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Extracellular endo-mannanase from *Bacillus* sp. CFR1601: Economical production using response surface methodology and downstream processing using aqueous two phase system

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ABSTRACT

Bacillus sp. CFR1601, isolated from decaying plant litter, produced an extra-cellular endo-mannanase (198.0 IU/g) under solid state fermentation (SSF) using defatted coconut residue as the prime solid substrate. In order to enhance endo-mannanase production, three component, five level central composite design (CCD) of response surface methodology (RSM) was used. Based on contour plots and variance analysis, optimum conditions for endo-mannanase production from *Bacillus* sp. CFR1601 were attained when defatted coconut residue was supplemented with sesame oil meal (10.0, w/w), Tween-80 (0.2%, v/v) and inoculated with bacterial cells from log phase (12 h old; $OD_{600\,nm} \approx 3.6$). The empirical model developed through RSM brought about 4.04–4.39-fold (800.0–870.0 IU/g) improvement in endo-mannanase yield as compared to un-optimized growth conditions. Downstream processing of endo-mannanase from SSF media was carried out for the first time using polyethylene glycol (PEG)/salt aqueous two phase system (ATPS). ATPS system consisting of a combination of PEG 3350 12.0% (w/w), Na_2SO_4 12.0% (w/w), protein load 10.0% (w/w) and pH 5.0 resulted in one-sided partitioning of endo-mannanase towards bottom phase with 3.8-fold purification and 95.4% recovery. Second stage ATPS with fresh top phase further improved purification of endo-mannanase to 12.32-fold. Our overall results suggest a cost-effective and integrated process for production and downstream processing of endo-mannanase.

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Keywords: *Bacillus* sp.; Endo-mannanase; Solid state fermentation; Response surface methodology; Aqueous two phase system; Downstream processing

1. Introduction

Endo-mannanase (EC 3.2.1.78) belongs to glycosyl hydrolase family (GH 5 and 26) of enzymes and randomly hydrolyses internal glycosidic bonds present in linear chain of hetero-1,4-β-D-mannans releasing manno-oligosaccharides (MOS) with different degrees of polymerization (DP). The growing interest in endo-mannanases from microorganisms like bacteria, fungi and yeast is due to their unrivalled potential in food, feed, bio-fuel, oil and gas as well as pulp and paper industries. However, a major bottleneck preventing their full industrial exploitation is the high cost of production as they are majorly produced under submerged fermentation utilizing expensive pure mannans like locust bean gum, guar gum and konjac mannan. Thus, it is pertinent to explore and develop cost-effective enzyme production processes using low-cost substrates (Chauhan et al., 2012; Zyl et al., 2010). SSF using economical agro-industrial residues represents an energy conserving alternative for ameliorated production of industrial enzymes when compared to submerged fermentation (Barrios-González, 2012; Beg et al., 2000; Heck et al., 2005; Heerd et al., 2012; Kapoor et al., 2000; Natarajan and Rajendran, 2012; Pandey et al., 1999; Regalado et al., 2000; Yin et al., 2013). Agro-industrial residues are generated in huge quantities from diverse economic activities across the globe and when not properly discharged or used lead to

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environmental pollution. Coconut residue, a by-product of virgin coconut oil industry in south East Asian countries like India, Philippine, Thailand, Indonesia and Malaysia (Marina et al., 2009), can serve as an excellent low-cost substrate for endo-mannanase production from microorganisms under SSF as it contains substantial quantity of mannan-rich hemicelluloses, micronutrients for bacterial metabolism and good water holding capacity (Naik et al., 2012).

Downstream processing of endo-mannanases from fermentation media is largely dependent on costly and multi-step conventional procedures like ammonium sulphate precipitation, acetone precipitation, ultrafiltration followed by either gel filtration, ion exchange or hydrophobic interaction chromatography's (Chauhan et al., 2012; Zyl et al., 2010) which are not only labour intensive [as many reports cite usage of many chromatographic/concentration steps ranging between two (Puchart et al., 2004) to five (Hrmova et al., 2006) for mannanase purification] and time consuming but also result in low recoveries. As separation and purification accounts for major portion of total cost incurred in generation of purified enzyme, there is an urgent need for effective and inexpensive purification technique, which can achieve dual objectives of high purity and yield. Liquid–liquid extraction using ATPS has been reported as a bio-compatible technique for separation, concentration, and partial purification of enzymes and offers multitude of benefits like ease of operation, high yield, selectivity, scope for scale-up and continuous operation (Dembczynski et al., 2013; Karkas and Onal, 2012; Ng et al., 2011; Yang et al., 2010; Yucekan and Onal, 2011; Zhi et al., 2004). As the partitioning and purification of enzymes in ATPS is largely governed by their physico-chemical properties like size, shape, surface charge, molecular weight, hydrophobicity and bonding interaction (electrostatic, Vander waals and hydrogen bonding) with phase forming components, optimization of operating parameters is pivotal for obtaining high recovery and purity (Benavides et al., 2011).

In the present study, we report (1) utilization of defatted coconut residue as prime solid substrate for endo-mannanase production from indigenous bacteria *Bacillus* sp. CFR1601 using response surface methodology. (2) Downstream processing of endo-mannanase using ATPS.

2. Materials and methods

2.1. Microorganism

The bacterial strain CFR1601 used in the present study was isolated from lignocellulose-rich plant litter at CFTRI campus, Mysore and was maintained on mannan-agar containing (%, w/v) guar gum 0.5, yeast extract 1.0, peptone 1.0, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.01 and agar-agar 1.8, pH 6.0 and was maintained at culture collection of CFTRI. The identification of morphological and physiochemical characteristics of *Bacillus* sp. CFR1601 was carried out according to Bergey's manual of systematic bacteriology (Sneath, 1986). The 16S rDNA gene was amplified by PCR using forward (AGAGTTTGATCCTGGCTCAG), reverse (TACGGYTACCTTGTTACGACTT) primers and with the following cycling parameters: 94 °C for 1.0 min, 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1.0 min final extension at 72 °C for 5.0 min. The amplified fragment was purified from agarose gel using QIA quick gel extraction kit (Qiagen, Germany), ligated into PGEMT Vector (NEB, USA) and transformed into *Escherichia coli* DH5α. Plasmid DNA was isolated from positive bacterial clone and sequenced using universal primers at Merck, Bangalore, India. The sequence deposited in the Genbank database (Accession No. JQ796718) was analyzed for homology using BLAST search with similar existing sequences available in data bank at National Centre for Biotechnology Information (NCBI). The phylogenetic tree was constructed by the neighbour-joining method using multiple sequence alignment program of clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.2. Endo-mannanase production under SSF

2.2.1. Substrate

Coconut residue, procured after extraction/removal of oil and other products (coconut milk, proteins) by wet processing methodology as described elsewhere (Naik et al., 2012), was defatted (for 24 h) in percolator by solvent extraction using n-hexane in 1:15 ratio (coconut residue: n-hexane). Thereafter, spent solvent was decanted and defatted coconut residue obtained was sun dried for 24–48 h and used as substrate for endo-mannanase production.

2.2.2. Growth conditions

Bacillus sp. CFR1601 was aseptically inoculated from fresh mannan agar plate into seed media [(%, w/v) glucose 0.5; yeast extract 1.0; peptone 1.0; $MgSO_4$ 0.01; KH_2PO_4 0.1, pH 6.5] in 250 ml Erlenmeyer flasks and incubated at 45 °C under shaking conditions (180.0 rpm) for 12 h. Basal media for enzyme production under SSF contained 5.0 g defatted coconut residue hydrated with mineral salts solution (MSS) [(g/l) KH_2PO_4, 1.0; NaCl, 2.5; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; $CaCl_2$ 0.1; pH 6.0 adjusted using 10.0% (w/v) Na_2CO_3] in 250 ml Erlenmeyer flasks to get an initial solid substrate-to-moisture ratio of 1:10.5 and was sterilized by autoclaving at 121 °C for 15 min. MSS was completely absorbed by defatted coconut residue resulting in moist/damp solid-substrate lacking any free flowing water. Thereafter, basal media were inoculated with 10.0% (v/v) inoculum (12 h old) and flasks were incubated at 45 °C for 24 h in a humidified incubator (Tempo Industrial Corporation Pvt. Ltd. India). The contents of the flasks were mixed intermittently by gentle thumping so as to ensure uniformity during the course of fermentation.

After completion of incubation period, endo-mannanase was extracted from fermented substrate using citrate-phosphate buffer (50.0 mM, pH 6.0, 1:10 solid-to-liquid ratio) under shaking conditions (200.0 rpm), 15.0 min, room temperature followed by filtration (using Whatman No. 1) to remove solids. The separated solids after filtration were oven dried (60 °C) until constant weight was attained and endo-mannanase activity obtained in filtrate was finally expressed as IU/g of dry solid substrate.

2.3. Enhanced endo-mannanase production from *Bacillus sp. CFR1601* using response surface methodology (RSM)

Significant variables like pH, carbon source, nitrogen source and additives (amino acid and their analogues, vitamins and surfactants) were optimized using one variable at a time approach (data not shown). Subsequently, variables like sesame oil meal, Tween-80 and inoculum age that had the maximum influence on final response (endo-mannanase production) were optimized through RSM following central composite design. The minimum and maximum range of

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