ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2017; 3(6): 1028-1031
www.allresearchjournal.com
Received: 10-04-2017
Accepted: 12-05-2017

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Biodegradation of pigment yellow-73 (PY-73) by *Rhizopus stolonifer* (Ehrenb.: Link) Lind

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Abstract
Several dyes are employed in construction industry for providing aesthetic appearance. Among the many dyes that find application in the civil constructions belong to the class of azo-dyes. The quantity of azo-dyes employed is at an enormous extent and may pose a public health concern if disposed in large quantities. The azo-dyes are reported to initiate several health issues which may include allergic reactions and carcinogenesis. The azo-dye, Pigment Yellow-73 (PY-73) finds extensive application in construction of buildings for painting surfaces. Therefore its usage poses an environmental hazard to construction workers and the general public. A fungal strain was isolated from PY-73 painted surface and was identified as *Rhizopus stolonifer* (Ehrenb: Link) Lind. *R. stolonifer* decolorized PY-73 and it was degraded through the involvement of chlorophenol and aniline as the metabolic intermediates. The biodegradation of PY-73 by *R. stolonifer* was enhanced by increased agitation and in the presence of glucose (80%), starch (85%), and sucrose (60%) as the co-metabolic carbon sources for the decolorization. The biodegradation of PY-73 by *R. stolonifer* was assessed by spectral, TLC and enzyme analysis.

Keywords: Paints, Azo dyes; Decolorization; Biodegradation; Aromatic amines; chlorophenol

1. Introduction
Micro-organisms are simple in nutrient requirements. They use whatever is available as a food source, multiply and build up biomass. Biodegradation and biodeterioration processes are both positive and negative activities that are mediated by microbial activity [Role of Microbes in Environment]. Biological processes are dependent upon many factors that include light, temperature, microbial population, degree of acclimatization, accessibility of nutrient, cellular transport properties and chemical portioning of growth medium and so on [Guptha, 2012]. Many groups of microorganisms have been shown to be involved in paint deterioration. Bacteria and fungi can grow on applied paint films and solvent and water-based coatings. Most commonly isolated fungal species in paints have been reported [Resende *et al*., 1996; McCormack *et al*. 1996] [9, 6]. The deterioration of paints and paint-products is under the influence several factors which may include among others the anaerobic environment, the organic nature of the paint components, and the microbial quality of the packaging materials and the hygiene level of the manufacturing plant. The consequences of paint microbial deterioration include foul smell, viscosity loss, discolouration and visible surface growth that cause serious economic implication on paint industry [Obidi *et al*., 2009] [8]. The present study is to investigate the microbial action on paints with a view to improving the shelf life of paint and paint products.

Materials and Methods
Chemicals
Azodye Pigment Yellow 73 (PY-73) was procured from local market and used for experiments without further purification.

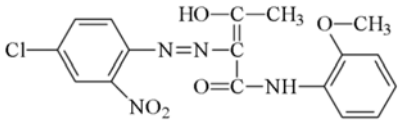

Fig 1: Chemical Structure of Pigment Yellow-73 (CAS No. 13515-40-7)

The chemical constituents used in fungal culture media and for the metabolic pathway investigations were of analytical grade.

Fungal culture media

Potato-Dextrose medium (PD) was used for routine culturing of fungus and Sabouraud Dextrose Agar (SDA) for maintenance. Alternatively, Byrde mineral salt medium (BMSM) composed of (g/L) of K_2HPO_4 (1.6), KH_2PO_4 (0.2), NH_4Cl (1.0), $MgCl_2$ (0.2), NaCl (0.1), $CaCl_2.2H_2O$ (0.02), $FeSo_4.H_20$ (0.01), $Na_2MoO_4.2H_20$ (0.05), $MnSO_4H_20$ (0.05), and $Na_2W0_4.2H_20$ (0.05) was used for some experiments [Byrde *et al*., 1956] [1]. The pH of media was adjusted to 5.5 and autoclaved.

Isolation of fungus degrading Pigment Yellow-73

Fungus degrading PY-73 was isolated from building scrapings by enrichment culture technique [Wei *et al*., 2010] [10]. Briefly, about 5g of building scrapings were suspended in 10 ml of distilled water and the suspension was swirled for about 1-2 hours. The soil particles from the suspension were removed by filtration. Then about 2 ml of this suspension was used to inoculate into100 ml of PD medium containing 1g PY-73 in a 250 ml Erlenmeyer flask. The flasks were incubated on a rotary shaker (110 rpm) at 35 ± 1^0C for a period of 2-3 days. When a good growth was observed, about 5-10 ml aliquot from the flask was transferred to BMSM supplemented with 0.01% PY-73 and incubated as before. After 3-4 such transfers, heavy growth of fungus was observed in the flasks and the transfers were repeated for 2-3 times. The enriched fungus was purified further and characterized based on its morphological and spore characteristics. The culture was preserved on SDA at 4^0 C and regenerated by inoculating on fresh SDA slants every 5-7 days.

Growth and Decolorization Studies

The biodegradation studies were initiated on medium incorporated with PY-73 (0.1mg/ml) into BMSM as a sole source of carbon and energy. The decolorization studies were carried out both under aerobic and anaerobic conditions. The poor growth of fungus in culture flask was enhanced by inclusion of additional carbon substrates (0.5-1%) like glucose, sucrose or starch as co-substrates and some cofactors like yeast extract and peptone. The experiments for decolorization studies of PY-73 by fungus were typically performed in 250 ml Erlenmeyer flasks containing 100 ml BMSM supplemented with 1% starch as co-substrate, 0.025% yeast extract, and 0.025% peptone. PY-73 at 0.05-0.10 mg/ml was included as a growth substrate. The fungal spent medium was withdrawn at regular time intervals and the biomass weight was determined to measure growth. The decolorization of the dye was determined by measurement of the absorption maxima of PY-73 at 580nm. Suitable blanks were prepared from aliquots of centrifuged medium. At high biomass densities, samples were diluted with water by the same factor.

Extraction and Characterization of Metabolites

Metabolites from the spent medium were extracted and characterized by Thin Layer Chromatography (TLC) followed by their detection with Ultraviolet torch. Briefly, the spent medium was acidified and extracted with ethyl acetate (1:3 v/v) three times and the extract obtained was dried over anhydrous sodium sulphate. After drying the residue was dissolved in methanol and characterized by TLC (Macherey-Nagel GmbH & Co, Germany), using the solvent system, benzene: methanol (95:5 v/v). After development of the chromatogram, the metabolite spots were scrapped from the plate, and extracted in methanol. The UV spectrum of the isolated sample was compared with authentic samples. The metabolic fate of PY-73 was monitored by solvent extraction of spent medium and by analysis of the components by TLC and UV analysis.

Preparation of cell free extract

The fungus was harvested in its log phase (3 days) of growth by filtration. The fungal mat was repeatedly washed with 50mM Citrate buffer of pH-5.5. After final suspension of the cells in 0.1M citrate buffer, (pH-5.5) containing 1mM ascorbic acid, 10% acetone, 10% glycerol and 100µM ferrous sulphate, they were macerated. After maceration, the fungal debris and unbroken cells were separated by centrifugation at 10,000 rpm for 20 minutes. The resulting supernatant was used as the crude source of enzymes. The protein in the enzyme preparation was estimated according to Folin-Ciocalteu method [Lowry *et al*., 1951] [5].

Assay of Azoreductase

Determination of the azoreductase activity was carried out spectrophotometrically by monitoring the decrease in absorbance of PY-73. The media components were dissolved in 50 mM sodium acetate buffer (pH 5.5) and degassed with nitrogen for 10 min. The assay mixture (3.5 ml) comprised of 20 mM of sodium acetate buffer (pH 5.5), 2mM NADH, 0.005 mM FAD and 100 µl enzyme extract, protein content (200 µg). After preincubation at 37^0C for 5 min, the reaction was initiated by adding 0.01-0.05 mM dye solution. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the decolourization of 1µmole of substrate per min.

Assay of Deaminase

The deaminase activity of the fungal extracts was determined [Chaney and Marbach, 1962]. Fungal cultures grown for 48h on a rotary shaker and fungal mycelia were then harvested by filtration, washed with Phosphate-citrate buffer (pH 5.5) and used for enzyme extractions. The deaminase activity in the extracts was determined by phenol and indophenol method. Deaminase assay mixture contained about 0.5 ml of 100 mM asparagine, 50 mM Phosphate-citrate buffer (pH 5.5) and enzyme to give a final volume of 1.5 ml were incubated for various intervals of time (0-30 min) at 37^0 C. The reaction was stopped by adding 0.1 ml TCA and centrifuged. The resulting supernatant was added to 6ml of water then treated with sodium phenate and sodium hypochlorite solutions and finally absorbance was read at 625 nm. One unit of enzyme activity was defined as the amount of ammonia in µmoles produced per min per milliliter enzyme solution under experimental conditions.

Assay of Dehalogenase activity

The Dehalogease activity was measured in a reaction mixture (5 mL) containing: 25mM phosphate buffer (pH 7.2), 1 mM chlorophenol (substrate) and fungal extract (0.04mg). Before initiation of the reaction, the reaction mixture was equilibrated at 30ºC in a water bath for 10 min.

The reaction was then initiated by the addition of enzyme, after which the free halide was determined. Enzyme activity unit is defined as the amount of enzyme that catalyses the formation of 1µmol halide ion/minute [Jing *et al*., 2008] [3].

Results and discussion

Screening and isolation of fungi degrading Pigment Yellow-73

Fungus degrading PY-73 was isolated from painted building scrapings by adopting enrichment culture technique. Among various fungi obtained the most potent and efficient pigment degrading members were further purified by adopting standard mycological procedures. The fungal isolates were characterized based on their morphological and spore characteristics on solid selective media following mycological manuals.

Fig 2: Fungi isolated from painted surfaces on PDA.

Table 1: Nomenclature and spectral properties of Pigment Yellow-73

Paint	Common name	C.i. no	Λ_{max}	Molar coefficient(ε)
Pigment Yellow -73	Yellow-73	11738	580	$6.41X10^3M^{-1}Cm^{-1}$

Table 2: Characteristics features of fungus degrading Pigment Yellow-73

Features	Properties
Macroscopic	Cotton-candy like, the colony is white initially and turns grey to yellowish brown in time, reverse is white to pale.
Microscopic	Brown in color and usually un branched, round with flattened bases, unicellular, round to ovoid in shape, hyaline to brown in color, and smooth.
Temperature response	Can grow well at 37°C.

Degradation of pigment yellow-73 by *Rhizopus stolonifer* (Ehrenb.: Link) Lind

The decolourization of PY-73 was monitored by following change in its absorbance maxima (580nm) consequent to fungal growth at different fermentation times. The dye concentration greatly decreased with increasing incubation periods. The decolorization assays indicated that the exponential growth phase was initiated after 1 day of incubation. The fungal growth and decolorization curves are depicted in Fig.3. The rate of decolorization was highest during the later part of the exponential phase of growth. It was demonstrated that a color reduction of 96% was achieved after 4 days.

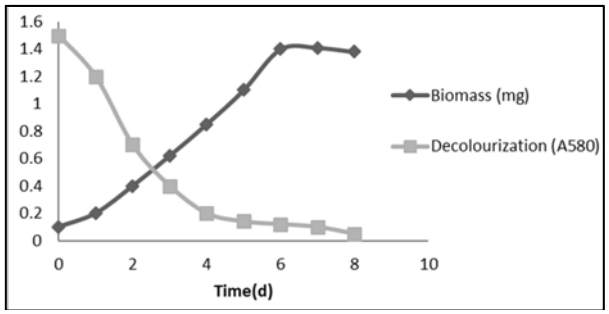

Fig 3: Growth and decolorization of Pigment Yellow-73 by *Rhizopus stolonifer* (Ehrenb.: Link) Lind

The initial biodegradability assays indicated that the dye was not readily utilized as a carbon source by *Rhizopus stolonifer* (Ehrenb.: Link) Lind. Therefore other carbon substrates like glucose, sucrose and starch were used as co-substrates. Among various co-substrates starch was found to assist greater decolourization (Fig.4).

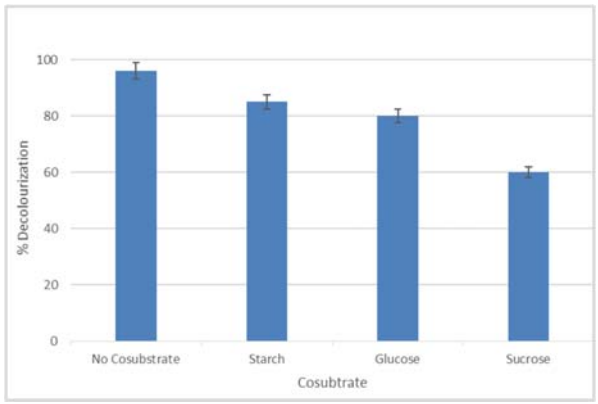

Fig 4: Decolourization of Pigment Yellow-73 in presence of co-substrates by *Rhizopus stolonifer* (Ehrenb.: Link) Lind

The metabolic pathway of PY-73 was investigated by isolation of metabolites that accumulate during the growth of *Rhizopus stolonifer* (Ehrenb.: Link) Lind in medium supplemented with PY-73 as substrate. The metabolites from the spent medium were isolated by solvent extraction and characterized by TLC and UV spectral analysis using authentic samples. The experimental results of metabolite characterization indicated the accumulation of metabolite having the λ_{max} and R_f values that matched to those of aniline (Table.3).

Table 3: TLC Analysis of metabolite isolated from spent medium of *Rhizopus stolonifer* (Ehrenb.: Link) Lind in utilizing Pigment Yellow-73

Growth substrate	Metabolite	R_f Authentic	R_f Isolated
Pigment yellow-73	Aniline	0.77	0.77

Degradation of Pigment Yellow-73 follows through the involvement of aniline

The possible fate of PY-73 was investigated by conducting the utilization studies (Fig.5). The involvement of aniline as an intermediate was proved by its isolation and characterization by TLC and UV studies of the spent extracts. The experiments showed that the aniline was further catabolized to non-aromatic end products as evidenced by the decrease in the absorbance specific to aromatic groups with increasing incubation times.

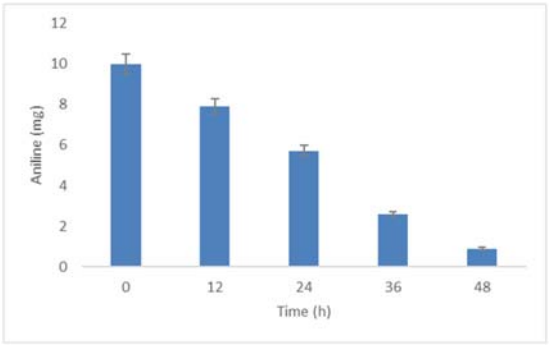

Fig 5: Utilization of aniline by *Rhizopus stolonifer* (Ehrenb.: Link) Lind

Enzymes of *Rhizopus stolonifer* (Ehrenb.: Link) Lind involved in the degradation of PY-73

The paint pigment was able to induce specific enzymes required for metabolism of PY-73. On growth of *Rhizopus stolonifer* (Ehrenb.: Link) Lind under aerobic conditions elaborated an azoreductase, a deminase and a dehalogenase.

Table 4: Enzyme activities in cell free extracts of Pigment Yellow-73 degrading *Rhizopus stolonifer* Ehrenb: Link) Lind.

Enzymes	Specific activity (µ moles min^{-1} mg^{-1} protein)
Azoreductase	0.36
Deaminase	0.43
Deholagenase	0.31

Discussion

Paints are released into the environment as construction and industrial effluents. The paint effluents are highly visible even at low concentrations (1mg\L). Added to this, certain paints, paint precursors and aromatic amines have been shown to be toxic, carcinogenic and mutagenic. Thus, appropriate treatment of paint wastewaters to remove both color and the paint compounds is clearly an important issue. Different physical, chemical and biological techniques can be used to remove paints from wastewater. Most physico-chemical dye removal methods have drawbacks because they are too expensive, have limited versatility, are greatly interfered by other wastewater components and/or generate other waste products that need to be handled further. Alternatively, biological treatment holds promise as a relatively inexpensive way to remove paints from wastewater.

The present study was directed towards characterization of the mechanism of decolourisation of PY-73 mediated by *Rhizopus stolonifer* (Ehrenb.: Link) Lind. The decolourization involves reduction of azo group, forming the corresponding amines, in agreement with other studies [Tan, *et al*., 2005]. The cell free extracts obtained from that *Rhizopus stolonifer* (Ehrenb.: Link) Lind growing and metabolizing PY-73 under aerobic conditions showed the presence of azoreductase, deaminase and dehalogenase activities. These enzymes supported the reduction of azo group of PY-73, removal of same as ammonia by deaminase and possible removal of halogen by the dehalogenase. The TLC analysis of the metabolic intermediates indicated the accumulation of aniline in the spent medium. Thus the experiments establish that azo dye PY-73 undergoes complete mineralization due to the activity of *Rhizopus stolonifer* (Ehrenb.: Link) Lind.

Conclusion

The fungus *Rhizopus stolonifer* (Ehrenb.: Link) Lind decolourized and degraded PY-73 efficiently. The fungus also elaborated enzymes required for decolourization and biodegradation of PY-73. On the basis of the utilization studies of the dye PY-73, isolation of the metabolites and the assay of enzymes, *Rhizopus stolonifer* (Ehrenb.: Link) Lind is concluded to metabolize PY-73 to innocuous substance.

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