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ORIGINAL ARTICLE

Isolation, screening and molecular identification of novel bacterial strain removing methylene blue from water solutions

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Abstract The potentially deleterious effects of methylene blue (MB) on human health drove the interest in its removal promptly. Bioremediation is an effective and eco friendly for removing MB. Soil bacteria were isolated and examined for their potential to remove MB. The most potent bacterial candidate was characterized and identified using 16S rRNA sequence technique. The evolutionary history of the isolate was conducted by maximum likelihood method. Some physiochemical parameters were optimized for maximum decolorization. Decolorization mechanism and microbial toxicity study of MB (100 mg/l) and by-products were investigated. Participation of heat killed bacteria in color adsorption have been investigated too. The bacterial isolate was identified as *Stenotrophomonas maltophilia* strain Kilany_MB 16S ribosomal RNA gene with 99% sequence similarity. The sequence was submitted to NCBI (Accession number = KU533726). Phylogeny depicted the phylogenetic relationships between 16S ribosomal RNA gene, partial sequence (1442 bp), of the isolated strain and other strains related to *Stenotrophomonas maltophilia* in the GenBank database. The optimal conditions were investigated to be pH 5 at 30 °C, after 24 h using 5 mg/l MB showing optimum decolorization percentage (61.3%). Microbial toxicity study demonstrated relative reduction in the toxicity of MB decolorized products on test bacteria. Mechanism of color removal was proved by both biosorption and biodegradation, where heat-killed and live cells showed 43 and 52% of decolorization, respectively, as a maximum value after 24-h incubation. It was demonstrated that the mechanism of color removal is by adsorption. Therefore, good performance of *S maltophilia* in MB color removal reinforces the exploitation of these bacteria in environmental clean-up and restoration of the ecosystem.

Keywords Decolorization · Methylene blue · *Stenotrophomonas maltophilia*

Introduction

Disposal of synthetic dyes improperly in wastewater depicts acute toxic effects on aquatic flora and fauna, causing severe environmental problems (Singh et al. 2014). Therefore, the increasing amount of dyes like methylene blue (MB) because of industrial activities indicates a probable hazard to the ecosystem. Alongside, the urgent need for water and decreasing supply made the treatment and recycle of industrial effluents are of great importance. Methylene blue is a thiazine (cationic) dye exploited in the coloring paper, hair, cotton and wools (Han et al. 2010). Notably, about 10–15% of the dyes are lost in the effluents of textile units, making them highly colored. Maas and Chaudhari (2005) reported that 280,000 tons of textile dyes are inflow in such industrial effluents every year worldwide (Maas and Chaudhari 2005). Once they released into receiving water bodies, they cause environmental problems where they affect water transparency and gas solubility blocking the permeation of oxygen and sunlight necessary for the survival of various aquatic forms (Crini 2006). Dye removal is of a particular concern because it is mostly unaffected by traditional treatment systems. Dyes;

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however, are more difficult to treat because their synthetic origin where they consist mainly of complex aromatic molecular structures, often synthesized to resist fading on exposure to soap, sweat, water, light or any oxidizing agents (Khan and Husain 2007). This renders them more stable and less amenable to degradation (Seshadri et al. 1994). Moreover, chemical and physical methods for removing dyes from waste water were not widely applied in the textile industries because of the high cost and disposal problems (Mazmanci and Unyayar 2005). Wastewaters have been known to be harmful to the microorganisms involved in wastewater treatment; thus, dye waste waters cause failure of the treatment plants (Altaher and El Qada 2011). Concentration of methylene blue used is within the limits found in polluted waters (50–1000 mg/dm^3) (Sarioglu and Atay 2006). In recent years, the biological treatment took attraction in removing the undesired color and toxicity of dyes than other ordinary treatment processes. A considerable attention has been directed in evaluating the capability of microorganism in decolorization and degradation of dyes. The removal of MB by different microorganisms was investigated (Ong et al. 2005; Fulekar et al. 2013; Singh et al. 2014; Ranga et al. 2015). Therefore, microbial processes play a vital role in the safe clean-up of environmental messes (Abbas et al. 2014). However, the effluent because of biotransformation of dyestuffs could be toxic (Chung and Stevens 1993). For environmental safety, the microbial toxicity of the by-products produced during the decolorization process should be evaluated. Microorganisms have the ability to decolorize the dye solution through two ways: either adsorption on the microbial biomass or biodegradation of the dyes by the cellular enzymes. Therefore, the biological method is the focus of recent studies on dye degradation and decolorization. Therefore, the objective of this study was to evaluate the ability of soil-isolated bacteria in the Southern area of Saudi Arabia to remove MB.

Methods

Chemicals

All chemicals and reagents used for experiments were of analytical grade.

Decolorization experiment

About 0.1 ml of bacterial culture was inoculated in 250 ml Erlenmeyer flask containing 50 ml nutrient broth media and incubated at 30 °C for 24 h. MB was added at a concentration of 10 mg/l (0.5 mg/50 ml) and 3 ml of the culture media was withdrawn at different time intervals to be centrifuged at 5000 rpm/min for 20 min to separate the bacterial cell mass. Decolorization potential was ascertained by measuring the absorbance of culture supernatants at the maximum absorption wavelength of MB (665 nm). The percentage of decolorization (D%) was calculated by the following equation Eq. (1) (Parshetti et al. 2010):

$$\text{Decolorization } (\%) = (A_0 - A_t)/A_0 \times 100 \quad (1)$$

where, A_0 denote the initial absorbance of sample and A_t denotes the absorbance at different time intervals.

Isolation and characterization of soil bacteria

Bacterial strains were isolated from soil rhizosphere in Abha/Kingdom of Saudi Arabia by serial dilution method (Benson 2002). A sample of 5 g soil was suspended in 100 ml water. 1 ml of soil suspension was serially diluted in distilled water. A volume of 0.1 ml of soil suspension was spread onto nutrient agar plates and incubated at 30 °C for 24 h. The different colonies observed were selected and characterized.

Screening of bacterial isolates towards MB decolorization

The screening was carried out for different bacterial isolates individually towards MB decolorization according to Parshetti et al. (2010) as described above.

Molecular identification of the selected isolate

The selected candidate was identified by sequencing the 16S rRNA. Total genomic DNA was extracted from pure bacterial cultures using DNeasy Blood and Tissue Kit (Qiagen, West Sussex, UK). PCR amplification of the16S rRNA gene from bacterial isolate was conducted using the universal primers: 785F (5′-GGATTAGATACCCTGGTAGTC-3′) and 907RA: (5′-CCGTCAATTCCTTTGAGTTT-3′) (Morales and Holben 2009). Thermal cycling comprising the following steps: 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s, and a final step at 72 °C for 10 min. The PCR products submitted for sequencing. The amplified DNA fragments gel-purified using QIA quick™ Gel Extraction Kit (Qiagen, USA) following the instructions of the manufacturer and sequenced by Macrogen Inc (Seoul, Korea) using an ABI3730 XL Automatic DNA Sequencer (Applied Biosystems, Renton, USA). Multiple alignments of sequences and the evolutionary history were deduced with other sequences downloaded from the GenBank database using Mega 6 (Molecular evolutionary genetic analysis) software (Tamura et al. 2013). The maximum likelihood method was adopted to construct the phylogenetic tree.

Effect of physiochemical parameters on decolorization process

To evaluate the impact of environmental factors, various concentrations of MB (2.5, 5, 10, 15, 20 g/l) used to determine the effect of concentration on the decolorization process. The incubation period was evaluated by incubating the bacterial isolate for different times (0, 1, 2, 3, 4, 5 days). The effect of the temperature and pH was also investigated by incubating bacterial isolate at temperature range (4, 20, 28, 30, 37 and 40 °C) and various initial pH values (4, 5, 6, 7, 8, 9), respectively (Parshetti et al. 2010).

Microbial toxicity study

For assessment of the impact of MB (100 mg/l) and its degradation products, microbial toxicity tests performed by agar well diffusion technique using Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*P. aeruginosa, Shigella sp, Klebsiella sp* and *Proteus sp*). Test bacterial cultures were grown overnight in nutrient broth and standardized to OD_{600} nm = 0.1. Aliquots of bacterial cultures were then spread-plated onto nutrient agar plates. Wells of diameter 6 mm done in the agar plate where they loaded 100 µl of MB (100 mg/l) and by-products, separately. The plates were left for 2 h for diffusion before incubation at 30 °C for 24 h. The plates were observed for inhibition zones.

Mode of action of decolorization

The change in the peak of absorption spectrum reveals the mechanism of decolorization process. Variation of UV—visible spectra of MB- containing supernatant of bacterial culture was checked at 0, 24 and 48 h spectrophotometrically where the maximum wavelength at 665 nm (Shah et al. 2013).

Decolorization of MB by live and heat-killed cells

Heat-killed bacteria were prepared according to Zuany-Amorim et al. (2002). Bacterial culture was grown overnight in MB supplemented-nutrient broth, centrifuged at 6000 rpm for 5 min and then washed three times with KK_2 buffer (6.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2).The pellet is suspended in 1/10th of the initial volume of KK_2 and an aliquot is diluted to measure the OD_{650}. The volume of the bacterial suspension is then adjusted to a density of 10^{12} cells/ml (assuming that an OD_{650} of 0.1 corresponds to 10^8 cells/ml). Cells are heat-killed at 70 °C for 10 min and then added to 100 ml NB media containing 100 mg/l MB. Consequently, live bacterial cells inoculated to another 100 ml NB media containing 100 mg/l MB. Both flasks were incubated at 37 °C. Aliquot 3 ml of each flask withdrawn at zero time and every 12 h and checked spectrophotometrically at 665 nm.

Statistical analysis

The statistical analysis performed using the one-way ANOVA. A P value < 0.05 was statistically significant. The biochemical data are recorded and expressed as mean ± SD (Zar 2007).

Results

Isolation and screening of bacterial isolates

Total of 40 bacterial isolates were isolated from soil rhizosphere, of them three isolates showed the decolorization activity. Figure 1 depicts that there is no significant difference between them towards decolorization activity meanwhile the most potent isolate has the ability to remove 53% of MB was chosen in the present study.

Identification of the selected isolate

The results cleared that the bacterial isolate is identified as *Stenotrophomonas maltophilia* strain Kilany_MB 16S ribosomal RNA gene, exhibiting 99% sequence similarity. The sequence was submitted to NCBI (Accession number = KU533726). Figure 2 shows the phylogenetic relationship of 16S ribosomal RNA gene, partial sequence (1442 bp) of the isolated strain and other strains and species related to *Stenotrophomonas maltophilia* in the GenBank database.

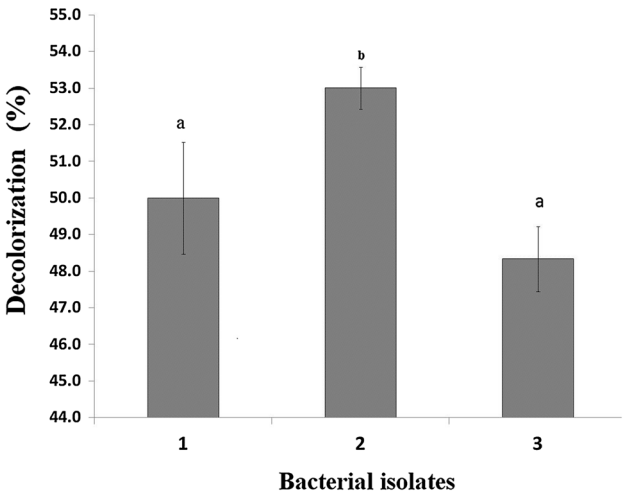

Fig. 1 Screening of bacterial isolates towards MB decolorization

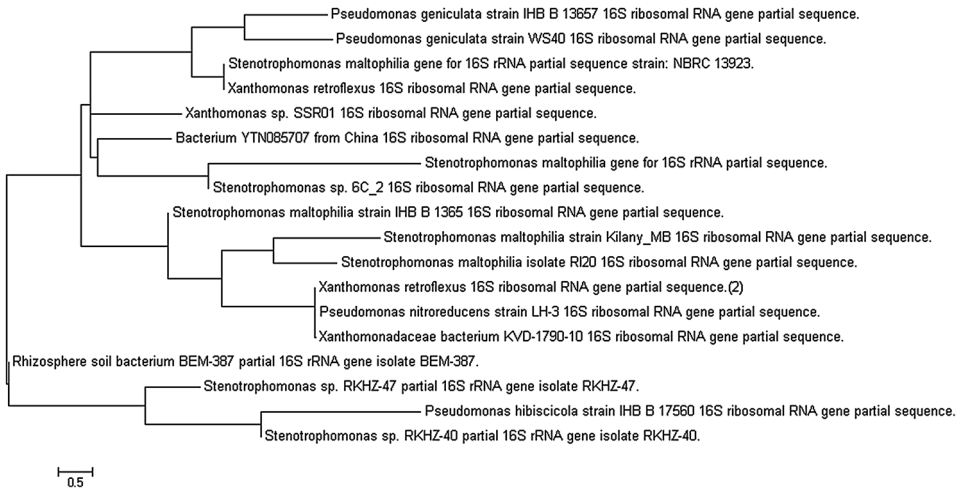

Fig. 2 Rooted phylogenetic tree based on the 16S rRNA sequences of *Stenotrophomonas maltophilia* strain Kilany_MB and related bacteria. This tree was made using the maximum likelihood method. The *bar* represents 50 nucleotide substitutions per 100 nucleotides

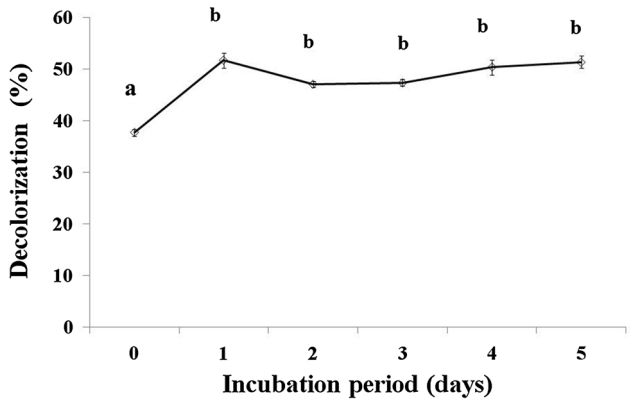

Fig. 3 Effect of incubation period

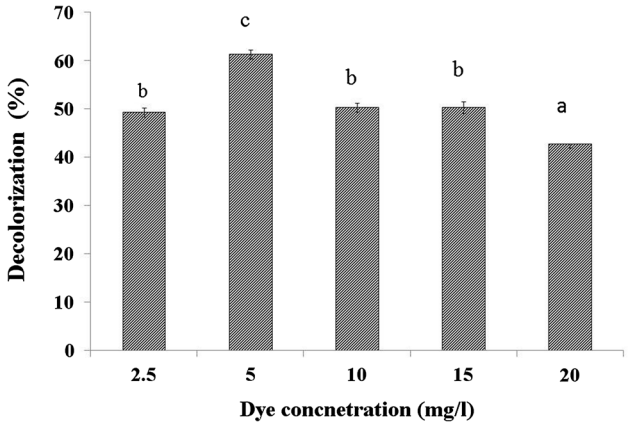

Fig. 4 Effect of methylene blue concentration

Effect of incubation period

The results revealed that the rate of decolorization was very fast where it begins just after bacterial inoculation reaching the maximal value (51.7%) after 1st day of incubation and there was non-significant difference in decolorization with the increase of incubation period (Fig. 3).

Effect of MB concentration

The results showed that the percentage of decolorization significantly increased with the increase in MB concentration reaching the maximum value at 5 mg/l (61.3%); further increase in concentration exhibited no significant increase in percentage of decolorization (Fig. 4).

Effect of temperature

The decolorization activity significantly increased with the increase in temperature reaching the maximum value at 30 °C (60.3%) then began to decline (Fig. 5).

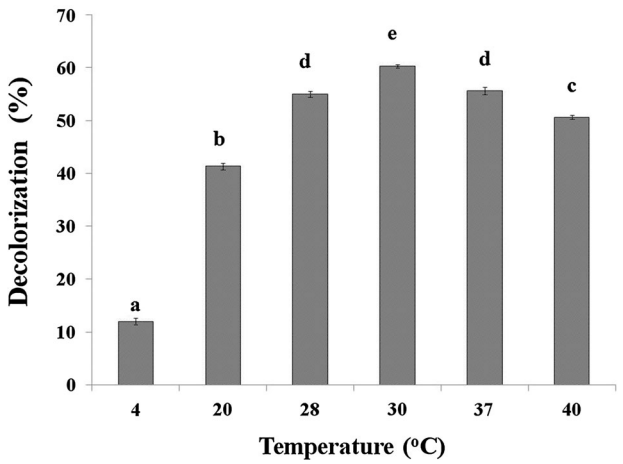

Fig. 5 Effect of Temperature

Effect of initial pH value

In acidic conditions, the percentage of decolorization increased significantly with the increase in pH from 4 to 5

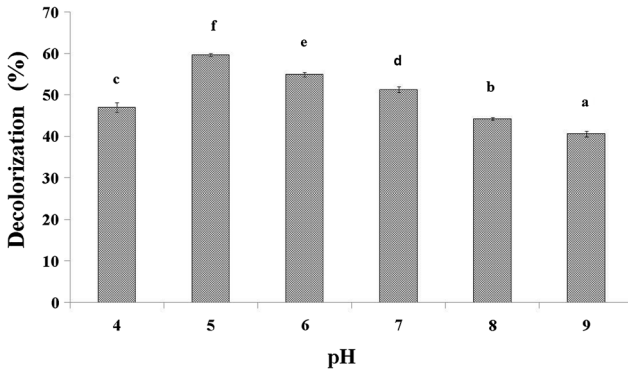

Fig. 6 Effect of initial pH value

showing the maximum value at pH 5 (59.6%) then began to drop (Fig. 6).

Microbial toxicity study

The results revealed that MB exhibited more inhibitory effect than the by-products against all tested bacterial strains (Table 1). Subsequently, the degradation products were less toxic than the MB to an exploited microorganism. As well as, Gram-positive bacteria were more sensitive to MB than Gram-negative bacteria.

Mode of action of MB decolorization

Figure 7 displays the decrease of UV–visible spectra over periods of time (0, 24, and 48 h). In addition a new peak was appeared at 550 nm that is might be corresponding to a new compound. Noteworthy, Fig. 8 illustrated that heat-killed bacterial cells and live bacterial cells showed 43 and 52% of decolorization as a maximum value after 24-h incubation, respectively.

Decolorization of MB by live and heat-killed cells

Heat-killed bacteria were prepared according to Noethe and Manstein (1998). Bacterial culture was grown overnight in MB doped-nutrient broth, centrifuged at 6000 rpm for 5 min and then washed three times with KK_2 buffer (6.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2). The last pellet is resuspended in 1/10th of the initial volume of KK_2 and an aliquot is diluted to measure the OD650. The volume of the bacterial suspension is then adjusted to a density of 1012 cells/ml (assuming that an OD650 of 0.1 corresponds to 108 cells/ml). Cells are heat-killed at 70 °C for 10 min and then added to 100 ml NB media containing 100 mg/l MB. Consequently, live bacterial cells inoculated to another 100 ml NB media containing 100 mg/l MB. Both flasks were incubated at 37 °C. Aliquots of 3 ml of each flask withdrawn at zero time and every 12 h and checked at 665 nm.

Discussion

In recent years, a number of studies have focused on some microorganisms, which are able to biodegrade and biosorb dyes in wastewaters. A wide variety of microorganisms capable of decolorizing a wide range of dyes includes bacteria, fungi, and algae. The use of bacteria for the removal of synthetic dyes offers considerable advantages. Bioremediation is relatively inexpensive, simple and the by-products of complete mineralization are less or non-toxic. Considering this fact, the present study aimed to test decolorization of MB by bacteria isolated from soil. Bacteria showing maximum MB decolorization efficiency (53%) was selected and identified as *Stenotrophomonas maltophilia*. It is an aerobic, non-fermentative, Gram- negative bacterium and ubiquitous in aqueous environments, soil, and plants. It has also been used in biotechnology applications (Ryan et al. 2007) such as metabolizing the explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and degradation of acrylamide (Kitahara et al. 2012). In a recent study, Arshi et al. (2013) investigated that *Aspergillus niger* decolorized MB by 22.44% whereas *T. lignorum* can decolorized MB by 48.3%. In the current study, the rate of decolorization is very fast reaching the maximal value of MB decolorization (51.7%) after the 1 st day of incubation. This result is matching with what obtained by Ong et al. (2005) and Shah et al. 2013 who found that the color of MB begins to disappear within a few minutes after incubation due to reduction by bacterial biomass reaching 28% after 24-h incubation. On the other hand, *P putida* decolourized 69% of MB after 7 days of incubation (Fulekar et al. 2013). In this study, MB decolorization significantly increases with the increase in MB concentration reaching the maximum value at 5 mg/l (61.3%). This may be explained by the fact that the available binding sites on the surface of bacteria quickly reached saturation at a very high initial concentration of MB. This result is inconsistent with the findings reported by other researchers (Mohamed et al. 2012; Rao et al. 2013) who observed that the removing of methylene blue decreased by increasing the initial concentration of MB in aqueous solution. However, Ong et al. (2005) investigated that the increase of MB concentration had resulted in the increase of maximum dye removal rate. Notably, the temperature is an important factor for the activity of bacteria. Where the temperature required to the maximum rate of color removal tends to correspond with the optimum bacterial growth tempera-

Table 1 Microbial toxicity study of methylene blue and metabolites obtained after decolorization

Bacteria	Diameter of inhibition zone (mm)	
	Methylene blue at 100 mg/L	By-product at 100 mg/L
S. aureus	30	10
P. aeruginosa	25	10
Sheigella sp	23	11
Klebsiella sp	20	N.I.
Proteus sp	20	12

N.I. no inhibition

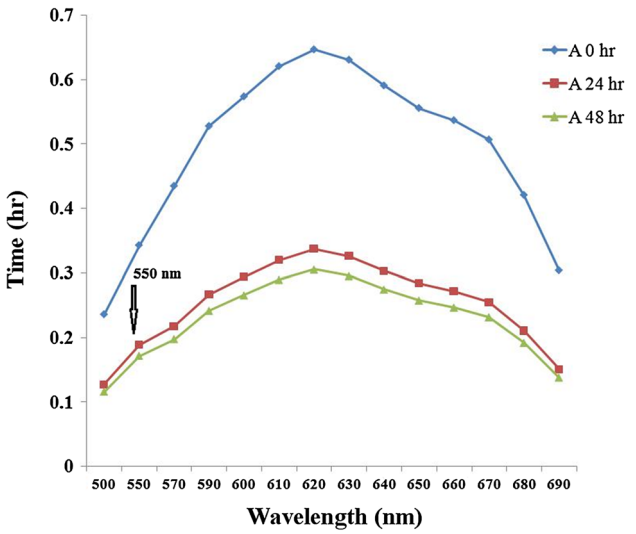

Fig. 7 Spectral analysis of MB after 0, 24 and 48 h with *Stenotrophomonas maltophilia*

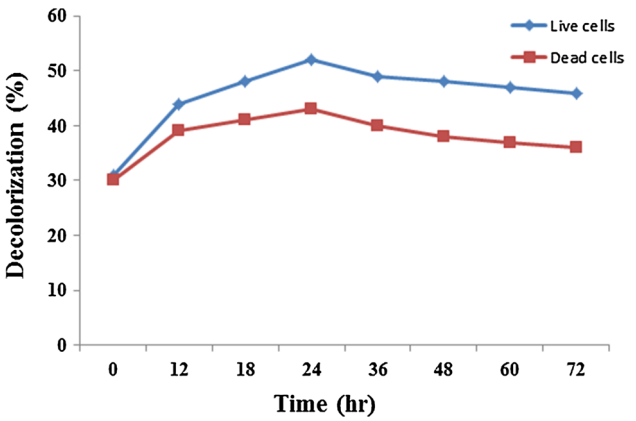

Fig. 8 Decolorization of methylene blue by both live and heat-killed cells of *Stenotrophomonas maltophilia*

ture of 30 °C (60.3%). This result is in concordant with Benetoli et al. (2011). While Liu et al. (2014) suggested that the maximum dye removal is achieved at 40 °C. At lower temperatures, the Brownian motion of bacteria is weak, which reduces the contact of bacteria with the MB, leading to less bio association. On the other hand, much higher temperatures also weaken the decolorization, which may be due to inactivation of intracellular enzymes assisting in the active transport of the bacteria, reducing the bacterial activity. Appropriately, raising the temperature could enhance the bacterial adsorption activity, thereby improving the decolorization efficiency (Benetoli et al. 2011). Bacterial culture generally exhibits maximum decolorization at pH 5 (59.6%) whereas the rate of color removal tends to decrease rapidly at highly acidic or alkaline pH. The results are in agreement with Rao et al. (2013) who reported that the percentage of MB decolorization significantly increased from pH 4 to 7 then it is began to decrease. This may explained by the fact that at low pH, protons in solution compete with the cationic dye for the binding sites on the surface of bacteria. At higher pH, hydroxyl groups in solution complex with cationic dye preventing their adsorption by microbial sorbents (Rao et al. 2013). In this study, the degradation products were less toxic compared to the MB to the exploited microorganism (*S. aureus, P.aeruginosa, Shigella sp, Klebsiella sp and Proteus sp*). These results are consistent with what reported by Kalyani et al. (2008) and Przystas et al. (2015) who observed that metabolites were non-toxic as compared to dye sample. In spite of the effectiveness of the biological treatments, in some cases microorganisms can transform dyes into compounds more toxic than the original compound (Karigar and Rao 2011). Consequently, there is an urgent need for assessing the toxicity of product after the biological treatments. For this purpose, a toxicological assay such as microbial toxicity used to determine the toxicity of degradation products (Kalyani et al. 2008). In this work, mechanism of decolorization was confirmed by both biosorption and biodegradation (Aravindhan et al. 2007; Kumar et al. 2007). In the former case, MB was adsorbed onto the surface of bacterial cells, this is judged by the examination of the absorption spectrum where most peaks decrease approximately with time in proportion to each other. Meanwhile, the other part of MB removal is attributed to biodegradation, because a new UV–Vis absorbance peak was appeared at 550 nm (Chen et al. 2003; Yu and Wen 2005). In the case of heat-killed bacterial cells, 43% of decolorization was recorded after 24-h incubation, which might be due to the adsorption on dead bacterial cells, and this was also confirmed by the presence of blue colored cell pellets. Biological adsorption is the result from electrostatic attraction between the bacteria and MB where bacteria are negatively charged due to the presence of (PO_4) groups on their surfaces, while MB is positively charged. Therefore, the

electrostatic attraction causes MB to adhere onto the bacterial surface. Additionally, Uv- Vis spectrum shows the migration and decline of the peaks of carboxyl groups, HOH and $–NH_2$ during the decolorization process, indicating that these functional groups can react with MB (Liu et al. 2014). While in the case of live cells, 52% of decolorization was achieved after 24 h and the cell pellets were not pigmented. These findings provided an obvious evidence for biodegradation of MB by *Stenotrophomonas maltophilia*, rather than inactive surface adsorption. In this work, the decrease of UV–visible spectra of MB with time revealing adsorption of MB on *Stenotrophomonas maltophilia*. Biological adsorption is resulted from electrostatic attraction between the bacteria and MB where bacteria are negatively charged due to the presence of PO_4- groups on their surfaces, while the MB is positively charged. Therefore, the electrostatic attraction causes MB to adhere onto the bacterial surface. Additionally, the UV-Vis spectrum shows the migration and decline of the peaks of carboxyl groups, HOH and $–NH_2$ during the decolorization process, indicating that these functional groups can react with MB.

Conclusion

It is concluded that *Stenotrophomonas maltophilia* strain kilany_MB has the potential to MB decolorization at 30 °C at pH 5 using 5 mg/l after the 1st day of incubation by adsorption. MB is removed by two ways biodegradation and adsorption on bacterial surface. The most economical importance from the narrow decolorization potential difference between live and dead cells is that dead bacterial cells can be used to remove MB without environmental pollution as done by live cells. Hence, *Stenotrophomonas maltophilia* is one of the most promising strain that help in the low-cost and naturally renewable technology.

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Compliance with ethical standards

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