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Effect of physical and chemical mutagenesis on protease production from *Aeromonas hydrophila*

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Abstract: Secondary metabolite production from wild strains is very low for economical purpose therefore certain strain improvement strategies are required to achieve hundred times greater yield of metabolites. The present study was conducted for enhanced production of protease from *Aeromonas hydrophila* A4 by mutagenesis using ultraviolet (UV) radiation, and Mitomycin C (MitC) as mutagens. Results showed that a protease over producer mutant symbol *A. hydrophila* A4-78 was obtained after mutagenesis with UV irradiation with higher enzyme specific activity (47.6 U/mg protein) in comparison to the wild type (30.01U/mg protein). On the other hand another overproducer mutant symbol *A. hydrophila* A4-127 was obtained after chemical mutagenesis with MitC characterized with high protease production. The enzyme specific activity in its crude filtrate was 38.4 U/mg protein in comparison with the wild type. it could be concluded that physical mutagenesis using UV irradiation was more efficient than the chemical mutagenesis by MitC to enhance the ability of *A. hydrophila* A4 in protease production.

Key words: *Aeromonas hydrophila*, mutant, UV, Mitomycin C

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Introduction:

Aeromonas species usually inhabit aquatic environments including fresh water, brackish water, and seawater (1,2). *Aeromonas* produced various extracellular proteins containing hemolysin, protease, and lipases (3,4). Protease enzyme constituted two third of the total enzyme used in various industries (5). It is applied in pharmaceutical, food, detergent industries, waste treatments and others (6,7). Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used (8).

Many species of the genus *Aeromonas* produce a variety of extracellular factors include an array of exoenzymes and exotoxins, enterotoxins, hemolysins, lipases, gelatinase, caseinase, elastase, lecithinase, deoxyribonuclease and proteases. Many of the proteins involved in pathogenicity are reliant on the general secretory pathway for export (9,10). On the other hand, direct evolution tools have been increasingly used to improve enzymes and whole genomes for various bioprocessing applications, and numerous molecular biological techniques have been developed to create genetic diversity through mutagenesis. In this study, we try in attempt to increase protease production from *A. hydrophila* by

inducing genetic mutations using physical and chemical mutagens.

Materials and Methods:

Media and culture conditions:

A. hydrophila A4 was obtained from previous study (11). This isolate was cultured and maintained on MacConky agar medium at 30 ºC for 24h under aerobic conditions.

Protease production:

Aliquot of 100 µl of fresh bacterial culture was taken and used to inoculate nutrient broth medium in conical flasks and incubated at 30 ºC for 24 hrs. with shaking at 150 rpm. After incubation, cultures were centrifuged, pellets were discarded, and supernatant as crud enzyme was assayed for protease activity.

Enzyme assay:

Activity of protease was assayed by measuring the release of trichloroacetic acid soluble peptides from 1% (w/v) casein solution at 280 nm (12). One unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 280 nm equal to 0.01 in one minute under experimental conditions.

Protein concentration:

Protein concentration was determined according to the method described by Bradford, (13).

Physical mutagenesis:

Mutagenesis by UV irradiation was achieved according to Khattab and Mohamed (14). *A. hydrophila* A4 was exposed to UV rays at a distance of 10 cm from the UV lamp for 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 seconds. All these exposures were performed in a dark room to avoid any photoreaction in the production of mutants. Aliquot of 0.1 ml of irradiated cell suspension was then spread on nutrient agar plates, and were incubated at 35 ^{o}C for 24 hours.

Chemical mutagenesis:

Chemical mutagenesis by Mitomycin C was achieved according to Borwring and Morris (15), by incubating 1 ml fresh culture of *A. hydrophila* A4 with 1 ml of 0.1 mg/ml Mitomycin C at 30°C for 120 minutes, then aliquots of 0.1 ml of cell suspension was taken every 20 minutes of incubation, streaked on nutrient agar plates and incubated for 48 hour at 35°C.

Results and Discussion:

In order to enhance the ability of *A. hydrophila* A4 in protease production by random mutagenesis, this isolate was subjected to physical and chemical mutagens. Results of physical mutagenesis illustrated in figure (1) showed that the LD90 was reached after 70 seconds of irradiation by UV-ray, and then cultivability of most *A. hydrophila* A4 was lost after 80 seconds of irradiation. Survivals of irradiated bacterial cells obtained after subjection to LD90 of UV- ray were selected and screened according to their ability to produce protease.

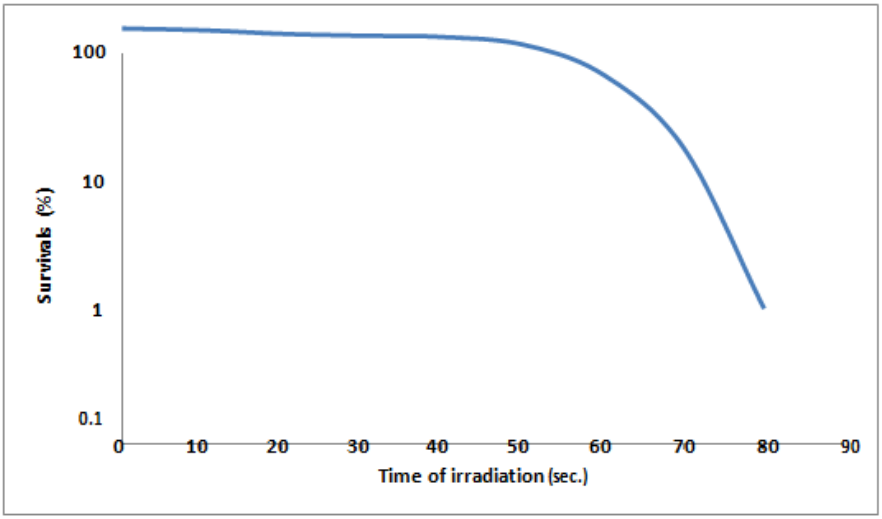

Figure (1): Survivals of *A. hydrophila* A4 after UV irradiation for different periods of times.

Results indicated in table (1) showed that only three mutants out of one hundred were improved their ability in protease production. The most efficient mutant in protease production was A4-78 when its specific activity of protease in culture filtrate was 47.6 U/mg compared with 31.01 U/mg for the wild type. The strain improvement strategies especially mutagenesis and screening of hyper-producing mutants are very important in the production of secondary metabolites during the fermentation process (16). UV radiation and chemical mutagenesis have been employed to obtain the protease hyper-producing mutants. In the present study, physical and chemical mutagenesis has been employed to mutate *A. hydrophila* A4 to obtain protease hyper-producing mutants. Physical mutagenesis was done using UV radiation. In a previous study, it was reported that to have powerful mutations and effective screening of mutants, lethality rate should be very high (17). It is reported that the death rate of 70-95% has been optimized for enhanced secondary metabolite production (18). It is reported that a decrease in the survival rate of *hydrophila* A4 from 100% to 8% occurred as a result of UV mutagenesis when the time of UV exposure was increased from 0 to 25 minutes (19). In another study, survival rates of 8% and 5% were observed at UV exposure of 100 and 120 seconds, respectively, showing a gradual reduction in the survival rate with increasing UV exposure on *A. hydrophila* A4. Mutants at these exposure times resulted in enhanced protease production, compared to the wild type strains (20).

Table (1): Specific activity of protease produced by mutants of *A. hydrophila* A4 after subjection to LD_{90} of UV irradiation

Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)
A4-1	30.6	A4-34	29.6	A4-67	31.0
A4-2	31.0	A4-35	31.0	A4-68	28.9
A4-3	28.9	A4-36	31.0	A4-69	29.7
A4-4	31.0	A4-37	31.0	A4-70	30.2
A4-5	26.6	A4-38	31.0	A4-71	31.1
A4-6	30.9	A4-39	31.0	A4-72	30.6
A4-7	30.6	A4-40	21.0	A4-73	23.0
A4-8	29.1	A4-41	26.3	A4-74	29.9
A4-9	26.8	A4-42	31.1	A4-75	31.0
A4-10	30.1	A4-43	31.7	A4-76	31.0
A4-11	25.8	A4-44	22.5	A4-77	30.7
A4-12	29.0	A4-45	29.7	A4-78	47.6
A4-13	31.0	A4-46	30.6	A4-79	30.9
A4-14	29.1	A4-47	30.0	A4-80	31.0
A4-15	29.7	A4-48	31.0	A4-81	45.1
A4-16	31.0	A4-49	30.7	A4-82	30.1
A4-17	31.0	A4-50	30.8	A4-83	25.5
A4-18	27.9	A4-51	28.0	A4-84	29.0
A4-19	30.9	A4-52	23.3	A4-85	31.0
A4-20	30.5	A4-53	29.1	A4-86	31.0
A4-21	23.8	A4-54	28.2	A4-87	38.8
A4-22	27.9	A4-55	30.7	A4-88	31.0
A4-23	21.1	A4-56	31.0	A4-89	30.0
A4-24	29.9	A4-57	27.5	A4-90	28.8
A4-25	19.8	A4-58	30.0	A4-91	29.0
A4-26	31.0	A4-59	31.1	A4-92	29.3
A4-27	31.9	A4-60	31.1	A4-93	30.0
A4-28	30.0	A4-61	30.2	A4-94	31.2
A4-29	29.9	A4-62	29.9	A4-95	31.0
A4-30	29.8	A4-63	31.0	A4-96	28.1
A4-31	28.5	A4-64	30.6	A4-97	31.0
A4-32	30.4	A4-65	31.0	A4-98	24.2
A4-33	30.1	A4-66	20.7	A4-99	30.1
A4-100	30.9	Wild-type	31.01		

Results of the present study indicated that the enhanced production of protease might be due to some changes in the genetic code of *A. hydrophila* A4-78 as a result of UV radiation. Over-mutagenesis should therefore be avoided while screening for hyper-producing mutant strain. Optimum dose of mutagen is required in order to get positive mutation. According to the Poisson model of mutagenesis, 37% of the survival rate would be unaffected for enhanced metabolic production (21).

Chemical mutagenesis was also achieved by using Mitomycin C to induce random mutations in the double stranded DNA helix. MitC was used as mutagenic agent to generate mutants with higher protease production. For this purpose fresh culture of *A. hydrophila* A4 was incubated with MitC in a concentration of 0.1mg/ml at 30°C for different periods of time. Results illustrated in figure (2) showed that survival of the bacterial cells was decreased with increasing time of incubation of the mutagen till complete death after 120 minute of incubation with MitC, while the LD_{90} was reached after 80 min. with the mutagen. Aliquots of cell culture were taken after this time of incubation to evaluate the overproducer protease production. It is well known that the most efficient mutants were raised after reaching the LD_{90} (15).

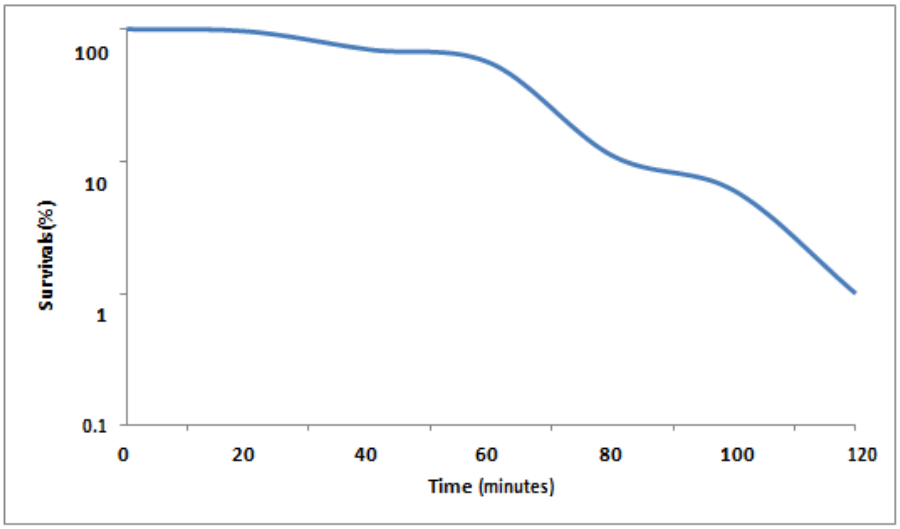

Figure (2): Survival curve of *A. hydrophila* A4 after mutagenesis with 0.1mg/ml Mytomycin C for different periods.

Results indicated in table (2) showed that many over-producer mutants were obtained after 80 minutes of incubation with MitC. Among them was mutant designated A4-127 specific activity of protease in its culture filtrate in comparison with the productivity of the wild type of *A.hydrophilia* (31.01 U/mg).

Chemical mutagenesis is time and concentration dependent. In the present study, *A. hydrophila* A4 was treated with 0.1mg/ml of Mitomycin C for time intervals ranging from 20 to 120 minutes. Protease hyper-producing mutant was obtained after incubation for 80 min. of mutagen, some of the obtained mutants showed almost similar activities (29-31 U/mg) as was shown in table (2). In the present study, it is also observed that A4-127 higher over-producer mutant (38.4 U/mg) was obtained.

Table (2): Specific activity of protease enzyme produced by mutants of *A. hydrophila* A4 after subjection to LD_{90} of Mitomycin C mutagenesis.

Mutant symbol	Specific activity (U/mg)	Mutant symbol	Specific activity (U/mg)
A4-101	31.1	A4-120	29.0
A4-102	31.0	A4-121	31.1
A4-103	28.3	A4-122	26.8
A4-104	30.9	A4-123	28.0
A4-105	22.9	A4-124	29.3
A4-106	31.0	A4-125	29.1
A4-107	26.6	A4-126	35.1
A4-108	20.1	A4-127	38.4
A4-109	29.8	A4-128	30.9
A4-110	30.0	A4-129	25.0
A4-111	32.7	A4-130	31.0
A4-112	27.0	A4-131	31.1
A4-113	30.0	A4-132	31.1
A4-114	29.1	A4-133	30.9
A4-115	31.0	A4-134	29.9
A4-116	31.0	A4-135	26.0
A4-117	31.0	A4-136	31.0
A4-118	21.9	A4-137	31
A4-119	31.9	A4-138	20.7
Wild-type	31.01		

According to these results it could be concluded that physical mutagenesis using UV irradiation was more efficient than the chemical mutagenesis by MitC to enhance the ability of *A. hydrophila* in protease production, highest specific activity of protease produced by this mutant (A4-78) was 47.6 U/mg in comparison with 38.4U/mg for the mutant A4-127 obtained after mutagenesis by MitC, so that the over producer mutant *A. hydrophila* A4-78 was selected and used for determining the optimum conditions for protease production.

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