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Optimization production of thermo active levansucrase from *Bacillus subtilis* Natto CCT 7712

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ABSTRACT

Levansucrase is a microbial enzyme, classified as fructosyltransferase and responsible for levan polymerization. Levan is a polyfructan widely used in pharmaceutical, industrial and food field. Enzymatic synthesis of polymers on industrial scale requires the use of stable enzymes during process variations, such as temperature, which can promote enzyme denaturation. In addition, high yield of enzyme production by microorganism is required. Although several studies report empirical levansucrase production, there are few information about statistical optimization levansucrase production. Furthermore, most levansucrase reported are high temperature intolerant. This is first study on levansucrase production by *Bacillus subtilis* Natto CCT 7712 using response surface methodology. Substrate concentration, pH and agitation were variables evaluated using Box-Behnken 2^3 design and levansucrase obtained was partially characterized. Levan production and levansucrase activity were quantified after fermentation. The best levan production was 205 g/L and the higher levansucrase activity was 8.53 UA/mL. Levansucrase demonstrated to be thermo-stable and thermo-active at 30°C and optimum temperature was 50°C. *B. subtilis* natto CCT 7712demonstrated high potential to produce levan by levansucrase for industrial application.

1.INTRODUCTION

Levansucrase (E.C.2.4.1.10) is an enzyme produced by gram-positive bacteria such as *Bacillus subtilis* [1], *Lactobacillus reuteri*[2], gram-negative bacteria, such as *Zymomonas mobilis*[3], *Halomonas* sp. [4], *Erwiniaamyl ovora* [5], *Pseudomonas syringae*e *P. chlororaphis* [6] and fungi, such as *Streptococcus mutans* [7] and *Actinomyces viscosus* [8]. *B. subtilis* is considered as a model to study gram-positive bacteria due to their genetic and physiological characteristics and for not pathogenic potential, non-colonizing tissues and good ability to form spores. Furthermore, it is naturally found in soil and for these reasons it has been the microorganism of choice for obtaining levansucrase [9]. In biotechnological and industrial fields, *B. subtilis* has been very attractive due to multiplication potential and ability to secrete extracellular proteins [10]. *B. subtilis* Natto variety is commonly used in natto preparation for over 1000 years, a typical Japanese fermented food and based on soy.The levansucrase synthesizes levan polymer using sucrose as substrate. Levan is formed by fructose residues polymerized, linked by β-(2→6) bonds in linear chain and β-(2→1) bonds in ramifications. Applications in therapeutic area, due to antiviral [11], antidiabetic [12] and antitumor [13] activity has been tested. Antitumor activity is related to molecular weight of levan chain [14] and the size chain can be adjusted by medium ionic strength [15]. In food industry, levan is used as a source of fructose in fructo-oligossacharide (FOS) production [16], stabilizing and encapsulating agent, colors and flavors fixer [17, 18]. Levan can be used as energy reserves and protection mechanisms to the microorganism producer and when cultivations conditions are limiting levansucrase is activated and hydrolyze the polymer, so their products act as carbon source to replace scarce intracellular sources [19].

Due to innumerous levan applications, empirical studies relating to levansucrase production have been developed. These studies take a long time and require a large number of experiments to evaluate different fermentation condition, once several factors such as pH, temperature, medium aeration, substrate concentration and medium supplementation, are able to influence the microbial metabolism, affecting enzyme synthesis. According Belghith [20], levansucrase is significantly influenced by carbon sources and an exploratory study determined sucrose was the best carbon source tested. Berté [21] reported influence of sucrose concentration, pH, fermentation time and agitationin levansucrase production by *Bacillus subtilis*. Response surface methodology, a statistical tool, has been an alternative to the

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empirical study for enzyme [22, 23] and polysaccharides production optimization, such as levan [24,25] evaluating different culture conditions using an limited experiment numbers [26].

In the 80s, Lyness and Doelle [27-29] evaluated the enzyme production by *Z. mobilis* and the results demonstrated that sucrose hydrolysis rate is dependent of temperature, pH and sucrose concentration. Cote [30] investigated the production of levansucrase by *Erwinia herbicola* NRRL B-1678 and reported a reduction in microbial growth and production of enzyme in fructose and glucose medium content. Levansucrase activity was maximal after stationary phase and sucrose was not required for extracellular enzyme secretion. Do not [31] reported inhibition of levansucrase by glucose at temperatures above 45°C. Abdel-Fattah Mahmoud and Esawy [32] tested the effect of different nitrogen sources and baker's yeast with 2% concentration gave the highest levansucrase activity and addition of 0.15 g/L $MgSO_4$ was the most favorable for levansucrase production. In addition, levan production was directly proportional to enzyme concentration.

This study was conducted in three steps. In the first step production of levansucrase from*B. subtilis* NattoCCT 7712was optimized using the Box and Behken 2^3designvarying fermentation conditions of sucrose concentration (200-300 g/L), pH (5.5 to 9.5) and agitation (160-240 rpm), and production of levan was determined at the end of the assays. Optimized condition was used for the remaining steps. In the second step the influence of salts KCl, NaCl and Na_2SO_4 in production of levansucrase was studied. The third step was performed partial characterization of levansucrase crude extract with optimum pH and temperature, thermal stability, effect of salts on their activity and K_m determination.

MATERIAL AND METHODS

Microorganism, culture medium and inoculum

B. subtilis Natto CCT7712 was isolated in the Department of Biochemistry and Biotechnology, State University of Londrina and identified by André ToselloInstitution Foundation. Microorganismwas incubated at 37°C in culture medium containing (g/L): peptone, 50; meat extract, 30; agar, 20 for 48 h and stored at 4°C. Inoculum was prepared by batch fermentation in culture medium containing (g/L) sucrose 100; yeast extract 2; KH_2PO_4 2, $(NH_4)_2SO_4$, 1; $MgSO_4$ ($7H_2O$), 0.5 [14] in150 mL Erlenmeyer flasks with 25 mLof inoculum medium. Culture medium was autoclaved at 1 atm for 15 min, inoculated with *B. subtilis* NattoCCT 7712and incubated at 37°C, 150 rpm for 48 h. After this time, cultures were harvest by centrifugation at 9050 xg at 4°C for 15 min. Cells were resuspended in saline solution (NaCl 0.9%) and concentration was standardized to 0.2 g/L for all fermentations. Cell concentration was determined by turbidimetry at 400 nm.

Levansucrase Production

Box Behnken 2^3statistical model was applied to optimize production of levansucrase, varying fermentation conditions of sucrose concentration (200-300 g/L), pH (5.5 to 9.5) and agitation (160-240 rpm) (Table 1).Fermentations were carried out in batch culture medium containing (g/L): yeast extract 2, KH_2PO_4, 1; $(NH_4)_2SO_4$, 3; $MgSO_4$ ($7H_2O$), 0.6, $MnSO_4$, 0.2; citrate ammonium, 0.25 [33] in 150 ml Erlenmeyer flasks containing 25 mLculture medium and autoclaved at 1 atm for 15 min. Cultures were harvest by centrifugation at 9050xg for 15 min at 4°C, after 24 h. Supernatantwas defined as cell-free extract (CFE) and was used to determine levan and levansucrase.

Responses variables studied: Y_1 (levansucrase activity) and Y_2 (levan production) were analyzed using STATISTICA 7.0 software [34] with 5% confidence interval.

Effect of salts

Fermentations were carried out to evaluate the influence of NaCl, KCl, and Na_2SO_4 (0.2 to 0.8 M)using pre-defined optimal conditionsof levansucraseproduction.

LevanDetermination

Levanwas precipitated from CFE with 3 volof absolute ethanol at 4°C for 12 h, centrifuged at 18.060xg at 4°C for 20 min and hydrolyzed with 0.1 M HCl at 100°C for 1 h [35], neutralized with NaOH 2M and determinated by reducing sugars according to Somogyi and Nelson [36] with fructose as standard.

Assay of Levansucrase

Levansucrase activity was estimating by levan-forming. The reaction mixture: CFE (250 µL), sucrose 0.1 M (250 µL) and acetate buffer (50 mM, pH 5)1 M (500 µL)at 30°C for 2 h [37]. Levan was estimated.One unit of enzyme activity (AU) was defined expressed as the amount of enzyme thatpolymerize 1 µmol of reducing sugar per 1 min, under experimental conditions.

Determination of the optimum temperature and pH

Rotational Central Composite Design (RCCD) 22 with four axial points and two replicates in center point was applied to determine optimum temperature and pH. Citrate buffer and tris-aminomethane were used at pH 3.2 to 8.8 and temperatures from 29oC to 71oC. Levansucrase activity was expressed as AU (µmol/min).

Thermal Stability

Thermal stability of levansucrase was examinatedin two steps. First,the enzyme was incubating at different temperatures (30, 50, 70 and 90oC) and pH 5.0 for 16 h and in second step the enzyme was incubated at 30 and 50°C and pH 5.0 for 10 days. Experiments were performed in triplicate, levansucrase activity were determined and the results expressed as a percentage of residual activity compared to control.

Effect of different salts on levansucrase activity

Effect of salts NaCl, KCl, $ZnCl_2$, $BaCl_2$, $CaCl_2$, $MnCl_2$, Na_2SO_4, $FeSO_4$, $CuSO_4$ (0,2 to 0,8 M) in levansucrase activity was determined using acetate buffer (50 mM), pH 5.0.

Effect of substrate concentration (Km)

Effect of sucrose concentration (0.1 to 2.0 M) in levansucrase activity was determined at 50°C, pH 6.0 and 2 h incubation. The K_m value was determined by Lineweaver-Burk method.

RESULT AND DISCUSSION

Optimization of levansucrase and production of levan

According to Table 1, levansucrase activities rangedfrom 1.20 to 8.53 AU/mL in sucrose medium and the highest activities observed were 8.45, 8.47 and 8.53 AU/mL in experiments 5 (200 g/L, pH 7.5, 160 rpm), 8 (300 g/L , pH 7.5, 240 rpm) and 6 (300 g/L, pH 7.5 and 160 rpm), respectively. Abdel-Fattah, Mahmoud andEsawy[32] reported activities of 14.5 and 14.1 AU / mL for levansucrase from*B. subtilis*NRC33a in sucrose and glucose presence as carbon source. Cote [30] investigated levansucraseproduction from*Erwinia herbicola* NRRL B-1678, and the yield was higher in corn mash and sorbitol culture medium, with 0.7 U/ml activity, a value 92% lower than the observed in this study. The most favorable enzyme production occurred at pH 7.5 and lower production were observed at pH 9.5, in agreement with the study of Lyness andDoelle[29] and Doelleand Greenfield [38], who reported that pH values above 8.0 inhibit the production of levansucrase from*Z. mobilis*.

Table. 1: Box & Behnkendesing for investigation of the factors: sucrose concentration, pH and agitation on the levansucrase activity and levan production by *B. subtilis* NattoCCT7712.

Run	Variables			Response	
	X_1	X_2	X_3	Enzyme Activity (AU/mL)	Levan Production (g/L)
1	-1	-1	0	6.06	133.33
2	+1	-1	0	4.66	188.74
3	-1	+1	0	1.20	20.23
4	+1	+1	0	1.36	26.67
5	-1	0	-1	8.45	101.63
6	+1	0	-1	8.53	195.51
7	-1	0	+1	5.56	93.40
8	+1	0	+1	8.47	194.71
9	0	-1	-1	6.65	180.75
10	0	+1	-1	2.62	28.46
11	0	-1	+1	7.35	205.92
12	0	+1	+1	1.61	78.64
13	0	0	0	7.48	162.12
14	0	0	0	7.31	157.93
15	0	0	0	7.21	144.38

Code	Factors	Levels		
		-1	0	+1
X_1	Sucrose concentration (g/L)	200	250	300
X_2	pH	5.5	7.5	9.5
X_3	Agitation(rpm)	160	200	240

Resultscontrary to our study, Castillo and Lopez-Mungia[39] reported levansucrase from *B. subtilis*was produced in extracellular environment with acidic pH. An increase of only 1% of levansucrase activity was observed with agitation reduction from 240 to 160 rpm, indicating that agitation does not significantly influence levansucrase production; Berté [21] reported that agitation was statistically significant in production of levansucrase from *B. subtillis*ATCC 6633. Experimental results were analyzed using the program STATISTICA 7.0 [35] and from the regression analysis for enzyme activity, it was possible to define the second order polynomial model:

$$Y = 7.33 + 0.22x_1 - 2.24x_2 - 0.41x_3 - 0.41x_1^2 - 3.60x_2^2 + 0.83x_3^2 \quad \text{(Eq 1)}$$

where Y corresponding enzyme activity (AU/ml) and x_1, x_2 and x_3 are the variables sucrose concentration, pH and agitation.

Determination coefficient (R^2) was 0.97, indicating that equation can be used for predictive purposes. In Table 2 is observed F calculated valuesfor linear and quadratic terms in production of levansucrase from*B. subtilis* CCT7712, which is approximately five-fold times greater than F tabulated for F distribution, with their respective degrees of freedom at 5% level, indicating that the model is adjusted and describes variables function. The best conditions for levansucrase production was 300 g/L of sucrose, pH 7.5 and 160 rpm, with predicted activity of 8.53 AU/mL, resulting in an increased of levan production (195.51g/L).

Figure 1a shows response surface generated from the results of Table 1 by the polynomial model.There are two optimal levansucrase production influenced only by agitation, so the lowest agitation is preferred due to energy low cost.

Significant variables (p = 5%) to levan production were sucrose concentration (p=0.016484) and pH (p=0.000608). The model can be used for predictive purposes, since the lack of fit was not significant (p = 0.077).

From the data was possible to generate a response surface relating sucrose and pH shown in Figure 1b. Levan production by levansucrase varied between 20.23 and 205.92 g/L (Table 1) and the highest yields were 180.75, 188.74, 194.71, 195.51 and 205.92 g/L, obtained in experiments 9 (250 g/L, pH 5.5, 160 rpm), 2 (300g/L, pH 5.5, 200 rpm), 8 (300 g/L, pH 7.5, 240 rpm), 6 (300 g/L, pH 7.5, 160 rpm) and 11 (250 g/L sucrose, pH 5.5, 240 rpm), respectively.

Levan highest yields were obtained in higher sucrose concentrations and lower pH values used, showing the positive influence of sucrose and acidic pH.*B. subtilis* NattoCCT7712 is promising for levan production with values higher than those related by other authors.

Shih [40], obtained 50 g/L of levan from *B. subtilis*(natto) Takahashi in culture medium containing 200 g/L sucrose, and Shih, Chen & Wu [41] obtained 86.3 g/L of levan using immobilized cells of *B. subtilis* Nattoin calcium alginate and although the performance was increased after immobilization of microorganism cells, were respectively 75% and 60% lower than those observed in this study. *B. subtilis* has demonstrated improved performance when compared to *Z. mobilis*, which yielded 14.67 g/L of levan [25] and 40.14 g/L [24].

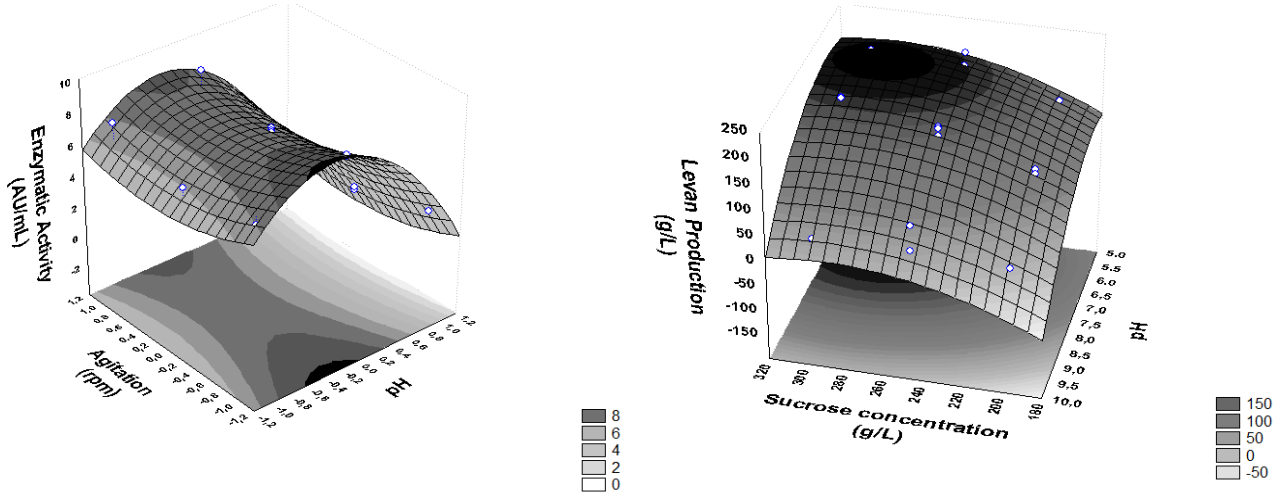
Table. 2: ANOVA to levansucrase production by *B. subtilis* Natto CCT7712.

	Degrees of Freedom	Sum of Squares	Medium Square	Fcal	Ftab 5%
Regression	9	102.9204	11.4356	16.4840	4.77
Linear	3	41.8968	13.9656	20.1301	5.41
Quadratic	3	51.1539	17.0513	24.5788	5.41
Interation	3	9.8697	3.2899	4.7423	5.41
Experimental error	5	3.4687	0.69374		
Total Sum of Squares	14				

Table. 3: Central Composite Rotacional Design 2^3 for investigation of pH and temperature influence on the levansucrase activity.

Run	Variables		Response
	pH	**T (°C)**	**Enzymatic Activity (UA/mL)**
1	-1	-1	2.11
2	-1	+1	1.47
3	+1	-1	1.45
4	+1	+1	1.40
5	-1.41	0	0.60
6	+1.41	0	1.54
7	0	-1.41	4.80
8	0	+1.41	3.13
9	0	0	5.17
10	0	0	5.38
11	0	0	5.61

Code	**Factors**	**Levels**				
		-1.41	**-1**	**0**	**+1**	**+1.41**
X_1	pH	3.2	4.0	6.0	8.0	8.8
X_2	Temperature (°C)	29	35	50	65	71



Fig, 1: Response surface showing effect of independent variables: agitation and pH on levansucrase activity (a) and sucrose concentration and pH on levan production (b).

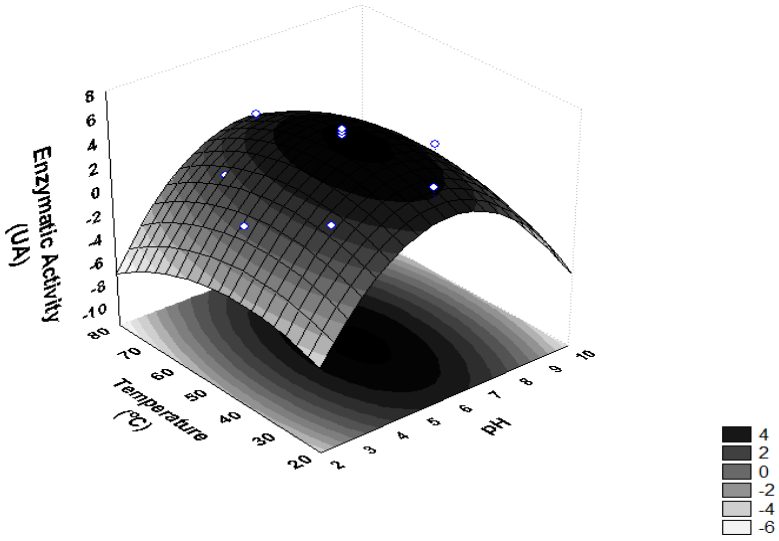


Fig. 2: Response surface showing effect of temperature and pH on levansucrase activity.

Effect of different salts on levansucrase activity

Influence of salts NaCl, KCl and Na_2SO_4 was evaluated in production of levansucraseobtained (data not shown)from the optimal condition pre-defined (300 g/L of sucrose, pH 7.5 and 160 rpm).

All of salts tested induced increase in enzyme activity and production of levan. NaCl induced 26% levansucrase activity (0.8 M) and 60% levan production (0.6 M). These data are similar to those obtained by Vigants[42], who observed the stimulation of levan synthesis from*Z. mobilis*withNaCl during fermentation and in supernatant after the end of fermentation, increasing productivity in 1.2 times-foldat a concentration of 0.4 M, suggesting anenzymicactivating. When used KCl0.8 M, there was an increase of 71%in production of levansucrase and 90% in production of levan. Na_2SO_4 0.4 M was the salt that most influenced the activity reducing significantly of cell growth, inducing 200% of levansucrase activity and 291.82% of production of levan. Important characteristic for the industrial application with low microbial growth facilitating separation of the biomass.

Effects of Temperature and pH on levansucrase activity

Optimal temperature and pH for levansucrase activity was 50°C and6.0, respectively with 5.38 AU/mL, corresponding with the average of the results of the central points (Table 3). The data were treated statistically and generate a response surface (Figure 2). Temperature and pH were significant factors ($p<0.05$), although with a negatively influence in levansucrase activity.

The pH interferes with enzymatic activity, altering the distribution of electrical charges of the enzyme, influencing the conformation of its active site and consequently its interaction with the substrate. Optimum pH of mostlylevansucrases studied is between 5.5 and 6.0 [43-46].

Tian, Inthanagov and Karboune[47] observed increase levansucrase activity from*B. amyloliquefaciens* in pH range 6.0-6.5. Belghith[20] reported optimum pH of 6.5 for levansucrase from *Bacillus* sp. Goldman et al. [48] studied levansucrase from *Z. mobilis* and noted that the enzyme exists in two active forms, dependent on pH and ionic strength. At pH higher than 7.0 the protein present in dimeric form and less than 6.0, in well-ordered microfibrils. These forms to interconvert pH change, and the particular arrangement of the same affects the production of polysaccharides and their kinetic properties. So at pH higher than 7.0 the enzyme hydrolyzes sucrose and synthesizes FOS and at pH below 6.0 catalyzes the synthesis of levan.

Optimum temperature (50°C) obtained in this study was similar to temperature reported in literature to thermostable levansucrase from *Bacillus* sp. TH4-2 [44], levansucrase from *Bacillus* natto stabilized with glucomannan [49], levansucrase from *B. amyloliquefaciens* [47] and levansacarase from *Bacillus* sp [20]. However, the optimum temperature for enzyme activity is dependent of micro-organism producer and fermentation conditions. Purified levansucrase from *Z. mobilis* showed optimum temperature in action range of 5-15°C [42]. For levansucrase from *Microbacterium laevaniformans* [45], *B. subtilis* NRC 33rd [32] and *Acetobacter nitrogenifigens*[46], optimum temperature was 30°C.

Thermal Stability

Levansucrase showed an increase of 50% in activity at 30°C and 50°C after 1 h of incubation. After 16 h of incubation, approximately 80% of the activity was maintained at these temperatures. Levansucrase activity decreased by 72% at 70°C and was completely inactivated at 90°C after 16 h of incubation (Figure 3a). Belghith[20] observed 100% increase of levansucrase from *Bacillus* sp original activity at 50°C for more than 1 hincubation at pH 6.5.

After this result, enzyme stability at 30 °C and 50 °C were tested for 10 days of incubation (Figure 3b). At 30 °C was observed an average increase of 50% in the first 4 days incubation. In a previous study (Figure 3a), this increase was observed only in first hour. At 50 °C and 1 day of incubation was maintained activity compared to control. In 2 days of incubation there was 40% activity reduction. After 10 days,at 30 °C and 50 °C, 30% of levansucrase activity was maintained. Thermal stability of this enzyme was greater than levansucrase produced by *Bacillus* sp. TH4-2 with stability at 50 °C for 1 h of incubation and 50% activity reduction after incubation at 60 °C for 30 min [44].

Effect of different salts on levansucrase activity

Effect of salts on activity of levansucrase from*B.subtilis* was assessed in presence of 0.2 to 0.8 M of each ion (data not shown). Monovalent cations Na^+, K^+ and Zn^+ increased enzyme activity at 0.4 to 0.8 M concentrations, and Na^+ bounded to the anion (SO_4^{-2}) showed the largest increase (57%).

Divalent cations Ba^{+2}, Ca^{+2}, Mn^{+2}, Fe^{+2} and Cu^{+2} (0.2 to 0.8 M) partially inhibited enzyme activity, except Zn^{+2}, which inhibited the activity at 0.2 M, and activated gradually in other concentrations. Levansucrase from *Acetobacterdiazotrophicus*[50] was weakly inhibited by Fe^{+2}.

However, levansucrase from *Bacillus* sp. TH4 showed significantly different results, once was inactivated by Zn^+ (5 mM $ZnCl_2$) and activated by Fe^{+2} (5 mM $FeSO_4$) four-fold times [44], as well as levansucrase from *Bacillus*spwhich activity was four-fold times activated by Fe^{+2} (50 mM $FeSO_4$) and with a decrease of activity in presence of $Zn^{+2}$and Cu^{+2}[20].

The results of this study have revealed that the activation or inhibition of the enzyme activity is not related only with cation but also with anion of the salt, and a possible synergism between them. In addition, literature shows conflicting results regarding the effects of different salts on enzyme activity, as well as differences in concentrations used for the determinations that can vary from 0.05 M to 0.8 M.

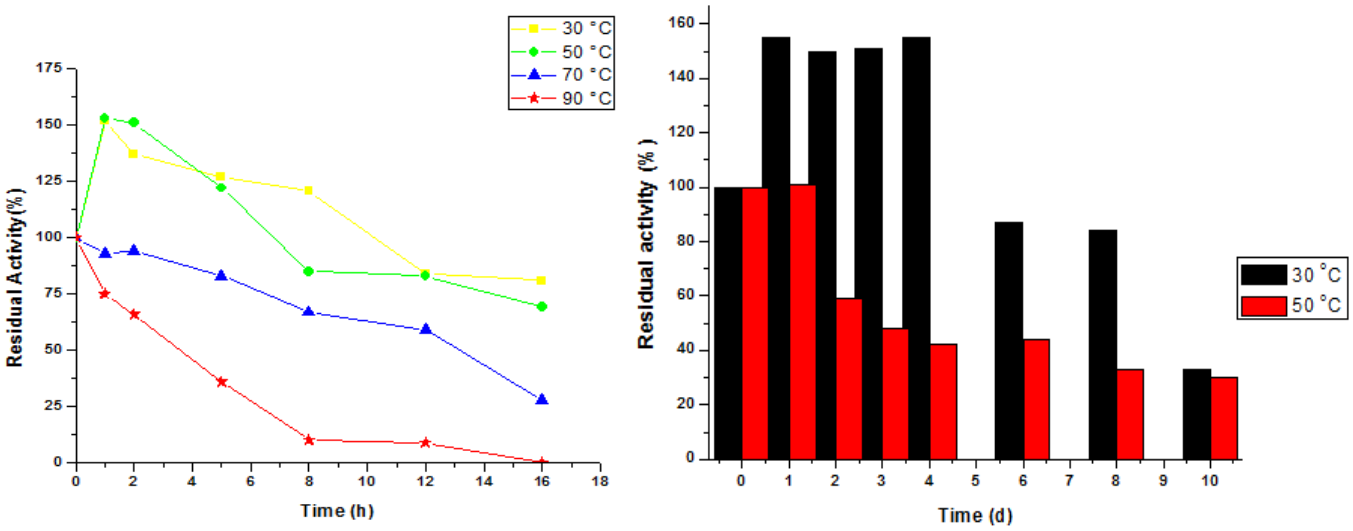


Fig. 3: Thermal stability of levansucrase in optimal conditions of pH and temperatures of 30, 50, 70 and 90°C incubation for 16 h (a) and 30 and 50°C incubation for 10 days (b).

Effect of substrate concentration and determination of the enzyme kinetics

Results observed in Figure 4 indicate that kinetics of levansucrase from *B. subtilis* NattoCCT7712 follows the Michaelis-Menten model. The Michaelis constant (K_m) for fructose polymerizing activity was determined in the optimal condition of pH 6.0 and 50°C and was found 313 mM sucrose. This result was higher than that reported in literature, since its determination was related to the activity of fructose polymerization, in agreement with the research of Tian, and InthanavongKaboune [47] who obtained K_m values 7.3 mM for hydrolytic activity and 1556.4mM for transfructosilationactivity, for extracellular levansucrase from *B. amyloliquefaciens*, indicating lower affinity of the enzyme for sucrose during the polymerization activity. $K_m$values, whereas the activity of sucrose hydrolysis were 4.0 mM and 160mM for *B. megaterium* and *B.subtilis* [2,51,52], 11.8mM for *Gluconobacter diazotrophicus* [50], 122 mM for *Z. mobilis* [43] and 160.0 mM for *Pseudomonas phaseolicola* [53].

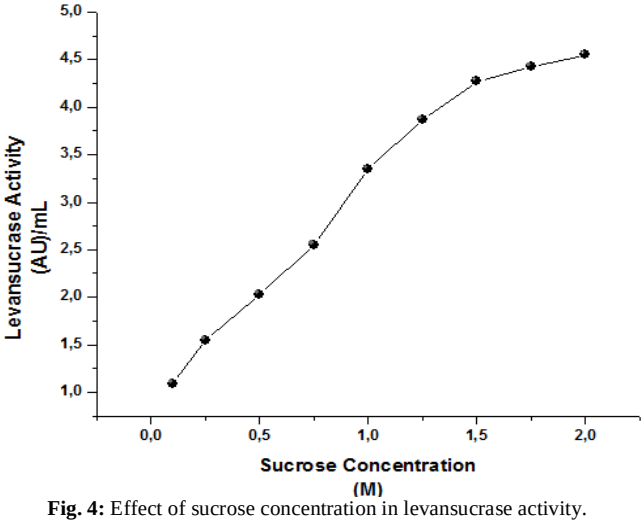


Fig. 4: Effect of sucrose concentration in levansucrase activity.

CONCLUSION

B. subtilis Natto CCT7712 demonstrated to be an efficient levan producer. This micoorganism showed superior performance when compared to others authors strains tested, reaching yields up to 75%. The best conditions observed for levansucrase production was 300 g/L of sucrose, pH 7.5 and 160 rpm, with predicted activity of 8.53 AU/mL and levan production of 195.51g/L. NaCl, KCl and Na_2SO_4 influence in production of the enzyme and promoted increase of levansucraseactivity andlevan production and decrease of cell growth. Na_2SO_4inhibited by 80% microorganismgrowthand increased 100% enzyme activity and 200% levan production. KCl and Na_2SO_4 were also tested and demonstrated inducers. In addition levansucrase from *B. subtilis* Natto may be considered thermostable.

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