BIODEGRADATION OF METHYL RED BY *ACINETOBACTER BAUMANNII* SM01 AND *KLEBSIELLA PNEUMONIAE* SM27 AMONG OTHER EGYPTIAN ISOLATES

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ABSTRACT

Azo dyes are xenobiotic pose a long-term effect on human life. The capability of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolated from petroleum oil contaminated soil sites in Egypt for degradation of methyl red (MR) was investigated. The physicochemical parameters which affect MR degradation were examined. The maximum degradation of MR by both isolates was attained when 1.0 % (w/v) of glucose, 0.1 % (w/v) of yeast extract and 1.0 mM of $CaCl_2$ were added to the culture media, and incubated for 6.0 h at 35℃ with the highest speed of shaking (150 rpm).

The mechanism of degradation of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates was studied through analysis of MR degradation products by FT-IR and double beam UV-visible spectrophotometer. Their results confirmed that MR underwent azo reduction and further degradation. The enzymes responsible for biodegradation of MR by *Acinetobacter baumannii* SM01 isolate were investigated, in the cell free medium obtained after 6.0 h of incubation and a control. The results showed a significant increase in the activities of laccase, tyrosinase, azoreductase and NADH-DCIP reductase enzymes. The outcomes of phytotoxicity studies on seeds of *Triticum aestivum* illustrated that MR degradation metabolites produced by *Acinetobacter baumannii* SM01 inhibited the germination rate by only 10 % and decreased root and shoot length by 27.8 % and 23.9 % respectively correlated to the inhibition exhibited by the intact MR. Consequently, the high efficiency of MR degradation enables *Acinetobacter baumannii* SM01 to be a potential candidate for bioremediation of MR containing wastewater.

Keywords: Biodegradation, *Acinetobacter baumannii, Klebsiella pneumoniae,* Methyl red, Biodegradation products, Phytotoxicity.

INTRODUCTION

Azo dyes are the largest class of synthetic aromatic dyes composed with one or more azo (N=N) groups and sulfonic ($-SO_3^-$) groups with lots of commercial interest. There are more than 3000 azo dyes which are widely used by the textile, leather, cosmetics, food colouring and paper production industries **(Sudha *et al.,* 2014)**. Azo dyes are xenobiotic pose a long-term effect on human life. Toxic compounds of azo dyes mix with water bodies, swallowed by fish or other aquatic creatures which are further taken up by humans causing hypertension, sporadic disorder, cramps, etc. with prolonged effect **(Sarkar *et al*., 2017)**.

Physical and chemical treatment methods such as precipitation, coagulation, adsorption, flocculation, flotation, electrochemical destruction, and mineralization processes minimize the toxicity level not to neutralize the toxicity (**Maier *et al.,* 2004)**. Microorganisms are the best alternatives for detoxification of azo dyes since they can completely degrade the azo dyes by secreting enzymes such as laccase, azo reductase, peroxidase and hydrogenase which reduce them. The reduced forms of azo dyes are further mineralized into simpler compounds and are utilized as energy sources **(Sudha *et al.,* 2014)**.

Methyl red which is also named acid red2 is a mono-azo dye. It has the molecular formula $C_{15}H_{15}N_3O_2$, molecular weight 269.29 and IUPAC name 2-(N,N-Dimethyl-4-aminophenyl)azo benzene carboxylic acid. Its structure is illustrated in Figure (1) **(Shah, 2014).** Methyl red (MR) is not practiced as a textile dye in industry, but it is used as a model azo dye to know or underline decolourization and degradation attributes. **Hsueh and Chen (2007)** reported MR as the most difficult dye to degrade due to charged carboxyl group on methyl red at ortho position to azo bond, inhibiting decolourization activity **(Patil *et al*., 2016)**.

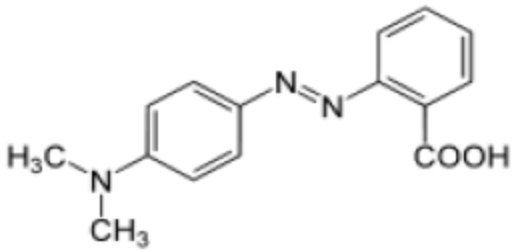

Figure (1): Structure of methyl red (Shah, 2014).

Decolourization of azo dyes may take place by two methods either adsorption on the microbial biomass (biosorption) or biodegradation of the dyes by the living cells. Most of the azo dyes have high molecular weight and cannot pass through bacterial membranes so dye removal activity is not dependent on the intracellular uptake of the dye. Effective biosorption depends on several conditions such as pH, temperature, ionic strength, time of contact, dye concentration, dye structure and type of used microorganism **(Singh and Singh, 2017). Sari and Simarani (2019)** found that *Lysinibacillus fusiformis* W1B6 could remove methyl red through biosorption to dead bacterial cells, but the main mechanism of methyl red removal was through its

degradation by oxidoreductive enzymes and formation of new by-products. While **Sharma *et al.* (2016)** investigated that the complete decolourization or removal of MR by immobilized bacterial beads with Fe_3O_4 nanoparticles was due to biodegradation of methyl red through reductive cleavage of azo bond by *Aeromonas jandaei* strain SCS5, but not due to adsorption.

Microbial degradation of MR was catalysed by synergy of oxidoreductase enzymes **(Singh and Singh, 2017).** The predominant enzymes are azoreductase, laccases, lignin peroxidase, manganese peroxidase, and hydroxylases **(Sudha *et al.*, 2014).**

Azoreductase and NADH-dichlorophenol indophenol (NADH-DCIP) reductases catalyse the reductive cleavage of azo bond (-N=N-) in MR to produce colourless aromatic amine products, these enzymes were involved in MR degradation by *Saccharomyces cerevisiae* MTCC 463, *Bacillus* spp. UN2 and *Bacillus megaterium* ITBHU01 **(Jadhav, 2007; Zhao *et al.*, 2014; Tripathi *et al.*, 2016 and Sari and Simarani, 2019)**. Laccase was also known to catalyse the oxidation of phenolic and several aromatic compounds, and they prevent the formation of toxic aromatic amines **(Sudha *et al.*, 2014).** Laccase was involved in MR degradation by *Saccharomyces cerevisiae* MTCC 463, *Bacillus* spp. strain UN2, *Bacillus megaterium* MTCC 8371 and *Lysinibacillus fusiformis* W1B6 **(Jadhav, 2007; Zhao *et al.*, 2014; Nair *et al.*, 2017 and Sari and Simarani, 2019).** Tyrosinase catalysed oxidation of phenolic compounds, it was involved in mineralization of MR by *Saccharomyces cerevisiae* MTCC 463 **(Jadhav, 2007).**

It is very important to know whether biodegradation of a dye leads to detoxification of the dye or not. This can be done by performing phytotoxicity test of the original dye and its biodegradation products **(Ali, 2010)**.

This study aimed for screening for most potent MR- and other dyes degrading bacterial isolates obtained from different soil sites in Egypt. In addition, the physicochemical factors which affect MR degradation and the mechanism of MR biodegradation were studied.

MATERIALS AND METHODS

Soil samples:

Thirty three soil samples were collected from various environmental sites including the surface of petroleum oil-contaminated sites (24 samples), livestock waste (3.0 samples), agriculture soil (3.0 samples) and mud from irrigation canals (3.0 samples). The specimens were collected using a spatula and placed in clean new plastic bags. The samples were inserted into an ice box (4.0°C) and transferred to the microbiological laboratory as soon as possible.

Dyes, chemicals and media used in this study:

Methyl red (MR) was purchased from Universal Fine chemicals, India. Ferric chloride ($FeCl_3$) from Alfa Chemika, India, lead acetate ($Pb(CH_3COO)_2$) and manganese sulphate ($MnSO_4$) from Universal Laboratories, India; calcium chloride ($CaCl_2$) and magnesium sulphate ($MgSO_4$) from Lobal Chemie, India.

The experimental **medium MI** was composed of 5.1 g/L K_2HPO_4, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 100 mg/L of MR and filter-sterilized glucose (1.0 w/v), it was used for screening of MR-degrading isolates **(Zhao *et al.*, 2014)**. The experimental **medium MII** was composed of glucose-free MI medium to which 0.1% (w/v) yeast extract was added and the pH was adjusted to 7.0. It was used to study the effects of added various carbon sources on the biodegradation of MR. The **MIII medium** was composed of MI medium to which 0.1% (w/v) yeast extract was added. Its pH was adjusted to 7.0 and it was used for studying the effect of addition of various metal ions on the biodegradation of MR. The **MIV medium** was composed of MR-free MIII medium to which 1.0 mM of $CaCl_2$ was added. Its pH was adjusted to 7.0 and it was used to study biodegradation of different concentrations of MR. **Bushnell Haas (BH) medium** was used for isolation of petroleum oil degrading isolates, it was composed of 1.0 g/L K_2HPO_4, 1.0 g/L KH_2PO_4, 1.0 g/L NH_4NO_3, 0.2 g/L $MgSO_4$, 0.02 g/L $CaCl_2$ and 0.05 g/L $FeCl_3$, the final pH was adjusted 7.0 ± 0.2 **(Bushnell and Haas, 1941)**.

Isolation of MR-degrading microorganisms:

Twenty crude petroleum oil degrading bacterial isolates had been isolated from different thirty three soil sites in Egypt by enrichment on 1.0% (v/v) crude oil as described by **Obi *et al.* (2016)**. The isolates were conventionally characterized according to the scheme of **Bergey's** manual of determinative bacteriology **(Holt *et al.*, 1994)** and **Procop *et al.* (2017)**. Definitive identification of the bacterial isolates was performed using VITEK2 system (BioMérieux, France) at Ain Shams University Hospital, Cairo, Egypt. These isolates were screened for their ability to degrade methyl red as described by **Singh *et al.* (2015).** In brief, two mL of fresh bacterial culture in nutrient broth with $OD_{\lambda\ 600\ nm}$ ($A°_{600}$)= 0.5 were inoculated into 250 mL conical flask containing 100 mL of MI medium pH 7.0. The flasks were incubated at 37℃ with shaking at 120 rpm for 24 h. The culture media were visually observed for decolourization of MR after 24 h compared to a control of un-inoculated medium. The most potent MR-degrading isolates were then selected on the basis of which isolate showed the fastest and highest percent of MR degradation after only 10h of incubation as described by **Sari and Simarani (2019).** Two mL of culture medium were withdrawn aseptically every 2.0 h and examined for the percent of MR-degradation and the growth rate of the bacterial isolate.

Determination of bacterial growth was carried out by measuring the absorbance (OD at λ_{600} nm) and compared with un-inoculated flask as a control.

Determination of the concentration of MR:

The concentration of MR at any specific time during the degradation experiment was determined by withdrawing 2.0 mL of the bacterial culture and were centrifuged at 10,000 rpm for 15 min. The pH value of cell-free medium was measured by a pH meter, then diluted by a phosphate buffer solution of the same pH. The absorbance of the diluted cell-free medium was measured using the UV-spectrophotometer **(Optima, Japan)** at the corresponding wavelength suitable for the detected pH value. The final concentration of MR was calculated from the following equations which were deduced from the previously prepared standard curves:

$$\mathbf{X = 4.0453Y + 0.7427} \qquad \text{If pH} \leq 4.4$$

$$\mathbf{X = 43.6681Y - 13.3930} \qquad \text{If pH 4.4 - 6.2}$$

$$\mathbf{X = 42.0168Y - 12.0294} \qquad \text{If pH} \geq 6.2$$

Where **X** is the concentration of MR at certain time and **Y** is absorbance at this time at the corresponding wavelength.

Determination of the percent of MR-degradation (Tripathi *et al.*, 2016):

The percentage of MR degradation was calculated from the difference between the initial and the final concentrations using the following formula.

$$\text{Degradation \%} = \frac{C_0 - C_1}{C_0} \text{ x } 100$$

Where C_0 is initial concentration, and C_1 is final concentration for the dye.

Gene amplification of 16S rRNA and sequence determination:

Genomic DNA of the selected most potent MR-degrading isolates were extracted according to the method described by **Al-Ahmadi and Roodsari (2016)** and the 16S rRNA genes were amplified. Amplification of 16S rRNA genes was carried out with universal primers (Metabion, Germany) 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGCTACCTTGTTACGACTT-3') in a final volume of 25µL containing 10 µL Dream Taq Green PCR master mix (2x) (Thermo Scientific, USA), 2.0 µL template DNA, 11µL of nuclease free water (Ambion, USA) and 1.0 µL of each primer at a concentration of 10 pmol/µL. PCR programme for 16S rRNA gene amplification was 1.0 cycle for pre-denaturation at 95℃ for 5.0 min followed by 35 cycles of 1.0 min denaturation at 94℃, 1.0 min annealing at 57℃ and 1.0 min extension at 72℃ and a final extension step of 7.0 min at 72℃. The amplified 16S rRNA gene was separated on an agarose gel using 100 bp DNA marker (Intron, Korea), then extracted from agarose gel using Megaquick-spin plus total fragment DNA purification kit (Intron, South Korea). The forward sequences of 16S rRNA genes of the isolates were identified by Macrogen (Korea).

The forward sequence of 16S rRNA gene of SM01 and SM27 isolates were aligned and compared with the known 16S rRNA gene sequences in Genbank database

using the BLAST search at NCBI. The taxonomic position and the phylogenetic tree of the isolates were constructed with MEGA-X. These isolates were registered in Genbank database with specific accession numbers.

Methyl red standard curves:

To assess the concentration of methyl red (MR) in the growth medium; three standard curves of MR were performed at three different pH values i.e. 3.0, 5.0 and 7.0. For each curve: Different sets of concentrations of MR (1.0, 2.0, 3.0, 4.0, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, 90.0, 100.0, 110.0, 120.0, 130.0, 140.0, 150.0 and 160.0 mg/L) were prepared from a stock solution containing 400 mg MR/L by a dilution buffer solution having the intended pH. The absorbance of each set of concentrations was determined at the three different wavelengths 430 nm, 451 nm and 520 nm using the UV-Visible spectrophotometer (Optima, Japan). The wavelength which gave the maximum absorbance values of MR solutions at each of the tested pH values (3.0, 5.0, or 7.0) was determined. The absorbance values at the wavelength found for maximum absorbance were used to draw the standard curve and to deduce its standard equation **(Helal, A-S. A-H. Personal communication).**

Degradation of MR using MR as a sole source of carbon:

***Inoculum preparation*:** Two mL of fresh bacterial suspension in nutrient broth were centrifuged at 10,000 rpm using cooling centrifuge 4.0℃ for 15 min. The supernatant was removed, and the bacterial pellet was washed 3.0 times with sterile phosphate buffer saline (PBS) (pH 7.0) then resuspended in 2.0 mL PBS to obtain 0.5 absorbance at λ_{600} nm ($A°_{600}$) **(Sarkar *et al*., 2011)**.

Two mL of inoculum were added to 100 mL BH medium containing 100 mg MR/L in 250 mL conical flask then incubated at 37℃ shaking at 120 rpm for 48 h. The bacterial growth rate and the percentage of MR degradation were calculated every 24 h in comparison with a control. The bacterial growth was evaluated by measuring the absorbance ($A°_{600}$).

The concentration of MR at any specific time was determined by withdrawing 2.0 mL of the bacterial culture and were centrifuged at 10,000 rpm for 15 min. The pH value of cell-free medium was measured by a pH meter, then suitably diluted by a phosphate buffered saline of the same pH. The absorbance of the diluted cell-free medium was measured using the UV-spectrophotometer **(Optima, Japan)** at the corresponding wavelength suitable for the detected pH value. The final concentration of MR was calculated from the standard equations which were deduced from the previously prepared standard curves. The percentage of MR degradation was calculated from the following formula stated by **Tripathi *et al.* (2016)**.

$$\text{Degradation } \% = \frac{C_0 - C_1}{C_0} \text{ x } 100$$

Where C_0 is initial concentration, and C_1 is final concentration of MR.

Factors affecting MR degradation by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates:

Two mL of fresh bacterial culture with $A°_{600}$ of 0.5 were added into 250 mL conical flask containing the specific medium (mentioned under each factor) and incubated for 6.0 h under the specified conditions. At the end of the incubation, both the bacterial growth and the percentage of MR degradation were measured and compared with an un-inoculated control medium **(Tripathi *et al.*, 2016)**.

1. Effect of initial pH value on MR degradation:

The pH of the M1 medium was adjusted to different pH values (5.0 to 8.0) with 1.0 N HCl or 1.0 N NaOH. This experiment was carried out at 37 °C and 120 rpm for 6.0 h using a shaking water bath **(Zhao *et al.*, 2014)**.

2. Effect of incubation temperature on MR degradation (Zhao *et al.*, 2014):

MI medium pH was adjusted to 7.0 and was incubated at either 25, 30, 35 or 40 ℃. The inoculated media were oscillated at 120 rpm for 6.0 h in a shaking water bath.

3. Effect of the incubation shaking speed on MR degradation (Zhao *et al.*, 2014):

MI medium pH 7.0 was incubated at 35℃ using different shaking rates i.e. 0.0, 50, 100 and 150 rpm for 6.0 h.

4. Effect of different added nitrogen sources on MR degradation (Zhao *et al.*, 2014):

Yeast extract (0.1 % w/v) and ammonium nitrate (0.1 % w/v) were added separately to MI medium. A medium without any added nitrogen source was also used as a control. All media were adjusted to pH 7.0 and incubated at 35℃ with shaking at 150 rpm for 6.0 h.

5. Effect of different added carbon sources on MR degradation(Zhao *et al.*, 2014):

Millipore filter (0.22 µm) sterilized solutions of glucose, maltose, sucrose or starch were added separately to MII medium at a final concentration of 1.0 % (w/v). These experiments were carried out at 35 ℃ with shaking at 150 rpm and incubated for 6.0 h.

6. Effect of metal salts on MR degradation (Zhao *et al.*, 2014):

Either one mM of $CaCl_2$, $MgSO_4$, $Pb(CH_3COO)_2$, $MnSO_4$ or $FeCl_3$ were added separately to an MIII medium. A medium without added metal salts was used under the same conditions for comparison. The media were shaken at 150 rpm at 35°C for 6.0 h.

Degradation of different concentrations of MR (Zhao *et al.*, 2014):

These experiments were carried out using 100 mL of MIV medium to which either 200, 300, 400, or 500 mg MR/L were added. The inoculated media were incubated at 35℃ shaking at 150 rpm for 48 h. The bacterial growth and percent of MR-degradation were evaluated every 12 h in comparison with the corresponding un-inoculated control.

Biodegradation of 100 mg/L of MR under optimum growth conditions:

This experiment was carried out using 100 mL of MIV medium to which 100 mg MR/L were added. The inoculated media were incubated at 35℃ with shaking at 150 rpm for 6.0 h. The bacterial growth and percent of MR-biodegradation were measured every 1.0 h in comparison with un-inoculated controls.

Analysis of MR degradation products produced by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates:

Extraction of MR degraded products from the growth media was carried out using ethyl acetate after incubation for 6.0 h as described by **Patil *et al.*, 2016**. The extracts were used as samples for FT-IR analysis.

1. Fourier transform infrared (FT-IR) spectroscopy of methyl red degraded products (Yaman and Durakli Velioglu, 2019):

The ethyl acetate extract residues were placed directly on the crystal diamond of Alpha II **(Bruker, Germany)** The samples were analyzed by attenuated total reflectance at mid IR range (400-4000 cm^{-1}).

2. Determination of Ultraviolet-visible (UV-vis) absorption of methyl red and its degraded products:

At the end of incubation time (6.0 h), 2.0 mL of the culture medium of *Acinetobacter baumannii* SM01 or *Klebsiella pneumoniae* SM27 were centrifuged at 10,000 rpm for 15 min. The supernatants were screened for their maximum absorbance wavelength using a double-beam UV-1800 spectrophotometer **(Shimadzu, Japan)** against a control medium **(Tripathi *et al.*, 2016)**.

Evaluation of the activity of some enzymes produced by the isolate *Acinetobacter baumannii* SM01 grown in MIV medium in presence of MR:

Acinetobacter baumannii SM01 was grown on MIV medium containing 100 mg/L MR and incubated at 35 °C with shaking at 150 rpm for 6.0 h. An identical control experiment was performed under the same conditions but without addition of MR.

1. Azoreductase activity:

The azoreductase activity was assayed by the method mentioned by **Nakanishi *et al.* (2001)** through measuring the decrease in optical density of methyl red at 430 nm using a Hewlett UV-visible spectrophotometer at room temperature. The reaction mixture (2.0 mL) contained 25 mM potassium phosphate buffer (pH 7·1), 25 µM MR, 0.1 mM NADPH and 100 µL of cell-free MIV growth medium of *Acinetobacter baumannii* SM01 mentioned above. The reaction was initiated by addition of the growth medium containing the produced enzyme. Initial velocity was determined by monitoring the change in the amount of MR substrate in the first 2.0 min in a glass cuvette of 1.0 cm light path. Several molar absorption coefficients were used (23 360 M^{-1} cm^{-1} for methyl red at 430 nm). One unit (U) of the detected enzyme activity was defined as the amount of enzyme required to degrade one µmol MR per minute. Proteins were quantified using the bicinchoninic acid assay (Pierce) with BSA as standard.

2. Determination of nicotinamide adenine dinucleotide hydrogen-2,6-dichlorophenol indophenol (NADH-DCIP) reductase activity (Zhao *et al.*, 2014):

The activities of NADH-dependent 2,6- dichlorophenol indophenol (NADH-DCIP) reductase involved in MR decolourization were assayed spectrophotometrically in the crude MIV cell-free growth medium of *Acinetobacter baumannii* SM01 isolate by monitoring the decrease in the DCIP concentration at 590 nm in a reaction mixture of 3.0 mL containing 50 µM DCIP, 50 mM potassium phosphate buffer (pH 7.4), 50 µM NADH and 100 µL of cell-free MIV growth medium of *Acinetobacter baumannii* SM01 mentioned above. One unit of enzyme activity was defined as a microgram of DCIP reduced/ min/mg of protein. The enzyme assay was conducted in triplicate, and the average rates were calculated to represent the enzyme activity.

3. Determination of laccase activity (Faraco *et al.*, 2009):

Spectrophotometric determination of laccase activity was carried out at 25ºC by measuring the oxidation of 2.0 mM 2,20-azino-bis(3-ethylbenzthiazoline- 6-sulphonic acid) (ABTS) in 100 mM phosphate-citrate buffer pH 4.0. The formation of cation radical $ABTS^{\bullet+}$ was followed by absorbance measured at 420 nm (ε_{420} = 36 mM^{-1} cm^{-1}) using an UV-Vis spectrophotometer. One enzyme unit (U) is defined as the amount of enzyme which oxidizes 1.0 µmol of substrate per minute in the assay conditions. The enzyme assay was conducted in triplicate, and the average rates were calculated to represent the enzyme activity.

4. Assay of tyrosinase activity (Rooseboom *et al*., 2004):

Tyrosinase activity was assayed using 15 mM L-DOPA and 2.0 mM/L. tyrosine as substrates. The reaction mixture was carried out in 0.1 M sodium phosphate buffer (pH 7.0) at 25°C. The reaction was initiated by addition of 10 µM of cell-free MIV growth medium of *Acinetobacter baumannii* SM01 mentioned above to the substrate solution. Dopachrome formation was monitored at 470 nm. The enzyme activity unit was expressed as the amount of enzyme required to oxidize 1.0 µM of the substrate in one min under standard assay conditions. Specific activity [U/mg] = enzyme activity [U/mL]/protein [mg].

Phytotoxicity study of MR and its biodegradation products:

The MR degradation products produced by the isolate *Acinetobacter baumannii* SM01 were extracted by ethyl acetate as described by **Patil *et al.* (2016)**. The ethyl acetate extract of MR degradation products and undegraded methyl red were tested for phytotoxicity on wheat seeds (*Triticum aestivum*) as described by **Zhao *et al.* (2014).** The germination percentages of the seeds were determined after one week and the shoot and root lengths of the germinated seeds were also measured and compared with control sterile distilled water. The percent of germination was calculated using the formula mentioned by **Lade *et al.* (2012).**

$$\textbf{Germination (\%)} = \frac{\textit{Number of seeds germinated}}{\textit{Number of seeds sowed}} \text{ X } \mathbf{100}$$

Biostatistics (Mouafi *et al.*, 2016):

Biostatistics of the results in this study were carried out by GraphPad Prism 8.0.2 using one way ANOVA, followed by Tukey-Kramer for multiple comparison.

RESULTS

Standard curves of MR:

Standard curve of MR solutions at pH 3.0 was used to determine unknown concentrations (X) of MR solutions of pH ≤ 4.4 by measuring the absorbance (Y) at λ520 nm and using the standard equation $X = 4.0453Y + 0.7427$. Whereas the standard curve of MR solutions at pH 5.0 was used to determine unknown concentrations (X) of MR solutions of pH 4.4-6.2 by measuring the absorbance (Y) at λ450 nm and using the equation X= 43.6681Y-13.3930. In addition, the standard curve of MR solutions at pH 7.0 was used to determine unknown concentrations (X) of MR solutions of pH ≥ 6.2 by measuring the absorbance (Y) at λ430 nm and using the equation $X = 42.0168Y - 12.0294$.

Screening and identification of MR-degrading isolates:

The twenty crude petroleum oil-degrading isolates were screened for their ability to degrade 100 mg methyl red/L MI medium. The visual observations showed that five

isolates were capable to induce a degree of decolourization on MR after 24 h. These isolates were identified as *Bacillus slashline* SM04, *Acinetobacter baumannii* SM01, *Achromobacter denitrificans* SM21, *Klebsiella pneumoniae* SM27 and *Stenotrophomonas maltophilia* SM28.

Further screening exhibited that, *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates were the most potent MR-degrading isolates since they completely (100%) degraded MR after 10 h of incubation. Whereas the isolates *Stenotrophomonas maltophilia* SM28, *Achromobacter denitrificans* SM21 and *Bacillus slashline* SM04 showed partial degradation of MR (51.8%, 12.2% and 1.8% respectively) after incubation for 10 h (Table 1).

Table (1): Biodegradation values of MR by *Acinetobacter baumannii* SM01, *Bacillus slashline* SM04, *Achromobacter denitrificans* SM21, *Klebsiella pneumoniae* SM27 and *Stenotrophomonas maltophilia* SM28 isolates grown in MI medium.

Time (h) / Bacterial isolates	At zero time		2nd h		4th h		6th h		8th h		10th h	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
***Acinetobacter baumannii* SM01**	95.7	**0.0**	92.0	**3.9**	76	**20.6**	21.5	**77.5**	4.8	**95**	0.0	**100.0**
***Bacillus slashline* SM04**	95.7	**0.0**	95.0	**0.7**	95.0	**0.7**	94.0	**1.8**	94.0	**1.8**	94.0	**1.8**
***Achromobacter denitrificans* SM21**	95.7	**0.0**	95.0	**0.7**	95.0	**0.7**	94.0	**1.8**	90.0	**6.0**	84.0	**12.2**
***Klebsiella pneumoniae* SM27**	95.7	**0.0**	87.0	**9.1**	71.0	**25.8**	16.1	**83.2**	2.2	**97.7**	0.0	**100.0**
***Stenotrophomonas maltophilia* SM28**	95.7	**0.0**	95.0	**0.7**	95.0	**0.7**	77.2	**19.3**	67.9	**29.0**	46.1	**51.8**

C_{MR} (concentration of MR mg/L); D%(Percent of Degradation wt./vol.)

Phylogenetic analysis of SM01 and SM27 isolates:

The sequence blast showed that SM01 isolate had a high similarity (97.5%) to *Acinetobacter baumannii* strain st10 (MF102141.1), 97.49% similarity to *Acinetobacter baumannii* strain 309-14 (MG557814.1), 97.4% similarity to *Acinetobacter baumannii* strain TERI SID6 (KX822160.1) and 97.41% similarity to *Acinetobacter baumannii* strain AIMST.Pbst1 (KM087101.1). Phylogenetic analysis of 16S rRNA gene sequence of the SM01 isolate showed that, it was closely related to *Acinetobacter baumannii* strain st10. The SM01 isolate was definitely identified as *Acinetobacter baumannii* and submitted to Genbank with accession number MT444986.1 (Figure 2).

The sequence blast showed that SM27 isolate had a high similarity (99.3%) to *Klebsiella pneumoniae* strain KP1553 (MK386778.1), 99.2% similarity to *Klebsiella* spp. strain InAD-063 (MF401269.1), 99.2% similarity to *Klebsiella pneumoniae* strain ZG26 (MF767582.1), 99.2% similarity to *Klebsiella pneumoniae* strain YYR54-1 (MF767579.1). Phylogenetic analysis of 16S rRNA gene sequence of the SM27 isolate showed that, it was closely related to *Klebsiella pneumoniae* strain KP1553 (Figure 3).

The SM27 isolate was definitely identified as *Klebsiella pneumoniae* and submitted to Genbank with accession number MT444988.1.

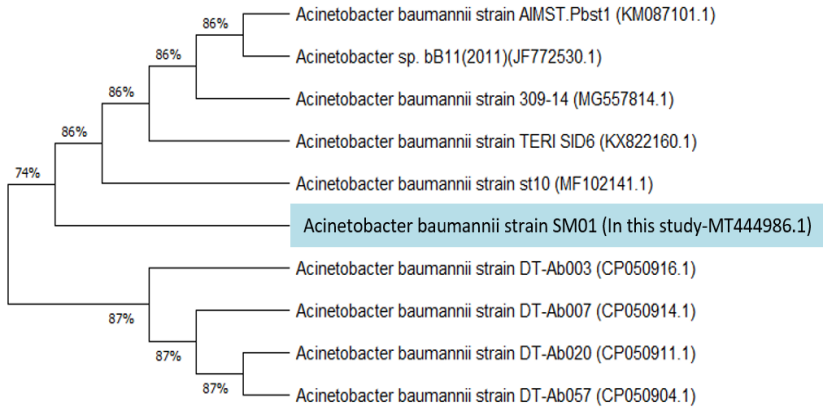

Figure (2): Phylogenetic tree of *Acinetobacter baumannii*, i.e. the SM01 isolate based on 16S rRNA gene sequence analysis. Genbank accession numbers are given in parentheses.

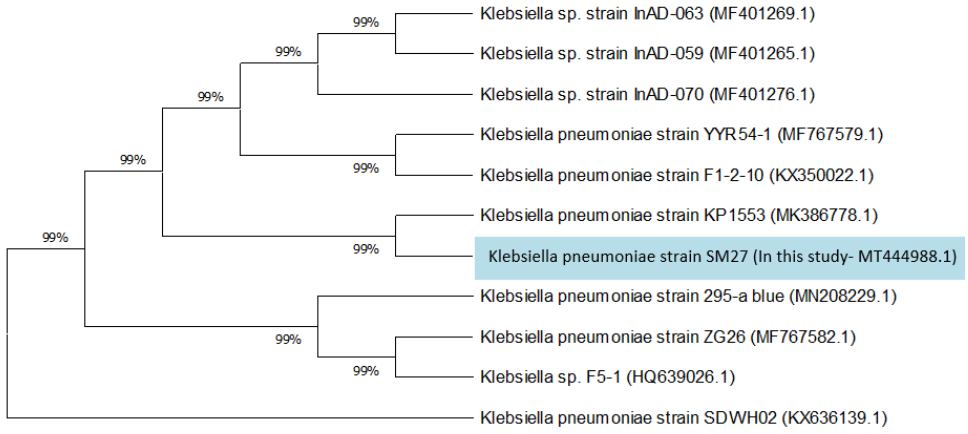

Figure (3): Phylogenetic tree of *Klebsiella pneumoniae*, i.e. the SM27 isolate based on 16S rRNA gene sequence analysis. Genbank accession numbers are given in parentheses.

Utilization of MR as a sole source of carbon by the isolates *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 grown in BH medium containing 100 mg MR/L:

Both *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates were able to utilize MR as a sole source of carbon showing slow growth rates (Figure 4). *Acinetobacter baumannii* SM01 completely degraded (100%) MR after incubation for 48 h whereas *Klebsiella pneumoniae* SM27 only degraded 65 % of MR (Table 2).

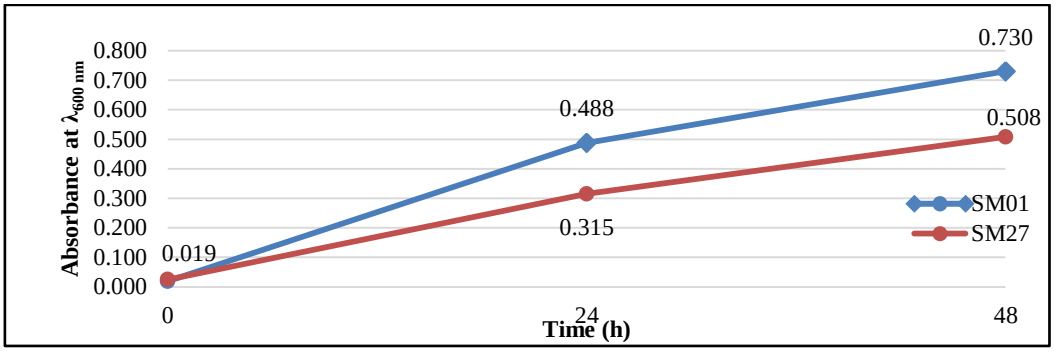

Figure (4): Growth rates of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates in BH medium using MR (100 mg/L) as a sole source of carbon.

Table (2): Biodegradation values of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 on using MR as a sole source of carbon 100 mg/L in BH medium.

Time (h) / Bacterial isolates	At zero time		24 h		48 h	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
***A. baumannii* SM01**	95.0	**0.0**	56.7	**40.3**	0.0	**100.0**
***K. pneumoniae* SM27**	95.0	**0.0**	85.6	**9.9**	33.2	**65.0**

C_{MR} (Concentration of MR mg/L); D%(Percent of Degradation wt./vol.)

Factors affecting MR biodegradation by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates:

1. Effect of initial pH on MR degradation:

Acinetobacter baumannii SM01 and *Klebsiella pneumoniae* SM27 isolates exhibited their maximal growth density in presence of MR (MI medium) at initial pH 8.0, followed descendingly by pH 7.0, 6.0 and 5.0 after 6.0 h of incubation (Figure 5).

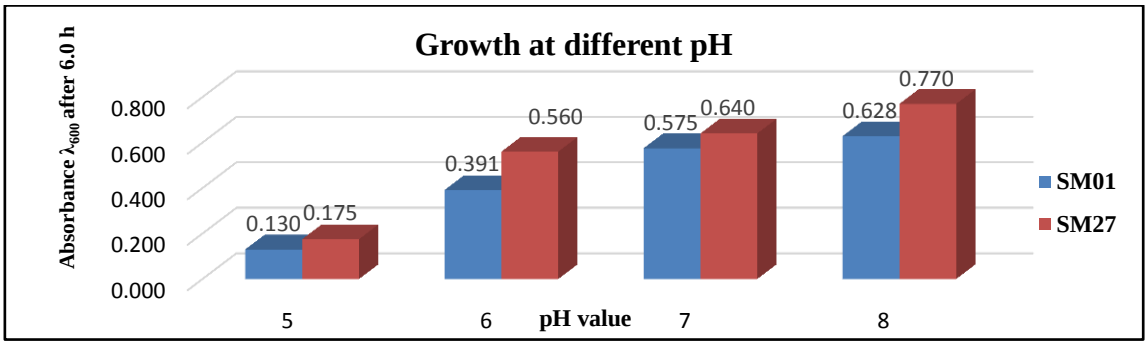

Figure (5): Growth densities of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 in presence of MR (MI medium) at different initial pH values after 6.0 h of incubation.

As shown in Table (3), *Acinetobacter baumannii* SM01 isolate degraded 44.2 % of MR at initial pH 8.0, 77.8 % at initial pH 7.0, 56.8 % at initial pH 6.0 whereas only 1.1 % of MR was degraded at initial pH 5.0 after 6.0 h of incubation. Similarly, *Klebsiella pneumoniae* SM27 was capable of degrading 52.8 % of MR at initial pH 8.0, 83.4 % at initial pH 7.0, 43.2 % at initial pH 6.0, whereas only 2.1 % of MR was degraded at initial pH 5.0 after 6.0 h of incubation.

Table (3): Biodegradation values of MR at different pH values by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 grown in MI medium.

Initial pH values / Bacterial isolates	5.0		6.0		7.0		8.0	
	C_{MR} (Final pH, Absorbance, dilution factor)	D%	C_{MR} (Final pH, Absorbance, dilution factor)	D%	C_{MR} (Final pH, Absorbance, dilution factor)	D%	C_{MR} (Final pH, Absorbance, dilution factor)	D%
Control	94.0 (5.0, 0.523, 1/10)	-	95.0 (6.0, 0.525, 1/10)	-	97.0 (7.0, 0.512, 1/10)	-	93.2 (8.0, 0.507, 1/10)	-
***A. baumannii* SM01**	93.0 (4.9, 0.520, 1/10)	**1.1**	41.0 (5.4, 0.400, 1/10)	**56.8**$^{(a)}$	21.5 (5.77, 0.800, 0.0)	**77.8**$^{(a, b)}$	52.0 (6.5, 0.411, 1/10)	**44.2**$^{(a, b, c)}$
***K. pneumoniae* SM27**	92.0 (4.7, 0.517, 1/10)	**2.1**	54.0 (4.5, 0.430, 1/10)	**43.2**$^{(a)}$	16.1 (6.7, 0.670, 0.0)	**83.4**$^{(a, b)}$	44.0 (6.3, 0.390, 1/10)	**52.8**$^{(a, b, c)}$

C_{MR} (concentration of MR mg/L); D%(Percent of Degradation wt./vol.)

Data are presented as means

a= Significantly different from pH=5.0 at $P \leq 0.05$; ***b***= Significantly different from pH=6.0 at $P \leq 0.05$; ***c***= Significantly different from pH=7.0 at $P \leq 0.05$ using one way ANOVA, followed by Tukey-Kramer for multiple comparison.

2. Effect of incubation temperature on MR degradation:

Acinetobacter baumannii SM01 and *Klebsiella pneumoniae* SM27 exhibited their ability to grow at a wide range of incubation temperatures (25-40℃) however their maximum growth density was at 35℃ ($A°_{600}$ ~ 0.630 and 0.670 respectively) (Figure 6).

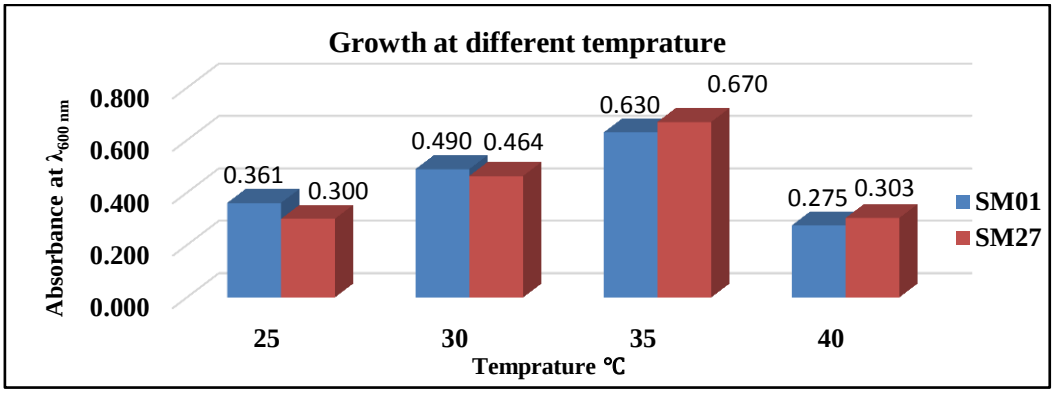

Figure (6): Growth densities of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates in presence of MR (MI medium) at different temperatures after 6.0 h of incubation.

Acinetobacter baumannii SM01 showed the maximum MR-degradation 80.7% at 35 ℃ followed in a descending order by 51.3 % at 30 ℃, 20.2 % at 25 ℃ and the least percent of MR degradation was at 40 ℃ (Table 4). On the other hand, *Klebsiella pneumoniae* SM27 isolate degraded 89.8 % of MR at 35℃, followed in a descending order by 37.8 % at 40 ℃, 35.8 % at 30 ℃ and 33.0% at 25 ℃ (Table 4).

Table (4): Biodegradation values of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates grown in MI medium at different incubation temperatures for 6.0 h.

Temperature (℃) / Bacterial isolates	25 ℃		30 ℃		35 ℃		40 ℃	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
MR Control	96.5	-	96.5	-	96.5	-	96.5	-
***A. baumannii* SM01**	77.0	**20.2**	47.0	**51.3**$^{(a)}$	18.6	**80.7**$^{(a, b)}$	90.0	**6.7**$^{(a, b, c)}$
***K. pneumoniae* SM27**	64.6	**33.0**	62.0	**35.8**$^{(d)}$	9.8	**89.8**$^{(a, b)}$	60.0	**37.8**$^{(a, c, e)}$

C_{MR} (Concentration of MR mg/L); D%(Percent of Degradation wt./vol.)

Data are presented as means.

a= Significantly different from 25 ℃ at $P \leq 0.05$; ***b***= Significantly different from 30 ℃ at $P \leq 0.05$; ***c***= Significantly different from 35 ℃ at $P \leq 0.05$; ***d***= Not significantly different from 25 ℃ at $P \leq 0.05$; ***e***= Not significantly different from 30 ℃ at $P \leq 0.05$ using one way ANOVA, followed by Tukey-Kramer for multiple comparison.

3. Effect of shaking speed on MR degradation:

Increasing the agitation rate resulted in increasing the growth density of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates in MI medium, and the maximum growth density occurred at 150 rpm shaking speed (Figure 7). Biodegradation of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 at a static incubation (zero rpm) were significantly low i.e. 29.5% and 37.8% respectively. While gradual increase of shaking speed resulted in gradual increases in MR-biodegradation by both isolates until maximum degradation values of

86.0 and 86.3 % by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 respectively were attained at 150 rpm shaking speed (Table 5).

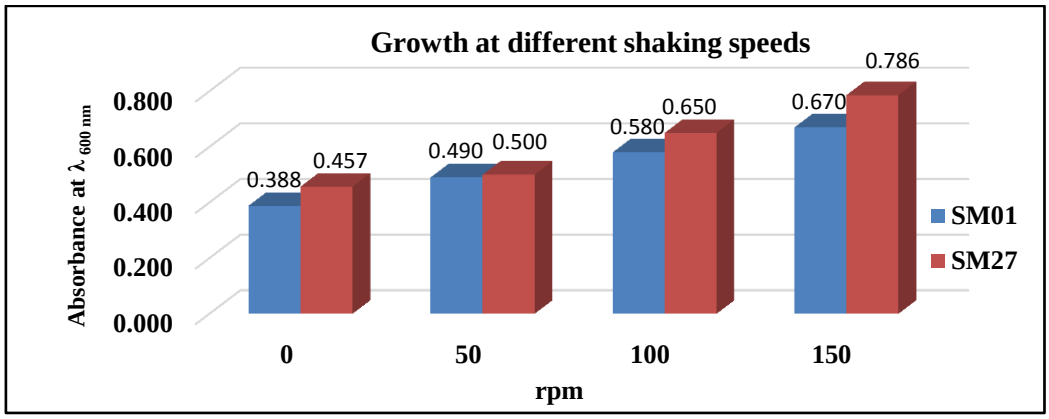

Figure (7): Growth densities of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates in presence of MR (MI medium) at different shaking speeds after 6.0 h of incubation.

Table (5): Biodegradation values of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates by incubation for 6.0 h at different shaking speeds.

Shaking speed (rpm) / Bacterial isolates	0 rpm		50 rpm		100 rpm		150 rpm	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
Control	96.5	-	96.5	-	96.5	-	96.5	-
***A. baumannii* SM01**	68.0	**29.5**	26.0	**73.1**$^{(a)}$	20.5	**78.8**$^{(a, b)}$	13.5	**86.0**$^{(a, b, c)}$
***K. pneumoniae* SM27**	60.0	**37.8**	49.0	**49.2**$^{(a)}$	24.6	**74.5**$^{(a, b)}$	13.2	**86.3**$^{(a, b, c)}$

C_{MR} (concentration of MR mg/L); D%(Percent of Degradation wt./vol.).

Data are presented as means

a= Significantly different from zero rpm at $P \leq 0.05$; ***b***= Significantly different from 50 rpm at $P \leq 0.05$; ***c***= Significantly different from 100 rpm at $P \leq 0.05$ using one way ANOVA, followed by Tukey-Kramer for multiple comparison.

4. Effect of different added nitrogen sources in growth media on MR degradation:

Either yeast extract or ammonium nitrate added to MI medium resulted in increasing the growth rate of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 as compared to their growth in media free from any added nitrogen source. Their maximum growth occurred in presence of yeast extract (Figure 8).

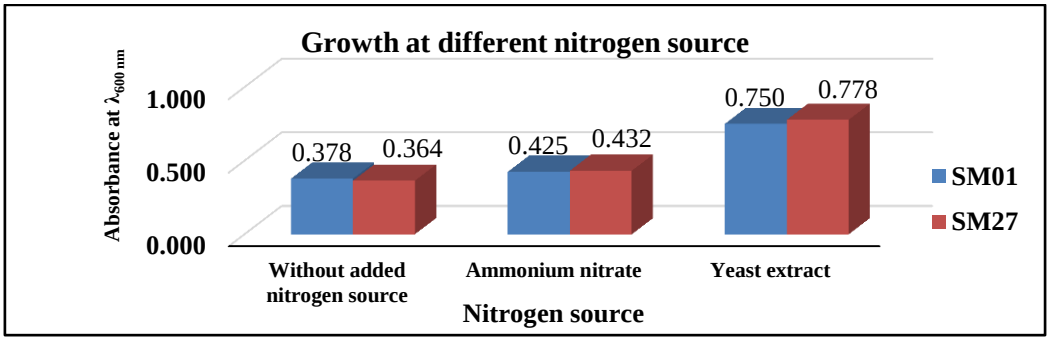

Figure (8): Growth densities of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 in presence of MR (MI media) and different added nitrogen sources after 6.0 h of incubation.

Addition of 0.1 % (w/v) yeast extract enhanced MR-degradation values to 87.4% and 84.5% respectively. While addition of 0.1% (w/v) ammonium nitrate to MI medium increased MR degradation values exhibited by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 at relatively low extents (63.4 % and 67.5 % respectively) (Table 6). *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 degraded MR at lower extents (32.0 % and 38.1 % respectively) in absence of any added nitrogen source (Table 6).

Table (6): Biodegradation values of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 on growing in presence of different added nitrogen sources.

Nitrogen source / Bacterial isolates	Without added nitrogen source		Ammonium nitrate		Yeast extract	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
MR Control	97.0	-	97.0	-	97.0	-
***A. baumannii* SM01**	66.0	**32.0**	35.5	**63.4**$^{(a)}$	12.2	**87.4**$^{(a, b)}$
***K. pneumoniae* SM27**	60.0	**38.1**	31.5	**67.5**$^{(a)}$	15.0	**84.5**$^{(a, b)}$

C_{MR} (concentration of MR mg/L); D%(Percent of Degradation wt./vol.).
Data are presented as means.

a= Significantly different from medium without added nitrogen source at $P \leq 0.05$; ***b***= Significantly different from ammonium nitrate at $P \leq 0.05$ using one way ANOVA, followed by Tukey-Kramer for multiple comparison.

5. Effect of different added carbon sources on MR degradation:

Both *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 were able to grow well in MII medium in presence of different added carbon sources (glucose, maltose, sucrose or starch). Their maximum growth occurred in presence of 1.0 % (w/v) glucose (Figure 9).

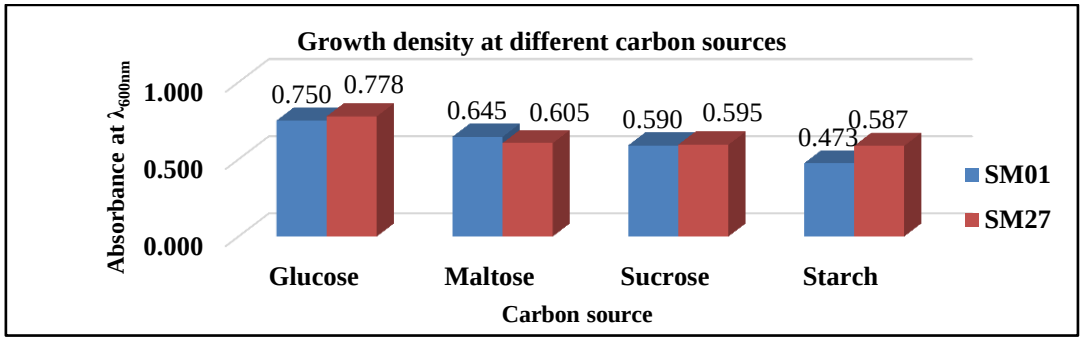

Figure (9): Growth densities of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 in presence of MR in MII medium with different added carbon sources after 6.0 h of incubation.

Acinetobacter baumannii SM01 utilized glucose as the most efficient carbon source on which MR degradation reached 87.4 % followed in a descending order by maltose (77.7%), sucrose (70.1%) and starch (56.1%) (Table 7). Similarly, *Klebsiella pneumoniae* SM27 was able to degrade MR with maximum efficiency in presence of glucose (84.5%), followed in a descending order by maltose (81.4%), sucrose (67.5%) and starch (49.5%) as shown in Table (7).

Table (7): Biodegradation values of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 on growing on MII medium in presence of different added carbon sources.

Carbon source / Bacterial isolates	Glucose		Maltose		Sucrose		Starch	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
Control MR	97.0	-	97.0	-	97.0	-	97.0	-
***A. baumannii* SM01**	12.2	**87.4**	21.6	**77.7**$^{(a)}$	29.0	**70.1**$^{(a, b)}$	42.6	**56.1**$^{(a, b, c)}$
***K. pneumoniae* SM27**	15.0	**84.5**	18.0	**81.4**$^{(a)}$	31.5	**67.5**$^{(a, b)}$	49.0	**49.5**$^{(a, b, c)}$

C_{MR} (concentration of MR mg/L); D%(Percent of Degradation wt./vol.)

Data are presented as means.

a= Significantly different from glucose at $P \leq 0.05$;***b***= Significantly different from maltose at $P \leq 0.05$;***c***= Significantly different from sucrose at $P \leq 0.05$ using one way ANOVA, followed by Tukey-Kramer for multiple comparison.

6. Effect of different metal salts added to MIII medium on MR degradation:

Degradation of MR by *Acinetobacter baumannii* SM01 in absence of any metal salts reached 73.7%. Whereas addition of 1.0 mM $CaCl_2$ resulted in the highest increase of MR degradation by *Acinetobacter baumannii* SM01 to 92.3 % followed in a descending order by 1.0 mM $FeCl_3$ (86.7%) and 1.0 mM $MgSO_4$ (84.7%). On the other hand, both 1.0 mM $Pb(CH_3COO)_2$ and 1.0 mM $MnSO_4$ decreased MR degradation to 66.0 % and 62.9 % respectively (Table 8). *Klebsiella pneumoniae* SM27 isolate degraded MR up to 84.5% of its initial concentration in absence of any added metal. One mM $CaCl_2$ enhanced MR biodegradation up to 93.1%, followed in a descending

order by 1.0 mM $FeCl_3$ (91.2%) and 1.0 mM $MgSO_4$ (86.2%). On the other hand, both 1.0 mM $Pb(CH_3COO)_2$ and 1.0 mM $MnSO_4$ decreased MR degradation to 59.3% and 73.1% respectively (Table 8).

Table (8): Biodegradation values of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 grown in MIII medium in presence of 1.0 mM of different types of metal ions.

Metal salts / Bacterial isolates	Medium without metal		$CaCl_2$		$MgSO_4$		$Pb(CH_3COO)_2$		$MnSO_4$		$FeCl_3$	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
Control	97.0	-	97.0	-	97.0	-	97.0	-	97.0	-	97.0	-
***A. baumannii* SM01**	25.5	**73.7**	7.5	**92.3** (a)	14.8	**84.7** (a, b)	33.0	**66.0** (a, b, c)	36.0	**62.9** (a, b, c, g)	12.9	**86.7** (a, b, d, e, f)
***K. pneumoniae* SM27**	15.0	**84.5**	6.7	**93.1** (a)	13.4	**86.2** (a, b)	39.5	**59.3** (a, b, c)	26.1	**73.1** (h ,b, c, d)	8.5	**91.2** (a, c, d, e, i)

C_{MR} (concentration of MR mg/L); D%(Percent of Degradation wt./vol.).

Data are presented as means.

a= Significantly different from medium without metal at $P \leq 0.05$; ***b***= Significantly different from $CaCl_2$ at $P \leq 0.05$; ***c***= Significantly different from $MgSO_4$ at $P \leq 0.05$;***d***= Significantly different from $Pb(CH_3COO)_2$ at $P \leq 0.05$; ***e***= Significantly different from $MnSO_4$ at $P \leq 0.05$; ***f***= Not significantly different from $MgSO_4$ at $P \leq 0.05$; ***g***= Not significantly different from $Pb(CH_3COO)_2$ at $P \leq 0.05$; ***h***= Not significantly different from medium without metal at $P \leq 0.05$; ***i***= Not significantly different from $CaCl_2$ at $P \leq 0.05$ using one way ANOVA, followed by Tukey-Kramer for multiple comparison.

Degradation of different concentrations of MR in MIV medium:

Acinetobacter baumannii SM01 could tolerate up to 500 mg/L of MR, the growth rate of *Acinetobacter baumannii* SM01 was enhanced by increasing the concentration of MR in MIV growth medium from 200 mg/L to 300 mg/L. The growth rate decreased on increasing MR concentration to 400 mg/L and deteriorated on increasing it to 500 mg MR/L (Figure 10). This isolate was able to completely degrade 100 % of 200 mg/L and 300 mg/L of MR after 12 h and 400 mg/L after 24 h of incubation. Whereas it was only able to degrade 8.4 % of the 500 mg MR/L after 48 h (Table 9).

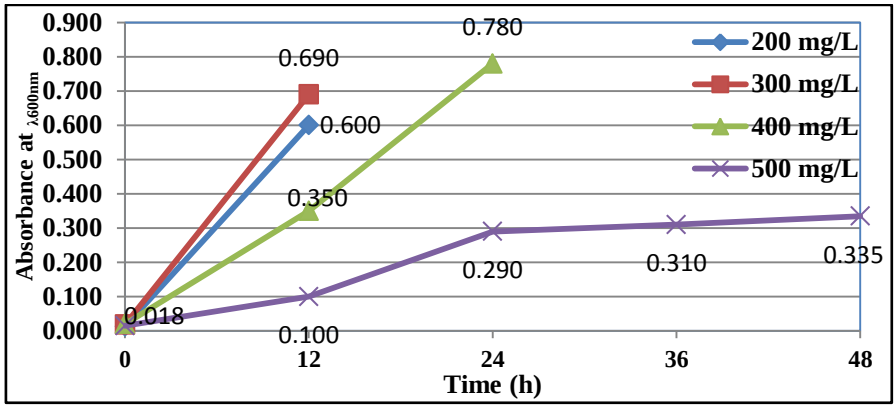

Figure (10): Growth rates of *Acinetobacter baumannii* SM01 in MIV medium in presence of different concentrations of MR.

Table (9): Biodegradation rates of different concentrations of MR in MIV medium by *Acinetobacter baumannii* SM01.

Time (h) / MR Conc. (mg/L)	Zero time		12 h		24 h		36 h		48 h	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
200	199.0	**0.0**	0.0	**100.0**	-	-	-	-	-	-
300	297.0	**0.0**	0.0	**100.0**	-	-	-	-	-	-
400	398.0	**0.0**	130.0	**67.3**	0.0	**100.0**	-	-	-	-
500	499.0	**0.0**	495.0	**0.8**	478.0	**4.2**	478.0	**4.2**	457.0	**8.4**

C_{MR} (concentration of MR mg/L); D%(Percent of Degradation wt./vol.)

Klebsiella pneumoniae SM27 isolate was able to grow in MIV medium in presence of MR concentrations up to 500 mg/L. Its growth rate declined with increasing the concentration of MR in the growth medium (Figure 11). This isolate completely degraded (100%) of 200 and 300 mg MR/L after 24 h and 36 h of incubation respectively. It degraded 78.9% of 400 mg MR/L after 48 h. Whereas it was able to degrade 500 mg MR/L at a very low rate of 0.8% after 48 h of incubation (Table 10).

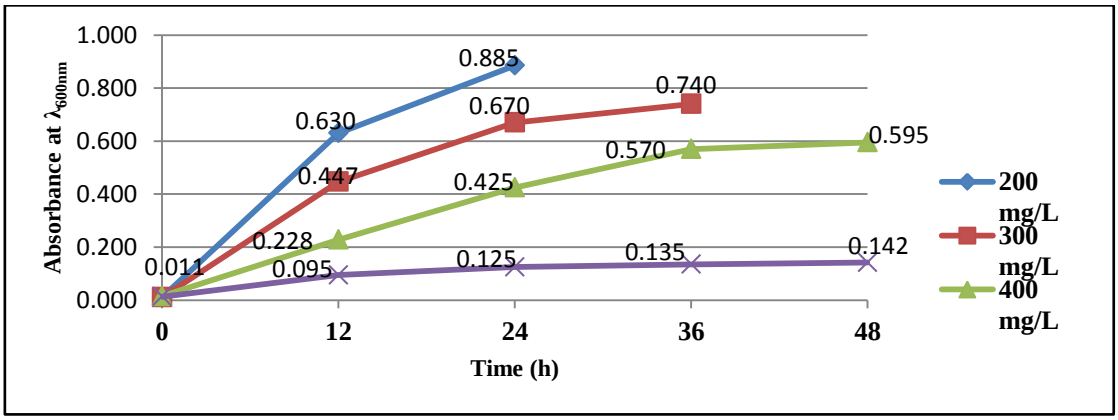

Figure (11): Growth rates of *Klebsiella pneumoniae* SM27 in MIV medium in presence of different concentrations of MR.

Table (10): Degradation rates of different concentrations of MR in MIV medium by *Klebsiella pneumoniae* SM27.

Time (h) / MR (mg/L)	Zero		12 h		24 h		36 h		48 h	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
200	199.0	**0.0**	22.5	**88.7**	0.0	**100.0**	-	-	-	-
300	297.0	**0.0**	226	**23.9**	95.6	**67.8**	0.0	**100.0**	-	-
400	398.0	**0.0**	325.6	**18.2**	275.2	**30.9**	164.0	**58.8**	84.0	**78.9**
500	499.0	**0.0**	495.0	**0.8**	495.0	**0.8**	495.0	**0.8**	495.0	**0.8**

C_{MR} (Concentration of MR mg/L); D%(Percent of Degradation wt./vol.)

Biodegradation of 100 mg/L of MR in MIV medium by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates under the deduced optimum growth conditions:

Under the optimum growth conditions (pH 7.0, 35℃, 150 rpm, 1.0% (w/v) of glucose, 0.1% (w/v) of yeast extract and 1.0 mM of $CaCl_2$) deduced in this study, *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates were able to grow well in presence of MR (Figure 12), and were capable to completely degrading (100 %) 100 mg MR/L after incubation for 6.0 h (Table 11).

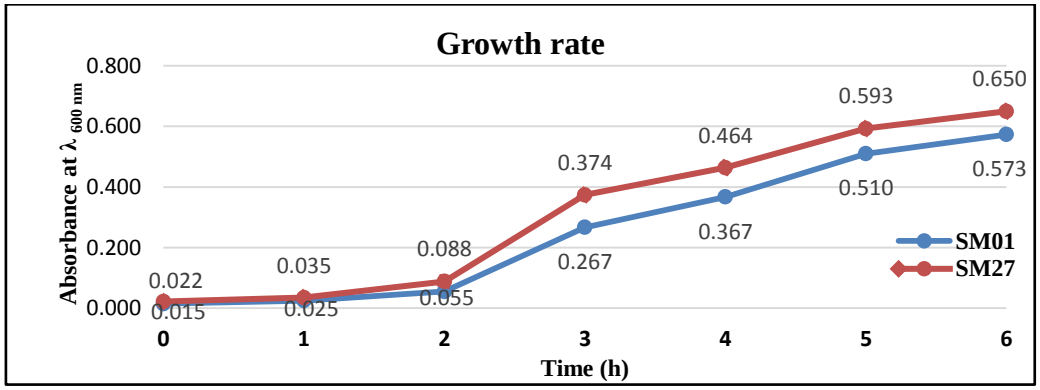

Figure (12): Growth curves of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 in presence of 100 mg MR/L of MIV medium under the deduced optimum growth conditions.

Table (11): Rates of degradation of 100 mg MR/L of MIV medium by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates under the deduced optimum growth conditions.

Time (h) / Bacterial Isolates	Zero		1st h		2nd h		3rd h		4th h		5th h		6th h	
	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%	C_{MR}	D%
***A. baumannii* SM01**	98.6	**0.0**	96.1	**2.5**	80.0	**18.9**	71.3	**27.7**	47.3	**52.0**	10.1	**89.8**	0.0	**100.0**
***K. pneumoniae* SM27**	98.6	**0.0**	97.8	**0.8**	84.4	**14.4**	83.1	**15.7**	62.6	**36.5**	16.14	**83.6**	0.0	**100.0**

C_{MR} (concentration of MR mg/L); D%(Percent of Degradation wt./vol.).

Analysis of methyl red degradation products obtained by the effects of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates:

1. Spectrophotometric analysis of MR degradation products:

The spectrum profile of control MR showed characteristic absorption peak in the visible region (λ_{max} = 430 nm) for methyl red (Figure 13A). Whereas the culture supernatant of the isolates *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 at the end of incubation period (6.0 h) in presence of 100 mg MR/L MIV medium showed complete disappearance of the peak at 430 nm and a new major peak appeared in UV region (200-300 nm) with a maximum absorption at λ_{max} of 252 nm (Figure 13B).

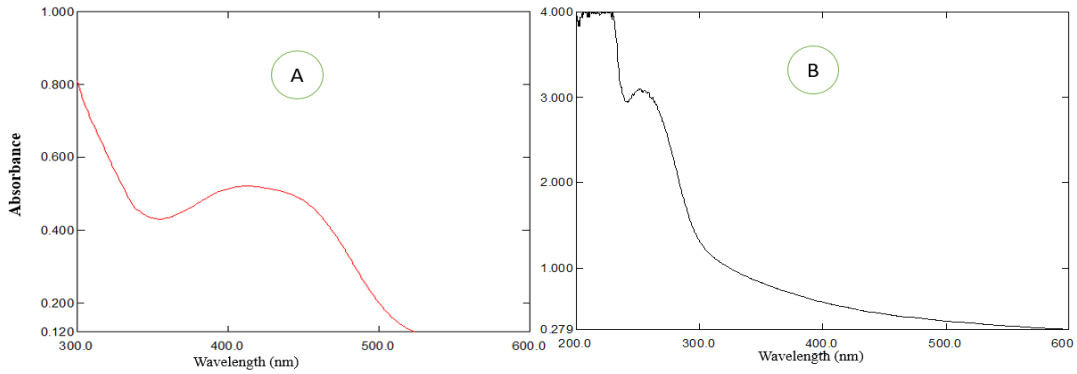

Figure (13): UV-visible spectrum of (A) control methyl red (λ_{max} 430 nm) and (B) MR degradation products by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 (λ_{max} 252 nm) after 6.0 h of incubation.

2. Analysis of MR-degradation products by Fourier transform infrared (FT-IR):

The FT-IR spectra obtained from culture supernatant of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 after 6.0 h exhibited significant changes in position of signals when compared to control MR confirming its degradation. The spectrum of control MR displayed a signal at 1598 cm^{-1} for azo bond (-N=N-), a signal at 1705 cm^{-1} for carbonyl (C=O) of carboxylic group, a signal at 1274 cm^{-1} for C-N stretch of aromatic amines, a signal at 1483 cm^{-1} designates C-C stretch in aromatics and a signal at 940 cm^{-1} for O-H bend of carboxylic acid that confirmed the

structure of MR (Figure 14). Whereas The analysis of FT-IR of MR degradation products by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 revealed that; absence of a signal at 1598 cm^{-1} indicates breakdown of azo bond. The absence of signals at 686 cm^{-1}, 725 cm^{-1}, and 764 cm^{-1} indicates loss of aromaticity, lack of peak at 1274 cm^{-1} represents absence of C-N stretch of aromatic amines. Disappearance of carbonyl signals (1705 cm^{-1}) means that the carboxylic group is no longer available. The appearance of a signal at 1375 cm^{-1} indicated CH2, CH3 bending. Prominent signals at 2852cm^{-1} and 2922 cm^{-1} are for CH aliphatic (Figures 15 and 16).

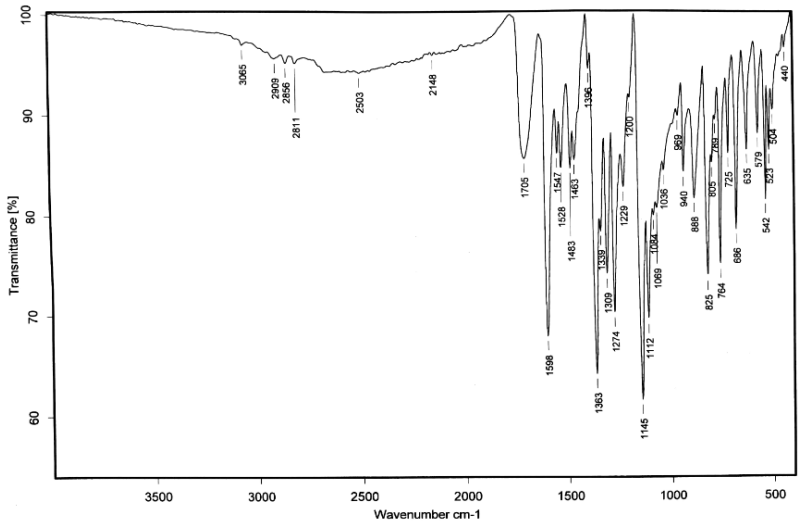

Figure (14): FT-IR spectrum of methyl red.

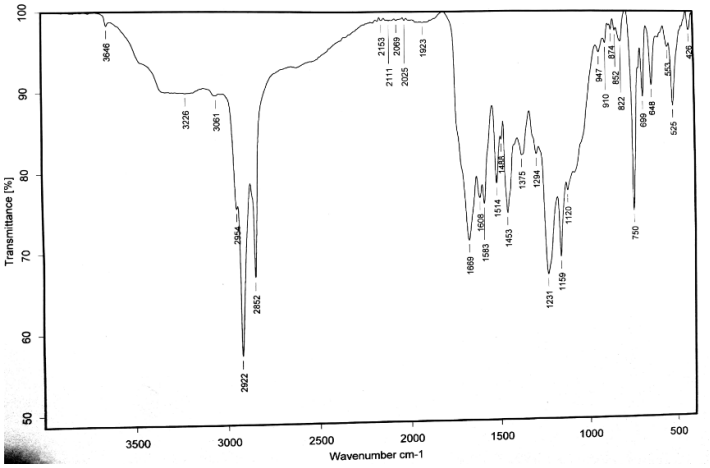

Figure (15): FT-IR spectrum of MR degradation products by *Acinetobacter baumannii* SM01 after 6.0 h of incubation.

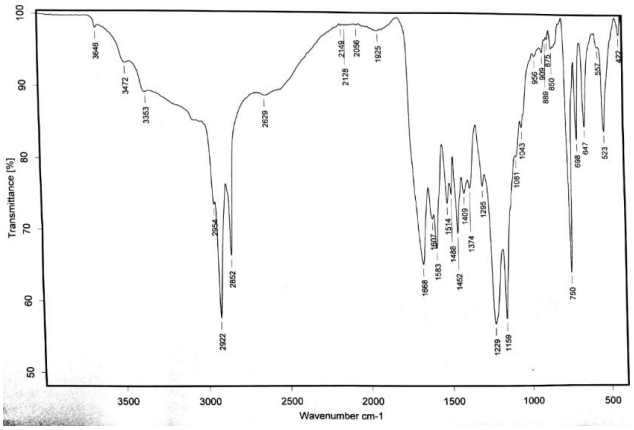

Figure (16): FT-IR spectrum of MR degradation products by *Klebsiella pneumoniae* SM27 after 6.0 h of incubation.

Analysis of enzymes involved in MR degradation by the isolate *Acinetobacter baumannii* SM01:

The results in Table (12) indicate significant ($p<0.001$) increases of enzymatic activity of azoreductase, NADH-DCIP reductase, laccase and tyrosinase in the MR-containing solution compared to the control (MR-free) medium. The cell-free medium containing MR exhibited higher azoreductase activity (7.74 µg MR reduced/ mg of protein/min) compared to control medium (1.57 µg MR reduced/ mg of protein/min). As for NADH-DCIP reductase, the enzymatic activity was higher in MR-containing solution (27.52 µg DCIP reduced/mg of protein/min) compared to control solutions (22.98 µg DCIP reduced/mg of protein/min). Similarly, the activity of laccase was higher in MR-containing solution (2.29 U/mg of protein/min) compared to control one (0.93 U/mg of protein/min). Also the activity of tyrosinase was higher in MR-containing solution (6.11 U/mg of protein/min) in comparison to that of the control (1.77 U/mg of protein/min). All of the four enzymes are important candidates for degradation of methyl red.

Table (12): Activity of oxidoreductase enzymes produced by *Acinetobacter baumannii* SM01 isolate grown in MIV medium in presence and absence of MR.

Sample / Enzyme	Enzyme activity of control grown without MR	Enzyme activity in presence of MR
Azoreductase (µg MR reduced/mg of protein/min)	1.57±0.05	7.74±0.04
NADH-DCIP reductase (µg DCIP reduced/mg of protein/min)	22.98±0.40	27.52±0.37
Laccase (U/mg of protein/min)	0.93±0.04	2.29±0.06
Tyrosinase (U/mg of protein/min)	1.77±0.04	6.11±0.06

The data are means of triplicate experiments ± SE. Significantly different from control sample at $p\leq0.001$ by two-tailed P values comparison.

Phytotoxicity studies of MR-degradation products of *Acinetobacter baumannii* SM01 isolate:

The outcomes of phytotoxicity studies on seeds of *Triticum aestivum* indicated that undegraded MR was more toxic to crop plant seeds, as it showed 30 % inhibition of germination rate, compared to MR degradation metabolites produced by *Acinetobacter baumannii* SM01 which inhibited the germination rate by only 10 %. MR sample decreased root and shoot length by 42.4 % and 38.3 % respectively whereas MR degradation metabolites decreased root and shoot length by 27.8 % and 23.9 % respectively (Table 13).

Table (13): Phytotoxicity tests of MR and its degradation metabolites produced by *Acinetobacter baumannii* SM01 on wheat (*Triticum aestivum*) seeds.

Parameters studied		Seeds of wheat (*Triticum aestivum*)		
		Water (Control)	Undegraded MR	Degradation metabolites of MR
Germination rate (%)	Number of germinated seeds	10/10	7/10	9/10
	Percent of germination	100 %	70 %	90%
	Percent of inhibition of germination	0.0 %	30 %	10 %
Root	Length (cm)	9.55 ±0.55	5.5 ± 0.53$^{(a)}$	6.9 ± 0.29$^{(a, b)}$
	Percent of root length (%)	100 %	57.6 %	72.2 %
	Percent of reduction of root length (%)	0.0 %	42.4 %	27.8 %
Shoot	Length (cm)	9.72 ± 0.53	6.0 ± 0.45$^{(a)}$	7.4± 0.42$^{(a, b)}$
	Percent of shoot length (%)	100 %	61.7%	76.1%
	Percent of reduction of shoot length (%)	0.0 %	38.3 %	23.9 %

Data are presented as means ± SD.

a= Significantly different from seeds treated with water at $P \leq 0.05$;***b***= Significantly different from seeds treated with undegraded MR at $P \leq 0.05$ using one way ANOVA, followed by Tukey-Kramer for multiple comparison.

DISCUSSION

The release of azo dyes containing effluent into the environment is of great concern due to its aesthetic value, toxicity, mutagenicity and carcinogenicity. Therefore, effluents from textile industries have serious environmental concern and the removal of dyes from effluent is necessary prior to their disposal **(Singh and Singh, 2017)**. Preliminary screening of MR-degrading isolates showed that, only five of the selected crude petroleum oil-degrading isolates i.e. *Acinetobacter baumannii* SM01, *Bacillus* slashline SM04, *Achromobacter denitrificans* SM21, *Klebsiella pneumoniae* SM27 and *Stenotrophomonas maltophilia* SM28 were capable of complete decolourization of 100 mg MR/L after incubation for 24 h. *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates were selected as the most efficient MR-degrading isolates since they exhibited complete (100%) biodegradation of MR after incubation for 10 h. Whereas, the isolates *Stenotrophomonas maltophilia* SM28, *Achromobacter denitrificans* SM21 and *Bacillus* slashline SM04 showed partial degradation of MR (51.8%, 12.2% and 1.8% respectively) after incubation for 10 h. These results are consistent with **Cui *et al*. (2014)** who reported that *Klebsiella* spp. strain Y3 which gave

the highest degradation ability was used for further study. However, **Patil *et al.* (2016)** selected *Bacillus circulans* NPP1 as potential dye decolourizing strain because it illustrated a rapid dye decolourization activity and a broad decolourization profile. The present results showed fair agreement with **Sari and Simarani (2019)** who reported that *Lysinibacillus fusiformis* strain W1B6 was the most efficient in decolourizing the MR where 94 % of decolourization was achieved within 4.0 h under aerobic condition.

The isolates *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 were able to utilise MR as sole source of carbon though they showed very slow growth rates. *Acinetobacter baumannii* SM01 completely (100%) degraded MR after incubation for 48h whereas *Klebsiella pneumoniae* SM27 degraded only 65 % of the initial concentration of MR after incubation for the same period of time. Similarly, **Sari and Simarani (2019)** found that low amount of dye decolourization by *Lysinibacillus fusiformis* W1B6 was observed in the medium on using MR as the sole source of carbon. That could indicate that there is difficulty in utilizing MR as a sole source of carbon and energy. **Sari and Simarani (2019)** explained that the low degradation rate was due to poor cell growth, and that the growth rate increased gradually maybe due to the absorption of MR and/or utilizing new by-product metabolite resulting from the reductive cleavage of azo bond for bacterial growth as a carbon and energy source.

The effects of physicochemical parameters, influencing biodegradation of MR, was performed based on adjustment of initial pH, incubation temperature, shaking speed, added nitrogen and carbon sources and metal salts.

Acinetobacter baumannii SM01 and *Klebsiella pneumoniae* SM27 isolates showed their maximum growth densities in presence of MR at initial pH 8.0 and their maximum MR degradation capabilities were at initial pH 7.0. This could indicate that the optimum activity of the enzyme system responsible for biodegradation of MR by these isolates was achieved at initial pH 7.0 of the medium.

The results of this study illustrated that, *Acinetobacter baumannii* SM01 degraded amounts of MR which varied from 44.2 % to 77.8% after incubation for 6.0 h at variable initial values from pH 6.0 to 8.0. The maximum degradation was 77.8 % at pH 7.0. Whereas *Klebsiella pneumoniae* SM27 was capable for degrading MR at a range of 43.2% to 83.4% after incubation for 6.0 h at initial pH range from 6.0 to 8.0, and its maximum degradation power was 83.4 % at pH 7.0. Meanwhile, there were significant decreases in MR degradation at initial pH 5.0 by *Acinetobacter baumannii* SM01 (1.1 %) and by *Klebsiella pneumoniae* SM27 (2.1%). This is explained by the inability of both isolates to grow in extreme acidic medium. These results show fair agreement with **Sari and Simarani (2019)** who mentioned that *Lysinibacillus fusiformis* W1B6 was capable of decolourizing MR from 90% to 97% within the pH range of 5.5 to 8.5 within 4.0 h and that the decolourization declined significantly at pH values of 5.0, 9.0, and 10 to 63%, 34%, and 30%, respectively. Similar results were obtained by **Sharma *et al.* (2016)** who reported that, *Aeromonas jandaei* SCS5 could decolourize (100 %) MR at pH 7.0 within 6.0 h and decolourized partially (60%) within 6.0 h at pH 8.0. However, **Zhao *et al*. (2014)** study showed that MR degradation by *Bacillus* spp. UN2 was higher in a pH range of 7.0 to 9.0 than under acidic conditions.

This means that MR is easily reduced at slight acidic and neutral pH, whereas extreme acidic and alkaline pH may deactivate reductive and oxidative enzymes.

The present results are inconsistent with those of **Jadhav *et al*. (2008)** study who reported that MR decolourization by *Galactomyces geotrichum* increased for the acidic pH values of 3.0 to 5.0 and decreased under alkaline conditions. This may be related to the optimum pH for the microbial growth.

Acinetobacter baumannii SM01 and *Klebsiella pneumoniae* SM27 could grow and degrade MR at incubation temperature range from 25℃ to 40℃. The maximum MR degradation by *Acinetobacter baumannii* SM01 was 80.7% and by *Klebsiella pneumoniae* SM27 was 89.8% at 35℃ after 6.0h of incubation. MR degradation by both isolates was significantly decreased at 40℃. These results are consistent with **Sharma *et al.* (2016)** who found that *Aeromonas jandaei* strain SCS5 was an effective MR decolourizing agent within a temperature range of 25 to 50℃, whereas the optimum temperature for MR decolourization was 35℃. They stated that at higher temperature (50℃), the decolourization rate was found to be lower. In addition, the present results show fair agreement with **Zhao *et al.* (2014)** who reported that *Bacillus* spp. strain UN2 could degrade more than 90% of MR in a temperature range of 20 to 40℃ within 6.0h, while only 45% degradation was achieved at 45℃.

On the other hand, the current results are inconsistent with **Sari and Simarani (2019)** who reported that the maximum decolourization of MR (93%) was achieved at 30℃ within 4.0h. That could indicate that the effective degradation of MR maybe related to the optimum temperature for the microbial growth. Whereas **Sharma *et al.* (2016)** mentioned that the effective degradation is an attribute of the optimum activity temperature of the degradation enzymes, and that the decline in degradation activity at higher temperature (50℃) may be due to the partial denaturation or inactivation of responsible enzymes or proteins related to MR decolourization.

Biodegradation of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 at a static incubation (zero rpm) were significantly low i.e. 29.5% and 37.8% respectively. Gradual increase of shaking speed resulted in gradual increase in bacterial growth and MR-biodegradation by both isolates until maximum degradation values of 86.0 and 86.3 % by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 respectively were attained at 150 rpm shaking speed. That could indicate that the increasing of shaking rate may increase the availability of oxygen and nutrients to the microorganisms and enhance growth of microorganisms and degradation of MR. These results differ from those of **Sharma *et al.* (2016)** study who reported that there was no significant difference on the rate of MR degradation by *Aeromonas jandaei* SCS5 under static and shaking conditions.

Acinetobacter baumannii SM01 and *Klebsiella pneumoniae* SM27 degraded MR at lower extents (32.0 % and 38.1 % respectively) in absence of any added nitrogen source. Addition of yeast extract enhanced the bacterial growth and MR-degradation rate to 87.4% by *Acinetobacter baumannii* SM01 and to 84.5% by *Klebsiella pneumoniae* SM27. Whereas, addition of ammonium nitrate increased MR degradation rates by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 by relatively

low extents (63.4 % and 67.5 % respectively). These results show fair agreement with those of **Zhao *et al* (2014)** who reported that yeast extract was the optimal nitrogen source which resulted in 98% MR degradation within 4.0h of cultivation and that in the absence of any added nitrogen source, MR degradation was 36 % after 5.0 h by *Bacillus* spp. strain UN2. This could indicate that addition of an organic nitrogen source such as yeast extract enhances bacterial growth and increases production of enzymes required for biodegradation of MR. It was also, reported that addition of yeast extract is essential to the regeneration of NADH, which acts as an electron donor for the reduction of azo dyes **(Khan *et al.*, 2013)**.

Biodegradation of azo dyes requires a suitable carbon source and the biodegradation of dyes without any supplement of carbon or nitrogen sources is very difficult **(Singh *et al.*, 2020**).

The impact of different added carbon sources (glucose, maltose, sucrose or starch 1.0% (w/v) in MII medium) on growth of isolates and the ability to degrade MR was investigated. *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 utilized glucose as the most efficient carbon source for their growth and for MR degradation 87.4% and 84.5% respectively. These results are consistent with **Zhao *et al*. (2014)** who found that, glucose was especially effective for improving MR degradation percentage to 93 and 98 % within 2.0 and 3.0 h respectively. D-glucose is known to be more easily taken up in living cells and is more readily metabolized than other sugars. In addition, **Chen *et al.* (2003) and Khehra *et al.* (2005)** reported that, the presence of glucose enhances the reduction rate of the azo compound by increasing the rate of formation of reduction equivalents (NADH, FADH), which are reported to be redox mediators involved in the reduction of azo compounds.

The current study illustrated that biodegradation of MR by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 in absence of any metal salts reached 73.7% and 84.5% respectively. $CaCl_2$, $FeCl_3$ and $MgSO_4$ had significant positive impact on MR degradation by *Acinetobacter baumannii* SM01 (84.7% to 92.3%) and *Klebsiella pneumoniae* SM27 (86.2 to 93.1%). Whereas $MnSO_4$ and $Pb(CH_3COO)_2$ showed negative effect on MR degradation by both isolates. These results are consistent with the findings of **Xu *et al.* (2007)** with respect to the effects of Fe^{+2} and Fe^{+3} and with the findings of **Zhao *et al.* (2014)** with respect to Fe^{+2}, Fe^{+3} and Mg^{+2} since they all reported that addition of these ions to the growth media enhanced MR biodegradation. The present results are also consistent with what was reported by **Zhao *et al.* (2014)** concerning the negative effects of addition of Pb^{+2} on MR biodegradation.

However, contraversions arise with both **Xu *et al.* (2007)** and **Zhao *et al.* (2014)** concerning the effects of Mn^{+2} and Ca^{+2}. Both authors reported that addition of Mn^{+2} had significant positive effects on MR biodegradation whereas, **Zhao *et al.* (2014)** reported that addition of Ca^{+2} had significant negative effect on MR biodegradation. That could indicate that metal ions may directly and variably affect activities of enzymes responsible of MR degradation or indirectly have their effect on the bacterial growth.

Acinetobacter baumannii SM01 and *Klebsiella pneumoniae* SM27 could grow in presence of MR up to 500 mg/L and the growth rates declined with increasing the concentration of MR. *Acinetobacter baumannii* SM01 was an efficient MR degrading isolate, it completely degraded (100 %) of 200 mg MR/L and 300 mg MR/L after 12h and 400 mg MR/L after 24 h of incubation, whereas it was only able to degrade 8.4 % of the 500 mg MR/L after 48h. In addition, its growth was enhanced by increasing the concentration of MR in MIV growth medium from 200 to 300 mg/L and decreased with further increases. While *Klebsiella pneumoniae* SM27 isolate completely degraded (100%) of 200 and 300 mg MR/L after 24h and 36h of incubation respectively, and degraded 400 mg MR/L by 78.9% after 48 h, whereas it was able to degrade 500 mg MR/L at a very low rate (0.8%) after 48 h.

These results show fair agreement with **Zhao *et al.* (2014)** who found that *Bacillus* spp. strain UN2 completely degraded MR within a range of 100 mg/L to 400 mg/L and the degradation rate decreased with increasing the initial concentration of MR. These results are also, consistent with **Sari and Simarani (2019)** who reported that MR decolourization by *Lysinibacillus fusiformis* W1B6 increased with the increase of MR concentration from 10 to 100 mg/L, but decreased rapidly at the highest concentration of 1000 mg/L. In addition, **Sharma *et al.* (2016)** reported that *Aeromonas jandaei* SCS5 achieved 100% decolourization of 200-600 mg MR/L, within 8.0-10 h. Moreover, 100% and around 90% decolourization were found within 12 h at a dye concentration of 800 mg/L, and about 70% within 12 h at 1000 mg MR/L under aerobic conditions.

On the other hand, **Sarkar *et al*. (2011)** reported that a more efficient MR degrading *Staphylococcus arlettae* PF4 could completely decolourize higher MR concentrations of 600 mg/L and 800 mg/L within 24h and 48h respectively and partially decolourized 1000 and 1200 mg/L of MR within 72h. That could indicate that the response of each bacterial isolate to different concentrations of MR is attributed to the limited inherent ability of the isolate to the bio-elimination process of the dye at the higher concentration (**Sari and Simarani, 2019)**. In addition, it was concluded that the high concentrations of dye were toxic and inhibited the growth of the bacterium **(Cui *et al*., 2014)**.

To confirm and illustrate MR biodegradation, the degradation products were analyzed by the UV visible spectrophotometry and Fourier transform infrared (FT-IR) in comparison with intact MR.

The spectrum profile of intact MR showed characteristic absorption peak in the visible region (λ_{max} = 430 nm) for methyl red which conforms with the findings of **Sarkar *et al*. (2011); Sharma *et al.* (2016)** and **Sari and Simarani (2019).** Meanwhile, the culture supernatant of *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates at the end of incubation period (6.0h) showed complete disappearance of the peak at 430 nm and a new major peak appeared in UV region (200-300 nm) with a maximum absorption at λ_{max} = 252 nm. These results go along with those of **Sari and Simarani (2019**) who found that, the spectrum analysis of intact and treated MR revealed that the major peak of MR(430 nm) disappeared, and a new major peak appeared at (300-350 nm) after 2.0h of incubation with *Lysinibacillus fusiformis* W1B6.

In addition, **Sharma *et al.* (2016)** showed that peaks for MR at 430 nm decreased gradually until complete decolourization of MR within 6.0h and new peaks appeared within the 200-300 nm range. Disappearance of the major absorbance peak (at λ_{430} nm) indicates complete removal of MR, whereas appearance of a new peak at end of incubation may be attributed to formation of new by-products. This confirms biodegradation rather than bioaccumulation as the principal mechanism of decolourization of MR by microbial isolates as documented by **Sari and Simarani (2019)**. In addition, **Shah *et al.* (2013)** and **Patil *et al.* (2016)** explained the red colour of culture medium consequently turned to colourless due to the reductive breakdown of azo (-N=N–) bond, while the presence of new peak was probably due to the production of metabolites.

Fourier transform infrared (FT-IR) spectrum of intact MR displayed a signal at 1598 cm^{-1} for azo bond (-N=N-), a signal at 1705 cm^{-1} for carbonyl (C=O) of carboxylic group, a signal at 1274 cm^{-1} for C-N stretch of aromatic amines, a signal at 1483 cm^{-1} designates C-C stretch in aromatics and a signal at 940 cm^{-1} for O-H bend of carboxylic acid that confirmed the structure of MR. This result shows fair agreement with **Patil *et al.* (2016); Tripathi *et al*. (2016) and Nair *et al.* (2017)** who determined these major peaks for configuring the structure of MR.

The current study illustrated that, the analysis of FT-IR of MR degradation products by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 revealed absence of the signal at 1598 cm^{-1} which indicates breakdown of azo bond. The absence of signals at 686 cm^{-1}, 725 cm^{-1},764 cm^{-1} indicates loss of aromaticity, lack of a peak at 1274 cm^{-1} represents absence of C-N stretch of aromatic amines. Disappearance of carbonyl signals (1705 cm^{-1}) means that the carboxylic group is no longer available. The appearance of a signal at 1375 cm^{-1} indicated CH2, CH3 bending. Prominent signals at 2852cm^{-1} and 2922 cm^{-1} are for CH aliphatic. These results are consistent with those reported by **Patil *et al*. (2016).** This means that *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 could effectively decolourize methyl red due to biodegradation through azo reduction and formation of aliphatic amines as end products. In addition, **Patil *et al*. (2016)** mentioned that FT-IR spectra of treated MR indicate biodegradation by reduction of azo bond by azoreductase enzyme. The azo reduction products were further degraded into aliphatic amines possibly through the mediation of oxidative enzymes such as lignin peroxidase and laccase.

The microbial biodegradation of azo dyes is mediated by enzymatically catalyzed reactions. Bacterial cell could produce enzymes in the absence of dye as a constitutive enzyme. Also, the presence of dye stimulates the cell to produce more of these enzymes as inducible enzyme **(Sari and Simarani, 2019)**.

In this study, the activity of oxidative (laccase and tyrosinase) and reductive enzymes (azoreductase and NADH-DCIP reductase) were determined during degradation of MR to ascertain if they accounted for MR biodegradation by *Acinetobacter baumannii* SM01. Significant ($p<0.001$) increases of enzymatic activity of azoreductase (7.74 µg MR reduced/ mg of protein/min), NADH-DCIP reductase (27.52 µg DCIP reduced/mg of protein/min), laccase (2.29 U/mg of protein/min) and tyrosinase (6.11 U/mg of protein/min) in the MR-containing solution compared to the

control (MR-free) solution. These results agree with those reported by **Zhao *et al* (2014)** who showed a significant increase in the activities of azoreductase, laccase, and NADH-DCIP reductase produced by *Bacillus* spp. strain UN2 during MR degradation. In addition, **Patil *et al.* (2016)** reported that *Bacillus circulans* NPP1 cell-free growth medium had shown the presence of significant activities of lignin peroxidase, azoreductase, laccase and tyrosinase in presence of MR. Moreover, **Jadhav *et al.* (2007)** found a significant increase in the activities of lignin peroxidase and NADH-DCIP reductase, azoreductase and tyrosinase during biodegradation of MR by *Saccharomyces cerevisiae* MTCC 463. In addition, a number of studies had reported the involvement of different oxidoreductase enzymes such as azoreductases, laccases, lignin peroxidases, Mn peroxidases, DCIP–NADH reductases, tyrosinase, aminopyrine N-demethylase and riboflavin reductases in the degradation of azo-dyes **(Agrawal *et al.*, 2014; Imran *et al.*, 2014; Zhao *et al* 2014; Zheng *et al*., 2014; Tripathi *et al.*, 2016).** This proves that, oxidative and reductive enzymes are important candidates for degradation of MR, and that the difference in the type of induced enzyme during MR biodegradation is related to the difference in the bacterial genus and species.

The predicted MR-degradation pathway by *Acinetobacter baumannii* SM01 based on FT-IR results -in accordance with the reports of **(Moutaouakkil *et al*. (2003)**, **Jadhav *et al.* (2008)** and **Zhao *et al.* (2014)** - which involves induced enzymes activities is illustrated in **Figure (17).** The azo bond (-N=N-) in MR may be reduced by reductase enzymes (azoreductase and NADH-DCIP reductase) forming aromatic amines; 2-amino benzoic acid (2-AMA) and N, N-Dimethyl p-phenylene diamine (DMPD). The produced aromatic amines were further oxidized by laccase and tyrosinase with loss of their aromaticity producing aliphatic amines.

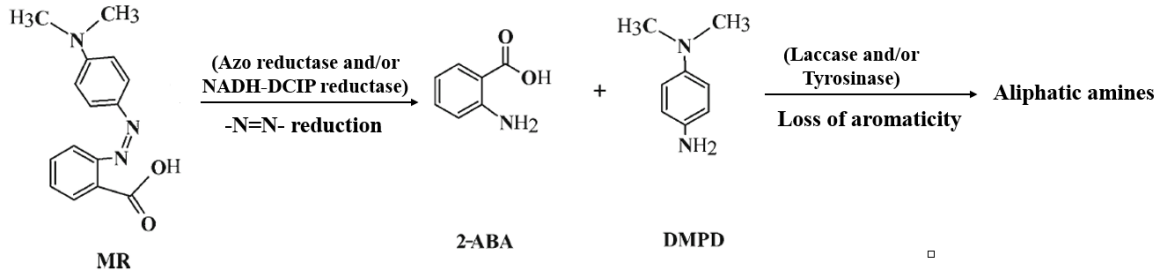

Figure (17): The predicted MR degradation pathway by *Acinetobacter baumannii* SM01.

Some authors had reported 2-amino benzoic acid (2-AMA) and N, N-Dimethyl p-phenylene diamine (DMPD) as the metabolic products obtained after degradation of MR due to microbial activity such as in the case of *Enterobacter agglomerans* **(Moutaouakkil *et al*., 2003**), *Galactomyces geotrichum* **(Jadhav *et al.*, 2008)**, *Bacillus* spp. strain UN2 **(Zhao *et al.*, 2014)** and *Bacillus circulans* NPP1 **(Patil *et al*. 2016).**

The current study investigated, the relative sensitivity of plant seeds (*Triticum aestivum*) against MR (300 mg/L) and MR degradation metabolites (300 mg/L) produced by *Acinetobacter baumannii* SM01 after treatment for one week. The outcomes of phytotoxicity studies indicated that undegraded MR was more toxic to crop plant seeds, as it showed 30 % inhibition of germination rate, as compared to MR

degradation metabolites which inhibited the germination rate by only 10 %. MR sample decreased root and shoot lengths by 42.4 % and 38.3 % respectively while MR degradation metabolites decreased root and shoot lengths by 27.8 % and 23.9 % respectively. These results show fair agreement with those reported by **Patil *et al*. (2016)** who studied the phytotoxicity of MR (300 mg/L) and its biodegradation metabolites (300 mg/L) obtained by *Bacillus circulans* NPP1 on the seeds of *Sorghum bicolor* and *Pennisteum Americanum.* Their results showed that, there was no germination inhibition of both the plant seeds by MR metabolites (300 mg/L). Moreover, the extracted metabolites of MR showed insignificant toxic effect on roots and shoots lengths in seed assay compared to seeds grown in presence of MR. In addition, **Zhao *et al* (2014)** reported that the intact MR (100 mg/L) showed 24.0% and 18.0% germination inhibition in *Triticum aestivum* and *Sorghum bicolor* respectively. They also reported no germination inhibition by metabolites of MR (100 mg/L) produced by *Bacillus* spp. strain UN2. In addition, the untreated MR solution greatly inhibited the shoot and root elongation of *Triticum aestivum* and *Sorghum bicolor* whereas, the inhibition effect was considerably counteracted after degradation by *Bacillus* spp. strain UN2. Moreover, **Tripathi *et al.* (2016)** reported that, the degradation products of MR (258.54 mg/L) produced by *Bacillus megaterium* ITBHU01 on the seeds *of Sorghum bicolor, Triticum aestivum* and *Salanum lycopersicum* did not exhibit any inhibitory outcomes but supported good seed germination with good shoot and root. This means that *Acinetobacter baumannii* SM01 is a promising antidote for MR and produces relatively less toxic biodegradation metabolites.

CONCLUSION

Bacterial degradation of MR used as a sole source of carbon is very slow and difficult. Optimization of the culture conditions for *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 isolates shortens the duration required for complete degradation (100%) of MR to 6.0 h.

The exact mechanism of MR removal by *Acinetobacter baumannii* SM01 and *Klebsiella pneumoniae* SM27 is by biodegradation and formation of new by-products. Both isolates could effectively degrade MR through azo reduction and formation of aliphatic amines as end products. This was due to a combination of both reductive (azoreductase and NADH-DCIP reductase) and oxidative enzymes (laccase and tyrosinase) which are efficient for removing MR toxicity.

Acinetobacter baumannii SM01 isolate is an efficient MR degrading isolate. It was able to completely degrade (100 %) up to 400 mg MR/L within 24h of incubation. The MR degradation products produced by *Acinetobacter baumannii* SM01 were relatively less toxic for *Triticum aestivum* seeds.

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التدرك البيولوجى للمثيل الأحمر بجراثيم الراكدة البومانية س.م.٠١ و الكلبسيلة الرئوية س.م.٢٧ بين عزلات مصرية أخرى

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الملخص:

تعد الأصباغ الآزوية من أكثر المواد ذات الأثر المديد على حياة الإنسان، وقد تم بحث قدرة جراثيم الراكدة البومانية س.م.٠١ و الكلبسيلة الرئوية س.م.٢٧ – المعزولتين من تربة ملوثة بزيت النفط من مواضع فى مصر – على التدرك البيولوجى للمثيل الأحمر، و قد تم فحص المتثابتات الفيزيوكيميائية التى تؤثر فى تدرك المثيل الأحمر و قد بلغ هذا التدرك أقصاه بهاتين العزلتين عند إضافة الجلكوز (١.٠% وزن/حجم)، خلاصة الخميرة (٠.٠١% وزن/حجم)، كلوريد الكالسيوم (١.٠ ملليمول) للمستنبت الزرعى و الحضانة عند ٣٥ درجة مئوية لمدة ست ساعات مع أعلى سرعة اهتزاز عند ١٥٠ دورة/دقيقة.

كما تمت دراسة آلية تدرك المثيل الأحمر بواسطة الراكدة البومانية س.م.٠١ و الكلبسيلة الرئوية س.م.٢٧ من خلال تحليل نواتج تدرك المثيل الأحمر بمطيافية الأشعة تحت الحمراء و مقياس الطيف المرئى مزدوج الشعاع للأشعة فوق البنفسجية فأكدت النتائج أن المثيل الأحمر حدث له إختزال آزوى مع مزيد من التدرك.

و قد تمت دراسة الإنزيمات المسئولة عن التدرك البيولوجى للمثيل الأحمر بجراثيم الراكدة البومانية س.م.٠١ فى المستنبت الخالى من الخلايا المتحصل بعد ست ساعات من الحضانة فى وجود مستنبت ضابط، و قد أوضحت النتائج حدوث زيادة بارزة معتدة فى أنشطة إنزيمات لكاز، تيروزيناز، مختزلة آزو و مختزلة ثنائى كلوروفينول إندوفينول معتمد ثنائى نيوكليوتيد النيكوتيناميد و الأدنين المختزل.

كما بينت نتائج دراسات السمية النباتية على بذور القمح أن مستقلبات تدرك المثيل الأحمر التى أنتجتها جراثيم الراكدة البومانية س.م.٠١ قد ثبطت إنبات ١٠% فقط من البذور و أنقصت أطوال جذر وساق النبتة ٢٧.٨% و ٢٣.٩% على التوالى منسوبة لتثبيط المثيل الأحمر السليم،

وعليه فإن الكفاءة العالية فى تدرك المثيل الأحمر تؤهل بكتريا الراكدة البومانية س.م.٠١ لتكون مرشحا محتملا للتدرك البيولوجى لمياه الصرف المحتوية على المثيل الأحمر.

كلمات مفتاحية: التكسير الحيوي، الراكدة البومانية، الكلبسيلة الرئوية، المثيل الأحمر، نواتج التكسير، السمية النباتية.