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Production and Characterization of Galactose Oxidase Produced by Four Isolates of *Fusarium graminearum*

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ABSTRACT

A screening aimed to find new galactose oxidase producer isolates and to evaluate the production among Fusarium graminearum strains was conducted. Thirty-five isolates out of 39 analysed produced the enzyme at several levels. The data indicated a wide distribution of galactose oxidase within F. graminearum and also revealed new producer isolates. The enzyme produced by different isolates showed similar thermal activity and stability and were active on same substrates. However, the optimum pH ranged from 7.0 to 7.5. Thus, all evaluated isolates were suitable for the production of galactose oxidase.

Key words: Galactose oxidase, Fusarium graminearum, Enzyme

INTRODUCTION

The copper metal enzyme galactose oxidase (GO) (D-galactose: oxygen 6-oxidoredutase, EC 1.1.3.9) catalyses an oxidation reaction of alcohols to aldehydes, with concomitant reduction of O_2 to H_2O_2 (Whittaker, 2003; Rogers and Dooley, 2003; Whittaker, 2002). The production of extra cellular GO has been detected in a few species of filamentous fungi, including *Fusarium graminearum* (Avigad et al., 1962; Tressel and Kosman, 1982; Barbosa-Tessmann et al., 2001), *Gibberella fujikuroi* (Aisaka and Terada, 1981), and *F. acuminatum* (Barbosa-Tessmann et al., 2001). The majority of the studies with GO have been done with *F. graminearum* (NRRL 2903; ATCC 46032), isolated as a mycoparasite of the basidiomicete *Polyporus circinatus*, and initially identified as *Dactylium dendroides* in Southern Brazil (Avigad et al., 1962; Nobles and Madhosingh, 1963; Ögel et al., 1994; Niessen and Vogel, 1997). This isolate has been used for the industrial production of GO.

GO of *F. graminearum* is described to occur in a monomeric mature active form with size and molecular weight, calculated from the gene (*gao*A) sequence of this enzyme, of 639 amino acid residues and 68.5 kDa, respectively (McPherson et al., 1992, Baron et al., 1994). This enzyme, which is very basic (pI = 12; Ettinger and Kosman, 1981), is reported to be inactive below pH 5.0 and to have its maximal activity at pH 6.7 - 7.3 (Cooper et al., 1958; Ettinger and Kosman, 1981). This protein is reported to be stable at temperatures below 60 ^{o}C with a T_{50} of 67 ^{o}C (Sun et al., 2001). Substrates for GO include dihydroxyacetone, raffinose, melibiose, lactose, guar gum, galactose, and its derivatives as

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galactosamine and methyl-galactopyranosides (Whittaker, 2002; Mazur, 1991; Chiu et al., 1996). Applications of GO include determination of D-galactose and lactose concentrations (Tkac et al., 2000; Karube et al., 1990); enzymatic synthesis of carbohydrates (Franke et al., 2003; Andreana et al., 2002; Basu et al., 2000; Liu and Dordick, 1999; Mazur, 1991); histochemical staining studies (Schulte and Spicer, 1983; Roberts and Gupta, 1965); biotransformation of glyco-biopolymers into paper strength additives (Chiu et al., 1996); and early detection of colon cancer by a cytochemical staining method used to identify a dissacharide (Gal-β(1,3)-GalNAc) expressed in the rectal mucus (Vucenik et al., 2001; Carter et al., 1997; Said et al., 1999).

One problem associated with the use of GO regards the availability of sufficiently pure enzyme. The purification procedures are laborious and the commercial preparations are very expensive (Mazur, 1991). Because of this reason, there are reports about the use of recombinant DNA technology to improve the GO production (Sun et al., 2001; Xu et al., 2000; Whittaker and Whittaker, 2000).

The applications of GO would be beneficial if microorganism is able to produce high levels of enzyme or if an enzyme with better biochemical characteristics as thermal stability, high substrate affinity, and high catalytic efficiency is produced. Considering this, the objective of this work was to do a screening of several strains of *F. graminearum* isolated from the South of Brazil for GO production and to compare some biochemical characteristics of the enzyme produced by four different isolates, which were considered as good producers.

MATERIALS AND METHODS

Microorganisms

Thirty-nine *F. graminearum* isolates were used in the screening studies. These microorganisms were isolated from diseased ears or seeds of small cereal crops (wheat and triticale) from several counties of the States of Paraná, Santa Catarina, and Rio Grande do Sul, Brazil. To isolate the microorganisms, a conidial mass of infected ears was transferred to culture plates containing potato dextrose agar medium supplemented with 300 ppm streptomycin (PDA/S). Seeds were previously disinfected in 2% sodium hypochloride, washed twice in sterilized water, and transferred to a culture plate containing three layers of sterilized wet paper. After germination, the pathogen was isolated from diseased seedlings by transferring conidia or mycelia from the diseased tissues to a culture plate containing PDA/S. Single spore cultures were obtained according to procedures described by Nelson et al. (1983).

For the comparative analyses of the biochemical characteristics, the following isolates of *F. graminearum* were used: UEM 02 and UEM 10, selected in the performed screening as good producers, and the isolates IAPAR 2218 and UEL R2118, selected as good producers in a previous work (Barbosa-Tessmann et al., 2001). The original galactose oxidase producer strain (GOPS) of *F. graminearum* (Cooper et al., 1958; Avigad et al., 1962) was kindly provided by Dr. Glaci T. Zancan (Universidade Federal do Paraná, Curitiba, PR, Brazil) and used as a reference. All isolates were maintained in potato dextrose agar (PDA) slants with trimestral transfer and in stocks under mineral oil (Booth, 1971).

Screening of *F. graminearum* isolates for GO production

The culture liquid medium used was as described by Markus et al. (1965) with 122.5 mM phosphate buffer, pH 7.0. The original medium described by Markus et al. (1965) had 122.5 mM phosphate buffer, pH 6.79. However, Shatzman and Kosman (1977) reported that a medium pH of 7.0 is better for GO production by *F. graminearum*. The medium composition was (mM) 15.14 $(NH_4)_2SO_4$, 12.5 NH_4NO_3, 0.1% yeast extract, 51.4 KH_2PO_4, 71.2 Na_2HPO_4, with pH corrected to 7.0 with NaOH. Prior to inoculation, sterile solutions of mineral ions (final concentration: 1.62 mM $MgSO_4 \cdot 7H_2O$, 11.83 µM $MnSO_4 \cdot H_2O$, 10 µM $CuSO_4 \cdot 5H_2O$) and D-glucose (final concentration: 1%) were added to the medium.

A fragment of a fresh PDA culture was used as inoculum for 125 ml Erlenmeyer culture flasks containing 25 ml of the sterile liquid medium. The cultures were grown for four days at 25 ^oC under orbital shaking (110 rpm), homogenized through passage first through a sterile sieve and then through an 18-gauge needle attached to a sterile syringe, and used as inoculum (2% vol/vol) to new 125 ml culture flasks, containing 25 ml of the same liquid medium supplemented with 1% glucose. These subcultures were incubated in the

same way for 72 h and then filtered through filter paper. The enzymatic activity was analysed in the filtrates of two or three flasks and the mycelia on the filter paper were dried to have weight estimated. The results represented the average of the data obtained in two or three culture flasks analysed independently.

Determination of enzyme activity

Filtrate (0.5 ml pure or diluted with 50 mM phosphate buffer, pH 7.0); 1.4 ml of the reactive mixture [50 mM phosphate buffer, pH 7.0; 0.2 mg/ml *o*-dianisidina (Sigma D-3252; previously dissolved in methanol 2 mg/ml); 0.04 mg/ml (6.0 U/ml) peroxidase (Sigma P-8125)]; and 0.1 ml of 0.5 M D-(+)-galactose were mixed and incubated for 10 minutes at 30ºC (Avigad et al., 1962; Tressel and Kosman, 1982). In this reaction, the hydrogen peroxide produced in the GO reaction was reduced to water by the peroxidase, with concomitant oxidation of the *o*-dianisidine to a brown colored product, which was read at 460 nm. For these conditions, one enzymatic unit corresponded to an absorbance of 1.0.

Biochemical properties

For these analyses, the cultures were cultivated in the same liquid medium and in the conditions described above, except that the liquid medium phosphate buffer concentration was 100 mM (pH 7.0). To find out the best temperature for activity, the enzyme activity was determined as described above at 30, 40, 50, 60, and 70 ^{o}C. To determine the thermal stability of the enzyme, the filtrates were incubated for 30 minutes at 30, 40, 50, 60, or 70 ^{o}C and submitted to the enzyme activity test as described above. To determine the optimum pH, the enzymatic activity was determined by using the phosphate buffer with the following pHs 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. To determine the GO activity on different substrates, galactose was substituted by the following sugars: D-(+)-glucose, raffinose, guar gum, lactose, and dihydroxyacetone (all at 0.5 M, except guar gum that was used at 1%). Analysis were performed independently in the filtrate of three culture flasks.

RESULTS AND DISCUSSION

The results for growth (mycelia dry weight) and for the ability to produce the GO are presented in Table 1. The majority of the tested isolates (89.7%) were able to produce the enzyme. Dias and Kemmelmeier (1987) screened 48 *F. graminearum* isolates obtained from wheat and maize plants from Southern Brazil for GO production and found 18 producers (37.5%). The present data agreed with this about the wide distribution of GO production by *F. gramineraum* in this geographic area.

The isolates UEM 02 and UEM 10 were able to produce high level of the enzyme. Isolates 04, 15, 16, and 32 did not produce the enzyme, although these were able to grow in the used culture medium, as shown by the mycelia dry weight (Table 1).

Fig. 1 shows the temperature effect on the GO activity at pH 7.0. From 30 to 50 ^{o}C the enzyme was active and at 60 ^{o}C the activity reduced to about 50% when compared to the activity at the optimum temperature (40 ^{o}C).

Studies of thermal stability of the GO from the *F. graminearum* strains showed that the enzyme maintained nearly most of the initial activity after preincubation at temperatures of 30 to 60 ^{o}C for 30 minutes at pH 7.0 (Fig. 2), but they were fully inactivated after 30 minutes incubation at 70 °C. With regard of the pH effect, the enzyme was inactivated at pH 5.5 and was most active over the pH range of 7.0-7.5 (Fig. 3). In comparison with the GOPS enzyme pH curve, the pH curve of the enzyme produced by the isolate UEM 10 had a similar profile, whereas the enzyme of the UEL R2118 isolate had a pH curve deviated to the right, being less than 50% active at pH 6.5. The pH curves of the isolates IAPAR 2218 and UEM 02 had the same format as the isolate UEL R2118 curve (not shown).

Table 2 shows the relative activity of the GO enzyme from the isolates toward galactose derivatives and alternative substrates. The best substrate for all tested GO appeared to be dihydroxyacetone. The activity of the enzymes on raffinose, lactose, and guar gum indicated that they could oxidize galactose derivatives with substitutes at the carbon-1site.

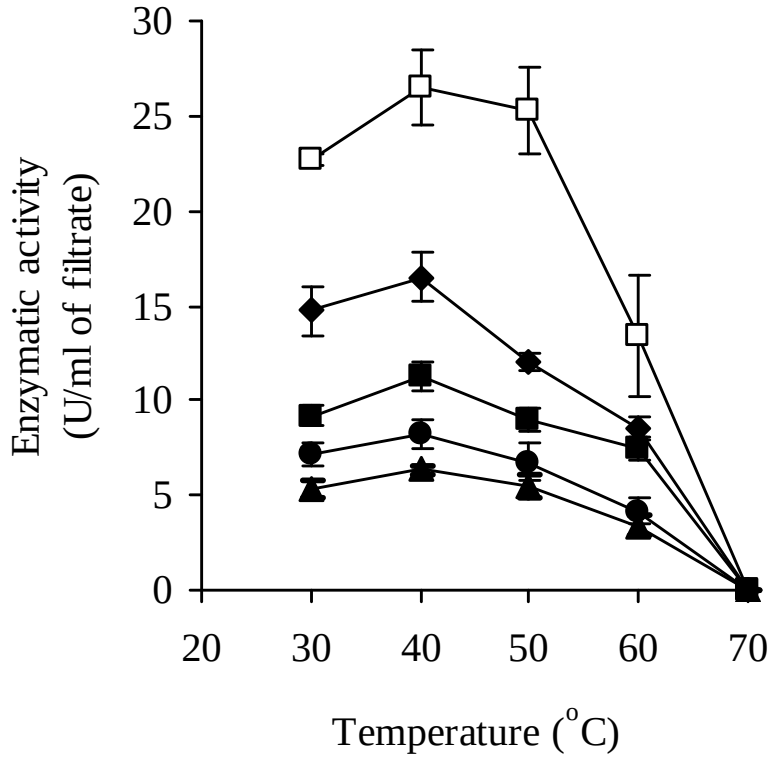

Figure 1 - Effect of the temperature in the activity of the galactose oxidase from the different isolates of *F. graminearum*. GOPS □, UEM 02 ◆, UEM 10 ■, IAPAR 2218 ●, and UEL R2118 ▲.

In contrast, the lack of activity of the enzymes on glucose indicated that the stereo configuration of the OH-group at the carbon-4 was important for their activities.

The results for the GO from the GOPS were in agreement with the reports in the literature (Whittaker, 2003; Wittaker, 2002; Mazur, 1991; Chiu et al., 1996; Sun et al., 2001; Amaral et al., 1966; Cooper et al., 1958; Avigad et al., 1962; Ettinger and Kosman, 1981). The optimum pH, temperature, thermal stability, and substrate specificity for the enzyme of the four tested isolates were similar when compared with the GO from GOPS. Considering this, the GO from those isolates could substitute the enzyme from GOPS in its possible biotechnological applications.

In conclusion, the results suggested a wide GO production distribution among different strains of *F. graminearum* from the southern region of Brazil.

Table 1 - Galactose oxidase production by isolates of *F. graminearum* from the South region of Brazil

***F. graminearum* isolate**	**Geographic origin (County, State)**	**Plant organ where it was isolated**	**Host**	**Enzymatic activity (U/ml of filtrate)***	**Mycelia dry weight (g/25 ml of medium)***
UEM 02	Campo Mourão, PR	Ear	Wheat	14.85	0.22
UEM 03	Mamborê, PR	Ear	Wheat	3.53	0.15
UEM 04	Mamborê, PR	Ear	Wheat	0	0.12
UEM 05	Mamborê, PR	Ear	Wheat	1.78	0.27
UEM 06	Peabiru, PR	Ear	Wheat	0.86	0.15
UEM 08	Francisco Beltrão, PR	Ear	Wheat	11.96	0.37
UEM 09	Francisco Beltrão, PR	Ear	Wheat	8.64	0.38
UEM 10	Francisco Beltrão, PR	Ear	Wheat	18.31	0.43
UEM 11	Iguaraçú, PR	Ear	Wheat	0.83	0.25
UEM 12	Iguaraçú, PR	Ear	Wheat	0.38	0.36
UEM 13	Jacarezinho/PR	Ear	Wheat	12.2	0.40
UEM 14	Jacarezinho PR	Ear	Wheat	2.29	0.31
UEM 15	Carambeí, PR	Seed	Triticale	0	0.34
UEM 16	Carambeí, PR	Seed	Triticale	0	0.33
UEM 17	Carambeí, PR	Seed	Triticale	0.34	0.25
UEM 18	Carambeí, PR	Seed	Triticale	0.31	0.22
UEM 21	Carambeí, PR	Seed	Wheat	2.07	0.27
UEM 22	Tibagi, PR	Seed	Wheat	7.11	0.29
UEM 27	Abelardo Luz, SC	Seed	Triticale	6.59	0.37
UEM 28	Abelardo Luz, SC	Seed	Triticale	1.18	0.30
UEM 29	Abelardo Luz, SC	Seed	Triticale	9.6	0.26
UEM 30	Abelardo Luz, SC	Seed	Triticale	3.15	0.36
UEM 32	Abelardo Luz, SC	Seed	Triticale	0	0.21
UEM 34	Pato Branco, PR	Seed	Triticale	3.34	0.29
UEM 35	Pato Branco, PR	Seed	Triticale	5.81	0.27
UEM 36	Pato Branco, PR	Seed	Triticale	5.81	0.27
UEM 37	Seberi, RS	Seed	Wheat	0.67	0.34
UEM 38	Seberi, RS	Seed	Wheat	4.71	0.35
UEM 39	Seberi, RS	Seed	Wheat	10.68	0.37
UEM 40	Palmeira das Missões, RS	Seed	Wheat	6.92	0.50
UEM 41	Palmeira das Missões, RS	Seed	Wheat	10.06	0.39
UEM 43	Ijuí, RS	Seed	Wheat	2.59	0.37
UEM 44	Ijuí, RS	Seed	Wheat	2.44	0.41
UEM 45	Ijuí, RS	Seed	Wheat	5.47	0.36
UEM 46	Não-Me-Toque, RS	Seed	Wheat	1.54	0.44
UEM 47	Não-Me-Toque, RS	Seed	Wheat	1.37	0.35
UEM 56	Campos Novos, SC	Seed	Wheat	7.26	0.35
UEM 60	Maringá, PR	Seed	Wheat	0.18	0.41
UEM 61	Maringá, PR	Seed	Wheat	0.75	0.35

*Data are the average of the results obtained independently in two or three culture flasks.

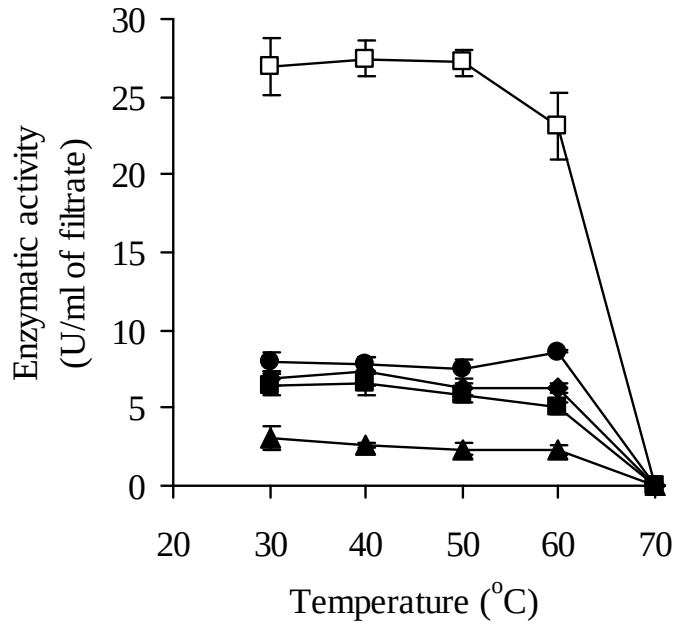

Figure 2 - Thermal stability of the galactose oxidase from the different isolates of *F. graminearum*. GOPS □, UEM 02 ◆, UEM 10 ■, IAPAR 2218 ●, and UEL R2118 ▲.

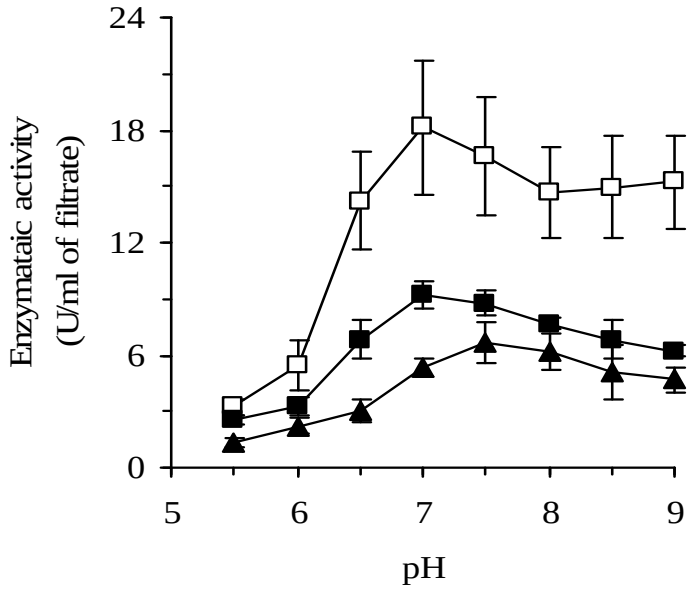

Figure 3 - The pH dependence of the galactose oxidase from the different isolates of *F. graminearum*. GOPS □, UEM 10 ■, and UEL R2118 ▲.

Table 2 - Relative activity (%) of the galactose oxidase from the different isolates of *F. graminearum* toward various substrates

Substrate	*F. graminearum*				
	GOPS	**UEM 02**	**UEM 10**	**UEL R2118**	**IAPAR 2218**
D-(+)-Galactose	100	100	100	100	100
Dihydroxyacetone	213.3	188.5	176.2	233.7	118.2
Raffinose	172.2	124.7	151.4	151.2	112.9
Lactose	2.4	0.2	0.5	0.5	0.5
D-(+)-Glucose	0	0	0	0	0
Guar gum	1.8	0.9	0.7	0.4	0.7

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RESUMO

Uma análise visando encontrar novos isolados produtores de galactose oxidase (GO) e avaliar a distribuição da produção entre cepas de *Fusarium graminearum* foi conduzida. Trinta e cinco isolados de 39 testados produziram a enzima em diferentes níveis. Os dados indicaram uma ampla distribuição da produção da galactose oxidase por *F. graminearum* e revelaram novos isolados produtores. A enzima produzida por diferentes isolados apresentou atividade e estabilidade térmicas similares e foi ativa sobre os mesmos substratos. No entanto, o pH ótimo variou de 7,0 a 7,5. Assim, todos os isolados são adequados para a produção de galactose oxidase.

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