Comparative Studies on *In-Vitro* and *In-Vivo* Production of Cellulase by Storage Molds of Rice (*Oryzae Sativa L*). *Aspergillus niger* and *Aspergillus flavus*

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Abstract

Assay for the production of cellulase in vitro and in vivo by the storage molds Aspergillus niger and Aspergillus flavus was investigated. Investigated also was the effect of temperature and pH on the activity of cellulase secreted by the two storage molds. The results of the assay showed that the two fungi produced the enzyme in vitro and in vivo, at 25 °C. The results also showed that the optimum temperature for the activity of the cellulase was 30 °C, while the pH for the optimum activity was in the range of 5.0 – 6.0.

Keywords: Activity, Cellulase, Enzyme, Optimum, pH, Temperature

Introduction

Several workers have associated cellulase secreted by many microbes with rot diseases of such crops like potatoes, yam and decay of wood and textiles. Cellulase bring about the breakdown of cellulose fibres into short insoluble particles. They also convert cellulose to glucose. Cellulase activity has also been reported in bean hypocotyl tissue infected by *Rhizoctonia solani* by(Bateman, 1963, 1966, and 1976), *In vitro* and *in vivo* secretion of cellulase by *Botryodiplodia theobromae* Pat, pathogenic to yams and sweet potato have been demonstrated by (Arinze *et al*, 1975 and Arinze, 1976). Cellulolytic enzymes from the fungus *Pleurotus ostreatus* grown on media with pulp, cotton, stems, rice hulls, or hemp fibre have been extracted ((Akhmedova, 1992).

Several fungal pathogens have been implicated in post harvest deterioration of rice seeds in storage. The possible involvement of *Fusarium moniliforme* and *Bipolaris oryzae* in the post-harvest deterioration of rice seeds in storage has been reported by (Ibiam and Arinze, 2003).This research seeks to determine the possible *in vitro* and *in vivo* production of cellulase by storage molds of rice, *Aspergillus niger* and *Aspergillus flavus,* and its involvement in the deterioration of rice seeds in storage.

Materials and Methods

Culture filtrate for the study of *in vitro* production of enzymes: Culture medium(25 mls) (Reeze and Levinson, 1952), containing 10g carboxy methyl cellulose (CMC 90), 4.6 g. $NaNO_3$, 1.0g KH_2PO_4, 0.5g $MgSO_4$ $7H_2O$ and 0.1g yeast extract per litre of distilled water was introduced into 250ml Erlenmeyer conical flasks. Disc (5 mm) of five–day old culture of each of *Aspergillus niger* and *Aspergillus flavus* grown on potato dextrose agar was introduced into each flask. These were incubated for four days at 25 °C. Three replicates were made for each test fungus. After four days, the enzyme filtrate of each test fungus was obtained by removing the mycelia, and filtering with two layers of sterile muslin cloth.

Culture filtrate for the study of *in vivo* production of enzymes: Homogenates of culture filtrates were obtained by removing the tissues of rice seeds rotted by the test fungi isolated, with sterile carpel. The rotted tissues were mixed with 0.1M phosphate buffer pH 7.0 (1g tissue/10 ml buffer) containing 0.2M NaCl (to de-absorb proteins from the tissues), and 10^{-3}M 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.

Partial purification of enzymes for *in vitro* and *in vivo* studies: The method of (Spalding, 1969), was used to purify enzymes from culture filtrate of the test fungi, *A niger* and *A flavus*. The filtrates were centrifuged at 2,500g for 15 minutes, and the deposited insoluble pellets discarded. Cold acetone (kept in the refrigerator) was added to the supernatant to precipitate the protein fraction. The precipitate was collected by centrifuging at 2,500g 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 cellulase activity *in vitro* and *in vivo*: Cellulase activity was assayed viscometrically. The reaction mixture contained 4 mls of 1% carboxy methyl cellulose (CMC 90) in 0.2M acetate buffer pH 5.0, 1 ml water and 2 mls of purified enzyme. Cellulase activity was expressed in relative viscometric units (RVU), defined as 1000/t, where t= time in minutes for 50% loss of viscosity (Arinze, 1985a and Arinze, 1985b), and used to determined the activity of cellulase in culture and homogenates, and at different temperatures and pH levels.

Effect of temperature and pH on cellulase activity *in vitro* and *in vivo:* Samples of the reaction mixtures were the same as used for viscometric assay for cellulase activity. They were incubated at temperatures of 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C in a water bath for ten minutes. The activity of the enzyme was determined viscometrically as in the assay for cellulase activity described earlier. The influence of pH on cellulase activity was tested at nine levels: 2, 3, 4, 5, 6, 7, 8, 9 and 10. The reaction mixture, which were the same as that for viscometric assay for cellulase activity was incubated at 25 °C for 10 minutes. Cellulase activity was determined viscometrically as in the assay for cellulase activity.

Results

The result of assay for *in vitro* production of cellulase by *A niger,* and *A flavus* showed that the activity of cellulase from *A niger* was 55.6 relative viscometric units (R.V.U), and 58.6. relative viscometric units (R.V.U), for *A flavus,* and that of boiled enzyme 18.2 relative viscometric units (RVU), while the result of the assay for *in vivo* production of cellulase by *A niger,* and *A flavus* showed that the activity of cellulase from *A niger,* and *A flavus* were 58.6 relative viscometric units (R.V.U), and 62. 5 relative viscometric units (R.V.U.) each, and that of boiled enzyme was 20.0 relative viscometric units (R.V.U), all at 25 °C.

Effect of temperature and pH on the activity of cellulase secreted by *Aspergillus niger,* and *Aspergillus flavus in vitro* and *in vivo:* As shown in Fig. 1, optimum the activity of cellulase secreted *in vitro* by *A niger,* was at 30 °C, with a value of 62.5 relative viscometric units (R.V.U), and least at 50 °C, with a value of 22.7 relative viscometric units (R.V.U), while the optimum activity of cellulase secreted *in vivo* was at 30 °C, with a value of 71.4 relative viscometric units (R.V.U), and least at 50 °C, with a value of 34.5 relative viscometric units (R.V.U.).

The activity of the enzyme at other temperatures in the *in vitro* studies was, 20 °C, 40 °C, and 45 °C, 34.5, relative viscometric units (R.V.U) each, and 25 °C and 35 °C 55.6 relative viscometric units (R.V.U) each. The activity of the enzyme at other temperatures in the *in vivo* studies was, 20 °C and 45 °C, 55.6 relative viscometric units (R.V.U), 25 °C, 58.6 relative viscometric units (R.V.U), 35 °C, 66.7 relative viscometric units (R.V.U), and 40 °C, 62.5 relative viscometric units (R.V.U).

As shown in Fig. 2, the activity of cellulase secreted by *A niger in vitro* was optimum at pH 5, with a value of 66.7 relative viscometric units (R.V.U), and least at pH 2, 8, 9 and 10, with a value of 34.5 relative viscometric units (R.V.U.) , while The activity of the enzyme secreted by the fungus *in vivo* was optimum at pH 5, with a value of 76.9 relative viscometric units (R.V.U), and least at pH 2 and 10 with a value of 55.6 relative viscometric units (R.V.U).

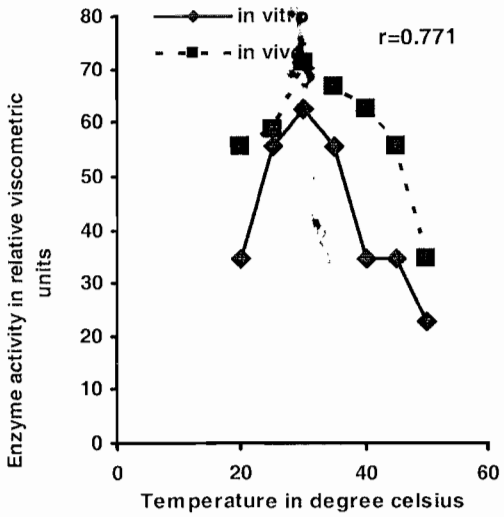

Fig. 1: Effect of temperature on the activity of cellulase secreted by *Aspergillus niger* in vitro and in vivo

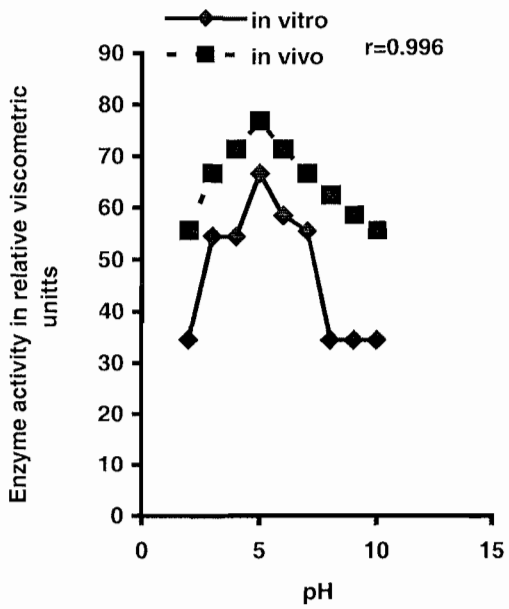

Fig. 2: Effect of pH on the activity of cellulase secreted by *Aspergillus niger* in vitro and in vivo

The activity of the enzyme at the other pH levels in the *in vitro* studies was pH 3, and 4, 54.5 relative viscometric units(R.V.U) each, pH 6, 58.6 relative viscometric units (R.V.U) and pH 755.6 relative viscometric units (R.V.U). The activity of the enzyme at the other pH levels in the *in vitro* studies was, pH 3 and 7 66.7 relative viscometric units (R.V.U) each, pH 4 and 6, 71.4 relative viscometric units (R.V.U) each, pH 8, 62.5 relative viscometric units (R.V.U), and pH 9, 58.8 relative viscometric units (R.V.U.).

As showed in Fig. 3, the result of the activity of the enzyme secreted *in vitro* showed that the activity of the enzyme secreted by *A flavus* was optimum at 30 °C, with a value of 66.7 relative viscometric units (R.V.U), and least at 20 °C, 45 °C, and 50 °C, with a value of 31.3 relative viscometric units (R.V.U), while the optimum activity for the cellulase secreted *in vivo* by the fungus was at 30 °C, with a value of 76.9 relative viscometric units (R.V.U), and least at 50 °C, with a value of 31.3 relative viscometric units (R.V.U) . The activity at other temperatures in the *in vitro* studies was, 25 °C and 40 °C, 58.6 relative viscometric units (R.V.U), and 35 °C, 62.5 relative viscometric units (R.V.U). The activity of the enzyme secreted *in vivo* by the fungus was optimum. The activity at other temperatures in the *in vivo* studies was, 20 °C, 55.6 relative viscometric units (R.V.U), 25 °C, 62.5 relative viscometric units (R.V.U), 35 °C 71.4 relative viscometric units (R.V.U), 40 °C, 66.7 relative viscometric units (R.V.U), and 45 °C, 40.0 relative viscometric units (R.V.U).

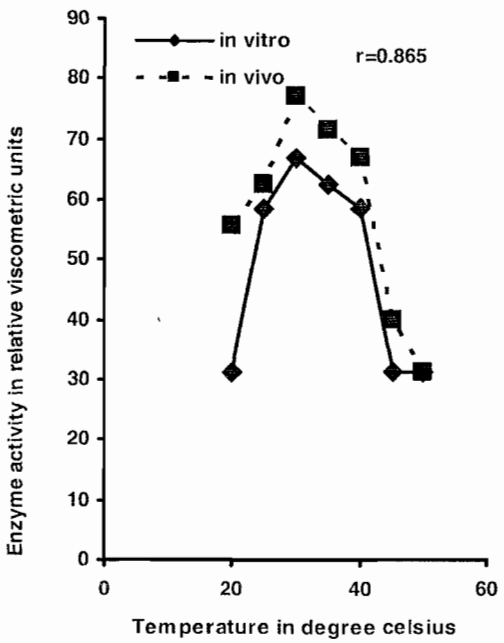

Fig. 3 Effect of Temperature on the activity of cellulase secreted by *Aspergillus flavus* in vitro and in vivo

The result as shown in Fig. 4., indicate that the activity of the enzyme secreted *in vitro* by *A flavus* was optimum at pH 5, with a value of 62.5 relative viscometric units (R.V.U) and least at pH 2, 9, and 10, with a value of 31.3 relative viscometric units (R.V.U), while the activity of the enzyme was optimum at pH 5, with a value of 71.4 relative viscometric units (R.V.U) and least at pH 2, 8, 9 and 10, with a value of 55.6 relative viscometric units (R.V.U) each in the *in vivo* studies. The activity of the enzyme at the other pH levels in the *in vitro* studies was pH 3, and 4, 40.0 relative viscometric units (R.V.U), pH 6, 58.6 relative viscometric units (R.V.U), and pH 7 and 8, 55.6 relative viscometric units (R.V.U). The activity of the enzyme at the other pH levels in the *in vivo* studies was pH 3 and 7, 58.6 relative viscometric units (R.V.U), pH 4, 62.5 relative viscometric units (R.V.U), and pH 6, 66.7 relative viscometric units (R.V.U).

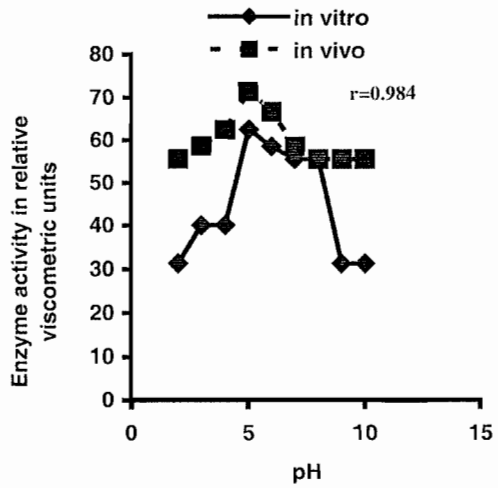

Fig.4 Effect of pH on the activity of cellulase secreted by *Aspergillus flavus* in vitro and in vivo

Discussion

The two storage molds, *Aspergillus niger* and *Aspergillus flavus,* produced cellulase *in vitro* and *in vivo.* The activity of cellulase produced by *A niger in vitro* was 55.6 R.V.U, and 58.6 R.V.U, for that produced by *A flavus;* while that produced *in vivo* by *A niger* was 58.6 R.V.U, and 62.5 R.V.U for that produced by *A flavus,* all at 25 °C. Cellulase activity has been reported by (Bateman, 1963, 1964, 1976 and Ibiam and Arinze, 2003).

Cellulolytic enzymes have been extracted from the fungus *Pleurotus ostreatus* grown on media with pulp, cotton stems, rice hulls, or hemp fibre as reported by(Arinze *et al,* 1975), from celluloytic fungi *Trichoderma viride, Sclerotium rolfsii, Schizophyllum commume, Corticum rolfsii* as reported by (Vipan *et al,* 1994) and from microbes, and fungi in composts, according the report of (Kang *et al,* 1995). Cellulases produced by these fungi might have been induced. This is because, according to(Mandels and Reeze, 1960), cellulases are rarely constitutive, as most are induced by cellubiose, the end product of celluloase degradation. (Goodman *et al,* 19860 also stated that cellulase production might not occur as a result of catabolic repression until the disaccharide is nearly completely metabolized. It follows that the cellubiose which was produced due to degradation of the cellulose in the cell wall of the seeds of rice might have been induced by the secretion of the enzyme by the storage molds A *niger,* and *A flavus.*

The optimum temperature for the activity of cellulase from the two molds was 30 °C *in vitro* and *in vivo (*Figs. 1 and 3).There was significant difference in the activity of the enzyme secreted by *A flavus,* $r < 0.01$, and $r < 0.05$ for the enzyme by *A niger, in vitro* and *in vivo (* Figs 1 and 3). The difference in the activity of the enzymes secreted *in*

vitro and *in vivo* by each of these fungi, suggests that the type of cellulase secreted by them were different. The medium in which the enzyme operated were also different, with respect to solute concentration and chemical content. (Kang *et al*, 1995), demonstrated that the activity of cellulase was optimum at the temperature range 30 ^{o}C – 40 ^{o}C. It is worthy of note, that their activity decreased with increase in temperature, both *in vitro* and in *vivo*. 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 optimum pH for the activity of cellulase from the two fungi *A niger* and *A flavus* was pH 5 *in vitro* and *in vivo* (Figs. 2 and 4). (Kang *et al*, 1995), reported that the pH range for optimum activity of cellulases was pH 5.0-5.5. There was significant difference in the activity of the enzyme secreted by *A niger* $r < 0.01$, and $r < 0.05$ for the enzyme from *A flavus*, *in vitro* and *in vivo* (Figs. 2 and 4). This indicates that the activity will decrease above or below this pH level. The difference in the activity of the enzymes secreted *in vitro* and *in vivo* by each of these fungi, could suggests that cellulase secreted by each fungus *in vitro* and *in vivo* were different from the other. 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).

From the result obtained, it could be suggested that the two storage mold could be implicated in the post-harvest deterioration of seeds in storage by secretion of these enzymes which breakdown the cellulose fibre of the seed-coat paving way for the entrance of mycelia of the molds or even paving way for other more virulent pathogens. It is important that some control measures which could inhibit the growth of these fungi in storage without any detrimental effect on the germinability effect of the seeds, and on human when consumed should be explored.

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