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Degradation of cereal bran polysaccharide-phenolic acid complexes by *Aspergillus niger* CFR 1105 ☆

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Abstract

Cereal brans such as wheat and rice are abundant sources for obtaining bioactive phenolic compounds such as ferulic and coumaric acids, in turn these can be bio-transformed into high value flavour compounds such as vanillin. *Aspergillus niger* CFR 1105, found to induce greater amounts of cell wall degrading enzymes, was inoculated into wheat and rice brans, and grown for 24, 48, 72 and 96 h to understand the degradation pattern of non-starch polysaccharides and phenolic acid complexes. Native wheat bran polysaccharides mainly consisted of arabinose, xylose, galactose, and glucose, in % of 27:39:2:30, with traces of mannose, whereas rice bran consisted of arabinose, xylose, galactose, and glucose in % of 9:27:31:32, respectively. Both the cereal bran arabinoxylans were degraded extensively by *A. niger* at 96 h, whereas the degradation of 1,3/1,4-β-D-glucans and cellulose was negligible. Bound phenolic acids of rice and wheat brans identified by HPLC were found to be mainly ferulic, coumaric, syringic acids in % of 93.6:6.3:0 (wheat), 34.7:55.8:9.5 (rice) and were drastically degraded/utilized at 96 h. The above results have indicated preferential degradation of arabinoxylans of wheat bran by *A. niger*, which can be exploited to obtain bioactive compounds such as ferulic acid.

Keywords: Non-starch polysaccharides; Arabinoxylans; Ferulic acid; Vanillin; Fungal transformation

1. Introduction

Plant cell wall polysaccharides are the most abundant organic compounds found in nature. They can be divided into three groups: cellulose, hemicellulose and pectin (McNeill, Darvill, Fry, & Albersheim, 1984). The major hemicellulose polymer in cereals is arabinoxylan. It consists of a xylan backbone, containing β-1,4-linkages, with side chains of α-L-arabinose substituted either at C-2/C-3 (Wilde & Woo, 1977) and 4-*O*-methyl D-glucuronic acid (Brillouet & Joseleau, 1987). The arabinose side chains are substituted at the 5′-hydroxyl group with ferulic acid (4-hydroxy-3-methoxy cinnamic acid), an antioxidant (Saulnier, Marot, Elg orriaga, Bonnin, & Thibault, 2001) which is an ideal potential precursor for the universally used flavour vanillin (4-hydroxy-3-methoxy benzaldehyde) (Falconnier et al., 1994; Gasson et al., 1998). Wheat bran contains (%): Polysaccharides, 66.0; Lignin, 12.0; protein, 6.0; ash, 4.0; Moisture, 12.0 (Ring & Selvendran, 1980). Rice bran contains (%): polysaccharide, 62.4; Lignin, 12.0; protein, 9.0; ash, 2.7; Moisture, 15.0 (Maningat & Juliano, 1982). Ferulic acid comprises of about 0.5–1% w/w dry matter of wheat bran (Hartley & Jones, 1977), 0.9% w/w dry matter of rice bran (Shibuya, 1984) and 3% of maize bran

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(Saulnier, Vigouroux, & Thibault, 1995). Rice and wheat brans are abundant in India and serve as ideal sources for the production of ferulic acid, which can be converted into vanillin by fungi (Lesage-Meessen et al., 1996).

Among microorganisms known for their ability to produce plant cell wall degrading enzymes, fungi are the most interesting group (Maria, Bieley, & Vrsanska, 1989). The genus *Aspergillus* is a group of filamentous fungi with a large number of species. Black aspergilli such as *Aspergillus niger* and *Aspergillus tubingensis* are the most important for industrial applications due to their good fermentation capabilities, high levels of protein secretion, wide range of cell wall degrading as well as phenolic acid esterase enzymes (De Vries & Visser, 2001; De Vries, 2003).

In a recent study we have evaluated *A. niger* CFR 1105, *Aspergillus oryzae* CFR 232 and *Rhizopus arrhizus* NICM 997 for the induction of cell wall degrading enzymes, and among the three, *A. niger* was found to be the best with respect to the induction of starch/cell wall degrading enzymes (Kavitha, Shyamala, Muralikrishna, Varadaraj, & Rati Rao, in press). In this communication, we have evaluated and compared the degradation of cell wall polysaccharide-phenolic acid complexes of wheat and rice brans by *A. niger* which would allow us to choose the appropriate bran for the degradation studies by *A. niger*.

2. Materials and methods

2.1. Materials

Cereal brans such as rice and wheat were obtained in bulk quantity from a local market. All the chemicals and sugar standards were of analytical grade. Phenolic acid standards such as caffeic, coumaric, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids, and Amberlite (IR 120-H^+) were purchased from Sigma Chemical Company, St. Louis, MO, USA. GC (OV-225) and HPLC (Shimpak C-18) columns were obtained from Pierce Chemical Company, Rock ford, IL, USA and Shimadzu Corporation, Tokyo, Japan, respectively.

2.1.1. Fungal strain and preparation of inoculum

The culture of *A. niger* CFR 1105 was obtained from the stock culture collection of Food Microbiology Department, CFTRI, Mysore, India. The culture was maintained at 6 °C on malt extract agar, with regular subculturing at an interval of 30 days. Inoculum of *A. niger* CFR 1105 was prepared by harvesting the spores from a 5-day old malt extract agar grown slant with 20 ml of sterile water. The spore count of the suspension was determined by plating on malt extract agar, which showed 1×10^8 spores/ml.

2.2. Methods

2.2.1. Medium and culture conditions

The medium used in the present study includes wheat and rice brans. Quantities of 10 g each of wheat and rice brans taken in 500 ml Erlenmeyer conical flasks, to which were added 10 ml distilled water and autoclaved at 121 °C for 30 min. The sterile medium was cooled to ambient temperature and inoculated with 1 ml spore suspension of *A. niger* CFR 1105 containing 1×10^8 spores and incubated at 30 °C for a period of 96 h. Growth and biochemical parameters were arrested at intervals of 24, 48, 72, and 96 h and for each incubation period, a separate set of flasks with medium were used. Inoculated and uninoculated bran samples were designated as experimental and native, respectively. Moisture content of both native and experimental bran samples of varying incubation periods was determined by established procedure (AOAC, 2000).

2.2.2. Characterization of non-starch polysaccharides (NSP)

Twenty milligram quantities of uninoculated and *A. niger* grown wheat and rice bran samples of varying incubation periods were suspended in water (0.5 ml) and solubilized with concentrated sulphuric acid (0.6 ml) at ice cold temperature, after which the concentration of sulphuric acid was brought down to 8% by the addition of water. The above mixture was refluxed in a water bath for 10–12 h; neutralized with barium carbonate, filtered, concentrated, deionized with Amberlite (IR 120-H^+) and reduced with sodium borohydride. Alditol acetates were prepared according to the method of Sawardekar, Slonekar, and Jeanes (1965). The component sugars were separated and identified on a 3% OV-225 ($1/8'' \times 6''$) column using a Shimadzu 14-B gas liquid chromatograph equipped with flame ionization detector.The parameters used in GC analysis were 200 °C column temperature and 250 °C injector and detector port temperatures. Nitrogen (40 ml/min) was used as carrier gas. A sugar mixture consisting of rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose was used as reference and inositol as internal standard.

2.2.3. Isolation and characterization of bound phenolic acids

Bound phenolics were extracted according to the method of (Nordkvist, Salomonsso, & Aman, 1984). One gram quantity of uninoculated and *A. niger* grown wheat and rice bran samples of varying incubation periods were defatted with chloroform and methanol (3×50 ml) in the ratio of 2:1. Sugars and free phenolics were removed with 70% alcohol (3×50 ml). The samples were dried sequentially with 80%, 90%, absolute alcohol and finally with diethyl ether. The dried samples were extracted with 1 M NaOH (2×100 ml, 2 h each)

containing 0.5% sodium borohydride under nitrogen atmosphere and the clear supernatants were collected upon centrifugation and processed as described earlier (Subba Rao & Muralikrishna, 2001) and analyzed on a C-18 HPLC column (4.6 × 250 mm) using a diode array detector (operating at 280 and 320 nm) and a solvent system of water:acetic acid:methanol (isocratic, 80:5:15). For identification and quantification of phenolic acids present in the samples, standards such as protocatechuic, syringic, gentisic, vanillic, caffeic, ferulic and coumaric acids were used.

2.2.4. Isolation and characterization of free phenolic acids

One gram quantity of uninoculated and *A. niger* grown wheat and rice bran samples of varying incubation periods were defatted as mentioned in the case of bound phenolic acids and extracted with 70% ethanol (3 × 50 ml, 2 h each); and the supernatants obtained by centrifugation were concentrated, and their pH was adjusted in the range of 2–3 with 4 N HCl. Phenolic acids were separated by ethyl acetate phase separation (3 × 50 ml), and analyzed by HPLC as mentioned in the case of bound phenolic acids (Subba Rao & Muralikrishna, 2002).

2.2.5. Assay of ferulic acid esterase (EC 3.1.1.73)

One gram quantity of uninoculated and *A. niger* grown wheat and rice bran samples of varying incubation periods were extracted with extraction medium (6.6 ml); containing tris (hydroxymethyl) aminomethane hydrochloride, 50 mM, pH 8.0; reduced glutathione, 25 mM; Triton-X-100, 1% (wt\vol); polyvinylpolypyrrolidone, 0.2 g for 2 h at 4 °C, centrifuged, dialyzed against tris buffer (50 mM, 8.0 pH). Ferulic acid esterase activities were assayed using ethyl ferulate as the substrate, incubating at 37 °C for 1 h (Donaghy, Kelly, & McKay, 1999). Reaction was stopped by the addition of methanol. Ferulic acid released was analyzed and quantified by HPLC using C-18 column (4.6 × 250 mm) using a diode array detector and a solvent system of water:acetic acid:methanol (isocratic, 70:5:25).

Enzyme activity was defined as the increase in ferulic acid concentration per hour (μmol/h) (Humberstone & Briggs, 2000).

All experiments were carried out in duplicates and the results given are the average of the two.

3. Results and discussion

3.1. Degradation of non-starch polysaccharides

The major sugars identified in the non-starch polysaccharides (NSP) in native and experimental samples of wheat and rice brans were arabinose, xylose, galactose and glucose. Mannose and rhamnose are present as minor constituents (Table 1).

Native wheat bran polysaccharides mainly consisted of arabinose (27%), xylose (39%), galactose (2%) and glucose (30%) with traces of mannose. As against the native wheat bran, in the experimental samples, there was 9- and 19-fold decrease in the arabinose and xylose contents, respectively, in a 96 h growth period. Similarly a marginal decrease was observed in the rhamnose/fucose content (Table 1).

The substantial decrease in the arabinose and xylose content indicates the degradation of arabinoxylan backbone of the wheat bran polysaccharide. This is in good agreement with our earlier work pertaining to the various levels of cell wall degrading enzymes, wherein, xylanase activity is found to be higher, indicating extensive degradation of the xylan backbone (Kavitha et al., in press).

Native rice bran consisted of arabinose (9%), xylose 27 (%), galactose (30%) and glucose (32%). More than 4-fold decrease was observed in the arabinose content in the experimental sample of rice bran, whereas xylose was degraded by 13-fold. These results indicated extensive degradation of arabinoxylans rather than hexosans during the growth of the fungus, which can be correlated with higher activity of xylanase (Kavitha et al., in press). There was a significant amount of glucose in the native

Table 1
Comparison of degradation of non-starch polysaccharides by *Aspergillus niger* grown on wheat and rice brans as determined by GLC analysis

Sugar	Wheat bran					Rice bran				
	Native	24 h	48 h	72 h	96 h	Native	24 h	48 h	72 h	96 h
Rha/Fuc	1	6	1	3	ND	1	1	1	5	3
Ara	27	8	7	4	3	9	7	7	4	2
Xyl	39	11	5	3	2	27	10	12	4	2
Man	1	ND	2	3	2	ND	2	7	7	1
Gal	2	ND	9	14	8	31	35	34	3	7
Glu	30	75	76	73	85	32	45	39	77	85

Sugars are represented in %.
ND, not detectable.
Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, Galactose; Glu, glucose.

bran as well as in all experimental samples, which originated from cellulose (Bonnin et al., 2002) and β-D glucan (Brillouet, Joseleau, Utille, & Lelievre, 1982) and starch.

In the above experiments, the mass utilized at different time intervals of *A. niger* are as follows: wheat bran → 24 h – 1%; 48 h – 6.3%; 72 h – 17%; 96 h – 17% and rice bran → 24 h – 6.3%; 48 h – 7.3%; 72 h – 11.2%; 96 h – 25.8%. The % of arabinose and xylose remaining in the 96 h experimental sample compared to the control is as follows: wheat bran → arabinose – 11%; xylose – 5%. Rice bran → arabinose – 22%; xylose – 12%.

It can be concluded that arabinoxylan degrading enzymes (pentosanases) are induced, which is prerequisite for the induction of esterase to cleave ferulic acid from the cell walls. Increase in esterase activity was directly influenced by the rate of polysaccharide degradation, which also dependent on the activity of the xylanase (Faulds, Bartolome, & Williamson, 1997).

3.2. Changes in bound phenolic acids

Ferulic and coumaric acids are the major bound phenolic acids present in the native wheat bran. However, rice bran in addition to the above phenolic acids, consisted of substantial amounts of syringic acid. Ferulic acid decreased by 8- and 4-fold in the experimental samples of wheat and rice brans respectively at 96 h. A 7-fold decrease in the content of bound coumaric acid was observed in the experimental sample of wheat bran at 96 h. A 2-fold decrease was observed with respect to coumaric acid in the experimental sample of rice bran at 24 h. Thereafter the degradation was not substantial. This may be perhaps due to the inter-conversion of liberated ferulic acid to coumaric acid at 72 and 96 h. Decrease in bound syringic acid was substantially less than ferulic and coumaric acids. Degradation of these bound phenolic acids clearly indicates the induction of various phenolic acid esterase(s) (Table 2). Degradation of bound ferulic acid can be correlated with maximum activity of ferulic acid esterase in the 72 h experimental sample of wheat bran (Fig. 1).

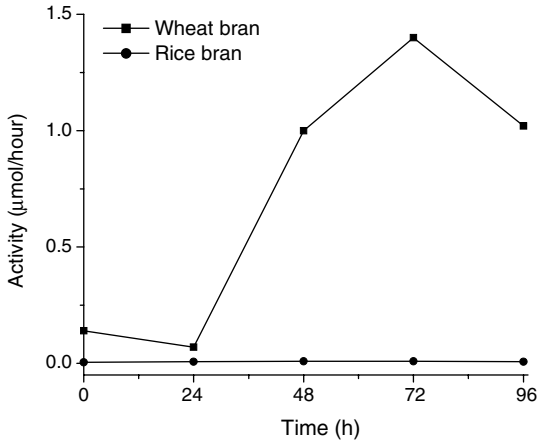

Fig. 1. Activity of ferulic acid esterase in samples of wheat and rice brans grown with *A. niger* CFR 1105 for varying incubation periods.

3.3. Changes in free phenolic acids

Wheat bran consisted of both benzoic and cinnamic acid derivatives. Gallic, protocatechuic and vanillic acids were present in the ratio of 2.2:3:1. Cinnamic acid derivatives such as coumaric and ferulic acids are present in 1:3 ratios. Gallic and protocatechuic acids are completely degraded in 96 h experimental sample. Coumaric acid is almost completely degraded at 96 h, whereas the decrease in ferulic acid content at 96 h was negligible. A direct correlation with the decrease in the bound ferulic acid with a concomitant increase in the free phenolic acids could not be correlated from these results. This may be perhaps due to the oxidation or interconversion of benzoic as well as cinnamic acid derivatives (Fig. 2(a)).

Rice bran exclusively consisted of benzoic acid derivatives such as gallic, protocatechuic and vanillic acids. Cinnamic acid derivatives are completely absent. Gallic and protocatechuic acids are completely degraded after 24 h of incubation of *A. niger* grown on rice bran. At 96 h, a 3-fold increase was observed with respect to vanillic acid. Gallic and protocatechuic acids are completely disappeared in the experimental samples of rice bran (Fig. 2(b)).

From the above studies, it was clear that there is a preferential degradation of arabinoxylans rather than

Table 2
Degradation of bound phenolic acids by *A. niger* in the experimental samples of wheat and rice brans

Phenolic acid	(mg/100 g sample on dry weight basis)									
	Wheat bran					Rice bran				
	Native	24 h	48 h	72 h	96 h	Native	24 h	48 h	72 h	96 h
Ferulic acid	227.8	133.2	60.1	35.0	30.1	179.1	64.8	39.2	44.0	37.8
p-Coumaric acid	15.3	15.1	7.4	6.2	2.1	287.5	164.7	142.4	146.1	143.8
Syringic acid	ND	ND	ND	ND	ND	49.0	48.6	46.5	44.5	40.4

ND, not detectable.

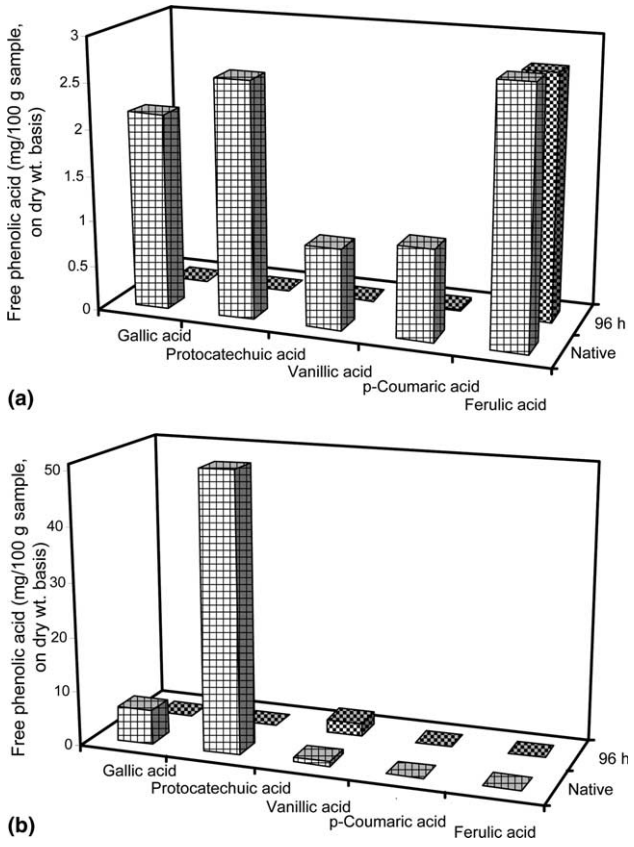

Fig. 2. Free phenolic acids in native and 96 h grown samples of wheat (a) and rice (b) brans with *A. niger* CFR 1105.

hexosans by *A. niger*. Substantial decrease in bound ferulic acid was also noticed in experimental samples of wheat and rice brans, indicating the possible induction of ferulic acid esterase. The amount of free ferulic acid was comparatively very low in rice bran compared to wheat bran. All the free phenolic acids were degraded in the 96 h experimental sample.

4. Conclusion

Our work proved wheat bran as a better substrate for the growth of *A. niger* with respect to the induction of arabinoxylan degrading enzymes as well as phenolic acid esterases as compared to rice bran. The degradation of cereal bran polysaccharide-phenolic acid complexes can be exploited to obtain bioactive compounds such as ferulic acid as well as xylooligosaccharides for various end uses.

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