Pak. J. Phytopathol.,
Vol. 21 (1): 77-82, 2009.

STATUS OF *ASPERGILLUS NIGER* STRAINS FOR PECTINASES PRODUCTION POTENTIAL

Abdul Hannan, Rukhsana Bajwa and Zakia Latif
Institute of Mycology and Plant Pathology, University of the Punjab, Lahore 54590, Pakistan
mpplpu@yahoo.com

ABSTRACT

A multistep evaluation of 52 strains of *Aspergillus niger* was performed on the basis of polygalacturonase production. Plate method was employed for preliminary screening of isolates. The strains exhibiting relative clear zone with diameter above 1.386 were selected for final screening. Out of 52 strains, 23 were selected for further screening by using optimized enzyme assay method. The enzyme production by 23 selected strains was studied in submerged fermentation with pectin as the only carbon source. Maximum activity was observed in two isolates of *A. niger* (H12 and H51) showing more than 0.700 units after 96 hrs incubation period. The four isolates (H06, H13, H45, and H46) gave the maximum activities after incubation for 72 h whereas decline was observed at 96 h.

Key words: *Aspergillus niger* pectinase

INTRODUCTION

Pectinases are a heterogeneous group of enzymes that catalyze the breakdown of pectin containing substrates. In commercial terms pectinases refer to a mixture of primarily three different enzymatic activities: polygalacturonase (PG), pectinesterase (PE) and pectinlyase (PL). All three activities contribute to the breakdown and modification of pectins from wide variety of plant materials. Recently pectinases have become more widely used in food, textile and other industrial sectors (Semenova *et al*., 2003). These are widely used in the fruit and vegetable processing industries to increase the juice production and to clarify juices, to improve the cloud stability of juices and nectars, for depectinization in order to produce high density fruit juice concentrate, and for haze removal from wines. (Alkorta *et al*., 1998; Corredig and Wicker, 2002). Several microorganisms such as bacteria, yeasts and fungi are known to produce a wide range of pectinases with varying physico-chemical and enzymatic properties (Alkorta *et al.*, 1998). *Aspergillus niger* is one of the most important microorganisms used in biotechnology. The synthesis of pectinases can be induced or stimulated by the presence of pectin, and for economic reasons this is normally supplied by adding sugarbeet or apple bagasse or citrus fruit rind and wheat bran to the culture medium (Rombouts and Pilnik, 1980).

Keeping in view the importance of pectinases in fruit and vegetable processing industries, a multistep screening of collected strains of *Aspergillus niger* from different sources was employed to evaluate their natural enzyme production potential. Previously many waste products from agricultural industry have been used as carbon source to induce pectinases production by different microorganisms. With increased application of pectinases, low production cost has become one of the most important target. For this purpose citrus peel was used as a carbon source for the induction of pectinases by *A .niger*. It is also required to adapt further measures to increase the production potential to meet the local demand that is increasing day by day.

MATERIALS AND METHODS

Isolates and culture conditions: *A .niger* strains were isolated from different sources (Table 1) at Institute of Mycology and Plant Pathology, University of the Punjab, Lahore, Pakistan.

Isolation of *Aspergillus niger:* Infected materials were washed thoroughly with tap water and sterilized by immersion in 0.1% NaOCl for 10 minutes. After washing three times in sterilized distilled water, the infected material was covered with distilled water and teased with sterilized needle to allow discharge of spores. The resulting spore suspension was used to inoculate 2% water agar plates. Plates were incubated at 30 ±2ºC for 3-4 days. Colonies arising from single spores were used to inoculate plates containing Czepak Dox medium ($FeSO_4$ 0.01, KCl_2 0.5, $MgSO_4$ 0.5, K_2 HPO_4 1.0, $NaNO_3$ 3.00, glucose 20, agar 20 g/L with pH 6.5). These plates were incubated at 30 ±2°C for six days. Purified and identified colonies arising from single spore culture were selected for further studies.

Preparation of substrate: Citrus peel was collected from the local market of Lahore. It was dried at 70^0C, for 3 days and ground to powder (CPP-citrus peel powder) by using an electrical grinder. This powder substrate was fractionated using sieve of Mesh No. 20. The sieved powder substrate was stored at room temperature and used in further studies. Chemicals of analytical grade were used in all experiments.

Preparation of inoculum/spore suspension: In order to produce copious number of spores for inoculum, fresh citrus peels were chopped into small pieces. About 20 g of chopped citrus peel were autoclaved in a 100 mL flask for 30 min. A loopfull of mycelium from Czepak Dox medium plates was added to the flask and was shaken to ensure equal distribution of inoculum. After incubation at 30 ±2°C for 8 –10 days, the infected citrus peels were agitated with distilled water to give a resulting spore suspension of 10^6 spores/mL with the help of haemacytometer.

Preliminary screening of *A. niger* strains by plate method: Petri plates containing Czepak Dox medium supplemented with 2% pectin instead of glucose as carbon source were prepared. Three wells were made with sterilized cork borer in each petri plate under aseptic conditions. Conidial suspension of *A. niger* was adjusted by haemacytometer @ 10^{6} spore/mL. This conidial suspension was poured into each well @ 50µL and plates were incubated at 30 ±2°C. After 6 days of incubation period, the plates were flooded with 0.05% ruthenium red solution for one hour and then washed with tap water. The diameter of colonies and clear zones were measured. The relative clear zones were calculated with the following formula:

$$\text{Relative clear zone} = \frac{\text{Dia. of clear zone}}{\text{Dia. of fungal colony}}$$

Submerged fermentation: Erlenmeyer flasks containing 30 mL Czepak Dox liquid medium with 2% pectin instead of glucose was inoculated with 3 mL of spore suspension (10^6spore/mL) and incubated at 30 ±2 °C on orbital shaker at 150 rpm for 4 days. The experiment was performed in four replicates and one flask after every 24 h was used to perform the assay for determining the enzyme activity. Culture filtrates (CF) were harvested by passing the incubated mixture through Whatman No. 1 filter paper. The crude culture filtrates (CF) were processed immediately for enzyme assay.

Enzyme assay: Polygalacturonase activity in cell free culture filtrate (s) of screened *A. niger* was assayed by quantifying reducing sugars using DNS (3-5-dinitrosalicylic acid) method (Miller, 1959) and galacturonic acid as a reference. The reaction mixture containing 1mL of CF, 1mL of 0.9% polygalacturonic acid in 0.1M sodium acetate buffer (pH 4.5) was incubated at 45^0C for 30 min. (One unit of polygalacturonase was defined as the amount of enzyme that liberates one µmol of galacturonic acid per minute).
Data were statistically analysed by applying ANOVA followed by Duncan`s Mutiple Range Test, (Steel and Torrie, 1980)

RESULTS

Aspergillus niger colonies were isolated from different sources (Table 1) as described in the materials and methods. The identification of *A.niger* was established by morphological characteristics as observed under the microscope (Onions *et al*. 1981). The ability of the fungus to degrade pectin was evaluated through plate assays as well as by enzyme assay under sterile environment. The fungus was grown on freshly chopped citrus peel for the production of multiple inoculums up to 8-10 days at 30 ± 2^oC (Fig.1). When 8-10 days old infected citrus peel was agitated in sterilized water, a heavy spore suspension devoid of mycelium contamination was obtained
Isolates of *A.niger,* were grown in Czapek Dox medium supplemented with 2% citrus peel powder (CPP), as described in the materials and methods. Enzyme activity of fungus free CF was investigated by well-plate method and enzyme assay (DNS method, Miller, 1959). All isolated strains of *A. niger* were evaluated by preliminary screening. Under the given experimental conditions pectin degradation by *A.niger* in well plate was evident by clear zone around the fungal colony when 6 days old culture plates were flooded with 0.05% ruthenium red solution (Fig.1). The strains exhibiting relatively large clear zones were selected for further evaluation. The strains having diameter of relative clear zone below 1.386 were discarded, and the strains showing diameter above 1.386 were selected for final screening by using optimized enzyme assay methods. Out of 52 strains, 23 were selected for further studies (Table 1). The enzyme production by 23 selected strains of *A. niger* was studied in submerged fermentation with pectin as carbon source.

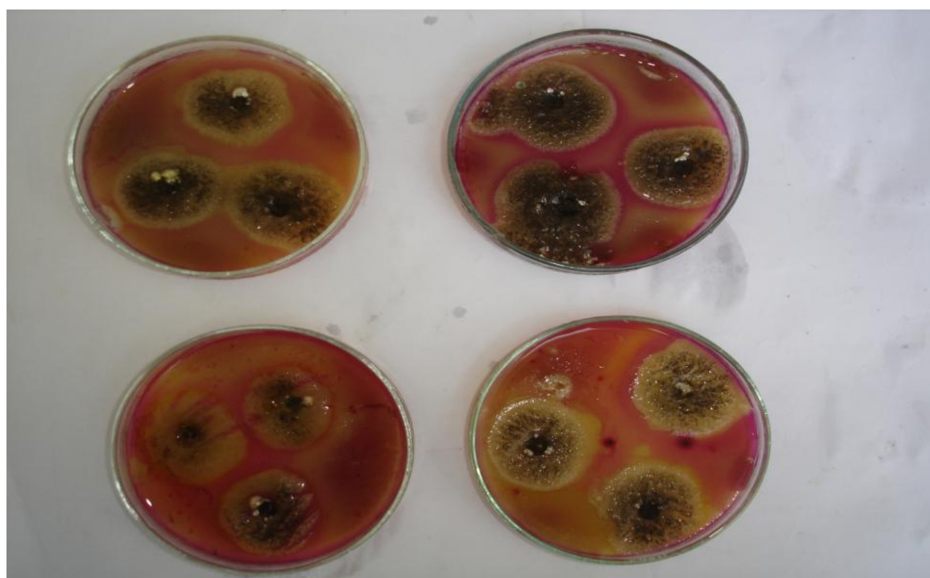
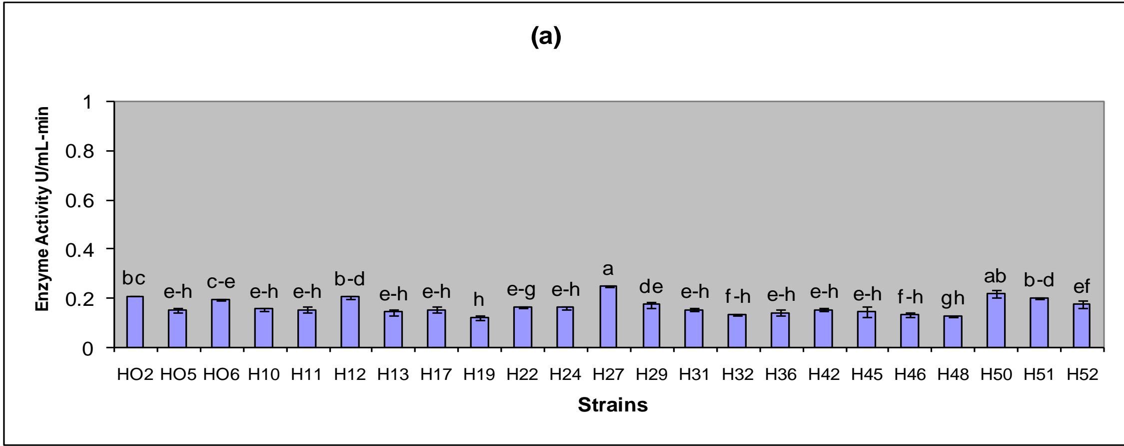


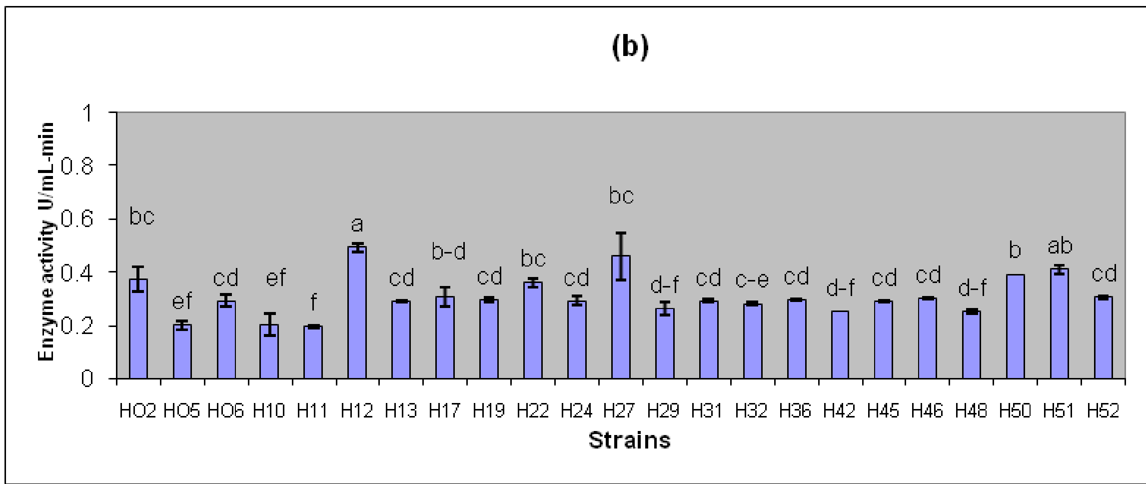
Fig. 1: Clear zone formation on plates by the growth of *Aspergillus niger* showing evidence of pectin degradation.

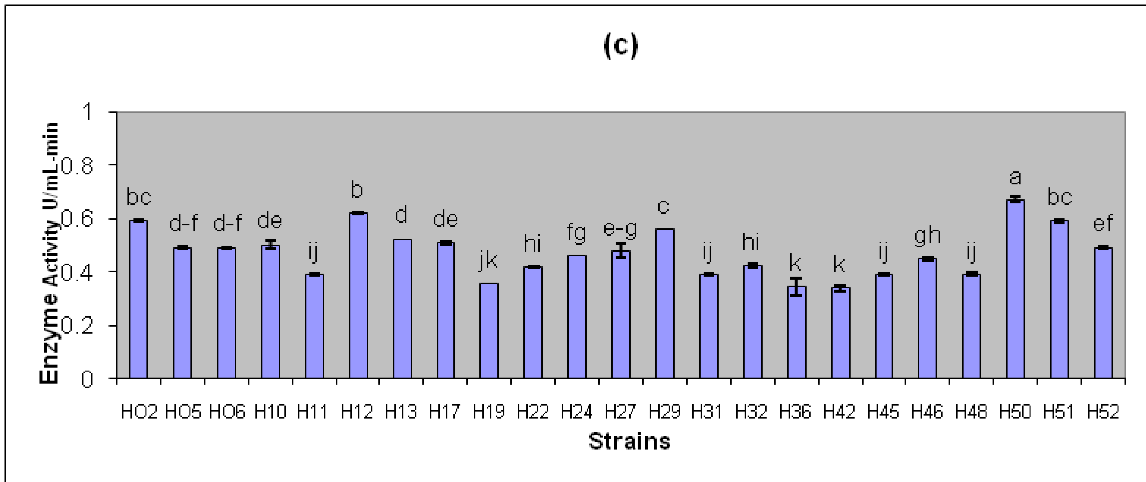
Table1: Diameter of clear relative zones by *Aspergillus niger* in descending order

Strain No.	Habitat	Relative clear zone	Strain No.	Habitat	Relative clear zone
H 27	Apple fruit debris	1.905 a*	H 09	Maize seeds	1.340 e-o
H 50	Rotten apple	1.761 ab	H 08	Air	1.338 e-p
H 51	Rotten apple	1.743 ab	H 18	Fruit debris	1.288 f-q
H 02	Citrus	1.742 ab	H 14	Air	1.283 f-q
H 12	Rotten citrus	1.740 ab	H 15	Stale bread	1.276 g-q
H 05	Apple	1.625 bc	H 25	Citrus debris	1.275 g-q
H 45	Citrus	1.570 b-d	H 01	Air	1.238 h-q
H 29	Rotten fruit	1.540 c-e	H 23	Stale bread	1.235 i-q
H 06	Rotten apple	1.520 c-e	H 39	Water	1.223 i-q
H 10	Drain water	1.490 c-g	H 03	Canal water	1.221 i-q
H 31	Fruit debris	1.461 c-g	H 38	Maiz seed,	1.210 j-q
H 17	Rotten fruit	1.446 c-g	H 04	Stale bread	1.206 K-q
H 32	Water	1.426 d-i	H 35	Water	1.206 k-q
H 42	Water	1.425 d-i	H 44	Stale bread	1.198 l-q
H 46	Apple	1.420 d-j	H 20	Water	1.195 m-q
H 48	Air	1.411 d-k	H 37	Air	1.190 m-q
H 22	Air	1.410 d-k	H 33	Air	1.86 m-q
H 19	Stale bread	1.406 d-l	H 30	Stale bread	1.168 n-q
H 24	Stale bread	1.406 d-l	H 47	Soil	1.146 n-q
H 13	Stale bread	1.395 d-m	H 34	Peanut (dry fruit)	1.146 n-q
H 36	Soil	1.393 d-m	H 21	Air	1.145 o-q
H 52	Maize seeds	1.386 d-m	H 26	Air	1.130 p-q
H 11	Water	1.386 d-m	H 43	Water	1.118 q
H 7	Fruit debris	1.355 e-n	H 49	Water	1.118 q
H 16	Air	1.351 e-o	H 40	Air	1.113 q
H 28	Fruit debris	1.343 e-o	H 41	Air	1.090 q

*Values with different letter showing significant difference (p=0.05) as determined by DMR Test.


(a)
Enzyme Activity U/mL-min
1
0.8
0.6
0.4
0.2
0
bc e-h c-e e-h e-h b-d e-h e-h h e-g e-h a de e-h f-h e-h e-h e-h f-h gh ab b-d ef
HO2 HO5 HO6 H10 H11 H12 H13 H17 H19 H22 H24 H27 H29 H31 H32 H36 H42 H45 H46 H48 H50 H51 H52
Strains


(b)
Enzyme activity U/mL-min
1
0.8
0.6
0.4
0.2
0
bc ef cd ef f a cd b-d cd bc cd bc d-f cd c-e cd d-f cd cd d-f b ab cd
HO2 HO5 HO6 H10 H11 H12 H13 H17 H19 H22 H24 H27 H29 H31 H32 H36 H42 H45 H46 H48 H50 H51 H52
Strains


(c)
Enzyme Activity U/mL-min
1
0.8
0.6
0.4
0.2
0
bc d-f d-f de ij b d de jk hi fg e-g c ij hi k k ij gh ij a bc ef
HO2 HO5 HO6 H10 H11 H12 H13 H17 H19 H22 H24 H27 H29 H31 H32 H36 H42 H45 H46 H48 H50 H51 H52
Strains

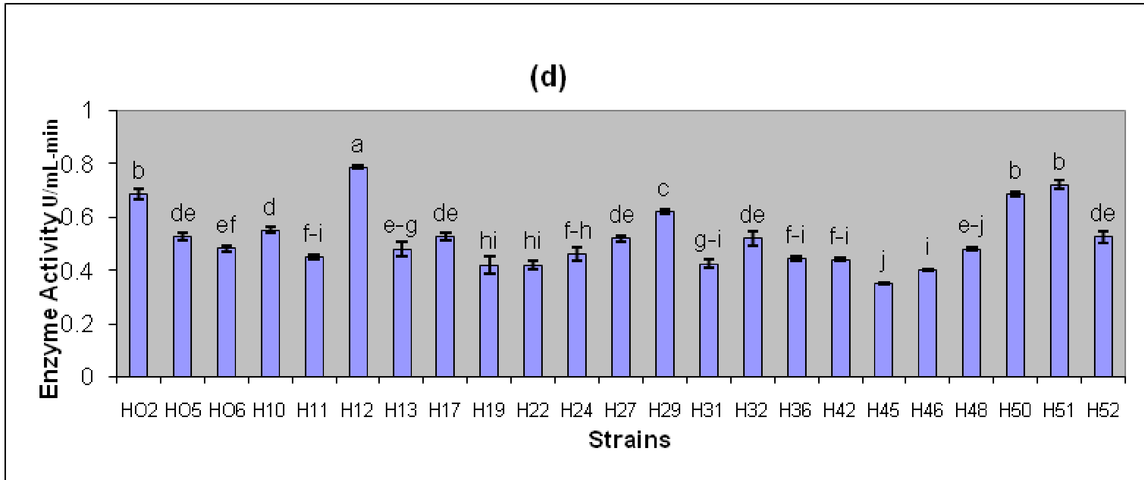


Fig. 2a,b,c & d: Polygalacturonase activity after different incubation periods by 23 strains of *Aspergillus niger*: a) 24 h, b) 48 h, c) 72 h & d) 96 h. Vertical bars show standard error of means of three replicates. Bars with different letters show significant difference ($P \leq 0.05$) as determined by DMR test.

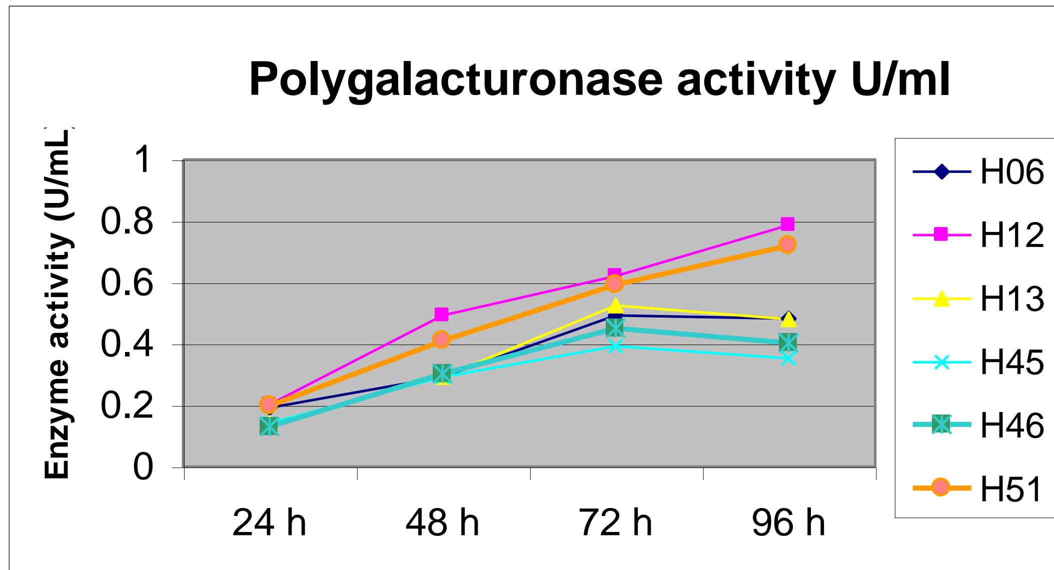


Fig.3: Kinetics of six isolates of *A. niger* showing production of polygalacturonase. Culture filtrates of strains H06, H13, H45 and H46 showed maximum activity after 72 hours incubation period whereas in case of H12 and H51, maximum activity was observed at 96 hours.

Table 2: ANOVA for the evaluation of activity of polygalacturonase by 23 strains of *Aspergillus niger* after 96 hours.

Sources of variation	df	SS	MS	F values
Treatments	91	44.109	0.485	540.530***
Strain (S)	22	1.200	0.055	60.870***
Time (T)	3	5.467	1.822	2033.418***
S × T	66	0.561	0.009	9.492***
Error	184	0.165	0.001	
Total	275	44.274		

***= significant at $P \leq 0.001$.

In order to investigate the quantitative levels of polygalacturonase at different stages of fungal growth, the content of static cultures were resolved in Czepak Dox medium supplemented with CPP. *A. niger* was grown for 96 h and samples of fungus-free culture filtrates were collected after every 24 h. It was observed that production of enzyme varied considerably among different isolates of *A. niger* (Fig.2). Greatest variation in the polygalacturonase activity was recorded by 23 isolates of *A. niger*. Culture filtrates of strain No. H13, H19, H22, H31and H45 gave the least activity < 0.4 units per mL. Strain No. H12 showed the highest average activity after 96 hrs of incubation period whereas strain No. H02 and H50 were also good for the production of polygalacturonase. In remaining isolates, there was continuous increase in activity up to 96 h. It was interesting to note that subsequent incubation of the fungus cultures lead to gradual increase in activity except in few isolates. The present data demonstrate that maximum enzyme activity was exhibited at 96 h incubation period. It was observed that maximum activity varied significantly from one isolate to the other. Four isolates i.e. H06, H13, H45 and H46 gave the maximum activity after incubation for 72 h and then decline was observed at 96 h. (Fig.3) during kinetic studies. Maximum activity (0.786 and 0.700 units/ mL) was observed in H12 and H51 after incubation of 96 h. These strains were isolated from rotten citrus fruit and rotten apple respectively. Analysis of variance clearly demonstrated evaluation of activity of polygalacturonase by 23 strains of *Aspergillus niger* after 96 h (Table 2).

DISCUSSION

The polygalacturonase production by *A. niger* studied by submerged fermentation in the medium with pectin as a sole carbon source, strain H12 isolated from rotten citrus fruit indicated the best enzymatic activity of polygalacturonase i.e. 0.786 units/mL.The same results have been reported by Maldonado *et al* (1986) in which *A. niger* isolated from rotten lemons was able to produce good quality of extra-cellular pectinesterase and polygalacturonase. Studies on the selection of *Aspergillus niger* strains for production of pectinases have previously been made by several workers including Dinu and Dan (1994). They collected 109 strains of *A. niger* and subjected them for qualitative and quantitative screening to get the best producers for pectinases. All strains were tested for their exopolygalacturonase, pectinesterase and pectinlyase activities and four strains were chosen. The advantage of using microorganisms for the production of enzymes is that these are not influenced by climatic and seasonal factors and can be subjected to genetic and environmental manipulation to increase the yield (Boing, 1987). Cotty *et al*., (1995) determined the pectinases production by *Aspergillus flavus* by measuring the clear zones formed around the colonies stained with ruthenium red. Out of 87 isolates tested, 15 produced red zone after staining with dye. Major achievement of this study was to screen out most efficient pectinases producing isolates of *A. niger* from the local environment to fulfill the requirement of local demand.

REFERENCES

Alkorta I., Garbisu C., Llama M.J. and Serra J. L. (1998), " Industrial application of pectic enzymes: a review", Process Biochem. 33(1) 21-28.

Corredig M. and Wicker L. (2002), "Juice clarification by thermostable fractions of marsh grapefruit pectinmethylesterase", J. Food Sci 67 (5), 1668-1671.

Boing J. T. P. (1987), Enzyme production. In (G. Reed,ed) Prescott and Dunn,s Industrial Microbiolog, 4th ed (CBS Publishers and distributors, Delhi) 678-681.

Cotty P. J., Medronho R.A., Leite S. G. F. and Couri S. (1995), Partial purification of a polygalacturonase produced by solid state cultures of *Aspergillus niger* 3T5B. Revista de Microbiologia. 26**:** 318-322.

Dinu D. and Dan V. (1994), Selection of *Aspergillus niger* strains producing pectic enzymes. Analele Universitatii Biologie. 43: 33-37.

Miller L. G. (1959), Use of dinitrosalicylic acid reagent for determination of reducing sugars Anal Chem, 31. 426-428.

Maldonado M.C., Navarro A. R. and Callieri D. A. S. (1986), Production of pectinases by *Aspergillus* spp using differently pretreated lemon peel as the carbon source. Biotechnol Lett. 8: 501-504.

Onions A. H. S., Allsop D. and Eggins H. O. W. (1981), Smith′s introduction to industrial mycology . 7th ed., Edward Arnold London.

Roumbouts F. M. and Pilnik W. (1980), Pectic enzymes In: Economic Microbiology (Rose A. H., ed.). Aca- demic Press, London, Vol. 5, 227-282.

Semenova M. V., Grishutin S. G., Gusakov A.V., Okunev O. N. and Sinitsyn A. P. (2003). Isolation and properties of pectinases from the fungus *Aspergillus japonicus*, Biochemistry (Moscow) 68 (5) 559–569.

Steel R. G. D. and Torrie J. H., (1980), In: *Principles and procedures of statistics*. McGraw Hill Book Co., Inc. New York, U.S.A.

Pakistan Journal of Phytopathology,

Published By:
Pakistan Phytopathological Society
www.pakps.com
info@pakps.com