Bioresource Technology 154 (2014) 67–73

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Efficacy of binary combinations of botanical pesticides for rotifer elimination in microalgal cultivation

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HIGHLIGHTS

- Binary toxicity of celangulin, matrine and toosendanin for rotifers were studied.
- Celangulin/matrine and celangulin/toosendanin at a 1:9 ratio exhibited synergism.
- Celangulin/toosendanin (1:9) exterminated rotifers and allowed microalgae survive.
- Application of celangulin/toosendanin (1:9) reduces biocide dosages and the cost.
- Celangulin/toosendanin (1:9) is a potential practical rotifer-control combination.

ARTICLE INFO

Article history:
Received 29 September 2013
Received in revised form 26 November 2013
Accepted 30 November 2013
Available online 15 December 2013

Keywords:
Botanical pesticides
Binary interaction
Rotifer contamination
Microalgal cultivation

ABSTRACT

Binary interactions of celangulin, matrine and toosendanin against the rotifer *Brachionus plicatilis* were studied. Types of interactions (antagonism, synergism and addition) were dependent on the biocides themselves and their ratios in combinations. Mixtures of matrine/toosendanin mainly produced addition owing to their similar modes of action aiming at the nervous system. Combinations of celangulin mixed with matrine or toosendanin at 1:9 exhibited synergism, which is attributed to the interference of matrine or toosendanin with the detoxification enzymes of celangulin. Both the synergistic combinations were inappropriate for rotifer extermination in *Isochrysis* sp. cultivation owing to the high phytotoxicity resulting from the absence of cell walls. However, the celangulin/toosendanin (1:9) mixture decreased rotifer reproduction without damaging cells of *Chlorella* and *Nannochloropsis* sp. Application of frequent, low doses of celangulin/toosendanin (1:9) mixture also reduced the dosage of biocides, thereby reducing the cost of exterminating rotifers, and indicating a considerable practical application in microalgal cultivation.

1. Introduction

Microalgae are potential biomass feedstock for proteins, pigments, pharmaceuticals and biofuels, owing to their high efficiency of solar energy utilization (theoretical ~10% of the total captured solar energy), fast growth rate and ability to accumulate a high quantity of lipid (Day et al., 2012; Rawat et al., 2013). Furthermore, microalgal cultivation could be coupled with CO_2 capture from industrial flue gases and removal of nitrogen and phosphorous compounds during wastewater treatment (Li et al., 2008). There are several parameters, which should be considered for realizing these potential applications of microalgae, such as large-scale cultivation, extraction economics, high-value chemicals production and post-processing of residual biomass (Šoštarič et al., 2012). Grazing of cultivated cells by rotifers is an especially serious threat for the microalgal large-scale, biomass production (Wang et al., 2013). The rotifer *B. calyciflorus* can graze the microalga *Chlamydomonas reinhardtii* at a feeding rate of >500 cells rotifer^{-1} h^{-1} (Fischer et al., 2012). The high grazing capacity of rotifers leads to a rapid rise of rotifer density, resulting in the inevitable clearing of an algal suspension in a few days. The grazing activity of rotifers also leads to the over-growth of non-target microalgae and the development of bacteria-algae-flocs (Schlüter et al., 1987).

To date, filtration is commonly used to remove the majority of the adult rotifers (>200 μm in length). However, rotifer eggs and developing neonate individuals can still pass through the mesh, together with the microalgal cells (Borowitzka, 2005; Wang et al., 2013). It is essentially impossible to obtain rotifer-free algal cultures. Changes of growth conditions such as a pH shift to pH 3.0 for 1–2 h are recommended for controlling rotifer contamination

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http://dx.doi.org/10.1016/j.biortech.2013.11.098

(Becker, 1993). It may be effective in freshwater microalgal cultivation, but is difficult to carry out in marine microalgal cultivation owing to the seawater's buffering capacity. Adding therapeutic agents is a feasible method for controlling rotifer contamination. However, most of chemical therapeutic agents that have lethal toxicity to rotifers are also phytotoxic to microalgae. Trichlorphon (274 to 318 mg/L) and pyrethroids (0.30 to 1.28 mg/L) had lethal toxicity to *Brachionus* sp. (Wang et al., 2013; Sánchez-Fortún and Barahona, 2005). The lethal dosages of these pesticides for rotifers decreased cell growth and pigment content significantly or disrupted the photosynthesis of microalgae (Chen and Jiang, 2011; Sáenz et al., 2012).

Therapeutic agents that inhibit rotifer contaminants without damaging the target microalgae are the preferred choices. Celangulin, matrine and toosendanin are considered to be potential botanical pesticides for controlling rotifers in microalgal mass cultivation (Huang et al., 2013). However, exposure to an individual pesticide for a long time would soon result in the development of insecticide resistance in pests (Elbert et al., 2012). Some individual biocide like the celangulin showed the problems of shortened half-life and fast degradation under the high temperature or pH shift conditions, thereby decreasing the efficacy for exterminating rotifers. Rational pesticide mixtures can avoid the development of resistance, also lead to increased efficacy, reduced dosage and thereby reduced cost of contamination control (Ahmad et al., 2009). In the present study, the joint-action toxicity of binary mixtures of celangulin, matrine and toosendanin against *Brachionus plicatilis* was investigated. Two binary mixtures that showed synergistic activity against rotifers were selected to assess their possible phytotoxicity on three large-scale-cultivated microalgae. The possible use of synergistic binary mixtures for rotifer extermination in microalgal cultures was also evaluated.

2. Methods

2.1. Interaction toxicity of binary mixtures of celangulin, matrine and toosendanin against the rotifer B. plicatilis

Binary mixture experiments were conducted following a fixed ratio design (Greco et al., 1995). All ratios were based on the individual 24 h LC_{50} values of celangulin, matrine and toosendanin (Huang et al., 2013). For each binary combination, the components was mixed in nine ratios (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1). Seven toxicant concentrations were used in dose-mortality testing of each ratio. The various concentrations, each with four replicates (10 neonates of *B. plicatilis* per replicate), were randomly arranged in 24-well plates, with each well containing 1 ml toxicant medium. Rotifers in each well were fed with *Nannochloropsis oculata* at a density of 3×10^6 cells/ml and incubated at 26 °C. The mortality of rotifers in each treatment was assessed after a 24 h exposure. Dose-mortality response was performed by probit analyses to estimate 24 h LC_{50} values and 95% confidence intervals (CI) of each mixed ratio. The co-toxicity coefficient (CTC) based on 24 h LC_{50} of each mixture was used to determine the interaction toxicity: A CTC higher than 120 was considered as synergism, between 80 and 120 as addition, and less than 80 as antagonism (Sun and Johnson, 1960). If a mixture (M) was formulated of part A and part B, then the CTC of the mixture (M) was calculated as follows:

Toxicity index (TI) of A = 100;
Toxicity index (TI) of B = $LC_{50}$of A/LC_{50} of B × 100;
Actual TI of M = LC_{50} of A/LC_{50} of M × 100;
Theoretical TI of M = TI of A × %(W) of A in M + TI of B × %(W) of B in M;
Co-toxicity coefficient (CTC) = Actual TI of M/Theoretical TI of M × 100.

2.2. Toxicity assessment of synergistic binary mixtures on microalgae

Isochrysis, *Nannochloropsis* and *Chlorella* sp. at the exponential growth stage were incubated in 60 ml L_1 medium (Guillard and Hargraves, 1993) into 100-ml glass flasks with aeration tubes and perforated polyethylene film covered. The initial pH of medium was 8.2 ± 0.2. All the materials and culture medium were previously autoclaved for 25 min at 121 °C and 1.5 atm. The initial cellular density was set to 2.0–2.5 × 10^6 cells/ml aiming at the best compromise between maximizing bioassay sensitivity and having sufficient viable cells. The algae were then exposed to various concentrations of synergistic binary mixtures for a period of 72 h. Each concentration and control had three replicates. The toxicity experiment was carried out in a light incubator at 26 ± 1 °C under a light density of 80 μ mol photons m^{-2} s^{-1} with a 14:10 h light/dark photoperiod. Illumination was provided using PAR fluorescence lights. The algal medium was aerated at an airflow rate of 3.5 L/h. The percentage of cell growth inhibition was used to assess the toxicity of binary mixtures on microalgae. It was calculated as: Growth inhibition (%) = [(cell density of control) − (cell density of treatment)]/(cell density of control) × 100. The cell growth inhibition data and synergistic mixture concentrations were fitted to a Weibull model according to: Growth inhibition (%) = $C_\infty \times [1-\exp(-(C_m/\beta)^\alpha)]$, where C_∞, α and β were the maximum value of cell growth inhibition, the shape parameter of the curve and the related concentration parameter, respectively (Monteiro et al., 2011). Set the percentage of growth inhibition to 50%, the 72 h EC_{50} was calculated with regression analysis of the binary mixture concentrations versus percentage inhibition.

2.3. Safety evaluation of the synergistic binary combination celangulin:toosendanin (1:9) for microalgal cultivation

After microalgae were inoculated into the L_1 medium, *B. plicatilis* rotifers were added into the algal culture medium to reach an initial density of 3–6 individuals/ml. The culture characteristics such as temperature, light and aeration were consistent with those in Section 2.2. After adaptation for 2 days, the synergistic mixture celangulin:toosendanin (1:9) was added into the algae-rotifer culture. A rotifer-free algal culture was used as control. Flasks containing 2.5 L microalgal culture were set up in triplicate for each group. Samples were taken at a day interval. The rotifer density was estimated using a 1-ml zooplankton counter under the dissection microscope. After that the algal culture was separated from rotifers by a 74-μm mesh. The microalgal cell density was determined by direct microscopic counting of cells using a haemocytometer. The egg to female rotifer ratio was calculated as: Egg ratio = N_e/N_f, where N_e and N_f were the number of total eggs and female rotifers respectively. Total pigments of microalgae were estimated after methanol extraction (Hartmut, 1983). The effective quantum yield of PSII (φPSII) and photosynthetic efficiency (α) of microalgal cells were measured according to Serôdio et al. (2006).

2.4. Statistical methods

The data on cell growth inhibition and synergistic combination concentrations were fitted to the Weibull model with the Sigmaplot software v.10.0 (Systat Software Inc. 2006). Significant differences between treatments and controls were determined using a one-way ANOVA followed by Dunnett's test. Values were considered significantly different at $P < 0.05$.