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BIOREMEDIATION OF CHEMICAL POLLUTANTS- CONTAMINATED WATER

A-BIODECOLORIZATION OF CRYSTAL VIOLET CONTAMINATED WATER BY *Pseudomonas geniculata*

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ABSTRACT

Biodcolorization of crystal violet (C V) was investigated in aquatic system. C V decolorizing bacterium (designated isolate AT 17) was isolated from effluent sample using enrichment technique by clear zone formation. Based on morphological, physiological and 16S rDNA, this bacterium was identified as *Pseudomonas geniculata*. It was capable of using C V as a sole source of carbon. Nutrient agar medium inhibited C V decolorization but mineral salt medium (MSM) increased C V decolorization. C V decolorizing by *P. geniculata* was found to be optimum at pH 7 and 35°C. Additional carbon sources (i.e., glucose) and nitrogen sources (i.e., peptone, yeast extract and beef extract) inhibited completely C V decolorization. On the ather hand, ammonium chloride and ammonium sulphate increased the C V decolorization. Ammonium chloride was the best inorganic nitrogen source in C V decolorization. Incubation period for 7 days was the optimum for C V decolorization. *P. geniculata* was able to decolorize 99% of C V completely in liquid medium at pH 7 and 35 °C after 7 days comparing with uninoculated medium(control). There was no toxicity of detected C V after 7 days of incubation with *P. geniculata* on *Bacillus subtilis* as microbial bioassay test. The dissipation of C V was coinciding with increasing *P. geniculata* biomass in C V contaminated water. This study has shown that *P.geniculata* C V could be applied to remediate Chemical pollutants - contaminated water.

Keywords: Crystal violet, decolorization, Microorganisms.

INTRODUCTION

Crystal violet falls in the class of triphenylmethane dyes which are commonly used in the clothing industry to dye wool, silk and cotton (Kim *et al*., 2005). It is well known as a biological stain, yet also possesses medicinal properties. It has been used in the treatment of pinworms and as a topical agent as well as being added to feed to prevent fungal growth (Azmi *et al*., 1998). The toxicity of effluent is because of the presence of dye or its degraded products which are mutagenic or carcinogenic. Therefore, the treatment of industrial effluents contaminated with dye becomes necessary prior to their final discharge to the environment. Various kinds of physico-chemical methods are in use for the treatment of wastewater contaminated with dye. These methods are not environment friendly and cost-effective and hence become commercially unattractive (Nigam *et al*., 1996 and Azmi *et al*., 1998).

Therefore, the purpose of this study was to isolate and characterize of the Crystal violet decolorizing microbial strain and its use in bioremediation of Crystal violet - contaminated water.

MATERIALS AND METHODS

Chemicals:

C V was donated by Agric. Microbiol. branch, Agric. Botany Dep., Fac. of Agric., Kafrelsheikh Univ., Egypt.

Microbial decolorization of the crystal violet:

Sampling and analysis of the effluent

The effluent samples (the sludge and the wastewater) were collected from the Station Exchange Aldoakhlah and Nasr Company for Textile orchard each of these areas Almehalla Alkobra. The samples were transported to the laboratory at 4°C. Minimal Medium as mineral salt medium (MSM) and Nutrient agar medium were used through this study as described by (Brunner *et al*., 1980).

Isolation, screening and identification of dye decolorizing microorganisms from effluent.

Crystal violet decolorizing bacterium was isolated from effluent sample using enrichment technique use 100 ml sterilized mineral salt liquid medium (MSL) in 500 ml glass bottle containing 50 mg /L from C V as a sole source of carbon. Dilution series were prepared after the final time from enrichment culture in a glass test tube containing 9 ml MSL liquid medium up to $1{:}10^{-6}$ and then 100 µl of the third enrichment culture was transferred onto MSA plates containing crystal violet (50 mg/L) and spread evenly with sterilized glass beads and were incubated at 28◦C for 7days monitored for appearance of colonies by clear zone formation. (Belal, and El-Nady, 2013 and Shah *et al*., 2013)

Screening for Crystal violet decolorizing isolates by applying the clear zone test method.

Mineral salt agar medium (MSA) plates containing C V (50 mg /L) were inoculated with a loop full of bacterial culture from cultures of the strains. The increase in clear zone diameters developing on the MSA plates was followed up periodical (3 day and 7 day) and measured by slide gauge. Experiments were made in tri-plicates. At least three replicates experiments were performed with the unspotted plate as a control.

Identification:

The efficient selected Crystal violet (C V) degrading bacterial isolate was identified depending upon morphological and physiological characteristics as described by John (1984) and Bergy's manual of systematic bacteriology. (1984) as well as 16S rDNA (Boye *et al*., 1999). This technique was performed by sigma, Cairo, Egypt and GATC Company.

Effect of different culture conditions on decolorization of Crystal violet (C V) by *P. geniculata* strain AT 17

1-Media

MSA and NA were containing C V (50 mg /L) were inoculated a loop full of cultures from the strain and incubated at 28°C for 7days.

2-pH

The pH of the inoculated MSA was adjusted to (4, 5, 7, 8 and 9). The effect of pH on dye decolorization was checked after 7 days.

3-Temperature

The inoculated MSA was incubated at various temperatures (20, 30, 35 and 40°C). The effect of temperature on dye decolorization was checked after 7 days.

4-Nitrogen sources

To study the effect of nitrogen sources on decolorization of C V, MSA with concentrations of nitrogen such as $(NH_4)_2SO_4$, (NH_4Cl) (0.5, 1, 1.5 and 2%) and peptone(5g/L), Beef extract(3 g/L) as well as yeast extract(1g/L) at pH 7 and 35ºC.

5-Incubation Time

To determine the effect of incubation period, at the optimum culture conditions (pH 7 and 35 ºC) for different incubation period (3, 5, 7, 9 and 11 days).

Decolorization of Crystal violet (C V) in MSL by *P. geniculata* strain AT 17

The bacterial cell suspension (10^7cfu/ml) was then used to inoculate 100 ml MSL containing (50 mg/L) of the dye. The cultures were incubated at 35 °C, pH 7 and 150 rpm for 7 days. The test principle is based on the decrease of the optical density of the dye during the dyes decolorization. The percentage of decolorization of the dye was determined photometrically using UV–vis pectrophotometer at OD_{590nm} for C V. Control flasks of equal volume of MSL and dye without any microbial inoculation were incubated in parallel at all intervals to assess abiotic loss. During the experiment, samples were collected (1 ml) periodically at 0, 1, 3, 5 and 7 day for estimation of viable cell count (cfu/ml) by using dilution series onto MSA containing of the dye and to determine the decolorization using UV–vis pectrophotometer. All the experiments were done in triplicates. The percentage of decolorization was calculated as by (**Yatome *et al*., 1993).**

Toxicity test Crystal violet (C V) decolorizing products

The bioassay of the remaining C V toxicity was performed on the aqueous solutions after 7 days of incubation with *P. geniculata* strain AT 17. *Bacillus subtilis*, as gram positive bacterium was used as the test organism. The toxicity was determined by recording of inhibition zone in growth of *B. subtilis* comparing to control treatment (untreated). Plates were incubated at 35 °C for 7 days.

Statistical analysis

Data were calculated as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA). Probability of 0.05 or less was considered significant. The statistical package Program was used for all chemometric calculations (Parshetti *et al*., 2011).

RESULTS AND DISCUSSION

The most widely used screening method for Crystal violet (C V) decolorizing organisms is the so called “clear zone” method. The extracellular hydrolyzing enzymes secreted by the target organism decolorize the suspended dye in the agar medium into water soluble products thereby producing zones of clearance around the colony. The main advantage of this test is that it is generally fast, cheap and simple, and allows the simultaneous performance of a great number of parallel tests (Belal, 2003).

Isolation and screening of dye decolorizing microorganisms

From the microbial sources (textile effluent) a total of 19 morphologically different C V decolorizing isolates were obtained.

Among 19 bacterial isolates, One bacterial isolate designated as AT 17 achieved higher C V decolorization (wider clear zone) comparing with the other isolates (Fig.1).

The results were compared with those obtained with noninoculated medium (controls).

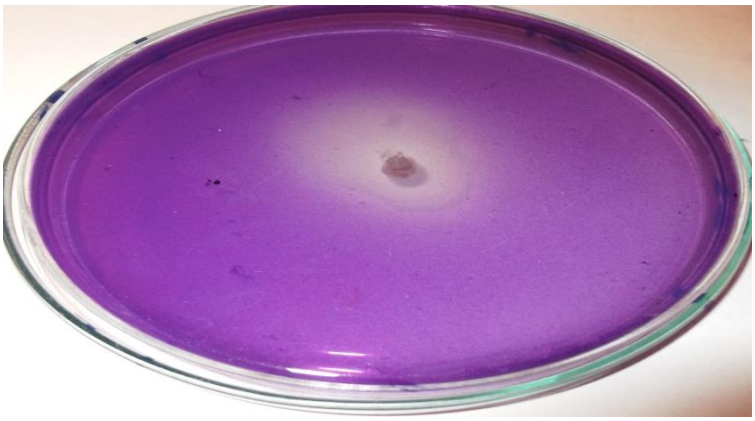


Fig. 1: Clear zone formation on MSA containing Crystal violet (C V) by The isolated bacterium.

Identification of the efficient of Crystal violet (C V) decolorizing isolate.

This bacterial isolate (AT 17) was identified according to morphological, physiological as well as using analysis of 16S rDNA **(Boye *et al*., 1999).** This efficient C V decolorizing isolate (AT 17) was gram-negative, motile, short rods and oxidase positive. According to the 16S rDNA analysis, the phylogenetic tree of the C V decolorize bacterium isolate AT 17 and related bacterial species based on the 16S rDNA sequence is provided in Fig (2). It can be clearly seen that the *Pseudomonas* sp AT 17. as C V decolorize bacterium was included in the genus *Pseudomonas* and closely related to the

species *geniculata*. It showed the highest sequence similarities with *Pseudomonas geniculata* strain ATCC 19374 (100 %) Figure (2).

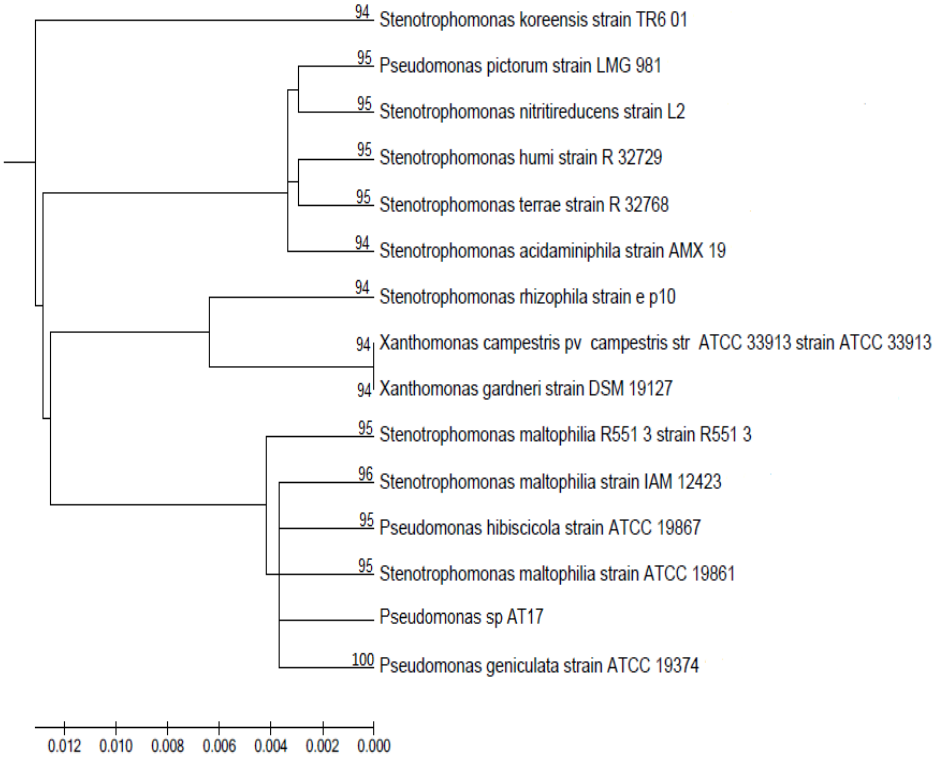


Fig. (2): Phylogenetic dendrogram obtained by distance matrix analysis of 16S rDNA sequences, showing the position of strain *Pseudomonas geniculata* AT 17 among phylogenetic neighbors. The scale bar indicates 0.02 substitutions per nucleotide position.

Effect of different Media on decolorization of Crystal violet (C V) by *P. geniculata* AT 17

MSA and Nutrient agar containing C V (50 mg/L) media were used to know the effect of the media on C V decolorization by clear zone formation. *P. geniculata* strain AT 17 formed only clear zone on MSA but it was observed that no clear zone was formed on nutrient agar medium containing C V and this may be due to presence of nutrient components in nutrient agar medium suppressed C V decolorization enzymes which could be decolorize C V.

The results were compared with those obtained with noninoculated medium (control) incubated in the respective media. The noninoculated control showed no clear zone. MSA was selected for the further experiments.

Effect of different pH on decolorization of Crystal violet (C V) by *P. geniculata* AT 17

The influence of pH on decolorization of C V in MSA with *P. geniculata* strain AT 17 by using clear zone formation is shown in Table (1). The highest C V decolorization with strain was achieved at pH 7 with wider clear zone formation followed by PH 8 C V was not decolorized by strain at pH4 and 5 (acidic pH) as well as at pH9 (alkali pH). This results are in agreement with Adedayo *et al*. (2004) who showed that *Klebsiella pneumonia* RS-13, which completely degraded Methyl Red in the pH range of 6–8**.**

Table (1). Effect of different pH on decolorization of Crystal violet (C V) by *P. geniculata* strain AT 17

pH / Strain	Diameter of decolorization zone (clear) zone (mm) at different pH				
	4	5	7	8	9
Pseudomonas geniculata AT 17 + C V	0	0	37±0.05	25.5±0.05	0
Control (un-inoculated)	0	0	0	0	0

Effect of different Temperature on decolorization of Crystal violet (C V) by *P. geniculata* strain AT 17

The effect of different temperatures on decolorization of C V is shown in Table (2). while the highest decolorization was achieved at 35 °C, (wider clear zone). The dye decolorization activity of the strain was found to decrease with increasing incubation temperature over 35°C. and least decolorization at 30, respectively. This can be explained that temperature can influence enzyme conformations which in turn effects catalytic activity (Staub and Denes, 1969).

Table (2): Effect of different Temperature on decolorization of Crystal violet (C V) by *P. geniculata* strain AT 17.

Temperature / Strain	Diameter of decolorization zone (clear) zone (mm) at different temperature (°C)			
	20	30	35	40
P. geniculata AT 17 + C V	0	32±0.5	43±0.5	22.5±0.05
Control (un-inoculated)	0	0	0	0

Effect of mineral nitrogen sources and components of nutrient broth medium on decolorization of Crystal violet (C V) by *P. geniculata* strain AT 17.

The effect of additional Nitrogen sources (such as 1% ammonium sulphate and ammonium chloride) and components of nutrient medium (organic nitrogen) (peptone 5gm/L, beef extract 3gm/L and yeast extract 1gm/L) is shown in Table (3). Mineral nitrogen sources (such as 1% ammonium sulphate and ammonium chloride) were the best in C V decolorization (clear zone formation) by *P. geniculata* strain AT 17. Decolorization was achieved with ammonium chloride more than ammonium sulphate. Components of nutrient broth medium suppressed the decolorization of C V by *P. geniculata* strain AT 17.

Table (3). Effect of nitrogen sources on decolorization of Crystal violet (C V) by *P. geniculata* strain AT 17.

Treatments / Strain	Diameter of decolorization zone (clear zone) (mm) on different nitrogen sources				
	MSA + Pepton (5gm/L)	MSA + Beef extract (3g/L)	MSA + Yeast extract (1g/L)	MSA + NH_4Cl (1%)	MSA + $(NH_4)_2SO_4$ (1%)
P.geniculata AT 17 +C V	0	0	0	32±0.1	11±0.1
Ctrol	0	0	0	0	0

Effect of different concentrations from ammonium sulphate and ammonium chloride on decolorization of Crystal violet (C V) by *P. geniculata* strain AT 17

Crystal violet (C V) decolorization by *P. geniculata* strain AT 17 when grown on different concentrations (0.5, 1, 1.5 and 2%) of the best N source (ammonium sulphate and ammonium chloride) in presence of the best carbon source (C V) are presented in Tables (4). It was revealed that ammonium chloride at 1.5% was the optimum level for P. geniculata AT 17. Parshetti *et al.* (2011) who showed that Crystal Violet decolorization of 100% was observed with 0.1% NH4Cl within 5 hr.

Nigam *et al*. (1996**)** reported on how the highest decolorization was addition of inorganic nitrogen source (ammonium chloride or ammonium sulphate) but it decreased or inhibited completely addition organic nitrogen source (peptone or yeast extract). With fungal studies concerning dye degradation, additional nitrogen sources can either have a positive or adverse effect on decolorization. They proposed that nitrogen suppressed the enzymatic system and found that a nitrogen limiting environment stimulates enzyme production. Although it's clear that additional nitrogen reduces or inhibits decolorization.

Table (4): Effect of different concentration from ammonium chloride and ammonium sulphate on decolorization of Crystal violet by *P. geniculata* AT 17.

Treatment / Strain	Diameter of decolorization (clear) zone (mm) at different NH_4Cl or $(NH_4)_2SO_4$ concentrations (%)			
	0.5	0.1	1.5	2.0
NH_4Cl				
P.geniculata strain AT 17 + C V	11±0.2	32±0.17	57±0.2	20.5±0.15
$(NH_4)_2SO_4$				
P.geniculata strain AT 17 + C V	11±0.17	11±0.17	11±0.1	12.5±0.05
Control (un-inoculated)	0	0	0	0

Effect of different incubation period on decolorization of Crystal violet (C V) by *P. geniculata* strain AT 17

Results in Tables(5) show that the effect of incubation time on C V decolorization under optimum conditions (pH7, 35°C and NH_4Cl 1.5%). The C V decolorization (clear zone formation) increase with time dependent manner and highest C V decolorization was obtained after 7 days for growth of *P. geniculata* strain AT 17. The C V decolorization was similar at 7, 9 and 11 days after incubation time, respectively. Incubation period on decolorization of Crystal violet by *Pseudomonas putida* is 7 days **(Chen *et al*.**, 2007).

Table (5): Effect of different incubation period on decolorization of C V by *P. geniculata* AT 17.

Treatments / Strain	Diamatar of decolorization (clear) zone (mm)				
	Incubation period (day)				
	3	5	7	9	11
P. geniculata + C V	50±0.3	52.5±0.08	63±0.17	63±0.5	63±0.5
Control (un-inoculated)	0	0	0	0	0

Effect of decolorization of Crystal violet (C V) in MSL by *P. geniculata* strain AT 17 in aquatic system.

Additionally to the qualitative clear-zone tests, the degradation potential of *P. geniculata* strain AT 17 was characterized via percentage of decolorization determination of dyes in MSL. The decolorization potential obtained for C V which obtained it with *P. geniculata* strain AT 17 at 35°C pH7 after 7 days. The results in Table (6) summarize the differences of decolorization for C V by *P. geniculata* strain AT 17 in aquatic system. In the present study, dyes decolorization was increased with increasing the incubation period in the medium amended with *P. geniculata* strain AT 17 in all cases. The application of C V decolorizing strain increased the number of cultivable C V - decolorizing cells in the the aquatic system during the 7 days of incubation (Table 6). The experimental results were compared with the noninoculated (control), which showed less decolorization of the dye due to a biotic stress.

Table (6). Decolorization of C V by *P. geniculata* strain AT 17 in aquatic system

Treatments / Strain		% Remaining of dyes and CFU/ml				
		C V				
		0 day	1 Day	3 day	5 day	7 Day
Control (un-inoculated) (C V)	%Remaining	100±0	100±0.0	98±0.3	96.9±0.2	95.5±0.2
P. geniculata AT 17+ C V	%Remaining	100±0	61.4±0.2	47.7±0.2	30±0.2	1 ±0.2
	CFU/ml	١٥٥*10^{٧}	٢٦٦*10^{٧}	٢٤٧*10^{٧}	١٩٠*10^{٧}	١٥٥*10^{٧}

Chengalroyen (2011) found that, Bacterial strain *Amycolatopsis orientalis* SY6 was able to decolorize amido black, janus green and several triphenylmethane dyes effectively. This suggests that the same enzyme/s might be involved in the reduction of all these dyes. In general, it was noticed that bacteria capable of degrading C V were also able to mineralize other triphenylmethane dyes such as malachite green, brilliant green and basic fuchsin. This allows for the speculation that either the enzymes responsible for triphenylmethane class degradation are more lenient in their substrate

interactions or the dye structural differences are minor and hence easier to accommodate. Additionally, research done on *P. pseudomallei* found that decolorization of triphenylmethane dyes was not linked to the molecular weight or permeability of the compound through the membrane (Azmi *et al*., 1998).

Toxicity evaluation:

Fig. (3). Show 99 % decolorizing of C V by *P. geniculata* strain AT 17 under the optimum conditions. Toxicity of the remaining C V in the aqueous solution after 7 days of incubation with the tested microbial strain was evaluated using *B. subtilis* as a microbial bioassay test. *P. geniculata* strain AT 17 exhibited the highest decolorization for CV. The results showed that the supernatant of C V after 7 days of incubation with *P. geniculata* AT 17 had no toxicity which could be detected against *B. subtilis* as a test organism. The obtained results were compared with control treatment (C V only) which revealed 100% of inhibition against *B. subtilis* under the same conditions (Fig. 4). This implies that the aqueous solution spiked with C V was completely detoxified after 7 days of treatment with *P. geniculata* strain AT 17. It has been reported that these dyes inhibit cell growth by interfering with nucleic acid synthesis thus decreasing protein synthesis (Azmi *et al*., 1998). Yatome *et al*. (1993) were the first to elucidate the degradation of C V by *Nocardia* spp.

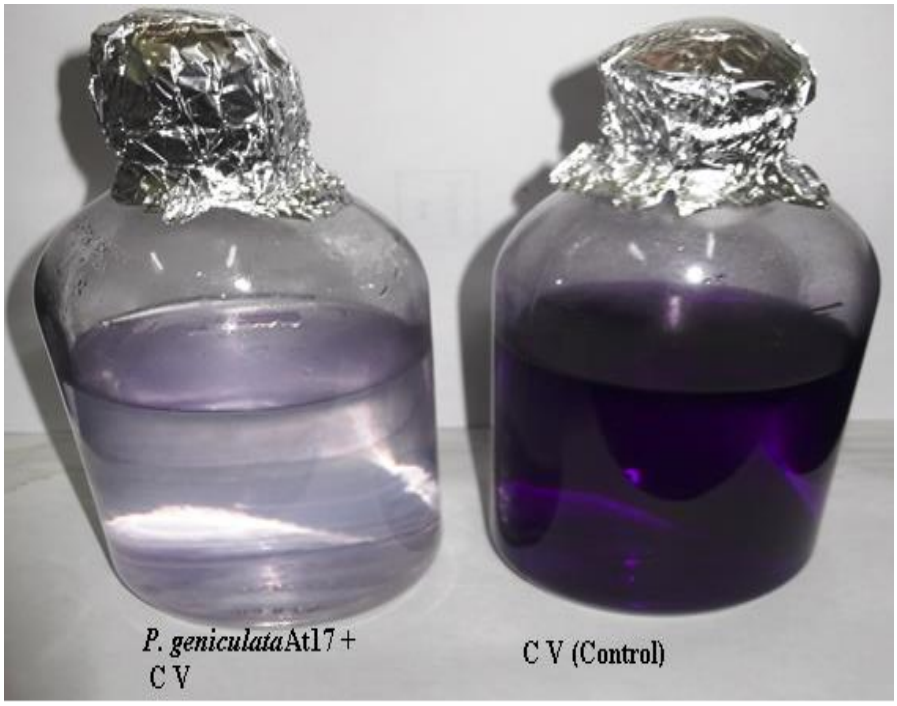
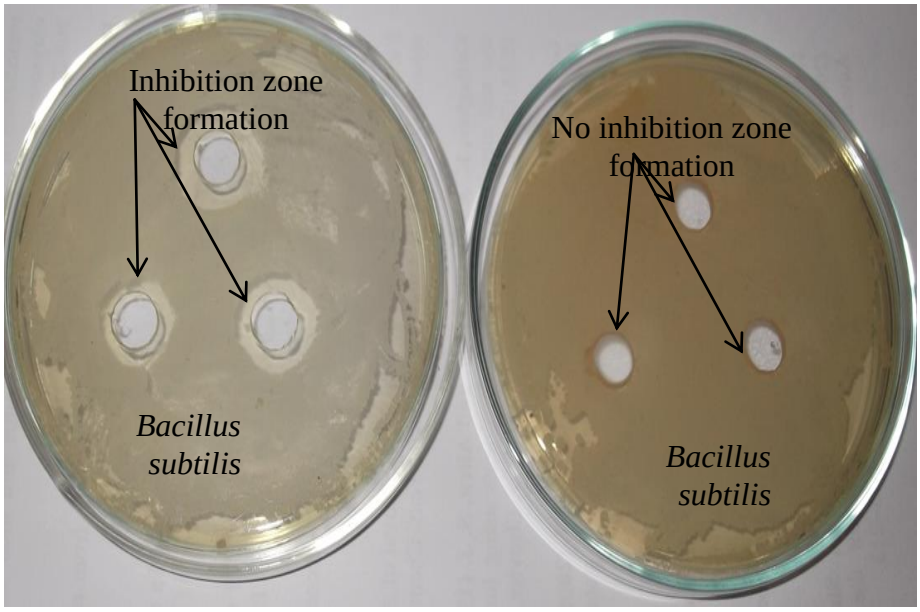


Fig. (3). Decolorization of C V by *P. geniculata* AT 17 in aquatic system.



C V (Control) Inhibition zone formation

C V + *P. geniculata* At17

Fig. (4). Biodetoxfication of C V by *P. geniculate* AT 17 in aquatic system against *B. subtilis* as bioassay organism test on nutrient agar medium.

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المعالجة البيولوجية للملوثات الكيماوية فى المياه
أ- الإزالة البيولوجية لصبغة الكريستال البنفسجي الملوثة للمياه بواسطة بكتيريا بسيدوموناس جينكيولاتا

فتحي إسماعيل حوقه[١] ، السيد بلال عبد المنطلب بلال[٢] ، محمد عبد الله العوضى سليم[١] و عطيه ابراهيم جاد[١]

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٢ - قسم النبات الزراعي ، كلية الزراعة ، جامعة كفر الشيخ ، مصر.

تم دراسة عملية إزالة اللون لصبغة الكريستال البنفسجي الملوثة للماء من مصادر مختلفة مثل مصانع النسيج بواسطة البكتيريا حيث أنه تم عزل بكتيريا مزيلة للون صبغة الكريستال البنفسجي (رقم كودي AT 17) بطريقة الامداد الغذائي من عينة مياه صرف باستخدام الهالة الشفافة (منطقة التحلل الشفافة). وتم تعريف البكتيريا إعتماداً على الصفات المورفولوجية والكيموحيوية والوراثية باستخدام 16S rDNA على أنها بسيدوموناس جينكيولاتا *Pseudomonas geniculata*. وتستطيع هذه السلالة استخدام صبغة الكريستال البنفسجي كمصدر للكربون . ولدراسة أفضل الظروف لعملية إزاله اللون بواسطة هذه السلالة وجد أن أفضل بيئة هي البيئة المعدنية لإزالة الكريستال البنفسجي بعكس بيئة الأجار المغذي التي منعت إزالة اللون تماما ، كما وجد أن رقم الحموضة ٧ ودرجة حرارة ٣٥°م هى الأفضل لنشاط الميكروب فى عملية الإزالة. كما ثبط الجلوكوز أيضا كمصدر للكربون والبيبتون ومستخلص الخميرة ومستخلص اللحم كمصدر للنيتروجين العضوي لعملية إزالة لون الكريستال البنفسجي تثبيطا كاملا ولكن وجد من ناحية أخرى أن كل من كبريتات الأمونيوم وكلوريد الأمونيوم زادت من عملية إزالة لون الكريستال البنفسجي كلوريد الأمونيوم كان أفضل مصدر نيتروجين غير عضوى فى ازالة اللون لصبغة الكرستال البنفسجى.

كما اتضح أيضا أن افضل فترة تحضين لعملية الإزالة اللونية لصبغة الكرستال البنفسجى هى ٧ أيام. أظهرت النتائج أيضا أنه قد تم إزالة لون الكريستال البنفسجي بنسبة تصل إلى ٩٩% بيكتيريا بسيدوموناس جينكيولاتا *Pseudomonas geniculata* في البيئة السائلة مقارنة بالبيئة السائلة غير الملقحة والمستخدمة ككنترول. كما نلاحظ عدم وجود سمية لصبغة الكريستال البنفسجي في بيئة الأجار المغذى التي تم تحضينها مع بكتيريا بسيدوموناس جينكيولاتا *Pseudomonas geniculata* لمدة ٧ أيام على بكتيريا باسيليس سابتليس *Bacillus subtilis* كميكروب مستخدم للتقييم الحيوي.

وأوضحت النتائج أن إزالة اللون كان متوافقا مع زيادة الكتلة الحيوية لبكتيريا بسيدوموناس جينكيولاتا *Pseudomonas geniculate* في البيئة السائلة.

وتبين هذه الدراسة أن بكتيريا بسيدوموناس جينكيولاتا *P. geniculata* يمكن استخدامها تطبيقيا للمعالجة البيولوجية للملوثات الكيماوية فى المياه.