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Isolation of a novel soil fungus VT-NSK capable of utilizing the distillery spentwash and synthetic melanoidin – a preliminary report

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Soil samples collected from Mc Dowell's Distillery Ltd, Aleppey, India were screened for potent melanoidin degrading fungus. Seven fungi were obtained in pure culture. Out of the seven isolates obtained, one strain showing highest rate of melanoidin degradation was coded as VT-NSK and was characterized in detail. The isolate VT-NSK was systematically identified by microscopy, phylogeny by molecular techniques like 18 S rRNA gene sequencing followed by Blast analysis. The isolate VT-NSK was further screened for the melanoidin degrading activity on 1% synthetic melanoidin and 10% distillery effluent amended Czapek dox medium. The isolate showed a decolourization zone of 61mm diameter in 1% synthetic melanoidin and 69mm diameter in 10% distillery effluent amended czapek dox medium after 48hours of incubation. 18S r RNA gene sequencing of the isolate showed maximum alignment with *Cunninghamella blakesleeana* sp belonging to zygomycetes class. The sequence has been deposited in GenBank with Accession number JN570507.

Today, industries are the major source of pollutants to the ecosystem. Techniques such as distillation and fermentation were known to human civilization since ages. Distilling industries are those concerned with the production of ethanol and distilled spirits such as rum, whisky, brandy, gin and cordials and liquors. These days alcohol distilleries have emerged as a prominent sector world wide due to the large scale industrial applications of alcohol in pharmaceuticals, food, perfumery etc. Moreover alcohol is used as an alternate fuel. There are more than 319 distilleries producing 3.25 x 10^9 l of alcohol and generating 40.4 x 10^{10} l of wastewater annually in India alone (Pant and Adholeya 2007). Apart from other industries distillery is one of the industries which produces wastes characterized by high organic matter, disagreeable colour and odour. In fact alcohol distilleries are listed at the top in the " Red Category" industries as per the Ministry of Environment and Forests (MoEF) due to their high polluting potential (Tewari et al. 2007).

Today the most serious pollution threat caused by melanoidin to the environment is eutrophication in natural water bodies, reduction of sunlight penetration leading to decreased photosynthetic activity and dissolved oxygen concentration in lakes, rivers or lagoons etc. (Kumar et al, 1997a; 1997b). Nevertheless melanoidin pollution on land leads to reduction in soil alkalinity, inhibition of seed

germination etc. (Kannabiran and Pragasam, 1993). Hence, pretreatment of wastewaters is required in order to ensure its safe disposal into the environment (Mohana et al., 2007; Kumar and Chandra, 2006). Therefore, in order to characterize the chemical structure and bioremediation aspects the degradation and decolourisation of molasses melanoidins by chemical (Kim et al, 1985) and biological means (Ohmomo et al, 1987; Kumar et al, 1997b) have been attempted.

In order to abide by the stringent government policies on pollution control, distillery industries have now been forced to look for more effective treatment technologies which would not only be environment friendly but also cost effective. Due to high cost and generation of secondary pollutants, treatment of distillery wastewaters by physical or chemical methods was found not feasible and also difficult to treat by conventional biological methods (Pant and Adholeya 2007; Miyata et al. 2000) due to the antioxidant properties of melanoidins which render them toxic to aquatic macro and microorganisms (Chandra et al. 2008). In 2003 Central Pollution Control Board (CPCB) , the national agency responsible for environmental compliance, stipulated that distilleries should achieve zero discharge inland surface water courses by the end of 2005 (Tewari et al. 2007). Consequently, the wastewater needs to undergo extensive treatment in order to meet the stipulated environmental demands. Treating the spent wash, up to the standards prescribed by the respective regulatory authorities, still remains a challenge today.

Materials and methods

Preparation and characterization of synthetic Melanoidin

Synthetic melanoidin was prepared by dissolving equimolar amounts of analytical grade glucose and glycine (1mole each) and sodium carbonate (0.5 moles) in 1L deionized water. The sample was heated in an autoclave for 3 h at 121^{o} C (Migo et al. 1993). The resulting brown stock solution was lyophilized and stored in the refrigerator prior to use. Physical, chemical and biological characterization of the effluent was performed as per standard methods (APHA, 1995). Parameters analysed were pH, electrical conductivity, TDS, D.O, chlorides, nitrates, Biological Oxygen Demand (BOD 3 days), Chemical Oxygen Demand (COD) and reducing sugars.

Isolation of melanoidin degrading fungi

Soil samples were taken from various sites of Mc Dowell's Distillery Ltd., Aleppey, India such as the dumping areas of distillery effluent. Isolation of soil fungi was carried out in Czapek Dox broth (sucrose, 30g ; $NaNO_3$, 3g ; K_2HPO_4, 1g and $MgSO_4$ _$7H_2O$, 0.5 g ; KCl , 0.5 g ; $FeSO_4$, 0.01 g ; in 1L at pH 7.0) amended with 10% distillery effluent. Thus 100ml of Czapek dox broth containing 10% distillery effluent was prepared in a 500ml conical flask and five grams of soil sample were transferred to it. The conical flask was incubated at 30^{0}C for 2hours in an orbital shaker at 120rpm. An aliquot (0.1 ml) was spread on melanoidin-amended Czapek Dox agar plates and incubated at 30^{0}C overnight. The same sample was inoculated again into agar plates with basal mineral media containing 10% distillery effluent and incubated at 30^{0}C for 24-72h.

The colonies grown on primary solid media were subcultured on Sabouraud Dextrose Agar plates with 10% distillery effluent, pH (7.0). Cultures were further purified by subsequent subculturing and maintained on plain SDA slants at 4^0 C. Cultural characteristics were then examined.

Screening of the most potent isolate for melanoidin degradation

A total of seven cultures were obtained. Screening of the best isolate with reference to their melanoidin degrading activity (MDA) in 1% synthetic melanoidin and 10% distillery

effluent amended Czapek dox agar medium were done.

Czapek dox agar with 1% synthetic melanoidin and `10% distillery effluent were prepared and inoculated with an agar block of about 6mm diameter cut out from the tip of actively growing mycelium on a Sabouraud Dextrose Agar (SDA) plates . Plates were incubated at 30^0C for 48 hours. Zones of decolourization were measured in mm.

Characterization of VT-NSK

Selection of the most potent isolate showing the melanoidin degrading activity was done by measuring the diameter of the zone of clearance indicating decolourization around colonies. The isolate was coded as VT-NSK and was screened on production medium and subcultured on growth, maintenance medium. VT-NSK was used for further characterization.

Microscopic morphology of VT-NSK:

All the cultures were subjected to routine morphological tests and presence of spores using standard protocols. Smears were prepared from the colonies, stained with Lacto Phenol Cotton Blue and were examined under the microscope.

Molecular identification of VT-NSK by 18 S r-RNA gene sequencing:

This was carried out by standard protocols as follows (Chromous Biotech pvt Ltd, Bangalore, India) (William et al, 2000 and E O Wiley et al, 1991)

1. **Genomic DNA was isolated** from the pure culture pellet (Chromous Genomic Fungal DNA isolation kit RKT13) and loaded on 1 % agarose gel. Standard used was 1kb DNA Ladder (Chromous Cat. No. LAD03).The ~1.4kb rDNA fragment was amplified using high –fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward (ITS 5), reverse (ITS 4) Primer-18s rDNA primers. The PCR product was gel eluted and the purified PCR product was sequenced using 2 primers.

2. PCR Amplification conditions:

DNA: 1 µl; 18s Forward Primer 400ng; 18s Reverse Primer 400ng; dNTPs (2.5mM each) 4 µl; 10X Taq DNA Polymerase Assay Buffer 10 µl; Taq DNA Polymerase Enzyme (3U/ µl) 1 µl; Total reaction volume: 100 µl. All PCR reagents were of Chromous Make. Profile: Initial denaturation: 94 for 5 min; Denaturation: 94 for 30 sec; Annealing: 55 for 30 sec Extension: 72 for 2 min; Final extension: 72 for 15 min; $MgCl_2$: 1.5mM final conc. Numbers of Cycles were 35.

3. Forward and reverse primer sequence which was used for amplification of 18s rDNA sequence: Eukaryotes: 18s rRNA specific primer 18s Forward Primer: 5′-ggaagtaaaagtcgtaacaagg-3'; 18s Reverse Primer: 5′- tcctccgcttattgatatgc-3'. The PCR product size 1.5kb:

5. Thermal Cylcler ABI2720

6. Sequencing Reaction:

The Sequencing mix Composition and PCR Conditions were as follows: 10µl Sequencing Reaction: Big Dye Terminator; Ready Reaction Mix: 4µl. Template (100ng/ul): 1µl; Primer (10pmol): 2µl; Milli Q Water: 3µl

7. PCR Conditions: (25 cycles); Initial Denaturation : 96°C for 1min; Denaturation : 96°C for 10 sec; Hybridization : 50 °C for 5 sec; Elongation : 60 °C for 4 min;

8. Instrument and Chemistry Details: ABI 3130 Genetic Analyzer, Big Dye Terminator version 3.1".Cycle sequencing kit: Polymer & Capillary Array: POP_7 polymer; 50 cm Capillary Array.; Analysis protocol: BDTv3-KB-Denovo_v 5.2; Data Analysis: Seq Scape_ v 5.2 Software; Reaction Plate: Applied Biosystem Micro Amp Optical 96-Well Reaction plate

Phylogenetic Analaysis of VT-NSK

Phylogentic Tree Builder uses sequences aligned with System Software aligner. A distance matrix was generated using the Jukes-Cantor corrected distance model. When

generating the distance matrix, only alignment model positions were used, alignment inserts were ignored and the minimum comparable position was 200. The tree was created using Weighbor with alphabet size 4 and length size 1000.

Results

Characterization of synthetic melanoidin

Synthetic melanoidin was characterized as per the Standard Methods (APHA, 1995. It had a dark brown colour, highly acidic with a low pH (5.5) , electrical conductivity 3390 µS/cm, total solids 1750 mg/l , dissolved oxygen nil ,Chloride 354 mg/l, Nitrate 2mg/l , BOD 90-95,000 mg/l and COD 1,50,000 mg/l.

Isolation of melanoidin degrading fungi

Soil samples inoculated into basic mineral media with 10% distillery effluent at pH 7.0 and incubated at 30^0C overnight showed highly frothy and heavily turbid growth with good decolourization (Fig 1).

Screening of the most potent isolate for melanoidin degradation

Growth on Czapek dox agar with 10% distillery effluent after 48 hours of incubation at 30^0C showed seven types of fungal colonies varying in colony morphology and decolourization zone indicating melanoidin degradation. Cultures were maintained on sterile Sabourauds Dextrose Agar slants at 4^0C. The melanoidin degrading fungi (MDF) were coded as MDF1, MDF2, MDF3, MDF4, MDF5, MDF6 and MDF 7 (Fig 2). Colony characters are given in Table 1.

Fig 1: Soil samples inoculated in Mineral media containing distillery effluent, 30^0C/ 24 hours.

Table 1. Colony characters

Sl. No	Colony Code	Colony characters on Czepek dox agar with 10% distillery effluent and 1% synthetic melanoidin	Decolorization Zone Distillery Effluent	Synthetic Melanoidin
1	MDF1	Opaque and whitish cream colour circular, non sporing colonies no pigmentation/mucus around colony.	negligible	negligible
2	MDF2	Small pin tip colonies, orange coloured , circular, entire margin, smooth slightly convex and opaque. No mucilage or pigmentation around the colony	negligible	negligible
3	MDF3	Small pin tip colonies, creamy, smooth, entire margin, convex, circular and opaque, numerous. No mucilage or pigmentation	negligible	negligible
4	MDF4	Whitish cream, circular with smooth and wavy margin convex and opaque colony. No mucilage or pigment present	negligible	negligible
5	MDF5	Large sized (3-4 mm) colonies, cream colour, smooth, entire margin slightly convex, opaque and circular, no mucus or pigment present.	negligible	negligible
6	MDF6	Opaque, gray, thick, raised powdery colonies	negligible	negligible
7	MDF7	White sporing filamentous long thread like, hyphae	69mm	61mm

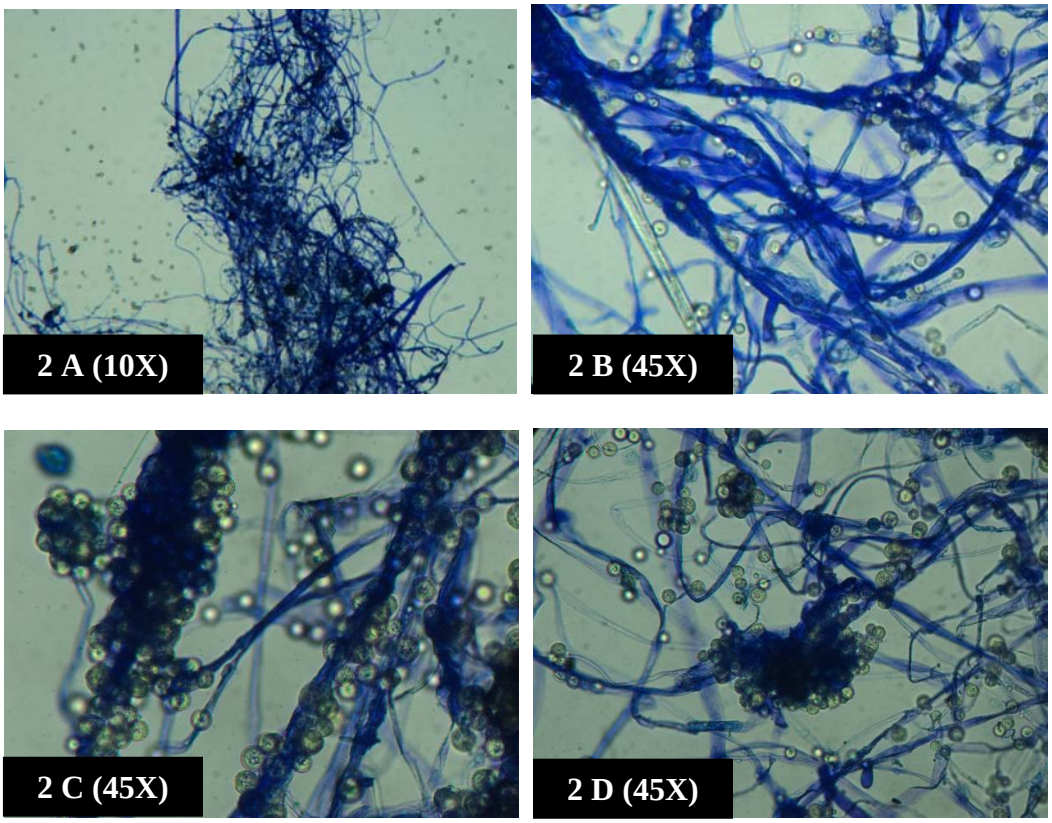


Fig 2 (A-D): Microscopic morphology of VT-NSK stained with Lactophenol Cotton Blue stain

Characterization of VT-NSK

Microscopic morphology of VT-NSK

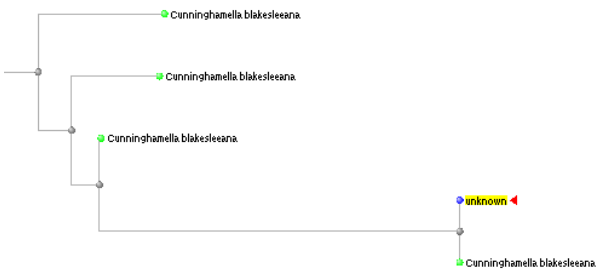
VT-NSK was a highly sporing fungus, darkly stained, non septated, highly branched.

Screening of VT-NSK for melanoidin degradation

Out of the seven isolates obtained, MDF 7 showing highest rate of melanoidin degradation was characterized in detail. The isolate was further screened for the melanoidin degrading activity on 1% synthetic melanoidin and 10% distillery effluent amended Czapek dox medium. Their growth and melanoidin degradation were monitored over 24-48 hours via decolourization zone. The isolate showed a decolourization zone of 61mm diameter in 1% synthetic melanoidin and 69mm diameter in 10% distillery effluent amended Czapek dox medium after 48hours of incubation.(Fig 3 and 4).

Phylogeny of VT-NSK

VT-NSK was found to be 95% similar with the 18s rRNA of *Cunnighamella blakesleeana*. Hence it was concluded that the isolate VT-NSK was a sporing zygomycete coming under the genus *Cunnighamella sp*



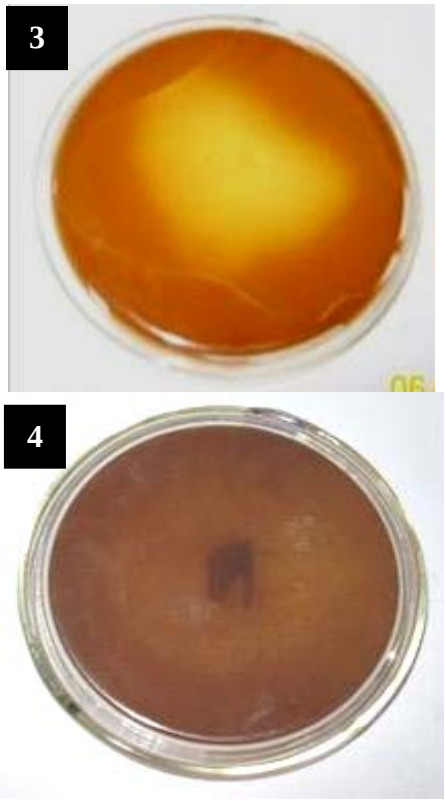


Fig 3 and 4: Decolourization zone of hydrolysis of MDF 90 Czapek dox agar + 1% synthetic melanoidin and 10 % distillery effluent respectively

Discussion

Ghosh et al. (2004) have also isolated some bacterial strains such as *Pseudomonas, Enterobacter, Stenotrophomonas, Aeromonas, Acinetobacter* and *Klebsiella* capable of degrading anaerobically digested spent wash from soil of effluent discharge site which brought about 44% COD reduction either singly or collectively. Jain et al. (2002) isolated three bacterial strains from the activated sludge of a distillery effluent identified as *B. megaterium, B. cereus* and *B. fragairae* which removed colour and COD from the distillery effluent in the range of 38-58 and 55-68%, respectively. Cibis et al. (2002) achieved biodegradation of potato slops (distillation residue) by a mixed population of bacteria under thermophilic conditions up to 60^{o} C which reduced COD removal up to 77% under non-optimal conditions. An Acetogenic strain was isolated by Sirianuntapiboon et al. (2004) from vegetables and juice samples which decolorized molasses pigment medium and anaerobically treated distillery effluent to 73-76% with in 5 days when supplemented with glucose and nitrogen sources. Kumar and Chandra (2004) successfully treated distillery effluent by a bacterium Bacillus thuringiensis followed by subsequent reduction of remaining load of pollutants by a macrophyte Spirodela polyrrhiza. In yet another investigation, two aerobic bacterial strains TA2 and TA4 isolated from sites contaminated with anaerobically treated distillery effluent (Asthana et al., 2001) resulted in 66% and 62% BOD reduction in anaerobically treated spentwash.

Similarly Kumar and Viswanathan (1991) had isolated some bacterial strains from sewage which were able to reduce COD by 80% in 4–5 days without any aeration. *Pseudomonas fluorescence* decolourised melanoidin wastewater (MWW) up to 76% under non-sterile conditions and up to 90% in sterile samples (Dahiya et al., 2001a).

Similarly, in 1985, Ohmomo et al. used *Coriolus versicolour* Ps4a for MSW decolourization and obtained 80% decolourization in darkness under optimum conditions. Jimnez et al. (2003) reported the treatment of distillery spent wash with ascomycetes group of fungi such as Penicillium sps. for example *P. decumbens, P. lignorum* which resulted in about 50% reduction in color and COD and 70% phenol removal. Sirianuntapiboon et al. (1995) studied that Rhizoctonia sp. D-90 decolourized molasses melanoidin medium and a synthetic melanoidin medium by 87.5 and 84.5% respectively, under experimental growth conditions. Recently, Pant and Adholeya (2007a,b) isolated three fungal strains and identified them by molecular methods as *Penicillium pinophilum* TERI DB1, *Alternaria gaisen* TERI DB6 and *Pleurotus florida* EM 1303 which were found to produce

liglotic enzymes and decolourized the effluent upto 50, 47 and 86 % respectively. Shayegan et al. (2005) used an *Aspergillus* species isolated from the soil for decolourization of anaerobically digested (UASB) and aerobically treated distillery wastewater which brought about 75% decolourization of diluted wastewater and 40% on using undiluted wastewater. Another strain of *Hansenula anomala* J 45-N-5 and I-44 isolated from soil, resulted in 74% reduction in total organic carbon (TOC) (Moriya et al., 1990).

Conclusion

The present work reports isolation, phylogenic identification, and, basic characterization of a highly potent uncommon fungus producing melanoididn degrading enzymes in significant levels. The isolate was identified as with *Cunninghamella blakesleeana sp* Accession number GeneBank JN570507. The isolates are unusual in both occurrence as well as enzymatic potential for melanoidin degradation. The isolate VT-NSK holds enormous prospects for more extensive molecular characterization and application in a wide range of industries from food, textile, confectionary, environmental, tannery, detergent, pharmaceutical, medical biotechnology as well as molecular biology and genetic engineering.

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