Biotechnol Lett (2011) 33:763–768
DOI 10.1007/s10529-010-0496-1

ORIGINAL RESEARCH PAPER

Evaluation of inert and organic carriers for *Verticillium lecanii* spore production in solid-state fermentation

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Received: 24 October 2010 / Accepted: 1 December 2010 / Published online: 18 December 2010

Abstract Growth and sporulation of *Verticillium lecanii* on inert and organic carriers (sugar-cane bagasse, corncob, rice straw, polyurethane foam and activated carbon) in a solid-state fermentation process was studied. Sugar-cane bagasse and polyurethane foam produced 10^{10} spores g^{-1} dry carrier whereas corncob, rice straw, and activated carbon yielded, respectively 8×10^9, 4×10^9, and 3×10^8 spores g^{-1}. Chitinase activity of the conidia was in the following order: sugar-cane bagasse (3.3 U mg^{-1}) > wheat bran (3.0 U mg^{-1}) > polyurethane foam (2.7 U mg^{-1}). There was no significant difference (2.5–2.7 U mg^{-1}) in the proteinase activity among the conidia from the three cultures. Scanning electron microscopy shows that aerial mycelium freely penetrated into the internal area of polyurethane foam. Sugar-cane bagasse provided enough area for vegetative hyphae to attach. Of the carriers analyzed, polyurethane foams and sugar-cane bagasse were the best carriers for *V. lecanii* growth and spore production.

Keywords Chitinase and proteinase activity · Inert and organic carriers · Solid-state fermentation · Spore production · *Verticillium lecanii*

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Introduction

Verticillium lecanii is an entomopathogenic fungus with a broad range of hosts including insects: Coleoptera, Orthopteta, Homoptera, and Lepidoptera. *Verticillium lecanii* also parasitizes some plant pathogens such as cucumber powdery mildew and chrysanthemum rust fungi.

For biological control by *V. lecanii*, large numbers of spores are needed. Solid-state fermentation (SSF) and submerged fermentation (SmF) are current systems for fungal spore production. Volumetric spore productivity by SmF is relatively low compared with SSF. In addition, some fungi produce few spores under SmF conditions. SSF can use relatively cheap agricultural byproducts such as wheat bran. Natural substrates in SSF have some disadvantages including excessive thickness of the substrate layer, low porosity, and inadequate internal structures that disturb the aeration, heat removal, and inefficient nutrient uptake (Hölker and Lenz 2005; Marin-Cervantes et al. 2008). Therefore, SSF with inert carriers such as ion exchange resins and polyurethane foams (PUF) were developed. Inert carriers present high porosity, low density, relatively high water absorption, and a satisfactory environment for fungal growth, where the nutrients from a liquid medium are absorbed. The use of inert carrier allows direct biomass determination, renewable use, cleaner extractions, good aeration, and heat removal (Marin-Cervantes et al. 2008). Recently, we used sugar-cane bagasse as an organic carrier in SSF for *V. lecanii* spore

production (Shi et al. 2009). With optimized growth medium and carrier surface parameters by response surface methodology, spore production on sugar-cane bagasse reached 10^{10} spores g^{-1} dried carrier (DC), much higher than on natural substrates such as wheat bran (2×10^9 spores g^{-1} initial dry matter) (Shi et al. 2009).

In this paper, we evaluated inert and organic carriers, i.e., sugar-cane bagasse, corncob, rice straw, PUF, and activated carbon to compare *V. lecanii* spore production in SSF. Fungal growth, spore production, spore germination rate, chitinase and protease activity, and effect of carrier microstructure on fungi growth were investigated.

Materials and methods

Materials

Polyurethane foam with 300–400 holes cm^{-2} was purchased from Tianjin Yiwu Science Company Limited (Tianjin, China). Activated carbon with granularity of 0.8–1.2 mm and hole volume of 0.8 cm^3 g^{-1} was purchased from Hangzhou Microbiology Institute (Hangzhou, China). Sugar-cane bagasse, corncob, and rice straw were obtained from a local farmland.

Microorganism and inoculum preparation

Verticillium lecanii (CBS 102071) was grown in potato/dextrose/agar (PDA) at 25°C for 7 days. The spores were harvested from the surface by pouring sterile 0.1% (v/v) Tween 80 to wash off the spores. The spore suspension of 10^7 cfu ml^{-1} was used immediately to inoculate the subsequent fermentation.

Solid-state fermentation

Sugar-cane bagasse was washed and corncob was immersed in distilled water for 4 h to ensure that sugar-cane bagasse and corncob were free of sugar and surface dirt, and then both were dried at 80°C. The bagasse, corncob, and rice straw were cut into 4 mm pieces. Each carrier (2 g) was mixed with 7 ml liquid medium. Polyurethane foams was washed by distilled water, dried at 80°C, and cut into 4 mm pieces. Two grams of the PUF was mixed with 8 ml liquid medium. The activated carbon (10 g) was mixed with 4 ml liquid medium.

Liquid medium contained (g l^{-1}): corn flour, 35.79; yeast extract, 8.69; KH_2PO_4, 1.63; K_2HPO_4, 0.325; and $MgSO_4$, 0.325 (Shi et al. 2009), pH 6.0. A 0.5 ml spore suspension (10^7 spores ml^{-1}) was added in the liquid medium and inoculated at 25°C for 8–14 days under relative humidity of 90%.

Spore counting

The final cultural sample (0.5 g) was mixed with 15 ml sterile water and shaken for 5–8 min to separate spores from the carriers. The spore suspension was diluted appropriately to a proper concentration that could be identified by microscopic counting with a hemocytometer. Spore production was expressed as spores per gram DC.

Assessment of spore germination

The spore suspension was diluted with sterile water to 10^7 cfu ml^{-1}. The diluted suspension was spread on Sabouraud agar (containing per liter of water: peptone, 10 g; glucose, 40 g; and agar, 15 g, pH 6.0 ± 0.2) plate. Spore germination was counted every 2 h using a microscope during cultivation at 25°C. Germination rate was calculated by counting four visual fields (about 300–500 spores) each time.

Analysis of enzyme activity of conidia

The spore suspension was diluted with sterile water to 10^7 cfu ml^{-1}. The diluted suspension was separately inoculated into a chitin medium [a mixture of A/B (v/v, 1:1), A (w/v): 2% colloidal chitin, and B (w/v): 0.05% KH_2PO_4; 0.05% $MgSO_4$; 0.05% NaCl; and 0.001% $FeSO_4$] or a casein medium [a mixture of A/B (v/v, 1:1), A (w/v): 2% casein, and B (w/v): 0.05% KH_2PO_4; 0.05% $MgSO_4$; 0.05% NaCl; and 0.001% $FeSO_4$]. Folin and DNS methods were used to analyze chitinase and proteinase activity (Jackson et al. 1998).

The specific enzyme activity was expressed as enzyme activity per unit cell dry weight.

Examination of carrier microstructure and fungi growth using scanning electron microscopy (SEM)

The final fermentation sample was fixed by immersion in 5% (v/v) glutaraldehyde for 24 h at 4°C,

post-fixed with 1% (w/v) osmium tetroxide for 2 h at 4°C, and dehydrated in a gradient alcohol series. Then, the sample was covered with carbon and gold prior to examination by SEM (Sarhy-Bagnon et al. 2000).

Statistical analysis

Experimental results were means ± SD of three independent samples. The data were analyzed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Duncan's multiple range tests at $P = 0.05$ or independent sample t test ($P = 0.05$).

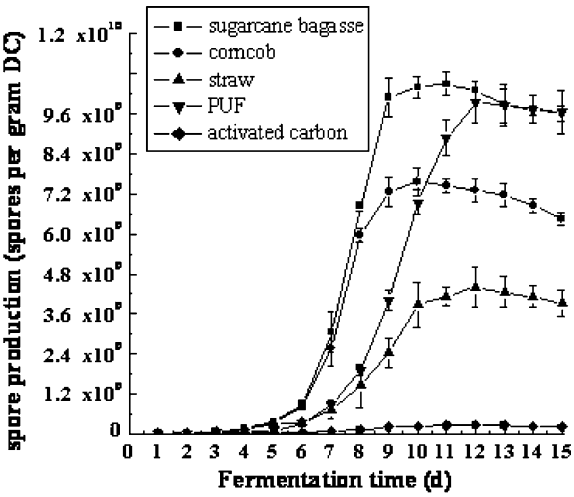

Fig. 1 *Verticillium lecanii* time-course spore production during fermentation as affected by carrier type. *Filled square*, *circle*, *triangle*, *inverted triangle*, and *diamond* represent fermentation in sugar-cane bagasse, corncob, rice straw, polyurethane foam, and activated carbon, respectively. Values represent the mean of three replications ± SD, *DC* dried carrier

Results and discussion

Figure 1 shows the spore production of *V. lecanii* in SSF in the different inert and organic carriers. From the 4th day onwards, spore production increased and showed difference associated with the different carriers. The fungi in the sugar-cane bagasse yielded the highest sporulation in the shortest fermentation time among the five carriers. At the end of fermentation, SSF in the sugar-cane bagasse and PUF produced 10^{10} spores g^{-1} dry carrier whereas the corncob, rice straw, and activated carbon yielded 8×10^9, 4×10^9, and 3×10^8 spores g^{-1}.

Germination rate of *V. lecanii* conidia from the five carriers increased with time (Table 1) and differed according to the carrier but was consistently and significantly higher from the sugar-cane bagasse than from the others, reaching 91% after 12 h. During 12–14 h, the conidia from the sugar-cane bagasse, corncob, and PUF had better germination than those from the rice straw and activated carbon.

Chitinase and proteinase activities contribute to *V. lecanii* conidia pathogenic effect in pest control (Askary et al. 1998). The chitinase activity of the conidia from the three cultures was in the following order: sugar-cane bagasse (3.3 U mg^{-1}) > wheat bran (3 U mg^{-1}) > PUF (2.7 U mg^{-1}) (Fig. 2). The protease activity of the conidia was in the following order: wheat bran (2.7 U mg^{-1}) > PUF (2.6 U mg^{-1}) > sugar-cane bagasse (2.5 U mg^{-1}) (Fig. 3). The results demonstrated that the quality of conidia from the inert carriers were comparable with that from the wheat bran. The fermentation time to produce maximal chitinase and proteinase activities in the two carriers was longer than that in the wheat bran. The carriers had

Table 1 Germination rate of conidia from different carriers

Time (h)	Germination rate (%)				
	Sugar-cane bagasse	Corncob	Rice straw	Polyurethane foam	Activated carbon
6	4.8 ± 1a	3.5 ± 0.7b	2.2 ± 0.8c	2.9 ± 0.5b	2 ± 0.5c
8	19.9 ± 3.2a	10.5 ± 2.1b	7.3 ± 1.4c	9.8 ± 1.8b	5.4 ± 1.1c
10	51 ± 6.2a	42 ± 5.5b	31 ± 3.8c	40 ± 5.3b	30 ± 3.5c
12	91 ± 1.1a	89 ± 1.3a	79 ± 2.0b	90 ± 2.1a	73 ± 1.8b
14	97 ± 0.5a	95 ± 0.9a	91 ± 1.1b	98 ± 0.4a	90 ± 2.1b
16	100	99 ± 0.4	97 ± 0.3	100	97 ± 1.0
18	100	100	100	100	100

Data based on three trials. Values are the mean ± SD, and *alphabet letters* indicate the *same letters* in the same row are not statistically significantly different according to Turkey-HSD ($P < 0.05$)

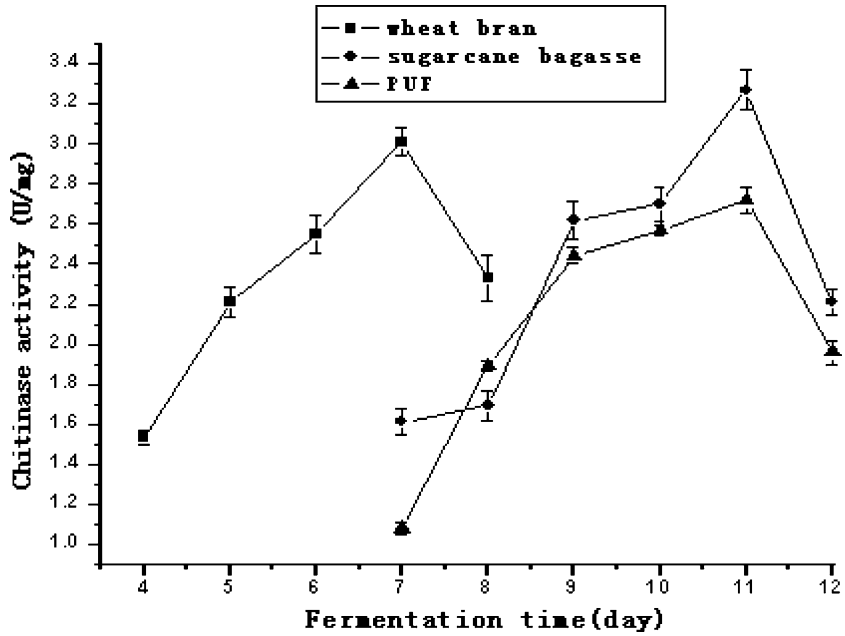

Fig. 2 Effect of carrier type on chitinase activity of *Verticillium lecanii* conidia. *Filled square*, *diamond*, and *triangle* represent fermentation in wheat bran, sugar-cane bagasse, and polyurethane foam, respectively. Values represent the mean of three replications ± SD

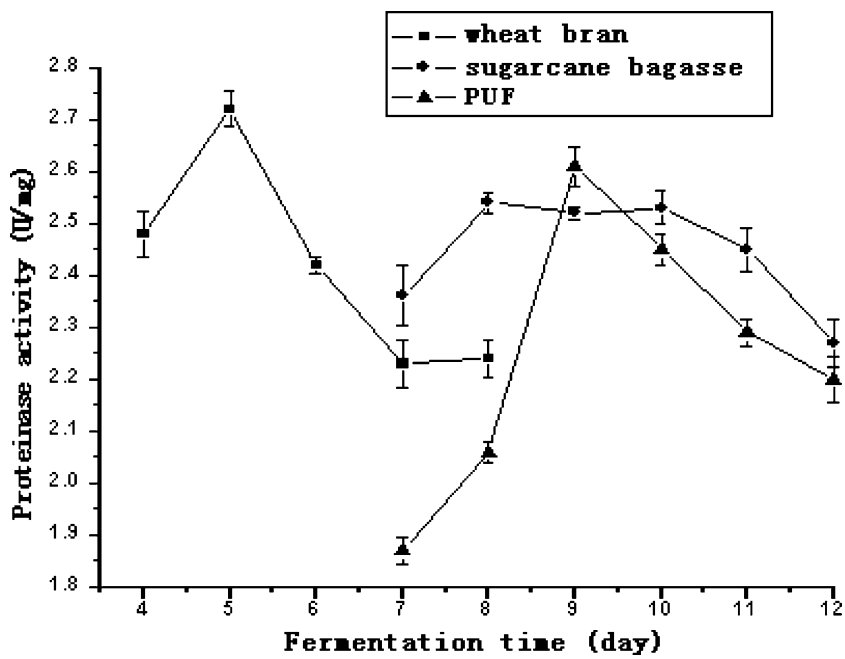

Fig. 3 Effect of carrier type on proteinase activity of *Verticillium lecanii* conidia. *Filled square*, *diamond*, and *triangle* represent fermentation in wheat bran, sugar-cane bagasse, and polyurethane foam, respectively. Values represent the mean of three replications ± SD

some disadvantages compared to the wheat bran in this aspect.

In order to determine why sugar-cane bagasse and PUF appeared to be the best carriers for spore production, we observed *V. lecanii* growth in the carriers at the micro- and macro-levels by scanning electronic microscopy. Figure 4 shows the SEM imaging of the fungi growing in the sugar-cane bagasse or PUF after 8–12 days of cultivation. There were a large number of aerial hyphae in the PUF

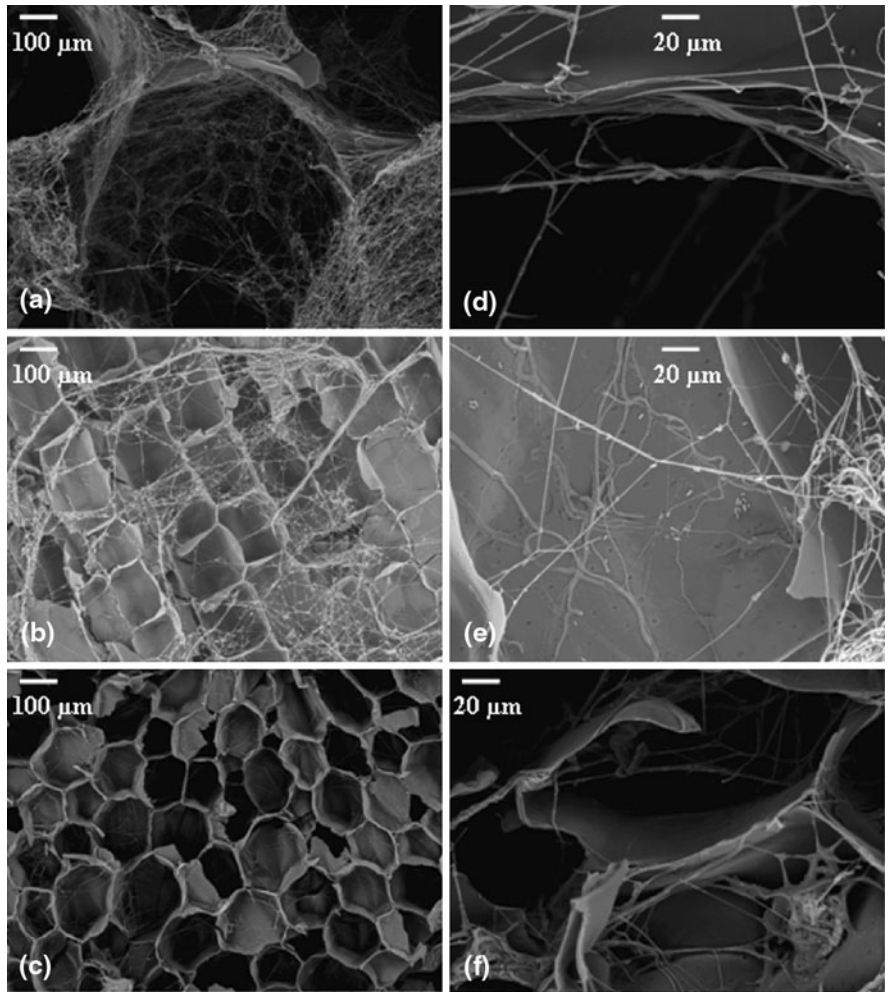

Fig. 4 Scanning electron micrographs of *Verticillium lecanii* growth in the carriers after 8–12 day fermentation. **a**, **b** Aerial hyphae on the surface of the polyurethane foam; **c**, **d** vegetative hyphae on the surface of the sugar-cane bagasse; **e**, **f** vegetative hyphae inside the sugar-cane bagasse

(Fig. 4a), whereas fewer aerial hyphae on the surface of the sugar-cane bagasse (Fig. 4b) and almost not any aerial hyphae inside the sugar-cane bagasse (Fig. 4c). In addition, most hyphae on the surface and inside the sugar-cane bagasse were the vegetative hyphae (Fig. 4e, f), which was distinguished based on its sturdier mycelia climbing on the surface like plant branches and roots. More aerial hyphae in the PUF indicated larger spore production, while more developed vegetative hyphae in the sugar-cane bagasse could provide *V. lecanii* with more nutrition to grow high-quality conidia (Hallsworth and Magan 1995).

The pore diameter of the PUF was larger and arrangement of pores was more regular. *Verticillium lecanii* could well wind its surface and then penetrate into the internal area (Fig. 4d). As a result, more aerial hyphae developed in the PUF. Aerial mycelia that developed from the hyphae produced more conidiophores and more conidia (Kazmierczak et al. 2005). On the other hand, the PUF provided a relatively small area for vegetative hyphae to attach, resulting in fewer vegetative hyphae. This may explain why *V. lecanii* sporulation in the PUF entered the stationary phase later than on the sugar-cane bagasse. Insufficient vegetative hyphae and a large number of aerial hyphae slowed down its growth in the PUF.

In contrast, the pores of the sugar-cane bagasse were smaller and less regularly developed than those

of the PUF. The pore was more like a cube opening one side (Fig. 4b). The aerial hyphae grew only on the surface of the sugar-cane bagasse and produced only a small amount of mycelia. Furthermore, the vegetative mycelia attached to the surface as well as internally. Well-developed vegetative mycelia provided enough nutrition for conidia, and produced more vigorous sporulation (Table 1). This may explain why the conidia from the sugar-cane bagasse germinated earlier than the conidia from the PUF.

Conclusions

Of the five carriers analyzed, the synthetic material PUF and sugar-cane bagasse as a natural resource proved to be the best carriers for *V. lecanii* growth and spore production in SSF. Corncob was the next suitable carrier for *V. lecanii* spore production, but rice straw and activated carbon were the poor carriers.

Acknowledgment This work was supported by a research grant from the Science and Technology Department of Zhejiang Province, China (2007C24016).

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