Isolation and improvement of some proteolytic enzymes (lipase, amylase and protease) produced from local Aspergillus sp.

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Isolation and improvement of some proteolytic enzymes (lipase, amylase and protease) produced from local *Aspergillus sp.*

Abstract:

Fourteen fungal isolates were isolated from soil, ten were found as *Aspergillus niger* and four *Aspergillus terreus.* Three enzymes (lipase, amylase and protease) were determined qualitatively and quantitively by chemical methods. Tow isolates from *A. niger* and *A. terreus* highly productive was genetically modified by ultraviolet (UV). From each isolate, 50 mutants were randomized obtained and qualitatively tested. Ten of them were quantitatively determined. Different response was obtained for the three enzymes. After UV irradiation lipase was the most producer, then protease showed higher production in *A. terreus* only, while production decreased in *A. niger*, while amylase it showed a slight difference in both isolates compared to the wild. Genetic relationships of the mutants of *A. niger* and *A. terreus* were analyzed with a randomly amplified polymorphic DNA–polymerase chain reaction (RAPD–PCR). Results obtained 61.22% genetic similarity (49 total bands) was calculated between parental and mutant strain of *A. niger* and 48.88% genetic similarity (45 total bands) for *A. terrues*.

Introduction

There is an increased demand for use microbial enzymes. *Aspergillus* is one of a filamentous fungus which is used for industries, such as production of extracellular enzymes. It is most commonly found in decaying vegetation, soil, or seeds. *Aspergillus niger*, the most abundant mold found in the environment, it been source of several industrial enzymes **(Schuster *et al.*, 2002).** *A. terreus*, also known as *Aspergillus terrestrius*, is a fungus found in soil worldwide. It found commonly in soil *A. terreus* is a storehouse of several industrial

enzymes (**De Vries *et al.*, 2004).** *Aspergillus* species biosynthesize a large variety of extracellular enzymes of which α-amylases are of world-wide interest in fermentation, food, pharmaceutical, textile, and paper industries **(Pandey *et al.*, 2000).** Lipases are the most widely used enzymes in bio transformations. *Aspergillus* has been described as a producer of several lipases **(Pokorny et *al.*, 1997).** Protease's enzymes that catalyze the hydrolysis of protein molecules into peptides and amino acids. Proteases constitute one of the most important groups of enzymes in industrial field, and account for at least 60% of the total world-wide enzyme sales and constitute two thirds of the total enzymes used in various industries. **(Germano *et al.*, 2003 and Özkan and Figen 2012).** For efficient enzyme production strains can be improved by mutagenesis which is successful method. This process is mostly trial and error process **(Iftikhar *et al.*, 2010)**. For improved yield of enzymes production from different fungi by treating it with chemicals such as ethyl methyl sulphonate (EMS), N – methyl – N – nitro – Nnitrosoguanidine (MNNG) and (HNO_2) nitrous acid **(Guerola *et al.*, 1971).** Sub-lethal concentration of mutagens in fungus can increases the rate of enzyme production **(Rudravaram *et al.*, 2003)** Ultraviolet (UV) and gamma (γ) irradiations can also be used to obtain mutants yielding higher enzymes production from of thermophilic fungi **(Witkin 1969).**

The present study was concerned to isolation different isolates of *Aspergillus sp.,* improvement by random mutagenesis (UV Irradiation) and genetic identification for *Aspergillus* strains highly producers for protease, lipase and amylases and studying the characteristics of the different enzyme. Present study uses the molecular method of random amplification of polymorphic DNA (RAPD) to study the environmental sources of Aspergillus species.

MATERIALS AND METHODS

Shake flask studies: Twenty-five milliliter of the fermentation media containing (g/l) Starch 10 $MgSO_4.7H_2O$ 0.005, $CaCl_2.2H_2O$ 0.2, $FeSO_4$ 0.1, $(NH_4)_2SO_4$ 2, was transferred to separate 250 ml conical flasks. The flasks were sterilized in an autoclave for 15 min and cooled at room temperature. One milliliter of inoculum was transferred to each flask. The flasks were placed in the orbital shaking incubator for incubation at 30°C with shaking speed of 200 rpm. After 72 h of incubation, content of flasks was filtered and filtrate was used for the estimation of enzyme activity while the residue was used for the estimation of cell mass. All the experiments were run parallel in triplicates.

Fungal isolation and identification: Fungal isolation was done by the dilution plate method, similar to the methodology used by other authors. **(Horn and Dorner 1998).** Briefly, each soil sample (10 g) was diluted with 90 ml of 1 g l^{-1} peptone water and kept at room temperature (25 ± 2 °C) for approximately 20 min. The suspension was then shaken and diluted by decimal dilution to 10^{-2} and 10^{-3}. A 0.1 ml aliquot of each dilution per sample was spread on the surface of two solid media, dichloran/rose Bengal/chloramphenicol (DRBC) and dichloran/glycerol 18% (DG18), in triplicate **(Pitt and Hocking 1997)**. The plates were incubated in darkness for 5–7 days at 30 °C. At the end of the incubation period the average number of colonies (triplicate) was determined and the results were expressed as colony–forming units per gram (cfu g^{-1}) of soil. The identification of *Aspergillus* section *Flavi* was done according to Pitt and Hocking**. (Pitt and Hocking 1997).**

Ultraviolet (UV) irradiation: From the parental fungal isolate (5 days old culture), 1ml of the conidial suspension was added to a cotton wool plugged Erlenmyer flask containing 25ml of sterilized medium. The conidia were allowed to grow at 30°C on

a shaker incubator with 200 *rpm* for about 6 h to get fresh growing fungal mycelia. Five milliliter of the medium containing mycelial suspension was poured to a sterilized Petri plate and these mycelia were exposed to ultraviolet (UV) irradiation for 5-40 minutes under the beam (λ=253 nm and 220 V at 50 c/s) of UV lamp. (**Azin and Noroozi, 2001).**

Qualitative estimation of lipase: The preliminary screening of lipolytic fungi was carried out using Tween 80 agar plates and the phenol red staining technique after isolation using the serial dilution process **(Singh *et al.*, 2006).**

Qualitative estimation of amylase: The isolates were tested for amylolytic activity after being cultured on starch agar medium. Using the I2/KI reagent, the clearance zone established around the colonies was examination. **(Olakusehin *et al*.,2021)**

Qualitative estimation of protease: A plate assay using skim milk agar plate was used to screen the fungal isolates for extracellular protease activity (**Abdel Galil 1992).**

Quantitative estimation of lipase: Lipase was produced by Submerged Fermentation (SmF) using medium based on **(Adham and Ahmed 2009).** The medium consisted of peptone 3%, MgSO4.7H2O 0.05%, KCl 0.05%, K2HPO4 0.2%, olive oil 0.5% and glucose 0.5%. Fifty ml medium was placed in 250 ml flask sterilized by autoclave at 1 atm, 121℃ for 15 min. The sterile media in Erlenmeyer flasks were inoculated with spore or hypha suspension **(Lima *et al*., 2019**). Each isolate inoculated into 4 flasks. The flasks were incubated at room temperature with 200 rpm shaking for 96 h. Lipase activity was assayed based on colorimetric method **(Kwon 1986).** One hundred ml of filtrate was mixed with 1 ml substrate olive oil for 20 min at 30℃. The reaction was stopped by placing the mixture in the ice bath for 5 min. One hundred ml of sample from top layer (organic phase) of previous mixture was then added to 1900 ml

isooctane and 400 ml Cupric-Acetate Pyridine (CAP) reagent. The mixture was homogenized with vortex for 5 sec and allowed to stand for 20 min until the aqueous phase (bottom layer) and organic phase (top layer) separated. Absorbance value of the organic phase was then measured at 715 nm. **(Ilmi *et al*., 2017)**

Quantitative estimation of amylase: Five gram of substrate (Cassava) was taken into a 250 ml Erlenmeyer flask and to this a fungal amylase production containing (g/l) KH2PO4 – 1.4; NH4NO3 – 10; KCl – 0.5; MgSO4.7H2O – 0.1; FeSO4.7H2O – 0.01; starch – 20 gm; and distilled water was added to adjust the require moisture level. The contents of the flaks were mixed thoroughly and autoclaved at 121°C for 20 min. Solid state fermentation was carried at 30°C with substrate initial moisture content of 64% for 72 hours using 2 ml *Aspergillus niger* suspension as inoculum.

Enzyme activity assay the digestive activity of alpha-amylases is measured via several colorimetric methods, including dinitrosalicylic acid method (DNS), Nelson–Somogyi, and Iodine method. **(Ahmed *et al.*,2015)** In iodine method, Lugol interacts with starch and forms complexes and alpha-amylase degrades starches and reduces UV absorbance at 580 nm. **(Karim *et al.*, 2018)** Starch solution 1% prepared in 0.1 M phosphate buffer (pH 7.0) add to 1 ml of crude enzyme mixed and incubated at 50 C for 30 min, sop reaction by adding 1ml 1 M HCL ,1 ml of iodine reagent (5 mM 12- and 5-mM KI).

Quantitative estimation of protease: The fungal isolates were inoculated in 100 mL of protease specific fermentation broth containing (%, w/v): yeast extract 1.0, MgSO4 0.02, glucose 2.0, K2HPO4 0.1, pH 7.0. Flasks were incubated at 28 ˚C for 5-6 days. At the end of incubation, the contents of flasks were filtered through Whatman filter paper No. 1 and then the filtrates were centrifuged at 8,000 rpm at 4˚C for 10 minutes. Pellet was discarded after centrifugation and clear supernatant was used as

source of protease enzyme. The supernatant of crude enzyme was further used for subsequent studies **(Josephine *et al*., 2012).** To measurement of enzyme activity Protease activity in the crude enzyme extract was determined according to the method of **(Carrie Cupp-Enyard 2008)** by using casein as substrate. Five mL of 0.65% casein solution was added in test and blank tubes and test tubes were placed at 37 ˚C for 5 minutes. One mL of enzyme solution was added in test tube. It was mixed properly and incubated at 37 ˚C in a water bath for 30 min for allowing the enzymatic reaction to occur. The reaction was terminated by addition of 5 mL of Trichloroacetic acid (TCA) solution in both test and blank tubes. One mL of enzyme solution was added in blank test tube only and it was allowed to stand for 15 minutes at room temperature. Solution from both test tubes was filtered using Whatmann's No 1 filter paper. Two mL of test and blank filtrate were taken in two new test tubes and labeled as test (T) and blank (B). Five mL of sodium carbonate was added in both test tubes followed by addition of 1 mL of 2-fold diluted Follin phenol reagent. The resulting solutions in both test tubes were incubated in dark for 30 minutes at room temperature for the development of blue color. The absorbance of the blue color compound was measured at 660 nm.

DNA extraction: Aspergillus mycelial mass was harvested from the 12 to 24 hr fungal liquid cultures, filtered and purified. The genomic DNA was extracted by glass beads and phenol-chloroform method, (a solution of 1mM EDTA, 1% SDS, 100mM NaCl, 10 mM Tris-HCl and 2% Triton X-100, in distilled water, pH 8.0 was used as lysis buffer). The extracted DNA was checked by using 1.5% agarose gel electrophoresis **(Rath *et al., 2002*).**

PCR for identification: The PCR assay was performed using 5 µl of the DNA template in a total reaction volume of 25 µl

(consisting of PCR buffer [20 mMTris- HCl at pH 8.0], 50 ml KCl, 0.1mM) using six RAPD primers **Table (1)**. The reactions were performed in a thermo cycler (XP Cycler, BIOER, China). Thermal program included an initial DNA denaturation at 95°C for 5 min that followed by 30 cycles, consisting of the stages; denaturation at 95°C for 30 sec, annealing at 36°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min following the last cycle. The DNA fragments were length separated by electrophoresis through 1.5% agarose gels in Tris Borate EDTA (TBE) buffer and 0.50 mg of ethidium bromide per ml. Results were documented by using a UV tarns illuminator (Syn Gene SYDR4/ 680X, UK) **(Mirhendi *et al.*, 2007).**

Table (1): List of the primer names and their nucleotide sequences used in the study for RAPD procedure:

	Name	Sequence
1	OP-C3	5´ GGG GGT CTT T 3`
2	OP-C9	5´ CTC ACC GTC C 3`
3	OP-C15	5´ GAC GGA TCA G 3`
4	OP-D1	5´ ACC GCG AAG G3`
5	OP-D9	5´CTC TGG AGA C 3`
٦	OP-K2	5` CAC GAG TCT C 3`

Results and Discussion

Isolation of Aspergillus: fourteen fungal isolates were isolated from soil and identified according to Pitt and Hocking**. (Pitt and Hocking 1997**). Of the fourteen isolates, ten were classified as *Aspergillus niger* and four *Aspergillus terreus*. The isolates were coded after being defined with AN for isolates of *Aspergillus niger* and took numbers from 1 to 10. Whereas the

isolates of *Aspergillus terreus*. are coded with AT and taken numbers from 1 to 4.

Production of enzymes: Three enzymes (lipase, amylase and protease) were determined qualitative and quantitative, all the examined strains of *Aspergillus sp*. After five days of incubation, slow growth and produce cnidogenesis beginning in the centre of colonies were observed in all cultures except strains. After five days of incubating the strains were tested for their ability to produce lipase, a discrepancy was found in the amount of its analysis of olive oil for quantitively and tween 80 for qualitatively. The best strains were *A. niger* AN10 of *A. terreus* AT3. These isolates showed in **Table (3)** the highest activity of amylase. *A. niger* AN1, AN5 &AN8 and *A. terreus* AT1 showed moderate activity. *A. niger* AN2, AN4& AN6 and *A. terreus* AT4 showed low activity. No lipase activity was observed in isolates *A. niger* AN3, AN7 and AN9 showed in **Table (2).**

All *Aspergillus sp.* showed activity for starch analysis, and in order, *A. niger isolate* AN10 had the highest activity, then *A. niger* AN2, AN3, AN4, *A. terreus* AT1, AT2, and AT3 then *A. niger* AN1and AN9 then *A. niger* AN5 respectively. All strains showed protease enzyme activity except for the strain *A. niger* AN6. As it is clear from **Table (2),** the best strain producing the three enzymes at a distinct level are *A. niger* AN10 and *A. terreus* AT3.

Table (2): Qualitative screening of *A. niger* and *A. terreus* isolates for lipase, amylase and protease activity.

	Enzyme → Isolate ↓	Lipase	Amylase	protease
A. niger isolate	AN 1	++++	++	+
	AN 2	+++	++++	+
	AN 3	-	++++	+++
	AN 4	+++	++++	+++
	AN 5	++++	+	++++
	AN 6	+++	+++	-
	AN 7	-	++	++
	AN 8	++++	++++	+++
	AN 9	-	++	+
	AN 10	+++++	+++++	++++
A. terreus isolate	AT 1	++++	+++	++++
	AT 2	++	+++	++
	AT 3	+++++	++++	++++
	AT 4	++	+++	+++

Table (3): Results quantitative to lipase, amylase and protease activity from Aspergillus niger and Aspergillus terreus isolates.

	Isolates	Lipase (U\ml)	Amylase(U\ml)	Protease(U\ml)
A. niger isolate	A.N 1	0.60	1.16	0.14
	A.N 2	0.47	0.84	0.04
	A.N 3	0.73	1.02	0.07
	A.N 4	0.69	0.88	0.37
	A.N 5	0.70	1.13	0.48
	A.N 6	0.63	0.99	0.34
	A.N 7	0.49	0.95	0.91
	A.N 8	0.56	0.93	1.07
	A.N 9	0.79	1.12	0.87
	A.N 10	0.91	1.16	1.11
A. terreus	A.T 1	0.87	0.85	0.32
	A.T 2	0.37	0.90	0.36

isolate	A.T 3	0.92	0.99	0.33
	A.T 4	0.39	0.64	0.48

UV mutagenesis: In the present study an attempt was made to improve the yield of enzymes production from highest isolates *Aspergillus niger* (A.N10) and *Aspergillus terreus* (A.T3) by induction of UV mutation 50 mutants were randomized obtained from each isolate and qualitatively tested. Highly ten mutants of them were quantitatively determined. The results of average of three replicate of each mutant that revealed improved production of enzymes as compared to the parental showed it in Table (4). The production was described of lipase, amylase and protease. When the fungus was exposed type to UV light for induction of variation, it was found that it had a significant effect, as the rate of enzyme activity in some mutants increased and others decreased. The maximum increasing was 1.85 in mutant A. terreus (AT 3 M.T15) with lipase, 1.47 in mutant A. niger (A.N 10 MN30) and 2.79 in A. terreus (AT 3 M.T28).

For strain improvement, induction of mutation by UV is the Main effect of this physical mutagen is to modify the structure of pyrimidine causing the formation of pyrimidine and mainly thymine dimmers which distort the structure of DNA heliex and block the further replication process **(Sambrook and Russell 2001)**. In most cases UV mutation are very harmful but sometime it may lead to better adaptation of an organism to its environment with improved biocatalytic performance. **(Sandana *et al.*, 2001).** According to **(Agrawal *et al.*, 1999)** UV radiation was a potent mutagen. UV irradiation was found to be best for the improvement of strains like *Aspergillus niger* for maximum production of various enzymes **(Kang *et al.*, 1999).**

Table (4): Enzymes activity for A. Niger and A. terreus isolates after induction of mutations exposure of different interval times of UV irradiation.

		Lipase (U\ml)	Amylase (U\ml)	Protease (U\ml)	rate of increases		
A. niger (A.N 10)	Wild	0.92	0.96	1.106	Lipase	Amylase	Protease
	M.N16	1.32	1.01	0.09	1.43	1.05	0.08
	M.N18	1.36	0.89	0.08	1.48	0.93	0.07
	M.N25	0.97	0.8	0.14	1.05	0.83	0.13
	M.N30	0.6	1.41	0.66	0.65	1.47	0.60
	M.N34	1.37	0.94	0.08	1.49	0.98	0.07
	M.N35	0.91	0.85	0.65	0.99	0.89	0.59
	M.N41	1.16	0.92	0.02	1.26	0.96	0.02
	M.N44	0.95	0.91	0.05	1.03	0.95	0.05
	M.N47	0.94	0.86	0.61	1.02	0.90	0.55
	M.N49	1.42	0.85	0.53	1.54	0.89	0.48
A. terreus (AT 3)	Wild	0.91	0.92	0.33			
	M.T9	0.99	0.74	0.55	1.00	0.80	1.67
	M.T14	1.06	0.75	0.36	1.07	0.82	1.09
	M.T15	1.83	0.84	0.42	1.85	0.91	1.27
	M.T23	1.62	0.93	0.75	1.64	1.01	2.27
	M.T28	1.05	0.73	0.92	1.06	0.79	2.79
	M.T30	0.94	0.88	0.76	0.95	0.96	2.30
	M.T34	1.17	0.99	0.66	1.18	1.08	2.00
	M.T35	1.34	0.91	0.59	1.35	0.99	1.79
	M.T47	1.24	0.84	0.76	1.25	0.91	2.30
	M.T50	1.7	0.83	0.7	1.72	0.90	2.12

Genetic variability studies

The genomic DNA of the parental isolates AN10 and AT3 and four of their best mutational isolates (from *Aspergillus Niger* (M.N18, M.N30, M.N41 and M.N49) and *Aspergillus Terreus* (M.T9, M.T23, M.T35 and M.T50)) were identified using RAPD-PCR technique, to study the genetic differences

between two isolates of *Aspergillus* species and their best 4 resulting mutants. A total 6 primers were employed (OP-C3, OP-C9, OP-C15, OP-D1, OP-D9, OP-K2). Fieger (1)

Data generated from PCR analysis based on the RAPD PCR assay showed genetic polymorphism between the isolates. The first isolate *Aspergillus niger in* Table (5), amplified 49 bands, 30 bands were polymorphic with average (3-8) polymorphic bands. the OP-D1 primer produced 8 polymorphic bands, which considered the highest percentage of polymorphism (80%), Meanwhile, the Op-D9 primer generated 3 polymorphic band, that consider the least polymorphic percentage (42.85%). Moreover, there were 19 monomorphic bands, whereas primer OP-C3, OP-C9 and OP-D9 primers generated the highest percentage of monomorphic band. In addition, there were 18 unique bands, where Primer OP_C9, and OP-C15 has the pic part. which were almost generated OP-C15 in sample (A.T "parental", M.T49), when mutant (M.T 49) most of samples appeared unique bands in primers OP-C9, OP-15 and OP- D13.

Table (5): Total bands, polymorphic bands and unique bands of each RAPD primers that observed in parental isolates AN10 and AT3 and four of their best mutational isolates A. niger (M. N18, M. N30, M. N41 and M. N49) and A. terreus (M.T9, M.T23, M.T35 and M.T50)

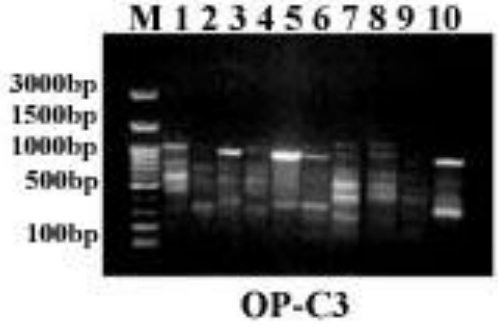
	Primer Name	Total Band	Monomorphic Band	Polymorphic Band	Unique Band	Polymorphic %
A. niger	OP-C3	9	4	5	3	55.55%
	OP-C9	8	4	4	4	50%
	OP-C15	8	2	6	4	75%
	OP-D1	10	2	8	3	80%
	OP-D9	7	4	3	2	42.85%
	OP-K2	7	3	4	2	57.14%
	Total	49	19	30	18	61.22%
A. terreus	OP-C3	9	3	6	3	33.33%

	OP-C9	6	4	2	2	33.33%
	OP-C15	7	4	3	1	42.85%
	OP-D1	7	1	6	3	85.71%
	OP-D9	9	4	5	4	55.55%
	OP-K2	7	2	5	2	71.42%
	Total	45	23	22	15	48.88%

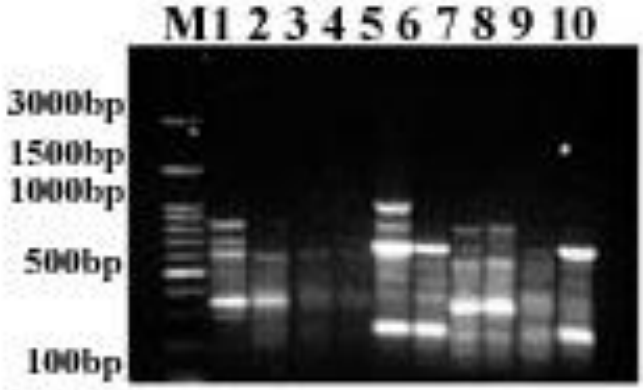
The RAPD molecular marker profile of *Aspergillus terreus* isolate and their best resulting mutants was different, all six RAPD primers generated 48.88% of polymorphism and the highest percentage of polymorphism was recorded 85.71% and 71.42% by OP-D1 and OP-K2 primers, respectively. The highest percentage of monomorphism were shown in equal proportion in OP-C9, OP-C15 and OP-D9. the least percentage of polymorphism was recorded by OP-C9 primer, but in monomorphism was recorded by OP-D1. the highest number of bands was generated by OP-C3 and OP-D9, it amplified nine bands. Four unique bands were generated in primer OP-D9 in sample A.T and M.9 it high produced in lipase and protease. Whereas the least number of bands were generated by primer OP-C9, showed in Table (5).

RAPD is a powerful tool to study variation and relatedness between species **(Zhang et al., 2009).** Random primers have been successfully applied to differentiate isolates of *A. niger* and other strains worldwide **(Yoshimitsu 2010).** It is therefore suggested that RAPD assay is sufficient to differentiate the wild and the mutant strains which are low and high enzyme producing strains, respectively. In future, these polymorphic DNA fragments can be utilized for converting into sequence characterized amplified regions (SCARs), which would help to overcome the concern of lack of reproducibility of the RAPD assay. RAPD analysis can be utilized efficiently **(Zhang et al., 2009)** to differentiate wild or mutant cells, intra-specific and

inter-specific strains of organisms **(Yoshimitsu 2010)** in developing countries where complex facilities are not available. However, reproducibility in amplification of RAPD markers is a major concern **(Zhang *et al.*,2009).** Random primers have been successfully applied to differentiate isolates of *A. niger* and other strains worldwide **(Zhang *et al.*,2009– Yoshi *et al.*, 2010).** This technique was capable to readily differentiate between parent and mutant strain **(online supplementary data Table 1)** and production pattern of the tested strains. Both strains with some primers showed a correlation of genetic variability with enzyme production. Similar RAPD markers were also used to verify genetic diversity and phenotypic correlation in other studies **(Zhang *et al.*,2009 and Yoshi *et al.*, 2010).** In this study 61.22% genetic similarity (49 total bands) was calculated between parental and mutant strain of *A. niger* and 48.88% genetic similarity (45 total bands) for *A. terrues*. It is therefore suggested that RAPD assay is sufficient to differentiate the wild and the mutant strains which are low and high enzyme producing strains.



OP-C3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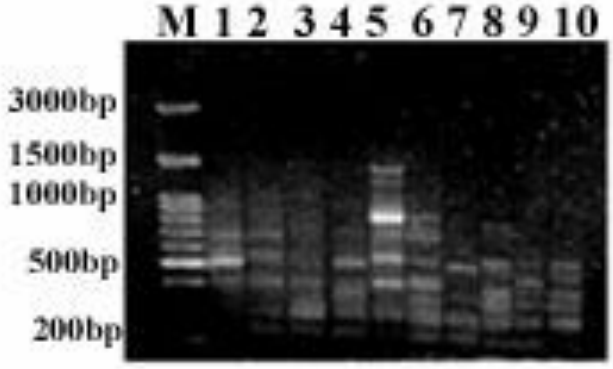
OP-C15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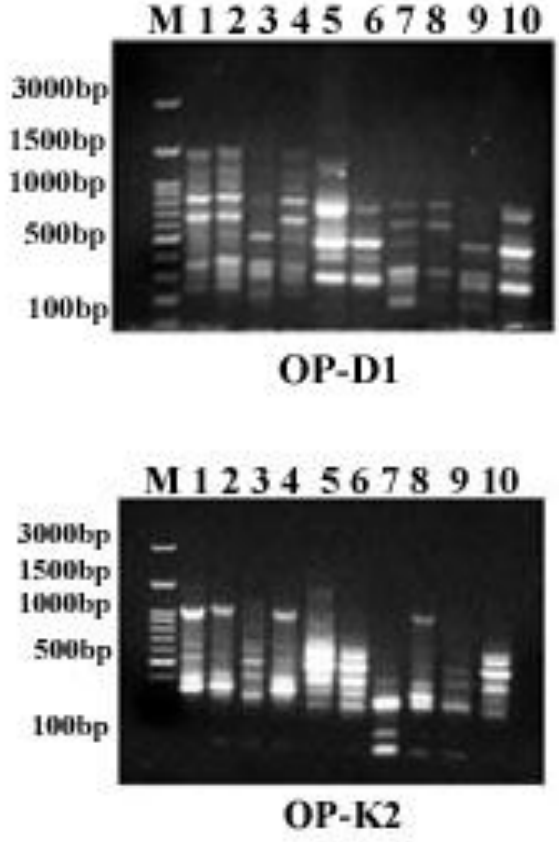

Figer (1): Agarose gel electrophoresis with ethidium bromide of RAPD-PCR reaction 6 primers *Aspergillus niger* (parental isolates (lane 1), M.N18 (2), M.N30(3) , M.N41(4) ,M.N49(5)) *Aspergillus terreus* (wild isolate(6), M.T9(7), M.T23(8),M.T35(9) ,M.T50(10))and M (ladder)

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