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Full Length Research Paper

Determination of bacterial and fungal numbers in floats of pre-tanning operations

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This study was aimed at determining the numbers of bacteria and fungi in the floats of main soaking, liming, deliming-bating, degreasing and pickling stages. To this end, the numbers of total aerobic mesophilic, proteolytic, lipolytic and aerobic spore-forming bacteria and, the numbers of total aerobic fungi (yeast and mould), proteolytic and lipolytic fungi were determined on the media containing different concentration of NaCl (0, 5, 10, 15 and 25%). Sheep skins were processed up to the end of pickling stage by adding a commercial bactericide (Derbio® DB 99), whose active ingredient is composed of quarternized compounds, into main soaking float. According to the results of the study, while most of the bacteria displayed growth from the main soaking process to the end of deliming-bating, most of the fungi showed growth from the main soaking process to degreasing. It is found that main soaking and deliming-bating processes came to the fore in terms of bacterial growth. It is also remarkable that the numbers of proteolytic bacteria were higher in degreasing float as well as in main soaking float. In the study, fungal numbers were found to be lower in all the stages than bacterial numbers in general. Moreover, fungi displayed growth in all NaCl concentrations (0, 5, 10 and 15%) in all the processes up to the end of the degreasing process except on the media containing 25% NaCl for the main soaking float. As for in pickling float, only proteolytic fungi displayed growth on the media containing 0 and 5% NaCl.

Key words: Beamhouse operations, proteolytic bacteria, lipolytic bacteria, fungi, leather industry.

INTRODUCTION

Hides or skins which are waste materials of the meat industry are converted into durable and useful products by different processing stages. They are prepared for tanning by pre-tanning operations and to this end, undergo a series of processes. These are pre-soaking, main soaking, unhairing-liming, deliming-bating, degreasing and pickling, respectively (Kumar, 2006). While beamhouse processes from soaking to bating are carried out with the purpose of cleansing the skin and removing unwanted interfibrillary matrix components and foreign substances, pickling operation is done so as to get the pelt ready for subsequent chrome tanning (Ramasami et al., 1997). Skin being the main raw material of the leather industry is susceptible to growth of bacteria, mould and other micro-organisms due to the fact that it is a protein source (Tancous, 1986). While the animal is alive, its skin is resistant to microbial attacks due to its metabolic defences. But following slaughter, microbial activities start just after flaying (Thorstensen, 1993). Micro-organisms cannot digest such large molecules as proteins and polysaccharides. Hence, they reduce these molecules to amino acids and simple sugars, respectively. Micro-organisms reduce these large molecules with the help of extracellular enzymes (Didato et al., 1999). Collagen, the main component of fibrous structure of skin, may get damaged from proteolytic enzymes secreted by micro-organisms. Water content of skin is principally decreased in order to prevent the damage caused by putrefactive bacteria (Bienkiewicz, 1983). For this reason, the skins are commonly conserved with salt. However, almost 70% of halophilic micro-organisms isolated from conserved hides are known to be proteolytic and damage the skin (Birbir et al., 1996). These bacteria

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may form red and violet pigments by getting active in the pre-soaking and main soaking floats regarded as a kind of culture medium (Orlita, 2004). In a study carried out by Mericli Yapici et al. (2004), it was found that the numbers of halophilic and halotolerant bacteria were dominant at the end of the pre-soaking process. The presence of some bacteria, such as *Proteus vulgaris*, *Bacillus* and *Micrococcus* species with high proteolytic activity in unhairing and liming floats is a warning. Moreover, it is known that alkalifilic *Bacillus* spores isolated from these floats survive for a long time (Pfleiderer and Reiner, 1988). In a study where unhairing- liming floats of hides were used ten times through regeneration, the fact that the numbers of fungi were found to be higher than those of bacteria is an important finding (Mericli Yapici et al., 2008).

In case of lasting of bating time, many bacteria especially proteolytic bacteria could display growth and, pelt pieces which remained in the float and decomposed proteins in this process could damage the structure of the pelt by prompting bacterial growth (Karaboz, 1994). Even if bacterial growth is prevented during pickling, the probability of fungal growth is high especially in cases in which pelt pieces remain in the drum (Mericli Yapici and Karaboz, 1997).

Generally, pre-tanning process stages were separately studied by different researchers in former studies and these mostly contained bacterial findings. However, in some recent studies, it has been seen that there are important findings about fungi both in hides (Bitlisli et al., 2004) and liming liquor (Mericli Yapici et al., 2008). Thus, it is important to determine in what density bacteria and fungi that can adversely affect the quality of leather in all the pre-tanning process stages. This study was aimed to determine the numbers of total aerobic mesophilic, proteolytic, lipolytic and aerobic spore-forming bacteria along with total numbers of aerobic fungi (yeast and mould), the numbers of proteolytic and lipolytic fungi in all pre-tanning process floats from main soaking process to pickling. It is expected that data obtained from the study will contribute to the solution of microbiological problems of the leather industry and to other studies.

MATERIALS AND METHODS

Skins used in the research were obtained freshly from the slaughterhouse and conserved properly with 50% NaCl for protection till production. They were then processed on the basis of the principles of clothing leather production (Thorstensen, 1993; Sharphouse, 1989) in the Leather Practice Unit of Biga Vocational College, Canakkale Onsekiz Mart University. A commercial bactericide which is composed of quarternized compounds was added at 0.4% into the main soaking float and the skins were processed to the end of pickling (Table 1). A parallel trial was done without the bactericide.

Both samples with bactericide (experimental) and without bactericide (control) were taken from each pre-tanning float at the same time and microbiological examinations were performed all together.

Media and solutions

Halophile medium and modified malt extract agar containing different concentration of NaCl (0, 5, 10, 15 and 25%) were used to count bacteria and fungi, respectively. The halophile medium contained 5.0 g each of KCl, $MgCl_2.6H_2O$, NH_4Cl and $MgSO_4.7H_2O$; 5.0 ml trace element solution; 10.0 ml of 1% ferric-citrate solution; 30.0 ml of yeast extract solution (150 g/l); 30.0 ml of peptone solution (150 g/l); 10.0 g agar; and 925.0 ml distilled water. Trace element solution (per litre of distilled water) comprised of 1.0 mg $CuSO_4.5H_2O$, 220.0 mg $ZnSO_4.7H_2O$, 10.0 mg $CoCl_2.6H_2O$, 180.0 mg $MnCl_2.4H_2O$ and 6.3 mg $Na_2MoO_4.H_2O$ (Anonymous, 2006). Modified malt extract agar consisted of 30.0 g malt extract, 5.0 g peptone, 15.0 g agar, 10.0 g glucose, 1.0 g yeast extract, streptomycin (100 µg/ml) and 1000.0 ml distilled water (Bitlisli et al., 2004).

Halophile medium and modified malt extract agar containing 10% skim milk or 2% Tween 80 were used to detect proteolytic and lipolytic activity, respectively. While the media containing 0, 10, 15 and 25% NaCl were used to count micro-organisms in the main soaking float and the media containing 0, 5 and 10% NaCl for all other pre-tanning processes floats.

pH values of the media with different NaCl contents were adjusted to be the same as those of process stages (main soaking pH: 7.0, unhairing-liming pH: 12, deliming-bating pH: 8.5, degreasing pH: 5.5, pickling pH: 3) sterilized with 1 N HCl or 1 N NaOH (Temiz, 1996).

All counting were done using the spread plate technique which is one of the counting methods (Prescott et al., 2002). For this, liquid samples were put into sterile boxes at the end of every pre-tanning process. The samples were serially diluted in sterile saline solutions, and 0.1 ml of the diluents were transferred to sterile culture plates and spread with a sterile Drigalski spatule. In order to detect aerobic spore-forming bacteria, after heating the diluted samples at 80°C for 10 min, they were plated on the media (Pichhardt, 2004). For bacterial counts, inoculated plates without NaCl were incubated at 37°C for 48 h. For the same purpose, while the plates containing 5, 10 and 15% NaCl were incubated at 41°C for 72 h, the plates containing 25% NaCl were incubated at 41°C for 3 weeks. On the other side, inoculated plates for fungal growth were incubated at 27°C for 3 weeks (Birbir et al., 1996). At the end of the incubation, while only the colonies forming zone were counted on the media with skim milk or Tween 80, all of the colonies were counted in all of the other media (Pichhardt, 2004; Bitlisli et al., 2004).

RESULTS AND DISCUSSION

Results of bacterial counting obtained from experimental (E) samples in the main soaking float are given in Table 2 together with the ones obtained from control (C) samples. It is clearly seen in Table 2 that bacteria numbers of E samples are lower than those of C samples in all of the pre-tanning processes. This case showed that the bactericide used in the main soaking process was effective in controlling bacterial activities. This effect was observed more apparently especially in the main soaking float (Table 2).

Findings obtained from E samples displayed that bacterial growth was more intense in the main soaking and deliming-bating floats. Contrary to the other media containing NaCl, no bacterial growth took place on the media containing 25% NaCl for the main soaking float.

The numbers of proteolytic bacteria in the main soaking

Table 1. Treatment methodology of wet-salted sheep skins (% based on the skin weight).

Process	%	Chemical	Temp (°C)	Duration (min)	pH
Pre-soaking	500	Water	20	240	7.0
Drain					
Main soaking*	500	Water	20		
	0.5	Non-ionic emulsifier			
	0.4	Bactericide**		30	7.0
Run on automatic (stop 55 min/run 5 min for 18 h)					
Painting: 15 °Be' Na_2S- 25 °Be' Ca $(OH)_2$ (solution is applied onto the flesh side of the skin) Unhairing					
Liming*	400	Water	20		
	2	Na_2S			
	4	$Ca(OH)_2$		30	12
Run on automatic (stop 55min/run 5 min for 24 h)					
Fleshing, trimming and weighing (referred to as pelt weight) % based on pelt weight					
Washing	300	Water	35	15	
Drain					
Deliming bating*	300	Water	35		
	1.5	$(NH_4)_2SO_4$		30	8.5
	1	Proteolytic enzyme		60	
Washing	200	Water	20	10	
Degreasing*	100	Water	35		5.5
	5	Degreasing agent		90	
Washing	100	Water	35		
	2	NaCl		30	
Drain					
Washing	100	Water	35		
	2	NaCl		30	
Drain					
Washing	100	Water	35	30	
Pickling*	150	Water	20		
	5	NaCl		10	
	0.5	HCOOH (1:10)		30	
	0.8	H_2SO_4 (1:10)		90	3.0

*Processes from which float samples were taken for microbiological counting.
**Only used in experimental group.

float, the control of which is crucial for the leather industry, were found to be 4.1 × 10^4, 5.4 × 10^4 and 3.4 × 10^4 cfu ml^{-1} respectively on the media containing 0, 10 and 15% NaCl and 2.9 × 10^3, 3.5 × 10^3 and 3.0 × 10^3 cfu ml^{-1}, respectively, on the media containing 0, 5 and 10% NaCl for deliming-bating. It was determined that only proteolytic bacteria could grow in degreasing float and their numbers were found to be 2.0 × 10^4, 2.8 × 10^4 and 3.0 × 10^4 cfu ml^{-1}, respectively, at 0, 5 and 10% NaCl.

In the study, lipolytic bacteria were only detected in the main soaking and deliming-bating floats and their numbers were found to be similar to those of proteolytic bacteria. In the same way, the numbers of lipolytic bacteria in the main soaking float were detected to be 2.9 × 10^4, 5.3 × 10^4 and 2.4 × 10^4 cfu ml^{-1}, respectively, at 0, 10 and 15% NaCl and in deliming-bating to be 2.8 × 10^4, 1.5 × 10^4 and 2.2 × 10^2 cfu ml^{-1}, respectively, in the same NaCl content. For the deliming-bating floats, the numbers of lipolytic bacteria at 0 and 5% NaCl content were determined to be higher than those of proteolytic bacteria. In the liming, degreasing and pickling floats, no lipolytic bacteria growth was observed. The fact that lipolytic bacteria were detected in some pre-tanning stages at a considerable level was another significant finding of the study. On the other hand, no bacterial growth was detected in the samples taken from the pickling float.

In the study, it was determined that the growth of total aerobic mesophilic and spore-forming bacteria was remarkable in the main soaking and deliming-bating

Table 2. Numbers of bacteria in the pre-tanning process floats.

Processing stages	NaCl (%)	Numbers of bacteria (E) ($cfu\ ml^{-1}$)				Numbers of bacteria (C) ($cfu\ ml^{-1}$)			
		Total aerobic mesophilic bacteria	Aerobic spore forming bacteria	Proteolytic bacteria	Lipolytic bacteria	Total aerobic mesophilic bacteria	Aerobic spore forming bacteria	Proteolytic bacteria	Lipolytic bacteria
Main soaking	0	6.9×10^4	2.9×10^3	4.1×10^4	2.9×10^4	4.0×10^7	8.5×10^4	2.7×10^8	6.7×10^7
	10	7.2×10^4	2.0×10^4	5.4×10^4	5.3×10^4	1.5×10^5	2.5×10^4	3.5×10^7	1.4×10^7
	15	5.8×10^4	-	3.4×10^4	2.4×10^4	4.5×10^5	1.7×10^1	3.0×10^7	1.2×10^5
	25	-	-	-	-	-	-	-	-
Liming	0	2.0×10^1	1.0×10^1	2.0×10^1	-	1.1×10^2	3.0×10^2	3.0×10^2	2.6×10^1
	5	-	-	1.0×10^1	-	1.0×10^1	2.1×10^1	1.2×10^2	1.1×10^1
	10	-	-	-	-	-	-	-	-
Deliming-bating	0	2.3×10^3	1.2×10^2	2.9×10^3	2.8×10^4	7.8×10^4	1.1×10^4	6.0×10^4	3.0×10^4
	5	3.0×10^3	5.0×10^1	3.5×10^3	1.5×10^4	1.6×10^5	2.8×10^4	5.6×10^4	2.5×10^4
	10	1.6×10^3	2.2×10^2	3.0×10^3	2.2×10^2	2.0×10^4	3.4×10^4	4.5×10^3	2.5×10^2
Degreasing	0	-	-	2.0×10^4	-	-	-	3.0×10^4	-
	5	-	-	2.8×10^4	-	-	-	3.5×10^4	-
	10	-	-	3.0×10^4	-	-	-	4.8×10^4	-
Pickling	0	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-

- : No growth.

stages. In addition, the highest numerical values in terms of NaCl content were obtained on the media containing 10% NaCl for the main soaking float (Table 2) which proved that halophilic bacteria that can grow at 10% NaCl were intensively present in the main soaking float.

Mericli Yapici et al. (2004) added 0.5% of a formulation composed of quarternized compounds into the main soaking float based on skin weight and determined the number of total aerobic mesophilic, aerobic spore-forming and proteolytic bacteria and of the bacteria on the media containing 10% NaCl to be 2.6×10^5, 1.1×10^3, 2.3×10^5 and 7.6×10^3 $cfu\ ml^{-1}$, respectively.

In the present study, the same bactericide was used at the ratio of 0.4% on the basis of skin weight and numerical data obtained from bacterial groups and were not found to be much different from the ones the researchers obtained in the former study. Thus, it can be stated that the results obtained from the main soaking float in the study were parallel with the findings of the former study.

Even though an effective bactericide was used, when the bacterial loads of all the pre-tanning process floats examined in the study were taken into consideration, bacterial growth was observed at various levels in different stages except for the pickling stage. The most remarkable point among these is that proteolytic bacteria survive from the first process to the end of the degreasing process. According to the experimental results, lipolytic bacteria were observed in considerable numbers only in the main soaking and deliming-bating floats and displayed no growth in the other three stages. Nevertheless, the growth of the lipolytic bacteria in the aforementioned stages should not be ignored.

Especially, the control of the proteolytic bacteria

Table 3. Numbers of fungi in the pre-tanning process floats.

Processing stages	NaCl (%)	Numbers of fungi (E) (cfu ml^{-1})			Numbers of fungi (C) (cfu ml^{-1})		
		Total aerobic fungi	Proteolytic fungi	Lipolytic fungi	Total aerobic fungi	Proteolytic fungi	Lipolytic Fungi
Main soaking	0	2.6×10^3	9.0×10^3	3.5×10^2	5.6×10^6	7.5×10^6	9.3×10^6
	10	1.2×10^3	4.2×10^3	9.0×10^2	1.0×10^4	1.0×10^4	4.0×10^4
	15	2.0×10^2	3.0×10^2	2.5×10^2	3.0×10^3	1.2×10^3	1.3×10^3
	25	-	-	-	-	-	-
Liming	0	7.5×10^1	$1,0 \times 10^2$	$1,0 \times 10^2$	$3,0 \times 10^2$	$3,0 \times 10^3$	$2,0 \times 10^2$
	5	$1,5 \times 10^2$	$1,7 \times 10^2$	$5,5 \times 10^1$	$1,0 \times 10^3$	$2,0 \times 10^3$	$6,0 \times 10^1$
	10	$1,0 \times 10^1$	$6,0 \times 10^1$	$2,5 \times 10^1$	$1,1 \times 10^2$	$1,0 \times 10^2$	$4,0 \times 10^1$
Deliming-bating	0	$3,0 \times 10^2$	$2,0 \times 10^1$	$9,0 \times 10^1$	$1,0 \times 10^3$	$1,5 \times 10^3$	$2,6 \times 10^2$
	5	$2,9 \times 10^3$	$6,0 \times 10^1$	$7,0 \times 10^1$	$3,0 \times 10^3$	$3,1 \times 10^2$	$1,5 \times 10^2$
	10	$1,7 \times 10^2$	$7,0 \times 10^1$	$5,0 \times 10^1$	$2,2 \times 10^2$	$2,4 \times 10^2$	$1,1 \times 10^2$
Degreasing	0	$1,4 \times 10^2$	$3,0 \times 10^1$	$6,0 \times 10^1$	1.0×10^3	1.0×10^2	1.4×10^2
	5	4.0×10^1	7.0×10^1	8.0×10^1	1.5×10^2	3.0×10^2	3.2×10^2
	10	1.0×10^1	1.0×10^1	4.0×10^1	1.2×10^2	2.0×10^2	2.6×10^2
Pickling	0	-	5.0×10^2	-	-	1.4×10^4	-
	5	-	1.0×10^2	-	-	1.8×10^3	-
	10	-	-	-	-	-	-

- : No growth.

in the pre-tanning processes is of great significance in the production of leathers of high quality because it is known that most of the halophilic bacteria isolated from hide are proteolytic and damage the leather (Birbir et al, 1996). Additionally, in a study it was stated that micro-organisms decrease the suede quality due to their lipolytic and proteolytic activities on the flesh side of the sheep skin and this leads to economic losses (Bitlisli et al., 2004). For that reason, during pre-tanning processes, primarily in the soaking process, the growth of proteolytic and lipolytic bacteria should be controlled and the probability that they might damage the skin should always be considered.

Fungal counting results obtained from E and C samples in the study are presented in Table 3. Fungi were detected in considerable numbers in C samples especially in the main soaking float. Further, fungi were counted in almost every processing step and on the media with different NaCl contents up to the end of the pickling stage. It was observed that the bactericide composed of quarternized compounds that was used in the main soaking float decreased not only the bacterial numbers but also the fungal numbers in all the pre-tanning processes. In the same way, it was emphasized by Russell and McDonnel (1999) that this compound is a membrane active agent aiming at the cytoplasmic membrane of bacteria and on the plasma membrane of yeasts. This result is significant in terms of the fact that the used commercial bactericide also controls fungal growth along with bacterial growth in pre-tanning processes.

According to the findings obtained from E samples, proteolytic fungi displayed growth at all NaCl concentrations and in all of the pre-tanning floats except for on the media containing 25% NaCl for the main soaking and on the media containing 10% NaCl for the pickling process. However, fungal numbers in the main soaking floats were found to be lower than bacterial numbers (Tables 2 and 3). Similar to proteolytic bacteria, the most intense growth for proteolytic fungi was observed in the main soaking float and the numbers of proteolytic fungi detected at 0, 10 and 15% NaCl were determined to be 9.0×10^3, 4.2×10^3 and 3.0×10^2 cfu ml^{-1}, respectively. The numbers of proteolytic fungi detected at 0, 5 and 10% NaCl for the deliming-bating float were found to be 2.0×10^1, 6.0×10^1 and 7.0×10^1 cfu ml^{-1}, respectively.

The numbers of proteolytic fungi in the degreasing float were generally similar to those detected in the deliming-bating float. On the media containing 0 and 5% NaCl, only proteolytic fungi displayed growth in the pickling stage in which no bacterial growth was observed and their numbers were found to be a bit higher than the ones obtained from deliming-bating and degreasing floats. Since the pickling process possessing acidic pH values is favourable for fungal growth (Didato et al., 1999), the rise in fungal numbers was considered normal. Although no lipolytic bacteria were observed in the liming and degreasing floats, lipolytic fungal growth was detected on the media for all the concentrations of NaCl in these process floats (Tables 2 and 3). While the numbers of lipolytic fungi on the media containing 0, 5 and 10% NaCl in liming float were found out to be 1.0×10^2, 5.5×10^1

and 2.5 × 10^1 cfu ml^{-1}, respectively, these numbers were 6.0 × 10^1, 8.0 × 10^1 and 4.0 × 10^1 cfu ml^{-1}, respectively, in the same concentrations of NaCl for the degreasing float.

The detection of fungal growth in all of the process floats in the pre-tanning processes laid open that these floats are favourable not only for bacterial growth but also for fungal growth and the fact that they were determined to be of proteolytic and lipolytic characteristics is a remarkable finding. The reason is that pre-tanning processes which are gravely important in the manufacture of leathers of good quality could affect each other as well as tanning and post-tanning processes (Thorstensen, 1993).

Conclusion

In this study, a commercial bactericide composed of quarternized compounds was used in the main soaking process and changes in bacterial and fungal numbers in the pre-tanning processing floats were investigated on the media containing different concentrations of NaCl. It was determined that population density changed depending on the characteristics of the processing floats. It was found out that proteolytic and lipolytic bacteria displaying growth in different NaCl concentrations were more intense especially in the main soaking and deliming-bating processes. Although the commercial compound used as bactericide controlled fungi growth to some extent, they were detected in all the NaCl concentrations (0, 5, 10, and 15%) from the main soaking (except for the 25% NaCl) up to the end of degreasing processes. Only proteolytic fungi grew at 0 and 5% NaCl in the pickling float. In C samples, it was remarkable that fungi were detected in high numbers in the main soaking float primarily, liming, deliming-bating and pickling floats in different NaCl concentrations.

Skin and its processing floats are fairly favourable media for microbial growth. Different micro-organisms could grow in each process stage depending on different characteristics (temperature, pH, NaCl content, presence of various chemical agents, e.t.c.) of the process floats in leather production. Moreover, micro-organisms can manage to survive by being tolerant to some changing factors. When the conditions become appropriate for them again, they increase their numbers in short time and could damage the skin.

Consequently, control of microbial activities in pre-tanning processes is crucial to manufacturing leathers of high quality. Especially, the use of an effective fungicide for the security of the post-tanning processes is gravely important besides the use of an effective bactericide for the proteolytic and lipolytic bacteria at the beginning of the main soaking.

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