

Plasmid-Mediated Dimethoate Degradation by *Bacillus licheniformis* Isolated From a Fresh Water Fish *Labeo rohita*

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The *Bacillus licheniformis* strain isolated from the intestine of *Labeo rohita* by an enrichment technique showed capability of utilizing dimethoate as the sole source of carbon. The bacterium rapidly utilized dimethoate beyond 0.6 mg/mL and showed prolific growth in a mineral salts medium containing 0.45 mg/mL dimethoate. The isolated *B. licheniformis* exhibited high level of tolerance of dimethoate (3.5 mg/mL) in nutrient broth, while its cured mutant did not tolerate dimethoate beyond 0.45 mg/mL and it was unable to utilize dimethoate. The wild *B. licheniformis* strain transferred dimethoate degradation property to *E. coli* C600 (Na⁺, F⁻) strain. The transconjugant harbored a plasmid of the same molecular size (approximately 54 kb) as that of the donor plasmid; the cured strain was plasmid less. Thus a single plasmid of approximately 54 kb was involved in dimethoate degradation. Genes encoding resistance to antibiotic and heavy metal were also located on the plasmid.

INTRODUCTION

Dimethoate is used extensively in agriculture and public health as an effective replacement of its oxygen analog, omethoate, which has been banned in many countries because of its higher mammalian toxicity. Dimethoate is of particular concern to those exposed occupationally during manufacture, formulation, and use [1]. It is acutely toxic [2], is a suspected human teratogen, and has possible links to cancer [3]. Although the concentration of dimethoate required to cause acute toxicity in humans would generally be higher than this found in the environment, the presence of even low levels might pose chronic health problems for both human being and natural faunas.

The microbial degradation of hazardous waste offers a promising strategy by which such chemicals may safely be detoxified. Low level of degradation of dimethoate (0.005 mg/mL) by *Proteus vulgaris* from the Ganges at

Sreerampore, India, has been reported [4]. Dimethoate has been reported to exert selection pressure in aquatic bodies giving rise to dimethoate-resistant bacterial population in the environment [5]. Herein, we isolated a *Bacillus licheniformis* from the intestine of a fresh water fish, *Labeo rohita*. The aim of the present work was to characterize the isolated bacteria for its capability to utilize a high level of dimethoate as the sole source of carbon and energy.

MATERIALS AND METHODS

Isolation of microorganism and enrichment medium

A freshly killed fish, *L. rohita* (about 80 gm), was taken as the source of bacterial strain. The fish was treated with 3% lysol followed by repeated washing with sterile distilled water. The intestine of the fish was aseptically dissected out and homogenized in sterile normal saline, and 0.5 mL of the homogenate was inoculated into 50 mL mineral salt (MS) solution (see [6]) in a sterilized 250 mL conical flask containing KH₂PO₄ (0.2g), K₂HPO₄ (0.8g), MgSO₄·7H₂O (0.2 g), CaSO₄ (0.1g), (NH₄)₆Mo₇O₂₄·4H₂O (0.001 g), and (NH₄)₂SO₄ (5.0 g/100 mL). The medium was supplemented with 0.1 mg/mL of dimethoate as a carbon source, and incubated at 28°C for 24 hours. Bacterial growth in the medium was determined initially by measuring optical density at 600 nm (OD₆₀₀) and by streaking the culture on nutrient agar and MacConkey agar plates.

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Establishment of the identity of the isolate

The isolated bacterium was identified on the basis of cultural, microscopical, and biochemical characteristics [7].

Preparation of bacterial inocula

The bacterial strain *B. licheniformis* was pregrown in MS solution containing dimethoate (0.1 mg/mL) and incubated at 28°C for 24 hours. The culture was centrifuged at 2000 \times g for 20 minutes. To remove residual nutrients and dimethoate, cells were washed twice by centrifugation at 2000 \times g for 20 minutes using 10 mL of MS solution. Washed cells were resuspended in MS solution, diluted serially, and inoculum was adjusted to approximately 5×10^5 CFU/mL by colony count technique. Following the same method, inocula were also determined for other strains used in the study.

Dimethoate degradation test

The degradation of dimethoate was observed by bioassay method. *B. licheniformis* isolate was grown in nutrient broth (Hi-media, India) supplemented with dimethoate (1 mg/mL) at 28°C for 72 hours, and culture filtrate was prepared. The culture filtrate was then mixed with fresh nutrient broth at various ratios, namely, 1:15, 1:7, 1:3, 3:5, 1:1, 5:3, 3:1, 4:1, and 9:1. Thus the media containing an increasing concentration of dimethoate: 0.0625, 0.125, 0.250, 0.375, 0.500, 0.625, 0.750, 0.800, and 0.900 mg/mL were obtained.

Another set was prepared with sterile nutrient broth mixed with fresh dimethoate giving final concentration in the above range (0.0625–0.750 mg/mL). The cured *B. licheniformis* strain was then inoculated to all sets and incubated at 28°C for 24 hours, and growth was confirmed by spot inoculation (10^4 CFU per spot) of the broth culture on nutrient agar (Hi-media) plates without dimethoate.

Tolerance of dimethoate

Dimethoate tolerance level for *B. licheniformis*, its transconjugant, cured mutant, and recipient were determined in nutrient broth containing dimethoate at concentrations ranging from 0.025 to 4.0 mg/mL, and an initial inocula of approximately 5×10^5 CFU/mL. After 24 hours of incubation at 28°C, bacterial growth was observed by plating from the broth culture and counting CFU.

Utilization of dimethoate

Tests for the utilization of dimethoate were carried out by growing *B. licheniformis* for 24 hours in MS solution amended with different concentrations of dimethoate (0.025–2.5 mg/mL). The rate of dimethoate utilization by *B. licheniformis* was determined at 28°C in dimethoate (0.1 mg/mL) mixed MS solution with or without yeast extract supplementation (0.01%) for up to 10 days.

Effect of yeast extract on the utilization of dimethoate

The effect of varying the levels of yeast extract on the rate of dimethoate utilization was studied for the isolated bacteria. The bacterial isolate was allowed to grow for 24 hours at 28°C in MS solution amended with dimethoate (0.1 mg/mL) plus yeast extract (0%–1% w/v) (MSDY), and in MS solution supplemented only with yeast extract (MSY). The results were interpreted in terms of viable CFU/mL in MS solution by agar dilution technique.

Antimicrobial susceptibility

Antimicrobial susceptibility tests were performed by disk diffusion method [8] in Mueller-Hinton agar (Hi-media) with an inoculum of about 10^4 CFU/spot, following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) [9]. Microbial sensitivity disks (Hi-media) were used to find out the drug resistance pattern using polymyxin B (PB) (300 unit/disk), amoxicillin (Ax) (10 μ g/disk), ceftriaxone (Rp) (30 μ g/disk), gentamicin (G) (10 μ g/disk), amikacin (Ak) (10 μ g/disk), norfloxacin (Nx) (10 μ g/disk), chloramphenicol (Ch) (30 μ g/disk), ciprofloxacin (Cp) (5 μ g/disk), tetracycline (Te) (30 μ g/disk), ofloxacin (Ofx) (5 μ g/disk), nalidixic acid (Na) (30 μ g/disk), cefotaxime (Cf) (30 μ g/disk), erythromycin (Er) (15 μ g/disk), and cefazolin (Cz) (30 μ g/disk).

Metal resistance

Sterile solutions of different metallic compounds were incorporated into Mueller-Hinton agar medium. The strains were examined by growth on plates incorporating different metals, and the inocula used were about 10^4 CFU/spot. The metal ions which were used in this study were $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}[\text{Cu}^{2+}]$ (1 mM), $\text{K}_2\text{Cr}_2\text{O}_7[\text{Cr}^{2+}]$ (0.2 mM), $\text{BaCl}_2[\text{Ba}^{2+}]$ (20 mM), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}[\text{Zn}^{2+}]$ (1 mM).

Plasmid curing

The *B. licheniformis* strain was subjected to plasmid curing at 40°C to check the loss of dimethoate resistance trait according to Anjanappa et al [10], with slight modifications mentioned in our earlier publication [4]. In this study the curing agent used was ethidium bromide (0.1 mg/mL).

Conjugation experiment

To further establish the role of the plasmid of *B. licheniformis* in dimethoate degradation, a mating experiment was performed following Miller [11] with some modifications mentioned in our earlier publications [12]. In the present study, *Escherichia coli* C600 strain (Na^+F^-) was used as a recipient, and the mating mixture in nutrient broth was incubated for 24 hours at 28°C. The transconjugants were selected on MacConkey agar mixed with Na (30 μ g/mL) and Ch (30 μ g/mL). The transconjugants were tested for the acquisition of dimethoate

resistance and its utilization as a carbon source in MS media. The transfer frequency was calculated as the ratio of the number of transconjugants obtained per recipient cell [13]. The transconjugant strain was tested for resistance to antibiotic and heavy metals.

Plasmid Isolation and agarose gel electrophoresis

B. licheniformis, its transconjugant, and cured derivative were subjected to plasmid isolation following the protocol of Kado and Liu [14], and Birnboim and Doly [15], with slight modification of both.

In the former method, heat treatment was performed at 56°C for 30 minutes and the clear lysate, obtained after treatment with phenol-chloroform, was mixed with an equal volume of isopropyl alcohol, kept at 4°C for overnight, and centrifuged at 2000 $\times g$ in 4°C. The plasmid DNA pellet after washing with 200 μL of 70% chilled ethyl alcohol was dried and dissolved in 50 μL TE buffer.

In the latter method, the harvested cells from 10 mL culture were suspended in 0.4 mL glucose-tris-EDTA solution and cells were lysed with 1 mL NaOH-SDS solution. To the lysate 0.8 mL glacial acetic acid-potassium acetate solution was added and kept in ice for 30 minutes. The plasmid DNA of *E. coli* V517 was employed as a molecular size marker.

Electrophoresis was performed on 0.8% agarose gel (Sigma, USA) in Tris-borate buffer following Maniatis et al [16]. Gel was stained with ethidium bromide (Sigma) in (0.0005 mg/mL) and documented with Gel-Doc system.

Statistical analysis

Tests of significance between initial inocula used and growth at concentrations ≥ 0.75 mg/mL in MS solution, and also the growth between MSDY and MSY, of the original *B. licheniformis*, were compared statistically using χ^2 -test.

RESULTS

The only bacterium, isolated from the fish intestine, capable of utilizing dimethoate as a sole source of carbon was a spore-forming Gram-positive rod (6–12 $\mu m \times 2 \mu m$), and it grew both aerobically and anaerobically. The strain was a lactose fermenter and showed positive results for urease, gelatin (12%) hydrolysis and production of acid from glucose and mannitol. Utilization of citrate and production of hydrogen sulfide could not be detected. Based on the above description the strain was identified as *B. licheniformis*. When treated with ethidium bromide (0.1 mg/mL in nutrient broth) for 7 days at 40°C the isolated strain no longer utilized dimethoate in MS solution.

Figure 1 shows tolerance level of dimethoate for *B. licheniformis* and its cured mutant in nutrient broth. The original strain tolerated up to 3.5 mg/mL of dimethoate; however, the growth was prolific up to 0.75 mg/mL showing high peaks in the presence of 0.45–0.75 mg/mL. Beyond 2 mg/mL, the pesticide started showing inhibitory

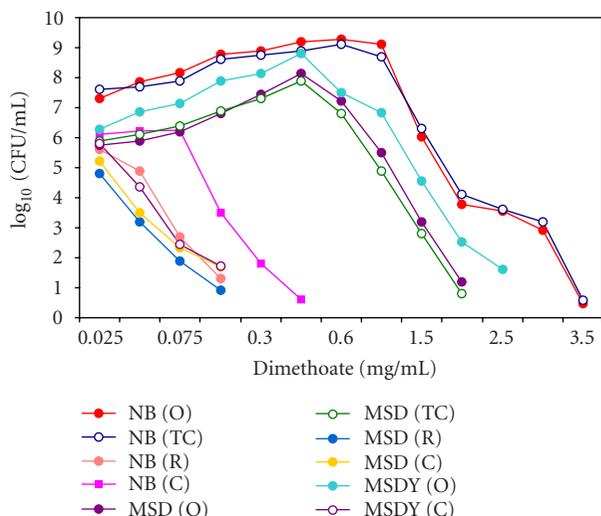


FIGURE 1. Utilization and tolerance level of isolated *Bacillus licheniformis* (O), its transconjugant (TC), cured derivative (C), *E. coli* C600 recipient strain (R), nutrient broth with dimethoate (NB), mineral salt solution amended with dimethoate plus yeast extract (MSDY), mineral salt solution supplemented only with yeast extract (MSY).

effects. Although the cured strain tolerated 0.45 mg/mL of dimethoate, 0.15 mg/mL dimethoate was inhibitory.

Growth on dimethoate provided as the sole carbon source was studied in MS solution (Figure 1). The *B. licheniformis* was found to show capability of utilizing dimethoate up to 2 mg/mL in MS solution, with maximum growth at 0.45 mg/mL, after an incubation for 24 hours at 28°C. The initial inoculum used in the method was approximately 5×10^5 CFU/mL. Based on the appearance of the number of CFU, the original *B. licheniformis* strain showed no significant growth at concentrations ≥ 0.75 mg/mL of dimethoate. When the bacterial colonies that appeared in presence of 0.75 mg/mL, 1.5 mg/mL and, 2.0 mg/mL dimethoate were compared with the initial inoculum, a significant decrease in the number of CFU was observed at 1% level of significance for 1 degree of freedom using χ^2 -test.

In the presence of 0.01% yeast extract in MS solution amended with 0.1 mg/mL dimethoate, the growth of *B. licheniformis* was accelerated. In such condition, the original and cured strains tolerated 2.5 mg/mL and 0.15 mg/mL dimethoate, respectively (Figure 1).

The strain when used for the utilization of dimethoate in the presence of yeast extract showed higher degrading activity with 0.1% yeast extract. It showed significant growth, at 1% level for 1 degree of freedom using χ^2 -test with all available concentrations (0.001–1%), in MSDY compared to MSY (Figure 2). The interesting finding of this study was that the rate of utilization of dimethoate by *B. licheniformis* increased progressively with the increase in the concentration of yeast extract up to 0.1%, but the growth was effected at concentration $\geq 0.5\%$.

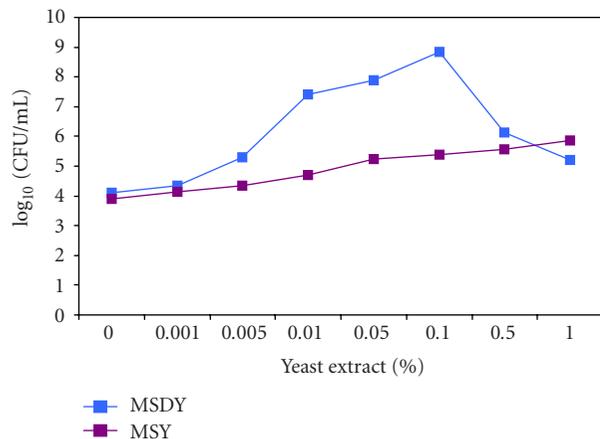


FIGURE 2. Effect of yeast extract on bacterial density as a measure of dimethoate degradation in mineral salt solution supplemented with 0.1 mg/mL dimethoate. Mineral salt solution amended with dimethoate plus yeast extract (MSDY), mineral salt solution supplemented only with yeast extract (MSY).

The growth curve for the enrichment isolate, *B licheniformis*, cultivated on 0.1 mg/mL of dimethoate is shown in Figure 3. The bacterium showed its maximum growth rate in between days 2 and 4 in MS solution not supplemented with yeast extract. However, the *B licheniformis* strain exhibited high rate of dimethoate utilization on day 3.

The results of bioassay methods for dimethoate degradation are represented in Table 1. In set 1, the cured strain of *B licheniformis* tolerated dimethoate up to 0.150 mg/mL or 0.450 mg/mL, while the higher concentrations were inhibitory. In set 2, the strain showed growth in nutrient broth with culture filtrate at ratios matched with the concentration of dimethoate up to 0.750 mg/mL mentioned in column 1 of Table 1.

Figure 4 represents the zone diameter of growth inhibition around antibiotic disks. Following NCCLS recommendation for the interpretive zone diameters, *B licheniformis* strain exhibited resistance to PB, Ax, Ch, Te, Cf, Er, and Cz antibiotics.

The *B licheniformis* isolate was conjugated with plasmidless *E coli* C600 strain to determine whether the dimethoate degradation ability could be transferred and expressed in the recipient strain (Figure 1). Originally, the recipient *E coli* C600 strain was not able to degrade dimethoate, and this strain tolerated dimethoate up to 0.15 mg/mL in nutrient broth. However, following conjugation, the recipient strain acquired, from the *B licheniformis* strain, dimethoate resistance property and showed dimethoate tolerance up to 3.5 mg/mL in nutrient broth. The transconjugant strain also acquired the ability to grow in the presence of dimethoate (2 mg/mL) as the sole source of carbon and energy and thus showing dimethoate degradation activity. The transfer frequency of the resistance properties was 3.7×10^{-8} .

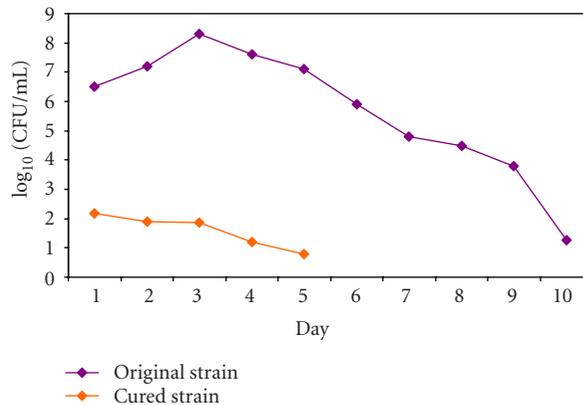


FIGURE 3. Growth curve of *Bacillus licheniformis* in mineral salt solution supplemented with 0.1 mg/mL dimethoate.

A comparison of the antibiotic and heavy metal resistant traits between *B licheniformis*, its transconjugant, and cured strain, is shown in Table 2. The transconjugant was resistant to Na, Er, Ch, Cz, Cf, Ba²⁺, and Zn²⁺. The cured derivative was sensitive to Er, Ch, Cz, Cf, Ba²⁺, and Zn²⁺.

The *B licheniformis* isolate showed a single plasmid by both the method and the plasmid band comigrated with 54 kb plasmid of *E coli* V517 marker (Figure 5). All the transconjugants harbored a plasmid of the same molecular size as that of the donor plasmid. The cured mutant strain of *B licheniformis* failed to show any plasmid band.

DISCUSSION

In the present study, a single strain of *B licheniformis*, which is capable of growth on dimethoate as a sole source of carbon and energy, has been isolated from the intestine of *Labeo rohita*. Pesticide toxicity to various biochemical, physiological, and other aspects of fishes has been studied [17, 18]. Based on the capability of pesticide utilization, the microorganisms, which live in association with fish and become resistant to a wide range of such xenobiotics exposed on them, are considered the foremost line of defense to combat the pesticide toxicity [19]. Ranjitsingh [20] reported the pesticide resistance of bacterial floras in different organs of fresh water fish *Mystus vittatus* to parrylsulfan and sicocil. Walker et al [21] reported the utilization of the pesticide, by resistant bacterial isolates, as the sole source of carbon.

The discovery of microorganisms capable of tolerating or growing in high concentrations of pesticides provides a potentially interesting avenue for treating hazardous wastes [22, 23]. In this study, *B licheniformis* tolerated dimethoate up to the concentration of 3.5 mg/mL in nutrient broth and 2 mg/mL in MS solution. The strain showed maximum growth of log_{8.13} CFU/mL (in MS solution) at concentrations 0.6 mg/mL and 0.45 mg/mL of dimethoate, respectively, after 24 hours incubation at 28°C. The maximum

TABLE 1. Results of dimethoate degradation by bioassay method. + denotes growth and – denotes no growth.

SN	Dimethoate (mg/mL) in nutrient broth	Growth of cured <i>B. licheniformis</i>	Ratio of fresh nutrient broth and culture filtrate [#]	Growth of cured <i>B. licheniformis</i>
1	0.0625	+	1:15	+
2	0.125	+	1:7	+
3	0.250	–	1:3	+
4	0.375	–	3:5	+
5	0.500	–	1:1	+
6	0.625	–	5:3	+
7	0.750	–	3:1	+
8	0.800	–	4:1	+
9	0.900	–	9:1	+

[#] refers to the ratios matched with the expected concentrations in column 1 if no degradation occurs.

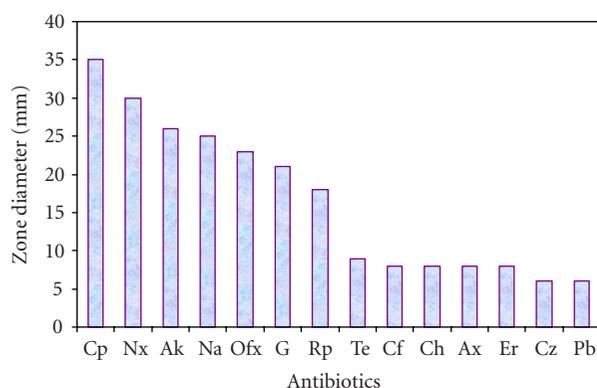


FIGURE 4. Zone diameter of growth inhibition around antibiotic disks for the strain *Bacillus licheniformis*. Polymyxin B (PB), amoxicillin (Ax), ceftriaxone (Rp), gentamicin (G), amikacin (Ak), norfloxacin (Nx), chloramphenicol (Ch), ciprofloxacin (Cp), tetracycline (Te), ofloxacin (Ofx), nalidixic acid (Na), cefotaxime (Cf), erythromycin (Er), and cefazolin (Cz).

growth of *B. licheniformis* ($\log_{8.3}$ CFU/mL), in MS solution amended with 0.1 mg/mL of dimethoate, was noted on day 3. In our previous study, we reported the capability of dimethoate degradation by an isolate of *P. vulgaris* from the Ganges river water [4]. Such different pattern of pesticide utilization might be due to variation in the ecological niches or in the biochemical nature of pesticide degradation [19, 24, 25, 26].

Interestingly, the degradation of dimethoate in the enrichment culture of *B. licheniformis* was governed by yeast extract supplement to the medium. The growth rate of the bacterium in MS solution containing 0.1 mg/mL of dimethoate increased progressively with the increase in the concentration of yeast extract; concentrations $\geq 0.5\%$, however, reversed the growth rate in the medium with dimethoate. But progressive increase of log CFU was recorded with increase in the concentration of yeast extract in the medium without dimethoate (Figure 4). An increase in $\log_{2.29}$ CFU/mL was noted between the growth of *B. licheniformis* in dimethoate containing MS

solution with yeast extract and that without yeast extract. Sharmila et al [27] reported earlier that the rate of degradation of organophosphorus pesticides (parathion, methyl parathion and fenitrothion) by soil bacteria, *Bacillus* species, was regulated by the amount of yeast extract in the medium. *B. licheniformis* is the first isolated bacteria, from the intestine of a fresh water fish, utilizing dimethoate at very high concentrations.

Bacterial plasmid plays a role in the degradation of the pesticide [4, 28]. The cured strain, in the present study, was unable to utilize dimethoate in MS medium with or without yeast extract, and the growth of the cured mutant was completely inhibited by dimethoate at concentrations 0.5 and 0.2 mg/mL, respectively, in nutrient broth as well as MS solution.

Plasmid carrying antibiotic resistance was isolated from clinical bacteria. Plasmid conferring resistance to chromate, nickel, and cadmium was recorded in bacteria from aquatic environments [29, 30, 31]. We also reported plasmid-mediated chloramphenicol, tetracycline, cadmium, and mercury resistance in *P. vulgaris* isolated from the Ganges water [4]. In the present study, the isolated *B. licheniformis* strain exhibited resistance to PB, Ax, Ch, Te, Cf, Er, Cz, Cu^{2+} , Cr^{2+} , Ba^{2+} , Zn^{2+} , and the cured derivative was sensitive to Ch, Cf, Er, Cz, Ba^{2+} , and Zn^{2+} . Plasmid-mediated antibiotic resistance and heavy metal resistance in *P. vulgaris* from a fresh water fish *Channa punctatus* have been reported earlier [26].

The isolation of plasmid DNA from the original as well as the cured *B. licheniformis* strains allowed us to achieve the characterization of R-plasmids. Despite various studies on pesticide resistance in aquaculture systems [20], no data is available on the plasmid of fish bacteria. Plasmid-mediated dimethoate degradation was reported earlier by Deshpande et al [28], employing *Pseudomonas aeruginosa*. In this study, a single plasmid of approximately 54 kb conferring dimethoate degradation property was isolated from fish bacterium *B. licheniformis*. This plasmid that encodes dimethoate degradation property was a conjugative plasmid, which transferred too the

TABLE 2. Antibiotic resistance and metal tolerance of the isolated *B licheniformis*, its cured derivative, and transconjugant strain. PB: polymyxin B; Ax: amoxicillin; Rp: ceftriaxone; G: gentamicin; Ak: Amikacin; Nx: norfloxacin; Ch: chloramphenicol; Cp: ciprofloxacin; Te: tetracycline; Ofx: ofloxacin; Na: nalidixic acid; Cf: cefotaxime; Er: erythromycin; Cz: cefazolin; Cu²⁺: CuSO₄.5H₂O; Cr²⁺: K₂Cr₂O₇; Ba²⁺: BaCl₂; Zn²⁺: ZnSO₄.7H₂O.

Agents	<i>B licheniformis</i> strain					
	Original		Cured		Transconjugant	
	Resistance	Sensitive	Resistance	Sensitive	Resistance	Sensitive
Antibiotics	PB, Ax,	Rp, G,	PB, Ax,	Rp, G,	Ch, Cf,	PB, Ax,
	Ch, Te,	Ak, Nx,	Te	Ak, Nx,	Er, Cz, Na	Te, Rp,
	Cf, Er, Cz	Cp, Na,		Cp, Na,		G, Ak,
		Ofx		Ofx, Ch,		Nx, Cp,
			Cf, Er,		Ofx	
			Cz			
Heavy metals	Cu ²⁺ , Cr ²⁺ , Ba ²⁺ , Zn ²⁺	—	Cu ²⁺ , Cr ²⁺	Ba ²⁺ , Zn ²⁺	Ba ²⁺ , Zn ²⁺	Cu ²⁺ , Cr ²⁺

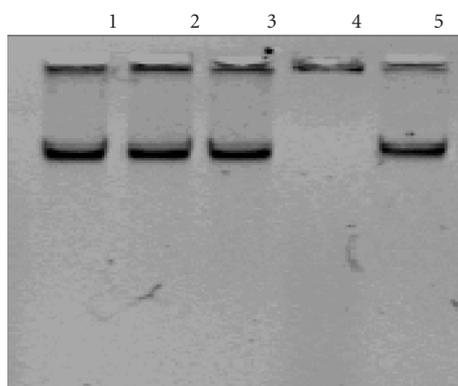


FIGURE 5. Agarose gel electrophoresis of isolated plasmid DNAs. Lane 1: *Bacillus licheniformis* ([14]), lane 2: *B licheniformis* ([15]), lane 3: transconjugant *E coli* C600, lane 4: cured *B licheniformis*, lane 5: *E coli* V517 (54 kb).

resistance traits for antibiotics and heavy metals to the recipient *E coli* C600 strain. Grohmann et al [32] also reported conjugative plasmid mediating antibiotic resistance of Gram-positive bacteria.

Based on the above fact it can be concluded that the utilization of high concentrations of dimethoate, resistance to antibiotics Er, Ch, Cf and heavy metals Ba²⁺ and Zn²⁺ by the *B licheniformis* was mediated by plasmid, of approximately 54 kb.

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