Micología Aplicada Internacional
Colegio de Postgraduados (Campus Puebla, México)
dcarrera@colpos.mx
ISSN (Versión impresa): 1534-2581
MÉXICO

2007
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Micología Aplicada Internacional, año/vol. 19, número 001
Colegio de Postgraduados (Campus Puebla, México)
Puebla, México
pp. 7-14

Red de Revistas Científicas de América Latina y el Caribe, España y Portugal

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Micologia Aplicada International, 19(1), 2007, pp. 7-14
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Comparative spore germination of filamentous fungi on solid state fermentation under different culture conditions

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Accepted for publication November 6, 2006

ABSTRACT

Environmental factors influencing spore germination of three filamentous fungi (*Aspergillus niger*, *Myceliophthora thermophila*, *Rhizopus microsporus*) were studied. Four substrates, all containing sugar cane bagasse as support, were tested for spore germination. Different temperature, moisture, aeration and humidity conditions were evaluated for each species. Spore germination was assessed by observing emergence of the germ tube. Germination rates in *A. niger*, *M. thermophila*, and *R. microsporus* cultivated in solid state fermentation were affected by the substrate, incubation temperature, and initial moisture. A mixture of sugar cane bagasse and wheat bran (80:20 w/w) resulted in high germination rates for all species tested, and was selected for additional experiments. The optimal temperature for spore germination and initial growth was 30 C for *A. niger* and 45 C for *M. thermophila* and *R. microsporus*. Higher germination rates were recorded in all species at 85% (a_w 0.99) initial moisture after 6 h of incubation at optimal temperature. Forced humid aeration increased germination rates and decreased the time required for complete spore germination in all species studied.

Key words: Germination, temperature, initial moisture, aeration, sugar cane bagasse, *Aspergillus niger*, *Myceliophthora thermophila*, *Rhizopus microsporus*.

INTRODUCTION

Fungal spore germination consists of all processes and modifications that the spore undergoes during initial stages of its development or growth from dormancy. A spore undergoes structural transformations and is considered as germinated when the germ tube reaches a length at least equal to its widest diameter[7,16]. Spore germination is an essential stage in the development of filamentous fungi. It is divided into three main stages: 1) Activation of the resting spore in response to environmental conditions; 2) Swelling growth characterized by the absorbance of water and the reactivation of fungal metabolism; and 3) The emergence of the germ tube, also known as polarized growth[5]. Recent studies on the morphology of the spore surface by scanning electron microscopy and molecular genetics brought new information about the participation of various cellular processes, notably the metabolism of trehalose as well as gene expression[5]. In addition, numerous physiological processes concerning the initiation of protein synthesis and the role of Ca^{2+} have been reported[6,13]. When environmental factors, such as temperature and water activity, are favourable, filamentous germination and growth can occur[9,10,18].

Fungal spores contain nutrient reserves that can support growth for a limited period; however, medium composition affects the process of germination. Spore germination of *Aspergillus flavus* and *A. niger* in media containing fructose or glucose was always greater than in those having glycerol[4]. The effect of aeration on spore germination in moulds has been poorly studied, although aeration could be a good tool to accelerate germination[15].

In this study, we evaluated first the effect of substrate on spore germination of three species of filamentous fungi. After that, the effects of temperature, water availability, forced aeration, and humidity on spore germination were studied.

MATERIALS AND METHODS

Microorganisms. Three species, *Aspergillus niger* Tiegh. (A), *Myceliophthora thermophila* (Apinis) Oorschot (B), and *Rhizopus microsporus* Tiegh. (C), were isolated from an olive press cake. They were maintained at 4 C on potato-dextrose-agar (PDA), and deposited at the IRD research culture collection.

Inoculum preparation. Each species was inoculated in flasks containing 30 ml of PDA. After 7 days incubation at 35 C for *A. niger*, and at 45 C for *M. thermophila* and *R. microsporus*, 100 ml of sterile distilled water containing 0.01% Tween-80 was added to flasks and were shaken vigorously under aseptic conditions for inoculum production.

Preparation of solid substrates. Three culture media were tested and prepared for solid state fermentation (SSF), according to the following proportions: sugar cane bagasse alone, sugar cane bagasse and olive press cake (80:20 w/w), and sugar cane bagasse and wheat bran (80:20 w/w). Sugar cane bagasse and potato dextrose broth (PDB) (50:50 w/w) was used as a control for comparison. Sugar cane bagasse was sieved to reach a size of 0.70-2 mm, then washed with distilled water 2-3 times, and dried at 50 C for 5 days. Olive press cake was pitted. Wheat bran was sieved to eliminate wheatmeal. Substrates containing wheat bran and olive press cake were homogenized for few seconds using a domestic mill (Moulinex, France).

All solid supports were adjusted at 40% moisture content with distilled water, and were sterilized at 121 C for 20 min. The initial moisture was adjusted with a spore suspension in sterile water (inoculum at 2 x10^7 spores/g of initial dry matter) as previously described[16]. The spore suspension was added gently as required to reach an initial moisture of 45%, 55%, 65%, 75%, and 85%. After that, 5 g of each inoculated substrate was tested in triplicate to confirm initial moisture.

Temperature, moisture, aeration and humidity . The effect of temperature on fungal germination when grown on sugar cane bagasse and wheat bran was studied at five different temperatures (19 C, 30 C, 45 C, 50 C, and 60 C). Initial moisture was tested at five water activity levels: 45% (a_w 0.92), 55% (a_w 0.95), 65% (a_w 0.97), 75% (a_w 0.98), and 85% (a_w 0.99). Aeration is used in SSF as a source of oxygen and a means to evacuate heat. The influence of aeration was studied on column bioreactors (3 cm in diameter and 20 cm in length). Sugar cane bagasse and wheat bran-filled columns were aerated at 25-30 ml/min with humidified air (98% relative humidity), and with ambient air (46% relative humidity). Other columns were maintained without forced aeration.

Spore germination. Spore germination for each species was assessed according to Frossard and Oertli[7]. It consisted in collecting a sample (fragment) of the fermented medium with a scalpel, previously soaked in sterile water. The sample was placed on a slide, and colored with 2-3 drops of Lactophenol Cotton blue (Fluka 61335). A cover slide was added over the fragment carefully to avoid air bubbles. Under the microscope (40x), for every zone selected, germinated and non-germinated spores were counted. Ten representative zones were selected at random. The sum of germinated spores over the total spores present determined the percentage of germination for each species at the time of sampling. Sampling was carried out in triplicate.

Statistical analysis. Data were subjected to repeated measures analysis of variance following the Wilks' Lambda Test, using the formula $\eta^2 = 1-\Lambda^{1/s}$. Tests of significant differences were made at alpha=0.01%.

RESULTS AND DISCUSSION

Influence of solid substrates on spore germination. All substrates tested resulted in germination of spores from species studied (**Table 1**). The effect of substrates on the time of germination for all species was significant ($Pr > F\ 10^{-4}$). Sugar cane bagasse and wheat bran showed higher germination rates. After 8 h of incubation, 81% of *A. niger* and *R. microsporus* spores germinated on this substrate. It is to be noted that only 21% of spores from *A. niger* germinated on bagasse and PDB after 8 h. *M. thermophila* spores showed similar germination rate (45-46%) when grown either on bagasse and wheat bran or bagasse and PDB. After 11 h of incubation, 72% of spores from *M. thermophila* germinated on bagasse and wheat bran while complete germination (100%) was observed for *A. niger* and *R. microsporus* spores. Complete germination was not reached by *M. thermophila*, whose highest germination rate was 86% on bagasse and wheat bran after 13 h of incubation. However, on bagasse and olive press cake, germination rates were only 63% for *A. niger* and 44% for *M. thermophila* during the same incubation period. *R. microsporus* showed complete germination (100%) on all substrates after 13 h of incubation.

On bagasse and olive press cake, only *R. microsporus* showed good germination rate

Table 1. Effect of solid substrates on spore germination (%) of *Aspergillus niger* (A), *Myceliophthora thermophila* (B), and *Rhizopus microsporus* (C) incubated in SSF for 13 h (temperature, A: 30 C, B-C: 45 C).

Time (h)	Sugar cane bagasse and PDB			Sugar cane bagasse and wheat bran			Sugar cane bagasse and olive press cake			Sugar cane bagasse		
	Species			Species			Species			Species		
	A	B	C	A	B	C	A	B	C	A	B	C
0	0	0	0	0	0	0	0	0	0	0	0	0
6	3	27	63	52	26	61	5	3	32	31	25	45
8	21	45	82	81	46	81	22	16	58	62	30	69
11	47	76	100	100	72	100	55	28	90	84	44	100
13	73	89	100	100	86	100	63	44	100	100	64	100

Data were significantly different between substrate and time (F= 41.42); species and time (F= 186.13); and substrate, species and time (F= 43.74). Pr > F 0.0001.

(32%) after 6 h of incubation. This substrate did not allow fast germination rates for *A. niger* (22%) and for *M. thermophila* (16%) after 8 h of incubation. Olive press cake is rich of polyphenols and toxic fatty acids, which may affect spore germination[2,8].

Bagasse alone as substrate resulted in spore germination of *A. niger* (62%) and *R. microsporus* (69%), but germination rates were lower than those of bagasse and wheat bran after 8 h of incubation. The germination rate of *M. thermophila* was lower (30%) during the same incubation period.

In fact, sugar cane bagasse and wheat bran was a favourable solid substrate for spore germination in all species tested. Sugar cane bagasse is an excellent support for solid state fermentation, allowing good absorption of spores and nutriments, and providing a good free-water reserve[14,16]. Bagasse and wheat bran was therefore selected for further studies on environmental parameters affecting spore germination.

Effect of incubation temperature and initial moisture on spore germination. The effect of temperature on spore germination of species studied is shown in **Fig. 1**. The optimal temperature for spore germination and initial growth was 30 C for *A. niger*, while 45 C for *M. thermophila* and *R. microsporus*. Germination rates of *A. niger* were similar at 19 C (100%), 30 C (100%) and 45 C (92%), although much lower at 50 C (11%) and 60 C (1%), after 12 h of incubation. *M. thermophila* and *R. microsporus* showed variable germination rates at all temperatures, being lower at 19 C after 12 h of incubation. In both cases, at least 45% of spores had germinated after 12 h of incubation. Higher germination rates were recorded at 30 C for *A. niger* and 45 C for *M. thermophila* and *R. microsporus*, which agrees with their mesophilc, thermophilic or thermotolerant nature, respectively. The significant influence of temperature

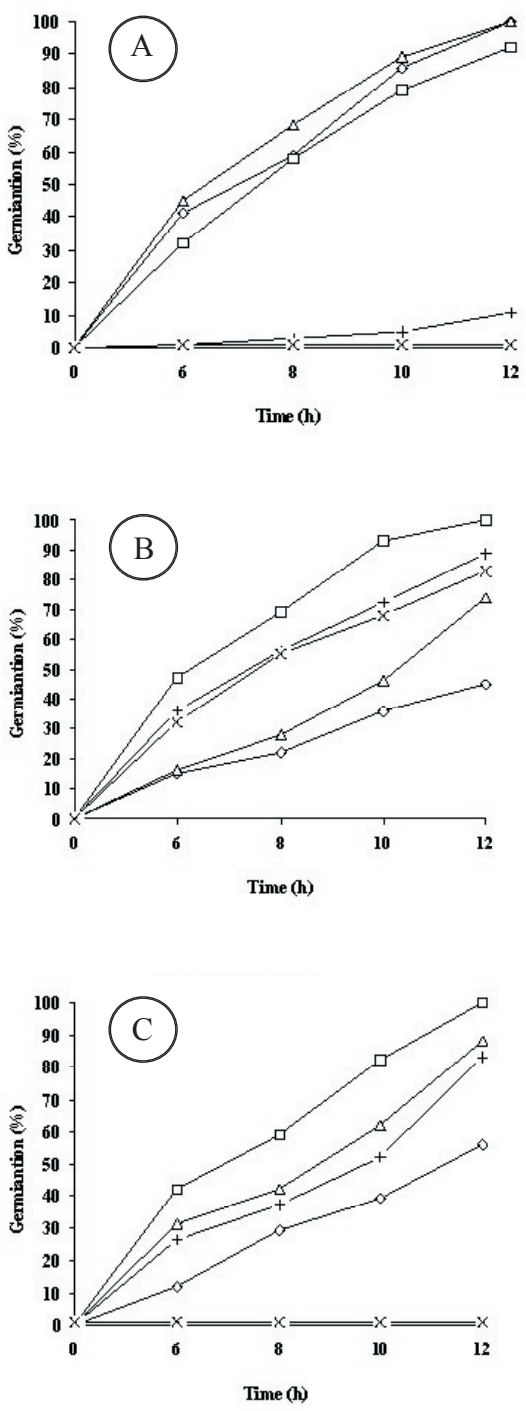

Fig. 1. Influence of incubation temperature on spore germination (%) of *Aspergillus niger* (A), *Myceliophthora thermophila* (B), and *Rhizopus microsporus* (C) cultivated on sugar cane bagasse and wheat bran (80:20 w/w). Temperature: ◇ = 19 C; △ = 30 C; □ = 45 C; + = 50 C; X = 60 C.

on germination and growth has also been studied in other filamentous fungi, such as *Erynia neoaphidis*, *Actinomucor elegans*, *A. penicillioides*, *Penicillium roqueforti*, and *Rhizopus oligosporus*[10,11,12].

Higher germination rates were recorded in all species at 85% (a_w 0.99) initial moisture after 6 h of incubation at 30 C for *A. niger*, and 45 C for *M. thermophila* and *R. microsporus* (**Fig. 2**). A gradual decrease of initial moisture from 85% to 45% (a_w 0.92) was correlated with decreasing germination rate in *Aspergillus niger* (70% to 8%), *Myceliophthora thermophila* (63% to 2%), and *Rhizopus microsporus* (65% to 5%). After 8 h of incubation, at 85% initial moisture, germination rates were 99% for *A. niger*, 70% for *M. thermophila*, and 88% for *R. microsporus*.

It is to be noted, however, that 65% (a_w 0.97) initial moisture was sufficient to achieve 100% germination rate in *A. niger* and *R. microsporus* after 12 h of incubation. By contrast, in the same condition, *M. thermophila* reached only a germination rate of 60%. Similar results were obtained in *P. roqueforti*, whose spores germinated completely (100%) at 0.99 a_w, or partially (89.7%) at 0.88 a_w, after 24 h of incubation at 22 C[3].

Optimal incubation temperature may be associated with water activity in all species tested, as water availability at optimal temperature has been directly linked to spore germination. Gock *et al*.[10] showed that optimal temperature for spore germination varied from 25 C to 37 C according to the water activity variation, from 0.92 to 0.82 for *P. roqueforti* and from 0.92 to 0.7 for *A. penicillioides*. The same phenomenon was observed in *Actinomucor elegans*, *Aspergillus flavus*, *Penicillium* spp., and *R. oligosporus*[1,11]. Recently, temperature and water activity were mathematically modeled[17].

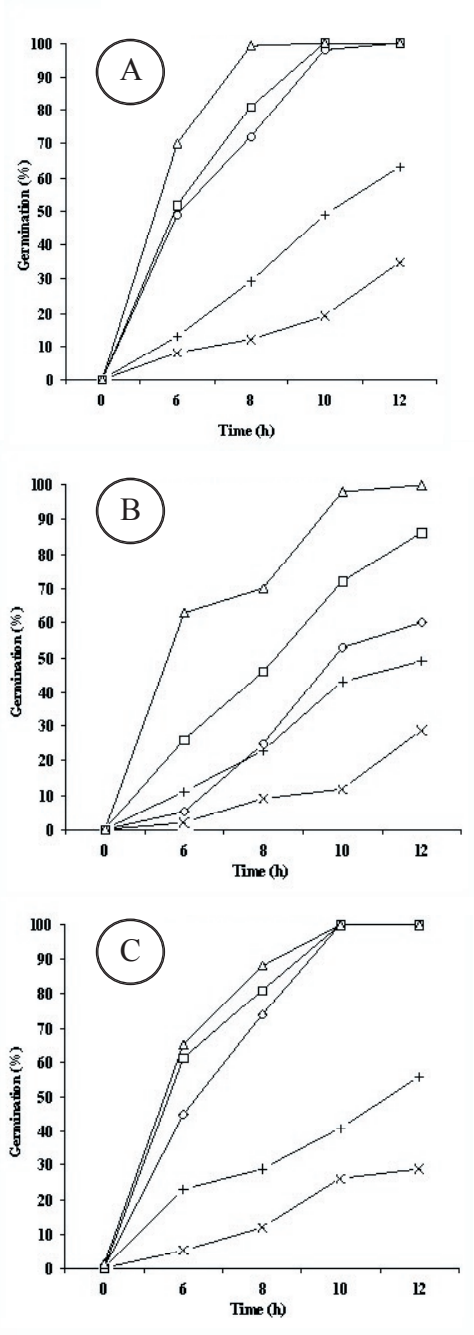

Fig. 2. Influence of substrate initial moisture (%) on spore germination of *Aspergillus niger* (A), *Myceliophthora thermophila* (B), and *Rhizopus microsporus* (C) cultivated on sugar cane bagasse and wheat bran (80:20 w/w). Incubation temperatures: 30 C for *A. niger*, and 45 C for *M. thermophila* and *R. microsporus*. Moisture: **x** = 45%; **+** = 55%; ◇ = 65%; □ = 75%; △ = 85%.

Influence of forced aeration and humidity on spore germination. All three species germinated without forced aeration (**Table 2**). However, when ambient air (46% relative humidity) was forced through SSF cultures, higher germination rates were observed in all species. Moreover, when the air was humidified (98% relative humidity), germination rates were highest. After 4 h of incubation without aeration, less than 10% of spores germinated. When cultures were not aerated, all species needed 11 h of incubation in order to achieve complete germination (100%). If forced air was humidified, time required for germination rates ranging from 96-100% in *Aspergillus niger*, *Myceliophthora thermophila*, and *Rhizopus microsporus* was reduced to 8 h of incubation, as compared to non-aeration which took 11 h. In fact, aeration significantly affected the germination rate of all species, at each time of sampling (Pr > F 10^{-4}).

Similar results were obtained by Prenerová[15] who demonstrated that aeration increased and accelerated spore germination in *Paecilomyces farinosus*. Han *et al*.[11] also showed that the optimal growth temperatures and relative humidity for *Actinomucor elegans* and *Rhizopus oligosporus* were 25 C at 95–97%, and 35 C at 95–97%, respectively.

In conclusion, spore germination in *A. niger*, *M. thermophila*, and *R. microsporus* cultivated in solid state fermentation was affected by the substrate, incubation temperature, and initial moisture. The initial moisture and water availability were main factors influencing germination rate. Lower availability of free water during germination resulted in a low reactivation of metabolism even at optimal temperature. These two parameters markedly determined germination rate. Forced humid aeration

Table 2. Influence of aeration and humidity on spore germination (%) of *Aspergillus niger*, *Myceliophthora thermophila*, and *Rhizopus microsporus* cultivated on sugar cane bagasse and wheat bran (80:20 w/w). Incubation temperatures: 30 C for *A. niger*, and 45 C for *M. thermophila* and *R. microsporus*.

Time of incubation (h)	*A. niger*			*M. thermophila*			*R. microsporus*		
	Forced aeration		Without forced aeration	Forced aeration		Without forced aeration	Forced aeration		Without forced aeration
	Humid air	Ambient air		Humid air	Ambient air		Humid air	Ambient air	
0	0	0	0	0	0	0	0	0	0
4	36	29	2	39	18	6	67	23	8
6	79	53	31	68	39	29	96	62	38
8	96	86	52	98	67	49	100	89	59
10	100	92	83	100	83	79	100	100	73
11	100	100	100	100	100	100	100	100	100

Data were significantly different between species and time (F= 44.35); air and time (F= 206.18); and species, air and time (F= 36.65). Pr > F 0.0001.

increased germination rates and decreased the time required for complete spore germination on sugar cane bagasse and wheat bran. During the spore germination phase, forced aeration could be a valuable tool for further industrial applications.

ACKNOWLEDGEMENTS

Hicham Hassouni is grateful to IRD for financing and supporting this work. Authors thank M. Cheheb for excellent technical assistance.

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