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Full Paper

Cellulase Production by *Trichosporon laibachii*

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Abstract: The ability of twenty-three yeast strains isolated from decayed wood and the fungus garden of attine ants to produce hemi- and cellulolytic hydrolases in a chemically defined medium was acessed. Xylanase, β-glucosidase and cellulase activities were found in yeasts strains in the genera of *Cryptococcus*, *Trichosporon*, *Debaryomyces* and *Pichia*. Among the isolated microorganisms, two strains of *Trichosporon laibachii* MG270406-1A14 strain showed higher cellulase titers. Several biochemical and physiological parameters were investigated for optimum cellulase production under submerged fermentation. Enzyme induction was also examined using different carbon and nitrogen sources. Cellobiose and ammonium sulphate were found best carbon (C) and nitrogen (N) sources to enhance carboxymethylcellulase production. A 2^2- factorial design with center points was developed to optimize enzyme production, and data analysed by the response surface method. Maximal enzyme titres (0.3 U mL^{-1}) occurred at initial pH 6.0, C/N ratio 9.0 and 5 days.

Keywords: cellulase; fermentation; *Trichosporon*; yeast

1. INTRODUCTION

Sustainable technologies are being developed for the economic ethanol production using lignocellulosic biomass worldwide. It is necessary to depolymerize the carbohydrate fraction of lignocellulosic biomass into simple sugars for their use as preferred carbon source by microorganisms for ethanol production [1]. However, pretreatment of lignocellulosic biomass is necessary to overcome the biomass recalcitrance. Pretreatment enables holocellulose (cellulose+hemicellulose) for the cellulase-mediated saccharification into fermentable sugars [2, 3]. The efficient hydrolysis of cellulosic fraction of plant cell wall requires the synergistic action of the cellulolytic enzymes- exoglucanase, endoglucanase, cellobiohydrolase and β-glucosidase, and some hemicellulolytic enzymes [4, 5].

Three different classes of enzymes have been required for the complete hydrolysis of cellulose to glucose. The two major activities involved in cellulose solubilization are endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91). For the production of glucose a third type of activity, cellobiase or β-glucosidase (EC 3.2.1.21) is also required. In a hemicellulolytic material, xylanases (endo-1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) are also required, to catalyze the hydrolysis of 1,4-beta-D-xylosidic linkages in xylans constituents of hemicellulose, a structural component of plant cell walls [6]. Microbial cellulolytic enzymes have shown their potential application in various industries including pulp and paper, textile, bioethanol, wine, brewery, olive oil, carotenoid, feedstuffs, detergents and waste management. The growing interest in cellulase production has been focused in improving the process economics of various biotechnological industries [7].

Yeasts are widely used in fermentative processes, mainly in the baking, brewing and wine factories. In recent years, the exploration of new

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biotechnological aspects related to the production of renewable fuels stimulated the use of these microorganisms to produce cellulolytic enzymes and ethanol [8]. Fungi and some bacteria, some yeast are also capable of producing filter paperase (FPase), carboxymethylcellulases (CMCases) and β-glucosidases [9]. The strains belonging to the genus *Trichosporum, Cryptococcus, Candida, Debaryomyces, Kluyveromyces* have been described for producing cellulases and xylanases constitutively [10, 11, 12], and also β-glucosidases when grown in defined media inducers, such as cellobiose [13].

Screening of fermentation media is an important qualitative test to microorganisms to produce metabolites. Jiménez et al. [14] evaluated the production of cellulases (CMCases, β-glucosidases and β-xylosidases) for fifty-one species of yeasts from the genera *Candida*, *Kluyveromyces*, *Filobasidium* and *Pichia,* isolated from decaying wood, grown in CMC and wood chips. Production of cellulase, xylanase and β-glucosidases was also assessed from several strains isolated from grapes and grape juice belonging to the genera *Kloeckera, Candida, Debaryomyces, Rhodotorula, Pichia, Zygosaccharomyces, Hanseniaspora* and *Kluyveromyces* [15].

This study evaluated the production of cellulolytic enzymes by several yeast strains isolated from Brazilian natural sources. Carbon and nitrogen sources, C/N ratio and initial pH were optimised for the production of CMCase by *Trichosporon laibachii*, under submerged fermentation conditions using statistical designs. This is the first report on cellulolytic enzymes production by the yeast strain *Trichosporon laibachii* MG270406-1A14.

2. MATERIAL AND METHODS

Yeast strains and cultivation Microorganisms

Twenty three yeast strains used in these studies belonged to *Cryptococcus*, *Trichosporon*, *Debaryomyces* and *Pichia* genera. These strains were isolated from decaying leaves, wood and ant nests from Brazilian biodiversity [16, 17]. These strains were previously selected by growing in solid medium containing carboxymethyl cellulose (CMC) as sole carbon source in Department of Biochemistry and Microbiology (UNESP, Rio Claro-SP, Brazil) and Department of Microbiology (UFMG, Belo Horizonte-MG, Brazil). The strains are preserved in 15% glycerol at -80°C and during the assays they were maintained on medium contained (g L^{-1}):glucose, 10.0; malt extract, 3.0; yeast extract, 3.0; peptone,5.0; and agar, 20.0; at 4 ºC and sub-cultured at fortnightly intervals.

Screening of fermentation medium for cellulase production

Two different screening experiments were performed in chemically defined media to assess cellulases production. In the first screening, twenty-three yeast strains were assessed individually for their ability to grow in medium containing CMC, Avicel®, cellobiose and xylan from Birchwood (Sigma) as sole carbon sources (Table 1). Falcon (50 mL) conical centrifuge tubes containing 10 ml of Vogel minimum salts medium [18] and different carbohydrate substrates (10.0 g L^{-1}) were incubated at 30 ºC for two weeks. The five strains indicated in Table 4 and that showed higher activity growing on each carbon source were selected for the evaluation of the production of cellulases, xylanases and β-glucosidases under submerged cultivations.

In the second screening, yeast strains were assessed for both growth and cellulases production in submerged conditions. Pre-cultures were developed by growing the yeast strains in Erlenmeyer flasks (125 mL) containing 50 mL of glucose (10.0 g L^{-1}), malt extract (3.0 g L^{-1}), yeast extract (3.0 g L^{-1}) and peptone (5.0 g L^{-1}) at 30 ºC and 200 rpm for 24 hours. The experiments were carried in Erlenmeyer flasks (125 mL) containing 50 mL of Vogel's medium and CMC, Avicel®, cellobiose and xylan (10.0 g L^{-1}) as sole carbon sources separately. The flasks were inoculated with 1.2 mg cells/ml for 24 hours, and incubated in a rotary shaker (200 rpm) for 120 hours at 30 ºC.

Evaluation of CMCase production by T. laibachii MG270406-1A14

The inoculum preparation and culture cultivation were carried out as described above. Conditions to evaluate the best carbon source for CMCase production by *T. laibachii* MG270406-1A14 were performed following a statistical mixture-design matrix [19] with three carbon sources in the media formulation. A total of 15 experimental runs were performed at 30 °C and 200 rpm. The independent variables for the optimization of CMCase production (Y_1, Um L^{-1}) were: x_1 (Avicel®, g L^{-1}); x_2 (CMC, g L^{-1}), and x_3 (cellobiose, g L^{-1}) as shown in Table 2.

Table 1. Screening of various yeasts for the production of carboxymethylcellulases (CMCase), avicellases, β-glucosidases and xylanases grown under static submerged cultivation conditions.

	Strains	CMC	Avicel	Cellobiose	Xylan
1	*T. moniliiforme* UFMG-CLM51.2a	+	+	+	+
2	*T. mycotoxinivorans* UFMG-CLM68.1	+++	+++	++	++
3	*T. mucoides* UFMG-CLM42.4	-	++	+++	+++
4	*T. laibachii* UFMG-CLM53.1a	++	+++	++	++
5	*T. mycotoxinivorans* UFMG-CLM61.3	+++	+++	++	++
6	*T. mucoides* UFMG-CLM41.1b	-	++	+++	++
7	*T. mucoides* UFMG-CLM48.1b	++	+	+	+
8	*T. mucoides* UFMG-CLM21.1	++	+	+++	+++
9	*T. moniliiforme* UFMG-CLM48.1a	++	+	+	+++
10	*T. laibachii* UFMG-CLM46.3	++	+	+	+
11	*T. mycotoxinivorans* UFMG-CLM62.1	++	-	-	-
12	*C. humicola* UFMG-CLM57.5	+	++	++	++
13	*T. laibachii* UFMG-CLM59.3	+++	+++	++	+++
14	*T. laibachii* UFMG-CLM47.3	++	+	+	+
15	*C. humicola* UFMG-CLM9.1	+	-	-	-
16	*C. humicola* UFMG- CLM53.2	-	++	+	-
17	*C. humicola* UFMG-CLM56.1	-	-	-	-
18	*T. laibachii* UFMG-CLM54.2	++	+	+	++
19	*C. humicola* UFMG-CLM44.3	-	-	-	-
20	*C. laurentii* MG1603067A	+++	++	++	+++
21	*T. laibachii* MG270406-1A14	+++	+++	+++	+
22	*T. laibachii* MG2704061A8	++	+	+	+
23	*D. hansenii* MG2704062B-55	++	+++	+++	+

Table 2. Statistical mixture-design matrix defining carbon sources formulation for the optimization of CMCase production by *T. laibachii* MG270406-1A14 grown for 5 days.

Run number	Carbon source (g/l)			CMCase (U/ml)
	Avicel (x_1)	CMC (x_2)	Cellobiose (x_3)	
1	10	0	0	0.056
2	0	10	0	0.348
3	0	0	10	0.139
4a	5	5	0	0.033
4b	5	5	0	0.089
5a	5	0	5	0.332
5b	5	0	5	0.408
6a	0	5	5	0.249
6b	0	5	5	0.241
7	6.7	1.7	1.7	0.109
8	1.7	6.7	1.7	0.257
9	1.7	1.7	6.7	0.042
10a	3.3	3.3	3.3	0.040
10b	3.3	3.3	3.3	0.053
10c	3.3	3.3	3.3	0.014

Growth profile using cellobiose (10.0 g L^{-1}) as sole carbon source was followed over 168 hours, and 2 Erlenmeyer flasks were interrupted at time intervals of 24 hours. The effect of some nitrogen sources as NH_4Cl, $(NH_4)_2SO_4$, yeast extract, peptone and NH_4NO_3, N source of Vogel's medium as well on enzyme production using cellobiose (10.0 g L^{-1}) was evaluated after 120 hours of incubation.

The C/N ratio and initial pH to enhance CMCase production by *T. laibachii* using cellobiose (10.0 g L^{-1}) as sole carbon source were studied as variables in a factorial design. Modified Vogel's medium was prepared using different concentrations of

$(NH_4)_2SO_4$. A 2^2-factorial central-composite experimental design, with three replicates at the central-point, summarizing 12 experimental runs. Independent variables for optimization of CMCase production (Y_1, U/ml) were: x_1 (C/N ratio) and x_2 (initial pH) as shown in Table 3.

Table 3. Central-composite experimental-design matrix defining conditions for C/N ratio and initial pH for optimised CMCase production by *T. laibachii* MG270406-1A14 grown for 5 days.

Experimental run	Variables in coded levels		CMCase
	x_1	x_2	(U mL^{-1})
1	-1	-1	0.045
2	1	-1	0.065
3	-1	1	0.101
4	1	1	0.098
5	-1	0	0.325
6	1	0	0.254
7	0	-1	0.036
8	0	1	0.187
9	0	0	0.043
10	0	0	0.021
11	0	0	0.222
Factors	**Real levels**		
	-1	**0**	**+1**
x_1, C/N ratio	3	9	15
x_2, initial pH	5	6	7

All experiments were carried out in duplicate to check the reproducibility, and the results represent the mean ±SD. Analysis of variance (ANOVA) and multiple regression analysis was performed using STATISTICA (data analysis software system) Version 6. www.statsoft.com (StatSoft, Inc. 2001).

Enzyme assay

Cell-free supernatants were obtained by centrifugation (1250 g, 5 min at 4 ºC) of freshly grown yeast cultures and used as the source of extracellular enzymes. Assay for total cellulase and endoglucanase activities using the substrates filter paper (FPase) and CMC (CMCase), respectively, was carried according to Ghose [20]. Avicelase activity was determined according to Wood and Bhat [21]. Xylanase activity was measured using xylan from birch wood (Sigma, 0.1 g L) as substrate, and incubated at 50 °C for 20 min. Reducing sugars released were measured according to Nelson [22] and Somogyi [23] methodology. The unit of enzyme activity was defined as the number of µmol of reducing sugars produced per minute and per ml of test solution under the standard assay conditions. β-glucosidase activity was measured using *p*-nitrophenyl-β-glucopyranoside (4 mM) as substrate, and incubated at 50 °C for 20 min. The reaction was stopped by the addition of 1.0 ml of 0.25 M Na_2CO_3 solution. The unit of β-glucosidase activity was defined as the number of µmol *p*-nitrophenol liberated/min [24]. The specific activities of all enzymes are expressed as the rate of substrate consumption (or product formation) per unit of protein [µmol min^{-1} (mg protein)$^{-1}$].

Analytical techniques

Cell concentration was determined spectrophotometrically at 600 nm. Reducing sugars were determined by the cuproarsenate method [22, 23]. Protein was determined by the Lowry modified method [25].

3. RESULTS AND DISCUSSION

Twenty-three yeast strains belonging to the genera of *Cryptococcus, Trichosporon, Pichia* and *Debaryomyces* were screened for the cellulolytic and hemicellulolytic enzymes activities. These strains were isolated from leaves, decaying wood and ant nests [16,17]. Strains were previously selected on the basis of their ability for cellulases production after growing on solid growth medium (CMC + agar) (data not shown). *T. laibachii* UFMG-CLM59.3 showed the highest activity of CMCase, xylanase and avicelase when grown in static liquid medium containing the inducers CMC, xylan and Avicel, respectively, as sole carbon sources (Table 1). *T. laibachii* MG270406-1A14 showed higher activity of CMCase, avicelase and β-glucosidase when grown in static liquid medium containing CMC, Avicel and cellobiose, respectively, as sole carbon sources. Among the twenty-three yeast strains, *C. humicola* UFMG-CLM56.1 and UFMG-CLM44.3 did not show any enzyme activity on any carbon source.

From these results, five yeast strains that showed increased activity on each carbon source were further chosen for the second screening under submerged liquid cultivations. From the Table 4, it can be noted that the isolate *T. laibachii* MG270406-1A14 showed higher levels of CMCase (≈ 0.75 U mL^{-1}) when grown on CMC as sole carbon source in comparison to other evaluated strains. *T.laibachii* MG270406-1A14

was the only yeast strain that exhibited xylanolytic activity in this condition. The highest β-glucosidase activities were detected in cultures of yeasts *T. mycotoxinivorans* UFMG-CLM 61.3 and *T. laibachii* MG270406-1A14 (≈ 0.92 U mL^{-1}). None of the isolates showed FPase activity in any culture medium. Absence of FPase activity regardless of positive CMCase activity have been also described to *Penicillium* sp. fungi and can be explained by the lowest affinity with this kind of cellulose substrate [26].

Table 4. Screening of various yeasts for the production of carboxymethylcellulases (CMCase), avicellulose, β-glucosidases and xylanases grown under agitated submerged cultivation conditions.

Carbon source	Strains	Enzyme activity (U mL^{-1})				
		FPase	CMCase	Avicellulose	Xylanase	β-glucosidase
CMC	*T. mycotoxinivorans* UFMG-CLM68.1	N.D.	0.304	0.016	0.000	0.315
	T. mycotoxinivorans UFMG-CLM61.3	N.D.	0.398	0.012	0.000	0.991
	T. laibachii UFMG-CLM59.3	N.D.	0.451	0.022	0.000	0.075
	C. laurentii MG1603067A	N.D.	0.427	0.012	0.004	0.918
	T. laibachii MG270406-1A14	N.D.	0.752	0.010	0.021	0.335
Avicel	*T. mycotoxinivorans* UFMG-CLM68.1	0.002	0.020	0.004	0.012	1.450
	T. mycotoxinivorans UFMG-CLM61.3	0.019	0.030	0.003	0.011	1.525
	T. laibachii UFMG-CLM59.3	0.028	0.023	0.003	0.013	1.166
	T. laibachii UFMG-CLM53.1a	0.031	0.022	0.005	0.024	1.465
	T. laibachii MG270406-1A14	0.033	0.028	0.005	0.012	1.439
Xylan	*T. moniliiforme* UFMG-CLM48.1a	0.004	0.012	0.036	0.149	0.003
	T. mucoides UFMG-CLM42.4	0.002	0.022	0.031	0.178	0.011
	T. laibachii UFMG-CLM59.3	0.003	0.040	0.020	0.404	0.004
	T. mucoides UFMG-CLM21.1	0.004	0.051	0.017	0.381	0.008
	C. laurentii MG1603067A	0.005	0.034	0.036	0.137	0.006
Cellobiose	*T. mucoides* UFMG-CLM21.1	0.001	0.045	0.015	0.064	1.445
	T. mucoides UFMG-CLM41.1b	0.001	0.059	0.011	0.057	1.525
	T. mucoides UFMG-CLM42.4	0.002	0.058	0.012	0.054	1.166
	D. hansenii MG2704062B-55	0.002	0.065	0.014	0.054	1.465
	T. laibachii MG270406-1A14	0.001	0.090	0.019	0.057	1.439

Avicel and cellobiose, as sole carbon sources, induced the production of β-glucosidase by all strains tested, while decreased CMCase and xylanase activities in all cultures. The yeast isolates when grown on birchwood xylan showed low activity of FPase. The strain *T. mucoides* UFMG-CLM 21) presented higher activities of β-glucosidase (≈ 0.01 U mL^{-1}), CMCase (≈ 0.05 U mL^{-1}) and xylanase (≈ 0.4 U mL^{-1}). The enzyme levels found to evaluated yeasts are in according to the cellulolytic activities described in literature for a non-fungi species as bacteria [27] and yeasts [12].

As Avicel, CMC and cellobiose were found cellulase titres promoters by yeast isolates, a statistical mixture-design was developed to evaluate the best concentration of each substrate for CMCase production by *T. laibachii* MG270406-1A14 strain. The response was obtained as a function of proportions of the mixture of components (Table 2) and the model was given by Equation 1:

$$\hat{Y}_1 = 0.232194x_2 + 0.227823x_3 \quad (1)$$

Equation 1 showed that use of Avicel (x_1) to the culture medium was not significant to enhance CMCase production by *T. laibachii* MG270406-1A14. However, CMC (x_2) and cellobiose (x_3) promoted enzyme production at similar levels. The response surface plot was depicted as a contour (Figure 1). The maximum enzyme production value was predicted for a pure CMC and pure cellobiose in nutrient medium. An additional run was made to test the predictive ability of the model. The predicted result was 0.18 U $mL^{-1}$of maximum activity of CMCase using cellobiose as sole carbon source.

Another factor reported to be crucial for enzymes production is the N source for yeast cultivation [23]. We examined the ability of three inorganic (NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$) and two organic (yeast extract, peptone) N sources to stimulate CMCase production by *T. laibachii* MG270406-1A14 using cellobiose as sole carbon source (Figure 2). Highest CMCase activity was detected in $(NH_4)_2SO_4$ (0.22 U mL^{-1}), while NH_4NO_3 increased avicellase

production (0.12 U mL^{-1}). NH_4NO_3 was reported as a best N source for β-glucosidase production by *Debaryomyces pseudopolymorphus* grown on cellobiose (10 g L^{-1}) as sole carbon source [13] as well as for cellulases production by *Penicillium occitanis* [28]. A profile of yeast growth on cellobiose (10 g L^{-1}) and $(NH_4)_2SO_4$ over 168 h showed that after 72 h the yeast reached stationary phase although CMCase production has a maximum activity at 120 h and decreased after this period (data not shown).

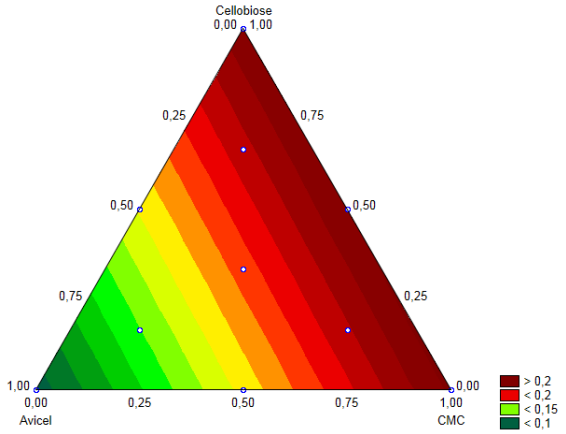

Figure 1. Response surface contours for CMCase (U mL^{-1}) production by *T. laibachii* MG270406-1A14.

Highest CMCase specific activity was detected in $(NH_4)_2SO_4$ and NH_4Cl (~22 U mg^{-1}), while NH_4NO_3 and NH_4Cl presented highest avicellase specific activity (~9.5 U mg^{-1}). Xylanase specific activity (3.7 U mg^{-1}) was favored by the use of $(NH_4)_2SO_4$ as N source. The specific activities of all hydrolases was low in the culture medium containing yeast extract and peptone due the high content of protein derived from these organic N sources, which was around 1.5 and 1.0 mg mL^{-1}, respectively.

Earlier, Thongekkaew et al. [11] observed the CMCase production by the yeast *Cryptococcus* sp. S-2 when grown on cellobiose and yeast extract, as carbon and N sources respectively. *Pichia etchelsii* JFG-2201 strain was reported to be producing two intracellular β-glucosidases induced by the addition of 10 mM cellobiose in the medium, and one of these enzymes had a higher affinity for the cellobiose and p-NPG [29].

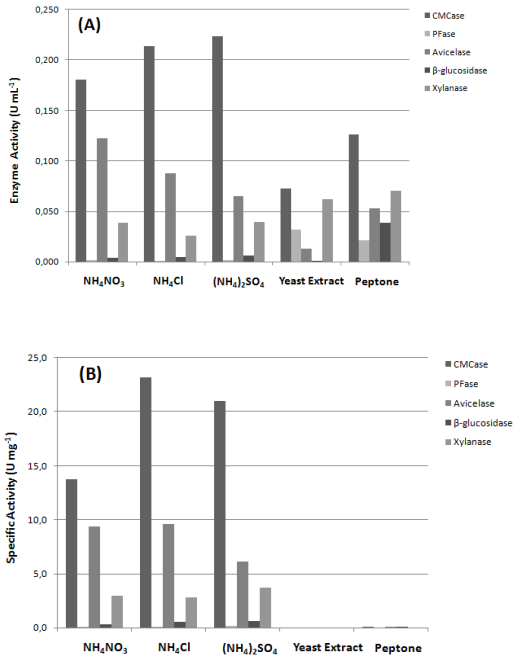

Figure 2. CMCase production profile by *T. laibachii* MG270406-1A14 grown in the presence of different N-sources: (A) enzymatic activity, (B) specific activity.

Furthermore, to study the effect of important factors and their interaction on CMCase production by *Trichosporon laibachii* MG270406-1A14, a 2^2-factorial central-composite experimental design was developed. Through multiple regression analysis of the experimental data, a second-order polynomial equation was obtained for CMCase production and is given by Equation 2.

$$\hat{Y}_2 = 0.269250 + 0.075637x_1 - 0.155000x_1^{\,2} - 0.234500x_2^{\,2} \quad (2)$$

Linear effect term of the variable x_2 was discarded as being non-significant. According to the results, an intercept was significant indicating that the central-points (C/N ratio 9.0 and initial pH of 6.0) were correctly chosen. The variable more important for CMCase production by *T. laibachii* MG270406-1A14 was initial pH, followed by C/N ratio (Table 3). The analysis of variance (ANOVA) showed the lack-of-fit ($p > 0.05$) was not significant, indicating that the model was predictive.

The R-squared value implies 90 % of the variability in the observed response values can be explained by the model, or by experimental factors and

their interactions. The pure error was low, indicating good reproducibility of the experimental data. According to our experimental data, maximum CMCase production by *T. laibachii* MG270406-1A14 (0.3 U mL^{-1}) occurred within 5 days of growth, initial pH of 6.0 and C/N ratio of 9.0. The response-surface plots are depicted as a contour and a 3-dimensional surface shown in Figure 3.

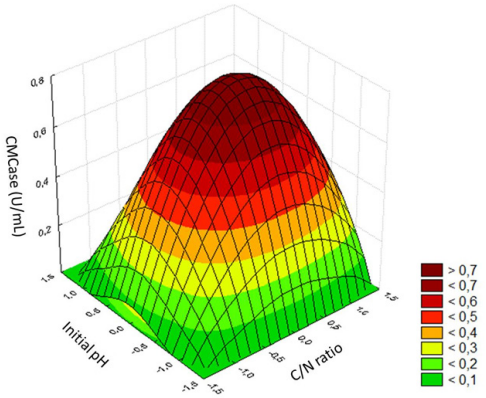

Figure 3. Response-surface plots depicted as a three-dimensional surface for CMCase production (Y_1) by *T. laibachii* MG270406-1A14 as a function of C/N ratio (x_1) and initial pH (x_2) according to a 2^2-factorial central-composite experimental design.

These results are in agreement to the cellulose production by yeast strains in the literature. Otero et al. [12] described the production of endo-xylanase (2.7 U mL^{-1}), FPase (0.1 U mL^{-1}) and CMCase (0.1 U mL^{-1}) by the yeast strain, isolated from chicory, *Cryptococcus laurentii*. In other study, 82 yeast strains were screened on their capabilities of degrading CMC as a sole carbon source, and three different strains belonging to the *Candida* sp. genus showed higher levels of CMCase production (0.2 U/mL) [30]. Similar amounts of CMCase (0.3 U mL^{-1}) was described to yeast-like fungus *Acremonium strictum* [31].

4. CONCLUSION

This is the first report on cellulases production by *Trichosporon laibachii* MG270406-1A14 strain. The results clearly demonstrated the potential application of this strain for the production of cellulolytic enzymes, which can be further applied in the hydrolysis of lignocellulosic biomass to obtain clean sugars which are the building blocks of second generation biofuels production.

5. ACKNOWLEDGMENTS

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