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Optimization of Aquaculture Wastewater Bioremediation and Harvesting Utilizing Response Surface Methodology (RSM)

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Microalgae are known as microorganisms which is highly concentrated with chlorophyll making them very effective bio-absorbents in treating inorganic nutrients present in the wastewater. The current study was initiated by utilizing biological-based treatment approach using microalgae, *Chlorella* sp. bioremediation to treat aquaculture wastewater. Bioremediation proceeds with various different pH of *Chlorella* sp. (pH 5- pH 10) of wastewater. While regular biomass harvesting is required for maintaining suitable biomass density and effective nutrient recycling. This study proceeded with optimization by using response surface methodology (RSM) on the evaluation of harvesting using bio-flocculant. This study proposed filamentous fungus, *Aspergillus niger* as sustainable bio-flocculant for that purpose. In bioremediation, the optimum inoculation dosage was recorded at 30 vol% and pH 7 with effluent concentration of ammonia 0.014 mg L^{-1} at Day 10. Optimum dosage, pH and mixing rate for bio-flocculation process was achieved at 30 mg L^{-1}, pH 7 and rpm 125 with 97.2 % harvesting efficiency. The development of bioremediation with bio-harvesting would promote sustainable green technology for effective wastewater treatment.

1. Introduction

Aquaculture contributes to the global food supply and main source of animal protein. Aquaculture production was forecast of 93 Mt of production by 2030 which up from 74 million in 2014 (Kobayashi et al., 2015). Based on the projected model, the largest expansion is expected in India, Latin America, Caribbean and Southeast Asia. However, rapid development and growth of intensive aquaculture industry requires a continuous management to protect adversely negative impacts towards the environment. Normally, wastewater discharged from aquaculture industries classified into organic matter and nitrogenous compounds such as ammonia, nitrite and nitrate, phosphorus and dissolved organic carbon. The untreated nutrient content of the wastewater may contribute to the environmental impact such as eutrophication and deterioration of the natural coastal ecosystem. There are various physical and chemical technologies used for wastewater treatment including the de-nitrification process to remove nitrogenous compounds by releasing them to the atmosphere (Mook et al., 2012). However, this method is not environmentally friendly since it produced toxic sludge as a by-product that will affect human health. Proper wastewater treatment is desired to mitigate uncontrolled pollution and environmental impacts before it is discharged into the water bodies while sustaining the development of aquaculture industry. Terrestrial plants were used in aquaponics system as pytoremediation agent (Li et al., 2019). However, it was continuously replaced by unicellular plants such as microalgae and known as phycoremediation. Phycoremediation refers to the use of green plants for bio-remediate contaminants in the wastewater and the emerging technology that offers an environmentally safe, cost-effective and non-intrusive technique (Ajala and Alexander, 2020; Lam et al., 2017). Recent studies reported microalgae as promising alternative in the phycoremediation of wastewater (Ajala and Alexander, 2020; Lam et al., 2017). During phycoremediation treatment, microalgae remove nitrogen and phosphorus via utilizing nutrients for their growth without using chemical and additional mixing requirements, and offers a cost-effective solution for wastewater treatment (Lananan et al., 2014).

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Numerous authors have reported that the microalgae biomass released of absorbed nutrients to the water bodies as entering the declining growth phase (Nasir et al., 2015). However, the sustainable harvesting technique is required to control biomass density of the microalgae. The most difficult step in microalgae cultivation and utilization is the harvesting process. They are obstacles to harvest microalgae cells since it has specific characteristics for example low density (range of 0 – 5 g L^{-1}) and small size (the range 3 – 30 µm diameter) (Molina Grima et al., 2003). There are several commercialized harvesting techniques such as centrifugation, flotation, filtration and coagulation-flocculation (Laamanen et al., 2016; Phoochinda and White, 2003). Among those, coagulation-flocculation is one of the most appropriate and low-cost methods for harvesting microalgae. Relatively, the potential of filamentous fungus isolated from aquaculture wastewater namely *Aspergillus niger* (*A.niger*) in harvesting microalgae biomass density was investigated. Filamentous fungus is known as grown in dispersed mycelia, clumps and form pellets in submerged culture. On top of that, the molecularly sticky hyphae of fungi in pellet form are able to attach and entrap microalgae cells in the harvesting process. Successful use *A.niger* as bio-flocculant would develop low-energy and chemical-free for future applications for microalgae harvesting and high mass production of biofuels from microalgae based (Pradana et al., 2017). Realizing the importance of *Chlorella* sp. as biological agent for bioremediation and *A.niger* as bio-flocculant in harvesting technique, efforts were made to find an optimum condition for maximizing the production with highest reduction of nutrient as well as highest harvesting efficiency by using response Surface Methodology (RSM). In addition, RSM can reduce required time and effort to differentiate the interaction effects between those individual factors compared to conventional methods which may investigate one independent factor with other factors fixed at one time (Jeong and Park, 2006). However, the limitations of this technique lie when too high nutrient content in wastewater and the various concentration microalgae for harvesting that do not examine by this approach. This research provides a solid background for future application of this technology for microalgae harvesting.

2. Methodology

2.1 Microalgae cultivation and bioremediation

Green microalgae, *Chlorella* sp. was isolated and incubated at a constant temperature of 20 ± 2 °C and maintained at pH of 6.9 ± 2 under continuous illumination. The microalgae were cultivated in Bold's Basal Medium (BBM) until reached early stationary and maturity phase of growth before undergoing the phycoremediation. Chlorophyll-a (Chl-a) analysis was used as an indirect method to determine microalgae concentration in water sample throughout the phycoremediation. This method was applied based on chl-a analysis of microalgae carried out by (Method No. 10200 Section F) from Standard Method for the Examination of Water and Wastewater (APHA, 1997). Water quality testing is an integral part of environmental monitoring in aquaculture industry and also highlighted in this study. The water quality analyses were carried out every 2 d collection of 50 mL water samples from each treatment within 14 d treatment period. The water sample was centrifuged at 6000 rpm for 10 min by Eppendorf 5702 Variable Speed Multi-Purpose Centrifuge to obtain a clear supernatant. The supernatant was analyzed to monitor the ammonia concentration using a Dual-Beam UV-Vis spectrophotometer. Ammonia determination were carried out in accordance standard methods, Phenate method based on APHA (1997).

2.2 Fungus cultivation and harvesting

The second phase of this study was performed on harvesting freshwater microalgae, *Chlorella* sp. by using filamentous fungus, *A.niger*. The *A.niger* was isolated and allowed to grow on the potato dextrose agar (PDA). Potato dextrose broth (PDB) growth medium was used for fermentation purposes and *A.niger* was cultivated for 48 h at a constant temperature of 30 ± 2 °C in an incubator shaker (Lab Companion SI-600, Korea) with constant agitation speed. The formation of spherical pellets with relatively homogenous size could be observed after two days of cultivation period. The formation of pellets was prepared based on method reported by Nasir et al. (2019). The fungus was prepared in pellet form for flocculating microalgae for harvesting process. Prior to run for optimization harvesting using RSM, the effects of flocculation parameters, namely bio-flocculant concentration, pH and mixing rate were individually experimented by analyzing bio-flocculation efficiency.

2.3 Experimental design for evaluation using RSM

With reference from primary investigations, the algal biomass production process was optimized by applying the response surface methodology. This investigation involved the utilization of Central Composite Design (CCD) of RSM and values for bio-flocculation parameters were fixed. CCD was experimented to optimize the three variables that significantly influenced the bio-flocculation process.

2.4 Evaluation for harvesting

The experimental was designed and statistically analyzed using Design Expert Software. The three independent five levels (-α, -1, 0, +1, +α) with 20 experimental runs were performed. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan concerning their values in actual form are listed in Table 1. The harvesting efficiency was used as dependent variable or response (Y).

Table 1: Levels of the three independent variables (factors) used in RSM.

Variables	Units	Range of levels				
		-α	-1	0	+1	+α
Dosage (concentration)	g L^{-1}	21.59	25	30	35	38.41
pH	-	5.32	6	7	8	8.68
Mixing rate	rpm	82.96	100	125	150	167.04

2.5 Statistical analysis and modelling

All experiments in this study were conducted in triplicate. Besides, the process with two independent variables for bioremediation and three independent variables for harvesting were evaluated using one factor at a time and statistically optimized by Response Surface Methodology (RSM). The experimental data obtained was subjected to analysis one-way of variance (ANOVA), appropriate to the design of experiments. In order to ascertain the observation of variation in growth rates, nutrient removal and harvesting efficiency were statistically significant, the probability (p) values were determined. 95 % confidence level ($p \leq 0.05$) was applied for all analyses.

3. Results and discussion

Based on the results of 'one-at-a-time' method in harvesting, the influencing factors for high yield and quality of harvesting using *A.niger* were concentrations of bio-flocculant, pH and mixing rate. To achieve the maximum harvesting efficiency, interaction of these factors was studied at constant temperature (27 ± 0.2 °C) throughout the experiment. Before proceeded to harvesting process, optimum conditions for microalgae growth in aquaculture wastewater were determined.

3.1 Microalgae, *Chlorella sp.* bioremediation varying pH

Bioremediation treatment utilizing *Chlorella* sp. was carried out in batch culture with six different pH 5, pH 6, pH 7, pH 8, pH 9 and pH 10 with fixed microalgae concentration, 30 vol% based on previous study reported by Nasir et al. (2015). Table 2 shows the performance of phycoremediation on aquaculture wastewater within 14 d treatment period. Reduction pattern of ammonia concentration steadily observed from Day 2 until *Chlorella* sp. reached its stationary and decline phases for all treatment. After 10 d treatment period, the nutrients concentration achieved the lowest value. It is suggested that the treatment period of 10 d is sufficient to achieve the maximum nutrient reduction of up to 98 % from its initial concentration. The initial concentration of ammonia for all treatment was maintained at 1.0 ± 0.05 mg L^{-1}. The first two days which is early stage of each treatment showed the steadily decreased in ammonia concentration after the *Chlorella* sp. inoculation. As shown in Table 2, the ammonia concentration reduced gradually until Day 6 for all inoculations except for pH 5. The ammonia concentration in pH 5 was fluctuated throughout the treatment period. pH 7 had achieved a faster rate of ammonia reduction at Day 6 which remove about 95 % from the water sample. As compared to other treatments, pH 8 and pH 9 also showed higher percentages removal, at Day 12 yielding 95 % and 81 %, but requires a significantly longer treatment period. The ammonia concentration was reduced because of nitrification process where ammonia was converted to nitrite and nitrate by oxidizing bacteria (Ruiz et al., 2003).

Figure 1 shows the effect of nutrients in aquaculture wastewater on the growth of microalgae, *Chlorella* sp.. Similar growth patterns were observed for all treatment with a relatively long lag phase, followed by the exponential phase in the first six days. It was observed that the death phase began on Day 10 towards the end of the treatment period. As shown in Figure 1, the microalgae exhibit lag phase of two to four days and the cell concentration were increased about two-folds of the initial biomass density. A few days of lag phase indicated that *Chlorella* sp. adapts well to the aquaculture wastewater. Rolfe et al. (2012) reported microalgae population synthesize new metabolites and perform homeostatic regulation as it was transferred to the new environment exhibiting lag growth phase. At Day 2 of inoculation, the microalgae cell density rapidly increased

and began entering the exponential growth phase. Danquah et al. (2009) reported that this growth phase has the highest differential biomass growth per unit time. According to the result obtained, the maximum microalgae concentration was reached at 8 d of treatment period. The microalgae cell density in pH 7 was increased about two-fold of the density in the lag phase, from 7.80 mg L^{-1} to 14.82 mg L^{-1}. However, as the pH decreased to pH 5, exponential phase becomes less apparent. The decreasing trend of growth phase was observed at Day 8 in all different pH except pH 5 and pH 7 due to the depletion of available nutrients to sustain microalgae propagation leading to the stationary growth phase. In addition, long exposure period to nutrient depletion, the microalgae biomass underwent the death phase endogenous catabolism started leading to cell autolysis. The microalgae biomass density was started to decline as treatment period approached Day 12 – 14.

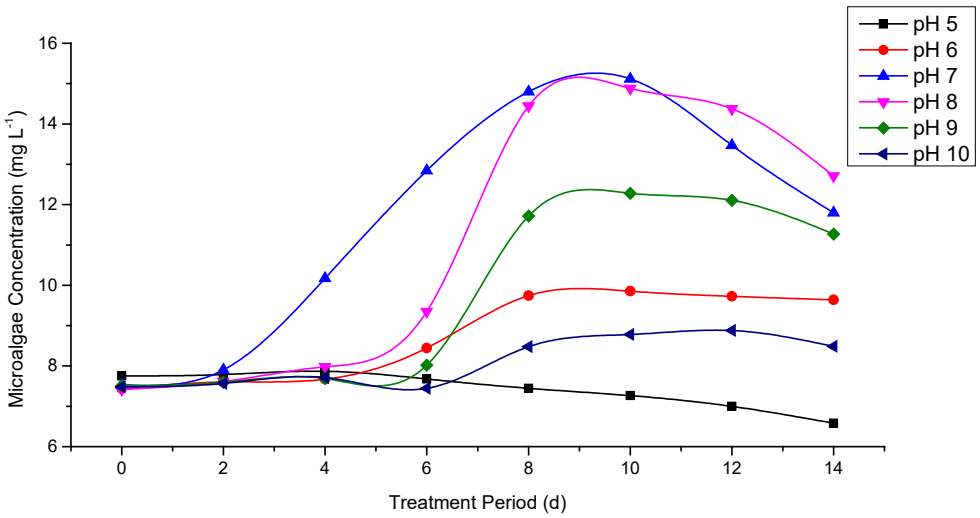

Figure 1: Microalgae cell density growth at various pH; pH 5, pH 6, pH 7, pH 8, pH 9 and pH 10

Table 2: Microalgae Growth and ammonia reduction for various pH (pH 5, pH 6, pH 7, pH 8, pH 9, pH 10) throughout 14 d treatment period.

	pH 5		pH 6		pH 7		pH 8		pH 9		pH 10	
Day	NH_4^+	Grw	NH_4^+	Grw	NH_4^+	Grw	NH_4^+	Grw	NH_4^+	Grw	NH_4^+	Grw
0	1.01	7.76	0.97	7.44	1.05	7.80	0.98	7.41	0.97	7.51	0.99	7.48
2	0.92	7.79	0.77	7.60	0.69	7.90	0.43	7.61	0.53	7.60	0.62	7.57
4	0.93	7.87	0.60	7.67	0.15	10.18	0.20	7.98	0.36	7.69	0.47	7.71
6	0.91	7.68	0.59	8.44	0.02	12.84	0.09	9.44	0.28	8.02	0.39	7.45
8	0.92	7.45	0.56	9.74	0.02	14.82	0.06	14.44	0.25	11.72	0.29	8.48
10	0.90	7.27	0.47	9.85	0.01*	15.12*	0.04	14.88	0.18	12.28	0.30	8.78
12	0.94	6.70	0.44	9.73	0.03	13.47	0.04	14.38	0.15	12.11	0.31	8.88
14	0.94	6.60	0.45	9.64	0.05	11.80	0.09	12.71	0.16	11.27	0.32	8.48

NH_4^+ = Reduction of Ammonia Concentration (mg L^{-1}), Grw = Growth of Microalgae Concentration (mg L^{-1})

3.2 Response surface methodology (RSM) of bio-flocculation of microalgae

To assess flocculation efficiency, *A.niger* pellets were mixed at the various parameters representing the actual high cell density cultures with the microalgae culture. From this optimization study, the optimal values bio-flocculant concentration, pH and mixing rate were found as 30 g L^{-1}, pH 7 and 125 rpm. Table 3 shows that the maximum efficiency was obtained at 97.2 % which is in complete agreement with the prediction of the model. Low concentration of *A.niger,* 25 g L^{-1} show less significant on the microalgae biomass harvesting achieving only 50 % of harvesting efficiency. However, an increasing concentration of bio-flocculant from 30 g L^{-1} to 38 g L^{-1}, may increase harvesting efficiency. The optimum concentration of 30 g L^{-1} obtained in this study agreed with Nasir et al., (2015). Since the pH does not significantly affect the harvesting efficiency for this study, pH natural (pH 7) was chosen as optimum pH. The pH of aquaculture wastewater is fluctuated due to diurnal changes and seasonal timeframes.

Table 3: Design sheet with the experimental runs and the observed values of flocculation efficiency.

Run	Dosage (g L^{-1})	pH	Mixing rate (rpm)	Predicted Harvesting Efficiency (%)	Actual Harvesting Efficiency (%)
1	30	7	125	85.33	93.86
2	21.59	7	125	93.25	88.07
3	30	7	82.96	38.25	45.56
4	30	7	125	93.25	97.19
5	30	5.32	125	72.81	89.05
6	25	8	150	79.37	83.87
7	25	6	150	61.85	54.55
8	25	8	100	52.24	52.54
9	30	7	125	93.25	92.98
10	30	7	167.04	47.36	42.54
11	25	6	100	64.81	51.60
12	35	6	100	79.57	73.38
13	30	7	125	93.25	95.17
14	38.41	7	125	99.79	93.64
15	30	7	125	93.25	92.51
16	35	6	150	63.15	61.17
17	30	8.68	125	77.93	64.07
18	30	7	125	93.25	93.16
19	35	8	150	81.81	93.34
20	35	8	100	68.13	73.74

Most of the aquaculture wastewater that contains highly concentrated microalgae also typically in range pH 7 to pH 7.5. Besides, the effect of mixing rate was analyzed in the range of 100 rpm to 150 rpm. Harvesting efficiency was not significant at a slow rate. The microalgae began to absorb into bio-flocculant as the rate was 100 rpm and dispersed again at 150 rpm. This phenomenon happens due to the restabilization of the microalgae cell at high mixing rate. This result showed that the microalgae harvesting was strongly dependent on the concentration of bio-flocculant and mixing rate during harvesting process. Figure 2 shows the three-dimensional response surface plots and illustrate the interactions between responses an experimental levels of each variable. These graphs can locate us the optimum levels of each variables to obtain the maximum harvesting efficiency.

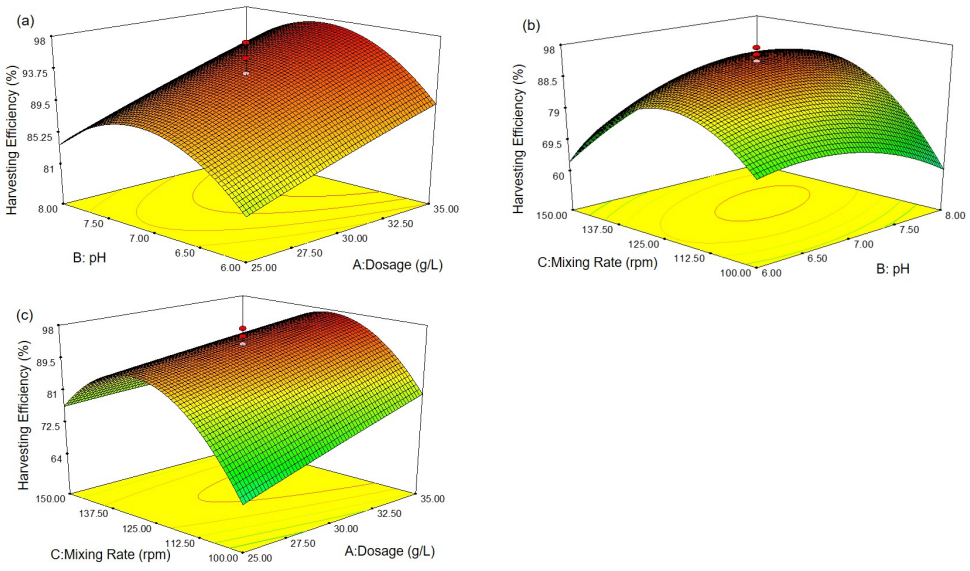

Figure 2: 3D Response surface plots depicting the effects of three independent variables: dosage, pH and mixing rate.

4. Conclusion

The present study dealt with bioremediation using *Chlorella* sp. and optimizing the influential parameters of bio-flocculation namely bio-flocculant concentration, pH and mixing rate by the statistical tool, Response Surface Methodology (RSM). This study confirms that the *Chlorella* sp. can be used to remove nutrients, ammonia from aquaculture wastewater. In addition, to best of knowledge, this is the first attempt using *A.niger* for harvesting microalgae with the optimization by RSM. Optimal conditions were found to be 30 g L^{-1} bio-flocculant concentration, pH 7 and rpm 125 agitation speed yielding a maximum efficiency of 97 %. The filamentous fungus, *A.niger* could be an advantageous and novel bio-flocculant in algal technology and be helpful in microalgae harvesting for cost-effective production of biodiesel from algal lipids without increasing the environmental impacts.

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