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Original Research Paper

# Design-of-experiment strategy for the production of mannanase biocatalysts using plam karnel cake and its application to degrade locust bean and guar gum

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ABSTRACT

Production of β-mannanase from thermotolerant *Aspergillus terreus* FBCC 1369 was optimized by response surface methodology (RSM) using plam kernel cake (PKC) as substrate. Effect of particle size of substrate, pH, moisture content and carbon and nitrogen supplements was studied. Under unoptimized conditions i.e. 2 mm particle size of PKC, moisture level 1:1 and pH 6.5 fungus produced only 41 U/gds. Initial optimum conditions i.e. 0.5 particle size of PKC, locust bean gum (1% w/v) and urea (1% w/v) at pH 6.5 and moisture level 1:1 resulted in 159 U/gds of β-mannanase yield. The effect of pH and moisture content on production was further optimized using RSM. Rotatable central composite design (RCCD) was employed to investigate the effects of pH (4–10) and moisture content (5–20 ml) on mannanase yield during solid state fermentation (SSF). Results revealed statistical significance of model as evidenced from high value of coefficient of determination ($R^2 = 1.000$) and $P < 0.05$. Optimized medium consisted of 5 g PKC of 0.5 particle size, LBG 1% (w/v) and urea 1% (w/v) as carbon and nitrogen supplementation with 12.8 ml moisture content and pH 8.0 resulting in 417 U/gds β-mannanase, which was 10- fold higher than the original value. The β-mannanase production obtained in this study using *A. terreus* was significantly higher than those reported in the literature. Activity of accessory enzymes, α-galactosidase (13 U/gds) and β-glucosidase (21 U/gds) in this preparation indicated that it can be used to achieve complete hydrolysis of galactoglucomannan by synergistic action of these enzymes. The end product analysis of mannanolytic action analyzed using HPLC revealed formation of mannose and mannobiose from galactomannan (locust bean and guar gum).



## 1. Introduction

Mannan is the second major hemicellulose after xylan that appears as hemicellulose fraction of soft and hard wood. Mannan is a heteropolysaccharide chiefly consisting of mannose along with glucose in main chain and galactose in side chain. Complete degradation of mannan requires main chain breaking enzymes, β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25) and β-glucosidase (EC 3.2.1.21 ) and side chain breaking enzyme α-galactosidase (EC 3.2.1.22) (Soni and Kango, 2013). Mannan degrading enzymes have various applications in the paper, food and feed, oil drilling and detergent industries (Chauhan et al., 2012; Soni and Kango, 2013). Many fungi like *Aspergilli* spp., *Penicillium* spp., *Streptomyces* spp., and *Trichoderma reesei* and bacteria such as *Acinetobacter* sp., *Bacillus* spp., *Cellulomicrobium* sp., and *Klebsiella* sp. have been reported as potential mannanase producers (Chauhan et al., 2012; Soni and Kango, 2013). Production of mannanase by microorganisms is influenced by the medium composition and mode of production as well. Some filamentous fungi give better result in solid state cultures due to low $a_w$ requirement. Beside carbon and nitrogen source in SSF, type and condition of substrate with initial pH and moisture are key factors that influenced final yield of enzymes (Kamra and Satyanarayana, 2004; Mohamad et al., 2011; Chauhan et al., 2012; Yin et al., 2013; Sadaf and Khare, 2014).

A mathematical method, RSM is a useful statistical method for studying the influence of parameters in terms of response by varying them simultaneously using a limited number of experiment runs (Neter et al., 1996; Rao and Satyanarayana 2003; Rao

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and Satyanarayana 2007). The main objective of this study was to utilize an agro-industrial waste (PKC) for its use in mannanase production in SSF. Agro-industrial residues are generated and accumulated in large quantities from various economic activities across the world and due to their improper discharge or use lead to environmental pollution. SSF also provides some advantages over submerged culture, for instance its relatively simple, lower cost investment, low moisture level and easy downstream processing. PKC is residue of palm oil industry that is produced as by-product after oil extraction from palm tree (*Elaeis guineensis*) fruit (Keng et al., 2009). According to Dusterhoft et al. (1992) report, PKC contains more than 70% mannan and therefore can serve as an excellent low-cost substrate for mannanase production from microorganisms under SSF. The present study was aimed to optimize the process parameters for mannanase production on PKC and understand interaction of some of the important factors using RSM. Mannanase production using expensive pure mannans like LBG, guar gum, konjac mannan etc. leads to high cost of the enzyme and renders their application economically difficult (Chauhan et al., 2012). In this work, RSM was applied to study the combined effects of two important independent variables of SSF, pH and moisture content on β-mannanase production by *A. terreus* FBCC 1369.

## 2. Materials and methods

Mannobiose ($M_2$) and oligo-mannans ($M_3$, and $M_4$) standards were purchased from Megazyme (Bray, Ireland). LBG, solka floc, glucose, mannose, guar gum, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenol (pNP) and other chemicals were sourced from Sigma-Aldrich, USA. PKC was purchased from M/s Meh Impex, Tamil Nadu, India. Fenugreek seed meal (seed meal of *Trigonella foenum-graecum*), rice husk, *aloe vera* pulp, wheat bran and wheat straw were purchased from local market.

## 3. Microorganism

Thermotolerant *A. terreus* FBCC 1369 was isolated from Sagar, M.P. India during a survey of thermophilic fungi from litter and decaying wood (Maijala et al., 2012). It was identified on the basis of cultural and morphological characteristics. Its identity was also confirmed using ITS sequence (GenBank: FN811183.1). The strain was maintained on Czapek Dox slants at 4 °C and sub-cultured after every 30 days.

## 4. Inoculum preparation and solid state fermentation

Spores of *A. terreus* from 5 days old culture grown on Czapek Dox agar at 37 °C were harvested using 5 ml sterile 0.01% (w/v) Tween 80 with aid of wire loop. Various substrates like wheat bran, wheat straw, rice husk, PKC, fenugreek seed meal, and aloe vera pulp were used for mannanase production. Different sizes of sieve were used to obtain 0.5, 1.0 and 2.0 mm particle size of PKC. Fermentation was carried out in Erlenmeyer flask (250 ml) with 5 g substrates with 1:1 ratio of moisture content. Selected substrate was supplemented with locust bean gum, guar gum, konjac gum, glucose, mannose and solka floc as carbon supplements (1% w/v) and yeast extract, peptone, urea and ammonium sulfate used as nitrogen supplements (1% w/v). Flasks were then autoclaved at 121 °C for 30 min and inoculated with 1 ml of spore suspension ($2 \times 10^6$ spores/ml) of *A. terreus* and incubated at 37 °C for five days.

## 5. Extraction of mannanase

After incubation of 5 days, 50 ml of 0.05 M citrate buffer (pH 5.0) was added to each flask and kept for shaking at 150 rpm for 1 h at 4 °C temperature. For extraction, entire content of flask was squeezed through a muslin cloth and the extract was centrifuged at 9000 *g* for 15 min at 4°C. Clear supernatant was decanted and used as source of mannanase.

## 6. Enzyme assays

### 6.1. β-Mannanase

β-Mannanase activity was measured using locust bean gum (0.5% w/v) as substrate. LBG was dissolved in 0.05 M Na-Citrate buffer (pH 5.0) by stirring constantly for 1 h at 60 °C clear solution was obtained by centrifugation at 10,000 for 10 min. 100 μl enzyme sample was added to 900 μl substrate and incubated at 50 °C for 10 min. The reaction was stopped by adding 3 ml dinitrosalicylic acid (DNS) reagent and boiling for 5 min. Reducing sugar was measured at 540 nm against the blank (Miller, 1959). One unit (1 U) of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of mannose per min under experimental conditions.

### 6.2. α-Galactosidase and β-glucosidase

*p*-Nitrophenyl-α-D-galactopyranoside (2 mM in 0.05 M Na-citrate buffer pH 5.0) 900 μl was incubated with 100 μl enzyme sample at 50 °C for 10 min. The reaction was stopped by adding 0.5 ml of 1 M $Na_2CO_3$, and absorbance of released *p*-nitrophenol was determined at 400 nm (Bailey and Nevalainen, 1981). One unit (1U) of α-galactosidase was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol in 1 min. β-glucosidase assay was same as described for α-galactosidase except that *p*-nitrophenyl-β-D-glucopyranoside (1 mM) was used as substrate.

### 6.3. Experimental design for the determination of optimum pH and moisture content

Both physical components (A) pH and (B) moisture content were selected to find their optimum values for mannanase production by *A. terreus* using RCCD. The ranges and levels of the variables taken for RSM are listed in Table 1. According to RCCD, the total number of experimental combinations is $2^k+2k+n_o$, where $k$ is the number of independent variables and $n_o$ is the number of repetitions of the experiments at the center point. A total of 13 set of experiments including five center points were conducted along with different combinations of the two physical parameters. Each numeric factor was varied over 5 levels, that is, plus and minus alpha (axial point), plus and minus one (factorial points), and zero (center point).

**Table 1**
Experimental ranges and levels of the independent variable used in RSM for mannanase production by *A. terreus* FBCC 1369.

| Variables | | Experimental ranges and levels | | | | |
|---|---|---|---|---|---|---|
| | | $-\alpha$ | $-1$ | 0 | 1 | $+\alpha$ |
| pH | Unit | 3.76 | 5 | 8 | 11 | 12.24 |
| Moisture | ml | 1.89 | 5 | 12.5 | 20 | 23.11 |