Engineering in Life Sciences

Eng. Life Sci. **2017**, *17*, 392–401 www.els-journal.com

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Research Article

Screening, statistical optimized production, and application of β-mannanase from some newly isolated fungi

Eighty-eight fungi isolated from soil and decaying organic matter were screened for mannanolytic activity. Twenty-eight fungi produced extracellular mannanase on locust bean gum as evidenced by zone of hydrolysis produced on mannan agar gel. Six prominent producers, including four *Fusarium* species namely *Fusarium fusarioides* NFCCI 3282, *Fusarium solani* NFCCI 3283, *Fusarium equiseti* NFCCI 3284, *Fusarium moniliforme* NFCCI 3287 with *Cladosporium cladosporioides* NFCCI 3285 and *Acrophialophora levis* NFCCI 3286 produced the β-mannanase in the range of 84–140 nkat/mL. All these grew well on particulate substrates in solid-state fermentation (SSF), producing relatively higher titers on mannan-rich palm kernel cake (PKC) and copra meal. Two high yielding strains, *F. equiseti* (1747 nkat/gds) and *A. levis* (897 nkat/gds) were selected for statistical optimization of mannanase on PKC. Interaction of two critical solid state fermentation parameters, pH and moisture on mannanase production by these two molds was studied by response surface method. Optimized production on PKC resulted in three- to fourfold enhancement in enzyme yield was observed in case of *F. equiseti* (5945 nkat/gds) and *A. levis* (4726 nkat/gds). HPLC analysis of mannan hydrolysate indicated that *F. equiseti* and *A. levis* mannanase performed efficient hydrolysis of konjac gum (up to 99%) with exclusive mannooligosaccahride (DP of 4) production. A seminative SDS-PAGE revealed that *A. levis* and *F. solani* produced three isoforms, *F. moniliforme* produced two isoforms while *F. fusarioides*, *F. equiseti*, and *C. cladosporioides* produced a single enzyme.

Keywords: *Acrophialophora levis* / *Fusarium equiseti* / Hemicellulose / Mannanase / Solid-state fermentation

Additional supporting information may be found in the online version of this article at the publisher's web-site

Received: May 19, 2016; *revised:* August 3, 2016; *accepted:* September 19, 2016

DOI: 10.1002/elsc.201600136

1 Introduction

Enzymes sourced from fungi occupy pivotal role in various biotechnological applications such as bioconversion, biotransformation etc. Mannan-degrading enzymes find applications in the area of functional food, animal feed, and lignocellulose biotechnology [1, 2]. Ubiquitous and diverse fungal forms present a great opportunity for their screening as source of mannan-degrading enzymes. Forests and woods of central India situated near tropic of cancer are rich in biodiversity and have much less explored niches, which harbor myriad fungal forms. Lignocellulose hydrolysis has been a challenging area and fungi being natural lignocellulose degraders are therefore being explored for this purpose. This has led to identification of many potential cellulase and xylanase producers [3].

Mannans comprise a large share (up to 10%) of hemicellulosic fraction of lignocellulosic plant biomass [4]. Mostly

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Abbreviations: ANOVA, analysis of variance; **GG**, guar gum; **LBG**, locust bean gum; **MOS**, mannooligosaccharides; **PHGG**, partially hydrolyzed guar gum; **PKC**, palm kernel cake; **RCCD**, rotable central composite design; **RSM**, response surface methodology; **SSF**, solid-state fermentation

mannans are recovered from softwood trees such as spruce and also observed in specialized structures such as plant seeds and fruits [5].

Mannanolytic enzymes are involved in the conversion and modification of mannans. Using a retaining mechanism, they can in addition to hydrolysis, also potentially perform transglycosylation reactions, synthesizing new glycoconjugates. Generation of reducing sugars by enzymatic saccharification of agroresidues and its use in production of biofuel ethanol may lead to development of cost-effective bioprocess. Complete degradation of mannan requires synergistic action of multiple enzymes viz. β-mannanases (1,4-β-mannan mannohydrolase EC 3.2.1.78) that release mannooligosaccharides (MOS), β-mannosidases (1,4-β-mannopyranoside hydrolase EC 3.2.1.25) that release mannose, β-glucosidases (1,4-β-glucoside glucohydrolase EC 3.2.1.21) that break glycosidic bond between mannose and glucose and side-chain cleaving enzyme, α-galactosidase (1,6-α-galactoside galactohydrolase EC 3.2.1.22) [6, 7].

Currently, many workers have focused on devising novel cost-effective enzyme production medium by employing inexpensive mannan-rich agroresidues or agrowaste materials that are abundantly available, e.g. palm kernel cake (PKC), copra meal (CM) etc. [3, 8] In the present investigation, we have employed several agroresidues in solid state fermentation (SSF) as sole substrate for production of mannanase. Production using such substrates in SSF is cost effective and also presents a way of managing environmental pollution originating due to dumped agroresidues.

Recent surge in importance of prebiotic oligosaccharides has led to the development of several methods for their economical production. Enzymatic generation of MOS using cheap and easily available substrate such as konjac gum can be a low-cost competitive method. Certain MOS possess nutritional values; MOS-based nutrition supplements are widely used in nutrition as feed additives. Various reports reveal the beneficial effects of MOS on the intestinal microflora, intestinal structure, and function [9]. Many feed manufacturers add MOS in diet preparations of animals such as horses, dogs, cats, rabbits, and poultry [10]. MOS are an important poultry feed additive that prevent infection by interfering in bacterial attachment in intestinal tract. Also, MOS selectively promote probiotic flora, especially *Lactobacillus* and *Bifidobacterium* [11, 12].

Owing to these benefits, considerable efforts on isolation and screening of fungi producing β-mannanase are being made. Some strains of *Trichoderma*, *Penicillium*, *Sclerotium,* and *Aspergillus* are reported as potential producers of mannanase [6, 13–15]. However, there is a strong need to explore fungal diversity for identification of new strains for mannanase production containing endoactivity for MOS generation with less or no monomeric sugars. Present communication describes for the first time, some native fungal isolates producing mannanase belonging to *Acrophialophora, Cladosoprium,* and *Fusarium* genus useful in generation of MOS. We also describe generation of MOS from cheap and readily available substrate konjac gum and production of partially hydrolyzed guar gum (PHGG), which is useful in clinical nutrition and in treatment of irritable bowel syndrome.

2 Materials and methods

Mannobiose (M_2) and oligomannans (mannotriose M_3 and mannotetraose M_4) standards were purchased from Megazyme (Bray, Ireland). Locust bean gum (LBG), solka floc, glucose, mannose, guar gum (GG), *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenol and other chemicals were sourced from Sigma–Aldrich (St. Louis, MO, USA). CM was obtained as sample from Parker Biotech Private Ltd., Chennai, India. PKC was purchased from M/s Meh Impex, Chennai, India. Food grade konjac gum (glucomannan) was obtained from New Foods, Bloomingdale, IL, USA. Fenugreek seed (*Trigonella foenum-graecum*) meal, *Aloe vera* pulp, wheat straw, rice husk, and wheat bran were purchased from local market.

2.1 Isolation and screening of fungi

Eighty-eight strains of fungi were isolated from various ecological niches (Supporting information Table S1) and screened for mannanolytic activity. All the isolated fungi were grown in mannan containing medium (g/L): yeast extract 14.0, $(NH_4)_2SO_4$ 2.1; $MgSO_4 \cdot 7H_2O$ 3.0, $CaCl_2 \cdot 2H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.5, and KH_2PO_4 10.0, pH 5.0 and LBG (0.5% w/v) as mannan source [6]. Each flask was inoculated with two discs (6 mm) of 6-day-old culture and incubated for 5 days at 28°C with agitation of 150 rpm. After incubation, cell-free culture filtrate was obtained by filtration and centrifugation at 9000 $\times$ *g* for 20 min at 4°C. The supernatant was used as the enzyme source for assessment of mannanase activity by mannanase assay.

2.2 Detection of mannanase activity

Mannan agar gel was prepared by adding agar (2% w/v) to mannan solution (0.5% LBG in 50 mM sodium citrate buffer, pH 5.0). It was autoclaved and dispensed in Petri dishes of 12 cm diameter. Wells (10 mm) were loaded with 50 μL of culture filtrate and plates were incubated for 12 h at 50°C. Zone of hydrolysis was visualized by staining with Congo red (1% w/v) for 30 min. The gels were destained with 1 M NaCl to remove unbound stain and formation of a clear zone around well was used to score a given fungi as positive for mannanase production [16], while heated crude enzyme (100°C) served as a negative control.

2.3 Enzyme assay

2.3.1 β-Mannanase, α-galactosidase, β-mannosidase, and β-glucosidase assay

Mannanase activity was quantified using LBG (0.5% w/v) as substrate [6]. LBG was dissolved in 50 mM Na-citrate buffer (pH 5.0) under constant stirring for 1 h at 60°C. Hundred microliters of culture filtrate was incubated with 900 μL substrate at 50°C for 10 min. The reaction was stopped by adding 1.5 mL dinitrosalicylic acid reagent and subsequent boiling for 5 min. Reducing sugar was measured at 540 nm against appropriate

blank prepared using 100 μL citrate buffer instead of culture filtrate was incubated with 900 μL substrate at 50°C for 10 min and mannanase activity was expressed as nkat/mL. One nano katal (nkat) of mannanase was defined as the amount of enzyme that liberated 1 nano mole of reducing sugar per second.

For α-galactosidase assay 900 μL of *p*-nitrophenyl-α-D-galactopyranoside (2 mM in 50 mM Na-citrate buffer pH 5.0) 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. β-Mannosidase and β-glucosidase were assayed similarly except that *p*-nitrophenyl-β-D-mannopyranoside (2 mM) and *p*-nitrophenyl-β-D-glucopyranoside (1 mM), respectively, were used as substrate [6]. All activities were expressed as nkat/mL in case of submerged fermentation and nkat/gds in case of SSF.

2.4 Identification, culture conditions, and maintenance of strains

The identity of potential isolates was confirmed on the basis of their colonial and morphological characteristics using available literature [17,18]. These were deposited with National Fungal Culture Collection of India (NFCCI), Pune, India, a national facility. Filamentous fungi *Fusarium fusarioides* NFCCI 3282, *Fusarium solani* NFCCI 3283, *F. equiseti* NFCCI 3284, *Cladosporium cladosporioides* NFCCI 3285, *A. levis* NFCCI 3286, and *Fusarium moniliforme* NFCCI 3287 were grown and maintained on potato dextrose agar slants at 4°C and subcultured after every 30 days. The identity of *F. equiseti* and *A. levis* was confirmed on the basis of internal transcribed spacer sequencing (Supporting information) and submitted to NCBI with GenBank accession number *A. levis* (*KU568179*.1) and *F. equiseti* (*KU568180*.1).

2.5 Inoculum preparation, submerged, and SSF

Submerged fermentation was carried out in 250 mL Erlenmeyer flasks containing 50 mL of basal medium [(g L^{-1}): yeast extract 14.0, $(NH_4)_2SO_4$ 2.1; $MgSO_4 \cdot 7H_2O$ 3.0, $CaCl_2 \cdot 2H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.5 and KH_2PO_4 10.0, pH 5.0]. Different carbon sources used in preparation of production media included 0.5% w/v LBG, GG, or konjac gum. Flasks were then autoclaved at 121°C for 30 min and inoculated with two mycelial discs of 5-day-old fungal cultures and incubated in a rotary shaker (Lark Innovata, Germany) at 28°C for 4 days at 120 rpm. Subsequently, content was filtered through Whatman No. 1 filter paper and the filtrate was centrifuged (9000 × *g*, 4°C) for 15 min. Clear filtrate thus obtained was used as source of mannanase.

For SSF, various substrates such as wheat bran, wheat straw, rice husk, CM, PKC, fenugreek seed meal, and *Aloe vera* pulp were used for β-mannanase production. Fungal spores from 5-day-old culture grown on Czapek Dox agar at 28°C were harvested in 5 mL sterile Tween 80 0.01% w/v solution. Particle size of CM or PKC solid substrate was fixed (0.5 mm) as described earlier [15]. SSF was carried out in Erlenmeyer flasks (250 mL) with 5 g substrate with distilled water as moistening agent (1:1). To enhance β-mannanase yield, first "one variable at a time" approach was used for screening of suitable nitrogen and carbon supplement. Selected substrates were supplemented with LBG, GG, konjac gum, glucose, mannose, and solka floc as inducers (1% w/v). Yeast extract, peptone, urea, and ammonium sulphate at a concentration of 1% w/v were supplemented as nitrogen source. Flasks were then autoclaved at 121°C for 30 min and inoculated with fungal spore suspension (2×10^6 spores/mL) and incubated at 28°C for 6 days.

2.6 Statistical optimized production of β-mannanase using response surface methodology (RSM) and validation of experimental modeling

Two important parameters for SSF (A) pH and (B) moisture content were selected to find their optimum values for enhance β-mannanase production by selected fungi using rotable central composite design (RCCD) approach of response surface methodology (RSM). The range and levels of the variables taken for RSM are listed in Supporting information Tables S2–4. According to RCCD, the total number of experimental combinations were $2k + 2^k + n_o$, where k is the number of independent variables and n_o is the number of repetitions of the experiment at the centre point. Total 13 experiments including five center points were conducted along with different combination of parameters for selected fungi. Each numeric factor was varied over five levels, that is, plus and minus alpha (axial point), plus and minus one (factorial points), and zero (center point).

Data obtained from RSM were subjected to analysis of variance (ANOVA) for analysis of regression coefficient, prediction equations, and case statistics. Analysis of data was performed using Design-Expert software (Version 9.0). The experimental results of RSM were fitted using the second-order polynomial equation:

$$Y = \beta 0 + \sum_{i} \beta i X i + \sum_{ii} \beta i i X i 2 + \sum_{ij} \beta i j X i X j \qquad (1)$$

In this polynomial equation, Y is the predicted response, Xi and Xj are independent variables, β0 is the intercept term, βi is the linear coefficient, βii is the quadratic coefficient, and βij is the interaction coefficient. The statistical models of each selected fungus were validated with respect to all variables within design space. Five random optimized experimental combinations were used to study β-mannanase production under SSFfor each statistical model.

2.7 Enzyme extraction and protein precipitation

Mannanase was extracted from solid-state cultures by mixing dry fermented substrate with 50 mL of 50 mM citrate buffer (pH 5.0) and stirring at 120 rpm for 1 h in cold 4°C. The slurry was filtered through glass filter paper and the filtrate was centrifuged (9000 × *g*, 4°C) for 15 min. Cell-free filtrate thus obtained was used as source of mannanase and assayed for enzyme activities as described earlier.

β-Mannanases from fungal strains were partially purified. Total protein from culture filtrates was precipitated by adding a

double volume of chilled ethanol under constant stirring at 4°C. The mixture was then kept at −20°C overnight to allow complete precipitation. The precipitate was collected by centrifugation at 8000 × *g* for 15 min. Precipitate thus obtained was resuspended in 20 mL citrate buffer pH 5.0 and dialyzed using dialysis membrane (10 000 MWCO, Hi-Media, Mumbai) against the same buffer at 4°C overnight [19]. Dialyzed samples were analyzed for detection of β-mannanase activity by seminative SDS-PAGE and partially purified β-mannanase used for hydrolysis of mannans.

2.8 Detection of mannanase activity by seminative SDS-PAGE (zymography)

Protein precipitate was resolved on SDS-PAGE (12%) containing 2 mL of konjac gum (0.2% w/v) (75 V for 3 h). The gel was washed with 25% v/v propane in Na-citrate buffer (pH 5.0) followed by washing with double-distilled water. It was incubated at 50°C for 5 min in 50 mM Na-citrate buffer (pH 5.0) and subsequently stained with 0.1% w/v Congo red solution for 20 min. The gel was destained using NaCl solution (1% w/v) for 1 h [20].

2.9 Hydrolysis experiments and end-product analysis

Both branched (LBG, GG) and linear (konjak gum) mannans (0.5 % w/v in citrate buffer, pH 5.0) were hydrolyzed by mixing equal volumes of mannanase (400 nkat/mL) and substrate followed by incubation at 50°C with constant shaking. Samples were withdrawn at time intervals of 2, 5, 10, and 20 h and boiled to stop the reaction. Samples were filtered through membrane filter (0.45 μm, Merck) and analyzed by HPLC (Waters, USA) using Sugar Pak column, RI detector 2414 and injection valve with capacity of 20 μL [21]. HPLC grade water was used as mobile phase with flow rate of 0.5 mL/min and column temperature was 90°C. Mannose (M_1), mannobiose (M_2), mannotriose (M_3), and mannotetraose (M_4) were used as the standards and analysis was done by Empower 2 Build software 2154.

3 Results

3.1 Isolation and screening of mannanase-producing fungi

Eighty-eight strains were isolated from the various habitats (Supporting information Table S1). Culture filtrate of 28 fungi produced clear zone of hydrolysis around wells in mannan agar plate (Supporting information Fig. S1). Positive strains belonged to seven genera viz. *Aspergillus*, *Curvularia*, *Fusarium*, *Alternaria*, *Penicillium*, *Cladosporium*, and *Acrophialophora* (Table 1). Among positive strains, six strains produced zone of hydrolysis in the range of 20 to 25 mm. Their activity on gel was also corroborated by high mannanase activity in the range of 65–140 nkat/mL. These six strains covered three genera, *Fusarium* (70–140 nkat/mL), *Cladosporium* (65 nkat/mL), and *Acrophialophora* (104 nkat/mL). Colonial and morphological characteristics of these six fungal strains are given in Fig. 1.

3.2 Enzyme production

All six selected molds were grown in submerged fermentation on media containing three different mannans LBG, GG, and konjac gum. Cell-free clear filtrates were analyzed for β-mannanase and accessory mannanolytic enzymes. *Fusarium fusarioides* NFCCI 3282 produced maximum β-mannanase (171 nkat/mL) on medium containing konjac gum (linear mannan) as the sole C-source. High β-mannanase activity was noticed in *F. moniliforme* NFCCI 3287 (140 nkat/mL) on LBG as C-source followed by *A. levis* (104 nkat/mL) on the same medium. The results of this experiment are presented in Table 2. High β-glucosidase activity was noticed in *F. equiseti* NFCCI 3284 (7.8 nkat/mL), *F. fusarioides* NFCCI 3282 (6.2 nkat/mL), and *F. moniliforme* NFCCI 3287 (5.5 nkat/mL) while *F. fusarioides* and *C. cladosporioides* NFCCI 3285 produced high titers of α-galactosidase, 20 and 8.4 nkat/mL, respectively. β-Mannosidase activity was not detected in any case except *C. cladosporioides* NFCCI 3285 (0.12 nkat/mL).

Low-value mannan-rich agroresidues such as PKC and CM were found as suitable alternatives over pure mannans for β-mannanase production [15]. In this study, seven agroresidues were used as substrate in SSF for mannanase production. Among these, PKC was observed to be the best for β-mannanase production followed by CM in all cases (Table 3). *F. equiseti* NFCCI 3284 and *A. levis* NFCCI 3286, based on high mannanolytic activity, were further used for SSF studies. Prior to optimization, *F. equiseti* NFCCI 3284 and *A. levis* NFCCI 3286 produced 1747 and 897 nkat/gds mannanase with PKC as substrate in SSF, respectively.

Among the carbon supplements examined, LBG supported maximum mannanase yield (2450 nkat/gds), which was much higher than unsupplemented control (2124 nakt/gds, with 0.5 mm particle size of PKC). LBG has been used widely as an inducer of β-mannanase [22, 23]. Glucose and mannose supplementation clearly repressed β-mannanase production as evidenced by low mannanase titres (Supporting information Fig. S2). Among the nitrogen supplements examined, yeast extract supported maximum yield as compared to the unsupplemented control (Supporting information Fig. S3).

3.3 Optimization of factors by RSM

Compared to conventional methods, statistical approach saves time, cost, and is more efficient. RCCD approach of RSM was adopted to optimize two parameters, moisture, and pH, for β-mannanase production by two selected fungi. Thirteen experiments including five center points were carried out for both fungi. Each numeric factor was varied over five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$). The full experimental plan with respect to their actual and coded values is listed in Supporting information Tables S2–4. The response values (Y = mannanase production) in each trial were the average of the triplicates. ANOVA was used for analysis of regression coefficient, prediction equations, and case statistics. The experimental results of RSM were fitted using the following second-order polynomial equation (1). In this study, the independent variables were coded as A (pH) and B (moisture). Thus, the second order polynomial equations for

Table 1. Summary of results of mannanase activity of the mannanolytic test fungi as noted by mannan-agar plate assay and dinitrosalicylic acid method

SN	Fungus	Isolate no.	Range of hydrolysis zone (mm)	Activity (nkat/mL)
Samples collected from Sagar, MP				
1	*Aspergillus* sp. I	SG	+	11.0
2	*Aspergillus* sp. II	SG-1	+	9.4
3	*Aspergillus* sp. III	SG-4	++	43.0
4	*Fusarium* sp. I	SG-6	++++	120
5	*Acrophialophora* sp.	VFS-1	++++	104
6	*Penicillium* sp. I	VFS-22	++	38.0
7	*Alternaria* sp.	VFS-11	+	1.4
8	*Aspergillus* sp. IV	VFS-3	+	4.2
9	*Fusarium* sp. II	MULTI-V	++++	71.0
10	*Aspergillus* sp. V	SGD	+	12.0
11	*Penicillium* sp. II	SG-2	+	11.0
12	*Malbranchea cinnamomea*	L-64	+++	43.0
13	*Melanocarpus albomyces*	DOM-65	++	45.0
14	*Myceliophthora thermophila*	CM-24	++	37.0
15	*Aspergillus* sp. VI	SGD	++	12.0
Samples collected from Bhopal, MP				
16	*Penicillium* sp. III	BPL-088	++	40.0
17	*Fusarium* sp. III	BWS-2	++++	56.0
Samples collected from Amarkantak, MP				
18	*Fusarium sp. IV*	AM03	++++	84.0
19	*Cladosporium sp.*	AM05	++++	65.8
20	*Aspergillus sp. VII*	AM06	+	2.6
21	*Aspergillus* sp. VIII	AM07	+	2.9
22	*Fusarium* sp.V	AM08	+	3.4
23	*Fusarium* sp.VI	AM09	+	1.6
24	*Aspergillus* sp. IX	AM04	++	36.3
25	*Fusarium* sp. VI	AM4W	++	26.0
26	*Curvularia* sp.	AM4B	+++	38.8
27	*Aspergillus* sp. X	AM2B	+++	31.3
28	*Aspergillus* sp. XI	AM-3S	++	10.9

+Less than 5 mm, ++ 5 to 10 mm, +++ 10 to 20 mm, ++++ More than 20 mm, - No zone

mannanase production in different substrates by these fungi can be represented as follows:

$$\beta\text{-Mannanase production by } F.\ equiseti \text{ with PKC as solid substrate} = +5899.00 + 390.85 \times A + 413.70 \times B - 2029.65 \times A^2 - 1799.65 \times B^2 + 234.00 \times A \times B \quad (2)$$

$$\beta\text{-Mannanase production by } F.\ equiseti \text{ with CM as solid substrate} = +3549.00 + 66.48 \times A + 86.68 \times B - 1223.87 \times A^2 - 1266.87 \times B^2 - 8.50 \times A \times B \quad (3)$$

$$\beta\text{-Mannanase production by } A.\ levis \text{ with CM as solid substrate} = +4725.60 + 15.80 \times A + 30.22 \times B - 1506.55 \times A^2 - 1688.05 \times B^2 + 24.00 \times \mathrm{A} \times \mathrm{B} \quad (4)$$

The statistical significance of the second-order polynomial equations (Eqs. (2)–(4)) was checked by Fisher distribution (*F*-test) ANOVA and the results are presented in Supporting information Tables S5–7. For each case of β-mannanase production, the correlation coefficient (R^2) of polynomial equation was found to be 1.0. The R^2 value indicated a measure of variability in the observed response values which was described by the independent factors and their interactions over the range of the corresponding factor. Thus, a quadratic model was suggested for this analytical work with these two fungi. In all the three cases, the "Predicted R^2" value of 0.997 was in reasonable agreement with the "Adjusted R^2" value of 0.998. The predicted R^2 and the adjusted R^2 was within 0.10 of each other [24, 25] indicating a good agreement between the observed and predicted values. The coefficient of variation percent (CV %) was a measure of residual variation of the data relative to the size of the mean and a low value of CV indicated reliability of the above experiment.

The Model *F*-value in all three cases implied that models were significant. There is only a 0.01% chance that a "model *F*-value" this large could occur due to noise. Values of "Prob > *F*" less than 0.0500 indicated model terms were significant. In all cases *A*, *B*, A^2, B^2 and AB were significant

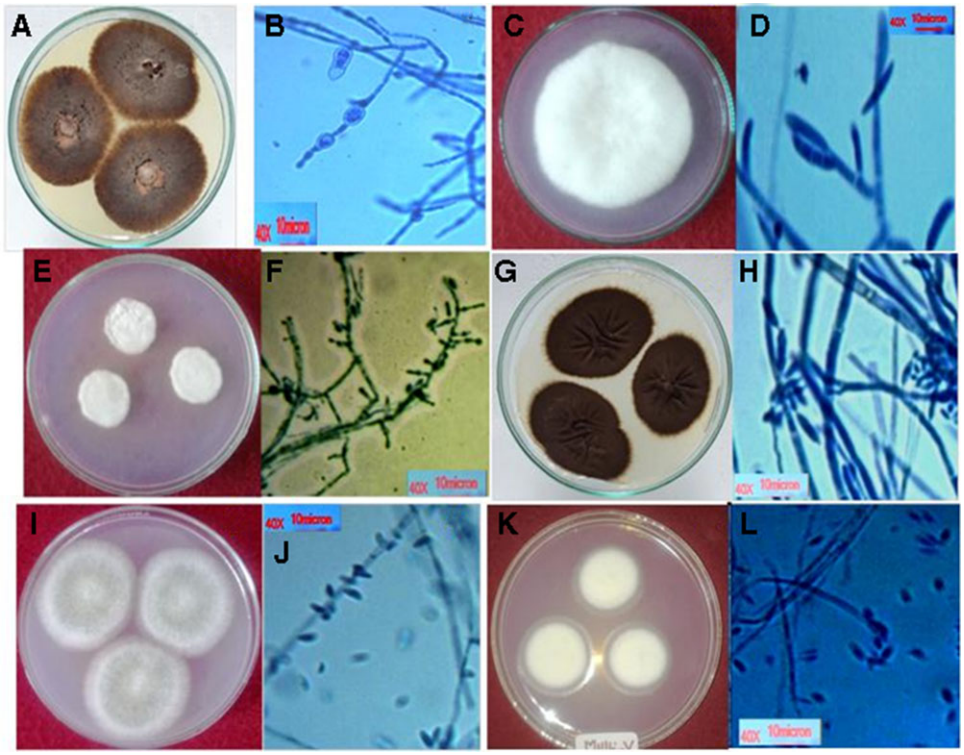

Figure 1. Colonial and morphological (400×) characteristics of mannanolytic fungi. (A and B) *Fusarium fusarioides* NFCCI 3282, (C and D) *Fusarium equiseti* NFCCI 3284, (E and F) *Acrophialophora levis* NFCCI 3286, (G and H) *Cladosporium cladosporioides* NFCCI 3285, (I and J) *Fusarium solani* NFCCI 3283, (K and L) *Fusarium moniliforme* NFCCI 3287.

Table 2. Mannanolytic enzyme profiles (nkat/mL) of fungi cultivated under submerged fermentation on three different mannan substrates (Data represent average value of three experiments ± SD)

Fungus	C-source[a)]	β-Mannanase	β-Mannosidase	β-Glucosidase	α-Galactosidase
F. fusarioides	LBG	84 ± 1.6	BD	2.6 ± 0.3	20 ± 1.5
	GG	26 ± 1.2	BD	2.7 ± 0.7	1.4 ± 0.9
	KG	171 ± 21.3	BD	6.2 ± 1.1	5.1 ± 1.2
C. cladosporioides	LBG	53 ± 7.2	BD	0.6 ± 0.1	14 ± 1.7
	GG	23 ± 1.9	BD	1.1 ± 0.5	8.4 ± 1.3
	KG	71 ± 2.1	0.12	0.18 ± 0.02	0.21 ± 0.01
F. equiseti					
	LBG	71 ± 11.3	BD	2.5 ± 0.03	2.4 ± 0.03
	GG	34 ± 3.7	BD	5.5 ± 0.07	5.4 ± 0.9
	KG	95 ± 9.7	BD	7.8 ± 0.7	0.03 ± 0.001
F. solani	LBG	36 ± 3.5	BD	0.5 ± 0.02	0.3 ± 0.001
	GG	3.7 ± 0.7	BD	0.6 ± 0.02	0.23 ± 0.01
	KG	4.0 ± 1.2	BD	0.29 ± 0.07	BD
F. moniliforme	LBG	140 ± 19.7	BD	3.7 ± 0.13	0.16 ± 0.02
	GG	33 ± 2.7	BD	5.5 ± 0.7	0.2 ± 0.001
	KG	69 ± 11.2	BD	0.3 ± 0.03	0.03 ± 0.001
A. levis	LBG	104 ± 23.3	BD	0.23 ± 0.03	2.4 ± 0.08
	GG	62 ± 2.8	BD	0.4 ± 0.01	7.2 ± 1.2
	KG	25 ± 2.3	BD	0.23 ± 0.03	6 ± 1.3

[a)] KG, konjac gum; BD, below detection.

model terms. The "lack-of-fit *F*-value" of more than 1 implied that lack of fit was not significant and indicated a good fitness of models.

Predicted versus actual plots (Supporting information Fig. S4) represented a high degree of similarity that was observed between the predicted and experimental values. From the diagnostic plots, it can be concluded that the model satisfied the assumptions of the ANOVA and also reflected the accuracy and applicability of RSM to optimize the process for mannanase production. The three-dimension response surface curves and their respective 2D contour plots (Fig. 2) of all three cases determined the interaction of the factors and optimum value of each factor for maximum mannanase production. Figure 2 shows the effect of pH and moisture on mannanase production by *F. equiseti*

Table 3. β-Mannanase production profile of various fungi cultivated under SSF using different agroindustrial residues as solid substrates (data represent average value of three experiments, nkat/gds ± SD)

Particulate substrates							
Fungus	PKC	CM	FSP	WB	WS	RH	AP
F. fusarioides	152 ± 2.3	18 ± 4.2	10 ± 1.2	16 ± 2.5	13 ± 0.5	1.0 ± 0.1	BD
C. cladosporioides	129 ± 3.2	12 ± 1.2	10 ± 6.1	20 ± 1.2	8.0 ± 0.7	0.8 ± 0.2	BD
F. equiseti	1747 ± 22.3	871 ± 12.1	24 ± 2.3	24 ± 2.2	21 ± 3.2	5.0 ± 0.5	4.0 ± 0.3
F. solani	243 ± 8.8	22 ± 4.2	10 ± 0.8	22 ± 2.4	15 ± 1.2	2.0 ± 0.5	0.8 ± 0.2
F. moniliforme	557 ± 18.1	26 ± 2.3	15 ± 1.1	25 ± 0.8	14 ± 2.2	1.5 ± 0.2	0.8 ± 0.1
A. levis	897 ± 17.5	280 ± 15.8	17 ± 0.6	28 ± 0.9	12 ± 1.1	4.0 ± 0.8	2.5 ± 0.3

FSP, fenugreek seed powder; WB, wheat bran; WS, wheat straw; RH, rice husk; AP, *Aloe vera* pulp; BD, below detection.

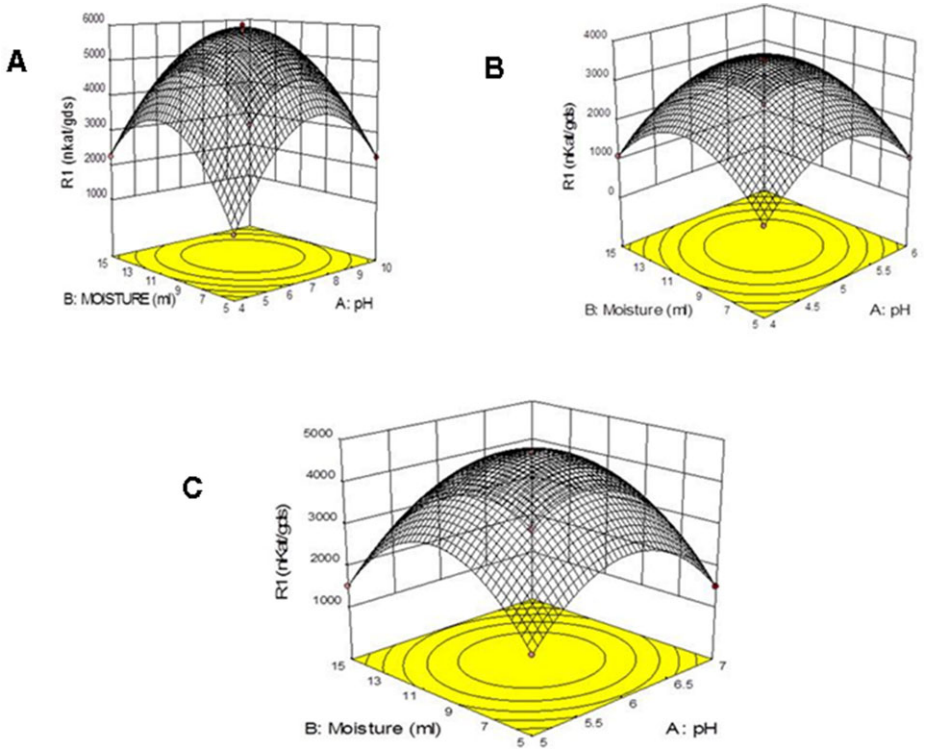

Figure 2. Three-dimensional response surface curves for mannanase production (nkat/gds) under SSF by (A) *F. equiseti* NFCCI 3284 with PKC as solid substrate, (B) *F. equiseti* NFCCI 3284 with CM as solid substrate, (C) *A. levis* NFCCI 3286 with PKC as solid substrate, as a function of (A) pH and (B) moisture content (culture conditions: temperature 28°C, incubation time 6 days).

(Fig. 2A) and *A. levis* (Fig. 2C) in SSF with PKC. Figure 2B depicts response surface curve for *A. levis* grown on CM. It was obvious that when moisture was increased beyond level 0, mannanase yield decreased in all the three cases.

3.4 Validation of the optimum condition defined by the models

An attempt was made to maximize mannanase production in all three cases by keeping the moisture and pH "in range." By using these criteria, solutions (moisture 10.5 mL and pH 7.2 in case of *F. equiseti* with PKC as substrate, moisture 10.17 mL and pH 5.03 in case of *F. equiseti* with CM as substrate, and moisture 10.04 mL and pH 6.00 in case of *A. levis* with PKC as substrate) with maximum production response were selected and experiments were conducted. The observed response of mannanase production yield in all three cases (5950, 3558, and 4728 nkat/gds, respectively) were more than the predicted outcome (5939, 3551, and 4725 nkat/gds, respectively). Mannanase production predicted by the quadratic model equation and the production recorded experimentally were in good agreement and thus the models were valid. The production in all three cases attained after statistical optimization was twofold higher than production under unoptimized conditions (Fig. 3).

Titers of accessory mannanolytic hemicellulases were also recorded from different cultivations of these fungi. *F. equiseti* produced 120 nkat/gds α-galactosidase and 73.5 nkat/gds β-glucosidase on PKC, while relatively lower yield of α-galactosidase (86.2 nkat/gds) and β-glucosidase (40 nkat/gds) were noticed on CM. *A. levis* produced 53 nkat/gds α-galactosidase and 83.5 nkat/gds β-glucosidase under optimized conditions on PKC. Presence of these accessory enzymes is understood to play a significant role in hydrolysis of

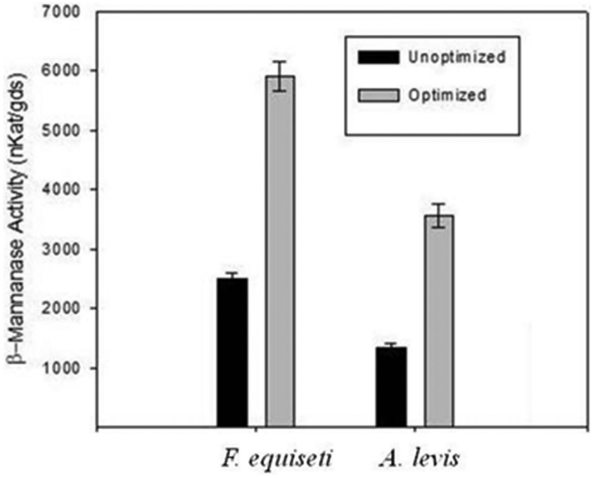

Figure 3. Mannanase production by *F. equiseti* NFCCI 3284 and *A. levis* NFCCI 3286 on PKC under optimized and unoptimized conditions under SSF. (conditions: particle size of PKC 0.5 mm, Tem 28°C, moisture agent distilled water, incubation time 6 days). Data are presented as mean ± standard error of the mean (SEM); $n = 2$.

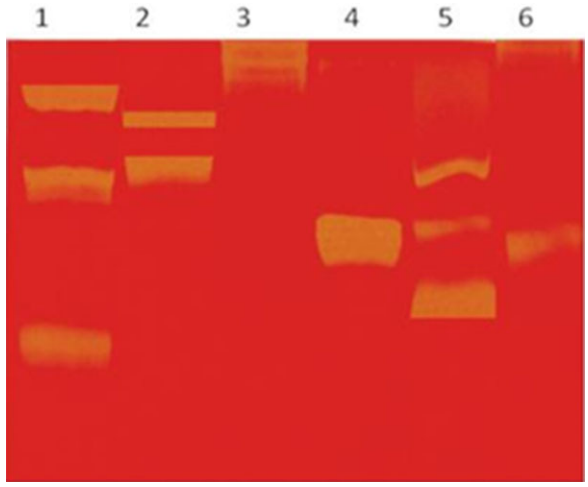

Figure 4. Detection of mannanase on seminative SDS-PAGE (substrate, konjac mannan 0.5%) lane 1 culture filtrate of *Acrophialophora levis*, lane 2 *Fusarium moniliforme,* lane 3 *Fusarium fusarioides*, lane 4 *Fusarium equiseti*, line 5 *Fusarium solani*, lane 6 *Cladosporium cladosporioides*.

heteromannans with high percentage of galactose and glucose in composition [4].

3.5 Detection of mannanase enzyme activity by seminative SDS-PAGE

Seminative SDS-PAGE study revealed that some of the tested fungal strains produced different isoforms of mannanases. Among the tested strains, *A. levis* NFCCI 3286 and *F. solani* NFCCI 3283 produced three isoforms, *F. moniliforme* NFCCI 3287 produced two isoforms while *F. fusarioides* NFCCI 3282, *F. equiseti* NFCCI 3284, and *C. cladosporioides* NFCCI 3285 showed presence of a single mannanase on seminative SDS-PAGE (Fig. 4).

3.6 Hydrolysis experiments and end-product analysis

Different mannans konjac gum, LBG, and GG were subjected to hydrolysis by mannanase preparation from these fungi and end products were analyzed by HPLC (Supporting information Fig. S5). Mannanase preparation from *A. levis* hydrolyzed GG and LBG into mannose, mannobiose (M_2), and a DP 3 oligosaccharide as the major end products (12–13%). However, in case of linear konjac mannan the enzyme action yielded a DP 4 oligosaccharide (22%) as the exclusive end product indicating endo-mannanase action (Table 4). Mannanase sourced from *C. cladosporioides* showed no mannose generation and only oligosaccharide (DP 3) was observed after hydrolysis of both LBG and GG. DP 4 mannooligosaccharide was the only major end product (24%) released after hydrolysis of konjac gum by this preparation. Different *Fusarium* species showed similarity in end-product profile indicating similar nature of their mannanases. All the strains predominantly generated a DP 3 oligosaccharide (10–15.4%) from hydrolysis of LBG and GG, while much higher amounts of oligosaccharides DP 3 (19-28%) and DP 4 (70-80%) were generated from linear konjac mannan by *F. equiseti* and *F. fusarioides* mannanase (Table 4). *Fusarium moniliforme* and *F. solani* produced DP 4 oligosaccharide after hydrolysis of linear konjac mannan. The action pattern of fungal mannanases indicated hydrolysis in endo-fashion liberating oligosaccharide DP 3 and DP 4 as the major end products. Mannose generation was noticed in case of branched mannans (LBG and GG) while it was altogether absent in case of linear konjac mannan. This pattern can be attributed to typical endo-β-mannanase activity and absence of β-mannosidase. Hydrolysis of three different mannans by all these fungal mannanases indicated the versatility of mannanases and their potential in gum hydrolysis for generation of value added MOS.

4 Discussion

The present study presents a detailed account of mannanolytic activity of newly isolated *Fusarium* spp., *C. cladosporioides* and *A. levis*. Our results indicated metabolic diversity in fungi with reference to mannanolytic enzymes. *Fusarium equiseti*, *F. moniliforme*, *F. fusarioides,* and *F. solani* produced varied levels of mannanase and also exhibited different isoforms indicating mannanolytic potential of *Fusarium* spp.

Several strains of *Aspergillus*, *Trichoderma,* and *Sclerotium* have been reported to be efficient mannanase producers utilizing a variety of mannan-rich crude substrates as carbon source, for instance, coffee waste [26], wheat bran [13], defatted copra [27] etc. Recently, apple pomace and PKC were explored for production of fungal β-mannanase in SSF by Yin et al. [28] and Soni et al. [15], respectively. Apple pomace has also been used as substrate for xylanase production using *Aspergillus niger* [29].

In contrast to several reports of mannanase production under submerged fermentation [27, 30, 31] our premise of using

Table 4. End-product profile of mannan hydrolysis by fungal mannanase

Fungus	Name of hydrolysis substrates and generated end products							
	LBG			GG			Konjac gum[a)]	
	M	M_2	DP-3	M	M_2	DP-3	DP-3	DP-4
F. fusarioides	—	—	10%	—	—	13%	19%	80%
F. solani	—	—	14.7%	—	—	12.4%	—	14%
F. equiseti	1.7%	—	15.6%	—	—	14%	28%	70%
C. cladosporioides	5.4%	—	14.1%	—	—	14%	—	24%
A. levis	8.1%	7.5%	13.2%	5.8%	6.2%	12.5%	—	22%
F. moniliforme	—	3%	15.4%	—	—	13.6%	—	25%

[a)] No monosugar was detected.
DP, degree of polymerization; M, mannose; M_2, mannobiose.

cheaper mannan-rich agroresidues is intended to develop a cost-effective SSF bioprocess. This was demonstrated successfully as two fungal strains, *F. equiseti* NFCCI 3284 and *A. levis* NFCCI 3286 produced high mannanase titers with PKC and CM as solid substrates under optimized conditions. In this study *F. equiseti* NFCCI 3284 produced 356 U/gds when grown on PKC while *Aspergillus niger* USM F4 [32] and *A. niger* [33] produced 119 and 297 U/gds mannanase, respectively, in SSF. Chin [34] has reported 334 U/g mannanase yield by *Bacillus subtilis* grown on PKC under SSF.

In hydrolysis study, mannanase from all strains generated oligosaccharides (DP 3–4) without generation of mannose from konjac gum. Zhang et al. [35] have shown formation of oligosaccharides (DP 2–6) with negligible mannose from konjac flour using bacterial mannanase. Absence of mannose with exclusive generation of oligosaccharides is highly desirable for prebiotic preparations [36]. Absence of mannose after linear mannan hydrolysis confirmed that these fungal β-mannanase possess exclusive endo-β-mannanase activity. HPLC results showed generation of oligosaccharide with DP 3 in hydrolysis of LBG by all fungal mannanases. The oligosaccahride yield was higher as compared to *Aspergillus awamori* K4 β-mannanase [37].

Major product released by GG hydrolysis was also oligosaccharide (DP 3), indicating that fungal β-mannanase can efficiently produce oligosaccharide (DP 3) from higher galactose containing mannan (GG has about 30% galactose) and can be used in preparation of PHGG, which is reported to be useful in clinical nutrition. Earlier reports of GG hydrolysis by *Aspergillus niger* and *Bacillus circulans* K-1 mannanase report 5% yield of smaller oligosaccharides [38, 39], whereas in the present study much higher yield (10 to 18%) was noticed. PHGG reduces the incidence of diarrhea in septic patients, increases population of Bifidobacteria in the gut, and has low viscosity as compared to GG. This makes it useful in treatment of irritable bowel syndrome and in manufacturing enteral products and beverages [40, 41]. The study also provides an approach for utilization and management of agro industry residues which causes pollution. CM and PKC are interesting alternatives for β-mannanase production, since these are readily available in India. Moreover, the residues left after SSF have a reduced galactomannan content that may be useful in monogastric animal feed.

Author N.K. is thankful to University Grants Commission (UGC), New Delhi for financial support (grant no. 42–474/2013SR) and author H.S. expresses his gratitude to UGC, New Delhi for providing SRF.

The authors have declared no conflict of interest.

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