

Characterization of single spore isolates of *Volvariella volvacea* (Bulliard: Fries) Singer

A. Kaur* and H.S. Sodhi

Department of Microbiology, Punjab Agricultural University, Ludhiana-141004, India.

*E-mail: amritpalkaur171@gmail.com, drhssodhi@rediffmail.com

Abstract

Volvariella volvacea is a tropical mushroom with variable biological efficiency which may be stabilized through isolation of single spore cultures. Eighty seven single spore cultures (VAT-1 to VAT-87) of *V. volvacea* strain VV132 were isolated from three fruit bodies. The single spore cultures were characterized for their growth and enzyme producing capability followed by yield trials. Maximum growth rate was recorded in VAT- 81 and maximum biomass production was recorded in VAT-15 and VAT-82. On the basis of growth characteristics, twelve single spore cultures were selected for enzyme activity and cultivation trials along with the parent strain VV-132. Maximum endo-1,4- β - glucanase (EC 3.2.1.4) activity was recorded in VAT-82 (0.158U/h/mg). Maximum xylanase (EC 3.2.1.8) activity and exo-1,4- β -glucanase (EC 3.2.1.91) activity were recorded in VAT-15 (0.155U/h/mg and 0.083U/h/mg, respectively). The laccase (EC 1.10.3.2) activity was maximum for VAT-73 (3.66U/min/mL). In comparison to parent strain *V. volvacea* VV132, five single spore cultures VAT-15, VAT-26, VAT-33, VAT-73 and VAT-81 had shown higher yield whereas the number of fruit bodies was higher for single spore culture VAT-81. During the present study, these five *V. volvacea* single spore cultures have been identified as high yielding strains.

Key words: *Volvariella volvacea*, single spore cultures, growth, enzyme activity, yield

Introduction

Volvariella volvacea, also known as paddy straw or Chinese mushroom is an edible mushroom of tropical and subtropical regions. It can be cultivated at a temperature range of 28 to 38°C and relative humidity of 57-80 %. Paddy straw is most common substrate for this mushroom but it can also be cultivated on cotton waste, banana leaves, corn stovers, sugarcane baggase, oil palm pericarp, sawdust, oil palm bunch and fibers, pulses straw and crop wastes (Ahlawat and Kumar, 2005). It is a fast growing mushroom as it takes 10-12 days from spawning to harvesting. It is third most important mushroom cultivated in the world with an annual production of 287 million tons (Thakur *et al.*, 2003). In 2010, output of the mushroom on the Chinese mainland was 330,000 tons (Bao *et al.*, 2013). It has significant pharmacological properties, including anti tumor polysaccharides, immunosuppressive proteins and immunomodulatory lectins (Kishida *et al.*, 1992; She *et al.*, 1998). The productivity of *V. volvacea* is very low (about 10-15% on rice straw) as compared to other cultivated species of mushroom (Chang and Miles, 2004). The hydrolytic enzyme production potential and quality of substrate used for cultivation has direct bearing effect on its mushroom production potential.

This mushroom produces a multicomponent enzyme system consisting of endo-1,4- β - glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), β -glucosidase (EC 3.2.1.21) and laccase (EC 1.10.3.2) (Cai *et al.*, 1999). The cellulases play role during substrate colonization stage, while laccase during sporophore development stage (Ding *et al.*, 2001; Chen *et al.*, 2003). Cultivation of *V. volvacea* in Punjab has been successfully practiced but with a lot of variation in its biological yield

efficiency which could probably be stabilized through selection of single spore cultures. The single spore isolates have been used for strain improvement of mushroom. Present study was planned to collect single spore isolates of *V. volvacea* to characterize the cultures for growth and enzyme producing capability with an impact of variable parameters on mushroom yield potential.

Materials and methods

V. volvacea, strain VV132 was procured from the Culture Collection Bank, Mushroom Research Complex, Punjab Agricultural University, Ludhiana. The culture was maintained on potato dextrose agar medium at 30 \pm 2°C.

Cultivation of *V. volvacea* strains: Cultivation of *V. volvacea* was conducted during the summer season (May-September). The cultivation was carried out indoor under natural climatic conditions with temperature ranging from 28 to 40 °C and relative humidity of about 70-90 per cent at Mushroom Research Complex, Punjab Agricultural University, Ludhiana. Proper ventilation and diffused light in growing rooms were maintained. Paddy straw was used as substrate for growing *V. volvacea* and it was used in the form of bundles tied at two ends, chopped from the side and each bundle was of 500 g. These bundles were soaked in 1.5 % CaCO₃ mixed with water for 16 hours and excess of water was drained off, so as to attain 65-68% moisture. Then these bundles were arranged in the form of a bed, 5 bundles in 4 layers + 2 *i.e.* 22 bundles per bed (5 \times 4+2). Then the substrate was thoroughly spawned with grain spawn (@ 1.5 % of dry weight of paddy straw). Spawn beds were kept in growing rooms for spawn run (Khanna and Kapoor, 2007). After 5-7 days of spawning, mushrooms started appearing in flushes for about 15-20 days. The mushrooms were harvested by gentle twisting of the fruit

body, till the end of crop. The number and weight of fruit bodies from each bed were recorded.

Collection and germination of basidiospores: A healthy and mature, but unopened *V. volvacea* fruit body was collected; its hymenium was cut exposed with sterile blade and was supported to the top needle of the spore collection apparatus, to allow its free hanging with its hymenium facing downwards. Thereafter, the jar was covered with sterilized lid of the petri plate. Spore prints were stored at 28 °C in incubator until germination trials were conducted. A loopful mass of spores was suspended in 10 mL water blank and serially diluted to contain 50 spores per mL. The spore count was monitored with a haemocytometer (Heubauer, Germany). Spore suspension (0.1 mL) was added onto the potato dextrose agar plate and spread using a sterilized glass spreader. The plate was incubated at 30 ± 2 °C and observed until small colonies appeared. As soon as the first appearance of colony was noticed, it was picked and transferred to potato dextrose agar slants and incubated at 30 ± 2 °C to grow as single spore culture. Eighty seven single spore cultures VAT-1 to VAT-87 of *V. volvacea* were obtained from different fruit bodies. Single spore cultures of *V. volvacea* were maintained on Potato Dextrose Agar (PDA) slants at 30 ± 2 °C by sub-culturing them forthrightly.

Characterization of single spore cultures

Growth study: The single spore cultures of *V. volvacea*, VV 132 were grown on complete yeast extract medium (CYM) to prepare master plates. To estimate the linear growth, bits of equal size (10 mm) were placed from master plates on to the CYM agar plate, incubated at 30 ± 2°C. Linear growth was measured as mm per day along with the growth pattern and colony. The single spore cultures of *V. volvacea* were grown on complete yeast extract medium (CYM) broth (50 mL/flask) that was autoclaved, cooled and inoculated with *V. volvacea* mycelia agar bit (10 mm diameter) and incubated at 30 ± 2°C. Each flask was filtered after 10 days of incubation onto preweighed Whatmann No.1 filter paper. The mycelium was dried in an oven at 55°C, till it attained constant weight. The weight of dry mycelium was recorded, as the difference of the final and initial values.

Enzyme assay: *V. volvacea* single spore cultures were screened for enzyme activity. Enzyme activity was estimated by growing the culture in Mushroom Minimal Media broth (MMM). Basal broth (30 mL) was supplemented with cellulose (@ 0.3 g/flask as inducer for extracellular enzyme production. The medium was autoclaved at 20 psi for 30 minutes. Flasks with 25 mL MMM broth were inoculated with 10 mm agar bits of single spore cultures from the master plates using cork borer. These flasks were then incubated at 30 ± 2° C for 10 days. Flasks were shaken daily. Each flask was filtered onto Whatmann No.1 filter paper. The filtrate was collected in capped vials and stored at -4° C. This filtrate was used to estimate the cellulase, xylanase and laccase enzymes activity. Cellulases and xylanases activity was measured by estimation of reducing sugars released during incubation of substrate with enzyme extract according to the methods of Sandhu and Kalra (1982). The reaction mixture for exoglucanase (FPase, EC 3.2.1.91) comprised of eight filter paper (Whatman No. 1) discs of 0.6 cm diameter in 0.5 mL acetate buffer of pH 5.0 and 0.5 mL of the enzyme source. The reaction mixture was incubated at 45 °C for 6 h and the reducing sugars

released were measured by Nelson Somogyi method (Nelson, 1944). The endoglucanase (CMCase, EC 3.2.1.4) activity was measured following the above method, replacing filter paper discs with 0.5 mL of carboxy methyl cellulose. Xylanase (EC 3.2.1.8) was assayed at 45°C following the method described as Sandhu and Kalra, (1986). Laccase was assayed following the method of Dhaliwal *et al.* (1991). Laccase (EC 1.10.3.2) was assayed by adding 1 mL enzyme source to 3 mL of 0.02 M guaiacol in phosphate buffer (0.1 M) of pH 6.0 and change in absorbance was recorded for every 15 sec. upto 120 sec. at 495 nm. Total protein was estimated according to method of Lowry *et al.* (1951). One unit of laccase activity was calculated as change in absorbance of 0.001 min/ mL of enzyme source at 25 °C, while that of FPase, CMCase and xylanase as the μ mol glucose released h⁻¹ mg⁻¹ of enzyme source.

Cultivation of *V. volvacea* single spore cultures: Cultivation of twelve single spore cultures of *V. volvacea* VAT-1, VAT-15, VAT-18, VAT-26, VAT-31, VAT-33, VAT-34, VAT-37, VAT-73, VAT-74, VAT-81 and VAT-82 along with the parent strain VV-132 was carried out using paddy straw bundles following the same methodology as given in cultivation of parent VV132.

Results and discussion

Cultivation of *V. volvacea*, VV-132 strain: Spawn run was completed in 5-7 days of spawning. The first harvest was made between 7-9 days to give a total of 13.9 kg fresh mushrooms/q paddy straw giving 1871 number of fruit bodies in 3 weeks harvest. The average weight of fruit body was 7.5 g.

Isolation of single spores cultures of *V. volvacea*, VV-132 strain: Three spore prints were made and counts of spores per 100 μL were 110, 102 and 95 respectively. The percentage germination of three spore prints was 21.8, 32.3 and 31.6 % (Table 1). Eighty seven spore germinants were collected up to 3 days of incubation. Each culture was assigned a number from VAT 01 to VAT 87 and maintained as pure culture on CYM agar slants.

Table 1. Isolation of single spore cultures of *V. volvacea*, VV-132 strain

Spore Print	Haemocytometer count of spores per 100 μL	Volume of 100 spores (μL)	No. of single spore cultures (%germination)	Assigned no. for each single spore cultures
1	110	85	24 (21.8%)	VAT 1-VAT 2 4
2	102	92	33 (32.3%)	VAT 25-VAT 57
3	95	101	30 (31.6%)	VAT 58-VAT 87

Characterization of single spore cultures

Growth study: Eighty seven single spore cultures, VAT-1 to VAT-87, along with the parent culture VV-132 were subjected to growth on CYM media broth and grouped into lots with identical growth pattern. Two representatives from each group were randomly selected for further study. On CYM agar the growth rate (mm/day) was highest for lot-6 (17.0 mm/day) on day 1. On 2nd day the growth was highest for lot-4 (26.5 mm/day), while it was highest for lot-6 (37.3 mm/day) on 3rd day. On day 4, the growth rate was lowest for lot-6 (11.6 mm/day) (Table 2). On the basis of linear growth characteristics, twelve cultures were selected for further study. In broth cultures, the biomass harvested was between 5.3 to 12.5 g/L with maximum for VAT-1, VAT-15, VAT-18, VAT-26, VAT-31, VAT-73 and VAT-82.

Table 2. Linear growth characteristics of *V. volvacea* monospore cultures

Lot	Monospore cultures	Growth in diameter (mm per day)				Colony morphology	
		Day 1	Day 2	Day 3	Day 4	Colour	Morphology
1	VAT 10,17,74,81,84,87	12.0	29.2 (17.2)	47.2 (18.0)	67.2 (20.0)	Off-white	Aerial thin
2	VAT 28,31,34,35,36	12.0	29.2 (17.2)	47.2 (18.0)	68.4 (21.2)	Creamy-White	Cottony aerial thin
3	VAT 29,33,37,38	13.0	36.5 (23.5)	56.0 (19.5)	72.6 (16.6)	Creamy-White	Aerial thin
4	VAT 4,5,6,11,19,26,27,30,32,39-48,50, Parent VV132	14.0	40.5 (26.5)	67.8 (27.3)	90 (22.2)	White	Aerial thin
5	VAT 2,3,12, 15,16,18,20,21,23, 25,49, 51-57, 66-72,75-80,82	13.5	38.7 (25.2)	61.6 (22.9)	83.7 (22.1)	Off-White	Apprised thick
6	VAT 1,13,14,24,58,60-65, 73	17.0	35.0 (18.0)	72.3 (37.3)	83.9 (11.6)	White	Aerial thin
	LSD ($P=0.05$)	0.178	0.178	0.178	0.177	-	-

Table 3. Biomass production and enzyme assay of monospore cultures of *Volvariella volvacea*

<i>V. volvacea</i> monospore cultures	Biomass production (g/L)	Specific Activity (U h ⁻¹ mg ⁻¹)			Laccase (U min ⁻¹ mL ⁻¹)
		Endoglucanase	Exoglucanase	Xylanase	
VAT 1	11.8	0.023	0.074	0.110	1.33
VAT 15	12.5	0.036	0.083	0.155	1.75
VAT 18	11.2	0.009	0.029	0.122	1.83
VAT 26	9.3	0.054	0.018	0.083	1.33
VAT 31	12.1	0.049	0.031	0.064	1.50
VAT 33	8.2	0.046	0.056	0.071	1.33
VAT 34	6.0	0.040	0.015	0.098	1.16
VAT 37	7.3	0.056	0.033	0.129	1.16
VAT 73	12.3	0.009	0.052	0.054	3.66
VAT 74	5.3	0.039	0.080	0.085	2.75
VAT 81	8.6	0.040	0.036	0.094	1.50
VAT 82	12.5	0.158	0.069	0.088	1.66
Parent VV132	11.3	0.051	0.080	0.124	3.66
LSD ($P=0.05$)	0.2	0.161	0.168	0.168	0.167

Table 4. Cultivation of *V. volvacea* single spore cultures

Strain Number	Spawn run (Days)	First harvest (Days)	Yield (kg/q of dry straw)	NFB ^a (number/q of dry straw)	Average weight of fruit body (g)
VAT 1	9	12	12.8	2206	5.8
VAT 15	8	11	16.5	2323	7.1
VAT 18	10	13	08.7	1280	6.8
VAT 26	8	10	15.1	1840	8.2
VAT 31	-		No fruiting		
VAT 33	7	10	17.6	1934	9.1
VAT 34	10	13	09.8	1195	8.2
VAT 37	9	11	13.2	1808	7.3
VAT 73	7	09	18.2	2413	7.5
VAT 74	-		No fruiting		
VAT 81	8	10	16.7	2455	6.8
VAT 82	7	09	11.5	1666	6.9
Parent VV132	6	8	13.9	1871	7.5
Mean	8	10	13.6	1890	7.2
LSD ($P=0.05$)	-	-	0.2	1.09	-

Date of sowing: 20 Aug, 2014, Rate of spawn: 300g/bed. ^aNFB: number of fruit bodies

Biomass production was maximum in VAT-15 and VAT-82 (12.5 g/L). Biomass production was minimum in VAT-74 (5.3 g/L) (Table 3). Variation in growth morphology and cultural characters among spore isolates were detected in *V. volvacea* as reported earlier (Ahlawat *et al.*, 2014). Within the group, the single spore cultures also varied in their type of growth and colony characteristics.

Enzyme assay of monospore cultures of *V. volvacea*: On the basis of growth characteristics, twelve monospore cultures VAT-1, VAT-15, VAT-18, VAT-26, VAT-31, VAT-33, VAT-34, VAT-37, VAT-73, VAT-74, VAT-81 and VAT-82 were selected for enzyme (cellulases, xylanase and laccase) activities. The enzyme assay of monospore cultures indicated that maximum enzyme activity of endoglucanase (EC 3.2.1.4) activity was found in VAT-82 (0.158U/h/mg) and minimum was found in VAT-73 (0.0096U/h/mg). While exoglucanase (EC 3.2.1.91) activity was maximum in VAT-15 (0.083U/h/mg) and minimum in VAT-34 (0.015U/h/m). Maximum xylanase (EC 3.2.1.8) activity was found in VAT-15 (0.155U/h/mg). The laccase activity was maximum for VAT-73 (3.66U) followed by VAT-74 (2.75U) and VAT-18 (1.83U) with lowest in VAT-34 and VAT-37 (1.16U) (Table 3). The variability in activities of extracellular enzymes in *V. volvacea* strains along with role of cellulases in mycelial colonization and laccase in sporophore formation have also been reported earlier by other workers (She *et al.*, 1998).

Cultivation of *V. volvacea* single spore cultures: On the basis of growth characteristics, twelve monospore cultures VAT-1, VAT-15, VAT-18, VAT-26, VAT-31, VAT-33, VAT-34, VAT-37, VAT-73, VAT-74, VAT-81 and VAT-82 were selected for yield trials. The spawn run was complete within 7-10 days and mushrooms were first harvested between 9-13 days after sowing. The number of fruit bodies and yield was harvested for 20 days in high yielding strains (Fig.1). In comparison to parent strain *V. volvacea* VV132 (13.9kg/q), five single spore cultures VAT-15 (16.5kg/q), VAT-26 (15.1kg/q), VAT-33 (17.6kg/q), VAT-73 (18.2kg/q) and VAT-81 (16.7kg/q) had shown higher yield. The yield potential for two single spore cultures VAT-18 and VAT-34 was lowest. The number of fruit bodies was higher for single spore culture VAT-81 (2455). There was no fruiting in two single spore cultures, VAT-31 and VAT-74. The average weight of fruit body ranged between 5.8-9.1 (Table 4). These five *V. volvacea* single spore cultures VAT-15, VAT-26, VAT-33, VAT-73 and VAT-81 have



Fig. 1. Cultivation of parent strain VV132 along with single spore cultures VAT-73, VAT-26, VAT-33, VAT-81 and VAT-15 on paddy straw

been identified as high yielding strains. Yield evaluation trials for selecting a better performing strain or single spore isolate have also been performed earlier by several workers (Ahlawat *et al.*, 2005, 2008 and 2011; Graham, 1975; Garcha *et al.*, 1986) but only few of them have correlated the morphological and biochemical characteristics of a strain with its yield potential and have helped in selecting several high yielding strains (Ahlawat *et al.*, 2005, 2011, 2014).

Eighty seven single spore cultures of *Volvariella volvacea* strain VV132 were isolated. On the basis of linear growth and biomass production, twelve single spore cultures were selected for enzyme activity and cultivation trials. In comparison to parent strain *Volvariella volvacea* VV132, five single spore cultures VAT-15, VAT-26, VAT-33, VAT-73 and VAT-81 had shown higher yield whereas the number of fruit bodies was higher for single spore culture VAT-81.

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