CHEMICAL ENGINEERING TRANSACTIONS
VOL. 27, 2012
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Guest Editors: Enrico Bardone, Alberto Brucato, Tajalli Keshavarz

ISBN 978-88-95608-18-1; **ISSN** 1974-9791
DOI: 10.3303/CET1227049

Application of fungal endophytes in biotechnological processes

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The term “endophytes” includes a family of microorganisms that grow intra-and/or intercelullarly in the tissues of higher plants without causing over symptoms on the plants in which they live. These microorganisms represent a potential source of novel natural products for medicinal, agricultural and industrial uses, such as antibiotics, anticancer agents, biological control agents, and other bioactive compounds. The aim of the present work was to investigate the biotechnological potential of fungal endophytes isolated from Baru (*Dipteryx alata* Vog.). Accordingly, the antimicrobial activity, the enzymatic profile and the biotransformation of terpenes in aroma compounds were evaluated. A total of 5 fungal strains were isolated from Baru and identified in the present work. Screening of the antimicrobial activity of endophytic extracts revealed a considerable activity against the pathogenic cultures tested. Most of the extracts inhibited the growth of *Candida albicans*, *Escherichia coli* and *Sthaphylococcus aureus*. Meanwhile, the production of proteases was not detected, however all showed potential of amylase and lipase production. The preliminary results obtained from the biotransformation process showed that three fungal strains bioconverted α-pinene into verbenol (85 % similarity in MS results), compound with great industrial interest. The use of endophytic microorganisms isolated from Brazilian biome demonstrates their potential for application in biotechnological processes.
Keywords: Endophytes, antimicrobial activity, enzymes profile, biotransformation process, terpenes.

1. Introduction

The term “endophytes” includes a suite of microorganisms that grow intra-and/or intercelullarly in the tissues of higher plants without causing over symptoms on the plants in which they live, and have proven to be rich sources of bioactive natural products (Li et al., 2008; Tan and Zou, 2001). Mutualism interaction between endophytes and host plants may result in fitness benefits for both partners (Kogel et al., 2006). The endophytes may provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicine (Strobel, 2003).
Approximately 300.000 plant species growing in unexplored area on the earth are host to one or more endophytes (Strobel and Daisy, 2003), and the presence of biodiverse endophytes in huge number plays an important role on ecosystems with greatest biodiversity, for instance, the tropical and temperate rainforests (Strobel, 2003), which are extensively found in Brazil and possess almost 20 % of its biotechnological source (Souza et al., 2004). Considering that only a small amount of endophytes have been studied, recently, several research groups have been motivated to evaluate and elucidate

Please cite this article as: Molina G., Pimentel M., Bertucci T. and Pastore G., 2012, Application of fungal endophytes in biotechnological process, Chemical Engineering Transactions, 27, 289-294 DOI: 10.3303/CET1227049

the potential of these microorganisms applied on biotechnological processes focusing on the production of bioactive compounds.

Endophytes provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, benzopyranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others (Tan and Zou, 2001). Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents (Strobel, 2003).

The discovery of novel antimicrobial metabolites from endophytes is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens, the insufficient number of effective antibiotics against diverse bacterial species, and few new antimicrobial agents in development, probably due to relatively unfavorable returns on investment. Many bioactive compounds, including antifungal agents, have been isolated from the genus *Xylaria* residing in different plant hosts, such as “sordaricin” with antifungal activity against Candida albicans (Pongcharoen et al., 2008); “mellisol” and “1,8-dihydroxynaphthol 1-O-a glucopyranoside” with activity against herpes simplex virus-type 1 (Pittayakhajonwut et al., 2005); “multiplolides A and B” with activity against *Candida albicans* (Boonphong et al., 2001).

The biotransformation process provides a number of advantages over chemical synthesis. Therefore, biotransformation is a useful method for production of novel compounds; enhancement in the productivity of a desired compound; overcoming the problems associated with chemical analysis; leading to basic information to elucidate the biosynthetic pathway (Suresh et al., 2006). The biotransformation of terpenes has been extensively employed for the production of volatile compounds with great industrial interest, since it allows the production of enantiomerically pure flavors and fragrances under mild reaction conditions (Krings and Berger, 1998).

The aim of the present work was to investigate the biotechnological potential of fungal endophytes isolated from Baru (*Dipteryx alata* Vog.). Accordingly, the antimicrobial activity, the enzymatic profile and the biotransformation of α-pinene were evaluated.

2. Material and methods

2.1 Isolation of fungal endophytes

After proper sterilization of the surface, parts of Baru fruits (including the seeds, pulp and internal part of peel) were evenly spaced in Petri dishes containing potato dextrose agar (PDA) medium. The Petri dishes were incubated at 30 °C and monitored every day to check the growth of microorganism colonies from the fruit segments. After isolation, the strains were cultivated and preserved by periodic replications (once a week) on Yeast-Malt agar (YM: 10 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ peptone, 3 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ malt extract, pH 6.7).

2.2 Antimicrobial capacity

The pathogenic microorganisms *Escherichia coli* CAT 0547, *Pseudomonas aeruginosa* ATCC 13388, *Staphylococcus aureus* CTC 2740, *Salmonella choleraesuis* CCT 4296 and *Candida albicans* ATCC 10231, obtained from the Culture Collection of the Laboratory of Microbiology CPQBA (Unicamp, Brazil), were inoculated in Petri dishes containing solid BHI (Brain Heart Infusion) medium and incubated at 30 °C. After 24 h, one full loop of each pathogenic culture was transferred to 50 mL Erlenmeyer flasks containing 10 mL of liquid BHI medium, and then was placed on the shaker at 30 °C and 150 rpm. After 24 hours, the absorbance was adjusted with pure liquid BHI between 0.080 and 0.100 (625 nm). Then, 100 µL of each microorganism was spread in Petri dishes containing solid BHI, using a sterile Drigalsky spatula.

Thereafter, three filter paper discs (5 mm diameter) were placed on each BHI Petri dish previously inoculated with pathogenic microorganisms. Ten (10) µL of the extracts of endophytic cultures were dispensed to each disc. After incubation at 30 ºC for 24 h, the presence of inhibition zones around the discs was analyzed adapted from (Rabanal et al., 2002). Chlorine was assayed as positive antimicrobial reference, and dimethyl sulfoxide (DMSO) as negative control.

2.3 Screening of extracellular enzymes

The isolated strains were tested for their ability to produce extracellular enzymes that degrade starch, proteins and olive oil, for a screening of enzymes amylase, protease and lipase production, respectively. The production of all enzymes was determined by the observation of color intensity and diameter of degrading halos formed in the solid cultivation media.

Amylase

The endophytic microorganisms were inoculated on Petri dishes containing YM medium and incubated at 30 °C for 24 h. The production of amylase was determined using starch medium agar with 0.5 % soluble starch (Bastos, 2005). After 48 h, 10 mL of iodine solution (30 % iodine) was applied in each plate, and the halos around the colonies were measured. All assays were performed in triplicate.

Lipase

The medium used for the determination of lipase production contained the following compounds (per liter): peptone, 3.0 $g.L^{-1}$; K_2HPO_4, 2 $g.L^{-1}$; $MgSO_4$, 1 $g.L^{-1}$, rhodamine B, 0.01 $g.L^{-1}$; yeast extract, 2 $g.L^{-1}$; agar, 18 $g.L^{-1}$ and 20 $g.L^{-1}$ olive oil (Lin et al., 1995). In order to obtain colonial growth, Inoculated plates were incubated at 30 °C for 48 h. The presence of orange fluorescent halos around colonies under UV rays observation indicated the presence of positive lipase-production. All assays were performed in triplicate.

Protease

Protease activity was determined by inoculating the microbial strain in a culture medium containing triptone, 46.43 $g.L^{-1}$; yeast extract, 2.79 $g.L^{-1}$; skim milk powder, 23.21 $g.L^{-1}$ and agar, 18.57 $g.L^{-1}$ adapted from (Tang et al., 2008). After 48 h at 30 °C, the halos around the colonies were measured. All assays were performed in triplicate.

2.4 Biotransformation procedure

A 72 h culture grown on agar in a Petri dish was divided amongst 50 mL of YM medium and homogenized under sterile conditions using an Ultra-Turrax® T18 (Ika, Wilmington, NC, USA) until complete disruption of the solid matter. After 72 h of incubation at 30 °C and 150 rpm, the cell mass was concentrated an placed in 50 mL of mineral medium. Biotransformation was started by adding 3 $g.L^{-1}$ of α-pinene and incubated at 30 °C and 150 rpm. Samples were extracted with the same volume of ethyl acetate (with decane as internal standard) at 0, 24, 48, 72 and 96 h for analysis by gas chromatography and mass spectrometry (GC-MS) for detection, identification and quantification of the products formed and substrate consumption.

3. Results and discussion

3.1 Screening of endophytic microorganisms and antimicrobial activity

A total of 5 fungal strains were isolated from Baru and named as LBBR01, LBBR02, LBBR03, LBBR04 and LBBR05. Initially, the disk diffusion method was used to determine the spectrum of antimicrobial activity of the endophytic microorganisms. This test is accepted by the FDA (Food and Drug Administration) and it is established as standard by NCCLS (National Committee for Clinical Laboratory Standards). The diameters of the inhibition zones obtained with the extracts are presented in Table 1.

Table 1: Antimicrobial results for the Baru endophytes that inhibited the pathogenic microorganisms tested. The value is equal to the average of the 3 halos diameter in mm, discounting the disc diameter (5 mm)

Strain	*C. albicans*	*E. coli*	*P. aeruginosa*	*S. choleraesuis*	*S. aureus*
LBBR01	8.0	5.0	13.3	9.0	7.0
LBBR02	9.7	0.0	9.0	7.0	10.0
LBBR03	10.3	0.0	7.3	0.0	7.0
LBBR04	9.7	0.0	11.0	10.7	12.7
LBBR05	9.3	0.0	10.7	10.7	11.7
Chlorine	20.0	20.0	20.0	20.0	20.0

Studies evaluating the inhibition of the extracts of endophytes were reported by Wiyakrutta et al. (2004), Teles et al. (2006) and others.
In the present work, the screening of the antimicrobial activity of the endophytic extracts revealed a considerable activity against the bacteria and yeasts tested. Most of the extracts inhibited the growth of *Candida albicans* and *Sthaphylococcus aureus*. The most significant activity was observed for the LBBR01, which inhibited the growth of all the pathogens tested.
Identification of the secondary metabolites responsible for this activity, studies involving other solvents for its extraction and the MIC (minimum inhibitory concentration) should be performed in future studies.

3.2 Screening of extracellular enzymes

The results obtained suggest that the microorganisms have the ability to use starch and lipids as energy sources, while the production of protease enzymes was not detected, as shown in Table 2.

Table 2: Enzymatic index of fungal strains from Baru

	Halo diameter (mm)		
Strains	Amylase	Lipase	Protease
LBBR01	22.0	20.0	0.0
LBBR02	27.0	25.0	0.0
LBBR03	24.0	24.0	0.0
LBBR04	20.0	19.0	0.0
LBBR05	0.0	18.0	0.0

To date references containing the screening of enzymes from endophytic microorganisms in Petri dishes are still scarce, but former studies used these strains to degrade xylan and mannan, as reported by Tomita (2003). It is assumed that during the incubation period, the bacteria and fungi tested released enzymes (amylases, proteases and oxidases) that actively degraded components of the culture medium. Comparative analysis of the production of extracellular enzymes detected variability among isolates of Baru, which can be very useful information for the identification of isolates.

3.3 Biotransformation procedure

Endophytes were evaluated in biotransformation procedure using α-pinene ($C_{10}H_{16}$) as substrate, one of the most studied terpene in biotransformation process. This hydrophobic organic volatile compound is emitted from the forest products industry (*e.g.*, wood products, pulp and paper industries). Because of their economic advantage, pinenes represent an ideal substrate for biotechnological processes and have been extensively employed in microbial conversion experiments (Yoo et al., 2001).

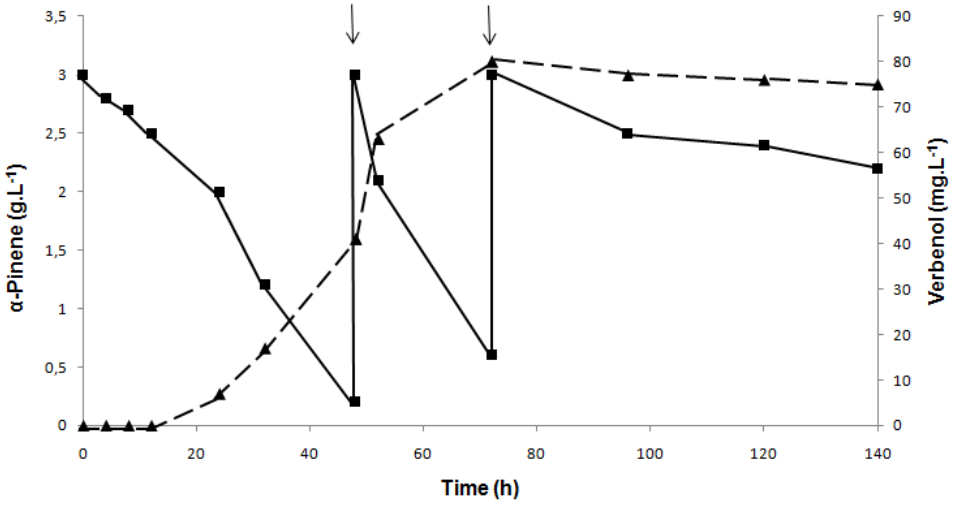

Figure 1: Biotransformation of α-pinene for the production of verbenol by the strain LBBR02, where: -■- represents α-pinene consumption (in g.L^{-1}),--▲-- represents verbenol production (in mg.L^{-1}) and the arrows indicates the injection of α-pinene

The preliminary results showed that the fungal strains LBBR01, LBBR02 and LBBR04 bioconverted the α-pinene into verbenol (85 % similarity in MS results and confirmed with commercial standard), after 24 h of contact with the terpene.
Although this product was recurrent from three fungal strains, the highest concentration of this compound was achieved by LBBR02. The quantification of verbenol showed a maximum production around 72 h, reaching 80 $mg.L^{-1}$.
Figure 1 shows the biotransformation of α-pinene for the production of verbenol by the strain LBBR02 during 140 h of fermentation. Product concentration remained stable after 72 h of fermentation, which probably indicates that the accumulation of product and substrate in culture media may have had an inhibitory effect, due to the toxicity of the terpenes to the microorganism (Van der Werf et al., 1997). Techniques of successive product removal should be studied and further work with fed-batch could be considered to enhance production.
The biotransformation to verbenol occurred based on the biochemical reaction of hydroxylation of α-pinene (Figure 2), and this reaction was reported in some articles. Maróstica et al. (2007) showed the biotransformation of α-pinene from the turpentine oil, by fungal strains, and a microextraction in solid phase was used for extraction of the aroma compounds. The production of 50 $mg.L^{-1}$ of verbenol and 70 $mg.L^{-1}$ of verbenone from α- and β-pinenes, respectively, was performed by *Mucor* sp. 2276. The production of verbenol/verbenone from α-pinene by *Aspergillus* sp. and *Penicillium* sp. strains was also reported by Agrawal et al. (1999). Further studies should be performed to optimize the process parameters to increase the production of this compound.

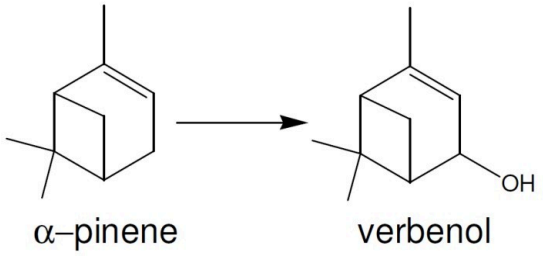

Figure 2: Biochemical reaction of hydroxylation of α-pinene to verbenol

4. Conclusion

This paper describes the use of fungal endophytes isolated from the Brazilian Cerrado Biome and demonstrates a partial use of these microorganisms in biotechnological processes and their potential as source of bioactive compounds. Further studies are still needed to identify the compounds related to the antimicrobial activity, and also to verify the minimum inhibitory concentration (MIC) and estimate the enzyme activity. Interestingly, this appears to be one of the first works where the production of verbenol was achieved by an endophytic microorganism. Although the high potential of these microorganisms, studies using endophytes in the field of biotransformation are still limited.

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