Appl Biochem Biotechnol (2014) 172:2412–2424
DOI 10.1007/s12010-013-0683-3

Production and Characterization of β-Glucanase Secreted by the Yeast *Kluyveromyces marxianus*

Mariana R. Lopes · Carlos J. A. de Souza · Marina Q. R. B. Rodrigues · Daniela A. Costa · Ancély F. dos Santos · Leandro L. de Oliveira · Humberto J. O. Ramos · Valéria M. Guimarães · Wendel B. Silveira · Flávia M. L. Passos · Luciano G. Fietto

Received: 12 September 2013 / Accepted: 15 December 2013 /
Published online: 4 January 2014
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Abstract An extracellular β-glucanase secreted by *Kluyveromyces marxianus* was identified for the first time. The optimal conditions for the production of this enzyme were evaluated by response surface methodology. The optimal conditions to produce β-glucanase were a glucose concentration of 4 % (*w*/*v*), a pH of 5.5, and an incubation temperature of 35 °C. Response surface methodology was also used to determine the pH and temperature required for the optimal enzymatic activity. The highest enzyme activity was obtained at a pH of 5.5 and a temperature of 55 °C. Furthermore, the enzyme was partially purified and sequenced, and its specificity for different substrates was evaluated. The results suggest that the enzyme is an endo-β-1, 3(4)-glucanase. After optimizing the conditions for β-glucanase production, the culture supernatant was found to be effective in digesting the cell wall of the yeast *Saccharomyces cerevisiae*, showing the great potential of β-glucanase in the biotechnological production of soluble β-glucan.

Keywords β-glucanase · *Kluyveromyces marxianus* · Production · Optimization · Yeast lysis

Introduction

β-Glucanases are enzymes that hydrolyze the β bonds present in polymers of linear or branched glucose residues. According to the cleavage site in the substrate, they are classified

Mariana R. Lopes and Carlos J. A. de Souza contributed equally to this work.

M. R. Lopes · C. J. A. de Souza · M. Q. R. B. Rodrigues · A. F. dos Santos · H. J. O. Ramos · V. M. Guimarães · L. G. Fietto (✉)
Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Av. PH Rolfs s/n, Campus universitário, Viçosa, MG 36571-000, Brazil
e-mail: lgfietto@ufv.br

D. A. Costa · W. B. Silveira · F. M. L. Passos
Departamento de Microbiologia, Universidade Federal de Viçosa, Av. PH Rolfs s/n, Campus universitário, Viçosa, MG 36571-000, Brazil

L. L. de Oliveira
Departamento de Biologia Geral, Universidade Federal de Viçosa, Av. PH Rolfs s/n, Campus universitário, Viçosa, MG 36571-000, Brazil

as exo- or endo-glucanases [1–3]. β-Glucanases can be classified into four categories: (1) β-1,3,β-1,4-glucanases, which cleave β-1,4 glycosidic bonds adjacent to β-1,3 glycosidic bonds; (2) β-1,4-glucanases, which cleave β-1,4 glycosidic bonds; (3) β-1,3(4)-glucanases, which cleave polymers of β-1,3-1,4-glucan and β-1,3-glucan; and (4) β-1,3-glucanases, which cleave the glucosidic linkages in β-1,3-glucan [4–7].

β-Glucanases have great biotechnological potential. They are used in a variety of industrial applications, such as an additive in animal feed [8, 9]. They are also employed in biological pest control due their ability to cleave the cell walls of pathogenic microorganisms and in obtaining bioactive oligosaccharides, β-glucan in particular, which is considered a potent immunomodulator [10–13]. β-Glucan has been shown to possess antitumor, anti-inflammatory, antimutagenic, hypocholesterolemic, and hypoglycemic activities and to protect organisms against infection [14–17]. Kim and Yun [18] demonstrated that the β-glucan obtained from the yeast cell wall appears to be more effective than that obtained from other sources. Thus, *Saccharomyces cerevisiae*, a yeast widely used in industrial fermentation processes, is a major source of bioactive β-glucan [19].

The biological activity of a polysaccharide is related to its molecular weight, configuration, and glycosidic linkages, among other characteristics [20]. Acid hydrolysis has been the most widely used route to obtain oligosaccharides; however, this method yields a large amount of mono-, di-, and trisaccharides as well as by-products, such as furfural. For this reason, the enzymatic hydrolysis of β-glucan has been evaluated to obtain different gluco-oligosaccharides as it allows the production of higher molecular weight oligomers (degree of polymerization≥4) [17]. In this context, enzymatic lysis of the yeast cell wall using β-glucanase has a potential application in the treatment of residual cell mass to obtain β-glucan in the fermentation industry.

Kluyveromyces marxianus shows some interesting traits that would be useful in industrial applications, including the capacity to grow in a wide variety of substrates and at high temperatures [21], a high specific growth rate, and a natural ability to secrete enzymes, which is a desired property for the cost-effective industrial process. Moreover, different strains of *K. marxianus* have generally recognized as safe status, similar to *S. cerevisiae* and *Kluyveromyces lactis* [21].

In this study, a new extracellular β-glucanase produced by *K. marxianus* was identified in addition to the intracellular β-glucosidase that has been previously described for this yeast [22, 23]. Response surface methodology was used to optimize the production process and to determine the optimal parameters (pH and temperature) for its enzymatic activity. Furthermore, after optimizing the conditions for β-glucanase production, the resulting culture supernatant was found to be effective in digesting the cell wall of the yeast *S. cerevisiae*, indicating the great potential of this enzyme in the biotechnological production of soluble β-glucan and protoplasts from yeast cells.

Materials and Methods

Microorganisms and Growth Conditions

The yeast *K. marxianus* UFV-3 was maintained on YPD agar medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar) at 28 °C and subcultured at nightly intervals. The media used for enzyme production consisted of 1 g/L yeast extract and 2 g/L peptone (0.1× YP), containing different glucose and Tween 20 concentrations and pH values, as indicated by the factorial design (Table 1). The pH of the medium was kept constant by

Table 1 Central composite experimental design matrix defining conditions for optimization of β-glucanase production by *K. marxianus* grown in shake flasks: influence of concentrations of glucose, Tween 20, medium pH, and temperature of growth and respective observed and adjusted values for enzyme activity (units per milliliter) and residuals

Treatment	Independent variables				Enzymatic activity (U/mL)	$\hat{y}$ (U/mL)	ε
	Temperature (°C)	pH	Tween 20 (% *v*/*v*)	Glucose (% *w*/*v*)			
1	31.5 (−1)	4.75 (−1)	0.05 (−1)	3.0 (−1)	5.23	5.6984	−0.46983
2	38.5 (+1)	4.75 (−1)	0.05 (−1)	3.0 (−1)	7.04	6.1205	0.92111
3	31.5 (−1)	6.25 (+1)	0.05 (−1)	3.0 (−1)	4.10	5.1915	−1.09269
4	38.5 (+1)	6.25 (+1)	0.05 (−1)	3.0 (−1)	2.73	5.6137	−2.88416
5	31.5 (−1)	4.75 (−1)	0.15 (+1)	3.0 (−1)	5.13	5.6137	−2.88416
6	38.5 (+1)	4.75 (−1)	0.15 (+1)	3.0 (−1)	6.68	6.1205	0.55798
7	31.5 (−1)	6.25 (+1)	0.15 (+1)	3.0 (−1)	4.43	5.1915	−0.76613
8	38.5 (+1)	6.25 (+1)	0.15 (+1)	3.0 (−1)	2.81	5.6137	−2.80851
9	31.5 (−1)	4.75 (−1)	0.05 (−1)	5.0 (+1)	4.22	6.7505	−2.52818
10	38.5 (+1)	4.75 (−1)	0.005 (−1)	5.0 (+1)	4.22	6.7505	−2.52818
11	31.5 (−1)	6.25 (+1)	0.05 (−1)	5.0 (+1)	5.39	6.6659	−1.27342
12	38.5 (+1)	6.25 (+1)	0.05 (−1)	5.0 (+1)	5.39	6.6659	−1.27342
13	31.5 (−1)	4.75 (−1)	0.15(+1)	5.0 (+1)	4.31	6.7505	−2.43992
14	38.5 (+1)	4.75 (−1)	0.15 (+1)	5.0 (+1)	6.92	7.1727	−0.25212
15	31.5 (−1)	6.25 (+1)	0.15 (+1)	5.0 (+1)	4.87	6.2437	−1.37702
16	38.5 (+1)	6.25 (+1)	0.15 (+1)	5.0 (+1)	6.54	6.6659	−0.12351
17	28.0 (−α)	5.5 (0)	0.10 (0)	4.0 (0)	6.11	3.3950	2.71610
18	42.0 (+α)	5.5 (0)	0.10 (0)	4.0 (0)	5.12	4.2394	0.88071
19	35.0 (0)	4.0 (−α)	0.10 (0)	4.0 (0)	3.21	2.8690	0.33834
20	35.0 (0)	4.0 (−α)	0.10 (0)	4.0 (0)	5.11	1.8553	3.25848
21	35.0 (0)	5.5 (0)	0.00 (−α)	4.0 (0)	13.45	10.8913	2.56308
22	35.0 (0)	5.5 (0)	0.20 (+α)	4.0 (0)	10.76	10.8913	2.56308
23	35.0 (0)	5.5 (0)	0.10 (0)	2.0 (−α)	8.36	6.6056	1.75617
24	35.0 (0)	5.5 (0)	0.10 (0)	6.0 (+α)	10.55	8.7100	1.84065
25	35.0 (0)	5.5 (0)	0.10 (0)	4.0 (0)	10.75	10.8913	−0.14272
26	35.0 (0)	5.5 (0)	0.10 (0)	4.0 (0)	11.41	10.8913	0.52302
27	35.0 (0)	5.5 (0)	0.10 (0)	4.0 (0)	11.37	10.8913	0.47763
28	35.0 (0)	5.5 (0)	0.10 (0)	4.0 (0)	11.10	10.8913	0.09937
29	35.0 (0)	5.5 (0)	0.10 (0)	4.0 (0)	10.99	10.8913	0.09937

addition of 50 mM sodium citrate buffer. Erlenmeyer flasks (150 mL) containing 50 mL of culture medium were inoculated by adding yeast pre-culture to an OD_{600} of 0.6 and incubated at different temperatures on a rotary shaker (180 rpm) for 48 h.

Enzyme Assays

Cell-free supernatants were obtained by centrifugation of freshly grown yeast cultures and used as the source of extracellular β-glucanase. The enzymatic activities of the supernatants were determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG, Sigma) as a substrate.

The *p*-NPG-hydrolyzing activity was estimated by incubating 0.250 mL of each supernatant with 0.250 mL of 2 mM *p*-NPG in 0.05 M sodium citrate buffer (pH 5.5) at 50 °C for 40 min. The reaction was stopped by the addition of 1.0 mL of 0.5 M Na_2CO_3. The release of *p*-nitrophenol was determined at 410 nm using the molar extinction coefficient (ε) 18,300 M^{-1} cm^{-1}. The enzyme activity was expressed in units per milliliter, and one unit (U) was defined as the nanomoles of *p*-nitrophenol released per minute.

Optimization of Enzyme Production

A central composite design with four independent variables (concentration of glucose, concentration of Tween 20, pH of the medium, and incubation temperature) was examined for the shake flask cultivation of *K. marxianus* UFV-3. The effects of these factors on the production of extracellular β-glucanase by this yeast strain were evaluated using a central composite experimental design ($2^4+2\times4+5$) with five replicates at the central point, summarizing 25 different combinations and 29 experimental runs. The independent variables and respective levels of variation for β-glucanase production (Y_1=units per milliliter) are shown in Table 1. Analysis of variance (ANOVA) and multiple regression analysis were performed using Minitab 16 software, and surface plots were constructed using SigmaPlot 10.0 software.

Optimization of Enzyme Activity

The yeast *K. marxianus* UFV-3 was grown for 48 h in 0.1× YP under the conditions determined by the experimental optimization. After centrifugation (10,000×*g* for 10 min), the cell-free supernatant was characterized for β-glucanase activity. To evaluate the effect of temperature, the supernatant was incubated with the substrate *p*-NPG at 28, 37, 42, 50, 55, 60, 65, or 70 °C. To evaluate the effect of pH, the enzymatic activity assays were performed using McIlvaine buffer (citrate/phosphate) with pH values ranging from 3.0 to 7.5 (0.5 intervals). Based on these results, a factorial experiment was devised for the optimization of enzyme activity using a central composite design ($2^2+2\times2+5$) with five replicates at the central point, summarizing eight different combinations and 13 experimental runs. The independent variables and respective levels of β-glucanase activity (Y_1=units per milliliter) are shown in Table 2. ANOVA and multiple regression analysis were performed using Minitab 16 software, and surface plots were constructed using SigmaPlot 10.0 software.

Partial Purification of the Extracellular β-Glucanase

The extracellular β-glucanase was purified by ion exchange chromatography. First, a crude extract was obtained by growing the yeast *K. marxianus* UFV-3 under the optimal conditions determined from the response surface analysis. The extract was concentrated by ultrafiltration using a Vivaspin column (10 kDa molecular weight cutoff (MWCO), GE Healthcare) at 4,500×*g* to a final concentration of 10 % of the initial volume. The concentrated crude extract was adjusted to a pH of 7.0 and then subjected to anion exchange liquid chromatography using a HiTrap Capto Q column (GE Healthcare) that was previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 1.0 mL/min. Stepwise elution was performed with NaCl concentrations from 0.1 to 1 M. The fractions that were positive for enzyme activity were pooled and concentrated using a Vivaspin column (10 kDa MWCO, GE Healthcare). For all steps, the enzyme assay was performed and the concentration of total soluble protein was determined by the bicinchoninic acid method [24].

Table 2 Central composite experimental design matrix defining conditions for optimization of β-glucanase activity: effect of pH and temperature and respective observed and adjusted values for enzyme activity (units per milliliter) and residuals

Treatment	Independent variables		Enzymatic activity (U/mL)	$\hat{y}$ (U/mL)	ε
	Temperature (°C)	pH			
1	40.0 (−1)	4.5 (−1)	25.22	16.59	−0.84
2	40.0 (−1)	6.5 (+1)	12.24	20.82	4.40
3	66.0 (+1)	4.5 (−1)	12.36	14.07	−1.83
4	66.0 (+1)	6.5 (+1)	41.32	20.67	0.67
5	53.0 (0)	4.0 ($-\alpha$)	21.35	10.55	0.84
6	53.0 (0)	7.0 ($+\alpha$)	11.39	44.54	−2.22
7	35.0 ($-\alpha$)	5.5 (0)	15.75	44.54	−2.74
8	71.0 ($+\alpha$)	5.5 (0)	8.73	44.54	−3.13
9	53.0 (0)	5.5 (0)	42.32	18.34	−5.98
10	53.0 (0)	5.5 (0)	41.80	27.82	13.50
11	53.0 (0)	5.5 (0)	41.41	44.54	0.17
12	53.0 (0)	5.5 (0)	44.71	44.54	1.59
13	53.0 (0)	5.5 (0)	46.13	13.15	−4.42

Protein Digestion and Identification by Mass Spectrometry (MS)

Protein Digestion

Protein spots were removed manually from the gels, reduced with DTT, and alkylated with iodoacetamide. The digestion was carried out with trypsin in 50 mM ammonium bicarbonate buffer (pH 7.8) containing 20.0 ng/μL sequencing grade trypsin (Promega) at 37 °C overnight. Peptides were extracted from the spots with 30 μL of 50 mM ammonium bicarbonate, followed by incubation for 10 min with occasional vortex mixing. The supernatant was collected and transferred to a 0.5-mL plastic microcentrifuge tube. This extraction was performed two more times. The extract was completely dried by evaporation in a SpeedVac. The tryptic peptides were solubilized in 30 μL of MS grade water (Sigma-Aldrich) containing 0.1 % (*v*/*v*) formic acid.

Protein Identification and Sequencing

Matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF)/TOF (Ultraflex III, Bruker Daltonics) and quadrupole-time-of-flight (Q-TOF) (micrOTOF-Q III, Bruker Daltonics) were used to analyze the proteins. For MALDI-TOF/TOF mass spectrometry, the tryptic peptides were mixed with α-cyano-4-hydroxyl cinnamic acid (Bruker Daltonics) at a 1:1 ratio. The mass spectra obtained were processed using Flexi Analysis software (Bruker Daltonics), and a peak list (mascot generic format (mgf) format) was used for peptide mass fingerprinting and de novo sequencing.

For Q-TOF, 10 μL of the tryptic digest was used for LC-MS analysis using a HPLC system (Prominence UFLC, Shimadzu) and a C18 capillary column (5 Å, 300 μm × 100 mm) at a flow rate of 3.0 μL/min. The eluted peptides were injected into a micrOTOF-Q mass spectrometer using a micro-ESI ionization needle. The mobile phase buffers used for the gradient program

were water with 0.1 % (*v*/*v*) formic acid (A) and acetonitrile with 0.1 % (*v*/*v*) formic acid (B). The gradient program consisted of 5 % B for 5 min, linear ramping to 50 % B over 35 min, linear ramping to 95 % B over 10 min, holding at 95 % B for 10 min, ramping back to 5 % B over 5 min, and, finally, holding at 5 % B for 5 min. The mass spectrometer scanned between 300 and 1,500 *m*/*z* in positive mode, and data were acquired for 70 min for each LC-MS/MS run. Data acquisition by both MS instruments was managed using the Hystar package (Bruker), and the spectrums were processed with the Data Analysis package (Bruker) using the default settings for proteomics. The mass spectrometer operated in auto-MSn mode, which collected MS2 spectra for the more intense ions in each full scan spectrum, excluding single charge ions. The scan time was set to 0.5 s for the survey scan, and the MS2 spectra were recorded for five spectrums to enable the collection of the maximum number of MS2 spectra during the analysis. Peak lists were generated in an mgf by the Data Analysis package (Bruker) and used for protein identification with the Mascot algorithm (Matrix Science). Batch Mascot searches were performed locally against the protein database from NCBI using the Mascot Daemon client with a precursor tolerance of 0.1 Da for the product ions, allowing for methionine oxidation and deamidated (NQ) as a variable modification, carbamido methylation as a fixed modification, one missed cleavage, charge states of 2, 3, and 4, and trypsin as the enzyme. The peptide and protein identifications were statistically evaluated and validated using the PeptideProphet and ProteinProphet algorithms from the Scaffold package (Proteome software) using a threshold of >90 % probability. The sequences were verified manually by de novo sequencing.

Enzyme Specificity to Different Substrates

To determine the specificity of the enzyme on various chromogenic substrates, the partially purified β-glucanase was used in the enzymatic reaction described above with each of the following substrates: *p*-nitrophenyl (*p*NP) β-D-glucopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-cellobioside, *p*NP-α-glucopyranoside, *p*NP-α-mannopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-α-L-arabinopyranoside, *o*-nitrophenyl (oNP) β-D-glucopyranoside, oNP-α-D-galactopyranoside, and *m*-nitrophenyl (mNP) α-D-galactopyranoside.

The concentrated culture supernatant was used to test the enzyme activity on cellobiose, carboxymethylcellulose, and β-glucan. To measure the enzyme activity on cellobiose, the enzyme extract was incubated with 100 mM cellobiose in 50 mM citrate buffer (pH 5.5) at 37, 42, or 50 °C for 30 h. Aliquots were withdrawn every 6 h and boiled for 5 min. The glucose released was measured by HPLC. To measure the enzyme activity on carboxymethyl cellulose (CMC) and β-glucan, the reaction was incubated for 4 h at 50 °C in a total volume of 750 μL, which contained 225 μL of sample, 375 μL of 2 % (*w*/*v*) CMC or β-glucan, and 150 μL of 50 mM sodium citrate buffer (pH 5.5). The reducing sugar released was quantified using the dinitrosalicylic acid method [25]. Furthermore, the glucose concentration was also determined in this assay using HPLC.

Lytic Activity Against S. cerevisiae

Lytic activity was quantified by measuring the OD_{600} of a cell suspension of *S. cerevisiae* in the presence and absence of the enzyme. Yeast cells were collected by centrifugation, washed with 50 mM citrate buffer (pH 5.5), and suspended in the same buffer to an OD of 1.4. Enzyme activity assays were performed using 50 μL of the crude extract concentrate with or without 50 mM β-mercaptoethanol and 50 μL of yeast culture (final OD of 0.7). The assay was performed for 16 h at 45 °C in microplates.

Results and Discussion

Optimization of β-Glucanase Production

Factorial design and response surface methodology are efficient strategies to obtain optimal conditions for systems containing multiple variables [26]. These techniques have been successfully employed in several areas of biotechnology, especially in the optimization of culture media for enzyme production [27, 28] and in the optimization of conditions that favor enzymatic activity [29, 30]. Thus, to optimize the production of extracellular β-glucanase by *K. marxianus* UFV-3, these techniques were used to determine the relationship of enzyme production with the following controllable factors: pH, temperature, Tween 20 concentration, and glucose concentration. The range of values tested for each factor was determined according to the literature for Tween 20, as well as from data that were previously obtained by our laboratory (data not shown). Table 1 shows the combinations of factors, the levels assayed, and the respective observed and adjusted ($\widehat{y}$) activity (units per milliliter) of the secreted β-glucanase and the residual error obtained for each treatment.

Multiple regression analysis of the experimental data enabled the creation of a second-order polynomial model for *K. marxianus* extracellular β-glucanase production (Eq. 1).

$$\mathrm{EA} = -295.92 + 10.1661T + 41.3599P + 6.99310G - 0.144369T^2 - 3.79071P^2 - 0.808376G^2 \quad (1)$$

where EA corresponds to enzymatic activity (units), T corresponds to temperature, P corresponds to the pH of the medium, and G corresponds to the concentration of glucose.

All of the effect terms in Eq. 1 significantly influenced ($p<0.05$) the extracellular β-glucanase activity. The linear and squared effect terms for the Tween 20 concentration and the effect terms for the interactions between the experimental factors were discarded because they were not significant according to the t test ($p>0.05$). The model showed a R^2 of 73.53 %, indicating that most of the variation observed in the extracellular β-glucanase production could be explained using the adjusted model.

The response surface and contour plots of the adjusted extracellular β-glucanase production obtained from Eq. 1 are shown in Fig. 1a–c. The maximum extracellular β-glucanase production was observed in the temperature range of 32.5 to 37.5 °C, at a glucose concentration between 3.5 and 5.5 %, and at a pH value between 5.0 and 6.0, covering the central point of the experiment (35 °C, 4 % glucose, and pH 5.5). Thus, these conditions, which are close to the central point, were considered to be optimal for the production of extracellular β-glucanase by *K. marxianus* UFV-3. Moreover, according Eq. 1 and Fig. 1a–c, there is an optimal range in which the combination of several factors provides the same optimal result, enabling the selection of an economical level of each factor. The coefficients and t test allowed us to conclude that out of all of the tested factors, pH had the greatest influence on the production of active extracellular β-glucanase by *K. marxianus*, followed by cultivation temperature, and the glucose concentration provided a minor contribution.

Effect of pH and Temperature on β-Glucanase Activity

The factors of pH and temperature significantly influence enzymatic activity. Thus, to determine the pH and temperature that results in the highest activity of the β-glucanase secreted by *K. marxianus* UFV-3, initial tests were performed on the culture supernatant to evaluate the effect of these parameters on the β-glucanase activity. The maximum activity was found at a pH of 5.0, and the activity decreased when the pH increased from this point, indicating that acidic pH values are most appropriate for the performance of this enzyme. In addition,

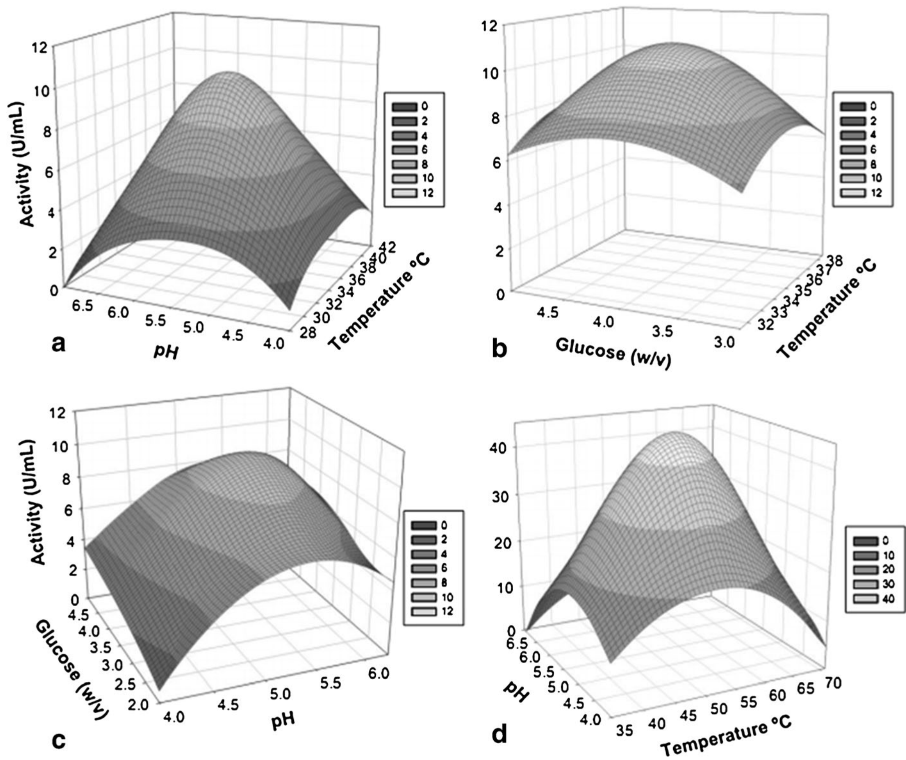

Fig. 1 Response surface plot for the optimization of β-glucanase production (**a**–**c**) and activity (**d**). Response surface plot for the adjusted activity (units per milliliter) of the β-glucanase in the culture supernatant and the following factors: pH (4.0–7.0) and temperature (28–42 °C) (**a**), glucose (2–5 % (*w*/*v*)) and temperature (28–42 °C) (**b**), and glucose (2–5 % (*w*/*v*)) and pH (4.0–7.0) (**c**). **d** Response surface plot for the adjusted activity (units per milliliter) of β-glucanase and the pH (4.0–7.0) and temperature (28–42 °C)

increasing the temperature had a positive effect on the enzyme activity up to 60 °C, at which point there was a negative effect, suggesting thermal denaturation (data not shown).

These independent experiments, which were performed with different pH and temperature values, do not show the effect of the possible interactions between these parameters, and therefore, these results may not match the optimal conditions of this bioprocess. Therefore, to determine the optimal conditions (temperature and pH) for the activity of this β-glucanase, these results were used as the basis for planning a central composite design and to create a response surface model for β-glucanase activity. Table 2 shows the combinations of the independent variables (temperature and pH) and the respective observed and adjusted β-glucanase activity (units per milliliter) and residual error obtained for each treatment. The second-order polynomial model adjusted for β-glucanase activity is given in Eq. 2.

$$\mathrm{EA} = -578.069 + 138.07P + 9.61257T - 12.8586P^2 - 0.0915863T^2 \tag{2}$$

where EA corresponds to enzymatic activity (units), T corresponds to temperature, and P corresponds to pH.

ANOVA indicated that this model was significant (p=0.001), and the R^2 value indicated that 84.08 % of the observed variation in the β-glucanase activity can be explained by this model. The contour plot and response surface plot generated from the data are shown in

Fig. 1d. For the reaction conditions used, the maximal enzymatic activity was observed in the temperature range of 45 to 60 °C and at a pH value between 4.8 and 6.0, spanning the central point of the experiment. Therefore, the combination of the temperature and pH central point (53 °C and pH 5.5) was considered optimal for the activity of this β-glucanase. This broad area of maximum performance allows the exploitation of many combinations of temperature and pH, indicating the potential versatility of this enzyme in various processes.

Fungal β-glucanases exhibit optimal activity at temperatures between 40 and 60 °C and at pHs between 4.5 and 6.0 [3, 4, 7, 31]. The exo-β-(1,3)-glucanase from *Pichia guilliermondii* is most active at pH 4.5 and 45 °C. The endo-β-(1,3)-glucanases from *S. cerevisiae* and *Schizosaccharomyces pombe* show optimal activity at pH 6.0 and 6.5, respectively [32, 33], values close to those found for the extracellular β-glucanase in this study.

Partial Purification of β-Glucanase

The concentrated crude extract showed a specific activity of 113 U/mg protein, and the specific activity obtained at the end of the partial purification was 3,927.6 U/mg protein, representing a purification factor of 35, with 50 % recovery of the enzyme.

The concentrated extract was applied to a HiTrap Capto Q column. The fractions with the highest activity were pooled, concentrated in a Vivaspin column (10 kDa MWCO), and analyzed by SDS-PAGE (Fig. 2). A significant concentration of two proteins approximately 45 kDa was subsequently identified as being similar to β-glucanases. However, even at low concentrations, four contaminating proteins were observed in the sample. Three proteins had a molecular weight of approximately 97 kDa, and one protein was approximately 31 kDa.

Analysis by Mass Spectrometry

The two bands observed near 45 kDa (Fig. 2) were excised from the gel, subjected to tryptic digestion, and analyzed by MALDI-TOF/TOF mass spectrometry. The MS1 mass spectra

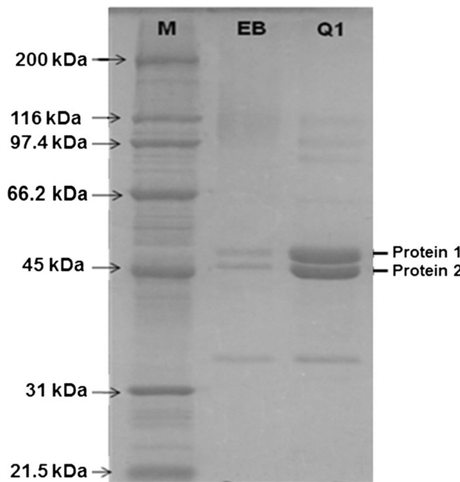

Fig. 2 Anion exchange chromatography of the concentrated culture supernatant using a HiTrap Capto Q column. SDS-PAGE analysis of the purified β-glucanase secreted from *K. marxianus*. The gel was stained with Coomassie brilliant blue G-250. *Lane 1*, molecular mass marker; *Lane 2*, culture supernatant concentrated by ultrafiltration; *Lane 3*, proteins obtained after Capto Q ion exchange and concentration by ultrafiltration

were processed and compared using the Flex Analysis package (Bruker Daltonics). The peptide mass fingerprinting was the same for both proteins, indicating the presence of two β-glucanase isoforms. The tryptic digestion was also analyzed by micrOTOF-Q (Bruker Daltonics), and the MS2 spectra were used for protein sequencing using the Mascot algorithm against a public database. The sequences of the identified peptides were additionally verified manually by de novo sequencing and aligned with the β-glucanases in the database, with 28 MS/MS spectra identified for 6 peptide sequences and 35 % of putative protein coverage. A NCBI BLAST search revealed the presence of domains of the cellulase and endo-glucanase superfamilies. The amino acid alignment using the Clustal algorithm showed this sequence as a new glucosidase, sharing similarities with 1,3-β-glucanases (Fig. 3).

Substrate Specificity of the β-Glucanase

The partially purified β-glucanase showed low rate of hydrolysis (0–4 %) for the substrates *p*NPαG, *p*NPβMan, *p*NPαMan, *p*NPβGal, *p*NPαGal, mNPαGal, oNPαGal, and *p*NPαAra compared to *p*NPβG (100 %) (Table 3). The hydrolysis of the substrates *p*NPβCb, *p*NPβXyl, and *p*NPβG was 28, 16, and 6.5 %, respectively.

These results show the high specificity of this β-glucanase for substrates with a β-anomeric configuration as it was not efficient in hydrolyzing any of the α-glycosidic substrates tested.

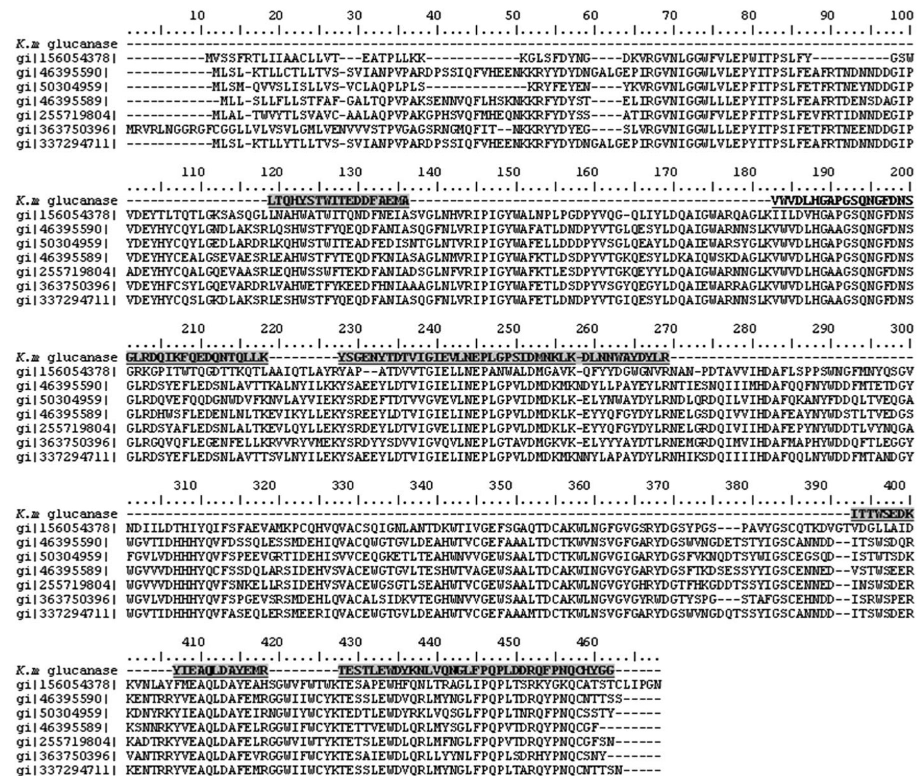

Fig. 3 Alignment of data bank sequences presenting similarity to the six peptides obtained by MS/MS sequencing of the *K. marxianus* β-glucanase. *Dashed boxes* indicate de novo peptide sequences. Other sequences and their accession numbers are shown in the figure

Table 3 Beta-glucanase specificity

Substrates (2 mM)	Relative activity (%)[a]
*p*NP-β-D-glucopyranoside (control)	100
*p*NP-β-D-cellobioside	28.2±4.45
*p*NP-β-D-xylopyranoside	16.2±0.03
*p*NP-β-D-mannopyranoside	4.0±0.17
*p*NP-α-D-galactopyranoside	3.8±0.35
*p*NP-α-D-glucopyranoside	3.2±0.12
*p*NP-α-D-mannopyranoside	2.9±0.78
*p*NP-β-D-galactopyranoside	2.7±0.03
*p*NP-α-D-arabinopyranoside	2.6±0.12

[a]Each value is the mean of duplicate experiments

The results also show that the β-glucanase from *K. marxianus* has a high specificity for β-glycosidic bonds, a low specificity for β-xylosidic bonds, and no activity on sugars containing mannose, galactose, or arabinose (Table 3).

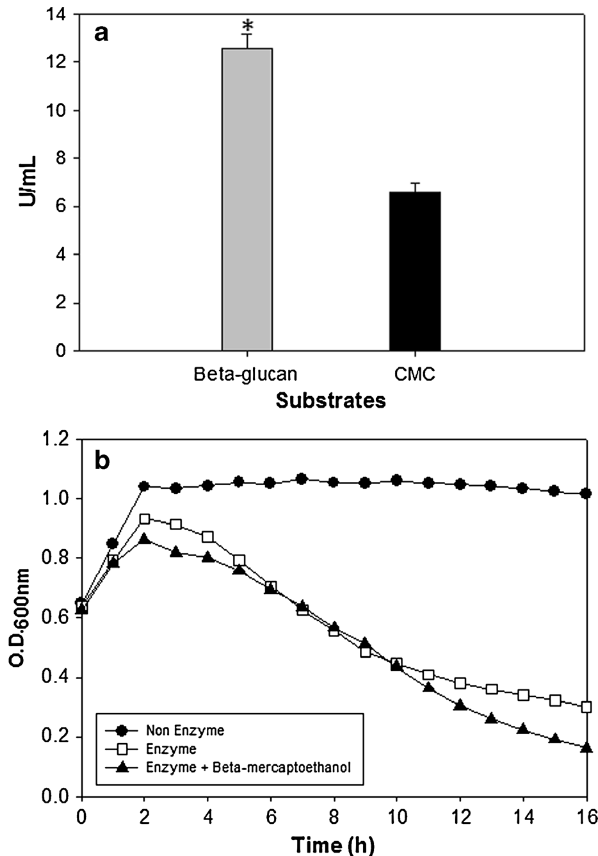

Fig. 4 **a** β-Glucanase activity on β-glucan (β-1,3:β-1,4) and CMC (β-1,4). Data are presented in nanomoles Glc per minute per milliliter of culture media. **b** Lytic activity of the culture supernatant on *S. cerevisiae* with or without β-mercaptoethanol

When evaluating the activity of the β-glucanase on CMC and β-glucan, the activity for β-glucan was almost twofold greater than that for CMC, indicating its greater specificity for β-1,3 and β-1,4 bonds (Fig. 4a). In these tests, there was no glucose production, which suggests that the secreted enzyme is an endo-glucanase. These results suggest that this enzyme is β-1,3(4)-glucanase. This type of glucanase hydrolyzes β-1,3-glucans, β-1,4-glucans, and β-1,3-glucans [5]. The lytic activity of the culture supernatant on *S. cerevisiae* was accompanied by a reduction in the optical density at 600 nm. As shown in Fig. 4b, the optical density of the *S. cerevisiae* culture was reduced when it was incubated in the presence of the enzyme extract. The activity of the β-glucanase on the yeast cell wall can be explained by the specificity of this enzyme to hydrolyze of β-1,3 glycosidic linkages [18].

Conclusions

An extracellular β-glucanase expressed and secreted by *K. marxianus* was identified for the first time. The optimal conditions to produce β-glucanase were a glucose concentration of 4 % (*w*/*v*), a pH of 5.5, and an incubation temperature of 35 °C. The highest enzyme activity was obtained at a pH of 5.5 and 55 °C. After optimizing the culture conditions for β-glucanase production, the resulting culture supernatant was found to be effective in digesting the cell wall of the yeast *S. cerevisiae*, indicating that this β-glucanase possesses great potential for the biotechnological production of soluble β-glucan and protoplasts from yeast cells.

Acknowledgments This research was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Amparo a Pesquisa do Estado de Minas Gerais, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

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