Advanced Blast Algorithm for Molecular Identification, Biodegradation and Decolorization of Synthetic Melanoidins Using Fungal Species Isolated from Soil and Spent Wash

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Introduction/Importance of Study: Distillery spent wash contains a high organic load as Melanoidins. It is generated due to the Millard reaction, which produces sugar and amino acids, leading to extensive water and soil pollution. Anaerobic digestion removes 60-70% COD and color, so post treatment is required for degradation by using fungal species as biological process.
Objectives and Novelty statement for this study: The study aims to isolate and identify fungal species for the degradation of synthetic melanoidins from spent wash using a cost-effective, low-toxicity, and environmentally friendly fungal-based biological process.
Material and Method: Three mixed fungal culture inoculums (spent wash, wet, and dry soil) and seven isolated fungal strains were examined on solid media that degraded and decolorized melanoidins at controlled pH 5.5, 25°C, 160 rpm for 3-5 days.
Result and Discussion: The results showed that mixed culture of spent wash removed the highest COD 91.8 %, color removal was 75.7 %, F-S6 isolate identified as *Penicillium* showed maximum soluble COD removal was 96.7 %, and F-S5 isolate identified as *Syncephalastrum* showed a maximum color removal was 98.8 %.
Concluding Remarks: It was concluded that the microbial process using fungal species was successfully applied to enhance degradation and decolorization to remove melanoidins. Furthermore, Gompertz Modeling was done to check the fitting of the curve at 680 nm Optical Density (OD) analysis for seven fungal strains with the following five factors significantly estimating maximum specific growth rate μM, Asymptote A, coefficient of determination R2, lag time λ, and goodness of fit.
Keywords: Synthetic Melanoidins, Biodegradation and Bioremediation, Indigenous Fungal Species, Spent Wash, Molecular Identification

Introduction:

Distillery industries are significant polluters in the world, generating spent wash waste by-products [1]. Spent Wash (SW) is characterized by high COD (80000 -190000 mg/L), BOD5 (30000-60000 mg/L), low pH (3-4.5), and a dark brown color with bad odor which leads to soil level pollution in case of seed germination, soil alkalinity, and damage crops [2-9]. The dark brown color is due to the presence of Melanoidins [6]. Melanoidins are dark brown to black pigments formed through the Maillard reaction between reducing sugars and amino acids [10], as shown in Figure 1.

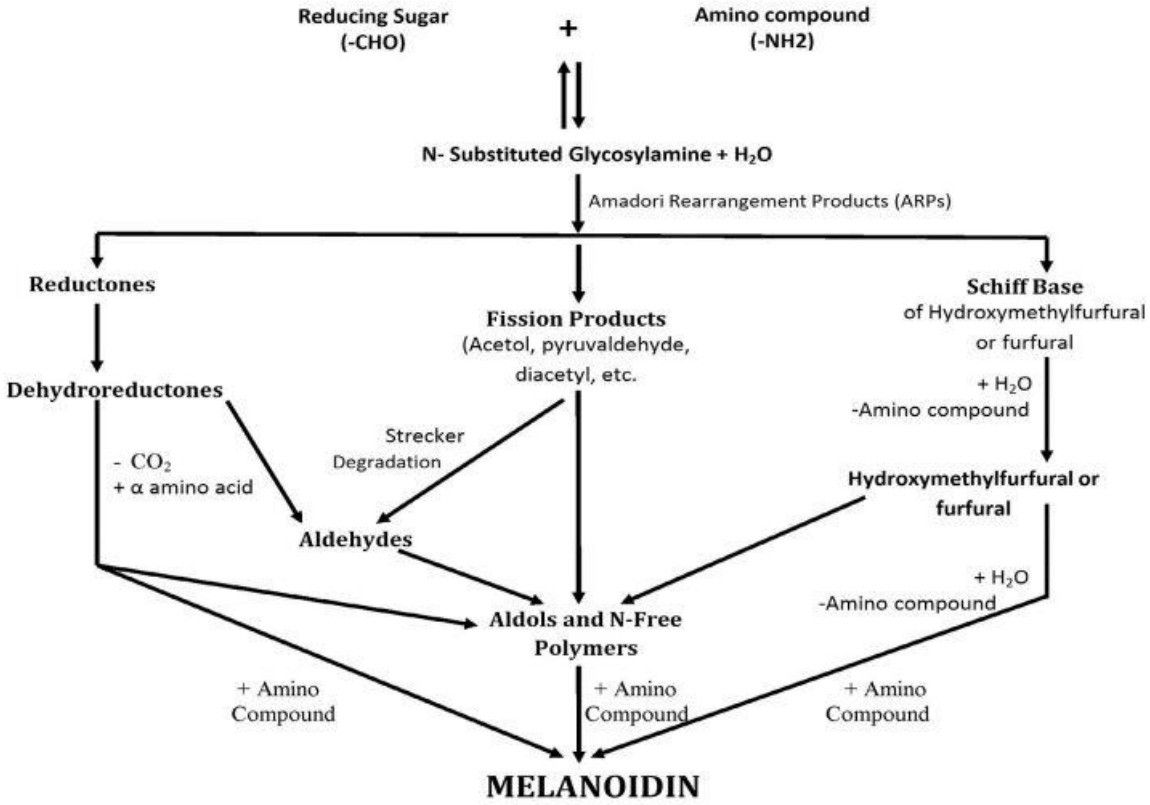


Figure.1. The basic structure of Melanoidins by [11].

Melanoidins are non-enzymatic polymers with a heterogeneous structure that contain nitrogen pigments [12]. It is an organic substance with the empirical formula $C_{17\text{-}18}H_{26\text{-}27}O_{10}N$ [13] and a molecular weight of Melanoidins polymers ranging between 30000-60000 Da [14].The Melanoidins have usually net negative charge [15]. Melanoidins create an antimicrobial activity to affect microbial growth [16]. Several physicochemical treatment methods were employed to treat Melanoidins from spent wash such as ozonation, chlorination, electrocoagulation, activated carbon, and advanced oxidation process. However, these methods are costly, time-consuming, energy-intensive, and require extensive optimization [7, 17-19]. In recent decades, bioremediation techniques for the removal of melanoidins from spent wash have emerged as cost-effective, eco-friendly solutions that also serve as biofertilizers for sustainable agriculture. [2] investigated that *Aspergillus* species founded from the abandoned site of discarded effluent spent wash for isolation and identification for degradation of melanoidins and compared with the different bacterial consortium and fungal species which show the highest decolorization (84.3%) and also a reduction in COD and BOD. [20] observed the *Phragmites australis* associated *rhizosphere* bacterial species isolated which shows the highest decolorization up to 75.5% of distillery effluent from 15 culturable bacterial sp. and its identification based on the 16S rRNA sequencing from the *rhizosphere* soil. The sole medium used as a carbon source for a level

reduction in COD and BOD. [21] study *acetogenic* bacteria from 170 strains, it displayed the highest decolorization yield (76.4 ± 3.2%) at pH 6.0 and 30 oC for five days in spend wash medium and the supplements used as 3% glucose, 0.5% extract yeast, 0.1% KH_2PO_4 and 0.05% $MgSO_4.7H_2O$ for additional decolorization as 32.3 ± 3.2% and 73.5 ± 3.5%, respectively

Many researchers have tried to use microbial organisms which might have less toxic and safe devices for bioremediation and management of spent wash with potential enzyme and ligninolytic activity [22, 23]. At present time, few reports on the fungal application are available for bioremediation [24-26]. In this research paper study, the use of spent wash, wet soil, dry soil, and fungal strains was done for degradation and de-colorization of Melanoidins from spent wash using 7 isolated fungal species with their morphological characteristics.

Objectives:

The objectives of this study to degrade synthetic Melanoidinds from distillery effluent spent wash using indigenous microbial species, and the novelty of this study is that it is cost-effective and environmentally friendly process with less toxicity.

Materials and Methods:

This study focused on the degradation of synthetic Melanoidins using indigenous microbial species found in spent wash and soil of digested distillery effluent site that has been generated after ethanol production as by product. The manifest of this experiment starts from sample collection of spent wash, and soil to degradation of microbial species to optimize different parameters like optical density, pH, Soluble Chemical Oxygen Demand (SCOD) and color. Furthermore, synthetic Melanoidin was prepared and identification of microbial species. The experimental overview of the methodology as shown in Figure 2.

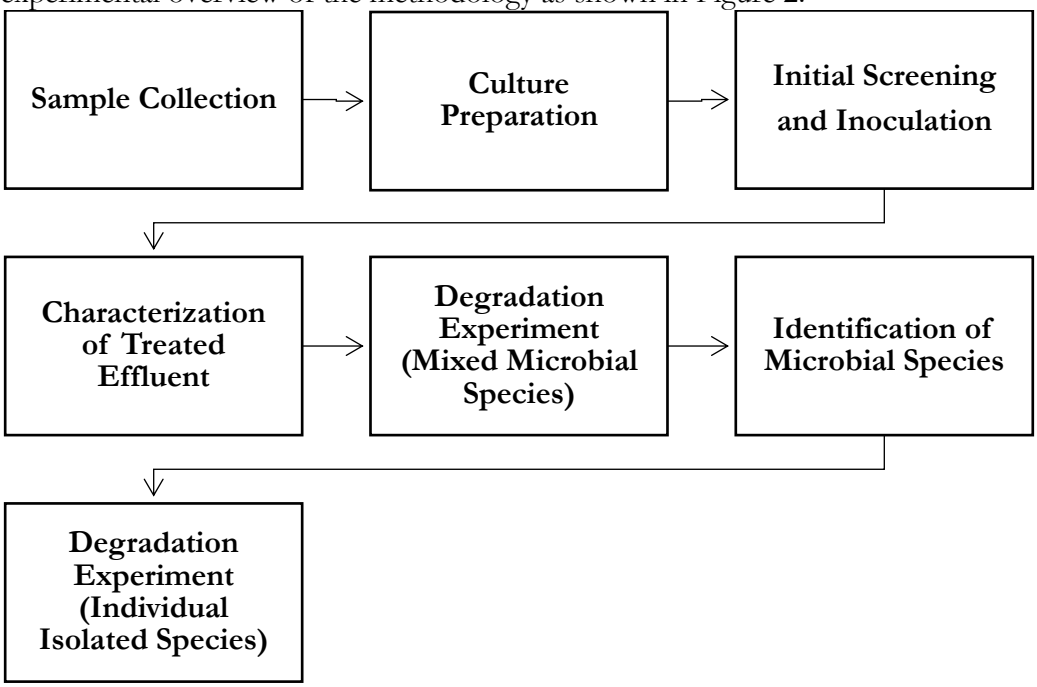


Figure.2. Process flow diagram of research methodology.

Sample collection:

Distillery Spent Wash (DSW) sample was aseptically collected in a sterilized plastic bottle from an outlet of an anaerobic digester, wet and dry soil samples were also collected in airtight plastic bags from the dumped site of Matol Distillery at Matiari district near Hyderabad, Sindh, Pakistan and were stored at 4°C in a refrigerator (Dawlance, Pakistan). The dark brown color of spent wash and 3.5-4.0 pH and parameters were determined using standard methods for the examination of water and wastewater [27-30].

Preparation of synthetic Melanoidins solution:

The synthetic Melanoidins solution was prepared by adding 4.5 g of $C_6H_{12}O_6$ (Scharlau's, Spain), 1.88 g $C_2H_5NO_2$ (Sam Chun, South Korea), and 0.42 g $NaHCO_3$ (Sam Chun, South Korea) with 100 mL of de-ionized water and then heated at 95°C for 7 h. After heating, 100 mL de-ionized water was added [31, 32]. The prepared synthetic Melanoidins had 25000 to 30000 mg/L COD value and pH was adjusted, 0.1 N NaOH (Dae-Jung, South Korea) and 0.1 N HCl (RCI Lab scan, Thailand) were exploited.

Preparation of liquid media for the degradation process:

The screening of liquid media was prepared for degradation experiment in 250 mL Erlenmeyer flask containing 100 mL medium solution of 5 g NH_4Cl (Dae-Jung, South Korea), 1 g KH_2PO_4 (Dae-Jung, South Korea), 0.5 g $MgSO_4$ (Dae-Jung, South Korea), and 0.5 g KCl (Dae-Jung, South Korea) with 12.5 g synthetic Melanoidins. The mixture was autoclaved at 121°C for 15 min and pH was adjusted to 5.5. For the growth of fungal cells in 50 mL falcon tube containing 25 mL Sabouraud dextrose broth (SDB) solution composition 1 g glucose (Oxoid, Ireland), 0.25 g peptone (Oxoid Ireland), 1 mL preserved colonies in 25 mL de-ionized water incubated in shaking incubator (Optic Ivy men, Spain) at 25°C, 160 rpm for 48 h [33, 34].

Degradation experiment of mixed isolated fungal culture:

Screening of fungal species having the ability to decolorize synthetic Melanoidins from the spent wash, wet soil, and dry soil was done by enrichment of mixed culture comprising of 5 g wet soil/ dry soil (WS/DS), 5 mL Spent Wash (SW) with 12.5 mL Synthetic Melanoidins (SM) in 100 mL with prepared medium solution then solution was incubated in an incubator shaker at 25°C, 160 rpm for 5 days. For pure 7 fungal species, the enrichment was done by adding a loopful colony from Petri plate, 12.5 mL synthetic Melanoidins with 87.5 mL prepared medium in 250 mL Erlenmeyer flask at pH 5.5, 25°C, 160 rpm for 50 h of incubation [1, 2].

Preparation of solid media for isolation and streaking of fungal species:

Solid media was prepared for initial screening of fungal species using two agar media one was Sabouraud dextrose agar (SDA, Oxoid, Ireland) containing 6.5 g SDA in 100 mL de-ionized water, and the other was technical agar (T.A, Oxoid, Ireland) containing 2 g T.A in 100 mL prepared medium solution then the medium was sterilized (Memmert, UK) at 121°C for 15 min. For isolation of fungal species the media was poured in Petri plates (90 mm) then take loopful degraded sample and streaks into agar plates in the laminar hood (Bio base, China) after that, plates were kept in an incubator for further analysis at 25°C for 5 days [12, 35].

Preservation of fungal culture:

Fungal colonies were preserved in a 1.5 mL Eppendorf tube containing 1 mL solution of 0.4 mL broth culture and 0.6 mL of 40% prepared glycerol stock (40 mL glycerol in 100 mL de-ionized water) at -20°C refrigerator (Selecta, Spain) for short term and at -80°C for long term preservation in a refrigerator (Panasonic, Japan) [2, 36, 37].

Degradation experiment of pure indigenous fungal species:

A pure single colony of an isolated fungal species was introduced into a 250 mL Erlenmeyer flask containing 100 mL of a solution composed of 12.5 mL synthetic Melanoidins (SM) and 87.5 mL prepared media. The pH of media was adjusted at 5.5 by using 1 N solution of KOH and 1 N solution of NaCl the solution was put in incubator shaker (Optic Ivymen) at 25°C and 160 rpm for 50 hrs. The experimental samples were taken at different time intervals 0, 2, 4, 6, 22, 24, 26, 46, 48, and 50 h [38, 39]

Analysis of synthetic Melanoidins degradation experiment:

The degradation experiment using indigeneous fungal species was conducted to determine the reduction in SCOD removal using COD digester and analyzer (Lovibond, UK) [29]. The decrease in color was measured using a double beam UV-visible spectrophotometer (Perkin Elmer, US) at 475 nm absorbance. A pure loopful culture from SDA and TA plates was transferred into a 250 mL Erlenmeyer flask containing 12.5 mL synthetic Melanoidins with 87.5 mL liquid medium composition.

Formula for COD and Color removal of synthetic Melanoidins:

The degradation and decolorization percent removal was analyzed using the following formulas [38, 39].

$$\text{SCOD Removal \%} = \frac{\text{C in} - \text{C out}}{\text{C in}} \text{X } 100$$

$$\text{Color absorbance removal \%} = \frac{\text{C in} - \text{C out}}{\text{C in}} \text{X100}$$

Morphological identification of pure isolated fungal species:

Pure fungal isolated species were identified through colony morphology, simple staining, macroscopic and microscopic observation on fluorescence microscopy (Zeiss, Germany) using the slide culture method of SDA and TA as shown in Table-2. All the seven fungal isolates were analyzed, characterized, identified and compared with different manual of the literature of Bergey's manual and Microbiological applications for identification and characterization purpose [40-42].

Molecular Identification of fungal genes:

DNA Extraction:

The extraction of DNA of 6 fungal strains FS-1 to FS-07 were carried out from isolated cultured liquid media by using CTAB buffer method [43]. In order to remove RNA contamination, the extracted DNA samples were reacted with RNase solution of 0.4 mg/mL and quantity was checked with Qubit 4.0 Fluorometer of Invitrogen USA.

Agarose Gel Electrophoresis:

To separate fragments of DNA 1% agarose gel was used and prepared with different composition added 0.5 g agarose of MoleQule in 10x 50 mL TAE buffer then Shaked well, and heated at 60°C for 5 min in oven then cool down and add 2.5 µL of Ethidium Bromide. The comb was fixed in racks and solution was poured then 2% dsDNA HS reagent dye were added and mixed. Moreover, 6 µL ladder was used to find size of fragment and samples were put in Electrophoresis at 80 V for 40 min of Mercury Alpha then taken the image in UV gel studio analytic-gen SA[2].

PCR (Polymerase Chain Reaction) Amplification of Fungal strains:

The extracted DNA from unknown fungal strains were amplified by PCR (Polymerase Chain Reaction). Oligonucleotide universal primers of forward and reverse were used for amplification of DNA for Fungal species. The forward primers ITS1 5' TCC GTA GGT GAA CCT GCG G 3' and reverse primers ITS4 5' TCC TCC GCT TAT TGA TAT GC 3' [44]. A typical mixture of PCR 50 µL having composition 28 µL sterile Milli-Q water, 2.5 µL of 50 mM MgCl2, 5 µL of 10x Taq buffer, 4.0 µL forward Primer, 4.0 µL Reverse primer, 5.0 µL genomic DNA, 1.0 µL dNTPs, and 0.5 µL Taq polymerase were added into fungal samples. In this experiment the thermal cycle for initial denaturation were done at 94°C for 2 min for 35 cycles of denaturation DNA for 0 sec, annealing of primers at 56 °C for 10 sec, the elongation strand was done at 72 °C for 30 sec, the final elongation a 75°C for 2 min in BIOMETRA TRIO DLAB. PCR product was checked in the gel for size confirmation by using 10 µL PCR product added with 2 µL loading dye in 1% gel electrophoresis at 6µL Hyper ladder size at 100 bps (base pairs).

Phylogenetic Tree:

Fungal genes of ITS1/ITS4 primers were compared in greedy algorithm BLAST (Basic Local Alignment Search Tool) aligning DNA sequence using NCBI Blasting of morphological identified species with ITS1/ITS4 region and phylogenetic tree relationship were prepared in Mega11 software.

Fitting kinetic model using statical analysis to optimize parameters of optical density (OD) data:

A kinetic model was fitted using statistical analysis to optimize the parameters of optical density (OD) data. The modified Gompertz model explained the lag phase independent of the

growth of the kinetics. This model interprets microbial parameters and plots the time to detection against initial inoculum size under known environmental conditions, yielding a straight-line relationship with a specific growth rate [45]. This model tells the relationship of a variable with lag time, specific growth rate, and coefficient of determination which reparametrized [46] and goodness of fit (GoF) value also used as the null hypothesis to compare the observed data with expected data under model through statical fit as Chi-square.

Goodness of fit Equation: The goodness fit model formula as shown below.

$$GoF(x)^2 = \sum_{i=1}^{k} \left(\frac{O_i - E_i}{E_i} \right)$$

Where Oi is the initial optical density value, Ei is the expected optical density value, k is no of intervals (experiments), and 'i' is the sum of several values.

Modified Gompertz Modeling Equation: The modified Gompertz model formula as shown below.

$$Modified\ Gompertz\ Model\ (P) = A \times exp\left\{-exp\left[\frac{\mu m \times e}{A}(\lambda - t) + 1\right]\right\}$$

Where A is the Asymptote, which means predicted value, μm is the specific growth rate, λ is the lag time, and t is the experimental time in h.

Results:

Optimization results of degraded mixed fungal isolates experiment:

The optimization characteristics of three different fungal compositions with controlled one for treating synthetic Melanoidins were determined after five days and compared with controlled condition. The concentrations of different distillery effluents spent wash, wet soil, and dry soil (12.5 g, 5 g, and 5 g), respectively, were tested for COD reduction and decolorization in all three inoculums as shown in Figure 3a. These effluents had initial COD 44100, 40400, and 43400 mg/L respectively, while the initial color absorbance was 3.43, 3.36, and 4.40 nm respectively. The other parameters were kept constant; pH 5.5, RPM 150, and 25°C temperature. In all fungal inoculated spent wash showed a maximum COD reduction of 91.8 % in the spent wash as compared to wet soil and dry soil, which were 81.2 % and 87 % respectively at controlled rpm 150, 25°C temperature, and acidic pH range 5.5. These results show that substantial inoculum is required for synthetic Melanoidins before actual COD reduction or color removal can be achieved because mix culture didn't remove all Melanoidins compounds due to 2% Melanoidins in spent wash and 98% other compounds which were identified by using GC-MS analysis.

Optimization parameters on the degradation process of indigenous fungal species:

Change in pH on the degradation process of fungal species:

Different final pH variation values of 5.5-6.5 were observed after the degradation of Melanoidins by fungal species at optimal temperature, revolutions, and incubation periods, as presented in Supplementary Table 1. The highest Melanoidins degradation (COD) removal occurred at pH 6.08 for isolate F-S6 as *Penicillium*, and the highest Melanoidins decolorization (color) removal occurred at pH 6.07 for isolate F-S5 as *Syncephalastrum,* indicating these cultured isolates were at optimal growth condition. The preferred pH variation at every 2 h interval for the growth of individual consortia was shown in Figure 3b. The remarkable observation is that pH is more towards acidic (5.9-6.19) condition during three days of incubation for degradation, which validates acidity of the medium through degradation process. Further, it should reflect maximum degradation and decolorization activities for carrying out enzymes.

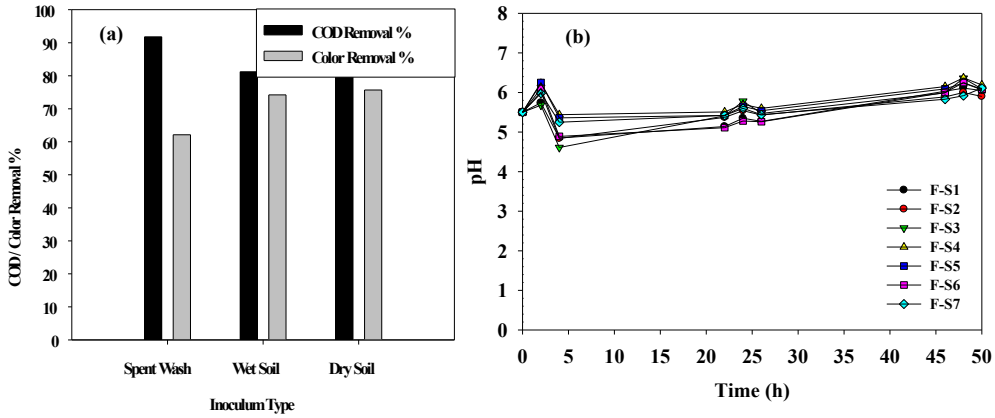


Figure 3. Optimization parameters using **a** mixed fungal culture of spent wash, wet soil, and dry soil on Melanoidins process **b** change in pH on degradation process.

Change in optical density on the degradation process of fungal species:

The final change in optical density was measured to examine the growth pattern of each fungal isolate over time for degradation and decolorization. Initial measurements were taken within 24 hours, as the samples had already transitioned from the lag phase to the log phase. A gradual increase of 24-50 h, the growth was increased, which ultimately enhanced the decolorization and degradation of Melanoidins (Supplementary Table 1). The best optical density at 680 nm absorbance for maximum COD removal by F-S6 isolate and color removal by F-S5 isolate was observed at 1.38 and 1.64, respectively. The growth pattern every two h intervals of time is shown in Figure 4a, and Figure 4b. This variation examined those organisms efficiently utilize metabolizable compound components to degrade melanoidins.

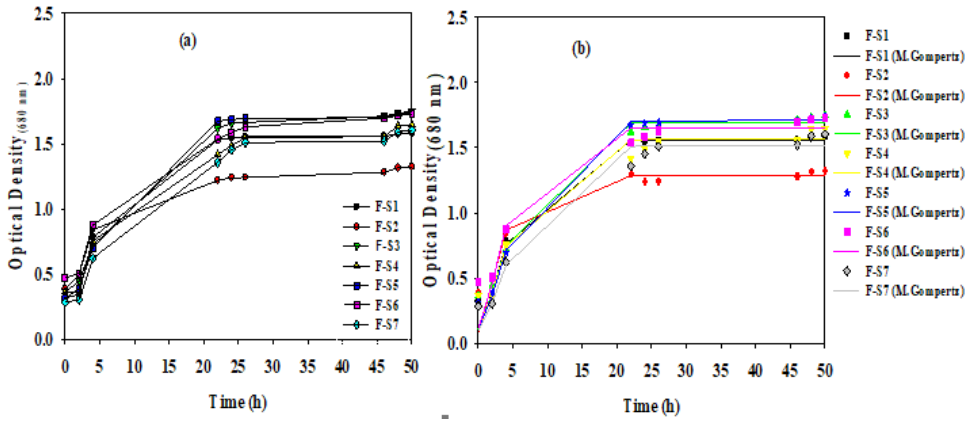


Figure 4. Summary of estimated optical density parameters using fungal species on Melanoidins degradation process: **a** change in optical density, **b** changes in optical density using M. Gompertz model.

Removal % of SCOD using fungal isolates:

The reduction in COD was analyzed as an increase in the time function. A 50-h degradation process with 12.5 % of synthetic Melanoidins medium at 25 °C was used, and it indicates more than 50 % COD reduction was achieved, which is presented in Supplementary Table 1. From 7 fungal isolates, four isolates removed COD more than 80 %. The maximum COD reduction was obtained from F-S6, identified as *Penicillium*, which removed 96.7% of COD. This significant reduction occurred between 2 and 50 hours, as shown in Figure 5a. These results showed that an increase in the growth of fungal species would increase COD reduction. The spent wash has only 2% melanoidins and 98% other compounds, so SCOD removed dew compounds as compared with color.

Removal % of de-colorization using fungal species:

The decolorization of synthetic melanoidins was monitored by measuring the decrease in optical density at 475 nm over time. Its yield was obtained as a reduction in absorbance against the initial value. Out of 7 isolates, each isolate decolorized synthetic Melanoidins above 90 % removal with 50 h incubation, as shown in Supplementary Table 1. It was observed that all isolates had decolorized ability, and three isolates named F-S4, F-S5, and F-S6 decolorized more than 90 %removal within 24 h of incubation, as shown in Figure.5b. It was clearly shown that isolate F-S5 identified as Penicillium has a maximum decolorization of 98.8 %. The color removed more efficiency than SCOD due to more degradation efficiency to degrade other compounds.

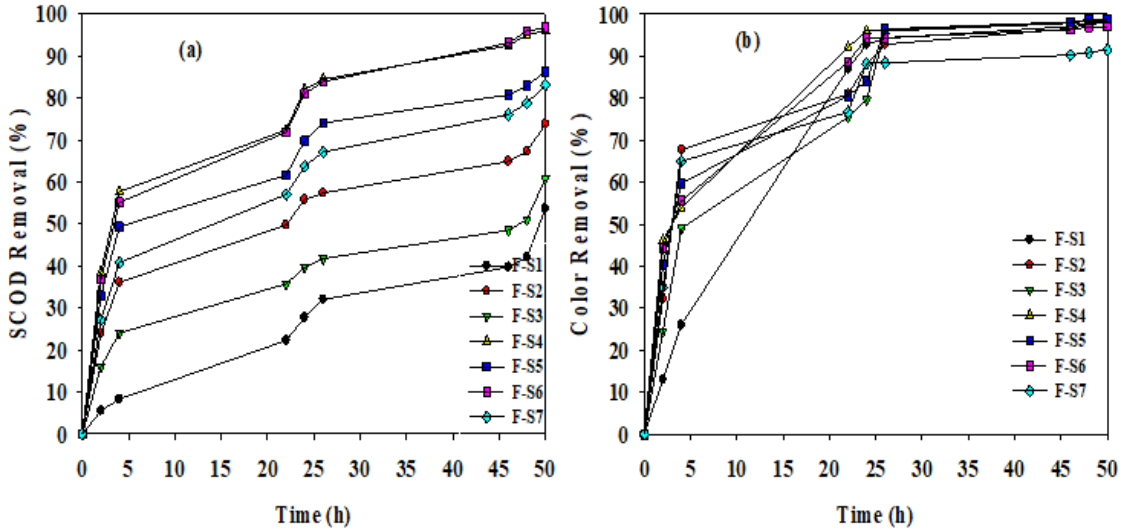


Figure.5. Degradation of COD and color of synthetic Melanoidins using fungal species: **a** SCOD removal % **b** color removal % at various time intervals**.**

Table 1. Parametric characterization of fungal species to degrade and decolorize Melanoidins

Species	pH		Optical density		Color Absorbance			SCOD		
	In	Fi	In	Fi	In	Fi	Re (%)	In	Fi	Re (%)
F-S01	5.5	6.05	0.32	1.54	1.18	0.02	98.0	7960	3690	53.6
F-S02	5.5	5.90	0.39	1.14	0.92	0.02	97.8	6280	2130	73.8
F-S03	5.5	6.09	0.36	1.57	1.84	0.03	98.5	8620	3380	60.8
F-S04	5.5	6.19	0.36	1.38	1.84	0.03	98.4	7560	300	96.0
F-S05	5.5	6.07	0.31	1.64	1.90	0.02	98.8	8760	1240	86.3
F-S06	5.5	6.08	0.47	1.38	1.77	0.05	97.0	6640	220	96.7
F-S07	5.5	6.11	0.29	1.25	1.85	0.16	91.5	6160	1160	83.0

*(F-S) =Fungal species, SCOD= Soluble chemical oxygen demand, In= Initial, Fi= Final, Re= Removal.

Morphological Identification of pure fungal isolates:

Determining pure fungal species was done using simple staining, motility, morphological characteristics, and microscopic observations, as shown in Table 2. Colonies were grown on SDA and TA agar within five days of incubation. Among seven isolates, only one isolate, F-S5 identified up to species level as *Syncephalastrum* with zygomycetes class; three were identified as genera levels of F-S1, F-S6, and F-S7 were class of *Eurotiomycetes*, and remaining three genera

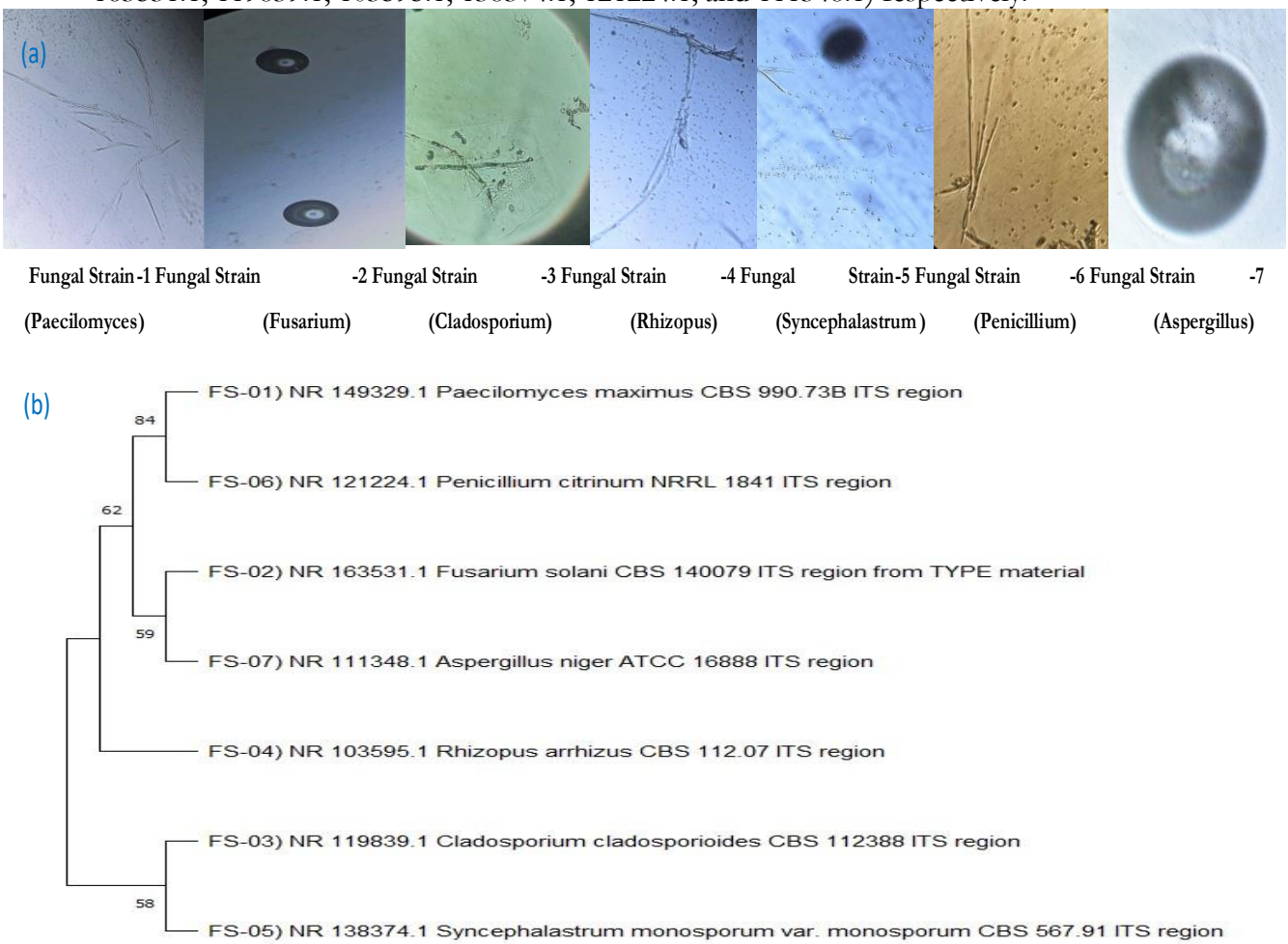
levels of F-S2, F-S3, and F-S4 were class of *Sordariomycetes*, *Dothideomycetes*, and *Zygomycetes* respectively. The structure of these fungal isolates with different shapes is shown in Figure.6.

Molecular Identification of Unknown Fungal Genes:

DNA Concentration: The concentration of isolated DNA was checked in Qubit Fluorometer Invitrogen of 7 fungal species FS-01 to FS-07 were original concentration (12.5, 15.2, 10.1, 18.4, 12.0, 9.3, and 8.4) ng/mL respectively as shown in Figure 7a and 7b.

PCR (Polymerase Chain Reaction): PCR with help of ITS1/ITS4 region for fungal gene amplification results through DGGE analysis from Illumina sequencing and compared isolated band of sequence image of agarose gel electrophoresis as shown in Fig.7d. DGGE analysis method were used for PCR amplification as shown in Figure 7c to separate fungal strains from FS-01 to FS-07 to expected the diverse organism population and 1kb ladder size as shown in Figure 7e was used to check correct PCR products size on UV gel studio SA^2 as shown in Figure 7f.

Phylogenetic Tree: The amplified product was sequenced in BLAST (The basic local alignment search tool) database in online NCBI (National center for biotechnology information) NIH (National Institutes of Health). The sequence analysis of isolated fungal species showed that FS-01 to FS-07 belonged to gene *Paecilomyces, Fusarium, Cladosporium, Rhizopus, Syncephalastrum, Penicillium, and Aspergillius* respectively. In addition, seven isolated produced phylogenetic group with above seven genes of Fs-01 to Fs-07 with different accession number NR (149329.1, 163531.1, 119839.1, 103595.1, 138374.1, 121224.1, and 111348.1) respectively.



.**Figure 6.** Morphological identification and Phylogenetric tree of fungal species: FS-01 to FS-07 **a** staining and fluorescence microscopy **b** Phylogenetric tree.

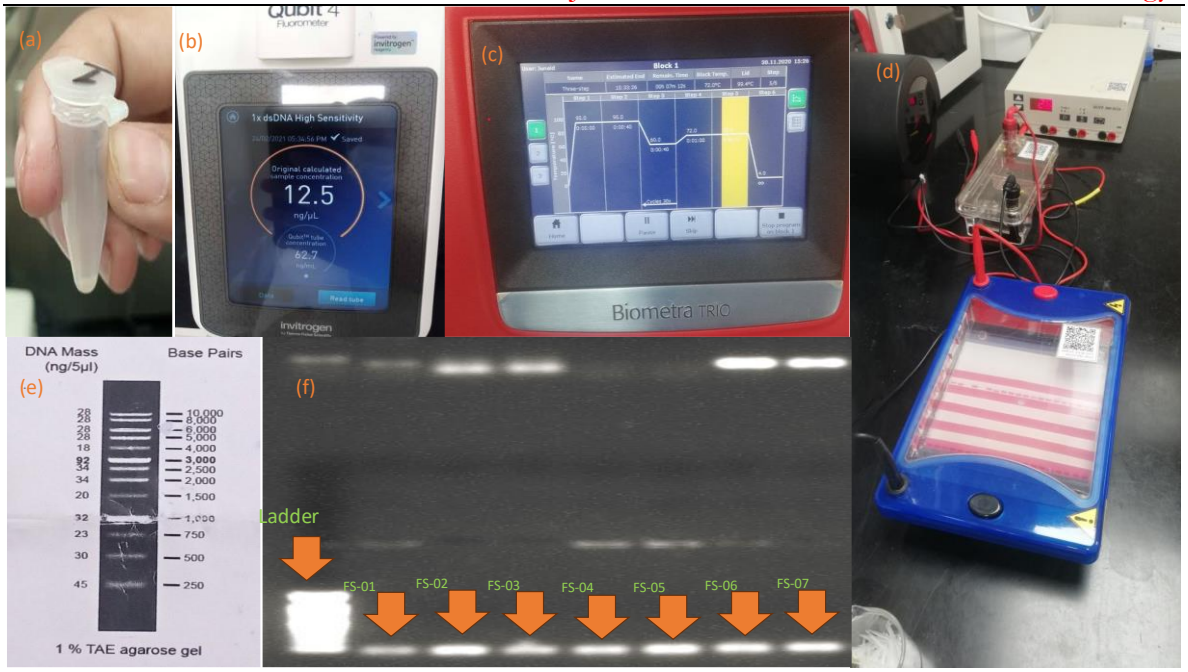


Figure.7. Molecular Identification of fungal species: FS-01 to FS-07 **a** Extracted DNA **b** Concentration of DNA **c** PCR Amplification **d** Agarose gel electrophoresis **e** 1kb base pair of 1% TAE agarose gel **of** DNA fragment and PCR Product on UV gel studio SA[2]**.**

Table 2. Staining of fungal species using Fluorescence microscope and biochemical tests.

Microbial Species	Color	Configuration	Specie like	DNA (ng/µl)	Sample type	Composition
F-S01	Yellow-brown	Molds	*Paecilomyces*	2.04	WS	SDA
F-S02	Purple	Macroconidia (Spores)	*Fusarium*	1.95	DS	Media+ T. A
F-S03	Grayish	Blastoconidia (Spores)	*Cladosporium*	12.5	WS	Media+ T. A
F-S04	Grey-Yellowish	Zygomycete (spores)	*Rhizopus*	-	SW	SDA
F-S05	Grey to blackish	Sporangiospore	*Syncephalastrum*	-	WS	SDA
F-S06	Bluish-green	Phialospores	*Penicillium*	-	DS	SDA
F-S07	Sulfur Yellow	Molds	*Aspergillus Niger*	-	DS	Media+ T. A

*(F-S) =Fungal species, SW=Spent wash, DS=Dry soil, WS=Wet soil, G+ve=Gram positive, T. A=Technical agar, and SDA=Sabouraud dextrose agar

Summary result of estimated parameters using Modified Gompertz model:

The modified Gompertz model was applied to the individual growth data sets to fit the kinetic parameters for biodegradation and decolorization of synthetic melanoidins as a function of time related to microbial activity (Table 2). The fit of the modified Gompertz model related to optical density growth for Melanoidins degradation is shown in Figure 3b. Among all seven

isolates, the coefficient of determination was R2= 0.95-0.99, which indicated that the modified Gompertz model must be used for maximum optical density growth to degrade and decolorize Melanoidins. The following values of different parameters were obtained through an algorithm run on experimental growth data for fitting the modified Gompertz model. A = 1.25-1.75, μm = 0.15-0.25, λ = 0 due to overnight growth (lag phase) sample taken. Furthermore, the goodness of fit Chi-square was also used for the best fitting value, and it fits the best model, which showed GoF = 0.99 as shown in Table 3.

Table 3. Summary result of estimated parameters using modified Gompertz model

Species	Asymptote *(A)*	Specific growth rate (μm)	Lag time *(λ)*	R^2 value	Goodness of fit	Average optical density (680nm)
F-S01	1.56	0.19	0	0.98	0.999899	0.30±1.60
F-S02	1.28	0.23	0	0.95	0.997528	0.38±1.40
F-S03	1.69	0.19	0	0.99	0.999761	0.36±1.75
F-S04	1.56	0.19	0	0.98	0.999551	0.36±1.65
F-S05	1.71	0.18	0	0.99	0.999965	0.30±1.75
F-S06	1.65	0.23	0	0.98	0.996466	0.45±1.75
F-S07	1.52	0.15	0	0.99	0.999959	0.28±1.65

*(F-S) =Fungal Strain, R^2=Coefficient of determination

Discussion:

Wastewater poses a significant pollution threat, particularly when untreated waste is directly discharged into the environment. Distilleries generate substantial amounts of waste as spent wash, which has a high organic load, including melanoidins [44, 45]. Melanoidin is a recalcitrant and dark brown colored compound which is a complex compound formed through Millard reaction, making it difficult to degrade [38]. Traditional physico-chemical methods for treating distillery effluent are unsuitable for industrial purposes due to the resilient nature of melanoidins, making decolorization challenging [47, 48]. Recently, many researchers focused on microbial treatment using microorganisms as fungi for degradation and decolorization of Melanoidins because this method is cheaper, safer, and efficient [49]. The present study aimed to treat melanoidins using fungal isolates for bioremediation. Spent wash, wet soil, and dry soil samples from a distillery dump site were collected, containing fungal strains capable of melanoidin degradation [50, 51].

Physico-chemical characteristics of effluent after degradation process had low COD and less color with acidic pH in nature [3]. The identification of 7 fungal isolates was done through simple staining, morphology, and biological tests [2]. After streaking on Petri plates, the fungal isolates were grown on Sabouraund dextrose agar with minimal salt media and incubated at 25°C for 14 days [52]. The multiple colony growth results of screened fungal isolates were shown on Petri plates and identified as *Aspergillus*, *Rhizopus*, *Cladosporium*, *Paeclomyces*, *Penicillium*, *Fusarium*, and *Syncephalastrum* [2, 35, 38, 53, 54]. The change in pH from 4-9 after 24-48 h did not significantly affect the degradation process, while temperature from 20-40 °C within the same period had more degradation activity and pH was more acidic [55-57]. The increase in a fungal dry cell due to an increase in cell volume or utilize more sole carbon then decreases after 2-7 days [46, 58]. After 2 days the increase in optical density at 590 nm reached from 1.6-1.8 which

ultimately increased the growth of yeast for a higher degradation rate of synthetic Melanoidins [59].

Many workers had researched COD reduction at various parameters, temperature 20-30 °C, pH 5-7, rpm 160, time 2-21 days, and different glucose and carbon sources added to the process for degradation of Melanoidins and obtained favorable results using pure fungal isolates as COD reduction by *Aspergillus Niger* 53.4 %, *Aspergillus Terreus* 84 %, and *Fusarium* 19.32 % [2, 33, 38]. The Melanoidins decolorization was expressed as a decrease in the final absorbance at 475 nm against its initial wavelength, hence the color reduction was achieved by different fungal species as *Aspergillus Fumigates* 70 %, *Paeclomyces* 80+7.5 %, *Fusarium* 37.65 %, and *Penicillium* 41 % with 4-15 days of incubation [35, 38, 60]. The modified Gompertz model was applied, demonstrating a good fit with a coefficient of determination was $R^2 = 0.99$ and the lag time was calculated by subtracting the observed value to theoretical value [61, 62]. The goodness of fit test was used to compare the observed data with expected data and it was achieved by Bayesian p-value [63]. Fungal treatment of distillery effluent spent wash using indigenous fungal species has successfully been achieved for bioremediation. Hence the present study was taken for Melanoidins removal from distillery effluent spent wash using indigenous fungal species. [64] Examined that distillery effluent spent wash is not suitable for discarding into the environment due to their toxicity and recalcitrant nature. *Pediococcus acidilactici* thermotolerant bacterium identified and it was isolated which showed the highest decolorization 79 %, COD 85 %, and BOD 94 % decrease at 113°F, pH 6.0 within one day under inert condition. In addition, carbon and nitrogen supplements used were glucose 0.1 %, peptone0.1 %, MgSO4 0.05 %, K_2HPO_4 0.05 % for additional removal percentage.

The author in [65] reported decolorization of dark brown colored distillery spent wash using white-rot basidiomycete fungus as *Flavodon flavus*, its isolate from an aquatic environment which decolorizes the distillery spent wash using *Flavodon flavus* by immobilization containing 10% diluted distillery spent wash which able to decolorized from 60 to 73% by 5 to 7 days, respectively, so this study more focus and future work will be done by enhancing its experiments with different perspectives. As compared with bacterial species few authors had worked on them and the results were [66] studied Bacillus sp. isolate had the highest decolorization (85) % and COD reduction (90) % with 12 hours from distillery spent wash using Taguchi method then it is compared with 16S rDNA based mutate gradient gel electrophoresis and magnified random DNA restriction analysis (ARDRA) isolate has less capability to reduce color (21%) and (COD) (30%), *Bacillus* sp. identified by 16S rDNA sequence analysis. [67] observed that the isolation and identification of *Pseudomonas* sp. show the highest decolonization (56-63) % and COD reduction at 10% diluted spent wash following through different parameters pH 6.8 to 7.2, Temperature 30 to 35 °C in three days incubation time but still not good results achieved by bacterial species as compared with fungal species.

Conclusions:

The mixed culture of fungal isolates from three inoculums (spent wash, wet soil, and dry soil), spent wash showed maximum COD removal of 91.8 %, while dry soil removed a maximum color of 75.7 % with controlled pH and temperature. The seven isolated indigenous fungal species were used for degradation and decolorization of Melanoidins from which F-S6 as Penicillium removed a maximum SCOD of 96.7 % while F-S5 as Syncephalastrum removed a maximum color of 98.8 %; further, it was concluded that all fungal species have more color removal efficiency comparative to SCOD removal, these species might utilize all intermediate compounds formed from Millard reaction products.

Future Research: This research not ends here more work needed with different ratios as time variation, smooth process, inoculum dosage variation, and identified compounds by using GC-MS analysis which clarifies that which specific specie can degrade which type of compounds.

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Consent to Participate. This consent was taken from all individual participants included in the study.

Consent to Publish. The authors declare that the human research participants provided informed consent to publish images in Figure (s) 1a, 1b,1c.

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