Egypt. J. Chem. **Vol. 65,** No. 10 pp. 723 - 732 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Enhancement of Lipase Production based on improved *Bacillus Licheniformis* for Catabolizing of Edible Oil Wastes

Abd El-Nasser A. Khattab[1*], El-Shahat H. A. Nashy[2], Adel G. Abdel-Razek[3], and Azza M. Abdel-Fattah[4]

[1]*Genetics and Cytology Dept., National Research Centre, Dokki 12622, Cairo, Egypt.*
[2]*Chemical Industries Research Institute, National Research Centre, Dokki 12622, Cairo, Egypt.*
[3]*Fats and Oils Dept., National Research Centre, Dokki 12622, Cairo, Egypt.*
[4]*Chemistry of Natural and Microbial Products Dept., National Research Centre, Dokki 12622, Cairo, Egypt.*

Abstract

Due to the applied rules by various pollution control authorities to maintain the discharged norms of wastes to treated water, the food business has a difficult challenge. The wastewater contains high levels of fats, grease, and oils increased every year. So, this work was devoted to isolating and investigating microorganisms that could be used to treat wastewater as an alternative to chemical treatment. Seven Bacilli were locally isolated from soil and tested for lipase production. The AMG-Kh-B2 was the most potent isolate that produced the highest lipase activity (36.4 $U.mL^{-1}$) after 3 days of fermentation. The *Bacillus* isolate (AMG-Kh-B2) was identified as *Bacillus licheniformis* through the BLAST analysis of the amplified 16S ribosomal RNA gene sequence. Mutation induced by ethyl methane sulfonate (EMS) was performed to improve lipase enzyme production. The mutant M-9 was the highest enzyme producer since it produced 262.64 % lipase enzyme higher than the wild-type strain (WT). The use of the random amplified polymorphic DNA (RAPD) approach using polymerase chain reaction (PCR) on certain excellent mutants resulted in a correlation between the genetic characteristics of the excellent mutants (M-9 and M-27) and the genetic characteristics of the wild type strain (WT). The evidence of genetic diversity created in *B. licheniformis* DNA following EMS-mutagenesis was corroborated by differences in RAPD patterns. As a result, cluster analysis was utilized to classify the strains under analysis into clusters that potentially recognize the genetic variety of lipase-producing mutants.

Keywords: Edible oil wastes, Biodegradation, *Bacillus*, Lipase improvement, EMS-mutants, Molecular-characterization.

1. Introduction

According to FAO statistics, the quantitative loss of edible oils amounts to 20% of world production annually **[1].** Every year, huge volumes of lipid-containing effluent are released in Egypt, with food companies and restaurants accounting for over 75% of this pollution **[2].** The amounts of wastewater containing high levels of fats, greases and oils increase each year due to the progressing of industrial plants **[3, 4].**

In general, Development of various food industries and restaurants rapidly leads to the accumulation of more wastes. Wastes usually emitted from restaurants, hotels, dairy, food processing and edible oil refining are rich in fats and oils, that bring potential problems in the management of wastewater due to the high content of fats, oils and greases (FOG) **[5,6].** FOG at low temperature will found in a waxy or a solid form that clogs drains and causes problems in wastewater treatment systems **[7, 8].** Moreover, untreated fatty materials and waxes which are emitted into the environment through the wastewater, is one of the greatest contributors to environment pollution.

Animal fats and vegetable oils are the primary components of FOGs, which are hydrolyzed to produce a mixture of glycerol and free fatty acids **[5]**. It is well known that, lipids in wastewater are difficult to be removed and/or degraded because of the reason that they're insoluble in water and have been shown to stop methane production **[9-12]**. Almost, the amount of fats in wastewater measured 30 – 40% of the total organic matter, this is based on chemical oxygen demand (COD**) [13].** FOGs interfere with activated oxygen transfer rate and hinder the diffusion of oxygen and substrates in waste water, due to the oil film which can be formed on the surface of reservoir. Moreover, lipids promote the growth of filamentous microorganisms which impetus bulking and foaming (*Sphaerotilus natans*, *Thiothrix* sp., *Beggiatoa* sp., *Nocardia* sp., *Microthrix* sp.) **[14-16].**

*Corresponding author e-mail: ab.khattab@nrc.sci.eg (**Abd El-Nasser A. Khattab**)
Receive Date: 18 May 2022, Revise Date: 02 June 2022, Accept Date: 04 June 2022
DOI: 10.21608/EJCHEM.2022.138927.6116

Wastewater released from edible oil processing, dairy products industry and slaughterhouse contains a high concentration of lipids (> 100 mg/L). The increased concentrations of lipids in the waste water, leads to major problems in biological wastewater treatment processes. Lipids hardening at lower temperature cause operational requirements damage, such as clogging and development of unpleasant odours **[2, 17-18].** Emulsification, degradation, and breakdown into glycerol and fatty acid are all phases in the lipid metabolism process. Through the beta-oxidation route, fatty acids are transformed to acetyl-CoA, which participates in the TCA cycle. In wastewater treatment systems, wastewater pipes are often clogged by lipids **[19].**

This meaning that, wastewater usually contains large amounts of hazardous wastewater with high concentration of salts, suspended materials, colloidal, oil & grease, BOD and COD and surplus chemicals. Therefore, biodegradability of vegetable oils is the preferred solution for their industrial use. From the viewpoint of environmental impact, the use of industrial fuels, chemicals and lubricants, offer the most reasonable solution from the issue of obtaining sustainable and eco-friendly oleo-chemicals **[20].**

Lipases (triacylglycerol acyl-hydrolases, EC 3.1.1.3) are multifunctional hydrolytic enzymes that catabolize and generation acyl-glycerol at the lipid-water interface [21]. Mutations that increase enzyme features such as selectivity, activity, alternative catalytic activity, and thermal stability have been found by a number of research groups **[22-27].**

The maximum utilization of the FOG wastes not only for environmental pollution treatment but also it can be used for lipase production which can be considered as added value. In some research, residual bovine non-marketable fat was converted to meat-like aroma which can be considered as less expensive products based on fat as a natural source of carbonyl groups [28]. Also, biodiesel was produced by *Aspergillus niger* KY401431 from cooking oil using solid-state fermentation **[29].**

So, effective by-product usage has a direct influence on our country's economy and pollution levels. Therefore, the present work was devoted to apply many *Bacillus* isolates collected from soil that can catabolize and lysis wastewater lipids through the production of lipase enzymes. For environmental protection and pollution prevention, microbial functions have been explored in terms of their potential to remove wastewater lipids. Furthermore, the present study has been undertaken to induce the mutants with high lipase activity after EMS-mutagenesis of the potent *Bacillus* isolate. Also, the molecular identification and characterization of the most potent *Bacillus* isolate and its excellent mutants, respectively, were applied.

2. Materials and Method

2.1. Culture Media and Growth Conditions:

Nutrient agar (Oxoid, Basingstoke, UK) was used for maintaining the microbial strains and nutrient broth (Oxoid, Basingstoke, UK) was used for sub-culturing and pre-culturing the strains. The enrichment medium (EM) composed of (g/l) 1, edible oil waste; 0.5, $(NH_4)_2SO_4$; 0.5, KH_2PO_4, 0.1, $MgSO_4$ and 1, yeast extract was used for the enrichment of lipid-degrading microorganisms. Tributyrin -peptone agar (TBA) containing (g/l) 10.0, peptone; 5.0, NaCl; 1.0, $CaCl_2$; 18, agar and 5 ml/l tributyrin and was used as the production medium for the screening of lipid-degrading strains **[12].** To investigate the ability of the strains to degrade fatty substrates, two fermentation media were used for the production of lipase enzyme by *Bacillus* strains as follow; fermentation medium 1 (FM1) which is composed of (g/l) 0.48, KH_2PO_4; 1.12, K_2HPO_4; 0.1, $MgSO_4.7H_2O$; 5, NaCl; 2.0, $(NH_4)_2SO_4$; 0.001, EDTA and all the constituents dissolved in distilled H_2O containing 1% edible oil waste as the natural substrate **[30].** And fermentation medium 2 (FM2) composed of (%, w/v) 0.325, nutrient broth; 0.1, $CaCl_2$; 1.0, edible oil waste and 1, gum Arabic **[31].** All media were adjusted to pH 6.5.

2.2. Isolation and screening of lipid-degrading bacterial isolates:

The samples from soil were harvested in sterile reagent made in small culture tubes marked appropriately and kept at 4ºC till investigation in Kotoor, Gharbia Governorate (Egypt). One gram of soil sample was dissolved in 9 mL sterile distilled water and strongly agitated. Bacteria were isolated using a series of repeated dilutions up to 10^{-10}. Each dilution's aliquot of 0.1 ml was distributed equally over the surface of FM1 and incubated overnight at 37ºC. Plates were checked after 24 hours. To select the high-producing lipase isolate, colonies with grew over the surface of TBA medium containing tributyrin (0.5 %, v/v) were selected **[12].**

2.3. EMS-mutagenesis and lipase production:

Five ml of overnight bacterial cells was centrifuged at 8000 rpm for 5 min and the bacterial biomass pellets were dissolved in a phosphate buffer with a pH of 7 and a concentration of 0.1 M. To select the superior generating lipase colonies, the isolated bacterial cells were then treated with EMS-mutagen for 20, 40, and 60 minutes, then diluted and dispersed across the surface of TBA medium containing tributyrin (0.5 percent v/v). The plates were then incubated at 37°C for 3 days. For further research, the screening colonies with high clear zones were kept on Nutrient agar slants. At this point, the viability had dropped by nearly 90%. Colonies with superior clearing zones were re-cultured on the above medium and the plates were incubated at 37°C for 3 days **[35-36]**.

2.4. Ribosomal gene sequences of *Bacillus* strain (AMG-Kh-B2):

The 16S-ribosomal gene was amplified by using Thermo K1051's Maxima Hot Start PCR Master Mix, and the 16S-primers' nucleotide sequences are as described in the following: forward primer [27F], 5'-AGAGTTTGATCCTGGCTCAG -3'; reverse primer [1492R], 5'- GGTTACCTTGTTACGACTT -3'. DNA was extracted from the *Bacillus* strain (AMG-Kh-B2) with Gene Jet genomic DNA purification Kit (Thermo K0721, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA). PCR Purification Kit was used to clean up the PCR product (Thermo K0701). The ABI 3730xl DNA sequencer (GATC Company, Germany) was used to sequence the PCR product's DNA using forward and reverse primers.

2. 5. Constructed phylogenetic tree:

The isolate's 16S rRNA sequence was matched to sequences found in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) using a BLAST search. The sequence was compared to that of reference data obtained from public databases. The evolutionary distances were calculated using a parameter model, and the neighbor-joining method was used to build the phylogenetic tree **[32].**

2.6. Extracellular lipase activity:

Lipase was assayed by p-nitro phenyl-palmitate (pNP-P). Extracellular extract (enzyme) was treated in a final volume of 1ml with pNP-P (8.0 mM, in isopropanol), 0.05M sodium phosphate buffer (pH 8.0), and Triton X- 100 (0.4 %, w/v) at 35°C for 30 minutes. At 410 nm, the developing color was measured. The enzyme solution was not present in the blank tube, which was identical to the active test tube. The release of 1µmol of pNP per minute is one unit of lipase activity. The standard was followed **[25].**

2.7. Molecular characterization of original and mutant strains by RAPD:

i-genomic BYF-DNA extraction Mini-Kit, iNtRON Biotechnology Inc., South Korea, was applied to extract the bacterial DNA. The concentration and purification of the acquired DNA were recorded at 260 and 280 nm using a Shimadzu UV-VIS spectrophotometer model UV-240 **[33].** For PCR tests, we utilized 2xPCR Master mix Solution (i-StarTaq), Hot-Start (iNtRON Biotechnology Inc., South Korea Product Catalog No: 25166). Each bead includes all of the materials required for 25 µl PCR amplification operations, with the exception of primer and DNA template. In this investigation, three distinct random primers were employed. 5'- GGG GTTTGC CACTGG -3' was the sequence of the first primer (P1). The sequence for the second primer (P2) was 5'-GTGTTGTGGTCCACT -3.' 5'-AACCTCCCCCTGACC -3' was the sequence of the third primer (P3). Operon Technologies Company in amplification process was reduced to 25 µl. The following was the amplification procedure: Five minutes of denaturation at 95°C. Each of the thirty-five cycles is made up as follow: Denaturation at 95°C for one minute; primer annealing at 55°C for two minutes depending on the GC ratio of each primer; and DNA polymerization at 72°C for two minutes. Finally, the PCR was kept at 4°C until it was analyzed. The amplified DNA products from RAPD analysis were electrophoresed for roughly 2 hours on a 1.0 percent agarose gel with 1 X TBE buffer at a constant 100 volt. The band sizes were determined using the Sizer TM 1000 plus DNA ladder (iNtRON Biotechnology Inc., South Korea Product Catalog No: 24075), and the distinct bands were recorded by using Gel Documentation System with UV Transilluminator after being discovered with 0.5 g/ml ethidium bromide.

2.8. RAPD-data and statistical analysis:

The amplification waveforms for the strains that were employed as a consequence of the RAPD system were compared to each other and to a standard DNA marker. DNA bands were assessed as binary data, with (1) indicating band existence and (0) indicating band absence. The following statistical equation was used to compute the distance coefficients: $F= 2Nxy/ (Nx + Ny)$. Where Nx and Ny are the counts of bands in genotypes x and y, respectively, Nxy is the number of fragments bands that differ between the two genotypes, and F is the distance coefficient **[34].**

For analysis, the electrophoretic pattern of each primer's repeatable banding patterns obtained by RAPD was chosen. All the other observed results were analyzed by one-way ANOVA test, using version 18-SPSS program. The significant variances between means were measured by Duncan at $p < 0.05$.

3. Results and Discussion

3.1. Screening of the obtained strains and lipase production using two fermentation media:

To screen for highly lipase producers, the seven isolated bacterial strains from many isolates were selected on TBA medium based on their growth rate and produced clearing zone around the growth area. The obtained results showed that all the bacterial strains had clearing zones and/or large dotted zones due to lipase activity and free fatty acids precipitation around the growth area **(Fig. 1).**

Table 1 shows the influence of two distinct fermentation media (FM1 and FM2) on lipase production of the *Bacillus* isolates obtained, as well as the incubation durations (2, 3, and 5 days). Table 1 clearly revealed that fermentation medium No. 1 (FM1) was the best since all tested isolates generated more lipase on this medium after various incubation times. The greatest lipase productivity records were 36.4 $U.mL^{-1}$ achieved after 3 days from the original *Bacillus* (AMG-Kh-B2) isolate. Isolate AZ-3 on the FM1 for 5 days was the second strain for lipase productivity. On the other side, the isolate of *Bacillus*

(AMG-Kh-B3) on FM2 had the lowest record of lipase synthesis after two days of incubation, with 22.8 $U.mL^{-1}$. In comparison to the other investigated isolates, *Bacillus* (AMG-Kh-B2) demonstrated to be the highest lipase producer on FM1 based on the above data. As a result, it was chosen as the starting point for all of the genetic approaches used to boost lipase production on FM1.

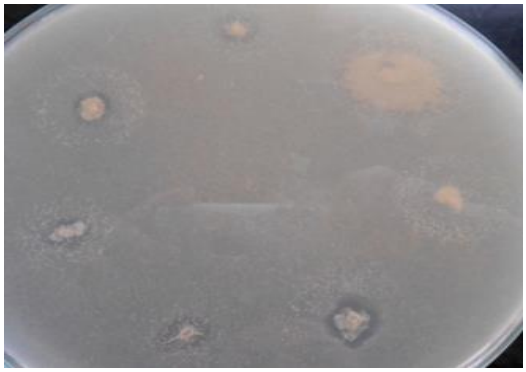

Fig. 1. The growth and lipase activity of seven bacterial strains on TBA medium after 5 days incubation.

3. 2. Identification of *Bacillus* (AMG-Kh-B2):

The 16S-primers F and R were used to amplify the area of the 16S-ribosomal RNA gene from *Bacillus* genomic DNA (AMG-Kh-B2). A product of about 1500 bp was produced after PCR amplification, as illustrated in **Fig. 2.**

The amplified 16S-ribosomal RNA gene sequence matched 99% similarity through BLAST analysis with the partial 16S-ribosomal RNA gene of *Bacillus licheniformis* strain (accession number: GQ430476.1). The strain of *Bacillus* (AMG-Kh-B2) was identified as *Bacillus licheniformis* as a result of this finding, as shown in **Fig. 3.** The isolate of *Bacillus* (AMG-Kh-B2) was identified as *Bacillus licheniformis* and submitted under accession number (MF150039).

3. Mutation induction and of lipase productivity:

When *Bacillus* (AMG-Kh-B2) was exposed to EMS for 20 minutes, the lipase production of 31 chosen mutants out of the surviving mutants was compared to the W.T. strain. (Table 2). These findings demonstrated that the majority of the mutants examined generated more lipase than the W.T. strain. Meanwhile, just three mutants, E-19, E-21, and E-29, were found to have somewhat lower lipase productive efficiency than the W.T. strain. E-26 was the greatest lipase-producing mutant, with a 237.64 percent increase in the output above the parent strain. E-9 was the next best mutant for lipase productivity, with a production rate of 234.34 percent greater than the W.T. strain.

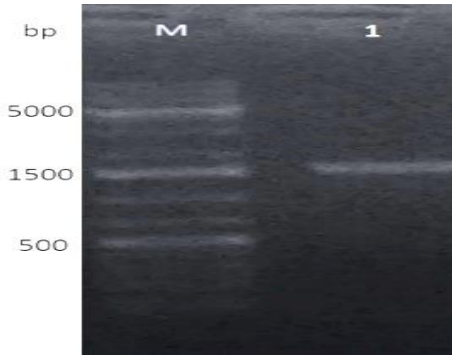

Fig. 2. Amplified band photograph of 16S-RNA for bacterial strain (AMG-Kh-B2) against DNA ladder which has three remarkable bands: 500, 1500, 5000 bp (lane M).

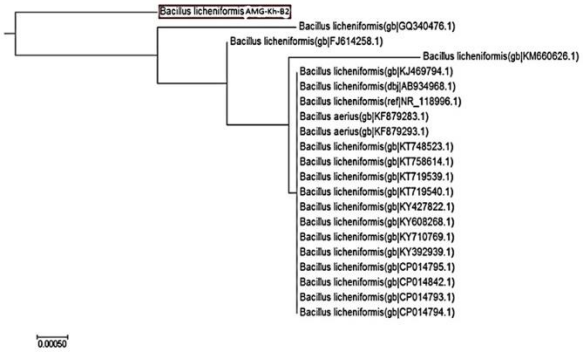

Fig. 3. Phylogenetic tree of the superior lipase (AMG-Kh-B2) strain against to the most closely related bacterial strains in GenBank.

Table (3) compares the lipase production of chosen 31 mutants to that of the W.T. strain after 40 minutes of exposure to EMS-mutagen. Only one of the examined generated lipase at a lower level than the parental strain. The mutant M-9 produced the largest amount of lipase, with 95.6 $U.mL^{-1}$ (262.64% more than the W.T. strain). Meanwhile, after the same treatment, the other mutants produced lipase that was higher than the W.T. strain but lower than the superior mutant M-9. M-27 was the next best mutant for lipase productivity, with a production rate of 254.12 percent greater than the normal strain.

TABLE 1. Effect of varied fermentation media and incubation durations on lipase production in batch fermentation of the original strains cultivated in shaking flasks.

Strain code	FM1			FM2		
	2d*	3d	5d	2d	3d	5d
AMG-Kh-B1	31.2^{d}±0.07	34.0^{c}±0.02	34.1^{c}±0.05	26.4^{c}±0.04	27.1^{d}±0.03	27.2^{f}±0.05
AMG-Kh-B2	34.5^{b}±0.08	36.4^{a}±0.04	36.2^{a}±0.04	28.5^{a}±0.05	30.2^{b}±0.04	30.0^{c}±0.11
AMG-Kh-B3	28.5^{e}±0.01	34.6^{c}±0.05	34.4±0.05	22.8^{e}±0.06	26.5^{d}±0.06	26.4^{g}±0.05
AZ-1	34.2^{b}±0.09	35.4^{b}±0.05	35.2^{b}±0.03	26.4^{c}±0.08	28.2^{c}±0.07	28.0^{e}±0.09
AZ-2	32.2^{c}±0.06	34.5^{c}±0.08	34.2^{c}±0.03	24.8^{d}±0.06	34.5a±0.08	34.2^{a}±0.06
AZ-3	35.0^{a}±0.03	36.2^{a}±0.06	36.4^{a}±0.07	27.5^{b}±0.04	30.2^{b}±0.09	31.4^{b}±0.07
AZ-4	32.5^{c}±0.14	35.4^{b}±0.03	35.0^{b}±0.04	26.7^{c}±0.1	28.8^{c}±0.07	29.0^{d}±0.06

***d=days, ** U.mL^{-1}**

TABLE 2. Lipase productivity of selected EMS-mutants of *Bacillus* obtained after 20 min.

Mutant No.	Lipase (U/mL)	Percent to W.T	Mutant No.	Lipase (U/mL)	Percent to W.T
W.T	36.4^{h}±0.12	100.00	E-16	38. 6^{g}±0.07	106.04
E-1	56.2^{l}±0.06	154.40	E-17	38.2^{g}±0.03	104.95
E-2	71.5^{e}±0.07	196.43	E-18	53.8^{o}±0.04	147.80
E-3	48.7^{q}±0.05	133.79	E-19	34.6^{h}±0.07	95.05
E-4	70.2^{f}±0.08	192.86	E-20	62.5^{j}±0.06	171.03
E-5	64.5^{i}±0.05	177.20	E-21	34.5^{h}±0.05	94.78
E-6	55.8^{m}±0.05	153.30	E-22	74.5^{d}±0.05	204.67
E-7	62.5^{j}±0.06	171.03	E-23	46.5^{f}±0.06	127.75
E-8	48.7^{q}±0.08	133.79	E-24	54.7^{n}±0.07	150.27
E-9	85.3^{b}±0.09	234.34	E-25	52.2^{p}±0.10	143.41
E-10	74.5^{d}±0.10	204.67	E-26	86.5^{a}±0.04	237.64
E-11	38.5^{h}±0.07	105.77	E-27	71.5^{e}±0.03	196.43
E-12	53.8^{o}±0.05	147.80	E-28	55.8^{m}±0.08	153.30
E-13	75. 9^{c}±0.08	208.52	E-29	33.2^{h}±0.05	91.21
E-14	65.3^{h}±0.04	179.40	E-30	66.5^{g}±0.06	182.69
E-15	58.5^{k}±0.07	160.71	E-31	45.5^{f}±0.07	125.00

*** W.T.**

TABLE 3. Lipase productivity of selected EMS-mutants of *Bacillus* obtained after 40 min.

Mutant No.	Lipase (U/mL)	Percent to W.T	Mutant No.	Lipase (U/mL)	Percent to W.T
W.T	36.4^{i}±0.12	100.00	M-16	63.8^{k}±0.05	175.27
M-1	41.3^{h}±0.04	113.46	M-17	84.5^{d}±0.09	232.14
M-2	45.5^{g}±0.03	125.00	M-18	54.7^{o}±0.11	150.27
M-3	62.5^{l}±0.04	171.03	M-19	48.5^{q}±0.06	133.24
M-4	74.7^{g}±0.06	150.27	M-20	76.5^{f}±0.07	210.16
M-5	45.5^{g}±0.08	125.00	M-21	53.8^{p}±0.05	147.80
M-6	67.2^{j}±0.05	184.62	M-22	72.4^{h}±0.03	198.90
M-7	67.8^{i}±0.08	186.26	M-23	45.5^{h}±0.05	125.00
M-8	84.5^{d}±0.09	232.14	M-24	54.7^{o}±0.06	150.27
M-9	95.6^{a}±0.03	262.64	M-25	84.5^{d}±0.12	232.14
M-10	54.7^{o}±0.06	150.27	M-26	56.2^{n}±0.08	154.40
M-11	47.8^{g}±0.08	131.32	M-27	92.5^{b}±0.06	254.12
M-12	77.7^{e}±0.10	213.46	M-28	59.5^{m}±0.08	163.46
M-13	45.5^{g}±0.07	125.00	M-29	34.6^{i}±0.05	95.05
M-14	43.5^{gh}±0.03	119.51	M-30	84.7^{c}±0.06	232.69
M-15	54.7^{o}±0.06	150.27	M-31	47.2^{h}±0.02	129.67

Finally, compared to the untreated parent strain, the results in Table (4) shows that lipase production of 31 chosen mutants increased when *Bacillus* (AMG-Kh-B2) was exposed to EMS for 60 minutes. Only two of

the examined mutants generated lipase at a lower unit than their W.T strain, according to the findings. S-22 was the highest lipase producer mutant, with a production rate of 226.65 percent greater than the parent strain. S-23 was the next best mutant for lipase productivity, with a production rate of 221.15% greater than the normal strain.

The results were consistent with those of **Ashwini et al. 2013 [35],** who isolated *Bacillus subtilis* from the Chilli rhizosphere, capable of expressing three mycolytic enzymes (chitinase, glucanase, and cellulase), and altered it with the EMS-mutagen. The antifungal potential of mutants was tested using a dual plate assay against *Colletotrichum gloeosporioides*, the causative agent of anthracnose disease in fruit crops. On NA plates, the EMS-treatments produced 60 isolated mutants, three of which showed loss of antagonism against *C. gloeosporioides* and six mutants showed enhanced antagonism due to increased activity of associated enzymes. On the other hand, **Haq et al. 2009 [36]** used EMS to enhance the strain of *Bacillus licheniformis* that produces alpha-amylase. On the basis of clear zones of starch hydrolysis, twenty-eight isolates were chosen. Only one isolate, *B. licheniformis* EMS-20040, had enzyme activity of 102.78±2.01 U/ml/min. Using a *Pseudomonas* mutant produced by UV, HNO_2, and NTG, **Caob and Zhanga [37]** observed a 3.25-fold increase in lipase synthesis. **Ellaiah et al. 2002 [38]** also found a 200 percent rise in lipase output by an *Aspergillus niger* mutant after UV and NTG treatments .The strain enhancement of indigenous isolate A. japonicas by induced mutations using UV, HNO_2, and NTG resulted in a 276 percent increase in lipase synthesis in the current study. Around the globe. *Reproductive biology and endocrinology*, **13** (1), 37 (2015)

Barratt C.L., Björndahl L., De Jonge C.J., Lamb D.J., Osorio Martini F., McLachlan R., Oates R.D., van der Poel S., St John B. and Sigman M., **3. 4. Molecular analysis of the four isolated strains:**

The genetic diversity of the two mutant strains on the level of DNA nucleotide sequence was observed when compared to W.T strain. Three random primers supplied from Operon Technologies were utilized to determine genetic heterogeneity among the two mutant strains against to the W.T strain. All of the primers effectively amplified particular genomic DNA fragments. To see if genetic markers may be linked to strains, researchers utilized the RAPD method. All three primers were successful in producing polymorphic and counted bands that were repeatable. In comparison to W.T strain, the polymorphism patterns of the three RAPD primers enumerated among the two mutant strains are displayed in **(Table 5 and Figs. 4-6).** The fragment patterns of RAPD fragments employing these primers with the two mutant strains in contrast to W.T strain **(Figs. 4-6 and Table 5)** revealed 29 amplified fragments that differed among the three primers employed, with 4 polymorphic fragments out of total bands, 6 monomorphic loci, and 7 unique loci. The use of primer No.1 **(Fig. 4)** with the DNA of the two mutant strains in comparison to W.T revealed the presence of six bands with sizes of 1900, 1800, 1200, 900, 500, and 350 bp for the original strain and the mutant strains, respectively (M9 and M-27). Furthermore, mutant strain-9 discovered four unique bands with sizes of 3000, 2800, 1500, and 600 bp (lane 2). In the mutant strain-27, a novel unique band with a size of 2000 bp was also discovered (lane 3). In contrast, when primer No.2 was employed **(Fig. 5)**, two amplified bands with sizes of 900 and 650 bp were identified in lanes 1 and 2. Furthermore, with mutant strain-27, no amplified bands were seen (lane 3). Finally, using primer No.3 **(Fig. 6)**, two amplified bands with sizes of 600 and 400 bp were observed in lane 3 of the mutant strain M-27.

Furthermore, neither mutant strain M-9 (lane 2) nor the original strain (lane 1) showed any amplified bands (lane 1). Using the Gel Analyzer 19.1 software, the molecular weight of each band of the isolated bacterial strains was estimated.

3. 5. Genetic linkages and phylogenetic tree produced by RAPD-analysis:

As demonstrated in, the matrices distance generated from the RAPD-data were used to construct accurate associations according to RAPD-

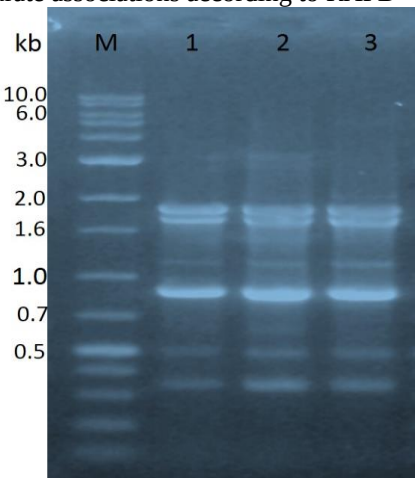

Fig. 4. DNA amplified bands based on RAPD for the two mutant strains compared to W.T strain against 100bp DNA-ladder (lane M) using primer No.1. The strains are arranged as follows: (lane 1 to 3), original (W.T), M-9 and M-27.

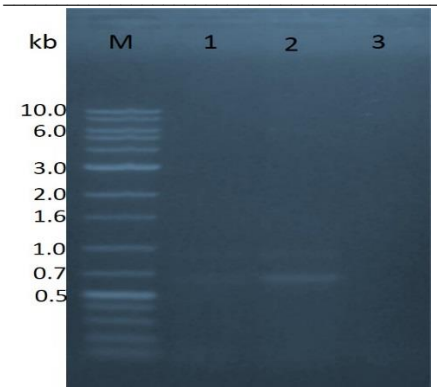

Fig. 5. DNA amplified bands based on RAPD for the two mutant strains compared to W.T strain against 100bp DNA-ladder (lane M) using primer No.2. The strains are arranged as follows: (lane 1 to 3), original (W.T), M-9 and M-27.

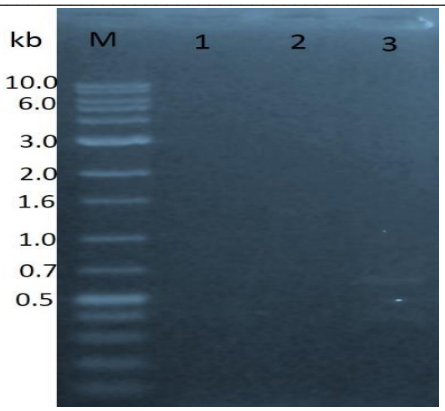

Fig. 6. DNA amplified bands based on RAPD for the two mutant strains compared to W.T strain against 100bp DNA-ladder (lane M) using primer No.3. The strains are arranged as follows: (lane 1 to 3), original (W.T), M-9 and M-27.

TABLE 4. Lipase productivity of selected EMS-mutants of Bacillus obtained after 60 min.

Mutant No.	Lipase (U/mL)	Percent to W.T	Mutant No.	Lipase (U/mL)	Percent to W.T
W.T	36.4^{x}±0.12	100.00	S-16	53.8^{o}±0.09	147.80
S-1	51.5^{p}±0.06	140.66	S-17	72.7^{e}±0.04	199.73
S-2	55.8l±0.08	153.30	S-18	44.5^{t}±0.06	122.25
S-3	45.8^{s}±0.04	125.82	S-19	62.5^{j}±0.07	171.03
S-4	65.5^{h}±0.13	179.95	S-20	70.0^{f}±0.06	192.31
S-5	77.2^{c}±0.06	212.09	S-21	43.4^{u}±0.08	119.23
S-6	56.2^{k}±0.07	154.40	S-22	82.5^{a}±0.07	226.65
S-7	48.6^{q}±0.02	133.52	S-23	80.5^{b}±0.03	221.15
S-8	64.5^{i}±0.01	177.20	S-24	34.4^{y}±0.05	94.51
S-9	75.3^{d}±0.05	206.87	S-25	54.3^{n}±0.08	149.18
S-10	47.9^{r}±0.02	131.59	S-26	55.8^{l}±0.04	153.30
S-11	39.6^{w}±0.10	108.79	S-27	62.5^{j}±0.06	171.03
S-12	56.2^{k}±0.04	154.40	S-28	45.8^{s}±0.06	125.82
S-13	65.8^{g}±0.06	180.77	S-29	40.7^{v}±0.08	111.18
S-14	36.5^{x}±0.05	100.27	S-30	34.5^{y}±0.05	97.52
S-15	65.8^{g}±0.11	180.77	S-31	55.5^{m}±0.04	152.47

TABLE 5. The overall polymorphism %, as well as the number and kinds of amplified DNA bands generated by RAPD-primers.

Primer	Total bands	Loci of polymorphic	Loci of monomorphic	Loci of Unique	Polymorphic bands	Polymorphic percentage
1	23	0	6	5	0	00.00 %
2	4	2	0	0	4	100.00 %
3	2	0	0	2	0	00.00 %
Total	29	2	6	7	4	-

TABLE 6. A proximity matrix of the superior mutants and the W.T strain according to RAPD-analysis.

Strain code	W.T	M-9	M-27
W.T	1.000		
M-9	0.800	1.000	
M-27	0.706	0.571	1.000

The genetic distance between original strain W.T and mutant strain M-9 was found to be the greatest (80%), followed by the genetic distance between original strain W.T and mutant strain M-27 (70.6%), and finally the genetic distance between mutant strain M-9 and mutant strain M-27 (70.6%). (57.1 percent).

The dendrogram according to RAPD-results split the two superior strains into two major groups, as shown in **(Fig. 7)**. The strains W.T and M-9 were found in the first cluster. M-27 mutant was found in the second cluster. In comparison to W.T, the genetic linkages between the two mutant strains and the proximity matrix that was applied to split the examined strains into groups might reflect the genetic variety of the two mutant strains. Furthermore, the dendrogram revealed that the genotypes (W.T and M-9) that were thought to be substantially connected to genetic distance were clustered together in one cluster, while the other mutant M-27 and W.T were greatly divergent and were situated in a different cluster. **Chun and Kyung (2000) [39]** determined partial gyrA gene sequences for twelve *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus licheniformis*, *Bacillus mojavensis*, *Bacillus subtilis* subsp. *subtilis*, *Bacillus subtilis* subsp. *spizizenii*, *Bacillus subtilis* subsp. *vallismortis* strains, and the strains were divided into four groups. The RAPD-assay was used by **Renders et al. (1996), De Vos et al. (1997),** and **Rajarajan et al. (2018) [40-42]** for DNA fingerprinting and phylogenetic relationship of *P. aeruginosa* strains, demonstrating its utility in the lab when combined with other protocols for detecting phylogenetic relationships of the tested strains belonging to the same species.

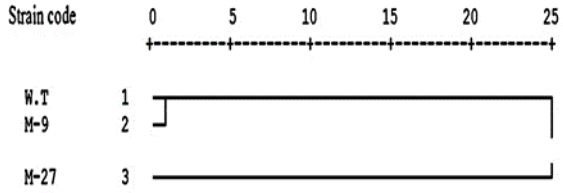

Fig. 7. Dendrogram created by Average Linkage of the superior mutants against W.T strain based on RAPD-analysis.

Conclusion:

The efficient use of edible oil wastes has a strong influence on the country's pollution levels and economy. The development of microbial strains is an essential part of the improvement process of the fermentation products. Superior strains can save costs by increasing production and possessing specific desired traits; these enhanced strains can be obtained through mutation. In this investigation, certain *Bacillus licheniformis* EMS-mutants were created. This contribution would be very great when the fat of animal by-products was efficiently utilized after treatment with lipase produced by the superior *B. licheniformis* EMS-mutants.

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