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Recovery of Cellulase by HPMC-Salt Precipitation

Analysis by Statistical Experimental Design

Sebastião Avelino, Adriano R. Azzoni, Paulo T. V. Rosa, Everson A. Miranda, and Cesar C. Santana*

Department of Biotechnological Processes,
School of Chemical Engineering, State University of Campinas,
CP 6066, CEP13083-970, Campinas, SP, Brazil

Abstract

Production of industrial enzymes including cellulases requires minimum cost with the downstream processing. The objective of this work was to analyze the precipitation of cellulases by ammonium sulfate in the presence of hydroxypropyl(methylcellulose) as a co-precipitant through the use of statistical experimental design. The model generated with the experimental results showed that high protein recovery can be achieved at high levels of temperature, aging times, and rate of salt-solution addition, and at a low mixing level. The results also allowed the observation that activity recovery was improved at high levels of temperature, rate of salt addition and mixing level, and a low level of aging time.

Index Entries: Hydroxypropyl(methylcellulose); precipitation; downstream processing; cellulase.

Introduction

Precipitation of proteins is widely used in downstream processing of proteins even though purification is not achieved in many cases. What makes precipitation a powerful recovery technique is the concentration it can provide. This is specially important regarding industrial enzymes owing to the high cost of "transporting water."

We developed a method to recover and concentrate cellulase by a two-step process involving precipitation with ammonium sulfate and hydroxypropyl(methylcellulose) (HPMC), followed by flotation. This process is based on the process developed by Miranda and Berglund *(1)* for amylases. The introduction of HPMC is done to provide floatability to the precipitate

*Author to whom all correspondence and reprint requests should be addressed.

because efficient floatation can be obtained only with flocs of precipitates having strong hydrophobic character. Because salt precipitation does not guarantee the required minimum hydrophobicity, we used HPMC as a co-precipitant to impart a hydrophobic character to the protein precipitate. Also, the authors claim that there is a possibility of a biospecific interaction between the enzyme and HPMC owing to the similarity of the amylase substrate, amylose, and the linear structure of HPMC, a derivative of a linear carbohydrate, cellulose. The same could happen with the cellulase that has cellulose as a substrate.

In this work, statistical experimental design was used to study the variables related to the physical environment of cellulase precipitation by ammonium sulfate in the presence of HPMC:

1. Incubation temperature;
2. Aging time;
3. Rate of salt addition; and
4. Level of mixing.

The statistical experimental design is a powerful tool used to decrease the amount of experimental work as the effect of variables is studied on a process independently as well the combined effect of different variables.

Materials and Methods

Materials

Crude preparation of cellulase from *Trichoderma reesei* (dried preparation with 15% of protein) was kindly provided by Biobrás-Bioquímica (Brasil S.A.) Brazil. Hydroxypropyl(methylcellulose) was produced by Dow Qu'mica (Brazil). All other chemicals were at least of reagent grade.

Methods

Determination of Protein Concentration

Protein concentration was determined by the Coomassie Blue technique according to the method developed by Bradford *(2)* and at an absorbance at 280 nm.

Cellulase Activity

Cellulase activity was measured by hydrolysis of Whatman's filter paper in 50 m*M* acetate buffer at pH 5.0 at 30°C for 150 min. The increase in reducing power owing to the hydrolysis products was quantified by the 3, 5 dinitrosalicylic acid method *(3)*.

Experimental Design

A factorial design was used to determine the influence of rate of salt addition (from 0.2–30 mL/min), time (from 1–21 min) and temperature of aging (25–45°C), and level of mixing (from 75–255 rpm) on the recovery of

the total protein and activity by the precipitation. The experimental design was done according to Barros Neto and collaborators *(4)*.

Cellulase Precipitation

The enzyme precipitation (batches of 20 mL) was carried out in 50-mL Erlenmeyer flasks inside an orbital shaker with temperature control. Equal volumes of 2 mg/mL solutions of HMPC and cellulase were incubated at the desired temperature for 10 min. After this temperature equilibration, 10 mL of the mixtures were pipeted into Erlenmeyer flasks and the precipitation was carried out by the addition of 10 mL of a 80% saturation ammonium sulfate solution under the desired flow rate with the use of a peristaltic pump. After aging the precipitate at the precipitation temperature for different times, the precipitate was removed by centrifugation at 15,000*g* for 20 min. The precipitate was dissolved in 50 m*M* acetate buffer, pH 5.0, and analyzed for protein concentration and activity.

Results and Discussion

Table 1 shows the result of the precipitation experiments that were carried out according to the conditions also displayed in the same table. The design used was a 2^4 cube plus star (central composite design), with the four independent variables (incubation temperature, aging time, rate of salt addition, and level of mixing), and two dependent variables (protein and activity recoveries). The errors associated with the coefficients were determined by the variability of five experiments at a central point. The values for the minimum and maximum levels of the independent variables were set based on the literature and preliminary experiments. Statistical analysis of the data using the software Modreg (software that comes with the publication Barros Neto and collaborators *[4]*) and Statistica (Statsoft, EUA) allowed the fitting of the data to the following quadratic model:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_1^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \qquad (1)$$

where Y is the dependent variable, β_i the model coefficients, and X_i the independent variables. The values of the coefficients of the quadratic model are presented in Table 2. The dependence of activity and protein recoveries on the variables studied could then be displayed in three-dimensional graphs. The most significant are shown in Figs. 1–3. Despite the low value of the correlation coefficient for activity recovery, the graphs for protein recovery and activity have similarity in shape. Therefore, the tendencies indicated were analyzed.

Preliminary experiments were carried out to determine the conditions of precipitation that would allow the experiments that followed to detect changes in precipitation recoveries. These changes would indicate tendencies for maximum precipitation efficiency regarding the variables of incu-

Table 1
Statistical Experimental Design Results

Assay	Variable				% of Recovered protein	% of Recovered activity
	X1[a]	X2[b]	X3[c]	X4[d]		
1	30	6	7.5	120	54.30	58.85
2	40	6	7.5	120	56.98	54.83
3	30	16	7.5	120	60.33	74.89
4	40	16	7.5	120	64.57	75.73
5	30	6	22.6	120	55.85	62.53
6	40	6	22.6	120	58.08	55.92
7	30	16	22.6	120	60.05	66.58
8	40	16	22.6	120	60.72	68.00
9	30	6	7.5	210	49.24	53.79
10	40	6	7.5	210	57.43	67.48
11	30	16	7.5	210	53.52	69.35
12	40	16	7.5	210	55.86	59.41
13	30	6	22.6	210	52.27	63.11
14	40	6	22.6	210	65.50	70.58
15	30	16	22.6	210	54.03	68.31
16	40	16	22.6	210	58.34	66.55
17	35	11	15.1	165	56.82	64.64
18	35	11	15.1	165	56.14	70.35
19	35	11	15.1	165	56.71	71.06
20	35	11	15.1	165	55.98	65.84
21	35	11	15.1	165	56.07	69.04
22	25	11	15.1	165	55.26	63.50
23	45	11	15.1	165	55.31	70.88
24	35	1	15.1	165	57.79	72.48
25	35	21	15.1	165	61.45	74.77
26	35	11	0.2	165	59.22	68.21
27	35	11	30.0	165	70.99	78.18
28	35	11	15.1	75	58.28	68.79
29	35	11	15.1	255	52.42	65.48

[a]X1, incubation temperature (°C).
[b]X2, aging time (h).
[c]X3, rate of salt addition (mL/min).
[d]X4, level of mixing (rpm).

bation temperature, aging time, rate of salt addition, and level of mixing (an optimization should be the objective of another work). This required conditions that would allow recovery in the range of 50–80%: lower values for recovery would imply large relative errors and they could mask negative effects on the process efficiency; higher values could mask positive effects because recovery higher that 100% is not expected. The set of conditions selected were: final ammonium sulfate concentration, 40% saturation; mass ratio of HPMC to enzyme preparation, 1:1; enzyme preparation concentration, 0.5 mg/mL; pH 5.0). These conditions resulted in recoveries between 60–70% (data not shown).

Table 2
Coefficients of the Quadratic Model

Coefficient[a]	% Protein recovery	*p*	% Activity recovery	*p*
β_0	56.34*	0.00000	68.19*	0.00000
β_1	1.58*	0.00888	0.66	0.58485
β_2	1.05	0.06465	–1.17	0.31938
β_3	1.50*	0.01188	2.76*	0.03464
β_4	–1.51*	0.01141	0.43	0.70670
β_{11}	–0.52	0.31850	1.13	0.35368
β_{22}	0.56	0.27861	0.32	0.77658
β_{33}	1.94*	0.00172	–0.22	0.85245
β_{44}	–0.50	0.33337	–1.18	0.31394
β_{12}	–0.92	0.17023	–1.25	0.40272
β_{13}	0.19	0.77405	0.00	0.99830
β_{14}	1.14	0.09568	1.11	0.45383
β_{23}	–0.93	0.16701	–1.70	0.26063
β_{24}	–1.45*	0.03982	–2.77	0.07560
β_{34}	0.97	0.14973	1.86	0.21888
r^2	0.83		0.55	

[a]1, Temperature.
2, Aging time.
3, Rate of salt addition.
4, Mixing level.
*Most important coefficients ($p < 0.04$).

It was possible to recover up to 71% of the total protein and 78% of the total cellulase activity by the precipitation of protein using ammonium sulfate and hydroxypropyl(methylcellulose) (Table 1). According to the data in Table 2, the most important variable is the rate of salt addition because it is statistically significant for both protein and activity recovery and both increase with increases of this variable.

The aging time of the precipitate has a small but opposite effect on protein and cellulase activity recovery, showing that probably the cellulase is one of the first proteins to precipitate and that it can be replaced from the precipitate by other proteins. Despite being the single variable less important on the protein recovery, its effect is significant when coupled with the effect of level of mixing (Table 2). At long aging times, the recovery of both protein and activity decrease as the level of mixing increases (Fig. 1). Probably, the high shearing at high level of mixing breaks up the aggregates or hinders their formation. At short aging times, the effect is the opposite: despite the short time for particle growth, the high level of mixing may allow the formation of many nuclei that even at small sizes contribute to high precipitation recovery.

High temperatures at short aging times lead to high recoveries of both protein and enzyme activity. This can be owing to the unusual property of HPMC: lower solubility at higher temperature. This low solubility may

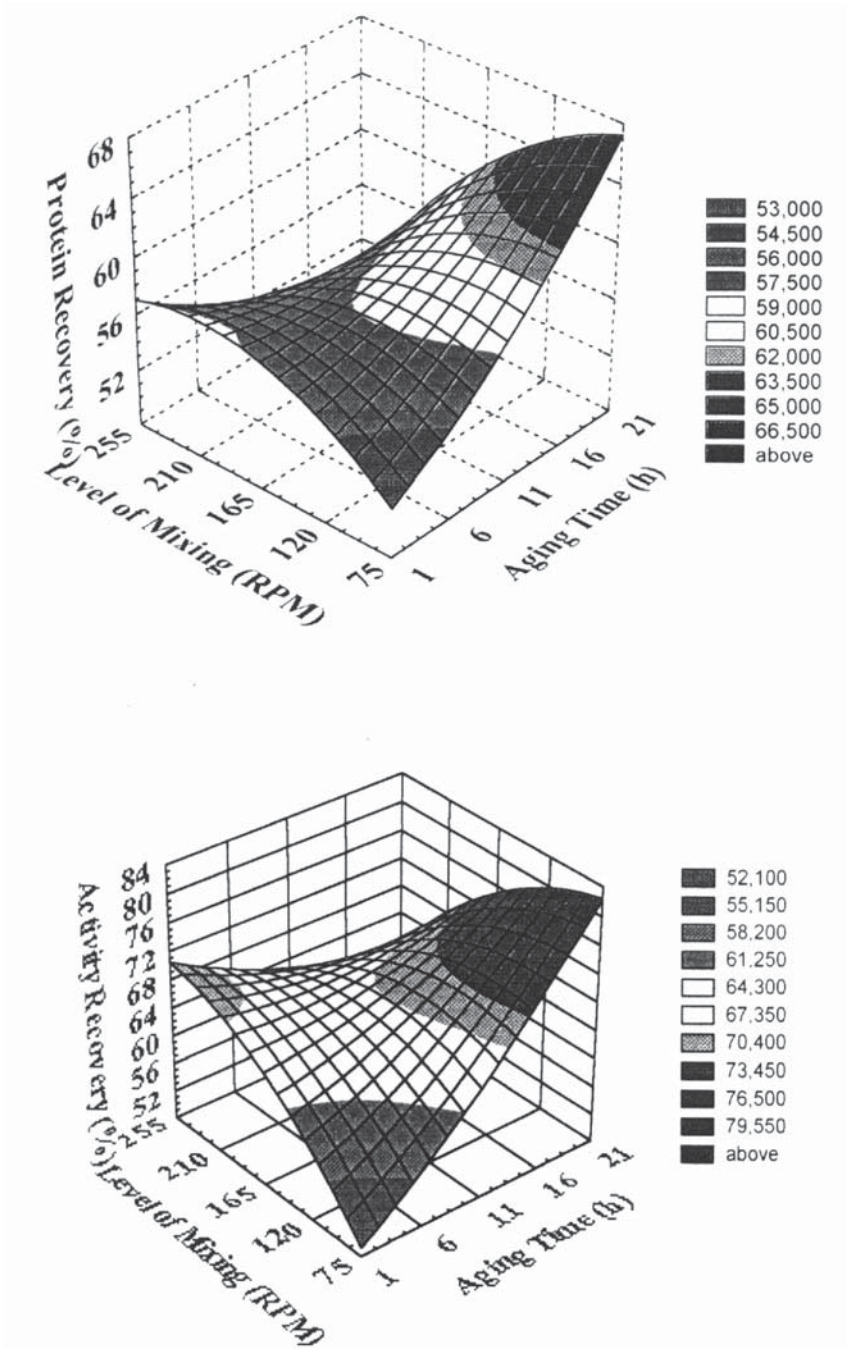

Fig. 1. Mixing level and aging time effect on cellulase precipitation by ammonium sulfate and HPMC.

balance a slow kinetics. At long aging times, high recovery of protein can be achieved through the whole range of temperature studied because there is time for particle formation and growth even with a slow kinetics. How-

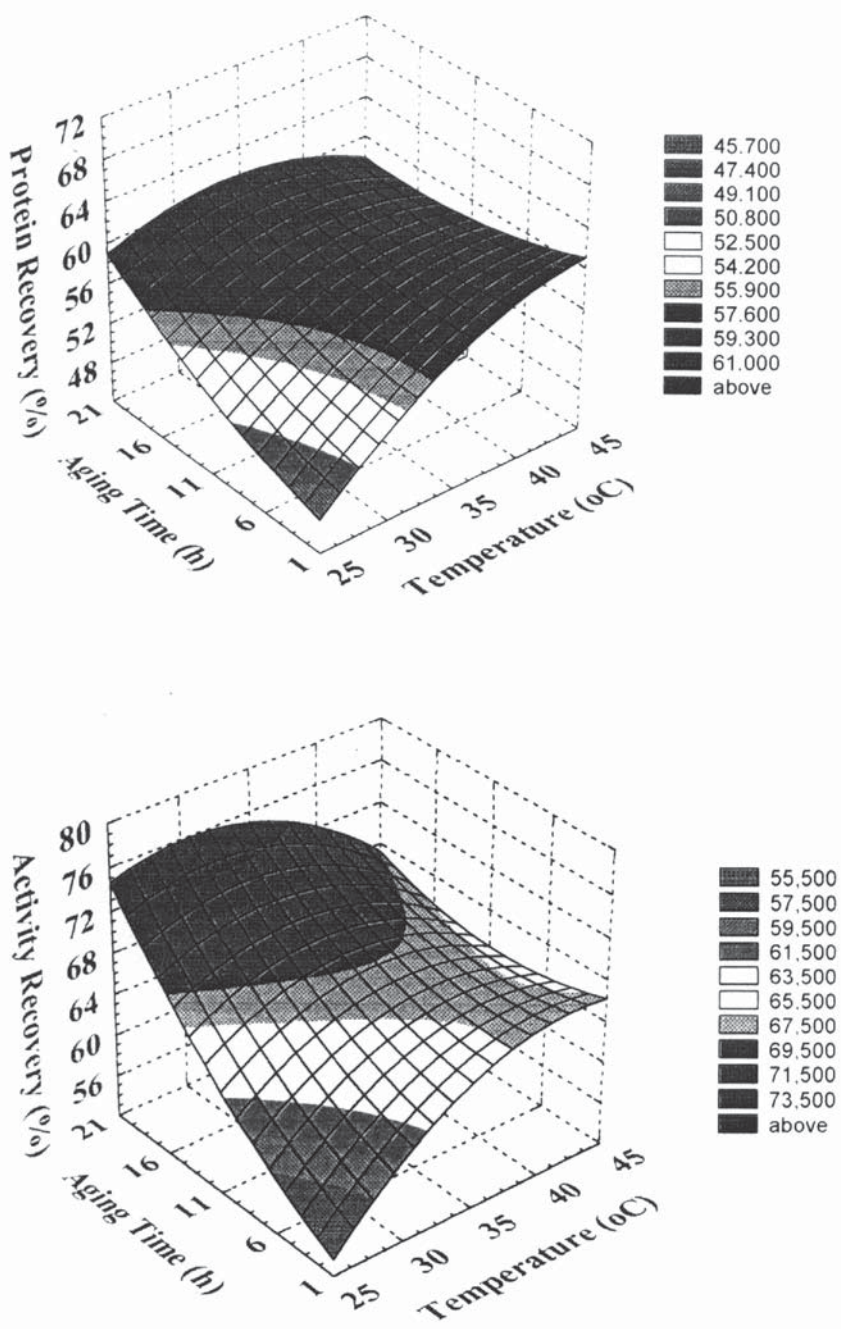

Fig. 2. Temperature and aging time effect on cellulase precipitation by ammonium sulfate and HPMC.

ever, there is a decrease in activity recovery at high temperatures for long aging times (Fig. 2). Enzyme denaturation may be the reason for this decrease in recovery.

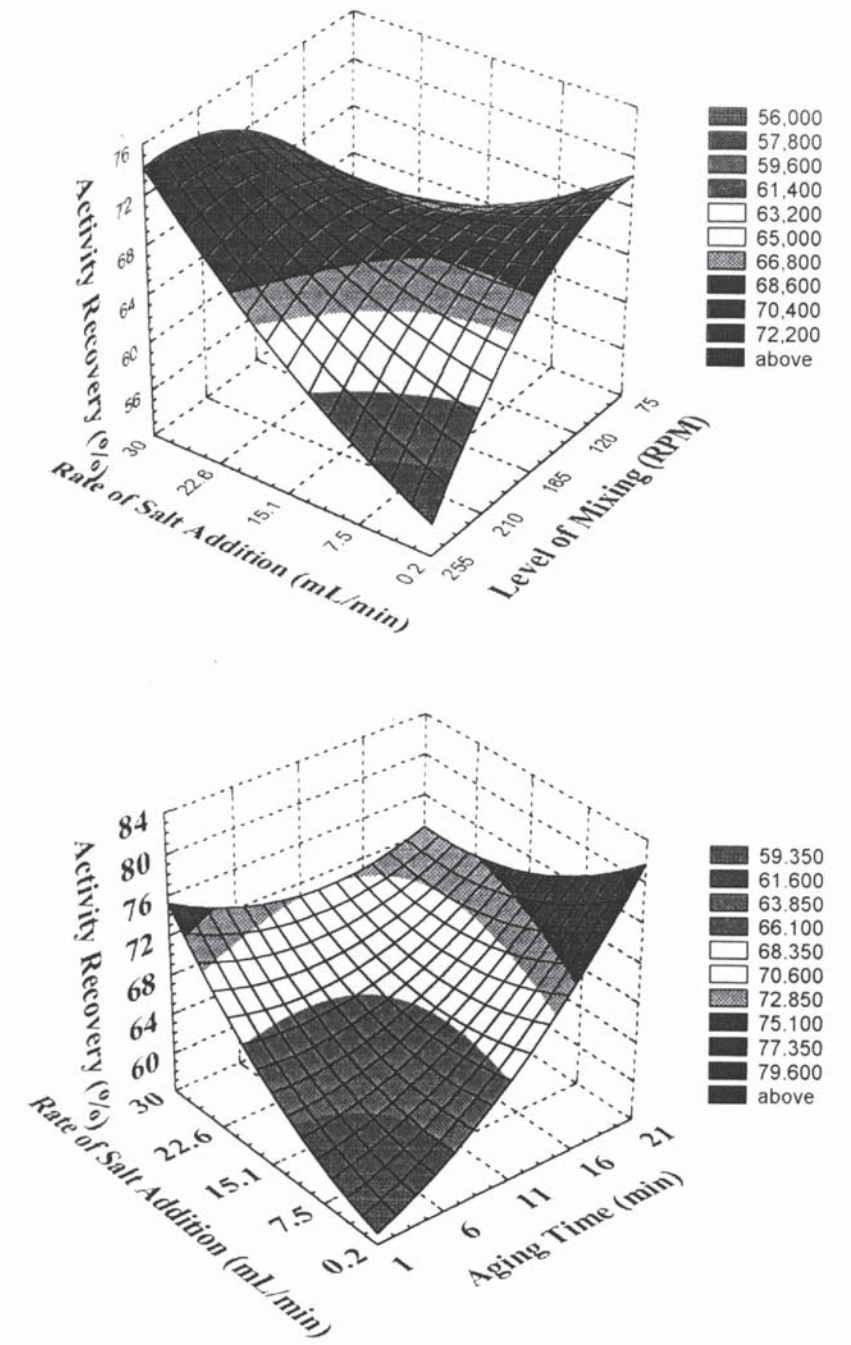

Fig. 3. Effects of rate of salt addition associated with level of mixing and aging time on the activity recovery on cellulase precipitation by ammonium sulfate and HPMC.

As said before, the increase in the rate of salt addition affected positively the recovery of activity in the precipitate (Fig. 3). Large precipitate particles were formed at a high rate of salt addition. These large particles

were certainly more efficiently separated from the liquid phase by centrifugation, leading to higher recovery. The analysis of the effect of mixing and aging times on the activity recovery corroborates with this explanation. At low rate of salt addition (and low aging time and level of mixing), small particles were formed leading to low activity recovery. However, as the level of these two variables were increased, allowing the formation of larger particles, the activity recovery also increased.

Conclusions

The effects of the four key precipitation variables on the recovery of protein and activity of cellulase by ammonium sulfate and HPMC could be assessed by batch experiments through the use of statistical experimental design. This study determined the most important variables in the process and analyzed the tendency of response of the process regarding activity and protein recovery for changes in the four precipitation variables studied. The most important variable was the rate of salt addition and this phenomena seems to be related to the particle size formed under different addition rates. Temperature affects the precipitation owing to the unusual characteristic of HPMC of lower solubility at higher temperatures.

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