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Decolorization of the azo dye methyl red by an isolated bacterium *Enterobacter hormaechei* strain CUIZ1

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Abstract

Microorganisms play a major role in the degradation of organic matter, and a simple adaptation to a polluting molecule can lead to its biodegradation or biotransformation. This is the bioremediation principle which is one of the important biotechnologies who generates important results in the management of waste such as the removal of textiles dyes, which are considered to be xenobiotic compounds and recalcitrant to biodegradation. In the present bioremedial approach, a novel bacterium was isolated from dye-contaminated argan soil of the region of Souss Massa, Agadir, Morocco. Through 16S rRNA sequence matching and morphological observation it was identified as *Enterobacter hormaechei*. This strain has the ability to decolorize more than 100mg/L of the toxic azo dye methyl red (MR) just after 24hours of incubation. Several parameters as agitation, pH, temperature and initial dye concentrations were optimized to develop total decolorization process. The high MR decolorization ability and low nutrient requirement of this bacterium make it very useful in the biological treatment of industrial effluent containing azo dyes. The phytotoxicity assay with pepper seeds (*Capsicum annuum*) very cultivated in the region of Souss Massa, revealed that the decolorization of Methyl Red produced non toxic metabolites.

1. Introduction

Azo dyes are the most common types of synthetic dyes and constitute the largest class of dyes used commercially because of the ease and cost effectiveness of their synthesis, their stability and the variety of colors available compared to natural dyes [1]. Synthetic azo dyes are used extensively as dyes for textiles, food, cosmetics and pharmaceutical industries [2]. Most of the azo dyes, which are released into the environment, originate from the textile industry and the dyestuff manufacturing industry [3].

Azo dyes are a group of compounds characterized by the presence of one or more azo bonds (-N=N-) in association with one or more aromatic systems [4]. This makes them relatively resistant to biological and chemical degradations. However, several studies have shown azo dyes to be toxic and/or carcinogenic [5].

Because of the high quantities of water used in the dyeing processes, the textile industry is one of the greatest generators of liquid effluent pollutants. These pollutants become very toxic once diverted into the environment, with the increase in temperature and in association with other substances like reducing and oxidizing agents. Many synthetic azo dyes and their metabolites are toxic, carcinogenic, and mutagenic [6,7]. Moreover, numerous reports indicate that textile dyes and effluents have toxic effects on the germination rates and biomass of several plants [8].

Therefore, treatment of industrial effluents containing azo dyes and their metabolites is necessary prior to their final discharge to the environment, in view of their high Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), color, pH, and also it contains metal ions [9]. Dye wastewaters are usually treated using physicochemical methods such as flocculation, coagulation, adsorption, membrane filtration,

precipitation, irradiation ozonization and Fenton's oxidation [10]. These methods require more energy and chemicals, are unable to remove completely the recalcitrant azo dyes and/or their organic metabolites, and may generate significant amounts of chemical sludge, whose disposal in secure landfill increases process cost [11].

Among the important biological solutions, bioremediation, including biostimulation and bioaugmentation, is being used to enhance natural biodegradation rates through optimization of limiting environmental conditions, and has been shown to be an economic, versatile and ecologically acceptable cleanup approach [12]. In such, microbes acclimatize themselves to the toxic wastes and new resistant strains develop naturally, which then transform various toxic chemicals into less harmful forms [7,13]. However, Bacterial decolorization of azo dyes under certain environmental conditions has gained momentum as a method of treatment, as these are eco-friendly, can be applied to wide range of dyes and requiring less water consumption compared to physicochemical methods [14].

In the present study, we have focused our attention on the isolation of methyl red decolorizing bacterial strains from dye-acclimatized argan soil, having a specific flora since it is a virgin soil and distant from any source of contamination. Also we have studied the decolorization ability of the isolated bacteria under various assay conditions. The toxicity of the decolorization products was tested by phytotoxicity assay on the seeds of *Capsicum annuum*.

2. Material and Methods

2.1. Dye

Methyl red (MR) were purchased from the Loba Chemie (India). The solutions were prepared by dissolving the powder products in distilled water to obtain the desired concentrations. The absorbance was measured with spectrophotometer (JASCO V630 spectrophotometer), and Samples were centrifuged at 14,000 rpm for 20 min to remove bacteria before measuring absorbance.

2.2. Microorganism isolation and culture conditions

The microorganism used in this study was isolated from the argan soil in Souss-Massa region, Morrocco. The soil sample was added (1g/100 mL) in mineral salt medium containing dyes (100 mg/L) and incubated at 30 °C under shaking conditions. The mineral salts medium (MSM) used in all experiments contained: K_2HPO_4 (1.6 g L^{-1}), KH_2PO_4 (0.2 g L^{-1}), $(NH_4)_2SO_4$ (1.0 g L^{-1}), $MgSO_4$ (0.2 g L^{-1}), $FeCl_3$ (0.01 g L^{-1}), NaCl (0.1 g L^{-1}), $CaCl_2$ (0.02 g L^{-1}) and was autoclaved for 20 min at 121°C before using [15]. Five versions of MSM were prepared as, MSM without carbon and nitrogen source, MSM with 1 g L^{-1} of yeast extract as nitrogen source, MSM with 3 g L^{-1} and 1 g $L^{-1}$of glucose respectively as carbon source, and finally MSM with 3 g $L^{-1}$of glucose and 1 g $L^{-1}$of yeast extract.

After 3 days in 30°C incubation, 1.0 ml of the culture was serially diluted. Aliquots of 0.1 ml of 10^{-3}, 10^{-4} and 10^{-5} dilutions were spread on the MR agar plates containing 100 mg/L of dyes. After 2 days of incubation at 30°C, the colonies were screened for their ability to form a clear zone around them [16]. To confirm their abilities to decolorize MR, colonies surrounded by decolorized zones were picked and re-streaked onto MR plates. The plates were re-incubated at the same conditions.

One isolated bacteria was chosen for their high performance and identified using 16Sr RNA method.

Pre cultures of the selected bacterium were prepared by growing a single colony in 10 ml of MSM and an aliquot of 1 ml were taken for inoculated the samples.

2.3. 16S rRNA sequencing and phylogenetic analyzis

The isolate was identified using 16S rRNA sequence analyzis. 16S rRNA gene sequencing of isolated bacteria was carried out at Secugen S.L society, Spain (http://www.secugen.es/en_index.php). The sequences were uploaded from the EzTaxon server and aligned by Muscle program [17].

The bootstrap consensus tree inferred from 1000 replicates [18]. The phylogenetic tree is constructed from the aligned sequences by bioinformatics sites (http://www.bioinformatics.org/sms/rev_comp.html) and (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html) using the UPGMA method [19] and Jukes-Canter method for taxon distances in MEGA7 software [20]. The accession number is MF774495 after submission in Genbank databases.

2.4. Decolorization assay

Decolorization experiments of MR were executed in Erlenmeyer flasks (capacity 250 mL). The culture medium used was MSM containing 100 mg/L of MR and the inoculated cultures were incubated at 30 °C under shaking condition (120 rpm), except other indications.

At several time intervals, 2 mL aliquots of each culture were sampled and their densities were measured spectrophotometrically at 600 nm in order to determine the bacterial growth. After centrifugation the supernatant was 10-fold diluted and the concentration of MR was determined spectrophotometrically. All assays were conducted in triplicate and the average rates were calculated to represent the results. The percentage of decolorization was determinate by using following formula:

$$\text{Decolorization } \% = \frac{(\text{Initial Absorbance} - \text{Observed Absorbance})}{\text{Initial Absorbance}} \times 100 \quad [21]$$

2.5. Effect of physico-chemical parameters

We have studied the decolorization ability of the isolated bacteria under various conditions. Thus, effects of physicochemical parameters on the MR decolorization and on the bacterial growth are studied especially: incubation temperature, glucose concentration, yeast extract concentration, pH, initial concentration of dye, and effect of shaking.

2.6. Phytotoxicity study

The phytotoxicity test was performed to evaluate the toxicity of dyes and metabolites obtained after their decolorization [22,23]. The test was carried out on pepper seeds: *Capsicum annuum* commonly cultivated in Souss Massa. 15 surface sterilized seeds were sown in a Petri dishes containing moist filter paper. Toxicity study was carried out by watering (2 ml per day) the seeds with the original dye solution (100mg/L) and with metabolites of MR decolorization product. Control was run by watering the seeds with MSM solution. Germination percentage (%), roots and shoots lengths were measured after 15 days of incubation at 25°C [24].

3. Results and discussion

3.1. Isolation of methyl red decolorizing bacteria

Preliminary selection of MR degrading bacteria was based on the decolorization of soil-MR suspension. The MSM amended with 3g/L of glucose with or without yeast extract caused a complete decolorization of culture medium. But, the MSM amended with 1g/L of glucose or yeast extract induces only 60% of decolorization. In the other hand, the MSM without carbon neither nitrogen source failed to decolorize the culture medium as is illustrated in the Table 1.

Table 1: Table of the decolorization percentage after 3 days of incubation in each soil-MR suspensions used

Sample	Percentage of decolorization (%)
Soil-MR in MSM	9.18
Soil-MR in MSM+1G/L of YE	60.01
Soil-MR in MSM+1G/L of G	59.64
Soil-MR in MSM+3G/L of G	97.95
Soil-MR in MSM+1G/L of YE+3G/L of G	97.13

YE: Yeast Extract, G: Glucose

Secondly, after seeding the discolored suspension in MR agar we found that grew colonies formed a clear zone around them. Three different colonies were isolated and compared according to their ability to decolorize 100 mg/L of MR in erlen flask (Fig. 1). Among the three isolated bacterial strains, one strain (CUIZ1) has resulted in 99% decolorization percentage.

3.2. Identification and phylogenetic position of the isolates

To analyze the phylogenetic position, the 16SrDNA sequence of the strain CUIZ1 (1247bp, MF774495) was determined. The phylogenetic relationship between the strain CUIZ1 and other related microorganisms found in the GenBank database is shown in Fig. 2. The homology assay result indicated that the strain CUIZ1 was in the phylogenetic branch of the Enterobacter, CUIZ1 exhibited a maximum identity (99.68%) to *Enterobacter hormaechei subsp. Steigerwaltii* DSM 16691. The sequence NR 075005.1 was used as an outgroup.

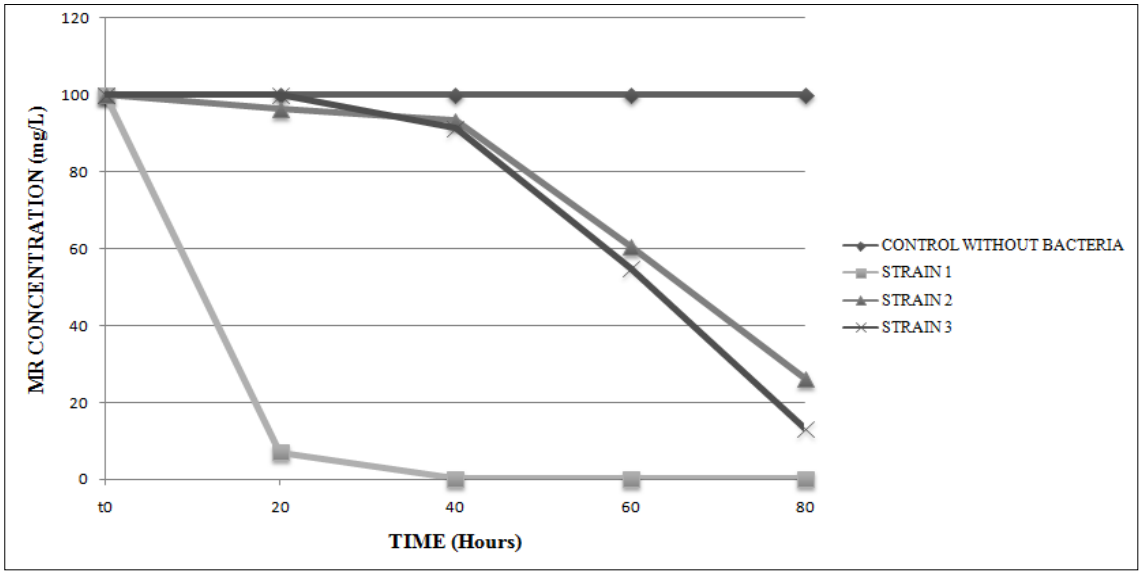

Figure 1: decolorization comparison between 3 bacteria: (♦) control without bacteria, (■) strain CUIZ1, (▲) strain 2, (×) strain 3.

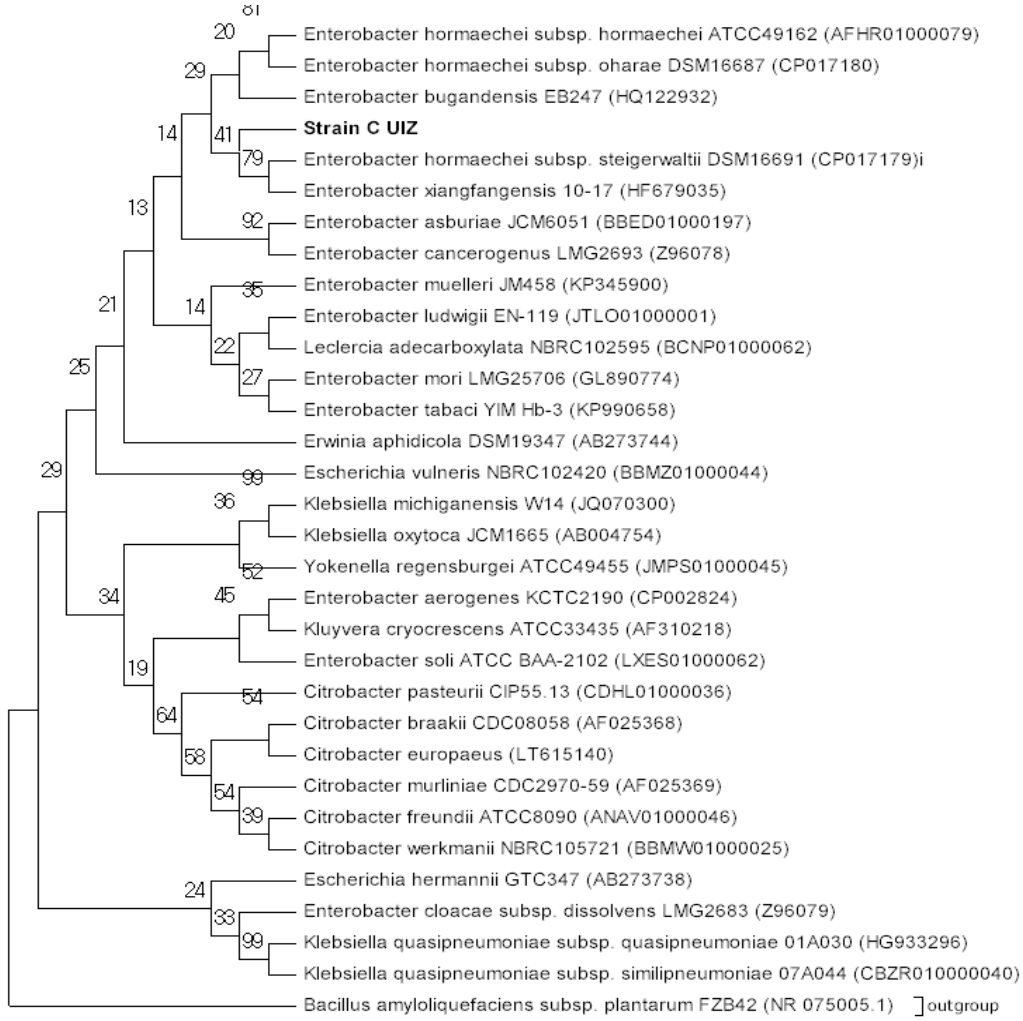

Figure 2: Phylogenetic tree of the *Enterobacter hormaechei CUIZ1* and related organisms, tree constructed using the UPGMA method based on the 16S rDNA sequences. Bootstrap values based on 1000 replicates are presented on the nodes of the tree. Distances between taxa are calculated by the Jukes-Cantor method and are expressed in units of the number of base substitutions per site.

The earlier report showed that some species of Enterobacter have a role in biodegradation of dyes [5]. But to the best of our knowledge, there was no literature available on the use of *E. hormaechei* in dye degradation. However, this microorganism have been used in the degradation of other toxic compounds such as neonicotinoid pesticide[25].

3.3. Effects of physicochemical parameters on methyl red decolorization

The effect of various physicochemical conditions on the decolorization of MR by using *E. hormaechei* was studied in detail.

a) Effect of temperature

As shown in Table 2, decolorization of MR was obtained between 21°C and 37°C. But, the maximum potential of *E. hormaechei* to decolorize this dye was at 30°C. Decolorizing activity was significantly suppressed at 44°C, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 44°C [26,27].

Table 2: Effect of incubation temperature on *E. hormaechei* growth and MR decolorization after 24 hours.

Incubation temperature	Concentration of bacteria - control without MR- (CFU / mL)	Concentration of bacteria -presence of MR- (CFU / mL)	Percentage of decolorization (%)
21°C	51872500	43346250	85.60
25°C	47026250	48500000	87.40
30°C	48827500	37536250	99.30
37°C	52007500	42210000	98.60
44°C	23461250	5981250	1.30

CFU: Colony Forming Unity

Current results can be explained by the variations of enzyme activity at different temperatures. Contrary to results obtained by Saratale [23] and Moutaouakkil [5] for Remozal Red and Methyl Red, with *Lysinibacillus sp* and *Enterobacter agglomerans* respectively, who confirmed that only a larger population of cells could decolorize more molecules.

b) Effect of shaking

The culture medium incubated under static conditions showed a decolorization of 100% after 24 hours of incubation, whereas the agitated medium did not exceed 94%. That is to say, the shaking of the sample reduces the decolorization of MR by *E. hormaechei* with a low difference.

Decolorization was not only related to biomass concentration but was significantly correlated with the presence of oxygen, despite the contrary effect on bacterial growth as mentioned by Chang [1]. In other words, there could be competition of oxygen and the azo compounds for the reduced electron carriers under aerobic condition [21]. Therefore, facultative or obligate anaerobes are necessary for azo dye reduction because the microbial reduction of these dyes is an enzymatic reaction inhibited by oxygen [28].

This result is confirmed by several authors like Kalme [21], Isik [28], Jadav [29], Phugare [30], Saratale [23] and Telke [31] who have found that agitation can retarded up to 80% of biodegradation.

c) Effect of glucose concentration

After 24h of incubation more than 99% of MR was decolorized when the glucose concentration was greater than or equal to 1g/L, whereas, in absence of glucose, the decolorization percentage of MR does not exceed 43% (Table 3). This can be explained by the difficulty of the dyes decolorization without any supplement of carbon [32], by the very weak growth of bacteria in absence of glucose or by the bacterium use of the energy generated from the glucose for the reductive cleavage [5].

Table 3: Effect of different glucose concentration on decolorization of MR after 24hours of incubation

Glucose concentration (mg/L)	Decolorization percentage (%)
0	42.97±1.15
0.5	72.65±2.93
0.75	98.80±1.11
1	99.36±0.55
1.25	100±0.00
2.5	99.47±0.46
5	99.57±0.51
10	98.58±1.57

d) Effect of yeast extract concentration

In presence or absence of yeast extract, the percentage of decolorization after 24 hours of incubation reached 99%. However, most of the previous studies like that of Chen [33] showed that increasing the concentration of yeast extract results in higher decolorization rates.

In our case, we deduce that nitrogen had no significant effect on MR decolorization, which is obvious since our studied strain was isolated from a low medium in yeast extract and this bacterium did not need a nitrogen source to grow neither to discolor the MR.

e) Effect of initial pH

pH was found to be the crucial parameter during the decolorization [23, 24, 33]. By studying the decolorization percentage in the different pHs from 5 to 9, we found that the optimal pH was 8.5 with a percentage of 97.54% just after 24 hours, followed by pH 6 which required 2 days to reach 99.87%.

Several studies confirmed that neutral pH was favorable for decolorization, but extreme acidic (3 and 5) or alkaline pH (9 and 11) retards this process. This could be explained by the inhibition of the bacterial growth at these pHs [5].

f) Effect of initial concentration of dye

To assess the maximum decolorization ability of *E. hormaechei*, it was tested against different concentrations of the dye. Fig.3 depicts that after 24 hours of incubation, the MR concentration from 10 to 100mg/L showed 100±0.00% of decolorization, followed by the concentrations of 200 mg/Lwith 98±1.63% of decolorization. And, for 1000 mg/L the decolorization achieved 97±1.75%.

This slight decrease in decolorization efficiency at high dye concentrations may be due to the toxicity of the dye towards bacteria [34]. These results indicated a high decolorizing performance even in high initial concentrations of dyes but they were not in good agreement with other studies such that of Wang et al. [27] who found that *Citrobacter sp*. can decolorize up to 41.69% to 1000 mg/L of Reactive Red 180 after 120 h of incubation, and Jadhav et al. [37] who showed that the specific decolorization rate increases with increase in initial dye concentration and then it slowed down.

3.1. Effect of E. hormaechei cells autoclaved on adsorption

Autoclaved cells of *E. hormaechei* were incubated with 100mg/L of MR under the same conditions and following the same protocol. No variation in decolorization was observed during the next 3 days. In contrast, the living cells of *E. hormaechei* were able to decolorize MR completely within 24 h of incubation. These results clearly indicated that the overall decolorization was due to biological mechanisms but not due to adsorption. Similar observations were reported earlier by Jadhav et al. [36, 37] and Khehra et al. [38].

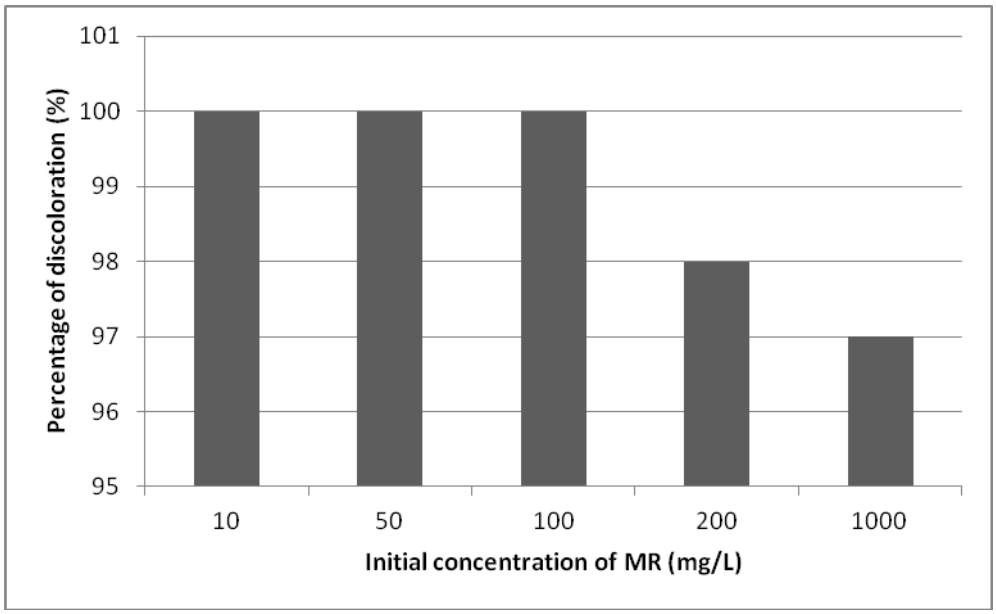

Figure 3: Percentage of decolorization of MR after 24hours according to initial concentration of dye.

3.2. Monitoring of E. hormaechei decolorization of MR

After optimizing the favorable conditions of decolorization (100mg/L, pH 8.5, 1.25 g L^{-1} of glucose, without nitrogen source, at 30 °C and under static conditions) this monitoring is carried out to follow the steps of decolorization during the 24 hours every 2hours. Fig. 4 shows that the rate of decolorization was slow up to 4h of incubation and accelerate after, this indicate that the organisms required some time to acclimatize in that environment. Total decolorization was exactly after 24hours.

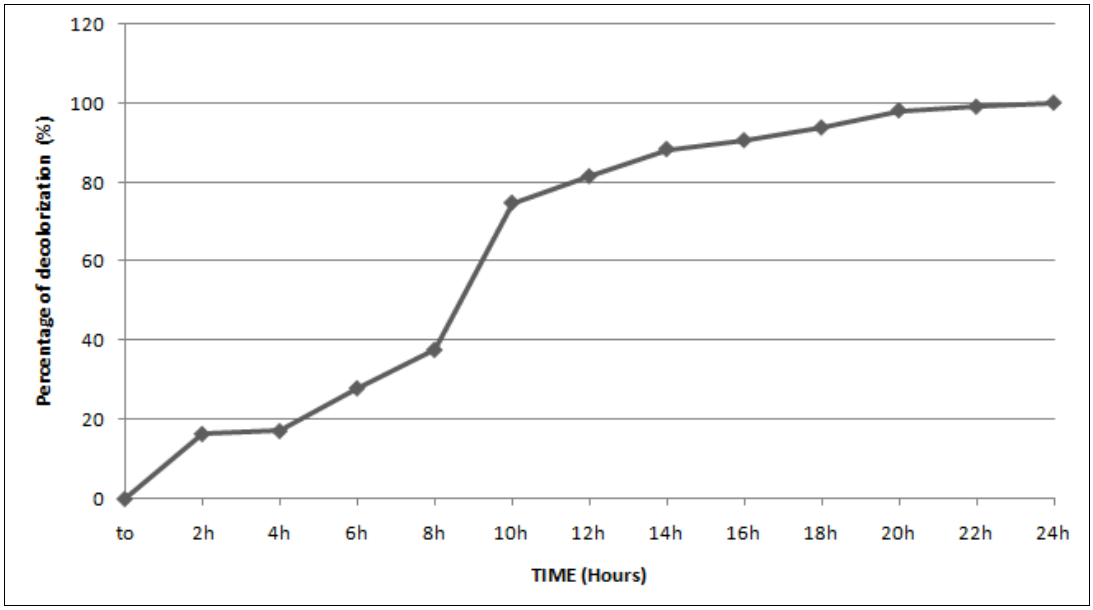

Figure 4: Monitoring *of E. hormaechei* decolorization of MR during 24hours.

3.3. Phytotoxicity test

Because the use of untreated and treated dyeing effluents in agriculture has direct impact on fertility of soil, it is necessary to assess the phytotoxicity of ME bacterial decolorization products. The Table 4 shows the comparison between the MR phytotoxicity and its metabolites on *C. annuum* seeds after 15 days of incubation. MSM was used as a control in addition to distilled water to eliminate the effect of glucose and minerals elements on germination.

Table 4: Phytotoxicity study of Methyl Red and its decolorization products on *C. annuum* seeds.

Studied parameters	Distillated Water	MSM	Methyl Red	Metabolites
Germination%	100	100	76	100
Shoot length(cm)	3.00±0.19	2.65±0.17	1.91±0.12**°	3.41±0.20°
Root length(cm)	2.90±0.12	2.25±0.20**	1.25±0.06°°	3.05±0.14°°

*Values are mean of three experiments, SEM (±), significantly different from the control (seeds germinated in distilled water) at *P < 0.05, **P < 0.001, significantly different from the control (seeds germinated in MSM) at °P < 0.05, °°P < 0.001, by one-way analyzis of variance (ANOVA) with Tukeye Kramer multiple comparisons test.*

Obtained results indicate that the germination of *C. annuum* seeds was reduced with MR treatment, whereas the decolorization products accorded to the plant a highly significant growth. In fact, the shoot and root length of *C. annuum* was enhanced compared with controls (MSM and distilled water). This observation suggests that the metabolites obtained after MR decolorization may have a nutritive role in the germination and growth of plants, in addition to their non-toxic nature compared with the untreated dye.

Conclusion

This study demonstrates that *Enterobacter hormaechei CUIZ1* was able to decolorize and detoxify the toxic Methyl Red into nontoxic metabolites.

This bacterial strain, isolated from dye-contaminated Argan soil collected from SOUSS MASSA region, completely decolorized 100 mg/L of MR after 24hours of incubation in the mineral salt medium with 1.25g/L of glucose at 30°C under static condition. These advantages allow the use of this bacterium in the treatment of azo dyes in industrial effluents with ever-changing physicochemical properties.

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