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Türk Hijyen ve Deneysel Biyoloji Dergisi

Determination of biofilm formation capacity of *Metschnikowia reukaufii* strain

Metschnikowia reukaufii suşunun biyofilm oluşturma kapasitesinin belirlenmesi

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ABSTRACT

Objective: Biofilm is a community of microorganisms attached to biotic or abiotic surfaces. Microorganisms that form biofilms cause much more serious medical and industrial problems than their planktonic forms. In this sense, the ability of microorganisms to form biofilms must be known and precautions must be taken. The aim of this study is to create stress in the *Metschnikowia reukaufii* MN622824 strain, whose inhibitory substance production increases under stress conditions, by trying different environmental conditions and to determine its effect on biofilm formation. In addition, to observe its effect on biofilm formation on stainless steel (SS) surfaces, which are frequently used in food machines.

Methods: Biofilm formation was compared with the tube method and the microtitration plate method. For this purpose, 5 different media compositions containing 5% glucose, 10% glucose, 5% NaCl, 10% NaCl, containing no glucose and NaCl (sodium chloride), and 3 different incubation periods consisting of 3, 5 and 7 days were tested. Additionally, scanning electron microscopy imaging was performed to examine the adhesion of biofilm to stainless steel coupons.

ÖZET

Amaç: Biyofilm, biyotik veya abiyotik yüzeylere tutunan mikroorganizmalardan oluşan bir topluluktur. Biyofilm oluşturan mikroorganizmalar, planktonik formlarına göre çok daha ciddi tıbbi ve endüstriyel sorunlara neden olmaktadır. Bu nedenle mikroorganizmaların biyofilm oluşturma yeteneklerinin bilinmesi ve önlem alınması gerekmektedir. Bu çalışmanın amacı stres koşullarında inhibitör madde üretimi artan *Metschnikowia reukaufii* MN622824 suşunun, farklı çevre koşullarını deneyerek stres oluşturmak ve bunun biyofilm oluşumuna etkisini belirlemektir. Ayrıca, gıda makinelerinde sıklıkla kullanılan paslanmaz çelik (SS) yüzeylerde biyofilm oluşumuna etkisini gözlemlemektir.

Yöntem: Biyofilm oluşumu tüp yöntemi ve mikrotitrasyon plak yöntemi ile karşılaştırılmıştır. Bunun için glikoz ve NaCl (sodyum klorür) içermeyen, %5 glikoz, %10 glikoz, %5 NaCl, %10 NaCl içeren beş farklı ortam kompozisyonu ve 3, 5 ve 7 günlük periyotlardan oluşan üç farklı inkübasyon süresi denenmiştir. Ayrıca, biyofilmin paslanmaz çelik kuponlara yapışmasını incelemek için taramalı elektron mikroskobu görüntülemesi yapılmıştır.

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Results: In the tube method, the highest biofilm formation was observed after three days of incubation in tubes with 5% glucose added. No biofilm was formed in the tubes that were kept for seven days or 10% NaCl was added. In the microtitration plate method the strong biofilm formation (1.3022) was obtained after three days of incubation in a medium containing 5% glucose. In the control group without glucose and NaCl, moderate biofilm formation were observed on the 3rd day (0.7385) and the 5th day (0.6882). Biofilm formation was evaluated as absent or weak in media containing 10% glucose or NaCl. As glucose and NaCl concentrations increased, biofilm formation decreased. Reticular bond structure showing biofilm formation were imaged with a scanning electron microscope in the control group on days 3-5-7, 5% NaCl and 5% glucose concentration on the 3rd day, and 5% glucose concentration on the 5th day.

Conclusion: Biofilm formation in food factories poses a significant health problem. It was observed that increasing NaCl, glucose concentration and incubation time negatively affected the biofilm formation of the *Metschnikowia reukaufii* strain. Biofilm formation decreases or disappears in yeasts exposed to stress conditions. It is thought that the data obtained will help in biofilm control.

Key Words: *Metschnikowia reukaufii*, biofilm, glucose, sodyum chloride, biocontrol

Bulgular: Tüp yönteminde en yüksek biyofilm oluşumu %5 glikoz ilaveli tüplerde üç günlük inkübasyon sonrasında gözlenmiştir. Yedi gün bekletilen veya %10 NaCl eklenen tüplerde biyofilm oluşmamıştır. Mikrotitrasyon plak yönteminde, %5 glikoz içeren bir ortamda üç günlük inkübasyonun ardından güçlü biyofilm oluşumu (1.3022) elde edilmiştir. Glikoz ve NaCl bulunmayan kontrol grubunda 3. gün (0.7385) ve 5. gün (0.6882) orta derecede biyofilm oluşumu gözlenmiştir. %10 glikoz veya NaCl içeren ortamlarda biyofilm oluşumu yok veya zayıf olarak değerlendirilmiştir. Glikoz ve NaCl konsantrasyonları arttıkça biyofilm oluşumu azalmıştır. Biyofilm oluşumunu gösteren retiküler bağ yapısı taramalı elektron mikroskobu ile kontrol grubunda 3., 5. ve 7. günlerde, %5 NaCl ve %5 glukoz konsantrasyonunda 3. günde, %5 glukoz konsantrasyonunda ise 5. günde görüntülenmiştir.

Sonuç: Gıda fabrikalarında biyofilm oluşumu önemli sağlık sorunu oluşturmaktadır. Artan NaCl, glikoz konsantrasyonu ve inkübasyon süresinin *Metschnikowia reukaufii* suşunun biyofilm oluşumunu olumsuz etkilediği gözlenmiştir. Stres koşullarına maruz kalan mayalarda biyofilm oluşumu azalmakta veya ortadan kalkmaktadır. Elde edilen verilerin biyofilm kontrolünde yardımcı olacağı düşünülmektedir.

Anahtar Kelimeler: *Metschnikowia reukaufii*, biyofilm, glikoz, sodyum klorür, biyokontrol

INTRODUCTION

The complex matrix containing EPS (exopolysaccharide or extracellular polymeric substance), proteins, eDNA (extracellular DNA), various enzymes and the microorganism itself secreted by bacteria, yeast, mold, algae and protozoa is called biofilm (1). Biofilm can be found attached to a surface or embedded in an extracellular matrix (2). This biofilm matrix makes them resistant to harsh conditions and resistant to antibacterial drugs (3,4). Additionally, this resistance makes infections difficult to treat and causes a wide variety of chronic diseases (5). At the same time, biofilm formation protects microorganisms against various harsh environments (ultraviolet radiation, extreme temperature, extreme pH, high salinity, high pressure, poor nutrients, antibiotics, etc.), increases microbial competitiveness in environments, and is also used in some cellular functions (6,7). In particular, biofilms

formed by pathogenic microorganisms (8) and decaying microorganisms are inappropriate sources of microbial contamination. Such microbial cells are likely to contaminate raw materials and food during processing, leading to food spoilage and economic losses for producers (9). They are also major obstacles in the food industry and healthcare industry, as their ability to form biofilms protects them from ordinary cleaning procedures and allows them to persist in the environment. This persistence results in increased microbial load in the food processing environment and the final food product; this leads to spoilage and shortened shelf life, as well as increased risks from infectious disease outbreaks from food sources (10). Therefore, biofilm formation must be prevented at the initial stage or the resulting biofilm structure must be eliminated in the food industry. As biofilm-related infections become common, knowing the various aspects and functionality of biofilm formation will facilitate the implementation of methods to prevent these structures and will help determine the measures to be used to combat these infections (11).

Yeasts form biofilms by adhering to abiotic surfaces such as wood, stainless steel, glass and plastic polymers. Many yeast species such as *Saccharomyces cerevisiae*, *Candida albicans* and *Cryptococcus neoformans* are known to form biofilms. Some strategies, such as good hygiene practices, including regular cleaning and disinfection of surfaces, can help reduce biofilm formation. Using antimicrobials and alternative methods to eliminate yeast biofilms can also help ensure food safety (12).

Metschnikowia reukaufii (Ascomycota, Saccharomycetales) are yeasts commonly found in flowers and flower nectar, environments with high sucrose concentrations (400 g/l). It is found predominantly in the nectar of plants such as *Helleborous foetidus* (13,14). Although the ecological function of this yeast is relatively unknown, many studies relate it to nectar sugar composition, synthesis of volatile compounds and even increased temperature of nectars, all factors that can influence the behavior of pollinators (15,16).

The objectives of this study were to compare the biofilm formation ability of the *Metschnikowia reukaufii* MN622824 strain, which is not pathogenic but increases the production of inhibitory substances under stress conditions, using the tube method and the microtitration plate method, and its biofilm formation capacity was evaluated. Its effect on stainless steel (SS) surfaces, which are frequently used in the food industry, was determined by observing the scanning electron microscope (SEM).

MATERIAL and METHOD

Strains and growth conditions

In this study, *M. reukaufii* (Genbank Accession Number: MN622824, http:// www.ncbi.nlm.nih.gov/blast)) strain isolated from flower at Süleyman Demirel University, Faculty of Engineering, Department of Food Engineering was used. *M. reukaufii* strains were activated in Malt Extract broth (Merck, Germany) adjusted to pH 5.5. The inoculated tubes were incubated at 30°C for 24 hours. Then, the absorbance was adjusted to 0.5 at 570 nm on the spectro-photometer for each culture.

Testing different glucose and NaCl concentrations

Malt Extract broth was used as a medium to observe the effect of different stress conditions on biofilm formation. After adjusting the pH value of the media, different concentrations of glucose and NaCl were added. Five different media compositions including without glucose and NaCl, 5% glucose, 10% glucose, 5% NaCl, 10% NaCl and three different incubation periods as 3, 5 and 7 days were applied.

Biofilm analysis with tube method

Biofilm formation was examined by adding different amounts of glucose and NaCl to Malt Extract broth, whose pH value was adjusted to 5.5. Yeast strains growing in these media were discharged from the tubes after 3-5-7 days of incubation. The evacuated tubes were washed with sterile phosphate

buffer saline (pH 7.3) and dried. Then, 10 mL of the solution prepared with distilled water containing 0.1% crystal violet was added to the tubes and incubated for 10 minutes. After incubation, the crystal violet solution in the tubes was drained and the excess dye was washed with ionized water. After this process, the tubes were left to dry. After the tubes dried, values of absent/weak (1), medium (2) and strong (3) were given according to the density of the crystal violet solution adhering to the tube (17).

Biofilm analysis with microtitration plate method

Biofilm formation of *M. reukaufii* yeast strain by the microtitration plate method was performed as previously described in Zhang et al. (18) with a few modifications. Overnight culture was diluted into Malt Extract broth prepared with different specifications (without glucuse and NaCl, %5 glucose, %10 glucose, %5 NaCl, %10 NaCl) at a ratio of 1:100. Diluted cultures were added to a 96-well plate, 100 µL per well. It was then incubated at 30°C for 3-5-7 days. Additionally, negative control wells contained Malt Extract broth only. After incubation, total cell-mass was measured as absorbance at 630 nm by spectro-photometer.

After the measurement, the plates were emptied, the wells were washed with sterile water and then the drying process was carried out. Following the drying process, 125 µL of 0.1% crystal violet solution was added to each well and incubated for 20 minutes at room temperature. It is known that the dye bound to adhered cells in the wells can be resolubilized and measured in optical density with a spectro-photometer. With this method, the unbound crystal violet solution was washed with distilled water and dried. 100 µL of 95% ethanol was transferred to each well to dissolve and release the bound dye. The dye solution dissolved in ethanol in each well was transferred to a clean plate, respectively. Finally, absorbances at 492 nm were measured with a spectro-photometer. The results obtained were calculated with the formula B= A492/A630 and the degrees of biofilm formation were determined: No biofilm producer ($B<0.1$), Weak biofilm producer ($0.1\leq B\leq 0.5$), Moderate biofilm producer ($0.5\leq B\leq 1$), Strong biofilm producer ($B\geq 1$).

Scanning electron microscope analyzes

The yeast strains were then tested to form biofilms on SS surfaces in food machines frequently used in the fermentation industry. SS coupons with biofilm formation were examined with SEM and biofilm structures were visualized. For this purpose, steel coupons were cleaned with 70% ethanol for 10 minutes. Then, it was washed with sterile distilled water and dried at 60 ºC for 2 hours. The dried coupons were placed in heat-resistant glass containers, covered first with aluminum foil and then with their own lids, and sterilized in an autoclave at 121°C for 15 minutes. Steel coupons were placed in 24-well microplates. 2 mL of the media in different compositions including without glucose and NaCl, 5% glucose, 10% glucose, 5% NaCl, 10% NaCl was added to each well. Then, the absorbance of the freshly prepared cultures was adjusted by incubating them at 30°C for 24 hours using Malt Extract broth. For each different concentration, it was incubated at 30°C for 3, 5 and 7 days. After incubation, the steel coupons removed from the plates were washed three times with 10 mL of sterile distilled water to remove non-adherent cells.

Steel coupons were prepared to be examined with SEM as described previously Kaya et al. (19). For this purpose, the coupons were fixed in 2.5% glutaraldehyde (prepared with 0.1 M phosphate-buffered saline (PBS), pH 7.4) overnight at 4°C. They were washed twice with 0.1 M PBS. Then, it was fixed in 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, Pa) for 1 hour and then washed with distilled water. For dehydration, the samples were passed through ethyl alcohol series for 15 minutes (30%, 50%, 70%, 90%, and 96%), and in the final stage, they were treated with absolute alcohol for 30 minutes and dried. Steel coupons are placed on aluminum stabs and plated with gold. It was examined with the FEI / Quanta 450 FEG brand SEM within the Hitit University Scientific Technical Application and Research Center.

RESULTS

Tube method

To observe the effect of different stress conditions on biofilm formation, different concentrations of glucose and NaCl were added after adjusting the pH value of the media. Biofilm formation was compared with the tube method in five different media compositions including without glucose and NaCl, 5% glucose, 10% glucose, 5% NaCl, 10% NaCl and 3 different incubation periods as 3, 5 and 7 days. The results obtained are shown in Figure 1.

According to the tube method results, the highest biofilm formation was observed after three days of incubation in tubes with 5% glucose added. No biofilm formation was observed in the tubes incubated for seven days. Additionally, biofilm formation was prevented in tubes to which 10% NaCl was added.

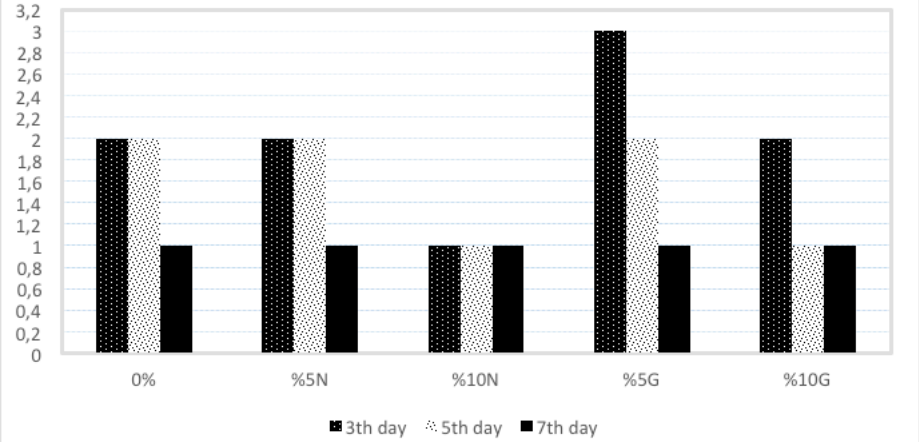

Figure 1. Biofilm formation of *M. reukaufii* strain by tube method

0%: without glucose and NaCl, %5N: 5% NaCl, %10N: 10% NaCl, %5G: 5% glucose, %10G: 10% glucose

Microtitration plate method

In detecting biofilm formation with the tube method, visual qualitative analysis is performed according to the density on the bottom and/or walls of the tubes (17). Since grading this staining in the tubes may vary from person to person, biofilm formation should be tested with the microtitration plate method as well as the tube method. The microtitration plate method is a quantitative method that is based on the optical density of the crystal violet solution added to the biofilm matrix and gives numerical results by measuring spectrophotometrically (18). To observe biofilm formation under different conditions, absorbances were measured with a spectrophotometer first at 630 nm and after fixation at 492 nm. The obtained absorbance values were calculated with the formula and the results are given in Table 1.

According to the microtitration plate method results, moderate biofilm formation was observed on day 3 and day 5 in a glucose and NaCl-free environment, while no biofilm formation was observed on day 7. Biofilm formation was observed on the 3rd day in the medium supplemented with 5% NaCl, but no biofilm was formed as the incubation period was prolonged. While *M. reukaufii* strain produced the most biofilm in the medium supplemented with 5% glucose, no biofilm was observed in the medium supplemented with 10% glucose. As the amount of added glucose increased, biofilm formation decreased. Comparable results according to biofilm formation are shown in Figure 2.

Table 1. Biofilm formation of *M. reukaufii* strain by microtitration plate method

	0%	%5N	%10N	%5G	%10G
3th day	0.739±0.25	0.627±0.07	0.240±0.05	1.302±0.15	0.255±0.01
5th day	0.688±0.31	0.510±0.05	0.396±0.06	0.603±0.19	0.124±0.01
7th day	0.275±0.05	0.374±0.02	0.414±0.02	0.658±0.14	0.118±0.01

Data are expressed as means ± standard deviation

0%: without glucose and NaCl, %5N: 5% NaCl, %10N: 10% NaCl, %5G: 5% glucose, %10G: 10% glucose

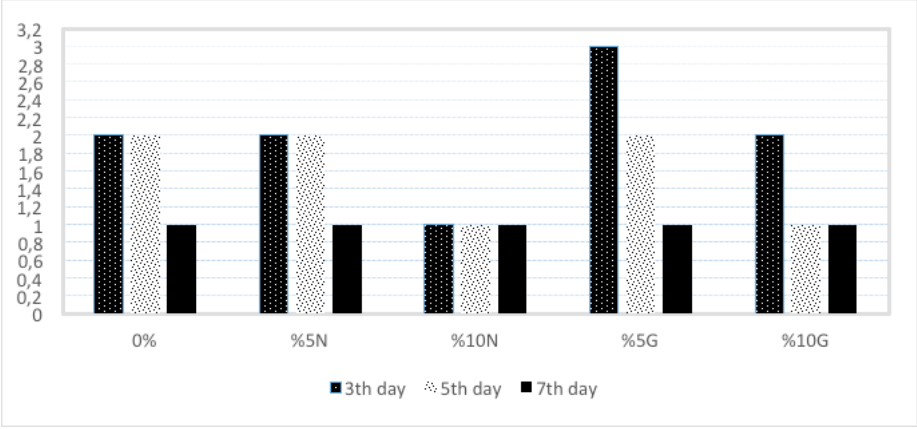

Figure 2. Biofilm formation of *M. reukaufii* strain by microtitration plate method

0%: without glucose and NaCl, %5N: 5% NaCl, %10N: 10% NaCl, %5G: 5% glucose, %10G: 10% glucose

Scanning electron microscopy analysis

The effect of incubation time on the adhesion of *M. reukaufii* strain grown in Malt Extract Broth in different environments (without glucose and NaCl, 5% glucose, 10% glucose, 5% NaCl, 10% NaCl) to SS surfaces was investigated. Biofilm conditions and SEM images formed on steel coupons are given in Figure 3.

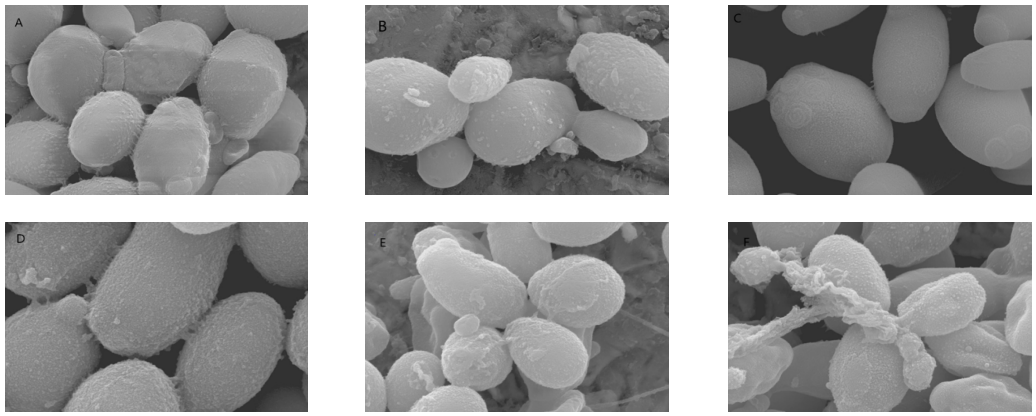

Figure 3. SEM images of biofilm formation of M. reukaufii strain on SS coupons

(A) 0%-3th day, (B) 0%-5th day, (C) 0%-7th day, (D) 5% NaCl-3th day, (E) 5% glucose 3th day, (F) 5% glucose 5th day

DISCUSSION

The majority of yeast species operate between water activity values of 0.9-1.0. It has been stated that the optimum water activity value for the growth of *S. cerevisiae* is between 0.975 and 0.999, and when this value falls below 0.94, the yeast cannot develop. It is also known that the ethanol produced by yeast causes water stress in the cell by reducing the water activity value. In this case, the hydrogen bonds in the hydrated cell components interact and the enzyme and membrane structure in the cell is disrupted (20). In light of this information, as the amount of NaCl and glucose increased, water activity decreased, which prevented biofilm formation.

The effect of glycerin concentration, also known as sugar alcohol, on biofilm formation of *S. cerevisiae* strain was investigated. It was observed that by adding glycerin to the liquid medium, the water activity of the medium decreased and the yeast could not grow. According to this information, it is thought that as the concentration of glycerin increases to 5%, the metabolic activities of the yeast slow down and they cannot form biofilms. It is also known that environmental factors are very important for the development of microorganisms and biofilm production (21). In another study, biofilm formation of the *A. hydrophila* strain was investigated at different glucose concentrations using the microtitration plate method. Biofilm formation decreased with the addition of glucose to the medium. While the addition of 0.05% glucose did not significantly reduce biofilm formation compared to the control (0% glucose), biofilm formation was significantly inhibited at 0.25% glucose concentration (22).

In a study, biofilm formation of *S. cerevisiae* strains was investigated at different NaCl concentrations. The highest biofilm formation was observed when the pH of the medium was 5.0 and in the tube without NaCl addition. It was determined that biofilm formation decreased as the NaCl ratio increased, and biofilm did not form when the ratio reached 10% (21). It has been reported that high NaCl concentration prevents cells from adhering to surfaces (23). Similarly, it was determined that biofilm formation of *Salmonella* enterica strains was restricted by the increase in the amount of NaCl (24). Increasing the NaCl concentration in the environment causes a decrease in cell hydrophobicity. Therefore, the presence of different amounts of NaCl in the environment changes cell surface properties and biofilm distribution. It has been reported that high NaCl ratio inhibits cell adhesion (25). It is thought that yeast cell adhesion is inhibited by the increase in NaCl concentration, as a result of which the cells cannot attract each other and form biofilms. Giaouris et al. (23) showed that high sodium chloride concentration (10.5% NaCl; aw 0.95) prevented cells from adhering to the plate. Mizan et al. (26) reported that Vibrio parahaemolyticus produced the best biofilm at 2% NaCl concentration. It has been observed that when this rate reaches 5%, it produces the least. Studies show that increasing NaCl ratio at the same temperature disrupts the structure of the cell and prevents biofilm formation.

It is known that increasing the NaCl concentration in the environment disrupts the ion balance in the cell. High amounts of NaCl intake causes an increase in the amount of Na+ and Cl ions in the cell. This situation causes low water potential and ion toxicity (27). Although the sensitivity of the microorganism to NaCl increased at higher osmotic pressures, the harmful effect of salts on the development of this microorganism was determined to be due to the specific ion effect rather than the osmotic effect (28). In the light of this information, it is understood that as the amount of NaCl ions in the cell increases, yeast activity is affected and biofilm formation decreases. It has been observed that the growth of yeast slows down as the NaCl ratio increases in the medium (29). Since the high amount of NaCl in the medium prolongs the development time of the yeast, it is thought that after 24 hours of incubation, the yeast cannot grow much in the medium and cannot reach sufficient cell density for biofilm formation.

Compared to this study, this study supports the conclusion that as the NaCl concentration increases in the medium, the yeast cannot complete its development and the level of biofilm formation decreases. According to the study, it was observed that microorganism cells in the biofilm were under more osmotic stress than planktonic cells and that high osmotic potential prevented biofilm formation (30).

According to the SEM analysis, the reticular bond structure indicating biofilm formation was visualized in the SS coupons of the control group incubated for 3-5-7 days. However, biofilm formation gradually decreased in coupons incubated for 5 and 7 days. This showed that 3 days of incubation period was sufficient for the *M. reukaufii* strain to form a biofilm. This can be explained by the fact that the cells lose or decrease their viability after 3 days. Similarly, biofilms were observed on day 3 in the tube with 5% glucose addition. No biofilm was observed on SS coupons in environments containing NaCl, except for the tube containing 5% NaCl (3 days incubation). As in the tube and microtitration plate method, no biofilm formation was observed on SS coupons in media prepared at 10% NaCl concentrations. This showed that NaCl concentration prevented the growth of yeasts. Betts et al. (29) concluded in a study that the *S. cerevisiae* strain could not grow at 8% NaCl, whereas the maximum NaCl rate suitable for the growth of the yeast was 4.8%. It is stated that S. cerevisiae needs high water activity (0.65) to carry out its metabolic activities. This yeast needs water to ferment. It has been noted that environments containing high sugar can impose osmotic stress to negatively affect cell physiology. It is thought that a decrease in the amount of biofilm formation is observed because the metabolic activities of the yeast are limited by reducing the water activity of the environment (31). Microorganisms and biofilm formation are affected by changes in environmental conditions. The ability of *Salmonella enteritidis* to form biofilms on SS surfaces was examined at different temperatures (5, 20 and 37°C), pH (4.5, 5.5, 6.5 and 7.4) and water activity values (0.5, 1.5, 5.5 and 10.5% NaCl) (23). It has been reported that maximum biofilm formation is reached in 6 days at 20°C. It has been reported that biofilm formation after seven days of incubation at 20°C was not dependent on pH change and that high sodium chloride concentration (10.5% NaCl, aw 5 ± 0.94) clearly inhibited the adhesion of cells to the coupons.

In conclusion; microorganisms produce a gel-like layer defined as biofilm in order to be more resistant to adverse conditions. In the food industry, heat treatments to extend the shelf life of foods and chemicals used to clean the materials in the process do not affect the biofilm formed by microorganisms. For this reason, biofilm formation in food factories poses a health problem. It was observed that increasing NaCl, glucose concentration and incubation time negatively affected biofilm formation. It is very important for food factories that biofilm formation disappears under these stress conditions. The data obtained as a result of this study is intended to shed light on practices to prevent biofilm formation.

ETHICS COMMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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