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Effect of pesticide residues in grapes on alcoholic fermentation and elimination of chlorothalonil inhibition by chlorothalonil hydrolytic dehalogenase

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ABSTRACT

The effect of different kinds of pesticide residues in grapes on alcoholic fermentation by *Saccharomyces cerevisiae* was evaluated. Among four types of pesticides added into the grape slurry, omethoate, triadimefon and cyhalothrin did not inhibit the alcoholic fermentation at their proposed spraying concentration of 0.21, 0.10 and 0.10 g L^{-1}, respectively, whereas chlorothalonil concentration above 0.03 g L^{-1} behaved significantly negative influence on both *S. cerevisiae* growth and alcoholic fermentation efficiency. When the chlorothalonil concentration was lower than 0.01 g L^{-1}, the fermentation proceeded smoothly without any degradation of chlorothalonil. Considering the cumulative toxicity and adverse effect of chlorothalonil on fermentation, chlorothalonil hydrolytic dehalogenase (Chd) extracellularly expressed from the recombinant *Bacillus subtilis* WB800 was used to pretreat the chlorothalonil-contaminated grape slurry. After treatment by the Chd enzyme in an activity of 7.25 mU L^{-1} slurry for 60 min, the inhibition effect could be substantially eliminated even at an initial concentration of 0.10 g L^{-1} chlorothalonil. This study provides a potential approach for solving the conflict in fermentation industry with pesticides inhibition.

1. Introduction

Pesticides are used extensively to protect crops against pests, diseases and weeds in modern agricultural practices. It would arouse serious food safety issues inevitably. More attention has been paid to food safety, particularly the problem of pesticide residues on fruits and vegetables (Bai, Zhou, & Wang, 2006; Bourn & Prescott, 2002). Grapes, citrus fruits and potatoes are most likely to be contaminated by pesticides among fruits and vegetables (Fenik, Tankiewicz, & Biziuk, 2011). For grapes, chemical pesticides including fungicides, insecticides and herbicides, are being increasingly used. Thus, pesticide residues are always detected in grapes, grape juices and wines (Čuš, Česnik, Bolta, & Gregorčič, 2010) and consequently product quality of wine will be affected. To obtain high-quality and healthy wines, it is necessary to limit the quantity of pesticides from grape ripening to wine brewing. Many works have detailed the fates of various pesticides from vine to wine (Cabras et al., 1997; Cabras et al., 1995; Rose, Lane, & Jordan, 2009; Ruediger, Pardon, Sas, Godden, & Pollnitz, 2005; Čuš et al., 2010), mostly focusing on the persistence, distribution and degradation of pesticides. Pesticide residues in grape juice and wine were reported to be significantly diminished through separation of the solid and liquid phases by degradation or absorption during the vinification process, especially during the pressing of crushed grapes and wine racking after alcoholic fermentation (Čuš et al., 2010). However, sometimes pesticides could not be degraded or removed because some oxidative degradation process is inhibited by the native antioxidants in grape juices (Picó & Kozmutza, 2007).

A few studies assessed the effects of pesticides on food-processing including winemaking process. A three-year study about the effects of phthalimides on fermentation microorganisms showed that all of the tested fungicides, particularly folpet delayed fermentation even at a concentration of 0.1 mg L^{-1} (Gaia, Tarantola, & Barbero, 1978). The presence of folpet in the grapes skin inhibited the alcoholic fermentation by *Saccharomyces cerevisiae* and *Kloeckera apiculata*. On the contrary, phthalimide had no negative effect on the alcoholic fermentation. Dicofol was reported to inhibit malic acid catabolism by *Oenococcus oeni* whereas chlorothalonil

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had only a minor effect (Ruediger et al., 2005). However, in another study, chlorothalonil was confirmed to inhibit alcohol fermentation by yeast (Li et al., 2012). Additionally, mancozeb, folpet and hexaconazole were proven to be highly toxic to yeast activity (Caboni & Cabras, 2010), and presence of pesticides always decreased the growth rate of fermentation microorganisms including yeasts and bacteria (Jorge, Olalla, Raquel, Beatriz, & Jesús, 2015).

Thus, the inhibition of alcoholic fermentation by pesticides should be eliminated before fermentation. Presently, many methods have been tried for the removal of pesticide residues during or before food processing. Membrane, ozone, washing, fermentation, drying, blanching and sterilization were chosen to remove different kinds of pesticide residues (Bonnechère et al., 2012; Chen, ShangGuan, Wu, Xu, & Fu, 2012; Kusvuran, Yildirim, Mavruk, & Ceyhan, 2012; Plakas & Karabelas, 2012; López-Fernández, Rial-Otero, & Simal-Gándara, 2013). Most of the treating methods could remove the pesticides effectively. For example, 96% of the mancozeb on lettuces was removed under the optimum conditions of washing treatment by Amukine solution or hydrogen peroxide solution (López-Fernández et al., 2013). However, shortages of these physical and chemical treatments existed. The pesticides were only just transferred from one system into another through an expensive or even more energy consumption processing. Therefore, biodegradation of pesticides by microorganisms or degrading-enzymes rather than absorption onto the microbial cell surface is one of the most feasible ways for removing the pesticide residues during fermentation (Meng et al., 2015; Ruediger et al., 2005).

In this study, different pesticides were used to evaluate their effects on alcoholic fermentation and growth of the yeast *S. cerevisiae*. Omethoate, triadimefon, chlorothalonil and cyhalothrin were chosen as the model pesticides mainly because their wide use on grape planting and their residues were easily detected in grapes and grape juices. Roughly speaking, omethoate is an organophosphorus systemic insecticide; triadimefon is a triazole systemic fungicide; chlorothalonil is an organochlorine non-systemic fungicide, and cyhalothrin is a pyrethroid and non-systemic insecticide. Then the pesticide with inhibition effect was pretreated by its corresponding degrading-enzyme secreted from recombinant *Bacillus subtilis* WB800 to investigate the elimination of its adverse effect on winemaking.

2. Materials and methods

2.1. Chemicals and strains

The analytical standard of omethoate, triadimefon, chlorothalonil and cyhalothrin were purchased from J&K Scientific Ltd. (Shanghai, China). Grapes (*Vitis vinifera* L. *var. Muscat*, a kind of red grape) were purchased from a local market. The wine yeast *S. cerevisiae* was bought from Angel Yeast Co. Ltd. (Yichang, China). All other analytical grade chemicals were purchased from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Alcoholic fermentation

Grapes were first immersed and washed by 1% $NaHCO_3$ solution, scrubbed to remove dirts, pesticide residues and microorganisms attached to the surface, and then wiped using absorbent paper to remove the excess liquids. Cleaned grapes were crushed to form slurry with a grinder, adjusted to a 17% reducing sugar concentration by sucrose addition, and then sterilized at 115 °C for 15 min. Omethoate, triadimefon, chlorothalonil and cyhalothrin were added into the sterilized grape slurry, respectively. The added amount was consistent with the proposed spraying concentration of each pesticide. The final concentrations of omethoate, triadimefon, chlorothalonil and cyhalothrin in slurry were 0.21, 0.10, 0.10 and 0.10 g L^{-1} grape slurry (Li et al., 2012), respectively. Sample without addition of pesticide was performed as the control. The fermentation conditions were the same as previously reported except for 26 °C (Heard & Fleet, 1988). All experiments were carried out in triplicate. Samples were collected at intervals for alcohol, biomass and reducing sugar analysis.

2.3. Crude chlorothalonil hydrolytic dehalogenase preparation and enzymatic activity assay

A patented chlorothalonil hydrolytic dehalogenase (Chd) gene (Wang, Li, Li, & Jiang, 2010) was cloned into the vector pP43NMK under the control of P43 promoter and NprB signal peptide-encoding sequence to achieve its extracellularly expressed protein in a protease-deficient strain *B. subtilis* WB800 (Meng et al., 2015). Then, submerged fermentation was performed in 250-mL Erlenmeyer flasks with a 30-mL working volume. The constructed recombinant strain was cultured at 37 °C, 175 rpm for 24 h in medium of 25 g L^{-1} tryptone, 31.2 g L^{-1} yeast extract, 50 g L^{-1} glucose, 3 g L^{-1} K_2HPO_4, 3 g L^{-1} NaCl, 3 g L^{-1} $MgSO_4$, 50 mg L^{-1} kanamycin, pH 7.0 and 10% (v/v) inoculum, 20 mg L^{-1} chlorothalonil was added into the medium at 12 h.

After fermentation, the broth was centrifuged at 8000 rpm for 10 min to achieve solid–liquid separation. The supernatant obtained after centrifugation was used as crude enzyme and its activity was assayed as previously described (Meng et al., 2015). One unit of Chd activity was defined as the amount of enzyme needed to decrease 1 μmol chlorothalonil per minute under optimal conditions.

2.4. Elimination effect of chlorothalonil by Chd secreted from B. subtilis WB800

Crude enzyme was used to treat grape slurry which contained 0.1 g L^{-1} chlorothalonil at 40 °C before fermentation. The Chd activity was 14.50 U L^{-1} and the amount added was 0.5 mL L^{-1} slurry. Different treat times (10 min, 30 min and 60 min) were applied to test the elimination effect. Sample without addition of chlorothalonil was performed as control. Sample without addition of Chd was also studied. All experiments were carried out in triplicate.

2.5. Analytical procedures for winemaking

The amount of reducing sugar was assayed by DNS method (Ghose, 1987). Alcohol was measured using a gas chromatograph (GC-2010, Shimadzu) equipped with a flame ionization detector (FID) and an RTX-Wax capillary column (30 m × 0.25 mm). Oven temperature was held at 85 °C for 4.5 min, programmed at an increment of 20 °C min^{-1} until 170 °C for 2.5 min. Both injector and detector temperatures were set at 250 °C. Nitrogen was used as the carrier gas and isopentanol was used as the internal standard. The biomass in the fermentation broth was measured at OD_{600nm} using an ultraviolet–visible spectrophotometer (UV-2450, Shimadzu Co., Kyoto, Japan). The measured OD was correlated with dry weight using an established proportional constant (He & Chen, 2013). The *S. cerevisiae* viability was rapidly evaluated by the alkaline methylene blue staining method (Sami, Ikeda, & Yabuuchi, 1994) using a microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan). The concentration of chlorothalonil was analyzed by high-performance liquid chromatography (HPLC) after extraction with dichloromethane and then tested as previously described (Meng et al., 2015).