Biodegradation of CladinafopPropargyl by *Bacillus* sp. and *Bacillus cereus* Isolated from Field Crop

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Abstract: Objectives: to remove clodinafoppropargyl (CF) residue from wheat soil field by using CF as a sole carbon, nitrogen and energy source by two CF-degrading bacterial strains. **Results**: Two bacterial strains were identified as *Bacillus* sp. strain HT31 and *B. cereus* strain HT44. 88.7 and 87.5% CF were degraded out of initial provided 80 mg/L CF by the strains HT31 and HT44, respectively. Degradation of CF was accompanied by release of chloride ion. In the same time, the two strains exhibited esterase activity during the degradation at 12 and 24 hours. The optimal pH and temperature for the growth of *Bacillus* sp. and *B. cereus* were between pH from 7 to 8 and temperature from 30-35ºC in the mineral salts medium supplemented with CF. An actively growing culture of the two strains degraded CF to clodinafop acid and 4-(4-Chloro-2-fluoro-phenoxy)-phenol within 12 h, as determined by GC-MS analysis. **Conclusions**: Those strains could be a potential candidate to remove CF from contamination sites due to its high degradation efficiency.
[Ismail Ahmed, Omer Mohammed, Magdy Bahgat, Heba Montaser. **Biodegradation of Cladinafop Propargyl by *Bacillus* sp. and *Bacillus cereus* Isolated from Field Crop.** *J Am Sci* 2017;13(4):72-78]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). http://www.jofamericanscience.org. 10. doi:10.7537/marsjas130417.10.

Keywords: *Bacillus* sp. strain HT31, *Bacillus cereus*strain HT44, Biodegradation, Clodinafoppropargyl.

1. Introduction

Environment preservation is one of the aims of the sustainable development. With the modernization of agriculture in the 60's, a massive use of agrochemicals, fertilizers and machinery began, aimed at improving field productivity. These goals have been achieved, although the side effects of this situation include an intense and abusive utilization of these chemicals, creating many kinds of problems (Fay *et al.,* 1997). The agrochemical impact on the environment is clear, but measuring its effects is difficult. Residues can cause damage and bring about disease to plants in a rotation culture system in a cumulative manner, restricting microbial growth (Rickman *et al.,* 2002).

Biodegradation is the enzyme-mediated transformation of a xenobiotic by living microbial cells. In soil systems, biodegradation is a fundamental attenuation process for pesticides and is controlled by biotic factors (i.e. microbial activity) and a number of physicochemical processes such as sorption and desorption, diffusion, and dissolution (Chen *et al*., 2009). Microbial degradation is an important environmental biotechnology for elimination of organic pollutants (Harbottle*et al*., 2009).

Cladinafoppropargyl (CF) (prop-2-ynyl (R)-2-[(5-chloro-3-fluoro-2-pyridyloxy) phenoxy]propanoate) is a recently introduced aryloxyphenoxypropionate herbicide, used for post emergence control of annual grasses in cereals (Singh, 2013). Egypt is an agriculture based country and today, high-yielding agriculture heavily depends on chemical weed control. The Government of Egypt has given provisional registration to cladinafop along with other herbicides to control annual grasses in cereals. The widespread use of CF has resulted in the discharge of large amounts of the compound into the environment, which eventually reach the biosphere (Vazan*et al*., 2011). CF is absorbed by the leaves and rapidly translocated to the growing points of leaves and stems. It interferes with the production of fatty acids needed for plant growth in susceptible grassy weeds (Singh, 2013). CF acts by targeting the enzyme acetyl coenzyme-A-carboxylase, essential for lipid biosynthesis.

Clodinafop-propargyl (CF) and other aryloxyphenoxypropanoate (AOPP) analogs in the environment are degraded by both abiotic and biotic processes (Lin *et al*., 2008), microorganisms make the largest contribution. AOPP herbicide-degrading microorganisms have been isolated from various genera (Hou*et al.,* 2011; Singh, 2013; Kumar *et al.,* 2014; Dong *et al.,* 2015; Zhang *et al.,* 2016). The degradation pathway of AOPP herbicides has also been investigated and proposed based on metabolite identification. However, degradation of CF by aerobic bacteria involves esterase activity that results in the formation of phenols as metabolites (Hou*et al*., 2011; Singh *et al.,* 2013).

2. Materials and Methods

Enrichment and isolation of CF degrading bacteria

Soil samples were collected from crop field area with a previous history of CF application, located in South Port Said and South Hussinia plane, El-Sharkia Governorate, Egypt. Isolation of clodinafoppropargyl degrading bacteria was done according to Singh (2013). A selective mineral salt medium (MS) was prepared as follows: 40 mg/L CF (99.2 % purity) purchased from Sigma Aldrich (PESTANAL, Fluka analytical), as a sole source of carbon, nitrogen in addition to 4 g $Na_2HPO_4.2H_2O$, 2 g KH_2PO_4 (0.025 %), $MgSO_4.7H_2O$ (0.05 %), and 1 mL of trace element solution (0.1 g of $ZnSO_4.7H_2O$, 0.03 g of $MnCl_2.7H_2O$, 0.3 g of H_3BO_3, 0.2 g of $CoCl_2.6H_2O$, 0.01 g of $CuCl_2.2H_2O$, 0.02 g of $NiCl_2.6H_2O$, in 1 L of the solution). Five grams of soil sample were inoculated into Erlenmeyer flask (250 mL) containing 100 mL autoclaved water. Processed soil sample (0.5 mL) was spread on MS medium plates and incubated at 30°C for 3 days until bacterial colonies became visible. Colonies grown on these plates were evaluated for their CF degrading capabilities. Single colony types were separated and subcultured on fresh plates to purity using identical growth conditions at each transfer, except that the CF concentration was increased stepwise from 40 to 120 mg/L. Two strains, designated as strain HT31 and strain HT44, which possessed the highest CF-degrading ability and could utilize CF as the sole carbon source for growth, was purified and selected for further investigation. The two isolated strains HT31 and HT44 were classified by Gram staining and 16S rRNA analysis. The 16S rRNA gene of the bacterial isolates was amplified with a set of universal primers (Invitrogen, USA). The primers 5'-ATTCCGGTTGATCCTGCCGG-3' (positions 6–25 in *Escherichiacoli* numbering) and 5'AGGAGGTGATCCAGCCGCAG-3' (positions 1540–1521).

Evaluation of cladinafop-propargyl degradation by bacterial isolates

During degradation, 1 mL sample was removed from each vial at regular intervals to measure the inoculants growth of the cell and CF concentrations. To analyze CF and its metabolites sample aliquots (1 mL) were taken at regular intervals. The culture broth was centrifuged (10,000 rpm, 4°C for 10 min) and supernatant was acidified with 1 M H_2SO_4 to a pH<5. The solution was extracted with two 1 mL portions of hexane and derivatized by adding 0.1 mL diazoethane (Sigma Aldrich). Diazoethane is an ethylating agent that facilitates the simultaneous analysis of CF and its derivatives'. Excess diazoethane was removed under a stream of cold nitrogen and the solution was dried over anhydrous Na_2SO_4. A cold stream of nitrogen was used to evaporate the hexane and to allow a solvent exchange to toluene. The final volume was adjusted to 2 mL with toluene. Samples were analysed by using high-pressure liquid chromatography (HPLC). HPLC analysis was performed in Central Agric. Pesticides Laboratory (CAPL) with an Agilent 1100 HPLC system, with photodiode array detector. The chromatographic column was C8 zorbax SB (250 x 4.6 mm, 5 um film thickness) at 220 nm. The mobile phase was acetonitrile/methanol (65:35 v/v) and flow rate was 1 mL/min. To calculate % degradation, peak areas were measured to quantify the CF.

Determination of chloride released from CF degradation

The chloride ion concentration was determined using Mohr method (Singh, 2013). Two hundred microliters of a sample diluted so that the chloride concentration was up to 0.1 mM was added to 50 mL of 0.25 M potassium chromate. The reaction mixture was titrated with 0.1 M silver nitrate solution. Chloride ion concentrations were calculated by using volumetric analysis.

Identification of clodinafoppropargyldegradation metabolites

The CF concentration and its metabolites produced by biodegradation were determined by HPLC and GC-MS as described by Singh (2013). The HPLC analysis was performed using system (DionexUltiMate® 3000) consisting of P680 HPLC pump, a C18 reversed-phase analytical column (Acclaim 120, 4.6 mm X 250 mm, dp= 5 µm) with suitable guard column and a D170U UV-detector using acetonitrile: water (50:50) as mobile phase at flow rate 1.0 ml min-1 and an injection volume 20 µl. For GC-MS analysis, GCMS-QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) analysis was used. Capillary column used in the GC was Rtx-1MS (30 m x 0.25mm ID x 0.25µm df) supplied by Restek U.S. (Bellefonte, PA, U.S.A.). GC column oven temperature was programmed for an initial hold of 1 min at 100 ºC; then temperature was increased at 10 ºC min^{-1} to 200 ºC; then up to 260 ºC at the rate of 15 ºC min^{-1}; followed up to 300 ºC at the rate of 3 ºC min^{-1} and then hold at 300 ºC for 2 min. The gas flow rate was 1 mL min^{-1} in splitless mode with injection temperature of 270 ºC.

Esterase Activity Determination of Bacterial Isolates

Each bacterial inoculum containing 10^8 cells from the isolates were allowed to grow in 10 ml MS medium supplemented with 80 mg/L CF at 30°C for 24 hr., on a rotary shaker at 150 rpm. Samples of previous media were taken at 0, 12 and 24 hr., of incubation. All samples were centrifuged at 7000 rpm for 15 min at 4°C to collect bacterial pellets and then

samples supernatants were separated and stored at -20°C until measurement of the general esterase activity. Esterase activity assay was determined Colorimetrically using 96–well microplate of Microplate Autoreader EL 311S (Bio Instrument, Highland Park, Winoosky, VT). The general substrate as α-Naphthyl acetate (α-NA) was used in determination as described by Gomori (1953). Briefly, 0.02% of α –NA, and 0.05% fast blue B salt were prepared in 50 mM of sodium phosphate buffer pH 6.8. In a glass test tube, 480 µl of α -NA was added to 20 µl of sample, then 500 µl of fast blue B salt was added to the previous mixture and mixed. Optical densities at 600 nm were recorded for determining the activity of general esterase in triplicates of 300 µl / replicate.

Effect of incubation temperature and initial pH on clodinafoppropargyl biodegradation by bacterial isolates

Bacterial isolates HT31 and HT44 were tested for their optimum incubation temperature and initial pH for biodegradation of CF at concentration 80 mg L^{-1}. Solutions of CF were freshly prepared in methanol at concentration of 1 mg/mL. A 1 mL aliquot of CF solution was transferred into sterile 12 mL glass vials. The vials were left open in a fume hood to allow the solvent to evaporate. A 100 µL pre-grown single bacterial clone (OD_{600} = 0.5) was inoculated into each vial to give an initial CF concentration of 80 mg/L. The cultures were incubated at 30°C and 100 rpm on a rotary shaker for 12 h. In order to evaluate the effect of temperature and pH, MS medium containing 80 mg/L CF was incubated for 12 h at different temperatures (15, 20, 25, 30, 35, 40, and 45°C) and under different pH conditions (4.0–10.0, in increments of 1.0 pH units). Uninoculated MS medium was used as control. Each treatment was performed in three replicates, and the control experiment without microorganism was carried out under the same conditions.

Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) using SPSS (version 14.0) for windows. Probability of 0.05 or less was considered as significant.

3. Results and Discussion

The fate of aryloxyphenoxypropionate herbicides in soil is affected by many factors, and the microbial community is believed to be one of the most important factors. Soil samples were collected from wheat fields of South Husania (El Sharkia Governorate) and South Port Said (Port Said Governorate) that had used clodinafop-propargyle (CF) as the main herbicides to control weed for over three years.

For the purpose to identify the microbial species and to understand the process that involved in CF degradation, efforts were made to isolate CF-degrading bacteria from the soil samples. CF-degrading bacteria were enriched by consecutive supplements of CF (80 mg L^{-1}, this was the suitable CF concentrations to isolates grow) to soil suspension in MS medium. After six times of transfer (6 weeks), the enrichment was used to isolate CF-degrading bacteria. In total, 38 CF-degrading bacterial strains were isolated.

Two pure isolates that could grow by using CF as the sole source of carbon were obtained from the soil samples. The ability of these strains to degrade CF was confirmed in liquid MS media supplemented with CF. The two isolates, designated as strains HT31 and HT44, showed the highest CF-degrading ability and were selected for subsequent experiments.

Blastn analysis of the sequencing results provided by Macrogen's sequencing service for the two isolates were as follow:

Isolate HT31: *Bacillus* sp. strain (CP010322).

Isolate HT44 *Bacillus cereus* strain NIT 7 (KM885306).

They are gram positive, rod shape and spore forming bacteria.

The degradation of CF by strains *Bacillus* sp. and *B. cereus* was shown in Figure (1). The amount of CF was decreased by the increasing time and the lowest amount was estimated at 12 and 16 hours for the two isolates as compared with the uninoculated medium.

HPLC chromatograms of control and test reactions were recorded and CF peak was observed after retention time 2.611min for the standard CF and after 2.614 min for the control samples. However, with the strains *Bacillus* sp. and *B. cereus,* the CF was not recorded.

At the degradation process the bacterial biomass was measured for the two isolates, however, the bacterial biomass was increased with increasing the time. The highest bacterial biomass was shown at 12 hours for the two strains *Bacillus* sp. and *B. cereus* (Figure 2).

The chloride ion concentration increased in the media inoculated with strains *Bacillus* sp. and *B. cereus* as combared with uninoculated medium. The concentration of chloride ion increased in the media with increasing the time. The highest concentration of chloride ion was observed at 12 hours and was 1.75 & 1.65 mg/L for *Bacillus* sp. and *B. cereus* strains, respectively (Figure 3).

The bacterial strains *Bacillus* sp. and *B. cereus* degrade clodinafop-prpargyl and used it as a sole carbon source. However, the bacterial and fungal degradation and utilization of pesticides as sole

carbon sources have been reported by many investigators (Mohamed *et al.*, 2010; Singh, 2013; Mohan and Naveena, 2015).

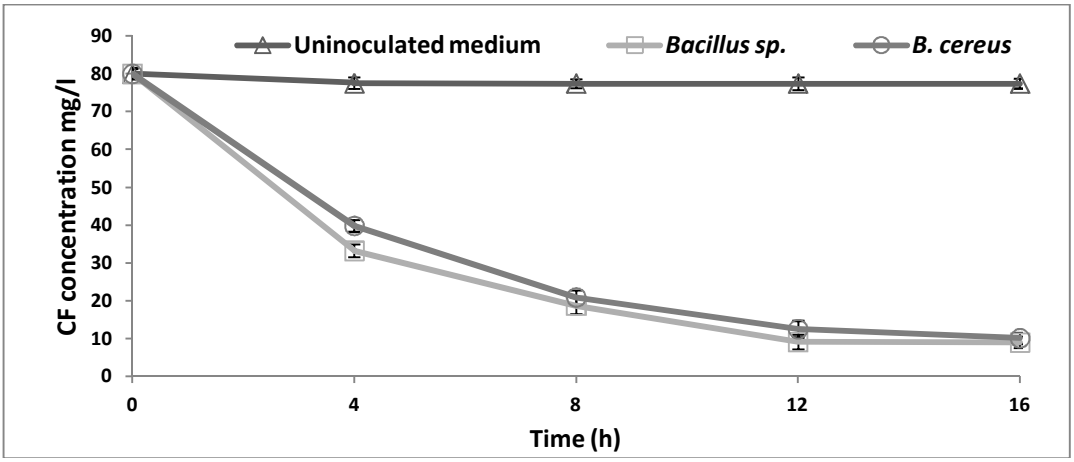

Figure (1): Clodinafop-propargyl biodegradation by bacterial strains *Bacillus* sp. and *B. cereus* by the time in MS medium supplemented with 80 mg/L CF.

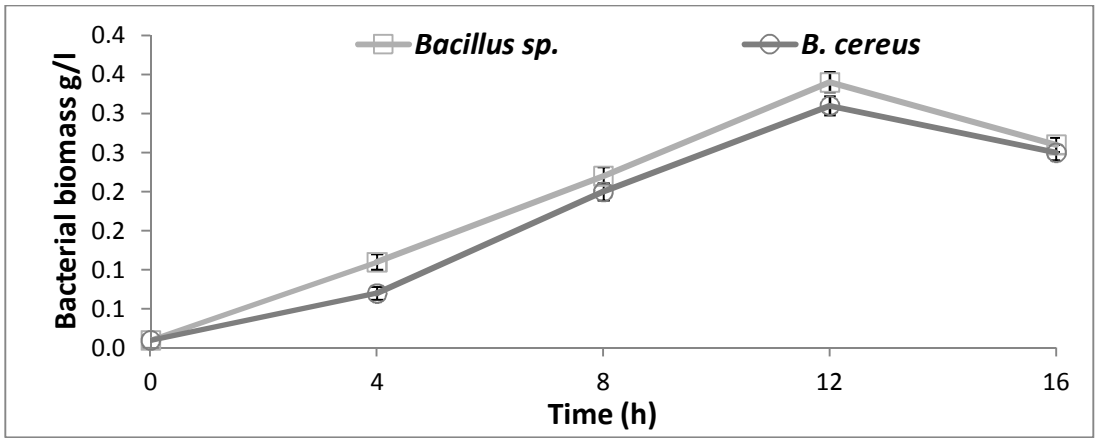

Figure (2): Bacterial isolates HT31 and HT44 biomass after inoculation on MS medium supplemented by CF as carbon and nitrogen source.

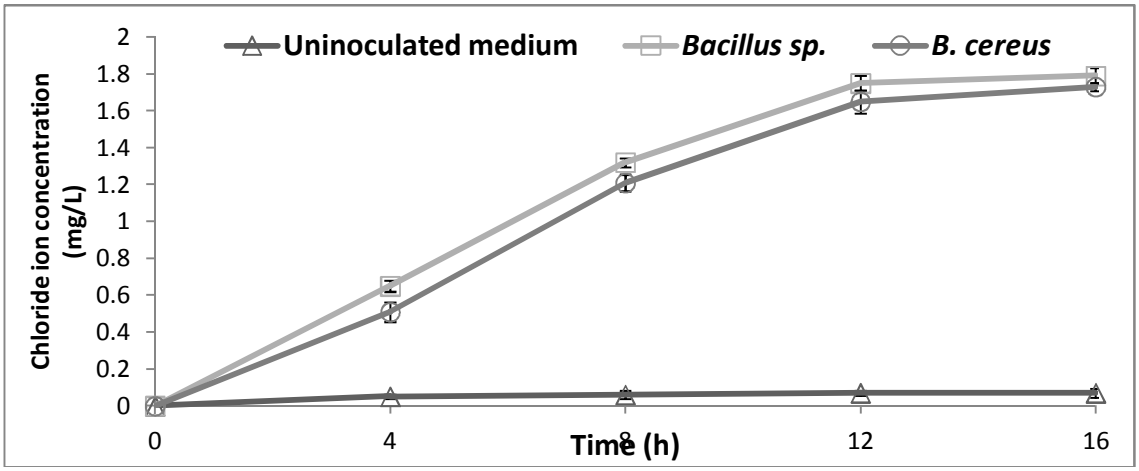

Figure (3): Chloride ion concentration relazed from clodinafop-propargyl biodegradation by *Bacillus* sp. and *B. cereus* inoculated to MS medium supplemented with CF.

With CF as the carbon, nitrogen and energy source, *Bacillus* sp. and *B. cereus* produced a typical sigmoidal growth curve consisting of a relatively very short lag phase and an exponential phase of approximately 12 h, followed by abrupt transition to the stationary phase. The GC-MS spectrum pattern of standard (without inoculum) and its metabolites were recorded. The major metabolites of cladinafop-propargyl degradation wereclodinafop acid and 4-(4-Chloro-2-fluoro-phenoxy)-phenol. No change in CF concentration was observed in culture that was inoculated with heat-killed strainsHT31 and HT44. Singh (2013) reported that higher intracellular CF concentration would result in slower degradation rate and this is in consistent with our observation. Standard exhibited molecular ion peak (M+) at 349 m/z and characteristic fragment ions at 323 m/z, 266 m/z, and 238 m/z. 4-(4-Chloro-2-fluoro-phenoxy)-phenol displayed a molecular ion at *m/z* 240 (M+) and characteristic fragment ions at 183 m/z, 165 m/z and 100 m/z. Only trace amounts of 4-(4-Chloro-2-fluoro-phenoxy)-phenol were detected during the early stages of growth (1-2 h), high concentrations of this metabolite in the growth medium during the log and stationary phases (15-30 h) suggested that 4-(4-Chloro-2-fluoro-phenoxy)-phenol was the major degradation product. Other possible breakdown product, including clodinafop acid was also observed. These metabolites were in accordance with previous study (Singh, 2013). During the reaction amounts of chloride ion (1.75±0.04 and 1.65±0.06 mg/L were released from initial provided 80 mg/L CF within12 h for the two strains, respectively. Therefore, it is possible that the chloride ion release leads to catabolism of the pyridyl moiety in CF (Singh, 2013). The increase in chloride concentration was accompanied with decrease of 4-(4-chloro-2-fluoro-phenoxy)-phenol concentration and support further degradation of 4-(4-chloro-2-fluoro-phenoxy)-phenol metabolite (Figure 4). However, no any other metabolites were observed by adopted methods of GC-MS detection. Strains *Bacillus* sp. and *B. cereus* grew in MS medium containing CF with 4-(4-Chloro-2-fluoro-phenoxy)-phenol as metabolite was observed during growth and was in agreement with previous observations (Smith-Grenier and Adkins, 1996; Singh, 2013; Kumar *et al*., 2014).

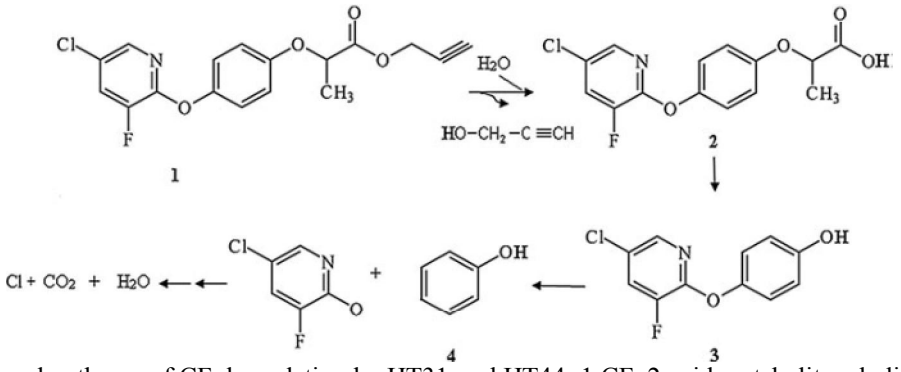

Figure (4): Proposed pathway of CF degradation by HT31 and HT44. 1 CF, 2 acid metabolite, clodinafop acid, 3 4-(4-Chloro-2-fluoro-phenoxy)-phenol, 4 phenol

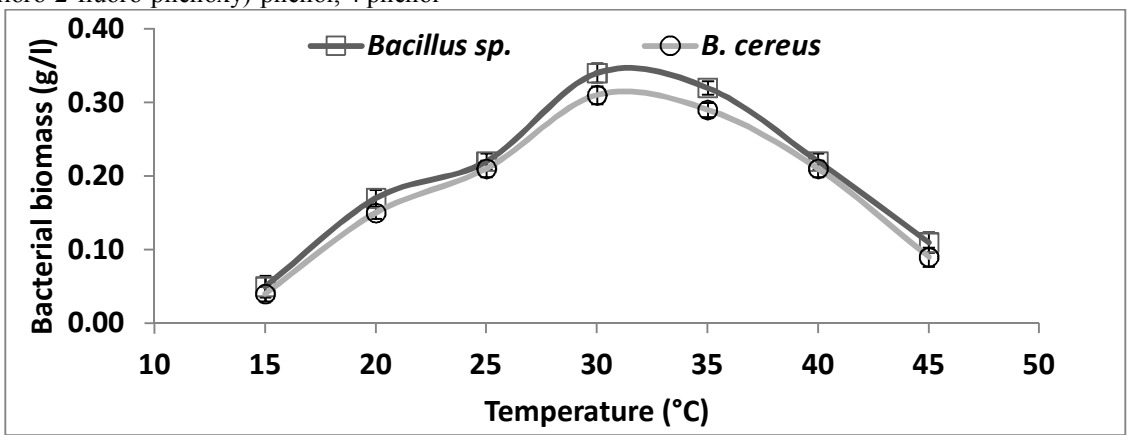

Figure (5): Effect of incubation temperature on the bacterial biomass of bacterial strains *Bacillus* sp. and *B. cereus* after 12h incubation on MS medium suplemented with 80 mg/l CF.

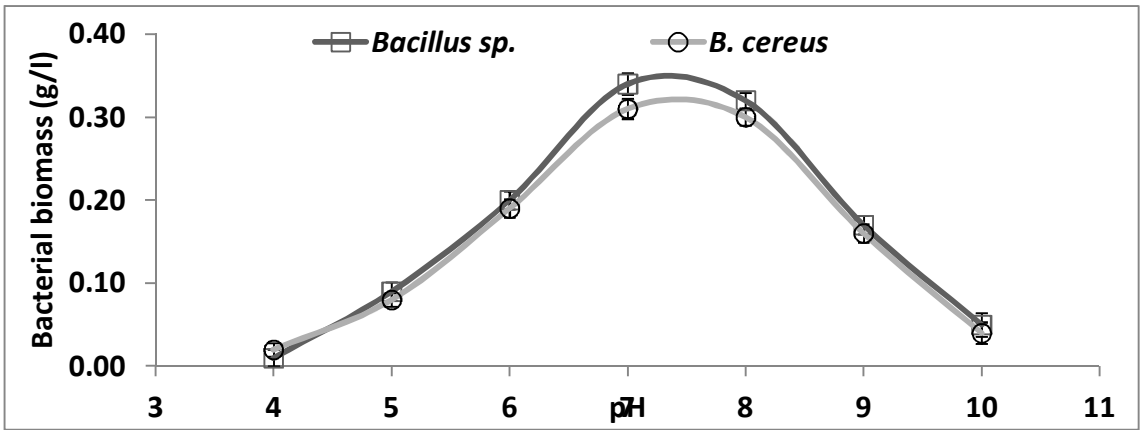

Figure (6): Effect of pH on the bacterial biomass of bacterial strains *Bacillus* sp. and *B. cereus*after 12h incubation on MS medium suplemented with 80 mg/l CF.

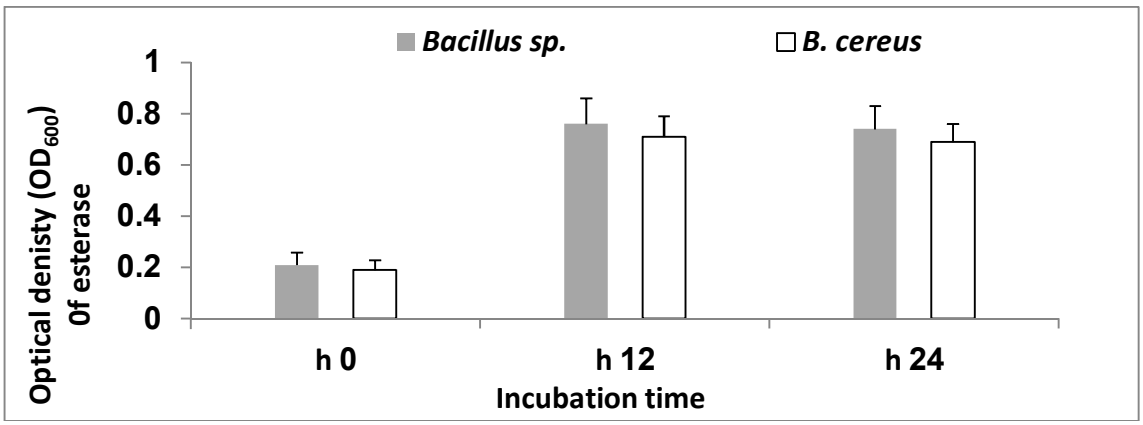

Figure (7): Esterase activity in MS medium supplemented with 80 mg/L CF of bacterial isolates after 12 and 24 hours.

The effect of incubation temperature on the bacterial biomass of the selected strains *Bacillus* sp. and *B. cereus*inoculated on MS medium suplemented with CF was shown in Figure (5). As illustrated from the figure the optimum temperature for bacterial growth of the two selected isolates HT31 and HT44 were between 35-40ºC. However, before 35ºC and after 40ºC the bacterial growth decreased for the two isolates.

The effect of pH medium on the bacterial growth of *Bacillus* sp. and *B. cereus*inoculated on MS medium suplemented with CF was presented in Figure (6). As shown from the diagram the optimum pH for bacterial growth and subsequently degradation of clodinofop-propargyl by *Bacillus* sp. and *B. cereus* was between pH 7 and 8 for the two strains.

Esterase activities were determined in growth medium at 12 and 24 hr of incubation (Figure 7). In general, the isolates at 12 hr of incubation to CF showed esterase activities and those activities non-significantly decreased at 24 hr of incubation to CF as compared to 0 h. However, bacterial strain *Bacillus* sp. showed non-significant increase in esterase activity higher than *B. cereus* at 12 and 24 hr of exposure to 80 mg/L CF. This suggested that esterase may be involved in the tested pesticides degradation as previously reported by different bacterial isolates (Mohamed *et al*., 2010; Goda*et al*., 2010; Ibrahim *et al*., 2015).

In summary, the results indicate that bacterial strains *Bacillus* sp. and *B. cereus* are capable of rapidly hydrolyzing the ester bond of CF to pro-duce clodinafop acid, which in turn may either be directly hydrolyzed to form 4-(4-Chloro-2-fluoro-phenoxy)-phenol.

In this report, a CF-degrading strains, *Bacillus* sp. and *B. cereus*, were isolated from wheat field area. The degradation of CF by those strains was simple, rapid and highly effective. Those strains could be a potential candidate to remove CF from contamination sites due to its high degradation efficiency.

References

1. Chen H, He X, Rong X, Chen W, Cai P, Liang W, Li S, Huang Q (2009) Adsorption and biodegradation of carbaryl on montmorillonite, kaolinite and goethite. Applied Clay Science46: 102–108.
2. Dong W, Jiang S, Shi K, Wang F, Li S, Zhou J, Huang F, Wang Y, Zheng Y, Hou Y, Huang Y, Cui Z (2015) Biodegradation of fenoxaprop-*P*-ethyl (FE) by *Acinetobacter* sp. Strain DL-2 and cloning of FE hydrolase gene *afeH*. Bioresource Technology 186: 114-121.
3. Fay EF, Souza CMG, MeloIS (1997) Degradaçãoabiótica de xenobióticos. In: Microbiologia Ambiental, Ed. by Melo IS and Azevedo JL, EMBRAPA-CNPMA Jaguariúna pp.125.
4. Goda SK, Elsayed EE, Khodair TA, El-Sayed W, Mohamed ME (2010) Screening for and isolation and identification of malathion degrading bacteria: cloning and sequencing a gene that potentially encodes the malathion-degrading enzyme, carboxylestrase in soil bacteria. Biodegradation 21(6): 903-913.
5. Gomori G (1953) Human esterase. J Lab Clinical Med 42: 445-453.
6. Harbottle MJ, Lear G, Sills GC, Thompson IP (2009) Enhanced biodegradation of pentachlorophenol in unsaturated soil using reversed field electrokinetics. J Environ Manage 90 (5): 1893–1900.
7. Hongming L, Xu L, Zhaojian G, Fan Y, Dingbin C, Jianchun Z, Jianhong X, Shunpeng L, Qing H (2015) Isolation of an aryloxyphenoxy-propanoate (AOPP) herbicide-degrading strain *Rhodococcusruber* JPL-2 and the cloning of a novel carboxylesterase gene (*feh*). Brazilian J of Microb 46(2): 425-432.
8. Hou Y, Tao J, Shen W, Liu J, Li J, Cao H, Cui Z (2011) Isolation of the fenoxaprop-ethyl (FE)-degrading bacterium *Rhodococcus* sp. T1, and cloning of FE hydrolase gene feh. FEMS MicrobiolLett 323:196–203.
9. Ibrahim GGA, Amin MK, Hassan AA, El-Sheikh EA (2015) Identification of pesticides degrading bacteria isolated from Egyptian soil. Zagazig J Agric Res 42(5): 1129-1143.
10. Kumar A, Kaur H, Kaur S, Singh K, Singh B (2014) Biodegradation of cladinafop-propargyl by *Aeromonas* sp. isolated from field crop. Frontiers of Biolo and Life Sci 2(4): 67-70.
11. Lin J, Chen J, Wang Y, Cai X, Wei X, Qiao X(2008) More toxic and photoresistant products from photodegradation of fenoxaprop-P-ethyl. J Agric Food Chem 56: 8226-8230.
12. Mohamed KZ, Ahmed MA, Fetyan NA, Elnagdy SM (2010) Isolation and molecular characterisation of malathion-degrading bacterial strains from waste water in Egypt. J Adv Res 1:145–149.
13. Mohan N and Naveena L (2015) Isolation and determination of efficacy of acephate degrading bacteria from agricultural soil. J Environ Sci Toxicol and Food Technol 9 (3): 10-20.
14. Rickman OB, Ryu JH, Fidler ME, Kalra S (2002) Hypersensitivity pneumonitis associated with *Mycobacterium avium* complex and hot tube use. Mayo Clin Proc 77: 1233-1237.
15. Singh B (2013) Degradation of clodinafop propargyl by *Pseudomonas* sp. strain B2. Bull Environ Contam Toxicol 6:730–733.
16. Singh B, Kaur J, Singh K (2013) Microbial degradation of an organophosphate pesticide, malathion. Crit Rev Microbiol 40: 146-154.
17. Smith-Grenier L and Adkins A (1996) Degradation of diclofop-methyl by pure cultures of bacteria isolated from Manitoban soils. Can J Microbiol 42: 227–233.
18. Vazan S, Oveisi M, Baziar S (2011) Efficiency of mesosulfuron-methyl and clodinafop-propargyl dose for the control of *Loliumperenne* in wheat. Crop Protection 30: 592-597.
19. Zhang H, Li M, Dai C, Wang G, Xiong M, Li F, Liu Y, Xu D (2016) Characterization of EstQE, a new member of esterase family VIII from the quizalofop-P-ethyl-degrading bacterium *Ochrobacterium* sp. QE-9. J of Molecular Catalysis B: Enzymatic 133: 167-175.

4/5/2017