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Research Article

COMET ASSAY TO STUDY DNA DAMAGE CAUSED BY DIFFERENT STRESS INDUCERS IN THE RIBOFLAVIN OVERPRODUCER *EREMOTHECIUM ASHBYII*

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ABSTRACT

Objectives: The present study focuses on investigating morphological changes and genotoxicity effects due to physical and chemical mutagens such as riboflavin, ethidium bromide (EtBr), pH, and ultraviolet (UV).

Methods: Riboflavin doses were given at 0.2 mM. 0.4 mM, 0.8 mM, and 1.0 mM concentration, pH stress doses were given by adjusting the pH of the medium to pH 3.0, pH 5.0, and pH 7.0 whereas EtBr stress doses were 1.0 µL and 0.5 µL and UV radiation stress doses were given for 5 min, 10 min, 15 min. Our study reports at using a modified alkaline yeast comet assay methodology to detect DNA damages in *Eremothecium* ashbyii caused by stress inducers. Fluorescence microscopy was used to detect the comets and CaspLab software to quantify the intensity of DNA damages.

Results and Conclusion: The metrics employed for the quantification of DNA damage under the specified stressful circumstances were, tail DNA, olive tail moment (OTM), and the tail moment (TM). The statistical analysis of the DNA damage doses with CaspLab parameters to detect DNA damage shows maximum DNA damages at pH 5.0 with the highest peak for OTM value followed by UV damage at 15 min. Our study reports the first case using the comet assay technique to detect the DNA damages in the filamentous *E. ashbyii* cells.

Keywords: Cell genotoxicity, Chemical mutagen, Olive tail moment, Radiation stress, Single-cell electrophoresis, Tail moment.

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INTRODUCTION

Ostling and Johanson performed a comet assay for the 1st time in 1984 to quantify DNA damage in individual cells [1]. Hence this technique is also referred to as single-celled gel electrophoresis. The degree of DNA damage is estimated by several parameters, of which the most commonly used, is tail length calculation or the DNA percentage in the tail [2,3]. The Comet assay is applied to genotoxicity testing, molecular epidemiology, and human biomonitoring, as well as preliminary studies of DNA damages and repair [2]. Several physiological and environmental factors are responsible for chromosomal DNA aberrations oxidizing DNA bases and eventually causing DNA strand breakage [3]. Single-cell gel electrophoresis assesses the damages to the DNA by correlating the distance of the migrated chromosomal DNA. The purpose of this research is to use comet assay to determine the extent of DNA damages in *Eremothecium ashbyii* under different stress conditions.

Riboflavin is one of the most important water-soluble vitamin also known as Vitamin B_2, which is actively involved in the cellular oxidation and reduction process and carbohydrates, proteins, and lipids metabolism [4]. Riboflavin overproduction by *E. ashbyii* is one of the significant features of this filamentous hemiascomycete fungus that serves as a defense mechanism in response to the production triggered by the biotic and abiotic stress.

E. ashbyii is haploid and has a small genome making it suitable for being used as a model for studying filamentous fungal growth. The mycelium of *E. ashbyii* under the effect of environmental stresses, such as riboflavin, pH, ultraviolet (UV), oxidative stress, and chemical mutagens undergoes morphological alterations and inducing the production of riboflavin in an early stage [5]. Excess amount of riboflavin production induces reactive oxygen species (ROS) accumulation as a result of oxidative stress which may lead to many secondary complications, such as DNA and protein oxidation, and lipid peroxidation [6].

In the initial stages of growth, the organism exhibits a filamentous morphology, producing long filaments. As the growth progresses, the filaments become swollen and bulbous with hyaline granules visible under the bright field microscope [5]. A declining growth rate of the organism induces overproduction of riboflavin.

E. ashbyii under the influence of stressors such as Ethidium bromide (EtBr) as chemical mutagens and riboflavin itself have been studied to exhibit morphological changes [5]. There was a report showing riboflavin administration as a stressor spikes riboflavin production at an early stage which eventually induces a feed-forward impact on riboflavin production [5]. The organism expressed resilience to 0.8 mM concentration of riboflavin; however, the application of EtBr triggered a toxic response in the organism that eventually led to a decline in the production of riboflavin [5]. The different stressors induce excess riboflavin production, causing DNA damage to the riboflavin-overproducing cells [7]. Oxidative stress can be induced exogenously into the cells by the external addition of chemical stressors (H_2O_2, methadione) or by gassing to raise aerobic conditions highly by increasing O_2 enrichment. In the case of *Aspergillus niger*, the recombinant protein yielding was reported to decreased due to over-oxygenation resulted from oxidative stress [8,9]. Raimondi and his co-workers in 2008 reported alleviating oxidative stress induces overexpression of the *SOD1* gene in *Kluyveromyces lactis* and *Arxula adeninivorans* promoting the production of recombinant protein HSA (Human serum albumin) and glucoamylase. The oxidative burst stimulates *Fusarium graminearum* to yield secondary metabolic compounds, for example, type B trichothecenes [10]. Fungal survival is aided by the production of secondary metabolic compounds (riboflavin) which functions as a scavenging mechanism against ROS [11-13]. Furthermore, it's speculated that the fungal cell maintains optimum levels of intracellular ROS with the assistance of mycotoxin production. The secondary metabolism of the fungal

cell in response to the oxidative stress was regulated by several transcriptional factors.

Declined growth rate and depleting nutritional supplements trigger stress signals inducing riboflavin overproduction by its natural overproducer *E. ashbyii* and *Ashbya gosspyii* [14]. The fact that riboflavin overproduction initially became apparent during the latter stages of the growth phase and that it has been linked with sporulation, it seems likely that growth stress is the root cause of this phenomenon [14,15]. While exogenous riboflavin shields the spores, excessive endogenous riboflavin accumulation has been shown to trigger photo-induced damage that is equivalent to UV damage, inducing damages to DNA, RNA, and proteins [7,16].

In the present study, the Comet assay was performed to investigate the genotoxic effects on *E. ashbyii* grown under different concentrations of stressors. The cells were grown at various time intervals under the stressed conditions for morphological studies and riboflavin production was observed under the fluorescence microscope. The feed-forward growth mechanism of *E. ashbyii* helps in determining decreased cell growth and comet assay has been performed at this stage to quantify the extent of DNA aberrations in the individual cells. The CaspLab software, which detects the comets and calculates the tail moment, head and tail DNA content, and percentage of each, was used in the present research to assess and quantify the degree of DNA damage in *E. ashbyii*.

METHODS

Culture and growth conditions

The riboflavin overproducer *E. ashbyii* 366 was procured from MTCC Chandigarh and was maintained at 25°C on sterilized Potato Dextrose Agar plates (PDA g/L: Potato infusion 200 g, Dextrose 20 g, Agar 20 g) and was sub-cultured every 15 days on (PDA) slants.

Pre-inoculum was obtained by inoculating mycelium from a 7 to 10-day-old slant in the 50 mL of the pre-inoculum medium (g/L: Glucose-3, Yeast Extract-0.8, Peptone-0.2) into a 250 mL Erlenmeyer flask and the flask was incubated at 25°C and 120 rpm on a rotary shaker.

1% of a 36–48 h old pre-inoculum was inoculated into the 50 mL of the production medium (g/L: Yeast Extract 0.8, Peptone 0.2, Sodium molybdate 0.1, Zinc Sulphate 0.1, Magnesium Sulphate 0.1, Potassium dihydrogen phosphate 0.2, Sodium chloride 0.1, Tween80 20 mL, Glucose 3 pH ± 6.0) and the flasks were incubated at 25°C and 120 rpm on a rotary shaker.

Stress inducers were added into the production medium at different concentrations as described below, followed by growing the fungal cells in the medium.

Stress-inducers

Riboflavin, EtBr, pH, and UV radiation were used as stress inducers to study the DNA damage in *E. ashbyii*.

Riboflavin stress

A 100 mM stock solution of riboflavin was prepared, sterilized by filtration, and diluted under aseptic conditions into the 250 mL Erlenmeyer flasks with 50 mL of the sterilized production medium to obtain the final concentrations of 1.0 mM, 0.8 mM, 0.4 mM, and 0.2 mM. The inoculated flasks were incubated at 25°C and 120 rpm in a rotatory shaker. Samples were withdrawn under aseptic conditions at 24 h and 48 h intervals to study the stress response.

EtBr stress

A 10 mg/mL stock solution of EtBr was prepared and 0.5 µL and 1.0 µL of the stock solution were added separately to the 50 ml of the sterilized production medium in the 250 mL Erlenmeyer flasks as stress. The flasks were incubated in a rotatory shaker at 25°C and 120 rpm for 24 h and 48 h at the end of which the cells were harvested to study the stress response.

pH stress

To study pH stress the production medium's pH was adjusted to pH 7.0, pH 5.0, and pH 3.0, and the inoculated flasks were incubated for 24 h and 48 h, respectively, at 120 rpm and 25°C in the rotatory shaker at the end of which the cells were harvested to study the stress response.

UV stress

The inoculated production medium, was transferred into a sterile Petri dish and exposed to UV radiation in a UV trans-illuminator at 312 nm for the following time intervals

Flask 1/Dish 1–05 min

Flask 2/Dish 2–10 min

Flask 3/Dish 3–15 min

The samples were retransferred to the flasks under aseptic conditions and incubated for 24 h at 25°C temperature and 120 rpm speed in the rotatory shaker and the under-stressed grown mycelium was harvested later to study the stress response.

Comet assay

The stressed cells were subjected to the comet assay according to Oliveira and Johansson [17] to investigate DNA damage. The stressed cell undergoes centrifugation for 10 min at 10,000 rpm and 1 mL of cell suspension was extracted and diluted in 1 mL of S-buffer (25 mM KH_2PO_4, 1 M sorbitol, pH 6.5), followed by centrifugation for 20 min at a force of 10,000 rpm in 4°C. An equivalent volume of 1.5% w/v low melting agarose that had been melted in the S-buffer was mixed with the retrieved pellet. 40 µL of the sample was immediately layered on pre-coated 0.5% w/v of agarose slides and kept for solidification. The slides were kept for overnight incubation in lysis buffer (1 M NaCl, 30 mM NaOH, 10 mM Tris-HCl, 500 mM EDTA pH 10) and were washed off in electrophoresis buffer (10 mM Tris-HCl, 30 mM NaOH, 10 mM EDTA, pH 10) 3 times for 20 min each. Electrophoresis of each slide was done at 50kV for 20 min with electrophoresis buffer and the slides were incubated in neutralization buffer (Tris-HCl-10 mM, pH 7.4) for 10 min. The slides were further incubated for 10 min in 70% and 90% ethanol, followed by staining with EtBr (20 µL of 10 mg/mL), and allowed to dry in the air. After the slides were dried completely, the comets were visualized using the Carl Zeiss Axioplan Epi Fluorescent Microscope with a blue excitation filter and 40× magnification. The same microscope and excitation filter were used to observe morphological changes.

RESULTS

This study reports the morphological changes of *E. ashbyi*i only under UV and pH stress. In an earlier publication, we have already reported on the morphological changes when *E. ashbyii* was exposed to various concentrations of riboflavin and EtBr [5]. Therefore, for these two stressors, DNA damage alone was studied. The olive tail moment (OTM), tail moment (TM), and tail DNA (TDNA) were the three measures used to analyze the Comet assay data to determine the extent of DNA damages in all the stressed cells.

Morphological changes of the pH-stressed cells

The proper functioning of the cells depends on the pH of the growth medium and variations in pH cause abnormal growth of the mycelial cells and affect cellular functions. *E. ashbyii* grows well naturally at pH 6.0. Mycelial morphological changes were studied in an acidic medium, at pH 3.0 and at 24 and 48-h time intervals (Fig. 1). At pH 3.0, very condensed hyphal growth was observed at 24 h with the least riboflavin production, and mycelial degradation could be seen without any riboflavin production for the cells grown for 48 h. At pH 5.0 (Fig. 2), thick, defined rod-like hyphal growth was observed at 24 h, whereas at

48 h, opaque, tightly packed hyphal growth could be seen with the least riboflavin production. At pH 7.0 (Fig. 3), mycelial growth was found at 24 h with almost no riboflavin production, and the cells degraded completely at 48 h.

Morphological changes of the UV-stressed cells

The organism was subjected to UV stress using a UV trans-illuminator at 312 nm for three different durations of 5 min, 10 min, and 15 min. At 5 min, riboflavin production was initiated, and the riboflavin was evenly distributed throughout the mycelium. At 10 min, cell lysis was observed, and riboflavin started to burst out of the cell due to UV stress, whereas at 15 min, complete cell lysis was observed (Fig. 4).

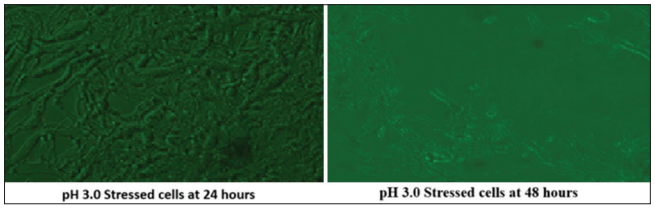

Fig. 1: Morphological changes of *Eremothecium ashbyii* at pH 3.0 at 24 h and 48 h: Almost no riboflavin production was observed in the acidic medium at 24 h, with the cells being degraded completely at 48 h. Green fluorescence shows the distribution of riboflavin throughout the mycelium under fluorescence microscopy

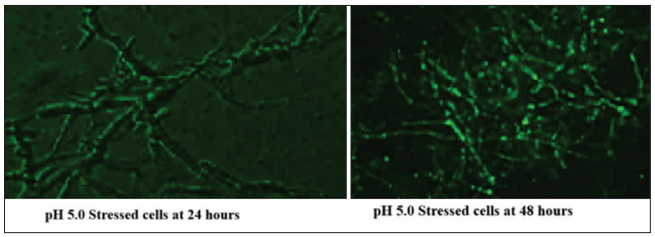

Fig. 2: Morphological changes of *Eremothecium ashbyii* at pH 5.0 at 24 h and 48 h: The hyphal nodes observed were rod-shaped with no riboflavin production, whereas, at 48 h, the hyphae were found to be tightly packed with the least riboflavin production (green fluorescence)

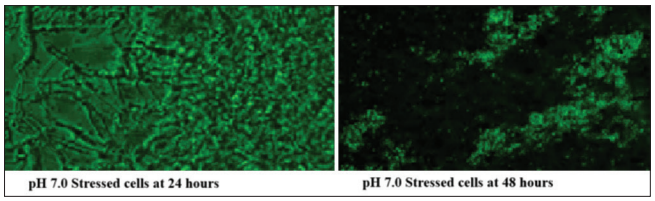

Fig. 3: Morphological changes of *Eremothecium ashbyii* at pH 7.0 at 24 h and 48 h: Mycelial growth was observed at 24 h with no riboflavin production, whereas the cells started degrading at 48 h. The absence of green fluorescence under the fluorescence microscope depicts no production of riboflavin

In the wild-type control, at 24 h, the mycelium was very thick with high riboflavin production, whereas, at 48 h, the mycelium was much more defined and narrow-shaped with distributed riboflavin production inside the hyphae (Fig. 5).

DNA damage study under different stressed conditions

The correlation between riboflavin production and DNA damage under different stress conditions was analyzed in the present study using the comet assay method. The appearance of comets at pH 5.0, at all the selected concentrations of riboflavin stress, for both EtBr stress conditions, and UV exposure indicated DNA damage under these conditions (Figs. 6-9). No comets were observed at pH 3.0 and pH 7.0. EtBr was found to be toxic to the cells and doesn't induce riboflavin production, such as UV radiation. At high concentrations of EtBr, the number of viable cells decreased with time. Riboflavin production worked in a feed-forward and feedback mechanism, and riboflavin acted as a stress signal at a particular concentration. At high concentrations of externally added riboflavin, very little riboflavin was produced, and at 1.0 mM concentration, it became toxic to the cell. The apoptosis of the cells under these conditions led to the formation of DNA lesions which was analyzed using the method of comet assay.

Comet assay of the riboflavin and EtBr stressed cells

Riboflavin production was impacted when *E. ashybii* was grown in the presence of riboflavin as an additional supplement (0.2 mM, 0.4 mm, 0.8 mM) in the medium for stress studies. At 0.2 mM concentration, there was the least disruption, and the comet was clearly visible with the least DNA damages. DNA damage was observed with larger disrupted comets at the concentration of 0.8 mM and 0.4 mM, with an increased DNA damages at concentrations above 0.2 mM, as shown in (Fig. 6 a-d). At a concentration above 0.8 mM, the riboflavin itself became toxic to the organism, and the cells died out. Thus, no comets were observed at riboflavin concentrations above 0.8 mM. In contrast to the cells stressed at 0.2 mM and 0.4 mM, the *TM* and the OTM were found to be highest at 0.8 mM riboflavin concentration.

EtBr showed observable DNA damage in both the concentrations of 0.5 µL and 1.0 µL (Fig. 7a and b). At higher concentrations, a great degree of cell disruption was observed.

Comet assay of the pH stressed cells

A relative amount of DNA damages has been observed for *E. ashbyii* under pH stress at pH 5.0 (Fig. 8), whereas no comets were visible at pH 3.0 and pH 7.0.

Comet assay for UV-stressed cells

UV-stressed cells when subjected to comet assay, gave positive results for all 3 time periods indicating DNA damages. The size and dispersion of the comet rays and diameter being proportional to the extent of DNA damage, the comet of the cells exposed to UV (312 nm) for 15 min showed the most DNA damage (Fig. 9).

STATISTICAL ANALYSIS AND DISCUSSION

A dose-to-damage ratio with respect to different parameters has been plotted (Fig. 10). The comets were analyzed using CaspLab software where detected OTM, TM, and TDNA values were compared with the comets formed under different stress inducers. TDNA and TM are more

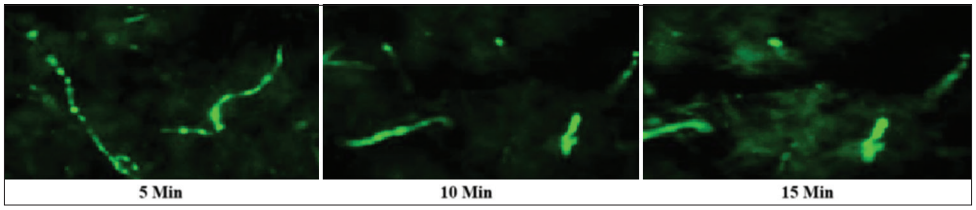

Fig. 4: Morphological changes of *Eremothecium ashbyii* when subjected to ultraviolet stress at 312 nm: At 5 min, riboflavin production was initiated, and the riboflavin was evenly distributed throughout the mycelium. At 10 min, cell lysis was observed, and riboflavin started to burst out of the cell due to UV stress, whereas, at 15 min, complete cell lysis was observed

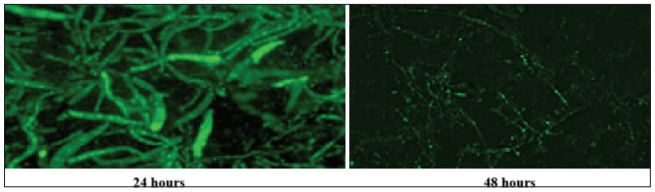

Fig. 5: Morphological changes of wild-type *Eremothecium ashbyii*: At 24 h mycelial cells were observed to be thick with riboflavin distinctly distributed throughout the cells, whereas at 48 h the cells were found to be narrowly defined with localization of riboflavin into vacuolar structures. The green fluorescence of riboflavin shows it distribution throughout the cell

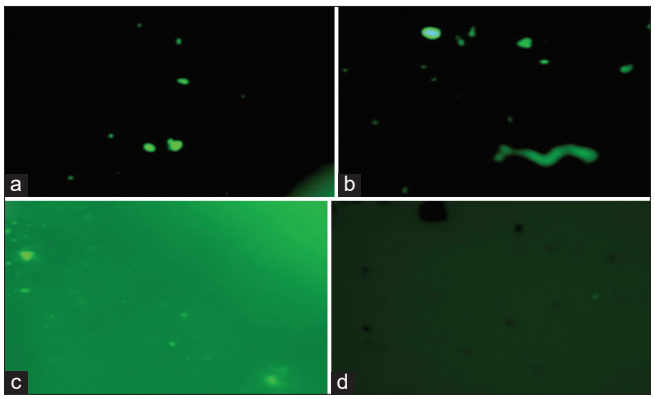

Fig. 6: DNA damage analysis by comet assay due to riboflavin stress: The presence of green fluorescence (Dot-like) of the ethidium bromide stained stressed cells shows the comet, quantifying the DNA damage of the cells. The least DNA damage was observed for riboflavin at a concentration of 0.2 mM (a), which subsequently increased when the riboflavin concentration was raised to 0.4 mM (b) and 0.8 mM (c). At 1.0 mM (d) concentration, riboflavin became toxic to the cells, resulting in apoptosis with no visible comets. The comets identified were analyzed with the help of *CaspLab* software

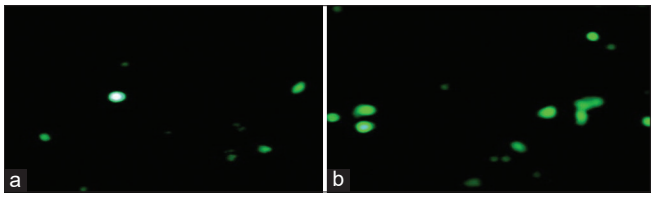

Fig. 7: DNA damage observed by comet assay due to ethidium bromide (EtBr) stress: With increasing concentrations of the EtBr, DNA damage also increased with more comets being identified. (a) depicts a comet at 0.5 µL EtBr stress and (b) shows a comet of 1.0 µL EtBr stressed *E. ashbyii* cells. The presence of green fluorescence (Dot-like) of the stained stressed cells shows the comet, quantifying the cells with DNA damages

significantly used for detecting the extent of DNA damages but in our study, we have considered OTM value as well as it helps in explaining the heterogeneity and DNA distribution throughout the comet tail within the cell population [18]. TDNA is estimated by multiplying the tail length and tail intensity whereas OTM is calculated by multiplying %TDNA and the length between the centers of the tail and head of the comet.

DISCUSSION

The stressed *E. ashbyii* cells underwent using comet assay detection technique to quantify the level of DNA damages in the cells. The comet

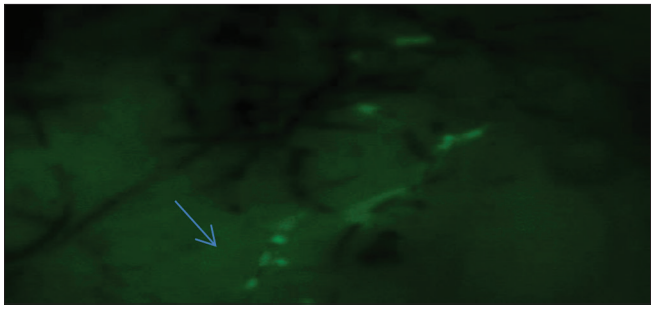

Fig. 8: Analysis of comets observed when *Eremothecium ashbyii* was grown at pH 5.0: A small comet (green fluorescent) was observed for the pH-stressed cells at pH 5.0, which was analyzed further to determine the DNA damage. The head and tail of the identified comet were analyzed

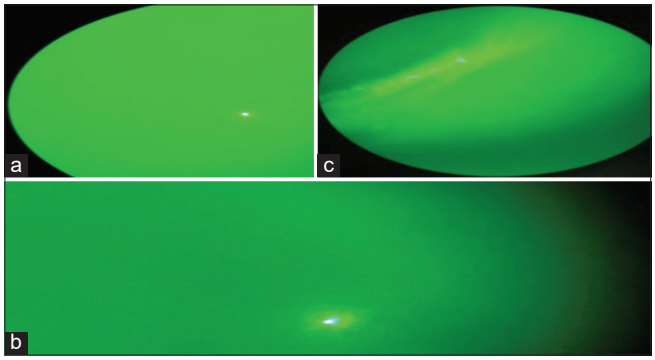

Fig. 9: DNA damage observed by comet assay due to UV stress: The presence of green fluorescence (Dot-like) of the ethidium bromide stained stressed cells shows the comet quantifying the DNA damage of the cells. The comets were observed for the UV-stressed cells at 5 min (a), 10 min (b), and 15 min (c). The comets showed that the extent of DNA damage is directly linked to the radiation doses

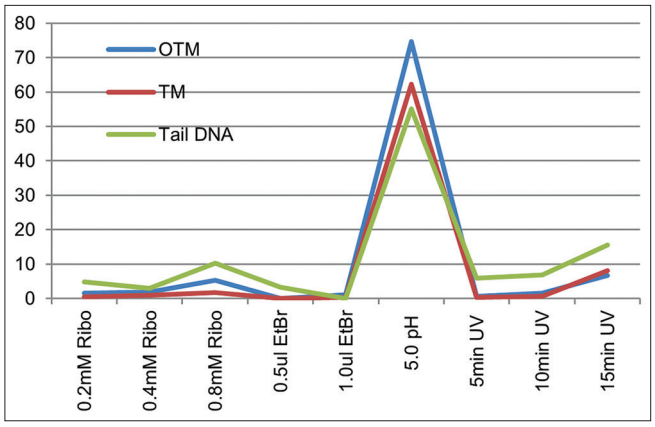

Fig. 10: Statistical comparison of DNA damages in proportional to different stress studies using comet data

assay parameters of the CaspLab software, that is, *TM*, OTM, and %Tail DNA [18], were used to detect the damage in the exposed population and found to be higher than the control population [19]. OTM parameter is used for the quantification of DNA fragmentation [20] which particularly describes the heterogeneity within a cell population [18]. When a comparative analysis of the data was made (Table 1), it was observed that OTM and %Tail DNA were higher for the cell under pH stress (pH 5.0) and least under EtBr stress (0.5 µL) showing higher heterogeneity of the pH 5.0 stressed cell population. The %Tail DNA

Table 1: Comparative analysis of comet assay data readings showing DNA break quantification (TM and olive moment) was done using CaspLab software in the stressed cells. The data depicts the TM and OTM values of comets at each induced stressed condition and the result has been interpreted accordingly

Stress inducers	TM	OTM	TDNA	Interpretation
Riboflavin stress at 0.2 mM	0.433	1.6242	4.81	Heterogeneity level is 1.6 and % DNA damage is 4.81
Riboflavin stress at 0.4 mM	0.9374	1.89202	3.02	Heterogeneity level is 1.9 and % DNA damage is 3.0
Riboflavin stress at 0.8 mM	1.65238	5.35015	10.32	Heterogeneity level is 5.35 and % DNA damage is 10.32
Ethidium Bromide stress at 0.5 µL	0.000151	0.0020	3.3	Heterogeneity level is 0.002 and % DNA damage is 3.3
Ethidium Bromide stress at 1.0 µL	0.236	1.04	−0.005	Heterogeneity level is 1.04 and the % DNA damage is very minute 0.005
pH 5.0	62.2609	74.614	55.09	Heterogeneity level is 74.6 and % DNA damage is 55.
UV exposure for 5 min	0.29821	0.6345	5.96	Heterogeneity level is 0.63 and % DNA damage is 5.9
UV exposure for 10 min	0.6849	1.48276	6.8	Heterogeneity level is 1.48 and % DNA damage is 6.8
UV exposure for 15 min	8.05	6.66	15.48	Heterogeneity level is 6.6 and % DNA damage is 15.5.

UV: Ultraviolet, M: Tail moment, OTM: Olive tail moment, TDNA: Tail DNA

value for pH 5.0 stressed cells was 55.09 and for EtBr cells, it was 0.005. DNA percentage of the comet tails (%Tail DNA) is directly proportional to the DNA damage [21]. The riboflavin overproduction by *E. ashbyii* is an ecological phenomenon in response to a defense mechanism triggered by environmental stress [7] such as UV stress. The endogenous riboflavin induces oxidative DNA damages by accumulating the generated ROS, upon reacting with the light [7]. The rate of DNA damages is directly linked with the dose of radiation exposure [18]. Analysis of comet assay data (Table 1) reveals that as the concentration of added riboflavin was increased, damage to the DNA also increased (Fig. 6). Riboflavin added at 0.8 mM had a higher TM and % Tail DNA value (1.65238 and 10.32) in comparison to 0.2 mM (0.433 and 4.81) and 0.4 mM (0.9374 and 3.02) which showed that riboflavin itself may act as a stress inducer, damaging the DNA. The UV-stressed cells exhibited maximum damage when exposed for 15 min, with higher OTM and TM values, as compared to the cells exposed for 5 min and 10 min. Our comet analysis statistical data (Fig. 10) showed that DNA damage due to different stresses was in the order of pH 5.0 stress > UV stress for 15 min > Riboflavin stress at 0.8 mM > EtBr stress (Table 1).

CONCLUSION

The purpose of this study was to investigate the potency of the comet assay method to analyze the extent of DNA damages and repair mechanism in *E. ashbyii*. Our results show conclusively that it is indeed possible. Quite possibly, the ease of application of the comet assay in *E. ashbyii* is linked to certain unique features of the cell wall of this organism, affecting cell homogeneity and genotoxicity, and this could bear further investigation. This research work provides the insight of the comet assay application for the first time for analyzing DNA damages in *E. ashbyii.*

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AUTHOR'S CONTRIBUTIONS

Simadri contributed toward molecular genetics and microscopy studies, data analysis and interpretations, and drafting the manuscript, Vishal contributed toward molecular genetics and microscopy studies, both Simadri and Vishal carried out the experiments. Rajagopal and Vijayalakshmi conceived the study and participated in its design and coordination and helped to draft the manuscript. Vijayalakshmi provides financial assistance in carrying out the research from her DST-funded project.

CONFLICTS OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

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