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Full Length Research Paper

Influence of physical and chemical mutagens on dye decolourising *Mucor mucedo*

Bhargavi Moturi[1]* and M. A. Singara Charya[2]

[1]Centre for Biotechnology, Jawaharlal Nehru Technological University, Hyderabad – 5000085, AP, India.
[2]Department of Microbiology, Kakatiya University, Warangal – 506009, AP, India.

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The decolourisation efficiency of the wild fungal strain *Mucor mucedo* was investigated by the treatment with physical mutagen ultraviolet radiation (UV) and chemical mutagens [ethyl methyl sulfonate (EMS), diethyl sulfonate (DES) and colchicines]. The mutants that were exposed to 12 and 15 min UV radiation showed reduction in extension and branching of hyphae. EMS at concentrations of 100 and 150 mg inhibited the germination of spores in *M. mucedo*. Surprisingly, at concentrations of 100 and 150 mg DES, spores were converted to yeast like cells which produced buds instead of hyphae. Colchicine at higher concentrations abolished the germ tube and hyphal elongation. There was stimulatory effect on growth at lower concentrations only. With respect to enzyme productions and decolourisation activity, there was increase in protease (1.48 U/ml) and peroxidase (1000 U/ml) production in *Mucor mucedo* when exposed to 9 min UV radiation and showed maximum decolourisation activity of crystal violet (90%) and malachite green (70%). There was an increase in the peroxidase enzyme (1200 U/ml) production at 50 mg concentration relating to the maximum decolourisation activity of crystal violet (90%) and malachite green (71%). Also, there was a decrease in the production of all enzymes in *Mucor mucedo* when treated with different concentrations of DES and so, there was no improvement in decolourisation activity. There was an increase in protease (1.86 U/ml) and peroxidase (1000 U/ml) production only at 10 mg concentration of colchicine proving that the higher enzymatic secretions were responsible for the decolourisation efficiency of 89% in crystal violet and 74% in malachite green. The wild strain isolated from dye effluent amended soils when exposed to different physical and chemical mutagens showed improvement in the decolourisation of crystal violet and malachite green except in the case of DES.

Key words: *Mucor mucedo*, ultraviolet radiation, ethyl methyl sulfonate, diethyl sulfonate, colchicine, protease, peroxidase, laccase, decolourisation activity.

INTRODUCTION

Fungal biomass has huge capability of treating effluents discharged from various industries. Tein and Kirk (1984) visualized that the biodegradable ability of crystal violet was due to the extracellular lignin peroxidase secreted by *Phanerochaete chrysosporium*. Spadaro and Renganathan (1994), Knapp and Newby (1995), Chivukula and Ranganathan (1995), studied the decolourisation and biodegradation of triphenyl methane group of dyes using live and dried fungal mycelium and immobilized forms of fungal cells in the process of bioadsorption of the dye. Nagarathnamma and Bajpai (1999) noticed that *Rhizopus oryzae* could decolourise and detoxify industrial effluents at lower co-substrate concentrations than the basidiomycetes. Decolourisation of dyes by fungi is mainly ascribed to extracellular activity, which is in agreement with results reported previously for *Trametes hispida* (Rodriguez et al., 1999). A few reports have been presented concerning the microbiological degradation of triphenyl methane dyes (Nagai et al., 2002; Jones and Falkinham, 2003).

The use of microorganisms for the removal of synthetic

*Corresponding author: E-mail: moturi.bhargavi@yahoo.co.in. Tel: +91 9663479559.

dyes from industial effluents offers considerable advantages. The process is relatively inexpensive, it is a simple method and the running costs are low and the end products of complete mineralization are not toxic (Forgacs et al*.,* 2004) . Their enzyme producing activity makes them effective decolourizers; they remove toxic metals by biosorbtion ultimately rendering the effluents more ecofriendly (Erkurt et al., 2007). *Aspergillum niger* was the most efficient fungus to cause biodegradation of the color direct red 81 dye while *Penicillium* spp. was the most efficient fungus to cause biodegradation of the colour reactive red (Husseiny et al*.,* 2008). Recent advances and future potential on the decolourisation of dye wastewaters through fungi via two processes (biosorption and bioaccumulation) was analyzed (Kaushik et al., 2009).

Improvement of dye decolourisation may be achieved by using particularly the soil fungi isolated from dye-amended effluents. Attempts have been made to improve the efficiency of the decolourisation of the isolated strain *Mucor mucedo* by subjecting it to mutagenic changes by physical mutagen (ultraviolet radiation) and chemical mutagens (ethyl methyl sulfonate, diethyl sulfonate and colchicine).

MATERIALS AND METHODS

M. mucedo used in this study was isolated from dye amended soils around textile dye industries on Tenali road at Mangalagiri, 10 km away from Vijayawada, Andhra Pradesh. As *Mucor mucedo* was able to decolorize both the selected triphenyl methane group dyes more efficiently when compared to other fungi, this fungi was used for stable strain improvement by exposing the strain to physical as well as chemical mutagens.

Growth

The growth of the isolated soil fungi was measured in potato dextrose broth after 5, 10, and 15 days of incubation in the presence of two dyes; crystal violet and malachite green. Mycelial biomass was weighed on preweighed filter papers. Initial and final weights were measured to get the actual weight of fungal biomass. 25 ml of potato dextrose broth was poured into a conical flasks and 0.5 ml of 0.02% dye solution was added. The flasks were inoculated with 7 days old fungal culture after sterilizing the media. After appropriate incubation period, the mycelial mass was separated with Whatmann filter paper. The culture extract obtained was centrifuged and used for the assay of three enzymes; protease, peroxidase and laccase (phenol oxidase) and analysis of decolourisation percentage.

Estimation of protease, peroxidase and laccase (phenol oxidase)

Protease

Protease enzyme was estimated by the procedure suggested by Nanniperi et al. (1980). To 1 ml of culture extract, 1 ml of 1% casein solution [2 g casein in 20 ml of 1 M NaOH and 80 ml of DW (distilled water)] was added and incubated for 1 h at 37°C. This reaction mixture was added to 2 ml of 0.4 M of TCA (0.163g TCA in 1000 ml DW) and incubated for 20 min at 37°C. The contents were filtered through Whatmann No.1 filter paper. 1 ml of filtrate was taken and added to 5 ml of 0.4 M Na_2CO_3 (105.99 g of Na_2CO_3 in 1000 ml of DW) and 1 ml of diluted Folin and Ciocalteau's reagent (diluted in 1:2 ratio with DW). The contents were incubated at 37°C for 1 h and the developed blue colour was read at 660 nm. Blank was prepared without culture extract and activity was expressed in terms of casein denaturation optical density at 660 nm as U/ml.

Peroxidase

The peroxidase enzyme from the fungal cultures was estimated by the method suggested by Tein and Kirk (1984). An amount of 0.5 ml of culture filtrate was taken as an enzyme source and added to 0.5 ml of 0.01 M phosphate buffer (pH 4.5) followed by 0.5 ml of guaiacol (0.04 M). The reaction was initiated by the addition of 1.0 ml of 1% hydrogen peroxide. In the presence of peroxidase enzyme, guaiacol was oxidized to tetraguaiacol, which gave a brownish red colour. The developed colour was read at 470 nm for 90 s with 10 s time interval and the activity was expressed in enzyme units increase in optical density (1.0 digit increase in 1 ml at 470 nm is equal to 1 unit).

Laccase (Phenol oxidase)

The laccase enzyme from the fungal cultures was estimated by the method adopted by Lobos et al. (1994). 7 days incubated culture filtrate was used as enzyme source for laccase estimations. An amount of 0.5 ml of enzyme was added to 1.0 ml of 0.1 M phosphate buffer (pH 4.5) followed by 0.5 ml guaiacol (0.4 M). The activity gave a brownish red colour, which was read at 440 nm by ELICO UV visible spectrophotometer. Units were expressed as the amount of tetraguaiacol formed per minute.

Decolourisation assay

Decolourising activity was expressed in terms of percent decolourisation as described by Yatome et al. (1993). The decrease in absorbance was monitored at A_{530} and A_{420} nm for crystal violet and malachite green, respectively. Decolourisation was calculated according to the following formula:

$$D\% = 100 \times \frac{(A_{ini} - A_{fin})}{A_{ini}}$$

Where D = decolourisation, A_{ini} = initial absorbance, and A_{fin} = final absorbance of dye, after incubation time.

RESULTS AND DISCUSSION

M. mucedo was selected for the present study among many soil fungi based on dye agar plate method where it showed very good decolourisation capability. With a view to enhance its selective decolourisation capacity, mutations were induced by both physical and chemical mutagens.

Table 1. Enzyme production by *Mucor mucedo* exposed to UV irradiation for different time intervals.

Fungus	Exposure to UV radiation (min)	Protease (U/ml)	Peroxidase (U/ml)	Laccase (U/ml)
Mucor mucedo	Wild strain	1.38	800	300
	3	1.39	800	300
	6	1.40	900	200
	9	1.48	1000	100
	12	1.42	700	100
	15	1.39	400	0

Growth

The mutants that were exposed to UV radiation for a time period of 3, 6 and 9 min did not vary much from the wild strain in mycelial morphology as well as in the growth pattern, instead they showed mild increase in growth rate, but the mutants that were exposed to extensive period of time such as 12 and 15 min to UV radiation showed reduced growth rate as well as reduction in extension and branching of hyphae.

When the wild strain was treated with ethyl methyl sulfonate, it was clear that there was not much improvement up to 50 mg of EMS, in the growth of *Mucor mucedo* when compared to the wild strain. Initially, 1, 10 and 50 mg of EMS prevented either spore swelling or germ tube emergence, whereas when the concentration was increased to 100 and 150 mg, it inhibited the elongation of germ tube and hyphae and also spore germination. This showed 6.25 and 20.88% of reduction of the growth relative to control, but *Mucor mucedo* that was treated with 50 mg of EMS showed 26% increase in the growth rate indicating that EMS is stimulatory in mild concentrations. The results clearly indicated that the high concentrations of EMS had negative effect on the growth pattern of *Mucor mucedo*.

When the wild strain was treated with 10 mg of diethyl sulfonate, there was not much differentiation in the growth pattern of the mutants when compared to the parental strain. At the concentration of 100 and 150 mg, there was inhibition in the formation of hyphae and decrease in growth. After 10 days of incubation, surprisingly, at the concentrations of 100 and 150 mg, spores of the mutants were converted to yeast like cells which produced buds instead of hyphae.

With respect to the wild strain, when treated with 10 mg of colchicine, it was observed that there was no such morphological change as well as changes in the growth pattern. When the concentration of colchicine was increased to 50, 100 and 150 mg, it abolished the germ tube and hyphal elongation. Colchicine at 100 and 150 mg inhibited the germination of spores into hyphae. The mutants that were treated with 1and 10 mg of colchicine showed mild increase in growth rate. But, the mutants that were treated with high concentrations (50, 100 and 150 mg) of colchicine showed reduced growth rate. They showed 1, 18.8 and 33.3% of reduction of the growth compared to parental strain, respectively. These results indicated that when the strain was treated with low concentrations of colchicine, the effect was stimulatory whereas when the strain was treated with high concentrations it had an inhibitory effect on the growth pattern.

Estimation of protease, peroxidase and laccase by mutants

To investigate the effect of mutagenesis on the production of decolourising enzymes, the mutants were inoculated in PDB and incubated at 37°C for 10 days. The obtained data are presented in Tables 1– 5.

With respect to enzyme productions, there was increase in protease (1.48 U/ml) and peroxidase (1000 U/ml) production and decrease in laccase production in *Mucor mucedo* when exposed to 9 min UV radiation. Regarding the peroxidase enzyme production, wild strain secreted 800 U/ml whereas the strain that was treated with 50 mg of EMS showed maximum enzyme production (1200 U/ml). There was decrease in protease and laccase production in *Mucor mucedo* when it was treated with different concentrations of EMS. Also, there was a decrease in protease, peroxidase and laccase production in *Mucor mucedo* when treated with different concentrations of DES. There was an increase in protease (1.86U/ml) and peroxidase (1000U/ml) enzyme production only at 10 mg concentration of colchicine proving that the higher enzymatic secretions were responsible for the decolourisation activity.

Decolourisation activity of the mutants with crystal violet and malachite green

The decolourisation efficiency of the mutants during the decolourisation of two triphenyl methane group dyes (crystal violet and malachite green) was studied and the results are presented in Tables 5 - 8. The enzymatic

Table 2. Enzyme production by *Mucor mucedo* treated with different concentrations of ethyl methyl sulfonate.

Fungus	Ethyl methyl sulfonate (mg)	Protease (U/ml)	Peroxidase (U/ml)	Laccase (U/ml)
Mucor mucedo	Wild strain	1.38	800	300
	1	1.35	800	200
	10	1.29	1000	200
	50	1.24	1200	100
	100	0.39	1100	0
	150	0.20	700	0

Table 3. Enzyme production by *Mucor mucedo* treated with different concentrations of diethyl sulfonate.

Fungus	Diethyl sulfonate (mg)	Protease (U/ml)	Peroxidase (U/ml)	Laccase (U/ml)
Mucor mucedo	Wild strain	1.38	800	300
	1	1.26	800	200
	10	1.14	700	100
	50	0.98	500	0
	100	0.60	100	0
	150	0.30	0	0

Table 4. Enzyme production by *Mucor mucedo* treated with different concentrations of colchicine.

Fungus	Colchicine (mg)	Protease (U/ml)	Peroxidase (U/ml)	Laccase (U/ml)
Mucor mucedo	Wild strain	1.38	800	300
	1	1.39	800	300
	10	1.86	1000	100
	50	1.66	900	100
	100	1.23	600	100
	150	1.20	400	0

Table 5. Decolourisation of crystal violet and malachite green by wild and mutants of *Mucor mucedo.*

Fungus	Exposure to UV (min)	Decolourisation (%)	
		Crystal violet	Malachite green
Mucor mucedo	Wild strain	78	65
	3	82	66
	6	88	68
	9	90	70
	12	85	65
	15	83	65

activity was directly related to the decolourisation activity. When the wild strain was exposed to 9 min UV radiation, maximum decolourisation of crystal violet (90%) and malachite green (70%) was witnessed relating to the higher enzyme secretions and also to the exposure time period. The organism treated with 50 mg of EMS showed the maximum decolourisation efficiency in both crystal violet (90%) and malachite green (71%). The strain that was treated with 1 mg of DES showed the same decolourisation activities just like wild strain in the case of

Table 6. Decolourisation of crystal violet and malachite green by *Mucor mucedo* after 10 days of incubation in the presence of EMS.

Fungus	Ethyl methyl sulfonate (mg)	Decolourisation (%)	
		Crystal violet	Malachite green
Mucor mucedo	Wild strain	78	65
	1	82	68
	10	84	68
	50	90	71
	100	83	70
	150	75	67

Table 7. Decolourisation of crystal violet and malachite green by *Mucor mucedo* after 10 days of incubation in the presence of DES.

Fungus	Diethyl sulfonate (mg)	Decolourisation (%)	
		Crystal violet	Malachite green
Mucor mucedo	Wild strain	78	65
	1	78	65
	10	78	63
	50	76	60
	100	74	58
	150	65	58

Table 8. Decolourisation of crystal violet and malachite green by *Mucor mucedo* after 10 days of incubation in the presence of colchicine.

Fungus	Colchicine (mg)	Decolourisation (%)	
		Crystal violet	Malachite green
Mucor mucedo	Wild strain	78	65
	1	85	70
	10	89	74
	50	85	73
	100	75	68
	150	70	60

crystal violet (78%) and malachite green (65%), and when the concentration was increased, the decolour-isation efficiency gradually decreased. As there were no active enzymatic secretions, there was no decolourisation activity also. With 1 mg of colchicine, decolourisation efficiency of 89% in crystal violet and 74% in malachite green was observed. The wild strain isolated from dye effluent amended soils when exposed to different physical and chemical mutagens showed improvement in the decolourisation of crystal violet and malachite green except in the case of DES.

The mutagenesis was dose-dependent and time-dependent and high doses proved to be lethal and this strain was found to be to some extent radiation resistant when subjected to irradiation at small doses for short duration. There was no or marginal improvement of decolourisation when the strain was exposed to ultraviolet irradiation for seconds. Improvement of deco-lourisation efficiency resulted from successive transfers of radiation treated strains. Chemical mutagens like diethyl sulfonate invariably inhibited decolourisation effi-ciency and the inhibition was dose dependent. But there was great improvement in the dye decolourisation efficiency with ethyl methyl sulfonate and colchicine.

Improvement of biomethane production was achieved by subjecting the methanogens to mutagenic changes by physical (irradiation) and chemical (colchicine, acridine orange) mutagens (Chakrahorty et al., 2003). The production of biomedically important enzyme lipase was enhanced by subjecting the indigenous lipase producing strain *Rhizopus* sp. BTS-24 to improvement by natural selection and random mutagenesis (UV and N-methyl-N'-

nitro-N-nitroso guanidine, NTG) (Bapiraju et al., 2004). *Candida tropicalis* was treated with ultraviolet (UV) rays, and the mutants obtained were screened for xylitol production. One of the mutants (CT- OMV5) produced 0.85 g/g of xylitol from xylose (Rao et al., 2005). A mutant, *Fusarium maire* K178 with high production of paclitaxel (taxol) was selected by protoplast mutation of UV radiation and diethyl sulfate (DES). After the strain improvement and optimization of the media, the yield of taxol increased from 20 to 225.2 g/l (Xu et al., 2006). Jeennor et al. (2008) identified a *Mucor rouxii* mutant with high accumulation of an unusual trans-linoleic acid by the use of chemical mutagens. All the above works proved that mutagenesis played a very good and effective role in strain improvement supporting our results.

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