GLOBAL JOURNAL OF PURE AND APPLIED SCIENCES VOL. 10, NO. 1, JANUARY 2004: 75-79
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ASPECTS OF THE ISOLATION AND CHARACTERIZATION OF THERMOSTABLE α -AMYLASE FROM *Alternaria alternata.*

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(Received 15 May 2003; Revision accepted 7 August 2003)

ABSTRACT

A local isolate of *Alternaria alternata* isolated from soil under the decaying cassava peels heap was screened for the production of α-amylase and hydrolysis of starch. The maximal dextrinizing amylolytic activity of the partially purified enzyme was obtained at temperature of 60°C and pH 7.0. Apart from glucose, sucrose and maltose, several other unknown products of cassava starch hydrolysis were obtained at elevated temperatures (60 – 90°C). The thermostable property of the enzyme is discussed in relation to its potential in improving the nutritional quality of starchy foods and industrial usefulness in the future.

KEYWORDS: Thermostable α - amylase; Starch hydrolysis products; *Alternaria;* Starchy foods; Thin Layer Chromatography.

INTRODUCTION

Starch is one of the most abundant polysaccharides in nature where it serves as energy storage in plant seeds and tubers (Swinkels, 1985). In other to access the chemical energy stored in starch, organisms need starch-hydrolyzing enzymes (Duedahl-Olesen *et al.*, 2000). Since carbohydrates are widely used in various industries such as food, beverages, paper and textile production, and increasing use for medical purposes, there have been considerable desire for research into the synthesis of novel oligosaccharides and polysaccharides (Chitradon *et al.*, 2000). The best method for the synthesis of such oligosaccharides is the use of *in vitro* enzymes, especially those isolated from microorganisms (Chaplin and Bucke, 1990).

The genus *Alternaria* is ubiquitous and abundant on decaying plant materials and damp indoor areas (Jay, 2000). Species of *Alternaria* are plant pathogens and several diseases caused by them are common throughout the world (Agrios, 1997). They have been known to kill plant cells through the production of enzymes and other by-products (Alfieri *et al.*, 1994), and these include endo-polygalacturonase and pectate lyase (Perez *et al.*, 1991; Auba *et al.*, 1993), which are responsible for the hydrolysis of pectin components of the plant cell wall (Collmer and Keen, 1986). The use of a by-product, the red phytotoxic pigments of *Alternaria eichhorniea* for the biocontrol of water hyacinth in Egypt has been reported (Shabana *et al.*, 2001). The genus *Alternaria* has no positive use in the food industry (Jay, 2000) and the intensive literature and Internet search indicate that there are no previous reports on the use of α-amylase of *Alternaria alternata* in the production of oligosaccharides.

This paper is a report of observation and work in progress on the production, isolation and characteristics of a thermostable α-amylase produced by a local isolate of *Alternaria alternata.*

MATERIALS AND METHODS

Isolation of *Alternaria* and Culture conditions.

The strain of *Alternaria alternata* used in this study was isolated from soil sample collected from cassava peel dump on 1% starch minimal agar (1% soluble starch, and 2% bacteriological agar). About 100mg/ml of ampicillin was added to the medium to inhibit bacterial growth (Nwachukwu, 2000). The fungus was black to grey-black in colour with moderately abundant and dense aerial mycelium. It had characteristics dark, tadpole shaped conidia with walls in two dimensions. The amylase production and starch hydrolysis by the fungus were assayed by culturing on starch minimal agar at 28°C for 24hours. The plates were then flooded with weak Iodine solution to stain any unhydrolysed starch. Pure colonies of *Alternaria alternata* were maintained on starch minimal agar, yeast extract agar and potato dextrose agar.

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Production of α- amylase.

Production of α-amylase was done by inoculating about 8mm block of 24hr-old culture of the fungus into 100ml of the production medium (1,2,3,4 or 5% soluble Starch, 0.005% KH_2PO_4, 0.005% Peptone and 0.002% Yeast extract). The culture was incubated at 28°C for 5-7 days. After the harvest of the mycelial mass, the broth culture was brought to 30% ethanol concentration and centrifuged at 1000xg for 15minutes. The supernatant was further brought to 70% ethanol concentration and recentrifuged at 1000xg for 15minutes (Kundu and Das, 1970). The precipitate, which was formed after the second centrifugation, was taken as the crude extracellular α-amylase. The crude protein content of the enzyme was determined accordingly (Lowry *et al.*, 1951).

Assay for amylolytic activity

Amylase dextrinizing activity was determined in a reaction system containing 5ml of 1% starch solution, 3ml of phosphate buffer (pH 7.2), 1ml of 0.5M NaCl, and 1ml of the partially purified enzyme. The reaction was carried out at 55°C for 1hr, after which the reaction was stopped by boiling at 100°C for 5minutes. The amount of starch hydrolyzed was determined according to the methods of Smith and Roe (1949). One unit (U) of dextrinizing amylase activity was defined as the amount of the enzyme that will hydrolyze 10mg of starch per ml under the conditions of this procedure. Enzyme assay was carried out in triplicate. In all cases, the standard deviation was smaller than 10% of the mean.

Effects of pH and temperature on the amylolytic activity of the enzyme.

The effect of pH on the activity of the enzyme was determined by replacing the phosphate buffer (pH 7.2) used for the enzyme assay with phosphate buffer solution of pH values 4.0, 6.0, 7.0, 8.0, and 9.0. To investigate the effect of the temperature on the amylolytic activity of the enzyme, the temperature of the reaction mixture was held at 45, 55, 60, 65, 75 and 85°C, while other assay procedure earlier described were kept constant.

TLC analysis of the products of starch hydrolysis

The enzymatic hydrolysis of cassava starch by the α-amylase of *Alternaria alternata* was undertaken according to the methods of Bernfeld (1955). One milliliter of precipitated enzyme was added to 1ml of 1% cassava starch at 40, 60 70 and 90°C for 30minutes. The reaction was stopped by holding the reaction mixture in boiling water at 100°C for 5minutes. In each case, the hydrolyzed cassava starch was centrifuged at 1000xg for 20minutes to obtain the hydrolysate containing the

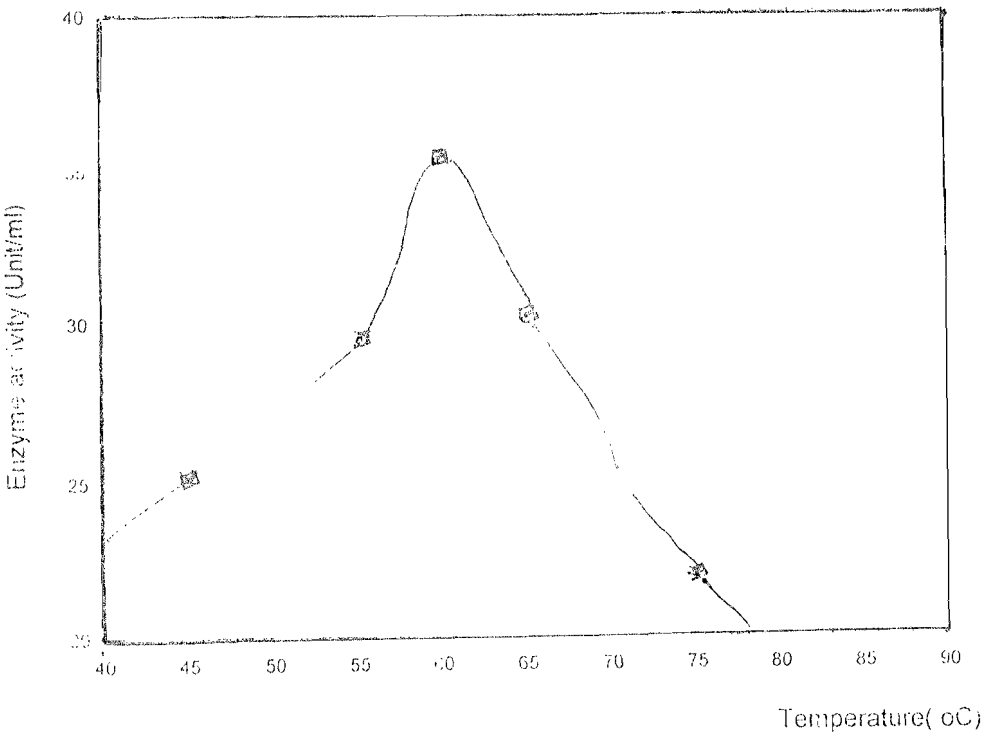

Fig.1:The effect of Temperature on the activity of the enzyme .

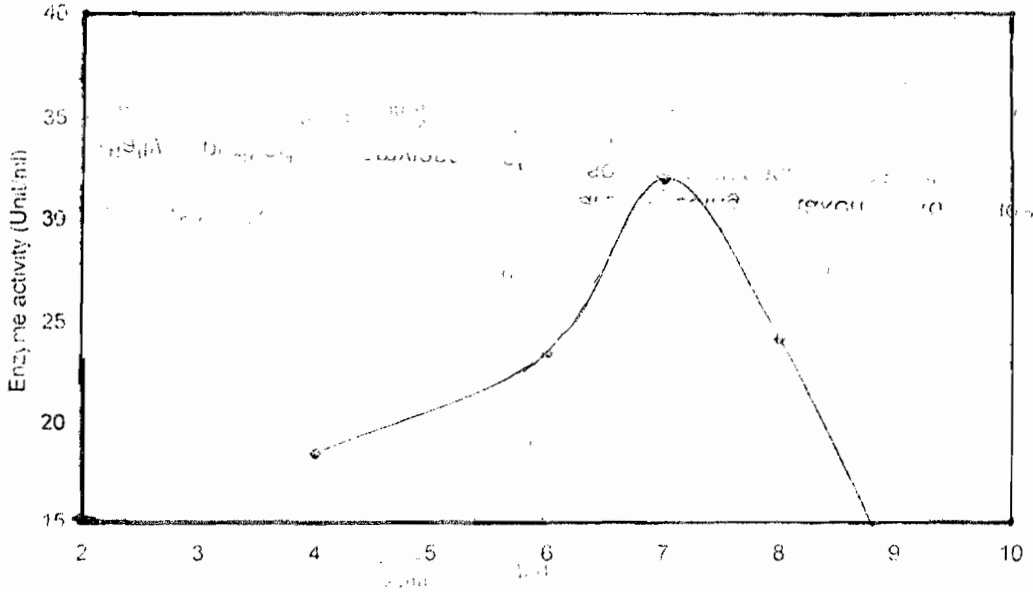

Fig.2: The effect of pH on the activity of the enzyme

products of hydrolysis. The products of hydrolysis of starch were analyzed on TLC plates using the methods of Prakash *et al.* (1989).

RESULTS AND DISCUSSION

The partially purified α-amylase of *Alternaria alternata* had maximal dextrinizing amylolytic activity with soluble starch at 60°C and considerable lower activities were detected at temperature below or above 60°C (Fig. 1). The optimum temperature (60°C) is the same as that reported for starch hydrolysis by the glucoamylase from *Humicola grisea* (Campos and Felix, 1995) and *Trichoderma harzianum* (Azevedo *et al.*, 2000). The activity of the enzyme at 55°C was maximal at pH 7, but substantial activity was detected in the pH ranges of 4 – 6 and 8 – 9 (Fig. 2). The result of the TLC analyses of products is as shown in Table 1. The TLC is a simple and relatively high sensitive method (Hayashi *et al.*, 1988a, 1988b) that can separate maltooligosaccharides up to DP 10 (Deudahl-Olesen *et al.*, 2000). Different types of products were produced under the various reaction conditions. Except in C, two products of starch hydrolysis were produced in each of the reaction mixtures. Glucose was produced in A alone, whereas maltose and sucrose were produced in B. The hydrolysis of starch to glucose by α-glucosidase has been previously reported (Forgarty and Kelly, 1979; Robyts, 1984). α-amylases are capable of hydrolyzing starch to maltose, a disaccharide and this is widely reported in literature. It is not well understood how sucrose was formed under this condition. The plausible explanation is that possibly other enzymes are present in the enzyme preparation. One unknown product was produced in A and C. It was the only product in C. In D, two unknown products were produced. These unknown products are likely oligosaccharides and further analytical measures are being taken to identify them. Isomaltooligosaccharides are produced by the combined action of α-amylase and α-glucosidase (Prapulla *et al.*, 2000).

The reaction conditions in A, B, and D could be exploited in food industries where mixtures of food-grade oligosaccharides with different degrees of oligomerization are desired (Prapulla *et al.*, 2000). The ability of the enzyme to be active within a broad temperature range (40 – 85°C) and hydrolyze cassava starch at elevated temperatures of 60, 70 and 90°C into the products earlier mentioned is noteworthy. The thermostability of the enzyme clearly show that it will be a good candidate for industrial process and immobilization, where multiple re- use of enzyme and the establishment of a continuous process yielding a product with a constant quality are desired (Lopez-Leiva and Guzman, 1995). Also this property suggests that the enzyme can be incorporated in future in to the programme on the enhancement of the nutritional quality of starchy foods. This is important in view of the fact that starchy foods particularly cassava – based foods still remain the staple foods in developing nations of the world. In this perspective, feeding experiments are underway to study some aspects of the toxicological, haematological and nutritional effects of the products in experimental rats. In C, where the reaction was held at 70°C, a monoproduct that is presumably an oligosaccharide was formed.

Table 1: The Rf values of reference standards and the products of hydrolysis.

Spot	Rf value	Reference standards	Rf value
A	0.23_1, 0.53_2	Fructose	0.14
B	0.36_1, 0.84_2	Sucrose	0.35
C	0.23	Glucose	0.53
D	0.48_1, 0.78_2	Galactose	0.66
		Maltose	0.84

1-2 corresponds to the products as appeared on the plates; temp. in °C (A: 40; B: 60; C: 70 & D: 90)

Thus, under this condition, the starch can be hydrolyzed into a monoproduct, thereby limiting the problems of purification of the product to make product recovery and downstream process easier. New oligosaccharides are being continuously produced, and recently several new oligosaccharides were obtained in a hydrolysis process using α-amylase from *Bacillus licheniformis* (Chitradon *et al.*, 2000). Investigations into the purification of the α-amylase of *Alternaria alternata* and the nature of the new oligosaccharides are being undertaken.

In this study, we have reported for the first time, the usefulness of α-amylase from *Alternaria alternata* in the production of oligosaccharides. We also suspect the presence of other enzymes such as α-glucosidase and isomerase in the enzyme preparation. Thorough purification of the enzyme might reveal this in the nearest future. Enzymes from similar fungal phytopathogens such as *Aspergillus oryzae* and *Fusarium oxysporum* have been used in the production of oligosaccharides (Singh *et al.*, 1995; Tsitsimpikon *et al.*, 1996).

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