Original Article

Bacterial Decolorization of Textile Azo Dye Acid Orange by *Staphylococcus hominis* RMLRT03

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ABSTRACT

A bacterial strain RMLRT03 with ability to decolorize textile dye Acid Orange dye was isolated from textile effluent contaminated soil of Tanda, Ambedkar Nagar, Uttar Pradesh (India). The decolorization studies were performed in Bushnell and Haas medium (BHM) amended with Acid Orange dye. The bacterial strain was identified as *Staphylococcus hominis* on the basis of 16S rDNA sequence. The bacterial strain exhibited good decolorization ability with glucose and yeast extract supplementation as cosubstrate in static conditions. The optimal condition for the decolorization of Acid Orange dye by *Staphylococcus hominis* RMLRT03 strain were at pH 7.0 and 35°C in 60 h of incubation. The bacterial strain could tolerate high concentrations of Acid Orange dye up to 600 mg l^{-1}. The high decolorizing activity under natural environmental conditions indicates that the bacterial strain has practical application in the treatment of dye containing wastewaters.

Key words: 16S rDNA, BHM, cosubstrate, decolorization, textile dye

INTRODUCTION

Synthetic dyes are poly-aromatic molecules that give a permanently color to materials like textile fabrics. Over 100,000 commercial synthetic dyes including several classes have been generated worldwide with an annual production of around 280,000 tons.[1] These synthetic dyes are widely used in textile, paper, food, cosmetics, and pharmaceutical industries with the textile industry as the largest consumer.[2] Among all the available synthetic dyes, azo dyes are the largest group of dyes used in textile industry constituting 60–70% of all dyestuffs produced because of the ease and cost effectiveness of their synthesis, their stability and the variety of colors.[3] Azo dyes are aromatic compounds with one or more –N=N– groups and is the most common synthetic dyes released into the environment.[4] Azo dyes are considered as electron deficient xenobiotic compounds because they possesses electron withdrawing groups, generating electron deficiency in the molecule and making them resistant to degradation. All dyes do not bind to the fabric and inefficiency in dyeing processes resulted in 10-50% of unused dyestuff entering the wastewater directly.[5] Improper textile wastewater disposal in aqueous ecosystems affects the aesthetic merit, obstructs light penetration, oxygen transfer in water bodies and depicts acute toxic effects on aquatic flora and fauna, causing severe environmental problems.[6] In addition to their visual effect many synthetic azo dyes and their degradation intermediates are toxic and in some cases these compounds are carcinogenic and mutagenic to humans as well as other animals.[7] Several physico-chemical methods such as adsorption, coagulation/flocculation, membrane filtration, precipitation, filtration and oxidation have been used for the treatment of textile wastewater but these methods are expensive and producing large amounts of sludge which requires safe disposal.[8] It is, therefore, important to develop efficient and cost-effective methods for the removal

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of azo dyes in textile effluents and contaminated soil. As a viable alternative, biological process including different taxonomic groups of microorganisms such as bacteria, fungi, yeast, and algae received increasing interest due to their cost effectiveness, ability to produce less sludge, and eco-friendly nature.[9] Dye decolorization by fungi is mainly attributed by adsorption rather than degradation but slow growth and low decolorization efficiency limits the use of fungi for the treatment of textile effluent.[10,11] In contrast different trophic groups of bacteria can achieve a higher degree of degradation and even complete mineralization of dyes under optimum conditions.[12] The biodegradation of azo dyes may occur either aerobically, anaerobic or by a combination of both. The initial step in the bacterial degradation of azo dyes is the reductive cleavage of azo bond by an enzymatic biotransformation reaction under static or anaerobic conditions which leads to the formation of colorless aromatic amines.[5] The resulting toxic products such as aromatic amines are further degraded to simpler non-toxic forms by multiple-step bioconversion occurring aerobically or anaerobically.[5]

The present study was aimed to isolate and identify a bacterial strain capable of decolorizing Acid Orange dye commonly used in textile industries of Tanda, Ambedkar Nagar, Uttar Pradesh (India). In addition, the effect of various physical and nutritional conditions on decolorization of this dye by isolated bacterial strain was studied.

MATERIALS AND METHODS

Dye and Chemicals

The azo dye Acid Orange used in this study was of industrial grade and purchased from local market of Tanda, Ambedkar Nagar, Uttar Pradesh (India). All the other chemicals used were of analytical grade.

Culture medium

Bushnell and Haas medium (BHM) containing $MgSO_4$ 0.2, K_2HPO_4 1.0, $CaCl_2$ 0.02, $FeCl_3$ 0.05, NH_4NO_3 1.0 (g l^{-1}) supplemented with or without glucose (0.1% w/v) and yeast extract (0.05% w/v) was used for all the studies.[13] The final pH of the medium was adjusted to 7.0.

Isolation and identification of dye decolorizing bacteria

Soil samples collected from the sites around the different dyeing houses of Tanda, Ambedkar Nagar, Uttar Pradesh (India) were used for isolation and screening of dye decolorizing bacteria using BHM amended with dye Acid Orange (100 mg l^{-1}). Aliquots (10 ml) of soil suspension (10% w/v) were inoculated into 100 ml of dye containing media (in 250 ml Erlenmeyer flask) and incubated at 30°C under shaking and static condition. Repeated transfers were carried out several times in fresh dye containing media until the dye containing media was decolorized. After incubation the samples were 10-fold serially diluted and 100 µl aliquots of each dilution were spread on BHM agar plates with 100 mg l^{-1} Acid Orange dye. The morphologically distinct bacterial isolates showing clear zones around their colonies due to decolorization of dye were streaked on BHM agar plates with 100 mg l^{-1} Acid Orange dye for purification. The pure culture stocks of these isolates were stored at 4°C on BHM agar with 100 mg l^{-1} Acid Orange dye. These isolates were screened for their ability to decolorize Acid Orange dye (100 mg l^{-1}) in liquid culture. The bacterial strain with efficient decolorizing ability was designated as RMLRT03.

Genomic DNA Extraction and 16S rRNA gene amplification

Fifty ml of Luria Bertani (LB) broth was inoculated with a fresh single colony of bacterial strain RMLRT03 and incubated for 24 hrs at 37°C in a shaker incubator (150 rpm). Broth culture was centrifuged at 8000 rpm and cell pellet was processed for genomic DNA extraction by Qiagen genomic DNA purification kit. Purified genomic DNA was quantified and integrity checked on 0.8% agarose gel. 50 ng of genomic DNA was taken for the amplification of 16S rRNA gene with primers pA (5'AGAGTTTGATCCTGGCTCAG3') and pH (5'AAGGAGGTGATCCAGCCGCA3').[14] All the steps were performed on ice. The PCR mixture contains 50–90 ng DNA template, 1X Taq buffer, 0.2 mM each of dNTPs, 10 pmol of each primer, 1.5 mM $MgCl_2$, and 2U of Taq DNA polymerase (Promega). The PCR amplification was performed in a G-Strom Thermal Cycler System using following conditions: Initial denaturation of 5 min at 94°C, followed by 40 cycles consisting of 30 s at 94°C (denaturation), 45s at 56°C (annealing) and 1 min 30s at 72°C (extension) and a final extension of 7 min at 72°C. Amplified products were resolved in 1.5% agarose gel stained with ethidium bromide (EtBr) and gel image was digitalized (Alpha-Imager).

Sequencing of 16S rDNA and BLAST analysis

Nucleotide sequence of gene 16S rDNA was sequenced on both sides through BigDye chain termination cycle sequencing kit (ABI) and the sequence was deciphered on 3130XL genetic analyzer (ABI) by Sanger's di-deoxy chain termination method and then assembled by Cap3 program to get the sequence of genes. Gene sequences were identified through similarity search against database through BLASTn for 16S rDNA. Evolutionary analyses were conducted by Neighbor-Joining method using MEGA 4.0 software.[15,16]

Effect of shaking and static condition

Decolorization of Acid Orange was studied under continuous shaking and static conditions. For decolorization study under continuous shaking condition, 30 ml MSM along with glucose (0.1% w/v) and yeast extract (0.05% w/v) amended with Acid Orange dye (100 mg l^{-1}) was taken in 100 ml Erlenmeyer flasks, inoculated with bacterial strain (5% v/v) and incubated at 30°C for 60 h under shaking condition (150 rpm). For static condition, the above mentioned inoculated medium was taken in 30 ml screw capped tubes and incubated at the same temperature and incubation period under static condition.

Effect of pH and temperature

Decolorization was studied at varying pH (4.0–9.0) and temperature (20°C-40°C). The pH of the medium was adjusted using 0.1 N HCl or 0.1 N NaOH. Experiments were performed in 30 ml screw capped tubes containing 30 ml of above mentioned inoculated medium under static culture condition.

Effect of carbon and nitrogen source

To find the most suitable carbon and nitrogen source, various carbon (glucose, fructose, starch, sucrose) and nitrogen (ammonium sulphate, ammonium chloride, yeast extract, peptone) source were evaluated under above optimized conditions.

Effect of initial dye concentration

The effect of varied initial dye concentrations on decolorization were determined by using a range (100-600 mg l^{-1}) of Acid Orange dye in the MSM and incubated under optimized static culture condition for 60 h.

Measurement of decolorization efficiency

The samples were collected at every 12 h intervals during 60 h of incubation and centrifuged at 10,000 rpm for 10 min at 4°C. Decolorization efficiency was analyzed by measuring the absorbance of culture supernatant at 484 nm. The decolorizing efficiency was expressed as percentage of decolorization.[17]

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Statistical analyses

Each experiment was performed in triplicate. The standard deviation (SD) was calculated using Microsoft Excel, and results presented as mean ± SD value.

RESULTS AND DISCUSSION

Isolation and identification of dye decolorizing bacteria

Isolation of dye decolorizing bacteria was carried out from soil samples collected from the sites contaminated with wastewater from dyeing houses of Tanda. A bacterial strain RMLRT03 with efficient decolorization ability against Acid Orange dye was isolated using BHM with glucose and yeast extract as co substrate. RMLRT03 strain was observed to be gram positive spherical cells in cluster.

Identification of bacterial strain RMLRT03 was done on the basis of 16S rRNA gene sequence. The sequence of RMLRT03 strain showed maximum similarity (99%) with *Staphylococcus hominis* during similarity search at RDPII database and identified as *Staphylococcus hominis*. The 16S rDNA partial sequence of *Staphylococcus hominis* RMLRT03 has been deposited in GenBank under the accession number KF900123. In phylogenetic analysis [Figure 1], RMLRT03 strain fall in the cluster of *Staphylococcus hominis*. The evolutionary history was inferred using the Neighbor-Joining method using MEGA 4.0 software.[15,18] The *Escherichia coli* have been taken out of group. The optimal tree with the sum of branch length (0.41634370) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.[19] The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.[16] The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 877 positions in the final dataset.

Effect of shaking and static condition

Oxygen has a significant effect on the physiological characteristics of the cells during the growth stage of cell.[20] The presence of oxygen can either favor or inhibit the microbial degradation of azo dyes. The *Staphylococcus hominis* RMLRT03 strain showed 85.52% decolorization of Acid Orange in static condition whereas in shaking condition only 32.47% decolorization was observed [Figure 2]. Similar results using *Acinetobacter calcoaceticus* showed maximum decolorization of azo dye Amaranth under static condition.[21] Several researchers reported efficient dye decolorization under static culture as compared to shaking (aerobic) conditions.[7,9] Telke *et al.*,[22] reported that in static condition 90% decolorization of Direct Black 38 by *Rhizobium radiobacter* was observed while only 6% decolorization was observed in shaking condition. Tripathi

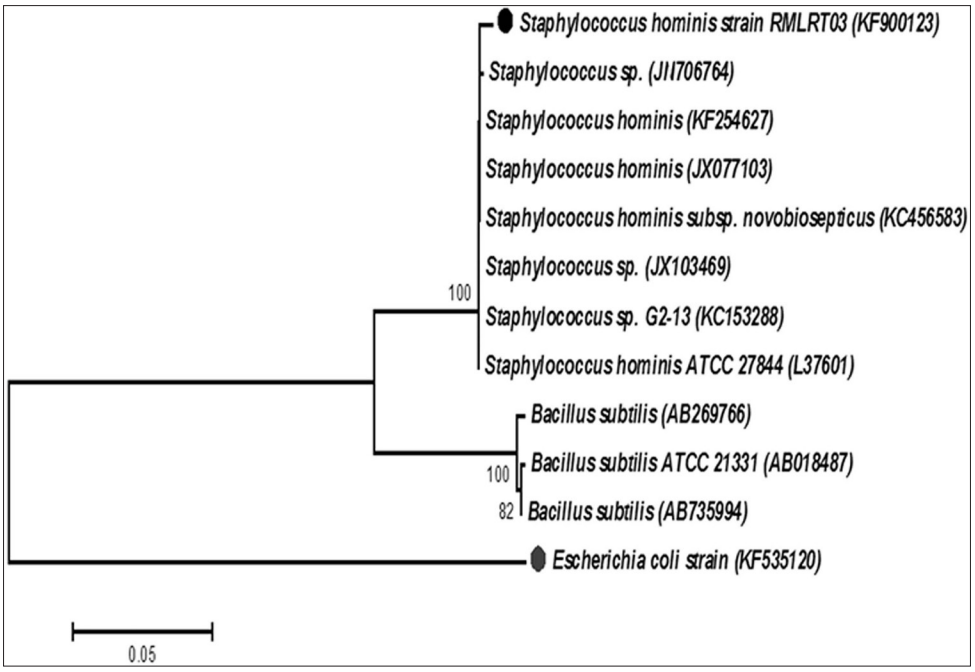

Figure 1: Phylogenetic tree of the *Staphylococcus hominis* RMLRT03 strain based on 16S rDNA partial sequences

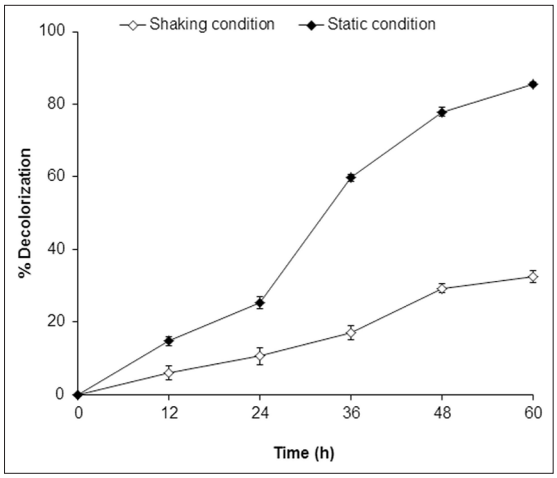

Figure 2: Effect of shaking and static conditions on decolorization of Acid Orange (100 mg l^{-1}) by *Staphylococcus hominis* RMLRT03 strain

and Srivastava[23] found 90% decolorization of Acid Orange 10 under static condition by *P. putida* strain as compare to 6% decolorization in shaking condition. The mechanism of bacterial degradation of azo dyes to their corresponding amines is initiated by a reduction of azo linkage with the aid of low specificity cytoplasmic azoreductase. Azoreductase mediated degradation of azo dyes is inhibited by the presence of oxygen because oxygen was a preferable terminal electron acceptor over the azo groups in the oxidation of reduced electron carriers such as NADH.[24] Under shaking conditions, the presence of oxygen deprives the azoreductase from receiving electrons required for azo bond cleavage, whereas under static conditions, these electrons are readily available to the enzyme from NADH to decolorize the azo dyes.[25]

Effect of pH and temperature

The pH and temperature are important factor for the optimal physiological performance of microbial cultures and decolorization of dyes. These factors affect the cell growth and various biochemical and enzymatic mechanisms. The decolorization of Acid Orange dye by *Staphylococcus hominis* RMLRT03 strain was found in the pH range of 6.0-8.0 [Figure 3]. The maximum decolorization (94%) was observed at pH 7.0. A further increase or decrease in pH from the optimum value decreases the decolorization rate. Our results are in good agreement with Olukanni *et al*.,[26] who achieved maximum decolorization of Methyl Red by *Micrococcus* strain R3 in pH range of 6.0–8.0. Shah *et al*.,[27] reported that the decolorization of Remazol Black B by *Bacillus* sp. ETL-2012 was found in the pH range of 5.0–8.0. The optimum pH for decolorization of dyes is often at a neutral pH value or slightly acidic/alkaline pH. The rate of dye decolorization tends to decrease rapidly at strongly acid or strongly alkaline pH values.[20]

The decolorization of Acid Orange dye by *Staphylococcus hominis* RMLRT03 strain was increased with an increase in temperature from 20–35°C [Figure 4]. A further increase in temperature to 40°C drastically affected the decolorization activity of the bacterial strain RMLRT03. The optimum temperature for decolorization of Acid Orange dye by *Staphylococcus hominis* RMLRT03 strain was found to be 35°C with 92.38% decolorization. Similar to our result, Cetin and Donmez[28] reported that the maximum decolorization of Reactive Red dye by mixed cultures was observed at 35°C. *A. hydrophilla* decolorize Red RBN dye in the range of 20–35°C.[29] Moosvi *et al*.[13] found that bacterial consortium

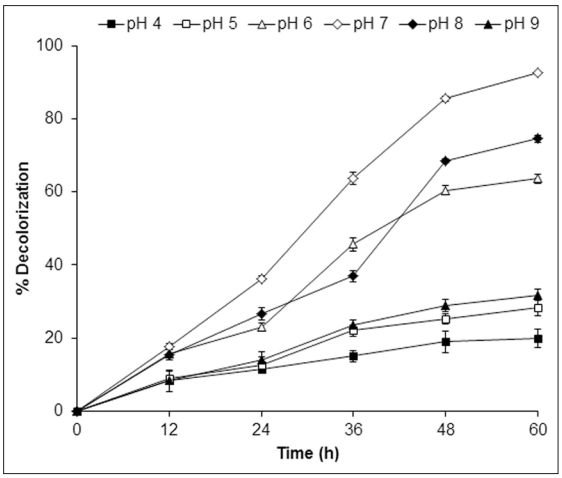

Figure 3: Effect of pH on decolorization of Acid Orange (100 mg l^{-1}) by *Staphylococcus hominis* RMLRT03 strain under static condition

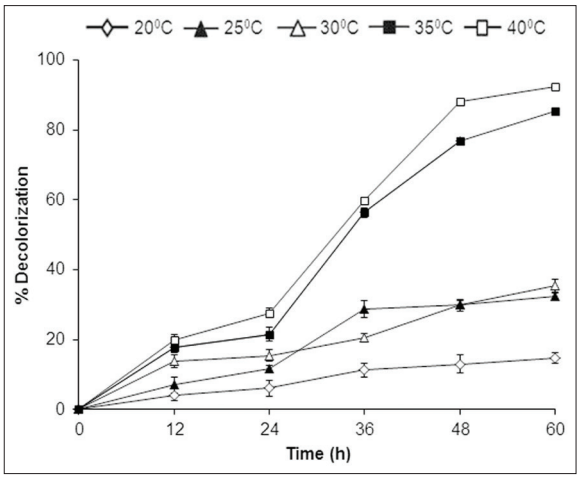

Figure 4: Effect of temperature on decolorization of Acid Orange (100 mg l^{-1}) by *Staphylococcus hominis* RMLRT03 strain under static condition

JW-2 showed maximum 93% decolorization of Reactive Violet 5R at 37°C. They also reported that further increase or decrease in temperature from optimum, decreases the decolorization rate of Reactive Violet 5R dye. In many bacterial systems, the decolorization rate of azo dyes increases with increasing temperature up to the optimal temperature, within a defined range. Afterwards there is a marginal reduction in the decolorization activity. The reduction in decolorization rate at higher temperature may be attributed to thermal deactivation of azoreductase enzymes or loss of cell viability.[3]

Effect of carbon and nitrogen source

Azo dyes are deficient in carbon sources, and microbial degradation of dyes without any supplement of carbon or nitrogen sources is very difficult.[30] The bacterial strain *Staphylococcus hominis* RMLRT03 showed maximum decolorization of Acid Orange in the presence of carbon and nitrogen source as co-substrate. Among different carbon sources tested for efficient decolorization of Acid Orange, glucose was found to be better carbon source with maximum decolorization (89.81%) whereas fructose, sucrose and starch was observed as poor carbon source showing only 69.98%, 54.65%, and 42.54% decolorization of Acid Orange dye, respectively [Figure 5]. Several reports are available for the decolorization of dye in presence of additional carbon sources. Joe *et al*.,[31] reported that the addition of glucose to the medium enhance the decolorization rate of Reactive Red 3B-A and Reactive Black 5 by *Clostridium bifermentans* strains. Wang *et al*.,[32] reported that in the presence of glucose, 90% decolorization of Reactive Red 180 dye by *Citrobacter sp*. CK3 was observed whereas in absence of glucose only 26.72% decolorization was found. Carbon sources provide energy for the growth and survival of the microorganisms and as electron donors, which are necessary for the breakage of the azo bond.[33] These sources generate reducing equivalents which are transferred to the dye during decolorization process. In the electron transport chain of the bacterial metabolism these reducing equivalents such as flavin nucleotide (FAD) works as an electron shuttle between a dye and an NADH-dependent azo reductase.[34]

The decolorization efficiency of *Staphylococcus hominis* RMLRT03 strain in the presence of different nitrogen source (yeast extract, peptone, ammonium sulphate and ammonium chloride) was studied. The maximum decolorization (93.24%) was observed in the presence of yeast extract, whereas in the presence of peptone, ammonium sulphate and ammonium chloride only 49.72%, 64.19%, and 36.97% decolorization of Acid Orange dye was observed respectively [Figure 6]. Similar to our finding, Telke *et al*.,[22] reported that yeast extract was effective co-substrates for decolorization of Reactive Red 141 by *Rhizobium radiobacter* which is in agreement to our studies. Moosvi *et al*.,[35] reported that maximum decolorization of Reactive Violet 5 by bacterial consortium RVM 11.1 was observed in presence of peptone and yeast extract as co-substrate. Moreover Mendez-Paz *et al*.,[36] used inorganic nitrogen source (NH_4Cl) in anaerobic treatment of azo dye Acid Orange 7 under fed-batch and continuous culture conditions. The metabolism of organic nitrogen sources regenerate NADH which acts as an electron donor for the reduction of azo dyes by bacterial system.[7]

Effect of initial dye concentration

The decolorization of dye was greatly influenced by concentration of dye. The decolorization rate of Acid Orange by *Staphylococcus hominis* RMLRT03 strain was decreased with an increase in dye concentration. Maximum decolorization (92.32%) of Acid Orange by RMLRT03 strain was observed at 100 mg l^{-1} of dye concentration. Further increase in dye concentration (200–600 mg l^{-1}) showed reduction in decolorization rates [Figure 7]. Rate of dye decolorization was gradually decreases with

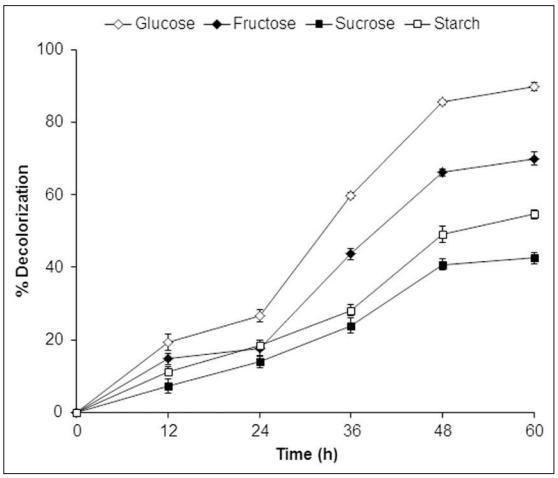

Figure 5: Effect of different carbon source on decolorization of Acid Orange (100 mg l^{-1}) by *Staphylococcus hominis* RMLRT03 strain under static condition

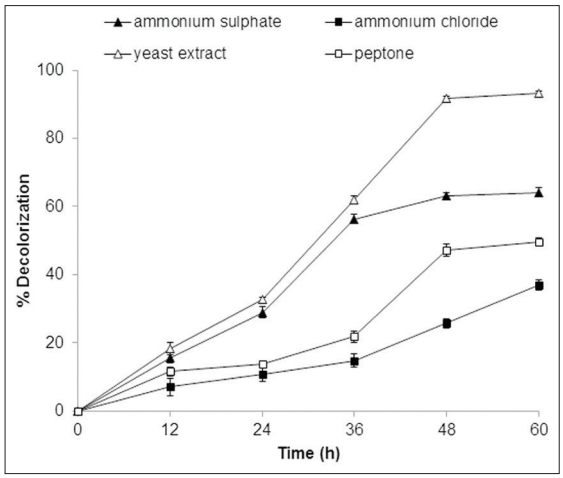

Figure 6: Effect of different nitrogen source on decolorization of Acid Orange (100 mg l^{-1}) by *Staphylococcus hominis* RMLRT03 strain under static condition

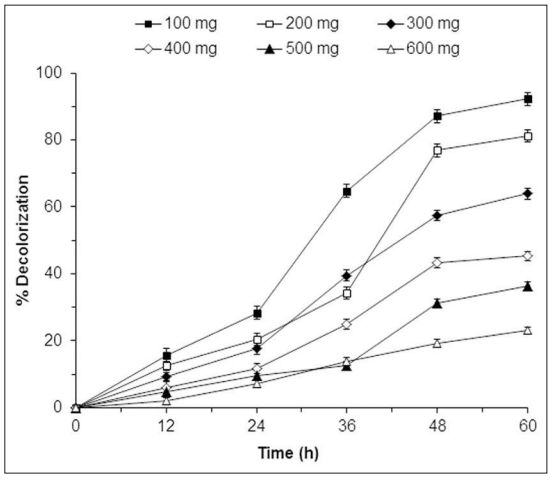

Figure 7: Effect of initial dye concentration on decolorization of Acid Orange (100 mg l^{-1}) by *Staphylococcus hominis* RMLRT03 strain under static condition

increasing concentration of dye due to the toxic effect of dyes on degrading microorganisms or the blockage of active sites of azoreductase enzymes by dye molecule with different structures.[37] Wang *et al*.,[10] reported that the decolorization rate of Reactive Black 5 by *Enterobacter* sp. EC3 was decreased with increase in initial dye concentration. Moreover Cetin and Donmez[28] reported that higher the dye concentration, longer the time required to remove the dye. They observed that at 125.6-206.3 mg l^{-1} initial dye concentrations of Remazol Blue, 100-90% dye was decolorized at the end of 30 h incubation period but when concentration of dye was increased upto 462.5 mg l^{-1}, only 90% decolorization was found by mixed cultures after 50 h incubation period. At higher dye concentrations (593.8 and 1031.3 mg l^{-1}), in spite of longer incubation periods decolorization yield could only reach about 85%.[28]

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