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Phylloplane yeasts as a source of industrially interesting enzymes

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ABSTRACT: (Phylloplane yeasts as a source of industrially interesting enzymes). The objective of this study was to evaluate the phylloplane of tropical plants as a source of yeasts capable of producing industrially relevant enzymes. A total of 446 yeast and yeast-like strains were isolated from the phylloplanes of *Hibiscus rosa-sinensis* (Malvaceae), *Coussapoa microcarpa* (Urticaceae), and species of *Ficus* (Moraceae) and Bromeliaceae, which were collected in southern Brazil and tested for the production of extracellular enzymes. Esterase activity was predominant (202 strains), while 71 isolates had amylase activity, 170 were caseinolytic, and 72 degraded gelatin. Only three strains were capable of producing all enzymes. The phylloplane appears to be a good substrate to look for and isolate yeasts with enzyme production potential. There were differences in yeast enzyme profiles among the plants analyzed: gelatinase was more common among the isolates from *H. rosa-sinensis* (36.6%), while esterase was predominant among the isolates from *C. microcarpa* and the species of *Ficus* (71.3%). This study suggests the phylloplane as a new potential source of yeasts with industrially interesting properties.

Key words: biotechnology, amylase, protease, esterase.

RESUMO: (Leveduras do filoplano como fonte de enzimas de interesse industrial). O objetivo deste estudo foi avaliar o filoplano de plantas de habitats tropicais como fonte de leveduras capazes de produzir enzimas de relevância industrial. Um total de 446 cepas de leveduras e fungos semelhantes a leveduras foram isoladas do filoplano de *Hibiscus rosa-sinensis*, bromélias e figueiras, coletadas no Sul do Brasil, e testadas para a produção de enzimas extracelulares. A atividade de esterase foi predominante (202 cepas), enquanto 71 isolados tiveram atividade amilolítica, 170 foram caseinolíticos e 72 degradaram gelatina. Apenas três cepas foram capazes de produzir todas as enzimas. O filoplano parece ser um bom substrato para o isolamento de leveduras com potencial de produção de enzimas. Existiram diferenças nos perfis enzimáticos das leveduras entre as plantas analisadas: gelatinase foi mais comum entre os isolados de *H. rosa-sinensis* (36,6%), enquanto esterase foi predominante entre os isolados de figueiras (71,3%). Esse estudo chama a atenção para o filoplano como uma nova fonte potencial de leveduras com propriedades de interesse industrial.

Palavras-chave: biotecnologia, amilase, protease, esterase.

INTRODUCTION

The enzyme market is expanding, and microbial enzymes are now required and searched for by various industries. Sources of microbial enzymes with new interesting properties are culture collections, genomic libraries, and natural habitats. Whenever microbial isolates are available, functional screening assays are used to select the enzyme producers, which usually involves easily recognized reactions on a specific medium housed in Petri dishes (Marrs *et al.* 1999, Schäffer *et al.* 2007).

Most studies that involve the screening of yeasts for enzyme production target proteinase or pectinase, mainly because these are important enzymes used to clarify fruit juices (Braga *et al.* 1998, Trindade *et al.* 2002, da Silva *et al.* 2005, Oliveira *et al.* 2006). Other industrially interesting enzymes that are screened for in yeasts are lipase / esterase, amylase, cellulase and β -glucosidases (Middelhoven 1997, Strauss *et al.* 2001, Buzzini & Martini 2002, Rodrigues *et al.* 2004, Laitila *et al.* 2006,

Kudanga *et al.* 2007). The search for new habitats has led to extreme environments as a source of new microbes and, consequently, new enzymes (Nakagawa *et al.* 2004, Brizzio *et al.* 2007). Some studies have focused on tropical habitats, although most of these have dealt with tropical fruits, pulps or juices (Abranches *et al.* 1997, Braga *et al.* 1998, Buzzini & Martini 2002, Trindade *et al.* 2002, da Silva *et al.* 2005, Kudanga *et al.* 2007).

The phylloplane is considered an extreme environment because of limited nutrients, exposure to solar radiation, and high temperature fluctuations (Fonseca & Inacio 2006). It is also considered a good substrate to look for new yeast species (Sampaio *et al.* 2004, Péter *et al.* 2007, Inacio *et al.* 2008, Landell *et al.* 2009), but few studies have evaluated the biotechnological potential of these yeasts. Therefore, the objective of the present study was to evaluate the phylloplanes of plants from tropical habitats to see if they could be a source of yeasts capable of producing industrially relevant enzymes.

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MATERIAL AND METHODS

Isolation of yeast and yeast-like strains

Leaves of *Hibiscus rosa-sinensis* (Malvaceae) were sampled at Farroupilha Park, Porto Alegre, and leaves of *Coussapoa microcarpa* (Urticaceae), 2 species of Moraceae (*Ficus cestripholia*, *F. luschnathiana*) and 11 species of Bromeliaceae (*Aechmea recurvata*, *Bilbergia nutans*, *Bromelia antiacantha*, *Dyckia* sp., *Tillandsia crocata*, *T. gardneri*, *T. geminiflora*, *T. stricta*, *Vriesea friburgensis*, *V. gigantea*, *V. procera*) were sampled at Itapuã Park, Viamão; both sites are located in the state of Rio Grande do Sul, in southern Brazil (Fuentefria & Valente 2004, Landell *et al.* 2005). Mature leaves were aseptically collected in polyethylene plastic bags, transported to the laboratory, and washed with sterile distilled water. Isolates of *H. rosa-sinensis* were obtained by leaf imprinting in YEPG agar (yeast extract 1%, glucose 2%, peptone 1%, agar 2%), which was supplemented with 0.04% chloramphenicol (pH 4.0) and incubated at 25°C for 3 days. The leaves of *C. microcarpa* and the *Ficus* and Bromeliaceae species were cut into 9–10 cm² fragments, put in Erlenmeyer flasks with 50 mL of sterile distilled water and placed on a shaker for 10 minutes. The water was then discarded and replaced with 30 mL of 0.5% Tween 20, which was followed by vigorous shaking for 30 minutes. This last step was repeated once, and decimal dilutions of the final wash were spread on acidified YM agar (1% glucose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 2% agar, acidified to pH 4.0, and supplemented with 0.04% chloramphenicol) plates. After incubation at 20–25°C for up to 7 days, representative colonies of each morphological type were purified, and maintained on GYMP (2% glucose, 2% malt extract, 0.5% yeast extract, 0.2% sodium monobasic phosphate and 2% agar) slants at 4°C, which were covered with sterile mineral oil.

Enzymatic screening procedure

The strains that were previously grown on YEPG agar were diluted in sterile distilled water to about 4×10^5 cells/mL, kept at 25°C for 24 h, and used to inoculate the solidified agar surface of pre-poured plates, or tubes containing liquid or solid media. Results were scored as negative (-), weak (W), positive (+) or highly productive (++). Positive results were repeated for confirmation. No discrepant results were found in the repeated experiments.

Amylase screening: Cultures were screened for their ability to hydrolyze starch on a medium containing 0.67% Yeast Nitrogen Base (YNB), 0.2% soluble starch and 2% agar, at pH 6.0 (Buzzini & Martini 2002; Strauss *et al.* 2001). The plates were incubated for 7 days at 25°C. After cell growth, the Petri dishes were flooded with an iodine solution. A pale yellow zone around the colony, which was surrounded by a blue medium, indicated starch-degrading activity. The level of enzyme production in

the Petri dishes was evaluated by the corresponding halo diameter around the colony, which was measured in centimeters. Haloes ≤ 1.5 cm diam. were interpreted as weak production, between 1.5 cm and 2.4 cm as positive, and ≥ 2.5 cm as highly productive. To exclude the hypothesis that the high concentration of agar used (2%) could have resulted in false negatives, strains that presented weak activity were tested using media containing two other agar concentrations (1.5% and 1%); however, this did not improve the sensitivity of the test (data not shown). **Esterase screening:** Esterase production was determined in tubes with liquid media or Petri dishes with solid media (2% agar), both containing 0.67% YNB with 0.5% Tween 20. Results were scored after incubation at 25°C for up to 7 days. Enzyme production in the liquid media was determined by the increase in cell mass estimated from the turbidity level, which was measured with a Wickerham card. The degree 1 on the card was considered as weak enzymatic production, the degree 2 as positive production, and the degree 3 as highly productive. Enzyme production in the solid media was estimated from colony growth in the esterase medium in comparison with colony growth in 0.67% YNB + 0.5% glucose + 2% agar and growth in YNB + agar without any carbon source. Growth that was better than that on the glucose plate was considered highly productive, similar growth was considered positive production, and intermediate growth as weak production (Landell *et al.* 2006).

Protease screening: Extracellular protease production was tested by inoculation on Casein agar (0.67% YNB, 0.5% glucose, 0.5% casein and 2% agar), adjusted to pH 7.0 (Braga *et al.* 1998; Strauss *et al.* 2001; Trindade *et al.* 2002). After incubation at 25°C for 7 days, protein precipitation was achieved with 1 M HCl and incubation for 1 hour, and protease production was indicated by the presence of a clear zone around the colonies. The level of enzyme activity was evaluated as described for amylase, but the ranges considered were the following: a halo ≤ 0.5 cm diam. was considered as weak, between 0.5 cm and 0.9 cm as positive, and ≥ 1.0 cm as highly productive.

Gelatin hydrolysis was also used to detect protease production. Yeasts growing in tubes containing a medium of malt extract and gelatin (10% malt extract and 12% gelatin) were incubated at 25°C for up to 21 days (Abranches *et al.* 1997). After incubation, the tubes were kept at 4°C for 2 hours. Gelatin hydrolysis results in liquefaction of the medium. A strong liquefaction was considered as positive, and an intermediate liquefaction as weak enzyme production.

RESULTS

A total of 446 yeast and yeast-like strains were isolated from the phylloplanes of *H. rosa-sinensis*, *C. microcarpa*, and the species of *Ficus* and Bromeliaceae, which were tested for the production of industrially relevant extracellular enzymes (Table 1). Esterase activity was predominant (202 strains), while 71 isolates had amylase

Table 1. Extracellular enzymatic profile of yeasts isolated from the phylloplanes of *Hibiscus rosa-sinensis*, *C. microcarpa*, and species of *Ficus* and Bromeliaceae.

	Coussapoa & Ficus n = 175	Bromeliaceae species n = 189	Hibiscus n = 82	Total n = 446
Each enzyme				
Amylase (A)	18 (10.3%) ^a	35 (18.5%)	18 (21.9%)	71 (15.9%)
Caseinase (C)	70 (40.0%)	74 (39.1%)	26 (31.7%)	170 (38.1%)
Gelatinase (G)	18 (10.3%)	24 (12.7%)	30 (36.6%)	72 (16.1%)
Esterase (E)	119 (71.3%) ^b	57 (33.9%) ^b	26 (31.7%)	202 (48.4%) ^b
Two enzymes				
A + C	6 (3.4%)	23 (12.2%)	12 (14.6%)	41 (9.2%)
A + G	2 (1.1%)	3 (1.6%)	6 (7.3%)	11 (2.5%)
A + E	10 (6.0%) ^b	6 (3.6%) ^b	6 (7.3%)	22 (5.3%) ^b
C + G	18 (4.6%)	16 (8.5%)	11 (13.4%)	45 (10.1%)
C + E	57 (34.1%) ^b	21 (12.5%) ^b	13 (15.8%)	72 (17.3%) ^b
G + E	14 (8.4%) ^b	5 (3.0%) ^b	11 (13.4%)	30 (7.2%) ^b
Three enzymes				
C + G + E	14 (8.4%) ^b	4 (2.4%) ^b	5 (6.1%)	23 (5.5%) ^b
A + C + G	2 (1.1%)	1 (0.5%)	5 (6.1%)	8 (1.8%)
A + C + E	4 (2.4%) ^b	5 (3.0%) ^b	3 (3.7%)	12 (2.9%) ^b
A + G + E	2 (1.2%) ^b	0	2 (2.4%)	4 (1.0%) ^b
Four enzymes				
A + C + G + E	2 (1.2%) ^b	0	1 (1.2%)	3 (0.7%) ^b

a. Positive results (percentage).

b. Number of isolates tested for esterase was 167 for *Coussapoa* and *Ficus* species and 168 for Bromeliaceae species. Percentage was calculated based on the number of isolates.

activity, 170 were caseinolytic, and 72 degraded gelatin. Caseinase and gelatinase activities were not correlated, although some isolates were capable of both.

DISCUSSION

Although some phylloplane yeasts are capable of producing plant cell wall-degrading enzymes, such as cellulases, pectinases and xylanases, this is not believed to be their main nutrient source. They are believed instead to survive on the leaf surface using plant exudates as nutrients (Fonseca & Inacio 2006). This means the yeast are versatile in relation to the nutrients they uptake and utilize, because plant exudates are present in low concentrations and are ephemeral. Thus, phylloplane yeasts should be capable of producing a variety of hydrolytic enzymes, some of them with potential industrial uses.

Most enzyme-producing microorganisms are not capable of high-yield production, and are thus not tailored for industrial uses, but the advent of genetic engineering makes it possible to indirectly use them. Genes that code for enzymes with interesting properties may be cloned and expressed in suitable expression vectors, overcoming the problem of yield limitation (Fernandez *et al.* 2006). This has raised interest in novelties from microorganisms isolated from natural habitats.

We found a higher percentage of amylolytic yeasts (15.9%) compared to the approximately 9% found in other studies focused on yeasts isolated from natural habitats (Buzzini & Martini 2002, Brizzio *et al.* 2007). Although it is improbable that phylloplane yeasts use starch as a nutrient in their natural habitat, it is interesting to note that amylolytic capability is higher in yeasts isolated from plant substrates. If these microorganisms play a posterior role in plant decomposition and nutrient

turn-over, the amylolytic activity may be important.

Most studies dealing with proteolytic yeasts screen for enzyme producers using casein as the protein source. We found 38.1% of the yeasts isolated were caseinolytic, compared to 37–38.5% found in tropical fruits and frozen pulps (Trindade *et al.* 2002), 26.4% in *Parahancornia amapa* and associated drosophilids (Braga *et al.* 1998), and 17.8% in water, soil, insects and plant materials from Brazilian rain forests (Buzzini & Martini 2002). Proteases are represented by a wide diversity of enzymes, each with different specificities. In this context, the use of different protein substrates (e.g., gelatin and casein) may provide relevant insights into the specificity of the enzymes produced. Gelatin liquefaction was scarcer among our isolates (16.1%) in comparison to casein degradation, but protease production in both casein and gelatin media could mean that these strains produce proteases with broad substrate specificities or produce more than one kind of protease with different specificities. Forty-five strains (10.1%) isolated from the phylloplanes could be selected based on this broad specificity, and may be industrially useful.

Esterase/lipase activity seems to be the commonest enzymatic activity found in yeasts isolated from several substrates. Brizzio *et al.* (2007) found between 58.7% and 76% esterase/lipase activity, depending on the lipidic substrate used, in cold-adapted yeasts tested at 4°C. Buzzini & Martini (2002) found between 46% and 49.4%, Kundang *et al.* (2007) found 48% among *Aureobasidium pullulans* isolates in Zimbabwe, and Middelhoven (1997) found 100% in yeasts isolated from plant material from the Canary Islands; however, the total number of strains tested was low (44 isolates). Our results are comparable to these (48.4% of the strains), and seem to suggest that esterases/lipases are possibly the main enzymes that these

yeasts can contribute to industries.

We found some important differences in yeast enzyme profiles among the plants analyzed. The most striking were gelatinase and esterase production. While gelatinase was more common among *H. rosa-sinensis* isolates (36.6% of the strains compared to 10.3% in *C. microcarpa* and the *Ficus* spp., and 12.7% from the species of Bromeliaceae), esterase was found in 71.3% of the isolates from *C. microcarpa* and the *Ficus* species, 33.9% of the isolates from Bromeliaceae species, and 31.7% of the isolates from *H. rosa-sinensis*. This may be explained by the difference in microbial species composition among the studied substrates, because *H. rosa-sinensis* isolates were predominantly yeast-like (filamentous fungi capable of yeast-like growth in media usually employed for yeast cultivation), while the isolates from the other species were mostly true yeasts. Alternatively, the difference may be explained by the fact that different plants possess different leaf and exudate compositions, meaning that the search for a specific enzyme must take into account these differences. The authors are not aware of studies concerning the chemical composition of *H. rosa-sinensis*, *C. microcarpa*, *Ficus*, or Bromeliaceae leaves, but studies about other plants suggest differences among them (Mongrand et al. 2005, Wayman et al. 2010). This is especially relevant for esterase activity because leaf wax acts as an inducer of this enzyme.

Strains capable of producing a large variety of enzymes are extremely interesting because they are versatile and can be used in a variety of industrial processes (Panke & Wubbolts 2002, Van Beilen & Li 2002). Although phylloplane yeast strains found on leaf blades did not prove to be capable of producing many enzymes, we were able to select some interesting isolates. Strains FI125 and FI163, isolated from species of Moraceae, and HB11b, isolated from *H. rosa-sinensis*, produced all four enzymes tested (amylase, the proteases caseinase and gelatinase, and esterase). Some other strains were capable of producing 3 or 2 of these enzymes, which could mean they are industrially useful. More studies are necessary in order to evaluate the optimum conditions for the production of each enzyme by each isolate. The phylloplane appears to be a good substrate for the isolation of yeasts with enzyme production potential, comparable or better than other substrates analyzed in the literature, especially when considering the enzymes tested in this study.

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