Bull. Environ. Contam. Toxicol. (1999) 62:70-78

Bulletin of Environmental Contamination and Toxicology

Nitrate, Ammonium, and Phosphate Uptake by the Immobilized Cells of *Dunaliella salina*

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Received: 30 April 1998/Accepted: 1 November 1998

Eutrophication of rivers & lakes is the major cause of pollution worldwide. Nitrogen and phosphorus through agricultural effluents and industrial outpour are the chief cause of eutrophication. In recent years microalgae have been largely used as test systems for the removal of pollutants from wastewater Codina *et al* (1993). Many advanced bioreactors use immobilized microorganisms as biocatalysts since they reduce cell washout and increase the biocatalyst concentration with optimum contact with the substrate. Also the use of immobilized cells instead of free cells offers increased stability and the ease of separation of algal biomass after waste water treatment.

Immobilization is the process which limits the free migration of cells by aggregating them to a solid support or entrapping them into a fibrous or porous material or a membrane. Immobilized cells are being used for various applications (Scott, 1987). Chief potential uses are for the production of pharmaceuticals and reagents, biosensors, chemicals, food and beverages. Wastewater treatment is one of the chief and significant areas of application for immobilized cell processes. There are reports of nitrification (Lemoine *et al* 1988) and denitrification (Vossoughi *et al* 1982) of wastewater and also phosphate removal (Chevalier and Noue 1985) have been studied in different organisms. Proulx & de la Noue (1988) have reported the use of immobilized algae in the removal of nitrate and phosphate from wastewater. Both natural and synthetic polymers have been employed for immobilization of the cells. For an immobilized system to be viable and long lasting there should be no irreversible, structural, physiological or metabolic damage to the cells. The choice of alginate as a matrix seemed to be effective as the algae remained metabolically active longer in an immobilized system Brouers *et al* (1989).

Due to increasing human population, upcoming industries and agriculture practices there is an increase in eutrophication of aquatic ecosystem by the release of chemicals, fertilizers in the aquatic ecosystem. Removal of ions is highly expensive and energy consuming effort. Use of biological systems serve a cheap

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and efficient way of nutrient removal from wastewater.

The present study aims to investigate the nitrate, ammonium and phosphate uptake capacity of the immobilized cells of *D. salina.*

MATERIALS AND METHODS

Stock cultures of *Dunaliella salina* were raised in 250ml Erlenmeyer flasks containing 100ml of liquid medium (Johnson *et al* 1968). Illumination was provided by cool white fluorescent light (14 W/m^2) for a light/dark period of 14/10 hrs. The temperature was maintained at 26±1°C and any drop in the pH of the medium was adjusted by adding sterilized 1N NaOH. A dense homogeneous 100 ml sterile suspension of alga (absorbance at 660nm= 1.0) was mixed with 4% (w/v) sodium alginate with continuous manual shaking for 20 min. A 10 ml syringe fitted with a needle of 1mm bore was used for extruding beads of algae entrapped in alginate dropwise from a height of 10 cm into a beaker having 100 ml of 0.2M calcium chloride. The resulting alginate beads (diameter 2.8 ± 0.11) were allowed to harden in calcium chloride solution for 30 min. Stabilized beads were washed 2-3 times with culture medium and finally resuspended into the basal medium for further growth.

Logarithmic algal cells with same initial cell density (absorbance at 660mn= 1.0) were divided into two. One part was kept as free and other was immobilized and grown in 250ml conical flasks in triplicates. At regular intervals the free and immobilized algae were withdrawn and placed in 10ml of 0.1M trisodium citrate. The cells were released from the beads after 15 min which was then centrifuged. Methanol was added and the cells incubated for 48hrs.The suspension was centrifuged and the clear supernatant was used to estimate chlorophyll *a* and the absorbance was noted in a calorimeter (Mackinney, 1941).

For dry weight determination the algal cells were harvested by centrifugation by using a Sorvall RC centrifuge. The cells were washed 2-3 times with Johnsons medium. 100ml of culture was filtered through Whatman No. 1 filter paper, weighed and dried in vacuum oven at 80°C for 24 hrs. Thereafter the final weight was recorded after cooling. Uptake of phosphate was estimated calorimetrically by Stannous chloride method (APHA, 1985) & nitrate was determined by brucine sulphuric acid method (Nicholas & Nason, 1957) and ammonium was assayed by Nessler's reagent (Herbert *et al* 1971). Stastistical analysis was done by one way Analysis of Variance(ANOVA) and means compared using Duncan's new multiple range test ($p< 0.05$).

RESULTS AND DISCUSSION

Time course effect of growth (biomass) of free and immobilized cells of *D. salina* is depicted in Table 1. After 25 days of cultivation immobilized cells exhibited 71% increased growth rate than free living cells. *D. salina* in free and immobilized states. Immobilization could facilitate recycling or regeneration of cells. Our results agree with the findings of Chevalier and Noue (1986) who worked with *Scenedesmus obliquus* for nutrient removal from the wastewater. It is also possible to achieve higher cell densities with cultures grown in immobilized cell systems than in free cell suspensions (Mitsui *et al* 1985).

Table 1. Biomass (Dry weight) (mg/l) of *D. salina* cells in free living (F) and immobilized cells (I).

		Biomass (Dry weight)	
Days	Fre	F	I
0		20.1 ± 0.11^{a}	20.1 ± 0.11^{a}
5		25 ± 0.15^{b}	58.2 ± 0.22^{b}
10		55.2 ± 0.17^{c}	80 ± 0.11^{a}
15		60.1 ± 0.21^{b}	105 ± 0.27^{c}
20		65 ± 0.25^{ac}	110 ± 0.19^{c}
25		70 ± 0.17^{ab}	120 ± 0.19^{b}

Values are mean ± SEM of three replicates. a, b, c means in the horizontal row followed by different letters are significantly different ($p<0.05$).

Immobilization offers a unique advantage over free cells due to increased reaction rates due to increased cell density, no cell washout from matrix, increased cell metabolism (Brouers *et al* 1989). *Dunaliella salina* has been used as a test organism for the detection of pesticide residues in water and soil (Yarden *et al* 1993).

In order to test the effectiveness of the nature of the growth medium, the growth was tested in batch and semicontinuous mode of culture conditions. In the semicontinuous mode the medium was replaced after 10 days. It was noticed that semicontinuous mode showed 66% higher growth than cells under batch mode after 25 days of growth Table 2. It has been seen that hydrogen production in *Anabaena cylindrica* that if fresh media was provided to the system, the deteriorated cells would be replenished and the growth of new cells stimulated thus enhancing the rate of production (Jeffries *et al.* 1978). Cells immobilized in matrix could be subjected to regular media exchange without washout (Philips and Mitsui, 1985).

Table 2. Effect of replacement of growth medium (after 10 days) on the growth of immobilized *D. salina* cells under batch and semicontinuous mode of culture conditions.

	Chlorophyll a (µg/ml)	
Days	Batch	Semicontinuous
0	2 ± 0.3^{a}	2 ± 0.3^{c}
5	5 ± 0.4^{b}	4 ± 0.4^{a}
10	6 ± 0.5^{ab}	7 ± 0.3^{b}
15	8 ± 0.3^{c}	10 ± 0.5^{a}
20	9 ± 0.4^{a}	15 ± 0.6^{ab}
25	9 ± 0.4^{b}	15 ± 0.6^{c}

Values are mean ±SEM of three replicates. Means within a column for each treatment followed by same letters do not differ significantly from each other by Duncan' s new multiple range test ($p<0.05$).

Table 3 illustrates the uptake of nitrate, ammonium & phosphate by the free living immobilized cells. The uptake rate of nitrate was 44% in free-living and 62% in immobilized algae after 36 hrs. Uptake of ammonium was 25% in free living and 42.2% in immobilized cells. Similarly phosphate uptake was 34.7% in free-living and 64.7% in immobilized cells. Immobilized cells show a better capacity of ions uptake than free living ones probably because of their mass aggregation inside the matrix.

Effect of pH on the rate of ions uptake elicited pH 8 favorable for nitrate, ammonium and phosphate uptake (Table 4). The uptake was 15.3%, 11.6% and 25% higher for nitrate, ammonium and phosphate uptake in immobilized cells than their free living counterparts. Effect of pH is important in the study because any deviation on either side of pH 8 would inhibit the uptake rates probably due to precipitation of ions in the medium.

Table 5 compares the biomass of alginate beads on the rate of ion uptake. At the

Table 3: Time course effect of nitrate, ammonium & phosphate uptake by the immobilized cells of *D.salina.*

Time (hrs)	Nitrate uptake (µg/ml)		Ammonium uptake (µg/ml)		Phosphate uptake (µg/ml)	
	F	I	F	I	F	I
0	180 ± 0.16^{b}	210±0.21a	380 ± 0.20^{a}	450 ± 0.25^{ac}	115 ± 0.23^{ac}	142 ± 0.17^{ac}
6	145 ± 0.17^{a}	175 ± 0.14^{c}	362 ± 0.21^{b}	425 ± 0.21^{a}	110 ± 0.33^{b}	130 ± 0.15^{a}
12	119 ± 0.15^{ab}	160 ± 0.17^{ab}	341 ± 0.22^{ab}	480 ± 0.29^{b}	100 ± 0.35^{ab}	121 ± 0.20^{ab}
18	115 ± 0.21^{c}	155 ± 0.20^{ab}	330 ± 0.31^{ac}	375 ± 0.32^{c}	95 ± 0.31^{b}	103 ± 0.31^{b}
24	110 ± 0.22^{b}	150 ± 0.19^{c}	325 ± 0.19^{c}	310 ± 0.29^{a}	87 ± 0.27^{a}	70 ± 0.24^{c}
36	100 ± 0.15^{a}	180 ± 0.24^{a}	260 ± 0.31^{ab}	$260=0.31^{ab}$	75 ± 0.25^{c}	50 ± 0.25^{ac}

Values are mean ± SEM of three replicates. Means within a column for each treatment followed by the same letters do not differ significantly from each other by Duncan's new multiple range test ($p< 0.05$).

Table 4: Effect of pH on the rate of nitrate, ammonium and phosphate uptake by the free living (F) and immobilized cells (I) of *D. salina.*

pH values	Nitrate uptake (µg/ml)		Ammonium uptake (µg/ml)		Phosphate uptake (µg/ml)	
	F	I	F	I	F	I
4	25.2±0.2^{a}	41.4±0.11^{ab}	80.1±0.19^{ab}	97.2±0.33^{a}	55±0.14^{b}	70=0.17^{b}
6	120.1±0.11^{ab}	139.1±0.33^{ab}	179.4±0.24^{a}	215.3±0.42^{ab}	115±0.31^{ab}	130=0.39^{ac}
8	174.4±0.31^{c}	201.2±0.24^{c}	394.2±0.12^{c}	440.1±0.14^{c}	120±0.22^{a}	150=0.41^{c}
10	131±0.21^{b}	172.3±0.17^{ab}	315.2±0.20^{b}	391.7±0.51^{ab}	70±0.17^{c}	85=0.25^{ab}

Values are mean ± SEM of three replicates. Means within a column for each treatment followed by the same letters do not differ significantly from each other by Duncan' s new multiple range test.

Table 5: Effect of cell density of the beads on the rate of nitrate, ammonium and phosphate uptake by the free living (F) and immobilized (I) cells of *D. salina.*

Cell density µg chl a/bead	Nitrate uptake (µg/ml)		Ammonium uptake (µg/ml)		Phosphate uptake (µg/ml)	
	F	I	F	I	F	I
0.8	135 ± 0.27^{ab}	142 ± 0.15^{a}	119 ± 0.17^{c}	130 ± 0.23^{ac}	95 ± 0.14^{a}	116 ± 0.21^{a}
0.6	179 ± 0.21^{c}	215 ± 0.17^{c}	390 ± 0.21^{b}	450 ± 0.15^{a}	117 ± 0.29^{ac}	140 ± 0.41^{b}
0.4	110 ± 0.14^{b}	130 ± 0.21^{a}	160 ± 0.19^{ab}	195 ± 0.31^{ab}	95 ± 0.25^{ab}	110 ± 0.21^{ab}
0.2	90 ± 0.11^{a}	65 ± 0.14^{ab}	229 ± 0.23^{ab}	279 ± 0.29^{ac}	50 ± 0.14^{b}	75 ± 0.17^{ab}

Values are mean ±SEM of three replicates. Means within a column for each treatment followed by the same letters do not differ significantly from each other by Duncan' s new multiple range test ($p< 0.05$).

biomass of 0.6µg chl a/ bead nitrate, ammonium and phosphate uptake was 20%, 15.3% and 19.6% higher in immobilized cells than free living ones.

Finally our results elicit the capability of immobilized *D. salina* cells for ions uptake that can lead to the development of bioreactor for removal of ions through immobilization.

Acknowledgments: AT thanks the Council for Scientific and Industrial Research, New Delhi for financial support.

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