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Production and Characterization of Extracellular Amyloglucosidase from *Aspergillus niger* CA-19 by Solid-State Fermentation

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Abstract: The study was conducted with the aim of producing amyloglucosidase enzyme from *A. niger* using Solid State Fermentation (SSF) and to carry out preliminary characterization of the enzyme produced. Amylolytic *A. niger* CA-19 was isolated from the soil on Remazol Brilliant Blue-starch agar and used for enzyme production using rice bran supplemented with soya bean flour in SSF process. The crude enzyme extract had optimal temperature and pH activities at 60°C and pH 4, respectively. With the exception of cocoyam starch, the enzyme preparation was able to hydrolyse both the cereal (maize) and root starches (yam, cassava, sweet potatoes) tested. Hydrolysis was significantly ($p<0.05$) dependent on starch source.

Key words: Amyloglucosidase, starch, fungi, solid state fermentation, rice bran, soya bean

INTRODUCTION

Starch-degrading enzymes like amyloglucosidase are gaining more importance among the industrial enzymes because of the importance of starch, sugars and other products in modern biotechnological era (Prakasham *et al*., 2007). Amyloglucosidase also known as glucoamylase hydrolyzes α-1,4 and α-1,6 linkages and produce glucose as the sole end-product from starch and related polymers. Amyloglucosidases have applications for dextrose production, confectionery, baking and in pharmaceuticals (Rose, 1980; Pandey *et al*., 2000).

Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques (Burhan *et al*., 2003). Both SSF and Submerged Fermentation (SmF) could be used for the production of amyloglucosidase. However, the cost of enzyme production in submerged fermentation especially in developing countries is high, which necessitates reduction in production cost by alternative methods. Baysal *et al*. (2003) have reported alpha-amylase production in solid-state fermentation with wheat bran and rice husk as substrates. In our laboratory, we have formulated a suitable low cost fermentation medium for the production of amylase by using cheap rice bran supplemented with peanut cake and soya bean flour (Akpan *et al*., 1999b).

In spite of the wide distribution of amylases, microbial sources are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (Burhan *et al*., 2003). Microbial amylases have completely replaced chemical hydrolysis in the starch processing industry (Gupta *et al*., 2003). They are also potential candidates in the medicinal, clinical and fine chemical industries (Becks *et al*., 1995; Gupta *et al*., 2003).

The objective of this study was the production of amyloglucosidase from *A. niger* by SSF and to carry out preliminary characterization of the enzyme.

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MATERIALS AND METHODS

Screening and Isolation of Amylolytic *A. niger*

An amylolytic *A. niger* was isolated from soil samples collected from different areas within the premises of the University of Agriculture, Abeokua (UNAAB), Nigeria using Remazol Brilliant Blue (RBB)-starch agar medium prepared by the method earlier described (Akpan *et al*., 1999a). Amylase production was detected by the disappearance of the blue colour of the medium around microbial colonies after incubation. Evaluation of the clear zones of each colony was estimated as radius (mm) of the clear zone minus the radius of the colony.

A. niger colonies producing large clear zones were picked up and purified by streaking on malt extract agar. Identification of the isolate was based on cell and colonial morphological characteristics with references to the method of Rasper and Fennel (1965).

Amyloglucosidase Production by SSF

The fermentation was carried out in 250 mL Erlenmeyer flasks using the procedures described by Akpan *et al*. (1999b). The medium consisted of rice bran (10 g) and soya-bean flour (3 g) moistened to 55% moisture content with aqueous mineral salts solution [$MgSO_4.7H_2O$, 0.1%; KH_2PO_4, 0.1%; $CaCl_2$, 0.1%; $FeSO_4$, 0.05% and $(NH_4)_2\ SO_4$, 0.1%]. Sterilization was at 121°C for 15 min. After cooling the flasks were inoculated with 1 mL of conidial suspension of *A. niger* CA19 (2×10^6 spores/100 mL and incubated (30°C, 72 h).

Extraction of the enzyme from the mouldy bran was according to the procedures described by Omemu *et al*. (2003). To 1 g of the mouldy bran, 10 mL of 0.01M citrate buffer (pH 4.5) was added and the mixture was shaken on an orbital shaker (LAB-LINE, UK) at 150 rpm and 28°C for 1 h. The filtrate was used as crude enzyme source.

Assay of Amyloglucosidase

Amyloglucosidase activity was assayed at 60°C for 1 h by the method of Ramakrishna *et al*. (1982) using 1% (w/v) corn starch in 0.1 M citrate buffer (pH 4.5). Reducing sugars were estimated by the DNS methods (Miller, 1959). One unit of amylase activity (U) was defined as the amount of enzyme that liberated 1.0 μmole of D-glucose from starch in 1.0 μL reaction mixture under the assay conditions.

Enzyme Characterization

Effect of Temperature

To check optimum temperature for amyloglucosidase activity, enzyme was incubated with soluble starch in 0.1 M acetate buffer, at various reaction temperatures ranging from 20 to 90°C.

Effect of pH

Amyloglucosidase activity was measured at various pH ranging from 4 to 10. The pH of the reaction mixture was varied using different buffers (citrate buffer for pH = 3-6, phosphate buffer for pH = 7-8 and borate buffer for pH = 9-10).

Enzyme Stability

The enzyme solution was kept at room temperature (28±2°C) and at 4°C (±2°C) for 34 days. At different time intervals, aliquots of the crude amylase was taken and analyzed for amylase activities.

Digestion of Raw Native Starches

The ability of the crude enzyme to hydrolyze raw starch was studied using maize, cassava, sweet potato, yam and cocoyam starches. Commercial soluble starch was used as the standard. Enzyme

solution, (1 mL; acetate phosphate buffer (pH 4.5, 1 mL) and 3 mL each of the raw starch solutions were incubated at 60°C for 1 h. Susceptibility of the raw starches to hydrolysis was determined in terms of the quantity of reducing sugars (mg g^{-1}) produced.

Thin Layer Chromatography

Sugars produced by the hydrolysis of the raw native starches with amyloglucosidase of *A. niger* CA-19 were identified by Thin Layer Chromatography (TLC). Chromatograms were developed by the ascending method with commercially prepared TLC plates (Polygram, UK) as the stationary phase and n-butanol: acetic acid: diethyl ether: water (9:6:3:1) as solvent. Reducing sugars were detected by spraying with p-anisidine hydrochloride (Schellart *et al*., 1976).

RESULTS AND DISCUSSION

Isolation and selection of suitable organisms is necessary for maximum production of amylase. Several amylolytic mold cultures were isolated from the soils of different areas of UNAAB by observing clear zones of hydrolysis on the RBB-agar medium. These cultures were further tested for amyloglucosidase production. Of all the cultures tested, the culture No. 19 gave the maximum production of amyloglucosidase. The strain was identified as *Aspergillus niger* and assigned the code *Aspergillus niger* CA-19. This organism was used for further studies.

Result of this study showed the occurrence of amylolytic organisms from the soil and this agrees with earlier reports that several amylolytic microorganisms are found in the soil (Omemu *et al*., 2005). The ability of the isolate to produce amyloglucosidase supports the reports of Sivaramakrishnan *et al*. (2006) that many microorganisms including bacteria, yeast and fungi are capable of producing amyloglucosidase and that filamentous fungi like *Aspergillus* constitute the major source among all microorganisms and are often used for commercial production of the enzyme using SSF.

In SSF, the selection of a suitable solid substrate is a critical factor and thus involves the screening of a number of agro industrial materials for microbial growth and product formation (Ellaiah *et al*., 2002). Since rice bran is an agricultural by product, cheap and readily available, its use as substrate for enzyme production makes the waste an ideal raw material for amylase production by solid-state fermentation.

Characterization of the Enzyme

Preliminary attempt made to characterize the enzyme for its enzymatic activity showed that the amyloglucosidase produced by *Aspergillus niger* CA-19 was optimally active at 60°C. The activity of the enzyme decreased drastically at temperatures above 60°C and was totally lost at 90°C (Fig. 1). This is in agreement with the findings of James and Lee (1995) who reported that the temperature optima of amyloglucosidases generally fall in the range of 30-60°C.

Result showed that the activity of the enzyme was optimal when the assay was carried out at pH 4.0. After pH 4, a continuous decrease in enzyme activity was observed (Fig. 2). Fogarty (1983) reported that most amylases are stable between pH 4.0-8.0. The pH profiles appeared to be similar to the amylase-produced by *Talaromyces flavus*, with maximum glucoamylase activity in pH values of 4.0-4.8 (Hang and Woodams, 1993).

It was observed that the enzyme extract was very stable and retained about 95% of its initial activity after storage at 4°C for 10 days. Even at 28°C, it retained 78% of its activity at 10 days (Fig. 3). Enzymes are mostly proteins with a labile nature. Inactivating agent such as temperature impair the native conformation of an enzyme thus affecting its catalytic activity. Since most enzymes are not used immediately after production, the utility of an enzyme depends mainly on its operational

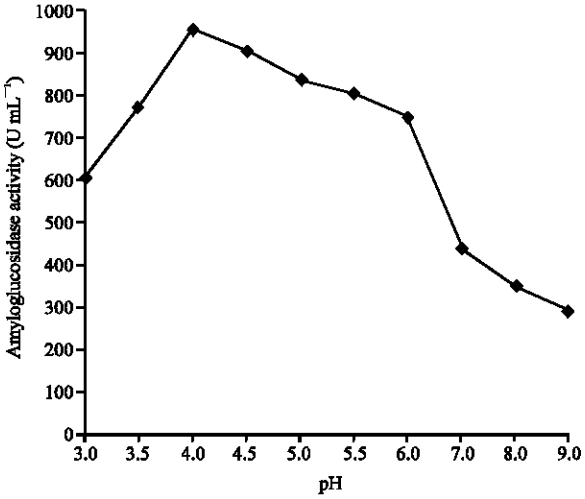

Fig. 1: Effect of pH on Amloglucosidase activity (U mL^{-1})

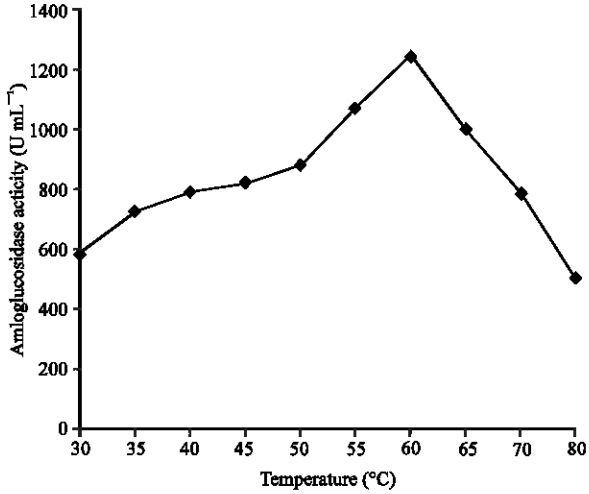

Fig. 2: Effect of temperature on Amlogucosidase activity (U mL^{-1})

and storage stability (Fagain, 2003). The stability of the enzyme is an advantage especially for industrial purposes. According to Fagain (2003) enzyme stability is a crucial factor in the application of enzymes.

Hydrolysis of Raw Starch

Studies on the ability of the crude enzyme to hydrolyze raw starch was carried out using both cereal and tuber starches. The results are presented in Table 1 in terms of the amount of reducing sugars (mg g^{-1}) produced. The susceptibility of each starch to amyloglucosidase hydrolysis was described as the conversion efficiency and it was calculated relative to the corn derived soluble starch used as the standard. The ability of the enzyme extract to degrade the raw starches was significantly ($p<0.05$) dependent on the starch source.

Table 1: Raw starch hydrolysis by amyloglucosidase of *A. niger* CA-19

Starches	Reducing sugars (mg g^{-1})*	Relative conversion efficiency (%)
Cassava	3.6^{b}	35.0
Cocoyam	0.0^{a}	00.0
Maize	10.2^{e}	99.0
Sweet potato	6.6^{c}	64.1
Yam	8.9^{d}	86.4
Soluble starch	10.3^{f}	100.0

*: Mean of triplicate determinations, Values with different superscript are significantly ($p \leq 0.05$) different from one another

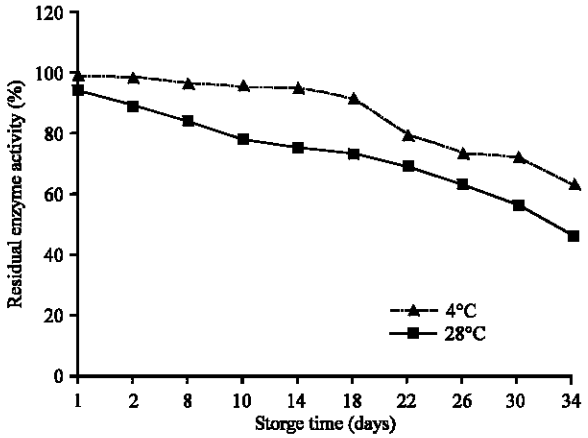

Fig. 3: Stability of crude amloglucosidase of *A. niger* CA-19 stored at 28 and 4°C

With the exception of cocoyam starch, the crude amyloglucosidase of *A. niger* CA-19 hydrolysed all the other starches tested. Statistical analysis showed that there was no significant ($p<0.05$) difference between 10.3 and 10.2 mg g^{-1} reducing sugars obtained for the corn derived soluble starch used as the standard and the maize starch. These were followed by yam starch (8.9 mg g^{-1}) and sweet potato starch (6.6 mg g^{-1}). Cassava starch was least hydrolysed to give 3.6 mg g^{-1} of reducing sugars. The conversion efficiency (Table 1) revealed that relative to the soluble starch used as the standard (100%), maize starch had conversion efficiency of 99.0% while yam, potato and cassava starches had 86., 64.1 and 35.0%, respectively.

This study showed that the susceptibility of the raw starches to the crude enzyme of *A. niger* AM07 was significantly dependent on the starch source. This agrees with earlier reports of Okolo *et al*. (1995) and Omemu *et al*. (2005) that the susceptibility of starch granules to digestion by amylase is dependent on starch source and the length of amylase treatment.

In contrast to the reports of Taniguchi *et al*. (1982) and Okolo *et al*. (1995) that potato starch was considered as one of the less susceptible starch to enzyme hydrolysis, the amylase of *A. niger* CA19 was able to hydrolyse the raw potato starch to give a conversion efficiency of 64.1% relative to the corn soluble starch used as the control.

The ability of the amloglucosidase of *A. niger* CA-19 to hydrolyze especially cassava starch presents a remarkable property since these root starches are abundantly available in the tropics.

Although cassava starch showed the least conversion efficiency (35%), further studies may be carried out to exploit the possibility of obtaining higher conversion efficiency for cassava starch. This will be an added advantage since cassava is readily perishable after harvesting and this has led to the loss of over 30 million tonnes of cassava yearly (Anthony *et al*., 1996; Oluwole *et al*., 1999). The conversion of raw cassava by this enzyme means that some of the cassava could be used as raw materials by the starch industry for value added products. This will reduce wastage and improve economic gain.

Thin Layer Chromatography

The end products determined by thin layer chromatography, under optimum conditions (pH 4.0 at 60°C) showed that glucose was the sole product released in all samples tested after 10, 20, 40 and 60 min of hydrolysis, indicating that the action pattern of the enzyme was entirely of the amyloglucosidase type. This is a major advantage in the industrial production of glucose syrup. Thus our observation on amyloglucosidase production by *A. niger* CA-19 have shown promising results

CONCLUSIONS

This study has confirmed that *A. niger* is a good producer of extracellular amyloglucosidase Due to the fact that these microorganisms are among the microorganisms Generally Recognized As Safe (GRAS) for food, brewing and pharmaceutical applications, more research is necessary to optimize the fermentative process in order to obtain higher amyloglucosidase production through this strain.

REFERENCES

Akpan, I., M.O. Bankole and A.M. Adesemowo, 1999a. A rapid plate culture method for screening of amylase producing microorganisms. Biotech. Tech., 13: 411-413.

Akpan, I., M.O. Bankole, A.M. Adesemowo and G.O. Latunde-Dada, 1999b. Production of amylase by *A. niger* in a cheap solid medium using rice bran and agricultural materials. Trop. Sci., 39: 77-79.

Anthony, O.E., C. Yusuf and M. Murray, 1996. Culture of *Saccharomyces cerevisae* on hydrolysed waste cassava starch for production of baking quality yeast. Enzyme Microb Technol., 18: 519-525.

Baysal, Z., F. Uyar and C. Aytekin, 2003. Solid-state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water. Process Biochem., 38: 1665-1668.

Becks, S., C. Bielawaski, D. Henton, R. Padala, K. Burrows and R. Slaby, 1995. Application of a liquid stable amylase reagent on the Ciba corning express clinical chemistry system. Clin. Chem., 41: 186.

Burhan, A., U. Nisa, C. Gokhan, C. Omer, A. Ashabil and G. Osman, 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. Isolate ANT-6. Process Biochem., 38: 1397-1403.

Ellaiah, P., K. Adinarayana, Y. Bhavani, P. Padmaja and B. Srinivasulu, 2002. Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus species*. Process Biochem., 38: 615-620.

Fagain, C.O., 2003. Enzyme stabilization-Recent experimental progress. Enzyme Microb Technol., 33: 137-149.

Fogarty, W.M., 1983. Microbial Enzymes and Biotechnology. In: Microbial Enzymes and Biotechnology. Fogarty, W.M. (Ed.), Applied Sciences Publishers, Barking, pp: 1-92.

Gupta, R., G. Paresh, H. Mohapatra, V.K. Goswami and B. Chauhan, 2003. Microbial α-amylases: A biotechnological perspective. Process Biochem., 38: 1599-1616.

Hang, Y.D. and E. Woodams, 1993. Thermophilic glucoamylase from *T. flavus*. Lett. Applied Microbiol., 17: 156-157.

James, J.A. and B.H. Lee, 1995. Cultural conditions for production of glucoamylase from *Lactobacillus amylovorus* ATCC 33621. J. Applied Microbiol., 79: 499-505.

Miller, G.L., 1959. Use of dinitro-salicylic acid reagent for determination of reducing sugars. Anal. Chem., 31: 426-428.

Okolo, B.N., L.I. Ezeogu and C.N. Mba, 1995. Production of raw starch digesting amylase by *Aspergillus niger* and *Bacillus alvei* grown on native starch sources. J. Sci. Food Agric., 69: 109-115.

Oluwole, O.B., A.A. Joaquim, A.U. Olatunji, A.U. Ozumba and S.A. Odunfa, 1999. Assessment of the pasting and organoleptic qualities of cocoyam flour from selected *Xanmthomonas* sp. Proceedings of 23rd Annual Conference of NIFST.

Omemu, A.M., I. Akpan and M.O. Bankole, 2003. Amylase recovery from mouldy bran of *Aspergillus niger* AM 07. Nig. Food J., 22: 60-64.

Omemu, A.M., I. Akpan, M.O. Bankole and O.D. Teniola, 2005. Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil. Afr. J. Biotechnol., 4: 19-25.

Pandey, A., P. Nigam, C. Soccol, V. Soccol, D. Singh and R. Mohan, 2000. Advances in microbial amylases. Biotechnol. Applied Biochem., 31: 135-152.

Prakasham, R.S. Subba Rao, R. Ch. Sreenivas Rao and P.N. Sarma, 2007. Enhancement of acid amylase production by an isolated *Aspergillus awamori*. J. Applied Microbiol., 102: 204-211.

Ramakrishna, S.V., T. Suseela, N.P. Ghilyal, A. Jaleel, P. Prema, B.K. Lonsane and S.Y. Ahmed, 1982. Recovery of amylo glucosidase from mouldy bran. Indian J. Technol., 20: 476-480.

Rasper, K.B. and D.J. Fennel, 1965. The Genus *Aspergillus*. Baltimore: Williams and Wilkins.

Rose, H.A., 1980. Microbial Enzymes and Bioconversions. Economic Microbiology, Academic Press, 5: 116-117.

Schellart, J.A., F.M.W. Visser, T. Zandstra and W.J. Middelhoven, 1976. Starch degradation by the mould *Trichoderma viride*. I. The mechanism of starch degradation. Antonie Van Leewenhoeck 42: 229-238.

Sivaramakrishnan, S., D. Gangadharan, K.M. Nampoothiri, C.R. Soccol and A. Pandey, 2006. α-amylases from microbial sources. Food Technol. Biotechnol., 44: 173-184.

Taniguchi, H., F. Odashima, M. Igarashi, Y. Maruyama and M. Nkamura, 1982. Characterization of a potato starch digesting bacterium and its production of amylase. Agric. Biol. Chem., 46: 2107-2115.