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Effects of process conditions on exopolysaccharide produced by *Mesorhizobium* sp. in whey permeate

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ABSTRACT. Exopolysaccharides (EPS) produced by diazotrophic bacteria are promising biomolecules with commercial potential; however, effects of cultivation conditions on their yield have remained underexplored. This study aimed to maximize EPS production by *Mesorhizobium* sp. SEMIA 816 in whey permeate (WP) as the carbon source. A Plackett–Burman (PB) design was applied to assess the impact of 12 variables, namely K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, yeast extract (YE), $MnCl_2 \cdot 4H_2O$, $CaCl_2 \cdot 2H_2O$, WP, pH, medium-to-reactor volume ratio (V_M:V_R), agitation and temperature, on EPS and biomass concentrations. EPS production ranged from 0 to 9.28 g L^{-1}; the WP concentration exerted the most positive influence. Biomass production ranged from 0.6 to 7.15 g L^{-1}; YE exerted the greatest effect, although it was negatively correlated with EPS production. Maximum EPS concentration (9.28 g L^{-1}) was achieved after 96 h under the central point conditions of the experimental design, whereas the highest biomass concentration (7.15 g L^{-1}) was reached after 72 h under a different set of conditions. Agitation and temperature influenced both responses negatively, a fact that highlighted the need to control them precisely. This study provides insights into the maximization of EPS production since it shows the potential of WP as an effective carbon source and identifies key factors affecting both EPS and biomass yields.

Keywords: Biopolymer; Diazotrophic bacteria; Experimental design.

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Introduction

Many microorganisms synthesize polysaccharides in response to stress and have their production favored by excess carbon sources (Castellane et al., 2017; Freitas et al., 2010). Biopolymers, classified into intracellular and extracellular, exhibit unique physicochemical properties and have versatile applications (Hussain et al., 2017; Nwodo et al., 2012; Paulo et al., 2012). Exopolysaccharides (EPS), which are extracellular polymers produced by bacteria, molds and yeasts, are found either attached to cells or secreted into the medium as capsules or slime (Moscovici, 2015; Seesuriyachan et al., 2012; Silva et al., 2006; Suresh Kumar et al., 2007). Their diverse chemical and structural characteristics enable various applications since they develop viscous solutions in aqueous media (Bomfeti et al., 2011; Silva et al., 2006).

Due to their flexible properties, EPS have garnered increasing interest. They share characteristics with plant-derived gums and offer other advantages, such as faster production, independence from climate factors and reproducible properties in diverse raw material (Suresh Kumar et al., 2007). Commercial production depends on optimizing cultivation conditions, determining chemical structures and assessing certain physicochemical properties, such as rheology. EPS serve as thickeners, gelling agents and stabilizers in food, pharmaceutical, cosmetic and oil industries (Barreto et al., 2011; Ribeiro & Burkert, 2016).

Diazotrophic bacteria, which are biological nitrogen-fixing ones, produce substantial amounts of EPS. They belong to the Rhizobiaceae family, which includes *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium* and *Azorhizobium* (Bomfeti et al., 2011; Janczarek et al., 2015; Monteiro et al., 2012).

Most studies of rhizobial EPS production use carbohydrates, such as glucose (Castellane & Lemos, 2007; Razika et al., 2012; Staudt et al., 2012), sucrose (Barreto et al., 2011; Castellane & Lemos, 2007; Staudt et al., 2012) and mannitol (Castellane & Lemos, 2007; Sayyed et al., 2011; Staudt et al., 2012). However, high costs of substrates hinder commercialization. To mitigate costs, agro-industrial residues, such as residual glycerol (Oliveira et al., 2018), rice bran hydrolysate (Devi et al., 2012), whey (Zhou et al., 2014), fish-processing wastewater (Sellami et al., 2015) and soybean molasses (Oliveira et al., 2020), have been investigated.

In this scenario, whey permeate (WP), a dairy byproduct that results from cheese whey ultrafiltration, is a promising alternative. Powdered WP contains approximately 76–85% (w/v) lactose, 2–7% protein, minerals and 5% moisture (American Dairy Products Institute, 2023). Its high lactose content makes it a viable carbon source for biotechnological applications, such as microbial cultivation. WP has been valued in organic acid, biofuel and biochemical production since it offers environmental and economic benefits. In food, pharmaceuticals and animal feed, WP supports waste reduction and circular bioeconomy practices. Its low cost and high availability enable it to be a viable substrate for EPS production (Mukherjee et al., 2023; Nham et al., 2024; Risner et al., 2020).

EPS yields depend on growth conditions, such as concentrations of carbon sources, temperature, pH and microorganism strains. However, there are few data on their effects on rhizobial EPS production. Experimental designs, such as the Plackett-Burman (PB) screening, are valuable for evaluating multiple factors by minimal experiments and for guiding process optimization. Optimizing EPS production enhances industrial competitiveness by reducing waste and supporting circular bioeconomy and makes it a viable alternative in food, pharmaceutical and cosmetic industries.

Therefore, we aimed to investigate EPS production by *Mesorhizobium* sp. SEMIA 816 in WP as the carbon source by employing the PB design to identify the most critical variables in the process. *Mesorhizobium* sp. was previously selected by a study that investigated the potential of different rhizobial strains to produce EPS in WP (González, 2019).

Material and methods

Microorganism

The bacterial strain *Mesorhizobium* sp. SEMIA 816, which was used as the EPS-producing microorganism, was provided by the SEMIA Rhizobia Collection (DDPA, SEAPI, Porto Alegre, Brazil). It was maintained at 8 °C and reactivated through successive transfers on Yeast Mannitol (YMA) agar, which contained mannitol (10 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), NaCl (0.1 g L^{-1}), yeast extract (0.4 g L^{-1}) and agar (15 g L^{-1}). After reactivation, cultures were incubated at 30°C for 48 h (Oliveira et al., 2020).

Whey permeate

Bovine WP powder was provided by Arla Foods Ingredients (Viby, Denmark) with approximately 85.15% carbohydrate (mainly lactose), 6.41% ash, 5.22% moisture, 2.58% protein and 0.64% fat.

Inoculum preparation

In inoculum preparation, 10 mL 0.1% (*w/v*) peptone diluent was added to the reactivated culture, scraped and transferred to a 500 mL Erlenmeyer flask containing 90 mL YMA broth with mannitol (10 g L^{-1}), K_2HPO_4 (0.1 g L^{-1}), KH_2PO_4 (0.4 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), NaCl (0.1 g L^{-1}) and yeast extract (0.4 g L^{-1}). pH was adjusted to 7 and incubation occurred at 30°C in a rotary shaker (Tecnal TE-420, Brazil) at 200 rpm until optical density (OD) of 0.8 at 600 nm was reached (Staudt et al., 2012).

Shake flask cultivation

EPS production in WP as the carbon source was evaluated by the PB design with 20 assays, including 4 central points (Table 1), following Rodrigues and Iemma (2014). Variable levels were based on González (2019), considering higher, lower and absent components. Cultures were conducted in 500 mL flasks in medium volumes adjusted to PB design ratios (V_M:V_R). Concentrations of K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, yeast extract, $MnCl_2 \cdot 4H_2O$, $CaCl_2 \cdot 2H_2O$ and WP varied in the assays. pH was adjusted accordingly and 10% (v/v) inoculum was added. Flasks were incubated under PB-defined temperature and agitation conditions for 96 h.

Biomass concentration

Samples (4 mL) were taken every 24 h and centrifuged at 13,000 × g (4°C, 30 min). After 15 min, the supernatant was removed for pH measurement, the pellet was washed with distilled water and centrifuged again for 15 min. The pellet was resuspended and absorbance at 600 nm was measured by a visible spectrophotometer (Bioespectro SP-22, China). Biomass concentration (g L^{-1}) was determined by converting absorbance values with the use of a calibration curve specific to the microorganism (Staudt et al., 2012).

pH

pH of the supernatant was measured by a previously calibrated pH meter (Marte MB-10, Brazil), in agreement with Association of Official Analytical Chemists (2000) guidelines.

Recovery and quantification of EPS

EPS were recovered from the remaining flask content. The supernatant was mixed with ethanol (1:3 v/v) and stored at 4°C for 24 h to enable precipitation. EPS quantification was performed gravimetrically by drying the precipitate in pre-weighed Petri dishes at 45°C until constant mass was achieved.

Statistical analysis

Data were analyzed by Statistica 5.0 software (StatSoft, Inc., USA) at 90% confidence interval ($p < 0.1$), as recommended for PB designs (Rodrigues & Iemma, 2014).

Results and discussion

PB design

Table 1 shows maximum biomass and EPS concentrations after 96 h of cultivation under the various process conditions proposed by the PB design. EPS production ranged from 0 g L^{-1} (Assays 2 and 13) to 9.28 g L^{-1} (Assay 20), whereas biomass production ranged from 0.6 g L^{-1} (Assay 2) to 7.15 g L^{-1} (Assay 6). Since the main goal of this study was to maximize EPS production, the highest biopolymer yields were observed in the assays corresponding to the central points (17-20). In contrast, the lowest EPS production was recorded in Assay 12 (0.06 g L^{-1}) while, in Assays 2 and 13, EPS production was undetectable.

In an experimental design, the effect measures how changes in an independent variable – from its lowest (-1) to its highest (+1) levels – impact the dependent variable (response) (Rodrigues and Iemma, 2014). Variables with the highest effect values exert the greatest influence. When responses at central points deviate significantly from others, a high standard error may indicate that results do not follow a first-order model, suggesting significant curvature (Rodrigues and Iemma, 2014). EPS production at central points exceeded other conditions, a fact that emphasizes the need to check for curvature. Accounting for curvature lowers standard error, increases the t value and decreases the p value. Thus, it reveals statistically significant variables otherwise obscured by high error. Since this approach minimizes incorrect decisions in process optimization, it was applied to the statistical analysis below.

Effects of variables on maximum biomass concentration

The highest microbial growth was observed in Assays 6 (7.15 g L^{-1}) and 10 (5.03 g L^{-1}), which produced low EPS concentrations (1.98 g L^{-1} and 1.65 g L^{-1}, respectively). Biomass concentrations found by this study are significant by comparison with values reached by diazotrophic bacteria and reported by the literature. It shows the effectiveness of the conditions under investigation. Roesler et al. (2021) reported maximum biomass of 1.44 g L^{-1} produced by Rhizobium tropici SEMIA 4080 and 3.93 g L^{-1}, by Mesorhizobium sp. SEMIA 816, while Devi et al. (2012) found that Sinorhizobium meliloti MTCC 100 reached 7.45 g L^{-1} after 72 h under optimized conditions.

High microbial growth and high EPS production do not always correlate due to various factors. Microorganisms often prioritize biomass formation over secondary metabolite synthesis and divert resources from EPS production. Additionally, EPS biosynthesis is typically induced under stress conditions, which shift metabolic fluxes toward polymer accumulation instead of cell proliferation (Ates, 2015). Genetic and metabolic regulation further influence the process, which means that high cell density does not always activate EPS synthesis (Feng et al., 2022). EPS yields also vary among strains since some reach high production under low-growth conditions (Ates, 2015; Feng et al., 2022).

The analysis of main effects, standard deviations and t and p values (Table 2) showed that the curvature was not significant for biomass response. Increasing YE, $MgSO_4.7H_2O$ and WP levels significantly enhanced biomass by 2.03, 1.30, and 0.77 g L^{-1}, respectively ($p < 0.1$). Conversely, increasing V_M:V_R and temperature had negative effects since they decreased biomass by 1.23 and 0.98 g L^{-1}, respectively ($p < 0.1$). Other variables had no significant impact ($p > 0.1$).

Table 1. Coded levels and real values (in parentheses) for the Plackett–Burman design and responses

Assay	X1 (g L^{-1})	X2 (g L^{-1})	X3 (g L^{-1})	X4 (g L^{-1})	X5 (g L^{-1})	X6 (g L^{-1})	X7 (g L^{-1})	X8 (g L^{-1})	X9	X10 (rpm)	X11	X12 (°C)	X_{MAX} (g L^{-1})	EPS (g L^{-1})
1	+1 (0.2)	-1 (0)	-1 (0)	-1 (0)	+1 (4.0)	-1 (0)	-1 (0)	+1 (40)	+1 (7.5)	-1 (100)	+1 (0.25:1)	-1 (25)	2.88 (72h)	4.45
2	+1 (0.2)	+1 (0.8)	-1 (0)	-1 (0)	-1 (0.4)	+1 (0.24)	-1 (0)	-1 (10)	+1 (7.5)	+1 (200)	-1 (0.15:1)	+1 (35)	0.60 (72h)	0
3	+1 (0.2)	+1 (0.8)	+1 (0.4)	-1 (0)	-1 (0.4)	-1 (0)	+1 (0.3)	-1 (10)	-1 (6.5)	+1 (200)	+1 (0.25:1)	-1 (25)	1.18 (96h)	1.98
4	+1 (0.2)	+1 (0.8)	+1 (0.4)	+1 (0.2)	-1 (0.4)	-1 (0)	-1 (0)	+1 (40)	-1 (6.5)	-1 (100)	+1 (0.25:1)	+1 (35)	1.67 (96h)	5.71
5	-1 (0)	+1 (0.8)	+1 (0.4)	+1 (0.2)	+1 (4.0)	-1 (0)	-1 (0)	-1 (10)	+1 (7.5)	-1 (100)	-1 (0.15:1)	+1 (35)	2.02 (96h)	1.12
6	+1 (0.2)	-1 (0)	+1 (0.4)	+1 (0.2)	+1 (4.0)	+1 (0.24)	-1 (0)	-1 (10)	-1 (6.5)	+1 (200)	-1 (0.15:1)	-1 (25)	7.15 (72h)	1.98
7	-1 (0)	+1 (0.8)	-1 (0)	+1 (0.2)	+1 (4.0)	+1 (0.24)	+1 (0.3)	-1 (10)	-1 (6.5)	-1 (100)	+1 (0.25:1)	-1 (25)	2.37 (96h)	0.96
8	+1 (0.2)	-1 (0)	+1 (0.4)	-1 (0)	+1 (4.0)	+1 (0.24)	+1 (0.3)	+1 (40)	-1 (6.5)	-1 (100)	-1 (0.15:1)	+1 (35)	4.73 (72h)	6.24
9	+1 (0.2)	+1 (0.8)	-1 (0)	+1 (0.2)	-1 (0.4)	+1 (0.24)	+1 (0.3)	+1 (40)	+1 (7.5)	-1 (100)	-1 (0.15:1)	-1 (25)	1.93 (96h)	6.35
10	-1 (0)	+1 (0.8)	+1 (0.4)	-1 (0)	+1 (4.0)	-1 (0)	+1 (0.3)	+1 (40)	+1 (7.5)	+1 (200)	-1 (0.15:1)	-1 (25)	5.03 (96h)	1.65
11	-1 (0)	-1 (0)	+1 (0.4)	+1 (0.2)	-1 (0.4)	+1 (0.24)	-1 (0)	+1 (40)	+1 (7.5)	+1 (200)	+1 (0.25:1)	-1 (25)	2.21 (96h)	2.29
12	+1 (0.2)	-1 (0)	-1 (0)	+1 (0.2)	+1 (4.0)	-1 (0)	+1 (0.3)	-1 (10)	+1 (7.5)	+1 (200)	+1 (0.25:1)	+1 (35)	1.58 (72h)	0.06
13	-1 (0)	+1 (0.8)	-1 (0)	-1 (0)	+1 (4.0)	+1 (0.24)	-1 (0)	+1 (40)	-1 (6.5)	+1 (200)	+1 (0.25:1)	+1 (35)	1.97 (24h)	0
14	-1 (0)	-1 (0)	+1 (0.4)	-1 (0)	-1 (0.4)	+1 (0.24)	+1 (0.3)	-1 (10)	+1 (7.5)	-1 (100)	+1 (0.25:1)	+1 (35)	0.83 (96h)	1.42
15	-1 (0)	-1 (0)	-1 (0)	+1 (0.2)	-1 (0.4)	-1 (0)	+1 (0.3)	+1 (40)	-1 (6.5)	+1 (200)	-1 (0.15:1)	+1 (35)	2.26 (96h)	2.43
16	-1 (0)	-1 (0)	-1 (0)	-1 (0)	-1 (0.4)	-1 (0)	-1 (0)	-1 (10)	-1 (6.5)	-1 (100)	-1 (0.15:1)	-1 (25)	0.78 (72h)	2.33
17	0 (0.1)	0 (0.4)	0 (0.2)	0 (0.1)	0 (2.2)	0 (0.12)	0 (0.15)	0 (25)	0 (7.0)	0 (150)	0 (0.2:1)	0 (30)	3.13 (72h)	8.52
18	0 (0.1)	0 (0.4)	0 (0.2)	0 (0.1)	0 (2.2)	0 (0.12)	0 (0.15)	0 (25)	0 (7.0)	0 (150)	0 (0.2:1)	0 (30)	3.24 (72h)	8.03
19	0 (0.1)	0 (0.4)	0 (0.2)	0 (0.1)	0 (2.2)	0 (0.12)	0 (0.15)	0 (25)	0 (7.0)	0 (150)	0 (0.2:1)	0 (30)	3.16 (72h)	8.63
20	0 (0.1)	0 (0.4)	0 (0.2)	0 (0.1)	0 (2.2)	0 (0.12)	0 (0.15)	0 (25)	0 (7.0)	0 (150)	0 (0.2:1)	0 (30)	2.64 (72h)	9.28

X1: K_2HPO_4; X2: KH_2PO_4; X3: $MgSO_4.7H_2O$; X4: NaCl; X5: yeast extract; X6: $MnCl_2.4H_2O$; X7: $CaCl_2.2H_2O$; X8: whey permeate; X9: pH; X10: agitation; X11: medium- to-reactor volume ratio (0.15:1, 0.2:1 and 0.25:1 correspond, respectively, to volumes (mL) of 75:500, 100:500 and 125:500); X12: temperature; X_{MAX}: maximum biomass concentration (time in parentheses); EPS concentration after 96 h of cultivation.

Table 2. Estimated effects when the maximum biomass concentration was used as the dependent variable, without and with (in bold) the analysis of curvature.

Factor	Effect (g L^{-1})		Standard error		*t* (7)	*t* (6)	p value	
Mean	2.57	2.45	0.18	0.19	14.41	13.17	< 0.0001*	< 0.0001*
Curvature	-	1.18	-	0.83	-	1.42	-	0.2041
K_2HPO_4	0.53	0.53	0.40	0.37	1.34	1.43	0.2232	0.2023
KH_2PO_4	- 0.70	- 0.70	0.40	0.37	- 1.77	- 1.89	0.1201	0.1069
$MgSO_4.7H_2O$	1.30	1.30	0.40	0.37	3.27	3.51	0.0136*	0.0127*
NaCl	0.40	0.40	0.40	0.37	1.00	1.07	0.3488	0.3236
YE	2.03	2.03	0.40	0.37	5.10	5.46	0.0014*	0.0016*
$MnCl_2.4H_2O$	0.55	0.55	0.40	0.37	1.38	1.48	0.2099	0.1898
$CaCl_2.2H_2O$	0.08	0.08	0.40	0.37	0.20	0.21	0.8466	0.8368
WP	0.77	0.77	0.40	0.37	1.94	2.08	0.0937*	0.0831*
pH	- 0.63	- 0.63	0.40	0.37	- 1.57	- 1.69	0.1593	0.1426
Agitation	0.59	0.59	0.40	0.37	1.49	1.60	0.1790	0.1608
V_M:V_R	- 1.23	- 1.23	0.40	0.37	- 3.08	- 3.30	0.0178*	0.0164*
Temperature	- 0.98	- 0.98	0.40	0.37	- 2.47	- 2.65	0.0427*	0.0381*

YE: yeast extract; WP: whey permeate; V_M:V_R: medium-to-reactor volume ratio. * $p < 0.10$.

Factors that exerted significant effects were ranked by their influence on the maximum biomass concentration. They are shown by the Pareto chart (Figure 1): YE (+ 2.03), $MgSO_4.7H_2O$ (+ 1.3), V_M:V_R ratio (- 1.23), temperature (- 0.98) and WP (+ 0.77).

YE positively influences biomass, since nitrogen, along with carbon, is a key macronutrient for microbial growth and metabolism. It is essential to synthesize nucleotides, amino acids and other metabolites (Sharma et al., 2018) which are linked to microbial reproduction. YE also contains vitamins and growth factors (Pokhrel & Oga, 2007). Freitas et al. (2017) reported that extra nitrogen favors cell growth.

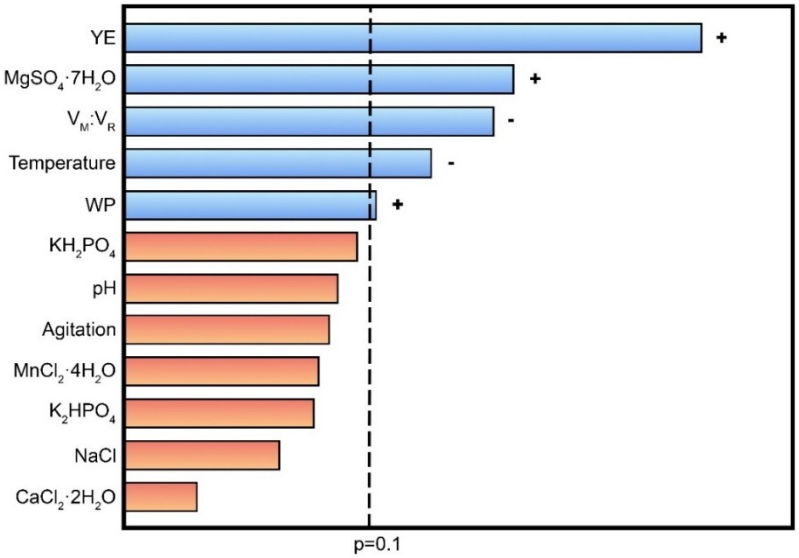

Figure 1. Pareto chart of estimated effects in the analysis of the Plackett–Burman design for maximum biomass concentration.

Torres et al. (2014) studied the effects of different glycerol and nitrogen concentrations on *Enterobacter* A47 growth and EPS production. They found that initial nitrogen concentrations above 1.05 g L^{-1} hindered EPS synthesis and decreased productivity to 0.35-0.62 g L^{-1} d^{-1}, by comparison with 1.89–2.04 g L^{-1} d^{-1} at lower nitrogen levels (0.68–1.05 g L^{-1}). Additionally, the EPS composition was altered, i. e., lower fucose content (14–17 mol% by comparison with 36–38 mol%) was observed at higher nitrogen levels. They stated that similar patterns have been observed in the cases of other EPS-producing bacteria, such as *Aeromonas salmonicida*, *Sphingomonas paucimobilis* GS1 and *Xanthomonas campestris*, in which high contents of nitrogen tend to favor cell growth but decrease EPS production. It is explained by the fact that nitrogen is primarily used for cell growth and enzyme production, thus, fewer resources are available for EPS synthesis.

In this study, the positive effect of increasing $MgSO_4.7H_2O$ concentration from 0 to 0.4 g L^{-1} is due to its role as a microbial growth supplement, linked to enzyme activity and carbohydrate metabolism (Freitas et al., 2017). Su et al. (2007) found that the addition of $MgSO_4$ stimulated marine diazotrophic bacterium *Cyanothece* sp. 113 growth, since it reached 0.9 g L^{-1} at 0.2 g L^{-1} $MgSO_4$.

The V_M:V_R ratio influences aeration of the culture medium directly and affects oxygen availability, which is crucial for microbial growth. The larger the ratio, the larger the initial volume of the medium, and consequently, less available oxygen in the flask (Freitas et al., 2017; Liu et al., 2017). Mahapatra & Banerjee (2013) optimized EPS production by *Fusarium solani* SD5 and explained that, although the cotton plug does not prevent air from penetrating into the culture flasks, it may restrict free air passage. Therefore, the volume of air in the headspace may influence oxygen levels in the liquid medium. Other factors related to the V_M:V_R variable that also influence it are the surface area and depth of the liquid medium, which control oxygen diffusion and circulation; a large surface area and shallow depth increase diffusion. In this study, considering the surface area, headspace volume and average depth, it may be assumed that the lower the medium volume, the higher the content of dissolved oxygen content, while the higher the medium volume, the lower the content of dissolved oxygen. Thus, in this study, the negative effect of the V_M:V_R variable may be linked to low aeration in the medium.

Serrato et al. (2006) also concluded that low aeration affected biomass concentration when culture conditions for EPS production by the diazotrophic bacterium *Burkholderia tropica* were studied. Different aeration levels were tested at different medium volumes in 500 mL Erlenmeyer flasks: 400 mL for low aeration,

250 mL for moderate aeration and 100 mL for high aeration. All flasks were shaken at the same rotation and temperature (120 rpm and 30°C) and optical density at 600 nm was measured after 72 h. Results showed that high aeration promoted intense microbial growth (OD600 ~ 8), whereas low aeration affected growth; OD600 values were ~4 and ~1.4 (moderate and low aeration, respectively).

The incubation temperature is one of the limiting factors of microbial growth since every bacterial species has an optimal temperature. According to Liu et al. (2017), the optimal incubation temperature for most endophytic species, such as rhizobia, ranges from 24 to 30°C. Therefore, the negative effect of temperature may be explained by the fact that the highest temperature (35°C) was not within the recommended range. Additionally, lower temperatures result in lower energy consumption during the process.

Regarding the positive effect of WP, carbohydrates are essential components for microbial growth because they are the main sources of carbon and energy. Therefore, the following conditions were established to maximize biomass: 4 g L^{-1} YE, 40 g L^{-1} WP, 0.4 g L^{-1} $MgSO_4.7H_2O$, pH 6.5, 100 rpm agitation, V_M:V_R of 0.15:1 and 25°C, with the possibility of eliminating five components from the medium (KH_2PO_4, K_2HPO_4, NaCl, $MnCl_2.4H_2O$ and $CaCl_2.2H_2O$) to decrease costs.

Although high biomass production does not necessarily result in high EPS production, it may be important to increase this parameter in agricultural inoculant production, for instance. Inoculation of plants with symbiotic and growth-promoting microbes, in an attempt to mitigate the use of fertilizers and pesticides, is a potential alternative to minimize damage to soil health (Iturralde et al., 2020). The production of plant growth-promoting microbes offers some advantages, such as easy production, the possibility of being cultivated under laboratory conditions with the use of different strategies and scalability. Some studies have evaluated the positive effects of bacterial biomass inoculation, such as high bean yield (Pastor-Bueis et al., 2021), better strawberry productivity (Flores-Félix et al., 2018) and enhanced plant resistance in highly calcareous soils (Ipek et al., 2014).

Effects of variables on EPS production

Table 3 shows the analysis of the main effects, including standard deviations, t values and p values, both with and without curvature. Numerical values of the main effects remained consistent in both analyses; however, the curvature effect, at 12.36 g L^{-1}, emerged as the largest and statistically significant one ($p < 0.0001$). As a result of the analysis of the curvature, the standard error of the independent variables decreased from 2.10 to 0.20, which led to higher t values and lower p values and highlighted the significance of most factors under evaluation. Rodrigues and Iemma (2014) stated that it confirms the presence of curvature and indicates that results do not fit a first-order model.

Table 3. Estimated effects when EPS concentration was used as the dependent variable, without and with (in bold) the analysis of curvature.

Factor	Effect (g L^{-1})		Standard		*t* (7)	*t* (6)	p value	
Mean	3.67	2.44	0.94	0.10	3.91	23.95	0.0058*	< 0.0001*
Curvature	-	12.36	-	0.45	-	27.18	-	< 0.0001*
K_2HPO_4	1.82	1.82	2.10	0.20	0.87	8.95	0.4141	0.0001*
KH_2PO_4	- 0.43	- 0.43	2.10	0.20	- 0.20	- 2.11	0.8439	0.0796*
$MgSO_4.7H_2O$	0.73	0.73	2.10	0.20	0.35	3.57	0.7394	0.0118*
NaCl	0.35	0.35	2.10	0.20	0.17	1.74	0.8709	0.1327
YE	- 0.76	- 0.76	2.10	0.20	- 0.36	- 3.72	0.7292	0.0099*
$MnCl_2.4H_2O$	- 0.06	- 0.06	2.10	0.20	- 0.03	- 0.30	0.9775	0.7735
$CaCl_2.2H_2O$	0.40	0.40	2.10	0.20	0.19	1.97	0.8538	0.0960*
WP	2.41	2.41	2.10	0.20	1.15	11.84	0.2887	< 0.0001*
pH	- 0.54	- 0.54	2.10	0.20	- 0.26	- 2.64	0.8056	0.0387*
Agitation	- 2.27	- 2.27	2.10	0.20	- 1.08	- 11.18	0.3144	< 0.0001*
V_M:V_R	- 0.65	- 0.65	2.10	0.20	- 0.31	- 3.21	0.7644	0.0183*
Temperature	- 0.63	- 0.63	2.10	0.20	- 0.30	- 3.08	0.7740	0.0217*

YE: yeast extract; WP: whey permeate; V_M:V_R: medium-to-reactor volume ratio. * $p < 0.10$.

Based on data shown in Table 3, independent variables that showed no significant effects when moving from the lowest level (- 1) to the highest level (+ 1) were NaCl ($p = 0.1327$) and $MnCl_2{\cdot}4H_2O$ ($p = 0.7735$). Thus, these variables were set at level -1 (0 g L^{-1}) (i.e., removed from the medium) and led to decrease in production costs. On the other hand, variables agitation, yeast extract (YE), V_M:V_R ratio, temperature, pH and KH_2PO_4 exerted significant negative effects ($p < 0.1$). Increase in these variables from level -1 to + 1 resulted in

decrease in EPS concentration. Average decreases were 2.27, 0.76, 0.65, 0.63, 0.54 and 0.43 g L^{-1}, respectively. Therefore, these variables should be maintained at their lowest levels: 0.4 g L^{-1} YE, pH 6.5, agitation at 100 rpm, V_M:V_R ratio of 0.15:1, 25°C and no addition of KH_2PO_4. Notably, decrease in YE concentration and temperature also contributes to lower production costs. The remaining variables – K_2HPO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$ and WP – showed significant positive effects ($p < 0.1$) when they increased from the lowest to the highest levels. Therefore, the best conditions for these variables were 0.2 g L^{-1} K_2HPO_4, 0.4 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.3 g L^{-1} $CaCl_2{\cdot}2H_2O$ and 40 g L^{-1} WP.

The Pareto chart (Figure 2) highlights the most significant variables that influence responses, specifically those that exceed the critical p value threshold of 0.1. Among the main effects, WP, used as the carbon source, stands out as the variable with the greatest positive impact on EPS concentration (+ 2.41). It was followed by agitation, which had a significant negative effect (- 2.27), and K_2HPO_4, which also influenced EPS concentration positively (+ 1.82). These results show that WP and K_2HPO_4 play key roles in promoting EPS production while high agitation tends to decrease it.

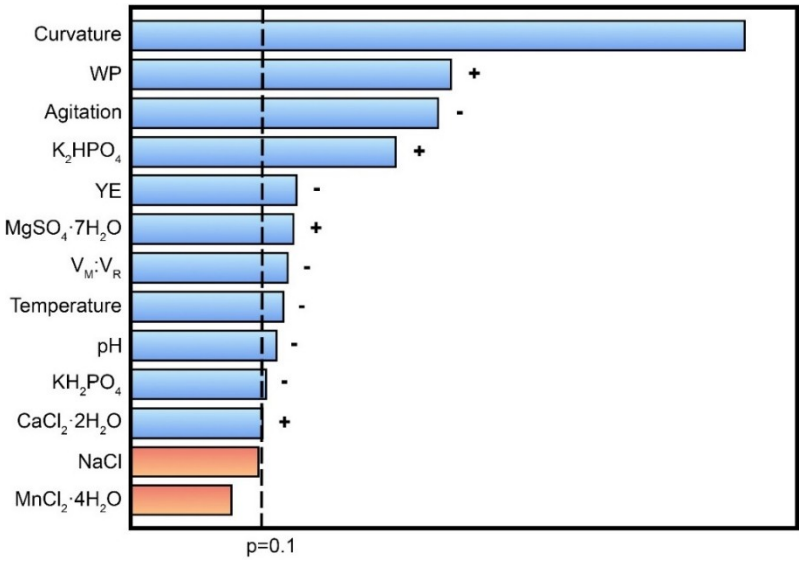

Figure 2. Pareto chart of estimated effects in the analysis of the Plackett–Burman design for EPS concentration.

The comparison of the effects of variables on increases in EPS concentration (Table 3) and maximum biomass concentration (Table 2) highlights that the only variable that had a significant opposite effect on the different responses was YE, the nitrogen source concentration. Low YE concentration (0.4 g L^{-1}) favored biopolymer production while high YE concentration (4 g L^{-1}) promoted high biomass production.

The significant influence of WP concentration in the culture medium on biopolymer production may be attributed to the fact that carbohydrates are considered the primary nutrient and energy source for microbial growth and polysaccharide production. The carbon source affects the catabolic repression of secondary metabolism directly, thereby promoting polysaccharide production through anabolic pathways (Ruiz-Villafán et al., 2022). Liu et al. (2017) reported that increasing the initial concentration of the carbon source in the medium generally leads to high EPS production, as shown by several studies.

For example, in EPS production by *Bacillus* sp. EPS003, optimization of carbon sources, particularly sucrose, resulted in 2.5-fold increase in EPS yield (Marimuthu et al., 2023). Similarly, in the case of *Halomonas xianhensis* SUR308, maximum EPS production of 5.70 g L^{-1} was achieved with the use of 3% glucose concentration, a fact that highlights glucose as a favorable carbon source (Biswas & K. Paul, 2017). *Chryseobacterium indologenes* MUT.2 showed the highest EPS production since it reached 8.32 g L^{-1} at optimized concentrations of glucose and sucrose (Khani et al., 2016). Moreover, in cultivation of *Methylobacterium* strains, high EPS production, up to 75%, was observed when a high-carbon medium was used; it pointed out the positive correlation between carbon levels and EPS yield (Woo et al., 2012). Increase in carbon concentrations generally increases EPS production, but excessively high levels may either lead to metabolic imbalances or inhibit growth in some strains, a fact that emphasizes the importance of careful optimization (Pal & Paul, 2013).

Joshi et al. (2013) used a PB design to study EPS production by the fungus *Schizophyllum commune* AGMJ-1. The most influential factors in this process were xylose and YE since both exerted positive effects. These

results align with those of the current study regarding the carbon source but differ concerning the nitrogen source. According to Sengupta et al. (2018), the role of nitrogenous compounds in a medium is quite complex. For example, in the case of rhizobacterium *Cupriavidus pauculus*, increasing the nitrogen concentration had a positive effect on EPS yield, whereas in the case of *Streptococcus thermophilus*, gradual increase in nitrogen levels led to decrease in the molecular weight of resulting bacterial EPS.

Feng et al. (2010) optimized the medium to enable growth of mycelia and EPS production by *Lentinus edodes* and observed that YE is frequently used for providing essential growth factors, but excessively high concentrations may inhibit the use of other carbon sources and accumulation of metabolites. The balance between nitrogen and carbon is vital for microbial growth and metabolite production. Excessive nitrogen, such as the one from YE, may shift metabolism toward biomass formation, rather than biopolymer synthesis, and potentially limit consumption of carbohydrates needed for EPS production (Alsafadi et al., 2020). Since EPS is often induced under nutrient stress, high nitrogen levels may reduce its accumulation, which might explain why increase in YE concentration exerted a negative effect in this study. This hypothesis is further supported by Barbosa et al. (2004), who reported that low nitrogen concentrations stimulate EPS production, while high ones inhibit it. Freitas et al. (2011) also highlighted that carbon availability, coupled with nitrogen limitation, typically favors EPS production in microorganisms.

In this study, temperature (25–35°C) and pH (6.5–7.5) exerted less pronounced effects on EPS production than the other variables. It may have happened because the selected range, which has been widely used in processes involving diazotrophic bacteria, is considered optimal for EPS production (Liu et al., 2017).

Thus, conditions that maximized EPS production in this study were 0.4 g L^{-1} YE, pH 6.5, agitation at 100 rpm, V_M:V_R ratio of 0.15:1, temperature of 25°C, 0.2 g L^{-1} K_2HPO_4, 0.4 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.3 g L^{-1} $CaCl_2 \cdot 2H_2O$ and 40 g L^{-1} WP. This formulation enabled the elimination of three components from the production medium: NaCl, $MnCl_2 \cdot 4H_2O$ and KH_2PO_4. In this condition, EPS reached 7.15 g L^{-1}. This value is notably higher than the ones reported by other studies that used rhizobia and agroindustrial substrates. For example, *Mesorhizobium loti* produced 4.91 g L^{-1} when residual glycerol was used (Oliveira et al., 2018) while *Ensifer meliloti* achieved 4.12 g L^{-1} in soybean molasses (Oliveira et al., 2020). Results of the study reported by this paper exceed them by over 40%. Additionally, the simplified medium, which eliminates some components, such as NaCl, MnCl2·4H2O and KH2PO4, represents a more economical and sustainable approach. The combination of high yield and medium optimization makes the study a promising reference for further studies of biopolymers and their applications.

Conclusion

The PB design enabled to identify how cultivation parameters significantly affect both EPS production and microbial growth. The key factors influencing these responses are concentrations of WP, yeast extract (YE), $MgSO_4 \cdot 7H_2O$ and the medium-to-reactor volume ratio (V_M:V_R).

In terms of biomass production, YE had the highest positive effect. However, increase in YE concentration, which favored growth, inhibited EPS formation. Additionally, $MgSO_4 \cdot 7H_2O$ positively influenced biomass, whereas higher V_M:V_R ratios and temperature negatively affected this response. It suggested that decrease in the medium volume and maintenance of moderate temperature (25°C) lead to maximization of biomass concentration.

Regarding EPS production, WP was the most important variable. On the other hand, high agitation, YE concentration and temperature influenced EPS production negatively. It suggests that decrease in these factors may increase biopolymer yield. The best conditions for EPS were low YE (0.4 g L^{-1}), low agitation (100 rpm) and high WP levels (40 g L^{-1}).

This study highlights the delicate balance among variables to maximize both EPS and biomass production. WP and YE concentrations play critical roles, but their effects on each response differ. It shows the need for a nuanced approach in further optimization efforts. In addition, the composition of the medium may be adjusted to the focus of the bioprocess, e.g., production of either biopolymers or agricultural inoculants.

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