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Isolation and characterisation of theobromine-degrading filamentous fungi

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ABSTRACT

Strategies for achieving global food security include identification of alternative feedstock for use as animal feed, to contribute towards efforts at increasing livestock farming. The presence of theobromine in cocoa pod husks, a major agro-waste in cocoa-producing countries, hinders its utilisation for this purpose. Cheap treatment of cocoa pod husks to remove theobromine would allow largescale beneficial use of the millions of metric tonnes generated annually. The aim of this study was to isolate theobromine-degrading filamentous fungi that could serve as bioremediation agents for detheobromination of cocoa pod husks. Filamentous fungi were screened for ability to degrade theobromine. The most promising isolates were characterized with respect to optimal environmental conditions for theobromine degradation. Secretion of theobromine-degrading enzymes by the isolates was investigated. Theobromine degradation was monitored by HPLC. Of fourteen theobromine-degrading isolates collected and identified by rDNA 5.8S and ITS sequences, seven belonged to *Aspergillus* spp. and six were *Talaromyces* spp. Based on the extent of theobromine utilization, four isolates; *Aspergillus niger*, *Talaromyces verruculosus* and two *Talaromyces marneffei*, showed the best potential for use as bioagents for detheobromination. First-time evidence was found of the use of xanthine oxidase and theobromine oxidase in degradation of a methylxanthine by fungal isolates. Metabolism of theobromine involved initial demethylation at position 7 to form 3-methylxanthine, or initial oxidation at position 8 to form 3,7-dimethyuric acid. All four isolates degraded theobromine beyond uric acid. The data suggest that the four isolates can be applied to substrates, such as cocoa pod husks, for elimination of theobromine.

1. Background

In cocoa-producing countries, millions of metric tonnes of cocoa pod husks (CPH) are discarded annually as by-products of the cocoa industry. There is the ever-present potential of producing CPH-incorporated feed material that can be cheap and readily-available for livestock, poultry and horses. Significant use of CPH in animal feed is however limited by problems with digestibility due to its high fiber content (Alemawor et al., 2009), palatability and adverse effects of some of its chemical constituents. The problems with palatability and adverse chemical effects have mainly been linked to the presence of theobromine in CPH (Gans, 1982; Adeyina et al., 2008).

Theobromine exerts adverse effects on animal physiology, including reduction in cattle milk yield, thymus atrophy in rats, retarded growth and lethargy in pigs and delayed egg-laying in chicken (Braude and Foot, 1942; Gans et al., 1980; Tarka, 1982; Odunsi and Longe, 1995; EFSA, 2008). Besides the expenses involved with the cost of chemicals and physical conditions required, existing methods of theobromine removal from various substrates, including CPH, have resulted in decreased nutritional value of treated material (Odunsi et al., 1999; Adeyina et al., 2010). Introducing the novel application of microorganisms as bio-tools for detheobromination, Adamafio et al. (2011) reported a 71.8% reduction in theobromine content after fermenting CPH with *Aspergillus niger* for 7 days. The results from the study suggested that biodetheobromination could be a successful route to reducing theobromine content in CPH to levels tolerable to livestock (Adamafio et al., 2011).

An important setback with the agent adopted however is the fact

Abbreviations: CPH, cocoa pod husks; AnTD, *Aspergillus niger*; TmTD, *Talaromyces marneffei*; TvTD, *Talaromyces verruculosus*; ThAM, theobromine agar medium; ThLM, theobromine liquid medium; ITS, internal transcribed spacer; PCR, polymerase chain reaction; dGTP, deoxyriboguanidine triphosphate; dATP, deoxyriboadenosine triphosphate; dCTP, deoxyribocytidine triphosphate; dTTP, deoxyribothymidine triphosphate; nBLAST, nucleotide basic local alignment search tool; HPLC, high performance liquid chromatography

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that the majority of fungi of the *Aspergillus* spp. produce ochratoxin A (Varga et al., 1996). Like theobromine, ochratoxins are toxic to animals, exhibiting immunosuppressive, nephrotoxic, teratogenic, and carcinogenic properties (Lea et al., 1989; Varga et al., 1996). The identification and application of other microorganisms which are not ochratoxigenic, and yet are able to degrade theobromine at rates comparable to or even better than *A. niger*, as reported by Adamafio et al. (2011), is therefore of keen interest. The aim of this study was to prospect for theobromine-degrading filamentous fungi that may be applied in biodetheobromination.

2. Materials

Fresh husks of *Theobroma cacao* (cocoa) pods were collected from the Cocoa Research Institute of Ghana (CRIG), Akim-Tafo. CPHs that had been sun-dried for 3 days were broken into pieces and used in preparation of inoculum for isolation of theobromine-degrading fungi.

3. Methods

3.1. Isolation of theobromine-degrading filamentous fungi

Inoculum was prepared by shaking approximately 50 g of CPH in 100 ml sterile distilled water for 30 min. Theobromine-degrading fungi were selected on three differently constituted theobromine agar media (TAM) modified from Hakil et al. (1998). The modifications used 0.6 g theobromine (Sigma-Aldrich) as sole carbon and nitrogen source in the base medium (TAM-T) and either 2.0 g sucrose (Sigma-Aldrich) or 1.01 g ammonium sulphate (Fluka AG) as carbon source or nitrogen source respectively in the theobromine-sucrose (TAM-S) or theobromine-ammonium sulphate (TAM-N) media respectively. All theobromine media were adjusted to pH 5.8 with 0.5% (v/v) H_2SO_4 (Sigma-Aldrich) and 1% (w/v) KOH (Fluka AG). Potato dextrose agar (Park Scientific) was also prepared according to the manufacturers guidelines. All media were sterilised by autoclaving.

Plates of the three theobromine agar media were inoculated with 50 µl of inoculum and incubated at room temperature (25–29 °C). The plates were monitored every 12 h and filamentous fungal colonies were sub-cultured on potato dextrose agar (PDA) plates. Fungal colonies were differentiated from each other based on their colony characteristics; shape, texture, colour and exudate formation. Pure isolates were stored on PDA slants at 4 °C for up to 4 weeks.

3.2. Molecular identification of isolates

The theobromine-degrading filamentous fungi were identified by nuclear rDNA ITS sequencing. Genomic DNA was extracted from the isolates using a Norgen Biotek Plant/Fungi DNA isolation kit (Sigma-Aldrich). The DNA was used as template for ITS-PCR with the primer pair ITS-1 (TCC GTA GGT GAA CCT GC) and ITS-4 (TCC TCC GCT TAT TGA TAT GC). PCRs were performed in 25 µl reaction mixtures with 0.2 µM of each primer, 5 µl of DNA template and reagents from a QIAGEN TopTaq PCR Master Mix kit. The cycling profile was as follows: initial denaturation at 94 °C for 5 mins followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min, extension at 72 °C for 1 min 15 s, and a final extension step at 72 °C for 7 min. Reaction products were separated on 1.5% agarose gel alongside a GeneRuler 100 bp DNA ladder (Thermo Scientific). Bands were stained with ethidium bromide and visualized under UV transillumination. PCR products were purified with a QIAquick PCR purification kit (QIAGEN) and eluted in 50 µl elution buffer and stored at −20 °C. Cycle sequencing was carried out with a Big Dye Terminator cycle sequencing kit version 3.1 (ABI, USA). Sequencing primers were the same as used for amplification by PCR. Cycling conditions were 94 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. Products from the sequencing cycles were purified using an AgenCourt CleanSeq Dye Terminator Removal kit and loaded onto a 3130 ABI Genetic Analyser. Generated sequences were analysed using POP-7 polymer in a 3130 ABI Genetic Analyser. Sequence data were recalled using the Sequence Analysis software version 5.2 and analysed with CodonCode Aligner software version 5.1.5. The generated sequences were matched by nBLAST to library sequences on GenBank database.

3.3. Preparation of spore suspensions

Slants of TAM-S were prepared, inoculated and incubated at room temperature. A maximum of 30 ml sterile distilled water was used to wash spores from each culture (Castellani, 1967) into sterile falcon tubes and the spore suspensions used as inocula for subsequent experiments. Where there was no sporulation after 14 days, bits of hyphae were rubbed-off into distilled water.

3.4. Evaluation of growth of isolates on the different theobromine agar media

For each isolate, plates of each of the three theobromine media were inoculated with spore suspension and incubated at room temperature. At 24-h intervals for 5 days, colony diameters were measured (Spadaro et al., 2010). The isolates were categorized as follows based on their total colony diameters: low theobromine utilisation = diameter ≤ 5.9 cm; moderate theobromine utilisation = diameter 6.0–11.9 cm; high theobromine utilisation = diameter ≥ 12.0 cm. Isolates selected for further study were those that showed at least moderate theobromine utilization (score of ≥ 6.0 cm) on agar media. The medium with the highest total colony diameter was selected as common growth medium for subsequent experiments.

3.5. Assessment of theobromine utilisation in liquid medium

Cultures for subsequent experiments were conducted in theobromine liquid medium. Theobromine liquid medium (TLM) and trace element solution were prepared according to Gutierrez-Sanchez et al. (2012). Theobromine was absent from control TLM. The pH values were adjusted to 5.8 with H_2SO_4/KOH. Filter-sterilised trace element solution was added at 10 ml per liter of TLM. For each isolate, control and experimental media (20 ml each) were inoculated with spore suspension at 10 µl of spore suspension per ml of medium. Cultures were incubated for 5 days at RT, with or without agitation at 90 strokes/min. At the end of incubation, each culture was autoclaved and homogenized using a PRO 200 hand-held homogeniser (PRO Scientific). Turbidity of each homogenate was measured by recording transmittance at 800 nm, and taken as an index of fungal biomass to reflect growth of the isolate. Transmittance readings were converted to absorbance values and growth differences (theobromine-dependent growth) between each isolate and its control were calculated as:

Growth difference = Control absorbance − Experimental absorbance

Using the values in absorbance units (AU) obtained for theobromine-dependent growth, the isolates were categorized as follows: low theobromine utilsation = ≤ 0.09 AU; moderate theobromine utilization = 0.1–0.49 AU; high theobromine utilization = ≥ 0.5 AU. Only isolates that showed growth difference ≥ 0.5 AU (high theobromine utilization) in TLM were included in subsequent experiments.

3.6. Effect of pH

Effect of pH on theobromine-dependent growth of the selected isolates was investigated within the range of pH 3–8 in increments of one unit. Exact initial pH values were measured after media were ready for inoculation. For each isolate, TLM for the different pH environments were inoculated as described. Culture tubes were slanted and incubated without agitation at RT. After 5 days, the cultures were autoclaved,