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Production of Lipase by Certain Soil Fungi. I: Optimization of Cultural Conditions and Genetic Characterization of Lipolytic Strains of *Aspergilli* Using Protein Patterns and Random Amplified Polymorphic DNA (RAPD)

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Abstract: Filamentous fungi isolated from soil were screened for their ability to produce extracellular lipase. Among ten filamentous fungi tested, two strains identified as *Aspergillus niger* and *Aspergillus terreus* were selected as the highest lipase producer. Maximum lipase production was obtained in 5 days cultures utilized 0.4% (w/v) corn oil as a carbon source. Optimum pH for crude lipase production by the tested fugal strains was 6.0, while L-glutamic acid as a nitrogen source gave the highest lipase production. PCR-RAPD analysis of genomic DNA using 5 primers (OPB-05, OPB-17, OPA-09, OPA-12, OPA-13) showed that DNA of genus *Aspergillus* was characterized by the presence of 14, 10, 14, 4 and 3 fragments in case of primer OP-05, OPB-17, OPA-09, OPA-12 and OPA-13 respectively. All these fragments could be considered as *Aspergillus* specific fragments because they have been detected with both species. Results obtained from the comparison between two species of *Aspergillus* showed differences in sizes and numbers of amplified fragments per primer for each species. DNA extracted from *Aspergillus niger* species was characterized by having 3 special fragments more than *Aspergillus terreus* with primer OPB-05, one fragment more with primer OPB-17, two fragments more with primer OPA-12 and 4 fragments with the primer OPA-13. Thus, some bands were found to be associated with both fungal strains, but some others were specific for *Aspergillus niger*. Analysis of protein bands showed that some bands were specific to the tested *Aspergilli* and other more specific to *Aspergillus niger*. It was concluded that the excess of DNA fragments through both the PCR-RAPD reaction and bands of protein patterns of *Aspergillus niger* could be responsible for their highest activity of lipase enzyme.

Key words: Lipase, fungi, PCR-PAPD, genetic characters, culture conditions

Introduction

The production of lipase by microorganisms is apparently important from the economic and industrial standpoints (Arnold *et al.*, 1975; Mohawed *et al.*, 1985; Mutua and Akoh, 1993). Fungi are the important enzyme producers since their enzymes are produced extracellularly (Borgstrom and Brockman, 1984; Venkateshwarlu and Reddy, 1993; Hang and Woodams, 1990; Ferreira Costa and Peralta, 1999). The production of lipase by fungi was found to be affected by many factors such as pH (Ammar and McDaniel, 1984; Elgamal and El-Sheikh, 1989; Abd-Alla, 1999; Ferreira Costa and Peralta, 1999), temperature (Ferreira Costa and Peralta, 1999; Venkateshwarlu and Reddy, 1993), carbon source (George *et al.*, 1999; Gao and Breuil, 1995) and nitrogen source (Chander *et al.*, 1980; Alford and Pierce, 1963; George *et al.*, 1999).

Concerning the effect of oil and fat on lipase production by fungi, it was found that addition of butter oil, corn oil or olive to the cultivation medium was inhibitory of lipase production by *Penicillium roqueforti* (Eitenmiller *et al.*, 1970). Tributyrin, tricaprylin, butter and olive caused reduction in activity of *Penicillium chrysogenum* lipase (Chander *et al.*, 1980). On the other hand, corn oil was also reported to the best substrate for different microbes such as *Thermoactinomyces vulgaris* and *Aspergillus sydowi* (Mohawed, 1983).

Regarding to the fungi species, which produce lipase, are differentiated from each other by microscopic morphology. However, such methods do not have the ability to differentiate between *Aspergillus* perfectly. Accordingly, a proper technique will be needed for differentiation between the two species of *Aspergillus (A. niger, A. terreus)* easily and perfectly. Therefore, this work was directed to use RAPD analysis (random amplified polymorphic DNA) (Welsh and McClelland, 1990; Williams *et al.*, 1990) which is considered a sensitive and quick technique to distinguish the differences between such *Aspergillus* spp.

Molecular markers can be definitive in confirming the correctness of any morphological taxonomic system and provide an additional tool to characterize fungal genotypes. Manicom *et al.* (1990), reviewed the potential used of molecular markers in the identification and taxonomy of filamentous fungi.

The rise in DNA technology has opened several new avenues of investigation. The utility of DNA-based diagnostic markers is determined to a large extent by the technology that is used to reveal DNA-based polymorphisms. Restriction fragment length polymorphism (RFLPs) can potentially provide a large number of genetic markers (Michelmore and Hlbert, 1987).

RAPD-PCR could be considered as powerful tool to reveal extensive DNA polymorphism between the genomes of different species under the same genus. Keinath *et al.* (1995) used the RAPD technique to distinguish between *Phoma cucurbitacearum*, the causal agent of gummy stem blight of cucurbits and other *Phoma* spp.

RAPD markers can obtain measurement of genetic relatedness and genetic variation within and between natural fungal populations. McDermott *et al.* (1994) used RAPD markers to detect genetic variations of *Erysiphe graminis* f.sd. Hordei isolated collected from through out the Europe and demonstrated that these markers segregated in specific crosses. They reported that PCR was suitable to detect genetic variation in populations of obligate fungal pathogens where the difficulty of collecting tissue imposes constraints on the number of individuals sampled.

The present study aimed to select some lipoplytic fungi isolated from fertile soils in Dakhlia regions. Optimum cultural conditions for lipase production (pH, incubation periods, corn oil concentration and nitrogen sources) and genetic characterization of selected most active lipolytic isolates of *Asperilligi (Aspergillus niger, Aspergillus terreus)* were studied. The last characterization was achieved by detected and analyzed the electrophoretic gel of both protein and RAPD-PCR genomic DNA extracted from the two selected fungal species.

Materials and Methods

Organisms: All fungal strains tested for lipase activity were isolated from fertile soils in different regions at Dakhlia Governorate and identified according to the dilution plate method as described by Moubasher and Abdel-Hafez (1978).

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Lipase production medium: This medium was prepared according to Elwan *et al.* (1977). It contained (%, w/v): tributyrin emulsion, 0.2; $NaNO_3$, 0.2; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; $FeSO_4.7H_2O$, 0.001; distilled water up to 1L. The pH was adjusted at 6.8 before autoclaving. The medium was distributed in 50 ml portions in Erlenmeyer flasks (250 ml capacity). Flasks were sterilized at 121°C for 15 min. After cooling, flasks were inoculated with standardized 5 ml aliquots spore suspension from 7 days old stock fungal cultures and incubated at 30°C for 7 days under static conditions. Three flasks were used for each treatment and the lipase (s) produced were assayed at the end of the incubation period.

Lipase assay: The tributyrin clearing zone (T.C.Z.) technique as mentioned by El-Sheikh (1982) was applied for assaying all lipolytic activities expressed by $\mu g\ ml^{-1}$ for lipase concentration. The clear zone obtained in each test (mm) was translated to mg lipase per ml using a standard curve. In this assay 0.2% tributyrin was emulsified and added to 2% agar (Difico) for solidification. pH was adjusted at 8.8 using tris buffer. Holes were made in each plate using sterile cork-borer. Equal amounts (0.1 ml) of the enzyme were plated in each hole and plates were incubated immediately at the required temperature for 6 h. Diameters of clear zones were measured by an accurate scale. Standard curve using assaying pancreatic lipase (Merck) was prepared as follows: 0.1 ml of each concentration of the enzyme was transferred to a hole as mentioned above and the clear zones were measured after 6 h of incubation at 30°C. Logs of concentrations were plotted against clear zone diameters in mm.

DNA extraction: The extraction of DNA from two isolates of *Aspergillus* spp. were performed according to the method given by Lee and Taylor (1990) with some modifications. The mycelium of each isolate was harvested and filtered from a colony growing on a PDA liquid media, mycelium was ground in liquid nitrogen mixed with buffer. DNA was extracted and purified according to the CTAB method described by Nicholson *et al.* (1996). UV spectrophotometer and following gel electrophoresis estimated DNA concentration, by comparison with DNA standard (USA). Gels were viewed under UV light on a 'Gel Doc 1000' system and analyzed using molecular analyst software (Bio-Rad, Hercules, CA)

SDS polyacrylamide gel electrophoresis: Electrophoresis was carried to the method described by Hams (1981). The total protein was estimated and the SDS polyacrylamide gel coomassie blue ($0.5\ g\ l^{-1}$) was extraction of protein was carried out with fresh of both *Aspergillus* spp. used for staining. Destined gels were photographed while wet and the protein patterns were analyzed quantitatively using computerized gel documentation system (SDS).

Table 1: The nucleotide sequences and guanine-cytosine (GC) ratio of the five primers used for RAPD-PCR analysis

Primer code	Sequences	%GC
OPB-05	TGCGCCCTTC	60
OPB-17	AGGGAACGAG	60
OPA-09	GGGTAACGCC	60
OPA-12	TCGGCGATAG	60
OPA-13	CAGCACCCAC	60

Extraction of genomic DNA: Genomic DNA was extracted according to the method described by Doyle and Doyle (1987). DNA isolated from 1g of mycelia 3 weeks old of both species were used for PCR amplification. Five primers (Operon Tech. Inc., Kit A and B, USA) were used (Table 1). A total volume of 50 μl was used for PCR reaction which contained: 100 mg DNA, 50 pmol primer, 200 M dNTP (dATP, dGTP, dCTA and dTTP), 3Mm $MgCl_2$ and 0.5 unit Taq polymerase T-gradient PCR thermal cycler (Biometra-Germany) was used for the DNA amplification. Thermal conditions were 6 min at 94°C, 2 min at 36°C and 3 min at 72°C) and finally 10 min at 72°C (post-extension).

Results and Discussion

Screening of lipolytic fungal isolates: Nine fungal species obtained from the collected soil samples were screened for lipase production. The data shows that all the tested strains were capable of producing lipase but in varying degrees (Fig. 1). The most favorable species for lipase production were *A. niger* followed by *A. terreus*, *A. fumigatus* and *Fusarium moniliform*. On the other hand, the least productive organisms of lipase were *Penicillium chrysogenum* and *Alternaria alternata*. These findings agree with those reported by previous investigators. Ogundero (1981) stated that *A. fumigatus* and *A. nidulans* were able to degrade vegetable oils and triglycerides. Mohawed *et al.* (1985) pointed out that 24 strains of *Aspergillus*, which were isolated from Egyptian soils, showed lipolytic activity and the most active was *A. niger* (Abd-Alla, 1999). The difference in lipase production not only occurred within the different genera but also within the

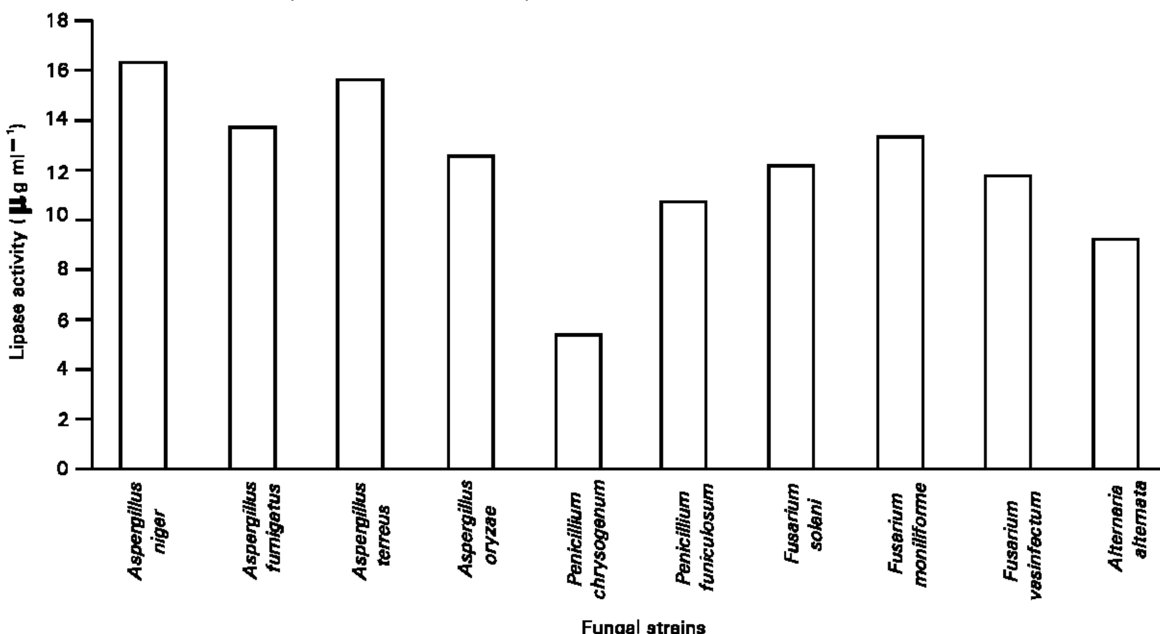

Fig. 1: Lipase activity of fungal strains isolated from Egyptian soil samples

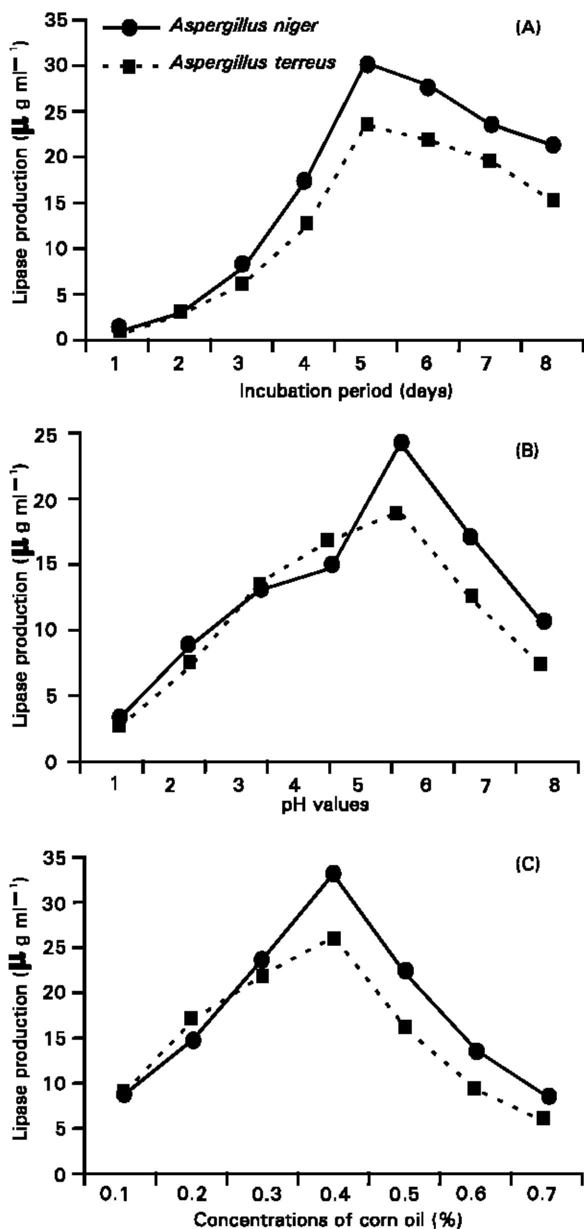

Fig. 2: Effect of different incubation periods (A), pH values (B) and concentrations of corn oil © on lipase production by the tested fungal strains

different species of the same genus (Fig. 1). Based on the above-mentioned results, cultural optimization and genetic characterization were restricted on the most active species (*A. niger, A. terreus*) for lipase production.

Cultural conditions

Effect of incubation periods: Results show that as incubation period progressed the lipase production increased reaching its maximal value after 6 days. Afterwards, the lipase activity decreased by increasing the fermentation period (Fig. 2a). Elwan *et al.* (1978) found that *Thermoactinomyces vulgaris* produced its maximal yield of lipases after 48–72 h. The maximal yield of lipases production by *Penicillium roqueforti* (Eitenmiller *et al.*, 1970),

Table 2: Results of total protein SDS-PAGE profile as indicated by using computer gel documentation system (Gel Pro V 3.0) for *Aspergillus niger* and *Aspergillus terreus*

Mol. wt	*A. niger*	*A. terreus*
132.67	3.64	
114.99	2.26	2.76
101.42	2.38	3.58
85.53	4.00	4.85
71.59	3.02	4.50
67.94	2.41	2.80
52.07	3.67	5.33
49.18	3.05	4.79
45.24	3.09	
40.71	3.47	3.52
38.69		3.37
36.22	3.24	4.77
35.17	2.12	
32.72	1.73	1.94
30.71	2.35	1.85
27.38	1.41	0.84
26.39	1.46	0.47
24.34	2.89	
23.41	2.57	3.41
22.72	4.09	1.77
21.52	2.35	1.34
18.27	3.69	2.31
15.68	2.43	2.13
12.61	4.59	3.76
10.03	4.98	1.91
Total	24.00	21.00

Saccharomyces lipolytica (Jonson and Snygg, 1974) was achieved within 2–7 days. In this connection, Abd-Alla (1999) indicated that *Aspergillus niger* produced relatively lipase yields at pH 5-6 with a maximum at pH 6.0 after 5 days of incubation period.

Effect of different pH values: Increasing the pH range till it reaches its maximal value at 6, increased the activity of the tested isolates for lipase production (Fig. 2b). As the pH increased over 6, lipase production decreased. These results agree those obtained by many investigators who claimed that optimal pH value for lipase production by microorganisms varied. *Penicillium roqueforti* produced the maximal lipase yield at pH 5.5 (Eitenmiller *et al.*, 1970), while the maximal yield of lipase was produced at pH 9.5 by *Saccharomyces lipolytica* (Jonson and Snygg, 1974). In addition, Ferreira Costa and Peralta (1999) reported that the optimum pH for crude lipase activity produced by *Penicillium wortmanii* was 7.0.

Effect of different concentrations of corn oil: Seven concentrations of corn oil (v/v) 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 were chosen to elucidate the best of them which is suitable for maximal lipase production. Maximal lipase production was attained at 0.4% concentration of corn oil by *A. niger* (32.70 μg ml^{-1}) and *A. terreus* (25.65 μg ml^{-1}). Corn oil was selected as a lipid source based on the previous investigators (El-Gamal and El-Sheikh, 1989; El-Sheikh, 1982; Mohawed, 1983) who reported that corn oil was the best lipid substrate for lipase production by different microbes. In this connection, our results are in agreement with those reported by Mohawed *et al.* (1985) and Elgamal and El-Sheikh (1989) who found that the optimum concentration of both oil and fats ranged to 0.2-0.8% for lipase activity of various microbes. These results also declare that the variation of lipolytic activity of different microorganisms was actually due to the concentration of the lipid source and the nature of the respective organism.

Effect of different nitrogen sources: Results revealed that KNO_3 was the inorganic nitrogenous compound, which exerted the highest lipase yield (Fig. 3). This result is in agreement with those obtained by Mohawed *et al.* (1988) who found that KNO_3 was the best inorganic nitrogen source for lipase production by *Aspergillus fumigatus*. On the other hand, the use of organic nitrogen

Table 3: Relative concentration of DNA fragments detected by the PCR-RAPD of genomic DNA extracted from two *Aspergillus* spp. Shown in Fig.1. Data were identified by using 5 primers OPB-(5 and 17) and OPA-(9,12 and 13) and analyzed by the computerized gel documentatin system

	OPB-05		OPB-17		OPA-09		OPA-12		OPA-13	
Mol. wt	*A. niger*	*A. terreus*	*A. niger*	*A. terreus*	*A. niger*	*A. terreus*	*A. niger*	*A. terreus*	*A. niger*	*A. terreus*
6557	0.125									
4686	4.089									
4361			0.92		1.04	0.95				
3557	4.34				14.41	9.27				
3491							2.59			
2900									0.47	
2847										
2795	14.94	2.35								
2322			3.18	0.96						
2287					20.26	20.82				
2186	3.7	0.21								
2153							8.44	4.39		
2089									2.52	
2027			8.27	2.72	8.92	9.99				
1891							33.82	22.68		
1839	11.15	8.72	15.99	13.75						
1691							12.38	5.68		
1599					14.01	16.1				
1513							11.71	4.23		
1471	11.16	15.04							8.08	
1372					1.52	4.55				
1353			2.015	1.39	11.27	10.75				
1223	1.63	3.6								
1208										
1163									6.01	
1092	0.24	0.31								
1078			10.98	19.24	0.19	0.28				
1013										
1000					12.08	7.91				
928					6.42	9.66				
917	1.92	1.8								
905										
872			8.32	9.61	0.22	0.15				
860									31.17	26.45
778			8.78	6.97						
735							0.93			
695	9.78	19.82								
666			10.75	8.97						
657					0.299	0.039				
647									23.97	37.32
629	0.013	0.24								
603					1.32	2.97				
499	20.08	19.63								
466					0.003	0.21				
453			21.18	18.13						
441									4.56	0.45
424	5.12	8.49								
407			5.53	6.6						
370	0.049	0.089								
319	1.028	3.01								
263	2.68	6.56								

compounds revealed that urea and peptone were less available compounds, while L-glutamic acid was the most available to the two fungal isolates for high lipase production. In this connection, suitability of nitrogen sources to lipase biosynthesis differs according to different microorganisms. Ammonium ions were found to increase lipase production when used as nitrogen source by *Penicillium roquefori* (Goa and Breuil, 1995), but it inhibited completely lipase production by *Pseudomonas fluorescens* (Cutchins *et al.*, 1952). Elgamal and El-Sheikh (1989) who found that both DL-aspartic acid and L-glutamic acid were prominent nitrogen sources for biosynthesis of lipase in accordance with those report our results by *Rhizobium Japonicum*. On the other hand, Mohawed *et al.* (1985) found that L-arginine or DL-serine was the best nitrogen source for lipase production by *Aspergillus anthiceus*.

Molecular results

SDS polyacrylamide gel electrophoresis: Electrophoretic profile of their proteins was detected (Fig. 4, Table 2) to distinguish between two *Aspergilli* (*Aspergillus niger*, *Aspergillus terreus*). Scanning of the SDS-PAGE gel indicated the occurrence of about 19 protein bands with molecular weights ranging from 114.99 to 10.03 Kda in proteins extracted from each of both *Aspergillus* spp. These bands were fixed in both, it could indicate that these bands are a characterize to the genus of *Aspergillus*. There were several proteins bands appeared in the extract of *Aspergillus niger* and disappeared in *Aspergillus terreus*. These bands were 132.67, 45.24, 35.17 and 26.39 Kda. These bands could be considered as a specific bands of *Aspergillus niger* species because they have not been detected with *Aspergillus terreus* and it could be responses for increasing high yield of lipase with *A. niger* more than *A. terreus*.

Detection of PCR products: To elucidate genetic markers of two *Aspergillus* spp. (*Aspergillus niger*, *Aspergillus terreus*), five arbitary primers were used for the PCR-RAPD analysis of genomic

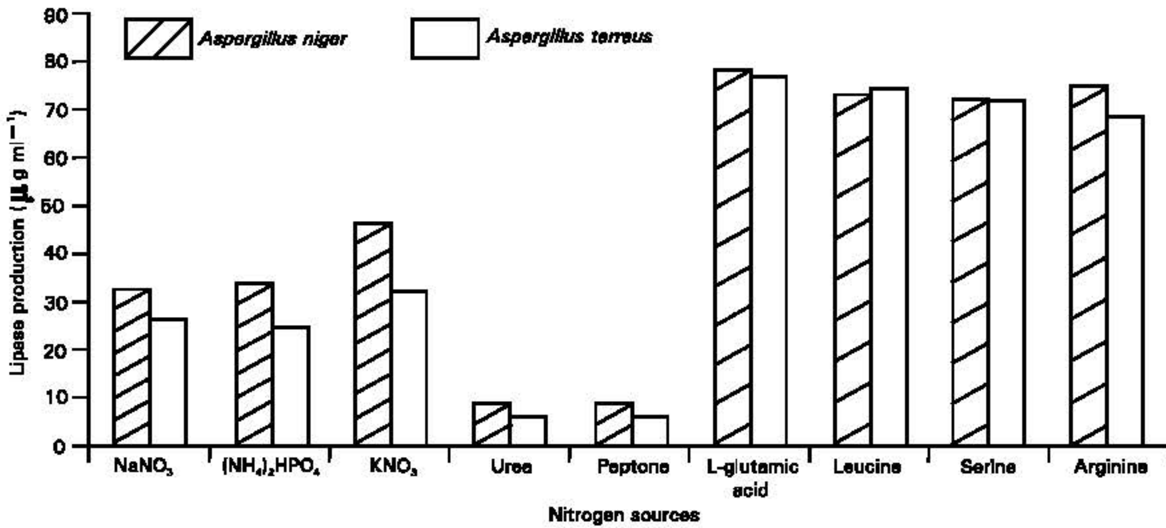

Fig. 3: Effect of different nitrogen sources on lipase production by the tested fungal strains

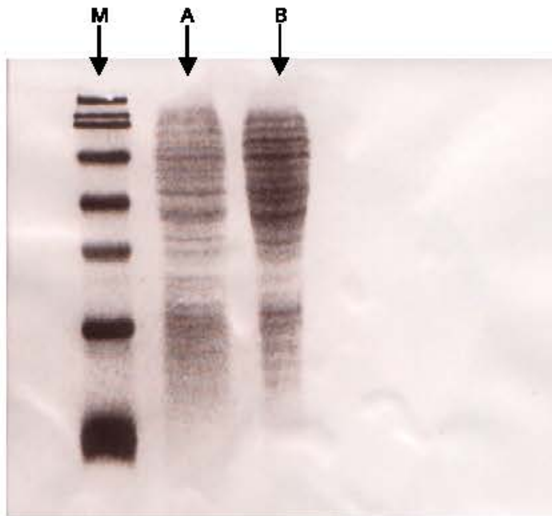

Fig. 4: SDS-PAGE of proteins from *Aspergillus niger* (A), *A. terreus* (B) and M = genetic markers

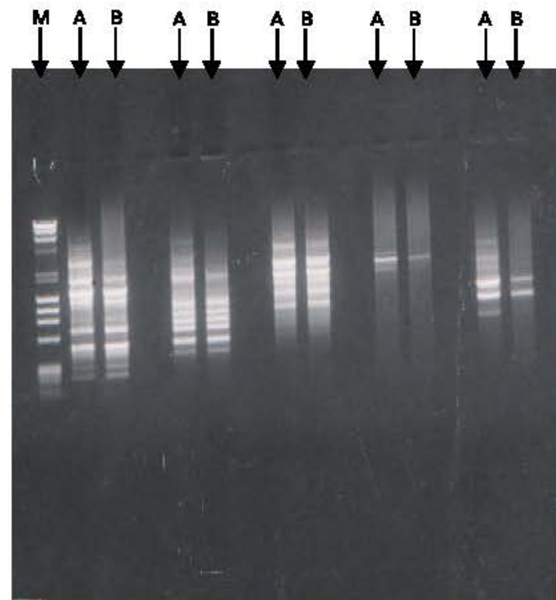

Fig. 5: PCR-RAPD of genomic DNA extracted of *Aspergillus niger* (A) and *A. terreus* (B). DNA fragments were detected by using 5 primers: OPB-05, OPB-17, OPA-09, OPA-12 and OPA-13. M = genetic markers

DNA isolated one week old mycelia (Fig. 5, Table 3). Analysis of DNA extracted of both species showed major fragments having base pairs at 2795, 2186, 1839, 1471, 1223,1092, 917, 695, 629, 499, 424, 370, 319 and 263 with the primer OPB-05; 2322, 2027, 1839, 1353, 1078, 872, 778, 666, 453 and 407 with the primer OPB-17;4361, 3557, 2287, 2027, 1599, 1372, 1353, 1078, 1000, 928 , 872, 657, 603 and 466 BP with the primer OPA-09; 2153, 1891, 1691, 1513 BP with the primer OPA-12 and 860, 647 and 441 BP with the primer OPA-13. These fragments could be considered as a specific for *Aspergillus* as agenus because they were detected in the DNA isolated from *Aspergillus niger* and *Aspergillus terreus*. DNA extracted from *Aspergillus niger* showed additional 3 fragments having sizes at 6557, 4686 and 3557 BP which was not detected with *Aspergillus terreus*. Moreover, several fragments that present in DNA isolated from mycelium of *Aspergillus niger* at 6557, 4686 and 3557; 4361 with primer OPB-17; 3491 and 735 BP with OPA-12 and 2900, 2089, 1471 and 1163 BP with primer OPA-13 and disappeared with *Aspergillus terreus*.

The RAPD data can detect genetic diversity between related species (Harvey and Botha, 1996) and also within species (Van Oppen *et al*., 1996) and capable of detecting differences between interesterility group (Crowhurst *et al*., 1991; Garbelotto *et al*., 1993) and individuals (Aufauvre-Brown *et al*., 1992).

From the above results, it was concluded that among all soil fungi tested for lipase production, *Aspergillus niger* and *A. terreus* were selected as the highest lipase producers. Maximum lipase production was obtained in medium containing 0.3% (w/v) corn oil, L-glutamic acid as a nitrogen source at pH 6.0 after 5 days of incubation period. In this connection, *A. niger* was the most efficient for lipase production rather than *A. terreus*. These results could be attributed to the genetic differentiation (DNA fragments and protein bands) between both species.

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