RELATIONSHIP BETWEEN INITIAL WATER CONTENT OF THE SUBSTRATE AND MYCELIAL GROWTH AND SPORULATION OF THE NEMATOPHAGOUS FUNGI, *PAECILOMYCES LILACINUS* AND *POCHONIA CHLAMYDOSPORIA*

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Summary. Experiments were undertaken to relate the effects of three rates of initial water content (1.0:1.0; 1.5:1.0; and 2.0:1.0) in a solid substrate (wheat bran) with mycelial growth and conidia production of the nematophagous fungi *Paecilomyces lilacinus* PDBC PL55 and *Pochonia chlamydosporia* PDBC PC57, and to relate the effects to substrate characters. Increase in water content of the substrate resulted in increased moisture content and saturation in terms of water activity. A negative relationship was observed between the proportion of initial water content in wheat bran and spore yield of *P. lilacinus* PDBC PL55 and *P. chlamydosporia* PDBC PC57. The increase in water content of the substrate also increased the duration of mycelial growth, time to completion of sporulation and total time for one cycle of production. Increased moisture content of the wheat bran resulted in a higher final moisture level in the substrate (74-76%), which in turn resulted in a longer time (8-9 days) required to dry the substrate to 15-18%, compaction and bacterial contamination. The optimum proportion of water to be added initially to wheat bran for conidial production by *P. chlamydosporia* and *P. lilacinus* was 1:1(v/w).

Key words: Mass production, moisture content, solid-state production, water activity.

Among the most widely researched nematophagous fungi, *Paecilomyces lilacinus* (Thom) Samson and *Pochonia chlamydosporia* Zare, Gams *et* Evans (ex- *Verticillium chlamydosporium*) have reached the status of potential nematode biocontrol agents and commercialization (Stirling *et al*., 1998). Paradoxically, the protocols for their production, post-production processing, formulations and enhanced shelf-life have yet to be finalised. For the development of ideal amorphous formulations, aerial conidia produced on solid substrates were observed to be robust and to have high viability (Nagesh *et al.*, 2003).

Solid-state fermentation involves the growth of microorganisms on moist solid substrates in the absence of free-flowing water. In solid-state fermentation, the water content is quite low and the microorganism is almost in contact with gaseous oxygen in the air (in contrast to liquid fermentation) for its growth and sporulation. Various workers have used solid-state fermentation techniques for mass production of spores needed in the transformation of organic compounds (Singh *et al.*, 1967, 1968; Vezina *et al.*, 1968; Sansing and Ciegler, 1973). Vezina and Singh (1975) reported that the solid-state fermentation technique for production of fungal spores had advantages over submerged culture methods because the former was simple and gave higher yields of homogenous and pure spores. Linderfelser and Ciegler (1975) reported that the initial moisture level was the most critical factor in solid-state fermentation. Nevertheless, there is hardly any information on the proportion of water to be added to the solid substrates for optimized production of conidiospores of the fungi. Excess moisture in the media is known to often cause compaction, limiting the air space and surface area of substrate available for reproduction of the organism under culture (Moo-Young, *et al*., 1983), and a problem that is compounded during scale-up.

In this investigation, three levels of initial water content in wheat bran were examined to find the optimal water content : quantity of substrate (wheat bran) combinations for optimized spore production and short production cycles for *Paecilomyces lilacinus* and *Pochonia chlamydosporia.*

MATERIALS AND METHODS

Fungal isolates and maintenance. The isolates of *Paecilomyces lilacinus*, PDBC PL55 (Nagesh *et al.,* 2006) and *Pochonia chlamydosporia* PDBC PC57 (Nagesh *et al*., 2007), are maintained at the Project Directorate of Biological Control, Bangalore, India, at -20 °C in glycerol. They were thawed, streaked onto PDA and incubated at 28 °C for 5 days to restore and stabilize the fungi prior to experimentation.

Solid substrate for conidia production. As wheat bran is the commonest substrate used in solid-state fermentation (SSF) for conidial production in most laboratories,

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we also used it as substrate for mass production of both fungal isolates in our experiments but with three different initial water contents. Wheat bran was obtained from a local whole grain and bran market.

The three terms frequently used throughout the text are water content (WC), moisture content (MC) and water activity (a_w) and they are defined as follow: Water content refers to the volume of water added to 100 g of a solid substrate and expressed as a percentage. To obtain a substrate of 1% water content, 1 ml of sterile distilled water would be added to 100 g of wheat bran and mixed thoroughly. Volumes of water per 100 g substrate necessary to obtain the desired water contents were added and the substrates were left to soak for 30 minutes in closed plastic containers after the water was added.

Moisture content refers to the weight loss or difference in weight of the substrate after drying to a constant weight divided by the weight of the dry substrate and multiplied by 100 to express it as a percentage.

Water activity is the relative availability of water in a substance. It is defined as the vapour pressure of water in the substrate divided by that of pure water at the same temperature. Water activity was measured by using a HygroPalm AW1 (ROTRONIC AG, Bassersdorf) and calculated according to the formula

$$a_w = p / p_0$$

where, p is the vapour pressure of water in the substance, and p_0 is the vapour pressure of pure water at the same temperature.

The initial water contents (WC) examined were 1:1; 1.5:1 and 2:1 (volume of water in ml : 100 g wheat bran). The MC and a_w of the wheat bran prior to the addition of water were recorded as explained above.

Inoculation of P. lilacinus *and* P. chlamydosporia *to the substrates*. Thirty g of each of the substrates of different initial water contents were spread out in plates, autoclaved (at 120 °C and 15 PSI for 1 hour), inoculated with aqueous spore suspension of *P. lilacinus* and *P. chlamydosporia*, and incubated at 28 °C in specially designed containers (Patent in process in India, Reg. No. 417/CHE/2006).

A method was devised to spread the fungal inoculum onto the solid substrates without altering the moisture content of the autoclaved wheat bran. The fungi were freshly cultured on autoclaved PDA, incubated at 28 °C and, after complete sporulation, used to prepare conidiospore suspensions for inoculation. Conidiospores of *P. lilacinus* PDBC PL55 and *P. chlamydosporia* PDBC PC57 were harvested individually by carefully scraping the surface of the PDA medium, transferred to 100-ml capacity Erlenmeyer flasks containing 25 ml of sterile 2% glucose solution with 0.2% sodium dodecyl sulphate (SDS), and vortexing vigorously for 30 minutes to make near-homogeneous spore suspensions. The spore suspensions were then transferred into autoclaved plastic (kitchen garden) sprayers of 250 ml capacity under laminar flow. A suitable atomizing spray nozzle was used to dispense fungal spores in a minimum quantity of moisture (about 50 µl of spray suspension per plate containing 30 g of substrate) uniformly on the surface of the substrate. To ensure uniform distribution of the inoculum, the substrate was mixed using a sterile spatula, and sterile gloves were worn throughout the process of inoculation to prevent cross-contamination.

The inoculated substrates were examined under a laminar flow hood at 24-hour intervals, starting five days after inoculation for a period of ten days, to record the duration of sporulation from initiation to completion. Completion of sporulation was indicated by a complete colour change of the mycelial mat, to lilac in the case of *P. lilacinus* and yellowish (creamy) white in the case of *P. chlamydosporia*. The spore-laden wheat bran substrates were then carefully removed from their plates in a laminar flow hood, wrapped in saran in which small holes were made with a fine sterile needle, and dried for four days in a hood with an exhaust fan on. Cotton bags containing 50 g each of dry calcium carbide and silica were placed in the hood to help absorb moisture. The holes in the saran wrap facilitated aeration and drying. Fan-drying of substrates in ambient conditions was continued until individual spore-laden substrates reached a moisture content of 8.0 to 10.0% (monitored using Infrared Moisture Analyzer Sartorius MA 100C).

To record spore production per g substrate, one g of dried substrate was harvested per treatment and mixed thoroughly with 10 ml of sterile distilled water (2% SDS). Serial dilution in sterile distilled water allowed 100 µl of aqueous spore suspension at 10^{-3} dilution to be plated onto PDA. The numbers of individual colonies that developed were counted and multiplied by the dilution factor and the volume of the initial spore suspension (10 ml) to calculate the number of colonies per g for each substrate of different moisture level. Spore production per g substrate at different moisture levels was cross-checked by counting the spores in aqueous spore suspensions (at serial dilution of 10^{-3}) using a Neubauer haemocytometer. Three replications were prepared per treatment and the mean values were log transformed. The data were subjected to analysis of variance and the means compared by Duncan's multiple range test.

RESULTS

Data on the effect of initial moisture content on duration of mycelial growth, time for completion of sporulation and spore yield of *P. lilacinus* PDBC PL55 and *P. chlamydosporia* PDBC PC57 are presented in Table I. With the increase in initial water content from 1:1 to 2:1, the time taken for mycelial growth increased from 5 ± 1 to 10 ± 2 days for *P. lilacinus*, and from 8 ± 1 to 11 ± 2 days for *P. chlamydosporia*. Similarly, the time taken

for apparent completion of sporulation increased from 6 ± 1 days to 15 ± 1 days for *P. lilacinus*, and from 7 ± 1 days to 14 ± 2 days for *P. chlamydosporia,* respectively. The total time required for one growth cycle, i.e., completion of mycelial growth and sporulation, at 2:1 water content was approximately twice that at 1:1 for both fungi (Table I).

Log values of spore yield per g of wheat bran for both fungi were greatest at a moisture content of 1:1 and decreased significantly with the increase in water content of the wheat bran (Table I). Spore production at an initial moisture content of 2:1 was less than half that at 1:1 for both *P. lilacinus* and *P. chlamydosporia*.

Moisture content of the substrate recorded before autoclaving increased by approximately 60-72% as WC increased from 1:1 to 2:1 (Table II). After the completion of sporulation of the nematophagous fungi, the moisture content of wheat bran ranged between 44 and 48% at 1:1 initial water content, while the water activity of the substrate remained saturated (a_w = 1.00). With increasing water content of the wheat bran (to 1.5:1 and 2:1), the MC was very high (56-58% and 74-76%, respectively) at the end of the process.

Initial water content of the wheat bran had a direct effect on the time required to reduce moisture content of the substrate to the optimal level of 15-18% (Table II). Wheat bran with initial water content of 1:1 had required 3-4 days of drying, while bran at 1.5:1 and 2:1 initial water content required 5-7 and 8-9 days, respectively, of drying. Initial water contents of wheat bran of 1.5:1 and 2:1 resulted in very high compaction of the substrate after the sporulation of the fungi (Table II), with the substrate showing sogginess, wet patches and bacterial contamination.

Table I. Effect of initial water content (WC) of wheat bran on growth and sporulation of mycelia of *Paecilomyces lilacinus* PDBC PL55 (PL) and *Pochonia chlamydosporia* PDBC PC57 (PC) at 28 °C.

Water content (ml/g)[1]	Duration of mycelial growth (days)		Time for sporulation (days)		Number of days per production cycle		Log value of conidiospores/g substrate	
	PL	PC	PL	PC	PL	PC	PL	PC
1.0:1.0	5±1	8±1	6±1	7±1	11±1	15±2	9.8921 c	8.5344 c
1.5:1.0	7±1	10±2	10±2	11±1	17±3	21±2	6.7243 b	6.6812 b
2.0:1.0	10±2	11±2	15±1	14±2	25±2	25±3	4.5315 a	4.2142 a
F Test							S	S
SEM ±	-	-	-	-	-	-	0.0165	0.0757
CD (P = 0.05)	-	-	-	-	-	-	0.0498	0.2312

S = Significant at P = 0.05.
Values are means of three replicates. Means followed by the same letter within a column are not significantly different according to modified Duncan's multiple range test.
[1]Volume of water/g substrate.

Table II. Effect of initial water content (WC) of wheat bran on moisture content (MC) at completion of sporulation of mycelia of *Paecilomyces lilacinus* PDBC PL55 and *Pochonia chlamydosporia* PDBC PC57 at 28 °C, and number of days required for drying the substrate to 15-18% MC.

Water content (ml/g)[1]	MC of wheat bran (%)		Water activity (a_w)		Number of days for drying to 15-18%	Compaction		Remarks
	Before autoclaving	End of sporulation	Before autoclaving	End of sporulation		PC	PL	
1.0:1.0	50-52	44-48	1.00	1.00	3-4	Low	Low	No contamination No sogginess
1.5:1.0	68-72	56-58	1.00	1.00	5-7	Medium	Medium	Substrate was wet even after sporulation
2.0:1.0	80-86	74-76	1.00	1.00	8-9	Very high	Very high	High bacterial contamination

[1]Volume of water/g substrate.

DISCUSSION

Linderfelser and Ciegler (1975) and Gonzalez *et al.* (1988), investigating solid-state fermentation of *Aspergillus ochraceus,* demonstrated that of all fermentation conditions the initial moisture content was among the most critical and had a two-fold benefit. First, the initial water content gave the water activity that was required for fungal growth, and second, it caused swelling of the substrate thereby making for better utilization of space and nutrition. The initial amount of water added to solid substrates such as wheat bran greatly influences the production of aerial conidia. Murthy *et al.* (1993) and Gervais and Molin (2003) extensively reviewed the role of water in solid-state fermentation and found that the dynamics of water content, expressed in terms of water activity, were critical at different stages of production. In our study, at a 1:1 proportion of water, water activity reached saturation before autoclaving and continued at this level until completion of sporulation on wheat bran. A higher initial water content (1.5:1 and above) provided a very high MC (>80% and saturated water activity), which adversely affected spore production, duration of the cycle of conidial production, drying of media, and consistency of the substrate, and also led to bacterial contamination. Higher water content in the substrate also increased the time taken for mycelial growth, sporulation and therefore, in turn, the number of days per production cycle. Beuchat (1981) reported that the optimal water activity for fungal growth was about 0.7, for yeast about 0.8 and for bacteria about 0.9. In our work, an initial proportion of water of 1:1 (v/w) to wheat bran was best for production of conidia of *P. lilacinus* PDBC PL55 and *P. chlamydosporia* PDBC PC57 at the given set of conditions on wheat bran. This water proportion reduced the number of days required for sporulation and days per production cycle of the fungi, reduced the number of days required to lower the moisture content of the substrate to the optimal of 15-18%, and resulted in production of the largest number of conidiospores per g of substrate.

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LITERATURE CITED

Beuchat L.R., 1981. Microbial stability as affected by water activity. *Cereal Foods World*, *26*: 345-349.

Gervais P. and Molin P., 2003. The role of water in solid-state fermentation. *Biochemical Engineering Journal, 13*: 85-101.

Gonzalez J.B., Tomasini A., Gonzalez G.V. and Lopez J., 1988. Penicillin production by solid state fermentation. *Biotechnology Letters, 10*: 793-798.

Linderfelser L.A. and Ciegler A., 1975. Solid-substrate fermentor for ochratoxin A production. *Applied Microbiology*, *29*: 323-327.

Moo-Young M., Moriera A.R. and Tengerdy R.P., 1983. Principles of solid state fermentation. Pp. 117-144. *In*: The Filamentous Fungi, Vol.4, Fungal Biotechnology (Smith J.E, Berry D.R. and Kristianser B., eds). Edward Arnold Publishers, London, UK.

Murthy M.V.R., Karanth N.G. and Rao K.S.M.S.R., 1993. Biochemical engineering aspects of solid-state fermentation. *Advances in Applied Microbiology, 38*: 99-147.

Nagesh M., Hussaini S.S., Singh S.P. and Biswas S.R., 2003. Management of root-knot nematodes *Meloidogyne incognita* (Kofoid & White) Chitwood in Chrysanthemum using *Paecilomyces lilacinus* (Thoms) Samson in combination with neem cake. *Journal of Biological Control, 17*: 125-132.

Nagesh M., Hussaini S.S., Ramanujam B. and Rangeswaran R., 2006. Management of *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *lycopersici* wilt complex using antagonistic fungi in tomato. *Nematologia Mediterranea*, *34*: 63-68.

Nagesh M., Hussaini S.S., Ramanujam B. and Rangeswaran R., 2007. Molecular identification, characterization, variability and infectivity of Indian isolates of the nematophagous fungus, *Pochonia chlamydosporia. Nematologia Mediterranea, 35*: 47-56.

Patent No.417/CHE/2006. A simple and novel design for small-scale solid-state mass production unit for antagonistic fungi. Nagesh M., Hussaini S.S., Rabindra R.J., Vidya H.S. and Chidanandaswamy B.S., Project Directorate of Biological Control, P.B. No.2491, H.A. Farm Post, Bellary Road, Bangalore 560024, Karnataka, India. (Applied for Indian patent).

Sansing G.A. and Ciegler A., 1973. Mass propagation of conidia from several A*spergillus* and *Penicillium* species. *Applied Environmental Microbiology*, *26*: 830-831.

Singh K., Sehgal S.N. and Vezina C., 1967. Transformation of steroids by *Mucor griseo-cyanus*. *Canadian Journal of Microbiology*, *13*: 1271-81.

Singh K., Sehgal S.N. and Vezina C., 1968. Large-scale transformation of steroids by fungal spores. *Applied Environmental Microbiology*, *16*: 393-400.

Stirling G.R., Licastro K.A., West L.M. and Smith L.J., 1998. Development of commercially acceptable formulations of the nematophagous fungus, *Verticillium chlamydosporium. Biological Control*, *11*: 217-223.

Vezina, C., Sehgal, S.N. and Singh, K. 1968. Transformation of organic compounds by fungal spores. *Advances in Applied Microbiology, 10*: 221-268.

Vezina C. and Singh K., 1975. Transformation of organic compounds by fungal spores. Pp. 158-192. *In*: The Filamentous Fungi, Vol. I, (Smith J.E. and Berry D.R., eds.). Edward Arnold Publishers, London, UK.