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Analyzing the Efficiency of Foam as an Immobilization Matrix for the Storage of Cyanobacterial Cells

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ABSTRACT

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The present study aimed to analyze the efficiency of preserving cyanobacterial cultures by immobilizing them in foam. Preserving these photosynthetic organisms is advantageous as it reduces the researchers' load of repeated isolation, purification, and maintenance of batch cultures. Cyanobacterial cells were immobilized for a period of one, two, and three years and various biochemical aspects such as energy production, carbohydrate, and protein synthesis were evaluated. Activities of different enzymes like nitrogenase, glutamine synthetase, nitrate reductase, and nitrite reductase were also checked. The morphological studies carried out using scanning electron microscopy (SEM) revealed that cyanobacterial filaments adhered well to the foam matrix and regenerated into healthy filaments when conditions became favorable. The results obtained after the investigation suggested that cyanobacterial cells retained all of the characteristics analyzed for a maximum period of twenty-four months. Thus, foam serves as a good immobilization matrix for the preservation of cyanobacteria for at least two years.

Introduction

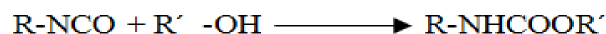
Cyanobacteria constitute the most primitive and well-diversified group of gram-negative and photoautotrophic prokaryotes. They inhabit almost every conceivable terrestrial and aquatic habitat on earth, including soil, rocks, and fresh and saltwater (Stal, 1995; Nayak and Prasana, 2007). Morphologically, cyanobacteria exist in diverse forms ranging from unicellular to branched and non-branched, filamentous to colonial types (Rippka *et al.,* 1979). Cyanobacteria are characterized by the ability to carry out two intrinsically incompatible processes: oxygenic photosynthesis and oxygen-intolerant nitrogenase-dependent nitrogen fixation by restricting the latter process to specialized, nearly anoxic cells termed heterocysts. Owing to their ability to fix atmospheric nitrogen, cyanobacteria contribute significantly to all ecosystems in which they occur by supplying combined nitrogen. Also, they are known to increase the concentration of oxygen in the environment where they grow (Meeks and Elhai, 2002). The tremendous amount of available knowledge on the diversity and physiology of cyanobacteria provides an excellent base for exploring their biotechnological applications. Over the years,

cyanobacteria have gained attention as a rich source of bioactive compounds. Cyanobacteria produce a wealth of secondary metabolites which are antibacterial (Jaki *et al.,* 2000), antifungal (Kajiyama *et al.,* 1998), antiviral (Patterson *et al.,* 1994), anticancer (Gerwick *et al.,* 1994), antiplasmodial (Papendorf *et al.,* 1998), algicide (Papke *et al.,* 1997), and immunosuppressive agents (Koehn *et al.,* 1992). It has long been known that cyanobacteria have applications in agriculture as biofertilizers (Watanabe *et al.,* 1951). They are also involved in the production of laboratory and commercial chemicals, wastewater treatment (Shah *et al.,* 2001), and are being used as agents for bioremediation (Raghukumar, 2001). Further, certain strains of cyanobacteria can synthesize polyhydroxyalkanoates that can be used as biodegradable plastics. This has immense implications for environmental remediation as cyanobacteria-derived polyhydroxyalkanoates have the potential to replace non-biodegradable petrochemical-based plastics (Sudesh *et al.,* 2001).

Taking into consideration the vast applications of cyanobacteria in various fields of human and environmental welfare, different cyanobacterial strains need to be isolated, purified, and characterized for their specific potential applications. However, these processes usually are very tedious and time-consuming. Moreover, maintaining cyanobacterial cultures in their axenic forms is always challenging as there is a lingering possibility of contamination, and this has risen as a matter of concern among researchers. It has led to efforts in developing methods for cyanobacterial preservation for successful and effective maintenance of these organisms under laboratory conditions. Immobilization is one such method, which involves the attachment of cells to some solid matrix so as to inhibit their independent movement when placed in a fluid environment. The process can be carried out by physical means such as adsorption or entrapment in a gel or foam matrix as well as by chemical methods such as covalent binding (Hall *et al.,* 1991).

Entrapment in porous gels and foams is, by far, the most recurrently used method of immobilization of chloroplasts, chromatophores, plant, and algal cells as it does not modify or cause any damage to the organelle or cell (Hall *et al.,* 1991). The basis of the entrapment methods is the confinement of cells in a three-dimensional lattice. The cells move freely within their compartments and the pores present in the material facilitate the diffusion of substrates and products to and from the cells. Several natural polymers (agar, agarose, alginate, cellulose, etc.), proteins (collagen, gelatine, etc.), and synthetic polysaccharides (acrylamide, polyvinyl, polyurethane, etc.) have been used for this purpose. The most frequently used matrices for immobilizing photosynthetic cells are agar and alginate gels, and polyurethane and polyvinyl foams. However, the use of agar and alginate comes with the disadvantage of low mechanical stability for long-term use in bioreactors. Polyurethane and polyvinyl foams have better mechanical properties and are unreactive to most commonly used ions (Brouers and Hall, 1986).

Polyurethanes constitute a class of synthetic polymers used to produce foams of variable structural properties like porosity, flexibility, hydrophobicity, and toxicity with varied domestic and industrial applications. Urethane is synthesized by the reaction between an isocyanate and a hydroxyl group:



The condensation of the urethanes with other isocyanates, amines, and alcohols produces a cross-linked polymer. CO_2 is evolved in such hydrolytic condensation processes. The overall structure of the polyurethane matrix depends upon the structure and functionality of the polyol resin, the amount of CO_2 entrapped, the temperature, and the presence of catalysts (Rao and Hall, 1984; Gisby *et al.,* 1987). In the present study, we analyzed the growth and metabolic parameters of a cyanobacterial species after specific periods of immobilization in polyvinyl foam to gain knowledge of the efficiency of the immobilization process. For this purpose, a heterocystous filamentous cyanobacterial species, namely, *Nostoc muscorum* was selected. To get an in-depth understanding of the impact of immobilization on the organism, different physiological parameters like energy production, synthesis of carbohydrates and proteins, the ability to utilize fixed nitrogen, and growth were evaluated in cells regenerated after one, two, and three years of immobilization respectively.

Materials and Methods

Organism and growth conditions

The cyanobacterial strain used in the present study, identified as *Nostoc muscorum,* was obtained from

Banaras Hindu University, Varanasi, India. The cyanobacterial culture was grown in BG-11_0 medium of pH 7.4 in a culture room maintained at 25±2 ˚C and with continuous illumination at a photon fluence rate of 50 µmol photons m^{-2} s^{-1} (Rippka *et al.,* 1979).

Immobilization in foam

The ordinary foam used for packaging was cut into twenty cubes of uniform size and washed a few times with sterile distilled water. These were then dried, soaked in media, and autoclaved. The excess media was drained in the laminar flow. 30 ml of exponentially growing culture (chlorophyll *a* content of 10 µg ml^{-1}) was centrifuged, and 0.5 ml of the concentrated cyanobacterial culture was injected into each of the twenty foam cubes. These were left to air dry in sterile conditions inside the culture room. When dried, the foam cubes were stored in sterile containers, and periodically, some of the cubes were regenerated in a liquid medium. This was done in order to analyze the stability of the distinctive characteristics of the cyanobacterial cells when regenerated from an immobilized state as compared to the control culture, which will provide insight into the efficacy of the immobilization process.

Electron microscopic studies

The morphology of the cyanobacterial filaments immobilized within the foam matrix was viewed under a Scanning Electron Microscope, SEM (JSM, 6360, JEOL, Tokyo, Japan) at Sophisticated Analytical Instrumentation Facility (SAIF), NEHU. The samples were first treated with 4% glutaraldehyde and kept at 4 ˚C for 24 hours. The samples were washed three times with sodium cacodylate buffer and were dehydrated with 30, 50, 70, 80, 90, 95, and 100% acetone. Dehydrated samples were mounted on brass stubs coated with gold and viewed under SEM.

Evaluation of energy production

Respiratory and photosynthetic ETC activities

The energy production in a cell that is required to carry out various cellular activities can be described in terms of respiratory electron transport chain (ETC) activity. For this purpose, a Clark-type oxygen electrode is used, which measures respiratory ETC as the rate of oxygen consumption in the regenerated cells (Robinson *et al.,* 1982). This electrode is installed in a 3 ml Plexiglass container with a magnetic stirrer (Rank Brothers, England). 3 ml of cyanobacterial culture was added to the sample chamber of the non-polarized electrode. The sample chamber was wrapped in aluminum foil to allow respiration to occur in the dark, and 5 minutes were given for the sample to equilibrate. The electrode was then polarized to obtain a linear rate of oxygen consumption. The oxygen consumption rate is expressed as nmol O_2 consumed µg^{-1} chlorophyll *a* min^{-1}.

For analyzing the photosynthetic rate of cyanobacterial cells, a Clark-type oxygen electrode (Rank Brothers, England) is used, which measures photosynthetic ETC as the rate of oxygen evolution in the regenerated cells (Robinson *et al.,* 1982). The same procedure described above was employed, except that the sample chamber was illuminated with a 100 W tungsten filament bulb to allow photolysis of water molecules by cyanobacterial cells that can be measured in terms of oxygen evolved under illuminated conditions. The rate of oxygen evolution is expressed as nmol O_2 evolved µg^{-1} chlorophyll *a* min^{-1}.

Measurement of chlorophyll *a* content

Photosynthesis can also be assessed in terms of the increase in chlorophyll *a* content, as it is the main photosynthetic pigment that plays a major role in the process (Mackinney, 1941). For this, 3 ml of culture was taken from each sample (both control and regenerated cultures) and centrifuged at 2500x g. The supernatant was discarded, and 3 ml of 100% methanol was added to the pellet. Chlorophyll *a* was extracted by incubation of the tubes containing the samples at 60 ˚C for 15 minutes. After incubation, the solutions in the tubes were thoroughly mixed using a vortex mixer and again subjected to centrifugation at 2500x g. The supernatant obtained was used for the spectrophotometric determination of chlorophyll *a* content at 663 nm. Chlorophyll *a* content was measured using the following formula:

Chlorophyll *a* (µg/ml) = Absorbance at 663 nm x 12.63

Estimation of carbohydrate content

Carbohydrate concentration was determined following the protocol described by Roe (1955). 3 mL of culture was centrifuged at 2500 rpm for 3 minutes. The pellet was washed with distilled water and subjected to sonication, after which 1 mL of the cell extract was

taken, and 4 mL of anthrone reagent (0.2% H_2SO_4) was added. The mixture was mixed and incubated in a boiling water bath for 10 minutes. After cooling, the solution was again centrifuged at 2500 rpm for 5 minutes, and the absorbance of the supernatant was read at 630 nm using a spectrophotometer. A calibration curve prepared using glucose as a standard solution with concentrations ranging from 10 to 100 µg/mL was used to calculate the carbohydrate content of the cells.

Nitrogen fixation and assimilation

Heterocyst frequency

Olympus BX 51 microscope was used to study the heterocyst frequency of the regenerated cell samples, which was expressed as a percentage of total cells. At least 1,000 cells were counted for each study.

$$\text{Heterocyst frequency} = \frac{\text{Number of heterocystous cells}}{\text{Total number of cells}} \times 100$$

Nitrogenase activity

Nitrogenase (EC 1.18.6.1) activity was determined in vivo using the acetylene reduction assay method as described by Stewart *et al.,* (1967). 5 ml of cyanobacterial culture was taken in 10 ml vials and acetylene gas at a final concentration of 10% (v/v) of air phase was injected into the vial. The vials were then incubated at 25±2˚C under light at a photon fluence rate of 50 µmol m^{-2} s^{-1} with constant shaking for one hour. To estimate the amount of acetylene reduced, a gas chromatograph (Tracor 540) fitted with a Porapak T column (stainless steel column 6’ X 1/8”, packed with a Porapak T of mesh size 80/100) and a flame ionization detector was used. Nitrogenase activity was expressed as nmol C_2H_2 produced μg^{-1} chlorophyll *a* h^{-1}.

Glutamine synthetase activity

Glutamine synthetase (transferase) activity was measured by assaying γ-glutamyl hydroxamate as described by Sampaio *et al.,* (1979). 3 ml of culture was centrifuged, and the pellet was washed with 50 mM Tris-HCl buffer (pH 7.5) and resuspended in 3 ml of the same. The cells were subjected to sonication to release the enzyme into the solution. The assay mixture contained 40 mM Tris-HCl buffer (pH 7.5), 3 µmol $MnCl_2$, 20 µmol potassium arsenate, 0.4 µmol ADP (sodium salt), 60 µmol hydroxylamine and 30 µmol glutamine. 0.5 ml of this assay mixture was added to 0.5 ml of the enzyme and incubated in the dark at 30 ˚C for 10 minutes, after which the reaction was terminated by the addition of a stop mixture (10% $FeCl_3$, 24% TCA, and 6 N HCl in distilled water). The final mixture was then centrifuged at 2500 rpm for 5 minutes, and the absorbance of the supernatant was read at 540 nm. The concentration of the γ-glutamyl hydroxamate formed was estimated using a standard curve prepared in the range of 100-1000 nmol γ-glutamyl hydroxamate ml^{-1}.

Nitrate reductase activity

The activity of nitrate reductase (NR) was measured *in situ* following the procedure described by Manzano *et al.,* (1976). 5 ml of cyanobacterial culture was taken and centrifuged, and the pellet obtained was washed with and resuspended in NR buffer (50 mM Tris-HCl buffer, pH 7.5, 0.1 M NaCl, 0.3 M sucrose, 1 mM KNO_3, 1 mM EDTA and 5 mM $MgCl_2$).

The cells were then sonicated, and the reaction mixture was raised to a volume of 1 ml by adding 20 Mm KNO_3, 100 mM Glycine-KOH (pH 10.5), 4 mM methyl viologen, and 10 mM sodium dithionate freshly dissolved in 0.1 ml of 0.23 M $NaHCO_3$. The solution was incubated in the dark at 30 ˚C for 10 minutes, and the reaction was terminated by adding 0.2 ml of 1 M zinc acetate. Subsequently, the nitrite formed during the reaction was estimated by the method of Snell and Snell (1959).

Nitrite reductase activity

For assaying the activity of nitrite reductase (NIR), the method of Arizmendi and Serra (1990) was employed. 5 ml of cyanobacterial culture was centrifuged, washed with 50 mM Tris-HCl buffer (pH 7.5), and sonicated.

In addition to the permeabilized cells, the reaction mixture volume was raised to 1 ml by adding 2.5 mM KNO_3, 90 mM Tris-HCl buffer (pH 7.5), 3 mM methyl viologen, 20 mM sodium dithionate freshly dissolved in 0.3 M $NaHCO_3$. The solution was pre-incubated at room temperature for 5 minutes, and the reaction was carried out at 30 ˚C for 10 minutes. To terminate the reaction, the mixtures were vigorously shaken to oxidize the excess reductant. The remaining nitrite was estimated by the method of Snell and Snell (1959).

Nitrite estimation

The method of Snell and Snell (1959) was used to colorimetrically estimate the amount of nitrite present. To 1 ml of the sample, 1 ml of sulphanilamide (1% (w/v) sulphanilamide in 3 M HCl) and 1 ml of NED (0.02% (w/v) N-1-Napthyl ethylene diamine dihydrochloride) were added. The solution was thoroughly mixed, and after 15 minutes, absorbance was read at 540 nm. A calibration curve was prepared using KNO_2 as standard.

Protein estimation

The total protein content in the free-living and regenerated cyanobacterial cells was estimated following the procedure of Bradford (1976). 3 mL of culture was taken and centrifuged at 3000x g, followed by resuspension in 50 mM Tris buffer. The culture was sonicated, and 100 µL of cell extract was taken, to which 3 mL of Bradford reagent was added. The solution was vortexed and incubated in the dark for 5 minutes. The absorbance was then read at 595 nm. Bovine serum albumin (BSA) with concentrations ranging from 10-200 µg mL^{-1} was used as standard.

Measurement of biomass content

The growth of cyanobacterial cultures was measured in terms of biomass production. The biomass content was determined by taking 3 mL of cell culture and drying it in the oven at about 45 ˚C to a constant weight and expressed in terms of mg mL^{-1}.

Results and Discussion

Structure of the foam matrix and regeneration of cyanobacteria in media after immobilization

Fig.1a shows the foam cubes inoculated with fresh cyanobacterial culture. Fig.1b shows the scanning electron micrograph of the large pores of the foam matrix. After a year of storage, the dried cells started growing into viable filaments immediately after reintroducing into a liquid medium (Fig.1c).

Both viable and dried filaments can be seen on the foam matrix under SEM one day after transferring into fresh medium (Fig.1d-e) whereas morphologically healthy filaments were observed regenerating from foam after four days of transfer (Fig.1f).

Effect of immobilization on energy production

Respiratory and Photosynthetic ETC

Table 1 shows the rates of respiratory O_2 consumption and photosynthetic O_2 evolution of cyanobacterial cells regenerated after 1, 2, and 3 years of immobilization in foam as compared to their free-living counterparts.

Effect of immobilization on chlorophyll *a* content

Fig.2 gives a comparative study of the growth curves of regenerated *N. muscorum* Meg1 cells with that of the control cells. Cells allowed to grow in fresh media for seven days showed chlorophyll contents of 2.94 µgml^{-1} (control), 3.48 µgml^{-1} (after the first year of immobilization), 2.90 µgml^{-1} (after the second year), and 1.70 µgml^{-1} (after the third year).

Effect of immobilization on carbohydrate content

The estimation of the total carbohydrate content of the cyanobacterial cells provides an indication of carbon fixed by these photosynthetic organisms. Fig. 3 shows that the total carbohydrate content of the control culture was 27.06 µg ml^{-1}. After the first year of immobilization, the carbohydrate content was around 27.48 µg $ml^{-1,}$ and after the second year, the concentration of carbohydrates was 23.74 µg ml^{-1}. However, the total carbohydrate estimated after the third year of immobilization dropped to 13.92 µg ml^{-1}.

Effect of immobilization on nitrogen fixation and assimilation

Heterocyst frequency and Nitrogenase activity

The heterocyst frequencies of *Nostoc muscorum* samples regenerated after the first and second years of storage showed an increase of 4.17% and 1.39% as compared to their control cultures, respectively (Fig. 4). There was a drop in the heterocyst frequency by 29.17% after the third year of storage. In the case of the enzyme nitrogenase, the activity in the control cells was 9.12 nmol C_2H_4 produced µg^{-1} Chl *a* h^{-1} and that of the immediate regenerates of cyanobacteria stored for one year was 9.23 nmol C_2H_4 produced µg^{-1} Chl *a* h^{-1}. On the other hand, the nitrogenase activity in cells regenerated after the second year was 7.57 nmol C_2H_4 produced µg^{-1} Chl *a* h^{-1} and after the third year of storage, the activity was 5.1 nmol C_2H_4 produced µg^{-1} Chl *a* h^{-1} (Fig. 4).

Table.1 Comparison of the rates of O2 consumption and evolution of free-living and immobilized cyanobacterial cells (Mean ± SD)

	Respiratory O_2 consumption (nmol O_2 consumed μg^{-1} chl *a* h^{-1})		Photosynthetic O_2 evolution (nmol O_2 evolved μg^{-1} chl *a* h^{-1})	
	Control	**Regenerated sample**	**Control**	**Regenerated sample**
1st year	413 ± 3.5	411 ± 2.2	437 ± 3.3	444 ± 2.7
2nd year	415 ± 3.3	397 ± 2.1	439 ± 2.8	424 ± 2.3
3rd year	413 ± 3.1	378 ± 2.1	439 ± 2.8	420 ± 2.3

Table.2 GS activity of Nostoc muscorum (Mean ± SD)

Time period	GS activity (nmol γ-glutamyl hydroxymate formed min^{-1} mg^{-1} protein)	
	Control	**Regenerated sample**
1st year	767 ± 5.3	765 ± 5.1
2nd year	763 ± 5.2	635 ± 5.0
3rd year	763 ± 5.2	600 ± 5.0

Table.3 Enzyme activities of free-living and immobilized cyanobacterial cells (Mean ± SD)

Time period	NR activity (nmol NO_2 formed min^{-1} mg^{-1} protein)		NIR activity (nmol NO_2 consumed min^{-1} mg^{-1} protein)	
	Control	**Regenerated sample**	**Control**	**Regenerated sample**
1st year	2.4 ± 0.1	2.4 ± 0.07	547 ± 3.1	531 ± 3.1
2nd year	2.3 ± 0.13	2.0 ± 0.07	542 ± 3.1	520 ± 3.1
3rd year	2.3 ± 0.9	1.5 ± 0.08	546 ± 2.8	500 ± 3.0

Figure.1 (a) Foam cubes inoculated with cyanobacterial culture (b) Matrix of a foam cube as seen under SEM (c) Regeneration of cyanobacteria from one-year-old dried foam (d,e) adhered cyanobacterial filaments seen regenerating upon return of favorable growth conditions (f) healthy cyanobacterial filaments obtained after regeneration.

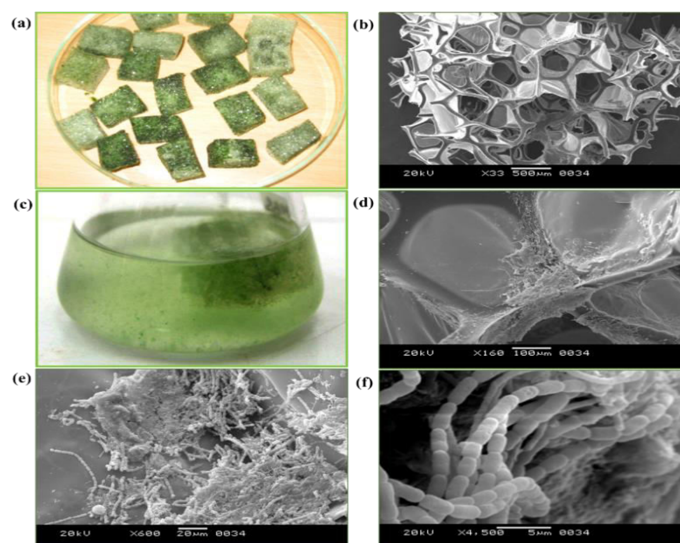


Figure.2 Growth curve of Nostoc muscorum regenerated from foam cubes as compared to the free-living counterpart. [In the figure, "F" refers to foam cubes, and "1, 2, 3" indicate samples regenerated after the first, second, and third years of immobilization. An initial inoculum of 0.4 µg ml^{-1} was used].

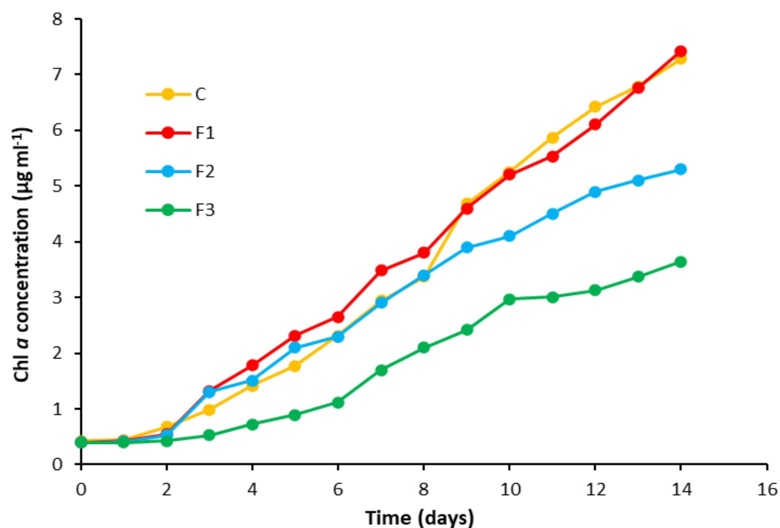


Figure.3 Total carbohydrate content of control and foam-immobilized cyanobacterial cells. [In the figure, column "C" represents the control, and columns "F1, F2, F3" represent the samples after immobilization for 1, 2, and 3 years in foam, respectively]. All values are in Mean ± SD.

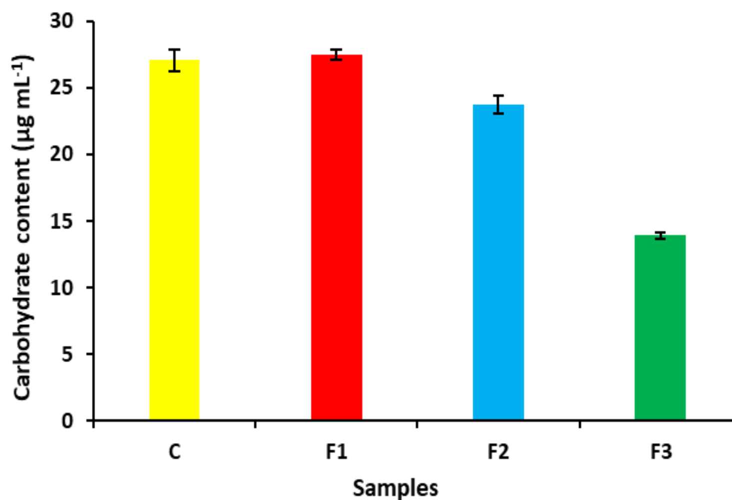


Figure.4 Heterocyst frequency and Nitrogenase activity of control cells and those regenerated from foam cubes. [In the figure, column "C" represents the control, and columns "F1, F2, F3" represent the samples after immobilization for 1, 2, and 3 years in foam, respectively]. All values are in Mean ± SD.

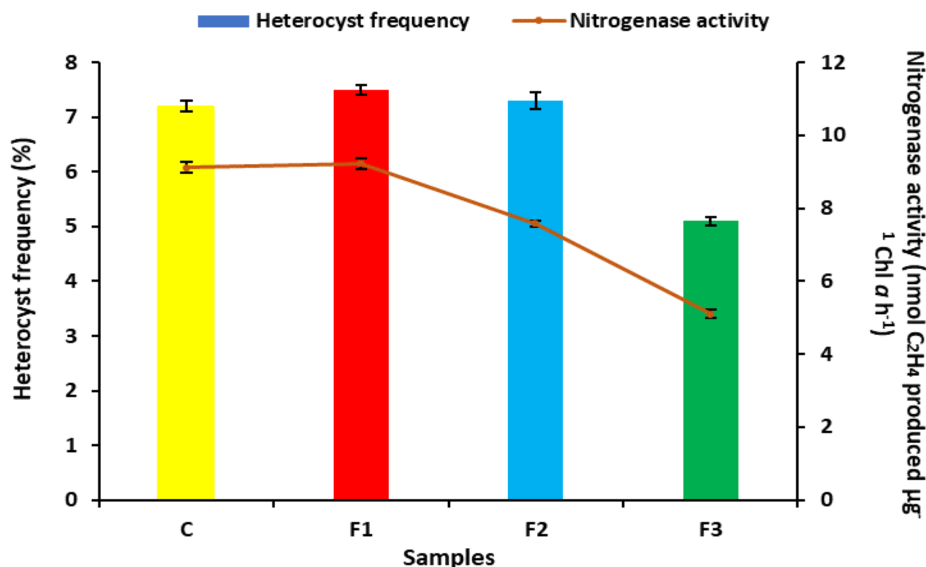


Figure.5 Protein concentrations of control and regenerated cyanobacterial cells. [In the figure, column "C" represents the control, and columns "F1, F2, F3" represent the samples after immobilization for 1, 2, and 3 years in foam, respectively]. All values are in Mean ± SD.

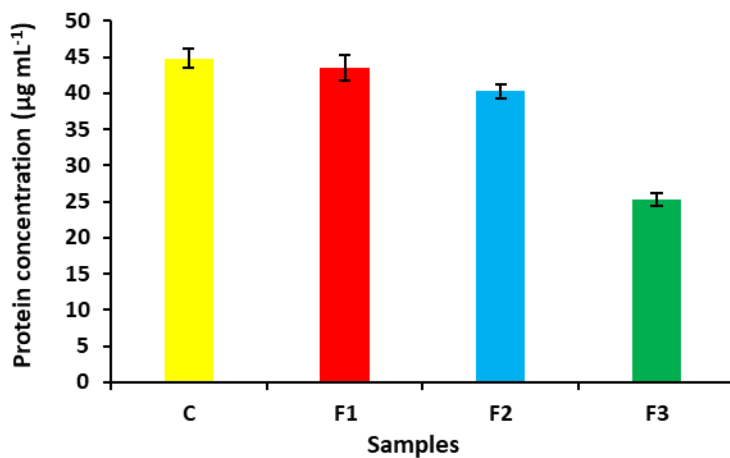


Figure.6 Biomass content of free-living cyanobacterial cells and those regenerated from cubes. [In the figure, column "C" represents the control, and columns "F1, F2, F3" represent the samples after immobilization for 1, 2, and 3 years in foam, respectively]. All values are in Mean ± SD.

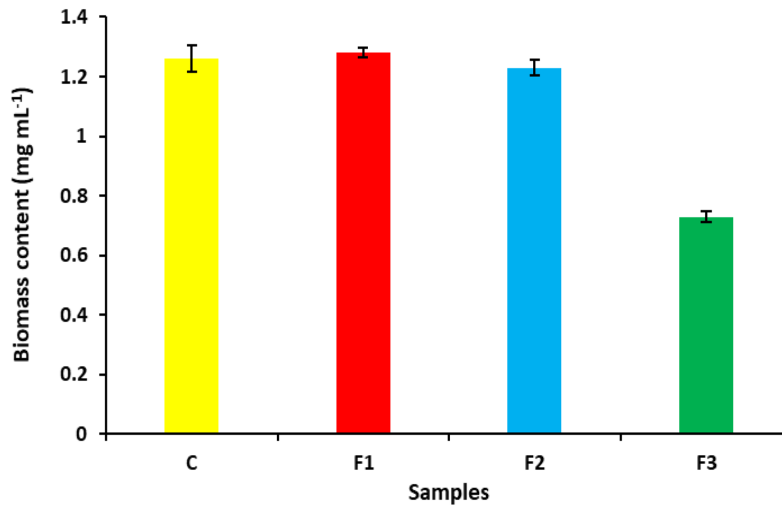
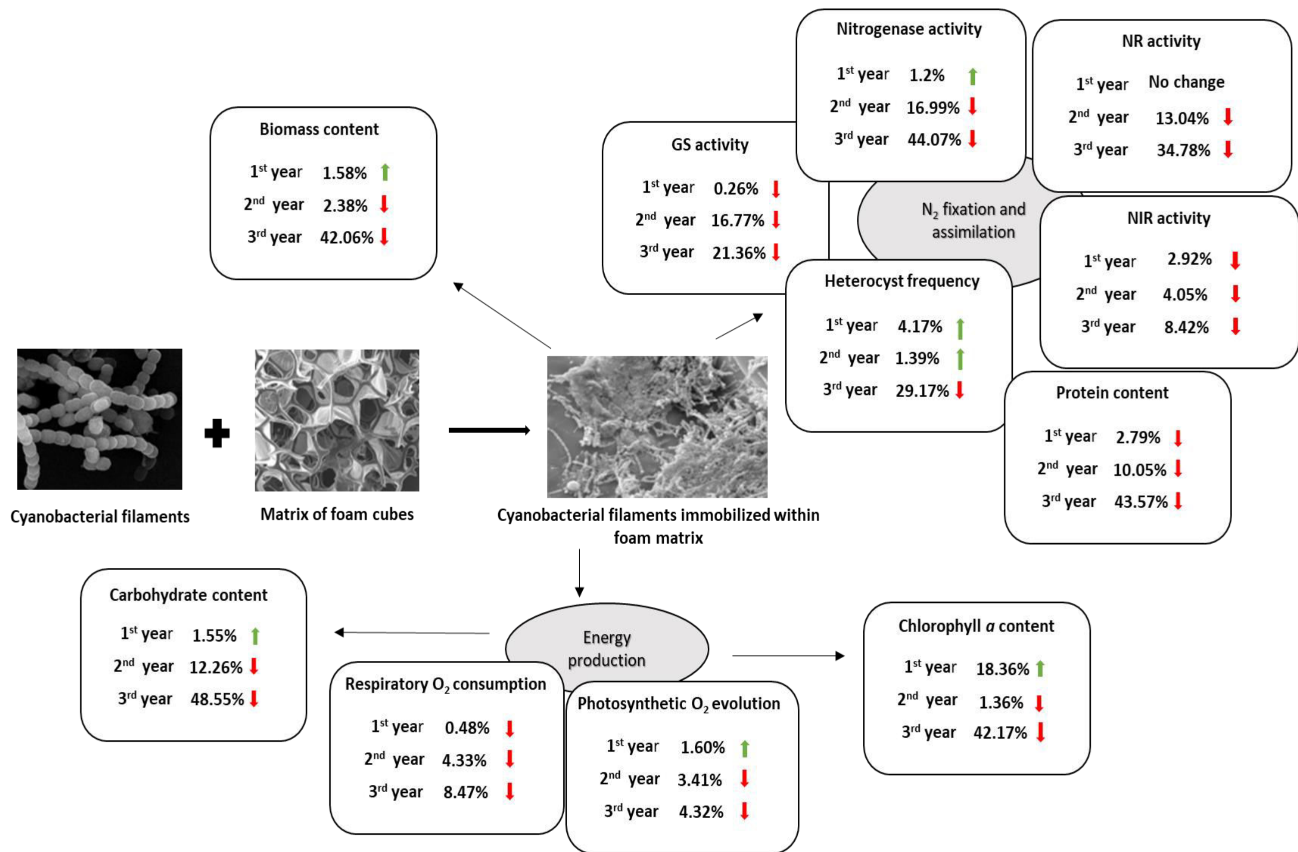


Figure.7 Schematic representation of the effect of immobilization on various biochemical parameters of cyanobacterial cells after a period of one, two, and three years. ↑ indicates an increase and ↓ indicates a decrease in the values recorded after the respective periods of immobilization.



Glutamine synthetase activity

Table 2 shows the retention of GS activity in cyanobacteria regenerated from foam.

Activities of NR and NIR

The activities of nitrate reductase and nitrite reductase retained after immobilization in foam for 1, 2, and 3 years respectively are shown in table 3.

Effect of immobilization on total protein content

The protein concentration of the control cyanobacterial culture was 44.75 µg ml^{-1} and in the regenerated cultures at the end of the first, second, and third years of immobilization, the protein concentrations were 43.5 µg ml^{-1}, 40.25 µg ml^{-1}, and 25.25 µg ml^{-1} respectively (Fig. 5).

Effect of immobilization on total biomass content

The growth of control and regenerated cell cultures was monitored in terms of the increase in biomass content. Fig. 6 shows that the biomass content of the control cells was 1.26 mg ml^{-1} and the cells regenerated from foam cubes after the first, second, and third years of immobilization had biomass contents of 1.28 mg ml^{-1}, 1.23 mg ml^{-1}, and 0.73 mg ml^{-1} respectively.

Cyanobacteria have long been studied for their interesting morphology, physiology, and diversity, but extensive work in the last decades has revealed the potential of these microbes in biotechnology-relevant fields (Abed *et al.,* 2009).

With the latest advances in metabolic and genetic engineering technologies and the availability of more than 300 cyanobacterial genome sequences, there is substantial progress in research leading toward realizing the maximum potential of these photosynthetic organisms (Lau *et al.,* 2015).

Consequently, an increasing number of efforts are being made in order to preserve cyanobacterial cells in their native forms. By doing so, researchers will be relieved from the maintenance of batch cultures which is not only labor intensive but also involves the risk of contamination due to routine handling inside the culture room. The process is expensive as regular sub-culturing is required and can lead to mislabelling or loss of cultures due to overcrowded racks. Preservation of purified cultures is also indispensable to avoid chances of cross-contamination between various cyanobacterial cultures that are kept in close proximity.

In this study, cyanobacterial cells were immobilized in ordinary packing foam. This method of preservation was chosen because foam is readily available in any laboratory that purchases the usual consignment of research equipment, thus making the immobilization process a very cost-effective one. So, for the purpose of evaluating the efficiency of foam as an immobilization matrix, different biochemical parameters such as respiratory and photosynthetic ETC activities; heterocyst frequency; activities of various enzymes like nitrogenase, glutamine synthetase, nitrate reductase, and nitrite reductase; chlorophyll *a*, total carbohydrate, protein, and biomass contents were analyzed in cyanobacterial cultures regenerated after preservation.

For analyzing energy production in the immobilized cyanobacterial cells, the respiratory and photosynthetic ETC activities were examined. In PS II, protons released upon water splitting, as well as formed during plastoquinol oxidation by the cytochrome b_6f complex are released into the thylakoid lumen, creating a proton gradient across the thylakoid membrane that is used for ATP synthesis by the enzyme ATP synthase. These ATPs are then utilized for CO_2 fixation and other cellular functions (Vermaas, 2001). The respiratory ETC activity after the first year of immobilization was almost the same as that of the control culture. In contrast, the photosynthetic ETC activity showed a slight increase of 1.6%, suggesting that the immobilization process had no negative impact on the energy-generating processes of the organism. A look into chlorophyll *a*, the main photosynthetic pigment that plays a role in capturing light energy, and into carbohydrate content, the end product of the entire process indicated that the content of chlorophyll *a* of the cells regenerated after the first year was elevated by 18.36%. However, it dropped by 1.36% after the second year and by 42.17% after the third year of immobilization. Following a similar pattern, the carbohydrate content increased by 1.55% in the regenerated cells after the first year but decreased by 12.26% and 48.55% after the second and third years respectively. The slight increase seen after the first year of immobilization may be due to the frantic cellular activities revived when water and nutrients became available. However, the significant drop in the parameters especially after the third year can be

rationalized from the fact that the cubes were stored under dehydrated conditions during prolonged immobilization and most cells probably perished due to lack of nutrition and water during the three years of storage. The nitrogen fixation and assimilation capacities of the regenerated cells were assessed by evaluating heterocyst frequency and nitrogenase and glutamine synthetase activities. Mature heterocysts provide the microoxic environment required for nitrogen fixation, spatially separating this process from the oxygen-evolving photosynthesis in vegetative cells (Golden and Yoon, 1998).

The heterocyst frequencies of cells regenerated after the first and second years of immobilization recorded an incline of 4.17% and 1.39%, respectively; after the third year, it dropped by 29.17%. The increased heterocyst frequencies after the first two years of immobilization indicated a surge in the differentiation of vegetative cells into heterocysts to meet the growing demand for fixed nitrogen, thus maintaining the C/N ratio at 5:1, which is indispensable for cyanobacterial growth (Wolk, 1973).

The activity of nitrogenase showed an upsurge of 1.2% after immobilizing for one year. This may be attributed to elevated photosynthetic electron transport and stable enzyme activity (Brouers and Hall, 1986). The accessible form of nitrogen that is most widely used by cyanobacteria is nitrate (Musgrave *et al.,* 1982).

Nitrate is assimilated after being reduced to ammonium by nitrate reductase (which showed no change in activity after the first year of immobilization) and nitrite reductase (which recorded a slight decrease of 2.92%).

The incorporation of the fixed nitrogen into organic nitrogen compounds is taken care of by the enzyme glutamine synthetase, which witnessed a comparable value to un-immobilized control cells after a year of immobilization. The effect of the reduced enzyme activities was also evident in the decrease in total protein content by 2.79%, 10.05%, and 43.57% after the first, second, and third years respectively.

The overall effect of the immobilization process can finally be analyzed in terms of the total biomass content of the regenerated cells, which marked an increase of 1.58% after the first year. However, the biomass content decreased by 2.38% after the second year, and by 42.06% after the third year of immobilization. Further subculturing of the immobilized cells led to the complete recovery of all parameters to their original values. Fig. 7 below shows a diagrammatic representation of the changes observed in various biochemical parameters of the regenerated cyanobacterial cultures.

The investigation revealed that the packing foam could effectively be employed as a means of preservation of cyanobacteria for a period of at least 24 months. The immediate regenerate of the samples preserved for one year showed growth parameters and enzyme activities quite similar to those of the control cultures.

Even after preservation for two years, the decrease in the various biochemical parameters was almost negligible and could be recovered with further sub-culturing. However, samples regenerated after three years of immobilization in foam displayed a significant reduction in all the parameters evaluated.

This indicated that although foam may be used as a means of preservation of cyanobacterial cultures for one or two years, it cannot serve the same purpose if a longer preservation time is required. To preserve cyanobacterial cells for more than two years, an alternative immobilization matrix, such as calcium alginate, would be a better choice (Flores *et al.,* 2005; Syiem and Bhattacharjee, 2015).

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Author Contributions

Prof. Mayashree B. Syiem: Conceived the original idea, designed the model, and reviewed and edited the manuscript.; Dr. Amrita Bhattacharjee: Designed the model, and collected and analysed the data.; Ms. Lanakadaphi R. Chullai: Curated the data and prepared the original draft

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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