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Research article

Improvement of thermophilic α-amylase productivity through UV mutagenesis and *Amy*E gene amplification and sequencing

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Key words: *Bacillus licheniformis,* α-amylase, *Amy*E gene, UV mutation, Genomic environmental interaction.

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Abstract

Induction of mutation with UV was used to improve α-amylase enzyme production by *Bacillus licheniformis* MK90, an Egyptian isolate. One hundred eighty mutants were isolated after UV treatment for 3, 5, 7 and 10 min. Three mutants (UV-5-M3, UV-3-M17, and UV-5-M121) were higher α-amylase producers than parental strain and mutant UV-5-M121 was the highest producer one with 150.8% of WT productivity. Parental α-amylase has molecular weight equal 64 kDa while it was 65 kDa with UV-5-mutant 121 and 61 kDa with UV-5-mutant 3 through SDS-PAGE analysis. SDS-PAGE showed a high variance between the two mutants and WT, mutant UV-M3 showed 15 bands, UV-M121 showed 17 bands, while WT showed 18 bands. The best two producer mutants UV-5-m121 and UV-5-m3 proved its maximum production after 72h of fermentation at temperature 55 and 65°C with pH 7 and 8. Starch at 1.5% was the best concentration for the most mutants to reach their maximum productivity after 72h of fermentation. *Amy*E gene was amplified, and sequenced. It was 1539 bps in the three sequences. Mutant UV-5-m1 21 contains the lowest nucleotide substitution sites; it reached 5 only. While mutant UV-5-m3 contains seven substitutions compared with the parental sequence.

Introduction

Bacterial amylases have longer shelf life and can be stored for weeks without significant loss of activity [1]. α-Amylases produced from thermophilic microorganisms were the ideal ones [2,3] since they are not usually denatured by high temperatures and are even active at elevated temperatures [4]. Production of amylases from bacteria is beneficial for human population as their starch degrading ability can be exploited for preparation of special food items, easily digestible for infants, patients, and elderly people [5-9]. Utilization of bacterial strains specifically from genus *Bacillus* is gaining momentum because of their ability to resist and survive under harsh industrial conditions [10].Genetically modified organisms are also being used for production of α-amylase. There are various methods by which microorganisms can be manipulated at a genetic level in order to improve and optimize the production of this enzyme [11].

Physical and chemical mutagens are promising and are used for inducing the high yielding strains [12]. For industrial usage, enzyme must be produced at low cost, reusable, and reproducible. To achieve this target many techniques have been developed for strain improvement. Strain improvement is usually carried out by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, ɣ-rays, UV rays, etc., and chemical mutagens such as NTG, EMS, EtBr, [13].

The PCR amplification of the α-amylase gene was 1887bp with an approximately 93.65% similarity with standard bacterial strain *Bacillus subtilis* [8]. The coding region for the AmyE gene from the *Bacillus sp.* BBM1 was identified by means of the BLASTX software [14].

Experimental

Materials and methods

Qualitative screening for α-amylase production

Screening for the enzyme productivity done on agar medium composed of 0.5g peptone, 0.1g KCl, 0.5g $MgSO_4$.$7H_2O$, 0.1 g $(NH_4)_2SO_4$ 0.1g NaH_2PO_4 [15], 2g starch was added as [16] recommended.

Amylase production was carried out in production medium containing (w/v) 6g bacteriological peptone, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 1g Starch and 1000 ml distilled water [17]. The flasks were incubated at 50°C for 24 h under shaking. Whole broth was centrifuged at 10000 rpm for 15 minutes at 4°C. The clear supernatant (crude enzyme) was used for estimation of amylase activity. The isolates were routinely maintained on nutrient agar slants at 4°C.

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Amylase assay

The enzyme activity was assayed following the method of [18] using 3,5-dinitrosalicylic acid. An enzyme blank with 3,5-dinitrosalicylic acid added before the addition of enzyme was used as control. The amount of reducing sugar released was quantified using 3,5- dinitrosalicylic acid with maltose as standard at 540 nm with a UV spectrophotometer. One unit of α-amylase activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute from soluble starch at pH 7.0 when incubated at 50°C.

Partial purification of α-amylase

Partial purification of α-amylase method modified from [19] in which 4:1 cold acetone to sample added with constant stirring. The mixture was allowed to stand for 1h at 4°C and the enzyme fraction was dried over anhydrous calcium chloride under decreased pressure at room temperature. The fraction tested for enzyme activity and used for SDS step.

SDS-PAGE of whole cell proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by [20-22]. Electrophoresis was performed at the constant current of 20 mA, using a 12 % separating gel (pH= 8.8) and 4 % stacking gel (pH=6.8).

UV-Mutagenesis

The UV irradiation was used to induce mutations in *Bacillus licheniformis* according to modified method of [23-26] where, cells suspension of overnight culture was prepared by shaking for five min. Cells were exposed to Ultraviolet Irradiation (UV) at a distance of 20 cm for 3, 5, 7 and 10 minutes. After irradiation the treated suspension protected from light for 1h by keeping in dark place. One ml from treated cells with suitable dilution was plated on LB, LB supplemented with starch and minimal starch [27].

Effect of substrate concentration on α-amylase production

Different substrate concentrations (0.5-1.5%) added to 250 ml conical flasks containing medium composed of (w/v) 6g peptone, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, with 1.5 % maize starch. The conical flasks incubated for 24 hours at optimal pH and temperature for each mutant.

Effect of temperature and pH optima

The temperature and pH activity profiles for amylase from UV mutants was studied in the pH range of 6.0 to 9.0 by mixing the enzyme with sodium phosphate buffer (pH 6.0 and 7.0), Tris buffer (pH 8.0), and glycine-NaOH (pH 9.0 and 10.0) in the ratio 1:1 at different temperatures (35-75°C) using optimal concentration starch for each mutant.

Genomic DNA extraction

Genomic DNA was isolated according Alkaline Method Kit [28]. In an eppendorf, 1.5 ml from overnight culture were taken, centrifuged at 8,000 x g for 1 min, pellet was kept and 250 µl of solution A was added, mixed by automatic pipette up and dawn. Then 250 µl of solution B was added and mixed by moving up and down three times. Then 250 µl of solution C was added and centrifuged at 13,000 x g for 5 min. Finally, the upper phase was removed into new eppendorf. After extraction of the DNA samples, an appropriate amount was transferred (about 25 µl) of each sample to a fresh eppendorf and 5µl of loading buffer was added.

Amplification and sequencing of α-amylase *(AmyE)* gene

Thermo cycler PCR used to amplify α-amylase coding gene (*AmyE*) with two primers, forward (MK-am.F) 5'-ATGAAACAACAAAAACGGCTTT-3' and Reverse (MK-am.R) 5' -CTATCTTTGAACATAAATTGAAACCG-3'. *Bacillus licheniformis* MK90 and the two mutants used as DNA template for amplification.

PCR cycle profile

The PCR performed with the following cycling profile: initial denaturation at 94°C for 5minutes, followed by 35 cycles of 1minute denaturation at 94°C, annealing at 52°C for 1 minute, and extension at 72◦C for 3minutes. The time for the final extension step was extended to 15 minutes. The PCR product was electrophoresed in 1% agarose horizontal slab gels. Gels were run at 112 V, and stained with Ethidium bromide for 30 minutes. The PCR product was purified and sequenced.

DNA sequence analysis

The sequencing reactions were carried out by Sanger Sequencing Technology on Applied Bio-systems automated DNA sequencer, model ABI 3730XL DNA Analyzer (Applied Bio-systems, USA; service provided by Macrogen Inc., South Korea). The sequence analyses and alignments were performed by NCBI-BLAST programs of the National Center for Biotechnology Information [14, 29-31] and DNA alignment was performed to compare between DNA of wild strain and the two selected mutants which show the best α-amylase productivity using Cluster W online software.

Results and Discussion

Induction and selection of mutation using UV irradiation

Local Egyptian Strain *Bacillus licheniformis* MK90 isolated from the internal soil of Pharaoh pools which characterized by its thermophilic nature, used as a Parental stain or WT coded on Genebank with accession number kt387748 was treated with UV radiation at wave length 254.5 for different periods of time 3, 5, 7 and 10 minutes and all the available

colonies appeared after UV treatment (about 180 colonies) were picked up. Surviving ratio was decreased as the time of UV exposure increased; it recorded 100% without UV exposure, 69% after 3 min, 55% after 5min, 41% after 7min, and 33% after 10 min (Figure 1).

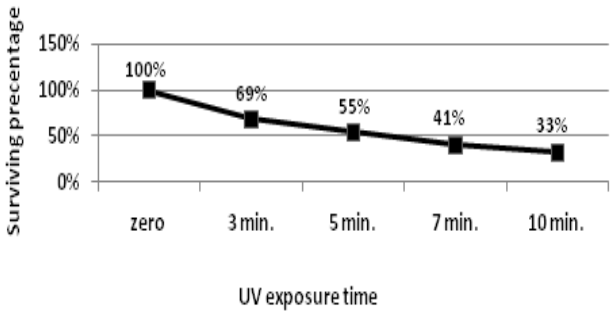


Figure 1. percentage of surviving single colonies at different time of UV- exposure 3, 5, 7, and 10 min respectively at concentration 10^{-1}.

All colonies grown on Starch agar medium tested for their ability to produce α-*amylase*. Mutants divided in to active and inactive α-amylase ones, inactive producers present 8.8% of the total selected mutants while active ones represent 91.2% (Figure 2).

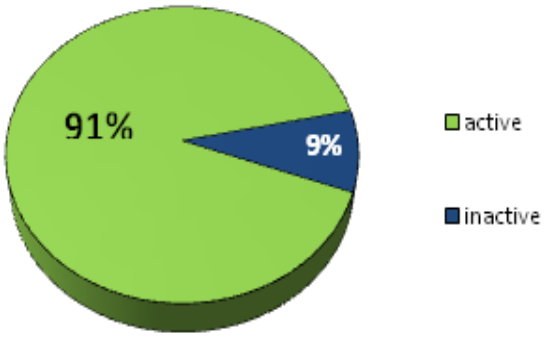


Figure 2. Percentage of active and inactive mutants isolated after UV treatment.

As the second cycle of selection, mutants with clear zone diameter near or higher than parental strain tested again in starch agar and fermentation medium. Clear zone measured in starch agar medium and DNS chemical assay method used with mutants grown in fermentation medium **(Table 1).**

Twenty mutants were screened for their enzyme productivity for 72h at 50°C; Productivity was varied than parental strain as shown in **(Table 1)** Out of twenty; five mutants were higher producer than parental strain after 24h of incubation, UV-3-M1, UV-5-M3, UV-5-M6, UV-3-M17 and UV-5-M121 with clear zone recorded as 28.1 mm (104%), 36 (133.3%), 29 (107.4%), 30 (111%) and 41mm (151.8%) respectively while parental strain had clear zone diameter 27mm (100%) **(Table 1).**

Table 1. Productivity of α-amylase by active UV-mutantson minimal Starch agar for 72h.

UV- mutants	Incubation time		
	24h	**48h**	**72h**
UV-3-m1	28.1±0.3	36 ±0.1	45 ±0.2
UV-10-m2	25 ±0.1	26 ±0.1	27 ±0.3
UV-5-m3	36 ±0.1	32 ±0.1	29 ±0.2
UV-5-m4	22 ±0.2	27 ±0.2	26 ±0.3
UV-10-m5	26 ±0.2	24 ±0.1	28 ±0.1
UV-5-m6	29 ±0.1	28 ±0.1	33 ±0.2
UV-5-m7	22 ±0.1	27 ±0.2	32 ±0.2
UV-3-m17	30 ±0.1	34 ±0.4	37 ±0.1
UV-10-m20	26 ±0.1	25 ±0.4	28 ±0.2
UV-3-m40	26 ±0.2	26 ±0.3	27 ±0.2
UV-7-m41	19 ±0.2	19 ±0.1	21 ±0.3
UV-5-m42	26 ±0.1	25 ±0.1	25 ±0.1
UV-7-m66	24 ±0.2	28 ±0.2	28 ±0.2
UV-10-m70	26 ±0.1	22 ±0.1	24 ±0.2
UV-3-m90	25 ±0.1	25 ±0.1	22 ±0.4
UV-3-m92	22 ±0.3	21 ±0.3	26 ±0.2
UV-3-m118	23 ±0.2	24 ±0.4	24 ±0.2
UV-5-m121	41 ±0.1	45 ±0.2	45 ±0.3
UV-3-m123	24 ±0.1	26 ±0.3	25 ±0.1
UV-3-m130	23 ±0.1	23 ±0.2	25 ±0.2
WT	27±0.1	29±0.3	29±0.3

Clear zone was determined after addition iodine reagent within mm

Twenty mutants out of 180 mutants isolated after UV treatment grown on fermentation medium supplemented with 1.5% starch as a carbon source. Three mutants (UV-5-M3, UV-3-M17, and UV-5-M121) were higher α-amylase producers, 133.3%, 111% and 151.8% respectively compare with parental strain as 100% (Figure 3).

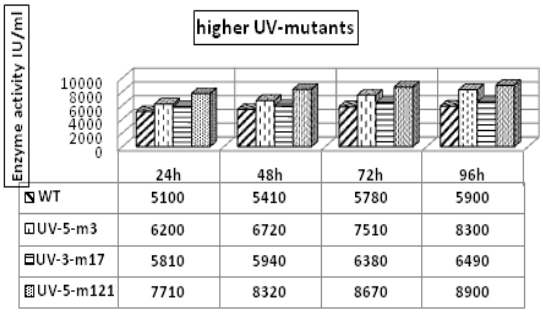


Figure 3. Productivity of α-amylase for 4 days by the three higher producer UV-mutants.

The mutant U 2-6 was more thermostable and more enzyme productivity than parental strain *Bacillus subtilis* with 36% [32]. It was reported that Keratinase productivity by *mutant Bacillus Subtilis* FUN 30.2 was improved 75.93% than parental strain through UV mutation [33]. *Bacillus* mutant strain RS1 isolated from UV irradiation showed clearing

zones measuring 14-15 mm on the milk casein agar compared with wild strain RS zones 10-12 mm [34]. Enhancement of amylase, cellulase, and lipase activity was observed in all the strains after fungal strain exposure to UV radiation [35].

SDS PAGE

Extracellular protein analysis

UV-m-121 α-amylase has molecular mass of 65 kDa while α-amylase from UV-m-3 has molecular mass of 61 kDa and α-amylase from WT of 64 kDa by SDS-PAGE, It similar to [36] result**.** Amylase detected as a single band with 65 kDa by SDS polyacrylamide gel electrophoresis [37]. The α-amylase from *Bacillus licheniformis* EMS-6 was a single band and when purified, it showed 55 kDa by FPLC [11]. The purified α-amylases from *Bacillus subtilis* and mutant U 2-6 strain were 56 kDa molecular weight [32] (Figure 4).

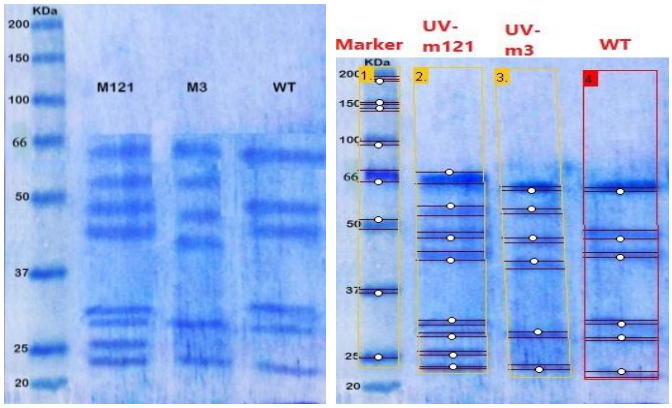


Figure 4. SDS-PAGE for extracellular proteins of parental strain and the two higher producer UV-mutants, were Lane 1: marker broad range, Bio-Rad within α-amylase has 66 KDa); Lane 2: extracted proteins of UV-5-M121; Lane 3: extracted proteins of UV-5-M3; Lane 4: extracted proteins of parental strain (WT).

Total cellular protein analysis

Total protein bands of mutants and parental strain on SDS-PAGE were analyzed by Gel Analyzer 2010 software. A high variance was notice between the two mutants and WT, mutant UV-M3 showed 15 bands, UV-M121 showed 17 bands. The WT showed 18 bands. SDS-PAGE with protein marker performed and results presented in (Figure 5).Total protein bands of mutants and parental strain on SDS-PAGE analyzed by Gel Analyzer 2010 software. A high variance was notice between the two mutants and WT, mutant UV-M3 showed 15 bands, UV-M121 showed 17 bands, and the WT showed 18 bands.

The interaction between genomic of selected mutants and different environmental conditions

Effect of incubation period in α-amylase production

Six higher producing mutants resulted from UV treatment selected as promising mutants beside parental strain, tested for their enzyme productivity in the production medium supplemented with 1.5% starch.

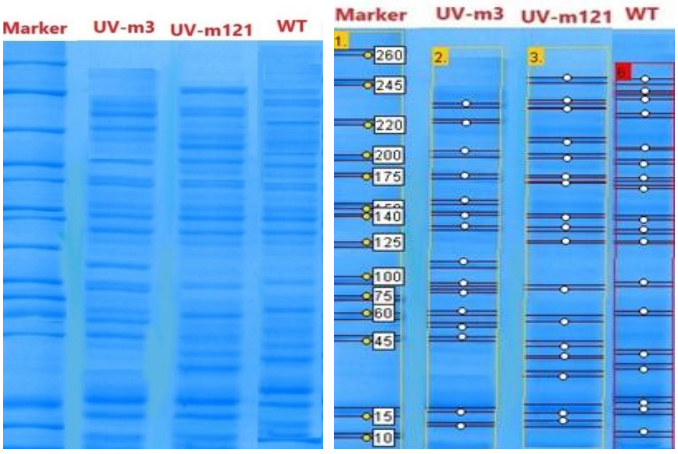


Figure 5. SDS-PAGE for total proteins extracted from W.T. and the best two mutants resulted from UV and EtBr manipulation was: Lane 1: marker broad range, Bio-Rad); Lane 2: extracted proteins from UV-M3; Lane 3: extracted proteins from UV-M121; Lane 4: extracted proteins from parental strain.

Three mutants proved their higher productivity than parental strain after 24h, UV-mutant-3, UV-mutant-17, UV-mutant-121, their productivity were 6010 (127.8%), 5510 (127.87%), and 6800 (144.68%), respectively. These three mutants showed slightly decreasing in their productivity after 48h till the end of incubation, except UV-mutant-121, increased after 48h of incubation and slightly decreased till 7200IU/ml till the end of incubation (Table 2).

Mutant UV-5-m121 was the best enzyme producing mutant with 7210 (180.2%) compared with the parental strain with 4000 IU/ml (100%). It reached its maximum productivity after 72h. While, maximum α-amylase productions were achieved at the end of 12 h of growth for alkaline, and thermophilic *Bacillus sp.* strain KH-13 (WT) and its mutant KH13-M3 [38].

Mutant UV-5-m 121 was the best enzyme producer with 7300 IU/ml (155.31%) compared with the parental strain with 4700 IU/ml (100%). It reached its maximum productivity after 72h.

Effect of temperature in α-amylase production

The selected mutants tested using five different temperatures for maximum production of α-amylase production, 35, 45, 55, 65, and 75°C. Parental strain selected as a thermophilic isolate isolated from internal soil of Pharaoh Pools, the selected mutants from it keep on their character, none of tested mutants beside the parental strain prove its maximum productivity at 35°C (Table 3).

Only one mutant, UV-3-m130 showed its maximum productivity at 45°C while two mutants UV-3-m17, and UV-3-m92 reached their maximum productivity at 55°C (Table 3). Three mutants beside parental strain proved their maximum productivity when incubated at 65°C, moreover

the highest producer mutant showed its maximum productivity at this temperature was UV-5-m3 (Table 3)

The optimum temperature for α-amylases of alkaline and thermophilic *Bacillus sp.* strain KH-13 (WT) and its mutant KH13-M3 was 40 and 50°C, respectively [38]. Better α-amylase enzyme activity of *Bacillus amyloliquefaciens* EMS-6 mutant was at 37ºC for 24 to 72h incubation times [11]. The enzyme activity was increased with fermentation period increased. The maximum α-amylase production (60.9 U/ml/min) was at 37ºC in 48 h. When the temperature was increased up to 43ºC, the enzyme activity markedly declined.

The α-amylase production by the Streptomyces clavifer AM-7-1-4 mutant was affected by different environmental factors. The optimum condition was 60°C. Sufficient activity (≥ 86%) was also observed at temperature ranged from 50 to 65°C, respectively [39].

UV Mutant number 17 of *Bacillus mojavenis* PTCC 1723 produces 330.56 IU/ml xylanase. It was 3.45 times more enzyme than the wild strain with 95.73 IU/ml. Optimization resulted 575 IU/ml xylanase, with wheat bran as the best carbon source, corn steep liquor as the best nitrogen source accompanied with natural bakery yeast powder, in a medium with pH 7, after 48 hr incubation at 37°C, and the shaking rate of 230 rpm [40].

Effect of pH in α-amylase production

Six mutants beside WT tested for their ability to produce α-amylase in different pH, 6, 7, 8, and 9.

Four mutants reached their maximum productivity at pH 7 beside WT, it also prove its maximum productivity at this pH. Mutant UV-5-m121 prove its maximum productivity at pH 7, it's also the third highest producing mutant in the level of all tested thirteen mutants. Two mutants out of six, proved their maximum productivity at pH 8. They were UV-5-m3 and UV-3-m92 Table (4). The highest producing mutant out of them at pH 8, was UV-5-m3, it produce 5620 IU/ml which nearly 138% compared with WT.

The α-amylase production by the Streptomyces clavifer AM-7-1-4 mutant was affected by different environmental factors, such as pH and temperature [39]. It was observed that the optimum condition was pH 6 and 60°C. Sufficient activity (≥ 86%) was also observed at pH range 5.5 to 6.5.

Table 2. Enzyme productivity by the best selected UV- mutants and their parental strain for 96 h.

Mutants	24h	48h	72h	96h
UV-5-m3	6010 (127.8%)	6050 (143.7%)	6110 (152.7%)	5960 (149%)
UV-3-m17	5510 (117.2%)	5440 (129.1%)	5380 (134.5%)	5090 (127.2%)
UV-3-m40	4630 (98.5%)	5020 (119.2%)	5020 (125.5%)	5000 (125%)
UV-3-m92	4090 (87%)	4700 (116.3%)	4600 (115%)	4310 (107.7%)
UV-5-m121	6800 (144.6%)	7300 (173.3%)	7210 (180.2%)	7200 (180%)
UV-3-m130	3910 (83.1%)	4300 (102.1%)	4680 (117%)	5020 (125.5%)
Parental strain	4700 (100%)	4210 (100%)	4000 (100%)	4000 (100%)

Table 3. Enzyme productivity of WT and the best UV mutants at different temperature.

Mutants	35°C	45°C	55°C	65°C	75°C
UV-5-m3	5200	5620	6010	6050	4100
UV-3-m17	5100	5370	5700	5240	4630
UV-3-m40	2720	3420	3970	4100	4010
UV-3-m92	3940	4350	4700	4160	3420
UV-5-m121	4000	4600	5160	5730	5410
UV-3-m130	3750	4620	4600	4510	4020
WT	3630	4600	5100	5190	2980

Table 4. enzyme productivity of WT and the selected mutants at different pH values

Mutants	pH6	pH7	pH8	pH9
UV-5-m3	3520	4910	5620	5230
UV-3-m17	3470	4030	3410	2890
UV-3-m40	2600	3920	3210	3010
UV-3-m92	2630	3740	4410	3460
UV-5-m121	3860	5830	5460	4680
UV-3-m130	3120	4010	3700	2060
WT	2100	5300	4070	3600

Table 5. Enzyme productivity by the best UV-mutants and their parental strain at different starch concentrations.

Mutants	0.50% Starch			1% Starch			1.50% Starch		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
UV-5-m3	4000	4170	4170	4630	4790	4850	6300	6210	6100
UV-3-m17	3100	3340	3520	5100	5470	5300	5600	5640	5790
UV-3-m40	3040	3200	3410	4300	4100	3520	3970	4300	4570
UV-3-m92	3850	3740	3600	4100	4100	4200	4410	4570	4720
UV-5-m121	3900	4100	4210	4700	5300	5700	6000	6170	6230
UV-3-m130	3620	3430	3070	3600	3750	3840	4860	4430	4010
WT	3400	2900	2530	3540	4030	4100	3740	3980	4200

Effect of starch concentration in α-amylase production

Production medium with different starch concentrations 0.5, 1.0, and 1.5 g/l was used to study the effect of starch concentration on the enzyme productivity by WT and the selected mutants (Table 5).

1.5% starch concentration was the best one for the maximum enzyme productivity by all mutants and UV-5-m3 was the highest producing mutant; it reached its maximum productivity after 24h with 6300 IU/ml (168.4%) compared with W.T (Table 5).

Bacillus mutant strain RS1 isolated after UV irradiation showed 14-15 mm clearing zones on the milk casein agar indicating the presence of a protease, after the plates spotting with 10 µl cultures and incubated at 30°C for 48 hr but wild strain zones were 10-12 mm [34].

***AmyE* gene amplification and sequencing**

In this study, parental strain *Bacillus licheniformis* MK90 and the best two mutants isolated after UV treatment, used for AmyE sequencing gene, coding for α-amylase enzyme. The designed forward (MK-am.F) 5ATGAAACAACAA AAACGGCTTT3 and reverse (MK-am.R) 5-CTATCTT TGAACATAAATTGAAACCG3 primers used for amplifying *AmyE* gene by PCR using the two mutants and their parental strain DNA as a template. PCR products loaded in agarose gel electrophoresis with DNA ladder. Gel analysis showed that, the amplified fragments have about 1539 bps (Figure 6).

The resulting three PCR products of parental strain beside the two mutants sequenced. The resulted sequences showed in Figure (7).

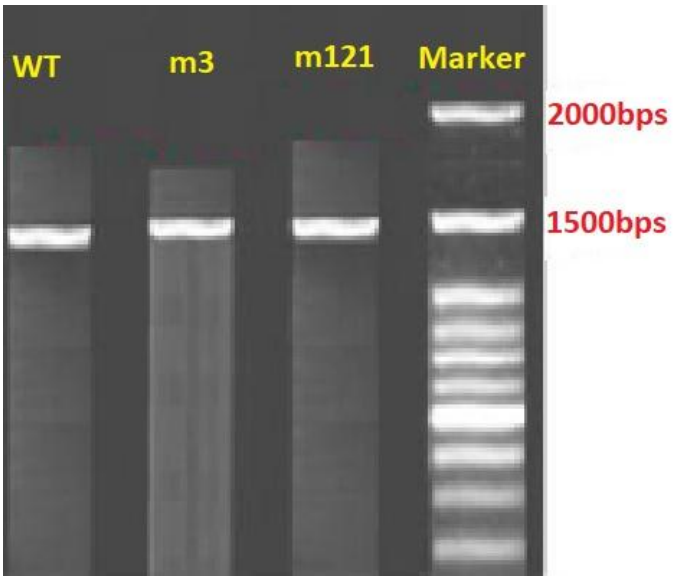


Figure 6. Agarose gel electrophoresis showed PCR product of *amyE* gene (1500-1600 bps) using specific Forward (MK-am.F) and Reverse primers (MK-am.R). Lan 1: WT, Lan 2: UV-5-m3, Lan3: UV-5-m121 and Lan 4: DNA marker.

(A) WT

ATGAAACAACACAAACGGCTTTATGCCCGATTGCTGCCGCTGTTATTTGCGCTCATCTTCTTGCTGTCTCACTCTGCAGC
AGCGGCGGCAAGTCTTAATGGGACGCTGATGCAGTATTTTGAGTGGTACATGCCAAATGATGGCCAACATTGGAAACG
CTTACAAAATGACTCGGCATATTTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGT
CAAGACGATGTAGGCTACGGCGCTTACGATCTGTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAG
TACGGCACAAAGGGAGAACTGCAATCTGCGATCAACAGTCTTCATTCCCGGGACATCAACGTTTACGGCGATGTAGTCA
TCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACGGCTGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAA
CATCAGGAGAACAGCGAATCAAAGCGTGGACACATTTTCAATTCCCGGGGCGCGGCAGCACATACAGCGATTTCAAAT
GGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAGG
CATGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTACTTGATGTATGCCGACATCGATTATGATCATCCTGA
TGTCACGGCAGAAATAAAGAGATGGGGAACGTGGTATGCCAATGAGCTGCAATTGGACGGATTCCGCCTTGATGCCGT
CAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTCAATCATGTCAGGAAAAAACAGGGAAAGGAAATGTTTACGGTA
GCTGAATATTGGCAGAATGACTTAGTGCGCTGGAAAACTATTTTGAACAAAACAAACTTTAATCATTCAGTGTTTGACG
TGCCGCTTCATTACCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGAACAGT
CGTTTCCAAGCATCCTGTGAAAGCGGTTACGTTTGTTGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
GTCCAAACATGGTTTAAGCCGCTGGCTTACGCTTTTATTTTGACAAGAGAAGCAGGCTACCCGCAGATTTTCTACGGGG
ATATGTACGGGACGAAAGGAGCCTCGCAGCGCGAAATTCCTGCCCTCAAACACAAAATCGAACCGATCTTAAAAGCGA
GAAAACAATATGCGTACGGAGCACAGCATGATTATTTCGATCATCATAACATTGTCGGCTGGACGAGAGAAGGCGACA
GCTCGGTTGCAAATTCTGGTTTGGCGGCGTTAATAACAGACGGACCCAGCGGGACAAAGCGAATGTATGTCGGCCGGC

AAAACGCCGGTGAGACATGGCATGACATCACCGGAAACCGTTCCGATTCTGTTGTCATCAATGCAGAAGGCTGGGGAG
AGTTACACGAAAACGGCGGATCGGTTTCGATCTATGTTCAAAGATAG

(B)UV-5-m3

ATGAAACAACACAAACGGCTTTATGCCCGATTGCTGCCGCTGTTATTTGCGCTCATCTTCTTGCTGTCTCACTCTGCAGC
AGCGGCGGCAAGTCTTAATGGGACGCTGATGCAGTATTTTGAGTGGTACATGCCAAATGATGGCCAACATTGGAAACG
CTTACAAAATGACTCGGCATATTTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGT
CAAGACGATGTAGGCTACGGCGCTTACGATCTGTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAG
TACGGCACAAAGGGAGAACTGCAATCTGCGATCAACAGTCTTCATTCCCGGGACATCAACGTTTACGGCGATGTAGTCA
TCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACGGCTGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAA
CATCAGGAGAACAGCGAATCAAAGCGTGGACACATTTTCAATTCCCGGGGCGCGGCAGCACATACAGCGATTTGAAAT
GGCATTGGTACCATAATGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAGG
CATGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTACTTGATCTTTGCCGACATCGATTATGATCATCCTGA
TGTCACGGCAGAAATAAAGAGATGGGGAACGTGGTATGCCAATGAGCTGCAATTGGACGGATTCCGCCTTGATGCCGT
CAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTCAATCATGTCAGGACGCCACAGGGAAAGGAAATGTTTACGGTA
GCTGAATATTGGCAGAATGACTTAGTGCGCTGGAAAACTATTTTGAACAAAACAAACTTTAATCATTCAGTGTTTGACG
TGCCGCTTCATTACCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGAACAGT
CGTTTCCAAGCATCCTGTGAAAGCGGTTACGTTTGTTGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
GTCCAAACATGGTTTAAGCCGCTGGCTTACGCTTTTATTTTGACAAGAGAAGCAGGCTACCCGCAGATTTTCTACGGGG
ATATGTACGGGACGAAAGGAGCCTCGCAGCGCGAAATTCCTGCCCTCAAACACAAAATCGAACCGATCTTAAAAGCGA
GAAAACAATATGCGTACGGAGCACAGCATGATTATTTCGATCATCATAACATTGTCGGCTGGACGAGAGAAGGCGACA
GCTCGGTTGCAAATTCTGGTTTGGCGGCGTTAATAACAGACGGACCCAGCGGGACAAAGCGAATGTATGTCGGCCGGC
AAAACGCCGGTGAGACATGGCATGACATCACCGGAAACCGTTCCGATTCTGTTGTCATCAATGCAGAAGGCTGGGGAG
AGTTACACGAAAACGGCGGATCGGTTTCGATCTATGTTCAAAGATAG

(C) UV-5-m121

ATGAAACAACACAAACGGCTTTATGCCCGATTGCTGCCGCTGTTATTTGCGCTCATCTTCTTGCTGTCTCACTCTGCAGC
AGCGGCGGCAAGTCTTAATGGGACGCTGATGCAGTATTTTGAGTGGTACATGCCAAATGATGGCCAACATTGGAAACG
CTTACAAAATGACTCGGCATATTTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGT
CAAGACGATGTAGGCTACGGCGCTTACGATCTGTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAG
TACGGCACAAAGGGAGAACTGCAATCTGCGATCAACAGTCTTCATTCCCGGGACATCAACGTTTACGGCGATGTAGTCA
TCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACGGCTGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAA
CATCAGGAGAACAACGAATCAAAGCGTGGACACATTTTCAATTCCCGGGGCGCGGCAGCACATACAGCGATTTCAAAT
GGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAGG
CATGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTACTTGATGTATGCCGACATCGATTATGATCATCCTGA
TGTCACGGCAGAAATAAAGAGATGGGGAACGTGGTATGCCAATGAGCTGCAATTGGATGGATTCCGCCTTGATGCCGT
CAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTCAATCATGTCAGGAAAAAACAGGGAAAGGAAATGTTTACGGTA
GCTGAATATTGGCAGAATGACTTAGTGCGCTGGAAAACTATTTTGAACAAAACAAACTTTAATCATTCAGTGTTTGACG
TGCCGCTTCATTACGAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGAACAGT
CGTTTCCAAGCATCCTGTGAAAGCGGTTACGTTTGTTGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACT
GTCCAAACATGGTTTAAGCCGCTGGCTTACGCTTTTATTTTGACAAGAGAAGCAGGCTACCCGCAGATTTTCTACGGGG
ATATGTACGGGACGAAAGGAGCCTCGCAGCGCGAAATTCCTGCCCTCAAACACAAAATCGAACCGATCTTAAAAGCGA
GAAAACAATATGCGTACGGAGCACAGCATGATTATTTCGATCATCATAACATTGTCGGCTGGACGAGAGAAGGCGACA
GCTCGGTTGCAAATTCTGGTTTGGCGGCGTTAATAACAGACGGACCCAGCGGGACAAAGCGAATGTATGTCGGCCGGC
AAAACGCCGGTGAGACATGGCATGACATCACCGGAAACCGTTCCGATTCTGTTGTCATCAATGCTTGGGGCTGGGGAG
AGTTACACGAAAACGGCGGATCGGTTTCGATCTATGTTCAAAGATAG

Figure 7. The draft of AmyE gene sequences from the WT and the best two UV producing mutants

Online BLAST analysis through NCBI site revealed that, 99 % homology between PCR products and standard α-amylase gene. In (Table 6), strain *Bacillus licheniformis* with accession number AF438149.1, has identity 99% in its sequence with the four mutants and their parental stain sequences, its *AmyE* gene coded for thermostable α-amylase enzyme, like the gene sequences under study.

The three sequences then analyzed using BLAST program to detect the differences between the two mutants and the parental strain due to UV effect, using online multiple alignment ClusterW and Jalview software.

Nucleotide sequences of *AmyE* gene of the parental strain and its two mutants compared to each other using online Cluster W and the results analyzed via jalview Version 2 (a multiple sequence alignment editor and analysis workbench) and many nucleotide substitutions were present in each mutant and these substitutions differ from parental strain nucleotide sequence. Mutant UV-5-m121 contains the lowest substitution sites reached 5 only between 486 and 765, beside range between 958 and 1480, while mutant UV-5-m3 contains substitutions reached seven between nucleotide number 546 and 837 (Figure 8).



Figure 8. DNA alignment between *AmyE* gene sequence from parental strain and the two mutants using Jalview software which showed that *AmyE* gene composed of 1539 bps.

Table 6. Top 10 hits description on the query of Sanger sequence for α-amylase gene *(AmyE) of Bacillus licheniformis* MR90 on BLAST.

	Description[a]	Max score[b]	Total score[c]	Query cover[d]	E value[e]	Identitiy[f]	Accession[g]
1	*Bacillus licheniformis* from Iran hyperthermostable α- amylase gene, complete cds	2721	2721	100%	0.0	99%	AF438149.1
2	*Bacillus licheniformis* strain UTM118 α- amylase gene, complete cds	2771	2771	100%	0.0	99%	KP893116.1
3	*Bacillus licheniformis* α- amylase (amy) gene, complete cds	2771	2771	100%	0.0	99%	JX897677.1
4	*Bacillus licheniformis* strain 3TB2 α- amylase (bla) gene, complete cds	2760	2760	100%	0.0	99%	GQ284655.1
5	*Bacillus licheniformis* strain NH1 α- amylase gene, complete cds	2754	2754	100%	0.0	99%	EF125542.1
6	*Bacillus licheniformis* strain MSG α- amylase precursor, gene, complete cds	2726	2726	100%	0.0	99%	GQ262779.1
7	*Bacillus licheniformis* gh13A gene for α- amylase , complete cds	2809	2809	100%	0.0	99%	AB643493.1
8	*Bacillus licheniformis* strain SHG10 α- amylase gene, complete cds	2676	2676	100%	0.0	98%	JN853583.1
9	*Bacillus licheniformis* strain AR1 α- amylase (amyL) gene, complete cds	2289	2289	100%	0.0	94%	KJ508878.1
10	*Bacillus licheniformis* strain ATCC 27811 α- amylase (amyA) gene, complete cds	2283	2283	100%	0.0	93%	AY630336.1

[a]the description/title of matched database, [b] the highest alignment score (Max score) from that database sequence, [c] the total alignment scores(Total score) from all alignment segments, [d] the percentage of query covered by alignment to the database sequence, [e] the best (the lowest) Expect value (E value) of all alignments from that database sequence, [f] the highest percent identity (Max identity) of all query-subject alignments and [g] the accession of the matched database sequence.

The *amyA*gene from thermophilic *Halothermothrixorenii* was 1545 bps long, and encoded a 515 residue protein composed of a 25 amino acid putative signal peptide and a 490 amino acid mature protein. It possessed the five consensus regions characteristic of the α-amylase family and showed the greatest homology to the *Bacillus megaterium* group of *a*-amylases [41]. The nucleotide sequence of DNA fragments of gene coding heat-stable and pH-stable α-amylase of *Bacillus licheniformis* 584 (ATCC 27811) was 1948 bps containing the entire amylase gene was determined [42].

Amino acid sequence analysis of *AmyE* gene expression

The three sequences of parental strain and its two mutants analyzed via Snapgene viewer version 3.1.2. Software in which, nucleotide sequence of genes translated into amino acid sequences to observe the effect of UV in the gene expression and resulted enzyme.

The results showed that, the three sequences coding 513 amino acids but with some amino acid substitutions in the sequences of two mutants when compared with parental strain (Figure 9).

Sequence of mutant UV-5-m121 showed only one amino acid substitution in amino acid number 494, it replaced by Tryptophan while it was Glutamic acid in the parental sequence (Figure 9).

Mutant UV-5-m3 sequence showed three amino acid substitutions in sites 189, 279 and 280, these amino acids changed to Asparagine, Threonine, and Proline while they were in the parental sequence, Phenylalanine, Lysine and Lysine respectively (Figure 9).

We concluded that, all substitutions in amino acid replacement with different amino acid as the effect of UV treatment could be classify as missense mutation (Table 7).

As inferred from the DNA sequence, the *B. licheniformis* α-amylase had a signal peptide of 29 amino acid residues and the mature enzyme comprised 483 amino acid residues, giving a molecular weight of 55,2 kDa. The amino acid sequence of *B. licheniformis* α-amylase showed 65.4% and 80.3% homology with those of heat-stable *Bacillus stearothermophilus* α-amylase and relatively heat-unstable *Bacillus amyloliquefaciens* α-amylase, respectively [42].

Table 7. Amino acids substitution in UV-mutants in comparison with WT

Amino acid No	WT	UV-5-m3	UV-5-m121
150	P	P	P
189	F	N	F
279	K	T	K
280	K	P	K
300	K	K	K
301	T	T	T
310	H	H	H
320	Q	Q	Q
326	T	T	T
329	G	G	G
346	P	P	P
494	E	E	W

P: Proline, D: Aspartic acid, F: Phenyl alanine, N: Asparagine, K: Lysine, I: Isoleucine, H: Histidine, T: Threonine, G: Glycine, Q: Glutamine, L: Leucine, A: Alanine, E: Glutamic acid and W: Tryptophan.

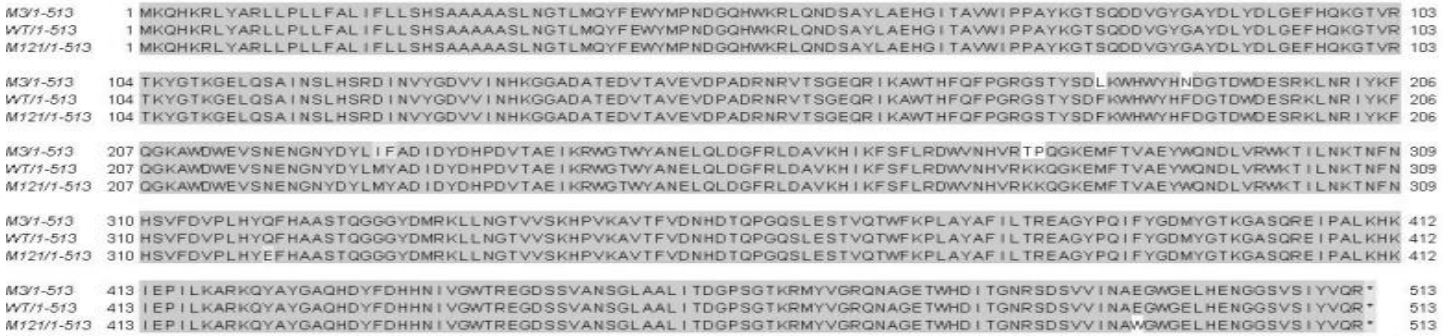


Figure 9. Multiple alignments of Amino acid sequence for parental strain and the best four mutants.

Conclusion

Selected mutants isolated from *Bacillus licheniformis* MK90 after UV treatment showed higher α-amylase productivity. UV-5-M121 mutant was the highest producer with 150.8% compared with WT productivity. UV mutants produced a thermophilic α-amylase. The interaction between genomic of selected mutants and different environmental conditions were studied. Optimum condition for α-amylase production was at 55 to 65°C with pH 7 and 8 in 1.5% Starch. SDS-PAGE analyzed showed high variance between the two mutants and WT. Mutant UV-M3 showed 15 bands, UV-M121 showed 17 bands and WT 18 bands. A thermophilic α-amylase gene (*Amy*E) of in *Bacillus licheniformis* MK90 and its two mutant's sequences showed 1539 bps and this sequence coding for 513 amino acids. Many nucleotide substitutions were present in each mutant and differ than parental strain nucleotide.

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