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Rodrigues Araújo, Thaíssa; de Oliveira Petkowicz, Carmen Lúcia; Cardoso, Vicelma Luiz; Coutinho Filho, Ubirajara; Vieira, Patrícia Angélica

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Biopolymer production using fungus *Mucor racemosus Fresenius* and glycerol as substrate

Thaíssa Rodrigues Araújo[1], Carmen Lúcia de Oliveira Petkowicz[2], Vicelma Luiz Cardoso[1], Ubirajara Coutinho Filho[1] and Patrícia Angélica Vieira[1]*

[1]Faculdade de Engenharia Química, Universidade Federal de Uberlândia – UFU, Uberlândia, MG, Brazil
[2]Departamento de Bioquímica, ACF Centro Politécnico, Universidade Federal do Paraná – UFPR, Curitiba, PR, Brazil
**patriciavieira@feq.ufu.br*

Abstract

This study evaluated ***extracellular production of biopolymer*** using fungus *Mucor racemosus Fresenius* and glycerol as a carbon source. Initially employing conical flasks of 500 mL containing 100 mL of cultive medium with 0.18 ± 0.03 g.L^{-1} of microorganisms, the results showed that the best conditions of the variables studied were: initial concentration of glycerol 50 g.L^{-1}, fermentation time of 96 h, inoculum cultivation time of 120 h, and aeration in two stages–the first 24 hours without aeration and 72 hours fermentation with aeration of 2 vvm and 2 g.L^{-1} of yeast extract. The experiments conducted in a Biostat B fermenter with a 2.0 L capacity that contained 1.0 L of medium showed production of 16.35 g.L^{-1} gum formed and 75% glycerol consumption. These conditions produced a biopolymer with the molecular weight and total sugar content of 4.607×10^6 g.mol^{-1} (Da) and 89.5%, respectively.

Keywords: *biopolymer, Mucor racemosus Fresenius, glycerol, aeration, fermentation, productivity.*

1. Introduction

The excess of glycerol in the market, generated by the production of biodiesel, is a problem that has been discussed over the years. Researchers are seeking an alternative for the bioconversion of this by-product. According to Moralejo-Garate et al.[1] the crude glycerol is a by-product of the biodiesel industry and a potentially good substrate for the production of biopolymers.

Biopolymers are complex chains of polysaccharides of microbial origin, synthesized by bacteria, fungi and yeasts, and are also known as gums due to their ability to form viscous solutions and gels in aqueous media. The biopolymers produced by fungi have not been adequately explored, and only some of them have been produced on an industrial scale. However, several of these biopolymers have attracted attention due to their physico-chemical and rheological properties, and have found a wide range of applications, including use in pharmaceutical therapy for action-tumors, anti-virals and anti-inflammatories[2].

Biopolymers have unique chemical and physical properties that are superior to those of traditional polysaccharides, such as higher viscosity and gelling power, compatibility with a wide variety of salts in a large range of pH and temperature, stable in high ion concentrations, high solubility water, and also synergistic action with other polysaccharides[3,4].

The increasing interest in biopolymers compared to traditional polymers is due to its scarcity and high oil prices in addition to the environmental impact that is determined by its extraction, refining and difficult biological degradation[5].

The species of the *genus Mucor* has been reported in the literature as a potential producer of high value bioproducts. According to Alves et al.[6] the *mucor* species genera are a large group of fungi with potential biotechnological importance, which is responsible for the production of industrial enzymes. The high production capacity of lipase, protease and phytase by *Mucor racemosus*[6-7] are mentioned in the literature. Furthermore, the strains *Mucor racemosus* are cited in the literature as producing various bioproducts, such as the production of chitosan, which is extracted from the mycelium of this strain in various growth stages[8]. In the literature, there are few papers based on the use of this fungus for the production of biopolymers. Nevertheless, none of them report the production of the biopolymer applying glycerol as substrate.

The goal of this study was to evaluate the biopolymer production employing the fungus *Mucor racemosus Fresenius* and glycerol as substrate and to select the best operational conditions among those studied. The subsequent objective was to evaluate the ability of the fungus to produce biopolymer using a 2 L capacity Biostat B fermenter.

2. Materials and Methods

2.1 Microorganisms, medium culture for maintenance and production process

2.1.1 Microorganisms

In this work we used a fungus isolated from a central area in Brazil (State of Minas Gerais) identified by the Foundation André Tosello for Research and Technology (Campinas, SP, Brazil) and Laboratory Exame (Uberlândia, MG, Brazil) as *Mucor racemosus Fresenius*. This microorganism was selected among other fungi in preliminary trials as a potential producer of biopolymer.

2.1.2 Maintenance of microorganisms: cultivation of inoculums

The fungus was grown in Petri dishes containing Czapeck medium (pH=6) with the following composition in $g.L^{-1}$: 20.0 glucose, 2.0 sodium nitrate, 1.0 potassium phosphate dibasic, 0.5 magnesium sulfate, 0.5 potassium chloride, 0.01 ferrous sulfate, 20.0 agar-agar. The medium was sterilized at 110 °C and 1 atm for 20 minutes. The incubation was performed at 28 ± 1 °C for 120 h (initial standard time).

2.1.3 Biopolymer production

The culture medium used in all experiments, obtained in preliminary tests, showed the following composition in $g.L^{-1}$: 0.5 K_2HPO_4, 1.4 KH_2PO_4, 1.0 NH_4NO_3, 1.0 $MgSO_4.7H_2O$, 0.02 $CaCl_2$ .H_2O, 0.03 $MnSO_4.H_2O$, 2.0 yeast extract and varying concentrations of glycerol in accordance with each assay. The medium had its pH adjusted to 6.5 with subsequent sterilization at 110 °C and 1 atm for 20 minutes.

2.2 Carbon source

Commercial glycerol (Vetec, Brazil) with a purity of 99.5% was used as substrate.

2.3 Preliminary tests: evaluation of process variable

All experiments were performed in conical flasks of 500 mL containing 100 mL of culture medium, and glycerol concentrations varied according to each experiment. The amount of inoculum used corresponded to 0.18 ± 0.03 $g.L^{-1}$.

The variables evaluated in chronological sequence were: initial concentration of glycerol (without aeration process), fermentation time, cultivation time of the inoculum (inoculum age), aeration flow, aeration mode and the effect of yeast extract in the biopolymer production.

All experiments were conducted twice and triplicate.

Determination of glycerol concentration, biopolymer production time and inoculum cultivation time

The experiments for evaluating the optimal concentration of glycerol, biopolymer production time and inoculum cultivation time were performed without additional aeration.

The response variables monitored in the experiments were the following: concentration of the biopolymer, glycerol consumption, productivity (P_p) and the product yield constant (g-product/g-substrate) ($Y_{P/S}$). All assays were performed under agitation in an oscillatory shaker (New Brunswick) at 150 rpm and 30.0 ± 2 °C. The productivity and product yield constant is given by Equations 1 and 2, respectively:

$$P_P = \frac{P_m - P_0}{t_{fp}} \quad (1)$$

$$\frac{Y_P}{S} = \frac{P_m - P_0}{S_0 - S} \quad (2)$$

Where, P_p: productivity;

P_0: initial product concentration;

P_m: maximum product concentration (final);

t_{fp}: product formation time;

S_0: initial substrate concentration (glycerol);

S: final substrate concentration (glycerol);

$Y_{P/S}$: product yield constant (g-product/g-substrate).

The glycerol initial concentrations tested were 25, 50, 100 and 125 $g.L^{-1}$. For the fermentation time, 48, 72, 96, 120, 144 and 168 hours were tested. In order to verify the effect of inoculum cultivation time (age of inoculum) in the biopolymer production process, the following times were evaluated: 48, 72, 96 and 120 hours.

2.3.1 Evaluation of aeration mode in the production process of biopolymer

Initially, a preliminary test was performed with continuous aeration of 3 vvm. In this operation (3 vvm) was observed the decrease in the medium volume in the reactor (the liquid medium was entrained in air). And the production process of gum was impaired. Subsequently, three additional tests were performed. The first test was performed without aeration; the second test with continuous aeration of 2 vvm. The third test was carried out without aeration in the first 24 hours and with continuous aeration of 2 vvm in 72 hours of assay.

2.3.2 Evaluation of the effect of yeast extract addition on biopolymer production

After selecting the initial concentration of glycerol (without aeration process), fermentation time, cultivation time of the inoculum (inoculum age), aeration flow, and aeration mode, the effect of yeast extract concentration in the production of the biopolymer was valued.

To evaluate the effect of yeast extract (YE) in biopolymer production, different concentrations were tested: 0; 0.5; 1; 2; 2.5, 3.0 and 4.0 $g.L^{-1}$. It should be emphasized that a trial was also carried out using only yeast extract as the source of carbon at concentrations of 1, 2, 3 and 4 $g.L^{-1}$, i.e., without glycerol.

2.4 Biopolymer production in Biostat B fermenter

After optimization of variables (inoculum cultivation time, glycerol concentration, level of aeration, fermentation time and yeast extract concentration) kinetic assays were performed in Biostat B fermenter to evaluate the performance of the fungus in the extracellular production of biopolymer. The experiment was performed using a Biostat B fermenter with a 2.0 L capacity that contained 1.0 L of medium, with the first 24 h without aeration and with 72 h with aeration of 2 vvm. The above procedure was used, because preliminary tests showed that after 24 h fermentation had visible change in the viscosity of the fermentation broth. This fact show that gum production occurred after that time. Therefore, it was tested the addition of aeration after 24 hours of fermentation.

The process was evaluated by monitoring the amount of formed gum, glycerol consumption and cell concentration. For the characterization of the biopolymer produced, the following analyses were performed: rheologic behavior evaluation, monosaccharide composition and molecular parameters by steric exclusion chromatography.

2.5 Analytical determinations

2.5.1 Recovery and purification of biopolymer

The fermented broth was diluted 1:1 with deionized water and centrifuged in a Beckman Coulter Avanti J-25 centrifuge at 18,900×g for 40 min to remove cells.

This procedure of dilution was necessary, because the medium was very viscous. This facilitated the withdrawal of the fermented broth cells. This procedure was the same adopted by Faria et al[9]. The supernatant was filtered and treated with a saturated solution of KCl, and the polymer was recovered by precipitation with ethanol. Finally, the product was dried under vacuum system at 30 ± 1 °C[10].

2.5.2 Analysis of glycerol concentration

Glycerol concentration in the cell-free supernatant was determined by liquid chromatography (HPLC) with an Aminex HPX-87H column (Bio Rad), coupled to a refractometer. The analysis was performed at 50 °C, with sulphuric acid (H_2SO_4 0.01 N) as eluent, at a flow rate of 0.6 mL/min[11].

2.5.3 Determination of biomass

After fermentation, the fermented culture medium was diluted 1:1 in water, and the cells were separated by centrifugation Beckman Coulter Avanti J-25-18900 g for 30 minutes. The precipitate (cells) was washed three times in distilled water for complete removal of medium components and metabolites[10].

2.5.4 Evaluation of rheological behavior of the biopolymer

The rheology of gum was obtained by preparing a polymer solution of 0.5%, 0.75% and 1% under magnetic stirring for approximately 10 h. The gum used to prepare this solution was dried at room temperature, and then crushed and hydrated[10,12].The rheology was measured using a Brookfield rheometer RVDVIII coupled with a water bath mark Brookfield, model TC-502P, using the small sample adapter, spindle 18. Following the template Ostwald Waele or power-law, data on shear stress, measured from shear rates, were used. The units of measurement were apparent viscosity in Pa.s and s^{-1} to shear rate.

2.5.5 Determination of monosaccharide composition and molecular parameters

To determine the monosaccharide composition of the fractions in terms of neutral sugar samples were hydrolyzed with 2 M trifluoroacetic acid in two conditions: for 8 h at 100 °C or 120 °C for 2 h. For each hydrolysis condition, four replications were performed. Upon completion of the hydrolysis, the excess of acid was removed by evaporation[13]. After total acid hydrolysis, the monosaccharides were solubilized in about 5mL of distilled water and reduced by adding approximately 10 mg of sodium borohydride for 16 h at 4 °C. Later, strongly acidic cation exchange resin was added to remove the Na^+ ions.

The solutions were filtered and the solvent evaporated in vacuo. Methanol (1 mL) was added to remove boric acid and borate of methyl formed was evaporated in vacuo. This process was repeated three times. The formed alditols were acetylated by adding 0.5 mL of acetic anhydride and 0.5 mL of pyridine in sealed tubes, standing for 12 h at room temperature. The reaction was stopped by the addition of ice, and then the extraction of ethyl alditols was performed by the addition of chloroform and subsequent elimination of pyridine in successive treatments with 5% copper sulphate and distilled water. After evaporation of the solvent, the acetates and the alditols were subjected to gas-liquid chromatography-mass spectrometry (GC-MS) in order to determine the composition of the neutral monosaccharides. The monosaccharide composition was calculated as the average of eight replications.

2.5.6 Determination of the content of acid monosaccharides (uronic acids)

Dosing of uronic acid concentration was accomplished by the method of Blumenkrantz and Asboe-Hansen[14], with the standard solution galacturonic acid in concentrations of 10-100 μg. mL^{-1} and reading of 520 nm. A total of eight replications were carried out.

2.5.7 Identification of uronic acids

For the identification of the uronic acids, hydrolysed and free acid samples were filtered through cellulose acetate membranes with pores of 0.22 μm, and for anion exchange chromatography using a Dionex ICS-5000 and CarboPac PA20 column, according to the methodology described by Nagel et al.[15].

2.5.8 Liquid-gas chromatography mass spectrometry (LGC-MS)

The analyses were performed on a Varian 3300 gas chromatograph coupled with a mass spectrometer FINNIGAN-MAT, with injector the 50 °C and "ramp" of 40 °C per minute to 220 °C, equipped with a fused silica capillary column (30 m × 0.25 mm d.i) coated with DB-225, and helium as the carrier gas (1 $mL.min^{-1}$).

2.5.9 Sample analysis by steric exclusion chromatography coupled with the detector of laser light of multi-angle and differential detector of index of the refraction (HPSEC-MALLS/RI)

Analyses were performed on a device consisting of an HPLC pump (Waters 515), injector, four columns of Ultrahydrogel–120, 250, 500 and 2,000–with limits of exclusion 5.103, 8.104, 4.105 and 7.106 respectively, DAWN DSP Light Scattering (Wyatt Technology), and an index detector of differential refractive model 2410 (Waters). The eluent used was a 0.1 M $NaNO_2$ solution containing 200 ppm $NaNO_3$.

The samples were solubilized at a concentration of 1 $mg.mL^{-1}$ in the solution of the eluent. Before analysis, the samples were filtered through cellulose acetate membranes with pores of 0.22 micrometers. The average molar mass was determined by light scattering method.

2.5.10 Thin layer chromatography (TLC)

The monosaccharides were identified by Thin-Layer chromatography using a silica gel plate (20×20 cm) as stationary phase, previously activated in a greenhouse at 100 °C for 1 h. Monosaccharides were visualized using orcinol-H_2SO_4 (specific for carbohydrates) and then with ninhydrin (for amino group)[16].

3. Results and Discussion

3.1 Experiments employing stirred conical flasks

Figure 1 shows the influence of the initial concentration of glycerol in the formation of the biopolymer.

It can be observed that the glycerol concentration that promoted the higher formation of the biopolymer was 50 $g.L^{-1}$. In this concentration, the content of gum

was 2 g.L^{-1} with productivity of 0.012 g.L^{-1} h^{-1}, product yield constant ($Y_{P/S}$) of 0.104 g of biopolymer/g glycerol and glycerol consumption of 38.4%. Furthermore, it can be seen by these results that higher concentrations of glycerol at 100 and 125 g.L^{-1} inhibited the production of gum. Nevertheless, at such concentrations, consumption of substrate was superior, achieving 42.75% and 46.12%, respectively. This suggests that consumed glycerol was used mainly for further purposes, besides the production of the biopolymer. The final concentration of cells for assays with glycerol concentration of 25, 50, 100 and 125 g.L^{-1} were: 0.34, 0.67, 1.01 e 1.21 g.L^{-1}, respectively. This fact confirms the earlier hypothesis.

Antônio et al.[17], in their study of biopolymer production using bacteria *Gluconoacetobacter hansenii* and 25 g.L^{-1} of glycerol as substrate, obtained 6.9 g.L^{-1} of biopolymer after 14 days of fermentation. Comparing this result with what was found by the present work, it can be seen that despite a lower result presented in this study (2 g.L^{-1} gum and 50 g.L^{-1} glycerol in (7 days) 168 h of process), the studied microorganism showed a potential gum production capacity, since at this stage no further optimization of production conditions was carried out. Therefore, the concentration of 50 g.L^{-1} glycerol was chosen to be used in the following stages of this work.

Figure 2 shows the results of the amount of the produced gum and glycerol consumption in assays performed on fermentation times of 48, 72, 96, 120, 144 and 168 h.

Figure 2 shows that the maximum production of gum was obtained in the fermentation time of 96 h producing 2.96 g.L^{-1} of gum, productivity of 0.031 g.L^{-1} h^{-1}, and product yield constant of $Y_{P/S}$ of approximately 0.10 g of biopolymer/g of glycerol.

At 120, 144 and 168 h a decrease in the amount of gum formed was observed, corresponding to the values of 2.87, 2.26 and 2 g.L^{-1}, respectively. This behavior suggests that enzymes produced by the own microorganism may have degraded the gum. This assumption may be confirmed by the consumption of glycerol according to the fermentation times illustrated in Figure 2, showing that glycerol consumption was enhanced despite the small amount of formed gum. This suggests that the gum begins to be degraded and the glycerol is consumed for the formation of other by-products. Based on these results, the fermentation time chosen to attain an increased amount of biopolymer production was 96 h.

The influence of fermentation time in producing gum is reported by some authors who argue that a longer fermentation time promotes higher productivity[18].

Figure 3 shows the results of the amount of formed gum and glycerol consumption using inoculum of different ages (incubation times). This study has become necessary since the incubation time is associated with the age of the inoculum, which can directly influence the process of fermentation, product generation and gum formation.

Figure 3 shows that the higher time of incubation, the higher the content of biopolymer produced up to 120 h. The use of an inoculum incubated for 120 h allowed a production of 3 g.L^{-1} (of fermentation time of 96 h), productivity of 0.031g.L^{-1} h^{-1}, product yield constant of approximately, 0.100 g of biopolymer/g of glycerol and consumption of glycerol of 33.9%. By applying an inoculum of 144 h, there was an increase of only 0.2 g.L^{-1} of gum formed, which is not statistically different. Thus, the age of the inoculum culture adopted for the development of this work was 120 h.

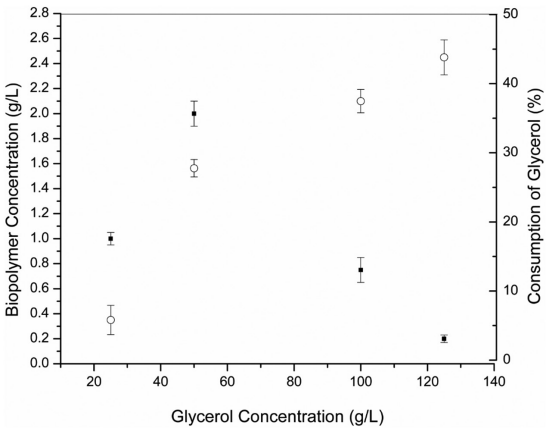

Figure 1. Amount biopolymer formed (■) and glycerol consumption (○) for different concentrations initial of glycerol substrate (25, 50, 100 and 125 g.L^{-1}).

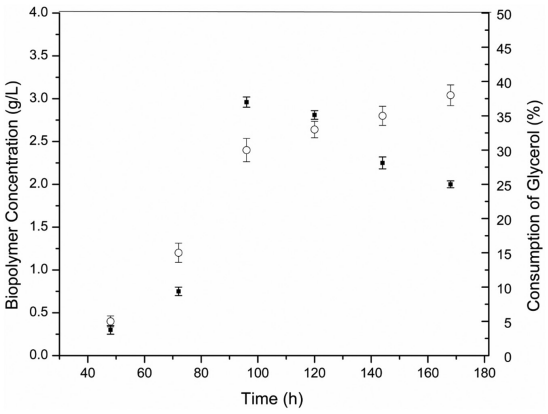

Figure 2. Study of the effect of fermentation time on the amount of formed gum (■) and glycerol consumption (○).

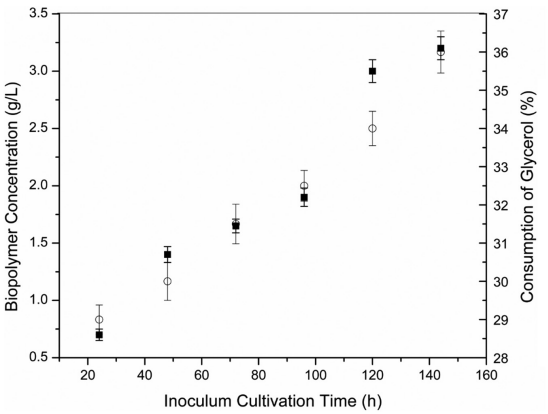

Figure 3. Effect of the incubation time of the inoculum about on the amount of formed gum (■) and glycerol consumption (○).

In preliminary tests, in order to verify the effect of the aeration level values on the system of production of biopolymer, aeration of 3.0 vvm was tested along with other pre-established conditions. In this aeration maximum production of 4.04 $g.L^{-1}$ gum and productivity of 0.084 $g.L^{-1}$ were attained in 48 h. These results demonstrated that the aeration is an important variable in the gum formation by this fungus. Furthermore, it was observed that the air flow rate used was high for the 96 hours of processing, since drying of the culture medium occurred (the liquid medium was entrained in air). Another important point, checked with the aeration, was an increased productivity, which increased from 0.031 $g.L^{-1}\ h^{-1}$ in the 96 h process to 0.084 $g.L^{-1}h^{-1}$ in 48 h. Therefore, aeration proved to be an important parameter to be explored and evaluated in different ways.

Therefore, in order to determine the best condition of aeration on the production of biopolymer, three experiments were performed, each one using 50 $g.L^{-1}$ glycerol, cultivation time (inoculum age) of 120 hours, room temperature 30 ± 2 °C and fermentation time of 96 hours. The results are shown in Table 1.

The results shown in Table 1 indicate the importance of aeration in the metabolism of the fungus to provide increased production of the biopolymer. The results using the air flow of 2 vvm after 24 h of process (Experiment 3: 72 h with continuous aeration) showed better results for biopolymer production (14.15 $g.L^{-1}$), productivity (0.147 $g.L^{-1}\ h^{-1}$), product yield constant (0.89 g of biopolymer/g of glycerol consumed) and glycerol removal (68.34%), among the evaluated experiments.

It can be seen from Table 1 that the aeration promoted in Experiment 3 caused an increase in the production of the biopolymer of 372% compared to Experiment 1 (without addition oxygen), and 33.0% compared to Experiment 2 (with continuous addition of oxygen for 96 h). This shows that there is no need for aeration in the phase where there is no production of gum (first 24 hours of the process). In addition, aeration promotes an increase in the cost of the process. Thus, it was determined that fermentation would be carried out with the addition of air after the 24 hour process.

The results of determination of the effect of yeast extract addition on gum production are shown in Figure 4.

From Figure 4, it is possible to conclude that yeast extract is used by the microorganism as a carbon source to produce the gum, since the increase of the concentration of this compound led to increases in the gum production. Without added yeast extract, the microorganism produced 0.25 gL^{-1} of biopolymer and it exhibited low consumption of glycerol—only 15.53%.

Souza et al.[19] studied the effect of yeast extract on the biopolymer production by probiotic lactic acid strains, *Lactobacillus acidophilus* (La-5) and *Lactobacillus casei* (LC-1). The optimum concentration of the yeast extract obtained was of 0.58% (w/v).

Faria et al.[10] also studied the effect of the addition of yeast extract on the production of xanthana gum by *Xanthomonas campestris pv.* using broth cane sugar as a carbon source, and found that the optimum condition of the yeast extract concentration was 1.8 $g.L^{-1}$. As a result, in the next tests, it was chosen the amount of 2 g. L^{-1} yeast extract used in previous trials, which generated an amount of 14.2 $g.L^{-1}$ gum and productivity of 0.148 $g.L^{-1}\ h^{-1}$ (Figure 4).

The experiment to evaluate the amount of gum produced using only yeast extract as a carbon source in the medium provided the following results of 0.05 0.15, 0.55 and 1.35 $g.L^{-1}$ of gum, for the concentration of *YE* of 1, 2, 3 and 4 g. L^{-1}, respectively. This test confirmed that the fungi applied yeast extract as a carbon source to produce the biopolymer.

3.2 Biopolymer production in fermenter

Previous studies were important to the success of the process in the production of gum in a fermenter. According to Garcia-Ochoa et al.[20], operating conditions such as the configuration of the bioreactor operation mode (batch or

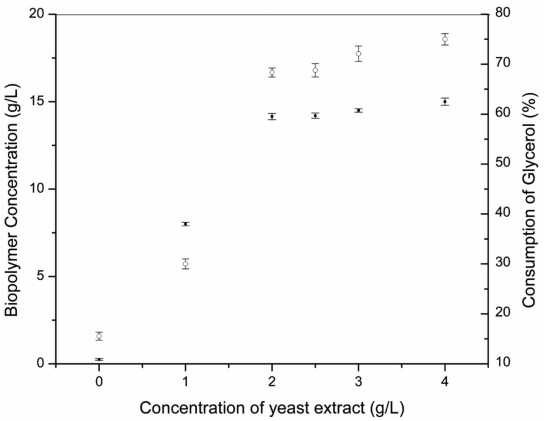

Figure 4. Effect of the amount of yeast extract in the formation of the polymer, amount of formed gum (■) and glycerol consumption (○).

Table 1. Results obtained of formed biopolymer concentration, productivity, product yield constant $Y_{P/S}$ and glycerol removal to the performed experiments.

Experiments	Biopolymer concentration ($g.L^{-1}$)	Productivity ($g.L^{-1}\ h^{-1}$)	Product yield constant $Y_{P/S}$
Experiment 1: without additional aeration (96 hours)	3	0.031	0.100
Experiment 2: with continuous aeration at 2.0 vvm for 96 hours	10.63	0.111	0.494
Experiment 3: without aeration 24 h and 72 h of process with continuous aeration of 2 vvm	14.15	0.147	0.890

$Y_{P/S:}$ g of biopolymer formed/g of glycerol consumed.

continuous), medium composition, temperature, pH, agitation speed, aeration rate and the fermentation time influence not only the growth of the microorganism and the production of xanthan gum, but also the structure and rheological properties of the polymer. Thus, the optimization of fermentation conditions on a bench scale is absolutely necessary before the expansion of the production scale.

Once the conditions were set, yeast extract concentration of 2 $g.L^{-1}$, glycerol concentration of 50 $g.L^{-1}$, fermentation time of 96 h at 100 rpm, culture time of inoculum of 120 h, and continuous aeration of 2 vvm after 24 h of process, a kinetic study was performed in Biostat B fermenter. Figure 5 shows the curves obtained for cell growth, the amount of formed gum and glycerol consumption as functions of fermentation time.

According to Figure 5, after 16 h of fermentation, the production of biopolymer was 1.1 $g.L^{-1}$ and productivity of 0.069 $g.L^{-1}h^{-1}$. Moreover, there is a relationship between cell growth and the amount of gum formed, since the amount of gum formed increased with the growth of the microorganism.

In 74 h there was a maximum production of gum, 16.35 $g.L^{-1}$ (productivity of 0.221 $g.L^{-1}h^{-1}$) with a maximum cell growth of 6.5 $g.L^{-1}$. Another finding was the reduction in the content of biopolymer after 74 h, along with an increase of glycerol consumption. After 74 h of fermentation, it was verified visually that the fibers were shattered, presenting a different structure from those fibers produced in 74 h of process where fibers were compact and long. This fact indicates that the fungus

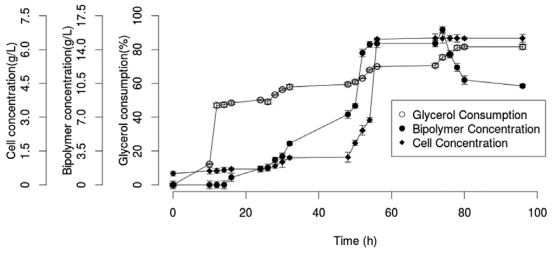

Figure 5. Profile Kinetic of the biopolymer production in the bioreactor, amount of formed gum (●), glycerol consumption (○) and cell growth (♦).

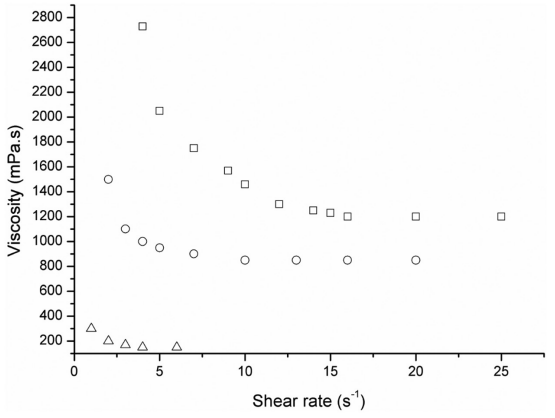

Figure 6. Variation of viscosity versus shear rate of aqueous solutions of the polymer in concentrations of *0.5; 0.75 and 1% at 25 °C.*

could have produced enzymes capable of degrading the gum using the remaining glycerol as a substrate for production of other by-products.

Faria et al.[10], studied the kinetic profile of gum production using *Xanthomonas campestris pv. campestris* NRRL B-1459 in the bioreactor and it obtained 16.4 $g.L^{-1}$ of gum.

The values found in this study were next compared to Faria et al.[10], as was shown above, proving that the study of the variables is extremely important to the produced biopolymer.

Knowing about the properties of polymers, especially the viscosity and rheological behavior, are important for future industrial applications because it allows us to obtain information about the deformation and flow properties of materials[21].

The rheological properties were evaluated by the apparent viscosity analysis to verify the quality of the gum produced in the previously selected conditions. Figure 6 shows the behavior of viscosity of the gum as a function of shear rate for different gum concentrations (0.5, 0.75 and 1%). According to Faria et al.[12], an important feature of biopolymers is their ability to cause thickening at a given concentration, generally less than 1% (w/v), promoting higher viscosities to the known strain rates.

The results indicated that the apparent viscosity decreased with increasing the shear rate. According to the literature, this behavior has been found in polymer solution microbial polysaccharides[22].

Beyer et al.[23] found that 1% solutions of polysaccharide produced by *Rhizobium* CB744 at 25 °C, also promoted a decrease in viscosity with increasing shear rate.

In the comparative graphs of the apparent viscosity readings of aqueous solutions of gum produced, it can be seen that the concentration of 0.5% has the lowest viscosity values, of 143 mPa.s at a shear rate of 4 s^{-1} (Figure 6).

The solutions of concentrations from 0.75% to 1% at (under) shear rate of 4 s^{-1}, had viscosities of about 2,730 to 1,010 mPa.s, respectively (Figure 6). This shows that at a low shear rate the produced gum showed higher viscosity.

3.2.1 Characterization of the formed goma: determination of monosaccharide composition and molecular parameters

The presence of amino sugars was investigated by thin layer chromatography (TLC). The patterns of amino sugars (glucosamine, and mannosamine) were revealed with ninhydrin and orcinol. This was negative for the presence of amino sugars in the sample.

Table 2 shows the monosaccharide composition of the gum formed by the fungus *Mucor racemosus Fresenius.*

Table 2. Monosaccharide composition of the formed gum.

Components	Composition (%)
Rhamnose	6.6 ± 0.52
Manose	28.3 ± 1.79
Galactose	32.1 ± 0.81
Glucose	22.5 ± 1.08
Glucuronic acid	10.0 ± 0.01
Galacturonic acid	0.5 ± 0.01

Table 3. Molecular parameters of the formed gum.

Variables	Results
M_w	4.607×10^6 g.mol^{-1}
M_n	4.600×10^6 g.mol^{-1}
M_z	4.613×10^6 g.mol^{-1}
Polydispersity index (M_w/ M_n)	1.002 ± 0.011
R_n	56.0 nm
R_w	56.3 nm (error 1.7%)
R_z	56.5 nm (error 1.7%)

As Table 2 shows, the total sugar content for the biopolymer produced was 89.5%. It can be seen that the gum is composed of larger amounts of sugars mannose, galactose and glucose, and minor amounts of rhamnose and uronic acids.

TLC confirmed the presence of neutral sugars and indicated that the main sugar acid is glucuronic acid at 10%. The ratio of components glucose, mannose and glucuronic acid obtained in the present study was 2.25: 2.83: 1. The ratio of these components (glucose, mannose and glucuronic acid) in the xanthan gum obtained by Rosalam and England[24] was 2: 2: 1 and Faria et al.[12] was 1.79: 1:33: 1. The ratios different found in this paper. Furthermore, the gum obtained in this paper presents sugars (rhamone and galactose) that are not in significant quantities in the structure of the xanthana gum. Therefore, the gum produced in the present study using of the cerrado fungus (*Mucor racemosus Fresenius*) and glycerol as raw material, showed different structure from the Xanthan gum.

Analyzing the results of unimodal distribution of mass obtained was observed value of polydispersity index (Mw / Mn) equal to 1.002 (Table 3). This polydispersity value shows homogeneity in the analyzed material.

The molecular parameters obtained to the sample from the elution profile using the Zimm method are shown in Table 3.

The weighted average molecular weight (Mw) obtained of the gum was 4.607×10^6 g.mol^{-1} (Da). By analyzing this parameter, it can be seen that this value is within the molecular weight range provided for the xanthan gum can vary from 5.0×10^7 to 2×10^6 Da (Daltons)[25].

The variations in the fermentation conditions are factors that influence the molar mass of xanthan. The use of xanthan gum as a reference if should the interest of the physico-chemical properties that surpass all other polysaccharides available. The property that stands out is its high viscosity at low concentrations (0.05-1%), being induced by their branched structure and high molecular weight and stability in a wide range of temperature and pH[20,26].

4. Conclusions

Glycerol showed a promising substrate for the production of biopolymer using the fungus *Mucor racemosus Fresenius*. The results showed production of 14.15 g.L^{-1} gum and consumption of 68.34% glycerol at selected conditions –50 g.L^{-1} glycerol in the initial table shaker at 100 rpm, with air flow of 2 vvm during production (without aeration in the first 24 hours and with continuous aeration in 72 hours of assay), age inoculum of 120 hours and room temperature of 28 ± 2 °C. The use of aeration increased the production of gum to 371%. Under conditions previously determined, assays carried out in a reactor of 2 L with a useful volume of 1 L, at 74 h of processing obtained 16.35g.L^{-1} gum (productivity of 0.221 g.L^{-1} h^{-1}) and 75% glycerol consumption. The gum presented a pseudoplastic behavior with higher viscosity than the xanthan gum in the shear rate of 4 s^{-1}, similarly to the biopolymer reported by the literature. The weighted average molecular weight (Mw) obtained for the gum was 4.607×10^6 Da. The value of the polydispersity index (Mw/Mn) equal to 1.002 shows the homogeneity of the produced biopolymer. The monosaccharide composition of the polymer was 6.6% of rhamnose, mannose 28.3%, 32.1% galactose, 22.5% glucose, 10% glucuronic acid and 0.5% galacturonic acid. It is worth mentioning that the production of a large amount of biopolymer using the fungus *Mucor racemosus Fresenius* and employing glycerol as substrate is not easily found in the literature.

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