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SUBERIN UTILIZATION BY *Chrysonilia sitophila* : EVIDENCE FOR LIPOLYTIC ENZYMES PRODUCTION

UTILIZAÇÃO DE SUBERINA POR. *Chrysonilia sitophila*: EVIDÊNCIA DA PRODUÇÃO DE EMZIMAS LIPOLÍTICAS

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SUMMARY

The role of the development of *Chrysonilia sitophila* on cork slabs, during the manufacturing process of cork stoppers, is not clearly understood. This work describes the first results demonstrating the potential of that mould to metabolize suberin. Lipolytic activity was clearly observed in solid and liquid cultures of *C. sitophila*.

RESUMO

A função do desenvolvimento de *Chrysonilia sitophila* em pranchas de cortiça, durante o processo de fabrico de rolhas de cortiça, não está ainda claramente clarificada. Neste trabalho são descritos os primeiros resultados que demonstram a potencialidade daquele fungo para metabolizar suberina (um constituinte da cortiça constituido por uma parte aromática ligada a cadeias alifáticas de ácidos gordos, esterificadas ou não). A actividade lipolítica de *C. sitophila* foi claramente demonstrada em culturas líquidas e em culturas sólidas do fungo.

Key Words: *Chrysonilia sitophila*, Lypases, suberine, cork
Palavras Chave: *Chrysonilia sitophila*, lipases, suberina, cortiça

INTRODUCTION

The special properties of the cork tissue produced by *Quercus suber* L., like insulation, elasticity and impenetrability to water, are mainly attributed to the presence of suberin which contributes to about 47% of the cell wall composition (Marques and Pereira, 1987). Those properties stand for the base of cork industry importance. One of the best-known and valuable applications of cork is the manufacture of cork stoppers for sealing wine bottles. The cork stopper manufacturing process, summarized by Lee and Simpson (1993), includes a stabilization period of the cork slabs, after boiling, during which mould growth completely covers them. *Chrysonilia sitophila* was identified as the dominant mould present at this stage (Danesh, *et al*., 1997). The role of this mould development is not clearly understood, however, its systematic development on cork slabs points to the enzymatic ability to grow on the available substrates (cellulose, lignin and suberin. Walton JD (1994) wrote: "since all the polymers of a plant cell wall are eventually degraded by microorganisms, for every type of chemical bond there must be an enzyme that can cleave it". In a previous work, we have shown the ability of *C. sitophila* to produce cellulases, which can degrade cellulose, one of the constituents of cork, (results not yet published).

Graça and Pereira (1997) have proposed that suberin is an aliphatic polyester where glycerol is the cross-linking monomer esterified with the the long suberinic fatty acids. Suberized tissues contain both poly (aliphatic) and poly (phenolic) domains. Consequently, different terms must be used in reference to either domain when they are considered separately from suberin as a whole (Bernards, 2002). At the most basic level, both aliphatic and phenolic components are derived from the products of carbohydrate metabolism, most notably pyruvate, phosphoenolpyruvate, and erythrose-4-phosphate. A clear point of divergence occurs where the fatty acid synthesis and shikimate pathways begin, with the former ultimately giving rise to the 16:0 and 18:0 fatty acids that are the precursors to all of the aliphatics in suberized tissues (Bernards, 2002).. Lipase and esterase are characterized by the ability to hydrolyze glyceryl esters of long chain fatty acids under interfacial activation (lipase) or short chain fatty acids (esterase) (Junge, *et al*, 1984). Therefore, they appear as the first enzymes to be studied in order to investigate the ability of *C. sitophila* to metabolize suberin hydrolyzing the glycerol/poly aliphatic acids.

Fungi, characterized by being ubiquitous in distribution, are highly successful in survival because of their great plasticity and physiological versatility.

Fungi thrive well in harsh habitats with environmental extremes because of their efficient enzyme systems. Among the varied mechanisms for fungi adaptability to environmental extremes and for the utilization of their trophic niche, their ability to produce extra cellular enzymes is of great survival value (Gopinath et al, 2005).

In this report, some results concerning the investigation of the aptitude of *C. sitophila* to produce lipase, one of the necessary enzyme systems to metabolize suberin, the main constituent of cork, are described. However, it should be kept in mind that those enzymes are not the only one allowing suberin degradation. Therefore, this work presents the preliminary results concerning a depth research of the mechanisms involving cork suberin degradation actually ongoing in the laboratory.

The production of lipases along the fungal growth was monitorized in media containing either cork powder or cork powder free of its polysaccharide and major phenolic compounds (condensed tannin fraction) so the major component of this extract is suberin.

MATERIAL AND METHODS

Microbes and culture conditions: Suspensions of spores of *C. sitophila*, (from IBET fungi cultures) in 8 g l^{-1} NaCl, were prepared from 72 h old cultures on Potato Dextrose Agar (PDA) under 27ºC. Spores concentration was standardized by adjusting the absorbence to 0.1 at 450 nm, and this suspension was used for the assays. Two ml of the suspension referred to above were added to 500 ml Erlenmeyer flasks containing 48 ml of liquid culture media prepared according to the experiments described below. Cultures were incubated at 27ºC, at 60 rpm for 50 h with a 5 h light per day. After this time, the fungal mycelium was removed and the liquid media analyzed for enzymatic activities.

Screening for lipase and esterase activities: The activities were firstly investigated in liquid cultures, using the basal medium modified by Sterneberg *et al*., (1976) containing per liter of distilled water: 1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.3 g $CaCl_2$, 0.3 g $MgSO_4$, 5 mg $FeSO_4.7H_2O$, 1.6 mg $MnSO_4.H_2O$, 1.4 mg $ZnSO_4.7H_2O$, 3 mg $CoCl_2$, 0.3 g urea. To this medium was added microcrystaline cellulose 7.5 g L^{-1}, glucose 7.5 gL^{-1}, tannic acid, 0.08 % (w/v) or cork powder 10 gL^{-1}, independently or simultaneously. A control was done growing the fungus in culture medium without cellulose and glucose. Before sterilization at 120 ºC for 15 min, the pH was adjusted to 6.0 with 0.1 M NaOH. All the experiments were done in duplicate.

Fungal growth was monitorized, evaluating the dry mycelium weight along the time in the different liquid culture medium. At fungus stationary phase of growth, the fungus mycelium was removed and the production of lipase and esterase was investigated in the different liquid media, using the API ZYM system (bioMérieux, Lyon). This system is composed by 20 micro wells containing different dried synthetic substrates and the respective buffers. Each of the enzymatic crude extracts (culture medium), obtained as described above, was added to each micro well of the API ZYM stripes that were incubated at 30 ºC for 6 and 24 h, according to supplier instructions. The enzymatic reaction products produced during the incubation period were detected through colored reactions promoted by the addition of reagents according to the instructions furnished by the supplier. A similar method was previously used by Centeno and Calvo (2001) to detect some enzymatic activities produced by *C. sitophila*.

Lipase and esterase activity research in solid cultures: a portion of *C. sitophila* mycelium obtained from fungus previous 72 h cultures on PDA, was inoculated onto Petri dishes containing a culture medium (peptone, 2 gL^{1}, meet extract 1.5 gL^{1}, NaCl 2 gL^{-1}, agar 5 gL^{-1}) supplemented with 1% (w/v) trioleine (olive oil) and 0.001% (w/v) Rhodamine B (Kouker, and Jaeger, 1987). The results obtained were confirmed using the Spirit Blue Agar test commercialized by Difco (which contains olive oil as substrate). The medium was inoculated as described above. Both tests are based on the ability to release fatty acids by enzymatic hydrolysis of the glyceryl ester. This acid release originates the formation of orange fluorescent halos, in the presence of the Rhodamine B (under UV, 350 nm) or dark blue halos in the presence of Spirit Blue, around the fungal mycelium.

Another assay was performed according to Jacobsen *et al*. (1989), also using fungal mycelium obtained from previous culture as described earlier, to inoculate a solid nutritive medium (peptone 5 gL^{1}, yeast extract 3 gL^{-1}, agar 10 gL^{-1}) containing tributirine (1% w/v) as the enzyme substrate.

RESULTS AND DISCUSSION

Enzymes activity screening using API ZIM: In addition to lipase and esterase, acid and alkaline phosphatase, α and β-glucosidase, N-acetyl-β-glucosaminidase activities were detected using this system, in the extracts from *C. sitophila* cultures in the presence of cellulose, tannic acid, and cork, but not in the medium containing glucose. These results make evidence for the ability of *C. sitophila* to hydrolyze the suberin glyceryl esters of cork and suggest that the enzymes are repressed by the presence of glucose in the medium. Results suggest that *C. sitophila* can produce the enzymes when cultured in basal media but its culture in the same media added of cork or some cork constituents seems to increase the activities.

Enzymes activities in solid cultures: The results described above were complemented by investigating the production of the enzymes in solid cultures. Further assays, either using Rhodamine B that becomes yellow fluorescent under UV radiation (350nm) after *C. sitophila* culture (Fig.1) or Spirit Blue (Fig. 2), where a blue halo can be observed around the *C. sitophila* mycelium, indicating that the fungus had hydrolyzed trioleine and olive oil and the resulting products had reacted with the dye in the medium. The reported results clearly indicate that *C. sitophila* is able to synthesize lipase. The results shown in Fig. 3, also confirm the enzyme synthesis by *C. sitophila* that shows to be able to hydrolyze tributirine, originating a transparent halo around the fungal mycelium. These results and those reported above showed that lipase and/or esterase are synthesized bound to the mycelium and excreted into the culture medium. Although several works referring to the ability of filamentous fungi to produce lipolytic enzymes have been published, the production of those enzymes by *C. sitophila* had not been reported before. *C. sitophila* appears to be equipped with all the enzymatic activities essential to its colonization of cork slabs. First, in the early stage of growth, a lipase is produced (30h of growth) (Fig 4) allowing the

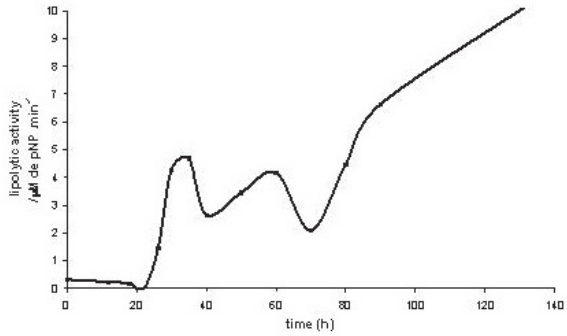

Fig. 4 – Lipases production by *Chrysonilia sitophila* along growth in liquid culture containing trioleine

Produção de lipases por Chrysonilia sitophila em meio de cultura líquido contendo trioleina

fungal adhesion to the surface layers of the substrate. Cellulases and proteases activities were earlier partly characterized in the laboratory (results not shown), allowing the access of the mould to the carbon and protein sources available on cork slabs. After their exhaustion, a new lipolytic activity appears to be synthesized in order to guarantee fungal survival, now using the suberinic cork tissue. Further studies are ongoing in the laboratory aiming to go deep on the characterization of all the enzymatic systems involved in cork slabs fungi colonization.

Ultrastructural studies have shown that some fungi can penetrate suberized cell walls**.** However, it appears that the suberized walls are penetrated only extremely slowly and few organisms can readily utilize suberized walls as a carbon source (Kolattukudy, 1985) . Silva Pereira *et al*, (2006) observed that fungi growth occurred primarily on the surface of the cork pieces and that fungal mycelium reached deeper cork cell wall layers, due to hyphae penetration of the lenticular channels and the cork cell wall. The same authors reported the observation of specific correlation between fungi colonisation and mechanical properties of cork, so they made evidence for fungi influence on cork slabs quality and consequently on cork stoppers quality.

The results reported on this paper reinforce the idea that the growth of *C. sitophila* on cork slabs modifies cork properties contributing to cork stoppers quality. Furthermore, these findings represent additional scientific foundation for the cork stopper manufacturing process that advises to favour the dominance of that fungus on cork slabs after boiling.

CONCLUSIONS

C. sitophila appears to be equipped with all the enzymatic activities essential to the colonization of cork slabs. First, in the early stage of growth, a lipase is produced allowing the fungal adhesion to the surface layers of the substrate. After the access of the mould to the carbon and protein sources available on cork slabs, and after their exhaustion, a new lipolytic activity appears to be synthesized in order to guaranty fungal survival, now mortifying at least a part of the suberin cork tissue. The results here presented were observed using liquid and specific solid cultures. Although these results need to be complemented doing the study of all the enzymatic system of the fungus, they validate previous observations reporting fungal penetration throughout cork structure. To our knowledge is the first time that similar data are published.

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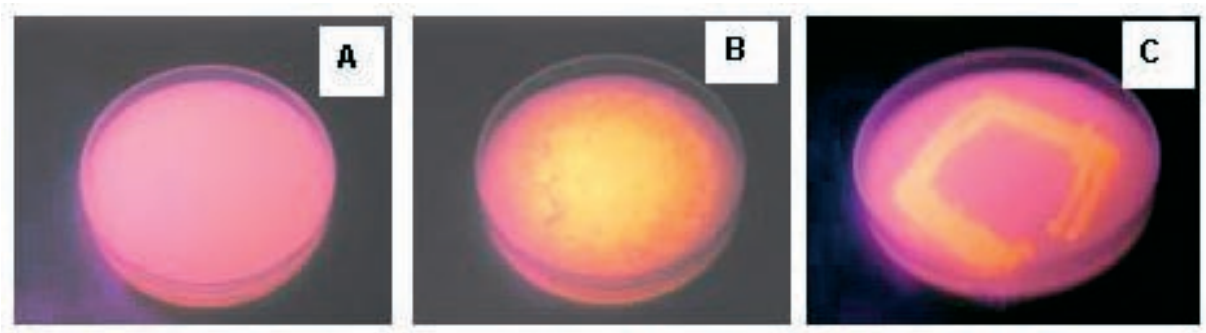

Fig. 1 - Detection of lipase production by *Chrysonilia sitophila* on solid culture medium containing trioleine and rhodamine B, observed under UV radiation at 350nm. **A**: Not inoculated medium; **B**: *Chrysonilia sitophila* culture; **C**: Positive control *(Staphylococcus aureus* culture).

Detecção da produção de lipase por Chrysonilia sitophila em meio de cultura sólido contendo trioleina e rhodamina B, observados sob radiação UV a 350nm. ***A****: Meio controlo não inoculado* ***B****: Meio após cultura de Chrysonilia sitophila* ***C****: Controlo positivo (usando uma cultura de Staphylococcus aureus)*

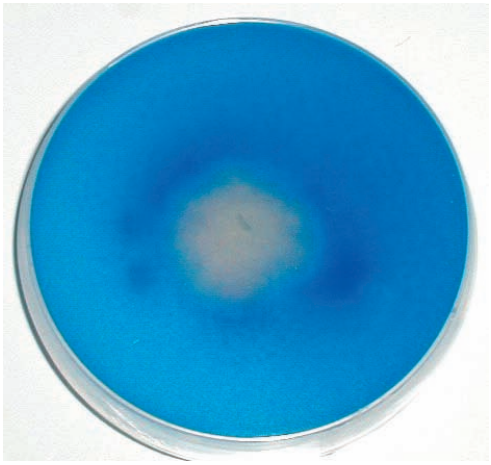

Fig. 2 - Detection of lipase production by *Chrysonilia sitophila* cultured in Spirit Blue Agar (containing olive oil)

Detecção da produção de lipase por Chrysonilia sitophila em meio de cultura Spirit Blue Agar (usando azeite como substrato)

Fig. 3 - Detection of lipase production by *Chrysonilia sitophila* in culture medium containing tributirine as substrate.

Detecção da produção de lipase por Chrysonilia sitophila em meio de cultura sólido contendo tributirina como substrato.