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EFFECT OF UV IRRADIATION AND ETHYL METHYL SULPHATE ON CARBOXY METHYL CELLULASE PRODUCTION BY *BACILLUS PUMILUS*

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ABSTRACT

The aim of this study was strain improvement of *Bacillus pumilus* for Carboxy Methyl Cellulase production by physical mutation (UV irradiation) and Chemical mutation (Ethyl Methyl Sulphate).The wild *Bacillus pumilus* strain isolated from earthworm gut produced 0.665 ± 0.004 IU/ml of CMCase. After UV irradiation *Bacillus pumilus*(UVR10) exhibited high efficiency of CMCase production (1.330 ± 0.075 IU/ml).The UV mutant *Bacillus pumilus* was further subjected to chemical mutation in EMS (200 µg/ml)at different time intervals. The least survival was recorded at an exposure period of 120 minutes. In this regard, *Bacillus pumilus* CR24 exhibited high efficiency on the basis of the CMCase production (2.610 ± 0.093 IU/ml).The mutant strain of *Bacillus pumilus* effectively produced higher amount of CMCase than wild *Bacillus pumilus* strain.

Keywords: UV irradiation, EMS, *Bacillus pumilus*, CMCase, Cellulose.

INTRODUCTION

Cellulases have wide range of industrial applications such as starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry and textile industry [1]. The homopolymeric cellulose made of anhydro-D-glucose linked by β-1,4 bonds constitutes the most abundant biopolymer on earth [2]. The degradation of the cellulose to glucose requires a combined or cooperative action of at least three enzymes namely an endo-1,4-β-glucanase (also referred to as carboxy methylcellulase or CMCase; EC 3.2.1.4), an exo-1,4-β-glucanase (EC 3.2.1.91) and a β-glucosidase (EC 3.2.1.21). Studies have highlighted the need for cellulase and its application in various industries (wine, textile, detergent, pulp and paper industries), in agriculture and in animal feeds [3].

DNA may be modified, either naturally or artificially, by a number of physical, chemical and biological agents, resulting in mutations. Mutation can be either spontaneous or induced; arise because of changes in the base sequence of the nucleic acids or organisms genome. Mutagenic agents, such as ultra violet (UV) light, ionising radiation or chemicals can randomly induce DNA lesions in the genome [3]. Strain improvement for cellulase production via mutagenic agents has attracted great attention owing to their efficiency. The use of different mutagenic agents including ultraviolet (UV), X-rays, gamma radiation, ethyl methyl sulfonate (EMS), N-methyl-N-nitro-N-nitrosoguanidine (NTG) and mustards were demonstrated [4]. This investigation mainly focused on CMCase production by *Bacillus pumilus* using UV irradiation and EMS.

MATERIALS AND METHODS

CMCase producing *Bacillus pumilus* EWBCM1 (Gen Bank accession number JF906499) isolated from the gut of Earthworms (*Eudrilus eugeniae*) [5-8] was mutated for strain improvement using physical mutation (UV irradiation) and Chemical mutation (Ethyl Methyl Sulphate).

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Determination of endoglucanase activity

The supernatant of the culture broth centrifuged at 5000 rpm for 20 minutes at 4°C served as the enzyme source. Endoglucanase was assayed as per method of Johnveslyet *al*. (2002) using CMC as the substrate. This enzyme solution 0.5 ml was added to 0.5 ml of 1% substrate (CMC) taken in 0.2 M citrate phosphate buffer (pH 7) and incubated at 45°C for 30 minutes. The reaction was stopped by the addition of 2 ml dinitrosalicylic acid reagent by keeping for 5 minutes in boiling water bath and quick cooling to room temperature. The degree of enzymatic hydrolysis of the CMCase was determined the spectrophotometrically (UV-Vis Spectrophotometer, Systronics, India) by measuring the absorbance at 540nm. The enzyme activity was expressed in units as the amount of enzyme required to release 1µ mol of reducing sugar as glucose equivalent min/g of the enzyme sample [9].

IU/ml = Concentration of the glucose / 0.18 x 0.5 x 30

Physical mutagenesis and mutant selection

Overnight nutrient broth *B. pumilus* culture was prepared and nutrient agar plate was prepared. Then serially diluted 10^{-6} dilution was spreaded on the plate 0.1 ml culture was added. Petri dish containing *B. pumilus* strain was placed under an ultraviolet lamp (UV C, 15W and 2537 A) with a distance of 30 cm and was irradiated for different seconds on the 7 plates one as control (20, 40, 60, 80, 100 and 120 seconds). After that black cloth was covered on the irradiated plates. The plate was incubated for 24 hours at 37^0C. The 80 seconds irradiated mutants plates were selected. Mutated colonies were marked as UVR1 - UVR30. This UVR1 - UVR30 strains were streaked on slants. CMCase assay was performed for these strains. Best strain was selected by CMCase assay. CMCase was determined by spectrophotometrically (UV-Vis spectrophotometer) by measuring the absorbance at 540 nm.

Chemical mutagenesis and mutant selection

The best UV mutant (UVR10) was further used for chemical mutagenesis. The culture suspension was prepared in the same manner as described earlier. To 5 ml of cell suspension with viability of 10^6/ml, 5 ml of sterile solution of Ethyl methyl sulphonate (EMS) (200 µg/ml) was added. The reaction was allowed to proceed. Samples were withdrawn from the reaction mixture at intervals of 15, 30, 60, 90, 120 and 150 minutes. Immediately, all the samples were resuspended in sterile phosphate buffer (pH 7). The suspended sample was again centrifuged at 5,000 rpm for 10 min. The supernatant was discarded and cells were washed thrice with sterile distilled water to remove traces of EMS. The samples were serially diluted in the same buffer and plated on screening medium as mentioned earlier. A total of 32 colonies (designated as EMR1 - EMR32) were selected from the plates and they were similarly tested for CMCase production ability.

RESULTS

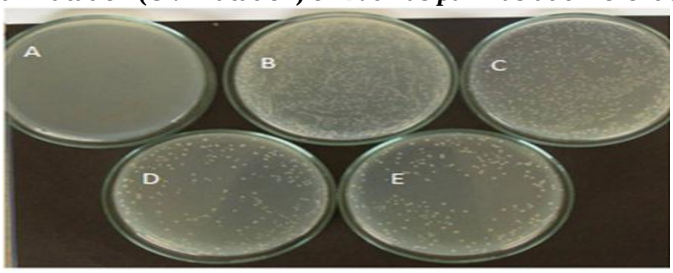
Physical mutation through UV irradiation

The survival of *Bacillus pumilus* after different periods of exposure to UV light was assessed and the results are furnished in Table. 1. The increased period of exposure at the distance of 10 cm from the UV source and the parent to be mutated is directly correlated with the mortality rate. The survival was observed at an exposure period of 120 min. This optimized UV irradiation dosage was used for the induction of mutants in *Bacillus pumilus*. Out of the total 30 mutant (UVR1 to UVR30) strains (Fig. 1), survived UV treatments were assayed for CMCase production. Only *Bacillus pumilus* UVR10 exhibited high efficiency of CMCase production (1.330 ± 0.075 IU/ml).

Chemical mutant through EMS

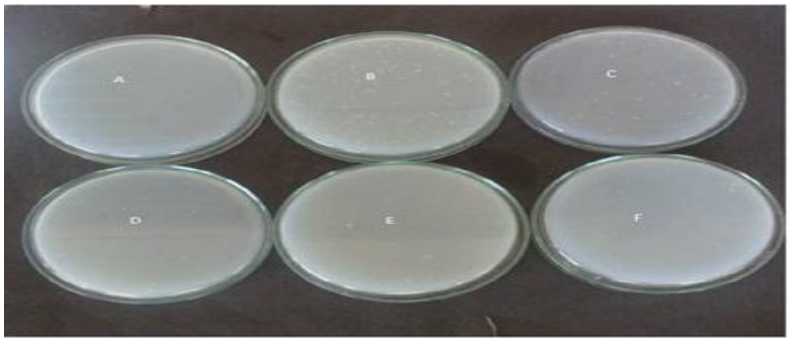
The suspension of *Bacillus pumilus* (UVR10) was exposed to 200 µg/ml of EMS for different time intervals and the results were furnished in Table. 2. The least survival was recorded at an exposure period of 120 min. Out of the total 32 mutant (CR1 to CR32) strains (Fig. 2), survived EMS treatments were assayed for CMCase production. Only CR24 *Bacillus pumilus* strain exhibited high efficiency in production on the basis of the CMCase production (2.610 ± 0.093 IU/ml). This CR24 optimized EMS exposure time was used for the induction of mutation in *Bacillus pumilus*.

Fig: 1. Physical mutation (UV mutation) of *Bacillus pumilus* at different time intervals



A. control plate; B. 20 seconds UV exposure plate; C. 40 seconds UV exposure plate; D.60 seconds UV exposure plate; E.80 seconds UV exposure plate.

Fig 2. Potential UV mutated strains *Bacillus pumilus* (UVR10) subjected to chemical mutation (EMS) at different times of intervals



A. 15 minuties chemical mutated plate; B.30 minuties chemical mutated plate; C.60 minuties chemical mutated plate; D.90 minuties chemical mutated plate; E.120 minuties chemical mutated plate; F.150 minuties chemical mutated plate.

Table. 1. Induction of mutation through UV irradiation

S. No	Isolates	CMCase IU/ml
1	UVR1	1.035 ± 0.005
2	UVR2	1.110 ± 0.012
3	UVR3	1.125 ± 0.034
4	UVR4	1.145 ± 0.048
5	UVR5	1.070 ± 0.023
6	UVR6	1.090 ± 0.031
7	UVR7	1.110 ± 0.012
8	UVR8	0.960 ± 0.003
9	UVR9	1.130 ± 0.036
10	UVR10	1.330 ± 0.075
11	UVR11	0.096 ± 0.003
12	UVR12	0.098 ± 0.005
13	UVR13	1.165 ± 0.015
14	UVR14	0.096 ± 0.004
15	UVR15	0.085 ± 0.002
16	UVR16	0.925 ± 0.004
17	UVR17	0.925 ± 0.003
18	UVR18	0.096 ± 0.004
19	UVR19	1.035 ± 0.045
20	UVR20	0.925 ± 0.004
21	UVR21	0.885 ± 0.002
22	UVR22	0.096 ± 0.003
23	UVR23	1.145 ± 0.046
24	UVR24	1.110 ± 0.038
25	UVR25	0.925 ± 0.006
26	UVR26	1.220 ± 0.046
27	UVR27	0.905 ± 0.004
28	UVR28	0.885 ± 0.002
29	UVR29	1.220 ± 0.023
30	UVR30	1.110 ± 0.039

Table. 2. Induction of mutation through EMS

S. No	Isolates	CMCase IU/ml
1	CR1	1.850 ± 0.045
2	CR2	1.500 ± 0.029
3	CR3	0.700 ± 0.002

4	CR4	1.885 ± 0.048
5	CR5	1.905 ± 0.052
6	CR6	1.905 ± 0.052
7	CR7	1.905 ± 0.052
8	CR8	1.850 ± 0.049
9	CR9	1.885 ± 0.048
10	CR10	1.885 ± 0.048
11	CR11	1.850 ± 0.039
12	CR12	1.850 ± 0.040
13	CR13	1.850 ± 0.042
14	CR14	1.885 ± 0.053
15	CR15	1.850 ± 0.039
16	CR16	1.850 ± 0.041
17	CR17	1.850 ± 0.046
18	CR18	1.885 ± 0.063
19	CR19	2.035 ± 0.096
20	CR20	1.850 ± 0.048
21	CR21	1.570 ± 0.019
22	CR22	1.885 ± 0.056
23	CR23	1.570 ± 0.039
24	CR24	2.610 ± 0.093
25	CR25	1.570 ± 0.042
26	CR26	1.570 ± 0.038
27	CR27	1.850 ± 0.042
28	CR28	1.625 ± 0.068
29	CR29	2.035 ± 0.092
30	CR30	1.850 ± 0.056
31	CR31	1.570 ± 0.052
32	CR32	1.500 ± 0.023

Table 3. CMCase production by wild, UV and EMS mutant *Bacillus pumilus*

S. No	Strains	CMCase (IU/ml)
1	Wild strain	0.665 ± 0.004
2	Physical mutant (UVR10)	1.330 ± 0.075
3	Chemical mutant (CR24)	2.610 ± 0.093

DISCUSSION

The present study reveals that the *Bacillus pumilus* wild strain was subjected to UV irradiation at the distance of 10 cm from the UV source at an exposure period of 120 seconds for physical mutation. Survived UV mutants were assayed for CMCase production. From the study *Bacillus pumilus*UVR10 have the highest enzyme production (1.330 ± 0.075 IU/ml). Similar work was also done by Jaivel and Marimuthu (2010), Myasnik *et al*. (2001). Sangkharak *et al*. (2012) reported that UV mutant strain produced 1884 U/mg protein in CMC medium. Maximum cellulolytic activities of 67.02 IU/ml and 35.8 IU/ml as reported by Sangbriba *et al.* (2006) for *Bacillus circulans* and *Bacillus megaterium* respectively is also far higher than those obtained in present study [10-13].

Similarly, the potential use of cellulosic materials as renewable sources of carbon was recognized and accelerated after cellulose degrading enzymes or cellulases were identified [14]. Up to date, the production of cellulase, which is one of the key enzymes for cellulose biodegradation, has been found to be the most expensive step. This step takes approximately 40% of the total cost during ethanol production from cellulosic biomass [15]. The high cost of the enzyme production limits its industrial use in the production of soluble sugars. Therefore, several approaches including chemical mutations, UV irradiations and genetic engineering to obtain enhanced cellulase producing strains have been given a high priority in the last decade [16]. In view of Sanchez *et al*. (1992) stated that the mutation induction and or selection techniques, together with cloning and protein engineering strategies have been exploited to develop enzyme production [17-18].

Ultraviolet radiation is one of the well-known and most commonly used mutagen. It is universally used to induce genetically improved strains indicated that in *B.*

megaterium, the formation of both thymine dimmers in germinated spores and spore photoproducts in dormant spores requires much less UV dose than that in germinated *gpr B. subtilis* spores. Kotchoni and Shonukan, (2002) indicated that UV treatment was more effective as a mutagenic agent than N-methyl- N-nitro- N-nitrosoguanidine (MNNG) in isolation of spore less mutants in *Bacillus sphaericus* [19]. Nieves *et al*.(1998) revealed that *B. anthracis* spores may be three to four times more resistance to UV than spores of commonly used strains of *B. subtilis.* Mutant repair is carried out by an enzyme called photolyase and also by complex combination of more than a dozen enzymes which they reverse the UV induced damage [20-21].

In chemical mutation *Bacillus pumilus* (UVR10) were exposed to 200 µg/ml of EMS for different time intervals. The least survival was recorded at an exposure period of 120 min. survived EMS treatments were assayed for CMCase production. Only CR24 *Bacillus pumilus* exhibited high efficiency of CMCase production (2.610 ± 0.093 IU/ml). This CR24 optimized EMS exposure time was used for the induction of mutants in *Bacillus pumilus*. Kotchoni and Shonukan, (2006) reported that the similar mutation work and their result up to 6.2 mg cellulase and 11.4 mg cellulase per gram of dry cell mass respectively. Similar work also done by Jaivel and Marimuthu (2010) [22].

Snustad*et al*. (2000) observed that the alkylating agentsare chemicals that donate alkyl groups to other molecules. They include the nitrogen, sulfur and mustards. Methyl methyl sulfonate (MMS), ethyl methyl sulfonate(EMS) and nitrosoguanidine (NTG) chemicals that have multiple effects on DNA. Mechanism of mutagenisis by alkylating agents involves the transfer of methyl or ethyl groups to the bases, resulting in altered base pairing potentials. For examples, EMS causes ethylation of the bases in DNA at the 7-N and the 6-O positions. When 7-ethyl guanine is produced, its base pairs with thymine to cause G:C→ A:T transitions. Other base alkylation product activates error prone DNA repair processes that introduce transitions, transevertions, and frame shift mutations during the repair process. Some alkylating agents (those with two relative alkyle groups), cross link DNA strands or molecules and induce chromosome breaks, which result in various kinds of aberrations. Alkylating agents as a class therefore exhibit less specific mutagenic effects than do base analogs, nitrous acid or acridiens. Alkylating agents induce all types of mutations, including transitions, transevertion, frame shifts and even chromosome aberrations, with relative frequencies that depend on the relativity of the agent involved

Strain improvement of *Aspergillus terreus* was carried out by physical and chemical mutagenesis for increased lovastatin production (Jaivel and Marimuthu, 2010). The spore survival of *Aspergillus terreus* strain JPM3 after different periods of exposure to UV light and Ethyl Methyl Sulfonate (EMS) was assessed. Four mutant clones were obtained by the mutagenesis programme. In which two mutants were derived by UV irradiation and another two mutants derived by EMS treatment. The yield of lovastatin varied from mutant to mutant and the mutant strain JPM3-UV1 produced the maximum lovastatin yield (1553.02 mg/l) followed by strain JPM3-EMS2 (948.5 mg l/1). It was correlated to the genetic alterations in ethyl methyl sulfonate (EMS) induced *ad-$_3$B* mutants of *Neurospora crassa* have been identified by tests for specific reversibility after treatment with *O*-methyl-hydroxylamine (OMHA), nitrous acid (NA), ethyl methyl sulfonate (EMS) and a monofunctional acridine mustard (ICR-170). Mutants classified as base pair substitutions reverted after treatment with at least 1 of the first 3 compounds, whereas mutants classified as base-pair insertions or deletions reverted only after treatment with ICR-170 [23-24].

CONCLUSION

This study enlightens the CMCase production by strain improvement through UV irradiation and EMS treatment. The promising strain *Bacillus pumilus* can be used for various industrial applications with economically feasibility.

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