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Stabilization Strategies of β-glucosidases Produced by Yeasts *Rhodotorula oryzicola* and *Saccharomyces cerevisiae*

Geise Camila Ribeiro [1, †], Pedro Fernandes [2, 3, 4, †], Floriatan Santos Costa [5, †], Sandra Aparecida de Assis [1, †, *]

1. Enzymology and Fermentation Technology Laboratory, Health Department, State University of Feira de Santana, Av. Transnordestina s/n, Novo Horizonte, 44036-900, Feira de Santana, Bahia, Brazil; E-Mails: ribeirocamila0207@gmail.com; sandraassis@uefs.br
2. iBB—Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal; E-Mail: pedroefe@yahoo.com
3. Associate Laboratory i4HB—Institute for Health and Bioeconomy, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
4. Lusófona University, BIORG—Bioengineering and Sustainability Research Group, Av. Campo Grande 376, Lisbon, 1749-024, Portugal
5. Department of Chemistry, Federal University of Paraná, Curitiba, Paraná, Brazil; E-Mail: floriatansc@gmail.com

† These authors contributed equally to this work.

* **Correspondence:** Sandra Aparecida de Assis; E-Mail: sandraassis@uefs.br

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Abstract

The rising demand for enzymes with improved properties has driven continuous developments in enzyme technology, including enzymatic formulation. Enzyme formulation primarily aims to minimize losses in enzymatic activity during transport, storage and use. One strategy to mitigate enzyme inactivation is to include stabilizing agents or metal ions that

preserve enzyme structure and activity. Thus, in this work, we evaluated stabilization strategies for β-glucosidase produced by the yeasts *Rhodotorula oryzicola* and *Saccharomyces cerevisiae* using various chemical additives. The most effective additives were then applied to obtain a formulation with improved enzymatic activity. KCl, $ZnSO_4$, EDTA, SDS, Triton X-100, Tween 20 and Tween 80 were tested as potential stabilizers for β-glucosidase. The three best stabilizers for each enzymatic sample were used in a simplex-centroid mixture design to evaluate possible synergistic effects and obtain a stable enzyme formulation. The formulations that yielded the highest enzymatic activity contained 2.5 mM of $ZnSO_4$ and 0.375% of Tween 20 for β-glucosidase from *R. oryzicola*, and 0.5 mM of $ZnSO_4$ and 2.5 mM of EDTA for the enzyme from *S. cerevisiae*, showing relative activities of 313.60% and 302.11%, respectively. The results obtained in our study show that both yeasts produce β-glucosidases with good tolerance to chemical additives, and that some of these additives can be used to obtain more stable enzymatic formulations with enhanced catalytic efficiency.

Keywords
Chemical additives; cellulases; enzymatic formulation; enzymatic hydrolysis; optimization

1. Introduction

The emergence of new applications, alongside conventional ones that face increasing performance requirements and growing market competitiveness, has created a strong demand for enzymes with improved properties, driving continuous advances in enzymatic formulation technologies influenced by developments in biotechnology and bioinformatics [1, 2].

The development of enzymatic formulations aims to preserve proper enzyme folding (the active, functional structure of the protein), thus improving catalytic efficiency under industrial conditions. Several protocols to stabilize enzymes have been developed, enabling easier handling and providing greater product versatility on the market. These advances allowed enzymes to better adapt to processing conditions and environmental requirements, resulting in significant improvements in catalytic efficiency and facilitating their broader application in industrial processes [3, 4].

The main objective of an enzymatic formulation is to minimize the loss of enzymatic activity during transport, storage, and use. Formulations for enzymatic stabilization increase stability by counteracting primary deactivation mechanisms such as denaturation, catalytic site deactivation and proteolysis. The most common strategies to prevent primary deactivation include maintaining adequate levels of essential cofactors, incorporating reversible inhibitors, eliminating reactive or oxidizing species from the formulation, and immobilizing enzymes. Additionally, the inclusion of chemical stabilizers can help preserve the three-dimensional structure of the enzyme, an approach often referred to as medium engineering [1, 2, 5].

By definition, enzymes bind to substrates and catalyze reactions that occur confined to an active site, also called a catalytic site [6]. Many researchers have studied how cellulases bind to their substrates. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), cellulases are classified as glycosidases (EC 3.2.1), which are enzymes that hydrolyze glycosidic bonds involving O- or S-glycosyl groups. All cellulases target the

β-1,4 glycosidic bonds of the cellulose polymer, catalyzing their cleavage through either exo- or endo-acting mechanisms [7, 8]. Cellulases typically hydrolyze substrates through two primary acid–base mechanisms, the retaining and inverting mechanisms, which differ in whether the anomeric configuration of the substrate is preserved or inverted during hydrolysis.

Some enzymes perform their function without the need for other chemical compounds. However, others require an additional component to enable or improve substrate binding and/or maintain structural stability under reaction conditions. These components may bind to the enzyme at an allosteric site, often near the catalytic site, thus inducing a conformational change. This conformational modification in the catalytic site can either activate or inhibit the formation of the enzyme-substrate complex and consequently affect catalysis [9]. Thus, it is essential to identify potential activators (stabilizers) and inhibitors of an enzyme of industrial interest, such as cellulases.

The effects of ion salts, chelating agents, and surfactants on cellulase activity have been studied to identify the best additives for improving the catalytic efficiency of these enzymes, as well as to predict which potential inhibitors may impair the reaction yield. However, the effect of these additives on enzymes varies, and empirical research is required to determine how each additive influences the activity of the enzyme produced by the microorganism under study [10-12]. Since β-glucosidase activity is a limiting factor in the enzymatic hydrolysis of lignocellulosic materials, any additive used in the enzymatic reaction should not inhibit this enzyme [13, 14].

Thus, the present work aimed to evaluate stabilization strategies for β-glucosidase produced by yeasts *Rhodotorula oryzicola* and *Saccharomyces cerevisiae* using metal ions, chelating agents, and anionic and nonionic surfactants as additives. The chelating agent and surfactants used were selected based on previously published studies on this topic. The metal ion salts (KCl and $ZnSO_4$) were selected based on preliminary results obtained in the present work.

2. Materials and Methods

2.1 Materials and Reagents

2.1.1 Reagents

The YM Agar medium was obtained from Acumedia® (Indaiatuba, São Paulo, Brazil). Bovine serum albumin, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPβG) and 3,5-dinitrosalicylic acid were obtained from Sigma-Aldrich® (St. Louis, Missouri, United States). Coomassie Brilliant Blue was obtained from Merck® (Darmstadt, Germany). Acetone p.a. was obtained from Synth® (Diadema, São Paulo, Brazil).

2.1.2 Microorganism

Samples of *Rhodotorula oryzicola* and *Saccharomyces cerevisiae* were obtained from the Culture Collection of Microorganisms of Bahia (CCMB) of the State University of Feira de Santana (UEFS, Brazil). Strain identification was performed by isolating genomic DNA and amplifying the D1/D2 domain of the 26S rRNA gene using polymerase chain reaction [15].

The cultures were maintained in plates containing YM Agar medium (Acumedia®) and stored in a refrigerator (4-8°C) until use.

2.1.3 Yeast Growth

Yeast cultures were streaked on YM agar medium (Acumedia®) and incubated in a B.O.D. ABC-LAB incubator (São Bernardo do Campo, São Paulo, Brazil) at 28°C. Activation was performed through three consecutive transfers, with an interval of 24 h between each.

2.1.4 Obtaining β-Glucosidase by Submerged Fermentation of *Saccharomyces cerevisiae* and *R. oryzicola*

The stock culture was maintained on YM agar plates at 4°C until use. *S. cerevisiae* CCMB 520 and *R. oryzicola* were grown on YM Agar (Acumedia®, Indaiatuba, São Paulo, Brazil) previously sterilized in a vertical autoclave (AVM Marte Científica, São Paulo, Brazil) at 121°C for 15 min. The yeast was activated on YM agar through three consecutive subcultures, incubated at 28°C for 24 h (BOD Incubator ABC-LAB, São Caetano do Sul, São Paulo, Brazil).

Yeast cells were pre-inoculated in sterile saline solution (0.45% w/v NaCl) that had been autoclaved at 121°C for 15 min. A 10% (v v^{-1}) aliquot of the resulting pre-inoculum (10^8 CFU mL^{-1}) was then transferred to the appropriate liquid induction medium. For *R. oryzicola*, YP broth was used, supplemented with 1% (w v^{-1}) yeast extract, 2% (w v^{-1}) peptone, and 2% (w v^{-1}) cellobiose as the inducer [16]. For *S. cerevisiae*, the induction medium consisted of YPD broth containing 1% (w v^{-1}) yeast extract, 2% (w v^{-1}) peptone, and 2% (w v^{-1}) D-glucose as the inducer [17].

The enzyme-rich supernatant was collected by centrifugation (14,000 × *g*, 15 min at 4°C) using a Hettich® Universal 320R centrifuge (Tuttlingen, Germany). The supernatant was filtered through filter paper (Whatman n. 1) and stored at 0°C until use. This cell-free supernatant was used as a crude extract with no further purification; therefore, it contained β-glucosidase and likely also other secreted proteins and residual medium components. Accordingly, the observed stabilization effects correspond to the crude enzyme preparation.

2.1.5 Enzymatic Assays

β-glucosidase activity in the supernatant was determined using a chromogenic substrate, 4-nitrophenyl-β-*D*-glucopyranoside (ρNPβG), in a reaction mixture containing 1 mL of 4 mM ρNPβG solution in 0.1 M citrate-phosphate buffer pH 5.0 and 0.25 mL of enzyme sample incubated at 50°C for 20 min. The reaction was subsequently stopped by adding 1.25 mL of 100 mM sodium carbonate solution [18, 19].

The enzymatic activity (U) was calculated using a ρ-nitrophenol calibration curve (Sigma-Aldrich®). One β-glucosidase activity unit was defined as the amount of enzyme required to release 1 µmol of ρ-nitrophenol per minute under the reaction conditions. The released ρ-nitrophenol was measured at 420 nm using a UV/Vis spectrophotometer (Cary 50 UV-Visible spectrophotometer, Varian Inc., São Paulo, São Paulo, Brazil).

Total protein content (mg) was determined by the Bradford method using a bovine serum albumin (Sigma-Aldrich®) calibration curve in a concentration range of 0 to 100 µg/mL [20]. The specific activity (U/mg) was calculated as the ratio of β-glucosidase activity to total protein concentration. All experiments were performed in triplicate.

2.1.6 Stabilization of β-Glucosidase by Chemical Additives

For the stabilization of β-glucosidase, the salts KCl and $ZnSO_4$, the chelating agent EDTA, and SDS (sodium dodecyl sulfate) were used at concentrations ranging from 1 to 20 mM. The surfactants Triton X-100, Tween 20 and Tween 80 were tested at concentrations ranging from 0.10-1.0% (v v^{-1}). All additives were solubilized in 0.1 M phosphate buffer, pH 6 [21-23]. The enzyme was mixed with the respective buffers containing the additives at different concentrations and incubated at 25°C for 24 h in a shaking incubator at 100 rpm. Enzymatic activity was expressed as relative activity (%) using the β-glucosidase activity of the enzyme without stabilizing treatment as the control [24].

2.1.7 Optimization of Enzyme Stabilization

The three most effective stabilizers previously identified for each enzymatic sample were selected to evaluate their combined ability to retain β-glucosidase activity, including potential synergistic effects. A simplex-centroid mixture design was used to assess the effect of the additives on retention of enzymatic activity, by varying the volume (mL) of each additive in the assays. Each enzymatic extract was diluted 1:4 (extract:water), and in all experiments, 4 mL of water, 1 mL of enzymatic extract, and a total of 1 mL of additives were used. The proportion of each additive within the mixture varied from 0 to 1 mL (central point, 0.33 mL), according to the experimental design. The study was conducted for 72 hours at 25°C, providing a measure of short-term formulation stability under the tested conditions.

2.1.8 Statistical Analysis

The results obtained were statistically evaluated using analysis of variance (ANOVA) and the means were compared using the F-test ($p < 0.05$) with Statistica software, version 7.0.

3. Results

3.1 Stabilization of β-Glucosidase

The yeasts were cultivated by submerged fermentation using cellobiose (*R. oryzicola*) and glucose (*S. cerevisiae*) as inducers, as described in previous publications [16, 17]. The crude, non-purified β-glucosidase preparations showed initial enzymatic activity of 2.17 ± 0.04 U for *R. oryzicola* (BGL *oryzicola*) and 1.41 ± 0.03 U for *S. cerevisiae* (BGL cerevisae). Table 1 presents the relative activity (%) of both enzymes after 24 h of treatment with different additives.

Table 1 Relative activity of β-glucosidase from *R. oryzicola* (BGL *oryzicola*) and *S. cerevisiae* (BGL cerevisiae) in the presence of various chemical additives.

Chemical additives	Concentration of chemical additives	Relative activity (%)	
		BGL *oryzicola*	BGL *cerevisiae*
KCl	1 mM	202.39 ± 1.71	58.18 ± 0.43
	5 mM	179.90 ± 3.02	60.61 ± 7.70
	10 mM	186.48 ± 1.33	39.47 ± 1.24
	15 mM	177.39 ± 4.21	46.54 ± 1.62
	20 mM	189.76 ± 3.15	49.40 ± 0.80
$ZnSO_4$	1 mM	145.83 ± 6.24	108.16 ± 3.32
	5 mM	168.67 ± 10.10	95.62 ± 3.19
	10 mM	91.57 ± 8.91	65.33 ± 6.97
	15 mM	63.16 ± 7.10	0.0
	20 mM	13.31 ± 6.43	0.0
EDTA	1 mM	119.46 ± 4.61	112.48 ± 5.24
	5 mM	31.59 ± 3.85	116.02 ± 1.27
	10 mM	40.85 ± 1.46	63.77 ± 2.17
	15 mM	31.98 ± 0.20	39.35 ± 0.39
	20 mM	32.45 ± 4.56	32.76 ± 0.30
SDS	1 mM	115.95 ± 5.24	31.71 ± 4.65
	5 mM	115.82 ± 1.71	33.32 ± 6.56
	10 mM	92.63 ± 0.05	33.44 ± 0.15
	15 mM	92.16 ± 1.95	33.52 ± 0.34
	20 mM	87.39 ± 3.44	35.81 ± 2.77
Tween 20	0.10%	192.07 ± 0.57	113.75 ± 6.30
	0.25%	189.94 ± 4.84	127.61 ± 4.41
	0.50%	107.47 ± 3.42	108.76 ± 3.07
	0.75%	208.52 ± 1.89	120.52 ± 2.60
	1.00%	125.46 ± 2.34	101.02 ± 1.62
Tween 80	0.10%	126.14 ± 3.74	46.70 ± 5.28
	0.25%	130.45 ± 7.93	105.75 ± 3.71
	0.50%	155.69 ± 5.80	92.58 ± 7.01
	0.75%	150.22 ± 4.98	56.15 ± 2.01
	1.00%	107.82 ± 5.58	78.18 ± 2.71
Triton X-100	0.10%	129.65 ± 7.84	55.33 ± 3.23
	0.25%	123.66 ± 0.81	44.53 ± 3.47
	0.50%	121.18 ± 2.17	34.91 ± 2.51
	0.75%	102.19 ± 1.90	39.55 ± 4.09
	1.00%	69.01 ± 3.33	31.89 ± 2.77

BGL *oryzicola* displayed high tolerance to KCl at all tested concentrations, showing an increase in enzymatic activity of up to 202.39% at 1 mM. Moreover, activity remained high (177-190%) across the remaining concentration range [24]. In the presence of $ZnSO_4$ and SDS, enzymatic activity

increased at low concentrations (1-5 mM) but decreased as the concentration of these additives increased. EDTA increased enzymatic activity only at the lowest concentration, 1 mM.

In contrast, BGL cerevisae displayed improved activity in the presence of 1 mM $ZnSO_4$ (108.16%), 5 mM EDTA (116.02%), and 0.25% Tween 20 (127.61%). All concentrations of KCl, SDS and Triton X-100 inhibited enzymatic activity, reducing it by more than 40% relative to the control.

The synergistic effects of the most successful additives for BGL *oryzicola*, KCl, $ZnSO_4$ and Tween 20, were further assessed using a simplex-centroid mixture design. Different proportions of these additives, at initial concentrations of 1 mM (KCl), 5 mM ($ZnSO_4$), and 0.75% (Tween 20) were evaluated, and their effects on relative activity are shown in Table 2. The highest activity was obtained using the formulation containing 2.5 mM $ZnSO_4$ and 0.375% Tween 20, which yielded a relative activity of 313.60 ± 9.59%.

Table 2 Simplex-centroid mixture design and enzymatic activity obtained for BGL *oryzicola*.

Essay	KCl (mM)		$ZnSO_4$ (mM)		Tween 20 (%)		Relative Activity (%)
1	1 mM	(1.0)	0	(0.0)	0	(0.0)	129.95 ± 1.74
2	0	(0.0)	5 mM	(1.0)	0	(0.0)	154.59 ± 0.26
3	0	(0.0)	0	(0.0)	0.75%	(1.0)	167.31 ± 5.69
4	0.5 mM	(0.5)	2.5 mM	(0.5)	0	(0.0)	240.66 ± 9.49
5	0.5 mM	(0.5)	0	(0.0)	0.375%	(0.5)	173.95 ± 3.88
6	0	(0.0)	2.5 mM	(0.5)	0.375%	(0.5)	313.60 ± 9.59
7 (CP)[a]	0.33 mM	(0.33)	1.65 mM	(0.33)	0.25%	(0.33)	143.25 ± 10.77
8 (CP)[a]	0.33 mM	(0.33)	1.65 mM	(0.33)	0.25%	(0.33)	143.17 ± 10.42
9 (CP)[a]	0.33 mM	(0.33)	1.65 mM	(0.33)	0.25%	(0.33)	148.03 ± 9.83
10 (CP)[a]	0.33 mM	(0.33)	1.65 mM	(0.33)	0.25%	(0.33)	147.93 ± 9.50

[a] CP = Central Point.

Figure 1 shows the Pareto chart summarizing the linear (L) and quadratic (Q) effects of the studied variables on enzyme activity after 72 h of treatment with additives. The corresponding surface plot retrieved from the cubic model is shown in Figure 2.

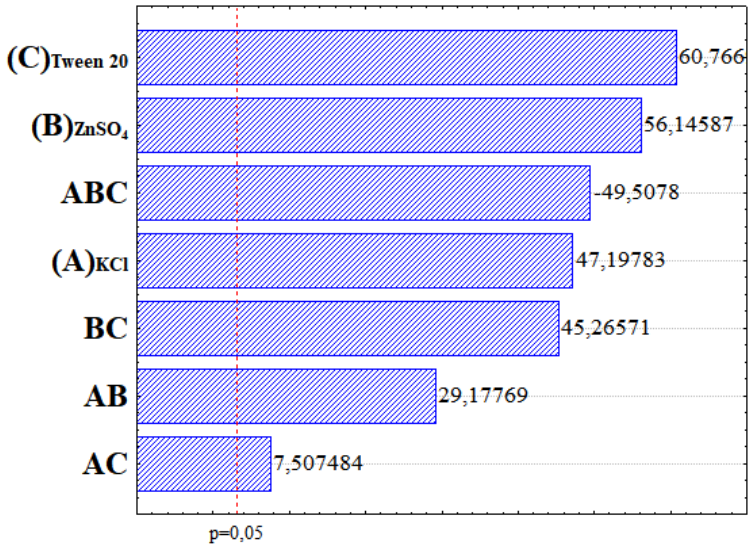

Figure 1 Pareto plot showing the influence of linear (L) and quadratic (Q) effects on the optimization of BGL *oryzicola* activity, illustrating the interaction among the different additives in the enzyme formulation.

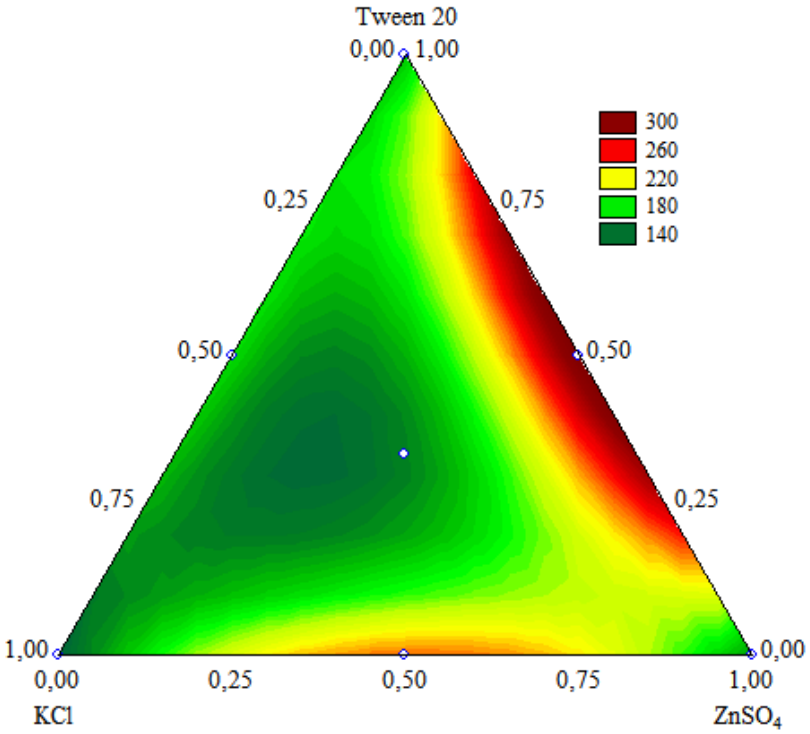

Figure 2 Response surface plot for the optimization of BGL *oryzicola* activity showing the interaction among the different additives of the enzyme formulation.

The Pareto chart shows that the three selected additives influenced enzyme activity. The pronounced $ZnSO_4$–Tween 20 interaction observed in the response surface (Figure 2) aligns with a mechanism suggested here by analogy with previous reports and not directly tested in this work, in which Zn^{2+} would enhance structural stability. At the same time, Tween 20 would reduce interfacial denaturation and improve substrate accessibility. The negative cubic term in Equation (1) indicates that excessive additive concentrations may disrupt enzyme conformation, highlighting the

relevance of balanced proportions [25-27]. Table 3 shows the Analysis of Variance (ANOVA) for the studied model. The F test yielded a value of 647.87, which is higher than the tabulated F-value (9.01), with an R^2 of 0.99 at a 95% confidence level, showing that the model fits the experimental data well.

Table 3 Analysis of variance for BGL *oryzicola* activity optimization with various enzyme formulation additives.

	Sum	Degrees of freedom	Average	F	R^2	P
Model	29469.22	6	4911.537	647.8745	0.99	0.000094
Total error	22.74	3	7.581			
Lack of adjustment	0.00	0	0.000			
Pure error	22.74	3	7.581			
Adjusted total	29491.96	9	3276.885			

Equation 1 shows the correlation between the variables of the model under study, where *A* is the volume of 1 mM KCl, *B* is the volume of 5 mM $ZnSO_4$, and *C* is the volume of 0.75% Tween 20.

$$\begin{aligned} Relative\ Activity\ (\%) = {} & 129.95 \times A + 154.59 \times B + 167.31 \times C \\ & +393.57 \times A \times B + 101.26 \times A \times C + 610.57 \times B \times C \\ & -3451.84 \times A \times B \times C \end{aligned} \quad (1)$$

The synergistic effect of the most successful additives for BGL cerevisiae, $ZnSO_4$, EDTA, and Tween 20, was further assessed using a simplex-centroid mixture design. Different proportions of these additives, with initial concentrations of 1 mM ($ZnSO_4$), 5 mM (EDTA), and 0.25% (Tween 20), were tested, and the impact on relative activities shown in Table 4. The highest activity was obtained with the formulation containing 0.5 mM ZnSO4 and 2.5 mM EDTA, yielding 4.36 ± 0.17 U, roughly three times the initial enzymatic activity (1.41 ± 0.03 U), with a relative activity of 302.11 ± 3.67%.

Table 4 Simplex-centroid mixture design and enzymatic activity obtained for BGL cerevisae.

Essay	$ZnSO_4$ (mM)		EDTA (mM)		Tween 20 (%)		Relative Activity (%)
1	1 mM	(1.0)	0	(0.0)	0	(0.0)	253.36 ± 7.86
2	0	(0.0)	5 mM	(1.0)	0	(0.0)	151.09 ± 0.76
3	0	(0.0)	0	(0.0)	0.25%	(1.0)	102.85 ± 2.73
4	0.5 mM	(0.5)	2.5 mM	(0.5)	0	(0.0)	302.11 ± 3.67
5	0.5 mM	(0.5)	0	(0.0)	0.125%	(0.5)	85.58 ± 2.26
6	0	(0.0)	2.5 mM	(0.5)	0.125%	(0.5)	136.09 ± 6.07
7 (CP)[a]	0.33 mM	(0.33)	1.65 mM	(0.33)	0.082%	(0.33)	241.79 ± 2.86
8 (CP)[a]	0.33 mM	(0.33)	1.65 mM	(0.33)	0.082%	(0.33)	241.67 ± 3.11
9 (CP)[a]	0.33 mM	(0.33)	1.65 mM	(0.33)	0.082%	(0.33)	241.52 ± 4.18
10 (CP)[a]	0.33 mM	(0.33)	1.65 mM	(0.33)	0.082%	(0.33)	240.80 ± 1.15

[a] CP = Central Point.

Figure 3 shows the Pareto chart and the influence of linear (L) and quadratic (Q) effects of the variables under study on enzyme activity after 72 h of treatment with additives. The surface plot of the cubic model under study is shown in Figure 4.

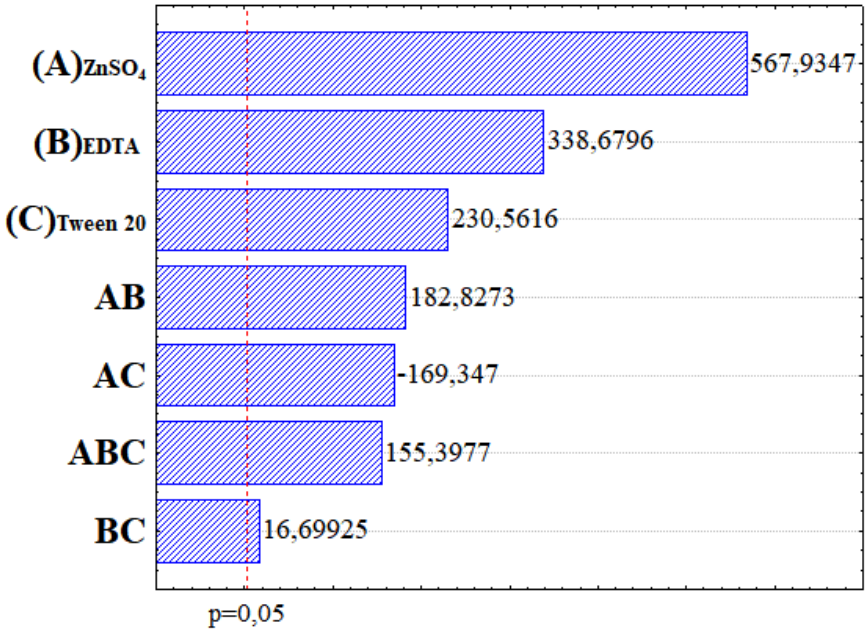

Figure 3 Pareto plot and the influence of linear (L) and quadratic (Q) effects on the optimization of BGL cerevisae activity showing the interaction among the different additives of the enzyme formulation.

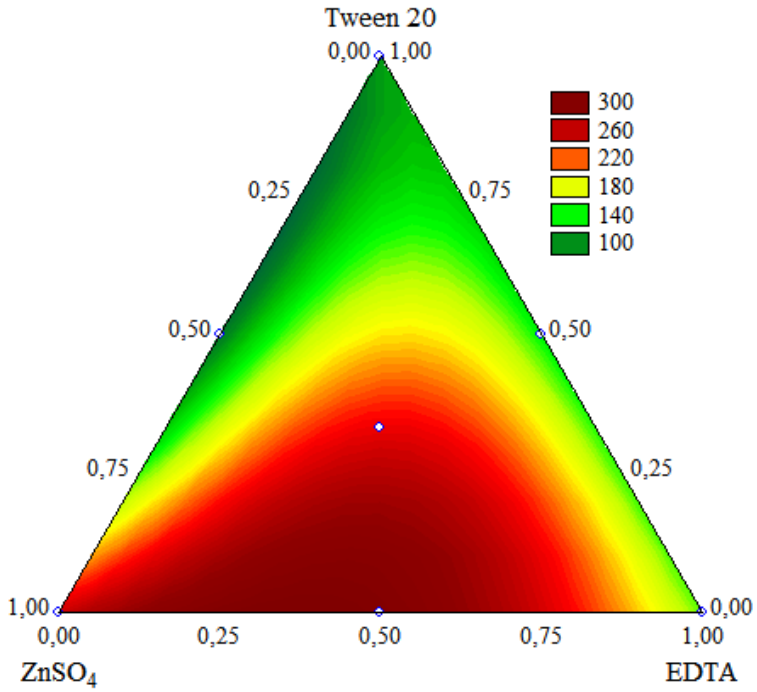

Figure 4 Response surface plot for the optimization of BGL cerevisae activity showing the interaction among the different additives of the enzyme formulation.

Equation 2 shows the correlation between the variables of the model under study, where *A* is the volume of $ZnSO_4$, *B* is the volume of EDTA, and *C* is the volume of Tween 20.

$$\begin{aligned} Relative\ Activity\ (\%) = {} & 253.36 \times A + 151.09 \times B + 102.85 \times C \\ & +399.56 \times A \times B - 370.09 \times A \times C + 36.50 \times B \times C \\ & +1755.47 \times A \times B \times C \end{aligned} \quad (2)$$

The positive $ZnSO_4$–EDTA interaction (Equation 2) suggests a complementary dual mechanism, again proposed by analogy with the literature, rather than confirmed experimentally. Thus, while EDTA would remove inhibitory metal contaminants, Zn^{2+} would stabilize the active conformation of the enzyme. Tween 20 had a smaller influence, possibly enhancing solubility while also promoting partial enzyme unfolding at higher concentrations, again highlighting the relevance of the balanced proportions noted previously for the BGL *oryzicola* formulation [25-27].

Table 5 shows the Analysis of Variance (ANOVA) for the model under study. The F test yielded a value of 41161.81, which is higher than the tabulated F-value (9.01), with an R^2 of 0.99 at a 95% confidence level, showing that the model fits the experimental data well. The Pareto graphs showed that the three selected additives influenced enzyme activity.

Table 5 Analysis of variance for BGL cerevisiae activity optimization with various enzyme formulation additives.

	Sum	Degrees of freedom	Average	F	R^2	P
Model	49149.27	6	8191.545	41161.81	0.99	0.000000
Total error	0.60	3	0.199			
Lack of adjustment	0.00	0	0.000			
Pure error	0.60	3	0.199			
Adjusted total	49149.87	9	5461.096			

The results obtained in our study show that *R. oryzicola* produced an enzyme with greater stability in the presence of additives, maintaining or increasing its enzymatic activity in most of the tested formulations. The divergence in activity between the two enzymatic samples was expected, since the amino acids sequence present in the catalytic site of the enzyme may vary depending on the species used to obtain the enzymatic extract. These amino acids may or may not contain chemical groups that interact differently with the additives under study.

Dikshit and Tallapragada [28] studied the effect of 5 mM KCl on the β-glucosidase activity of *Monascus sanguineus,* reporting a 69% inhibition of enzymatic activity. Conversely, Silva *et al.* [29] studied the influence of 5 mM KCl on the β-glucosidase activity of *Pichia guilliermondii* G1.2, reporting a positive modulating effect, with a 7% increase in activity. Similarly, when assessing the effect of KCl and $ZnSO_4$ (1-20 mM) on the β-glucosidase activity of *Trichoderma reesei,* Obeng *et al*. [23] found that the relative activities peaked at 15 mM KCl (112.2%) and 20 mM $ZnSO_4$ (102.8%). Aligned with these observations, Li *et al*. [30] reported that the activity of a β-glucosidase isolated from *Acidothermus cellulolyticus* increased more than twofold in the presence of 5 mM KCl.

Salts that contain strongly hydrated kosmotropic anions such as SO_4^{2-}, and, to a much lesser extent, Cl^-, can alter water structure and protein hydration, while increasing solution viscosity and

decreasing fluidity. In many aqueous systems, this tends to favor protein compaction, stabilize the native folded state relative to the unfolded counterpart, and promote salting-out [31, 32].

These characteristics may or may not favor enzyme-substrate binding, provided that the structural modifications do not alter the catalytic site or cause steric hindrance to enzyme-substrate interaction. At lower salt concentrations, the anions' polarizability is reduced, which tends to favor the reaction. At low concentrations (<0.1 M), ions affect protein stability and enzymatic activity primarily through electrostatic interactions. At higher salt concentrations (usually >0.1-0.3 M, but not extremely concentrated, up to about 3.0 M), ion dispersion forces exceed the electrostatic forces [32].

The effects of SDS and EDTA on the β-glucosidase activity of *M. sanguineus* were studied, both compounds caused more than 60% inhibition of enzyme activity [28].

When evaluating the effect of non-ionic surfactant agents, all samples in the present study showed increased β-glucosidase activity in the presence of Tween 20, Tween 80 and Triton X-100, except for the sample containing 1% Triton X-100. The greatest increase in activity was observed with 0.75% Tween 20, yielding an enzymatic activity of 4.83 ± 0.04 U, twice the initial activity.

Tween 20 and Tween 80, also known as polysorbate 20 and polysorbate 80, are nonionic, amphipathic surfactants, consisting of polyoxyethylene sorbitan esters of fatty acids. Tween 20 is the laurate derivative of polyoxyethylene (20) sorbitan, whereas Tween 80 is the monooleate derivative, and both are commonly used in biotechnological applications [33, 34]. Polysorbates are effective at low concentrations, exhibit relatively low toxicity, are relatively inert when mixed with proteins and can strongly inhibit protein surface adsorption [35].

Similarly, Obeng *et al.* [23] reported a positive effect of surfactants on β-glucosidase activity of *Trichoderma reesei*, with the highest relative activity (145.6%) observed in samples containing 0.5% Tween 20. The surfactant concentration strongly influences enzyme solubilization and catalytic efficiency, with higher surfactant concentrations leading to decreased catalytic activity, as under such environments, surfactant–protein interactions promote partial unfolding, active-site disruption, or aggregation [28, 36, 37].

4. Conclusion

Comparing the results obtained for the two yeasts under study with those from previous studies, the β-glucosidase from *R. oryzicola* showed a favorable modulation in response to most additives across the tested concentrations. Conversely, the enzyme produced by *S. cerevisiae* showed lower tolerance and retention of activity when exposed to the same additives. These differences likely result from distinct interactions between additive molecules and amino acid residues at the enzyme surface, leading to conformational changes that may either favor or hinder substrate binding and catalytic efficiency. Among the additives assessed, $ZnSO_4$ at low concentration (<5 mM) proved to be a good chemical additive for both enzymes. Tween 20 also showed positive effects on both enzymes, both when considered individually and when combined with other additives, suggesting its potential in enzyme formulations, particularly for applications in the hydrolysis of lignocellulosic biomass, since this surfactant decreases the non-productive adsorption of cellulases to lignin. The observed synergistic effects, namely between $ZnSO_4$ and Tween 20 for *R. oryzicola*, and between $ZnSO_4$ and EDTA for *S. cerevisiae*, highlight the importance of rational additive combinations in enhancing enzyme performance.

Overall, these formulations improved catalytic activity and suggested practical use in developing more efficient and stable enzyme systems for industrial bioconversion processes.

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Author Contributions

Geise Camila Ribeiro: methodology, writing – original draft. Floriatan Santos Costa: methodology, statistical analyses, writing – original draft. Pedro Fernandes: writing – review and editing. Sandra Aparecida de Assis: Conceptualization, methodology, writing – review and editing. All authors have read and approved the published version of the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

Data Availability Statement

Data sharing will be provided by requested.

References

1. Patel AK, Dong CD, Chen CW, Pandey A, Singhania RR. Production, purification, and application of microbial enzymes. In: Biotechnology of microbial enzymes. Cambridge, MA: Academic Press; 2023. pp. 25-57.
2. Singhania RR, Patel AK, Thomas L, Goswami M, Giri BS, Pandey A. Chapter 13 - Industrial enzymes. In: Industrial biorefineries & white biotechnology. Amsterdam, Netherlands: Elsevier; 2015. pp. 473-497.
3. Wahab WAA. Review of research progress in immobilization and chemical modification of microbial enzymes and their application. Microb Cell Fact. 2025; 24: 167.
4. Martins M, Silva C, Cavaco-Paulo A. Enzyme stabilization for biotechnological applications. In: Advances in textile biotechnology. Cambridge, UK: Woodhead Publishing; 2019. pp. 107-131.
5. Khan MF. Enhancing stability of enzymes for industrial applications: Molecular insights and emerging approaches. World J Microbiol Biotechnol. 2025; 41: 362.
6. Robinson PK. Enzymes: Principles and biotechnological applications. Essays Biochem. 2015; 59: 1-41.
7. Bhardwaj N, Kumar B, Agrawal K, Verma P. Current perspective on production and applications of microbial cellulases: A review. Bioresour Bioprocess. 2021; 8: 95.

8. Sharma A, Tewari R, Rana SS, Soni R, Soni SK. Cellulases: Classification, methods of determination and industrial applications. Appl Biochem Biotechnol. 2016; 179: 1346-1380.
9. de Cassia Pereira J, Giese EC, de Souza Moretti MM, dos Santos Gomes AC, Perrone OM, Boscolo M, et al. Effect of metal ions, chemical agents and organic compounds on lignocellulolytic enzymes activities. In: Enzyme inhibitors and activators. London, UK: IntechOpen; 2017. pp. 139-164.
10. Chauhan AS, Patel AK, Chen CW, Dong CD, Singhania RR. Strategies for overcoming the inhibition of cellulose hydrolysis. In: Handbook of biorefinery research and technology: Biomass logistics to saccharification. Dordrecht, Netherlands: Springer; 2024. pp. 1001-1021.
11. Campos J, Almqvist H, Bao J, Wallberg O, Lidén G. Overcoming extended lag phase on optically pure lactic acid production from pretreated softwood solids. Front Bioeng Biotechnol. 2023; 11: 1248441.
12. Sánchez-Muñoz S, Balbino TR, de Oliveira F, Rocha TM, Barbosa FG, Vélez-Mercado MI, et al. Surfactants, biosurfactants, and non-catalytic proteins as key molecules to enhance enzymatic hydrolysis of lignocellulosic biomass. Molecules. 2022; 27: 8180.
13. Ouyang B, Wang G, Zhang N, Zuo J, Huang Y, Zhao X. Recent advances in β-glucosidase sequence and structure engineering: A brief review. Molecules. 2023; 28: 4990.
14. Erkanli ME, El-Halabi K, Kim JR. Exploring the diversity of β-glucosidase: Classification, catalytic mechanism, molecular characteristics, kinetic models, and applications. Enzyme Microb Technol. 2024; 173: 110363.
15. Oliveira RQ, Rosa CA, Uetanabaro AP, Azeredo A, Neto AG, Assis SA. Polygalacturonase secreted by yeasts from Brazilian semi-arid environments. Int J Food Sci Nutr. 2009; 60: 72-80.
16. de Araujo Ribeiro GC, Assis SA. Production of β-glucosidase by *Rhodotorula oryzicola* and use of enzyme for hydrolysis of sugarcane bagasse delignified. J Food Sci Technol. 2023; 60: 2761-2771.
17. Almeida LE, Ribeiro GC, Aparecida de Assis S. β-glucosidase produced by *Moniliophthora perniciosa*: Characterization and application in the hydrolysis of sugarcane bagasse. Biotechnol Appl Biochem. 2022; 69: 963-973.
18. da Silva Almeida LE, Fernandes P, de Assis SA. Immobilization of β-glucosidase from *Moniliophthora perniciosa* on different supports by adsorption. Braz J Chem Eng. 2025; 42: 883-892.
19. Matsuura M, Sasaki J, Murao S. Studies on β-glucosidases from soybeans that hydrolyze daidzin and genistin: Isolation and characterization of an isozyme. Biosci Biotechnol Biochem. 1995; 59: 1623-1627.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-254.
21. Kamsani N, Salleh MM, Basri SA, Mohamad SE, Aziz SA, Kamaruddin K. Effects of surfactant on the enzymatic degradation of oil palm empty fruit bunch (OPEFB). Waste Biomass Valor. 2018; 9: 845-852.
22. Li K, Wang X, Wang J, Zhang J. Benefits from additives and xylanase during enzymatic hydrolysis of bamboo shoot and mature bamboo. Bioresour Technol. 2015; 192: 424-431.
23. Obeng EM, Budiman C, Ongkudon CM. Identifying additives for cellulase enhancement-A systematic approach. Biocatal Agric Biotechnol. 2017; 11: 67-74.

24. das Neves CA, de Menezes LH, Soares GA, dos Santos Reis N, Tavares IM, Franco M, et al. Production and biochemical characterization of halotolerant β-glucosidase by *Penicillium roqueforti* ATCC 10110 grown in forage palm under solid-state fermentation. Biomass Conv Bioref. 2022; 12: 3133-3144.
25. Shi X, Zhao L, Pei J, Ge L, Wan P, Wang Z, et al. Highly enhancing the characteristics of immobilized thermostable β-glucosidase by Zn^{2+}. Process Biochem. 2018; 66: 89-96.
26. Li R, Chang Y, Zhang Y, Chen XE, Zhu Y. Role of a Tween 20-containing antifoaming agent in renaturation of foam-denatured pepsin during defoaming. J Mol Liq. 2018; 265: 740-746.
27. Srinivasulu S, Rao AA. Kinetic and structural studies on the interaction of surfactants with lipoxygenase L1 from soybeans (*Glycine max*). J Agric Food Chem. 1993; 41: 366-371.
28. Dikshit R, Tallapragada P. Partial purification and characterization of β-glucosidase from *Monascus sanguineus*. Braz Arch Biol Technol. 2014; 58: 185-191.
29. da Silva RR, da Conceição PJ, de Menezes CL, de Oliveira Nascimento CE, Bertelli MM, Júnior AP, et al. Biochemical characteristics and potential application of a novel ethanol and glucose-tolerant β-glucosidase secreted by *Pichia guilliermondii* G1. 2. J Biotechnol. 2019; 294: 73-80.
30. Li Y, Bu M, Chen P, Li X, Chen C, Gao G, et al. Characterization of a thermophilic monosaccharide stimulated β-glucosidase from *Acidothermus cellulolyticus*. Chem Res Chin Univ. 2018; 34: 212-220.
31. Silva C, Martins M, Jing S, Fu J, Cavaco-Paulo A. Practical insights on enzyme stabilization. Crit Rev Biotechnol. 2018; 38: 335-350.
32. Zhao H. Protein stabilization and enzyme activation in ionic liquids: Specific ion effects. J Chem Technol Biotechnol. 2016; 91: 25-50.
33. Kerwin BA. Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: Structure and degradation pathways. J Pharm Sci. 2008; 97: 2924-2935.
34. Kishore RS, Pappenberger A, Dauphin IB, Ross A, Buergi B, Staempfli A, et al. Degradation of polysorbates 20 and 80: Studies on thermal autoxidation and hydrolysis. J Pharm Sci. 2011; 100: 721-731.
35. Ha E, Wang W, Wang YJ. Peroxide formation in polysorbate 80 and protein stability. J Pharm Sci. 2002; 91: 2252-2264.
36. Holmberg K. Interactions between surfactants and hydrolytic enzymes. Colloids Surf B Biointerfaces. 2018; 168: 169-177.
37. Aguirre-Ramírez M, Silva-Jiménez H, Banat IM, Díaz De Rienzo MA. Surfactants: Physicochemical interactions with biological macromolecules. Biotechnol Lett. 2021; 43: 523-535.