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Comparison of Methods for the Detection of Biofilm Formation in Yeast and Lactic Acid Bacteria Species Isolated from Dairy Products

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Abstract**—**Lactic acid bacteria (LAB) and some yeast species are common microorganisms found in dairy products and most of them are responsible for the fermentation of foods. Such cultures are isolated and used as a starter culture in the food industry because of providing standardisation of the final product during the food processing. Choice of starter culture is the most important step for the production of fermented food. Isolated LAB and yeast cultures which have the ability to create a biofilm layer can be preferred as a starter in the food industry. The biofilm formation could be beneficial to extend the period of usage time of microorganisms as a starter. On the other hand, it is an undesirable property in pathogens, since biofilm structure allows a microorganism become more resistant to stress conditions such as antibiotic presence. It is thought that the resistance mechanism could be turned into an advantage by promoting the effective microorganisms which are used in the food industry as starter culture and also which have potential to stimulate the gastrointestinal system. Development of the biofilm layer is observed in some LAB and yeast strains. The resistance could make LAB and yeast strains dominant microflora in the human gastrointestinal system; thus, competition against pathogen microorganisms can be provided more easily. Based on this circumstance, in the study, 10 LAB and 10 yeast strains were isolated from various dairy products, such as cheese, yoghurt, kefir, and cream. Samples were obtained from farmer markets and bazaars in Bursa, Turkey. As a part of this research, all isolated strains were identified and their ability of biofilm formation was detected with two different methods and compared with each other. The first goal of this research was to determine whether isolates have the potential for *biofilm* production, and the second was to compare the validity of two different methods, which are known as "Tube method" and "96-well plate-based method". This study may offer an insight into developing a point of view about biofilm formation and its beneficial properties in LAB and yeast cultures used as a starter in the food industry.

Keywords**—**Biofilm, dairy products, lactic acid bacteria, yeast.

I. Introduction

BIOFILM can be defined as a community of microorganisms which are attached and adhered to the biotic or abiotic surfaces. Several microbial strains occur co-culture in the biofilm layer. The most important effect of biofilm is changing of microbial behaviours and therefore they become more resistant to inadequate and/or adverse conditions [1]. Drug resistance and resistant pathogen occurrence are some results of mutualism among the microorganisms embedded in the biofilm matrix. The biofilm formation increases the virulence of pathogen microorganisms. Therefore, in most cases, biofilm formation is considered as a reason of infections [1], [2]. In the food industry, foodborne pathogens and food spoilage microorganisms can be accumulated on the surfaces especially such as elbow-shaped pipes, corners of the conveyor bands and etc. that the places hard-to-reach for cleaning. This microbial accumulation results in the attachment of the microbial cells to the surfaces. Biofilm formation and cell-to-cell communication make the community more resistant to the disinfectants. The biofilm layer formed throughout the pipelines causes the decrease of liquid flow and with the increase in the volume of biofilm matrix the flow is blocked completely. Furthermore, the liquid foodstuff is contaminated by microorganisms embedded in biofilm layer [2], [3].

The biofilm formation is observed in lactic acid bacteria and yeast cultures which are used as starter cultures in the production of especially traditional foods. Dairy products have been known for their beneficial properties on human health and the dairy products include several lactic acid bacteria and yeast strains.

In this study, two methods were performed to determine the biofilm formation capacity of selected and identified 10 LAB and 10 yeast strains. The results obtained from both "Tube method" and "96-well plate-based method" and the validity of the methods were compared. It has been known that "Tube method" is a qualitative method for biofilm detection whereas the 96-well plate method is a quantitative method [4].

II. Material and Methods

A. Cultures

Yeast and LAB strains were isolated from various kinds of cheeses, unpacked yoghurt and Kefir samples which are exposed for sale in bazaars. One of the kefir samples was obtained from Uludag University Food Engineering Department. The microbial strains were identified as *Enterococcus durans, Enterococcus hirae, Enterococcus feacium, Lactobacillus curvatus, Lactobacillus plantarum, Lactococcus lactis, Lactobacillus graminis, Lactobacillus sakei* and *Leuconostoc mesenteroides*. Different *Saccharomyces cerevisiae* strains were isolated from kefir and some cheese samples.

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B. Isolation and Identification of Yeast and Bacteria Strains

Dairy food samples were obtained from bazaars and the Uludag University Food Engineering Department. The isolation of strains was carried out by surface plate method. Bacteria cultures were spread on MRS Agar (Merck, Germany), and yeast cultures were spread on Malt Extract Agar (Merck, Germany). After the appropriate incubation period of each culture, the colonies were picked and the macroscopic and microscopic morphological properties examined. Gram and Catalase reactions of bacterial cultures were also determined. Regarding their microscopic properties, colonies were purified by streaking on their individual agar medium, followed by transferring them to the cryovials including individual broth mediums and glycerol at 30% concentration. These stock cultures in cryovials were stored in the freezer at -80 °C [5].

Isolated bacteria and yeast strains were identified by the PCR amplification described by [6], [7].

C. In-vitro Assay for Determination of Biofilm Forming Capacity of Yeast and LAB Strains

Monitoring biofilm formation of each selected strains in the present work was performed by two different methods described by [4], [8], with slight modifications. The applied methods were known as “Tube Method” and “Tissue Culture Plate (96-well plate) Method”.

1. Tube Method

In this study, “Tube method” was performed described by [4].

Isolated and identified test microorganisms were kept and stored at -80°C as a stock culture in cryovials until they were examined to determine their biofilm forming capacity. Test microorganisms were activated by transferring a loopful from each stock culture to sterile individual broth medium. All inoculated tubes were incubated at 30 °C for 18-24 hours. After the incubation period, the biofilm formation capacity of each fresh culture was examined. According to the method, 100 µL of each fresh culture was inoculated in 10 mL of Malt Extract Broth (MEB) for yeast strains and MRS broth for LAB strains in test tubes. The tubes were incubated at 30 °C and 37 °C for 24 hours and 48 hours. After incubation, the tubes were decanted and washed with sterile phosphate buffer saline (pH 7.3) and dried. The tubes were then stained with crystal violet (0.1%), and excess stain was washed with deionized water. The tubes were dried in the inverted position. The scoring for tube method was performed according to the results of the negative control tubes including only individual broth medium. Biofilm forming ability was detected regarding occurrence of a visibly stained film at the bottom or/and on the walls of the tubes. Such strained layer formation was accepted as biofilm positive. The amount of biofilm formed was scored as 0-none, 1-weak, 2-moderate, 3-high. The experiment was repeated in triplicate.

2. Tissue Culture Plate (96 well-plate) Method

In this study, 96 well-plate method was performed, as described by [8]. According to the method, fresh cultures of each yeast and LAB strain were diluted 1:100 into sterile MEB and MRS broth, respectively. Diluted cultures were transferred to each well as 100 µL, and were grown in 96-well microtiter plates at 30 °C and 37 °C for 24 hours and 48 hours. Negative control wells contained individual broth medium only. After the incubation, total cell mass was measured as absorbance at 630 nm in a spectrophotometer (BioTek, PowerWave HT Microplate Spectrophotometer). The plates were decanted and the wells were washed by submerging into the distilled water, followed by drying at 37 °C for 30-45 min. Dried plates were stained by addition 125 µL of a 0.1% crystal violet solution (in deionized water) to each well of the microtiter plate. Stained microtiter plates were incubated at room temperature for 20 min. During the staining period, the biofilm layer formed by test microorganisms in the wells held the crystal violet solution in different amounts according to the thickness of the biofilm layer. It has been known that the dye bound to adherent cells in the wells can be resolubilized and measured in optical density with the spectrophotometer [9]. In this method, unbound crystal violet solution was removed completely from the wells by rinsing the plates with distilled water, followed by drying at 37 °C for 30-45 min. 100 µL of 95% ethanol was transferred to each well for the resolution of the attached dye. After 30 min., dissolved dye solution in ethanol found in the each well was transferred, respectively, to a clean microplate and the absorbance at 492 nm was measured by microplate spectrophotometer. The experiment was repeated in triplicate. The ratio of absorbance at 492 nm and 630 nm was named as “B” represents the level of biofilm formation [8].

The ratio was measured according to the formula:

$$“B = A_{492}/A_{630}”$$

Test microorganisms were categorized according to the scale values of B. The scale values were considered as:

- $B < 0.1$ (non-biofilm producer),
- $0.1 \leq B < 0.5$ (weak biofilm producer),
- $0.5 \leq B < 1$ (moderate biofilm producer),
- $B \geq 1.0$ (strong biofilm producer).

III. Results and Discussion

A. Biofilm-Forming Ability of LAB and Yeast Cultures by Tube Method and Tissue Culture Plate (96-well plate) Method

In-vitro determination of biofilm forming capacity of the strains was carried out by two different methods. The first one is a qualitative method (tube method) grounded on naked-eye observation of the visibly stained biofilm layer on the wall and the bottom of the tube. The second one is a quantitative method (96-well plate method) which gives numerical results measured spectrophotometrically, which is grounded on the optical density of the attached crystal violet solution to the biofilm matrix.

1. Comparison of the Methods for the Detection of Biofilm Formation in LAB Strains

In this research, biofilm formation capacity of LAB strains

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was observed by both Tube method and 96-well plate method. Biofilm forming scores and their explanations are seen in Table I.

The LAB strains and their biofilm formation capacities were demonstrated as "B" ratio (A492/A630) in Table I.

TABLE I
COMPARISON OF BIOFILM FORMATION LEVELS OF LAB STRAINS

Strain Codes	24h-Incubation Biofilm formation level				48h-Incubation Biofilm formation level			
	Tube 30°C	TCP 30°C	Tube 37°C	TCP 37°C	Tube 30°C	TCP 30°C	Tube 37°C	TCP 37°C
LB1	1^+	$0,0^-$	1^+	$0,1^+$	0^-	$0,0^-$	0^-	$0,0^-$
LB2	1^+	$0,0^-$	1^+	$0,1^+$	0^-	$0,0^-$	0^-	$0,0^-$
LB10	1^+	$0,0^-$	1^+	$0,1^+$	0^-	$0,0^-$	0^-	$0,0^-$
25	1^+	$0,1^+$	1^+	$0,7^{++}$	1^+	$0,3^+$	1^+	$0,3^+$
29	3^{+++}	$0,2^+$	1^+	$0,4^+$	3^{+++}	$0,4^+$	2^{++}	$0,9^{++}$
32	1^+	$0,1^+$	1^+	$0,4^+$	1^+	$0,4^+$	2^{++}	$0,9^{++}$
40	1^+	$0,1^+$	1^+	$0,4^+$	3^{+++}	$0,2^+$	2^{++}	$0,6^{++}$
41	1^+	$0,1^+$	0^-	$0,0^-$	3^{+++}	$0,2^+$	1^+	$0,9^{++}$
42	1^+	$0,1^+$	2^{++}	$0,8^{++}$	3^{+++}	$0,2^+$	1^+	$0,7^{++}$
43	1^+	$0,0^-$	0^-	$0,0^-$	1^+	$0,0^-$	1^+	$0,4^+$

The level of the biofilm formations of each strain for both methods (tube method, 96-well plate method (TCP)) were demonstrated as; "-" (non-biofilm producer), "+" (weak biofilm producer), "++" (moderate biofilm producer), "+++" (strong biofilm producer); LB1: *E. durans*, LB2: *E. hirae*, LB10: *E. feacium*, 25: *Lb. curvatus*, 29: *Lb. plantarum*, 32: *L. lactis*, 40: *L. lactis*, 41: *Lb. graminis*, 42: *Lb. sakei*, 43: *L. mesenteroides*

As it has been demonstrated in Table I, some results of biofilm forming capacity in the same LAB strains was observed similarly in both methods, whereas some of them were different from each other. According to the results of tube method, one of a LAB strains *E. durans* (LB1) formed a weak biofilm layer at 30°C after 24-hour incubation period; however, the rational result of absorbance in the 96-well plate method demonstrated that *E. durans* (LB1) could not form the biofilm layer. Similar results have been observed in *E. hirae* (LB2), *E. feacium* (LB10) and *L. mesenteroides* (43). On the other hand, the biofilm formation level of LB1, LB2, LB10 and 43 was found similar in both methods at 37°C after a 24-hour incubation period.

According to the tube method results, some strains could form a biofilm layer at the 24th hour of the incubation whereas, after 48 hours, their biofilm layer was not observed in the tubes. LAB have the ability to ferment carbon sources and invert them into mainly lactic acid. The extension of the incubation period could cause the inhibiting effect on the biofilm layer because of the accumulation of lactic acid in the medium. It has been reported that lactic acid reduced the biofilm formation. This reduction is a result of decreasing pH in the medium. Thus, all organic acids produced by microorganisms such as citric, malic, gallic acids have a role of removal the biofilm layer formed on the surfaces [10], [11].

In the present experiment, it has been determined that the biofilm layer formation increased in some cultures incubated at 37°C compared with 30°C, whereas some could not form the biofilm layer when they were incubated at 37°C. The biofilm formation required various conditions which have to be changed regarding the microbial characteristics.

2. Comparison of the Methods for the Detection of Biofilm Formation in Yeast Strains

In this research, biofilm formation capacity of yeast strains was observed by Tube method and 96-well plate method. Biofilm forming scores and their explanations are seen in Table II.

In some yeast strains, various differences have been observed between the Tube and 96-well plate method for the biofilm forming level of the same strains such as Y1, Y2, Y3, Y4 and Y5 incubated at both 30°C and 37°C for especially 24 hours.

The yeast strains and their biofilm formation capacities by 96-well plate method were demonstrated as "B" ratio (A492/A630) in Table II.

In this experiment, the biofilm formation levels of different *S. cerevisiae* strains were examined. Biofilm layer seems to be removed after 48-hour incubation period at 37°C for all strains of *S. cerevisiae*. It has been thought that the biofilm layer formed by yeast strains could increase under stress conditions.

The temperature changes are one of stress conditions. As it has been demonstrated in Table II, the increment of temperature from 30°C to 37°C was likely a stress factor for *S. cerevisiae* strains incubated for 24 hours, because the biofilm formation level increased during this period. However, after 48 hours at 37°C, biofilm formation levels of almost all yeast strains showed a slight decrease.

TABLE II
COMPARISON OF BIOFILM FORMATION LEVELS OF YEAST STRAINS

Strain Codes	24h-Incubation Biofilm formation level				48h-Incubation Biofilm formation level			
	Tube 30°C	TCP 30°C	Tube 37°C	TCP 37°C	Tube 30°C	TCP 30°C	Tube 37°C	TCP 37°C
Y1	1^+	$0,2^+$	0^-	$0,5^{++}$	0^-	$0,2^+$	1^+	$0,1^+$
Y2	1^+	$0,3^+$	0^-	$0,6^{++}$	0^-	$0,2^+$	0^-	$0,3^+$
Y3	1^+	$0,2^+$	0^-	$0,6^{++}$	0^-	$0,2^+$	0^-	$0,2^+$
Y4	1^+	$0,1^+$	1^+	$0,5^{++}$	1^+	$0,1^+$	0^-	$0,2^+$
Y5	0^-	$0,2^+$	0^-	$0,4^+$	0^-	$0,2^+$	1^+	$0,1^+$
Y7	0^-	$0,3^+$	0^-	$0,6^{++}$	1^+	$0,1^+$	1^+	$0,1^+$
Y9	1^+	$0,1^+$	1^+	$0,3^+$	1^+	$0,1^+$	0^-	$0,1^+$
Y16	0^-	$0,1^+$	0^-	$0,0^-$	1^+	$0,1^+$	0^-	$0,1^+$
Y27	1^+	$0,1^+$	1^+	$0,1^+$	1^+	$0,1^+$	0^-	$0,0^-$
Y28	1^+	$0,1^+$	1^+	$0,2^+$	1^+	$0,1^+$	0^-	$0,1^+$

The level of the biofilm formations of each strain for both methods (tube method, 96-well plate method (TCP)) were demonstrated as; "-" (non-biofilm producer), "+" (weak biofilm producer), "++" (moderate biofilm producer), "+++" (strong biofilm producer); "Y1, Y2, Y3, Y4, Y5, Y7, Y9, Y16, Y27, Y28": *Saccharomyces cerevisiae*

S. cerevisiae strains produce alcohol because of their fermentative activity on carbon sources such as maltose, glucose, sucrose, and fructose, etc. The carbon sources have been metabolized by *S. cerevisiae* strains into alcohol and carbon dioxide, basically [12]. According to the previous studies, the morphological properties and biofilm forming capacities of yeast strains can change regarding the presence of various alcohols in the growth medium. Attachment to the biotic/abiotic surfaces in yeast cells is affected by methanol, ethanol, propanol, and butanol even the alcohols are known as

own metabolites of the yeast strains [13]. Thus, the alcohol production was going on during the incubation period. An increasing the amount of alcohol in the medium could degrade the biofilm layer formed by yeast cells at 48th hour although the biofilm levels of the strain had been measured as moderate/high levels at the 24th hour.

B. Validity of Tube Method and 96-well Plate Method

In extreme or stress conditions, some microorganisms may survive, but they may lose their biofilm forming ability. Therefore, the optical density of cell mass increase during the incubation period, but the microbial biofilm formation might be inhibited by stress factors [14]. The progressing microbial growth and definite blocking the biofilm formation could result in a decrease of biofilm forming levels at the 48th hour compared to the 24th hour.

The differences of the results between the Tube and 96-well plate method in biofilm formation abilities of the same strain could be explained that; in tube method, the enumeration was not performed after the incubation periods. The visibly stained biofilm layer could be observed as a moderate/high level by a naked-eye view. However, it has been thought that the visibly stained layer may not mean that the strain could be accepted as an appropriate biofilm producer. The ability of the biofilm forming level is dependent directly on the ratio of the volume of attached biofilm layer and the cell mass found in the medium. The method was not found as the appropriate method to determine precise and reliable results. Fig. 1 presents the visibly stained biofilm layer formed by one of the LAB strains determined by Tube and 96-well plate method.

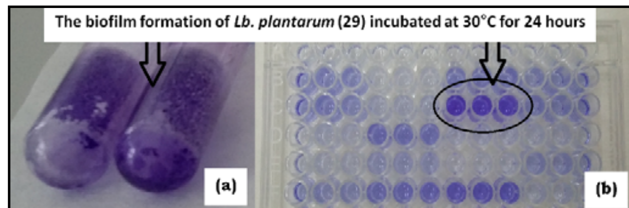

Fig. 1 Biofilm formation capacity of *Lb. plantarum* (29) incubated at 30°C for 24-hours; (a): Tube method; (b) 96-well plate method

Biofilm formation could be a desired property in LAB growth; LAB strains are commonly used as a starter culture in, especially traditional food products. They promote the sensorial and technological values of the food product. Furthermore, the food product becomes more resistant against microbial spoilage, and therefore, shelf-life could be extended by using the LAB cultures and their metabolites in foods. On the other hand, most LAB have been known as *probiotic* cultures. Because of these properties of LAB strains, the increase of their resistance and viability under harsh conditions (lack of nutritional compounds, extreme temperature, pressure and etc.) make them more suitable to use as a starter culture in the food industry. Additionally, the biofilm formation by probiotic microorganisms could promote the colonization and provide the permanence as dominant microflora over time in host mucosa [15]. In this regard, the biofilm brings LAB strains different properties and behaviours to survive under adverse conditions. Thus, the biofilm forming capacity becomes more important in the food industry. In the human gastrointestinal system, probiotic LAB strains in particular have an important role. More resistant probiotic strains are required to create the microbial flora found in the intestinal system to fight against pathogens. Microbial strains have more resistance in the biofilm layer; in this circumstance, the biofilm formation should be continuous.

In the present study, the most resistant strains against acidic, alcoholic conditions were determined by Tube and 96-well plate methods. According to the results of the 96-well plate method, resistant strains were selected among the yeast and LAB strains which are formed biofilm aggregates remained constant after 48-hour incubation period. Thus, the permanent and resistant biofilm formation produced by yeast and LAB strains has been determined. In this regard, more resistant strains could be determined and marked because they are able to form biofilm matrix even after incubation period for 48 hours. Fig. 2 represents the results of the biofilm forming capacities of *S. cerevisiae* and LAB strains examined by 96-well plate method.

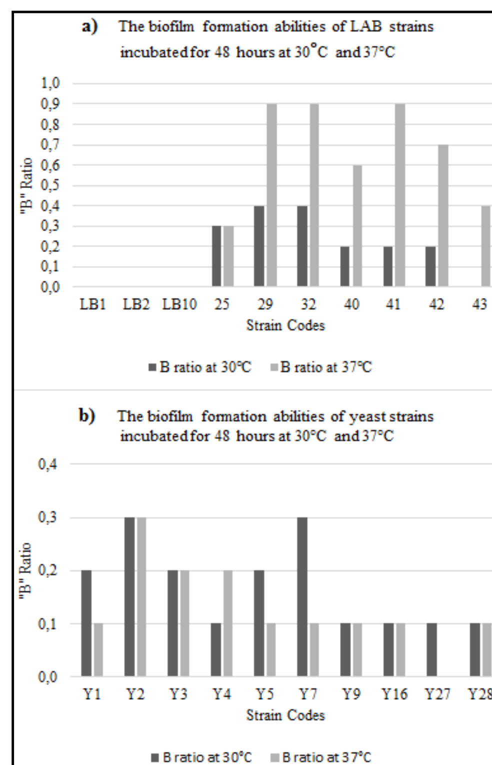

Fig. 2 Biofilm formation levels of all selected microorganisms determined by microplate plate method ((a): LAB strains; (b): *S. cerevisiae* strains) incubated at 30°C and 37°C for 48 hours

The results of 96-well plate method demonstrated that biofilm forming abilities of LAB strains increased at 37°C, whereas *S. cerevisiae* strains could create biofilm matrix in optimal growth conditions. In the stress conditions, yeast

strains had less ability to produce a biofilm layer.

In conclusion, when the phenotypic methods (Tube and 96-well microplate) were compared, it was observed that the sensitivity was different between the two methods. It was difficult to determine moderate, weak and none of biofilm formation by Tube method because it is based on the observation by a naked eye. According to the results of this research, 96-well plate (Tissue culture plate) method was an ideal biofilm detection method because of its sensitivity and reliability. However, it has been thought that the Tube method could be modified by measuring the cell mass density before examination for their biofilm forming capacity. Thus, the level of stained biofilm layers formed the wall and bottom of the test tubes may be explicated by correlating the cell concentration during the examination of the cultures for their biofilm forming capacity.

The next step in studies may be comparison between the phenotypic methods and molecular methods to determine the most sensitive phenotypic method in biofilm formation of microbial strains.

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