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Exopolysaccharides From Lactobacillus Plantarum Tsas Corrosion Inhibitors.

TS Ignatova-Ivanova[1]*, Ibryamova S[1], and R Ivanov[2].

[1]Department of Biology, Shumen University, Shumen, Bulgaria Shumen University "Konstantin Preslavski" Department of Biology 115 Universitetska Str., Shumen, Bulgaria.
[2]Department of Organic chemistry and technologies, Shumen University, Shumen, Bulgaria, Shumen University "Konstantin Preslavski" Department of Biology 115 Universitetska Str., Shumen, Bulgaria.

ABSTRACT

Bacterial EPSs (exopolysaccharides) 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. In this paper, data on EPS production and the effect of EPS on corrosion of steel produced by Lactobacillus plantarum Ts are presented and discussed. It was tested for its ability to produce EPS when cultivated in a media containing 10% glucose, 10% sucrose; 10% fructose, 10% lactose, 10% galactose and 10% maltose. The different black staining of Congo Red Agar method (CRA)is caused by different mechanisms for synthesis of EPS of the species depending on the presence of different carbohydrates – into and extracellular. The rate of corrosion, the degree of protection, and coefficient of protection have been calculated. The structure of layer over steel plates was analyzed by SEM and AFM. When used as inhibitor of the protection strain L. plantarum Ts cultivated in the presence of 10% sucrose the protection of corrosion was highest.

Keywords: Corrosion, Inhibitor, Lactic acid bacteria, SEM, AFM

Corresponding author

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INTRODUCTION

Corrosion of metals is a serious and challenging problem faced worldwide by industry. It affects almost every industry in an economy from electronics to healthcare, energy, and infrastructure, and it causes exorbitant losses [1]. Thin polymer films are the subject of intensive study as cheap and efficient alternatives to traditional anti-corrosive coating technology. There is an increasing demand for bio-based polymers for industrial applications and production of polymers by microorganisms is especially attractive. Many microorganisms produce biofilms composed of proteins, fatty acids, and carbohydrates. Carbohydrate polymers excreted by microbes (exopolysaccharides) have been commercialized [2].A vast number of microbial EPSs were reported over the last decades, and their composition, structure, biosynthesis and functional properties have been extensively studied. LAB can produce a large structural variety of EPS and oligosaccharides from glucose that differing in size, molecular organization, chemical composition, structure, and genetic determinants [3] through the activity of glucansucrase and glycosyltransferase (GTF) enzymes. Examples of commercially available microbial exopolysaccharides are xanthan produced from Xanthamonas spp; cellulose from Acetobacter spp; and dextran from Leuconostoc mesenteroides. Recently, some Chryseobacterium and Klebsiella species endemic to the Middle East environment have been shown to have anti-corrosive properties. Lactobacillus delbrueckii and L. rueteri [4,5] both produce exopolysaccharides that were shown to inhibit corrosion of metal substrates submerged in electrolyte solutions [6]. In addition, sulfur-reducing bacteria (SRB) exopolysaccharides were shown to have anti-corrosive properties. In this paper, data on EPS production and the effect of EPS on corrosion of steel produced by L. plantarum Ts are presented and discussed.

MATERIALS AND METHODS

StrainL. plantarum Ts was obtained from the collection of the Department of Biology, Shumen University. Molecular analysis in LAB (lactic acid bacteria) was performed by molecular identification (16S rRNA gene sequencing) in GeXP Genetic Analysis System (Beckman Coulter, USA) [7].

Media The strain cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia) in composition, g/L: Tween 80—1; pepton from casein—10.0; meat extract—8.0; yeast extract—4.0; K2HPO4—2.0; sodium acetat—5.0; amonium citrate—2.0; $MgSO_4 \cdot 7H_2O$—0.2 and $MnSO_4$—0.05. The pH of media was adjusted to 6.5 with 1 M NaOH. The basic media 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% glucose, 10% sucrose; 10% fructose, 10% lactose, 10% galactose and 10% maltose to be tested.

Media for study of microbial biofilm with congo red agarCRA: The strain cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia) with 10% glucose,10% sucrose, 10% fructose, 10% galactose, 10% lactose and 10% maltose and congo red. CRA plates were inoculated with test organisms and incubated at 37° C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production[8]. The experiment was performed in triplicate and repeated three times.

STUDY OF THE CORROSIVE STABILITY

The study of the corrosive stability of steel samples was conducted with the gravimetrique method [9]. 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 desiccators, weighed on a balance and kept in a desiccators unit used. The weight of the samples was measured using analytical balances. The dimensions of the samples were measured with micrometer. Three types of experimental series were performed:

(a) cultivation of the studied strain in mMRS media with 10 % of glucose;
(b) in mMRS media with 10% fructose;
(c) in mMRS media with 10% sucrose;
(d) in mMRS media with 10% galactose;
(e) in mMRS media with 10% lactose;
(f) in mMRS media with 10% maltose.

Initially the steel samples were added in two variants: deproteinised supernatant and free cell supernatant. Then the steel samples were added in seawater 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 SEM (scanning electron microscopy) JSM 5510.

PARAMETERS OF CORROSION

After retrieval, the corrosion products were removed when washed with water. They were dried in an oven. After the removal of corrosion, steel plates were cleaned and reweighed as above to estimate weight loss. The rate of corrosion, the degree of protection, and coefficient of protection were calculated.

The corrosion rate K (g/cm2·h) was presented as follows: $K = \Delta G / S \cdot \tau$ (1)

Where, Δ is the corrosion rate; ΔG—losses of mass consequence of corrosion, g; S—is the area of plates, m2; τ—is duration of the corrosion, h.

In order to track out the inhibitor properties of EPS synthesized in media, the degree of protection (Z) and coefficient of protection (γ) have been calculated using the formulas:

$$Z = (K0 - Ki) / K0 \times 100, \% \quad (2)$$

$$\gamma = K0 / Ki \quad (3)$$

Where, K0 is the corrosion rate in control media; Ki—the corrosion rate in test media

ANALYSIS BY MEANS OF SEM MICROSCOPY

The steel plates made of low carbon steel are weighed with an allowance of 0,0001g with an assay-balance. They are put sterilely in a liquid ambient which contains a L. fermentum Ts. The samples were incubated at 37°C for 24 h. The structure of the layer over the metal plates was analyzed by SEM (scanning electron microscopy) JSM 5510 and atomic force microscopy (AFM). All experiments were performed in triplicate [10].

ANALYSIS BY MEANS OF AFM MICROSCOPY

An AFM Anfatec Instruments AG, Germany was usedfor characterization of surface topology. The measurements were realized in non-contact mode when the tip was scanning over the studied surface at a distance of few nanometers. A silicon nitride tip with a curvature radius of about 10 nm and force constant about 43 N/m was used. The three-dimensional images of scanned samples were created with ANFATEC PRESENT software.

RESULTS AND DISCUSSION

The corrosion of iron and its alloys causes severe economic loss resulting in a yearly cost of billions of dollars or Euros. The use of heavy metals and heavy metal containing compounds, such as chromate, has to be reduced in coatings for some are known to be very toxic, even carcinogenic, and cause great environmental damage. Prevention of or reduction in the rate of corrosion may be accomplished by the use of a biological, environmentally friendly anti corrosive layer at the metal interface. The presence of EPS associated with bacterial cells can be recognized by the formation of colonies in mucous solid medium [11]. Therefore, the presence of a translucent or creamy material involving a mucoid colony is indicative of EPS production potential. When cultivated in a media with high content of saccharides such as 10% sucrose solutions, 10% fructose solutions, 10% glucose solutions, 10% galactose solutions, 10% lactose solutions and 10% maltose solutions, strain L. plantarum Tssynthesizes exopolysaccharides (Fig. 1).

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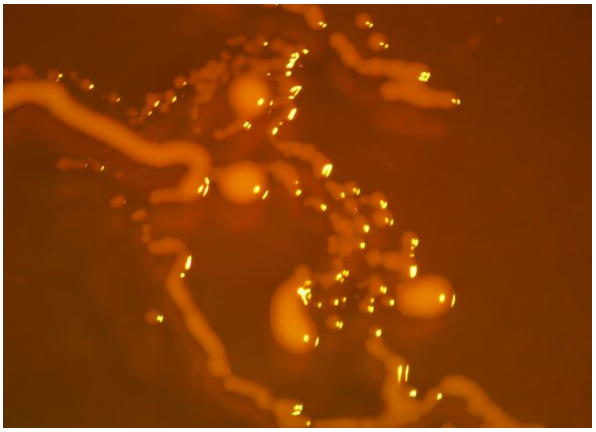

Fig 1: EPSs (exopolysaccharides) produced by L. plantarum Ts cultivated in a media containing 10% glucose, which are secreted in the culture medium.

The pictures were taken using stereomicroscope OPTIKA (Italy).

For chemical analysis of the formed microbial biofilm was used congo red agar CRA method and the results are represented on Figure 2.

Figure 2: EPSs (exopolysaccharides) produced by L. plantarum Ts Ts Congo red agar Black colonies shows biofilm formation of LAB, cultivated in media contained 10% glucose. The pictures were taken using stereomicroscope OPTIKA (Italy).

Homopolysaccharides produced by GRAS (Generally Recognized as Safe) lactic acid bacteria are often synthesized by a single extra-cellular sucrose enzyme, using only sucrose as substrate [12]. Composition of the culture medium and other fermentation conditions such as pH, temperature, oxygen concentration and agitation greatly affect the quantity and quality of bacterial produced biopolymers [13]. Biopolymers chemical and physical characteristics are highly related to their cultivation conditions [14].

Strain L. plantarum Ts was cultivated in a media containing 10% sucrose,10% glucose, 10% lactose, 10% fructose, 10% galactose and 10% maltose for 12 h. The steel samples were placed in seawater as control probe and a dilution (3: 100) of the cultural media of the studied strain was added as inhibitor of the corrosion.

The received results are presented in Table 1 and figure 3.

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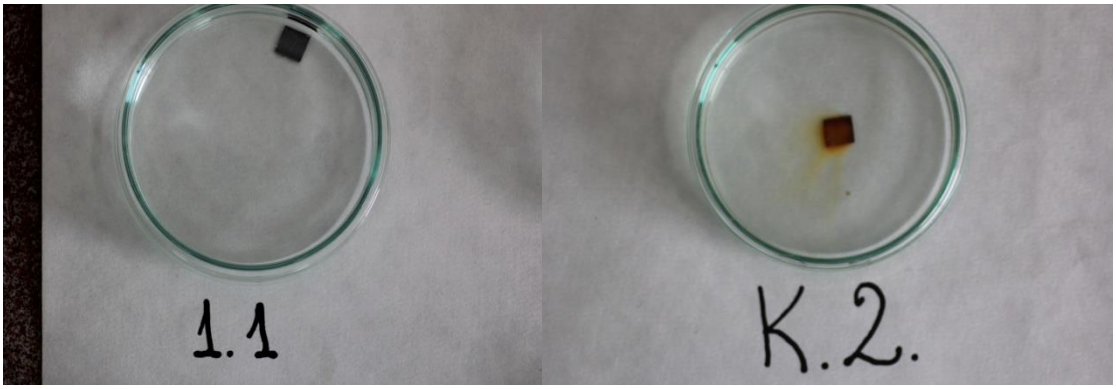

A) B)

Fig 3: Steel samples immersed in seawater for a period of 120 h. (A) Steel plates in seawater with inhibitor supernatant obtained of mixed 10% glucose; (B) control—steel plates in seawater.

Table 1: Characterizationoftheprotectiveproperties in seawater with added supernatant.

№ Sample	media	The quantity of the supernatant in Seawater, %	K.10^{-4}, g/cm^2.h	Z, %	γ
1.	10% glucose*	3.0	0.730	14.13	1.16
2.	10% fructose	3.0	0.684	19.51	1.24
3.	10% sucrose	3.0	0.461	**45.79**	1.84
4.	10% galactose	3.0	0.641	24.56	1.32
5.	10% lactose	3.0	0.657	22.75	1.29
6.	10% maltose	3.0	0.539	36.61	1.58
7.	control	-	0.851	-	-

*The steel plates were photographed afterwashing; Results are mean ± SEM of three separate trails.

When used as inhibitor of the protection strain L. plantarum Ts cultivated in the presence of 10% sucrose the protection of corrosion was highest (table 1). It could be underlined that 10% carbohydrates in the media stimulated the process of protection of corrosion.

The structure of the layer over the steel plates was analyzed by Scanning electron microscopy. The results from this procedure areshown in Fig. 4.

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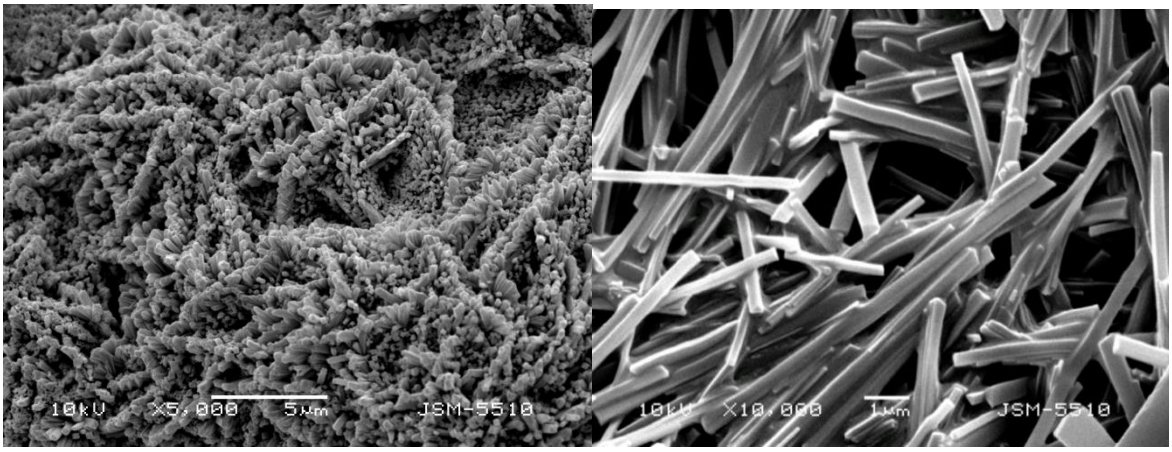

A) B)

Fig 4: Biofilm formed by L. plantarum Ts on the surface of mild steel, visualized using SEM. (A) Steel plates after corrosion in seawater with inhibitor supernatant obtained of mixed 10% glucose; (B) control—steel plates after corrosion in seawater.

AFM imaging of biopolymer as polysaccharides was generally conducted in air or under a liquid in order to avoid excessive dehydration. The topographical AFM images of L. plantarumTsEPS were shown in Fig.5.

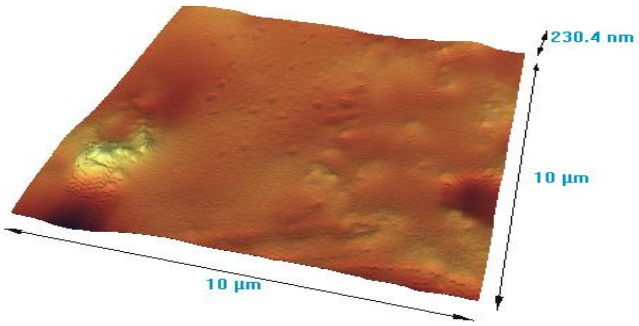

Figure 5: Atomic force microscopy (AFM) images of molecular structure of L. plantarum Ts EPS. Biofilm formed by lactic acid bacteria cultivated with 10% glucose.

L. plantarum Ts EPS deposited from 10 g/mL aqueous solution, roundness lumps and chains can be seen (Fig. 5).

DISCUSSION

In our previous studies [15-19], it was shown that at the presence of high concentration of lactose (5% to 15%), high concentration of sucrose 4%, mixed sucrose 4% and 2% maltose and mixed sucrose 5% and 5% maltose, mixed 5% sucrose and 5% fructose and mixed 5% sucrose and 5% fructose the strains Lactobacillus delbrueckii B5, L. delbrueckii K27, L. delbrueckii B8, L. delbrueckii O43, L. delbrueckii K3, L. delbrueckii K17, and L. delbrueckii K15 synthesized exopolysaccharides which have inhibitory properties. It is well known that some lactobacillus strains such as genus Leuconostoc secreted trans glucosidases after cultivation in the presence of sucrose. The screening of EPS-producing bacterial strains basically depends upon the development of mucoid characteristics of bacterial colonies. The more mucoidal form of colonies produces more EPS. The release of

EPS is an interesting process for the bacterium, which comprises hydrophilic, high molecular weight polymer assembly in the cytoplasm and its traverse through the cell envelope, without affecting the critical barrier to transport. It is known that the production of EPS takes place because of cellular stress. Therefore, as expected, adding high concentration of carbohydrates to the growth media increased bacterial EPS production.

The biofilm makesit noteasily corrodible in seawater, supplemented with cultivated ambient from the same strain grown in a composite of 10% glucose (figure 3A). Figure 3B shows a picture of a steel surface sample treated directly with seawater. The observed lamellae are most probably $FeCl_2$ crystals, product of the corrosion.The corrosion of mild steel starts with generation of ferrous ions by anodic oxidation at the surface because of the reaction ($Fe \rightarrow Fe^{+2} + 2\ e^-$) which may undergo further oxidation producing Fe^{+3} species ($Fe^{+2} \rightarrow Fe^{+3} + e^-$). Ferric ions are particularly deleterious for mild steel as they tend to accelerate corrosion by the reaction ($Fe + 2Fe^{3+} \rightarrow 3\ Fe^{2+}$). If ferric ions are immobilized then it may be possible to control the corrosion of mild steel. Some polysaccharides are reported to exhibit strongest stability constant for Fe^{3+} ions [20]. Such a complex may serve as a corrosion inhibitor. 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 sea water.Microscope techniques provide information about the morphology of microbial cells and colonies, their distribution on the surface, the presence of EPS (Fig. 4A) and the nature of corrosion products (crystalline or amorphous; Fig. 4B). The SEM images of the EPS showed a stacked flakes with relatively uniform shapes Fig.4A. Similar porous web microstructure of EPS was reported earlier with the EPSs produced by Streptococcus thermophilus GST-6 [21] and Lactobacillus plantarum strains [22-23]. Chen M., and colleagues [24] showed that after sulphonation the appearance of polysaccharide fragments without uniform size could be changed into regular even structure similar to tile. This was in agreement with the similar micro structural change described above for the EPS from strain L. plantarum Ts of this study. The maximal height of lumps at 10% solution of glucose is 166,2 nm, at 10% solution fructose is 193nm and 10% solution maltose is 201,6nm. This result suggested that L. plantarum Ts EPS could combine water in the aqueous. Furthermore, it showed pseudo plastic behavior because the strong interaction between water molecules and the hydroxyl groups (−OH) of L. plantarum Ts EPS. The forms of corrosion which can be promoted by the interaction of microorganisms with metals are numerous, including general pitting, crevice attack, stress corrosion cracking, enhancement of corrosionfatigue, intergranular stress cracking and hydrogen embrittlement and cracking. Most cases of microbially-influenced corrosion (MIC) are associated with localized attack [25]. The role of EPS in MIC of stainless steel remains obscure. The ability of EPS to bind specific metal ions strongly influences its adhesion to metal surface and its ability to concentrate metal ions from surfaces and bulk media. Binding of metals may be important in both passivation and activation reactions. The observed inverse relationship between EPS and the corrosion rates of mild steel suggests that similar reactions may be occurring in the natural environment leading to the formation of a protective film on the metal surface. Biofilm [20] 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%. From this, it is evident that some microorganisms and/or their polysaccharides can act as a strong corrosion inhibitors. Our data suggest that biofilm EPS inhibits the corrosion of mild steel in natural marine waters.Amin Alipour,[20] showed that the corrosion resistance results from one constant carbon source (sucrose) and different nitrogen sources nearly coincide each other. Changing the medium ingredients can influence the biopolymer production amount, but the biopolymer anticorrosive quality approximately depends on carbon sources alone.The adhesion ofmicroorganisms to the surfaces and the subsequent biofilmdevelopment are very complex processes, affected by severalvariables. Lactic acid bacteria and other secreteextracellular polymeric substances (EPS) which anchors to thesubstratum, thereby modifying the surface chemistry which canstimulate further growth and the support and emplacement ofmacroorganisms [26]. EPSs also contain divalent metal cations that act as ionic bridges linking adjacent polysaccharide chains. The acidic nature of the EPS is a result of the presence of uronic acids, pyruvate, and inorganic residues such as phosphate or sulfate. Uronic acid plays an important role in metal binding [27].The characteristics of the functional groups present on EPS from varying microorganisms are unique and need to be considered to understand the mechanism of metal chelation by EPS.

Exopolys accharides and other biopolymers exhibit excellent metal-binding properties with adistinct degree of specificity and affinity; for example, cadmium may have a higher affinity for EPS than copper and zinc. Even though EPSs have different affinities for different metals, the modification could be observed in EPS confirmation.

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CONCLUSION

From the received results it was evident that a mixture of 10% sucrose,10% glucose, 10% lactose, 10% fructose, 10% galactose or 10% maltose maltose stimulated the formation of microbial biofilm inhibiting the corrosion of steel. Our data showed that L.plantarum Ts produce EPS, which serve as corrosion inhibitor for mild steel. When used as inhibitor of the protection strain L. fermentum Ts cultivated in the presence of 10% sucrose the protection of corrosion was highest. Further studies are needed to evaluate the potential of the biofilm exopolysaccharides as anticorrosive agents.

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