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Theobromine-degrading Potential of Yeast Strain Isolated from Tomato (*Lycopersicon esculentum*) Fruit

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ABSTRACT
At high dietary concentrations of cocoa (*Theobroma cacao*) pod husk, theobromine exerts deleterious effects on animals, thereby limiting the utilization of the husk as animal feed. The development of simple and efficient procedures for the elimination of the methylxanthine from the husk would be of tremendous benefit to both the cocoa and animal production sectors. Investigations aimed at sourcing for biological agents suitable for the detheobromination of cocoa pod husk resulted in the isolation of a yeast strain from tomato (*Lycopersicon esculentum*) fruit. The isolate was biochemically, physiologically and morphologically characterized and identified as *Candida krusei*. The dependence of its growth on pH, carbon and nitrogen sources was studied. Optimal pH was found to be 5.5. Growth was most vigorous when glucose was used as a source of carbon. Isocratic reversed-phase HPLC using 0.01 M KH_2PO_4: methanol (88:12; v/v) with UV detection was performed to identify theobromine metabolites. Demethylation at position 3 appears to be a major catabolic route for theobromine in the yeast isolate. The isolate holds promise as a tool for the biodetheobromination of cocoa pod husk. Further investigation into its ability to completely utilize theobromine is a subject of ongoing research in our group.

Key words: *Candida krusei*, theobromine, cocoa pod husk, bioprocessing

INTRODUCTION
Millions of metric tonnes of cocoa pod husk, generated from the production of more than 70% of world's cocoa beans (ICCO, 2011), are allowed to decompose on farm dump sites in the West African sub-region annually. One of the strategies for unleashing the huge economic potential of this bio-resource would be to convert it to much-needed complementary feed for the livestock sector (Alemawor *et al.*, 2009; Oddoye *et al.*, 2010; Adeyina *et al.*, 2010). This would address, in part, the perennial challenges of inadequate dry-season forage and the spiraling cost of commercial feed.

Although studies have shown that the pod husk contains significant amounts of soluble and structural carbohydrates that could provide energy for both non-ruminants and small ruminants, the presence of theobromine, 3,7-dimethylxanthine, imposes limitations on its use as a feed ingredient (Owusu-Domfeh, 1972; Adamafio *et al.*, 2004; Rhule *et al.*, 2005). At high dietary concentrations, the bitter purine alkaloid accumulates in animals because it is metabolized very

slowly (Alexander *et al.*, 2008) and causes an array of deleterious effects including spontaneous abortions and reduced weaning weight (Gartrell and Reid, 2007; Ashihara *et al.*, 2008; Belscak *et al.*, 2009).

The use of the pod husk as feed would, therefore, require the development of affordable, efficient procedures for the elimination of theobromine from cocoa pod husk. Chemical methods are available for the removal of theobromine but are inappropriate because they require the use of expensive, sophisticated equipment, solvents and adsorbents (Chiovini *et al.*, 1983; Odunsi and Longe, 1998). Bioprocessing methods are worth pursuing because they are more affordable and environment-friendly (Adamafio *et al.*, 2011). In a previous study, *Aspergillus niger* was found to effectively degrade more than 70% of the theobromine present in cocoa pod husk (Adamafio *et al.*, 2011). However, the possibility of concomitant ochratoxin A production makes its use less than ideal (Varga *et al.*, 1996; Klich *et al.*, 2009; Copetti *et al.*, 2012). The identification of atoxigenic theobromine-degrading microorganisms would greatly facilitate the development of biodetheobromination processes. The present study was, therefore, aimed at bioprospecting for non-pathogenic theobromine-degrading microorganisms.

MATERIALS AND METHODS

HPLC-grade theobromine, 7-methylxanthine, 3-methylxanthine and xanthine were purchased from Sigma-Aldrich and tomato fruits were purchased locally.

Isolation of microorganism: Tomato fruits were allowed to decompose naturally at room temperature for 16-22 days when whitish colonies were visible on the surface of the fruit. A single white colony, which was subsequently identified as *Candida krusei*, was transferred aseptically from decomposing tomato fruit to sterile broth containing 25 mg mL^{-1} chloramphenicol. This was incubated at 37°C for 3 day on a shaking incubator. Samples of the broth were then used in inoculating Sabouraud dextrose agar plates and Potato dextrose agar and sub-cultured to obtain pure cultures.

Identification of microorganism: Microscopic and morphological examinations of colonies were done with the aid of reference mycological guide (Samson and Van Reenen-Hoekstra, 1988). Yeast cell-containing samples were tested for germ-tube formation after the Gram's reaction was carried out as modified by Hucker (1921). Inoculation of pure culture from a negative germ tube test was done on Cornmeal-Tween 80 plates and incubated at 25°C for 72 h. Sugar fermentation test (dextrose, maltose, sucrose, lactose and galactose) was carried out using the Durham's tube in broth cultures for acid and gas production. Isolates were subjected to the urease test and the carbon assimilation reactions of the organisms were also investigated at 25°C incubation.

Effect of pH and carbon source: The influence of different carbon sources on the growth rate of the cells was investigated by growing cells in Salts-citrate medium, pH 5.5, containing the following:- (in g L^{-1} 3.0 KH_2PO_4, 6.0 Na_2HPO_4, 3.0 NaCl, 0.04 $MgCl_2$, 0.11 Na_2SO_4, 2.0 Trisodium citrate). The culture flasks were incubated at 25°C for 16 h in an orbital incubator at 150 rpm, after which turbidity was measured spectrophotometrically at 520 or 600 nm. To determine the optimum pH for growth, similar experiments were conducted in which the pH was varied from 4.0 to 6.5.

Utilization of theobromine as nitrogen source: The yeast isolate was grown on agar plates containing theobromine as either the sole nitrogen or carbon source as previously described

(Adamafio *et al.*, 2011). Subsequently, growth experiments were conducted essentially as described by Lakshmi and Nilanjana (2010) using liquid media containing theobromine instead of caffeine. Aliquots were withdrawn at intervals and optical density was measured at 600 nm. In other experiments, xanthine was used in place of theobromine as the nitrogen source.

HPLC analysis of theobromine and its metabolites: Aliquots of incubation medium were centrifuged and stored at -20°C until analyzed. Supernatants were filtered (0.45 μm) and then subjected to isocratic reversed-phase high-performance liquid chromatography. Separation was performed on a Shim-Pack CLC-C8 (M) column (4.6×250 mm, 5 μm) using a Shimadzu ultrafast LC-20 system with 0.01 M KH_2PO_4: methanol (88:12; v/v) at a flow rate of 1 mL min^{-1} at 25°C and eluates were monitored by absorbance at 275 nm.

Statistical analysis: Analysis of variance (ANOVA) tests along with Least Significant Difference (LSD) post-hoc comparisons were conducted using Excel Data Analysis Statistical Software and Statgraphics-plus Software Programme (Version 3.0). The level of significance was set to $p<0.05$. Differences among means were regarded as statistically significant if p was less than 0.05.

RESULTS

Identification and characterization of microorganism: Microscopic examination of the cells following Gram staining revealed large Gram positive cocci colonies. The isolate grew rapidly and matured in 3 days. Colonies were cream-colored, flat, dry and dull, developing a mycelial fringes. Pseudomycelia formation with elongate blastospores forming a 'cross-match-sticks' or 'tree-like' appearance on Cornmeal-Tween 80 agar was observed microscopically (Fig. 1). Biochemical characterization of the isolate revealed that it assimilated dextrose and ferments urea at room temperature. The isolate did not produce pseudomycelium as revealed by the germ tube test (Table 1). Experiments conducted to further characterize the isolate showed that the optimal pH for growth in liquid medium was 5.5 (Fig. 2). The mean values obtained at pH 5, 5.5, 6 and 6.5 were significantly higher than values obtained at pH 4.0 and 4.5 ($p<0.05$) but were not significantly different from each other. Glucose was the preferred carbon source of the isolate as

Table 1: Biochemical and physiological characterization of the isolate

	Assimilation	Fermentation (acid and gas)	Other tests	Result
Dextrose	+	+	Cycloheximide @ 25°C	-
Maltose	-	-	Urease @ 25°C	+
Sucrose	-	-	Capsule	-
Lactose	-	-	SDA @ 37°C	+
Galactose	-	-	Germ tubes	-
Melibiose	-	ND		
Cellobiose	-	ND		
Inositol	-	ND		
Xylose	-	ND		
Raffinose	-	ND		
Trehalose	-	ND		
Dulcitol	-	ND		
KNO_3	-	ND		

Key: +: Positive, -: Negative, ND: Not determined

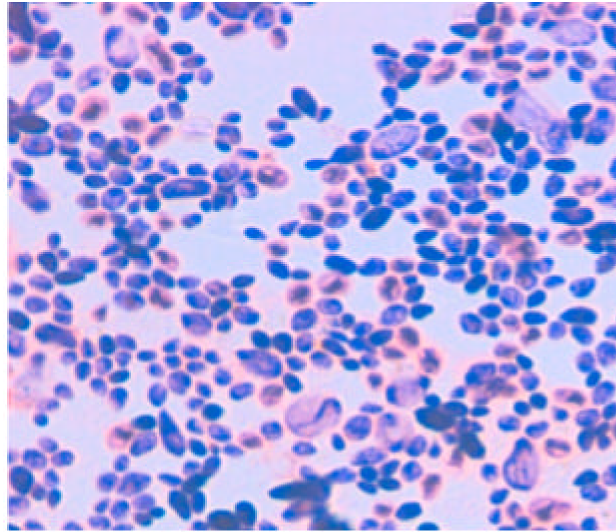

Fig. 1: Gram positive large cocci of the isolate at x1000 magnification

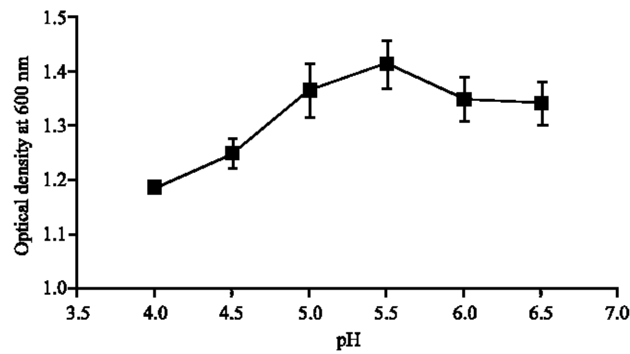

Fig. 2: Effect of pH on growth of yeast isolate

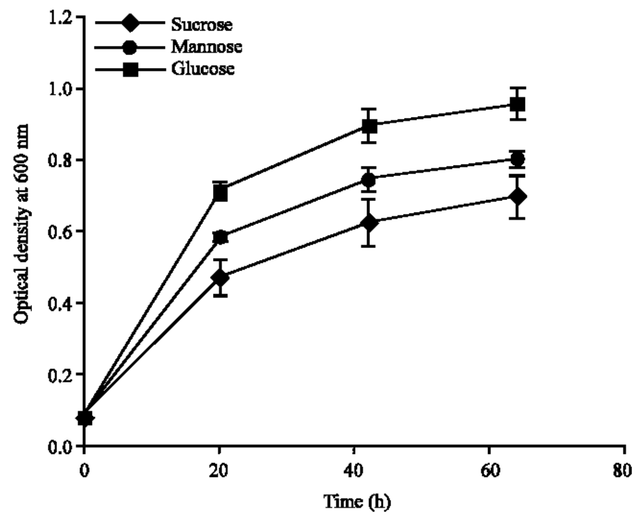

Fig. 3: Effect of carbon source on growth of yeast isolate

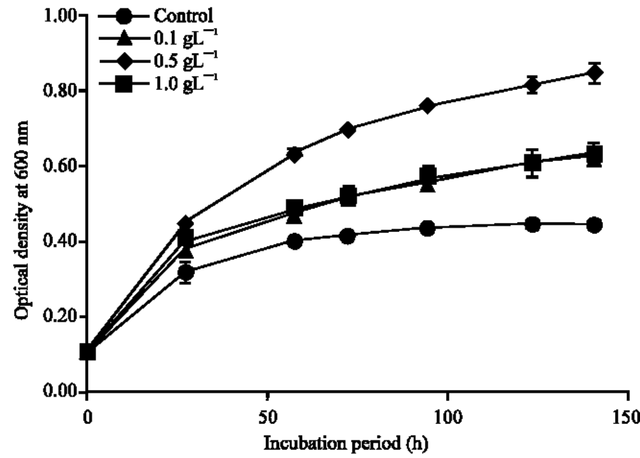

Fig. 4: Utilization of theobromine as a nitrogen source

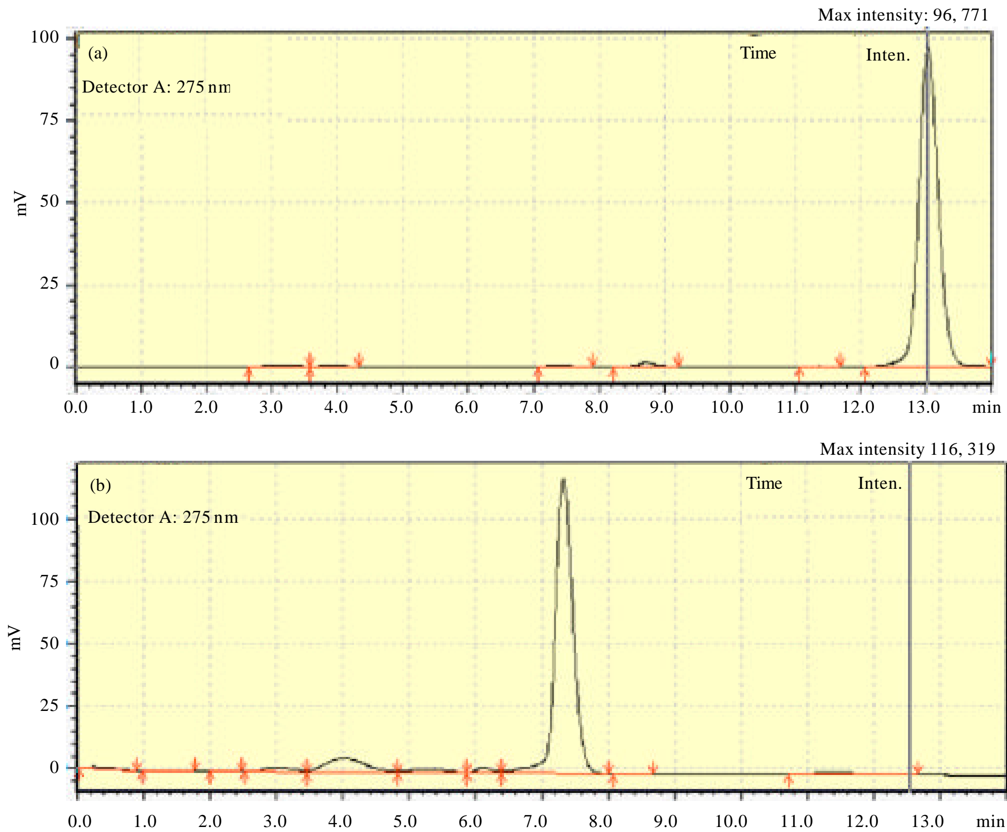

Fig. 5(a-b): Reversed-phase HPLC analysis of liquid media (a) Before and (b) After inoculation with yeast isolate

evidenced by the high growth rate relative to the rates on mannose and sucrose (Fig. 3). The difference between growth rates in glucose and sucrose medium was statistically significant ($p<0.05$).

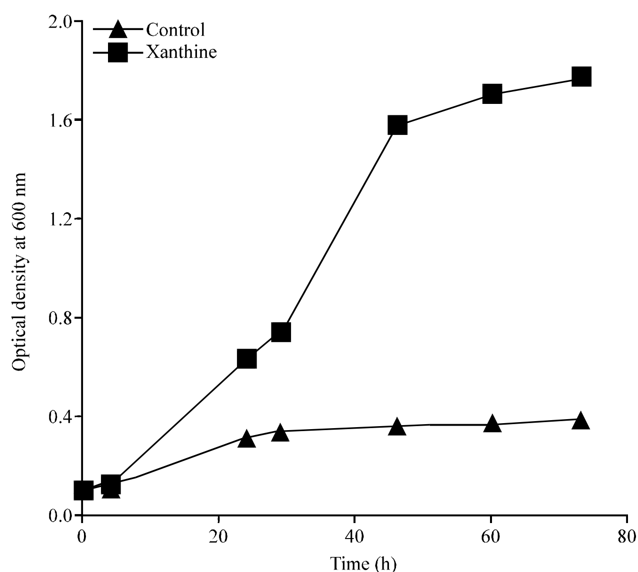

Fig. 6: Utilization of xanthine as a nitrogen source

The isolate grew on agar plates when theobromine was the sole nitrogen source, but growth was sparse when the purine was used as the sole carbon source (results not shown). As shown in Fig. 4, in experiments conducted using liquid media containing a carbon source, the presence of theobromine caused a marked increase in growth at all concentrations tested ($p<0.05$). The rate of growth increased when the concentration of theobromine was raised from 0.1 to 0.5 g L^{-1}. However, a further increase in concentration to 1 g L^{-1} resulted in a decline in the rate of proliferation. The mean values obtained for 0.1 and 1 g L^{-1} theobromine were not significantly different from each other.

Reversed-phase HPLC analysis of liquid culture media showed the disappearance of the theobromine peak and the consequent appearance of a major 7-methylxanthine peak as well as several minor unidentified hydrophilic peaks (Fig. 5). As depicted in Fig. 6, growth on xanthine as a sole nitrogen source was profuse and highly significant ($p<0.01$).

DISCUSSION

The present study was aimed at bioprospecting for non-pathogenic, theobromine-degrading microorganisms, particularly from edible sources such as tomato fruit, which has been shown to be associated with a wide variety of yeasts, filamentous fungi and bacteria (Samson and Van Reenen-Hoekstra, 1988). It is anticipated that, ultimately, an atoxigenic substitute for *A. niger*, suitable for use as a biodetheobromination tool, will be identified.

On the basis of microbiological and biochemical characterization, the theobromine-degrading isolate from tomato fruit was tentatively identified as *Candida krusei* pending definitive characterization preferably by means of 18S rRNA analysis. Ideally, an effective biological tool to be used for the detheobromination of cocoa pod husk must thrive at pH 5.8, the pH value of both fresh and dried cocoa pod husk. The fact that growth of the isolate peaked at pH 5.5 suggests that pH should not pose a problem in the colonization of cocoa pod husk by the isolated microorganism. The use of this yeast isolate for detheobromination purposes would eliminate the possibility of ochratoxin A contamination associated with the use of *A. niger*. The use of a yeast strain would

have an added advantage: Faster colonization of substrates because yeasts grow rapidly like bacteria. The present findings are consistent with the observation that several yeast strains have the ability to degrade caffeine, a related purine alkaloid (Lakshmi and Nilanjana, 2010).

Utilization of theobromine as carbon source: Growth studies were conducted on theobromine infusion plates to determine whether or not the yeast cells could utilize theobromine as a sole carbon source. The results showed that growth was sparse when the methylxanthine was the sole carbon source. Because theobromine is only sparingly soluble in water, the concentrations of theobromine used in the present study were unavoidably low. Given the fact that yeasts require a high carbon to nitrogen ratio of 8:1 to 25:1, the sparse growth observed might be attributable to insufficient carbon rather than an inability to express the enzymes required for the utilization of theobromine as a carbon source.

Utilization of theobromine as nitrogen source: In contrast, growth of the cells on agar was profuse when theobromine was the sole nitrogen source. The difference between growth rates in the presence and absence of theobromine in liquid media confirmed the ability of the isolate to utilize theobromine as a nitrogen source. Presumably, the absence of a primary nitrogen source led to the activation of genes encoding specialized proteins and enzymes needed for the transport and metabolism of the only available secondary nitrogen source. Experiments were also conducted to investigate whether or not theobromine promoted growth in a concentration-dependent manner. Interestingly, although theobromine supported growth of the cells at all concentrations tested, a higher growth rate was recorded at 0.5 g L^{-1} compared with 1.0 g L^{-1}. This raises the possibility that the cells might exhibit a limited tolerance for theobromine in liquid medium. It will be important to investigate the underlying causes of this phenomenon. These findings are similar to the reported inhibitory actions of high concentrations of caffeine, a related purine alkaloid, on the growth of a number of microorganisms (Al-Janabi, 2011; Maletta and Were, 2012).

Theobromine catabolic pathway: Several methylxanthine degradation pathways have been described in microorganisms. Theobromine can undergo initial N-demethylation at either position 7 or 3 and further demethylation to xanthine, which then enters purine intermediary metabolism. Alternatively, the purine alkaloid can be oxidized at position C-8 to 3,7-dimethyluric acid or to 6-amino-5[N-methylformylamino-l-methyluracil when oxidation is coupled to ring-opening (Mazzafera, 2002; Agarwal and Banerjee, 2009). High performance liquid chromatographic analysis of culture media was performed in order to gain insight into the preferred pathway for the degradation of theobromine in the yeast cells. The results showed that 7-methyxanthine is a prominent metabolite. Thus, 3-N-demethylation of theobromine and subsequent demethylation to xanthine appears to be a major catabolic pathway in the yeast cells. The prolific growth observed when xanthine was used as the sole nitrogen source supports this view. It has been demonstrated that in all animal species studied except the rat and dog, demethylation at position 3 predominates over demethylation at position 7 (Walton *et al.*, 2001).

Yeast species are widely used in the food and beverage industries. *C. krusei* is one such species involved in food fermentations. For instance, studies on microbial succession during the fermentation of maize dough have identified *C. krusei* as the dominant yeast species after 72 h possibly due to its ability to tolerate high concentrations of organic acids (Annan *et al.*, 2003; Halm *et al.*, 2004). Studies have also shown that *C. krusei* features prominently during the

fermentation of cocoa beans for the production of chocolate (Tarka, 1982; Jespersen *et al*., 2005; Galvez *et al*., 2007). These examples of the participation of *C. krusei* in food fermentation suggest that issues regarding toxicity and deleterious effects on livestock are unlikely to arise in the use of *C. krusei* as a biodetheobromination tool for cocoa pod husk.

The present study has clearly demonstrated the ability of a *C. krusei* isolate to degrade theobromine and has provided useful information about the isolate. Future studies will focus on the determination and optimization of process parameters for the elimination of theobromine from cocoa pod husk using the isolate.

CONCLUSION

We have successfully isolated a strain tentatively identified as *Candida krusei* that has the capacity to utilize theobromine as a nitrogen source and should be relatively innocuous compared with *A. niger*. The efficacy of this novel yeast isolate as a biodetheobromination tool will depend on several factors: its capacity to colonize cocoa pod husk, the extent to which it will eliminate theobromine during solid-state fermentation of the husk and the alterations it might produce in the nutrient profile of the husk.

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