Bistablity in Fluorescence from a purple non-sulfur bacteria

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(Dated: May 1, 2017)

Bistability has been observed for photosynthetic purple non-sulfur bacteria *Rhodobacter capsulatus* SB1003. The microbes respond to UV excitation (at 365nm) in a switchable manner. A time dependent increase in fluorescence (590-685nm) following roughly a first order kinetics is observed at normal temperature (298K). When either concentration is lowered the state switches to a steady state first order bleaching. Similar bistability is also observed with respect to temperature variations , as amplification occuring at say 298K disappears below 280K. One can synthetically control bistability and force a re-emergence of fluorescence amplification by exposing the system to a static magnetic field (0.25T). Differential excitability of singlet and triplet states and selective sensitivity of triplets to static magnetic field may explain this result. The triplets dominate at low temperature preventing amplification of emission, and the reversal may be caused by the Zeeman splitting of the triplets , wherein the excitable singlets re-emerge. Evolutionary and ecological implication of bistable photosynthetic system is discussed in the text.

PACS numbers: 87.18.Fx, 31.50.Df, 33.50.-j,87.50.C-

I. INTRODUCTION

Bistability in photochemical processes has been reported earlier in several photochromic compounds, polymers and derivatives [1–4]. However such bistability has rarely been reported for natural photosynthetic systems for instance in cellular system. In general we find different uses of fluorescent biomolecules that are [5, 6] mostly susceptible to photo-bleaching. We show that for the bacterium *Rhodobacter capsulatus* SB1003, a bistable fluorescent emission, the switchability determining parameters are concentration and temperature. We observed this bistable behaviour while studying the fluorescence recovery after photobleaching (FRAP) experiment[7]. FRAP is based on the high propensity of bleaching by fluorophores and its recovery by diffusion of unbleached molecules. Our study shows the fluorescence from *Rhodobacter capsulatus* SB1003 became brighter instead of bleaching, under a confocal microscope during relatively prolong UV excitation.

Photosynthetic purple non-sulfur bacteria *Rhodobacter capsulatus* SB1003 is one of the most primitive photoheterotroph that contain photoreceptors in the form of porphyrin derivatives like non-covalently bound cofactors, bacterio-chlorophyll and bacterio-pheophytin in the reaction centres of their photosynthetic membrane complexes[8]. This class of α-Proteobacteria has been employed as a model system for studying photosynthesis[9] and different classes of porphyrin production under various growth conditions [10]. Therefore bistability of the photosynthetic machinery of this purple bacteria seems to have important ecological and evolutionary implications.

These bacteria possess behavioral competencies to respond external stimuli by altered electron transport system which modulate their intra-cellular energy level to obtain optimal metabolic activity known as energy-taxis [11]. Such behavioral response is very essential survival strategy for obtaining highest metabolic yields at different external energy levels. The photosynthetic microbes are of particular interest as they induce a thermodynamically favorable condition for supporting the ecological network of different life forms by contributing negative entropy in the form of photon capture [12]. The general conjecture is that a sensory perception for species like *Rhodobacter* can respond to external stimuli like light energy through electron transport system in their photosynthetic apparatus [13]. Numerous models have been reported where the role of microbes in overall metabolic energy transfer circuitry of different ecological niche have been emphasized. [14–17].

Notable point that we report in the present paper is that spin perturbations agents like static magnetic field [18–20] can play important role in this bistable behaviour. Possible roles of radical pair formation [21, 22] or triplet fusion [23, 24] has also been investigated that could help one to truly embed the photon utilization and regulation machinery in designing and device level implementation of artificial photosynthesis.

II. MATERIAL AND METHODS

All chemical reagents were used of analytical grade Sigma-Aldrich (USA) or SRL (India) products.

Bacterial Growth Studies

The bacteria *Rhodobacter capsulatus* strain SB1003 was a gift from Dr. Patrick Hallenbeck, Department of Microbiology and Immunology, University of Montreal, Canada. For experimental purposes *Rhodobacter capsulatus* SB1003 were grown photoheterotrophicaly in anaerobic condition in yeast extract supplemented RCV medium (4g Malic acid, 1g NH_4Cl, 120 mg $MgCl_2$, 75 mg $CaCl_2$, 20 mg Sodium EDTA salt, 1mg Nicotinic acid, 10 mM KPO_4 buffer, 1000 ml dH_2O supplemented with 0.3g Yeast extract powder, $_P$H 6.8 $\pm$0.2) under continuous illumination for 5-6 days[25] at room temperature. The full grown bacterial culture and the exhausted medium were used for monitoring fluorescence properties.

Basic Spectroscopic Studies

Fluorescence properties of bacterial cells and cell free extract were measured. Spectroscopic measurements of samples were obtained by scan method setting, absorption range from 300 nm to 1000 nm, at a bandwidth 1.0 and 600 scan speed using Thermo Scientific^{TM} Evolution 300 UV- Vis spectrophotometer. The fluorescence properties of samples were studied with Photon Technology International (PTI) fluorimetric setup (Quantamaster ^{TM}40). A 72 W Xenon lamp was used as an illumination source and the detection was preceded by passing the emitted beam through an optical chopper and emission monochromator. The slit width was set at 4 nm for both excitation and emission. For temperature dependent fluorescence measurement a software controlled Peltier was employed. The magnetic field application is illustrated in Fig. **??**. A small square shaped magnet (field strength 0.25T) was placed under the quartz cuvette, in the cuvette-holder of above mentioned fluorimetric set up (see Fig. **??**) to study the effect of static magnetic field (SMF).The temperature variation experiment in presence and absence of SMF was performed using this setup.

Determination of kinetic parameters: Spectroscopic Data Analysis

Graphs were plotted considering the initial fluorescence amplification rate along Y axis and other variables such as temperature or concentration along the X axis. Change in fluorescence intensity (count) with per unit time was expressed by a first order kinetic parameter k (s^{-1}). In case of amplification, k value is positive. A negative K value could be obtained if only bleaching occurs. To obtain k, only the initial rate was evaluated using first 20 seconds of data. Solution to the first order equation $\frac{dF}{dt} = \pm kF$, representing fractional the rate of fluorescence intensity change. Matlab (Mathworks USA) was used to obtain the best value of k. Multiple k values were determined from 5 independent experiments using log linear regression and this was followed by a box plot representation of data.

Fluorescence Life Time Measurement

Fluorescence lifetime measurements were performed using 370nm sub-nanosecond pulsed LED source from HORIBA Scientific Single Photon Counting Controller: FluoroHub. Fluorescence Lifetime histogram was obtained using MicroTime 200, PicoQuant GmbH. Detail description of the instrument is given in the experimental set up section of Single Molecule Spectroscopy.

Live Cell Imaging

Microscopic fluorescence study was initially done using bacterial biofilm [26] in an inverted confocal microscope (Olympus, FV 1000). A 405 nm laser was used as excitation source and fluorescence emission was observed between 570-670 nm. Time lapse imaging of the samples were acquired using continuous laser at 1 minute time interval for a period of 10 minutes. Further study was carried out with full intensity laser for a period of one minute.

III. RESULTS

Time scan and concentration dependent bistability

The basic left and right panel of Figure(1) represents the basic setup design employed and a typical emission spectrum (excitation maxima being 395nm) respectively .The whole cell as well as the cell free extract was found to fluoresce near 613 nm when excited. The time scan reveals a time dependent increase in fluorescence emission intensity for both whole cells and the cell free extract containing the photosynthetic complex the rate in the two cases being different and being dependent on solvent polarity (data not shown). The spectral behavior of the membrane bound photosynthetic complex was found to be strongly sensitive to solvent perturbation. When acetone was selected as solvent two additional emission peaks appeared at 594nm and 635nm. Notably, the excitation is also red shifted to 411nm and 403 nm for the respective emissions.
The split of original spectral pattern into two peaks reveals the fact that the integrity of the membrane bound complex is sensitive to solvent perturbation. Notably, only one peak (635nm) among the two (split peaks originating as a result of solvent perturbation) is amplified with time when there is a prolonged excitation. There is some variations of exact peak position depending on the bacterial growth conditions , the robust result remaining similar.

Figure 2 reveals the bistable behavior of fluorescent emission. If we assume the emitted fluoresecnce F as a function of time i.e. F=F(t), one finds either an amplification state where $\frac{1}{F}\frac{dF}{dt}$ is a positive constant (representing first order kinetics of amplification) prevails or, a negative constant representing bleaching like first order decay of F(t) follows. This transition is concentration dependent as evident from the concentration values in the figure 2. The bleaching happens at lower concentration whereas the amplification at higher. It may be noted that we have observed the first order kinetics in the limit of initial measurements (0-20s). The first order rate constants was computed from linear regression of log(F) and t. Beyond 20 s , however the kinetic is zero order (dF/dt) is constant. Even the zero order kinetic constant has opposite signs in the amplifying and the bleaching domain.

An alternative way to understand the mentioned switching behavior is to plot the kinetic constant against the fluorophore concentration (see Fig. 3) .Kinetic constant has a systematic dependence on the concentration; rate of emission changing from negative to positive with increasing fluorophore concentration. The kinetic constant profile is akin to excitable system e.g.excitable membranes [43].

Temperature dependence and static magnetic field effect

Fig. 4 illustrates the effect of Static Magnetic Field (SMF) on fluorescence property of the system. The fluorescence amplification process was found best at 298K. But at low temperature (below 280K), the amplification process is attenuated (see upper panel of Fig. 4). The comparison of upper and lower panels of Fig. 4 clearly indicates that at lower temperature, the amplification reappears in presence of SMF. While there is bleaching in absence of SMF at such temperature, the statistical significance of SMF effect at low temperature (278 K)is validated by unpaired t-Test. The null hypothesis was rejected at 95% confidence level with P value 0.0002.

Confocal microscopy

The microscopic data (Fig. 5) is particularly relevant as the whole bacterial cells showed amplified fluorescence emission over a longer time period up to 10 minutes. The panels numbered 1-10, corresponds to minutes of excitation exposure of the same sample. When a FRAP (Fluorescence Recovery After Photo-bleaching) experiment was carried out with bacterial cells, the fluorescence emission intensity gradually increased instead of photo-bleaching. The behavior is similar to what is found in Fig. 2). For further study the intensity of the incident laser (405 nm) beam was increased from 25%to 100% and the sample was exposed for one minute. The result is shown in the lower panel of Fig. 5. The indices A and B refer to preexcitation and post-excitation states of the sample. This time there was a dual effect, the central part showing a bleaching (see B of the lower panel) and the peripheral region showing an intense fluorescence.

Fluorescence Life time

Life time studies revealed a few characteristics of the amplitude gain phenomenon. Dilution of sample with solvent shows that the life time value decreases with increasing order of dilution, higher dilutions have low life time values (see Table I).
The evaluation of life time values for the excreted fluorophores revealed order of 2 nanoseconds and 15 nanoseconds which indicate formation of two species in excited states. Where the shorter one is prone change life time upon perturbation of surrounding environment which could be due to alteration of structural changes on the other hand the longer one is more or less static. So, the notable point is that during fluorescence emission there should be a simultaneous competition between two upon excitation for long time as one species is found to show varying lifetime and another is not.

The switchability of the excitation and decision making

Hence the two switches present here plays the same role as an logical OR gate. If we consider high temperature (O1) and presence of magnetic field (O2) are the two input logical operands, the logical output, namely the fluorescence amplification (A) can be expressed by A=O1.*OR*.O2 (see the truth table in Table II). In Table II, O1=1, O2 = 0 , means high temperature and no field , and such condition A=1. Similarly, O1=0, O2 = 1, means low temperature and presence of field , and A=1 prevails in such conditions too. The other entries of the Table II similarly follows.

IV. DISCUSSION

Bistability in Photosynthetic System

The paper reports sustained increment of fluorescence intensity upon prolonged illumination at UV excitation wavelength. The kinetics of fluorescence emission is regulated with temperature and magnetic field.
The following facts may be put together while we suggest any mechanism. (a) Amplification of fluorescence at higher concentration (b) Photo-bleaching at higher dilutions and lower temperature. (c) Onset of The confocal imaging studies (Fig. 5) shows some notable features of fluorescence amplification with time. the upper panel shows gradual amplification while lower panel shows gradual decrease with time. The difference between two is that in the former the laser beam intensity is moderate (25%) where as in lower the intensity is maximum (100 %). The matter can be regarded as a bistability with respect to excitation level , higher photon flux causing a predominance of bleaching , whereas lower flux being associated with amplification.

The photosynthetic unit of the purple bacteria contains reaction centre protein pigment complex. The pigments are a particularly positioned bacteriochlorophyll dimer at the centre which act as primary electron donor upon excitation, two accessory monomeric bacteriochlorophyll at their antenna complex and carotenoid [34]. Upon excitation, bacteriochlorophyll dimer undergo primary charge separation [35] and certain population in the excited singlets forms triplet. As a result there is a decrease in overall fluorescence yield. However this triplets are being reactive to molecular oxygen and produce reactive oxygen species that also triggers fluorescence bleaching [36, 37]. In presence of carotenoid the long lived triplet states are rapidly quenched which in turn lead to greater fluorescence yield by inhibiting above mentioned reactions that causes fluorescence bleaching [38]. Considering the fact that lower temperature favors radical pair formation [22], the rate of triplet population formation rate will be higher in low temperature. This supports the observed data in Fig. 4 that is fluorescence bleaching at lower temperature. Though in presence of magnetic field the fluorescence amplification reappears. The presence of SMF destabilizes the triplet state by facilitating the formation of fused triplets that have same energy level like singlets. Alternatively Zeeman splitting may occur in which the spin degeneracy is removed and singlet states can reappear. In either case we could get enriched singlet population resulting in fluorescence enhancement.
It follows that spin thermalization [40] is facilitated at room temperature and less favored at low temperature. The increase in local electrostatic potential of the surrounding medium upon prolong photo-excitation is also effective at moderate temperature [39] which may facilitate the enrichment of singlets from fused triplets resulting in formation of brighter species that contribute to higher fluorescence. However, radical pair formation is favored at lower temperature [22]. The radical pair once formed can have a singlet triplet exchange (interconversion). It is often suggested that local magnetic field driven thermalization of spin [41] (that is often countered by external magnetic field) may thus comprehensibly explain the observed result [42].
To explain the mechanism behind amplified fluorescence emission broadly we can assume two distinct hypothesis.

- We can postulate self assembly of porphyrin derivatives residing in the photosynthetic complex. Ordered self-assembly of porphyrin derivative e.g. magnesium porphyrin and protoporphyrin, a likely candidate of the photosynthetic complex [27]. Porphyrins contain tetrapyrroles which could easily interact non-covalently because of the presence of pi bonds, commonly known as pi stacking and lead to self assembly [28, 29]. The fact is that the crowding of the photosynthetic molecule would favor self assembly which could explain the concentration dependent rise in fluorescence. We may assume that a kinetically controlled self assembly gradually causes the amplification process by accelerating the ground state energy transfer to the excited state.

- The second hypothesis involves radical pair formation. Our preliminary flash photolysis experiments (data not shown) validates presence of triplet and singlet states, their ratio sensitive to UV excitation(data not shown). In fact, as singlet triplet inter system conversion can lead to higher life time of the excited states, [30] any kinetic process that leads to enrichment of the excited singlet state can explain the amplification of fluorescence. The self assembly is known to be favored at low temperature [31] therefore fluorescence amplification should be more intense at low temperature. Magnetic field induced changes in the amplification pattern could imply the role of stacked pi ring assemblies which are known to respond to static magnetic field, [32].

- We thus have some verifiable way of exploring the exact mechanism by which this concentration , photon flux , or temperature driven bistability is induced , but the ecological and evolutionary implications seems evident.

V. CONCLUSION

Photon capture and its subsequent bio-energetic management is in the bottom of the ecological pyramid. We demonstrate multiplication of fluorescence emission in the red region for excitation at near UV region by a photosynthetic bacterium *R.capsulatus*. Importantly, the multiplicative emission occurs only if there is a critical bacterial population. A thermal switch puts off the multiplicative emission at lower temperature. Such low temperature driven repression of the emission gain is reversed in presence of a moderate dose of static magnetic field. The work suggests that one can employ the microbe as Maxwell's demon to minimize ecological damage caused by UV, as it absorbs UV and emits photo-chemically inert red light.

The amplification or gain of fluorescence with time in photosynthetic system *Rhodobacter capsulatus* is explainable by relative variations of singlet and triplet population upon continuous excitation. The single states are excitable while triplets are not , but a radical pair mechanism can lead to inter-convertibility of the two. The increase in formation of brighter species with average number of fluorochrome remaining constant can be explained from such considerations. At lower temperature there is higher propensity of triplet states. Hence no amplitude gain is observed with time and the bleaching phase dominates. As triplet states are sensitive to static magnetic field in presence of such field singlet like states can reappear and thus the amplitude gain mode re-appears. Overall one obtains an OR gate like situation when either high temperature or low temperature plus magnetic field can induce the amplitude gain mode.

We must mention the ecological significance of the result. It is intriguing to note that the principle of population inversion on which the lasers are made is exploited by one of the ancient life forms. The amplification of fluorescence can serve as a photonic agent of quorum sensing (which also involves induced excretion of chemical substances, in this case the inducer being photon).

The practical use of this concentration temperature and magnetic field sensitive system is evident and it may be possible to construct synthetic biological circuits by appropriate extrapolation of the above.

Acknowledgments

This work was supported by DBT (BT/PR3957/NNT/28/659/2013).

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TABLE I: **Life time values of the fluorophore present in photosynthetic complex at different concentrations (function of absorbance at 395 nm)**

Conc.mg $\cdot$ ml^{-1}	**Life time (ns)**
0.039	1.78, 15.7
0.113	1.90, 15.0
0.590	2.59, 15.6
1.206	2.74, 15.6
2.984	2.99, 15.6

Life time variation with respect to fluorophore concentration.

TABLE II: **The truth table shows OR gate mechanism between T and B switch of the system**

T>278K (O1)	**B (SMF 0.25 T) (O2)**	**Amplification (A)**
0	0	0
0	1	1
1	0	1
1	1	1

The table represents onset of amplification by either thermal switch or by magnetic switch. "1" denotes signal of amplified fluorescence whereas "0" means no amplification in fluorescence signal.)

FIGURE LEGENDS

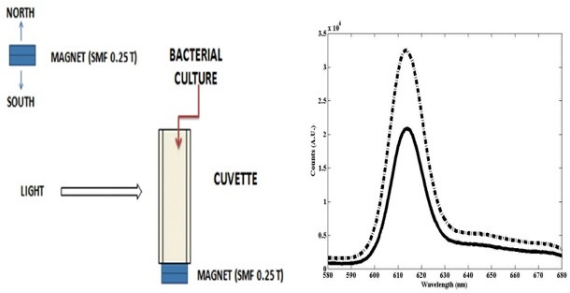

FIG. 1: **Left :Schematics for experimental set up of magnetic incubation during fluorimetric measurements. Right: Fluorescence emission spectra of the whole cells (solid line) and photosynthetic membrane complex isolated from bacterial cells (dotted line) with excitation at 395 nm**

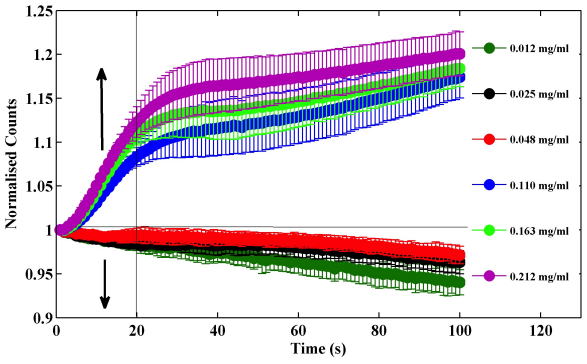

FIG. 2: **The figure shows how changing the fluorophore concentration switches the staeady states from bleaching to amplifying states of fluorescent emission. Fluorescence time scan normalized fluorescence intensity is plotted against concentration, error level being represented by the vertical lines. The bistability occurs between the critical concentration range** > 0.048 **but** $< 0.1mg \cdot ml^{-1}$**. The bleaching zone is marked by the concentration range** ≤ 0.048 **whereas the amplifying zone is marked by the range** $> 0.11mg \cdot ml^{-1}$

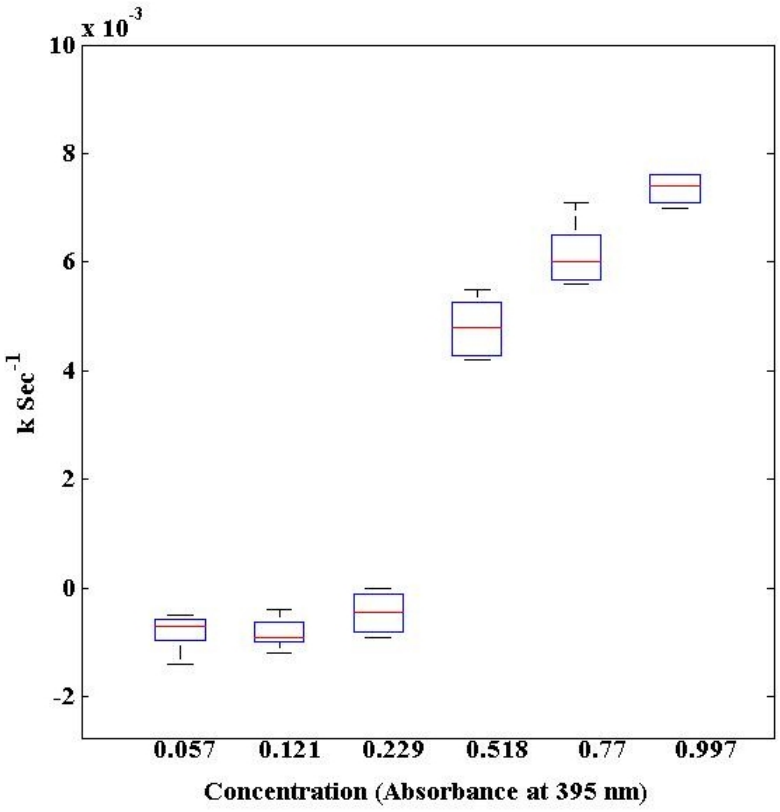

FIG. 3: **Fluorescence amplification rate is evaluated in dimensionless unit** $k(s^{-1}) = \frac{1}{concentration} \cdot \frac{d(concentration)}{dt}$ **for emission at 613 nm. k(** s^{-1} **) is plotted against concentration (the 395 nm absorbance in the abcissa corresponds to concentration 0.012,0.025,0.048,0.110,0.163,0.212** $mg \cdot ml^{-1}$ **respectively).**

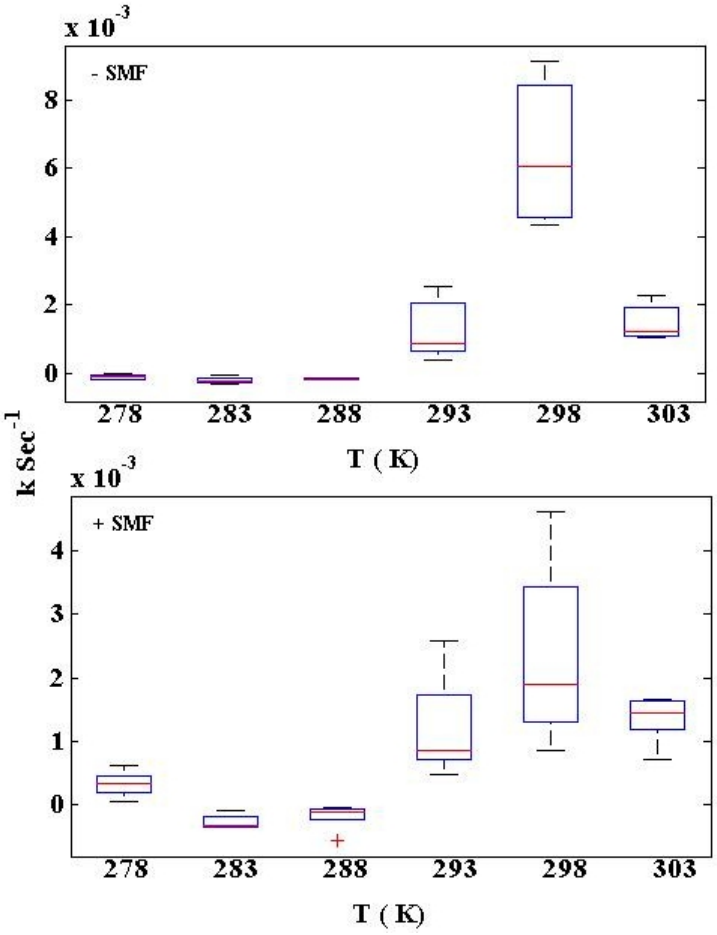

FIG. 4: **Fluorescence amplification rate at 613 nm (k s^{-1}) for excitation at 395 nm as function of T(in ^oK) in absence (upper panel) and presence (lower panel) of magnetic field.**

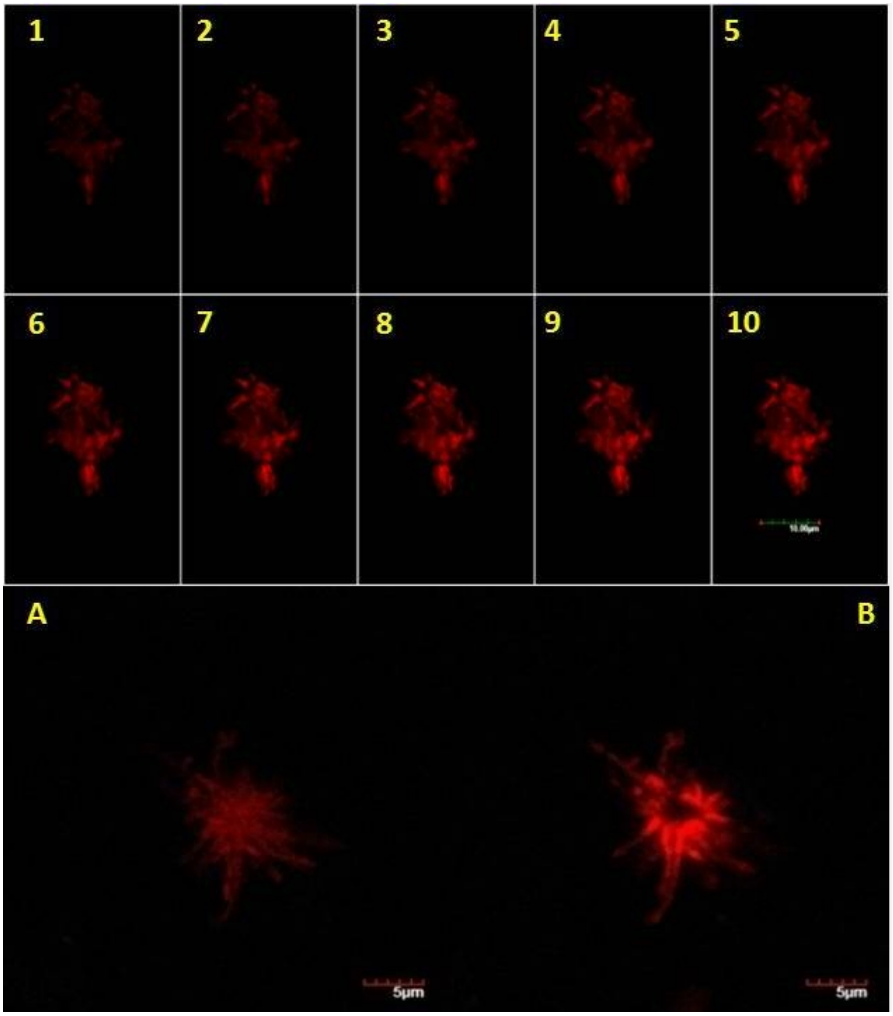

FIG. 5: **The upper panel shows gradual increase in fluorescence emission of bacterial cell cluster (100X magnified confocal image). Cells were exposed to 405 nm laser (25%intensity) up to 10 minutes, after each minute image was captured. The lower panel shows image of a cell cluster exposed for 60 seconds to 405 nm laser (100%intensity); A is the initial image of the cell cluster and B is image of the same, taken after 60 seconds of laser exposure. Where the central zone is bleached due to intense laser exposure whereas the perimeter that is comparatively less exposed is more illuminated.**