Study of levan productivity from *Bacillus subtilis* Natto by surface response methodology and its antitumor activity against HepG2 cells using metabolomic approach

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Abstract: Levan productivity of *Bacillus subtilis* Natto was evaluated in submerged culture varying the pH, temperature and culture time, using factorial design and response surface methodology. The characterization of levan molecular weight was performed by HPSEC and its antitumor activity against HepG2 cells using metabolomic approach was also evaluated. At first, the variables investigated, as well as their interactions, demonstrated significant effect. Further, a second design using the same variables at different levels was developed. Thus, according to the model, an optimized value corresponding to 5.82g.L^{-1}.h^{-1} was achieved at pH 8, 39.5°C in 21 hours, the highest value reported so far. After analysis by HPSEC, two molecular weights were obtained corresponding to 72.37 and 4146 kDa. The levan promoted an increase of acetate, alanine, lactate and phosphocreatine in HepG2 cells suggesting an alteration in the bioenergetics pathways and cellular homeostasis by intracellular accumulation of lactate, justifying its antitumor activity.

Keywords: Levan, *Bacillus subtilis* Natto, Antitumor activity.

INTRODUCTION

Many polysaccharides have been receiving attention lately due to their potential antitumor activity. Several studies involving fungal, vegetables and bacterial polysaccharides have shown impressive results against cancer cells *in vivo* and *in vitro* (Tong *et al.*, 2009; Rui *et al*., 2010; Zhao *et al.*, 2010).

The levan, an exopolysaccharide composed of fructose mainly linked by β-(2→6) glycosidic bonds with some β-(2→1) branch chains (Han and Clarke, 1990), synthetized by levansucrase (EC 2.4.1.10) in medium with high amount of sucrose (Meng and Fütterer, 2003), has also been studied for antitumor activity. Various studies have reported its activity *in vivo* against sarcoma 180 and Ehrich carcinoma (Calazans *et al.*, 1997; Calazans *et al.*, 2000); also, *in vitro*, against gastric (SNU-1) and hepacellular (HepG2) human carcinomas (Oh *et al*., 2004; Yoo *et al*., 2004; Yoon *et al*., 2004), using levans with different molecular weights and degrees of branching.

Analysis of antitumor activity can be studied by metabolomics, which is a science that studies the metabolic content of a cell or an organism in a determined time reflecting the actual physiological state of a biological material (Griffiths, 2003). This contributes to the development of early illness diagnosis (Kussmann *et al*., 2006). Studies on toxicity and drug metabolism (Keun, 2006). Tumor metabolic maps (Vizán *et al*., 2008). among others.

Nuclear magnetic resonance (NMR) can be used to analyze the metabolic content originated from tissues, cells or their extracts, monitor changes in metabolic profiles in response to a chemical stress, including the administration of biological active compounds (Harrigan *et al*., 2005).

Among the microorganisms capable of synthesizing levan the *Bacillus subtilis* Natto, which is used in the fermentation of Japanese food natto, has demonstrated great potential in converting sucrose into levan (Shih *et al.*, 2005; Shih *et al*., 2010).

To obtain high levan production it is important to study the factors that influence the process, as the concentration of carbon source in the medium (Shih *et al*., 2010), temperature (Abdel-Fattah *et al*., 2005; Euzenat *et al*., 1997), culture time (El-Refai *et al*., 2009; Oliveira *et al*., 2007) and pH (Shih *et al.*, 2005; Shih *et al*., 2010). The use of statistical planning is important to study all these factors together, since it reduces the number of experiments (Box and Behnken, 1960), and improves the quality of the result obtained, reducing time and costs at the end of the process. Coupled with statistical planning,

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the surface response methodology statistical technique widely used in processes involving optimization - also contributes to minimize empiricism of the theory of trial and error, providing reliable data on the process (Barros *et al*., 1995).

The aim of this work was to study levan productivity by *B. subtilis* Natto using response surface methodology and investigate its antitumor activity in hepacellular human carcinoma (HepG2) by metabolomics approach.

METHODS

Microorganisms and culture media

The *B. subtilis* Natto CCT 7712 strain was isolated from the Japanese food natto from the Department of Biochemistry and Biotechnology and identified by Tropical Culture Collection André Toselo Foundation - Campinas - SP - Brazil. The strain was maintained at 4°C in a medium containing ($g.L^{-1}$): peptone 50, meat extract 30 and agar 20 and sub-cultured every 2 months. The inoculum medium contained in ($g.L^{-1}$): sucrose 100; yeast extract 2; KH_2PO_4 2; $(NH_4)_2SO_4$ 1; $MgSO_4.7H_2O$ 0.5 (Calazans *et al*., 2000). The fermentation medium contained in ($g.L^{-1}$): sucrose 300, yeast extract 2, KH_2PO_4 1, $(NH_4)_2SO_4$ 3, $MgSO_4.7H_2O$ 0.6, $MnSO_4$ 0,2 e ammonium citrate 0,25 (Euzenat *et al*.,1997, modify).

Statistical planning

Growing conditions may interfere in levan production, therefore a factorial design with 15 runs and 3 repetitions at the central point (Box e Behnken, 1960) was applied, using the following variables: (X_1) pH 5, 6 e 7; (X_2) temperature 25, 31 and 37°C and (X_3) culture time at 12, 18 e 24 hours (table 1). The sucrose concentration and shaking speed were fixed at $300g.L^{-1}$ and 100 rpm respectively, according to previous tests.

Based on results of the first design, a second Box and Behnken (1960) design was proposed and the variables were maintained, but their values were modified and corresponding to: (X_1) pH 7, 8 e 9, (X_2) temperature 32, 37 e 42°C (X_3) culture time 18, 24 e 30 hours (table 2). The response analyzed was levan productivity (Y_1) in $g.L^{-1}$.

The values of levan productivity (Y_1) obtained were analyzed by ANOVA method and response surface methodology using STATISTICA 7.0 software. *P*-values below 0.05 were regarded as statistically significant.

Production and evaluation of levan

The strain was grown in inoculum medium for 48 hours at 37°C. Further the cells were centrifuged, resuspended aseptically in saline (NaCl a 0.9g/%) and measures at 605 nm. The inoculum was standardized at $0.2g.L^{-1}$.

The fermentations were carried out in 125mL flasks containing 25ml of fermentation medium according to statistical planning. Eventually the media were centrifuged for 15 minutes, 9056g at 4°C separating the supernatant from the biomass.

Levan was precipitated from the supernatant by the addition of absolute ethanol to 75% (v/v) at low temperature (4±1°C). Afterwards levan was centrifuged at 21382 g for 20 minutes at 4°C, rewashed with absolute ethanol (4±1°C) and centrifuged again.

Further levan was hydrolyzed with HCl 0.1N at 100°C, cooled and neutralized with NaOH 2N (Ananthalakshmy and Gunasekaran, 1999). Levan concentration was estimated according to the Nelson (1944) and Somogyi (1952) method, using glucose as standard (0 to 100 $\mu g.mL^{-1}$) and λ = 540nm.

Molecular weight of levan

To define the molecular weight, the levan was dialyzed against running and deionized water and lyophilized, dried and determined by size exclusion chromatography. A high performance size exclusion chromatography (HPSEC) apparatus (Waters, MA, USA) coupled to a differential refract meter (RID) and Wyatt Technology (Saint Barbara, CA, USA) Dawn-F Multi-Angle Laser Light Scattering detector (MALLS) was used for testing Levan (0.2% (w/v) in H_2O). Four Water's columns, Ultrahydrogel 2000/500/250/120, were connected in series and coupled to refractive index and light scattering detectors. A 0.1 M $NaNO_3$ solution, containing NaN_3 (0.5 $g.L^{-1}$) was used as solvent.

Assessment of levan antitumor activity

Human hepatoma cells (HepG-2) were obtained from the Rio de Janeiro Cell Bank (Brazil) and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, Inc.) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37°C, 5% CO_2 atmosphere in a concentration of 1 $x10^7$ cells (Laboratory of Toxicological Genetic, Department of Biology, State University of Londrina). The media were renewed every 2 days until the cells reached confluence. Thus the levan obtained from the best culture condition was subcultured with treatment in culture flasks ($175cm^2$).

The cell cultures were incubated, in triplicate, with levan or phosphate buffered saline (PBS) at a concentration of $1000\mu g.mL^{-1}$ (Yoo *et al*, 2004) for 48 hours, this concentration was previously tested and it was found absence of cytotoxicity and mutagenicity effects (data not shown). The cell lines were then detached from the culture flasks by adding 1.5mL of 0.025% trypsin solution (Invitrogen). The trypsin was inactivated by adding 5mL of 10% FBS in DMEM medium. Single-cell suspensions were gently pipetted into tubes and centrifuged. The supernatant was discarded and the remaining pellet was cautiously re-suspended in PBS. The suspension was centrifuged and the cell culture pellets were immediately frozen in liquid nitrogen and stored at -80°C.

Cell extraction

The metabolites were extracted from the cell pellets according to Le Belle *et al.* (2002). Reagent-grade methanol and chloroform (4°C) in a ratio of 2:1 (v/v, 250 µl/cell pellet) were added to the frozen cell pellets and then sonicated. After 15 minutes, chloroform and Milli-Q water were added to the cold samples in a ratio of 1:1 (250µl/cell pellet). The samples were then centrifuged at

Table 1: First factorial design box benhken (3^3) to evaluate the effects of pH, temperature and culture time on levan productivity (Y_1) of *Bacillus subtilis* Natto in 300g.L^{-1} of sucrose at 100rpm.

Runs	Variables in coded levels			Y_1 (g L^{-1} h^{-1})	
	X_1	X_2	X_3	Experimental	Predicted
1	-1	-1	0	0.35	0.49
2	1	-1	0	0.51	0.55
3	-1	1	0	0.48	0.43
4	1	1	0	4.80	4.65
5	-1	0	-1	0.88	1.03
6	1	0	-1	1.19	1.44
7	-1	0	1	0.39	0.13
8	1	0	1	4.15	3.99
9	0	-1	-1	1.02	0.73
10	0	1	-1	1.96	1.85
11	0	-1	1	0.54	0.65
12	0	1	1	3.29	3.58
13	0	0	0	0.66	0.78
14	0	0	0	0.83	0.78
15	0	0	0	0.86	0.78
Factors			Real levels		
			-1	0	1
X_1	pH		5	6	7
X_2	Temperature (°C)		25	31	37
X_3	Culture time (hours)		12	18	24

Table 2: Second factorial design Box -Benhken (3^3) for maximum levan productivity (Y_1) of *Bacillus subtilis* Natto in 300g.L^{-1} of sucrose at 100 rpm

Runs	Variables in coded levels			Y_1 (g L^{-1} h^{-1})	
	X_1	X_2	X_3	Experimental	Predicted
1	-1	-1	0	2.30	2.85
2	1	-1	0	2.26	2.98
3	-1	1	0	4.10	3.38
4	1	1	0	4,90	4.35
5	-1	0	-1	3,80	3.72
6	1	0	-1	4,69	4.43
7	-1	0	1	2,47	2.73
8	1	0	1	3,04	3.12
9	0	-1	-1	4,26	3.79
10	0	1	-1	4,89	5.69
11	0	-1	1	4,40	3.60
12	0	1	1	3,12	3.59
13	0	0	0	5,54	5.57
14	0	0	0	5,78	5.57
15	0	0	0	5,38	5.57
Factors			Real levels		
			-1	0	1
X_1	pH		7	8	9
X_2	Temperature (°C)		32	37	42
X_3	Culture time (hours)		18	24	30

12000g for 20min at 4°C. The upper phase (methanol and water) was separated from the lower phase (organic) using a glass syringe. The solvents of both fractions were then removed at room temperature under reduced pressure in a speed vac device. The extracts containing the metabolites were dissolved in 0.6mL of sodium trimethylsylilpropionate ($0.625mMol.L^{-1}$) in D_2O solution, sonicated and transferred to NMR tubes.

^{1}H-NMR spectroscopy

^{1}H-NMR spectra were acquired with a Varian INOVA-500 spectrometer (B0=11.7 T), operating at 499.886MHz for ^{1}H using a 5mm triple resonance (H, C, N) inverse probe with a z-gradient on the campus of National Synchrotron Light Laboratory (LNLS) -Campinas-SP-Brazil. Each experiment was acquired with 256 transient at 25°C. The intense residual water signal was suppressed using pre-saturation pulse sequence. ^{1}H NMR chemical shifts were expressed in ppm and referenced relative to sodium d_4-trimethylsylilpropionate calibrated to 0 ppm using the Origin 6.0 software to acquire the data. All results were assigned using MDL Magnetic Resonance Metabolomics Database (Lundberg *et al*., 2005).

RESULTS

Evaluation of levan productivity

The carbon source and its concentration are factors that have the most influence on levan production. Thus, sucrose has shown a great performance on levan formation (El-Refai *et al*., 2009; Shih *et al*., 2005). Based on preliminary tests, sucrose had a positive and significant effect (p=0.035), when used as carbon source at 300, as well as shaking speed at 100 rpm. Therefore both variables were set on those respective values. Taking into account, to define the best levan productivity by *B. subtilis* Natto, a Box and Behnken (1960) experimental design with 15 experiments and 3 replicates at central points was developed, at first, evaluating the influence of (X_1) pH, (X_2) temperature and (X_3) culture time (table 1).

The results obtained by analysis of variance indicated that all variables had significant (p=0.05) and positive effect in their interactions and linear terms, while they had negative effect in squared terms. The pH (X_1) and temperature (X_2) were the most significant variables (p=0.01), as well as their interaction (p=0.02). Shih *et al.* (2005) and Shih *et al*. (2010). demonstrated that initial pH directly interferes on levan production. Abdel-Fattah *et al.* (2005) studied temperature effect on levan production and observed that it also had a significant contribution in its formation.

According to the results an intercept was significant (P= 0.007) indicating that the centrals points (pH 6, 31°C e 18 hours) were correctly chosen. After multiple regression analysis, a second order polynomial equation is given by:

$$Y_1=0.78+1.07x_1+1.01x_2+0.41x_3+1.04\ x_1x_2+0.86x_1x_3+0.45x_2x_3+0.35x_1^2+0.40x_2^2+0.52x_3^2$$

where Y_1 is levan productivity, X_1, X_2 and X_3 are variables pH, temperature and culture time. In this first evaluation, the highest levan productivity was $4.8\ g.L^{-1}.h^{-1}$ (table 1) under pH 7, 37°C for 18 hours. When the pH was decreased to 5, setting temperature and culture time in the best values, a 10-fold reduction on levan productivity ($0.48g.L^{-1}.h^{-1}$) was observed, which indicates the importance of this variable to the process. Likewise, when the temperature was reduced from 37°C to 25°C it promoted an almost 10-fold reduction on levan productivity ($0.51g.L^{-1}.h^{-1}$) either, confirming the significant and positive effect of this variable. This large variation reflects the importance of an optimization of culture conditions, aiming for an optimum desired response (Liu *et al*., 2010).

The high R-squared value (p=0.97) with a not significant lack of fit (p=0.087), confers to the model its use for predicted purposes, once the experimental and predicted values of levan productivities were in agreement.

Levan productivity tends to increase, since the temperature (X_2) and culture time (X_3) increased at pH 7. Considering these results with the best levan productivity achieved ($4.80g.L^{-1}.h^{-1}$) a second Box and Behnken (1960) design with 15 experiments and 3 replicates at central points was proposed, aiming an increase of this value. Therefore, to achieve an optimization, the variables had their values increased and their levels at the best condition found was fixed as the lower levels for (X_1) pH and (X_2) temperature, while the central point was fixed for (X_3) culture time.

The results obtained, for the second design, were applied to ANOVA (P=0.05) and Student t-test, and demonstrated that only temperature, culture time and their interaction were significant, however pH (X_1) was considered for further analysis once it had a closed significant value (p=0.06). The culture time (X_3) was the variable that most interferes in the process (p=0.015) and had a negative effect. Therefore, while pH and temperature were fixed and the culture time was ranged from 18 to 30 hours, comparing runs (6 and 8) and (10 and 12), a drop corresponding to 35% on levan productivity occurred. Shih *et al.* (2005) had the highest levan productivity at 21 hours, decreasing after that. Abdel-Fattah *et al.* (2005) produced the greatest amount of levan at 18 hours, but at lower times the production still had not reached the maximum, while after 18 hours the values began to drop.

Regarding the temperature (X_2), there was an increase of 54% on levan productivity, when this variable was ranged from 32 to 42°C, as can be seen in runs (1 and 3) and (2 and 4), justifying its significant and positive effect. Shih *et al.* (2005) using *B. subtilis* (natto) Takahashi, found an optimum range for levan production that extends from 25

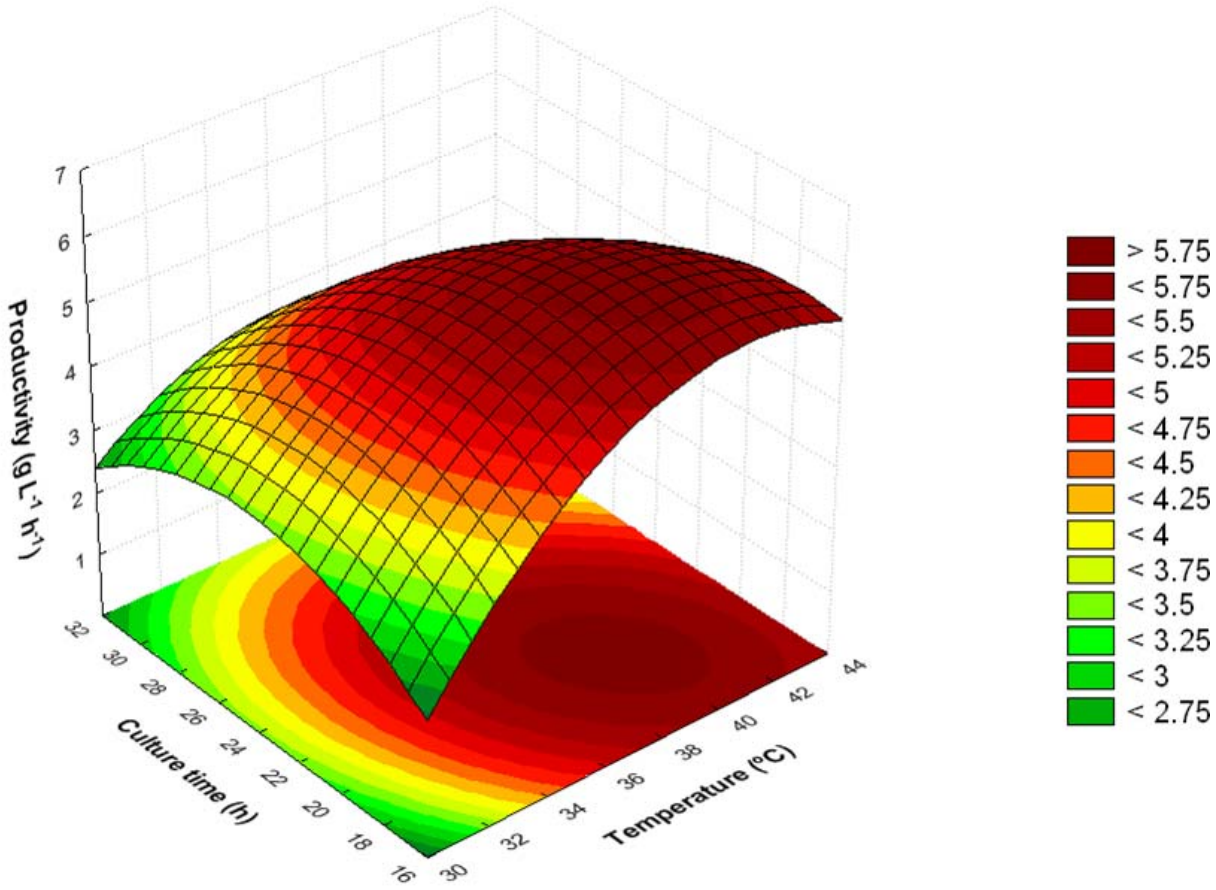

Fig. 1: Surface response of levan productivity for temperature (X_2) and culture time (X_3) at pH (X_1) 8 after optimization of the process.

to 40°C. Ammar *et al.* (2002) also using the same microorganism defined the optimum temperature for levan formation at 40°C.

An intercept was significant again (p = 0.0004), indicating the central point (pH 6, 21°C and 18 hours) was correctly chosen. After multiple regression analysis, a second order polynomial equation is given by:

$Y_1=5.57 + 0.47x_2-0.58x_3-0.48x_2x_3-1.42x_1^2-0.75x_2^2-0.64x_3^2$

The R – squared value (p=0.81) was acceptable again and the lack of fit of the model was not significant (p=0.05), attributing to the model high confidence.

The model obtained predicted that the maximum levan production would be $5.86 g.L^{-1}.h^{-1}$ at the points (0; 0,5; -0,5), where the variables corresponding to: pH 8, 39.5°C and 21 hours. The validation of this optimum point was tested in triplicate and the Student t-test was applied. The results showed no significant difference (p=0.54) between the predicted optimum value ($5.86 g.L^{-1}.h^{-1}$) and the average of experimental optimum values ($5.82 g.L^{-1}.h^{-1}$).

Therefore the condition found corresponding to $5.82 g.L^{-1}.h^{-1}$ at pH 8, 39.5°C in 21 hours, was the maximum levan productivity value achieved using this model, which means that the process was optimized, as can be seen the surface response curvature at the central point of the variables (fig. 1).

Analysis of levan molecular weight by HPSEC-RID-MALLS

Levan corresponding to better productivity ($5.82 g.L.h^{-1}$), obtained from the culture condition at pH 8, 39.5°C in 21 hours, was applied to HPSEC-RID-MALLS. The results are represented by the chromatograms in fig. 2. Through this analysis, two peaks were obtained corresponding to the molecular weight average, as well as their percentage.

Peak 1 corresponds to a molecular weight of 4146 kDa, equivalent to 5% of the sample. Peak 2 has a higher signal in RID with low light scattering, suggesting a presence of one major component with low molecular weight 72.37 kDa, corresponding to 95% of the sample.

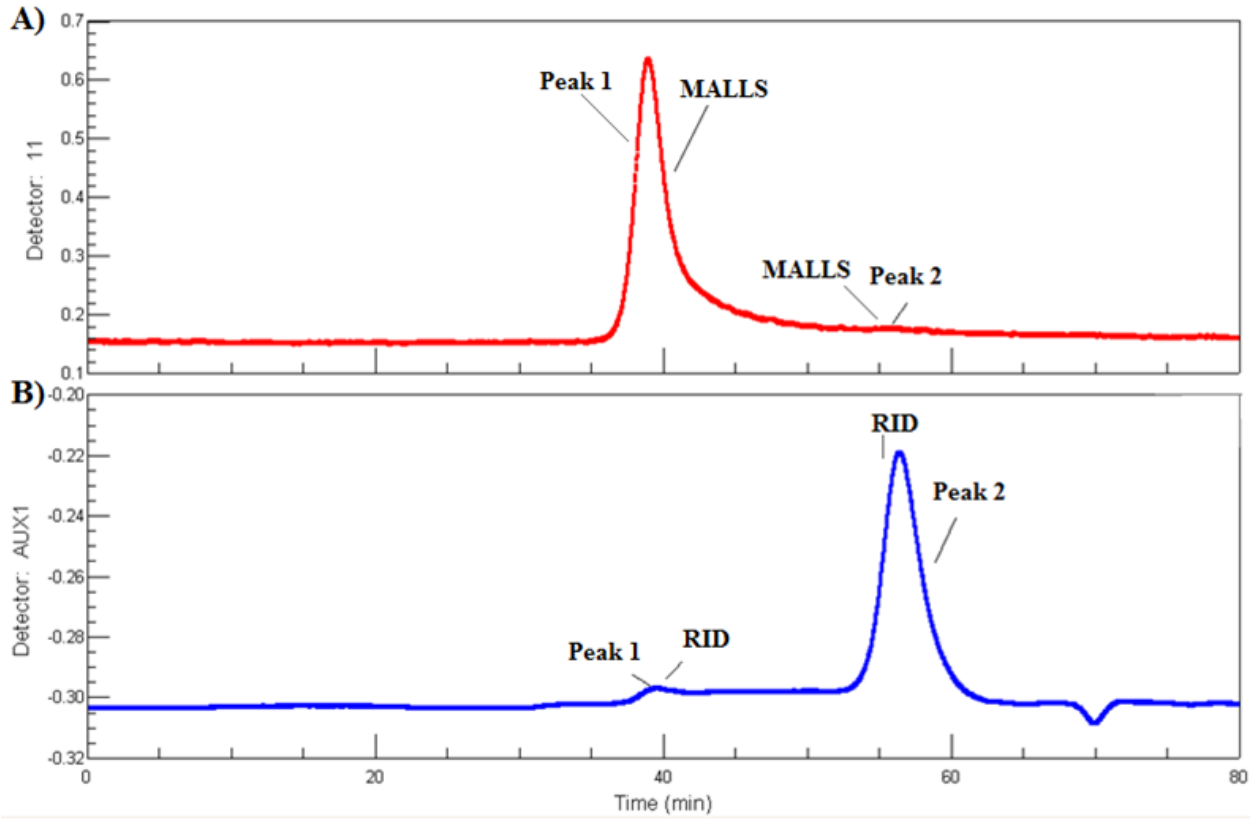

Fig. 2: Chromatograms of levan from *Bacillus subtilis* Natto obtained in the best culture conditions (pH 8; 39.5°C; 21 hours), analyzed by High Performance Size Exclusion Chromatography (HPSEC). A) sample detected by Multi-Angle Laser Light Scattering detector (MALLS); B) sample detected by refractive index detector (RID).

Metabolomics analysis of levan antitumor activity by ^{1}H-RMN

The levan of *B. subtilis* Natto obtained from the best culture condition (72.37 and 1146 kDa) in this work was applied on HepG2 culture cells at 1000µg.mL^{-1} for 48 hours, aiming to study at a metabolic level, changes caused by levan antitumor activity. Therefore, the analysis of HepG2 cell line metabolome was performed by ^{1}H - NMR.

The treatments with and without levan are shown in fig. 3 and demonstrated that four metabolites had its concentration increased when levan was added, they are: acetate (single et at 1.92 ppm), alanine (doublet at 1.46 and quartet at 3.76ppm), lactate (doublet at 1.31 and quartet at 4.09) and phosphocreatine (single et at 3.03 and 3.92ppm).

DISCUSSION

Levan productivity

Levan productivity found in this work (5.82 g.L^{-1}.h^{-1}), using *B. subtilis* Natto, was the highest value described in literature. Borsari *et al*. (2010) e Oliveira et al. (2007) used *Zymomonas mobilis* to produce levan in medium containing sucrose as a carbon source varying from 15 to 25 g.L^{-1}, pH 6 and temperature ranging from 20 to 25 °C, obtained a maximum levan production of 14,67 e 13,37 g.L^{-1}, corresponding a maximum productivity of 2 g.L^{-1}.h^{-1}. Liu *et al.* (2010) utilized *Paenibacillus polymixa* in a medium with 20 g.L^{-1} of sucrose, pH8 and 24 °C, and produced 36,26 g.L^{-1}corresponding a productivity of 0.6 g.L^{-1}.h^{-1}.

The highest levan production values reported so far were achieved using *B. subtilis* strains. Takesue *et al*. (2009) used a variety PHT - D36 and obtained a production of 79,6 g.L^{-1}, correspondig a productivity of 2.84 g.L^{-1}.h^{-1} in a medium containing 25 g.L^{-1} of sucrose, pH 8 and 37 °C. Shih *et al*. (2005) e Shih *et al*. (2010) utilized the variety (natto) Takahashi, the same used in this work, and produced 50 e 70,6 g.L^{-1} respectively, corresponding a productivity of 2.5 g.L^{-1}.h^{-1} using 20 g.L^{-1} of sucrose, pH 6, ranging the temperature from 25 to 40 °C. These results demonstrated a high potential for levan production by the strain used in this work as well as the efficiency of the optimization process.

Bergeron *et al.* (2002) reported that in media with high sucrose concentrations, the microorganism begins to produce levan in larger quantities to protect it from the stressful condition imposed, therefore one explanation for

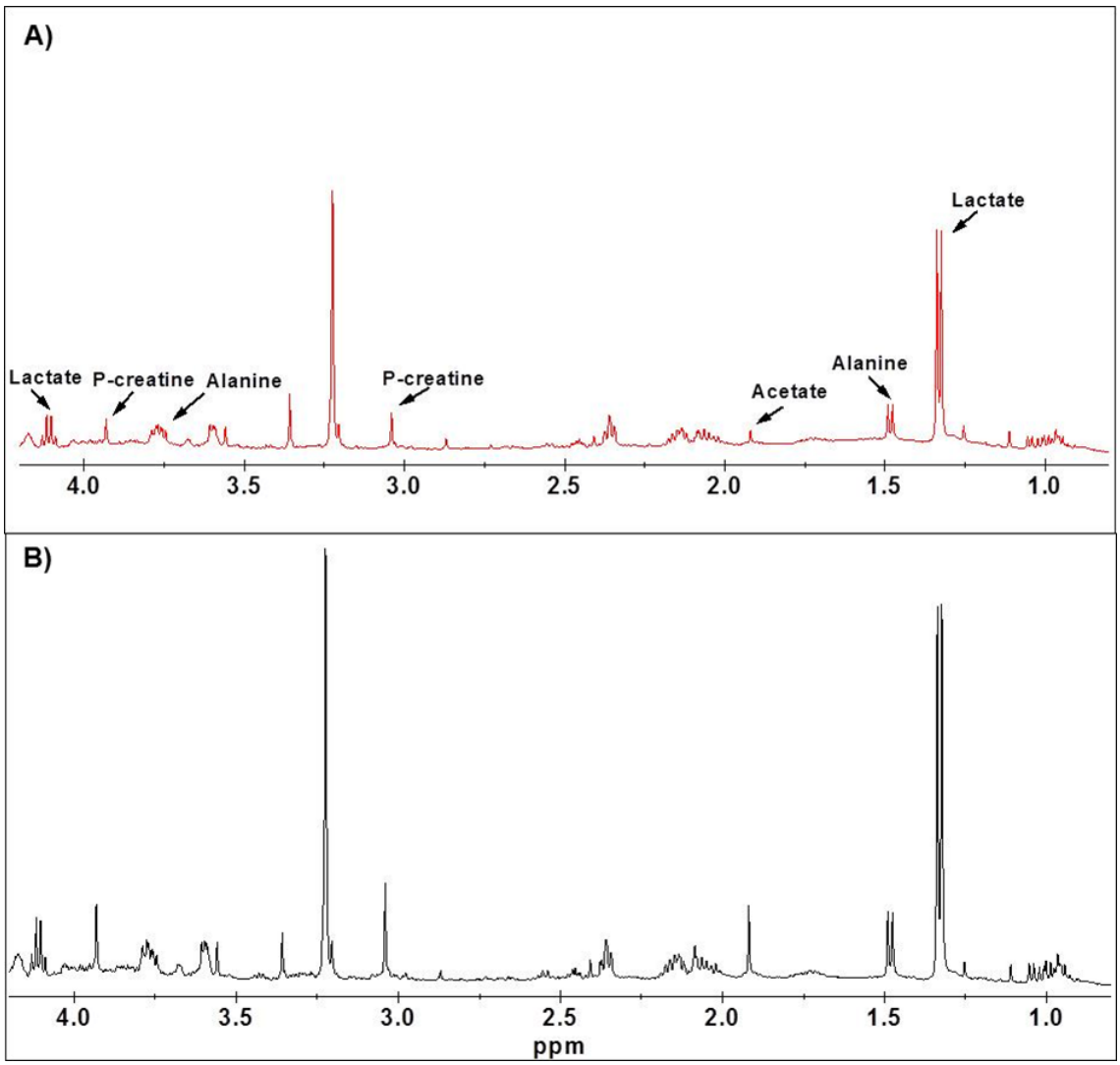

Fig. 3: Metabolomic data analysis using Varian INOVA-500 spectrometer (B0=11.7 T), operating at 499.886 Hz for ^{1}H at pH 7 and 25°C. Spectrum relating to chemical shifts between 1 and 4.2 ppm, in the region which were found variations in metabolites concentrations produced by HepG2 cells in the absence (A) and presence (B) of levan.

this high levan productivity is the strain potential allied to high sucrose concentration used (30%), when concentrations below 30% are used, it favors hydrolysis activity, whereas up to 30%, it favors transferase activity (Meng and Fürtterer, 2003). Levan formation is closely related to the rate of sucrose hydrolysis, which depends on pH and temperature (Lyness and Doelle, 1983), both variables optimized in this work. The microorganism also has an important contribution to the process, likewise observed for Shih *et al.* (2005) e Shih *et al.* (2010), once they achieved one of the highest levan productivity values reported so far, using the same microorganism used in this work.

Levan molecular weight

In this study we were able to find two different peaks of levan. Shih *et al.* (2005) and Shih et al. (2010), also produced two types of levan, of high and low molecular weight, likewise Euzenat *et al.* (1997) who conducted the fermentation of sucrose using *B. subtilis* and observed during the process that a high and a low molecular weight were formed, however over the course of fermentation, a lowest molecular weight became predominant over the highest one. One explanation for this major production of low molecular weight levan, can be related to initial pH at 8, which according to Shu and Lung (2004), when the initial pH was increased, a production of levan with low mass was favored. Another factor that may have contributed to this levan production was the medium, which consisted of 30% of sucrose. Shih *et al.* (2005) used a strain of *B. subtilis* Natto, concentration of sucrose corresponding to 20%, obtained large quantities of levan with high molecular weight (1794 kDa) rather than the low molecular weight (11 kDa), however, during the process the lowest equaled with the highest and surpassed it after 21 hours, i.e. culture time is an important factor to

mass distribution either. Utilizing the same micro-organism, Shih *et al.* (2010), found two peaks, a high (2000 kDa) and a low (6.7 to 8.4 kDa), when the sucrose concentration ranged from 0 to 250 $g.L^{-1}$. Moreover, while the sucrose concentration increased, the peak of high molecular weight decreased and the lowest one began to increase.

Regarding the use of *B. subtilis*, Abdel-Fattah *et al.* (2005), used sucrose for levan and fructoligosaccharides production and observed that sucrose concentration together with temperature were decisive factors to distribution of molar mass, since they used sucrose concentration below to 5% and levan with high molecular weight was formed, while concentrations greater than 5% favored levan with low molecular weight. Thus temperatures ranging between 15 and 20 °C and greater than 40 °C, a predominance of low levans also occurred, a fact observed previously by Euzenat *et al.* (1997), which produced levan with low molecular weight at temperatures above 37°C and high sucrose concentration (2.6 M).

Levan antitumor activity

Studies about antitumor activity using microbial levan have shown promising results. Calazans *et al.* (1997) and Calazans *et al.* (2000) using levan from *Zymomonas mobilis*, in a concentration of 200$mg.kg^{-1}$ of body weight, in mice and Swiss mice with induced Sarcoma 180 and Ehrlich carcinoma, promoted a reduction of tumor masses ranging between 22 and 74%. Moreover, authors attributed this variation to different levans with molar masses ranging between 350 and 1070 kDa. However, in all bands reductions in tumor mass were observed, but the molar mass corresponding to 457 kDa was the most efficient.

Researches using human gastric (SNU-1) and hepatocellular carcinoma (HepG2) cell lines have been conducted to study the effect of different levans from *Microbacterium laevaniformans* achieving promising results. Yoo *et al*. (2004) e Yoon *et al.* (2004) applied in these cancerous culture levans with different molecular weights (40-710 kDa) and degrees of branching (4.3 - 12.3%), in a concentration of 1000 $\mu g.mL^{-1}$ and observed, regardless of the type of levan, inhibition and reduction in the number of cancer cells, which ranged between 39 and 68%. Oh *et al.* (2004) also using different levans (10 - 1000 kDa), at a concentration of 400 $\mu g.mL^{-1}$, in the same tumor cell lines, this levan promoted reductions on cell growth ranging of 18 and 84%, noting that the molecular weight of 80 kDa was the most efficient in reducing this antitumor activity.

The results demonstrated in fig. 3 suggest that levan antitumor activity may be associated with an increased amount of intracellular lactate, compared to the control tests, since its accumulation can reduce the intracellular pH inactivating enzymes and proteins essentials for cell function (Maxwell *et al*., 1988).

Lactate is usually produced in hypoxic conditions, where the tricarboxylic acid cycle (TCA) is inhibited by lack of oxygen, resulting in increased anaerobic glycolysis (Vaupel, 2010). Levan, as a polymer could not inhibited gas exchange directly through plasmatic membrane, although as a polysaccharide it could provide cellular signals, mainly because it was observed that phosphocreatine levels increased instead of reduce in this particularly anoxic condition, once this metabolite is considered a primary energy reserve in various tissues such as heart (ten Hove e Neubauer, 2006) .

Scharte *et al.* (2006) observed hepatic cells in rats and demonstrated that lipopolysaccharide (LPS) antigen increases the expression of Hypoxic Inducible Factor (HIF-1α) gene. A portion of polysaccharide chain of LPS is recognized by innate immune system by toll like receptors that starts a cytosolic signal transduction, promoting as well the activation of HIF-1. The HIF-1 influences the intracellular levels of H+ suppressing TCA and oxidative phosphorylation by inducing pyruvate dehydrogenase kinase (PDK), which inhibited pyruvate dehydrogenase blocking the conversion of pyruvate into acetyl-CoA, resulting an increased in lactate, alanine and acetate levels observed in this work. The HIF-1 promoted an increase of protein expression responsible for glucose uptake (e.g. glucose transporter 1 [GLUT1]) and accelerates glycolysis pathway (Simon, 2006).

Huang *et al*. (2004) demonstrated that HIF-1α gene deletion in cardiomyocytes leads to reduced ATP, phosphocreatine, and lactate levels collaborating to the idea that HIF-1 stimuli must be involved in biological responses promoted by levan treatment.

The results of metabolomic analysis revealed a possible antitumor activity of levan, however, it is important to note that lactate levels increased is also related to metastasis genes expressions (Briezel *et al*., 2001), and the HIF-1 activation triggers cellular adaptive responses, for this reason the levan use as antitumor agent should be regarded cautiously. Therefore, more studies are needed to identify the properly levan antitumor activity mechanisms.

CONCLUSIONS

Productivity of levan of *B. subtilis* Natto was optimized reaching 5.82 $g.L^{-1}.h^{-1}$, one of the greatest values reported so far, indicating high production potential of the strain. The optimized levan showed two molecular weights equal to 4186 kDa (5%) and 72.37 kDa (95%). The metabolomic analysis revealed alterations in the

metabolic profile of cells treated with levan, which promoted deregulation of energy pathways and cellular homeostasis, justifying levan antitumor activity by excessive intracellular accumulation of lactate.

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