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Biodegradation of Chlorpyrifos by Soil-derived Aerobic Consortia and Bacterial Isolates

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Abstract—In present study, the development of aerobic consortia and subsequent screening of bacterial isolates were carried out from pesticide-contaminated soil of Rajasthan (India) by selective enrichment technique. The biodegradation potential of consortia and bacterial isolates were evaluated using chromatographic analysis. The developed consortia labelled as C1, C2 and C3 were found to degrade chlorpyrifos (100 mg/L) in basal medium to 51, 25, 38 and 43, 61, 68% after 14 and 28 days of incubation, respectively. Six cultures identified as *Alcaligenes sp.*, *Bacillus subtilis*, *Enterobacter sp., Klebsiella sp., Micrococci sp.* and *Pseudomonas aeruginosa* and obtained from these consortia showed degradation of chlorpyrifos (50 mg/L) after 7 and 15 days of incubation to concentration of 34, 38, 23, 38, 26, 43 and 71, 76, 52, 82, 63, 89%, respectively. The success of biodegradation was followed by monitoring the formation and disappearance of 3,5,6-trichloro-2-pyridinol which is a major metabolite of chlorpyrifos biodegradation. The significant findings in present investigation could be a strong potential of soil derived consortia and bacterial isolates. The characterized bacterial strains may be promising candidates for their future applications in the bioremediation of chlorpyrifos-contaminated sites.

Keywords: biodegradation, pesticide, chlorpyrifos, consortia, soil

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Pesticides are agrochemicals which are used throughout the world as keynote strategy to control pest problem in staple crops. Globally, around 2 million tonnes of pesticides are used annually and this usage further has been estimated to increase up to 3.5 million tonnes by 2020 [1]. The use of pesticide has considered as mandatory requirement as a loss in about 45% of the food production has been reported to occur annually due to pest infestation [2]. However, their increased and indiscriminate use has raised serious concerns with regard to environment, food safety and public health in past decades. In chemically diverse groups of pesticides, the organophosphorus (OP) group account for about 38% of total pesticide consumption [3]. This group includes highly toxic chemicals with broad spectrum activity against various insects/pests.

Chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) is one of most widely used, moderately toxic OP pesticide that is used to target both outdoor and indoor categories of pests [4]. It is widely used against pests in agriculture (cereal grains, cotton, sugarcane, fruits, vegetables and fodder crops), lawns, households and public health [5]. Continuous manufacturing and extensive use of chlorpyrifos have resulted in contamination of soil, air, surface and ground water bodies. The accumulation of pesticide residues in crops, soils, and biosphere has further resulted in several types of ecological stress [6]. Due to its neurotoxic nature, chlorpyrifos is also known to inhibit the acetylcholine esterase activity which in turn causes severe nervous system disorders in mammals and humans. The fate of chlorpyrifos in environment has been studied by several researchers. Chlorpyrifos has been reported to have increased soil absorption coefficient with decreased water solubility (2 mg/L) [7]. The half-life of chlorpyrifos has been found to vary from <1–10 day to >100–120 days depending on the type of pesticide formulation, soil temperature, moisture, pH, organic carbon, microorganisms and climatic conditions [6–9]. This indicates an urgent need and particular concern for decontamination of chlorpyrifos-contaminated sites.

Various physical, chemical and biological approaches have been attempted for chlorpyrifos detoxification. Among these, biodegradation is considered as an efficient, most advantageous and cost-effective biological approach to clean up pesticides

polluted environment [4, 10, 11]. Till now, many microorganisms including bacteria, fungi, algae and yeasts have been isolated and studied for chlorpyrifos biodegradation. Several studies have shown the role of bacteria such as *Bacillus pumilus* [12]; *Flavobacterium* sp. [13] and *Escherichia coli* [14]; fungi such as *Acremonium* sp. [15]; *Phanerochaete chrysosporium* [16], yeast like *Saccharomyces cervisiae* [17], and algae like *Chlorella vulgaris* [18]. In chlorpyrifos degradation. In many studies, biodegradation of chlorpyrifos has led to the accumulation of 3,5,6-trichloro-2-pyridinol (TCP), its hydrolytic product. TCP has been found to have anti-microbial properties and thereby preventing the proliferation of chlorpyrifos-degrading microorganisms and biodegradation to remain continued [7]. Till date, several studies that have focused on chlorpyrifos/TCP biodegradation have made the use of microbial isolates either alone as pure culture or in combination with other isolates (co-metabolism). However, fewer studies have been carried out on the development of microbial consortia to study biodegradation of chlorpyrifos and TCP. Keeping in view, the present study was undertaken with the aim of developing aerobic consortia and subsequent isolation/screening of bacteria capable of degrading chlorpyrifos and its major metabolite TCP from pesticide-contaminated soil.

MATERIALS AND METHODS

Collection of soil samples. Three soil samples (labelled as A, B and C) were collected from semi-desert soil of agricultural fields of Rajasthan in Northern India. These fields were under extensive pesticide spray over last 2–3 years. Top soil samples ranging in depth from 10–15 cm were collected in each field from 3 different spots within 2 × 2 m zone using sterilized spatula in sterile bags. Samples from different spots were then mixed to make a composite sample of each field. All soil samples were air-dried, sieved through 2 mm mesh sieve (Zhangxing, China) and kept at 4°C until further use.

Chemicals and reagents. Chlorpyrifos and TCP (HPLC grade, 99% pure) were purchased from Sigma-Aldrich (USA). Organic solvents of analytical grade were purchased from Merck, Germany. Microbiological media were procured from Himedia laboratories, India.

Development of aerobic consortia. Collected soil samples A, B and C were used to develop 3 different aerobic consortia namely C1, C2 and C3 respectively by repeated enrichment technique. To develop each consortium, 5 g soil sample was added in 100 mL of Luria broth containing 10 mg/mL of chlorpyrifos and incubated at 37°C in shaker incubator at 150 rpm for one week. After incubation, 10% of inoculum was used to inoculate 100 mL Bushnell Haas broth (BHB) containing (g/L): magnesium sulphate—0.2; calcium chloride anhydrous—0.02; potassium dihydrogen phosphate—1.0; dipotassium hydrogen phosphate—1.0; ammonium nitrate—1.0; ferric chloride—0.05 (pH 7.0 ± 0.2) supplemented with 30 mg/mL of chlorpyrifos as the only added carbon source and incubated. The process of transferring 5% inoculum to fresh BHB was repeated thrice to successfully develop all aerobic consortia. Further, all the developed consortia were used for cellular growth and total protein estimation, tolerance and degradation of chlorpyrifos studies.

Isolation and screening of bacterial isolates. Bacterial strains were isolated by spreading serially diluted aliquots of developed consortia on Bushnell Haas Agar (BHA, basal medium) plates supplemented with 30 ppm of chlorpyrifos as sole source of carbon. After spreading, plates were incubated at 37°C for 5 days. Following incubation, isolated and morphologically distinguishable colonies were picked up and purified by sub-culturing. Purified cultures were further grown in 2 mL BHB and BHA with bromothymol blue with 30 ppm chlorpyrifos. The selected isolates were further evaluated for their growth, total protein, tolerance and degradation of chlorpyrifos.

Identification of bacterial isolates. The identification of selected purified bacterial isolates was made by morphological, biochemical and physiological characterizations as per the procedure described in Bergey's Manual of Determinative Bacteriology [19]. Morphology was studied using microscopic Gram and endospore staining procedure. Further, the biochemical and physiological characterization was performed using standard IMViC, oxidase, catalase, urease, gelatin hydrolysis, starch hydrolysis, carbohydrate fermentation, H_2S production and nitrate reduction tests.

Tolerance studies. The developed consortia and screened bacterial isolates were inoculated in BHB medium tubes containing different concentrations of chlorpyrifos in the range from 30 to 500 mg/L. The tolerance was examined by visually observing the growth at each concentration.

Estimation of growth and total protein. The increase in growth was determined in terms of measuring cellular biomass and total protein for cultures (consortia/isolates) growing in basal medium containing chlorpyrifos and incubated on a rotary shaker at 37°C for 7 days. Cellular biomass was measured at regular time intervals at OD_{600} using spectrophotometer (Hitachi, Japan). On the other hand, total protein was determined by the procedure as described by Itzhaki and Gill [20].

Biodegradation studies. The soil-derived consortia and bacterial isolates were investigated for chlorpyrifos utilization by carrying out shake flask biodegradation studies. One hundred mL of sterile BHB containing 50 and 100 mg/L of chlorpyrifos was inoculated with 5% of bacterial isolates and consortia, respectively, and incubated at 37°C on a rotary shaker at 150 rpm. Samples were withdrawn after incubation of 7, 14, 21 and

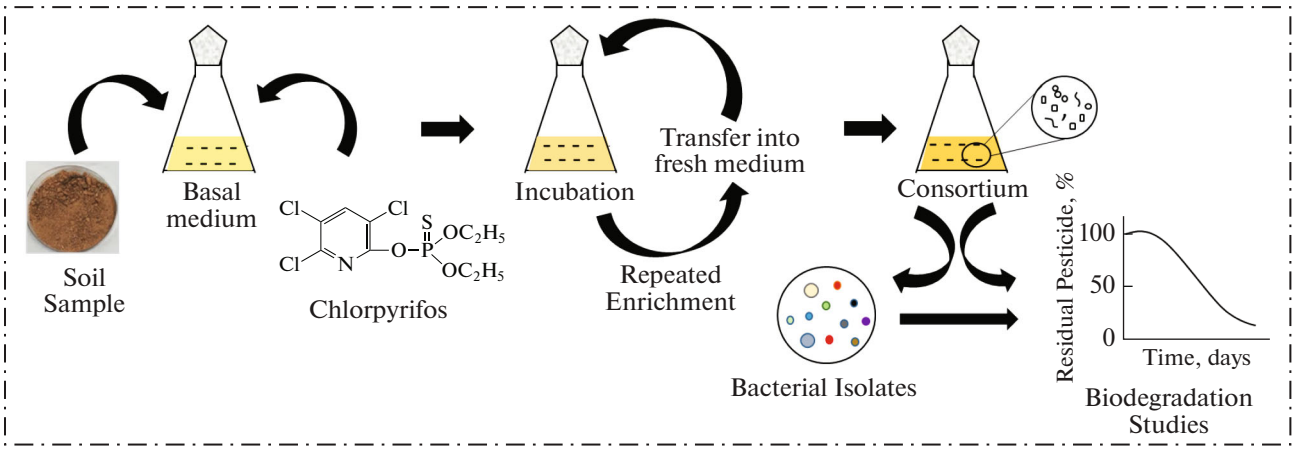

Fig. 1. Schematic diagram illustrating the steps involved in soil enrichment for development of chlorpyrifos-utilizing cosortia and bacterial isolates.

28 days for consortia and after 10 and 15 days for isolates, in duplicate to analyze chlorpyrifos utilization by extraction of samples using equal volume of ethyl acetate: phosphoric acid (97 : 3, vol/vol). Extracted organic solvent layer was evaporated and the dried residues were dissolved in acetonitrile (HPLC grade) and final volume was adjusted to 5 mL for further analysis. The extracted samples were analyzed by HPLC (Shimadzu, Japan) in isocratic mode using Atlantis C18 column 150 × 4.6 mm). Methanol: water (85 : 15, vol/vol) mixture was used as mobile phase for isocratic elution. It was pumped through the column at a flow rate of 1 mL/min. Control flask containing basal medium with chlorpyrifos was also extracted in order to determine the abiotic loss of chlorpyrifos. Lab Solutions software was used for data collection and acquisition.

Statistical analysis. All the experiments were performed in triplicate. All the data shown in figures and tables represent the mean and standard error of the findings obtained from 3 independent measurements.

RESULTS AND DISCUSSION

Enrichment culture technique is a powerful tool which was previously employed to obtain microbial consortium and isolate bacteria to degrade chlorpyrifos in several studies [21–23]. In present investigation, this technique was used to develop aerobic consortia capable of utilizing chlorpyrifos from soil which were used further to isolate pure bacterial cultures (Fig. 1). The significant findings obtained on development of consortia and screening of bacterial isolates and their subsequent use in carrying out degradation of chlorpyrifos are discussed below.

Consortia development. Three aerobic bacterial consortia namely C1, C2, C3 capable of growth on basal medium containing 30 mg/L chlorpyrifos as sole carbon source were developed using pesticide-contaminated soil samples by repeated enrichment. All consortia were tested for their ability to tolerate chlorpyrifos from 30 to 500 mg/L for an incubation period of 7 days (Table 1). The findings showed tolerance level of C1 and C3 to a maximum of 200 mg/L as inhibition in growth was observed at higher concentration i.e. 350 and 500 mg/L. On the other hand, C2 was found to tolerate even to 350 ppm of chlorpyrifos. Bacterial growth was found to decrease with increase in chlorpyrifos concentration. The best growth was observed for all consortia at 100 mg/L of chlorpyrifos by measuring the OD_{600} during a period from 0 to 28 days of incubation (Fig. 2, line graph). The results demonstrated a slight increase in growth from 0 to

Table 1. Tolerance level of developed consortia and isolated bacteria to chlorpyrifos

Culture	Chlorpyrifos, mg/L*					
	30	50	100	200	350	500
Consortia						
C-1	+	+	++	+	–	–
C-2	+	++	++	+	+	–
C-3	+	++	++	+	–	–
Control	–	–	–	–	–	–
Bacterial isolates						
415	+	++	+	+	–	–
116	++	++	++	+	+	+
301	+	++	++	+	+	–
403	+	+	+	+	–	–
113	++	++	+	+	+	+
610	++	+	+	+	–	–
Control	–	–	–	–	–	–

* "+" tolerance observed; "++" increased tolerance observed; "–" tolerance was not observed.

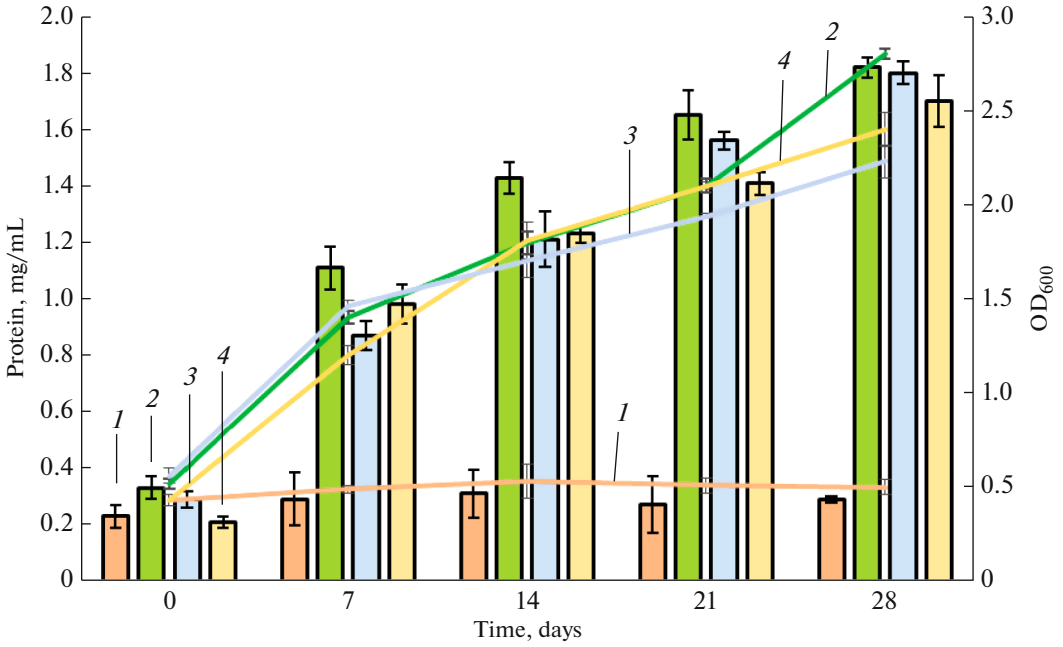

Fig. 2. Growth profile (OD_{600}, line graph) and total protein content (mg/mL, bar graph) of consortia (C1, C2 and C3) in basal medium containing chlorpyrifos (100 mg/mL) as sole carbon source. *1*—Control; *2*—C1, *3*—C2 and *4*—C3 consortia (mean ± SE, $n = 3$).

7 days with an exponential increase in growth of each consortium from 7 to 28 days. Similarly, their protein content was also found to increase over a period of 28 days incubation (Fig. 2, bar graph). These observations evaluated the capability of each consortium to utilize chlorpyrifos in order to support their growth. Similar to this work, development of aerobic consortia containing chlorpyrifos-degrading microbial communities through soil enrichment has also been reported by Lakshmi et al. [22]. Consortia developed in this study were found to degrade chlorpyrifos in the range from 54 to 61%.

Isolation, screening and identification of bacterial isolates. A total of 67 bacterial isolates were obtained from 3 consortia on BHA medium supplemented with 30 ppm of chlorpyrifos. Out of these, 15 bacterial isolates with morphologically distinguishable colonies were further purified and tested for their ability to grow after inoculating them in BHB and BTB media containing 30 ppm of chlorpyrifos. Among them, 6 bacterial isolates, namely 113, 116, 301, 403, 415 and 610, were selected as most efficient (data not shown). The efficacy of selected bacterial isolates to tolerate varying concentrations of chlorpyrifos in the range from 30 to 500 ppm was tested for an incubation period of 7 days (Table 1). The findings showed tolerance level of 301 to a maximum of 350 ppm and 415, 610 and 403 to 200 ppm level as inhibition in bacterial growth was observed at higher concentration i.e. 350 ppm and 500 ppm in BHB and BTB medium, respectively. On the other hand, 113 and 116 were found to show growth even up to 500 ppm of chlorpyrifos. Further, the growth of these isolates was studied at 50 mg/L chlorpyrifos and found to increase in a period from 0 to 15 days of incubation in terms of the OD_{600} (Fig. 3a). At the same time, total protein content was also found to increase from 0.061 to 1.7 mg/mL over a period of 15 days incubation (Fig. 3b). Based on their morphological, biochemical and physiological characterization, bacterial isolates namely 113, 116, 301, 403, 415 and 610 were identified as *Klebsiella* sp., *Pseudomonas aeruginosa*, *Micrococci* sp., *Bacillus subtilis*, *Alcaligenes* sp. and *Enterobacter* sp., respectively (Table 2). These findings are in accordance with those obtained by other researchers isolated these bacteria and evaluated their potential for utilization of chlorpyrifos from pesticides contaminated soil [24, 25].

Biodegradation studies. Biodegradation receives attention towards the cleaning of pesticide polluted agriculture land because of its cost effectiveness and ecofriendly nature [26]. Degradation dynamics of chlorpyrifos was studied by growing experimental consortia and selected isolates in basal medium containing 100 and 50 mg/L chlorpyrifos, respectively. The chlorpyrifos degradation efficiency of each consortium and isolate was estimated by extraction with ethyl acetate-phosphoric acid and subsequent quantification of samples withdrawn at regular intervals using HPLC.

Among three consortia, C1 and C3 degraded 51 and 38% of chlorpyrifos after 14 days and the degradation further increased to 43 and 68%, respectively, after 28 days when compared to un-inoculated control

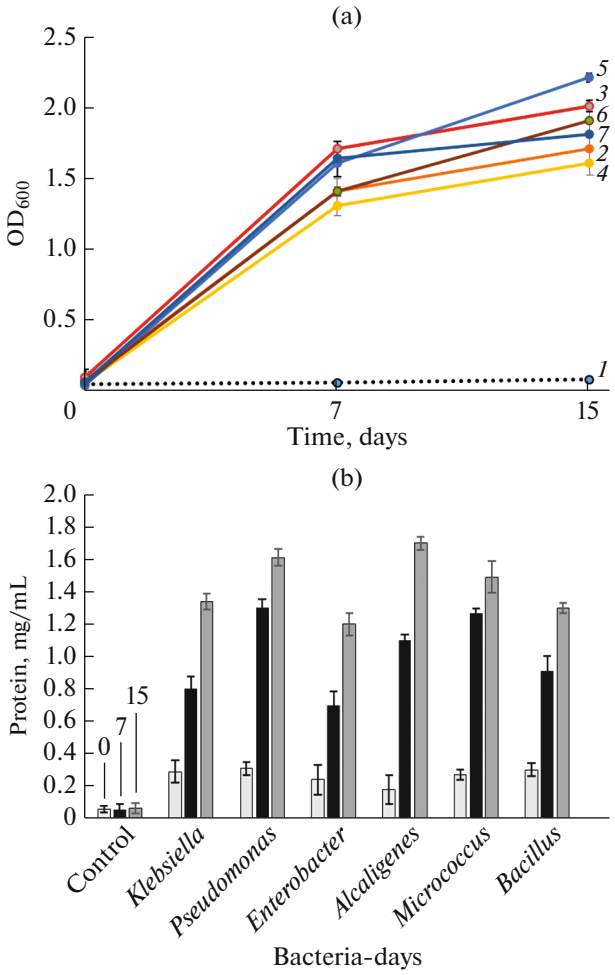

Fig. 3. Growth profile of bacterial isolates in basal medium containing chlorpyrifos (50 mg/mL) as sole carbon source. Increase in biomass (a) and total protein content (b) were estimated as an indicator of growth (mean ± SE, $n = 3$). In (a): *1*—control, *2*—*Klebsiella*, *3*—*Pseudomonas*, *4*—*Enterobacter*, *5*—*Alcaligenes*, *6*—*Micrococcus*, *7*—*Bacillus*. In (b): 0—0 days; 7—7 days; 15—15 days.

(Fig. 4). However, C2 showed increase in degradation up to 25% on day 14, and the degradation further elevated up to 61% after the completion of 28 days. The findings obtained in our work are in line with Lakshmi et al. [22] who also reported degradation of chlorpyrifos by bacterial consortia developed from soil samples collected from Punjab, India.

On other hand, selected 6 isolates, i.e. *Alcaligenes* sp., *B. subtilis*, *Enterobacter* sp., *Klebsiella* sp., *Micrococci* sp. and *P. aeruginosa* showed 34, 38, 23, 38, 26, 43% degradation of chlorpyrifos after 7 days of incubation and the degradation increased further to 71, 76, 52, 82, 63, 89% after 15 days of incubation, compared to 5 and 7%, respectively, in the uninoculated control (Fig. 5). Further, during biodegradation experiments, the process of bacterial biodegradation was also evaluated by monitoring the appearance and disappearance of peaks representing TCP in obtained spectra and

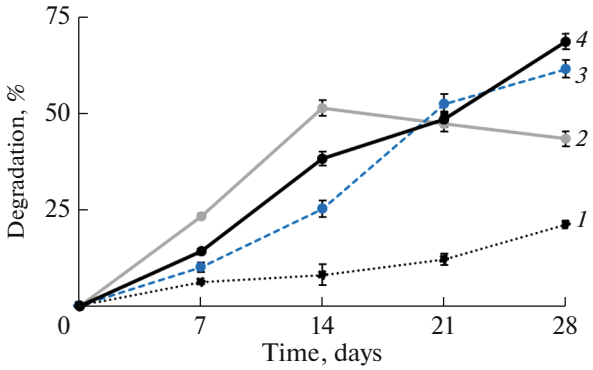

Fig. 4. Biodegradation of chlorpyrifos (100 mg/L) using developed aerobic consortia. *1*—Control; *2*—C1, *3*—C2 and *4*—C3 consortia (mean ± SE, $n = 3$).

Table 2. Morphological, biochemical and physiological characterization of bacterial isolates

Biochemical Tests	Bacterial isolates*					
	113	116	301	403	415	610
Gram staining	–ve	–ve	+ve	+ve	–ve	–ve
Shape	Rod	Short rods	Cocci	Rod	Rod	Rod
Spores	Absent	Absent	Absent	Present	Absent	Absent
Pigment	–	Green	Yellow	–	–	–
Indole	–	–	–	–	–	–
MR	–	–	–	–	–	–
VP	+	–	–	+	–	+
Citrate	+	+	+	+	–	+
Oxidase	–	+	–	–	+	–
Catalase	+	+	–	+	+	+
Starch hydrolysis	–	–	–	+	–	–
Gelatin	–	+	+	+	–	–
Urease	+	–	+	–	–	–
H_2S production	–	–	–	–	–	–
NO_3 reduction	+	+	+	+	–	+
Acid production from glucose	+	–	–	+	–	+
Acid production from sucrose	+	–	–	+	–	+
Identified bacteria	*Klebsiella* sp.	*P. aeruginosa*	*Micrococci* sp.	*B. subtilis*	*Alcaligenes* sp.	*Enterobacter* sp.

* "–ve" Gram negative; "+ve" Gram positive; "+" positive reaction; "–" negative reaction.

thereby confirming the success of chlorpyrifos biodegradation. The *Pseudomonas* is well known as a highly versatile organism with an ability to metabolize even the most complex polymers. Our results were in agreement with the findings of other scientists who isolated *Pseudomonas* spp. which were able to degrade chlorpyriphos [25, 27–31]. Lakshmi et al. [22]. Also worked on development of aerobic bacterial consortia and reported 84% degradation of chlorpyrifos by *P. aeruginosa* isolated in their study in liquid medium after 20 days of incubation. Hence, the outcome of current work clearly represents the high potential of soil-derived bacterial consortia and isolates, especially *Pseudomonas* sp. for efficient biodegradation of chlorpyrifos. The findings obtained in presented study could be explored in future to achieve on-field applications for biodegradation of organophosphorous pesticides.

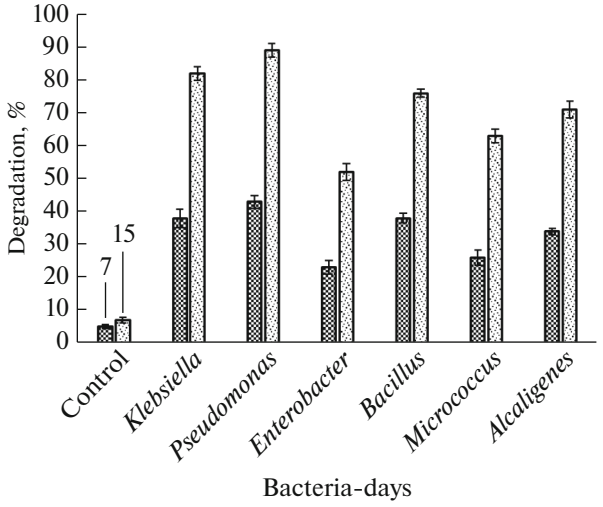

Fig. 5. Chlorpyrifos (50 mg/L) degradation efficiency using bacterial isolates obtained by enrichment culture (mean ± SE, $n = 3$). 7—7 days; 15—15 days.

* *

Technological challenges faced by the existing physical and chemical clean up strategies during the chlorpyrifos degradation process underscore the urgency to develop the more efficient degradation system. Significant findings obtained in this study successfully demonstrated the development and screening of chlorpyrifos-degrading consortia and bacterial isolates. The developed consortia and screened isolates were found to degrade from 49 to 61% and from 39 to 93% of chlorpyrifos, respectively, in basal medium during laboratory study. The outcome of this study can explore the potential of such cultures for

future application in bioremediation of chlorpyrifos in soil, water and other contaminated sites and, thus, minimizing the adverse toxicological effects of chlorpyrifos on other non-targeted life forms including human beings. Characterizing the degradation pathway and studying the enzymatic and genetic basis involved therein represent the areas of further investigation.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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