Chapter 15

Exopolysaccharides from Bacteria with Novel Application

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Additional information is available at the end of the chapter

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Abstract

The physiological role of EPS depends on the ecological niches and the natural environment in which microorganisms have been isolated. In this chapter, data on EPS production and the effect of EPS on corrosion of steel produced by *Lactobacillus* sp. are presented and discussed. *Lactobacillus plantarum* Ts was obtained from the Collection of Department of Biology, Shumen University. It was tested for its ability to produce exopolysaccharides when cultivated in a medium containing 10% sucrose. It could be underlined that 10% sucrose in the medium stimulated the process of protection of corrosion. Also, the biofilm in vitro in the combined cultivation of *Staphylococcus aureus* and the *Lactobacillus plantarum* Ts probiotic bacterium on the surface of different metal materials for fixed dental prostheses was investigated [unpublished results]. The structure of layer over steel plates was analyzed by scanning electron microscopy (SEM) JSM 5510. In our opinion, more detailed research is needed to be done in the future, and the possibilities should be analyzed for the creation of a thin biofilm from a probiotic bacterium or an exopolysaccharide this bacterium has produced, which would protect the implants against the growth of a pathogenic biofilm.

Keywords: exopolysaccharides, corrosion, microbial biofilms

1. Introduction

According to Reyes et al. reported one of the most complex topics within bacterial anatomy and physiology is that of exopolysaccharides (EPSs). These molecules have various structures and functions and also provide different types of advantages to their producing microorgan-

isms, including surface variability, resistance to innate and acquired immunity mechanisms, the ability to adhere to different surface and cell types, and resistance to antibiotic activity [1].

Exopolysaccharides (EPSs) are a term first used by Sutherland [2] "to describe high-molecular-weight carbohydrate polymers produced by marine bacteria." EPSs can be found as in capsular material or as dispersed slime in the surrounding environment with no obvious association to any one particular cell [3].

Many microorganisms produce exopolysaccharides as a strategy for growing, adhering to solid surfaces, and surviving adverse conditions.

Considerable progress has been made in discovering and developing new microbial EPSs that possess novel industrial significance [4].

Bacterial EPSs by Reyes [1] are "believed to play an important role in the environment by promoting survival strategies such as bacterial attachment to surfaces and nutrient trapping, which facilitate processes of biofilm formation and development. These microbial biofilms have been implicated in corrosion of metals, bacterial attachment to prosthetic devices, fouling of heat exchange surfaces, toxicant immobilization, and fouling of ship hulls." Corrosion of metals is one of the most serious and challenging problems faced by industries worldwide. Biofilms composed of a secreted polymeric substance containing microbial population have shown to inhibit corrosion in metals [5, 6]. Fang et al. and Chongdar et al. reported that "kinetics of corrosion processes of metals, mineral, and polymeric materials can be influenced by biofilms. Products of their metabolic activities including enzymes, exopolymers, organic and inorganic acids, as well as volatile compounds such as ammonia or hydrogen sulfide can affect cathodic and/or anodic reactions, thus altering electrochemistry at the biofilm/metal interface. This phenomenon is often referred to as 'biocorrosion' or 'microbially influenced corrosion'. Microbiologically, influenced corrosion has been documented for metals exposed to sea water, fresh water, demineralized water, process chemicals, food stuffs, soils, aircraft fuels, human plasma, and sewage" [7, 8].

In this paper, data on EPS production and the effect of EPS on corrosion of steel produced by *Lactobacillus* sp. are presented and discussed. The adhesion in the combined cultivation of *Staphylococcus aureus* and the *Lactobacillus plantarum* Ts probiotic bacterium on the surface of different metals is presented and discussed. Also, the biofilm in vitro in the combined cultivation of *Staphylococcus aureus* and the *Lactobacillus plantarum* Ts probiotic bacterium on the surface of different metal materials for fixed dental prostheses (Magnum Splendidum; Magnum Ni-Cr-Fe, Ruby Alloy–P, Ruby Alloy–C, and Ruby Alloy) is investigated [unpublished results].

2. Materials and methods

Strains: *Staphylococcus aureus* 745 were obtained from the Collection of the Department of General and Applied Microbiology, Sofia University. The isolate was checked for purity and

maintained in slant of Nutrient agar. Nutrient agar (Biolife 272-20128, Milano, Italia) was the medium used as the growth medium for the microbe.

Lactobacillus plantarum Ts was obtained from Collection of Department of Biology, Shumen University. The strain cultivated in medium of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia). The pH of medium was adjusted to 6.5 with 1M NaOH. The basic medium was sterilized by autoclaving at 121°C for 20 min.

Media for study of corrosion protection: The strain cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia) in composition, g/l: Tween 80–1; peptone from casein–10.0; meat extract–8.0; yeast extract–4.0; K_2HPO_4–2.0; sodium acetate–5.0; ammonium citrate–2.0; $MgSO_47H_2O$–0.2; and $MnSO_4$–0.05. The pH of the medium was adjusted to 6.5 with 1 M NaOH. The basic medium was sterilized by autoclaving at 121°C for 20 min, and carbohydrates supplemented were sterilized using 0.22-μM filters (Manisart®). The basic MRS broth was supplemented with 10% sucrose to be tested [9–11].

Media for study of microbial biofilm: The strain cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia) with 5% sucrose and congo red. Positive results are indicated by black colonies with a dry crystalline consistency.

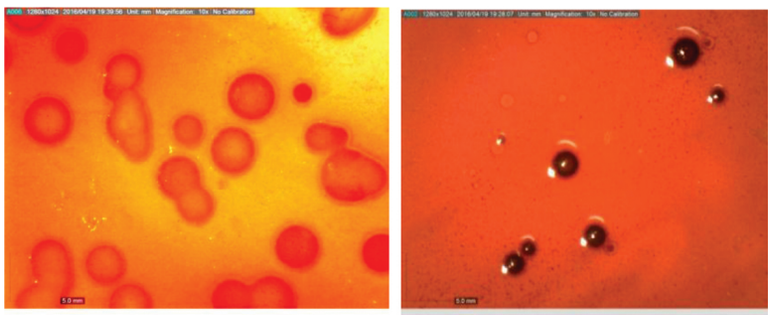
Study of the corrosive stability: The study of the corrosive stability of steel samples was conducted with the gravimetric method. Before use, steel panels (10 × 4 × 0.2 mm) were treated with 70% C_2H_5OH, washed with water and dried in an oven, cooled in a desiccator, weighed on a balance, and kept in a desiccator unit used. The weight of the samples was measured using analytical balances. The dimensions of the samples were measured with micrometer. Initially, the steel samples were added in two variants: deproteinized supernatant and free cell supernatant. Then, the steel samples were added in HCl as control probe, and a dilution (3:100) of the cultural media of the studied strain was added as inhibitor of the corrosion. The duration of the procedure was 120 h at 18°C. After the treatment, the steel samples were washed with water and dried to constant weight. The structure of layer over steel plates was analyzed by scanning electron microscopy (SEM) JSM 5510 [9–11].

Study of bacterial adhesion: Before the assays, the strains *L. plantarum* Ts and *S. aureus* 745 were twice pre-cultured in MRS broth and Nutrient broth, respectively, for 24 h at 37°C. Exponential cultures in broths were used as inoculum for the adhesion analysis.

Preparation of the metal samples: The steel plates made of low carbon steel are weighed with an allowance of 0.0001 g with an assay balance. The precise weighing (with an allowance of 0.0001 g) of the metal plates before and after the treatment found a minimum negative change in their weight, which may be caused by reduction resulting from corrosion processes, on one hand, or growth because of the forming of a biofilm, on the other. They are put sterilely in a liquid ambient which contains a mixture of *L. plantarum* and *S. aureus* 745 in a proportion 1:1. The samples were incubated at 37°C for 24 h. The structure of the layer over the metal plates was analyzed by scanning electron microscopy (SEM) JSM 5510. All experiments were performed in triplicate [12].

3. Results and discussion

Corrosion process causes great economic losses in various industries, shipbuilding, jewelry, archaeological monuments, railway, water channels, and all countries of the world. For handling this problem are normally applied different physical and chemical methods, but they often prove toxic. A perspective in this regard can be the application example of exopolysaccharides produced by the so-called good bacteria–probiotics. The presence of EPS associated with bacterial cells can be recognized by the formation of colonies in mucous solid medium. Therefore, the presence of a translucent or creamy material involving a mucoid colony is indicative of EPS production potential. When cultivated in a medium with high content of saccharides such as 10% sucrose solutions, strain *L. plantarum* Ts synthesized exopolysaccharides (**Figure 1A**).



A B

Figure 1. Congo red agar method exopolysaccharides (EPSs) produced by *L. plantarum* Ts cultivated in a medium containing 10% sucrose, which are secreted in the culture medium. (A) Red colonies show non-EPSs producers; (B) black colonies show biofilm formation of EPSs *L. plantarum* Ts. The pictures were taken using stereomicroscope OPTIKA (Italy).

When they develop microbial biofilm, the organisms are much more resistant and treating them is much more difficult. For investigation of the microbial biofilm, using different methods had been proposed, but in our work, we used the method by using the staining congo red.

"Qualitative assessment of biofilm formation is the microorganisms are grown in agar with 5% sucrose and congo red" [13]. Positive results are indicated by black colonies with a dry crystalline consistency. When cultivated in a medium with high content of saccharides such as 10% sucrose solutions, with 5% congo red, strain *L. plantarum* Ts formed biofilm (**Figure 1B**).

In the presence of high concentrations of sugars (as in our case 10% sucrose), lactic acid bacteria synthesize extracellular exopolysaccharide (**Figure 1A**), which is displayed as mucoid colonies on agar medium. By adding the staining congo red, the exopolysaccharides produced by lactic acid bacteria are displayed in black (**Figure 1B**).

Our studies also show that the use of sugar supplementation (sucrose was normally used though similar results were obtained using 5% glucose) is essential for the detection of slime production using the congo red medium. "The congo red method is rapid, sensitive, and reproducible and has the advantage that colonies remain viable on the medium" [13].

Similar experiments have also been demonstrated by other authors [14, 15]. Homopolysaccharides produced by generally recognized as safe (GRAS) lactic acid bacteria are often synthesized by a single extracellular sucrose enzyme, using only sucrose as substrate [15]. The structure of the layer over the steel plates was analyzed by scanning electron microscopy. The results from this procedure are shown in **Figure 2**.

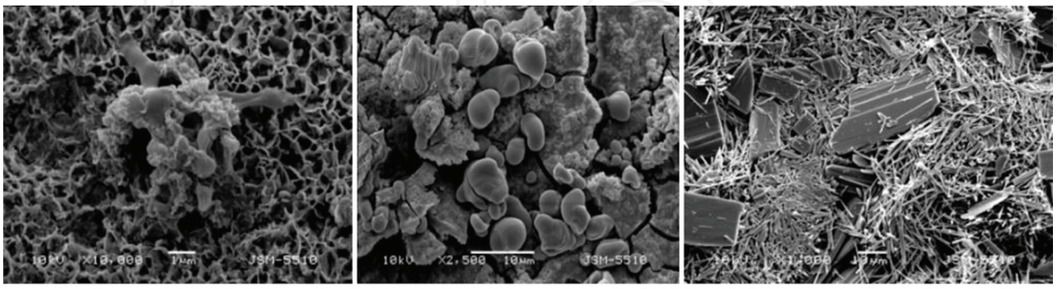


Figure 2. Biofilm formed by *L. plantarum* Ts on the surface of mild steel, visualized using SEM. (A) Biofilm formed by EPS from lactic acid bacteria; (B) biofilm formed by lactic acid bacteria; (C) control–steel plates after corrosion in HCl.

Microscope techniques provide information about the morphology of microbial cells and colonies, their distribution on the surface, and the nature of corrosion products (crystalline or amorphous). They can also reveal the type of attack (e.g., pitting or uniform corrosion) by visualizing changes in microstructure and surface features after removal of the covering and corrosion products (**Figure 2**).

The pictures in **Figure 2B** show that there is a biofilm formed on the steel surface which is an indicator of the good adhesive capacity of *L. plantarum* Ts type. The biofilm makes it not easily corrodible in 10% HCl, supplemented with cultivated ambient from the same strain grown in a composite of 10% sucrose (**Figure 2A**). **Figure 2C** shows a picture of a steel surface sample treated directly with 10% HCl. The observed lamellae are most probably $FeCl_2$ crystals, product of the corrosion.

Microorganisms can interact with the metal surfaces differently. Most often they form biofilms on contacting surfaces, but can also react with various metals to form complex compounds. For this reason, we think that different techniques have to be used to clarify the corrosion process influenced by microorganisms. When the corrosion process starts, the surface of metals is deposited large quantities of ferrousions, which are very harmful for all steel materials. If lactic acid bacteria can immobilized in the microbial biofilm, these ferrousions that could explain why exopolysaccharides produced by these bacteria protect the metal surface from corrosion. Similar to our van Geel-Schutten "biofilm of a polysaccharide-producing culture", delta marina was found to act as a strong corrosion inhibitor with almost complete passivation of mild steel, reducing the corrosion rate by 95% [16].

In our previous studies [9–11, 17–20], it was shown that the presence of high concentration of lactose (5 to 15%), high concentration of sucrose 4%, mixed sucrose 4 and 2% maltose, mixed sucrose 5 and 5% maltose, mixed 5% sucrose and 5% fructose, and mixed 5% sucrose and 5%

fructose, high concentration of lactose, sucrose and fructose (10%) the strains *Lactobacillus delbrueckii* B5, *L. delbrueckii* K27, *L. delbrueckii* B8, *L. delbrueckii* O43, *L. delbrueckii* K3, *L. delbrueckii* K17, and *L. delbrueckii* K15 and *Lactobacillus fermentum* Ts synthesized exopolysaccharides which have inhibitory properties. Moreover, we have shown that some of the end products of the fermentation process are also able to form a protective layer on the metal surfaces [20]. The observed inverse relationship between EPS and the corrosion rate of mild steel suggests that such a metal-polysaccharide complex was probably involved in developing a protective film on the metal surface in natural environment.

In recent years, the development of new technologies in medicine and dentistry leads to the production of various medical materials and prostheses.On these materials, however, when introduced in the human body, are deposited large number of microorganisms. According to van Geel-Schuten, 'biofilms are a major cause of systemic infections (e.g., nosocomial infections) in humans [16]. It is known that the human body consists about 3–4 kg microorganisms–mostly useful–but there are also the so- called 'pathogens'. In surgery, the probability of contamination with microorganisms and especially with *Staphilococcus* sp is rather big. The microorganisms, in order to survive and to form stable microbial population, create a microbial biofilm, which, however, makes them much more stable against antibiotics compared to when they are in a free state, thereby causing biomaterial-centered infections (BCI). The ability of the microorganisms to adher to different surfaces is determined on the one hand for the species and their metabolism and on the other by the type and elemental composition of the material itself. A powerful tool for the removal of BCIs is could be the use of nanocover of 'good bacteria' —probiotics.

The search for biomaterials that are able to provide for the optimal resistance to the infection can be based only on the deep understanding of the interactions between bacteria and biomaterials' [23].

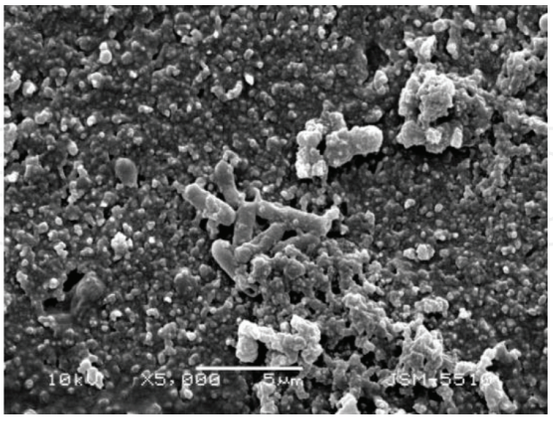


Figure 3. SEM of the tested samples of steel plates.

The adhesion in the combined cultivation of *Staphylococcus aureus* and the *Lactobacillus plantarum* Ts probiotic bacterium on the surface of different metals is investigated [12]. Also, the biofilm in vitro in the combined cultivation of *Staphylococcus aureus* and the *Lactobacillus*

plantarum probiotic bacterium on the surface of different metal materials for fixed dental prostheses (Magnum Splendidum; Magnum Ni-Cr-Fe, Ruby Alloy–P, Ruby Alloy–C, and Ruby Alloy) is investigated [unpublished results].

The results obtained from the SEM analysis of the adhesion ability of the tested microorganisms on the different metals are shown in **Figure 3**. When a combined culture is used on the surface of the steel plates, only the probiotic bacterium adheres.

The results obtained from the SEM analysis of the adhesion ability of the tested microorganisms on the different dental prostheses are shown in **Figure 4**.

The ability of microorganisms to adhere to the surface of various surfaces is determined by various physicochemical interaction of forces, such as–Lifshitz –van der Waals forces, Brownian motion forces, and electrostatic forces. These results are discussed by other authors [21, 22]. On the other hand, the microbial adhesion may be due to the presence of specific active group sin the microbial exopolysaccharides.

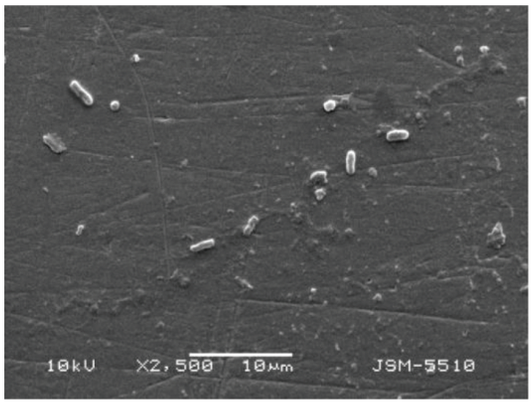


Figure 4. SEM of the tested samples of implants for tooth prosthesis.

After adhesion to biomaterials, most microorganisms start secreting slime and embed themselves in a slime layer, the glycocalix, which is an important virulence factor for BCI and which explains the extraordinary prevalence of slime producing *S. epidermidis* in BCI" [21]. According to Costerson et al., "the glycocalix provides protection against humoral and excreted cellular immune components, as these can not readily diffuse through the slime layer" [23]. Why it is only on the some plate that a biofilm of the beneficial bacteria is formed is a question difficult to answer at this stage. In our opinion, differences are also likely to appear in the adhesion process under in vitro and in vivo conditions because other processes are going to have an impact in the living organism, too. Kristopher P. et al. [24] concluded that "hydrophobic and photo-induced superhydrophilic surface coatings both have potential as a means of reducing microbial fouling of surfaces."

According to "the updated paradigm for biocompatibility", as redrawn by Williams, a biomaterial should perform its designed function eliciting the most appropriate tissue response [25].

The various metabolic ways and the various end metabolic products of the two types of bacteria: *Staphylococcus aureus* and *Lactobacillus plantarum*, could explain to a certain extent the different biofilms formed on the different metal surfaces. Different types of complex compounds are probably formed between the secreted exopolysaccharides or the end metabolic products and the metal surfaces. The mechanism of this process is still to be explained. The adhesion according to Page et al. "of microbes to surfaces can be affected by numerous physicochemical factors, and the complexity of microbial adhesion has been demonstrated. There is no one clear explanation for the behavior of all of the materials with regard to adhesion of microbes to their surface" [3]. Anti-infective biomaterials need to be tailored according to the specific clinical application. All their properties have to be tuned to achieve the best anti-infective performance together with safe biocompatibility and appropriate tissue interactions. The lack of well-structured prospective multicenter clinical trials hinders the achievement of conclusive data on the efficacy and comparative performance of anti-infective biomaterials [12].

4. Conclusion

In our opinion, more detailed research is needed to be done in the future and the possibilities should be analyzed for the creation of a thin biofilm from a probiotic bacterium or an exopolysaccharide this bacterium has produced, which would protect the implants against the growth of a pathogenic biofilm.

On the other hand, conduction of more detailed studies on the application of exopolysaccharides and the development of nanolayers as potential inhibitors of the corrosion process are needed.

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