Antibacterial and Anti–Biofilm Activities of Acetone Extracts of *Usnea* sp. against Mixed Cultures of Bacteria from Soak Liquor Samples and Tank Surfaces

by

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Abstract

Long-term or improper use of antibacterial agents utilized in the soaking process has led to the resistance of some bacteria in the leather industry. New agents may be the solution to combat these antibacterial resistant bacteria in the soaking process. As a natural resource, lichens are known to have many biological activities. In previous studies, we demonstrated that the acetone extracts of several lichen species including *Usnea* sp. may have potential antibacterial and anti–biofilm properties against some *Bacillus* species, which were isolated from different soak liquor samples. In the present study, it was questioned whether the same bioactivities of acetone extracts of *Usnea* sp. can be seen in the mixed cultures of tank surface samples and pre-and main soak liquor samples, which were obtained from different tanneries. Although the extracts did not show noteworthy antibacterial effect against one of the tank surface samples (inhibition ratios; 6.5–16.22 %), inhibition percentages were detected as 69.32 and 46.33 at the concentrations of 240 and 120 µg/mL for the other tank surface sample. The anti-biofilm potential of the extracts was tested on the sample where the antibacterial activity of the extracts was not observed. One of the mixed culture of samples from the tank surface could not be inhibited by the extracts in terms of bacterial growth. However, the extracts were tested on this biofilm-forming sample and detected more than 50% inhibition. Furthermore, the extracts inhibited the growth of the mixed culture of bacteria from pre-soak liquor by the percentages of 78.96, 61.5, 51.3, 45.1, and 33.4 at the concentrations of 240, 120, 60, 30 and 15 µg/mL, respectively. On the other hand, the same antibacterial efficacy could not be observed in the other mixed culture from pre-soak liquor sample obtained from a different tannery whereas this sample formed a biofilm structure. The mixed culture of samples from the main soaking process was inhibited by the extracts at the inhibition percentages of 62.13–78.17 at the concentrations of 240- 30 µg/mL. Similar results were also obtained for the other sample (64.6–76.5%) from main soak liquor sample obtained from a different tannery. In conclusion, lichen extracts may have potential antibacterial and anti-biofilm properties against the mixed culture of bacteria from tank surface, pre-and main soak liquor samples and maybe alternatively utilized in the leather industry.

Introduction

The leather industry has great economic value as one of the oldest industries in the world.[1] Bacterial microflora on hides/skins composes of many resident or transient bacteria which come from the animal itself or environmental sources. Whereas these bacteria are not harmful and do not cause any defects on hides of live animals under normal circumstances, the flaying process and a possible delay between the flaying and curing process trigger bacterial population growth. These bacterial population may have proteolytic and also other catabolic activities which cause to break down collagen network and diffuse into the hide and also bacterial attack to the grain surface. It was reported that protease and lipase producing bacteria cause hair-slip, putrefaction, grain peeling, loose grain, holes on the hides/skins, uneven dyeing, etc. Especially extremely halophilic archaea may cause red heat resulting in sueded grain.[2-5] It is well known that many bacteria can multiply in 1–3 h under optimal conditions and bacterial deterioration of hides/skins may occur within 5–6 h after flaying.[1, 6-8] It is obvious that bacterial density on raw hides/skins and in soak liquor may rapidly reach high levels in a short time period.[6] Hide preservation techniques ensures the destruction of harmful bacteria, prevention of bacterial activities and contamination; and resistance of hides/skins against putrefaction during transport and storage.[1] However, the adverse effects of bacterial activities on hides/skins in the production of high–quality leather have been emphasized in many studies.[4-6, 9-13] In this context, the accuracy of the preservation methods of raw hides/skins and also the proper soaking process gains importance. The researchers investigated the bacterial numbers from salt samples which were utilized for salt–curing, fresh, salted, and soaked hides and also from soak liquor samples.[2, 6, 9, 14, 15] The number of bacteria from fresh hides were reported to be 10^8 CFU/g.[14] Furthermore, the numbers of non–halophilic bacteria and extremely halophilic archaea were reported to be 10^4–10^8 CFU/g and 10^3–10^8 CFU/g on 36 salted hides. In the same study, the number of non-halophilic bacteria on soaked hides (10^5–10^8 CFU/g) and in soak liquors (10^5–10^7 CFU/mL) was also high.[2] On the other hand, it was reported that the number of bacterial populations in soak liquors should be up to 10^5 CFU/mL.[16] Therefore, the number of bacteria do not seem to be efficiently

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controlled by curing methods and the use of common antibacterial agents. Unfortunately, the presence of a large number of bacteria on the hides/skins and soaking liquor also reveals some consequences. Most bacteria, which were isolated from the leather industry, have been reported to secrete degrading enzymes such as protease and lipase. These enzymatic activities may cause degradation of the hide substance.[2, 4, 5, 11, 17]

The researchers emphasized that the mixed culture of bacteria on salted hides/skins may act synergistically and may cause unwanted defects on leather quality.[6] It is well known that many bacteria coexist by interacting with other bacteria in their surrounding milieu and utilize various functions.[18] To survive or dominate, bacteria must compete with each other because of their nutritional needs.[19] Recently, researchers have reported that bacterial social behaviors such as intraspecies, interspecies or interkingdom interactions are regulated by quorum sensing system (QS) when bacterial population density reaches to higher levels in the environment. This bacterial communication is achieved by signaling molecules called autoinducers (AIs). Three types of AIs were identified: AI–1, also called homoserine lactones (HSLs) for Gram–negative bacteria, autoinducing peptides (AIP) for Gram–positive bacteria, and autoinducer–2 (AI–2) for Gram–positive and Gram–negative bacteria for interspecies interaction.[20-23] In some circumstances, aggregated multicellular communities can form a three-dimensional biofilm structure, which is associated with the QS system, via extracellular polymeric substances (EPS).[24-27] The biofilm structure ensures resistance to disinfectants, antibiotics, U.V., etc. in favor of bacteria so that these bacteria can easily escape from these conditions and continue to survive.[28] Furthermore, this biofilm form has more resistance up to 10–1000 times against antibiotics when compared to its planktonic (free–swimming) forms.[29] In general, research on biofilm structures are performed by using monocultures. However, these mono-cultures of biofilm communities are reported to be rarely encountered in nature.[30] Moreover, it has been reported that mixed–culture biofilms are more resistant to disinfectants than monoculture biofilms.[30] This antimicrobial resistance property of bacteria in such biofilm structures also leads to the multidrug resistance problems.[31] In this respect, high bacterial density on the hides/skins and/or in soak liquor may activate the QS system followed by secretion of some virulence factors (protease, etc.) and/or biofilm formation. Then, unwanted defects may be seen due to all these features.

Due to resistance (intrinsic or acquired) problem of most bacteria to commonly utilized antibacterial agents, it has been suggested that some bactericides may not have efficient inhibitory effects against both total bacteria, proteolytic and lipolytic bacteria in soak liquors because of high organic content in soak liquors.[2, 32] In a previous study supporting this suggestion, the presence of many non-halophilic bacteria was demonstrated despite the antimicrobial agent at a twofold increased concentration (0.8 g/L).[32] The mixed cultures are sub–samples of complex natural communities consisting of two or more bacterial strains and can be utilized as model communities.[33] Since bacterial cells can survive despite the use of antibacterial agents, the difficulty of inactivation in mixed bacterial cultures with these agents has been reported.[34] Moreover, the physiological behaviors of bacteria in their natural community, i.e. in a mixed culture can change.[33] Furthermore, the efficacy of antimicrobials may change in multi-species biofilms in comparison to single-species cultures and planktonic cells.[35]

Recently, new bioactive compounds from natural sources are thought to be an alternative to overcome the potential antimicrobial resistance of many bacteria. Lichens, symbiotic organisms between fungus and one or more algae or cyanobacteria, can synthesize many novel secondary metabolites with biological activities such as antibacterial, anti–biofilm, anti–oxidant, etc. against some Gram–positive and Gram–negative bacteria.[36-38] In the past decade, it was reported that preservation and storage and/or early stages of beam house processes are suitable for biofilm formation on hides/skins.[39] More recently, the potential antibacterial and anti–biofilm activities of several lichen species against some *Bacillus* species from soak liquor samples were demonstrated in our previous studies.[40, 41]

In this way, we aimed to examine the potential antibacterial and anti–biofilm activities of *Usnea* sp. on mixed culture, which is similar to the natural environment but is an uncomplicated and also sufficient community. For this purpose, the acetone extracts of *Usnea* sp. were tested for their antibacterial and anti–biofilm properties on mixed cultures of samples from tank surfaces, as well as mixed cultures of pre– and main soak liquor samples collected from different tanneries.

Experimental

Lichen Samples

The lichen samples belonging to the *Usnea* sp. were collected from fir trees of Kastamonu province in the north–west of Turkey by all of the authors. The identification of the samples was confirmed by classical taxonomic methods based on microscopic examination by Çobanoğlu G.[42-43]

Usnea sp.: Turkey, Kastamonu province, Kapaklı Village, 41.24492, 34.18330.

Extraction of Lichen Samples

The lichen samples were washed, dried in air and weighed. After the samples were taken into sterile bottles, acetone (ACS, ISO, Reag. Ph Eur) was added and kept in a dark place for 24 h followed by filtration through filter paper. The acetone was evaporated in a rotary evaporator and crude lichen acetone extracts were obtained.[40, 41, 44]

Samples

The mixed culture samples from pre– (two samples) and main (two samples) soak liquors, as well as tank surfaces (two samples) were tested in the present study. Each pre- and main-soak liquor sample and tank surface sample was collected from different tanneries in Leather Organized Tannery Region, Tuzla–İstanbul, Turkey to obtain comparative results amongst the tanneries. The mixed culture samples of pre- and main soak liquor were taken into sterile glass bottles. The mixed culture samples were taken from the tank surfaces with transport swabs. Then, these samples were immediately placed into sterile sample bags and carried on ice during transportation. The samples were brought to the laboratory as soon as possible and the experiments were started. The pre- and main soak liquor samples were transferred into 50 mL sterile falcon tubes in the sterile cabinet. After these samples were centrifuged twice at 10000 rpm for 10 minutes, the supernatants in the tubes were removed. Then, tryptic soy broth (TSB) medium added onto cell pellets. The optical density (OD) values for each sample was checked at 600 nm for the antibacterial assays. On the other hand, the mixed culture of tank surface samples was also inoculated into TSB medium and the OD values at 600 nm were measured. In this way, bacterial suspensions with 0.02 OD values were prepared to examine antibacterial and anti–biofilm assays.

Antibacterial Assays

The antibacterial assays were performed in 96–well microplates (Greiner Bio–One, Cell Star, F–bottom, with lid). TSB medium was added to each well and five-fold serial dilutions of the acetone extracts of *Usnea* sp. were made. The final concentrations of lichen extracts were 240, 120, 60, 30 and 15 µg/mL. The prepared bacterial suspensions were added to obtain a total volume of 100 µL. The untreated (TSB medium and test bacteria) and blind wells (only the TSB medium) were included in the study. The tests were performed in three replicates. The bacterial growth was evaluated at 24th h using Cytation 3 multimode microplate reader (Biotek), by measuring the absorbance. The antibacterial effects of acetone extracts of *Usnea* sp. against the test samples were compared with the untreated ones.[40]

Anti–biofilm Assays

The anti–biofilm potential of the acetone extracts of *Usnea* sp. were also tested against the mixed culture samples from pre– and main soak liquors and tank surfaces. The assays were performed in 96–well microplates (Greiner Bio–One, Cell Star, F–bottom, with lid). TSB medium was added to each well and fivefold serial dilutions of the acetone extracts of *Usnea* sp. were made. The final concentrations of lichen extracts were 240, 120, 60, 30, and 15 µg/mL. The prepared bacterial suspensions were added to obtain a total volume of 100 µL. These samples were incubated overnight at 37°C in TSB medium in 96–well microplates without shaking. After incubation, we confirmed that the extracts did not kill bacterial strains in the samples tested. For this purpose, bacterial growth ratios at OD 600 nm were checked in the microplates before biofilm staining procedure in a microplate reader (Cytation 3-BioTek). Then, the microplates, in which no antibacterial effects were observed, were washed by physiological saline solution and dried at 60^{0}C. Then, 200 µl of 0.1 % crystal violet was added to each well for biofilm staining. The microplates were kept at room temperature for 10 min and washed three times. Following the second drying step, 200 µl ethanol was added and absorbance values were measured at OD 590 nm in a microplate reader (Cytation 3-BioTek). The experiments included untreated (the TSB medium and test bacteria) and blind wells (only the TSB medium). The anti–biofilm effect of acetone extracts of *Usnea* sp. against the test samples was compared with the untreated isolates. The tests were performed in three replicates.[38, 41, 45]

Results and Discussion

To the best of our knowledge, antibacterial and anti–biofilm effects of *Usnea* sp. against mixed–culture of bacteria from soak liquor and tank surface samples of leather processing plant have not been studied yet. In this study, we examined the acetone extracts of *Usnea* sp. for their antibacterial and anti–biofilm properties against mixed culture of bacteria from pre-and main–soak liquor samples and tank surface samples obtained from different tanneries.

The potential biological activities of various extracts obtained by different solvents from some lichen species have been indicated in the literature. In a recent study, the antibacterial properties of acetone extracts of *Hypogymnia physodes, Evernia divaricata, Pseudevernia furfuracea* and *Usnea* sp. at different concentrations were evaluated against *Bacillus toyonensis, Bacillus mojavensis, Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus velezensis, Bacillus cereus,* and *Bacillus licheniformis*, which were isolated from different soak liquor samples. In the same study, *Usnea* sp. acetone extracts were determined to have considerably high inhibitory effects on *Bacillus* species (86.6–97.9 %) even at low concentrations.[40] In a continuation of this work, biofilm–forming *Bacillus* species were detected as *B. subtilis, B. amyloliquefaciens,* and *B. velezensis* and the acetone extracts of *Usnea* sp. have been detected to have potential anti–biofilm properties depending on the tested bacteria or concentrations applied.[41]

In this study, we firstly examined the potential antibacterial and anti–biofilm efficacies of acetone extracts of *Usnea* sp. against a mixed culture of bacteria, which were obtained from tank surfaces. As mentioned earlier, it is very important to control the number of bacteria on raw hides/skins or in soak liquor samples to produce high–quality leather because the number of bacteria from raw and soaked hides as well as soak liquor samples has been reported considerably high in the literature.[2, 6, 9] A high number of bacteria means that the bacterial activity will be high and, as a result, hide or skin will be damaged. It is quite difficult to overcome any bacterial degradative activity, which occurs during the storage period or

soaking process, in the subsequent stages of tanning operations until the production of finished leather. We suggested that many bacteria may also colonize and form a biofilm structure on the tank surfaces and these bacteria may cause spontaneous contamination in every soaking process. From this point, two mixed–culture samples (A1 and A2) of tank surfaces from different tanneries were tested. According to our results, the acetone extracts of *Usnea* sp. had almost no antibacterial effect against the sample A1. The inhibition ratios were detected between 6.5 and 16.22%. On the other hand, the potential antibacterial efficacy was observed in the sample A2. The maximum inhibition percentages were calculated as 69.32 and 46.33 at the concentrations of 240 and 120 μg/ml. At the lower concentrations (60, 30 and 15 μg/ml), inhibition ratios were between 38.34 and 43.86%. These results demonstrate that the acetone extracts of *Usnea* sp. may have a potential antibacterial effect against the bacterial population, which colonized on the tank surfaces, depending on the bacterial communities. Considering our previous studies, the mixed culture of the tank surface samples may have *Bacillus* species dominancy.[40, 41] Therefore, we could detect the antibacterial activity of the extracts in the sample A2, but not in the sample A1 (Figure 1).

We also tested these mixed cultures of samples obtained from tank surfaces for the capability of forming a biofilm. The experiments showed that a mixed culture sample of A1 formed a biofilm but the sample A2 did not. The difference between the two samples may be related to the bacterial composition of mixed culture. This result pointed out that disinfection techniques have to be performed regularly on tank surfaces. The anti–biofilm potential of the acetone extracts of *Usnea* sp. was only evaluated on the sample A1 because the growth of sample A2 was already inhibited by the extracts at varying percentages. It is well known that anti-biofilm assays are performed at sub-inhibitory concentrations of antibacterial agents below the minimum inhibitory concentration (MIC) value. To evaluate the anti-biofilm potential of the extracts, the same test concentrations were utilized because we detected that these concentrations had no antibacterial effects in the sample A1. The extracts suppressed more than 50% biofilm formation of the sample A1 at concentrations of 240, 30 and 15 μg/ml (Figure 2).

According to the results of our anti-biofilm experiments, the fluctuating effect by the varying concentrations of the extracts was observed. A similar effect was also determined in the biofilm formation of some *Bacillus* species in our previous study.[41] Therefore, the bacterial population that forms a biofilm structure could probably have caused this effect. Since biofilm formation is a multi-step process, it may also be suggested that the tested extracts might affect different targets. It is well known that the soaking process consists of two stages as pre–soaking and main soaking.[16] It has been reported that the duration of the main soaking process generally changes from 1.5 to 24 h, depending on curing methods and countries.[2, 16] In our previous questionnaire study, we determined that the

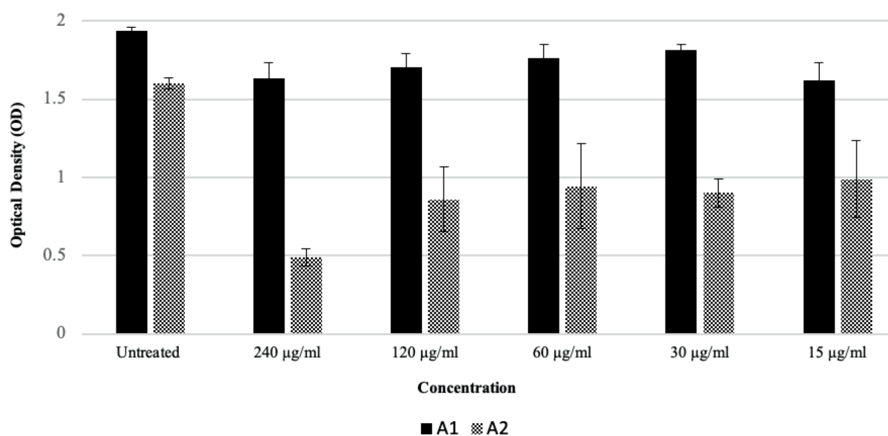

Figure 1. The antibacterial effects of the extracts against the samples A1 and A2.

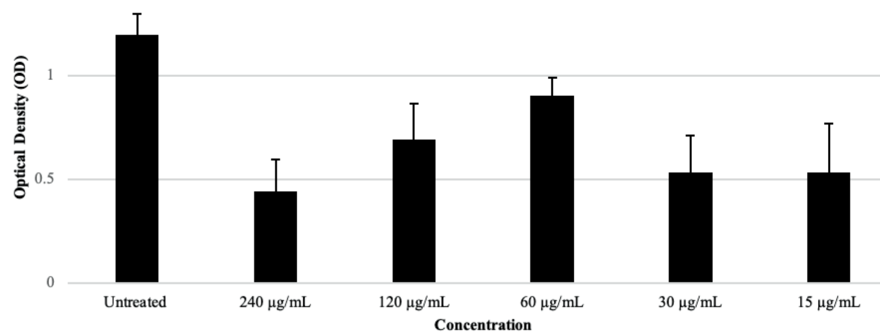

Figure 2. Anti–biofilm effect of the extracts against the biofilm–forming sample A1.

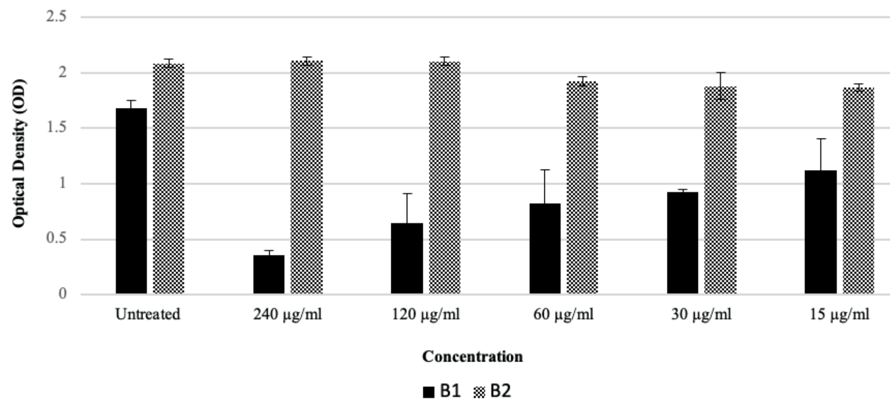

Figure 3. The antibacterial effects of the extracts against the samples B1 and B2.

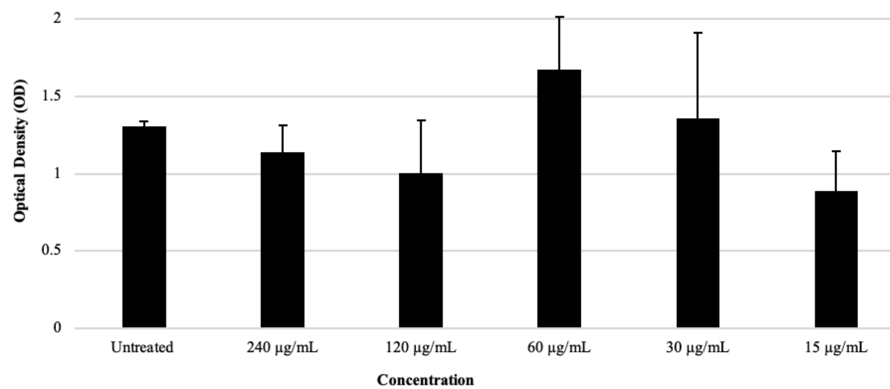

Figure 4. Anti–biofilm effect of the extracts against the biofilm–forming sample B2.

soaking process was generally carried out for 12–18 h in Turkey.[46] Such a quite long soaking process will inevitably trigger the growth of bacteria in the presence of high organic matter. Previously, the antimicrobial agent including didecyldimethylammonium chloride was found to be ineffective to decrease the number of bacteria in soak liquor samples at the manufacturers' recommended dose.[32] In the present study, we analyzed the antibacterial and anti–biofilm potential of the acetone extracts of *Usnea* sp. in two different samples of mixed culture bacteria from pre–soaking process. According to the results of the experiments evaluating antibacterial efficacy of the extracts, the sample B1, which was isolated from pre–soaking process, was inhibited by the percentages of 78.96, 61.5, 51.3, 45.1, and 33.4 at the tested concentrations of 240, 120, 60, 30 and 15 μg/ml, respectively. On the other hand, the same efficacy could not be observed in the sample B2. At the initial three concentrations, there was no antibacterial effect by the extracts. At the last three concentrations (60, 30 and 15 μg/ml), there was a slight inhibition on the bacterial growth of the sample B2, which was recorded as below 10.6 % (Figure 3).

Also, the samples B1 and B2 were tested for the capability of biofilm formation. Our findings showed that the mixed culture sample of B1 did not form a biofilm structure whereas the sample B2 formed a biofilm. As in the mixed culture samples of tank surfaces, the difference between the two samples was probably due to the bacterial composition of mixed cultures. Moreover, the acetone extracts of *Usnea* sp. could not efficiently inhibit the biofilm formation of the sample B2. Slight inhibition ratios were determined and these ratios were recorded under 50% (Figure 4). These data suggest that a mixed culture of bacteria can survive in a biofilm structure despite the utilized antibacterial agents. Although our natural extracts do not have a remarkable effect on the inhibition of biofilm formation of mixed culture of bacteria from pre–soaking process, further studies for antibacterial agents in combination with lichen extracts, which are known to have several biological activities, may be performed for the evaluation of their antibacterial and anti–biofilm efficacies against bacteria in soak liquor.

We also evaluated the antibacterial and anti–biofilm potential of the acetone extracts of *Usnea* sp. on two different samples of mixed culture bacteria from main–soaking process, which were obtained from different tanneries. The mixed culture of sample C1 from the main–soaking process was inhibited by the extracts at the inhibition percentages of 62.13–78.17 at the initial four concentrations (240, 120, 60, 30 μg/ml). The inhibition ratio for the concentration of 15 μg/ml was recorded as 48.24. Similarly, the inhibitions by the

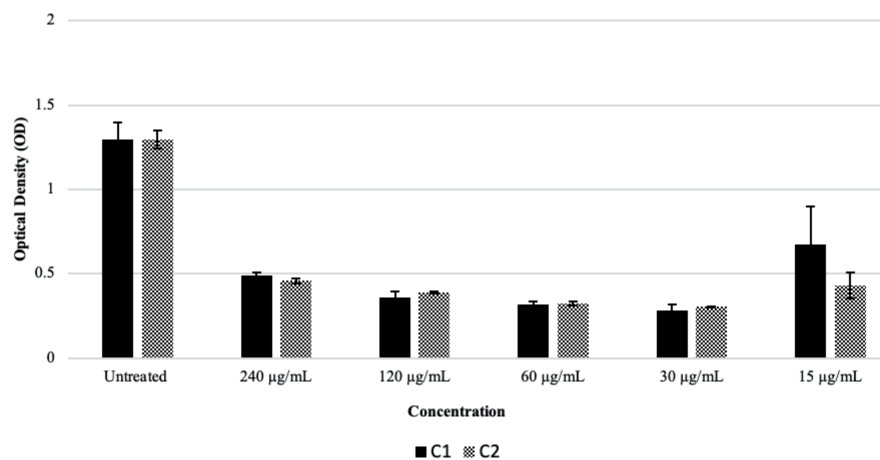

Figure 5. The antibacterial effects of the extracts against the samples C1 and C2.

acetone extracts of *Usnea* sp. were detected in the mixed culture of sample C2 from main soaking process. The inhibition percentages were between 64.6 and 76.5 (Figure 5). However, these samples did not form any biofilm structure (data not shown). These results might be related to the timing of sample collection because the main soak liquor samples were taken approximately at the 4 h of the main soaking process from the leather processing plant. Taking samples from the soak liquor periodically at certain intervals may be more useful to assess the potential of these extracts. Also, the differences in bacterial composition or the presence of resistant bacteria in the soak liquor samples may affect the biofilm formation.

There are also several studies in the leather industry that investigate the potential antibacterial effects of other natural products against bacterial growth. In a study evaluating the antibacterial effects of essential oils of *Lavandula officinalis* and *Eucalyptus globulus* on leather specimens, it was reported that samples treated with essential oil of *L. officinalis* had a more protective effect when compared to the samples cured with 2–(thiocyanomethylthio) benzothiazole (TCMTB) after 24 weeks. Also, the essential oil of *L. officinalis* was found to have a better antibacterial effect than *E. globulus*.[47] Furthermore, there are studies examining potential antibacterial agents from natural resources, especially in the soaking process. For example, myrtle oil (1%), which was added to soak liquor, was examined on the bacterial growth after 7 and 24 h soaking, and its effect was compared against the tested bactericide including 7–25% phenol, 4–chloro–3–methyl. The researchers detected similar bacterial counts in both treatment groups.[48] In another study, the potential antibacterial effect of *Origanum minutiflorum* was reported in comparison to the commercial bactericide, including 7–25% phenol, 4–chloro–3–methyl.[49] In this study, the potential antibacterial and anti-biofilm efficacies were also demonstrated for the acetone extracts of *Usnea* sp. In a previous study, it has been reported that biofilm formation causes dyeing defects in the leather industry.[39] Therefore, biofilm formation may have an important role in the production of the best value of leather. More recently, novel approaches such as anti-virulence strategy have come into prominence because most bacteria may secrete virulence factors such as protease and elastase. The production of virulence factors and also biofilm formation is associated with the QS system. The anti-QS potential of lichens was reported in the literature.[44] In this manner, the potential effects may be investigated in detail for anti-QS and anti-virulence properties of lichen extracts. These extracts and/or their compound(s) with anti-biofilm, anti-bacterial, and anti-QS properties may be applicable in the leather industry.

Conclusion

In the present study, it has been demonstrated that the acetone extracts of *Usnea* sp. may have antibacterial and anti–biofilm properties against a mixed culture of bacteria from tank surface and pre–or main soak liquor samples. The efficacy of the extracts varied depending on the samples. These differences may result from different bacterial compositions of mixed cultures obtained from soak liquor or tank surface samples. In this study, it has been also shown that mixed cultures of bacteria from the tank surface and pre-soaking process can form a biofilm structure. Biofilm formation may play an important role during leather–making processes. Further detailed studies may increase our knowledge about biofilm formation on hides/skins. Lichen extracts have advantages such as having potential antibacterial and anti–biofilm properties as well as being non–toxic, and ecological material. If the chemical(s) of these lichen extracts are comprehensively investigated, the major active ingredient may be discovered and may be utilized in the leather industry.

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