



MICROBIAL SPOILAGE IN BEER PROCESSING BY BIOFILMS

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SYNOPSIS

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disinfection.

We have study some aspects of biofilm's presence in brewery. The main objective was the characterization of microorganisms associated with these communities. This was study by cultivation techniques in selective medium to reveal the compositions of biofilms. They harbor Gram negative bacteria like: *Enterobacter spp*, *Escherichia spp*, *Pseudomonas spp* as well as Gram positive bacteria like *Actinomycetes*, *Bacillus*, *Micrococcus*, *Lactobacillus*. Yeasts were found to constitute an important part of community in many mature brewery biofilms.

Also the localization of some dangerous spots as well as their resistance against the common used disinfectants was study. Since the biofilms' microorganisms can affect directly to quality of beer, the beer-spoiling potential of isolated strains was defined through the microbiological analyses.

INTRODUCTION

Microbial spoilage is a constant concern in the food processing industry. Even breweries have the risk of contamination of their product, although beer is very restrictive to bacteria due to the low pH value (3.8-4.7), the concentration of hop bitters and the content of ethanol (0-4,5% w/v) (Jespersen & Jakobsen, 1996). Therefore, the beer-spoiling organisms are limited to a few genera. The most important of them are *Lactobacillus brevis*, which accounted nearly the half of the analyzed contaminated samples (Back, 2003), and *Pediococcus damnosus* which were regularly identified and constituted higher parts among the beer-spoiling organisms. An infection of beer during the production process is defined as a primary contamination (Back, 2003). Since all beer-spoiling bacteria are very sensitive in relation to heat and disinfectants, they are supposed to survive only in

combination with more resistant microorganisms (Storgards, 2000). The biofilms are considered as a reservoir of beer-spoiling organisms that potentially damage the final product. Out of the biofilm they can be carried off by persons, splash or movements of the air to all parts of the plant and they might get into the bottles. This would be secondary contamination, which takes place during the filling procedure. Biofilms study is part of the innovative strategies for an efficient and environmentally sound of food quality and safety improvement by minimization of microbial spoilage in food processing and in plant's environment.

Bacterial biofilms formed by *Pseudomonas*, *Escherichia*, *Lactobacillus*, *Acetobacter* and *Saccharomyces* yeast have been most frequently and well-known in breweries (Zottola & Sasahara 1994). Bacteria growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the same bacteria growing in a free-swimming state. For example, chlorination of a biofilm is usually unsuccessful because the biocide only kills the bacteria in the outer layers. The bacteria within the biofilm remains healthy, and the biofilm can develop again (Wirtanan, 1995). Repeated usage of antimicrobial agents can cause an increased resistance to biocides of bacteria within the biofilm. The huge doses of antimicrobials required to destroy biofilms are environmentally undesirable. So new strategies based on a better understanding of how bacteria is attached, grown and detached are urgently needed by many industries (Carpentier & Cerf, 1993).

The main purpose of this study was to reveal the composition of microbial communities of brewery biofilms. Also non beer-spoiling bacteria or yeast can be identified, furthermore, they can be harmful for the breweries, if they are able to form biofilms. In addition, the localization of some dangerous spots may lead to a more economic use of energy and chemicals in the brewing hygiene and food industry.

MATERIALS AND METHODS

SAMPLING. In the brewing plants, bottling area has complex machines with a lot of niches in which biofilms can develop. They can harbor beer-spoiling organisms and protect them against heat and disinfectants. Most part of samples for analyses were taken from machines and places in the filling area, in the conveyer, in the bottle washing machine or drainage pits, inside the filling machine. The conveyor from the bottling plant is in permanent contact with beer, and the transport activity of them might even distribute microorganisms throughout the brewery. For all these reasons, conveyors have been recognized as one of the most critical spots for potential contamination and therefore are considered in the present study. Often the sample materials may contain a high proportion of lubricants. To prevent abiotic compounds, these were removed by washing twice with 4 ml sterilized water and 4

ml decane on end-over-end rotator. The liquid phase was separated by centrifugation, rejected and the cells were stored.

Biofilm's samples were collected by sterilized spatulas, and transferred into sterile tubes and stored at 4°C until processing (Jespersen & Jakobsen, 1996). Samples were taken on two different periods, on August and on February from the same locations.

CULTIVATION AND ISOLATION. To reveal the composition of biofilm's samples microorganisms was used the cultivation techniques in standard and selective medium. The samples were suspended in 0.9% Ringer solution, plated on Petri dishes and isolated until pure cultures were achieved (Timke, 2004). Media used were TSA (trypticase soy agar), WA (wort agar), PDA (patato dextrose agar), WURTZ, Mac Concey, MRS (DeMan-Rogosa-Sharpe); NBB, Difco WL nutrient agar, DSM 254 medium -*Acetobacter peroxydans* medium (Oxoid manual, 1996).

MRS agar plates were incubated in an anaerobic jar at 30°C. The other media were used under aerobic conditions at 25°C. At first all samples materials were examined for growth on TSA, while the others medium were used as selective to detect the special groups of present microorganisms. Some different methods were used to get information about the diversity of the isolated yeasts. So WA medium was used for isolation and cultivating of all yeast and moulds, while WA¹ with cristal violet, is a selectiv one for wild *Saccharomyces* yeast, and WA² midium with CuSO₄ detect the wild non *Saccharomyces* yeast. This technique is widely used in breweries (Jespersen & Jakobsen, 1996).

MRS agar was recommended for isolation, enumeration and cultivation of *Lactobacillus* species. Wurtz medium was used for isolation and cultivating of *Enterobacteriaceae* members, and Mac Concey was used for cultivating of gram-negative, lactose-fermenting bacteria in water and foods as a presumptive test for coliforms (Oxoid Manual, 1996). NBB or Difco WL nutrient agar, was recommended for detect other beer spoilage potential bacteria during the fermentation. The presence of actidion make it a selektiv medium (Pirttjarvi et al., 1996).

The direct microscopic examination of biofilm's samples was done first to distinguish the type of microorganisms. Their morphological properties were study applied the Gram staying technique to differentiate Gram-positive and Gram-negative bacteria based on the chemical properties of their cell walls.

RESULTS AND DISCUSSIONS

Microbiological analyses of biofilm's samples was done first by direct microscopic examination of their slides. This is important to look the cells of microrganisms in nativ form and to detect the motility. The most of samples was located around the threat or dead areas of a screw with a diameter of approximately

4 cm. The biofilm was about 1-2 mm high and 6 mm in width, therefore it was considered as a mature one. In contrast to this sample location, the screw biofilm was only accessible by removing the screw. Because of its hidden position, it was not exposed directly to the cleaning procedures.

The samples presented slimy materials, light cream color to red until black. The biofilm's color change according to the age or the maturation state. Based on these examinations the most frequent microorganisms were the yeasts of fermentation and the wild yeasts with tendency to form pseudomycel. Also was detected in abundance the presence of filamentous bacteria Gram positive, mobil rods Gram negative bacteria, cocci Gram positive, and hyphae of moulds mycelium (fig 1).

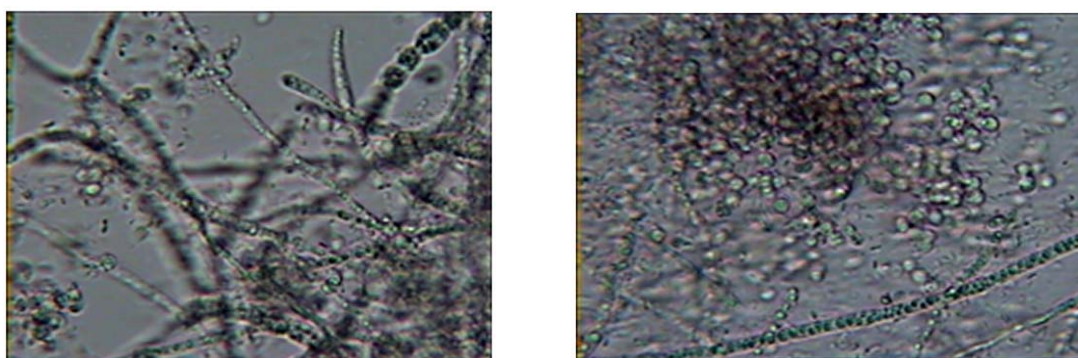


Figure 1: View of biofilms slides by microscopic examination (X 1500).

Regarding the results of cultivation method on respective mentioned media, the composition of biofilms was presented by the diversity of microorganisms. From the different media there was isolated lots of strains with different morphology of colony. They were grouped based on phenotypic and morphological characteristics. Isolates were the colonies developed in TSA media. Further isolations were obtained from turbid swab samples in NBB and they were grown on selective such as MRS or others mentioned medium, by plate count cultivation method (Wirtanan, 1995).

Then the morphological characteristics was evaluated from the microscopic examination of cells by pure isolated cultures. The most important were the physiological characteristics, for the identification of isolated bacteria which concern in their enzymatic activity of catalase and oxidase. These were done according to the rapid respective tests.

The data of analyses by cultivation method are presented in the table 1 and table 2, where the microorganisms divided in distinguish groups are listed. Samples were taken from the conveyor I that carries clean bottles to the bottling plant and from the conveyor II that transports filled bottles for packaging, also from washing and filling machines.

Table 1: Clasification of bacteria by biofilm's samples according to cultivation method.

Medium	Location of samples			
	Conveyor I	Washing machine	Conveyor II	Filling machine
TSA	Filamentous Gram+ <i>Actinomyces</i> rods/G-/kat+/ox+ <i>Pseudomonas</i>	Mobil rodsG-/kat+/ox+ <i>Pseudomonas</i> cocci G+/kat-/ox+ <i>Micrococcus</i>	<i>Actinomyces</i> <i>Bacillus</i> <i>Micrococcus</i>	<i>Diplococcus</i> sporogen/rods G+/ox+ <i>Bacillus</i>
WURTZ	rods/G-/kat-/ox+ <i>Enterobacter spp</i>	Mobil rods/G-/kat- <i>Enterobacter</i>	Mobil rods/G-/kat- <i>Enterobacter</i>	Mobil rods/G-/kat- <i>Enterobacter</i>
MRS	Non motil rods/G+/kat- <i>Lactobacillus</i>	rods/G+/kat- <i>Lactobacillus</i>	cocci/G+/kat- <i>Leuconostoc</i>	Bacil/G+/kat- <i>Lactobacillus</i>
Mc Conkey	Mobil rodsG-/kat-/ox- <i>Escherichia</i>	<i>Escherichia</i>	<i>Escherichia</i> <i>Aerobacter</i>	<i>Escherichia</i>

An abundant proportion among the isolates was constituted by members of *Enterobacteriaceae*.

Different microbial communities at the two conveyors may be related to variable conditions during the filling procedure, e.g. the influence of over foamed beer.

Table 2: Clasification of yeasts and moulds by biofilm's samples according to cultivation method.

Medium	Location of samples			
	Conveyor I	Washing machine	Conveyor II	Filling machine
WA ¹	<i>Wild yeasts</i> <i>Saccharomyces</i> pseudomycel	<i>Wild yeasts</i> <i>Saccharomyces</i> and moulds	<i>Wild yeasts</i> <i>Saccharomyces</i>	<i>Wild yeasts</i> <i>Saccharomyces</i> pseudomycel
WA ²	<i>Yeasts non</i> <i>Saccharomyces</i> <i>Candida</i>	<i>Yeasts non</i> <i>Saccharomyces</i>	<i>Candida</i> and moulds	<i>Yeasts non</i> <i>Saccharomyces</i>
PDA	moulds: <i>Geotricum</i> Fungi imperfecti	moulds <i>Tricoderma</i>	Moulds different genera	<i>Geotricum</i> and different genera

The variation in population composition from the same sample's site could be due to changes in operating conditions. Specifically, bottling capacity is reduced in wintertime, consequently there is less overflow of beer and the cleaning is more intense. The bottling plant was completely cleaned after taking the samples in August. Therefore, the biofilms from January were approximately 5 months old and were thus younger than those from August.

Relating of these data all biofilms were dominated by mycelium of different moulds, specially from *Geotricum* and *Trichoderma* genera, as well as wild yeasts *Saccharomyces* and non *Saccharomyces* yeasts.

The beer-spoiling potential of biofilm communities was investigated by inoculating them in beer. Growth of the isolates in a selective medium and in beer. The isolated strains of each group were tested for growth in NBB broth medium which contains a pH-indicator in order to detect acidification. The tubes were incubated at 25°C in an upright position without rotation. The test result was positive if the medium showed turbidity. In addition, acidification was noted (Storgards, 2000). Furthermore, the strains were inoculated in fermented wort and beer with 4.8% (v/v) ethanol and incubated in an anaerobic jar. The data of this analyze are shown in table 3.

Table 3: Determination of beer-spoiling potential microorganisms from biofilm's isolated.

Strains isolated from different medium	The cultivation test in NBB	The cultivation test in wort during fermentation	The cultivation test in beer
TSA 10 strains	8 strains A+/2 strains T+	+ 4 strains	-
WURTZ 10 strains	10 strains A +	+10 strains	-
MRS 10 strains	10 strains A +	+10 strains	7 strains - / 3 strains +
WA ¹ 10 strains	6 strains T+	+5 strains	-

T + is result positive, growth with turbidity

A+ result positive, growth with both turbidity and acidification

- is result negative - non growth in wort or beer + is result positive, growth in wort or beer.

Regarding these data the microbial analyzes indicated that the breweries biofilms contained microorganisms (bacteria and wild yeasts) which caused turbidity in NBB bouillon and some of them acidification. Particularly all isolated from WURTZ medium of the family *Enterobacteriaceae*, and from MRS of genus *Lactobacillus*, which were able to multiply all in NBB medium and in wort during the fermentation. So they can be denominated as beer spoiling potential. But these enrichments allowed the detection of minor proportions of beer-spoiling organisms. Only 3 strains by the biofilms isolated from MRS medium were able to multiply in beer with 4.8% of ethanol (v/v). By the identification test these represented strains of *Lactobacillus* genera (2 isolated) and one of *Leuconostoc* genera. The acetic acid bacteria, were supposed to be abundant brewery biofilm organisms, but this was not confirmed by any test of the cultivation used, even on selective medium.

CONCLUSIONS

The biofilms are present in brewery area, specially in filling plants and have the risk of contamination of their product. They harbor spoiling potential microorganisms.

The brewery biofilms were dominated by ubiquitous slime forming members of the *Enterobacteriaceae*, *Pseudomonadaceae* and filamentous bacteria of *Actinomycetes*, as well as accompanying organisms like *Micrococcus*, *Bacillus*.

At the beginning of this study, there was a suspicion concerning their beer-spoiling potential. Indeed, all isolated *Enterobacteriaceae* were found to be able to multiply in wort during the fermentation or non-alcoholic beer under access of oxygen but they represented no risk for bottle beer.

An abundant proportion among the isolates was constituted by members of *Lactobacillus* and *Leuconostoc* genera. This study picked up the fact that they represented the most important beer-spoiling organisms, because they were able to grow in beer with 4.8% of ethanol (v/v).

Others important members of biofilms were wild non *Saccharomyces* yeasts and certain moulds, like *Geotricum* and *Trichoderma*, specially in mature biofilms.

The biodiversity of biofilm's composition will mean the presence of various genotypes that make their populations to react in different ways against the cleaning and disinfection procedures. So the biofilms formed must be constantly disinfected and removed in order to prevent maturation that makes them more resistant. In that time they must be a risk for the contamination of the environment and the product.

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