Romanian Biotechnological Letters *Vol. 15, No.1, 2010*
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ORIGINAL PAPER

Optimization of culture conditions, partial purification and characterization of a new lectin from *Aspergillus nidulans*

Received for publication, August 20, 2009
Accepted, January 30, 2010

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Abstract

The effect of culture conditions on lectin activity of Aspergillus nidulans was determined. Higher lectin activity was observed in liquid medium as compared to mycelia from solidified agar cultures. Agitation mode of cultivation resulted in pellet formation of hyphal mass exhibiting only marginal lectin activity. Maximum lectin activity was detected in cultures growing at neutral pH and 30°C temperature for 7 days under stationary condition. Lectin was partially purified using $(NH_4)_2SO_4$ precipitation followed by dialysis and ultrafiltration. Lectin was precipitated at 50% saturation of $(NH_4)_2SO_4$. Ultrafiltration yielded 48% of the total activity with the specific activity of 46.04 ± 1.50 titre mg^{-1}. Partially purified lectin was characterized for specific agglutination of human type A, B, AB, O erythrocytes as well as rat, mice, sheep, goat and pig erythrocytes, and also lymphocytes, splenocytes and microbial cells. Agglutination was also tested with neuraminidase- and protease-treated erythrocytes. Lectin exhibited strong binding affinity to L-fucose, inulin, thiodigalactoside, mucin and asialofetuin.

Keywords: lectin, *Aspergillus nidulans*, agglutination, purification, carbohydrate specificity

Introduction

Lectins are polyvalent or univalent oligomeric proteins of non-immunoglobulin nature, playing an important role in cell recognition (SINGH et al. [1]). These bind reversibly with certain sugars and precipitate polysaccharides, glycoproteins and glycolipids bearing specific carbohydrate moieties on the apposing cells (TIWARY and SINGH [2]). This specificity of lectin-carbohydrate interaction has been exploited for targeting drugs to specific sites (TIWARY and SINGH [3]; BIES et al. [4]). The differential expression of lectins in normal and diseased tissues has served as clinical markers of cell differentiation and disease characterization (MURAMATSU [5]). Haemagglutination, which exploits the specific interaction of lectins with carbohydrates present on the surface of erythrocytes, is the most common method to determine the presence of lectins in any biological source (LIS and SHARON [6]).

Lectins are widely distributed in nature and have been reported in a variety of organisms including viruses, bacteria, fungi, invertebrates, vertebrates and plants (SINGH et al. [1]). Amongst the fungi, there are several reports on lectins in fruiting bodies (COOPER et al. [7]; YOSHIDA et al. [8]; KANEKO et al. [9]), but there are fewer reports on mycelial lectins (ODA et al. [10]; Rosen et al. [11]). Extracellular lectins have been reported in culture

filtrates of *Lentinus edodes* (TSIVILEVA et al. [12]) and *Sclerotium rolfsii* (INBAR and CHET [13]). Fungal lectins have been proposed to mediate interactions in fungal pathogenesis and mycoparasitism (MANOCHA and CHEN [14]). Fucose-containing glycoconjugates on cell surfaces are known to play an important role in cell-cell recognition and signaling. Sialyl lewis X on endothelial cells is a ligand for binding of selectins, which are involved in transport of leukocytes to the site of infection (LASKY [15]). Fucosyl glycoconjugates such as H-blood antigens on tumor cells modulate cell growth, invasion and metastasis (GORELIK et al. [16]). Fucose-specific lectins have been identified in mushroom *Aleuria aurantia* (KOCHIBE AND FURUKAWA [17]) and fungus *Rhizopus stolonifer* (ODA et al. [10]). Amongst the genus *Aspergillus*, fucose-specific lectins have been reported in *A. fumigatus* (ISHIMARU et al. [18]) and *A. oryzae* (MATSUMARA et al. [19]). As this vast genus was not much explored for the presence of lectins, earlier in a preliminary study our group screened 10 species of *Aspergillus* for lectin activity (SINGH et al. [20]). Four of them were found to possess lectins, all of which were specific for L-fucose. The present work was aimed to study the lectin activity of *Aspergillus nidiulans* under varied culture conditions and to partially purify the lectin for its characterization.

Materials and Methods

Organism and culture conditions

Fungal strain of *Aspergillus nidulans* MTCC 344 was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The culture was maintained on czapek agar slants containing czapek concentrate 1.0% (v/v), yeast extract 0.5% (w/v), K_2HPO_4 0.1% (w/v), sucrose 3.0% (w/v) and agar 3.0% (w/v), stored at 4 °C and subcultured at fortnight intervals. The culture was grown on solidified agar plates and in 250 ml Erlenmeyer's flasks containing 50 ml of the same liquid medium without agar, incubated at 30 °C for 10 days under stationary condition.

Optimization of culture conditions to enhance the lectin activity by *A. nidulans*

The effect of various culture conditions on the amount of lectin expressed was determined by inoculating a culture disc of 5 mm diameter per 50 ml of the medium and incubated at 30 °C for 10 days. The mycelium recovered from each of the medium was assayed for lectin activity. The pH of the medium was adjusted to 5.5-7.5 to determine its effect on lectin activity. To investigate the effect of temperature, cultures were grown at 25-35 °C. To determine the mode of cultivation in liquid medium, fungus was grown under stationary and agitation (150 rpm) conditions. The lectin activity was also determined as a function of culture age (3-14 days).

Preparation of fungal extract

The mycelium was recovered from solidified agar plates as well as from broth cultures. The mycelium from the surface of solidified agar plates was scrapped free of agar while mycelium from broth cultures was harvested by filtration, washed extensively with phosphate buffered saline (PBS, 0.1 M, pH 7.2) and briefly pressed dry. Extracts were prepared by homogenizing the recovered mycelium in PBS in the ratio 1:1.5 at high speed (22,000 rpm) for 3-5 min in an ice bath using a homogenizer (ULTRA-TURRAX® T25 BASIC IKA®-WERKE) and further ground in pestle and mortar with acidified river sand (40-200 mesh EP, SD FINE-CHEM LTD., INDIA) for 25 min on an ice bath as described earlier (SINGH et al. [20]). The extract was centrifuged (3,000 × g, 20 min, 4°C) and supernatant obtained was assayed for lectin activity.

Partial purification of lectins

Lectin was partially purified by ‘Salting out’ technique, using ammonium sulfate followed by dialysis and ultrafiltration. Ammonium sulfate is the salt of choice for precipitation due to its salting out effectiveness, low heat of solution and stabilizing effect on proteins. To the mycelial extract recovered after centrifugation, ammonium sulfate (10-100%) was added in small fractions with constant stirring on an ice bath. It was kept undisturbed overnight at 4°C. The resultant sample was centrifuged (3000 × g, 20 min, 4°C). The pellet obtained was dissolved in PBS (0.1M, pH 7.2) and dialysed extensively against PBS for 24 h using Snake Skin Dialysis tubing (10 KDa, PIERCE BIOTECH, ROCKFORD, USA). The dialysate was further concentrated using Amicon ultrafiltration centrifugal device (cutoff 10 KDa, MILLIPORE, USA). Lectin activity and protein content was estimated at each step (LOWRY et al. [21]).

Preparation of cell suspension

Erythrocyte suspension was prepared as described earlier (SINGH et al. [20]). For treatment of erythrocytes with neuraminidase (0.2 U ml,$^{-1}$ SIGMA TYPE V from *Clostridium perfringens*) and protease (2 mg ml,$^{-1}$ ICN BIOMEDICALS INC., USA, from *Streptomyces aureus*), one ml of washed 10% erythrocyte suspension was mixed with an equal volume of either enzyme and incubated at 37°C for 60 min (MENG et al. [22]). Reaction was stopped by adding excess of PBS and the reaction mixture was centrifuged (400 × g, 5 min, 4°C). Erythrocyte pellet obtained was washed five times in PBS to remove any traces of enzyme and the erythrocyte pellet was resuspended to a final concentration of 2% (v/v) in PBS.

Lymphocyte suspension was prepared by mixing 5 ml of heparinized blood with an equal volume of normal saline and layering it onto histopaque (SIGMA, USA) in the ratio 3:1. Upon centrifugation at 400 × g for 20 min, an opaque ring of lymphocytes formed at the interface of plasma and histopaque, was aspirated carefully and treated with 0.82% ammonium chloride at 37°C for 5 min to lyse any contaminating erythrocytes. Reaction was stopped by adding excess of PBS, followed by three washings in PBS. The lymphocytes were finally suspended in PBS to a final concentration of 5×10^6 cells ml.$^{-1}$

Splenocytes were obtained by teasing the spleen of Swiss albino mice and processed similarly. Splenocyte suspension was prepared in PBS to obtain 5×10^6 cells ml.$^{-1}$ Microbial cultures (*E. coli* and *Kluyveromyces marxianus*) of 24 h age were centrifuged (3,000 × g, 20 min, 4°C), pellet was washed thrice in PBS and resuspended in PBS to yield 1×10^7 cells ml.$^{-1}$

Agglutination assay

Agglutination assay was carried out as described earlier (SINGH et al. [20]). Agglutination of erythrocytes was recorded visually. For other cell types, agglutination was visualized using a phase contrast microscope. Lectin titre was defined as inverse of highest dilution capable of agglutination.

Characterization of lectin

Haemagglutination inhibition assay

Haemagglutination inhibition assay was performed as described earlier (SINGH et al. [20]). Formation of button in the presence of sugar indicated specific interaction between lectin and the sugar, while mat formation indicated non-specific sugars. Minimum inhibitory concentration (MIC) of specific sugars was determined by serial double dilution of the sugar solution. MIC was defined as the lowest concentration of sugar capable of complete inhibition of agglutination.

The sugars tested as inhibitors were: D-ribose, L-rhamnose, D-raffinose, D-xylose, L-fucose, D-mannose, D-arabinose, L-arabinose, D-galactose, D-trehalose, D-sucrose, D-

glucose, 2-deoxy-D-glucose, 2-deoxy-D-ribose, inositol, meso-inositol, melibiose, D-maltose, D-fructose, D-mannitol, D-lactose, D-glucuronic acid, D-galacturonic acid, D-glucosamine hydrochloride, D-galactosamine hydrochloride, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, inulin, bovine submaxillary mucin, porcine stomach mucin, asialofetuin, chondroitin-6-sulphate and pullulan (SIGMA, USA).

Bactericidal activity

Bactericidal activity of the partially purified lectin was assayed using *E. coli* MTCC 293 (procured from Institute of Microbial Technology, Chandigarh, India) as the test organism by agar diffusion method.

Statistical analysis

All experiments were carried out in triplicates and their mean was calculated. Data was analyzed using one-way ANNOVA followed by Tukey's test, wherever necessary. Values were considered significantly different when $P < 0.001$.

Results and Discussion

Specific haemagglutination activity of fungal extracts

The mycelial extracts obtained from solidified agar cultures exhibited only marginal lectin activity as compared to the mycelium recovered from broth cultures. The mycelium in broth did not produce large number of spores as compared to the mycelium growing on solidified medium. This indicates that lectin produced by *A. nidulans* is not spore specific. However, a high specific haemagglutination activity has been reported in swollen conidia and very low in 5 day-old mycelium of *A. fumigatus*, indicating that the lectin activity is exhibited by the spores (TRONCHIN et al. [23]). In further experiments, *A. nidulans* was inoculated into broth.

Determination of effect of culture conditions on lectin activity

Lectin activity was found to be highest at neutral pH. Further increase in pH decreased the amount of lectin expressed by the mycelium (Figure 1). Tukey's test revealed a statistically significant difference in specific activity at different pH ($P < 0.001$). An increase in extracellular lectin acivity of *Lentinus edodes* has been reported at pH 8-9 (TSIVILEVA et al. [24]). Highest lectin activity was observed in cultures growing at 30 °C. Temperature above or below 30 °C was accompanied by decrease in lectin activity. This decrease as analyzed by Tukey's test was statistically significant ($P < 0.001$) and greater than would be expected by chance. Temperature above optimum was more detrimental to the lectin activity. For the cultures incubated at 35 °C, four times lower activity was achieved while the activity dropped only two times for cultures grown at 25 °C (Figure 2). Similar results have been reported for *Lentinus edodes*, where a drastic reduction in lectin activity was observed at 32°C (optimum 26°C) and the activity was not much affected up to 16°C (TSIVILEVA et al. [12]). It has also been reported that lectin activity is not purely growth associated (NIKITINA et al. [25]). The broth cultures when grown under shaking conditions expressed only marginal lectin activity (haemagglutination titre 2).

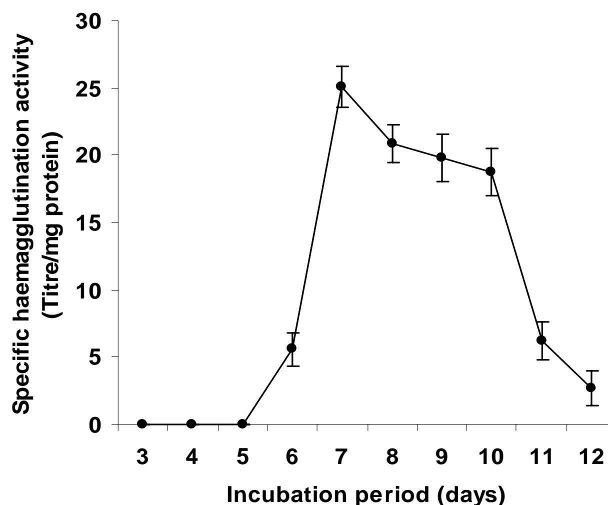

Figure 1. Effect of pH on lectin activity of *A. nidulans*

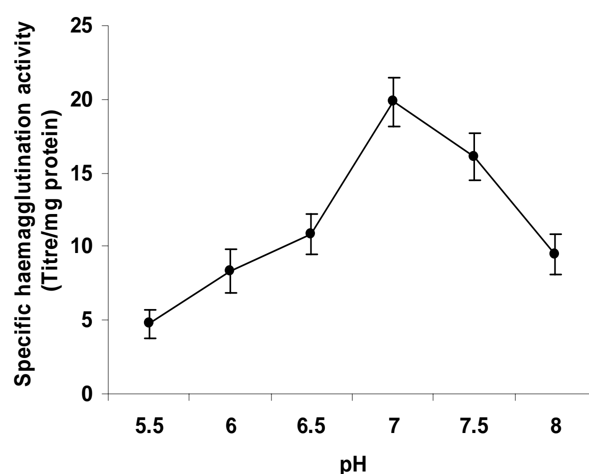

Figure 2. Effect of temperature on lectin activity of *A. nidulans*

Upon agitation, the culture forms a lump of hyphal mass and the mycelium did not reach maturity, suggesting that the lectin is produced primarily by the mature mycelium. A change in lectin activity with culture age was determined by examining the amount of lectin produced by 3-14 days old mycelium. Lectin activity began to be expressed in 5 days old mycelium, reaching its stationary value after 7 days of incubation (Specific activity 25.053 ± 1.521 titre mg^{-1} protein). No activity was observed in cultures incubated for 14 days or more. The results are depicted in Figure 3. These observations further confirm that lectin activity is not a function of growth rate alone (NIKITINA et al. [25]).

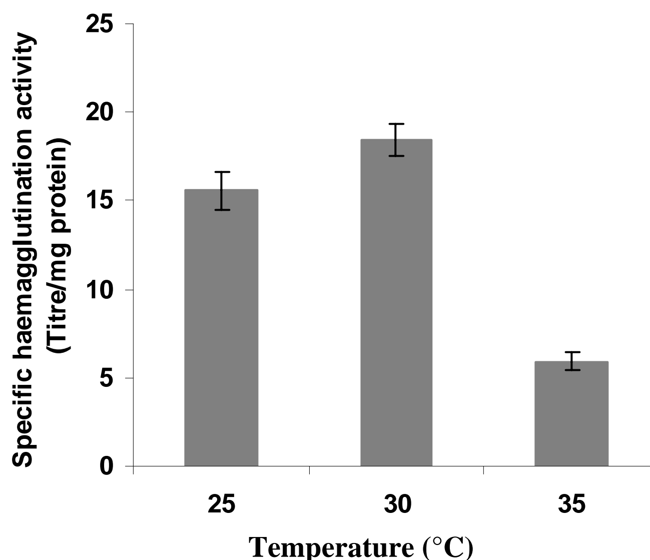

Figure 3. Effect of incubation time on lectin activity of *A. nidulans*

Partial purification of lectin

Mycelial extract was subjected to 10-100% saturation of ammonium sulfate at 4°C. The lectin was precipitated at 50% saturation. Specific activity of 40.18 ± 1.84 titre mg^{-1} was obtained in the pellet, with purification fold of 2.45 ± 0.16. The results of purification are summarized in Table 1. Almost similar precipitation patterns of fungal lectins have been observed by other workers. Lectin isolated from dry thalli of *Dictyonema glabratum* has been reported to be precipitated at 50-70% saturation of ammonium sulfate (ELIFIO et al. [26]). Lectin produced by *A. oryzae* has been reported to be precipitated at 30-75% saturation using ammonium sulfate (MATSUMARA et al. [19]). Dialysate yielded 56% of total titre with a specific activity of 32.98 ± 2.77 titre mg^{-1}. Upon concentration in Amicon ultrafiltration unit, the retentate showed specific activity of 46.04 ± 1.50 titre mg^{-1} protein and a purification fold of 2.81 ± 0.57 was obtained. The lectin of fungus *Arthrobotrys oligospora* has been partially purified following ultrafiltration after ammonium sulphate precipitation (ROSEN et al. [11]).

Table 1. Summary of partial purification of lectin from *A. nidulans*

Purification step	Total activity (Titre)	Total protein (mg)	Specific activity (Titre mg^{-1} of protein)	Fold purification	Yield (%)
Crude	6400	364.08 ± 9.1	16.38 ± 0.38	1	100
$(NH_4)_2SO_4$ precipitation	5120	127.82 ± 6.00	40.18 ± 1.84	2.45 ± 0.16	80
Dialysis	3584	109.17 ± 9.1	32.98 ± 2.77	2.01 ± 0.77	56
Ultrafiltration	3072	66.75 ± 2.13	46.04 ± 1.50	2.81 ± 0.57	48

Data are mean ± S.D. of three independent experiments.

Biological action spectrum of lectin

The partially purified lectin was characterized with respect to its ability to agglutinate human type A, B, AB, O erythrocytes as well as rat, mice, sheep, goat and pig erythrocytes. It was found that the lectin agglutinated all human, rat, mice and pig erythrocytes equally, while no agglutination was observed with sheep and goat erythrocytes. Its ability to agglutinate all erythrocytes to an equal extent suggests that this lectin falls under the category of non-specific lectins or panagglutinins (SHARON and LIS [27]). The non-specificity of the lectin towards different blood groups could be attributed to its interaction with saccharide units on the surface of erythrocytes other than the blood group determinants. The lectin activity was also determined with enzyme (neuraminidase and protease) treated erythrocytes. The lectin showed a higher titre against erythrocytes treated with neuraminidase, while protease treatment had no effect on agglutination compared to the untreated control (Table 2). Neuraminidase treatment reduces the net negative charge on the cell surface by removing sialic acid and exposes subterminal galactose-residues on the surface of erythrocytes (SCHAUER [28]). This suggests that galactosyl sugars or glycoproteins serve as receptors for the lectin and mediate the agglutination process. Lymphocytes, splenocytes, bacterial cells (*E. coli*) and yeast cells (*K. marxianus*) could also be agglutinated by the lectin as was determined microscopically. However, the titre was lower as compared to the haemagglutination titre.

Table 2. Agglutination of cells by *A. nidulans* lectin

Cells	Agglutination Titre
Untreated erythrocytes	1024
Neuraminidase-treated erythrocytes	8192
Protease-treated erythrocytes	1024
Splenocytes	64
Lymphocytes	64
Escherichia coli	16
Kluyveromyces marxianus	8

Haemagglutination inhibition assay

Agglutination inhibition of lectin was tested with a panel of carbohydrates: D-ribose, L-rhamnose, D-raffinose, D-xylose, L-fucose, D-glucose, D-fructose, D-mannitol, D-mannose, D-arabinose, L-arabinose, inositol, meso-inositol, D-trehalose, D-glucuronic acid, D-galacturonic acid, D-glucosamine hydrochloride, D-galactosamine hydrochloride, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, 2-deoxy-D-glucose, 2-deoxy-D-ribose, D-galactose, D-sucrose, D-maltose, D-lactose, melibiose, thiodigalactoside, inulin, bovine submaxillary mucin, porcine stomach mucin, asialofetuin, chondroitin-6-sulfate and pullulan. The results are depicted in Table 3. The lectin-mediated agglutination was inhibited by D-ribose, L-fucose, D-arabinose, D- sucrose, D-mannitol, D-maltose, inositol, D-glucuronic acid, D-galacturonic acid, melibiose, N-acetyl-D-galactosamine, thiodigalactoside, inulin, mucin and asialofetuin.

Table 3. Minimum inhibitory concentration (MIC) of sugars specific for *A. nidulans* lectin

Sugar	MIC
D-Ribose	> 50 mM
L-Fucose	> 6.25 mM
D-Arabinose	> 25 mM

D-Mannitol	> 12.5 mM
Inositol	> 25 mM
D-Glucuronic acid	> 50 mM
D-Galacturonic acid	> 50 mM
N-Acetyl-D-galactosamine	> 3.125 mM
D-Sucrose	> 12.5 mM
D-Maltose	> 25 mM
Melibiose	>50 mM
Thiodigalactoside	>1 mg ml^{-1}
Inulin	> 250 µg ml^{-1}
Bovine submaxillary mucin	> 1.97 µg ml^{-1}
Porcine stomach mucin	> 0.03 µg ml^{-1}
Asialofetuin	> 0.98 µg ml^{-1}

Other sugars did not show inhibition at the concentration tested (100 mM for simple sugars and 1 mg ml^{-1} for complex sugars and their derivatives). MIC of each of the specific sugar was determined. L- fucose could inhibit the lectin activity at a concentration greater than 6.25 mM. Fucose specific lectins have also been reported from other *Aspergillus* sp. as *A. fumigatus* (ISHIMARU et al. [18]) and *A. oryzae* (MATSUMARA et al. [19]). The lectin showed a higher affinity of binding to asialofetuin which has *O*-glycosidically linked and *N*-glycosidically linked sugar chains. Sugar specificity as well as our previous results that galactosyl-glycoproteins are involved in the haemagglutination process further indicates that galactose-containing residues may serve as receptors for the lectin. Lectin showed a very high specificity towards porcine stomach mucin (>0.03 µg ml^{-1}) and bovine submaxillary mucin (>1.97 µg ml^{-1}). Mucin-specific lectins have also been reported from other fungal species like *Pleurotus cornucopiae* (YOSHIDA et al. [8]), *Rhizoctonia crocorum, Athelia rolfsii* (KELLENS et al. [29]) and *Arthrobotrys oligospora* (ROSEN et al. [11]). The lectin from *Rhizopus stolonifer* has been reported to be specific for mucin as well as L-fucose (ODA et al. [10]). Sugar specificity of the lectin produced by *A. nidulans* is similar to earlier reports on *Aspergillus* lectins (ISHIMARU et al. [18]; MATSUMARA et al. [19]; TRONCHIN et al. [23]).

Bactericidal activity

The bactericidal activity was determined by the ability of the partially purified lectin to inhibit the growth of *E. coli* in nutrient agar. The lectin did not show any zone of inhibition in the test plates after 24 h of incubation.

Conclusions

This work reports a fucose-specific lectin from *Aspergillus nidulans* showing similarity in binding to lectins reported from other *Aspergillus* species as *A. fumigatus* (ISHIMARU et al. [18]; TRONCHIN et al. [23]) and *A. oryzae* (MATSUMARA et al. [19]). The lectin also showed high binding affinity to asialofetuin, N-acetyl-D-galactosamine and thiodigalactoside. Neuraminidase treatment of erythrocytes further enhanced their agglutinability by the lectin, which suggests that the lectin binds to galactosyl receptors on the cell surface. Porcine stomach mucin could inhibit the lectin-mediated haemagglutination at a very low concentration of 0.03 µg ml^{-1}. A lectin exhibiting similar specificity profile has been reported in *Rhizopus stolonifer* (ODA et al. [10]). There are two reports on lectins produced

by *Aspergillus fumigatus*, one specific for L-fucose (ISHIMARU et al. [18]) and other for mucin and sialic acid (TRONCHIN et al. [23]). This might suggest some degree of similarity between lectins produced by *Aspergillus* species and *Rhizopus stolonifer* lectin.

Acknowledgements

Authors are thankful to the Department of Biotechnology, Punjabi University, Patiala for providing the necessary facilities to execute this work. The financial assistance received from Department of Science and Technology, Govt. of India, New Delhi, under FIST programme, is duly acknowledged.

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