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RESEARCH ARTICLE OPEN ACCESS

Malathion Biodegradation by *L. casei* (NRRL1922) and *L. acidophilus* (NRRL 23431) in Fermented Skimmed Milk

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Abstract

The aim of this study was to investigate and trace the biodegradation products of the pesticide malathion in a comparative manner by two different lactobacilli strains; *L. casei* (NRRL1922) and *L. acidophilus* (NRRL 23431). The two strains were cultivated separately into skimmed milk supplemented with 5 ng/ml malathion. After incubation under the appropriate conditions, randomized samples were taken at intervals 24, 48, 72 and 120 hours along with control samples and analyzed for the presence of malathion and its degradation products by the GC-MS spectrometry; As well as, analyzed to record the level of phosphatase enzyme which suggested to be involved in the biodegradation process. The results showed a high ability of the two tested strains to degrade malathion with a superiority of *L. acidophilus* (NRRL 23431) over *L. casei* (NRRL 1922). The level of phosphatase enzyme was elevated in both strains in the presence of malathion and decreased gradually upon the depletion of malathion from the sample, which reflects the role of the phosphatase enzyme in the biodegradation process.

Keywords: Malathion, Biodegradation, Lactic acid bacteria, Metabolites

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INTRODUCTION

Organophosphorus has been widely used for eradicating pests. However, they are highly toxic to human health and cause serious public problems that are reported in many studies [1-9]. The OPPs are all esters of phosphoric acid which include aliphatic, phenyl and heterocyclic derivatives. So, most of organophosphorus can be used by microorganisms as carbon and/or phosphate source in the environment by carboxylesterase and phosphatase activities[10,11].

Malathion is wide-spectrum pesticide. It is widely used worldwide for agricultural, residential, and public health purposes, primarily to improve food production and provide protection against disease vectors. It has a major importance in Egypt due to its wide distribution, persistence and extensive use. Malathion led to contamination of soil, air, surface and groundwater.

Milk is an ideal fluid for dissolving environmental organic pollutants such as pesticide residues because most of them have lipophilic properties. Thus, milk is used as an indicator for determining the persistence of organic pollutants[12]. Their presence in dairy products occurs mostly due to the feeding of contaminated fodder, feedstuff and water to dairy cattle[13].

Technical-grade malathion which is usually used for agricultural purposes may contain up to 11 impurities formed during its production and/or storage, many of these impurities, such as isomalathion and malaoxon that have been found to be significantly more toxic than malathion itself or to potentiate malathion toxicity[14].

Biodegradation of pesticides by microorganisms is safer and cheaper than other ways to reduce pesticides level especially in the processed milk. But, the most crucial point is the type of the microorganism that can biodegrade the contaminant and being itself safe. Also, the biodegradation process that may resulted in products that may be toxic than the parent compound[15]

There are different genera have been used in the bioremediation of organophosphorus pesticides in the polluted environment including *Pseudomonas*[16] and *Paracoccus*[17]. For the sake of food safety guidelines many researches began to investigate the ability of Lactic acid bacteria – generally regarded as safe bacteria- to degrade toxic substances in raw food products or during manufacturing process[18]. Probiotic Lactic acid bacteria may be potential microbes for reducing the risk of pesticides in food and environment[19]. Unlikely, only very few reports have investigated the degradation of organophosphorus contaminant by microorganisms in food matrices[20].

Previous studies have studied LAB for the degradation of malathion for example, Zhang et al[21] assayed quantitatively the degradation of five organophosphorus including malathion in skimmed milk at 42°C by ten different species of lactic acid bacteria. Also, the phosphatase excreted by these microorganisms in the milk was also measured. Also, Zhou & Zhao[22] investigated different strains of lactic acid bacteria and yogurt starters that enhance the biodegradation of nine organophosphorus in skimmed milk and analyzed phosphatase enzyme activity. In this study, two different strains of lactic acid bacteria *L. casei* (NRRL 1922) and *L. acidophilus* (NRRL 23431) were investigated for malathion biodegradation by tracking its secondary metabolites during different intervals 24, 48, 72, 120 hours by GC-MS to evaluate precisely the degradation ability of the two strains along with the phosphatase enzyme activity excreted by these two strains in the same intervals. To the best of our knowledge, this is the first study to track the secondary metabolites of malathion with two different strains of lactic acid bacteria.

MATERIALS AND METHODS

Malathion utilized in this study was obtained from Kafr Elzayyat company, Egypt (95% active ingredient).

Two LAB strains *L. casei* (NRRL1922) -referred as (L1)- and *L. acidophilus* (NRRL 23431) – referred as (L2), were obtained in lyophilized form from the ARS Culture Collection (The National Center for Agricultural Utilization Research in Peoria, Illinois). Skimmed milk powder was obtained from a local market and stored at 4°C before use. All chemical reagents used were analytical grade, while solvent used were high-performance liquid chromatography-grade.

Strain activation

The two LAB strains were rehydrated in 1.0 mL sterilized skimmed milk medium (11g skimmed milk. 100 ml d.H_2O) then cultivated into

a normal MRS broth medium, (Oxoid Ltd., England) (5 %, v/v) and cultured at 37 °C for 24 h. The MRS medium re-culturing was repeated four times to ensure the full viability and purity of the strains[21,22].

Then, the strains were sub-cultured in a skimmed milk medium of 11% (w/w) for 12 h before use as the biodegradability experiment will be carried out in the skimmed milk as a medium.

Milk sample preparation and inoculation

Malathion was added into the skimmed milk (11%, w/w) at 5 ng/ml and stirred for enough time at room temperature to ensure well distribution of malathion. After sterilization of the spiked skimmed milk at the appropriate sterilization program (90°C for 15 min) and cooling to room temperature; LAB starters (L1 and L2) were inoculated separately into the treated milk at a level of 5% (v/v). The samples along with the control (malathion treated milk without inoculation of bacteria) were incubated at 37°C. for 24, 48, 72 and 120 hours, respectively; Some samples were selected randomly for analysis along with control samples at each interval time[21,22].

Extraction and purification of organophosphorus pesticides

The OPPs extraction and purification from milk samples were carried out three times. The process carried out by the addition equal volume of a 1:4, v/v acetone–acetonitrile mixture to 10 ml sample with hard shaking for a while then the samples were centrifuged for 400× g for 6 min. The collected upper liquid phases -containing the malathion- after the three times of extraction was transferred to a separation funnel for the purification with 50 ml dichloromethane with shaking for 20 minutes, then left enough time for phase separation. As a final step in purification and cleaning up process. The dichloromethane phase was separated, and filtered through anhydrous sodium sulphate (2 gm) for dehydrating the sample and collected as a purified pesticide extract. Pagliuca et al 23 A 15.0 ml sample of the purified extract was evaporated to dryness at 30°C with nitrogen gas in an evaporation station. The residue was reconstituted to a volume of 1.0 mL with acetone and filtered through a 0.45 µm microporous membrane filter to get rid of any impurities including bacterial cell before GC-MS analysis[24].

GC-MS analysis

For GC-MS analysis, 150 ml sample from the purified extract was evaporated to dryness. The residue was re-dissolved in 1 ml acetone and filtrated by syringe filter 0.45µm to remove any present impurities; thereafter 200 µl from it injected into the GLC system. Gas chromatographic analyses were carried out using Trace GC Ultra-TSQ Quantum mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The temperature of the column oven was held initially at 70°C hold for 5 min and then increased by 5°C/min to 280°C hold for 5 min. Temperature of the injector and MS transfer line were held at 250°C. Helium was used as a carrier gas at a constant rate of flow rate of 1 ml/min. The delay of the solvent was 4 min and 3 µl of diluted samples were automatically injected using Autosampler AS1310 coupled with GC in the split mode. The GC-MS was operated in a SIM/SCAN Mode[25].

Assay of phosphatase activity

The selected samples for malathion analysis were used for studying phosphatase activity along with malathion degradation in addition to three sets of control: the first set contains malathion only, the second one contains the tested bacteria L1 only, and the third set contains the tested bacteria L2 only. The samples as well as the controls were centrifuged 10,000 × g for 15 minutes at 4°C, the harvested bacterial cells were washed three times by 10 mmol L^{-1} sodium carbonate/bicarbonate buffer (which was prepared by dissolving 3.5 gm of anhydrous sodium carbonate and 1.5 gm of sodium bicarbonate in one liter of distilled water, pH 10.5). The harvested, washed bacterial cells were resuspended in 5 mL of 10 mmol L^{-1} sodium carbonate/bicarbonate buffer and agitated for 1 minute. The cells mixed with the buffer subjected to sonication for 10 seconds ten times with 15 seconds intervals to set the phosphatase enzyme free outside the bacterial cells; then the samples were centrifuged to get rid of the cell debris. The supernatant of each sample was used to evaluate the phosphatase activity separately. The phosphatase activity was measured according to Zhou & Zhao[22].

The level of serum alkaline phosphatase (ALP) was assayed by quantitative kinetic assay

Table 1. The relative abundance of Malathion and its metabolites fragment ions in L1 treatment

MF and MW of different fragments	Relative abundance of fragment ions at different intervals (%)					
	24 hours		48	72	120	
	Control	L1	L1	L1	Control	L1
Malathion MF: $C_{10}H_{19}O_6PS_2$ MW: 330.4 g/mol	42.89	30.14	15.14	3.99	23.89	0.95
Malaoxon MF: $C_{10}H_{19}O_7PS$ MW: 314.29g/mol	22.02	11.68	8.22	3.23	42.02	0.45
Isomalathion MF: $C_{10}H_{19}O_6PS_2$ MW: 330.4g/mol	11.56	5.22	3.55	1.89	29.56	1.0
2_mercaptosuccinic acid MF: $C_4H_6O_4S$ MW: 150.16g/mol		15.22	12.22	5.53		1.53
malathion monocarboxylic acid (MMA) MF: $C_8H_{15}O_6PS_2$ MW: 302.3g/mol		30.24	31.65	32.05		36.12
malathion dicarboxylic acid (MDA) MF: $C_6H_{11}O_6PS_2$ MW: 274.3g/mol		12.33	15.89	17.88		25.66

method using alkaline phosphatase kit (214001, Spectrum, Obour city, Cairo, Egypt) following the manufacturer's instructions by preparing the working solution according to the number of tests required by mixing 9 volumes of buffer solution (R1) which contain 2-Amino-2-Methyl-1-Propanol (pH 10.3) and $MgCl_2$ and 1 volume of substrate (R2) which contain p-Nitrophenylphosphate, e.g. 900 µl R1 + 100 µl R2 then a volume of 10 µl of the sample was added to 1 ml of the working solution, mixed carefully and read initial absorbance in a photometer DTN-405 Semi – automated chemistry analyzer (Austrian) at 405 nm after 1 minute and start timer simultaneously. Read again after 1, 2 and 3 minutes. Determine the mean absorbance change per minute (ΔA/min).

CalculationΔ

ALP activity (U/L) = ΔA405nm/min× 5454

Where (ΔA/min) is the average of the readings.

RESULTS

Investigation of malathion biodegradation by GC-MS

Data in Table 1, 2 showed considerable decrease in the relative abundance of malathion fragment ions after incubation with L1 and L2 strains over elapsed time. Data revealed that L2 treatment decreased the relative abundance of malathion fragment ions over L1 treatment.

The separation of the degraded products by GC-MS in control samples (95 % purity) showed the appearance of the molecular ion peak of malaoxon fragment ions and the molecular ion peak of isomalathion fragment ions (which formed normally upon storage). The relative abundance of malaoxon and isomalathion fragment ions were decreased in spiked milk as the culture time progressed in L1 treatment until it reach its lowest level in the 5th day by 1.0, 0.45 respectively when compared to control. Also, in L2 treatment the

Table 2. The relative abundance of Malathion and its metabolites fragment ions in L2 treatment

Fragment Chemical formula and Molecular wight	Relative abundance of fragment ions at different intervals (%)					
	24 hours		48 hours	72 hours	120 hours	
Relative abundance of fragment ions% in different hours	control	L2 sample			Control	L2 sample
Malathion $C_{10}H_{19}O_6PS_2$ M.wt 330	42.89	15.14	10.22	6.55	23.89	2.76
Malaoxon $C_{10}H_{19}O_7PS$ M.wt 314	22.02	15.55	12.24	8.13	42.02	2.18
Isomalathion $C_{10}H_{19}O_7PS$ M.wt 314	11.56	3.22	2.05	1.22	29.56	0.85
2-mercaptosuccinic acid $C_4H_6O_4S$ M.wt 150		9.55	7.22	4.05		1.11
malathion monocarboxylic acid (MMA) $C_{10}H_{19}O_7PS$ M.wt 314		45.24	52.21	60.24		68.66
malathion dicarboxylic acid (MDA) $C_6H_{11}O_6PS_2$ M.wt 274		10.23	15.44	19.42		23.52

relative abundance of malaoxon, isomalathion fragment ions decreased at 1st, 2nd and 3rd of incubation period until it reach the 5th day by 2.18, 0.85 respectively when compared to control due to the biodegradation process.

After the first 24 hours in L1 and L2 treatment, the molecular ion peak of malathion monocarboxylic acid (MMA) and malathion dicarboxylic acid (MDA) were detected. In L1 and L2 treatment, the relative abundance of MMA and MDA fragment ions were increased in spiked milk with malathion being decreased and it continued increasing until they reached their highest level in the 5th day of incubation period by 36.12 and 25.66 in L1 treatment and 68.66 and 23.52 in L2 treatment respectively. These data indicated that the main products resulted from bacterial biodegradation is malathion monocarboxylic acid which may convert to malathion dicarboxylic acid over time.

Also, the molecular ion peak of the low molecular weight 2-mercaptosuccinic acid fragment ions was detected which was reported as an environmental transformation product of malathion[26]. The relative abundance of 2-mercaptosuccinic acid fragment ions was decreased upon the L1 and L2 treatment over elapsed time as demonstrated in Table 1 and Table 2.

Determination of phosphatase enzyme activity

Malathion biodegradation to malathion monocarboxylic acid and malathion dicarboxylic acid may occur through the action of phosphatase enzyme. Phosphates activity was measured by using kinetic method over the intervals of incubation 24, 48, 72 and 120 hours; Results showed that phosphatase activity increased with increasing the incubation period as shown in Table 3, which indicate the continuous increase in phosphatase activity in skimmed milk in the

Table 3. The level of phosphatase enzyme activity (U/L)

Incubation period (hours)	A	B	C	D	E
24	0	8	78	16	170
48	0	12	106	45	200
72	0	20	170	56	215
120	0	23	185	75	220

(A): Phosphatase enzyme activity in malathion only group.,
(B): Phosphatase enzyme activity in L1 only.,
(C): Phosphatase enzyme activity in spiked milk with malathion inoculated with L1.
(D): Phosphatase enzyme activity in L2 only.,
(E): Phosphatase enzyme activity in spiked milk with malathion inoculated with L2.

presence of malathion and reflects its main role in malathion biodegradation. Generally, L2 produced higher levels of phosphatase enzyme in comparison with L1 whether in the presence or absence of malathion.

DISCUSSION

Due to the importance of milk in the diet of infants, children and also adults, the presence of significant amounts of residues is undesirable[27]. Consequently, residues limit for milk and dairy products tend to be more severe than those for other food stuffs. The annual reports of the WHO and FAO committee group represents tolerance level and limits for individual pesticides in different food commodities including milk and dairy products[28].

In the present study, the efficiency of two LAB strains L. casei 1922 (L1) L. acidophilus 23431 (L2), on the biodegradation of malathion in skimmed milk was investigated.

In the current study the two tested strain showed their ability to degrade malathion in skimmed milk. For L1, the malathion ion fragment was decreased in a nearly steady gradual rate as it decreased by about 30%, 65%, 91% and 97% over the different incubation periods respectively, while in case of L2, the degradation rate pattern was different where at the first 24 and 48 hours the degradation rate was higher than in L1 as the malathion ion decreased by 65 %, and 74 % respectively, then over the third and the fifth days the degradation rate was less than that of L1, it was about 85% and 88 % respectively. The ability of the tested two strains in this study to degrade OPPs including malathion was reported by Zhou & Zhao[22]. Also, the biodegradation of different OPPs including malathion during yoghurt processing (milk fermentation) by lactic acid bacteria was recorded by Bo & Zhao[24]. Also, biodegradation of malathion by Zhang et al[21] was investigated by different species of lactic acid bacteria.

The GC-MS analysis showed that Malathion and its two enantiomers breached forming 2-mercaptosuccinic acid and malathion mono- and di-succinic acids (The possible pathway for malathion biodegradation by microorganisms); These finding of metabolic products were reported by many studies[29-31]. In these studies they demonstrated the degradation of malathion into malathion monocarboxylic acid which partially transformed to malathion dicarboxylic acid and gradually to various phosphothionates by the action of some bacterial strains upon increasing incubation time.

The decrease in the degradation rate by L2 in the third and the fifth day may be due to the accumulation of the degradation metabolites malathion mono and di-malathion succinic acids which may confer the feedback effect on the substrate breakdown. Anyway; the ability of L2 in the biodegradation was higher than that of L1, this was concluded from the area percent of the metabolites in the analyzed samples where the final relative abundance of malathion mono-succinic acid which is the first degradation product of malathion after 120 hours incubation in L2 treated samples were more than that in L1 treated samples by about 53%, as well as the malathion di-succinic acid. Although L1 showed higher activity on the enantiomer malaoxon producing 2-mercaptosuccinate than L1 as shown in Table 1 and 2, it was clear from the final concentration of this compound upon the fifth day that L2 have a greater ability for further transformation of 2-mercaptosuccinate into other degradation products. But theses finding conflict those of Zhou & Zhao[22], as they reported in their study that *L. casei* have a little less ability to degrade malathion than *L. acidophilus*. This conflict may come from the time in the current study was about five days. on the other hand, in Zhou & Zhao, malathion was investigated in 24 hours only.

Anyway, in this study these finding were enhanced by the phosphatase level in the two

tested strains; where it was found that L2 has a higher enzyme level during all the incubation periods which explain the higher activity of L2 in the biodegradation process of the tested OPP malathion as well as its enantiomers. Where, many studies related the level of phosphatase enzyme with the ability of OPPs degradation[15,32,21].

CONCLUSION

Many Probiotic may have the ability to rescue our health from OPPs toxicity; so, different probiotics could be investigated for OPPs degradation ability in different food materials to reduce the toxicity risk of these harmful pesticides. Whole bacterial cells or their enzymes could be applicable. Also, probiotics which can degrade OPPs may play a role in our bodies if administrated regularly in reducing the effect of these pesticides, so in vivo studies are required. To the best of our knowledge, this the first study to track malathion metabolites with two different strains of lactic acid bacteria by GC-MS.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

HA conceived and designed the work. HAF collected the data. MAAE did the data analysis and interpretation. HAS critically revised the article

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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