ABSTRACT

This study utilizes *Spirulina* species NCIM 5143 procured from National Collection of Industrial Microorganisms, Pune, India. The phylogenetic analysis from 16S rRNA revealed its identity closer to *Spirulina subsalsa* BGLR6. The species was cultured in inorganic Zarrouk medium supplemented with trace elements in white light of 2500 lux at temperature of 32°C at 180 revolutions per minute. The specific growth rate and doubling time were found to be 0.0771 day^{-1} and 8.98 days respectively. The species was found to be a helical filament in structure with a diameter of 3 µm and pitch length of 1 µm. It exhibited gliding motility and moved towards light exhibiting phenomenon of positive phototaxis. This property influences the pattern of biofilm formation by the cyanobacteria and the configuration of filamentous cells within it, depending on the direction of illumination. The static culture always formed a network of filaments on walls that developed into biofilms over time and stuck to the walls. This natural biofilm formation capacity was utilized and the wet weight was inoculated into an H- type biofuel cell setup, leading to the formation of a biofilm over the electrodes. Interestingly, a subpopulation of bacteria was detected in the vicinity of the *Spirulina* filaments, and a few cells were directly attached to the filament structures. This close physical association indicates that crucial metabolite and nutrient exchange might occur between the host and bacterial cells. Four distinct heterotrophic colonies were isolated and identified through 16S rRNA sequencing as *Halomonas saliphila, Halomonas campaniensis, Alcanivorax pacificus,* and *Pelagibacterium lentulum.* We inferred, that these pure culture strains were the dominant members of the *Spirulina*-associated bacterial community as they could form colonies on the zarrouk medium plate devoid of any organic carbon source, thriving on residual extracellular organic carbon and metabolites exuded by the phototrophic host (*Spirulina*). These associations indicate a copiotrophic habitat typical of a phytoplankton bloom or matured phototrophic biofilm. We believe that this combination of predominant bacterial species isolated by us might be involved in efficient carbon and nitrogen cycling assisting the sustained growth of the host cyanobacterium (*Spirulina subsalsa*).

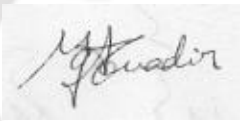
The *Spirulina* species when used as anodic catalyst in a microbial fuel cell (MFC) setup, the open circuit potential (OCP) was oscillated and synchronized with the 12-hours light-dark circadian pattern. However, unlike most of the previous report, a steep rise in voltage response with onset of each 12-hour dark phase was observed. Upon checking the contribution of individual anodes and cathodes, it was confirmed that these diurnal variations entrained to illumination regime were due to anodic contribution to total electromotive force. The cathode potential was stable at 270 mV throughout the operation. This observation indicates that more electrons were accumulated over the anode during the dark phase. Next, we focused on elucidating the mechanism behind this phenomenon following the *in-situ* voltammetry in MFC setups. We screened different materials and finally selected graphite plate as the anode considering its conducive nature

for forming biofilm and suitable electrochemical behaviors. The voltammetry of biofilms formed over glassy carbon electrodes showed a consistent reversible peak with a formal potential of 0.260 V and an irreversible oxidative peak with onset around 0.6 V. The photosynthetic production of oxygen was also identifies at - 0.25 V (oxygen reduction) in the voltamogram. The redox entity corresponding to peak at 0.260 V was present only with the biofilm and absent in the culture supernatant indicating its physical presence in the spaces closely associated with the cell wall. These voltammetric patterns were reproduced over graphite electrodes during in situ electrochemical investigations. From the guiding literature, we presumed that the redox species belongs to putative quinones, flavonoids or temperature sensitive redox peptides. The magnitude of the irreversible oxidation peak current at ~0.6 V obtained from the cell-free supernatant increased with the culture age, from 2 µA on day 4 to 42 µA on day 26. This potential was biotic in nature and distinct from the oxidative water splitting reaction identified at ~ 1.15V as revealed from an extensive electrochemical investigation. The increase in peak current at 0.6V coincided with the upsurge of total organic carbon content including proteins, carbohydrates and phenolic compounds in the supernatant.

Based on our results from macromolecular estimation, biofilm microscopy and electrochemistry we propose a hypothetical model. Firstly, any electron flux emanating out of cells (both cyanobacteria and symbionts) because of the primary metabolic activity like photosynthesis, respiration or fermentation will first end up with the supramolecular assembly of biofilm matrix containing redox moieties and metabolites interspersed in it. These redox moieties may be diverse and dynamic as these are formed because of microbial metabolic activities, macromolecular degradation, cell lysate recycling, assimilation and are akin to autochthonous humic and fulvic like substances in the composition. Further, many unknown exoproteome may also be present in this extracellular compartment with putative redox activity. While these diverse molecules may act as redox buffer storing electrical charges, their interactions with oxygen under oxic conditions may lead to partial electron quenching leading to peculiar voltage modulation patterns observed in our experiment. Further, the voltage output may also be influenced by the presence of artificially introduced redox mediator molecules and their chemical and physical interaction with the biofilm matrix components. The collective electrochemical output of these numerous possible redox reactions within the extracellular matrix and their interaction with oxygen may be responsible for peculiar increase of anode potential (more negative value) during dark period observed in our experiment. Secondly, alternate metabolic modes of *Spirulina* and associated symbionts in direct contact with electrode surface may be directly interacting with the electrodes through an exoelectrogenic process.

This study also aimed to investigate the effect of ferricyanide cross-over across nafion membrane on performance of cyanobacterial anodic biocatalyst during the long time operation of the MFC. These

experiments were performed in a specially designed MFC in OCP modes and identified the crossover of ferricyanide from the cathode to anode chamber. We confirmed that ferricyanide quenches cellular electrons at the biofilm-electrode interface, reducing the anodic redox potential. These conclusions were made on the basis of difference in anodic OCP variations between setups and enhanced magnitude of reductive currents in the Tafel plots. At its high concentration level the potentiometric response was though enhanced, simultaneously, a toxic effect of this redox mediator to the cells was also confirmed. Thus, the selection of an optimum concentration of the mediator is critical to realize a desired potential benefit in MFC. Moreover, direct contact of cells with the electrode is essential for generating the OCP oscillation between diel cycles and the interaction of ferricyanide with the biofilm matrix induces secretion of a oxidizable entity into the matrix. The results of our study demonstrate the role and associated pros and cons of biofilm extracellular matrix on electrochemical performance of a cyanobacterial biofilm based biophotovoltaic devices. Firstly, extracellular matrix provides nourishment to cyanobacteria for surviving prolonged dark conditions and harbors symbionts that make the biotic component of BPV resilient and durable for real time application. Secondly, it provides safety to the cells within it from stressful high mediator concentrations and toxic substances. Thirdly, it may act as reservoir of endogenously produced or artificially introduced redox molecules enhancing electrochemical performance. On the other side, it is difficult to achieve uniform mediator concentration and a predictable control over reaction dynamics within the biofilm matrix causing unpredictable variations that influence viability and performance of the BPV system. Moreover, the reaction dynamics within this matrix also changes with time and metabolic conditions that persist within biofilm.



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