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Full Length Research Paper

The effect of reuse of unhairing-liming residual floats through regeneration on the microorganism number

Binnur Meriçli Yapici[1]*, Ali Nail Yapici[2] and Ece Keçici[1]

[1]Çanakkale Onsekiz Mart University, Faculty of Science and Arts, Biology Department, Basic and Industrial Microbiology Section, 17100 Çanakkale, Turkey.
[2]Çanakkale Onsekiz Mart University, Biga Vocational College, 17200 Biga, Çanakkale, Turkey

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Even though microorganism load was mostly ignored in the unhairing-liming process due to extremely high pH values, it is a question to answer when unhairing-liming residual floats are reused through regeneration. The objective of this paper was to determine the number of total aerobic mesophilic bacteria and of proteolytic, lypolytic, aerobic spore-forming bacteria, and of total aerobic fungi (mould and yeast), proteolytic and lypolytic fungi in each unhairing-liming residual float reused ten times through regeneration. Enumeration of bacteria and fungi was done in three different concentrations of NaCl [0, 5, and 10% (w/v)]. The experiments were carried out with and without antimicrobial agents (experimental and control). In this study, generally, the values obtained from experimental samples were detected to be lower than control samples. For experimental samples, when all the NaCl concentrations were taken into account, the minimum and maximum numbers of total aerobic mesophilic bacteria and of proteolytic, lypolytic, aerobic spore-forming bacteria were found as follows: $2.0x10^1$ – $3.9x10^2$ cfu.mL^{-1}, $1.0x10^1$ – $4.1x10^2$ cfu.mL^{-1}, $2.0x10^1$ – $5.4x10^2$ cfu.mL^{-1} and $1.0x10^1$ – $2.0x10^2$ cfu.mL^{-1}, respectively. In these samples, it was found out that the minimum and maximum numbers of total aerobic fungi and of proteolytic and lypolytic fungi were $3.0x10^1$ – $2.8x10^3$ cfu.mL^{-1}, $1.0x10^1$ – $1.2x10^3$ cfu.mL^{-1}, and $5.0x10^1$ – $3.5x10^3$ cfu.mL^{-1}, respectively. In the study, there is a significant finding that the numbers of fungi were higher than those of bacteria. In addition, when bactericide and fungicide were added into the soaking processes and when unhairing-liming residual floats were reused ten times via regeneration, it was revealed that the numbers of bacteria and fungi can be controlled.

Key words: Unhairing-liming process, reusing, leather industry, environment, bacterial and fungal numbers.

INTRODUCTION

Leather production contains three main wet stages known as beamhouse (pre-tanning), tanning and post-tanning processes (Aravindhan et al., 2007). Salt, dirt, hair, non-structured protein, proteoglycan and fat are removed from hides in the beamhouse processes. In the tanning process, collagen which is a putrescible biological material is converted into a stable material with various tanning agents (Covington, 1997). In the post-tanning processes, some leather properties and aesthetic value are bestowed to the wet blue (Thanikaivelan et al., 2004). Beamhouse operations start with the soaking process (SP) and usually cover unhairing-liming process (ULP) and deliming-bating process (Thorstensen, 1993). Unhairing-liming and deliming-bating processes may be carried out separately, but they are increasingly common done simultaneously. Traditional beamhouse processes employ both enormous volume of water and hazardous chemicals (Saravanabhavan et al., 2007). Although sulphide is toxic, it is still the prime depilant in the ULP. Due to its high pollution potential, ULP is the most critical of all the beamhouse processes. There are a lot of possibilities to reduce the environmental impacts of the process, and many investigations have been carried out for this pur-

*Corresponding author. E-mail: yapici69@gmail.com. Tel: +(90) 286 2180018-1866. Fax:+(90) 286 2180533.

Abbreviations: ULP, Unhairing-liming process; and **SP**, soaking process.

pose. One of them is recycling of the residual lime liquor (Raghavo Rao et al., 2003). In a study on reducing the environmental impacts of ULP in leather production, ULP liquor has been used four times by means of regeneration. The researchers have determined that the method they employed have no negative effects on leather, is more economical and causes less environmental pollution (Nazer et al., 2006). In addition, Ludvik (2000) stated that unhairing spent liquor could be used for main soaking and liming spent liquor for the next liming process. When liming liquor is used ten times and unhairing liquor twenty times, it has been determined that S^{2-} decreased by 70% in pollution load and $Ca(OH)_2$ by 93%.

Hides and skins are rich in microorganism load. In a study, the number of the bacteria in hide samples was determined to be 10^8 cfu.g^{-1} (Oppong et al., 2006). It is certain that microorganisms within raw materials happen to be involved in the processes together and this may have undesired outcomes. Although the change in the pollutant parameters in waste water along with savings of water and chemicals has been studied in detail in many investigations on ULP, how the microbial load changes has been ignored. As well known, microbial growth is a significant problem in the processing of leather. Over 80 kinds of bacteria have been identified on animal skins, and the most dangerous ones are those that deteriorate proteins (Bienkiewicz, 1983). Although ULP is overlooked in terms of microorganism load due to extreme pH values (11 - 13), some bacteria that can cause loose grain or nubuck effect damage may grow even under these circumstances (Reeder, 1999). The primary aim of the study is to determine bacterial and fungal numbers in each ULP residual liquor when ULP residual liquor is used ten times consecutively through regeneration.

MATERIALS AND METHODS

Raw hide

In this study, conserved whole hides were chosen as material. Ten pieces of wet-salted hides with a weight range of 15 - 20 kg were obtained from local suppliers, Çanakkale, Turkey.

Chemicals used in the leather processing

Commercial grade of chemicals and tap water were used in both SP and ULP (10 cycles in total). Busan 30 WB® and Busan 85® (Buckman Laboratories Inc.) were added together into main soak liquor as the antimicrobial agents to control bacterial and fungal growth, respectively (termed experimental: E). No antibacterial agents were used for the control samples (termed control: C).

Media

Halophile medium was used to determine the number of total aerobic mesophilic bacteria and of proteolytic, lypolytic, aerobic spore-forming bacteria in the ULP residual liquor. It contained 5.0 g KCl, 5.0 g $MgCl_2.6H_2O$, 5.0 g NH_4Cl, 5.0 g $MgSO_4.7H_2O$, 5.0 mL of 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) consisted 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 $Na_2MoO_4.H_2O$ (Anon, 2006).

Modified malt extract agar [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] was used to determine the number of total aerobic fungi in ULP residual liquor (Kis-papo et al., 2001). Both of the media were modified with different concentrations of NaCl. Thus, media containing NaCl of 0, 5 and 10% were obtained. Since the pH value of ULP float ranged from 11.0 to 12.0, the pH value of the media was adjusted to 11.0 - 12.0 with sterilized 1N NaOH. Halophile medium and modified malt extract agar containing skim milk at 10 % or Tween-80 (Riedel-deHaën 63161) at 2% were also used to determine proteolytic and lypolytic microorganisms, respectively (Sanchez et al., 2003).

Dilution of solution

Saline solutions containing NaCl at 0.8% (w/v), 5% (w/v) and 10% (w/v) were used for dilutions. The pH values of solutions were adjusted to 11 - 12 with 1 N NaOH taking the pH value of ULP into consideration.

Processing hides

Ten pieces of wet-salted hides were cut off to obtain right and left side of hides. They were then weighted and numbered. The left ten sides were used for E and the right sides serving as C for comparison. The details of the process from SP to ULP for E are given in Table 1. Same formulation was used for C except for antimicrobial agents.

At the end of ULP, residual liquor samples were separately taken from both E and C for microbiological and chemical analyses. Percentages of sodium sulphide, lime and NaCl in the liquor were determined. Then, it was regenerated ten times by adding water, sodium sulphide and lime into the residual liquor during the experimental procedure for the next batch (Raghava Rao et al., 2003).

Samples

Liquid samples taking from each of the ULP residual liquors were used for microbiological and chemical analysis. The samples were immediately transported in an ice box (2 – 6^0C) from Leather Practice Unit of Biga Vocational College to Basic and Industrial Microbiology Laboratory for microbiological analysis and to Analytical Chemistry Laboratory for chemical analysis. Microbiological and chemical analyses were then carried out immediately.

Microbiological analysis of samples

Spread plate technique was used to determine the numbers of bacteria and fungi. 10 mL of each liquid sample was homogenized with 90 mL of sterile saline solution. Serial dilutions up to 10^{-5} were prepared and then 0.1 mL of the diluted samples was spread onto all of the media. For bacterial growth, while inoculated plates including NaCl of 5 and 10% were incubated at 41^0C for 72 h, the plates without NaCl were incubated at 37^0C for 48 h. For fungal growth, the plates were incubated at 27^0C for three weeks. After the incubation periods, the colonies were counted and recorded. All experiments were done in duplicate.

Table 1. Treatment methodology of wet-salted hide sides (% based on the raw hide weight).

Process	%	Chemical	Temperature (°C)	Duration (min)	pH
Pre-soaking	200	Water	22		
	0.2	Na_2CO_3		60	9.0 - 9.5
Run on automatic (stop 57 min/run 3 min for 5 h) **Drain**					
Main soaking	200	Water	22		
	0.5	non-ionic emulsifier			
	0.2	Busan 30WB®			
	0.2	Busan 85®		30	
Run on automatic (stop 95 min / run 15 min for 20 h) **Drain**					
Unhairing- Liming	200	Water	22		
	2	Sodium sulphide		30	
	4	Lime		30	11 - 12
Rest				30	
Run				30	
Rest				30	
Run				30	
Run on automatic (stop 57 min/ run 3 min for 21 h)					

Table 2. Minumum and maximum numbers of the bacteria in residual liquor samples obtained from E and C (10 cycles in total).

Counted microorganisms ($cfu.mL^{-1}$)	NaCl (%)	E		C	
		Minimum numbers	Maximum numbers	Minimum numbers	Maximum numbers
Total aerobic mesophilic bacteria	0	-	$3.9x10^2$	$1.6 x10^2$	$1.7 x10^3$
	5	$2.0 x10^1$	$2.2 x10^2$	$2.0 x10^1$	$8.0 x10^2$
	10	-	-	-	$4.5 x10^1$
Proteolytic bacteria	0	$4.0 x10^1$	$4.0 x10^2$	$3.0 x10^2$	$1.2 x10^3$
	5	$1.0 x10^1$	$4.1 x10^2$	$1.2 x10^2$	$1.4 x10^3$
	10	-	$2.0 x10^1$	-	$3.5 x10^1$
Lypolytic bacteria	0	-	$5.4 x10^2$	$2.0 x10^2$	$1.4 x10^3$
	5	$4.5 x10^1$	$4.0 x10^2$	$1.8 x10^2$	$1.1 x10^3$
	10	-	$2.0 x10^1$	-	$6.0 x10^1$
Aerobic spore-forming bacteria	0	-	$2.0 x10^2$	$1.0 x10^2$	$6.3 x10^2$
	5	-	$1.0 x10^2$	$2.0 x10^1$	$2.5 x10^2$
	10	-	$1.0 x10^1$	-	$4.0 x10^1$

-: No growth

Chemical analysis of samples

Chemical analyses of sodium sulphide and lime were done for the regeneration of ULP residual liquors. While preparing the media, NaCl content of ULP residual liquor was taken into consideration. Thus, NaCl in the residual liquor was analyzed as well (Hakimoğlu, 1989).

RESULTS AND DISCUSSION

Minimum and maximum numbers of the bacteria detected in the ULP residual liquor samples used ten times through regeneration are shown in Table 2. It is clear from Table 2 that the numbers of the bacteria detected in the C samples are higher in all the NaCl concentrations than those obtained in E samples. Because a bactericide is generally used in the SP of leather production, the results have been primarily evaluated in the light of data obtained from samples into which antibacterial substances have been added. While total aerobic mesophilic bacteria, lypolytic bacteria and aerobic spore-forming

Table 3. Minumum and maximum numbers of the fungi in residual liquor samples obtained from E and C (10 cycles in total).

Counted fungi ($cfu.mL^{-1}$)	NaCl (%)	E		C	
		Minimum numbers	Maximum numbers	Minimum numbers	Maximum numbers
Total aerobic mesophilic fungi	0	7.3×10^2	2.8×10^3	1.4×10^3	8.8×10^3
	5	2.4×10^2	7.0×10^2	3.5×10^2	2.2×10^3
	10	-	3.0×10^1	2.0×10^1	4.0×10^2
Proteolytic fungi	0	6.7×10^2	1.2×10^3	5.8×10^2	3.7×10^3
	5	2.4×10^2	7.8×10^2	4.4×10^2	7.1×10^3
	10	1.0×10^1	8.0×10^1	2.0×10^1	3.5×10^2
Lypolytic fungi	0	8.2×10^2	3.5×10^3	5.0×10^2	3.7×10^3
	5	2.5×10^2	8.4×10^2	4.6×10^2	4.2×10^3
	10	-	5.0×10^1	2.0×10^1	3.5×10^2

-: No growth

bacteria cannot be isolated in some media belonging to the samples containing NaCl at 0%, maximum numbers in these samples have been determined to be 3.9×10^2, 5.4×10^2 and 2.0×10^2 $cfu.mL^{-1}$, respectively. Minimum and maximum numbers of the proteolytic bacteria in the same percentage of NaCl were found to be $4.0 \times 10^1 - 4.0 \times 10^2$ $cfu.mL^{-1}$. However, total aerobic mesophilic, proteolytic and lypolytic bacteria were isolated in all of the residual liquor samples examined in the media containing just NaCl at 5% and minimum and maximum numbers determined for these bacteria were found to be $2.0 \times 10^1 - 2.2 \times 10^2$ $cfu.mL^{-1}$, $1.0 \times 10^1 - 4.1 \times 10^2$ $cfu.mL^{-1}$ and $4.5 \times 10^1 - 4.0 \times 10^2$ $cfu.mL^{-1}$, respectively. The numbers of aerobic spore-forming bacteria in the same NaCl concentration were found to be lower. As for the media containing NaCl at 10%, no bacterial growth took place in most of the samples. In this study, some amount of NaCl ranging from 5.10 to 5.59% was determined in the ULP residual liquor samples (Figure 1). In such a case, it was assumed that, to some particular degree, leather release-ed the NaCl used for conservation of the raw skin into ULP float.

In our study, the fact that total aerobic mesophilic, proteolytic and lypolytic bacteria were isolated in all of the residual liquor samples examined in the media containing just NaCl at 5% revealed that the bacteria has adapted to this NaCl concentration and they kept surviving in the ULP residual liquor in particular numbers.

Orlita (2004) stated that hides and skins were not exposed to degradation resulting from microorganisms in the liming and deliming processes following the SP. This is because the pH value is considerably high and thus no remarkable microbiological activity is expected (Dahl, 1956). In another study carried out by Bilgi (2007) to determine the number of bacteria and fungi in pre-tanning processes when bactericide was added into the SP, no lypolytic bacteria were observed and the numbers of proteolytic bacteria were determined to be $1.0 \times 10^1 - 2.0 \times 10^1$ $cfu.mL^{-1}$. When no bactericide was used, lypolytic bacteria ranging between the values $1.1 \times 10^1 - 2.6 \times 10^1$ $cfu.mL^{-1}$ were found and the numbers of the proteolytic bacteria in these samples increased to 1.2×10^2 -3.0×10^2 $cfu.mL^{-1}$.

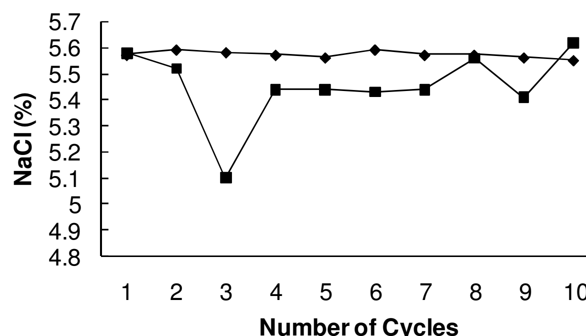

Figure 1. NaCl contents of ULP Liquors (♦: E and ■: C).

The aforesaid data were obtained as a result of the practice of that particular process once. The results of the bacterial enumeration obtained from the E samples in our study were found to be higher than the ones obtained from ULP residual liquor used only once by the resear-cher. This shows that the use of ULP liquor for 10 times through regeneration partially increased the bacterial load.

Minimum and maximum numbers of fungi observed in the ULP liquors are shown in Table 3. The numbers of fungi in the E samples were found to be slightly higher than those of bacteria (Tables 2 and 3). Almost all of the fungi numbers obtained from C residual liquor were figured out to be higher than those of E residual liquor (Table 3). In our study, according to the counting results obtained for E residual liquor, the highest fungi numbers were observed in the media containing 0% NaCl. Minimum and maximum numbers obtained in this concentration

from overall total aerobic mesophilic, proteolytic and lypolytic fungi were found to be varying between the values of $7.3x10^2$ – $2.8x10^3$ cfu.mL^{-1}, $6.7x10^2$ – $1.2x10^3$ cfu.mL^{-1} and $8.2x10^2$ – $3.5x10^3$ cfu.mL^{-1}, respectively. In a NaCl concentration of 5%, the values were $2.4x10^2$ – $7.0x10^2$ cfu.mL^{-1}, $2.4x10^2$ – $7.8x10^2$ cfu.mL^{-1} and $2.5x10^2$ – $8.4x10^2$ cfu.mL^{-1}, respectively. The numbers of fungi in the media containing NaCl of 10% were determined lower than the other NaCl concentrations and in some samples, fungal growth was not observed. Bilgi (2007) carried out an investigation just using bactericide, not only bacteria but also fungi were isolated in the ULP residual liquor. The researcher detected the numbers of proteolytic fungi as $6.0x10^1$ – $1.7x10^2$ cfu.mL^{-1} and the numbers of the lypolytic fungi as $2.5x10^1$ – $1.0x10^2$ cfu.mL^{-1}. Although bactericide was used in each SP as antimicrobial agent along with fungicide, the fact that the overall proteolytic and lypolytic fungi displayed growth to some extent in the residual liquor samples was one of the significant findings of our study. Fungal growth is a common problem in the leather industry and might occur in various stages of leather production (Annamalai et al., 1997). Halophilic fungi may appear in hides and skins stored in unfavourable conditions for a long time. It was expressed by some researchers that their proteolytic and lypolytic activities are eminent (Bitlisli et al., 2004; Bailey and Birbir, 1993). If bacterial and fungal growth cannot be efficiently controlled in the pre-tanning processes, when conditions are favourable, bacteria, aerobic spore-forming bacteria and fungi could display growth in particular numbers in the process of neutralisation, retanning, dyeing and fatliquoring (Durmuş, 2007). Even though the focus was primarily upon bacteria in pre-tanning processes and upon fungi in tanning and post-tanning processes (Didato et al., 1999), it is of great importance that both bacteria and fungi should be checked from the pre-tanning processes onwards due to probable proteolytic and lypolytic activities.

From this point of view, the study revealed that the use of not only bactericide but also fungicide in the SP could be beneficial in the case of the use of ULP residual liquor for many times

Conclusions

Since tanneries are rich in microbial load, microorganisms are considerably significant for leather quality in all of the processing stages. In some previous studies, both waste pollution load and volume of process water decreased with the use of ULP residual liquors for many times, and leather quality did not get affected negatively by this. In the present study, when ULP residual liquor was used ten times by adding bactericide and fungicide into the SP, it was revealed that the numbers of bacteria and fungi remained below a particular level. Consequently, it was figured out that the use of ULP residual float for many times, which is one of the methods suggested to reduce environmental impacts of ULP, will not pose danger in terms of microbial load.

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