Brazilian Journal of Microbiology 44, 4, 1059-1065 (2013)
ISSN 1678-4405

Research Paper

Extracellular polysaccharide production by a strain of *Pleurotus djamor* isolated in the south of Brazil and antitumor activity on Sarcoma 180

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Submitted: September 21, 2011; Approved: April 4, 2013.

Abstract

Polysaccharides with medicinal properties can be obtained from fruiting bodies, mycelium and culture broth of several fungus species. This work was carried out in batch culture using a stirred tank reactor with two different initial glucose concentrations (40-50 g/L) and pH values (3.0-4.0) to enhance extracellular polysaccharides production by *Pleurotus djamor* UNIVILLE 001 and evaluate antitumor effect of intraperitonial administration of *Pleurotus djamor* extract on sarcoma 180 animal model. According to factorial design, the low pH value (pH 3.0) led to a gain of 1.6 g/L on the extracellular polysaccharide concentration, while glucose concentration in the tested range had no significant effect on the concentration of polysaccharide. With 40 g/L initial glucose concentration and pH 3.0, it was observed that yield factor of extracellular polysaccharide on substrate ($Y_{P/S}$ = 0.072) and maximum extracellular polysaccharide productivity (Q_{Pmax} = 11.26 mg/L.h) were about 188% and 321% respectively higher than those obtained in the experiment performed at pH 4.0. Under these conditions, the highest values of the yield factor of biomass on substrate ($Y_{X/S}$ = 0.24) and maximal biomass productivity (Q_{Xmax} = 32.2 mg/L.h) were also reached. In tumor response study, mean tumor volume on the 21th day was 35.3 cm^3 in untreated group and 1.6 cm^3 in treated group (p = 0.05) with a tumor inhibition rate of 94%. These impressive results suggests an inhibitory effect of *P.djamor* extract on cancer cells.

Key words: *Pleurotus djamor*, extracellular polysaccharides production, antitumor activity.

Introduction

Natural products have been traditionally accepted as solutions to health, due to popular beliefs that they have fewer adverse effects (Mantovani *et al.*, 2008). Edible fungi have been widely used as functional foods and their extracts have been studied in natural therapy for prevention and treatment of tumors, raising commercial interest (Firenzuoli *et al.*, 2008). *Pleurotus* sp. antitumor activity has been focused by several studies (Zhang *et al.*, 2004; Gu and Sivam, 2006; Lavi *et al.*, 2006; Sarangi *et al.*, 2006; Jedinak and Sliva, 2008; Wolff *et al.*, 2008; Dalonso *et al.*, 2010).

The culture medium and conditions must be related to the nutritional needs of the microorganism and strongly in-

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fluence the metabolite formation, such as the intracellular polysaccharides (Shih *et al.*, 2006), the extracellular polysaccharides (Hwang *et al.*, 2003; Wisbeck, 2003; Gern *et al.*, 2008; Shih *et al.*, 2008; Furlan *et al.*, 2009), and the biomass formation (Hwang *et al.*, 2003; Wisbeck, 2003; Cho *et al.*, 2006; Shih *et al.*, 2008).

Several research groups have been developing experiments in shake flasks or bioreactors using different fungi species in order to optimize the production of polysaccharides by varying the concentration of nutrient sources and the growing conditions such as pH, temperature and aeration (Hwang *et al.*, 2003; Cho *et al.*, 2006; Shih *et al.*, 2006; Furlan *et al.*, 2008; Gern *et al.*, 2008; Shih *et al.*, 2008).

However, informations about nutritional requirements and environmental conditions for submerged culture of *Pleurotus djamor* are still limited. In the present study the biomass and extracellular polysaccharide production by *P. djamor* was evaluated in batch culture, using a stirred tank reactor. The influence of initial glucose concentration and pH control on mycelial growth and polysaccharide production were demonstrated. Inhibitory effect of *P.djamor* extract on cancer cells was proven.

Materials and Methods

Microorganisms and inoculum preparation

P. djamor UNIVILLE 001, isolated in the garden of the University of the Region of Joinville - UNIVILLE, was maintained in Petri dishes at 4 °C on WDA (Wheat Dextrose Agar) medium (Furlan *et al.*, 1997). The inoculum was prepared in a 2 L Duran flask containing 400 mL of POL medium: $(NH_4)_2SO_4$ 5.0 g; $MgSO_4.7H_2O$ 0.2 g; K_2HPO_4 1.0 g; yeast extract 2.0 g; peptone 1.0 g; $CaCO_3$ 1.0 g; glucose 20 g in 1 L of distilled water (Cavazzoni and Adami, 1992). pH value was 6.5 - 7.0, and the medium was sterilized at 15 psi and 121 °C. The entire content of one Petri dish was used to inoculate each Duran flask. The flasks were incubated at 30 °C on a rotary shaker at 120 rpm, for six days (Wisbeck, 2003).

Extracellular polysaccharides and biomass production

A factorial design 2^2 was used to evaluate the influence of initial glucose concentration (40 and 50 g/L) and pH control (3.0 and 4.0) on biomass (X) and extracellular polysaccharide (P) production in batch cultivation. These conditions were selected based on the results obtained by Wisbeck (2003) showing that *P. ostreatus* accumulated higher concentrations of extracellular polysaccharides with 40 g/L glucose and initial pH 4.

Culture medium composition was similar to that described in 2.1, except for glucose concentration (40 and 50 g/L). The medium was sterilized and inoculated with an inoculation ratio equal to 10% (v/v) and cultivated in a 5 L stirred tank reactor (Biostat B, B. BRAUN, Germany) with 4 L working volume. The cultivation was performed under the following conditions: 30 °C, 0.25 L/min air flow and 300 rpm (initial K_La equal to 15 1/h) and pH 3.0 or 4.0 according to the experiment.

All experiments were performed in duplicate and the calculation of the kinetic parameters was based on the kinetic profiles, using the data of both replications.

For the statistic analysis of the factorial design, the Paretos method was used (Barros Neto *et al.*, 1996).

The culture broth was filtered, washed with distilled water and the retained biomass was dried at 60 °C for 48 h for the determination of the biomass dry weight.

The glucose concentration was determinated by the Enzyme-Glucose Test (Wiener Laboratórios, Brasil).

After biomass separation, 10 mL of the broth were treated with acetone cooled to 8 °C (3:1, v/v) (Rosado *et al.*, 2003), and maintained for 24 h under refrigeration (4 °C) for extracellular polysaccharide precipitation. The precipitate was centrifuged at 3373 g for 5 min. The supernatant was separated and the precipitate was washed twice, using an acetone:ethanol:distilled water solution (3:1:1, v/v/v) (Cavazzoni and Adami, 1992). The extracellular polysaccharide concentration was estimated in the precipitate by the phenol-sulfuric method (Dubois *et al.*, 1956).

Polysaccharides extracts

Extracellular polysaccharides were produced in *Pleurotus djamor* submerged culture in POL medium (section 2.2) with 40 g/L initial glucose and pH 3.0. Extraction (Pokhrel and Ohga, 2007) in which ethanol PA (4:1, v/v) was added to the culture broth and left for 24 hours at 4 °C, with the formed precipitate being separated by centrifugation and lyophilized. The obtained material was prepared in concentration of 10 g/L for 30 mg/kg doses in phosphate buffered saline solution (PBS 0.01 M, pH 7.0). These parameters were established from a previous study comparing different methods of extraction, concentration and doses to determine the optimal antitumor effect.

Animal study

These were performed after following the protocol by the Ethics Committee of University of the Region of Joinville (No. 031/2008) and were carried out in accordance with current guidelines for the Care and Use of Laboratory Animals - Commission on Life Sciences, National Research Council, 1996.

Swiss albino mice were housed under standard conditions of temperature (21 ± 2 °C), relative humidity (60 ± 10%) and 12 h light/12 h dark cycle at the Cronic Treatment Laboratory. Thirty male Swiss albino mice were divided in two studies, named as Tumor Response Study and Survival Study.

Tumor response study

Ten male Swiss albino mice were divided in two groups of five animals each, named Untreated Group (tumor induction with no treatment) and Treated Group (tumor induction with treatment).

Tumor induction and treatment

Sarcoma 180 (S180) tumor culture was obtained by courtesy from the Pharmacology Department from UNIVALI (Itajaí/SC/Brazil), and maintained through weekly intraperitoneal injections in male Swiss albino mice (Pagno *et al.*, 2006). Tumor induction was performed subcutaneously, in the back of each mouse from both Treated and Untreated Groups in a concentration of $25x10^6$ cel/mL, in 0.2 mL volume (Mizuno *et al.*, 1999).

Polysaccharide extracts were administered intraperitoneally in the Treated Group for ten consecutive days, starting at 24 h after tumor induction (Zhang *et al.*, 2004), in daily doses of 30 mg/kg of body weight. A PBS solution was applied intraperitoneally to Untreated Group at a dose of 10 mg/kg.

Assessment of tumor response

Animals were observed from 11^{th} to 20^{th} post-induction days. All mice were sacrificed on the 21^{th} day and tumors were removed. Tumor weight (g) was obtained according to Misaki *et al.* (1984) and tumor volume (cm^3) was calculated as proposed by Ajith and Janardhanan (2003) and Lee *et al.* (2003), through the equation $(4/3)\pi(a^2b)/2$. Inhibition rate was calculated as [(C-T)/C]*100 (Zhang *et al.*, 2004).

Results and Discussion

Extracellular polysaccharides and biomass production

The kinetic profiles of glucose (S) consumption, biomass (X) growth and extracellular polysaccharide (P) production, obtained from experiments performed with 40 g/L initial glucose and pH 3.0, 40 g/L initial glucose and pH 4.0, 50 g/L initial glucose and pH 3.0, 50 g/L initial glucose and pH 4.0, are showed in Figure 1 (A and B) and 2 (A and B), respectively.

The time required for glucose consumption by *P. djamor* was lower using 40 g/L initial glucose concentration and pH value 3.0, when compared with the other tested conditions. It can also be observed that independent of the initial glucose concentration, the lower pH value (3.0) favors glucose consumption.

Table 1 shows the kinetic parameters obtained for the tested conditions. With 40 g/L initial glucose concentration, $Y_{P/S}$ and Q_{Pmax} obtained in the experiment conducted at pH 3.0 were about 188% and 321% higher than those obtained at pH 4.0. In culture conducted with 50 g/L initial glucose concentration, $Y_{P/S}$ and Q_{Pmax} obtained at pH 3.0 were about 190% and 242% higher than those obtained at pH 4.0.

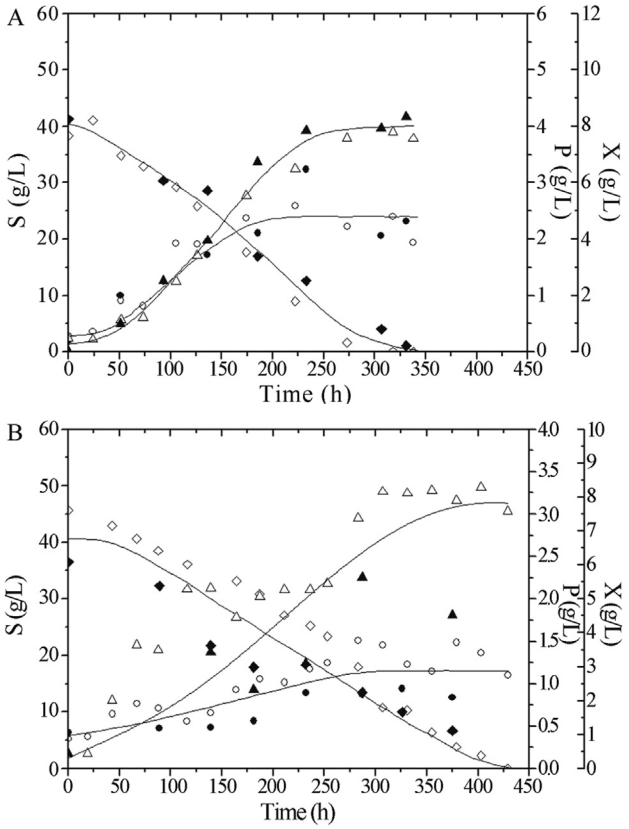

Figure 1 - Kinetics of glucose consumption (S ◆, ◊), biomass growth (X ▲, Δ) and extracellular polysaccharide production (P ●, ○) for the cultivation of *P. djamor* using 40 g/L initial glucose concentration and pH 3.0 (A) and pH 4.0 (B).

Comparing the kinetic parameters obtained at pH 3.0, it can be observed that 40 g/L initial glucose concentration promoted the highest yield factor and productivities in polysaccharides ($Y_{P/S}$ = 0.072, Q_P = 9.337 mg/L.h and Q_{Pmax} = 11.260 mg/L.h).

The initial glucose concentration equal to 40 g/L also favored Q_X and Q_{Xmax} independent of the pH value. Using this initial glucose concentration and pH 3.0, the highest values of $Y_{X/S}$ (0.244), Q_X (31.4 mg/L.h) and Q_{Xmax} (32.2 mg/L.h) were obtained.

Gern *et al.* (2008) investigated the extracellular polysaccharide production by *Pleurotus ostreatus* in a stirred tank reactor using K_La value equal to 10.2 1/h, pH 4.0 and 40 g/L initial glucose concentration. The kinetic parameters were: $Y_{P/S}$ = 0.047 and Q_P = 7.01 mg/L.h. The values reported by the authors for $Y_{P/S}$ and Q_P were about 35% and 25% lower respectively when compared to the best results obtained in this work (for S_0 = 40 g/L and pH 3.0). The authors did not evaluate the behavior of the microorganism in pH 3.0.

Bonatti *et al.* (2008) studied the production of extracellular polysaccharides by *Pleurotus ostreatus* in semi-

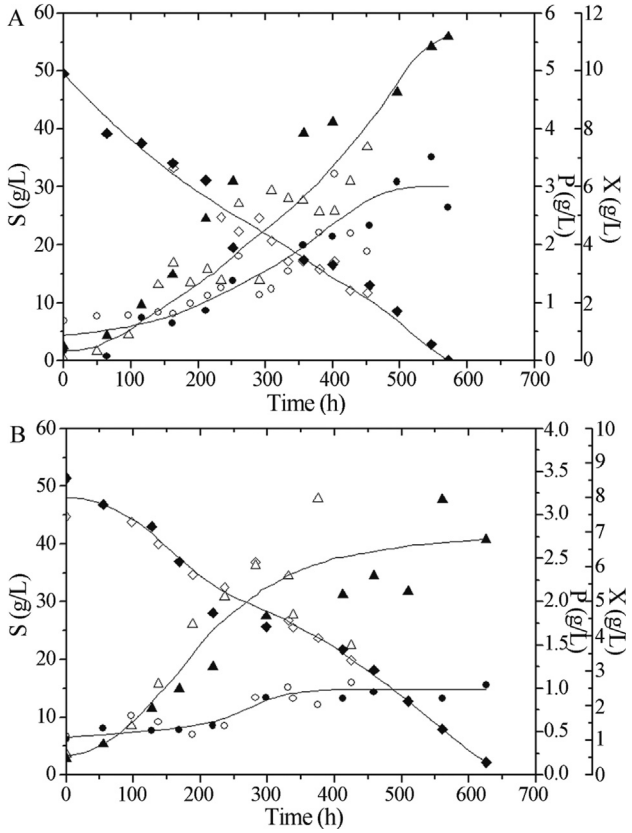

Figure 2 - Kinetics of glucose consumption (S ◆, ◊), biomass growth (X ▲, Δ) and extracellular polysaccharide production (P ●, ❍) for the cultivation of *P. djamor* using 50 g/L initial glucose concentration and pH 3.0 (A) and pH 4.0 (B).

continuous process with medium replacements of 50 and 75% in a bioreactor with 4 L working volume. 40 g/L initial glucose concentration, pH 4.0 and 50% medium replacement favored the production of polysaccharides ($Y_{P/S}$ = 0.09 and Q_{Pmax} = 9 mg/L.h) in the second cycle. According to the authors productivity was increased in nearly 30% compared to batch culture. $Y_{P/S}$ reached by the authors was higher than that obtained in this study (0.072) using 40 g/L glucose and pH 3.0. However, Q_{Pmax} reached in this study was about 25% higher than the value reported by the authors.

Wisbeck (2003) obtained with *P. ostreatus* cultivated in 40 g/L initial glucose concentration and pH 4.0: ΔP = 1.32 g/L, $Y_{P/S}$ = 0.081 and Q_P = 7.50 mg/L.h. Comparing the data obtained for the same culture conditions (40 g/L initial glucose concentration and pH 4.0) the author reached higher values than those obtained in this work. However, comparing the results reported by the author with those obtained in this study for pH 3.0, the last are higher for ΔP (59%) and Q_P (24%). The maximal specific growth rate obtained by the author (0.022 1/h) is lower than that reached in this work (0.041 1/h).

Cho *et al.* (2006) reached maximum biomass concentration equal to 10.4 g/L, maximal extracellular polysaccharide concentration of 3.05 g/L and $Y_{P/S}$ equal to 0.13 in 5 days cultivation of *Tremella fuciformis* in bioreactor. Hwang *et al.* (2003) obtained ΔX = 11 g/L after 15 days cultivation and ΔP = 3.3 g/L after 14 days cultivation of *Phellinus linteus* at pH 4.0. Both studies showed superior results than those observed in this study, but using different microorganisms.

In order to better evaluate the influence of initial glucose concentration (40 and 50 g/L) and pH value (3.0 and 4.0) on ΔX and ΔP a factorial design was done (Table 2).

Table 3 shows the effects of the variables S_0 and pH on the mycelial biomass (ΔX) and the extracellular polysaccharide (ΔP) formation. According to the results presented in this Table, S_0 in the tested range as well as the interaction between S_0 and pH, did not present any significant effect on both mycelial biomass and extracellular

Table 1 - Kinetic parameters for *P. djamor* cultivated with 40 and 50 g/L initial glucose concentration, pH 3.0 and 4.0, according to 2^2 factorial design.

Kinetic parameters	40 g/L pH 3.0	40 g/L pH 4.0	50 g/L pH 3.0	50 g/L pH 4.0
ΔP (g/L)	2.110	0.760	2.530	0.560
ΔX (g/L)	7.110	6.990	9.880	5.730
ΔP_m (g/L)	1.810	0.650	2.420	0.496
ΔX_m (g/L)	6.470	6.440	10.630	3.800
$Y_{P/S}$	0.072	0.025	0.058	0.020
$Y_{X/S}$	0.240	0.230	0.228	0.206
Q_P (mg/L.h)	9.340	2.380	5.040	1.320
Q_X (mg/L.h)	31.400	21.970	19.680	13.540
Q_{Pmax} (mg/L.h)	11.260	2.670	5.170	1.510
Q_{Xmax} (mg/L.h)	32.200	22.190	20.020	15.830
t (h)	226	318	502	423
t_{mp} (h)	161	242	467	328
t_{mx} (h)	201	290	531	240

Table 2 - Factorial design 2^2 to study the effect of initial glucose concentration (S_0) and pH values on the mycelial biomass (ΔX) and EPS (ΔP) concentrations.

Experiments	S_0 (g/L)	pH	ΔX ± sd* (g/L)	ΔP ± sd* (g/L)
1	40	3	6.86 ± 1.5	2.31 ± 0.4
2	40	4	6.28 ± 2.2	0.73 ± 0.3
3	50	3	7.86 ± 2.6	2.15 ± 0.9
4	50	4	5.60 ± 0.3	0.56 ± 0.02

*Standard deviation.

Table 3 - Effects of the variables S_0 and pH on the mycelial biomass formation (ΔX) and maximal extracellular polysaccharide concentration (ΔP) by the factorial design.

Variables	Effects	
	ΔX (g/L ± SE*)	ΔP (g/L ± SE*)
pH (1)	-1.42 ± 1.33	-1.58 ± 0.38**
S_0 (2)	0.16 ± 1.33	-0.16 ± 0.38
(1) and (2)	-0.83 ± 1.33	-0.00 ± 0.38

*Standard error, **Statistically significance effect (95% confidence limits).

polysaccharide concentration. However, pH has a negative significant effect on the extracellular polysaccharide formation. The lower pH value (pH 3.0) led to a gain of approximately 1.6 g/L on extracellular polysaccharide concentration. The highest values of extracellular polysaccharide concentration (Table 2) were obtained using 40 g/L initial glucose and pH 3.0 (2.31 ± 0.4 g/L) and 50 g/L initial glucose and pH 3.0 (2.15 ± 0.9 g/L).

Wisbeck (2003) evaluated the effect of initial glucose concentration (20 and 40 g/L) and pH (4.0 and 6.0) on extracellular polysaccharide production by *Pleurotus ostreatus* in submerged culture. The initial glucose concentration of 40 g/L and the pH 4.0 maximized the production of extracellular polysaccharides by that microorganism. In this work, using *Pleurotus djamor* it was observed the same behavior for pH. However, the initial glucose concentration in the tested range had no significant effect on the extracellular polysaccharide formation by the species *Pleurotus djamor*.

Several kinds of fungi have acidic optima pH during submerged cultures. Yang and Liau (1998) investigated the effects of environmental parameters on the extracellular polysaccharide production by *Ganoderma lucidum* in submerged cultures. For the optimal pH (4.0-4.5) the polysaccharide concentration reached 1.6 g/L, a value lower than those found in this work at pH 3.0. Shu and Lung (2004) studied the effects of pH (3.0-6.0) on extracellular polysaccharide concentration of *Antrodia camphorata* in a stirred tank fermenter. The optimum pH for this process was 5.0, with 0.118 g/L of extracellular polysaccharides, a very low value when compared to the finds of this study. Shih *et al.* (2008) cultivated *Grifola frondosa* in shake flasks, at different initial pH (4.0-6.0). After 9 days cultivation, the maximum extracellular polysaccharide concentration (0.82 g/L) was obtained in culture grown at an initial pH 5.0. This value is also lower than the best ones obtained in this investigation.

These results evidence that *P. djamor* is a potential microorganism for extracellular polysaccharide production mainly at pH 3.0 and the initial concentration of glucose 40g/L.

Tumor response

On the 21th day, mean tumor weight was 6.64 g in the Untreated Group and 0.37 g in the Treated Group ($p = 0.05$). Mean tumor volume was 35.3 cm^3 in the Untreated Group and 1.6 cm^3 in the Treated Group ($p = 0.05$). A tumor inhibition rate of 94% was found (Table 4).

Antitumor activity of mushroom extracts have been reported by several authors as described in the review published by Mantovani *et al.* (2008). Despite β-glucan polysaccharide has been regarded as the responsible agent for this effect, most of the related studies have used either crude extracts (Lee *et al.*, 2003; Wong *et al.*, 2007; Harhaji *et al.*, 2008) or a fraction of extracts (Ohno *et al.*, 2001; Nakamura *et al.*, 2004; Sarangi *et al.*, 2006; Unursaikhan *et al.*, 2006).

In a detailed biomolecular study, Jedinak and Sliva (2008) analyzed the inhibitory effect of four different mushroom extracts on proliferative activity of breast and colon cancer cell cultures. *Pleurotus ostreatus* extract was found to present a most potent suppression of both breast and colon cancer cell proliferation, but no effect was observed on normal mammary or colonic cells. β-glucans are glucose polymers that differ from each other by a highly variable structure of length and branching. Their immune function is apparently related to the conformational complexity and it has been suggested that intensity of anticancer effects is associated to a higher degree of structural complexity (Bohn and BeMiller, 1995).

This is the rational to assess the anti-tumoral effect of different species of β-glucan containing mushrooms. In the present study, we present the first investigation on the potential anticancer effects of *Pleurotus djamor* after its isolation.

Table 4 - Weight, volume tumor and inhibition rate for the treated and untreated groups with polysaccharides extract of *P. djamor.*

Groups	Tumor weight (g ± sd*)	Tumor volume (cm^3 ± sd*)	Inhibition rate (%)
Treated	0.37 ± 0.07	1.6 ± 0.15	94
Untreated	6.64 ± 1.19	35.37 ± 12.16	-

*Standard deviation.

The impressive results obtained in this pilot study suggests an inhibitory effect of *P.djamor* extract on cancer cells and have encourage us to pursued in further efforts to assess its role in future benefits in the treatment of malignant diseases.

Acknowledgments

The group wishes to thank the Research and Support Fund - FAP from UNIVILLE for the financial backing for the project. The authors wish to express their gratitude to Dr. A. A. Steil and Dr. D. Sato, from UNIVALI, for donating the tumor strain.

Nomenclature

a - The short diameter (mm^2)
b - The long diameter (mm^2)
C and T - The tumor weight of the untreated group and the treated group, respectively
K_La - volumetric oxygen transfer rate
Q_P, Q_X - global productivities in extracellular polysaccharides and biomass (for t = t)
Q_{Pmax}, Q_{Xmax} - maximum productivities in extracellular polysaccharides (for $t = t_{mp}$) and biomass (for $t = t_{mx}$)
P, S, X - Product, substrate and biomass concentrations
ΔP, ΔX - extracellular polysaccharides and biomass formed until the stabilization of extracellular polysaccharide concentration at its maximum value
ΔP_m - extracellular polysaccharide concentration formed by the time that productivity in extracellular polysaccharides is maximal
ΔX_m - mycelial biomass concentration formed by the time that productivity in biomass is maximal
t - time process, considered the time of the stabilization of extracellular polysaccharide concentration at its maximum value
t_{mp} - time for Q_{Pmax}
t_{mx} - time for Q_{Xmax}
$Y_{P/S}$, $Y_{X/S}$ - global yield factors of extracellular polysaccharides on substrate and biomass on substrate (for t = t)

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