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α-L-ARABINOFURANOSIDASE FROM AN EFFICIENT HEMICELLULOLYTIC FUNGUS *Penicillium janthinellum* CAPABLE OF HYDROLYZING WHEAT AND RYE ARABINOXYLAN TO ARABINOSE

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ABSTRACT

This study reports *Penicillium janthinellum* strain, producing high levels of α-L-arabinofuranosidase (AFase) as well as other components of hemicelluloytic enzyme system (endoxylanase, β-xylosidase and acetyl xylan esterase) on rice straw and wheat bran containing solidified culture medium. Optimization of culture conditions was carried out using Box–Behnken design of experiment to study the influence of process variables (ammonium sulphate, pH and moisture level) on AFase production. Analysis of data showed R^2 (0.9967) and adjusted R^2 (0.9925) indicating model to be good fit and robust to predict culture conditions for AFase production. Under optimal culture conditions *P. janthinellum* produced high levels of AFase (212 units/g dw substrate) in addition to xylanase (1800 units/g dw substrate), β-xylosidase (31 units/g dw substrate), acetyl xylan esterase (231 units/g dw substrate) and feruloyl esterase (27 units/g dw substrate). AFase from *P. janthinellum* culture extract was purified to homogeneity and characterized to be a 64 kDa protein with a *pI* of 3.8. The peptide mass fingerprinting showed the AFase belonged to family GH54. The enzyme was optimally active at 50° C at pH 5.5 and its activity was positively modulated in presence of Fe^{3+}ions. The enzyme preferentially catalyzed the hydrolysis of *p*NP- arabinofuranoside (*p*NPA) with *Km* and *Vmax* of 0.4mM and 260 unit mg^{-1} $protein^{-1}$, respectively. Hydrolysis with purified AFase (3.0 units/g substrate) released arabinose from rye arabinoxylans (29.5 mg/g substrate) and wheat arabinoxylan (13.4 mg/g substrate), as the sole product indicating *P. janthinellum* as the important source of α-L-arabinofuranosidase for bioconversion of agro-residue to value added products.

Keywords: *Penicillium janthinellum;* hemicellulase*;* α-L-arabinofuranosidase; GH54; arabinoxylan hydrolysis; response surface methodology

INTRODUCTION

Arabinoxylans are integral hemicellulosic components of cereal crop residues and bioprocessing by-products from rice, wheat, rye, sorghum, barley, etc., **Lagaert *et al.* (2013)**. Arabinoxylans are heteropolymeric with xylan backbone comprised of β-1,4 linked xylose residues few of which are singly or doubly substituted with arabinosyl residues. While arabinosyl residues are further ester linked to ferulolyl moieties of lignin. Additionally xylose is also linked to acetyl residues at C2 position. Owing to its complex structure efficient hydrolysis of arabinoxylan requires collective action of an array of hemicellulolytic enzymes namely, endo-1,4 β-xylanase (EC 3.2.1.8) that cleaves the xylan backbone and releases xylo-oligomers, whereas, β-xylosidases (EC3.2.1.37) mediated catalysis results in release of monomeric xylose from xylo-oligomers. The α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.39), acetyl xylan esterase (EC 3.1.1.72) and feruloyl esterases (EC. 3.1.1.73) are involved in debranching of the heteropolymeric structure (**Shallom and Shoham, 2003*;* Dodd and Cann, 2009**). The arabinoxylans derived from wheat and rye kernel are rich source of arabinose with xylose to arabinose ratio ranging between 0.49-0.5 **Guerfali *et al.* (2010)**. AFase plays an important role in hydrolysis of (1, 2), (1, 3) and (1, 5)-α-L-arabinofuranosyl moieties linked to xylan backbone of hemicellulose polymer and mitigate the steric hindrance for xylanase action **(Sorensen *et al*., 2006).** AFase derived from different microbial sources have been classified in GH families 3, 43, 51, 54, 62 and 93 (*Yang et al., 2015)*. AFase produced from *Aureobasidium pullulans, Trichoderma reesei* (**Numan and Bhosle, 2006**) and *Penicillium purpurogenum* (**Ravnal *et al*., 2012)** have been reported as useful in bioconversion of lignocellulosic biomass to biofuel, paper and pulp industry and in wine industry for enhancement of wine flavor, respectively. This paper for the first time reports *P. janthinellum*, isolated from montane alpine forests of Shivalik hills (**Monga and Chadha, 2014**) India, as rich source of AFase and other components of hemicellulolytic enzyme complex. Culture conditions for achieving optimal AFase production were studied employing response surface methodology. The enzyme was further purified and characterized its role in efficient hydrolysis of arabinoxylans derived from wheat and rye for production of arabinose was established.

MATERIAL AND METHODS

Production of hemicellulases by *P. janthinellum*

P. janthinellum isolated from decomposing leaf litters of oak trees from montane alpine forests of Shivalik hills (India) was identified on the basis of morphological/ molecular approach (**Sharma *et al.*, 2008**). The fungus was grown at 30°C for 5 days on yeast potato soluble starch (YpSs) agar (Cooney and Emerson, 1964) and maintained on the same medium at 4°C. For production of hemicellulases *P. janthinellum* was grown on solidified culture medium containing rice straw and wheat bran (3:2) as carbon source and 15 ml basal medium (KH_2PO_4 0.4%, CH_3COONH_4 0.45%, and $(NH_4)_2SO_4$ 1.3%, pH 7.0). The flasks were inoculated with 2ml spore suspension (6×10^7 spores/ml) prepared from a 7 days old YpSs agar slants and incubated at 30 ºC for 7 days. The enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM, pH 6.0) to the flasks and kept at 30 °C for 1 h under mild shaking. The resultant slurry was filtered and centrifuged at 8000×*g* for 20 min and the enzyme extract was used for assay of hemicellulolytic enzymes (xylanase, AFase, β-xylosidase and acetyl xylan esterase) as well as profiling AFase expression by SDS PAGE and 2 Dimensional gel electrophoresis, AFase activity staining of gels, and purification of enzyme.

The optimization of AFase was carried out using Box Benken Design of experiments employing response surface methodology. The production was studied using ammonium sulphate, pH and moisture level as independent variables at three levels (-1, 0, +1) using 17 flasks experiments. The experimental

data was analyzed using Statistical software (Design- Expert v 8.0.7, Stat-Ease Inc., USA) to calculate regression coefficient and generate response surface graphs.

Two dimensional gel electrophoresis (2DE)

The enzyme extract obtained after solidified culturing was subjected to ultrafiltration /desalting using 10 KDa membranes (Amicon). For 2DE protein sample (150 µg) was loaded onto IPG strips (7 cm) that rehydrated in buffer (150µl) containing 8 M urea, 2% CHAPS, destreak reagent, 1% IPG buffer (pH 3.0–10.0) and 0.005% bromophenol blue (BPB), for 16 h at room temperature. Ettan IGPhor 3 system (GE, Healthcare Biosciences) was used for IEF employing voltage program that increased linearly in a stepwise manner: 100 V, 4 h; 300 V, 2 h; 1,000 V, 2 h (gradient); 3,000 V, 3 h (gradient); 5,000, 3 h (gradient); 5000V, 6 h (step). Following IEF separation, IPG strips were equilibrated for 15 min in 7 ml of 0.05 M Tris Cl (pH 8.8), 8 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 60 mM dithiothreitol (DTT) and traces of bromo-phenol blue (BPB) followed by equilibration for 15 min in the same buffer where DTT was replaced with 50 mM iodo-acetamide. For second dimensional electrophoresis equilibrated IPG strips were placed onto 12% SDS, polyacrylamide gels and overlaid with 0.5 % low melting agarose solution that contains traces of BPB. The second dimension separation was carried using Hoefer mini VE system (GE Healthcare Biosciences) at a constant voltage of 25mA (**Kaur *et al*., 2013**). The protein spots were developed using silver staining method.

Identification of Protein

The well separated protein bands on SDS–PAGE were excised and subjected to peptide mass spectrometry analysis by 2D Nano LC/MS (Agilent 1100 series) at the TCGA (The Centre for Genomic Application, New Delhi). The data obtained by Mass spectrometry was compared with that present in Swiss Prot databases using the Mascot search algorithm.

Purification of AFase

The concentrated and desalted protein sample was loaded onto DEAE- Sepharose (fast flow) ion exchange column (24×2.6 cm; Pharmacia) pre equilibrated with sodium acetate buffer (50mM, pH 5.5). The column was first eluted with 2 bed volumes of equilibration buffer followed by a linear gradient of 1M NaCl in sodium acetate buffer (50mM, pH 5.5) at a flow rate of 1ml/min using (AKTA PRIME, Amersham Biosciences). Fractions corresponding to α-L-arabinofuranosidase active peak obtained during NaCl gradient elution were pooled, concentrated, desalted and equilibrated with 1.7M $(NH_4)_2SO_4$ in phosphate buffer (50mM pH 7.0) and applied onto phenyl sepharose (Amersham Biosciences) hydrophobic interaction (HIC) pre-packed column (5ml) that has been equilibrated with same buffer. The applied sample was eluted with a linear gradient of $(NH_4)_2SO_4$ (1.7M-0M) in phosphate buffer (50mM, pH 7.0) at a flow rate of 1ml/min. The fractions containing AFase activity were pooled, concentrated and desalted. AFase was further purified using sephacryl HR-200 (Amersham Biosciences) gel filtration (1.8× 90cm) column equilibrated with Tris-Hcl (20mM, pH 7.0) containing 0.15M NaCl. The protein in the fractions was determined by taking absorbance at 280 nm using Shimadzu-1240 spectrophotometer the fractions corresponding to purified AFase were pooled and characterized.

Assay of hemicellulolytic enzyme

Xylanase activity was assayed using 1% birchwood xylan (sigma) prepared in sodium citrate buffer (50mM, pH 6.0) as substrate. The assay was performed by incubating reaction mixture (1 ml) that contained equal amounts of appropriately diluted enzymes (500 µl) and substrate solution (500 µl) at 50 ^{o} C for 5 min. The reaction was terminated by adding 3ml DNS reagent and boiling for 10 minutes and developed colour was read at 540 nm. The reducing sugars were quantified using xylose as standard. One unit of xylanase activity was expressed as the amount of enzyme required to release 1 µmole of xylose per min under the assay conditions. The substrates, pNP-β-D-xylopyranoside, pNP-α-L-arabinofuranoside, (3 mM) prepared in sodium acetate buffer (50 mM, pH 5.0) were used to assay β-xylosidase, α-L-arabinofuranosidase (AFase), respectively using microtitre plate based method (Sharma *et al*., 2011). A reaction mixture (100µl) containing 50µl of sodium acetate buffer (50mM, pH 5.0), 25µl of suitably diluted enzyme, 25 µl of substrate (3mM) was incubated at 50^{o} C for 30 min. The reaction was terminated by adding 100µl of NaOH-glycine buffer (0.4M, pH 10.8) and developed color was read at 405 nm using an ELISA plate reader (BioRad). The Afase and β-xylosidase activity was expressed in units as the amount of enzyme required to release 1 µmole of pNP per minute under assay conditions.

Characterization of AFase

SDS PAGE and Isoelectric focusing (IEF)

The homogeneity and molecular mass of AFase was determined by SDS-PAGE (12% gel) using Mini-Protean II system (BIORAD). Isoelectric focusing (IEF) was performed using a 5% acrylamide gel that contained 2.4% broad range pH range (2-10) ampholine carrier servalyte (SERVA, Germany). L-lysine (0.29%) and L-arginine (0.35%) were used as cathode buffers whereas ortho-phosphoric acid (10 mM) was used as anode buffer. IEF was carried out at constant voltage of 100V (1h) and 200 V (1h) and 500V for 30 min (**Badhan *et al*., 2004**).

Detection of AFase activity in gel using 4-methylumbelliferyl α-L-arabinofuranoside (MUA)

AFase activitiy in the gel was detected using 10 mM MUA as substrate (prepared in 50 mM sodium citrate buffer pH 6.0). Upon completion of electrophoresis, the gels were incubated in renaturation buffer [(20mM piperazine–N,N-bis (2-ethanesulfonic acid), 2mM dithiothreitol (DTT), 2.5mM $CaCl_2$, 2.5% Triton X-100] for 1h at room temperature and then overnight at 4^{o} C in fresh renaturation buffer. After incubation the gel was thoroughly washed with sterilized double distilled water and incubated with overlaid substrate solution for 30 min at 50^{o} C. The AFase activity spots were observed under UV light using gel documentation system (Gene Genius, Cambridge, UK).

Temperature and pH optima

The activity profile of the purified AFase was determined at a temperature between 30 and 90 ºC with pNPA as substrate prepared in sodium acetate buffer (0.05 M, pH 5.0). The optimal pH was determined by measuring AFase activity between pH 2 and 10 using 0.1 M HCl-KCl (pH 2.0), sodium citrate (pH 3.0 to 6.0), sodium phosphate (pH 7.0 and 8.0), and Glycine-NaOH (pH 9.0 and 10.0 buffers at 50ºC.

Thermal and pH stability of AFase

The aliquots of purified AFase were incubated at 50 $^{\circ}$C and 60 $^{\circ}$C at pH 5.0 and 6.0 for 0 - 4 h, and subsequently assayed for residual enzyme activity.

Effect of the metal ions and other reagents

The purified AFase was incubated in 5 mM solutions of NaCl, KCl, $MnCl_2$, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $FeCl_3$, $CaCl_2$, EDTA, DTT, β-mercaptoethanol, NBS and SDS for 30 min at room temperature in sodium acetate buffer (0.05 M, pH 5.0). The residual AFase activity in the aliquots was assayed thereafter.

Substrate specificity

Substrate specificity of purified AFase was determined against 3mM *p*-nitrophenyl substrates, pNp-α-L-arabinofuranoside, pNp-α-L-arabinopyranoside, oNP-β-D-xylopyranoside, pNP-β-D-xylopyranoside pNP-β-D-glucopyranoside, oNP-β-D-galactopyranoside, pNP-β-D-glucoside, and pNP-cellobioside, pNP-α-D-mannopyranoside, 4-nitrophenyl-2-o-feruoryl-α-L-arabinofuranoside, 4-nitrophenyl-5-*O*-feruoryl-α-L-arabinofuranoside. (A reaction mixture (100µl) containing 25µl of substrate, 25µl of purified enzyme and 50µl of sodium acetate buffer (50mM, pH5.0) was incubated at 50^{o} C for 30 min). The $K_{m,}$ V_{max} and K_{cat} of purified AFase was determined against pNPA as substrate using Lineweaver Burke plot.

Analysis of Hydrolysis by Thin Layer (TLC) and High pressure liquid chromatography (HPLC)

900 µl of 1% w/v rye arabinoxylan (RAX), wheat arabinoxylan (WAX) and debranched arabinan (DA) prepared in sodium citrate buffer (50mM, pH 6.0) were incubated with 100µl of purified α-L-arabinofuranosidase at 50 ^{o} C for 72 h. Samples were withdrawn at interval of 24 h, freeze dried and redissolved in methanol. Thin layer chromatography (0.25-mm layers of silica gel F-254 plates, Merck, India) was carried out using ethyl acetate: acetic acid: water as solvent system in a ratio of (3:2:1 v/v). The TLC plates were then sprayed with diphenylamine reagent and air dried. Hydrolysis products were visualized by heating plates at 100^{0}C for 1-2 minutes. (1% w/v) Arabinose and xylose were run as standards. HPLC was carried out with DIONEX system (USA) using differential refractive index detector (RI-101, Shodex). The temperature of the Aminex column HPX-87P column (Bio-Rad) was maintained at 85^{o} C and water was used as mobile phase at a flow rate of 0.6ml/min. (1% w/v) arabinose, and xylose were used as standards.

RESULTS

Production of Hemicellulolytic enzymes by *Penicillium janthinellum*

The results (Fig 1) show hemicellulolytic production profile of *P. janthinellum* grown on wheat bran/rice straw containing solidified culture medium. Culture produced maximal levels of xylanase (1079 units/gds), AFase (140 units/gds), β-xylosidase (15.4 units/gds) and AXE (136 units/gds) after 7 days of incubation. SDS-PAGE and corresponding activity profiling of AFase by developing zymogram also indicated to gradual increase in AFase expression as observed from intensity of bands (Fig 2a). AFase active protein band was observed corresponding to 65 KDa (Fig 2b). The resolution of secretome by 2-dimensional gel electrophoresis (pI 3.0-10.0) revealed well separated protein spots (Fig. 2c) and zymogram developed using MUA as substrate, showed three distinct AFase active spots of high molecular weight and acidic pI (Fig 2d). The spots were identified using Peptide mass fingerprinting using LC-MS/MS approach in which two spots were identified as AFase belonging to GH 54 family showing close similarity to AFase from *A. niger(E=1e^{-09})*. The peptides matching were ADKWAIRGG, NSASVSLSTY.

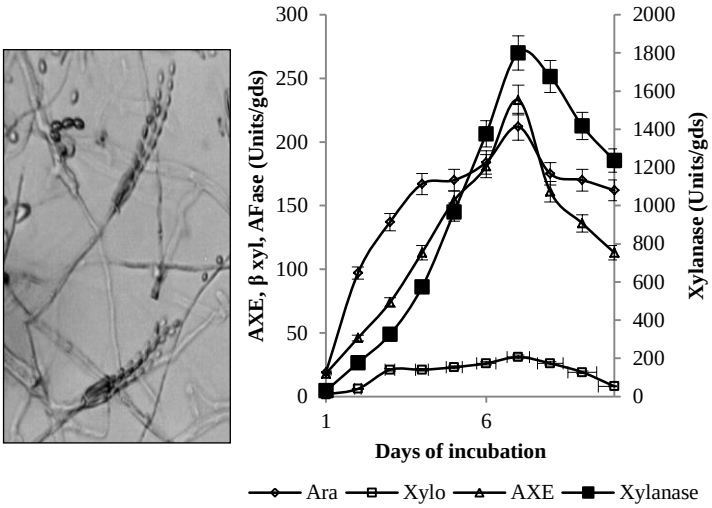

Figure 1 Production profile of hemicellulolytic enzymes (Arabinofuranosidase (AFase), β-xylosidase (β-xyl), Acetylxylan esterase (AXE) and Xylanase) produced by *Penicillium janthinellum* under unoptimized conditions. Error bars indicate SE @ 5% level.

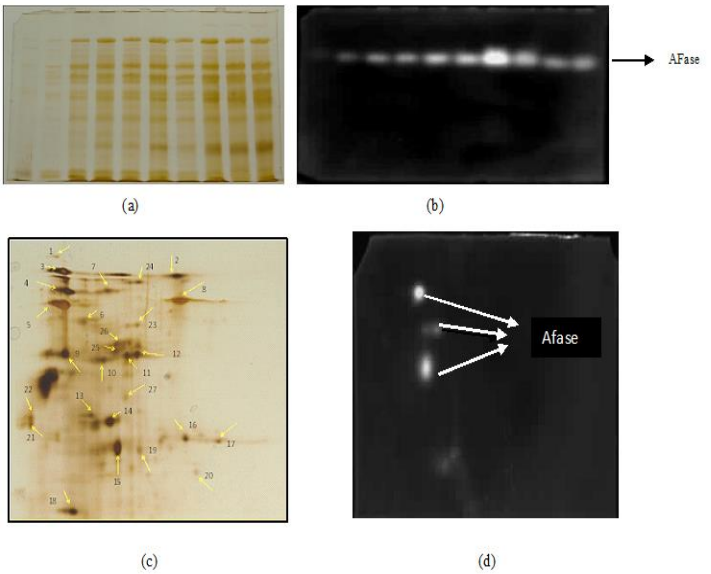

Figure 2 a) SDS-PAGE showing protein profile produced by *P. janthellium* under solid state fermentation under solid state fermentation at different day interval. b) Zymogram showing multiplicity of arabinofuranosidase developed by renaturation of SDS-PAGE. c) 2-DE secretome pattern of *P. janthinellum* grown on rice straw: wheat bran (3:2) containing solidified culture medium. Spots 3, 4 and 5 detected in 2D gel represents AFase activity d) Localization of α-L-arabinofuranosidase activity in 2DE gel.

Optimization of AFase production

The BBD experimental data showing effect of pH, moisture level and $(NH_4)_2SO_4$ on AFase production along with mean predicted and observed responses is presented in (Table 1). The model computed based on the data showed R^2 (0.997) and adjusted R^2 (0.992) along with high model F value of 236.43 and non significant lack of fit indicated it to be good fit to explain influence of effect of these variable on AFase production (Table 2). The production of AFase was significantly affected by $(NH_4)_2SO_4$, pH and moisture level in linear terms and squared terms ($p<0.005$). The iso-response contour plots (Fig. 3a, 3b) showed that maximal production of AFase (215 units/gds) could be achieved when the initial pH was in the range of 5.6-6.0, $(NH_4)_2SO_4$ concentration between 2.25-2.75 and moisture level was between 67-72%. In order to determine the accuracy of the model and to verify the optimization results, experiments were repeated three times under optimized culture conditions and AFase production of 211±3.2 (units/gds) was achieved that was 1.51 folds higher when compared to that achieved under unoptimized conditions. In addition to AFase, under optimized conditions production of other hemicellulolytic components (xylanase 1800 units/gds, β-xylosidase 31 units/gds, XAE 231 units/gds) (Fig 4) were also improved corresponding to 1.66, 2.01 and 1.73 fold increase, respectively. Table 3 shows the hemicellulolytic activities of different fungal strains under SmF and SSF. The enzyme activities obtained by *P. janthinellum* were comparatively higher to *Penicillium janczewskii*, *P. oxalicum*, *Aspergillus wortmanni*, *Talaromyces thermophilus*, *T. wortmanni* (under SmF) and *P. brasilianum and A. niger* NRRL 328 (under SSF).

Table 1 Box-Behnken design along with actual and predicted values of α-L-arabinofuranosidase.

	Independent variables						Response 1	
	A	B	C	A:(NH4)2SO4	B:pH	C:Moisture level	Arabinofuranosidase	
Std				%		%	U/g ds	
	Coded levels			Actual level			Actual	Predicted
1	-1	-1	0	0.5	4	67	48.20	48.04
2	1	-1	0	3.5	4	67	170.10	175.59
3	-1	1	0	0.5	10	67	70.10	64.61
4	1	1	0	3.5	10	67	89.00	89.16
5	-1	0	-1	0.5	7	59	42.40	43.64
6	1	0	-1	3.5	7	59	121.60	117.19
7	-1	0	1	0.5	7	75	77.80	82.21
8	1	0	1	3.5	7	75	162.00	160.46
9	0	-1	-1	2.0	4	59	119.00	117.90
10	0	1	-1	2.0	10	59	99.00	103.25
11	0	-1	1	2.0	4	75	183.50	179.25
12	0	1	1	2.0	10	75	123.00	124.07
13	0	0	0	2.0	7	67	203.00	206.20
14	0	0	0	2.0	7	67	205.00	206.20
15	0	0	0	2.0	7	67	205.00	206.20
16	0	0	0	2.0	7	67	212.00	206.20
17	0	0	0	2.0	7	67	206.00	206.20

Table 2 ANOVA statistics for fitted model for α-L- arabinofuranosidase produced by *P. janthinellum*

	F-value	p-value	
		Prob.>F	Significant
Source			
Model	236.43	<0.0001*	
A- (NH4)2SO4(%)	431.8	<0.0001*	
B-pH	91.01	<0.0001*	
C- Moisture level (%)	125.96	<0.0001*	
A^2- (NH4)2SO4(%)	792.61	<0.0001*	
B^2-pH	262.13	<0.0001*	
C^2- Moisture level (%)	184.25	<0.0001*	
A×B	99.01	<0.0001*	
A×C	0.23	0.6438	
B×C	15.31	0.0058	
R^2	99.67		
Adj R^2	99.25		
Lack of fit	0.27	0.1066	non-significant

* signficant at p<0.05,

not-signficant at p<0.05

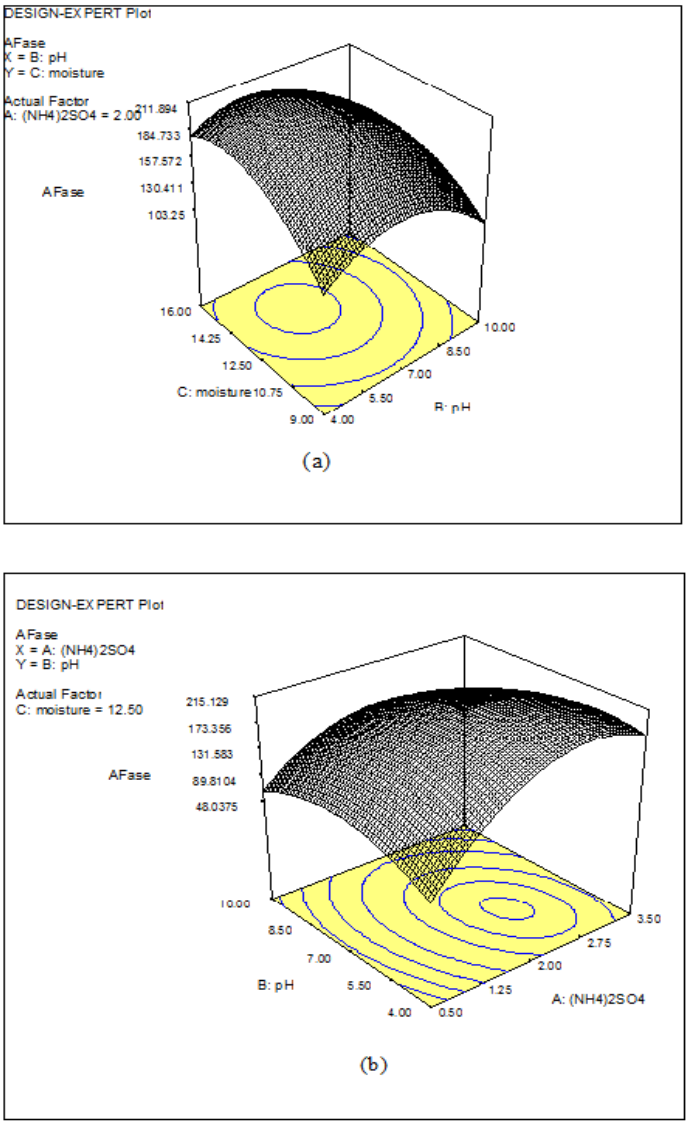

Figure 3 a) 3D contour plot showing the effect of interaction between a) pH and moisture level and b) ammonium sulphate and pH on α-L-arabinofuranosidase (AFase) production by *P. janthinellum*.

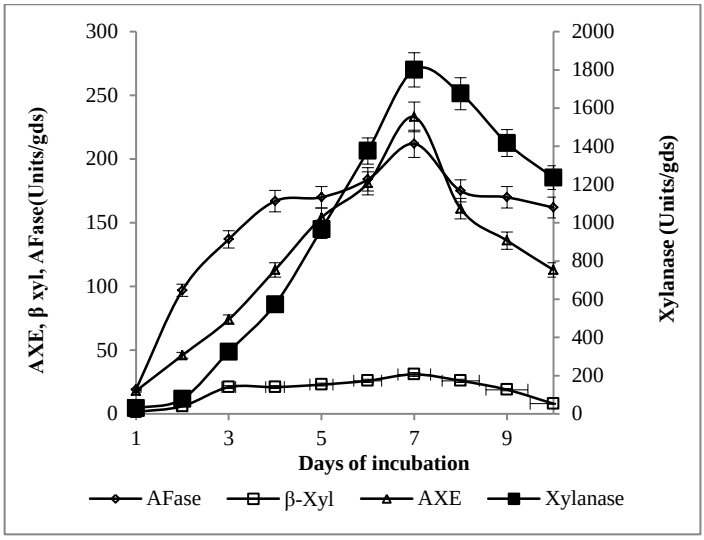

Figure 4 Production profile of hemicellulolytic enzymes (Arabinofuranosidase (AFase), β-xylosidase (β-xyl), Acetylxylan esterase (AXE) and Xylanase) produced by *P. janthinellum* under optimized conditions. Error bars indicate SE @ 5% level.

Table 3 Hemicellulolytic enzymes produced by different fungal strains under SmF and SSF

Fungi	Xylanase	β-xylosidase	α-L-ARAF	AXE	FAE	Reference
SmF (U/ml)						
Penicillium janczewskii	15.2	0.16	0.67	-	-	Terrasan et al .2010
Talaromyces thermophilus	22	1.2	0.85	-	-	Guerfali et al. 2011
Penicillium oxalicum	115.2	0.09	0.04	-	-	Liao et al. 2012
Aspergillus niger (Endophyte)	21.34	0.0	0.21	-	-	Robl et al. 2013
Talaromyces wortmanni (Endophyte)	4.85	2.85	0.91	-	-	Robl et al. 2013
SSF(U/gds)						
Penicillium brasilianum	709	-	3.5	-	1.5	Panagiotou et al. 2006
Aspergillus niger NRRL 328	950	-	-	-	-	Montibeller et al. 2014
Penicillium janthinellum	1800	31	212	233	27	Present work

Purification and characterization of AFase

AFase from *P. janthinellum* was purified to homogeneity using ultrafiltration, ion exchange, hydrophobic and gel chromatography. The purified enzyme exhibited specific activity of 9 $\mu mol^{-1}mg$ $protein^{-1}$ corresponding to 4.5 fold purification. The purified enzyme had a molecular mass and isoelectric point of 64 KDa and 3.8, respectively (Fig 5a & 5b).

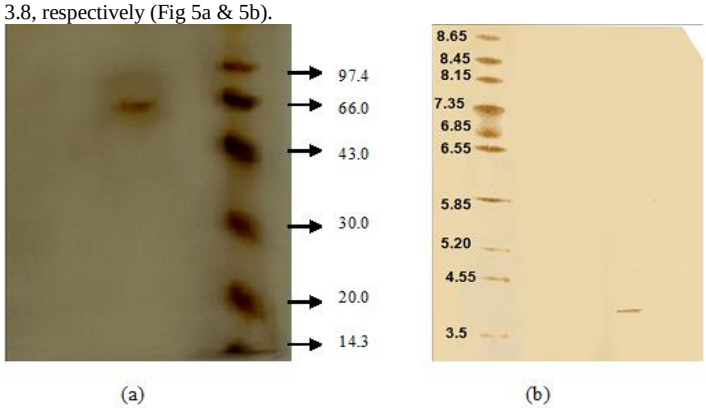

Figure 5 a) SDS-PAGE of purified α-L-arabinofuranosidase from *P. janthellium.* Lane M: standard protein markers of increasing molecular mass: soyabean trypsin inhibitor (20KDa); bovine serum albumin (43KDa); bovine serum albumin (66KDa); phosphorylase (97.4KDa), Lane 1: purified α-L-arabinofuranosidase. b) Isoelectric focusing of purified α-L-arabinofuranosidase (E). Lane M: standard pI markers(sigma). Lane1: purified α-L-arabinofuranosidase.

Temperature, pH optima and Stability

Purified AFase was optimally active at 50° C (Fig 6a). Further increase in temperature resulted in appreciable decline in its activity. The AFase from *P. janthinellum* was optimally active at pH 5.5 (Fig 6b). The AFase retained 98% of its activity after 2 hours at 50° C. (Fig. 6c). Furthermore, the AFase was appreciably stable showing half- life of more than 150 min at 50° C and pH 6.0 (Fig 6c). Table 4 summarizes the comparative properties of AFase from different fungal strains.

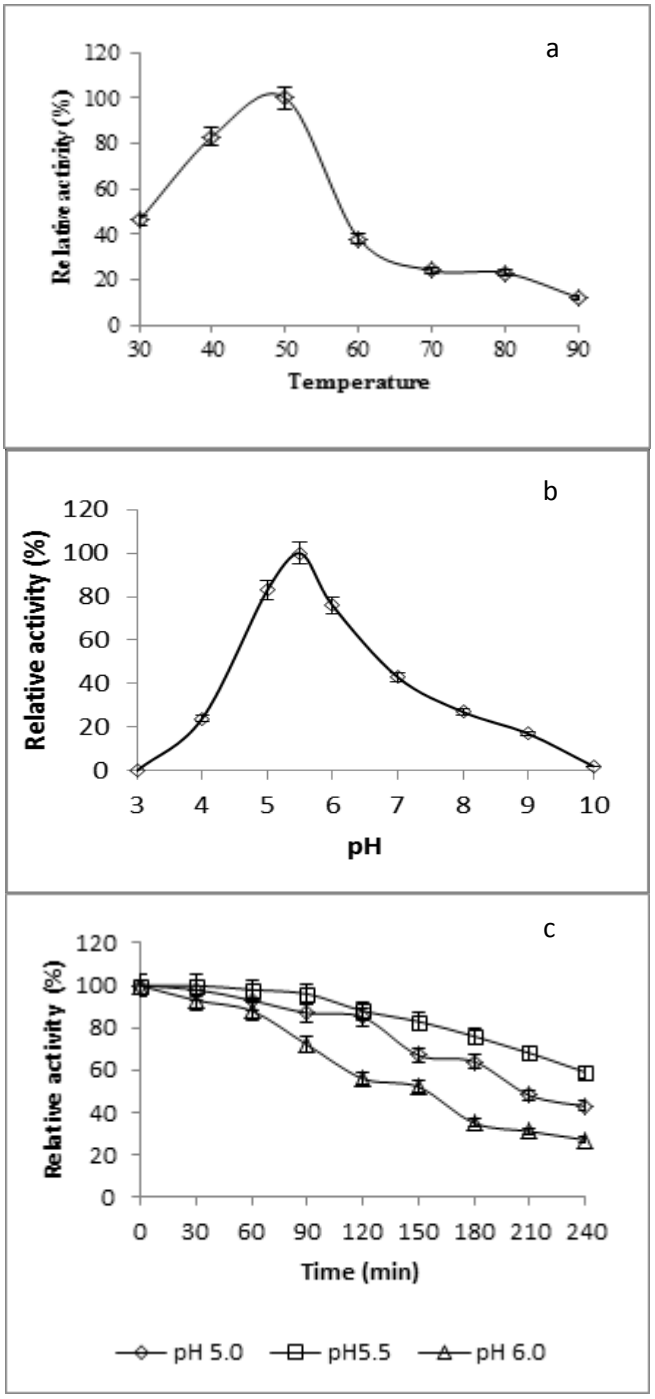

Figure 6 a) Effect of temperature. b) pH on α-L-arabinofuranosidase activity. c) Stability of α-L-arabinofuranosidase at pH 5.0, 5.5 and 6.0 at 50 °C as a function of time. Error bars indicate SE @ 5% level

Table 4 Properties of α-L-arabinofuranosidases (AFase) from different fungal strains

Microoganism	MW (kDa)	T_{opt} (°C)	pH_{opt}	Family	References
Fungi					
Penicillium canescens	*60* *70*	-	-	*54a* *51*	Gusakov et al. 2013
Penicillium janczewskii	-	*25*	*6.0*	-	Terrasan et al. 2010
Penicillium purpurogenum (ABF1)	*58*	50	4.0		De Ioannes et al.2000
Penicillium purpurogenum (ABF2)	*70*	*60*	*5.0*	*51*	Fritz et al.2008
Penicillium crysogenum	*52*	-	*3.3-5.5*	-	Sakamota and Kawasaki.2003
Penicilium brasilianum	-	25.5	6.0	-	Panagiotou et al.2005
Chaetomium sp.	*52.9*	*65*	*5.0*	-	Yan et al. 2012
Humicola insolens	-	*40*	*6.0*	*43*	Sorensen et al.2006
Penicillium janthinellum	64	50	5.5	54	Present work

Effect of metal ions

The activity of purified enzyme was positively modulated in the presence of Fe^{+3} (Fig 7). The activity of purified AFase was inhibited in the presence of Zn^{+2} and Cu^{+2}. N-bromosuccinate resulted in complete loss of enzyme activity.

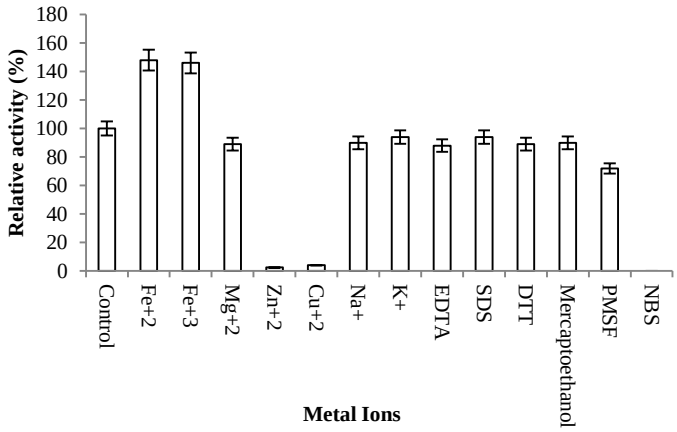

Figure 7 Effect of metal ions and other chemical reagents (10mM each) on the activity of α-L-arabinofuranosidase from *P. janthinellum.* Error bars indicate SE @ 5% level

Substrate specificity and Enzyme Kinetics

The AFase from *P. janthinellum* was maximally active against pNP-α-L-arabinofuranoside (pNPA) but was not able to degrade other pNP substrates. The purified enzyme did not show activity against p-nitrophenyl 2-*O* and 5-*O*-feruloyl α-L-arabinofuranoside. The AFase from *P. janthinellum* was more active on arabinoxylan (Wheat and Rye) than on debranched arabinan. AFase did not catalyse the hydrolysis of birchwoodxylan, CMC (low and high viscosity), and avicel. The purified AFase exhibited K_m and V_{max} of 0.4mM and 260µmol min^{-1}mg $protein^{-1}$against pNPA respectively.

Hydrolysis studies

Thin layer chromatography (TLC) and HPLC analysis of hydrolysis products of rye arabinoxylan, wheat arabinoxylan and debranched arabinan (Fig 8) indicated that AFase from *P. janthinellum* releases L-arabinose as the main sugar. The HPLC based quantification showed that hydrolysis with purified AFase (3.0 units/g substrate) released appreciable amounts of arabinose from rye arabinoxylan (29.5 mg/g substrate) followed by wheat arabinoxylan (13.4 mg/g substrate), and debranched arabinan (3.8 mg/g substrate mg/g) indicating exo-activity of AFase.

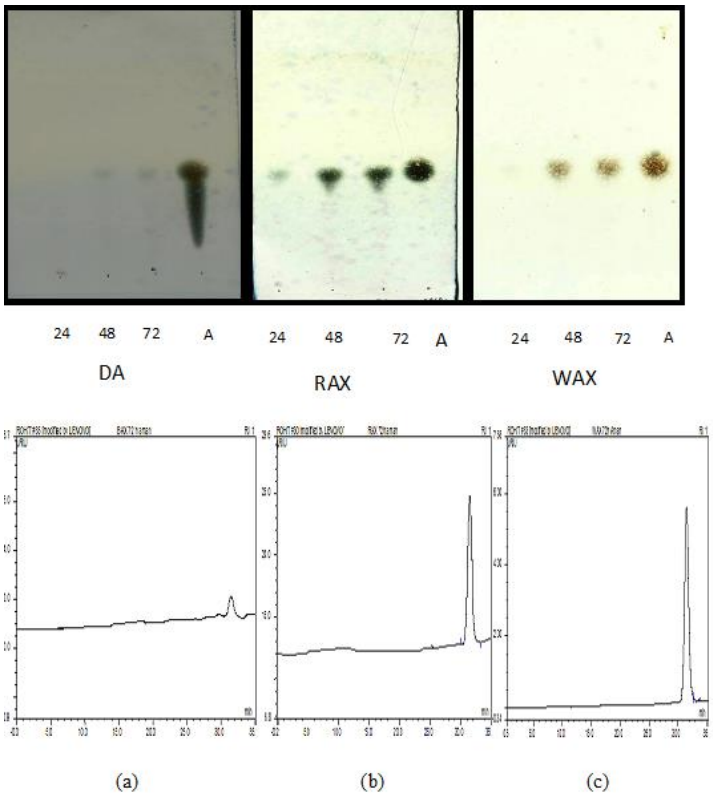

Figure 8 Thin layer chromatography and HPLC showing hydrolysis products obtained by action of α-L-arabinofuranosidase from *P. janthinellum* on a) RAX, b) WAX and c) DA

DISCUSSION

P. janthinellum strain grown on solidified medium containing rice straw and wheat bran as substrates produced high levels of AFase along with other hemicellulosic components (xylanase, β-xylosidase and acetylxylan esterase). The crude enzyme extract resolved by 2 Dimensional electrophoresis and its zymogram developed by using MUA as substrate revealed three distinct spots of high molecular weight and acidic pI. The observed multiplicity of AFase may be due proteolysis of the enzyme during processing that has also been observed previously for multi-domain enzymes such as GH5 β mananase (**Takasuka *et al.*, 2014**) or may be due to cross reactivity of β-xylosidase that can recognise MUA as substrate (**Wagschal *et al.*, 2009**). This is the first report for detection of AFase on 2DE gels by zymography techniques. Further the optimized culture conditions were established for enhancing AFase production. The model computed R^2 and adjusted R^2 for AFase production were 0.997 and 0.992. The R^2 value closer to 1 indicates that the model is robust to predict the response (**Babu and Satyanarayana, 1995**).The regression equation obtained after the analysis of variance (ANOVA) gives the level of enzymes produced as a function of initial value of $(NH4)_2SO4$, moisture level and pH. By applying multiple regressions analysis on the experimental data the second order polynomial equation was found to explain the enzyme production (**Jatinder *et al.*, 2006**). The purification and characterization of AFase from *P. janthinellum* were further

investigated. The results after purification suggest that the purified enzyme is a monomer with a molecular mass of 64 KDa, whereas AFase from *Penicillium* strains (*P. purpurogenum*, *P. chrysogenum* and *P. canescens*) showed a molecular weight of 58, 52 and 60 KDa, respectively (**De Ioannes *et al*, 2000**; **Sakamota and Kawasaki, 2003**; **Gusakov *et al*., 2013**) indicating diversity of AFase produced by different *Penicillium* strains which possibly influences their biochemical characterstics (Saha 2000). The maximal activity of the enzyme was observed at 50° C which is in concurrence with AFase from *Fusarium oxysporium* (50 ° C), *A. pullulans* (55 ° C), *T. thermophilus* (55 ° C) and *Clostridium thermocellum* (50 ° C) (**Chacon *et al*., 2004**; **Dewet *et al*., 2008**; **Guerfali *et al*., 2011**; **Ahmed *et al*., 2013**). The AFase from *P. janthinellum* was optimally active at pH 5.5, similar pH profiles have also been observed in AFase purified from *P. chrysogenum*, *P. purpurogenum and Chaetomium sp.* (**Sakamota and Kawasaki, 2003**; **Fritz *et al*., 2008**; **Yan et al., 2012**). The activity of purified enzyme was positively modulated in the presence of Fe^{+3}, Similar results were previously observed in *Arthrobacter sp*. (**Khandeparker *et al*., 2008**) whereas the addition of metal ions such as Zn^{+2} and Cu^{+2} inhibited the AFase activity significantly, suggesting that it is a thiol-sensitive enzyme because these heavy metals promote the oxidative process with thiol group and affect the native structure of enzyme thus destabilizing the conformational folding of the enzyme or lead to formation of disulfide bonds at irregular positions (**Ohmiya *et al*., 1995**). Inhibition in the presence of Cu^{+2} has also been previously observed (**Sakamota and Kawasaki, 2003**; **Guerfali *et al*., 2011**; **Yang *et al*., 2012**). Complete loss of the activity in the presence of N-bromosuccinate indicates to the role of tryptophan in the active site of the enzyme (**Adsul *et al*., 2009**). The purified enzyme was maximally active against pNP-α-L-arabinofuranoside as substrate however it did not recognize p-nitrophenyl 2-*O* and 5-*O*-feruloyl α-L-arabinofuranoside which are suitable substrates for determination and differentiation of FAE activity (**Mastihubova *et al*., 2010**). The AFase from *P. janthinellum* was more active on arabinoxylan (Wheat and Rye) than on debranched arabinan. Expectedly purified AFase did not recognised methylglucouronyl and acetyl substituted Birchwood xylan as substrate. The substrate specificity of *P. janthinellum* AFase is similar to GH family 54 AFase from *P. purpurogenum* and *P. funiculosum* (**De Ioannes *et al*., 2000**; **Guais *et al*., 2010**) that catalyzes the removal of α-L-arabinofuranosyl residues from singly substituted xylopyranosyl residues (**Sorensen *et al*., 2006**). The observed K_m (0.4mM) is lower than that of AFase from *P. purpurogenum* (1.23mM), *Aureobasidium pullulans* (3.7mM), *T. thermophilus* (0.77mM) and *Chaetomium* sp. CQ31(1.43mM) (**De Ioannes *et al*., 2000**; **Dewet *et al*., 2008**; **Guerfali *et al*., 2011**; **Yan *et al*., 2012**). Lower k_m indicates much higher affinity for the substrate. Furthermore the purified AFase showed higher rate of catalysis (V_{max}) when compared to AFase from *Chaetomium sp*. (**Yan *et al*., 2012**). The AFase hydrolyzed rye arabinoxylan, wheat arabinoxylan and debranched arabinan releasing arabinose, similar results have previously been observed in AFase of *Clostridium sp*. by (**Ahmed *et al*., 2013**) whereas GH54 AFase from *A. pullulans* (**Dewet *et al*., 2008**) was able to release arabinose from arabinoxylans but not from debranched arabinan. Debranched arabinan is mainly comprised of chain of α, 1,5-linked arabinofuranosyl residues whereas, arabinoxylans possess a xylan backbone that is substituted with arabinose and acetyl moieties through α,1,2 and α,1,3 linkages. The enzyme used in the present study seems to cleaves arabinose from α1, 2 and α1,3 linkages. The above results also indicate the exo-activity of AFase which has immense potential in bioconversion of agro-residues to value added products (**Guerfali *et al*., 2010**).

CONCLUSION

The study reports *P. janthinellum* as highly efficient source of AFase as well as other components of hemicellulolytic enzyme system. By employing response surface methodology optimal culture conditions for AFase production were established which were higher than reported earlier. Secretome analysis revealed *P. janthinellum* α-L-arabinofuranosidase belonging to family 54. This is the first report on identification of α-L-arabinofuranosidase on 2DE gels by zymography. Also purified AFase efficiently hydrolyses rye and wheat arabinoxylan to arabinose. Thus this AFase can be of great importance to food and bioconversion industry.

Conflict of interest: The author confirms that this article content has no conflict of interest.

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