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PRODUCTION OF HYDROLYTIC ENZYMES BY THE PLANT PATHOGENIC FUNGUS *MYROTHECIUM VERRUCARIA* IN SUBMERGED CULTURES

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ABSTRACT

The capability of the plant pathogenic fungus *Myrothecium verrucaria* to produce extracellular hydrolytic enzymes in submerged cultures was studied using several substrates. The fungus was able to produce different depolymerases and glycosidases, being xylanase, pectinase and protease the most important. Lipase was found in cultures developed in the presence of olive oil, while protease activity was detected in all cultures. Xylanase and pectinase were optimally active at pH 4.5-5.5, while protease was active in a large range of pH 3.5 to 11.0. All three enzymes were maximally active at 40ºC and they were stable for several hours at temperature up to 50ºC.

Key words: carbohydrases, hydrolytic enzymes, *Myrothecium verrucaria*, protease

INTRODUCTION

Plant pathogens produce a range of enzymes capable of degrading plant cell wall components (1,23). Among the economically important plant pathogens, *Myrothecium verrucaria* (Albertini and Schwein) Ditmar:Fr, is a ubiquitous phytopathogenic fungus which attacks a wide range of plants, including cucumber (20), soybean (16), upland cotton (11), sunflower (3), birdsfoot trefoil (6), tomato (25), rice (24) and corn (27).

Extracellular proteins secreted by fungus are able to macerate tissues and degrade cell wall components. They must thus contain the enzymes corresponding to the types of glycosidic linkages present in the cell wall polysaccharides. Extracellular enzymes are important to fungi not only for digestion but also in many instances for the pathogenic process: the enzymes may function in overcoming the natural resistance of the host as well as in providing soluble products that can be absorbed and used as food (9). *M. verrucaria* is known to produce endochitinase (8,26), xylanases (7), pectinases (5) and bilirrubin oxidases (10). The production of extracellular proteases by plant pathogenic fungi is also well documented, and it has been proposed that in some fungus-plant interactions these enzymes may function as pathogenic factors (4,17,19,20,23).

Studies of enzyme production by a phytopathogenic fungus are complicated by the presence of plant, particularly by the presence of plant enzymes and microbial enzyme inhibitors that occur in the plants. The most practical way to study the production of enzyme by a fungus is therefore to study the production of its enzymes on artificial growth media that contain no plant or enzyme inhibitors produced by the plant. Considering that, the aim of this study was to get an overview of the enzymes that are produced by *M. verrucaria* in submerged cultures possibly involved with its phytopathogenicity.

MATERIALS AND METHODS

Microorganism

Myrothecium verrucaria (Albertini and Schwein) Ditmar:Fr CCT 1886 was obtained from the Collection culture of Fundação André Tosello, Campinas, SP, Brazil and it was maintained on potato dextrose agar.

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Enzyme production

For enzyme production in submerged cultures, 1 x 10^9 spores were transferred to 50 mL of mineral media (15) containing 0.5% (w/v) of one carbon source, and pH adjusted to 6.0. The cultures were incubated at 28ºC on a rotary shaker at 120 rpm. After 7 days, the mycelia were removed from the culture media by filtration. To determine the dry weight of the mycelia, they were dried overnight at 60ºC. To study the effect of association of different substrates in the production of enzymes, each substrate or mixture of substrates were added to a final concentration of 1% (w/v).

Enzyme determinations

Glycoside hydrolase activities (β-glucosidase and β-xylosidase) were determined by measuring the rate of p-nitrophenol released from the appropriate p-nitrophenyl derivatives (p-nitrophenyl-β-glucopiranoside and p-nitrophenyl-β-xylopyranoside, respectively). The standard reaction mixture contained 2.0 µL of enzyme solution and 1 mg/mL of substrate in phosphate buffer 0.1 M, pH 6.0. After 15 min of incubation at 50ºC, reactions were stopped by the addition of 2 mL of 0.1 M Na_2CO_3 and the p-nitrophenol liberated was determined spectrophotometrically at 410 nm (22). Polysaccharidase activities (as xylanase, pectinase and carboxymethylcellulase, CMCase) were determined by measuring the amount of reducing sugar (xylose, galacturonic acid and glucose) released from various substrates (xylan, polygalacturonic acid and carboxymethylcellulose, respectively). A 2.0 µL volume of enzyme solution was incubated at 50ºC for 30 min. in 1.0 mL of substrate solution polysaccharide (2 mg/mL) dissolved in phosphate buffer, 0.1 M, pH 6.0. Reactions were stopped by the addition of 1 mL of dinitrosalicylic reagent (14). Tubes were placed in a boiled water bath for 5 min. The A_{540nm} was read with appropriate single sugars as standards (glucose, xylose and galacturonic acid, respectively to carboxymethylcellulase, xylanase and polygalacturonase activities). Lipase was determined using an olive oil emulsion as substrate. The liberated free fatty acids were titrated with 0.05 M NaOH and phenolphtalein as indicator (18). Protease was determined using casein as substrate. The released tyrosine was estimated by Lowry's method (12).

Effect of pH on the activity of xylanase, polygalacturonase and protease

The effects of pH on the activity of enzymes were determined in a series of McIlvaine's buffers with pH values from 3.5 to 8.3 (13), and 0.1 M glycine-NaOH (pH 8.5 to 10.0).

Effect of temperature on enzyme activity and stability

The effects of temperature on the activity of enzymes were carried out at temperature ranging from 25 to 70ºC. Thermal stability was investigated by incubating the enzyme at 30, 40, 50 and 60ºC for 1 hour. Immediately afterwards the enzymes were immersed in an ice bath and then the activities were tested under standard conditions.

Experimental design and statistical analysis

To study the effect of each carbon source in the enzyme production, the experimental outline used was a factorial experiment based on 1 x 13 block design (one culture time *versus* 13 different carbon sources) with three repetitions for each one. To study the effect of association of carbon sources in the enzyme production, it was used a 1 x 10 block design (one culture time *versus* 10 different carbon source combination) with three repetitions for each one. The analysis were done using the statistical pack program GraphPad Prism® (3.0). Tukey test ($P<0.05$) was applied for comparison of the averages.

Chemicals

The enzymatic substrates and carbon sources were obtained from Sigma Chemical Corp. (St. Louis, Mo). All other reagents were of analytical grade.

RESULTS

Enzymes capable of degrading a wide range of glucosides and polysaccharides were detected in cell-free culture supernatants (Table 1). Growth of *M. verrucaria* on various polysaccharides used as the sole carbon source demonstrated that the fungus secretes enzymes that convert cellulosic, pectinolytic and hemicellulolytic substrates to simple sugars. Glucose and xylose grown cultures did not exhibit polysaccharidase activities, suggesting the presence of simple sugars repressed the production of such enzymes. Among polysaccharide depolimerases, xylanase and polygalaturonase seemed to be the major enzymes secreted by the fungus.

Lipase was barely produced by the fungus when carbohydrates were offered as carbon source. However, its production was increased ten times when the culture medium was enriched with olive oil at 1% (Table 1). Protease was produced in all cultures and its production was not apparently affected by the carbon source ($P>0.05$).

Fig. 1 shows the production of xylanase, pectinase and protease when mixtures of different polymers were used as carbon source. An association of pectin and other carbon source (starch, xylan or casein) increased the production of pectinase from 3.2 U/mL (cultures using orange pectin as the only carbon source) to 8.1, 7.6, 9.9 and 10.1 U/mL when starch, casein, xylan and xylan plus casein were added in the pectin cultures, respectively. In fact, the highest levels of three enzymes were obtained in the cultures where wheat bran, a rich substrate containing several carbohydrates and proteins was the carbon source (12.9, 78.3 and 150 U/mL to pectinase, xylanase and protease, respectively).

Table 1. Production of enzymes by *M. verrucaria* in submerged cultures

Carbon source (0.5%)	Biomass (mg)	β-glu (U/mL)	β-xyl (U/mL)	Xylanase (U/mL)	Pectinase(**) (U/mL)	CMCase (U/mL)	Protease (U/mL)	Lipase (U/mL)
Xylose	58±7	4.2±1.3	0.9±0.1	1.4±0.5	Nd	Nd	45.9±4.6	0.11±0.05
Glucose	137±15	8.4±2.4	1.9±0.5	1.9±0.3	Nd	Nd	81.4±11.5	1.23±0.08
Maltose	83±9	9.1±2.6	1.5±0.2	6.1±0.3	1.3±0.2	Nd	58.1±6.8	0.83±0.07
Lactose	125±14	7.8±1.0	1.3±0.3	14.7±1.0	Nd	Nd	89.0±9.0	0.52±0.05
Cellobiose	77±9	8.3±3.8	3.7±0.5	7.9±0.6	1.5±0.6	0.3±0.1	56.86.0	0.37±0.04
Sucrose	55±9	5.1±1.1	0.9±0.2	5.4±0.7	1.2±0.4	Nd	35.3±4.4	0.35±0.03
Xylan	56±8	18.7±0.8 (*)	15.8±0.6 (*)	26.8±3.5 (*)	2.7±0.9	0.2±0.1	33.7±3.2	0.50±0.05
CMcellulose	21±3	3.2±0.3	0.1±0.04	1.7±0.2	0.4±0.04	0.2±0.04	10.10.9	0.07±0.01
Orange pectin	79±9	18.0±2.2 (*)	15.1±1.1 (*)	7.7±2.2	7.7±0.3 (*)	0.3±0.2	60.2±4.6	0.36±0.05
Starch	85±10	7.8±3.2	3.1±0.3	7.6±1.7	3.6±0.5	Nd	58.4±5.4	0.48±0.05
Casein	57±7	5.2±2.2	1.3±0.2	6.9±1.6	2.4±0.4	Nd	33.4±3.4	0.23±0.02
Ovoalbumin	66±8	4.0±1.8	4.1±0.7	10.4±4.0	1.3±0.1	Nd	43.4±3.7	0.30±0.04
Olive oil	139±19	6.6±2.5	5.0±0.8	14.2±1.9	3.3±0.8	0.5±0.3	69.55.8	7.42±0.4 (*)

The cultures were developed at 120 rpm and 28ºC for 7 days. The results represent the media±SD of three independent experiments. ND=not detectable activity; (*) significantly different among classes, $p<0.05$ (Tukey's test); (**) as polygalacturonase activity.

Some properties of the main extracellular enzymes (xylanase, pectinase and protease) from *M. verrucaria* were studied. Xylanase and pectinase was more active in an acidic of pH (4.5-5.5), while the best pH for protease activity was between pH 8.0 and 9.0 (Fig. 2). Fig. 2 suggest the existence of more than one group of protease, one with optimum pH between 6.0-7.0, and other with optimum pH between 8.0-9.0. All enzymes were optimally active at 40ºC and they retained more than 95% of initial activity after 60 min at 50ºC (data not shown).

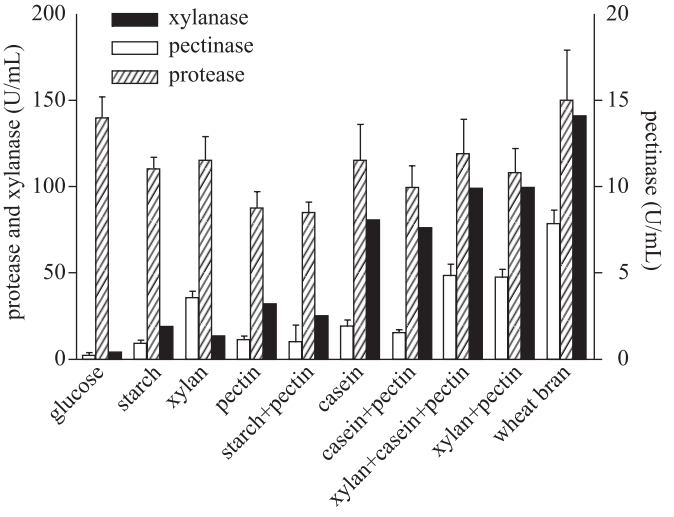

Figure 1. Effect of different mixtures of carbon source in the production of hydrolytic enzymes by *Myrothecium verrucaria*. The carbon source or mixture of carbon source was added to a final concentration of 0.5% (w/v). When more than one carbon source was used, they were used at the same concentration. The cultures were developed at 120 rpm and 28ºC for 7 days. The results represent the media ± SD of three independent experiments.

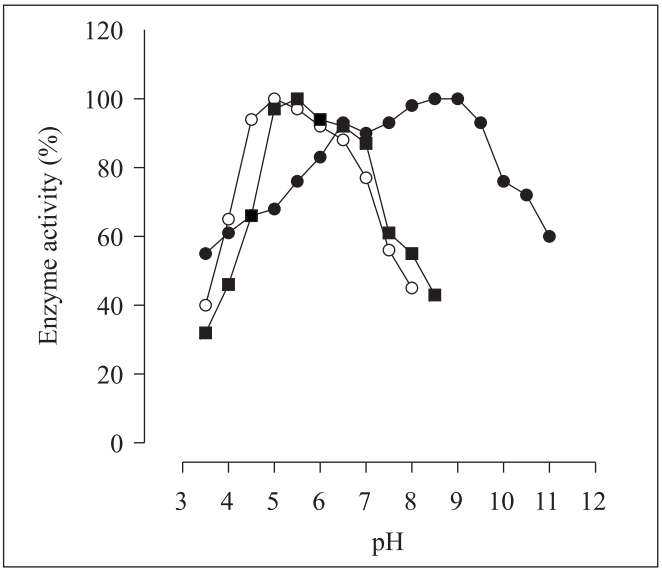

Figure 2. Effect of pH in the activity of *Myrothecium verrucaria* enzymes. Pectinase (○), xylanase (■) and protease (●) activities. The pH curves were done using the substrate for each enzyme in McIlvaine's buffer (pH 3.5 to 8.3) and 0.1 M glycine buffer (pH 8.5 to 10.0).

DISCUSSION

In the present work we have shown that *M. verrucaria* produces polysaccharide depolymerases and glucosidases necessary to degrade important structural cell wall polysaccharides, particularly pectin and hemicellulose (Table 1). The secretion of several enzymes provides this phytopathogenic fungus with the ability to attack hosts which differ in their polysaccharide cell wall compositions (22). The secretion of glycosidases combined with the polysaccharide depolymerases may also remove side groups of heteropolysaccharides, making easier the action of endoenzymes (21).

Our results showed the capability of *M. verrucaria* to produce other hydrolytic enzymes such as proteases and lipases. These enzymes may be involved in the capability of the fungus to invade vegetal tissues. *M. verrucaria* protease was identified as an alkaline protease (Fig. 2). However, differently from xylanase and pectinase that were active in a an acidic range of pH, protease from *M. verrucaria* showed high activity at alkaline pH, although it remained active in a large range of pH, what could indicate the presence of multiple isoenzymes, with different optimum pH.

It has been suggested that the proteases may facilitate located penetration of the plant cell wall by breaking down the fibrous glycoproteins that contribute to cell wall stability (2). Some phytopathogenic fungi such as *Fusarium*, *Alternaria*, and *Rhizoctonia* produced serine alkaline proteases, which are indispensable for their growth (9,19). They are probably nutrient-mobilizing enzymes whose primary function is the support of fungal growth after host cell death has occurred.

The highest levels of enzymic activities have been obtained when wheat bran was the main substrate (Fig. 1). Wheat bran is a very rich substrate consisting of a mixture of proteins, fat, soluble and insoluble carbohydrates. It is probable that the several hydrolytic enzymes secreted by the fungus present a synergy in the degradation of wheat bran components.

In conclusion, in the present study, it was observed the capability of *M. verrucaria* to produce and secrete different hydrolytic enzymes. Studies of production of enzymes using several plant materials are in progress in our laboratory. This study will serve to increase the understanding of factors that control the production, activity, and the role of *M. verrucaria* hydrolytic enzymes in its phyto-pathogenicity.

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RESUMO

Produção de enzimas hidrolíticas pelo fungo fitopatogênico *Myrothecium verrucaria* em culturas submersas

A capacidade do fungo fitopatogênico *Myrothecium verrucaria* produzir enzimas hidrolíticas extracelulares em culturas submersas foi estudada utilizando diversos substratos. O fungo foi capaz de produzir diferentes depolimerases e glicosidases, sendo xilanases, pectinases e proteases as mais importantes. Atividade lipase foi encontrada nos filtrados das culturas desenvolvidas na presença de óleo de oliva, enquanto atividade proteolítica foi detectada em todas as culturas. Xilanase e pectinase foram otimamente ativas em pH 4,5 a 5,5, enquanto protease foi ativa em ampla faixa de pH (3,5 a 11,0). As três enzimas foram otimamente ativas 40ºC e estáveis por várias horas a temperaturas até 50ºC.

Palavras-chave: carboidrases, enzimas hidrolíticas, *Myrothecium verrucaria*, protease

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