mathbin{|} A-\cdots 5'$$
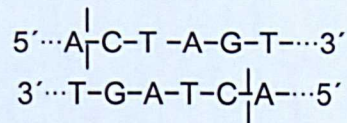
In this instance, it appears that the DNA of *P. aeruginosa* ATCC10145 (lane 2) has 15 *Spe*I sites, as the DNA was cleaved into 15 fragments. Based on the results obtained, the followed PFGE protocol was found to be appropriate for the *P. aeruginosa* DNA profiling. In addition, the DNA extracted from both of the *P. aeruginosa* ATCC10145 (lane 2) and *P. aeruginosa* isolated from the conventional pre-soaking effluent (lane 1) showed identical band patterns. This confirms that the origins of the DNA were not only the same species but also the same strain.
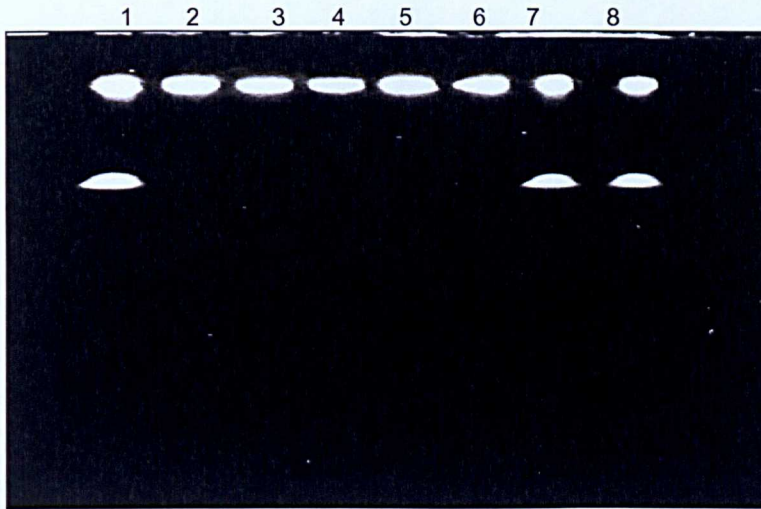
Figure 4.9: Pulsed-field gel electrophoresis of *P. aeruginosa* DNA (undigested). The DNA was extracted following a new method as described in Section 2.17.3. Lanes 1, 7 and 8: Lambda Ladder (45.8-970 kb) and lanes 2-6: undigested *P. aeruginosa* DNA.
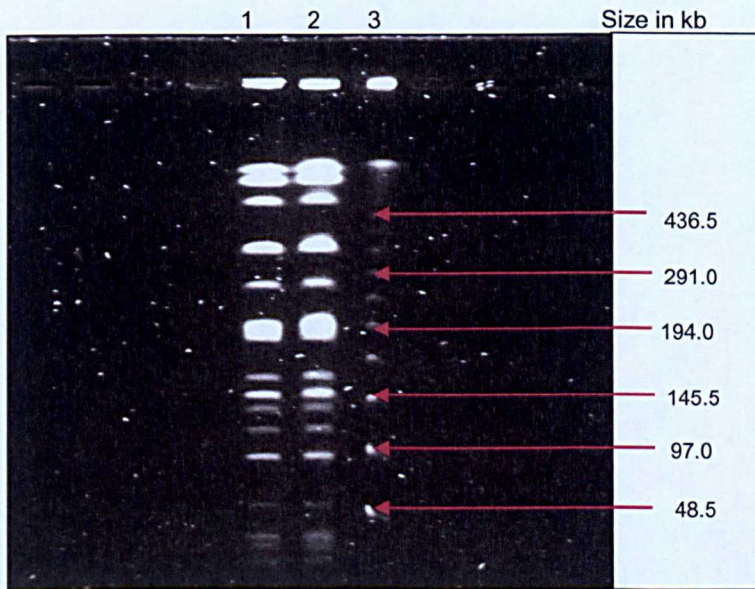
Figure 4.10: Pulsed-field gel electrophoresis of the *P. aeruginosa* DNA digested using *Spe*I. The DNA was extracted following a new method as described in Section 2.17.3. Lane 3: Lambda Ladder (45.8-970 kb). Lane 1: DNA of *P. aeruginosa* (isolated from conventional the pre-soaking effluent) and lane 2: DNA of *P. aeruginosa* ATTCC10145.

4.4.2. *Pulsed-Field Gel Electrophoresis (PFGE) of the <u>Bacillus cereus</u> DNA*

Figures 4.11 and 4.12 show the results obtained when PFGE was carried out with the DNA, extracted from *B. cereus,* following the protocol described in Section 4.3.1.3. It appears that *B. cereus* DNA did not migrate through the agarose gel (lanes 2-10) when electrophoresis of the undigested DNA was carried out (Figure 4.11), indicating the presence of un-degraded DNA.
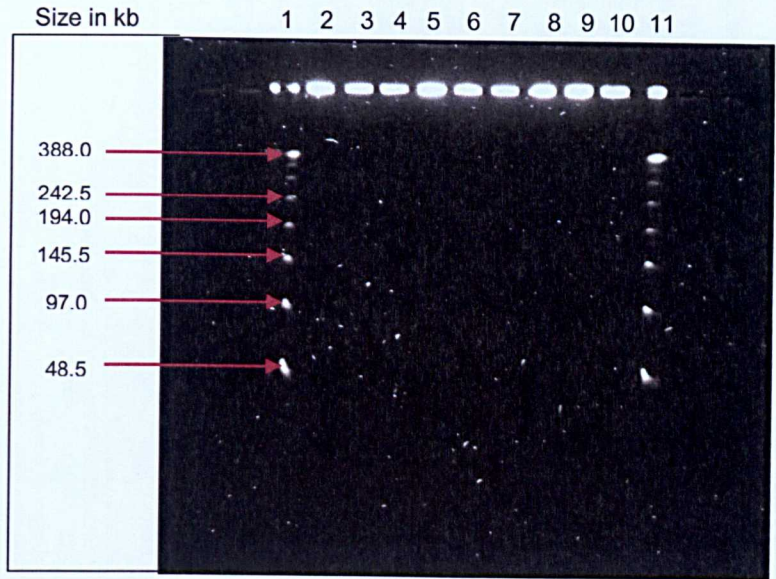
Figure 4.11: Pulsed-field gel electrophoresis of the *B. cereus* DNA (Section 4.3.1.3). Lanes 1 and 11: Lambda Ladder (45.8-970 kb). Lanes 2- 10: undigested *B. cereus* DNA.

Figure 4.12 shows, an image of the agarose gel after electrophoresis of the DNA that was extracted from *B. cereus* and was digested using a restriction enzyme *Sma*I. The DNA appeared to remain in the well. *Sma*I recognises a 6-base sequence (CCCGGG) with the cleave site G and C in a DNA molecule as shown bellow, and as result *Sma*I breakdowns a large DNA molecule into smaller fragments

$$5'\cdots C\text{-}C\text{-}C\text{-}G\text{-}G\text{-}G\text{-}\cdots3'$$
$$3'\cdots G\text{-}G\text{-}G\text{-}C\text{-}C\text{-}C\text{-}\cdots5'$$
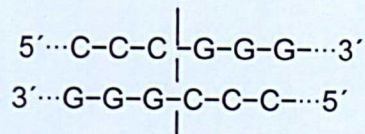
Electrophoresis of the digested DNA therefore separates the DNA fragments resulting in a distinctive band pattern. However, if the restriction enzyme fails to cleave the DNA into fragments, migration of DNA may not occur. Therefore, insufficient digestion was thought to be an obstacle for not obtaining DNA band separation.
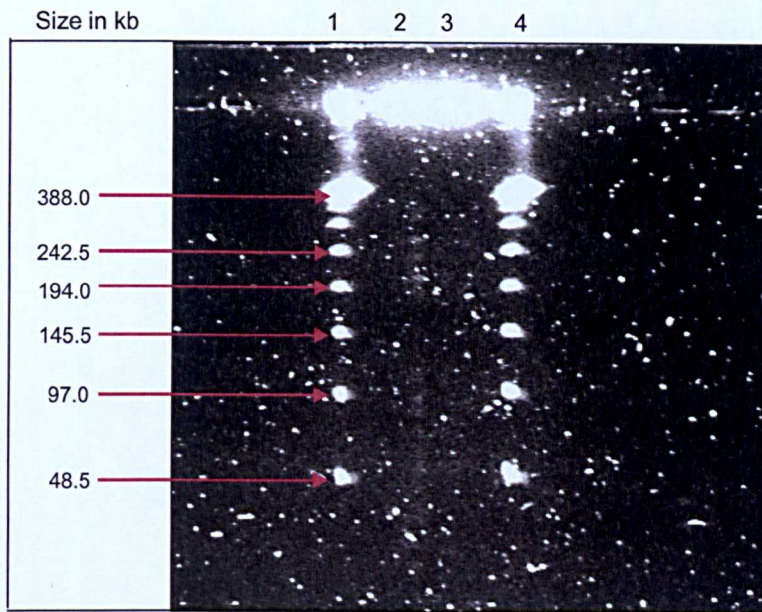
Figure 4.12: Pulsed-field gel electrophoresis of the *B. cereus* DNA digested with *Sma*I (Section 4.3.1.3). Lanes 1 and 4: Lambda Ladder (45.8-970 kb). Lanes 2 and 3: *B. cereus* DNA digested with *Sma*I.

*Sma*I available within the school was used for this experiment, which may have been stored for a long period of time. Prolong storage may cause a reduction in the enzyme activity. In order to investigate *Sma*I activity, a trial was conducted using a mini electrophoresis unit (see Section 4.3.2). The results obtained are shown in Figure 4.13.

Figure 4.13 shows the presence of two luminous bands (lane 2), when electrophoresis of the undigested pBluescript plasmid DNA was carried out. The pBluescript DNA was in super-coiled form (faster moving band). Due to the compact shape, a super-coiled DNA is able to migrate faster through the pores of an agarose gel than a linear or circular DNA (Madigan *et al.*, 1997). The pBluescript DNA may also contain circular DNA (Kang *et al.*, 2005; Lilley and Houghton, 1979; Zhang *et al.*, 2006). Figure 4.14 shows schematic structure of a circular and super-coiled DNA. Figure 4.13 shows the undigested pBluescript DNA (lane 2) was migrating faster than the digested pBluescript DNA (lane 3) suggesting that the restriction enzyme *Sma*I successfully cleaved the super-coiled structure of a pBluescript plasmid DNA into a linear structure (lane 3). However, this experiment showed that the restriction enzyme *Sma*I is active; therefore, the unsuccessful band separations of the *B. cereus* DNA was not due to the reduced activity of *Sma*I. It is possible that the followed protocol was not suitable for extraction of *B. cereus* DNA in this instance; therefore, a new method described in Section 4.3.1.4 was followed and the results obtained are shown in Figure 4.15.
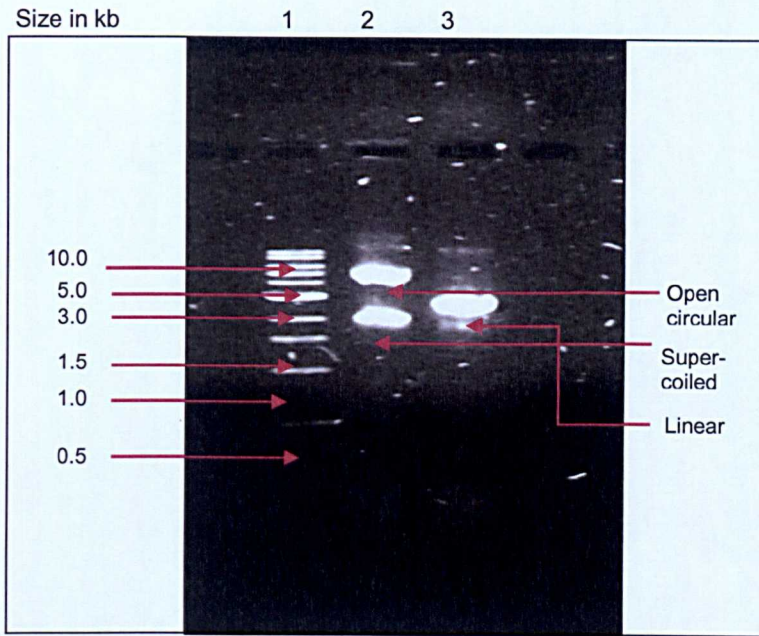
Figure 4.13: Electrophoresis of undigested and digested pBluescript DNA samples using a mini electrophoresis unit. Lane 1: DNA marker (1 kb), lane 2: undigested pBluescript DNA and lane 3: pBluscript DNA digested with *Sma*I.
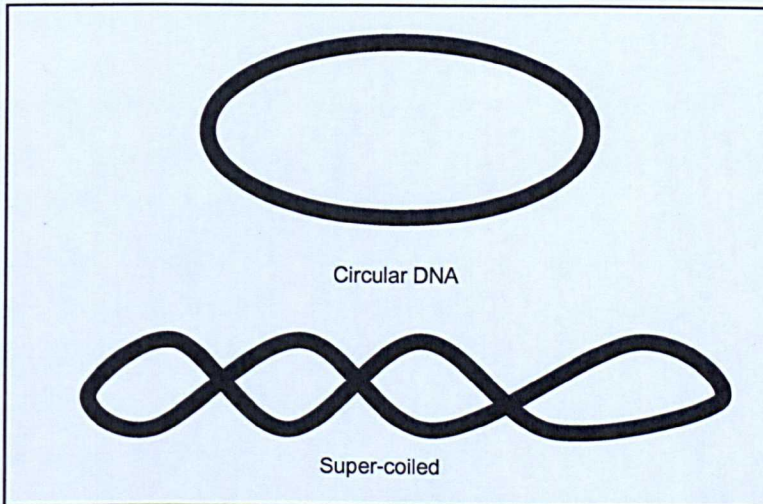
Figure 4.14: Schematic structure of a super-coiled and circular DNA (Madigan *et al.*, 1997).

It appears that both of the *Spe*I and *Sma*I cleaved *P. aeruginosa* DNA (lane 3 and 4) into smaller units, whilst no digestion was observed on the *B. cereus* DNA. Digested (lanes 6 and 7) as well as undigested (lane 5) *B. cereus* DNA remained in the wells instead of progressing through the agarose gel towards the bottom of the gel (Figure 4.15). It has already been shown that the *Sma*I was active and so far two different protocols have been followed, none-the-less, *B. cereus* DNA appeared not to be digested.
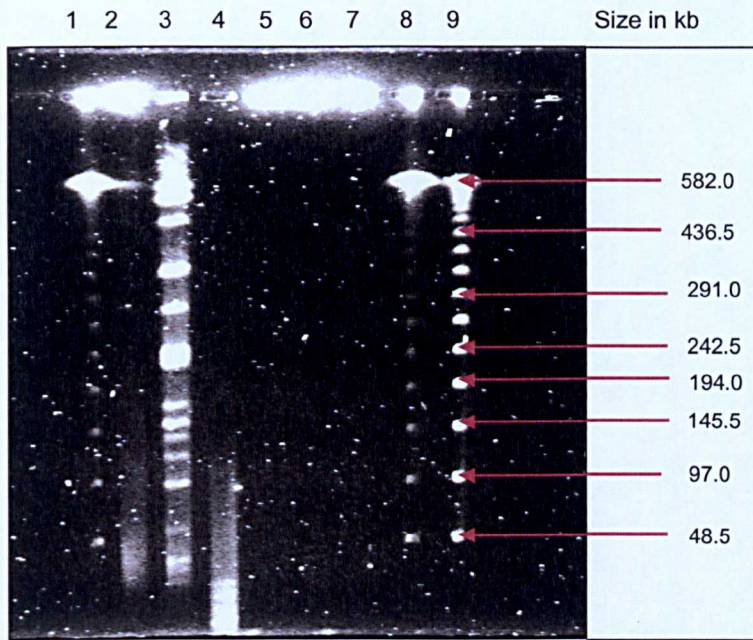
Figure 4.15: Pulsed-field gel electrophoresis of *P. aeruginosa* **and** *B. cereus* **DNA. The DNA was digested using** *Spe*I **and** *Sma*I. **Lanes 1, 8 and 9: Lambda Ladder (48.5-970 kb). Lane 2: undigested** *P. aeruginosa* **DNA. Lane 3 and 4:** *P. aeruginosa* **DNA digested with** *Spe*I **and** *Sma*I **respectively; lane 5: undigested** *B. cereus* **DNA; lanes 6 and 7:** *B. cereus* **DNA digested with** *Spe*I **and** *Sma*I **respectively.**

Inadequate lysis and proteolysis may be responsible for the unsuccessful band separation of the *B. cereus* DNA. Peptidoglycan is a major component of the bacterial cell wall. Lysozyme lysis or disrupts the cell walls by breaking the 1,4-glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid in peptidoglycan (Madigan *et al.*, 1997). Proteinase K may also assist the lysis of the bacterial cells. A complete cell lysis is important for extraction of quality DNA (Moré and Herrick, 1994). *Bacillus cereus* is a Gram-positive bacterium; generally, gram-positive bacteria have thicker cell walls than gram-negative bacteria (Madigan *et al.*, 1997). Therefore, difficulties may arise in lysis of the cell wall of Gram-positive bacteria. Incomplete lysis of a cell wall may prevent the release of DNA from the cells. The presence of a high volume of protein may also inhibit the enzymatic activity on the DNA molecules. Adequate proteolysis may be required to remove protein (Corkill *et al.*, 2000; Schwalbe *et al.*, 2007). Presence of a large quantity of bacterial cells may also lead to improper lysis and proteolysis. A trial was therefore undertaken in order to study the lysis or proteolysis activity, when *B. cereus* DNA was extracted using bacterial cells of various cell concentrations (see Section 4.3.1.5). The results obtained are shown in Figure 4.16.

The DNA concentrations may vary according to the cell concentrations used (Figure 4.16). A gradual increase in the brightness illustrates an increase in the DNA quantity and this occurred (lanes 2-12) with the increase in the bacterial cell concentrations. However, it

appears that the bacterial cell concentrations had no effect on the digestion of the *B. cereus* DNA. *Bacillus cereus* DNA remained in the wells regardless of the DNA/cell concentrations and did not migrated through the gel. On the other hand, DNA extracted from *P. aeruginosa* migrated to the bottom of the gel. Further experiments were undertaken in order to determine the effect of proteinase K, when used at various concentrations; during proteolysis of the *B. cereus* cells (see Section 4.1.3.5). In addition, a Bio-Rad DNA extraction kit was used (see Section 4.3.1.6) in order to extract *B. cereus* DNA. The results obtained are shown in Figures 4.17 and 4.18.
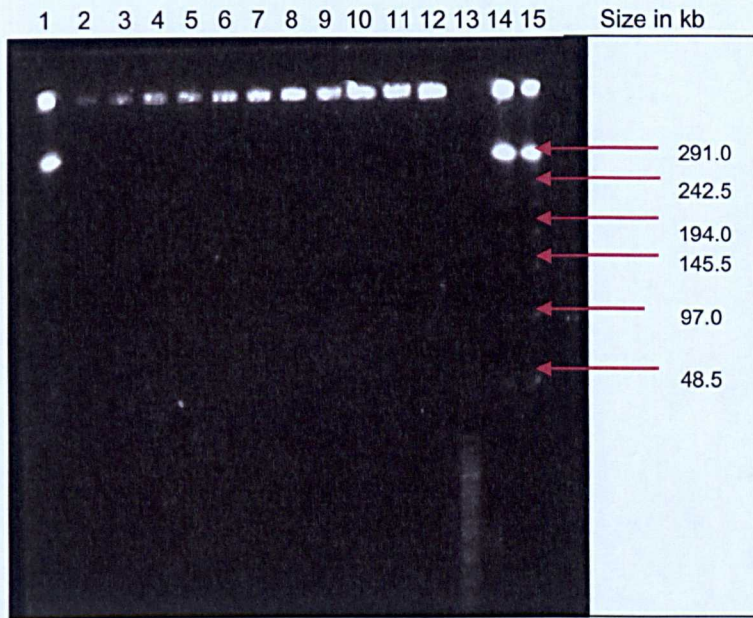
Figure 4.16: Pulsed-field gel electrophoresis of *B. cereus* DNA, when DNA was extracted using bacterial cells of various cell concentrations. Lanes 1, 14 and 15: Lambda Ladder (48.5-970 kb). Lanes 2, 4, 6, 8 are 10: undigested *B. cereus* DNA extracted using bacterial cells suspensions of 0.2, 0.5, 0.8, 1.0 and 1.2 absorbance value respectively. Lanes 3, 5, 7, 9, 11 and 12: digested *B. cereus* DNA extracted using bacterial cells suspensions of 0.2, 0.5, 0.8, 1.0 and 1.2 absorbance value respectively; Lane 13: *P. aeruginosa* DNA, digested using *Sma*I (15 units).

Figure 4.17 shows that *B. cereus* DNA (lanes 7 and 9) treated with 2 mg/ml proteinase K remained in the wells whereas, the *B. cereus* DNA (lanes 11 and 13), treated with 1.0 mg/ml proteinase K, migrated through the gel with definite band profiles. See Section 4.1.3.5 for the details of the protocol. However, it appears that only a limited quantity of the *B. cereus* DNA migrated through the gel. The appearance of a limited quantity of DNA in the gel was possibly due to an inadequate enzymatic digestion. A trial was therefore carried out in order to observe the effect of an increased *Sma*I concentration on DNA that was extracted from *B. cereus* and treated with 1 mg/ml proteinase K (see Section 4.1.3.5). The DNA was incubated with 20, 25, 30 and 35 units of *Sma*I (10000 units/ml). A trial was also undertaken (see

Section 4.1.3.5) to determine whether proteolysis using a smaller quantity of proteinase K assist in obtaining clear defined DNA band patterns. The results obtained are shown in Figure 4.18.
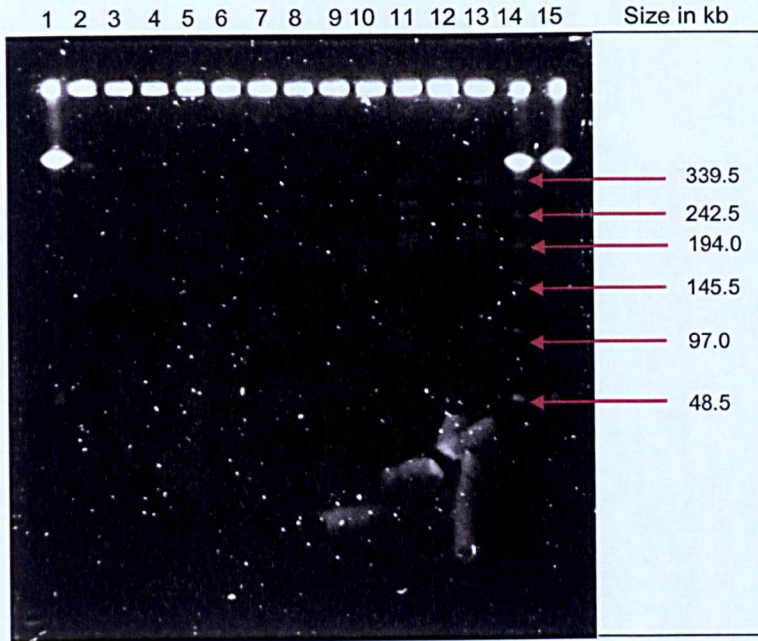
Figure 4.17: Pulsed-field gel electrophoresis of *B. cereus* DNA, when treated with either various proteinase K concentrations or extracted following new protocol as described in Section 4.3.1.6. Lanes 1, 14 and 15: Lambda Ladder (48.5-970 kb). Lanes 2 and 4: undigested *B. cereus* DNA (extracted using a Bio-Rad DNA extraction kit). Lanes 3 and 5: digested *B. cereus* DNA (extracted using a Bio-Rad DNA extraction kit). Lanes 6 and 8: undigested *B. cereus* DNA (proteolysed using 2 mg/ml proteinase K). Lanes 7 and 9: digested *B. cereus* DNA (proteolysed using 2 mg/ml proteinase K). Lanes 10 and 12: undigested *B. cereus* DNA (proteolysed using 1 mg/ml proteinase K). Lanes 11 and 13: digested *B. cereus* DNA (proteolysed using 1 mg/ml proteinase K). Digestion was carried out using 15 units of *Sma*I.

The presence of indistinct bands (lanes 4-8) in the gel shows that only a low concentration (visible as indistinct lines) of DNA migrated through the gel (Figure 4.18). This shows an increased quantity of the restriction enzyme *Sma*I, did not have an impact on the digestion of *B. cereus* DNA. It also appeared that proteolysis of the DNAs using a 0.5 mg/ml proteinase K (lane 1 and 2) did not improve the migration of the *B. cereus* DNA through the gel.
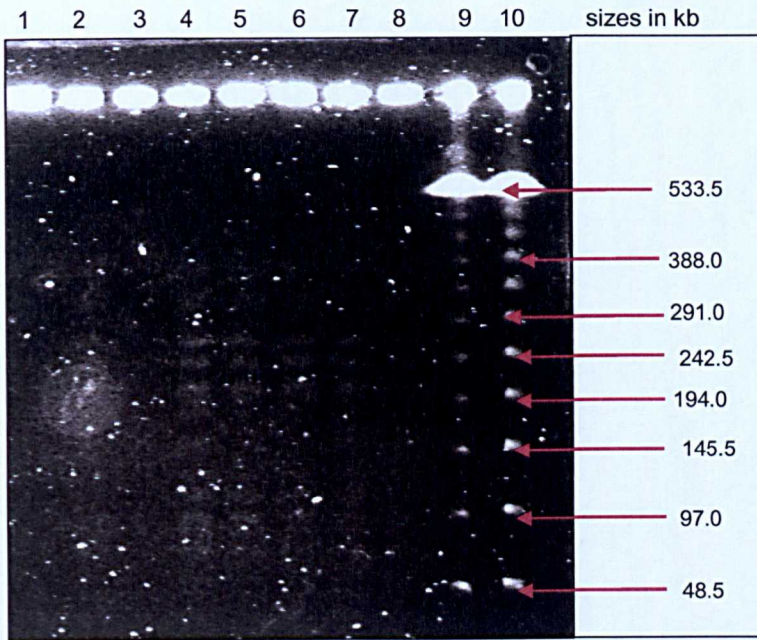
Figure 4.18: Pulsed-field gel electrophoresis of *B. cereus* DNA, when either treated with 0.5 mg/ml proteinase K or digested using various quantity of *Sma*I. Lanes 9 and 10: Lambda Ladder (48.5-970 kb). Lanes 1 and 2: undigested and digested (15 units *Sma*I) *B. cereus* DNA respectively (treated with 0.5 mg/ml proteinase K). Lanes 4-8: *B. cereus* DNA treated with 1 mg/ml proteinase K and digested with 20, 25, 30 and 35 units of *Sma*I respectively.

Bacillus cereus produces spores that are resistant to environmental stress and therefore, may cause difficulties during lysis of the cells to extract DNA. Overnight incubation of *B. cereus* on a nutrient media may provide sufficient time for the bacterial cells to produce spores. Additionally, spore formation may occur in adverse environmental conditions such as lack of nutrition (Pelczar *et al.*, 1993). Presence of too many bacteria cells, when bacteria are cultured in a closed system, may lead to nutrient depletion, which may encourage the bacterial cells to produce spores. Therefore, a new method as described in Section 4.3.1.7 was followed using a diluted *B. cereus* culture, which was incubated for a shorter period. In this instance, the bacterial samples were diluted to 10^{-3}, when cultured, to ensure presence of a low number of bacterial cells in TSB. This ensures that there is a sufficient nutrient supply and thus reduces the probability of spore formation. In addition, the incubation of *B. cereus* in TSB was carried out for 4 hours instead of overnight, to limit the generation of spores by the bacterial cells (Ferris *et al.*, 2004; Zhong *et al.*, 2007), as prolonged incubation may encourage the bacteria to produce spores. The results obtained are shown in Figure 4.19.

Electrophoresis of the DNA (lane 2, 3, 5 and 6) extracted from *B. cereus* following the above-mentioned protocol (Section 4.3.1.7) showed the presence of degraded DNA (Figure

4.19). A repetition of the protocol showed the same results (Figure 4.20). Degradation may have occurred during the DNA extraction or storage. However, this indicates complete lysis and proteolysis of *B. cereus* cells resulting in the release of DNA from the cells. A methodical approach was followed to determine the stage or stages of the DNA extraction and preparation for PFGE analysis that may have caused the DNA degradation. The final stage of the protocol involves a heat-shock of the extracted DNA at 70°C followed by 2 washes with preheated water at 70°C (Zhong *et al.*, 2007). The experiment was repeated and in this instance, the extracted DNA was not heat-shocked at 70°C; instead, the plugs were washed with TE buffer (see Section 2.17.2). The results obtained are shown in Figure 4.20.
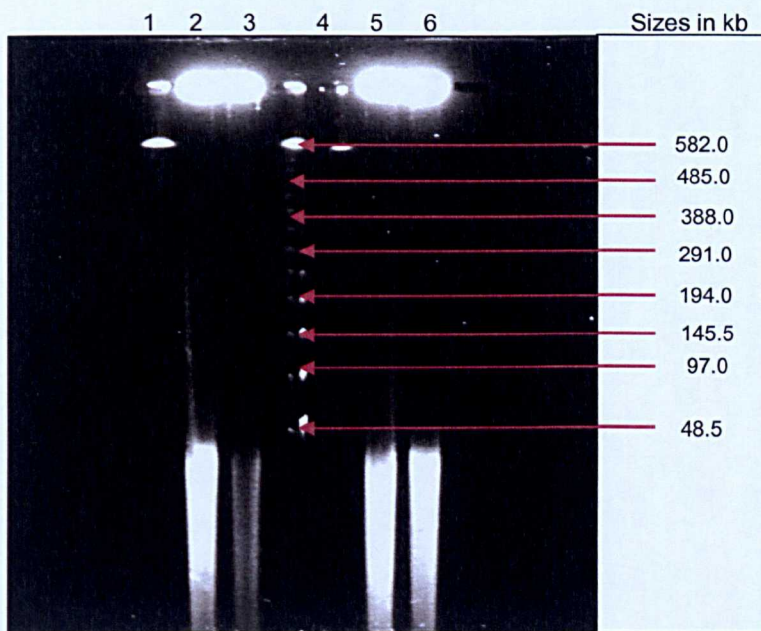
Figure 4.19: Pulsed-field gel electrophoresis of *B. cereus* DNA. The DNA was extracted following a protocol according to Ferris *et al.*, 2004; Zhong *et al.*, 2007 (see Section 4.3.1.7). Lanes 1 and 4: Lambda Ladders (48.5-970 kb). Lanes 2, 3, 5 and 6: undigested *B. cereus* DNA.

It appears that the *B. cereus* DNA not only migrated through the gel, but also distinctive band patterns were obtained after modifying the protocol that was described in Section 4.3.1.6 (Figure 4.20). Due to a successful experiment, the modified method (see Section 2.15.1) was considered to be suitable for the *B. cereus* DNA profiling. *Bacillus cereus* ATCC11778 DNA (lanes 3 and 4) showed identical band patterns indicating the DNA was extracted from the same bacterial strain.
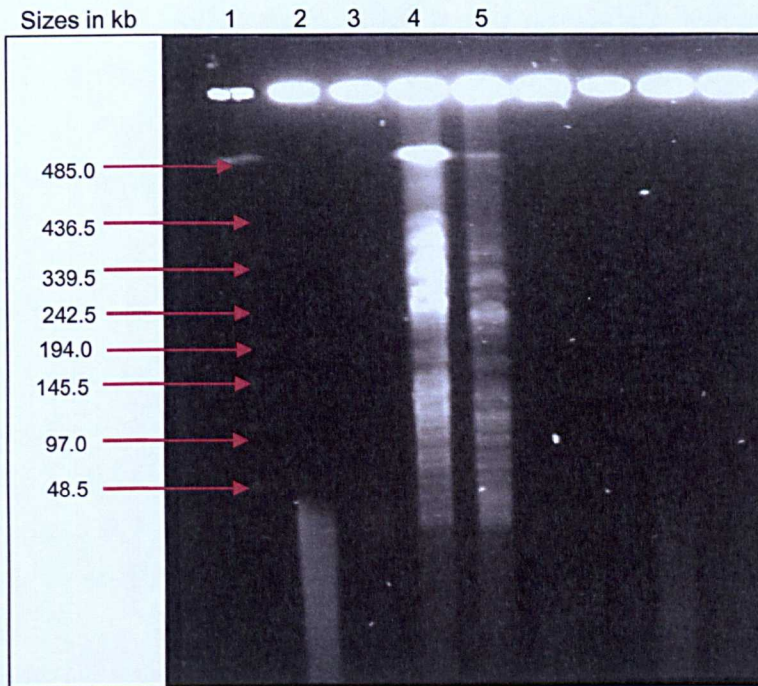
Figure 4.20: Pulsed-field gel electrophoresis of the DNA (lanes 4 and 5) extracted from *B. cereus* ATCC11778 following the modified protocol described in section 2.17.2. Lane 1: Lambda Ladder (48.5-970 kb), Lanes 2 and 3: *B. cereus* DNA extracted using a protocol described in section 4.3.1.7.

4.5. Summary

In this chapter, various PFGE protocols were optimised to obtain quality DNA from *B. cereus* and *P. aeruginosa* and so to achieve DNA separation with definite profiles. Separation of the Lambda Ladder was optimised. Initially, clear separation of the *P. aeruginosa* DNA was not achieved due to the degradation of the DNA. However, extraction of quality DNA from *P. aeruginosa* was obtained by following a new protocol resulting in a clear separate band patterns.

Difficulties arose in achieving band separation of the DNA extracted from *B. cereus* and various PFGE protocols were therefore trialled to achieve DNA band separation. It was observed that the bacterial cell concentration to extract DNA, the concentration of proteinase K for proteolysis, the utilisation of an increased amount of restriction enzyme did not assist in achieving DNA band separations. During the majority of the trials with the *B. cereus* DNA, the DNA was found to remain in the well instead of migrating through the gel. Improper lysis and proteolysis of the *B. cereus* cell wall was thought to be the cause of this phenomenon. The thicker proteoglycan layer and the ability of *B. cereus* to produce spores may be responsible for improper lysis or proteolysis. The problem was resolved by using a diluted and fresh (incubated for 4 hours) *B. cereus* culture to extract DNA. The PFGE method can

be used for the identification of the microbial species as well as determining the origin of a particular microbial species based on the DNA profiles. It may also be applied to monitor alteration in the DNA of the microbial species that have survived in tannery effluents or hides/skins during the leather manufacturing processes.

4.6.   Conclusion

A clear band separation was not obtained due to the extraction of degraded DNA from *P. aeruginosa*. Therefore, PFGE protocols were optimised in order to extract quality DNA from *P. aeruginosa* and so to achieve well-defined DNA band separations. On the other hand, difficulties arose due to improper lysis and proteolysis of the *B. cereus* cell to extract quality DNA. The problem was resolved using a diluted *B. cereus* culture containing vegetative cells for DNA extraction. The optimised PFGE protocols were described in Chapter 2, and used to investigate the DNA profiles of *B. cereus* and *P. aeruginosa* that were isolated at various stages of the leather-making processes (conventional and BAT).

Chapter 5: The Effects of the Leather Manufacturing Process on the Growth of *Bacillus cereus*

5.1. Introduction

Bacillus cereus, a member of the genus *Bacillus*, is described as a spore-forming, rod-shaped, aerobic or facultative anaerobic bacterium (Berkeley *et al.*, 2002; Hilliard *et al.*, 2003), and therefore is able to grow without oxygen. More than 60 species have been named in the genus *Bacillus* (Barrow and Feltham, 2003). These bacteria are mainly soil inhabitants (Todar, 2008). The spores produced by *Bacillus* are known as endospores as they are produced intracellularly. The mature spores are eventually released from the mother cell, known as a sporangium, as free spores. Majority of the species of genus *Bacillus* are motile, Gram-positive, but may appear as Gram-variable (Brock and Madigan, 1991, Barrow and Feltham, 2003; Hilliard *et al.*, 2003).

According to Bergey's Manual of Determinative Bacteriology, (1974) *B. cereus* occurs in chains, the cell size varies from 3-5 µm in length and 1-2 µm in width. It does not ferment arabinose, mannitol and xylose, but ferments glucose (Barrow and Feltham, 2003; Health Protection Agency (HPA), 2005c). *Bacillus cereus* produces an enzyme lecithinase, which hydrolyses lecithin in egg yolk, and as a result, production of a white precipitate of hydrolysed lecithin occurs. *Bacillus cereus* is often primarily identified based on the inability of fermenting mannitol and ability of forming lechithinase. *Bacillus cereus* is mesophile and requires a temperature range of 28-35°C, for optimal growth. Some psychotropic strains may grow at low temperatures as low as 5°C. The required pH range for the growth of *B. cereus* is 4.3-9.3 (Burgess and Horwood, 2006; Schneider *et al.*, 2004).

Bacillus cereus is known to be a bacterial pathogen to humans. It is one of the major causes of food-borne illnesses (Murray *et al.*, 2005; Schneider *et al.*, 2004). Infection doses (threshold limit) may vary from person to person. According to the PHLS Advisory Committee for Food and Dairy Products (2000), 10^4-10^5 colony forming units (cfu) per gram (g) ready-to-eat food is considered unsatisfactory and food containing equal or more than 10^5 cfu/g considered potentially hazardous for human consumption. However, in case of epidemic episodes a lower count of *B. cereus* colonies may be considered significant (HPA, 2005c). Research showed (Burgess and Horwood, 2006) that the infection doses vary from 10^3-10^{10} cfu/g during *B. cereus* outbreaks due to food poisoning.

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Various episodes of non-gastrointestinal infections due to *B. cereus* have also been reported. Non-gastrointestinal *B. cereus* infections may occur in immune-compromised patients. The infection may be contracted through open wounds (Arnold *et al.*, 2005, Hilliard *et al.*, 2003; Olszewski *et al.*, 1999; Ozkocaman *et al.*, 2006). However, the probabilities of contracting non-gastrointestinal *B. cereus* infection are low, whilst considering the general population (Olszewski *et al.*, 1999; Ozkocaman *et al.*, 2006).

The phenotypic and genotypic characteristics of *B. cereus* are similar to those of *B. anthracis* and *B. thuringiensis* (Berkeley *et al.*, 2002; Barrow and Feltham, 2003). *Bacillus anthracis* causes anthrax, which primarily infects herbivorous animals such as cattle, sheep or horses. Humans may contract anthrax through direct contact with infected carriers or inhalation of the spores, the infection may also occur through ingestion of contaminated meat. However, the latter mentioned mode of transmission is rather unusual (Schneider *et al.*, 2004). *Bacillus thuringiensis* is known as an insect pathogen and often used as an insecticide (Berkeley *et al.*, 2002).

Bacillus cereus and other *Bacillus* species such as *B. brevis*, *B. firmus*, *B. laterosporus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. sphaericus*, *B. brevis*, *B. pumilus* and *B. subtilis* have been isolated from fresh hides, pelts (unhaired hides or skins are known as pelt) and finished leather (Birbir and Ilgaz, 1996; Kayalvizhi *et al.*, 2008). *Bacillus subtilis* has also been isolated from tannery sludge and fleshings (Vasudevan and Ravindran, 2007).

This chapter includes the laboratory experiments, which were carried out in order to determine the effect of the conventional and BAT leather manufacturing processes on the growth of *B. cereus*.

5.2. Aims and Objectives

- To study the effect of the conventional and BAT leather-making processes on the growth of *B. cereus*.
  - To study the growth pattern of *B. cereus* ATCC11778 in order to calculate the mid-exponential phase for inoculation.

  - To undertake trials in order to evaluate the survival of *B. cereus* ATCC11778 on the sodium hypochlorite treated calf skin pieces.

- Enumeration of *B. cereus* in effluents that were collected from various stages of the conventional and BAT leather-making processes such as pre-soaking, soaking, unhairing, reliming, deliming, bating, pickling and chrome tanning in order to determine the presence of inoculated *B. cereus*.

- To determine the presence of the inoculated *B. cereus* on calf skins (by analysing swabs) at the end of the each above mentioned stages during the conventional and BAT leather-making processes.

- To carry out confirmatory tests to ensure presence of the inoculated *B. cereus* using biochemical assays (standard biochemical tests and the Biolog identification system).

- To undertake the PFGE method in order to determine the DNA profiles of *B. cereus* to ensure the isolated *B. cereus* colonies from the conventional and BAT leather-making processes were the same strain as inoculated *B. cereus* ATCC11778.

5.3. Materials and Methods

Bacillus cereus ATCC11778 (Oxoid, UK) was used for this experiment. Enumeration of *B. cereus* was carried out on a selective agar media, Polymixin Pyruvate Egg Yolk Blue Agar (PEMBA) (Oxoid UK) (see Section 2.5.1). In this study, the total number of replicates will be referred to as n. Growth curves were plotted using a spectrophotometer (CE1011, Cecil Instrument Ltd., UK) as described in Section 2.5 in order to calculate the required incubation time for the bacterial cells to reach mid-exponential phase. A trial was undertaken in triplicate to observe the growth and survival of the inoculated *B. cereus* colonies on sodium hypochlorite (w/v) decontaminated calf skin pieces (see Section 2.11).

Each of the conventional (see Section 2.12.1) and BAT (see Section 2.12.2) leather-making processes were conducted using five calf skin pieces (fleshed and salted), each weighing 100±6 g, and were taken from the same calf skin. In this instance, the five calf skin pieces will be referred to as experimental samples. Calf skin pieces were decontaminated using a 0.125 g/ml sodium hypochlorite (NaClO) solution (see Section 2.9). Microbial colonies in water and swabs, after the final washing cycle, following the decontamination procedure, was measured (see Sections 2.2 and 2.9). A pure culture of *B. cereus* ATCC11778 was collected from the mid-exponential phase and inoculated on decontaminated calf skin pieces (see Section 2.10). The inoculated calf skin pieces were incubated overnight at 30°C. The

following day a conventional or BAT leather-making processes were carried out with the inoculated calf skin pieces as described in Section 2.12. A control experiment was also undertaken without calf skin pieces, while, water and all the chemicals used for the leather manufacturing processes were added, and will be referred to as control sample for the purpose of this study.

Effluents were collected and skin samples were swabbed (see Section 2.13 for the details of the collection method) at the end of each stage of the conventional and BAT leather-making processes in duplicate. *Bacillus cereus* enumeration in effluent and swabs were carried out as described in Sections 2.14.1 and 2.14.2. Hair samples were also collected in duplicate from the conventional and BAT unhairing effluents, and incubated overnight in tryptone soya broth (TSB) at 30°C to allow recovery of the stressed or damaged bacterial cells (see Section 2.14.3 for details). *Bacillus cereus* colonies were enumerated and preliminary indentified as described in Sections 2.5.1 and 2.6.1 respectively. The isolated colonies will be referred to as presumptive *B. cereus* colonies. A Gram-staining method (see Section 2.7) and spore-staining method (see Section 2.8) was applied to observe the microscopic structure of *B. cereus*, using a light microscope (Nikon Eclipse 600, Nikon, Japan). Biochemical tests (see Section 2.15.1.1) and the Biolog identification method (see Section 2.15.2) were carried out for *B. cereus* ATCC11778 colonies and isolated *B. cereus* colonies during the leather manufacturing processes (conventional and BAT). In addition, the PFGE method was applied to determine the DNA profiles of the isolated *B. cereus* and compared with the DNA profile of *B. cereus* ATCC11778 (see Section 2.17.2).

5.4.   Results and Discussion

5.4.1.   *Bacillus cereus Growth Curve*

Bacterial cells in the exponential phase are most active (Prescott *et. al.*, 2008; Todar, 2008) with the physical and chemical properties being most stable, and therefore used in biomedical and physiological studies (Pelczar *et al.*, 1993; Prescott *et al.*, 2008). In this study, *B. cereus* inoculum was collected from the mid-exponential phase to ensure consistency and maximum efficiency. Growth curves were therefore plotted using the absorbance values against time and used to calculate the incubation period required to reach the mid-exponential phase. Figure 5.1 shows the growth pattern of a representative sample. The calculated incubation period was 379 minutes (±72.14, n=5).
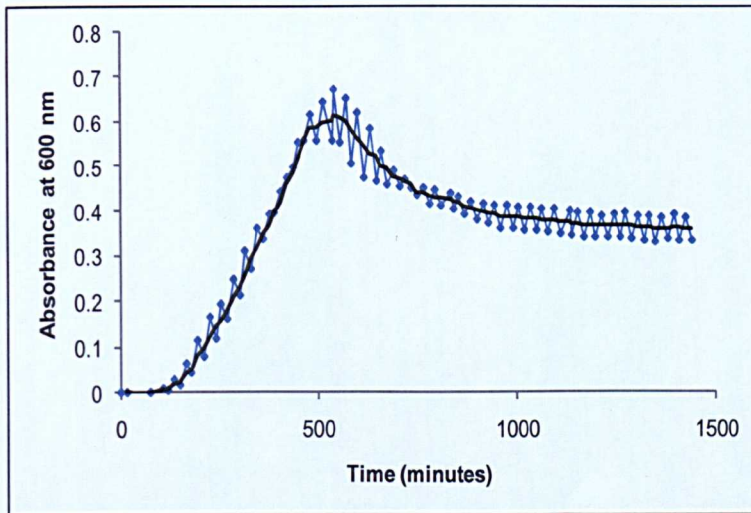
Figure 5.1: A *Bacillus cereus* ATCC11778 growth curve (a representative sample).

5.4.2.   Trials to Determine the Survival of the Inoculated <u>Bacillus cereus</u> ATCC11778 on the Sodium hypochlorite-Decontaminated Calf Skins

A trial was carried out in order to observe the survival of the inoculated *B. cereus* ATCC11778 on the NaClO (0.125 g/ml)-treated calf skin pieces. The average number of isolated bacteria in water and swabs, after the final washing cycle, following the NaClO-decontamination method, was log 0.58 (±0.69, n=3) cfu/ml and log 0.63 (±0.76, n=3) cfu/ml respectively. In addition, the isolated bacterial colonies were not identified as *B. cereus*. Due to the enumeration of a limited number of bacterial colonies from the water and swabs after NaClO-treatment, the decontamination effect of NaClO was considered to be satisfactory. The decontaminated calf skin pieces were inoculated with log 6.57 (±0.06) cfu of *B. cereus* ATCC11778.

After overnight incubation an average of log 6.48 (±0.15, n=3) cfu/ml and log 4.92 (±0.22, n=3) cfu/ml of the inoculated *B. cereus* was recovered from the water and swab samples respectively (see Figure 5.2). Due to the recovery of approximately 10^5 (=log 5.0) cfu/ml or above, the number of isolated *B. cereus* colonies from the water and swab samples was considered to be satisfactory. Therefore, the same decontamination and inoculation procedures were carried out during the conventional and BAT leather-making process experiments.
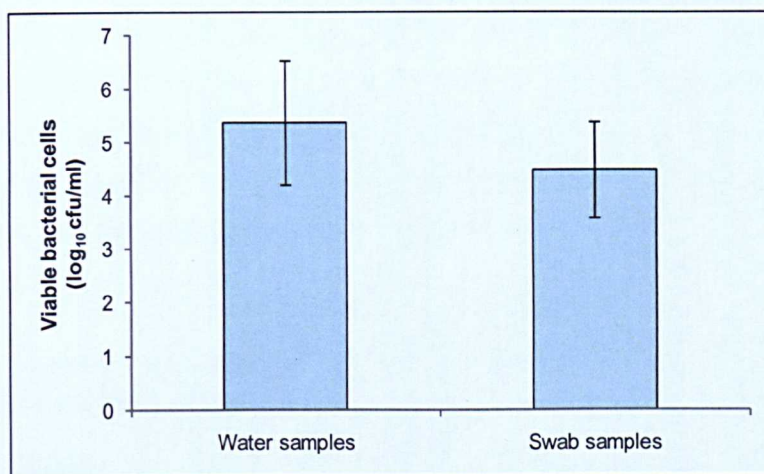
Figure 5.2: Recovery of the inoculated *B. cereus* on the sodium hypochlorite (0.125 g/ml) treated calf skin pieces, when water and swabs samples were analysed, n=3. Bacterial enumeration was carried out on PEMBA media in triplicate. The average number of inoculated *B. cereus* ATCC11778 was log 6.56 cfu. The error bars represent the standard deviation.

5.4.3. *Decontamination of Calf Skins Using Sodium Hypochlorite for the Conventional and BAT Leather-Making Process Experiments*

Calf skin pieces that were used for the conventional and BAT leather-making processes were treated with NaClO, 0.125 g/ml (see Section 2.10). Similar to the trial samples, bacterial growth was observed in water and swabs after the final washing cycle, following the NaClO-treatment. The average number of isolated bacteria from the water and swabs after the final washing cycle, following the decontamination of calf skin pieces (used for a conventional leather-making process), was log 0.17 (±0.46, n=5) cfu/ml and log 0.09 (±0.34, n=5) cfu/ml respectively. On the other hand, the average number of isolated bacteria from the water and swabs after the final washing cycle, following the decontamination of calf skin pieces (used for a BAT leather-making process), was log 0.18 (±0.48, n=5) cfu/ml and log 0.11 (±0.37, n=5) cfu/ml respectively. Additionally, the isolated bacterial colonies were not identified as *B. cereus*. Due to the enumeration of a limited number of bacterial cells (less than 10 cfu/ml=log 1), the decontamination of calf skin pieces was considered to be satisfactory.

5.4.4. *Inoculation of <u>Bacillus cereus</u>*

Bacillus cereus ATCC11778 inoculum (in TSB), 4 ml, was collected from the mid-exponential phase (see Sections, 2.5 and 5.4.3) and inoculated on the decontaminated calf skin pieces. The total number of inoculated *B. cereus* ATCC11778 colonies on the calf skin pieces that were used for the conventional and BAT leather-making processes were measured (see

Section 2.10.2). An average of log 8.61 (±0.15) cfu and log 8.75 (±0.3) cfu *B. cereus* ATCC10145 was inoculated on the calf skin pieces that were used for the conventional and BAT leather-making processes respectively. Statistical analysis showed there was no significant difference between the number of inoculated colonies for the conventional and BAT leather-making processes (t=-1.607, df=17, p=0.122).

5.4.5. Enumeration of *Bacillus cereus* from Effluent (Conventional and BAT Leather-Making processes)

5.4.5.1. Pre-soaking

Figure 5.3 shows the number of isolated presumptive *B. cereus* colonies from the conventional and BAT pre-soaking effluents. Analysis of the control samples shows no growth of *B. cereus* in the pre-soaking effluents for both of the conventional and BAT leather-making processes. Statistically bacterial growth in the experimental samples was significantly higher than the corresponding control samples (Mann-Whitney test: $U_{30, 6}$=0.00, p<<0.001 [conventional]; $U_{20, 4}$=0.00, p<<0.001 [BAT]).

Both of the conventional and BAT pre-soaking processes were carried out using 300% v/w water and 0.2% w/w of a sodium dimethyldithiocarbamate-based bactericide, Truposept BA (Trumpler, Germany), (see Section 2.12). However, analysis of the experimental samples shows a significant higher growth of *B. cereus* in the conventional pre-soaking effluent than the BAT pre-soaking effluent (t=15.012, df=47.273, p<<0.001). The average number of isolated *B. cereus* from the conventional and BAT pre-soaking effluents was log 4.75 (±0.45, n=5) cfu/ml and log 3.22 (±0.26, n=5) cfu/ml respectively.

Various environmental conditions such as the presence of nutrient, moisture, temperature, oxygen, light, pH and chemicals influence the growth of microorganisms in a particular habitat. In addition, the incubation period is also an important factor for microbial growth and regeneration (Maier *et al*, 2000; Murray *et. al.*, 2005; Wilson, 2005). Therefore, it was difficult to identify the exact cause of enumeration of a lower number of presumptive *B. cereus* colonies from the BAT pre-soaking effluent than the conventional pre-soaking effluent.
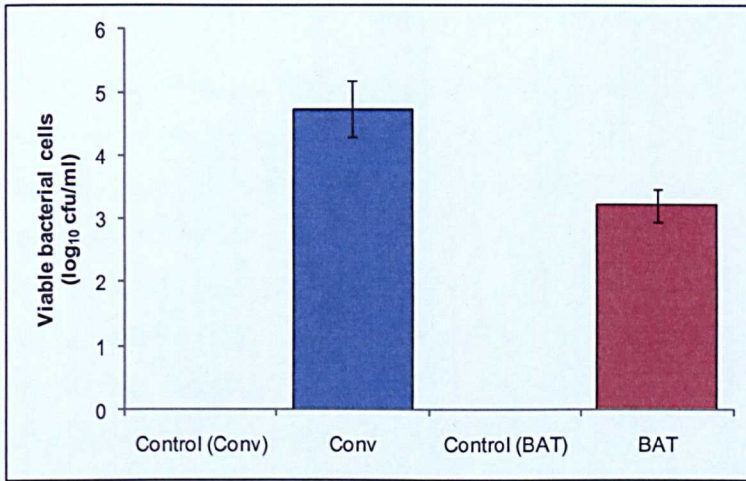
Figure 5.3: Number of isolated presumptive *B. cereus* colonies from the conventional (conv) and BAT pre-soaking effluents (n=5, t=15.012, df=47.273, p<<0.001). The average number of inoculated *Bacillus cereus* ATCC11778 was log 8.61 cfu (conv) and log 8.75 cfu (BAT). The error bars represent the standard deviation.

Due to proteolytic activity (Ghorbel *et al.*, 2003; Nilegaonkar *et al.*, 2007; Shede *et al.*, 2008), *B. cereus* is capable of breaking down protein to peptides and amino acids (Han *et al.*, 2006). *Bacillus cereus* is also able to hydrolyse lipids or fats (Loperena *et al.*, 2009; Shede *et al.*, 2008). In addition, *B. cereus* produces collagenase (Shede *et al.*, 2008) and therefore able to hydrolyse collagen (a structural protein). Since hides/skins largely consist of protein (mainly collagen), fat and water, *B. cereus* is capable of utilising hides/skins as a nutritional source. A small percentage of bactericide is generally added during the pre-soaking processes; this may control the microbial population (Kayalvizhi *et al.*, 2007) during the processing, but may not cause total inactivation.

During the pre-soaking processes, hides and skins gain moisture and this may assist bacterial recovery (Rangarajan and Didato 2003), as moisture is an important factor for microbial growth (Rushing *et al.*, 2006). The pH range of the conventional and BAT pre-soaking effluents was 7.6-8.3 and 8.0-8.2 respectively, which is within the pH range required for *B. cereus* growth. The room temperature during the BAT pre-soaking process was lower (approximately 22°C) than the room temperature during the conventional pre-soaking process (approximately 26°C). As mentioned in the introduction *B. cereus* grows optimally at 28-35°C, therefore, 26°C would be more suitable for *B. cereus* than 22°C, as it is nearer to the temperature range required for its optimum growth. However, due to the presence of protein, fat, moisture, a slightly alkaline pH range and moderate temperatures, the pre-soaking processes may be suitable the bacterial growth.

5.4.5.2. Soaking

Absence of *B. cereus* was observed in the control samples, whilst, *B. cereus* growth was observed in all effluent samples collected from the conventional and BAT soaking processes (Figure 5.4). Therefore, the difference between the number of bacterial colonies in the experimental sample was significantly high than the corresponding control samples (Mann-Whitney test: $U_{30,6}=0.00$, $p<<0.001$ [conventional]; $U_{19,4}=0.00$, $p<<0.001$ [BAT]). It appears a significantly reduced number of presumptive *B. cereus* colonies from log 4.75 (±0.45, n=5) to 3.56 (±0.62, n=5) cfu/ml was isolated from the conventional soaking effluent (t=8.457, df=58, $p<<0.001$). A significantly reduced number of presumptive *B. cereus* colonies from log 3.22 (±0.26, n=5) to 2.97 (±0.25, n=5) cfu/ml was also isolated from the BAT soaking effluent (t=3.127, df=37, p=0.003).

Figure 5.4: Number of isolated presumptive *B. cereus* colonies from the conventional (conv) and BAT soaking effluents, n=5. The average number of inoculated *B. cereus* ATCC11778 was log 8.61 cfu (conv) and log 8.75 cfu (BAT). The error bars represent the standard deviation.

As mentioned previously (Section 5.1.1.1), the growth of microorganisms is influenced by the various physical and chemical properties of an environment. The conventional soaking process was carried out using 300% v/w water, 0.2% w/w anionic wetting agent, Corilene®W385 (alkyletherphosphate, sodium salt) (STAHL Europe, The Netherlands), and 0.2% w/w bactericide Truposept BA, (see Section 2.12). Wetting agents, or surfactants, are generally used during the soaking processes to assist rehydration of hides/skins. Although, no information was available on the effect of sodium salt of alkyletherphosphate on microorganisms, however, the majority of the wetting agents used in the leather industry cause environmental pollution, by increasing the solubility of various compounds in water (Integrated Pollution Prevention and Control (IPPC), 2001). Due to the negative net charge, an anionic surfactant may interrupt cell function by binding with the positively charged

molecules. It exerts toxic effects to humans (mainly cause eye and skin irritation), microorganisms, plants, and many aquatic species including vertebrates and non-vertebrates (Tibor *et al.*, 2002).

In order to reduce the environmental impact due the presence of surfactants in the soaking effluents, a protease-based enzyme, Pelvit C (Together For Leather (TFL), Germany), (0.5% w/w) was used during the BAT soaking process, since enzymes are protein molecules and so have less of an environmental impact (IPPC, 2001; Thanikaivelan *et al.*, 2004). Pelvit C has an active pH range of 8.5-11.0, therefore, sodium carbonate (Na_2CO_3) (0.5% w/w) was added during the BAT soaking process to obtain the required pH range in order to ensure an optimal enzyme activity. A high pH also promotes rehydration of hides/skins. Little information was available on the toxic effect of Na_2CO_3 on the environment or microorganisms. However, Na_2CO_3 may have an antimicrobial activity and the antimicrobial activity may depend on the pH (Lakhanisky, 2002), since the pH of an environment is an important factor for microbial growth and proliferation.

A bactericide Truposept BA, 0.2% w/v, was added during the BAT-soaking process to control the bacterial growth. This may also have an influence on the growth of microorganisms. Truposept BA is a sodium dimethyldithiocarbamate-based compound and may have a low environmental impact (International Union of Leather Technologist and Chemists Societies (IULTCS), 2008). However, most of the biocides are considered potentially toxic as they are primarily applied to control or inhibit microbial growth (IPPC, 2001). Brözel *et al.* (1995) showed that *B. cereus* may be resistant to the toxic effects of sodium dimethyldithiocarbamate.

The pH of the conventional and BAT soaking effluents was 7.8-8.0 and 10.3-11.2 respectively. The pH of the conventional soaking effluent is within the pH range required for the growth of *B. cereus* whereas the pH of the BAT-soaking effluent is higher than the maximum pH required for *B. cereus* growth. A higher pH during the BAT soaking process may have lowered the growth of *B. cereus*. In addition, the room temperature during the conventional soaking process (approximately 25°C) was higher than the room temperature during the BAT soaking process (approximately 21°C). A temperature nearer to the required temperature range may have encouraged the growth of *B. cereus*.

Soaking processes are generally carried out for 5 to 24 hours depending on the condition of the preserved hides/skins (Heidemann, 1993). In this instance, calf skin pieces were soaked

overnight in the soaking liquid. This should have provided an adequate time for the bacterial cells to proliferate.

As the reduction of *B. cereus* cells was observed during both of the conventional and BAT soaking processes than the pre-soaking process, the presence of a wetting agent, Na_2CO_3, pH or temperature may not be the cause of enumeration of a lower number of *B. cereus* colonies from the soaking processes. Mechanical agitation during processing may cause the bacterial cells to transfer from the skin to the effluent and discharging the effluent consequently decreases the number of *B. cereus* cells present for enumeration (Birbir and Ilgaz, 1996). In addition, the bactericidal effect of the added bactericide cannot be disregarded. However, the isolation of approximately 10^3 (=log 3) cfu/ml indicates that *B. cereus* is capable of surviving in the soaking effluents.

5.4.5.3.   Unhairing

Figure 5.5 shows, *B. cereus* cells were absent in the control samples and present in the experimental samples when the conventional and BAT unhairing effluents were analysed. However, statistically there was no significant difference between the bacterial count obtained from the conventional unhairing effluent and the corresponding control sample (Mann-Whitney Test: $U_{30, 6}=60$, p=0.104). On the other hand, a significantly higher number of bacterial colonies were isolated from the BAT unhairing effluent than the respective control sample (Mann-Whitney Test: $U_{20, 4}=2.0$, p=0.003). A considerably decreased number of presumptive *B. cereus* colonies from log 3.56 (±0.62, n=5) to 0.48 (±69, n=5) cfu/ml was isolated from the conventional unhairing effluent (Mann-Whitney Test: $U_{30, 30}=0.00$, p<<0.001). In addition, growth of *B. cereus* was observed on 33.3% of the petri-dishes, which were inoculated with the conventional unhairing effluent.

The conventional unhairing process was carried out using 200% v/w water, 3% w/w sodium sulfide (Na_2S) and 2% w/w lime (calcium hydroxide). Due to the antimicrobial activity, lime is often used in dental surgery as an antimicrobial agent (Al-Nazhan, 2002; Estrela *et al.*, 2003) to destroy microorganisms. The presence of lime may therefore lower the number of *B. cereus* colonies in the conventional unhairing effluent. The antimicrobial activity of lime is probably due to its strong alkaline properties (George *et al.*, 2001) as it can raise the pH of an environment above the maximum pH range required for bacterial growth and survival. Sodium sulfide is also highly alkaline in nature as well as having strong reducing properties and therefore reduces the oxygen levels in water (Bajza and Vrcek, 2001, Taleb-Ahmed *et al.*, 2005; Tišler *et al.*, 2004) interrupting microbial activity of aerobic microorganisms. In

addition, due to the presence of lime and Na_2S, the pH of the conventional unhairing effluent was 11.1-12.2, which is higher than the required pH range for *B. cereus* growth. The conventional unhairing process did not provide favourable conditions for *B. cereus* growth. However, the study showed, although the presence of lime and Na_2S reduces the number of *B. cereus* cells in the conventional unhairing effluent, but total removal of *B. cereus* did not occur. Research carried out by Birbir and Ilgaz (1996), showed the presence of lime and Na_2S during an unhairing process inhibits bacterial growth, however, bacterial species in the genus *Bacillus* species were found to be survived on the unhaired hides. *Bacillus cereus* and other *Bacillus* species such as *B. licheniformis, B. megaterium, B. pumilus* and *B. subtilis* were isolated from hides after a conventional unhairing process (Birbir and Ilgaz, 1996). This phenomenon indicates that *B. cereus* may be able to survive extreme environmental conditions of a conventional unhairing process.

Figure 5.5: Number of isolated presumptive *B. cereus* colonies from the conventional (conv) and BAT unhairing effluents, n=5. The average number of inoculated *B. cereus* ATCC11778 was log 8.61 cfu (conv) and log 8.75 cfu (BAT). The error bars represent the standard deviation.

A significant reduction in the number of presumptive *B. cereus* colonies from log 2.97 (±0.25, n=5) to 2.44 (±0.75, n=5) cfu/ml was observed when the BAT unhairing effluent was analysed (t=2.962, df=23.366, p=0.007). In this instance, growth of *B. cereus* occurred on 95% of the petri-dishes that were inoculated with the BAT unhairing effluent. Results show that the number of the isolated *B. cereus* colonies from the BAT unhairing effluent was higher than the conventional unhairing effluent (see Figure 5.5).

To reduce the environmental pollution due to the conventional unhairing process, an enzyme-based hair-save unhairing method (see Section 1.2.5.2) was followed to remove intact or non-degraded hair. Enzymes are protein molecules and therefore may have less of

an environmental impact (Thanikaivelan *et al.*, 2004). In addition, enzymes are biological catalysts and require a low quantity to complete a reaction hence reduce pollution load. Therefore, use of enzymes or enzyme-assisted technology is considered as clean or cleaner technology (He *et al.*, 2005; Thanikaivelan *et al.*, 2004). A proteolytic-based unhairing enzyme (1.5% w/w) (Southern Petrochemicals Industries Corporation Ltd. (SPIC), India), was added during the BAT unhairing process.

A reduced amount of Na_2S, from 3% w/w to 0.5% w/w, and lime, from 2% w/w to 1.0% w/w, was also added. The solubility of Na_2S is 18.6 g/100ml, and only 20% v/w water was added during the BAT unhairing process, therefore, Na_2S concentration in the BAT unhairing liquid was higher than the conventional unhairing liquid. On the other hand, lime is sparingly soluble in water (0.185 g/100ml) thus addition of a reduced percentage of lime may not have an impact on its overall concentration during the BAT unhairing process. Due to the presence of lime and Na_2S, the BAT unhairing process may not provide suitable conditions for *B. cereus* growth. The pH of the BAT unhairing effluent was 10.3-11.2, which was lower than the pH of the conventional unhairing effluent. A lower pH may assist the survival of *B. cereus* in the BAT unhairing effluent. Due to the addition of a lower amount of water during BAT unhairing process, the *B. cereus* concentration per ml effluent may be higher.

Bacillus cereus produce spores under environmental stress such as lack of nutrition. The spores are capable of withstanding adverse environmental conditions, such as high temperatures, irradiation, strong acids and disinfectants for a long period of time (Pelczar *et al.*, 1993; Todar, 2008). The ability of *B. cereus* to produce spores may aid the survival of *B. cereus* cells (Birbir and Ilgaz, 1996) in the extreme environmental conditions during the BAT and conventional unhairing processes.

The growth of *B. cereus* in both of the conventional and BAT unhairing effluents may have been affected by the presence of lime and Na_2S resulting in the inconsistent growth of *B. cereus*. Some of the *B. cereus* colonies may have survived, and some may have produced spores, while others may have been inactivated or damaged or became dormant due to extreme environmental conditions during the unhairing processes. A large standard deviation may therefore due to the inconsistent growth of *B. cereus* during the unhairing processes.

5.4.5.4.   Reliming

The unhaired calf skins were relimed using 200% v/w water and 2% w/w lime.   All the samples for both of the conventional and BAT leather-making processes were treated following the same reliming method. It appears similar to the unhairing processes; the reliming process did not provide suitable conditions for *B. cereus* growth and proliferation. Although no bacterial growth was observed in the control sample during the unhairing processes however, the difference between the number of isolated bacterial colonies from the control and experimental samples was statistically insignificant (Mann-Whitney test: $U_{19,4}=30.00$, p=0.326 [conventional]; $U_{20,4}=38.00$, p=0.655 [BAT]).

The average number of isolated presumptive *B. cereus* colonies from the reliming effluent was log 0.42 (±0.86, n=5) cfu/ml and log 0.07 (±0.29, n=5) cfu/ml for the conventional and BAT leather-making processes respectively (see Figure 5.6). Statistical analysis showed there was no significant difference between the number of bacterial colonies isolated from the conventional unhairing and reliming effluent (Mann-Whitney test: $U_{30, 19}=264.00$, p=0.587). Alternatively, bacterial count in the BAT reliming effluent was significantly lower than the bacterial count in the BAT unhairing effluent (Mann-Whitney test: $U_{20, 20}=10.50$, p<<0.001).
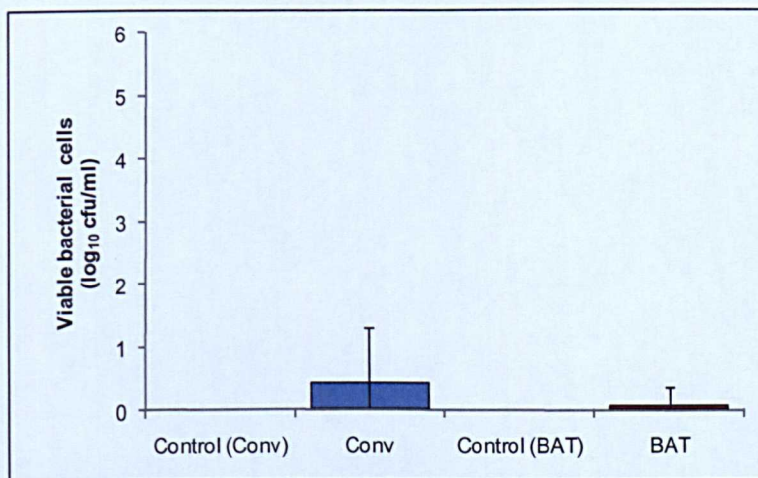
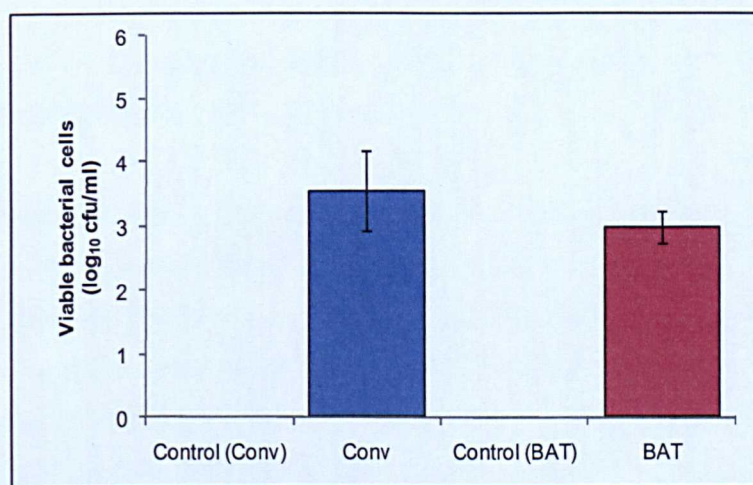**Figure 5.6: Number of isolated presumptive *B. cereus* colonies in the con (conventional) and BAT reliming effluents, n=5. The average number of inoculated *B. cereus* ATCC11778 was log 8.61 cfu (con) and log 8.75 cfu (BAT). The error bars represent the standard deviation.**

Bacillus cereus growth occurred on 21% and 5% of the petri-dishes that were inoculated with the conventional and BAT reliming effluents respectively, indicating inconsistent growth of *B. cereus* in the reliming effluents. Inconsistent growth of *B. cereus* may have occurred in the

reliming effluents due to the inhibitory action of lime towards microorganisms. The large standard deviation is probably due to the inconsistent growth of *B. cereus*.

The presence of lime during the reliming processes may have inhibited the recovery of *B. cereus*. The antimicrobial activity of lime may be due to its strong alkaline properties (George et al., 2001) as it increases the pH of a habitat to high levels generating an unsuitable pH for the microbial growth. The pH of the reliming effluents (for both of the conventional and BAT leather-making processes) was 12.0-12.5 due to the presence of lime, which is higher than the required pH range for *B. cereus* growth and proliferation.

5.4.5.5. Deliming

Figure 5.7 shows the average number of the *B. cereus* cells isolated from the conventional and BAT deliming effluents. *Bacillus cereus* was absent in the control samples for both of the conventional and BAT deliming processes. Bacterial count in the conventional deliming effluent was found to be significantly higher than the corresponding control sample (Mann-Whitney test: $U_{20,\ 4}=16.00$, $p=0.046$). Although bacterial growth was observed in the BAT deliming effluent, however, there was no significant difference between the bacterial count in BAT deliming effluent and the respective control sample (Mann-Whitney test: $U_{20,\ 4}=38.00$, $p=0.655$).
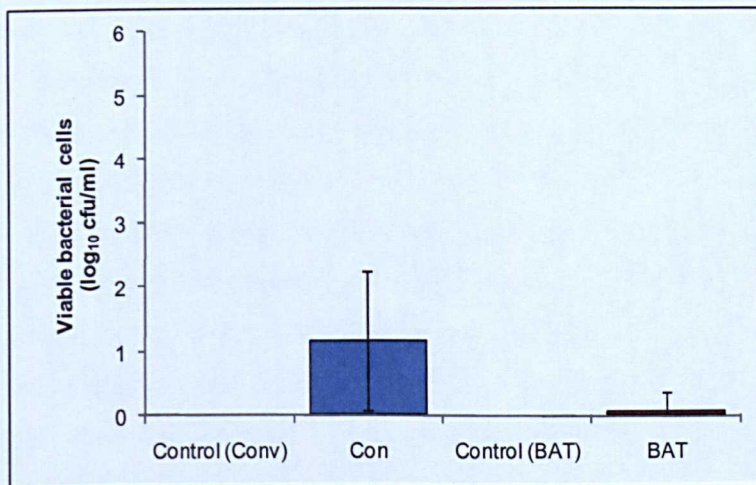
Figure 5.7: Number of isolated presumptive *B. cereus* colonies from the conventional (conv) and BAT deliming effluents, n=5. The average number of inoculated *B. cereus* ATCC11778 was log 8.61 cfu (conv) and log 8.75 cfu (BAT). The error bars represent the standard deviation.

The results show that the conventional deliming process may have provided suitable conditions for *B. cereus* growth since a significantly increased number of presumptive *B.*

cereus colonies, from log 0.47 (±0.86, n=5) to 1.16 (±1.08, n=5) cfu/ml, was enumerated from the conventional deliming effluent (Mann-Whitney test: $U_{19,20}$=117.00, p=0.021).

Ammonium salts (ammonium chloride and ammonium sulfate) are generally used during a conventional deliming process to neutralise alkalis that were absorbed by hides/skins during the unhairing and reliming processes (Leafe, 1999). In this instance, ammonium chloride (NH_4Cl 2% w/w) was used. Nitrogen is one of the essential elements for bacterial nutrition and the majority of bacteria are capable of using ammonia as a nitrogen source (Brock and Madigan, 1991; Pelczar *et al.*, 1993; Todar. 2008). Microorganisms also require carbon for their growth and multiplication, most of the bacteria are able to breakdown protein into amino acids and utilise it as a carbon and nitrogen source (Brock and Madigan, 1991; Pelczar *et al.*, 1993; Todar, 2008). Neutralisation of alkalis may have a positive impact on the growth and proliferation of *B. cereus*, since it lowers the pH (approximately from 12 to 8-9) of the deliming effluents to a range, which is suitable for bacterial growth. In this study, the pH of the conventional deliming effluent was 8.6-8.7, which is within the pH requirements for the growth of *B. cereus*. Therefore, the presence of ammonia, protein and the lowering of the pH, may encouraged the growth of *B. cereus* and spore germination.

On the other hand, a low number of *B. cereus* colonies, log 0.07 (±0.29, n=5) cfu/ml was isolated from the BAT deliming effluent. No significant difference was found in the bacterial count obtained from the reliming and deliming effluent during the BAT leather making process (Mann-Whitney test: $U_{20, 20}$ =200.00, p=1.00). The presence of nitrogen at high concentrations is toxic for the aquatic species as it reduces the oxygen levels in water, stimulating eutrophication. Addition of ammonium salts during a conventional deliming process increases the nitrogen levels in the effluents (IPPC, 2001). To reduce the nitrogen pollution load, the BAT deliming process was carried out using carbon dioxide (CO_2) instead of an ammonium salt. Due to the presence of CO_2, the pH of the BAT deliming effluent was lower, 6.3-6.6, than the pH of the conventional deliming effluent. The pH of the BAT deliming effluent is also within the pH requirement for *B. cereus* growth. Many bacteria have the potential to use CO_2 as a carbon source (Brock and Madigan, 1991, Pelczar *et al.*, 1993; Todar, 2008).

Carbon dioxide may or may not have an inhibitory effect on *B. cereus*. Research (Enfors and Molin, 1980; Molin, 1983; Noriega *et al.*, 2003) showed that CO_2 reduces the growth of *B. cereus*, which may be due to the acidification or replacement of oxygen (Devlieghere and Debevere, 2000) by CO_2. On the other hand, research carried out by Werner and Hotchkiss (2002) showed CO_2 did not inhibit or promote the germination of *B. cereus* spores. However,

an inhibitory effect of CO_2 on *B. cereus* may depend on the levels of exposure (HPA, 2005c; Enfors and Molin, 1980; Molin, 1983; Werner and Hotchkiss, 2002). In this study, CO_2 appeared to be inhibiting the growth *B. cereus* or spore germination. However, if the bacterial cells were damaged beyond repair, due to the environmental stress, culturing such damaged cells may not be feasible. Additionally, the time given (30 minutes) to undertake the BAT deliming process may also not be adequate for the recovery of *B. cereus*.

Inconsistent growth of *B. cereus* occurred on the petri-dishes that were inoculated with the conventional and BAT deliming effluents. This is probably due to the presence of a variable number of *B. cereus* colonies in the effluents that were culturable. Some *B. cereus* cells may have been damaged beyond recovery, whiles others may have repaired or recovered during the deliming processes resulting in inconsistent growth. The abrupt growth of *B. cereus* in the deliming effluents may be responsible of obtaining a large standard deviation in this instance.

5.4.5.6.   Bating

Figure 5.8 shows the results obtained when conventional and BAT bating effluents were analysed to enumerate *B. cereus*. During the bating process, the presence of *B. cereus* was not observed in the control samples for both of the conventional and BAT bating processes. Bacterial count in the conventional bating effluent was significantly higher than the respective control sample (Mann-Whitney test: $U_{20, 4}=12.00$, $p=0.022$), whilst there was no significant difference between the bacterial count obtained from the BAT bating effluent and the corresponding control sample (Mann-Whitney test: $U_{20, 4}=32.00$, $p=0.337$).

An increase in the number of presumptive *B. cereus* colonies from log 1.16 (±1.08, $n=5$) to 1.22 (±0.94, $n=5$) cfu/ml was observed, when the conventional bating effluent was analysed, however the difference was not statistically significant ($t=-0.180$, $df=38$, $p=0.858$). The conventional bating process was carried out using a pancreatic enzyme-based compound, Oropon ON 2, (TFL, Germany). Trypsin is one of the major pancreatic enzymes (Logsdon, 2001). Boschwitz *et al.* (1983) showed the presence of proteolytic enzymes, particularly trypsin, may be essential for the germination of *B. cereus* spores. The pH of the conventional bating processes was 8.6-8.7, which is within pH range required for *B. cereus* growth and therefore aid the survival of *B. cereus*. Moreover, the enzyme was added in the processing drums without complete removal of the deliming effluent or further addition of fresh water. This may have prevented removal of *B. cereus* cells that were present in the effluent.
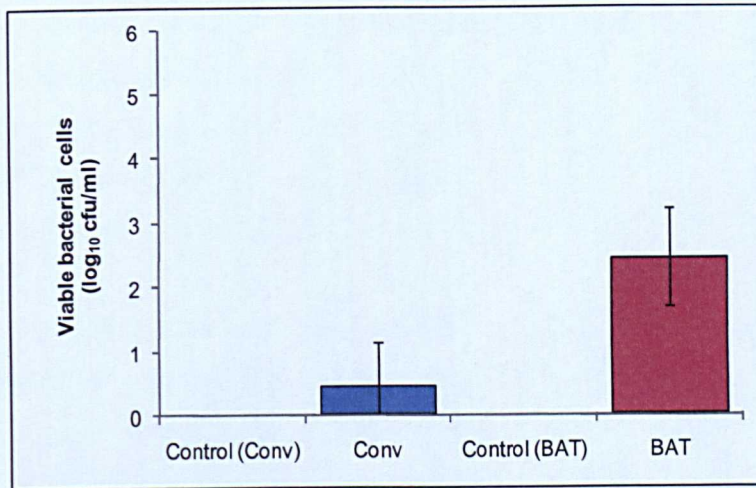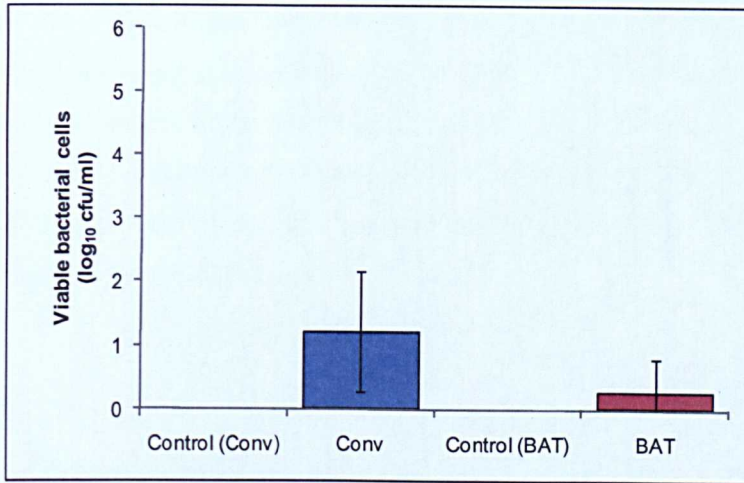
Figure 5.8: Number of isolated presumptive *B. cereus* colonies from the conventional (conv) and BAT bating effluents, n=5. The average number of inoculated *B. cereus* ATCC11778 was log 8.61 cfu (conv) and log 8.75 cfu (BAT). The error bars represent the standard deviation.

Although limited, however, an increased number of presumptive *B. cereus* cells from log 0.07 (±0.29, n=5) to 0.26 (±0.53, n=5) cu/ml was isolated from the BAT bating effluent. Statistical analysis showed that the increase in bacterial count was insignificant (Mann-Whitney test: $U_{20, 20}=170.00$, p=0.157). Oropon ON 2 (TFL, Germany) is an ammonium salt-free compound and may have less of an environmental impact; however, the active pH range of Oropon ON 2 is 7-9. Due to the use of CO_2, the pH of the BAT deliming bath (liquid) was equal to or below 7, which does not provide a suitable pH range to ensure the optimal activity of Oropon ON 2. Therefore, α-amylase (SPIC, India) 1.25% w/w, with an active pH range of 6-8, was used for the BAT bating process.

The inhibitory effect of α-amylase is disregarded in this instance since *B. cereus* produces α-amylase (Anto *et al.*, 2006). The pH of the BAT bating effluent was 6.9-7.1, which is also within the pH requirement for *B. cereus* growth. Before starting the BAT bating process, the deliming effluent was removed and 200% v/w water was added. Mechanical agitation may transfer the bacterial cells from the skin to effluent (Birbir and Ilgaz, 1996), and the consequent removal of the deliming effluent may reduce the number of *B. cereus* cells that were present on the calf skins. Additionally due to the use of a larger amount of water (200% w/v) during the BAT bating process than the conventional bating process (100% w/v), the net concentration of the *B. cereus* cells in the BAT bating effluent may have been lower.

The processing time is crucial for the growth of microorganisms. Microorganisms require adequate time for cell division, known as doubling time. The doubling time of a *B. cereus* cell is 18-27 minutes at an optimum temperature (28-35°C) and in the presence of a nutrient

medium (Novak *et al.*, 2002). In addition, microorganisms enter the lag phase when placed in a new environment. As mentioned previously (Section 1.1.3) cell division may not occur in the lag phase, as they adapt to the new environment or repair damage, thus the lag phase of stressed or damaged bacterial cells may be longer (Todar, 2008) than the healthy bacterial population. Inconsistent growth of *B. cereus* was also observed in the conventional and BAT bating effluents resulting in a large standard deviation.

5.4.5.7. Pickling

A limited number of presumptive *B. cereus* cells were isolated from the conventional as well as BAT pickling effluents. In addition, an equal number of *B. cereus* cells was isolated from both of the conventional and BAT pickling effluents, which was log 0.07 (±0.29, n=5) cfu/ml. *Bacillus cereus* was found to be absent in the control samples during the conventional and BAT pickling processes, but there was no significant difference between the bacterial count in the experimental sample than the corresponding control sample (Mann-Whitney test: $U_{20, 20}$=38.00, p=0.655 [conventional and BAT]). Additionally, bacterial count in the conventional pickling effluent was considerably lower than the conventional bating effluent (Mann-Whitney test: $U_{20, 20}$=66.5, p<<0.001). On the other hand, there was no significant difference between the number of bacterial colonies obtained from the BAT bating effluent and BAT pickling effluent (Mann-Whitney test: $U_{20, 20}$=170.00, p=0.157).

The pH of a conventional pickling liquid is reduced to 2-3 by adding acid, such as sulfuric acid and formic acid, in order to achieve chrome(III) penetration throughout the cross-section of the pelts. This may cause swelling of the pelts and therefore sodium chloride (NaCl), 6-8% w/v, is generally added to the pickling liquid to prevent the swelling (Leafe, 1999). In this instance, 1.2% w/w sulfuric acid and 1% w/w formic acid was added during the conventional pickling process and the pH of the pickling liquid was 2.8-3.0. Although *B. cereus* is capable of tolerating a high level of NaCl (Mahakarnchanakul and Beuchat, 1999), the growth of *B. cereus* was found to decrease at higher NaCl concentrations above 7% w/v (Raevuori and Genigeorgis, 1975).

In order to reduce the salt pollution load, a NaCl-free pickling procedure was followed during the BAT leather-making process. In this instance, the pH of the BAT pickling liquid was adjusted to 2.7-2.8 by adding an aromatic polysulfonic acid-based compound Sellatan P (2% w/w) (TFL, Germany) and formic acid (0.5% w/w). Sodium formate (HCOONa, 0.25% w/w) was also added to the BAT pickling liquid. Little information is available on the effect of various acids on *B. cereus*. Inorganic acids such as hydrochloric acid and organic acids,

such as acetic acid, may or may not inhibit the growth of *B. cereus* depending on the net pH of the solutions (Browne and Dowds, 2002; Chen *et al.*, 2009; Wong and Chen, 1988). The molecular properties of the organic acids may also be an important factor when considering the antimicrobial activities (Hsiao and Siebert, 1999). Scant information was found regarding the effect HCOONa on the microbial growth. However, HCOONa may not have an inhibitory effect on microorganisms instead it was found in several studies (Linton *et al.*, 1981; Peel and Quayle, 1961) that it may have a stimulatory effect on bacterial growth as many bacteria can utilise HCOONa as a carbon source.

It appears that the both of the pickling processed did not provide suitable conditions for bacterial growth and proliferation. The presence of acids during both of the conventional and BAT deliming processes as well as the presence of high amount of salt in the conventional pickling processes may have inhibited the growth of *B. ceruse*. Therefore, bacterial growth in the pickling effluent may be considered to be negligible.

5.4.5.8.   Chrome Tanning

Absence of *B. cereus* was observed in the chrome tanning effluent including the control samples for both of the conventional and BAT leather-making processes. Chromium (Cr), particularly Cr(VI) is toxic towards plants and animals (Department of Environment, 1978). The presence of high concentration of heavy-metal such as chrome and lead in soil found to have an adverse effect on soil microbial flora (Alvarez-Bernal *et al.*, 2006; Shi *et al.*, 2002). *Bacillus cereus* is a soil inhabitant (Todar, 2008), therefore presence of high chromium concentration may have an adverse effect on its growth. Kalantari (2008) found that Cr(III) has an inhibitory effect on the growth of *B. cereus* and therefore may prevent the growth or recovery of *B. cereus* during the chrome tanning process. In addition, a magnesium oxide (MgO)-based compound, Feliderm® MGO Powder (Clariant, Switzerland) was used to increase the pH during chrome tanning in order to increase the molecular size of the chromium(III) salt as well as the reactions sites within the skins to achieve chrome fixation (Covington, 2001). Research showed (Sawai and Yoshikawa, 2004; Sawai *et al.*, 2000) MgO has an antimicrobial property and this is may be due to the production of active oxygen (O^{-2}) by MgO, which interrupt microbial activity through oxidation of the cells (Klánová and Lajèíková, 2006). The presence of MgO therefore may also have an adverse effect on *B. cereus* growth during the chrome tanning process. The absence of *B. cereus* during the chrome tanning process may also be due to the presence of non-culturable bacterial cells or due to the inactivation of the bacterial cells during the previous stages (unhairing-pickling) of the leather manufacturing processes.

5.4.6. Enumeration of <u>Bacillus</u> <u>cereus</u> from Swabs (Conventional and BAT Leather-Making Process)

The flesh side of calf skin pieces were swabbed and the swabs were analysed in order to determine the presence of the inoculated bacterial colonies on calf skin pieces during the conventional and BAT leather-making processes. This would only provide qualitative data, as it was not feasible to measure the area of the swabbed location due to the impracticality of the task and to avoid potential contamination. No calf skin pieces were added during the control experiments therefore, no swabs were collected from the control samples (see Section 2.14 and 2.15).

5.4.6.1. Pre-soaking

The average number of isolated *B. cereus* colonies from swabs were log 3.61 ($\pm$0.54, n=5) cfu/ml and log 2.33 ($\pm$0.40, n=5) cfu/ml after the conventional and BAT pre-soaking processes respectively (Figure 5.9). Similar to the pre-soaking effluents, a lower number of *B. cereus* colonies were isolated from calf skins after the BAT pre-soaking process than the conventional pre-soaking process. Due the presence of a nutritional source, moisture, a suitable pH range and a moderate temperature, the pre-soaking process may assist survival of *B. cereus* (Ghorbel *et al.*, 2003; Nilegaonkar *et al.*, 2007; Rangarajan and Didato, 2003).
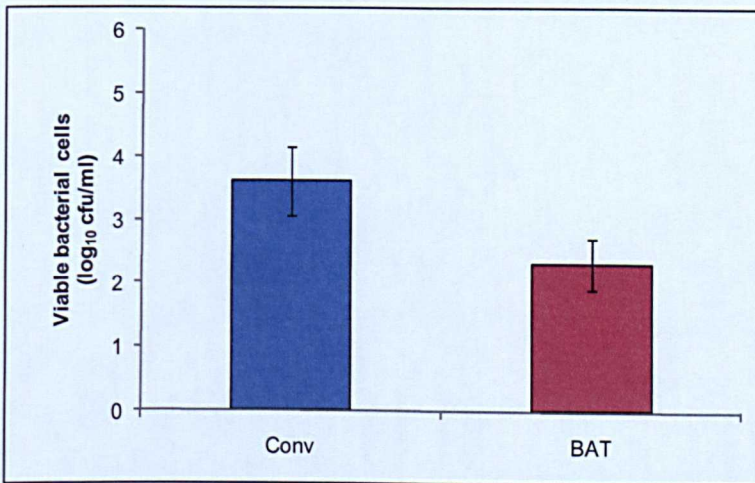
Figure 5.9: Number of isolated presumptive *B. cereus* colonies from swabs after the conventional (conv) and BAT pre-soaking processes, n=5. The average number of the inoculated *B. cereus* ATCC11778 was log 8.61 cfu (conv) and log 8.75 cfu (BAT). The error bars represent the standard deviation.

5.4.6.2.   Soaking

The average number of presumptive *B. cereus* colonies isolated from swabs after the conventional and BAT soaking processes was log 3.56 (±0.91, n=5) cfu/ml and log 2.00 (±0.82, n=5) cfu/ml respectively (Figure 5.10). Similar to the soaking process, due to the presence of protein, moisture, a suitable pH, moderate temperatures and an adequate incubation period, both the conventional and BAT soaking processes may also assist the survival of *B. cereus* (Birbir and Ilgaz, 1996).
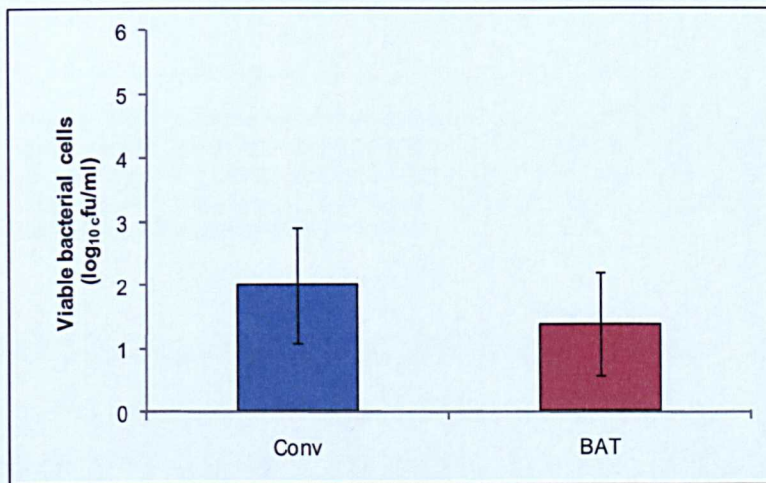
Figure 5.10: Number of isolated presumptive *B. cereus* colonies from swabs after the conventional (conv) and BAT soaking processes, n=5. Total number of inoculated *B. cereus* ATCC11778 was log 8.61 cfu (conv) and log 8.75 cfu (BAT). The error bars represent the standard deviation.

5.4.6.3.   Unhairing

Figure 5.11 shows the results obtained when swabs after the conventional and BAT unhairing processes, were analysed to determine the presence of *B. cereus* cells on unhaired calf skins. The average number of enumerated *B. cereus* colonies was log 1.17 (±0.89, n=5) cfu/ml and log 1.34 (±0.84, n=5) cfu/ml from swabs after the conventional and BAT unhairing processes respectively. In this instance, the presence of *B. cereus* was monitored in all calf skin samples for both of the conventional and BAT unhairing processes. The presence of Na_2S and lime during the unhairing processes may have suppressed the growth of *B. cereus* (Birbir and Ilgaz, 1996). However, the study showed the presence of viable *B. cereus* cells on calf skin pieces after the conventional and BAT unhairing processes, indicating that the unhairing processes did not cause total inactivation of the microorganisms.
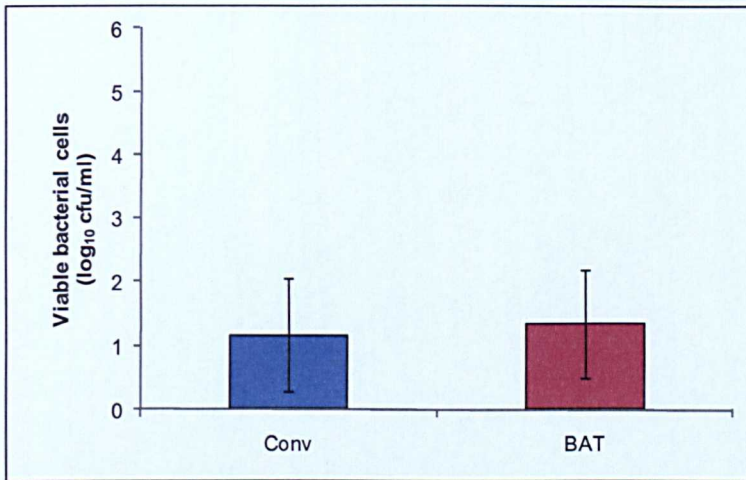
Figure 5.11: Number of isolated presumptive *B. cereus* colonies from swabs after the conventional (conv) and BAT unhairing processes, n=5. Total number of inoculated *B. cereus* ATCC11778 was log 8.61 cfu (conv) and log 8.75 cfu (BAT). The error bars represent the standard deviations.

5.4.6.4.   Reliming, Deliming, Bating, Pickling and Chrome Tanning

An average of log 0.13 (±0.40, n=5) cfu/ml of *B. cereus* colonies was isolated from swabs after the conventional reliming process, whereas, no *B. cereus* colonies were isolated from swabs after the BAT reliming process. The presence of lime during the reliming processes may have inhibited the recovery of *B. cereus*. In addition, absence of *B. cereus* was observed in swabs after both the conventional and BAT deliming, bating, pickling and chrome tanning processes. The absence of bacterial cells on the pelts does not necessarily indicate total inactivation of microorganisms; this could suggest that there was absence of microorganisms on the swabbed area or the presence of non-culturable microorganisms.

5.4.7.   *Enumeration of Bacillus cereus from Hair Samples Collect from the Conventional and BAT Unhairing Effluents*

An unexpected result was obtained when hair, which was collected from the conventional and BAT unhairing effluents, was incubated overnight in TSB at 30°C. The results obtained are shown in Figure 5.12. It was assumed that the unhairing processes particularly the conventional unhairing process, would destroy the majority of the bacteria (Department of Agriculture, Fisheries and Forestry, Australia, 2001; Biosecurity, New Zealand, 2008) resulting in a low recovery of the *B. cereus* colonies. However, the results show recovery of a minimum 10^2 (=log 2) cfu/ml of *B. cereus*. An overnight incubation in TSB may provide an adequate time, a suitable temperature and nutrition to repair the damaged cells and germination of spores.
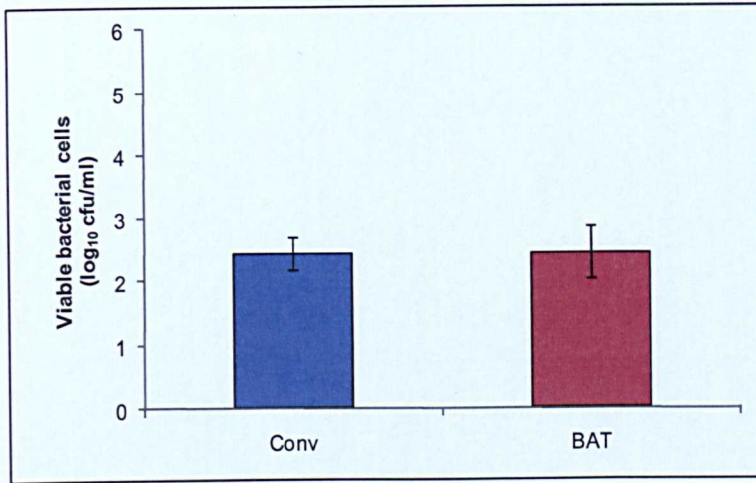
Figure 5.12: The average number of recovered presumptive *Bacillus cereus* colonies in hair collected from the conventional (con) and BAT unhairing process. Hair samples were incubated overnight in TSB at 30°C in order to assist the recovery of *Bacillus cereus* colonies, n=3. The error bars represent standard deviation.

5.4.8. Confirmation Tests for Presumptive <u>Bacillus</u> <u>cereus</u>

The isolated bacterial cells were purified by streaking to a single colony on PEMBA media. The purified colonies were then re-streaked on the media to ensure an adequate supply of the purified colonies for various confirmatory tests. The bacterial cells and spores were stained using a Gram-staining (see Section 2.7) and spore-staining (see Section 2.8) techniques, and the microscopic structure of the bacterial cells were observed under a light microscope (Nikon Eclipse 600; Nikon, Japan). Figure 5.13 shows Gram-positive and rod-shaped bacterial cells that occurred in chains. Figure 5.14 shows green endospores (malachite green) against red (safranin) vegetative cells.
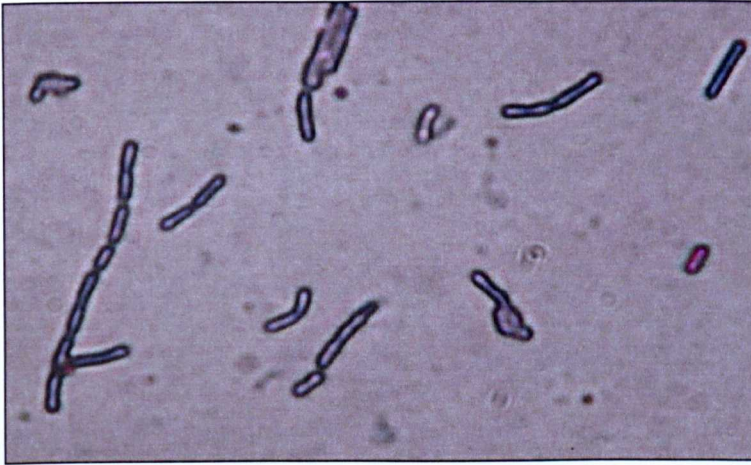
Figure 5.13: Gram-positive and rod-shaped *B. cereus* ATCC11778 cells under a light microscope (Nikon eclipses 600, Nikon, Japan), 1 k magnification. The cells were stained purple using a Gram-staining technique (see Section 2.7).
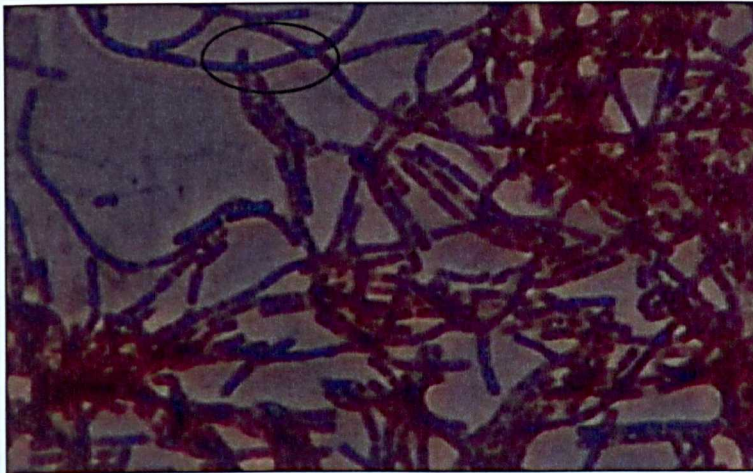
Figure 5.14: *Bacillus cereus* ATCC11778 endospores (circled area), when observed under a light microscope (1 k magnification, Nikon eclipse, 600, Nikon, Japan). The spores were stained using a malachite green dye, and the vegetative cells were stained using a red dye safranin (see Section 2.8).

Biochemical tests were carried out with *B. cereus* ATCC11778 colonies (Table 5.1). *Bacillus cereus* ATCC11778 colonies fermented dextrose, but did not ferment arabinose, mannitol and xylose. In addition, they also showed anaerobic growth, beta-haemolysis and the ability to reduce nitrate to nitrite. All the results from the biochemical tests are the characteristics of *B. cereus*.

Table 5.1: Biochemical tests for *B. cereus* ATCC11778 colonies.

Potentially suspected as	Carbohydrate fermentation				Nitrate reduction	Anaerobic growth	Beta haemolysis	Identified as
	Ara	Dex	Man	Xyl				
B. cereus	-	+	-	-	+	+	+	*B. cereus*
B. cereus	-	+	-	-	+	+	+	*B. cereus*
B. cereus	-	+	-	-	+	+	+	*B. cereus*
B. cereus	-	+	-	-	+	+	+	*B. cereus*
B. cereus	-	+	-	-	+	+	+	*B. cereus*

Note: Arb=arabinose, Dex=dextrose, Man=mannitol and Xyl=xylose; (+) sign indicates the positive reaction, whilst (-) sign indicates the negative reactions.

Tables 5.2 and 5.3 shows the results of biochemical test for the presumptive *B. cereus* colonies isolated during the conventional and BAT leather-making processes respectively. Presumptive *B. cereus* colonies isolated at different stages of the conventional leather-making process exhibited the same biochemical characteristics, when compared with *B. cereus* ATCC11778 (Table 5.2). These colonies produced an acid from dextrose but did not produce acids from arabinose, mannitol and xylose. In addition, the bacterial cells exhibited beta-haemolysis, anaerobic growth and reduced nitrate to nitrite. On the other hand, The presumptive *B. cereus* colonies isolated during various stages of the BAT leather-making process were beta-haemolytic, showed anaerobic growth, fermented dextrose and did not ferment mannitol, arabinose and xylose. In addition, 61.3% of the tested samples gave positive results for nitrate reduction tests. The results of the biochemical tests are shown in Table 5.3. The phenotypic characteristics of microorganisms are often dependent on the media and growth conditions such as temperature, pH, osmolarity, nutrient depletion, vitamin and mineral availability, growth cycle, water activity and colony density on plates (Sutton and Cundell, 2004). Therefore, some colonies of the same strain may fail to exhibit the same phenotypic characteristics.

Table 5.2: Biochemical tests for the presumptive *B. cereus* colonies that were isolated during the conventional leather-making process.

Potentially suspected as	Isolated from	Carbohydrate fermentation				Nitrate reduction	Anaerobic growth	Beta haemolysis	Identified as
		Ara	Dex	Man	Xyl				
B. cereus ATCC11778	Control	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Pre-soaking	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Soaking	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Unhairing/liming	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Reliming	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Deliming	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Bating	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Hair	-	+	-	-	+	+	+	*B. cereus*

Note: Arb=arabinose, Dex=dextrose, Man=mannitol and Xyl=xylose; (+) sign indicates the positive reaction, whilst (-) sign indicates the negative reactions; hair was isolated from the unharing effluent.

Table 5.3: Biochemical tests for the presumptive *B. cereus* colonies that were isolated during the BAT leather-making process.

Potentially suspected as	Isolated from	Carbohydrate fermentation				Nitrate reduction	Anaerobic growth	Beta haemolysis	Identified as
		Ara	Dex	Man	Xyl				
B. cereus ATCC11778	Control	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Pre-soaking	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Soaking	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Unhairing/liming	-	+	-	-	-	+	+	-
B. cereus	Reliming	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Deliming	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Bating	-	+	-	-	+	+	+	*B. cereus*
B. cereus	Pickling	-	+	-	-	-	+	+	-
B. cereus	Hair	-	+	-	-	-	+	+	-

Note: Arb=arabinose, Dex=dextrose, Man=mannitol and Xyl=xylose; (+) sign indicates the positive reaction, whilst (-) sign indicates the negative reactions; hair was isolated from the unhairing effluent.

Table 5.4 shows the results obtained when the Biolog identification method was carried out for *B. cereus* ATCC11778 colonies. The Biolog identification system identified *B. cereus* ATCC11778 as *B. cereus/thuringienisis*, with 100% probability. Due to the similar phenotypic properties, the Biolog system was unable to distinguish between *B. cereus* and *B. thuringienisis*.

Table 5.4: Application of the Biolog dentification method for *Bacillus cereus* ATCC11778 colonies.

Potentially suspected as	Biolog Identification	Probability (%)
B. cereus	*Bacillus cereus/thuringiensis A*	100
B. cereus	*Bacillus cereus/thuringiensis A*	100
B. cereus	*Bacillus cereus/thuringiensis A*	100

Tables 5.5 and 5.6 show the results obtained when the Biolog identification system was applied to identify the presumptive *B. cereus* colonies. The majority (80%) of the tested colonies, which were isolated from the conventional leather-making process, were identified as *B. cereus/thuringiensis* with 100% probability. All the tested colonies that were isolated from the BAT leather-making process were identified as *B. cereus/thuringiensis* with 100% probability.

Table 5.5: The Biolog Identification of presumptive *B. cereus* colonies that were isolated during the conventional leather-making process.

Potentially suspected as	Isolated from	Biolog Identification	Probability (%)
B. cereus ATCC11778	Control	*B. cereus/thuringiensis A*	100
B. cereus	Pre-soaking	*B. cereus/ thuringiensis A*	100
B. cereus	Soaking	*B. cereus/ thuringiensis A*	100
B. cereus	Reliming	*B. cereus/thuringiensis A*	100
B. cereus	Unhairing	*B. cereus/thuringiensis A*	100
B. cereus	Deliming	*B. cereus/thuringiensis A*	100
B. cereus	Bating	*B. cereus/thuringiensis A*	100
B. cereus	Hair (isolated from the unhairing effluent)	*B. cereus/thuringiensis A*	-

Table 5.6: The Biolog identification of presumptive *B. cereus* colonies that were isolated during the BAT leather-making process.

Potentially suspected as	Isolated from	Biolog identification	Probability (%)
B. cereus ATCC11778	Control	*B. cereus/ thuringiensis A*	100
B. cereus	Pre-soaking	*B. cereus/ thuringiensis A*	100
B. cereus	Soaking	*B. cereus/ thuringiensis A*	100
B. cereus	Liming	*B. cereus/ thuringiensis A*	100
B. cereus	Reliming	*B. cereus/ thuringiensis A*	100
B. cereus	Deliming	*B. cereus/ thuringiensis A*	100
B. cereus	Bating	*B. cereus/ thuringiensis A*	100
B. cereus	Pickling	*B. cereus/ thuringiensis A*	100
B. cereus	Hair (Isolated from the unhairing effluent)	*B. cereus/ thuringiensis A*	100

As the biochemical tests depend on many variables of the growth media and conditions (Sutton and Cundell, 2004) thus the tests may not always produce a reliable result for microbial identification. Alternatively, the DNA of each species is unique and therefore may be used as an identification tool. In this study, the PFGE method was applied to determine the DNA profiles of *B. cereus* ATCC11778 and isolated *B. cereus*.

Figures 5.15 and 5.16 show the results, when a PFGE method was applied to determine DNA profiles of *B. cereus*, that were isolated during the conventional and BAT leather-making processes and compared with the DNA profiles of *B. cereus* ATCC11778. It appears that the isolated *B. cereus* had the identical DNA band patterns when compared to the *B. cereus* ATCC11778. This confirms that the isolated *B. cereus* was the same strain as the inoculated *B. cereus* ATCC11778. In addition, this ensures that the growth of inoculated bacterial species was not inhibited by the presence of other species that may have survived the sodium hypochlorite-treatments. Analysis of the *B. cereus* DNA also suggests that no alteration in DNA occurred during the conventional and BAT leather-making process. Figures 5.16 and 5.17 show, a background smearing indicating the presence of degraded DNA. Research (Corkill *et al.*, 2000; Leclair *et al.*, 2006; Zhang, *et al.*, 2004) showed, that the addition of thiourea (50 μM) provided protection against degradation of sensitive DNA.
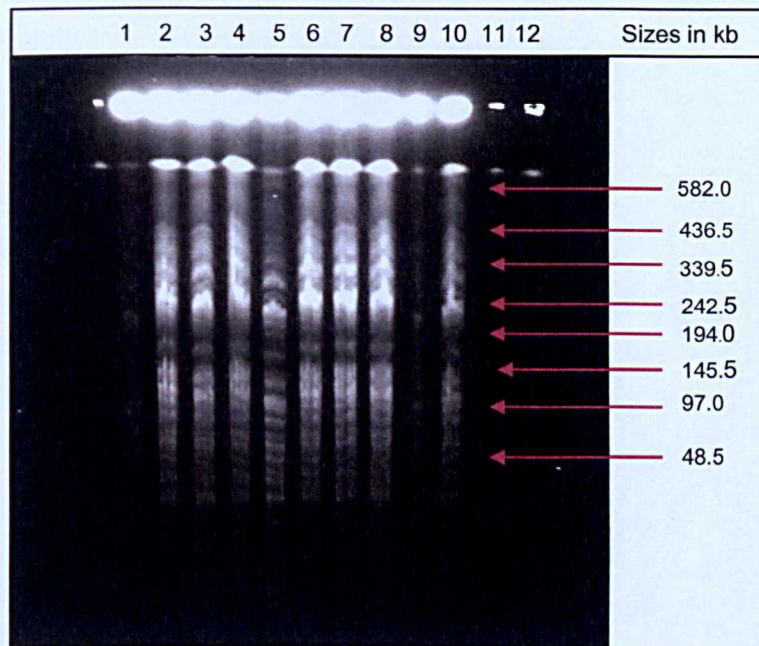
Figure 5.15: Pulsed-field gel electrophoresis of *Bacillus cereus* DNA (conventional leather-making process). Lane 1: *B. cereus* ATCC 11778 DNA; lanes 2, 3, 4, 5, 6, 7 and 8: *B. cereus* DNA isolated during the conventional pre-soaking, soaking, unhairng/liming, hair (after the unhairing/liming process), reliming, deliming and bating process respectively; lanes 11 and 12; Lambda Ladders (48.5- 970 kb).
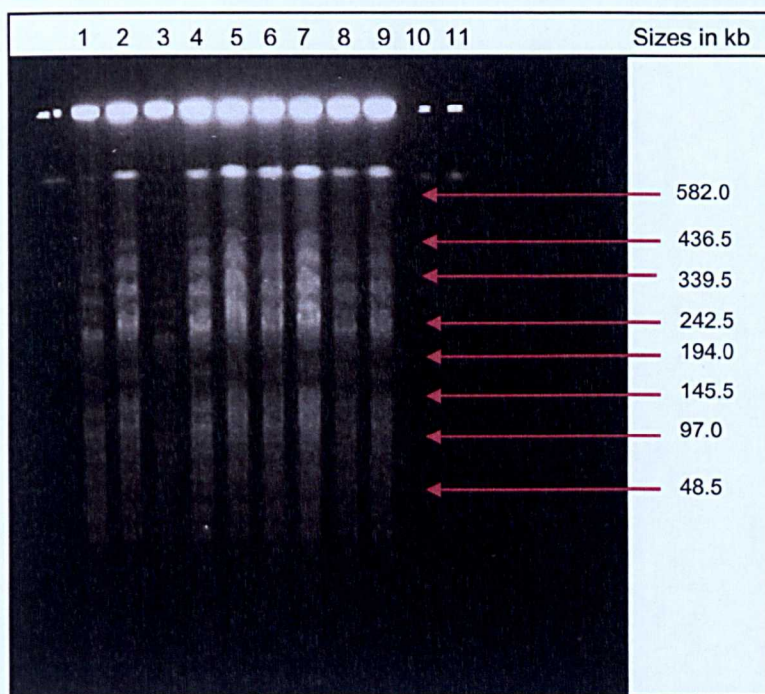
Figure 5.16: Pulsed-field gel electrophoresis of *Bacillus cereus* DNA. Lane 1: *B. cereus* ATCC11778 DNA; lanes 2, 3, 4, 5, 6, 7, 8 and 9: *B. cereus* DNA isolated during the BAT pre-soaking, soaking, unhairng/liming, hair (after the unhairing process), reliming, deliming, bating and pickling processes; lane 10: Lambda Ladders (48.5- 970 kb).

5.5.   Summary

The study showed that the conventional and BAT pre-soaking processes as well as the soaking processes, may favour *B. cereus* growth. Presence of nutrition, such as protein and fat, moisture, moderate pH and temperature may provide suitable conditions for the survival of *B. cereus* during the pre-soaking and soaking processes. Due to the presence of lime and sodium sulfide, the unhairing processes, particularly the conventional unhairing process, was found to be suppressing *B. cereus* growth. The presence of *B. cereus* colonies in the unhairing effluent, unhaired skins and hair from the unhairing effluents suggest that the unhairing processes did not sterilise the skins. Growth of *B. cereus* in the consequent reliming, deliming, bating effluents signifies that the total inactivation of *B. cereus* did not occur during the unhairing and reliming processes. Table 5.7 shows the average number of isolated *B. cereus* colonies from various stages of the conventional and BAT leather-manufacturing processes.

Bacterial species may became non-culturable, due to environmental stress such as lack of nutrients, inadequate temperatures, presence of salt, metals and other unwanted chemicals, but may still be viable (WS Atkins Environment, 2000; Rowan, 2004; Higgins *et al.*, 2007). In

addition, *B. cereus* produces spores when exposed to a harsh environment. Spores formed by *Bacillus* species are known to have survived in a dormant state for over 50 years (Brock and Madigan, 1991). However, recovery of the dormant bacterial cells may be possible when provided with suitable growth conditions (WS Atkins Environment, 2000; Rowan. 2004; Higgins *et al.*, 2007).

Table 5.7: The average number of *Bacillus cereus* colonies isolated from various stages of the conventional and BAT leather-manufacturing processes.

| Processing stages | Viable bacterial cells (log$_{10}$ colony forming units/ml) | | | |
| | Effluents | | Swabs | |
	Conventional	BAT	Conventional	BAT
Pre-soaking	4.75	3.22	3.61	2.33
Soaking	3.56	2.97	2.00	1.40
Unhairing	0.47	2.44	1.17	1.34
Reliming	0.42	0.07	0.13	0.07
Deliming	1.16	0.07	0.00	0.00
Bating	1.22	0.26	0.00	0.00
Pickling	0.07	0.07	0.00	0.00
Chrome tanning	0.00	0.00	0.00	0,00

During the standard biochemical tests, the majority of the isolated *B. cereus* exhibited the same biochemical properties as *B. cereus* ATCC11778. The Biolog system indentified the isolated bacterial colonies as *B. cereus*. In addition, the PFGE method of DNA fingerprinting verified that the isolated *B. cereus* were the same as *B. cereus* ATCC10145, indicating the presence of the inoculated *B. cereus* ATCC10145 during the leather manufacturing process experiments.

5.6.   Conclusion

The study showed that *B. cereus* is capable of surviving the environmental conditions found during the beamhouse processes for both of the conventional and BAT leather-making processes. Biochemical tests, the Biolog identification method and the PFGE method of DNA fingerprinting confirmed that the isolated bacterial colonies were the inoculated *B. cereus* ATCC11778.

Chapter 6: The Effects of the Leather Manufacturing Process on the Growth of *Pseudomonas aeruginosa*

6.1. Introduction

Pseudomonas aeruginosa is a Gram-negative, aerobic and rod-shaped bacterium. It belongs to the genus *Pseudomonas* and family *Pseudomonadaceae*. *Pseudomonas aeruginosa* is 1.5-3.0 μm in length and 0.5-0.8 μm in width (Todar, 2008). The majority of *P. aeruginosa* have 1-3 polar flagella (Pelczar *et al.*, 1993) and are therefore motile. It produces water-soluble pigments pyoverdin and pyocyanin. Due to the production of a fluorescent pigment pyoverdin *P. aeruginosa* fluoresces under UV lights. In addition, due to the formation of pyocyanin *P. aeruginosa* produces a distinctive blue-green appearance (Pelczar *et al.*, 1993; Barrow and Feltham, 2003; Todar, 2008), when cultured on a solid or a liquid medium. Formation of pyocyanin is a unique characteristic of *P. aeruginosa* (Barrow and Feltham, 2003) and therefore used for its identification. *Pseudomonas aeruginosa* grows at 37°C, also able to grow at higher temperatures such as 42°C (Barrow and Feltham, 2003; Health Protection Agency (HPA), 2007; Todar, 2008). Although *P. aeruginosa* is capable of growth at a pH range of 5.6-8.0, the optimum pH range is 6.6-7.0 (Todar, 2008).

Pseudomonas aeruginosa is a common inhabitant in soil, decaying organic compounds, water and plant surfaces. It may also occur on animal or human skins. It has minimal nutritional requirements, and is known to grow in distilled and mineral water (Legnani *et al.*, 1999; Todar, 2008). *Pseudomonas aeruginosa* is also able to utilise a wide range of organic compounds as a carbon source, including a variety of sugars, fatty acids, amino acids, amines and various organic compounds (Madigan *et al.*, 1997; HPA, 2007; Murray *et al.*, 2005; Todar, 2008). *Pseudomonas aeruginosa* is resistant to a wide range of chemicals such as high salt and dye concentrations. In addition, *P. aeruginosa* is resistant to weak antiseptics and many common antibiotics (Todar, 2008).

Pseudomonas aeruginosa is as an opportunistic pathogen. It does not infect healthy tissues, but may cause infection in immuno-compromised people. Therefore, it is primarily known as a nosocomial pathogen (nosocomial pathogens are mainly hospital-acquired pathogens). It is capable of penetrating through the skin via open wounds (Pelczar *et al.*, 1993; Madigan *et al.*, 1997; Blanc *et al.*, 2007; Todar, 2008). *Pseudomonas aeruginosa* causes a wide variety of infections such as pulmonary infections, skin infections, bacteraemia and septicaemia, central nervous system infections and gastrointestinal infections (Todar, 2008).

Research (Oppong *et al.*, 2006; Kayalvizhi *et al.*, 2008) showed the presence of *Pseudomonas* species on fresh hides/skins. *Pseudomonas* species were also isolated from the tannery wastewater by Kumar *et al.* (2008). Birbir and Ilgaz (1996) isolated *P. aeruginosa* from soaked hides. The ability to withstand a wide range of antibiotics and chemicals may assist the survival of *P. aeruginosa* in the extreme environmental conditions such as those found in leather manufacturing processes. In this chapter, the effect of a conventional and BAT leather-making process on the growth of *P. aeruginosa* was determined.

6.2.   Aims and Objectives

- To determine the effect of the conventional and BAT leather making processes on the growth of *P. aeruginosa*.

  - To study the growth patterns of *P. aeruginosa* ATCC10145 in order to calculate the mid-exponential phase for inoculation.

  - To undertake a trial in order to evaluate *P. aeruginosa* ATCC10145 growth on sodium hypochlorite decontaminated calf skins.

  - To enumerate and isolate *P. aeruginosa* in the effluent collected from various stages of the conventional and BAT leather-making process such as the pre-soaking, soaking, unhairing, reliming, deliming, bating, pickling and chrome tanning process.

  - To determine the presence of the inoculated *P. aeruginosa* on calf skins (by analysing the swabs) at the end of the each above mentioned stages of the conventional and BAT leather making processes.

- To carry out confirmatory tests with the isolated *P. aeruginosa* colonies using the Biolog identification system.

- To undertake the PFGE method in order to determine *P. aeruginosa* DNA profiles to ensure the *P. aeruginosa* colonies isolated during the conventional and BAT leather making processes were the same strain as the inoculated *P. aeruginosa* ATCC10145.

6.3.   Materials and Methods

Pseudomonas aeruginosa ATCC10145 (Oxoid, UK) was used in this experiment. A *Pseudomonas* CN media with glycerol, cetrimide and naildixate (Oxoid, UK) was used to enumerate *P. aeruginosa* (HPA, 2007). In this study the total number of replicates will be referred to as n. Growth pattern of *P. aeruginosa*, ATCC10145 was observed using a spectrophotometer (CE1011, Cecil Instrumetnt Ltd., UK) as described in Section 2.5 to calculate the mid-exponential phase. A trial was conducted in triplicate as described in Section 2.11 in order to evaluate the growth and survival of *P. aeruginosa* on the sodium hypochlorite (0.125 g/ml) treated calf skin pieces.

Calf skin pieces, 5, each weighing 100±4 g were obtained from the same calf skin and used for each of the conventional and BAT leather making processes. Calf skin pieces were decontaminated using a sodium hypochlorite (NaClO) solution (0.125 g/ml) (see Section 2.9) and inoculated with *P. aeruginosa* ATCC10145 (see Section 2.10). *Pseudomonas aeruginosa* ATCC10145 inoculum was collected from the mid-exponential phase. A conventional or BAT leather making processes was carried out using the inoculated calf skin pieces as described in Section 2.12. In this instance, the 5 calf skin pieces will be referred to as the experimental samples and the total number of replicates will referred to as n. A control experiment was also undertaken without calf skin pieces, whereas, water and other chemicals were added, and will be referred to as control sample for the purpose of this study.

Effluents were collected and calf skin pieces were swabbed in duplicate at the end of various stages during the conventional and BAT leather making processes (see Section 2.13). Swabs were not collected from the control samples as no calf skin pieces were added during the control experiments. Hair samples were also collected from the conventional and BAT unhairing effluents in duplicates and incubated overnight in tryptone soya broth (TSB) at 37°C to allow the recovery of damaged bacterial cells (see Section 2.14.3). Effluents, swabs and hair samples were analysed in order to determine the presence of *P. aeruginosa* at various stages of the conventional and BAT leather making processes (see Sections 2.5 and 2.14). Enumeration and primary identification of the isolated *P. aeruginosa* colonies were carried as described in Sections 2.5.3, 2.6.3 and 2.15.1.3. A Gram-staining (see Section 2.7) technique was applied to stain the bacterial cells and the microscopic structure was observed under a light microscope (Nikon Eclipse, Nikon, Japan). The Biolog identification system (see Section 2.15.2) along with the PFGE technique (see Section 2.17.3) was used

as a confirmatory test for the isolated *P. aeruginosa*. *Pseudomonas aeruginosa* ATCC10145 was used as a positive control for the Biolog and PFGE methods.

6.4. Results and Discussion

6.4.1. *Pseudomonas aeruginosa Growth Curve*
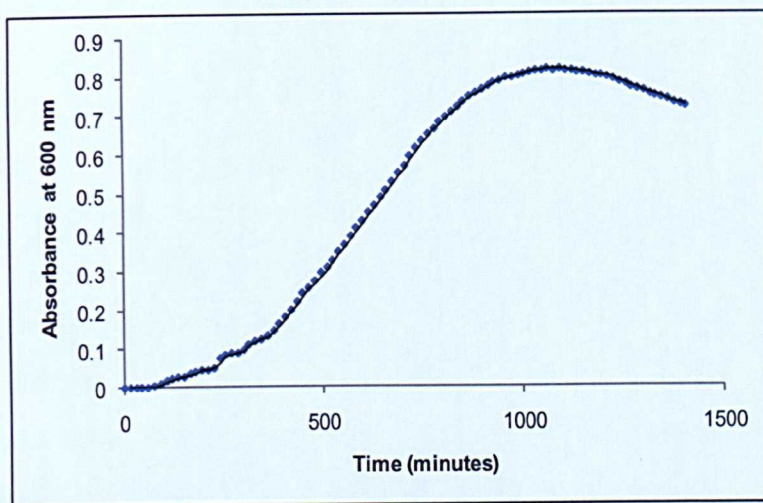
Figure 6.1: *Pseudomonas aeruginosa*, **ATCC 10145, growth curve (a representative sample).**

Pseudomonas aeruginosa ATCC10145 inoculum was collected from the mid-exponential phase, since bacterial cells in the exponential phase are the most active as well as being the most stable in physical and chemical properties (Pelczar *et al.*, 1993; Prescott *et al.*, 2008). Growth curves for *P. aeruginosa* were plotted using absorbance values against time (minutes) to calculate the incubation period required for the bacterial cells to reach the mid-exponential phase. Figure 6.1 shows the growth pattern of a representative sample. The calculated incubation period for *P. aeruginosa* ATCC10145 to reach the mid-exponential phase was 662 minutes (±178, n= 5).

6.4.2. *Trials to Determine the Survival of the Inoculated Pseudomonas aeruginosa ATCC10145 on the Sodium hypochlorite-Decontaminated Calf Skins*

A trial was undertaken in order to determine the survival and growth of *P. aeruginosa* on the NaClO (0.125 g/ml)-treated calf skin pieces. Analysis of the water and swabs after the final washing cycle, following a NaClO-decontamination of calf skin pieces, showed the absence of *P. aeruginosa*. Therefore, the decontamination procedure was considered to be satisfactory. The decontaminated calf skin pieces were inoculated with log 9.42±0.05 cfu of *P. aeruginosa* ATCC10145 followed by an overnight incubation at 37°C.
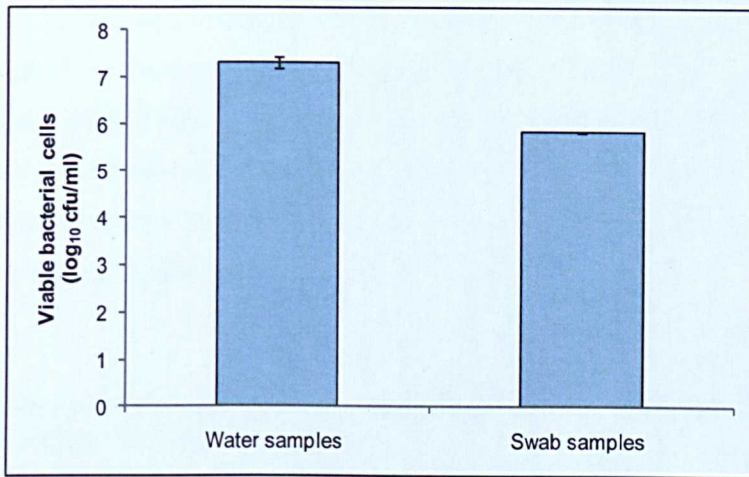
Figure 6.2: Recovery of the inoculated *P. aeruginosa* on the sodium hypochlorite (0.125 g/ml) treated calf skin pieces, when water and swabs samples were analysed, n=3. Bacterial enumeration was carried out on *Pseudomonas* CN media in triplicate. The average number of inoculated *P. aeruginosa* ATCC10145 was log 9.42 cfu. The error bars represent standard deviation.

Figure 6.2 shows, the recovery of the inoculated *P. aeruginosa* colonies on the decontaminated calf skin pieces after overnight incubation. An average of log 7.33 (±0.12, n=3) cfu/ml and log 5.85 (±0.12, n=3) cfu/ml of the inoculated *P. aeruginosa* was recovered from the water and swab samples respectively. Due to the recovery of more than 10^5 (=log 5.0) cfu/ml, the growth and survival of *P. aeruginosa* on the NaClO-decontaminated calf skin pieces was considered to be satisfactory. Therefore, the same decontamination and inoculation procedures were carried out for the conventional and BAT leather making process experiments.

6.4.3. Decontamination of Calf Skin Pieces Using Sodium Hypochlorite for the Conventional and BAT Leather Making process Experiments

Similar to the trial samples, the absence of *P. aeruginosa* was observed in water and swabs after the final washing cycle, following the NaClO (0.125 g/ml)-decontamination procedure of calf skin pieces, which were used for the conventional and BAT leather making processes. Due to the absence of *P. aeruginosa* in the water and swabs, the decontamination of calf skin pieces was considered to be satisfactory.

6.4.4. Inoculation of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa ATCC10145 culture (4 ml culture in TSB) were collected from the mid-exponential phase (see Section 6.4.1) and inoculated on the decontaminated calf skin pieces. The total number of inoculated *P. aeruginosa* ATCC10145 was measured (see

Section 2.10.2). The average number of inoculated *P. aeruginosa* colonies on calf skin pieces that were used for the conventional and BAT leather making processes was log 10.02 ($\pm$0.05) cfu and log 9.87 ($\pm$0.02) cfu respectively. Number of bacterial inoculate on calf skins that were used for the conventional leather-manufacturing process was significantly higher than the number of bacterial inoculate on calf skins that were used for the BAT leather-manufacturing process (t=5.424, d.f.=6.0, p=0.002).

6.4.5. Enumeration of <u>Pseudomonas aeruginosa</u> from Effluent (Conventional and BAT Leather Making Processes)

6.4.5.1. Pre-soaking

Figure 6.3 shows the average number of isolated *P. aeruginosa* colonies from the pre-soaking effluents during the conventional and BAT leather making processes. Analysis of the control samples during both of the conventional and BAT leather making processes showed absence of *P. aeruginosa*. It appears that the pre-soaking processes favoured *P. aeruginosa* growth. The number of isolated *P. aeruginosa* colonies from the conventional and BAT pre-soaking effluent were log 7.79 ($\pm$0.05, n=5) cfu/ml and log 7.69 ($\pm$0.11, n=5) cfu/ml, was isolated from the conventional and BAT pre-soaking effluent respectively. Evidently bacterial count in the pre-soaking effluent was significantly higher than the corresponding control samples (Mann-Whitney test: $U20_{20, 4}$=0.00, p=0.02 [conventional and BAT]). There was a significant difference between the bacterial count in the conventional and BAT pre-soaking effluent (t=3.876, df=28.580, p=0.001) although both of the conventional and BAT pre-soaking processes were carried out following the same method.

Both of the conventional and BAT pre-soaking process was carried out using 300% v/w water and 0.2% w/w of a sodium dimethyldithiocarbamate-based bactericide, Truposept BA (Trumpler, UK), which is may have a low environmental impact (International Union of Leather Technologist and Chemists Societies (IULTCS), 2008). As mentioned in Chapter 5, bactericides are generally added during the leather manufacturing processes to control the bacterial growth as the presence of a high number of bacterial colonies cause protein deterioration and hence reduce the quality of the leather production (Kayalvizhi *et al.*, 2007). However, the added bactericide may not cause total removal of the microorganisms present on the hides and skins.

Figure 6.3: Number of isolated *P. aeruginosa* colonies from the conventional (conv) and BAT pre-soaking effluents, n=5. The average number of inoculated *P. aeruginosa*, ATCC10145, cells was log 10.02 cfu (conv) and log 9.87 cfu (BAT). The error bars represent standard deviation.

Bacterial cells require nutrition for their growth and survival. Microorganisms are able to use organic compounds to derive essential elements, such as carbon, hydrogen, oxygen, nitrogen and sulfur, for microbial metabolism (Madigan *et al.*, 1997; Murray *et al.*, 2005). Many microorganisms also have the ability of utilising hide and skin proteins as a nutritional source (Shede *et al.*, 2008; Vankar and Dwivedi, 2009). *Pseudomonas aeruginosa* has proteolytic activity (Najafi *et al.*, 2005; Werthén *et al.*, 2004) and therefore is able to break down protein to amino acids and utilise it as a carbon source for the growth (Todar, 2008). The main structural protein of hides or skins is collagen (Bailey, 1998; Heidemann, 1993; Reich, 1986). Vasileva-Tonkova *et al.* (2007) found that *P. aeruginosa* are capable of utilising collagen hydrolysates obtained from calf skins. Therefore, hides or skins may serve as a nutritional source for *P. aeruginosa* metabolism during the leather manufacturing processes.

Factors such as moisture content, pH and temperatures of an environment are also important for the bacterial growth. During the pre-soaking process, hides/skins gain moisture promoting growth of microorganisms (Rangarajan and Didato, 2003). Microorganisms require a specific pH range for growth. The intracellular pH of microbial cells should maintain near to the neutral pH range, regardless of the extracellular pH, to prevent cell destruction. Various microorganisms are able to withstand various extracellular pH and therefore has various pH requirements (Madigan *et al.*, 1997). The pH of the conventional and BAT pre-soaking effluents was 7.6-8.0, which was within the pH range required for *P. aeruginosa* growth and proliferation (Todar, 2008). This may assist the growth of *P. aeruginosa* during the pre-soaking process. The room temperature during the conventional and BAT pre-

soaking processes was approximately 23°C. Although this was below the optimum temperature range required for the growth, however, Legnani *et al.* (1999) observed *P. aeruginosa* growth at 19-24°C. This suggests *P. aeruginosa* are capable of growth at a temperature that is lower than the required optimum temperature.

6.4.5.2.   Soaking

Figure 6.4 shows the results, when analysis of the conventional and BAT soaking effluents was carried out in order to enumerate *P. aeruginosa*. Analysis of the soaking effluents collected from the control samples showed no growth of *P. aeruginosa* during both of the conventional and BAT soaking processes indicating no contamination occurred during processing. Bacterial count in the experimental samples was found to be significantly higher than the bacterial count in the control samples (Mann-Whitney test: $U20_{20, 4}=0.00$, $p=0.02$ [conventional and BAT]). A significantly reduced number of *P. aeruginosa* colonies from log 7.79 (±0.05, n=5) to 7.64 (±0.07, n=5) cfu/ml and log 7.69 (±0.11, n=5) to 7.12 (±0.29, n=5) cfu/ml were isolated from the conventional (t=7.959, df=31.797, p<<0.001) and BAT (t=8.364, df=23.960, p<<0.001) soaking effluents respectively.
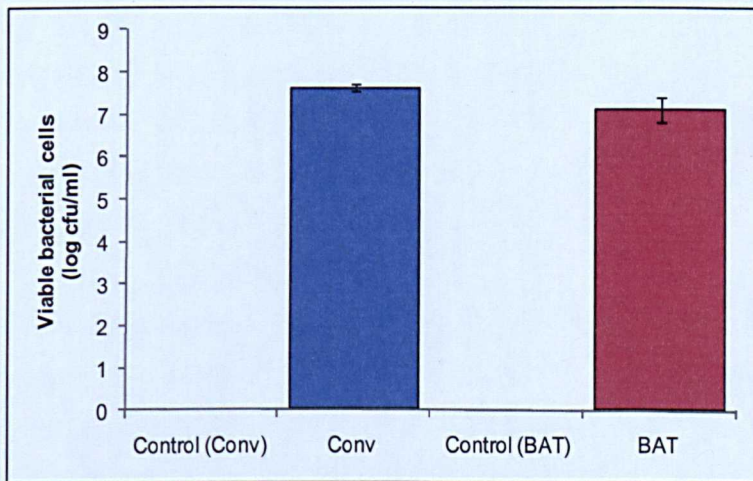
Figure 6.4: Number of isolated *P. aeruginosa* colonies from the conventional (conv) and BAT soaking effluents, n=5. The average number of inoculated *P. aeruginosa* ATCC10145, was log 10.02 cfu (conv) and log 9.87 cfu (BAT). The error bars represent standard deviation.

An anionic surfactant, sodium salt of alkyletherphosphate, Corilene®W385 (0.2% w/w) (STAHL Europe, The Netherlands) was added to the conventional soaking liquid to facilitate rehydration of calf skins. The majority of the surfactants that are used during the leather making processes cause environmental pollution and may also interrupt microbial activity by increasing the solubility of various compounds (Integrated Pollution Prevention and Control

(IPPC), 2001; Tibor *et al.*, 2002). A proteolytic enzyme-based compound Pelvit C, 0.5% w/w, (Together For Leather (TFL), Germany) was therefore used during the BAT soaking process to reduce the environmental pollution due to the presence of the surfactants. Enzymes are protein molecules (Madigan *et al.*, 1997) therefore may not have an inhibitory effect on *P. aeruginosa* growth, since many microorganisms are able to utilise protein as a nutritional source (Kayalvizhi *et al.*, 2007; Rangarajan and Didato, 2003; Wilson, 2005).

Sodium carbonate (Na_2CO_3) was added to increase the pH of the BAT soaking liquid, in order to achieve the required pH range (8.5-11.0) for the enzyme, Pelvit C. This also aids the water absorption by the skins (Heidemann, 1993). Sodium carbonate may have an inhibitory effect on the growth of microorganisms depending on the net pH of the solutions (Lakhanisky, 2002), was mentioned in Section 6.4.5.1, pH of a habitat is an important factor for microbial growth and multiplication.

A sodium dimethyldithiocarbamate-based bactericide, Truposept BA, 0.2% (w/w) was also added during the conventional and BAT soaking process. Bactericides are designed to control or destroy bacteria (IPPC, 2001); therefore, bactericides have an inhibitory effect on the bacterial growth. However, in this instance it appears that the presence of a bactericide, surfactant and Na_2CO_3 did not inhibit the growth of *P. aeruginosa* during the conventional and BAT soaking processes. *Pseudomonas aeruginosa* is resistant to a wide range of chemicals, which may assist the survival of bacterial cells during the soaking processes. In addition, the presence of proteins (i.e., skins/ enzymes) may serve as a nutritional source and therefore aid the growth of *P. aeruginosa*.

The conventional and BAT soaking processes were carried out overnight and this should provide adequate incubation period for proliferation of *P. aeruginosa*. The pH of the conventional soaking effluent was approximately 7.8, which is within the pH range required for the growth of *P. aeruginosa*. On the other hand, the pH of the BAT soaking effluent was higher, 9.2-9.5, than the conventional soaking effluent due to the presence of Na_2CO_3. The pH of the BAT pickling effluent is higher than the maximum pH range required for the growth of *P. aeruginosa*. However, isolation of a comparatively similar number of *P. aeruginosa* colonies from both the conventional and BAT soaking effluents indicates that a higher pH did not have a great deal of influence on *P. aeruginosa* growth in this regard.

6.4.5.3.   Unhairing

Analysis of the effluents collected from the control samples during the unhairing processes (both conventional and BAT) showed absence of *P. aeruginosa*. Absence of *P. aeruginosa* was also observed in the conventional and BAT unhairing effluents for the experimental samples. This suggests that both of the conventional and BAT unhairing process did not provide suitable environmental conditions for *P. aeruginosa* growth and proliferation. The conventional unhairing process was carried out using 200% w/w water, 2% w/w lime and 3% w/w sodium sulfide (Na_2S). To reduce environmental pollution a BAT unhairing process was carried out using 20% w/w water, 1.5% w/w proteolytic-based unhairing enzyme (Southern Petrochemicals Industries Corporation Ltd. (SPIC), India), 1% w/w lime and 0.5% w/w Na_2S. Lime (Al-Nazhan, 2002; Estrela *et al.*, 2003) and Na_2S (Bajza and Vrcek, 2001; Taleb-Ahmed *et al.*, 2005; Tišler *et al.*, 2004) have antimicrobial activity, which is mainly due to the strong alkaline properties and therefore inhibit the growth of P. aeruginosa during the unhairing processes. Due to the presence of lime and sodium sulfide the pH of the conventional and BAT unhairing effluent was 12.6-12.7 and 10.4-10.9 respectively, which are higher than the required pH range for *P. aeruginosa* growth. In addition, Na_2S is a reducing agent and therefore reduce the oxygen levels in water (Bajza and Vrcek, 2001; Bosnic *et al.*, 2000; Tišler *et al.*, 2004) and this may adversely affect the growth of aerobic microorganisms. Therefore, the presence of lime and Na_2S may prevent *P. aeruginosa* growth during the unhairing processes.

6.4.5.4.   Reliming

Similar to the unhairing processes, no *P. aeruginosa* colonies were isolated from the reliming effluents during the conventional and BAT leather making processes. The same reliming method, using 200% v/w water and 2% w/w lime, was followed during both of the conventional and BAT reliming processes. As mentioned previously lime has antimicrobial property (Sections 6.4.5.3, 5.4.5.3 and 5.4.5.4), which is probably due to its strong alkaline characteristics (Al-Nazhan, 2002; Estrela *et al.*, 2003; George *et al.*, 2001) and therefore may inhibit *P. aeruginosa* recovery during the reliming process.

6.4.5.5.   Deliming

Figure 6.5 shows the results obtained when the conventional and BAT deliming effluents was inoculated on *Pseudomonas* CN media in order to isolate *P. aeruginosa*. Microbial analysis of the control sample during the conventional deliming process showed no bacterial

growth. Although, bacterial growth was observed in the conventional deliming effluent, however, bacterial count in the effluent was not significantly different than the bacterial count obtained from the respective control sample (Mann-Whitney test: $U20_{20, 4}=24.00$, $p=0.138$). On the other hand, a limited number of *P. aeruginosa* colonies, log 0.33 cfu/ml (±0.65, n=5) was isolated from the control sample during the BAT deliming process. No further bacterial growth in the control sample occurred during the subsequent bating (Section 6.4.5.6), pickling (Section 6.4.5.7) and chrome-tanning (Section 6.4.5.8) processes. Bacterial count in the BAT deliming effluent was significantly higher than the bacterial count in the corresponding control sample (Mann-Whitney test: $U20_{20, 4}=9.00$, $p=0.015$).

It appears that both of the conventional and BAT deliming processes provided a suitable environment for the recovery of *P. aeruginosa*. An average of log 0.79 (±1.09, n=5) cfu/ml and log 2.68 (±1.70, n=5) cfu/ml presumptive *P. aeruginosa* colonies was isolated from the conventional and BAT deliming effluents respectively. Statistical analysis showed that the number of enumerated bacterial colonies from the deliming effluent was significantly higher than the number of the enumerated bacterial colonies from the reliming effluent (Mann-Whitney test: $U20_{20, 20}=120.00$, $p=0.02$ [conventional]; $U_{20, 20}=30.00$, $p<<0.001$ [BAT]).
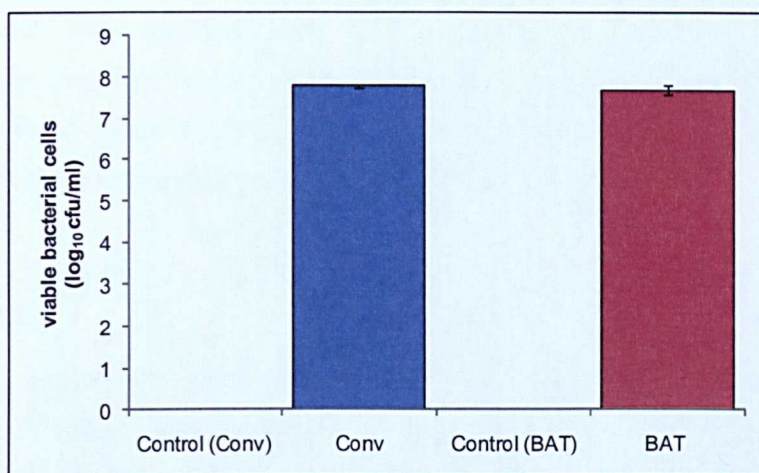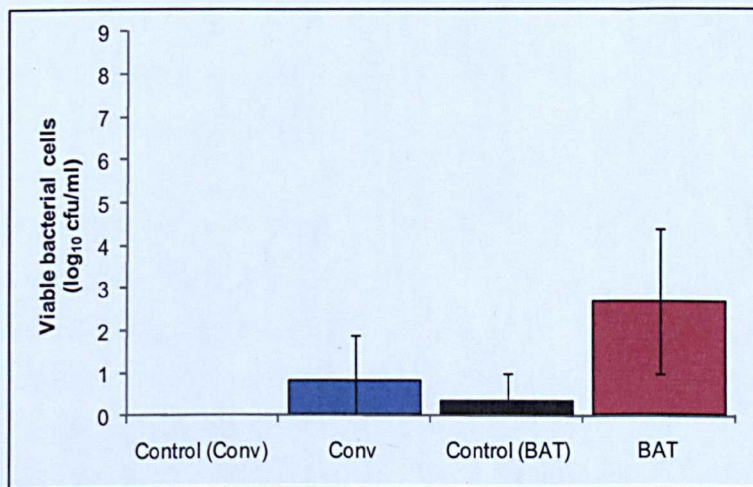

Figure 6.5: Number of isolated *P. aeruginosa* colonies from the conventional (conv) and BAT deliming effluent, n=5. The average number of inoculated *P. aeruginosa* ATCC10145, was log 10.02 cfu (conv) and log 9.87 cfu (BAT). The error bars represent standard deviation.

However, inconsistency in the number of the recovered *P. aeruginosa* colonies was observed. In addition, 10% and 60% of the petri-dishes that were inoculated with the conventional and BAT deliming effluent showed the presence of *P. aeruginosa*. The inconsistent growth of *P. aeruginosa* may be due to the recovery of some bacterial cells, while, other may have been injured beyond recovery or became non-culturable. The

inconsistent growth of *P. aeruginosa* on the inoculated plates is the cause of obtaining a large standard deviation.

Ammonium chloride (NH_4Cl), (2% w/w), was added to conventional deliming liquid to neutralise lime and other alkalise absorbed by skins during the unhairing and reliming processes, as well as to adjust the pH in order to ensure the maximum enzyme activity during the subsequent bating processes (Leafe, 1999). The pH requirement for the activity of the pancreatic-based enzyme Oropon ON 2 (TFL, Germany) that was used during the subsequent bating process is 7-9. Ammonium salts buffer the pH at 8-9 (Leafe, 1999), providing a suitable pH for Oropon ON 2 activity. In this instance, the pH of the conventional deliming effluent was 8.6-8.7, which was higher than the required pH range for *P. aeruginosa* growth. This may be the cause of isolation a lower number of *P. aeruginosa* colonies from the conventional deliming effluent than the BAT deliming effluent. Addition of NH_4Cl may promote *P. aeruginosa* growth and proliferation, since many bacteria are able to utilise ammonium salts as a nitrogen source. Nitrogen is one of the essential elements for microbial growth (Madigan *et al.*, 1997; Murray *et al.*, 2005). In addition, neutralisations of alkalis also lower pH of the deliming effluents assisting the microbial growth. A disadvantage of using ammonium salts during the deliming process is- it stimulates eutrophication and therefore causes oxygen depletion in water (Bosnic *et. al.*, 2000; IPPC, 2001). To reduce environmental pollution, NH_4Cl was replaced by carbon dioxide (CO_2) (IPPC, 2001; Leafe, 1999) during the BAT deliming process.

Carbon dioxide at a higher concentration may have an inhibitory action on the growth of *Pseudomonas* species (Bennik *et al.*, 1998). The inhibitory effect of CO_2 on *P. aeruginosa* may be due to the acidification or replacement of oxygen (Devlieghere and Debevere, 2000). However, some species of the genus *Pseudomonas* are capable of utilising inorganic compounds such as CO as a carbon source (Madigan *et al.*, 1997; Bergey's Manual of Determinative Bacteriology, 1974). In this regard, it appears that CO_2 did not have an inhibitory effect on *P. aeruginosa*; instead, CO_2 may have encouraged *P. aeruginosa* growth. Use of CO_2 reduces the pH of the deliming liquid to approximately 6.5 (Leafe, 1991). In this instance, the pH of the BAT deliming effluent was 6.3-7.5, which is within the pH requirements, and so promotes the growth of *P. aeruginosa*. Additionally calf skins may provide nutrition for microorganism (Brock and Medigan. 1991, Pelczar *et al.*, 1993; Murray *et al.*, 2005; Todar. 2008). The relimed calf skin pieces were washed with water before conducting both of the conventional and BAT deliming process. This reduces or neutralise the alkali that was absorbed by skins followed by the further neutralisation of alkalis during

the deliming process. Reduction in absorbed alkali reduces the alkalinity and this may had a positive influence on *P. aeruginosa*.

Enumeration of *P. aeruginosa* from the conventional and BAT deliming effluents suggests that the unhairing and reliming processes did not cause total elimination of *P. aeruginosa*. During the unhairing and reliming processes *P. aeruginosa* cells may became dormant or non-culturable, but still viable. Recovery of the dormant cells may have occurred when given suitable environmental conditions for the *P. aeruginosa* growth during the deliming processes.

6.4.5.6.   Bating

Figure 6.6 shows the results, when microbial analysis of the conventional and BAT bating effluents was carried out in order to enumerate *P. aeruginosa* colonies. Absence of *P. aeruginosa* colonies was observed in effluents that were collected from the control samples during the conventional and BAT bating processes. The number of bacterial colonies obtained from the conventional bating effluent was not significantly different than the corresponding control sample (Mann-Whitney test: $U20_{20, 4}$=26.00, p=0.177). On the other hand, bacterial count in the BAT bating effluent was significantly higher than the corresponding control sample (Mann-Whitney test: $U20_{20, 4}$=4.00, p=0.005).

A slightly reduced number of *P. aeruginosa* colonies, from log 0.79 (±1.09, n=5) to 0.66 (±0.93, n=5) cfu/ml was isolated from the conventional bating effluent (Figure, 6.6). On the other hand, a slightly increased number of *P. aeruginosa* colonies, from log 2.68 (±1.70, n=5) to 2.74 (±1.54, n=5) cfu/ml was isolated from the BAT bating effluent (Figure 6.6). However, both instances the difference between the bacterial counts in deliming and bating effluent was found to be insignificant (Mann-Whitney test: $U20_{20, 20}$=190.00, p=0.756 [conventional]; t=0.412, df=38.00, p=0.683 [BAT]). Similar to the deliming effluents, inconsistent growth of *P. aeruginosa* was also observed when the conventional and BAT bating effluents were analysed resulting in a high standard deviation. Appearance of *P. aeruginosa* occurred on 10% and 65% of the petri-dishes that were inoculated with the conventional and BAT bating effluents respectively.

Figure 6.6: Number of isolated *P. aeruginosa* colonies from the conventional (conv) and BAT bating effluent, n=5. The average number of inoculated *P. aeruginosa* ATCC10145 was log 10.02 cfu (conv) and log 9.87 cfu (BAT). The error bars represent standard deviation.

Similar to the deliming processes, the bating process, particularly BAT bating process appeared to be encouraging *P. aeruginosa* growth and proliferation. Neutralisation of the antimicrobial agents such as lime and Na_2S may promote the recovery of damaged microbial cells. In addition, available nutritional sources such as ammonium and proteins may have positive influences on the recovery of *P. aeruginosa*. The pH of the conventional bating effluent, 8.3-8.5, was higher than the required pH range for *P. aeruginosa* growth. A higher pH may be the cause of isolation of a lower number of *P. aeruginosa* colonies during the conventional bating process. Alternatively, a lower pH (6.9-7.4) of the BAT bating effluent may have promoted the *P. aeruginosa* growth.

The conventional and BAT bating process was carried out using a pancreatic-based enzyme Ororpn ON 2, 0.1% w/w, (TFL, Germany) and α-amylase, 1.25% w/w, (SPIC, India) respectively. Enzymes are considered as potentially non-hazardous chemicals, as these are protein catalysts (Madigan *et al.*, 1997) and therefore may not have had any toxic effect on the environment or on the growth of microorganisms. On the contrary, enzymes being protein, may act as a source of essential nutritional elements for microbial metabolism.

6.4.5.7. Pickling

A significant reduction in the number of bacterial colonies was occurred during both of the conventional and BAT pickling process (Mann-Whitney test: $U20_{20, 20}=130.00$, p=0.004 [conventional]; Mann-Whitney test: $U20_{20, 20}=20.00$ p<<0.001 [BAT]). Analysis of the effluents, which was collected from the control samples as well as from the experimental

samples during the conventional and BAT pickling processes, showed no bacterial growth. The conventional pickling process was carried out using 8% w/w sodium chloride (NaCl), 1.5% w/w sulfuric acid and 1.0% w/w formic acid. The pH of the pickling liquid is generally reduced to 2-3 to assist chrome(III) penetration throughout the cross-section during the chrome tanning process. However, a low pH may cause swelling of the collagen, and therefore to prevent the acid swelling, NaCl is generally added during the conventional pickling processes (Leafe, 1999).

Sivaprakasam *et al.* (2008), isolated salt-tolerant *P. aeruginosa* from tannery effluents and found that these bacteria are capable of withstanding a high NaCl concentration up to 5% (w/v). The presence of a higher quantity of NaCl (8% w/w) than the threshold limit may have a negative influence on the growth of *P. aeruginosa*.

The presence of salt (largely NaCl) in tannery effluents at high levels increases salinity of the soil and water, this may have adverse affects on plants and aquatic species particularly fresh water species (Bosnic *et. al.*, 2000; IPPC, 2001), when discharged to soil or water bodies. To reduce the salt pollution, the BAT pickling process was carried out using a non-swelling aromatic polysulfonic acid-based compound Sellatan P, 2.0% w/w, (TFL, Germany). As Sellatan P does not cause swelling of the skin therefore, NaCl is not required. In addition, 0.5% w/w formic acid and 0.25% w/w sodium formate (HCOONa) was added to the BAT pickling liquid. Sodium formate may serve as a carbon source for bacterial metabolism and therefore may have a stimulatory effect on bacterial growth (Linton *et al.*, 1981; Peel and Quayle, 1961). No information was available on the effect of an aromatic sulfonic acid on the growth of *P. aeruginosa*, however, it may inhibit microbial growth by lowering the pH of the pickling liquid.

In this instance, the pH of conventional and BAT pickling effluent was 2.6-2.8 and 2.5-2.8 respectively. The pH during the pickling process was lower than the required pH range for *P. aeruginosa* growth. *Pseudomonas aeruginosa* may be susceptible to a lower pH range. Tanner and James (1992) showed, at pH 2.5-3.0 in the presence of citric acid, lactic acid, phthalic acid, succinic acid, phosphoric acid, and sulfuric acid the viability of *P. aeruginosa* is reduced within 60 seconds. A low pH due to the presence of organic and inorganic acids during both of the conventional and BAT pickling process may therefore reduce the number of viable *P. aeruginosa* cells.

6.4.5.8. Chrome Tanning

Analysis of the chrome effluents during both of the conventional and BAT leather making processes showed the absence of *P. aeruginosa* in the control as well as in the experimental samples. Both of conventional and BAT process was carried out using a chrome (Cr)(III) sulfate, followed by the addition of a magnesium oxide (MgO)-based compound Feliderm®MGO Powder (Clariant, Switzerland). The pH of the tanning bath (liquid) is generally increased to approximately 4.0 in order to increase the molecular size of chrome salt and reaction sites within the pelts (Covington, 2001). In this instance, the pH of the conventional and BAT chrome tanning effluents was 4.1-4.5, which was lower than the required pH range for *P. aeruginosa* growth. Additionally, microorganisms are found to be sensitive towards Cr(III) (Chandra *et al.*, 2004) and MgO (Sawai and Yoshikawa, 2004; Sawai *et al.*, 2000). Therefore, the presence of Cr and MgO may have hindered the growth of *P. aeruginosa* during the chrome tanning processes. The mechanisms of chromium toxicity are not known. The antimicrobial activity of MgO is probably due to the formation of active oxygen (O_2^-) (Sawai and Yoshikawa, 2004; Sawai *et al.*, 2000), which may disrupt the bacterial cells through oxidation (Klánová and Lajèíková, 2006).

6.4.6. Enumeration of <u>Pseudomonas aeruginosa</u> from Swabs (Conventional and BAT Leather Making Processes)

6.4.6.1. Pre-soaking

As mentioned previously (Section 6.3), swabs were not collected from the control samples during the conventional and BAT leather making processes as no calf skins were used during the control experiments. Figure 6.7 shows the results obtained when swabs, after the conventional and BAT pre-soaking processes, were analysed. Similar to the effluent samples, approximately a similar number of *P. aeruginosa* colonies, which was log 5.83 (±0.07, n=5) cfu/ml and log 5.58 (±0.08, n=5) cfu/ml, were isolated from swabs after the conventional and BAT per-soaking processes respectively. Protein may provide the essential nutritional elements particularly carbon (Najafi *et al.*, 2005; Vasileva-Tonkova *et al.*, 2007; Werthén *et al.*, 2004) for *P. aeruginosa* metabolism. Additionally a suitable pH range, moderate temperatures and presence of moisture during the pre-soaking processes may promote the growth of *P. aeruginosa*.
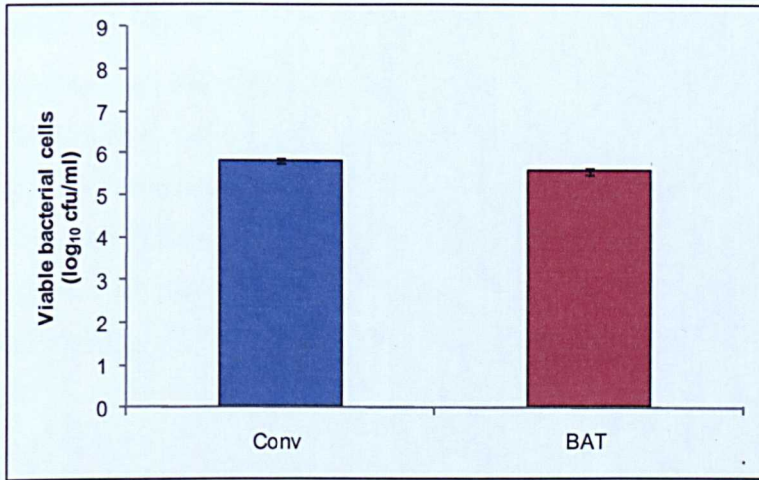
Figure 6.7: Number of isolated *P. aeruginosa* colonies in swabs after the conventional (conv) and BAT pre-soaking process, n=5. The average number of inoculated, *P. aeruginosa* ATCC10145 colonies was log 10.02 cfu (conv) and log 9.87 cfu (BAT). The error bars represent standard deviation.

6.4.6.2.   Soaking

Figure 6.8 shows the average of number of isolated *P. aeruginosa* from swabs after the conventional and BAT soaking processes and was of log 5.81 ($\pm$0.07, n=5) cfu/ml and log 5.43 ($\pm$0.18, n=5) cfu/ml respectively. Factors, such as nutritional source, moisture content, incubation period, moderate pH and temperature; maybe responsible for the growth and survival of *P. aeruginosa* on calf skin pieces during the soaking processes.
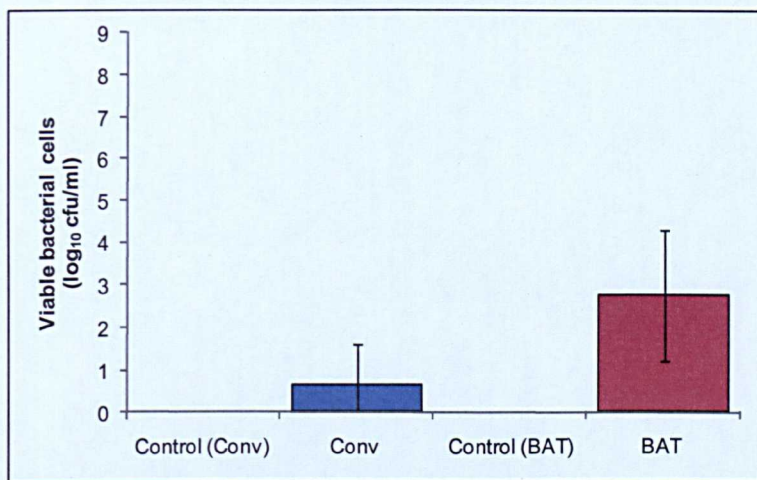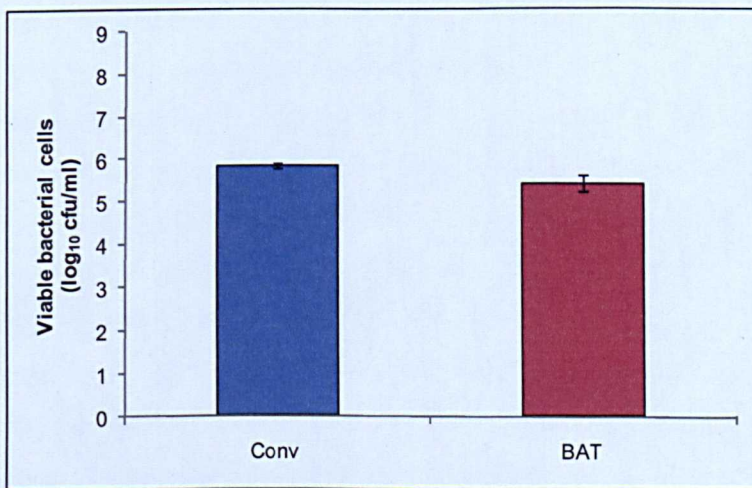

Figure 6.8: Number of isolated *P. aeruginosa* colonies from swabs after the conventional (conv) and BAT soaking processes, n=5. The average number of inoculated, *P. aeruginosa* ATCC10145, cells were log 10.02 cfu (conv) and log 9.87 cfu (BAT). The error bars represent standard deviation.

6.4.6.3. Unhairing and Reliming

Analysis of swabs after the conventional and BAT unhairing processes showed the absence of *P. aeruginosa*. Absence of *P. aeruginosa* was also observed in swabs after the conventional and BAT reliming processes. The presence of Na_2S and lime during the unhairing processes, and the presence of lime during the reliming processes may have inhibited *P. aeruginosa* growth (Birbir and Ilgaz, 1996; Biosecurity, New Zealand, 2008; Department of Agriculture, Fisheries and Forestry, Australia, 2001; Taleb-Ahmed *et al.*, 2005).

6.4.6.4. Deliming and Bating

An average of log 0.80 (±1.13, n=5) cfu/ml and log 0.75 (±1.06, n=5) cfu/ml *P. aeruginosa* was isolated from swabs after the BAT deliming and bating processes. Inconsistent growth of *P. aeruginosa* was observed during the deliming and bating processes resulting in a large standard deviation. *Pseudomonas aeruginosa* may have become dormant but still viable during the BAT unhairing and reliming processes. Presence of a protein source, moisture, CO_2, neutralisation of lime and Na_2S, a suitable pH and a moderate temperature during the BAT deliming and bating processes may have assisted the recovery of the dormant *P. aeruginosa*.

Absence of *P. aeruginosa* was observed in swabs after the conventional deliming and bating processes. Due to the presence of an ammonium salt, proteins, moisture, a moderate temperature and pH, the conventional deliming and bating processes may provide a suitable condition for *P. aeruginosa* growth. However, a low number of *P. aeruginosa* was isolated from the conventional deliming and bating effluents (Sections 6.4.5.5 and 6.4.5.6) indicating a recovery of a limited number of bacterial cells. It was observed in Chapter 5 as well as in this Chapter, enumeration of a lower number of bacterial cells from swabs than the corresponding effluent. This may explains the absence of *P. aeruginosa* colonies in swabs while limited growth of *P. aeruginosa* was observed in effluents during the conventional deliming and bating processes. Absence of *P. aeruginosa* may also be due to a number of reasons, such as the absence of the bacterial cells on the swabbed area or presence of the non-culturable bacterial cells.

6.4.6.5.   Pickling and Chrome Tanning

Analysis of the swabs after the conventional pickling process showed no bacterial growth. A limited number of *P. aeruginosa* (log 0.34 [±0.86, n=5] cfu/ml) was isolated from the swabs after the BAT pickling process and may be considered negligible. A low pH due to the presence of acids (sulfuric acid, formic acid and an aromatic polysulfonic acid-based compound) during the conventional and BAT pickling processes may be responsible for the decrease in the number of viable *P. aeruginosa* cells. In addition, no bacterial growth was observed in swabs after the chrome tanning process for both of the conventional and BAT leather making processes. Chrome (Chandra *et al.*, 2004) and MgO (Sawai and Yoshikawa, 2004; Sawai *et al.*, 2000) has antimicrobial properties and therefore the presence of Cr(III) and MgO may have inhibited the growth of *P. aeruginosa* during the chrome tanning process.

6.4.7.   Enumeration of Pseudomonas aeruginosa from Hair Samples Collect from the Conventional and BAT Unhairing Effluents

Analysis of the hair samples, that were collected from the conventional and BAT unhairing effluents, and were incubated overnight in TSB, showed absence of *P. aeruginosa*. This is probably due to the presence of lime and Na_2S. The strong alkaline properties of lime and Na_2S may have inactivated or damaged *P. aeruginosa* cells beyond recovery.

6.4.8.   Confirmatory Tests for Pseudomonas aeruginosa

The isolated bacterial cells were purified by streaking to a single colony on the *Pseudomonas* CN media and the purified colonies were then re-streaked on the media to ensure an adequate supply of the purified colonies for the Biolog and PFGE methods. A Gram-staining technique (see Section 2.7) was applied to stain *P. aeruginosa* cells and the stained cells were observed under a light microscope (Nikon Eclipse 600, Nikon, Japan) in order to determine the microscopic structure. The cells appeared red (Gram-negative) and rod-shaped when observed. Figure 6.9 and 6.10 show the microscopic structure of *P. aeruginosa* ATCC10145 cells (1 k magnification), when observed with a bright-field and dark-field background.
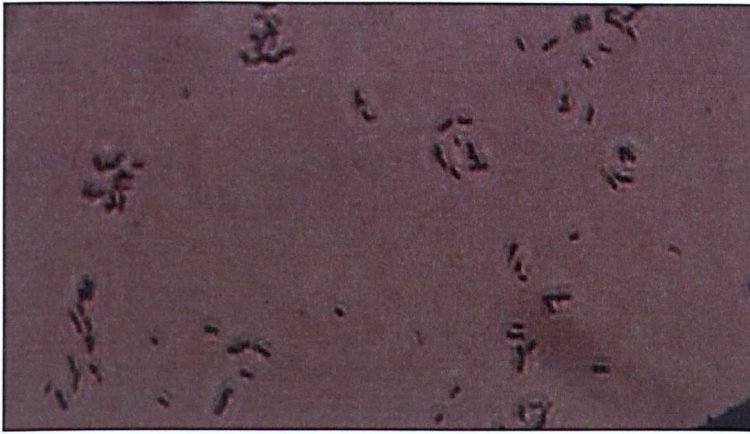
Figure 6.9: *Pseudomonas aeruginosa* **ATCC10145 cells under the light microscope at 1 k magnifications (Nikon Eclipse 600, Nikon, Japan). The rod shaped cells were appeared as Gram-negative when stained using a Gram-staining method as described in Section 2.7.**
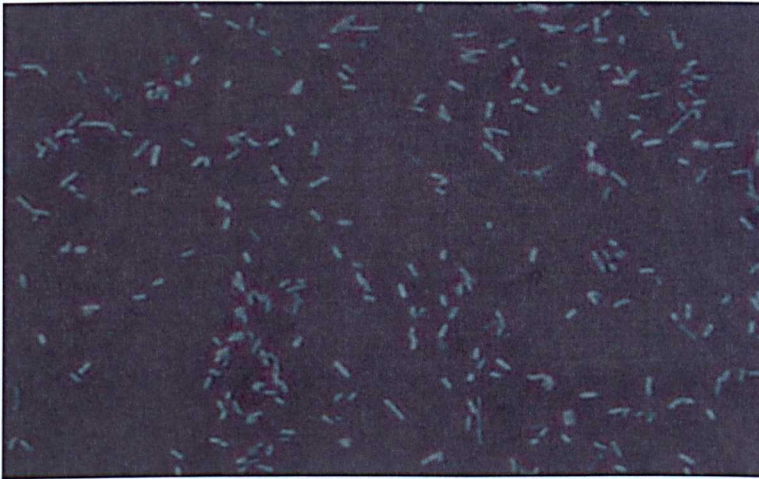
Figure 6.10: **Image of** *P. aeruginosa* **ATCC10145 cells with a dark-field background clearly showing the rod-shaped structure of the bacterial cells, when observed under a light microscope (Nikon Eclipse 600, Nikon, Japan).**

As mentioned previously (Section 2.17.1.3), according to the HPA protocol (2007), no further confirmation tests is necessary if *P. aeruginosa* produces blue or green coloured colonies on the *Pseudomonas* CN agar media. Since the isolated *P. aeruginosa* colonies during the conventional and BAT leather manufacturing processes appeared green on *Pseudomonas* CN media (Figure 2.4) and hence no further biochemical tests were undertaken for the isolated *P. aeruginosa* colonies.

The Biolog identification system was applied for the isolated *P. aeruginosa* colonies. Tables 6.1 and 6.2 show the results obtained when the Biolog identification method was used as a confirmatory test for the isolated *P. aeruginosa* colonies during the conventional and BAT

leather making processes respectively. All of the tested *P. aeruginosa* colonies were identified as *P. aeruginosa,* with 100% probability following the Biolog identification system.

Table 6.1: The Biolog identification of presumptive *P. aeruginosa* colonies that were isolated during the conventional leather making process.

Potentially suspected as	Enumerated from	Biolog Identification	Probability (100%)
P. aeruginosa, ATCC10145	Control	*P. aeruginosa*	100
P. aeruginosa	Pre-soaking	*P. aeruginosa*	100
P. aeruginosa	Pre-soaking	*P. aeruginosa*	100
P. aeruginosa	Pre-soaking	*P. aeruginosa*	100
P. aeruginosa	Soaking	*P. aeruginosa*	100
P. aeruginosa	Soaking	*P. aeruginosa*	100
P. aeruginosa	Soaking	*P. aeruginosa*	100
P. aeruginosa	Deliming	*P. aeruginosa*	100
P. aeruginosa	Bating	*P. aeruginosa*	100

Table 6.2: The Biolog identification of presumptive *P. aeruginosa* colonies that were isolated during the BAT leather making process.

Potentially suspected as	Enumerated from	Biolog Identification	Probability (100%)
P. aeruginosa, ATCC10145	Control	*P. aeruginosa*	100
P. aeruginosa	Pre-soaking	*P. aeruginosa*	100
P. aeruginosa	Pre-soaking	*P. aeruginosa*	100
P. aeruginosa	Deliming	*P. aeruginosa*	100
P. aeruginosa	Bating	*P. aeruginosa*	100
P. aeruginosa	Bating	*P. aeruginosa*	100
P. aeruginosa	Pickling	*P. aeruginosa*	100

The Biolog system is a useful tool for microbial identification, which identifies microbial species based on the utilisation of a specific carbon and nitrogen source. Therefore, it produces a phenotypic fingerprint, based on carbon and nitrogen utilisation (see Section 2.15.2) (WS Atkins Environment, 2000). However, the Biolog system is unable to produce a genetic fingerprint for a particular strain. A PFGE method was therefore applied for genetic fingerprinting of *P. aeruginosa*. The results obtained are shown in Figures 6.11 and 6.12.

The PFGE method was used to define *P. aeruginosa* DNA profiles that were isolated during the conventional and BAT leather making processes and compared with the *P. aeruginosa* ATCC10145 DNA profile. Figures 6.10 and 6.11 show the DNA band patterns of *P. aeruginosa,* which were isolated during the conventional and BAT leather manufacturing processes respectively.
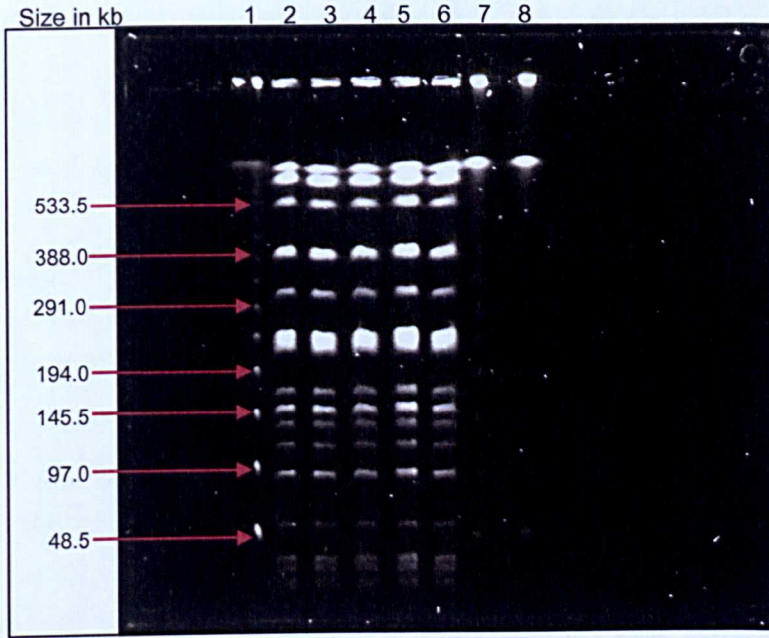
Figure 6.11: Pulsed-field gel electrophoresis of *P. aeruginosa* DNA (conventional leather making process). Lanes 1, 7 and 8: Lambda Ladders (48.5- 970 kb), lane 2: *P. aeruginosa*, ATCC10145, lanes 3, 4, 5 and 6: DNA of *P. aeruginosa* that were isolated during the conventional pre-soaking, soaking, deliming and bating processes respectively.
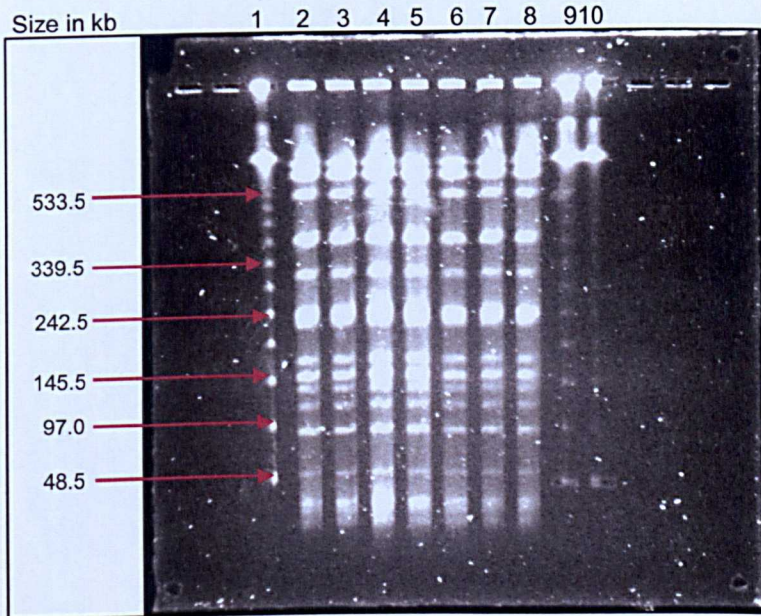
Figure 6.12: Pulsed-field gel electrophoresis of *P. aeruginosa* DNA (BAT leather making process). Lanes 1, 9 and 10: Lambda Ladders (48.5- 970 kb), lane 2: *P. aeruginosa* ATCC10145, lanes 3, 4, 6, 7 and 8: DNA of *P. aeruginosa* that were isolated from the BAT pickling, soaking, deliming, bating and pre-soaking processes respectively.

The experimented *P. aeruginosa* showed consistency in the DNA band patterns. The DNA profiles of the isolated *P. aeruginosa* colonies were identical match to the *P. aeruginosa* ATCC10145 DNA profile, indicating that all the tested *P. aeruginosa* was derived from the

same strain. This also suggests that no alteration in DNA have occurred during the conventional and BAT leather making processes. Additionally, this ensures that the growth of inoculated bacterial species was not inhibited by the presence of other species that may have survived the sodium hypochlorite treatments.

As mentioned previously (see page 10 and Figure 6.5) a limited number of *P. aeruginosa* colonies were isolated from effluents collected from the control samples during the conventional deliming process. In order to determine whether the origin of the isolated *P. aeruginosa* is *P. aeruginosa* ATCC10145, the PFGE method was undertaken and the results obtained are given in Figure 6.13. It appears that the isolated *P. aeruginosa* from the control sample have the identical DNA band patterns as *P. aeruginosa* ATCC1014, indicating that the tested bacterial DNA were derived from the same strain. The presence of *P. aeruginosa* ATCC10145 in the control samples (effluent) during the conventional deliming process was probably due to cross contamination during sample collection or analysis.
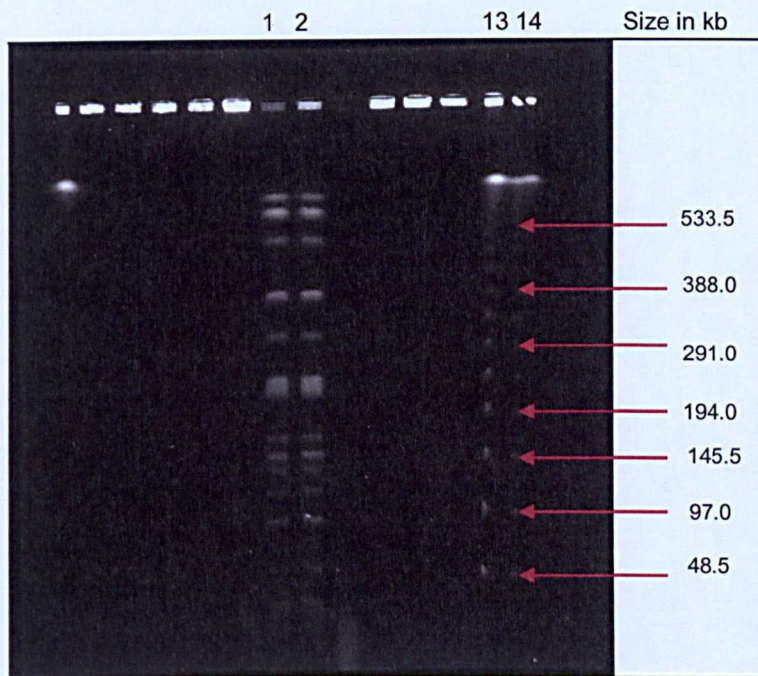
Figure 6.13: Pulsed-field gel electrophoresis of *P. aeruginosa* DNA isolated from a control sample during the BAT deliming process (lane 2), lane 1: *P. aeruginosa* ATCC10145, lanes 13 and 14: Lambda Ladders (48.5-970 kb).

6.5.   Summary

The study showed a higher *P. aeruginosa* growth during the pre-soaking and soaking stages for both of the conventional and BAT process than the subsequent processing stages indicating that the pre-soaking and soaking process may provide suitable growth conditions

for *P. aeruginosa*. This is probably due to the presence of a protein source, moisture, suitable pH, moderate temperature and adequate incubation period. Therefore, a high risk may be associated with the growth and transmission of pathogens to humans and environment during the pre-soaking and soaking processes. The growth of *P. aeruginosa* was found to be hindered during both of the conventional and BAT unhairing processes as well as reliming processes, indicating that the *P. aeruginosa* is sensitive towards Na_2S and lime.

The occurrence of *P. aeruginosa* colonies in the subsequent deliming and bating processes suggests that the unhairing and reliming process did not cause complete inactivation of *P. aeruginosa* cells. During the unhairing and reliming processes *P. aeruginosa* cells may became dormant but still viable. Recovery of the dormant and viable *P. aeruginosa* cells may have occurred due to the available nutritional source in combination with a suitable pH range due to the neutralisation of alkali and suitable temperatures during the deliming and bating processes (conventional and BAT). The pickling and chrome tanning process during both of the conventional and BAT process appeared to inhibit *P. aeruginosa*, indicating the susceptibility of *P. aeruginosa* towards a low pH, chromium and MgO. Table 6.3 shows the average number of isolated *P. aeruginosa* colonies from various stages of the conventional and BAT leather-manufacturing processes.

Table 6.3: The average number of *Pseudomonas aeruginosa* colonies isolated from various stages of the conventional and BAT leather-manufacturing processes.

| Processing stages | Viable bacterial cells (log_{10} colony forming units/ml) | | | |
| | Effluents | | Swabs | |
	Conventional	BAT	Conventional	BAT
Pre-soaking	7.79	7.69	5.83	5.58
Soaking	7.64	7.12	5.82	5.41
Unhairing	0.00	0.00	0.00	0.00
Reliming	0.00	0.00	0.00	0.00
Deliming	0.79	2.68	0.00	0.80
Bating	0.66	2.76	0.00	0.75
Pickling	0.00	0.00	0.00	0.34
Chrome tanning	0.00	0.00	0.00	0.00

The Biolog system identified the isolated bacterial colonies as *P. aeruginosa*. The PFGE method of DNA fingerprinting confirmed that the isolated *P. aeruginosa* were the same as *P. aeruginosa* ATCC10145, establishing the presence of the inoculated *P. aeruginosa* ATCC10145 during the leather making process experiments.

6.6.   Conclusion

The study showed that *P. aeruginosa* is capable of withstanding the leather manufacturing processes, particularly the beamhouse processes (both the conventional and BAT). The Biolog identification method and the PFGE method of DNA fingerprinting confirmed that the isolated bacterial colonies were *P. aeruginosa* ATCC10145.

Chapter 7: The Effects of the Leather Manufacturing Processes on the Growth of *Staphylococcus* species

7.1. Introduction

Staphylococcus (plural: Staphylococci) belongs to the family *Staphylococcaceae*. Staphylococci are non-motile, Gram-positive and occur in clusters (Barrow and Feltham, 2003; Madigan *et al.*, 1997; Murray *et al.*, 2005; Todar, 2008). Staphylococci are aerobes and require oxygen for growth, however many Staphylococci are facultative anaerobes and therefore capable of growth in the absence of oxygen. The spherical cells of *Staphylococcus* are 0.5-1.5 µm in diameter (Murray *et al.*, 2005). Many Staphylococci are common bacterial flora in humans and animals, whilst *S. aureus* and *S. epidermidis* are the two most common inhabitants in humans, and are largely found in nasal passages, skin and mucous membrane (Barrow and Feltham 2003; Madigan *et al.*, 1997; Murray *et al.*, 2005; Todar, 2008). The nasal passage of 10-50% of the healthy human population may carry *S. aureus* (Murray *et al.*, 2005). Staphylococci also found in the environment, such as in dust, air and water (Bremer *et. al.*, 2004).

The cell diameter of *S. aureus* may vary from 0.5-1 µm. *Staphylococcus aureus* produce yellow colonies on a nutrient rich medium and is able to grow at a wide temperature range of 7-45°C, but requires a temperature range of 30-45°C for optimal growth. In addition, *S. aureus* can withstand a wide range of pH, 4.4-9.7, but the optimum pH range is 6.0-7.5 (Bremer *et al.*, 2004; Murray *et al.*, 2005; Todar, 2008). Similar to *B. cereus*, *S. aureus* produces an enzyme lecithinase, which hydrolyses egg yolk. *Staphylococcus aureus* also produces enzymes coagulase and DNase. Due to the formation of coagulase it coagulates blood plasma and hydrolyses DNA due to the formation of DNase. Production of lecithinase, coagulase and DNase is often used for the identification of *S. aureus* (Health Protection Agency (HPA), 2005d). Coagulase-negative *S. aureus* have been isolated (O'Gara and Humphreys, 2001), though the coagulase test remains a reliable method for the identification of pathogenic *S. aureus* (Barrow and Feltham, 2003).

Staphylococcus aureus is an opportunistic pathogen and is often associated with nosocomial (hospital acquired) infections. Infections due to *S. aureus* may occur through ingestion, via damaged skin, wounds or hair follicles (Wilson, 2005). *Staphylococcus aureus* causes a wide range of diseases such as toxic shock syndrome, cutaneous infections, bacteraemia, pneumonia and septic arthritis (Murray *et al.*, 2005; Todar, 2008; Wilson, 2005).

Staphylococcus aureus is also a food poisoning bacterium and food containing 10^2 or more colony forming units (cfu)/ gram (g) is considered unsatisfactory for human consumption (HPA, 2005d).

Staphylococcus epidermidis is primarily considered as a non-pathogenic bacterium or a bacterium with low pathogenesis. However, it may act as an opportunistic pathogen in hospitalised patients due to their compromised immune system (Eiff *et al.*, 2002; O'Gara. and Humphreys, 2001; Todar, 2008; Wilson, 2005). *Staphylococcus epidermidis* causes bacteraemia and urinary tract infections, and may cause infections in orthopaedic joints, cardiac valves and vascular grafts. *Staphylococcus epidermidis* is found to be more resistant to antibiotics than *S. aureus* (Wilson, 2005).

Growth of *S. aureus* in salt (sodium chloride)-preserved hides and skin is feasible due to the ability of *S. aureus* to withstand high sodium chloride concentrations (Bremer *et al.*, 2004; Murray *et al.*, 2005; Todar, 2008). Various Staphylococci species including *S. aureus* and *S. epidermis* were isolated from raw hides/skins (Anderson, 1945; Birbir and Ilgaz, 1996; Hanlin *et al.*, 1995; McLaughlin and Rockwell, 1922; Oppong *et al.*, 2006) and leather (Birbir and Ilgaz, 1996). In this chapter the presence of *Staphylococcus* species at various stages of the conventional and BAT processes were determined. In addition, biochemical tests and the Biolog method of microbial identification were used as a confirmatory test for the isolated *Staphylococcus* species at various stages of the conventional and BAT leather-making processes.

7.2. Aims and Objectives

■ To determine the effect of the conventional and BAT leather manufacturing process on the growth of *S. aureus*.

- To study the growth patterns of *S. aureus* NCTC12981/ATCC25923 in order to calculate the mid-exponential phase for inoculation.

- To undertake a trial to study the survival of *S. aureus* NCTC12981/ATCC25923 on the sodium hypochlorite-treated calf skins.

- Microbial analysis of the effluents that were collected from various stages of the conventional and BAT leather-making processes to determine the presence of inoculated *S. aureus*.

- To observe the presence of inoculated *S. aureus* on calf skins by analysing swabs after various stages of the conventional and BAT leather-making processes.

▪ To carry out confirmatory tests to ensure the presence of the inoculated *S. aureus* using biochemical assays (standard biochemical tests and the Biolog identification system).

7.3.   Materials and Methods

Staphylococcus aureus NCTC12981/ATCC25923 (Prolab Diagnostic, UK) was used for the experiment. A Baird-Parker agar-based media (CM0275, Oxoid, UK) with egg yolk tellurite emulsion (SR0054, Oxoid UK) was used to enumerate *S. aureus*. In this study, the total number of replicates will be referred to as n. Growth curves of *S. aureus* were plotted in order to calculate the time required to reach the mid-exponential phase as described in Section 2.4. A trial was carried out in triplicate in order to study the survival of inoculated *S. aureus* on sodium hypochlorite (NaClO, 0.125 g/ml)-treated calf skin pieces (see Section 2.11).

Each of the conventional and BAT leather-making processes were carried out using 5 calf skins, each weighing 100±6 g and were taken from the same calf skin. In this instance, the calf skin pieces will be referred to as experimental samples. Calf skin pieces were decontaminated using a 0.125 g/ml NaClO solution, as described in Section 2.9. The water and swabs, after the final washing cycle, following the NaClO-decontamination, were enumerated to measure the number of microbial colonies present on the decontaminated-calf skin. *Staphylococcus aureus* NCTC12981/ATCC25923 inoculum was collected from the mid-exponential phase and inoculated on the NaClO-treated calf skin pieces (see Section 2.10).

Conventional (see Section 2.12.1) and BAT (see Section 2.12.2) leather manufacturing processes were conducted using the inoculated calf skin pieces. A control experiment was carried out during both of the conventional and BAT leather-making processes without the addition of calf skin pieces, while water and all the chemicals that were used for leather processing were added and will be referred to as control sample for the purpose of this study. Effluents were collected and calf skin pieces were swabbed in duplicates at the end of various stages of the conventional and BAT leather-making processes, such as pre-soaking, soaking, unhairing, reliming, deliming, bating, pickling and chrome tanning. Swabs were not collected from the control samples as calf skin pieces were not added during the control experiments. See Section 2.13 for the details of sample collection procedures. Effluent and

swabs were analysed as described in Section 2.14. Hair samples were also collected in duplicates from the unhairing effluents and incubated overnight in tryptone soya broth (TSB) at 37°C to allow recovery of damaged bacterial cells (see Section 2.14.1). Bacterial enumeration and preliminary identification was carried out as described in Sections 2.5.2 and 2.6.2 respectively. Biochemical tests (see Section 2.15.1.2) and the Biolog identification methods (see Section 2.15.2) were used as confirmatory tests for measuring the presumptive *S. aureus* colonies. *Staphylococcus aureus* NCTC12981/ATCC25923 was used as a positive control for the biochemical and Biolog experiments. The pulsed-field gel electrophoresis method of DNA profiling was not undertaken in this study due to time constraints.

7.4.   Results and Discussion

7.4.1.   *Staphylococcus aureus* Growth Curve
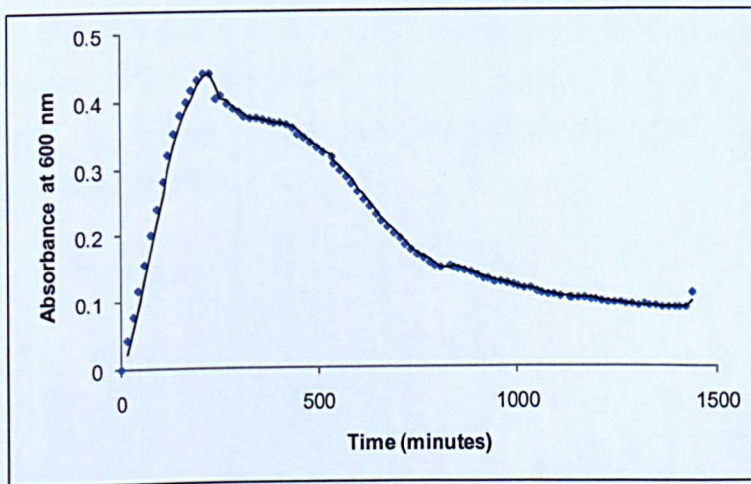
Figure 7.1: *Staphylococcus aureus* NCTC12981/ATCC25923 growth curve (a representative sample).

Staphylococcus aureus inoculum was collected from the mid-exponential phase, since bacterial cells in the exponential phase are most active (Prescott *et. al.*, 2008; Todar, 2008) and have stable physical and chemical properties (Pelczar *et al.*, 1993; Prescott *et al.*, 2008). In order to determine the incubation period required for the bacterial cells to reach the mid-exponential phase, *S. aureus* NCTC12981/ATCC25923 growth curves were plotted using the absorbance value against time (minutes). Figure 7.1 shows the growth pattern of a representative sample. It appeared that *S. aureus* colonies did not show the lag phase, which was may be due to the use of an overnight incubated bacterial culture to plot the growth curves. The calculated incubation period for the *S. aureus* NCTC12981/ATCC25923 to reach the mid-exponential phase was 144 minutes (±48.89, n=5). From the study, it was

decided that *S. aureus* was to be incubated in TSB for 2 hours and 25 minutes before inoculation on the NaClO- treated calf skin pieces for the trials as well as for the conventional and BAT the leather-making process experiments.

7.4.2. Trials to Determine the Survival of the Inoculated <u>Staphylococcus</u> <u>aureus</u> NCTC12981/ATCC25923 on the Sodium hypochlorite-Decontaminated Calf Skins

A trial was undertaken in order to determine whether *S. aureus* NCTC12981/ATCC25923 are capable of growing on NaClO-treated calf skin pieces. The calf skin pieces for the trials were treated with a NaClO (0.125 g/ml) solution and washed with sterilised water (see Section 2.11). Microorganisms present in the water and swabs after the final washing cycle were measured. An average of log 0.59 cfu/ml (±0.92, n=3) and log 0.32 cfu/ml (±0.64, n=3) was isolated from the water and swabs respectively after the final washing cycle. Due to the limited bacterial growth (<10 colonies≈log 1) in the water and swabs, the decontamination of calf skin pieces for this trial was considered to be satisfactory. The decontaminated calf skin pieces were inoculated with log 8.55±0.03 cfu of *S. aureus* NCTC12981/ATCC25923, and incubated overnight. The recovery of the inoculated *S. aureus* on the decontaminated-calf skin pieces is shown in Figure 7.2
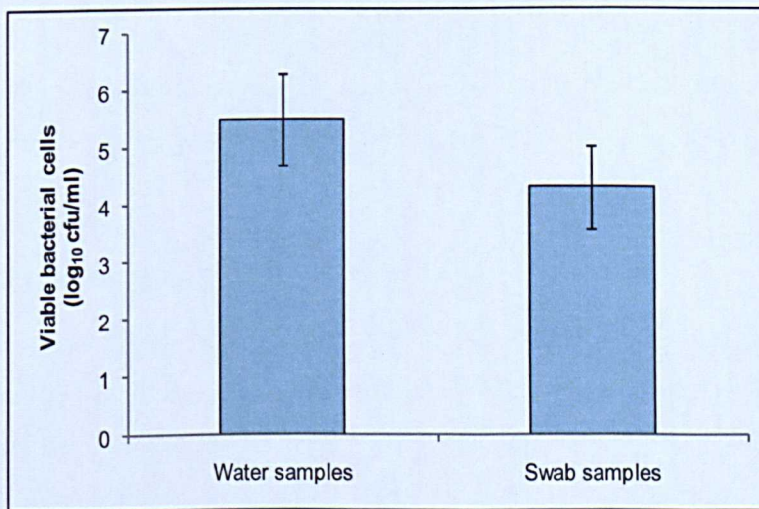
Figure 7.2: Recovery of the inoculated *S. aureus* on the sodium hypochlorite (0.125 g/ml) treated calf skin pieces, when water and swabs samples were analysed, n=3. Bacterial enumeration was carried out on Baird-Parker media in triplicate. The average number of inoculated *S. aureus* NCTC12981/ATCC25923 was log 8.55 cfu. The error bars represent the standard deviation.

An average of log 5.49 cfu/ml (±0.80, n=3) and log 4.30 cfu/ml (±0.7, n=3) of the inoculated *S. aureus* colonies were recovered in the water and swabs respectively after overnight

incubation. The infectious dose of *S. aureus* colonies for food poisoning is 10^2 cfu/g of food (HPA, 2005d). Due to the enumeration of more than 10^2 (=log 2) colonies, the recovery of *S. aureus* colonies was considered to be satisfactory and therefore the same decontamination and inoculation procedures were carried out for the conventional and BAT leather-making process experiments.

7.4.3. Decontamination of Calf Skin Pieces Using Sodium Hypochlorite for the Conventional and BAT Leather-Making Process Experiments

Calf skin pieces for the conventional and BAT leather-making processes were treated with NaClO (0.125 g/ml) and washed with sterilised tap water. Water and swabs after the final washing cycles were analysed in order to determine the presence of microorganism after the decontamination of calf skins. Absence of the microbial colonies was observed in water and swabs after the final washing cycle, following the decontamination of calf skin pieces that were used for the conventional leather-making processes. Therefore, the decontamination of calf skin pieces, in this instance, was considered to be satisfactory.

Absence of microorganisms was also observed in swabs after the final washing cycle, following the decontamination of calf skin piece that were used for the BAT leather-making process. On the other hand a limited number of microorganisms, log 0.07 cfu (±0.29, n=5) was isolated from the water samples after the final washing cycle and may be considered negligible. Therefore, the decontamination of calf skin pieces (used for the BAT leather-making processes) in this instance was also considered to be satisfactory.

7.4.4. Inoculation of <u>Staphylococcus aureus</u>

Staphylococcus aureus NCTC12981/ATCC25923 inoculum was collected from the mid-exponential phase and inoculated on the decontaminated calf skin pieces. The average number of inoculated *S. aureus* NCTC12981/ATCC25923 colonies on the decontaminated calf skin pieces was measured (see Section 2.10.2). The average number of inoculated colonies was log 8.37 (±0.10) cfu and log 9.09 (±0.01) cfu for the conventional and BAT leather-making processes respectively. Statistical analysis showed there was a significant difference between the number of the inoculated colonies on the decontaminated-calf skin pieces that were used for the conventional and BAT leather-making processes (t=14.559, df=6.0, p<<0.001).

7.4.5. Enumeration of <u>Staphylococcus</u> Species from Effluent (Conventional and BAT Leather-Making Processes)

7.4.5.1. Pre-soaking

Figure 7.3 shows the results obtained when the conventional and BAT pre-soaking effluents were analysed. Analysis of the control samples showed the absence of *Staphylococcus* colonies indicating contamination did not occur during the experiments. Growth of *Staphylococcus* spp. was observed in the experimental samples and was significantly higher than the bacterial count in the corresponding control sample (Mann-Whitney test: $U20_{20, 4}=0.000$, p=0.02 [conventional]; $U_{18, 4}=0.00$, p=0.02 [BAT]). An average of log 5.21 (±1.82, n=5) cfu/ml and log 6.10 (±0.47, n=5) cfu/ml of presumptive *Staphylococcus* spp. was isolated from the pre-soaking effluents during the conventional and BAT leather-making processes respectively. Although, bacterial count in the BAT pre-soaking effluent was found to be higher than the conventional pre-soaking effluent, however, statistically the difference was not significant (Mann-Whitney test: $U20_{20, 18}=134.00$, p=0.178).
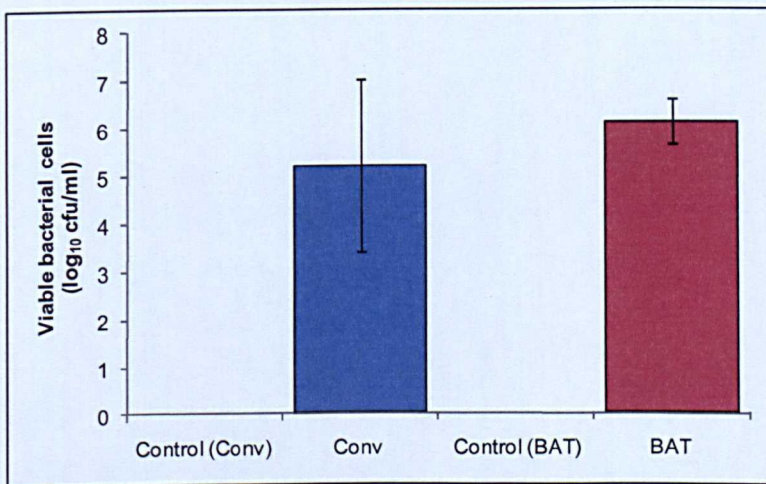
Figure 7.3: Number of isolated presumptive *Staphylococcus* colonies from the conventional (conv) and BAT pre-soaking effluent, n=5. The average number of inoculated *S. aureus*, NCTC12981/ATCC25923 colonies were log 8.37 cfu (conv) and log 9.09 cfu (BAT). The error bars represent standard deviation.

It appears that the conventional and BAT pre-soaking processes provided suitable environmental conditions for the growth of *Staphylococcus* spp. Both the conventional and BAT pre-soaking processes were carried out using 300% v/w water and 0.2% w/w of a bactericide Truposept BA (Trumpler, UK). As mentioned in Chapter 5 (Sections 5.4.5.1 and and 5.4.5.2) and Chapter 6 (Sections 6.4.5.1 and 6.4.5.2), Truposept BA is a sodium dimethyldithiocarbamate-based compound, which may a have low environmental impact (International Union of Leather Technologist and Chemists Societies (IULTCS), 2008). In general, bactericides are considered to be potentially toxic as these are designed to control

or inhibit bacterial growth (IPPC, 2001), and therefore may exerts toxic effects towards microorganisms. Research (Taylor *et al.*, 1987b) showed that dimethyldithiocarbamate is effective against *S. aureus*. However, bactericides are generally added during the leather manufacturing processes to control the microbial growth, which may not necessarily cause complete inactivation of the bacteria. During the pre-soaking processes, the hides/skins absorb water and consequently the microorganisms present on the hides/skins may also gain moisture, which therefore assists microbial growth (Rangarajan and Didato, 2003) during the pre-soaking process since microorganisms require moisture for the growth.

The pH and temperature are also important factors for microbial growth. The pH of the conventional and BAT pre-soaking effluents was 7.8-8.0, which is within the pH range required for the growth of *Staphylococcus* spp. The room temperatures were 25°C and 28.5°C during the conventional and BAT pre-soaking processes respectively, which were within the temperature range required for the growth of *Staphylococcus* spp.

Microorganisms require nutrition for the growth and survival. Many microorganisms have the ability to utilise proteins as a nutritional source (Shede *et al.*, 2008; Vankar and Dwivedi, 2009). The main structural protein in hides/skins is collagen (Bailey, 1998; Heidemann, 1993; Reich, 1986). *Staphylococcus aureus* are able to utilise collagen hydrolysates obtained from calf skins (Vasileva-Tonkova *et al.*, 2007). The presence of protein may therefore promote proliferation of *Staphylococcus* spp.

7.4.5.2.   Soaking

Figure 7.4 shows the results obtained, when the conventional and BAT soaking effluents were analysed. The analysis of the controls samples showed no growth of *Staphylococcus* spp. during both of the conventional and BAT soaking processes, and bacterial count in the experimental samples was significantly higher than the corresponding control samples (Mann-Whitney test: $U20_{20, 4}=12.00$, $p=0.024$ [conventional]; $U_{20, 4}=0.00$, $p=0.02$ [BAT]). A significantly decreased number of *Staphylococcus* colonies from log 5.21 (±1.82, n=5) to 2.38 (±1.84, n=5) cfu/ml, and from log 6.10 (±0.47, n=5) to 4.57 (±0.55, n=5) cfu/ml were isolated from the conventional (Mann-Whitney test: $U20_{20, 20}=48.00$, $p<<0.001$) and BAT (t=9.250, df=36.00, $p<<0.001$) soaking effluents respectively.

The conventional soaking process was carried out using 300% v/w water, 0.2% w/w of a sodium dimethyldithiocarbamate-based bactericide (Truposept BA; Trumpler, UK) and 0.2% w/w of a anionic surfactant, sodium salt of alkyletherphosphate, (Corilene®W385; STAHL

Europe, The Netherlands). Surfactants or wetting agents promote water absorption by hide/skins. Information on the toxic effect of sodium salt of alkyletherphophate on bacterial growth was not found. However, according to IPPC (2001), the majority of the surfactants used in the leather industry cause environmental pollutions and may also cause disturbance in microbial activity (Tibor *et al.*, 2002) by increasing the solubility of various compounds in water (IPPC, 2001). The presence of a dimethyldithiocarbamate-based bactericide (Taylor *et al.*, 1987b) and surfactant may have a negative impact on *Staphylococcus* spp. and therefore reduce the number of *Staphylococcus* colonies in soaking effluent. The cause of isolating a reduced number of bacterial colonies from the soaking effluents may also be due to the discharging of the pre-soaking effluents. During processing, the mechanical agitation may cause the microorganisms to transfer from the skin to the effluent and discharging the effluent consequently decreases the number of *B. cereus* cells present on hides/skins (Birbir and Ilgaz, 1996).
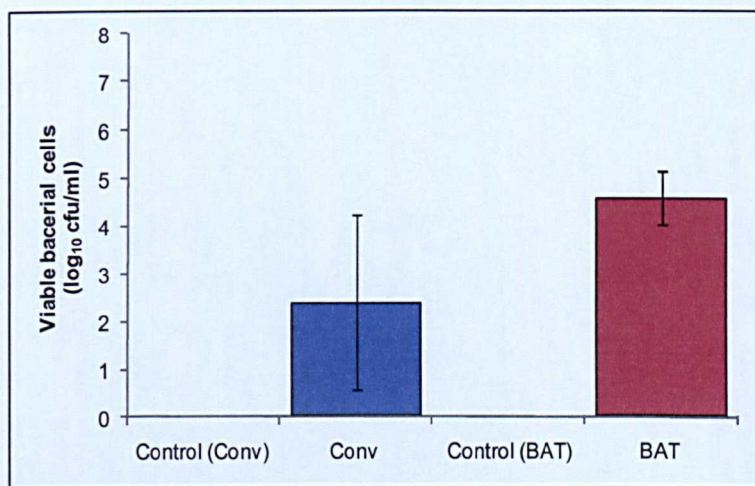
Figure 7.4: Number of isolated presumptive *Staphylococcus* colonies from the conventional (conv) and BAT soaking effluent, n=5. The average number of inoculated *S. aureus* NCTC12981/ATCC25923 colonies were log 8.37 cfu (conv) and log 9.09 cfu (BAT). The error bars represent standard deviation.

The BAT soaking process was carried out using 300% v/w water, 0.2% w/w of sodium dimethyldithiocarbamate-based bactericide (Truposept BA; Trumpler, UK), 0.5% w/w of a proteolytic enzyme-based compound (Pelvit C; Together For Leather (TFL), Germany) and 0.5% w/w sodium carbonate (Na_2CO_3). Pelvit C was added to the BAT soaking liquid instead of a surfactant to reduce the environmental pollution. In general, the use of enzymes or enzyme-assisted technology is considered as clean or cleaner technology, as enzymes are protein molecule and therefore may have less of an environmental impact (He *et al.*, 2005; Thanikaivelan *et al.*, 2004). Sodium carbonate was added to increase the pH of the BAT soaking liquid. This not only assists in achieving an optimum pH range for the enzyme

activity (the required pH range for pelvit C is 8.5-11.0) but also promotes absorbance of water by hides/skins. Sodium carbonate found to have an antimicrobial activity depending on the net pH in water (Lakhanisky, 2002), since every microorganism require a certain pH range for their growth and proliferation. As mentioned previously, discharge of the pre-soaking effluents may contribute to lowering the number of *Staphylococcus* spp. during the soaking process. Addition of a dimethyldithiocarbamate-based bactericide may also have a negative impact on the *Staphylococcus* growth (Taylor *et al.*, 1987b), lowering the number of *Staphylococcus* cells during the soaking process.

The presence of protein may serve as a nutritional source for *Staphylococcus* growth (Vasileva-Tonkova *et al.*, 2007) promoting the growth of *Staphylococcus* spp. Absorption of moisture during the soaking processes by skins may also assist *Staphylococcus* growth (Rangarajan and Didato, 2003), as bacteria require moisture for their growth and survival. The pH of the conventional soaking effluent was 7.9-8.1, which is within the pH range required for the growth of *Staphylococcus* spp. The pH of the BAT soaking effluent was 9.4-9.6, due to the addition of Na_2CO_3, is also within the pH range required for the growth of *Staphylococcus* spp.

The room temperature during the conventional and BAT soaking process was approximately 24°C, which is within the temperature requirement for *Staphylococcus* spp. An overnight soaking process may provide a sufficient incubation period for bacterial proliferation. It appears that over 10^4 (=log 4.0) cfu/ml *Staphylococcus* spp. were isolated from the conventional and BAT soaking effluents, which is higher than the threshold limit of *S. aureus* food poisoning (HPA, 2005d) (also see Section 7.1). This suggests that the soaking processes may provide suitable conditions for the survival of *Staphylococcus* spp. Factors such as available nutrition, moisture, pH, temperature and the processing period may assist the proliferation of the bacterial species during the soaking processes.

7.4.5.3. Unhairing

Analysis of the effluent collected from control samples during the conventional and BAT unhairing processes showed no bacterial growth. Analysis of the conventional unhairing effluent also showed the absence of bacterial colonies. However, a limited number of *Staphylococcus* spp., which was log 0.07 (±0.29, n=5) cfu/ml was isolated from the BAT unhairing effluent. In this instance, no significant difference was obtained between the experimental and the corresponding control sample (Mann-Whitney test: $U20_{20, 4}=38.00$, p=0.655). Evidently the reduction in bacterial count during both of the conventional and BAT

unhairing processes was significant (Mann-Whitney test: $U20_{20, 20}$=60.00, p<<0.001 [conventional]; $U_{20, 4}$=0.00, p<<0.001 [BAT]). Similar to *P. aeruginosa*, *Staphylococcus* spp. also did not prefer the environmental conditions of the conventional and BAT unhairing processes.

The environmental conditions of a certain habitat influence the growth of bacteria (Maier *et al*, 2000; Murray *et. al.*, 2005; Wilson, 2005). The conventional unhairing process was carried out using 200% v/w water, 2% w/w lime and 3% w/w sodium sulfide (Na_2S). To reduce environmental pollution due to the presence of Na_2S, a BAT unhairing process was carried out using 1.5% w/w of a protease-based unhairing enzyme (Southern Petrochemicals Industries Corporation Limited (SPIC), India). In addition, a reduced amount of water from 200% w/w to 20% w/w, Na_2S from 3% w/w to 0.5% w/w and lime from 2% w/w to 1% w/w was used during the BAT unhairing process. Lime and Na_2S have an antimicrobial effect due to the strong alkaline properties (George *et. al.*, 2001; Taleb-Ahmed *et al.*, 2005) and therefore increase the pH of the unhairing effluent to a level, which is unsuitable for bacterial growth. This may cause a reduction in the number of bacterial colonies during the unhairing processes. Due to the strong reducing properties, Na_2S reduces oxygen levels in water interrupting microbial activity (Bosnic *et al.*, 2000; Taleb-Ahmed *et al.*, 2005) of aerobic microorganisms, as they require oxygen for their growth and proliferation. However, some strains of *S. aureus* are able to utilise sulfide and thiosulfate as a sulfur source (Lithgow *et al.*, 2004). Similar to carbon and nitrogen, sulfur is an essential element for the microbial metabolisms (Pelczar *et al.*, 1993; Maier *et al*, 2000).

Due to the presence of lime and Na_2S, the pH of the conventional and BAT unhairing effluent was 12.6-12.7 and 10.6-11.5 respectively, which is higher than the pH range required for the growth of *Staphylococcus* spp. Grisi and Gorlach-Lira (2005) showed, that pH 11 effectively inactivates *S. aureus*. It can be concluded that the presence of lime and Na_2S were the cause of considerable reductions in the number of culturable *Staphylococcus* colonies during the unhairing processes.

7.4.5.4. Reliming

The analysis of the reliming effluent collected from the control samples during the conventional and BAT leather-making processes showed no bacterial growth. Although, no bacterial growth was observed in the conventional unhairing effluent, the presence of *Staphylococcus* spp. was observed in the subsequent reliming effluent. The average number of isolated presumptive *Staphylococcus* spp. from the conventional reliming effluent was log

0.72 (±0.76, n=5) cfu/ml and was found to be statistically insignificant when compared with the corresponding control sample (Mann-Whitney test: $U20_{20, 4}$=20.00, p=0.079). No bacterial growth was observed in the BAT reliming effluent. Additionally bacterial count in the reliming effluent was significantly higher than the bacterial count in the unhairing effluent during the conventional leather-making process (Mann-Whitney test: $U20_{20, 20}$=100.00, p<<0.001). On the other hand, no significant difference was obtained between the number of bacterial colonies obtained from the BAT unhairing and reliming effluent (Mann-Whitney test: $U_{20, 19}$=180.00, p=0.330).

However, isolation of *Staphylococcus* spp. in the conventional reliming effluent indicates the presence of viable *Staphylococcus* cells. Inconsistent growth of *Staphylococcus* spp. was observed, when the reliming effluent was analysed. A large number of *Staphylococcus* cells may become non-culturable or destroyed due to the environmental stress, while, others may have survived the unhairing and reliming processes resulting in abrupt growth. The large standard deviation was therefore due to the inconsistency in the growth of *Staphylococcus* spp.

A lower growth/no growth of *Staphylococcus* spp. in the reliming effluent shows that similar to the unhairing processes, the reliming processes did not provide suitable conditions for the bacterial growth. Both of the reliming processes were conducted using the 200% w/w water and 2% w/w lime. Lime, due to its strong alkaline properties may prevent microbial growth (George *et al.*, 2001) and hence reduce the number of viable *Staphylococcus* cells during the reliming process. Due to the presence of lime, the pH of the conventional and BAT reliming effluent was 12.4-12.6, which is higher than the pH range required for the growth of *Staphylococcus* spp., inhibiting the growth and recovery of *Staphylococcus* cells.

7.4.5.5.   Deliming

Figure 7.5 shows the results obtained when analysis of the conventional and BAT deliming effluents was carried out to measure the *Staphylococcus* growth. No *Staphylococcus* colonies were isolated in the control samples during the conventional and BAT deliming processes, indicating no contamination occurred during processing. *Staphylococcus* spp. was isolated from both of the conventional and BAT deliming effluent. The difference between the bacterial count in the experimental and corresponding control samples were found to be statistically insignificant (Mann-Whitney test: $U20_{20, 4}$=30.00, p=0.275) and significant (Mann-Whitney test: $U20_{20, 4}$=16.00, p=0.047) during the conventional and BAT deliming processes respectively.

Although a decreased number of *Staphylococcus* spp. from log 0.72 ($\pm$0.76, n=5) to 0.45 ($\pm$0.82, n=5) cfu/ml was isolated from the conventional deliming effluent; however, the difference was statistically insignificant (Mann-Whitney test: $U20_{20, 20}$=168.00, p=0.318). This also emphasises the presence of viable and culturable *Staphylococcus* colonies in the conventional deliming effluent. An average of log 1.39 ($\pm$1.22, n=5) cfu/ml *Staphylococcus* spp. was isolated from the BAT deliming effluent, which was significantly higher than the number of *Staphylococcus* spp. isolated from the BAT reliming effluent (Mann-Whitney test: $U20_{19, 20}$=76.00, p<<0.001). The BAT deliming process may have provided suitable conditions for the dormant bacterial cells to recover and proliferate.
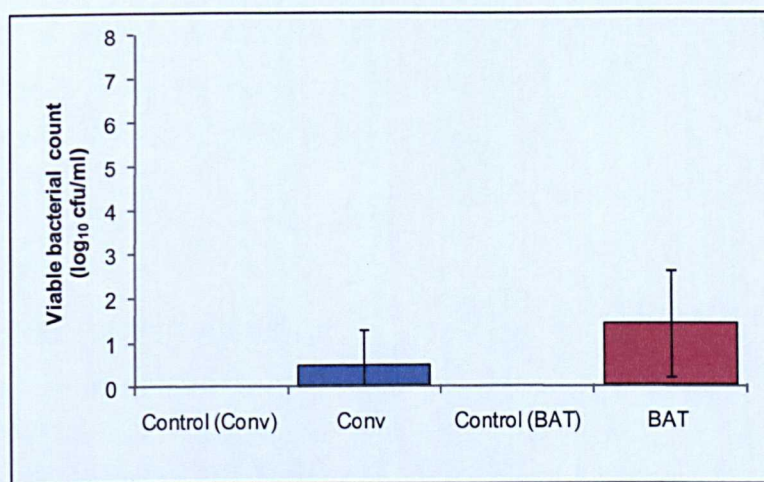
Figure 7.5: Number of isolated presumptive *Staphylococcus* colonies from the conventional (conv) and BAT deliming effluent, n=5. The average number of inoculated *S. aureus*, NCTC12981/ATCC25923, cells was log 8.37 cfu (conv) and log 9.09 cfu (BAT). The error bars represent standard deviation.

Abrupt growth of *Staphylococcus* spp. was observed in both of the conventional and BAT deliming effluents resulting in large standard deviation. This may be due to the presence of culturable and non-culturable bacterial cells in the deliming effluents. A number of *Staphylococcus* cells may have recovered during the deliming processes, but some still non-culturable or destroyed completely and therefore inconsistent growth of *Staphylococcus* occurred.

The conventional deliming process was carried out using 100% v/w water and 2% w/w ammonium chloride (NH_4Cl) to neutralise lime and other alkalis from the relimed skins. Many microorganisms are capable of utilising ammonium as a nitrogen source, which is an essential element for bacterial growth (Madigan *et al.*, 1997; Murray *et al.*, 2005) and may encourage the recovery of the injured bacterial cells. Ammonium salt stimulates eutrophication and therefore causes environmental pollution by reducing the oxygen levels in

water (Bosnic *et al.*, 2000; IPPC, 2001). In order to reduce environmental pollution due to the presence of ammonia, a carbon dioxide (CO_2) deliming method was followed during the BAT deliming process. Carbon is also an essential element for the microbial metabolisms and some microorganisms have the potential to derive carbon from CO_2 (Murray *et al.*, 2005).

The pH of the deliming effluent was 8.2-8.6, which is within the pH range required for the growth of *Staphylococcus* spp. Due to the presence of CO_2 the pH of the BAT deliming effluent (6.5-7.2), was lower than the conventional deliming effluent. The pH of the BAT effluent was within the optimum pH range required for the growth of *Staphylococcus* spp. and therefore may promote *Staphylococcus* proliferation. Valero *et al.* (2009) found that optimal growth of *S. aureus* occurs at a pH 7.0, and an increase of the pH from 7.0 to 7.5 caused a decrease in *S. aureus* growth. Therefore, a lower pH of the BAT deliming effluent may assist the growth and recovery of *Staphylococcus* spp.

7.4.5.6. Bating

The results obtained, when enumeration of *Staphylococcus* spp. in the conventional and BAT bating effluents was carried out, are shown in Figure 7.6. Similar to the previous results (Sections 7.4.5.1-7.4.5.5) no *Staphylococcus* growth occurred in the control samples for both of the conventional and BAT bating processes. Analysis of the experimental sample (for both of conventional and BAT bating effluent) showed growth of *Staphylococcus* spp. However, there was no significant difference between the bacterial count in the experimental and control samples (Mann-Whitney test: $U20_{20, 4}=26.00$, $p=0.176$ [conventional]; $U_{20, 4}=20.00$, $p=0.083$ [BAT]).

An increased number of *Staphylococcus* colonies, from log 0.45 (±0.82, n=5) to 0.74 (±1.04, n=5) cfu/ml, was isolated from the conventional bating effluent. A lower number of *Staphylococcus* colonies, from log 1.39 (±1.22, n=5) to 0.82 (±0.88, n=5) cfu/ml, was isolated from the BAT bating effluent. This increase and decrease in the number of isolated *Staphylococcus* colonies during the conventional (Mann-Whitney test: $U20_{20, 20}=169.50$, $p=0.309$) and BAT (Mann-Whitney test: $U20_{20, 20}=136.00$, $p=0.069$) bating processes respectively was found to be insignificant. Inconsistent growth of *Staphylococcus* spp. was also observed in the bating effluents resulting in a large standard deviation.
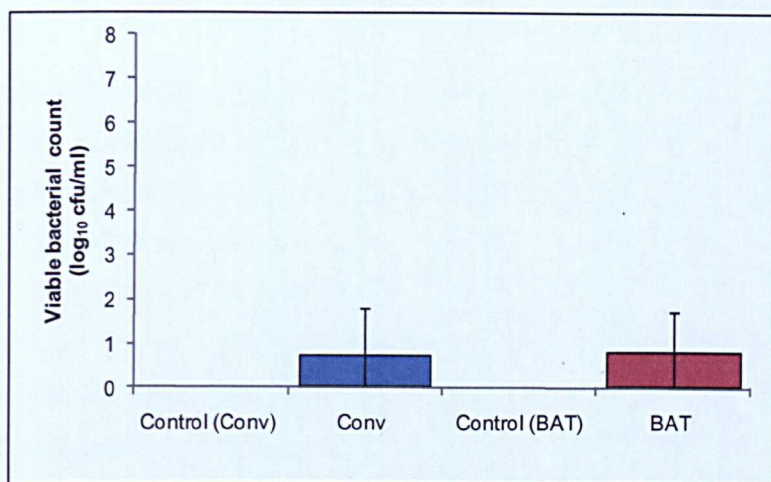
Figure 7.6: Number of isolated presumptive *Staphylococcus* colonies from the conventional (conv) and BAT bating effluents, n=5. The average number of inoculated *S. aureus*, NCTC12981/ATCC25923, cells was log 8.37 cfu (conv) and log 9.09 cfu (BAT). The error bars represent standard deviation.

The conventional and BAT deliming processes were carried out using a proteolytic enzyme-based compound Oropon ON 2 (TFL, Germany) and α-amylase (SPIC, India) respectively. Enzymes are protein molecules and therefore serve as a nutritional source encouraging the growth and recovery of the bacteria, as most of the bacteria have the potential to utilise protein to derive essential elements for their metabolism (Shede *et al.*, 2008; Vankar and Dwivedi, 2009). Additionally, the pH of the conventional and BAT bating effluents was 8.0-8.3 and 6.8-7.5 respectively, which is within the pH range required for the growth of *Staphylococcus* spp.

After the BAT deliming process, the deliming effluent was removed followed by the BAT bating processes using sterilised water (200% v/w) and α-amylase (1.25% w/w). As mentioned in Section 7.5.4.2 mechanical agitation during processing may cause bacterial cells to transfer from the skins to the effluents and consequent removal of the effluent may cause a reduction in the number of viable bacterial cells present on the skins (Birbir and Ilgaz, 1996). On the other hand, the conventional deliming liquid was not completely removed and Oropon ON 2 was added to the deliming liquid; this may have prevented the reduction or removal of the bacterial cells that were present in effluent or on pelts (unhaired hides/skins). However, recovery of a limited number of bacteria may also be due to inadequate incubation period. Both the conventional and BAT bating processes were carried out for 30 minutes, which may not provide an adequate incubation period for the injured bacterial cells to repair and recover.

7.4.5.7. Pickling

Analysis of the effluents collected from the control samples as well as from the experimental samples during the conventional and BAT pickling processes showed the absence of *Staphylococcus* spp. Statistically this reduction in the number of *Staphylococcus* colonies was significant (Mann-Whitney test: $U20_{20, 20}=130.00$, $p=0.004$ [conventional]; $U_{20,20}=100.00$, $p<<0.001$ [BAT]). This indicates that either the conventional or the BAT pickling process did not provide a suitable growth condition for *Staphylococcus* spp. The similar results were observed with *B. cereus* and *P. aeruginosa* (Chapters 5 and 6), where the growth of *B. cereus* and *P. aeruginosa* was hindered during the pickling processes.

The conventional pickling process was carried out using 100% v/w water, 8% w/w sodium chloride (NaCl), 1.2% w/w sulfuric acid and 1.0% w/w formic acid. In order to assist chrome (Cr) penetration within the skins during the subsequent chrome tanning process, the pH of the pickling liquid is generally adjusted to 2-3 by adding acids. This low pH causes swelling of the skin collagen and therefore NaCl is generally added to prevent acid swelling (Leafe, 1999). *Staphylococcus* can withstand high NaCl concentrations (Bremer *et al.*, 2004; Murray *et al.*, 2005; Todar, 2008). It was found by Mounier *et al.* (2007) that *S. saprophyticus* are able to grow at NaCl concentration 0-15% w/v, but optimal growth occurred when NaCl concentration was 0% w/v, indicating that the bacterial species may prefer a NaCl-free environment. Sivaprakasam *et al.* (2008) isolated NaCl-tolerant *S. aureus*, which adapt an environment containing 5% w/v NaCl. Therefore, the presence of a high quantity of NaCl 8% w/w may not favour the growth of *Staphylococcus* spp. in the pickling effluent.

The presence of a large quantity NaCl in the tannery effluents exerts toxic effects towards plants and animals particularly towards fresh water species (IPPC, 2001). In order to reduce NaCl-pollution, a NaCl-free BAT pickling process was followed using 80% v/w water, 2.0% w/w of a benzene poly sulfonic acid-based compound Sellatan P liquid (TFL, Germany), 0.5% w/w formic acid and 0.25% w/w sodium formate (HCOONa). Little information was available about the effect of HCOONa on bacterial growth. However, bacteria may utilise HCOONa as a carbon source and therefore HCOONa may have a stimulatory effect on bacterial growth (Linton *et al.*, 1981; Peel and Quayle, 1961). Barnum *et al.*, (1982) found that benzene (aromatic) sulfonic acid (dodecyl benzene sulfonic acid) have an inhibitory influence on *S. aureus*. The presence of a benzene sulfonic acid may therefore also hinder the growth of *Staphylococcus* spp. during the BAT pickling process. The inhibitory effect of aromatic sulfonic acid is may be due to the lowering of the pH to a level, which is unsuitable for the bacterial growth.

Haines and Harmon (1973) showed that the inhibitory effect of lactic acid on *S. aureus* increased below pH 4.27, also the growth of *S. aureus* was found to be reduced if the pH was lowered from 7 to 5.5 using hydrochloric acid. Eifert *et al.* (1997) found that the growth of *S. aureus* reduces at a pH of 5.0 when using acetic acid, lactic acid and hydrochloric acid. A recent molecular based study by Bore *et al.* (2007) established that a pH of 4.5 using hydrochloric acid reduced the growth of *S. aureus*. This evidence signifies that a low pH have an inhibitory effect on the growth of *Staphylococcus* spp. The pH of the conventional and BAT pickling effluent was 1.5-2.8 and 2.3-3.1 respectively and therefore may prevent the growth of *Staphylococcus spp.* during the conventional and BAT pickling processes.

7.4.5.8. Chrome Tanning

Analysis of the effluents collected from the control samples during the conventional and BAT chrome tanning processes showed the absence of *Staphylococcus* spp. A limited number of *Staphylococcus* spp., log 0.09 ($\pm$0.41, n=5) cfu/ml were isolated from the chrome tanning effluent during the conventional leather-making process and found to be statistically insignificant when compared to the control sample (Mann-Whitney test: $U20_{20,4}$=38.00, p=0.655). No significant difference was obtained between bacterial colonies isolated from the chrome tanning and pickling effluent (Mann-Whitney test: $U20_{20, 20}$=190.00, p=0.317) during the conventional leather-making process. Therefore, bacterial growth in this instance may be considered to be negligible.

No *Staphylococci* spp. was isolated from the chrome tanning effluent during the BAT leather-making process. This indicates that the chrome tanning processes did not provide suitable conditions for the recovery of the bacterial cells. Absence of *Staphylococcus* spp. may also be due to the complete inactivation of the bacterial cells or due to the presence of non-culturable cells in the chrome tanning effluent.

Both the conventional and BAT chrome tanning processes were carried out using chrome(III) sulfate (8% w/w). A magnesium oxide (MgO)-based compound, Feliderm®MGO Powder (previous name: Tanbase, Clariant, Switzerland) was used to raise the pH of the tanning liquid. The final pH of the chrome tanning effluent during the conventional and BAT leather-making processes were 4.1-4.4 and 4.2-4.7 respectively. The stability or survivable ability of *S. aureus* reduces at a lower pH, such as 4.5 (Haines and Harmon, 1973; Eifert *et al.*, 1997; Bore *et al.*, 2007) and therefore a low pH may inhibit the growth of *Staphylococcus* spp. during the chrome tanning process.

Microorganisms are known to be sensitive towards chrome (Cr). Chrome(VI) is considered more toxic than Cr(III) due to its higher solubility than Cr(III) as well as due to its strong oxidative properties. The solubility aids the diffusion of Cr(VI) through the cell membrane and Cr(VI) interrupt microbial activity by oxidation (Cervantes *et al.*, 2001; Jun *et al.*, 2008; Megharaj *et al.*, 2003; Verma *et al.*, 2001). Limited information was available regarding the effect of chrome(III) on the growth of *Staphylococcus* spp. Valenti *et al.* (1980) found that coagulase positive *Staphylococcus* is more resistant towards chromium chloride (CrCl$_3$) than coagulase negative *Staphylococcus* spp. and the sensitivity of *Staphylococcus* spp. towards CrCl$_3$ was found to be increased at higher concentrations of CrCl$_3$. In addition, MgO is found to have an inhibitory effect on the growth of *S. aureus*. The inhibitory effect of MgO is may be due to its ability to form active oxygen (O^{-2}) (Sawai *et al.*, 2000), therefore, disrupt the bacterial cells by oxidation. Due to the presence of high levels of Cr(III), MgO and a lower pH below 5.0, the chrome tanning process may prevent the growth of *Staphylococcus* spp.

7.4.6.   Enumeration of <u>Staphylococcus</u> Species from Swabs (Conventional and BAT Leather-Making Processes)

7.4.6.1.   Pre-soaking

The number of isolated presumptive *Staphylococcus* colonies from swabs after the conventional and BAT pre-soaking processes are shown in Figure 7.7. No calf skin pieces were added during the control experiments for both of the conventional and BAT leather-making processes, and therefore no swabs were obtained from the control samples. The average number of isolated presumptive *Staphylococcus* spp. from swabs after the conventional and BAT pre-soaking process was log 4.19 (±1.31, n=5) cfu/ml and log 5.36 (±0.15, n=5) cfu/ml respectively. The presence of protein, moisture, a suitable temperature and pH may assist the growth of *Staphylococcus* spp. during the pre-soaking process.

Figure 7.7: Number of isolated presumptive *Staphylococcus* colonies from swabs after the conventional (conv) and BAT presoaking processes, n=5. The average number of inoculated *S. aureus* NCTC12981/ATCC25923 colonies were log 8.37 cfu (conv) and log 9.09 cfu (BAT). The error bars represent standard deviation.

7.4.6.2.   Soaking

The results obtained, when swabs after the conventional and BAT soaking process were analysed, are shown in Figure 7.8. An average log 2.63 ($\pm$1.61, n=5) cfu/ml and log 3.81 ($\pm$0.65, n=5) cfu/ml of presumptive *Staphylococcus* spp. was isolated from swabs after the conventional and BAT soaking processes respectively. Factors, such as a suitable pH and temperature range, incubation period, available nutritional sources (such as protein) and moisture, may assist the survival of *Staphylococcus* spp. during the soaking processes.

Figure 7.8: Number of isolated presumptive *Staphylococcus* colonies from swabs after the conventional (conv) and BAT soaking process, n=5. The average number of inoculated *S. aureus*, NCTC12981/ATCC25923 colonies were log 8.37 cfu (con) and log 9.09 cfu (BAT). The error bars represent standard deviation.

7.4.6.3.   Unhairing

The average number of isolated presumptive *Staphylococcus* colonies enumerated in swabs after the conventional and BAT unhairing processes is shown in Figure 7.9. Although limited, *Staphylococcus* growth was observed in swabs, indicating the ability of *Staphylococcus* spp. to survive the extreme environmental conditions during both of the conventional and BAT unhairing processes. The average number of enumerated colonies was log 0.46 (±0.84, n=5) cfu/ml and log 0.59 (±0.84, n=5) cfu/ml for the conventional and BAT unhairing processes respectively. The reduction in the number of *Staphylococcus* colonies was due to the presence of lime and Na_2S. Inconsistent growth of *Staphylococcus* spp. was observed in swabs obtained after the unhairing processes resulting in a large standard deviation.
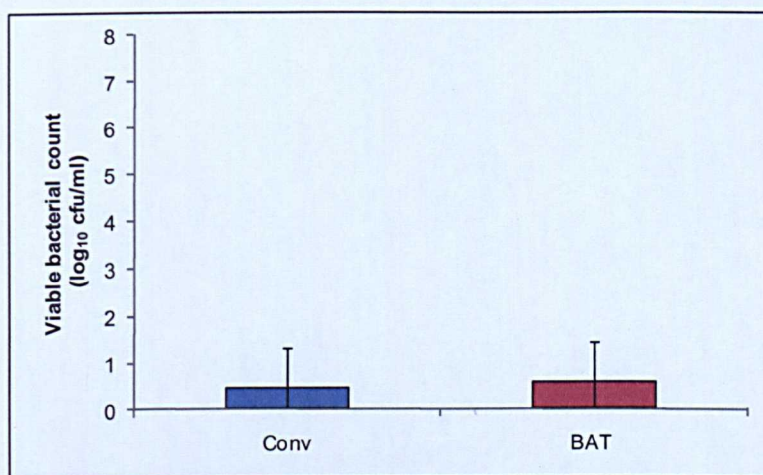
Figure 7.9: Number of isolated presumptive *Staphylococcus* colonies from swabs after the conventional (conv) and BAT unhairing processes, n=5. The average number of inoculated *S. aureus* NCTC12981/ATCC25923 colonies were log 8.37 cfu (conv) and log 9.09 cfu (BAT). The error bars represent standard deviation.

7.4.6.4.   Reliming

Similar to the effluent samples, growth of *Staphylococcus* spp. was observed in swabs after the conventional reliming process and the average number of isolated colonies was log 0.51 (±0.0.69, n=5) cfu/ml. No *Staphylococcus* growth was observed in swabs after the BAT reliming process. Due to the presence of lime, the reliming processes were found to suppress bacterial growth.

7.4.6.5.   Deliming

Figure 7.10 shows the number of enumerated presumptive *Staphylococcus* spp. in swabs after the conventional and BAT deliming processes. A low number of *Staphylococcus* colonies was isolated from swabs after both of the conventional and BAT deliming processes, which were log 0.15 (±0.48, n=5) cfu/ml and log 0.14 (±0.55, n=5) cfu/ml respectively. Due to the presence of protein, ammonia, moisture and neutralisation of alkalis, the deliming processes may provide suitable conditions for the bacterial growth. Isolation of a limited number of bacterial cells from the swabs after deliming processes indicates the presence of a limited number of culturable colonies on the calf skin pieces.
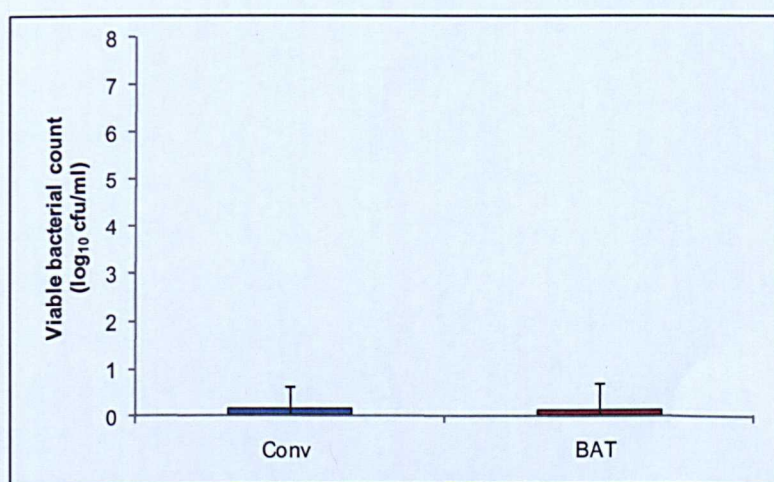
Figure 7.10: Number of isolated presumptive *Staphylococcus* colonies from swabs after conventional (conv) and BAT deliming processes, n=5. The average number of inoculated *S. aureus* NCTC12981/ATCC25923 colonies were log 8.37 cfu (conv) and log 9.09 cfu (BAT). The error bars represent standard deviation.

7.4.6.6.   Bating

A limited number of presumptive *Staphylococcus* colonies, log 0.08 (±0.36, n=5) cfu/ml was isolated from swabs after the conventional bating process and may be considered to be negligible. Absence of *Staphylococcus* colonies was observed in swabs after the BAT bating process. Due to the presence of proteins, moisture, ammonium salt and a suitable pH range, the bating processes should provide suitable conditions for the microbial growth and recovery. The continuous environmental stress during the leather-making process may have injured the cells beyond recovery. Absence or limited growth of *Staphylococcus* may also be due to the presence of non-culturable bacterial cells. As mentioned previously (Section 7.4.5.6) that both the conventional and BAT bating process was carried out for 30 minutes, which may not provide adequate incubation period for the bacteria to recover.

7.4.6.7.   Pickling

A limited number of *Staphylococcus* colonies, log 0.07 ($\pm$0.30, n=5) cfu/ml were isolated from swabs after the conventional pickling process and may be considered to be negligible. On the other hand, analysis of swabs after the BAT pickling process showed no *Staphylococcus* growth. The presence of sulfuric acid, formic acid and NaCl during the conventional pickling process and the presence of benzene sulfonic acid during the BAT pickling process may prevent the growth of *Staphylococcus* spp.

7.4.6.8.   Chrome Tanning

Figure 7.11 shows the number of isolated *Staphylococcus* colonies from swabs after the conventional and BAT chrome tanning process. The average number of isolated presumptive *Staphylococcus* colonies from swabs after the conventional and BAT chrome tanning processes was log 0.46 ($\pm$0.75, n=5) cfu/ml and log 0.28 ($\pm$0.57, n=5) cfu/ml respectively. The presence of a lower number of *Staphylococcus* spp. on calf skins may be due to the presence of MgO and chrome. The pH of the chrome tanning effluent was below 5, which may also have an adverse effect on the growth of *Staphylococcus* spp. However, this indicates the presence of *Staphylococcus* spp. even after the chrome tanning process. The unsuitable growth conditions during the chrome tanning process may cause inconsistent growth of *Staphylococcus* spp. resulting in a larger standard deviation.
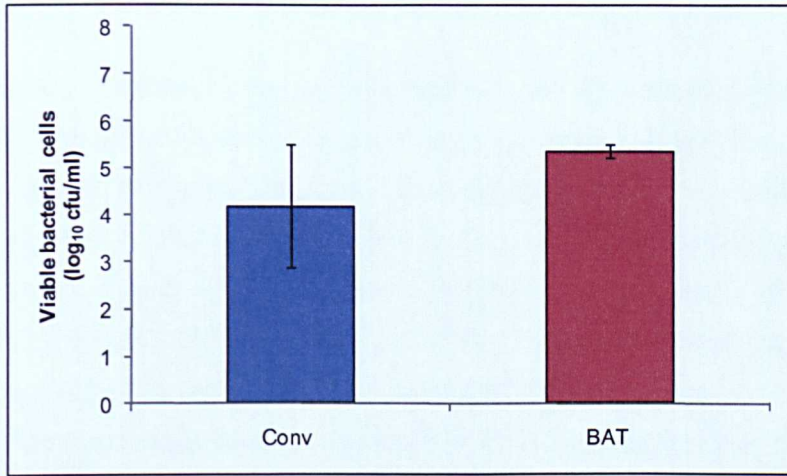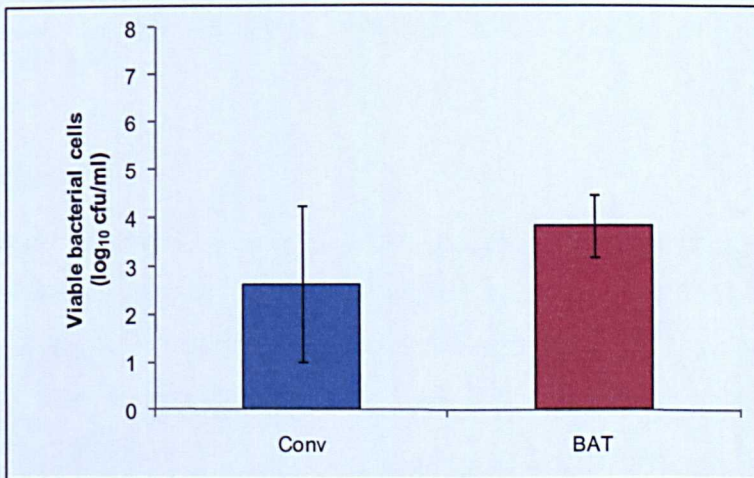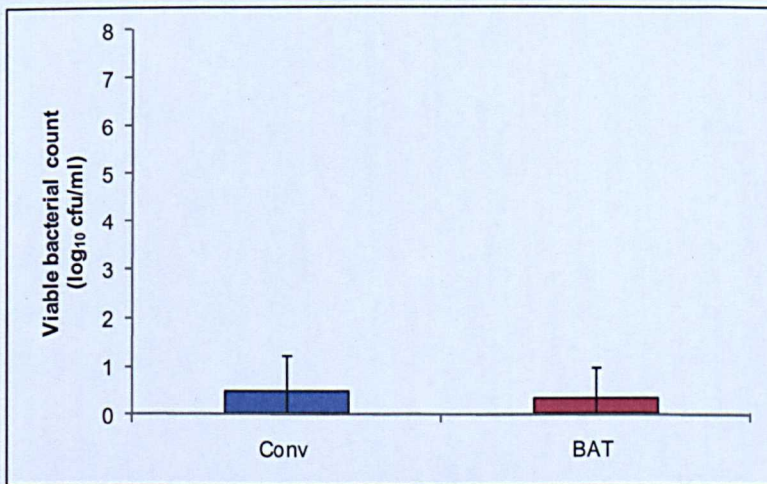

Figure 7.11: Number of isolated presumptive *Staphylococcus* colonies from swabs after the conventional (conv) and BAT chrome tanning process, n=5. The average number of inoculated *S. aureus*, NCTC12981/ATCC25923 colonies were log 8.37 cfu (conv) and log 9.09 cfu (BAT). The error bars represent the standard deviation.

7.4.7. Enumeration of <u>Staphylococcus</u> species from Hair Samples Collected from the Conventional and BAT Unhairing Effluents

Hair collected from the conventional and BAT unhairing effluents was incubated overnight in TSB in order to assist the recovery of injured bacterial cells. Overnight incubation may not only provide sufficient time for the bacterial cells to recover but also the cells may start multiplying. Therefore, determination of the initial bacterial colonies in hair is not possible using the followed method and hence this method was used as a qualitative test. Analysis of the overnight-incubated hair samples showed inconsistent growth or no growth of *Staphylococcus* spp. Therefore, the calculated standard deviation is high. An average of log 2.60 (±2.78, n=2) cfu/ml of *Staphylococcus* spp. was isolated from hair collected from the unhairing effluent.

On the other hand, the majority of the hair samples collected from the BAT unhairing effluent showed *Staphylococcus* growth. An average of log 4.81 (±0.95, n=4) cfu/ml of *Staphylococcus* spp. was enumerated in hair samples collected from the BAT unhairing effluent. The presence of *Staphylococcus* spp. in the hair after the unhairing processes suggests that some of the *Staphylococcus* spp. may have developed resistance towards lime and Na_2S.

7.4.8. Confirmation Tests for <u>Staphylococcus</u> Species

The isolated bacterial cells were purified by streaking to a single colony on a Baird-Parker agar-based media and the purified colonies were then re-streaked on the media to ensure sufficient supply of the purified colonies for various confirmatory tests. A Gram-staining technique, as described in Section 2.7, was used to stain the isolated *Staphylococcus* spp., and the stained cells were observed under a light microscope (Nikon Eclipse 600, Nikon, Japan) in order to determine the microscopic structure. The cells were appeared purple (Gram-positive) and spherical-shaped, when observed. Figure 7.12 shows spherical cells of *S. aureus* NCTC12981/ATCC25923 colonies (1 k magnification), which were arranged in clusters.
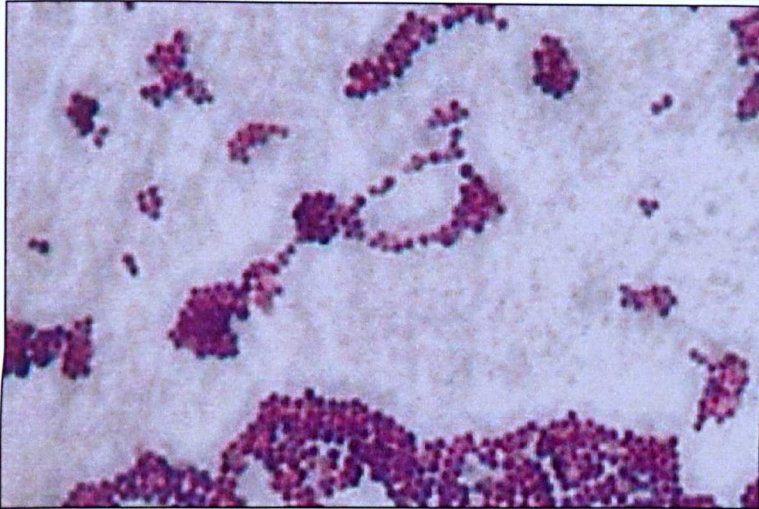
Figure 7.12: Gram-positive (purple) spherical *S. aureus* NCTC12981/ ATCC25923 cells, which are arranged in cluster (1 k magnification) The cells were stained using a Gram-staining method as describe in Section 2.7 and observed under the light microscope (Nikon Eclipse 600, Nikon, Japan).

Various biochemical tests (see Section 2.15.1.2) and the Biolog identification method (see Section 2.15.2) were used as confirmatory tests for the presumptive *Staphylococcus* colonies that were isolated during the conventional and BAT leather manufacturing processes. *Staphylococcus aureus* NCTC12981/ATCC25923 was used as a positive control for the biochemical and Biolog tests. *Staphylococcus aureus* NCTC12981/ATCC25923 and the isolated *Staphylococcus* spp. were grown on Columbia blood agar-based media (Oxoid, UK) with 5% w/v horse/sheep blood. Isolated *Staphylococcus* colonies were appeared as a silver sheen, similar to the *S. aureus* NCTC12981/ATCC25923. However, it was observed that some of isolated colonies, particularly, those enumerated from the unhairing-chrome tanning processes, were smaller than the *S. aureus* NCTC12981/ATCC25923 colonies, when grown on Columbia blood agar-based media.

DNase and coagulase tests were carried out in order to determine the ability of the tested bacterial cells to produce the enzymes DNase and coagulase. DNase and coagulase production are often used for the identification of *S. aureus* and therefore used in this study, since calf skin pieces were inoculated with *S. aureus* NCTC12981/ATCC25923. Tables 7.1 shows the results obtained when DNase and coagulase tests were carried out for *S. aureus* NCTC12981/ATCC25923. The results show that all the tested *S. aureus* colonies produced DNase and coagulase. All the results from the biochemical tests are the characteristics of *S. aureus*.

Table 7.1: DNase and coagulase tests for *S. aureus* NCTC12981/ATCC25923 colonies.

Staphylococcus aureus	DNase test	Coagulase test		
		2 hours	6 hours	Overnight
S. aureus NCTC12981/ATCC25923	+	+	+	+
S. aureus NCTC12981/ATCC25923	+	+	+	+
S. aureus NCTC12981/ATCC25923	+	+	+	+
S. aureus NCTC12981/ATCC25923	+	+	+	+
S. aureus NCTC12981/ATCC25923	+	+	+	+

Note: (+) sign indicates production of the enzymes DNase and coagulase by the bacterial cells.

Tables 7.2 and 7.3 show the results obtained when DNase and coagulase tests were carried out for presumptive *Staphylococcus* colonies that were isolated during various stages of the conventional and BAT leather-making processes respectively. Results obtained show that among the tested colonies, 27% and 22% of the *Staphylococcus* colonies that were isolated from the conventional and BAT leather-making processes respectively exhibited the ability to produce coagulase (coagulase-positive). This indicates that a large number of the isolated colonies from the conventional and BAT leather manufacturing processes were coagulase-negative (do not produce coagulase). On the other hand, 53% and 63% of the tested *Staphylococcus* colonies that were isolated during the conventional and BAT leather-making processes respectively produced DNase. Results obtained (Tables 7.2 and 7.3) suggest that although calf skin pieces were inoculated with *S. aureus*, however, *Staphylococcus* colonies, which were isolated from the various leather-making process stages may not be *S. aureus*.

Table 7.2: DNase and Coagulase tests for *Staphylococcus* species that were isolated during the conventional leather-making processes.

Isolated from	DNase test	Coagulase test		
		2 hours	6 hours	Overnight
Control (*S. aureus* NCTC12981/ATCC25923)	+	+	+	+
Pre-soaking	+	+	+	+
Soaking	+	+	+	+
Liming	-	-	-	-
Hair	-	-	-	-
Reliming	-	-	-	-
Deliming	-	-	-	-
Bating	-	-	-	-
Chrome tanning	+	+	+	+

Note: (+) sign indicates production of the enzymes DNase (Dnase positive) and coagulase (coagulase positive) by the bacterial cells, whilst (-) sign indicates that the bacterial cells failed to form DNase (DNase nagative) and coagulase (coagulase negative).

Table 7.3: DNase and Coagulase tests for *Staphylococcus* species that were isolated from the BAT leather-making processes.

Isolated from	DNase test	Coagulase test		
		2 hours	6 hours	Overnight
Control (*S. aureus* NCTC12981/ATCC25923)	+	+	+	+
Skin sample after decontamination	+	+	+	+
Pre-soaking	+	+	+	+
Liming	-	-	-	-
Hair	-	-	-	-
Deliming	±	-	-	-
Bating	-	-	-	-
Chrome tanning	+	+	+	+

Note: (+) sign indicates DNase and coagulase positive bacterial cells, whilst (-) sign indicates DNase and coagulase negative bacterial cells, and (±) may/may not from DNase/Coagulase.

Tables 7.4 shows the results obtained, when the Biolog identification test was carried out for *S. aureus* NCTC12981/ATCC25923. All of the tested *S. aureus* NCTC12981/ATCC25923 were identified as *S. aureus* with 97-100% probability, when the Biolog method was applied.

Table 7.4: Application of the Biolog Identification method for *S. aureus* NCTC 12981/ATCC25923 colonies.

Potentially suspected as	Biolog Identification	Probability (%)
S. aureus	*S. aureus*	100
S. aureus	*S. aureus*	100
S. aureus	*S. aureus*	97

Table 7.5 and 7.6 show the results obtained, when the Biolog identification test was carried out for the presumptive *Staphylococcus* spp. isolated during the conventional and BAT leather-making processes respectively. The majority of the isolated *Staphylococcus* colonies during the leather manufacturing processes were not identified as *S. aureus*. However, 86% of the isolated colonies from the conventional and BAT leather-making processes were identified as *Staphylococcus* spp.

The isolation of *Staphylococcus* spp., which were not *S. aureus* such as *S. epidermidis*, may be due to the contamination during the leather-making processes. However, precautions were taken to avoid contamination. In addition, similar results were obtained for both of the conventional and BAT processes. *Staphylococcus* contamination did not occur in the control samples during both the conventional and BAT leather-making process experiments, while the experimental and the control samples were treated following the same methods. Therefore, the occurrences of other *Staphylococcus* spp. except *S. aureus* may not be due to the contamination. The likelihood of the isolated *Staphylococcus* spp. to be the indigenous flora of calf skins cannot be disregarded.

Table 7.5: Application of the Biolog identification method for the *Staphylococcus* colonies that were isolated during the conventional leather-making process.

Potentially suspected as	Collected from	Biolog Identification	Probability (%)
S .aureus NCTC 12981/ATCC25923	Control	*S. aureus*	100
Staphylococcus sp.	Pre-soaking	*S. aureus*	100
Staphylococcus sp.	Soaking	*S. aureus*	100
Staphylococcus sp.	Unhairing	*S. epidermis*	97
Staphylococcus sp.	Reliming	*S. epidermis*	100
Staphylococcus sp.	Deliming	*S. arlettae*	88
Staphylococcus sp.	Bating	*Brochothrix campestris*	75
Staphylococcus sp.	Chrome tanning	*S. aureus*	100

Table 7.6: Application of the Biolog identification method for the *Staphylococcus* colonies that were isolated at various stages of the BAT leather-making process.

Potentially suspected as	Collected from	Biolog Identification	Probability (%)
S .aureus 12981/ATCC25923	Control	*S. aureus*	100
Staphylococcus sp.	Final washing cycle after decontamination	*S. aureus*	100
Staphylococcus sp.	Pre-soaking	*S. aureus*	100
Staphylococcus sp.	Unhairing	*S. warneri*	97
Staphylococcus sp.	Hair (collected from the unhairing effluent	*Enterococcus faecium*	100
Staphylococcus sp.	Deliming	*S. arlettae*	100
Staphylococcus sp.	Bating	*S. epidermis*	100
Staphylococcus sp.	Chrome-tanning	*S. aureus*	100

The isolation of *S. aureus* from water after the final washing cycle following NaClO-decontamination suggests that there is a possibility that the *Staphylococcus* spp. may withstand NaClO-treatments. The susceptibility of *S. aureus* towards chlorine is comparatively lower than *Bacillus* spp. (Sagripanti *et al.*, 1997). Sodium hypochlorite is a chlorine-based compound; therefore, some indigenous *Staphylococcus* spp. may have survived the NaClO-decontamination procedure. In a mixed microbial population, other faster growing microorganisms often found to suppress the growth of *S. aureus* (Bremer *et. al.*, 2004). Since *S. aureus* is a week competitor in a mixed microbial culture, hence this may provide the indigenous microbial flora (except *S. aureus*) of calf skins an opportunity to reappear. Isolation of *S. aureus* during the chrome tanning process shows the presence of viable and culturable *S. aureus* cells. This study implies that the *S. aureus* and other *Staphylococcus* species may be able to survive the extreme environmental condition during the leather-making processes. Due to time constraints, the PFGE method of DNA fingerprinting was not carried out during this study.

7.5. Summary

It was found that similar to *B. cereus* and *P. aeruginosa*, *Staphylococcus* spp. proliferated in the early stages of the beamhouse processes, such as pre-soaking and soaking. The presence of a nutritional source, moisture, moderate pH and temperature, and adequate incubation period may promote *Staphylococcus* growth during the pre-soaking and soaking processes. Sodium sulfide and lime during the unhairing process, and lime during the reliming process reduced the number of *Staphylococcus* spp. However, the presence of *Staphylococcus* spp. during the unhairing and reliming processes suggest the ability of the bacterial species to survive the extreme alkaline conditions.

Isolation of *Staphylococcus* spp. in the subsequent deliming and bating process signifies that the presence of viable and culturable *Staphylococcus* cells. This also indicates that the unhairing and reliming process did not cause total inactivation of the bacterial colonies. Reduction of the number isolated *Staphylococcus* spp. in the subsequent pickling and chrome tanning process shows that the pickling and chrome tanning processes, did not favour the growth of *Staphylococcus* spp. On the other hand, although limited, isolation of *Staphylococcus* spp. during the chrome tanning process shows that *Staphylococcus* spp. may withstand an acidic environment. Table 7.7 shows the average number of isolated *Staphylococcus* spp. colonies from various stages of the conventional and BAT leather-manufacturing processes.

Table 7.7: The average number of *Staphylococcus* spp. colonies isolated from various stages of the conventional and BAT leather-manufacturing processes.

Processing stages	Viable bacterial cells (log$_{10}$ colony forming units/ml)			
	Effluents		Swabs	
	Conventional	BAT	Conventional	BAT
Pre-soaking	6.80	6.10	4.82	5.36
Soaking	4.20	4.57	3.69	3.81
Unhairing	0.00	0.07	0.46	0.59
Reliming	0.72	0.00	0.51	0.00
Deliming	0.45	1.39	0.15	0.14
Bating	0.74	0.82	0.08	0.00
Pickling	0.00	0.00	0.07	0.00
Chrome tanning	0.09	0.00	0.46	0.34

The biochemical tests and Biolog identification method showed that the majority of the isolated bacterial colonies were not *S. aureus*, although the calf skin pieces were inoculated with *S. aureus* NCTC12981/ATCC25923. This suggests that the growth of the inoculate *S. aureus* NCTC12981/ATCC25923 was inhibited by the presence of other species that may

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8.1. Overall Summary

This study showed that various bacterial species exhibit different growth patterns during the conventional and BAT leather making processes (beamhouse and tanning). It was observed that both of the conventional and BAT pre-soaking process as well as the soaking process provide optimal conditions for bacterial growth. All of the tested bacterial species (*B. cereus*, *P. aeruginosa* and *Staphylococcus* spp.) were found to proliferate during the pre-soaking and soaking stages. Factors such as available nutrition, moisture, a moderate pH and temperature, and adequate incubation period may assist the growth and proliferation of the bacterial species.

Variable results were obtained when microbial analysis of the unhairing-chrome tanning effluents was carried out. Growth of *P. aeruginosa* was found to be completely inhibited during the conventional and BAT unhairing process as well as the reliming process. Both the conventional and BAT unhairing process were also found to suppress the growth of *Staphylococcus* spp., while limited growth and no growth of *Staphylococcus* spp. was evident in the reliming effluent during the conventional and BAT beamhouse processes respectively. The unhairing and reliming stages during the conventional beamhouse process reduced the growth of *B. cereus*, while a large number of *B. cereus* ($>10^2$=log 2 cfu/ml) was isolated from the BAT unhairing effluent, followed by a significant decrease in the subsequent reliming process. However, in the majority of instances, unhairing and reliming processes were found to provide unsuitable conditions for bacterial growth. The presence of sodium sulfide and lime during the unhairing and reliming processes was thought to have an adverse effect on bacterial growth. However, inconsistent growth of bacterial species showed that some of the bacterial cells survived the unhairing and reliming processes.

Variable results were also obtained during the deliming, bating, pickling and chrome tanning stages for both of the conventional and BAT leather making processes. The deliming and bating processes, particularly during the BAT beamhouse process, was found to encourage growth and survival of *P. aeruginosa*. Growth of *B. cereus* was observed in the BAT deliming and bating processes. However, growth of *B. cereus* was found to be increased during the conventional deliming and bating processes than the reliming process. Growth of *Staphylococcus* spp. also occurred during the deliming and bating stages for both of the conventional and BAT beamhouse processes. This suggests that the deliming and bating

process may assist bacterial growth and survival. Factors such as available nutritional sources, moderate temperature and pH, and the presence or absence of chemicals, which may have a stimulatory or inhibitory influence on the bacterial growth, may aid the survival of bacteria during the deliming and bating processes.

The pickling and chrome-tanning processes were found to reduce the bacterial growth. Due to the presence of sodium chloride at high concentrations, various acids (e.g., sulfuric acid, formic acid, benzene sulfonic acid), a magnesium oxide based-compound, and chrome(III) sulfate at high concentrations may suppress bacterial growth. However, limited bacterial growth occurred during the pickling and chrome tanning processes; therefore, it is hard to ensure the complete inactivation of bacterial cells,

The standard biochemical tests for *P. aeruginosa* and *B. cereus* showed that the isolated bacterial cells have similar characteristics to the inoculated *P. aeruginosa* ATCC10145 and *B. cereus* ATCC1778. The Biolog identification method in these instances identified the isolated bacterial species as *P. aeruginosa* and *B. cereus/ B. thuringiensis*. The PFGE method of DNA fingerprinting for *P. aeruginosa* and *B. cereus* confirmed that the isolated bacterial species during the leather-making processes were the same as the inoculated bacterial species (*P. aeruginosa* ATCC10145 and *B. cereus* ATCC1778), based on the DNA profiles. The PFGE method also established that no alteration in the *P. aeruginosa* ATCC10145 and *B. cereus* ATCC1778 DNA occurred during the conventional and BAT leather-making processes.

On the other hand, the biochemical tests for *S. aureus* showed that the characteristics of most of the isolated bacterial species were not the same as the inoculated *S. aureus* NCTC12981/ ATCC25923. The Biolog identification confirmed that majority of the isolated bacterial species were not *S. aureus*, but identified as *Staphylococcus* spp., indicating that the growth of *S. aureus* was inhibited by other bacterial species that may have survived the sodium hypochlorite decontamination procedure. Due to time constraints, the PFGE method of DNA fingerprinting was not carried out in this instance.

8.2. Conclusion

No considerable differences were observed between the effect of the conventional and BAT leather making processes on bacterial (*B. cereus*, *P. aeruginosa* and *Staphylococcus*) growth. A high risk of contracting pathogens to humans may occur during the pre-soaking and soaking stages, as a high bacterial growth and proliferation was observed during the

above-mentioned stages. Due to the isolation of a comparatively lower number of bacteria during the unhairing-bating processes, the chances of humans becoming infected with pathogens may be low. Growth of bacterial species was not only observed during the deliming and bating processes, but in some instances, assisted the recovery and the survival of bacterial cells. The pickling and chrome tanning processes were found to reduce bacterial growth to a very low level, hence the chances of developing bacterial infection during the processing stages may be considered to be unlikely.

The study showed the ability of the bacterial cells to recover and regenerate, and therefore, careful consideration is still necessary regarding pathogen-related health issues even though the bacterial (*B. cereus*, *P. aeruginosa* and *Staphylococcus*) count was found to be low. The risk of bacterial infections in humans may depend on many factors, such as the tannery environment, the leather making procedures and the personnel involved. There is a likelihood that pathogenic bacteria may be present and caution is recommended when dealing with hides/skins or effluents.

8.3.   Future Research

In this study, a culture-based method was followed for the enumeration of bacterial species from the effluent and skins, during the conventional and BAT leather-making processes. Recovery or enumeration of stressed bacteria may not always be feasible using a culture-based method, as the bacterial colony may change to a non-culturable but viable state due to environmental stress (Higgins *et al.*, 2007; Rowan, 2004; WS Atkins Environment, 2000). Various microscopic, DNA- and RNA-based techniques have therefore been developed for direct detection and identification of viable but non-culturable microorganisms (Kimura, 2006; WS Atkins Environment, 2000). The non-culturable but viable bacterial cells may become culturable and start proliferating when provided with suitable growth conditions. Therefore, further research is required to measure the presence of non-culturable but viable bacterial cells in the tannery effluent and to determine the potential impact of the bacteria on the quality of leather production, environment and human health.

Growth and proliferation of fungi in the tannery environment requires investigation, as various studies (Bitlisli *et al.*, 2004; Nigam, 1997; Özdilli *et al.*, 2007) have shown that the tannery environment may provide suitable conditions for fungal growth.

Investigation is required to determine the effect of effluent treatment plants on the growth of pathogens as microorganisms may develop resistance towards the chemicals used during

the leather manufacturing processes and so are able to survive tannery effluents. The effect of prolonged exposure on bacterial cells to constituents in the effluent may require further investigation.

Further qualitative and quantitative analysis is required to determine the presence of microorganisms in solid waste, such as sludge, fleshing, hair and trimmings during leather processing.

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Appendices

Appendix 1: Composition of the Microbial Media

Baired-Parker Agar Base (CM0275, Oxoid, UK).

Chemical Formula	gram/litre
Tryptone	10.0
'Lab-Lemco' powder	5.0
Yeast extract	1.0
Sodium pyruvate	10.0
Glycine	12.0
Lithium chloride	5.0
Agar	20.0
pH of the media 6.8 ± 0.2	

Egg Yolk Tellurite Emulsion (SR0054, Oxoid, UK).

Vial contents	Millilitres/litre
Egg yolk emulsion	47
Potassium tellurite	3

Egg yolk tellurite emulsion (50 ml) was added to 1 litter Baired-Parker agar base to prepare the media.

Brilliance *Bacillus Cereus* Agar (CM1036, Oxoid, UK).

Chemical Formula	gram/litre
Yeast extract	4.0
Peptone	10.0
Di-sodium hydrogen phosphate	2.52
Potassium di-hydrogen phosphate	0.28
Sodium pyruvate	10.0
Chromogenic mix	1.2
Agar	13.0
pH of the media 7.2 ± 0.2	

Brilliance *Bacillus Cereus* Selective Supplement (SR0230, Oxoid, UK).

Vial contents	Per vial	Per litre
Polymyxin B	53,000IU	106,000IU
Trimethoprim	5.0mg	10.0mg

One vial of polymixin B was added in 500 ml of sterilised agar solution, before pouring into sterilised Petri dishes.

Brilliance E. coli/Coliform Selective Media (CM1046, Oxoid).

Typical Formula*	gram/litre
Peptone	8.0
Di-sodium hydrogen phosphate	2.2
Sodium chloride	5.0
Potassium di-hydrogen phosphate	1.8
Sodium lauryl sulphate	0.1
Chromogenic mix	0.35
Agar	10.6

pH 6.7 ± 0.2 @ 25°C

Columbia Blood Agar Base (CM0331, Oxoid, UK).

Chemical Formula	gram/litre
Special peptone	23.0
Starch	1.0
Sodium chloride	5.0
Agar	10.0

pH of the media 7.3 ± 0.2

DNase Agar (CM0321, Oxoid, UK).

Chemical Formula	gram/litre
Tryptose	20.0
Deoxyribonucleic acid	2.0
Sodium chloride	5.0
Agar	12.0

pH of the media 7.3 ± 0.2

Nitrate Broth (72548, Biochemika, UK).

Chemical Formula	gram/litre
Meat extract	3.0
Peptone	5.0
Potassium Nitrate	1.0
pH of the media 7.0 ± 0.2	

Nutrient Agar (CM0003, Oxoid, UK).

Typical Formula	gram/litre
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
pH 7.4 ± 0.2	

Phenol-Red Broth Base (P8976, Biochemika, UK).

Chemical Formula	gram/litre
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	5.0
Phenol red	0.018
pH of the media 7.4 ± 0.2	

Plate Count Agar (CM0463, Oxoid, UK).

Typical Formula*	gram/litre
Yeast extract	2.5
Pancreatic digest of casein	5.0
Glucose	1.0
Agar	15.0
pH 7.0 ± 0.2 @ 25°C	

Polymyxin Pyruvate Egg Yolk Mannitol agar (CM0617, Oxoid, UK).

Chemical Formula	gram/litre
Peptone	1.0
Mannitol	10.0
Sodium chloride	2.0
Magnesium sulphate	0.1
Disodium hydrogen phosphate	2.5
Potassium dihydrogen phosphate	0.25
Bromothymol blue	0.12
Sodium pyruvate	10.0
Agar	15.0

pH of the media 7.2 ± 0.2

Polymyxin B Supplement (SR0099, Oxoid, UK).

Vial contents	Per vial	Per litre
Polymyxin B	50,000 IU	100,000 IU

One vial of polymixin B was added in 500 ml of sterilised agar solution, before pouring into sterilised Petri dishes.

Potato Dextrose Agar (CM0139, Oxoid, UK).

Chemical Formula	gram/litre
Potato extract	4.0
Glucose	20.0
Agar	15.0

pH of the media 5.6 ± 0.2

***Pseudomonas* CN Agar Base (CM0559, Oxoid, UK).**

Chemical Formula	gram/litre
Gelatin peptone	16.0
Casein hydrolysate	10.0
Potassium sulphate	10.0
Magnesium chloride	1.4
Agar	11.0

pH of the media 7.1 ± 0.2

Pseudomonas CN Selective Supplement (SR0102, Oxoid, UK).

Vial contents	Per vial	Per litre
Cetrimide	100.0mg	200.0mg
Sodium nalidixate	7.5mg	15.0mg

One vial was added in 500 ml of sterilised agar solution, before pouring into sterilised Petri dishes.

Salmonella Chromogenic Agar Base (CM1007, Oxoid, UK).

Chemical Formula	gram/litre
Special peptone	10.0
Chromogenic mix	28.0
Agar	12.0

pH of the media 7.2 ± 0.2

Salmonella Selective Supplement (SR0194, Oxoid, UK).

Vial contents	Per vial	Per litre
Cefsulodin	6.0mg	200.0mg
Novobiocin	7.5mg	15.0mg

One vial was added in 500 ml of sterilised agar solution, before pouring into sterilised Petri dishes.

Tryptone Soya Agar (CM0131, Oxoid, UK).

Chemical Formula	gram/litre
Tryptone	15.0
Soya peptone	5.0
Sodium chloride	5.0
Agar	15.0

pH of the media 7.3 ± 0.2

Tryptone Soya Broth (CM0876, Oxoid, UK).

Chemical Formula	gram/litre
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5

pH of the media 7.3 ± 0.2

Water Plate Count Agar (CM1012, Oxoid, UK).

Typical Formula	gm/litre
Tryptone	6.0
Yeast Extract	3.0
Agar	15.0
pH 7.2 ± 0.2	

Appendix 2: Chemicals and Instruments Used for PFGE Analysis

Chemicals used in this study for PFGE analysis.

Chemicals	Concentrations	Suppliers
Certified magabse agarose		Bio-Rad, USA
Low melting-point agarose		Bio-Rad, USA
Lysozyme	~70000 units/mg	Sigma-Aldrich, UK
Proteinase K	≥30 units/mg protein	Sigma-Aldrich, UK
Ethidium bromide	500 µg/ml	Sigma-Aldrich, UK
Tris borate EDTA (TBE) buffer (pH 8.3)	10x concentration in water	Sigma-Aldrich, UK

Instruments used in this study for PFGE analysis.

Instruments	Suppliers
Clamped homogenous electric field (CHEF-DR II) system	Bio-Rad, USA
Gel documentation (gel doc) system	Bio-Rad, USA
Centrifuge 5804R	Eppendorf, Germany
Spectrophotometer CE 1011	Cecil Instrument Ltd., UK
Plug mould	Bio-Rad, USA