Biological Forum – An International Journal **9**(2): 54-60(2017)

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Systemic Mutagenesis of *Bacillus* sp. APR-4 for Enhanced Production of Thermostable and Alkaline Protease

Neha Thakur*, Tek Chand Bhalla** and Dinesh Kumar*

**Faculty of Applied Sciences and Biotechnology, Shoolini University, Solan-173212, (H.P.), India.*
***Department of Biotechnology, Himachal Pradesh University, Summer Hill Shimla-171005, (H.P.), India.*

((Corresponding author: Dinesh Kumar, chatantadk@yahoo.com, neha29.thakur90@gmail.com)
(Received 17 June 2017, Accepted 27 July, 2017)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Proteases are the most important group of industrial enzymes and constitute 60% of the total worldwide enzyme sales. Strain improvement is an essential part of enhanced production of enzymes from wild strain available. In present study a wild strain *Bacillus* sp. APR-4 producing 2578±0.1 U/ml protease in GYP medium at pH 7.0 after 40h incubation at 50°C was used for random mutagenesis by physical, chemical and combined method to obtain a hyper producer mutant. The physical mutagenesis using UV-radiation resulted in 32.4% rate of survival of wild strain after 90 minute exposure which decreased gradually to 0% after 150 minutes. Isolate UV8 showed maximum zone of hydrolysis (13±0.1mm) and enzyme activity (2599±2.6U/ml). The chemical mutagenesis of *Bacillus* sp. APR-4 resulted in 28% survival rate after 90 min exposure with EMS at 200 µg/ml concentration. In EtBr (150 µg /ml), MMS (150 µg/ml), and FU (200 µg/ml) after 90 minutes exposure the survival rate was 38%, 20% and 25% respectively. In this study out of the 35 mutant isolates, EMS20 exhibited maximum zone of hydrolysis (18±0.2mm) and optimum enzyme activity (2678±1.5U/ml) at 200µg/ml of EMS. In combined mutagenesis the isolate UV8 with optimum zone of hydrolysis and protease activity was treated with chemical mutagens and out of total 71 isolates screened the mutant UVEMS52 exhibited maximum zone of hydrolysis (25±0.1mm) and optimum enzyme activity (3209±0.1U/ml). In overall a 1.24 fold increase in enzyme production over parent strain was observed after combined mutagenesis.

Keywords: *Bacillus* sp. APR-4, Thermostable alkaline protease, Physical, Chemical and Combined mutagenesis

INTRODUCTION

A continuous effort is being made on search for new range of enzymes to meet the increasing industrial demands for enzymes capable to withstand extreme industrial process conditions (Zhang and Kim, 2010). The current status of enzyme market is currently $5.1 billion and is expected to rise upto 6.3% annually (Cui *et al*., 2015). Proteases are among one of the three largest groups of industrial enzymes and account for 60 % of the total enzyme sales (Zambare *et al*., 2011). Proteases possess a wide range of industrial applications, mainly in detergents, leather processing, food processing, bioactive peptide synthesis, metal recovery, medical purposes, chemical industries, as well as in waste treatment (Lopez-Otin and Bond, 2008).

Despite massive use of enzymes in biotechnological applications, there is still significant need for improvement of enzymes in industrial processes. Most of the industrial strains currently used for the production of novel compounds are obtained from their natural habitats and are unable to synthesize desired products in bulk and thus affecting both cost and the productivity. The enzymes used in industrial applications should be reusable, reproducible with low cost (Sawant and Nagendran, 2014). Mutants of wild can reduce the costs with increased productivity and can possess some specialized desirable characteristics (Jeyasanta and Patterson, 2014). Mutagenesis through classical genetic approach involves the use of random mutations to improve the desired metabolite yield in a particular wild strain (Chen *et al*., 2008). A series of mutagenic treatments using either physical or chemical mutagens are given to parent strain for developing a better yielding strain (Soliman *et al*., 2016). In *Pantoea dispersa* for strain improvement gamma radiation and UV-mutagenesis were used as physical agents (Gohel *et al*., 2010). UV radiation was used as a physical mutagen in other study to improve the protease production in *Bacillus subtilis* (Mrudula *et al*., 2012). In a similar investigation either physical or chemical methods were used for hyper production of protease from *Bacillus* sp. (Raju and Divakar, 2013; Nadeem *et al*., 2010).

There are very few reports available on combined mutation studies of *Bacillus* sp. for hyper protease production. Therefore, the aim of present study was to use some physical, chemical and combined methods of mutagenesis to obtain hyper protease producer strain of wild culture *Bacillus* sp. APR-4 reported earlier (Kumar and Bhalla, 2002; Kumar *et al*., 2004) with potential in silver recovery from x-ray film (Kumar *et al*., 2002a), protein recovery from waste meat bone (Kumar *et al*., 2003), detergent supplement (Kumar *et al*., 2004a) and biologically active dipeptide synthesis (Kumar *et al*., 2003a).

MATERIALS AND METHODS

A. Chemicals and microorganism used

Chemical mutagens viz; Ethylmethanesulfonate (EMS), Methylmethanesulfonate (MMS), Ethidiumbromide (EtBr) and Flourouracil (FU) used in present study were purchased from International Scientific and Surgicals, Solan Himachal Pradesh and other chemicals were of analytical grade. The bacterial isolate *Bacillus* sp. APR-4 as mentioned earlier in introduction (Kumar *et al*., 2002) was used in the present study.

B. Preparation of cell suspension

Stock culture of *Bacillus* sp. APR-4 was maintained in GYP medium (glucose 10g, yeast extract 5g, peptone 5g, NaCl 5g, $MgSO_4.7H_2O$ 0.25g, $CaCl_2$ $2H_2O$ 0.5g/L) with 70% glycerol and stored at -20°C. A loopful culture of *Bacillus* sp. APR-4 was transferred into 50 ml of GYP medium and incubated at 55°C for 48h in an incubator shaker. After incubation it was centrifuged at 5,000 rpm for 30 minutes at 4°C to get the pellet of cells. The pellet was re-suspended in 10 ml sterile normal saline (0.85%) to prepare the cell suspension for further studies.

C. Physical, chemical and combined mutagenesis of Bacillus sp. APR-4

The improvement of culture was done by using physical and chemical methods of mutagenesis. For physical mutagenesis, 4 ml of cell suspension containing 1×10^7 cells/ml were pipetted aseptically into sterile petriplates and exposed to UV light in Laminar Air Flow cabinet for different time interval (0-150 minutes) and stored in dark overnight to avoid photoreactivation. After this the cell suspension were serially diluted in saline and plated on GYP agar medium and incubated for 48 h at 55°C.

For chemical mutagenesis different mutagens viz., ethylmethanesulphonate (EMS), ethidium bromide (EtBr), fluorouracil (FU) and methylmethane sulfonate (MMS) were used. A 2-5 ml of cell suspension were pipetted aseptically into equal volume of different concentration (50-250 µg/ml) of chemical mutagens and placed at room temperature (25-27°C) for different time interval (0-150 min). After this, the microbial culture was centrifuged at 5,000 rpm for 15 min and cell pellet thus obtained was resuspended in 10 ml of saline to stop the reaction. The washed cell suspension were further serially diluted in saline and plated on GYP agar medium and incubated at 55°C for 48 h.

In combined mutagenesis, a hyper mutant selected after physical mutagenesis with maximum zone of hydrolysis and enzyme activity was further treated at different concentrations (50-250 µg/ml) of above chemical mutagens and plated on GYP medium and incubated at 55°C for 48 h.

All mutants obtained through physical and chemical mutagenesis were compared with parent strain on the basis of zone of hydrolysis in GYP agar medium around colonies and also by assaying the enzyme activity in GYP production medium. Protease enzyme activity was determined by the method Manachini *et al.* (1988) and one unit of enzymatic activity is defined as the amount of enzyme required to release 1 micro gm of Tyrosine/ml/min under assay conditions.

RESULTS AND DISCUSSION

A. Strain Improvement using different mutagenesis and selection of mutants

The wild strain of *Bacillus* sp. APR-4 isolated earlier (Kumar *et al*., 2002) was evaluated for its improvement with physical, chemical and combined mutagens at different concentration and different time interval of exposure to obtain hyper protease producer mutant of the wild strain. The mutants thus obtained through systemic mutagenesis were compared for improvement with parent strain on the basis of zone of hydrolysis and production of protease in GYP medium as discussed in methodology.

B. Physical mutagenesis of Bacillus sp. APR-4

The physical mutagenesis using UV-radiation with parent strain resulted in 32.4% rate of survival after 90 minute exposure which decreased gradually to 0% after 150 minutes as shown in Fig. 1. Similar trend of decrease in survivability with increase in UV exposure time was reported in earlier studies on strain improvements (Mukhtar and Haq, 2008; Soliman *et al*., 2004; Dutta and Banerjee, 2006; Javed *et al*., 2013; Merz *et al*., 2006). Isolate UV8 showed maximum zone of hydrolysis (13±0.1mm) and enzyme activity (2599± 2.6U/ml) and minimum zone of hydrolysis and activity was present in isolate UV10 as shown in Table 1.

Similarly *Bacillus cereus* GD55 showed maximum enzyme production (14.60±1.15 U/ml) after 70 minute exposure to UV radiation (Raju and Divakar, 2013). In another study 1.44 fold increase in alkaline protease production over the parent strain *Bacillus pantotheneticu* was reported by Shikha and Darmwal (2007). A 2.5 fold increase in protease over parent strain was produced by UV mutant *of Pseudomonas* sp. JNGR242 (Dutta and Banerjee, 2006).

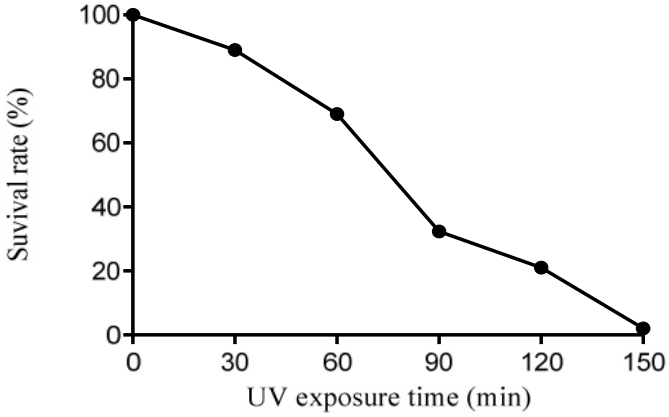


Fig. 1. Effect of UV radiation exposure on survival rate of *Bacillus* sp. APR-4 (1×10^7 cells) at room temperature.

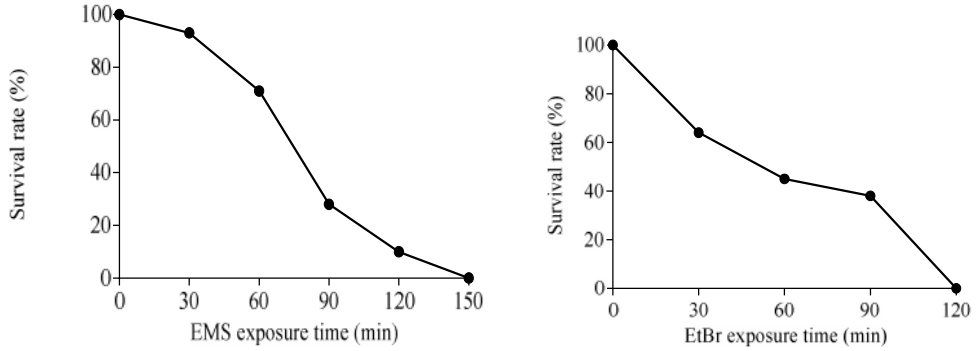
Table 1: Selection of mutants of *Bacillus* sp. APR-4 after treatment with UV radiation exposure at room temperature.

Physical Mutagen	Exposures time in UV radiation (min)	Selected mutant isolates with code	Zone of hydrolysis (mm)	Protease activity* (U/ml)
UV radiation		UV1	12±0.1	2581±1.5
		UV2	12±0.2	2580±3.6
	30	UV3	11±0.1	2475±3.2
		UV4	12±0.3	2579±2.0
		UV6	12±0.1	2470±1.5
		UV5	12±0.2	2582±3.6
	60	UV7	12±0.3	2584±1.7
		UV8	13±0.1	2599±2.6
		UV9	7±0.2	1449±3.0
	90	UV10	7±0.1	1337±3.6

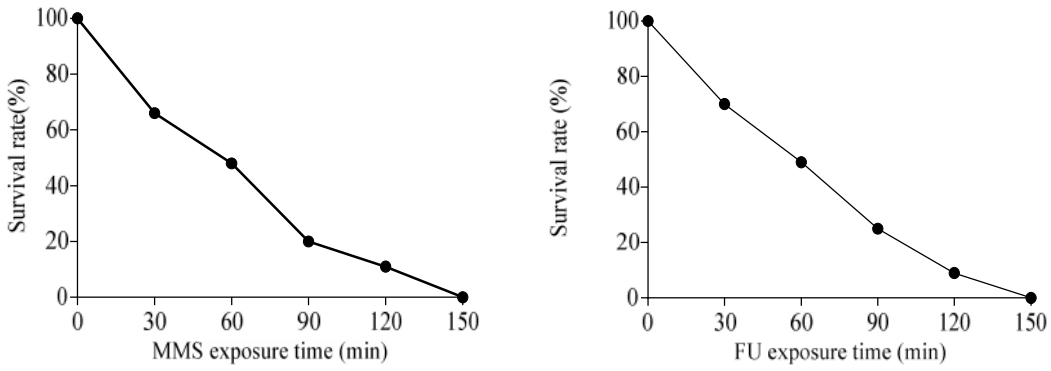
*Data: mean± SD (n=3)

C. Chemical mutagens of Bacillus sp. APR-4

On the other hand chemical mutagenesis of *Bacillus* sp. resulted in 28% survival rate after 90 min exposure with EMS at 200 µg/ml concentration. However with EtBr (150 µg/ml), MMS (150 µg /ml) and FU (200 µg/ml) after 90 minutes exposure the survival rate was 38%, 20% and 25% respectively. It decreased gradually with increase in exposure time to 0% after 150 min, 120 min, 150 min and 150 min respectively in above chemical mutagens as shown in Fig. 2.



(A) (B)



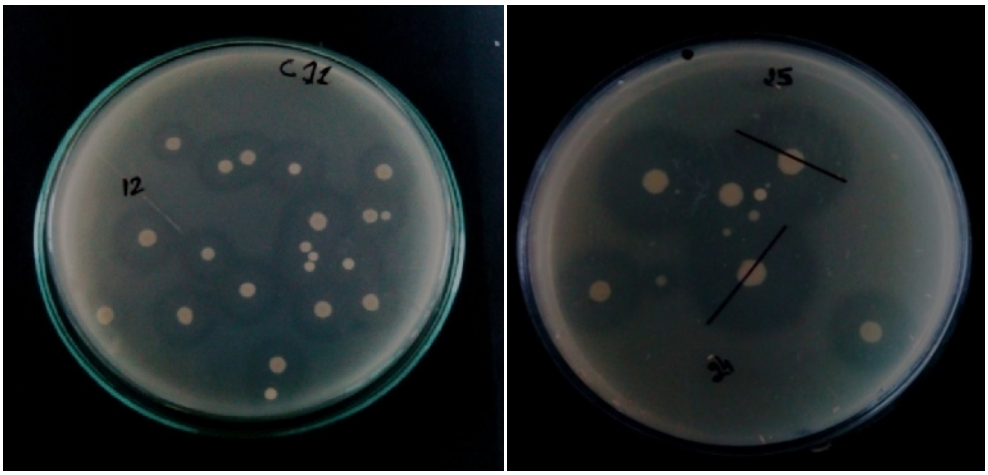
(C) (D)

Fig. 2. Effect of different chemical mutagens on survival rate of *Bacillus* sp. APR-4 (1×10^7 cells) at room temperature. [(A) Ethylmethanesulfonate (EMS); (B) Ethidiumbromide (EtBr); (C) Methylmethanesulfonate (MMS); (D) Flourouracil (FU)].

Similar pattern of decrease in survival rate with increase in exposure to chemical mutagen also been reported earlier (Nadeem *et al*., 2010; Dutta and Banerjee, 2006; Javed *et al*., 2013). In present study out of the 35 mutant isolates, EMS20 exhibited maximum zone(18±0.2mm) and optimum enzyme activity(2678±1.5U/ml) at 200µg/ml concentration of EMS and minimum zone of hydrolysis (7±0.1mm) and activity (936±2.0 U/ml) was observed in isolate FU47 as shown in Table 2.

D. Effect of combined mutagenesis

In combined mutagenesis the isolate UV8 with optimum zone of hydrolysis (13±0.1mm) and optimum protease activity (2599±2.6U/ml) was treated with different chemical mutagens for 90 min at (50-250 µg/ml) concentrations. In overall 71 isolates were screened and out of 71 mutants, isolate UVEMS52 exhibited maximum zone of hydrolysis (25±0.1mm) and optimum enzyme activity (3209±4.0U/ml) as shown in Table 3.



(A) (B)

Fig. 3. Zone of casein hydrolysis of cultures on skimmed milk agar plates after incubation at 55°C for 48h. [(A) wild culture of *Bacillus* sp. APR-4 (10mm±0.01) and (B); its mutant UVEMS-52 (25mm±0.01)].

Table 2: Mutants of *Bacillus* sp. APR-4 selected on the basis of zone of hydrolysis and enzyme activity after treatment with different chemical mutagen.

Chemical Mutagen	Conc. of mutagens (µg/ml) used	Selected mutant isolates with code	Zone of hydrolysis (mm)	Protease activity* (U/ml)
Ethylmethanesulfonate (EMS)	50	EMS11	13±0.4	1899±3.5
	100	EMS12	12±0.2	1612±2.0
	150	EMS13	12±0.3	1750±3.7
		EMS14	10±0.4	1209±3.2
		EMS15	13±0.1	2184±3.2
		EMS16	11±0.1	1438±2.6
		EMS17	12±0.2	1532±3.5
		EMS18	11±0.3	1369±1.5
	200	EMS19	17±0.1	2486±3.0
		EMS20	18±0.1	2678±1.5
		EMS21	17±0.2	2544±2.0
	250	EMS22	15±0.2	1894±1.0
Ethidiumbromide (EtBr)	50	EtBr25	11±0.1	1331±2.0
		EtBr26	11±0.1	1488±2.5
	100	EtBr27	12±0.1	1502±2.6
		EtBr28	12±0.2	1560±4.5
		EtBR29	16±0.1	1968±3.5
		EtBr30	10±0.1	996±2.6
	150	EtBr31	15±0.2	1641±4.5
	200	EtBr32	10±0.1	1180±4.5
		EtBR33	11±0.2	1252±4.5
Methylmethanesulfonate (MMS)	50	MMS34	12±0.4	1680±3.5
		MMS35	12±0.2	1536±1.5
	100	MMS36	12±0.1	1446±4.3
		MMS37	13±0.1	1651±3.4
	150	MMS38	17±0.3	2432±3.6
	200	MMS39	14±0.2	1809±4.3
	200	MMS40	12±0.2	1588±3.2
	250	MMS41	12±0.1	1507±2.9
Flourouracil (FU)	50	FU42	9±0.2	1339±3.2
	100	FU43	9±0.3	1372±3.2
	150	FU44	10±0.2	1446±3.0
	150	FU45	9±0.1	1481±2.0
	200	FU46	8±0.2	1243±2.0
	250	FU47	7±0.1	936±2.0

*Data: mean± SD (n=3)

Table 3: Use of chemical mutagens on UV exposed hyper mutant (UV8) of *Bacillus* sp. APR-4 as combined mutagenesis to evaluate its effect in hyper protease production.

Mutagen	Conc. of chemical mutagens (µg/ml)	Selected isolates and their codes	Zone of hydrolysis (mm)	Protease activity (U/ml)
Ethylmethanesulfonate (EMS)	50	UV+EMS48	18±0.2	2780±2.5
	100	UV+EMS49	21±0.1	2817±2.8
	150	UV+EMS50	21±0.2	2734±2.5
	200	UV+EMS51	24±0.2	3191±3.0
		UV+EMS52	25±0.1	3209±4.0
	250	UV+EMS53	12±0.3	1598±2.0
Ethidiumbromide (EtBr)	50	UV+EtBR54	21±0.1	2729±1.2
	100	UV+EtBR55	19±0.1	2140±2.0
		UV+EtBr 56	18±0.3	2251±4.3
	150	UV+EtBR57	13±0.2	1761±1.5
		UV+EtBR58	15±0.1	1836±2.0
	200	UV+EtBR59	13±0.2	1630±4.3
	250	UV+EtBR60	12±0.1	1512±4.5
Flourouracil (FU)	50	UV+FU61	9±0.2	1091±1.5
	100	UV+FU62	10±0.1	1140±1.5
	150	UV+FU63	9±0.2	1106±1.5
	200	UV+FU64	8±0.1	963±2.6
	250	UV+FU65	8±0.3	945±2.3
Methylmethanesulfonate (MMS)	50	UV+MMS66	10±0.1	1224±3.5
	100	UV+MMS67	12±0.2	1569±2.9
		UV+MMS68	13±0.1	1646±1.5
	150	UV+MMS69	21±0.1	2700±4.3
	200	UV+MMS70	15±0.2	1778±2.6

*Data: mean± SD (n=3)

The present finding are supported by earlier work by Nadeem *et al*. (2010) which reported 2.1 fold increase in enzyme activity when *Bacillus pumilus* was exposed to UV radiation followed by EMS and MMS. Similarly in *Bacillus cereus* GD55 mutant developed by combined mutagenesis using EtBr followed by EMS, showed 2 fold higher fibrinolytic protease production than wild strain (Raju and Divakar, 2013).

CONCLUSION

In the present study the improvement of *Bacillus* sp. APR-4 isolated earlier was carried out through systemic mutagenesis using physical, chemical and combined mutagens to isolate a hyper protease producer strain. For this UV- radiation was used as physical mutagen, and EMS, EtBr, FU and MMS were used as chemical mutagens individually. The protease activity increased to 2599±2.6U/ml in mutant (UV8) with UV exposure alone.

On the other hand chemical mutagens increased this production to 2678±1.5U/ml and rate of survival get decreased with increased exposure time after treatment of wild strain with different chemical mutagen. Further the UV optimized mutant UV8 was treated with individual chemical mutagens at different concentration to see the combined mutagenesis effect on protease production. However, subsequent treatment of UV optimized mutant with chemical mutagen, further increased this production to 3209±4.0 U/ml in mutant UVEMS52. This mutant showed 1.24-fold increase in enzymatic activity as compared to its wild strain. In conclusion this mutant strain with increased protease production will have more impact on enzyme economy for use in various industrial applications. The detailed characterization of this mutant of wild *Bacillus* sp. APR-4 is further under process to evaluate its potential.

ACKNOWLEDGEMENT

The authors are thankful to Shoolini University, Solan, H.P. for providing the facility to carry out the present research work.

REFERENCES

Chen, H.L., Wan, H.G., Zhang, J. and Zeng, X.X. (2008). Mutation-screening of *Pleurotus ferulae* with high temperature tolerance by nitrogen ion implantation. *Plasma Sci Tech*, **10**: 503-505.

Cui, H., Wang, L. and Yu, Y. (2015). Production and characterization of alkaline protease from a high yielding and moderately halophilic strain of SD11 marine bacteria. *J Chem*, **2015**: 1-8.

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

Gohel, V., Megha, C., Vyas, P. and Chhatpar, H.S. (2010). Evaluation and characterization of protease production by *Bacillus* sp. induced by UV-mutagenesis. *Annal Microbol*, **54**: 503-515.

Javed, S., Meeraj, M., Bukhari, S.A., Irfan, R. and Mahmood, S. (2013). Hyper production of alkaline protease by mutagenic treatment of *Bacillus subtilis* M-9 using agro-industrial wastes from submerged fermentation. *J Microb Biochem Technol*, **5**: 074-080.

Jeyasanta, K.I. and Patterson, J. (2014). Enhancement of alkaline protease production by *Bacillus* sp. through random mutagenesis. *Int J Microbiol Res*, **5**: 130-139.

Kumar, D., Gajju, H. and Bhalla, T.C. (2002). Production of thermostable protease by *Bacillus* sp. APR-4. *Asian J Microbiol Biotechnol Environ Sci*, **4**: 533-540.

Kumar, D., Chand, D., Sankhian, U.D. and Bhalla, T.C. (2004). Purification and characterization of a protease from *Bacillus* sp. APR-4. *Ind J Exp Biol*, **42**: 515-521.

Kumar, D., Chand, D., Sankhian, U.D. and Bhalla, T.C. (2002a). Application of *Bacillus* sp. APR-4 protease in silver recovery from used X-ray films. *Bull Biol Sci*, **1**: 39-41.

Kumar, D., Sankhian, U.D., Chand, D. and Bhalla, T.C. (2003). *Bacillus* sp. APR-4 protease in protein recovery from waste bones. *Nat Acad Sci Lett*, **23**: 133-136.

Kumar, D., Chand, D., Sankhian, U.D. and Bhalla, T.C. (2004a). *Bacillus* sp. APR-4 protease as a laundry additive. *Ind J Biotechnol*, **3**: 563-567.

Kumar, D., Chand, D., Sankhian, U.D. and Bhalla, T.C. (2003a). Utilization of *Bacillus* sp. APR-4 protease in peptide synthesis. *Ind J Microbiol*, **43**: 131-133.

Lopez-Otin, C. and Bond, J.S. (2008). Proteases: multifunctional enzymes in life and diseases. *J Biol Chem*, **45**: 30433-30437.

Manachini, P.L., Fortina, M.G. and Parini, C. (1988). Thermostable alkaline protease produced by *Bacillus thermoruber*. a new species of *Bacillus*. *Appl Microbiol Biotechnol*, **28**: 409-413.

Mrudula, S., Begaum, A. A., Ashwitha, K. and Pindi, P.K. (2012). Enhanced production of alkaline protease by *Bacillus subtilis* in submerged fermentation. *Int J Pharm Biol Sci*, **3**: 619-631.

Mukhtar, H. and Haq, I. (2008). Production of alkaline protease by *Bacillus subtilis* and its applications as depilating agents in leather processing. *Pak J Bot*, **40**: 1673-1679.

Merz, I.M., Choudhury, T. and Haq, M.M. (2006). Optimization of mutation conditions of *Bacillus* sp. to increase the yield of alkaline protease. *J. Human Life Sci*, **4**: 43-50.

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**: 1015-1025.

Nadeem, M., Syed, Q., Liaquat, A., Baig, S. and Kashmiri. A. (2010). Study on biosynthesis of alkaline protease by mutagenized culture of *Bacillus pumilus*. *Pak J Food Sci*, **20**: 1-4**.**

Raju, E.V.N. and Divakar, G. (2013). Non recombinant mutagenesis of *Bacillus cereus* for fibrinolytic protease production. *World J Pharmacy Pharm Sci*, **2**: 6189-6201.

Sawant, R. and Nagendran, S. (2014). Protease: an enzyme with multiple industrial applications. *World J Pharmacy Pharm Sci*, **3**: 568-579.

Soliman, E. A. M., El-Hamshary, O.I.M. and Batayyib, R. S. M. (2016). Enhancement of protease production of some *Bacillus* spp. Isolated from various regions in Jaddah city. *Int J Curr Microbiol App Sci*, **5**: 619-635.

Soliman, E. A. M., Hegazy, W.K. and Maysa, E.M. (2004). Induction of overproducing alkaline protease *Bacillus* mutants through UV irradiation. *Arab J Biotech*, **8**: 49-60.

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**: 881-885.

Zambare, V., Nilegaonkar, S. and Kanekar, P. (2011). A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. *New Biotechnol*, **28**: 173-181.

Zhang, C. and Kim, S.K. (2010). Research and applications of marine microbial enzymes: status and prospects. *Mar Drugs*, **8**: 1920-1934.