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Optimisation of Culture Conditions for Enhanced Decolourisation of Congo red by *Aspergillus* Species

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Abstract: *The present work was aimed to optimize different bio-physico-chemical parameters such as temperature, pH, additional carbon source, Nacl and biomass concentration for enhancing decolourisation of Congo Red, an Azo Dye by* Aspergillus *species isolated from the textile effluent amended soils. Our primary investigations revealed that the process of decolourisation was concomitant with the exponential growth phase of the fungi. The maximum extent of Congo Red decolourisation was recorded at 40°C temperature, pH-6, and at 0-0.5% Nacl concentration. The addition of 2% glucose as a co-substrate to the culture medium increased the rate of decolourisation by fungi than the addition of starch and mannitol, which may be attributed to the generation of redox equivalents (electron donors) as a result of glucose metabolism. Further, the raised mycelial biomass concentration on incubation exhibited much rapid decolourisation of culture media, thus confirming biosorption, as a promising way of decolourising textile effluents.*
Keywords: *Aspergillus, Biosorption, Congo Red , Decolourisation, Textile effluent.*

I. Introduction.

Increasing industrialization and urbanization leads to environmental pollution. Among various industries, textile dyeing industries discharge large volume of waste water after dyeing process [1]. Textile processing industries largely employ azo dyes and it had been estimated that about 10% of the dye stuff in the dyeing processes do not bind to fibers and are, therefore, released to the environment. They posses toxicity like lethal effect, genotoxicity, mutagenicity, and carcinogenicity to plants and animals. The discharge of these toxic effluents adversely affect water resources, soil fertility, aquatic organisms and disturb the integrity of ecosystem by altering the pH, increasing the biological oxygen demand (BOD) and chemical oxygen demand (COD) and there by greatly affect the water quality [2]. Azo dyes are considered as recalcitrant xenobiotic compounds, due to the presence of an N=N bond and groups such as aromatic rings that are not easily degraded. The discharge of these colored compounds into the environment causes considerable non aesthetic pollution and serious health risks. Once they are released , they produce toxic amines by reductive cleavage of azo linkages which causes severe effects on human beings through damaging the vital organs such as brain, liver, kidneys, central nervous and reproductive systems[3]. In Aquatic systems, these effluents affect the aesthetic merit, water transparency and gas solubility of the water bodies leading to the reduction in sun light penetration which in turn, decreases the photosynthetic activity, reduces dissolved oxygen concentration, depicts toxic effect on aquatic flora and fauna[4]. Thus several methods are adopted by textile industries for the reduction of azo dyes to achieve complete decolourisation before discharge.These include physico chemical methods, such as flocculation, filtration, specific coagulation etc . the implementation of these methods have inherent drawbacks of being economically unfeasible and generating sludge[5]. The microbial decolourisation and degradation of azo dyes has been of considerable interest since it is inexpensive, eco friendly and produces a less amount of sludge. A number of micro organisms have been found to be able to decolourise textile dyes including bacteria, fungi, and yeast [6]. Of these organisms extensive research work has been carried out on fungi. They developed certain enzyme systems for the decolourisation and mineralization of azo dyes, however various factors and environmental conditions may influence decolourisation. Thus our present work is mainly designed to study the effect of various physical, chemical and biological parameters on the rate of decolourisation and to find out the optimal conditions for enhancing decolourision of azo dyes by fungi.

II. Materials And Methods

2.1 Collection of soil samples and dyes

The soil samples were collected from nearby places where the dye effluents were discharged from the small and medium scale textile dyeing units. The structural and chemical information of the selected dye Congo Red was given in our earlier paper [7]. Concentration of dye used was 0.02 g/L as followed by [8].

2.2 Isolation and screening of soil fungi for dye decolourization

Soil fungi were isolated on Potato Dextrose Agar (PDA) medium following the standard method [11]. Morphologically different fungi were screened on PDA medium incorporated with the selected azo dye, by dye agar plate assay technique [10].

2.3 Colony morphology of selected fungi

High potential dye decolorizing fungi were selected by clear zone formation and characterized according to Gilmann [11]. These fungal cultures were streaked on PDA slants and stock cultures were sub cultured monthly and stored at $4^{o}C$ for further use.

2.4 Dye Decolourisation assay

The assay was performed following the method of Dias et al [12] The percentage of dye decolourisation was expressed in terms of degree of decrease in the absorbance at 506 nm for Congo Red against the initial absorbance at the same wavelength. Decolourisation yield was calculated by using the formula given by Yatome et al [13] and results were discussed in detail in our previous paper [7].

2.5 Optimization studies of fungal decolourisation of azo dyes

As different physico-chemical and biological parameters influence the ability to decolorize dyes, the following optimization studies were under taken.

2.5.1 Effect of temperature on fungal decolourisation of selected azo dyes

In the study of effect of temperature on dye decolourisation, 250 ml of sterile potato dextrose broth incorporated with selected dye was taken in different flasks and inoculated with the test fungi. These flasks were incubated under static conditions at different temperatures (20, 30,40, 50 and $60^{0}C$) for about 7 days. After incubation, the mycelial mat was removed and the culture filtrate was used for the dye decolourisation assay. For each dye decolourisation assay, a group of three flasks were operated along with the set of control flasks without fungal inoculum [14].

2.5.2. Effect of pH on fungal decolourisation of selected azo dyes

The decolorisation studies of the three fungal isolates with Congo Red were carried out at pH 2, 4, 6, and 8 by adjusting pH of the medium using 0.1 N NaOH & 0.1 N HCl. The fungal cultures were grown at the respective pH values for 7 days at $28^{0}C$ under static condition and used for decolourisation assay [15].

2.5.3 Effect of additional carbon source amendment on dye decolorisation

The effect of co substrates on dye decolourisation was investigated individually with glucose, starch and mannitol incorporated into the potato dextrose broth medium at the rate of 2% and percentage of decolourisation was assayed in fungal inoculated and control flasks at the end of 7 days [16] [17].

2.5.4 Effect of salt concentration on decolourisation activity

The ability of the fungi to decolourise the selected azo dye in presence of salt solution was tested in the present study as per the method described by Sumati and Manju [18]. Individual experiments were conducted by adding 0.5%, 1% and 1.5% of NaCl into the fungal growth medium containing 200 mg/lt of Congo Red and decolourisation assay was performed after 7 days of incubation.

2.5.5 Effect of microbial biomass on decolourisation activity

Fungal biomass measured in terms of dry weight produced on incubation for 7, 14 and 21days grown at $29^{0}C$ was used to study its effect on decolourisation of mono azo dye, Congo Red with 3 species of Aspergillus inoculated into the potato dextrose broth medium following the method suggested by [19].

III. Results And Discussion

3.1 Screening and identification of potent fungal isolates involved in dye decolorization

Nearly 17 fungal species have shown positive reaction for dye decolourisation in screening test. Among them, the most frequently encountered potential dye degrading fungi selected for different decolourisation studies were *Aspergillus niger, Aspergillus flavus* and *Aspergillus fumigatus*). The morphological characterisation of the selected fungi was described clearly in [7].

Among the 17 positive species, Similar to our observations, many researchers, [20] [21] [22] have investigated on various diversified fungal species and identified the species of *Aspergillus* capable of removing dyes from aqueous solution. Based on our results and going through the literature, it may be concluded that the

contaminated sites are the potential centers for the isolation of completely or partially mutated or adopted fungi capable of degradation and decolourisation of dyes.

3.2 Effect of temperature on the rate of decolourisation of Congo red

Table -1 Effect of temperature on Congo Red decolourisation at 7 days of incubation

Fungi	Temperature (^{O}c)	Decolourisation (%)
Aspergillus niger	20	47
	30	52
	40	58
	50	39
	60	31
Aspergillus flavus	20	51
	30	63
	40	69
	50	45
	60	36
Aspergillus fumigatus	20	53
	30	61
	40	67
	50	48
	60	37

Maximum decolourisation of 58 - 69% was observed at 40°C in all the three fungal cultures at the end of 7 days of incubation period (Table-1). At temperature below 40°C i.e, 20 and 30°C , 47-63% of moderate decolourisation occurred, but at high temperature range of 50 and 60°C, percentage of decolourisation was minimum (31-48%). With respect to *Aspergillus niger* 58% of decolourisation was observed at 40° C and less percentage of decolourisation (31%) was recorded at 60°C. Comparatively, in *Aspergillus flavus* decolourisation activity was seen at various temperatures but more significantly at 40°C (69%). Experimental results of *Aspergillus fumigatus* showed 67% of maximum decolourisation of Congo red at 40° C by the end of 7 days.

The data obtained from Table-1 revealed a gradual rise in level of decolourisation up to 40°C followed by a decreasing trend with further advancement of temperature. This may be due to the inactivation of enzymes involved in reduction of the selected mono and di azo dyes at higher temperature.

Coinciding with the above data, Ramalho et al [23] reported that the decolourisation rate of azo dyes was influenced by effect of temperature and other environmental factors suggesting the direct (or) indirect participation of enzyme activity. In similar to our experimental analysis , Zilly et al [24] investigated the ability of Pleurotus pulmonarius to decolourise 10 synthetic dyes like Congo red, Trypan blue, Methyl green etc and revealed that 40-45^{0}C range of temperature is the optimum to obtain the maximum extent of decolourisation of Congo red and Trypan blue.

Like wise, In a study made by Ali et al., [10] highest decolourisation of Acid red 151 (97.71-97.55%) by Aspergillus niger SA1 was observed at a mesophilic temperature range of 25-30^{0}C after 8 days of incubation. According to Singh et al [25] the rate of decolourisation of Congo red and Methyl green by Pleurotus sajor caju was positively effected by a temperature range of 30-35^{0}C , indicating that this temperature range is probably optimum for the enzymes responsible for decolourisation.

3.3 Effect of P^{H} on the rate of decolourisation of Congo red

Among the three fungal species, it was evident from Table-2 that the percentage of decolourisation of Congo red was maximum (65 %) in A.flavus at P^{H} 6 for 7 day interval, where as ,at acidic P^{H} 2 and alkaline P^{H} range 8-10, there is an abrupt decrease (25-28%) in the decolourisation activity of three *Aspergillus* members. The reason for variation in the percentage of decolourisation at different P^{H} range is, Congo red, an diazo dye is having less P^{k}a value at alkaline P^{H} range. So, azo dye reduction at lower and higher P^{k}a values becomes difficult where as, at neutral P^{H} range (P^{H}-6), the dye possess optimum P^{k}a value. Hence it is easily prone to reduction process [23].

The present experimental finding was in coincidence with the report made by Zilly et al [24]. As per his data, high level of decolourisation of Congo red by *Pleurotus pulmonarius* was noticed at pH 6.5. Singh et

al [25] reported greatest level of decolourisation of Congo red , Trypan blue , Crystal violet by *Pleurotus sajor caju* at a P^H range of 4.0 - 4.5.

Similarly Ramya et al [26] made a study on biodegradation of Reactive blue by *Aspergillus* species and found that decolourisation ability varied with the p^H ranging from 3-8. However p^H 3 was noted to be the optimal for the decolourisation activity. The above findings clearly indicate that the effect of p^Hdecolourisation is mainly associated with the type of micro organism and dyes used, and p^H dependence of the enzymes involved in decolourisation

3.3 Effect of carbon Source on decolourisation of Congo red

From the experimental data represented in Table-3, utilization of glucose as a carbon substrate for the decolourisation of the selected azo dye was more prominent when compared to starch and mannitol.The decolourisation activity was enhanced in all the three fungal cultures with glucose supplementation.

The percentage of decolourisation with glucose was 92% for *Aspergillus flavus*, 88% for *Aspergillus fumigatus,* 86% for *Aspergillus niger* . Where as the percentage of discolouration with starch was 77% for *Aspergillus flavus* 75% for *Aspergillus fumigatus* and 72% for *Aspergillus niger*, but with regard to mannitol there is a significant drop in decolourisation activity.

Table-2. Effect of p^H on Congo red decolourisation at 7 days of incubation

Fungi	P^H	Decolourisation (%)
Aspergillus niger	2	30
	4	48
	6	62
	8	54
	10	25
Aspergillus flavus	2	28
	4	49
	6	65
	8	47
	10	26
Aspergillus fumigatus	2	27
	4	46
	6	63
	8	49
	10	28

Table-3 Effect of Carbon source on Congo red decoloursation at 7 days of incubation

Carbon Source	Percentage of decolourisation		
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus fumigatus*
Glucose	88	92	86
Starch	75	77	72
Mannitol	66	69	65

Enhanced rate of discolouration of both glucose and starch was attributed to generation of electron donors as a result of metabolism of this co-substrates. Decrease in percentage of discolouration of mannitol might be due to the microbial inability to utilize mannitol as a suitable nutrient source, which inturn showed adverse effect on percentage of decolourisation. Similar finding was reported by [16]. According to them, decolourisation increased with glucose and starch for both Brilliant green and Crystal violet. In their experiment maximum decolourisation of 92.3% was observed for Brilliant green and 90.2% for Crystal violet at 0.2 mg/ml of glucose concentration. Ryu and Weon [17] confirmed that 2% glucose amendment supported the highest level of decolourisation of Congo red (91%) by *Aspergillus sojae B10* than that of other carbon sources (maltose, xylose and mannitol) tested.

3.4 Effect of sodium chloride concentration on growth and decolourisation of Congo red

Growth medium with different NaCl concentration (0, 0.5, 1.0, 1.5%) amended with Congo red is inoculated with *A.niger, A.flavus* and *A.fumigatus.* The growth pattern in correlation with percentage of decolourisation was observed and the results were presented in Table-4.

The growth of the three fungi was observed upto 1.5% concentration of NaCl after 7 days of

inoculation. However, the size of the fungal mat was more prominent at 0.5% NaCl concentration in all the three *Aspergillus* species. At 1.0% NaCl concentration the mycelial growth was moderate in both *A. niger* and *A. fumigatus*, where as significant growth was visualized in *A. flavus*. Minimum growth was observed at 1.5% of NaCl level in both *A niger* and *A. fumigatus* and moderate in A flavus. Coinciding with the growth pattern of the three fungal isolates, percentage of decolourisation was highest (41% - 61%) at 0.5% NaCl concentration and lowest (26-45%) at 1.5% NaCl concentration.

Supporting the above finding, Sumathi and Manju [18] have proposed that *Aspergillus foetidus* was able to grow and decolourise the Dimarene dye (38%) at 36 hrs of incubation in presence of 1% NaCl. Contradictory to the above statement, microbial growth was even observed at high concentration of 12% NaCl by a halophilic bacteria, Halomonas species isolated from alkaline black liquor according to Yang et al., [27]. This might probably due to the enzymatic tolerance of the organism at extreme condition.

Table-4. Effect of NaCl concentration on fungal growth and Congo red decolourisation at 7 days of incubation

Fungi	Nacl Conc (%)	Mycelial growth	Decolourisation (%)
Aspergillus niger	0	+++	47
	0.5	+++	41
	1.0	++	34
	1.5	+	26
Aspergillus flavus	0	+++	58
	0.5	+++	61
	1.0	+++	53
	1.5	++	45
Aspergillus fumigatus	0	+++	66
	0.5	+++	59
	1.0	++	48
	1.5	+	37

3.6 Effect of microbial biomass on the rate of decolourisation of Congo red

The data obtained from the effects of microbial biomass on rate of decolourisation was summarized in Table-5. As per the tabular data, *Aspergillus flavus* produced significant amount of mycelial biomass (3.3 gm) after 21 days of incubation and also exhibited 89% of highest decolourisation. Next to this, more biomass production and decolourisation was seen in *Aspergillus fumigatus*. Comparatively, biomass production and percentage of decolourisation was minimum in *Aspergillus niger* No toxic effect of Congo red was detected in all the three fungal species.

The growth of all the fungal species was meagre at 7 days, moderate at 14 days and highest at the end of 21 days, that means the increase in the incubation time resulted in production of remarkable mycelial biomass. This increase in the fungal biomass with the increase of incubation time may be attributed to that the fungal members utilized the azo dye in the medium as nutrient source for its mycelial growth and biomass development. It may also be due to that the increased biomass contributed to pronounced level of enzymatic activity that resulted in effective decolourisation of azo dye or it may be due to the absorption of most of dye on to the mycelia mat, (biosorption), which ultimately lead to increased rate of decolourisation.

Table-5. Effect of Microbial biomass on Congo red decolourisation at 7 days of incubation

Fungi	Days of incubation	Dry weight of mycelial bio mass (gm)		percentage of decolourisation
		Control	Test	
Aspergillus niger	7	1.41	2.2	50
	14	1.98	2.5	71
	21	2.34	2.8	80
Aspergillus flavus	7	1.15	2.3	61
	14	1.86	2.8	78
	21	2.25	3.3	89
Aspergillus fumigatus	7	1.10	2.3	68
	14	1.70	2.7	73
	21	2.25	3.0	82

In accordance with the present experimental data, Capalash and Sharma [28] revealed 42% increase in the rate of decolourisation of azo dye with increase in the microbial biomass of *Phanerochaete chrysosporium* from 0.2-2mg/ml. Shedbalkar et al [14] reported that *Penicillium ochrochloron* when used for decolourisation at 48 hours, as there was no sufficient growth, it could not show the decolourisation of Cotton blue which might

be due to insufficient amount of enzymes required for the degradation process. However maximum decolourisation i.e, 93% of Cotton blue was observed with the use of 96h grown biomass.

IV. Conclusion

The presence of dyes imparts an intense colour to the effluents, which leads to the environmental pollution problems. In tne present work, *Aspergillus* species werefound to be most effective in the decolourisation of azo dyes , other than the so far extensively studied white rot fungi. It may be concluded that, undoubtedly various environmental parameters may influence the rate of degradation process by indirectly influencing the microbial enzyme systems. It was observed that the rapid decolourisation can be achieved by providing the optimal conditions like culture media, temperature, pH, salt concentration, and addition of suitable co-substrates etc. Moreover, decolourisation by using microbial biomass (biosorption technique), proved to be an efficient, cost effective and a promising alternative approach in effluent treatment.

References

[1]. H. Zollinger. Colur chemistry, syntheses, properties and applications of organic dyes and pigments, VCH Publishers, New York, 1987, 92-102.

[2]. N. Puvaneswari, J. Muthukrishnan and P. Gunasekaran, Toxicity assessment and microbial degradation of azo dyes, Indian Journal of Experimental Biology, 44, 2006, 618-626.

[3]. Z. Aksu, Application of biosorption for the removal of organic pollutants: a review, Process Biochemistry, 40, 2005, 997-1026.

[4]. Archana, K. N. Lokesh, R. R. Siva Kiran, Biological mehods of dye removal from textile effluents- A Review, Journal of Biochemical technology, 3,(5), 2012,117-180.

[5]. F. Zhang., J.S. Knapp and K.N. Tapley, Decolourisation of cotton bleaching effluent with wood rotting fungus. Water Resources, 33(4), 919-928, (1999).

[6]. I. M. Banat, P. Nigam, D. Singh and R. Marchant, Microbial decolourisation of textile dyecontaining effluents: A Review, Bioresource Technology 58 (3), 1996, 217-227.

[7]. Ravuri jaya madhuri. And Gangavarapu vijaya lakshmi. Biodegradation of Congo Red Azo dye by *Aspergillus spp*. isolated from dye contaminated sites. Journal of Agricultural Science and Technology A_4, 2014, 422-434.

[8]. N. S. Raju, G.V. Venktaramana, S.T. Girish, V.B. Raghavendra and P. Shivashankar, Isolation and evaluation of indigenous soil fungi for decolourisation of textile dyes, Journal of Applied Science, 7(2), 2007, 298-301.

[9]. K.R. Aneja, Experiments in microbiology, Plant pathology and Biotechnology 4th edition, (New Age International Pvt. Ltd. Publishers), 2008, 69-71.

[10]. N. Ali, A. Hameed, S. Ahmed and A. G. Khan, Decolourisation of structurally different textile dyes by *Aspergillus niger* SA1, World. Journal of Microbiology and Biotechnology 24, 2008, 1067-1072.

[11]. C.J. Gilman, A Manual of Soil Fungi, Revised 2nd edition, Oxford and IBH Publications, India, 1957, 219-229.

[12]. A. A. Dias, R.M. Bezerra, P.M. Lemos, A.N. Pereira, Invivo and laccase catalysed decolourisation of xenobiotic azo dyes by a basidio mycetous fungus: Characterization of its ligninolytic system, World Journal of Microbiology and Biotechnology, 19, 2003, 969-975.

[13]. C. Yatome, S. Yamada, T. Ogawa and M. Matsui, Degradation of Crystal violet by *Nocardia coralline*. Applied Microbiology and Biotechnology 38, 1993, 565-569.

[14]. U. Shedbalkar, D. Rhishikesh and J. Jyoti, Biodegradation of triphenyl methane dye Cotton blue by *Penicillium ochrochloron* MTCC 517, Journal of Hazard Materials. 157, 2008, 472-479.

[15]. Swamy and J. A. Ramsay, The evaluation of white rot fungi in the decolourisation of textile dyes. Enzyme Microbial Technology, 24, 1999, 130-137.

[16]. N. A. Oranusi and C. J. Ogugbue, Effect of co substrates on primary biodegradation of triphenyl methane dyes by Pseudomonas. African Journal of Applied Zoology and Environmental Biology 7, 2005, 38-44.

[17]. B.H. Ryu and Y.D. Weon, Decolourisation of azo dyes by *Aspergillus sojae B10*, Journal of Microbiology and Biotechnology 2(3), 1992, 215-219.

[18]. **S.** Sumathi and B.S. Manju, Uptake of reactive textile dyes by *Aspergillus foetidus*. Enzyme Microbial Technology. 27, 2000, 347-355.

[19]. I. Seyis and T.Subasioglu. Comparison of live and dead biomass of fungi on decolourisation of Methyl orange, African Journal of Biotechnology 7(13), 2008, 2212-2216.

[20]. J. H. Kim, C.H. Rhee, C.J. Woo Park, G.J. Joo and S.k. Seo, 1999. Isolation and characterization of terephthalic acid degrading bacteria, Korean Journal of Applied Microbiology and Biotechnology 27, 118-123.

[21]. Y. Fu, T. Viraraghavan, Review on fungal decolourisation of dye waste waters, Bioresource Technology 79, 2001, 251-262.

[22]. M. Nagai, T. Saito, H. Watanabe, K. Saito, M. Kawata, H.Enei, Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes* and decolourisation of chemically different dyes, Applied Microbiology . Biotechnology, 60, 2002, 327-335.

[23]. P.A. Ramalho, M.H.Cardoso Cavaco, A. Paulo and M.T. Ramalho, Characterization of azo reduction activity in a novel ascomycete yeast strain, Applied Microbiology Biotechnology, 70, 2004, 2279-2288.

[24]. A. Zilly, C.G.M. Souza, I.P.B. Tessmann and R.M. Peralta, Decolourization of industrial dyes by a Brazilian strain of *Pleurotus pulmonarius* producing laccase as the sole phenol oxidizing enzyme. Folia Microbiology 47 (3) , 2002 , 273-277.

[25]. A. D, Singh, V. Sabaratnam, N, Abdullah, MSM Annuar and K.B. Ramachandran, Decolourisation of chemically different dyes by enzymes from spent compost of *Pleurotus sajor caju* and their kinetics. African Journal of Biotechnology 9(1), 2010, 041-054

[26]. M. Ramya, B. Anusha, S. Kalavathy and S. Devilakshmi Bio decolourisation and biodegradation of Reactive blue by *Aspergillus sp,* African Journal of Biotechnology 6(12), 2007, 1441-1445

[27]. C.Yang, W. Zhe, L.Yang, N. Yu, D. Miaofen, F.H. Xia, M. Cuiqing, J. Hongzhi and Y. Ping, Metabolic versatility of halotolerant and alkaliphilic strains of *Halomonas* isolated from alkaline black liquor, Bioresource. Technology 101, 2010 , 6778-6784.

[28]. N. Capalash and P. Sharma, Biodegradation of textile azo dyes by *Phanerochaete chrysosporium*. World Journal of Microbiology and Biotechnology 8, 1992, 309-312.