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Ethanol and Levan production by sequential bath using *Zymomonas mobilis* immobilized on alginate and chitosan beads

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ABSTRACT. Current study evaluates ethanol and levan production using *Zymomonas mobilis* immobilized on alginate and chitosan beads and assesses the capacity of the beads for reuse in sequential fermentations. Two experiments were carried out: In the first experiment $2^{(5-2)}$ design evaluated the following independent variables: sucrose concentration; pH; incubation time; temperature and agitation. In the second experiment, based on the best conditions observed in $2^{(5-2)}$, the capacity of the immobilization support reuse in subsequent fermentations was evaluated for 12 days. In experiments using fractionated factorial experimental design $2^{(5-2)}$ the best results by immobilized biomass (2.6 g L^{-1}) and immobilization efficiency was shown by alginate and chitosan beads showed only 0.22 g L^{-1}. Chitosan support provided the best rates to levan production (8.90 g L^{-1}). Alginate support showed the highest values by ethanol production (93.4 g L^{-1}). In batch sequential fermentation, re-using immobilization support, alginate support was efficient by maintaining the cellular viability on immobilized support (0.94 g L^{-1}), levan production (21.11 g L^{-1}) and ethanol production (87.21 g L^{-1}). Chitosan support was inadequate for sequential batches since the beads dissolved after the second cycle.

Keywords: biofuel, microbial polysaccharide, entrapment, fractionated factorial experiment.

Produção de etanol e levana por batelada sequencial utilizando *Zymomonas mobilis* imobilizadas em esferas de alginato e quitosana

RESUMO. O objetivo deste estudo foi avaliar a produção de etanol e levana por *Zymomonas mobilis* imobilizadas em esferas de alginato e quitosana e avaliar a capacidade de reutilização das esferas em fermentações sequenciais. Dois experimentos foram realizados: no primeiro, $2^{(5-2)}$ foram avaliadas as variáveis independentes: concentração de sacarose; pH; tempo de incubação; temperatura e agitação. O segundo experimento foi realizado com base nas condições ótimas do ensaio $2^{(5-2)}$, onde foi avaliada a capacidade de reutilização dos suportes durante 12 dias. Para o experimento $2^{(5-2)}$, o suporte mais eficiente para a imobilização foi o alginato, com 2,6 g L^{-1} de biomassa imobilizada enquanto o suporte quitosana apresentou apenas 0,22 g L^{-1}. A produção de levana foi maior nos experimentos realizados com o suporte quitosana (8,90 g L^{-1}). Os maiores valores de etanol foram obtidos nos experimentos com o suporte alginato (93,4 g L^{-1}) Nos experimentos por batelada sequencial, com reutilização dos suportes, o alginato foi mais eficiente para manutenção da viabilidade celular no suporte (0,94 g L^{-1}), produção de levana (21,11 g L^{-1}) e etanol (87,21 g L^{-1}). A quitosana não foi adequada para a fermentação sequencial, uma vez que as esferas desintegraram-se após o segundo ciclo de reutilização do suporte.

Palavras-chave: biocombustível, polissacarídeo microbiano, aprisionamento, planejamento fatorial fracionado.

Introduction

Zymomonas mobilis is highlighted in the literature as a promising microorganism for ethanol and other products of technological interest production such as the gum levan (or fructan) mainly due to its capacity to tolerate high ethanol and sugar concentrations, produce low biomass concentration, use the Entner-Doudoroff metabolic pathway for carbohydrate metabolism and show cell growth regardless of carbon consumption (Behera, Mohanty, & Ray, 2012; Pentjuss, Kostromins, Fell, Stalidzans, & Kalnenieks, 2013).

Despite the fermentation versatility of *Zymomonas mobilis*, its efficiency may be impaired by cells exposition to unfavorable conditions during conventional fermentation processes. To solve such difficulties, several researches have been carried out to improve this technology. In fact, the use of immobilized cells stands out amongst the more promising alternatives (Behera, Ray, & Monhanty, 2010).

Microbial immobilization offers a series of advantages when employing free cells, such as protection cells when submitted to unfavorable conditions, greater biomass concentration on the support and ease in recovering the biomass from the final product (Yamashita, Kurosumi, Sasaki, & Nakamura, 2008; Shih, Chen, & Wu, 2010).

Among the different methodologies for cell immobilization, entrapment on supports is used since it provides greater effectiveness and easier execution. In entrapment, the cells are inserted into rigid/semi-rigid matrix, and their diffusion in the reaction medium is limited by cell accumulation in porous material (Behera et al., 2010). This process confers cell protection against pH harsh conditions, temperature, inhibiting compounds, favor exchange of nutrients, metabolites and gases between the immobilized cells and reaction. The main supports include alginate, k-carrageenan, agar, chitosan and polyvinyl alcohol (El-Naas, Mourad, & Surkatii, 2013; Cheng, Ma, Deng, Xu, & Chen, 2014; Yang, 2015).

Moreover, immobilization may result in physical and chemical changes of microbial cells as modifying metabolism products, ionic charges, water activity, osmotic pressure, or surface tension (Rathore, Desai, Liew, Chan, & Heng, 2013). Due to the need to develop technologies that increase ethanol and levan yield production for the use of economically feasible, easily handled raw materials, current research evaluates the efficiency of immobilizing *Z. mobilis* on alginate and chitosan beads and ethanol and levan production and assesses the recycling capacity of the immobilized cell biomass by sequential batch fermentation.

Material and methods

Microorganism, culture media, fermentation conditions and immobilization

The microorganism *Zymomonas mobilis* CCT 4494 was obtained from the Tropical Culture Collection "André Tosello" Research and Technology Foundation - Campinas, São Paulo State, Brazil. Maintenance and pre-fermentation medium (*Z. mobilis* broth) was composed by 10 g L^{-1} yeast extract, 10 g L^{-1} peptone and 20 g L^{-1} glucose. Fermentation medium used was the basal medium composed by 5 g L^{-1} yeast extract; 1 g L^{-1} KH_2PO_4 1; 1 g L^{-1} $(NH_4)_2SO_4$ and 1 g L^{-1} $MgSO_4$. $7H_2O$.

Z. mobilis stock culture was seeded into tubes containing *Z. mobilis* broth and incubated at 30ºC for 24 hours to reactivate the culture. The tubes were added into pre-fermentation flasks containing *Z. mobilis* broth (50 mL) and incubated under the same conditions described below. For tests, the pre-fermentation medium was centrifuged at 3660 g 15 min^{-1} and the supernatant was discarded and the cell pellet re-suspended in 0.85% (m v^{-1}) saline solution to obtain a concentrated cell suspension which was standardized by spectrophotometry at absorbance 0.7 at 570 nm.

Z. mobilis cells at the pre-established concentration were added to a previously prepared and sterilized 3% (w v^{-1}) alginate solution and stirred for 10 min. The mixture was then dripped, drop by drop, using a hypodermic syringe, into a 0.2 M calcium chloride solution under constant agitation, obtaining beads with a diameter of approximately 3 mm. Immobilization on the chitosan beads was carried out in a similar way, with a 4% (m v^{-1}) chitosan solution, previously dissolved in 1% (v v^{-1}) acetic acid. Cell-chitosan suspension was maintained under agitation for 10 min, and then dripped into a 1 M NaOH solution under agitation, obtaining beads with a diameter of approximately 3 mm. Beads of the two supports were matured under refrigeration at 4ºC for 24 hours and then, washed with sterile distilled water before adding to flasks containing fermentation medium and a carbon source. Ten grams of beads were added to each flask which contained 50 mL fermentation medium under conditions described in Experiment 1.

Experiment 1: Optimum conditions for ethanol and levan production

A $2^{(5-2)}$ fractionated factorial experimental design determined the ethanol and levan production conditions, resulting in 27 experiments, with 2 repetitions at the central point, as shown in Table 1 (Montgomery, 2001). The parameters selected were based on previous experiments using free cells (Ernandes & Garcia-Cruz, 2011).

Table 1. Independent variables studied in the first $2^{(5-2)}$ experimental design.

Variables	Levels		
	-1	0	+ 1
X_1: sucrose (g L^{-1})	150	250	350
X_2: initial pH rate	4.0	5.5	7.0
X_3: time (h^{-1})	16	56	96
X_4: temperature (ºC)	30	35	40
X_5: agitation (rpm)	0	100	200

Experiment 2: Ethanol and levan production by sequential fermentation (support recycling)

Reuse supports capacity was based on the best conditions for levan and ethanol production obtained in the $2^{(5-2)}$ experiment, using response surface methodology: 350 g L^{-1} sucrose; pH 4; 30ºC and agitation at 200 rpm. Supports recycling were

carried out for 12 cycles with 24 hours of fermentation.

Calculation of kinetic parameters

The kinetic parameters were determined according to equations below as equations 1 and 2:

$$Y_{P/S} = \frac{Pf - P0}{Sf - S0} \text{ g g}^{-1} \quad (1)$$

$$P = \frac{Pf - P0}{tf - t0} \text{ g L}^{-1} \text{ h}^{-1} \quad (2)$$

where:
P = productivity;
P_0 = initial product mass (g L^{-1});
P_f = final product mass (g L^{-1});
S_0 = initial substrate concentration (g L^{-1});
S_f = final substrate concentration (g L^{-1});
$Y_{p/s}$ = coefficient of product yield in relation to substrate consumed;
t_f = time (h);
t_0 = initial time (h).

Analytical methods

Final pH rate was determined by Digmed DM20 pH meter. Free and immobilized biomasses were determined by spectrophotometry at 570 nm. Alginate beads were previously dissolved in 10 mL of 0.3 M sodium citrate (pH: 5, 1 M^{-1} citric acid) and chitosan beads in 5% (v v^{-1}) acetic acid to determine immobilized biomasses. Ethanol content was evaluated by gas chromatography (GC focus, TR-wax column 30 x 0.053, ID x1μm) and levan content was determined by technique described by Viikari (1984). Reducing sugars were evaluated according to copper-arsenate technique described by Somogyi (1952) and Nelson (1944), and total sugars by the phenol-sulfuric acid method described by Dubois, Gilles, and Hamilton (1956). Substrate consumption using alginate (SCA) or chitosan (SCC) was calculated by subtracting the initial substrate concentration (sucrose) from the final substrate concentration, with results in %. Factorial fractionated statistical design (2^{k-p}), described by Montgomery (2001), was used for the optimal experiments, using *Statistica* 6.0. Data were evaluated by variance analysis (ANOVA) for the best parameters to ethanol and levan production, using the contour curves produced by response surface methodology (RSM). Results obtained for the sequential batch fermentations during 12 days of support recycling were also evaluated by analysis of variance and Tukey's test, with Minitab 14. Electronic microscopy scanning was determined by fixing the samples in 3% (v v^{-1}) glutaraldehyde for 6 hours and 1% (v v^{-1}) osmium tetroxide during 1 hour. The samples fixed in OsO_4 were washed in distilled water and treated with increasing concentrations of ethanol (30, 50, 70, 90 and 100% (v v^{-1}) for 10 min. The samples which passed through the critical point were dried (K550, Emitech) and mounted on a SEM stub with copper tape and sputter coated with gold/palladium. Images of the samples were analyzed with scanning electron microscope (LEO 435 VPi SEM, Zeiss) according Madi-Ravazzi (2007).

Results and discussion

Experiment 1: Determination of best conditions for ethanol and levan production

Immobilized biomass, immobilization efficiency and substrate consumption (sucrose).

Parameters that showed significant statistical influence ($p < 0.05$) for increasing immobilized biomass on alginate support (2.60 g L^{-1}) were initial pH (approximately 7.0), sucrose (150 g L^{-1}) and without agitation of fermentation medium (Tables 2 and 4). On the other hand, in tests carried out using chitosan beads as the immobilization support (0.22 g L^{-1}), no variable evaluated had any significant statistical influence ($p < 0.05$), as showed in Tables 3 and 4.

Initial pH rate of fermentation medium was an important parameter for biomass production, with optimal rate for bacterial growth at approximately 7.0. Several authors have reported initial pH of the fermentation medium as an important factor for *Z. mobilis* growth, which grows at pH rates ranging between 3.5 and 7.5, although the optimum range between 5 and 7 (Swings & De Ley, 1977; Santos, Del Biachi, & Garcia-Cruz, 2014). Immobilization efficiency using cell concentration adhered on support and the free biomass in culture medium, demonstrated that alginate support was more efficient for immobilization (2.6 g L^{-1}) than chitosan support (0.22 g L^{-1}), as showed in Tables 2 and 3.

Also by substrate consumption the alginate support showed the highest conversion rate (90.7%) for experiments carried out at 30ºC, without agitation, 150 g L^{-1} of sucrose, initial pH at 7.0, and 96 hours of fermentation (Table 5).

Substrate was consumed by the microorganism, although it appeared that it had not been totally used for the synthesis of biomass, levan and ethanol, since the rates of these components together were below the total consumed. The above behavior suggests the synthesis of other compounds such as non-precipitating levan, cited by Viikari (1984), sorbitol, acetic acid, lactate, acetaldehyde, glycerol, acetoin,

di-hydroxy-ketone, mannitol and gluconic acid (Ernandes & Garcia-Cruz, 2011; Madigan, Martinko, Dunlap, & Clark, 2010).

Table 2. Mean rates for immobilized biomass, ethanol and levan production obtained in $2^{(5-2)}$ fractionated factorial experimental design for alginate support.

Test	X_1	X_2	X_3	X_4	X_5	IB	L	YL	PT L	E	YE	PTE
1	150	4.0	16	40	200	0.29	0.60	0.025	0.04	13.9	0.45	0.8
2	350	4.0	16	30	0	0.06	6.27	0.020	0.39	22.9	0.11	1.4
3	150	7.0	16	30	200	2.05	2.43	0.016	0.15	29.8	0.19	1.8
4	350	7.0	16	40	0	2.21	0.30	0.001	0.02	49.0	0.20	3.0
5	150	4.0	96	40	0	1.90	0.57	0.006	0.01	35.7	0.38	0.3
6	350	4.0	96	30	200	0.01	4.90	0.015	0.05	78.3	0.44	0.8
7	150	7.0	96	30	0	2.60	0.13	0.001	0.00	48.9	0.35	0.5
8	350	7.0	96	40	200	0.35	0.60	0.003	0.01	26.6	0.08	0.2
9C	250	5.5	56	35	100	1.64	0.33	0.001	0.01	93.4	0.41	1.6

(C): central point; x_1: sucrose (g L^{-1}); x_2: pH; x_3: time (h^{-1}); x_4: temperature (ºC); x_5: agitation (rpm); IB: immobilized biomass (g L^{-1}); L: levan production (g L^{-1}); YL: yield in levan (g g^{-1}); PTL: productivity for levan (g L^{-1} h^{-1}); E: ethanol production (g L^{-1}); YE: yield in ethanol (g g^{-1}); PTE: productivity for ethanol (g L^{-1} h^{-1}).

Table 3. Mean rates for immobilized biomass, ethanol and levan production obtained in $2^{(5-2)}$ fractionated factorial experimental design for chitosan support.

Test	X_1	X_2	X_3	X_4	X_5	IB	L	YL	PT L	E	YE	PT E
1	150	4.0	16	40	200	0.02	1.57	0.01	0.10	0.39	0.003	0.02
2	350	4.0	16	30	0	0.10	2.50	0.02	0.16	1.07	0.007	0.07
3	150	7.0	16	30	200	0.05	2.17	0.06	0.14	0.49	0.013	0.03
4	350	7.0	16	40	0	0.04	2.63	0.01	0.16	1.08	0.005	0.07
5	150	4.0	96	40	0	0.05	1.27	0.04	0.01	0.57	0.019	0.01
6	350	4.0	96	30	200	0.07	4.67	0.09	0.05	1.39	0.026	0.01
7	150	7.0	96	30	0	0.22	1.77	0.03	0.02	0.78	0.012	0.01
8	350	7.0	96	40	200	0.10	8.90	0.03	0.09	1.14	0.004	0.01
9C	250	5.5	56	35	100	0.02	2.53	0.20	0.05	5.24	0.489	0.11

(C): central point; x_1: sucrose (g L^{-1}); x_2: pH; x_3: time (h^{-1}); x_4: temperature (ºC); x_5: agitation (rpm); BI: immobilized biomass (g L^{-1}); L: levan production (g L^{-1}); YL: yield in levan (g g^{-1}); PTL: productivity for levan (g L^{-1} h^{-1}); E: ethanol production (g L^{-1}); YE: yield in ethanol (g g^{-1}); PTE: productivity for ethanol (g L^{-1} h^{-1}).

Table 4. ANOVA table for biomass concentration of *Zymomonas mobilis* immobilized in alginate and chitosan supports.

Variables	Immobilized biomass			
	Alginate		*Chitosan*	
	Effect	p	Effect	p
X_1	-1.05114	0.000009*	-0.005	0.844
X_2	1.23883	0.000001*	0.046	0.134
X_3	0.06327	0.730681	0.055	0.074
X_4	0.00854	0.962902	-0.059	0.056
X_5	-1.01661	0.000015*	-0.047	0.122

*Significant statistical influence ($p < 0.05$); x_1: sucrose (g L^{-1}); x_2: pH; x_3: time (h^{-1}); x_4: temperature (ºC); x_5: agitation (rpm).

Table 5. Results for free biomass, total sugars and substrate consumption obtained using *Zymomonas mobilis* CCT 4494 immobilized on alginate and chitosan.

Test	FBA	FBC	TSA	TSC	SCA	SCA	SCC	SCC
			(g L^{-1})			(%)	(g L^{-1})	(%)
1	0.01	0.28	119.2	22.47	30.8	20.6	127.5	85.00
2	0.01	0.01	133.8	197.83	216.2	61.8	152.2	43.47
3	0.21	0.25	119.2	110.90	30.8	20.5	39.1	26.07
4	0.01	0.01	108.1	124.87	241.9	69.1	225.1	64.33
5	0.26	0.05	56.2	119.80	93.8	62.5	30.2	20.17
6	0.22	0.01	173.2	296.17	176.8	50.5	53.8	15.37
7	0.01	0.01	12.1	84.23	137.9	92.0	65.8	43.83
8	0.01	0.04	33.7	47.57	316.3	90.4	302.5	86.43
9 (C)	0.09	0.14	23.4	237.17	226.6	90.7	12.8	5.13

FBA: free biomass alginate (g L^{-1}); FBC: free biomass chitosan (g L^{-1}); TSA: total sugars (final) alginate (g L^{-1}); TSC: total sugars (final) chitosan (g L^{-1}); SCA: sucrose consumed alginate (g L^{-1} and %); SCC: sucrose consumed chitosan (g L^{-1} and %); (C): central point.

Levan production by *Zymomonas mobilis* immobilized on alginate and chitosan

Specialized literature cited different optimum parameters for the synthesis of the polymer: sucrose in high concentration (between 250 and 350 g L^{-1}), temperature range between 25 and 37ºC, and optimum pH between 3.5 and 7.5 (Bekers, Laukevics, Upite, Kaminska, & Linde, 1999; Madigan, Martinko, Dunlap, & Clark, 2010). Current analysis evaluated these parameters (Table 1).

The highest rates for levan production were obtained using the bigger sucrose concentration (350 g L^{-1}) by Tests 2 and 5, using alginate (6.27 g L^{-1}) and chitosan in Tests 6 and 8 (4.67 and 8.0 g L^{-1}), as shown in Figure 1 and Table 2. Sucrose (350 g L^{-1}), lowest pH rate (4), temperature (30ºC) and fermentation time provided a significant statistical interference ($p < 0.05$) by levan production using alginate support (Table 6). Chitosan support revealed the same behavior with sucrose concentration, although pH 7, 40ºC and agitation (200 rpm) were statistically significant (Table 6).

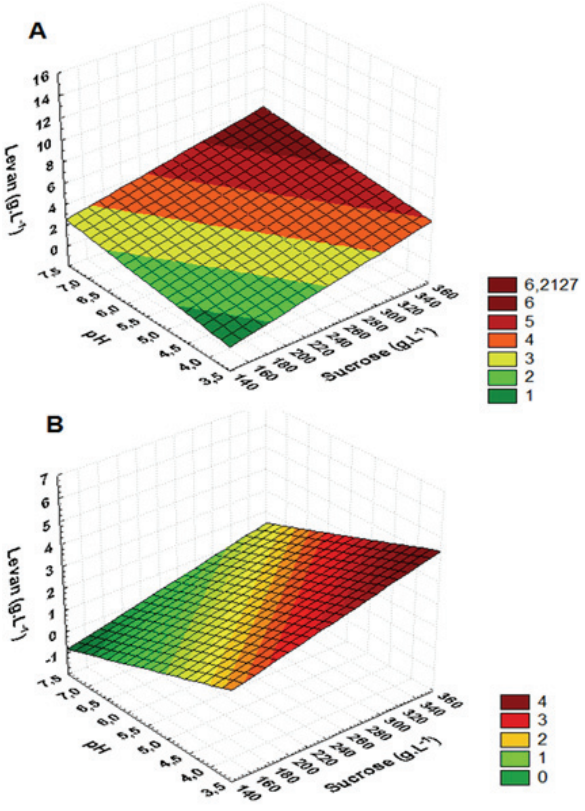

Figure 1. Response surfaces for levan production (g L^{-1}) using chitosan (A) *(*Levan = -4.0276+0.0166*x+0.5701*y*) and alginate (B) *(*Levan = 3.238+0.0104*x-0.7359*y*) at rates close to optimum for sucrose (g L^{-1}) and initial pH.

Regarding to sucrose concentration, a similar behavior was observed by Lorenzetti, Moro, and

Garcia-Cruz (2015) using immobilized *Z. mobilis* in alginate/PVA hybrid support. They obtained best levan production (18.84 g L^{-1}) using 300 g L^{-1} of sucrose. Santos, Del Bianchi, and Garcia-Cruz (2014) also obtained maximum levan production (23.5 g L^{-1}) by same microorganism immobilized in alginate using sucrose 350 g L^{-1}.

Silbir, Dagbagli, Yegin, Baysal, and Goksungur (2014) studied *Zymomonas mobilis* B-14023 immobilized on alginate beads by levan production with sucrose (300 g L^{-1}) as substrate and initial pH 6.0 and obtained a maximum levan concentration of 40.2 g L^{-1}. As shown in Tables 2 and 3, levan production decreased in the two evaluated supports but the greatest production did not present the highest yield rate. Chitosan revealed the highest rates in production yield with 0.2 g g^{-1} in run 9C, whereas alginate gave 0.02 g g^{-1} in experiments 1 and 2 (Table 3).

Variations obtained in levan production rates might be due to the biochemical mechanisms that regulate the polysaccharide synthesis since it is a reserve energy substance (Alegre, Wendt, & Rigo, 2005). It has also been observed that, since greater substrate consumption did not result in a proportionally greater levan production by alginate and chitosan supports (Tables 2, 3 and 5), the substrate might have been mainly used to produce biomass and other metabolites, such as ethanol, sorbitol and fructooligosaccharides (Swings & De Ley, 1977; Madigan, Martinko, Dunlap, & Clark, 2010).

Ethanol production by *Zymomonas mobilis* immobilized on alginate and chitosan beads

Alginate support presented the highest rates for ethanol production (93.4 g L^{-1}) in run 9 C and yield of 0.41 g g^{-1} (Table 2 and Figure 2). Maximum ethanol yield was obtained in runs 1 (0.45 g g^{-1}) and 6 (0.44 g g^{-1}), as shown in Table 2. However, no variable presented significant statistical interference ($p < 0.05$) (Table 6). Ethanol production efficiency using *Z. mobilis* immobilized on alginate beads was also reported by Yamashita, Kurosumi, Sasaki, and Nakamura (2008); Behera et al. (2010) and Behera, Mohanty, and Ray (2012). Chitosan support gave maximum ethanol production of 5.24 g L^{-1} and ethanol yield of 0.48 g g^{-1}, both in trial 9, and the variables sucrose, fermentation time and agitation presented significant statistical interference (Table 6 and Figure 2).

Optimal conditions for ethanol production by *Zymomonas mobilis* in immobilized systems are divergent in literature. Most cited parameters include initial pH between 4.5 and 7.5; temperature between 25 and 37ºC and sugar concentration between 150 and 250 g L^{-1} (Bandaru, Somalanka, Mendu, Medicherla, & Chityala, 2006). These parameters were evaluated in current study (Table 1).

Table 6. ANOVA table for levan and ethanol production of *Zymomonas mobilis* immobilized in alginate and chitosan supports.

Variables	Levan production				Ethanol production			
	Alginate		*Chitosan*		*Alginate*		*Chitosan*	
	Effect	p	*Effect*	p	*Effect*	p	*Effect*	p
X_1	2.07	0.000006*	3.31	0.000036*	12.13	0.27	3.31	0.000009*
X_2	-2.20	0.000003*	1.71	0.013562*	0.89	0.93	1.71	0.560940
X_3	-0.84	0.023620*	2.26	0.001782*	18.47	0.10	2.26	0.000081*
X_4	-2.91	0.000000*	1.15	0.084281	-13.65	0.22	1.15	0.250549
X_5	0.31	0.370630	2.63	0.000452*	-1.95	0.85	2.63	0.000020*

*Significant statistical influence ($p < 0.05$); x_1: sucrose (g L^{-1}); x_2: pH; x_3: time (h^{-1}); x_4: temperature (ºC); x_5: agitation (rpm).

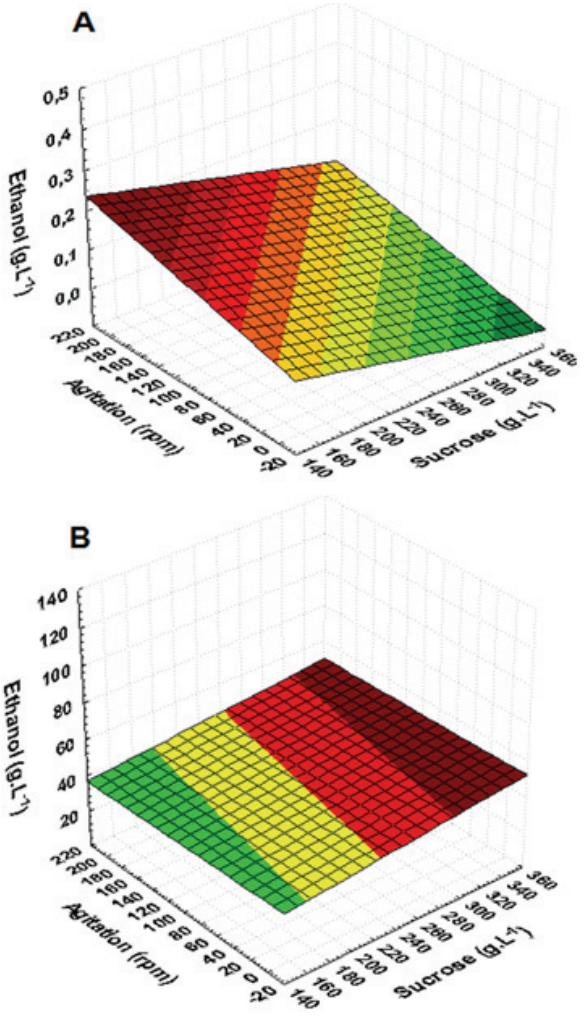

Figure 2. Response surfaces for ethanol production (g g^{-1}) using chitosan (A) *(Ethanol = 0.1922 – 0.0007*x+0.0006*y)* and alginate (B) *(*Ethanol = 30.1346+0.0607*x-0.0098*y*)* supports for ethanol production (g L^{-1}) at rates close to optimum for sucrose (g L^{-1}), pH and agitation (rpm).

Initial pH adjusted to 5.5 proved to be the best parameter evaluated for ethanol production by alginate and chitosan beads, even though without

any statistical interference (Tables 2 and 6). This behavior was also registered by Maiti, Rathore, Srivastava, Shekhawat, and Srivastava (2011); Pinilla, Torres, and Ortiz (2011). As mentioned above, agitation affects ethanol production by chitosan support (Table 6), also reported by Pinilla, Torres, and Ortiz (2011).

In current study, temperature at 30-35ºC (Tables 2 and 3 – Experiments 6 and 9) proved to be the best for ethanol synthesis on alginate and chitosan supports, although it did not statistically influence ethanol production (Table 6). This behavior was also cited by Behera et al. (2010) and by Bandaru et al (2006).

Similar to levan production, cell concentration on the support did not affect ethanol synthesis (Tables 2 and 3). This can occur because *Zymomonas mobilis* growth is independent of ethanol synthesis. Due to this peculiarity, *Z. mobilis* is considered to be an excellent ethanol producer (Yamashita et al., 2008; Pentjuss et al., 2013).

Experiment 2: Ethanol and levan production by sequential fermentation with immobilization support recycling

Behavior of immobilized biomass and final pH during alginate and chitosan supports recycling were carried out during 12 fermentation cycles, using the best production conditions obtained in the $2^{(5-2)}$ experiments (Table 7).

Immobilized biomass on alginate support remained viable throughout all the reused cycles, and was observed high affinity of this support by the microorganism. This was demonstrated in scanning electronic microscopy and the micrographs show a great number of immobilized cells on the surface and inside the beads (Figure 3A and B).

Table 7. Optimum conditions for levan and ethanol production using alginate and chitosan supports obtained by $2^{(5-2)}$ experiments using response surface methodology.

Variables	Alginate	Chitosan
Sucrose (g L^{-1})	350	350
pH	4	7
Temperature (ºC)	30	30
Agitation (rpm)	200	200

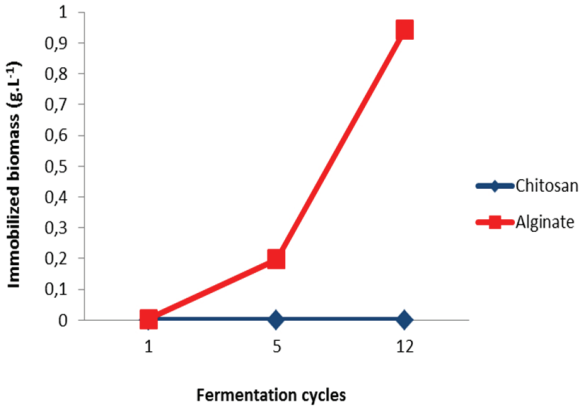

Figure 3. Immobilized biomass on chitosan and alginate supports during fermentation cycles of recycling support.

Figure 3 shows that the amount of immobilized biomass during the first fermentation cycle was 0.003 g L^{-1}; it increased to 0.19 g L^{-1} after 5 days; at the end of recycling it was 0.94 g L^{-1}. In fact, alginate beads remained intact from the first to the last fermentation cycle and only changed their texture and color possibly due to microbial growth or to levan presence inside the beads. Free biomass during 12 fermentation cycles ranged between 0.14 and 2.65 g L^{-1} (Table 9). Fermentation cycles were carried out by a hybrid system of free and entrapment cells. In fact, the above behavior has been observed in fermentative process with immobilized microorganisms.

On the other hand, chitosan beads showed 0.001 g L^{-1} immobilized biomass during the first fermentation cycle (Figure 3) even though they were not viable for immobilizing *Zymomonas mobilis* CCT4494. Chitosan beads would not withstand the fermentation conditions used or the fermentation cycles and dissolved after the second recycle. Free biomass was 1.10 and 1.30 g L^{-1} for first and second cycles, respectivelly (Table 8).

Zymomonas mobilis adhered to chitosan support after immobilization is showed in Figures 4C and D. Although reduced microbial adhesion there was microbial growth on the inside and on the outside of the beads, similar to alginate. Figure 4 also revealed the beads irregular external structure with the immobilized microorganism.

Table 8. Results for the production of levan, ethanol, substrate consumption, final pH and free biomass produced by *Zymomonas mobilis* CCT4494 immobilized on chitosan during sequential batch fermentation cycles.

Cycle	Immobilized biomass	Levan production	Levan yield	Ethanol production	Ethanol yield	Substrate consume	Free biomass	Final
	(g L^{-1})	(g L^{-1})	(g g^{-1})	(g L^{-1})	(g g^{-1})	(%)	(g L^{-1})	pH
1	0.001	3.47[a]	0.01	2.95[a]	0.12	56.69	1.10	7.55
2*	-	1.99[b]	0.007	0.96[b]	0.04	76.32	1.63	8.14
3-12*	-	-	-	-	-	-	-	-

a, b... (column) – means followed by the same small letters do not differ according to Tukey´s test ($p < 0.05$). *Chitosan beads were degraded by fermentation condition.

Table 9. Results for the production of levan, ethanol, substrate consumption and free biomass produced by *Zymomonas mobilis* CCT4494 immobilized on alginate during sequential batch fermentation cycles.

Cycles	L	LPT	YL	E	EPT	YE	SC	FB
(24 hours)	(g L^{-1})	(g L^{-1} $hour^{-1}$)	(g g^{-1})	(g L^{-1})	(g L^{-1} $hour^{-1}$)	(g g^{-1})	(%)	(g L^{-1})
1	3.63^{a}	0.15	0.02	19.32^{a}	0.81	0.09	61.14	0.18
2	$9.81^{b,c}$	0.41	0.04	$67.77^{b,c}$	2.82	0.26	74.57	1.04
3	$11.32^{b,c}$	0.47	0.06	25.1^{a}	1.05	0.14	51.41	0.29
4	$10.10^{b,c}$	0.42	0.07	50.03^{c}	2.08	0.33	43.58	0.14
5	8.78^{b}	0.37	0.03	69^{b}	2.88	0.26	74.78	1.66
6	8.29^{b}	0.35	0.04	$56.24^{b,c,d}$	2.34	0.30	54.14	1.17
7	$17.04^{c,d}$	0.71	0.06	$58.83^{b,c}$	2.45	0.21	78.96	2.65
8	$10.98^{b,c}$	0.46	0.05	$60.19^{b,c}$	2.51	0.27	64.16	1.36
9	8.63^{b}	0.36	0.03	$38.09^{a,c}$	1.59	0.14	75.75	1.64
10	21.11^{d}	0.88	0.09	$53.1^{b,c}$	2.21	0.23	64.87	1.23
11	$11.42^{b,c}$	0.48	0.05	$43.1^{c,d}$	1.80	0.19	64.03	1.45
12	$10.39^{b,c}$	0.43	0.04	87.21^{e}	3.63	0.37	66.66	1.97

^{a,b}... (column) – means followed by the same small letters do not differ by Tukey´s test ($p < 0.05$). L: levan production; LPT: levan productivity; YL: yield in levan; E: ethanol production; EPT: ethanol productivity; YE: yield in ethanol; SC: substrate consumption; FB: free biomass in the fermentation medium.

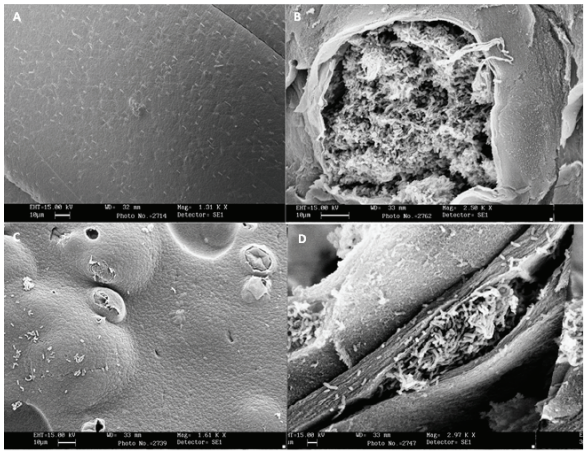

Figure 4. Scanning electronic microscopy of alginate (A & B) and chitosan (C & D) beads with immobilized *Zymomonas mobilis* CCT4494 (A & C: external surface with immobilized microorganism); (B& D: bead interior highlighting microbial growth).

Chitosan beads behavior during the fermentation cycles could have been due to support nature, which dissolves in acid media and too by metabolism of the microorganism those results in acids accumulation in culture medium and would have dissolved the chitosan beads (Xu, Huang, Zhu, & Ye, 2015). Final pH of fermentation media using chitosan beads was extremely basic with rates 7.55 and 8.14, respectively, in the first and second cycles (Table 8).

Levan production during sequential fermentation by immobilization support recycling

Levan production decreased when chitosan beads were used after the second reuse cycles. In fact, the highest production and yield occurred in the first cycle (3.47 g L^{-1} and 0.01 g g^{-1}) with significant statistical difference ($p < 0.05$) when compared to that of the second cycle (1.99 g L^{-1} and 0.007 g g^{-1}), as showed in Table 8. Levan production could not be evaluated for the remaining reuse cycles, since the beads dissolved due to the culture medium pH. This behavior was not observed during the batch fermentations in which the beads remained stable during the experiments, demonstrating that chitosan is not a viable support for sequential batch fermentations.

In spite of the reduced efficiency for the immobilization of *Z. mobilis* reported for chitosan support (Figure 3), the highest consumption rate of the sucrose substrate was not associated with the highest production of levan for the first (56.69%) and second (76.32 %) fermentation cycles (Table 8). *Zymomonas mobilis* immobilized in alginate beads showed the highest production and yield in the tenth (21.11 g L^{-1} and 0.09 g g^{-1}) and fourth (10.10 g L^{-1} and 0.07 g g^{-1}) cycles, without significant difference between them ($p < 0.05$). However, in relation to other cycles, the 10^{th} cycle showed the highest rates with significant difference ($p < 0.05$), as demonstrated in Table 8.

Levan production during twelve cycles was between 3.63 and 21.11 g L^{-1} and was almost constant after the tenth cycle, with substrate consumption (sucrose) between 43.58 and 78.96% (Table 9). The highest sucrose consumption was accompanied by the highest levan production and also by the largest amount of free biomass in the culture medium, as highlighted in Table 9. The maximum sucrose consumption was 78.96% in run 7 and 75.75% in run 9, which was not followed by a higher levan productivity (0.07 and 0.09 g L^{-1} $hour^{-1}$) respectively for runs 4 and 10, according to Table 9.

Lower production rates were observed in the first fermentation cycles, possibly due to microorganism adaptation. However, higher rates were reported for all reuse cycles, after the 2^{nd} recycle (8.05-12.11 g L^{-1}) which were higher than those results observed in batch cultivation (Table 2). Immobilized inoculum reuse demonstrated greater physiological stability and capacity to levan produce in larger amount (Table 9).

Silbir et al. (2014) also obtained good stability of alginate beads during continuous fermentation using

Zymomonas mobilis immobilized by levan production and obtained maximum concentration of 31.8 g L^{-1}. Using *Bacillus subtilis nato* immobilized on alginate beads, Shih, Chen, and Wu (2010) reported the best levan production (70.9 g L^{-1}) in the 3rd fermentation recycle. The above authors also registered the high stability of beads during five fermentation cycles.

High levan production observed by use of high sucrose concentrations has been reported by several authors. Under these conditions, the microorganism deviates its metabolic pathway and, after hydrolyzing the sucrose to fructose and glucose units, it initiates the transfructosylation of the fructose units with β (2.6) bonds, forming the levan chain (Shih et al., 2010; Silbir et al., 2014).

Ethanol production during sequential fermentation by immobilization support recycling

Chitosan support showed low ethanol yield and production during the two reuse cycles, with highest rate (0.12 g g^{-1}) and 2.65 g L^{-1} in the second cycle ($p < 0.05$) when compared to the first, in which production yield was only 0.04 g g^{-1} and 0.96 g L^{-1} (Table 8).

Ethanol production by alginate support oscillated from the second until the tenth fermentation cycles. There was an increase in ethanol production between the fourth and the eighth cycles (50.03 to 60.19 g L^{-1}), whereas the highest yield rate was obtained in the 12th cycle (0.37 g g^{-1} and 87.21 g L^{-1}) ($p < 0.05$), as shown in Table 9. Best rates in productivity were obtained in the 12th (3.63 g L^{-1} $hour^{-1}$) and 5th (2.88 g L^{-1} $hour^{-1}$) cycles (Table 9). Similarly to levan production, the sucrose consumption was not followed by the best ethanol production (Table 9). Such behavior (high variation in ethanol rates during 12 cycles) could be due to microorganism adaptation to stressful condition of exchanging culture medium and also to microbial population succession on the immobilization support.

Lower results were obtained by Watanabe et al. (2012) in ethanol production from hydrolyzed rice straw as a carbon source, using a mixed alginate and resin support during 5 fermentation cycles. The authors obtained production between 29.6 and 37.9 g L^{-1} from the first and fifth cycles, with support remaining viable up to the end of the last reuse cycle. Yamashita et al. (2008) also evaluated ethanol production by immobilized support recycling using alginate and *Zymomonas mobilis*. These authors reported 17.86 g L^{-1} of ethanol in first fermentation cycle and a reduction to 5.32 g L^{-1} in the last cycle.

The immobilization support needs to show stability for reuse after media replacement. According to Yamashita et al. (2008), this is the most important benefit of immobilized cells. Therefore, it is important to highlight that the immobilized system (alginate beads and *Zymomonas mobilis*) remained viable until the last fermentation cycle, with a high production of levan. In fact, it is interesting to use in semi-continuous production systems. Results comprise numerous benefits, such as low operational costs and greater production.

Conclusion

In experiments using $2^{(5-2)}$ fractionated factorial experimental design, the best results by immobilized biomass, immobilization efficiency and ethanol production was reported in alginate support. Chitosan support showed the highest rate in levan production. In sequential batches, experiments by support recycling, the greatest levan and ethanol production was obtained by alginate support. Chitosan support was not adequate for sequential batches since the beads dissolved after the second cycle.

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References

Alegre, R. M., Wendt, R., & Rigo, M. (2005). Levan production by isolated mutants of *Z. mobilis*. *Revista de Ciências Exatas e Naturais,* 7(1), 103-112.

Bandaru, V. V. R., Somalanka, S. R., Mendu, D. R., Medicherla, N. R., & Chityala, A. (2006). Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology. *Enzyme Microbiology and Technology, 38*(1-2), 209-214.

Behera, S. S., Mohanty, R. C., & Ray, R. C. (2012). Ethanol fermentation of sugarcane molasses by *Zymomonas mobilis* MTCC 92 immobilized in *Luffa cylindrica* L. sponge discs and ca-alginate matrices. *Brazilian Journal of Microbiology, 43*(4), 1499-1507.

Behera, S., Ray, R. C., & Monhanty, R. C. (2010). Comparative study of bioethanol production from mahula (*Madhuca latifolia*) flowers by immobilized cells of *Saccharomyces cerevisiae* and *Zymomonas mobilis* in calcium alginate beads. *Journal of Science and Industrial Research, 69*(6), 472-475.

Bekers, M., Laukevics, J., Upite, D., Kaminska, E., & Linde, R. (1999). Flame burned stainless steel wire spheres as carrier of *Zymomonas mobilis* cells and

extracellular levansucrase. *Acta Biotechnology, 19*(4), 341-348.

Cheng, Y., Ma, L., Deng, C., Xu, Z., & Chen, J. (2014). Effect of PEG-mediated pore forming on Ca-alginate immobilization of nitrilase-producing bacteria *Pseudomonas putida* XY4. *Bioprocess and Biosystems Engineering, 37*(8), 1653-1658.

Dubois, M., Gilles, K. A., & Hamilton, J. K. (1956). Colorimetric method for determination sugars and related substances. *Analytical Chemistry, 28*(3), 350-356.

El-Naas, M. H., Mourad, A. H., & Surkatii, R. (2013). Evaluation of the characteristics of polyvinyl alcohol (PVA) as matrices for the immobilization of *Pseudomonas putida*. *International Biodeterioration & Biodegradation, 85*(1), 413-420.

Ernandes, F. M. P. G., & Garcia-Cruz, C. H. (2011) Sucrose utilization by *Zymomonas mobilis*: Levan production optimization using submerged fermentation. *Journal of Engineering and Technology, 1*(2), 47-56.

Lorenzetti, M. F. S., Moro, M. R., & García-Cruz, C. H. (2015). Alginate/PVA beads for levan production by *Zymomonas mobilis*. *Journal of Food Process Engineering, 38*(1), 31-36.

Madigan, M. T., Martinko, J. M., Dunlap, P. V., Clark, D. P. (2010). *Microbiologia de Brock* (12a ed.). Porto Alegre, RS: Artmed.

Madi-Ravazzi, L. (2007). *Microscopia eletrônica de varredura com ênfase à análise de amostras biológicas* [Apostila]. São José do Rio Preto, SP: Ibilce/Unesp.

Maiti, B., Rathore, A., Srivastava, S., Shekhawat, M., & Srivastava, P. (2011). Optimization of process parameters for ethanol production from sugar cane molasses by *Zymomonas mobilis* using response surface methodology and genetic algorithm. *Applied Microbiology and Biotechnology, 90*(1), 285-395.

Montgomery, D. C. (2001). *Design and analysis of experiments* (5th ed.). New York, USA: Wiley.

Nelson, N. (1944). A photometric adaptation of Somogy method for determination of glucose. *Biochemistry, 153*(1), 375-380.

Pentjuss, A., Kostromins, A., Fell, D. A., Stalidzans, E., & Kalnenieks, U. (2013). Biotechnological potential of respiring *Zymomonas mobilis*: A stoichiometric analysis of its central metabolism. *Journal of Biotechnology, 165*(1), 1-10.

Pinilla, L., Torres, R., & Ortiz, C. (2011). Bioethanol production in batch mode by a native strain of *Zymomonas mobilis*. *World Journal of Microbiology and Biotechnology, 27*(11), 2521-2528.

Rathore, S., Desai, P. M., Liew, C. V., Chan, L. W., & Heng, P. W. S. (2013). Microencapsulation of microbial cells. *Journal of Food Engineering, 16*(2), 369-381.

Santos, V. A. Q., Del Bianchi, V. L., & Garcia-Cruz, C. H. (2014). Effect of initial pH in levan production by *Zymomonas mobilis* immobilized in sodium alginate. *Acta Scientiarum. Technology, 36*(2), 349-354.

Shih, I. L., Chen, L. D., & Wu, J. Y. (2010). Levan production using *Bacillus subtilis* natto cells immobilized on alginate. *Carbohydrate Polymers, 82*(1), 111-117.

Silbir, S., Dagbagli, S., Yegin, S., Baysal, T., & Goksungur, Y. (2014). Levan production by *Zymomonas mobilis* in batch and continuous fermentation systems. *Carbohydrate Polymers, 99*(2), 454-461.

Somogyi, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry, 195*(1), 19-23.

Swings, S., & De Ley, J. (1977).The biology of *Zymomonas*. *Bacteriology Review, 41*(1), 1-46.

Viikari, L. (1984). Formation of levan and sorbitol from sucrose. *Applied Microbiology and Biotechnology, 19*(4), 252-255.

Watanabe, I., Miyata, N., Ando, A., Shiroma, R., Tokuyasu, K., & Nakamura, T. (2012). Ethanol production by repeated-batch simultaneous saccharification and fermentation (SSF) of alkali-treated rice straw using immobilized *Saccharomyces cerevisiae* cells. *Bioresource Technology, 123*(1), 695-968.

Xu, L., Huang, Y. A., Zhu, Q. J., & Ye, C. (2015). Chitosan in Molecularly-Imprinted Polymers: Current and Future Prospects. *International Journal Molecular Science, 16*(8), 18328-18347.

Yamashita, Y., Kurosumi, A., Sasaki, C., & Nakamura, Y. (2008). Ethanol production from paper sludge by immobilized *Zymomonas mobilis*. *Biochemical Engineering Journal, 42*(3), 314-319.

Yang, Z. (2015). Immobilization of *Enterococcus faecalis* cells with chitosan: A new process for the industrial production of L-citrulline. *Process Biochemistry, 50*(7), 1056-1060.

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