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Preliminary Characterization of RV.F1.90 a Fungus with Respect to Biodegradation and Color Removal from Wood Kraft Effluent

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Abstract: RV.F1.90, a potent lignin degrading fungi was characterized in detail with respect to microscopic morphology, growth curve, optimization of growth parameters including degradation and color reduction in media containing varying concentration of paper kraft liquor and pure commercial lignin. The kraft effluent prepared in the laboratory was characterized with respect to pH, solids, BOD, COD, color, phenolics, lignins and tannins. Influence of parameters such as the concentration of lignins in kraft effluent pure commercial lignin, pH, temperature, aeration, static vs. shaking, carbon source, nitrogen source (organic and inorganic) and inoculum concentration on break down of lignins and polyphenols, biomass and color reduction was studied. The growth of RV.F1 was very rapid with sharp fall in pH within 18 to 20 hours. Color reduction was proportional to the decrease in concentration of kraft liquor and commercial lignin. Shaking and high temperature had an adverse effect on decolorization and build up of biomass while static incubation showed 94% color removal and biomass of 11.3 g/l. Glucose and peptone increased the rate of growth of organism and utilization of lignin significantly. The biodegradation and color removal was rapid and closely associated with primary metabolism of the fungal culture.

Key words: RV.F1.90, Fungus, Kraft effluent, Lignin degradation, Color removal

INTRODUCTION

The pulp and paper industry is one of the energy intensive and highly polluting sectors (Schumacher *et al*., 1999). The pulp and paper industry stands sixth largest polluter after oil, cement, leather, textile and steel industry which let out various pollutants to the environment (Ugurlu *et al*., 2006). The production of paper manufacturing consists of pulping, bleaching, chemical recovery and paper-making. Air emissions are: sulphur dioxide, nitrous oxides, particulate matter, methanol, polycyclic organic matter, hydrogen chloride, formaldehyde, chloroform, phenol and chlorinated phenolics, dioxins, furans and other chlorinated compounds, phosphates and suspended sediments (Ali *et al*., 2007). Solid wastes are sludge derived from their pulping and bleaching operations. Organic pollutants are cellulose fibre, dissolved organic compounds such as dissolved lignin compounds, carbohydrates, starch and hemi-cellulose (Lara *et al*., 2003).

Black color of the effluent is due to lignin and its derivatives which may increase water temperature and leads to decrease concentration of dissolved oxygen. Based on these problems, it is required to degrade lignin and color from paper and pulp industry (Nagarathnama *et al*., 1999). Physical and chemical method processes involve in lignin degradation are very expensive and economically not feasible and also removes only high molecular weight chlorinated lignins, suspended solids and COD but fail to remove BOD and low molecular weight lignin compounds which was fulfilled using biological methods (Singh *et al*., 2006).

Bacteria fail to degrade high molecular weight chlorolignin, the reason may be due to the fact that bacteria can produce intracellular enzyme capable of degrading lignin like structures, and the high molecular weight chlorolignin cannot penetrate the bacterial cell membrane. (Pallerla *et al*., 1996). Few microorganisms especially fungus *P.chrysosporium, Trametes, Phlebia*, *Aspergillus* sps, *Cladosporium* sps are commonly used for lignin degradation (Tuomela *et al*., 2000). In this paper we report the use of a new isolate for aerobic breakdown of synthetic black liquor and commercial lignin.

MATERIALS AND METHODS

Organism and Chemicals Used:

The pure culture used was previously isolated and reported by Dr.V.Thankamani. The fungal isolate was

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grown on SDA slopes and preserved at 4˚C. Medium for experiments consisted of basal mineral salt (Table.1) solution (Singh *et al*., 2007 and Singhal *et al*., 2009) nutrient broth, nutrient agar, SDA, SDB (Hi-Media), commercial lignin and wood extracts prepared in the laboratory.

Table 1: Composition of mineral salt solution

Media	gm/l
Na_2HPO_4. $2H_2O$	7.8 g
KH_2PO_4	6.8 g
$MgSO_4$	0.2 g
Ferric ammonium citrate	0.01 g
Sodium Nitrate	0.085 g

Synthetic Kraft Effluent Preparation:

Sawdust was suspended in distilled water containing bleaching powder (4 mg/l) and sodium hydroxide (0.1 M), pH adjusted to 10.0 to 11.0 autoclaved. The extract obtained was filtered through fine muslin cloth and stored in the cold room until completion of experiments. (Kortekaas *et al*., 1998)

Analytical Methods:

The basic characteristics of the synthetic waste such as pH, color (456 nm), phenols (280 nm), tannin and lignin (700 nm), total solids, volatile solids, fixed solids, BOD, COD were carried out as per APHA standard methods (APHA). All the experiments were done in triplicate for data consistency and accuracy.

Growth curve of RV.F1.90:

RV.F1.90 was inoculated into 100 ml of sterile SDB at pH 5.5 and incubated under static condition for 72 hours at ambient temperature (28 to 30ºC). Samples were drawn every four hours and analyzed for pH, wet biomass by centrifugation at 6000 rpm for 20 min at 20ºC.

Optimization of Growth Parameters for Maximal Biomass and Lignin Degradation:

Shake flask experiments were carried out using 100 ml of sterile SDB in 250 ml conical flasks inoculated with RV.F1.90 and incubated for 24 hours at ambient temperature with initial pH 9.0. Samples were analyzed for pH, biomass, color and lignin degradation as per standard APHA methods. Multiple flasks were setup to study the influence of various parameters such as pH (5.0, 7.0 and 9.0), temperature (ambient temperature, 37ºC, 50ºC), shaking (80 rpm) vs static condition, lignin (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1% w/v) and kraft effluent concentration (1, 2, 5, 7, 10, 20, 30, 40, and 50% v/v), inoculum concentration (2, 4, 6, 8, 10 and 12% v/v), various carbon sources (dextrose, maltose, lactose, sucrose and fructose at 1% w/v), organic sources (tryptone, peptone, yeast extract, beef extract and malt extract at 0.5% w/v) and inorganic nitrogen sources (ammonium nitrate, ammonium sulphate, sodium nitrate, sodium acetate and sodium phosphate at 0.5%w/v).

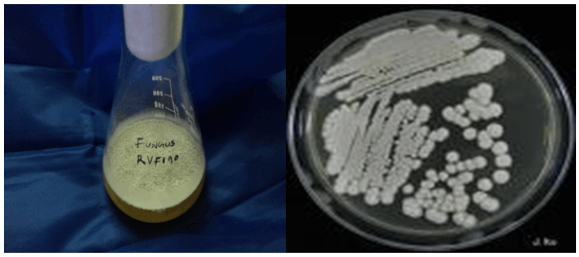
RESULTS AND DISCUSSION

Culture characteristics:

RV.F1.90 was inoculated on basal fungal medium and incubated at ambient temperature for 24 hrs. The colonies were creamy white dry, large, easily emulsifiable, non pigmented, opaque with delicate aerial mycelia in primary growth. It was non sporing and showed septate, slender, highly vacuolated cells in LPCB staining. There were no macroconidia, microconidia, thallospores or sporangiospores. In liquid medium it showed luxurious, rapid growth with a marked pellicle formation at the top of the medium and surface growth characteristics spread upwards on the walls of the tubes and flasks (Fig.1). The biomass was found to settle at the bottom leaving the column of broth clear. The isolate was identified by 18S rRNA sequencing and found to be *Trichosporon ashaii* (GU 323378) with 100% similarity shared by uncultured compost fungus (EMB/FM 173065) and uncultured Basidiomycete (AM901888) (Gen bank accession HQ197380) (Fig.2). The isolate is unique in being non sporing and with respect to the high rate of multiplication and requirement of oxygen in contradiction to most of the fungi reported.

Preliminary Characterization of Alkaline Extract:

The basic characteristics of the synthetic kraft effluent tested for amenability for microbial treatment showed high BOD, COD and volatile solids (Table.2)



(A) (B)

Fig. 1: Cultural characteristics of RV.F1.90 on both liquid and solid media. A - Liquid culture of RV.F1.90 showing heavy surface growth on the walls of the flask, B – Colony morphology of *Trichosporon ashaii* RV.F1.90 streaked on basal fungal medium (SDA)

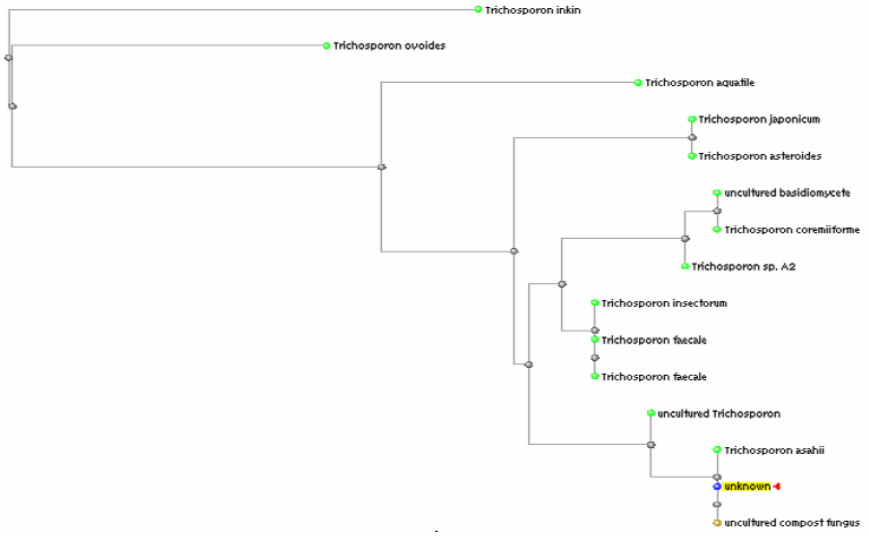


Fig. 2: Phylogenetic tree of RV.F1.90 culture

Table 2: Characteristics of Hot Alkaline extract of wood

Parameters	Values
pH	9 to 10
Total Solids	15.3 g/l
Fixed Solids	4.85 g/l
volatile Solids	10.45 g/l
BOD	5000 to 8000 mg/l
COD	40000 to 60000 mg/l
Color (456 nm)	2240 units
Phenolics(280nm)	20660 units
Lignin and Tannin (700 nm)	1530 µg/ml

Growth Curve: Influence of Initial Medium Ph on Growth and Lignin Degradation:

There was rapid fall in pH within 12 hr from 5 to 3.7 at initial pH 5. The observation was similar for pH 7.0 showing shift to pH 3.78 from 7.0 within 8 hours and 9.0 to 4.62 at initial pH 9.0 within 8 hours. Maximum growth (22.1 g/l) and color reduction (92%) was found in initial medium pH 9.0 compared to pH 7.0 (19.3 g/l biomass and 75.2% color reduction) and 5.0 (16.2 g/l biomass and 73% color reduction). This indicated that higher initial pH of medium favored the growth and degradation by the fungus (Fig. 3). A previous report (Sahoo *et al.*, 2005 and Damiano *et al*., 2003) showed alkaline pH as best suitable for lignin degradation by *Aspergillus fumigatus* and *Bacillus licheniformis* respectively. Since alkaline pH showed maximum decolorization, the lignin degrading enzyme might be expressed at this pH. In contrast some of the studies (Lara *et al*., 2003 and Belsare *et al*., 1988) reported that pH 4.0 to 6.0 range is best suitable for the treatment for paper and pulp effluent.

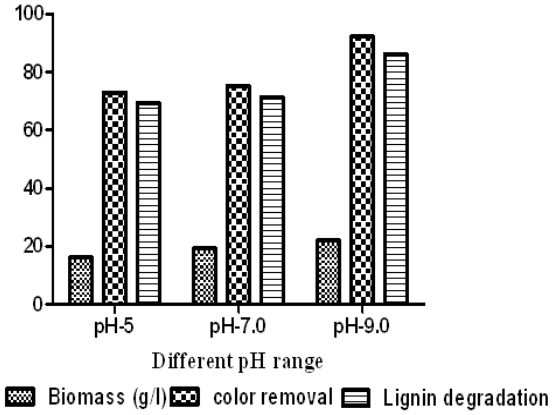


Fig. 3: Influence of initial medium pH on growth, color removal and lignin degradation

Influence of Temperature on Growth and Color Reduction:

Temperature plays major role in lignin degradation. Presence of lignin showed good growth with increase in biomass at ambient to 37 ºC from 12.85 to 15.7 g/l, but further increase in temperature (50ºC) showed marked reduction (9.65 g/l). Regarding color removal, ambient temperature showed 86.99% (83.25% lignin degradation), at 37ºC 80.9% (76.4% lignin degradation) and at 50ºC, 64.26% reduction (61.53% lignin degradation). Biomass and removal of color and lignin was adversely affected with increase in temperature. In SDB containing kraft effluent, effect of increase in temperature from ambient to 37 ºC was significant with respect to biomass from 19.65 to 17.3 g/l, further increase in temperature (50ºC) for growth caused decline of cell growth with a biomass of 10.6 g/l (Fig. 4 & 5). The biomass was closely associated with color reduction while degradation of the lignin components in the kraft effluent was enhanced markedly with increase in temperature of incubation. This strain prefer ambient temperature (30 to 35°C) for better growth and color reduction which is in associated with an previous report (Singhal *et al*., 2009) by *Emericella nidulans*. In contrast Tuomela *et al*., 2000 showed optimum temperature for lignin degradation as 40 to 50°C by fungal consortia.

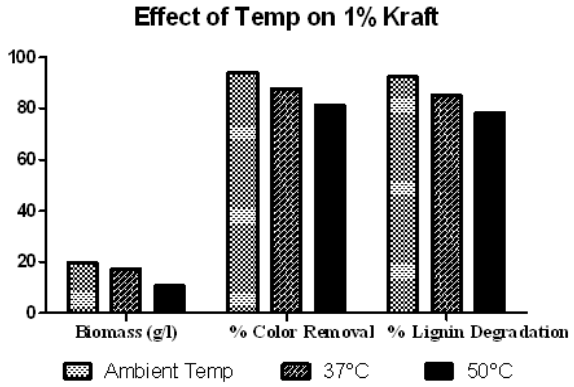


Fig. 4: Influence of different incubation temperature of RV.F1.90 with 1% kraft effluent on growth, color removal and lignin degradation.

Influence of Shaking vs Static:

Effect of shaking on growth and color reduction was determined by incubating the isolate at room temperature at 80 rpm (pH 9.0). In SDB with kraft effluent there was a uniform turbidity, good surface growth spreading high on the walls of the flask. Biomass ranged from 21.36 to 24.42 g/l in SDB and SDB containing kraft effluent respectively, while pure lignin suppressed the growth (12.84 g/l). pH dropped uniformly in all the three pH ranges. Color reductions achieved were 85.4 and 93.9% in lignin and kraft with 83.5 and 89.2% lignin breakdown in the lignin and kraft reaction mixture respectively (Fig. 6). Compared to shaking, static was found to be optimum (color reduction (94%) with biomass of 24.3 g/l may be due to the shear and tear of fungal mycelium at rapid moment (Singhal *et al*., 2009). This result is in contrast with Sahoo *et al*., 2005 showed maximum decolorization in shaking conditions by *Aspergillus flavus*.

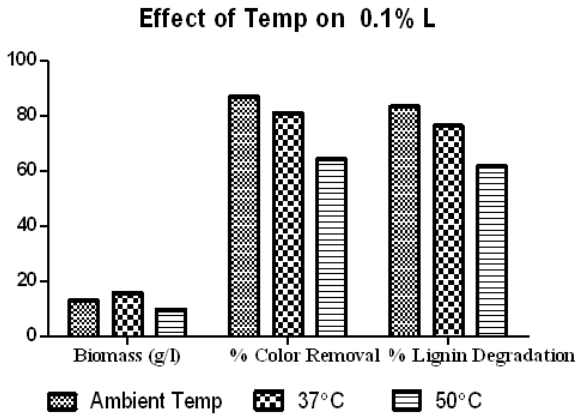


Fig. 5: Influence of different incubation temperature of RV.F1.90 with 0.1% commercial lignin on growth, color removal and lignin degradation.

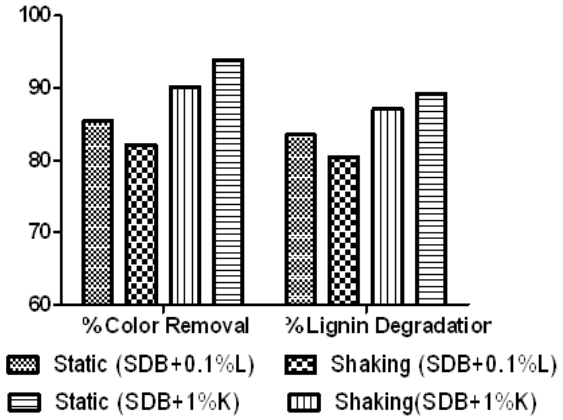


Fig. 6: Comparison of growth, color removal and lignin degradation of RV.F1.90 with respect to shaking and static condition

Influence of Inoculums Concentration:

To determine the effect of optimal inoculums concentration on growth and color reduction with 0.1% lignin, different size of inoculum (2, 4, 6, 8, 10 and 12%) was added to the medium. Growth and color reduction was increased with increase in inoculums concentration and maximum growth was found with 10% (27.66 g/l) which is coincided with color reduction (93% with 89% lignin degradation). Further increase of inoculums size there was not much difference with growth and color reduction. Less growth (10.2 g/l) and color reduction (59% with 57% lignin reduction) occur with 2% of inoculum (Fig.7).

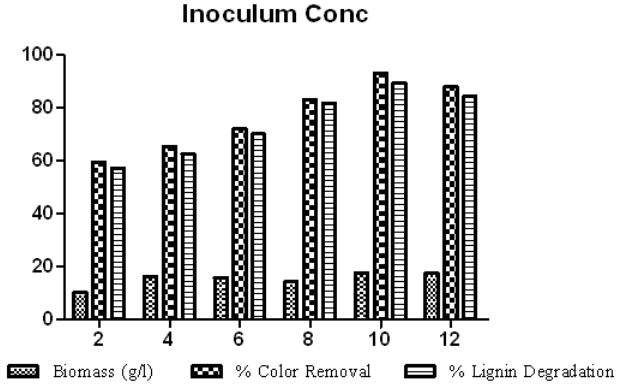


Fig. 7: Effect of different Inoculum concentration of RV.F1.90 on growth, color and lignin degradation

Influence of Carbon Sources:

To determine the effect of various carbon sources viz. dextrose, maltose, lactose, sucrose and fructose on growth, color removal and lignin degradation was done with 0.1% lignin. Fructose (28 g/l) and dextrose (17.95 g/l) were found to increase the biomass and showed 91% color reduction with 87% lignin degradation followed by dextrose showed 88% color reduction with 83.4% lignin degradation (Fig.8), this result is in accordance with Entry *et al*., 1993 showed maximum color reduction with dextrose and fructose by *Armillaria ostoyae.* When additional carbon source like glucose was added to the medium, color reduction was found to increase (Sahoo *et al*., 2005)

Influence of Organic and Inorganic Nitrogen Sources:

Various organic nitrogen sources (0.5%) like tryptone, peptone, yeast extract, beef extract and malt extract and different inorganic nitrogen sources like ammonium nitrate, ammonium sulphate, sodium nitrate, sodium acetate and sodium phosphate were added to the basal medium at pH 9.0. Maximum growth was found with tryptone (34 g/l), peptone (22.05 g/l), for inorganic nitrogen sources sodium nitrate (15.85 g/l) followed by ammonium nitrate (12.65 g/l) found to increase the biomass. Regarding color reduction same as that of biomass tryptone (92%) and peptone (89%) showed higher reduction in color (Fig.9). Similarly previous report with *Emericella nidulans* showed maximum color reduction with tryptone (Singhal *et al*., 2009).

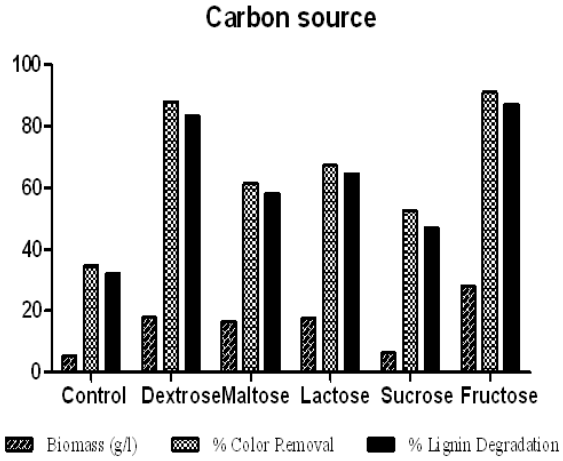


Fig. 8: Effect of different carbon sources on growth, color and lignin degradation of RV.F1.90.

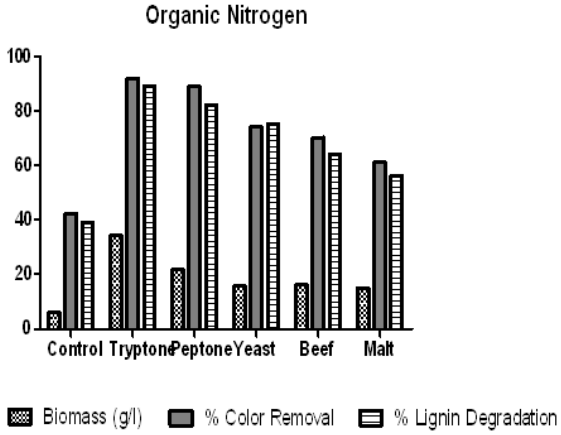


Fig. 9: Effect of Organic nitrogen sources on growth, color removal and lignin degradation of RV.F1.90

Influence of Increase in Substrate Concentration on Ph, Biomass and Lignin Degradation:

In 1% and 2% of kraft, there was heavy biomass, thick pellicle formation and almost complete color reduction. A clear gradation in color removal proportionate to gradual increase in concentration of kraft effluent was perceived. At 18-24 hours 24 hours irrespective of the concentration of lignin, pH dropped and reached a very low value. It varied from 3.66 to 5.7. The biomass ranged from 8.2 at 1% to 19.2 g/l at 0.2%. Cell

density showed a marked increase from 8.2 g/l to 19.2g/l with increase in substrate concentration from 0.1 to 0.2 g% lignin. Increase in the biomass during the first phase of growth from 0.1 up to 0.5 g% of lignin, though the concentration of glucose from SDB is similar in all tubes, clearly signifies utilization of lignin as a carbon source. At lignin concentration 0.6 – 1.0% growth and pH fall diminished reflecting on lignin breakdown as well as color removal (44 to 58%). Higher substrate concentrations inhibited growth of the organism. At lignin concentration 0.1% to 0.5 %, there was proportionate decrease in % color reduction from more than 95% to 60%. The formation of surface growth also decreased with increase in concentration of lignin (Fig.10 & 11). With lignin as a sole source of carbon, growth and decolorization was very minimal. A previous report (Perestelo *et al*., 1994) showed decline in growth rate and inhibitory effect of lignin when used as a sole source of carbon by *S.marcescens*. This may be due to the lack of adaptation on lignin (Hasen *et al*., 2009). In contrast, a report (Deschamps *et al*., 1980) showed 98% reduction of lignin by *Aeromonas* species when lignin used as a sole carbon source.

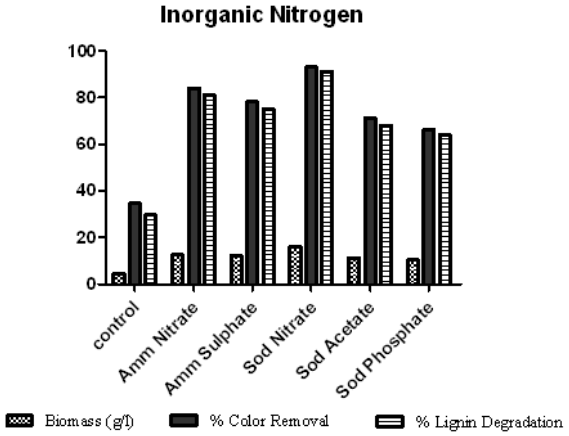


Fig. 10: Effect of inorganic nitrogen sources on growth, color removal and lignin degradation of RV.F1.90

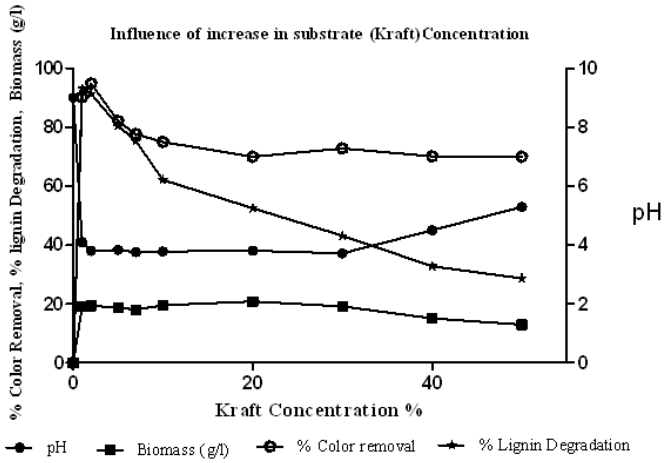


Fig. 11: Influence of Kraft concentration on pH, Biomass and % Color and lignin degradation of RV.F1.90

Conclusion:

A rapidly growing aerobic, non sporing highly alkalophilic Deuteromycete was isolated and characterized in detail with respect to degradation of pure commercial lignin as well as wood kraft effluent. There was complete degradation of the polyphenols compounds and lignin derivatives present in both the samples (within 18 to 24 hours). The uniqueness of the organism was preference for high alkalinity, production of high biomass, maximum rate of growth under static condition and ambient temperatures. These findings could make the isolate RV.F1.90 a potential candidate for large scale application in batch reactors for effective treatment of paper pulp industrial effluent especially the black liquor.

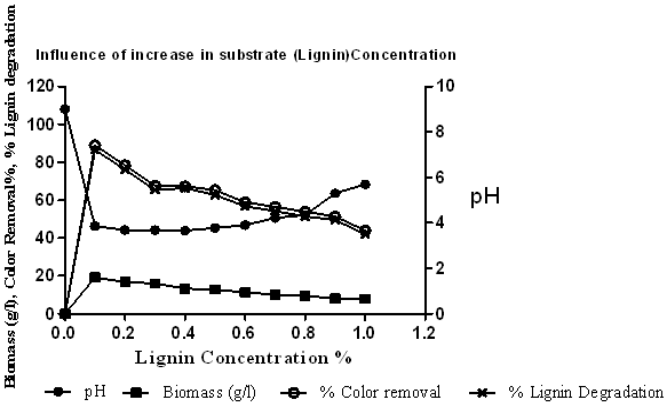


Fig. 12: Influence of Lignin concentration on pH, Biomass, % Color and lignin degradation of RV.F1.90

REFERENCES

Ali, M.T.R., Sreekrishnan, 2001. Aquatic toxicity from pulp and paper mill effluents: a review. *Adv. Environ. Res*, 5: 175-196.

APHA. 1995. "Standard Methods for the Examination of Water and Wastewater". American Public Health Association, American Water Works Association and American Water pollution Control Federation, 19th ed., Washington; USA;

Belsare, D.K., D.Y. Prasad, 1988. Decolorization of effluent from the bagasse-based pulp mills by white-rot fungus, *Schizophyllum commune*. Appl. Microbiol. Biotechnol., 28: 301-304.

Damiano, V.B., D.A. Bocchini, E. Gomes, R. Da Silva, 2003. Application of crude xylanase from *Bacillus licheniformis* 77-2 to the bleaching of eucalyptus Kraft pulp. World J. Microbiol.Biotechnol., 19: 139-144.

Deschamps, A.M., G. Mahoudeau, J.M. Lebeault, 1980. Fast degradation of kraft lignin by bacteria. Appl Microbiol Biotechnol., 9: 45-51.

Entry, J.A., P.K. Donnelly, K. Cromack, 1993. Effect of nitrogen and carbon sources on lignin and cellulose degradation by *Armillaria ostoyae*. *Euro J Forest Pathol.,* 23: 129-137.

Hassan, E., Abd-Elsalam., Amr A.El-Hanafy, 2009. Lignin biodegradation with ligninolytic bacterial strain and comparison of *Bacillus subtilis* and *Bacillus sp*. Isolated form Egyptiian soil. Amer Eura J. Agric Env Sci., 5(1): 39-44.

Kortekaas, S., G. Vidal, H.Y. Ling, G. Lettinga, A. Jim, 1998. Field, Anaerobic - Aerobic Treatment of Toxic Pulping Black Liquor with Upfront Effluent Recirculation. *J Ferm Bioeng.,* 86: 97-110.

Lara, M.A., A.J. Rodrguez-Malaver, O.J. Rojas, O. Holmquist, A.M. Gonzalez, J. Bullon, N. Penaloza, E. Araujo, 2003. Black liquor lignin biodegradation by *Trametes elegans. Int. Biodet. Biodegrad*., 52: 167-173.

Nagarathnamma, R., P. Bajpai, P.K. Bajpai, 1999. Studies on decolourization and detoxification of chlorinated lignin compounds in kraft bleaching effluents by *Ceriporiopsis subvermispora*. *Proces Biochem*., 34: 939-948.

Pallerla, S., P. Robert, Chambers, 1997. Characterisation of a Ca-Alginate-Immobilized *Trametes versicolor* bioreactor for decolorisation and AOX reduction of paper mill effluents. *Bioresour Technol*., 60: 1-8.

Perestelo, F., M.A. Falcon, A. Carnicero, A. Rodriguez, G.D. Fuente, 1994. Limited degradation of industrial, synthetic and natural lignins by *Serratia marcescens*. *Biotechnol. Lett*., 16: 299-302.

Sahoo, D.K., R. Gupta, 2005. Evaluation of ligninolytic microorganisms for efficient decolorization of a small pulp and paper mill effluent. *Proces Biochem.,* 40: 1573-1578.

Schumacher, K., J. Sathaye, 1999. India's Pulp and Paper Industry: Productivity and Energy Efficiency, Energy Analysis Program Environmental Energy Technologies Division Lawrence Berkeley National Laboratory Berkeley. CA 94720.

Singh, P., I.S. Thakur, 2006. Colour removal of anaerobically treated pulp and paper mill effluent by microorganisms in two steps bioreactor. *Bioresour Technol.,* 97: 218-223.

Singhal, A., I.S. Thakur,, 2009. Decolourization and detoxification of pulp and paper mill effluent by *Cryptococcus* sp. *Biochem Eng J*. 46: 21-27.

Ugurlu, M., M.H. Karaoğlu, I. Kula, 2006. Experimental Investigation of Chemical Oxygen Demand, Lignin and Phenol Removal from Paper Mill Effluents Using Three-Phase Three-Dimensional Electrode Reactor. *Polish J. of Environ.,* 15: 647-654.

Tuomela, M., M. Vikman, A. Hatakka, M. vaara, 2000. Biodegradation of lignin in a compost environment: a review. *Bioresour Technol*., 72: 169-183.