JASEM ISSN 1119-8362

Full-text Available Online at www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage. *June*, 2007
Vol. 11 (2) 99 - 103

Determination of *in vitro* and *in vivo* production of polygalacturonase (pg) by storage mold *aspergillus niger* v. Tieghem, during storage of rice (*oryzae sativa* l) seeds

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ABSTRACT: The *in vitro* and *in vivo* production of pectic enzyme polygalacturonase (PG) by storage mold of rice *Aspergillus niger* and the effect of temperature and pH on the activity of the mold were investigated. The result of the assay for the production of polygalacturonase (PG) by the mold *in vitro* and *in vivo* showed that the activity of the enzyme when tested viscometrically, using 10% pectin was 142.9 R.V.U (Relative viscometric units) at 25 ^{o}C. The optimum activity of the enzyme secreted *in vitro* by *Aspergillus niger* was obtained at a temperature of 25 ^{o}C, and at temperature of 25 ^{o}C and 30 ^{o}C for that secreted *in vivo*., with a value of 142.9 R. V.U. The least activity of the enzyme was obtained at 20 ^{o}C, 40 ^{o}C, 45 ^{o}C and 50 ^{o}C *in vitro*, and at 20 ^{o}C ,45 ^{o}C and 50 ^{o}C with a value of 111.1 R.V.U each. The optimum activity of the enzyme produced *in vitro* by the fungus obtained at pH 5 and 6 with a value of 125R.V.U, and at pH 5 with a value of 142.9 R .V. U. *in vivo*. The activity of the enzyme was least at pH 2, 3, 4, 7,8, 9 and 10 *in vitro*, and at pH 2,3,8,9 and 10. *in vivo* with a value of 111.1 R. V. U. @JASEM

Polygalacturonase (PG) E.C 3.2.1.15 is considered to be the main enzyme that degrades pectic substances. It catalyses the hydrolytic splitting of ά - 1,4 glycosidic linkages in the polygalachoronide chains of pectic (non-methyl esterified uronide chains) or pectinc acids of the middle lamella into polygalacturonic acids and galacturonic acid chains of smaller molecular size and eventually to monogalacturonic acid. It shows highest activity on pectic acids, whereas activity decreases considerably as the methoxyl content of the substrate increases. Several pathogens produce polygalacturonase (PG) in infected tissue, for example, Hancock and Millar (1965b), showed that *Colletotrichum trifolii* produced PG. It was also reported to have been produced by *Botryodiplodia theobromae* by Arinze and Smith, (1979) and Arinze (1985 a and b), by *F moniliforme* in carrot tissue (Akanu-Ibiam and Arinze, (1999), and in rice seeds (Ibiam and Arinze, 2005) .

Polygalacturonase operates optimally within a specific temperature .PG secreted by *Fusarium moniliforme* in carrot tissues *in vitro* as reported by Akanu-Ibiam and Arinze, (1999), and in tissues of rice *in vitro* and *in vivo*, by Ibiam and Arinze, (2005) operated optimally at a temperature of 25 ^{o}C Ibiam and Arinze, (2007), also reported that the temperature optimum for the activity of *Bipolaris oryzae* from rice seeds were 25 ^{o}C, *in vitro* and 25 ^{o}C and 30 ^{o}C *in vivo*. Barmore and Brown (1981), reported that the enzyme operated optimally at pH 6. Akanu-Ibiam, and Arinze, (1999), reported that the pH for the optimum activity of PG secreted from carrot tissues by *F moniliforme* was pH 5, while Ibiam and Arinze, (2005), reported that it was pH 5 and 6 for PG secreted by *F moniliforme in vitro* , and at pH 6 for that secreted *in vivo* by the same enzyme . Ibiam and Arinze, (2007), also reported that the optimum temperature the activity of PG secreted *B oryzae* from tissues of rice seeds were pH 6 *in vitro* and ph 5 and 6 *in vivo*.

This work is aimed at determining the *in vitro* and *in vivo* production of pectic enzyme by *Aspergillus niger*, and the temperature for and pH at which the enzyme operates optimally which will assist in the control of the storage mold.
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MATERIALS AND METHODS

This research was carried out at Mycology laboratory of the Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria

Enzyme Studies: In vitro production: 25mls of Reeze and Levinsen (1952) cellulsae medium containing 10g of pectin, 4.6g. $NaNO_3$, 1.0g KH_2PO_4, 0.5g $MgSO_4,7H_2O$ and 0.1g yeast extract per litre of distilled water was introduced into 250 ml Erlenmeyer conical flasks. 5mm disc of five–day old culture of *Aspergillus niger* was introduced into each flask. These were incubated for four days at 25 ^{o}C. Three replicates were made for the test fungus. After four days, the enzyme filtrate of the test fungus was obtained by filtering with two layers of sterile muslin cloth.

In vivo production: Homogenates were obtained by removing the tissues rotted by the test fungus with sterile carpel. The rotted tissues were mixed with 0.1M phosphate buffer, pH 7.0 (1g tissue/10ml buffer) containing 0.2M NaCl (to de-absorb proteins from the tissue), and 0.001M ascorbic acid (to prevent oxidation).The extract was prepared by

homogenizing the tissues in a sterile Warring blender, and straining the homogenate through sterile muslin cloth to obtain the filtrate.

Partial purification of enzyme for in vitro and in vivo studies: The method of Spalding (1969) was used to purify enzyme culture filtrates of the test fungus *A niger* . The filtrates were centrifuged at 2,500 x g for 15 minutes, and the deposited insoluble compounds discarded. Cold acetone was added to the supernatant to precipitate the protein fraction. The precipitate was collected by centrifuging at 2,500 x g for 15 minutes, and then dissolved in 0.1M phosphate buffer at pH 6.0. Fresh enzyme preparation was made on the day of each experiment. The precipitate was used for enzyme studies.

Assay for polygalacturonase activity: Activity of polygalacturonase from the storage mold *A niger was* assayed by the use of 300 size Oswald Cannon Fenske viscometer. This method is routinely used to determine the chain splitting reaction of culture filtrates and homogenates. The reaction mixture contained 4mls of 1% pectin in 0.1M citrate buffer pH 5.0, 1ml of water and 2mls of enzyme sample. Enzyme activity was expressed in viscometric units, defined as 1000/t, where t = time in seconds for 50% loss in viscosity of the reaction mixture (Arinze, 1985 a and b) at 25 ^{o}C, in a water bath. Viscometers were calibrated against water. The flow time for water represented 100% loss in viscosity.

Effect of temperature on polygalacturonase (PG) activity in vitro and in vivo: The influence of temperature on the activity of PG from the storage mold was investigated. Samples of the reaction mixtures, which were the same as described earlier in the viscometric assay was used for 20 ^{o}C, 25 ^{o}C, 30 ^{o}C, 40 ^{o}C and 50 ^{o}C in a water bath for ten minutes. The activity of the enzyme was determined viscometrically.

Effect of pH on polygalacturonase (PG) activity in vitro and in vivo: The influence of pH on the activity of PG from the storage mold was tested at nine pH levels 2, 3, 4, 5 , 6, 7, 8, 9, 10 ,using citrate, potassium chloride and boric acid buffer solutions prepared as described by Hale (1958).The reaction mixture was the same as that used to test for viscometric activity of PG. The reaction mixture at each level was incubated at 25 ^{o}C for 10 minutes, after which the enzyme activity was determined viscometrically.

RESULTS AND DISCUSSION

Assay for *in vitro* and *in vivo* production of the pectic enzyme polygalacturonase by *Aspergillus niger*, showed that the activity of the enzyme when tested viscometrically, using 10% pectin was 142.9 RVU at 25 ^{o}C. As shown in Fig. 1, the optimum activity of the enzyme secreted *in vitro* by *Aspergillus niger* was 142.9 RVU at 25 ^{o}C , while the least was 111.1 R.V.U . at 20 ^{o}C, 40 ^{o}C, 45 ^{o}C, and 50 ^{o}C each. The enzyme activity of PG at each of the other temperatures was 125 R. V. U at 30 ^{o}C and 35 ^{o}C . The enzyme activity of PG secreted *in vivo* was optimum at 25 ^{o}C and 30 ^{o}C, with a value of 142.9 R. V. U., and least at 20 ^{o}C, 45 ^{o}C, and 50 ^{o}C with a value of 111.1 R.V.U . At other temperatures, 35 ^{o}C and 40 ^{o}C, the enzyme activity was 125 R. V. U each. In Fig.2, the optimum activity of the enzyme produced by the fungus in vitro was 125 R. V. U at pH 5 and 6.The activity of the enzyme at each of the other pH levels was 111.1 R. V. U. at pH 2, 3, 4, 7, 8, 9, and 10 each. The activity of the enzyme secreted *in vivo* was optimum at pH 5 , with a value of 142.9 R. V. U, and least at pH 2, 3, 8, 9, and 10, with a value of 111.1 R. V. U each. The enzyme activity at each of the other pH levels pH 4 and 7 was 125 R. V. U each.

PG catalyses the hydrolytic splitting of ά-1,4 glycosidic linkages in the polygalacturonide chains of pectic (non-methyl esterified uronide chains) or pectinc acids of the middle lamella into polygalacturonic acids and galacturonic acid chains of smaller molecular size, and eventually to monogalacturonic acid. It shows highest activity on pectic acids, whereas activity decreases considerably as the methoxyl content of the substrate increases. PG of these fungi might have been involved in decay of the pectic acid or pectinic acids of the seed coat of the seeds of these varieties, making it easy for the penetration of the pathogens.

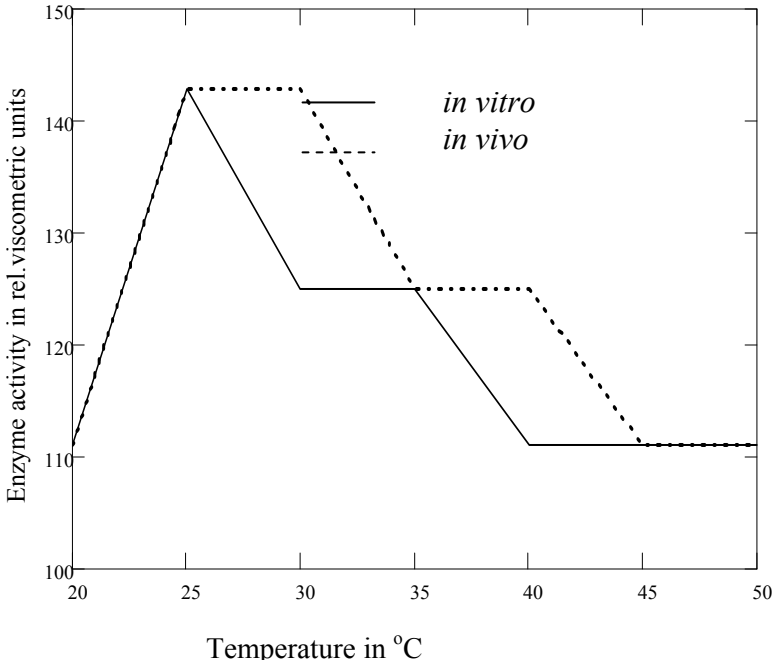

Fig 1 Effect of temperature on the activity of polygalacturonase
Secreted by *Aspergillus niger in vitro* and *in vivo*

According to Arinze and Smith (1979), PG was secreted in infected tissues by *Botryodiplodia theobromae.* It was reported to be secreted by *Aspergillus niger* (Cervone, *et al* (1987), and by *Penicillium italicum* Wehmer (Hershenhorn *et al,* 1990); in *Vigna ungunculata* Walp, by *F moniliforme* (Capari *et al,* 1993). Akanu-Ibiam and Arinze (1999), reported that PG from *Fusarium moniliforme in vitro*, was involved in cutical decay of carots. Ibiam and Arinze, (2007), also reported that *F moniliforme* and *Bipolaris oryzae* played some role in the rot of rice seeds.

There was significant difference in the activity of the enzyme secreted by the mold *in vitro* and *in vivo* $r < 0.01$ for the enzymes from the storage mold (Figs. 1 and 2). The result showed that the activity of PG from the mold decreased with increase in temperature for both *in vitro* and *in vivo* studies.

The optimum temperature for the activity of the enzyme *in vitro* was 25 ^{o}C , and 25 ^{o}C and 30 ^{o}C *in vivo.* This corroborates the report of (Akanu-Ibiam and Arinze, 1999), who stated that the temperature optimum for the PG activity *in vitro* was 25 ^{o}C, and also that of Ibiam and Arinze, (2006 and 2007) who reported that the temperature for optimum activity of PG was 25 ^{o}C *in vitro*, and 25 ^{o}C and 30 ^{o}C *in vivo*. The decrease in the activity of these enzymes as a result of increase in temperature , indicated that the enzymes were being denatured ,hence, decrease in their activity. Wiseman and Gould, (1971), stated that influence of temperature on the activity of enzymes was due to the effect on the stability of the enzyme and the enzyme substrate breakdown velocity.

The pH optimum for the activity of the enzyme secreted by this mold *in vitro* was pH 5 and 6, and pH 5 *in vivo*. On the other hand, Barmore and Brown (1981), Gao and Shain (1995), and Akanu-Ibiam and Arinze, (1999), reported that the pH for optimum activity of was pH 5,while Ibiam and Arinze, , (2005), reported that it was pH 5 and 6 for PG secreted *in vitro* and *in vivo*, and pH 6 for that secreted *in vivo* by the storage Ibiam and Arinze, (2006 and 2007) also reported that the pH for the optimum activity of PG was pH 6 *in vitro* and pH 5 and *6 in vivo.* There was significant difference in the activity of the enzyme secreted by the mold *in vitro* and *in vivo* $r<0.01$ as shown in Fig.2

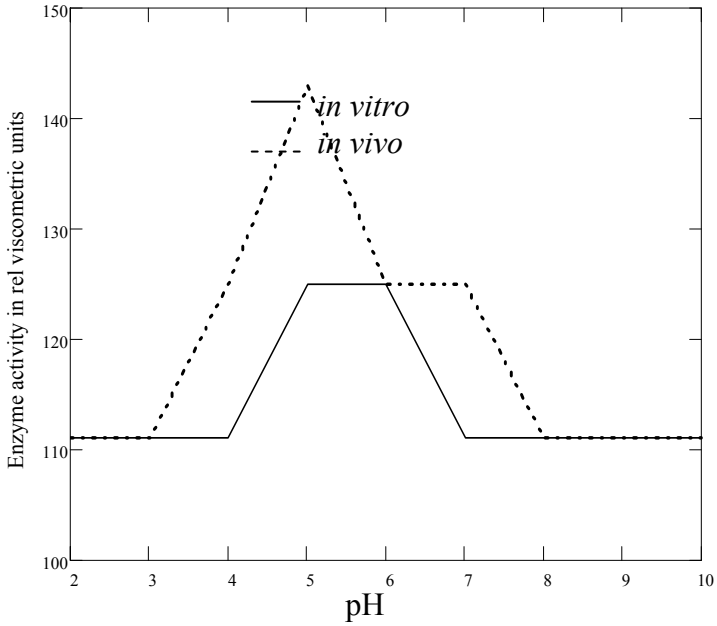

Fig 2 Effect of pH on the activity of polygalacturonase Secreted by *Aspergillus niger in vitro* and *in vivo*

However, the results obtained here could indicate, perhaps, that the pH optimum for the activity of pectic enzymes from rice mold is acid, pH 5 and 6, and that below or above this pH, their activity would not be very effective or could lead to de-naturation . The effect of pH on enzyme activity could be explained in terms of the relative molecular stability of the enzymes (Zefere and Hall, 1973), and partly on the state of ionization of the substrate, enzymes, or enzyme-substrate-complex as the pH changes (Lehninger 1973). Reduced enzyme activity reflects a lowered enzyme secretion due to participation of fewer fungal cells and unfavourable pH and temperature conditions, which prevented the enzyme from performing its task with maximum efficiency. However, it might be stated that profuse enzyme production does not necessarily guarantee pathogenicity, but minimal enzyme production could limit virulence or infective ability of the fungus. There must have been some regulators produced in the reaction medium that suppressed the production of enzymes in the reaction medium by the pathogens. Goodman *et al*, (1986), reported that extracts from a number of plants (potato, onion, carrot, and lettuce), could posses enzyme regulators, some of which could stimulate or repress severely, the production or synthesis of enzymes.

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