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# Action of proteases and carbohydrases in *Saccharomyces cerevisiae* deflocculation

*Ação de proteases e carbohidrases na defloculação de Saccharomyces cerevisiae*

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## Abstract

**Objective** – To present the study of proteases and carbohydrases in deflocculation of *Saccharomyces cerevisiae*. **Methods** – The enzymes were tested in different concentrations, time and pH, and the deflocculation ability of enzymes verified by spectrophotometry. **Results** – The results showed to require 4650UE of protease 642 in 175 min of reaction as 0.2UE of carbohydrase SP299 for 16h, so that deflocculation in pH 3.6 to be occurred. The yeast viability after the treatments did not suffer alteration (99%) demonstrating that the enzymes do not cause injury in the cells. The pH 2.7 showed the best performance of protease 642 and pH 4.0 was the best one for carbohydrase SP299, with maximum deflocculation, similar to the acid treatment pH 2.0. **Conclusion** – The results of this research suggest studies of alcoholic fermentation using the enzymatic technology in sulfuric acid replace, aiming at future industrial applications.

**Descriptors:** *Saccharomyces*; Proteases; Enzyme; Yeast

## Resumo

**Objetivo** – Apresentar o estudo de proteases e carbohidrases na defloculação de *Saccharomyces cerevisiae*. **Métodos** – As enzimas foram testadas em diferentes concentrações, tempo e pH, sendo a capacidade defloculadora das enzimas verificadas por espectrofotometria. **Resultados** – Os resultados revelaram a necessidade de 4650UE da protease 642 com 175 min. de reação e de 0,2UE da carbohidrase SP299 com 16 horas, para que ocorresse defloculação em pH 3,6. A viabilidade das leveduras após os tratamentos não sofreu alteração (99%) demonstrando que as enzimas não causam distúrbios nas células. O pH ótimo de atuação da protease 642 de 2,7 e da carbohidrase SP299 foi 4,0, com máxima defloculação, similar ao tratamento ácido pH 2,0. **Conclusão** – Os resultados dessa pesquisa sugerem estudos de fermentação alcoólica utilizando a tecnologia enzimática em lugar do ácido sulfúrico, visando futuras aplicações industriais.

**Descritores:** *Saccharomyces*; Proteases; Enzimas; Leveduras

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## Introduction

The flocculation of the yeast used in the ethanol industries leads to the yeasts settle in the deep of vat, it makes difficult the conversion of the sugar in ethanol and consequently it decreases the alcoholic yield<sup>1</sup>, because for a maximum conversion of sugar in ethanol and CO<sub>2</sub>, it is essential that the yeast remain suspended in the fermentation culture and not flocculates<sup>2</sup>. Besides causing loss of cells in the centrifuges and the consequent expense of substrate for the cell replacement, as contamination by *Lactobacillus fermentum* causes sharp drop in viability of yeast cells<sup>1,3-4</sup>.

The flocculation of the yeasts increases the contaminants bacteria population, through the consumption of sugar for growth and acid production (lactic acid, mainly) affecting the alcoholic yield and the productivity<sup>1,5</sup>, increasing in the cost of the production of fuel ethanol.

The flocculation occurs due to some factors as contact with gums synthesized for the bacteria<sup>6</sup>, direct contact between inductive bacteria of the flocculation and yeasts<sup>3</sup>, or contamination for flocculating yeasts<sup>7</sup>. In the alcoholic fermentation the lactics bacteria are the main promoters of undesirable fermentation, especially *Lactobacillus* resistant to ethanol<sup>1,8</sup>.

Proteinic factors associates with minerals and mannanas are involved in any type of yeast flocculation and the action of proteases is efficient in the deflocculation of yeast<sup>3,9-10</sup>.

For the development of this flocculation is necessary the presence of calcium<sup>11-12</sup> and also the contact between the cells, so that this mineral increases the flocculation process stimulating the adhesion<sup>13-14</sup>. The amino acid groups of yeast carbohydrates are responsible for the development of yeast flocculation by contaminants bacteria of the fermentation<sup>15-16</sup>.

At the moment it does not exist a definitive solution for this problem, since the yeast industrial treatment with sulfuric acid in pH 2.0 to 2.5 for 0.5 to 2 hours<sup>17-18</sup> before fermentation depend on the pH process and when the yeast is recycled to the vat the pH increase and the flocculation process return. Proteases or carbohydrases could be a more efficient alternative solution for the distilleries, since it is a method that causes yeast deflocculation independent of the pH changes in the process and it is more safety.

## Methods

### *Microorganisms*

The flocculation inductive bacteria *Lactobacillus fermentum* CCT 1396 (“André Tosello” Foundation – Cam-

pinas/Brazil) was a contaminant culture isolated from alcoholic distillery with serious microbiological problems 3 and the bakery yeast *Saccharomyces cerevisiae* (Fleishmann Royal Ltda) is a non flocculating strain. Both were preserved by freezing at -81°C (Hetofrig – High Technology of Scandinavia), in nutrient broth with 10% glycerol.

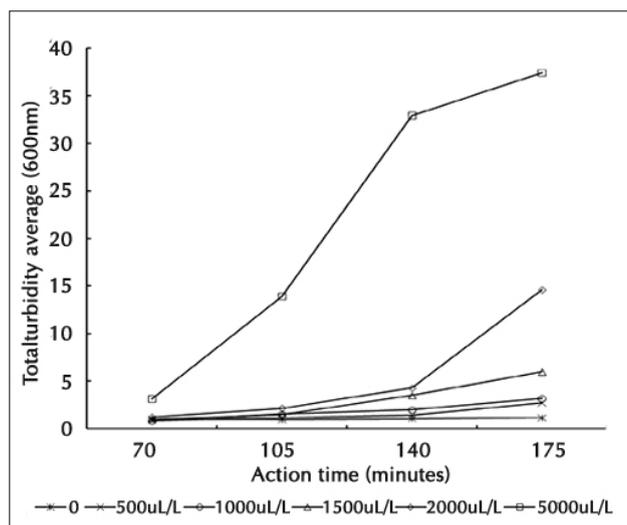
### Flocculation and deflocculation assays

Bacteria were cultivated in MRS broth liquid<sup>19</sup> at 30°C for 48 h. After the growth, these bacteria were washed three times with 0.025M EDTA by 1660 G centrifugation for 15 min (centrifuge mod. 215 – Fannem – Brazil) in buffer citrate pH 3.8. The cells of non-flocculating *Saccharomyces cerevisiae* after washed with EDTA were prepared in a 200g/L (wet biomass) solution at pH 3.8 in buffer citrate, correspondent to 65.4g/L – dry biomass<sup>18</sup>.

For the flocculation/deflocculation experiments were prepared the cells suspensions with: 20mL of 1.38g/L (dry biomass) bacteria suspension, 20mL of 65.4g/L (dry biomass) yeasts suspension and 10mL of 0.05 M calcium chloride in 250mL Erlenmeyer.

These suspensions were mixed during 15 min in a shaker (Tecnal/Brazil) at 30°C and 90 rpm and after that, they were replaced to a conical bottle, rested for 20min and 1mL samples withdrawn from the top of the cells suspensions. Concentrations of 50 to 5000µL/L proteolytic Novozyme 642 20 and 100 to 5000mg/L carbohydrase SP299 21 were added in the cells suspensions. 1500 µL/L Novozyme 642 is equivalent to 4650 enzymatic units (UE) by hydrolysis of casein and quantification by tyrosine released by Lowry method 22. 500mg/L of carbohydrase SP299 is equivalent to 0.2 UE 21.

The percentage of cell flocculation and deflocculation in the samples were determined by the turbidity (spectrophotometer Ultrospec III – Pharmacia – LKB) in 600



Graph 1. Quantification of theyeast flocculation (*Saccharomyces cerevisiae*) from induction by *Lactobacillus fermentum* CCT 1396, after the treatment with different concentrations of protease Novozyme 642

nm according respectively, Ludwig *et al.*<sup>18</sup> (2001) e Ludwig *et al.*<sup>23</sup> (2000).

The percentage of live cells (yeast viability) to the total cells was determined by light microscope with a Neubauer chamber. The yeast cells were dyed with erythrosine solution, in initial time (without enzyme) and at the end of assays<sup>24</sup>.

### The best range of pH for enzymes Protease and Carbohydrase

The bacteria and yeasts solution was fixed in the ratio of pH 2.0 to 5.5 with 0.1N HCl. After 35 minutes of reaction, a sample of the supernatant was removed it was determined the turbidity in spectrophotometry (600 nm). The turbidity in the samples of the treatments with Novozyme 642 and carbohydrase SP299 were determined respectively in 175 and 1360 minutes.

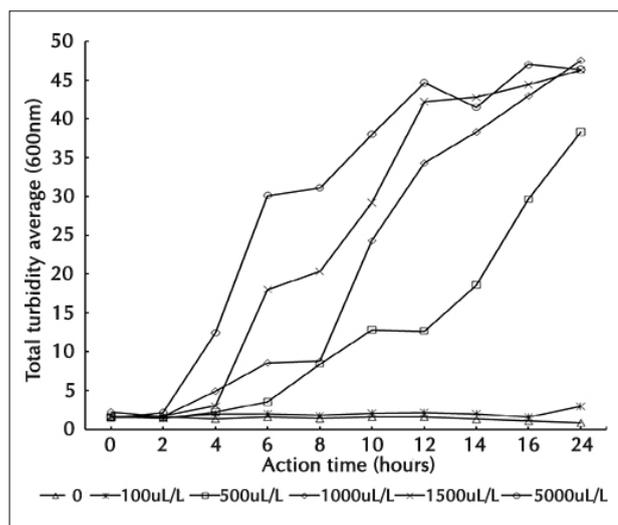
### Statistical Treatments

Statistics were effected using the program Graphad Instat (Rutgers University) and it was determined: matching between averages in the treatments by ANOVA and Tukey-Kramer. The percentage of the yeast deflocculation was determined by interpolation between extremities (100%) deflocculation to intense flocculation, it corresponding respectively, the total turbidity of 25 and 2, gotten experimentally through the average of five replications<sup>18</sup>.

## Results and Discussion

### Yeast deflocculation by different concentration of enzymes

The Graph 1 shows evaluation of the deflocculation *S. cerevisiae* by proteolytic enzyme action. The results have disclosed that in high concentration of this free enzyme (upper 6200 UE or 2000 µL/L) in 175 minutes occurred a strong and significant deflocculation (ANOVA F=15.4 e p< 0.0001, Tukey-Kramer p<0.05),



Graph 2. Performance of carbohydrase SP299 in the deflocculation of *Saccharomyces cerevisiae* flocculated by *Lactobacillus fermentum* CCT 1396 in the 0 a 5000 mg/L of concentrations in function of the time

**Table 1. Evaluation of the yeast viability (*Saccharomyces cerevisiae*) after treatment with the proteolytic enzyme Novozyme 642 (Novo Nordisk) and the Carbohidrase SP299, express in percentage**

Con. Enzyme (µL/L)	Novozyme 642		SP299	
	35 min.	175 min.	35 min.	175 min.
0.0	99.5	98.8	100	99.5
100	–	–	99.7	99.1
500	100	100	99.6	100
750	99.2	99.7	–	–
1000	99.7	99.5	99.8	99.9
1500	99.6	99.5	99.8	99.7
3000	99.8	99.5	–	–
5000	99.8	99.8	99.7	99.7

however with 1500µL/L (4650 UE), it had already a great deflocculation sufficiently to reduce the yeast precipitation after 175 minutes.

For the action of carbohidrase SP 299 (Graph 2) it was necessary only 500mg/L (0.2 UE) of this enzyme so that satisfactory and significant deflocculation occurred (ANOVA F=17.3 and p< 0.0001; Tukey-Kramer p<0.01), in 16 hours. In range of 1500-5000mg/L in 8 hours of performance already got 100% of deflocculation (turbidity of 20 to 30), and with 1000mg/L in 12 hours with 100% deflocculation (turbidity of 34). This enzyme was proved to be very efficient to rupt quematical connections and to decrease phases separation avoiding cell settle in the wort.

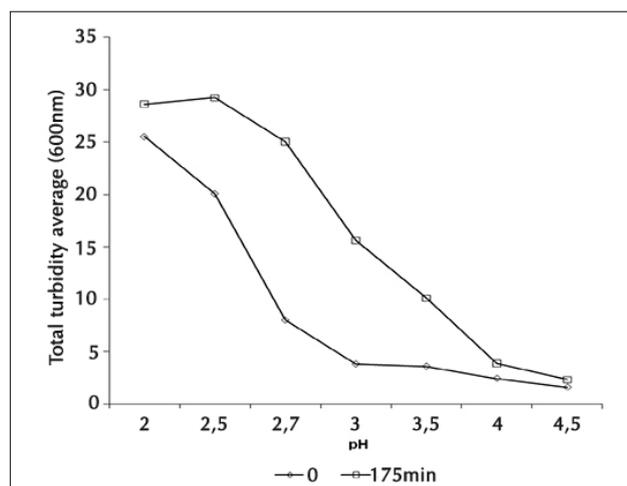
It can be affirmed that the enzyme protease needs to be in high concentration (4650 UE) to act in a short period of time (175 minutes), while the carbohidrase acts in lesser concentration (0.2 UE) in a minimal time of 16 hours so that occurs a satisfactory deflocculation and with the homogeneous solution, facilitating the fermentation process. These results confirm other works on the proteins and carbohydrates connection in the mechanism of cell flocculation<sup>3,9-11,16</sup>.

It did not have any change in yeast viability (99%) after enzymatic treatment, indicating that the handling with enzymes protease or carbohidrase do not cause injuries in the yeast cells (Table 1) in the concentration tested.

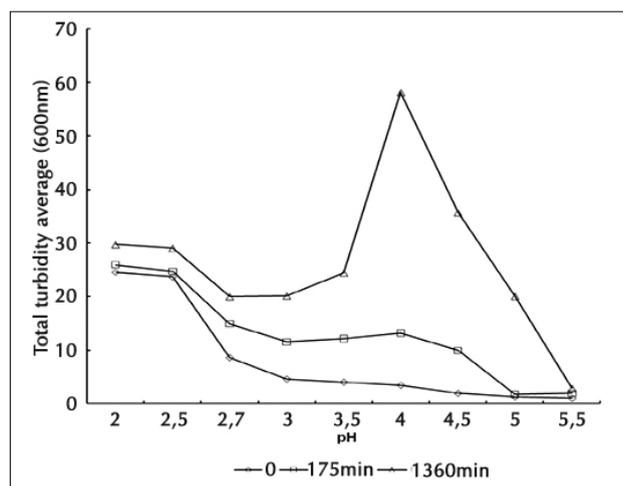
### The best range of pH for the Protease 642 and Carbohidrase SP 299 action

In the pH 2.0 to 2.7 occurred 100% deflocculation (turbidity 25-29) with protease 642, although influenced for the acid treatment. In the range of pH 4.0-4.5 the enzyme did not show any performance, the yeast remaining totally flocculated. The pH with the biggest difference in the turbidity between 0 and 175 minutes was pH 2.7. This great difference reflected the biggest deflocculation of the cells due the proteolytic action, since time 0 reflected the action of the acid (25-37% of deflocculation). In this pH occurred 100% deflocculation after 175 minutes of incubation with protease (Graph 3). According to statistics the turbidity differences between time 0 and after 175 minutes can be considered extremely significant (ANOVA F=16.362 and p<0,0001; Tukey-Kramer p<0.05).

The best pH for the carbohidrase SP 299 (Graph 4) was 4.0, leading to a high deflocculation and, consequently, reduction in the precipitation of the yeasts cells. 100% deflocculation (turbidity ≥ 25) also were gotten in the pH range of 3.5-5.0 with incubation time of 1360 minutes. In the pH range of 2.0-2.5 normally used in the acid treatment in the distilleries occurred a good deflocculation, although due to the acid treatment. Higher than pH 5.0 a total inactivity of the enzyme was observed. The carbohidrase SP 299 action indicates



**Graph 3. pH determination of 4650 UE Protease Novozyme 642 in deflocculation of *Saccharomyces cerevisiae* flocculated by *Lactobacillus fermentum* CCT 1396**



**Graph 4. pH determination of Carbohidrase SP 299 in deflocculation of *Saccharomyces cerevisiae* flocculated by *Lactobacillus fermentum* CCT 1396**

to be possibly the use of this enzyme in the currently pH range (3.8-4.5) of Brazilian distilleries wort. Oliva-Neto et al 25 (1999) indicate the appropriate use of this technology for industrial purpose.

## Conclusions

The proteolytic enzyme Novozyme 642 is efficient in the deflocculation of *Saccharomyces cerevisiae* and *Lactobacillus fermentum* CCT 1396 suspension becoming homogeneous with 1500µL/L (4650 UE) determining a deflocculation of 80-90%, while the carbohydrase SP299 acts in a concentration of 500 mg/L (0.2 UE), with 100% of deflocculation.

The minimum incubation time of the enzyme in the yeast and bacteria solution cells necessary to cause the cells deflocculation is 175 minutes with the minimum concentrations – 4650 UE for the proteolytic enzyme, with the best performance in pH 2.7. For 0.2 UE carbohydrase SP 299 the least time to enzyme action is 16 hours and the best pH 4.0 in environment temperature (25-30°C), similar to the used in distilleries so that could be used for this purpose.

The yeast viability is not modified by the treatment with two tested enzymes (protease and carbohydrase), therefore does not have any inhibition of the metabolism of *Saccharomyces cerevisiae* by these treatments.

This results suggest the necessity of a economic study of this enzymes to use in industrial scale, aiming at future applications in the alcoholic distilleries.

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