ASN, Vol 3, No 1, Pages 51-59, 2016

DE GRUYTER OPEN

Acta Scientifica Naturalis

Former Annual of Konstantin Preslavsky University – Chemistry, Physics, Biology, Geography
Journal homepage: http://www.shu.bg

Received: 30.10.2015
Accepted: 11.03.2016

Exopolysaccharides from lactic acid bacteria as corrosion inhibitors

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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 sp. are presented and discussed. Lactobacillus delbrueckii K27, Lactobacillus delbrueckii B8, Lactobacillus delbrueckii KO43, Lactobacillus delbrueckii K3, Lactobacillus delbrueckii K15 and Lactobacillus delbrueckii K17 was obtained from Collection of Department of General and Applied Microbiology, Sofia University. It was tested for its ability to produce exopolysaccharides when cultivated in a media containing 10% sucrose, 10% lacose and 10% maltose. The study of the corrosive stability of steel samples was conducted on the gravimetrique method. The rate of corrosion, the degree of protection, and coefficient of protection have been calculated. The structure of layer over steel plates was analysed by SEM (scanning electron microscopy) JSM 5510. It could be underlined that 10% sucrose, 10% lactose and 10% maltose in the media stimulated the process of protection of corrosion.***

Keywords*: **exopolisaccharides, corrosion, inhibitors***

Introduction

Lactic acid bacteria (LAB) are one of the microorganism groups widely distributed in the biosphere. They belong to a group of Gram-positive, nonsporing cocci or rods, anaerobic bacteria that excrete lactic acid as their main fermentation product into the culture medium. In a variety of ecological niches, microorganisms compete with each other for survival and through evolution form unique flora. In some food ecosystems, LAB constitute the dominant microflora. These organisms are able to produce antimicrobial compounds against competing flora, including food-borne spoilage and pathogenic bacteria [7]. Under unfavorable environmental conditions many species of LAB also produce EPSs, which protect themselves against desiccation, bacteriophage and protozoan attack [40,48,49]. EPSs is a term first used by Sutherland [44] to describe high-molecular-weight carbohydrate polymers produced by marine bacteria. Based on their sugar compositions, the EPSs can be divided into homopolysaccharides (HoPS), composed of a single type of monosaccharide, and heteropolysaccharides (HePS), containing several types of monosaccharide [12]. LAB can produce a large structural variety of EPS and oligosaccharides from glucose that differing in size, molecular organization, chemical composition, structure, and genetic

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DOI: 10.1515/asn-2016-0008

determinants [34] through the activity of glycansucrase and glycosyltransferase (GTF) enzymes. The main genes involved in HoPS production are glucansucrase and levansucrase genes [18,27,45,46].

HoPS are composed entirely of only one type of monosaccharide, i.e. D-glucose or D-fructose. As such, HoPS are α-glucans, β-glucans or fructans. HoPS synthesis is a relatively simple process and as there are no active transportation stages in the synthetic pathway, there is no energy expenditure, other than biosynthesis of the extracellular enzymes. These extracellular enzymes, collectively termed glycosyltransferases (or glycansucrases) include glucosyltransferase (GTF or glucansucrase) and fructosyltransferase (FTF or fructansucrase). These enzymes utilise glucose and fructose as the glycosyl donor during HoPS synthesis [32].

α-Glucans are found to be most common in LAB HoPS structure, with fewer β-glucans. There are four types of α-glucans – dextrans, mutans, reuterans and alternans – currently recognised. Dextrans are a large class of extracellularly formed glucans produced by the genus Lactobacillus, Leuconostoc, and Streptococcus, of which Leuc. mesenteroides and Leuc. dextranicum are the well-known dextran producers. Although each bacterial strain produces a unique glucan, a common structural feature of all dextrans is a high percentage (up to 95%) of α- 1,6 linkages with a smaller proportion of α-1,2, α-1,3, or α-1,4 linkages resulting in a highly branched molecule [16]. Dextrans are synthesized outside the cell by dextransucrase, which catalyzes sucrose to produce D-fructose and D-glucose, and transfers the latter to an acceptor to form dextran. The reaction is as follows:

sucrose + glucan acceptor dextran or mutans + D-fructose

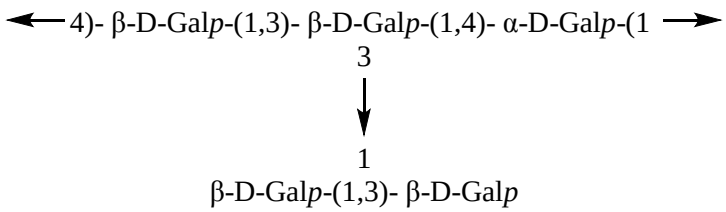
Dextran, synthesised by Ped. Pentosaceus CRAG3 Leuconostoc mesenteroides, Leuconostoc amelibiosum and Lb. curvatus, has an α-1,6-glucan repeating unit with occasional α-(1,3) branches [42]. Glucans with mainly α-(1,3) linkages are termed mutans and those with majority α-(1,4) linkages are reuterans. Glucans with alternating α-(1,3) and α-(1,6) linkages are referred to as alternans. Curdlan is an example of an insoluble, uncharged HoPS with a repeating unit of β-(1,3)-D-glucan [35]. This HoPS was first discovered to be produced by Alcaligenes faecalis var. Myxogenes 10C3, later renamed *Agrobactelium biosp*. 10C3 [31]. 10C3 is not a LAB, such a β-(1,3)-D-glucan is produced in high levels by *Lactobacillus suebicus* and at least three strains of *Pediococcus parvulus*, all originating from spoiled cider [9; 47]. In these strains, two distinct molecular species are found to be produced by each strain at different ratios; a β-(1,3)-D-glucan of molecular weight 10^4 Da and one of 10^7 Da.

Mutans are synthesized in a similar way by *S. mutans* and *S. sobrinus* [33]. However, mutans differ from dextrans in containing a high percentage of β-1,3 linkages, which are attributed to the insoluble nature of this type of polymers [19]. Some *S. salivarius* strains are able to produce fructans of the levan type with 2,6-linked β- fructofuranoside residues [3]. An extracellular enzyme levansucrase is involved in hydrolyzing sucrose and transferring D-fructose to growing fructan chains to form levans:

sucrose + fructan acceptor levan + D-glucose

Fructan HoPS are synthesized from sucrose by fructosyltransferase and can be divided into two groups, levan-type and inulin-type [32,36] Levan contains β-(2,6)-fructosyl linked units, with occasional β-(2,1) branches. Conversely, inulin contains β-(2,1)-fructosyl linked units, with β-(2,6) branches and is composed of linear chains of varying lengths of D-fructopyranosyl residues with a terminal D-glucose residue [41]. Aside from levan and inulin, alternan is an example of an α-glucan polymer often found in the food industry, produced by the alternansucrase enzyme of *Leuconostoc mesenteroides* and comprised of alternating α-1,6 and α-1,3 glycosidic bonds [38].

Another type of homopolysaccharide is the galactan produced by *Lc. lactis* ssp. *Cremoris* H414, which is composed of a branched pentasaccharide repeating unit as shown below [13].



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A Pediococcus strain produced a β-D-glucan with a trisaccharide repeating unit [28]. *Lactobacillus* spp. G-77 has been shown to produce a 2-substituted- (1□3)- β-D-glucan, identical to the EPS produced by P. damnosus 2.6 [14]. *Lactobacillus* spp. G-77 also produced a α-D-glucan composed of a trisaccharide repeating unit [15]. Recently, van Geel-Schutten et al. [10] reported for the first time the production of a fructan by *Lb. reuteri* strain LB121 with raffinose as a sugar substrate; this strain also produced both a glucan and a fructan on sucrose.

The majority of EPS produced by LAB are heteropolysaccharide, or HePS. The HePS synthesis mechanism is more intricate, with the precursor nucleotide units UDP-GalNac, GDP-fucose, dTDP-rhamnose, UDP-galactose and UDP-glucose being synthesised intracellularly from glucose-1-phosphate and fructose-6-phosphate. These sugar nucleotides are attached by priming GTF to an isoprenoid alcohol glycosyl carrier lipid, C55-polyprenyl phosphate [26]. In comparison with the homopolysaccharides, the production of heteropolysaccharides by LAB is much lower (60 to 400 mg L^{-1}) [43]. Generally, the heteropolysaccharides are synthesized intracellularly at the cytoplasmic membrane utilizing sugar nucleotides as precursors for the assembly of polysaccharide chains [4].

Kefiran is an example of a heteropolysaccharide synthesised by *Lactobacillus kefiri* and *Lactobacillus kefiranofaciens* and is found in the fermented dairy beverage Kefir. Kefiran is a water-soluble branched glucogalactan, composed of a hexasaccharide repeating structure with near equal quantities of glucose and galactose residues (1 : 1.05) [29]. The molecular weight of Kefiran is a matter of debate. A complex molecular organization is responsible for genes involved in HePS biosynthesis [8]. The structure, composition, and viscosity of EPS depend on several factors, such as the kind of strain, the composition of the culture medium, mineral salts, trace elements, and fermentation conditions (e.g., pH and temperature) [8]. A majority of exopolysaccharide backbones have repeating units composed of glucose, galactose, and rhamnose, which occur in different ratios and different anomeric configurations and are connected by different linkages. Occasionally, aminosugars such as N-acetyl-d-glucosamine and Nacetyl-d-galactosamine as well as non-carbohydrate substituents (sn-glycerol-3-phosphate, phosphate, and acetyl groups may also be present in EPSs [39].

Considerable progress has been made in discovering and developing new microbial EPSs that possess novel industrial significance [37]. Bacterial EPS 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 [5]. These microbial biofilms have been implicated in corrosion of metals [20-25], bacterial attachment to prosthetic devices, fouling of heat exchange surfaces, toxicant immobilization, and fouling of ship hulls [1,6].

In this paper, data on EPS production and the effect of EPS on corrosion of steel produced by different *Lactobacillus sp.* are presented and discussed.

Materials and Methods

Strains

Lactobacillus delbrueckii K27, Lactobacillus delbrueckii B8, Lactobacillus delbrueckii KO43, Lactobacillus delbrueckii K3, Lactobacillus delbrueckii K15 and Lactobacillus delbrueckii K17 was obtained from Collection of Department of General and Applied Microbiology, Sofia University.

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; K_2HPO_4—2.0; sodium acetat—5.0; amonium citrate—2.0; $MgSO_4 \cdot 7H2O$—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% sucrose; 10% lactose and 10% maltose to be tested.

Study of the Corrosive Stability

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The study of the corrosive stability of steel samples was conducted with the gravimetrique method. Before use, steel panels (10 × 4 × 0.2 mM) were treated with 70% C2H5OH, 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. Three types of experimental series were performed:
(a) cultivation of the studied strain in mMRS media with 10 % of sucrose;
(b) in mMRS media with 10% lactose;
(c) 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 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 analised 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:

$$K = \Delta G / S \cdot \tau \quad (1)$$

Where, Δ is the corrosion rate; ΔG—losses of mass consequence of corrosion, g; S—is the area of plates, m^2; 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

Results and Discussion

Corrosion of metals is a serious and challenging problem faced worldwide by industry. It has been estimated that the yearly corrosion damage costs are currently equivalent to 4.2% of the U.S. gross national product. These costs could be greatly reduced by better and wider use of corrosion protection techniques. Traditional methods of corrosion protection involve the use of organic coatings to protect metal surfaces through barrier and passivation mechanisms. Prevention of or reduction in the rate of corrosion may be accomplished by the useof 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% lactose solutions, and 10% maltose solutions, strain *L. bulgaricus K27* synthesizes exopolysaccharides (Fig. 1).

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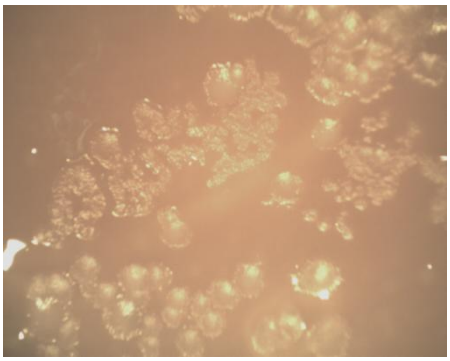

Figure 1. EPSs (exopolysaccharides) produced by *L. bulgaricus K27* cultivated in a media containing 10% maltose, which are secreted in the culture medium.

Similar experiments have also been demonstrated by other authors [17, 30]. Homopolysaccharides produced by GRAS (Generally Recognised as Safe) lactic acid bacteria are often synthesised by a single extra-cellular sucrase enzyme, using only sucrose as substrate [30]. They can be produced in largest quantities (bulk scale). Moreover, their structure can be modified allowing optimisation of their physicochemical properties. By means of cyclic voltammetry, impedance measurements and potential monitoring the electrochemical behaviour of a new type of anti-corrosive biopolymers has been studied, which can be deposited upon metal surfaces as layers.

The six strains *Lactobacillus sp.* was cultivated in a media containing 10% sucrose, 10% lactose, and 10% maltose for 12 h. The steel samples were placed 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 received results are presented in Table 1.

Table 1. Characterization of the protective properties in HCl with added supernatant.

№ sample	Media	The quantity of the supernatant in seawater, %	K$x10^{-2}$,g/m^2h	Z,%	γ
1	Control*		0.446	-	-
2	K27 sucrose	3	0,243	45,52	1,835
3	B8 sucrose	3	0,275	38,34	1,622
4	O43 sucrose	3	0287	35,65	1,554
5	K3 sucrose	3	0,302	32,29	1,477
6	K15 sucrose	3	0,350	21,52	1,274
7	K17 sucrose	3	0,318	28,70	1,403
8	K27 lactose	3	0,343	23,09	1,300
9	B8 lactose	3	0,289	35,20	1,543
10	O43 lactose	3	0,348	21,97	1,282
11	K3 lactose	3	0,350	21,52	1,274
12	K15 lactose	3	0,362	18,83	1,232
13	K17 lactose	3	0,323	27,58	1,381
14	K27 maltose*	3	0,227	49,10	1,965
15	B8 maltose	3	0,298	33,18	1,497
16	O43 maltose	3	0,197	55,83	2,264
17	K3 maltose	3	0,273	38,79	1,634
18	K15 maltose	3	0,285	36,10	1,565
19	K17 maltose	3	0,245	45,07	1,820

*The steel plates were photographed after washing; results are mean ± SEM of three separate trails.

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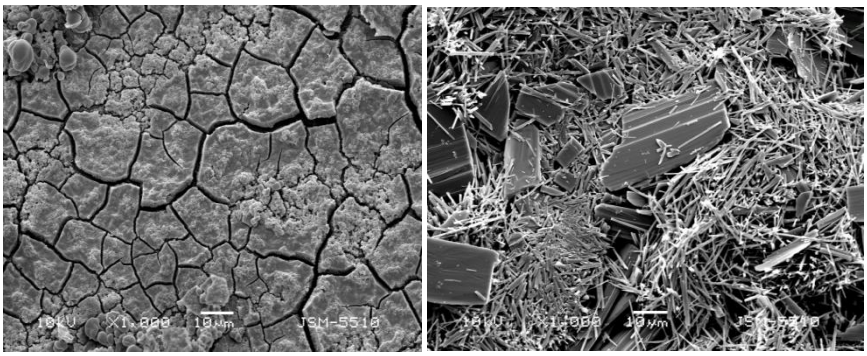
From the presented data in Table 1 the protective effect in all studied cases was proved. The coeffi cient of the protection of corrosion varied between 2,264 and 1,232. From the obtained results is clear that the protection of corrosion was higher in the case when 10% maltose for strain *L. delbrueckii K27* and a 10% sucrose for strain *L. delbrueckii K27* were used. In our previous studies [20-25], 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, 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. It is well known that some lactobacillus strains such as genus *Leuconostoc* secreted trans glucosidases after cultivation in the presence of sucrose. The structure of the layer over the steel plates was analyzed by Scanning electron microscopy. The results from this procedure are shown in Fig. 2.



A B

Figure 2. Biofilm formed by *L. delbrueckii* K27 on the surface of mild steel, visualized using SEM. (A) Steel plates after corrosion in HCl with inhibitor supernatant obtained of 10% maltose; (B) control—steel plates after corrosion in HCl.

The biofilm makes it not easily corrodible in seawater, supplemented with cultivated ambient from the same strain grown in a composite of 10% maltose (Fig. 2a). Fig. 2b shows a picture of a steel surface sample treated directly with HCl. The observed lamellaes are most probably FeCl2 crystals, product of the corrosion. Microscope techniques provide information about the morphology of microbial cells and colonies, their distribution on the surface, the presence of EPS (Fig. 2a) and the nature of corrosion products (crystalline or amorphous; Fig. 2b). 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 biofilm and corrosion products (Fig. 2b). Biofilm [11] 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. Some polysaccharides are reported to exhibit the strongest stability constant for Fe^{3+} ions [11]. 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. The data suggest that biofilm EPS inhibits the corrosion of mild steel in HCl.

Conclusions

From the received results it was evident that a 10% sucrose, 10% lactose or 10% maltose stimulated the formation of microbial biofilm inhibiting the corrosion of steel. The present research confirms the result of the pilot project [2] that polysaccharides made by microorganisms show anti-corrosive properties. Especially, homopolysaccharides showed interesting results for the protection of steel. Measurements indicate that it takes some time for layers of biopolymers on the metal to build a complete protective layer. The data showed that *Lactobacillus sp.* produce EPS, which serve as corrosion inhibitor

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DOI: 10.1515/asn-2016-0008

for mild steel. Further studies are needed to evaluate the potential of the biofilm exopolysaccharides as anticorrosive agents.

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Acknowledgements

We would like to express our gratitude for the support of this work by research grant of FSI RD-08-266/10.03.2015 of Shumen University.

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DOI: 10.1515/asn-2016-0008