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Exopolysaccharides Produced by *Rhizobium*: Production, Composition and Rheological Properties

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Abstract The use of exopolysaccharides (EPS) in industrial product formulations has increased in recent years due to their ability to increase the viscosity of solutions or cause the formation of gels, affecting the texture of products. In industry, EPS can be added as gelling, thickening and stabilizing agents in foods, pharmaceuticals and cosmetics. In this context, EPS from nitrogen-fixing rhizobial bacteria are emerging as potential biopolymers for industrial applications. However, the establishment of cultivation conditions, their chemical structure and physicochemical characteristics, such as rheological behavior, are essential to enable their use on a large scale. Furthermore, the possibility of using byproducts and agroindustrial wastes as substrates can contribute to the economic feasiability of the process. In this context, this article aims to present a review of EPS synthesized by different strains of Rhizobium in relation to their production, composition and rheological properties.

Keywords: *fixing-nitrogen bacteria, rhizobia, biopolymers, extracellular polysaccharides, rheological behavior*

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1. Introduction

The industrial use of bacterial polysaccharides has increased in recent years, due to their high potential for application in various industrial sectors. They are used as gelling, thickening and stabilizing agents during the processing of food and pharmaceutical products [13,16,21,27]. Moreover, they can present antioxidant and antibacterial activities [24] and be capable of acting against tumor cells [23].

The term exopolysaccharide (EPS) is used to name the biopolymers found attached to the surface of cells or secreted into the extracellular medium [2]. Studies have emphasized Rhizobia as potential producers of large quantities of EPS, highlighting the *Rhizobium* genus, that are capable of forming gels at low concentrations [1,28]. However, whereas xanthan gum, gellan gum and dextran are examples of bacterial EPS commonly used by industry and approved by the FDA (Food and Drug Administration) to be used as food additives [35], a commercial scale production of EPS from *Rhizobium* is not available.

The rheological characteristics of microbial polysaccharides depend on their physico-chemical properties, which are related to the bacterial strain, composition of the medium, pH, temperature, aeration rate, carbon/nitrogen ratio and other process parameters that directly influence the biosynthesis of the biopolymer [40]. Therefore, it is essential to have rigid control of these parameters during the cultivation and synthesis of EPS.

In this context, the purpose of this review is to provide subsidies that may be useful in studies involving the process design for EPS production from the *Rhizobium* genus.

2. EPS produced by *Rhizobium*

Rhizobia bacteria convert atmospheric nitrogen (N_2) into an assimilable form (NH_3) by plant when they are in symbiosis with plants in the Leguminosae family, which will be used for growth or cell maintenance of the plant [42]. This process is known as Biological Nitrogen Fixation (BNF) and is promoted by an enzyme complex called nitrogenase, with impacts on the environment and agriculture, associated with the reduced use of chemical fertilizers [6,7].

Rhizobia are bacteria belonging to class alpha-, beta- and gamma-proteobacteria, including species of the Rhizobiaceae, Phyllobacteriaceae, Methylobacteriaceae, Brucellaceae, Hyphomicrobiaceae, Bradyrhizobiaceae, Burkholderiaceae and Pseudomonaceae families [6]. Rhizobia are Gram-negative, aerobic bacteria, represented by the genera such as *Rhizobium*, *Sinorhizobium*, *Agrobacterium*, *Mesorhizobium* and *Bradyrhizobium*, which are known as producers of different EPS, from simple glycans to complex heteropolymers [8,28,29]. The secretion of EPS by Rhizobia is associated with the invasion process and bacteroid and nodule development, as well as being a response to environmental stresses [7].

Most research works on Rhizobia are directed to the study of genetics and the symbiotic interaction between bacteria and host plant. However, investigations of the synthesis, the production process and application of different types of rhizobial EPS have increased in recent years. They can be highlighted as potential producers of

EPS *Rhizobium* [40], *Mesorhizobium [28]*, *Agrobacterium* [25] and *Bradyrhizobium* [7].

Regarding the *Rhizobium* genus, in recent years several works can be quoted: *Rhizobium* sp. D1 10 [17], *Rhizobium* sp. KYGT 207 [20], *Rhizobium* sp. VMA301 [26], *Rhizobium tropici* [28], *Rhizobium tropici* CIAT899 [40], *Rhizobium radiobacter* S10 [43], *Rhizobium tropici* Semia 4080 [9], *Rhizobium tropici* Semia 4077 [10], Rhizobium leguminosarum bv. trifolii [19] and *Rhizobium leguminosarum* ATCC 10004 [37]. Table 1 presents some information about EPS produced by *Rhizobium*.

Table 1. Overview of cultivation conditions, structural and physicochemical properties of some EPS produced by *Rhizobium*

Microorganism	Studied variables	Established conditions	EPS production	Component characterization	Properties	Reference
Rhizobium sp. LBMP-C04	-	-	6.63 g L^{-1}	Rhamnose Glucose Galactose Mannose Glucuronic acid Galacturonic acid	Pseudoplastic behavior Emulsifying activity	[30]
Rhizobium sp. VMA301	Carbon source Nitrogen source Vitamins	Mannitol 1% w/v L-asparagine 0.3% w/v Biotin 1.5 mg L^{-1}	346 mg L^{-1}	-	-	[26]
Rhizobium D1 10	Carbon source Nitrogen source Vitamins Metal ions	Mannitol 2% w/v KNO_3 0.1% w/v Thiamine hydrochloride 1 mg mL^{-1}	1,122 mg mL^{-1}	Xylose Rhamnose Glucose Galactose Arabinose	-	[17]
Rhizobium sp. PRIM-18	-	-	2.1 g L^{-1}	-	Emulsifying, antioxidant and metal chelating activities Increase in cell proliferation (human dermal fibroblasts)	[34]
Rhizobium radiobacter S10	Carbon source	Whey 10 % w/w	2,834 mg L^{-1}	Galactose Glucose Glucosamine Mannose	Pseudoplastic behavior	[43]
Rhizobium tropici LBMP-C01	-	-	3.48 g L^{-1}	Rhamnose Glucose Galactose	Pseudoplastic behavior Emulsifying activity	[30]
Rhizobium tropici CIAT899	Carbon source pH C/N ratio	Sucrose 55 mM pH 6.9 C/N 20	4.08 g L^{-1}	-	-	[40]
Rhizobium tropici Semia 4077	-	-	7.45 g L^{-1}	Mannose Rhamnose Glucuronic acid Galacturonic acid Glucose Galactose	Pseudoplastic behavior Emulsifying activity	[10]

3. Production Parameters of EPS synthesized by *Rhizobium*

The methods used to obtain EPS from Rhizobia are similar, with some modifications. Figure 1 shows the general procedure for EPS production proposed by Ribeiro et al. [36], based on the works of Barreto et al. [3], Castellane and Lemos [8], Duta et al. [14] and Staudt et al. [40].

The EPS production process is influenced by the strain of the microrganism to operational conditions such as agitation and aeration rates, incubation time, temperature, pH, volume and age of inoculum and cultivation medium composition, acting directly on the synthesis, yield and composition of EPS [3,40].

The selection of microbial strains is a very important factor in the synthesis of these biopolymers and a constant challenge for researchers, in order to produce EPS with specific properties and characteristics [3].

Regarding the initial pH, for most bacteria, the pH should be maintained near neutrality [40]. In the work of Staudt et al. [40], EPS production by *Rhizobium tropici* CIAT899 was observed under near-neutral (pH 6.9) and slightly basic growth conditions (pH 8.0), reaching 2.35 and 2.96 g L^{-1}, respectively, while acidic conditions decreased microbial growth and EPS synthesis.

The carbon/nitrogen (C/N) ratio in the cultivation medium is an important parameter in the production of these biopolymers. The synthesis of EPS by microorganisms is enhanced by the limitation of an essential nutrient, other than carbon. In the work of Staudt et al. [40], the cultivation medium containing a high C/N ratio (20) favored the accumulation of EPS, because any sugar remaining could be directed to EPS synthesis. The same authors observed that the optimal C/N ratio for EPS production was different from the optimal C/N ratio for *Rhizobium tropici* CIAT899 growth. This behavior suggests that a two-stage process may be proposed: a stage of growth where higher nitrogen concentration is required for cell growth; and the production phase, where higher carbon concentration is required, for the purpose of product accumulation.

The cultivation medium composition is another essential factor in the synthesis of EPS, including different sources of carbon, nitrogen and potassium that are used for the growth of the microorganism. The presence of

trace elements such as sodium, potassium, calcium, magnesium and iron, are important because they are used as cofactors in the enzymatic production routes of polysaccharides. In the work of Duta et al. [14], the introduction of calcium carbonate in the medium, associated with high agitation and aeration, in the cultivation of *Rhizobium* EQ1, promoted a significant increase in the substrate/product yield. Breedveld et al. [5], in the cultivation of *Rhizobium meliloti* SU-47 in a medium containing 10 g L^{-1} mannitol, observed that the increase in osmotic pressure by the addition of NaCl (0.2 M) stimulated succinoglycan production, reaching 2.4 g L^{-1}. The effect of the presence of metal ions was evaluated by Ghosh et al. [17]. $HgCl_2$, $ZnSO_4.7H_2O$, K_2SO_4, $CuSO_4.7H_2O$, $MnCl_2.4H_2O$, KI, H_3BO_4, $FeSO_4.7H_2O$ and $NiCl_2$ were tested and no effect was observed in the production of EPS by *Rhizobium* D1 10. The same authors observed that the addition of thiamine hydrochloride improved EPS production.

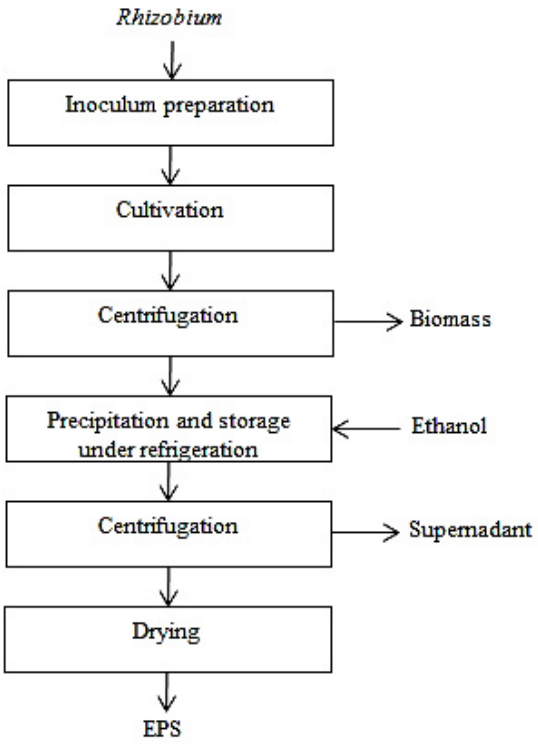

Figure 1. Schematic diagram for the production of EPS by Rhizobia (adapted from [36])

A key factor is the carbon source. Several sources of carbon and its influence on production of EPS have been investigated over the years. Navarini et al. [31] tested mannitol, glucose, galactose, lactose, sucrose and maltose as carbon sources, as well as the nitrogen source (glutamate, lysine, glycine, sodium nitrate, ammonium sulphate and yeast extract), in the cultivation of *Rhizobium hedysari*. A yield of 9.04 g L^{-1} was achieved using 15 g L^{-1} of mannitol and 1.5 g L^{-1} of glutamate.

Ghosh et al. [17] evaluated different carbon sources at 1% (lactose, maltose, L-arabinose, D-ribose, raffinose, glucitol, D-xylose, myo-inositol, D-manose, sucrose, D-galactose, D-fructose, D-glucose and D-mannitol) for the cultivation of *Rhizobium* D1 10. The best production was obtained using mannitol (765 mg L^{-1}), followed by D-glucose and D-galactose. Regarding the nitrogen source, potassium nitrate at 0.1% was the most effective for the production of EPS by *Rhizobium* D1 10 among other sources tested in the same concentration (ammonium chloride, L-asparagine, casamino acid, L-glutamic acid and sodium nitrate).

Kaci et al. [20] found an EPS production of 2.5 g L^{-1} by *Rhizobium* sp. KYGT207 using 20 g L^{-1} sucrose as a carbon source after three days of cultivation. Mandal et al. [26], in the cultivation of *Rhizobium* sp. VMA301, tested a number of carbon sources (lactose, L-arabinose, raffinose, D-ribose, mannitol, D-galactose, myo-inositol, D-xylose, maltose, glucose and glycerol) and nitrogen sources (glycine, ammonium sulphate, ammonium chloride, potassium nitrate, sodium nitrate, casamino acid, L-asparagine and glutamic acid) for EPS production. The yeast extract basal medium supplemented with mannitol (10 g L^{-1}), biotin (1.5 mg L^{-1}) and L-asparagine (3 g L^{-1}) resulted in a production of 346 mg L^{-1} of EPS.

Staudt et al. [40] observed an EPS production of 4.08 g L^{-1} with sucrose (10 g L^{-1}) as the carbon source and a C/N ratio of 20, in the cultivation of *Rhizobium tropici* CIAT899. Castellane et al. [9] achieved an EPS production of 2.52 g L^{-1} cultivating *Rhizobium tropici* Semia 4080 in a medium containing mannitol as a carbon source. Castellane et al. [10] produced 7.45 g L^{-1} EPS with *Rhizobium tropici* Semia 4077 using sucrose (30 g L^{-1}) as a carbon source.

Few authors have investigated EPS production using industrial wastes as a source of carbon. Devi et al. [11] achieved an EPS production of 1.2 g L^{-1} using *Sinorhizobium meliloti* MTCC 100, adding rice bran in the hydrolyzed form (20%) to the medium. Zhou et al. [43] achieved an EPS production of 2.83 g L^{-1} in the cultivation of *Rhizobium radiobacter* S10 in a medium containing whey, while Sellami et al. [37] produced 11.1 g L^{-1} of EPS by *Rhizobium leguminosarum* ATCC 10004, using residual water from the fish industry in the cultivation medium.

4. Composition of EPS from *Rhizobium*

The composition of EPSs produced by *Rhizobium* is reduced to a few monosaccharides including glucose, galactose, mannose, rhamnose, glucuronic acid and galacturonic acid [10,28].

Becker and Pühler [4] detected that a succinoglycan from *Rhizobium* sp. NGR234 is formed by repeated units containing one galactose and seven glucose molecules linked by β-1,3, β-1,4 and β-1,6 linkages, containing residues of succinyl, acetyl and pyruvyl. This microorganism synthesized another form of EPS, consisting of alternated units of glucose and galactose with α-1,3 and β-1,3 linkages and containing residues of acetyl and pyruvyl. Guentas et al. [18] analyzed the molecular structure of the EPS produced by a strain of *Rhizobium* sp. B isolated from nodules of alfafa and showed that this biopolymer consisted of high amounts of glucose and rhamnose (1:2) and traces of 2-deoxy-D-arabino-hexuronic acid. Staehelin et al. [39] verified that the acidic EPS synthesized by *Rhizobium* sp. NGR234 were composed by glucosyl, galactosyl, glucuronosyl and 4,6-pyruvylated galactosyl residues with glycosidic linkages β-1,3, β-1,4, β-1,6, α-1,3 and α-1,4.

Castellane and Lemos [8] studied the effect of different carbon sources (sucrose, glucose, glycerol and galactose) in the composition of EPS produced by *Rhizobium tropici* 4077 and 4080. They found the predominance of units of glucose, galactose and glucuronic acid with variations in their ratios according to the carbon source that was used. They also observed traces of mannose, rhamnose and galacturonic acid. Aranda-Selverio et al. [1] verified in the EPS produced by *Rhizobium tropici* the majoritary presence of glucose (79%), galactose (21%) and mannose (traces). Staudt et al. [40] observed evidence that the chemical composition of EPS produced by *Rhizobium tropici* CIAT899 varies with the carbon source, with the predominance of glucose and galactose residues when using D-glucose and L-arabinose as substrates. Castellane et al. [9] analysed EPS from *Rhizobium tropici* Semia 4080, mutant strains of *Rhizobium tropici* (MUTZC3 and MUTPA7) and rhizobial isolates (JAB1 and JAB6). They found as majority components the monosaccharides glucose (from 53.53 to 60.70%) and galactose (from 29.35 to 40.52%), with a low content of mannose, rhamnose, glucuronic acid and galacturonic acid.

Zhou et al. [43] noted that EPS synthesized by *Rhizobium radiobacter* S10 in a medium containing whey was formed by galactose and glucose at a ratio of (1:4.92), with traces of glucosamine and mannose, and a molecular weight of 3.03 x 10^6 Da. Priyanka et al. [34] produced an EPS consisting of glucose, galactose and mannose (6.1:1.8:1) in the cultivation of *Rhizobium* sp. PRIM-18, with a molecular weight of 9.33 x 10^6 Da.

In the work of Moretto et al. [30], the composition of EPS produced by *Rhizobium tropici* LBMP-C01 and *Rhizobium* sp. LBMP-C04, among other species, was evaluated. EPS from the LBMP-C04 strain showed glucose (54.17%), galactose (38.99%) and small amounts of glucuronic acid (3.57%), mannose (2.68%) and rhamnose (0.60%), while EPS from the LBMP-C01 strain showed glucose (65.25%), galactose (32.61%) and rhamnose (2.13%), without the presence of mannose and glucuronic acid.

Some chemical structures proposed for EPS produced by rhizobial bacteria are shown in Figure 2 and Figure 3.

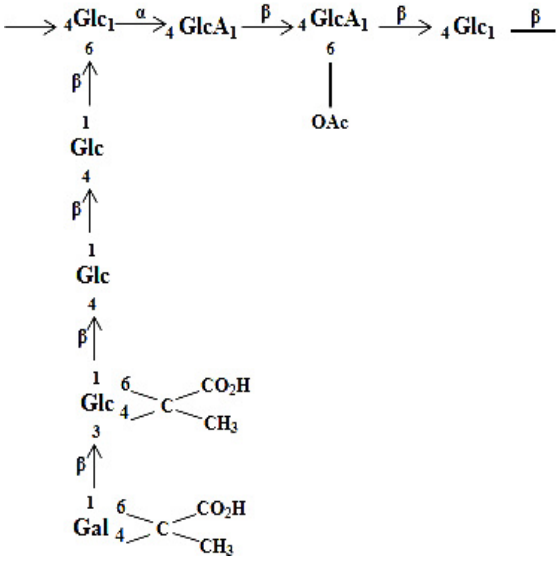

Figure 2. Chemical structure of the EPS produced by *Rhizobium leguminosarum* bv. *trifolii* [38]. Glc (glucose), Gal (galactose), Ac (acetyl)

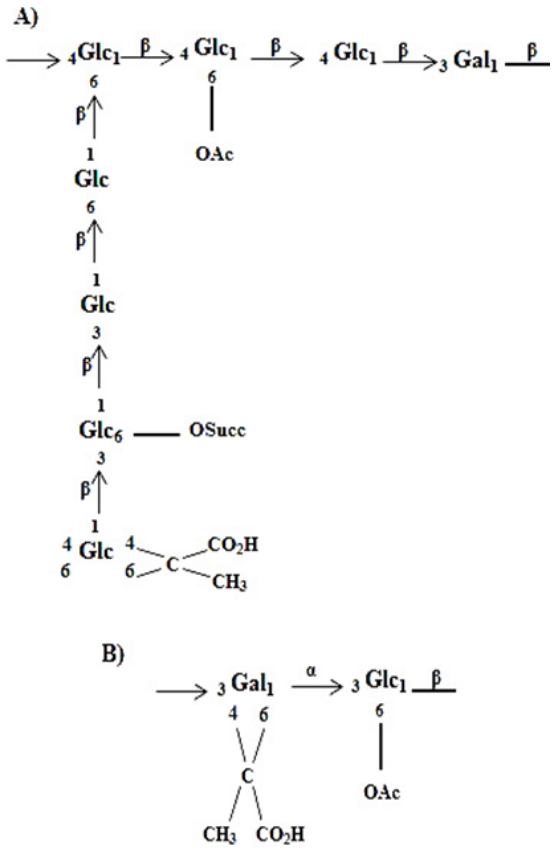

Figure 3. Chemical structures of the EPS produced by *Sinorhizobium meliloti*. (A) EPS I (succinoglycan); and (B) EPS II (galactoglucan) [38]. Glc (glucose), Gal (galactose), Succ (succinyl), Ac (acetyl)

5. Rheology of EPS Solutions Produced by *Rhizobium*

The rheology of EPS in an aqueous solution is influenced by molecular weight, chemical structure, concentration, temperature and shear rate [15,16,41].

The presence of ionizable groups leads to polyelectrolyte behavior, significantly affecting the aqueous properties [16]. The sequence of arrangement of sugars in the molecule and how their chains interact in the presence of ionic charged residues found in the branches, directly interferes in their rheological properties because the existing charges in acidic functional groups are present in these biopolymers, affecting the quality of conformation of molecular structure and increasing the solubility of the biopolymer [22].

Different EPS solutions with different concentrations, present different viscosities at shear rate equal to zero [41]. The effect of temperature on viscosity is described by the Arrhenius model and must be considered because the biopolymer can be submmited to different temperatures according to its processing [41].

The rheological behavior of materials may be described by mathematical models relating the shear stress and the shear rate. The most frequently used for food systems are the Ostwald De-Waele (Power Law), Casson, Herschel-Bulkley and Mizrahi-Berki models, the two first with two parameters and the others with three parameters [1].

The pseudoplastic fluids are those that exhibit non-Newtonian behavior characterized by the decrease of the viscosity rate with increasing shear rate [15]. The

pseudoplastic behavior has been observed in several EPS produced by *Rhizobium* [1,3,10,15,20].

Most articles using the Power Law model present the consistency coefficient (K) and the flow behavior index (n). The determination of "K" is important and can be applied for comparing different EPS concentrations, where an increase in "K" indicates a gradual increase in viscosity according to the concentration of the polymer. It can be useful to evaluate the effect of electrolytes on the viscosity of the EPS solution and to evaluate EPS from different sources. The determination of "n" indicates the degree of deviation of the Newtonian behavior. When the value of "n" is less than one, a pseudoplastic behavior is confirmed [1]. The value of "n" is obtained from the slope of the log-log plot of viscosity versus shear rate. The value of "K" is calculated from the intercept of the same graph [9].

Castellane et al. [9] studied the rheological behavior in solutions (0.5% w/v) of EPS produced by *Rhizobium tropici* Semia 4080 and isolates JAB1 and JAB6, and verified a pseudoplastic profile in all biopolymers. For EPS from *Rhizobium tropici* Semia 4080, JAB1 and JAB6, "K" values equal to 0.29, 2.1 and 1.9, respectively, were found, while the "n" values were 0.41, 0.25 and 0.26, respectively. An increment in "K" values was observed when concentration was changed from 0.5 to 1% w/v. Castellane et al. [10] observed the behavior of the EPS solution (0.5% w/v) produced by *Rhizobium tropici* Semia 4077 and a mutant strain 4070. They achieved values of 3.7 and 2.9 for "K", respectively, while the "n" values were 0.17 and 0.20, respectively, indicating a pseudoplastic behavior. Ribeiro et al. [36] also observed a pseudoplastic behavior in a solution of EPS (0.5% w/v) produced by *Rhizobium tropici* Semia 4077 using raw glycerol as carbon source (Figure 4).

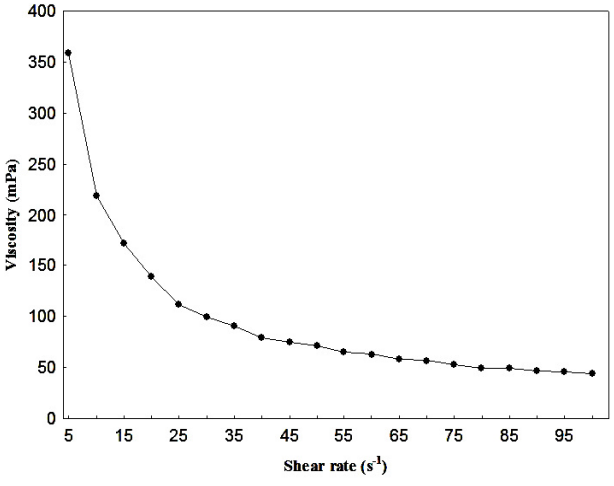

Figure 4. Variation of viscosity versus shear rate for aqueous EPS solution (0.5 % w/v) produced by *Rhizobium tropici* Semia 4077 [36]

Moretto et al. [30] studied the rheological behavior of an EPS solution at 0.5% (w/v) produced by *Rhizobium* LBMP-C01, LBMP-C02, LBMP-C03 and LBMP-C04. The values of "n" were 0.32, 0.17, 0.34 and 0.26, respectively, and "K" values were 1.00, 3.10, 1.00 and 1.90, respectively, confirming the pseudoplastic behavior of the EPS.

Aranda-Selverio et al. [1] observed that the presence of 0.2 M NaCl in EPS solutions from *Rhizobium* led to a small increase in the values of "K" when compared to the same concentrations of the respective aqueous solutions of polysaccharides, indicating that the presence of electrolytes favored the viscosity of the biopolymer. According to Diaz et al. [12], the addition of inorganic salts to the polysaccharide solution can promote changes in the viscosity, increasing or decreasing its value. According to Aranda-Selverio et al. [1], the variation in the degree of substituents of a polysaccharide changes their rheological properties in the solution, whose behavior can be attributed to the interaction of charged residues of uronic acid and ions from the dissociation of the salt when present in the solution.

The interest in biopolymers with pseudoplastic characteristics is due to their wide potential for industrial application in the food industry. They are used to improve the sensory qualities such as taste, mouthfeel and suspending properties of foodstuffs. They can also be used in the chemical industry and in the manufacturing of cosmetics, agricultural pesticides, paint and textiles, due to their high viscosity at low concentrations, compatibility with mineral salts and good stability over a wide range of pH, temperature and ionic strength [32,33,34].

6. Conclusion

The study of the cultivation conditions, in addition to the physical and chemical properties, especially the rheological behavior, is essential for the development of a production process and for the improvement of the quality of EPS from *Rhizobium*, establishing potential commercial applications. Furthermore, the use of byproducts and agroindustrial wastes as substrates for *Rhizobium* cultivation remains scarce and constitutes a promising field for future research.

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