http://dx.doi.org/10.22161/ijeab/2.5.10 *ISSN: 2456-1878*

Enhancement of protease production by Bacillus sp. and Micrococcus varians induced by UV-mutagenesis

Chibani Hiba Rahman*, Fellahi Soltana, Chibani Abdelwaheb

Laboratory of Microbiology and Plant Biology, Faculty of Natural and Life Sciences, University of Mostaganem, Algeria.
*Corresponding author

Abstract— *Microbial proteases contribute nearly 40% of the total worldwide enzyme market. Hence, with the view of this significance, the main objective of the present study was to enhance protease production of two bacterial strains, Bcillus sp. and Micrococcus varians using UV mutagenesis. Induction of mutation in both strains was carried out at different exposure times: 0, 3, 6, 9, 12, 15, 18 and 21 min at a distance of 10 between UV source and treated bacteria.Two best protease producer mutants for the two bacterial strains (UV-9 for Bcillus sp.and UV-18 forMicrococcus varians) were selected based on the clearance zone diameter of mutant colonies on 1% skimmed milk agar plates. UV-9 mutant showed 1.4 fold higher protease activity than the wild type in solid and liquid medium. However UV-18 mutant was found to produce 2.5 fold increases over the wild type on agar plates and 2.1 fold enhancement in liquid-medium assay.The two mutants were very effective in feather keratin-degrading in less than two days, UV- 18 was more efficient than UV-9.*

Keywords— Bcillus sp., Micrococcus varians,protease, UV-mutagenesis.

I. INTRODUCTION

Proteases are group of enzymes which catalyze hydrolysis of peptide bonds in proteins. They are also called as peptidases or proteinases or proteolytic enzymes (Rao*et al.,* 1998).Among the various proteases, bacterial proteases are the most significant,compared with animal and fungal proteases and among bacteria, *Bacillus* sp. are the most important producers of extra-cellular proteases (Boominadhan*et al.,* 2009).

Proteases are among the most important industrial enzymes due to their biotechnological interests. They account for about 60% of the total worldwide sale of enzymes(Reddy *et al.,* 2008), and are widely used in several industries that include detergent, food, pharmaceutical, leather, diagnostics,meat processing, waste management and silver recovery (Gupta *et al.,* 2002;Chellappan*et al.,* 2006). These enzymes also have potential to contribute in the development of high value added products due to their characteristic nature of aided digestion (Glazer and Nikaido, 1995). Due to their increased economic importance, research is being carried out throughout the world to isolate hyperactive strains for the production of proteases (Gupta *et al.,* 2002).

Microbial strain improvement plays a key role in the commercial development of microbial fermentation processes. As a role, the wild strains usually produce limited quantities of the desired enzyme to be useful for commercial application (Glazer and Nikaido, 1995). Mutation induction and/or selection techniques, together with cloning and protein engineering strategies have been exploited to develop enzyme production (Schallmey*et al.,* 2004). Ultraviolet radiation is one of the well-known and most commonly used mutagen and it is also very easy to take effective safety precautions against it. It gives a high proposition of pyrimidine dimmers and includes all types of base pair substitutions (Javed*et al.,* 2013). The present study highlights a possible enhancement of extracellular protease production from two bacterial strains, *Bcillus sp.* and *Micrococcus varians* via UV-mutagenesis.

II. MATERIAL AND METHODS

Bacterial strains used

Two bacterial strains *Bacillus sp.* and *Micrococcus varians*- producing protease were employed. *Bacillus sp.* was isolated from compost, whereas *Micrococcus varians* was isolated from soil. They were selected because of their high proteolytic activities.

Qualitative assay of proteolytic activity

The ability to produce protease enzyme was checked by transferring a single isolated colonies of both bacterial strains (wild and mutants) on 1% of skimmed milk agar plates. Plates were incubated at 37° C for 24h. The diameter (in mm) of the clear hydrolysed zone around each bacterial colony (X) was divided by the diameter of the same colony (Y). The ratio (X/Y) was taken as an indication of protease activity.

Quantitative assay of proteolytic activity

Protease activity was assayed by measuring the tyrosine released in culture supernatant from the action of protease on casein substrate bya modified Anson's method (Yang and Huang, 1994).The cell free supernatant of overnight cultures was used for protease assay.The reaction mixture contains 1 ml of enzyme was added to 1 ml of casein solution (1% w/v in 50 Mm potassium phosphate buffer, pH 7.5) and the mixturewas incubated for 10 min at 37ºC. The reaction was terminatedby adding 2 ml of 10% trichloroacetic acid reagent, keptfor 30 min incubation at room temperature and then centrifugedfor 15 min at 10,000 rpm. Then 2 ml of filtrate was mixed with3 ml of 500 mM sodium carbonate solution and absorbance was measured at 280 nm. One unit of enzyme activity is definedas the amount of enzyme required to liberate 1 µmol of tyrosineper min under the defined assay conditions. Enzyme units were measured using tyrosine (0-100µmole) as standard.

Preparation of cell suspension

Cell suspension was prepared by transferring colonies from 24h Luria- Bertani Agar culture of both strains into a 100ml-Erlenmeyer flasks containing 20ml of LB broth under aseptic conditions. Flasks were placed in a shaker incubator at 37ºC, 160 rpm for 24 h. after reaching an optical density of about 1,5 at 600 nm (corresponding to approximately 10^9- 10^{10}UFC/ml), it was used as source of cell suspension for irradiation.

UV mutagenesis

Ultraviolet (UV) irradiation as a physical mutagenic agent was used to select mutants which produce more protease than their parent strain. Mutagenesis was carried out according to Justin *et al.,* (2001) using different exposure times. 5ml of bacterial suspensions prepared previously were placed into 10-cm diameter-petri dishes at a distance of 10 cm from the UV lamp (30-W germicidal lamp, 2540-2550Å) and exposed to UV radiation for 0, 3, 6, 9, 12, 15, 18 and 21 min. Portions of 0.5 ml of suitable dilutions of bacterial suspensionsstrains were spread on five LBplates and incubated at 37ºC for 24 hr.Colonies developed after incubation werecounted and transplanted onto slants forfurther studies. The survival percentage was estimated for each treatment.

Screening of higher-proteolytic mutants

Plates having between 0.1 and10 % of survival rate were selected for isolation of mutants (Hopwood *et al.,* 1985). The isolates were selected on the basis of macroscopic differential characteristics. According to Solaiman*et al.,* (2005), for isolation of high protease producing mutants after UV irradiation, developed colonies inoculated into skim milk agar medium and incubated at 37ºC for 24h. Depending on the zone of clearance, mutants of the two bacterial strains exhibiting maximum zone of hydrolysis as compared to the wild type were selected.

Feather-degrading capacity of wild and mutant isolates

The wild type and the best mutant of *Bacillus sp.* and *Micrococcus varians*were tested for their ability to degrade feather by culturing both of them in modified basal medium II supplemented with 1% of chicken feather. Chicken feathers collected (medium size white hens) were chopped to small fragments, washed with distilled water and dried overnight at 60ºC (Bernhardt *et al.,* 1978; Johnvelsy, 2002). Cultures were incubated for 3 days at 37ºCwith shaking at 160 rpm. The feather-degrading capacity was assessed according to the physical appearance of feather pieces observed by naked eyes. The bacterial strain with high keratinolytic activity is the strain that degrade feather- keratin in shorter time.

III. RESULTS AND DISCUSSION

UV mutagenesis

The cost of enzymes in a bioprocess can be reduced by introducing hyper-productive strains after suitable mutagenic treatments. Results in Table 1 and 2 showed that thesurvival percentages for both isolatesdecreased by increasing the time ofexposure. The percentage of survivals has been sharply decreased from 100% to 15.07 and 35.62 % after 3min of UV treatment for *Bacillus sp.* and *Micrococcus varians*respectively.Thenitreaches 0.001 and 0.02 % after 20 minof UV treatment for *Bacillus* and *Micrococcus* respectively. This is maybe explained by the short distance from the UV lamp (10 cm). Similar trend of decrease in survivability with increase in exposure time has also been reported by Solaiman*et al.,* (2005) in which the distance from the UV lamp was 10 cm and the percentage of survivals was 0.12% after 10 min of UV treatment.However higher percentage of survivals has been reported by other investigations where the distance was 20 cm(Javed*et al.,* 2013; Karn and Karn, 2014).

After UV treatment plates having survival rate between 0.1 and 10 % corresponding to exposure time of 6, 9, 12 min for *Bacillus* and 9, 12, 15 for *Micrococcus*, were selected for isolation and screening of overproducing mutants. Based onmorphology and colour differencesbetween colonies, 27 and 18 mutants for *Bacillus* and *Micrococcus* respectively were selected and transferred to skimmed milk agar plates to test proteolyticcapacities. In case of *Bacillussp.* and depending on their proteolytic activity (X/Y) only four UV-mutants (9, 12, 16 and 21) did exhibit higherproteolytic activity compared to the wild type

(Table 3). Among the four mutants the most efficient strain (UV-9) was selected for further studies.*Bacillussp.* mutant 9(UV-9) showed 1.4 fold higher protease activity than the wild type.Similar fold increase in protease production was obtained by Nadeem*et al.,* (2010). Concerning *Micrococcusvarians*results showed that the majority of mutants were efficient in protease production. The superior protease producing mutants were 2, 8, 13, 16, 17 and 18. The X/Y values ranged from 03 to 08 for the mutant 18, so the improvement of *Micrococcus*was better than that of *Bacillus*(Table 4).*Micrococcus.*mutant 18(UV-18) showed 2.66 fold higher protease activity than the wild strain and was chosen for further studies. Shikha and Darmwal(2007) reported 1.44 fold increase in alkaline protease production over the wild strain of *B. pantotheneticus* while Dutta and Banerjee (2006) obtained 2.5 fold increase in protease production by UV-mutant *Pseudomonas sp.* Rao*et al.,* (1998) reported that mutagenesis either by conventional methods or by recombinant-DNA technology play an important role in improving the yield of protease. *Bacillus sp.* and *Micrococcus varians*mutantsshowed variable responses to UV radiation for protease production. These variations are more probably due to the differences induced in their genetic background.

Protease activity of the selected mutants in submerged culture

The best protease producing mutants UV-9 and UV-18 were further evaluated trough shack flask enzyme production studies over their wild strains. Results obtained proved that there is correlation between hydrolysis zone diameter and the ability to produce protease enzyme for *Bacillus sp.*and UV-9 mutant. UV-9 mutant produced almost 1.4 the yield of the wild strain. On the other hand, in the case of *Micrococcusvarians*and its mutant UV-18 therewas no correlation between the clearance zone on plates and proteolytic activity in liquid assay (2.5 fold increase in solid assay whereas it was 2.1fold increase in liquid assay) (Table 5). Similar results were found by Solaiman*et al.,* (2005), where some potent mutants had great in protease production in plates and were not able give any proteolytic activity in shack flask.

Feather-degrading capacity of selected mutants

The two UV mutants, UV-9 and UV-18 were used for testing their ability in feather degrading. The results showed that both mutants were able to degrade feather over their wild type. The mutants grew and produced protease using chicken feather as a source of carbon, energy and nitrogen. UV-18 was more effective in keratin-degrading than UV-9 and chicken feather completely disappearedin less than two days using UV-18 mutant (Figure 1). The ability of microorganism to grow and produce appreciable levels of protease using several wastes could offer tremendous potential for development of biological methods for the hydrolysis of such products. The use of these natural residues, especially in countries where they are generated in abundance, could results in a sustainable reduction in cost of enzyme production (Wang *et al.*, 2008).

IV. CONCLUSION

The results of the present investigation revealed that among different UV- mutants of *Bacillus sp.*and *Micrococcusvarians* UV- 9 and UV-18 were selected as higher-proteolytic mutants. UV- 9 and UV-18 mutants were able to increase protease production in plates and in liquid assay reaching 2.5 fold higher productions than the wild type.Hence these mutants were very effective in feather keratin degrading in two days presenting a potential use in keratin recycling and can result in a sustainable reduction in the cost of enzyme production.

ACKNOWLEDGEMENTS

The authors are thankful to the Laboratory of Microbiology and Plant Biology, Faculty of Natural and Life Sciences, University of Mostaganem, Algeriafor providing the facilities for the work.

Table.1: Survival data for Bacillus sp. after UV treatment at different exposure times.

Exposure time (min)	CFU/ml	Survival %
0	$65x10^8$	100
3	$98x10^7$	15.07
6	$22x10^7$	3.38
9	$47x10^6$	0.72
12	$113x10^5$	0.17
15	$59x10^5$	0.09
18	$39x10^4$	0.006
21	$104x10^3$	0.001

Table.2: Survival data for Micrococcus variansafter UV treatment at differentexposure times.

Exposure time (min)	CFU/ml	Survival %
0	$16x10^8$	100
3	$57x10^7$	35.62
6	$188x10^6$	11.75
9	$69x10^6$	4.31
12	$86x10^5$	0.53
15	$26x10^5$	0.16
18	$95x10^4$	0.05
21	$36x10^3$	0.02

Table.3: Protease production (X/Y) of Bacillus sp. mutants.

Mutant	Zone diameter X (mm)	Colony diameter Y (mm)	X/Y	Mutant	Zone diameter X (mm)	Colony diameter Y (mm)	X/Y
Wild	17	07	2.4	14	19	07	2.71
1	11	07	1.5	15	20	08	2.5
2	22	11	2	16	19	06	3.16
3	17	08	2.12	17	04	02	02
4	17	09	1.8	18	20	18	1.11
5	05	04	1.25	19	26	18	1.4
6	17	07	2.42	20	04	03	1.1
7	18	07	2.57	21	18	06	03
8	19	06	3.16	22	19	08	2.37
9	17	05	3.4	23	4.5	03	1.5
10	17	06	2.83	24	11	10	1.1
11	05	04	1.25	25	04	03	1.33
12	19	06	3.16	26	19	09	2.11
13	18	07	2.57	27	04	03	1.1

Table.4: Protease production (X/Y) of Micrococcus varians mutants.

Mutant	Zone diameter X (mm)	Colony diameter Y (mm)	X/Y	Mutant	Zone diameter X (mm)	Colony diameter Y (mm)	X/Y
Wild	09	03	03	10	21	04	5.25
1	20	04	05	11	19	04	4.75
2	18	03	06	12	19	04	4.75
3	20	04	05	13	19	03	6.33
4	09	03	03	14	19	04	4.75
5	08	04	02	15	18	05	3.6
6	20	05	04	16	18	03	06
7	19	04	4.75	17	19	03	6.33
8	18	03	06	18	24	03	08
9	22	04	5.5				

Table.5: Protease activity of the best Bacillus sp.and Micrococcus varians mutants.

Strain	Protease production (U/ml)	Protease activity (X/Y)
*Bacillus sp.*wild type	0.73	2.4
UV-9 mutant	1.02	3.4
*Micrococcus varians*wild type	0.65	03
UV- 18 mutant	1.37	08

http://dx.doi.org/10.22161/ijeab/2.5.10 *ISSN: 2456-1878*

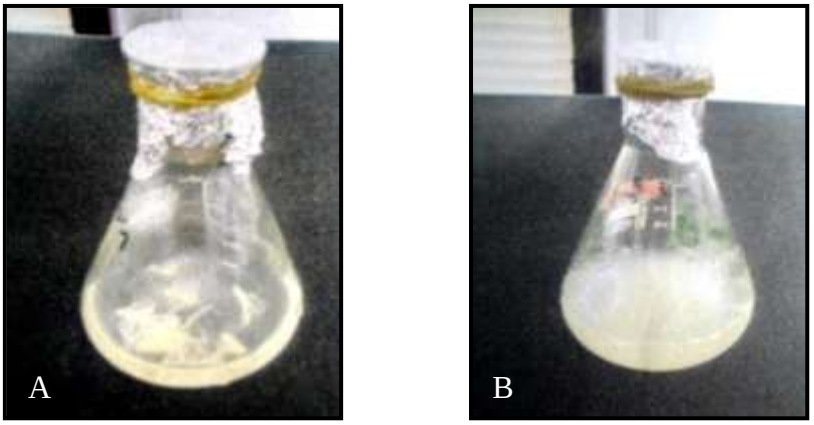

Fig.1: Chicken feather- degrading by Micrococcusvarians(A)and its mutant UV-18 (B).

REFERENCES

[1] Bernhard, K., Schrempf, H., and Goebel, W. 1978.Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*.J.bacteriol.,133(2), 897-903.

[2] Boominadhan, U., Rajakumar; R., Sivakumar, P.K.V. and Melvin, M.J. 2009. Optimization of protease enzyme production using *Bacillus* sp. isolated from different wastes.Bot Res Intl., 2: 83-87.

[3] Chellappan, S., Jasmin, C., Basheer, S.M., Elyas, K.K., Bhat, S.G., et al. 2006. Production, purification and partial characterization of a novel protease from marine *Engyodontium album* BTMFS10 under solid state fermentation. ProcBiochem., 41: 956-961.

[4] Hopwood, D.A., Bibb, M.J.,Chater,K.F.,Kieser, T.,Bruton,C.J., Kieser,H.M., Lydiate,D.J., Smith,C.P., Ward,J.M. and Schrempf,H. 1985. Genetic manipulation of Streptomyces: a laboratory manual, John Innes Foundation Norwich,pp, 356.

[5] Dutta, J. R.and Banerjee, R. 2006.Isolation and characterization of a newly isolated Pseudomonas mutant for protease production.Braz Arch Biol Technol., 49(1), 37-47.

[6] Glazer, A.N.andNikaido, H. 1995.Microbial enzymes. In: Glazer AN, Nikaido H (eds) Microbial Biotechnology, Freeman and Co,New York: W.H, pp. 24–263.

[7] Gupta, R., Beg, Q.K. And Lorenz, P.2002. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotech., 59:15-32.

[8] Javed, S., Meraj, M., Bukhari, S.A., Irfan, R. andMahmood, S. 2013. Hyper-production of Alkaline Protease by Mutagenic Treatment of *Bacillus subtilis*M-9 using Agroindustrial Wastes in Submerged Fermentation. J MicrobBiochem Technol., 5: 074-080.

[9] Johnvesly, B., Manjunatha, B.R. and Naik, G.R. 2002.Pigeon pea waste as a novel, inexpensive substrate for production of thermostable alkaline protease from thermoalklophilic*Bacillus* sp. JB-99.Bioresour Technol., 82, 61-64.

[10] Justin, C., Khodursky, A., Peter, B., Brown, P.O. and Hanawalt, P.C. 2001.Comparative gene expression profilesfollowing UV exposure in wild type and SOS-deficient *Escherichia coli*. Genetics., 158: 41-64.

[11] Karn, N.and Karn, S. K. 2014.Evaluation and Characterization of Protease Production by *Bacillus sp*. Induced By UV-Mutagenesis.Enz Eng.,3(119), 2.

[12] Nadeem, M., Qazi, J. I. and Baig, S. 2010. Enhanced production of alkaline protease by a mutant of *Bacillus licheniformis* N-2 for dehairing.Braz Arch Biol Technol., 53(5), 1015-1025.

[13] Rao, M.B., Tanksale, A.M., Ghatge, M.S. and Deshpande, V.V. 1998.Molecular and Biotechnological Aspects of Microbial Proteases**.**Microbiol. Mol. Biol. Rev., 62:597-635.

[14] Reddy, L.V., Wee, Y.J., Yun, J.S., Ryu, H.W. 2008. Optimization of alkaline protease production by batch culture of *Bacillus sp*. RKY3 through Plackett-Burman and response surface methodological approaches. Bioresour Technol., 99(7):2242-2249.

[15] Schallmey, M., Singh, A. and Ward, O.P. 2004.Developments in the use of *Bacillus* species for industrial production. Can. J.Microbiol., 50(1): 1-7.

[16] Shikha, S.A.and Darmwal, N.S. 2007.Improved production of alkaline protease from a mutant of alkalophilic Bacillus pantotheneticus using molasses as a substrate.Bioresour Technol., 98(4), 881-5.

[17] Solaiman, E.A.M., Hegazy, W.K. andMoharam, M.E. 2005.Induction of overproducing alkaline

protease *Bacillus* mutants through UV irradiation. Arab J. Biotech.,*8* (1): 49-60.

[18] Wang, S. L., Hsu, W. T., Liang, T. W., Yen, Y. H. and Wang, C. L. 2008.Purification and characterization of three novel keratinolyticmetalloproteases produced by *Chryseobacteriumindologenes*TKU014 in a shrimp shell powder medium.Bioresour Technol.,99(13), 5679-5686.

[19] Yang, S. and Huang, C. I. 1994.Protease production by amylolytic fungi in solid statefermentation. J. Chin. Agric. Chem. Soc., 32:589-601.