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# Optimization of spray-drying conditions for obtaining *Bacillus* sp. SMIA-2 protease powder

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**Abstract**

Microbial proteases, especially from *Bacillus* spp., have enormously been exploited for a broad variety of applications such as for physiological processes, food and feedstuff, detergents, as well as in the pharmaceutical and leather. In this work, proteases produced by the thermophilic *Bacillus* sp. SMIA-2 submerged cultures were spray-dried in an attempt to improve its stability for applications in industry. The enzymatic extract was dried using drying adjuvants, and optimal conditions for preserving enzymatic activity were studied following a statistical experimental design. The spray process factors studied were the drier air inlet temperature and the adjuvants concentrations. The responses analyzed were the enzymatic activity and mass recovered of the powder after spray drying. Additionally, the stability of the powder was assessed during 180 days at room temperature. The results revealed that satisfactory levels of enzymatic activity were obtained when 0.5% (w/v) carboxymethylcellulose and 1.0% (w/v) maltodextrin were incorporated to enzymes solutions and the spray drier inlet temperature was 110 ºC. Furthermore, this dried protease extracts showed potential for future commercial applications because of their stability at room temperature for 180 days.

**Keywords:** *Bacillus;* proteases; atomization; storage.

**Practical Application:** In this work, a durable protease-rich powder was obtained, allowing its use in future commercial applications.

## 1 Introduction

Proteases or peptidases constitute the class of enzymes with relevant role in a wide variety of industries, including leather, silk manufacturing, food and detergent industry (Freitas et al., 2012; Nasri et al., 2015; Singh & Bajaj, 2017). Thermostable proteases produced from thermophilic bacteria are advantageous in industrial processes that operate at higher temperatures (Barbosa et al., 2014; Barzkar et al., 2018) and bacteria from the genus *Bacillus* are promising sources for thermostable proteases production (Saggu & Mishra, 2017; Contesini et al., 2018; Zhou et al., 2020).

*Bacillus* sp. SMIA-2, a thermophilic and thermostable enzyme-producing bacterium was able to express a promising level of proteases in submerged cultures employing agricultural byproducts such as whey protein, corn steep liquor and passion fruit rind flour (Silva et al., 2007; Barbosa et al., 2014), which opened perspectives to generate high-value products from sustainable production processes. The bacterium was isolated from a soil sample collected in Campos dos Goytacazes City, Rio de Janeiro, Brazil. Phylogenetic analysis showed that this strain is a member of the *Bacillus* rRNA group 5 (Souza & Martins, 2001). Recently, Bernardo et al. (2020) revealed that SMIA-2 is 100% identical with the type strain *Bacillus licheniformis* Gibson 46 (ATCC 14580$^T$). The characteristics presented by the protease produced by SMIA-2, such as ability to function at high temperatures and pH levels and resistant to several surfactants and oxidizing agents (Silva et al., 2007; Nascimento & Martins, 2004) were very useful when thinking about industrial applications.

The performance of enzymes is influenced by its capacity for long term storage. Therefore, the development of formulations capable of preserving the activity of enzymes until their use is an important step in the manufacture of enzyme containing product (Costa-Silva et al., 2014). Spray drying is a cost-effective drying method widely used to stabilise heat sensitive ingredients, such as enzymes and probiotic bacteria (Schutyser et al., 2012; Assadpour & Jafari, 2019; Bajaj et al., 2021). This technique has been extensively used for the dehydration of many industrial enzymes such as cellulases (Belghith et al., 2001; Libardi et al., 2020), alpha amylases (Samborska et al., 2005; Abdel-Mageed et al., 2019), lipases (Costa-Silva et al., 2014; Utami et al., 2017; Mohtar et al., 2019) and proteases (Namaldi et al., 2006; Cabral et al., 2017; Hamin et al., 2018). However, when subjected to the high temperatures used in the spray drying process, some enzymes may be denatured and, consequently, lose their catalytic activity

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(Hamin et al., 2014). A variety of excipients are often used as bulking agents and protective additives in an attempt to stabilize the enzymes (Ohtake et al., 2011; Emami et al., 2018).

In this work, the proteases recovered from SMIA-2 strain were spray-dried in the presence of adjuvants, and optimal conditions for preserving enzymatic activity were studied using a statistical experimental design.

## 2 Material and methods

### *2.1 Microorganism and culture conditions*

The bacterial strain used in this study was a thermophilic *Bacillus* sp. previously isolated from a local soil sample. The culture medium for protease production contained ($gL^{-1}$): KCl-0.3, $MgSO_4$-0.5, $K_2HPO_4$-0.87, $CaCl_2$-0.29, ZnO-$2.03x10^{-3}$, $FeCl_3.6H_2O$-$2.7x10^{-2}$, $MnCl_2.4H_2O$-$1.0x10^{-2}$, $CuCl_2.2H_2O$-$8.5x10^{-4}$, $CoCl_2.6H_2O$-$2.4x10^{-3}$, $NiCl_3.6H_2O$-$2.5x10^{-4}$, $H_3BO_3$-$3.0x10^{-4}$, commercial corn steep liquor (Sigma Aldrich) -3.0, whey protein-1.0 and soluble starch-5.0 (Corrêa et al., 2011).

The pH was adjusted to 7.2 with 1.0 M NaOH and the medium was sterilized by steam-autoclaving at 121 °C, 1 atm for 15 min. The medium (50 mL in 250 mL Erlenmeyer flasks) was inoculated with 1mL of an standard overnight culture (initial number of cells $10^4$) and incubated at 50 °C in an orbital shaker (Thermo Forma, Ohio, USA) operated at 150 rpm. After 36 h of incubation culture, flasks were withdrawn and the contents were then centrifuged (HERMLEZ 382K, Wehingen, Germany) at 15,500 g for 15 min, at 4 °C, and the cell free supernatant was used as used as crude enzyme preparation.

### *2.2 Protease assay*

The activity of protease was assessed in triplicate by measuring the release of trichloroacetic-acid soluble peptides from 0.2% (w/v) azocasein prepared in 0.1 M Tris/HCl buffer (pH 8.0). The reaction mixture containing 0.5 mL of azocasein solution and 0.5 mL of appropriate concentration of enzyme solution was incubated at 70 °C for 10 min. The 1 mL reaction was terminated by the addition of 0.5 mL of 15% (w/v) trichloroacetic acid and then centrifuged at 20,600 g for 5 min, after cooling (Jenssen et al., 1994). The absorbance of the non-precipitable azopeptides produced as a result of the protease activity was measured at 420 nM and corrected using a reagent blank. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 420 nM equal to 1.0 in 60 minutes.

### *2.3 Spray drying of crude enzyme solution*

Spray-dried powders were produced through the atomization of crude proteases preparation in a lab-scale spray dryer (LAB-PLANT, MODEL SD-04, England). During operation, 100 mL prepared solution was fed at constant rate, 5.40 mL/min with a peristaltic pump to a nozzle, where atomization occurred by means of a pressurized air stream. Drying air entered the drying chamber in the same direction as the descending spray droplets. The process variable was the drier air inlet temperature and the adjuvants concentrations as showed in Table 1. According to the working inlet temperatures 70, 86, 110, 134 and 150 °C, observed drier outlet temperatures were 52, 65, 76, 92 and 102 °C, respectively.

In all of the experiments the air flow rate was constant. The adjuvants were incorporated into the enzyme solution before spray drying. The dried particles were collected and stored at room temperature (25-32 °C).

The protease activity was measured before and after drying. The activity measurement of the proteases after drying operation was accomplished by dissolving enzymes powder ($0.01\ g.mL^{-1}$) and the residual activity (%) determined and compared with the activity of the crude enzyme solution before drying.

**Table 1**. Matrix of CCD $2^3$ (real and coded values) used and their responses.

| Experiment number | Inlet air temperature (°C) | CMC (%, w/v) | MD (%, w/v) | Mass recovery (%) | Residual protease activity (%) |
|---|---|---|---|---|---|
| **1** | 86 (-1) | 0.32 (-1) | 1.6 (-1) | 15.06 | 78.58 |
| **2** | 134 (+1) | 0.32 (-1) | 1.6 (-1) | 19.60 | 85.23 |
| **3** | 86 (-1) | 0.68 (+1) | 1.6 (-1) | 19.15 | 80.32 |
| **4** | 134 (+1) | 0.68 (+1) | 1.6 (-1) | 23.38 | 81.91 |
| **5** | 86 (-1) | 0.32 (-1) | 3.4 (+1) | 20.67 | 67.80 |
| **6** | 134 (+1) | 0.32 (-1) | 3.4 (+1) | 26.20 | 74.67 |
| **7** | 86 (-1) | 0.68 (+1) | 3.4 (+1) | 17.51 | 64.69 |
| **8** | 134 (+1) | 0.68 (+1) | 3.4 (+1) | 19.72 | 63.10 |
| **9** | 70 (-1.68) | 0.5 (0) | 2.5 (0) | 16.72 | 76.99 |
| **10** | 150 (+1.68) | 0.5 (0) | 2.5 (0) | 23.37 | 74.24 |
| **11** | 110 (0) | 0.1 (-1.68) | 2.5 (0) | 19.61 | 79.88 |
| **12** | 110 (0) | 0.8 (+1.68) | 2.5 (0) | 22.01 | 76.70 |
| **13** | 110 (0) | 0.5 (0) | 1 (-1.68) | 17.54 | 86.69 |
| **14** | 110 (0) | 0.5 (0) | 4 (+1.68) | 27.49 | 72.94 |
| **15** | 110 (0) | 0.5 (0) | 2.5 (0) | 16.73 | 80.82 |
| **16** | 110 (0) | 0.5 (0) | 2.5 (0) | 17.46 | 79.45 |
| **17** | 110 (0) | 0.5 (0) | 2.5 (0) | 17.66 | 80.03 |

### *2.4 Experimental design and statistical analysis*

A central composite design (CCD) $2^3$ was constructed to evaluate the effects of the adjuvants concentrations and spray drier inlet air temperature on Residual protease activity and Mass recovery of the powder. The factorial planning encompassed three central points and yielded a total of 17 treatments for each experiment. The factors and factors levels studied are described in Table 1.

The results obtained were submitted to a regression analysis, initially calculating a polynomial equation, evaluating its lack of adjustment and the significance of each variable at $p < 0.05$. The predictive model was adjusted considering only the significant parameters and response surface graphs were drawn for those models with a high correlation coefficient, using the Statistica software, version 7.0.

Condition optimization was performed using CCD, and response surface was produced with fixed central points of 110 ºC to inlet air temperature, 0.5% (w/v) CMC and 2.5% (w/v) maltodextrin. The model of the experiment can be expressed as follows (Equation 1):

$$Y = b_o + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i \neq j=1} \sum b_{ij} x_i x_j \quad (1)$$

Where *bo, bi, bii* and *bij* are the intercept terms, linear, quadratic coefficient and interactive coefficient, respectively, and *xi* and *xj* are coded independent variables.

### *2.5 Mass recovery (%)*

The mass recovery was determined according to Belghith et al. (2001) (Equation 2).

$$\text{Mass recovery}(\%) = \frac{\text{Wt}}{\text{TSS}} \times 100 \quad (2)$$

Where, TSS is the total soluble solid of the crude protease solution in the presence of the adjuvant and Wt is the weight of powder mass (g) obtained from spray-drying process. TSS was determined from the crude extract protease solution using a hand-held digital refractometer (Pocket Refractometer Pal-1, Atago Co. Ltd, Tokyo, Japan). Three measurements were recorded from each sample.

### *2.6 Proteases powder stability (%)*

After drying, storage stability of obtained proteases powders at room temperature (25-32 ºC) up to 180 days was investigated. The stability of protease was determined by measuring protease activities right after drying and after six months of storage.

### *2.7 Water activity (aw)*

The water activity was determined in the dried samples by direct reading in an apparatus (water activity meter, HygroLab C1 Bench-Top Indicator, Rotronic AG, Switzerland).

## 3 Results and discussion

Considering the great industrial relevance of thermostable proteases produced by *Bacillus* sp SMIA-2, an attempt was done to increase their shelf life by using the spray drying process. The proteases were recovered from 36 hours of *Bacillus* sp SMIA-2 submerged cultures containing pectin, corn steep liquor and whey protein. The proteases were not purified before spray drying process because of their high cost. Besides, during the purification process ligand and/or proteins that have a protective effect on the crude enzyme could be removed and consequently, the enzyme properties could modify in such way that their kinetic and thermodynamic behaviour could also be different (Braga et al., 2013). Thus, the crude protease extract (13.82 $UmL^{-1}$) containing by-products of the fermentation process (other proteins, carbohydrates and salts) was subjected to spray drying process, in order to develop stable formulations of the dry enzyme.

It is known that an enzyme functions best at a certain temperature. According to Silva et al. (2007), the optimum temperature for activity of protease from *Bacillus* sp SMIA-2 was 70 °C and the enzyme retained 80% of the original activity after 2 h heat treatment at 60 ºC. An alternative to avoid loss of protease activity would be to use lower temperatures in the drying process. However, the use of low temperatures can lead to a reduction in the production rate and generate a product with higher moisture content and with low storage stability. The addition of excipients to the enzyme crude solution prior to spray drying process is another alternative usually taken for this purpose. Thus, carboxymethylcellulose and maltodextrin were incorporated into the enzyme solution before spray drying and the interactive effect between them and the inlet air temperature (ºC) were studied using a central composite design (CCD) $2^3$. Factor levels (inlet air temperatures and both maltodextrin and CMC concentrations) were chosen based on studies published in the literature focused on protease spray drying (Namaldi et al., 2006; Hamin et al., 2017) and in preliminary experiments. Table 1 shows the results of the Residual protease activity and Mass recovery of powder obtained, according to the experimental planning carried out.

The Residual protease activity values varied from 63.1% to 86.69%. In this case, the best result (Formulation 13) was obtained when 0.5% (w/v) CMC and 1.0% (w/v) MD were incorporated to enzymes solutions and the spray drier inlet temperature was 110 ºC. There are several studies of adding carbohydrates to enzymes containing formulations. The effect of air inlet temperature of the spray dryer between 70 ºC and 130 ºC and the effect of protective additives, glucose and maltodextrin, at 0-2% (w/v) on protease activity produced by recombinant *Bacillus subtilis* (BGSC-1A751) during spray drying were evaluated by Namaldi et al. (2006). Also, Hamin et al. (2018) evaluated the best conditions to remove the water by spray drying technique from collagenolytic proteases from *Myceliophthora thermophila* submerged bioprocess and Siqueira et al. (2013) selected the adequate adjuvant and evaluated the best parameters to use in drying the *Trichoderma harzianum* enzymatic extract containing peptidases and cellulases. According to all these authors higher activity preservation was obtained when the additives

were incorporated before spray drying the formulations and that the degree of improvement was dependent on the nature of the carbohydrate and its concentration.

No powder was recovered when the crude extract rich in proteases was dried in the absence of additives. The filtration of the fermented broth previously to the spray drying process, probably reduced the solids concentration. In fact, the culture supernatant generally contains very low amount of solids leading to high drying cost per unit weight of the product formed with very low product recoveries (Gupta et al., 2014). Therefore, the use of excipients is justified not only to avoid thermal denaturation leading to loss of enzyme activity (Silva et al., 2007; Piszkiewicz & Pielak, 2019; Giovannelli et al., 2021), but also to develop more stable enzyme formulations and to improve dry mass recovery purposes (Libardi et al., 2020).

As can be observed in Table 1, the maximum percentage of mass recovered obtained was about 27% (Essay 14). The low mass recovery may be related to the powder collector system performance (cyclone) used in the equipment. This problem could be solved during the scaling-up process, by choosing a more efficient collecting system (Costa-Silva et al., 2014).

An analysis of variance was performed for the experimental results presented in Table 1 in order to identify the variables that showed statistical significance. In this context, the results illustrated in the Pareto graph (Figure 1) revealed a negative, but statistically significant, influence of the three independent variables on Residual protease activity at the 95% confidence level. Increased concentrations of MD, CMC and inlet air temperature reduced protease activity. The concentration of maltodextrin was the variable that, originally presented a comparatively more pronounced effect (Figure 1A). On the other hand, increased inlet air temperature and increased MD concentration promoted a better mass recovery (Figure 1B). Thus, the conditions that led to higher levels of protease activity were different from those that provided the greatest Mass recovery.

Regression analysis was applied to the experimental results of the Residual protease activity and the Mass recovered. First, a model containing all linear and quadratic effects, as well as the interaction factors between the variables, was tested. Table 2 shows the results of Anova and Regression coefficient of the adjusted model.

The adjusted models were highly significant ($p < 0.05$), with a satisfactory value of determination coefficient ($R^2$). It was possible to state that 85.40% and 86.64% of the variability in the Residual protease activity (%) and Mass Recovered (%) response respectively, could be accounted by the model and that it was suitable to represent the real relationship among the independent variables studied. When the calculated F value is greater than the table for the adopted confidence level, there is sufficient statistical

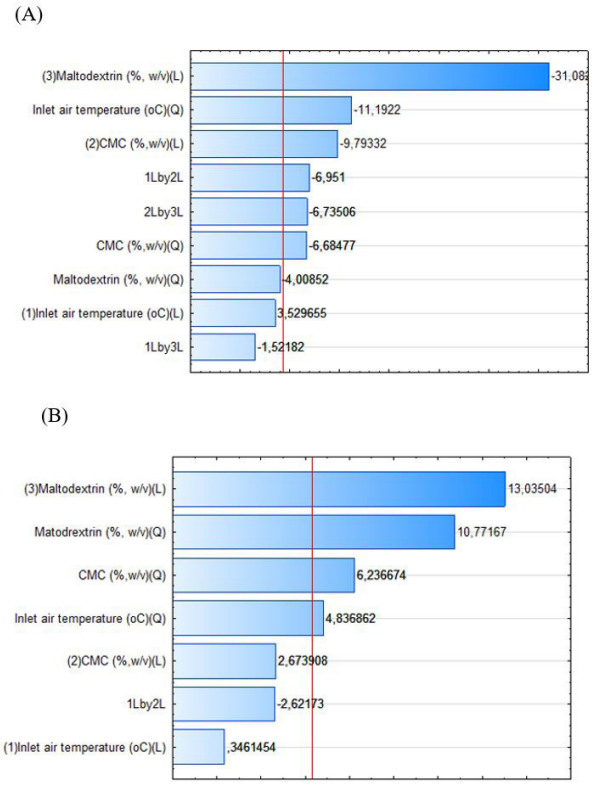


**Figure 1**. Effect of the studied independent variables and their interactions: (A) Residual protease activity (%); (B) Mass recovery, for a 95% confidence level ($p < 0.05$).

**Table 2**. ANOVA for the variables of response surface quadratic model.

| Variable | Sum of squares | (Degrees of freedom) | Mean square | Fcal. | Ftab0.05 | P<0.05 |
|---|---|---|---|---|---|---|
| **Residual activity (%)** | | | | | | |
| Regression | 602.2879 | 2 | 301.1440 | 41.1112 | 3.74 | 0.000689 |
| Residues | 102.5514 | 14 | 7.3251 | | | |
| Lack of adjustement | 101.6056 | 12 | 8.4671 | 17.9047 | 19.41 | |
| Pure error | 0.9458 | 2 | 0.4729 | | | |
| Total error | 694.8852 | 16 | 43.4303 | | | |
| **Mass recovery (%)** | | | | | | |
| Regression | 180.2790 | 2 | 90.1395 | 48.9591 | 3.74 | 0.000438 |
| Residues | 25.7756 | 14 | 1.8411 | | | |
| Lack of adjustement | 25.2964 | 12 | 2.1080 | 8.7969 | 19.41 | |
| Pure error | 0.4793 | 2 | 0.2396 | | | |
| Total error | 192.9564 | 16 | 12.0598 | | | |

evidence to suppose the existence of a relationship between the variables. The lack of adjustment was not significant ($F_{cal}$ less than the $F_{tab}$), indicating that the experimental data adjusted to the obtained models.

Subsequently, in order to conduct a comprehensive study of the influence of the independent variables on the Residual protease activity and Mass recovered of the powder, experimental and predicted values were compared with empirical equations adjusted with base on the analysis of variance (ANOVA). The equations obtained to predict the Residual activity of the protease (Equation 3) and the Mass recovered (Equation 4) of the obtained powder are:

$$\%Residual\ activity = 79.261 - 2.062\,X_1^2 - 1.582\,X_2 - 1.116\,X_2^2 - 5.778\,X_3 - 1.690\,X_1X_2 - 1.637\,X_2.X^3 \quad (3)$$

$$\%\ Mass\ recovery = 17.360 + 2.028\,X_1 + 0.711\,X_1^2 + 0.981\,X_2^2 + 1.731\,X_3 + 1.584\,X_3^2 - 2.189\,X_2.X_3 \quad (4)$$

Where $x_1$ is the Temperature ($^0$C), $x_2$ is the MD and $x_3$ is the CMC concentration (%, w/v).

The response surface and contour plot graphs (Figure 2) showed a relationship between two variables at time. The non-explicit variables were fixed at the central point (level 0) for the surface construction. An initial increase in CMC concentration in combination with lower concentrations of maltodextrin (< 1.75%; w/v) resulted in an increase of Residual protease activity (Figure 2A). In addition, it was observed that higher values of Mass recovery were found when higher concentrations of maltodextrin in combination with lower CMC concentrations were incorporated to enzyme solution. However, when lower concentrations of maltodextrin were combined with higher concentrations of CMC, higher values were also found (Figure 2D). As showed in Figure 2B, inlet air temperatures between 110-140 ºC and CMC concentrations lower than 0.45% (w/v) resulted in higher Residual protease activity. The Mass recovery increased with increasing the inlet air temperatures within the experimental level at both high and low concentrations of CMC (Figure 2E). Higher inlet air temperatures and maltodextrin concentrations lower than 1.75%

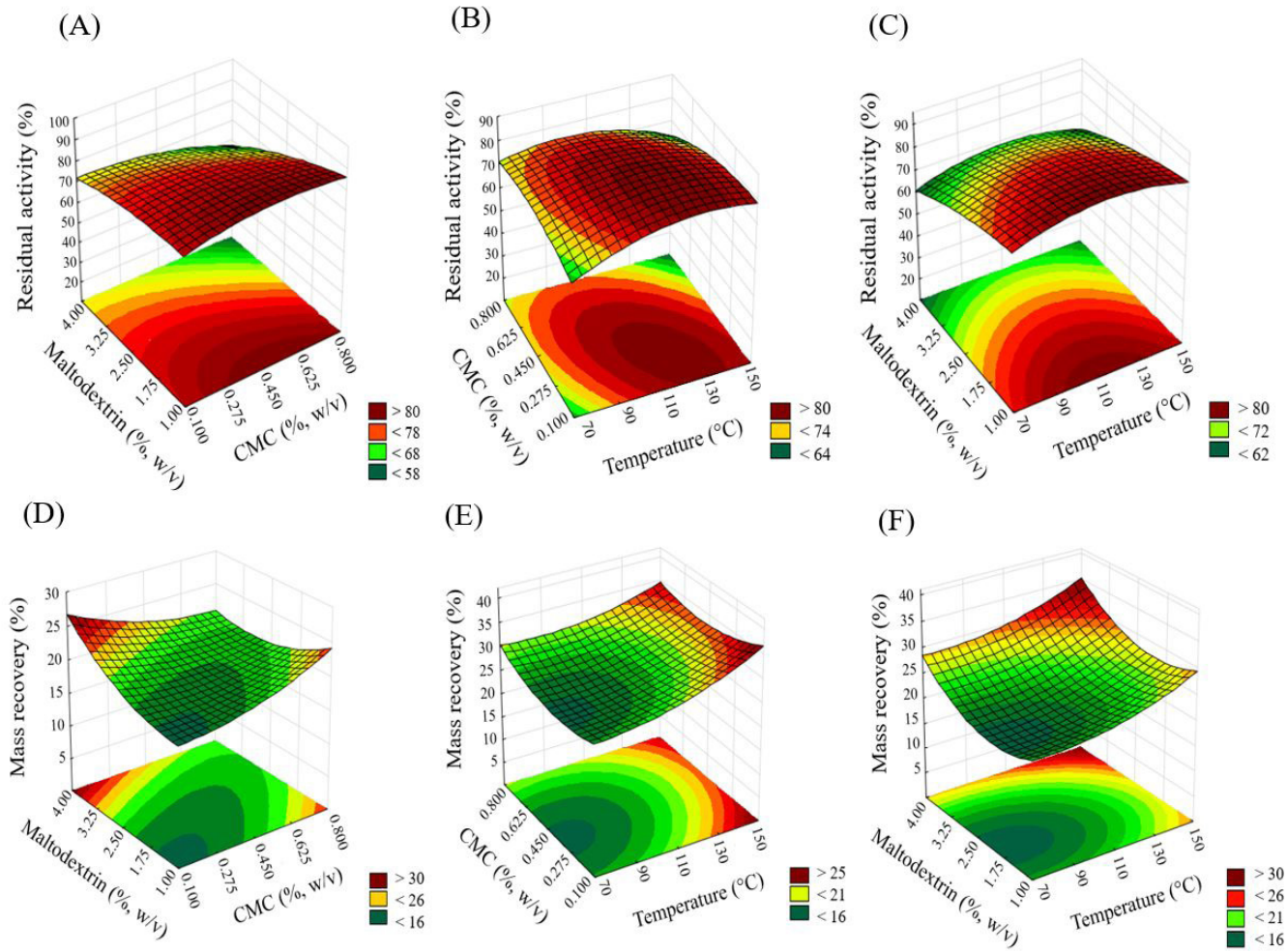


**Figure 2**. Three-dimensional response surface plot for: Effect of maltodextrin and CMC concentration on Residual protease activity (A) and Mass recovery (D) at a constant spray drier inlet temperature; Effect of spray drier inlet temperature and CMC concentration on Residual protease activity (B) and Mass recovery (E) at a constant maltodextrin concentration; Effect of spray drier inlet temperature and maltodextrin concentration on Residual protease activity (C) and Mass recovery (F) at a constant CMC concentration. Dark red color indicates high Residual protease activity and Mass recovery, while green and yellow color indicates low Residual protease activity and Mass recovery.

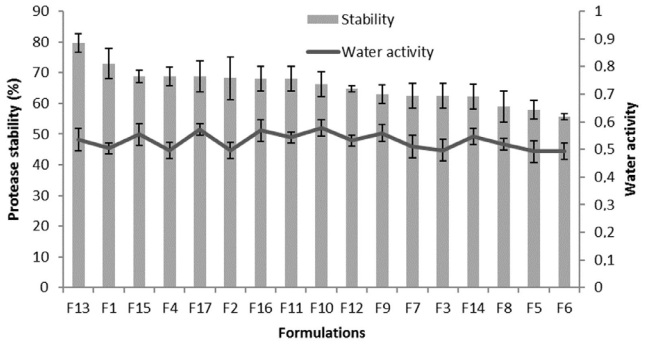


**Figure 3**. Effect of six months of storage on the protease stability (■) and the water activity (▬) of the powder.

(w/v) resulted in higher Residual protease activity (Figure 2C). Furthermore, high inlet air temperatures combined with high maltodextrin concentrations resulted in greater mass recovery (Figure 2F). Thus, the experimental design approach used in this study was found to be effective in rapidly assessing the spray drying conditions for obtaining protease powder at any point within the range of inlet air temperature and concentration of both CMC and maltodextrin tested.

### *3.1 Protease powder stability*

The stability of obtained proteases powders formulations during storage at room temperature for six months was investigated. The highest enzyme stability was maintained (80%) after 180 days for the Formulation 13, that was dried with 0.5% CMC and 1.0% MD and spray drier inlet temperature of 110 ºC (Figure 3). Under this condition, satisfactory levels of protease activity were found.

The water activity (aw) of the proteases dried formulations are also showed in Figure 3. Values below 0.6 were obtained for all formulations, indicating their potential for storage. The values of aw could indicate the possibility of microorganism growth and toxin production. According to Beuchat (1981) water activity values above 0.6 could promote microorganism growth and above 0.8 facilitate toxin production.

## 4 Conclusion

The study showed that the spray drying process used in this work was effective to dehydrate the proteases recovered from the submerged cultures of *Bacillus* sp. SMIA-2, maintaining good enzymatic activity when storage at room temperature for 180 days. The spray-dried protease generated could be very useful for industrial applications such as in the detergent industry, that not require the enzyme in pure form.

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