pAfrican Journal of Biotechnology Vol. 8 (24), pp. 6938-6944, 15 December, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Comparison of certain microbial counting methods which are currently commonly used in the soaking process

Eser Eke Bayramoğlu[1], Gonca Telli Yamamoto[2]* and Düden Uluç[3]

[1]Department of Leather Engineering, Faculty of Engineering Ege University 35100 Bornova-Izmir, Turkey
[2]Department of Information Systems, School of Applied Sciences, Okan University 34959 Tuzla/Istanbul, Turkey.
[3]Department of Leather Engineering, Faculty of Engineering Ege University 35100 Bornova-Izmir, Turkey.

Accepted 23 November, 2009

This study examines the interrelation of the test methods which are commonly applied in the leather industry for hide or skin soaking. For this purpose, five different bactericides were given during the soaking processes and after the presoaking process. The processes were measured at the first 20th min, 24th and 48th h. For this purpose, two different ATP test kits, dip slide and agar pour plate method were used in order to determine the microorganism load as RLU (Relative Light Units) or CFU (Colony Forming Units)/ml. The values with all methods were identified as highly different from each other, while the statistical analyses indicated positive Pearson correlation coefficients especially between the 24th and 48th h measurements. The appearance of a positive correlation between the different methods under constant conditions implied the effectiveness of the bactericides to the customer at the factory environment. The ATP test method and dip slide methods that are commonly used in the market are correct and convenient methods offered by some companies.

Key words: Leather industry, soaking process, bacteria number, bactericide.

INTRODUCTION

Bacteria develop rapidly during hide or skin soaking-wetting back processes. During this process, the hide may be damaged especially during the prolonged process, which might in turn affect the quality and processibility of the products. Certain studies have being carried out in order to prevent the hide from being damaged and affected from such external factors. While various types of bactericide are held in soaking float, microbial counts are made in order to determine their effectiveness. Although various counting methods are used in order to count the bacteria that develop during the soaking-wetting back process, naturally, those which are most practical and fastest are preferred in commercial practices. Among them, ATP (Bioluminescence Method) and dip slide method are most commonly applied. Rangarajan et al. (2003) reported that, these methods are employed, preferred and recommended by the seller companies to achieve effective sales of chemical substances in the leather industry. On the other hand, a classical process, the pour-plate method, is prominent under laboratory conditions.

The long time and effort requirement, as well as high amount of waste generated during the pour plate method led to a search for alternative methods under laboratory conditions. Performing the microbial counts with specially-developed devices for this has begun to gain prominence especially at laboratories that perform continuous food analyses. For example, the tempo device developed by Biomerieux Company is used for this purpose. Torlak et al. (2008) reported that Tempo EC is a practical and reliable alternative to the current standard plate method for the enumeration of *E. coli* in foods. However, purchasing a device and kit are also required in this method.

During the research, samples from the same soaking float were taken and the microbiological load was identified according to four different methods and the relation

*Corresponding author. E-mail: gonca.telli@okan.edu.tr. Tel: (90)2166771630/1132. Fax: +(90)2166771639.

between them was statistically analyzed.

MATERIALS AND METHODS

Raw skin

Brand sheep (salted-dry) raw skin was selected for the investigation. From the skin, six pieces (weighing about 100 g) were carefully cut and used in soaking tests run in parallel in the following order: the 1st row contained a known bactericidal agent, 0.3 g/lt (organo-sulfur compound), Bactericide B; the 2nd row contained a known bactericidal agent 0.35 g/lt (dimethyldithiocarbamic acid salt), Bactericide M; the 3rd, 4th and 5th soaking rows contained three different organic bactericides while the 6th row was the control.

Soaking process

Throughout the experiments, all washing procedures were performed in sterile distilled water (DW). Pre-washing steps (for 1 h) of the leather samples and the consecutive soaking steps (20 min, 24 to 48 h) took place in 1 L volumes of sterile DW. The aim of the pre-washing process was to let the water diffuse through the hides, removing the substances like dirt, blood, conservation salt, dust, among others. Necessary additions (the tester bactericides) were made prior to secondary soaking steps. The samples were mechanically agitated for 5 min with 1 h intervals.

ATP (Bioluminescence Method)

The ATP method was based on the principle of light creation (bioluminescence) as a result of the reaction between the ATP of microbial cells and Lucipherine-Lucipherase system (Karaboz et al., 2002). The result was digitally shown on the screen as related light units (RLU). Since all living cells contain ATP, the bacteria, fungus and algae cells can be measured with this method. Nonetheless, when the microbial-controlled sample emits too much light, the red light turns on and signals overload. That means the registered value is higher than 500,000 RLU.

The biggest advantage of this method is the very short process time. Despite a certain incubation period in other methods, this method provided result in a very short time. It also causes less waste. However the high cost of kits could be considered as a drawback.

ATP luminometer test kits

Multi trace water test kit

At the first method's trials multi trace water test kits were used.

ATP luminometer–aqua trace (water testing) with total ATP kit (self contained devices)

Here, the same ATP- luminometer was used. However, unlike the other one, the microbial measurements during the soaking process were made by using the total ATP kit.

Cultural count through pour plate method

In this counting method, the aerobic or facultative aerobic microorganisms were assigned. The method was applied as in Karaboz et al. (2002). The Petri dishes in the figures indicated bacteria colonies that develop in the dilutions from 101 to 107 (Karaboz et al., 2002). A Petri with 30 to 300 bacteria colony is ideal for counting.

This method requires an extensive effort and a long time. In addition to that, the process requires expertise. Also, the waste formation is high because disposable Petri dishes were used.

Dip slide-dipping method

Special kits were used for counting in this method. The advantage of this method compared to pour plate method is the elimination of agar medium preparation. The user is not required to spend an extra effort in this sense. These kits included a ready-made agar medium placed in a tube. With this method, it is possible to make an aerobic bacteria or fungus count. In the pour plate method, dilutions were prepared from the soaking bath; whereas dip slide includes directly testing through dipping in the soaking bath.
The dip slide kits of Merck Company are the common kits in the leather industry. It is one of the methods especially preferred by the sellers at factory conditions.

Although its preservation for 48 h is recommended in certain literature (Rangarajan et al., 2003), they are incubated at 37ºC for 24 h in this research since the room temperature varied according to climatic and other conditions. The establishment of the standard was considered as an important point for such studies. The environmental temperature and incubation period were very important elements for colony development and their standardization in the test methods was a key point. This test method also had certain drawbacks, in that the colonies may appear as small as the head of a needle or even as smaller dots when there are too many bacteria colonies as dilution cannot be made. As the number of bacteria increases further, dip slide became completely pink and counting became impossible. As a standard, the expression "uncountable" is placed when more than 400 colonies were observed during the counting processes.

RESULTS

Tables were drawn to indicate the values read on the luminometer at the 20th min, 24th and 48th h of the soaking processes. Figure 1 showed the effectiveness graphic of all bactericides according to time. According to these results, in the control group which does not contain bactericide, the most bacteria development occurred at the 24th h. However, the multiplication from that time to the 48th h was very little, even though it was almost unchanged. Biocide B (organo-sulfur compound) gave the worst results according to this method. Although the number of bacteria compared to the control group appeared lower until the 24th h, a rapid increase has been observed in the bacteria development after the 24th h. At Biocide S, an excessive increase was not observed in the number of bacteria from the beginning to the 48th h and the bacteria development occurred on a linear course.

The difference of ATP 2 test method from ATP 1 is the utilization of a ready-made swab. Therefore, the processes can be performed more practically. Although ATP 1 and ATP 2 values were not the same, they still yielded approximate results and the results had been

Table 1. ATP luminometer (multitrace water test kit) measured values (RLU).

Biocides	Beginning 20th min	24 h	48 h
Control	57016	154639	159650
Biocide B	71954	105038	263200
Biocide M	42505	4449	2235
Biocide K	50976	6462	77072
Biocide O	22092	9539	59634
Biocide S	39530	51293	66333

Table 2. ATP luminometer-total ATP kit measured values (RLU).

Biocides	Beginning 20th min	24 h	48 h
Control	115274	Overload (500001)	Overload (500001)
Biocide B	129499	190808	Overload (500001)
Biocide M	122302	9654	5242
Biocide K	107183	8599	190908
Biocide O	34879	17579	69973
Biocide S	77715	106125	105289

Table 3. Agar Pour Plate Method Measured Values (cfu/ml).

Biocides	Beginning (20th min)	24 h	48 h
Control	1.96×10^5	2.15×10^6	2.46×10^8
Biocide B	5.2×10^4	5.5×10^5	7.4×10^7
Biocide M	3.9×10^4	2.2×10^3	1.39×10^5
Biocide K	1.04×10^5	5.8×10^5	4.5×10^7
Biocide O	1.38×10^4	7.2×10^5	1.21×10^7
Biocide S	2.02×10^5	1.4×10^5	2.35×10^7

Table 4. Dip slide measured values (cfu/ml).

Biocides	Beginning (20th min)	24 h	48 h
Control	2.9×10^1	Uncountable (4.0×10^2)	Uncountable (4.0×10^2)
Biocide B	2.0×10^1	Uncountable (4.0×10^2)	Uncountable (4.0×10^2)
Biocide M	No development	0.2×10^1	3.5×10^1
Biocide K	0.4×10^1	2.1×10^2	
Biocide O	No development	8.9×10^1	9.7×10^1
Biocide S	0.9×10^1	3.08×10^2	Uncountable (4.0×10^2)

found as significantly positive compared to each other. Accordingly, all bactericides indicated an effective increase until the 24th h, but Biocide B showed a very fast bacteria increase after the 24th h. The most effective bactericide has been identified as Biocide M, while similar values to that of ATP 1 have also been measured on the other bactericides.

All bactericides were identified as effective also in the agar pour plate method. Unlike the ATP tests, the values were identified as the colony forming units (cfu)/ml. The most effective bactericide is Biocide M. For all groups, a fast bacterial increase was observed from the 24th h to the 48th h. However, the densest increase was observed for the control group.

As the measuring is limited with this method, the effectiveness of Biocide B at the 24th and 48th h cannot be clearly understood. However, the results clearly indicated that Biocide M is the most effective bactericide. After M,

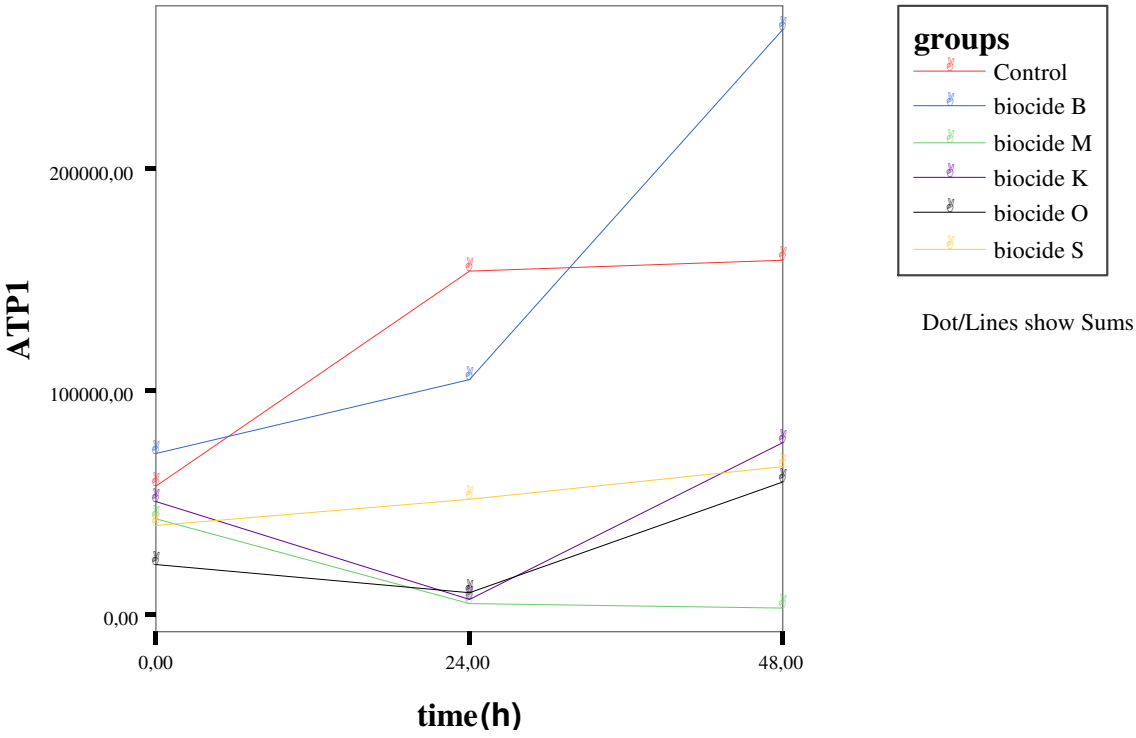

Figure 1. Activities of bactericides according to ATP 1 test.

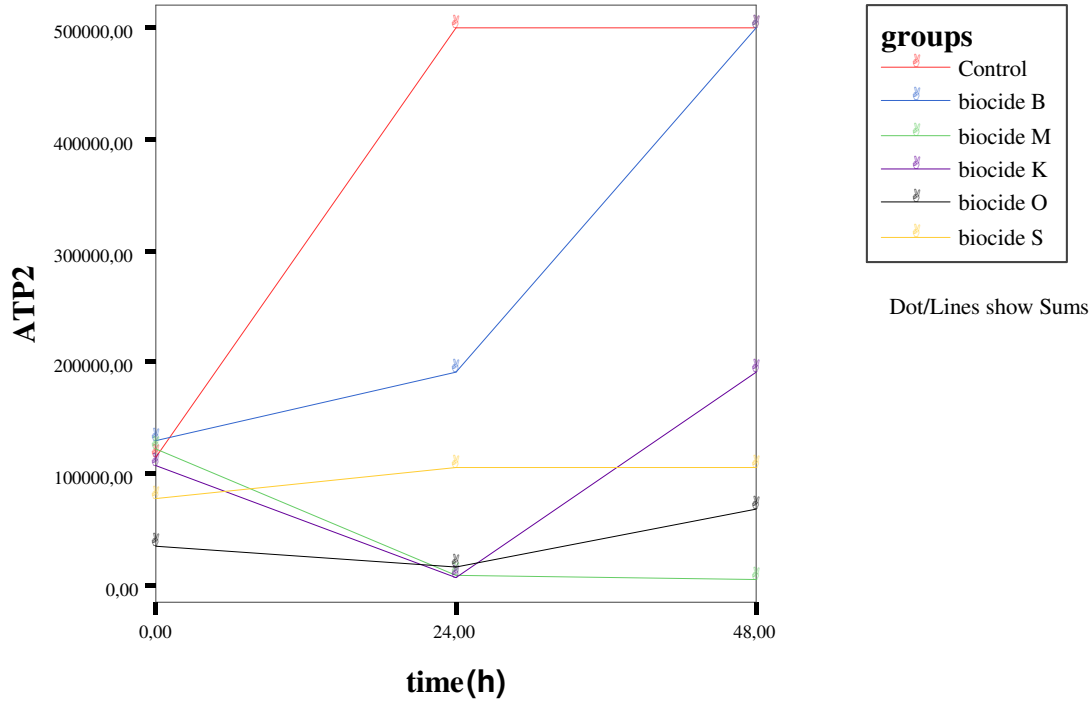

Figure 2. Effectiveness of bactericides according to ATP 2 test.

the second most effective bactericide is Biocide O. This method also indicated a positive correlation with the other methods.

DISCUSSION

In this research, the correlation between four of the com-

Table 5. The correlation between four of the commonly used counting methods in the leather industry in their soaking processes.

Time	Test methods	Correlations	ATP1	ATP2	Pour Plate	DIP Slide
20 min.	ATP1	Pearson Correlation	1	0.864*	-0.288	0.717
		Sig. (2-tailed)		0.026	0.580	0.109
	ATP2	Pearson Correlation	0.864*	1	-0.462	0.473
		Sig. (2-tailed)	0.026		0.356	0.343
	Pour plate	Pearson Correlation	-0.288	-0.462	1	0.342
		Sig. (2-tailed)	0.580	0.356		0.508
	Dip Slide	Pearson Correlation	0.717	0.473	0.342	1
		Sig. (2-tailed)	0.109	0.343	0.508	
24 h	ATP1	Pearson Correlation	1	0.958**	0.750	0.818*
		Sig. (2-tailed)		0.003	0.086	0.047
	ATP2	Pearson Correlation	0.958**	1	0.870*	0.699
		Sig. (2-tailed)	0.003		0.024	0.122
	Pour plate	Pearson Correlation	0.750	0.870*	1	0.460
		Sig. (2-tailed)	0.086	0.024		0.359
	Dip slide	Pearson Correlation	0.818*	0.699	0.460	1
		Sig. (2-tailed)	0.047	0.122	0.359	
48 h	ATP1	Pearson Correlation	1	0.926**	0.531	0.635
		Sig. (2-tailed)		0.008	0.278	0.176
	ATP2	Pearson Correlation	0.926**	1	0.802	0.683
		Sig. (2-tailed)	0.008		0.055	0.135
	Pour plate	Pearson Correlation	0.531	0.802	1	0.514
		Sig. (2-tailed)	0.278	0.055		0.296
	Dip slide	Pearson Correlation	0.635	0.683	0.514	1
		Sig. (2-tailed)	0.176	0.135	0.296	

* Correlation is significant at the 0.05 level (2-tailed); ** correlation is significant at the 0.01 level (2-tailed).

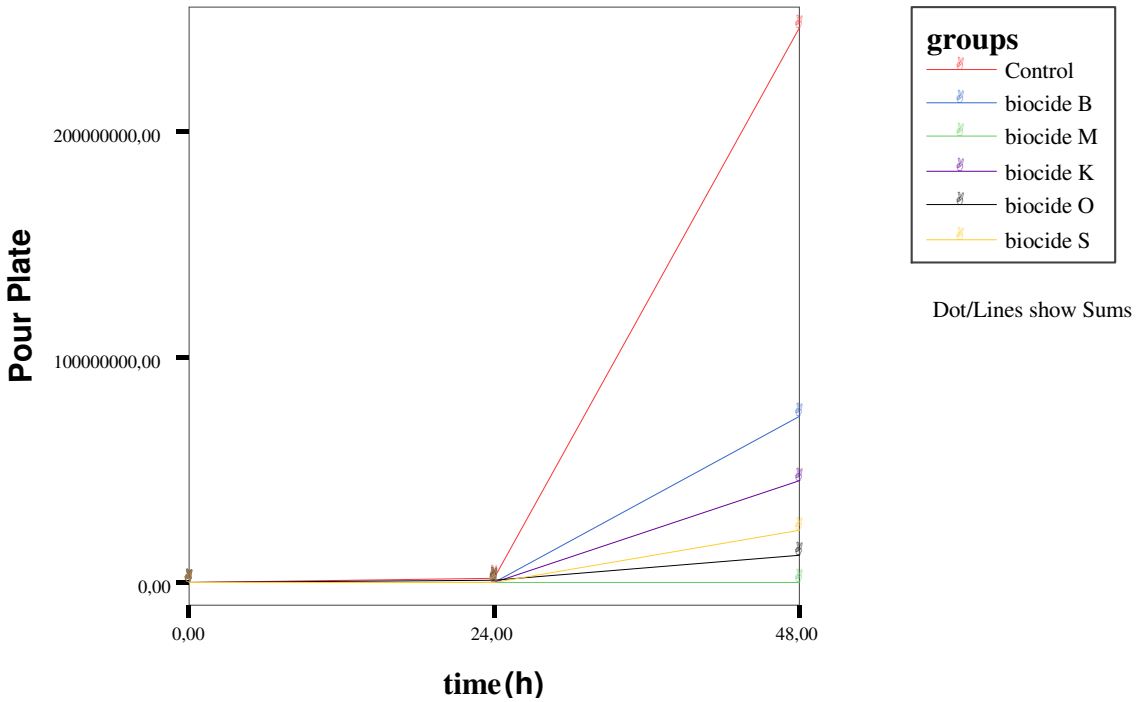

Figure 3. Activities of the bactericides according to agar pour plate method.

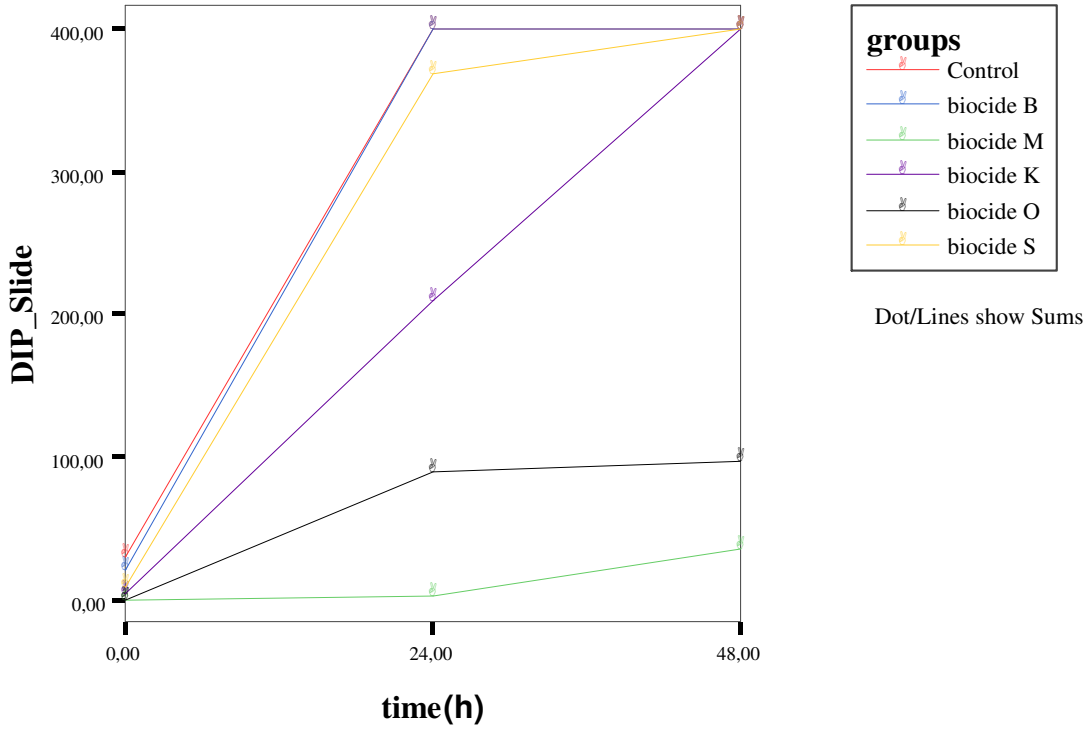

Figure 4. Activities of bactericides according to dip slide method.

Table 6. Microbial counting methods comparison table.

Microbial counting method	Time (short/long)	Ease of Use (easy/difficult)	Waste (much/little)	Sample Company	Cost (high/low)	Expertise to Apply (required/not required)
ATP1	Short	Easy	Minimum	Biotrace	High	Not required
ATP2	Short	Easiest (practical)	Little	Biotrace	High	Not required
Pour plate	Long	Difficult	Much	-	Medium	Required
Dip Slide	Medium	Easy	Little	Merck	Low	Not required

monly used counting methods in the leather industry in their soaking processes were taken into consideration. They were the ATP 1, ATP 2, pour plate and dip slide. According to research findings, a positive correlation was identified among the methods at the 24th and 48th h upon the comparison of all methods. Especially, the Pearson Correlation coefficient between ATP 1 and 2 was found to be highly meaningful with regard to time with 0.864, 0.958 and 0.926. Pour plate method indicated a negative correlation against the ATP methods during the initial measurements, while it indicated a positive correlation at the 24th and 48th h. This indicated that the methods yielded intercoherent results. Accordingly, the salesmen were able to provide quick presentations with ATP luminometer during the sales processes which also provided a positive idea in identifying the effectiveness of dip slide bactericides. However, in determining the effective amount (dose), certain pour plate or recently developed latest methods should be used and the quantities should be identified in accordance. Also, another important issue is working with different agar media especially in the conduct of researches related with halophilic bacteria, which are known not to be damaging to the skin, could be recommended. The comparison of the methods employed in the research, is shown in Table 6.

According to Table 6, although ATP1 and 2 counting methods were considered as positive in terms of time and ease, their high costs may be plausible for the industrialists in decision making. Pour plate and dip slide methods are respectively, considered as long and medium in terms of time. The use of dip slide is easy while expertise and laboratory are required for the pour plate method. From the point of cost, dip slide was identified as low cost. This research aimed to outline the relation between the different methods. It is believe that this study

will be a reference for sellers who are willing to sell by using ATP and dip slide methods and for the leather factories which are also willing to utilize effectiveness control by the bactericides.

According to all methods used for test processes, the most effective bactericide is biocide M (dimethyldithiocarbamic acid salt). Although a similar bacteria development has been observed for Biocide O and K, O has been identified as more effective. Despite the fact that Biocide B (organo-sulfur compound) was indicated as the most commonly sold bactericide in the world at present, they have been identified as not bearing as good results as Biocide M when they are used at the recommended dose by the company officials. For this reason, the sellers can recommend using dimethyldithiocarbamic acid salt containing bactericide especially in prolonged soaking processes. In identifying the effectiveness of a bactericide, the concentration of the active agent, the given dose, temperature, pH, conversation status of the hide and skin, among other issues should also be taken into consideration. Another point of consideration is the more or less harmful impact of all commonly used bactericides on human health at present (Kleban, 2008). In addition to that, as the prohibition of synthetic biocides is also expected with legal regulations in the future in order to prevent the environmental pol-lution, the conduct of more studies on the substitute organic biocides is recommended (Bayramoğlu, 2007; Bayramoğlu et al., 2006).

ACKNOWLEDGMENTS

We would like to thank Mrs. Özgür Şekeroğlu and Mr. Kemal Saral for their assistance and support in the realization of this study.

REFERENCES

Bayramoğlu EE (2007). Unique Biocide for the leather industry: Essential Oil of *Oregano*. JALCA, 102: 347-351.

Bayramoğlu EE, Gülümser G, Karaboz İ (2006). Ecological And Innovative Fungicide For Leather Industry: Essential Oil of *Origanum Minutiflorum*. JALCA, 101(3): 96-104.

Karaboz İ, Ateş M, Koçyiğit A (2002). Mikrobiyolojide Sayım Yöntemleri Teori ve Uygulamaları (Counting Methods Theory and Practices in Microbiology). EBILTEM yayınları, p. 45.

Kleban M (2008). Preserving agents in the leather production process. World Leather, May.

Rangarajan R, Didato DT, Bryant S (2003). Measurement of Bacterial Populations in Typical Tannery Soak Solutions By Traditional and New Approaches. JALCA, 98: 477-486.

Torlak E, Akan İM, Gökmen M (2008). Comparison of TEMPO EC and TBX medium for the enumeration of *Escherichia coli* in cheese. Lett. Appl. Microbiol. 47(6): 566-570.