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ANTAGONISTIC ACTION OF MUTANT *BACILLUS SUBTILIS* AGAINST PLANT PATHOGANDENIC FUNGI *ASPERGILLUS NIGER* AND *MICROSPORUM BULLARDI*

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Abstract:
Our research focused on a mutant strain of Bacillus subtilis and its potential to combat plant-pathogenic fungus like *Aspergillus niger* and *Microsporum bullardi*. Bacillus subtilis is widely recognized as a biocontrol agent that can inhibit the development of numerous plant diseases. In this study, mutagenesis was used to create a strain of B. subtilis with improved antifungal properties. Several in vitro tests were performed to measure the mutant strain's inhibitory potential. To investigate the mutant strain's inhibitory effects on fungal growth, the dual culture technique was used. Inhibition zones around mutant colonies were significantly larger than those around control colonies, demonstrating that the mutant might hinder the spread of both *A. niger* and *M. bullardi*. Radial growth of the fungi was measured with and without the mutant strain to demonstrate its antifungal action. Extracellular antifungal metabolites produced by the mutant strain were evaluated through GCMS to better understand the mechanisms driving the antagonistic effect. Significant antifungal activity against both infections was seen in the culture supernatant of the mutant strain, demonstrating the release of strong antifungal chemicals. The mutant strain significantly reduced the incidence of fungal infections, resulting in plants that were both healthier and more robust than their control counterparts. Overall, the Bacillus subtilis mutant strain was highly hostile to the plant-pathogenic fungus *Aspergillus niger* and *Microsporum bullardi*. The increased generation of antifungal metabolites was found to be responsible for the mutant strain's improved antifungal capabilities. These results suggest that the mutant B. subtilis strain has great promise as a biocontrol agent for the management of plant fungal infections, representing a greener alternative to chemical fungicides.

Keywords: Bacillus subtilis, Induction of mutation, NaN3 Antagonistic activity of mutant strain, GCMS analyses, Lowry Assay.

Abbreviations: NaN3: Sodium azide, GC-MS: Gas Chromatography Mass spectrometry, VOCs: volatile organic compounds, NMR: nuclear magnetic resonance, LC: Liquid Chromatography, EI: Electron ionization, CI: chemical ionization,

1. Introduction:

Bacillus is a genus that contains numerous economically useful species that are widely employed in the fermentation industry [1–3]. *Bacillus subtilis*, like many other members of the Bacillus genus, is a widespread microorganism. Decomposing plant matter, water, air, and soil all contain it [4]. *Bacillus* bacteria produce a spore by enclosing their DNA and other internal cell components in a protective wall. This makes them resistant to the effects of heat, cold, chemicals, and even some forms of radiation. Consequently, they can be implemented in manufacturing procedures. Because bacteria are

so amenable to genetic manipulation, they are often used in controlled laboratory experiments [5]. Proteins and metabolites can be effectively produced from it, and it is non-pathogenic, easily cultivated, and genetically modified [6], B. subtilis is a close relative of many other bacteria. In addition, many gram-positive bacteria found in soil and aquatic habitats produce a wide range of unique chemicals having medicinal and metabolic applications. Antibiotics like those made from Bacillus subtilis, such as difficidin, oxydifficidin, subtilosin A, bacillomyin B, bacitracin, and bacilysocin, are useful for treating and preventing bacterial skin infections and infections in minor cuts and burns [7–9]. Another application for Bacillus subtilis is as a fungicide [10]. Bacillus subtilis has been shown to be an effective biocontrol agent due to its ability to colonize the root system, leaving no space for fungal disease organisms. The bacteria are applied to vegetable seeds in agriculture, and their multicellular behaviour and production of a wide variety of toxic substances lend credence to this claim [11, 12]. The survival of organisms in complex ecological systems depends on their ability to adapt to their surroundings, and the creation of these metabolites may be vital for this adaptation. The genetically varied bacilli have shown to be effective in stimulating growth in a number of crops and in the biological control of a number of plant diseases [13,14,15]. For this reason, they have the potential as hostile organisms.

When it comes to agricultural plants, however, sodium azide (NaN3) has proven to be one of the most potent chemical mutagens. Azide compounds have a mutagenic effect because of the organic metabolites they produce. When this metabolite reaches the nucleus, it binds to DNA and causes a point mutation. Different mutagens have different effects depending on a variety of criteria, such as their characteristics, the length of treatment, the pH of the treatment solution, the pre- and post-treatment temperatures, the amount of oxygen present, etc. In every mutagenesis plan, the dose of a mutagen used is crucial. In most cases, it was found that the mutagen's toxicity increased in proportion to its concentration. Azide is a potent mutagen that produces high mutation yields with just mild effects on sterility, making it a potential top pick for the safest and most effective mutagen. Despite its established use in therapy, sodium azide has few physiological consequences, including the induction of a few chromosomal defects and a slowing of germination and growth. [16].

Different members of the *Bacillus* genus have been shown to promote plant development [17]. Bacteria can aid plant growth in a number of ways [18, 19]. These include enhancing plant nutrition, inducing systemic resistance, being poisonous to pests, and counteracting the effects of diseases. The antagonistic activity of *Bacillus* is linked to the production of a wide variety of antimicrobial peptides [20, 21], secreted enzymes, proteins, and VOCs [22, 23]. It has been established that many *Bacillus* isolates have antifungal activity against phytopathogenic fungi [24, 25–26], making them promising biocontrol options.

The virulence of different *B. subtilis* strains are affected by genetic and phenotypic variation within the species. Bacillus isolates are promising candidates for use as biocontrol agents due to their rapid growth, efficiency on low-cost media, and capacity to sporulate under unfavourable conditions. Since the global market for biopesticides is predicted to expand dramatically over the next 3–5 years, there is a growing demand for such agents. Major changes in the makeup of intracellular and extracellular proteins [27] that can be related to antibacterial characteristics were shown by a comparative investigation of proteomes of B. subtilis strains with antagonistic potential. The extraction of antifungal metabolites was performed after the detection of further antifungal activity, and GC-MS was used to determine protein concentration.

Studies in metabolomics draw from many different disciplines. The metabolomic analysis is the systematic study of metabolite patterns inside a biological system (such as a cell, tissue, organ, biological fluid, or organism) [28]. The results of well-executed metabolomics tests and the data they generate shed light on an organism's health and well-being at a given moment. GC-MS or LC-MS and

NMR spectroscopy are two of the most commonly mentioned analytical platforms in the scientific literature for metabolic profiling.

Most scientists agree that GC-MS is a flexible analytical tool [29]. This is because it is extremely reliable, has high selectivity, sensitivity, and repeatability, and can be used in a wide variety of settings [30]. EI and CI are the two primary ionization methods employed in GC-MS (CI). In addition to these benefits, it is also simple to use and can shed light on the identification of unknown compounds. For the latter, all you need is a basic GC unit and a single quadrupole MS detector. However, GC-MS is only capable of separating and identifying molecules that have a low molecular weight or are volatile [31].

In order to achieve this goal in this portion of our research, the B. subtilis strain MTCC 6808 will need to be exposed to chemical mutagens. To learn more about the proteins and fungi metabolites produced by *Bacillus subtilis*, GC-MS and the Lowry technique are employed.

Material and Methods:

Bacterial and fungal Strain:

From IMTEC Chandigarh, we obtained the bacterial strain *B.subtilis* (MTCC 6808). A sample of dirt from Prayagraj, Uttar Pradesh, was used. Other fungi, including *Aspergillus niger* (MTCC 404) and *Microsporum bullardi* (MTCC 6059), were obtained from IMTEC Chandigarh.

Conditions for the growth of the bacterial strain:

Two separate 250 ml Erlenmeyer flasks were used to prepare the inoculum by adding nutrient broth and nutrient agar media. The material was autoclaved at 121 degrees Celsius for 15 minutes. The broth medium was inoculated with bacteria and stirred for 48 hours while being stirred at 175 rpm and 28 degrees Celsius. After 48 hours of growth in a 28 Degree Celsius incubator, the bacterial culture was plated onto nutritional agar.

Culture conditions for fungal strain:

Two 250 ml Erlenmeyer flasks were used to inoculate with nutritional broth and agar medium, respectively. It was autoclaved for 15 minutes at 121 degrees Celsius. After inoculating the broth medium with the fungal culture (*Aspegillus niger* (MTCC 404), *Microsporum bullardi* (MTCC 6059), the flask was stirred at 175 rpm at 37 degrees Celsius for 48 hours. After 48 hours of growth in a petri dish at 37 degrees Celsius, the fungal culture was plated on nutritional agar.

NaN_3 Mutagenesis and isolation of mutant strains:

Separate stock solutions of the chemical mutagen NaN3 were made in water (1%). Mutagen was utilized in a range of concentrations, including 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, and 0.08%. In 9 mL of nutritional broth medium containing colonies of *Bacillus subtilis* (1×10^7 see unit spores per mL), the stock solution of NaN3 was added, and the mixture was then maintained at 30 °C in a shaker for 6 hours. The material was taken out after 6 hours and centrifuged for 1 minute at 10,000 rpm three times with nutritional broth to eliminate mutagens. After being cleansed, the cells were placed in a fresh broth of nutrients and allowed to grow for three to six days at 30^0C and 120 rpm of shaking. The colony was plated out on nutritional agar after numerous dilutions. The plates were incubated for three to five days at a temperature of 30^0C. Untreated colonies (parental/wild colonies) were also plated separately in the nutrient agar medium (Figure1).

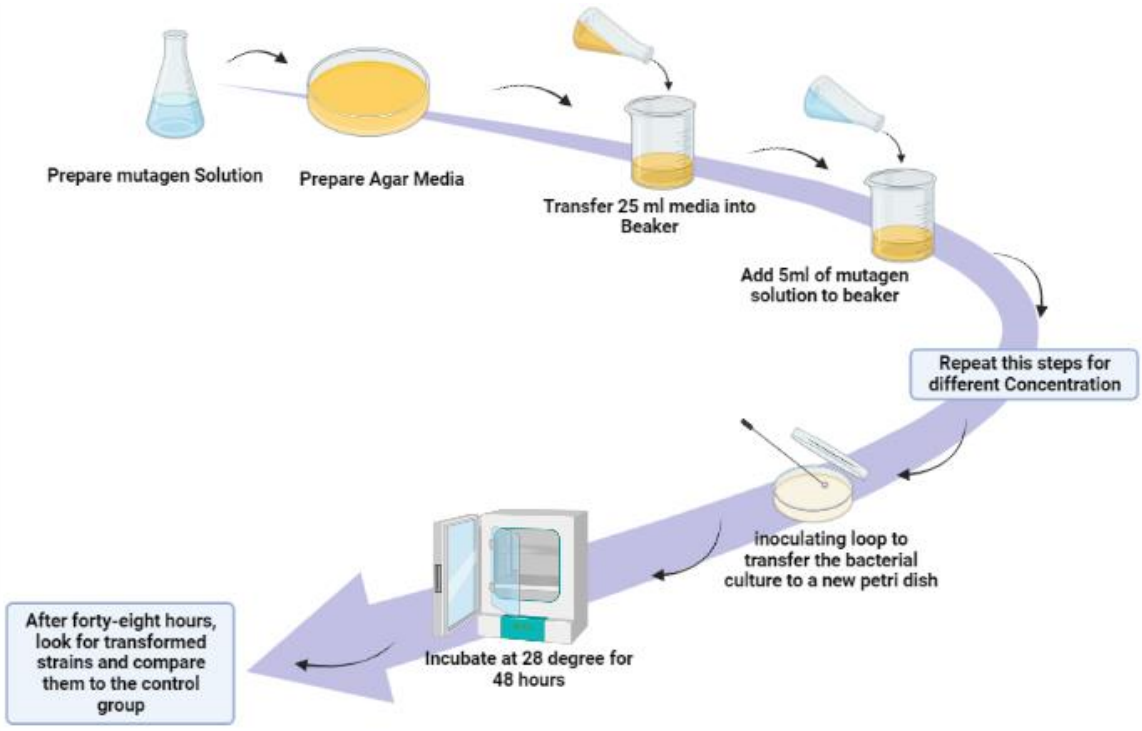

Figure 1: Experimental procedure of the chemical mutagenesis**.**

Screening of the mutant strains using dual culture with fungi:

As both organisms can grow on Nutrient Agar (NA) medium, it was employed to examine how the mutant *Bacillus subtilis* strain interacted with these phytopathogenic fungi. Inverting the nutritional agar plates, a line was drawn on the outside bottom of each plate to divide each palate into two pieces. Each *Bacillus subtilis* mutant isolated from NA slop was affixed to one side of the plate via zigzag streaking, while a portion of pathogenic fungal mycelium was kept on the other half using an inoculation loop and spaced at a minimum of 2 centimetres apart from each streak. On one side of the control plates, phytopathogens were infected, and on the other, sterile nutrient broth. All plates (control and treatment) underwent a 5-day incubation period at 25 ± 2°C. The percentage of growth inhibition was calculated using the following equation after the incubation time when the radial growth of the phytopathogens was evaluated in both the control and treated plates.

% inhibition = (A–B)/A × 100

where A = colony diameter of the phytopathogen in the control plate, B = colony diameter in the dual culture plate

Extraction of antifungal metabolites from selected mutant strains and their protein estimation:

The mutants of bacteria were inoculated into nutrient broth medium and incubated as described before. After reaching maximum growth, 5 ml culture was removed and remaining centrifuged at 10,000 rpm for five minutes at 4^0C. The supernatant was discarded, and the pellet was homogenized with 1 ml of saturated n-butanol using pestle and mortar. Centrifuged as above mentioned. The supernatant was divided into two parts, one for mutant and another for control strain. The solvent was allowed to evaporate in the petri dish and after evaporation, it was resuspended in 2 ml of saturated n-butanol, for each mutant strain. One ml from n-butanol was used for GC-MS analysis, and the remaining 1 ml of each was stored at 4^0 C.

Protein estimation by Lowry's Method:

Digestion of cell wall is achieved by the addition of lytic enzymes to a cell suspension. Enzymes are highly selective, gentle and most effective. Lysozyme is widely used to lyse bacterial cells. Take the 0.2 ml of supernatant and estimate the amount of protein present in each sample by Lowry's method. The steps are as follows:

- Take 1.5 ml of overnight grown bacterial culture in eppendorf tubes and centrifuge at 10000 rpm for 5 minutes.
- Discard the supernatant and suspend the biomass/ pellet in 1 ml of PBS (phosphate buffered saline) buffer.
- Add 200µl of lysis buffer to all the tubes.
- Incubate at different time intervals 10,20, 30, 40, 50 mins.
- Centrifuge all the eppendorf tubes at 10000 rpm for 5 minutes. (SRM university protocol)
- Take 0.2 ml of supernatant and estimate the amount of protein present in each sample by Lowry's method. (Lowry) [32]
- Plot the graph between time vs protein released.

The protein concentration in the sample can be estimated using the standard plot.

Results:

In present study various concentrations of the chemical mutagen NaN3 was used. In order to evaluate the wild-type *Bacillus subtilis* (MTCC 6808) alongside mutations and a control plate devoid of any mutagen. The outcomes of the control group and the experimental group were compared after 48 hours.

Toxicity of NaN_3 to induced mutation on *Bacillus subtilis* (MTCC 6808):

Toxicity of chemical mutagens sodium azide (NaN_3) at different concentrations against *B. subtilis* (MTCC 6808) cell was determined as a function of time and is presented in Figure. 2A-D. It was observed that cell's ability to survive was decreased as mutagen concentrations increased. NaN_3 at concentrations higher than 0.05% showed a decrease in survival of bacterial cells (Figure. 2 C and D), while the lower concentration (0.01%- 0.03%) appeared to be nontoxic to the cells (Figure. 2A and B).

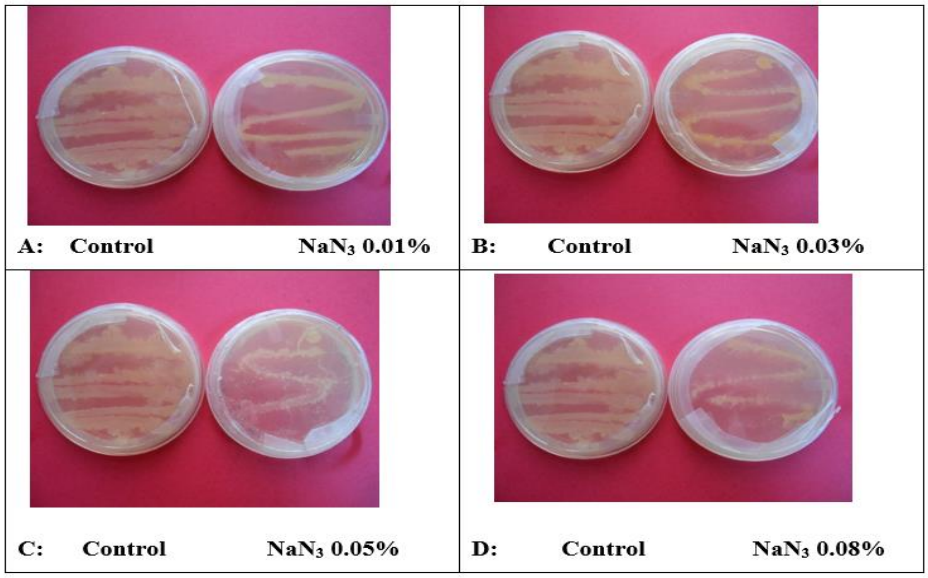

Figure 2: Colony morphology of the control strain (wild type) and NaN_3 treated mutants; (A) control vs NaN_3 0.01%; (B) control vs NaN_3 0 .03% EMS; (C) control vs NaN_3 0 .05% and (D) control vs NaN_3 0.08%.

Selection of mutants by antagonist assay:

After NaN3 treatment, colonies were picked from different concentration plates and streaked for dual culture assay. Antifungal activity against Aspergillus niger and Microsporum bullardi was evaluated in all of the chosen colonies. The findings showed that compared to their wild-type counterparts, mutant strains exhibited a higher inhibitory effect.

The zone of inhibition of *Bacillus subtilis* (MTCC 6808) control against *Aspergillus niger* (NaN3) was 6.7. However, the mutant bacteria from 0.01% NaN3 showed inhibition 8.3, with a 25% reduction in impact from control, followed by 0.03% concentration (zone of inhibition 11.3) with impact 70% more than control, 0.05% (zone of inhibition 5.0) with 25% reduction in impact as compared to control and 0.08% (zone of inhibition 12.3) with an 85% greater effect than the control (Table1, Figure. 3 and 4).

Table 1: Effect of mutant *Bacillus subtilis* on *Aspergillus niger*

	Mean	% Effect
Control	6.7	100
0.01%	8.3	25
0.03%	11.3	70
0.05%	5	-25
0.08%	12.3	85

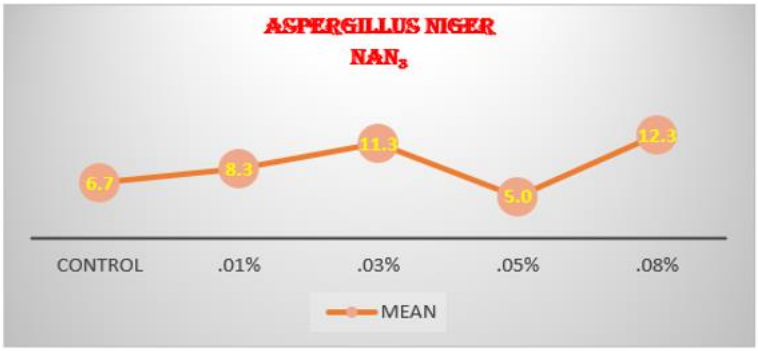

Figure 3: Effect of mutant *Bacillus subtilis* on *Aspergillus niger*

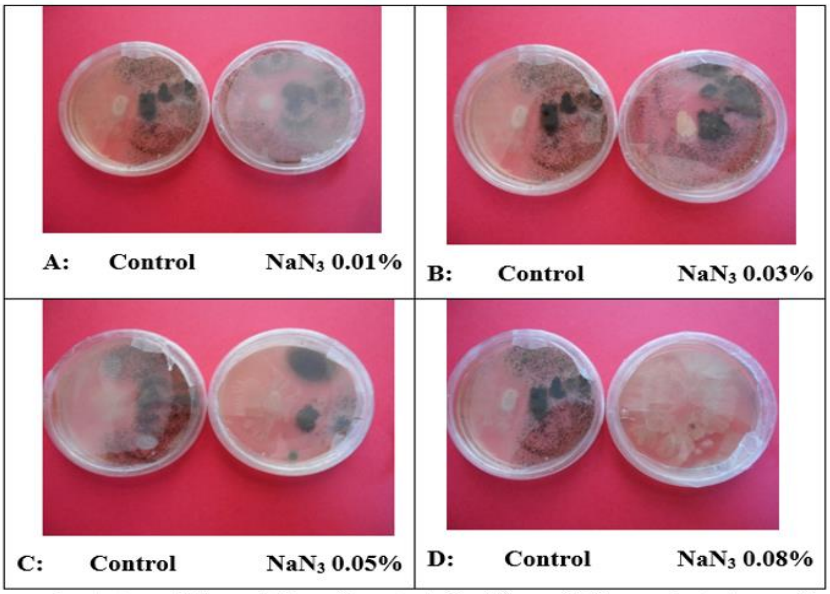

Figure 4: Antagonistic activity of mutant *Bacillus subtilis* against *Aspergillus niger* showing by zone of inhibition (A) control vs NaN_3 0.01%; (B) control vs NaN_3 0 .03% EMS; (C) control vs NaN_3 0 .05% and (C) control vs NaN_3 0.08%.

The antagonistic activity of *Bacillus subtilis* (MTCC 6808) control against *Microsporum bullardi* was checked and inhibitory effect was 9.7. However, mutagen treated *Bacillus* strain such as in 0.01% NaN3, zone of inhibition was 10.3, with a 6.90% higher effect than control, followed by 0.03% NaN3 (zone of inhibition 9.3) with 3.45% less effect than control, 0.05% NaN3 (zone of inhibition 11) with 13.79% raise in effect than control and 0.08% NaN3 (zone of inhibition 10.7) with 10.34% higher effect compared to control. The variation impact was found to diminish when concentration was increased from 0.05% to 0.08%, while it was found to rise when concentration was increased by 0.03% to 0.05% (Table 2, Figure 5 and 6).

Table 2: Effect of mutant *Bacillus* strain on *Microsporum bullardi*

	Mean	% Effect
Control	9.7	
0.01%	10.3	6.89655
0.03%	9.3	-3.4483
0.05%	11	13.7931
0.08%	10.7	10.3448

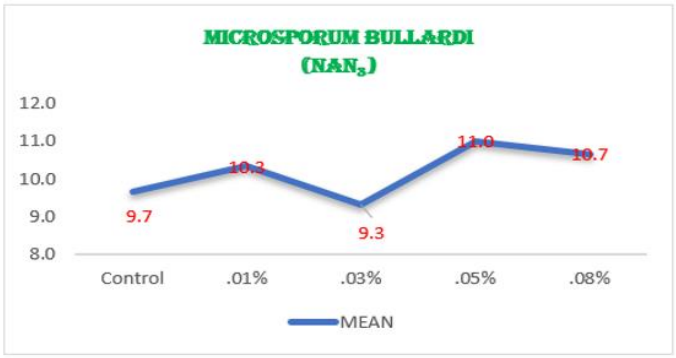

Figure 5: Effect of mutant *Bacillus subtilis* strain on *Microsporum bullardi*

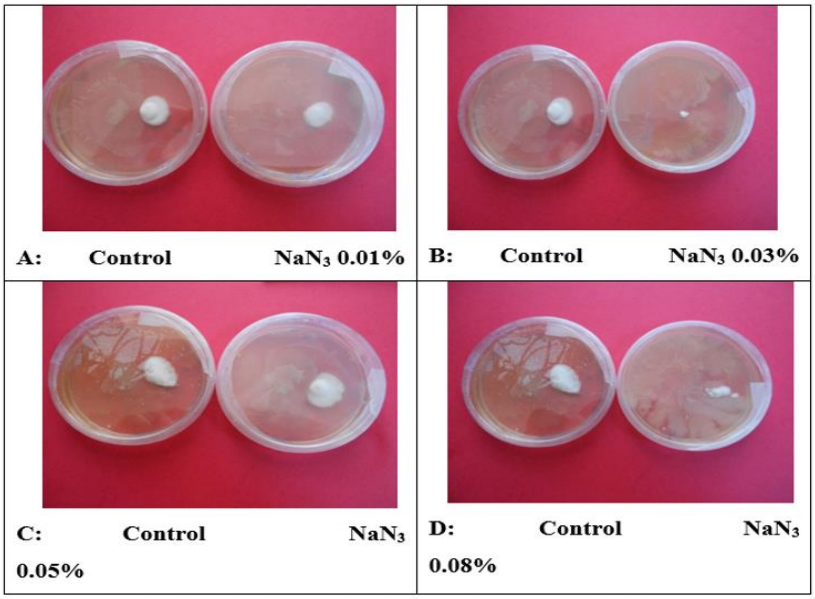

Figure 6: Antagonistic activity of mutant *Bacillus subtilis* against *Microsporum bullardi* treated with NaN_3 at various concentration; (A) control vs NaN_3 0.01%; (B) control vs NaN_3 0 .03% NaN_3; (C) control vs NaN_3 0 .05% and (d) control vs NaN_3 0.08%.

Estimation of protein content:

Bacillus subtilis strain (MTCC 6808) treated with different concentrations of sodium azide during various time intervals was analyzed for protein content. Results showed that in 0.01% treatment the highest protein content was observed in 50-minute (0.342 ug/ml) time interval followed by 0.03% found in 20 and 30 minute (277 ug/ml), in 0.05% treatment 30 and 40 minute (0.317 and 0.429 ug/ml respectively), however in 0.08% treatment it was shown in 10- 30 minutes (0.382 and 0.303 ug/ml) (Table 3 and Figure 7).

Table 3: Protein concentration in *Bacillus subtilis* strain (MTCC 6808) treated with different concentrations of sodium azide

Treatment	Protein concentration (ug/ml) with different time interval				
	10 min	**20 min**	**30 min**	**40 min**	**50 min**
Control	0.229	0.266	0.229	0.226	0.205
0.01%	0.2	0.209	0.268	0.13	0.342
0.03%	0.243	0.277	0.276	0.239	0.236
0.05%	0.252	0.221	0.317	0.429	0.182
0.08%	0.382	0.303	0.303	0.226	0.287

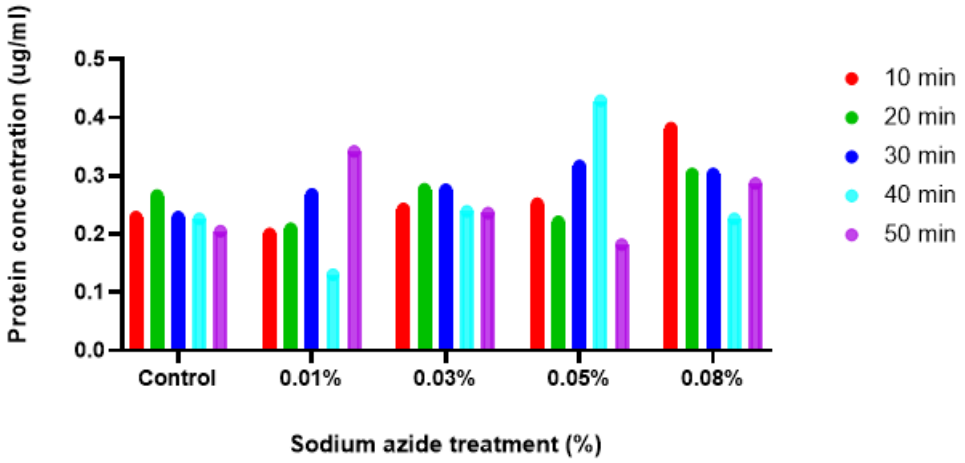

Figure 7: Effect of different concentration of sodium azide on protein content of *Bacillus subtilis* strain (MTCC 6808).

Determination of secondary metabolites by GC/MS

Bacillus subtills developed a number of antifungal chemicals, according to the GC-MS study. In order to identify the formation of antimicrobial chemicals, the crude metabolites of Bacillus subtills mutant strains created by treating them with sodium azide at concentrations of 0.01%, 0.03%, 0.05%, and 0.08% were examined by GC/MS. The compound identity was confirmed through NIST library. Most important antifungal compounds were detected such as Butanoic acid, Butyl ester Bis(2- ethylhexyl) Phthalate, and Cyclononasiloxane, Octadecamethyl. The highest amount of Butanoic acid, Butyl ester was observed in *Bacillus subtills* strain treated with 0.03% (38.25%) sodium azide, followed by 0.08% (32.52%), whereas control showed 30.32% (Table: 4; Figure 8). Similarly the highest concentration

of Bis(2- ethylhexyl) Phthalate was found in strain treated with 0.05% (94.6%) sodium azide, followed by 0.01% (91.91%) treatment. However, control had shown 69.67%. Though Cyclononasiloxane, Octadecamethyl were observed only in 0.01% (4.12%) and 0.08% (55.8%) treated strains, however absent in control (Table: 4; Figure 8).

Table 4: Identification of antimicrobial compounds from mutant *Bacillus subtilis* through GC/MS.

Concentration	Compound	Retention time	Area	% Area	% Composition	Activity	References
control	Butanoic acid, Butyl Ester	10.072	4323467	0.90	30.32	Antimicrobial	Hayashida-Soiza et al. (2008)[33]
	Propanoic acid, 2 Methyl Butyl Ester	absent	-	-	-	-	-
	Phenol	absent	-	-	-	-	-
	Pyrrolo [1,2-a] Pyrazine -1,4 - dione,hexahydro	absent	-	-	-	-	-
	Cyclononasiloxane, Octadecamethyl	absent	-	-	-	-	-
	Bis(2- ethylhexyl) Phthalate	43.112	9932315	2.06	69.67	Antimicrobial	Osuntokun and Cristina (2019)[34]
0.01%	Butanoic acid, Butyl Ester	10.066	301372	0.10	3.95	-	-
	Propanoic acid, 2 Methyl Butyl Ester	absent	-	-	-	-	-
	Phenol	absent	-	-	-	-	-
	Pyrrolo [1,2-a] Pyrazine -1,4 - dione,hexahydro	absent	-	-	-	-	-
	Cyclononasiloxane, Octadecamethyl	32.361	314724	0.10	4.12	Antifungal	Ahsan et al. (2017)[35]
	Bis(2- ethylhexyl) Phthalate	43.106	7008035	2.23	91.91	Antimicrobial	Osuntokun and Cristina (2019)[34]
0.03%	Butanoic acid, Butyl Ester	10.085	907876	0.19	38.25	Antimicrobial	Hayashida-Soiza et al. (2008)[33]
	Propanoic acid, 2 Methyl Butyl Ester	absent	-	-	-	-	-
	Phenol	absent	-	-	-	-	-
	Pyrrolo [1,2-a] Pyrazine -1,4 - dione,hexahydro	absent	-	-	-	-	-
	Cyclononasiloxane, Octadecamethyl	absent	-	-	-	-	-
	Bis(2- ethylhexyl) Phthalate	43.105	1465560	0.30	61.74	Antimicrobial	Osuntokun and Cristina (2019)[34]
0.05%	Butanoic acid, Butyl Ester	10.078	559814	0.13	5.39	Antimicrobial	Hayashida-Soiza et al. (2008)[33]
	Propanoic acid, 2 Methyl Butyl Ester	absent	-	-	-	-	-
	Phenol	absent	-	-	-	-	-
	Pyrrolo [1,2-a] Pyrazine -1,4 - dione,hexahydro	absent	-	-	-	-	-
	Cyclononasiloxane, Octadecamethyl	absent	-	-	-	-	-
	Bis(2- ethylhexyl) Phthalate	43.111	9811116	2.37	94.6	Antimicrobial	Osuntokun and Cristina (2019)[34]
0.08%	Butanoic acid, Butyl Ester	10.07	2339383	0.58	32.52	Antimicrobial	Hayashida-Soiza et al. (2008)[33]
	Propanoic acid, 2 Methyl Butyl Ester	absent	-	-	-	-	-
	Phenol	absent	-	-	-	-	-
	Pyrrolo [1,2-a] Pyrazine -1,4 - dione,hexahydro	absent	-	-	-	-	-
	Cyclononasiloxane, Octadecamethyl	41.21	4013197	0.21	55.8	Antifungal	Ahsan et al. (2017)[35]
	Bis(2- ethylhexyl) Phthalate	43.11	839197	1.00	11.66	Antimicrobial	Osuntokun and Cristina (2019)[34]

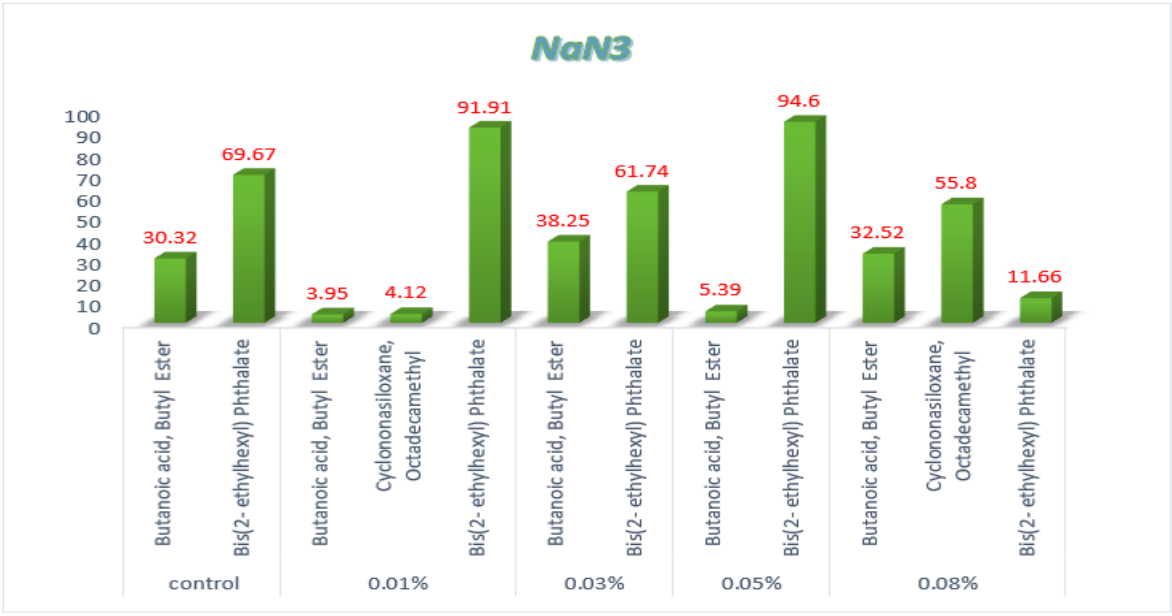

Figure 8: Antimicrobial compounds identified from mutant *Bacillus subtilis* through GC/MS.

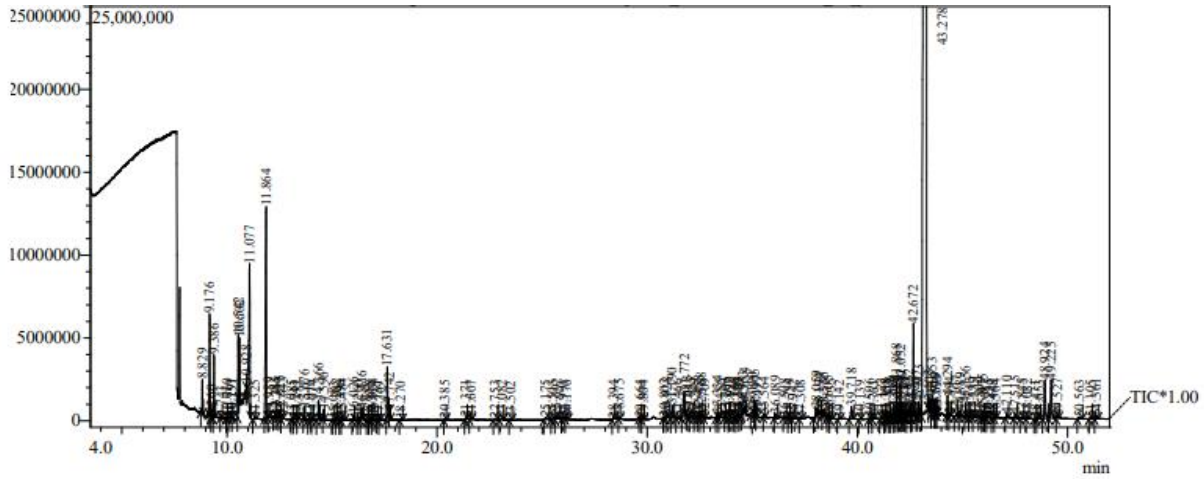

Figure 9: Chromatogram of Std.NaN3

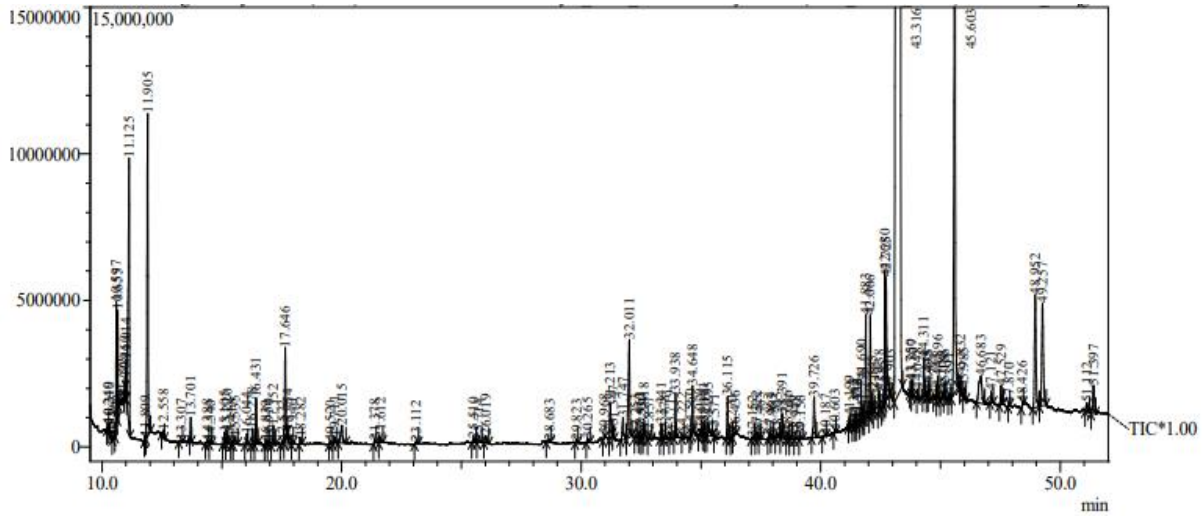

Figure 10: Chromatogram after Mutation

Discussion:

The process of commercializing natural products requires strain modification because wild-type strains' low titers can never meet the requirements of large-scale development [36]. Since its inception a century ago, random mutation and screening has consistently proven to be the most successful strategy for improving strains. Beneficial microorganisms frequently use mutation to create new strains that produce more secondary metabolites and have antagonistic effects on a variety of diseases [37]. The most often employed chemical mutagens include sodium azide, ethyl methane sulphonate, hydroxylamine, methyl methane sulphonate, and N-methyl N-nitro-N-nitroso guanidine. The most common method for producing high-yielding mutants is to administer mutagenic agents to a population until a particular "desired" death is achieved, plate off the survivors, and then test each resulting colony or a randomly chosen group of colonies for producing a particular product in flasks. [38].

The Bacillus subtilis strain (MTCC 6808) was subjected to a random mutation in this investigation using sodium azide at various dosages ranging from 0.01% to 0.08%. According to the findings, Aspergillus niger was more sensitive to higher sodium azide dosages, such as 0.03% and 0.08%. However, dosages of 0.05% and 0.08% led to Bacillus subtilis mutants that were hostile to Microsporum bullardi. These results are in line with those of Latif et al. (2018) [39], who found that treating Bacillus subtilis UTB1 mutants with ethyl methano sulfonate boosted biosurfactant synthesis and Aspergillus flavus control. Gamma radiation treatment of Trichoderma virens resulted in the

production of two to three times as many secondary metabolites [40]. In B. subtilis E8, ion beam implantation led to an increase in surfactin production. [41]

The *Bacillus subtilis* strain treated with various concentrations of sodium azide was analysed for protein contents and showed that with increasing concentration (0.08) of sodium azide the protein content was increased till 30 minutes exposure. However, 40 and 50 minutes depicted deviation and decrease protein content compared to control (Table.4 and Figure. 8). Similar results were reported by Hajduch et al. (2000) [42] and Hussain et al. (2017)[43].

Our GC-MS findings are consistent with those from previous research on biocontrol agents for several fungal diseases. Antifungal chemicals including Bis(2-ethylhexyl) phthalate and Pyrrolo[1,2-a]pyrazine-1,4-dione were found to be produced by the B. subtilis strain JNDKHGn-29-A, according to research by Bharose and Gajera (2018) [44]. According to Ramyabharathi and Raguchander (2014) [45], the Bacillus subtilis EPCO16 strain produced secondary metabolites, such as fatty acids with antifungal activity such dodecanoic acid, hexadecanoic acid, and pentadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester. Pyrrole shown antifungal effect when tested against the pathogen (Jiang et al., 2014). [46]

Conclusion:

In terms of biological control and disease suppression, the antagonistic effect of mutant Bacillus subtilis against plant pathogenic fungus, particularly Aspergillus niger and Microsporum bullardi, offers encouraging results.

Bacillus subtilis mutant strains that have been bred to have improved antifungal characteristics can stop the spread of harmful plant fungi. The mutant Bacillus subtilis strains successfully inhibit the growth and activity of Aspergillus niger and Microsporum bullardi. One of the most significant environmental challenges facing microbiologists and plant pathologists is the development of environmentally preferable alternatives to chemical pesticides for the treatment of crop illnesses. The utilization of beneficial bacteria is one of the most exciting new directions in the quest for safer, more effective crop management. In this respect, we used chemical mutagen sodium azide to produce *Bacillus subtilis* mutants with strong antifungal activity against *Aspergillus niger* and *Microsporum bullardi*. We screened mutants with 0.03% (11.3%), 0.0% (12.3%) against *Aspergillus niger* and 0.01% (10.3%), 0.05% (11.0%), 0.0% (10.7%) against *Microsporum bullardi* compared to control (wild type) strain. Thus, the selected *Bacillus subtilis* mutant has potential in biotechnological applications.

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