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Biological characterization of a fast growing non-sporing alkalophilic lignin degrading fungus MVI.2011

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MVI.2011 a rapidly multiplying alkalophilic non sporing fungus was isolated in 1990 and preliminarily identified as a Deuteromycete. The isolate was characterized in detail. The original isolate produced highly fluffy, cottony, fragile aerial mycelia on SDA and a similar growth in liquid SDB also. The organism grew out even on the surface of the conical flask containing the liquid medium inoculated indicating the high aerobic nature. With frequent sub culturing over 20 years the colony morphology on the same media appeared very confined with regular margin and dry surface. Yet there were no reproductive structures. LP staining showed dimorphism with apical fragmentation and no conidia, spores sexual or asexual etc. The pH range was very wide 5-11. The optimum cultural conditions for lignin degradation were pH 8.5, temperature 25-28°C, 12-18 hours and medium- 1% glucose, 0.5% peptone in basal mineral medium. The isolate could breakdown and decolourise commercial lignin (0.1-5%) and alkaline wood extract (1-50%) within 12-18 hours in static cultures evidenced by a clear reduction in absorption at 380 nm (lignin) and a marked shift to increased absorption at 360 nm and between 180 and 300 nm indicating appearance of lignin breakdown products. In optimised media containing commercial lignin (0.1%) and alkaline wood extract (10%), MVI.2011 secreted Lignin peroxidase (9.39 units/ml), Manganese peroxidase (2.093 units/ml) and laccase (3.5 units/ml) enzymes. The above data led us to conclude that the isolate was novel being highly alkalophilic, capable of rapid growth, decolourisation of lignins and secretion of lignin degrading enzymes. Based on microscopic morphology and colony features, the isolate coded MVI.2011 has been identified as "Uncultured Fungus" with NCBI Accession No JN606084. It has been deduced to be a member of *Mycelia sterilia* group.

Plant biomass is the most abundant renewable carbon source on earth mainly composed of cellulose, hemi cellulose and lignin. Complex formation between lignin and hemicelluloses provides an ideal cementing material in the secondary cell wall of the plant cell. The lignin with its heavy molecular weight and structural complexity provides mechanical stability and protection to plant cell wall (Del et al., 2002; Thompson et al., 2001; Venkata et al., 1997). Most of the agricultural crop based water consuming industries discharge very huge volumes of brown coloured effluents containing lignins into the environment and water bodies. The brown colour is attributed to the presence of polymers of lignin and its derivatives. Pulp and Paper industries are one of the major contributors to the discharge of untreated effluents (Thompson et al., 2001). This causes an imbalance in carbon exchange and poses a serious threat to the ecosystem disrupting the carbon balance (Hu et al., 2001).

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Accumulation of lignin also causes several health hazards like infertility, breathing ailments, damage of vital organs etc (Rappe et al., 1990; Sodergren et al., 1993; Swanson et al., 1988). The available chemical treatment methods are found to be inefficient compared to the microbial degradation of lignin. Though several investigators, for the past few decades have been working on the biodegradation of lignin the exact mechanism is still unclear. Hence there are no effective chemical or microbial processes. This has resulted in a demand for new efficient treatment methods using more powerful organisms for industrial application (Bajpai et al., 1996, 1997). Regarding biodegradation, Lignin, a highly recalcitrant compound, is decomposed only by a very small group of fungi belonging to class Basidiomycetes. There are also some bacteria that break down lignin into simpler units by conversion into CO_2 utilizing lignin as carbon source (Cameron et al., 2000; Cullen, 2001).

Some filamentous fungi, the white rot fungi in particular, have evolved to break down lignin by secreting potent extracellular enzymes like Lignin peroxidase, Manganese peroxidase and Laccase. These enzymes are capable of generating reactive Oxygen species which facilitate a series of spontaneous reactions to break down the heavy molecular weight lignin into simpler units that makes it easier for uptake by the organisms. The non specificity of these enzymes therefore has a wide application in paper pulp industries, breweries and textile industries that discharge gallons of effluents containing lethal dose of lignins (Cullen and Kersten., 2004; Kirk et al., 1986; Kirk and Farrell., 1987). Despite the extensive research of fungal lignin degradation since the mid-1980s, no commercial bio catalytic process for lignin depolymerisation is implemented till date, due to the practical challenges of fungal genetic manipulation and fungal protein expression (Bugg et al., 2010).

The present paper describes the detailed morphology, cultural characterisation and optimization for degradation of lignin by the indigenous fungal isolate coded MVI.2011, identified as a dimorphic Deuteromycete, surmised to be a *Mycelia sterilia sp*, reported for the first time in 1998 as a rapidly multiplying, highly alkalophilic melanoidin and lignin degrading fungus (Thankamani et al., 1998, 2002).

Materials and methods

Microorganism: A melanoidin degrading fungus isolated from soil samples and earlier studied for removal of colour from molasses based distillery spentwash (Thankamani et al., 1998, 2002) was used in this study. The purified fungal isolate was coded MVI.2011 and preserved on SDA slopes in the refrigerator. In the present study, MVI.2011 was characterized in detail and screened for its potential to secrete lignin degrading enzymes Lignin peroxidise, Manganese peroxidise and laccase.

Media and reagents: Standard culture media from Hi media Laboratories, India were. Used. Commercial lignin and natural lignins mixed with other components were prepared as follows. 150 g of saw dust was suspended in 1 litre of distilled water, pH adjusted to 10 using 1M NaOH, autoclaved for 40 min at 15 pounds per square inch and filtered. Using the hot alkaline wood extract was used as a source of natural lignins after suitable dilution (Venkatkumar et al., 2011).

Preliminary screening:

The isolate MVI.2011 was inoculated on SDA plates and SDB in 100 ml flasks containing 0.1 % commercial lignin (L) and 10% alkali wood extract each respectively were used. The density of growth and colony features on solid media and the development of turbidity and apparent reduction in colour in liquid media were observed.

Cultural characterization of MVI.2011

MVI.2011 was inoculated on routine media like NA, NB, SDA and SDB and incubated

at ambient temperature (25°C to 28°C) for 24 hours. Colonies on solid media and growth features in liquid media were observed and smears and films were stained by routine procedures such as Lacto Phenol Cotton Blue, Gram's, and, special stains (Mayer's Mucicarmine, Congo Red -Putchler's modification, Sudan Black) (Bayliss and Adams., 1972; Emmon et al, 1977) to detect cytoplasmic granules, polysaccharides, lipids etc., Growth on plain SDA as well as lignin containing media were subjected to scanning electron microscopy.

Scanning electron microscopy: The Scanning Electron Microscopy was carried out at the Department of Botany, University of Madras, India. MVI.2011 was inoculated on minimal mineral medium with peptone (0.5%), glucose (1%) and 0.1% lignin agar slopes and also on SDA without lignin. Blocks of the agar slants 2-3 mm thick along with the growth were cut out, placed on the aluminium grid and scanned under SEM (Hitachi S3400N). Details of cell morphology of the organism were noted and photographed.

Growth curve: The isolate was inoculated into 500 ml flasks containing NB (pH 7.0) and SDB (pH5.5) and incubated as described earlier. Also various combinations of basal media supplemented with additional 2% peptone and 0.1% lignin were prepared with initial pH 8.5. All the media were inoculated with MVI.2011 and incubated as static cultures at ambient temperature. Samples were drawn every 4 hours upto 24 hours, centrifuged at 4°C at 6000 rpm for 20 minutes and the supernatant was analysed for total protein, pH and biomass. The values were plotted against time to obtain the growth curves under various physiological conditions.

Factors influencing growth:

Influence of initial medium pH: Multiple flasks of SDB with initial pH 5,6,7,8,9,10 and 11 were inoculated with the organism and incubated at ambient temperature under static conditions for 18-24 hours. The broth was centrifuged and pH, total protein and wet biomass were estimated at defined intervals from 0-24 hours.

Influence of temperature: The isolate was inoculated into SDB pH 5.5 and incubated at varying temperatures ambient, 37, 40 and 50°C as static cultures for 24 hours. pH and biomass were monitored.

Influence of concentration of glucose, additional peptone and lignin on growth on solid and liquid media: The media consisted of NA, NA with various concentrations of glucose (1%, 2%, 3% and 4%) and Peptone (0.1%, 0.5%, 1%, 1.5% and 2 %) and SDA with 0.1%, 0.5%, 1% and 2% and SDB with 1%, 2%, 3%, 4% and 5% commercial lignin, pH 8.5 (Nagarathnamma et al., 1999). All were inoculated with the isolate MVI.2011 and incubated for 24 hours at ambient (25-30°C) temperature. The colony characteristics in each medium were recorded.

Potential of MVI.2011 to utilise lignins as sole carbon source: Duplicate sets of minimal mineral medium (0.01% each of sodium phosphate, potassium phosphate and magnesium sulphate) with peptone (0.5%), glucose (1%) and commercial lignin (0.1%) were prepared and one was inoculated with MVI.2011 the other served as control. Both were incubated for 24 hours at ambient temperature. Samples were drawn from both the tubes, centrifuged at 4°C, at 6000 rpm for 20 minutes and the absorption was measured in UV spectrophotometer at 380 nm for estimation of lignin and over 180 to 700 nm to screen for any new compounds formed. The absorption patterns of culture supernatant and medium control at 180 to 700 nm were compared (Venkat Kumar et al., 2011).

Production of extracellular lignin degrading enzymes in optimized conditions : A minimal mineral medium was formulated for studying lignin

utilization by MVI.2011 as a major carbon source. The optimized modified minimal medium consisting of Mineral salts (0.01% each of sodium phosphate, potassium phosphate and magnesium sulphate), 0.5% peptone, 1% glucose and 0.1% of lignin was adjusted to pH 8.5. The organism was inoculated and incubated at ambient temperature 25°C-28°C for 12 - 18 hours. The broth was centrifuged at 6000 rpm for 20 minutes at 4°C. The culture supernatant was analysed for all the lignin degrading enzymes as per standard protocols (Daljit et al., 2002).

Lignin peroxidase (LiP)

Lignin peroxidase was determined in a 12 hour culture supernatant as the source of crude enzyme. The reaction was performed at ambient temperature, the reaction mixture contained Azur B (32 µM), Sodium tartarate buffer (50mM, pH 3), and 0.5ml of crude enzyme. The reaction was initiated by adding 0.5ml of H_2O_2. Absorbance was measured every minute at 310 nm. One unit of enzyme activity was equivalent to an absorbance decrease of 0.1 unit $min^{-1}ml^{-1}$ (Archibald., 1992; Arora et al., 2001).

Manganese peroxidase (MnP)

MnP assay was performed on the basis of oxidation of phenol red. Reaction mixture contained 1 ml of sodium succinate buffer (50 mM, pH 4.5), 1 ml sodium lactate (50 mM, pH 5), 0.4 ml manganese sulphate (0.1mM), 0.7 ml phenol red (0.1 mM), 0.4 ml H_2O_2 (50 µM), gelatine 1mg ml^{-1} and 0.5 ml of crude enzyme. The reaction was initiated by adding H_2O_2 and incubated at 30°C. 40µl of 5N NaOH was added to 1 ml of the reaction mixture. Absorbance was measured at 610 nm. After every minute the same steps were repeated with 1ml of the reaction mixture up to 4 min. One unit of the enzyme activity was equivalent to an absorbance increase of 0.1 unit $min^{-1}ml^{-1}$ (Orth et al., 1993).

Laccase

Laccase activity was measured using Guaicol as substrate. The reaction mixture containing 3.8 ml acetate buffer (10 mM, pH 5), 1ml of Guaicol (2mM) and 0.2ml of the enzyme extract was incubated at 25°C for 2h. The absorbance was read at 450 nm. Laccase activity was expressed as colorimetric unit ml^{-1} (CU ml^{-1}) (Arora et al., 1985).

Results and Discussion

Microscopy

Light microscopy:

Colonies on SDA- Lacto phenol cotton blue staining:

LP mounts of MVI.2011 from SDA showed deep-blue stained cells characteristic of fungi. The yeast or cellular forms were predominant with a few mycelia like fragments. The cells were spherical, oval or cubical with the centre dense but weakly stained while the remaining cytoplasm around took up deep blue stain. The hyphae or mycelia forms were irregular, non uniform in diameter, size and shape, unbranched, non septate and showed swollen cells at apex. The mycelial forms showed beaded appearance, with close resemblance to apical fragmentation of hyphae – a phenomenon observed in *Mycelia sterilia*. The major portion of the cytoplasm was highly vacuolated with darkly stained condensations especially in the apical portions (Fig., 1A-B).

SDA with Peptone- LP:

Smears prepared from growth on SDA with 2% additional peptone stained by LP revealed a larger percentage of mycelia. The hyphae showed darkly stained discrete areas, granulations and swollen apical cells. There was no branching or any type of sexual or asexual reproductive bodies even in this medium. Frequently the hyphal elements were spindle shaped (Fig., 1C).

SDA- Gram Staining:

In Gram stained smears from colonies on SDA, the cells appeared stouter and more deeply Gram positive, compared to growth in nutrient agar or broth. This increase in cell mass would be due to the presence of

high percentage of glucose in the medium and being a solid medium. Microscopic morphology appeared quite unique (Fig., 1D). The isolate showed a high degree of dimorphism in one and the same medium at an identical temperature of incubation. This feature along with presence of yeast like and highly irregular slender fragile mycelia like forms, absence of both sexual and asexual reproductive structures and apical fragmentation appearing like beads in chain resembled members of Mycelia sterilia (Web).

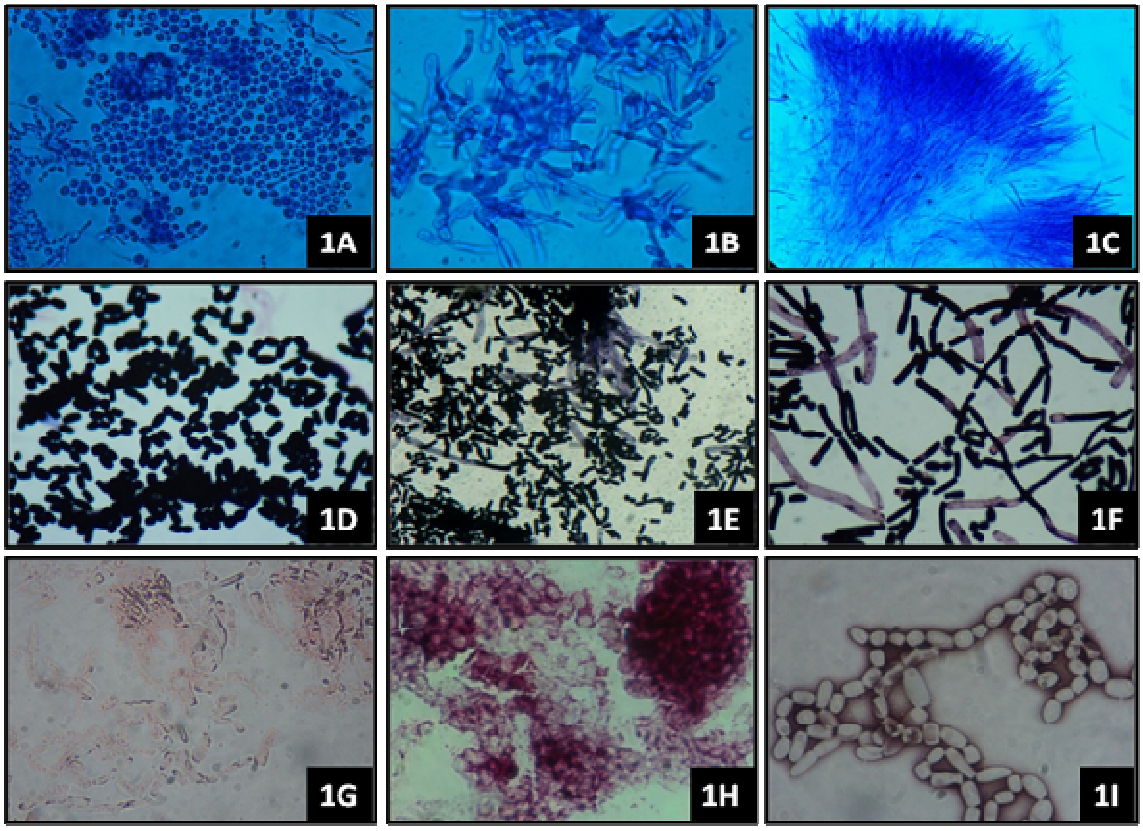

Figure 1 A - LP stained colony from SDA media; **1(B-C) –** LP stained colony from SDA media with additional 2% peptone; **1D** - Gram stained colony from SDA media; **1E** - Gram stained colony from Nutrient agar media with additional 10% Kraft liquor; **1F** -Gram stained colony from SDB media with additional 2% peptone and 0.1%Lignin; **1G** - Congo red stained colony from SDA media (100 X); **1H** - Mayer's Mucicarmine stained colony from SDA (100 X); **1I-** Sudan black positive colony from SDA media (100 X)

SDB:

In liquid media such as SDB, the mycelial forms were found in larger percentage in all fields along with cellular forms. A high medium pH (8.5) and incorporation of 0.1% lignin and 10% alkaline wood extract were found to enhance the growth of the isolate. Microscopic examination of these cultures demonstrated largely mycelia forms.

NB with glucose, peptone and lignin – Gram's: When the organism was grown on lignin in NB with 4% glucose, 2% additional peptone, the same transformation of yeast like cells into the brick shaped cells could be seen but the size of the rods were much larger and were also intensely stained. The longer mycelia like cells showed continuous protoplasm with no clear septa. The apical cells were swollen and club shaped. They

were also highly vacuolated and contained darkly stained granules (Fig., 1E and F). Production of hyphae, brick shaped cells, appearance of elongated cells with the entire protoplasm replaced by unstained vacuoles and secreted inclusion granules were a regular feature associated with the organism when cultured in lignin containing media. Hence it could be surmised that lignins induced the change in morphology to mycelia with abundant vacuoles which in turn were possibly mainly involved in the production of lignin degrading enzymes.

NB and NA with added peptone:

Gram Stain: The colonies on nutrient agar and broth contained mycelia mixed with shorter cell forms also. The NA plates with 0.1% to 2% peptone also showed only the elongated mycelia like growth but the cells were slender, regular and uniform in size. The mycelia were very slender, longer than those found in the basal media, highly granular and with large vacuoles filling the entire mycelia bits. Another notable feature about the morphology was the transformation. They were largely short regular brick shaped, deeply Gram positive cells replacing the typical yeast like cells found in SDA. Liquid media and solid media containing higher percentage of peptone promoted mycelia like forms. In brief incorporation of highly complex recalcitrant polymers like commercial lignins and alkali wood extract also induced high rate of mycelia formation. Another remarkable characteristic was that the mycelia formed in lignin containing media were thicker, larger, pleomorphic, irregular in shape with dense granulation even in very young cultures. Possibly these granules may be related to the metabolic utilization of lignins by the isolate. The young mycelia were highly vacuolated which also may have a bearing on production of lignin breakdown enzymes. Without any apparent sexual or asexual reproductive systems, the rapid growth and easy break-up of the hyphae enabled MVI.2011 to propagate and survive.

Special Stains: The presence of cytoplasmic poly-saccharides, proteins, lipids and other cell wall components on MVI.2011 were shown by positive reactions in special stains like Congo red, Mucicarmine and Sudan Black (Fig., 1 G-I). Literature shows that the majority of the lignin degrading fungi belong to basidiomycetes like *Phanerochete chrysosporium*, Trametes versicolor, Daedalea flavida and members of ascomycetes like Aspergillus sp (Arora D S, 2002). Although earlier studies reveal the potential of *Mycelia sterila* to decolourise waste water from molasses (Sirianuntapiboon et al., 1988 a, 1988b), there are hardly any reports of dimophic, nonsporing, rapid growers among fungi with physiological potentials to grow and break down lignin substrates so rapidly in 12-18 hours in high pH conditions, except Mycelia sterilia sp.

SEM

SEM analysis depict the clear dimorphism of MVI.2011. Samples from minimal media containing 0.5% peptone, 1% glucose, 2% agar and 0.1% lignin showed slender (1-2 μm), short (100-200 μm) mycelial like structures (Fig., 2 A-D). It was interesting to note formation of only yeast like cells when the isolate was grown on SDA. They were oval, spindle shaped or brick like forms. The size varied from 5-10 microns long and 2-5 microns in diameter (Fig., 2 E-F).

SEM of the isolate served to confirm the unique cell morphology and could be categorised as cellular or mycelia depending on the composition of the media particularly the organic nitrogen and complex polymers in media. This observation was quite significant since dimorphism is generally associated with primarily the temperature of incubation and pathogenesis in the case of human pathogenic fungi.

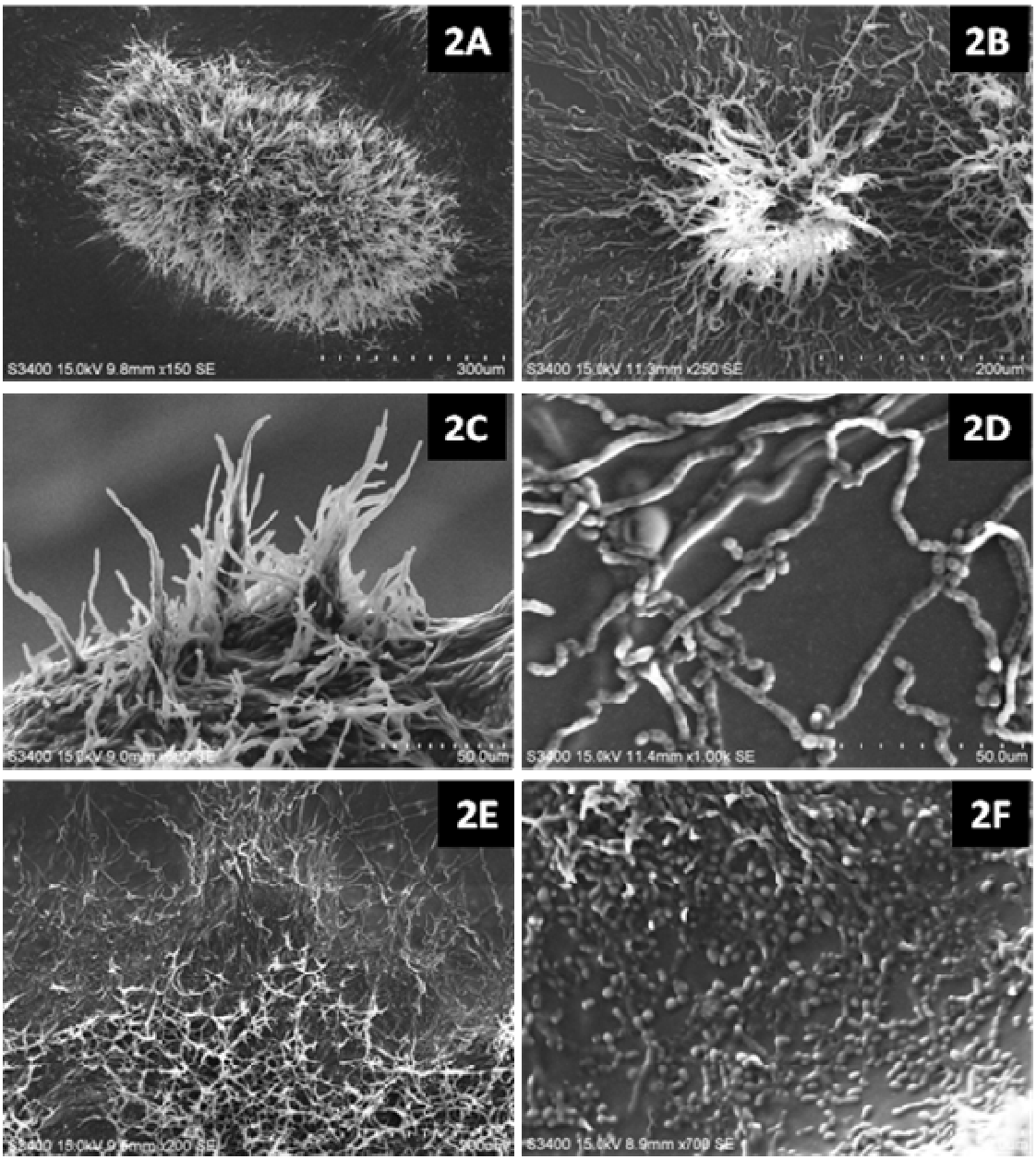

Figure 2: Scanning electron microscopy of MVI.2011- 2(A-D)- growth on minimal medium with 1% glucose, 0.5% peptone and 0.1% lignin showing predominant mycelia, slender, non uniform, highly irregular, pleomorphic beaded appearance with no specific reproductive structures; 2(E and F)-showing MVI.2011 on SDA without lignin. The morphology is predominantly cellular made up of spherical, oval, spindle shaped cells.

Screening for lignin degradation in solid and liquid media containing lignin and alkaline wood extract: On SDA with 0.1% lignin, growth could be perceived as early as eight hours. Within 18 hours the isolate produced large (3-5mm dia) dry looking dark brown colonies (Fig., 3A). On SDA with 1% alkaline wood extract also, there were similar large, creamy white, easily emulusfiable mucoid colonies (Fig., 3B).

MVI.2011 showed a drastic decolourization in SDB with 0.1 % (Fig., 3C) and 1% lignin, pH 8.5 (Fig., 3D). The organism grew rapidly producing more than 90% (apparent and by % absorption measurement at 380 nm) decolourisation, accompanied by heavy biomass in SDB with 10% alkaline wood extract, pH 8.5 (Fig., 3E). The remarkable property of the isolate was the decolourization within 12 hours of inoculation. The biomass was as dense and heavy at pH 8.5 as in the control SDB without lignin. Thus MVI.2011 was found to be capable of breaking down both commercial and natural lignins at a high pH.

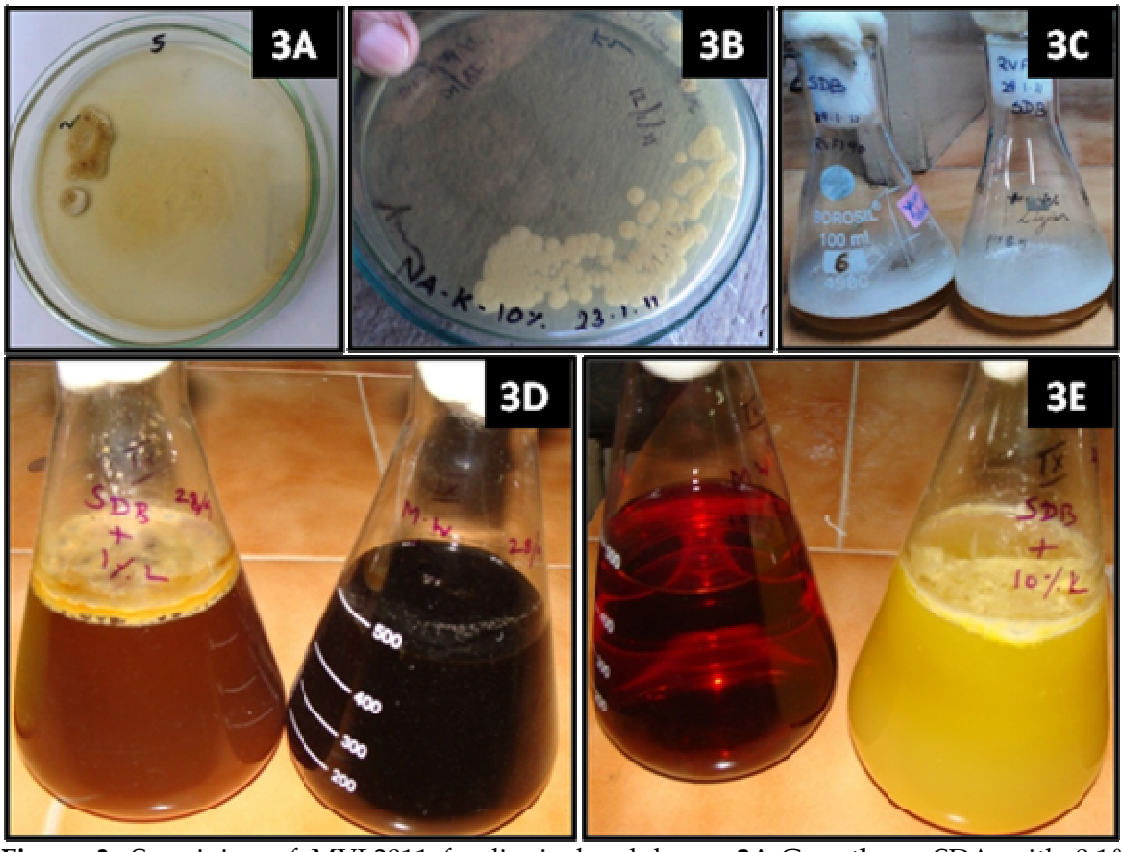

Figure 3- Screeining of MVI.2011 for lignin breakdown; **3A-**Growth on SDA with 0.1% lignin; **3B**- Growth on SDA with 1% alkali wood extract; **3C-** Growth in SDB and SDB + 0.1% lignin; **3D –** SDB with 1% lignin (pH8.5) with heavy surface growth, deposited biomass and high decolourisation; uninoculated medium control; **3E-** SDB with 10% alkaline wood extract (pH 8.5) control; 18 hours at ambient temperature, with high biomass, complete decolorisation and uniformly turbid and heavy biomass deposits.

Colony morphology on solid media and characteristics of broth culture:

NA & SDA: Growth of the isolate showed marked differences on various media. On NA the fungus showed moist, transparent, flat and pigmented colonies (Fig., 4A). The appearance, texture, size and colour were significantly distinct from those on SDA (Fig., 4B). The colonies on SDA were dry, white, opaque, larger, feathery with wavy margins, highly wrinkled but easily emulsifiable. They showed no soluble pigments any of the above media. In both the liquid media NB and SDB, there was uniform trurbidity with thick wrinkled surface growth, spreading along the walls of the tube above the surface.

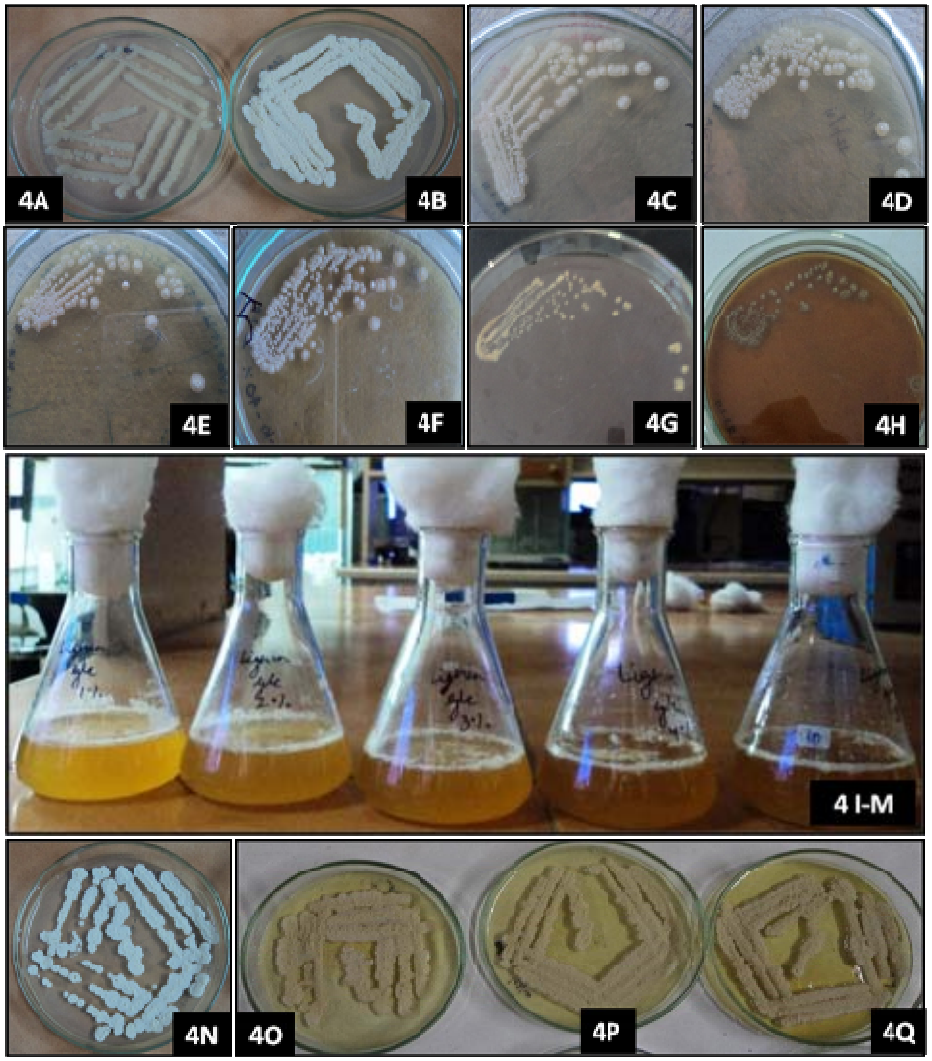

Figure 4: Surface growth of MVI.2011 on solid media, NA(**4 A**) and SDA(**4B)**; SDA with 10% Kraft liquor (**2C)**, 20% Kraft liquor (**2D)**, 30% Kraft liquor (**2E)** and 50% Kraft liquor (**2F)**; Nutrient agar media with 1% lignin (**4G),** 2% lignin (**4H)**; SDA with 0.1-2% peptone **(4I-L)**, Decolourisation of SDB containing 1%-5% lignin(**4M-Q)**.

NA with hot alkaline wood extract:
In NA with 1 to 50 % of Kraft liquor, the growth was large, creamy white, moist, mucoid yeast like, rapid, dense, forming large colonies 6-10 mm diameter within 12 to 18 hours. As the concentration of wood extract in the medium was increased from 1 to 50 %, the size, surface, texture and emulsifiability also showed marked changes with colonies showing increasing dryness, inhibited and size reduction. At 50%, the growth was minimal (Fig., 4 C-F). At 40%, the colonies were slightly smaller 4-6 mm diameter with regular margins.

NA with commercial pure lignin:
At 0.1% lignin concentration, the colonies were grey, 1-2mm diameter with entire margin and raised centre. When the concentration was increased the size of the colonies were very much reduced and growth was minimal at 2% lignin (Fig., 4 G-H). This finding viz. the unusual capability of the organism to tolerate and utilize lignins and other lignin derivatives at high

concentrations present in the crude hot alkaline wood extract is sure to make it a potent candidate for industrial application in the biodegradation of lignocellulosic waste as well as industrial effluents particularly pulp and paper, dye, textiles, distillery etc. In liquid media, the effect of increasing concentration of lignin was more evident. Growth as well as decolourisation was inversely proportional to the concentration (Fig., 4 I-M).

NA with glucose:

Colonies on NA plates with increasing concentrations of glucose (1, 2, 3 and 4%) and peptone (0.1, 1, 1.5 and 2%) (Fig 4 N-Q) were larger than the colonies on other plates. The NA plates showed production of yellowish brown diffusible pigment which was proportionate to the increase in peptone percentage. Increasing concentration of peptone showed production of diffusible pigment , richer, luxurious, moist and larger and smooth surfaced colonies. The yellow pigmentation and the moisture content increased with the increase in peptone concentration. The wrinkles started to disappear after 1% concentration. 1% peptone was found to support a healthy growth.

Till date, neither nitrogen supply nor nitrogen- limiting conditions in excess have been shown to enhance the decolourization rate. In contrast in *P. chrysosporium*, nitrogen limiting conditions stimulated decolourization (Nagarathnamma et al., 1999).

There are several reports on relation between time and concentration of colour in paper pulp effluent treatment. Liver et al. showed the decrease in colour reduction by *C. versicolor* by 65% after 3 days (Nagarathnamma et al., 1999). *T. versicolor* exhibited maximum AOX and colour reduction of 45% and 88% respectively in 48 h . Concentration of the Initial colour is a factor influencing colour reduction rate. Royer *et al.* reported maximum mycelia mean decolourization rate of 904 PCU g/l/day using *T. versicolor* (Basidiomycetes) mycelia pellets at an initial colour concentration of 3268 PCU (Berg., 1991; Royer., 1991)

The extra ordinary potential of the organism to grow and bring about >90% decolourisation in 1% lignin and 10% wood extract in basal minimal medium at initial pH 8.5 without being over grown by any environmental bacteria or fungi upto two years was proved experimentally. Growth, lignin break down (50-70%), colour reduction (>60-90%) in commercial lignin and wood extract respectively and drastic fall in pH (4-5) consequent to increase in biomass (19-20 g/l) occurred within 24 hours (fig., 3 D&E). There was thick surface growth in both substrates. The highest rate of lignin utilization, colour reduction and build up of biomass occurred in the first 12-18 hours. Further growth as well as colour reduction was low irrespective of volume of medium.

Influence of pH (medium SDB/24 hours static)

The fungus MVI 2011 exhibited remarkable capacity to grow at a rapid rate and built up high biomass at a wide pH range (5-11) with a maximum yeild of 35 g/l of biomass at pH 10. This was a highly significant feature useful for effluent treatment. This unique potential, in contradiction to the available fungi like fungus F3 that degrades only 65% of alkali lignin even after 8 days and the biodegradation of lignin by *Aspergillus sp* .F-3 occured only at initial pH 7.0 and excess acid or alkali conditions failed to support lignin degradation **(Table 1)** (Yang et al., 2011).

Influence of temperature

The biomass was highest at the ambient temperature 25^{0}C -32^{0}C (35-40 g/l biomass) and with increase in temperature upto 37^{0}C, the cell mass was decreased (16-20 g/l) and further increase to 40^{0}C reduced the biomass to 8-10 g/l and further increase inhibited the growth of the fungus. The pH also showed a gradation with final 3.5 at ambient temperature, 6.5 at 37^{o}C and 7.2 at

40°C. The inhibition of colour reduction beyond 45°C may be attributed to the denaturation of the chromophore degrading enzyme system (Nagarathnamma et al., 1999). Royer etal. also have reported that the decolourization of lignin by *C. Versisolor* pellets was inhibited at 40°C.

Potential of MVI.2011 to utilise lignin as sole carbon source

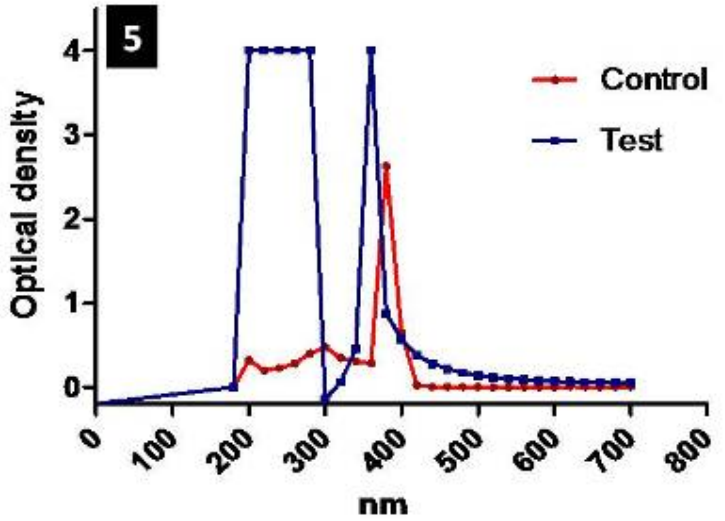

Figure 5; UV Vis spectrophotoscopic absorption pattern of uninoculated medium containing 0.1% lignin (Control) and culture supernatant from medium inoculated with MVI.2011, 18 hours

The control showed a single peak at 380 nm while the culture supernatant showed more than two clear peaks with absorption at 360 and between 200-300 nm. This indicates that the lignin substrate has been transformed into breakdown products whose absorption maxima are different from the substrate. The experiment demonstrates that the fungus MVI.2011 has utilised lignin as carbon source since the basal medium consisted of only minerals. The wet biomass in mineral medium with lignin was lesser (30%) than when grown in SDB or NB containing lignin (Fig., 5). No organism including white rot fungi is reported to utilize lignin as its carbon or energy source. Although it is believed that lignin degradation occurs to gain access to cellulose present within the cell (Cullen and Kersten., 2004).

Table 1: Influence of pH of medium on growth of MVI.2011

Time (hrs)	NB	SDB	NB + 2% P	NB + 0.1% L	SDB + 0.1% L	NB + 2% P + 0.1%L	SDB + 2% P + 0.1%L
0.	7.00	5.5	8.5	8.5	8.5	8.5	8.5
4.	7.07	5.0	7.1	8.0	7.5	7.3	7.4
8.	6.00	4.5	6.8	7.9	7.5	7.3	6.8
12.	6.03	4.5	6.5	8.0	7.5	6.9	6.0
16.	6.06	4.0	6.3	7.3	7.6	6.3	5.6
20.	6.09	3.5	6.3	7.6	7.6	6.3	4.9
24.	6.05	3.5	6.0	7.2	7.1	5.9	4.9

Growth curve

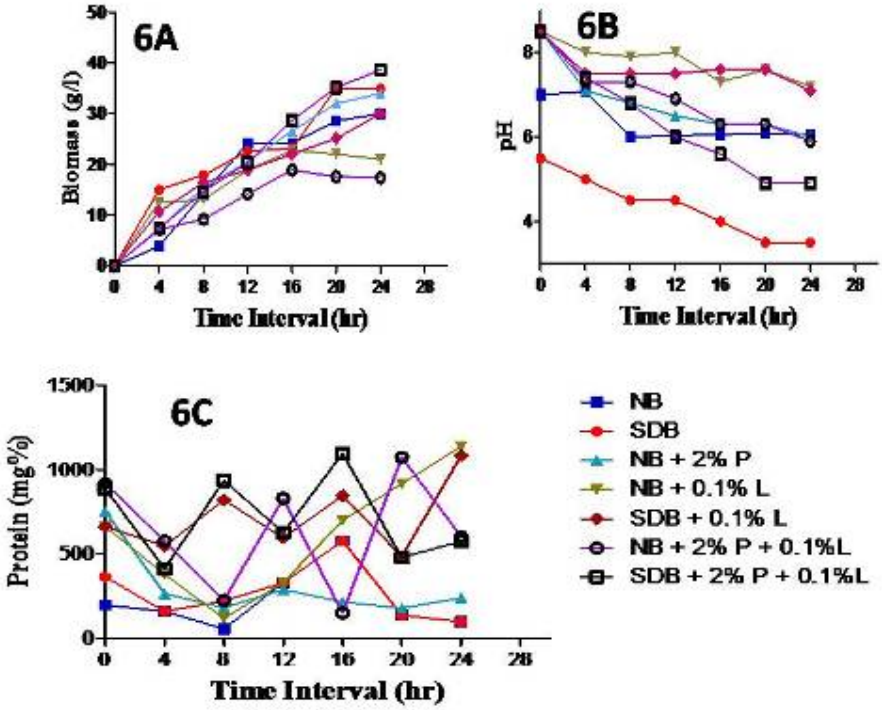

Figure 6 (A – C); Optimization of media containing lignin as a substrate attributed to wet biomass, pH and total protein content

The growth curve experiments were performed by inoculating 100 ml of different media SDB (pH 5.5), NB (pH 7), SDB+0.1% lignin(pH8), NB+0.1% lignin (pH8), NB+2% peptone(pH8), SDB+2% peptone+0.1% lignin(pH8) and NB+2% peptone+0.1% lignin(pH8) and incubation at ambient temperature in static condition for 24 hrs. At defined time intervals (every four hours) 5 ml of culture was drawn, centrifuged at 6000 pm for 20 min, the wet biomass was weighed and the culture supernatent was analysed for pH, total protein(Lowry etal) and wet biomass (Fig., 6 A-C).

NB: At four hours the biomass was 3.9g/l.The biomass buildup was also closely associated with decrease in pH and the maximum 30g/l was found at 20-24 hours . The pH fall was slow and reached 6.0 at 8 hours and there was no further decrease till 24 hours. The decrease in total proteins was more rapid in NB (57 mg%) than SDB (223 mg%) at 8 hours and later increased to 578 mg% at 16th hour and again lowerd to 100mg% at 24 hours in both media.

SDB: Growth was found to be very rapid with maximum biomass of 15 g/l within 4 hrs. At 24 hours the biomass reached maximum 35g/l. The protein content initially showed a drop followed by increase at different intervals. The pH dropped from 5.5 to 3.5 at 24 hours.

NB + 2% Peptone: Biomass was recorded maximum 34g/l at 24 hours. At four hours the biomass was 7.4g/l. The pH dropped from 8 to 5.9.

NB + 0.1% Lignin: At four hours the biomass was found to be 12.5g/l almost three times of that found in NB and the maximum biomass was only 21g/l compared to 30g/l in NB at 24 th hours. The pH dropped from 8 to 7.2. there was no further decrease over 24 hours which again

was clearly different from NB (pH 6.0). The protein value showed a steep decrease at 8 hours (120 mg%) and it was followed by a rapid steady increase to 1138mg% at 24 hours.

SDB + 0.1% Lignin: At four hours the biomass was 10.7g/l and reached 30g/l at 24 hours. The pH dropped from 8 to 7.1. The protein content was varied at different time intervals.

NB + 2% Peptone + 0.1% Lignin: The biomass was recorded with a maximum of 17.4 g/l at 24 hours. At four hours the biomass was 7.4 g/l. the pH dropped down from 8 to 5.9. the protein fluctuation was recorded as that of the other media

SDB+2% Peptone +0.1% Lignin: The biomass was 7.4 g/l at 4 hours and highest was 38.7 g/l at 24 hours of incubation. The pH dropped from 8 to 4.9. There was a drastic fluctuation in the protein level at various time intervals. *P. chrysosporium* also was reported to require a high concentration of sources of energy like glucose or cellulose (>10g/l) and high concentrations of oxygen to show significant decolourization. Archibald et al. reported a steady increase in decolourization with increase in glucose concentration from 1 to 3.5g/l with *Coriolus versicolor*. In the absence of glucose, no decolourization took place (Nagarathnamma et al., 1999).

Production of lignin hydrolysing enzymes

The enzyme activities were recorded for lignin peroxidase (9.39 units/ml), manganese peroxidase (2.093 units/ml) and laccase (3.5 units/ml) respectively at 12 hours of incubation. It has been reported that fungus like *Phlebia radiata* was an active wood degrader which produced important amounts of ligninolytic peroxidises and laccase. (Pavel et al., 1999). But the astonishing fact about MVI.2011 is the high quantity of enzyme synthesis, when compared to the enzyme production by other organisms studied till date like *T.versicolor, D.flavida, D.squalens, P.chrysosporium* etc which have been reported to synthesise negligible quantities of lignin degrading enzymes with maximum activity only after 8 days of incubation (D.S.,Arora et al., 2002).

Our isolate coded MVI.2011 resembled members of *Mycelia sterilia* described as the renegades that have dispensed with any kind of spore production whatsoever when isolated and cultured in the laboratory. *Mycelia sterilia* reproduce by hyphal fragmentation (Fig., Mycelia sterilia A, web)and access new sources of food. When cultured in the lab mycelia sterilia are often found to grow very quickly and produce a loose fluffy colony (Fig., Mycelia sterilia B, web)

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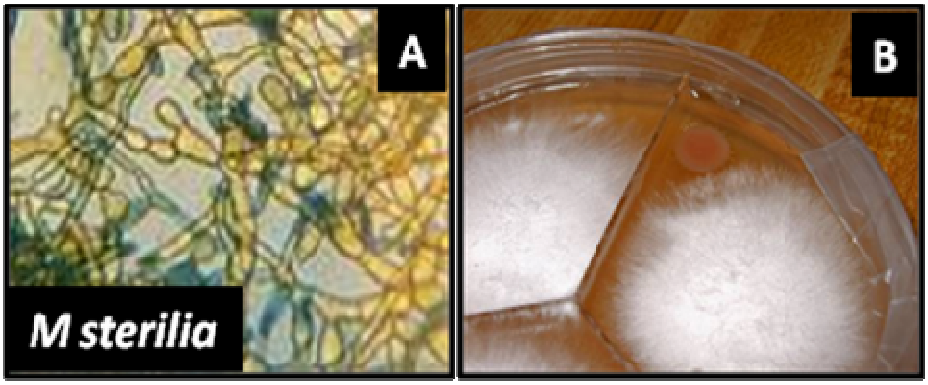
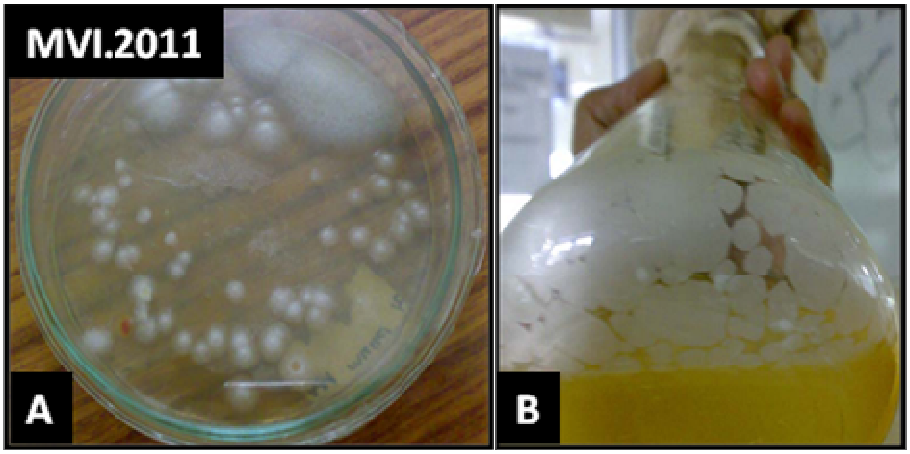


Mycelia sterilia on solid media and microscopic morphology (from web)

A similar phenomenon was observed with regard to MVI.2011.The hyphae (the basic vegetative cell type of fungi are aerial), which means they stick up into the air to a greater degree than is usually seen in other fungi. This growth habit increases the chance that the cells will be disturbed, break loose, and get carried away to start a new colony somewhere else (Fig.MVI.2011 A and B).



MVI.2011 original isolate **(A)** on SDA, 25^{o}C/12 hours and **(B)** colonies on the wall of the flask containing SDB, 25oC/Static incubation/12 hours (Thankamani V; 1998, 2002)

Although molecular classification of mycelia sterilia shows similarities with basidiomycetes, it is generally preferred to classify it under Deuteromycete. On a similar basis MVI.2011 has been concluded to be a Deuteromycete.

Conclusion

The MVI.2011, a rare dimorphic fungus with cellular and mycelia forms, reproduced asexually by breaking off from mycelia tips resembling budding and had a high lignin degrading potential. Based on the non sporing nature of the fungus it was classified as a Deuteromycete. The fungus in its mycelia form enhanced the lignin degradation. The optimum culture media was found to be minimal mineral medium with 0.5% peptone, 1% glucose and 0.1% lignin with an optimum temperature of 25-28^{0}C and pH 8.0-9.0. The wide range of pH and higher optimum pH for growth as well as utilization of lignin makes it industrially highly potent. The uniqueness of the organism was the rapid growth, tolerance to high concentrations of lignin, and, lignin degradation with maximum enzyme activity at 12 hours. The lignin degradation was clearly indicated by colour reduction within four hours of inoculation and was very closely associated with primary metabolism. Further detailed kinetics and optimization studies using immobilized cells of this Fungus in batch and continuous process will find wide application in the paper pulp industries, distilleries and tanneries in effluent treatment.

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This is dedicated to ***M**urali **V**aidyanatha **I**yer* (Former General Manager, Projects Africa, United Nations Mission In Sudan, UNMIS, Tri Star International) a genius, a synonym of benevolence, dynamism, excellence, diligence, sacrifice, commitment who could transform a desert into a lush green paradise, and who provided his personal money to fund this work).

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