Solid-state cultivation of Aspergillus niger NCIM 548 for glucoamylase production on groundnut shell

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Abstract

Glucoamylase is a well recognized amylolytic enzyme used in food industry, which has been produced by Aspergillus genus under solid-state and submerged cultivation. In this study, the strain Aspergillus niger NCIM 548 was examined for glucoamylase production on the solid surface of groundnut shell (0.5 mm particle size) supplemented with different nutrient factors in Aspergillus minimal medium. With 50% initial moisture content, a maximum glucoamylase activity (726 U/gdm) was achieved by this strain on groundnut shell in presence of sucrose (1%, w/v) and yeast extract (0.5%, w/v) at 84 hrs. However, when groundnut shell was combined with other starchy wastes this strain did not produce a considerable enzyme yield. This strain was produced more glucoamylase yield on groundnut shell alone. Since groundnut shell is a very cheap, easily available source from oil industry and more suited for solid-state cultivation of A. niger for glucoamylase production, a detailed study on the large-scale fermentation processes using groundnut shell and potential uses are suggested.

INTRODUCTION

Amylases are well known starch degrading enzymes, which are comprised in to □-, □-, and □-amylase. Among these, glucoamylase (□-amylase) is being a commercial interest in food industry. It has been exploited for the production of starch syrups, chocolate syrup and dextrose, the preparation of starch derived adhesives, grain alcohol and fruit cakes, improving utilization of enzymatically treated barely in poultry calf raising and the clarification of fruit juice for jelly manufacture in food processing industry (Joshi et al., 1999). Glucoamylase (amyloglycosidase, EC.3.2.1.3) is an exo-acting enzyme that yields □-D-glucose by hydrolyzing □-1-4 glycoside linkages from the non-reducing ends of amylose, amylopectin and glycogen (Forgarty and Kelley, 1980). The exclusive production of this enzyme is achieved by Aspergillus niger (Pandey et al., 1994a; Wang et al., 2006), A. oryzae (te Biesebeke et al., 1992), and A. terreus (Berka et al., 1992) in enzyme industry. These strains are already reported to produce substantial amount of glucoamylase in submerged (Berka et al., 1992) and solid-state fermentation (Alazard and Raimbault, 1981). Solid-state cultivation is a relatively good enzyme production procedure, but the growth of fungal strains on solid-surfaces of organic materials and or wastes has been poorly studied. In addition, parameters such as moisture, temperature and biomass have been difficult to measure. The production of amylolytic enzymes, particularly glucoamylase on solid substrate is more advantageous for the fermentation industry (Ghildyal et al., 1985; Joshi et al., 1999). Cereal bran and flours, potato residue and other starchy waste materials have been utilized as fermentation substrate for glucoamylase production by filamentous fungi (te Biesebeke et al., 2005; Joshi et al., 1999). Nevertheless, the selection of such raw material is important interest for enzyme production in solid-state cultivation; the selected raw material should be very cheap, easily available, and environment friendly. In this context, groundnut shell may be a prominent substrate for solid-state fermentation because abundant quantity of this material has been discharged by oil industry after the extraction of oil. India is the largest cultivator of groundnut in the world. Thus, this present study was aimed to evaluate the use of groundnut shell as solid substrate to cultivate A. niger NCIM 548 for improved production of glucoamylase. This work was also addressed on how different media ingredients along with groundnut shell have effected on A. niger for glucoamylase synthesis under solid-state conditions. Perhaps, in future our attempt would be helpful for researchers to exploit this organic material as a suitable substrate in enzyme industry.

MATERIAL AND METHODS

INOCULUM PREPARATION

The strain Aspergillus niger NCIM 548 was procured from National Collection for Industrial Microorganisms, National Chemical Laboratory (Pune, India). It was maintained on potato/dextrose/agar (PDA) slant at 4 ^{o} C with a periodic regeneration. The fungal inoculum was prepared by adding five mL of sterile distilled water with two drops of Tween-80 to 7 days old PDA slant. The conidiospores were slightly scraped with an inoculation loop and then homogenized.

SOLID-STATE CULTIVATION IN FLASK

Groundnut shell was collected from a local vegetable oil industry and grinded to obtain 0.5 mm particle size using a standard sieve, and preserved at room temperature. The chemical constituents of groundnut shell were analyzed according to the standard methods described by Ranade et al (1980). 10 g (w/w) of this substrate was distributed in a 250 mL wide-mouth flask and then moisturized with 40 mL of Aspergillus minimal medium. It has the following composition (g/L): $(NH_4)_2SO_4$, 23.93; $FeSO_4.7H_2O$, 3.35; $ZnSO_4.7H_2O$, 3.77; $CuSO_4.5H_2O$, 0.425. The initial pH of the medium was adjusted to 4.6 and sterilized at 121 ^{o} C for 20 min. The fermentation process was started by adding one mL (v/v) of conidial spore suspension (3 x 10 ^{6} spores/mL) as prepared above, whole content mixed thoroughly and then incubated at 30 ^{o} C for 7 days in a stationary condition. A similar experiment was conducted for glucoamylase production by replacing groundnut shell with 10 g (w/w) rice bran (Pandey et al., 1994a; 1994b). Every 12 hrs sample was withdrawn aseptically and used for determining the glucoamylase activity. Moisture and biomass were measured by constant dry weight basis.

EFFECT OF MEDIA SUPPLEMENTS ON ENZYME PRODUCTION

The effect of different media supplements including carbon, nitrogen and other starchy materials along with groundnut shell was studied in this work by using Aspergillus minimal medium. 1% (w/v) carbon source was separately tested in 40 mL of the above medium containing 10 g (w/w) groundnut shell for glucoamylase activity. For checking the effect of nitrogen sources on glucoamylase production, 0.5% (w/v) organic nitrogen source and 0.3% (w/v) inorganic nitrogen source were separately included in 40 mL of the above medium. The influence of different media ingredients on glucoamylase production was examined by replacing appropriate sources in the same medium with groundnut shell as a main substrate. 10 g of starchy material was used to replace the groundnut shell in the same minimal medium containing sucrose (1%) and yeast extract (0.5%). One mL of conidiospores (3 x 10 ^{6} spores/mL) was used as an inoculum to initiate fermentation and then all of the flasks were incubated at 30 ^{o} C for 4 days in a stationary condition.

SOLID-STATE CULTIVATION IN TRAY

One mL (v/v) spore suspension (3 x 10 ^{8} spores/mL) was transferred to a 500 ml wide-mouth flask containing 25 g (w/w) ground nut shell moisturized with 100 mL Aspergillus minimal medium and then sterilized at 121 ^{o} C for 20 min. After 7 days incubation in stationary condition, substrate was thoroughly mixed with fungal mycelia to obtain dough. In tray culture method, one Kg (w/w) pulverized (0.5 mm particle size) groundnut shell was distributed and uniformly layered on an aluminum tray (30 x 15 cm). Then, the same minimal medium was sprinkled on the surface of the groundnut shell until to acquire proper moister content. 25 g (w/w) dough was spreading on the surface of groundnut and dispersed thoroughly. This tray was closed invertically with another one tray by keeping a space between two trays with wooden strips and then incubated at 28 ^{o} C for 4 days.

EXTRACTION OF ENZYME

After 4 days incubation, 50 mM citrate buffer (pH-4.8) (1:10 ratio) was added to fermented dough and homogenized with a constant stirring. This suspension was filtered through Whatman filter paper number 1 and the filtrate was again centrifuged at 6000 rpm for 15 min. This solid-free supernatant was used as enzyme source for assaying glucoamylase activity.

GLUCOAMYLASE ASSAY

The enzyme activity was determined by a reactive mixture contains one mL of 50 mM citrate buffer (pH-4.8) and one mL starch solution (1 %, w/v) and 0.1 mL of crude enzyme. This reactive mixture was incubated at 50 ^{o} C for 20 min and then released reducing sugars were measured with 3, 5-dinitrosalicyclic acid (DNSA) reagent (Miller, 1959) using glucose as a standard. The protein concentration in crude enzyme was estimated by Folin-phenol method (Lowry et al., 1956) using BSA as standard. Glucoamylase activity was expressed as the amount of enzyme required to liberate one mole of reducing sugars from starch by multiplying the total amount of enzyme extracted from initial gram dry mass of substrate (gdm).

RESULTS AND DISCUSSION

The chemical constituents of groundnut shell were analyzed and are listed in Table 1. The high concentration of starch suggested the possible use of groundnut shell for amylase induction in solid-state conditions.

Figure 1

Table 1: Chemical constituents of groundnut shell

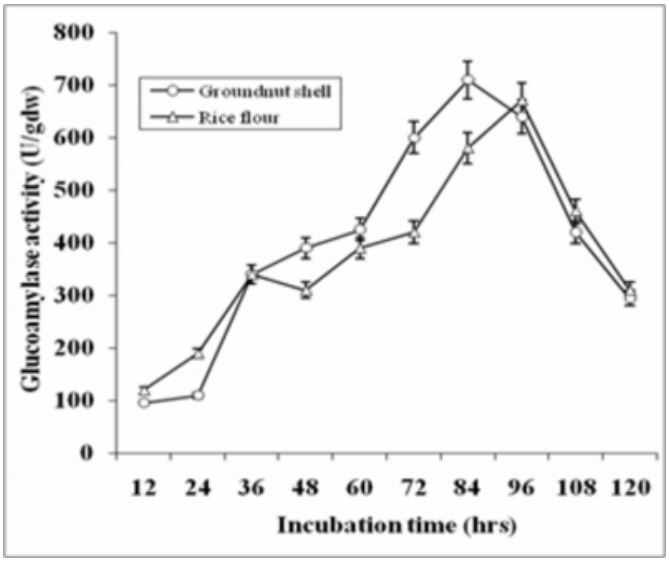
Constituents	Composition (%)
Starch	28 ± 5
Hemicellulose	11 ± 2
Cellulose	21 ± 3
Pectin	1.0 ± 0.3
Organic nitrogen	1.3 ± 0.2
Protein	8.0 ± 1
Oil and fat	3.0 ± 0.5
Coloring matter	4.2 ± 0.6
C:N ratio	22 ± 2

Aspergillus niger NCIM 548 was examined for amylolytic enzyme production on starch based minimal medium. The cell free-culture supernatant was analyzed by paper chromatography using n-butanol: pyridine: distilled water as mobile phase (50:28:32 ratio) for identifying which extracellular amylolytic enzyme is excreted in to broth. It indicated that A. niger NCIM 548 is capable of producing only glucoamylase with more extent in the fermentation media. Therefore, this strain was further studied to produce glucoamylase in solid-state fermentation conditions.

Fig.1 shows the effect of incubation time on glucoamylase production by A. niger NCIM 548 growing on groundnut shell and rice flour under the solid-state cultivation in flask. It revealed that glucoamylase activity slowly increased with fermentation time until 84-96 hrs, and thence maximum productivity sharply declined. Thus, the optimum incubation time for glucoamylase production reported as 84 hrs. This strain also showed better enzyme yield on groundnut shell than rice bran. Similarly, previous research work has shown that A. niger was yielded a maximum glucoamylase activity on wheat bran (142 U/gdm), and on wheat bran plus corn flour (500 U/gdm) after 96 hrs in flask culture (Pandey et al., 1994b).

Figure 2

Figure 1: showing the effect of incubation time on glucoamylase production when NCIM 548 growing on groundnut shell and rice bran under solid-state condition.



The optimum moisture content reported for maximum synthesis of extracellular amylases at 30 ° C was 50-55 % (Raimbault and Alazard, 1980; Wang et al., 2006). In this work, the similar moisture content was found to be favorable for glucoamylase synthesis. With moisture content below 30% was significantly lowering the formation of glucoamylase, and the biomass yield of A. niger as shown in Table 2. Water acts as a vehicle for substrate transport and as a reactant so it may be expected that aw (activity of water) effects enzymatic conversion during fermentation (Forgarty and Kelley, 1980; Alazard and Raimbault, 1981).

A maximum glucoamylase activity (726 U/gdm) was yielded by A.niger NCIM 548 on groundnut shell supplemented with yeast extract (0.5%) and sucrose (1%) with 50% moisture content at 84 hrs (Fig.1) and later on groundnut shell plus corn flour at 120 hrs. These results are comparatively better than glucoamylase (220 U/gdm) produced by A. niger on rice bran under the same culture conditions (Pandey et al., 1994b). Though Vogel's minimal medium also supported enzyme production on groundnut shell, an addition of sucrose and yeast extract found to enhance the growth of A. niger as well as enzyme yield in flask culture (Table 2).

Groundnut shell alone, without addition of minimal medium, served as a potential carbon source for the solid-state cultivation of A. niger NCIM 548 for glucoamylase formation (632 U/gdm). When it combined with different starchy raw materials, as shown in Table 2, the combination of groundnut shell (6.5 g) with corn flour (3.5 g) gave 696

U/gdm, followed by with rice flour resulted 672 U/gdm. Although a multi-combination of starchy materials with groundnut shells did not favor enzyme synthesis, they strongly supported for the mycelia growth of A. niger on solid surface.

Figure 3

Table 2: Showing the influence of groundnut shell based production media supplemented with different media supplements for glucoamylase production by NCIM 548.

Production media		Biomass (mg/gdm)	Glucoamylase activity (U/gdm)#
M1	Groundnut shell + MM	0.52 ± 0.3	600 ± 25
M2	M1 + Salicin	0.60 ± 0.2	400 ± 21
M3	M1 + Yeast extract	0.70 ± 0.2	248 ± 20
M4	M2 + M3	0.51 ± 0.2	384 ± 20
M5	M4 + Tween-80	0.55 ± 0.2	472 ± 22
M6	M5 + Vogel's minimal medium	0.62 ± 0.4	400 ± 25
M7	M1 + Potato starch	0.72 ± 0.3	494 ± 25
M8	M7 + Sucrose + Yeast Extract	0.65 ± 0.2	400 ± 20
M9	M7 + Sucrose	0.64 ± 0.2	384 ± 20
M10	M1 + Rice flour	0.70 ± 0.2	672 ± 25
M11	M1 + Corn flour	0.80 ± 0.3	696 ± 24
M12	M1 + Wheat flour	0.71 ± 0.3	320 ± 25
M13	M3 + Sucrose	1.10 ± 0.2	719 ± 21
M14	Groundnut shell + Distilled water	0.54 ± 0.3	632 ± 25
M15	Potato starch + MM	0.63 ± 0.3	560 ± 24
M16	M1 + Wheat straw + Rice flour + Potato starch + Corn flour (2 g each)	0.65 ± 0.4	144 ± 26

The concentration of sucrose and salicin, yeast extract and Tween-80 were 1.0, 0.5 and 0.03 % (w/v), respectively. 6.5 g of groundnut shell plus 3.5 g of other starchy materials were used in M7-M12.
'M' denotes production medium.
#The enzyme activity in culture supernatant was determined after 4 days incubation.

Additional carbon supplements, exclusively galactose, mannose and sucrose, were found to induce maximum glucoamylase activity where as its activity was lowered by adding xylose and fructose to minimal medium (data not shown). Similarly, media supplementation with sucrose in wheat bran increased enzyme production (271 U/gdm) as reported by Anto et al. (Anto et al., 2006). Glucose is a somewhat weaker inducer than maltose and starch, but xylose is apparently a strong inducer for glucoamylase synthesis (Fowler et al., 1990). Therefore, the selective additions of carbon supplements are only favored to glucoamylase enhancement under solid-state fermentation on groundnut shell.

Figure 4

Table 3: Showing the influence of nitrogen sources in minimal media incorporated with sucrose (1 %, w/v) for glucoamylase production when NCIM 548 growing on groundnut shell under solid-state fermentation.

Nitrogen source	Glucoamylase activity (U/gdm)#
Peptone	310 ± 23
Yeast extract	375 ± 22
Meat extracts	295 ± 22
Casein	425 ± 25
Ammonium chloride	375 ± 24
Di-ammonium hydrogen phosphate	370 ± 24
Ammonium sulfate	305 ± 23
Sodium nitrate	240 ± 25

The concentration of organic and inorganic nitrogen sources were 0.5 % (w/v) and 0.03 % (w/v), respectively.
#The enzyme activity in culture supernatant was determined after 4 days incubation.

Organic nitrogen source, corn steep liquor stimulated the growth of A. niger better than peptone, but gave a similar yield of enzyme (359 U/gdm and 339 U/gdm) (Pandey et al., 1994b) on wheat bran. Hence, the effect of additional nitrogen sources on glucoamylase production was studied and reported in Table 3. Casein and ammonium chloride exhibited stimulatory effects on glucoamylase formation by A. niger on groundnut shell. For fungi, organic nitrogen sources are better supplements than inorganic nitrogen sources. Similar results were found and explained in detail by Fogarty and Kelly (1980) and Anto et al. (2006) for glucoamylase production.

Among different starchy raw materials checked as shown Table 4, the maximum enzyme activity (704 U/gdm) with specific activity (15.08 U/mg proteins) was observed on groundnut shell followed on potato starch (687 U/gdm). In contrast, many of the raw materials used including cereal flours and straws when in combination failed to boost enzyme yield in presence of sucrose and yeast extract. These results revealed that on comparison to other expensive raw substrates groundnut shell exhibited more suitability for solid-state cultivation of A. niger.

Considering the results of solid-state cultivation of A. niger in flask, we attempted to produce enzyme in a tray culture method. It implied that the higher enzyme production was obtained to 600 U/gdm by A. niger at 60 hrs incubation. The bed thickness in tray was the most significant variable for having as response enzyme production and productivity. A good production yield of this enzyme was occurred by A. niger using pH-4.5 and bed thickness in the inferior limits at

2.0-4.2 cm (Siveira et al., 2006). Glucoamylase produced by A. niger in flask culture method is somewhat better than activity obtained in tray culture method, suggesting further optimization study to tray culture condition will be required to improve productivity of this enzyme interest.

Table 4: Showing the influence of production media supplemented with different starchy raw materials (10 g each) for glucoamylase production by A. niger NCIM 548.

CONCLUSIONS

niger NCIM 548 is a promising for industrial application since it gave a significant glucoamylase (726 U/gdm) activity on groundnut shell under solid-state conditions, which is comparatively better than A. niger on rice bran (359 U/gdm) (Pandey et al., 1994b) and Aspergillus sp. HA-2 for on wheat bran (264 U/gdm) followed by coarse waste (211 U/gdm) and medium waste (192 U/gdm) of rice flake manufacturing industry (Anto et al., 2006). As groundnut shell is a low cost substrate, easily available, and showing suitability for solid-state cultivation of filamentous fungi, it is suggested as a potential substrate for glucoamylase production. No additional nutrient supplements are required for improving glucoamylase productivity of A. niger in flask culture, it showing the noteworthy of using this substrate in enzyme industry. Moreover, the results of preliminary characterization of the crude enzyme revealed that enzyme produced by this strain has more stable (at pH-4.6, temperature 50 ^{o} C, in 5% SDS) even at adverse environmental conditions; it is expected to be stable in industrial use. Overall, we conclude that groundnut shell served as a more suitable substrate, compared to other available starchy materials, for solid-state cultivation of A. niger NCIM 548 in reference to glucoamylase synthesis and suggest the use of this strain to large-scale fermentation processes and in food processing industry.

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