ORIGINAL ARTICLE

Effect of mass concentration of immobilized *Spirulina platensis* on nitrogen removal from simulated shrimp pond water

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Abstract

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***Spirulina platensis* strain BP immobilized on fibrous polyester mat was applied for nitrogen removal from simulated shrimp pond water. Different *S. platensis* mass on mat was built up during immobilizing process by varying the amount of batch fed suspended *S. platensis* cells. During immobilization, 0.2 OD_{560} *S. platensis* was replenished to reactor and chlorophyll-a in the solution was monitored. The immobilized *S. platensis* mass was 63, 49 and 19 g dw-*S.* platensis/m^2 for Sp V-1, Sp IV-1 and Sp II-1 mat reactors adding five, four and two times of 0.2 OD_{560} *S. platensis* respectively. In accordance with statistic, the results of nitrogen removal tests by applying those immobilized mats in circulated batch system reactors loaded with 1.94 gN/m^2-d showed that there were significant differences on ammonia removal among the different initial *S. platensis* mass on mats (ANOVA; $P \leq 0.05$). The Sp V-1, Sp IV-1 and Sp II-1 mats could reduce the ammonia nitrogen concentration from 1 mg-N/L to the level as low as 0.18 mg-N/L within 2 weeks. Transformation of 75-81% ammonia nitrogen to organic nitrogen in microalgal cells, of which 27-43% were detached to solution, resulted to 44-58% total nitrogen removed from the system.**

Key words : immobilization, polyester, *Spirulina platensis*, simulated shrimp pond water

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บทคัดย่อ

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ผลของความเข้มข้นเซลล์ในการตรึงสาหร่าย *Spirulina platensis* เพื่อการกำจัดไนโตรเจนในน้ำนากุ้งสังเคราะห์

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การนำสไปรูไลน่าสายพันธุ์ BP มาใช้บำบัดน้ำเสียที่เลียนแบบน้ำนากุ้ง โดยใช้ในสภาพที่มีการตรึงสไปรูไลน่าบนตัวกลางเส้นใยโพลีเอสเตอร์ให้มีปริมาณต่างกัน ระหว่างการตรึงมีการเติมมวลสไปรูไลน่าในสภาพแขวนลอย และติดตามการเปลี่ยนแปลงปริมาณคลอโรฟิลล์ เอในน้ำ ผลการทดลองในช่วงการตรึงเซลล์ พบว่าสไปรูไลน่าที่ตรึงบนตัวกลาง Sp V-1, Sp IV-1 และ Sp II-1 มีปริมาณมวล 63, 49 และ 19 กรัมแห้ง/ตร.เมตร ตามลำดับ โดยที่มีการเติมสไปรูไลน่าความเข้มข้น 0.2 ที่ค่าการดูดกลืนแสง 560 นาโนเมตร 5, 4 และ 2 ครั้ง ตามลำดับ ผลการทดสอบการกำจัดไนโตรเจนในน้ำนากุ้งสังเคราะห์โดยใช้แผ่นตัวกลางตรึง Sp V-1, Sp IV-1 และ Sp II-1 ในปฏิกรณ์แบบกะที่มีการไหลเวียนน้ำ ซึ่งป้อน 1.94 กรัมN/ตร.เมตร-วัน พบว่าการกำจัดแอมโมเนียไนโตรเจนทั้งหมดมีความแตกต่างกันอย่างมีนัยทางสถิติ (ANOVA; $P \leq 0.05$) ปริมาณแอมโมเนียไนโตรเจนทั้งหมดในน้ำนากุ้งสังเคราะห์ลดลงจาก 1 มก.N/ลิตร ถึงระดับ 0.18 มก.N/ลิตรภายใน 2 สัปดาห์ ปริมาณแอมโมเนียไนโตรเจนทั้งหมดในช่วง 75-81% เปลี่ยนไปเป็นสารอินทรีย์ไนโตรเจนในเซลล์ สไปรูไลน่า ซึ่ง 27-43% ของเซลล์หลุดออกไปกับน้ำ ทำให้มีเพียง 44-58% ของปริมาณไนโตรเจนทั้งหมดที่ถูกกำจัด

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Unbalanced feed to shrimp pond results in the accumulation of organic nitrogen waste in the sediment in the pond. This waste is hydrolyzed to ammonia nitrogen later and can be toxic to shrimp. In addition, nutrients residue in effluent shrimp culturing water can cause a serious environmental problem due to the occurrence of eutrophication.

A biological wastewater removal process is a complex process involving many micro-organisms. Among these microbes, microalgae play a dominant role in nutrient removal and the process is less complicated. Fundamental studies on these microalgae structure and function are necessary to understand the factors affecting the removal process.

Microalgal immobilization has been used for wastewater treatment in various entrapment or attachment conditions because the algal cells are easily harvested (Chevalier and de la Noue, 1985, Cordoba and Hernandez, 1995, de la Noue and Prouix, 1986, Travieso *et al.*, 1996, Chen, 2001).

Recently, several studies have addressed the use of non-suspended microbes, either attached or immobilized, as a valid method for nutrient removal from shrimp culturing water (Bratvold and Browdy,2001; Thomson *et al.*,2002; Paniagua-Michel and Garcia, 2003, Wantawin *et al.*, 2004).

The important properties of microalgae, including *S.platensis*, applied in shrimp culturing water are salinity tolerance (Ketpokasiri *et al.*, 2003) and being non- toxic. Regardless of controlling *S. platensis* mass on mats, previous studies (Wantawin *et al.*, 2004; Chaowanapreecha *et al.*, 2004) found that even more than 90% of ammonia was removed from shrimp culturing water and the high nitrogen in suspended form was released from the reactor due to high *S. platensis* detached from mats. The detachment of new growth cells can be caused by too excess initially attached to the mats. Therefore, the objective of this research was to investigate whether the detachment of immobilized mass can be controlled by the method of immobilization as well as the optional amount of initial mass on the mat when applying *S. platensis*

mats to remove nutrient from simulated shrimp pond water. The efficiencies of nitrogen removal were also considered.

Materials and Methods

The Microalgae Culture

Culture of *Spirulina platensis* strain BP used in the study was obtained from the Algal Biotechnology research group, School of Bioresources and Technology, King Mongkut's University of Technology, Thonburi. The *S. platensis* seed from Zarrouk's agar slant was inoculated into 125 ml flasks containing 60 ml Zarrouk's medium about 7 days and then transferred to 700 ml in 1,000 ml flasks under conditions of 54 $\mu E/m^2/s$ light intensity at room temperature. The salinity in normal Zarrouk's medium is 9 ppt and was adjusted to 15 ppt by NaCl.

Attachment Media

Fibrous polyester mat size 3cm x 30cm x 3mm dimension (figure 1) was hung vertically in each 5 cm diameter and 60 cm length acrylic tube column to form the 1 lite reactor.

Simulated Shrimp Culturing Water

Simulated shrimp culturing water was prepare from modified Zarrouk's medium diluted at 0.1 time with tap water. The $NaNO_3$ in 0.1X Zarrouk's medium was replaced with NH_4Cl at a concentration of 1.0 mg as N/l. The salinity was adjusted to 15 ppt for salinity of shrimp culturing water by NaCl and the salinity measured by Salinity-Conductivity-Temperature Meter YSI model 33.

Nitrogen concentration was prepared according to that calculated from ammonia nitrogen balance in shrimp culturing water. With 2:1 food conversion ratio, the levels of nitrogen residue in 1600 m^3 water at 100 day shrimp cultivation were between 87 and 118 kgN (Lin *et al.*, 1991). On the assumptions that without any *in-situ* nutrient degradation occuring and all nitrogen being in soluble form, the calculated daily concentration of nitrogen should be about 0.54-0.74 mg-N/l.

Circulated Batch System Reactor

Cylindrical column made of acrylic (diameter 5 cm and height 60 cm) was jointed to a 35-L plastic tank. A submerged pump in this tank was used for circulating water to the 1 liter column (Figure 2). The *S. platensis* mat was hung in the middle of column and continuously illuminated under 54 $\mu E/m^2/s$.

Procedures

Immobilization

The periods as well as total cycles of cell replenishment used in the procedure for immobilization were evaluated first by doing a preliminary test. The *S. platensis* cell from the stock culture of age of 14 days was applied to a 250 ml flask in

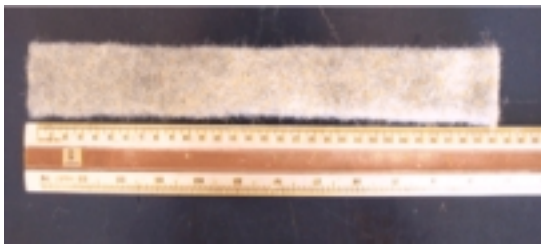

Figure 1. Attachment Media
(Color figure can be viewed in the electronic version)

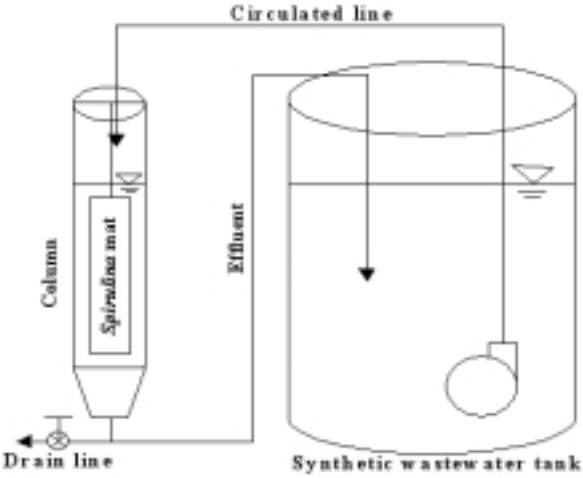

Figure 2. Circulation reactor

which was hung a 3 x 3 cm fibrous polyester mat. The suspended cells in the flask were monitored in order to be replenished with new stock culture when the remained cells dropped to 0.05 OD_{560}.

After the preliminary test, immobilized mats with different algal mass were built up by varying the amount of replenished suspended *S. platensis* cell based on preliminary data. The suspended *S. platensis* cells in Zarrouk's media without nitrogen source after 14 days in Zarrouk's medium culture have inoculated in 1,000 ml to obtain the optical density of 0.2 at 560 nm (OD_{560}). Suspended *S. platensis* cell at 0.2 OD_{560} in 15 ppt salinity solution was added in to the reactor containing 0.03 x 0.3 m fibrous polyester sheet under a light intensity of 18 $\mu E/m^2/s$.

There were three sets of immobilized mats named Sp V-1, Sp IV-1 and Sp II-1. Each set included two mats to act as duplicates. The number of adding suspended *S. platensis* cells at 0.2 OD_{560} in 15 ppt salinity solution was 5, 4 and 2 times for Sp V-1, Sp IV-1 and Sp II-1 mats, respectively. The immobilization was processed subsequent. All sets were completed in the same day.

Nitrogen Removal Test

After immobilization, the mats were used for testing the capability for nitrogen removal from

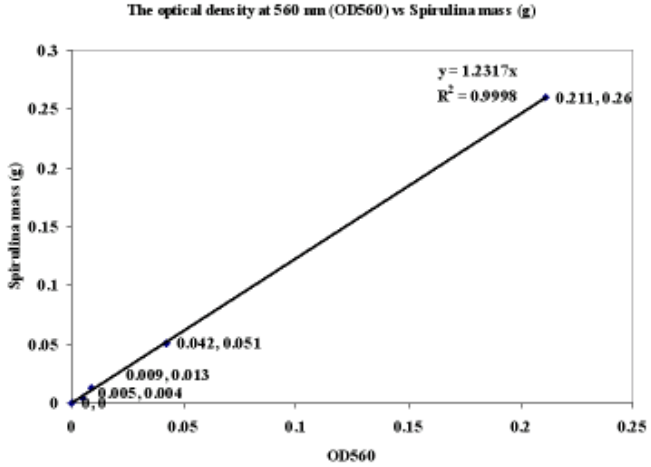

Figure 3. The standard curve of the optical density at 560 nm and *S. platensis* mass (g)

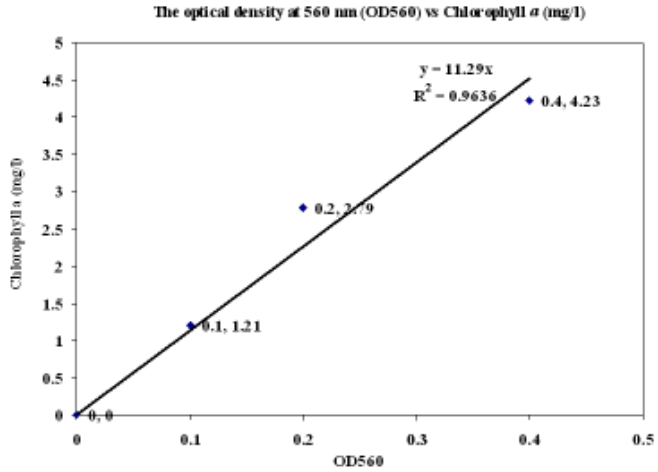

Figure 4. The standard curve of the optical density at 560 nm and Chlorophyll-a(g)

simulated shrimp pond water in circulated batch system reactors. The reactors were operated as one day fill and draw mode. Total ammonia nitrogen concentration in simulated shrimp pond water was 1 mg-N/L with loading of 1.94 g-N/m^2/d. The nitrogen removal test was conducted for 28 days. The flow rate was controlled by internal re-circulation of simulated shrimp cultural water at a velocity of 0.14 m/s. The detachment of *S. platensis* was also monitored by measurement of the optical density at 560 nm (OD_{560}) in solution.

Analytical Methods

Total Ammonia Nitrogen (TAN) and Total Kjeldahl Nitrogen (TKN) were measured by Phenate method and Titrimetric method respectively, according to Standard Methods for Examination of Water and Wastewater (APHA, 1992). Chlorophyll-a was analyzed according to Bennet and Bogard (1973) for transferring to the mass of *S. platensis*. The standard correlations of optical density at 560 nm (OD_{560}) with the suspended solids as well as with chlorophyll-a of *S. platensis* had been prepared for dry *S. platensis* mass calculation as shown in the Figure 3 and Figure 4, respectively. Results were analyzed using one way analysis of variance (ANOVA) with significance set at $P<0.05$.

Results and Discussion

Immobilization

The results from the preliminary study found that the density of suspended cell could be reduced after some period for every replenishment of 0.2 OD560 suspended *S. platensis* cell due to attachment on the mat until the at 5th addition. The *S. platensis* cells were attached to the mat in order to reduce their metabolism. The density of 0.2 OD_{560} suspended *S. platensis* cell at 5th addition remained

Table 1. Chlorophyll-a and calculated immobilized *Spirulina platensis* mass on mats in 1 L of reactor

Cycles of *S. platensis* adding		Dry mass (mg) at the end of cycle		
		Sp V-1	**Sp IV-1**	**Sp II-1**
1st	Chlorophyll-a in solution	1.48±1.15	*	*
	Chlorophyll-a on mat	1.79±1.15	*	*
	Mass of *S. platensis* on mat	190±30	*	*
2nd	Chlorophyll-a in solution	1.49±1.44	1.36±0.07	*
	Chlorophyll-a on mat	1.78±1.14	2.79±0.47	*
	Mass of *S. platensis* on mat	190±20	210±10	*
3rd	Chlorophyll-a in solution	0.97±0.35	0.84±0.62	*
	Chlorophyll-a on mat	2.29±0.35	2.43±0.62	*
	Mass of *S. platensis* on mat	250±40	260±70	*
4th	Chlorophyll-a in solution	0.27±0.19	0.47±0.47	1.5±0.3
	Chlorophyll-a on mat	3.00±0.19	2.38±0.05	2.92±0.02
	Mass of *S. platensis* on mat	260±20	300±50	190±40
5th	Chlorophyll-a in solution	0.92±0.32	2.3±1.5	1.9±0.2
	Chlorophyll-a on mat	2.34±0.32	2.66±0.86	1.42±0.2
	Mass of *S. platensis* on mat	240±40	160±110	150±20
Total Mass of *S. platensis* on mat		1,160±30	890±60	350±30

Remark * immobilization has not been started yet

nearly the same as initial even though the time was prolonged to more than 72 hours. In the first and second additions of suspended *S. platensis* solution, the *S. platensis* was attached on the mat and OD_{560} in the Zarrouk's medium was nearly zero within 6 and 12 hours respectively, while in the others, the time for attachment was more than 24 hours. Therefore immobilization was processed by applying a fifth addition of 0.2 OD560 suspended *S. platensis* solution to excess maximum build up of mass on the mats.

The mass of *S. platensis* on Sp V-1, Sp IV-1 and Sp II-1 mats as shown in Table 1 were calculated by subtracting the mass added (0.2 OD_{560}) from the remaining suspended *S. platensis* at the end of each cycle. The level correlation of chlorophyll-a corresponding 0.2 OD_{560} suspended *S. platensis* solution was 0.37 mg/L The mass of *S. platensis* was indirectly measured by multiplying chlorophyll-a detected in solution with 110 which is the correlated factor between *S. platensis* mass and chlorophyll-a analyzed from standard correlation graphs shown in Figures 3 and 4. The *S. platensis* mass on Sp V-1 mat was 1.3 and 3.3 times more than Sp IV-1 and Sp II-1, respectively. Based on the mat area, the immobilized *S. platensis* mass was 63, 49 and 19 g-*S.platensis*/m^2 for SpV-1, Sp IV-1 and Sp II-1, respectively.

Detachment of Cells during Removal Test

The time courses of OD_{560} representing solution suspended mass of Sp V-1, Sp IV-1 and Sp II-1 mat reactors during the nutrient removal test are shown in Figure 5. There were no significant differences among the mass (OD_{560}) detached from Sp V-1, Sp IV-1 and Sp II-1 mats (ANOVA; $P>0.05$). The correlation factor between gram of suspended solid concentration and OD_{560} from Figure 3 was 1.23. Calculated solution suspended solid concentration discharged as the effluent at steady state condition was in the range of 1.23-16 mg/L. The suspended *S. platensis* mass as high as 54 mg/L of Sp V-I detached from mats was found at the initial time during the first fifth days of nitrogen removal test even in the low mass mat reactor.

Nitrogen Removal

The time courses of remaining TAN fraction (C/C_0) are shown in Figure 6. The Sp V-1, Sp IV-1 and Sp II-1 could reduce the TAN in simulated shrimp pond water from 1 mg-N/L to the level as low as 0.18 mg-N/L in all mats. In accordance with statistical results, the TAN removal during 28 days in reactor with higher initial *S. platensis* mass tended to be greater than in reactor with the lower levels (ANOVA; $P \leq 0.05$). However, at steady state, there were no significant differences of TAN removal among the mats of different initial *S. platensis* mass. This implies that later microalgae can be developed on the mat to the optimize mass for the substrate existing in the solution. The steady state TAN removal was 85, 84 and 83 percent for Sp V-I, Sp IV-I and Sp II-I, respectively. Even when the previous culturing medium for stock *S. platensis* was nitrate nitrogen and *S. platensis* was exposed to a starvation period during immobilization for a long time, the rate of ammonia nitrogen uptake was high at the initial period and it took 17 days to reach to steady state for Sp V-I, Sp IV-I and 21 days for Sp II-I. Rapid uptake of nutrient by microalgae after the period of starvation incubation was found to occur in the other studies. Dy and Yap (2001) studied the surge ammonium uptake of cultured seaweed, *Kappaphycus alvarezii* (Doty) Doty (Rhodophyta: Gigartinales) and found that higher variability in

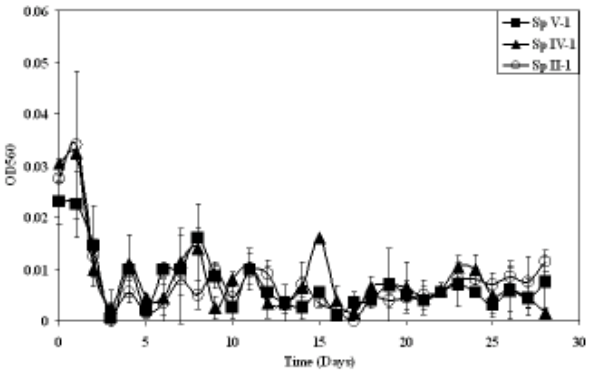

Figure 5. The OD_{560} in solution during the nitrogen removal tests at water velocity 0.14 m/s of Sp V-1, Sp IV-1 and Sp II-1 mats

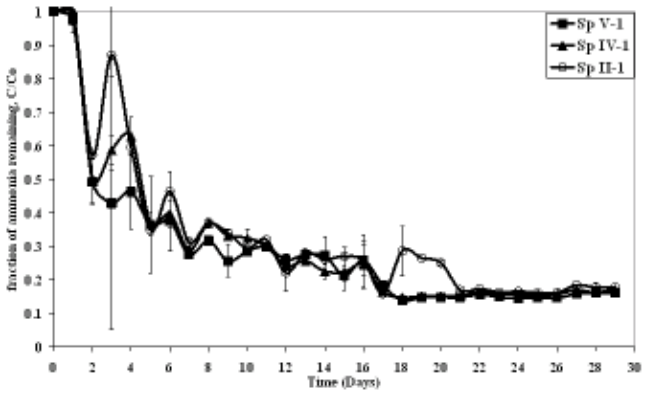

Figure 6. The time courses of remaining TAN fraction (C/C_0) by immobilized *S. platensis* mats during nitrogen removal test at water velocity 0.14 m/s

Table 2. Nitrogen for total experimental days (28 days) during nitrogen removal test of mat

Mat	Nitrogen in			Nitrogen out			Removal	
	TAN mg-N	Mass on mat mg-N	Average TKN in mg-N	TAN mg-N	Mass out mg-N	Average TKN out mg-N	TAN (%)	TKN (%)
Sp V-1	980	49.2	1029.2	216.2	216.0	432.2	79.0	58.0
Sp V-1'	980	34.3	1014.3	193.5	295.3	488.8	80.9	51.8
Sp IV-1	980	31.6	1011.6	225.4	339.1	564.5	77.7	44.2
Sp IV-1'	980	34.2	1014.2	216.1	247.5	463.6	78.7	54.3
Sp II-1	980	12.5	992.5	245.3	261.1	506.4	75.3	49.0
Sp II-1'	980	13.3	993.3	251.6	270.7	522.3	74.7	47.4

Remark Sp V-1', Sp IV-1' and Sp II-1' are the duplicate tests of Sp V-1, Sp IV-1 and Sp II-1, respectively.

uptake was noted during the first hour of incubation and surge uptake access of for incubations without additional enrichment than when algae were additionally exposed to enriched medium before the experiment.

The average efficiencies of TAN and TKN removal within 28 days and within steady state period (last 13 days) for all mats are shown in Tables 2 and 3, respectively. Nitrogen removal in aspect of TKN can be calculated and was compared with dissolved ammonia nitrogen removal. TAN in the range of 75-81% was transformed to organic nitrogen, in other words protein, in microbial cell of which 27-43% (Table 2) was detached to solution and discharged as effluent from the reactor.

According to the high *S. platensis* mass detached at the beginning of the removal tests as shown in Figure 5, the TKN removal efficiencies were only in the range of 44-58%. Because of the level on absense of detachment of cells in the steady state conditions, TKN removal (81.5-83%) was increased to nearly the same as TAN removal (87.4-89.6%) as shown in Table 3. The steady state TAN and TKN removal rates were 1.71 g-N/m^2/d and 1.59 g-N/m^2/d, respectively.

The high efficiencies of TAN removal in this study agree with the results from other investigation (De-Bashan *et al.*, 2002, Martinez *et al.*, 2000) was studied other types of microalgae. De-Bashan *et al.* (2002) studied the removal of ammonium ions from synthetic wastewater with TAN of 3 mg/L by the microalgae *Chlorella vulgaris* coimmobilized in alginate beads with the

Table 3. Nitrogen at steady phase (last 13 days) during nitrogen removal test of mat

Mat	Nitrogen in			Nitrogen out			Removal	
	TAN mg-N	Mass on mat mg-N	Average TKN in mg-N	TAN mg-N	Mass out mg-N	Average TKN out mg-N	TAN (%)	TKN (%)
Sp V-1	445	49.2	494.2	7.7	4.3	12	89.0	82.8
Sp V-1'	445	34.3	479.3	6.9	4.9	11.8	90.1	83.2
Sp IV-1	445	31.6	476.6	8.1	4.9	13	88.5	81.5
Sp IV-1'	445	34.2	479.2	7.7	4.8	12.5	89.0	82.1
Sp II-1	445	12.5	457.5	8.8	4.1	12.5	87.5	81.6
Sp II-1'	445	13.3	458.3	9.0	4.1	13.1	87.2	81.3

Remark Sp V-1', Sp IV-1' and Sp II-1' are the duplicate tests of Sp V-1, Sp IV-1 and Sp II-1, respectively.

microalgae growth-promoting bacterium *Azospirillum brasilense* and found that after the first 48 hour 100% of ammonia was removed. Martinez *et al.* (2000) studied nitrogen removal by the freshwater alga Scenedesmus obliquus and found that the highest percentage of ammonium removal (100%) resulted at the final culture time of 188.3 hour from initial nitrogen of 27.4 mg/L.

Conclusion

S. platensis cell can be attached on polyester mat and the immobilizing process for optimum mass was completed within 24 hours. According to low concentration of nitrogen in simulated shrimp culturing water, an initial *S. platensis* mass on the mat of about 20 g/m^2 was sufficient. Higher initial mass did not accelerate the rate of reaching to steady state. The uptake rate of ammonia to cell at steady state was 1.71 g-N/m^2/d. The detachment of cells to solution effluent during the acclimatization state resulted to low nitrogen removal; after that, TKN removal efficiency as high as 80 percent was obtained.

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