Methods of Isolation and Identification of Pathogenic and Potential Pathogenic Bacteria from Skins and Tannery Effluents

by

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Abstract

Currently there is no standard protocol available within the leather industry to isolate and identify pathogenic bacteria from hides, skins or tannery effluent. This study was therefore carried out to identify simple but effective methods for isolation and identification of bacterial pathogens from the effluent and skins during leather processing. Identification methods based on both phenotypic and genotypic characteristics were investigated. *Bacillus cereus* and *Pseudomonas aeruginosa* were used as indicator bacteria to evaluate the isolation and identification methods. Decontaminated calfskins were inoculated with a pure culture of the above mentioned bacterial species followed by pre-tanning and chromium tanning processes. Effluent samples were collected and skins were swabbed at the end of each processing stage. Bacterial identification was carried out based on the phenotypic characteristics; such as colony appearance on selective solid media, cell morphology following a standard Gram-staining and spore staining techniques, and biochemical reactions, e.g., the ability of a bacterial species to ferment particular sugars and ability to produce certain enzymes. Additionally, an identification system based on bacterial phenotypic characteristics, known as Biolog® system was applied. A pulsed-filed gel electrophoresis (PFGE) method for bacterial DNA fingerprinting was also evaluated and used for the identification of the inoculated bacteria. The methods described in the study were found to be effective for the identification of pathogenic bacteria from skins and effluent.

Resumen

En estos momentos no existen protocolos estandarizados disponibles en la industria del cuero para aislar e identificar bacterias patógenas en los cueros, pieles o en los efluentes de la curtiembre. Este estudio entonces se emprendió para la efectiva y simple identificación e aislamiento de bacterias patógenas en los efluentes y pieles en el proceso de curtición. Métodos de identificación basados en las características tanto de genotipos como las de fenotipos fueron investigados. *Bacilos céreos* y *Pseudomonas aeruginosa* se emplearon como bacterias indicadoras para evaluar los métodos de aislamiento e identificación. Pieles de ternero descontaminadas fueron inoculadas con culturas puras de las mencionadas especies de bacterias para luego ser sometidas a procesos de precurtido y curtido al cromo. Muestras de efluentes y pieles fueron muestreadas al final de cada paso del procesamiento. Identificación bacteriana fue efectuada en base a características fenotípicas tales como aspecto de colonias sobre medios sólidos selectivos, morfología de las células seguidas por técnicas de teñidos Gram standard y teñido de esporas, y reacciones bioquímicas específicas, v. g. la habilidad de una especie bacteriana en fermentar azúcares particulares y la habilidad en la producción de ciertas encimas. Adicionalmente un sistema de identificación de características fenotípicas bacterianas conocido como Biolog® se aplicó. Un método de electroforésis de gel por pulsaciones eléctricas (PFGE) de huellas digitales del ADN de la bacteria fue también evaluado y empleado para identificación de bacterias inoculadas. Los métodos descritos en este estudio se encontraron efectivos en la identificación de bacteria patogénica en pieles y efluentes de curtidos.

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Introduction

Animal skins naturally contain various types of microorganisms, which either are natural inhabitants or acquired from the environment.[1-7] Microorganisms present on the hides and skins are capable of rapid proliferation due to the presence of a nutritional source, high moisture content, suitable pH and temperature in the tannery environment.[2,4,6,8] The majority of the bacterial species isolated from raw hides, skins as well as from hides and skins during various stages of the leather making processes are non-pathogens;[9,10] however, a number of species that are considered as pathogens or potential pathogens, such as *Bacillus cereus*,[2,4,11] *Escherichia coli*,[11] *Staphylococcus aureus*,[1,2,4] and *Pseudomonas aeruginosa*,[3] were isolated from hides and skins. Pathogens present on hides and skins and in the tannery effluent may infect tannery personnel or contaminate the environment through discharge of the effluent or disposal of the solid waste. Moreover, 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 Best Available Technologies (BAT) leather-making processes may provide suitable conditions for bacterial growth. Since the leather industry is moving toward more environmentally-friendly methods of leather manufacturing, routine observation of the survival and growth of pathogens during the leather processing may be required. Therefore the presence of pathogens in tannery effluent and the pathogen related health issues requires investigation. Various methods may be used to identify pathogens in effluent and on hides and skins. In this study phenotypic as well as genotypic methods to isolate and identify pathogens from skins and tannery effluent are described.

Materials and Methods

Calfskins (Latco Ltd., UK) were used as raw materials. Soaked, fleshed and salted calfskins were cut into small pieces weighing approximately 100g each. The skin pieces were washed with autoclaved sterile tap water and decontaminated using sodium hypochlorite (NaOCl, 125 mg/ml). The skins were treated with NaOCl three times, for 3 minutes each time at room temperature.

The decontaminated samples were washed thoroughly with sterile water and the flesh side of the skin pieces were inoculated with pure cultures of *Bacillus cereus* ATCC11778 (Oxoid, UK) and *Pseudomonas aeruginosa* ATCC10145 (Oxoid, UK). The number of inoculated *B. cereus* and *P. aeruginosa* on each of the calfskin piece was approximately 10^8 colony forming units (cfu) and 10^{10} cfu respectively. Bacterial species were inoculated on the flesh side, as the epidermis layer (the outer side of the skin) may prevent penetration of the bacterial species through the cross-section of skins. Pure cultures of *B. cereus* ATCC11778 and *P. aeruginosa* ATCC10145 will be referred to as controls in this study. The bacterial inoculums were collected from the mid-exponential growth phase as the physical and chemical properties of the bacterial cells are most consistent during this phase[12-13]. The time required to reach the mid-exponential phase was determined by plotting growth curves for each of the bacterial species over time prior testing (see Figure 1 as an example of a growth curve).

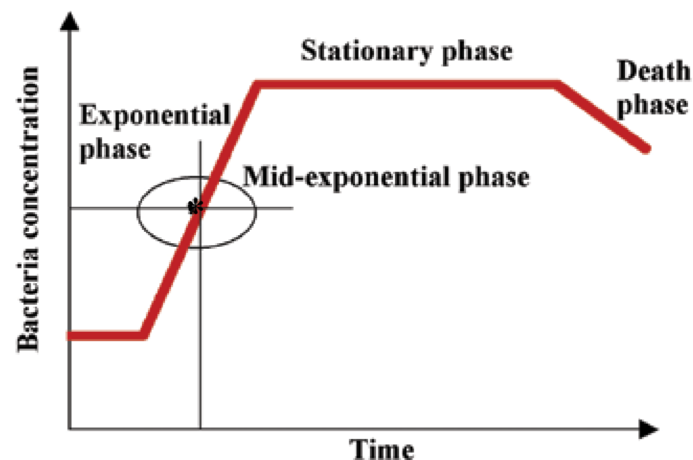

Figure 1. Bacterial growth curve, (*) showing mid-exponential phase.[12]

The inoculated calf skin pieces were incubated overnight at a most suitable temperature (see Table I) as recommended by the National Standard Methods, published by the Health Protection Agency (HPA), UK for the growth of *B. cereus*[14] and *P. aeruginosa*[15]. A conventional and a BAT pre-tanning and chromium tanning processes (see the Appendix 1; Tables VIII and IX) were carried out using the inoculated calfskin pieces. Effluent samples were collected at the end of the pre-soaking, soaking, unhairing, re-liming, deliming, bating, pickling and tanning processes aseptically in 15 ml sterilised centrifuge tubes (Fisher Scientific, UK). Centrifuge tubes were completely filled with the effluent to limit air contact with the collected samples and to reduce bacterial contamination from the environment. The flesh side of the calfskins were also swabbed using transport swabs (SWA3018, Scientific Laboratory Supplies Ltd., UK). These were undertaken to determine presence of the inoculated bacteria on skins and effluent. The effluent and swabs were stored in a refrigerator at 4°C and analysed within 24 hours of collection.

Isolation and Primary Identification of Inoculated Bacterial Species

Phosphate-buffered saline (PBS; pH 7.4±0.2) solution was used for dilution. The swabs were placed in a sterilised 5 ml bottle containing 2.5 ml PBS solution (Oxoid, U.K), and agitated at room temperature using a vortex mixer (Stuart®,

UK) at 2500 rpm for 20 seconds before plating. Hair after the unharing processes were also collected by centrifuging conventional and BAT unhairing effluents (10 ml) at 8228 *g* for 15 minutes. The supernatant was decanted carefully and 10 ml Tryptone soya broth (TSB; CM0876, Oxoid, UK) was added to the sediment (solid hair waste) then incubated overnight. An overnight incubation is thought to assist the recovery of damaged or stressed bacterial cells. However, this will also allow some of the bacterial cells to multiply increasing the initial number of bacterial cells in solid hair waste. After incubation, the samples were mixed using a vortex mixer 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.

Samples (50 μl)[16,17] were inoculated on respective solid media using a Whitley automated spiral plater (model WASP2, Don Whitley Scientific Ltd., UK).

In this study selective microbial media were used for primary identification and isolation of the selected bacterial species. Selective media prevents growth of certain microbial species while promoting growth of the target bacteria[18]. Various selective media are commercially available for the isolation and identification of pathogenic bacteria. The bacterial species were primarily identified based on their colony appearance on the selective solid media. The isolated bacteria will be referred to as presumptive bacterial colonies. The microbial media, incubation temperature and time for the growth of *B. cereus*[14]and *P. aeruginosa*[15], are stated in Table I. Microbial media were prepared according to the instructions provided by the suppliers (Oxoid, UK) (see Appendix 2). The prepared media was stored in a refrigerator at 4°C, and used within two weeks. Colony appearances on the selective media used in this study are also given in Table I.

Bacterial cultures were purified to a single colony through streaking. The purified single colony was re-streaked in order to produce more colonies and to obtain sufficient bacterial colonies to perform further tests as described in the following paragraphs. A pure culture of *B. cereus* ATCC11778 and *P. aeruginosa* ATCC10145 (Oxoid, UK) was used as a positive control for the tests.

Gram-staining and Spore-staining

Standard Gram-staining[19] and spore-staining techniques[20] were conducted on isolated bacteria to determine whether the bacteria are Gram-positive or negative and whether the bacteria produce spores.

Biochemical Reactions following Standard Methods

Bacteria colonies were subjected to various tests following standard methods published by the HPA[14-15]. In order to identify *B. cereus*, bacterial colonies were tested for anaerobic growth, carbohydrate fermentation ability, ability to reduce nitrate to nitrite and β-haemolysis[15]. According to the HPA standard methods[15], no further confirmation tests is necessary if *P. aeruginosa* produces blue or green coloured colonies on the *Pseudomonas* CN agar media. *P. aeruginosa* isolated in this study, appeared as green coloured colonies and therefore, no further biochemical tests were carried out.

The Biolog® System for Bacterial Identification

Samples for the Biolog® identification method were prepared according to the manual guide provided by Biolog Inc. (California, USA)[21]. A MicrologTM 4.2 database was used to interpret the data obtained on the Biolog® microplates. The Biolog® system was developed by Biolog Inc., and is used as an easier option of phenotypic identification[22]. The Biolog® technique of microbial identification is based on carbohydrate utilisation by microorganisms[22,23]. The Biolog® microplates consist of 96 wells that contain water and 95 various media of

TABLE I

Microbial media, incubation temperature and time for the growth of *B. cereus* and *P. aeruginosa* as well as colony appearance of the bacterial species on the selective solid media used in this study.

Bacterial species	Microbial media for bacterial incubation	Incubation temperature (°C)	Incubation time (hours)	Colony appearance on selective media
B. cereus	Polymyxin pyruvate egg yolk mannitol agar (PEMBA) (CM0617, SR0099, SR0047, Oxoid UK).	30	24	Turquoise or peacock-blue coloured colonies, which may or may not be surrounded by an egg yolk-precipitated zone.
P. aeruginosa	*Pseudomonas* CN media containing glycerol, cetrimide and nalidixic (CM0559, SR0102, Oxoid, UK).	37	24	Green colonies that fluoresce under UV-lights.

specific carbohydrates, and a redox indicator. The redox dye, tetrazolium, changes into a purple colour if microbial growth occurs in a particular well representing catabolism of the substrate [23]. The colour transformation of the dye is considered as a positive reaction. The well containing water is used as a control for the 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[21]. Figure 2 shows an image of a Biolog® microplate indicating the positive results by the colour transformation.

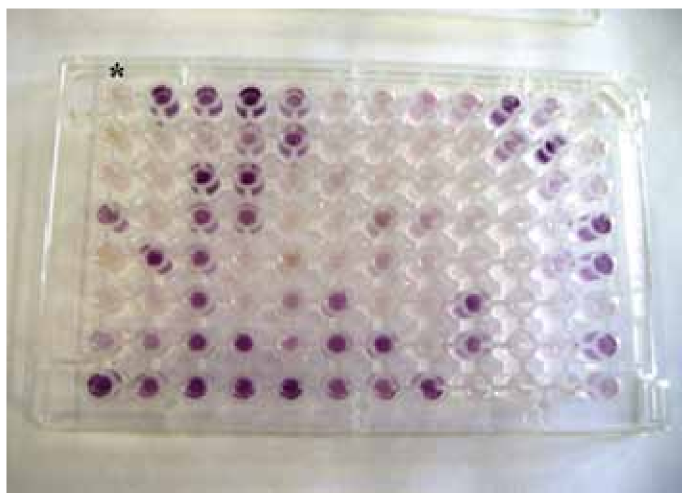

Figure 2. Photograph of a Biolog® microplate. The microplate was inoculated with *B. cereus* at 30°C for 18 hours. Utilisation of a particular carbohydrate is indicated by the transformation of the indicator dye to purple. Colour transformation did not occur (colourless wells) when the bacterial species did not use a carbohydrate. Well number 1 (*) contains water and used as a control. Photograph was taken using Pentax Optio 57 (7 MP).

Extraction and Purification of Bacterial DNA for Pulsed-Field Gel Electrophoresis

Bacillus cereus

The pulsed-field gel electrophoresis (PFGE) protocol for *B. cereus* DNA sequencing was provided by Dr. Babetta L. Marrone and Yulin Shou, Los Alamos National Laboratory, Los Alamos, USA[24,25]. *B. cereus* colonies were cultured overnight on a solid nutrient media (CM0003, Oxoid, UK). Single colony, collected from the overnight grown culture, was added to 5 ml TSB and incubated for 14-16 hours in a 37°C shaking water bath (water bath with orbital shaker) 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 8228 *g*. 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 at 4°C 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) and incubated overnight in 1 ml lysis buffer with lysozyme (2 mg/ml) (Sigma-Aldrich, UK) in a 37°C shaking water bath with an agitation speed of 200 rpm. The following day proteinase K (1 mg/ml) (Sigma-Aldrich, UK) 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.

Pseudomonas aeruginosa

P. 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 and centrifuged at 9289 *g* 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 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), transferred to a plug mould 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 [26-30].

Digestion of the Bacterial DNA

DNA extracted from *B. cereus* was digested using 20 units (10000 units/ml) of *Sma*I restriction enzyme (Sigma-aldrich, UK) in 200 µl 1 x concentration SH buffer (Sigma-Aldrich, UK) for 2 hours in a shaking water bath with orbital agitation speed of 200 rpm at 25°C. DNA extracted from *P. aeruginosa* was digested using 15 units (10000 units/ ml) of *Spe*I restriction enzyme (Sigma-Aldrich, UK) in 100 µl of 1x concentration SA buffer (Sigma-Aldrich, UK) overnight at 37°C [26-31].

Electrophoresis of Bacterial DNA

Electrophoresis was conducted using a PFGE system to separate the enzyme-digested *B. cereus* and *P. aeruginosa* DNA. The DNA band patterns obtained were compared with the DNA band patterns of the control bacterial species (*B. cereus* ATCC11778 and *P. aeruginosa* ATCC10145). A lambda ladder (48.5 - 970 Kb, Bio-Rad, USA) was used as a marker. A 1% w/v certified megabase agarose was prepared in TBE buffer (0.5 x concentrations). Ethidium bromide (50 µg/ml) was added to the molten agarose. Digested plugs and lambda ladder were loaded into the gel and sealed with 1% w/v molten agarose. The agarose 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 for analysing *P. aeruginosa* DNA 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 electrophoresis for *B. cereus* DNA was conducted 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 (Bio-Rad, USA) and photographed[27-30].

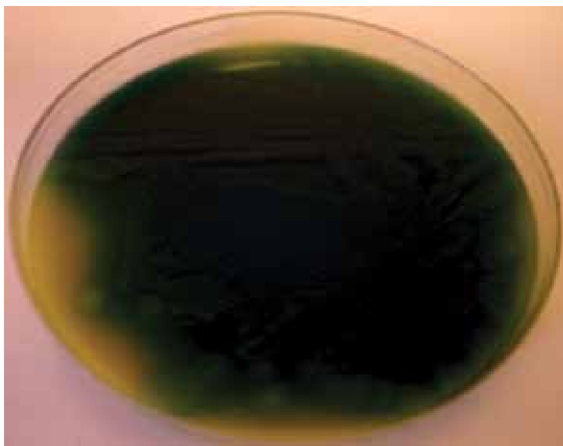

Figure 3. ***B. cereus*** ATCC11778 on PEMBA media. Photo was taken using a Pentax Optio 57, 7.0 Megapixel (MP).

Results and Discussion

Bacterial Growth on Selective Media

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. *B. 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 (such as *B. subtilis*) appear as straw coloured (acid production). *B. cereus* generally produces an enzyme lecithinase, which degrades lecithin in egg yolk causing precipitation of the hydrolysed lecithin. A photo of typical blue *B. cereus* colonies, on PEMBA media, surrounded by blue precipitated zone of hydrolysed lecithin is shown in Figure 3. However, some *B. cereus* strains may produce little or no lecithinase[18]. In this study all the presumptive *B. cereus* colonies showed the similar characteristics as to the control *B. cereus* ATCC11778.

Pseudomonas aeruginosa

P. aeruginosa produces two water soluble pigments pyoverdin and pyocyanin. Due to the production of pyocyanin, *P. aeruginosa* produces distinctive blue/green colonies. *P. aeruginosa* fluoresces under UV lights due to the production of pyoverdin[15,19]. Figure 4 shows green *P. aeruginosa* colonies on *Pseudomonas* CN media. Both the control (ATCC10145) and presumptive *P. aeruginosa* appeared green on the selective media.

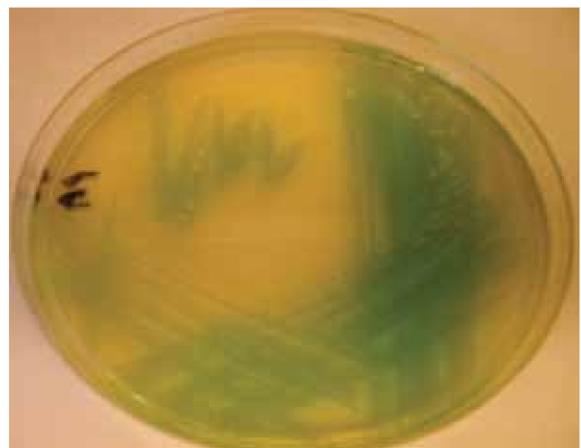

Figure 4. *P. aeruginosa* ATCC10145 on *Pseudomonas CN agar media*. Photo was taken using a Pentax Optio 57, 7.0 MP.

Gram-Staining and Spore-staining

Gram-staining and spore-staining provides useful information that assists primary identification of bacterial species. This information is also required to apply the Biolog® system for bacterial identification. When observed under a light microscope following Gram-staining, *B. cereus* appeared as a

rod-shaped Gram-positive bacterium while *P. aeruginosa* appeared as a Gram-negative and rod-shaped bacterium. *Bacillus* species also produce spores, which are known as endospores as they are produced intracellularly[19]. The spores are visible under microscope following a spore-staining technique. Similar to the Gram-staining, spore staining assists primary identification of bacterial species.

Biochemical Tests

Bacillus cereus

Typical *B. cereus* shows anaerobic growth. It ferments dextrose but does not ferment arabinose, mannitol and xylose. Due to the production of an enzyme nitrate reductase, *B. cereus* reduces nitrate to nitrite[20], and due to the production of an enzyme haemolysins, *B. cereus* destroys the red blood cells (cell envelopes) and appears as a clear, colourless zone of haemolysis on blood agar[20]. All the *B. cereus* colonies isolated from the conventional beamhouse process exhibited the same biochemical characteristics, when compared to the control *B. cereus* ATCC11778 (Table II). Although, the majority of the isolated *B. cereus* from the BAT beamhouse processes showed the same biological characteristics, when compared to the control, however, *B. cereus* isolated from the BAT unhairing and the pickling process failed to reduce nitrate to nitrite (Table III). The phenotypic characteristics of microorganisms are often dependent on the media and growth conditions such as temperature, pH, nutrient depletion, vitamin and mineral availability, growth cycle, water activity and colony density on plates[23]. Therefore, colonies of the same species may fail to exhibit the same phenotypic characteristics.

The Biolog® System for Bacterial Identification

Tables VI and V show the results obtained when a Biolog® system was used to identify isolated presumptive bacterial colonies from the conventional and BAT processing respectively. The majority (95%) of the presumptive *B. cereus* and *P. aeruginosa* isolated from both the conventional and BAT leather processing was identified as *B. cereus* and *P. aeruginosa* when a Biolog® identification method was used. The results obtained also show that the Biolog® system could not differentiate between *B. cereus* and *B. thuringienisis*. It may be difficult to distinguish between *B. cereus, B. thuringienisis* and *B. anthracis* using biochemical tests as the bacterial species have similar phenotypic characteristics [19,32].

Although biochemical tests may vary due to environmental conditions[23, 33] during incubation, biochemical tests are most commonly used methods for the identification of pathogenic bacteria[33]. These tests are comparatively inexpensive and are simple to conduct. In this study, the majority of the results obtained showed that with biochemical tests it is possible to identify bacterial pathogens effectively and therefore may be used in routine observation of pathogens present on hides and skins as well as within the tannery effluent.

TABLE II

Biochemical tests for the presumptive *B. cereus* colonies isolated during the conventional leather-making process (*B. cereus* ATCC11778 was used as a positive control).

Isolated from	Carbohydrate fermentation*				Nitrate reduction	Anaerobic growth	Beta haemolysis	Identified as
	Ara	Dex	Man	Xyl				
Control	-	+	-	-	+	+	+	*B. cereus*
Pre-soaking	-	+	-	-	+	+	+	*B. cereus*
Soaking	-	+	-	-	+	+	+	*B. cereus*
Unhairing	-	+	-	-	+	+	+	*B. cereus*
Re-liming	-	+	-	-	+	+	+	*B. cereus*
Deliming	-	+	-	-	+	+	+	*B. cereus*
Bating	-	+	-	-	+	+	+	*B. cereus*
Hair from the unhairing effluent	-	+	-	-	+	+	+	B. cereus

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

TABLE III
Biochemical tests for the presumptive *B. cereus* colonies isolated during the BAT leather-making process (*B. cereus* ATCC11778 was used as a positive control).

Isolated from	Carbohydrate fermentation*				Nitrate reduction	Anaerobic growth	Beta haemolysis	Identified as
	Ara	Dex	Man	Xyl				
Control	-	+	-	-	+	+	+	*B. cereus*
Pre-soaking	-	+	-	-	+	+	+	*B. cereus*
Soaking	-	+	-	-	+	+	+	*B. cereus*
Unhairing	-	+	-	-	-	+	+	-
Re-liming	-	+	-	-	+	+	+	*B. cereus*
Deliming	-	+	-	-	+	+	+	*B. cereus*
Bating	-	+	-	-	+	+	+	*B. cereus*
Pickling	-	+	-	-	-	+	+	-
Hair from the unhairing effluent	-	+	-	-	-	+	+	-

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

TABLE IV
The Biolog® identification of presumptive *B. cereus* and *P. aeruginosa* colonies isolated during the conventional leather-making processing (pure culture of *B. cereus* ATCC11778 and *P. aeruginosa*, ATCC10145 were used as positive controls).

Isolated from	*B. cereus*		*P. aeruginosa*	
	Biolog Identification	Probability (%)	Biolog Identification	Probability (%)
Controls	*B. cereus/ thuringiensis*	100	P. aeruginosa	100
Pre-Soaking	B. cereus/ thuringiensis	100	P. aeruginosa	100
Soaking	*B. cereus/ thuringiensis*	100	P. aeruginosa	100
Unhairing	*B. cereus/ thuringiensis*	100	None isolated	X
Re-liming	*B. cereus/ thuringiensis*	100	None isolated	X
Deliming	B. cereus/ thuringiensis	100	P. aeruginosa	100
Bating	*B. cereus/ thuringiensis*	100	P. aeruginosa	100
Chromium tanning	None isolated	X	None isolated	X
Hair from the unhairing effluent	B. cereus/ thuringiensis	-	None isolated	X

TABLE V
The Biolog® identification of presumptive *B. cereus* and *P. aeruginosa* colonies isolated during the BAT leather-making processing (pure culture of *B. cereus* ATCC11778 and *P. aeruginosa*, ATCC10145 were used as positive controls).

Isolated from	B. cereus		P. aeruginosa	
	Biolog Identification	Probability (%)	Biolog Identification	Probability (%)
Controls	*B. cereus/ thuringiensis*	100	P. aeruginosa	100
Pre-soaking	*B. cereus/ thuringiensis*	100	P. aeruginosa	100
Unhairing	*B. cereus/ thuringiensis*	100	None isolated	X
Re-liming	*B. cereus/ thuringiensis*	100	None isolated	X
Deliming	*B. cereus/thuringiensis*	100	P. aeruginosa	100
Bating	*B. cereus/ thuringiensis*	100	P. aeruginosa	100
Pickling	*B. cereus/ thuringiensis*	100	P. aeruginosa	100
Chromium tanning	None isolated	X	None isolated	X
Hair from the unhairing effluent	*B. cereus/ thuringiensis*	100	P. aeruginosa	100

Identification methods based on bacterial genotypic characteristics may be expensive and time consuming, however, methods based on genotypic identification will provide useful information that can be used to identify pathogenic bacteria accurately. Additionally single identification methods may not provide 100% accuracy and therefore identification methods based on phenotypic or genotypic characteristics may be combined for accurate identification. Studies based on microbial genotypic characterises have been carried out with the leather industry. For examples, Oppong *et al*. (2006)[6] and Shede *et al*. (2008)[11], have used a molecular-based method (16s ribosomal RNA sequencing) for bacterial identification from hides.

Electrophoresis of Bacterial DNA

Gel electrophoresis systems are the most commonly-used electrophoretic methods for microbial DNA profiling. As described in the methods, in order to conduct electrophoresis of the bacterial DNA, the bacterial cells were harvested by centrifugation and an appropriate buffer was added to prepare a cell suspension, followed by the preparation of plugs by mixing with molten agarose with the bacterial suspension and transferring it to a plug mould. The plug embedded bacterial cells were then lysed to extract DNA and proteolysed in order to remove remaining protein debris with the extracted DNA, followed by the digestion of the extracted DNA with an appropriate restriction enzyme. The restriction enzymes cleaved the DNA into fragments with specific sizes and shapes. Electrophoresis of such DNA fragments for several hours resulted in the separation of the DNA fragments producing a specific band pattern for the experimental bacterial DNA [18,34] . The obtained DNA band patterns were then compared to a positive control. This was undertaken to determine whether the isolated bacterial species at various stages of the beamhouse and tanning process were same as the inoculated bacterial species. The DNA pattern of the isolated bacteria should match with the DNA of the respective positive controls as the inoculated bacteria and the positive controls were form the identical source. However, if the early stages of the leather manufacturing process have had any impact on the bacterial DNA, some alternation in the bacterial DNA pattern may be exhibited when compared to the corresponding positive controls

Figures 5-8 show the results, when a PFGE method was applied to obtain DNA profiles of *B. cereus* and *P. aeruginosa* isolated during the conventional and BAT leather making process followed in the study. The results obtained showed that the isolated *B. cereus* and *P. aeruginosa* had the identical DNA band patterns when compared to the corresponding positive controls (*B. cereus* ATCC11778 and *P. aeruginosa* ATCC10145). This confirms that the isolated bacterial species

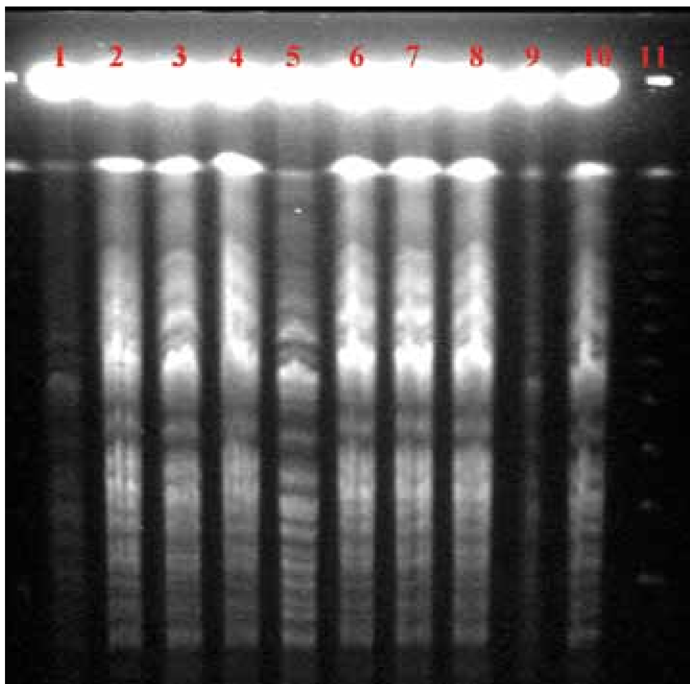

Figure 5. Pulsed-field gel electrophoresis of *Bacillus cereus* DNA (conventional leather-making process). Lane 1: *B. cereus* ATCC 11778 (control); lanes 2, 3, 4, 5, 6, 7 and 8: *B. cereus* DNA isolated during the conventional pre-soaking, soaking, unhairing, hair from the unhairing/liming effluent, re-liming, deliming and bating processes respectively; lane 11: Lambda Ladder (48.5- 970 kb).

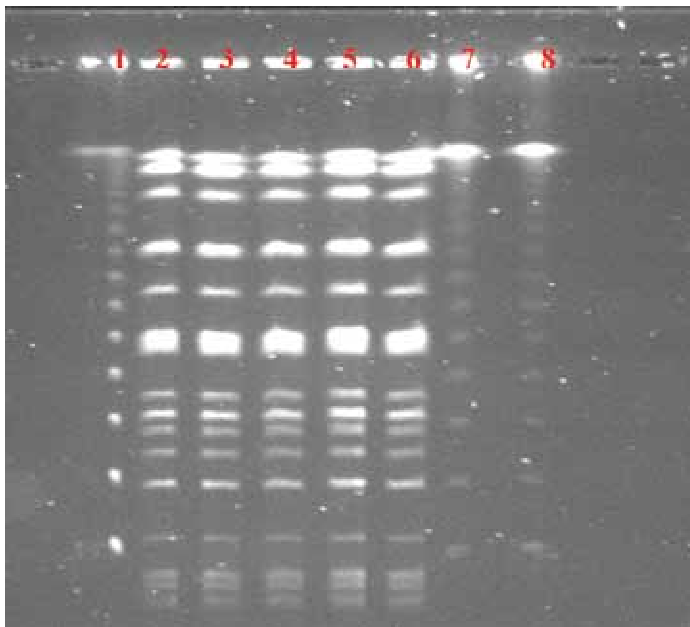

Figure 6. Pulsed-field gel electrophoresis of *P. aeruginosa* DNA (conventional leather making process). Lanes 1, 7 and 8: Lambda Ladder (48.5- 970 kb), lane 2: *P. aeruginosa* ATCC10145 (control), lanes 3, 4, 5 and 6: DNA of *P. aeruginosa* isolated during the conventional pre-soaking, soaking, deliming and bating processes respectively.

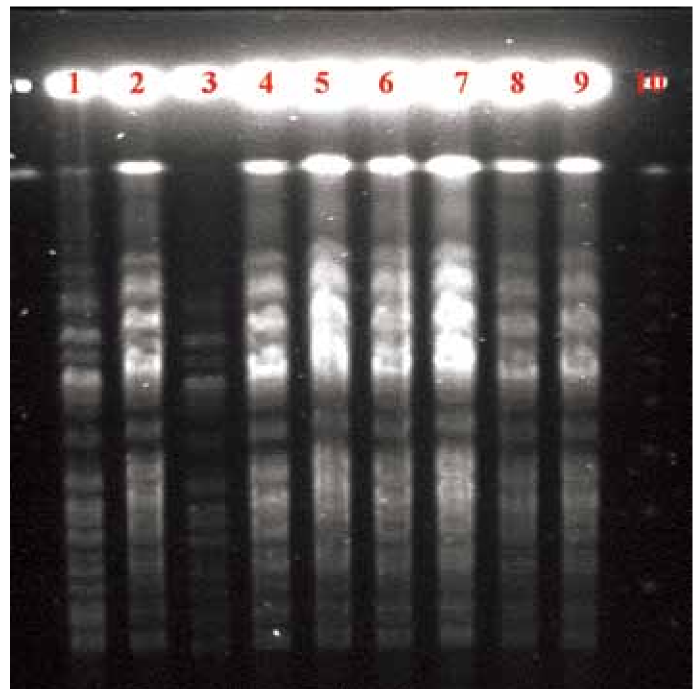

Figure 7. Pulsed-field gel electrophoresis of Bacillus cereus DNA (BAT leather-making process). Lane 1: *B. cereus* ATCC 11778 (control); lanes 2, 3, 4, 5, 6, 7, 8 and 9: *B. cereus* DNA isolated during the conventional pre-soaking, soaking, unhairing, hair from the unhairing effluent, re-liming, deliming, bating and pickling processes respectively; lanes 10: Lambda Ladder (48.5- 970 kb).

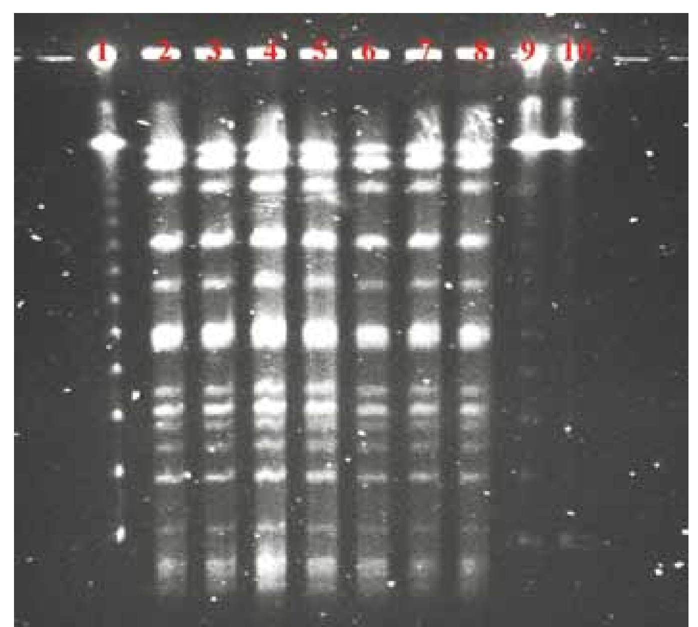

Figure 8. Pulsed-field gel electrophoresis of *P. aeruginosa* DNA (BAT leather making process). Lanes 1 and 9: Lambda Ladder (48.5- 970 kb), lane 2: *P. aeruginosa* ATCC10145 (control), lanes 3, 4, 5, 6, 7 and 8: DNA of *P. aeruginosa* isolated during the BAT pickling, soaking, hair from the unhairing effluent, deliming, bating and pre-soaking processes respectively.

were the same strain as the inoculated *B. cereus* and *P. aeruginosa*. This also suggests that no alteration in the bacterial DNA occurred during processing.

Tables VI and VII showed an estimated recovery of the inoculated bacterial species from various stages of the conventional and BAT pre-tanning as well as chromium tanning processes. The recovery rate was higher during the pre-soaking and soaking processes, decreasing during the unhairing/ liming stages. This is understandable, as the pre-soaking and the soaking processes provide the most suitable environment for the growth of bacterial species due to the presence of a nutritional source, moisture content, suitable pH, temperature and adequate incubation time for survival and proliferation. Although, similar to the pre-soaking and soaking processes, deliming and the bating processes also provide the bacterial species with a suitable environmental conditions for the growth and proliferation, however, the number recovered during these processes are low as majority of the bacterial colonies were destroyed during the unhairing/ liming and re-liming stages due to the extreme environmental conditions[35].

Generation or doubling time of the bacterial population may vary species to species. If provided with a most suitable environment many bacteria may multiply rapidly, some bacteria may have a replication time of 10 minutes[18]. Therefore, prolonged deliming and bating may potentially assist bacterial proliferation so increasing the bacterial concentration in the effluent. In this study majority of the bacterial colonies were recovered from the processing effluents. Mechanical agitation within the processing drums during the leather processing may have assisted bacterial colonies to transfer to the effluent from the skins[2].

Methods described in this study will be useful tools to determine the presence of pathogens in effluent or hides and skins. For primary identification and isolation of bacterial pathogens, selective media were used and further identified using biochemical tests based on phenotypic characteristics, and the utilisation of certain carbohydrates. Finally bacterial colonies were identified to strain level by using a DNA-based identification method.

TABLE VI
The estimated number of recover *B. cereus* colonies (cf/ml) from various stages of the pre-tanning and chromium tanning processes as well as from the hair collected from the unhairing/ liming effluent.

Processes	Estimated number of recovered *B. cereus* colony forming units/ ml			
	Effluent		Swabs	
	Conventional	BAT	Conventional	BAT
Pre-soaking	10^4-10^5	10^2-10^3	10^3-10^4	10^1-10^2
Soaking	10^3-10^4	10^2-10^3	0-10^2	0-10^2
Unhairing/ liming	0-10^1	10^1-10^3	0-10^2	0-10^2
Re-liming	0-10^2	0-10^1	0-10^1	0
Deliming	0-10^2	0-10^1	0	0
Bating	0-10^2	0-10^1	0	0
Pickling	0-10^1	0-10^1	0	0
Chrome-tanning	0	0	0	0
Hair from the unhairing effluent	10^2-10^5	10^1-10^3	-	-

TABLE VII
The estimated number of recover *P. aeruginosa* colonies (cf/ml) from various stages of the pre-tanning and chromium tanning as well as from hair collected from the unhairing/ liming effluent.

Processes	Estimated number of recovered *P. aeruginosa* colony forming units/ ml			
	Conventional	BAT	Conventional	BAT
Pre-soaking	10^7	10^7	10^5	10^5
Soaking	10^7	10^6-10^7	10^5	10^5
Unhairing/ liming	0	0	0	0
Re-liming	0	0	0	0
Deliming	0-10^2	0-10^4	0	0
Bating	0-10^2	0-10^4	0	0
Pickling	0	0	0	0-10^2
Chrome-tanning	0	0	0	0
Hair from the unhairing effluent	0	0-10^1	-	-

Conclusion

Due to presence of a large number of bacteria, particularly on raw hides/ skins, and during the soaking processes identification of individual species will be time consuming and expensive. Therefore, it will be an easier option to select a number of pathogens that are most likely to be present on hides, skins and in tannery effluent, and monitor their presence.

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Appendix

Conventional and BAT leather-making processes.

TABLE VIII
Conventional leather-making process.

Process	Chemicals	Amount (% w/w)*	Time	pH	Temperature (°C)
Pre-soaking	Water Truposept BA (Trumpler, Germany)	300 0.2	60 mins	7.6-8.0	21.1-25.8
Main-soaking	Water CorileneW385 (STAHL Europe, The Netherlands) Truposept BA	300 0.2 0.2	120 mins and left overnight	7.6-8.1	21.5-25.6
Unhairing	Water Sodium sulfide Lime	200 3 2	20 -24 hrs	11.1-12.7	21.2-27.8
Washing	Water	300	10 mins		
Re-liming	Water Lime	200 2	72 hrs	12.5-12.6	20.6-25.8
Washing (three times)	Water	300	10 mins		
Deliming	Zero float Ammonium chloride Water	2 100	15 mins 30-45 mins	8.2-8.8	24.5-26.8
Bating	Oropon ON 2 (TFL Germany)	0.1	30 mins	8.0-8.7	24.9-26.8
Washing (twice)	Water	300	10 mins		
Pickling	Water Sodium chloride Sulfuric acid Formic acid	100 8 1.2 1.0	3-4 hrs and left overnight	1.5-3.1	20.4-26.6
Tanning and Basification	Chromium (III) sulphate Feliderm®MGO (Clariant, Switzerland)	8 0.4	90 mins 4 hrs	4.1-4.9	22.8-27.7

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

TABLE IX
A BAT leather-making process.

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

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

APPENDIX II

Composition of the Microbial Media and Preparation

Polymyxin Pyruvate Egg Yolk Mannitol Agar (PEMBA, 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.

Preparation Method

PEMBA (CM0617), 20.5g was dissolved completely in 475ml of distilled water by heating gently to the boil, followed by autoclaving at 121°C for 15 minutes. The agar was then cooled to approximately 50°C and the contents of one vial of Polymyxin B Supplement (SR0099) was added aseptically to the agar. Sterile Egg Yolk Emulsion (SR0047), 25 ml was also added to the agar aseptically. The agar with supplement and egg yolk was mixed well and poured into sterile Petri dishes.

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.

Preparation Method

Pseudomonas CN agar base (CM0617) was prepared by suspending 20.5g of the agar base in 500 ml distilled water. Glycerol, 5 ml was added to the agar base and heated gently to the boil to dissolve the agar completely. The agar base was then sterilised by autoclaving at 121°C for 15 minutes and cooled to approximately 50°C. The contents of one vial of *Pseudomonas* CN Selective Supplement (SR0102) was added aseptically to the agar, mixed well and poured into sterile Petri dishes.