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REVIEW

Biodegradation Perspectives of Azo Dyes by Yeasts[1]

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Abstract—Azo dyes are the largest class of synthetic dyes, which are widely used in the textile industry. The amount of dyestuff does not bind to the fibers and is lost in wastewater during textile processing. The discharge of colored effluents into the environment is not only aesthetically unpleasing. Moreover, dyes and their breakdown products cause toxic effects and they affect photosynthetic activity of aquatic systems by reducing light penetration. A number of microorganisms belonging to different taxonomic groups of bacteria, algae, fungi and yeast have been reported for their ability to decolorize azo dyes. In the literature the ability to decolorize azo dyes by yeasts, compared to bacterial and fungal species, has been studied in a few reports. Within this review, an attempt is made to elucidate some basic biological aspects associated with the azo dye degradation by yeasts and enzymes involved that are responsible for degradation process.

Keywords: azo dyes, biodegradation, decolorization, textile wastewater, yeast

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INTRODUCTION

Azo dyes are the largest class of synthetic dyes. Approximately 70% of all the dyes used in industry are azo dyes. They are widely used in textile, cosmetic, leather, pharmaceutical, paper, paint and food industries [1–3]. The textile industry is one of the greatest generators of liquid effluent pollutants due to the high quantities of water used in dyeing processes [2]. The amount of dyestuff that does not bind to the fibers and is lost in wastewater during textile processing, varies from only 2–3% when using basic dyes to 50% when certain reactive dyes are used [4]. The azo dye concentration in wastewater produced by textile industries varies from 5 to 1.500 mg L^{-1} [5]. The discharge of colored effluents into the environment is not only aesthetically unpleasing. Moreover, dyes and their breakdown products cause toxic effects and they affect photosynthetic activity of aquatic systems by reducing light penetration [3, 6, 7]. Textile wastewaters are characterized by extreme fluctuations in many parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), total suspended solid (TSS), pH, color and salinity [6, 8].

Recalcitrant dyes, due to the complex chemical structure, are resistant to biological degradation processes and they are not completely removed in conventional aerobic wastewater treatment plants [3, 9–12]. Existing physical and chemical technologies (adsorption, coagulation-flocculation, oxidation, filtration, and electro-chemical methods) are expensive, time consuming and produce a large amount of sludge or cause secondary pollution. In contrast, the microbial decolorization and degradation of dyes has been of considerable interest due to their inexpensive and eco-friendly nature. Therefore, it is necessary to establish biological wastewater treatment of the azo dye considering enzymes involved in it [2, 12–14]. A number of microorganisms belonging to different taxonomic groups of bacteria, algae, fungi and yeast have been reported for their ability to decolorize azo dyes [15–25].

Decolorization generally occurs by adsorption of dyestuffs on bacteria, rather than oxidation in aerobic systems. Some anaerobic microorganisms can produce partial biodegradation of dyestuffs by azoreductase activity, with the side-effect of producing aromatic amines as dead-end by-products. In addition, microbial azo-reduction might also exist as a non-enzymatic reaction. As a result, effluents at the end of such biodegradation processes are usually toxic. Moreover, upon the exposure of anaerobic degradation products to oxygen, reverse colorization may take place. These problems limit large-scale application of bacterial decolorization. In order to find an eco-friendly alternative, the fungal biodecolorization ability has been widely reported and it is commonly asso-

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ciated with lignin-degrading exo-enzymes, such as lignin peroxidase (EC 1.11.1.14), manganese-dependent peroxidase (EC 1.11.1.13) or laccase (EC 1.10.3.2). The non-specific nature of these enzymes makes them able to transform, and eventually mineralize, a variety of persistent environmental pollutants, including dyes. However, the growth of filamentous fungi is slow compared with most single-cell microorganisms. In fact, they are poorly adapted to wastewater treatments and they trend to show an exuberant mycelium growth that aggravates disposal problems [14]. Also, the aging of fungal mycelium and the risk of contamination by bacteria under non-sterile conditions have hindered the application of white rot fungi in wastewater treatment [26]. Yeasts, on the other hand, have many advantages, not only because of their fast growth but also because, like filamentous fungi, they have the ability to resist unfavorable environments. Besides, yeasts have been found to be very efficient in treating high-strength organic wastewaters [14, 27].

The present review will focus upon the some basic biological aspects associated with the azo dye degradation by yeasts.

AZO DYE CHARACTERIZATION AND CLASSIFICATION

Dyes contain chromophores, delocalized electron systems with conjugated double bonds, and auxochromes, electron-withdrawing or electron-donating substituents that cause or intensify the color of the chromophore by altering the overall energy of the electron system. Usual chromophores are azo (–N=N–), carbonyl (–C=O–), methine (–CH=), nitro ($-NO_2$) and quinoid rings and common auxochromes include amine ($-NH_3$), carboxyl (–COOH), sulfonate ($-SO_3H$) and hydroxyl (–OH) [6].

All azo dyes are characterized by the presence of one or more azo groups (–N=N–), as chromophore, in their aromatic rings. The azo groups are in general bound to a benzene or naphthalene ring, but they can also be attached to heterocyclic aromatic molecules or to enolizable aliphatic groups. on the basis of the characteristics of the processes in which they are applied, the molecule of the dye is modified to reach the best performance; so they can be acid dyes, direct dyes, reactive dyes, disperse dyes, basic dyes, metal complex dyes or others. Detailed features of these classes were reviewed by O'Neill et al. [4].

BIODEGRADATION OF AZO DYE

As literature shows, different microorganisms belonging to different groups of bacteria, fungi and yeasts are involved in biodegradation of different recalcitrant azo dyes. Their metabolic pathways used for degradation may vary not only according to the environmental conditions (e.g. aerobic or anaerobic) but also according to the type of organism.

Degradation of azo dyes by yeasts is much less studied than the decolorization process mediated by bacterial and fungal species. It is worth to mention that dye bioremediation by yeasts generally occurs through three processes, namely, biosorption, bioaccumulation and biodegradation. In the following, we will try to cover the some biological aspects of azo dye biodegradation process by yeasts.

Azo Dye Degrading Yeasts

Since 1990s, a practical view on yeast azo dye degradation has been started and enzymatic decolorization was reported in several yeast species. In 1992, Kakuta and co-workers used an immobilized yeast strain, *Candida curvata*, for dye wastewater treatment [28]. Another study by Kim et al. showed that *Geotrichum candidum* Dec 1 decolorized different kinds of reactive, acidic and dispersive dyes [29]. Martins et al. demonstrated that liquid aerated batch cultures of the yeast *Candida zeylanoides* decolorized several azo dyes derived from 2-naphtholaminoazobenzene and *p*-N,N-dimethylaminoazobenzene [30]. Subsequent works demonstrated that cultures of *Debaryomyces polymorphus, Candida tropicalis* [17] and *Issatchenkia occidentalis* also decolorized several other azo dyes [31].

Recently, it has been shown that several other ascomycetous yeast species namely, *Pseudozyma rugulosa* [27], *Candida oleophila* [32], *Saccharomyces cerevisiae* [13], *Galactomyces geotrichum* [33], *Candida albicans* [7], *Yarrowia lipolytica* [3] and *Issatchenkia orientalis* [34], are able to decolorize rather complex azo dyes, such as Reactive Black5 [3, 14, 17, 32, 34], Direct Violet 51 [7], Reactive Briliant Red K–2BP [17, 27], Reactive Yellow84, Reactive Red141 [35], Reactive Blue171 [12].

The effectiveness of decolorization depends on the structure and complexity of the dye. Relatively small structural differences in dye, such as the different position of identical substituents, can affect the whole decolorization process [36]. The chemical structures of some azo dyes, used frequently in different studies, are shown in Fig. 1.

only a few reports involve basidiomycetous yeasts in azo dye degradation, including *Trichosporon multisporum, Trichosporon akiyoshidainum* [37, 38], *Trichosporon beigelli* [12] and *Trichosporon porosum* [35]. Yeast species capable of azo dye degradation are depicted in table.

Enzymes Responsible for Azo Dye Decolorizaton

In recent years, the enzymes involved in dye decoloriztion and degradation by yeast has received considerable attention in the literature. The yeast-mediated enzymatic biodegradation of azo dyes can be accomplished either by reductive reactions or by oxidative reactions [3].

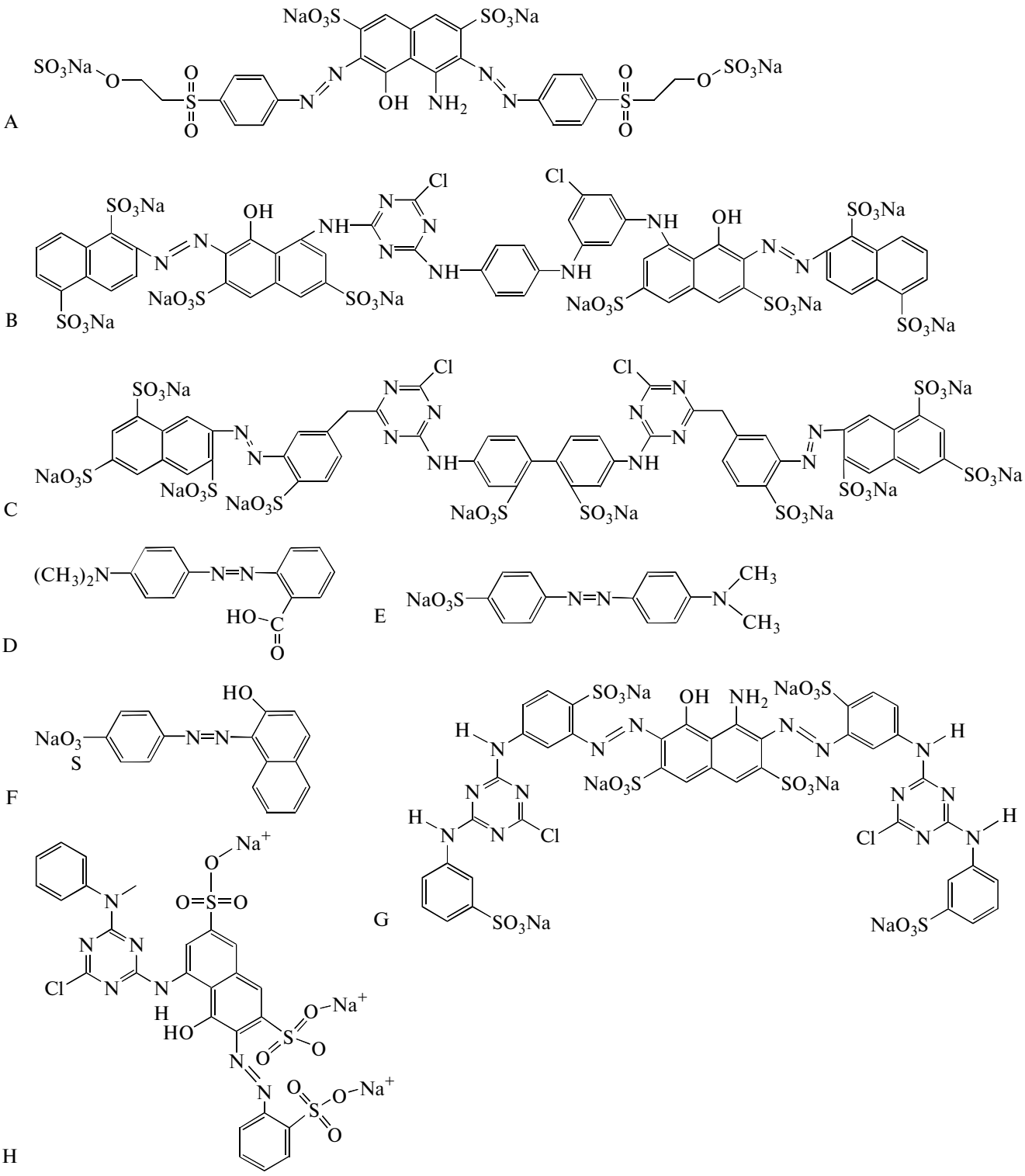

Fig. 1. Examples of azo dyes frequently studied in yeast degradation experiments: Reactive Black 5 (A); Reactive Red 141 (B); Reactive Yellow 84 (C); Methyl Red (D); Methyl Orange (E); Orange II (F); Reactive Blue 171 (G); Reactive Brilliant Red K–2BP (C.I. Reactive Red 24) (H)

Enzymes involved in reductive reaction. It seems that azo-reductase from the microorganisms catalyzes the reductive cleavage of the azo bond (–N=N–) and produces the metabolites [7]. The involvement of an azoreductase, externally directed to plasma membrane redox system (PMRS), was reported in *S. cere-*

Yeast species capable of azo dye decolorization by degradation process and their enzymes

Yeast	Azodye	Enzyme					Experiment condition	References
		Lac	MnP	LiP	Tyr	Azoreductase		
Candida zeylanoides	Several azo dyes derived from 2-naphtholaminoazobenzene and *p*-N,N-dimethyl aminoazobenzene	Nr	Nr	Nr	Nr	Nr	Shaking (150 rpm)	[30]
Debaryomyces polymorphous	Reactive Black 5	–	+	–	Nr	Nr	Aerobic	[17]
	Reactive Red M-3BE	–	+	–				
	Procion Scharlach H-E3G	–	+	–				
	Procion Marine H-EXL	–	+	–				
	Reactive Brilliant Red K-2BP	–	+	–				
	Reactive Yellow M-3R	–	+	–				
Candida tropicalis	Reactive Black 5	–	+	–	Nr	Nr	Aerobic	[17]
	Reactive Red M-3BE	–	+	–				
	Procion Scharlach H-E3G	–	+	–				
	Procion Marine H-EXL	–	+	–				
	Reactive Brilliant Red K-2BP	–	+	–				
	Reactive Yellow M-3R	–	+	–				
Issatchenkia occidentalis	Methyl Orange Orange II	Nr	Nr	Nr	Nr	+	Shaking (120 rpm)	[31]
Pseudozyma rugulosa Y-48, *Candida krusei*[a] G-1	Reactive Brilliant Red K-2BP, Weak Acid Brilliant Red B, Reactive Black KN-B, Acid Mordant Yellow	Nr	Nr	Nr	Nr	Nr	Shaking (200 rpm)	[27]
Saccharomyces italicus[b] CICC1201 *Saccharomyces chevalieri*[b] CICC1611	Reactive Brilliant Red K-2BP	Nr	Nr	Nr	Nr	Nr	Shaking (200 rpm)	[27]
Candida oleophila	Reactive Black 5	–	–	Nr	Nr	Likely	Shaking (120 rpm)	[32]
Saccharomyces cerevisiae MTCC 463	Methy Red	+	Nr	+	+	+	Static	[13]
Galactomyces geotrichumc MTCC 1360	Methyl Red Amido Black 10B	+	Nr	+	Nr	+	Shaking (150 rpm)	[33]

Table. (contd.)

Yeast	Azodye	Enzyme					Experiment condition	References
		Lac	MnP	LiP	Tyr	Azoreductase		
Trichosporon akiyoshidainum HP-2023	Reactive Red 141 Reactive Green Reactive Yellow 84	–	–	–	Nr	Nr	Shaking (200 rpm)	[38]
Candida albicans	Direct Violet 51	Nr	Nr	Nr	Nr	+	Shaking (150 rpm)	[7]
Trichosporon begeliid NCM-3326	Reactive Bluel71 Reactive Red 141 Reactive Yellow 17 Reactive Green 19A Reactive Orange 94	W	Nr	–	W	+	Static	[12]
Galactomyces geotrichum MTCC 1360	Mixture of structurally different dyes	+	Nr	Nr	+	–	Shaking (120 rpm)	[39]
Trichosporon porosum MM 4037	Reactive Yellow84 Reactive Black5 Reactive Red 141 Mixture	– – – –	– + – –	Nr	W + – –	Nr	Shaking (250 rpm)	[35]
Barnettozyma californica MM 4018	Reactive Yellow84 Reactive Black5 Reactive Redl41 Mixture	– – – –	– + – –	Nr	W + – –	Nr	Shaking (250 rpm)	[35]
Cyberlindnera satwnus MM 4034	Reactive Yellow84 Reactive Black5 Reactive Redl41 Mixture	– – – –	– + – W	Nr	– + – –	Nr	Shaking (250 rpm)	[35]

Table. (Contd.)

Yeast	Azodye	Enzyme					Experiment condition	References
		Lac	MnP	LiP	Tyr	Azoreductase		
Candida sp. MM 4035	Reactive YelIow84 Reactive Black5 Reactive Redl41 Mixture	– – – –	– + – –	Nr	W + – –	Nr	Shaking (250 rpm)	[35]
T. akiyoshidainum HP-2023	Reactive BIack5	Nr	+	Nr	+	Nr	Shaking (250 rpm)	[14]
Galactomyces geotrichum MTCC 1360	Remazol Red Direct Red 5B Blue GL Solo Navy Blue 3G Orange M2R Remazol Black 5 Direct Red 2B Remazol Orange 3R Green HE4B Reactive Blue HE2R Reactive Navy BlueRX	+	Nr	Nr	+	+	Static	[2]
Yarrowia lipolytica NBRC 1658	Reactive Black 5	–	–	Nr	Nr	Likely	Aerobic	[3]
Issatchenkia orientalis JKS6e	Reactive Black 5 Reactive Orange 16 Reactive Red 198 Direct Blue 71 Direct Yellow 12 Direct Black 22	_	Nr	Nr	Nr	Likely	Shaking (150 rpm)	[34]

The enzymatic studies commonly performed for one dye as a model. LiP: Lignin peroxidase; MnP: Manganese-dependent peroxidase; Lac: Laccase; Tyr: Tyrosinase; +: Positive; -: Negative; Nr: Not referred; W: Weak.

a: *Candida krusei* is anamorph name of *Issahchenkia orientalis*

b: *Saccharomyces italicus* and *Saccharomyces chevalieri* are synonyms of *Saccharomyces cerevisiae*

c: Obsolete synonyms of *Endomyces geotrichum* and *Dipodascus geotrichum* used for *Galactomyces geotrichum*

d: *Trichosporon begelii* has been synonymously used with *Trichosporon cutaneum*

e: Laccase production by this yeast strain was studied in solid media containing guaiacol or tannic acid as indicator.

visiae and was identified as a ferric reductase. The plasma membrane ferric reductase system participates in the extracellular reduction of azo dyes [40]. Other reductase enzymes were also reported that participate in dye degradation mediated by yeast such as NADH–DCIP (dichlorophenolindophenol) reductase in Methy Red degradation by *Saccharomyces cerevisiae* MTCC 463 [13] and *Galactomyces geotrichum* MTCC 1360 [33]. Saratale et al. observed a significant increase in NADH–DCIP reductase and azo reductase activities in the *Trichosporon begelii* NCIM–3326 cells obtained after complete decolorization of Navy blue HER (C.I. Reactive blue171) which presumably indicates the involvement of these enzymes in decolorization process [12]. The presence of NADH–DCIP reductase, riboflavin reductase and the induction in azoreductase activity during Remazol Red decolorization with *Galactomyces geotrichum* MTCC 1360 was reported by Waghmode et al. [2].

From the literature consultation it is worth to mention that *Galactomyces geotrichum* MTCC 1360 was used in decolorization of a mixture of structurally different dyes and also Remazol Red at two different conditions namely shaking (at 120 rpm) and static by Waghmode et al. 2011a and by Waghmode et al. 2012, respectively. The results of these two studies showed the absence of azoreductase and riboflavin reductase enzymes in the experimental condition of Waghmode et al. (2011a) work and, in contrast, the presence of these enzymes in Waghmode et al. (2012) report. Waghmode et al. (2011a) referred this event, i.e. absence of reductase enzymes, to the inhibition of azoreductase activity by oxygen at shaking condition. But it does not seem to be a more convincing reason because earlier, this strain, *Galactomyces geotrichum* MTCC 1360, was used in Methyl Red degradation process [33] at shaking condition (150 rpm) and the azoreductase production was reported in it. However, the structure of dyes used in the studies may be important in the enzyme production throughout the decolorization process.

The metabolites produced through reductase enzyme activity are further degraded into aliphatic amines, which may have been facilitated by oxidative enzymes.

Enzymes involved in oxidative reaction. Lignin modifying enzymes such as laccase (Lacc), manganese peroxidase (MnP), lignin peroxidase (LiP), tyrosinase (Tyr), and to some extent N-demethylase, have been widely related to dye biodecolorization and mineralization in filamentous fungi. However, the relative contribution of each enzyme to the overall biodegradation process has proved to be different for each microorganism [35]. The reactions catalyzed by these extracellular enzymes are oxidation reactions, e.g. lignin peroxidase catalyses the oxidation of non-phenolic aromatics, whereas manganese peroxidase and laccase catalyze the oxidation of phenolic compounds [11].

Neither laccase nor manganese-dependent peroxidase activities were detected in the culture medium treated by *Candida oleophila* and *Yarrowia lypolitica*. The observed growth-associated decolorization and concomitant absence of extracellular oxidative enzymes in batch cultures indirectly suggested the involvement of an enzyme for the azoreductase-like activity in azo dye reduction [3, 32], as previously was postulated in the other ascomycetous yeast species *C. zeylanoides* [41] and *I. occidentalis* [31]. Among the three tested ligninolytic enzymes, only MnP was detected by Yang and co-workers in *D. polymorphus* and *C. tropicalis* during Reactive Black 5 decolorization. This has confirmed that these two yeasts could not produce LiP or laccase. Also, the presence of the reactive azo dye was found to be an obligatory condition for MnP production. The MnP production abilities and color removal efficiencies of the two yeast isolates in other dye-bearing solutions were also reported. But MnP activities differed between the two yeast isolates and also differed among dyes used. In this study, at least four other azo dyes, Reactive Red M-3BE, Procion Scharlach H-E3G, Procion Marine H-EXL and Reactive Brilliant Red K-2BP, induced *D. polymorphus* to produce MnP. MnP activities were only detected in the culture of *C. tropicalis* containing Reactive Red M-3BE and Reactive Brilliant Red K-2BP, among all tested dyes [17, 26]. Although MnP was reported as an important enzyme responsible for degradation of different kinds of dyes in Yang and co-workers' study, they emphasized that the presence of only MnP was not enough for color removal of RB5. Their further experiments indicated that the decolorization process ceased immediately after glucose was depleted even MnP activity remained high in the culture [17, 26]. Furthermore, Martorell and coworkers, while working with Trichosporon akiyoshidainum, observed the obvious decoupling between dye decolorization and ligninolytic enzymes (MnP and/or tirosinase) production strongly suggesting that dye decolorization should be regarded as a complex multienzymatic process [14]. Later reports clearly indicated that the decolorization/degradation of the sulfonated azo dye Remazol Red occurred through the combined action of azo reductase and laccase enzymes by Galactomyces geotricum [2]. Yeast species capable of azo dye decolorization by degradation process and their enzymes were shown as table.

Mechanism of Azo Dye Degradation by Yeast

Induction of various oxidative and reductive enzymes during decolorization gives additional insight into the decolorization mechanism [2]. In general, reductive reactions lead to cleavage of azo dyes into aromatic amines, which are further mineralized by yeasts [13].

Earlier studies on biological decolourization of the azo dyes by yeasts mainly focused on the azoreduc-

tases presence in microorganisms since they catalyze reductive cleavage of azo groups (–N=N–) primarily responsible for the biodegradation. Azo bond cleavage by aerobic azo-reductase was reported in the yeast species such as *Candida oleophila* and *Candida zelanoides* [32, 41]. Ramalho et al. demonstrated that a reductive cleavage of the azo bond was involved in the decolorization process performed by *Candida zeylanoides*. The tested dyes were derived from the diazonium salts of metanilic and sulfanilic acids and N,N-dimethylaniline or 2-naphthol as coupling components. In the course of decolorization either metanilic acid or sulfanilic acid were detected in the supernatant fluid by HPLC (high-pressure liquid chromatography) analyses. None of those aminobenzenesulphonates supported microorganism growth as carbon and energy source but both could be used, to a limited extent, as nitrogen sources. This study showed that decolorization by this yeast strain is due to azo bond reduction, forming the corresponding amines, as observed with decolorizing anaerobic or facultative aerobic bacteria [41]. Subsequent studies with *I. occidentalis* also revealed the presence of metanilic acid or sulfanilic acid during decolorization of the same dyes, showing this yeast is capable of reducing several monoazo dyes to the corresponding amines and, therefore, promotes their decolorization through the cleavage of the azo bond. Thus, the azo-reductase activity of the NADH: ubiquinone oxidoreductase was suggested in *I. occidentalis* that acted in condition shaken at 120 rpm [31]. A study of the enzymes responsible for the biodegradation of Methyl Red by *S. cerevisiae* MTCC 463 showed different levels of the activities of laccase, lignin peroxidase, NADH–DCIP reductase, azoreductase, tyrosinase and aminopyrine *N*-demethylase. Enzymatic studies indicate the involvement of azoreductase as prominent enzyme for biodegradation. It seems, by results extracted from HPLC and FTIR (fourier transform infrared spectroscopy) analyses, that azoreductase catalyzes the reductive cleavage of the azobond of Methyl Red and produces 2-aminobenzenzoic acid and N,N'-dimethyl-*p*-phenylenediamine using NADH as electron donor. These products are further degraded into aliphatic amines that might be facilitated by oxidative enzymes such as lignin peroxidase and laccase [13].

It was reported that the strain *T. beigelii* NCIM–3326 degraded reactive azo dye Navy blue HER via enzymatic reduction mechanism. The produced metabolites of Navy blue HER biotransformation were analyzed by TLC (thin layer chromatography), HPLC, UV–vis spectral analysis and FTIR. It seems that azoreductase catalyzes the reductive cleavage of azo bond and further degradation of products might be facilitated due to oxidative enzymes such as laccase and tyrosinase [12]. Recently, the possible azo dye biodegradation pathway adapted by *G. geotrichum* MTCC 1360 was clearly illustrated by Waghmode and coworkers, as showed in Fig. 2. The enzymatic analysis of *G. geotrichum* showed a tremendous induction of laccase and azo reductase after 36 h of degradation. These results suggest the active participation of laccase and azo reductase in Remazol Red degradation. The degradation of the Remazol Red into different metabolites was confirmed using HPLC, FTIR and GC–MS (gas chromatography mass spectroscopy) analysis. This report shows that initial cleavage might start off with the reduction of azo bonds with action of azo reductase followed by subsequent oxidative and reductive cleavage with the help of various oxidative and reductive enzymes viz. laccase and DCIP reductase. Azo reductase catalyzed initial reductive cleavage of azo bond leads to the formation of two postulated reactive intermediates 3-amino [4,5 (6-chloro-1,3,5 triazine-2yl) amino] naphthalene 2, 4, 7 benzene trisulfonic acid (M.W. 638) and 2[3-aminophenyl) sulfonyl] ethane sulfonic acid (M.W. 265). Asymmetric cleavage of the first intermediate by laccase resulted in the formation of 2-amino naphthalene (*m/z*, 141) and N-phenyl-1,3,5 triazine (*m/z*, 170). Further to this, 2[(3-aminophenyl) sulfonyl] ethane sulfonic acid undergoes deamination to produce ethylsulfonyl-benzene having a mass peak of (*m/z*) 246 followed by a desulfonation reaction resulting in the formation of 2-ethylphenyl sulfone with a mass peak (*m/z*) of 168 (Fig. 2). The investigation showed *G. geotrichum* has the potential to detoxify sulfonated azo dyes because this reaction releases azo linkages as molecular nitrogen, which prohibits aromatic amine formation [2].

Effect of Glucose and Cometabolic Induction

It is clear that the decolorization depends strongly on the presence of glucose, as an alternative carbon and energy source, in the medium [26, 31, 34, 39, 41]. It was found that glucose and yeast extract act as electron donors for faster decolorization of dyes [39]. Studies showed that glucose utilization is corresponding to the quick decolorization stage of biodegradation. Moreover, an apparent close association between glucose depletion and color removal over time was evident [32, 42]. This may be due to the involvement of an enzyme that needs reducing power (NADH and/or FADH2) to azo dye reduction as reported in few works [31, 32] and probably, assimilation of a carbon source and its subsequent metabolism through glycolysis, tricarboxylic acids cycle and pentose phosphate pathway supplies yeast cells with these reducing powers [32]. Another reason is that the activity of the extracellular peroxidase, such as LiP and MnP, requires H_2O_2 as a co-substrate. It was mentioned that enzymes such as glucose-1-oxidase and glucose-2-oxidase are responsible for H_2O_2 generation, thus, it is possible that glucose is the substrate for H_2O_2 generation during decolorization [43]. In this point, glucose played two important roles in biodegradation of dyes: providing a carbon and energy source for yeast growth and subse-

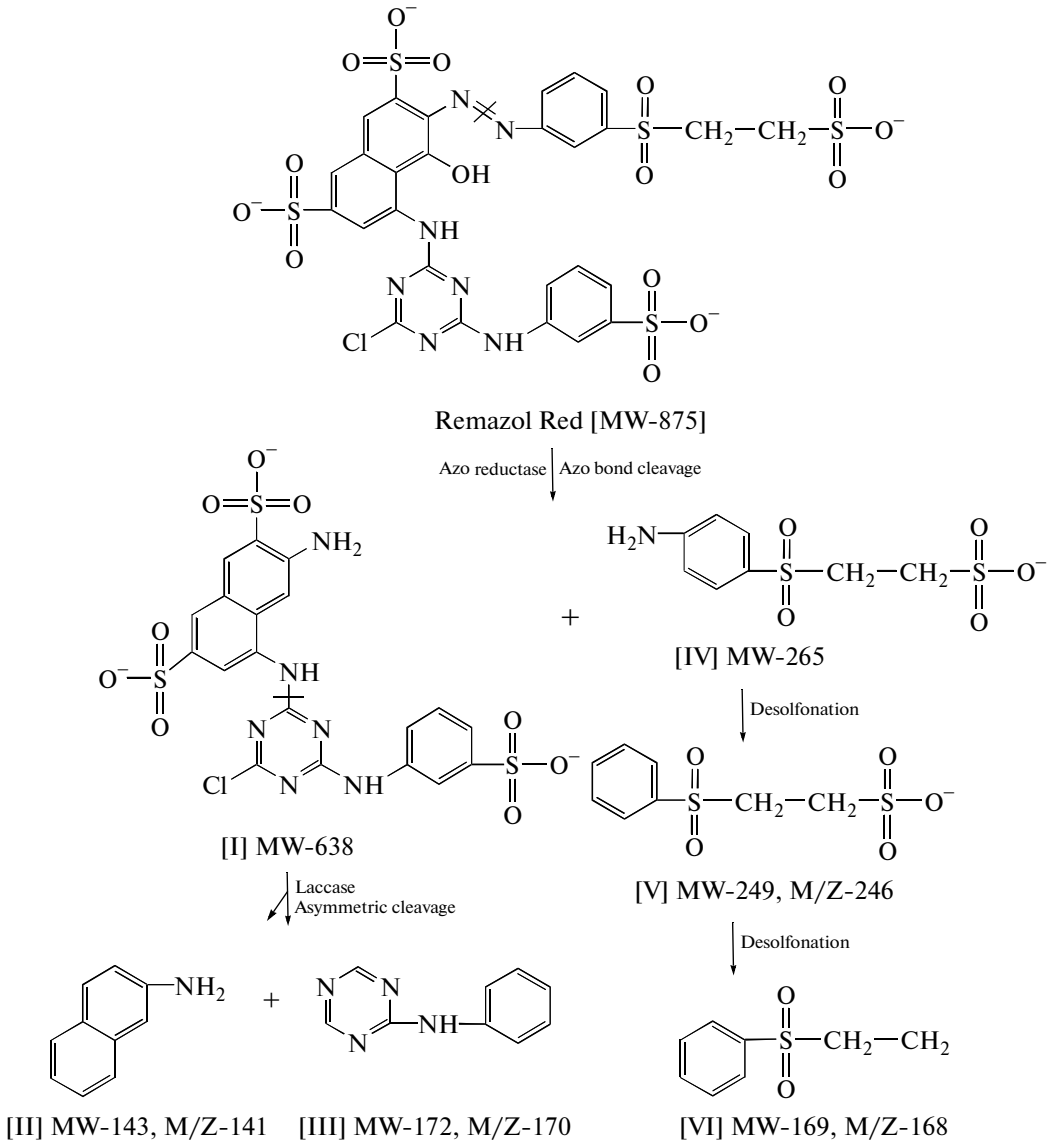

Fig. 2. The proposed pathway for the decolorization of Remazol Red by using *G. geotrichum* MTCC 1360 [2].

quently for reducing power (NADH and/or $FADH_2$) production and also acting as a substrate for H_2O_2 production.

Effect of Dissolved Oxygen

There are many studies that reported the bacterial decolorization occurred in microaerphilic or anaerobic conditions and decolorization performance in aerobic condition was less [19, 23, 44–46]. Dissolved oxygen is considered an inhibitor of the azo dye reduction process, since both molecules act as electron acceptors and oxygen is a much stronger oxidant. Hence, under aerobic conditions, the electrons are in limited supply for reductive cleavage. In contrast, under anoxic conditions, NADPH carries more electrons for reduction [19]. Thus, azo dyes are more readily reduced under anaerobic conditions by bacteria.

Among existing reports on azo dye decolorization performed by yeasts, most of them occurred under aerobic condition [3, 7, 14, 32, 34, 35, 39] and decolorization efficiency in static or anoxic conditions was lower. *Issatchenkia* sp. JKS6 could decolorize 200 mg RB5 L^{-1} up to 90% within 12–18 h under agitated condition (at 150 rpm). Lower decolorization of 15.6% was observed under static condition during the same incubation time [34]. *Galactomyces geotrichum* MTCC 1360 showed 88 and 49% decolorization of dye mixture within 24 h under shaking (120 rpm) and static condition, respectively. Also, COD reduction of 69 and 14% as well as TOC reduction of 43 and 3.5%

was observed under shaking and static conditions, respectively [39]. Ramalho and co-workers found that *Issatchenkia occidentalis* removed more than 80% of the tested dyes in flasks containing 0.2 mM dye, shaken at 120 rpm within 15 h. Under anoxic conditions, decolorization does not occur, even in the presence of pre-grown cells. They also conducted an experiment at different aeration conditions as follows: 120 rpm (microaerophilic), forced aeration (aerobic) and nitrogen flushed conditions (anaerobic). It was concluded that under aerobic condition and under microaerophilic (shaken at 120 rpm) condition the decolorization progress is similar showing that in this system oxygen does not compete with dye for electrons. The results also showed that under anaerobic conditions yeasts failed to grow [31]. Faster decolorization at shaking condition may be due to faster diffusion of oxygen and substrate [39]. Although there are some instances that yeast decolorization occurred under static anoxic condition as, *Saccaromyces cerevisiae* cells decolorize Methyl Red completely within 16 min under anoxic condition by biodegradation, and it took 60 min at shaking condition [13]. Decolorization performance of Navy blue HER with *T. begelii* NCIM–3326 was reported 100% under static condition and 30% under shaking condition within 24 h. The growth of *T. begelii* NCIM–3326 was also observed to be more under static (9.2 g L^{-1}) as compared to shaking condition (4.2 g L^{-1}) [12].

Degradation of azo dyes in aerobic condition can also be carried out by lignin degrading fungi, mainly white-rot fungi, and observed that shaking condition was more favorable than static condition for Reactive blue-25 degradation by using *Aspergillus ochraceus* NCIM 1146 [47], or by peroxide-producing bacterial strain, mainly *Streptomyces* species such as *Streptomyces chromofuscus* [48, 49].

Faster Dye Decolorization During the Exponential Growth Phase

Available literature showed that decolorization by yeasts is faster during the exponential growth phase in primary metabolism, which slowed down in the stationary phase. The quick decolorization stage by yeasts was strongly accompanied by the cells' growth [41, 42], as observed in reports with yeast species of *C. oleophila, C. tropicalis, D. polymorphus, C. zeylanoides, I. occidentalis, I. orientalis, Y. liplytica, Trichosporon* sp. HP–2023 [3, 17, 30–32, 34, 38, 41], showing decolorization produced by yeasts might depend on the actively growing cells [41]. As literature shows, the observed growth-associated decolorization is consistent with an azoreductase catalysed cleavage of azo bonds postulated in the ascomycetous yeast species [31, 32, 41] because intracellular formation of NAD(P)H, required for azoreductase activity, is a growth-dependent process. Thus, the decolorization process must be dependent on cell biomass and on actively growing cells [41]. It is worth to mention when dye decolorization is associated to primary metabolism, yeast should be kept in its exponential growth phase so that the bioremediation process occurs much more quickly, as the literature reported that azo dye decolorization by yeast species occurred in a short incubation time, commonly up to 24 h, [17, 30–32, 34, 41] compared to filamentous fungi. on the contrary, filamentous fungi especially of white-rot type typically remove azo dyes during secondary metabolism [32], which implies long incubation periods (usually several days) [36, 50].

Toxicity Studies of Produced Metabolites During Azo Dye Degradation

Chronic effects of dyestuffs, especially of azo dyes, have been studied for several decades. Azo dyes in purified form are seldom directly mutagenic or carcinogenic, except for some azo dyes with free amino groups. However, reduction of azo dyes leads to the formation of aromatic amines, several of which are known mutagens and carcinogens to human beings [51]. There are several reports, however, that show the metabolites produced through biodegradation phenomenon accomplished by yeast have a nontoxic or less-toxic nature when compared to the dye [2, 12, 33, 39]. Phytotoxicity tests were performed in order to assess the toxicity of dyes and metabolites obtained after their decolorization [52].

In a work carried out with *G. geotrichum* MTCC 1360, it was revealed that the yeast has the potential to detoxify sulfonated azo dyes because this reaction releases azo linkages as molecular nitrogen, which prohibits aromatic amine formation. The results indicated that the germination percentage, length of plumule and radicle of *Phaseolus mungo* (Family *Fabaceae*) and *Sorghum vulgare* (Family *Gramineae*) seeds was less with Remazol Red as compared to its formed metabolites and water. This study thus showed the less toxic nature of formed metabolites as compared to the dye sample [2]. Saratale et al. showed the detoxification of Navy blue HER by *T. beigelii* NCIM–3326. The mean of plumule length and radicle length of *P. mungo* were 12.86 ± 0.26 and 7.45 ± 0.17 cm, and 13.16 ± 0.41 and 17.00 ± 0.46 cm in case of *S. vulgare*, respectively of 10 seeds in distilled water as a control with 100% germination. The germination of both plant seeds inhibited 90% when seeds treated with 1500 ppm concentration of Navy blue HER, whereas the plumule length and radicle length was found in *P. mungo* (9.77 ± 0.31 and 5.28 ± 0.18 cm) and in *S. vulgare* (12.38 ± 0.28 and 13.38 ± 0.25 cm), respectively with 100% germination when treated with 1500 ppm degradation products. Thus, phytotoxicity studies showed good germination rate as significant growth in the plumule and radicle for both plants, in the metabolites extracted after decolorization, as compared to dye sample [12]. Waghmode et al. also

found that the germination (%) of both plants, *P. mungo* and *S. vulgare*, was less in the dye mixture (of seven dyes) solution (1000 ppm) as compared to the germination in ethyl acetate extracted metabolites of degraded dyes by *G. geotrichum* MTCC 1360 [39]. Earlier, it was also reported that untreated Methyl Red at 300 mg L^{-1} concentration showed 88 and 72% germination inhibition in *Sorghum bicolor* and *Triticum aestivum*, respectively, whereas there was no germination inhibition for both plants by Methyl Red metabolites formed through *G. geotrichum* MTCC 1360 degradation at 300 mg L^{-1} concentration [33].

There is a positive impact in terms of maintaining iron levels and growth of common agricultural crops through the process of bioremediation of organic dyes. It was showed that biodegradation of Remazol Red dye by *G. geotrichum* helps to improve iron absorption in plants by the inhibition of chemical chelation and immobilization of iron in soil caused by the Remazol Red dye [2].

INTEGRATED TECHNIQUES MEDIATED BY YEAST SPECIES FOR AZO DYE DEGRADATION

Various physical, chemical and biological pretreatment, main treatment and post treatment techniques can be employed to remove color from dye containing wastewaters. Several factors that determine the technical and economic feasibility of each single dye removal technique include dye type and its concentration, wastewater composition, operation costs (energy and material), environmental fate and handling costs of generated waste products. The use of an individual process may often not be sufficient to achieve complete decolorization and degradation [53]. Therefore, a combination of different techniques was reported.

Advanced Oxidation Process, followed by biological treatment, could be justified if bio-recalcitrant compounds are easily degradable by AOP and the resulting intermediates are easily degradable by the biological treatment [54], as previously reported in the degradation of different biorecalcitrant azo dyes [55–57]. Lucas et al. studied on the decolorization of azo dye Reactive Black 5 solution by combined chemical-biological process, namely, an advanced oxidation process (Fenton's reagent) followed by an aerobic biological process mediated by the yeast *Candida oleophila*. The major purpose of this integrated process was to reduce the operational costs, particularly the hydrogen peroxide concentration used in Fenton's reagent, to efficiently decolorize the RB5 dye at a high concentration of 500 mg L^{-1}. Initial experiments showed that Fenton's process alone, as well as aerobic treatment by *C. oleophila* alone, exhibited the capacity to significantly decolorize azo dye solutions up to 200 mg L^{-1}, within about 1 and 24 h, respectively. by contrast, neither Fenton's reagent nor *C. oleophila* sole treatments showed acceptable decolorizing abilities for higher initial dye concentrations (300 and 500 mg L^{-1}). However, it was verified that Fenton's reagent process lowered these higher azo dye concentrations to a value less than 230 mg L^{-1}, which is apparently compatible with the yeast action. The final decolorization obtained with Fenton's reagent process as primary treatment, and growing yeast cell as a secondary treatment, achieves a color removal of about 91% for an initial RB5 concentration of 500 mg L^{-1}. The results showed that Fenton's reagent alone requires 5 times more H_2O_2 and Fe^{2+} to achieve an identical level of color removal [1].

A few reports describe the application of photocatalytic process as a post-treatment in a combined process [54, 58]. These investigations were suggested since the concentration of organic compounds were high in wastewater and the use of photocatalytic process as a pre-treatment stage was not favorable, and/or, partial oxidation of original molecules had occurred under biological process and intermediate molecules produced through the biological process could be more toxic than original molecules [59].

Jafari et al. studied on the capacity of combined yeast *Candida tropicalis* JKS2 and a photocatalytic process (UV/TiO_2) to efficiently mineralize the Reactive Black 5 dye at 200 mg L^{-1} concentration. Mineralization of 50 mg L^{-1} RB5 solution was obtained after 80 min by photocatalytic process (in presence of 0.2 g L^{-1} TiO_2). However, photocatalytic process was not effective in the removal of the dye at higher concentrations (~200 mg L^{-1}). Complete decolorization of the culture containing 200 mg L^{-1} RB5 occurred under *Candida tropicalis* JKS2 treatment in less than 24 h. Also, successful application of the biological treatment in decolorization of high concentrated synthetic medium (up to 1000 mg L^{-1} RB5) was achieved but the remediation of aromatic rings produced from destruction of dye molecule did not occur, even with prolonged incubation time to 72 h. Therefore, photocatalytic process was used as a post– treatment for degradation of aromatic rings and was effective for this purpose, but COD reduction was not observed in the second step which could be due to the production of strongly oxidative by-products that are resistant to further oxidation by photocatalytic treatment. In this report, at 200 mg L^{-1} dye concentration, the results showed that application of biological-photocatalytic process was more efficient than the photocalalytic and biological process solely in aromatic by-product remediation, and also showed that the combined process was effective than biological treatment in remediation of aromatic rings (resulting from decolorization of the dye) and was cost-effective (in terms of the electrical power utilized) compared to the photocatalytic treatment only. However, it was suggested that further evaluation is required to improve the system in which three stages take place. The third step (i.e., biological treatment) subsequent to the photocatalytic step could facilitate further COD reduction [60].

Azo dyes are readily reduced by anaerobic bacteria and produce potentially toxic and mutagenic end products (aromatic amines) that are resistant to anaerobic biodegradation. Thus, aerobic phase is essential for complete biodegradation of colored effluents, as many works reported the using of microaerophilic/aerobic process for the complete biodegradation of azo dye [61–63]. An attempt was made to develop new batch process with the help of prepared consortium GG–BL, using two microbial cultures *Galactomyces geotrichum* MTCC 1360 and *Brevibacillus laterosporus* NCIM 2298, via aerobic/microaerophilic batch process for complete mineralization of reactive mono azo dye Golden Yellow HER (GYHER). Aerobic oxidation of GYHER is followed by microaerophilic reduction of metabolite formed during aerobic degradation. After development of consortia, the dye added at 50 mg l^{-1} concentration and the flask was incubated for aerobic condition (shaken at 120 rpm) for the first 12 h of decolorization and after aerobic treatment, the flask was transferred to microaerophilic condition (static condition) for subsequent decolorization for the next 12 h. Consortium GG–BL showed 100% decolorization of GYHER (50 mg L^{-1}) at aerobic/microaerophilic condition with significant reduction in COD (84%) and TOC (63%) as well as showing 85% decolorization at microaerophilic/aerobic condition. The same procedure was applied in case of individual strains. *G. geotrichum* and *B. laterosporus* showed 54 and 91% decolorization at aerobic/microaerophilic condition, with 18 and 72% of COD reduction along with 7 and 51% TOC reduction respectively. Consortium GG–BL also showed 91 and 70% decolorization at the concentration of 100 mg L^{-1} and 250 mg L^{-1} within 72 h respectively. Combined activities of oxidoreductive enzymes in consortium GG–BL resulted in an increased decolorization of GYHER as compared to individual strains [64].

CONCLUSION

Wastewater contained azo dye seems to be one of the most polluted wastewaters, which requires efficient treatment prior to release in the environment. Bioremediation is an important method in detoxifying and eliminating environmental contaminants. Many dyes are resistant to conventional wastewater treatment processes by activated sludge.

on the basis of the available literature, it can be concluded that bacterial ability to achieve complete mineralization of azo dyes generally depends on the control of the process in which initial decolorization takes place under microaerophilic conditions with low oxygen, followed by elimination of the dye metabolites using an aeration step. However, the yeasts capable of azo dye decolorization, particularly those with oxidative capabilities, can be very important in terms of industrial application because they achieve the complete degradation of the dyes under the same operational conditions.

Moreover, the selection of an appropriate method for achieving this purpose will depends on the different wastewater characteristics such as class and concentration of dye, pH, salinity and toxic compounds. Up to now, some biological and physiological aspects associated with the azo dye degradation by yeasts have been clarified, such as dye decolorization by yeasts is comparatively unspecific but is affected by the yeast strain used and by the medium composition, dye structure, and dissolved oxygen levels. It was also concluded that decororization is faster during the exponential growth phase. However, further information is required for successful application of yeasts in a wastewater treatment process and for revealing more practical aspects of this full-scale treatment system.

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