Innovative Romanian Food Biotechnology Vol. 16, Issue of March, 2015
 Received November 24, 2014/Revised February 19, 2015/Accepted February 20, 2015

RESEARCH ARTICLE

ISOLATION AND MOLECULAR CHARACTERIZATION OF INULINASE-PRODUCING THERMOPHILIC FUNGI FROM WASTE DUMPSITE LOCATED AT APETE-AWOTAN IN IBADAN, SOUTH-WESTERN NIGERIA

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Abstract: In this study, attempts were made to isolate and characterized inulinase-producing thermophilic fungi from waste dump site located at Awota-Apete, Ibadan, South-Western Nigeria. The thermostability of the inulinase produced was also investigated by incubating inulinase produced at 65 °C for three hours and thereafter measuring the residual inulinase activity. Eight different fungi capable of growing on inulin agar at 50 °C were isolated. They were identified as *Aspergillus flavus*, *A. tamarii*, *A. parasiticus*, *A. niger*, *A. oryzae*, *Rhizopus* sp., *Penicillium citrinum* and *Neurospora* sp. using cultural, microscopic and the sequence of the Internal Transcribed Spacer regions 1 and 2 (ITS1/ITS2) of the fungal genome. Thermostability studies on the produced inulinase revealed that inulinase from *A. tamarii*-INU4 retained 13.7U/ml of its activity after incubation at 65°C for 3 hr. This was followed by extracellular inulinase produced by *A. flavus*-INU2 with 5.4U/ml residual activity after incubation at 65°C for 3hrs. Extracellular inulinase by *Penicilium citrinum* had the lowest residual activity of 0.2U/ml after incubation at the same condition. Conclusively, this paper reports potential sources of thermostable inulinase- a property which is necessary for the complete hydrolysis of inulin by inulinases in the production of fructose and fructo-oligosaccharides.

Keywords: inulinase, thermostability, thermophilic, fructose

Introduction

Fructose as an alternative sweetener to sucrose in the food and allied industries has received so much attention due to the several health benefits associated with the consumption of fructose; fructose does not trigger the production of insulin by the pancreatic β Cells hence ideal for diabetic patients (Sanchez-Lozada *et al.*, 2008). Fructose has also been found to increase iron absorption in children and also to stimulate calcium absorption in postmenopausal women (Heuvel *et al.*, 2000). Furthermore, studies have shown fructose to prevent colon cancer due to the stimulation of the growth of Bifidogenic organisms in the small and large intestine (Durieux *et al.*, 2001).

The current industrial production of fructose involves the combined action of three enzymes (α-amylase, amyloglucosidase and glucose isomerase) acting on starch in succession yielding about 55% fructose and 40% glucose. Fructose from this process is costly and practically not sufficient to meet the demand of the various fructose-utilizing

industries, therefore microbial inulinases (β-fructan frutanohydolases E. C. 3.2.1.7) enzyme capable of hydrolyzing inulin (polysaccharides of β-(2,1)-linked fructose residues terminated by a sucrose residue, accumulated in the underground organs of chicory, dahlia and Jerusalem artichoke) to fructose and fructo-oligosaccharide in a single step reaction, yielding 95% fructose have been proposed as an alternative to the combined action of these three enzymes (Vandamme and Derycke, 1993).

However, to obtain a high fructose and fructo-oligosaccahride hydrolysate the process of hydrolysis is usually carried out at elevated temperatures (60°C) due to the relative solubility of inulin at room temperature and high ample chance of microbial contamination (Gill *et al.*, 2006). At this temperature, most of the reported inulinases lose their activity after a few hours hence, a need for the replenishment of the inulinase enzyme. This therefore leads to increased cost of fructose production by this process. Based on this, there is therefore an urgent need to isolate and characterize thermostable inulinases from various sources that will be able to meet the high temperature demand of fructose and fructo-oligosaccahride production from inulin.

In this paper we report the isolation and characterization of extracellular inulinase-producing thermophilic fungi isolated from waste dump site Awotan-Apete, Ibadan, south-western Nigeria and the thermal stability of the extracellular inulinases.

Materials and Methods

Screening procedure for inulinase-producing fungi

Soil samples collected from waste dumpsite located at Awotan-Apete within Ibadan, south-western Nigeria were serial diluted and appropriate dilutions plated on the medium of Kim (1975) which contain the following inulin 2.0%; K_2HPO_4 0.1%; $MgSO_4 \cdot 7H_20$ 0.05%; $NaNO_3$ 0.15%; $(NH_4)H_2PO_4$ 0.20%; KCl 0.05%; $FeSO_4 \cdot 7H_20$ 0.01% (initial pH 5.0); agar 1.8%. Plates were incubated at 50 °C for 5 days and observed daily for fungal growth. Distinct colonies were sub-cultured on Potato Dextrose Agar (PDA) and pure cultures maintained on PDA slants at 4°C. Secondary screening for inulinase-producing fungi was done using the medium described above (without agar) as follows; sterile 100 ml of the medium in 250 mL Erlenmeyer flasks was inoculated with 1 ml of spore suspension (containing $5.8x10^7$ spores) and incubated at 50°C for 5 days. Thereafter, the whole culture was centrifuged at 15,000 rpm for 15 minutes and the supernatant taking as the crude inulinase enzyme.

Fungal Identification

Conventional Identification. Active inulinase producers were identified using both cultural and microscopic characteristics.

DNA extraction, PCR amplification and Sequencing

The total genomic DNA of the fungal isolates was extracted and purified as described by Binder and Hibbett (2004). The specific primers ITS1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´) as described by White *et al.* (1990) were used. The PCR reaction mix was made up of 0.5 µM of each primer, 10 µM deoxynucleotides, 1.5 mM MgCl2 and 1 x buffer (Promega). As described Korabecna (2007), the suspension was heated at 95°C for 15 min in thermocycler (T100™, Bio Rad) followed by the addition one unit of the Taq Polymerase (Promega). PCR conditions were as follows: 35 cycles of denaturing at 94°C for 1 min; annealing at 55.5°C for 2 min and extension at 72°C for 2 min with final extension at 72°for 10 min. PCR products (10µl) were digested without further purification using restriction endonucleases CfoI, HaeIII and HinfI. Sequencing of the purified PCR amplicon was done using ABI Bigdye 3.1 cycle sequencing kit (Applied Biosystems, California, USA) on ABI 3730XL and the nucleotide sequence determined by automated sequencer by Laragen Inc., Culver City California. The nucleotide sequences of ITS1/ITS2 of the isolates were aligned and analyzed by using Basic Local Alignment Search Tool (BLAST) program available at http://www.ncbi.nlm.nih.gov and phylogenetic relationship between the isolates done using MEGA software version 5.

Measurement of Inulinase activity

Inulinase activity was determined as follows; culture filtrate obtained by the centrifugation of the enzyme

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extract (0.1 mL) was incubated at 50°C for 15 min with 0.9 mL sodium acetate buffer (0.1 M, pH 5.5) containing 2% inulin. The enzyme was further inactivated by incubating the reaction mixture at 90°C for 10 min.

Table 1. *Cultural and Microscopic characteristics of inulinase-producing thermophilic fungi isolated from waste-dump site located at Awota-Apete Ibadan, south-western Nigeria.*

Isolate codes	Characteristics	Identity
INU1	Wooly texture with characteristic dirty green colour. Highly branched and well developed mycelia with septate hyphae. Vessicle are Flask-shaped bearing coarse and globose conidia.	*Aspergillus parasiticus*
INU2	Highly branched, woolly mycelia with characteristic lime colour. Conidia are globose	*Aspergillus flavus*
INU3	White wooly, highly branched and well developed mycelia with septate hyphae. There is the formation of Rhizoids. Presence of sporangiophores	*Rhizopus* sp.
INU4	Wooly and highly branched mycelium consisting of aseptate hyphae conidiphores end with the formation of a vesicle at its tip. Conidia are brownish and globose.	*Aspergillus tamarii*
INU5	Wooly and highly branched mycelium made up of septate hyphae. Vesicles are flask-shaped bearing black coloured and globose conidia.	*Aspergillus niger*
INU6	Wooly, highly branched mycelium with characteristics white green center and pale yellow on the reverse. The conidia are yellowish green and globose.	*Aspergillus oryzae*
INU7	Well branched mycelia with a characteristic dark green colour (pale yellow on the reverse) made up of sepate hyphae. Conida are spherical	*Penicilium citrinum*
INU8	Wooly mycelia consisting of septate hyphae which later develop to form the conidiphores bearing the conidiospore. The plate is usually orange coloured both on the surface and the reverse.	*Neurospora* sp

This was followed by estimation of reducing sugar liberated using fructose using the DNSA method (Miller, 1959). Absorbance of the reaction mixture was measured using a Jenway Spectrophotometer at 540 nm. One unit of inulinase activity was defined as the amount of inulinase enzyme that produced 1 µmol fructose per minute under standard assay conditions.

Thermostability of the crude inulinase

This was done as described by Garuba and Onilude (2012). Crude extracellular inulinase produced the fungal isolates were incubated at 65°C in the absence of inulin and thereafter the residual activity measured as described above. All experiments were carried out in triplicates and data obtained were subjected to ANOVA at $p \leq 0.05$ using SPSS software ver 17.0.

Results and Discussion

Eight different fungi that can grow at 50°C on inulin-agar medium were isolated. Based on the results of the cultural and Microscopic examination as presented in Table 1, the isolates were identified as *Aspergillus niger*, *Penicillium citrinum*, *Rhizopus* sp., *Aspergillus tamarri*, *Aspergillus flavus*, *Aspergillus oryzea*, *Aspergillus parasiticus* and *Neurospora* sp.

The identity of the isolates was further confirmed comparing the sequence of the ITS1 and ITS2 of the isolates with the fungal genome in the GenBank.

The result of the identification using the NCBI online database is presented in Table 2. Of the eight thermophilic organisms isolated in this study, five were found to belong to the genus *Aspergillus (Aspergillus niger, Aspergillus tamarri, Aspergillus flavus, Aspergillus oryzae,* and *Aspergillus parasiticus*).

Table 2. *Identity of the fungal isolate using NCBI online database*

Isolate Code	Length of Nucleotide Sequence	Accession number of nearest Homology	% of similarity	Name of organism
INU1	567	KJ175439.1	97	*Aspergillus parasiticus*
INU2	623	JF951750.1	100	*Aspergillus flavus*
INU3	574	HE962369.1	100	*Rhizopus* sp.
INU4	614	JX110981.1	100	*Aspergillus tamarii*
INU5	570	KF737865.1	99	*Aspergillus niger*
INU6	648	DQ155287.1	100	*Aspergillus oryzae*
INU7	717	KC665721.1	100	*Penicillium citrinum*
INU8	494	JN572055.1	100	*Neurospora* sp

The genus *Aspergillus* has been reported to compose of several known inulinase-producers (Sharma *et al.*, 1998; Singh and Gill, 2006) with *Aspergillus niger* been one of the most explored fungus for the production of inulinase (Kango and Jain, 2011). Although there seems to be paucity of information on inulinase production by *Aspergillus tamarii*, the inulinase production by *Aspergillus tamarii*-INU4 in this study is among the highest reported in literatures for various wild strain of *Aspergillus* spp (Singh and Gill, 2006; Chand and Jain, 2011). Inulinase production by some species of *Penicillium* has also been reported (Onodera and Shioni, 1992; Sharma *et al.*, 1998; Nakamura *et al.*, 1997; Pessoni *et al.*, 1999 and Hazaa, 1999) while Ohta *et al.* (2002) and Moriyama *et al.* (2002) documented the inulinase production by species of *Rhizopus* sp. Figure 1 shows the phylogenetic relationship between the isolates constructed with Molecular Evolution Genetics Analysis (MEGA) version 5.

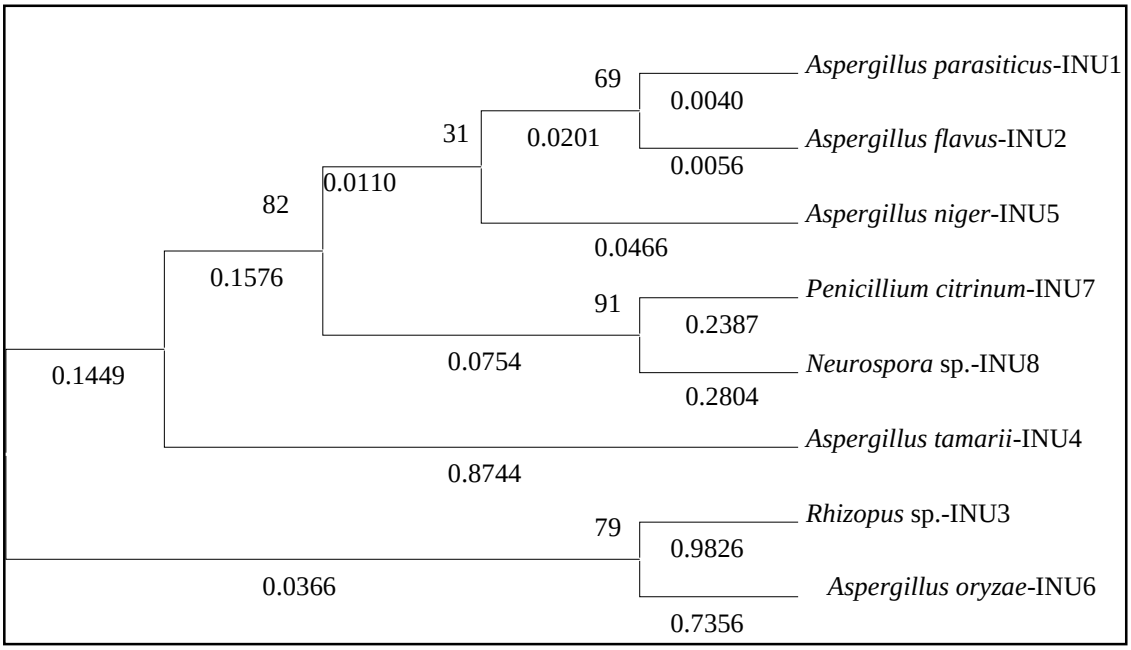

Figure 1. *Phylogenetic relationship of inulinase-producing fungi isolated from waste dump site located at Awotan-Apete within Ibadan Metropolis, Oyo state, Nigeria*

The phylogenetic analysis of the isolates using the neighbour joining method based on maximum composite likelihood revealed that the isolates clustered into three regions with *Aspergillus parasiticus*-INU1, *Aspergillus flavus*-INU2, *Aspergillus niger*-INU5, *Penicillium citrinum*-INU7

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and *Neurospora* sp-INU8 forming a cluster. *Rhizopus* sp-INU3 together with *Aspergillus oryzae*-INU6 also formed another cluster while *Aspergillus tamarii*-INU4 represents the third group. The result of inulinase production in submerged cultures and thermostability of the extracellular inulinase is presented in Table 3.

Table 3. *Inulinase production by Fungi isolated from soil sample from Awotan-Apete in Ibadan, South-Western Nigeria.*

Isolate code	Inulinase activity (U/mL)	Residual inulinase activity at 65 °C for 3 hours
Aspergillus parasiticus-INU1	*11.10±0.12^{d}	2.4±0.71^{d}
Aspergillus flavus-INU2	12.62±0.81^{c**}	5.4±0.37^{b}
Rhizopus sp-INU3	2.40±0.29^{f}	0.9±0.46^{f}
Aspergillus tamarii-INU4	22.21±0.19^{a}	13.7±0.21^{a}
Aspergillus niger-INU5	13.81±0.11^{b}	5.8±0.71^{b}
Aspergillus oryzae-INU6	10.21±1.22^{e}	1.5±0.69^{e}
Penicillium citrinum-INU7	0.91±1.14^{g}	0.2±0.46^{g}
Neurospora sp-INU8	10.81±0.23^{e}	3.2±0.31^{c}

*Data are means of three replicates
**Means with different letters within each column differ significantly ($p \leq 0.05$)

The results showed that inulinase from *Aspregillus tamarii* had the highest residual activity of 13.7 U/mL after incubation at 65 °C for three hours. This was followed by inulinase from *Aspergillus flavus* with 5.4 U/mL residual activity. Extracellular inulinase from *Aspergillus flavus* was followed by inulinase produced by *A. niger* with a residual activity of 5.8 U/mL. The lowest residual activity (0.2U/mL) was observed with inulinase from *Penicillium citrinum*. Similar variation was previously reported by Singh and Gill, (2006). This variation could be as a result of increased salt bridges and or hydrogen bonds within the protein molecule. Also, tighter packaging of the hydrophobic core coupled with higher amino-acids making up the inulinase molecule could also be responsible for the increased thermostability (Berezovsky and Shakhnovich, 2005). High thermal stability observed by *Aspergillus tamarii*-INU4 in this study makes this organism of a potential importance from the industrial point of view. In conclusion, this paper documents the presence of inulinase-producing thermophilic strains of fungi isolated from waste dump site. However, optimization of inulinase production by the various organisms and characterization of the inulinases will need to be carried out in other to determine the suitability of the inulinases for industrial application.

Acknowledgments

The authors wish to thank Mrs Bukola Atobatele of the Department of Biological Sciences, Bowen University, Iwo for the Technical assistance provided during the course of the work.

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