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RESEARCH ARTICLE

Optimal Conditions for the Treatment of Shrimp Culture Effluent Using Immobilized Marine Microalga *Picochlorum maculatum* (PSDK01)

S. Dinesh Kumar[1,2] · P. Santhanam[1] · P. Prabhavathi[3] · B. Kanimozhi[4] · M. Abirami[4] · Min S. Park[5] · Mi-Kyung Kim[2]

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Abstract A significant environmental concern has been raised over the wastewater produced from aquaculture including shrimp farms. In order to evaluate the potential of microalgae to treat the wastewater from a shrimp aquaculture, response surface methodology (RSM) was applied to identify optimal conditions for various parameters. *Picochlorum maculatum* immobilized beads were used to remove excessive nutrients (phosphate, nitrate, nitrite and ammonia) from a 90 days old shrimp (*Litopenaeus vannamei*) cultured wastewater. The effects of number of algal cells per bead, density of beads per given volume of wastewater, pH, and retention time were investigated. A significant maximum nutrient removal was obtained at pH 7, 24 h of retention time, 150 beads of density and 111,200 cells/ml of algal cell concentration. The primary experimental results were used to RSM for optimizing the variables statistically for maximum nutrient removal. A 'minimum run resolution V' central composite design with four variables (pH and retention time, different bead density and algal cell concentrations in beads) was applied to optimize the process. The results showed good fits with the proposed statistical model for the removal of nutrients.

Keywords Wastewater treatment · Shrimp culture · Beads · Immobilization · *Picochlorum maculatum* · Response surface methodology

Introduction

High concentrations of nitrogen and phosphorus in wastewater are the main cause for eutrophication of estuaries and seas. It is imperative to remove these substances from wastewater in order to maintain water quality in the environment [1, 2]. Removal of nutrients from wastewater using physical and chemical methods is highly expensive and energy consuming. However, use of biological systems serves as a cheap and efficient method of nutrient removal from wastewater [3–6]. In recent decade, microalgae have received more attention, especially in the tropical and subtropical regions, as an alternative biological system for wastewater treatment [6–8]. One of the major issues in using microalgae for wastewater treatment is their recovery from the treated wastewater. Immobilization technology, which entraps the microalgal cells into a matrix, solves the harvest problem. This technology can offer operational flexibility and easy separation of algae from the treated wastewater. Moreover authors reported that higher nutrient removals were achieved in immobilized algal matrix than suspension culture of algal species [4, 9].

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✉ P. Santhanam
sanplankton@yahoo.co.in; santhanamcopepod@gmail.com

[1] Marine Planktonology and Aquaculture Laboratory, Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu 620 024, India

[2] MCK Biotech Co. Ltd, Daegu R&D Fusion Center, Daegu 704 948, South Korea

[3] Department of Microbiology, Nadar Saraswathi College of Arts and Science, Theni, Tamil Nadu 625 531, India

[4] PG and Research Department of Microbiology, PSG College of Arts and Science, Coimbatore 641014, Tamil Nadu, India

[5] Center for Microalgal Technology and Biofuels, Institute of Hydrobiology, Chinese Academy of Science, Wuhan 430072, China

As the result of rapid growth of aquaculture industry including shrimp farms throughout Southeast Asian countries such as Thailand and India, water quality has been detraining due to the incompletely treated wastewater (culture water) discharged from these farms. In order to investigate the potential of algae to treat wastewater from shrimp farms, the authors tested various parameters that might affect the applicability of immobilized microalgae for nutrient-removal from the waste water. Effective removal of nutrients using immobilized algal system is dependent on various factors such as algal species, type of matrix for immobilization, pH, treatment time or hydraulic retention time (HRT), algal cell density, bead concentrations and the degree of aeration.

Response surface methodology (RSM) has an important application in the process design and optimization as well as the improvement of an existing design. In last few years, RSM has been used to optimize and evaluate the interactive effects of independent factors in numerous chemical and biological wastewater treatment such as advanced treatment of olive oil processing wastewater using Fenton's peroxidation [10] and, acidogenesis of cheese-whey wastewater to acetic and butyric acids [11], powdered activated carbon augmented activated sludge process for the treatment of semi-aerobic landfill leachate [12], Fenton and photo-Fenton treatment of distillery wastewater and optimization of treatment conditions [13].

Several workers attempted removal of nutrients from various wastewaters using fresh water microalgal beads [6, 14–17]. However, very few attempts have been made using marine microalgae for wastewater treatment [4, 7, 17]. Therefore, the present study was undertaken for estimating removal efficiency of nutrients (phosphate, nitrate, nitrite and ammonia) from wastewater from a shrimp farm by using immobilized marine microalgae *Picochlorum maculatum*. *Picochlorum maculatum* (*Nannochloris maculata* Butcher 1952) is a group of marine microalgae and their size varies between 1.5 and 3 microns diameter. Zeaxanthin and Canthaxanthin are most dominant pigments in *P. maculatum* [18]. It has been investigated independent variables of pH, retention time, beads density and algal cell concentration in beads using response surface analysis and presented the results from comprehensive process modeling efforts.

Material and Methods

Collection and Composition of Wastewater

The wastewater was collected from an individual shrimp (*Litopenaeus vannamei*) culture pond located at Ennoor, Chennai, Tamil Nadu, India (13°19′43″N; 80°19′54″E) and transported to the laboratory. At laboratory, wastewater was kept undisturbed to settle the suspended particles. The composition of effluent was analyzed according to standard methods [19–21].

Immobilization of Microalgae

The isolation and cultivation methods for marine microalgae *P. maculatum* were previously described [5]. Microalga *P. maculatum* was harvested during the exponential phase by using Milliphore filtering system (Model No. X10422050, 50 Hz) and were immobilized according to Santos et al. [22] with minor modifications in respect to alginate and cation solution ($CaCl_2$) concentrations. The total volume of alginate mixture was 100 mL (70 mL alginate + 30 mL NaCl mixture). Sodium chloride was included since saline microalgae used for immobilization treatment. In brief, 3 g sodium alginate (sodium alginate is a natural polysaccharide product extracted from brown seaweed) was first dissolved by slow stirring in 70 ml of distilled water. In the remaining 30 ml of distilled water, 3.5 g sodium chloride was dissolved to obtain a 35 psu salinity final solution. When the alginate was completely dissolved, the two parts were mixed using a magnetic stirrer. After preparation of alginate mixture, the equal amounts of centrifuged algae were added to the mixture and the mixture has been added drop-wise into the cation solution. While alginate algae mixture added to the cation solution, the thin layer has been formed around the alginate algae mixture. The cation solutions (2 g $CaCl_2$ to 100 mL of nanopure water) were prepared in nanopure water. The beads were prepared by adding algae alginate mixture drop-wise to the cation solution ($CaCl_2$) by using 20 ml syringe (0.8 mm × 40 mm needle; Braun, Melsungen, Germany) from a height of approximately 15 cm and at a rate of approximately one drop per second. The distance between syringe tip and cation solution determined the beads size. After forming of beads, the beads were kept stirring in the cation solution for 45 min to allow the complete hardening of the alginate, and washed three times with filtered (0.45 μm) natural seawater to eliminate the remaining cation solution.

Experimental Variables

In order to find out the effect of pH and retention time, the different pH levels from 1 to 10 and retention time viz., 4, 8, 12, 16, 20 and 24 h on biosorption were maintained. The pH of the wastewater was adjusted to the desired value using 0.1 N HCl or 1 N NaOH. The effect of bead-density on the nutrient removal was studied by employing 50, 100, 150, 200, 250, 300, 350 and 400 beads. The algal cells were suspended in 50 ml deionized water to produce various concentrations of microalgae of 21,500, 35,300, 49,200, 65,700, 81,400, 92,600, 111,200, 131,800, 169,700, 210,400, 262,400, and 286,600 cells/ml. All experiments were performed in triplicates.

Experimental Setup

The experiments were conducted in 1000 ml conical flask filled with 500 ml of shrimp cultured wastewater. Immobilized microalgal beads were added to culture flasks and water sampling was made every 4 h for analyses. After experiments, wastewater was separated from the immobilized microalgal beads through filter paper. The amounts of nutrients in the samples were measured spectrophotometrically using Shimadzu Model-2450 as per the standard protocol [19–21].

RSM Design

A design of experiment (DOE) statistical software (Design-Expert version 9, Stat-Ease, Minneapolis, MN) was employed to design statistically the smallest number of experiments using ‘‘minimum run resolution V Design (Min–Run Characterize)’’ design. The response surface design was preferred to carry out removal of nutrients (phosphate, nitrate, nitrite and ammonia). Four operating factors were selected as independent variables and the interaction of variables on nutrient removal was evaluated using a limited number of designed experiments by MinRes V [23]. It is an optimal design which predicts the possible effects and interactions of the four chosen factors with a minimum number of experimental runs.

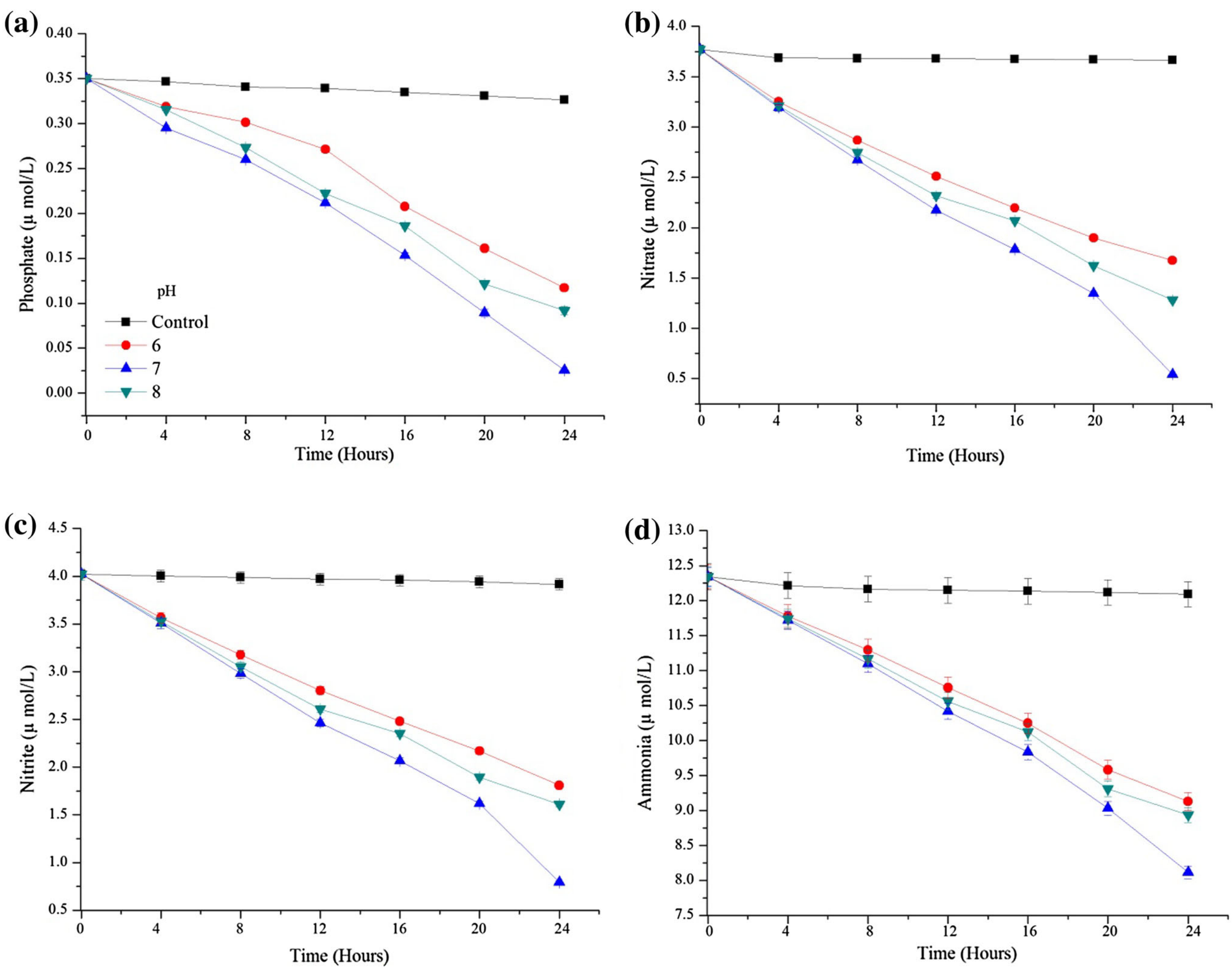

Fig. 1 Effect of pH and retention time on **a** phosphate removal, **b** nitrate removal, **c** nitrite removal, and **d** ammonia removal

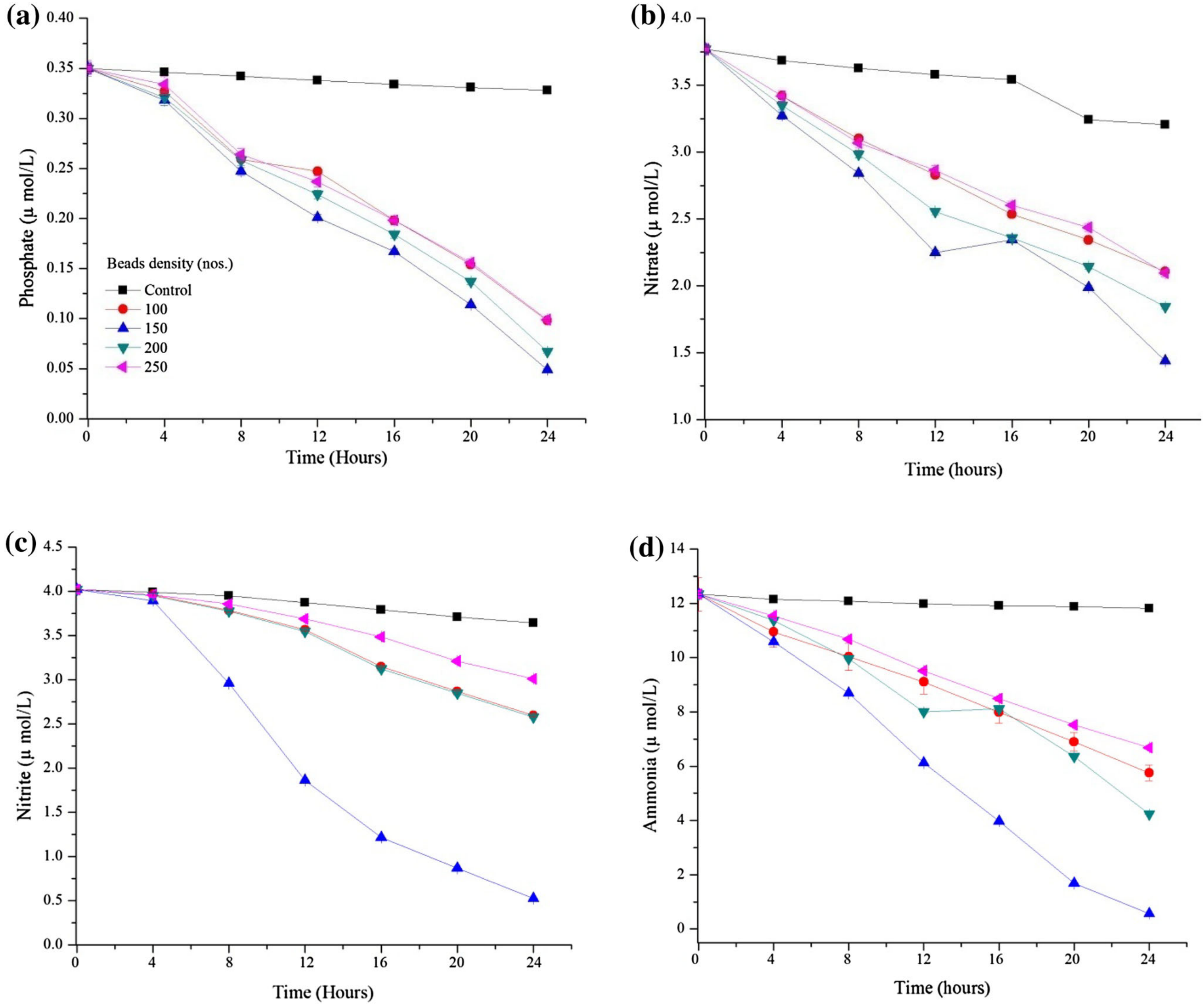

Fig. 2 Effect of beads density on **a** phosphate removal, **b** nitrate removal, **c** nitrite removal, and **d** ammonia removal

Results and Discussion

Effect of pH, Retention Time, Bead-Density and Algal Cell Density on Nutrient Removal

The recorded initial concentrations of physico-chemical parameters viz., pH, salinity, dissolved oxygen, phosphate, nitrate, nitrite, ammonia, silicate, total nitrogen and total phosphorus in 90 days shrimp (*L. vannamei*) cultured wastewater were 7.8, 31 psu, 5.38 mg/L^{-1}, 0.35, 3.77, 4.02, 12.34, 20.18, 19.59, 0.53 µmol/L^{-1} respectively. Immobilized *Picochlorum maculatum* beads were efficient in removing nutrients from aquaculture wastewater suggesting that microalgae-imbedded bead can be used to remove nutrients in excess in waste water from shrimp farm. The significant decrease in the concentration of PO_4^{3-}, NO_3^-, NO_2^- and NH_3 was observed in the pH, ranging from 1 to 10 and time ranging from 4 to 24 h (Fig. 1a–d) (detailed results were showed in supplementary files). The maximum phosphate, nitrate, nitrite and ammonia removal that was obtained at pH 7 at 24 h were 0.33, 3.23, 3.22 and 4.23 µmol L^{-1}, respectively. The efficiency of removal (%) was 92.8, 85.6, 80.3, and 34.2 for phosphate, nitrate, nitrite and ammonia, respectively. The minimum reduction of nutrients (PO_4^{3-}, NO_3^-, NO_2^- and NH_3) was observed at pH 1 during 24 h retention period, where the concentrations were 0.02, 0.10, 0.11 and 0.25 µmol L^{-1} respectively. Figure 1a–d shows that nutrient removal was pH-dependent and was optimal at neutral pH range, while it was negatively affected by either acidity or alkalinity. The pH dependence of nutrient uptake can be largely related to the functional groups that are present in algae and also on the nutrients and metallic chemistry in solution [24]. At low pH, the concentration of

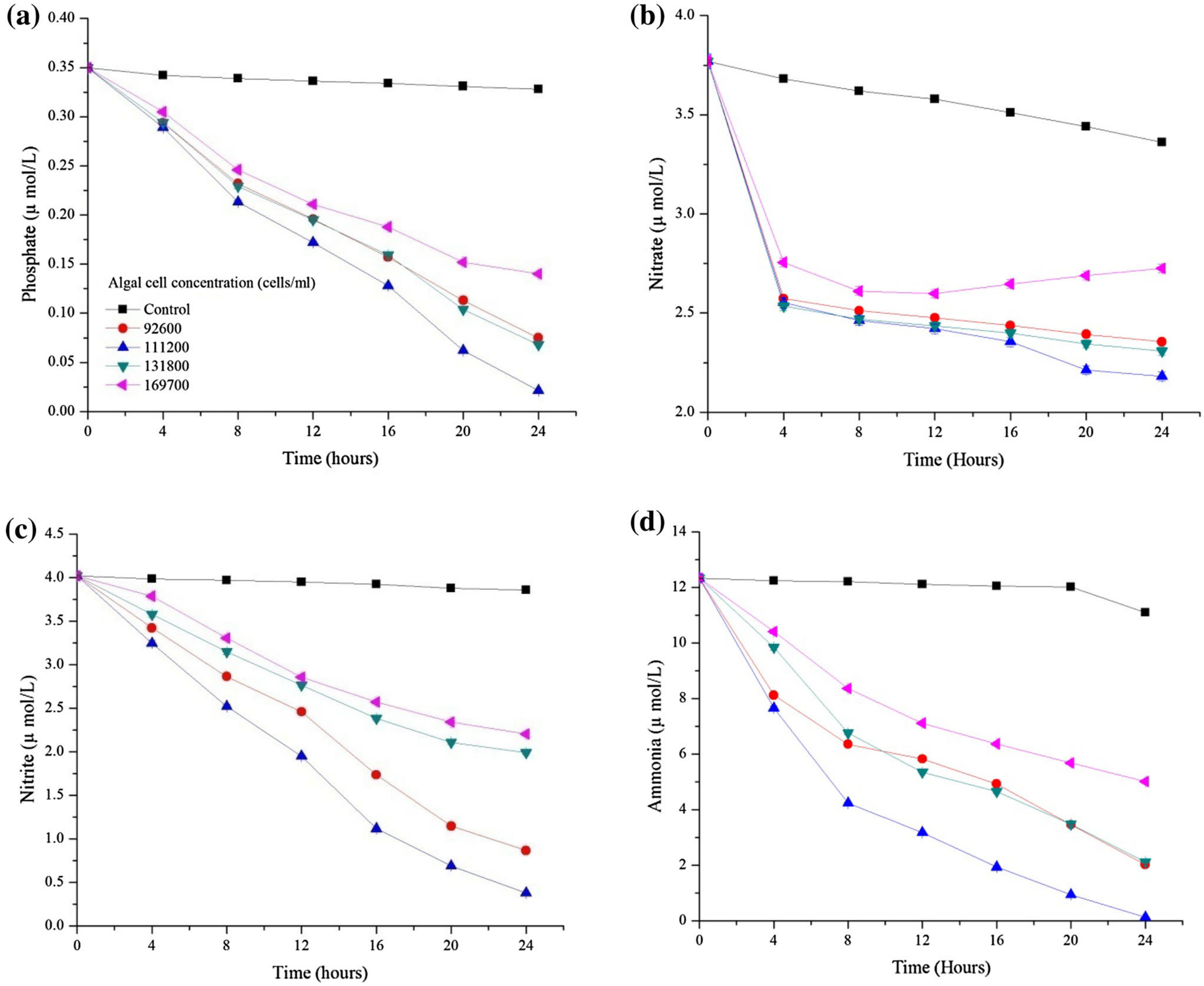

Fig. 3 Effect of algal cell density in beads on **a** phosphate removal, **b** nitrate removal, **c** nitrite removal, and **d** ammonia removal

the hydrogen protons in the solution far exceeds that of ions and hence, these protons competes with these nutrient and metal ions by forming a bond in the active sites (functional groups) on the surface of the algae, leaving the metal ions free in solution. These bonded active sites thereafter become saturated and thus are inaccessible to other cations. When the pH was increased, the competing effect of hydrogen protons decreased and the positively charged metal ions took up the free binding site [7].

In the present experiment, the effect of bead-densities (50, 100, 150, 200, 250, 300, 350 and 400) was also studied for nutrient removal. Results showed a significant reduction of nutrients from the shrimp farm wastewater (Fig. 2a–d) (see supplementary files for detailed results). The removal of nutrients (PO_4^{3-}, NO_3^-, NO_2^- and NH_3) was maximum in the experiment with the bead-density of 150 after 24 h-retention time and the removal efficiency was 86, 62, 87, and 94%, for PO_4^{3-}, NO_3^-, NO_2^- and NH_3, respectively. The concentrations of nutrients reduced from 0.35 to 0.049 µmol L^{-1} (phosphate), 3.77–1.44 µmol L^{-1} (nitrate), 4.02–0.52 µmol L^{-1} (nitrite) and 12.34–0.57 µmol L^{-1} (ammonia) when 150 numbers of beads were maintained. Regarding the effect of beads density on biosorption, moderate number of beads (150 beads) showed good biosorption when compared to both high and low density of beads (Fig. 2a–d). Higher density of beads in treatment reactor resulted in a significantly low nutrient removal. Higher density of beads would shrink the penetration of light through the reactor, and enhance the self-shading effects, which might have limited the metabolic activities of algal cells. Due to heavy weight of high density of beads (200, 250, 300, 350 and 400 numbers), the beads were settled in bottom. Under this condition, the supplied air was not sufficient to completely suspend all of the beads in the reactor. As a result, low nutrient removal was observed as have been previously observed

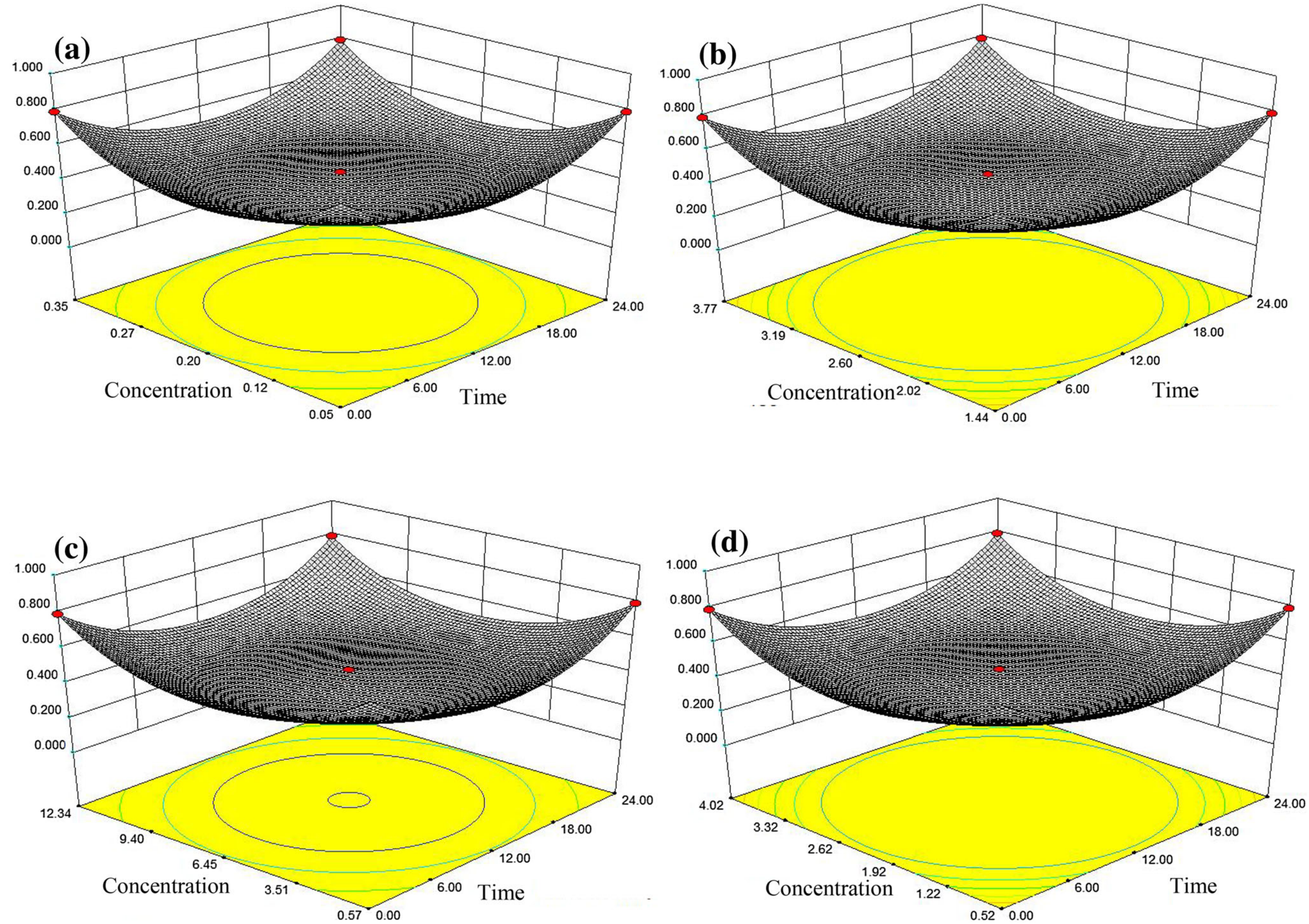

Fig. 4 Response surface methodology (RSM) model for **a** phosphate removal, **b** nitrate removal, **c** nitrite removal, and **d** ammonia removal

[6, 7, 16, 25, 26]. Pakshirajan and Singh [27] also stated that higher stocking density decreases the biosorption due to cell loading in gel which weakens the membrane of gel [28].

The influence of algal cell density per bead (21,500, 35,300, 49,200, 65,700, 81,400, 92,600, 111,200, 131,800, 169,700, 210,400, 262,400, and 286,600 cells/ml) on removal of nutrients is depicted in Fig. 3a–d (see supplementary files for detailed results). As expected, removal of nutrients was dependent on the number of algae per individual bead, although the removal kinetics was different among types of nutrients. Phosphate and nitrite were removed in similar manner (Fig. 3a, c), while nitrate and ammonia were removed rapidly in the early hours of incubation. The recorded removal of nutrients were 94% (PO_4^{3-}), 42% (NO_3^-), 90% (NO_2^-), and 99% (NH_3) achieved after 24 h of retention time in 111,200 cells/ml algal cell concentrations. The beads without algae (control) had resulted in lower amount of adsorption of nutrients (PO_4^{3-}-6.28%; NO_3^--10.88%, NO_2^--3.98%, NH_3-10.06%) as compared to the treatment beads (beads with algae). Phosphate (0.35–0.021 μmol L^{-1}), nitrate (3.77–2.18 μmol L^{-1}), nitrite (4.02–0.37 μmol L^{-1}) and ammonia (12.34–0.11 μmol L^{-1}) were effectively removed in the presence of algae at various densities. The influence of algal cell density in beads on removal of nutrients from wastewater (Fig. 3a–d), moderate algal cell concentration (111,200 cells/ml) resulted in higher nutrient removal (94% of PO_4^{3-}, 42% of NO_3^-, 90% of NO_2^-, 99% of NH_3) as compared to high (131,800, 169,700, 210,400, 262,400, 286,600 cells/ml) and low algal cell concentrations (21,500, 35,300, 49,200, 65,700, 81,400, 92,600 cells/ml). Increasing the algal cell concentrations in beads appeared to reduce their efficiency in nutrient removal and caused cell leakage. The algal cell leakage was monitored in the treatment reactor during each sampling period by counting the cell number in 10 ml culture medium. Earlier researchers [7, 14, 16] have seen that, increasing algal cells concentration in beads did not improve nutrient removal and this might be due to a significant algae leakage from the beads when too many cells were used to create imbedded-beads [14, 29]. It could be also due to the limited diffusion of nutrients when the algal cell density is high [30]. The same microalgae species were

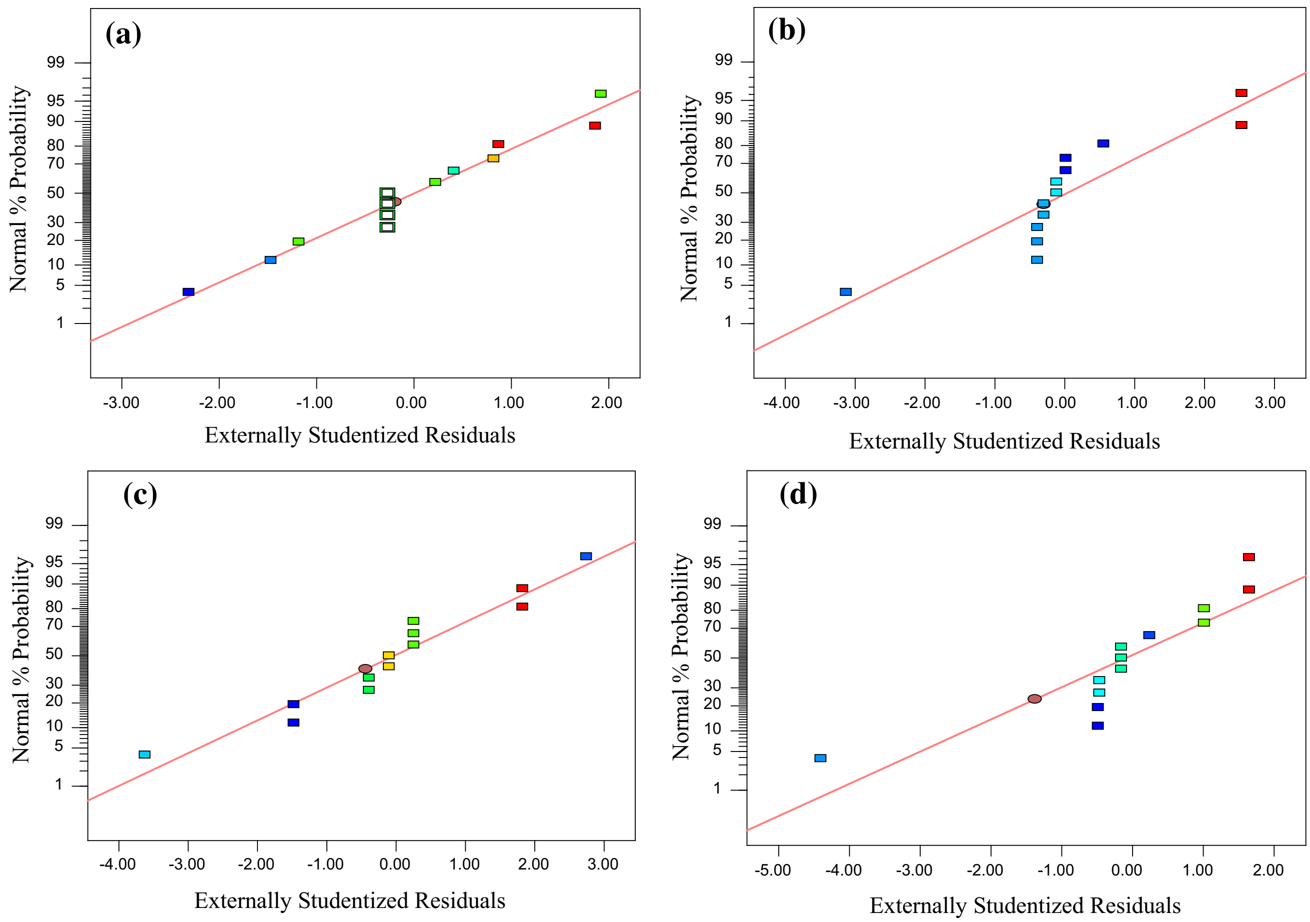

Fig. 5 Normal probability plot of residual for **a** phosphate removal, **b** nitrate removal, **c** nitrite removal, and **d** ammonia removal

used for nutrient elimination and the removal rate was lower (PO_4^{3-}-89.08%, NO_2^--56.66%, NH_3-72.94%) than the present investigation except for nitrate (59.33%) while using the mobilized form [31]. While immobilized algal beads are used for the removal of nutrients in wastewater, phosphate and ammonia are adsorbed to the surface of beads and then penetrate slowly into alginate beads and continuously absorbed by algal cells [32]. As it has been demonstrated by de-Bashan et al. [15]. It has been also observed that moderate algal cell concentration (111,200 cells/ml) was effective in nutrient-removal from the wastewater from shrimp aquaculture.

Response Surface Methodology

All experimental data were obtained from 13-individual-experiments and the predicted data from a response surface analysis model (results were given in supplementary Table 1) and analyses of variance with reference to pH, density of beads and algal cell concentration results were analyzed (results were given in supplementary Table 2, 3 and 4). The model F-value of all treatments is less than 4 ($F < 4.00$) indicating that the model is significant except nitrate and nitrite with reference to pH; and phosphate and nitrate with reference to density of beads. While values of ''Prob > F'' >0.05 indicate that model terms are insignificant. In all responses, B–B is significant model term. The highest lack of fit F-value of 19.10 implies that the lack of fit is significant. To investigate the significant interactive effects of different variables on nutrient removal (µmol L^{-1}), the three-dimensional profiles of multiple nonlinear regression models were made and results were shown in Fig. 4a–d. Analysis on the normal probability plot (Fig. 5a–d) of the residual showed a rather straight line residual distributions, which refer that error are evenly scattered and hence support adequacy of the model. In order to quantify the curvature effects, the data from the experiments were fitted to higher degree polynomial equations, i.e. four factor interaction (4FI) and quadratic equations. In the design expert software, the response data were analyzed by default. The model terms in the equations are those that remained after the elimination of insignificant variables and their interactions. Based on the statistical analysis, the models were highly significant with very low probability values (<0.0001). It was shown that the model terms of independent variables were significant at

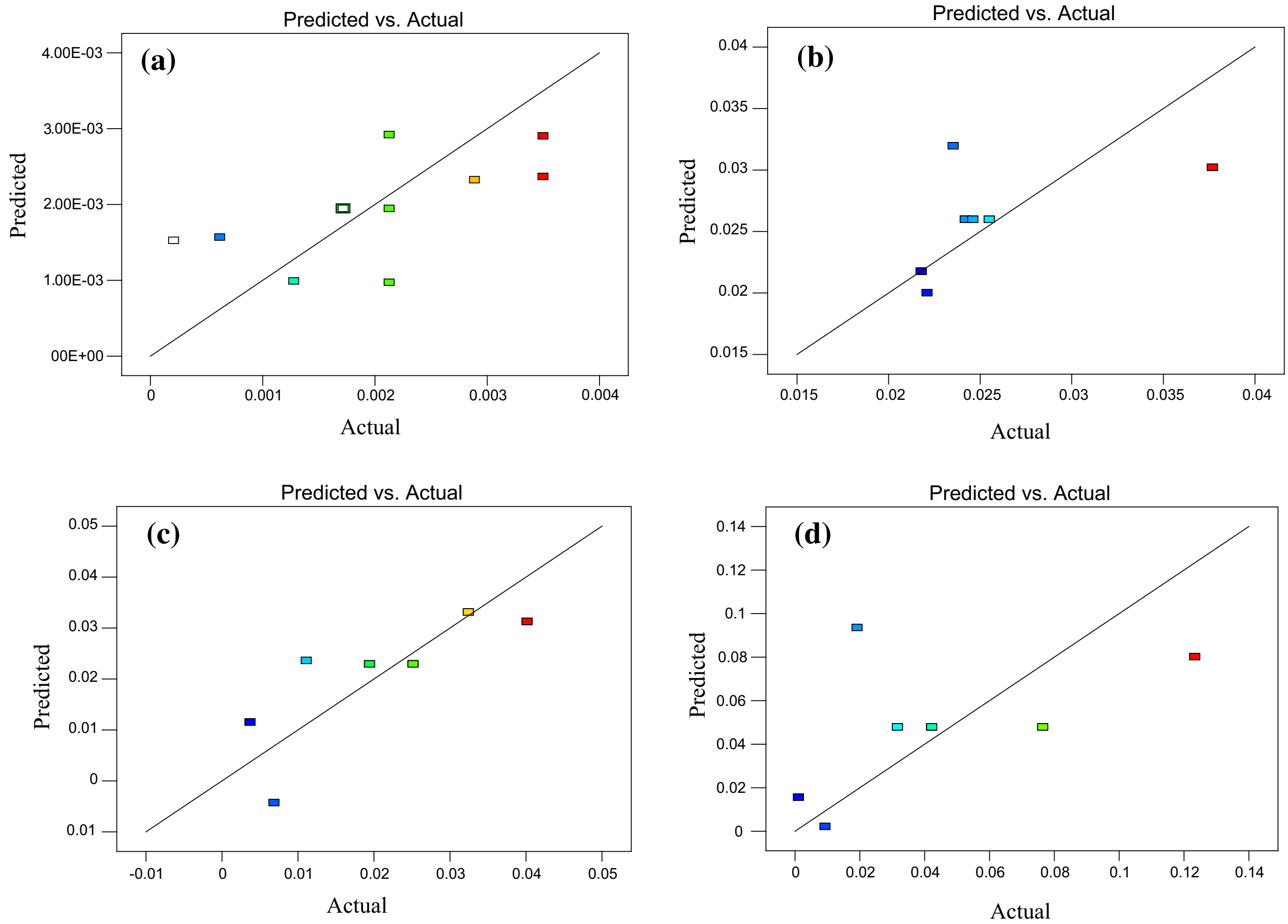

Fig. 6 Design-expert plot; predicted versus actual values plot for **a** phosphate removal, **b** nitrate removal, **c** nitrite removal, and **d** ammonia removal

the 99% confidence level. The square of the correlation coefficient for each response was computed as the coefficient of determination (R^2) at 95% confidence level. The predicted values were in very good agreement with the experimental values, as shown in Fig. 6a–d. Hence, the quadratic model appears to be well suited for this experimental set up. By carrying out parameter optimization based on the built mathematical models, the present work indicated that the majority of nutrients in the wastewater of shrimp farm can be removed to the minimum concentrations of nutrients (μmol L^{-1}) like phosphate-1.52, nitrate-0.022, nitrite-0.011 and ammonia-0.015, which are equivalent to the percentage removal of 93.4, 99.4, 97.2 and 99.8%, respectively.

Conclusion

The present findings revealed that *Picochlorum maculatum* immobilized in alginate beads can be used to remove nutrients in excess from the wastewater from a shrimp farm (control). Further, the authors also observed that pH 7, retention time of 24 h, density of beads 150, and 111,200 cells/ml of algal cell concentration per bead were found to be optimum for maximum removal of nutrients from the wastewater. RSM results demonstrated the effects of operating variables as well as their interactive effects on the response. Therefore, pH, retention time, density of beads and algal cell concentration in beads appeared to be some of the important parameters for immobilized microalgae-based wastewater treatment.

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Compliance with Ethical Standards

Conflict of interest There is no conflict of interest among the authors for publishing this manuscript.

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