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# Detection of Extracellular Enzymes Produced by Fungi Isolated from Dried Fruits

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**ABSTRACT**

Screening of 57 fungal isolates isolated from dried fruits for the production of lipase, cellulase, invertase, and protease showed that there is a variation in enzyme production not only among the different genera and species, but also among the different isolates in the same species. Thirty two of tested fungi (66%) belonging to *A. flavus, A. fumigatus, A. niger, A. terreus, A. tamarii, Cochliobolus spicifer, Humicola insolens, F. oxysporium, P. glaprum, P. oxalicum, P. stekii, P. variabile* and *Phoma sp.* had a high ability to produce lipase. Ten of tested isolates representing 24.39% of tested fungi had a moderate ability to produce cellulase enzyme. These isolates were belonging to *Aspergillus flavus, A. niger, A. parasiticus, A. versicolor, Curvularia lunata, Penicillium griseofulvum, P. oxalicum, P. stekii, P. variabile* and *Ulocladium atrum.* Twenty seven isolates representing 37.64 % of tested isolates belonging to *A. flavus, A. fumigatus, A. niger, A. parasiticus, A. terreus, A. versicolor, Fusarium oxysporium, Mucor fuscus, P. glaprum, P. griseofulvum, P. oxalicum, P. variabile, Phoma sp.,* and *U. atrum* were moderate invertase producers. Sixteen fungal isolates representing 28.07 % of tested isolates were moderate protease producers, these isolates were belonging to *A. flavus, A. niger, A. terreus, A. tamarii, A. versicolor, C. lunata, F. oxysporium, H. insolens, P. corylophilum, P. griseofulvum, P. oxalicum, P. stekii, P. vinaceum, Rhizopus stolonifer.*

## INTRODUCTION

In recent years, the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated a renewed interest in the exploration of extracellular enzymatic activity in food grade yeasts (Bilinski and Stewart, 1990; Burden and Eveleight, 1990; De Mot, 1990; Ratledge and Tan, 1990).

Fungi secrete proteins which are protective in function to the fungi themselves or which can be exploited for the welfare of mankind (Ng, 2004). Lipases (tricylglycerol hydrolyses EC 3.1.1.3) are serine hydrolyases of considerable physiological significance and industrial potential, which

catalyze the hydrolysis of triglycerides at the oil water interface. They are produced by animals, plants and microorganisms (Sztajer, *et al.,* 1988; Aires-Barros, *et al.,* 1994; Ionita, *et al.,* 1997; Ueda, *et al.,* 2002). To date, a large number of lipases produced by filamentous fungi has been extensively studied, both from the biochemical and genetic point of view. The most productive species belong to the genera *Geotrichum, Penicillium, Aspergillus* and *Rhizomucor* (Stocklein, *et al.,* 1993 and Miura and Yamane, 1997). Cellulose is the most abundant component of all photosynthetic land plants and thus represents the main organic food source for heterotrophic decomposers. On land, the major biological agents of cellulose breakdown are fungi, with aerobic and anaerobic cellulolytic bacteria playing a minor role (Swift, 1982). Invertase (1, 2-β-D-fructofuranosidase fructohydrolase, EC3.2.1.26), an important enzyme used in food industry, is usually synthesized constitutively by yeasts (Costaglioli, *et al.,* 1997) while, in certain filamentous fungi, it is inducible (Chen 1996 and Romero-Gomez, *et al.,* 2000). Many phytopathogenic fungi are known to produce extracellular proteinases (Kalashnikova, *et al.,* 2003) and Sara and Heale, (1990), suggested that proteinases play an active role in the development of plant diseases. Proteases with different molecular masses, optimum pH values and optimum temperatures are produced by different fungal species (Chou, *et al.* 2001; Paoletti, *et al.* 2001; Pekkarinen, *et al.* 2002; Poza *et al.* 2001).

## MATERIALS AND METHODS

Fifty seven fungal isolates isolated from dried fruits were screened for their ability to produce extracellular enzymes on solid and liquid media. The following fungal organisms were tested: *Aspergillus flavus, A. tamarii*, *A. niger*, *A. terreus*, *A. versicolor*, *A. parasiticus*, *A. fumigatus*, *Cochliobolus spicifer*, *Curvularia lunata*, *Emericella quadriline, Fusarium oxysporium*, *F. verticillioides*, *Humicola insolens*, *Mucor fuscus*, *Myrothecium roridum*, *Penicillium corylophilum, P. expansum*, *P. glaprum, P. griseofulvum*, *P. oxalicum*, *P. variabile*, *P. stekii*, *P. vinaceum, Phoma sp.*, *Rhizopus stolonifer*, *Ulocladium atrum* and *U. botrytis*.

### 1. Lipase production:

Lipase production was measured according to Ulman and Blasins, (1974). The basal medium was composed of: Peptone, 10.0 g; Magnesium sulphate, 2.0 g**;** Calcium chloride, 0.2 g; 1% Tween 20, 10.0 ml.; Agar, 15.0 g and Distilled Water 1000.0 ml, pH 6.0.The medium was sterilized by autoclaving at 121 ºC for 30 minutes. The Tween 20 was autoclaved separately and 10.0 ml. was added to 1000.0 ml. of cooled basal medium. The isolated fungi were separately inoculated on the surface of agar basal medium and incubated at 28 ºC for 10 days. Occurrence of a visible precipitate due to the formation of calcium salt crystals of the oleic acid liberated by enzyme indicates a positive lipolytic production as shown in plate 1.a.

### 2. Cellulase production:

Cellulase production was screened using CMC agar (carboxymethylcellulose agar medium) which was composed of: Carboxy methyl cellulose (a soluble form of cellulose) 5.0 g, Sodium nitrate 1.0 g, Potassium dihydrogen phosphate 1.0 g, Potassium chloride 1.0 g, Magnesium sulphate 0.5 g,Yeast extract 0.5 g, Agar 17.0 g, Distilled water 1000.0 ml. The medium was sterilized by autoclaving at 121 ºC for 30 minutes. The isolated fungi were inoculated on the surface of agar medium and incubated at 28 ºC for 10 days. All

plates of our isolates were flooded with Congo red solution (1.0 mg of Congo red per 1.0 ml of water) for 15 minutes, and then de-stained with salt solution (1M Sodium chloride) for 10-15 minutes. Unstained areas indicate where the CMC has been broken down to β1-4 glucans which contains seven or fewer glucose residues as shown in plate 1.b. The diameter of the clear zone was measured (Teather and Wood, 1982).

**3. Invertase (sucrase) production:**
Sucrose hydrolysis by fungal isolates was tested on 20% sucrose Czapek's liquid medium (Sucrose 200.0 g, Sodium nitrate 3.0 g, Potassium dihydrogen phosphate 1.0 g, Magnesium sulphate 0.5 g, Potassium chloride 0.5 g, Ferrous sulphate 0.01g and Distelled Water 1000.0 ml. at pH 4.5) . The isolates were inoculated and incubated at 28 ºC for 10 days. The sucrase (invertase) production was determined in the culture medium filtrate using Benedict solution method. About 0.5 ml. of Benedict solution was added to culture filtrate and heated in water bath at temp. 100 ºC, a positive result was indicated by a yellow, green, or brown precipitate as shown in plate 1.c. (Abdel-Sater and Saber, 1999).

**4. Protease production:**
The fungal proteolytic producer was tested by using a casein hydrolysis medium (Paterson and Bridge, 1994). This medium was intended for presumptive protease production, and contains skim milk, which gives an opaque of final medium. Hydrolysis of the casein results as a clear zone around the colony.

The composition of the medium was: Potassium dihydrogen phosphate, 1.0 g; Potassium chloride, 0.5 g; Magnesium sulphate, 0.2 g; Calcium chloride, 0.1 g; 15% skim milk, 25.0 ml; Glucose, 10.0 g; Agar, 12.0 g and Distilled water 1000.0 ml. The cooled medium was poured into 9 cm Petri-dishes (about 20 ml. for each). The tested fungi were separately inoculated in the centre of Petri-dishes and incubated at 28 ºC for a week. After incubation, complete degradation of milk protein was seen as a clear zone in a some what opaque agar around colonies indicating of protease production.

## RESULTS

**1. Lipase production:**
Fifty isolates comprising 87.71 % of tested fungi were recorded as lipase producers (Table, 1). Thirty two of tested fungi (66%) belonging to *A. flavus, A. fumigatus, A. niger, A. terreus, A. tamarii, C. spicifer, H. insolens, F. oxysporium, P. glaprum, P. oxalicum, P. stekii, P. variabile* and *Phoma sp.* had a high ability to produce lipase. Eleven isolates representing 24% of tested isolates were recorded as moderate lipase producers. These fungal isolates were *A. flavus, A. niger, A. terreus, A. tamarii, E. quadriline, F. oxysporium, M. fuscus, P. corylophilum, P. glaprum, P. verrucosum* and *P. vinaceum.* Five isolates comprising 10% of tested fungi were recorded as weak lipase producers. These fungi were *A. flavus, F. verticillioids, P. expansum, P. griseofulvum* and *R. stolonifer*.

**2. Cellulase production:**
Forty one fungal isolates representing 71.93% of tested isolates were cellulase producers (Table 1). Five isolates (12.19%) had a high ability to produce cellulase. These isolates were *A. parasiticus, A. terreus, P. expansum, P. glaprum* and *P. stekii*. Ten of tested isolates representing 24.39% of tested fungi had a moderate ability to produce cellulase

enzyme. These isolates were belonging to *A. flavus, A. niger, A. parasiticus, A. versicolor, C. lunata, P. griseofulvum, P. oxalicum, P. stekii, P. variabile* and *U. atrum.* Twenty six of fungal isolates representing 63.41 % belonging to *A. niger*, *A. terreus, A. tamarii, H. insolens, M. fuscus, P. glaprum. Phoma sp.,* and *R. stolonifer* were a weak cellulase producers.

**2. Invertase production:**

The ability of fungal isolates to produce invertase (sucrase) enzyme in liquid medium were studied. It was observed that there is a variation in enzyme production not only among the different genera and species, but also among the different isolates in the same species presented in Table (1). Out of the 57 isolates studied, there are 56 (98.24%) were able to produce invertase enzyme. From these producing isolates, only one isolate had a high degree of invertase production *(A. tamarii).* Twenty seven isolates representing 37.64 % of tested isolates belonging to *A. flavus, A. fumigatus, A. niger, A. parasiticus, A. terreus, A. versicolor, F. oxysporium, M. fuscus, P. glaprum, P. griseofulvum, P. oxalicum, P. variabile, Phoma sp.,* and *U. atrum* had a moderate degree of invertase production. Twenty three fungal isolates belonging to *A. flavus, A. niger, A. parasiticus, A. tamarii, C. spicifer, C. lunata, E. quadriline, F. oxysporium, F. verticillioids, H. insolens, M. roridum, P. corylophilum, P. stekii, P. verrucosum, P. vinaceum* and *R. stolonifer* were a weak invertase producers. Only one isolate was non invertase producer, this isolate was *P. expansum.*

**4. Protease production:**

Twenty three of fungal isolates were tested for their ability to produce protease enzyme and recorded as protease producers (Table 1). These isolates belonging to *A. flavus, A. niger, A. terreus, A. tamarii, A. versicolor, C. lunata, F. oxysporium, H. insolens, P. corylophilum, P. griseofulvum, P. oxalicum, P. stekii, P. variabile, P. vinaceum, Phoma sp., R. stolonifer* and *U. atrum.* Sixteen fungal isolates representing 28.07 % of tested isolates were moderate protease producers, these isolates were belonging to *A. flavus, A. niger, A. terreus, A. tamarii, A. versicolor, C. lunata, F. oxysporium, H. insolens, P. corylophilum, P. griseofulvum, P. oxalicum, P. stekii, P. vinaceum and R. stolonifer* whereas, seven fungal isolates belonging to *A. flavus, A. terreus, P. stekii, P. variabile, Phoma sp.,* and *U. atrum* were weak protease producers.

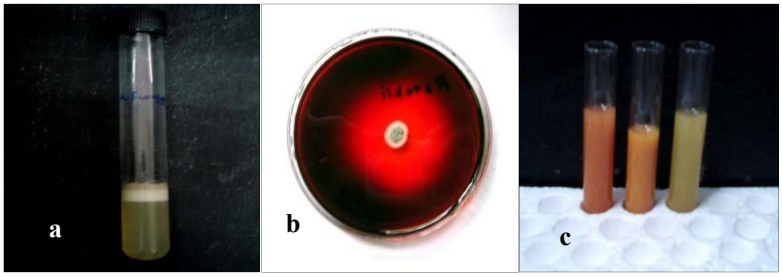


**Plate (1): a. White precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by lipase enzyme.**
**b. Unstained area indicates CMC degradation by cellulase to ß 1- 4 glucan.**
**c. Formation of different color precipitates indicating an invertase production.**

**Table (1): Enzymatic activity of fungal isolates isolated from dried fruit samples.**

| Organisms | NTI | Lipase | | | | Cellulase | | | | Invertase | | | | Protease | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NPI | Degree of production | | | NPI | Degree of production | | | NPI | Degree of production | | | NPI | Degree of production | | |
| | | | H | M | W | | H | M | W | | H | M | W | | H | M | W |
| *Aspergillus flavus* | 13 | 11 | 9 | 1 | 1 | 10 | 0 | 1 | 9 | 13 | 0 | 8 | 5 | 5 | 0 | 3 | 2 |
| *A. fumigatus* | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *A. niger* | 10 | 10 | 8 | 2 | 0 | 10 | 0 | 0 | 10 | 10 | 0 | 5 | 5 | 1 | 0 | 1 | 0 |
| *A. parasiticus* | 2 | 2 | 0 | 0 | 2 | 2 | 1 | 1 | 0 | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| *A. terreus* | 2 | 2 | 1 | 1 | 0 | 2 | 1 | 0 | 1 | 2 | 0 | 2 | 0 | 2 | 0 | 1 | 1 |
| *A. tamarii* | 3 | 3 | 3 | 0 | 0 | 1 | 0 | 0 | 1 | 3 | 1 | 0 | 2 | 1 | 0 | 1 | 0 |
| *A. versicolor* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| *Cochliobolus spicifer* | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *Curvularia lunata* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Emericella quadriline* | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *Fusarium oxyspoirum* | 3 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 1 | 2 | 1 | 0 | 1 | 0 |
| *F. verticillioides* | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *Humicola insolens* | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Mucor fuscus* | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *Myrothecium roridum* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *Penicillium corylophilum* | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *P. expansum* | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *P. glaprum* | 2 | 2 | 1 | 1 | 0 | 2 | 1 | 0 | 1 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| *P. griseofulvum* | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| *P. oxalicum* | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| *P. stekii* | 3 | 2 | 2 | 0 | 0 | 3 | 1 | 2 | 0 | 3 | 0 | 0 | 3 | 2 | 0 | 1 | 1 |
| *P. variabile* | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| *P. verrucosum* | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *P. vinaceum* | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Phoma sp.* | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| *Rhizopus stolonifer* | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 |
| *Ulocladium atrum* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| Total Isolates | 57 | 50 | 32 | 11 | 7 | 41 | 5 | 10 | 26 | 56 | 1 | 27 | 28 | 23 | 0 | 16 | 7 |

NPI: Number of positive isolates and NTI: Number of tested isolates. W: Weak <0.5; M: Moderate 0.5- 0.9; H: High ≥ 10 m

# DISCUSSION

The data in Table (1) clearly showed that 50 of tested isolates were lipase producers. Buzzini and Martini (2002) screened 196 strains of ascomycetes, 155 of basidiomycetes, and 46 of yeast-like organisms for their ability to produce extracellular enzymes; they found that about 60.7%, 43.5% and 13.5% of ascomycetes, yeast-like organisms and basidiomycetes respectively, were lipase producers. Cardenas *et al.* (2001b), screened 960 microorganisms isolated from soil samples, including yeast (100 strains) and filamentous fungi (860 strains) for their ability to produce lipase. They reported that 440 microorganisms produced a clear halo around them in plates containing tributyrin, whereas only 92 microorganisms showed hydrolysis on the olive oil plates. Shatter, (2004), screened 68 fungal isolates for their lipolytic activity, and reported that about 87.88 % of their isolates were lipase producers. Mohammed and Hussein (2004) screened 54 isolates of fungi isolated from luncheon meat for their lipolytic ability; of these isolates, 81.5% were able to produce lipase enzyme. Fotedar and Al-Hedaithy (2005) tested 87 isolates of *Candida dubliniensis* and 52 isolates of *C. albicans* for their ability to produce phospholipase. None of the 87 isolates of *C. dubliniensis* were phosphoplipase producers whereas, in contrast all the 52 *C. albicans* isolates showed varying degree of phospholipase activity, with 35 of them eliciting a higher phospholipase activity.

Another result presented in Table (1) showed that 41 of the tested isolates were reported as cellulase producers. Schlegel (1996) reported that species of the genera *Fusarium* and *Chaetomium* are prominent. Others known to be cellulolytic are *Aspergillus fumigatus, A. nidulans, Botrytis cinerea*, *Rhizoctonia solani*, *Trichoderma viride*, *Chaetomium globosum* and *Myrothecium verrucaria*. Strauss *et al.* (2001) screened 245 yeast isolates isolated from four wine production regions of the Western Cape, South Africa, for their cellulytic activity. They found that only 11 isolates of *Candida stellata*, *C. pulcherrima* and *Kloeckera apiculata* showed some cellulase activity, but they reported that *C. pulcherrima* showed only activity on medium containing glucose only.

Also, results in Table (1) showed that 98.24% of the studied isolates were able to produce invertase enzyme. This result agrees with Abdel-Sater and Ismail, (1993), where they found that all their studied isolates were able to produce invertase. Abdel-Sater and Saber (1999) reported that 86.9% of their tested isolates isolated from dried fruits could produce invertase enzyme.

Finally, data in Table (1) showed that 40.35% of the tested isolates were able to produce protease. This result agrees with Buzzini and Martini (2002) who found that about 3.6, 15.2 and 31.0% of ascomycetes, yeast-like organisms and basidiomycetes, respectively were protease producers. Mohammed and Hussein (2004) screened 54 isolates of fungi for their proteolytic ability; of these isolates, 72.2% of isolates were able to produce protease enzyme. Shatter (2004) screened 68 fungal isolates for their proteolytic activity, and found that about 85.42% of isolates were protease producers.

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# التحري عن الانزيمات الخلوية الخارجية المنتجة بواسطة الفطريات المعزولة من الفواكه المجففة

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**ملخص**

تم في هذه الدراسة الكشف عن قدرة ٥٧ عزلة فطرية تم عزلها من الفواكه المجففة للكشف عن قدرتها على انتاج انزيمات اللايبيز، السيليوليز، انفرتيز والبروتيز.حيث أظهرت هذه الدراسة تنوعاً للعزلات في انتاجها لهذه الانزيمات ليس خلال الأنواع و الأجناس المختلفة فحسب ، بل من خلال السلالات من النوع نفسه.

اظهرت الدراسة ان ٣٢ عزلة فطرية كان لها قدرة عالية على انتاج انزيم اللايبيز(٦٦٪) تنتمي الى: اسبرجلس فلافس، اسبرجلس فوميقاتس، اسبرجلس نيجر، اسبرجلس تيرريس، اسبرجلس تاماري،كوتشيلوبولس سبيسفير، فيوزاريم اوكسيسبوريوم، هيوميكولا انسولنس ، بنسيليوم قلابرم،بنسيليوم اوكساليكم، بنسليوم فاريابيل،بنسيليوم ستيكي و فاوما كان لها قدرة عالية على انتاج انزيم اللايبيز.

كما ظهرت ١٠ عزلات تمثل ٢٤,٣٩٪ من الفطريات المختبرة لها قدرة متوسطة على انتاج انزيم السيليوليز،وهذه العزلات تنتمي الى الانواع التالية: اسبرجلس فلافس، اسبرجلس نيجر، اسبرجلس باراسينكس، اسبرجلس فيرسيكولور،كرفيولاريا لوناتا، بنسيليوم قريسوفولفم، بنسيليوم اوكساليكم،بنسيليوم ستيكي، بنسليوم فاريابيل والوكلاد يوم اترم.

وجد ان ٢٧ عزلة تمثل٣٧,٦٤٪ من العزلات التي تم اختبارهاتملك قدرة متوسطة لانتاج انزيم الانفرتيزو تنتمي الى: اسبرجلس فلافس، اسبرجلس فوميقاتس، اسبرجلس نيجر، اسبرجلس باراسيتكس، اسبرجلس تيرريس، اسبرجلس فيرسيكولور، فيوزاريم اوكسيسبوريوم، ميوكر فاسكس، بنسيليوم قلابرم، بنسيليوم قريسوفولفم، بنسيليوم اوكساليكم، بنسليوم فاريابيل، فاوما والوكلاديوم اترم تملك قدرة متوسطة لانتاج انزيم الانفرتيز.

واخيراً بالنسبة لانزيم البروتيز فقد ظهرت ١٦ عزلة فطرية ممثلة ٢٨,٠٧٪ من العزلات المختبرة كانت متوسطة الانتاج لانزيم البروتيز، هذه العزلات تنتمي الى: اسبرجلس فلافس، اسبرجلس نيجر، اسبرجلس تاماري، اسبرجلس تيرريس، اسبرجلس فيرسيكولور، كرفيولاريا لوناتا، فيوزاريم اوكسيسبوريوم ،هيوميكولا انسولنس، بنسيليوم كوريلوفيللوم، بنسيليوم قريسوفولفم، بنسيليوم اوكساليكم، بنسليوم ستيكي، بنسيليوم فيناسيم و رايزوبس ستولونيفير.