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Optimization of Media for Enhanced Glucoamylase Production in Solid-State Fermentation by *Fusarium solani*

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Summary

Solid-state cultivation of *Fusarium solani* was carried out for enhanced production of glucoamylase (GA) using different substrates like wheat bran, rice bran, green gram bran, black gram bran and maize bran. The SSF medium containing wheat bran as a substrate yielded the highest enzyme activity. The physical and chemical parameters were optimized. Maximum enzyme activity (61.35±3.69) U/g of dry wheat bran was achieved under optimum growth conditions. The optimum conditions were fructose as carbon and energy additive 1 % (by mass), urea as nitrogen additive 1 % (by mass), initial moisture content of solid substrate 70 % (by mass per volume), incubation period 96 h, inoculum size 15 % (by mass per volume) having 10^6–10^7 spores/mL, incubation temperature (35±1) °C and pH=5.0. It was further observed that the addition of surfactants caused a decrease in enzyme biosynthesis by *F. solani* in SSF of wheat bran under optimum process conditions.

Key words: glucoamylase, wheat bran, optimization, solid-state fermentation, moisture content

Introduction

Glucoamylase (GA, 1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3) is an exoacting enzyme that yields β-D-glucose from the nonreducing chain ends of amylose, amylopectin and glycogen by hydrolyzing α-1,4 linkages in a consecutive manner (*1–4*). GA also hydrolyses α-1,6 and the rare α-1,3 linkages, although at much slower rate (*5*). GAs have got extensive biotechnological applications in food and fermentation industries, baking, brewing and starch processing (*6*).

GAs have widely been reported to occur mostly in microorganisms, and also in animals and plants. A large number of microbes, including bacteria, yeast and fungi are capable of producing GAs. Filamentous fungi, however, constitute the major source among all microorganisms. Microbial strains of genera *Aspergillus* and *Rhizopus* are mainly used for commercial production of GAs (*1,6*). Thermostability is a desired characteristic of most of the industrial enzymes and each application of industrial enzymes requires unique properties with respect to specificity, stability, temperature and pH dependence (*7*). Keeping in view the extensive industrial applications, it is inevitable to investigate new microbial strains as potential producers of thermostable GAs.

Traditionally, GA has been produced by submerged fermentation (SmF) and used in a one way process in solution. In recent years, however, the solid-state fermentation (SSF) processes have been applied increasingly for the production of GA. SSF holds tremendous

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potential for the production of enzymes (*8*). The cost of production in SmF is high and it is comparatively uneconomical. On the other hand, SSF is a low-cost technology having high productivity per reactor volume and easier downstream processes. Moreover, it can be of special interest in processes where the crude fermented product may be used directly as an enzyme source (*9–11*).

Solid-state fermentation (SSF) is receiving a renewed surge of interest, primarily because of increased productivity and prospects of using a wide range of agroindustrial residues as substrates (*12,13*). The selection of a particular strain, however, remains a tedious task, especially when commercially significant enzyme yields are needed. Virtually all commercial interest in GA production is directed at making forms that will produce more glucose at the expense of byproducts without loss of activity. There are few reports on GA production in SSF systems (*6,14–18*). In order to obtain higher enzyme titers, a number of factors need to be optimized, including a suitable microorganism and process parameters (*13,14*).

Pakistan imports GA from industrially advanced countries for its industrial needs and it involves a huge amount of foreign exchange. The production process for GA has not been developed and commercialized in Pakistan. In this manuscript, we describe the production and characterization of GA from a locally isolated *F. solani* strain. This will lead to the development of an indigenous technology for the production of this industrially important enzyme.

Materials and Methods

Substrates

Commercially available wheat bran, rice bran, green gram bran, black gram bran and maize bran were obtained from the local market. These substrates were dried in an oven at 70 °C to a constant mass, ground in a Wiley mill to 4 mm particle size and used as inducer substrates for GA production in SSF.

Microorganism and inoculum preparation

Pure culture of newly isolated *F. solani* obtained from National Fungal Culture Collection of Pakistan (NFCCP), Department of Plant Pathology, University of Agriculture, Faisalabad was maintained on potato dextrose agar (PDA) slants at 4 °C. Inoculum was prepared by transferring spores from 5- to 6-day-old slant culture into 500-mL Erlenmeyer flask containing 150 mL of sterile Vogel's medium. The composition of inoculum medium was (in g/L): glucose 20.0, trisodium citrate 2.5, KH_2PO_4 5.0, NH_4NO_3 2.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4{\cdot}7H_2O$ 0.2, peptone 2.0, microelement solution 10 mL and vitamin solution 5.0 mL. The solution of trace elements had the following composition (in g/L): $CuSO_4$ 0.08, H_2MoO_4 0.05, $MnSO_4{\cdot}4H_2O$ 0.07, $ZnSO_4{\cdot}7H_2O$ 0.043 and $Fe_2(SO_4)_3$ 0.05, whereas the vitamin solution contained (in g/L): biotin 0.2, folic acid 0.2, thiamine-HCl 0.5, riboflavin 0.5, pyridoxin HCl 1.0, cyanocobalamine 0.1, nicotinic acid 0.5, Ca-pantothenate 0.5, *p*-aminobenzoic acid 0.5 and thioctic acid 0.5. The pH of the medium was adjusted to 5.0 using 1 M HCl/1 M NaOH. The inoculum flasks were incubated at 30 °C (150 rpm) for 48 h to get homogenous spore suspension (10^6–10^7 spores/mL).

Enzyme production in solid-state fermentation

The static experiments were conducted in 250-mL Erlenmeyer flasks containing 10 g of substrate moistened with mineral salt solution of the following composition (in g/L): trisodium citrate 2.5, KH_2PO_4 5.0, NH_4NO_3 2.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4{\cdot}7H_2O$ 0.2 to 50 % (by mass per volume) moisture content. Flasks were plugged with cotton and sterilized by autoclaving for 15 min at 121 °C. After sterilization, the flasks were cooled and inoculated with 10 % (by mass per volume) inoculum (unless otherwise mentioned) having 10^6–10^7 spores/mL. The flasks were incubated at (30±1) °C and initial pH=5 under still culture conditions for a desired time period. The contents of the flasks were gently shaken after every 12 h.

Initial time course study for substrate selection

Triplicate SSF media of wheat bran, white gram bran, black gram bran and maize bran (10 g of each) were moistened with mineral salts solution (pH=5) and subjected to still culture fermentation by *F. solani* for 144 h at (30±1) °C in an incubator**.** Triplicate samples were harvested after every 24 h and production of GA was monitored.

Isolation of GA

At the end of fermentation, the whole fermented medium was treated with 50 mL of distilled water and agitated thoroughly on orbital shaker at 100 rpm for 30 min. The crude enzyme was filtered through muslin cloth. The residue was again treated with another 50 mL of distilled water in the same way and filtered. The filtrates were pooled and centrifuged at 18000 rpm (39200 × *g*) for 30 min at 4 °C to remove the suspended particles.

GA assay

GA activity was determined by following the method of Iqbal *et al*. (*19*). Appropriate amount of the enzyme was reacted with 1 % soluble starch solution in 50 mM 2-morpholinoethanesulphonic acid (MES) buffer (pH= 5.5) at 40 °C for 40 min. The reaction was quenched by placing tubes in boiling water bath for 5 min, and then immediately cooled in ice. The released glucose was measured using a glucose oxidase method. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose equivalent/(mL·min) at pH=5.5 and 40 °C. The enzyme activity was given as units per gram of dry substrate (U/g).

Optimization of the production process

The SSF of the selected substrate (wheat bran) was optimized by varying process conditions like moisture level, pH, temperature, inoculum size, nitrogen additives, carbon additives and substrate concentration. The traditional classical method involved varying one parameter at a time by maintaining pre-optimized SSF conditions (*19–22*).

Effect of initial moisture content

To study the effect of moisture content of the substrate on GA production, the SSF of wheat bran was carried out with varying initial moisture levels (50, 60, 70, 80 and 90 %, by mass per volume) of wheat bran, which was adjusted with salt solution. The other condition was 10 % (by mass per volume) inoculum and fermentation was carried out for 96 h (optimum) at (30±1) °C. The optimum initial moisture content of the solid substrate achieved by this step (70 %) was used in subsequent studies.

Effect of initial pH

Wheat bran was moistened to optimum moisture level with mineral salt solutions of varying pH to study the effect of pH on GA production. The pH of the salt solution was varied from 3.0 to 7.0 with 1 M HCl or 1 M NaOH. The media were inoculated (10 %) and fermentation was carried out at (30±1) °C for 96 h. The optimum initial pH of the solid substrate achieved by this step was fixed for subsequent experiments.

Effect of temperature

The growth media of wheat bran were subjected to SSF for 96 h at temperatures ranging from (25–45±1) °C. The pre-optimized conditions of moisture content and pH were kept constant.

Effect of inoculum size

The media of wheat bran were inoculated with varying inoculum levels (5, 10, 15 and 20 %, by mass per volume) and SSF was carried out for 96 h under pre-optimized growth conditions at (35±1) °C (optimum).

Effect of substrate concentration

The effect of the amount of substrate on GA production was studied by using different amounts (5, 10, 15 and 20 g) of wheat bran in 250-mL flasks. The study was carried out at (35±1) °C, keeping all other conditions at their optimum levels.

Effect of carbon and nitrogen additives

The optimum SSF medium of wheat bran was supplemented with different carbon (glucose, fructose, sucrose and starch) and nitrogen sources (urea, yeast extract, soybean meal and peptone) at 1 % (by mass) level to study their effect on GA production by *F. solani*. The fermentation was carried out at pH=5 and (35±1) °C for 96 h.

Effect of surfactants

Different surfactants (SDS, CTAB and Triton X-100) were employed at pre-miceller ($8.3 \cdot 10^{-4}$ M), critical miceller ($8.3 \cdot 10^{-3}$ M) and post-miceller ($8.3 \cdot 10^{-2}$ M) concentrations to study their effect on the production of the enzyme by *F. solani* in SSF medium of wheat bran under optimum conditions.

Results and Discussion

The production of extracellular GA by *F. solani* was studied in solid-state fermentation (SSF). Solid-state fermentation has numerous advantages over submerged fermentation (SmF), including superior productivity, simple technique, low capital investment, low energy requirement and less wastewater output, better product recovery and lack of foam build-up (*6*,*12*,*23–25*).

In SSF, the selection of a suitable solid substrate is a critical factor and thus involves the screening of a number of agroindustrial materials for microbial growth and product formation (*17*). In the present study, five different substrates, *viz*. black gram bran, green gram bran, wheat bran, rice bran and maize bran were used for growth and GA production by the *F. solani*. Culture media containing 10 g of each substrate were subjected to fermentation for 144 h at pH=5, temperature (30±1) °C, moisture 50 % (by mass per volume) and inoculum level 10 % (by mass per volume). All the substrates supported growth and enzyme formation by the culture, but wheat bran proved superior to other substrates (Fig. 1). Wheat bran medium gave high titer of GA activity ((22.56±1.80) U/g) after 96 h, followed by maize bran ((18.70±1.34) U/g). Black gram bran gave the minimum enzyme yield ((12.72± 0.54) U/g). Wheat bran had previously been reported to be the best substrate for GA production (*17*,*26*,*27*). The biosynthesis of GA decreased after 96 h. Ramadas *et al.* (*28*) reported that 96 h is the optimum period for amyloglucosidase synthesis by *Aspergillus niger* in SSF.

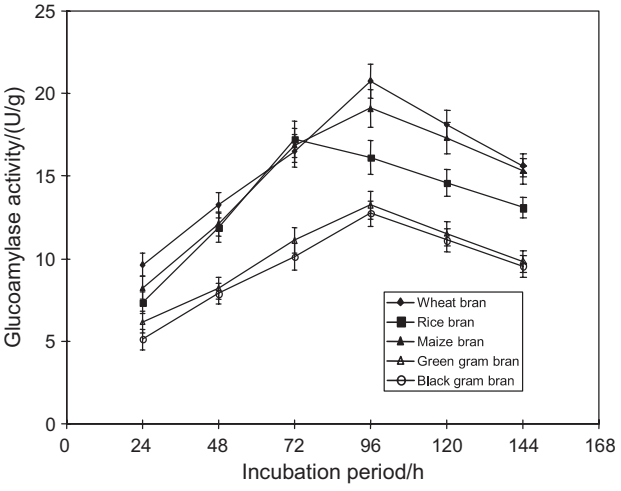

Fig. 1. Initial time course study on GA production by *F. solani* in SSF of different substrates: moisture 50 %, pH=5.0, temperature (30±1) ºC, inoculum size 10 % (by mass per volume), substrate 10 g

Enzyme production profiles with varying moisture levels (Fig. 2) showed that SSF medium adjusted at 70 % moisture level resulted in higher GA synthesis ((25.67± 2.13) U/g). Lower or higher moisture levels yielded lower GA activities. The critical importance of moisture level in SSF media and its influence on the biosynthesis and secretion of the enzyme can be attributed to the interference of moisture with the physical properties of the so-

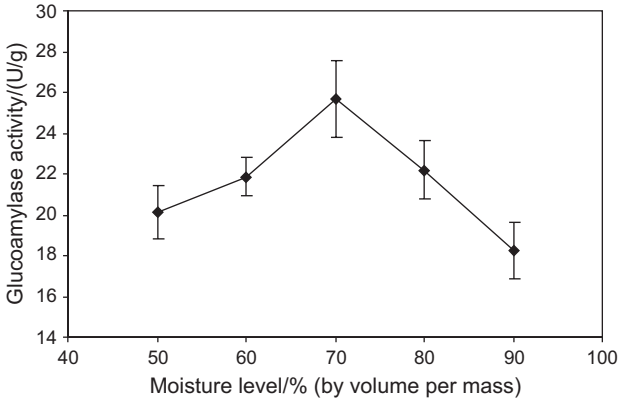

Fig. 2. Effect of initial moisture content and incubation period on GA production by *F. solani*: fermentation time 96 h, pH=5.0, temperature (30±1) ºC, inoculum size 10 % (by mass per volume), substrate 10 g

lid particles. High substrate moisture results in decreased substrate porosity, which in turn prevents oxygen penetration (*9*). At the same time, low moisture level leads to poor microbial growth and poor accessibility to nutrients (*10*). Different microorganisms show better performance at different moisture levels. *Aspergillus* sp. A3 produced maximum GA with 80 % moisture content (*17*), whereas *Rhizopus nigricans* PCSIR-18 gave optimum GA yield at 60 % moisture level (*27*).

Investigated effect of initial pH of the medium is shown in Fig. 3. The maximum GA production ((24.48± 1.71) U/g) was obtained at pH=5.0 after 96 h of incubation at (30±1) °C. Optimal pH is very important for growth of the microorganism and its metabolic activities. As the metabolic activities of the microorganism are very sensitive to changes in pH, GA production by *F. solani* was affected by varying pH of the medium. Our findings are comparable to previously reported results from literature. Pandey and Radhakrishnan (*16*) reported maximum GA production by *Aspergillus niger* NCIM--1245 at pH=4.7. In line with our work, Ellaiah *et al.* (*17*) also optimized the culture conditions for GA production

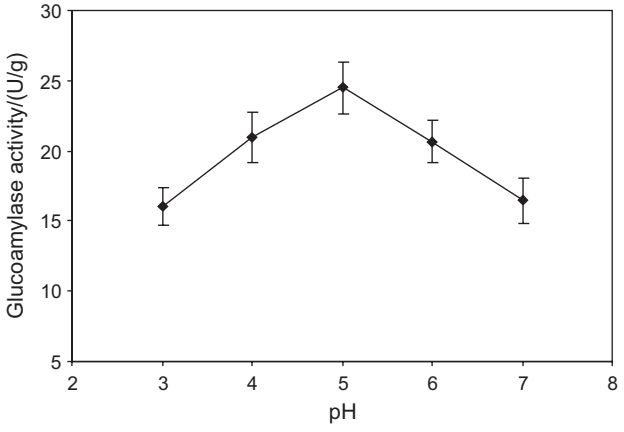

Fig. 3. Effect of pH on GA production by *F. solani* under optimum conditions: fermentation time 96 h, moisture content 70 %, temperature (30±1) ºC, inoculum size 10 % (by mass per volume), substrate 10 g

by *Aspergillus* sp. A3 under SSF and noted optimum enzyme yield at pH=5.

Fig. 4 shows the effect of temperature on GA production by *F. solani* under pre-optimized conditions. Maximum GA yield of (28.87±1.87) U/g was observed in the medium incubated at (35±1) °C. Higher incubation temperatures resulted in lower enzyme synthesis by the fungus. Growth temperature is a very critical parameter which varies from organism to organism and slight changes in growth temperature may affect GA production (*29*). At higher temperature, due to the production of large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation (*12*). It has previously been reported that *Aspergillus awamori* NRRL 3112 and *Aspergillus niger* NRRL 337 produced maximum GA at 35 °C (*30*). *Aspergillus* sp. A3 gave GA titres at 30 °C under optimum SSF process conditions (*17*).

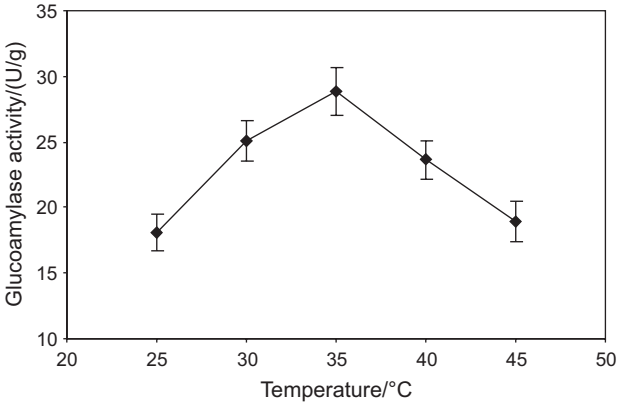

Fig. 4. Effect of initial incubation temperature on GA production by *F. solani* under optimum conditions: fermentation time 96 h, moisture content 70 %, pH=5, inoculum size 10 % (by mass per volume), substrate 10 g

Varying inoculum levels were tested to study their effect on GA production by *F. solani*. Under optimum conditions, GA production increased with an initial increase in inoculum size and maximum ((32.64±2.61) U/g) GA activity was noted in the medium receiving 15 % (by mass per volume) inoculum level. A further higher spore density caused a decrease in GA synthesis in SSF of wheat bran (Fig. 5). Inoculum density is an important factor in an SSF processes since higher inoculum levels, besides increasing spore concentration, also increase water content of the solid substrate, thereby inhibiting fungal growth and enzyme induction (*23*). On the other hand, lower inoculum levels require more time for fermenting the substrates in SSF still cultures. In contrast to our results, 10 % inoculum level for GA production in SSF of wheat bran by *Aspergillus* sp. A3 has previously been reported as optimum (*17*).

Different amounts (5, 10, 15 and 20 g/250 mL) of wheat bran were used to study their effect on GA production. The highest enzyme production of (42.57±3.62) U/g was observed when the substrate mass to flask volume ratio was 1:50 (Fig. 6). The concentration of substrates is vital in solid-state fermentation. The level of

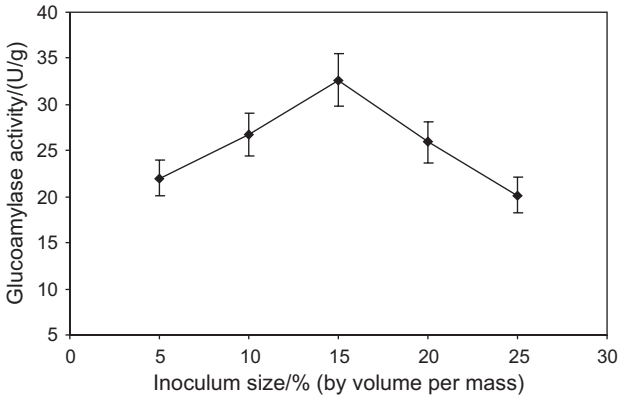

Fig. 5. Effect of inoculum size on GA production by *F. solani* under optimum conditions: fermentation time 96 h, moisture content 70 %, pH=5, temperature (35±1) ºC, substrate 10 g

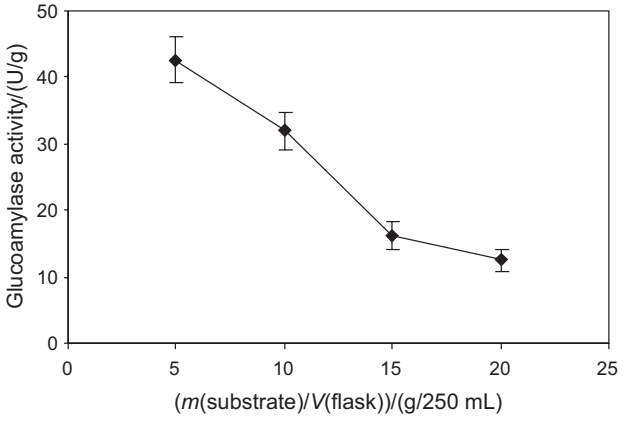

Fig. 6. Effect of the amount of substrate on GA production by *F. solani* under optimum conditions: fermentation time 96 h, moisture content 70 %, pH=5, temperature (35±1) ºC, inoculum size 15 % (by mass per volume)

substrate per unit area of working volume of the flask influences the porosity and aeration of the substrate (*15*). In line with our results, Ellaiah *et al*. (*17*) also reported 1:50 of substrate mass to flask volume ratio for optimum GA production by *Aspergillus* sp. A3.

Addition of different sugars as additives to wheat bran medium enhanced GA production by *F. solani*. Optimum GA yields ((50.56±3.42) U/g) were recovered from the medium supplemented with fructose (Fig. 7). Supplementation of wheat bran and rice powder SSF media with sucrose, glucose (*29*) and fructose (*17*) had also been reported. Among various nitrogen sources, addition of urea exhibited better GA production ((59.72±3.01) U/g, Fig. 7), followed by peptone and yeast. The production of GA is strongly influenced by the nature and concentration of N and C sources. A 100 % increase in GA production by *Aspergillus awamori* has been reported (*31*) when $(NH_4)_2SO_4$ was replaced by urea as a nitrogen source in SSF of wheat bran. Bertolin *et al*. (*29*) reported an enhancing effect of yeast extract and peptone addition on GA production in SSF of wheat bran. In accordance with our results, urea as nitrogen supplement in

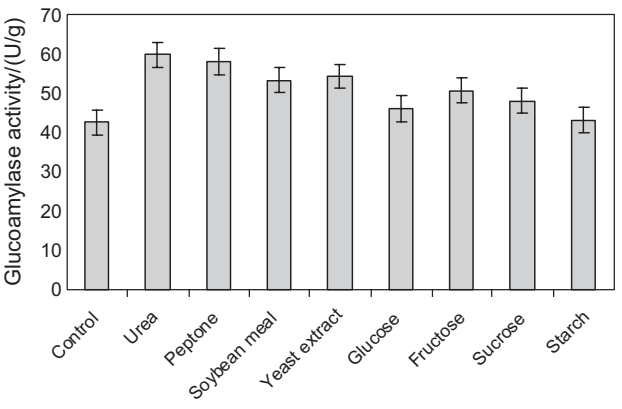

Fig. 7. Effect of different carbon and nitrogen additives on GA production by *F. solani* under optimum conditions: fermentation time 96 h, moisture content 70 %, pH=5, temperature (35±1) ºC, inoculum size 10 % (by mass per volume), substrate 5 g

wheat bran medium has also been reported to have enhancing effect on GA synthesis (*17*).

It was further observed that the addition of various surfactants at all levels decreased the GA synthesis, revealing that these have inhibitory effect on growth of microorganisms (data not shown). Surfactants have variable effects on SSF of different substrates and in most cases they depend on the composition of the solid substrate. Surfactants have the potential to enhance microbial growth in SSF by promoting the penetration of water into the solid substrate matrix, leading to an increase in surface area (*23*). However, this is not true with all substrates and microbial strains; surfactants and detergents have been found to show inhibitory effects on the production of amylolytic enzymes (*13*,*32*).

Conclusions

It was concluded that the nature of the substrate, incubation time, temperature, pH, *etc*. all affect the production of GA in solid-state fermentation of wheat bran. The results provided valuable information for the production of GA by *F. solani* using relatively inexpensive substrate wheat bran.

Acknowledgements

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