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Bioremoval of Cylindrospermopsis raciborskii cells and cylindrospermopsin toxin in batch culture by the yeast Aureobasidium pullulans

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Abstract

This study describes the ability of a yeast strain, *Aureobasidium pullulans* KKUY0701 isolated from eutrophic lake to eliminate *Cylindrospermopsis raciborskii* and cylindrospermopsin (CYN) toxin. The anti-cyanobacterial activity of this yeast strain was evaluated by growing with living cells and filtrate of *C. raciborskii*. CYN bioremoval was assayed using living and heat-inactivated yeast cells. Both Living cells and filtrate of this yeast strain were able to suppress the growth of *C. raciborskii*, with total cell death occurring at day 2 and day 3, respectively. Living and inactivated yeast cells, but not yeast filtrate, reduced CYN concentrations released into cyanobacterial cultures, indicating that this toxin might be removed from the culture medium via absorption onto yeast surface rather than enzymatic biodegradation. The adsorption experiments also confirmed the elimination of CYN by living and heat-inactivated yeast. Nevertheless, inactivated yeast exhibited higher capacity (K = 3.3) and intensity (n = 1.4) than living yeast (K = 1.9, n = 1) for CYN adsorption. The study suggests that this yeast strain could be employed for bioremediation of *Cylindrospermopsis* blooms in freshwaters. Additionally, heat-inactivated yeast biomass could be used in slow sand filters for elimination of CYN in drinking water treatment plants.

Introduction

Harmful cyanobacterial blooms (HCBs) are a worldwide phenomenon in eutrophic water sources and expected to increase in the next decade because of climate change (Paerl and Otten 2013). HCBs are mostly constituted by harmful species, which produce wide array of cyanotoxins including heptatoxins, neurotoxins and skin irritant toxins (Codd et al. 2005). These toxins may cause negative impacts on aquatic ecosystems and deteriorate drinking water quality (Mohamed and Al-Shehri 2007; Mohamed et al.2015). *C. raciborskii* is one of the most bloom-forming species, which can survive a wide range of light, temperature and nutrients (Burford et al. 2016). C. *raciborskii* can produce two types of cyanotoxins viz; the hepatotoxin cylindrospermopsin and the neurotoxin saxitoxin that caused fish and cattle mortality, and has been implicated in outbreaks of human poisoning (Svircev et al. 2016). CYN is a low -molecular weight (415Da) alkaloid cyanotoxin, and its toxic effects include inhibition of protein synthesis and potential genotoxicity and carcinogenicity (Van Apeldoorn et al. 2007; Bazin et al. 2010). Unlike other cyanotoxins, CYN is naturally excreted into the aquatic environment with large quantities (98% of total toxin) because of its high water solubility and the apparent permeability of *Cylindrosperompsis* cell membrane (Wormer et al. 2008). As it is chemically stable and slowly degradable, CYN shows high persistent in the aquatic environment (Wormer et al. 2008). This may represent a challenge for water authorities during CYN removal from drinking and irrigation waters. Several methods were demonstrated for removing cyanobacteria and their toxins from drinking water (Mohamed and Al-Shehri 2010; Shao et al. 2013). Nevertheless, these methods have serious disadvantages including, where they cost high energy and produce secondary pollutants that leadto the collapse of aquatic ecosystem (Nishu et al. 2019). Conversely, biological methods using anti-algal microorganisms (e.g. bacteria and fungi) are relatively cost effective for elimination of cyanobacteria in water (Hou et al. 2019). Taken that

cyanobacterial blooms contain associating bacteria and fungi (Van Wichelen et al. 2016) that may have interrelationship with them or show strong antagonistic activities (Li et al. 2011; Van Wichelen et al. 2016), these microorganisms could be exploited in controlling HCBs in in freshwaters. Several studies have been conducted on algicidal effects and cyanotoxin biodegradation by bacteria (Ndlela et al. 2018; Nishu et al. 2019; Weiss et al. 2019; Massey and Yang 2020). A few studies have also demonstrated the antialgal activity of some fungal species against harmful cyanobacteria (Jia et al.2010 a,b; Mohamed et al. 2014; 2020) and their degrading capability of cyanotoxins (Jia et al. 2012; Mohamed et al. 2014). However, most of these studies have concentrated only on *M. aeruginosa* and microcystin toxins. To the best of our knowledge, no research has been made on anti-cyanobacterial activity of fungal species on *C. raciborskii* and CYN toxin, though C. *raciborskii* frequently coexists in the same environment with *M. aeruginosa* (Lei et al. 2020). *A. pullulans* is a yeast-like fungus, which can resist a wide range of temperatures and survive in dry and wet environments, and (Di-Francesco et al. 2015). *A. pullulans* produces lytic enzymes (Vero et al. 2002) that exert antibacterial activity and inhibit pathogens growth (Prasongsuk et al. 2018). Moreover, *A. pullulans* and its metabolites were found to be safe for biotechnological and environmental applications (Prasongsuk et al. 2018), and thereby can be employed in preservation of vegetables and fruits after harvest (Di-Francesco et al. 2017). This indicates that this fungus would be a promising candidate to control HCBs in water bodies. Recent study showed that the yeast *A. pullulans* sppresed the cell proliferation of *M. aeruginosa* and eliminated microcystin toxins via adsorption onto its cell walls (Mohamed et al. 2020). As *C. raciborskii* frequently coexists in the same environment with *M. aeruginosa*, we hypothesized that *A. pullulans* yeast could have the same inhibitory effects on *C. raciborskii*. Therefore, the present study aimed to investigate the capability of *A. pullulans* for growth inhibition of *C. raciborskii* and eliminatation of CYN toxin produced by this cyanobacterium.

Materials And Methods

Microorganisms

KKUY0701 yeast strain used in experiments was isolated previously from Saudi eutrophic lake, identified as *Aureobasidium pullulans* based on 26S rDNA gene analysis, and assigned as KKUY0701 strain (accession number MK141707) (Mohamed et al. 2020). The axenic strain was inoculated into a 250-ml sterile conical flask containing sterile nutrient yeast extract-malt extract agar medium (YMA; 100 mL). The flasks were incubated in a shaking incubator (150 rpm) at 30˚C for 72 h according to Kurtzman and Fell (1998). The axenicity of yeast cultures was verified by inoculation on nutrient agar. Yeast culture was centrifuged (8,000 rpm for 15 min) to separate cell-free supernatant from cells. The pellets were washed twice and re-suspended in sterilized water to the required yeast cell density ($2.1x10^5$ cells mL^{-1}) by counting the cells in a haemocytometer chamber. Both yeast cell suspension and filtrate were used immediately in the experiments of anti-cyanobacterial activity. *C. raciborskii* strain used in our experiments was isolated from a Saudi eutrophic lake (17°03'N and 42°57'E), and reported as CYN producer (568 μg g^{-1}) dry weight (Mohamed and Al-Shehri 2013).To obtain axenic culture, the strain was grown in nitrogen –depleted BG-11 medium containing a mixture of antibiotics (rifampicin – 300 mg L^{-1},

streptomycin/penicillin – 25mg L^{-1}) and antifungal nystatin (10mg L^{-1}) (Wilkens and Maas 2012). The cultures were grown at 25ºC and continuous illumination at approximately 90 µmol photons m^{-2} s^{-1} for 48h. The axenicity of *C. raciborskii* cultures was assessed by inoculation on nutrient agar, and axenic cells were then used for inoculation in the experiments of yeast anti-cyanobacterial activity. Exponentially growing *C. raciborskii* cells were transferred under aseptic conditions into a 250 mL flask containing BG-11 medium without nitrogen (100 mL) to give an initial cell concentration of 4.3×10^5 cells mL^{-1}.

Anti-cyanobacterial activity of A. pullulans

A. pullulans cells in sterile distilled water were co-cultivated at a final concentration of 2.1x 10^5 cells mL^{-1} with *C. raciborskii* (cell density, 4.3×10^5 cells mL^{-1}) in BG-11 medium without nitrogen for 5 days under the same conditions described above. YMA medium was not added in the anti-cyanobacterial experiment to assess the capability of *A. pullulans* for lysis of cyanobacterial cells and using their contents as a nutrient source. *C. raciborskii* cultures not-containing yeast were employed as a control. Yeast cells in BG11 medium was used as negative procedural control. To study the anti-cyanobacterial activity of yeast filtrate, different proportions of cell- free supernatant of yeast culture (1%, 10% and 50%) were added into a 250-ml sterile conical flask containing exponentially growing *C. raciborskii* (4.3×10^5 cells ml^{-1}). Control *C. raciborskii* culture did not contain yeast filtrate. All cultures were incubated under the same growth conditions outlined above. *C. raciborskii* growth was monitored daily and measured as a cell density using a Sedgewick-Rafter counting chamber under a microscope. Meanwhile, *A. pullulans* cells in treated and control cultures were also counted by a hemocytometer after staining with methylene blue (0.1%) according to Thomson et al. (2015). The anti-cyanobacterial activity was appertained as the decrease in *C. raciborskii* cell density of treated cultures in comaprsion to control. All samples were withdrawn from cultures under aseptic conditions. Each experiment was run in triplicate.

Cylindrospermopsin removal

To assess the capability of *A. pullulans* for removing CYN in the medium of yeast cyanobacterial cultures, an aliquot (5 mL) was sampled daily from yeast-treated and filtrate-treated *C. raciborskii* cultures. The samples were centrifuged)6000 x g for 15 min(and concentration of CYN remaining in the supernatant was detected by Enzyme-linked immunosorbent assay (ELISA) using commercial kit (Abraxis, 54 Steamwhistle Drive Warminster, PA 18974).

Potential adsorption of CYN by yeast

To test whether the decrease of CYN concentrations in the medium of yeast-*Cylindrospermopsis* cultures over the incubation period is due to biodegradation by living yeast cells or adsorption onto yeast surfaces, the adsorption experiment was carried out using heat-inactivated yeast. Pellets of yeast cultures grown in YMA medium for 24 h at 37ºC were collected and washed with 0.9% NaCl. Part of these pellets was used as viable yeast cells, while the other part was inactivated by heating in an oven (80°C for 12 h), and the inactivity was verified by inoculation on solid YMA medium, with no yeast growth appearing on the agar. Using inactivated yeast was to exclude the implication of metabolic and enzymatic degradation

of CYN by viable cells of *A. pullulans* (Petruzzi et al. 2015). Inactivated and viable yeast cells (100 mg dry weight) were inoculated separately in 100 ml-conical flasks containing YMA medium (50 ml, pH 7.2) and different concentrations of crude CYN (1,10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg L^{-1}), and incubated in a thermostatically controlled shaker (200 rpm) at 30°C for 1h. Flasks containing YMA medium and CYN only, but without yeast cells were assigned as control. The contents of flasks were then centrifuged at 6000 g for 15min at 30ºC. CYN concentrations not-bound or degraded was determined directly in the supernatants by ELISA as mentioned above, whereas the pellets were used in bound CYN release assay. The CYN adsorption efficiency (R %) by yeast was calculated using the following equation:

$$R\% = \frac{(C_0 - C_f)}{C_0} x\ 100$$

Where C_0 and C_f are the initial and final concentrations of CYN (µg L), respectively. Freundlich isotherm equation was used to fit adsorption isotherm data obtained during this study.

$$q_e = KC_e^{1\backslash n}$$

Where qe = toxin concentration adsorbed onto yeast cells (µg g^{-1}), Ce = Toxin concentration remaining in bulk solution (µg L^{-1}) at equilibrium, K is the adsorption capacity of yeast cells for CYN, and n is the affinity of yeast cells to adsorb CYN. The data were plotted on logarithmic scale and the regression isotherm coefficients were calculated.

Bound CYN release assay

To test whether the yeast-CYN binding is reversible or irreversible (i.e., the firmness of CYN adsorption to the adsorbent), the experiment of potential release of CYN bound to yeast cells was conducted according to Petruzzi et al. (2015) with some modifications. Briefly, pellets of inactivated and viable yeast cells with adsorbed CYN collected from adsorption experiment were suspended in BG-11 medium (pH 7.2), vigorously agitated for one min., and incubated at 30°C for 1 h, while shaking (200 rpm). The solution was centrifuged (6000g, 15 min), and CYN concentrations potentially released back into the washing solution was determined directly in supernatant by ELISA as described above.

Statistical analysis

Differences in *C. raciborskii* growth and CYN concentrations between treated and control cultures were estimated by ANOVA followed by Tukey's post-hoc test ($P < 0.05$).

Results

KKUY0701 yeast strain exhibited anti-cyanobacterial activity against *C. raciborskii* in batch cultures. The cell density of *C. raciborskii* decreased remarkably (53%) within one day of co-cultivation with living yeast compared to control ($P < .05$). The cells of these cultures were totally lysed after 4 days (Fig. 1). The filtrate of this yeast also inhibited the growth *C. raciborskii*, and this inhibition increased along with the proportion of filtrate in the cultures ($P < 0.05$) (Fig. 2). The highest filtrate proportion (50%) exhibited

complete growth inhibition of *C. raciborskii* within two days of the incubation period, while complete growth inhibition of *C. raciborskii* after 3 days by the 10% filtrate proportion (Fig. 2). The lowest filtrate proportion (1%) decreased the cyanobacterial growth but without complete cell lysis (Fig. 2). The growth curve of *A. pullulans* showed that the cell number of yeast co-cultivated with *C. raciborskii* cells increased significantly compared to control culture ($P < 0.05$) grown in YMA medium (Fig. 3). The toxin analysis revealed an elevation in CYN concentrations released into the medium of living –yeast treated *C. raciborskii* cultures and yeast-filtrate treated cultures compared to control cultures ($P < 0.05$) during the first two days of incubation period (Fig. 4). Thereafter, the released CYN decreased sharply in the medium of living-yeast treated cultures and became undetectable at day 4 (Fig. 4). Conversely, CYN amounts released into the medium of yeast- filtrate treated cultures did not differ significantly ($P > 0.05$) all over the incubation period (Fig. 4).

The results of adsorption isotherm experiments revealed the capability of both living and heat-inactivated yeast for binding CYN onto their surfaces. However, heat-inactivated cells had higher CYN adsorption efficiency (32–83%) than living ones (24–63%) (Fig. 5). This adsorption efficiency differed significantly ($P \leq 0.05$) with initial concentrations of CYN used in our experiments (Fig. 5). The highest adsorption percentage of CYN by living (63%) and inactivated (83%) yeast was obtained at CYN initial concentration of 40 μg L^{-1} and 50 μg L^{-1}, respectively. After that, these adsorption percentages decreased with increasing toxin concentrations (Fig. 5). The adsorption isotherms of CYN by living and inactivated yeast were well fitted to with Freundlich equation (Table 1). However, inactivated yeast showed higher adsorption capacity (i.e., high K value) and intensity (i.e., low 1/n value) for CYN than viable yeast (Table 1). The toxin release assay data showed that CYN was much firmer to inactivated yeast than viable yeast. About 74% of CYN bound to living yeast released into the washing solution (Table 2) whereas no CYN was detected in the washing solution of the complex of inactivated yeast-CYN (Table 2).

Table 1. Values of Freundlich isotherm parameters (K = adsorption capacity and n= adsorption intensity) for cylindrospermopsin adsorption by living and heat- inactivated cells of *A. pullulans*

	K	n	R^2
Living yeast	1.9	1	0.8
Inactivated yeast	3.3	1.4	0.7

Table 2. Potential release of cylindrospermopsin adsorbed on the surfaces of living and inactivated yeast upon washing with BG-11 medium.

Adsorbent	Bound CYN (μg g^{-1})	Released CYN (μg g^{-1})
Living yeast	264	195.4± 31 (74%)
Inactivated yeast	455	*ND

*ND = non-detectable at ELISA limit

Discussion

This study provides clearly demonstrated the growth inhibition of the harmful cyanobacterium *C. raciborskii* and adsorption of CYN toxin adsorption by the yeast *A. pullulans*. Co-cultivation of *C. raciborskii* with yeast cells showed decline in *C. raciborskii* cell density, with total cell lysis within 4 days. This is the first study on the anti-cyanobacterial activity of fungi against *C. raciborskii.* Nevertheless, KKUY0701 strain was found to remove all *M. areuginosa* cells within 72 hours in batch experiment (Mohamed et al. 2020). Additionally, the removal efficiency of *C. raciborskii* cells by KKUY0701 strain can be compared to those of other anti-cyanobacterial fungi such as *Trichoderma abietinum*, *Trichoderma citrinoviride* and *Lopharia spadicea*, which eliminated all *M. aeruginosa* cells within 48h (Jia et al. 2010b; Mohamed et al. 2014). Our study also showed that the filtrate of *A. pullulans* could suppress *C. raciborskii* growth, indicating involvement of anti-cyanobacterial substances excreted by this yeast into the medium. Thus, our results support the finding of other studies stating that *Trichoderma citrinoviride* and *A. pullulans* inhibited cyanobacterial growth through the extracellular excretion of anti-cyanobacterial substances into the medium in lieu of direct attack (Mohamed et al. 2014; 2020).

In this study, *A. pullulans* growth increased after one day of co-incubation with *C. raciborskii* cells and became greater than in control cultures (i.e., YMA medium). The ability of *A. pullulans* to grow in medium without carbon source suggests that it could decompose and utilize cell constituents of *C. raciborskii* for its growth. These results support the finding of previous studies that some yeast strains e.g., *Saccharomyces cerevisiae* and *A. pullulans* lysed cyanobacterial cells and used their contents as a carbon source (Möllers et al. 2014; Mohamed et al. 2020).

The anti-cyanobacterial activity of *A. pullulans* towards *C. raciborskii* may be attributed to the production for lytic enzymes (e.g., N-β-acetylglucosaminidase), which can digest peptidoglycan, the main constituent of *Cylindrospermopsis* cell wall (Di Francesco et al. 2015). However, such inhibitory and lytic compounds produced by *A. pullulans* should be further isolated and characterized.

In addition to growth inhibition of *C. raciborskii*, *A. pullulans* removed the released CYN toxin in the culture medium. This was manifested by the decline in CYN concentrations in the medium of yeast-treated cultures but without significant change in filtrate-treated cultures. This observation indicates that CYN toxin could be eliminated through biodegradation by living yeast cells or adsorption onto yeast surfaces. Our adsorption experiment revealed a reduction in CYN concentrations in the presence of heat-inactivated and living yeast. However, inactivated yeast caused greater decrement in CYN concentrations than living yeast. This suggests that the metabolic and enzymatic activities were not involved in the reduction of CYN concentrations (i.e., not biodegradation), but the decline in toxin concentrations was rather due to its binding to yeast cell walls. Concomitantly, other studies demonstrated the adsorption of mycotoxins (Luo et al. 2015) and microcystins (Mohamed et al. 2020) by yeast strains. Our results also showed that Freundlich isotherm parameters including K (i.e., adsorption capacity) and 1/n (i.e., the

adsorbent affinity) are correlated, and therefore, the adsorption of CYN toxin by KKUY0701 strain is suitable. In our study, the data of isotherm parameters (K = 3.3, and 1/n = 0.7), can be compared with those (K = 2.9, 1/n = 0. 84, respectively) recorded in the literature for CYN adsorption by activated carbon (Liu 2017). The adsorption capacity of KKUY0701 yeast strain for CYN toxin increased with the increase of initial CYN concentrations, up to a certain concentration (40–50 μg L^{-1}) and then decreased at higher concentrations (60–100 μg L^{-1}). This reflects that CYN adsorption this yeast strain is a process reaching saturation. The highest adsorption capacity of CYN by inactivated (83%) and living yeast (63%) was obtained at initial concentrations of 50 and 40 μg L^{-1}, respectively. This means that each gram of heat-inactivated yeast would remove 455 μg CYN, while the amount of toxin removed by viable yeast would be 264μg toxin g^{-1} yeast. Given that the highest level of CYN reported in the aquatic environment is about 100 μg L^{-1} (WHO 2020), and the World Health Organization recommended a maximum allowable level of 3 μg L^{-1} CYN in drinking water (WHO 2020), the dose of heat-inactivated yeast required for CYN removal to below the proposed guideline value would be 200 mg L^{-1}.It is noteworthy in our study that CYN binding to inactivated yeast was very stable. Conversely, about 74% of CYN bound to living yeast released back into the washing solution, indicating CYN binding to living yeast was not stable. Previous studies also demonstrated the firmness of mycotoxin and microcystin adsorption to heat-inactivated yeast (Luo et al. 2015; Baptista et al. 2004; Mohamed et al., 2020). Those authors attributed the capability of yeast for toxin adsorption to β-D-glucans present in the yeast cell wall.

In conclusion, this study clearly demonstrates the capability of *A. pullulans* for inhibiting *C. raciborskii* growth. Taken the selective antagonistic activity of *A. pullulans* against cyanobacteria rather than other eukaryotic algae reported in our earlier study (Mohamed et al. 2020), living cells or filtrate of *A. pullulans* would be employed to selectively control *C. raciborskii* growth in the aquatic environment. However, biological control measures are still needed to evaluate the feasibility of antagonistic yeast for application in the natural environment. Furthermore, the heat -inactivated cells of *A. pullulans* had a firmer and higher adsorption of CYN toxin than living cells. Such inactivated yeast would be used to eliminate CYN in drinking water treatment plants as it is non-toxic, pathogenic or infective. Nevertheless, urgent *in situ* study is needed to commercialize the utilization of heat-inactivated yeast as an adsorbent.

Declarations

Ethical Approval and Consent to Participate: Not applicable

Consent to Publish: Not applicable

Authors Contributions: MH, SA and YM cultured *A. pullulans* strain and followed its growth in bioremoval experiments. ZM collected and tabulated data of anticyanobacterial and toxin- degrading yeast. ZM set up the experiments of anti-cyanobacterial activity of *A. pullulans* and cylindrospermopsin adsorption, and was a major contributor in writing the manuscript. All authors participated in analysis and interpretation of the data, read and approved the final manuscript.

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Availability of data and materials: Not applicable

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Conflict of interest: The authors declare that there is no conflict of interest.

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Figures

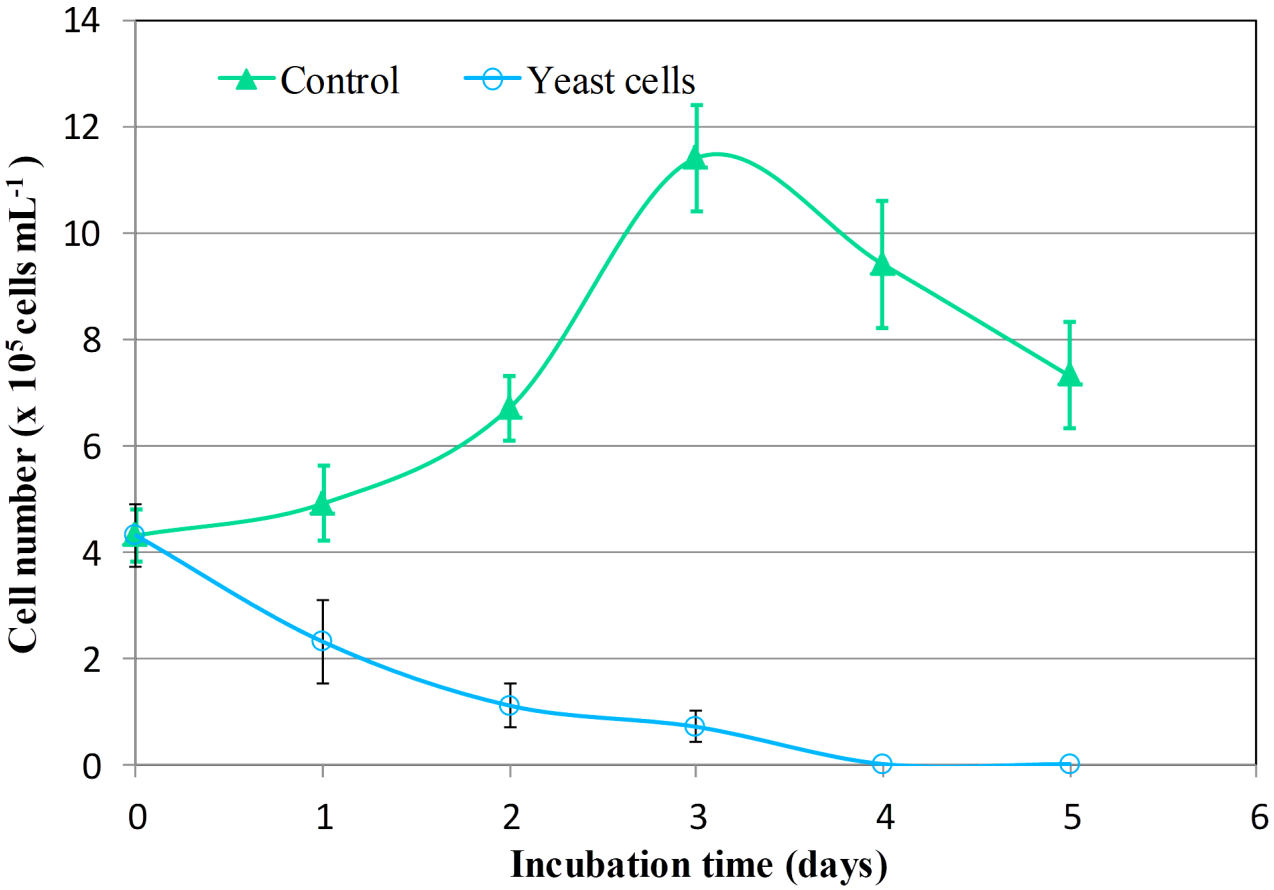

Fig.1

Figure 1

Variation in the growth of *Cylindrospermopsis raciborskii* co-cultivated with *A. pullulans* living cells

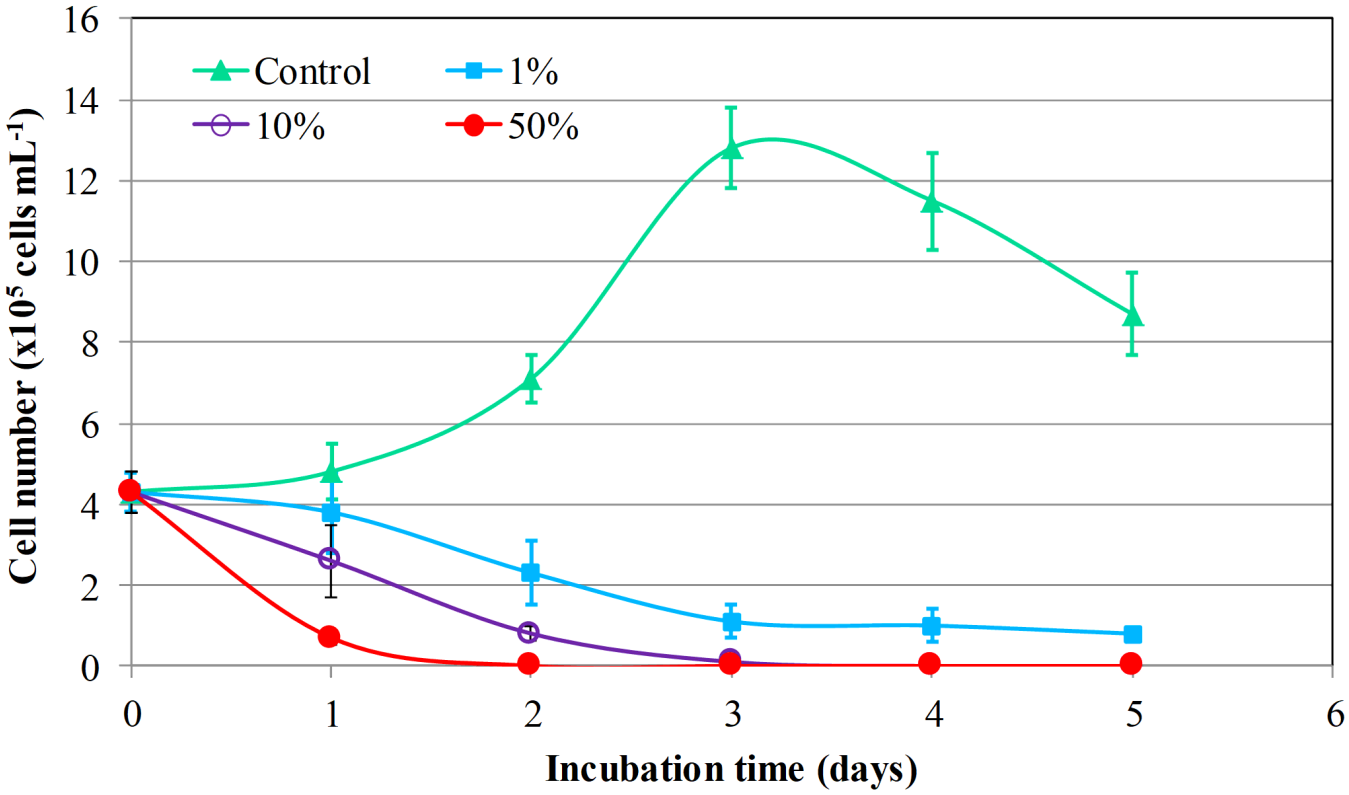

Fig.2

Figure 2

Variation in the growth of *Cylindrospermopsis raciborskii* co-cultivated with *A. pullulans* filtrate at different proportions (1%, 10%, 50%, v/v)

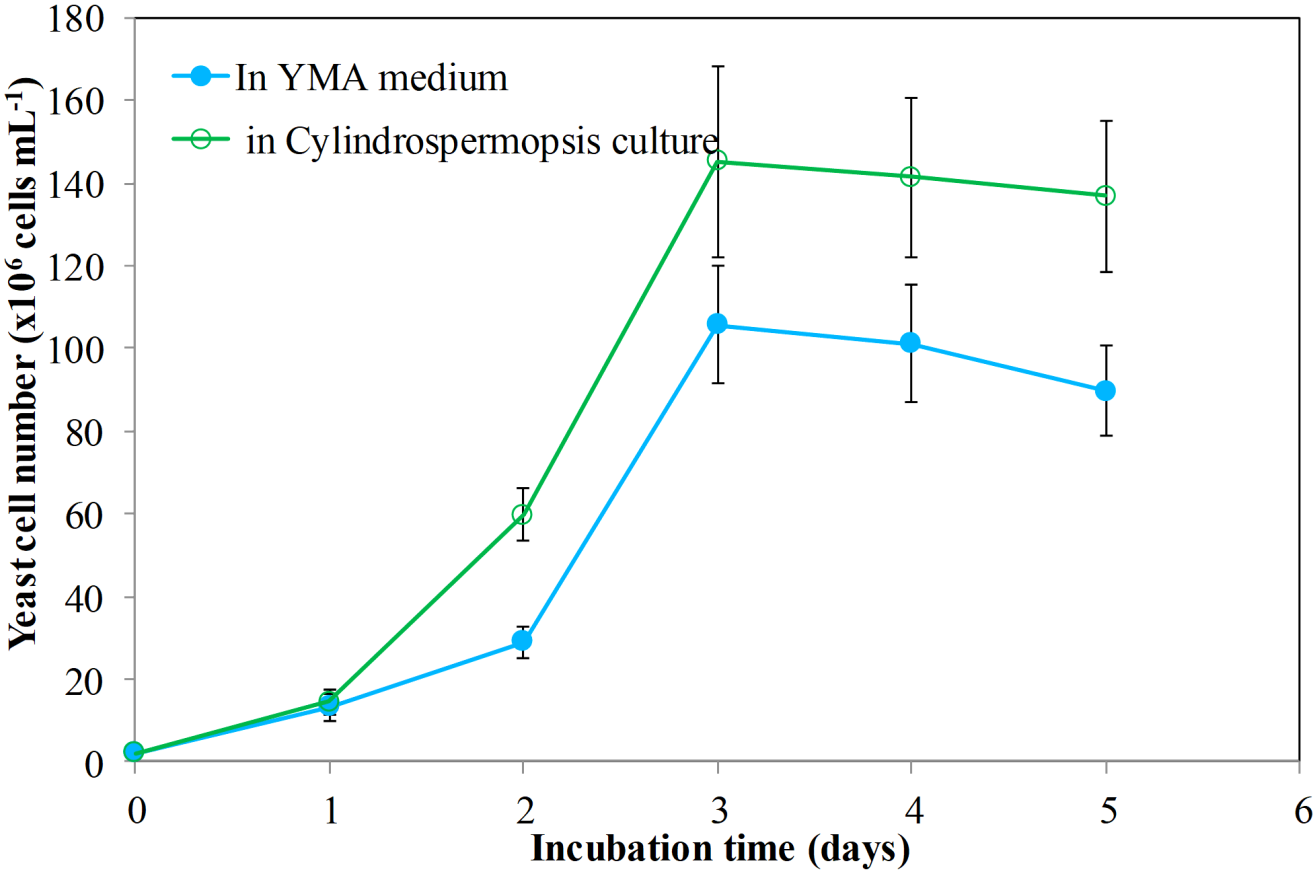

Fig.3

Figure 3

Growth curve of *A. pullulans* co-cultivated with living cells of *Cylindrospermopsis raciborskii* and YMA medium.

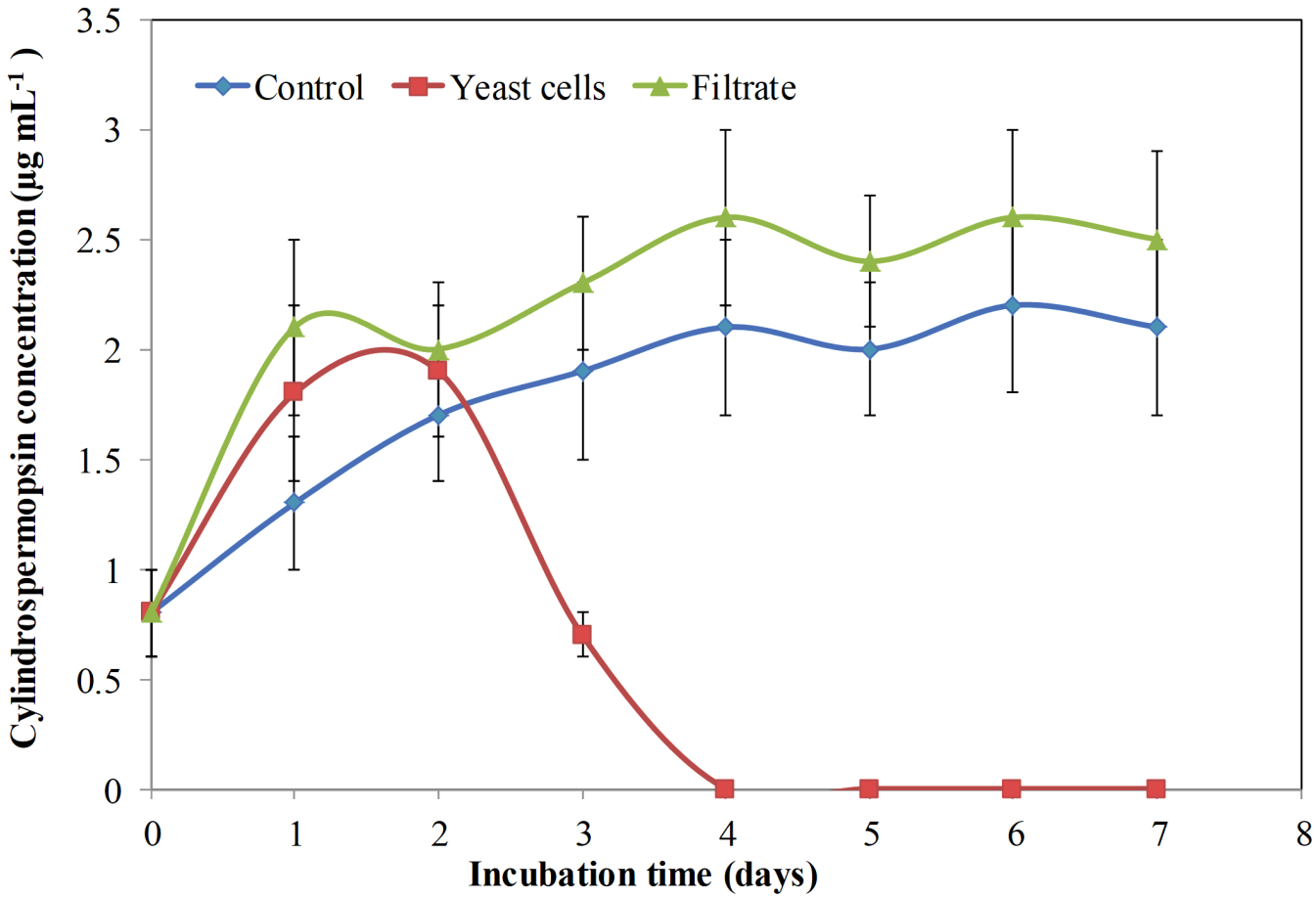

Fig.4

Figure 4

variation in cylindrospermopsin concentrations released into the medium of *Cylindrospermopsis raciborskii* cultures during incubation with living cells and filtrate of *A. pullulans*

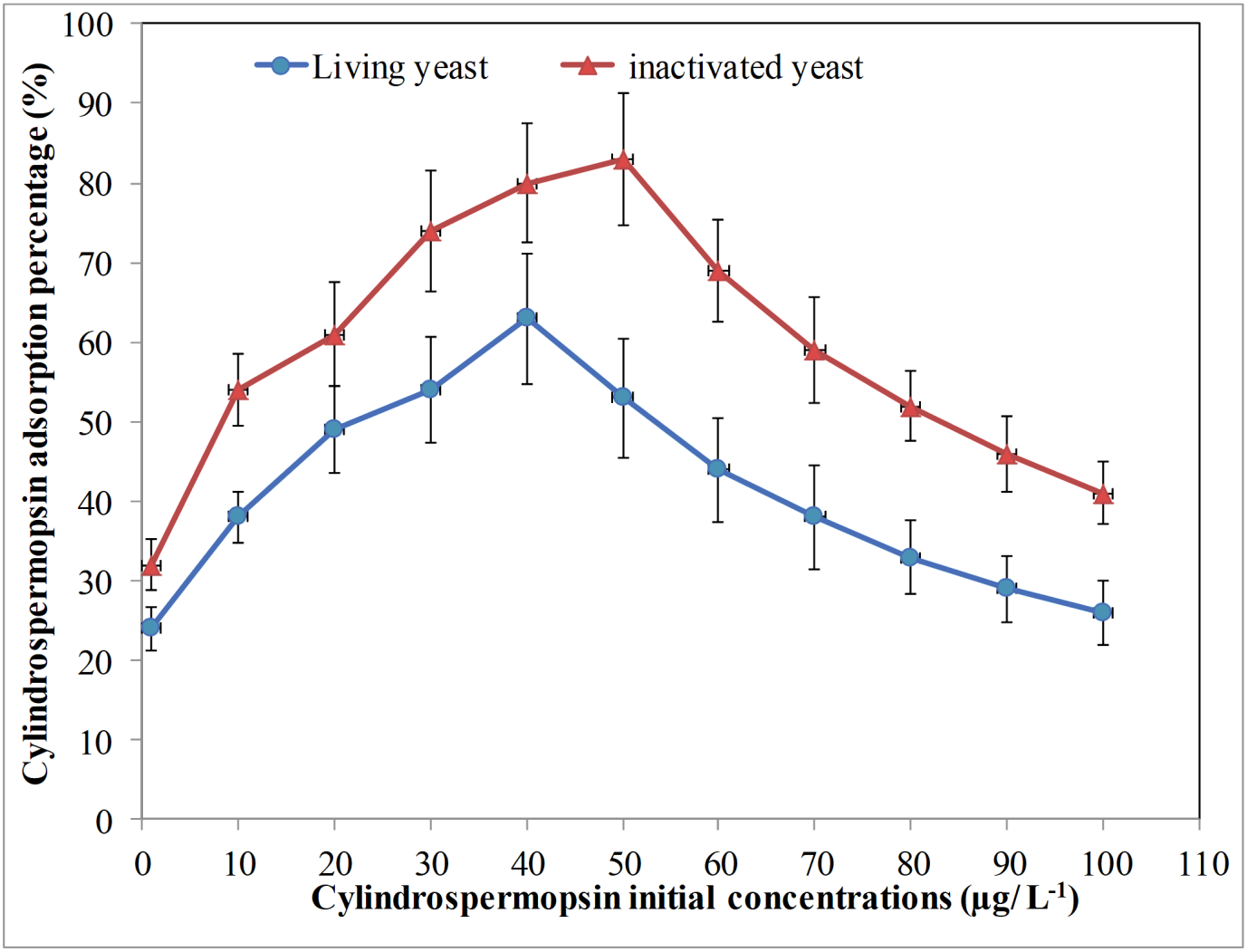

Fig.5

Figure 5

Adsorption efficiency (%) of cylindrospermopsin by living and heat-inactivated cells of *A. pullulans*