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Enhancement of Protease Production of Some *Bacillus* spp. Isolated from Various Regions in Jeddah City

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A B S T R A C T

In this study, we aimed to increase the protease production of some *Bacillus* isolates obtained from Jeddah City. Among fifty-four isolates, four isolates were the highest productive, J1, KA6, F5 and M4. They gave 10.76, 10.50, 10.30 and 9.36 U/ml, respectively. They were assigned as Bacillus BS, BP, BT and BL. The highest productive strains were subjected to mutagenesis using UV irradiation and Ethidium bromide. Out of fifty-two mutants, four mutants exhibited high efficiency of protease production. They were BL4-1, RS6-1, BETL0-1 and BETP40-2.They gave 25.98, 22.10, 19.74 and 14.92 U/ml, respectively. BL4-1 gave productivity threefold than *Bacillus* BL parental strain while RS6-1 gave two fold than Bacillus BS. Generally, UV irradiation was more efficient than EtBr in obtaining mutants with higher efficiency. Top highest mutants, BL4-1, RS6-1, and their parental strains were subjected to plasmid curing. Curing of Bacillus BL by elevated temperature has no effect on production while curing with acridine orange; production was increased. BL4-1 mutant showed great decrease in productivity by acridine orange compared to elevated temperature. In case of Bacillus BS parental strain, productivity was increased after curing with acridine orange while with elevated temperature the increase was medium. Production was not significantly affected by acridine orange in RS6-1 mutant while decrease sharply by elevated temperature.

Introduction

To obtain high yielding productive strains, chemical and physical mutagens are promising agents to achieve this goal (Sidney and Nathan, 1975). The productivity of microorganisms, that is known to be important in producing economic enzymes, is controlled by the organism's genomeal though their yield may been enhancing and increased by optimizing their cultural conditions (Stanbury *et al.,* 1995). Ultraviolet irradiation (UV) is the best mutagenic agent in prokaryotic organisms. It also characterized as the most proper mutagens to be employed in obtaining high yielding productive strains. UV irradiation covers all kinds of base pair substitutions and gives a high ratio of pyrimidine

dimmers (Roja and Prasad, 2012). On the other hand, chemical mutagens are powerful mutagenic agents that responsible in the occurring of changes in DNA sequence (Miller, 1972). They also affect the replication of DNA (Haq *et al.,* 2002, Besoain *et al.,* 2007). Ethidium bromide was used as a mutagen because itreacts with DNA bases and insert among them (Waring, 1965). Due to this insertion, DNA become more stretch and as a result, frame shift mutations occurred. This result affects the replication and transcription of DNA as reported by (Suribabu *et al.*, 2014). Great number of articles reported and explained the efficient use of physical and chemical mutagens. They proved the importance and effectiveness of these mutagens to be applied to improve the productivity of bacterial strains that produce various important industrial enzymes like protease and lipase (Soliman *et al.,* 2004, Nadeem *et al.,* 2006, Jamal *et al.,* 2011).Alkaline proteases are one of the most widely studied groups of enzymes that have commercial value because of their use in multiple applications in various industrial sectors (Jasvir *et al.,* 2004; Javed *et al.,* 2013). Their sales exceeding more than 60% of the total worldwide enzymes sales. They widely used in many various industries like leather processing, meat processing, dairy, detergent additives and for many other uses (Chellappan *et al.,* 2006, Prakasham *et al.,* 2006). Today, proteases are mainly used in detergent industry and originated from the genus of *Bacillus* (Roger *et al.,* 2002).Bacteria belong to genus of *Bacillus* are participated in the enzyme industry and known to produce proteolytic enzymes efficiently and effectively (Boominadhan *et al.,* 2009). Microbes producing proteases enzymes serve as the preferred source of proteases due to several factors. These factors include their rapid growth, limited spaces that needed for their cultivation, and can be genetically manipulated easily to produce new enzymes with altered properties. The present study mainly focused to strain improvement of some *Bacillus* strains isolated from various regions in Jeddah City through mutation for enhanced production of protease enzyme.

Materials and Methods

Media

Luria- Bertani broth (LB) used to propagate bacterial strains at 370C with vigorous aeration. For solidification the medium, 1.5 % of agar was added (Davis *et al.,* 1982). LB agar plates plus 1% skim milk was used to detect the production of alkaline proteases, i.e., a clear zone, that illustrate and express the hydrolysis of skim milk, gave a marker for protease production after incubation at 370C for 40 hours under alkaline conditions (Adinarayana and Ellaiah, 2003). Alkaline agar medium was used to subculture bacterial strains from frozen stock cultures (Takami *et al.,* 1989). GYP medium used as production medium of alkaline protease (Roja and Prasad, 2012). Media were sterilized by autoclaving at 1210C for 20 minutes under pressure of 15 lb/square inch.

Isolation and Screening of Bacterial Isolates

Samples of agricultural soil were collected from nine different districts distributed widely between south, center and north of Jeddah province, Saudi Arabia. We tried as much as possible to collect soil samples from diverse locations. Soil samples were collected by scraping off approximately 100 gmfrom 5-10 cm below surface layer with a sterile spatula and then stored at 40C until use. About 1.5 gm from each soil sample was added to 10 ml sterilized distilled water

in a glass tube. The glass tube containing soil suspension was heated at 80°C for 15-20 min in a water bath. They immediately cooled in ice-cold water.LB plus skim milk agar plate was used to detect the alkaline protease production according to Adinarayana and Ellaiah (2003).

It is worth mentioning that skim milk was added to LB agar medium after sterilized and cooling at 370C.Protease production in plates was demonstrated by the clearing of opaque milk proteins surrounding colonies that growing on the plate surface. Samples of 100 µl of soil suspension were spread on LB agar plus 1% skim milk plate and then incubated at 30°C for 48 h. Colonies forming diaphanous zones due to partial hydrolysis of skim milk were selected, picked and purified by streaking on skim milk agar. The purified proteolytic selected isolates were examined microscopically, maintained in LB agar slants by sub culturing at monthly intervals, and stored at 4°C.

Identification of Selected Bacterial Isolates

Fifty-four local isolates were isolated in this study as shown in Table (1).These isolates representing different regions isolated from nine different districts distributed widely between south, center and north of Jeddah province, Saudi Arabia. We coded isolates according to the locality, i.e., places from which the soil sample originated. Selected isolates, which showed good ability to produce protease enzyme were subjected to some morphological, physiological and biochemical studies in accordance with Bergey's Manual of Systematic Bacteriology (Khan *et al.,* 2011). Gram stain, capsule stain, motility, growth with 5% NaCl, glucose fermentation, utilization of citrate, catalase activity and starch hydrolysis were determined. Pure stock cultures of these selected bacterial isolates were maintained in LB broth medium with 70% glycerol and preserved at -200C.

Alkaline Protease Production

GYP production medium (50 m1 in 250 m1 conical flask) was inoculated with 1 ml of an overnight culture of tested strains and incubated at 370C in a rotary shaker operated at 150 rpm for 12-hour at time intervals; the turbidity of the culture was determined by measuring the increase in optical density at 450 nm with a spectrophotometer. After incubation, production medium was centrifuged at 8000 rpm for 15 min to separate the cells. The supernatant was collected as it contained the crude enzyme and stored at 40C until further use.

Alkaline Protease Assay

One ml of 2% casein solution was incubated with 0.1 ml of enzyme solution and 0.9 ml of sodium phosphate buffer (pH 7) for 10 minutes at 370C. The reaction mixture was stopped by using Trichloroacetic acid solution (10%). After 20 minutes, the mixture was centrifuged at 8000 rpm/5 min. Color intensity of supernatant was read at 280 nm. The enzyme activity was calculated from standard curve of tyrosine already prepared. One unit enzyme activity was taken as the amount of enzyme producing 1µg of tyrosine under standard assay conditions and expressed as unit/ ml enzyme (Boominadhan *et al.,* 2009).

Strain Improvement Techniques

Microorganism and Inoculum Preparation

The selected tested bacterial strains were subjected to mutagenesis via physical and chemical mutagens. A loopful of each stock

tested strain culture was transferred to 100 ml of sterile LB broth medium then incubated overnight at 350C on a rotary shaker before being used to inoculation (Rajuand Divakar, 2013).

Physical Mutagenesis using UV Radiation

Best production strains were subjected to mutagenesis using UV radiation according to Justin *et al.,* (2001) using different exposure times and different distances from irradiation source. A 24-hour old loopful culture of each strain was transferred from slant into 50 ml LB broth and incubated at 370C for 12-hour. After that, ten ml of the inoculated broth was centrifuged at 4000 rpm for 10 min at 40C. The supernatant was removed and the cell pellets was washed with sterile saline followed by sterile distilled water. Finally the cell mass was suspended in sterile saline and used as a source of cell suspension in irradiation experiment and later experiments. Various serial dilutions of cell suspension were prepared and the dilution of both 10-5 and 10-7/ml distributed into different round sterilized disposable petri dishes (2 ml in each petri plate) in dark. Then, they irradiated for 0, 10, 20 and 40 min in UV chamber keeping the distance of UV source fixed at 5, 11.5 and 20 cm. 15-W germicidal lamp (254 nm) used. To avoid any repair in DNA, which damaged because of UV light, the treated samples were transferred into sterile test tubes covered with a black paper and kept in the refrigerator overnight. Half ml of suitable dilution of each strain was spread on skim milk agar plates and incubated for 24-hour at 370C. Colonies that developed after incubation was counted and the survival percentage was calculated over the control, where a less than 10% survival rate was observed. Werandomly selected the colonies, transferred onto skim milk plates and checkedfor production of protease based on the zone formation compared to what has been obtained by parental strains. Mutants of hyperactive production that showing bigger zones were picked up. The mutants were cultured at least five times to emphasize incapability of reverse mutation.

Chemical Mutagenesis using Ethidium Bromide (EtBr)

Mutagenesis carried out according to (Fakhraddin *et al.,* 2014). Five ml of the cell suspension containing 1×107 /ml of each strain was pipette aseptically into separate individual sterilized centrifuged tubes and EtBr was added at concentration of (75 and 150 mg/ml) and kept them in water bath at 370C for different time intervals, 0, 30, 60 and 120 min. After a specific time interval, sample of one ml was pulled, centrifuged at 10,000 rpm for one minute and cell pellet washed three times with normal in order to get rid of mutagen from sample. We dilute the suspension of the washed cells serially in saline, spread on LB skim milk agar plates and incubated for 48 hours at 370C. In each plate, number of colonies was counted and the survival percentage was calculated over the control. Mutants of hyperactive production of protease that showing bigger zones compared to the parental strain were picked up.

Plasmid Curing

Plasmid curing is a process of eliminating bacterial plasmids through means of methods including physical agents such as elevated temperature and chemical agents such as acridine orange that used in this study. Parental strains and their best-derivatived mutants were subjected to curing to find out the effect of these two agents on their enzyme productivity. Curing of plasmids with elevated temperature was carried out following the method of (Soliman *et al.,* 2003).To achieve curing by heat treatment, each strain was grown in LB

broth medium at temperature 5-7°C above its optimum growth temperature on a rotary shaker at 150 rpm/min. When culture arrived to late log phase, it diluted to a dilution ratio of 1:20. Then we re-incubated sample culture at the same incubation degree until late log phase reached again. We prepared and plated a serial dilution to obtain single colonies. The colonies are individually tested on LB plus 1% skim milk plates for wastage of plasmids that causing any shortage in the ability of cured strains to produce protease enzyme. Curing of plasmids using acridine orange (AO) was conducted in accordance with the method of Zaman *et al.,* (2010). To achieve curing by (AO), two different concentrations of acridine orange (50 µg/ ml and 75 µg/ml) using different exposure times (30 and 60 min) were used. An overnight culture of each tested strain was diluted to 104 cells/ml using freshly LB broth medium by serial dilution technique. From this diluted culture, 0.5 ml was added with 4.5 ml LB containing (AO) separately in different concentrations. Thus, the concentration became 103 cells /ml. The cultures were then incubated at 370C in an orbital shaker at 150 rpm for 24 hours. After incubation, the broth culture was again diluted to 103 cells/ml with sterile normal saline to avoid the chemical effects. Appropriate volume was spread on LB plus 1% skim milk plates. After 24-hour incubation at 37°C, the plates were observed for growth. Some well-isolated colonies from this plate culture were randomly selected and simultaneously patched with sterile toothpick on LB agar plus 1% skim milk. They tested for loss of its ability to produce alkaline protease enzyme.

Results and Discussion

Isolation, Identification and Screening of Protease -producing Bacterial Strains

Fifty-four bacterial isolates were isolated from soil samples collected from nine different regions and coded according to the locality, i.e., districts from which the soil sample originated as shown in (Table 1). Results shown in (Table 2) explained the enzyme activities of these isolates, which showed a broad range of productivity. Morphological, physiological and biochemical properties of the highest productive isolates were investigated in accordance with Bergey's Manual of Systematic Bacteriology (Khan *et al.,* 2011). Gram stain, motility, starch hydrolysis, glucose fermentation, nitrate reduction, catalase activity and growth with 5% NaCl were determined. Finally, they were identified as *Bacillus* spp. and assigned as *Bacillus* BS, BP, BT and BL as shown in (Table 3).

Determination of Enzyme Activity of Selected Bacterial Strains

To detect the alkaline protease production for all tested bacterial isolates, LB plus 1% skim milk plates were used following the method of Adinarayana and Ellaiah (2003). Alkaline protease production was assayed for all isolates(Boominadhan *et al.,* 2009) and results shown in (Table 2) showed a broad range of productivity, i.e., 1.34 to 10.76 U/ml, The highest four isolates were J1, KA6, F5 and M4, i.e., 10.76, 10.50, 10.30 and 9.36 U/ml, respectively.

Strain Improvement and Selection of Overproducing mutants

Both mutagen agents UV irradiation and ethidium bromide (EtBr) were employed in systematic manner to obtain mutants that yield higher protease production. The parent strains BT, BL, BS and BP were treated with UV irradiation for 0, 10, 20 and 40 min in UV chamber keeping the distance of UV source fixed at 5, 11.5 and 20 cm. They also subjected to EtBr at concentration of (75 and 150 mg/ml) for different time intervals, 0,

30, 60 and 120 min. For the isolation of overproducing mutants, developed colonies were picked, inoculated into LB plus 1% skim milk plates and incubated at 370C for 24 h. Depending upon the zone of clearance, mutants with high activity compared to parental strains were selected and the enzyme activity was assayed. Results in (Table 4 and 5) revealed that after UV irradiation, the survival rate in all strains were decreased gradually whatever with the increase of exposure time, in both cell dilutions 10-5 or 10-7 and radiation intensities, i.e., whether distances was 5, 11.5 or 20 cm. On the other hand, results showed that the decreasing in survival percentages was highest in a distance of 5 cm when compared to other distances. In addition, the decreasing was also highest at a dilution of 10-5 compared to 10-7.Generality, survival was sharply reduced and became 0% in most results. Similar trend of decrease in survivability with increase in exposure time has also been reported by some other studies (Soliman *et al.,* 2005; Shikha and Darmwal, 2007; Mukhtar and Haq 2008; Javed *et al.,* 2013). All these findings indicated that the survivability of parent strains depended on the nature of the microorganisms, treatment period and the type of mutagens (Javed *et al.,* 2013). Regarding to EtBr treatments, results in (Table 6) revealed that the survival percentage in all strains was decreased gradually with the increase of exposure time in both concentration of EtBr whether in concentration of 75or 150 mg/ml. Survival percentage at exposure time of 120 min became 0 % in both concentration of EtBr. In case of exposure time 60 min, we found various results, which proved that the survival rate in all strains decreased gradually with150 mg/ml concentration of EtBr compared to 75 mg/ml. Results in (Table 7) for UV irradiation treatment and (Table 8) for EtBr treatment showed the values of enzyme activities for both parents and their resulting mutations. Mutant strains showed different responses to UV irradiation for alkaline protease production. Results showed a broad range of productivity and the highest four mutants were, BL4-1, RS6-1, BETL0-1 and BETP40-2, i.e., 25.98, 22.10, 19.74 and 14.92 U/ml, respectively. Mutant BL4-1 that resulted from *Bacillus* BL where it gave productivity threefold than parental strain while RS6-1 mutant that resulted from *Bacillus* BS gave two fold obtained highest enzyme productivity (25.98 U/ml). Results also showed that treatments with UV irradiation were more efficient than EtBr to increase the productivity of protease enzyme. These results may be due to increasing in gene copy number and amplification of DNA region. The variations are more probably due to the differences in genetic background of strains. Therefore, the results that obtained revealed variation in gene expression, i.e., enzyme activity as reported by Justin *et al*. (2001). Variations perhaps also due to some factors, e.g., deterioration or mutation occurs in gene(s), differences in the ability to repair their DNA, damage of the repair enzymes and the repair mechanism is not universal, as a result, replication cannot occur again (Ben, 2003).Uehara *et al*. (1974) suggested the existence of regulator genes that responsible for protease production. In addition, mutations that led to the higher production of proteases may also encompass those genes, which could alter the productivity, instead of the structure genes themselves. Nagami and Tanaka (1986) proved that mutation of genes controlling the composition of cell membrane led to the hyperactive 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 hyperactive producing cultures (Qadeer *et al.,* 1980).Similar results were consistent with the results obtained in this study. Jamal *et al*. (2011) use UV irradiation in strain improvement. They revealed thatthe percentages of survival were decreased by increasing both of radiation intensities, i.e., distances and time of exposure. They obtained three mutants with high lipase production after being treated with UV, where these mutants gave production equivalent to 1.2, 2.6 and 4 folds than obtained by original strain. On another hand, Soliman *et al*. (2005) used *Bacillus pumilus* and *Bacillus alvei* to investigate the changes in gene expression that responsible for protease production after conducting UV irradiation. They indicated that no enzyme activity was scored with derived mutants resulting from *B. pumilus*, while the activity was pronounce, in derived mutants resulting from *B. alvei.* They obtained three mutants that yield higher production in comparison to parental strain. These activities were 2.6, 3.02 and 3.7 folds than production obtained from the original strains. In addition, Hungund and Gupta (2010)proved the efficiency of UV irradiation for strain improvement. They obtained higher cellulose production mutants resulted from *Gluconacetobacter xylinus* NCIM 2526 wild strain.

Table.1 *Bacillus* Isolates

Region	Isolate No.	Code	Region	Isolate No.	Code
SOUTH JEDDAH				28	SH5
MADAIN AL-FAHD	1	M1		29	SH6
	2	M2		30	SH7
	3	M3	**AL- KANDARAH**	31	KA1
	4	M4		32	KA2
AL-HINDAWIYA	5	H1		33	KA3
	6	H2		34	KA4
	7	H3		35	KA5
	8	H4		36	KA6
	10	H6	**NORTH JEDDAH**		
	11	H7	**AL- FAISALYA**	37	F1
AL-WAZIRIYAH	12	W1		38	F2
	13	W2		39	F3
	14	W3		40	F4
	15	W4		41	F5
	16	W5	**AL- SAFA**	42	S1
	17	W6		43	S2
	18	W7		44	S3
CENTER JEDDAH				45	S4
AL-JAMAA	19	J1		46	S5
	20	J2		47	S6
	21	J3		48	S7
	22	J4	**AL- KHALIDIYA**	49	K1
	23	J5		50	K2
AL-SHARAFYA	24	SH1		51	K3
	25	SH2		52	K4
	26	SH3		53	K5
	27	SH4		54	K6

Table.2. Alkaline Protease Activity of *Bacillus* isolates at pH 9.

Isolates code	Enzyme activity U/ml	Isolates code	Enzyme activity U/ml	Isolates code	Enzyme activity U/ml
M1	--	**J1**	**10.76**	F1	--
M2	2.50	J2	--	F2	--
M3	3.00	J3	8.65	F3	1.34
M4	**9.36**	J4	5.87	F4	5.82
H1	--	J5	--	**F5**	**10.30**
H2	1.75	SH1	--	S1	
H3	--	SH2	7.00	S2	--
H4	--	SH3	--	S3	--
H5	--	SH4	5.88	S4	2.98
H6	--	SH5	3.55	S5	--
H7	4.00	SH6	--	S6	5.34
W1	2.80	SH7	1.34	S7	3.82
W2	7.90	KA1	6.82	K1	--
W3	5.04	KA2	4.87	K2	--
W4	--	KA3	7.43	K3	--
W5	--	KA4	3.40	K4	5.74
W6	1.90	KA5	--	K5	8.82
W7	8.76	**KA6**	**10.50**	K6	--

(--): undetected amount of enzyme production. One enzyme unit was defined as the amount of enzyme that yields an increase of 0.1 OD at 420 nm /30 min under standard reaction conditions.

Table.3 Morphological and Biological Characterization of *Bacillus* isolated strains

Isolate Code	Morphological characteristics		Biological characteristics					Name
	Gram stain	Motility	Starch hydrolysis	Glucose fermentation	NO_3^- reduction	Catalase activity	Growth at 5% NaCl	
KA6	+	Present	+	+	-	+	-	*Bacillus* BP
M4	+	Present	+	+	+	+	+	*Bacillus* BL
F5	+	Present	+	+	+	+	+	*Bacillus* BT
J1	+	Present	+	-	+	+	-	*Bacillus* BS

*Spore shape (O = oval and R= round), **Spore location(T= Terminal, C= central and S=sub-terminal)

Fig.1 Zones of clearance on skim milk agar plates produced by parent strain *Bacillus* BL (BL0-1) and some of its various mutants after UV irradiation

Table.4 Survival Percentage of *Bacillus* strains after UV Irradiation at 10-5cell dilution

Strains	Exposure time inmin.	No. of cells after irradiation	Survival (%)	No. of cells after irradiation	Survival (%)	No. of cells after irradiation	Survival (%)
		Distance of 5 cm		Distance of 11.5 cm		Distance of 20 cm	
***Bacillus* BT**	0	301	100	295	100	285	100
	10	68	22.59	109	36.94	169	59.29
	20	7	2.32	13	4.40	23	8.07
	40	0	0	0	0	3	1.05
***Bacillus* BL**	0	244	100	234	100	223	100
	10	45	18.44	65	27.77	84	37.66
	20	4	1.63	10	4.27	17	7.62
	40	1	0.40	1	0.42	5	2.24
***Bacillus* BS**	0	304	100	322	100	340	100
	10	48	15.78	70	21.73	98	28.82
	20	5	1.64	7	2.17	19	5.58
	40	0	0	0	0	4	1.17
***Bacillus* BP**	0	205	100	180	100	190	100
	10	30	14.63	54	30	70	36.84
	20	4	1.95	19	10.55	13	6.84
	40	0	0	2	1.11	3	1.57

Table.5 Survival percentage of *Bacillus* strains after UV irradiation at 10-7 cell dilution

Strains	Exposure time inmin.	No. of cells after irradiation	Survival (%)	No. of cells after irradiation	Survival (%)	No. of cells after irradiation	Survival (%)
		Distance of 5 cm		Distance of 11.5 cm		Distance of 20 cm	
***Bacillus* BT**	0	120	100	109	100	118	100
	10	25	20.83	33	30.27	45	38.13
	20	1	0.83	4	3.66	9	7.62
	40	0	0	0	0	0	0
***Bacillus* BL**	0	88	100	90	100	98	100
	10	16	18.18	24	26.66	32	32.65
	20	0	0	3	3.33	5	5.10
	40	0	0	0	0	0	0
***Bacillus* BS**	0	95	100	105	100	100	100
	10	20	21.05	25	23.80	36	36
	20	0	0	2	1.90	3	3
	40	0	0	0	0	0	0
***Bacillus* BP**	0	75	100	83	100	85	100
	10	12	16	15	18.07	21	24.70
	20	0	0	0	0	1	1.17
	40	0	0	0	0	0	0

Table.6 Survival Percentage of *Bacillus* strains after EtBr treatment

Strains	Exposure time inmin.	No. of cells after EtBr treatment	Survival (%)	No. of cells after EtBr treatment	Survival (%)
		Concentration of 75 µg/ml		Concentration of 150 µg/ml	
***Bacillus* BT**	0	533	100	550	100
	30	87	16.32	18	3.27
	60	20	3.75	1	0.18
	120	0	0	0	0
***Bacillus* BL**	0	480	100	453	100
	30	61	12.70	22	4.85
	60	12	2.5	0	0
	120	0	0	0	0
***Bacillus* BS**	0	560	100	577	100
	30	70	12.5	31	5.37
	60	19	3.39	4	0
	120	0	0	0	0
***Bacillus* BP**	0	404	100	420	100
	30	91	22.52	27	6.42
	60	32	7.92	2	0.47
	120	0	0	0	0

Table.7 Enzyme Activities of Parental Strains and their best Mutants arising after UV Treatment

Strains	Enzyme activity U/ml	Strains	Enzyme activity U/ml
***Bacillus* BL parental strain and their best derived mutants**		***Bacillus* BS parental strain and their best derived mutants**	
BL (B0-1)	9.36	*Bacillus* BS	10.76
BL1-1	9.88	RS6-1	12.28
BL4-1	25.98	RST5	22.10
BL8-1	12.02	RS18-1	12.66
BL33-1	13.50	RS24-1	15.62
BL42-1	12.34	RS47-1	13.60
***Bacillus* BP parental strain and their best derived mutants**		***Bacillus* BT parental strain and their best derived mutants**	
Bacillus BP	10.50	*Bacillus* BT	10.30
BP5-1	12.02	BT5-1	12.26
BP20-1	14.04	BT13-1	12.18
BP22-1	13.12	BT42-1	10.14
BP32-1	10.90	BT48-1	11.10
BP55-1	12.00	BT58-1	11.28

Table.8 Enzyme Activities of Parental Strains and their best mutants arising after EtBr treatment

Strains	Enzyme activity U/ml	Strains	Enzyme activity U/ml
***Bacillus* BL parental strain and their best derived mutants**		***Bacillus* BS parental strain and their best derived mutants**	
Bacillus BL	9.36	*Bacillus* BS	10.76
BETL1-2	9.80	BETS12-2	12.32
BETL8-2	10.38	BETS 23-2	11.98
BETL0-1	19.74	BETS33-2	13.48
BETL28-2	12.32	BETS37-2	13.42
BETL36-2	11.30	BETS45-2	12.10
***Bacillus* BP parental strain and their best derived mutants**		***Bacillus* BT parental strain and their best derived mutants**	
Bacillus BP	10.50	*Bacillus* BT	10.3
BETP13-2	13.74	BETT9-2	11.2
BETP15-2	13.42	BETT14-2	12.34
BETP35-2	14.08	BETT28-2	12.56
BETP40-2	14.92	BETT36-2	11.4
BETP45-2	13.30	BETT41-2	11.54

Table.9 Enzyme activity (EA) of parental strains (*Bacillus* BL and *Bacillus* BS) and some of best resulting mutants after plasmid curing by elevated temperature (ET) and acridine orange (AO) at concentration of 75 µg/ml.

Strains	EA U/ml	EA after ET treatment	EA after AO treatment
Bacillus BL	9.36	9.48	20.06
Mutant BL4-1	25.98	23	19.69
Bacillus BS	10.76	19.86	21.86
Mutant RST5	22.10	9.66	23.12

Fig.2 Zones of clearance on skim milk agar plates produced by parent strain *Bacillus* BS (RS0-1) and some of its various mutants after UV radiation

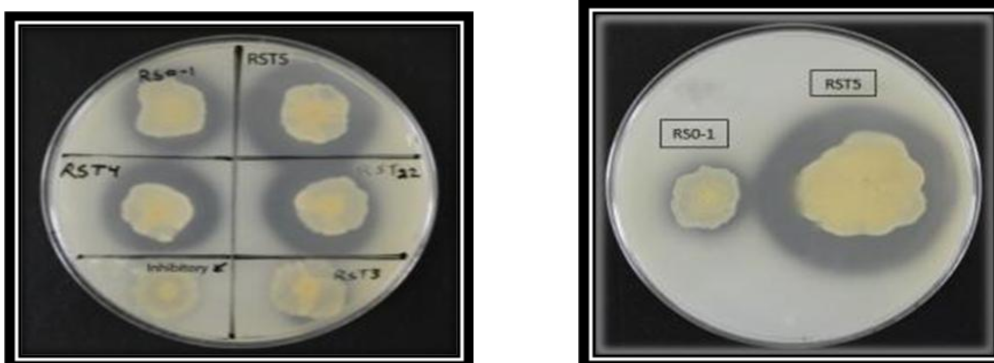


Fig.3 Zone of clearance of (A): *Bacillus* BL (B4-1) mutant, (B): Mutant after treatment with (AO) and (C): Mutant after treatment with (ET)

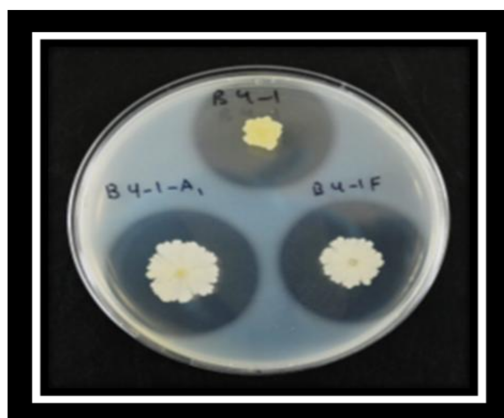


Fig.4 Zone of clearance of (A): Parental *Bacillus* BL (B0-1) strain, (B): Parental strain after treatment with (AO) and (C): Parental strain after treatment with (ET)

Fig.5 Zone of clearance of: (A) Mutant resulted from parental *Bacillus* BL strain after treated with EtBr ,(B) Mutant after curing by (ET), (C) Mutant resulted from parental *Bacillus* BL after treated with UV and (D) Mutant after curing by (ET).

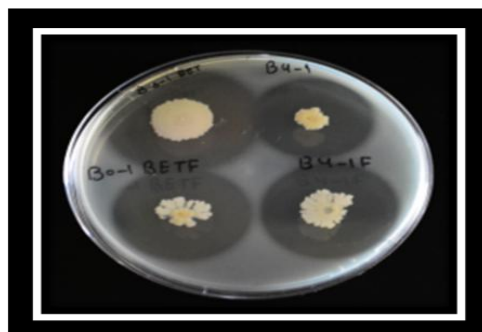


Fig.6 Zone of clearance of: (A) Parental *Bacillus* BS strain (RS0-1), (B) Parental strain after treated with (AO), (C) RST5 mutant resulted from parental after treated with UV and (D) after curing by (ET).

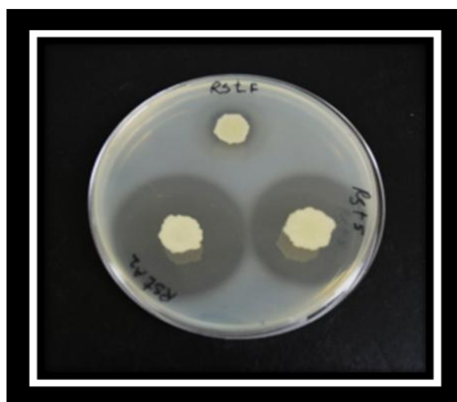
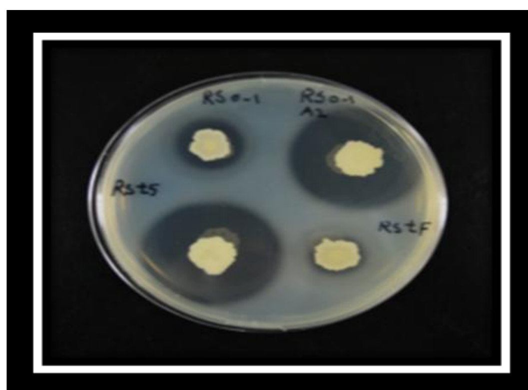


Fig.7 Zone of clearance of: (A) RST5 mutant resulted from parent *Bacillus* BS strain after treated with UV, (B) Mutant after curing by ET and (C) Mutant after curing by AO



Beatriz *et al*. (2010) also obtained mutant from *Aspergillus oryzae* IPT-301 with highest value of total fructosyl transferase activity after treating with UV irradiation. Javed *et al.,* (2013) improved the productivity of protease enzyme by subjecting *Bacillus subtilis* M-9 parental strain to UV-irradiations. They obtained BSU-5 mutant strain that showed the hyperactive proteolytic activity of alkaline protease over parent strain and other mutants. In contrast, Raju and Divakar (2013) enhanced the production fibrinolytic protease by subjecting *Bacillus* cereus to strain improvement by random mutagenesis using UV irradiation and ethidium bromide treatment. They proved that ethidium bromide was the best and most efficient for obtained optimum production of fibrinolytic protease and this was not compatible with our finding.EB-15 mutant strain was found to produce 2-4 fold more enzyme. Kumar *et al.,* (2009) revealed that the best mutant, *Bacillus* sp. FME 2, selected from UV irradiation and EtBr, was shown to be the most promising and the yield of

glucoamylase generated by the mutant strain was approximately 3.0 fold which was larger than the yield generated by the wild-type strain. Basavaraju *et al.,* (2014) were subjected the wild strain *Bacillus* isolate 7 to strain improvement through UV irradiation which gave protease productivity less than mutant *Bacillus* isolate 7. In addition to the above listed, the same results obtained in this study were consent with other results reported by (Khodayari *et al.,* 2014; Ghazi *et al.,* 2014; Arotupin *et al.,* 2014).

Plasmid Curing Response

Results in (Table 9) showed the effect of the removal of plasmids by elevated temperature (ET) and acridine orange (AO) on the enzyme activity (EA) in the two highest mutants, BL4-1 and RS6-1, compared to their parental strains. In the case of *Bacillus* BL parental strain, plasmid curing by elevated temperature has not any impact on production while production increased after treatment with acridine orange, reaching twice-parental production. Production in the case of BL4-1 mutant showed greater decrease by acridine orange compared by elevated temperature. Moreover, in parental strain *Bacillus* BS, productivity was increased two fold after curing with acridine orange while with elevated temperature the increase was medium. On the other hand production was not significantly affected after curing with acridine orange in the case of mutant RS6-1 while decrease sharply in case of elevated temperature from 22.10 to 9.66 U/ml. The variations in response for curing in *Bacillus* strains may be due to the difference in their genome genes. Therefore, results obtained revealed variation in gene expression, i.e., enzyme activity. Jaiswal and Singh (1990) proved that the virulence of *Clostridium perfringens* type B was affected by curing due to the shortage, which occurred in toxin production while Beg and Ahmad (2000) proved that the curing of plasmid from E. coli x+ was confirmed by defining the loss of resistance markers in the cured culture obtained after removal of this plasmid. It may be suggested that the increasing which detected in enzyme productivity can be resulted from elimination of genes existed on plasmids which have a negative impact on chromosomal genes that responsible for productivity, i.e., repression. The noticed decrease in protease productivity may be due to natural regulatory relations between indigenous existence plasmids and chromosomal gene. These strains might become a novel and interesting source of proteases enzymes with important economic features, therefore might be of potential applications in many important industries.

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