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Improved yield of α-L-arabinofuranosidase by newly isolated *Aspergillus niger* ADH-11 and synergistic effect of crude enzyme on saccharification of maize stover

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Abstract

Background: In the view of depleting resources and ever-increasing price of crude oil, there is an urge for the development of alternative sources to solve the issue of fuel in the coming years. Lignocellulosic biomass is considered to be the most potential alternative resources for fossil fuel. Bioconversion of cellulosic and hemicellulosic components into fermentable sugars is the key step in producing fuel ethanol from lignocellulose. The enzymatic hydrolysis of lignocellulosic biomass needs a highly balanced composition of cellulases and hemicellulases. Commercial enzymes are usually poor in accessory hemicellulolytic enzymes like α-L-arabinofuranosidase. The addition of such accessory enzymes in combination with cellulase or hemicellulase plays a vital role in improving the total yield of fuel ethanol by enhancing the saccharification yield.

Results: The newly isolated fungal strain *Aspergillus niger* ADH-11 produced a maximum of 22.14 U/g of α-L-arabinofuranosidase under solid-state fermentation using wheat bran as the substrate and modified Mandels-Weber medium at 30°C after 180 h of incubation. The optimization of various fermentation parameters was performed by response surface methodology employing Plackett-Burman design followed by Box-Behnken design. The yield of α-L-arabinofuranosidase was enhanced by 2.34-fold after executing statistical optimization of various fermentative parameters. Crude α-L-arabinofuranosidase was found to be highly stable for 3 h at its optimum temperature (55°C) and pH (4.0). The assessment of the crude enzyme extract in saccharification of alkali-treated maize stover revealed that the supplementation of crude α-L-arabinofuranosidase to commercial cellulase and crude xylanase mixture increased the saccharification yield up to 730 mg/g of maize stover.

Conclusions: The newly isolated *A. niger* ADH-11 was found to be a potential producer of α-L-arabinofuranosidase. The crude enzyme was active at low pH and high temperature which makes it suitable for various industrial applications such as enzymatic saccharification of lignocellulosic biomass. The supplementation of α-L-arabinofuranosidase enzyme to commercial cellulases and hemicellulases improves the bioconversion of lignocellulosic biomass to a greater extent.

Keywords: *Aspergillus niger* ADH-11; α-L-Arabinofuranosidase; Solid-state fermentation; Response surface methodology; Maize stover

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Background

Hemicelluloses are the second most abundant renewable source on the earth after celluloses representing up to 30 to 35% of the total dry weight of the carbohydrate polymers, out of which xylan is the major constituent [1,2]. Xylan-rich agroindustrial wastes are among the most important biomass sources in the world, representing an annual generation of 40 million tons causing considerable damage to economic activities in the agroindustrial sector and environment as well [3]. As xylan is the complex structure, its complete degradation requires the action of accessory hemicellulolytic enzymes out of which α-L-arabinofuranosidase is one of the most important enzymes [2,4].

α-L-Arabinofuranosidase is an exo-type enzyme that generally catalyzes the cleavage of the terminal α-L-arabinofuranosyl residues of arabinoxylan, arabinan, and arabinogalactan present in the hemicellulose structure. The arabinose side chains on hemicelluloses participate in cross-linking within the plant cell wall structure. L-Arabinose substitutions on xylan strongly inhibit the action of xylan-degrading enzymes, thus preventing the complete degradation of the polymer to its basic xylose units [5]. It is now well established that the action of α-L-arabinofuranosidase alone or in the combination with other hemicellulolytic enzymes is inevitable for enzymatic bioconversion of lignocellulosic biomass in to sugars which can be further fermented to ethanol. This enzyme is also finds a wide range of applications in food, feed, paper pulp, and pharmaceutical industries.

The production of α-L-arabinofuranosidase is widespread among bacteria and fungi. However, filamentous fungi are more attractive than bacteria as potential producers of these enzymes as fungi secrete higher enzyme levels in the culture medium than bacteria [6]. α-L-Arabinofuranosidase production has been reported by many fungal strains, such as *Trichoderma reseei* (142 nkat/mg), *Aspergillus awamori* (22.0 U/mg), *Aspergillus nidulans* (30.0 mIU/ml), *Aspergillus terreus* (6.0 nkat/ml), *Penicillium purpurogenum* (1.0 U/ml), *Aspergillus oryzae* (0.02 U/mg), and *Penicillium chrysogenum* (0.34 U/mg) [7-13].

Solid-state fermentation (SSF) is a microbial process in which solid material is used as the substrate which may provide necessary nutrients and serves as an anchorage for the cell. SSF can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source [14]. Fungi grow well on moist substrates in the absence of free-flowing water [15] and produce large amount of extracellular enzymes like cellulases, hemicellulases, and ligninases. The production of enzymes using fungi under SSF is many a times higher than submerged fermentation (SmF), since SSF processes reproduce the natural living conditions of such fungi [16]. The use of SSF for production of enzymes has many economic advantages over SmF like no need for complex and sophisticated machinery, easy product recovery, simple and inexpensive substrate for the fermentation, low energy demand, high volumetric productivity, and often a high yield of product [17]. Response surface methodology has been extensively used in optimizing various fermentative parameters for enzyme production using solid-state fermentation [18-21].

Considering the potential and future prospects of α-L-arabinofuranosidase, the present study was aimed at production of α-L-arabinofuranosidase under SSF by a newly isolated fungal strain of *Aspergillus niger* ADH-11 employing response surface methodology. The properties of crude α-L-arabinofuranosidase were also evaluated to predict its end application, and the crude enzyme was supplemented to cocktails containing cellulase and xylanase to check synergistic action during saccharification of maize stover.

Methods

Materials

All the reagents, media, and chemicals used under study were of analytical grade purchased from Qualigens Fine Chemicals Pvt Ltd, HiMedia Laboratories Pvt Ltd, Merck & Co., and Loba Chemie Pvt Ltd from Mumbai, Maharashtra, India. *p*-Nitrophenyl-α-L-arabinofuranoside was obtained from Sigma-Aldrich, St. Louis, MO, USA. Wheat straw, rice straw, corn cobs, and maize bran were provided by a local farmer, whereas wheat bran and rice bran were procured from local market. The raw materials were washed thoroughly with water, dried at 80°C, and stored at room temperature in air tight plastic bags until use. The commercial cellulase used in this study was provided by MAPs Enzyme Limited (Ahmedabad, Gujarat, India).

Microbial strain

The α-L-arabinofuranosidase-producing fungal strain was isolated from decaying custard apple and it was identified as *A. niger* ADH-11 by the Agharkar Research Institute (ARI) Pune, India on the basis of its molecular characteristics. The sequence of the strain was submitted to NCBI gene bank with accession no. KF026012. The culture was grown on potato dextrose agar (PDA) slant and store at 4°C. It was subcultured every 15 days.

α-L-Arabinofuranosidase production under SSF using different agro residues by *A. niger* ADH-11

The α-L-arabinofuranosidase production was carried out in 250-ml Erlenmeyer flasks containing 5 g of washed dried agro residues (wheat bran, wheat straw, rice bran, rice straw, corn cobs, and maize bran) moistened with 10 ml of modified Mandels-Weber medium containing the following (g/l): urea, 0.3; ammonium sulfate, 1.4; KH_2PO_4, 0.3; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; proteose

peptone. 1.0 and (mg/l) $MnSO_4.7H_2O$. 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2; Tween 80, 0.1%; and initial pH 5.3. The medium and the substrate were sterilized separately at 121°C for 15 min at 15 lbs. The inoculum was prepared using 7 days old *A. niger* ADH-11 slants. The spore suspension was prepared by adding 5 ml of 1% Tween 80 to each slant. The spore count was carried out using Neuber's chamber. The medium and the substrate were mixed at the time of inoculation at 10^8 spore/ml and incubated at 30°C under static condition. The contents of the flasks were mixed intermittently (twice a day), and the crude enzyme was extracted from each flask at regular interval of time (at every 24 h).

Enzyme extraction

The contents from each flask were extracted using a minimum quantity (30 ml) of 50 mM sodium citrate buffer (pH 5.3) along with 0.2 ml of Tween 80. The flasks were kept at 30°C with 150 rpm for 30 min for thorough mixing of the contents, followed by filtration through a wet muslin cloth with thorough squeezing, and the filtrate was centrifuged at 8,000 rpm for 30 min. The clear supernatant thus obtained was used as a crude enzyme for further studies.

Enzyme assays

The α-L-arabinofuranosidase activity was determined according to the method reported by Yanai and Sato [22] with some modifications. The reaction mixture consisting of 1 mM *p*-nitrophenyl-α-L-arabinofuranoside in 50 mM sodium citrate buffer (pH 5.3) was incubated with enzyme at 50°C for 10 min in total volume of 0.5 ml. The reaction was terminated by adding 1 ml of 2 M sodium carbonate solution. The amount of *p*-nitrophenol released was determined by measuring absorbance at 410 nm. One unit of α-L-arabinofuranosidase activity is defined as amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute under assay condition. The xylanase activity was measured using 1% birch wood xylan solution as reported earlier [23]. The substrate was prepared in 50 mM sodium citrate buffer of pH 5.3. The enzyme reaction was carried out at 50°C for 10 min. After incubation, the reaction was terminated by adding 1 ml of DNS reagent. The enzyme activity was determined by measuring the release of reducing sugar using xylose as standard [24]. One unit of xylanase activity is defined as the amount of enzyme releasing 1 μmol of xylose per minute under assay condition. The filter paper activity was measured according to IUPAC recommendation employing filter paper (Whatman no. 1) as a substrate [25]. The release of reducing sugars in 60 min at 55°C and pH 4.8 (50 mM sodium citrate buffer) was measured as glucose equivalent using DNS method. One unit of filter paper activity is defined as amount of enzyme releasing 1 μmol of glucose per minute under assay condition.

Protein estimation

The soluble protein was estimated by Folin's method using bovine serum albumin as a standard [26].

Optimization of α-L-arabinofuranosidase production using response surface methodology

The optimization of the physicochemical parameters for α-L-arabinofuranosidase production was performed in two stages. Initially, ten variables were considered for screening using Plakett-Burman design (PBD) to identify the variables, which significantly influenced α-L-arabinofuranosidase production, and in the second stage the significant variables screened from PBD were further optimized using a Box-Behnken design (BBD).

Screening of parameters affecting α-L-arabinofuranosidase production by PBD

In this study, urea, ammonium sulfate, proteose peptone, yeast extract, KH_2PO_4, $CaCl_2$, $MgSO_4$, pH, moisture ratio, and fermentation time were selected as the independent variables. Each variable was set at two levels, higher and lower (Table 1). The experimental design is given in Table 2. The significance of regression coefficients was tested by *T* test for α-L-arabinofuranosidase production (Table 3).

Optimization of significant parameters for α-L-arabinofuranosidase production by BBD

BBD involves full factorial search by observing simultaneous, systematic, and efficient variation of important components on the fermentation process. Urea, $MgSO_4$, $CaCl_2$, pH, ammonium sulfate, and fermentation time were selected as individual variable for the production of α-L-arabinofuranosidase. BBD in six factors having three center runs (with a total of 54 experimental runs) was

Table 1 Actual and coded level of variables tested with Plakett-Burman design

	Code	−1	1
Urea	X1	0.1	5
$MgSO_4$	X2	0.1	3
$CaCl_2$	X3	0.1	3
pH	X4	3	8
Ammonium sulfate	X5	1	10
Fermentation time	X6	48	216
Protease peptone	X7	1	5
Yeast extract	X8	1	5
KH_2PO_4	X9	0.1	3
Moisture ratio	X10	1:1	1:3

Table 2 PBD matrix for the screening of variables influencing α-L-arabinofuranosidase production

Run	X1	X2	X3	X4	X5	X5	X7	X8	X9	X10	α-L-Arabinofuranosidase (U/g)
1	0.1	0.1	0.1	3	1	48	1	1	0.1	1	8.75
2	5	0.1	0.1	8	10	216	1	5	0.1	3	35.41
3	5	0.1	3	3	1	216	5	5	0.1	1	35.01
4	0.1	3	3	3	10	48	5	5	0.1	3	9.4
5	0.1	3	3	8	1	48	1	5	3	3	4.97
6	5	0.1	0.1	3	10	48	1	5	3	1	12.35
7	0.1	3	3	8	10	216	1	1	0.1	1	19.85
8	0.1	0.1	0.1	8	1	216	5	5	3	1	35.08
9	0.1	0.1	0.1	3	10	216	5	1	3	3	30.41
10	5	3	3	3	1	216	1	1	3	3	32.15
11	5	3	3	8	10	48	5	1	3	1	10.79
12	5	0.1	0.1	8	1	48	5	1	0.1	3	11.81

used for the optimization of α-L-arabinofuranosidase production.

The design allowed to evaluate the main and interactive effects of urea (X1), $MgSO_4$ (X2), $CaCl_2$ (X3), pH (X4), ammonium sulfate (X5), and fermentation time (X6) for α-L-arabinofuranosidase yield (U/g). The α-L-arabinofuranosidase yield (U/g) corresponding to the combined effects of six variables was studied in their specific range as shown in Table 4. The temperature was kept constant at 30°C throughout the experiment. All flasks were analyzed for α-L-arabinofuranosidase yield at specific time intervals as planned in BBD. The plan of BBD in the coded levels of six independent variables is shown in Table 5.

For statistical calculation the independent variables were coded as follows:

$$x_i = (X_i - X_o)/\delta X_i \qquad (1)$$

Where X_i is the experimental value of the variable; X_o is the midpoint of X_i, δX_i is the step change in X_i, and x_i is the coded value for X_i, $i = 1 - 6$.

The α-L-arabinofuranosidase yield was fitted using response surface methodology applying Equation 2 and was analyzed using MINITAB 16.0 version:

$$Y = \beta_o + \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_i \qquad (2)$$

where Y is the predicted response variable, β_o, β_i, β_{ii}, β_{ij} are fixed regression coefficients of the model, x_i, x_j ($i = 1$, 2, 3, 4, 5 and 6, $i \neq j$, $i < j = 1, 2, 3, 4, 5, 6$) represent independent variables in the form of original values.

Table 3 Regression coefficient for α-L-arabinofuranosidase production

Term	Effect	Coefficient	Standard error Coefficient	*T* value	*P* value
Constant		26.02	1.579	16.48	0.039
Urea	15.88	7.94	1.579	5.03	0.025
Ammonium sulfate	−12.63	−6.32	1.579	−4.00	0.050
Protease peptone	4.21	2.10	1.579	1.33	0.410
Yeast extract	4.12	2.06	1.579	1.30	0.417
KH_2PO_4	−0.12	−0.06	1.579	−0.04	0.059
$CaCl_2$	−28.81	−14.40	4.737	−3.04	0.002
$MgSO_4$	36.24	18.12	5.470	3.31	0.047
pH	−12.73	−6.37	1.579	−4.03	0.050
Moisture Ratio	−0.65	−0.33	1.579	−0.21	0.870
Fermentation time	32.68	16.34	1.579	10.35	0.047

Interpretation and data analysis

The results of the experimental design were analyzed and interpreted using Minitab 16 (Minitab Inc, State College, PA, USA) statistical software. The prediction of fermentation parameters and generation of response contour plot by the model were also done by the same software. Analysis of variance (ANOVA) was used to establish the significance of the model parameters.

Table 4 Actual and coded level of variables tested with Box-Behnken design for α-L-arabinofuranosidase production

Process variable	Coded level of variable		
	−1	0	+1
Urea (X1)	0.1	2.55	5
$MgSO_4$ (X2)	0.1	1.55	3
$CaCl_2$ (X3)	0.1	1.55	3
pH (X4)	3	5.5	8
Ammonium sulfate (X5)	1	5.5	10
Fermentation time (X6)	48	132	216

Table 5 Full factorial BBD matrix for α-L-arabinofuranosidase production by *Aspergillus niger* ADH-11

	Urea	$MgSO_4$	$CaCl_2$	pH	Ammonium sulfate	Fermentation time	α-L-Arabinofuranosidase	
Run	X1	X2	X3	X4	X5	X6	Predicted activity (U/g)	Experimental activity (U/g)
1	−1	−1	0	−1	0	0	24.803	23.750
2	1	−1	0	−1	0	0	23.506	21.940
3	−1	1	0	−1	0	0	21.359	24.070
4	1	1	0	−1	0	0	22.517	20.990
5	−1	−1	0	1	0	0	21.465	22.510
6	1	−1	0	1	0	0	22.089	19.860
7	−1	1	0	1	0	0	20.756	21.840
8	1	1	0	1	0	0	23.835	25.370
9	0	−1	−1	0	−1	0	18.686	20.850
10	0	1	−1	0	−1	0	18.814	20.140
11	0	−1	1	0	−1	0	19.025	19.720
12	0	1	1	0	−1	0	14.183	12.240
13	0	−1	−1	0	1	0	16.684	17.410
14	0	1	−1	0	1	0	19.828	20.350
15	0	−1	1	0	1	0	21.293	18.750
16	0	1	1	0	1	0	19.467	18.520
17	0	0	−1	−1	0	−1	20.493	17.850
18	0	0	1	−1	0	−1	17.673	19.390
19	0	0	−1	1	0	−1	12.448	13.680
20	0	0	1	1	0	−1	16.328	15.430
21	0	0	−1	−1	0	1	22.543	23.930
22	0	0	1	−1	0	1	18.641	16.920
23	0	0	−1	1	0	1	21.868	20.640
24	0	0	1	1	0	1	24.666	26.820
25	−1	0	0	−1	−1	0	16.175	16.160
26	1	0	0	−1	−1	0	17.414	17.340
27	−1	0	0	1	−1	0	14.703	12.370
28	1	0	0	1	−1	0	17.861	17.410
29	−1	0	0	−1	1	0	19.621	19.590
30	1	0	0	−1	1	0	18.245	21.060
31	−1	0	0	1	1	0	17.154	17.710
32	1	0	0	1	1	0	17.697	17.230
33	0	−1	0	0	−1	−1	15.256	15.060
34	0	1	0	0	−1	−1	15.484	14.150
35	0	−1	0	0	1	−1	15.089	14.820
36	0	1	0	0	1	−1	18.333	16.440
37	0	−1	0	0	−1	1	22.735	23.410
38	0	1	0	0	−1	1	17.793	19.280
39	0	−1	0	0	1	1	23.168	25.720
40	0	1	0	0	1	1	21.242	20.220
41	−1	0	−1	0	0	−1	16.569	15.160
42	1	0	−1	0	0	−1	16.174	18.470
43	−1	0	1	0	0	−1	13.391	15.960

Table 5 Full factorial BBD matrix for α-L-arabinofuranosidase production by *Aspergillus niger* ADH-11 *(Continued)*

44	1	0	1	0	0	−1	20.412	21.240
45	−1	0	−1	0	0	1	24.726	23.410
46	1	0	−1	0	0	1	19.487	16.430
47	−1	0	1	0	0	1	20.467	18.660
48	1	0	1	0	0	1	22.643	24.540
49	0	0	0	0	0	0	18.998	21.490
50	0	0	0	0	0	0	18.998	16.630
51	0	0	0	0	0	0	18.998	20.180
52	0	0	0	0	0	0	18.998	17.460
53	0	0	0	0	0	0	18.998	18.670
54	0	0	0	0	0	0	18.998	19.560

Wheat bran was used as the substrate at 30°C under SSF.

Effect of temperature and pH on α-L-arabinofuranosidase activity

The optimum temperature for α-L-arabinofuranosidase was determined by assaying relative activity at different temperatures ranging from 40°C to 75°C. The optimum pH for α-L-arabinofuranosidase was determined by assaying relative activity at different pH (3 to 7) using 50 mM sodium citrate buffer for pH 3, 4, 5, and 50 mM sodium phosphate buffer for pH 6 and 7.

Temperature and pH stability of α-L-arabinofuranosidase

To determine the thermal stability of α-L-arabinofuranosidase, the enzyme solution was treated at different temperatures (45°C, 55°C, and 65°C) in 50 mM sodium citrate buffer (pH 4.0) in a temperature-controlled water bath and the residual activity was measured at different time intervals (60 min) up to 3 h. To determine the pH stability of α-L-arabinofuranosidase, the enzyme solution was appropriately diluted at different pH buffers (4, 5, 6 and 7) and left to room temperature and the residual activity was measured at different time intervals (60 min) up to 3 h.

Pretreatments of maize stover

The substrate (maize stover) was washed, dried, and sieved to get uniform particle size before its use. The maize stover was given two separate pretreatments. In one case, pretreatment was given by preparing 15% slurry of maize stover in 4% NaOH solution and was incubated at 30°C for 12 h. The second pretreatment was given by 15% ammonia solution in the ratio of (1:4.5 (*w*/*v*)) to prepare the slurry. The slurry was autoclaved at 121°C and 15 lbs for 1 h. The substrate was washed completely with distilled water until neutral and dried in oven at 80°C until moisture was evaporated.

Enzymatic hydrolysis of maize stover

The enzymatic saccharification of pretreated and untreated maize stover was carried out using crude α-L-arabinofuranosidase from *A. niger* ADH-11, crude xylanase from *Aspergillus foetidus* MTCC 4898 [18], and commercial cellulase individually. The crude enzyme from *A. niger* ADH-11, crude enzyme from *A. foetidus* MTCC 4898, and commercial cellulase also have other cellulolytic and xylanolytic enzymes. Crude α-L-arabinofuranosidase was used at 4.0 U/g, crude xylanase was used at 300 U/g, while commercial cellulase was used at 5.0 FPU/g during saccharification of maize stover. The enzymatic hydrolysis was performed in 150-ml screw cap Erlenmeyer flasks containing 2.5% pretreated and untreated maize stover and diluted enzyme, as mentioned earlier, in 50 mM sodium citrate buffer (pH 4.8) containing 0.1% Tween 80 in a final volume of 40 ml. Controls were kept for each reaction in which the active enzyme was replaced with heat-inactivated enzyme. The reaction system was fortified with 10 mg% sodium azide. The reaction was carried out at 50°C in water bath with mild shaking. The samples were withdrawn every 4 h and incubated in boiling water bath (100°C) to inactivate the enzyme; the reaction mixture was then filtered through wet muslin cloth by thorough squeezing and centrifuged to collect the clear supernatant. This supernatant was used for further analysis to estimate total reducing sugar by DNS method.

Enzymatic hydrolysis of pretreated maize stover using commercial cellulase supplemented with crude xylanase and crude α-L-arabinofuranosidase

The enzymatic hydrolysis of alkali (NaOH)-treated maize stover was performed using commercial cellulase (5.0 FPU/g), crude xylanase from *A. foetidus* MTCC 4898 (300 U/g), and crude α-L-arabinofuranosidase from *A. niger* ADH-11 (4.0 U/g) individually and cocktail of all. The reaction system was same as described in 'Enzymatic hydrolysis of maize stover' section. The hydrolysed products were analyzed by high-performance liquid chromatography (HPLC; Phenomenex, Rezex ROA-organic acid H+,

column; Phenomenex Inc, Torrance, CA, USA). The degree of synergy or synergy is defined as 'the ratio of the yield of product released by enzymes when used together to the sum of yield of these products when the enzymes are used separately in the same amounts as they were employed in the mixture'.

Results and discussion

α-L-Arabinofuranosidase production using different agro residues under SSF

The production of α-L-arabinofuranosidase was carried out using various agro residues like wheat bran, wheat straw, rice bran, rice straw, corn cobs, and maize bran as a sole carbon source and Mandels-Weber medium as a moistening agent at 30°C under SSF. It was observed that all six substrates supported production of α-L-arabinofuranosidase but wheat bran was found to be the most appropriate substrate under SSF, yielding a maximum yield of α-L-arabinofuranosidase up to 9.45 U/g after 192 h of incubation (Figure 1); hence, wheat bran was selected as the substrate for production of α-L-arabinofuranosidase. The biochemical composition of wheat bran indicates that it contains predominantly non-starch carbohydrate polymers (approximately 58%), starch (approximately 19%), and crude protein (approximately 18%). The non-starch carbohydrate polymers are being primarily approximately 70% arabinoxylans, approximately 24% cellulose, and approximately 6% β-(1,3) (1,4)-glucan [27]. The presence of higher amount of arabinoxylans in wheat bran may have induced α-L-arabinofuranosidase production. Relatively very few attempts have been reported on the production of these enzymes under SSF. Khandeparker et al. [28] reported wheat bran was the best substrate for α-L-arabinofuranosidase production by *Arthrobacter* sp. MTCC 5214 under SSF.

Optimization of α-L-arabinofuranosidase production under SSF using response surface methodology

Response surface methodology has been successfully applied for the optimization of fermentation medium components, conditions for enzymatic hydrolysis, and fermentation processes [29-32]. It predicts the maximum enzyme production among the selected range of variables and also studies the interaction among independent variables.

In the present study, the effect of urea, ammonium sulfate, proteose peptone, yeast extract, KH_2PO_4, $CaCl_2$, $MgSO_4$, pH, moisture ratio, and fermentation time was evaluated on the basis of α-L-arabinofuranosidase production using PBD (Table 1). Among these variables, urea, ammonium sulfate, KH_2PO_4, $CaCl_2$, $MgSO_4$, pH, and fermentation time were identified as the most significant and contributing variables (Table 3) for α-L-arabinofuranosidase production (Figure 2a). These parameters were further analyzed as variables using BBD for better production of α-L-arabinofuranosidase.

The result of 54 run BBD in six variables urea, ammonium sulfate, KH_2PO_4, $CaCl_2$, $MgSO_4$, pH, and fermentation time for the optimization of α-L-arabinofuranosidase production is shown in Table 5.

The experimental results suggest that the variables selected for the fermentation process had a strong effect on α-L-arabinofuranosidase production. On the basis of these experimental values, statistical testing was carried out using Minitab 16.0. The analysis of the model was tested by Fisher's *F* test and Student's *T* test. ANOVA of α-L-arabinofuranosidase production showed that the response surface model was significant ($P = 0.002$) (Table 6) due to the square portion of the regression model. A *P* value below 0.05 indicates that the test parameters are significant. In common, the larger the magnitude of *T* and smaller the value of *P*, more significant is the consequent coefficient term [33]. The fitted second-order response surface model as specified by Equation 2 for α-L-arabinofuranosidase yield in coded process variables is as follows:

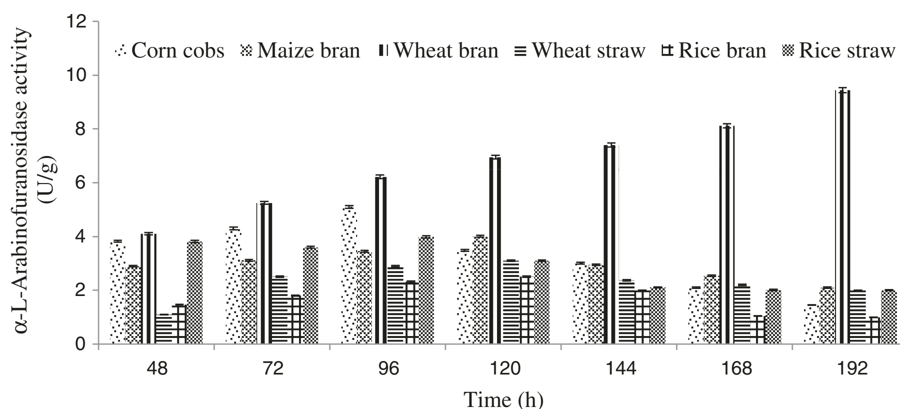

Figure 1 α-L-Arabinofuranosidase production using agro-residues under solid-state fermentation at 30°C.

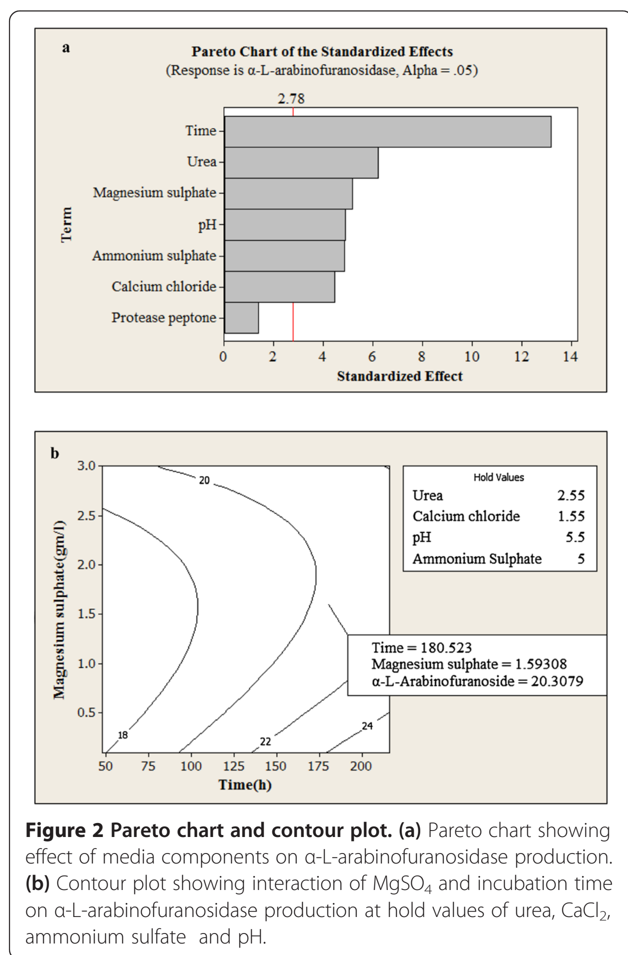

Figure 2 Pareto chart and contour plot. (a) Pareto chart showing effect of media components on α-L-arabinofuranosidase production. **(b)** Contour plot showing interaction of $MgSO_4$ and incubation time on α-L-arabinofuranosidase production at hold values of urea, $CaCl_2$, ammonium sulfate and pH.

$$Y = 18.9983 + 0.4454x_1 - 0.4446x_2 - 0.0054x_3 - 0.5050x_4 + 0.8204x_5 + 2.5971x_6 + 0.5010{x_1}^2 + 2.4422{x_2}^2 - 0.2028{x_3}^2 + 5997{x_4}^2 - 2.7403{x_5}^2 - 0.0628{x_6}^2 + 0.6138x_1x_2 + 1.8538x_1x_3 - 1.2113x_1x_6 + 0.7537x_2x_5 - 1.2925x_2x_6 + 0.6838x_3x_4 + 1.0675x_3x_5 - 0.2487x_4x_6 + 0.1500x_5x_6 \quad (3)$$

where Y is α-L-arabinofuranosidase yield square root of the predicted response, and x_1, x_2, x_3, x_4, x_5, and x_6 are the coded values of urea, $MgSO_4$, $CaCl_2$, pH, ammonium sulfate, and fermentation time, respectively. A comparison of the experimentally obtained values of the enzyme with the predicted values indicated that these data are in reasonable agreement with predictive values (Table 5).

The corresponding P value showed that the independent variable $MgSO_4$ (X2) and fermentation time (X6) had significant effect (0.003, 0.000) on α-L-arabinofuranosidase yield (Table 7). A significant interaction was also observed between urea (X1) and $CaCl_2$ (X3) and between pH (X4) and ammonium sulfate (X5) (0.034, 0.035) contributing to the response at significant level for α-L-arabinofuranoside production (Table 7). The R^2 value provides a measure of variability in the observed response value that can be explained by the experimental factors and their interactions. The coefficient R^2 of α-L-arabinofuranoside was 76.4%.

The fitted response for the above regression model was plotted in contour plot (Figure 2b) for the pairs of interactive variables while keeping other variables at their predicted optimum level. Among the variables, $MgSO_4$ (X2), $CaCl_2$ (X3), pH (X4), and fermentation time (X6) were the most significantly linear and had a positive effect on enzyme production. The significant interaction was observed between $MgSO_4$ (X2) and fermentation time (X6) for α-L-arabinofuranosidase production. Increasing the fermentation time (X6) up to certain level may lead to an increase in α-L-arabinofuranosidase yield, while increasing the $MgSO_4$ (X2) up to 2.55 g/l may lead to a drastic decrease in the α-L-arabinofuranosidase yield. $MgSO_4$ is considered as a good stimulator of mycelial growth which decreases the dormancy of the spores and can affect the enzyme yields [34]. Guerfali et al. [35] performed PBD followed by CCD and found that $MgSO_4$ played an important role in α-L-arabinofuranosidase production.

The application of RSM with BBD predicted that the maximum α-L-arabinofuranosidase production will be up to 20.30 U/g at decoded values of process parameters as $MgSO_4$ 1.59, urea 2.55, $CaCl_2$ 1.55, and ammonium

Table 6 ANOVA for the response surface quadratic model for α-L-arabinofuranosidase production

Source	Degree of freedom	Sequential sums of squares	Adjusted sums of squares	Adjusted mean squares	*F* value	*P* value
Regression	27	464.93	464.93	17.220	3.12	0.002
Linear	6	193.24	193.24	32.207	5.84	0.001
Square	6	123.23	123.23	20.539	3.73	0.008
Interaction	15	148.46	148.46	9.897	1.80	0.092
Residual error	26	143.28	143.28	5.511		
Lack-of-fit	21	127.27	127.27	6.061	1.89	0.249
Pure error	5	16.00	16.00	3.201		
Total	53	608.21				

Table 7 Regression coefficient for α-L-arabinofuranosidase production

Term	Coefficient	Standard error coefficient	*T* value	*P* value
Constant	18.9983	0.9583	19.824	0.000
Urea	0.4454	0.4792	0.930	0.361
$MgSO_4$	−0.4246	0.4792	−0.886	0.384
$CaCl_2$	−0.0054	0.4792	−0.011	0.991
pH	−0.5050	0.4792	−1.054	0.302
Ammonium sulfate	0.8204	0.4792	1.712	0.099
Fermentation time	2.5997	0.4792	5.420	0.000
Urea × Urea	0.5010	0.7319	0.684	0.500
$MgSO_4$ × $MgSO_4$	2.4422	0.7319	3.337	0.003
$CaCl_2$ × $CaCl_2$	−0.2028	0.7319	−0.277	0.784
pH × pH	0.5997	0.7319	0.819	0.420
Ammonium sulfate × Ammonium sulfate	−2.7403	0.7319	−3.744	0.001
Fermentation time × Fermentation time	−0.0628	0.7319	−0.086	0.932
Urea × $MgSO_4$	0.6138	0.8300	0.739	0.466
Urea × $CaCl_2$	1.8538	0.8300	2.234	0.034
Urea × pH	0.4800	0.5869	0.818	0.421
Urea × Ammonium sulfate	−0.6538	0.8300	−0.788	0.438
Urea × Fermentation time	−1.2113	0.8300	−1.459	0.156
$MgSO_4$ × $CaCl_2$	−1.2425	0.8300	−1.497	0.146
$MgSO_4$ × pH	0.6838	0.8300	0.824	0.418
$MgSO_4$ × Ammonium sulfate	0.7537	0.5869	1.284	0.210
$MgSO_4$ × Fermentation time	−1.2925	0.8300	−1.557	0.131
$CaCl_2$ × pH	1.6750	0.8300	2.018	0.054
Ammonium sulfate × Fermentation time	1.0675	0.8300	1.286	0.210
$CaCl_2$ × Ammonium sulfate	−0.2706	0.5869	−0.461	0.649
$CaCl_2$ × Fermentation time	−0.2487	0.8300	−0.300	0.767
pH × Ammonium sulfate	1.8425	0.8300	2.220	0.035
pH × Fermentation time	0.1500	0.8300	0.181	0.858

sulfate 5 g/l with initial pH 5.5 after incubation 180 h of fermentation time.

Validation of the experimental model

A repeat fermentation for α-L-arabinofuranosidase production was carried out under optimal conditions to validate the parameters predicted by the model. The α-L-arabinofuranosidase production under optimized parameters *viz.* $MgSO_4$ 1.59, urea 2.55, $CaCl_2$ 1.55, and ammonium sulfate 5 g/l at pH 5.5 and fermentation time of 180 h yielded α-L-arabinofuranosidase activity of 22.14 U/g (3.16 U/ml). This was significantly higher than the predicted value (20.30) by the model. Crude enzyme extract also contained 140 U/g (20.0 U/ml) endo-xylanase, 70.0 U/g (10.0 U/ml) β-xylosidase, and 0.14 U/g (0.02 U/ml) filter paper activity. Thus, it was proved that the statistical optimization increased α-L-arabinofuranosidase production by 2.34-fold as compared to their initial production which was 9.45 U/g (1.35 U/ml) before statistical optimization. Khandeparker [28] reported a maximum 3 U/g of α-L-arabinofuranosidase production after 120 h of incubation by *Arthrobacter* sp. MTCC 5214 under SSF. Guerfali et al. [35] reported a maximum 0.6 U/ml of α-L-arabinofuranosidase production after 120 h of incubation by *Talaromyces thermophilus* using response surface methodology under submerged fermentation. Hence, newly isolated *A. niger* ADH-11 was found to be a strong producer of α-L-arabinofuranosidase; however, the fermentation time was higher as compared to other reports.

Effect of temperature and pH on α-L-arabinofuranosidase activity

The influence of temperature on α-L-arabinofuranosidase activity was evaluated in the range of 40°C to 75°C. The results revealed that the optimum temperature for α-L-arabinofuranosidase activity was 55°C (Figure 3a). α-L-

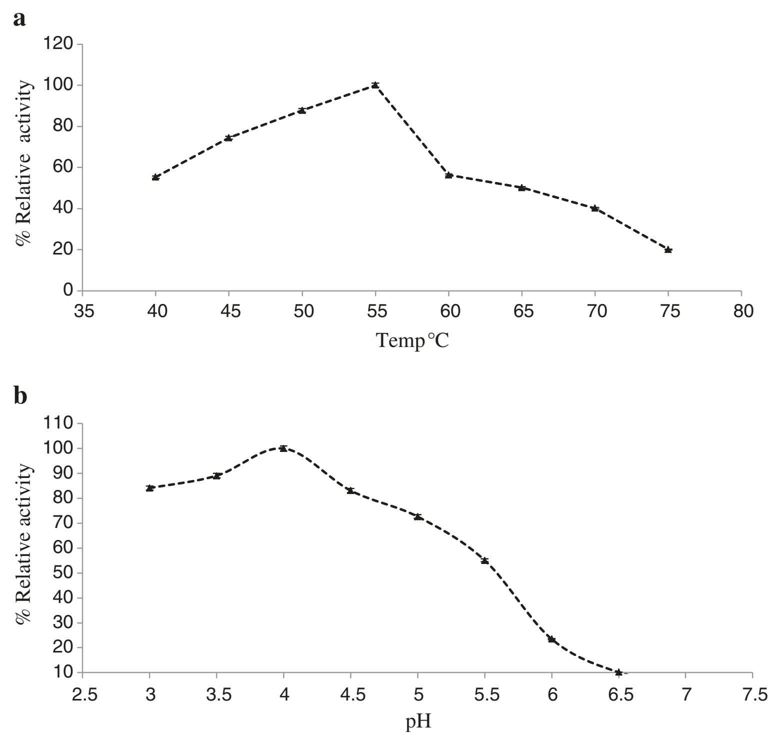

Figure 3 Effect of temperature (a) and pH (b) on α-L-arabinofuranosidase activity.

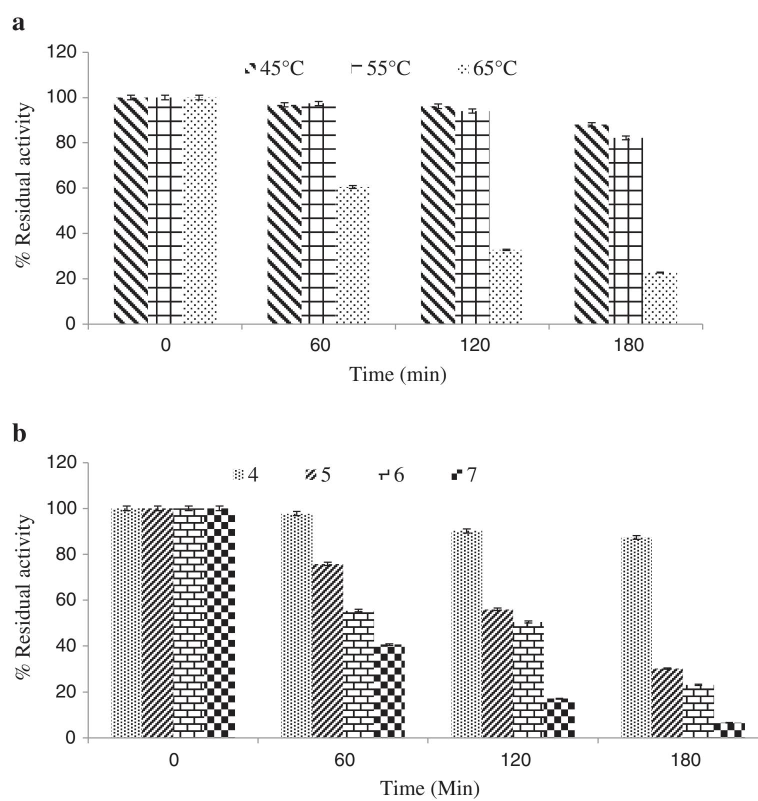

Figure 4 Thermal stability (a) and pH stability (b) of α-L-arabinofuranosidase.

Arabinofuranosidase activity was reduced by only 13% and 44% at 50°C and 60°C, respectively. The result was similar to many α-L-arabinofuranosidases from different strains of fungi [36]. Guerfali et al. [35] also reported that the optimum temperature of α-L-arabinofuranosidase from *Talaromyces thermophilus* was 55°C.

The α-L-arabinofuranosidase activity at various pH (3 to 7) was measured using *p*-nitrophenyl-α-L-arabinofuranoside as a substrate at 55°C. The optimum pH for α-L-arabinofuranosidase activity was found to be at 4 (Figure 3b). The enzyme was remarkably active even at pH 3.5 and 4.5, with loss of only 11% and 17% activities. Kaneko et al. [37] also found the optimum pH as 4.0 for α-L-arabinofuranosidase from *A. niger* 5-16. Most of the fungal α-L-arabinofuranosidases exhibited acidic pH in the range of 4.0 to 5.0. The enzyme activity is markedly affected by variation in pH outside its optimum pH. This may be due to substrate binding and catalysis, which are often affected by charge distribution on both substrate and enzyme molecules [38].

Temperature and pH stability of α-L-arabinofuranosidase

The α-L-arabinofuranosidase from *A. niger* ADH-11 was found to be highly stable in the range of 45°C to 55°C (Figure 4a). At 55°C the enzyme retained 82.38% of its activity after 3 h. Yan et al. [39] reported that α-L-arabinofuranosidase from *Chaetomium* sp. was stable at 55°C for 30 min. Guais et al. [40] found that α-L-arabinofuranosidase activity was dropped by 50% at 60°C after 12 min. The α-L-arabinofuranosidase could retain its activity up to 87.37% at pH 4 after incubation of 3 h (Figure 4b). The activity of α-L-arabinofuranosidase was gradually

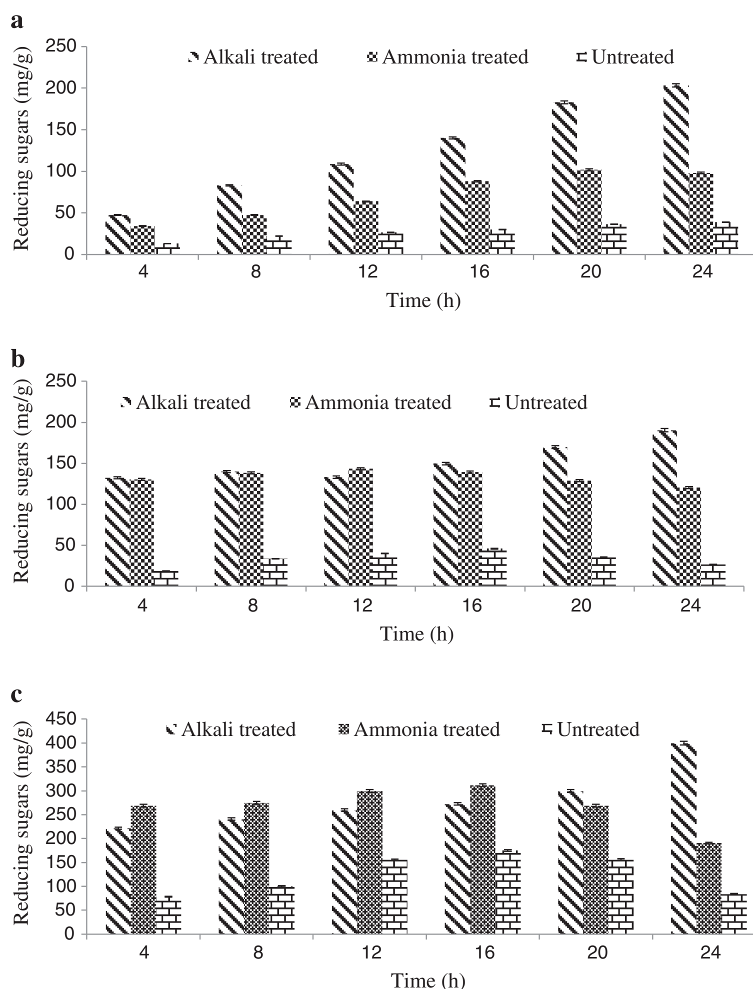

Figure 5 Enzymatic hydrolysis of untreated and alkali (NaOH)- and ammonia treated maize stover using different enzymes. (a) Enzymatic hydrolysis of untreated and alkali- and ammonia-treated maize stover using 4.06 U/g crude α-L-arabinofuranosidase at 50°C. **(b)** Enzymatic hydrolysis of untreated and alkali-, and ammonia-treated maize stover using 300 U/g crude xylanase at 50°C. **(c)** Enzymatic hydrolysis of untreated and alkali- and ammonia-treated maize stover using 5.0 FPU/g commercial cellulase at 50°C.

Table 8 Composition of enzymes in each cocktail

Enzyme	β-Xylosidase (U/ml)	α-L-Arabinofuranosidase (U/ml)	Feruloyl esterase (U/ml)	Xylanase (U/ml)	Filter paper activity (U/ml)	Endoglucanase (U/ml)	β-Glucosidase (U/ml)
Crude α-L-arabinofuranosidase[a]	10.0	3.16	1.0	20.0	0.02	5.56	0.18
Crude xylanase[b]	0.2	ND	ND	375.0	0.12	4.5	0.20
Commercial cellulase[c]	ND	ND	ND	300.0	43.9	2,798.0	43.0

[a]Crude α-L-arabinofuranosidase from *A. niger* ADH-11; [b]Crude xylanase from *A. foetidus* MTCC 4898; [c]Commercial cellulase MAPs 450. ND, not detected.

reduced to 30.21% and 23.07% at pH 5 and 6 after 3 h, respectively, while only 6.59% activity was retained at pH 7 after 3 h. Filho et al. [41] reported that α-L-arabinofuranosidases I and II from *Penicillium capsulatum* retained 50% activity at pH 4.0 after 9 and 17.5 min, respectively. Yan et al. [39] reported that α-L-arabinofuranosidase from *Chaetomium* sp. retained 80% activity at pH 4.0 after 30 min. In comparison to the above reports, the crude α-L-arabinofuranosidase from *A. niger* ADH-11 was found to be more stable at high temperature and low pH, so it can be suitable for saccharification of lignocellulosic biomass along with commercial cellulases.

Enzymatic hydrolysis of maize stover

The major conversion steps in biochemical production of cellulosic biofuels are pretreatment and enzymatic hydrolysis. Together, these steps work to breakdown the cellulose and hemicelluloses found in the cell walls of plants (biomass) to simple sugars which can then can be fermented to ethanol. Maize stover is currently the largest waste biomass in the India, consisting of more than one-third of the total solid waste produced. Maize stover typically contains 70% cellulose and hemicellulose and 15% to 20% lignin. Ammonia and NaOH-pretreated maize stover were used to test the ability of the crude α-L-arabinofuranosidase, crude xylanase, and commercial cellulase for the production of fermentable sugar by saccharification. Maximum reducing sugars of 203.6 mg/g were produced using crude α-L-arabinofuranosidase, while crude xylanase and commercial cellulase yielded 190.7 and 400.0 mg/g respectively using NaOH-pretreated maize stover (Figure 5a,b,c). A higher yield of reducing sugars was achieved when the substrate was pretreated with NaOH as compared to ammonia.

The maximum bioconversion of lignocellulosic biomass requires action of not only efficient cellulolytic enzymes but also main chain depolymerizing and debranching hemicellulolytic enzymes at appropriate levels. It is evident from Table 8 that the commercial enzyme used in this study was deficient in β-xylosidase, α-L-arabinofuranosidase, and feruloyl esterase and hence, the indigenously produced crude α-L-arabinofuranosidase was supplemented to form a balanced enzyme cocktail for saccharification of alkali-pretreated maize stover. There was a remarkable increase in β-xylosidase, α-L-arabinofuranosidase, feruloyl esterase, endo-xylanase, and β-glucosidase loading upon supplementation of crude α-L-arabinofuranosidase to commercial cellulase-crude xylanase cocktail (Table 9). As shown in Figure 6, the cocktail produced maximum 730.1 mg/g of reducing sugar after 24 h which was higher as compared to each enzyme used alone (crude α-L-arabinofuranosidase 203.6 mg/g, crude xylanase 134.4 mg/g, and commercial cellulase 294.1 mg/g, respectively) for saccharification. The co-action of crude α-L-arabinofuranosidase with commercial cellulase-crude xylanase produced a superior saccharification yield. The HPLC analysis of enzymatic hydrolysates revealed synergistic rise in glucose, xylose, and arabinose (degree of synergy 1.21, 1.00, and 1.33, respectively). The levels of glucose, xylose, and arabinose were 5.57, 4.99, and 0.64 mg/ml when the cocktail was used (Table 10). Thus, the supplementation of crude α-L-arabinofuranosidase (4.0 U/g) with commercial cellulase and crude xylanase increased the glucose yield up to 176.8% and xylose yield up to 166.8% (Table 10). Although supplementation of crude xylanase with commercial cellulase increased the

Table 9 Enzyme loading for saccharification of pretreated maize stover

Enzyme	β-Xylosidase (U/g)	α-L-Arabinofuranosidase (U/g)	Feruloyl esterase (U/g)	Xylanase (U/g)	Filter paper activity (U/g)	Endoglucanase (U/g)	β-Glucosidase (U/g)
Crude α-L-arabinofuranosidase[a]	12.7	4.0	1.27	25.4	0.025	7.06	0.22
Crude xylanase[b]	0.16	ND	ND	300	0.09	3.6	0.16
Commercial cellulase[c]	ND	ND	ND	33.0	5.0	307.7	4.78

[a]Crude α-L-arabinofuranosidase from *A. niger* ADH-11; [b]Crude xylanase from *A. foetidus* MTCC 4898; [c]Commercial cellulase MAPs 450. ND, not detected.

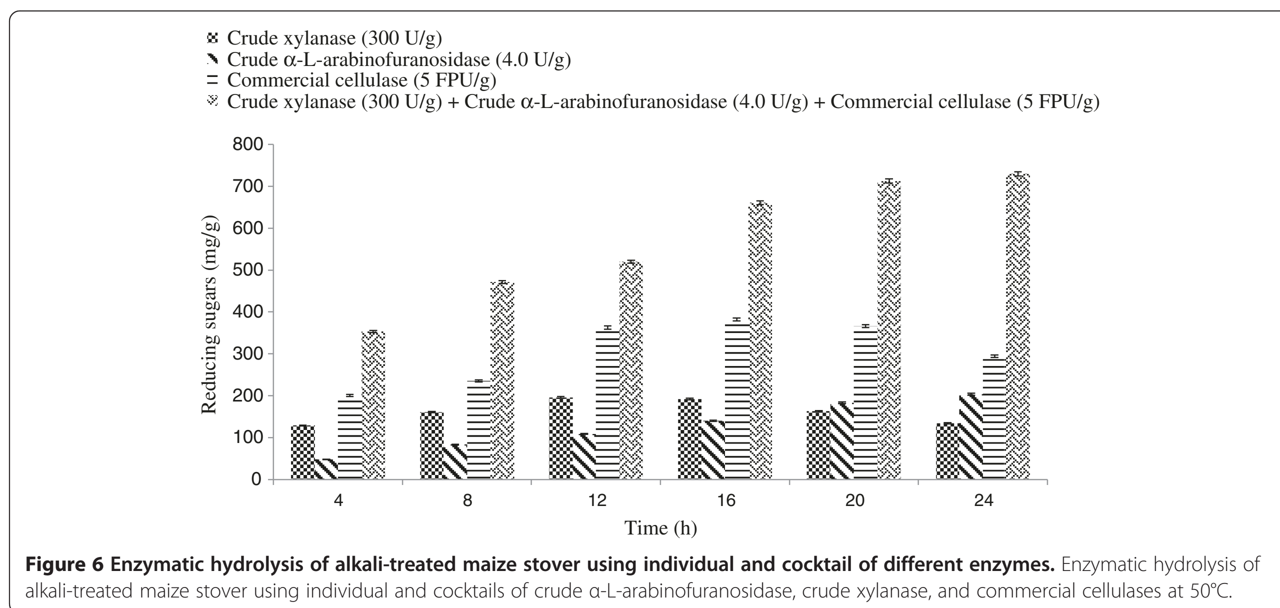

Figure 6 Enzymatic hydrolysis of alkali-treated maize stover using individual and cocktail of different enzymes. Enzymatic hydrolysis of alkali-treated maize stover using individual and cocktails of crude α-L-arabinofuranosidase, crude xylanase, and commercial cellulases at 50°C.

loading of endoglucanase, β-glucosidase, and endoxylanase (Table 9), the yield of glucose, xylose, and arabinose was lesser compared to the cocktail of crude α-L-arabinofuranosidase (4.0 U/g) with commercial cellulase (5 FPU/g) and crude xylanase (300 U/g) cocktail. These results clearly suggest that the higher yield of reducing sugars obtained by this cocktail was due to higher amount of α-L-arabinofuranosidase and β-xylosidase enzymes which were contributed by the crude α-L-arabinofuranosidase produced by *A. niger* ADH-11. The presence of α-L-arabinofuranosidase plays an important role by removing arabinose from the side chains of xylan, and β-xylosidase plays a crucial role in reducing the end product inhibition of xylanases by hydrolyzing xylobiose and hence facilitates better bioconversion of maize stover. A similar observation was reported by Delabona et al. [42]. Their studies on sugarcane bagasse strongly suggested that the supplementation of α-L-arabinofuranosidase with commercial cellulase resulted in greater sugar release. The present study clearly demonstrated that the crude α-L-arabinofuranosidase-rich enzyme obtained from *A. niger* ADH-11 can be used to enhance the efficiency of commercial cellulase for saccharification of biomass.

Table 10 Monomer sugar composition of each cocktail used for enzymatic saccharification of NaOH-treated maize stover

Enzyme cocktails	Glucose (mg/ml)	Xylose (mg/ml)	Arabinose (mg/ml)
Commercial cellulase (5 FPU/g)	2.98	1.69	ND
Crude α-L-arabinofuranosidase (4.0 U/g)	0.69	1.59	0.48
Crude xylanase (300 U/g)	0.93	1.70	ND
Commercial cellulase (5 FPU/g) + Crude xylanase (300 U/g)	3.15	2.99	ND
Commercial cellulase (5 FPU/g) + Crude α-L-arabinofuranosidase (4.0 U/g) + Crude xylanase (300 U/g)	5.57	4.99	0.64

ND, not detected.

Conclusions

The present investigation showed that newly isolated *A. niger* ADH-11 is a potential α-L-arabinofuranosidase producer. The statistical optimization for α-L-arabinofuranosidase production by SSF was highly advantageous as enzyme production was enhanced by 2.34-fold. To the best of our knowledge, this report describes the highest level of α-L-arabinofuranosidase production under SSF. The crude enzyme was highly active and stable at 55°C and pH 4.0, which makes it suitable for application in enzymatic saccharification of biomass. Moreover, it was evident from the present investigation that the supplementation of crude α-L-arabinofuranosidase can play significant role in the enzymatic hydrolysis of maize stover.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HP designed and performed the experiments along with the data analysis and organized the manuscript draft. JD designed and analyzed the statistical data for optimization of enzyme production. DC helped in designing the experiments and preparing the manuscript draft. AS supervised the complete study along with experimentation, interpretation, and manuscript preparation. All authors read and approved the final manuscript.

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