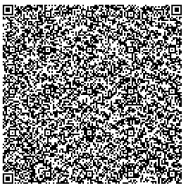

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Non-Recombinant Mutagenesis of Bacillus Tropicus CUIMW1718 for Hyper Production of Alginase

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Abstract

Microbial extracellular enzymes occupy a prominent place in the industrial sector due to their multifunctional ability. Due to their extensive application, the gap between production and demand is widening. In order to fulfil this gap, researchers focusing on various strain improvement methods such as recombinant and non-recombinant mutagenesis. In the present research, we made an attempt to screen high yielding industrial important extracellular alginase producing *Bacillus tropicus* CUIMW1718 strain by treating with inexpensive mutagens such as UV, EMS and EtBr. In this, the indigenous strain was subjected to Ultraviolet (UV) irradiation, Ethidium bromide (EtBr), Ethyl Methane Sulfonate (EMS) mutagenesis, followed by cross mutation of Ultraviolet (UV) irradiated strain with Ethyl Methane Sulfonate (EMS) and Ethidium bromide (EtBr). High yielding mutant strains were selected based on the zone of clearance.

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1. Introduction

Microorganisms are considered to be the preferred source of extracellular enzymes due to their rapid growth, less space required for cultivation, and the ability to be genetically mutated to generate new enzymes with preferred properties. Alginate is the most abundant polysaccharide (about 40% of dry weight) of brown algae, which consists of β-D-mannuronate (M) and α-L-guluronate (G) as monomeric units. These units are linked in 3 different blocks, poly β-D mannuronate (poly M), poly α-L-guluronate (poly G) and the heteropolymer (poly MG). Commercial alginates are produced by extraction from biomass of brown algae such as *Laminaria Hyperborea*, *Macrocystis pyrifera*, *Laminaria japonica* etc. Alginate oligosaccharides are depolymerisation products of alginate by alginate lyase. Alginase attracted due to its wide applications in food and pharmaceutical industry. In the present study, an attempt was made to mutate *Bacillus tropicus* CUIMW1718 by exposing it to physical (UV) and chemical mutagens (EtBr, EMS). Ethyl Methane Sulfonate (EMS) a mutagenic, teratogenic, and possibly carcinogenic organic compound produces random mutations in genetic material by nucleotide substitution; particularly by guanine alkylation. EMS is used as mutagens since it produces random mutations in genetic material by nucleotide substitution; particularly by guanine alkylation which produces only point mutations.

2. Materials and Methods

2.1Experimental Chemicals

All chemicals and reagents used in this research were of analytical grade and are mostly purchased from sigma USA and Hi-media Mumbai.

2.2 Microorganism and Inoculum Preparation

The *Bacillus tropicus* CUIMW1718 strain that produces alginase was employed in the present study. Stock cultures were maintained in nutrient broth with 70% glycerol, and cultures were preserved at -20°C. The inoculum was prepared by transferring a loopful of stock culture to a certain volume (100 ml) of sterile nutrient broth. Then it was incubated overnight at 37°C on a rotary shaker with 200 rpm. Before using it for inoculation, a stock suspension was prepared by adjusting it to 1×10^3 cells/ml.

2.3 Mutagenesis by UV Irradiation

20 ml of broth which contains $1x10^{-3}$ cells/ml was taken into sterile Petri dish and was exposed at different heights (7, 14, 21, 28, 35, 42, and 49 cm) to UV irradiation. At regular intervals of 10 min (10, 20, 30, 40, and 50 min), a 1 ml sample containing $1x10^{-3}$ cells was taken and centrifuged. The supernatant was discarded, and the pellet was resuspended with 1000 µl of saline to stop the cell division. From the resuspended cell solution, 100 µl was plated onto sodium alginate agar media using the spread plate method. The plates were incubated for 24 h at 37°C. Mutants with hyperproduction of alginase were detected visually by the intensity of the zones after the addition of iodine.

2.4 Mutagenesis by Ethidium Bromide (EtBr)

One day prior the wild strain was inoculated into 100 ml of alginate broth. 100 µl of the culture was pipetted into a microcentrifuge tube of 2 ml, thereafter 0.75 µl of ethidium bromide (0.5mg/ml) was transferred and kept for incubation. The sample was dispensed with the intervals of 10, 20, 30, 40, 50, 60, 70, and 80 min and centrifuged at 10,000 rpm for 10 min. Thereafter the supernatant was discarded, and the pellet was resuspended with 1000 µl of saline and serially diluted to 10^{-4} which constitutes $7X10^3$ cells[18,] and then 100 µl from that was plated on an alginate agar plate. The plates were incubated for 24 h at 37°C. Mutants with hyperproduction of alginase were detected visually by the intensity of the zones after the addition of iodine.

2.5 Mutagenesis by Ethyl Methane Sulfonate (EMS)

One day prior the wild strain was inoculated into 100 ml of alginate broth. 100 µl of the culture was pipetted into a microcentrifuge tube of 2 ml, thereafter 0.75 µl of ethyl methane sulfonate (0.5mg/ml) was transferred and kept for incubation. The sample was dispensed with the intervals of 10, 20, 30, 40, 50, 60, 70 and 80 min and centrifuged at 10,000 rpm for 10 min (Zhu et al., 2018; Raju, & Divakar, 2013). Thereafter the supernatant was discarded, and the pellet was resuspended with 1000 µl of saline and serially diluted to 10^{-4} which constitutes $7x10^3$ cells and then 100µl from that was plated on an alginate agar plate. The plates were incubated for 24 h at 37^oC. Mutants with hyperproduction of alginase were detected visually by the intensity of the zones after the addition of iodine.

2.6 Cross mutation of Ultraviolet (UV) irradiated strain with Ethidium Bromide

One day prior, the UV mutated strain was inoculated into 100 ml of alginate broth. 100 µl of the culture was pipetted into a microcentrifuge tube of 2 ml, thereafter 0.75 µl of ethidium bromide (0.5mg/ml) was transferred and kept for incubation. The sample was dispensed with the intervals of 10, 20, 30, 40, 50, 60, 70, and 80 min and centrifuged at 10,000 rpm for 10 min. Thereafter the supernatant was discarded, and the pellet was resuspended with 1000 µl of saline and serially diluted to 10^{-4} which constitutes $7x10^3$ cells, and then 100 µl from that was plated on alginate agar plate[5,8,13]. The plates were incubated for 24 h at 37^oC. Mutants with hyperproduction of alginase were detected visually by the intensity of the zones after the addition of iodine.

2.7 Cross mutation of Ultraviolet (UV) irradiated strain with Ethyl Methane Sulfonate

One day prior, the UV mutated strain was inoculated into 100 ml of alginate broth. 100 µl of the culture was pipetted into a microcentrifuge tube of 2 ml, thereafter 0.75 µl of ethyl methane sulfonate (0.5mg/ml) was transferred and kept for incubation. The sample was dispensed with the intervals of 10, 20, 30, 40, 50, 60, 70,

and 80 min and centrifuged at 10,000 rpm for 10 min. Thereafter the supernatant was discarded, and the pellet was resuspended with 1000 µl of saline and serially diluted to 10^{-4} which constitutes $7x10^{-3}$ cells, and then 100 µl from that was plated on alginate agar plate[5,8]. The plates were incubated for 24 h at 37^{O}C. Mutants with hyperproduction of alginase were detected visually by the intensity of the zones after the addition of iodine.

2.8 Production of Alginase from Mutant Strains

The alginase production was carried out in a 250 ml conical flask, containing 100 ml modified production medium composed of $Fecl_{3:}$ 0.05g, NH_4SO_4: 1g, $K_2HPO_{4:}$ 0.38g, $MgSO_{4:}$ 0.2g, Sodium alginate: 10g, pH 7. A 1% (v/v) level of inoculums was added. The fermentation was carried out at 37^{O}C for 24 h. After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4^{O}C, and the clear supernatant (crude enzyme) was subjected to biochemical assay.

2.9 Alginase Assay

The supernatant was used as a crude enzyme source for Alginase assay. A solution of 0.6 g sodium alginate dissolved in phosphate buffer (1.75 g NaCl in 100 ml of 50 mM NaHPO4, pH 7) was used as the substrate. Incubation was performed in a water bath at 37°C for 20 min. The reaction was terminated by the addition of 1 ml of DNS reagent and tubes were kept in a boiling water bath for 10 min. After cooling the tubes at room temperature, 1 ml of distilled water was added to each tube. The intensity of the color was read at 490 nm in a UV-Vis spectrophotometer. The standard curve was performed with glucose solution. Enzyme activity was expressed in international units.

3. Results and Discussion

The present investigation was undertaken to improve the alginase quantitatively from *Bacillus tropicus* CUIMW1718 strain through exposure to physical and chemical mutagens. For industrial use,

the enzyme must be produced at a low cost and should be reusable and reproducible. To achieve these many techniques have been developed for strain improvement. Improvement of strain is usually made by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, UV rays, etc. and chemical mutagens such as NTG, EMS, and EtBr etc. The best strain of UV-treated mutant strains of *Bacillus tropicus* CUIMW1718 was isolated based on a bigger zone of clearance due to the alginase production in the Petri plates and named it as UV mutants. This mutant showed improvement in the production of the alginase enzyme at the height of 49 cm and 50 min (Table: 1 and Fig: 1) of the UV treatment gave the best result when mutated at different heights (7, 14, 21, 28, 35, 42, and 49 cm) and different timings (10, 20, 30, 40, and 50 min) for each height 5 different timings were mentioned, and the best of them were chosen to treat with chemical mutagens, i.e. EtBr and EMS. Best colonies that showed more clearing zone at the time specified: UV mutated with EtBr showed more clearing zone at 60 min (Fig: 6 and 7). UV mutated with EMS showed more clearing zone at 60 min (Table: 5 and Fig: 8 and 9). Wild strain treated with EtBr showed more clearing zone at 20 min (Table: 2 and Fig: 2 and 3). Wild strain treated with EMS showed more clearing zone at 10 min (Table: 3 and Fig: 4 and 5) but this mutant was not stable. It may be because the mutant produced by UV irradiations had undergone back mutations when they were exposed to light. The best mutant strains of *Bacillus tropicus* CUIMW1718 obtained from UV, EtBr, and EMS mutagenesis were inoculated in the optimized media for assessing the quantity of enzyme produced (Fig: 6). The production of alginase enzyme following the growth of the organism was found to be highly significant than other mutant derivatives. Enzymes are ubiquitous in occurrence, they have been found in all living organisms, and are necessary for cell growth and differentiation. Of these enzymes, strains of *Bacillus sp.* dominate the industrial sector. The genus *Bacillus* contains a number of industrially important species, and approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus*. There are a great number of literature reported to use the strain

improvement process for producing various industrial enzymes like lipase, chitinase, cellulase, glucoamylase, protease, and fibrinolytic protease. But there was no report available on mutation studies of *Bacillus tropicus* CUIMW1718 for alginase production.

Table 1: Selected Bacillus Tropicus Cuimw1718 Uv Mutants At Different Heights And Time

Time (Min) /Height (Cm)	10	20	30	40	50
Control	-	-	-	-	-
7	23	8	10	16	20
14	-	6	-	13	21
21	2	4	13	2	22
28	10	-	6	12	26
35	-	-	11	6	30
42	9	-	3	12	20
49	12	-	8	-	5

Table 2: Selected Bacillus Tropicus CUIMW1718 ETBR Mutants

Time (Min)	Clearing Zone Radius (Cm)	Selected Colonies
10	-	-
20	1.2	5
30	0.8	9
40	-	-
50	0.5	-
60	0.8	9
70	0.4	-
80	0.1	-

Table 3: Selected Bacillus Tropicus CUIMW1718 EMS Mutants

Time (Min)	Clearing Zone Radius (Cm)	Selected Colonies
10	1.7	6
20	1	4
30	-	-
40	1.2	11
50	0.5	6
60	1	28
70	-	-
80	1	13

Table 4: Selected Bacillus Tropicus CUIMW1718 UV-ETBR Mutants

Time (Min)	Clearing Zone Radius (Cm)	Selected Colonies
10	0.5	6
20	-	-
30	0.5	8
40	0.5	5
50	0.7	4
60	2.1	47
70	1.5	20
80	1.7	11

Table 5: Selected Bacillus Tropicus CUIMW1718 UV-EMS Mutants

Time (Min)	Clearing Zone Radius (Cm)	Selected Colonies
10	0.6	3
20	0.9	11
30	-	-
40	0.5	2
50	0.7	8
60	2	30
70	0.5	2
80	1	28

Figure 1: Selected Bacillus Tropicus CUIMW1718 UV Mutants

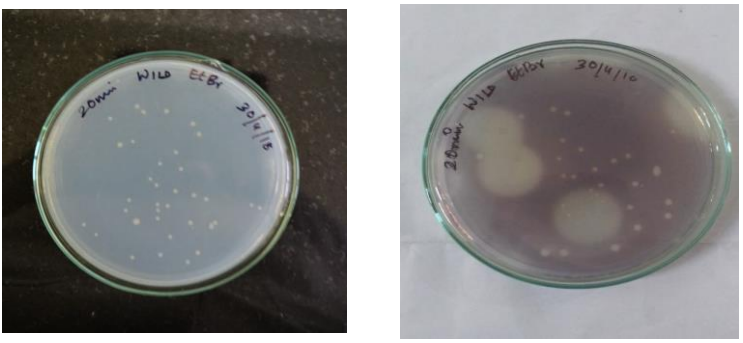

Figure 2: Selected Bacillus Tropicus CUIMW1718 ETBR Mutants

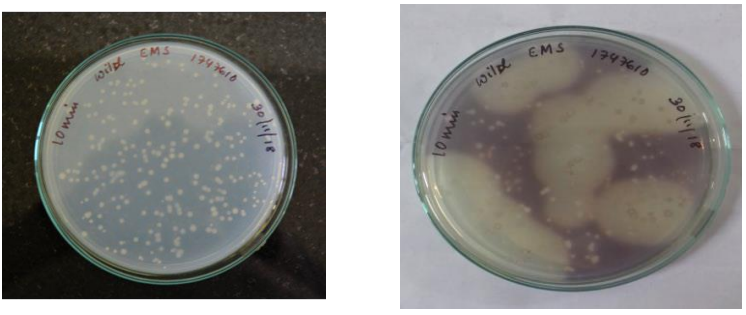

Figure 3: Selected Bacillus Tropicus CUIMW1718 EMS Mutants

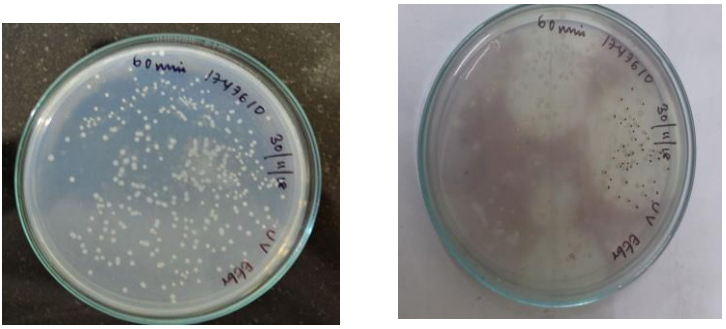

Figure 4: Selected Bacillus Tropicus CUIMW1718 UV-ETBR Mutants

Figure 5: Selected Bacillus Tropicus CUIMW1718 UV-EMS Mutants

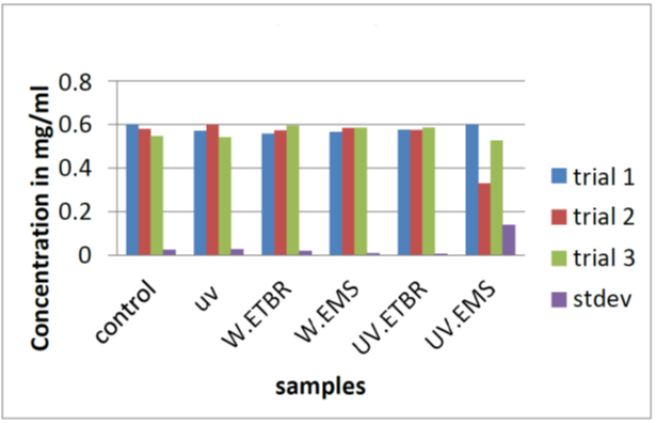

Figure 6: Selected Bacillus Tropicus CUIMW1718 Mutants Biochemical Assay

4. Conclusion

In this study, the wild strain of *Bacillus tropicus* CUIMW1718 was improved for alginase production by using physical and chemical mutagens. Best strains were obtained based on the zone of clearance by adding iodine solution to the sodium alginate agar plates. Based on the results obtained, we are concluding that the mutants may help in minimizing the cost of alginase production.

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Conflict of Interest

The authors declare no conflict of interest.

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