Algicidal activity of an actinomycete strain, *Streptomyces rameus*, against *Microcystis aeruginosa*

Kanchariya Phankhajon, Anchana Somdee and Theerasak Somdee

**ABSTRACT**

An actinomycete strain (KKU-A3) with algicidal activity against *Microcystis aeruginosa* was isolated from soil in Khon Kaen Province, Thailand. Based on its phenotypic characteristics and 16S rDNA sequence, strain KKU-A3 was identified as *Streptomyces rameus*. Strain KKU-A3 also exhibited algicidal activity against the cyanobacteria *Synechococcus elongatus*, *Cylindrospermum* sp. and *Oscillatoria* sp. A mathematical and statistical technique was used to optimize the culture conditions and maximize its anti-*Microcystis* activity. The single factor experiments indicated that glucose and casein were the most effective carbon and nitrogen sources, respectively, and produced the highest anti-*Microcystis* activity. Response surface methodology indicated that the optimum culture conditions were 19.81 g/L glucose and 2.0 g/L casein at an initial pH of 7.8 and an incubation temperature of 30°C. The anti-*Microcystis* activity increased from 82% to 95% under optimum conditions. In an internal airlift loop bioreactor, the removal of *M. aeruginosa* KKU-13 by the bacterium was investigated in batch and continuous flow experiments. In the batch experiment, KKU-A3 displayed maximum anti-*Microcystis* activity of 95% at day 7, whereas in the continuous flow experiment, KKU-A3 displayed maximum anti-*Microcystis* activity of 95% at day 10.

**Key words** | algicidal activity, internal airlift loop bioreactor, *Microcystis aeruginosa*, response surface methodology, *Streptomyces rameus*

**INTRODUCTION**

*Microcystis* is a non-nitrogen-fixing cyanobacterial genus that usually causes *blooms* in warm, calm, nutrient-enriched waters (Chorus & Bartram 1999). In eutrophic waters, *Microcystis* blooms are regular, extensive, persistent, and dominant throughout the year (Zurawell et al. 2005). *Microcystis* is the most cosmopolitan of the toxic cyanobacteria, and *M. aeruginosa* has been connected to animal and human illness and even death, worldwide (Apeldoorn et al. 2007). *Microcystis* was the first genus (from *M. aeruginosa* strain NRC-1) shown to produce cyclic-peptide hepatotoxins, which were later referred to as microcystins (MCs). To date, more than 100 MC variants have been isolated, and approximately 60 variants of the toxins have been produced from species and strains of *Microcystis* (Zurawell et al. 2005).

There is an urgent need for the development of methods for controlling *Microcystis* blooms. A wide range of methods have been used to control this cyanobacterium (Zurawell et al. 2005). A rapid and efficient method for eradicating *Microcystis* blooms is the application of chemical algicides that contain copper salts (e.g. copper sulphate). This method is normally used to reduce cyanobacteria in small bodies of water, such as ponds and small lakes (Sigee 2005). The disadvantage of using copper sulphate is that it results in the detrimental release of the intracellular *Microcystis* toxins into the surrounding water (Apeldoorn et al. 2007). Jones & Orr (1997) demonstrated the control of toxic cyanobacteria with copper sulphate. The treatment of a *M. aeruginosa* bloom in Lake Centenary, Australia caused cell lysis and the release of MCs into the water. Dissolved MCs were detected within 24 h of spraying, and the toxins persisted at high levels (1,300–1,800 μg/L) for nine days.

It is widely accepted that biological control has an important role in regulating the phytoplanktonic biomass, including *Microcystis*, in freshwater environments (Shunyu et al. 2006). The application of biocontrols with algicidal activity against cyanobacteria, using naturally occurring...
aquatic microorganisms, is a potentially safer and more natural way to eliminate *Microcystis* cells (Sigee 2005). In freshwater ecosystems, biological agents, including bacteria, serve as potential suppressors of cyanobacterial blooms (Bai et al. 2011). The aim of the present study was to: (1) isolate an algicidal bacterium with high anti-*Microcystis* activity, (2) identify the algicidal bacterial strain based on its morphological and biochemical characteristics and 16S rRNA gene sequence, (3) investigate the algicidal activity of the algicidal bacterial strain against other cyanobacteria strains, (4) optimize the media components, initial pH and incubation temperature to obtain the highest anti-*Microcystis* activity using a sequential statistics-based experimental design, and (5) determine the removal of *Microcystis* cells in an internal airlift loop (IAL) bioreactor under batch and continuous operation.

**MATERIALS AND METHODS**

**Cyanobacterial species**

*M. aeruginosa* strain KKU-13 was previously isolated from Chulabhorn Dam, Chaiyaphum, Thailand, using the micropipette washing method (Somdee et al. 2013). The cultures were grown in BG-11 medium in a shaking incubator at 150 rpm at 25 °C under a light intensity of 20 μmol photons m⁻² s⁻¹ from fluorescent tubes (Philips TL65 W/25; white) with a 12/12 h light/dark cycle.

**Isolation and screening of the anti-** *Microcystis** bacteria

The bacterial strains were isolated from the surface water and soil near the edge of the Bueng Nong Khot reservoir, Khon Kaen, Thailand during a bloom in June 2012 using the double-layer algal-lawn method (Furu-sawa et al. 2005). Each purified bacterial strain was cultured on nutrient agar (5 g/L beef extract, 5 g/L peptone and 15 g/L agar, pH 7.5) for nonspecific bacteria or starch casein agar (10 g/L soluble starch, 0.3 g/L casein, 2 g/L K₂HPO₄, 2 g/L KNO₃, 0.05 g/L MgSO₄·7 H₂O, 0.02 g/L CaCO₃, 0.01 g/L FeSO₄·7 H₂O and 15 g/L agar, pH 7.3) for actinomycetes.

**Testing of the anti-** *Microcystis** activity on *M. aeruginosa*

The anti-*Microcystis* activity was investigated by agar well diffusion (Perez et al. 1990) and a liquid-culture method (Hua et al. 2009). In the first method, a culture of *M. aeruginosa* KKU-13 was grown in BG-11 broth for seven days, using the conditions described above. The cells were harvested by centrifugation at 5,000 g for 20 min at 4 °C. The cell pellet was mixed with the BG-11 agar medium and poured on to a plate. After incubation under illumination for seven days, a well was drilled with a sterile cork borer (6 mm in diameter), and 100 μL of each bacterial broth culture was transferred into each well on the algal lawn. The inoculated plates were incubated under illumination for seven days at 25 °C. The anti-*Microcystis* activity was determined by measuring the diameter of the inhibition zone formed in the algal lawn. The inhibition zones were expressed as the means of three independent experiments. The bacterial strains that created a clear zone with a width >10 mm were selected as the primary candidates.

In the second method, the primary candidates (actinomycete strains KKU-A3, KKU-D4, KKU-A6, KKU-C3 and KKU-E5) were screened in liquid culture. *M. aeruginosa* strain KKU-13 was grown in the conditions described above for seven days. The bacterial culture (10% v/v) was inoculated into a liquid culture of *M. aeruginosa* KKU-13 and incubated under the *M. aeruginosa* culture conditions for seven days. The anti-*Microcystis* activity was determined by the removal efficiency (%), as indicated by the changes in the chlorophyll-α concentration compared with the control. Sterile BG-11 broth and starch casein broth were used as controls, and three replicates were performed for each test. The chlorophyll-α concentration was measured at 630, 645, 665 and 750 nm with a spectrophotometer (UV-2550, Japan) after extraction with 90% methanol (Mu et al. 2007). The removal efficiency (%) was calculated with the following equation:

\[
\text{Removal efficiency} = \left[ 1 - \left( \frac{C_1}{C_0} \right) \right] \times 100
\]

where *C₀* and *C₁* are the chlorophyll-α concentrations of *M. aeruginosa* strain KKU-13 in the control and test groups at the initial time and time *t*, respectively (Kong et al. 2014).

**Identification of strain KKU-A3**

Strain KKU-A3 was identified using standard procedures and International Streptomyces Project (ISP) protocols, using tryptone–yeast extract agar (ISP1; 5.0 g/L tryptone, 3.0 g/L yeast extract and 20.0 g/L agar, pH 7.3), yeast extract-malt extract agar (ISP2; 4.0 g/L yeast extract, 10.0 g/L malt extract, 4.0 g/L dextrose and 20.0 g/L agar, pH 7.3), oatmeal agar (ISP3; 20.0 g/L oatmeal, 1.0 mg/L...
FeSO$_4$.7H$_2$O, 1.0 mg/L MnCl$_2$.4H$_2$O, 1.0 mg/L ZnSO$_4$.7H$_2$O and 18.0 g/L agar, pH 7.3), inorganic salts-starch agar (ISP4; 10 g/L soluble starch, 1.0 g/L K$_2$HPO$_4$, 1.0 g/L MgSO$_4$.7H$_2$O, 1.0 g/L NaCl, 2.0 g/L (NH$_4$)$_2$SO$_4$, 2.0 g/L CaCO$_3$, 1.0 mg/L FeSO$_4$.7H$_2$O, 1.0 mg/L MnCl$_2$.4H$_2$O, 1.0 mg/L ZnSO$_4$.7H$_2$O and 20.0 g/L agar, pH 7.3), peptone yeast extract iron agar (ISP6; 20 g/L peptone, 0.5 g/L ferric ammonium citrate, 20.0 g/L agar, pH 7.3), peptone yeast extract agar (ISP5; 10.0 g/L glycerol, 1.0 g/L K$_2$HPO$_4$, 1.0 mg/L FeSO$_4$.7H$_2$O, 1.0 mg/L MnCl$_2$.4H$_2$O, 1.0 mg/L K$_2$HPO$_4$ and 20.0 g/L agar, pH 7.3), peptone yeast extract iron agar (ISP6; 20 g/L peptone, 0.5 g/L ferric ammonium citrate, 0.08 g/L Na$_2$S$_2$O$_3$, 1 g/L yeast extract, 1 g/L K$_2$HPO$_4$ and 20.0 g/L agar, pH 7.3) (Holt et al. 1994). The morphological characteristics of this strain were also studied under a light microscope and a scanning electron microscope, according to Somdee et al. (2013). The analysis of 2,6-diaminopimelic acid (DAP) pattern was carried out according to Staneck & Roberts (1974) using thin layer chromatography with a methanol/water/6 M HCL/pyridine (80:26:4:10 by volume) solvent system.

Additional identification was performed using 16S rRNA gene sequencing and the procedure described by Somdee et al. (2013). The obtained 16S rDNA nucleotide sequence (615 bp) was deposited at the National Center for Biotechnology Information (NCBI) GenBank database under accession number KF857225. Closely related homologues were identified by BLAST analysis by comparing the partial 16S rDNA sequences with sequences deposited in the GenBank database. The sequence of the 16S rRNA gene from KKU-A3 was aligned with other nucleotide sequences. A phylogenetic and distance analysis of the aligned sequences was performed with the MEGA program (version 5.05). The resulting unrooted tree topologies were evaluated by bootstrap analysis using the neighbour-joining method based on 1,000 resamplings.

Algicidal activity of KKU-A3 on other cyanobacteria strains

The algicidal range of KKU-A3 was examined in liquid culture. Using a starch casein medium broth, KKU-A3 was cultured at 30 ºC with orbital shaking at 150 rpm for four days. Samples of each tested cyanobacteria were cultured in BG-11 broth under the M. aeruginosa conditions and collected from individual exponential-phase cultures of the following: Anabaena sp. TISTR 8076, Synechococcus elongatus TISTR 8500, Cylindrospermum sp. TISTR 8158, Nostoc commune TISTR 8160 and Oscillatoria sp. TISTR 8491. The bacterial culture (10% v/v) was inoculated into the liquid culture of each cyanobacterial species and incubated under the M. aeruginosa culture conditions for seven days. The anti-cyanobacteria activity was determined by the removal efficiency, using the changes in the chlorophyll-a concentrations after co-cultivation for seven days. Triplicate experiments were performed for each treatment.

Optimization of medium composition and conditions to improve the anti-Microcystis activity using response surface methodology

Effects of different carbon and nitrogen sources on the anti-Microcystis activity

The effects of carbon and nitrogen sources on the anti-Microcystis activity were investigated. Various carbon sources (soluble starch, glucose, fructose, sucrose, xylose and lactose: 10 g/L) and nitrogen sources (casein, yeast extract, peptone, skimmed milk, malt extract and ammonium nitrate: 0.3 g/L) were individually tested for their effect on the anti-Microcystis activity in starch casein medium broth at 30 ºC. The anti-Microcystis activity was determined by the removal efficiency percentage after cultivation for seven days. The experiments were performed in triplicate for each treatment. Later, glucose and casein were chosen as the optimum carbon and nitrogen sources, respectively, and produced high anti-Microcystis activity.

A completely randomized design was used to determine the effect of carbon and nitrogen sources on the removal efficiency of M. aeruginosa KKU-13 by strain KKU-A3. The results are expressed as the mean ± one standard error (SE) of three replicates and data were analyzed using one-way analysis of variance (one-way ANOVA) and multiple comparisons with Duncan’s multiple range test to determine the significance relative to the control, using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Single factor experiments for determining the optimal range of four factors

Single factor experiments were performed to establish the optimal range for each of four factors (glucose, casein, initial pH and temperature) in producing the highest anti-Microcystis activity. Different levels of glucose (1.0, 5.0, 10.0, 15.0, 20.0 and 25.0 g/L), casein (0.1, 0.3, 0.6, 1.0, 1.5 and 2.0 g/L), initial pH (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) and temperature (25, 30, 35, 40 and 45 ºC) were investigated. The anti-Microcystis activity was assessed after seven days of co-culture. All experiments were performed in triplicate.
Response surface methodology for optimizing the medium composition, initial pH and temperature

Before establishing the regression model, the lower and upper values of each variable were identified by single factor experiments as described above. The single factor experiment data were regressed using Design Expert software version 7.0.3 to obtain a second-order polynomial. The regression model can be used to identify the most effective levels of each variable for maximizing anti-\textit{Microcystis} activity and, therefore, provide sufficient information to construct a contour and a surface. In this study, a Box-Behnken design (BBD) was used to optimize the conditions for maximizing the anti-\textit{Microcystis} activity of KKU-A3. The experimental range and the levels of the four independent variables from the BBD are shown in Table 1.

Removal of \textit{M. aeruginosa} KKU-13 in an IAL bioreactor

\textit{M. aeruginosa} removal was investigated using a 10-L capacity glass-cylinder bioreactor. The bioreactor comprised a 10-L \textit{M. aeruginosa} culture in BG-11 medium (a late exponential growth phase for strain KKU-13), a plastic medium with a height of 180 mm and a diameter of 75 mm, an air pump and a 0.22 \textmu m air filter. The bacterial cells were immobilized on the plastic medium, which was placed in the centre of the bioreactor unit. Clean air was constantly introduced into the bioreactor via a 0.22 \textmu m sterile filter. An air flow rate of 5 L/min was applied into a diffuser placed inside the bioreactor at its base, causing the \textit{M. aeruginosa} culture circulation to flow upward in a loop over the reactor.

Cell immobilization on a plastic medium

The strain KKU-A3 was cultured in 50 mL of a modified starch casein medium broth, determined by the response surface methodology (RSM) optimization in a shaking incubator at 150 rpm and 30 °C for four days. The four-day culture was inoculated in 1,500 mL (2% v/v) of fresh modified starch casein medium broth and was incubated in a shaking incubator at 30 °C and 150 rpm for two days. After two days of incubation, the bacterial culture was poured directly into the plastic medium, such that the plastic medium immobilized the cells. The bioreactor, complete with the plastic medium unit containing immobilized cells, was then incubated at 30 °C for two days on a shaking incubator at 90 rpm. Therefore, the entire bacterial culture was incubated for a total of four days (which corresponds to a late exponential growth phase for strain KKU-A3 and promoted sufficient biofilm formation on the plastic medium).

Table 1 | Levels of the four variables in the experimental design

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Code</th>
<th>Non code</th>
<th>Code levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose A</td>
<td>X₁</td>
<td>15 g/L</td>
<td>20 g/L</td>
</tr>
<tr>
<td>Casein B</td>
<td>X₂</td>
<td>1 g/L</td>
<td>1.5 g/L</td>
</tr>
<tr>
<td>Initial pH C</td>
<td>X₃</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Temperature D</td>
<td>X₄</td>
<td>25 °C</td>
<td>30 °C</td>
</tr>
</tbody>
</table>

Removal of \textit{M. aeruginosa} KKU-13 in the batch experiment

After strain KKU-A3 was immobilized onto the plastic medium for four days, 10 L sterile phosphate buffer was used to wash and remove the excess culture medium and loose bacterial biofilm from the plastic medium. The sterile bioreactor (Figure 1(a)) packed with the plastic medium (Figure 1(c)) contained a culture of \textit{M. aeruginosa} KKU-13 that had been grown in BG-11 broth for seven days (which corresponds to the late exponential growth phase for strain KKU-13). The bioreactor was incubated in a 30 °C water bath under a light intensity of 20 \textmu mol photons m⁻² s⁻¹ with a 12/12 h light/dark cycle for 14 days. The removal efficiency (%) was calculated by the change in the chlorophyll-a concentration.

Removal of \textit{M. aeruginosa} KKU-13 in the continuous experiment

A similar scheme as that used in the batch experiments was employed in the continuous experiment, with the addition of a \textit{M. aeruginosa} KKU-13 culture reservoir, a recipient flask and a peristaltic pump. The culture of \textit{M. aeruginosa} KKU-13 was grown in BG-11 broth for seven days and continually flowed from an adjacent 'reservoir' through the bioreactor to accumulate in a recipient flask. Figure 1(b) shows a schematic diagram of the flow-through mode experiment. The continuous bioreactor was incubated in a 30 °C water bath under light. The water flow was maintained at 2 mL/min with a hydraulic retention time (HRT) of 8 h. An aliquot of the solution was withdrawn from the effluent tube every day for 14 days. The change in the chlorophyll-a concentration was used to analyse the removal efficiency. The bioreactor cultured with \textit{M. aeruginosa} KKU-13 in BG-11
broth for 14 days in the absence of bacteria on the plastic medium was used as the control.

RESULTS

Isolation and identification of anti-\textit{Microcystis} bacterium strain KKU-A3

A total of 28 bacterial strains were isolated from the Bueng Nong Khot reservoir, Khon Kaen, Thailand. However, only five actinomycete strains (KKU-A3, KKU-D4, KKU-A6, KKU-C3 and KKU-E5) exhibited anti-\textit{Microcystis} activity. Strain KKU-A3 displayed the strongest anti-\textit{Microcystis} activity and highest removal efficiency (%) compared with the growth of the control and the growth under the conditions used for other strains (Table 2). Therefore, KKU-A3 was chosen for further characterisation and experimental testing.

Strain KKU-A3 was cultured on different ISP media and displayed the typical morphology of \textit{Streptomyces}: aerobic, slow growing, glabrous or chalky, heaped and folded. The colour of the aerial mycelium was white to yellow, and the substrate mycelium was yellow or brown, depending on the ISP medium (data not shown). In addition, strain KKU-A3 had an earthy odour and produced soluble brownish pigments. Smooth, oval shaped spores occurred in long, straight to rectiflexible chains, with 10–20 spores per filament. Of the range of media tested, KKU-A3 grew well on most ISP media, with the exception of ISP6 media (data not shown). These results indicate that KKU-A3 is a Gram-positive and non-acid-fast bacterium. The optimal growing conditions were pH 7–8 and 30°C. An analysis of the whole-cell hydrolysates revealed the presence of LL-diaminopimelic acid (LL-DAP), leading to the classification of the cell wall as type I. The identification was confirmed using the 16S rDNA sequence. The sequence of KKU-A3 (GenBank KF857223) highly matched the sequence of \textit{Streptomyces rameus} (KF006406), with 99% sequence homology.

Algicidal activity of KKU-A3 against other cyanobacteria strains

Strain KKU-A3 exhibited strong anti-cyanobacterial activity against not only \textit{M. aeruginosa} strain KKU-13 but also \textit{Synechococcus elongatus} TISTR 8500 (Table 3). Strain KKU-A3 exhibited moderate anti-cyanobacterial activity against \textit{Cylindrospermum} sp. TISTR 8158 and weak anti-cyanobacterial activity against \textit{Oscillatoria} sp. TISTR 8491. However,
strain KKU-A3 possessed no anti-cyanobacterial activity against *Anabaena* sp. TISTR 8076 and *Nostoc commune* TISTR 8160.

**Effects of different carbon and nitrogen sources on the anti-** *Microcystis* **activity**

The effect of carbon and nitrogen sources on the anti-*Microcystis* activity was determined using the removal efficiency of *M. aeruginosa* strain KKU-13 and is shown in Figures 2 and 3. Statistical analysis by one-way ANOVA and Duncan’s multiple range test to determine the significance between treatments at the significance level 0.05 (data not shown) indicated that significant differences in the removal efficiencies of the strain KKU-13 were not detected utilizing six different carbon sources. As illustrated in Figure 2, glucose, utilized as the sole carbon source, was most effective in enhancing the potential of the isolate KKU-A3 to inhibit *M. aeruginosa* strain KKU-13, with inhibition percentages of 74% and 82% on days 5 and 7, respectively. In contrast, the use of starch as the sole carbon source resulted in low removal efficiencies of 62% and 72% after five and seven days of co-culture, respectively. Similar results were noted for the nitrogen sources, as suggested by the statistical analysis. Figure 3 shows that casein was the most effective sole nitrogen source in inhibiting the growth of *M. aeruginosa* strain KKU-13, showing removal efficiencies of 74% and 82% after five and seven days of co-culture, respectively. On the other hand, the lowest removal efficiencies of 51% and 61% on days 5 and 7, respectively, were obtained when ammonium nitrate was used as the sole nitrogen source.

**Optimization of medium composition and conditions for anti-** *Microcystis* **activity using single factor and RSM experimental designs**

The RSM experimental design was used to obtain the optimum levels of glucose, casein, initial pH and temperature for highest removal efficiency of *M. aeruginosa* strain KKU-13 by KKU-A3 during a four-day incubation. Single factor experiments were conducted to identify the optimum range for each parameter. The optimum ranges of glucose, casein, initial pH and temperature were 15.0–25.0 g/L, 1.0–2.0 g/L, 6.0–10.0 and 25–35 °C, respectively. Using the data listed in Table 1, 29 experiments were designed using the BBD method, as shown in Table 4. The results from an ANOVA on the regression analysis presented in

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**Table 3** The anti-cyanobacterial activity of KKU-A3 on other cyanobacterial species

<table>
<thead>
<tr>
<th>Cyanobacterial strains</th>
<th>Anti-cyanobacterial activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anabaena</em> sp. TISTR 8076</td>
<td>–</td>
</tr>
<tr>
<td><em>Synechococcus elongatus</em> TISTR 8500</td>
<td>++ +</td>
</tr>
<tr>
<td><em>Cylindrospermum</em> sp. TISTR 8158</td>
<td>++</td>
</tr>
<tr>
<td><em>Nostoc commune</em> TISTR 8160</td>
<td>–</td>
</tr>
<tr>
<td><em>Oscillatoria</em> sp. TISTR 8491</td>
<td>+</td>
</tr>
</tbody>
</table>

The anti-cyanobacterial activity was determined by removal efficiency % after seven days of co-culture.

+++ >80% = strong anti-cyanobacterial activity; ++ >50% = moderate anti-cyanobacterial activity; + >20% = weak anti-cyanobacterial activity; – 0% = no anti-cyanobacterial activity (TISTR = Thailand Institute of Scientific and Technological Research).

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**Figure 2** Effects of different carbon sources on the anti-*Microcystis* activity of KKU-A3, as indicated by the removal efficiency percentage. The error bars represent the standard deviation of the means for the replicates.

**Figure 3** Effects of different nitrogen sources on the anti-*Microcystis* activity of KKU-A3, as indicated by the removal efficiency percentage. The error bars represent the standard deviation of the means for the replicates.
Table 5 specified that the P-value of almost all variables was significant at 0.05. Therefore, the following quadratic regression equation that provides the response value as a function of the four tested variables was obtained:

\[
\hat{Y} = 95.26 - 3.06X_1 + 3.05X_2 - 1.64X_3 + 1.68X_4 \\
- 2.80X_1X_2 + 3.34X_1X_4 - 3.14X_2X_3 - 3.16X_2X_4 \\
+ 5.11X_3X_4 - 7.29X_1^2 - 5.75X_2^2 - 9.07X_3^2 - 7.35X_4^2 
\]

(1)

where \(\hat{Y}\) is the removal efficiency of \(M. \ aeruginosa\) strain KKU-13 and \(X_1, X_2, X_3\) and \(X_4\) are the coded values of the four tested variables, i.e. glucose concentrations, casein concentrations, temperatures and pH values, respectively. The regression model (Equation (1)) fits the experimental data very well, with an \(R^2\) value of 0.9661, indicating that the equation could be used to predict the removal efficiency of strain KKU-13 with an accuracy of 97%.

The optimum conditions for the maximum removal efficiency could be obtained using the regression model (Equation (1)). According to the RSM experimental design, the optimum levels of glucose, casein, temperature and pH for maximum removal efficiency were 19.81 g/L, 2.0 g/L,
30°C and 7.8, respectively. Under these optimum conditions, the predicted maximum removal efficiency was 95%, while the experimental value was 95% in a four-day culture period under the optimum conditions, being 13% higher than that (82%) observed using the non-optimum conditions. The RSM method is appropriate for evaluating effective factors, building models to study the interactions between factors and selecting the optimum conditions of medium variables and the physicochemical parameters for the maximum removal efficiency of M. aeruginosa strain KKU-13 by KKU-A3.

The removal of M. aeruginosa KKU-13 in the bioreactor

The removal of M. aeruginosa KKU-13 in the bioreactor using plastic medium for KKU-A3 cell immobilization was performed in batch and continuous modes. In the batch experiment, the removal of M. aeruginosa began at day 1 and reached a maximum at seven days, with a maximum removal efficiency of 95% (Figure 4(a)). The removal remained consistent from days 7 to 14. In the continuous flow test, the removal efficiency of M. aeruginosa exhibited a similar pattern as the batch experiment, starting at day 1 until day 10 and remaining consistent from day 10 until day 14, with a maximum removal efficiency of 95% (Figure 4b).

**DISCUSSION**

A wide range of bacteria have been reported to exhibit algicidal activity against Microcystis aeruginosa, including Myxococcus, Cytophaga, Alcaligenes, Pseudomonas and Bacillus (Sige 2005; Shunyu et al. 2006; Mu et al. 2007). Several actinomycetes in the genus Streptomyces, that possess anti-Microcystis activity, have also been isolated from both freshwater and soil environments (Choi et al. 2005; Hua et al. 2009; Somdee et al. 2013). In this study, the bacterium KKU-A3 exhibited the strongest anti-Microcystis activity among the 28 strains that were isolated from the Bueng Nong Khot Reservoir, Khon Kaen, Thailand. Based on the physiological and biochemical characteristics and the 16S

**Table 5** | Analysis of variance (ANOVA) for the regression model

<table>
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<th>Source</th>
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<th>MS</th>
<th>F-value</th>
<th>P-value</th>
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<td>28.54</td>
<td>&lt;0.0001</td>
<td>Significant</td>
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<td>B-Casein</td>
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<td>111.87</td>
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R² = 0.9661.
R²adj = 0.9323.
rDNA sequencing analysis of strain KKU-A3, it is affiliated to the genus Streptomyces, with Streptomyces rameus as the closest species. The bacterium S. rameus has been reported to show antimicrobial activity against pathogenic bacteria and fungi (Varalakshmi et al. 2014). The bacterium exhibited high activity against Gram-positive bacteria such as Bacillus subtilis and Staphylococcus aureus and against Gram-negative bacteria such as Pseudomonas aeruginosa and the fungi Saccharomyces cerevisiae and Candida albicans but less activity against Escherichia coli, Micrococcus luteus and Klebsiella pneumoniae. However, to the best of our knowledge, there are no reports on the anti-cyanobacterial activity of S. rameus.

To assess its potential as an algicide in natural waters, the algicidal activity of strain KKU-A3 against other cyanobacteria was investigated. In this study, strain KKU-A3 exhibited anti-cyanobacterial activity against not only M. aeruginosa but also other potentially toxic cyanobacteria. Strain KKU-A3 inhibited Synecochoccus elongatus TISTR 8500, Cylindrospermum sp. TISTR 8158 and Oscillatoria sp. TISTR 8491, but not Anabaena sp. TISTR 8076 and Nostoc commune TISTR 8160. A similar result was shown by Hua et al. (2009), who reported that Streptomyces strain NT0401 inhibited M. aeruginosa and Synecocystis sp. PCC6803, but not Anabaena sp. PCC7120. Therefore, species-specific algicidal activity of bacteria may be beneficial for the selective removal of some toxic cyanobacteria in bodies of water.

Media with glucose as the carbon source and casein as the nitrogen source provided the highest anti-Microcystis activity in this study. Based on the BBD approach, the optimum levels of glucose, casein, temperature and pH that maximized the removal efficiency were 19.81 g/L, 2.0 g/L, 30 °C and 7.8, respectively, with 95% anti-Microcystis activity. A similar study was performed by Kong et al. (2014), who used RSM to optimize the medium for the production of anti-cyanobacterial substances by Streptomyces sp. HJC-D1. They found that sucrose and KNO3 were the most suitable carbon and nitrogen sources, respectively. Sucrose, KNO3 and the initial pH were the major factors that affected the anti-cyanobacterial activity of the isolated strain. The optimum culture conditions were 22.73 g/L sucrose, 0.96 g/L KNO3, and an initial pH 8.82, and the chlorophyll-a, removal efficiency by strain HJC-D1 increased from 63 ± 2% to 78 ± 2% under optimum conditions.

The removal of M. aeruginosa was previously tested in a small bioreactor. A fluidized bed bioreactor with a 6 L working volume was used to remove Microcystis cells with high density of the microanimals Monas guttula, Aeolosoma hemprichi and Philodina erythrophthalma (Iwami et al. 2000). The bioreactor reduced the viable Microcystis cells by 56% at a HRT of 8 h. In this study, a 10 L IAL reactor bioreactor was examined for M. aeruginosa removal. Previously, this type of bioreactor was effectively used to remove various pollutants, including 2,4-dichlorophenol and phenol, high carbohydrates, printing ink, and quinoline, from wastewater (Zhang et al. 2002a, 2002b, 2005; Quan et al. 2004). A bioreactor containing plastic medium for KKU-A3 cell immobilization reduced the number of viable Microcystis cells at a HRT of 8 h, with a M. aeruginosa removal efficiency above 95% at seven and ten days in the batch and continuous flow experiments, respectively. From all of these results, it appears that the removal of M. aeruginosa KKU-13 in a bioreactor (in which the KKU-A3 cells were immobilized onto plastic medium) is an effective and promising process for the removal of M. aeruginosa and other potentially toxic cyanobacteria.

![Figure 4](https://iwaponline.com/wst/article-pdf/74/6/1398/458353/wst074061398.pdf)
The application use of the IAL bioreactor technology for large scale water treatment plant may be adapted as follows. (1) A flat water-flow concrete tank (approximately 1 m wide × 10 m long × 1 m deep) used as the bioreactor. (2) Large plastic medium, as the bacterial carrier support. (3) KKU-A3 used directly in the form of freeze-dried material (cells), or by suspending it in the modified starch casein medium broth. The bacterial cells would be immobilized onto the plastic medium by soaking them together at ambient temperature for five to seven days, depending on how biofilm formation progresses (in controlled laboratory conditions, KKU-A3 was incubated at 30 °C for four days). (4) A flow-through principle used to expose water to KKU-A3 isolate. (5) Electronic operation of inflow-outflow of water in the bioreactor (concrete tank) to maintain HRT in place of peristaltic pump. However, pilot studies of proposed design would need to be carried out to obtain preliminary data on performance, and improve efficiency of the bioreactor for bigger scale water treatment. In addition, the measurement of cyanobacterial MCs in the bioreactor after reactor for bigger scale water treatment. In addition, the measurement of cyanobacterial MCs in the bioreactor after

ACKNOWLEDGEMENTS

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