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Thermo-acid-stable phytase-mediated enhancement of bioethanol production using *Colocasia esculenta*

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HIGHLIGHTS

- Batch and fed-batch production of phytase by *Thermomyces lanuginosus.*
- The purified enzyme is thermostable and acid-stable.
- The enzyme reduced phytate content in *Colocasia esculenta* starch.
- The enzyme improved availability of fermentable sugars.
- Immense potential for application in bioethanol producing industries.

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ABSTRACT

Phytase production by the thermophilic mould *Thermomyces lanuginosus* SSBP was enhanced 8.56-fold in submerged fermentation, which was further improved in fed-batch cultivations. The protein was purified to homogeneity using ammonium sulphate precipitation, Resource Q anion exchange and Superdex gel-filtration chromatography, with an overall purification of 24.7-fold and a yield of 5.16%. The purified 49 kDa protein was optimally active at 55 °C and pH 5.0, and was stable between 50 and 90 °C from pH 3.0–6.0, with a half-life of 138.6 min at 70 °C. It was moderately stimulated by Ba^{+2} and Mg^{+2}. The enzyme reduced phytate content in *Colocasia esculenta* starch (from 1.43 mg/g to 0.05 mg/g) that resulted in an improvement in the availability of fermentable sugars with a concomitant reduction in viscosity and 1.59-fold improvement in ethanol production. Thermo-acid-stable phytase from *T. lanuginosus* SSBP could be of major biotechnological interest, especially due to its robustness and wide applicability.

1. Introduction

Despite a major research shift towards the second, third and now, fourth generation of biofuels, starch-based feedstocks still constitute a major percentage of the global bioethanol production. To address the issue of global food insecurity due to the use of major food crops such as corn in the US, sweet potato in China, and wheat in the EU as feedstocks for bioethanol production, there is an urgent need to identify alternate sources of starch that do not compete with major crops. Underutilized crops could be used as feedstocks for several applications and as a substitute for major crops.

Amadumbe or taro (*Colocasia esculenta*), the fifth most harvested root crop in the world, is cultivated traditionally by rural farmers in Africa and throughout the subtropical and tropical regions with an estimated global production of 11.8 million tons per annum (Nath et al., 2013). In South Africa, amadumbe is grown especially along the KwaZulu-Natal coastline. *C. esculenta* is rich in starch (Naidoo et al., 2015) and several essential elements including Ca, Mg, Al, Mn, Cu, Fe, Co, Cr, Zn, Ni and Se. The bioavailability of these essential nutritional components is, however, limited due to the high phytic acid (PA) content, which also contributes to the lower yield of ethanol from starch fermentation. PA complexes with starch, dietary proteins and lipids and inhibits a number of important digestive enzymes due to negatively charged phosphates on the *myo*-inositol ring. The stable phytin complex interferes with mineral absorption in the digestive tract of monogastrics and is therefore classified as an anti-nutrient. Recently, the PA hydrolysing phytases (EC 3.1.3.8), which are currently the top feed enzymes in the multibillion dollar enzyme market, have also been included in commercial enzyme cocktails routinely used for bioethanol production, although reports on the use of phytases to enhance bioethanol production are scarce

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(Mikulski et al., 2014, 2015; Chen et al., 2015). Moreover, phytase used in these reports was only from mesophilic *Aspergillus niger*, which is not robust enough to withstand the harsh processing conditions. It is, therefore, important to investigate the applicability of thermo-acid-stable phytases that can withstand the high temperature of gelatinization and acidic pH during the enzymatic saccharification and liquefaction steps.

Thermophilic filamentous fungi such as *Thermomyces lanuginosus*, which is a source for the commercial production of thermostable xylanase, lipase and phospholipase, have natural advantages over mesophilic counterparts to secrete high titres of industrial enzymes with increased stability and suitability for harsh processing conditions. The recent genome (Mchunu et al., 2013) and secretome analyses (Winger et al., 2014) of *T. lanuginosus* by our group have shown the presence of many enzymes of major commercial importance including 1431 bp and 1539 bp encoding two phytases.

This study investigated batch and fed-batch production, characterization and application of one of the phytases of *T. lanuginosus* SSBP for improving bioethanol production from underutilized *C. esculenta* starch. This is the first report on a fed-batch production of a unique phytase from a compost-dwelling thermophilic filamentous fungus *T. lanuginosus* SSBP, which is capable of reducing the viscosity and improving the amount of fermentable sugars from *C. esculenta*. The uniqueness of this thermostable phytase is further demonstrated by its utilization immediately after gelatinization.

2. Materials and methods

2.1. Microorganism, culture conditions and raw materials

The filamentous thermophilic fungus used in this study was previously isolated and identified by Singh et al. (2000) as *T. lanuginosus* SSBP, and deposited in the MIRCEN Culture Collection, Bloemfontein (accession number PRI 0226). The fungus was maintained and sub-cultured on potato dextrose agar (PDA) plates at 50 °C. Spores were harvested after 7 days by flooding with normal saline containing 0.1% (v/v) Tween-80 and the spore suspension was adjusted to 1×10^7 CFU/ml. The spores were preserved at 4 °C on PDA slants and as 30% glycerol stocks at -80 °C. The yeast *Saccharomyces cerevisiae* was obtained from the Culture Collection at the Department of Microbiology, Stellenbosch University, South Africa. The strain was maintained at 4 °C on agar slants prepared with yeast malt extract (YM) agar (g/l: yeast extract, 3; malt extract, 3; peptone, 5 and agar, 16) and cryopreserved in YM medium supplemented with 15% glycerol.

To study the effect of phytase on bioethanol production, the purple variety of *C. esculenta* tubers were obtained from a local farm in Durban, KwaZulu-Natal, South Africa. All reagents were of analytical grade and were obtained from commercial sources.

2.2. Enzyme preparation and phytase assay

The *T. lanuginosus* inoculum was prepared using a 2% spore suspension in 250 ml Erlenmeyer flasks containing 50 ml of seed medium containing (g/l): glucose, 15; yeast extract, 1; KH_2PO_4, 2; K_2HPO_4, 2.3; and $MgSO_4 \cdot 7H_2O$ 1, for 36 h at 50 °C and 200 rpm. The composition of the production media and growth conditions varied as per Plackett-Burman and response surface designs (Tables S1a and S2b). The mycelia were harvested by centrifugation at 8800*g* for 20 min and the cell-free supernatant was used to assay total phytase by determining inorganic phosphate (Pi) liberated according to the method described by Heinonen and Lahti (1981). One unit of phytase activity was defined as the amount of enzyme required to liberate 1 nmol of Pi per second under standard assay conditions (Chanderman et al., 2016). The protein concentration was determined by the Bradford method (Bradford, 1976) using Bio-Rad protein assay reagent. Biomass was determined by filtering 10 ml culture samples through pre-weighed membrane filters (0.45 μm; Millipore Corp., USA) and then drying them to constant weight at 80 °C.

2.3. Phytase production

A two-level Plackett-Burman design (PBD) was used to identify the critical cultural variables affecting phytase production by *T. lanuginosus* SSBP. A total of 11 trials with 9 variables and 2 unassigned variables were screened in the present design (Table S1a). Significant factors identified by PBD were further optimized by Response Surface Methodology (RSM) using Design-Expert software (Stat-Ease, Inc., USA) and their interactions were studied (Table S2). The responses obtained from randomly-selected solutions provided by the software were repeatedly compared with the predicted values and the optimized conditions were validated empirically.

2.4. Batch and fed batch fermentation for phytase production

Batch and fed-batch fermentations were conducted in a 5 L glass fermenter (Minifors, Infors HT, Switzerland) containing 2 L of optimized medium with 2% (v/v) inoculum at 47.7 °C. Batch fermentation was carried out for 72 h, while fed-batch fermentation continued for up to 120 h with dissolved oxygen (DO) maintained at 20% air saturation using a cascade controller.

2.4.1. Selection of time of feed

Feeding is generally performed in the exponential phase of microbial growth. In order to find the actual time of feed, fed-batch addition points were evaluated in the exponential phase after 24, 30, 36 and 42 h with 10 ml of glucose (300 g/l). The initial glucose concentration in the production medium was 20 g/l.

2.4.2. Effect of glucose addition

The concentration of the feed can also affect phytase production. Therefore, it was necessary to determine the optimal concentration of feed (glucose as C-source), which could result in the increased production of phytase. Ten ml of concentrated glucose solutions (100, 300, 500, 700 and 900 g/l) were fed to the production medium at 30 h.

2.5. Purification, PAGE and zymography

The crude extract was concentrated by 55–85% ammonium sulphate precipitation and the precipitate was suspended in 0.1 M sodium acetate buffer (pH 5.5). The sample was desalted through a Hi-Prep 26/10 column (GE Healthcare, USA) against 20 mM sodium acetate buffer (pH 5.5) using AKTA purifier 100 (GE Healthcare, USA). This was followed by tangential ultrafiltration using a Sartocon Slice holder with 10 kDa molecular weight cut-off membrane cassettes (Sartorius, Germany). The concentrated and desalted sample was loaded onto a 6 ml Resource Q (GE Healthcare, USA) anion-exchange column equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The proteins were eluted with linear 0–1 M NaCl gradient in 20 mM Tris-HCl buffer (pH 7.5) at a flow rate of 1 ml/min using a combination of isocratic and gradient elution steps. The active fractions were pooled and concentrated using a vacuum centrifuge (Concentrator Plus, Eppendorf, Germany). Subsequently, it was applied to Superdex 200 Increase 10/300 column (GE Healthcare, USA), and eluted with 50 mM sodium acetate buffer (pH 5.5) at a flow rate of 0.5 ml/min. Elution of the protein was