ROLE OF ALKALINE-TOLERANT FUNGAL CELLULASES IN RELEASE OF TOTAL ANTIOXIDANTS FROM AGRO-WASTES UNDER SOLID STATE FERMENTATION

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The alkaline-tolerant marine-derived fungus *Chaetomium globosum* was tested for the production of enhanced levels of cellulases and free phenolics under highly alkaline conditions using agro wastes (cotton seed, sugar cane bagasse) as substrates under solid state fermentation (SSF) processes. In both the agro wastes used, an increase in cellulases (β-endoglucanase, β-Glucosidase, and β-exoglucanase) production was observed with increase in pH. This enhanced carbohydrate-hydrolyzing enzymes (β-endoglucanase, β-Glucosidase and β-exoglucanase) and thereby enriched the total phenolic release from agro-wastes under SSF conditions of higher pH. A linear correlation was observed between released total phenolic contents of agro-wastes and total antioxidant property. The increased antioxidant activity on free radical scavenging was also observed with the increase in pH. Thus, the present study makes it possible to produce nutraceutical ingredients cost-effectively from agricultural wastes.

Keywords: Agro wastes; Alkaline pH; Antioxidant; Cellulases; C. globosum; Free radical scavenging

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INTRODUCTION

Phenolic compounds of plant materials have numerous health applications, as they are recognized as potent antioxidants, exerting antioxidative function as terminators of free radicals, and for chelating metals that are capable of catalyzing lipid peroxidation (Liu et al. 2007). Furthermore, the carcinogenic properties of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), provide motivation to consider the use of plant materials as important possible sources of compounds with the potential to be developed into highly efficient and safe antioxidants with a multitude of biological effects (Cheung et al. 2003). Agro-industrial byproducts such as oilseed meals sesame, soybean, canola, peanut, cotton seed, mustard, wheat bran, and rapeseed have been reported to be rich sources of natural antioxidants (Wettasinghe et al. 2002). However, most phenolic compounds of plant materials primarily occur in the bound form as conjugates with sugars, fatty acids, or proteins (Correia et al. 2004). Thus, enzyme hydrolysis of phenolic glycosides seems to be a promising way to increase the concentration of free phenolics, thereby enhancing the nutraceutical activity of these compounds (Zheng and Shetty 2000). The process, however, is not considered as

economically feasible because the commercial enzymes used for this process are not cost-effective. Studies have shown that fungi grown in solid-state systems are able to produce high amounts of enzymes that are capable of hydrolyzing phenolic glycosides. This enzyme-linked release is suggested to play an important role in the development of antioxidant functionality of phenolic compounds (Bhanja et al. 2008; Lee et al. 2008; Lin et al. 2006; McCue and Shetty 2003; Vattem and Shetty 2002). Although several studies have been carried out to address solid state fermentation process as an alternative way to improve the phenolic content and antioxidant potential in fermented foods (Bhanja et al. 2008; Lee et al. 2008; Lin et al. 2006; McCue and Shetty 2003; Vattem and Shetty 2002), only very few studies have been done with agro-wastes (Lateef et al. 2008; Bhanja et al. 2009).

Marine mangrove fungi have proven to be an important source for xylanolytic and cellulolytic activity on lignocellulosic substrata in the recycling of lignocellulose in the marine environment (Luo et al. 2005). Thus, in the present study, the marine derived fungus *C. globosum*, which was found to grow within a wide range of pH (4 to 12), was isolated from mangrove wood litters. The fungus was investigated for its production of alkaline cellulases, endoglucanase (EC 3.2.1.4, 1,4-β-D-glucan-4-glucanohydrolase), exoglucanse (EC 3.2.1.91, 1,4-β-D-glucan-cellobiohydrolase), and β-1,4-glucosidase (EC 3.2.1.21), that can degrade the cell wall constituents of agro wastes, thus assisting in the release of intracellular contents such as phenols under alkaline stress in SSF. There was no report available on the enrichment of total phenols and antioxidant activity using alkaline stress, marine fungal enzymes, and the agro wastes (cotton seeds, sugarcane bagasse and rice bran) with SSF. This solid-state bioprocessing approach, using the alkaline stress as the enrichment source may help to add phenolic functional value to the agro waste by-products and may be of useful in pharmaceutical industries and other biotechnological applications.

MATERIALS AND METHODS

Collection of Plant Material and Isolation of Wood Litter Fungi

The wood pieces were collected in sterile plastic bags from mangrove swamps of Goa, India and washed with sterile sea water, dried, and incubated (25 ± 2 °C). They were screened under a stereomicroscope for the fungal structures at different intervals of incubation (2, 8, 16, and 32 weeks). The isolated fungal strains was cultured in plates and slants containing Malt extract Agar medium (MEA) and maintained at room temperature and stored in 4 °C.

Enzyme Production: Solid State Fermentation (SSF)

A mixture of substrates (cotton seed, sugarcane bagasse, rice bran) with or without mineral salt solution (gL^{-1}, w/v): $NaNO_3$, 3.0; KH_2PO_4, 1.0; Tween 80, 1.0 mL; $MgSO_4.7H_2O$, 0.5; KCl. 0.5; $FeSO_4.\ 7H_2O$, 0.01 was added to the 500 mL flask, moistened with ca. 20 mL of water. The flasks were cotton plugged and sterilized at 115 °C for 60 min. After sterilization, each flask was seeded with 3 plugs of inoculums. The final moisture content was about 80% w/w. The solid state fermentation were analysed

with different parameters such as pH, with and without nutrients and moisture conditions. The non-fermented substrates (cotton seeds, sugarcane bagasse and rice bran) were prepared without the addition of inoculums. The culture flasks were incubated at room temperature for 8 days. The flasks were shaken twice a day. All the experiments were run parallel in triplicate.

Enzyme Harvesting and Enzyme Assay

The enzyme was extracted by adding 100 mL of 0.05 M citrate buffer (pH 4.8) to the fermented substrate in each flask. The flasks were rotated on a rotary shaker at 200 rpm for 1 hr at 30 °C. The fermented broth was filtered by using Whatman No.1 filter paper, and the filterate was analysed for cellulase activity. Different enzyme assays for cellulase were performed using various substrates. Exo-β-1,4-glucanase was estimated in terms of filter paper (FP) activity and endo-β-1,4-glucanase as carboxy methyl cellulase (CM cellulase) activity by using filter paper (1-6 cm strips of Whatman No. 1) and carboxy methyl cellulose (CMC) sodium salt as the respective substrates, while β-1,4-glucosidase was measured using cellobiose (Sandhu and Puri 1989). One unit of enzyme activity has been defined as the amount of enzyme required to produce 1 µmol of the glucose in 1 min under assay conditions (pH 5.0; 50 °C). The released reducing sugar (glucose) was estimated by the dinitrosalicylic acid (DNS) method. Specific activity of the enzyme is defined as the units of enzyme per mg of protein. Protein was estimated using Bradford's method. The pH stability of the enzyme was measured by incubating 5 IU of enzyme for one hour, at 50 °C, in a buffer of desired pH. The temperature stability was determined by incubating 5 IU of enzyme, at varying temperatures for different time intervals, and then estimating the residual activity under standard assay conditions.

Gel Electrophoresis Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% polyacrylamide gel. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. The molecular weight standards used were from a high-molecular-weight calibration kit (Fermentas). CMCase zymograms were prepared using SDS-10% polyacrylamide gels containing 0.2% CMC, which was incorporated into the separating gel prior to the addition of ammonium persulphate and polymerisation. After electrophoresis, the gel was stained with Coomassie Blue R dye in methanol-acetic acid-water solution (4:1:5, by volume) for 1 h and destained in the same solution without dye.

For the determination of CMCase activity, SDS was removed by washing the gel at room temperature in Solution A (sodium phosphate buffer, pH 7.2, containing isopropanol 40%) for 1 h and Solution B (sodium phosphate buffer, pH 7.2) for 1 h, respectively. Renaturation of the enzyme proteins was carried out by leaving the gel in Solution C (sodium phosphate buffer, pH 7.2, containing 5 mM β-mercaptoethanol and 1 mM EDTA) at 4°C overnight. The gel was then transferred onto a glass plate, sealed in a film, and incubated at 37°C for 4 to 5 h. The gel was stained in a solution of 1% Congo Red for 30 min, and destained in 1 M NaCl for 15 min. Clear bands indicated the presence of CMCase activity (Ratanakhanokchai et al. 1999).

Determination of Total Phenolic Content

The total soluble phenolic content was estimated for each extract using a modified version of the Folin assay (Vattem and Shetty 2002), and gallic acid as the phenolic standard. Sample of 100 µL and 2 mL sodium bicarbonate mix were incubated at room temperature for 2 mins, followed by addition of 100 µL of Folin-Ciocalteu reagent, and incubated in dark for 30 mins. The samples were vortexed for absorbance at λ=725 nm using a spectrophotometer. Gallic acid 1 mg/mL was used as standard and standard curve was obtained using various concentrations of gallic acid.

Antioxidant Activity Assay

The antioxidant activity of each extract was determined as the ability of the extract to scavenge 1, l-diphenyl-2-picrylhydrazyl (DPPH) radicals (Yildirim et al. 2001). Briefly, a 1 mM DPPH radical solution in 95% ethanol was prepared. DPPH solution of 800 µL was mixed with 200 µL of sample extract, vortexed well, and then incubated for 30 min at room temperature at dark. The control sample was 1 mL of 95% EtOH. After the 30 min incubation, the samples were poured into microcentrifuge tubes and centrifuged for 5 min at 13,500 rpm (at room temperature). Then, the absorbance (*A*) of each sample at λ = 517 nm was measured. This antioxidant activity is given as % DPPH scavenging, calculated as $[(A_{control} - A_{extract}) / (A_{control}) \times 100]$.

Statistical Analysis

The mean values and the standard deviations were calculated from the data obtained from three separate experiments. Analysis of variance (ANOVA) was performed using Microsoft excel (MS). Statistical differences at $P < 0.05$ were considered to be significant coefficient of determination (R^2) to determine the relationship between two variables were calculated using MS Excel.

RESULTS AND DISCUSSION

Production of Cellulases using Solid State Fermentation Conditions (SSF)

Degradation of biomass using cellulases has not yet become regarded as economically feasible due to the cost of enzymes or to the lack of efficiency of cellulases (Ysohihiko and Takaisha 2002). Many researchers have tried to improve the catalytic efficiency of cellulase by domain shuffling and site-directed mutagenesis (Ysohihiko and Takaisha 2002). In the present study, the marine fungus *C. globosum* was found to grow within a wide range of pH (Ravindran and Naveenan 2011). Thus, the fungus was tested for the celluase production using various agro wastes (cotton seed, rice bran, and sugarcane bagasse) as substrates under non-alkaline (with and without nutrition) and alkaline-treated (with and without nutrition of pH 10 and 12) conditions. The production of cellulases in the culture filtrates of *C. globosum* showed marked variation depending upon the changes in cultural conditions (Fig. 1a to 1c). β-endoglucanase initiates the cellulose degradation by attacking amorphous region on cellulose and converts the polymeric form of cellulose into oligosaccharide form. Thus, β-endoglucanase, as an important enzyme in the degradation process, was found at a higher level in alkaline-

treated than the non-treated conditions in all three substrates used (Fig. 1a to 1c). The highest secretion levels of β-endoglucanase were observed when using cotton under SSF conditions (Fig.1b). The exoglucanase, which separates cellobiose by acting on the non-reducing end, showed some low levels of secretion in rice bran and sugarcane bagasse compared with that of the non-alkaline treated (Fig 1a and 1c). But the exoglucanase levels were found high with the alkaline-treated rice bran at pH 12 (Fig 1a), cotton seeds (Fig. 1b), and sugar cane bagasse at pH 10 (Fig. 1c). β-glucosidase, which completes the hydrolysis by converting cellobiose into glucose, was found to be highest with the alkaline-treated SSF conditions (Fig. 1a to 1c). Thus, all similar changes in distribution of cellulases in various fractions have also been reported earlier by several workers for different fungi (Soni et al. 1999). For all three cellulase components, the optimum temperature was 50° C, while the optimum pH ranged between 5.0 and 12. However, in different *Chaetomium* species, 50-60 °C and acidic pH (4.0–5.0) have been reported to be optimal for enzyme production (Lakshmikant and Mathur 1990). In *Neurospora crassa*, the optimum pH for β-glucosidase and endoglucanase activity was found to be 6.0 while, it was 7.0 for exoglucanase (Yazadi 1990). In several other fungi, especially *Trichoderma* and *Aspergillus* sp., pH optima are variable but in acidic (3.4–6.3) range (Soni et al. 1999). The concentration of substrate required for maximum enzyme production varied depending upon the cultivation conditions. Because different substrates differ in their composition, they need different pretreatments to increase their biodegradability. The electrophoretic studies with extracellular fractions of cotton seeds, revealed two forms. Each may be of endoglucanase, EG I, EG II/ β- glucosidase, β-Glu I, β-Glu II (Fig.2) as shown by previous studies *Chaetomium erraticum* (Soni et al. 1999) and *Chaetomium fusisporale* (Sanhu and Puri, 1989) under SSF, which exhibited a variation in the intensities of the bands corresponding to the enzyme activity in the filtrates.

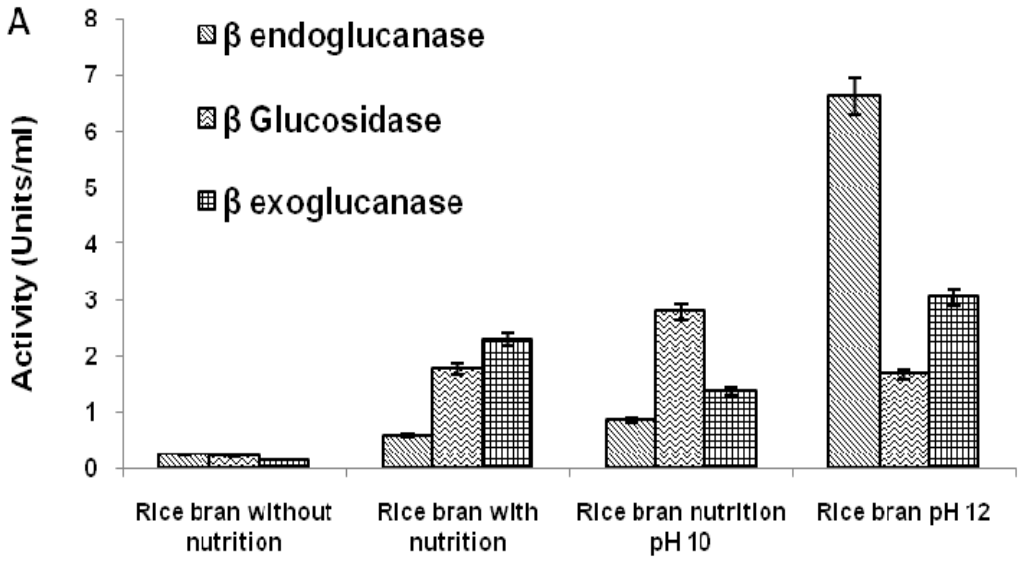

Fig. 1A. Production of exoglucanase, endoglucanase, and β-glucosidase by *Chaetomium globosum* under SSF (solid state fermentation) using three different substrates (Rice bran (A); cotton seeds (B), Sugarcane bagasse (C)) at different conditions. pH 10 and pH 12 of cotton seed and sugar cane bagasse produced maximum secretion levels of β-exoglucanase, β-endoglucanase, and β-glucosidase. Values are means ± S.D. (n=3).

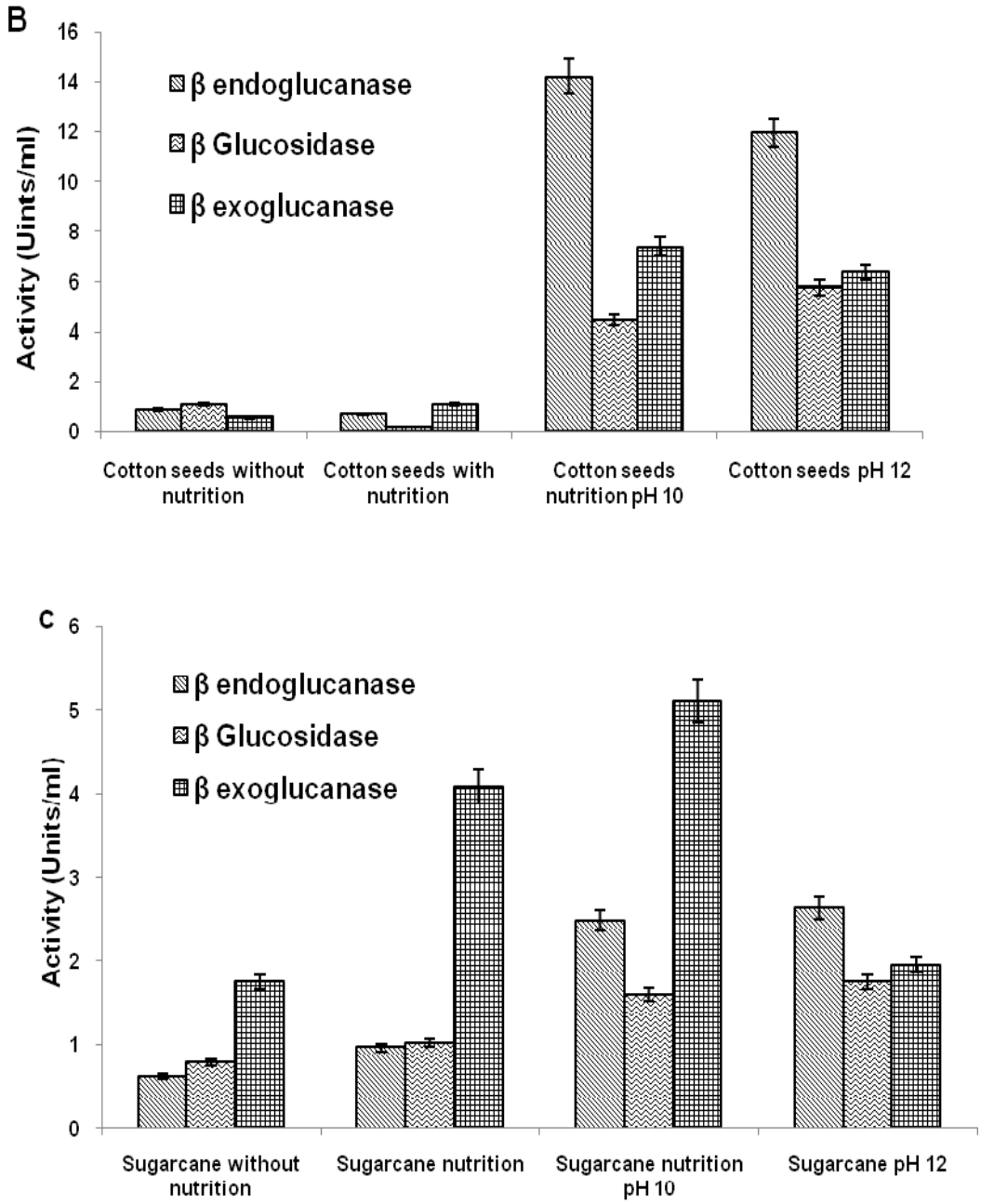

Fig. 1B-C. Production of exoglucanase, endoglucanase and β-glucosidase by *Chaetomium globosum* under SSF (solid state fermentation) using three different substrates (Rice bran (A); cotton seeds (B), Sugarcane bagasse (C)) at different conditions. pH 10 and pH 12 of cotton seed and sugar cane bagasse produced maximum secretion levels of β-exoglucanase, β-endoglucanase and β-glucosidase. Values are means ± S.D. (n=3).

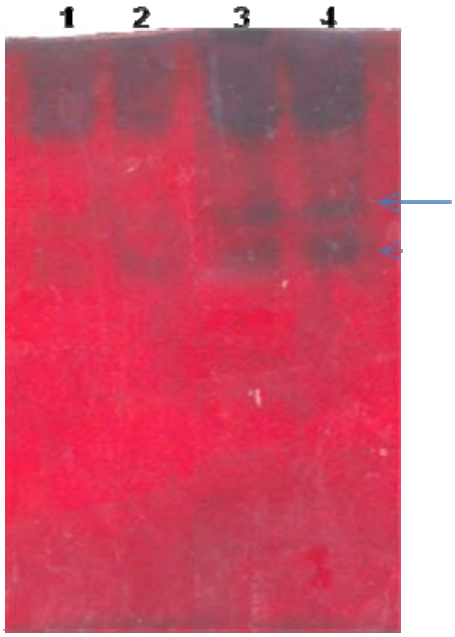

Fig. 2. Zymograms showing isozymes of cellulase enzymes. Crude extracts of alkaline treated sugarcane bagasse (1 and 2) and cotton seeds of SSF (3 and 4)

Influence of Cellulase on Poly-Phenol Release and Total Phenolic Content

Earlier studies have shown that fungal enzymes are involved in the phenolic mobilization during solid-state growth (McCue and Shetty 2003; Vattem and Shetty 2002). Thus, in this study we elucidate that the cellulolytic enzymes of *C. globosum* would play an important role in release of poly phenolic compounds from agro wastes (cotton seeds, sugarcane bagasse, and rice bran). Low phenolic content was observed under conditions other than alkaline pH among the different experimental conditions used (Fig. 3a). This low phenolic content suggests that most natural phenolics were in bound form and only a relatively small part was in free phenolics form (soluble). The alkaline treatments showed similar release of cellulase enzymes that is increase with the increased pH (Fig. 3a). This significant correlation of enhanced levels of cellulase enzymes of *C. globosum* and phenolic content of agro wastes under alkaline condition suggest that alkaline stress also can play an important role with that of the cellulase enzymes in release of free phenolic compounds from agro-waste residues. These results also show that during fermentation of agro wastes with *C. globosum*, cellulases appears to be the major carbohydrate-cleaving enzymes stimulated for increased phenolic mobilizing activity. In addition, the phenolic content increased after fermentation may be also due to the fact that hydrolytic enzymes produced by fungi, catalyze the release of aglycones from the substrate, thereby resulting in an increase in their phenolic content as well as antioxidant potential. It was reported that in comparison to the non-fermented condition, the total phenolic content and antioxidant activities were increased to a maximum after fermentation (Lin et al. 2006). Therefore, we analysed the three different substrates for their total phenolic content and antioxidant activities with and without fermentation conditions. We found increased levels of total phenolic content with fermentation (Fig. 3a) when compared to that of without fermentation (Fig 3b).

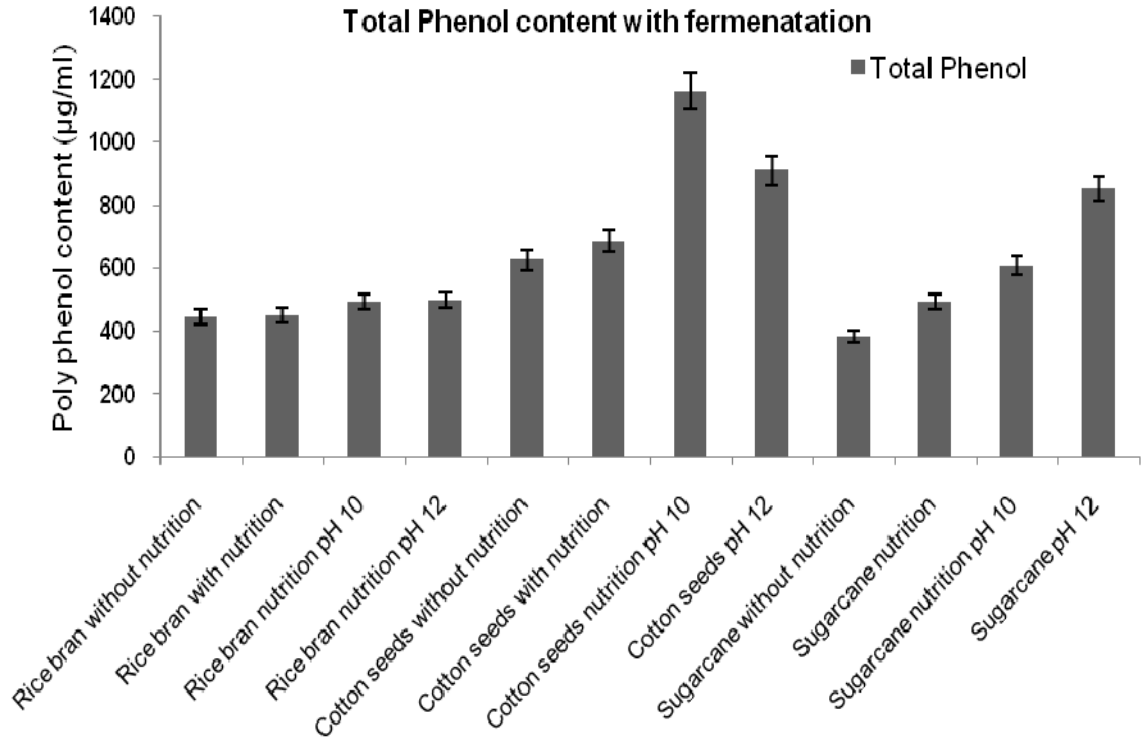

Fig. 3a. Total phenol content released from the different agro-wastes (rice bran, cotton seed, and sugarcane bagasse) under normal and alkaline stress conditions in SSF with fungus, *C. globosum.* Values are means ± S.D. (n=3)

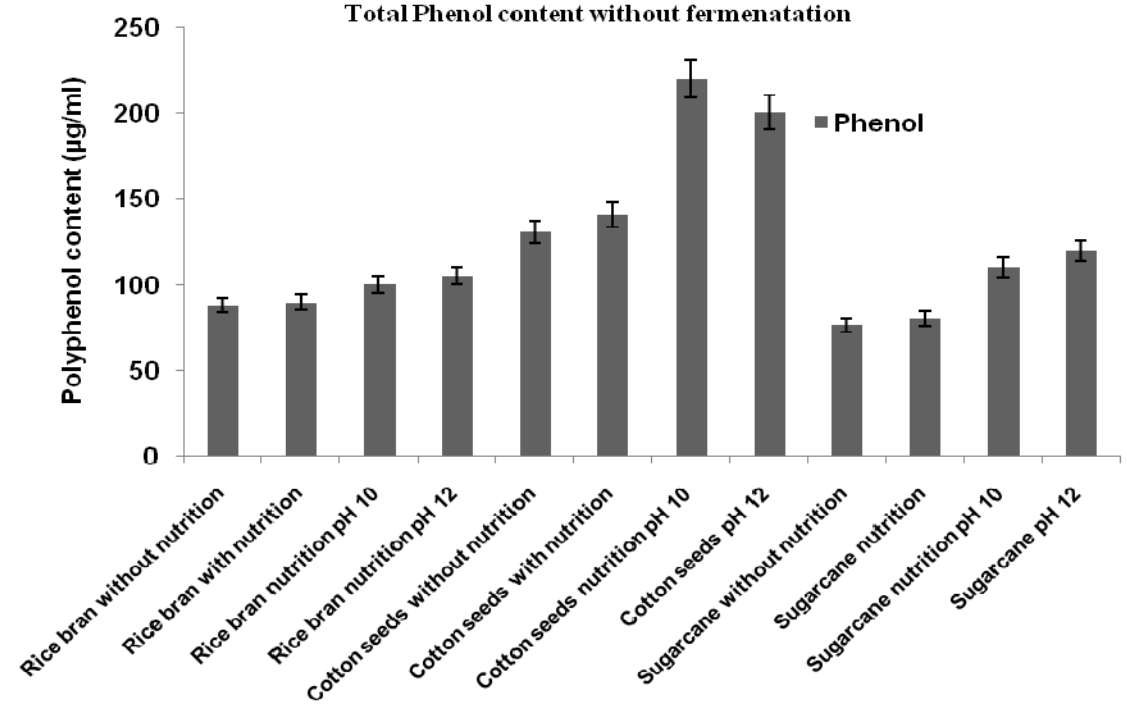

Fig 3b. Total phenol content released from the different agro-wastes (rice bran, cotton seed and sugarcane bagasse) under normal and alkaline stress conditions without fermentation. Values are means ± S.D. (n=3)

This supports the previous results of Lee et al. (2006), Adom et al. (2003), and Bhanja et al. (2008). Earlier, it was also shown that phenolic material resistant to alkali remained associated with wall polysaccharides and was not released from until over 50% of cellulose (and hemi-cellulose) had been degraded (Chesson 2006). Therefore, in the SSF system the alkaline treatment and the cellulase enzymes actively play an important role in release of phenols with more free hydroxyl groups, which can improve the antioxidant property or nutraceutical potential.

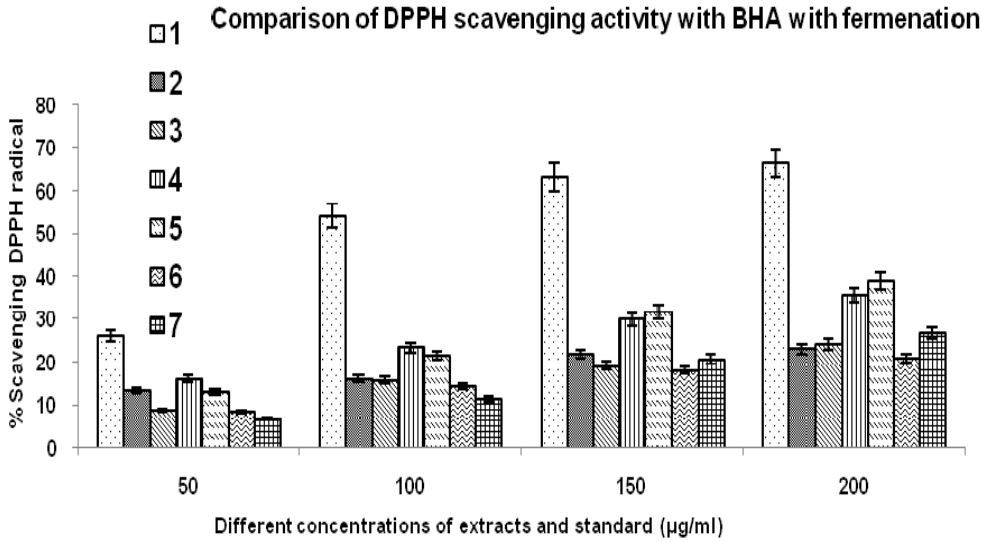

Fig. 4a. DPPH˙ scavenging activity of agro wastes compared with standard, BHA. (1) BHA (2) Rice bran nutrition pH 10, (3) Rice bran nutrition pH 12, (4) Cotton seeds nutrition pH 10, (5) Cotton seeds pH 12, (6) Sugarcane nutrition pH 10, and (7) Sugarcane pH 12

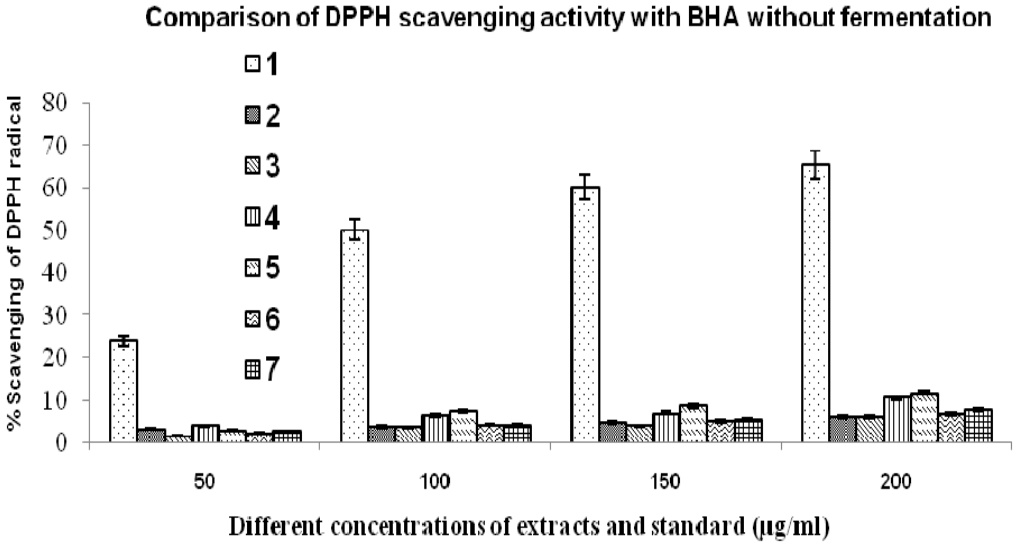

Fig. 4b. DPPH˙ scavenging activity of agro wastes compared with standard, BHA without fermentation. (1) BHA, (2) Rice bran nutrition pH 10, (3) Rice bran nutrition pH 12, (4) Cotton seeds nutrition pH 10, (5) Cotton seeds pH 12, (6) Sugarcane nutrition pH 10, and (7) Sugarcane pH 12

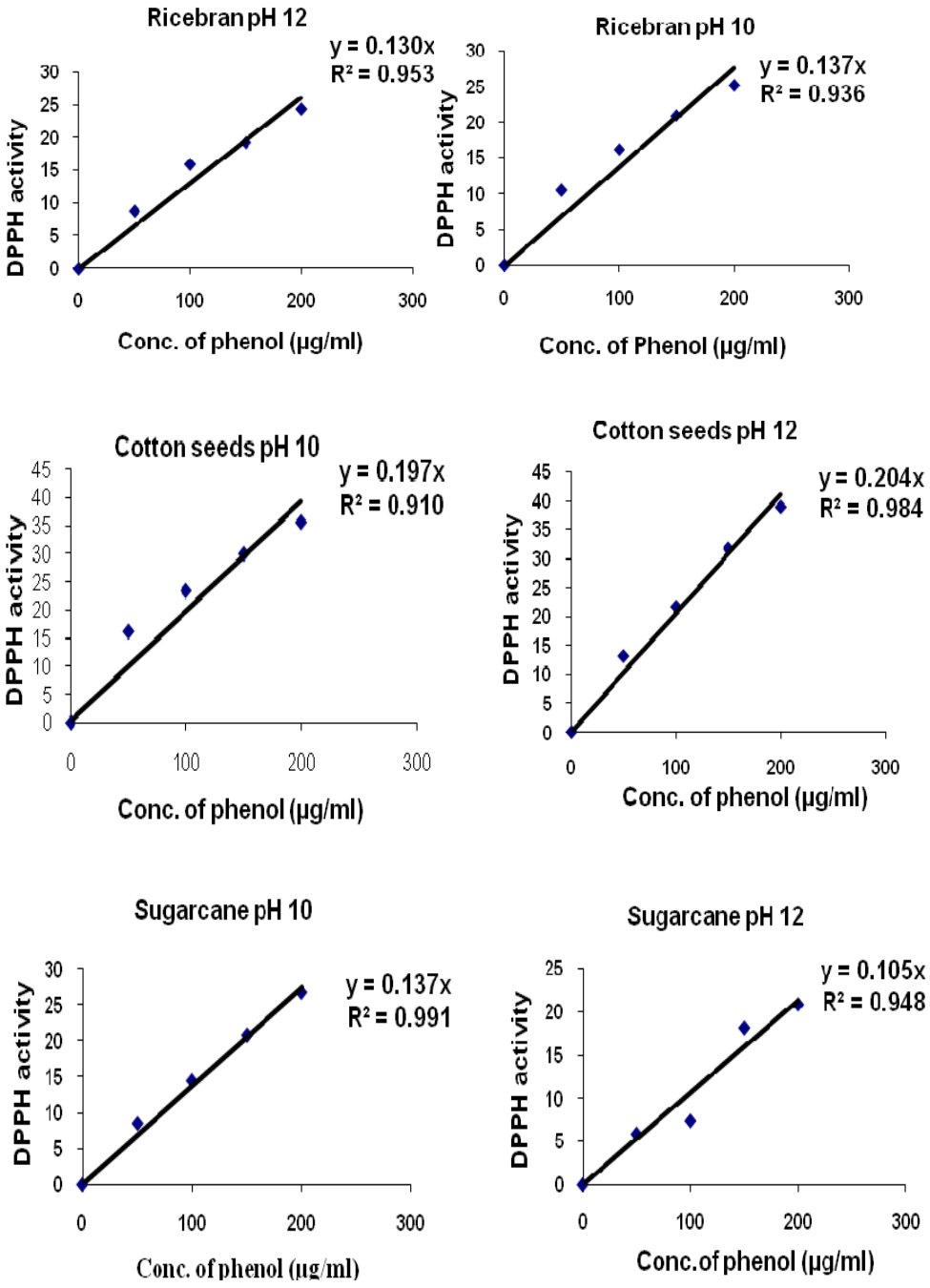

Fig. 5. Relationship between total phenolic content and DPPH˙ scavenging property of agro-wastes (rice bran, cotton seed and sugarcane bagasse) under SSF using *C. globosum.* Total phenolic content and DPPH radical scavenging activity of agro-waste fractions were highly correlated [(R^2 = 0.936 (rice bran pH 10) and 0.953 (rice bran pH 12); R^2 = 0.910 (cotton seed pH 10) and 0.984 (cotton seed pH 12); R^2 = 0.991 (sugar cane pH 10); and 0.948 (sugar cane pH 12)]

DPPH Radical Scavenging Activity

DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants (Oyaizu 1986). The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g., p-phenylene diamine, p-aminophenol), reduce and decolorise DPPH by their hydrogen donating ability (Blois 1958). The free radical-scavenging activities of alkaline stress extracts along with reference standard BHA were determined using DPPH. The alkaline stress condition showed concentration-dependent antiradical activity by inhibiting the DPPH radical (Fig. 4a). Antioxidant activity was increased with fermentation condition, compared to that of non-fermentation (Lin et al. 2006). Thus, we analysed the DPPH radical scavenging activity with the extracts of non-fermentation conditions (Fig. 4b). The results showed very much less antioxidant activity compared to that of the fermented extracts (Fig. 4b). Total phenolic content and DPPH radical scavenging activity of agro waste fractions were highly correlated (Fig. 5), providing strong evidence that the predominant source of antioxidant activity is derived from phenolic compounds in agro wastes.

CONCLUSIONS

The results of this study demonstrate that alkaline stress can result in the release of an increased level of cellulase enzymes from fungus and total phenolics from agro-wastes under SSF, and the *in vitro* antioxidant activity may be of useful in improving the bioavailable nutraceutical and nutritional properties of other agrowastes and nutraceutical ingredients. However, further investigation of individual compounds is needed, including the study of *in vivo* antioxidant activities in different antioxidant mechanisms.

ACKNOWLEDGMENTS

The authors thank Director, National Institute of Oceanography (NIO), Council of Scientific and Industrial Research (CSIR) Goa, for providing the lab facilities. This is NIO contribution (number 5016).

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Article submitted: April 30, 2011; Peer review completed: June 18, 2011; Revised version received: June 27, 2011; Accepted: July 1, 2011; Published: July 5, 2011.