Induction and mass sporulation in lignin degrading fungus *Ceriporiopsis subvermispora* for its potential usage in pulp and paper industry

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Induction of sporulation was successfully achieved in otherwise non-sporulating fungus *Ceriporiopsis subvermispora*, a potential lignin degrader with application in biopulping. Manipulation of different culture parameters revealed the role of both nutritional as well as osmotic stress in sporulation. The spores produced were chlamydospores. Among the different stress media tried, D-glucose calcium chloride medium at pH 3 resulted in maximum chlamydospore production (308×10^4 spores/ml) within 60 h under static culture conditions. The spores could be easily separated from the mycelium by enzymatic treatment. The spores colonized wood chips and were found to be viable for 6 months at room temperature after packaging in a suitable carrier.

CERIPORIOPSIS subvermispora, a white rot basidiomycetous fungus has gained a lot of importance in the past few years for its use in biopulping and biobleaching for paper-making[1–4]. The organism is very selective for lignin degradation with very low cellulolytic activity compared to *Phanerochaete chrysosporium*[3,5,6]. The bottleneck for commercial exploitation of *C. subvermispora* is its non-sporulating nature. This creates a lot of problem in preparation of mass inoculum of the fungus for large-scale inoculations and for its transfer to distantly-located paper industries. Here, we report our results on induction of large amounts of spores in *C. subvermispora* which could potentially be used as an inoculum for pulp and paper industry.

C. subvermispora was obtained from the Department of Agriculture, University of Wisconsin, Madison, USA. The organism was grown for 5 days at 25°C on potato dextrose agar slants and subsequently maintained at 4°C in a BOD incubator.

The growth pattern of *C. subvermispora* was studied and compared in different liquid broths, viz. potato dextrose, Czapek Dox complete, minimal and various stress media. Stress media were prepared by modification of the medium D-glucose soluble starch[7]. Starch in the above medium was replaced with salts, sugar alcohols and inert compounds, viz. $CaCl_2 \cdot 2H_2O$, NaCl, Na_2SO_4, mannitol, glycerol and PEG-6000. pH of the medium was adjusted to 6.2 ± 0.2 using 1 N NaOH or 1 N HCl. The fungus was grown in 50 ml of liquid broth in 250 ml Erlenmeyer flasks. The flasks were incubated at 25°C. The growth and the morphology of the fungus were observed under Nikon compound microscope (40 ×). The selected best stress medium D-glucose $CaCl_2$ (D-GCC), was further optimized for enhancing sporulation in *C. subvermispora* by studying the effect of various physico-chemical factors on sporulation. These were: (i) effect of varying concentrations of $CaCl_2.2H_2O$ in D-GCC medium; (ii) effect of incubation time; (iii) effect of temperature; and (iv) effect of pH.

To separate chlamydospores from the mycelium, *C. subvermispora* culture (72 h-old) containing maximum number of spores was subjected to chitinase treatment. This enzyme was prepared by growing chitinase-producing strain of *Streptomyces aureofaciens* on fermentation waste biomass of *Aspergillus terreus*. The fungal lysate was assayed for chitinase by the DNSA method[8]. One unit of chitinase activity is the amount of enzyme that catalyses the release of 1 µmol of N-acetyl glucosamine per ml in 60 min. One gram of the mycelium was treated with different amounts of the fungal lysate (0.022 U/ml) and the mixture was incubated at 37°C for 10–12 h. Microscopic observations were recorded every hour in order to study the extent of mycelial degradation and spore separation.

The separated spores were analysed for their potential to infect and colonize bamboo wood chips by inoculating them with spore suspension and incubating at 25°C. Microscopic observations were made after 24, 48, 72 and 96 h. The extent of colonization of the wood chips was further determined by scanning electron microscopy.

Two different carrier media, silica gel and activated charcoal were used for spore preservation. Ten gram of each carrier was heat-sterilized at 80°C for 48 h. Fifteen ml of the spore suspension was mixed with the respective carriers and left at room temperature. The viability of chlamydospores was checked periodically for 6 months by inoculating the carrier onto the PDA plates.

The growth pattern of *C. subvermispora* was studied by inoculating the fungus in minimal, complete and potato dextrose media for 360 h. The results clearly showed that in potato dextrose and complete media, predominantly vegetative mycelium was formed (Figure 1 *a* and *b*). However, in minimal medium by 72 h, there was formation of terminal and intercalary spores (75×10^4 spores/ml, Figure 1 *c*). This is due to the fact that in minimal medium nutritional stress is achieved much earlier, leading to sporulation. Similar reports on sporulation in fungi have been reported[9,10]. Microscopic observations showed that the spores were large, about 18–20 µm in size, double-walled and contained dense cytoplasmic material (Figure 1 *f* and *g*). The spores were both terminal and

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intercalary and were formed throughout the length of the hyphae, clearly showing that it was the mycelium itself that had transformed into these large and thick-walled structures, which are referred to as chlamydospores.

In order to enhance sporulation, *C. subvermispora* was grown in different stress media. Results show that maximum sporulation was observed in D-GCC medium (Figure 1 *e*), followed by D-glucose mannitol (D-GM) medium (Figure 1 *d*, Table 1). Maximum sporulation was achieved in 60 h in these stress media in comparison to minimal medium, where it took nearly 120 h. Further, microcycle conidiation was observed, which significantly enhanced the extent of sporulation (Figure 1 *h*). Thus, it could be inferred that both salt and sugar alcohol caused requisite osmotic stress for induction of sporulation in *C. subvermispora*.

Studies on the effect of different concentrations of $CaCl_2 \cdot 2H_2O$ on chlamydospore formation in D-GCC medium showed that a concentration of 1% $CaCl_2 \cdot 2H_2O$ was optimal to achieve maximum sporulation (151×10^4 spores/ml). This must be most probably due to the fact that maximum stress is perceived by the fungus at this concentration.

An incubation period of 60 h has been found to be optimum to achieve maximum number of chlamydospores (151×10^4 spores/ml) of this fungus under these stress conditions. After this time period, there was no appreciable increase in their number.

The effect of pH on sporulation was examined in the range 2–10 both in D-GCC and D-GM media, because in D-GCC only pH range 2.0–7.0 could be studied due to precipitation of calcium at a higher pH. It was observed that a combination of pH and nutrient stress drastically

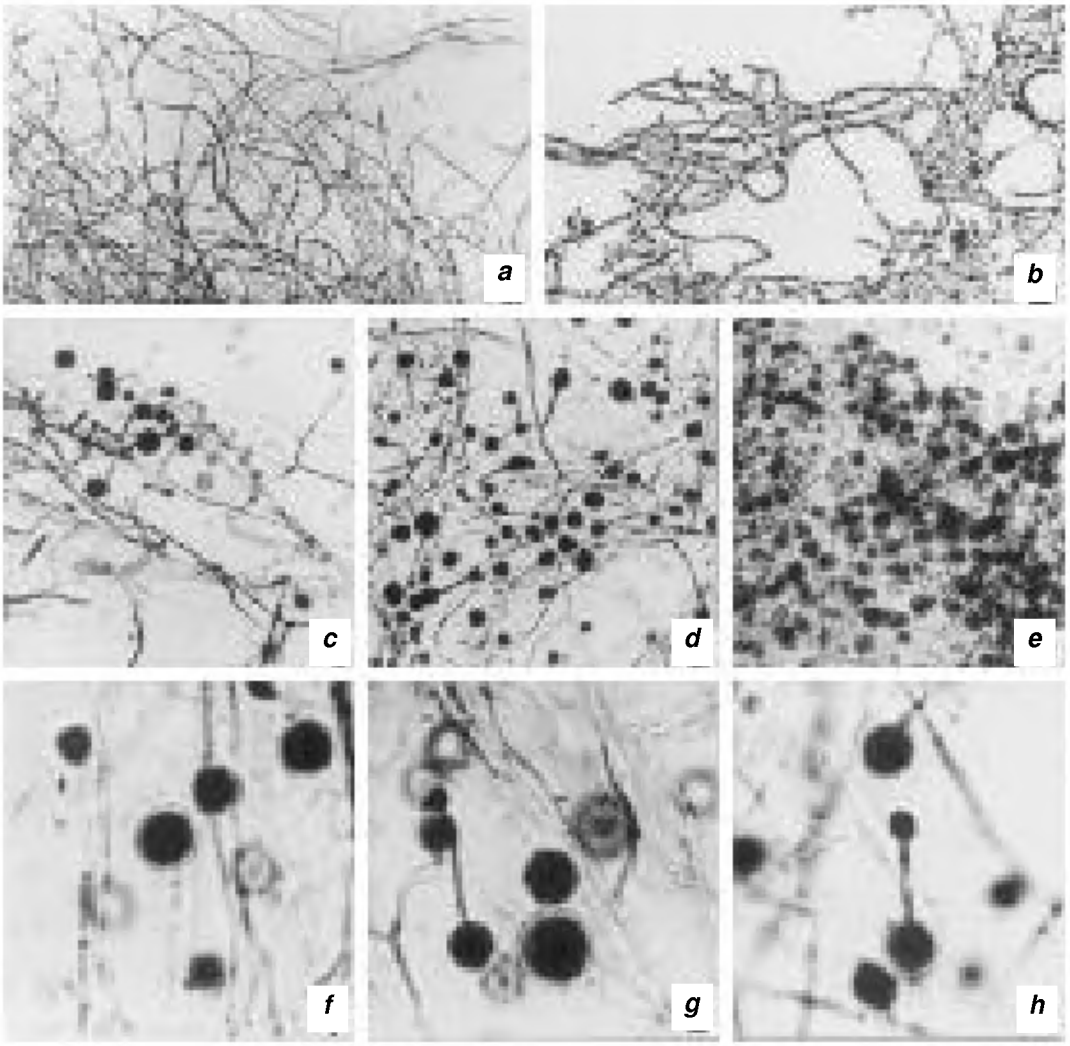

Figure 1. Growth of *C. subvermispora* as observed through light microscope (40 ×) on (***a***) potato dextrose agar, and (***b***) complete medium and formation of chlamydospores in different stress media: (***c***) D-glucose calcium chloride, (***d***) D-glucose mannitol, (***e***) D-glucose polyethylene glycol, after 72 h at 25 ± 1°C under static conditions. Morphology of chlamydospores shows double-walled spherical structures having dense cytoplasmic content (***f*** and ***g***) and microcycle conidiation (***h***).

increased the extent of chlamydospore formation. Acidic pH in the range 3.0–4.0 in both D-GCC and D-GM media led to production of a large number (308 and 278 × 10^4 spores/ml, respectively) of thick-walled, terminal and intercalary chlamydospores (Table 2).

As the chlamydospores produced are both terminal and intercalary, their separation from the mycelium is necessary in order to facilitate their easy storage and packaging. Amongst the different treatments used to separate the mycelium and the spores, it was observed that by freezing the mycelium or subjecting it to high temperatures, there was no significant mycelial killing and thus separation of the spores. It is established that the enzyme chitinase can lyse the fungal cell wall made of chitin[11,12]. The microscopic observations revealed the fragmentation of mycelium and its lysis due to enzyme action. Maximum separation was obtained with 1 : 20 ratio after 3 h (Figure 2). The whole process is thus economical as this result could be obtained with crude enzyme only and the enzyme is produced using fermentative waste biomass. The viability of the spores was determined by inoculation in minimal, complete and potato dextrose agar plates, where the spores germinated after 6 h at 25°C and subsequently formed the mycelium.

Table 1. Effect of different stress media on sporulation under static conditions at 25°C

Salt/sugar/inert compound (%)	Concentration (%)	Average number of spores (× 10^4) per ml
Soluble starch (control*)	0.7	109
$CaCl_2 \cdot 2H_2O$	1.0	151
NaCl	0.2	25
Na_2SO_4	0.9	24
Mannitol	4.0	115
Glycerol	10.0	0
PEG-6000	20.0	104

*Control: D-glucose soluble starch medium. Soluble starch was replaced with other components, i.e. salt, sugar alcohol or inert compound to prepare different stress media.

Table 2. Effect of pH on sporulation in D-GCC and D-GM media at 25°C under static conditions after 72 h

	Average number of spores (× 10^4) per ml	
pH	D-GCC medium	D-GM medium
2.0	79	52
3.0	308	278
4.0	320	282
5.0	172	120
6.0	151	115
7.0	138	103
8.0	–	–
9.0	–	54
10.0	–	27

However, the importance of these spores lies in their ability to colonize wood chips. To determine this, the chlamydospores free of the mycelium were inoculated on pretreated wood chips. Germ-tube formation was observed within 6–9 h followed by mycelial formation, as observed under light microscope (Figure 3 *a* and *b*). Further, confirmation was achieved by scanning electron microscopy (Figure 3 *c*). The scanning electron micrographs showed the emergence of infection threads from the inner walls of the spores. The surface of the wood chips was colonized by the fungal mycelium emerging from the chlamydospore.

Two inert carrier media were examined for storage of the chlamydospores, viz. silica gel and charcoal. Results showed that the preserved spores along with the carrier could efficiently grow on nutritional medium and could colonize wood chips under appropriate growth conditions within 4–6 h.

The findings of the present investigation are of immense importance as *C. subvermispora* could be successfully induced to sporulate. The chlamydospores formed could even be separated from the mycelium.

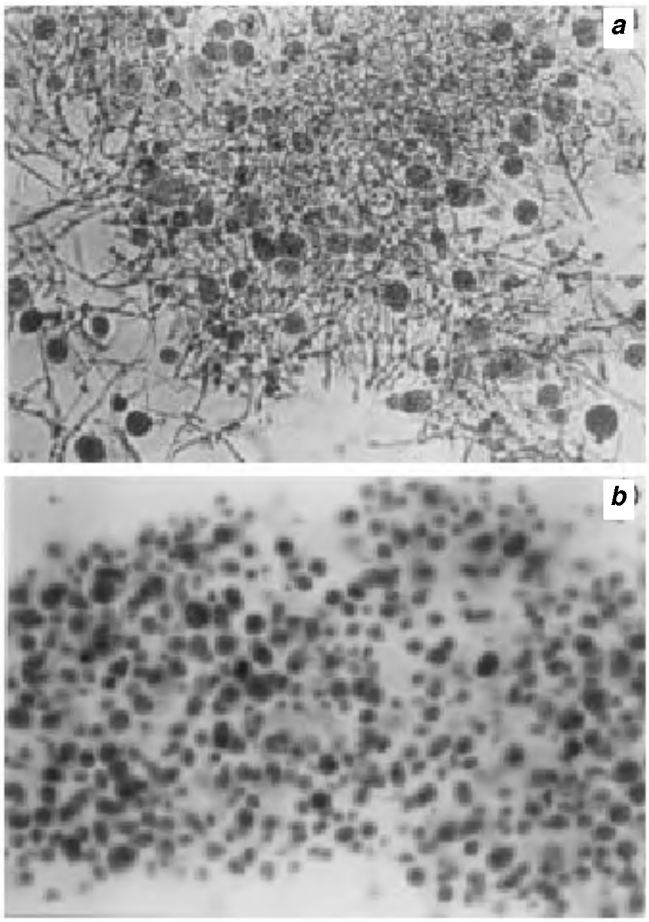

Figure 2. Separation of chlamydospores from the mycelium by enzymatic treatment (fungal lysate from *Streptomyces aureofaciens*). (*a*) Mycelium with chlamydospores prior to treatment; (*b*) Separated chlamydospores after treatment.

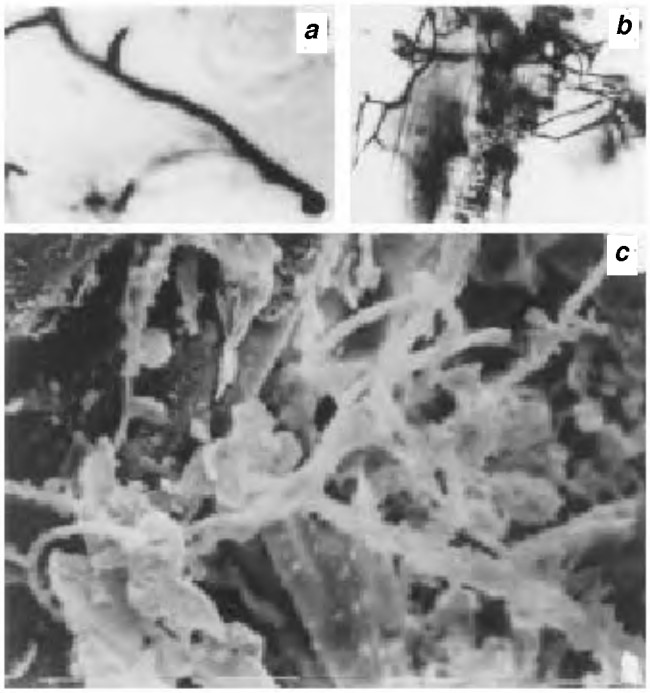

Figure 3. Growth and germination of chlamydospores of *C. subvermispora* on wood chips after 48 h incubation at 25°C. (*a*) Germ-tube formation from the chlamydospore and (*b*) mycelium formation and colonization as observed under light microscope (40 ×). (*c*) Chlamydospore germination and mycelium formation as observed under scanning electron microscope (5000 ×).

These spores could efficiently colonize wood chips and other agro-wastes which are used for paper manufacture. Thus, there is immense practical feasibility of the procedure for the production of chlamydospores in this fungus. First, their mass production is essential for the preparation of starter cultures for biopulping and biobleaching. Secondly, they can be transported in large quantities, remain viable for a long period of time and can be handled easily. Finally, since the spores formed are thick-walled, they have a prolonged shelf-life and are resistant to contamination.

1. Eriksson, K. E. and Kirk, T. K., *Comprehensive Biotechnology* (ed. Murray Moo-Young), Pergamon Press, 1985, vol. 3, pp. 271–294.
2. Blanchette, R. A., Burnes, T. A., Leatham, G. F. and Effland, M. J., *Biomass*, 1988, **15**, 93–101.
3. Blanchette, R. A., Burnes, T. A., Eerdmans, M. M. and Akhtar, M., *Holzforschung*, 1991, **46**, 109–115.
4. Akhtar, M., *Holzforschung*, 1994, **48**, 199–202.
5. Akhtar, M., Attridge, M. C. and Blanchette, R. A., *Biotechnology in the Pulp and Paper Industry* (eds Kuwahara, M. and Shimada, M.), UNI Publishers Co. Ltd, Tokyo, 1992, pp. 545–560.
6. Ruttimann, C., Salas, L., Vicuna, R. and Kirk, T. K., *Appl. Environ. Microbiol.*, 1993, **59**, 1792–1797.
7. Saxena, R. K., Khurana, N., Kuhad, R. C. and Gupta, R., *Mycol. Res.*, 1992, **96**, 490–494.
8. Monreal, J. and Reese, E., *Can. J. Microbiol.*, 1969, **15**, 689–696.
9. Saxena, R. K., Ph D thesis, University of Delhi, 1976.
10. Turian, G., *Annu. Rev. Phytopathol.*, 1974, **12**, 129–137.
11. Ordentlich, A., Elad, Y. and Chet, I., *Phytopathology*, 1988, **78**, 84–87.
12. Gupta, R., Saxena, R. K., Chaturvedi, P. and Virdi, J. S., *J. Appl. Bacteriol.*, 1995, **78**, 378–383.

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Late Quaternary changes in sea level and sedimentation rate along the SW coast of India: Evidence from radiocarbon dates

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We determined the ^{14}C dates of carbonized wood/peat and organic matter from two cores from the southwestern inner continental shelf of India, to understand late Quaternary changes in sea level and sedimentation rate. The ^{14}C ages of carbonized wood/peat samples from the two cores range from 10,760 ± 130 to 9,280 ± 150 yr BP, corresponding to Late Pleistocene–Early Holocene. These ages suggest that the sea level was much lower (> 50 m) than the present level during the Late Pleistocene–Early Holocene, when there was a luxuriant growth of mangrove vegetation and rainfall in the region. Later, due to transgression during Early Holocene, the mangrove vegetation was submerged and buried under a thick column of sediment, giving rise to carbonized wood/peat. The ^{14}C ages reported for the present-day onshore peat and shell material at various depths along the SW coast of India reveal that after 6400 yr BP, the sea level receded and stabilized at the present level. The results indicate that: (1) the present-day sedimentation rates in the study area are generally comparable to those reported for similar water depths off the Karnataka coast, but higher than that reported for the Kerala coast; (2) sedimentation rate at a site is controlled by its proximity to river mouths and it decreases offshore; and (3) sedimentation rate during the Pleistocene–Holocene transition period is 6–7 times higher compared to the Holocene.

SEDIMENTS of the western continental shelf of India may be categorized as: (1) modern clayey silt and silty clay

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