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Preliminary study on soil microbiota degrading plant latices and their protease and lipase enzymes

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Abstract. The present study aimed to isolate soil microorganisms-degrading plant latices and to screen their capablilities of producing protease and lipase. Many bacterial and fungal strains of mesophilic, thermotolerant or thermophilic nature were able to grow on sugar-free medium amended with crude latex of either *Euphorbia pulcherrima, Ficus elastica* or *F. nitida* and incubated at 25ºC, 36ºC or 45ºC. This growth revealed that these growing microorganisms were able to make use of the crude latex as a sole carbon source. The total mesophilic bacterial propagules recovered on mineral salt medium amended with F. elastica latex was much higher than those recovered on media amended with either *E. pulcherrima* or *F. nitida* latex. Some of the mesophilic bacteria formed clear zones around their colonies (some *Streptomyces* spp. recovered on *Ficus elastica* and *F. nitida* latices) while the others showed no clear zones. Also one thermophilic and another thermotolerant species related to the genus *Bacillus* were isolated on media amended with the three plant latices. The total numbers of mesophilic fungal propagules recovered on medium amended with either *Ficus* latex were nearly equal, but higher than that amended with *E. pulcherrima* latex. Thirty-one species of fungi were recovered on the three latex media. Of mesophiles, *Aspergillus* followed by *Fusarium, Penicillium* and *Cladosporium* were the most common fungi, while the remaining fungi were less frequent. Three truly thermophilic species, *Humicola grisea* var. *thermoidea, Malbranchea cinnamomea* and *Myceliophthora thermophile*, were isolated. All mesophilic bacterial isolates, except one related to *Streptomyces* sp., were capable of producing protease and lipase, while from the thermophilic/thermotolerant bacterial isolates tested, only one *Bacillus* isolate could produce both enzymes and the other two isolates of *Bacillus* could not. The thirty mesophilic fungal isolates tested were capable of producing lipase, while 25 only were protease producers. On the other hand, the eleven thermophilic fungal isolates tested were capable of producing protease, while 8 only were lipase producers.

Key words: Degradation, lattices of *Ficus* and *Euphorbia* spp., soil fungi and bacteria, production of lipase, protease.

Introduction

Over 2000 species of plants belonging to different families (Euphorbiaceae, Asclepiadaceae, Moraceae, Asteraceae, Caricaceae, Cannabinaceae and Apocyanaceae) can produce latex and natural rubber (Archer and Audley 1973 and Lewinsohn 1991). In addition, at least two fungal genera, *Lactarius* and *Peziza* were also found capable of producing latex and natural rubber with low molecular weights (Ohya and Tanaka 1998).

Latex is the cytoplasm of highly specialized cells known as laticifers, and is produced and stored in the tubular structures known as vessels. The latex vessel is located mainly in the secondary phloem of the trunk, and is intermingled with a sieve tube, although latex is also present in the flowers, fruits and leaves. The average composition of latex milk is as follows (wt/wt): 25 to 35 % polyisoprene, 1 to 1.8 % protein, 1 to 2% carbohydrates, 0.4 to 1.1% neutral lipids, 0.5 to 0.6% polar lipids, 0.4 to 0.6% inorganic components, 0.4% amino acids, amides, etc., and 50 to 70% water (Subramanian 1995). Within the latex milk, natural rubber (NR) occurs in the form of particles as an emulsion of droplets with a predominant size of 0.1 to 2 mm in diameter in water (Pendle and Swinyard 1991). These rubber particles are covered by a layer of proteins and lipids, which separate the hydrophobic rubber molecules from the hydrophilic environment.

Investigations of bacterial degradation of NR revealed two groups of NR-degrading bacteria according to their strategy for substrate utilization. (i) Members of the first group form clear zones on latex overlay-agar plates, indicating an extracellular enzyme activity. Representatives belong to the genus *Streptomyces* (Rose *et al.* 2005, Ibrahim *et al.* 2006 and Bröker *et al.* 2008). (ii) Members of the second group (non-forming clear zone) exhibit adhesive growth with direct contact of the cells with the NR material and extensive disintegration of the substrate. Representatives belong to the genera *Gordonia*, *Mycobacterium*, *Streptomyces* and *Nocardia* (Linos *et al.* 1999, 2002).

This work aimed to isolate and identify soil microorganisms (bacteria and fungi) which have the ability to degrade and metabolize plant

latices of *Euphorbia pulcherrima*, *Ficus elastica* or *Ficus nitida* when added to the carbon-free medium and to assess the ability of these microorganisms to produce protease and lipase enzymes that may enable them to grow on and metabolize such latices.

Materials and Methods

Collection of soil samples

Fifty samples of cultivated soils were collected from different sites in Assiut Governorate, Egypt.

Plant Latices used and their sampling

Latices chosen for this study were obtained from *Euphorbia pulcherrima* Willd. ex Koltzsch (family Euphorbiaceae), *Ficus elastica* Roxb. ex Hornem and *Ficus nitida* Thunb (family Moraceae). Their natural rubber is cis-1, 4 polyisoprene. Crude latices from the above plants growing in the farms of the Faculties of Science and Agriculture, University of Assiut were collected from plants by the tapping method (Wititsuwannakul and Wititsuwannakul 2001). They were obtained in sterile Eppendorf tubes by cutting leaves of *E. pulcherrima* or injuring trunk in case of *F. elastica* and *F. nitida*, thereafter the latex was drawn by sterile disposable syringe.

Media used for isolation of bacteria and fungi degrading crude latex

Sugar-free mineral salt medium (MSM) for bacteria (Heisey and Papadatos 1995) of the following composition (g/l); K_2HPO_4 8, KH_2PO_4 1, $(NH_4)2SO_4$ 0.5, $MgSO_4.7H_2O$ 0.2, NaCl 0.1, Ca $(NO_3)_2$ 0.1, $CaCl_2.2H_2O$ 20 mg, $FeSO_4.7H_2O$ 20 mg, $Na_2MoO_4.H_2O$ 0.5 mg, $MnSO_4$ 0.5 mg, yeast extract 30 mg, agar (for the bottom layer) 20, agar (for the soft top layer) 7, pH 7.5. Sterile crude latex was added at a rate of 2 ml/l of autoclaved soft agar medium.

Sugar-free Czapek agar medium (SFCZ) for fungi (Lynch *et al.* 1981) of the following composition (g/l): $NaNO_3$ 3, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.5, KCl 0.5, agar (for the bottom layer) 20 and agar (for the soft top layer) 7, pH 7. Sterile crude latex was added at a rate of 2 ml/l of autoclaved soft agar medium.

Isolation procedure of bacteria and fungi degrading crude latex

Bacteria and fungi were isolated on sugar-free agar media described above. Overlay technique was used (Braaz *et al.* 2004). About 20 ml of bottom agar media were poured into each of three replicates of sterile Petri-dishes, then overlayed with vortexed soft agar media (0.7%) containing a proper dilution volume of soil suspension plus crude latex (20 µl / 10 ml soft agar). After solidification, the plates were incubated for 15 days at 36ºC and 45ºC for bacteria and 25ºC and 45ºC for fungi. The developing bacterial and fungal colonies were counted, isolated and identified. Pure cultures were deposited in the Culture Collection of Assiut University Mycological Center (AUMC).

Identification of microorganisms

Bacterial isolates were identified on the basis of their phenotypic characters according to Bergey's Manual (2005). Gram staining, motility (Rhodes 1958), catalase test (Wittenberg 1964) and cytochrome C oxidase (Ewing and Johnson 1960) were carried out. Fungal isolates were identified based on their macro- and microscopic features following the keys of Raper and Fennell (1965), Moubasher (1993), Domsch *et al.* (2007), and Salar and Aneja (2007).

Enzyme production

Protease production: Bacterial and fungal proteolytic ability was tested using casein hydrolysis media, medium of O'reilly and Day (1983) for bacterial isolates and medium of Paterson and Bridge (1994) for fungal isolates. The media were sterilized by autoclaving at 121ºC for 15 minutes. The cooled medium was then poured into 9 cm Petri-dishes (~20 ml/plate). The inoculated plates were incubated at 36ºC and 45ºC for three days in case of bacteria and 25ºC, and 45ºC for 48 hours in case of fungi. After incubation complete degradation of milk protein was seen as clear zone around the colony. The diameters of the clear zone and fungal colony were measured (in mm). Enzyme index for caseinase (PI) was expressed according to Ho and Foster (1972) as follows:

Enzyme index = Diameter of the outer limit of the clear zone/ Diameter of the fungal colony.

Lipase production: Bacterial and fungal lipolytic capability was determined using the medium of Ullman and Blasins (1974) with slight modification. Tween 80 (sorbitan polyoxyethylene monooleate) was added instead of tween 20. The medium was dispensed aseptically in test tubes. The inoculated tubes were incubated for 48 hours at 36ºC and 45ºC in case of bacteria and at 25ºC and 45 ºC in case of fungi. The enzyme production was detected by measuring the depth of white precipitate formed at the top of the medium, due to the formation of crystals of the calcium salt of the oleic acid liberated by the enzyme.

Results and Discussion

Diversity of soil mesophilic bacteria recovered on carbon-free agar medium amended with crude latex

The total propagules of mesophilic bacteria recovered from the 50 soil samples at 36ºC on mineral salt medium amended with *Euphorbia pulcherrima* latex ranged from 16.66 to 150 colony-forming units (CFUs) per mg soil, however in 10 out of 50 soil samples bacteria were missing on this medium (Table 1). On medium amended with *Ficus elastica* latex, mesophilic bacteria ranged from 8.33 to 135 CFUs per mg soil and in 4 soil samples bacteria were missing. On medium amended with *Ficus nitida* latex, their counts ranged from 5 to 116.66 CFUs per mg soil, however in 7 soil samples no bacteria were recovered. It was noticed that the mean total bacterial propagules recovered on mineral salt medium amended with *Ficus elastica* latex was much higher than those encountered on media supplemented with either *E. pulcherrima* or *F. nitida* latex (Table 1). This may reveal that *E. pulcherrima* and *F. nitida* latex may impose some inhibitory compounds on soil bacteria.

Total mesophilic bacteria recovered from the 50 soil samples at 36°C on media amended each with one of the three types of latices of *E. pulcherrima*, *F. elastica* or *F. nitida* accounted for 523, 636.65 and 388.99 CFUs per mg soil, respectively. The percentage counts of Gram-positive and Gram-negative bacteria were 68.26% and 31.74%, 82.83% and 17.17%, and 80.72% and 19.28% on the above latices respectively (Table 2). It was clear that the percentage CFUs of Gram-positive bacteria was more than that of Gram-negative bacteria.

The Gram-positive and Gram-negative bacteria emerged from 74 %, 50 %; 92 %, 48 %; 68 % and 40 % of the soil samples on media amended with the latices of the three plants respectively (Table 2).

Two *Streptomyces* spp. formed clear zones around their colonies and these were isolated from *F. elastica* latex (one with white colour and the other with grey colour) accounting for 27.9% and 16.61% of the total propagules, and emerging from 86% and 68% of the samples. The third *Streptomyces* sp. with buff colour did not form clear zone and was isolated on the latices of *F. elastica* and *F. nitida* accounting for 25.83% and 53.2% of the total propagules, and emerging from 84% and 68% of the samples, respectively.

In this respect, Heisey and Papadatos (1995) isolated 14 isolates of bacteria from soil on pure natural rubber (pale crepe rubber) that had been surface coated as a thin film on mineral salts agar. Ten isolates were identified as species of *Streptomyces*, *Nocardia* and *Amycolatopsis*. This demonstrates that these bacteria can use the hydrocarbon of natural rubber as well as crude latices as a sole source of carbon and energy (Heisey and Papadatos 1995). Rubber (latex of *Hevea brasiliensis*)-degrading actinobacteria were also recorded from different ecosystems (soil, fresh water and its bottom sediments) by Rifaat and Yosery (2004). These were, in order of dominance, *Streptomyces griseus*, *S. rochei*, *S. coelicolor*, *S. halstedii*, *Micromonospora aurantiaca*, *Actinoplanes italicus*, *Gordonia* sp. and *Nocardia* sp. *Streptomyces* strains degrading natural rubber and forming clear zones on mineral salts agar media containing latex as a sole carbon source were also reported from soil in Germany (Gallert 2000 and Rose *et al.* 2005). They stated that the ability of microorganisms to degrade natural rubber and form translucent halos around their colonies depends on their capability of producing extracellular enzymes. On the other hand, other bacteria could grow on natural rubber latex overlaying agar without showing clear zones around their colonies, e.g. *Pseudomonas aeruginosa* (Linos *et al.* 2000), which exhibited strong rubber-decomposing properties. Also, a *Pseudomonas* sp. was isolated from soil sample through spread plate method on fresh natural rubber latex-agar medium (Roy *et al.* 2006).

Diversity of soil thermophilic and thermotolerant bacteria recovered on carbon-free agar medium amended with crude latex

The total propagules of thermophilic (or thermotolerant) bacteria recovered from 20 soil samples at 45ºC on medium amended with *E. pulcherrima* latex ranged from 0 to 35.33 CFUs per mg soil (Table 1). On medium amended with *F. elastica* latex, counts of soil thermophilic (or thermotolarent) bacteria ranged from 0 to 21.33 CFUs per mg soil and the mean was 8.43 CFUs. In case of *F. nitida* latex, their numbers ranged from 0 to 31.33 CFUs per mg soil. It is worthy to mention that in 6 out of 20 soil samples these bacteria were missing on the three latices. The total numbers of thermophilic and thermotolerant bacteria and percentages of occurrence recovered from the 20 soil samples at 45°C on the latices of *E. pulcherrima*, *F. elastica* and *F. nitida* were 112, 145 and 93.7 CFUs per mg soil respectively (Table 2).

Two species of Gram-positive thermophilic bacteria related to the genus *Bacillus* (one species grew well at 65°C and the other at 55°C) were recovered from 40%-70% of the soil samples on the latices of the above plants (Table 2). In this respect, Ibrahim *et al.* (2006) isolated thermophilic bacteria able to degrade natural

rubber from soil samples collected from Egypt, Germany and Cambodia. These bacteria were identified as *Actinomadura nitritigenes*, *Nocardia farcinica* and *Thermomonospora curvata.*

Diversity of soil mesophilic fungi recovered on carbon-free agar medium amended with crude latex

The total numbers of propagules of mesophilic fungi recovered from 50 soil samples at 25ºC on sugar-free medium supplemented with *E. pulcherrima* latex, ranged from 0 to 53 CFUs per mg of soil, however 2 soil samples were fungi-free on this medium. On the media supplemented with *F. elastica* and *F. nitida*, the counts of fungi ranged from 0.66 to 45 CFUs and 0.33 to 56 CFUs per mg soil respectively (Table 2).

The highest number of CFUs on medium amended separately with the three latices was recorded in a soil sample cultivated with *Triticum aestivum* and possessed the highest pH value (8.4) and nearly the lowest moisture content (3.6%) (data not shown). It was also noticed that the mean total CFUs recovered on medium amended with the latex of the two species of *Ficus* were nearly similar, however higher than those recorded on medium amended with *E. pulcherrima* latex. This indicates that latter latex may impose some antifungal compounds toxic to the soil mycobiota.

The results presented in Table (2) also revealed that 17 genera represented by 27 species were isolated on sugar-free Czapek agar medium amended with latex of either *E. pulcherrima* (21 species related to 14 genera), *F. elastica* (23 species related to 14 genera) or *F. nitida* (25 species related to 11 genera). From these fungi, *Aspergillus* (represented by 7 species) was the only fungus isolated in high frequency on the three media. From *Aspergillus* only *A. niger* and *A. terreus* were isolated in high frequency on the three media. *A. flavus, A. fumigatus, A. sydowii* and *A. ustus* were also isolated on the three media but in low frequency (except *A. ustus* in moderate frequency on *F. elastica* latex). *A. versicolor* was isolated also in low frequency but only on media amended with both *Ficus* species latices. In this respect, Roy *et al.* (2006) isolated an unidentified *Aspergillus* strain from a soil sample through spread plate method on latex-agar medium.

Fusarium (represented by 2 species) was encountered in high frequency on the latices of *E. pulcherrima* and *F. elastica* and in moderate frequency on *F. nitida* latex. *F. solani* was the most common species. It was isolated in moderate frequency on the three latices, while *F. verticillioides* was isolated in moderate frequency only on *E. pulcherrima* latex and in low frequency on both *Ficus* latices. On the other hand, *Penicillium* and *Cladosporium* (2 species each) were isolated in moderate frequency on the three latices in the following order: *P. chrysogenum* > *C. cladosporioides* > *C. sphaerospermum* > *P. aurantiogriseum.* In this respect, attempts to grow *Fusarium solani* upon vulcanized rubber tyres were reported by Faber (1972). Also, Borel *et al.* (1982) isolated some fungal strains from soil samples and deteriorated tyres and rubber by plate cultures on a mineral medium amended with powdered natural rubber and these were identified as *Fusarium solani, F. oxysporum, Cladosporium cladosporioides, C. sphaerospermum, Mucor hiemalis, M. plumbeus, Penicillium variabile, P. vinaceum, Paecilomyces lilacinus* and *Phoma eupyrena*.

Other remaining fungi (14 species) were isolated less frequently as shown in Table (2). Atagana *et al.* (1999) isolated five fungal species from natural rubber waste serum and these included *Mucor racemosus*, *Mucor* sp., *Rhizopus* sp, *Aspergillus niger* and *Aspergillus* sp.

Diversity of soil thermophilic and thermotolerant fungi recovered on carbon-free agar medium amended with crude latex

The total propagules of thermophilic and thermotolerant fungi recovered from 20 soil samples on medium amended with *E. pulcherrima, F. elastica* and *F. nitida* latex at 45ºC ranged from 0.066 to 0.7 CFUs, 0.133 to 0.8 CFUs per mg and 0.133 to 1.033 CFUs per mg soil respectively. It is worthy to mention that in 3 and 2 soil samples no fungi were recovered on medium amended with *E. pulcherrima* or *F. nitida* latex respectively.

Six fungal species were recovered from the 20 soil samples investigated on Czapek agar media at 45 °C coated with latex of *E. pulcherrima* (4 genera and 5 species), *F. elastica* (5 and 6) or *F. nitida* (5 and 6). *Aspergillus* (with *A. terreus* followed by *A. fumigatus*) was the most common fungus recovered on all media. *Emericella nidulans* was reported in high, moderate and low frequency on the three latices of *E. pulcherrima, F. elastica* and *F. nitida* respectively. The three truly thermophilic species, namely *Myceliophthora thermophila* emerged in moderate frequency on both *Ficus* latices only, *Humicola grisea* var. *thermoidea* in moderate frequency on *F. nitida* latex and in low frequency on *E. pulcherrima* and *F. elastica* latices and *Malbranchea cinnamomea* in low frequency on the three latices. In this respect, Allen and Emerson (1949) isolated several species of thermophilic fungi that had temperature limits extending up to 60°C and

Table 1: Minimum, maximum and mean of CFUs/mg dry soil of mesophilic (out of 50 soil samples) and thermophilic (out of 20 samples) bacteria and fungi recovered on sugar-free media amended with plant latices (20 µl latex/plate).

	Counts on media amended with latex of					
	E. pulcherrima		*F. elastica*		*F. nitida*	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
Mesophilic (n=50)						
Minimum	0.0	0.0	0.0	0.66	0.0	0.33
Maximum	150	53	125	45	116.66	56
Mean of total±SD	25.29±36.65	10.67±9.79	64.03±40.44	14.01±11.14	39.46±30.86	14.33±12.33
Thermophilic (n=20)						
Minmum	0.0	0.0	0.0	0.133	0.0	0.0
Maximum	35.33	0.7	21.33	0.8	31.33	1.033
Mean of total ±SD	11.43±10.65	0.32±0.20	8.43±7.63	0.35±0.18	9.57±9.79	0.40±0.27

demonstrated the utilization and reduction in the amount of resin in crude rubber of guayule shrub (*Parthenium argentatum*).

Protease and lipase enzymes production by mesophilic and thermophilic bacteria

Six mesophilic bacterial species represented by 10 isolates, in addition to three thermophilic bacterial isolates related to *Bacillus* were tested for their capabilities of producing protease and lipase enzymes (Table 4). All mesophilic isolates, except the one related to *Streptomyces* sp. ASU3, were capable of producing protease enzyme but with different capabilities. Gram-negative strain ASU13 and *Streptomyces* strain ASU2 and Gram-positive strain ASU6 exhibited high producing abilities (enzyme index >1.5). Also, all mesophilic strains (except *Stroptomyces* sp. ASU3) were capable of producing lipase enzyme but with different rates. From the positive isolates, seven strains assigned to Gram-negative short rods ASU13, ASU9 and ASU4, *Streptomyces* strains ASU5 and ASU8, Gram-positive strains ASU11and ASU12 showed high lipolytic activities (the depth of the visible precipitate was more than 1.5 cm). It was noticed that the low-producing isolates of lipase belong to *Streptomyces* strain ASU2 and Gram-positive strain ASU6 of spore-forming *Bacillus*. In this respect, actinomycetes are known to be good protease producers (Peczynska-Czoch and Mordarski 1988). Rifaat *et al.* (2007) in their screening of 317 isolates of different streptomycetes obtained from several sources and areas in Egypt, found that only 39 isolates produced proteases. Also, Vermelho *et al.* (1996) reported that streptomycetes hydrolyzed preferentially gelatin.

Christen and Marshall (1984) found that lipase and protease were produced simultaneously by *Pseudomonas fluorescens*. On the other hand, many microorganisms are known as good producers of extracellular lipase (Eom *et al.* 2006). It was reported that isolates of bacteria e.g. *Pseudomonas*, *Bacillus*, *Aeromonas* and *Staphylococcus* could produce lipase and the highest producers were *Pseudomonas* sp., *P. aeruginosa* (Haba *et al.* 2000) and *Bacillus stearothermophilus* (Abada 2008).

The present results showed that the three thermophilic isolates tested, only one (*Bacillus* sp. ASU14) exhibited high protease producing ablity (PI>1.94), but low lipase producing ability (LI=0.8). On the other hand, the two strains of *Bacillus* (ASU 7 and ASU 15) were incapable of producing protease and lipase (Table 3). In this respect, there have been many reports on thermophilic proteases produced by different strains of *Bacillus* spp., including the thermolysin from *B. thermoproteolyticus* (Endo 1962), an alkaline protease from *Bacillus* sp. (Takami *et al.* 1989), and a thermostable neutral protease from *B. stearothermophilus* (Fujio and Kume 1991). Protease production has also been manifested by thermophilic *Bacillus* strains (Nascimento and Martins 2004) and *Bacillus licheniformis* (Folasade *et al.* 2008) and a mesophilic *Bacillus* strain (Al-Nahdi 2012). Moreover, Atalo and Gashe (1993) found a thermophilic *Bacillus* species that was high-protease producer. Extracellular lipolytic activity was also shown by *Bacillus* sp. (Wang *et al.* 1995) and *Bacillus thermoleovorans* (Lee *et al.* 2001).

Table 2: Bacteria and fungi (meso & thermo) isolated from soil samples on sugar-free media amended with plant latices from *Euphorbia pulcherrmia*, *Ficus elastica* and *Ficus nitida* (20 µl latex/plate).

Microorganisms	***Euphorbia pulcherrima***				***Ficus elastica***				***Ficus nitida***			
	TC	**TC%**	**F%**	**O**	**TC**	**TC%**	**F%**	**O**	**TC**	**TC%**	**F%**	**O**
Mesophilic bacteria (at 36ºC)												
Gram positive bacteria	357	68.3	74	H	523	82.16	92	H	314	80.7	68	H
Bacilli	357	68.3	74	H	79.7	12.5	42	M	106.3	27.3	64	H
Streptomyces sp. 3 FCz (white)					177.7	27.9	86	H				
Streptomyces sp. 2 FCz (grey)					106	16.61	68	H				
Streptomyces sp. 5 NFCz (buff)					164.7	25.83	84	H	207.7	53.2	68	H
Gram negative short rods	166	31.7	50	H	109.3	17.17	48	M	75	19.3	40	M
Gross total count	523	100	80	H	636.7	100	92	H	389	100	86	H
Thermophilic and themotolerant bacteria (at 45ºC)												
Bacillus sp.	41.3	36.9	55	H	84.3	58.16	70	H	40.7	43.4	70	H
Bacillus sp.	70.7	63.1	55	H	60.7	41.83	70	H	53	56.6	40	M
Gross total count	112	100	70	H	145	100	70	H	93.7	100	70	H
Mesophilic fungi (at 25ºC)												
Acremonium strictum W. Gams					2	0.28	2	L	9.3	1.3	6	L
Alternaria alternate (Fries) Keissler	12.3	2.4	12	L	20.3	2.88	26	M	7	0.98	10	L
Aspergillus	186.7	36.3	86	H	365.3	51.82	84	H	347.7	48.8	98	H
A. flavus Link	9.3	1.8	18	L	12	1.7	18	L	18.7	2.6	20	L
A. fumigatuss Fresenius	1.3	0.3	2	L	1.7	0.23	2	L	1.7	0.2	2	L
A .niger van Tieghem	52.3	10.2	52	H	65.3	9.27	50	H	90.7	12.7	62	H
A. sydowii (Bainier & Sartory) Thom & Church	3.7	0.7	8	L	6.3	0.89	6	L	1.3	0.2	2	L
A .terreus Thom	115.7	22.5	66	H	240.7	34.14	80	H	209.3	29.4	88	H
A .ustus (Bainier) Thom & Church	4.3	0.8	10	L	26.3	3.74	28	M	7.7	1.1	20	L
A .versicolor (Vuillemin) Tiraboschi					13	1.85	2	L	18.3	2.6	4	L
Cladosporium	52.7	10.2	26	M	48.3	6.86	36	M	93	13.1	30	M
C. cladosporioides (Fresenius) de Vries	45.7	8.9	16	L	46.7	6.62	28	M	14.3	2.0	12	L
C. sphaerospermum Penzig	7	1.4	10	L	1.7	0.23	8	L	78.7	11.0	20	L
Cochliobolus lunatus R. Nelson & Haasis	14.3	2.8	16	L	18.7	2.65	20	L	5	0.7	6	L
Fusarium	116	22.6	64	H	104	14.75	50	H	45	6.3	44	M
F. solani (Martius) Saccardo	69.3	13.5	38	M	61	8.65	28	M	21.7	3.0	28	M

F.verticillioides (Saccardo) Nirenberg	46.7	9.1	26	M	43	6.1	22	L	23.3	3.3	20	L
Humicola grisea Traaen	9.3	1.8	4	L	11.3	1.61	10	L	22	3.1	14	L
Mucor circinelloides van Tieghem					4	0.57	8	L	2	0.3	6	L
Myrothecium verrucaria (Albertini & Schweinitz) Ditmar	10.3	2.01	8	L	20	2.84	14	L	26.7	3.7	22	L
Penicillium	55	10.7	36	M	63.3	9	28	M	48.3	6.8	44	M
P. aurantiogriseum Dierckx	16.3	3.2	10	L	25.7	3.64	6	L	2.7	0.4	6	L
P. chrysogenum Thom	38.7	7.5	26	M	37.7	5.34	22	L	45.7	6.4	38	M
Phoma sp.	11.7	2.3	8	L	7	1	6	L	17.3	2.4	6	L
Scopulariopsis candida (Gueguen) Vuillemin					2.7	0.38	6	L	3	0.4	8	L
Setosphaeria rostrata Leonard	1.7	0.3	4	L	1	0.14	4	L	2.3	0.3	4	L
Stachybotrys	43	8.4	26	M	37	5.25	20	L	81.3	11.4	46	M
S. chartarm (Ehrenberg) Hughes	43	8.4	26	M	37	5.25	20	L	79.3	11.1	42	M
S. elegans (Pidoplichko) W. Gams									2	0.3	4	L
Trichderma hamatum (Bonorden) Bainier									2	0.3	4	L
Trichothecium roseum (Persoon: Fries) Link	0.17	0.06	2	L								
Trichurus spiralis Hasselbring	1	0.2	2	L								
Gross total count	514.3	100	96	H	704.93	100	100	H	712	100	100	H
Thermophilic and thermotolerant fungi (at 45ºC)												
Aspergillus	4.57	72.49	85	H	3.37	50.26	85	H	3.73	47.7	85	H
A. fumigatus Fresenius	1.47	23.27	35	M	1.4	20.89	45	M	1.3	16.6	40	M
A. terreus Thom	3.1	49.22	70	H	1.97	29.36	70	H	2.43	31.1	75	H
Emericella nidulans (Eidam) Vuillemin	1.47	23.27	55	H	1.5	22.39	35	M	0.8	10.2	5	L
Humicola grisea var. *thermoidea* Cooney & R. Emerson	0.2	3.18	15	L	0.37	5.463	15	L	0.73	9.37	30	M
Malbranchea cinnamomea (Libert) van Oorschot & de Hoog	0.07	1.05	10	L	0.17	2.477	15	L	0.5	6.4	25	L
Myceliophthora thermophile (Apinis) van Oorschot					1.3	19.4	45	M	2.06	26.3	45	M
Gross total count	6.3	100	85	H	6.7	100	100	H	7.83	100	90	H

TC: Total count of each organism (per 50 samples; TC%: Percentage total count, calculated per total count of all bacteria and fungi; F%: Percentage frequency calculated per total number of samples (50 samples); O: Occurrence remarks: H=high occurrence, >50% (out of 50 samples); M=moderate occurrence, from 25-49%; L=low occurrence, less than 25%.

Protease and lipase production by mesophilic, thermotolerant and thermophilic fungi

Ten mesophilic fungal species represented by 30 isolates (3 isolates for each species were isolated on each of the three types of media), in addition to four thermophilic and thermotolerant species represented by 11 isolates were tested for their capabilities of producing protease and lipase enzymes. Twenty-five mesophilic isolates of the 30 tested organisms were capable of producing protease but with different degrees. Of the positive isolates only 6 were high producers and these were related to *Alternaria alternata* AUMC 4686, *Aspergillus ustus* (three strains AUMC Nos. 4797, 4798 and 4799, *Fusarium verticillioides* AUMC 4801 and *Penicillium chrysogenum* AUMC 4706. It was observed that the 5 non-protease producing strains belong to *Cladosporium cladosporioides* (3 strains) and *Stachybotrys chartarum* (2) (Table 3).

Proteins constitute one of the components of natural rubber serum. Some fungi readily decompose proteins, amino acids and other nitrogenous compounds to liberate ammonia as was reported by Alexopoulus (1962). Fungal genera whose proteolytic enzymes have received extensive studies include *Alternaria, Aspergillus, Mucor, Penicillium* and *Rhizopus* (Atagana *et al.* 1999). Djamel *et al.* (2009) tested a total of 253 *Penicillium* isolates on skimmed milk agar and found only 10 strains (3.9%) with proteolytic activities. Acid proteases are also synthesised by *Mucor miehei* (Fernandez-Lahore *et al.* 1999), *M. hiemalis, M. racemosus* and *M. bacilliformis* (Fernandez-Lahore *et al.* 1997, 1998) and by species of *Aspergillus* (Tremacoldi *et al.* 2004) and *Rhizopus* (Kumar *et al.* 2005).

On the other hand, the 30 mesophilic isolates were lipase-producing but with different capabilities. The high-producing 20 strains were related to *Alternaria alternata* (2 isolates), *Aspergillus flavus* (3), *A. niger* (1) *A. ustus* (2), *Cladosporium cladosporioidies* (3), *Fusarium solani* (3), *F. verticillioides* (2), *Penicillium chrysogenum* (2) and *Stachybotrys chartarum* (2). The low-producing strains of lipase belong to *Alternaria alternata*, *Aspergillus niger*, *A. terreus, A. ustus*, *Fusarium verticillioides*, *Penicillium chrysogenum* and *Stachybotrys chartarum* (Table 3). Cho *et al.* (2007) recorded that *Penicillium chrysogenum* and *Cladosporium cladosporioides* out of 9 strains tested for lipase production showed the highest activity. High lipolytic activity was also observed with *Penicillium citrinum* (Maliszewska and Mastalerz 1992), *P. restrictum* (Gombert *et al.* 1998), and *P. wortmannii* (Costa and Peralta, 1999). Different from our findings that *A. terreus* isolates were of low lipolytic activity, those of Gulati *et al.* (1999) were of high activity when corn oil was used as an inducer.

The eleven thermophilic and thermotolerant isolates tested were capable of producing protease. The three strains of *Aspergillus terreus* followed by the three strains of *Emericella nidulans* showed high protease-producing abilities. The two strains of *Myceliophthora thermophila* (one recovered on *F. elastica* and the other on *F. nitida* latex) showed also high production of protease. On the other hand, the three strains of *Aspergillus fumigatus* tested were with low ability (Table 3). The extracellular protease production by thermophilic moulds was studied by several workers (Ong and Gaucher 1976 and Satyanarayana and Johri 1983). Thermostable alkaline proteases were also identified on casein agar by *Malbranchea sulfurea, Chaetomium thermophile, Humicola lanuginose* (Ong and Gaucher 1976), *Aspergillus fumigatus* (Paneerselvam *et al.* 1978), *Mucor miehei* (Rickert and McBride-Warren 1974), and *Sporotrichum thermophile* (Adams and Deploey 1978).

Eight out of the 11 isolates tested were capable of producing lipase but with different rates while the three isolates of *A. terreus* were not. One strain of each of *Myceliophthora thermophila* AUMC 4652 and *Emericella nidulans* AUMC 4654 were high producers. *Aspergillus fumigatus* (the three strains), *Myceliophthora thermophila* AUMC 4653 and *Emericella nidulans* (two strains) were low producers (Table 3). In relation with our results, Krikstaponis *et al.* (2001) reported that some of the tested strains of *A. fumigatus* were able to produce extracellular lipases and hydrolyze fatty acids of vegetable oil. Others reported also lipase from *A. fumigatus* (Ogundero 1982). Moreover, Arima *et al.* (1972) purified an extracellular lipase from *Humicola lanuginosa* strain. Also, Omar *et al.* (1987) reported that production and thermostability of lipase differed with different strains of *H. lanuginosa*. Cold-adapted lipases were also produced by *Aspergillus nidulans* (Mayordomo *et al.* 2000).

Table 3: Protease and lipase production by most common mesophilic, thermotolerant and thermophilic bacteria and fungi recovered on sugar-free media amended with the lattices of *Euphorbia pulchermmia (*EP*)*, *Ficus elastica* (FE) and *Ficus nitida* (FN).

Bacterial and fungal species	ASU/ AUMC No.	Latex source	Protease			Lipase
			MCD±SD	MDCZ±SD	PI	
Mesophilic bacteria						
Gram positive bacilli	6	EP	1.1±0.11	2.35±0.17	2.13	1.3
	11	FE	4.65±0. 4	6±0	1.29	2.5
	12	FN	4±0.16	5.08±0.15	1.26	1.8
Gram negative short rods	4	EP	1.82±0.12	2.5±0	1.37	1.8
	9	FN	1.22±0.2	2.45±0.1	2	2.6
	13	FE	0.9±0.08	1.47±0.12	1.63	1.9
Stroptomyces spp.	3	FE	1±0	0	0	0
	5	FE	0.9±0	0.98±0.05	1.08	1.95
	8	FN	0.9±0	1.2±0.14	1.33	1.8
	2	FE	0.5±0	1.02±0.05	2.04	1.3
Thermophilic bacteria						
Bacillus spp.	14	EP	0.9±0	1.75±0.05	1.94	0.8
	7	FE	0.8±0	0	0	0
	15	FN	0.9±0	0	0	0
Mesophilic fungi						
Alternaria alternata	4686	EP	1.43±0.09	2.55±0.17	1.79	1.3
	4687	FE	2.6±0.14	3.25±0.2	1.25	1.95
	4684	FN	2.93±0.09	3.5±0	1.19	1.95
Aspergillus flavus	4794	EP	2.78±0.15	4.05±0.1	1.46	1.8
	4795	FE	3.98±0.12	4.45±0.17	1.12	2.25
	4793	FN	3.43±0.17	4.5±0	1.31	1.7
Aspergillus niger	4700	EP	3.4±0.14	4.28±0.32	1.26	1.15
	4702	FE	3.8±0.21	4.2±0.14	1.11	1.9
	4703	FN	3.75±0.13	4.1±0.2	1.09	1.1
Aspergillus terreus	4680	EP	3.1±0.11	4.15±0.28	1.34	0.9
	4682	FE	3±0	4.08±0.09	1.36	0.7
	4683	FN	3.03±0.09	4.2±0.08	1.39	0.8
Aspergillus ustus	4797	EP	2.08±0.09	3.25±0.12	1.57	1.1
	4799	FE	2.15±0.057	3.6±0.08	1.67	1.95
	4798	FN	2.1±0	3.3±0.11	1.57	1.8
Cladosporium cladosporioides	5063	EP	2.55±0.17	0	0	1.5
	4663	FE	3.25±0	0	0	1.7
	4665	FN	2.15±0.057	0	0	1.75
Fusarium solani	5066	EP	5.08±0.05	5.35±0.12	1.05	2
	5073	FE	3.25±0.05	3.55±0.05	1.09	2.3
		FN	3.55±0.05	4±0.18	1.13	2.15
Fusarium verticillioidies	4801	EP	2.8±0.24	4.2±0.08	1.5	1.2
	5065	FE	4.1±0	5.15±0.05	1.26	1.5
	4800	FN	4.35±0.17	5.25±0.17	1.21	1.95
Penicillium chrysogenum	4706	EP	1.53±0.5	2.33±0.17	1.52	1.2
	4704	FE	2.43±0.09	2.58±0.05	1.06	1.8
	4707	FN	2.3±0	2.45±0.057	1.06	1.95
Stachybotrys chartarum	4690	EP	2.4±0.08	2.5±0	1.04	1.15
	4691	FE	2.33±0.17	0	0	2
	4688	FN	2.55±0.17	0	0	2
Thermotolerant and thermophilic fungi						
Aspergillus.fumigatus	4658	EP	1.58±0.05	1.8±0	1.14	0.8

	4657	FE	2.05±0.1	2.23±0.05	1.08	1.1
	4659	FN	1.85±0.057	2.03±0.05	1.09	1
Aspergillus terreus	4662	EP	0.78±0.05	2.65±0.05	3.42	0
	4661	FE	0.65±0.09	2.78±0.18	4.26	0
	4660	FN	0.8±0.11	3.07±0.09	3.83	0
Emericella nidulans	4656	EP	1.08±0.09	2.58±0.05	2.93	1.1
	4654	FE	1.18±0.095	2.45±0.05	2.08	1.75
	4660	FN	0.95±0.057	2.13±0.05	2.24	1.3
Myceliophthora thermophila	4653	FE	1.83±0.09	3.53±0.05	1.93	1.1
	4652	FN	1.7±0.14	3.13±0.05	1.84	1.85

Figures in the table are mean diameters of the clear zones MDCZ ± SD of three replicates for protease (in cm after 5 days), or mean depths of the visible precipitate of two replicates for lipase (in cm after 7 days), >1.5 cm: high producer, <1.5 cm: low producer.
MCD=mean colony diameter, MDCZ= mean diameter of clear zone.
PI= protease index. 0= zero indicates negative result, H >1.5, L<1.5.
ASU: Assiut University bacterial collection numbers, AUMC: Assiut University Mycological center numbers

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