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Production of Pectinase Enzymes by Citrus Fruits Contaminant Fungi.*

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Abstract:

Seventy five fungal cultures were isolated from decayed citrus fruits and tested for their pectinolytic activity. Thirty six out of them proved to have ability for pectinase enzymes production. These isolates were identified and classified into twenty strains belonging to eleven species appertaining to six genera of fungi. *Aspergillus* was the first predominant genus followed by the genera of *Cladosporium* and *Penicillium*.

Evaluation of the twenty strains for their ability to produce pectinase enzymes indicated that the greatest clearing zones among the locally isolated fungi were observed with *Aspergillus niger* AUMC 4156 (22 mm) and *Paecilomyces variotii* AUMC 4149 (21 mm), which was mostly comparable with the pectinolytic activity of the imported strain *Aspergillus niger* NRRL 337 (23 mm). Studying the environmental and nutritional factors leading to maximum pectinase (s) production by these three strains showed that, the optimum incubation temperature and period for pectinase (s) production by *Aspergillus niger* NRRL 337, *Aspergillus niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149 were 30˚C and 5days, respectively. The optimum pH values were 5 – 6, 5 and 6 for *A. niger* NRRL 337, *A. niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149, respectively. The best inoculum size was 1% for *Aspergillus niger* NRRL 337, and 2% for both *Aspergillus niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149. Pectin at concentration of 2% proved to be the best carbon source followed by starch for all the studied fungal strains. Also, it was found that, the maximum enzyme production was attained by using of 2% yeast extract as the sole nitrogen source for all the studied fungi.

Introduction.

Pectic substances are glycosidic macromolecules of high molecular weight compounds that are widespread in the plant kingdom. They form the major components of the middle lamella, a thin layer of adhesive extracellular material found between the primary walls of adjacent young plant cells. In

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addition, they constitute an important part of the primary plant cell wall (Kertesz, 1951).

The enzymes that hydrolyze pectic substances are broadly known as pectic enzymes, pectinases, or pectinolytic enzymes, which include pectinesterase,polygalacturonase, pectin lyase and pectate lyase on the basis of their mod of action (Alkorta *et al*., 1998).

The production of pectinase has been widely reported in bacteria and filamentous fungi (Jenson and Olsen, 1999). On the other hand, pectinases are not produced commercially from bacteria because of the high costs of production, although highly productive constitutive strains are known (Lei *et al*., 1985 and Chatterjee *et al*., 1995). The production of pectinases is not widespread in yeasts, very few yeasts show this ability (Luh and Phaf, 1951; Vaugn *et al*., 1969; Winborne and Richard, 1978; Lim *et al*., 1980; Federici, 1985 and Barnby *et al*., 1990).

The most important enzymes of the pectinases complex are polygalacturnase (PG; EC 3.2.1.15), pectin lyase (PL; EC 4.2.2.10), pectate lyase (PAL; EC 4.2.2.2), and pectinesterase (PE; EC 3.1.1.11) (Godfrey and West, 1996). Pectinases production by filamentous fungi varies according to the strains, the composition of the growth medium and the cultivation condition (pH, temperature, aeration, agitation and incubation time) (Fiedurek *et al*., 1989).

The present study was aimed to evaluate the ability of the natural mycoflora isolated from different citrus fruits to produce the pectinase enzymes. The optimum environmental and nutritional conditions for the enzyme production were also studied.

Materials and methods.

Microorganisms used:

Twenty fungal strains, isolated from infected and decayed citrus fruits (oranges, lemons and mandarins) obtained from the local market of Assiut city, Assiut, Egypt, in addition to the imported strain, *Aspergillus niger* NRRL 337 which is known as a highly amylase-producing strain (Ragab, 1989), were used in the present study. All the isolated fungal cultures were identified at Assiut University Mycological Center (AUMC) Table 1. *Aspergillus niger* NRRL 337, was obtained from the fermentation laboratory of the Northern Regional Research Laboratory of U.S.A. All the fungal cultures were grown on slant surfaces of potato dextrose agar medium and stored in a refrigerator at 5˚C.

Pectic substances:

All the pectic substances were obtained from Sigma-Aldrich chemical company, Germany.

Microbiological procedure:

Isolation, screening and identification of pectinolytic fungi:

Pectinase–producing fungi were isolated from various types of citrus fruits such as oranges, lemons and mandarins, with special attention given to rotting and

decaying fruits. The dilution-plate method (Johnson and Curl, 1972) was applied for isolation of fungi using Czapek's agar medium. Chloromphenicol (20µg/ml) and rose Bengal (30 ppm) were used as bacteriostatic agents. Three plates of each fruit sample were prepared and incubated at 30˚C for one week. The developing fungal colonies were isolated and tested for their pectinolytic activities.

Seventy five fungal isolates were isolated from citrus fruits as previously described. All the isolated fungi, in addition to the imported strain, *Aspergillus niger* NRRL 337 were examined for their pectinolytic activity on the basis of utilization of pectin as a sole carbon source in the pectin mineral salt agar medium as described by Ammar *et al.* (1995).All isolates which proved to possess pectinolytic activity were identified by Assiut University Mycological Center (AUMC), as shown in Table (1).

Cultivation of fungal spores and preparation of crude pectinase extracts:

Microbiological procedure was carried out in 250 ml Erlenmeyer flask; each flask contains 100 ml of pectin mineral salt broth medium (Pectin, 10; $(NH_4)_2HPO_4$, 2; $NH_4H_2PO_4$, 0.9; $MgSO_4$, 0.1 and KI, 0.5 g/L) adjusted to pH 7 and autoclaved at 121˚C for 15 minutes. After cooling at room temperature, each flask was inoculated by 1.0 ml of fungal spores suspension containing 10^7 spores per ml. Then, the flasks were incubated for 5 days at 30˚C. All experiments were carried out in triplicate. At the end of incubation period, the content of each flask was filtered through Whatman No.1 filter paper. The culture filtrates served as the crude enzyme sources.

Pectinase clearing zone (P.C.Z.) technique:

For the purpose of pectinase (s) assay, the so called pectin cup plate clearing zone (P.C.Z) technique of Ammar *et al.* (1995) was applied, with some modification as follows:

Twenty ml aliquots of sterilized P.C.Z assay medium (pectin, 1.0; Arabic gum, 5.0 and agar-agar, 15 g/L as described by Ammar *et al.* (1991)) were poured aseptically in each sterilized Petri-dish, the medium were allowed to cool and solidify. One cup was made per each plate by a sterilized corkborer (10 mm diameter), 0.1 ml of the culture filtrate was introduced into the cup, and then, the plates were incubated for 24 hours at 30˚C. At the end of incubation period, plates were flooded with 10 ml of (0.02 N) iodine solution. The transparent zone diameters (mm) were measured and recorded as an indication of pectinase (s) activities, and then means as well as standard deviation (S.D.) were calculated.

Factors affecting the production of pectinase (s):

Environmental factors:

Effect of incubation temperature:

For the identification of optimum incubation temperature for the best production of exo-polygalacturonase, 100 ml of pectin mineral salt broth medium were inoculated with 1 ml of fungal spores suspension and then incubated at 20, 25, 30, 35, 40 and 45˚C (± 1˚C) for one week. Pectinase (s) activities were estimated at the end of the incubation period as described in 2.4.

Effect of incubation period:

Hundred ml of pectin mineral salt broth medium were inoculated with 1.0 ml of fungal spores suspension and then incubated for 2, 4, 6, 8, 10, 12 and 14 days at the optimum temperature which indicated from the previous experiment.

Effect of pH values:

Pectin mineral salt broth medium was inoculated with 1.0 ml of fungal spores suspension after adjustment its initial pH with (0.2 M) acetate buffer to different pH values ranging from 3 to 8. In all experiments, the inoculated medium was incubated at the optimum temperature for optimum incubation period which obtained from experiments.

Effect of inoculum size:

In this experiment, the pectin mineral salt broth medium was inoculated with different spores suspension concentrations (0.5%, 1%, 2% and 3% v/v) for the identification of optimum inoculm size required for the best production of pectinase (s).

Nutritional factors:

Effect of different carbon sources:

Different carbon sources such as, pectin, starch, sucrose and glucose were added separately at concentration of 1% to the pectin mineral salt broth medium, which was inoculated by 1%, 2% and 2% spore suspension and incubated for 5 days at 30˚C and at pH 6, 5 and 6 for *Aspergillus niger* NRRL 337, *Aspergillus niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149, respectively. Pectinase (s) activities were estimated at the end of the incubation period using P.C.Z technique.

Effect of different nitrogen sources:

The nitrogen source of the synthetic medium was replaced by different nitrogen sources namely, yeast extract, peptone, sodium nitrate and ammonium sulfate, that added separately at 1% concentration to the pectin mineral salt broth medium, containing 2% pectin. All flasks were inoculated by1%, 2% and 2% spore suspension and incubated for 5 days at 30˚C and at pH 6, 5 and 6 for *Aspergillus niger* NRRL 337, *Aspergillus niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149, respectively. Pectinase (s) activities were estimated at the end of the incubation period using (P.C.Z.) technique.

Results and discussion.

Isolation, screening and Identification of the pectinolytic fungi:

Seventy five fungal isolates were isolated from decaying citrus fruits. All of these isolates were screened for their pectinolytic activity. Thirty six out of seventy five isolates proved to have pectinolytic activity, since they showed varied ability to hydrolyzed pectin as a sole carbon source in the cultivation medium. The thirty six pectinolytic isolates were subjected to the identification tests at Assiut University Mycological Center (AUMC). Data of the identification program (Table 1) indicated that these isolates represent twenty strains belonging to eleven species appertaining to six genera of fungi. It could be observed that, *Aspergillus* was the first predominant genus encountering in 50% of the total isolated fungi. Five species of *Aspergillus* were identified of which, *A. flavus* was the most prevalent species, followed by *A. niger* and *A. sydowii*. The second higher incidence rate was recorded for the genera of *Cladosporium* and *Penicillium*. The genus *Cladosporium* was represented by two species, *C. cladosporioides* and *C. sphaerospermum*. Meanwhile, *Penicillium* was recorded by three strains of *P. oxalicum*. *Paecilomyces variotii* was occurred twice, while each of *Alternaria alternata* and *Emericella nidulans* was detected as a single representative of their genera and infrequently encountered.

Table (1): List of fungal strains isolated from decayed citrus fruits and their code numbers as recorded at Assiut University Myco logical Center (AUMC).

Fungal strains	code	number
Alternaria alternate	AUMC	4151
Aspergillus flavus	AUMC	4150
Aspergillus flavus	AUMC	4155
Aspergillus flavus	AUMC	5029
Aspergillus flavus	AUMC	5032
Aspergillus niger	AUMC	4156
Aspergillus niger	AUMC	5030
Aspergillus parasiticus	AUMC	4158
Aspergillus sydowii	AUMC	4152
Aspergillus sydowii	AUMC	4159
Aspergillus versicolor	AUMC	4157
Cladosporium cladosporioides	AUMC	5035
Cladosporium cladosporioides	AUMC	5037
Cladosporium sphaerospermum	AUMC	5033
Emericella nidulans	AUMC	5034
Paecilomyces variotii	AUMC	4149
Paecilomyces variotii	AUMC	4154
Penicillium oxalicum	AUMC	4153
Penicillium oxalicum	AUMC	5031
Penicillium oxalicum	AUMC	5036

Screening of fungal strains for pectinase (s) production:

The results in Table (2), illustrated by figure (1) showed that all the tested fungal strains were positive for pectinase (s) production in P.C.Z assay, as evidenced by the resulted clearing zones.

Variation from 9 mm (*Aspergillus versicolor* AUMC 4157) to 23 mm (*Aspergillus niger* NRRL 337) in diameter of clearing zone indicating pectinase (s) activities were observed. The greatest clearing zones among the locally isolated fungi were observed with *Aspergillus*

niger AUMC 4156 (22 mm) and *Paecilomyces variotii* AUMC 4149 (21 mm), which was mostly comparable with the pectinolytic activity of the imported strain *Aspergillus niger* NRRL 337 (23 mm).

These results are in rather agreement with those reported by Souza *et al*. (2003), who found that *Paecilomyces clavisporus* 2A.UMIDA.1, *Aspergillus* 2A.UMIDO and *Penicillium* 2A.SECO were the best pectinase (s) procedures with the largest clearing zone in diameter (26 mm, 25 mm and 24 mm), respectively.

Table (2): Screening of fungal strains for pectinase (s) production.

Fungal strains	Pectinase(s) activity(diameter of clearing zone, mm)
Aspergillus niger NRRL 337	23
Aspergillus niger AUMC 4156	22
Paecilomyces variotii AUMC 4149	21
Aspergillus niger AUMC 5030	20
Penicillium oxalicum AUMC 5031	20
Penicillium oxalicum AUMC 5036	20
Alternaria alternata AUMC 4151	19
Penicillium oxalicum AUMC 4153	19
Paecilomyces variotii AUMC 4154	19
Emericella nidulans AUMC 5034	19
Aspergillus sydowii AUMC 4152	18
Aspergillus flavus AUMC 4150	17
Aspergillus flavus AUMC 4155	15
Aspergillus sydowii AUMC 4159	15
Cladosporium cladosporioides AUMC 5035	15
Aspergillus parasiticus AUMC 4158	14
Aspergillus flavus AUMC 5029	14
Cladosporium sphaerospermum AUMC 5033	13
Cladosporium cladosporioides AUMC 5037	13
Aspergillus flavus AUMC 5032	11
Aspergillus versicolor AUMC 4157	9

Aspergillus niger

Cladosporium cladospor-

Emericella nidulans

Aspergillus versi-

Fig. (1): Screening of fungal strains for pectinase (s) production using pectinase clearing zone (P.C.Z) assay.

Factors affecting the pectinase (s) production by *Aspergillus* niger NRRL 337, *Aspergillus niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149:

Effect of incubation temperature:

Figure (2) showed that 30˚C was the most optimal temperature giving maximal pectinase (s) activities for all tested fungal strains. Production of pectinase (s) was increased gradually with increasing the incubation temperature from 20 to30˚C and then turned to the gradual decrease with increasing the incubation temperature from 30 to 45˚C.

These results agree with those obtained by **Ammar *et al.* (1995)** who found that *Aspergillus niger* S-48 TAT was the most potent pectinase (s) producer out of 13 fungal strains in P.C.Z technique using pectin mineral salt broth medium at 30˚C. while, **Souza *et al.* (2003)** found that incubation temperature of *Paecilomyces clavisporus* 2A.UMIDA.1, *Aspergillus* 2A.UMIDO and *Penicillium* 2A.SECO at 28˚C gave maximum pectinase (s) production in cup-plate assay with the largest clearing zone in diameter (26 mm, 25 mm and 24 mm), respectively

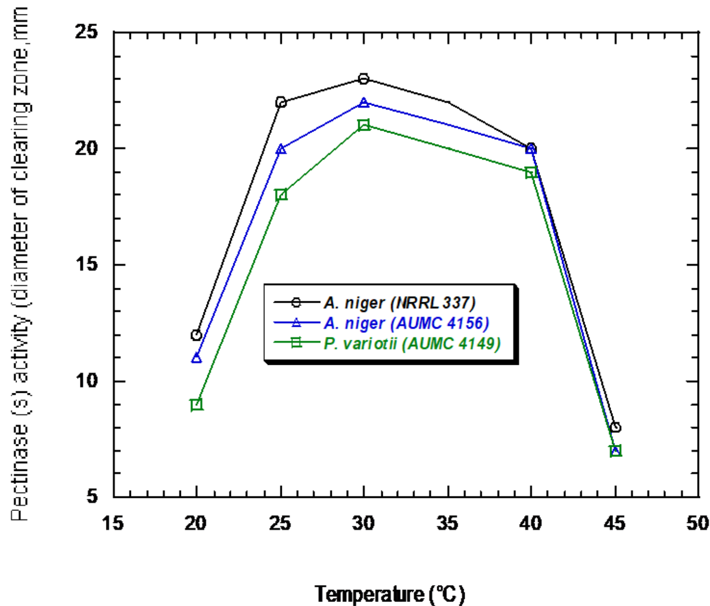

Fig.(2): Effect of incubation temperature on pectinase (s) production.

Effect of incubation period:

Data in fig (3) showed that, the rate of pectinase (s) production increased gradually as the incubation period increased, reaching the maximum value after 5 days, then dropped until it reached the minimum value after 14 days for all tested fungal strains. The reason for the decrease of pectinase production after 120 hours (5 days) may be related to the consumption of an essential compound or to the appearance of an inhibitor of enzyme biosynthesis (Mansour, 1996).

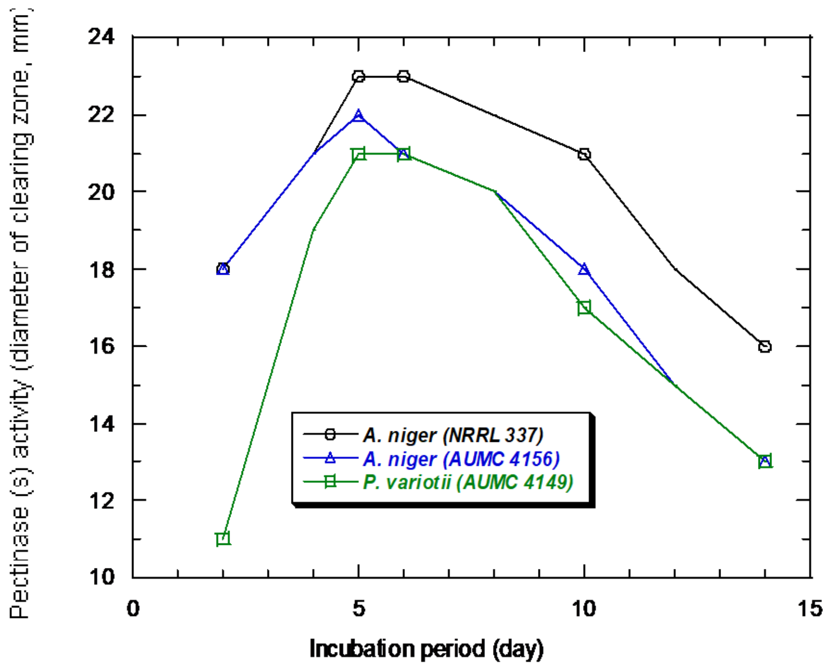

Fig. (3): Effect of incubation period on pectinase (s) production.

Results obtained in this study are similar to those observed by Ammar *et al*. (1995) who found that *Aspergillus niger* S-48 TAT produced high pectinase (s) activity out of 13 fungal strains in P.C.Z technique after 5days of incubation, Puchart *et al* (1999) found that maximum pectinolytic enzymes activities by 14 of 17 strains of *Thermomyces lanuginosus* on sugar beet pulp were recorded after 4 days of incubation. Only four strains were found to produce maximum pectinolytic enzymes after 4 days of growth on citrus pectin. Also, Souza *et al*. (2003) demonstrated that, the production of pectinases in cup-plate assay by *Paecilomyces clavisporus* 2A.UMIDA.1 reached its maximum level after 96 hours of incubation.

Effect of pH value:

Results in figure (4) indicated that, the optimum pH for the

production of pectinase (s) ranged between 5 to 6 for *Aspergillus niger* NRRL 337 while it was 5 for *Aspergillus niger* AUMC 4156 and 6 for *Paecilomyces variotii* AUMC 4149. Increasing the pH value above 6, 5 and 6 for *Aspergillus niger* NRRL 337, *Aspergillus niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149, respectively, showed decrease in pectinase (s) activity.

These results were rather in agreement with those obtained by Martins *et al*. (2002) who noted that, the optimum pH for maximum production of pectinase enzymes by *Thermoascus aurantiacus* 179-5 was 5.0. On the other hand, the present results are rather differed from those described by Ammar *et al*. (1995) who found that, the highest pectinase (s) activities was achieved at pH 7 in P.C.Z technique by *Aspergillus niger* S-48 TAT. While Souza et al. (2003) reported that, the highest production of pectinases in cup-plate assay by *Paecilomyces clavisporus* 2A.UMIDA.1 was achieved at pH 2.5.

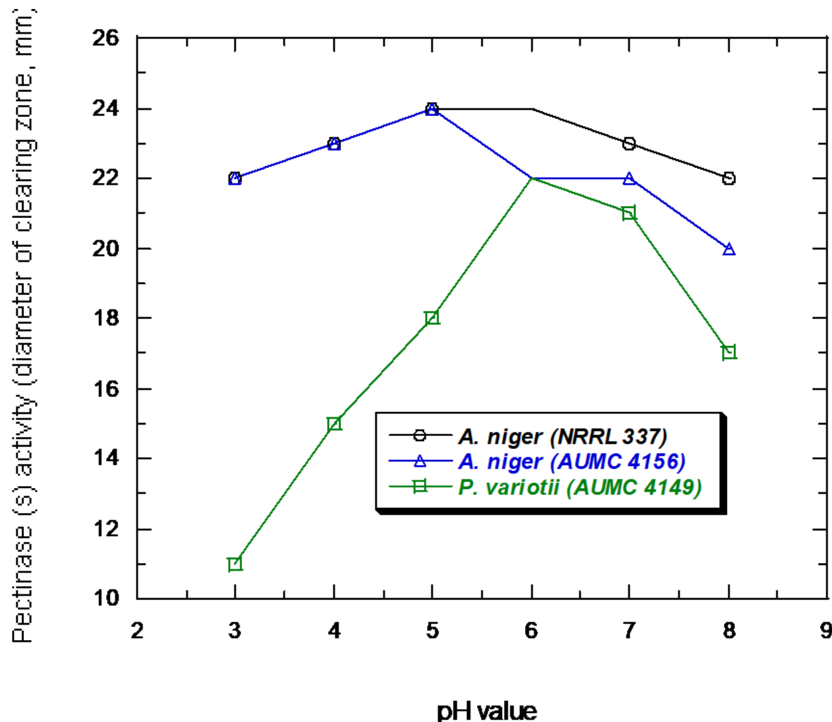

Fig. (4): Effect of different pH values on pectinase (s) production.

Effect of inoculum size:

Fig (5) showed that, the pectinase (s) activities increased as the inoculum size increased from 0.5 to 1%, in case of *Aspergillus niger* NRRL 337 and from 0.5 to 2%, in case of *Aspergillus niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149. Therefore, 1% and 2% were selected as the best economical inoculum sizes for *Aspergillus niger* NRRL 337, *Aspergillus niger* AUMC 4156 and *Paecilomyces variotii* AUMC 4149, respectively.

These results are in accordance with these obtained by Souza *et al.* (2003) who obtained high production of pectinases in cup-plate assay using liquid medium inoculated with 10^4 spores/ml of *Paecilomyces clavisporus* 2A.UMIDA.1.

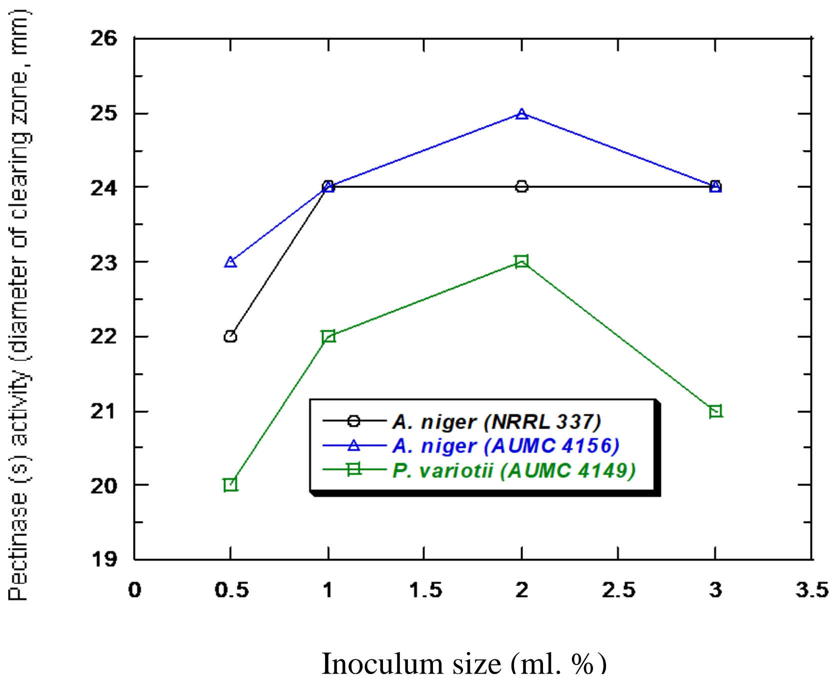

Fig.(5): Effect of inoculm size on pectinase (s) production.

Effect of different carbon sources:

The results given in fig (6) indicated that pectin was the best carbon source for pectinase (s) production followed by starch for all the studied fungal strains. These results are in agreement with those obtained by Aguilar and Huitron (1987) who reported that the production of pectinase enzymes from several mould was enhanced by the presence of pectic substances in

the medium. Lack of pectinolytic activity in cultures supplemented with glucose as a sole carbon source reflects the inducible nature of pectic enzymes from *Aspergillus niger* (Fawole and Odunfa, 2003).

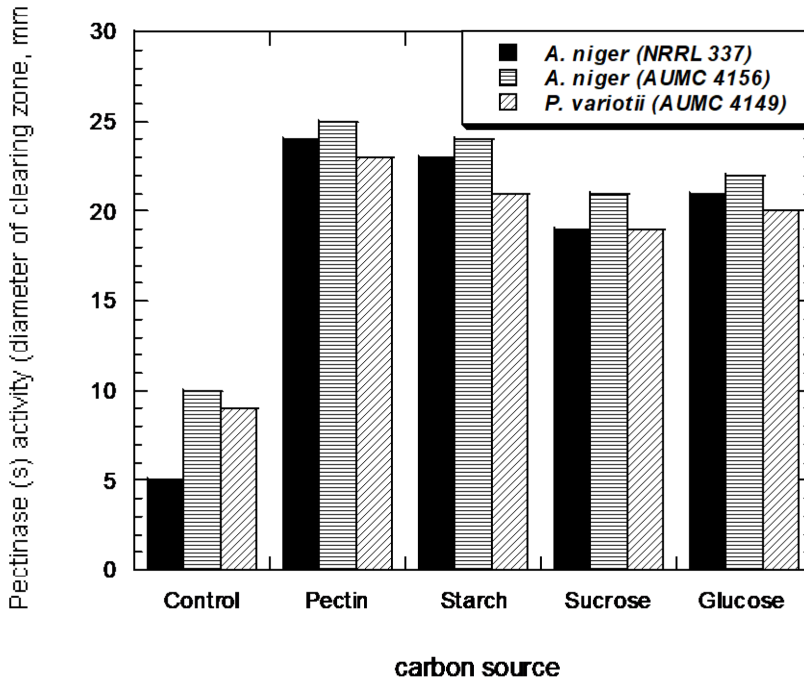

Fig.(6): Effect of different carbon sources on pectinase (s) production.

Effect of different pectin concentrations:

To study the effect of different concentrations of pectin that could stimulate the highest pectinase (s) production by the same fungal strains. Pectin was added to the pectin mineral salt broth medium at concentrations of 0.5, 1, 2, 3 and 4% (w/v).

Results recorded in fig (7) clearly showed that, the accumulation of pectinase (s) was increased gradually as the pectin concentration increased reaching the maximum level at 2% concentration for all tested fungal strains. The same stimulatory effect was observed at 3% pectin concentration, but at higher pectin concentration, the enzyme production tended to decrease.

The present results are rather differ from those described by Ammar *et al*. (1995) found that, the production of pectinase (s) enzymes by *Aspergillus niger* S-48 TAT was enhanced when the

pectin used a sole carbon source in the basal medium at concentration of 1%. While Souza *et al.* (2003) found that, 0.5% citrus pectin in the liquid medium as a sole carbon source promoted the highest production of pectinases in cup-plate assay by *Paecilomyces clavisporus* 2A.UMIDA.1.

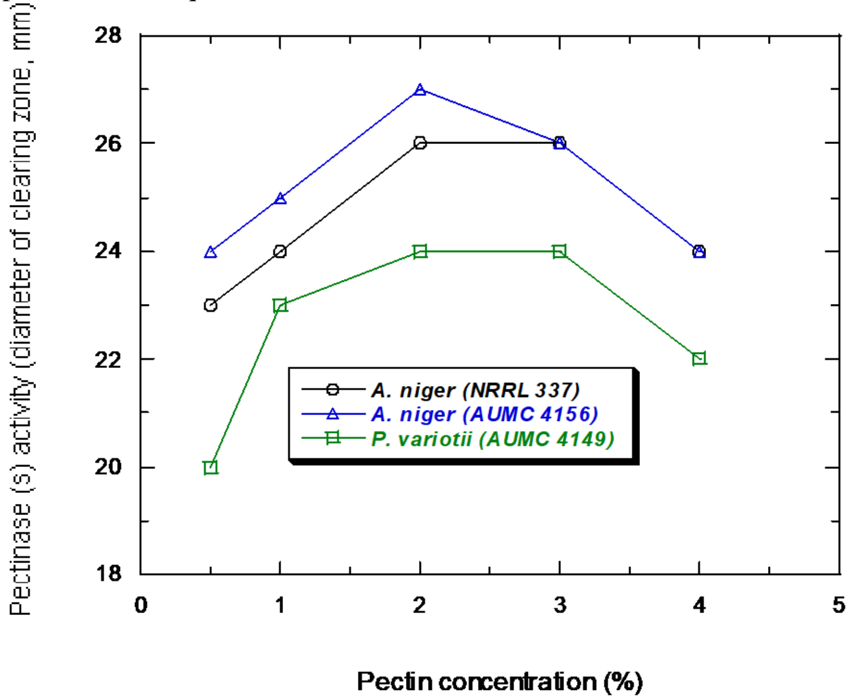

Fig.(7): Effect of different pectin concentrations on pectinase (s) production.

Effect of different nitrogen sources:

Data represented fig (8) indicated that yeast extract was the most favourable nitrogen source for the stimulation of Pectinase (s) production followed by peptone for all the studied fungal strains.

These results are agree to an extent with those obtained by **Souza *et al.* (2003)** who found that, the high pectinases production in cup-plate assay by *Paecilomyces clavisporus* 2A.UMIDA.1 was obtained with 1% yeast extract. However, these results differed from these obtained by Antov and Pericin (2003) who reported that, the higher ammonium sulphate concentration increased total pectinases production by *Polyporus sqamosus*.

Effect of different yeast extract concentrations:

An experiment was conducted to investigate the effect of different concentration of yeast extract as a sole source of nitrogen

on pectinase (s) production, yeast extract was added to the pectin mineral salt broth medium at concentrations of 0.5, 1, 2, 3 and 4% (w/v). Results in figp (9) showed that, the optimum concentration of yeast extract that secured the highest pectinase (s) activities was 2% for all tested fungal strains. It also showed that pectinase (s) activities increased gradually with increasing the concentration of added yeast extract from 0.5 to 2%. Addition of yeast extract at concentrations higher than 2% did not show any further stimulation for pectinase (s) production.

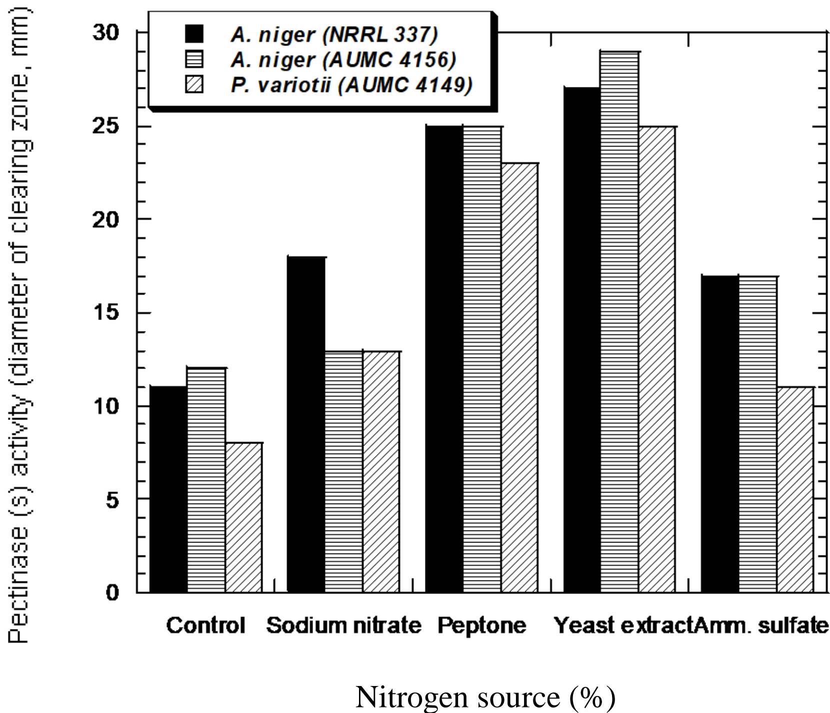

Fig.(8): Effect of different nitrogen sources on pectinase (s) production.

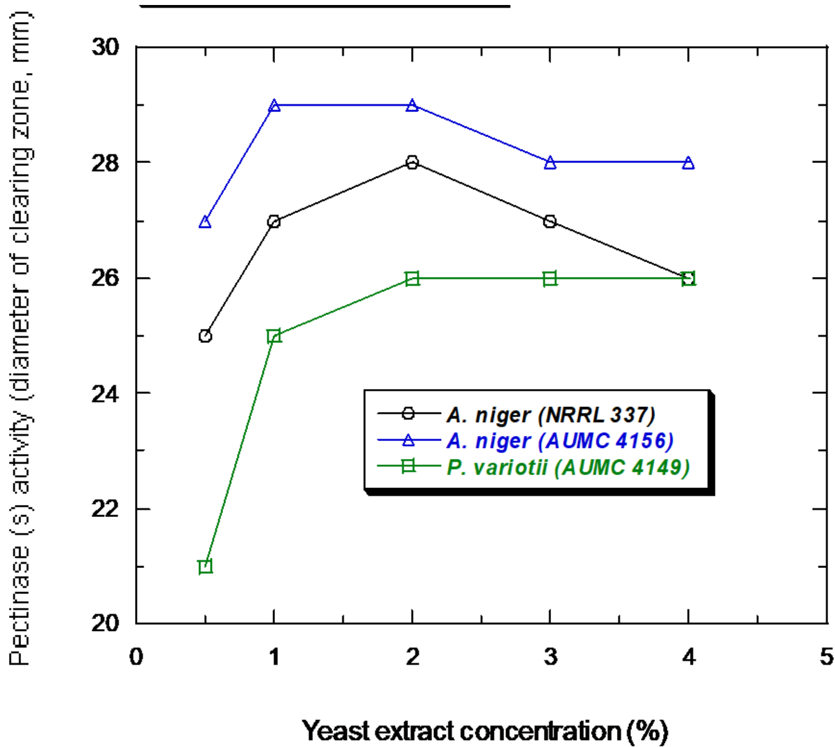

Fig.(9): Effect of different yeast extract concentrations on pectinase (s) production.

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إنتاج إنزيمات البكتينيز بواسطة الفطريات الملوثة لثمار الموالح

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الملخص العربي

تناول البحث اختبار خمسة وسبعون مزرعة فطرية معزولة من ثمار الموالح المصابة من حيث نشاطها في تحليل البكتين وأظهرت ستة وثلاثون مزرعة منها قدرتها على إنتاج إنزيمات البكتينيز.تم تعريف تلك المزارع ووجد أنها تمثل عشرون سلالة تنتمي إلى احد عشر نوعا تابعة لستة أجناس فطرية وكانت السيادة لجنس الاسبرجلس ويليه جنسي الكلادوسبوريم والبنسليوم.

تم تقييم قدرة السلالات العشرين على إنتاج إنزيمات البكتينيز وكانت السلالتان *Paecilomyces variotii* AUMC و*Aspergillus niger* AUMC 4156 4149هما الأعلى انتاجاً للإنزيم وكانت قدرتهما في ذلك مقارب قدرة السلالة المستوردة *A.niger* NRRL 337 المعروفة بكفاءتها كمنتج للإنزيم. اتضح من دراسة العوامل البيئية والتغذوية المؤدية إلى تعظيم إنتاجية الإنزيم بواسطة السلالات الفطرية الثلاثة سالفة الذكر ما يلي:-

- درجة الحرارة المثلى للإنتاج هي 30 درجه مئوية. وأفضل فترة تحضين هي 5 ايام لجميع السلالات المختبره.
- أفضل درجة pH لبيئة الإنتاج كانت في مجال5 – 6 في حالة سلالة فطر *Aspergillus niger* NRRL 337 وكانت 5 في حالة سلالة فطر *Aspergillus niger* AUMC 4156 وكانت 6 في حالة سلالة فطر *Paecilomyces variotii* AUMC 4149 pH.
- أفضل حجم لقاح هو 1 % في حالة فطر *Aspergillus niger* NRRL 337 بينما كان 2 % في حاله السلالتين *Aspergillus niger* AUMC 4156 و سلالة *Paecilomyces variotii* AUMC 4149.
- ثبت أن إضافة البكتين بتركيز 2% إلى بيئة الإنتاج تمثل أفضل مصادر الكربون لجميع السلالات ويلي ذلك النشا.
- تحققت أعلى إنتاجية للإنزيم عند إضافة مستخلص الخميرة بتركيز2% كمصدر وحيد للنيتروجين في بيئة الإنتاج لجميع السلالات الفطرية المختبرة.