Research article Available online www.ijsrr.org ISSN: 2279–0543

International Journal of Scientific Research and Reviews

A Study on decolorization and detoxification of commercial textile azo dye methyl orange by enterobacter cloacae mk418746.

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ABSTRACT

Synthetic dyes have increasingly been used in the textile industries. This has resulted in the discharge of highly polluted effluents into environment. Bio remediation of textile dyes has been of considerable interest since it is inexpensive, Eco friendly and produces a less amount of sludge when compared to physio chemical methods. In this work soil samples were collected from a textile industry (Arcade, Vellore Tamil Nadu, India), were plated on nutrient agar plate containing 0.1g of methyl orange. The isolated bacterium exhibited a remarkable dye degradation and decolourization under optimized conditions. FTIR and toxicity assays helped to perceive the extend of dye degradation.

KEY WORDS: Textile industry, Effluent, Methyl orange, Bio degradation.

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INTRODUCTION

Bright and colorful fabrics get its color through the use of textile dyes. Synthetic or man-made dyes are used to color our clothing and home furnishings. However these synthetic dyes cause many problems around the world. The effluents from dying process mainly discharge into water bodies. Which contain many harmful chemicals and traces of dyes, it is threaten to the aquatic life. Some dyes never degrade within water, but some are degraded to produce harmful by products. There are almost 4,50,000 tons dyes been produced per year and worldwide and > 11% are lost in processing and application[1]. Textile effluents are considered as unpleasant by product of economic development and technology. Improper handling of these waste product leads to harmful effects to both human and aquatic life.

Azo dyes are one of the largest commercials dyes available and most widely used in textiles, foods, cosmetics, plastic laboratory, leather, paper, printing, color photography (M.A, Syed et al 2009). Azo dyes are organic compound contain azo groups (N=N-), which contribute coloring function to the compound. Azo dyes having a nitro group, which cause mutagenicity of azo dye[]. However, differences in the toxicity and mutagenic activity of azo dyes are strongly dependent on their chemical structure[3].

Physio-chemical method such as adsorption, precipitation, chemical degradation or photo degradation are tried to treat textile effluents. But they are costly, methodologically damaging, time consuming and mostly not very effective. Other techniques have been used to remove dyes from textile effluent, including bio sorption and enzymatic treatment etc. which are collectively called bio remediation. In bio sorption, biomass of bacteria, yeast, filamentous fungi and algae has been used to remove toxic dyes[4]. The cell wall components are the major cause of property of bio sorption in these microbes such as hetero polysaccharides and lipids, which consists of different charged functional groups creating a force of attraction between the azo dye and cell wall.

Azoreductase, laccases, lignin peroxidase, manganese peroxidase, and hydroxylases are the enzymes involved in enzymatic degradation of azo dyes in microbes. In which azoreductase and laccases play prominent role in bio-degradation. Azoreductases depend upon reducing equivalents such as NADPH, NADH and FADH for de colorization of azo dyes[5]. During bio-degradation, azo reductases initially cleave azo bonds (N=N-), under anoxic condition. Laccases (a multi-copper oxidases) as non-specific oxidation capacity not require co-factors and do not use readily available oxygen as an electron acceptor.

Bio remediation of textile dyes has been of considerable interest since it is inexpensive, eco-friendly and produces a less amount of sludge when compared to physio chemical methods. There

for present study focus on isolate a bacterial strain from soils collected from areas of textile industry. Hence investigate the feasibility of isolate to degrade and decolorize the methyl orange under optimized conditions. FTIR and toxicity assays helps to perceive the extend of dye degradation.

MATERIAL AND METHODS:

Collection of Sample:

Soil samples were collected from a textile industry Arcade, Vellore, Tamil Nadu, India. It was collected in sterile airtight plastic container.

Isolation of Dye Degrading and Dye Decolorizing Bacteria:

Serially diluted soil samples of different dilutions (10^{-1} to 10^{-6}) were inoculated over sterilized nutrient agar medium and incubated for 37 °C for 2 days. The medium contained 1ml of 10ppm stock solution of Methyl Orange. A single colony obtained from 10^{-1} was isolated and re-streaked several times to obtain pure cultures.

Identification of Isolates:

A loop full of isolate obtained after incubation was streaked on nutrient agar medium supplemented with dye and incubated at 37^0C for 24 hours. Colony characterization was studied after incubation. polymerase chain reaction(PCR) amplified the16s rRNA sequence of the isolate using forward and reverse primers(27F/1492R primers). Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit. Purified PCR product was sequenced using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits. BLAST-n site at NCBI server www.ncbi.nlm.nih.gov/BLAST was used to analyze the nucleotide sequence. For multiple sequence alignment MUSCLE 3.7 was used. Using the program Gblocks 0.91b eliminated poorly aligned positions and divergent regions. PhyML 3.0 aLRT program was used for phylogeny analysis and HKY85 as Substitution model.

Dye Decolorization Assay:

Loop full of isolate was inoculated on nutrient broth supplemented with 10ppm of Methyl Orange and incubated at 150rpm at room temperature for 24 hours. To find the maximum decolorization condition, isolate with medium was also kept under static condition. A control without isolate was also maintained.

3ml of sample was withdrawn after different time intervals and centrifuged at 10000 rpm for 15 minutes. Extend of decolorization was detected by measuring the absorbance of supernatant of control and samples at 510nm using spectrophotometer.

Percentage of Dye decolourization = Initial OD – Final OD/Initial OD*100

Optimization of Physio-chemical parameters:

1. ***Effect of Shaking and Static conditions :*** Loop full of isolate was inoculated on nutrient broth supplemented with 10ppm of Methyl Orange was incubated at 150rpm and under static condition at room temperature for 24 hours. Maximum decolorization was detected by comparing the absorbance of samples at 510nm using Spectrophotometer. Percentage of dye decolorization was also determined.
2. ***Effect of pH :*** Nutrient broth with dye(10ppm) was adjusted at different pH (5,6,7,8 and 9).The flask were inoculated with the isolate and incubated under static condition and observed.Extend of decolorization was detected by measuring the absorbance at 510nm using Spectrophotometer. Percentage of dye decolorization was also determined.
3. ***Effect of Temperature :*** Nutrient broth with dye (10ppm) was inoculated with isolates under different temperature (30°C, 35°C and 40°C) under static condition. Absorbance and percentage of decolorization were determined.
4. ***Effect of Carbon Source :*** In addition to medium [Bushnell Hass Broth with dye(10ppm)], glucose and fructose were separately added in media,to which a loop full of isolate was inoculated. The Percentage of dye decolorization was determined from optical density values at 510nm.
5. ***Effect of Nitrogen Source :*** Bushnell Hass Broth with dye (10ppm) was supplied with different nitrogen sources (yeast extract and tryptone). Medium was inoculated with the isolates.Absorbance was measured at 510nm and percentage of dye degradation was calculated.

Analysis of Dye Degradation:

The biodegraded methyl orange dye samples were characterized by FTIR spectroscopy (Perkin-Elmer). The analysis results were compared with the FTIR spectra of control dye methyl orange. The biodegraded sample was mixed with chloroform in 2:1 ratio (chloroform: sample). The FTIR analysis (ATR) was done in the mid IR region (400-4000 cm^{-1}).

Toxicity Analysis by Brine Shrimp Larvae (nauplii):

Toxicity tests were performed in order to assess the toxicity of the untreated and treated dye samples. Expose the nauplii to both the biodegradable sample and control dye (stock solution of methyl orange) in test tubes. Count the number of survivors and calculate the percentage of death

after 24 hours. For each tube, count the number of dead and number of live nauplii, and determine the Percentage of death using the equation given below;

Percentage of death = Number of dead nauplii /Number of dead nauplii + Number of live nauplii x 100

RESULT AND DISCUSSION

The effluents from dying process mainly discharge into water bodies,which contain many harmful chemicals and traces of dyes, it is threaten to the aquatic life. Some dyes never degrade within water, but some are degraded to produce harmful by products. Moreover, these azo dyes and their intermediate aromatic amines are either toxic or mutagenic or carcinogenic, posing a potential health hazard to human kind[6]. So purification of textile effluents has become a matter of great concern[7] One promising strategy is the use of microbial strains that possess the ability to decolorize and mineralize synthetic dyes[8]

Collection Isolation and identification of Dye Degrading and Dye Decolorizing Bacteria:

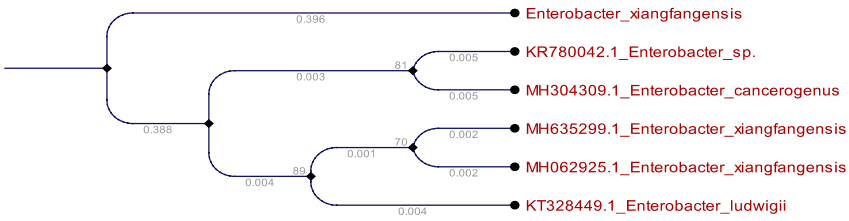
Its clearly observed that the textile industry play an important role in economical position[9]. Soil was collected from the textile industry site (Arcade, Vellore) so that the presence of dye degrading bacteria would increase. The isolate was identified by microscopic and biochemical characteristics (Table.1). By analyzing 16sr RNA sequence, the isolate had highest similarity with the species *Enterobacter cloacae* (99%). Also in the phylogenetic analysis, the sequence formed a cluster with in *Enterobacter spp* with 99 % identity. Thus confirming the isolate belongs to *Enterobacter cloacae* strain. The obtained sequence was submitted to Gen Bank with the accession number MK418746. PCR amplified sequence of *Enterobacter cloacae* is given below;

>_contig SAM-2

TTCCTTTTACAACCCACTCCCATGGTGTGACGGGCGATGTGTACAAGGCCCGGGAACGT
ATTCACCGTAGCATTCTGATCTACGATTACCAGCGATTCCCACTTCATGGAGTCGAGTTG
CAGACTCCAATCCGGACTACGACGCACTTTATGAGGACCGGTTGCTCTCGCGAGGTCGC
TTCTCTTTGGATGCGCCATTGTAGCTCGTGTGTGGGCCTGGTCGTAAGGGCCATGATGGC
TTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCT
AACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCA
CAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCACAGTTCCCGAAGGAACCAATC
CATCTCTGGAAAGTTCTGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAA
TTAAACCACATGCTCCACCGCTTGTGCGCGCCCCCGTCAATTCATTTGAGGTTTAACCTT
GCGGACGTACTCCCCAGGCGGTCGATTTAACGCGTTAGCTCCGGAAGCCACGTCTCAAG

GGCACCACCTCAAAATCGACATCGTTTACGGCGTGCACTACCAGGGTATCTCATCCTGT
TTGCTCACCGACGCTTTCGCACCTAAGCGTCAGTCTGTGACCAGGGGGACGTCCTCGCC
ACGGGAATTCCTCCAGAAATCTACGCATTTTAGCTGCTACCCCTGGAAGTCTACCTCCCC
TCTACAAAGACTCTTAGACTGCCAGCTTCGGAAGGCAGCTGCCCCGGGTGTAGCCCCGG
GAATTTAAATTACCGAGGTGGCCGAACCAACCTGCGTGGGCCATTTATGTCCAGTTAAT
TTCGAATAA

1.PCR Amplified 16 SrRNA sequence of isolate MK418746

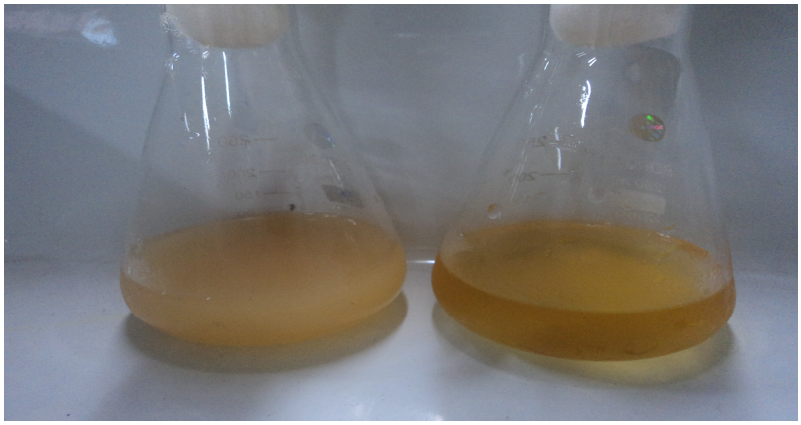


2 Phylogenetic Tree of the Isolate MK418746

Table 1 microscopic and biochemical characteristics

SL.No	Test	Result
1	**Gram staining : The test was performed to identify Gram positive and Gram negative organism.**	+
2	**Cell shape and arrangement**	**Single Rod**
3	**Colony size** **Colony shape** **Margin (edge)** **Elevation** **Color**	**Small round** **Circular** **Entire** **Convex** **Yellow \ Tan**
4	**IMViC**	**- - + +**

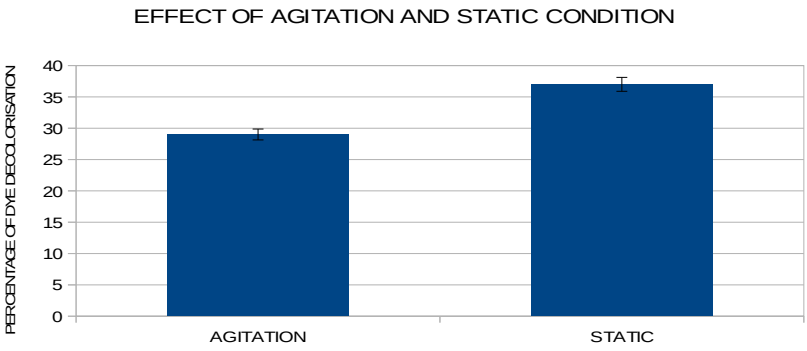
Dye Decolorization Assay: MK418746 strain exhibited remarkable efficiency in decolorizing Methyl Orange (Fig.3) under shaking and static conditions. From percentage of dye degradation equation it was proved that more dye degradation and decolorization occurs at static anoxic condition.



3.Decolorization of Methyl Orange by MK418746 Strain (Test sample and control Sample)

Optimization of Physiochemical Parameters:

Effect of static and agitation condition on dye degradation and decolourisation: Degradation and decolourisation of dye was observed maximum under static condition(37%)compared to agitation (29%).



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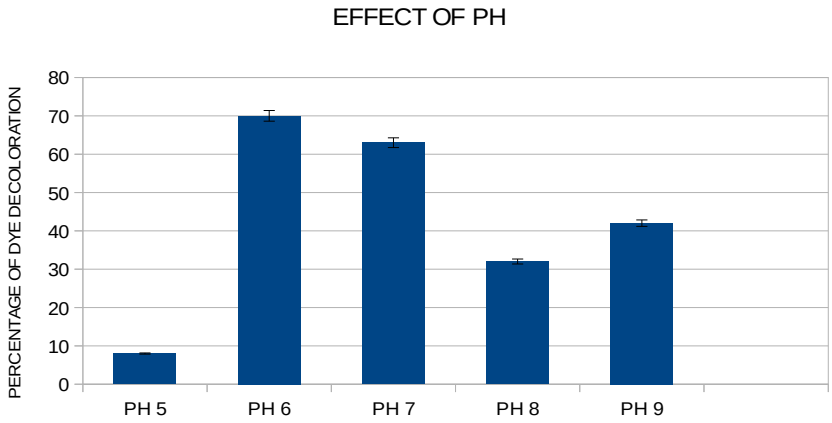
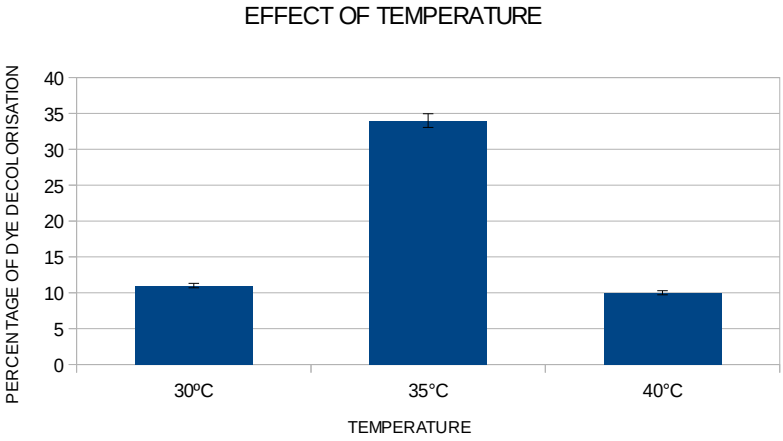
Effect of pH on dye degradation and decolourisation : The best decolourisation was achieved at pH 6.0 within 24 hours of incubation (70%).pH tolerance of dye degradation and decolourization is quite important because azo dye bind to cotton fibres by addition or substitution under alkaline condition.



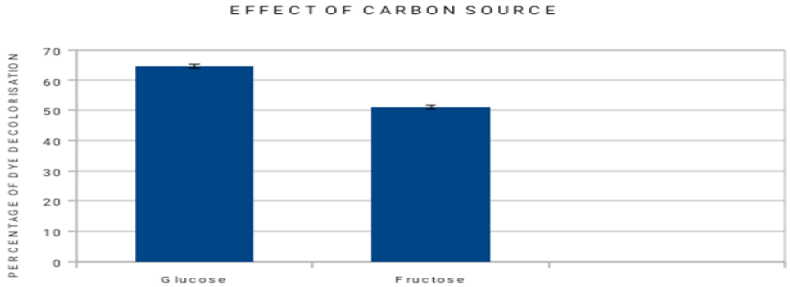
Effect of Temperature on Dye decolorisation and degradation :

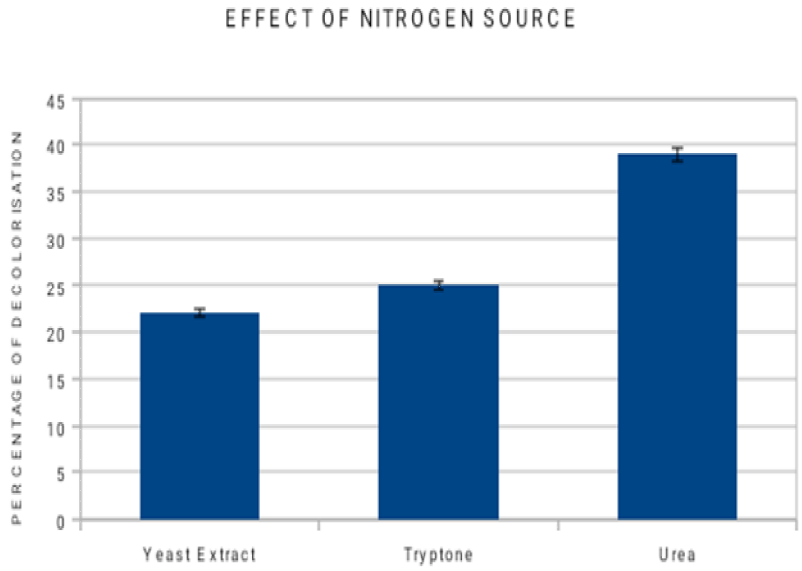
From the observations, MK418746 showed strong decolourization and degradation at 35°C (34%).



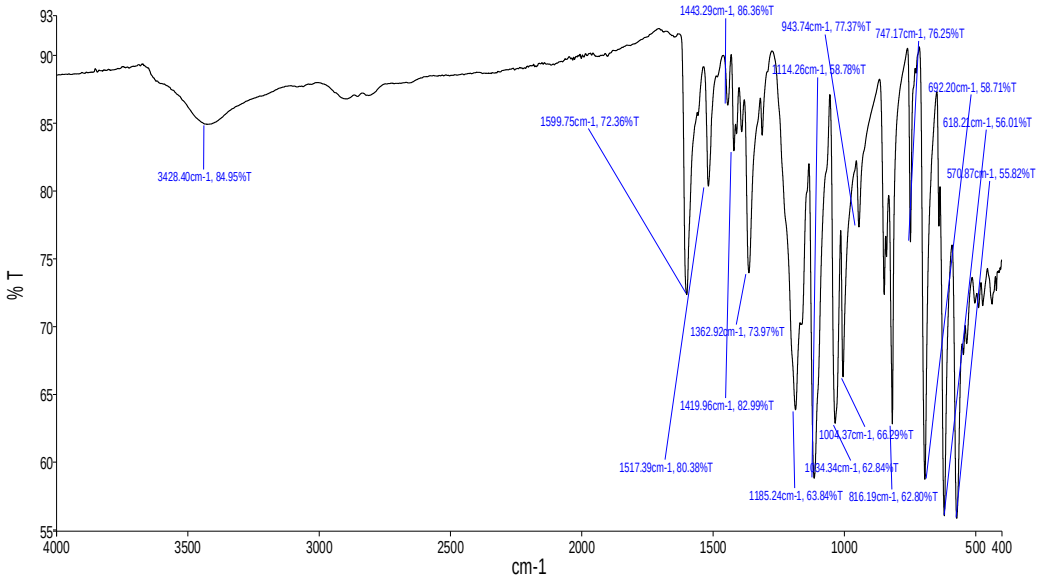
Effect of Carbon and Nitrogen Source on Dye decolorisation and degrdation:

The carbon sources subjected for observation were glucose and fructose.Glucose showed the optimum decolorisation activity (65%) after incubation,Among the nitrogen sources urea (39%) showed more activity when compared to yeast extract and tryptone.

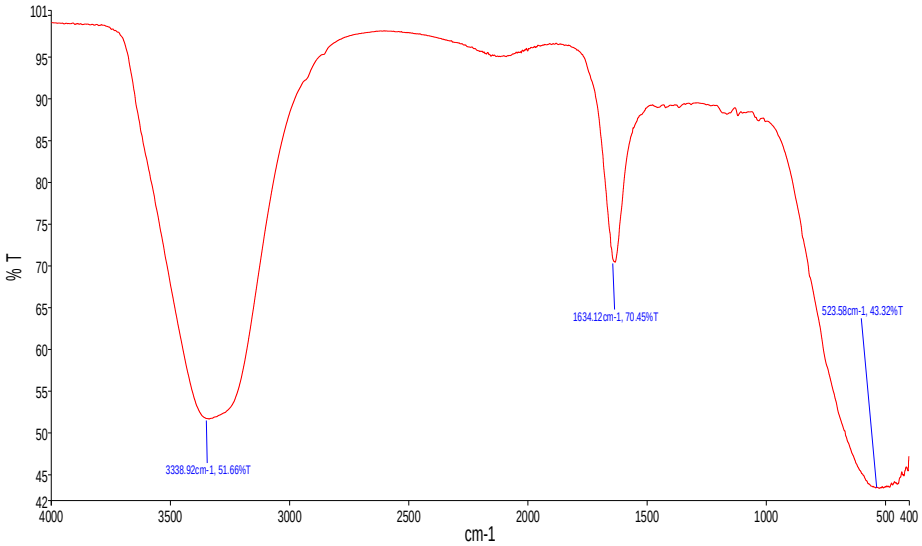




Analysis of Dye degradation FTIR : The FT-IR spectra of Methyl Orange dye displayed peaks at 3428.40 cm^{-1}, 1599.75 cm^{-1} ,1185.24 cm^{-1} 570.87 cm^{-1} .However the FT-IR spectra of degradation product displayed peaks at different positions indicating the complete breakdown of Methyl Orange. It means dye degradation and decolorization had taken place.



4 FT-IR Analysis of Methyl Orange



5. FT-IR Analysis of Degraded sample

Toxicity Analysis by Brine Shrimp Larvae (nauplii): *A.salina* has been used as a bio indicator of the toxicity of textile effluents, since the high degrees of salinity and conductivity in these effluents are critical parameters for fresh water species[10]

Death percentage of treated sample = Number of dead nauplii /Number of dead nauplii + Number of live nauplii x 100

= 1 / 1+9 x 100 = 10%

Death percentage of control = Number of dead nauplii /Number of dead nauplii + Number of live nauplii x 100

= 7 / 7+3 x 100 = 70%

CONCLUSION

Textile dye Methyl Orange one of the Azo dye, degraded under static conditions with a coordinated effect of bacteria, which was isolated from the textile dye effluent. The strain MK418746 was isolated from the textile effluent and was identified as *Enterobacter cloacae* using various morphological, biochemical and molecular identification (16s rRNA sequencing) techniques. In decolourizing assay, the strain MK418746 showed effective dye decolourization under static condition. Various factors were studied to see the effect of various physiochemical effect on dye degradation process and it was found that it gave better degradation with the factors of static condition, pH 6, Temperature 35 ℃, Carbon source as Glucose and Nitrogen Source as Urea. At this optimum condition the strain gave 70% degradation and decolourization within 24 hours of the incubation period. When comparing the peaks of FTIR spectrum of methyl orange and sample

obtained after complete decolorization clearly indicated the biodegradation of Methyl Orange by MK418746 was happened. The cytotoxicity assay with brine shrimp larvae (nauplii) clearly indicated the nontoxic nature of degraded and decolorized sample of Methyl orange.

ACKNOWLEDGEMENT

MCAR (Marian Center For Advanced Research,Thrissur,Kerala) for their constant support,guidance through out the work.

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