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Mutagenic Improvement of Some *Bacillus* Strains for Enhanced Alpha Amylase Production

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A B S T R A C T

The objective of this study was to enhance the production of α-amylase enzyme in some *Bacillus* strains through mutation techniques because of the exponential increase in demand for their usage in various fields. Seven *Bacillus* strains; *B. licheniformis*, *B. alvei*, *B. thuringensis*, *B. subtilis*, *B. pumilus*, *B. circulants*, and *B. cereus* were used in this study. The highest productive strains were *B. licheniformis* and *B. subtilis*. They were treated with UV irradiation and Ethyl methanesulfonate (EMS) as physical and chemical mutagens, respectively. Results revealed that the survival rate using either UV or EMS treatment was decreased gradually with the increase of exposure time. In case of UV mutagenesis, out of one hundred and fifty mutants, thirty-three were highly productive than parental strains. The highest two mutants resulted from *B. subtilis* were, BSUV5 and BSUV2 after 15 min. of exposure, i.e., 42.71 and 39.57 U/ml, respectively. They gave productivity thirty-three-fold and thirty-one-fold than parental strain. On the other hand, the highest three mutants resulted from *B. licheniformis* were, BLUV14, BLUV11(60 min. of exposure) and BLUV3 (15 min. of exposure), i.e., 42.86, 41.57 and 40.00 U/ml, respectively. The highest BLUV14 mutant gave productivity of thirty-seven-fold than parental strain. On the other hand, in case of EMS mutagenesis, out of twenty-two mutants resulted from *B. licheniformis* which was chosen to be treated with EMS, five potent mutants were higher in enzyme productivity. The highest two mutants were, BLEMS1 (at 250 µl /10 min.) and BLEMS5 (at 1500 µl /60 min.), i.e., 42.93 and 30.67 U/ml, respectively. Plasmid curing of parental strain by elevated temperature or EtBr has not any impact on enzyme production. Finally, these results suggest that induction of mutation was effective in getting super strains in productivity compared to what can be obtained by parental strains.

Introduction

Alpha amylases are answerable for the conversion of starch, widely distributed polysaccharide, into simple sugar. These enzymes have a myriad of diverse industrial applications like food, brewing, paper, detergent, textile, leather, starch liquefaction

and pharmaceutical industries. Therefore, they account for ~30% of global industrial enzyme production with an annual increase of ~4%. Commercially, alpha-amylase is collected mostly from different species of *Bacillus* (Paul *et al.,* 2017). The amelioration of microbial strains for high production of industrial microbial products is of great importance. To obtain high yielding productive strains, chemical and physical mutagens are promising agents to achieve this goal (Sidney and Nathan, 1975). This improvement is done by exposing the targeted microbes to physical mutagens such as ultraviolet and gamma rays in addition to chemical mutagens such as ethyl methane sulfonate and ethidium bromide (Queen *et al.,* 2016 and Khedr, *et al.,* 2017). 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). Ethyl methane sulfonate (EMS) is a mutagenic, tertagenic, and possibly carcinogenic organic compound with the formula $C_3H_8SO_3$. EMS is often used in genetics as a mutagen. Mutations induced by EMS can be studied in genetics screens or other assays. The mutations may also produce mutant proteins with intersecting properties or enhanced or novel functional that may be of commercial uses. 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 (Nadeem *et al.,* 2006, Jamal *et al.,* 2011, Effat *et al.,* 2016 and 2017). The present study aimed to enhance alpha amylase production of some *Bacillus* strains through mutation by using UV radiations and Ethyl methane sulfonate.

Materials and Methods

Materials

Bacterial strains

Seven bacterial strains; *Bacillus licheniformis*, *B. alvei*, *B. thuringensis*, *B. subtilis*, *B. pumilus*, *B. circulants*, and *B. cereus* were used in this study. Stock cultures of the strains were stored in 20% glycerol at -70^{0}C. Prior each experiment, the bacterium was sub-cultured from the frozen stocks onto LB agar plates. Purified cultures of strains were streaked on LB agar slants, pH 7.0 at 37^{0}C for maximum growth rate and stored at 4^{0}C.

Media

Luria broth medium (LB)

It was used to propagate bacterial strains at 37^{0}C with vigorous aeration (Davis *et al.,* 1980).

Starch agar medium

It was used in the primary screening of α-amylase producing-bacteria. It tests the ability of tested strains to produce α-amylase that is secreted out of the bacteria and diffuse into the starch agar. Since no color change occurs in the medium when organisms hydrolyze starch, we add iodine to the plate after incubation. Iodine turns blue, purple, or black (depending on the concentration of iodine) in the presence of starch. A clearing around the bacterial growth indicates that the organism has hydrolyzed starch (breakdown of starch)

due to its production of alpha-amylase enzyme (Suribabu *et al.,* 2014). On the contrary, the absence of any clearing indicates that the organism was not able to hydrolyze starch because of its inability to produce the enzyme.

To prepare this medium we suspend the first three ingredients in 1 liter of distilled water, mix thoroughly and heat with agitation and carefully bring to just boiling (Do not allow to boil because any increase in boiling may hydrolyze the starch). Final pH of the medium should be 7.5 at 25^0C. All media were sterilized by autoclaving at 121^0C for 20 minutes under pressure of 15 lb/square inch.

Lugol's iodine solution (Iodine reagent)

It is used to detect the presence of starch in a growth medium. When it is added to starch agar plates, the iodine reagent combines with starch in the medium to produce a blue-brown color. Bacteria producing α-amylase, an enzyme destroying starch, exhibit clear halos under their growth indicating that the starch has been digested. When iodine reagent is added to an overnight culture of a microbe unable to break down starch, the medium is converted to a uniformly blue-brown color. We dissolve potassium iodine in 100 ml of distilled water and slowly add the iodine crystals while shaking. Finlay, we filter and store in a tightly stoppered brown bottle for a period not exceeding one month (Suribabu *et al.,* 2014).

Mutagens

Ethyl methyl sulphonate (EMS) was used as chemical mutagen in concentration (1.17 g/ml) to take 0, 250 and 15000 µl/ml bacterial cell suspension for 0, 10 and 60 min while ultraviolet irradiation was used as physical mutagen at wave length of 254 nm for 0, 15 and 60 min at fixed distance of 25 cm.

Methods

Primary screening of highest α-amylase - producing strains

The collected bacterial strains samples were diluted by serial dilution technique. The diluted samples of 10^{-4} to 10^{-6} (0.1 ml) were spread with L-shaped glass rod by spread plate technique on the starch agar plates. After incubation at 37^0C for 24 hours, the plates were flooded with Lugol solution (1% iodine in 2% potassium iodide w/v). Colonies forming transparent zones, because of partial hydrolysis of starch which expressed and indicated alpha-amylase production by the organism were selected, picked and purified by streaking on starch agar (Suribabu *et al.,* 2014).

Microorganism and inoculum preparation

Twenty-four hours of each stock strain culture was inoculated in 100 ml Luria broth medium (LB) and kept on a rotary shaker at 200 rpm at 37^0C. Cultures giving an optical density of 0.50 ± 0.01 at 600 nm were used as stander inoculum.

Amylase extraction and assay by DNS method

Maltose produced by the hydrolytic activity of α-amylase on α-1, 4 linkages present in polysaccharides, reduce 3, 5 dinitro salicylate to an orange red colored 5-nitro 3-amino salicylate which can be measured at 620 nm. The starch substrate (0.5 ml of 0.5% in 0.1 M phosphate buffer (pH 6.8)) was mixed with 1% (0.2 ml) NaCl in a test tube and pre incubated at 37^0C for 10 minutes. The supernatant collected from the centrifugation of the production media was used as enzyme source, 0.5 ml of this was added to the reaction mixture. The reaction was terminated by the addition of 1.0 ml of 3, 5-

dinitrosalicylic acid reagent (1.0 gm DNS in 0.8% NaOH, 60% Na K tartrate) after incubation at 37^0C for 15 minutes. The contents were mixed well and kept in boiling water bath for 10 minutes. Then they were cooled and diluted with 10 ml of distilled H_2O. The color developed was read at 620 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions. Units of enzyme activity = (A_{620} control – A_{620} sample) ÷ A_{620}/mg starch ÷ 10 min ÷ 1ml, where, A_{620} sample refers to absorbance obtained for measured sample. A_{620} control refers to absorbance obtained from measured from control (un-inoculated), A_{620}/mg starch refers to absorbance obtained for 1mg of starch (from stander curve), 10 min refers to incubation time, 1 ml refers to the amount of crude enzyme added (Miller, 1959; Aullybux and Puchooa,2013; Suribabu *et al.,* 2014).

Stability studies of parental and mutant strains

Plasmid curing is a process of removing plasmids of bacteria by means of different chemical agents such as elevated temperature. Parental strains were chosen in terms of production efficiency to determine the effect of plasmid curing by elevated temperature on their enzyme productivities. Curing of plasmids with elevated temperature was performed by the method of (Soliman *et al.,* 2003). To achieve curing by heat treatment, each strain was grown at 40^0C and 43^0C in LB broth medium on a rotary shaker at 150 rpm/min for 24 and 48 h. Approximately 10^{10} cells from a log phase culture of each strain were evenly spread on the solid surface of LB plates. The plates were incubated as previously mentioned for 72-hour and observed for growth. From this plate culture, some well-isolated colonies were randomly selected and simultaneously patched with sterile toothpick on starch agar plates and tested for the loss of its ability to produce amylase enzyme.

On the other hand, the stability of parental and mutant strains for amylase production was tested. They were evaluated for ten repeated subcultures under uniform conditions via the plate assay method to determine the consistent of enzyme production (Kumar *et al.,* 2009).

Physical mutagenesis

Mutagenesis was carried out according to (Justin *et al.,* 2001) using different exposure times. A 24-hour old loopful culture of each strain was transferred from slant into 50 ml LB broth and incubated at 37^0C for 12-hour at 140 rpm on an incubatory shaker, at pH 9.5. After that, 10 ml of the inoculated broth was centrifuged at 4000 rpm for 10 min at 4^0C and the supernatant was decanted. The cell pellets were washed thoroughly with sterile saline (0.9% NaCl) followed by sterile distilled water. Finally the cell mass was suspended in sterile saline and used as a source of cell suspension for irradiation and subsequent experiments.

Dilutions of cell suspension were prepared and the dilution of 10^{-5}/ml was distributed into different round sterilized disposable petri dishes (2 ml in each petri plate) in dark and irradiated for 0, 15 and 60 min in UV chamber keeping the distance of UV source fixed at 25 cm. 15-W germicidal lamp (254 nm) was used. After UV radiation they were kept in dark for stabilization of thymine-thymine (T-T) dimers. We are protected by hanging the lamp behind a black curtain. Dishes are placed on a tray and the lids are removed immediately before being placed behind the curtain. It is imperative that lids are removed to permit UV exposure; it is also critical that the dishes (not the lids) are

labeled before irradiation to keep track of strains and treatments. The treated samples were transferred into sterile test tubes covered with a black paper and kept in the refrigerator overnight to avoid photo reactivation. 0.5 ml of suitable dilution of each strain was spread on starch agar media plates at pH 9.5 and incubated for 24-hour at 37^0C. Colonies developed after incubation was counted and the survival percentage was calculated over the control, where a less than 10% survival rate was observed. The colonies were randomly selected and transferred onto starch agar plates to check the production of α-amylase based on the zone formation. Mutants of hyper production of α-amylase that showing bigger zones compared to the parental strain were picked up. The mutants were cultured at least five times to assure incapability of reverse mutation.

Chemical mutagenesis

Ethyl methyl sulphonate (EMS) is alkylating agent that commonly used in laboratory mutagenesis of cells. It produces random mutations in genetic material by nucleotide substitution; particularly by guanine alkylation .This typically produces only point mutations .It can induce mutations at a rate of $5x10^{-4}$ to $5x10^{-2}$ per gene without substantial killing. The ethyl group of EMS reacts with guanine in DNA ,forming the abnormal base 61O-6-ethylguanine. During DNA replication, DNA polymerases that catalyze the process frequently place thymine, instead of cytosine, opposite O-6-ethylguanine. Following subsequent rounds of replication, the original G: C base pair can become an A: T pair (a transition mutation). EMS was used as mutagen in concentration (1.17g/ml) to take 0.0, 250, 500, 750 and 1000 µl/ml bacterial cell suspension .*Bacillus subtilis* and *B. licheniformis* were used for chemical mutagenesis following the method of (Hungund and Gupta, 2010) with some modifications. The culture suspension was prepared with viability of 10^5/ml. To 5 ml of cell suspension, 250 and 1500 µl of sterile solution of EMS (1.17g/ml) was added. The reaction was allowed to proceed. Samples were withdrawn from the reaction mixture at intervals of 10 and 60 min and immediately, the samples were resuspended in sterile buffer pH 7. The suspended sample was again centrifuged at 5000 rpm for 10 min. The supernatant was discarded. Cells were washed thrice with sterile distilled water to remove traces of EMS. The samples were serially diluted in the same buffer and plated on screening medium. A total of 15 colonies were selected from the plates and similarly tested for α-amylase production ability.

Results and Discussion

Screening and selection of best yielding bacterial strains

Isolated colonies of each strain were picked up from each plate containing pure culture and streaked in straight lines in starch agar plates with starch as the only carbon source. After incubation at 37^0C for 24-48 hrs., individual plates were flooded with Gram's iodine (Gram's iodine- 250 mg iodine crystals added to 2.5 gm potassium iodide solution, and 125 ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue color forms, which is the basis of the detection and screening of an amylolytic strain. The colonies which were showing zone of clearance in starch agar plates were maintained on to LB agar slants.

Determination of enzyme activity of selected bacterial strains

To detect the amylase production for all tested bacterial isolates; starch agar medium was used according to (Suribabu *et al.,* 2014).

Amylase production at pH 9 was assayed for all selective strains and showed a broad range of productivity, i.e., 0.42 to 1.29 U /ml. The highest four isolates were *B. subtilis*, *B. licheniformis*, *Pseudomonas aeruginosa* and *B. thuringensis*, i.e. 1.29, 1.16, 0.95 and 0.82 U/ml, respectively (Table 1, Fig. 1).

Strain improvement and selection of overproducing mutants

Ultraviolet irradiation (UV) as a physical agent and Ethyl methyl sulphonate (EMS) as a chemical agent were employed in systematic manner to obtain mutants that yield higher amylase production. Mutation induction and screening of commercially viable microorganisms are important for the successful development of various strains required in the fermentation industry. Isolation of overproducing mutants developed colonies were inoculated into starch agar medium under alkaline conditions (final pH of the medium should be 7.5) and incubated at 25^0C for 24 h.

Table.1 Enzyme activities of bacterial strains

Bacterial strain	Enzyme activity U/ml
Bacillus licheniformis	1.16
Bacillus subtilis	1.29
Bacillus thuringensis	0.82
Bacillus alvei	0.95
Bacillus circulans	0.54
Bacillus pumilus	0.62
Bacillus cereus	0.42

Table.2 Survival percentage of bacterial strains after UV exposure using different time periods

Exposure time in min.	No. of cells after irradiation		Survival (%)	
	B. licheniformis	***B. subtilis***	***B. licheniformis***	***B. subtilis***
0	634	764	100	100
15	389	432	61	57
60	56	49	0. 9	0.6

Table.3 Survival percentage of bacterial strains after EMS treatment at different concentrations using different time exposure periods

EMS	Exposure time in min.	No. of cells after irradiation		Survival (%)	
		B. licheniformis	***B. subtilis***	***B. licheniformis***	***B. subtilis***
250 µl	0	752	874	100	100
	10	350	476	46	54
	60	20	27	2.6	3.0
1500 µl	0	752	874	100	100
	10	376	432	50	49
	60	12	19	1.6	2.2

Table.4 Alpha-amylase enzyme activities of parent strains *B. subtilis*, *B. licheniformis* and their resulting mutants after UV treatment

Mutants code	Exposure time/ min	Enzyme activity U/ml	Mutants code	Exposure time/ min	Enzyme activity U/ml
B. subtilis		1.29	***B. licheniformis***		1.161
BSUV1	15	17.86	BLUV1	15	**33.77**
BSUV2	15	**39.57**	BLUV2	15	24.28
BSUV3	15	22.13	BLUV3	15	**40.00**
BSUV4	15	18.10	BLUV4	15	**37.14**
BSUV5	15	**42.57**	BLUV5	15	**35.92**
BSUV6	15	32.04	BLUV6	15	28.60
BSUV7	15	20.63	BLUV7	15	25.08
BSUV8	15	26.14	BLUV8	15	**39.53**
BSUV9	60	19.02	BLUV9	60	30.23
BSUV10	60	18.26	BLUV10	60	20.17
BSUV11	60	18.09	BLUV11	60	**41.57**
BSUV12	60	24.54	BLUV12	60	**35.12**
BSUV13	60	20.50	BLUV13	60	29.14
BSUV14	60	23.64	BLUV14	60	**42.86**
BSUV15	60	21.93	BLUV15	60	18.39
BSUV16	60	26.22	BLUV16	60	29..93
			BL UV17	60	26.22

Table.5 Alpha-amylase enzyme activities of parent strain *B. licheniformis* and their resulting mutants after EMS treatment

Mutants code	EMS con.	Exposure time/ min	Enzyme activity U/ml
B. licheniformis			1.161
BLEMS1	250 µl	10	42.93
BLEMS2	1500 µl	10	16.90
BLEMS3	250 µl	60	29.61
BLEMS4	250 µl	60	27.63
BLEMS5	1500 µl	60	30.67

Table.6 Enzyme activity (EA) of parent strains after plasmid curing by elevated temperature (ET) and ethidium bromide (EtBr)

Bacterial strain	EA*	EA after ET treatment	EA after EtBr treatment
B. licheniformis	1.16	1.51	1.33
B. subtilis	1.29	0.30	1.76

(*) Enzyme activity (U/ml)

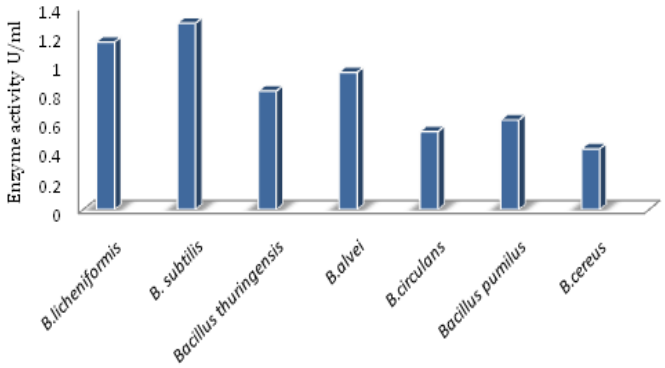

Fig.[1]. Enzyme activities of *Bacillus* bacterial strains

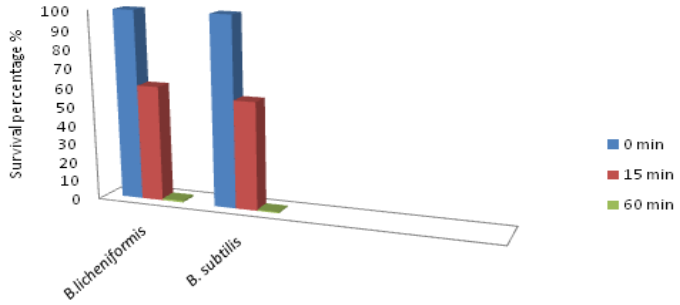

Fig. (2): Survival percentage of bacterial strains after UV exposure using different time periods

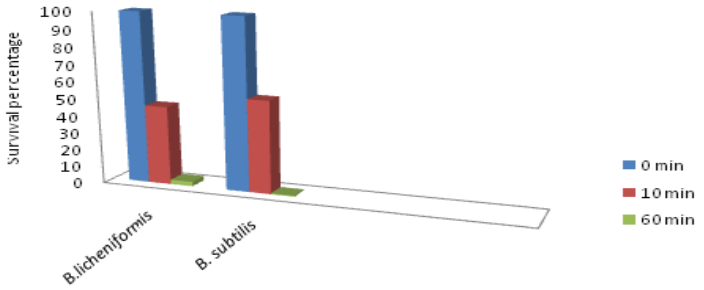

Fig. (3): Survival percentage of bacterial strains after EMS treatment at 250 µl using different time exposure periods

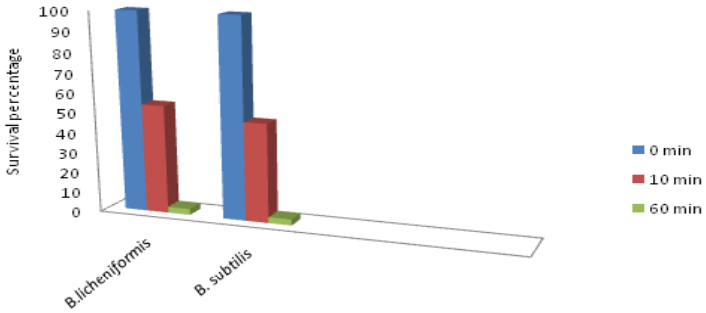

Fig. (4): Survival percentage of bacterial strains after EMS treatment at 1500 µl using different time exposure periods

Fig.[5]: Enzyme activity (EA) of parental strains after plasmid curing by elevated temperature (ET) and ethidium bromide (EtBr)

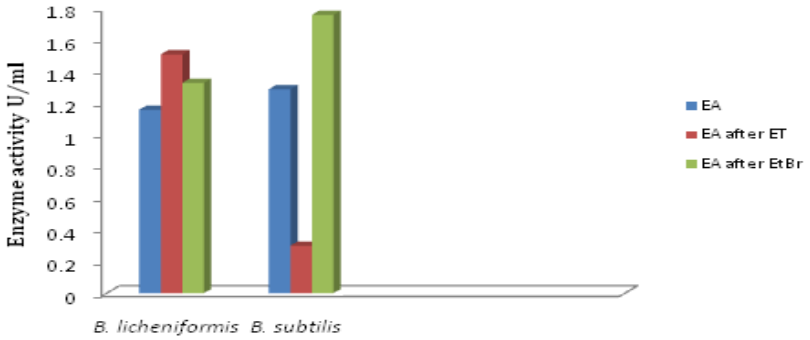

Fig. (6). Zone of clearance of *B. subtilis* mutants after 15 min UV irradiation

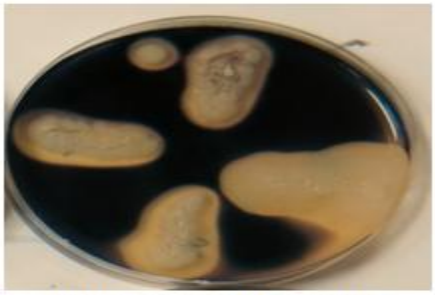

Fig. (7). Zone of clearance of *B. licheniformis* mutants after 60 min UV irradiation

Depending upon the zone of clearance, mutants with high activity compared to parental strains was selected. The enzyme activity of superior amylase mutants was assayed. Therefore, the parent strains *Bacillus subtilis* and *Bacillus licheniformis* were treated with UV irradiation as a physical agent and EMS as a chemical agent to improve its productivity. Potent UV radiation mutants which showed more zone of starch hydrolysis compared with parental strains were screened and selected at different minutes of exposure of irradiation. Out of one hundred and fifty mutants isolated, thirty-three mutants were higher in enzyme productivity than parental strains, sixteen mutants from *Bacillus subtilis* and seventeen from *Bacillus licheniformis*. On the other hand, out of twenty-two EMS *Bacillus licheniformis* mutants, five potent mutants were higher in enzyme productivity than parental strains.

Effect of UV exposure of *B. licheniformis* and *B. subtilis* for different exposure time (0, 15 and 60 min) was evaluated by constructing a survival curve itself. The results revealed a gradual decline in percentage of survivors with increase in UV exposure time. Results in table (2) and Fig. (2) revealed that the survival rate in all strains after mutagenesis by UV radiation was decreased gradually with the increase of exposure time. Similar trend of decrease in survivability with increase in exposure time has also been reported by some other studies as found in induction of overproducing alkaline protease *Bacillus* mutants through UV irradiation (Soliman *et al.*, 2005). All these findings indicated that the survivability of parent strains depended on the nature of the microorganisms, treatment period and the type of mutagens.

They also revealed that the survival rate in all strains after mutagenesis by EMS was

decreased gradually whatever with the increase of exposure time and in both concentration, i.e., 250 µl or 1500 µl, (Table 3, Fig. 3 and 4). Results in (Table 4) show the enzyme activities of parent strains and their resulting mutations after UV irradiation treatment. *B. subtilis* mutant strains showed different responses to UV irradiation for amylase production. Results showed a broad range of productivity and the highest two mutants were, BSUV5 and BSUV2 after 15 minutes of exposure, i.e., 42.57 and 39.57 U/ml, respectively (Fig. 6). Highest enzyme productivity (42.57 U/ml) was obtained by mutant BSUV5 where it gave productivity thirty three -fold than parental strain. On the other hand, *B. licheniformis* mutant strains showed different responses to UV irradiation for amylase production.

Results showed a broad range of productivity and the highest eight mutants were BLUV14,BLUV11, BLUV3, BLUV8, BLUV4, BLUV5, BLUV12, and BLUV1, i.e.,42.86,41.57,40.00,39.53,37.14,35.92,35.12 and 33.77 U/ml, respectively. These results are agreement with (Haq *et al.,* 2009) where they improved *Bacillus licheniformis* strain GCB- 30UCM for alpha amylase production using ethyl methane sulfonate. They obtained only one mutant designated as *B. licheniformis* EMS-20040 which yielded 102.78 U/ml/min compared to parental strain. The highest enzyme productivity (42.86 U/ml) was obtained by mutant BLUV14 (Fig.7), where it gave productivity thirty seven-fold than parental strain.

In case of EMS mutagenesis in *B. licheniformis*, we find that the results in table (5) have shown relatively different results. They showed a broad range of productivity and the highest two mutants were BLEMS1 after 10 minutes of treatment at concentration of 250 µl which yielded 128.83 U/ml. This value is equivalent of one hundred and ten times of the productivity of the parental strain. Results also revealed the increase in amylase productivity in mutagenesis using UV radiation or EMS treatment with a broad range of productivity and this may be due to increasing in gene copy number and amplification of DNA region. 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. 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 cannot take place again.

However, the application of UV irradiation, whatever the mutation(s) include either modifying or structural genes, led to the isolation of hyperactive enzyme producing cultures.

Stability studies and plasmid curing response of parental strains

Results in table (6) and fig. (5) showed the effect of the removal of plasmids by elevated temperature (ET) and ethidium bromide (EtBr) in enzyme activity (EA) of all tested strains. Results proved that there is no substantiality change in enzyme productivity in all strains either before or after curing treatment. Therefore, plasmid curing has not any impact on enzyme productivity using either elevated temperature (ET) or ethidium bromide (EtBr). Finally, results revealed that physical and chemical mutagens are promising and are used for screening of high yielding strains. Results also suggest that both local strains, *B. licheniformis* and *B. subtilis*, were genetically modified for its ability to reveal extra cellular alpha-amylase activity by subjected them to UV irradiation and EMS

treatment for hyper activity of an alpha-amylase enzyme compared to what can be obtained by parental strains.

Their derivatives mutants were quantitatively compared with the parental strain. UV and chemical mutagenesis brought about a dramatic enhancement in enzymatic activity. The mutant strains BSUV5 and BLUV14 exhibited up to 33 and 37 folds more enzyme activity than the native strains, *B. subtilis* and *B. licheniformis*, respectively. These results are in agreement with results from mutant strain obtained by (Singh *et al.,* 2011) who got maximum enzymatic activity up to 17 folds when they use60 minutes UV irradiation to mutagenized *Bacillus subtilis* (2620). Anyway, this improvement in enzyme activity of resulted mutants suggests that they are suitable strains to be used in various economic biotechnological applications.

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