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Full Length Research Paper

Production of β-glucanase enzyme from *Penicillium oxalicum* and *Penicillium citrinum*

Doughari, James Hamuel

Department of Microbiology, School of Pure and Applied Sciences, Federal University of Technology, PMB 2076, Yola 640002, Adamawa State, Nigeria. E-mail: jameshamuel@yahoo.com or doughari@gmail.com. Tel: +27(0) 7 33 550 274 or +234 (0)7 35 599 712.

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Two *Penicillium* species namely, *Penicillium oxalicum* and *Penicillium citrinum* cultivated by solid surface fermentation method using rice bran homogenized with 0.5% (w/v) $(NH_4)_2SO_4$ solution as nitrogen source and Whatman no. 1 filter paper (WFP1) as substrate for β-glucanase enzyme production medium were found to show a dense growth. Studies on the enzyme, using soluble cellulose (SC) and methyl cellulose (MC) as cellulose-glucan source and Somogyi titrimetric method, revealed optimum temperature for enzyme activity from the *Penicillium* species, ranging from 50 to 55°C and thermostability of up to 70°C after 15 to 30 min incubation in sodium phosphate buffer. It was found that the metal ions (0.5 to 5.0 M) namely: Zn^{2+}, Cu^{2+} and Hg^{2+} did not enhance β-glucanase activity. Cu^{2+} ions reduced the enzyme activity slightly, Zn^{2+} ions had no effect, while Hg^{2+} completely inhibited β-glucanase activity. β-Glucanase can be produced from some fungal species locally using the abundant agricultural wastes (such as rice bran) as substrates.

Key words: Fermentation, methylcellulose, metal inhibitors, soluble cellulose, *Penicillium* species.

INTRODUCTION

β-Glucanases are semi-constitutive hydrolytic enzymes that degrade glucan molecules found in the cell wall components of cereals and some fungi. They hydrolyze glucan molecules leading to the production of D-glucose, thus serving as carbon sources (Stahman, 1992; Sha-jun et al., 2001; Tang-Yao, 2002). β-Glucanases have wide industrial applications. They are used commercially in combination with other enzymes in the production of beer and in the brewery, for barley-beta glucan degradation for animal feed (Bajomo and Young, 1992). They can be used as effective additives in laundry detergents. They can also be used for saccharification of agricultural and industrial wastes to provide glucose syrups for animal use. Commercial β-glucanases are reported to be produced from *Aspergillus niger*, *Bacillus subtilis*, *Pseudomonas fluorescence*, *Disporotrichum dimorphosphorum* and *Penicillium* species. β-Glucanases from *Penicillium* species however, have been reported to be of greater advantage over glucanases from other sources. This is because *Penicillium* species are wide spread in nature, found most abundantly in soil and decaying fruits, not fastidious in their nutritional requirements and they can grow on different substrates and under a wide range of environmental conditions. In addition, β-glucanases from these fungi are highly thermostable and can withstand the processing temperatures in their industrial applications (Aono, 1995). Besides, industrial application of enzymes has been receiving attention throughout the world (Srinivasa, 2005). Consequently, because of the high potentials of β-glucanases as industrial enzymes, this study was carried out to produce, concentrate and partially purify this enzyme from very common fungal species of *Penicillium* and to determine the effect of pH, temperature and metal ions on the activity of the enzyme.

MATERIALS AND METHODS

Penicillium oxalicum and *Penicillium citrinum* isolates for β-glucanase production were obtained from the Biology Lab.IV of Abubakar Tafawa-Balewa University, Bauchi, Nigeria. The stock culture was maintained on PDA (Oxoid) slants at 4°C.

Media preparation and culture procedure

The substrate fermentation medium was composed according to Prokop et al. (1992) with minor modifications. It consisted of 20 g rice bran and 0.5 g Whatman no.1 filter paper (WFP 1) plate

suspended in 30 ml of 0.5% (w/v) $(NH_4)_2SO_4$ (pH 5.5). 200 g of rice bran was homogenized with 300 ml of 0.5% (w/v) $(NH_4)_2SO_4$ solution in 500 ml beaker. The pH of the solution was first adjusted to pH 5.5 using 1 N HCl solution. The moistened paste was distributed into 10 sterile plates with 20 g of the paste in each plate. WFP 1 was cut into average size of 2 mm diameter and grounded into smaller bits using pestle and mortar. Into each plate, 0.5 g of WFP 1 was incorporated and properly mixed. All the plates were packed into a can and autoclaved at 121°C and 15 Ib pressure for 15 min. The plates were allowed to cool and then inoculated with 10 ml each of loopful of spore suspension (1 x 10^7 spores/ml) of the *Penicillium* isolates previously subcultured on PDA (oxoid) slants. The cultures were allowed to grow and the substrate was fermented for 5 days at room temperature (30 to 32°C).

Production and separation of crude enzyme

After fermentation, the fungal culture was buffered to pH 5.0 with 50 ml of 1 N Na_2HPO_4 in a conical flask and agitated at 30°C on a shaker for 6 h. The culture fluid obtained after filtration of the 5-day cultures of *P. citrinum* and *P. oxalicum* was centrifuged at 10 000 *g* for 30 min to obtain the crude enzyme extract. This was then filtered using WFP 1 and the culture fluid obtained after filtration was then buffered to pH 5.0 (1N Na_2HPO_4) and once again centrifuged as earlier described. The clear supernatant was then concentrated using the rotary evaporator to 50 ml at 500 rpm (Prokop et al., 1992). To the concentrated solution, 5 g of $(NH_4)_2SO_4$ was added and the solution was left in the refrigerator (4°C) for 15 to 20 min and then removed and once again centrifuged at 10 000 *g* for 30 min. After centrifugation, the supernatant was discarded and the precipitate was redisolved in 20 ml distilled water. This was then poured into cellophane membrane tubes and dialyzed against Na_2HPO_4 buffer (pH 6.0) by immersing the tubes in a beaker of distilled water and left for 24 h in the refrigerator at 4°C after which any remaining precipitate was removed by centrifugation (10 000 *g* for 30 min) and the clear supernatant was used as the crude enzyme extract.

Assay of enzyme activity

Assay of β-glucanase activity was based on the rate of release of reducing sugars from soluble cellulose (SC) and methyl cellulose (MC) as cellulose-glucan source in Somogyi's titrimetric method (Plummer, 1978; Shao-jun Ding et al., 2001). One unit of enzyme activity (U) was defined as the amount of enzyme that produced one µmol of reducing sugar equivalents per min under the assay condition (Shao-jun et al., 2001).

Effect of pH on enzyme activity

This was determined at pH range of 3.5 to 7.5 using 1 N Na_2HPO_4 solution with enzyme-substrate mixture after 30 min of pre-incubation at 40°C.

Effect of temperature on enzyme activity

Optimal temperature and stability at different temperatures were determined by incubating the enzyme with the substrate at temperature ranges of 35 to 75°C for 30 min (Prokop et al., 1992).

Effect of inhibitors on enzyme activity

The effect of metal ions was studied by incubating enzyme samples with 0.5 to 5.0 M of metal ions at 40°C for 15 min and the activity was assayed.

RESULTS AND DISCUSSION

The two species of *Penicillium* (*P. oxalicum* and *P. ctrinum*) showed considerable growth on the growth and fermentation medium (rice bran). They also showed a high level of β-glucanase activity. Figure 1 shows the enzyme activity of β-glucanase at different temperatures and pH after incubation for 15 min. From the results obtained, there was no significant enzyme activity in *P. oxalicum* at 35°C but the activity started to rise with temperature increase from 40°C until it reached a maximum activity of 3.2 U at the optimum temperature of 50°C at pH 6.0. The temperature stability studies show that the enzyme was stable at 65°C for 30 min. For *P. citrinum*, there was no β-glucanase activity at 35°C but the enzyme activity increased with increase in temperature from 0.3 U (at 40°C) until the maximum activity of 2.5 U at 55°C.

Studies on the effect of pH on the enzyme activity of *P. oxalicum* showed that the activity rose with increase in pH from pH 4.0 until it reaches an optimum pH of 6.0 where the highest activity (3.0 U) was obtained (Figure 2). β-Gucanase maintained pH stability after incubation for 30 min at 50°C with pH range of 5.0 to 7.0 for both the two species of *Penicillium.* The β-glucanase activity of *P. citrinum* also showed a similar pattern as it started to rise steadily from pH 4 until it reached the optimum pH of 5.5 where the maximum activity of 1.6 U was recorded (Figure 2). The enzyme activity declined steadily and diminished at pH 7.5, but maintained pH stability up to pH 7.0 following incubation (pH 7.0) for 30 min.

Studies of the effect of metal ions and inhibitors on enzyme activity revealed that 5.0 M Cu^{2+} as $CuCl_2$ (aq) reduced β-glucanase activity at optimum pH for both *Penicillium* species. For *P. citrinum* at 55°C (pH 5.0), the activity reduced from 2.0 U to 1.8 µml while for *P. oxalicum* at 50°C (pH 6.0), the activity reduced from 3.0 to 2.7 U. The result showed that 5.0 M Zn^{2+} as $ZnSO_4$ (aq) had no effect on the enzyme activity, while 0.5 M Hg^{2+} as $HgCl_2$ (aq) completely inhibited the enzyme activity for all the *Penicillium* species (Table 1).

The abundant growth observed in the fermentation substrate indicated the ability of the *Penicillium* species to produce enzymes capable of degrading the medium. This has an immense economic advantage as the enzyme could be produced cheaply by culture of the fungi on the readily available substrate (rice bran). This will reduce cost with subsequent low price of the final products to consumers. The results of the temperature and pH effects on β-glucanase activity agreed with the earlier works of Tang-Yao (2002). The minimum temperature for the two species ranged from 50 to 60°C (Figure 1) and the pH optimal ranged from 4.5 to 6.0 (Figure 2). The rapid decline in β-glucanase activity above the optimum

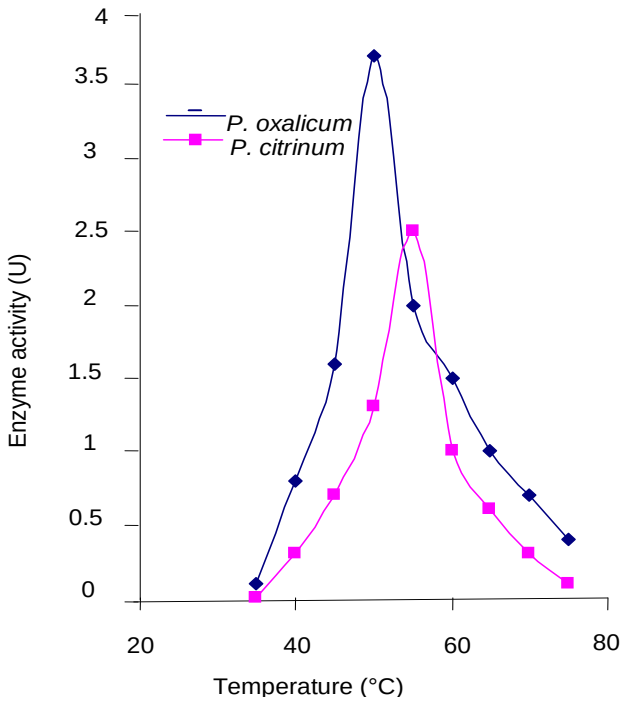

Figure 1. Effect of temperature (°C) on β-glucanase activity of *P. oxalicum* and *P. citrinum.*

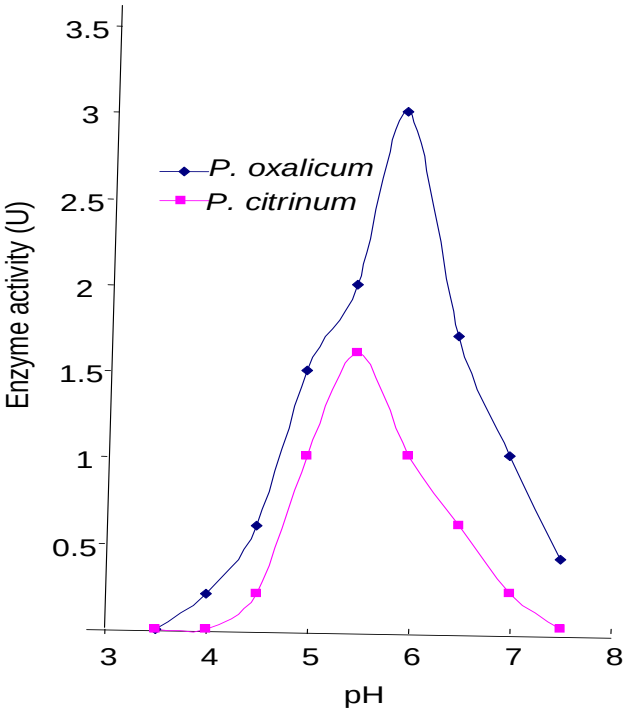

Figure 2. Effect of pH on β-glucanase activity of *P. oxalicum* and *P. citrinum.*

temperature may be due to denaturation of the enzyme molecules. Similar observations were reported by Kitamato (1987). β-Glucanase was found to be stable at 70°C. Similar results though with slight variations were

Table 1. Effect of metal ions on *Penicillium* β-glucanase activity after incubation for 15 min at optimum conditions.

Compound	Concentration (M)	Enzyme activity (U)			
		P_1N (50°C, pH 6.0)	P_1I (50°C, pH 6.0)	P_2N (55°C, pH 5.0)	P_2I (55°C, pH 5.0)
$CuCl_2$	5	3.0	2.7	2.0	1.8
$ZnSO_4$	5	3.0	3.0	2.0	2.0
$HgCl_2$	0.5	3.0	0.0	2.0	0.0

P_1N = *P. oxalicum* with no metal ion; P_1I = *P. oxalicum* with metal ion; P_2N = *P. citrinum* with no metal ion; P_2I = *P. citinum* with metal ion.

reported by Prokop et al. (1992) and Tangarone et al. (1993): they all reported optimum temperature ranging from 65 to 72°C. Different species however have different ranges of pH and temperature optimal. Copa-patino et al. (1987) had earlier reported pH optimal of 5.5 in some *P. oxalicum,* while enzyme stability at pH 6 and temperature of 40 to 50% was also reported by Hashem (1999). The effect of pH on the activity of β-glucanase from *Penicillium* species indicates that *Penicillium* β-glucanases are tolerant to lower pH ranges than β-glucanases from other sources; *Clostridium stercorarium* (pH 6.0 to 6.5) (Bronnenmeoer and Staduidenbaver, 1990), *Bacillus circulans* (pH 6.5 to 7.0) (Aono, 1995), *Candida albicans* and *Neurospora* (pH 5.5 to 6.0 and 5.0 to 7.0, respectively).

The various metal ions tested showed that 5.0 M Cu^{2+} as $CuCl_2$ (aq) at pH 5.0 and 6.0 and incubation temperatures of 50 and 55°C for a period of 15 min, slightly reduced the enzyme activities of both *P. oxalicum* and *P. citrinum.* Zn^{2+} ions (5.0M) as $ZnSO_4$ (aq) had no effect on the enzyme activity, 0.5 M Hg^{2+} ions as $HgCl_2$ (aq) however completely inhibited the enzyme activity of the β-glucanase. A similar finding was also reported by Prokop et al. (1992) and Tangarone et al. (1993) on β-glucanases isolated from *Rhizoctonia solani, Trichoderma longibrachiatum* and *Schizophyllum commune.*

Conclusion

The acidic value for β-glucanase optimum activity obtained from this study, revealed the great potential that the enzyme has towards detergent, beer and food fermentation industries whose manufacturing conditions are largely acidic. The high temperature optima and thermostability of the enzyme is significant in the brewery industries where high temperatures of up to 65°C are employed in their production process. Rice bran is a potential source of cheap substrate that can be used to produce the enzyme readily from *Penicillium* species locally in commercial quantities. Other cereal substrates such as sorghum, millet, wheat or barley brans should be investigated.

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