International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.8, No.12 pp 509-520. 2015

Evaluating the decolorization potential of genetically constructed *Pleurotus ostreatus* strains by protoplast fusion

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Abstract: Response of some *Pleurotus ostreatus* mutants to eleven metal ions with different concentrations were tested. Results showed that fusant (F6) was the most efficient one for dye removal followed by F4. All selected mutants and fusants exhibit enzyme activity and specific activity higher than the wild type strain. Positive relationship between laccase specific activity and decolorization capacity of some textile dyes was observed. SDS-PAGE analysis of water soluble proteins revealed 18 bands with different molecular weights ranged from 14 to 180 KDa. Nine bands were common in all samples while the other were varied among the isolated samples. The non-parental new bands observed in fusants indicating that there was an occurrence of interaction between the two parental genomes. When a real textile wastewater sample was treated using some selected mutants and fusants, maximum decolorization was recorded from2 to 5 days and afterword little change was recorded.
Key Words: Protoplast fusion, textile dyes**,** decolorization, laccase, SDS-PAGE.

Introduction

Over10.000 dyes are commercially available and around 15% of the global dye production is being discharged to the aquatic environment untreated or with partial treatment. Direct disposal of these toxic dyes cause deterioration of groundwater along with disruption of aquatic ecosystems and indirectly it enters into our food chain[1]**.** Besides all techniques, biological methods are highly versatile, cost effective and minimal sludge production. Now considerable research efforts have been devoted to the biological treatment processes with the use of microorganisms such as bacteria and fungi.

Protoplast fusion is an important tool in strain improvement for bringing genetic recombination and developing hybrid strains in filamentous fungi[2]. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties[3]. Although fungal protoplasts can be obtained from ungerminated fungal spores [4], most laboratories use mycelia as the starting material mainly because the spore wall is highly resistant to lysis with respect to hyphal age, results vary from fungus to fungus but in general, 18–48 h old mycelia, corresponding to the early to mid-exponential growth phase result in the best protoplast yield. It was found that while enzymes individually, or in combination, varied in their ability to produce protoplasts depending on the type of fungus, in general, Novozym 234, an enzyme mixture of chitinase, cellulase, and protease activities from *Trichoderma* species, was the most effective and produced sufficient numbers of protoplasts in 2–3 h [5].

Dye decolorization has been ascribed by several authors to ligninolytic enzymes produced by white rot fungi, namely lignin peroxidases (LIP), manganese peroxidases (MnP), manganese independent peroxidase

and laccases[6]. Some wood-degrading fungi contain all three classes of the lignin-modifying enzymes, while others contain only one or two of these enzymes. Lignin degrading enzymes have a low specificity which allow them to degrade a wide range of pollutants such as individual azo-, triphenylmethane, anthraquinone, phthalocyanine and heterocyclic dyes[7], polycyclic aromatic hydrocarbons PAH·s**,** as well as complex industrial effluents. The evaluation of decolorization potential of genetically constructed *Pleurotus ostreatus* strains by protoplast fusion has been investigated in this study.

Materials and Methods

1. Strains: *Pleurotus ostreatus* strain was obtained from Faculty of science, Ain Shams University in addition to eight UV-*Pleurotus ostreatus* mutants previously obtained by Ibrahim[8]**,** were used in this study. All cultures were maintained in malt extract agar (MEA) medium :(Malt extract, 30 g/l; Mycological peptone, 5 g/l; Agar, 20 g/l.)

2. Dyes: Six textile dyes were selected with consideration of prevalence in the textile industry. Methyl orange (6.8mg/l), Acid fast red (67mg/l), Reactive black 5(100 mg/l), Methylene blue (3.3mg/l) Congo red (20 mg/l) and Disperse Violet 31 (100 mg/l). All these dyes were obtained from S. D. fine-chemical ITD.

3. Resistance of heavy metal ions: Metal resistance of the parental strain and mutants during growth on hypertonic regeneration medium was studied with the use of Co^{+2}, Cu^{+2}, Fe^{+3}, Zn^{+2}, Cd^{+2}, Al^{+3} and Hg^{+2} as metal ions at different concentrations, while Cs^{+1}, $Se^{+2}$and Mn^{+2} metal ions was used at one concentration.

4. Protoplast fusion: Protoplasts were obtained according to Larraya[9]. One milliliter of each of freshly prepared protoplasting pellets of 7/30 and 14/30 mutants were fused using 30% PEG [10]. Fusion frequency was expressed as the ratio of the number of colonies formed on selective and nonselective media.

5. Screening for enzyme activity[11].

6. Enzyme activity using ABTS and DMPsubstrates[12, 13]**.**

7. Determination of protein concentration[14] using bovine serum albumin (BSA) as standard.

8. Extraction of protein for SDS-PAGE: Fungal mycelium was grown in liquid malt extract medium for six days at 30 °C in a rotary shaker at 150 rpm. Mycelium was collected by centrifugation at 4000 rpm for 5 min then about 1 g of mycelium transferred to a new Eppendorf tube. 200 µl of protein extraction buffer was added and vortex then stored overnight at 4 ºC. Samples were vortex again and centrifuged at 13000 rpm for 15 min at 4 ºC. The supernatant transferred to new Eppendorf tube and stored at -20 ºC.

9. Extraction of crude enzyme for Native-PAGE: Flasks containing 100 ml of liquid culture medium were inoculated with mycelium growing on MEA medium and incubated in a rotary shaker at 150 rpm at 30 °C for six days. Crude extract collected by centrifugation at 4000 rpm for 5 min. Extract was concentrated using dialysis back in glucose solution then the concentrated sample transferred to new Eppendorf tube and stored at -20 ºC.

10. Polyacrylmide Gel Electrophoresis (PAGE) [15, 16].

11. Gel staining: For protein, gel was covered overnight with staining solution. After removing the staining solution, gel was covered with a destaining solution and agitated gently for one hour and repeated several times until gel background becomes clear. For enzyme, gel was covered with the staining solution that contains DMP as a substrate and incubated for about 30-60 min. at room temperature until the color appeared [17].

12. Real textile wastewater biotreatment for decolorization: Fungal strains were used to inoculate 50 ml of liquid culture medium in 250 ml flasks, which were incubated at 30°C with shaking at 150 rpm. After six days, 50 ml of real textile wastewater sample obtained from Hessny Company at 10th Ramadan city was added to each flask and incubated at the same conditions. The decolorization was observed within 7 days at everyday interval.

Results and Discussion

In spite of the fact that protoplast fusion in fungi may yield new recombinants, which are different from the parental cultures, this technique offered the opportunity for overcoming the difficulties of the application of classical hybridization. As was mentioned previously, this study has explored an avenue for obtaining a new fusant(s) to decolorize all or most dyes used in this study

1. Response of mutants to different metal ions concentrations:

The metal ions was used to study their effects on the parental strain (*Pleurotus ostreatus)* and two mutants (7/30 and 14/30) isolated after UV treatment for 30 second, they were grown on MEA medium containing different concentrations of Co^{+2}, Cu^{+2}, Fe^{+3} , Zn^{+2} , Cd^{+2}, Al^{+2} and Hg^{+2}as metal ions, while one concentration of Cs^{+}, Se^{+} and Mn^{+2} metal ions was applied as described under materials and methods. Results showed that, *Pleurotus ostreatus* was resistant to all concentrations of Al^{+3}, Co^{+2} and As^{+5} metal ions in addition to resistant to all concentrations of Cu^{+2}, Fe^{+3} and Zn^{+2} except with 2000 µg/ml. Resistant to 25, 500 and 1000 µg/ml was also observed with Hg^{+2},$Mn^{+2}$and Cs^{+} respectively. On the other hand, the wild type strain showed sensitivity to all Cd^{+2} concentrations used in this study. Furthermore, the same trend of heavy metals response was detected with 14/30 mutant while7/30 mutant exhibited sensitivity to 25, 250 and 1000 µg/ml of Hg^{+2}, Co^{+2} and Cs^{+} respectively and resistance to 1000 µg/ml Cu^{+2}. Therefore, Hg^{+2} (25 µg/ml) and Cu^{+2} (1000 µg/ml) were selected as a selective marker for fusants isolation.

However, different methods were applied as marker genes detection for protoplast fusion. These methods included marker genes for drug resistance, auxotrophic mutants, morphological differences, catabolite repressors as well as heavy metal ions resistance. These complementary markers were used for fusants detection. Cobalt and mercury resistant markers were introduced in *Penicillium funiculosum* and *Trichoderma reesei*, respectively through induction of mutations. Protoplasts of these fungi were fused and screened for hybrids as Co^{R} and Hg^{R} resistant colonies[18].

2. Protoplast formation:

The examination of the two mutants with phase-contrast microscope showed that the gradual degradation of fungal mycelia started after the addition of lytic enzymes solution (Novozyme234 and Cellulase). The whole cell wall digestion was achieved following incubation at 28 ºC with gentle shaking for four hours. **Figure (1)** shows protoplast formation stages. Maximum release of protoplasts was obtained with 7/30 mutant that yielded 4.0 $x10^{6}$ protoplasts /ml. From the mycelium of 14/30 mutant, the highest yield of protoplasts of 3.1 x 10^{6} was obtained after three hours incubation period. The results obtained agree to some extent with those published by Kitamoto[19]. They reported that when protoplast isolations were performed in various filamentous fungi from all fungal subdivisions by using the enzyme prepared from *Trichoderma harzianum, P. ostreatus* gave the highest productions of protoplasts with the value of $1.3x10^{7}$ /ml. The results obtained by Dhitaphichit and Pornsuriya[10] nearly the same yield of protoplasts. However, production of protoplast varies with the factors used in the isolation process *e.g.* species and age of fungal mycelia, type and condition of the lytic enzyme and of the osmotic stabilizer.

3. Protoplast fusion and regeneration:

The protoplast fusion experiment was applied between 7/30and 14/30mutants according to resistance or sensitivity to one or more of the eleven metal ions. Equal amounts of protoplasts prepared from young mycelia of each two strains were fused and the fusion frequency obtained from the ratio of the number of colonies regenerating on nonselective medium and the number of colonies formed on selective medium was 1 x 10^{-3}. It was previously mentioned that the formation of protoplasts and regeneration are affected by different factors, *e.g.* enzymes, time of treatments, mycelial age and regeneration medium ….etc[20, 21].Protoplast fusion between *Pleurotus ostreatus* and *Schizophyllum commune*[22] *Pleurotus ostreatus* and *Pleurotussajor-caju*[23] were previously performed. However, colonies isolated after protoplast fusion of 7/30 and 14/30 mutants were selected as fusants and named as F1 to F15. Fusants stability was examined by successive subcultures on selective and nonselective media.

4. Evaluation of fusants for dye decolorization:

The frequency analysis of *pleurotus ostreatus* fusants according to dye decolorization in comparison with wild type strain and the two parental strains (P1 and P2) was presented in **table (1).** The differences between fusants could be due to their whole genomes not being identical but having only some genes in common. This result is coincide with those previously reported[10]. Anyhow, it could be concluded that fusantsF4 andF6 have the ability to decolorize most dyes used in this study. Therefore they were selected for enzymatic and protein determination.

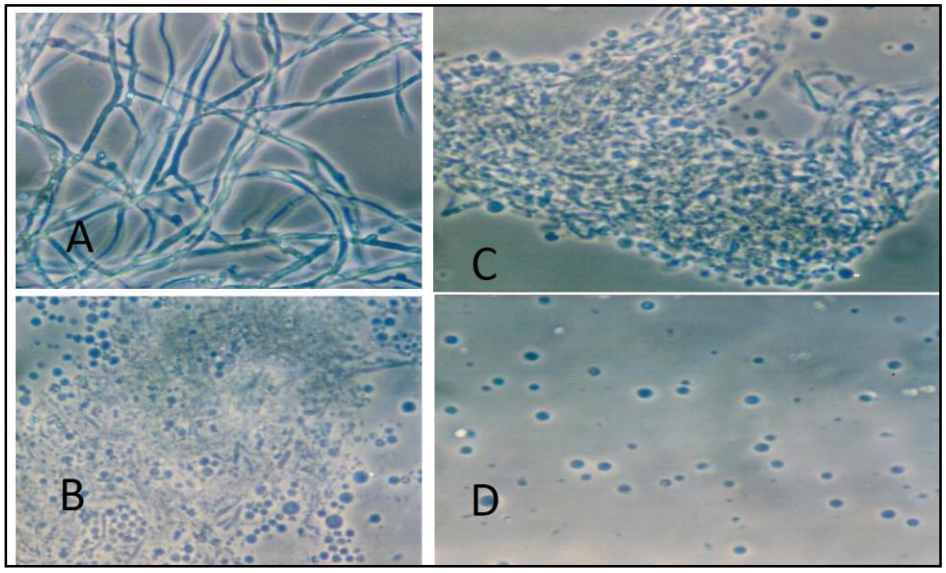

Fig (1): Micrographs representing protoplast formation stages. A: mycelia of *Pleurotus ostreatus*. B and C: degraded mycelia after treatment with Novozyme and cellulase. D: free protoplasts.

In this test, screening for ligninolytic enzymes production was carried out on agar plate containing nutrient salt medium using DMP as inducer. Orange-brown color indicates the enzymes production. **Figure (2)** showed laccase enzyme production by the original strain *Pleurotus ostreatus.* However, dye decolorization has been described by several authors to ligninolytic enzymes produced by white rot fungi[6].

5. Extracellular ligninolytic enzyme production:

White rot fungi are capable of producing extracellular enzymes such as lignin peroxidase, manganese peroxidase and laccase. Laccase is mainly responsible for the decolorization of aromatic compounds. It is able to oxidize substrates such as ABTS, DMP and guiacol[11].

6. Enzymatic activity of selected mutants and fusants:

Since the ability of *Pleurotus ostreatus* mutants and fusants to decolorize the dyes was evident in liquid medium, their capacity to produce extracellular oxidative enzymes was evaluated. The presence of substrates in this fungus was also necessary to detect this activity.

Table (1): Frequency analysis of *Pleurotus ostreatus* fusants according to dye decolorization.

Dyes	**Methyl orange**			**Acid fast red**			**Reactive black 5**			**Congo red**			**Methylene blue**			***Disperse violet 31***		
Incubation periods	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	*72h*
More than wild type	6	7	7	3	6	4	6	7	9	15	15	15	6	9	11	15	15	*15*
Less than wild type	9	8	8	12	9	11	9	8	6	0	0	0	9	6	4	0	0	*0*
More than P1(7/30)	4	1	2	3	3	1	15	14	14	0	0	0	12	13	13	12	15	*15*
Less than P1(7/30)	11	14	13	12	12	14	0	1	1	15	11	10	3	2	2	3	0	*0*
More than P2(14/30)	9	7	6	0	1	1	4	3	0	15	15	15	12	9	8	0	0	*1*
Less than P2(14/30)	6	8	9	15	14	14	11	12	15	0	0	0	3	6	7	15	15	14

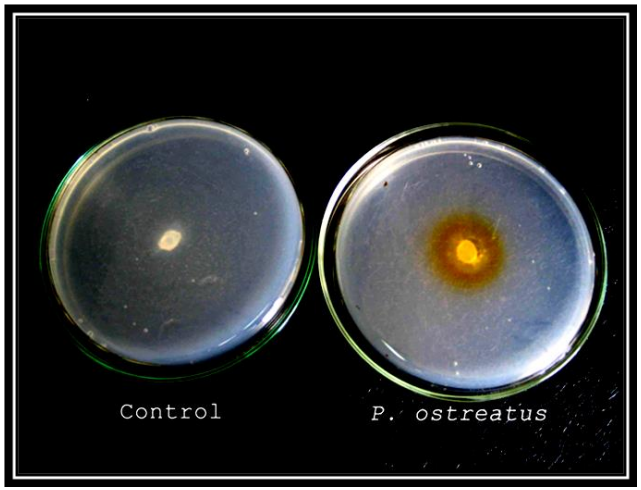

Fig (2): Laccase assay. Orange-brownish color indicates enzyme activity. Left: three days old mycelium without the inducer as a control. Right: DMP added as inducer, the assay carried out on agar plates containing nutrient salt medium.

Results showed that the oxidation of ABTS was detected in wild type, tested mutants and fusants but showing different production pattern **(Fig.3, A).** An intense oxidation of ABTS was observed in Fusant4 and Fusant 6. Similar levels of laccase (LAC) activity were found in. *Pleurotus ostreatus*, mutants and fusants in the presence of DMP as substrate **(Fig. 3, B).**

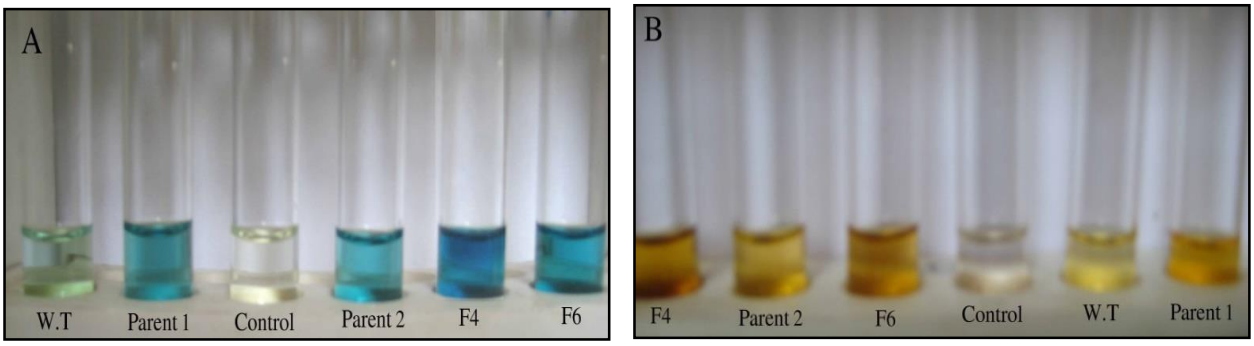

Fig. (3): Enzyme activity of *Pleurotus ostreatus* wild type, parental strains and two fusants by A: ABTS and B: DMP.

However, laccase activity of the culture filtrate was presented in **table (2).** When ABTS was used as a substrate, nearly all mutants and fusants exhibited higher, laccase activity than the wild type strain but not to the same extent. Among the mutants used, 12/30 and 22/30 showed the highest specific activity with 442.23 and 333.53IUmg$^{-1,}$ respectively while the wild type strain showed 58.05 IUmg^{-1}as listed in **table(2)**. On the other hand, mutant 26/30 showed considerably lower specific activity of 92.58IUmg^{-1}. However, mutants 12/30 and 22/30 exhibited enzyme activity of about 10 times higher than the parental strain with ABTS substrate. In addition, specific activity of 231.33 and 314.82 IUmg^{-1}was obtained with F4 and F6fusants which represents increase of 3.98 and 5.42 times, respectively higher than the original strain using ABTS substrate.

When using DMP as substrate, nearly the same trend of specific activity was obtained by all mutants, fusants in addition to the original strain. The high activity of laccase in the culture indicated that this enzyme may play an important role in the degradation of textile dyes. This result in accordance with Khelifi[24].The ability of decolorizing different dyes was positively related to the laccase activity. Induction in the activity of laccase during the decolorization process indicated that laccase played an important role in the efficient decolorization of different dyes by this fungus [25].

Table (2): Enzyme activity, specific activity and total protein concentration of selected mutants and fusants with ABTS and DMP substrates.

Strains	Total Protein conc.(mg/L)	ABTS*		DMP**	
		Enzyme activity (IU/L)	Specific activity (IUmg^{-1})	Enzyme activity (IU/L)	Specific activity (IUmg^{-1})
Pleurotus ostreatus	36.35	2110	58.05	1680	46.22
2/45	10	1527	152.7	1195	119.5
6/30	23.25	3177	136.64	2030	87.31
7/30	63.4	6850	108.4	3930	61.99
12/30	47.6	21050	442.23	13860	291.18
14/30	42.20	4720	111.85	3110	73.70
15/30	31	4994.44	161.10	4140	133.55
22/30	67.4	22480	333.53	17420	258.46
26/30	39	3611	92.58	2763	70.84
F4	45.1	10433	231.33	7330	162.53
F6	27.8	8752	314.82	6000	215.83

One unit of enzyme activity (IU) is defined as the amount of enzyme that transfers 1µM of substrate per mints. * ABTS, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). **DMP, 2, 6-dimethoxyphenol.

However, these results indicated that UV-mutagenesis is efficient in increasing variability of enzyme production, which is quite low in control. The differences observed, in decolorization obtained with the fungal strain and individual mutants suggested that different dye decolorization mechanisms might be involved [26].Moreover, the difference in enzyme production could be due to their whole genomes not being identical but having only some genes in common[10].

7. Protein estimation:

The amount of protein present in the culture filtrate was estimated using Bovine Serum Albumin (BSA) as standard. Data in **table (2)** showed total protein concentrations of eight UV-mutants in addition to two fusants. Protein concentrations of 63.4, 47.6, 42.2,67.4 and 39.0 mg/l were reached in mutants Nos. 7/30, 12/30, 14/30, 22/30 and 26/30, respectively Compared with the parental strain, the protein concentration was increased by 74.42%,30.95%, 16.09%,85.42 and 7.29 %.for these mutants. In contrast, three mutants,*i.e.*2/45, 6/30 and 15/30 showed lower protein concentrations than the original strain.

On the other hand, while 7/30 and 14/30 mutants which used as parental strains for protoplast fusion experiments produce 63.4 and 42.2 mg/ml protein, their fusants produce 45.1and 27.8mg/l for F4 and F6, respectively. These results indicate that the whole genomes of F4 and F6 were not identical and that the differences in their total protein concentrations resulted from gene recombination[10].

However, many strain development techniques are practiced that includes bio-engineering of organism focusing mainly on genetically improving required proteins. An important such technique, random mutagenesis, is used to induce mutation in organisms for better distinctiveness [27]. Mutagens such as UV light are employed for inducing mutation[28].

8. Laccases specific activity and decolorization capacity of some textile dyes:

The wild type strain *P.ostreatus*, two mutants and two fusants were examined for the decolorization of six textile dyes, and an attempt was made to correlate dye removal with enzyme production. From the isolates tested, it seems that laccase is involved in dye decolorization. Positive relationship between laccase specific activity and decolorization capacity for most dyesis clearly detected **(Table 3).** This result is accordance with Rodrıguez[29]**.** Spardaro[30] reported that the extent of color removal is not consistent with all the dyes. Decolorization depends on the laccase production, media and dyes. Results also showed that there are other enzymatic systems involved in dyedecolorization especially for Reactive black 5(diazo) and Methylene blue (heterocyclic). The mechanism of azo dye oxidation by laccase involves the oxidation of the phenolic group to produce a radical at the carbon bearing the azo linkage. Then, water attacks this phenolic carbon to cleave the molecule producing phenyldiazene. The phenyldiazene can be oxidized by one-electron reaction generating N_2[29]. However, mutation affects the production of various enzymes in the cell by distressing the metabolic cycle [31].

The improved decolorization for mutant is due to increased enzymatic production, which can be attributed due to the alteration of DNA sequence by mutagenic agents. Normally, mutations occur due to changes in DNA sequences by which codons get altered. By alteration of codons, amino acid groups are changed thereby overproducing certain enzymes[32]. Anyhow, only a limited number of studies have attempted molecular characterization of dye decolonization. There is still a gap in the degradation mechanisms of dyes by white-rot fungi and their ligninolytic enzymes[33].

Table (3): Dye decolorization and enzymatic activity of some mutants and fusants.

Strains	ABTS (Specific activity) ($IUmg^{-1}$)	% Decolorization					
		Methyl orange	Acid fast red	Reactive black 5	Congo red	Methylene blue	Disperse violet 31
wild type	58.05	44.29	50.33	89.56	91.19	42.49	91.52
p1(7/30)	108.4	96.85	99.05	71.96	100	18.46	92.23
p2(14/30)	111.85	63.78	99.81	99.21	89.56	63.09	98.59
F4	231.33	97.41	89.4	65.06	97.67	95.44	96.65
F6	314.82	96.12	100	98.74	100	96.67	97.42

9. Native-PAGE profile of extracellular crude enzyme

In order to distinguish between parental strains and fusants, native-PAGE was performed for wild type of *Pleurotus ostreatus*, two parental mutated strains (7/30and 14/30) and two fusants (F4 and F6).The Gel was stained by DMP as a substrate to detect the bands via enzymatic activity. **Figure (4)** verifies the hybridization relationship as the fusants showed band(s) common to either of the parental strains or to both. Furthermore, the non-parental new bands observed in the enzyme patterns of the fusants indicates that there was an occurrence of interaction between the two parental genomes .This experiment confirmed other previous works that protoplast fusion is an appropriate method in creating hybrids in mushrooms especially in the genus *Pleurotus*[10].

10. SDS-PAGE of water soluble proteins:

SDS-PAGE profile of water soluble proteins extracted from 6-dayes old mycelia from *Pleurotus ostreatus* wild type, the two parental strains of protoplast fusion (7/30 and 14/30) and) and two fusants (F4 and F6) is presented in **Fig (5).**

SDS-PAGE analysis of water soluble proteins revealed 18 bands with different molecular weights ranged from 180 to 14KDa as shown in **table (4).**Number of bands varied among the samples since wild type revealed 9 bands while P1 revealed 11 bands. In addition, P2, F4 and F6 showed16 bands. Nine bands were common in all samples at molecular weights of 16, 19, 25, 27, 29, 35, 43, 68 and 170 KDa while the other (nine bands) were varied among the isolated samples. Some of these bands Nos.3 and 5 (130 and 97KDa) appeared in the two parental strains (7/30 and 14/30) and disappeared in the wild type. Bands with molecular weight of 17, 21, 23, 27.5 and 180 KDa appeared in P2 and disappeared in P1. These bands induced after UV-light. Moreover, bands except the band with 17KDa appeared in both fusants F4 and F6 as presented in Table (4).

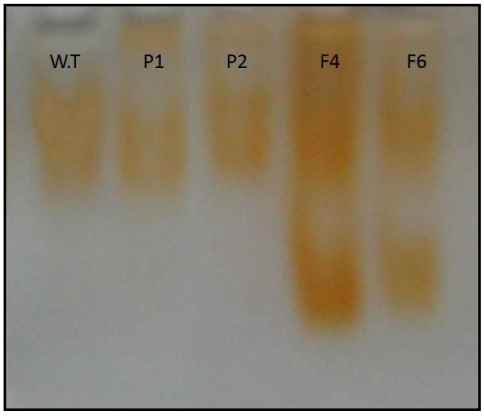

Fig (4): Native-PAGE profile of extracellular crude enzyme from *Pleurotus ostreatus* wild type, parent 1(7/30), parent 2 (14/30) and two fusants (F4 and F6). Staining was performed with 2.5mM2,6-dimethoxyphenol (DMP) in 100 mM acetate buffer (pH 4.5).

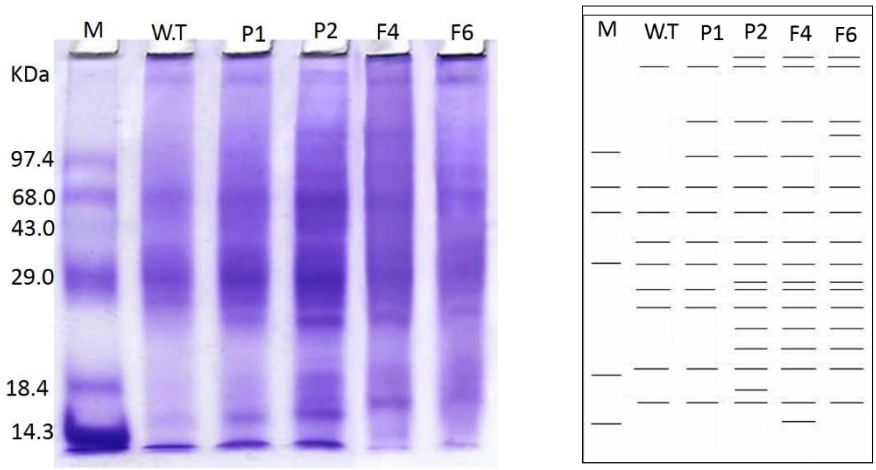

Fig (5): SDS-PAGE profile of water soluble proteins extracted from *Pleurotus ostreatus* (wild type), the two parental strains of protoplast fusion (7/30 and 14/30) and the most efficient fusants (F4 and F6).

On the other hand, the analysis showed the appearance of two unique bands. Band no.18 with molecular weight of 14KDa appeared only in fusant F4 while band No. 4 with 120KDa appeared in F6.Thenon-parental new bands observed in the protein patterns of the fusants indicate that there was an occurrence of interaction between the two parental genomes [10].

Table 4): SDS-PAGE analysis of water soluble proteins presenting band numbers, molecular weight, existence (+) or absence (-) of different bands.

Band No.	MW (KDa)	W.T	P1	P2	F4	F6	Polymorphism
1	**180**	-	-	+	+	+	**Polymorphic**
2	**170**	+	+	+	+	+	**Monomorphic**
3	**130**	-	+	+	+	+	**Polymorphic**
4	**120**	-	-	-	-	+	**Unique**
5	**97**	-	+	+	+	+	**Polymorphic**
6	**68**	+	+	+	+	+	**Monomorphic**
7	**43**	+	+	+	+	+	**Monomorphic**
8	**35**	+	+	+	+	+	**Monomorphic**
9	**29**	+	+	+	+	+	**Monomorphic**
10	**27.5**	-	-	+	+	+	**Polymorphic**
11	**27**	+	+	+	+	+	**Monomorphic**
12	**25**	+	+	+	+	+	**Monomorphic**
13	**23**	-	-	+	+	+	**Polymorphic**
14	**21**	-	-	+	+	+	**Polymorphic**
15	**19**	+	+	+	+	+	**Monomorphic**
16	**17**	-	-	+	-	-	**Unique**
17	**16**	+	+	+	+	+	**Monomorphic**
18	**14**	-	-	-	+	-	**Unique**

11. Real textile wastewater biotreatment for decolorization:

In order to evaluate the effectiveness of the genetically constructed strains obtained in this study,the textile wastewater effluent sample from Hessny Company at 10th Ramadan city, was treated using some selected mutants and fusants. Spectral absorption curve of real effluent before treatment showed large numbers and overlays of peaks from190-300 nm. This result reflects the fact that the complex mixed effluents are extremely variable in composition in one and the same factory, as is often the case in the textile industry. Thus, the decolorization of real effluents requires an appropriate choice of fungal strain as well as of reactor environment[34].

In this study, visual decolorization by the most efficient mutants and fusants was observed within 7days at everyday interval. When the real effluent was treated with mutants and fusants, effective color removal was observed with fusant F6 in comparison with the original strain and control without treatment **(Fig. 6).** Maximum decolorization was recorded from2nd to 5th day and afterward little change was recorded .This result is in accordance with Senthilkumar[35]. They noticed that maximum decolourization of 98% was achieved on the third day under normal conditions using *Phanerochaete chrysosporium* on synthetic dye bath effluent containing Amido black 10B.On the other hand, textile dye effluents contain not only dyes but also salts, sometimes at very high ionic strength and extreme pH values, chelating agents, precursors, by-products, surfactants, etc. [34].

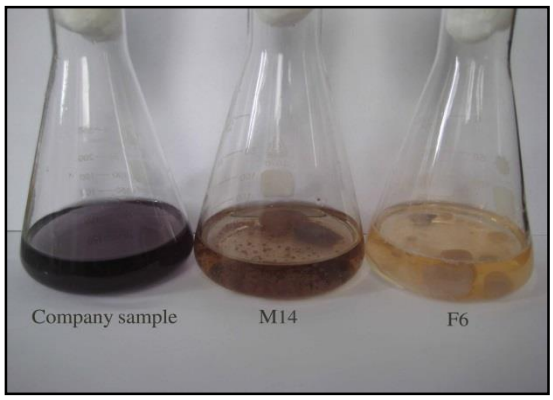

Fig (6): The effectiveness of the genetically modified strains (M14&F6) to decolorize textile wastewater effluent sample from Hessny Company at 10th Ramadan city.

As reported in a systematic study[36], certain white rot fungi strongly decolorize particular dyes but not others, whereas certain strains are more comprehensive in their decolorizing capacities. Small structural differences in dye mixtures can markedly affect decolorization, and this may be due to electron distribution and charge density, although steric factors may also contribute. In another report, Cu and Fe chelators as well as anionic detergents, which could be found in real textile industrial effluents, inhibited *Polyporus* sp. and *Trametes villosa* up to 20%, whereas *Schizophyllum commune* LMEs (lignin-modifying enzymes) were inhibited up to 70%[37]. However, a little decline in decolorization rate was observed when experiments were performed with real textile wastewater might be due to presence of other pollutants in textile waste water. Thus, decolorizing a real industrial effluent is quite troublesome[34]. However, the adsorption and degradation of dye molecules on living fungal hyphae might provide a mechanism for a feasible application of white rot fungi in continuous treatment of an industrial effluent. Furthermore, environmental pollution by textile dyes could be reduced by converting toxic substances such as azo dyes into harmless products[38].

On the other hand, the traditional textile finishing industry consumes about100 liters of water to process about 1 kg of textile materials [33]. New closed-loop technologies such as the reuse of microbial layer enzymatically treated dyeing effluents could help to reduce this enormous water consumption.

Conclusion:

The results of this study indicate that both the protoplasting and UV-mutagenesis are efficient methods for increasing dye decolorization. The differences observed, in decolorization obtained with the fungal strains, individual mutants and fusants suggested that different dye decolorization mechanisms might be involved.

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