Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Dissipation of pirimiphos-methyl during wheat fermentation by *Lactobacillus plantarum*

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Significance and Impact of the Study: Pesticide residues are an unavoidable part of the environment due to their extensive applications in agriculture. As wheat is a major cultivated cereal, the presence of pesticide residues in wheat is a real concern to human health. Reduction in pesticide residues during fermentation has been studied, but there is a lack of data regarding pesticide residues dissipation during cereal fermentation. Present work investigates the dissipation of pirimiphos-methyl during wheat fermentation by *L. plantarum*. Results are confirmation that food-processing techniques can significantly reduce the pesticide residues in food, offering a suitable means to tackle the current scenario of unsafe food.

Keywords
degradation, fermentation, lactobacilli, pesticide, wheat.

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2013/0848: received 30 April 2013, revised 12 June 2013 and accepted 28 June 2013

doi:10.1111/lam.12128

Abstract

In this study, the dissipation of pirimiphos-methyl during wheat fermentation by *Lactobacillus plantarum* was investigated. Sample preparation for GC/MS detection of pirimiphos-methyl residues from fermented wheat substrate was carried out by two steps: extraction with 25 mL of methanol : acetone = 1 : 1 solvent mix for 30 min, followed by clean-up procedure through a glass column with florisil coupled with elution by 25 mL of ethyl acetate : acetone = 4 : 1. To obtain the highest pesticide degradation level, the fermentation conditions were optimized according to response surface methodology. Our results showed that *L. plantarum* was able to reduce the level of pirimiphos-methyl in wheat. Although pirimiphos-methyl was partially labile during sterilization prior inoculation (~37–50%), and there was also spontaneous chemical degradation of pesticide (~6–11%), overall *L. plantarum* enhanced degradation from 15 to 34%, that is, to nearly 81%. Additionally, the effect of pirimiphos-methyl on the lactobacilli growth, and efficiency of fermentation, was studied where pirimiphos-methyl inhibit the growth of bacteria in concentrations higher than 5 mg kg^{-1}, while the presence of pirimiphos-methyl did not overall affect the lactic acid fermentation.

Introduction

Pesticide residues have become an unavoidable part of the environment due to their extensive applications in agriculture for pre- and postharvest pests control practices. Although remarkable progress has been made in the development of effective pesticides, still a very small fraction of all applied pesticides is directly involved in the pesticidal mechanism while most of the applied pesticides find their way as 'residue' in the environment, and, once the environment is contaminated with pesticides, they may easily enter into human food chain through the plants, creating serious acute health problems.

Wheat is a major cereal grain cultivated throughout the world, and this cereal in the form of flour and its processed products has become an essential part of human diet. Therefore, the presence of toxic pesticide residues in wheat and related food products is a real and important concern to human health. Generally, wheat grain becomes contaminated with pesticides through two principal

sources, pesticide residues originating from field spraying and the accumulated residues from pesticide treatments during storage (Iqbal and Ali 2006). One of the most commonly used pesticide for protecting wheat/grain against insect attack during storage is organophosphate pirimiphos-methyl as active substances with a long persistence of insecticidal activity (Fleurat-Lessard *et al.* 1998). The control of pesticide residues in grain is generally based on maximum residual limits (MRLs), which are below the highest expected residue level if the registered dosages are applied according to good agricultural practice. However, although MRLs are a credible and useful means for regulating the acceptable pesticide use, they are not sufficient for the assessment of human health risks from residue intake unless accurate knowledge about the loss of residues in the processing treatment is available (Fleurat-Lessard *et al.* 2007).

Kaushik *et al.* (2009) established that there were reductions in pesticide residue levels due to processing techniques in most of the food materials, including whole wheat grain, wheat and wheat flower. The processing of food commodities generally implies the transformation of the perishable raw commodity to value added product that has greater shelf life and is closer to being table ready (Chin 1997). Fermentation is one of the oldest simple biotechnological process, and reduction in pesticide residues during fermentation has been continuously studied in different food commodities (Abou-Arab 2002; Navarro *et al.* 2007; Rajashekar *et al.* 2007; Jung *et al.* 2009; Bo and Zhao 2010; Čuš *et al.* 2010), but there is a lack of data regarding pesticide residues in dissipation during cereal fermentation process.

Therefore, the objective of the present work is to investigate the dissipation of pirimiphos-methyl during wheat fermentation by lactic acid bacteria as one of the microbial community member from sourdough fermentation. To obtain the highest pesticide degradation level, the fermentation conditions were optimized according to response surface methodology (RSM). Additionally, the effect of pirimiphos-methyl on the lactobacilli growth, and further on the efficiency of wheat fermentation, was as well studied.

Results and discussion

Lactobacillus plantarum growth curve

Characterization of the growth parameters under laboratory conditions is the first and important step towards further experiment. Growth profile of *L. plantarum* used in this study showed a normal growth pattern under laboratory conditions. The curve on Fig. 1 depicts short lag phase (2 h) in the initial phase of growth, steep exponential phase up to 24–25 h, then a growth stage having a much slower speed. Obtained short lag phase is characteristic for this strain growing in different commonly accepted media, but it can be seen that the rate of reaching exponential phase of growth in MRS broth used in presented work was quite slower in comparison with growth in modified MRS broth supplemented with glucose and lactose (Georgieva *et al.* 2009). Growth rate constant (μ) for *L. plantarum* activated under laboratory conditions was $\mu = 0{\cdot}17\ h^{-1}$ while mean generation time was 4·04 h.

Further, when used for fermentation of wheat substrate, cells of *L. plantarum* were incubated for *ca*. 24 h (cells diluted 1 : 10 gave an $O.D._{620\ nm}$ of *ca*. 0·49, cell density of about 9 log CFU mL^{-1}).

Effect of pirimiphos-methyl on *Lactobacillus plantarum* growth

Figure 2 shows the effect of pesticide on the growth rate of *L. plantarum*. As can be seen, the results indicate that growth of bacteria was not inhibited by pirimiphos-methyl applied in less than MRL concentration. Inhibition started on MRL concentration, and in the presence of MRL, 2xMRL and 5xMRL, concentrations of pesticide remain on slightly elevated level (11–16% of inhibition). The effect on growth inhibition extremely appeared at 10xMRL (36%) and gradually enhanced to maximal applied concentration (20xMRL), with maximum 74% of inhibition. These results describing growth of lactobacilli negatively affected by higher concentrations of pirimiphos-methyl correspond with one obtained by Abou Ayana *et al.* (2011) who confirmed that lactic acid bacteria growth was affected by pesticides, whereas insecticides were the most effective inhibitors of lactobacilli growth compared with herbicides or fungicides. Obtained results are conformation of how different pesticides could impact on specific micro biodiversity including food interest micro-organisms like *Lactobacillus sp*. (Clair *et al.* 2012), resulting in lowering of viable counts in growth mediums contaminated with those agrochemicals (Abou-Arab 2002; Cho *et al.* 2009).

Regarding obtained results, three pirimiphos-methyl concentrations were further used for wheat fortification to evaluate influence of pesticide on fermentation as well as effect of fermentation on pirimiphos-methyl degradation. First fortification rate was 5 mg kg^{-1} (MRL), second was 25 mg kg^{-1} (5xMRL) and third one was 75 mg kg^{-1} (15xMRL).

Effect of pirimiphos-methyl on fermentation efficiency

Establishment of fermentation efficiency under laboratory conditions was conducted to determine effectiveness of

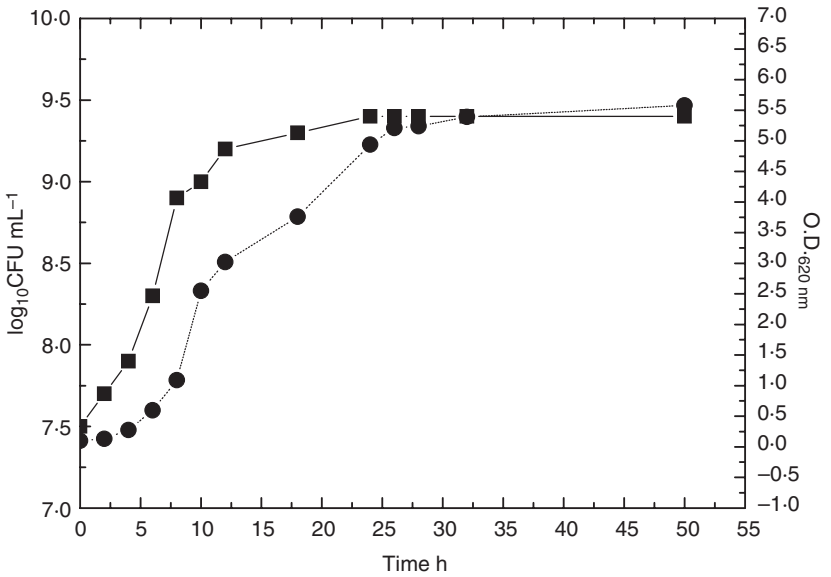

Figure 1 Growth curve of *Lactobacillus plantarum*. —■—Log_{10} CFU mL^{-1}; —●—$O.D._{620\ nm}$.

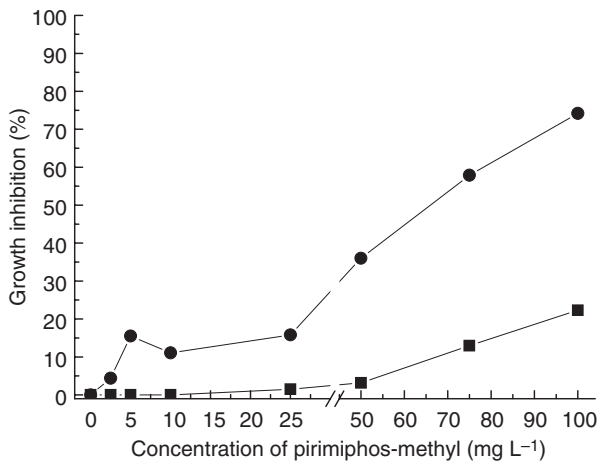

Figure 2 Effect of pirimiphos-methyl on growth of *Lactobacillus plantarum* in MRS broth. —■—$O.D._{620\ nm}$; —●—Log_{10} CFU mL^{-1}.

L. plantarum in fermentation of wheat contaminated with pirimiphos-methyl. Three levels for each parameter (inoculum size, fermentation time and temperature) were tested as independent variables, while pH, colony count (CFU) and determination of titratable acidity (TTA) were tested as fermentation responses.

Results presented in on Fig. 3 show that the presence of pirimiphos-methyl in all three applied concentrations did not affect the lactic acid fermentation in wheat by *L. plantarum*. Although presence of the smallest used amount of pesticide (5 mg kg^{-1}) seems to initially stimulate bacterial growth, probably due to stress response of lactobacilli cells, fortification with higher concentration leads to decrease in CFU with no statistical significant differences between 5xMRL and 15xMRL. Still, p-values (ANOVA, $\alpha = 0{\cdot}01$) for fortification at different concentrations (from 0 to 75 mg kg^{-1}) of 0·1403 for pH and 0·3288 for TTA indicate that, although in the samples fortified with 5xMRL and 15xMRL, the number of cells significantly decreased when compared with one in the control samples (*P*-value for CFU was $2{\cdot}86*10^{-8}$), the amount of lactic acid produced together with pH lowering was not significantly negatively affected. This could be explained due to the presence of a sufficient number of cells in all of the samples to allow a regular fermentative process.

Obtained results concerning lack of negative impact of pesticide on lactic acid fermentation efficiency are in correlation with one published by Cabras *et al.* (2000) and Cho *et al.* (2009), but disagreed with one obtained by Abou Ayana *et al.* (2011). It is obviously that different lactobacilli species respond differently in the presence of various pesticides; thus, those interactions should be always tested prior further step dealing with dissipation of pesticides during fermentation by lactic acid bacteria.

Effect of fermentation on dissipation of pirimiphos-methyl residues

With described analysis procedure, developed in our previous work (Đurović and Đorđević 2010), pirimiphos-methyl was efficiently extracted from fermented wheat substrate and measured accurately. Recovery test was performed at four fortification levels: 1, 5, 10 and 20 mg kg^{-1}, with obtained recoveries of 90·25, 89·96, 87·59 and 86·13% and good reproducibility, that is, RSD % of 7·7, 5·8, 3·2 and 4·8%. The limit of detection (LOD) and quantification (LOQ) were determined

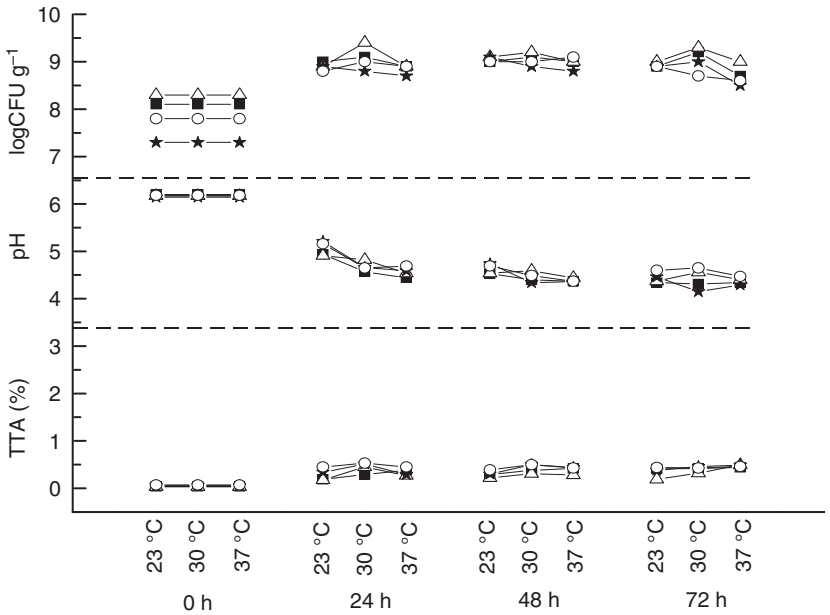

Figure 3 Effect of pirimiphos-methyl on fermentation activity of *Lactobacillus plantarum* in wheat substrate.
—■—0 mg kg^{-1}; —△—5 mg kg^{-1};
—★—25 mg kg^{-1}; —○—75 mg kg^{-1}.

according to IUPAC recommendations (Currie 1999). Obtained LOD and LOQ were 0·011 and 0·04 mg kg^{-1}; thus, it is obvious that the presented method is sensitive enough for determination of this pesticide at concentrations much below its MRL value.

From obtained results concerning evaluation of pirimiphos-methyl degradation during preparation of wheat substrate (Fig. 4), it is can be seen that after thermal processing by sterilization at 121°C for 15 min, reduction was 37·4, 44·86 and 49·58%, respectively, for samples fortified with MRL, 5xMRL and 15xMRL of pesticide. Mentioned results correspond with one obtained by Uygun *et al.* (2008) who point out that the rates of degradation and volatilization of residues were increased by the heat. It is obviously that, as Holland *et al.* (1994) mentioned, processes involving heat can increase volatilization, hydrolysis or other chemical degradation and thus reduce residue levels. Commonly, when substrate fortified with pesticides undergoes heating, the loss of pesticide residues obtain through physico-chemical processes, for example evaporation, co-distillation and thermal degradation, which may vary with the chemical nature of the individual pesticides (Sharma *et al.* 2005). During the process, the water contained in the sample could entrain pesticide molecules (codistillation) while heat causes evaporation and degradation (Cabras *et al.* 1998). Obtained high degradation of pirimiphos-methyl may be due to its somewhat higher vapour pressure in combination with confirmed degradation on temperature over 120°C (WHO Specifications). Thus, it could be concluded that possible pirimiphos-methyl contamination in wheat, in amount over MRL, could be reduced nearly up to 50% by autoclaving.

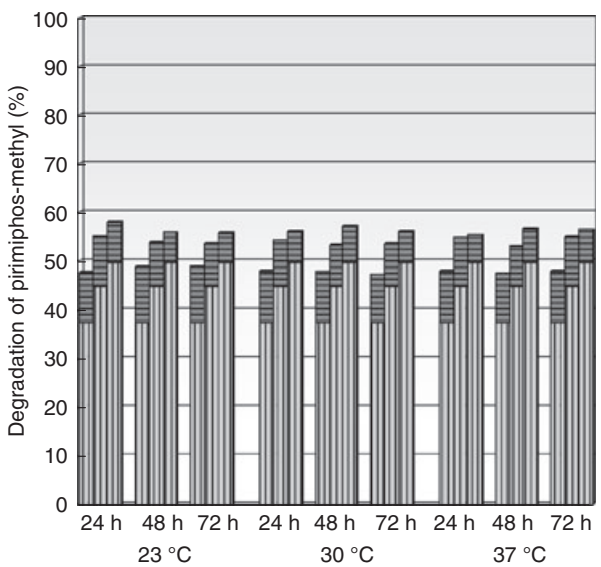

Figure 4 Degradation of pirimiphos-methyl residues during sterilization of wheat substrate and without lactobacilli under fermentation conditions during incubation time. First columns in series – samples fortified with 5 mg kg^{-1} concentration of pirimiphos-methyl; second columns in series – samples fortified with 25 mg kg^{-1} concentration of pirimiphos-methyl; third columns in series – samples fortified with 75 mg kg^{-1} concentration of pirimiphos-methyl. ▤ Degradation during incubation; ▥ Degradation during sterilization.

Further, during the incubation period, there was additional reduction from 5·7 to 11·5% (Fig. 4) without significant differences between variations in incubation parameters. Grains are commonly stored at ambient conditions for longer period of time, and numerous studies on insecticides persistence after postharvest treatments

showed that residues decline in time, although rather slowly (Holland *et al.* 1994). Pirimiphos-methyl was generally more persistent insecticide, but there were examples of its degradation when stored for longer period of time on ambient temperature (Wilkin and Fishwick 1981). Since in present experiment wheat was kept under conditions of elevated temperature and humidity, obtained low degradation of pirimiphos-methyl was not unexpected.

Overall, considering sterilization in autoclave and spontaneous chemical degradation of pirimiphos-methyl, there was reduction in pirimiphos-methyl residues of about 48, 54 and 56%, respectively, in samples fortified with MRL, 5xMRL and 15xMRL, without involving lactobacilli fermentation.

To examine influence of fermentation on pirimiphos-methyl degradation in wheat samples, the fermentation condition was optimized to obtain the highest pesticide degradation level. Optimization was carried out using a Box–Behnken design containing three levels for each parameter, where degradation of pesticides was response, and the optimization was performed using autoanalysis software. As can be seen from Fig. 5, further degradation by *L. plantarum* obtained at all three fortification level, reaching reduction in approximately 81·1, 80·8 and 70·8%, respectively, in samples fortified with MRL, 5xMRL and 15xMRL. Hence, considering already mentioned reduction in pesticide residues prior fermentation, it can be concluded that activity of *L. plantarum* has the highest influence in pesticide residue reduction in samples fortified with MRL concentration (approx. 34%), lower in samples fortified with 5xMRL concentration (approx. 27%) and the lowest in samples fortified with 15xMRL concentration (approx. 15%), which is in correlation with results concerning inhibition of lactobacilli growth by pirimiphos-methyl. Obtained results are in correlation with numerous publications concerning role of microbes in pesticide degradation in different food commodities (Abou-Arab 2002; Sharma *et al.* 2005; Rajashekar *et al.* 2007; Čuš *et al.* 2010). Boethling (1993) noted that if the microbial degradation does occur, it is likely to result from enzymatic activity and may occur either immediately or only after a period of adaptation to the chemicals, while, on the other hand, Ruediger *et al.* (2005) mentioned that reductions in pesticide concentrations could possibly be due to the absorption onto the bacterial cell walls, rather than chemical or biological degradation. Concerning that during the extraction procedure in presented study cells of lactobacilli remained in wheat samples, it is more likely that in studied lactic acid fermentation degradation of pirimiphos-methyl occurred as a result of enzymatic activity, that is, biological degradation, most likely by gene encoding

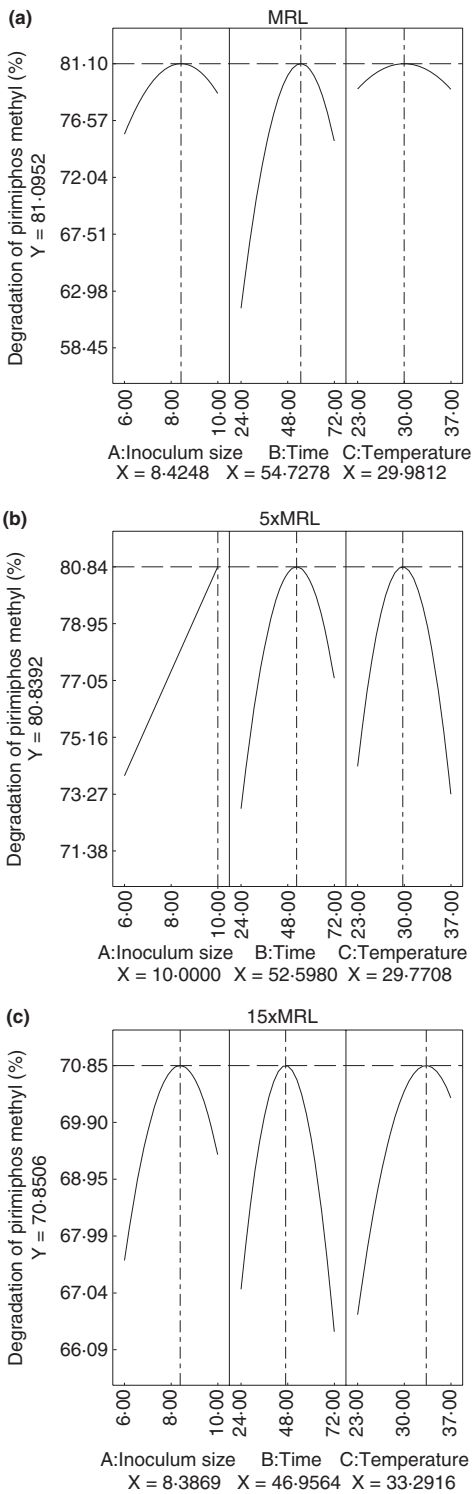

Figure 5 Degradation of pirimiphos-methyl residues during lactic acid fermentation of *Lactobacillus plantarum* in wheat substrate. (a) samples fortified with 5 mg kg^{-1} concentration of pirimiphos-methyl; (b) samples fortified with 25 mg kg^{-1} concentration of pirimiphos-methyl; (c) samples fortified with 75 mg kg^{-1} concentration of pirimiphos-methyl.

organophosphorus hydrolase enzyme isolated from lactic acid bacteria, which proved to decompose nine organophosphorus insecticides (Islam *et al.* 2010). Besides, as degradation by hydrolysis of pirimiphos-methyl is pH dependent, being more rapid at lower pH (pH 4) (WHO Specifications), acidity produced by *L. plantarum* during fermentation could be one more reason for significant reduce in this pesticide.

Overall, pirimiphos-methyl was partially labile during sterilization prior inoculation, and there was also some spontaneous chemical degradation of pesticide, while *L. plantarum* enhanced degradation for approximately 15–34%. Thus, lactobacilli fermentation processing methods of wheat could be effective tool for minimizing the residual contamination from most commonly used storage insecticide in final product through degradation to nearly 81%. Finally, this is one more confirmation that food processing techniques at domestic and industrial level can significantly reduce the pesticide residues in food, offering a suitable means to tackle the current scenario of unsafe food.

Materials and methods

Starter culture and growth conditions

A probiotic strain – *Lactobacillus plantarum* (DSMZ 20174) used in the study was maintained on MRS broth (Torlak Institute, Belgrade, Serbia) at 4–6°C. Starter culture was activated for 24 h at 30°C in MRS broth. Growth was monitored directly by plating on MRS agar (Torlak Institute, Belgrade, Serbia) and indirectly by measuring the $O.D._{620\ nm}$ (UV/visible spectrophotometer LKB Biochrom Novacpec II, Austria), during 54 h of incubation in MRS broth at 30°C. When used for wheat fermentation, cells of lactobacilli were incubated in MRS broth at 30°C until the exponential phase of growth was reached, harvested by centrifugation 9580 ***g*** for 10 min at 4°C (Velocity 14R, Dynamica, Salzburg-Mayrwies, Austria), washed with 50 mmol L^{-1} phosphate buffer pH 7·0 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and resuspended in sterile distilled water to its original volume.

Analytical standard and working solutions

Analytical standard of pirimiphos-methyl was obtained from Dr. Ehrenstorfer, Augsburg, Germany (purity 99·2%). Stock solution (2·0 μg mL^{-1}) of standard in acetone was stored at −18°C, and working solutions were prepared daily by diluting with sterile distilled water. Acetone, methanol and ethyl acetate, as well as anhydrous sodium sulfate (99·0% purity), were purchased from J.T. Baker (Deventer, Holland), while florisil (60–100 mesh) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). Before use, sodium sulfate was dried 24 h at 130°C, and florisil activated 4 h at 600°C and then 5 h at 130°C.

Effect of pirimiphos-methyl on *Lactobacillus plantarum* growth

Cells of lactobacilli were incubated on 30°C in MRS broth fortified with pesticide until exponential phase of growth was reached, and growth was determined as previously described. Fortification was at 2·5, 5, 10, 25, 50, 75 and 100 μg mL^{-1} rates, that is, $^1/_2$MRL, 1MRL, 2xMRL, 5xMRL, 10xMRL, 15xMRL and 20xMRL for pirimiphos-methyl in wheat, respectively.

Preparation and fermentation of wheat substrate

Wheat mash used as a substrate in this study was prepared from uncontaminated grain of *Triticum spelta* manufactured in organic production by Jevtić farm, Bačko Gradište, Serbia. Wheat grains were milled, fortified at 5, 25 and 75 mg kg^{-1} rates of pirimiphos-methyl and homogenized for 24 h by mechanical stirrer, so the pesticide was thoroughly absorbed. After 24 h, samples were sterilized in autoclave for 15 min and cooled, and slurry was prepared by adding distilled water (1 : 1). Culture of *L. plantarum* was inoculated at levels 6, 8 and 10% (v/w) into the flasks. Fermentation was carried out at 23, 30 and 37°C, for 24, 48 and 72 h. Three different controls were prepared: first consisted of sampling immediately after sterilization in autoclave to check the effect of sterilization on pesticide degradation; second consisted of wheat substrates without inoculums to check the pesticide chemical degradation; third consisted of inoculated wheat slurry made of uncontaminated wheat grains to check fermentation.

Sampling and analyses

Besides pesticide determination, the following analyses were made: pH, colony count (lactobacilli cells mg^{-1}) and determination of titratable acidity.

Enumeration of lactobacilli count

Lactobacillus plantarum cells present in fermented wheat mash were enumerated using MRS agar. 10 g of fermented slurry was added to 90 mL sterile distilled water. Further dilutions up to 10^7 were made. Each dilution (1 mL) was pour plated in sterilized MRS agar plates, incubated at 30°C for 48 h, and the colonies were counted using a colony counter (Scan 100 Manual Colony Counter, Interscience, France).

Titratable acidity and pH

Titratable acidity was determined as percentage of lactic acid using the standard method (AOAC, 2000). The acidity (%, as lactic acid) was calculated as: *0·0901 × mL of 0·1 N NaOH × F × 100/Sample (mL)*, where *F* is a factor of 0·1 N NaOH (1·010) and 0·0901 is factor for lactic acid. For the measurement of pH, InoLab 730 pH meter (WTW, Germany) was used.

Sample preparations for pesticide analysis

After fermentation, 10 g of subsamples was placed in polypropylene centrifuge tubes (Sarstedt, Germany) with 5 g of anhydrous sodium sulfate and extracted two times with 25 mL of methanol : acetone = 1 : 1 solvent mix for 30 min on a rotary stirrer and then centrifuged for 3 min at 4000 ***g*** (UZ 4, Iskra, Slovenia). The extracts were filtered through a filter paper containing 1 g of anhydrous sodium sulfate and evaporated to dryness at 35°C using a rotary evaporator (Devarot, Elektromedicina, Slovenia). The residues were further redissolved in 2·5 mL of ethyl acetate : acetone = 4 : 1 mixture, and 2 mL of obtained solutions were passed through a glass column containing 1 g of anhydrous sodium sulfate and 5 g of florisil transferred to a column with 25 mL of ethyl acetate : acetone = 4 : 1 mixture. The pesticide was eluted with 25 mL of ethyl acetate : acetone = 4 : 1. Eluates were evaporated to dryness and redissolved in 2 mL of acetone for GC–MS analysis.

For recovery assay, untreated fermented wheat grain samples were spiked prior to extraction with the appropriate volume of stock standard solution to reach 1, 5, 10 and 20 mg kg^{-1} of pirimiphos-methyl and further processed as above-described.

Pesticide analysis using GC/MS

A gas chromatograph–mass spectrometer (GC/MS) was used as a detection device (CP-3800/Saturn 2200, Varian Australia) with 30 m × 0·25 mm × 0·25 μm, VF-5 ms column (Varian, Australia). The GC was programmed as follows: initial temperature was 170°C, then increased to 260°C at 9°C min^{-1} and held for 3·5 min. The carrier gas (helium, 99·999%) flow rate was in constant flow mode at 1·1 mL min^{-1}. The ion trap mass spectrometer operated in the electron impact/selected ion monitoring (EI/SIM) mode. Ion (m/z) 290 was used for quantification, while ion (m/z) 234 was used for confirmation. The ion trap and transferline temperatures were set to 210°C and 250°C.

Statistical analysis

All experiments were performed in triplicates. Data from determination of microbial growth phases and growth rate constant, and data from determination of effect of pirimiphos-methyl on *L. plantarum* growth were processed by a statistical package Microcal Origin 6.0 (Microcal Software Inc., Northampton, MA, USA). The whole response surface analysis procedure, that is, data regarding effects of pesticide on fermentation efficiency and optimization of fermentation conditions to obtain the highest pesticide degradation level were analysed using ReliaSoft's DOE++ software (ReliaSoft Corporation, Tucson, AZ, USA).

Acknowledgements

Study was carried out as a part of the project No TR31043, supported by the Ministry of Education and Science of the Republic of Serbia.

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