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Effect of initial pH in levan production by *Zymomonas mobilis* immobilized in sodium alginate

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ABSTRACT. *Zymomonas mobilis* was immobilized using a cell suspension fixed to 8.6 x 10^7 CFU mL^{-1} by spectrophotometry. This biomass was suspended in sodium alginate solution (3%) that was dropped with a hypodermic syringe into 0.2 M calcium chloride solution. Was test two initial pH of fermentation medium (4 and 5) and different sucrose concentrations 15, 20, 25, 30 and 35% at 30°C, without stirring for 24, 48, 72 and 96 hours. The levan production to pH 4 was high in sucrose 25% for 24 (16.51 g L^{-1}) and 48 (15.31 g L^{-1}) hours. The best values obtained to pH 5 was in sucrose 35% during 48 (22.39 g L^{-1}) and 96 (23.5 g L^{-1}) hours, respectively. The maximum levan yield was 40.8% and 22.47% in sucrose 15% to pH 4 and 5, respectively. Substrate consumption to pH 4 was bigger in sucrose 15 (56.4%) and 20% (59.4%) and to pH 5 was in 25 (68.85%) and 35% (64.64%). In relation to immobilization efficiency, *Zymomonas mobilis* showed high adhesion and colonization in support, indicated by cell growth increased from 10^7 to 10^9 CFU mL^{-1} during fermentation time.

Keywords: entrapment cell, batch fermentation, biopolymer.

Efeito do pH inicial na produção de levana por *Zymomonas mobilis* imobilizada em alginato de sódio

RESUMO. Para a imobilização uma suspenção celular de *Zymomonas mobilis* padronizada a 8,6 x 10^7 UFC mL^{-1} foi adicionada em alginato 3%, sendo gotejada em $CaCl_2$ 0,2 M. Foram testados dois valores de pH inicial, sendo um ajustado para 4 e outro para 5; diferentes concentrações de sacarose: 15, 20, 25, 30 e 35% a 30°C, sem agitação, durante 96 horas. A maior produção de levana, nos experimentos com pH 4, foi observada em 25% de sacarose com 24 (16,51 g L^{-1}) e 48 (15,31 g L^{-1}) horas de fermentação. Nos experimentos realizados com pH 5, os melhores valores foram obtidos com 35% de sacarose em 48 (22,39 g L^{-1}) e 96 (23,5 g L^{-1}) horas. Os maiores percentuais de rendimento de levana foram de 40,8 e 22,47%, utilizando 15% de sacarose para pH 4 e 5, respectivamente. Os experimentos com pH 4, apresentaram maior consumo do substrato com 15 (56,4%) e 20% (59,4%) de sacarose e para o pH 5 os melhores valores foram observados em 25 (68,85%) 35% (64,64%). O microrganismo apresentou eficiente adesão e colonização das esferas, o que pode ser confirmado pelo aumento da população de 10^7 para 10^9 UFC mL^{-1} durante a fermentação.

Palavras-chave: aprisionamento celular, fermentação por batelada, biopolímero.

Introduction

Levan biopolymer is composed by fructose units linked in β (2-6), linear or branched form. It is synthesized by various microorganisms, particularly *Zymomonas mobilis*, in culture medium with sucrose, yeast extract and mineral salts (ERNANDES; GARCIA-CRUZ, 2010; SWINGS; DE LEY, 1977).

In food industry this polysaccharide has applications as stabilizer, thickener, flavor and color fixative, fat substitute, sweetening power, when hydrolyzed to fructose. Is efficient prebiotic agent for *Bifidus* spp. growth in functional foods (OLIVEIRA et al., 2007; TANO; BUZATO, 2002).

In pharmaceutical area have important applications such as hypocholesterolemic and anticarcinogenic agent and blood plasma substitute. Industrial scale for levan production is an alternative to replace xanthan and dextran gums, used widely in food that have high production cost (ERNANDES; GARCIA-CRUZ, 2005).

The *Zymomonas mobilis* is facultative anaerobic with optimum development temperature between 25 and 30ºC and pH values between 5 and 7. Is presented in form of Gram-negative large and wide rods, arranged in pairs or isolated, with mono or

lophotrichous flagella (MADIGAN et al., 2010; SWINGS; DE LEY, 1977).

According Behera et al. (2010) and Fu et al. (2009) this bacterium is considered promising for levan production in large scale. However, its effectiveness can be impaired by exposure to adverse environmental conditions during conventional fermentation that uses free cells in suspension. Biopolymers conventional production is performed using free cells in fermentation medium. However, this technology has limitations such as large exposure of microorganism in unfavorable conditions medium and difficulties in cells separation from the fermentation medium (KOURKOUTAS et al., 2004).

In view of these limitations, numerous studies have been conducted to improve this technology. Among the most promising techniques, it stands out the use of immobilized microorganisms.

Microorganism immobilization in alginate offers a number of advantages over use free cells. Among these, highlight the cell protection against environmental unfavorable conditions and higher biomass concentration in support (CARVALHO et al., 2006).

Entrapment in alginate gel has been studied widely, since it promotes the maintenance of cell activity and viability for much more time, and allows biomass reuse in multiple cycles of fermentation (BEHERA et al., 2010).

In front of the necessity to develop technologies that allow increasing levan production and availability levan for commercial level, using raw materials of easy manipulation, this study aimed to evaluate pH effect in levan production by *Zymomonas mobilis* cells CCT4494 immobilized in alginate spheres and monitor cell growth in alginate beads.

Material and methods

Microorganism, maintenance and pre fermentation medium

The microorganism used was *Zymomonas mobilis* CCT 4494 bacterium, obtained from Culture Collection of Tropical Foundation Research and Technology 'André Tosello' – Campinas, São Paulo State, Brazil.

This microorganism was maintained at 4ºC in liquid culture medium '*Zymomonas mobilis* broth' (ZM broth) containing in g L^{-1}: 10.0 yeast extract, 10.0 peptone and 20.0 glucose, being reactivated monthly.

Zymomonas mobilis CCT 4494, from a standard culture, was transferred to ZM broth tubes and incubated for 24 hours at 30ºC. After this period, these tubes were inoculated into pre-fermentation bottles containing the same medium and incubated under the same conditions.

Fermentation medium

For fermentation were used 250 mL^{-1} Erlenmeyer flasks contained 50 mL^{-1} of synthetic medium. This medium was proposed by Rodriguez and Callirieri (1986) with adaptations, consisting of yeast extract 5.0 g L^{-1}; KH_2PO_4 1.0 g L^{-1}; $(NH_4)_2SO_4$ 1.0 g $L^{-1}$and $MgSO_4$. 7 H_2O 1.0 g L^{-1}. In this medium, different sucrose concentrations were added (15, 20, 25, 30 and 35%), which were separately sterilized from salts solution at 121°C 15 $minutes^{-1}$. The process was conducted with initial pH of culture medium adjusted to 4 and 5, respectively, in triplicate, at constant temperature of 30ºC, without stirring, during 24, 48, 72 and 96 hours fermentation time.

Inoculum preparation, immobilization and Levan production

The inoculum was grown for 24 hours at 30°C in ZM broth and a cell suspension was obtained after centrifugation at 3660g for 15 minutes followed by cell mass re-suspension in 0.85% saline solution previously sterilized.

The inoculum was standardized by spectrophotometry to optical density of 0.7 at 570 nm. Thereafter the inoculum was added to 3% sodium alginate solution with stirring for 5 minutes. This solution was dropped with hypodermic syringe into 0.2 M calcium chloride solution, resulting in beads with mean diameter about 2-3 mm. The beads were kept for 24 hours at 4ºC for maturation, and then were washed in sterile distilled water to remove calcium chloride excess and then added in the fermentation medium.

10 grams of beads (2.9 x 10^7 CFU g^{-1}) were added to each Erlenmeyer flask containing 50 mL^{-1} of fermentation medium with initial pH adjusted to 4 and 5, and different sucrose concentrations (15, 20, 25, 30, and 35%). These flasks were incubated at 30°C, without stirring for 24, 48, 72 and 96 hours.

Analytical methods

After each fermentation period the pH was determined by potentiometry and cell growth in the support was determined by optical density at 570 nm, after dissolution in a solution of 0.3M sodium citrate. The levan was precipitated from fermentation broth without cells, with three volumes of cold ethanol, followed by centrifugation at 3660 g for 30 minutes.

The precipitate was further washed with distilled water and re-centrifuged under the same before mentioned conditions. The levan concentration was determined as fructose units by Somogyi (1952) and Nelson (1944) method after hydrolysis with 0.13 Mol L^{-1} HCl solution for 60 minutes at 100ºC, according Borsari et al. (2006) with adaptations.

The sucrose consume was determined by difference between initial sucrose (substrate) and residual sucrose after fermentation time. The sucrose concentration was determined by Dubois et al. (1956) after centrifuging the fermentation medium for cell removal, without precipitation of levan. The levan yield was determined according Behera et al. (2010).

Results and discussion

According to different authors the levan production by *Zymomonas mobilis* is directly related to sucrose concentration present in medium and the amount by cell metabolized. Another important factor is the initial pH of fermentation medium; that for *Zymomonas mobilis* should be between 4.5 and 6.0 (ERNANDES; GARCIA CRUZ, 2010; VIIKARI; GISLER, 1986).

In this study, sucrose concentration and initial pH values affected levan production during evaluated fermentation times. The higher production was observed with initial pH adjusted to 5 in sucrose concentration of 35% with 22.39 and 23.5 g L^{-1} of levan in 48 and 96 hours of fermentation, respectively. In fermentation with initial pH adjusted to 4, there was lower production, the best result was observed for sucrose 25%, with levan production of 16.51 and 15.31 g L^{-1} at 24 and 48 hours of fermentation, respectively, as shown in Table 1 and 2.

In relation to levan yield the highest percentage was observed in 15% sucrose during 24 (40.8%) and 72 (22.47%) hours, respectively (Tables 1 and 2). Considering this information can be said that was better production using sucrose 15% for both initial pH.

Table 1. Results to fermentation kinetics of *Zymomonas mobilis* immobilized in alginate beads with initial pH fermentation medium adjusted to 4.

	Fermentation time (h)							
	24		48		72		96	
Sucrose (%)	*P* (g L^{-1})	*Y* (%)	*P* (g L^{-1})	*Y* (%)	*P* (g L^{-1})	*Y* (%)	*P* (g L^{-1})	*Y* (%)
15	8.98	40.80	2.32	7.40	2.66	3.60	1.88	2.21
20	11.62	18.64	6.40	9.37	5.88	4.94	6.11	16.13
25	16.51	25.60	15.31	13.04	8.8	24.64	9.89	8.62
30	2.0	3.10	0.10	0.06	0.1	0.09	0.1	0.07
35	1.19	1.08	9.63	8.11	9.15	12.7	2.61	2.64

P: Levan production (g L^{-1}); Y: Levan yield (%).

Table 2. Results to fermentation kinetics of *Zymomonas mobilis* immobilized in alginate beads with initial pH fermentation medium adjusted to 5.

	Fermentation time (h)							
	24		48		72		96	
Sucrose (%)	*P* (g L^{-1})	*Y* (%)	*P* (g L^{-1})	*Y* (%)	*P* (g L^{-1})	*Y* (%)	*P* (g L^{-1})	*Y* (%)
15	3.28	4.38	3.04	3.87	6.87	22.47	11.36	12.36
20	11.07	9.84	7.33	6.50	9.46	11.80	5.88	5.20
25	1.69	2.23	3.35	2.82	2.08	1.20	5.60	6.81
30	7.54	4.65	4.58	2.94	5.78	3.39	11.60	7.42
35	14.33	9.71	22.39	9.94	16.56	9.20	23.50	10.38

P: Levan production (g L^{-1}); Y: Levan yield (%).

In relation to the best sucrose concentration by levan production, similarly at observed on this study, Oliveira et al. (2007) used different sucrose concentrations and obtained levan production of 21.68 and 18.24 g L^{-1} in 35 and 25% sucrose during 18 and 24 hours fermentation, respectively. These results show that the increase in levan production was parallel to increase on sucrose concentration as well as reduction on fermentation time.

Borsari et al. (2006) observed similar behavior to that obtained in this study for pH 4, at 30% sucrose concentration, there was 46.21% reduction in levan production. In the other hand, the optimal concentration for production was divergent with 15% of initial sucrose being the best parameter, whereas in the present study the best results were obtained with 25 and 35% at pH 4 and 5, respectively. This can be explained by higher resistance of immobilized cells, in relation free cells, to high sucrose concentrations in medium, since the beads serve as protection against adverse conditions medium. According to Vigants et al. (2001) the exposure of free microorganism to media with high osmotic pressure causes reductions in levan biosynthesis.

The lower levan production at pH 4 was too observed by Ananthalakshmy and Gunasekaran (1999), this authors did not get levan production. This can be explained because at this pH value, the levansucrase shows lower activity for levan formation than for the sucrose hydrolysis (TANO et al., 2000).

In relation to initial pH of fermentation adjusted by 5, Ananthalakshmy and Gunasekaran (1999), similarly to that observed in this work, obtained best levan production (14.5 g L^{-1}) on pH adjusted to 5. The best production at pH 5 can be explained because this is optimum pH for levansucrase synthesis, one of the enzymes responsible for levan formation (YANASE et al., 1992).

The higher production of levan in culture medium with initial pH adjusted to 5 is within the optimum range for levan production detected by Yokoya and Jerez (1996). These authors found that

Zymomonas mobilis grew well at pH between 4.5 and 6.0 in a sucrose medium, obtaining the highest yield in the first 24 hours of fermentation and after 48 hours there was a small decrease of levan due to hydrolysis of the biopolymer during fermentation.

The variations in levan production by *Zymomonas mobilis* obtained at this study can be explained by existence of biochemical mechanisms regulating the synthesis this polysaccharide, since levan is an energy reserve (SHIH et al., 2010). One of this mechanisms is the ability that extracellular levansucrase to degrade the levan when it is in high concentration (YANASE et al., 1992).

Comparing the levan production with the biomass present on the support, there was a direct relationship between the higher production and the greater number of cells per gram of alginate beads for medium with initial pH adjusted at 4 and 5, in sucrose 15 (1.9 x 10^9 CFU g^{-1}) and 25% (1.0 x 10^9 CFU g^{-1}) after 24 and 48 hours and 20 (4.8 x 10^8 CFU g^{-1}) and 35% (3.4x 10^9 CFU g^{-1}) after 96 hours, respectively (Figure 1 and 2).

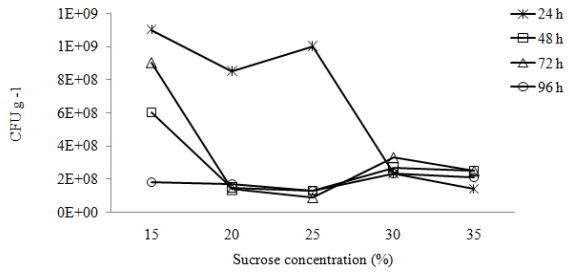

Figure 1. Effect of the fermentation time and sucrose concentration on the grown of *Zymomonas mobilis* immobilized in alginate beads using fermentation medium with initial pH adjusted to 4.

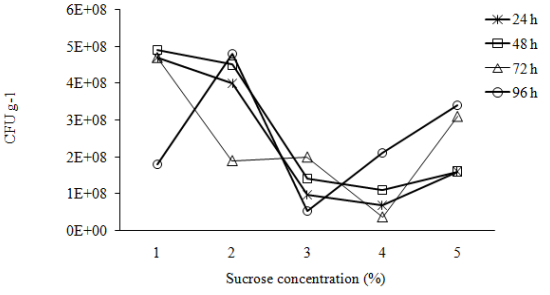

Figure 2. Effect of the fermentation time and sucrose concentration on the grown of *Zymomonas mobilis* immobilized in alginate beads using fermentation medium with initial pH adjusted to 5.

The sucrose utilization by *Zymomonas mobilis* immobilized in alginate beads not has association with cell grow or levan production, as shown in Tables 1, 2 and 3 and Figures 1 and 2. For fermentation medium with pH adjusted to 4 the higher consume was in sucrose 20% (59.4 %) and 15% (56.4%) at 72 and 96 hours, respectively. In fermentation realized with initial pH adjusted by 5 the high rates sucrose consume was in 25% and 35% during 72 (68.85%) and 96 (64.64%) hours.

Table 3. Total sugar and sucrose consumed by *Zymomonas mobilis* immobilized in alginate beads in fermentation medium with pH 4 and 5.

	Fermentation time (h)							
	24		48		72		96	
Sucrose (%)	*TS* ($g\ L^{-1}$)	*SC* (%)	*TS* ($g\ L^{-1}$)	*SC* (%)	*TS* ($g\ L^{-1}$)	*SC* (%)	*TS* ($g\ L^{-1}$)	*SC* (%)
15	128.02	14.65	118.81	20.78	76.44	49.03	65.26	56.49
20	137.67	31.16	131.75	34.12	81.02	59.48	162.1	18.93
25	185.53	25.78	132.65	46.93	214.2	14.28	135.7	45.70
30	236.83	21.05	150.09	49.96	190.3	36.53	173.8	42.03
35	240.08	31.4	231.36	33.89	278.4	20.43	251.2	28.20

TS: Total sugar; *SC*: Sucrose consume.

In relation to biomass on support immobilization there was increase in support cell mass for pH 4 and 5 to all analyzed times in relation to initial inoculum (8.6 x 10^7 CFU g^{-1}). For pH 4, the highest biomass in support was observed in 15, 20 and 25% sucrose concentration at 24 hours of fermentation. For 30 and 35% there was little variation in cell concentration at all times evaluated (Figure 1). In fermentation with initial pH 5, there was slight variation in cell growth between sucrose concentrations and fermentation times. The higher values were obtained in 15 and 20% after 24 and 48 hours and 35% after 72 and 96 hours of fermentation (Figure 2).

According to Kalnenieks (2006) *Zymomonas mobilis* is one of the bacteria with lower growth rate, because uses the Entner-Doudoroff fermentative pathway anaerobically for sugars metabolism, which has a low energetic efficiency, producing only 1 mol ATP per glucose mole consumed. This low rate of growth was not observed in that study possibly due to the evaluated pH values are within the optimum range for growth of this bacterium, as cited by different authors (MADIGAN et al., 2010; SWINGS; DE LEY, 1977).

For final acidification of the fermentation medium all evaluated concentrations after every fermentation times, there was a slight decrease, remaining the medium pH between 3.36 to 3.79 and 3.63 to 3.82 for medium with initial pH 4 and 5 respectively, as shown in Figures 3 and 4. This reduction of the final pH may be due to the ability of *Zymomonas mobilis* to produce typical metabolites production during cell grown and sugars consume. According Swings and De Ley (1977) this bacteria when in medium with sucrose can produce small amounts of lactate, acetaldehyde, glycerol, acetoin,

di-hydroxycetone, mannitol, acetic and gluconic acids (CRITTENDEN; DOELLE 1994).

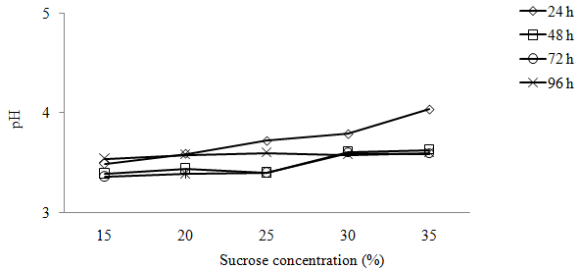

Figure 3. Acidification of fermentation medium with initial pH 4 by growth *Zymomonas mobilis* in different sucrose concentration and fermentation time.

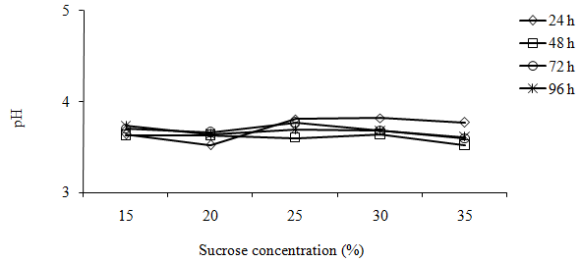

Figure 4. Acidification of fermentation medium with initial pH 5 by *Zymomonas mobilis* in different sucrose concentration and fermentation time.

Conclusion

The levan production using *Zymomonas mobilis* CCT4494 cells immobilized in alginate beads was higher in fermentation with initial pH adjusted to 5 at 35% sucrose concentration after 48 and 96 hours. There was a direct relationship between the higher production, high sucrose consume used and the greater number of cells per gram of alginate beads. The maximum levan yield was observed in sucrose 15% to pH 4 and 5. In relation to immobilization efficiency, *Zymomonas mobilis* showed good adhesion and colonization in support, due high biomass in beads observed during fermentation.

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