

Induction of overproducing alkaline protease *Bacillus* mutants through UV irradiation

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ABSTRACT

Bacillus pumilus and *Bacillus alvei*, among alkaline protease producing strains, were used to examine the changes in alkaline protease gene expression following UV irradiation. Induction of mutation in *Bacillus* mutant strains was carried out by 0, 5, 10, 15 and 20 min. exposure times of UV irradiation and different distances between the treated bacterial cultures and UV source. Results revealed that alkaline protease activity assayed under submerged culture conditions was more accurate than the relative growth production (C/G) method, because there was no proportional correlation between zone diameter and the ability to produce the enzyme in submerged cultures. No enzyme activity was scored with *B. pumilus* mutants, while the activity was pronounce, in case of *B. alvei* mutants. Mutants No.15, 55 and 34 were the most efficient in enzyme production under submerged conditions being 68.8, 81.1 and 99 U/ml, respectively. Their alkaline protease activities were 2.6, 3.02 and 3.7 folds than those of the original strains. The optimal enzyme production was achieved after 48 hr at air: medium ratio of 39: 1. Results also proved that the inoculum size in all tested mutant ranges had no significant effect on the enzyme production. The supplementation with glucose to growth medium gave the highest level of enzyme productivity, while lactose showed the lowest. The addition of arabinose and xylose completely inhibited the enzyme production by both tested strains, while incorporation into the culture medium of sucrose and maltose failed to produce the enzyme in *B. alvei*, but in mutant No.8, they enhanced high levels of productivity.

Key words: *Bacillus* spp, UV irradiation, alkaline protease.

INTRODUCTION

Microorganisms form an ideal source of industrial enzymes that could be produced in unlimited quantities. The production methods are relatively simple and well developed and microbial proteases with widely different properties can be produced. The detergent industry is the largest user of industrial enzymes, accounting for more than one third of the global market

which is considered to total 1.6 billion US dollars. The genus *Bacillus* is among the most important organisms for commercial protease production (Roa *et al.*, 1998). The enzymes used in the detergent industry today are mainly proteases and all are of *Bacillus* origin (Roger *et al.*, 2002). Many economic important industries are mainly depended on industrial enzymes.

Mutation induction and/or selection techniques, together with cloning and protein

engineering strategies have been exploited to develop enzyme production (Schallmeyer *et al.*, 2004). Ultraviolet radiation is one of the well-known and most commonly used mutagen. It is universally used to induce genetically improved strains. Sanchez *et al.* (1992) indicated that in *B. megaterium*, the formation of both thymine dimers in germinated spores and spore photoproducts in dormant spores requires much less UV dose than that in germinated *gpr B. subtilis* spores. Aly *et al.* (2001) indicated that UV treatment was more effective as a mutagenic agent than MNNG in isolation of sporless mutants in *Bacillus sphaericus*. Wayne and Belinda, 2003 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 (Ben, 2003).

The aim of this study was to examine the effect of UV irradiation on alkaline protease gene expression and induction of overproducing strains. The promising strains in this study may be used in various economic industrial applications and for successive genetic improvement as well.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Two *Bacillus* strains, *B. pumilus* and *B. alvei* producing alkaline protease under alkaline conditions were kindly provided by the Agricultural Microbiology Dept., NRC, Cairo and used in this study. Cultures of *Bacillus* strains were grown at 37°C in Lauria-Bertani (LB) broth with vigorous shaking or on LB agar (Davis *et al.*, 1980). The final pH of the medium was adjusted to pH 9 by the

addition of an appropriate volume of sterile 20% (w/v) sodium carbonate.

UV mutagenesis

Ultraviolet (UV) irradiation as a physical mutagenic agent was used with both *B. pumilus* and *B. alvei* for over production of alkaline protease. Mutagenesis of *Bacillus* strains was carried out according to Justin *et al.* (2001) using different exposure times and radiation intensities, i.e., distances from irradiation source.

Bacterial cells were grown in LB broth medium overnight with shaking at 37°C. A sample of each culture (ca. 10⁶ cells / ml) was centrifuged, resuspended in saline solution (0.9% NaCl). Two ml of *B. pumilus* suspension was placed into 15 cm diameter glass Petri dishes at a distance of 15 cm in dark and irradiated for 0, 5, 10, 15 and 20 min with gentle agitation while in case of *B. alvei*; the suspension was exposed at distances of 5 and 10 cm for 10 min. A 15-w germicidal lamp (254nm) provided the UV irradiation.

Portions of 0.5 ml of suitable dilutions of *Bacillus* strains were spread on five LB plates and incubated at 37°C for 24 hr. Colonies developed after incubation were counted and transplanted onto slants for further studies. The survival percentages were estimated for each treatment.

Enzyme detection

LB plus 1% skim milk agar was used to detect the alkaline protease production according to Kunammeni *et al.* (2003). Sterile skim milk was added after autoclaving and cooling the medium at 50°C. In situ protease production was demonstrated by the clearing of opaque milk proteins in the area surrounding colonies growing on the surface.

Isolation of alkaline protease over-producing mutants and enzyme assays

For isolation of overproducing mutants after UV irradiation, developed colonies were inoculated onto LB agar medium plus 1% skim milk under alkaline conditions and incubated at 37°C for 40 hr, then assayed for enzyme production according to Smith *et al.* (1952). Depending upon the zone of clearance, mutants with high activity were selected on the basis of the relative growth production (C/G) of the bacterial colonies. Three measurements were used to determine the enzyme assay, 1) enzyme production defined as lyses zone area, mm² (C). 2) colony growth area, mm² (G), and 3) relative growth production (C/G). The enzyme activity of superior alkaline protease producing mutants was assayed according to the method of Dumusois and Priest (1993) using nutrient yeast salt medium (NYSM) (Yousten and Davidson, 1982) and azocasein (0.4 %) as a substrate whereas one enzyme unit was defined as the amount of enzyme that yields an increase of 0.1 OD at 420 nm / 30 min under alkaline conditions. Soluble protein was determined by the method of Ohnisti and Barr

(1978) using bovine serum albumine as a standard.

Cultural and physiological studies

Some Cultural and physiological studies including incubation period, aeration, inoculum size and carbon sources were done according to Mehrotra *et al.* (1999).

RESULTS AND DISCUSSION

UV mutagenesis

Results in Table (1) showed that the survival percentages in case of *B. pumilus* were decreased by increasing the time of exposure whereas the survival percentage was very low (0.1 %) at exposure time for 20 min. On the other hand results in Table (2) showed that survival percentages in *B. alvei* were decreased by increasing the radiation intensities, i.e., distances. It also showed that at 5 cm, the bacterial survival was sharply reduced (0.006%).

Table (1): Survival of *Bacillus pumilus* after UV exposure using different doses at a distance of 15 cm.

Time (min)	Cells / ml	Survival %
0	60 x 10 ⁶	100.0
5	40 x 10 ⁶	66.7
10	60 x 10 ⁵	10.0
15	120 x 10 ⁴	2.0
20	60 x 10 ³	0.1

Table (2): Survival of *Bacillus alvei* after UV exposure at different distances for 10 minutes.

Distance (cm)	Cells / ml	Survival %
0	60 x 10 ⁶	100.000
5	40 x 10 ²	0.006
10	90 x 10 ³	0.129

A total of fifty *B. pumilus* strains, survived UV treatments, were assayed for alkaline protease production. Only twenty seven did exhibit high efficiency in production

on the basis of the relative growth production (C/G). Table (3) showed that the majority of strains were efficient in alkaline protease production. Considerable variations were

recorded in relation to cell productivity of the enzyme. The C/G values ranged from 10.2 for mutant No. 6 to 32.4 for the corresponding

No.3 which was 14.4 times more active than the original strain.

Table (3): The alkaline protease production (C/G) of the superior *Bacillus pumilus* mutants.

Mutant No.	G	C	C/G	Mutant No.	G	C	C/G
W.T	314	706.0	2.3	36	19.9	260.9	13.1
1	19.6	245.3	12.5	37	17.6	201.0	11.4
3	7.0	226.8	32.4	38	9.9	153.8	15.6
6	19.6	200.0	10.2	39	13.5	299.9	22.2
8	12.6	283.3	22.6	40	17.3	390.0	22.5
9	7.0	200.9	28.7	41	38.7	410.7	10.6
10	12.5	283.0	22.6	42	11.9	290.0	24.4
12	19.6	200.9	10.3	43	13.5	188.0	13.9
14	12.6	176.6	14.1	44	12.6	281.9	22.4
20	19.3	200.0	10.4	45	10.2	146.0	14.3
24	12.6	200.9	16.0	46	16.5	198.9	12.1
33	12.5	176.6	14.1	47	10.5	178.5	17.0
34	13.5	188.0	14.0	48	10.6	157.0	14.8
35	10.2	200.9	19.8	49	7.9	143.0	18.1

Table (4) indicates that out of twenty seven mutants, fourteen are falling in the production range of 10.1-15, five in 15.1-20,

six in 20.1-25 and one mutant in the remaining production ranges.

Table (4): The relative growth production range and the relative frequency of the superior twenty seven *B. pumilus* UV induced strains measured as (C/G).

Range of production	No. of strains	Relative frequency
0 – 5	-	-
5.1 – 10	-	-
10.1 – 15	14	51.9
15.1 – 20	5	15.5
20.1 – 25	6	22.2
25.1 – 30	1	3.7
More than 30	1	3.7

On the other hand, one hundred and fifty *Bacillus alvei* strains, survived UV treatments were assayed for enzyme production. Only twenty five did exhibit high efficiency in alkaline protease production on the basis of the relative growth production (C/G). Wide variations were detected in relation to cell

productivity of the enzyme, (Table 5). The C/G values ranged from 14 for mutant No.8 to 40.1 for mutant No.4 which was 10 folds more active than the original strain (Wild type).

Table (5): The alkaline protease production (C/G) of the superior *Bacillus alvei* mutants under two light intensity levels.

At a distance of 5 cm							
Mutant No.	G	C	C/G	Mutant No.	G	C	C/G
W.T	81	324	4.0	53	16	289	18
15	9	169	18.8	106	16	256	16
18	9	256	28.4	122	16	400	25
19	9	196	21.8	127	25	361	14
52	11	239	21.7	133	9	144	16
At a distance of 10 cm							
3	9	169	18.8	24	4	121	30
4	9	361	40.1	34	9	324	36
7	25	361	14.4	49	4	64	16
8	16	225	14.0	55	9	225	25
11	9	144	16.0	60	4	121	30
15	4	100	25.0	62	9	196	22
19	9	196	21.8	66	16	256	16
20	4	100	25.0	74	9	225	25

Results in Table (6) indicates that out of twenty five mutants, three mutants are lying in the production range of 10.1-15, eight mutants

in 15.1-20, nine mutants in 20.1-25, one mutant in 25.1-30, two mutants in 30.1-35 and one mutant in the rest production ranges.

Table (6): The relative growth production range and the relative frequency of the superior twenty five *Bacilli alvei* UV mutants measured as (C/G).

Range of production	No. isolates	Relative frequency
0 – 5	-	-
5.1 – 10	-	-
10.1 – 15	3	12
15.1 – 20	8	32
20.1 – 25	9	36
25.1 – 30	1	4
30.1 – 35	2	8
35.1 – 40	1	4
More than 40	1	4

The superior alkaline protease producing mutants of *Bacillus pumilus* and *Bacillus alvei* were chosen to assay their productivity according to Dumusois and Priest (1993). Negative results were found for enzyme

production in the case of *Bacillus pumilus* mutants while in *Bacillus alvei*, positive results were obtained and proved variations in enzyme activity.

Table (7): The alkaline protease activity of the highest *Bacillus alvei* mutants.

Mutant No.	C/G	Enzyme activity U/ml	Protein mg/ml	Specific activity expressed as U / mg protein	Mutant No.	C/G	Enzyme activity U/ml	Protein mg/ml	Specific activity expressed as U / mg protein
W.T	4.0	26.8	1.70	15.4	20	25.0	--	1.5	--
3	18.7	--	1.50	--	24	30.3	--	1.4	--
4	40.1	--	1.40	--	34	36.0	99.0	1.4	69.2
8	14.0	50.0	1.50	32.9	55	25.0	81.1	2.0	41.4
11	16.0	--	1.80	--	62	21.7	29.5	1.5	19.2
15	25.0	68.8	1.60	42.2	122	25.0	43.8	1.5	28.4
18	28.4	54.0	1.70	34.6	133	16.0	56.0	1.5	36.6
19	21.7	58.4	1.34	43.4					

(--): undetected amount of enzyme production.

Results in Table (7) proved that there is no correlation between zone diameter and the ability to produce the enzyme in submerged culture, e.g., mutant No.8 and 4 produced 14 and 40.1 in relative growth production (C/G) while mutant No. 8 was more efficient in enzyme activity (50 U/ml) than mutant No.4 which was not able to give any activity under submerged conditions and this was in agreement with the results reported by Aunstrup (1974). These results may be due to incompatibility or differences in growth conditions, e.g., depth of agar layer can be affected the diffusion of enzyme.

Bacillus alvei mutants showed different responses to UV radiation for alkaline protease production. These variations are more probably due to the differences induced in their genetic background. Therefore, results obtained revealed variation in gene expression, i.e., enzyme activity as reported by Justin *et al.* (2001). It is suggested that the increase in enzyme productivity might result from damage of genes located on plasmids which have a negative influence on the chromosomal alkaline protease production genes, i.e., repression, as reported by Solaiman *et al.* (2003). Variations may be also due to some factors, e.g., damage or mutation occurs in gene(s), differences in their ability of repairing their DNA, the repair enzymes themselves are damaged and the repair mechanism is not

universal, so replication can not take place again (Ben, 2003). Uehara *et al.* (1974) proposed the presence of regulator genes responsible for the production of alkaline protease. In addition, mutations led to the overproduction of proteases may also include those genes which could modify the productivity rather than the structure genes themselves. Nagami and Tanaka (1986) proved that mutation of genes controlling cell membrane composition led to the hyper production of proteases. They also pointed to the effect of mutations on regulatory genes which are associated with the structural genes. However, the application of UV irradiation, whatever the mutation(s) include either modifying or structural genes, led to the isolation of hyper enzyme producing cultures (Qadeer *et al.*, 1980). In general, results showed that alkaline protease production is a multigenic controlled system which support the finding of Kncerova *et al.* (1984) who reported that protease synthesis is regulated by repression and also Yang *et al.* (1984) and Takagi *et al.* (1990) who suggested that in addition to structural genes, regulatory genes also play an important role in the production levels.

Cultural and physiological studies of alkaline protease production

Some cultural and physiological studies including incubation period, aeration,

inoculum size and carbon sources, were done to test their effects on alkaline protease production obtained by the original strain *B. alvei* and mutant No.8.

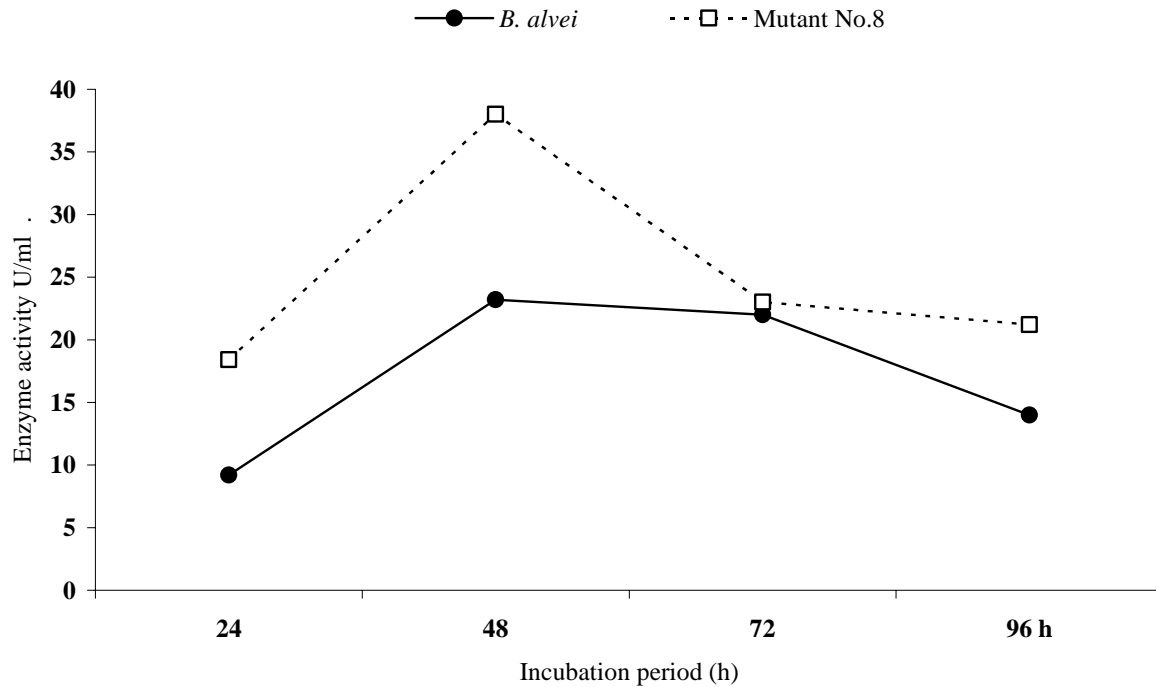


Fig. (1): Effect of incubation period on alkaline protease activity.

Results in (Fig.1) revealed that the optimal enzyme activity was achieved after 48 hr for both tested strains. The effect of aeration level proved that the optimal air: medium ratio for enzyme production was 39:1 for both tested strains Fig. (2). It is clear that enzyme activity increases progressively with increasing the air: medium ratio reaching maximum when the medium volume was reduced to occupy only about 2.5% of the total

volume of the experimental flask. Varying the inoculum size in the tested ranges had no obvious effect on the activity of enzyme produced by both strains (Fig. 3). In this respect, Rezkallah (1999) reported that the highest levels of enzyme activity were obtained at 4: 1 (air: medium ratio) under shake culture conditions after two days of incubation period using 4% inoculum size.

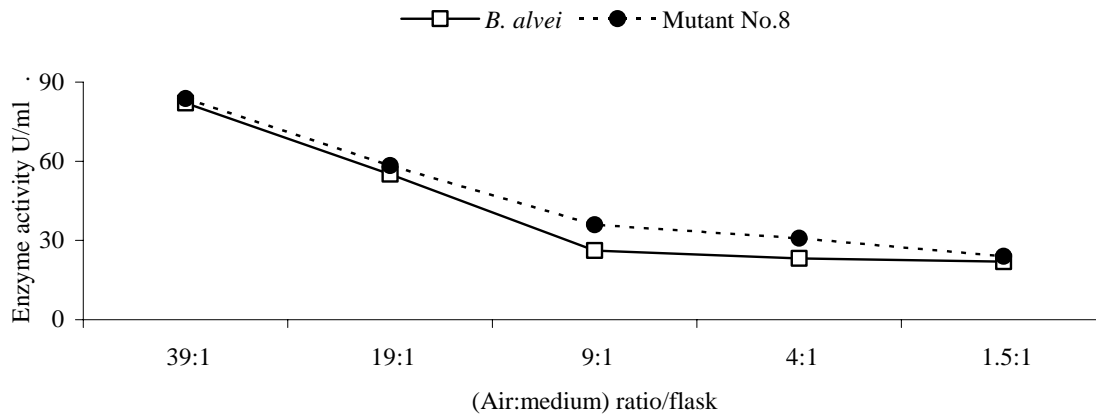


Fig. (2): Effect of aeration level on alkaline protease activity.

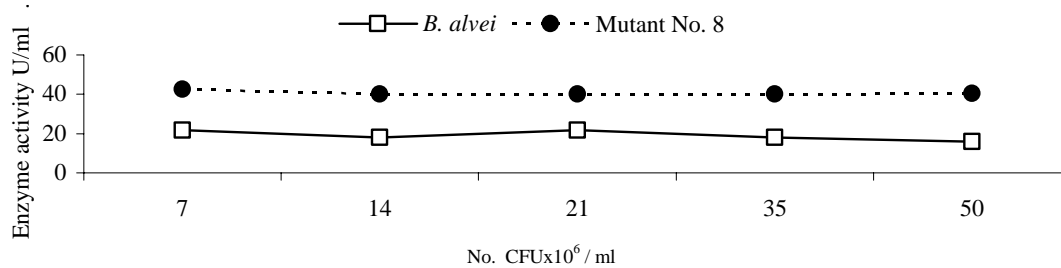


Fig. (3): Effect of inoculum size on alkaline protease activity.

The effect of supplementation with different carbon sources on the enzyme activity was determined (Fig.4). Results indicate that glucose gave the highest enzyme activity in case of mutant No.8 (140 U / ml) while lactose gave the lowest activity. On the other hand, arabinose and xylose completely inhibited the activity of the enzyme production by both tested strains. It is worthy to mention that sucrose and maltose failed to produce any levels of the enzyme activity in case of *B. alvei* while they enhanced the enzyme productivity

in case of mutant No.8. Mehrotra *et al.* (1999) reported that maximum alkaline protease activity produced by *Bacillus* spp was achieved in the presence of 1% (w / v) glucose over 20 hr incubation period. Mabrouk *et al.* (1999) reported that the highest yield of alkaline protease was achieved by using lactose (4%) and glucose (1.5%).

From the present results, it is concluded that protease enzyme-overproducing bacilli strains and UV-mutants are highly recommended for various applications.

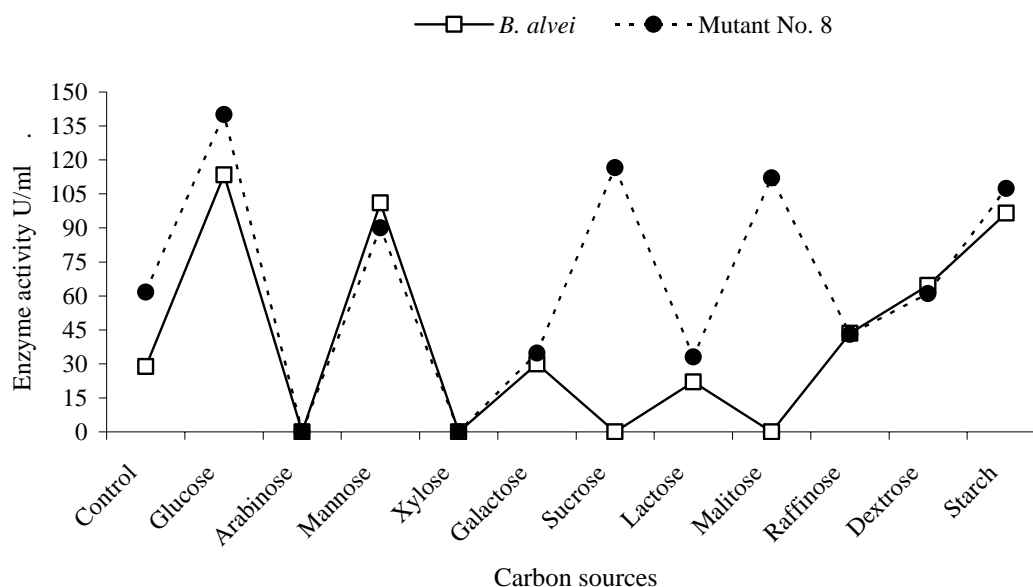


Fig. (4): Effect of different carbon sources on alkaline protease activity

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الملخص العربي

إنتاج طوافر عالية الإنتاجية للإنزيم القلوي المحلل للبروتين من الباسيلاس من خلال التشعيع بالموجات فوق البنفسجية

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