

PECTINASE AND POLYGALACTURONASE PRODUCTION BY A THERMOPHILIC *ASPERGILLUS FUMIGATUS* ISOLATED FROM DECOMPOSTING ORANGE PEELS

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ABSTRACT

A thermophilic fungal strain producing both pectinase and polygalacturonase was isolated after primary screening of 120 different isolates. The fungus was identified as *Aspergillus fumigatus* Fres. MTCC 4163. Using solid-state cultivation, the optimum levels of variables for pectinase and polygalacturonase (PG) production were determined. Maximal levels of enzyme activities were achieved upon growing the culture in a medium containing wheat bran, sucrose, yeast extract and $(\text{NH}_4)_2\text{SO}_4$ after 2-3 days of incubation at a temperature of 50°C. Highest enzyme activities of 1116 Ug^{-1} for pectinase and 1270 Ug^{-1} for polygalacturonase were obtained at pH 4.0 and 5.0, respectively.

Key words: thermophilic fungi, *Aspergillus fumigatus*, pectinase, polygalacturonase

INTRODUCTION

Pectinolytic enzymes catalyzing the degradation of pectic substances are of great industrial importance (28). The pectinases are required for extraction and clarification of fruit juices and wines, extraction of oils, flavors and pigments from plant materials, preparation of cellulose fibers for linen, jute and hemp manufacture (8), coffee and tea fermentations (29) and novel applications in the production of oligogalacturonides as functional food components (13). Fungal polygalacturonases used in industrial processes for juice clarification are mainly obtained from mesophilic aspergilli and penicillia (3) and the range of enzyme sources is being extended through new recombinant and non-recombinant fungal strains. Thermophilic fungi are potential sources of various industrially important thermostable enzymes, such as lipases, xylanases, proteases, amylases and pectinases. These enzymes have numerous applications in the detergent, starch, food, paper and pharmaceutical industries (17).

Higher cost of the production is perhaps the major constraint in commercialization of new sources of enzymes. Though, using high yielding strains, optimal fermentation conditions and

efficient enzyme recovery procedures can reduce the cost. In addition, technical constraint includes supply of cheap and pure raw materials and difficulties in achieving high operational stabilities, particularly to temperature and pH. Therefore, the understanding of various physiological and genetic aspects of pectinase is required for producing thermostable and acid stable strains of pectinolytic fungi.

Literature highlighting the optimization, biochemical characterization, genetics and strain improvement studies of pectinases from mesophilic fungi (7,11,18,19) is available. However, the studies on pectinases from thermophilic fungi are lacking. Considering the biotechnological importance of thermophilic fungi in the enzyme industry, the present paper reports the isolation of pectin degrading thermophilic fungi from various sources, their screening and process evaluation.

MATERIALS AND METHODS

Isolation of thermophilic fungi

Thermophilic fungi were isolated from different soil samples, compost and decomposed matter collected from the vegetable, fruit markets and composting soils of Amritsar, Jalandhar,

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Ludhiana, and Gurdaspur cities of Punjab (India). Isolation medium of following composition, gL⁻¹ (pectin, 10.0; sucrose, 10.0; tryptone, 3.0; yeast extract, 2.0; KCl, 0.5; MgSO₄.7H₂O, 0.5; MnSO₄.5H₂O, 0.01; (NH₄)₂SO₄, 2.0) supplemented with mineral salt solution of composition g/100 mL (CuSO₄.5H₂O, 0.04; FeSO₄, 0.08; Na₂MoO₄, 0.08; ZnSO₄, 0.8; Na₂B₄O₇, 0.004; MnSO₄, 0.008), 1 mL; distilled water to make 1L solution; pH 5.5-6.0 was used (27). To the above medium, ampicillin (100 mg/mL) was added to restrict bacterial growth. The inoculated plates were incubated at 50°C for 5-7 days. The cultures were further purified by sub culturing on YPSS (Yeast soluble starch agar) medium having composition, gL⁻¹ (starch, 15; yeast extract, 0.4; K₂HPO₄, 0.23; KH₂PO₄, 0.2; MgSO₄.7H₂O, 0.05; citric acid, 0.052; pH 5.5-6.0).

Screening of thermophilic fungal isolates for pectinolytic activity

Preliminary screening of isolates for pectinase production was carried out by disc plate method of Acuna-Arguelles *et al.* (2). The size of clearance zone formed around the colonies using 1% cetrimide solution corresponds to the enzymatic activity of a particular culture. The potency index of each isolate was calculated as the ratio of zone diameter to colony diameter. The cultures showing high potency index were further screened by semiquantitative plate assay method (22,23). The cultures were individually plated on YPSS medium (9) containing pectin in place of starch and incubated at 50°C. A 6 mm colony from the growing edge of the colony was transferred to 2mL citrate buffer (0.1M; pH 4.0), incubated for 1 h at 50°C under shaking conditions. The resultant extract was assayed for pectinase activity. The released sugars were estimated using DNS. On the basis of plate assay, eight cultures were selected for further studies and were compared along with three standard cultures for pectinase production by solid state culturing. The solid-state cultivation was carried out using basal medium of following composition (1), given as gL⁻¹: (pectin, 10; urea, 3.0; sucrose, 31.4; (NH₄)₂SO₄, 12.6; KH₂PO₄, 6.5; FeSO₄, 0.29; sugarcane bagasse, 231.0; pH, 5.0-5.5. The final moisture was adjusted to 70%. The flasks were inoculated with spore suspension (1 mL) containing (10⁶ spores mL⁻¹) and incubated at 50°C for 2-3 days. The crude enzyme was extracted by adding 100mL of citrate buffer (0.05M; pH 5.5) to each flask followed by filtration and centrifugation at 14400 g. The resultant extract was used for enzyme assay.

The isolate TF3, identified as *Aspergillus fumigatus* Fres. MTCC 4163 by Microbial Type Culture Collection (MTCC), Chandigarh, India, was taken up for further studies.

Production studies

The production of the pectinase was followed for 5 days; the contents of the flasks were harvested at regular intervals (24h) by adding 100ml citrate buffer (50 mM, pH 5.0) followed

by centrifugation. The resultant clear extract was assayed for pectinase and PG activities.

Effect of physico-chemical parameters

The effect of natural substrates like different complex carbon source (malt sprouts, wheat bran, rice bran and pectin from pomegranate, lemon, banana, orange and musambi) and purified commercial carbon sources (glucose, sucrose, galactose, trehalose, carboxymethyl cellulose and starch), nitrogen sources urea and (NH₄)₂SO₄ (U+N), yeast extract and (NH₄)₂SO₄ (Y+N), peptone and (NH₄)₂SO₄ (P+N), soybean meal and (NH₄)₂SO₄ (So+N), malt sprouts and (NH₄)₂SO₄ (M+N), yeast extract and NaNO₃ (Y+S), peptone and NaNO₃ (P+S), urea and NaNO₃ (U+S), incubation temperature between 30 and 80°C and effect of pH on pectinase and PG production were studied.

Analytical procedure

The pectinase and PG activities were determined using pectin and polygalactouronic acid as substrates (20). The reaction mixture (1mL) containing equal amounts of substrate (1%) prepared in citrate buffer (0.05 M pH4.4) and suitably diluted enzyme was incubated at 50°C for 30 minutes in water bath. After incubation 3 mL DNS solution was added to stop the reaction and tubes were kept in boiling water for 10 minutes. On cooling, the developed colour was read at 575 nm using UV-visible spectrophotometer (Shimadzu-Mini 1240). The amount of released reducing sugar was quantified using galactouronic acid as standard. The enzyme activity was calculated as the amount of enzyme required to release one micromole equivalent of galactouronic acid per minute under assay condition. The activities expressed as units/g of substrate. The protein in the supernatant was measured by the method of Lowry *et al.* (16)

Effect of pH and temperature on enzyme activities

The enzyme extract was pre-incubated at different temperatures ranging from 30-80°C for different time intervals and then assayed for pectinase and PG activity. The effect of pH on pectinase and PG activities were studied between pH 3.0 - 9.0 using citrate/phosphate (pH 3.0-7.2) and Tris-HCl (pH 7.2-9.0) buffers (50 mM). All the experiments were conducted in triplicate and the results show the mean values of the activities.

RESULTS

Screening of isolates

Thermophilic fungal strains isolated from various sources and sites of different cities of Punjab, were purified and their cultural and morphological characteristics were examined (9). Fungal cultures were further screened by disc plate method and the potency index was calculated. Fifteen cultures had a potency index above 3.5, thirty-five cultures had potency index between 3.0-3.5, and forty-three isolates had a potency index of

2.0-3.0. These isolates were categorized as high, moderate and low pectinase producers, respectively.

High pectinase producing strains were further screened semi-quantitatively by plate assay method. Results in Fig. 1 shows that the maximal pectinolytic activity was observed in isolate TF3 (O.D.value-0.978) closely followed by V50 (0.957) and V47 (0.802). Minimum enzyme activity was observed in TF40 (0.126). Eight isolates namely, TF3, V7, V44, V45, V47, V50, V52, V53 and three standard cultures *Thermoascus aurantiacus* 204492, *T. aurantiacus* 216529 and *Myceliophthora* sp. were further screened by solid state fermentation. The results shown in Fig. 2 indicate that isolate TF3 produced high pectinase 234 Ug^{-1} and PG 207 Ug^{-1} activities, followed by V7 producing 203 Ug^{-1} and 121 Ug^{-1} of pectinase and PG activities, respectively. Maximum protein concentration of 0.59 $mg\ g^{-1}$ of substrate was produced in isolate TF3, followed by V44 (0.48 $mg\ g^{-1}$). On the basis of these screening experiments, the isolate TF3 was found to be a potential source of pectinolytic enzymes. The isolate was identified as *Aspergillus fumigatus* Fres. MTCC 4163. This culture was used for optimization of pectinase production using solid substrate culturing.

Production of pectinase and PG by *A. fumigatus*

The production profile of pectinase and PG on pectin containing basal medium without sucrose was studied (Fig. 3). It was found that maximal production of pectinase (415 Ug^{-1}) and PG (473 Ug^{-1}) was obtained after 48 and 72 h of incubation, respectively. Further incubation resulted in the decline in enzyme activities up to 120 h.

Effect of substrate

The commercial pectin in the production medium was replaced with equal amounts of different natural substrates,

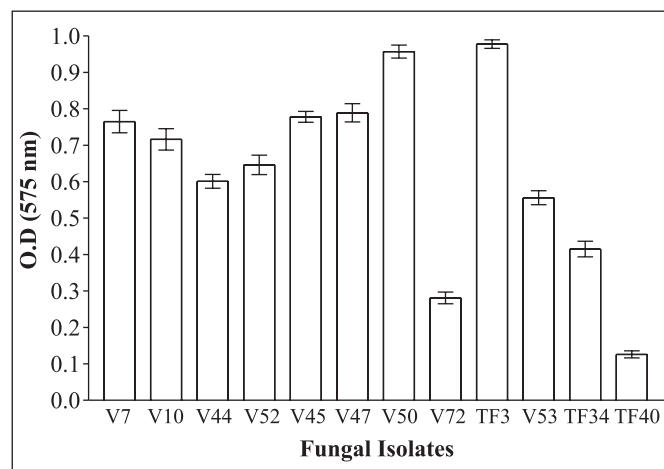


Figure 1. Semiquantitative analysis of thermophilic fungal isolates for pectinase production.

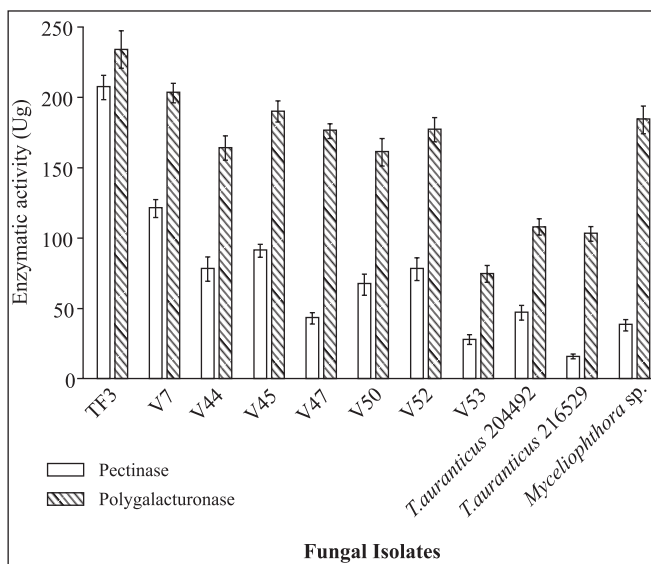


Figure 2. Screening of thermophilic fungal isolates by solid state culturing.

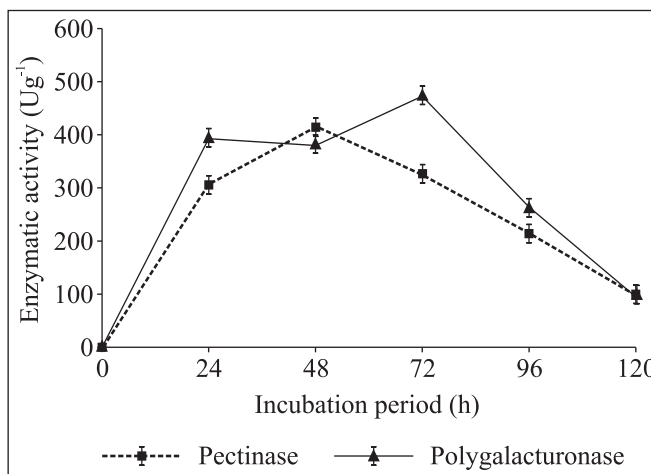


Figure 3. Enzymatic profile of *Aspergillus fumigatus* Fres. by solid state culturing.

i.e., malt sprouts, wheat bran, rice bran, pomegranate pectin, lemon pectin, banana pectin, orange pectin and *Citrus sinensis* (Var. Mussami) pectin. The results (Table 1) show that wheat bran supported maximum pectinase production (589 Ug^{-1}) and was closely followed by commercial pectin (569 Ug^{-1}). The PG activity however was maximally supported by pure pectin (642 Ug^{-1}) followed by wheat bran (625 Ug^{-1}). The minimum activity of 264 Ug^{-1} of pectinase and 283 Ug^{-1} of polygalacturonase was observed in *Citrus sinensis* (Var. Mussami) pectin.

Table 1. Effect of natural substrates on pectinolytic enzyme production of *Aspergillus fumigatus* Fres.

Natural Substrates	Pectinase activity (Ug ⁻¹)	Polygalacturonase activity (Ug ⁻¹)
Pure pectin	569.0 ± 35	642.0 ± 28
Malt sprout	452.0 ± 40	455.0 ± 31
Wheat Bran	589.0 ± 36	625.0 ± 33
Rice Bran	452.0 ± 30	489.0 ± 29
Pomegranate	437.0 ± 26	474.0 ± 25
Lemon	408.0 ± 29	401.0 ± 24
Banana	361.0 ± 25	387.0 ± 35
Orange	363.0 ± 21	299.0 ± 40
Citrus sinensis	264.0 ± 13	283.0 ± 23

S.E at 5% level.

Effect of supplementation of synthetic carbon sources

Addition of different carbon sources (glucose, trehalose, sucrose, galactose, carboxy methylcellulose (CMC) and starch to wheat bran containing production medium, on the pectinase production was studied. The supplementation of sucrose to the production medium resulted in marked increase in pectinolytic activities producing 792 Ug⁻¹ of pectinase and 868 Ug⁻¹ of polygalacturonase as compared to control, which produced 575 and 605 Ug⁻¹ of pectinase and polygalacturonase, respectively. The enzyme production however was strongly repressed in the presence of glucose, showing only 124 Ug⁻¹ and 136 Ug⁻¹ of pectinase and polygalacturonase activities, respectively (Table 2).

Effect of nitrogen sources

Of the various nitrogen sources used in the medium (Table 3), the presence of yeast extract+ (NH₄)₂SO₄ was found to support maximal production of pectinase (925 Ug⁻¹) followed by malt sprouts + (NH₄)₂SO₄ (785 Ug⁻¹), which also supported maximal PG activity (938 Ug⁻¹).

Effect of temperature

The results in Fig. 4 showed that the flasks containing basal medium with wheat bran, sucrose and yeast extract + (NH₄)₂SO₄ supported maximal pectinase (972 Ug⁻¹) and PG (1023 Ug⁻¹) activities at 50°C. The production of these enzymes however, was drastically affected at incubation temperatures of 35 or 55°C.

Effect of assay temperature on pectinase activity

The results (Fig. 5) showed that assay temperature of 50°C for 10 min was most suitable for maximum pectinase activity (1071 Ug⁻¹) followed by 60°C (925 Ug⁻¹). With the increase in incubation period, a constant decrease in enzyme activity was observed at their respective temperatures. At 60 min incubation

Table 2. Effect of synthetic carbon sources on pectinolytic enzyme production of *Aspergillus fumigatus* Fres.

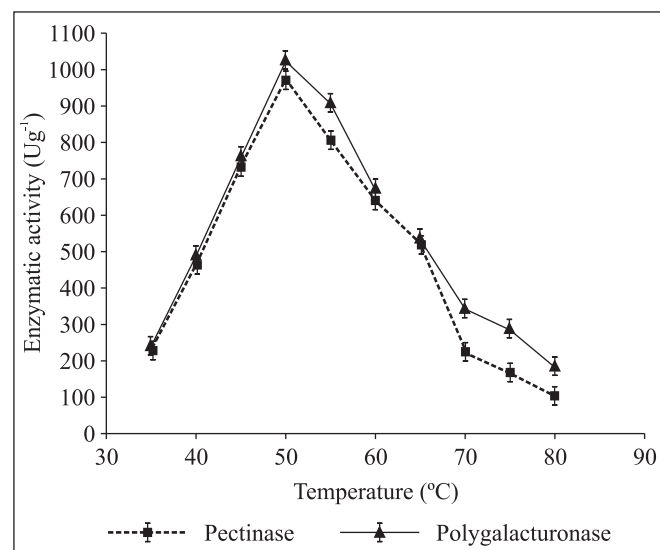
Synthetic carbon sources	Pectinase activity (Ug ⁻¹)	Polygalacturonase activity (Ug ⁻¹)
Control	575.0 ± 21	605.0 ± 28
Trehalose	467.0 ± 13	657.0 ± 32
Sucrose	792.0 ± 47	868.0 ± 39
Galactose	631.0 ± 16	554.0 ± 24
CMC	306.0 ± 19	372.0 ± 15
Starch	386.0 ± 27	415.0 ± 21
Glucose	124.0 ± 12	136.0 ± 9

S.E at 5% level.

Table 3. Effect of Nitrogen sources on pectinolytic enzyme production of *Aspergillus fumigatus* Fres.

Nitrogen sources	Pectinase activity (Ug ⁻¹)	Polygalacturonase activity (Ug ⁻¹)
U+N	467.0 ± 27	683.0 ± 37
Y+N	925.0 ± 10	865.0 ± 21
P+N	419.0 ± 19	613.0 ± 23
So+N	408.0 ± 22	372.0 ± 18
M+N	785.0 ± 16	938.0 ± 58
Y+S	372.0 ± 15	511.0 ± 18
P+S	452.0 ± 26	485.0 ± 26
U+S	350.0 ± 22	346.0 ± 20

S.E at 5% level.

**Figure 4.** Effect of incubation temperature on pectinolytic enzyme production of *Aspergillus fumigatus* Fres.

only 20% activity was quantified. But with the increase in temperature the decrease was not much pronounced. This is evident from the fact that incubating the enzyme at 80°C for 10 min resulted in only 25% loss of activity. These results clearly indicate that pectinase complex of this fungus is catalytically active with high rate of reaction at elevated temperatures. Similar profiles were obtained for PG where maximum activity (1122 U_g⁻¹) was obtained at assay temperature of 50°C after 10 minutes of incubation, the PG activity declined upon prolonged incubation.

Effect of pH

Results shown in Fig. 6 indicated that pH 4.0 was more suitable for pectinase (1116 U_g⁻¹) activity while PG was active

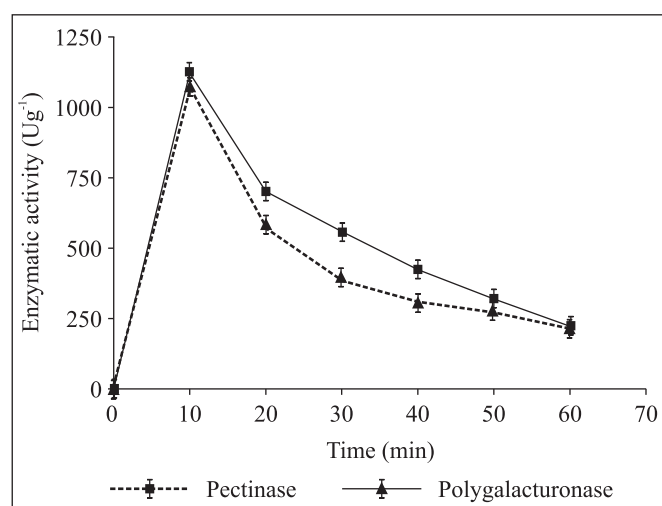


Figure 5. Effect of assay temperature (T=50°C) on pectinase and PG activity of *Aspergillus fumigatus* Fres.

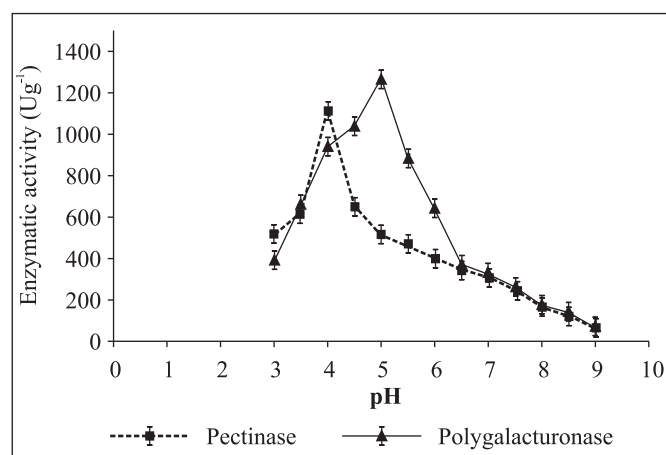


Figure 6. Effect of pH on pectinase activity of *Aspergillus fumigatus* Fres.

at pH 5.0 (1270 U_g⁻¹). Either increase or decrease in pH beyond the optimum value showed decline in enzyme activities.

From the above experiments it was concluded that the thermophilic fungi *A. fumigatus* Fres. expressed maximum pectinolytic activities after 2-3 days of incubation in a medium containing wheat bran, sucrose, yeast extract and (NH₄)₂SO₄ at 50°C. The enzyme was acid active and moderately thermostable.

DISCUSSION

There are very few reports available on thermostable acid active pectinase production by thermophilic fungi. This study reports the isolation and screening of pectinolytic thermophilic fungi. Using semi-quantitative plate assay approach, a thermophilic fungal strain TF3 (*Aspergillus fumigatus* MTCC 4163) isolated from decomposing orange peel waste, was selected as the best producer of pectinase and PG. The plate assay approach has been previously employed in our lab for isolating hyper amylase producing mutants/fusants of thermophilic fungus *Thermomyces lanuginosus* (22,23).

The incubation period to achieve peak pectinase activity by the isolate TF3 was 2 days, which is suitable from the commercial viewpoint. Earlier, Said *et al.* (24) have reported maximum pectinase activity in *Penicillium frequentans* after culturing for 48 h, whereas polygalacturonase activity was maximum on third day of incubation as reported by Hart *et al.* (14) and Crotti *et al.* (10) in *Rhizopus oryzae* and *Neurospora crassa*, respectively.

The pectinase production by *A. fumigatus* TF3 was ably supported by wheat bran, which is a cheap and readily available carbon source, similar findings were reported by Fujio *et al.* (12) in *Rhizopus* sp. When supplemented with sucrose the enzyme production was enhanced and this is in accordance with similar observations made by Crotti *et al.* (10), Baracat-Pereira *et al.* (6) and Minussi *et al.* (21).

The supplementation of yeast extract + (NH₄)₂SO₄ followed by malt sprouts + (NH₄)₂SO₄ greatly enhanced the enzyme production and promoted more than twice the enzyme activity as compared to Urea+ NaNO₃, in the present study. Our results are in concurrence with the observations of Sapunova (25) who also found that malt sprouts and ammonium salts stimulated the pectinolytic enzyme production in *A. alliaceus* BIM-83. Moreover, Sapunova *et al.* (26) has also observed that (NH₄)₂SO₄ stimulated pectinase synthesis, as in its absence fungus displayed a slight proteolytic activity and did not produce extracellular pectinases. Report of Aguilar *et al.* (4) showed yeast extract as the best inducer of exopectinases in *Aspergillus* sp. The strain *A. fumigatus* TF3 was able to grow optimally at 50°C and also produced maximum amount of pectinase at 50°C. Thermophilic fungi have been reported to optimally produce enzymes at 50°C (22,23). Loudiere *et al.*, (15) reported higher temperatures for optimum activity, 50-60°C for

polygalacturonase in *A. wentii* when grown on sugar beet pulp. An acidic pH of 4.0-5.0 was found to support high pectinase and PG activities. Alana *et al.* (5) also reported low pH values in *P. italicum* for obtaining high pectinase production. The study has highlighted that the novel *A. fumigatus* isolate is a good source for producing pectinases on cheap carbon source in short incubation period during solid-state cultivation. The produced pectinases are catalytically active at high temperatures and low pH. Further work on purification and characterization of pectinase from *A. fumigatus* TF3 is currently in progress.

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RESUMO

Produção de pectinases e poligalacturonase por *Aspergillus fumigatus* termofílico isolado de cascas de laranja em decomposição

Através da tiragem de 120 cepas de fungos, isolou-se uma cepa capaz de produzir tanto pectinase quanto poligalacturonase. A cepa foi identificada como *Aspergillus fumigatus* Fres. MTCC 4163. Empregando cultivo em estado sólido, determinou-se os níveis ótimos das variáveis para a produção de pectinase e de poligalacturonase. Os níveis máximos de atividade enzimática foram obtidos quando a cultura era realizada em meio contendo farelo de trigo, sacarose, extrato de levedura e $(\text{NH}_4)_2\text{SO}_4$ por 2-3 dias a uma temperatura de 50°C. A atividade máxima de pectinase (1116 U g^{-1}) e de poligalacturonase (1270 U g^{-1}) foi obtida em pH 4,0 e 5,0, respectivamente.

Palavras-chave: fungos termofílicos, *Aspergillus fumigatus*, pectinase, poligalacturonase

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