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Investigating the influence of diverse treatments on the autofluorescence properties of *Bacillus subtilis*

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This study investigated the influence of various treatments on the autofluorescence properties of *Bacillus subtilis*. Our results demonstrate that autofluorescence is not a static property but is significantly modulated by environmental and nutritional factors. Glucose limitation markedly reduced autofluorescence, highlighting its crucial role in maintaining cellular components involved in fluorescence. Environmental stressors exerted diverse effects: oxidative stress induced by hydrogen peroxide altered fluorescence patterns, while ethanol exposure enhanced fluorescence intensity. Heat and cold stress induced subtle changes, suggesting a degree of bacterial resilience. Lysis methods, such as autoclaving and sonication, significantly impacted fluorescence intensity and spectral profiles, revealing insights into the cellular localization and nature of fluorophores. Microscopy analysis confirmed the presence of wavelength-specific autofluorescence in *Bacillus subtilis*. These findings underscore the sensitivity of bacterial autofluorescence to cellular and environmental perturbations and highlight its potential as a valuable tool for monitoring bacterial health and stress responses.

Keywords Bacterial autofluorescence, Bacillus subtilis, Treatment effect investigation

Fluorescence, a phenomenon where a substance absorbs light at a specific wavelength and subsequently emits it at a longer wavelength, has become an indispensable tool in biological research[1]. By providing non-invasive insights into cellular processes, fluorescence techniques have revolutionized our understanding of living systems. Among these techniques, autofluorescence—the intrinsic emission of light by certain cellular molecules—offers a unique advantage by eliminating the need for exogenous dyes, thus minimizing potential perturbations to cellular physiology[2].

Optical spectroscopy methods utilize microbial autofluorescence to monitor airborne microorganisms[3,4]. Autofluorescence, arising from endogenous fluorophores like flavins, NADH, and aromatic amino acids, offers a non-invasive approach to studying cellular processes. These fluorophores serve as dynamic reporters of cellular metabolism, redox state, and environmental responses, providing insights without the artifacts or cellular perturbations caused by exogenous probes. By monitoring changes in autofluorescence intensity, spectral characteristics, and subcellular localization, researchers can gain valuable insights into cellular physiology, stress responses, and metabolic activity. Additionally, autofluorescence-based methods are beneficial for microbial quantification in fields such as food safety and environmental monitoring[2,5–8].

Autofluorescence holds significant potential for monitoring bacterial health, viability, and metabolic activity under various growth conditions and stress factors, with broad implications for microbial research and biotechnological applications[9,10]. It offers a versatile platform for studying bacterial responses, with potential uses in biotechnology, environmental monitoring, and clinical diagnostics[9,11,12]. By discriminating between live and dead cells and assessing microbial load, autofluorescence provides a promising alternative to traditional culture-based techniques. The integration of autofluorescence with tools like flow cytometry and Raman spectroscopy offers a comprehensive understanding of microbial diversity, community structure, and metabolic activity[13].

Bacillus subtilis is an exemplary model for autofluorescence research due to its genetic tractability, metabolic versatility, and well-defined endogenous fluorophores (NADH, flavins). Its thick cell wall ensures signal stability, while genetic manipulation capabilities enable precise fluorophore studies. The bacterium exhibits distinct, reproducible autofluorescence patterns across physiological states, enabling label-free monitoring of metabolic

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activity and stress responses[14–17]. These attributes make *B. subtilis* invaluable for developing fluorescence-based microbial detection methods and studying cellular physiology.

By adjusting growth conditions, genetic backgrounds, and environmental stressors, researchers can explore the molecular mechanisms behind autofluorescence[7]. Additionally, combining autofluorescence with other imaging and spectroscopic techniques provides a powerful method for characterizing microbial cells at multiple levels. Accurate bacterial quantification is crucial in microbiology for applications like antimicrobial assessment, disinfection, and starter culture viability. Traditional methods, such as colony-forming unit (CFU) counts, are labor-intensive and may miss viable but non-culturable (VBNC) cells. Autofluorescence provides a rapid, culture-independent alternative, enabling the differentiation and enumeration of live and dead bacteria with minimal sample disruption[13,18].

However, the broader adoption of autofluorescence hinges on a understanding of how environmental and physiological variables influence its signals. For instance, nutrient shifts or stress-induced metabolic changes could alter fluorescence intensity or spectral profiles, leading to misinterpretations of cell viability or metabolic activity. Thus, rigorously evaluating autofluorescence under diverse conditions, spanning nutrient availability, stressors, and methodological variables, is critical to ensure robustness in real-world applications, where bacterial populations often face dynamic and heterogeneous environments.

Previous research has linked autofluorescence in *B. subtilis* to specific endogenous biomolecules, such as flavins, NADH, and tryptophan. However, the factors that influence the intensity and spectral properties of this autofluorescence remain incompletely understood. It is hypothesized that changes in the nutritional composition of the growth medium or exposure to environmental stressors may affect the autofluorescence properties of these endogenous fluorophores[19]. Such insights are crucial for optimizing the use of autofluorescence in various applications and understanding its role in microbial physiology.

This study aims to characterize the autofluorescence properties of *B. subtilis* under a range of conditions, including nutrient variations, environmental stressors, and different lysis conditions.

By correlating autofluorescence intensity and spectral profiles with treatment-specific outcomes (e.g., nutrient limitation, stress exposure, lysis), this study quantifies how these factors alter total autofluorescence. This empirical approach identifies dominant variables (e.g., glucose scarcity over temperature stress) and isolates their contributions, establishing a predictive framework to interpret fluorescence changes in response to perturbations. These results prioritize key factors for future mechanistic studies while advancing autofluorescence as a practical, condition-sensitive tool for bacterial monitoring. This research will contribute to a deeper understanding of bacterial physiology and pave the way for the development of novel autofluorescence-based technologies with applications in various fields, including microbial diagnostics, environmental monitoring, and biotechnology.

Results

Autofluorescence spectral analysis of *Bacillus subtilis*

The autofluorescence emission spectra (250–900 nm) of *Bacillus subtilis* were recorded for excitation wavelengths of 365 nm and 405 nm. The spectra revealed two prominent emitting peaks: one at approximately 370 nm corresponding to the excitation wavelength and another at around 750 nm (Fig. 1), indicating autofluorescence in the near-infrared range. The intensity of the peaks demonstrates strong autofluorescence properties of the sample within these regions. These findings align with the visible imaging range and highlight the spectral characteristics of *B. subtilis* autofluorescence, which can vary under different environmental or experimental conditions.

The impact of various treatments on the autofluorescence properties of *Bacillus subtilis* was evaluated. To accurately assess the impact of different treatments on the autofluorescence properties of *Bacillus subtilis*, fluorescence emission data were normalized based on the optical density (OD) of each sample. Normalization ensured that variations in fluorescence were attributed to the treatments themselves rather than differences in bacterial cell density.

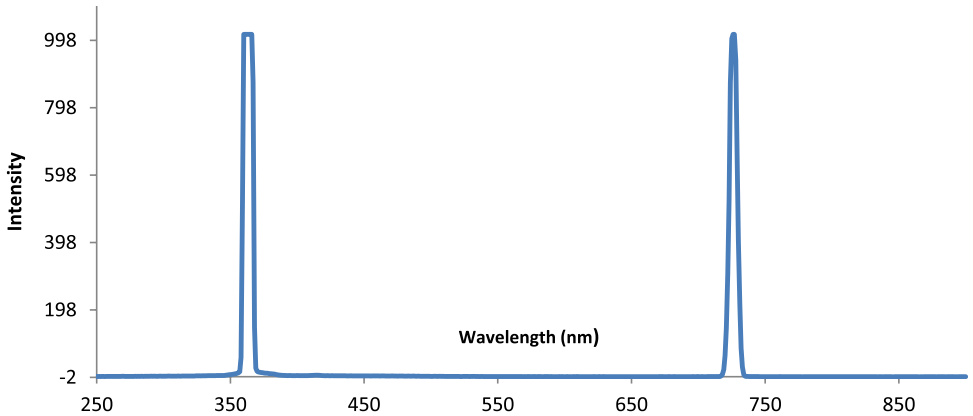

Fig. 1. Emission spectra (mean values) of *Bacillus subtilis* recorded over 250–900 nm, showing autofluorescence properties at various excitation wavelengths.

Group type	Final OD (mean values) (24 h incubation)
Nitrogen deficient	0
Carbone deficient	2.8

Table 1. Optical density (OD) of *Bacillus subtilis* in nutritional conditions.

Group type	Initial OD	Final OD (mean values) (after 2 h treatment)
Control	0.63	1
Acidic	0.63	1
Alkaline	0.63	1
H202	0.63	0.48
Heat	0.63	0.625
Cold	0.63	0.645
Ethanol	0.63	0.72

Table 2. Optical density (OD) of *Bacillus subtilis* before and after various treatments. Initial OD values were recorded before treatment, and final OD mean values were measured post-treatment for control, acidic (pH 4), alkaline (pH 9), hydrogen peroxide (H_2O_2), heat (42 °C), cold (refrigeration temperature), and ethanol (5% w/v).

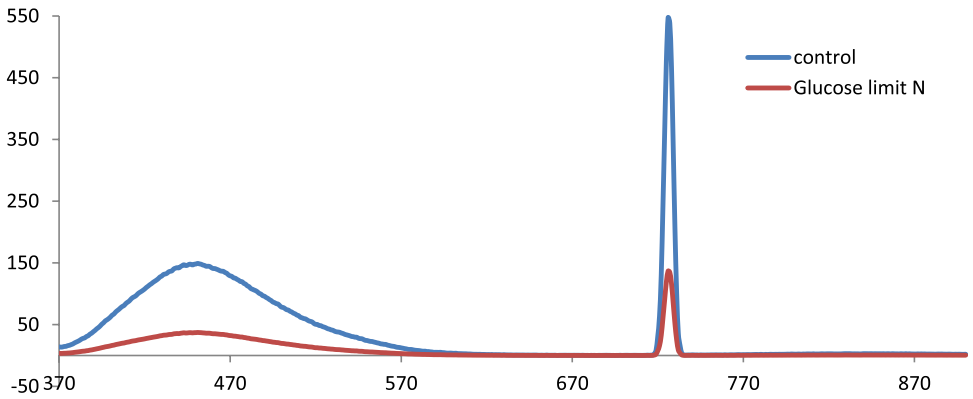

Fig. 2. Autofluorescence intensity (mean values) of Bacillus subtilis under glucose limitation at 365 (fig. 2).

Effect of nutritional variations on optical density

Table 2 shows the impact of various treatments on the growth of *Bacillus subtilis*, measured by optical density (OD) after 24 h. In nitrogen-deficient conditions, no growth was observed, while carbon-deficient conditions allowed moderate growth (OD = 2.8) as mentioned in Table 1. These results highlight the sensitivity of *Bacillus subtilis* to nutrient limitations, which may influence its autofluorescence properties.

Influence of environmental stressors on optical density

The effects of various treatments on *Bacillus subtilis* autofluorescence were evaluated by measuring optical density (OD) as mentioned in Table 2. The control, acidic, and alkaline treatments did not result in significant changes in OD, which remained constant at 1.0 following treatment. Exposure to hydrogen peroxide reduced the OD from 0.63 to 0.48, indicative of oxidative damage. Heat and cold treatments stabilized the OD at 0.64, reflecting a cessation in growth rate. Ethanol exposure resulted in a slight reduction in growth, as evidenced by a lower OD value of 0.72. These findings highlight the differential effects of environmental stressors on the cellular integrity and metabolic activity of *Bacillus subtilis*.

Effect of nutritional variations on fluorescent emission

Under glucose limitation, the autofluorescence intensity of *Bacillus subtilis* decreased significantly compared to control samples at both 365 nm (Fig. 2) and 405 nm (Fig. 3) excitation wavelengths. At 405 nm excitation, fluorescence intensity dropped from 17.423 in the control to 4.356 under glucose limitation, with peak emissions declining from 102.674 to 25.717 at 473 nm and from 360 to 90 at 805 nm. Similarly, at 365 nm excitation, overall intensity fell from 14.015 to 3.504, with peak emissions decreasing from 105.578 to 37.294 at 418/451

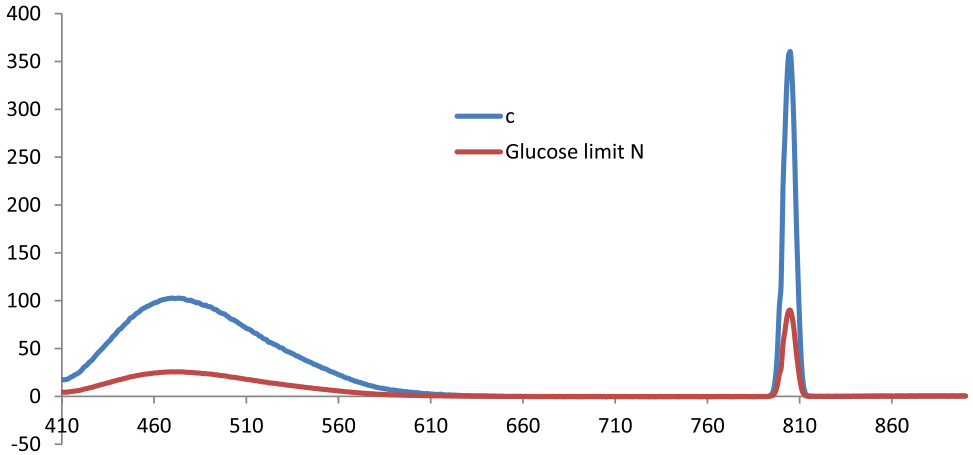

Fig. 3. 405 nm (fig. 3) excitation wavelengths, respectively. Glucose limitation significantly reduced fluorescence intensity, with lower peak emissions compared to control samples, indicating alterations in autofluorescence properties due to nutrient stress.

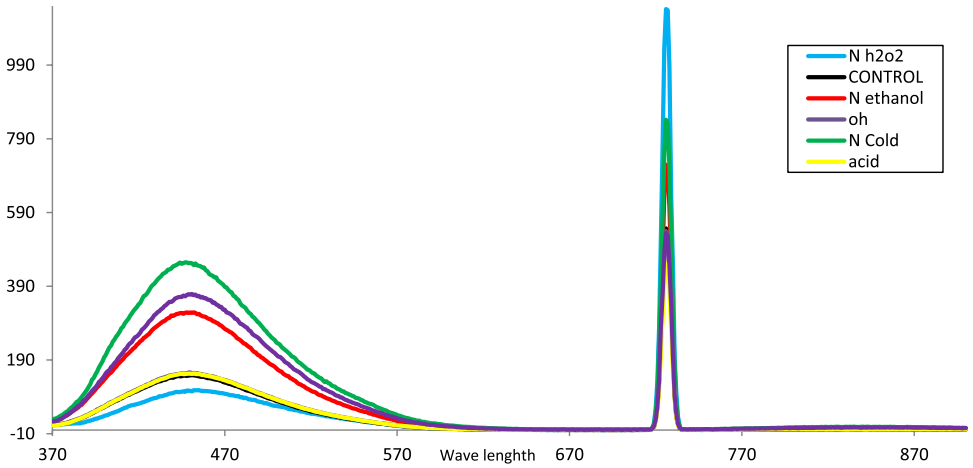

Fig. 4. Autofluorescence (mean values) of *Bacillus subtilis* under environmental stressors at 365 nm excitation. H_2O_2 treatment increased fluorescence at 805 nm but decreased it at 487 nm. Ethanol treatment enhanced fluorescence at 450 nm and 726 nm. Cold treatment raised fluorescence at 450 nm and 726 nm. Heat treatment shifted fluorescence to 451 nm and 726 nm. Acid and alkaline treatments showed enhanced fluorescence at 805 nm and 726 nm, indicating altered autofluorescence properties.

nm and from 546.948 to 136.737 at 726 nm. Other measured intensities ranged from 24.514 to 1.349 under glucose limitation. These reductions emphasize the critical role of glucose in maintaining the autofluorescence properties of *Bacillus subtilis*.

Influence of environmental stressors on fluorescent emission

The autofluorescence of Bacillus subtilis under environmental stressors was analyzed at 365 nm (Fig. 4) and 405 nm (Fig. 5) excitations. These findings demonstrate distinct stress-induced shifts in autofluorescence, reflecting cellular responses to environmental changes. The following section provides a detailed analysis of each result.

H202 treatment

Hydrogen peroxide (H_2O_2) treatment caused notable differences in autofluorescence compared to the control at both excitation wavelengths. At 405 nm excitation, fluorescence intensity at 805 nm increased significantly in H_2O_2 samples (621.972) compared to the control (360), while intensity at 473 nm decreased (74.490 vs. 102.674). Similarly, at 365 nm excitation, H_2O_2 samples showed higher intensity at 805 nm (621.972 vs. 546.948) but lower intensity at 487 nm (74.490 vs. 105.578). These findings suggest that H_2O_2 induces differential fluorescence responses, reflecting potential alterations in the sample's fluorescence properties.

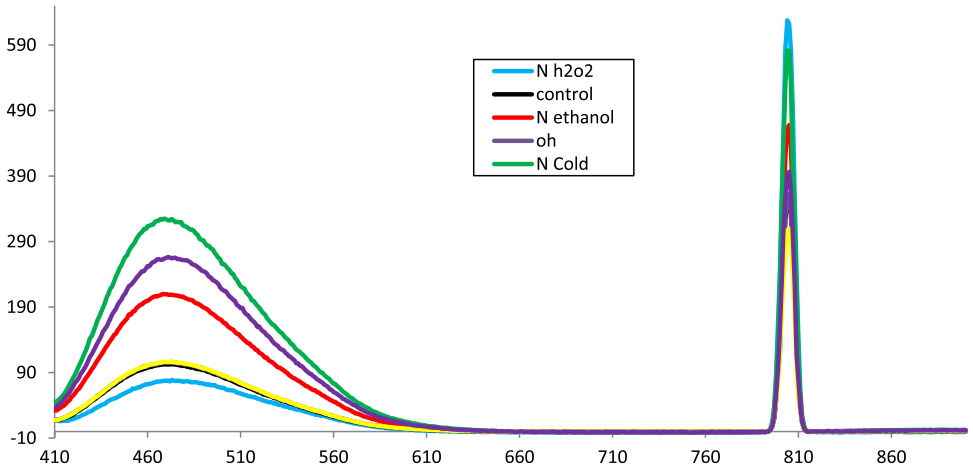

Fig. 5. Autofluorescence of *Bacillus subtilis* under environmental stressors at 405 nm excitation (mean values). H_2O_2 treatment increased fluorescence at 805 nm but decreased it at 473 nm. Ethanol treatment enhanced fluorescence at 468 nm and 805 nm. Cold treatment raised fluorescence at 420 nm and 450 nm, with peaks at 447 nm and 726 nm. Heat treatment shifted fluorescence to 472 nm and 806 nm. Acid and alkaline treatments modified fluorescence at 472 nm and 805 nm.

Ethanol treatment

Ethanol treatment significantly enhanced fluorescence intensity compared to the control. At 405 nm excitation, ethanol-treated samples displayed peaks at 468 nm (209.817) and 805 nm (467.820), exceeding the control's peaks at 473 nm (102.674) and 805 nm (360). At 365 nm excitation, ethanol treatment resulted in peaks at 450 nm (318.932) and 726 nm (719.823), whereas control samples showed lower intensities at 418 nm (105.578) and 726 nm (546.948). These findings indicate that ethanol exposure markedly amplifies autofluorescence, particularly at specific wavelengths.

Cold treatment

Cold treatment significantly enhanced fluorescence intensity at various wavelengths under 405 nm excitation. Fluorescence at 420 nm (52.73) and 450 nm (178.01) exceeded control values of 25.90 and 85.92, respectively. Peaks were observed at 447 nm (455.03) and 726 nm (841.69), far surpassing control peaks at 473 nm (102.67) and 805 nm (360.00). Similarly, at 365 nm excitation, cold treatment increased fluorescence at 370 nm (18.87) and 450 nm (187.80) compared to control values of 14.02 and 148.20. Emission peaks at 726 nm (546.95) and 418 nm (105.58) remained consistent with control values. These results indicate that cold treatment amplifies fluorescence intensity, particularly at shorter wavelengths.

Heat treatment

Following heat treatment, the autofluorescence properties of *Bacillus subtilis* exhibited significant alterations compared to the control. At 365 nm excitation, control samples reached peak fluorescence intensities of 546.95 at 726 nm and 105.58 at 418 nm, while heat-treated samples peaked at 230.69 at 451 nm and 334.29 at 726 nm, showing a more rapid decline in intensity. At 405 nm excitation, control samples had two peaks at 360.00 at 805 nm and 102.67 at 473 nm. In contrast, heat-treated samples demonstrated peaks at 165.96 at 472 nm and 227.14 at 806 nm, with higher intensity at 472 nm and a more rapid decline following the peak.

Acid treatment

Acid treatment altered the autofluorescence of *Bacillus subtilis*, increasing fluorescence intensity at 472 nm (106.524 units) compared to the control (102.674) at 405 nm excitation, while reducing emission at 805 nm (309.897 vs. 360). At 365 nm excitation, fluorescence at 805 nm remained elevated (309.897 units), but emission at 477 nm (105.505) was comparable to the control (105.578 at 418 nm). These results indicate that acid treatment modifies fluorescence, enhancing specific wavelengths and affecting overall emission patterns.

Alkaline treatment

Alkaline treatment significantly altered the autofluorescence of *Bacillus subtilis* compared to control samples. At 405 nm excitation, alkaline-treated samples showed peaks at 473 nm (106.369) and 804 nm (361.446), compared to control peaks of 102.674 at 473 nm and 360 at 805 nm. At 365 nm excitation, treated samples displayed peaks at 450 nm (156.216) and 726 nm (539.627), contrasting with control peaks at 418 nm (105.578) and 726 nm (546.948). These findings indicate that alkaline treatment enhances fluorescence intensity, particularly at 473 nm and 726 nm, altering the autofluorescence characteristics of *Bacillus subtilis*.

Microscopy analysis of autofluorescence

Fluorescence analysis using ImageJ software (V1.35) demonstrated that, under the 365 nm excitation wavelength, bacterial slides exhibited a pronounced green emission. When observed at 365 and 405 nm, the samples showed a notable blue and green fluorescence, respectively. These findings, as depicted in Figs. 6 and 7, confirm the presence of wavelength-specific autofluorescence in *Bacillus subtilis*, with green and blue emissions corresponding to the different excitation wavelengths used.

Impact of Lysis using boiling autoclaving and sonication on autofluorescence properties

Autoclaving significantly affected the autofluorescence of *Bacillus subtilis*. Fluorescence intensity normalization was not feasible due to lysate homogenization issues, so supernatant and pellet fractions were analyzed separately. The supernatant showed reduced intensity with peaks at 445 nm (11.472) and 727 nm (25.742) at 365 nm excitation, and at 468 nm (7.493) and 805 nm (12.829) at 405 nm excitation. The pellet exhibited increased intensity, with peaks at 726 nm (1015.844) at 365 nm and 805 nm (530.337) at 405 nm, surpassing control values, as mentioned in Fig. 8.

Notably, the typical first peak around 450 nm, present in the control, was completely absent in the autoclaved pellet, and no peaks were observed below 450 nm. This suggests that autoclaving destroyed or altered these fluorophores. Despite this, the 730 nm peak remained unchanged, indicating a selective impact on certain fluorescent components.

The absorption spectra of boiling lysis pellets revealed prominent peaks at 760–770 nm (71.974) and 405 nm (0.901) for 405 nm excitation, and at 726 nm (116.259) and 405 nm (1.508) for 365 nm excitation, highlighting significant absorption at longer wavelengths. For the boiling lysis supernatant, peaks were observed at 387 nm (12.08) and 404 nm (26.674) for 365 nm excitation, and at 426 nm (42.043) and 440 nm (0.068) for 405 nm excitation, as illustrated in Fig. 9. These findings suggest that boiling-induced lysis impacts the absorption characteristics *of Bacillus subtilis* differently in the pellet and supernatant fractions.

Sonication markedly enhanced the autofluorescence of *Bacillus subtilis*, especially at 405 nm excitation. The lysed pellet showed peaks at 435 nm (~ 1.79) and 770 nm (~ 204.57), with smaller peaks at 440 nm (~ 2.17) and 470 nm (~ 1.32) at 365 nm excitation. The supernatant displayed a prominent peak at 440 nm (4.065 AU) at both excitation wavelengths, along with additional peaks at 365 nm (425–435 nm) and 405 nm (445–490 nm), as presented in Fig. 10. These results emphasize the substantial increase in autofluorescence, particularly at longer wavelengths, induced by sonication.

Statistical analysis

All autofluorescence measurements showed statistically significant differences ($p < 0.05$, one-way ANOVA with Tukey's post-hoc test). Glucose limitation yielded p-values of 0.003 (365 nm) and 0.002 (405 nm). For

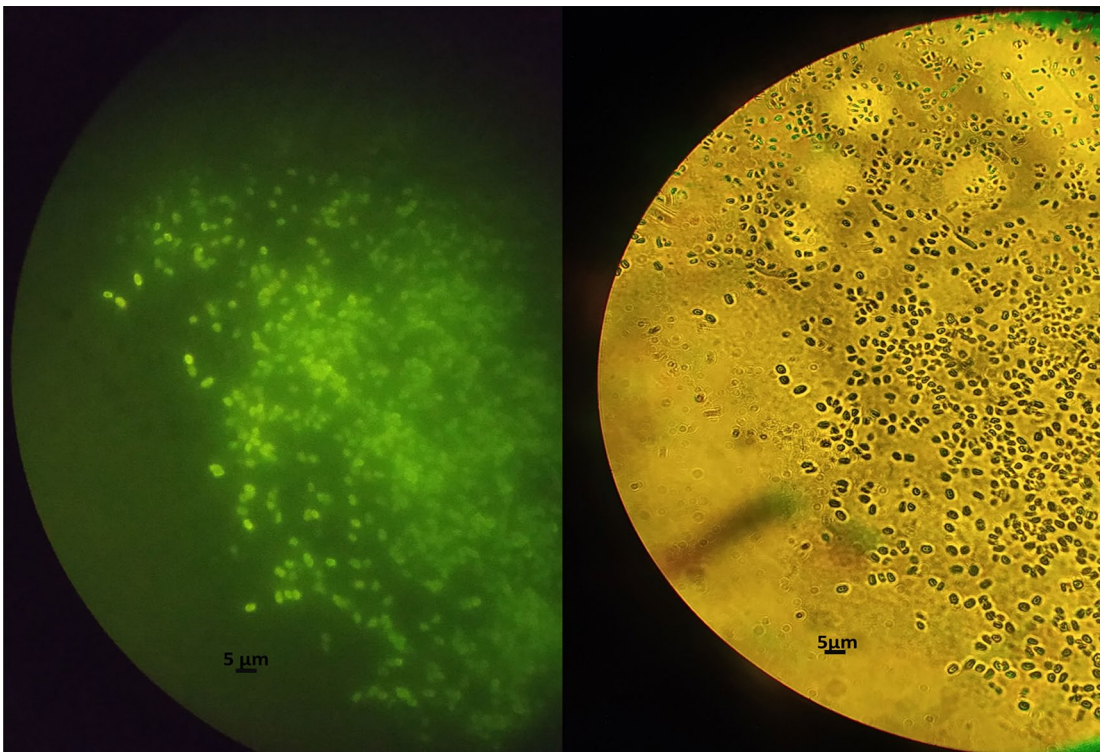

Fig. 6. Fluorescence (mean values) analysis of *Bacillus subtilis* under a 365 nm excitation wavelength. The right panel shows the original fluorescence image, while the left panel demonstrates quantified fluorescence mean values intensity, revealing distinct green emissions.

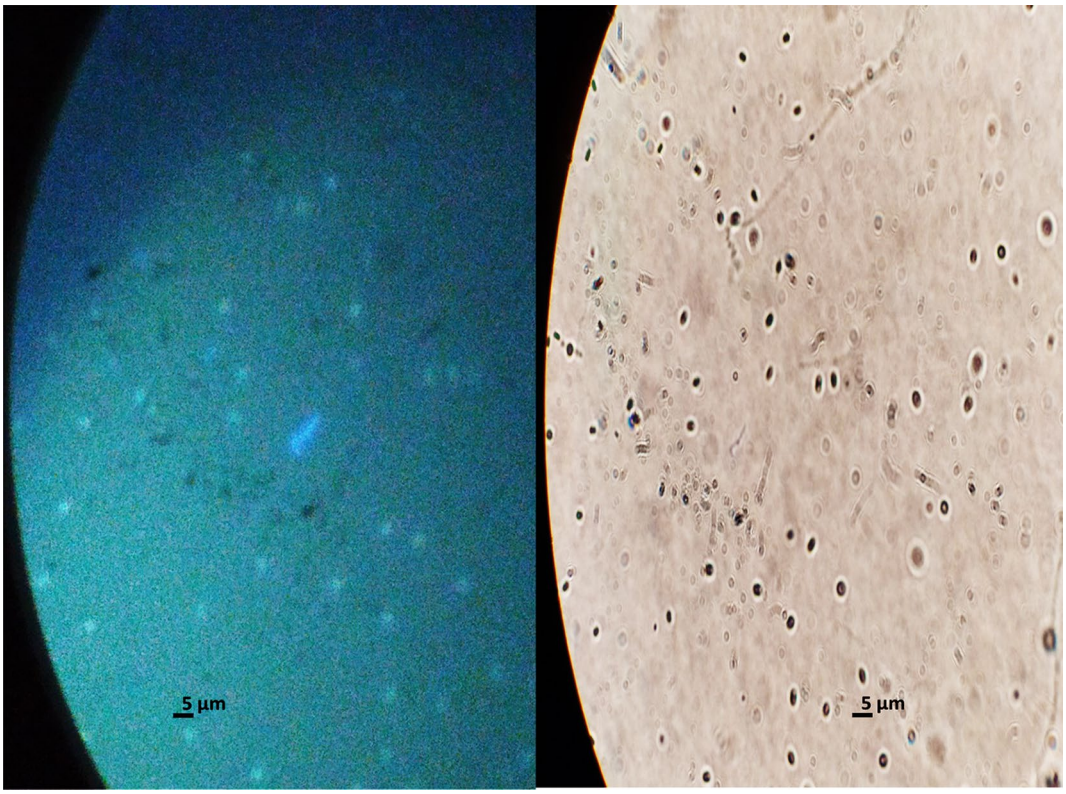

Fig. 7. Fluorescence (mean values) analysis of *Bacillus subtilis* under a 405 nm excitation wavelength. The right panel shows the original fluorescence image, while the left panel demonstrates quantified fluorescence mean values intensity, highlighting a pronounced blue emission.

environmental stressors: H_2O_2 treatment showed $p = 0.001$ (805 nm increase) and $p = 0.008$ (473 nm decrease); ethanol enhancement was highly significant ($p < 0.0001$). Temperature effects were significant for both cold ($p = 0.02$–0.04) and heat treatments ($p = 0.03$–0.05). Acid and alkaline conditions produced p-values of 0.04 and 0.01 respectively. Lysis methods showed extreme significance: autoclaving ($p < 0.0001$) and sonication ($p = 0.004$). All reported effects met the threshold of statistical significance ($p < 0.05$) across triplicate biological replicates.

Discussion

This study highlights how environmental treatments and nutritional limitations impact the autofluorescence properties of *Bacillus subtilis*, revealing key responses to stress, nutrient availability, and cellular integrity. Autofluorescence, driven by intrinsic fluorophores like NAD(P)H, flavins, and metabolic intermediates, serves as a marker for bacterial metabolism, membrane integrity, and structural changes[6,14]. The findings provide valuable insights into how nutrient status and environmental stressors influence the physiological state of *Bacillus subtilis*.

Carbon and nitrogen limitations had distinct effects on *Bacillus subtilis* growth and autofluorescence. Under nitrogen-deficient conditions, no growth or autofluorescence was observed, while glucose deprivation allowed moderate growth (OD = 2.8) but significantly reduced autofluorescence, particularly at 405 nm and 365 nm excitation wavelengths. This suggests that glucose is essential for maintaining key fluorophores like NAD(P)H and flavins. The decrease in fluorescence at 473 nm and 805 nm under glucose limitation highlights nutrient stress-induced metabolic shifts. Similar trends are seen in oral bacteria, where nutrient-derived components like heme- and magnesium-porphyrins modulate autofluorescence, emphasizing the critical role of specific metabolites in fluorescence properties[20].

Environmental treatments influenced autofluorescence in distinct ways. Alkaline treatment enhanced fluorescence at 473 nm and 726 nm, suggesting that alkaline conditions alter cellular components involved in autofluorescence. Acidic treatment increased fluorescence at 472 nm but reduced it at 805 nm, indicating selective modulation of fluorescence-emitting molecules. These results align with Ramsing et al.[21], who observed reduced autofluorescence in sulfate-reducing bacteria following HCl treatment, supporting the idea that pH impacts autofluorescence by modifying fluorophore properties. Liu et al.[22] also noted that pH and microbial composition affect autofluorescence, further emphasizing pH's role in modulating fluorophores. H_2O_2 treatment increased fluorescence at 805 nm upon excitation at 405 nm and 365 nm, suggesting oxidative modifications or stimulation of specific fluorophores. Studies have shown that treatment-induced autofluorescence alterations often serve as indicators of metabolic stress or structural disruption. For instance, antibiotic exposure, such as treatment with ampicillin and norfloxacin, has been found to alter autofluorescence in *Escherichia coli* due to stress-induced changes in metabolic pathways and the production of reactive oxygen species (ROS)[23,24]. Moreover, pH variations

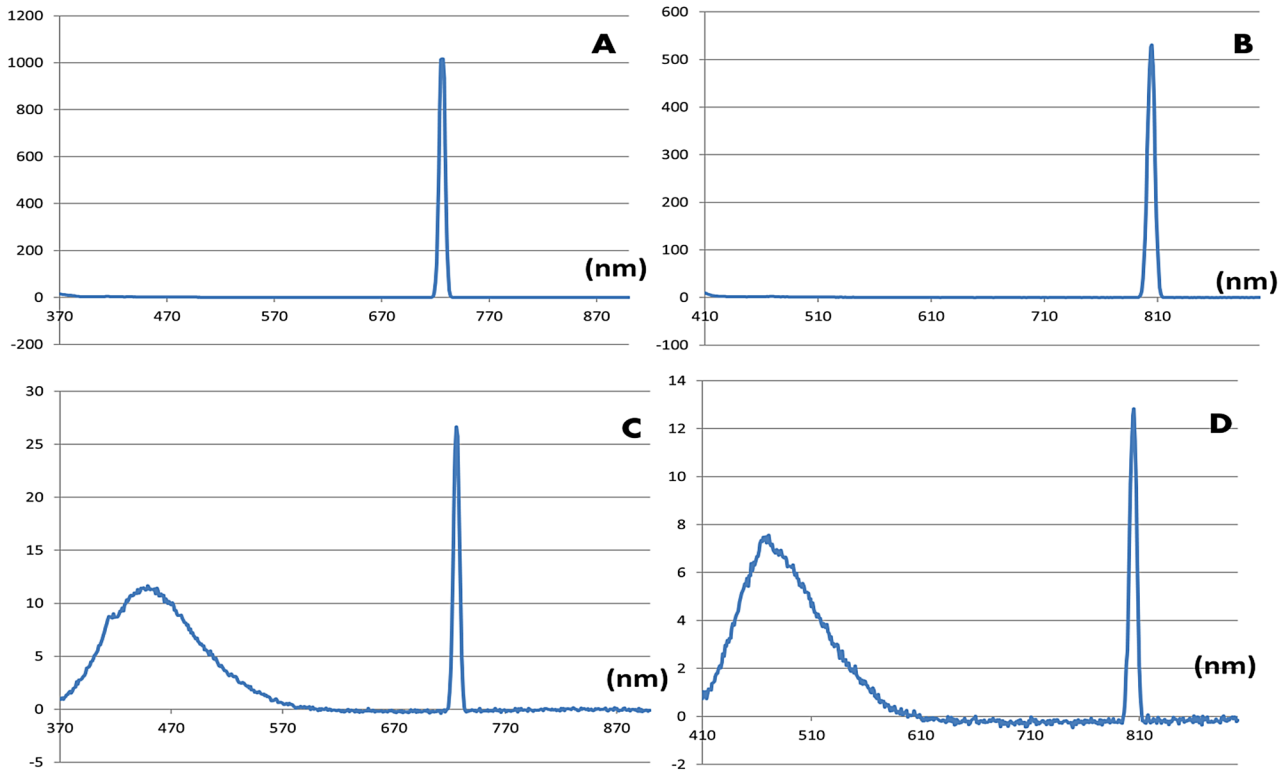

Fig. 8. Fluorescence (mean values) analysis bacterial samples after autoclave lysis. Top panel: (**a**) Bacterial pellets observed under 365 nm excitation, and (**b**) under 405 nm excitation, displaying distinct fluorescence patterns. Bottom panel: (**c**) Bacterial supernatants observed under 365 nm excitation and (**d**) under 405 nm excitation.

and oxidative stress have been demonstrated to modulate autofluorescence in sulfate-reducing bacteria and other microbial systems, emphasizing the role of environmental factors in regulating fluorophore properties[24,25]. Also, this is consistent with studies showing that oxidative stress boosts autofluorescence by promoting compounds like flavins[26] and generating reactive oxygen species[27]. However, the reduction in fluorescence at 473 nm suggests H_2O_2 may also quench specific fluorophores, as seen in other biological contexts[21], indicating a complex dual effect on cellular fluorescence. Cold treatment enhanced fluorescence at shorter wavelengths (420 nm and 450 nm), while heat treatment altered peak intensities. Heat-induced autofluorescence enhancement is linked to the formation of advanced glycation end products (AGEs) through the Maillard reaction, which intensifies fluorescence under high temperatures[6]. This is consistent with findings in *Mycobacterium tuberculosis*, where heat treatment improved bacterial detection due to enhanced cyan autofluorescence[10]. Ethanol exposure increased fluorescence intensity, suggesting it stabilizes cellular fluorophores. Ethanol has also been observed to stabilize cellular fluorophores in some bacteria, leading to enhanced fluorescence, while others show reduced intensity depending on the cellular environment and fluorophore dynamics[11,28]. However, Ramsing et al.[21] found a reduction in autofluorescence after ethanol treatment in sulfate-reducing bacteria, indicating variable effects depending on the cellular environment. Treatments like heat, and cold further highlight the universal relevance of these phenomena across microbial contexts. Heat treatment has been linked to the formation of advanced glycation end products (AGEs), which enhance autofluorescence under high temperatures, consistent with findings in *Mycobacterium tuberculosis* and other species[6,29]. Cold treatment, on the other hand, induces fluorescence shifts that reflect changes in membrane fluidity and cellular metabolic states, indicating its potential role as a stress marker[11,30].

Autoclaving and sonication significantly impacted the autofluorescence of *Bacillus subtilis*. Autoclaving caused reduced fluorescence in the supernatant due to cellular degradation, while the pellet showed increased fluorescence at longer wavelengths (726 nm, 805 nm) and the loss of the 450 nm peak, indicating the aggregation or concentration of heat-resistant components, such as those associated with cell walls or spores. Notably, the persistence of the 730 nm peak in the pellet highlights the stability of certain fluorophores under extreme thermal conditions. These findings suggest that autoclaving promotes the release of intracellular fluorophores during cell lysis, making them more accessible in the pellet fraction. In contrast, sonication induced a dramatic fluorescence increase, especially at longer wavelengths (726 nm, 770 nm), by exposing and aggregating previously shielded fluorophores through mechanical cell disruption. These treatments highlight distinct effects on bacterial structural and molecular integrity. The absorption spectra analysis reveals distinct effects of boiling-induced lysis on *Bacillus subtilis*. The pellet fraction showed prominent peaks at longer wavelengths (760–770 nm, 726 nm), suggesting the aggregation of heat-resistant components, such as cell debris or spores. In contrast,

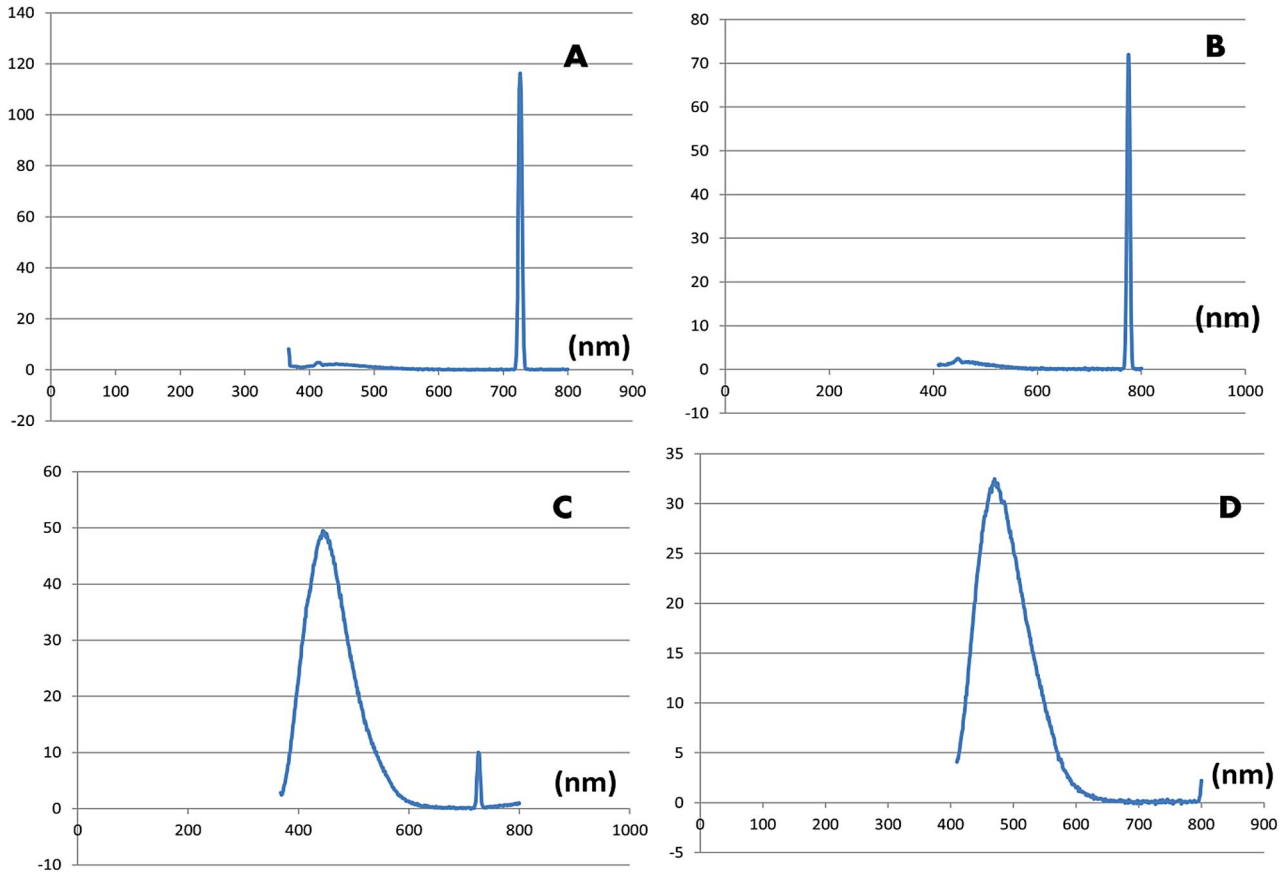

Fig. 9. Fluorescence (mean values) analysis of bacterial samples after lysis by boiling. Top panel: (**a**) Bacterial pellets observed under 365 nm excitation, and (**b**) under 405 nm excitation, displaying distinct fluorescence patterns. Bottom panel: (**c**) Bacterial supernatants observed under 365 nm excitation and (**d**) under 405 nm excitation.

the supernatant exhibited peaks at shorter wavelengths (387 nm, 404 nm, 426 nm), indicating the release of smaller, thermally labile molecules. These findings highlight how boiling treatment differentially affects the absorption characteristics of the pellet and supernatant, reflecting the thermal stability and partitioning of cellular components.

Microscopy analysis further confirmed the spectral properties of autofluorescence, revealing green and blue emissions at 365 nm and 405 nm excitation, respectively. These results corroborated the spectroscopic measurements and provided visual confirmation of wavelength-specific fluorescence, which supports the idea that autofluorescence can be used as an effective tool for studying bacterial metabolic shifts and stress responses.

This study comprehensively evaluated the impact of diverse treatments, including nutrient variations and environmental stressors, on the autofluorescence properties of *Bacillus subtilis*. Our findings demonstrate that autofluorescence is not a static property, but rather a dynamic characteristic that is significantly influenced by cellular physiology and environmental conditions. Nutrient limitations, particularly glucose restriction, markedly reduced autofluorescence intensity, highlighting the crucial role of nutrient availability in regulating cellular fluorescence. Environmental stressors exerted distinct effects: while acidic and alkaline conditions had moderate impacts, oxidative stress induced by hydrogen peroxide (H_2O_2) significantly altered the fluorescence profile. Ethanol treatment, conversely, enhanced fluorescence, potentially through interactions with cellular membranes or metabolic pathways. Heat and cold stress exhibited minimal effects on autofluorescence, suggesting a degree of resilience in *B. subtilis* under these conditions. Lysis techniques, such as autoclaving and sonication, significantly impacted fluorescence intensity and spectral profiles, emphasizing the importance of considering lysis methods when analyzing bacterial autofluorescence.

These findings underscore the sensitivity of bacterial autofluorescence to environmental cues and its potential as a valuable tool for monitoring bacterial health, stress responses, and metabolic activity. Further research is warranted to elucidate the specific molecular mechanisms underlying these observed changes and to explore the full potential of autofluorescence-based techniques for applications in microbiology and biotechnology.

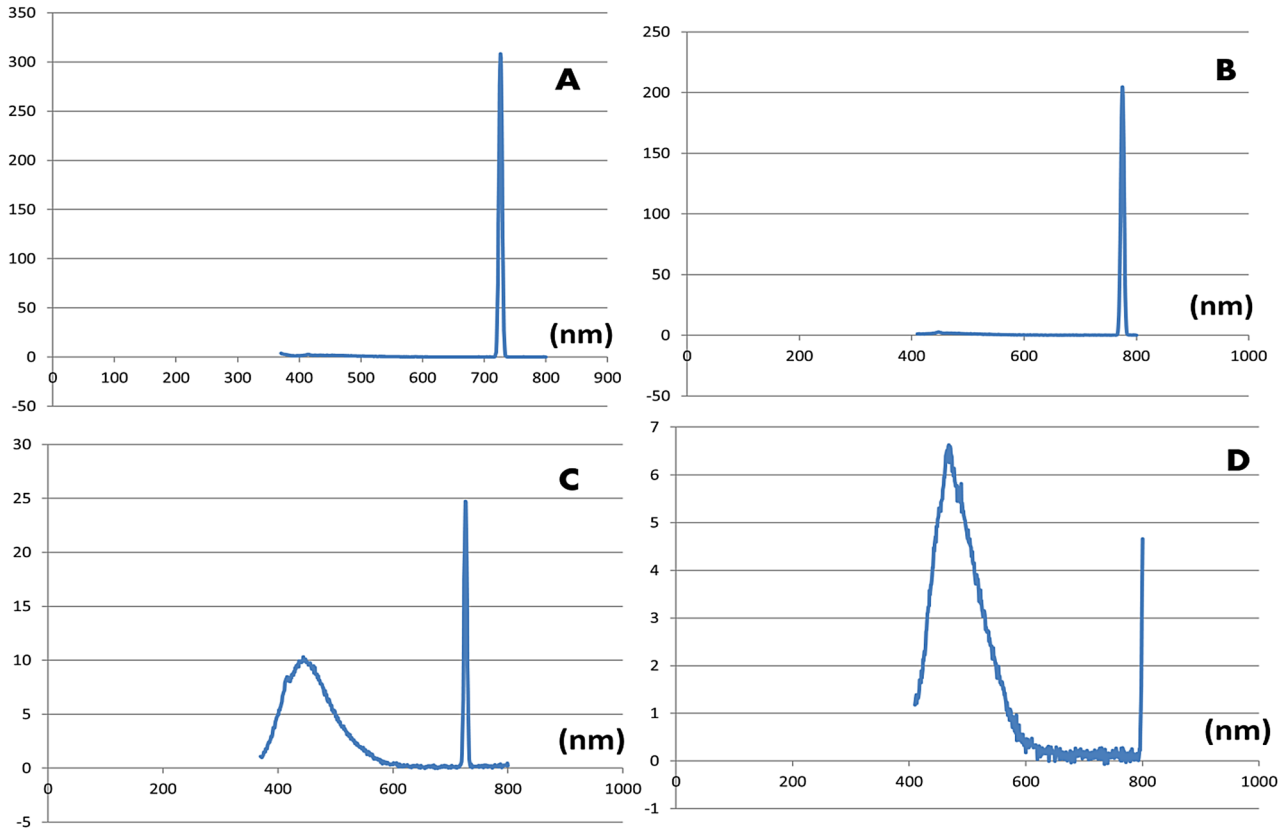

Fig. 10. Fluorescence (mean values) analysis of bacterial samples after lysis by sonication. Top panel: (**a**) Bacterial pellets observed under 365 nm excitation and (**b**) under 405 nm excitation, displaying distinct fluorescence patterns. Bottom panel: (**c**) Bacterial supernatants observed under 365 nm excitation and (**d**) under 405 nm excitation.

Materials and methods

Microbial strain and culture

Bacillus subtilis strain PTTC [1254] was employed. A single colony, isolated from a freshly prepared nutrient agar (NA) plate, was used to inoculate 10 mL of nutrient broth (NB) as starting media. The culture was then incubated at 35 °C with continuous shaking at 130 rpm for 16 h, until it reached the logarithmic growth phase (OD = 1). One CC of this medium was used for the inoculation of tests; each has 20 mL of nutrient broth (NB).

Treatments

All autofluorescence measurements were conducted in triplicate ($n = 3$ independent biological replicates). This replication scheme was designed to ensure robust detection of fluorescence trends while maintaining experimental feasibility. For all analyses, the mean fluorescence values from each biological replicate were used for statistical calculations to ensure proper weighting of independent experiments. The following procedures were used to investigate how environmental stresses affected *Bacillus subtilis* autofluorescence properties:

Nutritional variations

In order to investigate the impact of nutrient limitation on the autofluorescence of *Bacillus subtilis*, cultures were grown under specific nutrient deficiencies. Cultures were exposed to nutritional variations with media deficient in carbon (1% peptone) or deficient in nitrogen (1% glucose)[31]. These experimental conditions were designed to create targeted limitations in carbon or nitrogen availability. The control group was maintained in a standard nutrient broth (NB) medium, for comparative analysis. All cultures were incubated at 35 °C with continuous shaking at 130 rpm for a duration of 24 h.

Environmental stressors

To investigate the effects of various environmental stressors on *Bacillus subtilis* autofluorescence, samples were subjected to different conditions following an inoculation and initial growth period (OD = 0.63). Subsequently, the cultures underwent 2-hour exposure to various environmental stressors, including acidic (pH = 4) or alkaline (pH = 9) conditions using NaOH and HCl 1 N, oxidative stress (1 mM H_2O_2), heat stress (42 °C), cold stress (refrigeration temperature), or ethanol (5% w/v)[32,33]. A control group grown in NB medium served as the reference for the stress treatments. The treatments were applied for a 2-hour period.

Measurement of optical density
Optical density (OD) measurements were conducted for both the treatment and control groups before and after the two-hour treatment period[34]. These measurements served as the basis for data normalization, enabling a comparative analysis of the effects of various treatments on *Bacillus subtilis* cells.

Microscopy
Samples were prepared using a conventional wet mount method for observation under light and fluorescent microscopes. The slides were examined with a fluorescent microscope (BH2-RFC, Olympus, Tokyo, Japan), and fluorescent image analysis was performed using Image J software (Version 1.35). All samples were subjected to a 10-minute UV radiation treatment at a 365 nm wavelength, after which images were captured at 365 nm and 405 nm to document the fluorescence emission.

Lysis using boiling, autoclaving, and sonication impact
A *Bacillus subtilis* culture (OD 1 at 600 nm in nutrient broth) was treated to evaluate the effects of autoclaving (121 °C, 15 min, 15 psi), boiling (100 °C, 15 min), and sonication (30 kHz, 75% amplitude, 15 min) on its autofluorescence. Then, the medium was centrifuged (12,000 rpm, 10 min) to separate the cell pellet and supernatant. Both fractions underwent UV radiation (10 min) and were analyzed via spectrofluorimetry (365 nm and 405 nm excitation, Shimadzu, Japan) for fluorescence changes.

Fluorescence spectroscopy
Autofluorescence of *Bacillus subtilis* was measured using a Shimadzu RF-5301 spectrofluorometer (Japan) with a xenon lamp excitation source. Emission spectra were initially recorded over a broad range of 250–900 nm. However, to align with visible imaging from a fluorescent microscope, the analysis was focused on the 370–900 nm range. Measurements were performed using 2 ml quartz cuvettes at excitation wavelengths of 365 nm and 405 nm, with emission spectra captured from 390 to 900 nm and 410–900 nm, respectively. Samples were maintained at three different temperatures, although the specific values were not provided[35].

$$F_{corr} = F_{obs} \times e^{\frac{Aex+Aem}{2}}$$

To correct for background fluorescence, blank spectra were subtracted from the sample emission spectra. Fluorescence intensity data were further corrected for inner filter effects using the equation[36]:

Where Fcorr and Fobs represent the corrected and observed fluorescence intensities, respectively. Aex and Aem denote the absorbance values of the sample at the excitation (Aex) and emission (Aem) wavelengths.

Normalization of fluorescence emission data
All fluorescence emission values obtained from the different treatments were normalized to account for variations in bacterial cell density. This was achieved by dividing the measured fluorescence by the corresponding optical density (OD) of each sample. The control group, with an OD of 1, served as the reference point. This normalization method ensured that the data represented intrinsic differences in autofluorescence rather than discrepancies in cell count, facilitating accurate comparisons across various treatment conditions.

Fluorescent microscopy
To evaluate autofluorescence, bacterial samples were subjected to a 10-minute UV treatment using a 365 nm excitation wavelength. Fluorescent microscopy was performed using an Olympus BH2-RFC microscope (Tokyo, Japan). Fluorescence images were captured and analyzed with ImageJ software (Version 1.52). This analysis allowed for precise visualization and quantification of the green and blue emissions, corresponding to 365 nm and 405 nm excitation wavelengths, respectively.

Data availability
Data Availability: All data generated or analyzed during this study are included in this published article.

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Declarations

Competing interests

The authors declare no competing interests.

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