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Soybean β-Glucosidase Immobilisated on Chitosan Beads and its Application in Soy Drink Increase the Aglycones

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ABSTRACT

The objective of this study was to investigate the immobilisation efficiency of soybean β-glucosidase (181.6 U/mL; 23.8 mg protein/mL) on activated chitosan beads. Central Composite Rotational Design (CCDR) 2^3 was used and the application of immobilised enzyme in commercial soy drink was evaluated. The activation of chitosan beads was achieved with established 2.5% glutaraldehyde, pH 7.5, 8 h incubation time (6 h with agitation and 2 h without agitation) at 37ºC. The highest immobilisation efficiency (%) of soybean β-glucosidase on chitosan beads obtained was 37.74 U/mL and 18.84 mg protein/4 chitosan beads at pH 7.5 and 20 h coupling time of enzyme-matrix (7 h with agitation and 13 h without agitation) at 4ºC. The immobilised enzyme incubated at 50ºC, pH 5.5 resulted in 24% increase in the aglycones content in commercial soy drink after 60 min.

Key words: *β*-glucosidase soybean, immobilisation, chitosan, aglycones

INTRODUCTION

β-glucosidases (*β*-D-glucopyranoside glucohydrolases- E.C. 3.2.1.21) are enzymes that hydrolyze glycosidic bonds to release the non-reducing terminal glucosyl residues from glycosides and oligossaccharides. These enzymes are found widely in nature, can be synthetized by the plants or produced by the animals and microorganisms. Soy contains glucosidic isoflavones (daidzin, genistin and glycitin), which can be hydrolysed by *β*-glucosidases and then releases the aglycones (daidzein, genistein and glycitein). Aglycones forms exhibit higher biological activity and are more metabolically that can be absorved faster in higher amounts than glycosides (Izumi et al. 2000). The isoflavones are associated with the risk reduction or prevention of various diseases, such as breast cancer and prostate cancer (Liggins et al. 2000), osteoporosis, menopause symptoms (Levis et al. 2010), cardiovascular disease (Rimbach et al. 2008), improved memory (Lephart et al. 2002), estrogenic and antioxidant activity (Liu et al. 2010; Ma et al. 2010). Soy contains 2% of the aglycones in relation of the total isoflavones (Matsuura and Obata 1993). Thus, the application of endogenous β-glucosidase and its immobilisation can be an alternative to promote the bioconversion of the glycosidic isoflavones to aglycones with the aim to produce soyfoods with higher aglycones content. The immobilisation of soybean β-glucosidase and its application has not been described.

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The application of immobilised enzymes is a strategy for continuous bioprocesses and can improve the efficiency of biotechnological processes with reduction in production costs. This process increases enzyme stability and facilitates the separation of the reaction product (Chang and Juang 2007; Su et al. 2010). Several chemical and physical methods are used for immobilisation of an enzyme and must be materials of readily available and abudant, inexpensive, easy to operate on large scale, show high retention capacity (Nagashima 1984).

The enzyme immobilisation efficiency depends on various factors such as pH, agitation time and time-out (Cao 2006; Shing 2009; Sheldon 2011). Support activation with glutaraldehyde is a simple and efficient method that improves the stability of the enzyme due to multipontuais linkage or among the subunits of the enzyme (Betancor et al. 2006). Chitosan is a suitable support for the enzymes immobilisation because it is biocompatible, available in various forms (gel, membrane, fiber and film), nontoxic, biodegradable, and resistant to chemical modifications (Yi et al. 2009; Gomathi et al. 2010). Therefore, chitosan has potential for application as biomaterial (Arnaud et al. 2010).

The objective of this study was to investigate the immobilisation of soybean β-glucosidase on chitosan and evaluated its application in commercial soy drink to increase the aglycone.

MATERIAL AND METHODS

Material

Soybean cultivar BRS 213, developed at Empresa Brasileira de Pesquisa Agropecuária, Embrapa/Soja, Londrina, Parana, Brazil, was used. The grains were ground in a knife grinder (100 mesh) (TE 631, Tecnal, Brazil) to obtain a finely granulometric flour. The soymilk was obtained from a local commercial centre.

Extration of β-glucosidase of soy cotyledon flour

The conditions for β-glucosidase extration were as described by Matsuura and Obata (1993) with some modifications. Sixty grams of soy cotyledon flour and 100 mM sodium phosphate buffer, pH 6.6, in a 1:10 proportion (w/v) were mixed and slowly agitated at 4ºC for 1 h and then centrifuged at 4,000 *x*g at 4ºC for 15 min. The supernatant was acidified with 0.1 M HCl to pH 5.0, and the samples were centrifuged again under the same conditions. The supernatant obtained (extract crude) was precipitated by ammonium sulfate at 4ºC according to Santos et al. (2013). The crude extract was first precipitated by ammonium sulfate at 4ºC and 40% saturation. After centrifugation at 4,000 *x*g at 4ºC for 15 min, ammonium sulfate was added to the supernatant until 85% saturation was achieved and centifuged again under the same conditions. The precipitateds were resuspended in a 50 mM citrate phosphate bufer, pH 5.0 and dialysed with the same buffer at 4ºC for 14 h.

β-glucosidase activity

β-glucosidase activity was determined using ρ-nitrophenyl-β-D-glucopyranoside (ρ-NPG) substrate according to the procedure described by Matsuura and Obata (1993). The standard curve of ρ-nitrophenol (0.04-0.32 µmol) was prepared. One β-glucosidase activity unit (U) was defined as the amount of enzyme needed to liberate 1 µmol of ρ-nitrophenol/min under the assay conditions. The soluble protein content was quantified using the method described by Lowry et al. (1951) with a bovine serum albumin standard solution (40-400 µg/mL). The specific activity was determined as the relationship between the enzymatic activity and the protein content, which was expressed as U/mg.

Experimental design for the activation of chitosan beads with glutaraldehyde

Chitosan beads in the concentration of 1% glutaraldehyde (p/v) (Sigma-Aldrich) were prepared according to Kumar et al. (2009) and activated by incubation and agitation at 37ºC. Central Composite Rotational Design (CCDR) with 2^3 factorial, six axial points and three replicates at the central point was used, with a total of 17 randomized experiments. Table 1 presents the code and the real levels of independent variables X_1 (glutaraldehyde concentration, %), X_2 (pH of wash buffer) and X_3 (incubation time of activation system at 37ºC). The response function Y_1 (immobilisation efficiency of β-glucosidase on activated chitosan beads with glutaraldehyde, %) was evaluated according to mathematical model: $Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + e$ (Equation 1) where: Y = response function, x_1, x_2 *e* x_3 = levels of coded variables, β = estimated coefficients on the

response surface and *e* = pure error. The response function (immobilisation efficiency, %) was calculated as a relationship between the specific activity of immobilised and specific activity of free enzyme multiplied by 100.

Table 1 - Coded and real levels of independents variables used in a Central Composite Rotational Design (CCRD) 2^3 to activation of chitosan beads with glutaraldehyde.

Variables	Levels				
	−1.68	−1	0	+1	+1.68
X_1 = glutaraldehyde concentration (%)	0.0	1.7	2.5	4.2	5.0
X_2 = pH (wash buffer)	5.0	5.5	6.5	7.5	8.0
X_3 = incubation time at 37ºC*	0/8	2/6	4/4	6/2	8/0

* agitation time (h)/time without agitation (h).

Experimental design for the immobilization of β-glucosidase on activaded chitosan beads with glutaraldehyde

Soybean β-glucosidase immobilisation was conducted after determining the conditions of activation of the chitosan beads with glutaraldehyde using a new Central Composite Rotational Design (CCDR) with 2^3 factorial, six axial points and three replicates at the central point with a total of 17 randomized experiments. Table 2 presents the code and the real levels of independent variables X_4 (protein content (mg)/4 activated chitosan beads with glutaraldehyde), X_5 (pH of wash buffer) and X_6 (linkage time between enzyme and activated chitosan at 37ºC). The response function Y_2 (immobilisation efficiency (%) of β-glucosidase on activated chitosan beads with glutaraldehyde to 2.5%) was evaluated according to mathematical model expressed in the Equation 1. The response function Y_2 (immobilisation efficiency, %) also was calculated as a relationship between specific activity of immobilised and specific activity of free enzyme multiplied by 100.

Statistical analysis

After analysing the response functions (Y_1 and Y_2), the analysis of variance (ANOVA) of the regressions and the coefficients of determination (R^2) were determined and used to compare the fit of the model by the experimental data. Response surface graphs were generated for each response function evaluated. All the analyses were carried out and all the graphs were created using Statistica 7.0 software, version 4.0 (Statsoft, Inc., 2004, Tulsa, USA).

Commercial soy drink with immobilised soybean β-glucosidase

Commercial soybean drink (1.0 mL) and two chitosan beads with immobilised soybean β-glucosidase were incubated at 50ºC, pH 5.5 for 15, 20, 30, 45 and 60 min, then the commercial soy drink was filtered for the analysis. Isoflavones content were quantified by High Performance Liquid Chromatography (HPLC).

Table 2 - Coded and real levels of independents variables used in a Central Composite Rotational Design (CCRD) 2^3 to soybean β-glucosidase immobilisation.

Variables	Levels				
	−1.68	−1	0	+1	+1.68
X_4 = protein content (mg)/ chitosan beads*	6.0	10.0	15.0	20.0	24.0
X_5 = pH (wash buffer)	5.0	5.5	6.5	7.5	8.0
X_6 = linkage time at 4ºC**	0/20	7/13	10/10	13/7	20/0

*4 activated chitosan beads with 2.5% glutaraldehyde. **agitation time (h)/time without agitation (h).

Determination of isoflavones content by HPLC

The isoflavones were extracted according to Carrão-Panizzi et al. (2002) and quantified by the method of Berhow (2002) by HPLC (Model 2690, Waters, USA) with a reverse phase column ODS C18 (YMC-Pack ODS-AM S-5 µm, with a diameter of 4.6 mm and length 250 mm) and a diode array detector (model 996, Waters, USA) adjusted to a wavelength of 254 nm. A linear binary gradient system with methanol, trifluoroacetic acid and ultrapure deionized water was used for separation. The initial gradient was 20%, reached 80% at 35 min and returned to 20% at 40 min. The mobile phase flow rate was of 1.0 mL/min, and the temperature during the race was kept constant at 25ºC. Quantitation was performed with the external standard calibration curves of daidzin, genistin, glycitin, daidzein, genistein, glycitein, malonyldaidzin, malonylgenistin, malonylglycitin, acetyldaidzin, acetylgenistin and acetylglycitin purchased by Sigma Chemicals Co. (St. Louis, E.U.A.), and the results were expressed as µg isoflavones/ mL of commercial soy drink.

RESULTS AND DISCUSSION

The extract obtained from the cotyledon soybean flour with β-glucosidase activity of 181.6 U/mL and the protein content of 23.8 mg/mL was used as enzyme source for immobilization. Chitosan beads activation with glutaraldehyde increased significantly ($p \leq 0.05$) the immobilisation efficiency of soybean β-glucosidase. The high immobilisation efficiency (Y_1 = 41.55%) was observed in seven assays (Table 3) with 1.7% glutaraldehyde, pH 7.5 and 8 h incubation time (6 h with agitation and 2 h without agitation). This result was 11.5 times higher than the efficiency obtained for un-activated immobilised enzyme active. However, at 4.2 and 5.0% glutaraldehyde, a descrease in the immobilisation efficiency (22 and 44%, respectively) was observed. The increase in the concentration of glutaraldehyde increases the amount of free aldehyde groups on the support surface (Hua et al. 2009), promoting the immobilisation of the enzyme. However, the gradual increase of the concentration of glutaraldehyde can produce excessive crosslinking, which may cause aggregation, precipitation, distortion in the three-dimensional structure of the enzyme and decreased activity. In addition, at high concentrations of glutaraldehyde, the chitosan beads become fragile and brittle and the immobilisation efficiency of the enzyme decreases (Kumar et al. 2009).

Table 3 - Central Composite Rotational Design (CCRD) 2^3 with independent variables and the response function Y_1 and Y_2.

Experiments	X_1	X_2	X_3	Y_1	X_4	X_5	X_6	Y_2
1	1.7	5.5	2/6	10.59	10.0	5.5	7/13	32.60
2	4.2	5.5	2/6	22.70	20.0	5.5	7/13	53.48
3	1.7	7.5	2/6	32.66	10.0	7.5	7/13	70.27
4	4.2	7.5	2/6	39.10	20.0	7.5	7/13	73.75
5	1.7	5.5	6/2	12.19	10.0	5.5	13/7	11.46
6	4.2	5.5	6/2	32.56	20.0	5.5	13/7	20.64
7	1.7	7.5	6/2	41.55	10.0	7.5	13/7	22.90
8	4.2	7.5	6/2	32.26	20.0	7.5	13/7	45.37
9	0.0	6.5	4/4	3.62	6.0	6.5	10/10	17.57
10	5.0	6.5	4/4	23.39	24.0	6.5	10/10	60.15
11	2.5	5.0	4/4	11.88	15.0	5.0	10/10	13.65
12	2.5	8.0	4/4	31.64	15.0	8.0	10/10	22.72
13	2.5	6.5	0/8	25.03	15.0	6.5	0/20	46.10
14	2.5	6.5	8/0	33.99	15.0	6.5	20/0	39.41
15	2.5	6.5	4/4	24.92	15.0	6.5	10/10	55.33
16	2.5	6.5	4/4	26.06	15.0	6.5	10/10	57.70
17	2.5	6.5	4/4	26.78	15.0	6.5	10/10	52.65

Y_1 (immobilisation efficiency of β-glucosidase on activated chitosan beads with glutaraldehyde, %), Y_2 [immobilization efficiency (%) of immobilised β-glucosidase on activated chitosan beads with glutaraldehyde 2.5%], X_1 (glutaraldehyde, %), X_2 (pH), X_3 (incubation time, h at 37ºC), X_4 (protein content/4 chitosan beads activated with glutaraldehyde 2.5%), X_5 (pH) e X_6 [coupling time: agitation time (h)/time without agitation (h) at 4ºC]

The pH variation also significantly influenced ($p \leq 0.05$) the chitosan beads activation with glutaraldehyde. The immobilisation efficiency increased from 10.6 to 41.6% at pH from 5.5 to 7.5 at 1.7% glutaraldehyde. The pH variation can increase the amount of amino groups on the surface of chitosan, favoring the binding of glutaraldehyde and therefore, increasing the immobilisation efficiency (Dwevedi and Kayastha 2009).

The agitation of the immobilization system during the incubation time at 37ºC had a significant effect ($p \leq 0.05$) on the chitosan beads activation with glutaraldehyde. The immobilisation efficiency increased from 22.7 to 32.56% with the increase of agitation time 2 to 6 h. Thus, the relationship between the incubation time with and without agitation of the immobilisation system was important in the activation process of the support. The highest agitation time advance the contact of glutaraldehyde with chitosan beads.

According to regression parameters, independent variables X_1 (glutaraldehyde concentration, %) (β_1 = 4.61), X_2 (pH) (β_2 = 7.38) and X_3 (agitation time

of immobilisation system, h) ($\beta_3 = 2.09$) showed a significant linear positive effect at a 5% significance level on the response function Y_1 (immobilisation efficiency of β-glucosidase on chitosan beads activated with gluctaraldehyde). The quadratic effect of variable X_1 ($\beta_1^2 = -2.92$), X_3 ($\beta_3^2 = -2.76$) and the interation X_1X_2 ($\beta_1\beta_2 = -4.42$) had also significant effects on the response function Y_1. However, the quadratic effect of variable X_2 ($\beta_2^2 = 0.01$) and interation X_1X_3 ($\beta_1\beta_3 = -0.93$) and X_2X_3 ($\beta_2\beta_3 = -1.18$) were not significant at a 5% significance level. Thus, considering only the significant variables, the following mathematical model was developed: $Y_1 = 25.48 + 4.61\ x_1 - 2.92\ x_1^2 + 7.38\ x_2 + 2.09\ x_3 + 2.76\ x_3^2 - 4.42\ x_1x_2$. The lack-of-fit of the model was significant (95%) and 86% (R^2) of the experimental data was properly adjusted to the model. The significant lack-of-fit should not be considered relevant when the mean square of the pure error was low (1.759) (Box and Drapper 1987).

The response surface (Fig. 1) showed a region where the chitosan beads activation reflected on immobilisation efficiency. For the interaction between pH and glutaraldehyde, the ranges were 7.5- 8.5 and 1.7-4.0% (Fig. 1A) and for the interaction glutaraldehyde concentration and incubation time, the ranges were 1.7-4.0% and 7-8 h (Fig. 1B).

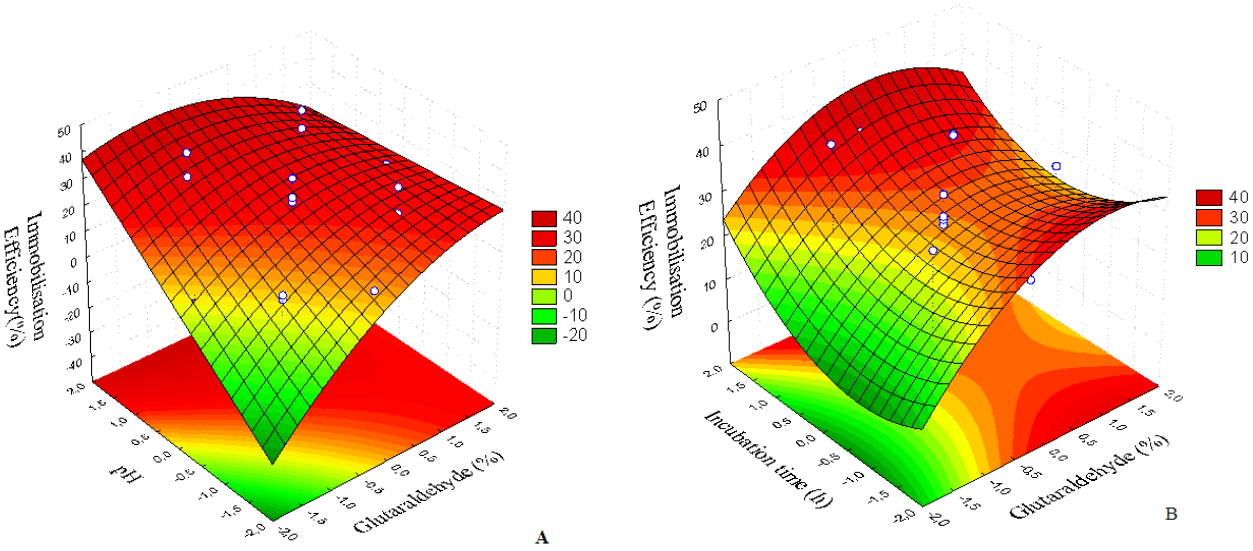

Figure 1 - Model of response surface for glutaraldehyde (%) and pH (A) and glutaraldehyde (%) and incubation time (h) (B) on the immobilisation efficiency of soybean β-glucosidase on chitosan beads activated with glutaraldehyde (Y_1).

After establishing the optimum conditions for chitosan beads with glutaraldehyde (2.5% glutaraldehyde, pH 7.5 and 8 h incubation time with agitation), the variables protein content (mg) per support (X_4), pH wash buffer (X_5) and coupling time of enzyme on the matrix at 4ºC (X_6) were investigated.

The higher immobilisation efficiency (%) (Y_2) on the chitosan beads activated with 2.5% glutaraldehyde was observed in the 4th assay (73.75%) (Table 3) with 20 mg of protein, pH 7.5 and coupling time of 20 h incubation time (7 h with agitation and 13 h without agitation). Reducing the pH from 7.5 to 5.5 and maintaining the other variables, the immoblisation efficiency decreased to 27%. The increase of agitation time from 7 to 13 h decreased it to 39%. With 10 mg of protein, pH 5.5 and 13 h of agitation time, 6.5 times reduction in the immobilisation efficiency (%) were achieved. These results suggested the importance of the pH and agitation time on the immobilisation efficiency. The variable X_4 (protein content per support, mg) ($\beta_4 = 9.34$), X_5 (pH wash buffer) ($\beta_5 = 8.00$) and X_6 (coupling time enzyme-matrix at 4ºC ($\beta_6 = 10.32$) showed a significant linear positive effect at a 5% significance level on the response function Y_2 (immobilisation efficiency (%) on the chitosan beads activated with 2.5% glutaraldehyde). The quadratic effect of variable X_4 ($\beta_4 = -3.71$) and

X_5 (β_5 = – 11.01) were significant and negative while the quadratic effect of variable X_6 (β_6 = – 10.32) and of interation (X_4, X_5, X_6) were not significant.

Considering only the significant variables, the following mathematical model was developed: $\hat{Y}_2 = 54.62 + 9.34\ x_4 - 3.71\ x_4^2 + 8.00\ x_5 - 11.01\ x_5^2 - 10.32\ x_6$. The lack-of-fit was not significant (at 95%) and 77% (R^2) of the experimental data was properly adjusted to the model.

The response surface (Fig. 2) showed a region where Y_2 was maximal. Therefore, the ranges of pH and protein content established were 7.5-8.5 and 20-24 mg (Fig. 2A) and pH and coupling time enzyme-matrix as 7.5-8.5 and 7 h with agitation and 13 h without agitation (Fig. 2B).

There were no reports on the immobilization of soybean β-glucosidase. However, an efficiency of 48.2% β-glucosidase immobilisation has been reported from almonds on chitosan activated with 0.5% glutaraldehyde (Chang and Juang 2007). Immobilisation of mature seeds soybean urease and of pea β-galactosidase on chitosan activated with 1.0% glutaraldehyde resulted in 65 and 67% efficiency, respectively (Kumar et al. 2009; Dwevedi and Kayastha 2009). These differences in immobilisation efficiencies could be attributed to the diversity of enzymes used and the type of interactions between the enzyme and matrix. Results showed that the soybean β-glucosidase immobilisation efficiency obtained in this work was higher than the others enzymes and matrix, e.g., α and γ-alumina (15%) and cellulose (18%) (Martino et al. 1996), Eupergit C (30%) (Tu et al. 2006), chitosan (25.1%) and alginate (31.4%) (Su et al. 2010). Thus, the the present results could be considered satisfactory, because it was possible to obtain 73.75% immobilisation efficiency. The soybean β-glucosidase immobilisation efficiency increased from 41.6 to 73.8%.

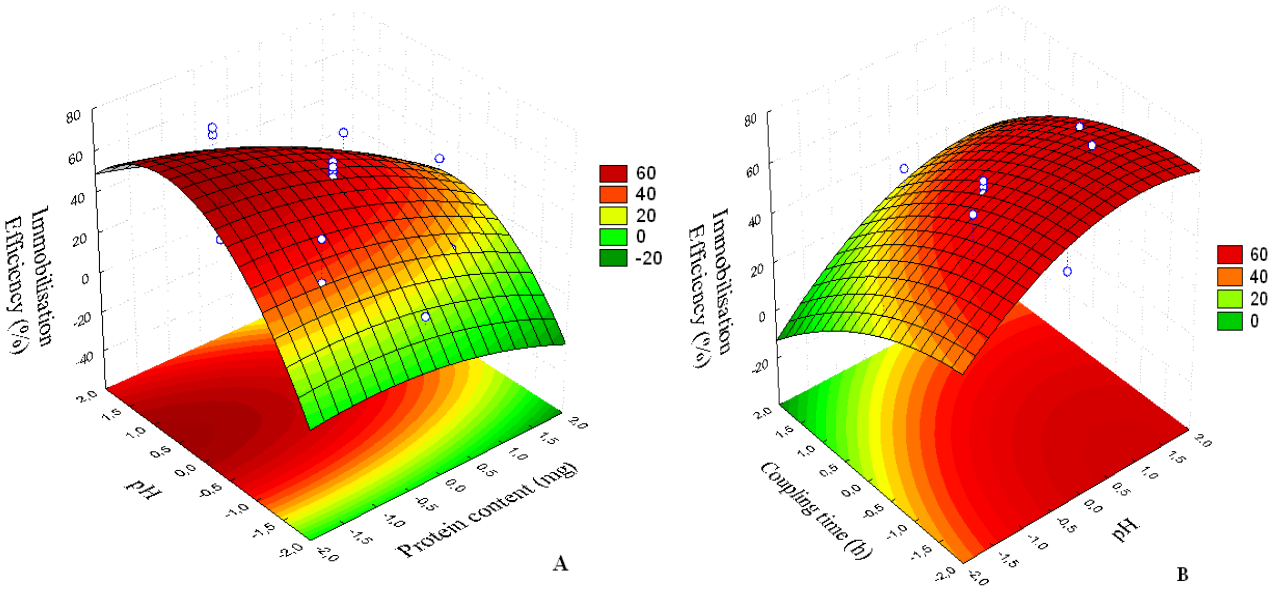

Figure 2 - Model of response surface for pH and protein content (A) and pH and coupling time enzyme-support (B) on the immobilisation efficiency of soybean β-glucosidase on chitosan beads activated with glutaraldehyde 2.5% (Y_2).

The β-glucosidase enzyme immobilised on chitosan beads activated with 2.5% glutaraldehyde was added in the commercial soy drink and isoflavones content were quantified (Table 4). The commercial soy drink without β-glucosidase enzyme immobilised showed 169.40 µg/mL of total isoflavones content (109.30 µg/mL β-glucosides, 27.90 µg/mL malonyl-glucosides and 32.20 µg/mL aglycones, respectively). After 60 min of incubation, an increase of 24% in the aglycones content was obtained, which was probably due the β-glucoside activity that hydrolyzed the β-glucosides forms in aglyconas. Thus, the addition of β-glucosidase enzyme immobilised in the commercial soy drink increased the aglycones content. No reports about

soybean β-glucosidase immobilisation and on the addition in soy drinks were found. Chen et al. (2013) reported that β-glucosidase of *Aspergillus niger* immobilised on spent coffee grounds was capable of catalysing the hydrolysis of isoflavones in black soymilk and aglycones content increased from 13.29 to 69.72% after 60 min of application of the enzyme.

Table 4 - Isoflavones content (µg de isoflavonas/mL) in commercial soy drink without and with soybean β-glucosidase immobilised on chitosan beads activated with 2.5% glutaraldehyde*.

Incubation time (min)	Glycosides	Malonyl-glycosides	Aglycones
0	109.30^{a}	27.90^{bc}	32.20^{d}
15	100.20^{c}	27.50^{c}	31.70^{d}
20	100.50^{bc}	27.90^{bc}	34.00^{c}
30	102.40^{b}	29.30^{a}	35.30^{bc}
45	98.20^{d}	29.20^{a}	36.10^{b}
60	88.70^{e}	26.10^{d}	40.00^{a}

*values obtained are an average of two repeats. Means followed by equal letters in the same column do not differ by Tukey's test at 5% probability.

CONCLUSION

The highest immobilisation efficiency (%) of soybean β-glucosidase on chitosan beads activated with 2.5% glutaraldehyde was obtained with 18.84 mg protein/mL and 37.74 U/mL per 4 chitosan beads at pH 7.5 and coupling time of enzyme-matrix of 7 h with agitation and 13 h without agitation at 4ºC. After incubation with immobilised enzyme, the commercial soy drink showed an increase of 24% in the aglycones contents after 60 min.

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