

## Economically viable mass production of lignocellulolytic fungal inoculum for rapid degradation of agrowaste

Livleen Shukla\*, Ansuman Senapati, S. P. Tyagi and Anil Kumar Saxena

Division of Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India

**A consortium of four hypercellulolytic fungal cultures, namely *Aspergillus awamori*, *Trichoderma viride*, *Aspergillus nidulans* and *Phanerochaete chrysosporium* was optimized for compost production on the basis of their lignocellulolytic enzyme production potential. The consortium has been effectively used for composting of diverse agricultural wastes such as paddy straw, soybean trash, pearl millet, maize residues and mustard stover. The inoculum of four fungi needs to be produced in bulk for talc-based consortium formulation. Routinely fungus is grown on potato dextrose agar medium, malt extract agar medium or Martin's Rose Bengal agar medium, which escalates the cost of production because of relatively expensive ingredients. Further, harvesting of fungal biomass from solid surface adds additional cost to the production schedule.**

**Keywords:** Compost production, fungal mats, jaggery, lignocellulolytic enzyme, spores.

THE foodgrain production in India has touched a landmark figure of 250.47 mt in 2011–12 (Economic Survey, 2011–12) by proper and efficient utilization of the resources. Consequently, the amount of agrowaste started to escalate and has reached about 889.71 mt annually<sup>1</sup>. Out of this, crops like wheat, maize, rice and sugarcane contribute 330 mt of organic waste. The recycling of these wastes is both an ecological necessity and economic compulsion. Globally, biomass is ranked fourth as an energy resource, providing approximately 14% of the world's energy needs and can be efficiently used as an alternative for fossil fuels. Currently, approximately 50% of the world's biomass is being used by humans for food, construction and fuel. In India, estimates of the share of biomass in total energy vary from nearly a third (36%) to a half (46%) of the total energy<sup>2</sup>.

The conventional and traditional method of burning agrowaste remains a constant hazard to human and animal health and represents a major source of environmental pollution<sup>3</sup>. However, the use of agricultural wastes as soil amendments on farmland instead of burning is an attractive alternative because it allows some cost recovery, prevents soil erosion<sup>4</sup>, improves soil physical, chemical and biological properties and recycles the nutrients into the soil<sup>5–16</sup>. Transforming agro-waste residues into

high-quality product as compost through fungal inoculation provides a prospective solution to its disposal problem. A consortium of four hypercellulolytic fungal cultures, namely *Aspergillus nidulans*, *Trichoderma viride*, *Phanerochaete chrysosporium* and *Aspergillus awamori* was optimized for compost production on the basis of their lignocellulolytic enzyme production potential. The consortium has been effectively used for composting of diverse agricultural wastes such as paddy straw, soybean trash, pearl millet, maize residues and mustard stover. For developing an effective compost technology, large quantity of inoculum is necessary. Several attempts have been made to multiply the fungus in general using natural and semi-synthetic media for development of spores<sup>17–19</sup>. The economics of mass production should be in favour of the technology so as to make it a viable option. It has been reported earlier that use of liquid medium in the mass production system encourages rapid mycelial growth of the fungal culture. Moreover the rate of growth of fungi is faster in liquid medium and is therefore the most economical method of production. The ingredients of the medium, particularly the carbon and nitrogen source should be selected judiciously to strike a balance between the growth rate and cost of the medium. This communication describes the development of an economical medium and an optimized protocol for mass culturing of fungal partners of the consortium for rapid composting.

*A. awamori*, *T. viride*, *A. nidulans* and *P. chrysosporium* were obtained from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi and maintained on potato dextrose agar (PDA) slants. The cultures were streaked individually on petri plates and incubated at  $30 \pm 2^\circ\text{C}$ , while special care was taken in the case of *P. chrysosporium* and the plates were incubated strictly at  $37^\circ\text{C}$ . After two days, the plates were visually observed and the spores were harvested with 0.9% sterilized normal saline in 30 ml screw-cap tubes. The spore titre of each fungus was estimated using a haemocytometer. The collected spores were stored in the refrigerator for further use.

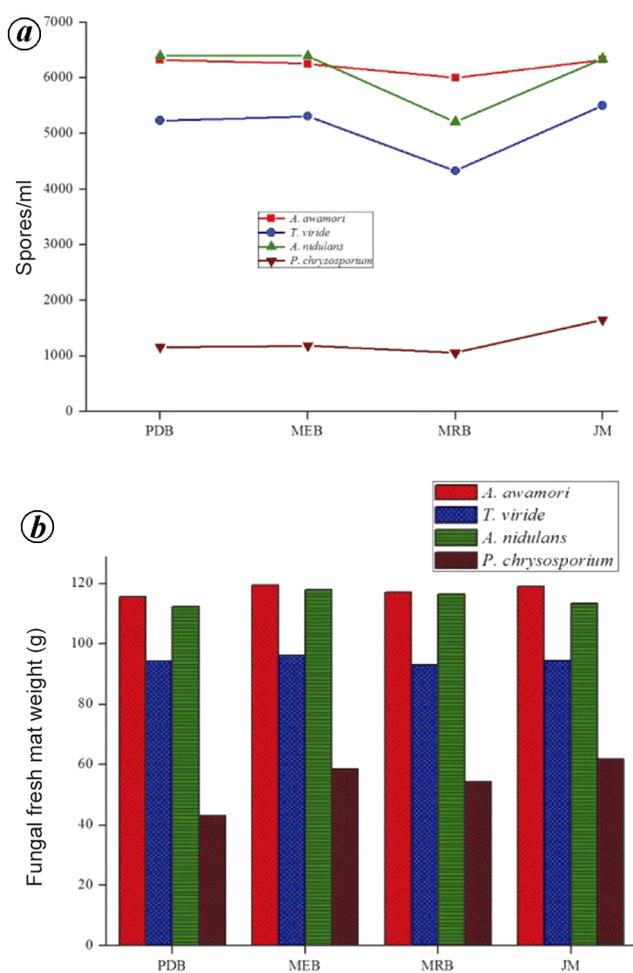
Culture grown on PDA plates, incubated at  $30^\circ\text{C}$  and  $37^\circ\text{C}$  for 7–8 days was used for preparation of spore suspension. Spore culture was washed twice with sterile saline (0.9%) water and stored at  $4^\circ\text{C}$ . This suspension was then charged in Neubauer's improved cell counting chamber to count the number of spores present. This number was adjusted to  $1 \times 10^9$  spores/ml, and the suspension was used as inoculum for further experiments.

The medium for mass multiplication of the four lignocellulolytic fungi was standardized and compared with routinely used media, that is, PDA, malt extract and Martin's Rose Bengal agar with regard to cost and time of cultivation. Jaggery was used as an alternate to conventional media for growing all the four fungal partners of the consortium. To optimize exact amount of jaggery

\*For correspondence. (e-mail: lshukla65@gmail.com)

that supports maximum growth, different quantities of jaggery varying from 5 to 50 g were used to make suspension in 1 litre of tap water. Next 300 ml of the above medium was dispensed in 500 ml conical flask and 0.25 g of yeast extract powder was added in each flask; pH of the medium was kept 6.5. All the flasks were sterilized at 15 lb pressure for 20 min. After cooling, the flasks were inoculated with different quantities of spores from 100 to 1000  $\mu$ l respectively, of the respective fungi. All the flasks were incubated in static condition at  $30 \pm 2^\circ\text{C}$  for three fungi, while for *P. chrysosporium* the flasks were incubated at  $37^\circ\text{C}$ . All the flasks were visually observed and on the seventh day, fungal mat was harvested and weight of the mat and spore count was taken.

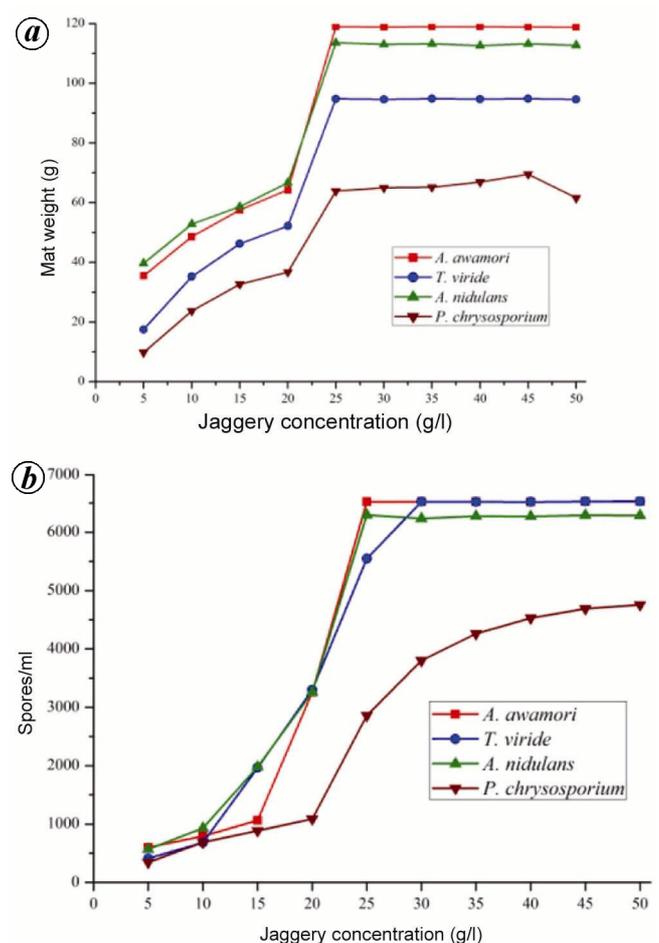
The fungal mat was removed from the liquid broth, washed with sterile distilled water and passed using 100 micron mesh sieve and washed them slightly with distilled water and then fresh mat weight was taken. All fungal mats were harvested separately in four containers and stored in plastic cans.



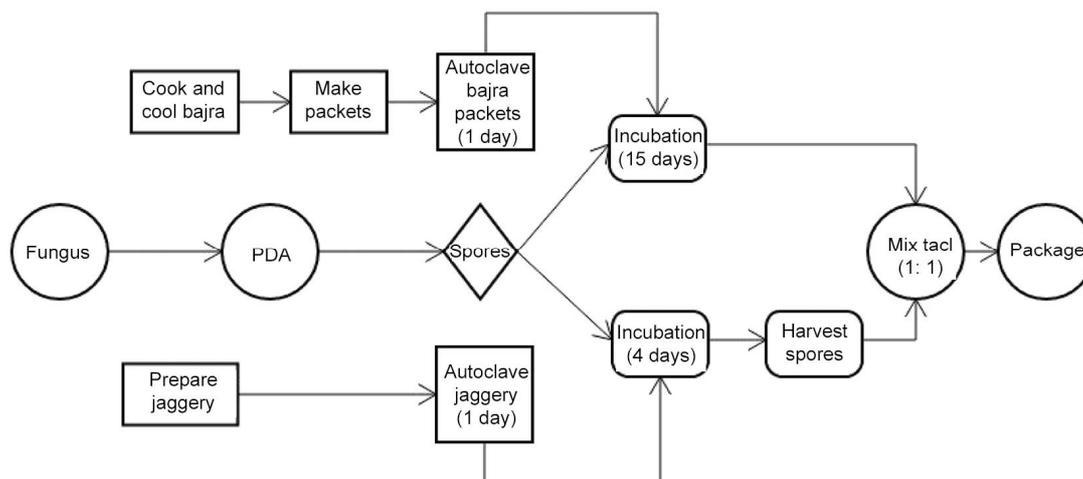
**Figure 1.** Evaluation of various media for (a) sporulation and (b) mycelial development of selected fungi. PDA, Potato dextrose broth; MEB, Malt extract broth; MRB, Martin Rose Bengal broth; JM, Jaggery medium.

Talc powder was kept in hot air oven for 30 min and then cooled at room temperature. The four fungi were mixed in the ratio of 1 : 1 : 1 : 2 (*A. awamori* : *T. viride* : *A. nidulans* : *P. chrysosporium*), followed by mixing of fungal mat with talc powder in 1 : 1 ratio to get 500 g as the final product. Another improvement has been added for mass multiplication using small-scale stir tank reactors (STRs). This STR with a fixed 125 RPM was developed with a working volume of 2.5 litres of jaggery medium in a 5 litre cylindrical vessel. This was inoculated with 5% fungal spore suspension and fermented for 48 h at  $30^\circ\text{C}$  and then kept for two more days for sporulation. Cost of each medium was calculated and compared with the jaggery medium to find the economic feasibility.

The four selected fungi are effective degraders of lignocellulosic agrowaste. Mass multiplication is important in order to produce spores as spore count in the inoculum is important for the composting process. Therefore, mass multiplication is an important criterion for development of inoculum. Since development of mycelia and spores represents two parts of the fungal lifecycle, it is important to define the media constituents so as to develop spores



**Figure 2.** Effect of jaggery concentration on (a) mat weight and (b) spore count.



**Figure 3.** Schematic representation of mass production of fungal inoculum.

**Table 1.** Comparison of cost of different routine media with newly formulated jaggery medium

Medium	Quantity (g)	Cost (Rs)	Quantity (g/l)	Cost/l (Rs)
Potato dextrose broth	500	1461	24.00	70.12
Malt extract broth	500	2250	20.00	90.00
Rose Bengal broth	500	1866	32.15	119.98
Jaggery	500	16.00	25.00	0.80
Yeast extract powder	500	1638	0.50	1.638
Jaggery medium				2.438

rather than mycelia. In such cases, carbon and nitrogen sources play an important role for initiating sporulation phase of the fungus. pH and temperature determine the vigour of growth. The pH was maintained at  $5.5 \pm 0.2$  for all fungi, whereas temperature was maintained at  $30^\circ\text{C}$  for all except *P. chrysosporium*, which had an optimal temperature range of  $37^\circ\text{C}$ . For mass production of the fungal biomass, seed culture was grown in PDA and the spores were harvested using sterile saline water. The final concentration of spores was found to be  $5 \times 10^6$  CFU/ml for *A. awamori*,  $4 \times 10^6$  CFU/ml for *A. nidulans*,  $2.3 \times 10^6$  CFU/ml for *T. viride* and  $1.6 \times 10^6$  CFU/ml for *P. chrysosporium*. The inoculum percentage for mass production of all the fungus was 5.

Important criteria such as nutrient content and economics are to be taken for selection of the medium needed for fungal multiplication. In a set of experiments carried out with the same fungus, we used four different routine media for mass production of fungal biomass – PDA, malt extract broth, Martin's Rose Bengal broth and jaggery medium. The fungi were inoculated separately in the broths as triplicate and their spore count as well as mycelial mat weight were determined after 96 h of incubation in the defined temperature range. It was inferred from the results that jaggery medium was the most economical and

gave enhanced spore count irrespective of the total fungal mycelia obtained.

The optimization of jaggery in the medium is an important aspect for determining the multiplication of fungus. Therefore in a set of experiments a gradient comprising increasing concentration of jaggery in a difference of 5 g/l was prepared in a range of 5–50 g/l with 0.25 g/l yeast extract and pH 5.5. Using the same parameters of incubation, the respective fungi were inoculated and their spore count determined with the help of a hemocytometer. Visual observation of all the flasks for four days showed that all four fungi were able to grow in the prepared jaggery medium. The growth of fungi in terms of mat formation increased in the medium with different amounts of jaggery, i.e. from 5 to 30, 35, 40, 45 and 50 g respectively. After four days weight of the mats was taken as well as spore count with the help of a haemocytometer. Table 1 shows that 30 g/l of jaggery was optimum for mass multiplication of all the four fungi.

The conventional method was based upon inoculating the fungus in sterilized bajra seeds. The problem associated with this method is the time constraint (15 days) and low spore count. However, in the formulation methodology utilizing jaggery medium, the spore count is more in a short time period. This is further increased through a STR in which the mycelia developed into beads 3–6 mm in diameter. This increased not only the surface area but also the fungal biomass in contrast to the stagnant mass multiplication, where the fungus develops mat on the surface of the broth and does not utilize the leftover medium space underneath the mat. After 48 h of agitation the cultured volume is kept stagnant for 24 h. This time is devoted for developing spores in the mycelia beads. As the surface area due to the agitation operation increases, the fungal spore volume also increases. This was found to be 300 g/l of biomass compared to 40 g/l biomass in the non-agitated fungal broths.

The cost of production of fungus on standard media ranges from about Rs 70 to 120  $\Gamma^{-1}$ , while the cost for the medium prepared by procuring every constituent from the market is Rs 2.43  $\Gamma^{-1}$ , which is much cheaper and from a single standard petri plate, one can prepare 40 flasks of each fungi.

## Rapid and molecular discrimination of host-specific fungal plant pathogens in pulse crops using genome profiling

Tripti Agarwal<sup>1</sup>, Shun Komazaki<sup>2</sup>,  
Harshita Sharma<sup>2</sup> and Manish Biyani<sup>1,3,\*</sup>

<sup>1</sup>Department of Biotechnology, Biyani Girls College, University of Rajasthan, Sector 3, Vidhyadhar Nagar, Jaipur 302 039, India

<sup>2</sup>Department of Functional Materials Science, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338-8570, Japan

<sup>3</sup>Green Devices Research Center, Japan Advanced Institute of Science and Technology, 1-1 Asahi-dai, Nomi, Ishikawa 923-1292, Japan

1. Singh, R., DARE/ICAR Annual Report, 2012–2013.
2. Ravindranath, N. H. and Hall, D. O., *Biomass, Energy and Environment: A Developing Country Perspective from India*, Oxford University Press, 1995.
3. Dincer, I., Energy and environmental impacts: present and future perspectives. *Energy Sources*, 1998, **20**(4–5), 427–453.
4. Rickson, R., Can control of soil erosion mitigate water pollution by sediments? *Sci. Total Environ.*, 2014, **468**, 1187–1197.
5. Atiyeh, R. *et al.*, Effects of vermicomposts and composts on plant growth in horticultural container media and soil. *Pedobiologia*, 2000, **44**(5), 579–590.
6. Senesi, N., Composted materials as organic fertilizers. *Sci. Total Environ.*, 1989, **81**, 521–542.
7. Hernando, S., Lobo, M. and Polo, A., Effect of the application of a municipal refuse compost on the physical and chemical properties of a soil. *Sci. Total Environ.*, 1989, **81**, 589–596.
8. Akiyama, H. *et al.*, N<sub>2</sub>O, NO and NH<sub>3</sub> emissions from soil after the application of organic fertilizers, urea and water. *Water, Air Soil Pollut.*, 2004, **156**(1), 113–129.
9. Våljamäe, P. *et al.*, Acid hydrolysis of bacterial cellulose reveals different modes of synergistic action between cellobiohydrolase I and endoglucanase I. *Eur. J. Biochem.*, 1999, **266**(2), 327–334.
10. Gardner, R. M., Doerner, K. and White, B. A., Purification and characterization of an exo-beta-1,4-glucanase from *Ruminococcus flavefaciens* FD-1. *J. Bacteriol.*, 1987, **169**(10), 4581–4588.
11. Christensen, B. T., Barley straw decomposition under field conditions: effect of placement and initial nitrogen content on weight loss and nitrogen dynamics. *Soil Biol. Biochem.*, 1986, **18**(5), 523–529.
12. Bangar, K. *et al.*, Preparation of nitrogen and phosphorus-enriched paddy straw compost and its effect on yield and nutrient uptake by wheat (*Triticum aestivum* L.). *Biol. Fertil. Soils*, 1989, **8**(4), 339–342.
13. Zayed, G. and Abdel-Motaal, H., Bio-active composts from rice straw enriched with rock phosphate and their effect on the phosphorous nutrition and microbial community in rhizosphere of cowpea. *Bioresour. Technol.*, 2005, **96**(8), 929–935.
14. Gaur, A. *et al.*, Role of mesophilic fungi in composting. *Agric. Wastes*, 1982, **4**(6), 453–460.
15. Tuomela, M. *et al.*, Biodegradation of lignin in a compost environment: a review. *Bioresour. Technol.*, 2000, **72**(2), 169–183.
16. Djonovic, S. *et al.*, Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. *Mol. Plant–Microbe Interact.*, 2006, **19**(8), 838–853.
17. Ibrahim, Y. and Low, W., Potential of mass-production and field efficacy of isolates of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* against *Plutella xylostella*. *Int. J. Pest Manage.*, 1993, **39**(3), 288–292.
18. Lewis, J. and Papavizas, G., Production of chlamydospores and conidia by *Trichoderma* spp. in liquid and solid growth media. *Soil Biol. Biochem.*, 1983, **15**(3), 351–357.
19. Camassola, M. and Dillon, A., Production of cellulases and hemicellulases by *Penicillium echinulatum* grown on pretreated sugar cane bagasse and wheat bran in solid–state fermentation. *J. Appl. Microbiol.*, 2007, **103**(6), 2196–2204.

**A rapid and accurate identification of potential plant pathogens below the species level is highly desirable to understand the genetic basis of host–pathogen interactions and thus to effectively manage plant diseases. In this study, a genome profiling (GP) technique was applied to identify 14 common seed-borne fungal pathogens from five different legume plant hosts in Rajasthan, India. Six species belonging to different taxonomic orders were successfully identified and classified topologically to the same position with their phenotypic traits. Next, we demonstrated that GP could be used to discriminate fungal pathogenic strains below the species level by classifying 10 different strains of *Aspergillus niger* and *Aspergillus flavus* based on plant host specificity. These results suggest that accurate identification of plant pathogenic subspecies is likely to become an easier task, and the resulting GP-based database can be an ideal platform for timely and unambiguous identification of fungal species, with pathogenic or beneficial relation to plant host.**

**Keywords:** Fungal plant pathogens, genome profiling, host–pathogen interaction, plant diseases, species identification.

THE high mortality associated with the increasing number of fungal and fungal-like diseases in recent times, has highlighted the need for rapid and accurate identification of fungal pathogenic species<sup>1</sup>. Classical identification of pathogenic isolates based on culture-based morphological, anatomical and biochemical analyses is not only inadequate in some instances but also time-consuming and laborious. Another important limitation of classical methods is the inability to differentiate pathogenic from nonpathogenic strains that belong to the same microbial species. In addition, most species of fungal plant pathogens are known to possess a broad host range, such as, two morphologically identical strains of a fungal species may have quite different infection capacity on two different hosts<sup>2</sup>. Pathogenic strains have therefore been assigned to

Received 6 May 2014; accepted 21 August 2014

\*For correspondence. (e-mail: biyani@jaist.ac.jp)