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Synthetic Production of Amylase from *Penicillium species* Isolated from Apple Fruit

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Abstract: *Pencillium species* isolated from decaying apple fruit was grown in a synthetic medium containing starch as sole carbon source. Culture filtrates exhibited amylase activity. Optimum enzyme activity was observed on the ninth day of incubation. Amylase activity was determined by measurement of dextrinized powder which is a measure of the change in the blue colour of starch-iodine complex due to decrease in the amount of starch. The enzyme was subjected to ammonium sulphate precipitation and dialysis. The activity of the enzyme was optimum at 35°C and pH 6.5. The enzyme was heat labile loosing its activity completely after twenty minutes of heating at 80°C. The presence of cations Mg^{++}, Ca^{++}, K^{+} and Na^{+} stimulated the activity of the enzyme.

Key words: *Penicillium* species · Synthetic production · Amylase · Decaying apple fruit

INTRODUCTION

Amylases are produced by bacteria and fungi [1, 2]. Although the degradation of starch is important in enzymatic hydrolysis, the main types of amylases commonly encountered in microbial degradation are α and β-amylases [3-5]. Starch molecules are hydrolysed into polymers of glucose units [6]. Degradation of substrate is important in enzymatic hydrolysis and starch is the substrate used in microbial assay [6].

This paper describes the synthetic production of amylase by a species of Penicillium isolated from apple fruit. Attempts were made to characterize the partially purified enzyme after dialysis as a means of maximizing output and maintaining optimum activity with the cheapest and least procedures of purification.

MATERIALS AND METHODS

Organism and Culture Conditions: The isolate of *Penicillium species* used in this research was part of a culture collection of the department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. It was isolated from decaying apple fruit. The stock was routinely grown and maintained on 1% malt yeast extract agar slants.

Preparation of Inoculum: A subculture of *Penicillium species* was prepared on 1% malt yeast extract agar slants and incubated at 25°C for 240 hours. After the incubation period, spore suspension was prepared from the inoculum by adding 10ml of sterile distilled water to each test tube slant containing the culture of the isolate. The spores on the surface of the agar medium were dislodged by carefully scraping them with sterile inoculating loop.

The spore suspension was diluted to have a final concentration of approximately 5×10^{7} spores per ml [7].

Inoculation: Fifty milliliter of growth medium, prepared as described by Adejuwon and Olutiola [7] but with starch as sole carbon source was dispensed into 250ml conical flasks. The final concentration of starch was 1% (w/v). Contents of each flask was inoculated with the spore suspension, prepared as described above. Growth temperature was at 25°C.

Enzyme Preparation: On the nineth day of inoculation, Daily, the contents of each flask was filtered using glass fibre filter paper (Wahtman G/A).This served as the enzyme preparation. Amylase activity [8] and protein content [9] of the preparation were determined.

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Ammonium Sulphate Precipitation: The crude enzyme was subjected to ammonium sulphate precipitation within the limits of 40-90% saturation. Precipitation during salt saturation was at 4°C for 24hr. it was subjected to cold centrifugation at 10,000g for 30 minutes at 4°C using a high speed cold centrifuge (Optima LE-80K Ultracentrifuge, Beckman, USA). The supernatant was discarded and the precipitate reconstituted in 0.02M citrate phosphate buffer pH 6.0.

Dialysis: The enzyme was dialysed using dialysis tubing (Visking dialysis tubing) which had earlier been acetylated as described by Whitaker *et al*. [10]. Dialysis was carried out in a multiple Dialyser (Pope Scientific Inc., Model 220, USA) at 4°C for 24 hours against four changes of the same buffer. Amylase activity and protein content of fractions were thereafter determined.

Amylase Assay: Amylase activity was assayed using the modified method of Pfueller and Elliott [8]. The reaction mixture was 2ml of buffered (0.02M citrate phosphate buffer pH 6.0) soluble starch (Sigma) and 0.5ml enzyme. Incubation was at 35°C for 30 minutes. The reaction mixture was terminated with 3ml of IN HCl. 2ml of the terminated reaction mixture was added to 3ml of O.1N HCl. Colour was developed by adding 0.1ml iodine solution. One unit of amylase activity was arbitrarily defined as the amount of enzyme which produced 0.1% reduction in the intensity of the blue colour of the starch-iodine complex under assay conditions.

RESUILTS

Penicillium species grew in synthetic medium containing starch as sole carbon source. Amylase activity gradually increased. Optimum activity was observed on the 9th day of incubation (Table 1).

There was a gradual increase in amylase activity as substrate concentration increased. Optimum activity was observed within 0.5 – 0.6mg/ml starch concentration (Table 2).

Within a temperature range of 20- 40°C, there was a gradual increase in amylase activity. Optimum activity was observed at 35^{0} C after which there was a gradual decline (Table 3).

When the enzyme was heated at 80°C for a period of 0-30 minutes, activity gradually declined. There was complete loss of activity at 20 minutes (Table 4).

Within a pH range of 4.0 to 8.0, amylase activity gradually increased. Optimum activity was observed at pH 6.5 after which there was a decline (Table 5).

Table 1: Amylase activity expressed on a daily basis

Days of Incubation	Amylase activity (Units)
1	20
2	98
3	350
4	560
5	680
6	720
7	770
8	890
9	920
10	810

Table 2: Amylase activity expressed with substrate concentration

Substrate concentration (mg/ml)	Amylase activity (Units)
0.1	650
0.2	720
0.3	850
0.4	860
0.5	920
0.6	920
0.7	780
0.8	650

Table 3: Amylase activity expressed with varying temperature

Temperature (°C)	Amylase activity (Units)
20	650
25	780
30	820
35	910
40	890

Table 4: Amylase activity expressed with heat at 80°C

Time (minutes)	Amylase activity (Units)
0	930
5	160
10	50
20	0
30	0

Table 4: Amylase activity expressed with varying pH

pH	Amylase activity (Units)
4.0	320
4.5	430
5.0	580
5.5	640
6.0	720
6.5	890
7.0	720
7.5	630
8.0	520

Table 5: Partial purification of amylase from *Penicillium species* isolated from apple fruit

Fraction	Amylase activity (Units)	Yield	Purification (fold)
Crude	930	100	1
Ammonium sulphate Precipitation	900	92.8	2.4

Within a concentration range of 0-25mM of Na^+, K^+, Mg^{2+} and Ca^{2+}, amylase activities gradually increased. Optimum activities were observed at 25mM for all the cations.

Amylase activity, yield and purification (fold) of fractions can be observed in Table 5.

DISCUSSION

Bacillus licheniformis produces an amylase which is widely used in various procedures of starch degradation in the food industry [11]. The optimum cultural medium for growth of *Streptomyces nigrifaciens* includes 4% potato starch, however, *Penicillium digitatum* which had an optimum temperature range of 25-30°C for mycelial growth could not thrive at 30°C [12].

Unmodified and low water activity (a(w))-tolerant cells of *Candida sake* applied before harvest and four months in cold storage of apple are effective against *penicillium expansum* infection on apple and therefore seem able to control blue mold of apples [13]. The application of antagonistic microrganisms such as *Aureobasidium pullulans* and *Rhodotorulla glutinis* in the field represents a promising alternative to fungicide treatments to control post harvest diseases of apple caused by *Penicillium expansum* [14]. However, both thiabendazole (TBZ) – sensitive and TBZ –resistant strains of *Penicillium expansum* can be used in the control of blue mold decay of Bosc pears [15]. Decay of pome fruits caused by *Penicillium expansum* can be reduced by vapors of acetic, formic and propionic acids. So also can the reduction of decay of citrus fruits by *Penicillium digitatum* be achieved by vapors of these three acids [16].Spraying apple trees with harpin, a few days before harvest, is a promising strategy for reducing blue mold decay (caused by *Penicillium expansum*) of apple fruits in storage [17].

According to Wang *et al*. (18), a filamentous fungus from the Huanghai sea sludge, *Pencillium species*, produced a cold active alpha amylase stimulated by Ca^{2+} but optimally active at 40°C.

Conclusively, amylase can be produced industrially using the specified growth medium used in this research. The species of penicillium employed is a cheap source of isolate. The properties of the enzyme as observed during characterization will be useful in maintaining activity and prolonging the shelf-life of the enzyme during storage procedures.

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