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Captan Utilization by a Soil Bacterium *Planomicrobium flavidum* Strain EF

(Penggunaan Captan oleh Bakteria Tanah Strain EF *Planomicrobium flavidum*)

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ABSTRACT

The fungicide captan, which is commonly used to control fungal diseases in many plants, causes soil infertility and cancer to human beings. Hence, this fungicide was tested for utilization as a sole carbon source by a newly soil isolate, Planomicrobium flavidum *strain EF. This bacterium resists captan up to 2000 ppm and showed higher growth patterns in minimum salt medium supplemented with captan only, if compared with minimum salt medium without captan. Moreover, almost 77.5% of captan has been utilized by* Planomicrobiu flavidum *after only 2 h of growth under shaking conditions and only 0.8% of the fungicide was remained after 24 h of bacterial growth. Captan residues in both soil samples and minimal salt medium were accurately estimated using GC-ECD (gas chromatography - electron detector) and GC-MS/MS (gas chromatography - mass spectrum) technologies. According to current results,* Planomicrobium flavidum *strain EF is highly recommended for captan and may be other fungicides bioremediation.*

Keywords: Captan; GC-ECD; GC-MS/MS; Planomicrobium flavidum

ABSTRAK

Racun kulat captan, yang biasanya digunakan untuk kawalan penyakit kulat dalam kebanyakan tumbuhan telah menyebabkan ketidaksuburan tanah dan kanser kepada manusia. Oleh yang demikian, racun kulat ini diuji untuk penggunaan sebagai sumber karbon tunggal melalui pencilan tanah baharu, strain EF Planomicrobium flavidum*. Bakteria rintang captan ini sehingga 2000 ppm dan menunjukkan corak pertumbuhan yang lebih tinggi dalam medium garam minimum yang ditambah dengan captan sahaja, jika dibandingkan dengan medium garam minimum tanpa captan. Selain itu, hampir 77.5% daripada captan telah digunakan oleh* Planomicrobiu flavidum *hanya selepas dua jam pertumbuhan dalam keadaan goncangan dan hanya 0.8% daripada racun kulat ini kekal selepas 24 jam pertumbuhan bakteria. Sisa captan dalam kedua-dua sampel tanah dan medium garam minimum telah dianggarkan secara tepat menggunakan teknologi GC-ECD (kromatografi gas - pengesan elektron) dan GC-MS/MS (gas kromatografi - spektrum jisim). Mengikut keputusan semasa, strain EF* Planomicrobium flavidum *amat disyorkan sebagai captan dan mungkin sebagai bio pemulihan racun kulat lain.*

Kata kunci: Captan; GC-ECD;GC-MS/MS; Planomicrobium flavidum

INTRODUCTION

In order to support human growing population, high crop production should be maintained and therefore, pesticides use will be expectedly increased. Fortunately, microorganisms are playing a vital role in degradation of such toxic compounds (Ye et al. 2004). Captan ($C_9H_8Cl_3NO_2S$) is a broad- spectrum, non-systemic fungicide which is commonly used to control disease in vegetables, fruits and other crops (Agriculture & Agri-Food Canada 1997). It is also used to inhibit fungal growth on many industrial products such as leather, paper, plastics, plasters and textiles (Rawan et al. 2008). Fungicides targeting non-specific binding sites can directly affect non target organisms and therefore, fungicide use may have negative impacts that are difficult to predict (Yang et al. 2011). Captan for instance, is mutagenic and carcinogenic to human beings and poisoning to some animals (Shirasu et al. 1976). It is a potent mutagen in both prokaryotes and eukaryotes (Hines et al. 2008; Kada et al. 1974; Rahden-Staron et al. 1994; Robert 1989). Besides, it is highly toxic to fish and aquatic life (Tomin 2000; U.S. Environmental Protection Agency 2009). Moreover, captan causes soil infertility by killing soil microorganisms necessary for nitrogen fixation and phosphate and other nutrient absorption (Martinez-Toledo et al. 1998).

Captan persistence in soil differs according to environmental conditions. Its stability increases with decrease in pH and soil moisture content (Agriculture & Agri-Food Canada 1997; Goring 1972). Accordingly, degradation of captan using safe and proper strategy is a necessary requirement. Few reports are available for captan degradation by freely suspended cells of soil microorganisms (More et al. 2014). However, Megadi et al. (2010) reported the use of captan by a strain of *Bacillus circulans* as a sole source of carbon and energy (Megadi et al. 2010). More recently, More et al. (2014) reported an enhanced degradation of captan by immobilized cells of *Bacillus circulans*.

In this investigation, we studied the ability of the newly isolated soil bacterium, *Planomicrobium flavidum* strain EF to utilize the fungicide captan as a sole carbon and energy source during its growth. Besides, bacterial resistance to captan was also tested using *Planomicrobium flavidum* and other common bacteria found in the environmental sample. Captan residues were accurately estimated using GC-ECD and GC-MS/MS technologies.

Materials and Methods

Source of sample and bacterial isolation

Three different soil samples (less than 1 cm in depth) were collected and mixed together, in May- 2016, directly after applying captan (125 gm/100 L, Arysta Life Science, France) on an apple farm to control apple scab disease. The farm located at Ahmad Oraby Village, Nubarya, Beheera Governorate, Egypt. One gram of the soil mixture was agitated in 100 mL of sterile and distilled water. 0.1 mL of soil suspension was mixed with melted nutrient agar in Petri-dishes after performing different serial dilutions. Total bacterial count was estimated after 48 h incubation at 33ºC. Bacterial colonies were then purified and maintained in glycerol.

Captan estimation in the soil sample and solutions

Soil mixture and minimal salt medium containing the pesticide were handled before captan estimation according to Attallah et al. (2012). Captan residues in the environmental sample and liquid minimum salt medium were quantified using GC-MS/MS (gas chromatography- mass spectrometry) and GC-ECD (gas chromatography- electron capture detector), respectively. For GC-MS/MS analysis, Agilent 7980 GC with 7000B Quadrupole equipped with Electron Impact (EI) ionization source was used for pesticide identification and quantitation. Separation was attained by injecting of 1 µL of the sample to Agilent DB-35ms Ultra Inert GC columns (35%-Phenyl)-methylpolysiloxane with dimensions 20 m × 0.18 mm × 0.18 µm. GC oven conditions were as follows: Initial oven temperature of 70°C for 2 min, heating from 70 to 135°C at 50°C/min, holding for 0 min at 135°C, heating from 135 to 200°C at 6°C/min, holding for 0 min at 200°C, heating from 200 to 310°C at 16°C /min and holding for 8.2 min. The total run time is 30 min. MS conditions were as follows, MS source: EI -70eV, quadrupole temperature: 180°C, transfer line temperature: 320°C, gain: 40, acquisition mode: MRM, dwell time: 10 ms and solvent delay time: 5 min. For GC-ECD, Gas Chromatograph HP 6890 equipped with two electron capture detectors was used, following the instructions mentioned in the instrument log book for operation, calibration and sequence procedures. The gas chromatograph instrument was adjusted for injector temperature of 225°C and detector temperature of 300°C. Flow rate of nitrogen is 1.3 mL/min and carrier total flow rate (carrier + makeup) is 55 mL/min. Oven program was as follows, initial temperature: 90°C and initial time: 2 min. For level 1, flow rate is 20°C/min, temperature is 150°C and time is 0 min. For level 2, flow rate is 6°C/min, temperature is 270°C and time is 15 min (Angioni et al. 2003; Gilvydis & Walters 1991).

Bacterial resistance to captan

The pure bacterial cells were cultured in nutrient agar plates supplemented with different concentrations of captan, 50 to 2000 ppm. The plates were incubated at 33°C for 48 h.

Captan biodegradation and growth monitoring

A promising bacterial colony was selected according to captan resistance test results and refreshed before subjecting to grow in the presence of captan as a sole carbon and energy source. Captan was added to 50 mL sterile minimum medium (MM) in a final concentration of 100 ppm. A separately sterilized yeast extract solution was added to captan MM in a final concentration of 1% for growth induction. Finally, the selected bacterium suspension was added to the mixture (1%) and its ability to grow in such minimum medium was tested under shaking conditions (150 rpm) at 33ºC. Bacterial growth (OD_{550}) and captan residues were estimated throughout time intervals till 30 h. The MM composition was as follows in g/L: Na_2HPO_4, 2.2; KH_2PO_4, 1.4; $MgSO_4.7H_2O$, 0.6; $(NH_4)_2SO_4$, 0.3; NaCl, 0.05; $CaCl_2$, 0.02; $FeSO_4.7H_2O$, 0.01 and pH, 7 (Tallur et al. 2008).

Bacterial growth in absence of captan was also monitored using the same previously mentioned nutritional and environmental conditions to compare between the growth patterns in absence and presence of the fungicide.

Phenotyping and 16S rDNA partial sequencing

Gram stain was used to detect cell morphology of the newly isolated bacterium. DNA was extracted using GeneJet PCR Purification Kit (Thermo Fisher Scientific). Amplification of the 16S rDNA and amplicons purification were done according to Mohamed (2016). After DNA partial sequencing (GATC Biotech), sequences were compared with those in the GenBank data base using BLAST search (Altschul 1997). The sequences were finally depositedin the GenBank and accession number was obtained as will be indicated in the results. Forward and reverse primers (Macrogene incorporation, Seoul, Korea) used in PCR reactions and sequencing are illustrated in Table 1.

TABLE 1. Forward and reverse primers used in PCR reactions and sequencing

Primer direction	primer sequence
Forward	5` - AGA GTT TGA TCC TGG CTC AG-3`
Reverse	5` - GGT TAC CTT GTT ACG ACT T-3`

RESULTS

The soil samples under test have been collected directly after applying captan (1.25 gm/L) on an apple farm to control apple scab disease. The mixed environmental sample contained captan residues of 70 mg/kg soil (average reading of 3 measurements). The same sample was analyzed for total bacterial count detection. Around 700 cfu were counted in nutrient agar Petri-dishes after 48 h of incubation at 33ºC and 8 different morphological types (data not shown) were detected.

The 8 different pure bacterial cultures were tested for their sensitivity to captan, 50-2000 ppm (Table 2). The most resistant isolate was C3 which with stands 2000 ppm of captan followed by C2 that showed weak growth at 500 ppm of the fungicide. On the other hand, C7 was the most sensitive isolate followed by C6 and C8.

Due to its high captan resistance (2000 ppm), C3 was chosen for further investigations including 16S rDNA partial sequencing. BLAST search indicated that C3 is *Planomicrobium flavidum* and its accession number in the GenBank is LC195269. This short rod-shaped, Gram- positive bacterium was allowed to grow in two different nutritional conditions. A minimum salt medium supplemented with only 1% yeast extract (growth inducer) and a minimum medium with 1% yeast extract and 100 ppm captan, a sole carbon and energy source (Figure 1).

TABLE 2. Sensitivity test of the soil bacterial isolates to different captan concentrations

Captan concentration (ppm)	Bacterial code							
	C1	C2	C3	C4	C5	C6	C7	C8
50	r	r	r	r	r	r	s	r
75	r	r	r	r	s	r	s	r
100	w	r	r	r	s	s	s	s
150	s	r	r	r	s	s	s	s
200	s	r	r	s	s	s	s	s
500	s	w	r	s	s	s	s	s
1000	s	s	r	s	s	s	s	s
2000	s	s	r	s	s	s	s	s

r=resistant; s= sensitive; and w= weak growth

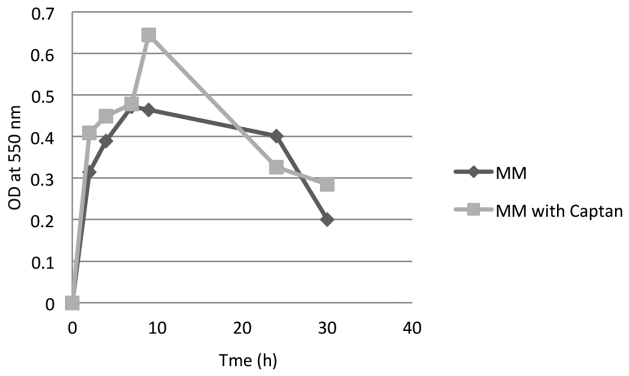

FIGURE 1. Growth curve of *Planomicrobium flavidum* in minimum medium (MM) and MM supplemented with 100 ppm of captan

TABLE 3. Captan content estimation (ppm) with growing *Planomicrobium flavidum* cells at different time intervals

Time (h)	Cell growth (OD_{550})	Captan content (ppm)
0	0.05	100± 0.24
2	0.409	22.46±0.11
4	0.449	21.99±0.13
7	0.479	14.68±0.12
9	0.645	12.18±0.13
24	0.327	0.8±0.08
30	0.285	0.52±0.07

In the presence of captan, cells growth was enhanced to reach an optical density of 0.645 at 550 nm after 9 h of shaking. On the contrary, cells optical density reached only 0.464 in the absence of captan after 9 h. This indicates the utilization of the fungicide by *Planomicrobium flavidum* cells as a carbon source.

To quantify the amount of captan that was utilized/ degraded by *Planomicrobium flavidum*, the fungicide residues were measured at different time intervals with the growing cells (Table 3). Surprisingly, only 22.46% of captan was detected in the liquid medium after only 2 h of growth under shaking conditions. The strain continued to utilize captan over time and only 0.8% and 0.52% of the fungicide were remained after 24 and 30 h of shaking, respectively.

Discussion

Captan is a multisite activity fungicide that controls a broad spectrum of plant disease. Unfortunately, it has side effects on other microorganisms due to its multiple biochemical sites impacts (Milenkovski et al. 2010). It inhibits growth of denitrifying bacteria may be due to its nonspecific effects on biochemical compounds that contain thiol in target cells (Kostov & Van Cleemput 2001). Interestingly, our new soil isolate, *Planomicrobium flavidum* EF, showed very high captan resistance, up to 2000 ppm, although the negative impact of captan on microbial flora including bacteria (Kostov & Van Cleemput 2001). This high resistance indicates the neglected effect of captan on *Planomicrobium flavidum* growth.

Captan residues may remain in soil from one day to several months according to soil type, temperature and moisture content (Li & Nelson 1985). Its persistence leads to destruction of soil microbial community (Martinez-Toledo et al. 1998). Accordingly, a safe, cost-effective and promising method for its removal is required, especially from aqueous solutions because it may leak to water bodies such as groundwater due to its mobility. In this study, *Planomicrobium flavidum* EF showed effective utilization of captan in short time, only 22.46% was remained after 2 h of growth. Moreover, only 0.8% of the fungicide was remained after 24 h of growth. In the minimum salt medium enriched with 1% yeast extract, *Planomicrobium flavidum* reached the log phase (OD_{550}= 0.472) in 7 h. On the other hand, *Planomicrobium flavidum* growth in captan minimum medium reached the log phase in 9 h (OD_{550}= 0.645) utilizing around 88% of captan in the medium. This emphasizes the ability of this bacterium to utilize captan as a soul carbon source for its growth. Megadi et al. (2010) have isolated a bacterial strain belonging to *Bacilluscirculans* that degrades captan to be used as a soul carbon source for its growth. They have also investigated in another study the degradation of captan into cis-1,2,3,6-tetrahydrophthalimide, a compound with no fungicidal activity. This later compound was further degraded to o-phthalic acid. This means a complete mineralization of captan by *Bacilluscirculans* (Megadi et al. 2010). Besides, Buyanovsky et al. (1988) have also investigated captan degradation by soil microbes under laboratory conditions. They declared the transformation of captan to tetrahydrophthalimide. In captan- treated soils, total count of fungi, bacteria and actinomycetes decreased at relatively high fungicide concentration (Banerjee & Banerjee 1987) and few articles have been published for captan biodegradation (More et al. 2014). Although *Planomicrobium flavidum* was not reported for captan degradation before, our results showed a dramatic decrease in captan levels in a minimum salt medium contains only one carbon source, captan, when *Planomicrobium flavidum* was used. This indicates the degradation of the fungicide by *Planomicrobium flavidum*. Accordingly, this promising bacterium that is commonly found in sediment, mud, marine and fermented sea foods (Dai et al. 2005; Zhang et al. 2009) may be recommended for removal of not only captan, but also other toxic fungicides from the agricultural environment. Moreover, Das and Tiwary (2013) isolated a novel strain of *Planomicrobium chinense* from diesel contaminated soil of tropical environment. This bacterium can degrade high concentration of diesel oil (up to 2.5% (v/v)). This means a need for more investigations in the bioremediation field using *Planomicrobium* spp.

Conclusion

Planomicrobium flavidum strain EF is highly efficient in captan utilization in a short time. This bacterium should be used in further study to optimize its captan degradation ability and to seek for the degradation products. However, this newly isolated bacterium which did not reported before for captan remediation, could be potentially used in bioremediation of soils and water contaminated with toxic fungicides that have negative impact on soil microflora, especially those involved in vital environmental roles in agricultural soils and water bodies. The pesticide residues were precisely estimated and accurate captan levels were successfully detected in soil and aqueous samples.

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REFERENCES

Agriculture and Agri-Food Canada. 1997. Regulatory Information on Pesticide Products (RIPP) Database (CCINFODISK). Produced by Agriculture and Agri-Food Canada and distributed by the Canadian Centre for Occupational Health and Safety. CD-ROM.

Altschul, S.F., Madden, T.L., Scaffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. 1997. Gapped BLAST and PSI- BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25(17): 3389-3402.

Angioni, A., Garau, V.L., Del Real, A.A., Melis, M., Minelli., E.V., Tuberoso, C. & Cabras, P. 2003. Determination and degradation of captan during the wine-making. *Journalof Agricultural Food Chemistry* 51: 6761-6766.

Attallah, E.R., Amer, M.E., Gomaa, A.M. & Elgohary, A.A. 2012. QuEChERS analytical method for determination of 93 pesticide residues in apples and potatoes using LC-MS/MS. *Journal of Applied Science Research* 8(12): 5690-5697.

Banerjee, A. & Banerjee, A.K. 1987. Influence of captan on some microorganisms and microbial procecessese related to the nitrogen cycle. *Plant Soil* 102: 239-245.

Buyanovsky, G.A., Pieczonka, G.J., Wagner, G.H. & Fairchild, M.L. 1988. Degradation of captan under laboratory conditions. *Bulletin of Environmental Contamination and Toxicology* 40: 689-695.

Dai, X., Wang, Y.N., Wang, B.J., Liu, S.J. & Zhou, Y.G. 2005. *Planomicrobium chinense* sp. nov., isolated from coastal sediment, and transfer of *Planococcus psychrophilus* and *Planococcus alkanoclasticus* to *Planomicrobium* as *Planomicrobium psychrophilum* comb. nov. and *Planomicrobium alkanoclasticum* comb. nov. *International Journal Systematic and Evolutionary Microbiology* 55: 699-702.

Das, R. & Tiwary, B.N. 2013. Isolation of a novel strain of *Planomicrobium chinense* from diesel contaminated soil of tropical environment. *Journal of Basic Microbiology* 53(9): 723-732.

Gilvydis, D.M. & Walters, S.M. 1991. Gas chromatographic determination of captan, folpet and captafol residues in tomatoes, cucumbers and appless using a wide-bore capillary column: Interlaboratory study. *Journal of Association Official Analytical Chemistry* 74(5): 830-835.

Goring, C.A.I. 1972. Fumigants, fungicides and nematicides. In *Organic Chemicals in the Soil Environment*, Vol. 2, edited by Goring, C.A.I. & Hamaker, J.W. New York: Marcel Dekker Incorporation.

Hines, C.J., Deddens, J.A., Jaycox, L.B., Andrews, R.N., Striley, C.A.F. & Alavanja, M.C.R. 2008. Captan exposure and evaluation of a pesticide exposure algorithm among orchard pesticide applicators in the agricultural health study. *Annuals in Occupying Huygens* 52(3): 267-278.

Kada, T., Moriya, M. & Shirasu, Y. 1974. Screening of pesticides for DNA interactions by rec-assay and mutagenesis testing and frameshift mutagens detected. *Mutation Research* 26: 243-248.

Kostov, O. & Van Cleemput, O. 2001. Microbial activity of Cu contaminated soils and effect of lime and compost on soil resiliency. *Compost Science Utilization* 9(4): 336-351.

Li, C.Y. & Nelson, E.E. 1985. Persistence of benomyl and captan and their effects on microbial activity in field soils. *Bulleten of Environmental Contamination and Toxicology* 34: 150-157.

Martinez-Toledo, M.V., Salmeron, B., Rodelas, C. & Pozo, J.G.L. 1998. Effects of fungicide captan on some functional groups of soil microflora. *Appllied Soil Ecology* 7: 245-255.

Megadi, V.B., Tallur, P.N., Mulla, S.I. & Harichandra, Z.N. 2010. Biodegradation of pendimethalin by *Bacillus circulans*. *Indian Journal of Biotechnolgy* 9: 173-177.

Milenkovski, S., Baath, E., Lindgren, P.E. & Berglund, O. 2010. Toxicity of fungicides to natural bacterial communities in wetland water and sediment measured using leucine incorporation and potential denitrification. *Ecotoxicology*19(2): 285-294.

Mohamed, E.A.H. 2016. Biophysical removal of some toxic heavy metals by Aeromonas strains. *Journal of Pure and Applied Microbiology* 10(1): 311-316.

More, V., Tallup, P., More, S.S., Niyozima, F.N. & Ninnekar, H. 2014. Enhanced degradation of captan by immobilized cells of *Bacillus circulans*. *Journal of Microbiology and Biotechnology of Food Science* 4(2): 108-111.

Rahden-Staron, I., Szumilo, M. & Ziemkiewicz, P. 1994. The effect of captan and captafol on different bacterial strains and on c-mitosis in V79 Chinese hamster fibroblasts. *Acta Biochimstry Polleten* 41: 45-55.

Rawn, D.F.K., Quade, S.C., Sun, W.F., Fouguet, A., Belanger, A. & Smith, M. 2008. Captan residue reduction in apples as a result of rinsing and peeling. *Food Chemistry* 109: 790-796.

Roberts, L. 1989. Pesticides and kids. *Science* 243: 1280.

Shirasu, Y., Moriya, M., Kato, K., Furuhashi, A. & Kada, T. 1976. Mutagenicity screening of pesticides in the microbial system. *Mutation Research* 40: 19-30.

Tallur, P.N., Megadi, V.B. & Ninnekar, H.Z. 2008. Biodegradation of cypermethrin by *Micrococcus sp*. strain CPN1. *Biodegradation* 19: 77-82.

Tomin, C.D.S. 2000.*The Pesticide Manual*: *A World Compendium*. 12th ed. British Crop Protection Council, Farnham, U.K. pp. 132-133.

U.S. Environmental Protection Agency. 2009. Registration Eligibility Decision (RED): Captan, EPA: Washington DC, http:// www.epa.gov/oppsrrdl/REDs/0120red.

Yang, C., Hamel, C., Vujanovic, V. & Yantai, G. 2011. Fungicide: Modes of action and possible impact on nontarget microorganisms. *ISRN Ecology*. Article ID. 130289.

Ye, J., Singh, A. & Ward, O.P. 2004. Biodegradation of nitroaromatics compounds and other nitrogen-containing xenobiotics. *World Journal of Microbiology and Biotechnology* 20: 117-135.

Zhang, D.C., Liu, H.C., Xin, Y.H., Yu, Y., Zhou, P.J. & Zhou, Y.G. 2009. *Planomicrobium glaciei* sp. nov., a psychrotolerant bacterium isolated from a glacier. *International Journal of Systematic and Evolutionary Microbiology* 59: 1387-1390.

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