Optimization of the drying parameters for an enzymatic extract, and an evaluation of the subsequent proteolytic and cellulolytic activities

Otimização dos parâmetros de secagem para extrato enzimático e a posterior avaliação de suas atividades proteolítica e celulolítica

Títulos abreviados:
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ABSTRACT

Enzymes reduce the activation energy needed to convert a substrate into product and are therefore called biological catalysts. Peptidases are enzymes that act on the peptide bonds present in protein substrates, whereas cellulases act in various ways to degrade cellulose. Two types of cellulases are endoglucanase and exoglucanase, called in this paper carboxymethylcellulase (CMCase) and Avicelase, respectively. *Trichoderma spp.* can produce cellulases and peptidases due to the versatility of their saprophytic characteristics. The drying of enzymes is an interesting procedure because it provides the highest long-term stability. The enzymatic extract produced by *Trichoderma harzianum* was submitted to spray drying, and the stabilities of the dried enzymes were verified using casein as the substrate for a peptidase assay and carboxymethylcellulose and Avicel as substrates for a cellulolytic activities assay. The aim of this work was to select the adequate adjuvant and to evaluate the best parameters to use in drying the *T. harzianum* enzymatic extract, which contained peptidase, CMCase and Avicelase. We also wanted to verify the stability of these enzymes after the dried microparticles were solubilized and stored at 4°C or 25°C for 30 days.

Keywords: spray drying, solid bioprocess, *Trichoderma harzianum*, experimental design.

RESUMO

Enzimas reduzem a energia de ativação necessária para converter um substrato em produto e são por esse motivo são chamadas de catalisadores biológicos. Peptidases são enzimas que agem sobre as ligações peptídicas presentes em substratos proteicos, enquanto que as celulases atuam em várias etapas da degradação da celulose. Dois tipos de celulases são a endoglucanase e a exoglucanase, denominadas nesse trabalho de carboximetilcelulase (CMCase) e Avicelase, respectivamente. *Trichoderma spp.* é capaz de produzir celulases e peptidases devido a versatilidade proporcionada por suas características saprofíticas. A secagem de enzimas é um processo interessante por ser capaz de proporcionar estabilidade por um maior período de tempo. O extrato enzimático produzido por *Trichoderma harzianum* foi submetido à secagem por *spray drying*, e a estabilidade das enzimas após a secagem foi analisada utilizando caseína como substrato para o ensaio de peptidase e carboximetilcelulose e Avicel como substratos para os ensaios de atividades celulolíticas. O objetivo deste trabalho foi selecionar o adjuvante adequado e avaliar os melhores parâmetros para a utilização na secagem do extrato enzimático de *T. harzianum*, que contêm peptidase, CMCase e Avicelase. Nós também verificamos a estabilidade destas enzimas depois que as micropartículas secas foram solubilizadas e estocadas a 4°C ou 25°C por 30 dias.

Palavras-chave: *spray drying*, bioprocesso sólido, *Trichoderma harzianum*, planejamento experimental.

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INTRODUCTION

Filamentous fungi are often used to generate biotechnological products due to their metabolic versatility and their capacity to produce organic acids, polysaccharides and enzymes, (EL-ENSHASY, 2007) which can include amylases, peptidases, (hemi-) cellulases, pectinases, lipases and others (VAN DEN HOMBERGH et al., 1997). *T. harzianum* is a filamentous fungus that is able to secrete different types of peptidases (MANCZINGER et al., 2002; SUAREZ et al., 2004) and cellulases (DELABONA et al., 2013).

Peptidases are enzymes that cleave the peptide bonds of protein structures, liberating amino acids and peptides (ESKIN; HENDERSON; TOWNSEND, 1971). This enzyme type is widely applicable and is used in detergent, cosmetic and food industries. Due to their commercial value, peptidases currently represent 60% of the world enzyme market (RAO et al., 1998; EL-ENSHASY et al, 2008; KRISHNA et al., 2009; GAUR et al., 2010).

Cellulases have been studied for applications in food, textile, paper, detergent and animal food industries as well as in agriculture. The advent of bio-refineries is largely responsible for the expansion of the cellulase market because this enzyme provides sugars that can be converted into bioethanol (MAMMA et al., 2009). Cellulose degradation occurs by the actions of three enzymes classes: endo-1,4-β-glucanases, exo-1,4-β-glucanases and 1,4-β-glycosidases (HORN et al., 2012).

Extract drying is based in liquid elimination and is used to make the enzymes as stable as possible (SHARAPIN et al., 2000). Spray drying is suitable to heat sensitive materials as enzymes because the solvent evaporation provides a cooling effect and exposes the dried product to relatively low temperatures (NAMALDI; ÇALIC; ULUDAG, 2006). Spray drying is used at the industrial scale because it is faster, has a higher productivity and is therefore cheaper (CASTELLO; MATTOCKS, 1962). The aim of this work was to select the adequate adjuvant and to evaluate the spray drying parameters used for the enzymatic extract of *Trichoderma harzianum*, as well as to examine the stability of enzyme samples stored at 25°C and 4°C after microparticle solubilization.

MATERIAL AND METHODS

Microorganisms

The fungus *Trichoderma harzianum* was isolated from silage and identified by the research group of Prof. Dr. André Rodrigues (Universidade Estadual Paulista "Julio de Mesquita Filho" Instituto de Biociências de Rio Claro). This fungus belongs to a collection of microorganisms in the Enzyme Technology Laboratory of the School of Pharmaceutical Sciences of Ribeirão Preto – University fo São Paulo. The fungus was kept in potato dextrose agar (PDA) slants at 4°C. To prepare the inoculum, the fungus was peaked in the slants and maintained for 7 days at 30°C, and the spores of the fungus were then scraped from the surface of the culture medium in the presence of saline solution (0.1% w/v $(NH_4)_2SO_4$, 0.1% w/v NH_4NO_3 and 0.1% w/v $MgSO_4 \bullet 7H_2O$).

Adjuvant selection

To select the adjuvant most suitable for the drying process, we tested the following adjuvants: carboxymethylcellulose RV 3000 (CMC), microcrystalline cellulose USP (MicC), Aerosil®, Farmal™ DT7483 dextrin, maltodextrin, mannitol and capsul MB153. The initial and final solids were determined via analytical balance. The total solids corresponding to solids of enzymatic extract and adjuvants. The yield was used to determine the best adjuvant and was calculated in the following manner:

$$Yield\ (\%) = \left(\frac{final\ solids\ (g)}{initial\ solids\ (g)}\right) \times 100$$

Drying

The first step was to determine the total solids contained in the enzymatic extract using a Ohaus® MB 45 moisture analyzer balance. Next, we mixed the enzymatic extract with the different adjuvants to select the appropriate adjuvant. The chosen adjuvant was tested in a Box-Behnken experiment to establish the proper assay proportions and drying parameters (Table 1). The assay control included no adjuvant. The drying process was performed by a spray dryer, model MSD 0.5 (Labmaq do Brasil). When selecting the adjuvant and developing the experimental design, the air flow was adjusted to 3 m^3/min and the drying parameters of outlet air temperature (named as drying temperature - DT), liquid flow rate and ratio of enzymatic extract to adjuvant (EE/Ad) were varied. The microparticles were resuspended in water and stored at 4°C or 25°C for 30 days. The peptidase activities were evaluated at days 0 and 30.

Table 1. The Box-Behnken experimental design for drying the enzymatic extract produced by *T. harzianum*.

	Coded factorial levels			Uncoded factorial levels		
Experiment	**EE/Ad***	**Liquid flow rate**	**DT**	**EE/Ad***	**Liquid flow rate**	**DT**
1	0	-1	-1	1:1	1 mL/min	40°C
2	0	-1	1	1:1	1 mL/min	80°C
3	0	1	-1	1:1	5 mL/min	40°C
4	0	1	1	1:1	5 mL/min	80°C
5	-1	0	-1	1:3	3 mL/min	40°C
6	-1	0	1	1:3	3 mL/min	80°C
7	1	0	-1	3:1	3 mL/min	40°C
8	1	0	1	3:1	3 mL/min	80°C
9	-1	-1	0	1:3	1 mL/min	60°C
10	-1	1	0	1:3	5 mL/min	60°C
11	1	-1	0	3:1	1 mL/min	60°C
12	1	1	0	3:1	5 mL/min	60°C
13	0	0	0	1:1	3 mL/min	60°C
14	0	0	0	1:1	3 mL/min	60°C
15	0	0	0	1:1	3 mL/min	60°C

***EE/Ad: Proportion of solids present in enzymatic extract to adjuvant (dextrin).**

Proteolytic activity

To evaluate the proteolytic activity of the enzyme extract, casein was used as a substrate in the protocol described by Sarath et al. (1996), with slight modification. The mixture of reaction tubes contained the sample and 1% (w/v) casein prepared in 50 mM monobasic sodium phosphate buffer at pH 6.5, which were then incubated together for 100 minutes at 45°C. At the end of the reaction time, we added 10% trichloroacetic acid (TCA) to stop the reaction. The blank tubes were prepared with denatured enzymes in the same reaction conditions. After the enzymatic reaction, the test tubes and blanks were centrifuged at 10,000 x *g* at room temperature for 10 minutes. The supernatants of the test tubes were measured in triplicate against their blanks at 280 nm in a Genesys 10S spectrophotometer (Thermo). One unit of activity was defined as the amount of enzyme required to cause an increase of 0.001 A_{280} within the reaction conditions, using casein as a substrate (SARATH et al., 1996). The peptidase activities of the respective experiments at day 0 were considered 100% relative activity. The solid quantity of enzymatic extract used was constant in all experiments.

Cellulase activities

To evaluate the endoglucanase/CMCase and exoglucanase/Avicelase activities, we used as substrates 1% carboxymethylcellulose (Synth) and 1% Avicel® (Sigma) prepared in 50 mM acetate buffer at pH 5.0. The cellulolytic activity assays were performed according to Daba et al. (2011) with modifications. The reducing sugars that were liberated by enzyme actions were estimated colorimetrically using 3,5-dinitrosalicylic acid as a reagent (MILLER, 1956). The samples were measured in triplicate against their blanks at 540 nm in a Genesys 10S spectrophotometer (Thermo). The blanks were prepared in the same manner than reaction tubes, however the samples had been boiled for 10 minutes to inactivate the enzymes. The CMCase and Avicelase activities of the respective experiments at day 0 were considered 100% relative activity. The solid quantity of enzymatic extract used was constant in all experiments.

Statistics analysis

Statistical analysis of the response surface methodology was performed by Minitab 14.0 software, assuming significance levels of 5%. We analyzed the influence of the drying temperature, liquid flow rate and ratio of enzymatic extract to adjuvant on the enzymatic activities and yields.

RESULTS AND DISCUSSION

Yields of spray drying with different adjuvants

The material used in the drying procedure was a crude extract produced in solid fermentation by *T. harzianum*, combined with either carboxymethylcellulose RV 3000 (CMC), microcrystalline cellulose USP (MicC), Aerosil®, Farmal™DT7483 dextrin, maltodextrin, mannitol, or capsul MB153. In Table 2, the yields obtained with the different adjuvants are shown. The adjuvant dextrin had the highest yield in the tested conditions (Table 2).

Table 2. The yields of a crude enzyme extract, produced in solid-state fermentation by *T. harzianum*, which was dried with different adjuvants.

Adjuvant	DT	Liquid flow rate	EE/Ad	Yield (%)
Control	40°C	5 mL/min	25%	12.6

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CMC	40°C	5 mL/min	25%	27.9
MicC	40°C	5 mL/min	25%	19.5
Aerosil	40°C	5 mL/min	25%	27.3
Dextrin	40°C	5 mL/min	25%	34.0
Maltodextrin	40°C	5 mL/min	25%	33.2
Mannitol	40°C	5 mL/min	25%	5.1
Capsul	40°C	5 mL/min	25%	20.0

CMC: carboxymethylcellulose; MicC: microcrystalline cellulose.

Spray drying causes water evaporation and may also change the chemical structure of some proteins due to a partial loss of water (NAMALDI; ÇALIC; ULUDAG, 2006). Dextrin is an adjuvant that can provide a decrease in the deposition of powder (BRANCO et al., 2010) and, consequently, an increase in the yield.

Yield of spray drying with dextrin as the adjuvant

Dextrin was the selected adjuvant because it had the best performance in the previous experiment (Table 2). The drying conditions were modified and tested by a Box-Behnken experimental design (Table 1) to determine the optimum set. The powder obtained from each experiment was measured to determine the total yield of the process (Table 3).

Table 3. The yields of a crude enzyme extract produced by *T. harzianum*, which was spray dried with dextrin as an adjuvant and with drying conditions that were varied by a Box-Behnken experimental design.

Experiment	EE/Ad*	Liquid flow rate**	DT***	Yield (%)
1	0	-1	-1	29.2
2	0	-1	1	52.9
3	0	1	-1	33.0
4	0	1	1	43.8
5	-1	0	-1	28.0
6	-1	0	1	39.6
7	1	0	-1	30.9
8	1	0	1	42.4
9	-1	-1	0	51.0
10	-1	1	0	42.2

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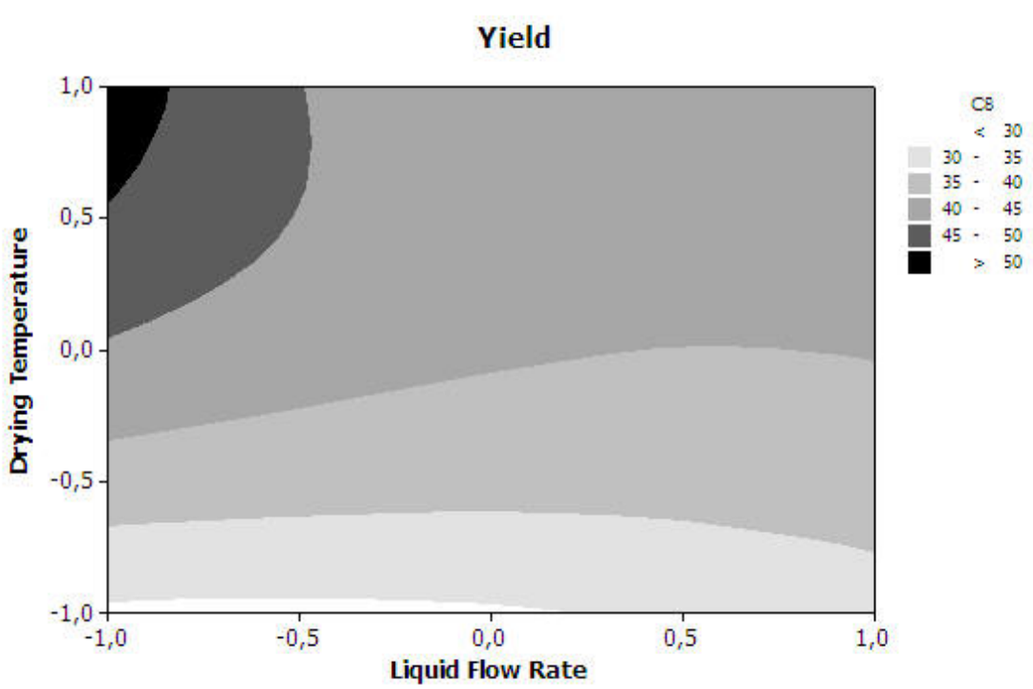
11	1	-1	0	37.9
12	1	1	0	38.2
13	0	0	0	44.1
14	0	0	0	43.0
15	0	0	0	34.5

***EE/Ad: 25% (-1), 100% (0) and 175% (1); **Liquid flow rate: 1 mL/min (-1), 3 mL/min (0) and 5 mL/min (1); ***DT: 40°C (-1), 60°C (0) and 80°C (1).**

The worst yields were obtained in experiments 1, 3, 5 and 7, which all had as a parameter the lowest drying temperature, whereas yield of above 50% were obtained with the lowest liquid flow rate (1 mL/min) and a high DT (60 and 80°C). Statistical analysis using Minitab 14.0 software demonstrated that the highest DT led to the best yields (Figure 1). These results are expected because highest outlet air temperature provides low humidity e best recovery.

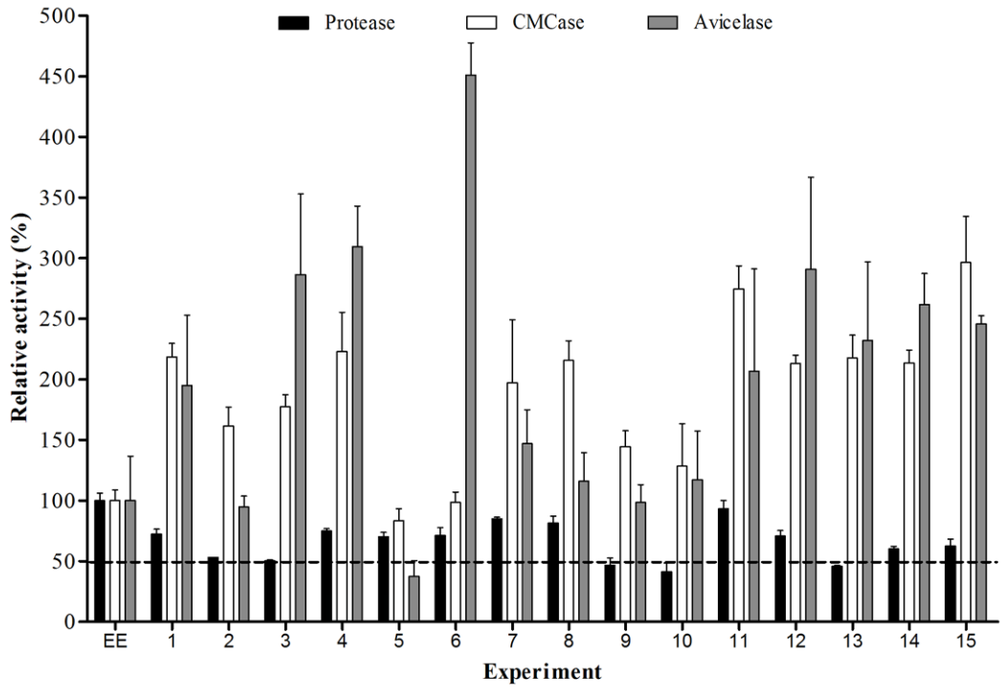
Figure 1. A contour plot of the yield versus the drying temperature and the liquid flow rate.



Enzyme activities after the spray drying process, using dextrin as the adjuvant

The powder obtained in each experiment was also used to determine the peptidase, CMCase and Avicelase activities (Figure 2). In Figure 2, the relative activity values are expressed as means and standard deviations. The value of the crude extract without drying was considered 100%.

Figure 2. Enzyme activities after the spray drying process. The activities presented for each enzymatic extract represent 100% of the relative activity. All of the values were calculated using the enzymatic extract as the control sample. The solid quantity of enzymatic extract used was constant in all experiments.



Note that all experiments had decreased proteolytic activity when compared to EE, though experiment 11 maintained activity levels above 90%. CMCase activity was sustained or increased by the drying process, and experiment 15 presented activity 3 times higher than EE. Avicelase activity was reduced in experiment 5 but was retained or improved in the others experiments, when compared to EE.

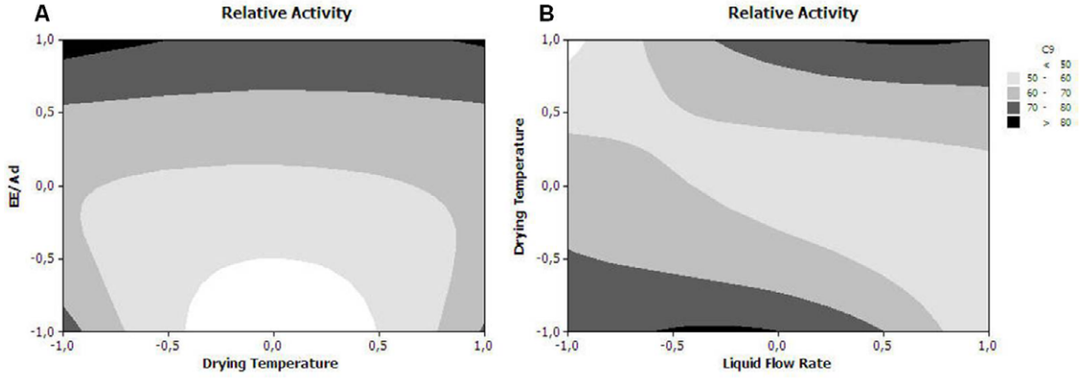
Dextrin is a polymer that is used as an adjuvant and a protective agent. In this study, dextrin was selected because it allowed for the best enzyme recovery, and the enzymes maintained their activities even after drying. However, Belghith, Chaabouni, Gargouri (2001) observed in studies with cellulases that the adjuvant with the best yield did not necessarily cause the best thermal stability.

CMCase (β-1,4-endoglucanase) and Avicelase (β-1,4-exoglucanase) have specificity for β bonds. However, *Trichoderma harzianum* also has great potential as a producer of others hydrolytic enzymes, such as peptidases, β-glucosidase (MARCO; VALADARES-INGLIS; FELIX, 2003) and amylase (MARCO; VALADARES-INGLIS; FELIX, 2003; AZEVEDO; MARCO; FELIX, 2000). The compound α-amylase is able to cleave α-1,4 bonds, and dextrins are polymer mixtures of D-glucose units linked by α-(1→4) or α-(1→6) glycosidic bonds; thus, dextrin can be a second substrate for α-amylase. The additional cleavage of dextrin could justify the increase in CMCase and Avicelase activities observed in some samples because the presence of α-amylase in the extract can generate more reducing sugars.

Here, the peptidase demonstrated higher thermolability than did the cellulases because the drying process, with dextrin used as a stabilizing agent, reduced peptidase activity but maintained cellulases activities. Millqvist-Fureby et al. (1999) purposed two mechanisms, based on other studies, to explain the stabilizing effects caused by carbohydrates such as dextrin.In the first hypothesis, carbohydrates replace the hydrogen bonds that had previously formed between the protein and the water molecules in an aqueous solution. In the second proposed explanation, a rigid amorphous matrix formed by carbohydrates prevents structural changes in the protein. In the same study, carbohydrates like dextrin that formed an amorphous matrix with different concentrations of trypsin better retained enzyme activity (MILLQVIST-FUREBY et al., 1999).

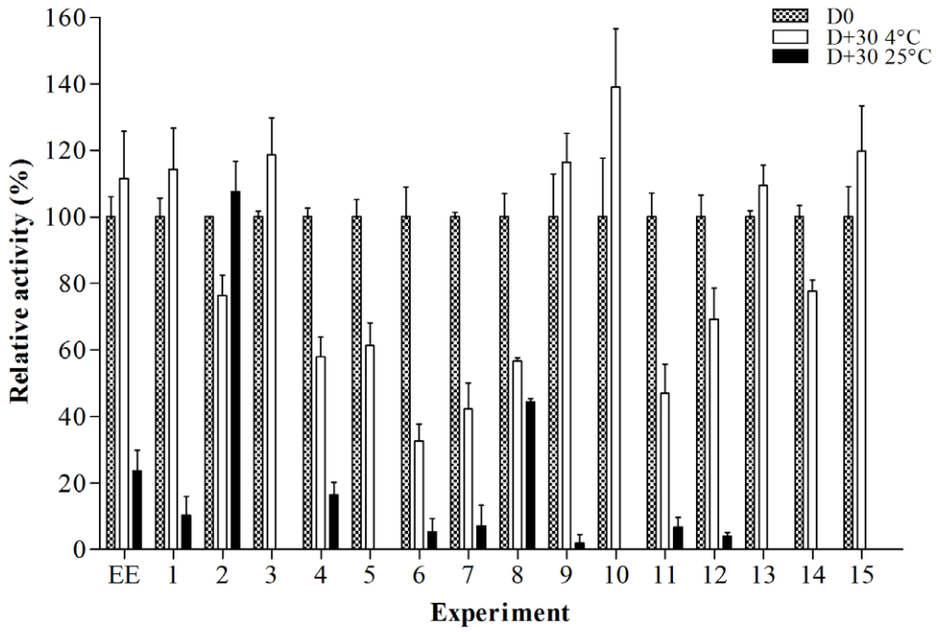
The enzyme activity results obtained after spray drying were statistically analyzed. Examining the EE/Ad ratio revealed that the highest amounts of dextrin provided the best peptidase activities (Figure 3A). Furthermore, the interaction between the other two parameters showed that the best peptidase activities were obtained with a combination of either the highest DT and liquid flow rate or the lowest DT and liquid flow rate (Figure 3B). An analysis of the parameters influencing the CMCase and Avicelase activities demonstrated that the DT, liquid flow rate and EE/Ad parameters had no significant effects ($p>0.05$).

Figure 3. A contour plot of peptidase relative activity. (A) Relative activity versus the EE/Ad ratio and the drying temperature. (B) Relative activity versus the drying temperature and the liquid flow rate. EE/Ad: ratio of enzymatic extract to adjuvantPeptidase activities after 30 days of storage at 4°C and 25°C



The solubilized samples were stored at two different temperatures, 4°C and 25°C, and were analyzed at days 0 and 30. Figure 4 illustrates the stability profile of the peptidase activities after the microparticles were solubilized in water and stored.

Figure 4. The stability of the peptidase activity of solubilized microparticles that were resuspended in water and stored at either 4°C and or 25°C for 30 days. The peptidase activities of the respective experiments at day 0 were considered 100% relative activity. The solid quantity of enzymatic extract used was constant in all experiments.

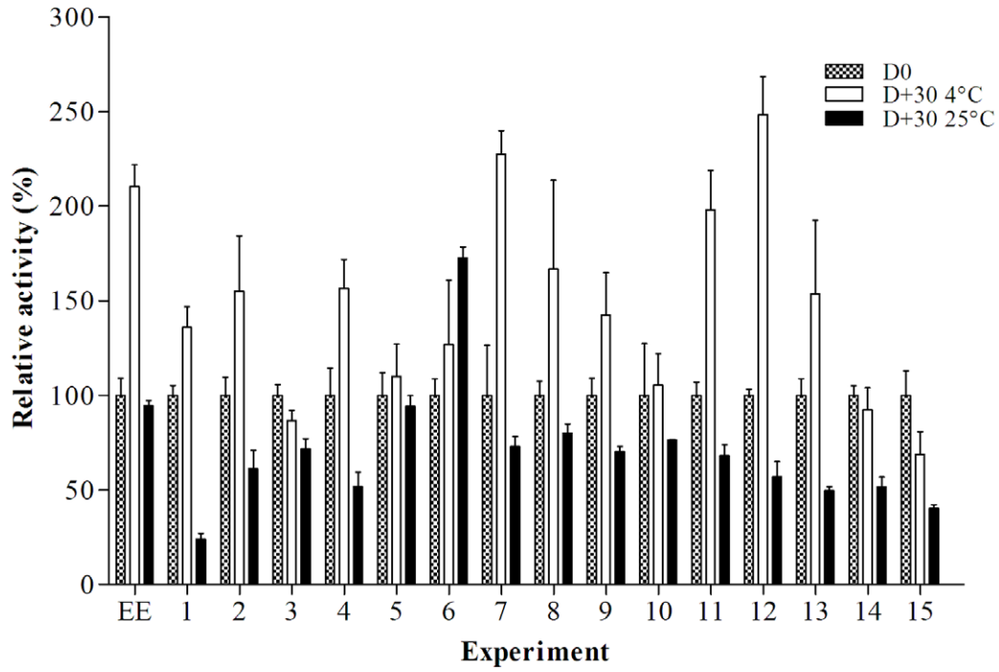


In Figure 4, we can observe that experiments 3, 5, 10, 13, 14 and 15 had a total loss of peptidase activity after 30 days at 25°C, while a residual activity remained after storage at 4°C. In experiment 2, no decreased activity occurred due to storage at either 4°C or 25°C. This protective effect may be due to the high yield and the low initial peptidase activity at day 0. In general, storing the solubilized microparticles at 4°C was more beneficial than at 25°C. It is important to note that the EE was not submitted to the drying process.

CMCase and Avicelase activities after 30 days of storage at 4°C and 25°C

The solubilized samples were stored at two different temperatures, 4°C and 25°C, and were analyzed at days 0 and 30. The CMCase (Figure 5) and Avicelase (Figure 6) activities were evaluated after this period. Figure 5 shows the stability profile of the CMCase activities after the microparticles were solubilized in water and stored.

Figure 5. The stability of the CMCase activity of solubilized microparticles that were resuspended in water and stored at either 4°C and or 25°C for 30 days. The CMCase activities of the respective experiments at day 0 were considered 100% relative activity. The solid quantity of enzymatic extract used was constant in all experiments.



Note that in Figure 5, the samples stored at 4°C for 30 days presented activities similar to those of day 0. The samples maintained at 25°C for 30 days demonstrated decreased endoglucanase levels.

Figure 6 illustrates the stability profile of the Avicelase activities after the microparticles were solubilized in water and stored.

Figure 6. The stability of the Avicelase activity of solubilized microparticles that were resuspended in water and stored at either 4°C and or 25°C for 30 days. The Avicelase activities of the respective experiments at day 0 were considered 100% relative activity. The solid quantity of enzymatic extract used was constant in all experiments.

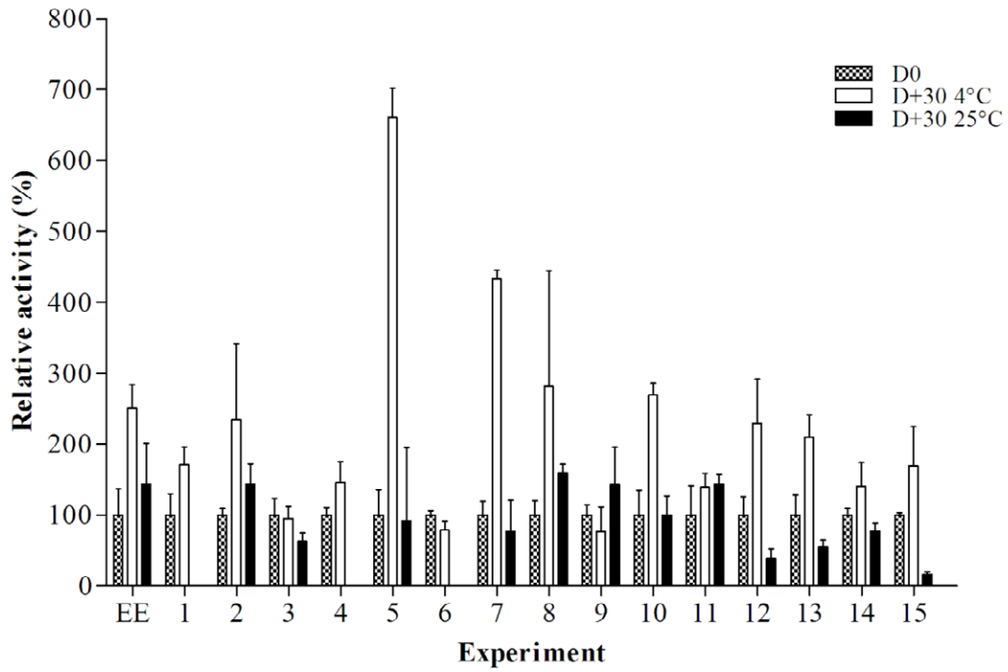


Figure 6 shows that in experiments stored at 4°C, there was no loss of activity when compared to day 0. Storage at 25°C presented different profiles when compared to day 0. In samples 2, 8, 9, 10 and 11, the Avicelase activity was maintained or increased, while in samples 3, 5, 7, 12, 13, 14 and 15, a decrease in Avicelase activity was shown. Samples 1, 4 and 6 completely lost their exoglucanase activity.

In the same way that the results observed in figure 2, the increase presented in some CMCase e Avicelase activities may be explained by additional cleavages in dextrin used as adjuvant.

CONCLUSION

In this work, dextrin was shown to be a better adjuvant for the spray drying process than other tested compounds. The best spray drying yields were observed in the highest temperatures (60°C e 80°C). The microparticles that were solubilized and stored at 4°C maintained the greatest stability of the tested peptidase, CMCase and Avicelase activities.

ACKNOWLEDGMENTS

This work received financial support provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (2011/06986-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (PQ-2).

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Received 02 April 2013
Accepted 16 May 2013