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Research Article

Biosynthesis of Extracellular Polymeric Substances by the Marine Bacterium *Saccharophagus degradans* under Different Nutritional Conditions

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The effect of carbon source, carbon to nitrogen (C/N) ratio, and limitation in nutrients (N, P, K, Ca, Mg, and Fe) on extracellular polymeric substances (EPS) synthesis by the marine bacterium *Saccharophagus degradans* was studied. This strain was able to grow in mineral medium and produce EPS with different efficiency according to the C source used (g EPS/L): glucose or starch (1.5 ± 0.2); galactose, sucrose, or xylose (0.7 ± 0.2); and fructose (0.3 ± 0.1). The C/N ratio (glucose/ammonium) had a significant effect on EPS biosynthesis due to its production rise as the C/N ratio increased from 3 to 100 (0.7 to 2.1 g EPS/L). It was also observed that limitation in nutrients such as N, P, K, Ca, Mg, and Fe also favored EPS biosynthesis. When taking into account both factors (C/N ratio, 100; nutrients limitation, 50%) a positive synergistic effect was noted on EPS production since under these conditions the maximum concentration obtained was 4.12 ± 0.3 g/L after 72 h of culture. The polymer was found to be a polysaccharide of mainly glucose, mannose, and galactose. This is the first report on EPS production by *S. degradans* which is a new feature of this versatile marine bacterium.

1. Introduction

In recent years, the demand for natural polymers has triggered the interest in the production of exopolymeric substances (EPS) by microorganisms [1]. As a result, the isolation and identification of microbial polysaccharides that might have novel uses as heavy metal removers [2] gelling agents, emulsifiers, texture enhancers, or source of specific monosaccharides is of particular interest [3]. EPS are also environmentally friendly since they are renewable in nature, nontoxic, and biodegradable [4]. EPS exist in a wide variety of chemical structures: hexoses sugars, such as D-glucose, D-galactose, D- and L-mannose; pentoses such as D-ribose, D-arabinose, and D-xylose; few heptoses; and branched-chain sugars [5].

The production of exopolymers in marine bacteria, such as *Alteromonas macleodii* [6] and *Halomonas ventosae* [7], is a common phenomenon. EPS increase the ability of such bacteria to adhere to surfaces which is an important feature to survive successfully in aquatic environment. Also, there is evidence they provide self-protection against desiccation, antimicrobial substances [8], and bacteriophages [3].

It has been mentioned that the production of exopolymers is promoted by a high C/N ratio and by nutritional limitations in P, Fe, K, N, O_2, and C [9]. Some bacteria are able

to synthesize simultaneously EPS and polyhydroxyalkanoates (PHA) since the conditions for PHA production are similar to those mentioned for EPS. This is the case of *Ralstonia eutropha* ATCC 17699 [10], for example.

Recently, González-García et al. [11] described the production of PHA in the marine bacterium *Saccharophagus degradans*. During its growth from glucose the production of exopolymeric substances was observed; nevertheless, there is lack of information about the chemical nature of this polymer, and its production from different C sources. *S. degradans* is one of the most versatile polysaccharide degrading marine bacteria reported so far; it was isolated from decaying salt marsh cord grass in the Chesapeake Bay watershed and it has been shown to depolymerize and metabolize polysaccharides including agar, alginate, cellulose, starch, and chitin [12]. Due to their ability to assimilate a wide variety of sugars including xylose and starch, such organisms could have an important use in biotechnological processes for the production of value added metabolites such as enzymes, bioplastics, and exopolysaccharides.

The present research was focused on assessing the ability of *S. degradans* to grow and produce EPS from various carbohydrates and the effect of the medium composition and the C/N ratio on its biosynthesis and to partially characterize the polymer obtained.

2. Materials and Methods

2.1. Strain and Inoculum Preparation of S. degradans. (DSM number 17024) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and was kept in fresh Difco Marine Agar 2216 plates. The inoculum was prepared from the agar plate culture in a 300 mL Erlenmeyer flask with baffles containing 100 mL of medium with the following composition (per liter): 2.5 g of NH_4Cl, 5 g of yeast extract, 23 g of sea salts (Red Sea), 50 mL of buffer Tris-HCl 1 M pH 7.6, and 10 g of glucose. The flask was incubated at 30°C during 18 hours.

2.2. Experimental Phases. Three different experiments were performed in order to assess the production of EPS by *S. degradans*: (A) production from different C sources in a nutrient balanced medium denominated as basal medium (BM); (B) effect of C/N ratio and combined nutrients limitation using N, P, S, Mg, Fe, and Ca limited medium (NL), with glucose as C source; (C) bioreactor culture from glucose, using the best culture condition according to experiment B. Mediums BM and NL are described in Table 1.

2.3. Production of EPS from Different C Sources in a Nutrient Balanced Medium. This experiment was conducted in 1 L Erlenmeyer flasks with baffles containing 400 mL of BM with different C sources (glucose, galactose, sucrose, starch, xylose, or fructose) and each flask was inoculated with 10 mL of the starter culture mentioned above. The flasks were incubated under the conditions previously described until the stationary growth phase was reached (48 hours); then 20 mL of the corresponding C source solution (200 g/L) was aseptically added to each flask and was incubated again for 48 h. Samples were withdrawn and analyzed for biomass, EPS, and C source concentration; experiment and analyses were duplicated. A one-way ANOVA (analysis of variance) test was performed (Statgraphics Centurion XVI.II) to determine if there were significant differences among the C sources used.

Table 1: Chemical composition of the culture mediums used: basal medium (BM) and nutrients limited medium (NL).

Compound (g/L)	Basal medium (BM)[a]	Limited medium (LM)[b]
Glucose	10	10
NH_4Cl	6.68	3.34
KH_2PO_4	2.8	1.4
NaCl	23	23
KCl	1.5	0.75
$CaCl_2$	0.3	0.15
$MgSO_4$	0.6	0.30
$FeSO_4$	0.16	0.08
EDTA	0.15	0.08
Tris-HCl[c] (mL/L)	100	100

[a]For experiment A the carbon sources used were also galactose, sucrose, starch, xylose, or fructose.
[b]Limitation consisted in reducing by 50% the amount of N, P, K, Ca, Mg, and Fe originally present in BM.
[c]pH 7.6.

Table 2: Experimental design (bifactorial with two replicates) to investigate the effect of nutrients and C/N ratio on EPS production.

Factor	Levels
(I) C/N ratio	3
	30
	50
	100
(II) Nutrients limitation	Limited (NL)[a]
	Not limited (BM)[b]

[a]Culture mediums all together limited by reducing in 50% the basal medium salts content (K_2HPO_4, KCl, $CaCl_2$, $MgSO_4$, and $FeSO_4$).
[b]Basal medium.

2.4. Effect of C/N Ratio and Limitation in Nutrients on EPS Biosynthesis. Batch cultures were performed, using a bifactorial experimental design (Table 2) with two replicates. This experiment was done in flasks in a two-step culture: the first step consisted in biomass production under nutritional balanced conditions during 30 hours (BM, glucose); for the second step, biomass was aseptically recovered by centrifugation and added to flasks containing 10 g/L of glucose and different concentrations of N source and other salts according to the experimental design (Table 2). They were incubated during 72 h under the conditions mentioned above and samples were taken and analyzed for biomass, EPS, and C source concentration. A multifactorial ANOVA test was conducted to analyze the effect of the variables (C/N ratio and nutrients limitation) on EPS production.

2.5. Production of EPS in Bioreactor. A 2 L Biolafitte fermenter (Pierre Guerin Technologies, France) was used with 1.5 L of culture medium (the composition was according to the best result of the previous experiment: C/N ratio, 100; medium, NL); pH was automatically controlled (7) by adding 1 N NaOH or H_2SO_4 solutions, and the temperature was kept at 30°C. The aeration and agitation speed was maintained in 1 VVM and 400 rpm, respectively. After initial glucose was depleted, the fermenter was fed with a concentrated solution of glucose to obtain a concentration of 10 g/L within the fermenter. Samples were periodically taken and analyzed for biomass, for EPS production, and for glucose, ammonium [13], and phosphate [14] consumption. The fermentation and analyses were duplicated.

2.6. Determination of Carbohydrates, Biomass, and EPS. Glucose, galactose, xylose, sucrose, and fructose were quantified from centrifuged and filtered supernatants by liquid chromatography using an Aminex HPX-87C column and a Shimadzu HPLC system consisting of SIL-20AC autosampler, LC-20AD pump, CTO-20AC column oven, and RID-10A refractive index detector. Water was used as the mobile phase with a flow rate of 0.6 mL/min, while column and detector temperature were maintained at 75°C and 50°C, respectively. Starch was hydrolyzed as reported by Kim and Chang [15] and then analyzed by HPLC.

Biomass was determined by dry cell weight. For this purpose, 10 mL of culture broth was centrifuged in preweighted tubes. The resulting cells pellet was washed twice with distilled water, dried, and weighted again to obtain the biomass weight.

For EPS determination, 30 mL of sample was centrifuged (5000 rpm, 30 min) and 25 mL of the free cells-supernatant was mixed with 4 volumes of cold ethanol and kept at 4°C overnight. The resulting precipitate was recovered by centrifugation and dried for weight determination. The rest of the culture broth received the same treatment; it was frozen and lyophilized for further characterization.

2.7. Characterization of EPS. The isolated EPS was analyzed by Fourier transform-infrared spectroscopy (FTIR) in the 400 to 4000 cm^{-1} region with a Perkin-Elmer Spectrum GX spectrometer. For the monosaccharide analysis, 10 mg of lyophilized EPS was hydrolyzed in 2 M Trifluoroacetic acid at 121°C for 3 h. The EPS hydrolysate was neutralized with a 1 M NaOH solution, and sugars were analyzed by HPLC as previously mentioned.

3. Results and Discussion

3.1. Production of EPS from Different C Sources in a Nutrient Balanced Medium. The production of exopolymeric substances was observed from all the C sources tested. The highest production (1.5–1.6 g/L) was noted when glucose or starch were used as C source (Figure 1). Those amounts represent almost twice the value of EPS compared to those produced from galactose, sucrose, and xylose. This result is similar to that obtained by Czaczyk and Myszka [16]; they worked with *Acetobacter xylinum* and reported the highest EPS production from starch while lower efficiency occurred under galactose and xylose. It was also noted that the growth of *S. degradans* and the EPS produced from fructose was less than that presented when growing on all the other C sources. It is worth mentioning that the difference between the C sources used is statistically significant at the 0.05 level (P value = 0.0001). The obtained yield values for biomass production on substrate ($Y_{X/S}$) were similar for all the C sources used (around 0.3 g biomass/g substrate). Meanwhile the g of EPS produced per g of substrate consumed ($Y_{EPS/S}$) or per g of biomass produced ($Y_{EPS/S}$) were higher when using glucose or starch as C source (Table 3). It has been reported that the type of C source in some cases affects the composition and molecular mass of the EPS produced, but it always affects the yield and the total amount of polysaccharide produced [3]. The use of a wide variety of C sources to produce microbial exopolysaccharides has been reported, predominantly carbohydrates such as glucose, sucrose, starch, maltose, sugar concentrates, and lactose [17]. In this study, lactose was also used as C source, but this source did not support growth nor produced EPS by *S. degradans*, the same result observed when glycerol was used.

Table 3: Biomass and product formation yields from the different C sources used by *S. degradans* growing on BM medium (two replicates).

Carbon source	$Y_{EPS/S}$ [a]	$Y_{EPS/X}$ [b]	$Y_{X/S}$ [c]
Glucose	0.17 ± 0.06	0.56 ± 0.11	0.30 ± 0.05
Galactose	0.10 ± 0.04	0.26 ± 0.07	0.37 ± 0.04
Sucrose	0.09 ± 0.02	0.36 ± 0.09	0.26 ± 0.00
Fructose	0.05 ± 0.01	0.40 ± 0.12	0.33 ± 0.03
Starch	0.18 ± 0.06	0.63 ± 0.01	0.28 ± 0.10
Xylose	0.12 ± 0.01	0.53 ± 0.13	0.23 ± 0.07

[a] $Y_{EPS/S}$ = g EPS produced/g carbon source consumed.
[b] $Y_{EPS/X}$ = g EPS produced/g biomass produced.
[c] $Y_{X/S}$ = g biomass produced/g carbon source consumed.

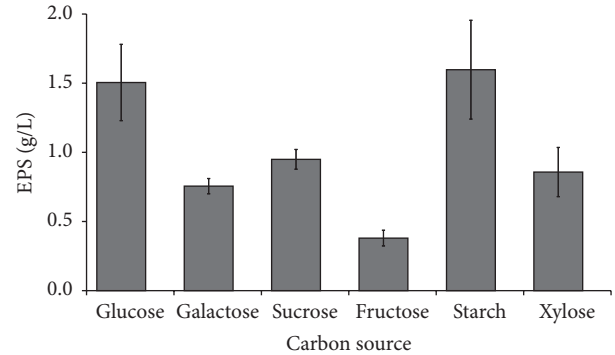

Figure 1: Maximum EPS production from each of the C sources tested in basal medium (BM). The values represent the media of three replicates.

3.2. Effect of C/N Ratio and Limitation in Nutrients on EPS Biosynthesis. Regarding experiment B, the effect of C/N ratio and the effect of combined nutrients limitation (Table 2) on

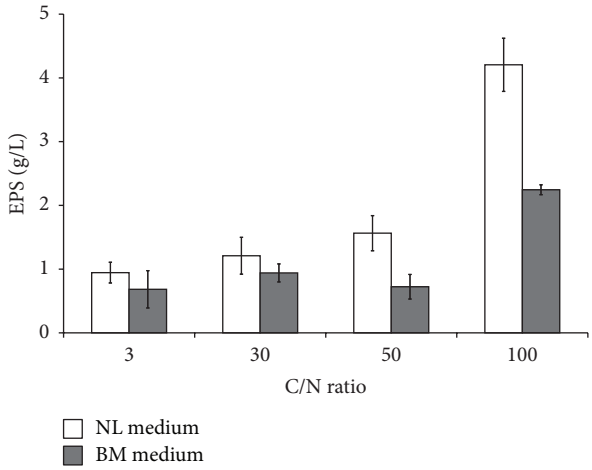

Figure 2: Effect of the C/N ratio and nutrient limitation on the EPS production by *S. degradans* from glucose as C source. The strain was cultured on the basal medium (BM) and on the nutrients limited medium (NL) which contains only 50% of N, P, S, Mg, Fe, and Ca regarding the BM.

EPS biosynthesis were studied (Figure 2). When using C/N ratios of 3, 30, and 50 the EPS production was practically the same (0.7–0.8 g/L) in BM medium; meanwhile in NL medium an increment in EPS production was observed as the C/N ratio increases from 3 to 50 (0.9 to 1.4 g/L). It was noticed that when using the C/N ratio of 100, the polysaccharide production was triggered in both mediums (limited or not limited) almost 3.5 times in comparison with the previous ratio tested (50). Therefore, a positive synergistic effect by the combination of factors (C/N ratio and nutrients limitation as depicted in Table 2) was observed, since the maximum EPS biosynthesis occurred at a C/N ratio of 100 when cultivated in NL medium (Figure 2). This value (4.12 g/L) is twice higher than that obtained at the same C/N ratio, but in BM medium, which demonstrates the importance of other nutrients besides N on the EPS biosynthesis in *S. degradans*. It is worth mentioning that, under these conditions, the EPS production was 2.7 times higher than that obtained using glucose in the regular BM medium (1.5 g/L) (Figure 1); this difference between treatments is statistically significant ($\alpha = 0.05$).

The importance of C/N ratio in EPS production has been reported previously by other researches using different bacterial strains; they all agreed that the extracellular biopolymers' synthesis by microbial cells depends on both the C and N availability in the culture medium [16]. Miqueleto et al. [18] conducted a study where the aim was to evaluate the influence of different C sources and the C/N ratio on the production of EPS by immobilized bacterial biomass and found that high C/N ratio favored the biopolymer production.

Sutherland [19] reported that under limited ammonium salts in the medium, 60% of the glucose was converted into EPS by *Sinorhizobium* spp., *Escherichia* spp., and *Pseudomonas* spp. According to Vargas-García et al. [20], microbial biomass production was higher as the N concentration increased in the medium, while EPS synthesis showed an opposite behavior since its production was higher at lower N concentration. Kimmel et al. [21] also mentioned this behavior by bacteria such as *Xanthomonas*, *Pseudomonas*, and *Rhizobium*.

Table 4: Kinetic parameters for EPS production from glucose (2 L Bioreactor; C/N ratio, 100; medium, NL) calculated using KaleidaGraph software (V4.0).

	First phase[a]		Second phase[b]	
	m^3: k (h^{-1})	R^2	m (h^{-1})	R^2
Consumption				
Glucose	0.1970	0.9974	0.1456	0.9913
NH_4^+	0.1752	0.9813	7.7×10^{-4}	0.9738
$PO_4^=$	0.2279	0.9976	3.9×10^{-4}	0.8377
Production				
Biomass	0.0895	0.9871	0.0189	0.9574
EPS	0.0134	0.9829	0.0896	0.9784

[a]Gompertz model (0–27 h).
[b]Lineal model (28–72 h).

On the other hand, it has also been observed that not only C/N ratio is important for EPS biosynthesis [22], since the concentration of P, O_2, and other ions such as Mg, Fe, Ca, and Zn also affects the conversion of the C source into polysaccharide [3]. In regard to P limitation, it has been observed that the maximum EPS production by bacteria such as *Klebsiella* spp. [23] and *Azotobacter vinelandii* [24] occurred in the absence or at low concentrations of phosphate ion. The impact of some cations on EPS production by different kinds of bacteria has also been studied, and contradictory results have been described, since some researchers found that the omission of some ions (Fe, Zn, Ca, and Mn) promoted microbial growth and polymer synthesis, as observed with *S. degradans*, while other studies demonstrated that presence of them (not the omission) stimulates the EPS production [3].

3.3. Production of EPS in Bioreactor. The fermentation profile using glucose as C source under the optimum conditions previously determined to produce EPS (C/N ratio, 100; medium, NL) was studied (Figure 3). The cell growth, substrates consumption, and EPS production are divided into two phases (0–27 h and 28–72 h). The first phase initiated with 10.12 g/L of glucose as C source and after 24 h of culture it was consumed almost completely (80%); therefore a glucose pulse was fed in order to rise the concentration again to 10 g/L (second phase). It is worth mentioning that neither any N nor P sources were added during this second phase. It was observed that after the pulse the glucose, ammonium, and phosphate consumption rate was considerably slower than in the first phase; the same behavior was observed regarding the cell growth rate which was 4.73 times lower than the growth rate presented during the first phase (Table 4). On the contrary, it was evident that under the second phase (nutrient limitation) the EPS production rate was higher (6.68 times) than in the first phase (Figure 3(a)). Related to these results, Bramhachari and Dubey [25] reported that the rate of EPS production in batch culture is the highest at the late log phase of growth when approaching stationary phase, which is the same behavior, observed with *S. degradans*. Czaczyk and Myszka [16]

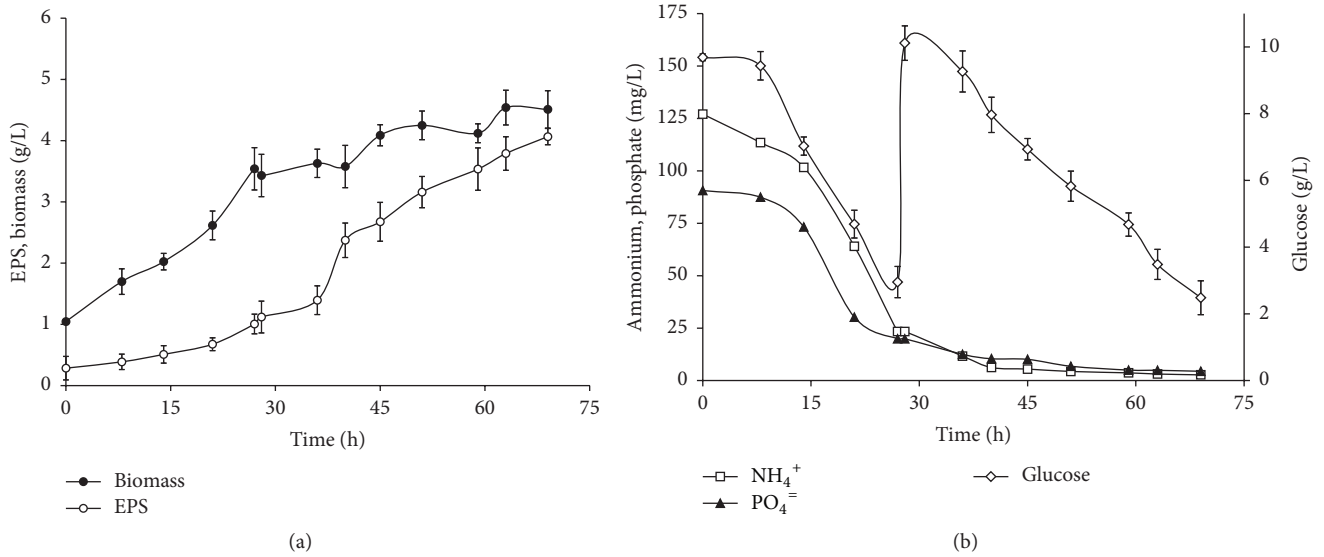

Figure 3: Fermentation profile of *S. degradans* with glucose as C source in NL medium at a C/N ratio of 100. (a) Biomass and EPS production. (b) Glucose, ammonium, and phosphate consumption.

reported that the dependence between EPS production and the stage of the microbial growth cycle is a feature of particular genera. In strains of *Pseudomonas* and *Staphylococcus*, high production of EPS was observed during the late logarithmic and early stationary phases of microbial growth [19, 26]. Mata et al. [27] described the same performance for three moderately halophilic bacteria (family Alteromonadaceae), and Poli et al. [28] observed in *Alteromonas macleodii* subsp. *fijiensis* that the production of EPS began at the end of the exponential phase and continued throughout the stationary phase.

As shown in the results (Figure 3(b)), the disappearance of N from the medium might also trigger exopolysaccharide synthesis, which was previously observed for other EPS such as pullulan and scleroglucan [17].

Regarding the amount of EPS produced by marine bacteria, strains such as *Bacillus thermodenitrificans*, *Bacillus licheniformis* B3–15, *Geobacillus* sp. 4004 [28], *Micrococcus* sp. [29], and *Aeribacillus pallidus* 418 [30] exhibit low production (less than 1 g/L). Bacteria such as *Halobacterium* sp. SM5 [31], *Idiomarina fontislapidosi* F32T, *Idiomarina ramblicola* R22T, and *Alteromonas hispanica* F23T [27] show a moderate EPS production (1 to 2.25 g/L).

On the other hand marine bacteria such as *Alteromonas macleodii*, *Pseudoalteromonas* sp. SM9913 [28], *Hahella chejuensis* [32], and *Zunongwangia profunda* SM-A87 [33] produce a significantly higher amount of EPS: 6, 5.5, 9.23, and 8.9 g/L, respectively. While *Pantoea* sp. BM39 (isolated from seafloor sediments) is the highest EPS producer so far reported (21.30 g/L) [34].

Concerning *S. degradans*, the maximum EPS production was 4.12 g/L, reached after 72 h of culture (C/N ratio, 100; culture medium, NL), which lies within the range previously mentioned.

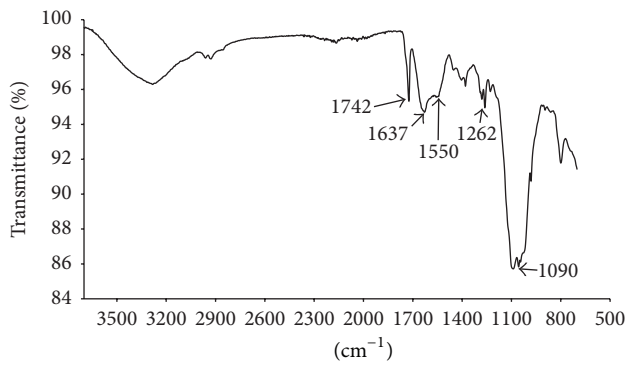

Figure 4: FTIR spectrum of the isolated EPS. It shows bands between 1000 and 1200 cm^{-1}; carboxyl group at 1637 and amide II at 1550 cm^{-1}; and bands at 1742 and 1262 cm^{-1}.

3.4. Characterization of EPS. The FTIR spectrum (Figure 4) presents a band between 1000 and 1200 cm^{-1} related to the presence of carbohydrates; carboxyl group at 1637 and amide II at 1550 cm^{-1}, which are related to the presence of proteins [25]; and bands at 1742 and 1262 cm^{-1} that might represent O-acetyl ester linkage bonds [34].

The HPLC analysis (Figure 5) indicates the presence of glucose, mannose, and galactose as the main sugars in the polymer structure. In this regard, it is known that the EPS released by marine bacteria have different chemical composition, structure, and properties. The sugar composition of the EPS produced by *S. degradans* is similar to that reported for other marine bacteria such as *Shewanella colwelliana* [35], bacterium strain 4001 [36], *Geobacillus* sp. [37], and EPS denominated as PS3a24 and PS3a35 produced by bacteria from the intertidal zone [38]. It is important to notice that the complete structure of the EPS synthesized by *S. degradans* has

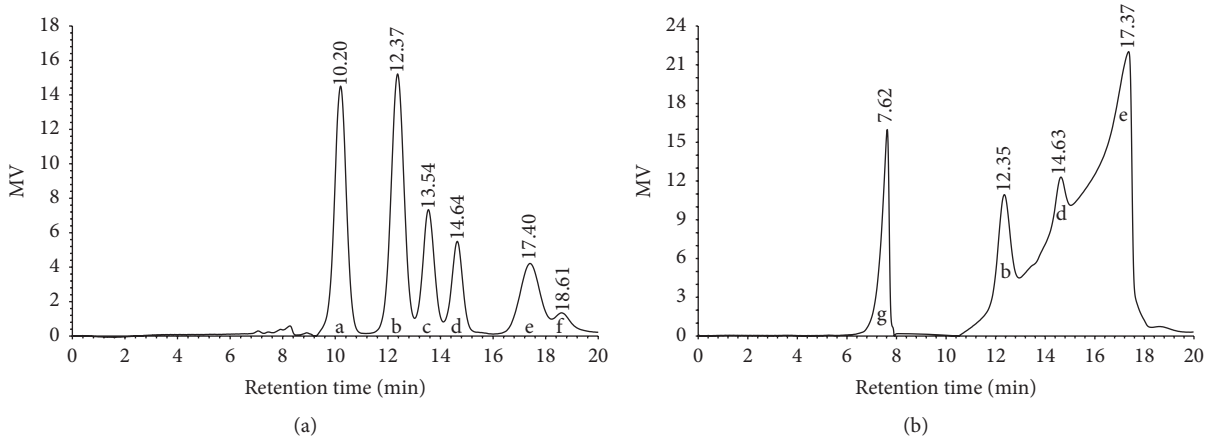

Figure 5: HPLC chromatograms of (a) a standard mixture of sugars and (b) EPS hydrolysate. Peaks a, b, c, d, e, and f correspond to sucrose, glucose, xylose, galactose, mannose, and fructose, respectively. Peak g corresponds to the EPS (not hydrolyzed).

not been elucidated, but based on the FTIR spectrum it might be acetylated or contain amino sugars.

4. Conclusions

It can be concluded from the results previously presented that the wide diversity of microorganisms found in marine environment offers vast potential resources for biotechnological applications. Among them *S. degradans* has proved to be a versatile bacterium able to produce EPS in high amounts from several carbohydrates sources including starch and xylose. This fact becomes very interesting since starch is widely available and cheaper than other substrates such as glucose and fructose; meanwhile xylose can be found in lignocellulosic biomass hydrolysates, which has a huge potential as renewable C sources for fermentation processes. Therefore, the production of EPS in *S. degradans* is enhanced by nutritional limitation; thus applying statistical optimization is recommendable to elucidate the effect of limitation in specific nutrients on the biopolymer biosynthesis and improve it. Work is ongoing to elucidate this EPS complete chemical structure and its potential application.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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