International Journal of Applied Environmental Sciences
ISSN 0973-6077 Volume 12, Number 5 (2017), pp. 999-1008

http://www.ripublication.com

Oxamyl Utilization by *Micrococcus luteus* OX, Isolated from Egyptian Soil

Eman A. H. Mohamed

Associate Prof. of Microbiology

Botany and Microbiology Department, Faculty of Science, Damanhour University, Damanhour city, Egypt.

Biotechnology Department, Faculty of Science, Taif University, Al-Hawya, Taif city, KSA.

Abstract

The extreme human poison, oxamyl, which is commonly used to control a broad spectrum of pesticides, including nematodes, was subjected to bioremediation using a newly isolated soil bacterium, *Micrococcus luteus* OX. This bacterium utilized 12.2% of oxamyl within 48h under shaking. Besides, growth was enhanced in the presence of the pesticide-containing MSM. Oxamyl residues were precisely estimated using HPLC/MS/MS system. The current study is the first to report utilization of oxamyl using *Micrococcus luteus*. Therefore, I recommend *M. luteus* OX as a bioremediator not only for oxamyl, but may be for other carbamate pesticides as well.

Keywords: Oxamyl, Bioremediation, *Micrococcus luteus*, HPLC/MS/MS, carbamate pesticides.

INTRODUCTION

Pesticides are chemical substances which are used to manage pest populations. Due to their mobility, pesticides represent a hazard for life [1]. Oxamyl, for instance, has been classified by the U.S. Environmental Protection Agency (EPA) as Restricted Use Pesticide (RUP) due to its acute toxicity to humans, birds, and mammals [2]. This insecticide controls a broad spectrum of insects including nematodes. It belongs to a family of pesticides called carbamates which work by blocking of cholinesterase, an essential nervous system enzyme [3]. This extreme human poison, oxamyl, can enter

the body throughout three different routes: inhalation, ingestion, or skin absorption [4, 5]. The EPA Lifetime Health Advisory (LHA) level for oxamyl is 200 ppb [6]. However, oxamyl soil half-life is one to 5 weeks, with residual levels found for up to 6 to 12 months later [7].

Bioremediation refers to any strategy can be used to remove undesirable effects of pollutants from any environment using living organisms. Bacteria have been extensively used in bioremediation due to their fast growth, easy handling, and low cost [1]. Osborn et al. (2010) have isolated and identified oxamyl-degrading bacteria from UK agricultural soils [8]. However, *Micrococcus* spp. are so effective bioremediators that can degrade carbaryl, carbofuran, naphthalene, and many other aromatic compounds to be used as growth substances [9]. In another study of Tallur et al. (2008), *Micrococcus* sp. strain CPN1 was effectively used for degradation of cypermethrine, a pyrethroid pesticide [10].

With the recent advances in HPLC/MS/MS (Mass Spectrometry) instrumentation, it can be used to simultaneously monitor hundreds of potential contaminants including those difficult to be detected by GC. The MS/MS detector allows for very specific and sensitive detection of the pesticides species. On the other hand, LC is important to ensure the highest data quality. Therefore, LC /MS/MS is a highly precise and specific technique to detect different types of pesticides [11].

In this study, a recently isolated bacterium (*Micrococcus luteus* OX) has been successfully used for bioremediation of oxamyl from a minimum salt medium. Pesticide residues were precisely quantified using MS/MS system combined with HPLC. The current study is the first to report utilization of oxamyl by *Micrococcus*.

MATERIALS AND METHODS

Sampling and bacterial isolation

Soil samples were collected, less than 1 cm in depth, in May- 2016, from a farm located at Nubarya, Beheera Governorate, Egypt. The soil samples were collected from different locations within the farm and mixed together. One gram of the soil mixture was agitated in 100 ml sterile and distilled water. Soil suspension (0.1 ml) was mixed with melted nutrient agar in Petri-dishes after different serial dilutions. In 48 h, the total bacterial count was estimated after incubation at 33^{o} C. Bacterial colonies were counted, purified and maintained in glycerol.

Pesticide estimation in the soil sample and aqueous solutions

Pesticide residues in the previously described environmental sample and liquid minimum salt medium were quantified using LC/MS/MS (liquid chromatography-mass spectrometry). LC/MS/MS was performed with an Agilent 1200 Series HPLC instrument coupled to an API 4000 Qtrap MS/MS from Applied Biosystems with an electrospray ionisation (ESI) interface. Separation was performed on an Agilent ZORBAX Eclipse XDB C18 column 4.6 x 150 mm, 5 Micron particle size. Necessary

reagents, preparations, extraction procedures and analytical steps were performed according to Attallah et al. (2012) [11].

Bacterial resistance to oxamyl, captan, and thiophonate methyl

Pure bacterial colonies were cultured in nutrient agar plates supplemented with different concentrations of oxamyl, captan, and thiophonate methyl, separately. The plates were incubated at 33° C for 48h. Pesticides names, mode of actions, and manufacturers are listed in Table 1.

Table 1: Pesticides names, mode of actions, and manufacturers

Pesticide chemical name	Brand name	Mode of action	Manufacturer
Captan 80% WG	Captan	Fungicide	Arysta Life Science, France
Oxamyl 24% SL	Nematex	Nematocide	Medmac, Jordon
Thiophonate methyl 70 WP	Topsin M	Fungicide	Wuxi xinan pesticides Co.Ltd., China

Oxamyl biodegradation and growth monitoring

A promising bacterial colony was selected according to oxamyl resistance test results and subjected to grow in the presence of oxamyl as a sole carbon and energy source. Nematex 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 Nematex 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 33° C. Bacterial growth (OD_{550}) and oxamyl residues were estimated throughout time intervals till 48 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 [10].

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

Phenotypic characterization

Gram stain and motility test were used to detect cell morphology of the newly isolated bacterium.

16S rDNA partial sequencing

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) [12]. After DNA partial sequencing (GATC Biotech), sequences were compared with those in the GenBank data base using BLAST search [13]. The

sequences were finally deposited in 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 2.

Table 2: Primers used in PCR reactions and sequencing

Direction	Sequence
Forward	5`- AGA GTT TGA TCC TGG CTC AG-3`
Reverse	5`- GGT TAC CTT GTT ACG ACT T-3`

RESULTS

The collected soil sample was subjected for analysis of pesticide residues including oxamyl, captan, thiophonate methyl, and others using LC/MS/MS system (Figure 1a). In Figure 1b, a sharp oxamyl peak was detected in a liquid minimal salt medium at a retention time of 3.5.

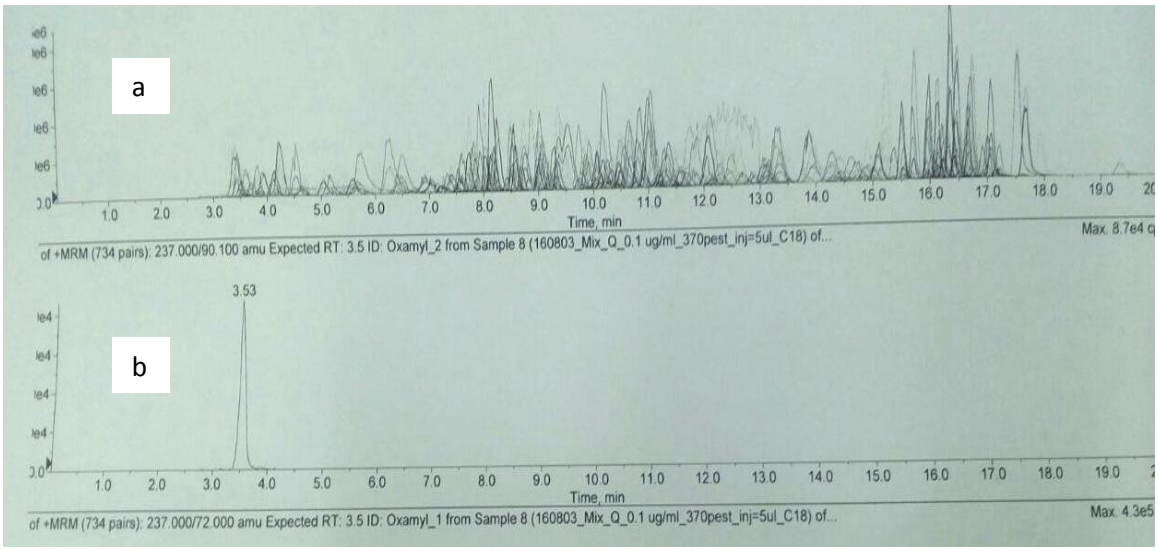

Figure 1 Pesticides analyses using HPLC/MS/MS for: a, the soil sample; and b, oxamyl in a MSM. Oxamyl retention time is 3.5.

In parallel with pesticide analysis, the total bacterial count was estimated and 697 cfu were detected with five different morphological types (data not shown). However, one phenotype, E1, was dominant (around 60%) among the others and characterized with yellow colonies on agar plates. Besides, bacterial resistance against 3 different

pesticides was recorded and E1 was found to be the most resistant phenotype (data not shown). The three pesticides are Captan, Nematex, and Topsin M. According to Table 3, E1 can resist Captan, Topsin, and Nematex till 150, 100, and 200 ppm, respectively. Therefore, Nematex was selected for bioremediation using E1 phenotype which was identified using the 16S rDNA partial sequencing (approx 1338 bp). Blast search indicated that the bacterium belongs to *Micrococcus luteus* (100 %) and its accession number which was deposited in the GenBank is LC209194. This bacterium, *Micrococcus luteus* strain OX, is a Gram- positive, non-motile cocci usually arranged in tetrads.

Table 3. Bacterial sensitivity against different pesticides

Pesticide concentration in ppm											
Captan				Topsin				Nematex			
50	100	150	200	50	100	150		50	100	150	200
R	R	R	S	R	R	S		R	R	R	W

- R, resistant; S, sensitive; and W, weak growth.

M. luteus was allowed to grow in two different liquid media. The first is a Minimum Salt Medium (MSM) amended with 1% yeast extract for growth induction, and the second is the same as the first one in addition to 100 ppm Nematex as a sole carbon source (Figure 1b). With Nematex medium, *M. luteus* OX growth was enhanced and reached an optical density of 0.62 at 550 nm in 24 h, if compared with the MSM without the nematicide ($O.D._{550}$= 0.483) (Table 4). This indicates the utilization of oxamyl by the bacterial cells as a carbon source for their growth. In 30 h of shaking incubation, cells continued to grow reaching an O.D. of 0.75.

Table 4: *Micrococcus luteus* OX growth in the presence and absence of oxamyl

Time (h)	Growth in MSM with oxamyl	Growth in MSM only
0	0.025	0.021
2	0.265	0.263
4	0.316	0.305
7	0.365	0.317
9	0.48	0.41
24	0.62	0.483
30	0.75	0.46

To quantify oxamyl concentration which was utilized by *M. luteus* OX, nematicide residues were precisely estimated by LC/MS/MS system at different time intervals

(Table 5). Surprisingly, only 12.2 % of oxamyl was detected in 48h and longer incubation periods did not show appreciable oxamyl reduction (data not shown).

Table 5. Oxamyl residues (ppm) estimated with the growing *Micrecoccus luteus* cells at different time intervals

Time (h)	Cell growth (OD_{550})	Oxamyl residues (ppm)
0	0.025	100
2	0.265	90.2
4	0.316	78.2
7	0.365	70.8
9	0.48	66.2
24	0.62	56.2
30	0.75	40.6
48	0.98	12.2

DISCUSSION

Pesticides can move through the air to reach other parts of the environment such as water. They can be washed off the soil into nearby water bodies including groundwater [14]. Hence, this study concerns with the bioremediation of oxamyl in aqueous solutions.

Resistance of *Micrococcus luteus* to the tested pesticides (Captan, Nematex, and Topsin M) and may be to other pesticides is expected due to its presence in a pesticide treated agricultural soil (Figure 1a). However, utilization of oxamyl by *Micrococcus luteus* as a sole carbon and energy source is efficient throughout time and cell growth is enhanced in the presence of the pesticide due to its degradation most probably into oxamyl oxime which is not further transformed [15]. The accumulation of oxamyl oxime in different bacterial cultures indicates that bacterial isolates cannot use it as an energy source [15]. However, the hydrolysis of oxamyl is a detoxification process itself, because oxamyl oxime is less toxic to mammals and aquatic organisms [16].

Oxamyl is a carbamoyloxime nematicide [8]. Thus, it seems that oxamyl-degrading bacteria utilize the methyl-carbamoyl which is released during oxamyl hydrolysis as a carbon and nitrogen source (Figure 2). Eventually, the unstable carbamic acid is rapidly broken down to formaldehyde and CO_2 [17, 18, 19]. This pathway is very common among carbamate-hydrolyzing bacteria [17, 20].

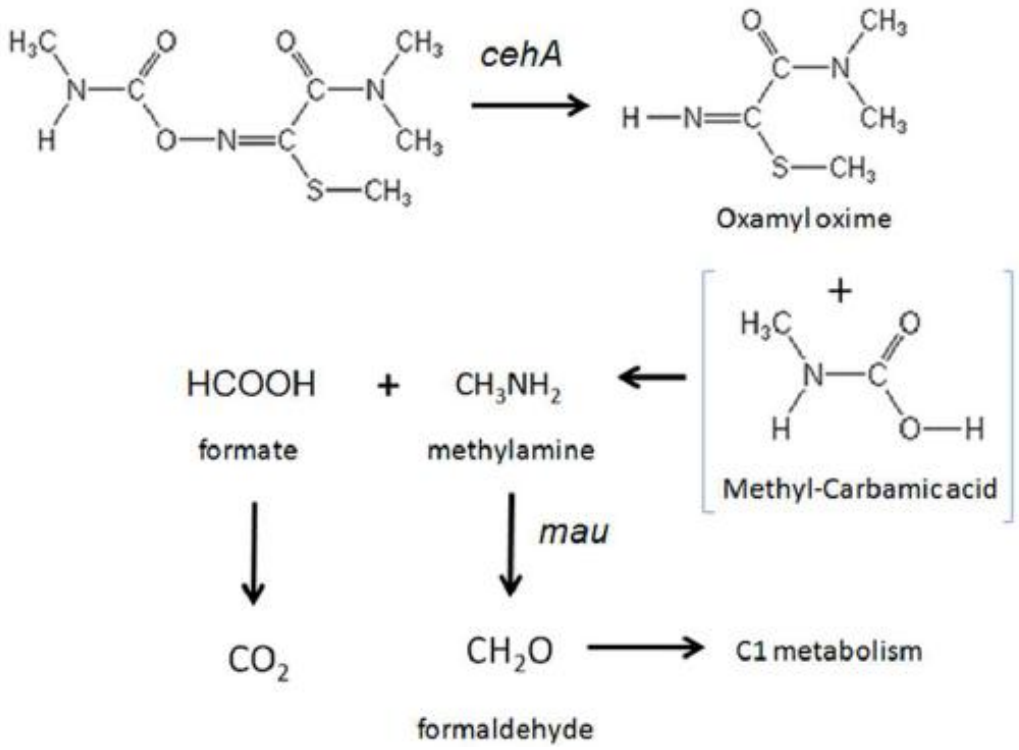

Figure 2 The proposed metabolic pathway of oxamyl by carbamate- hydrolyzing bacteria. The steps of the metabolic pathway controlled by the genes *cehA* and methylaminede hydrogenase (*mau*) are indicated (Rousidou et al. 2016).

In this study, 87.8% of oxamyl was utilized by *Micrococcus luteus* in 48h using MSM supplemented with the nematicide as a sole carbon source under shaking conditions. This result can be compared with those of Rousidou et al. (2016) [15]. They used four different newly isolated *Pseudomonas* strains to degrade oxamyl into oxamyl oxime. Three of these strains completed the oxamyl degradation within 96h. In my study, longer incubation than 2 days showed no appreciable degradation (data not shown). Besides, Osborn et al. (2010) reported oxamyl utilization by *Aminobacter* spp. and *Mesorhizobium sp* [8]. However, this is the first study reports the utilization of oxamyl by *Micrococcus luteus* or any other *Micrococcus* spp. On the other side, different *Micrococcus* species isolated from soils seem to be efficient bioremediators. They have been tested to degrade pyridine (a byproduct of cool gasification) [21], cypermethrine (a pesticide) [22], carbaryl (insecticide), and other aromatic compounds [9]. Carbaryl belongs to carbamate family of insecticide, like oxamyl, and a bacterium belongs to *Micrococcus sp.* which was isolated from a garden soil is capable to utilize carbaryl as a sole carbon source [9].

CONCLUSION

Oxamyl is an extreme human poison which shouldn't exceed 200ppb in water systems [6]. The current study is the first to report the utilization of oxamyl by *Micrococcus luteus*. This promising bacterium remediated 87.8% of 100ppm oxamyl within only 2 days in a poor medium containing one carbon source, oxamyl. Oxamyl residues in the liquid MSM were precisely measured using highly sensitive HPLC system, LC/MS/MS. Therefore, *Micrococcus luteus* strain OX is strongly recommended not only for oxamyl remediation, but may be for bioremediation of other pesticides, especially carbamates.

ACKNOWLEDGMENTS

I would like to express grateful thanks to Prof. Dr. Ashraf El-Mersafy and Dr. Emad Attallah, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Giza, Egypt, for their tremendous assistance in samples analyses using LC/MS/MS system. Many thanks are to be extended to Prof. Dr. Azza G. Farag, Prof. Dr. Sahar Yousef, and Dr. Fatma Moustafa (Institute of Plant Pathology Research, Agriculture Research Centre, Egypt) for their great help in providing the laboratory facilities.

REFERENCES

[1] Velazquez-Fernandez JB, Martinez-Rizo AB, Ramirez-Sandoval M, Dominguez-Ojeda D. Biodegradation and Bioremediation of Organic Pesticides, chapter 12. In: *Creative Commons Attribution License.* 2012 (http://creativecommons.org/licenses/by/3.0) doi.org/10.5772/46845. 2012

[2] DuPont Agricultural Products. Material Safety Data Sheet for "Vydate L" Insecticide/Nematicide. DuPont, Wilmington, DE. 1990.

[3] Hayes WJ. Pesticides studied in man. Baltimore, MD: Williams and Wilkins. 1982.

[4] Gosselin RE, Smith RP, Hodge C, Braddock JE. *Clinical toxicology of commercial products*, 5th ed. Baltimore: Williams and Wilkins. 1984.

[5] Sax NI. Dangerous properties of industrial materials. 6th edition. NY: Van Nostrand-Reinhold Co. 1984.

[6] US Environmental Protection Agency. Health Advisory Summary: Oxamyl. US EPA, Washington, DC. 1989.

[7] US Environmental Protection Agency. Health Advisory: Oxamyl. Office of Drinking Water, US EPA, Washington, DC. 1987.

[8] Osborn RK, Haydock PPJ, Edwards SG. Isolation and identification of oxamyl-degrading bacteria from UK agricultural soils. Soil Biol Biochem. 2010; 42: 998-1000.

[9] Doddamani HP, Ninnekar HZ. Biodegradation of carbaryl by a *Micrococcus* species. Current Microbiol. 2001; 43(1): 69-73.

[10] Tallur PN, Megadi VB, Ninnekar HZ. Biodegradation of cypermethrin by *Micrococcus sp*. strain CPN1. Biodegradation. 2008; 19: 77–82.

[11] Attallah ER, Amer ME, Gomaa AM, El Gohary AA. QuEChERS analytical method for determination of 93 pesticide residues in apples and potatoes using LC–MS/MS. J Appl Sci Res. 2012; 8(12): 5690-5697.

[12] Mohamed AH. Biophysical removal of some toxic heavy metals by *Aeromonas* strains. J Pure Appl Microbiol. 2016; 10(1): 311-316.

[13] Altschul SF, Madden TL, Scaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI- BLAST: A new generation of protein database search programs. Nucleic Acids Res. 1997; 25(17): 3389-3402.

[14] Tashkent, Part 1. Conditions and provisions for developing a national strategy for biodiversity conservation. Biodiversity Conservation National Strategy and Action Plan of Republic of Uzbekistan. Prepared by the National Biodiversity Strategy Project Steering Committee with the Financial Assistance of The Global Environmental Facility (GEF) and Technical Assistance of United Nations Development Programme (UNDP). 1998.

[15] Rousidou K, Chanika E, Georgiadou D, Soueref E, Katsarou D, Kolovos P, Ntougias S, Tourna M, Tzortzakakis EA, Karpouzas DG. Isolation of oxamyl-degrading bacteria and identification of *cehA* as a novel oxamyl hydrolase gene. Front Microbiol. 2016; **7**, Article 616.

[16] European Food Safety Authority [EFSA]. Conclusion Regarding the Peer Review of the Pesticide Risk Assessment of the Active Substance Oxamyl. EFSA, (Parma: European Food Safety Authority). 2005: 1–78.

[17] Karns JS, Mulbry WW, Nelson JO, Kearney PC. Metabolism of carbofuran by a pure bacterial culture. Pest Biochem Physiol. 1986; 25: 211–217.

[18] Feng X, Ou L-T, Ogram A. Plasmid-mediated mineralization of carbofuran by *Sphingomonas sp*. strain CF06. Appl Environ Microbiol. 1997; 63: 1332–1337.

[19] Bachman J, Patterson HH. Photodecomposition of the carbamate pesticide carbofuran: kinetics and the influence of dissolved organic matter. Environ Sci Technol. 1999; 33: 874–881.

[20] Hashimoto M, Mizutani A, Tago K, Kameyama M, Shimojo T, Hayatsu M. Cloning and nucleotide sequence of carbaryl hydrolase gene (*cahA*) from *Arthrobacter sp*. RC100. J Biosci Bioeng. 2006; 101: 410–414.

[21] Gerald K, LEE SIt, Sommers E, Konopka A. Degradation of pyridine by *Micrococcus luteus* isolated from soil. Appl Environ Microbiol. 1986; 51(5): 963-968.

[22] Tallur PN, Mulla SI, Megadi VB, Talwar MP, Ninnerkar HZ. Biodegradation of cypermethrin by immobilized cells of *Micrococcus sp*. strain CPN1. Braz J Microbiol. 2015; 46(3): 667-672.