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# Production of Polysaccharidases in Different Carbon Sources by *Leucoagaricus gongylophorus* Möller (Singer), the Symbiotic Fungus of the Leaf-Cutting Ant *Atta sexdens* Linnaeus

Aline Silva,[1] Maurício Bacci Jr.,[2] Fernando C. Pagnocca,[2] Odair C. Bueno,[2] Maria J.A. Hebling[2]

[1]Departamento de Produção Vegetal/Defesa Fitossanitária, Faculdade de Ciências Agronômicas, Universidade Estadual Paulista, Rua José Barbosa de Barros, 1780, Fazenda Lageado, Botucatu, São Paulo, Brazil, CEP 18610-307
[2]Centro de Estudos de Insetos Sociais, Universidade Estadual Paulista, Avenida 24-A, 1515, Bela Vista, Rio Claro, São Paulo, Brazil, CEP 13506-900

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**Abstract.** *Leucoagaricus gongylophorus*, the fungus cultured by the leaf-cutting ant *Atta sexdens*, produces polysaccharidases that degrade leaf components by generating nutrients believed to be essential for ant nutrition. We evaluated pectinase, amylase, xylanase, and cellulase production by *L. gongylophorus* in laboratory cultures and found that polysaccharidases are produced during fungal growth on pectin, starch, cellulose, xylan, or glucose but not cellulase, whose production is inhibited during fungal growth on xylan. Pectin was the carbon source that best stimulated the production of enzymes, which showed that pectinase had the highest production activity of all of the carbon sources tested, indicating that the presence of pectin and the production of pectinase are key features for symbiotic nutrition on plant material. During growth on starch and cellulose, polysaccharidase production level was intermediate, although during growth on xylan and glucose, enzyme production was very low. We propose a possible profile of polysaccharide degradation inside the nest, where the fungus is cultured on the foliar substrate.

*Leucoagaricus gongylophorus* is a fungus that lives in symbiosis with the leaf-cutting ant *Atta sexdens*, a crop pest that causes damage to agriculture in South America [2, 3, 10]. The symbiosis between these ants and their fungus has been object of many studies, among which the biochemical aspects are in relevance because one of the reasons for the interaction is the supply of fungal enzymes to the ants, including digestive enzymes. Martin and Weber [6], the first researchers to investigate the contribution of the fungus in the supply of these enzymes in the maintenance of the symbiosis, determined that cellulase was the main fungal enzyme in the interaction, derived from the ability of the fungus to metabolize the cellulose of the plants harvested by the ants, and supplying absorbable sugars to the ants.

*Correspondence to:* A. Silva; *email:* enila@estadao.com.br

However, most recent results regarding the metabolic potential of *L. gongylophorus* show that it metabolizes cellulose poorly in relation to others polysaccharides. In addition to amylase and xylanase, which are produced in larger amounts than cellulase, there is pectinase, which is the main enzyme produced by the fungus [9].

One of the questions about the metabolism of polysaccharides by the symbiotic fungus, the answer to which has remained unknown, is this: How are all these distinct polysaccharidases produced when the symbiotic fungus is cultured on a mixture of foliar carbohydrates. To address this question, this work quantified these enzymes when *L. gongylophorus* was cultured on pectin, starch, cellulose, xylan, or glucose.

## Materials and Methods

**Culture conditions of *L. gongylophorus* for the induction of polysacharidases.** A specific strain of *L. gongylophorus* (B1-97

strain, isolated from a laboratory *A. sexdens* nest, 1997) was cultured in (1) 75 mM citrate-phosphate buffer (pH 5.0) added to yeast nitrogen base (0.67 $g \cdot 100\ mL^{-1}$; Difco, catalog no. 100690)]; (2) 0.5 $g \cdot 100\ mL^{-1}$ glucose (J. T. Baker catalog no. 1916-01); or (3) 0.5 $g \cdot 100\ mL^{-1}$ each polysaccharide: starch, pectin, cellulose, or xylan (Sigma catalog numbers S-9765, P-2157, X-0502, and C-6288, respectively). The cells were grown for 30 days at 25°C in stationary cultures; our previous results showed that the cells develop in log-growth phase in these culture condition. Then, each of the culture flasks containing the secreted enzymes were filtered through a 0.45-μM pore sized membrane and dialyzed. Fungal mycelium was collected for cell mass determination, which was calculated in milligrams dry weight.

**Enzymatic assay.** Pectinase, amylase, cellulase, and xylanase were determined using pectin, starch, carboxymethylcellulose, and xylan, respectively. Two grams each polysaccharide was dissolved in 100 mL citrate–phosphate buffer 75 mM (pH 5.0). The samples and their respective substrates were mixed (1:1) and kept at 25°C for 2 hours. Aliquots of 200 μL were collected every 30 minutes of incubation, added to 400 μL dinitrosalicylic acid and 400 μL water, followed by a boil bath for 5 minutes and absorbance determination (540 nm). One unit of enzyme activity (U) was defined as the amount (in μmol) of hydrolysis product of each polysaccharide per minute of reaction time per biomass of *L. gongylophorus* (mg dry weight). Straight line-standards were used for calculations of enzyme activities: glucose (amylase and cellulase), xylose (xylanase), and galacturonic acid (pectinase).

**Protein assay.** Protein concentration was determined according to the Bradford method [1]. Serum albumin was used as standard.

**Polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15% polyacrylamide gels as described by Laemmli [5]. Samples containing 10 mg total proteins were loaded onto the gel, and the proteins bands were detected by silver staining [4]. Protein molecular weight was estimated based on standard proteins: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

**Statistics.** Each analysis was carried out 12 times, and the results, expressed as means, were subjected to the Tukey test, with a confidence interval of 95% [11], carried out on statistic software Prism 3.0.

## Results and Discussion

The efficiency of different carbon sources in stimulating fungal enzymes or biomass was evaluated in this work. After 30 days of culturing the fungus, pectin seemed the most important polysaccharide for enzyme production and glucose for stimulating mycelium growth in *L. gongylophorus*.

There was no correlation between biomass and enzyme production by the fungus when cultured on different carbon sources. However, the best substrate for biomass production was glucose followed by xylan, starch, pectin, and cellulose (Fig. 1, open squares). For enzyme production, the best substrates were, sequentially, pectin, starch, cellulose, glucose, and xylan (Fig. 1, closed squares).

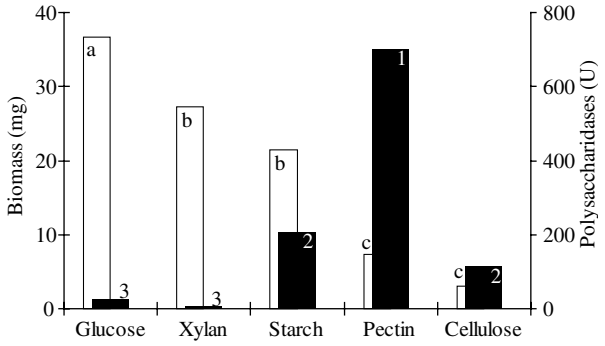


Fig. 1. Biomass (open squares; mg dry weight) and total units (U) of polysaccharidases (closed squares) produced by *L. gongylophorus* (B1-97 strain) on carbon sources after 30 days of cultivation. Values followed by distinct letters (for biomass) or numbers (for polysaccharidases) are significantly different (Tukey test, 95% confidence).

Pectinase, amylase, xylanase, and cellulase were produced by the fungus in all of the carbon sources tested, except for cellulase, which was not produced on xylan (Table 1). Similar abilities for polysaccharide metabolism by *L. gongylophorus* have already been reported for the strain CCT1 [9]. Pectinase appears in the largest quantities followed by amylase, xylanase, and cellulase. Totaling the amounts of enzyme units produced per mg of *L. gongylophorus*, pectinase production was approximately 5 times higher than amylase, 20 times higher than xylanase, and almost 58 times higher than cellulase. The values of enzymatic activities were submitted for statistical tests, which evaluated the efficiency of each carbon source in stimulating the production of polysaccharidases by *L. gongylophorus* (Table 1). Pectinase production was greatest on pectin, and starch or cellulose stimulated its production moderately, whereas glucose and xylan were the worst substrates for the production of this enzyme. Amylase was best induced by cellulose, pectin, and starch. The best substrates for xylanase production were pectin, cellulose, and starch, whereas glucose and xylan stimulated this enzyme poorly. There was no significant statistical difference in the production of cellulase among the carbon sources evaluated. This enzyme was poorly produced on all substrates tested, although its activity was not detected on xylan.

To evaluate the protein diversity produced on the different carbon sources tested, the samples were subjected to denaturing electrophoresis (SDS-PAGE), and the results are presented in Fig. 2. *L. gongylophorus* proteins with approximate molecular weights varying between 12 and 100 kDa were detected. A higher diversity of proteins was observed in samples containing starch or glucose as the only carbon source. In the other

Table 1. Units of polysaccharidases (pectinase, amylase, xylanase, and cellulase) produced by *L. gongylophorus* cultured for 30 days on carbon sources pectin, starch, cellulose, xylan, and glucose

| Carbon Sources | Enzymatic activity (mean ± SD) | | | | |
|---|---|---|---|---|---|
| (n = 12) | Pectinase | Amylase | Xylanase | Cellulase | Total |
| Pectin | 633 ± 96[a] | 48 ± 11[b] | 17 ± 2[a] | 3.0 ± 0.4[a] | 701.0 |
| Starch | 163 ± 13[b] | 29 ± 6[b] | 9 ± 2[a,b] | 5.7 ± 0.5[a] | 206.7 |
| Cellulose | 84 ± 10[b] | 120 ± 21[a] | 14 ± 3[a] | 5.6 ± 0.7[a] | 223.6 |
| Xylan | 3.1 ± 1.8[d] | 2.2 ± 0.4[c] | 0.7 ± 0.3[c] | 0.0 | 6.0 |
| Glucose | 17 ± 3[c] | 1.7 ± 1.0[c] | 3.3 ± 0.3[b] | 1.3 ± 0.6[a] | 23.3 |
| Total | 900.1 | 200.9 | 44.0 | 15.6 | – |

Note: Values followed by distinct letters in each column are significantly different (Tukey test, 95% confidence).

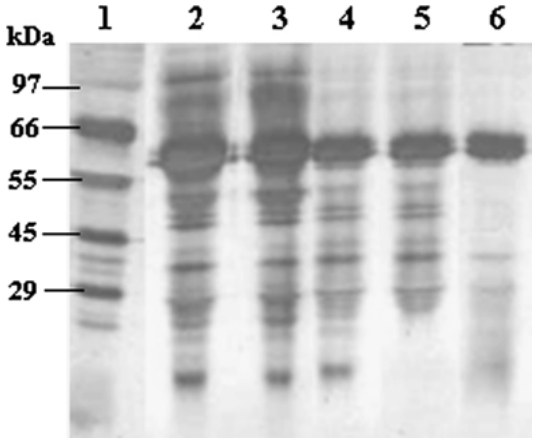


Fig. 2. SDS-PAGE of the samples with total proteins produced by *L. gongylophorus* after 30 days cultured in glucose (lane 2) starch (lane 3), pectin (lane 4), xylan (lane 5), and cellulose (lane 6). Lane 1 corresponds to the standard of molecular weight in kDa.

substrates tested, less protein diversity was detected in relation to starch or glucose. This result showed that pectin or cellulose, which were good substrates for enzyme production, were not good nutrients for the induction of a diversity of total proteins. Perhaps the explanation for this result is in the fact that both these polysaccharides are located outside the vegetal cell, stimulating the production of few proteins, in particular, the enzymes specialized in the immediate degradation of the substratum, promoting rapid access to the nutrients that promote the better fungal growth possible. This is an important factor in the establishment of the fungus in the substratum previous to other microorganisms which might be present at the moment of its inoculation in the leaf material, as described by Quinlan and Cherret [7].

Another interesting fact is that glucose appears as a substratum that does not inhibit the production of total proteins, which would seem to be an advantage because glucose is readily available within the nest [8]. However, despite the fact that glucose does not inhibit the enzymes totally, this carbon source appears to decrease their production, as listed in Table 1 and Fig. 1. This could be an important strategy for the regulation of soluble-nutrient production in the nest, perhaps even influencing leaf-cutting activities: new substrate would stimulate a higher enzymatic secretion by the fungus, and excess glucose would indicate low consumption of this sugar by the fungus or the ants, signalizing an excess of soluble nutrients. Thus, enzyme production would decrease and so would foraging activities. This hypothesis could be investigated in the future.

With the results on enzymes induction in the laboratory, a possible profile of polysaccharide degradation in the nests can be drawn as can the use of the products of hydrolysis. Pectinase was the enzyme most produced by the fungus, and pectin was its main inductor. This polysaccharide is located externally on the leaf cells, forming a superficial layer that acts as a cement that binds them together. By producing great amounts of pectinase in contact with the pectin, the fungus guarantees the rapid degradation of this polysaccharide, gaining access to other substrates that better stimulate its growth.

By breaking the barrier formed by pectin, the fungus has access to the other polysaccharides, such as cellulose and xylan, of the foliar cellular surface. Xylan was not a good inductor of enzymes, as listed in Table 1 and Fig. 1; however, it has already proven to be an excellent stimulant for fungal growth [9], most probably its natural role. The question of the role of cellulose then arises because is not a good inductor of the fungal biomass [9]; it could be considered to act as the main inductor of amylase as listed in Table 1. Thus, when the ants inoculate the fungal mycelium on the leaf pieces [7], this polysaccharide stimulates greater amylase pro-

duction, giving the fungus access to the starch within the leaf cell and the capacity to degrade it to producing glucose, a sugar that further stimulates fungal biomass production (Fig. 1).

The results presented in this work were intended to show the probable metabolic behavior of *L. gongylophorus* in the nest, where it is cultivated on leaf pieces taken by *A. sexdens* ants, which are composed of a mixture of polysaccharides. The fungus appears to show distinct behaviors on each carbon source, producing biomass, enzymes, or nutrients in the nest. These are some aspects of the nutritional balance of this symbiotic relationship.

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