The Journal of Microbiology (2011) Vol. 49, No. 1, pp. 58-65
DOI 10.1007/s12275-011-0288-x

Organic Acids Associated with Saccharification of Cellulosic Wastes During Solid-State Fermentation

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(Received August 2, 2010 / Accepted October 28, 2010)

Saccharification of five cellulosic wastes, i.e. rice husks, wheat bran, corn cobs, wheat straw and rice straw by three cellulytic fungi, i.e. *Aspergillus glaucus* MN1, *Aspergillus oryzae* MN2 and *Penicillium purpurogenum* MN3, during solid-state fermentation (SSF) was laboratory studied. Rice husks, wheat bran, and corn cobs were selected as inducers of glucose production in the tested fungi. An incubation interval of 10 days was optimal for glucose production. Maximal activities of the cellulases FP-ase, CMC-ase, and β-glucosidase were detected during SSF of rice husks by *P. purpurogenum*; however, α-amylase activity (7.2 U/g) was comparatively reduced. Meanwhile, the productivities of FP-ase, CMC-ase, and β-glucosidase were high during SSF of rice husks by *A. glaucus*; however, they decreased during SSF of corn cobs by *P. purpurogenum*. Addition of rock phosphate (RP) (75 mg P_2O_5) decreased the pH of SSF media. $(NH_4)_2SO_4$ was found to be less inducer of cellulytic enzymes, during SSF of rice husks by *A. glaucus* or *A. oryzae*; it also induced phytase production and solubilization of RP. The organic acids associated with saccharification of the wastes studied have also been investigated. The highest concentration of levulinic acid was detected (46.15 mg/g) during SSF of corn cobs by *P. purpurogenum*. Likewise, oxalic acid concentration was 43.20 mg/g during SSF of rice husks by *P. purpurogenum*.

***Keywords*:** *Aspergillus*, *Penicillium*, cellulase, organic acids, wastes

Lignocellulosic wastes are potential sources of ethanol, amino acids, and other products (Kang *et al*., 1999). Annually, over 150 billion tons of lignocellulosic materials are produced globally (Zhu *et al*., 2006). Lignocellulosic materials consist of three major components: cellulose, hemicellulose, and lignin. Cellulose is a linear homopolysaccharide of β-1,4 linked D-glucose residue; whereas, hemicellulose is a complex of polymeric carbohydrates with xylan as its major component (Badhan *et al*., 2007). Lignin is an aromatic polymer synthesized from phenylpropanoid precursors.

Cellulose is degraded by the synergistic action of three types of enzymes in the cellulase complex: endoglucanses (endo-1,4-β-glucanses, EC 3.2.1.4), cellobiohydrolases (exo-1,4-β-glucanses, EC 3.2.1.91), and β-glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21) (Kang *et al*., 1999). Several research studies have demonstrated the cooperative action between endo- and exo-glucanases during the solubilization of crystalline cellulose, and the release of various higher cello-oligosaccharides and cellobiose. β-Glucosidase completes the hydrolysis process by cleaving cello-oligosaccharides and cellobiose to glucose (Bhat and Bhat, 1997). Biosaccharification of lignocellulosic compounds has been recommended as successive bioremediation process, since several research studies have investigated saccharified cellulosic wastes during the fermentation process, and evaluated beneficial substances, such as ethanol, reducing sugars and organic acids (Lakshmikant, 1990; Bhat and Bhat, 1997; Saber *et al*., 2010). In general, the hydrolysis of lignocellulosic materials is achieved by cellulase enzymes that are produced, during the fermentation process, by bacteria or fungi. Chang *et al*. (2006) characterized the high-cellulase producing strain *A. glaucus* XC9, which was capable of an 81.9% bioconversion, of cellulose to reducing sugars rate. Low molecular weight CHO-containing compounds were found to be associated, as end products, with cellulosic hydrolysis by fungi (Cunningham and Kuiack, 1992). Saber *et al*. (2010) also found that the bioconversion of lignocellulosic materials is associated with the production of organic acids. The fermentation medium is supplemented with rock phosphate to induce the formation of organic acids, since a positive correlation was reported between the production of organic acids and the solubilization process (Saber *et al*., 2010).

Wherein, this solid-state fermentation (SSF) study has been conducted to include (i) saccharification of some Lignocellulosic wastes by some cellulytic fungi; (ii) selection of the waste products that best induce cellulase production; (iii) studying the effect of rock phosphate (RP) and $(NH_4)_2SO_4$ on cellulase production; and (iv) determination of the organic acid associated with biodegradation of the waste products.

Materials and Methods

Fungal strains

The cellulytic fungi *A. glaucus* MN1, *A. oryzae* MN2, and *Penicillium purpurogenum* MN3, were kindly obtained from the Microbiology Dept. of the Soils, Water and Environment Research Institute, Agric.

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Res. Center, Giza, Egypt. The cultures were maintained on PDA slants, and sub-cultured weekly.

Substrates and rock phosphate

Rice husks and wheat bran were purchased from a local market. Corn cobs, wheat straw, and rice straw were collected from Tag El-Ezz Agric. Res. Station, Dakahlia, Egypt. All substrates were dried at 70°C overnight, and then ground in an electric grinder. RP, containing 7.97% phosphorus (P), was kindly obtained from the Soils, Water and Environment Research Institute, Agric. Res. Center, Giza, Egypt.

Inocula preparation

Fungal organisms were inoculated on PDA plates and incubated at 28°C for 7 days. The fungal colonies were covered with 10 ml of sterile distilled water, and suspensions were made by gently probing the surface with the tip of a Pasteur pipette. Inoculum was adjusted to 3×10^6 spore/ml.

Solid-state fermentation (SSF)

The medium used in Chang *et al*. (2006) was used to provide optimum conditions for the hydrolysis of different substrates in a solid-state fermentation process. The medium was composed of 5 g of ground substrate and 15 ml of salt solution (4.0 g/L KH_2PO_4, 1.6 g/L $(NH_4)_2SO_4$, and 1.0 g/L $MgSO_4$). Inoculation was carried out using 5% (v/w) from the spore suspension of the tested fungi. The initial moisture content was adjusted to 65%, and the contents were mixed thoroughly. After incubation at 28°C, 50 ml of distilled water was added to each flask, shaken for 30 min on a rotary shaker at 140 rpm, and filtered through Whatman No.1 filter paper (Kumari *et al*., 2008). The residues of the fermented substrates were dried in an oven at 80°C to a constant weight, for the determination of the residual dry weight. The distilled water extracts of the decomposing substrates were used for the determination of pH and released glucose, using glucose oxidase kit (Spainreact Co., Spain), as well as, for the determination of soluble P by the method described in Jackson (1967). Amylase, invertase, cellulase and phytase production were also determined.

The tested parameters

The medium described above was used for (1) Screening for the best combinations of fungi and substrates; (2) studying the time course of substrate decomposition; (3) studying the effect of application of rock phosphate (75 mg P_2O_5) as the sole P source instead of KH_2PO_4; (4) investigating the effect of removing $(NH_4)_2SO_4$ from the fermentation medium; and (5) screening the organic acids associated with degradation of cellulosic materials.

Assay of amylase and invertase

Activities were assayed in a reaction mixture, which contained 0.9 ml of 1% soluble starch in a phosphate buffer (0.1 M, pH 6.5 for α-amylase and pH 5 for glucoamylase), 1% sucrose in a sodium acetate buffer (100 mM, pH 5.0 for invertase), and 0.1 ml of crude enzyme, for 15 min at 30°C. The reaction was terminated by adding 2 ml of dinitrosalicylic acid reagent (DNS), followed by incubation in a boiling water bath for 10 min. Before cooling, 1.0 ml Rochelle salt (40% sodium potassium tartarate) was added. The resulting colour, due to the reaction of DNS and reducing sugar, was measured at 540 nm (Miller, 1959). One unit of α-amylase, glucoamylase, or invertase was defined as the amount of enzyme that released 1 μmole of reducing sugar, measured as glucose or fructose per min, under the assay conditions.

Assay of cellulase

FP-ase, CMC-ase, and β-glucosidase activities were estimated by incubating 0.5 ml enzyme and 0.5 ml buffer (0.05 M citrate buffer pH 4.8) with 50 mg Whatman No. 1 filter paper, carboxymethylcellulose and 1% salicin, for 60, 30, and 15 min, respectively, at 50°C (Lakshmikant, 1990). Reducing sugars that were released in the assay mixture were measured using the DNS method (Miller, 1959). One unit of FP-ase, CMC-ase or β-glucosidase was defined as the amount of enzyme that released 1 μmole of reducing sugar, measured as glucose per min, under the assay conditions.

Assay of phytase

Phytase activity was assayed in the hydrolysate of fermented substrate, using sodium phytate as substrate (El-Sawah *et al*., 2001). One unit of phytase activity was defined as the amount of enzyme that released 1 μmole of inorganic phosphorus ml^{-1} min^{-1}.

High-performance liquid chromatography (HPLC)

Organic acid analysis was performed with high performance liquid chromatography (Agilent 1100 HPLC system), using an HyperREZ XP Carbohydrate H, 8 μm, Column, 300×7.7 mm with a mobile phase of 5 mM H_2SO_4, a flow rate of 0.6 ml/min, and a column temperature of 55°C. Injection volume of the samples used for HPLC analysis was 20 μl of the final extract. Detection was by UV absorbance at 280 nm. Detection and quantification of acetic acid was carried out using an Atlantis™ dC18 column (2.1 mm×150 mm) with a particle size of 5-μm. Two mobile phases were used to generate a linear gradient with a 0.2 ml/min flow-rate. Mobile phase A was water with 0.01% (v/v) formic acid, and mobile phase B was acetonitrile with 0.01% (v/v) formic acid. The linear gradient was from 0% to 10% B for the first 12 min, and from 10% to 50% B for the next 3 min; mobile phase B was kept at 50% for 3 min and then reduced to 0% for 1 min. The column was thermostated at 28°C, and the injection volume was 5 μl.

Standards and quantification

The organic acids were quantified by reference to the peak areas and retention times obtained, to the authentic standards for the nine organic acids in mobile phase. Oxalic acid, maleic acid, succinic acid,

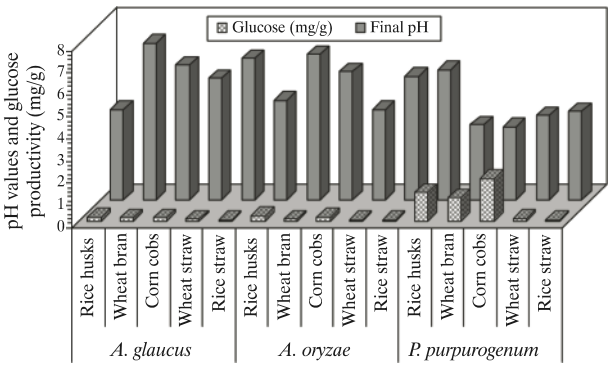

Fig. 1. pH values and glucose production of cellulytic fungi during SSF of lignocellulosic wastes.

Table 1. Time course profile of glucose production during SSF of lignocellulosic wastes

Fungus on substrate	Incubation (day)	Final pH	Loss in weight (%)	Glucose (mg/ml)
A. glaucus on rice husks	5	3.8	31	0.05
	10	4.1	36	0.18
	15	4.3	33	0.05
A. oryzae on rice husks	5	6.7	16	0.1
	10	4.5	38	0.25
	15	3.8	24	0.08
P. purpurogenum on rice husks	5	4.0	32	1.0
	10	5.9	39	1.31
	15	4.1	26	0.09
P. purpurogenum on wheat bran	5	3.5	43	0.08
	10	3.4	50	1.05
	15	3.7	35	0.08
P. purpurogenum on corn cobs	5	4.1	21	1.1
	10	3.3	27	1.92
	15	3.5	18	1.3

citric acid, ascorbic acid, itaconic acid, and acetic acid were analyzed individually at a concentration of 10 mg/ml. For levulinic acid and formic acid, the concentrations of the aqueous standard solutions were 16 mg/ml and 12 mg/ml, respectively.

Statistical analysis

Simple correlation coefficient (r) and linear regression analyses were performed to examine the relationships between individual properties, using the statistical analysis software CoStat v6.4.

Results and Discussion

Saccharification of cellulosic wastes

Selection of the best inducing substance : Saccharification of five lignocellulosic wastes by three cellulytic fungi, i.e., *A. glaucus* MN1, *A. oryzae* MN2, and *P. purpurogenum* MN3 is plotted in Fig. 1. Rice husks, corn cobs and wheat bran were the substrates that most induced glucose production. Results also indicate that the highest concentrations of glucose obtained during SSF by *P. purpurogenum* were, in descending order, 1.92, 1.31, and 1.05 mg/g substrate; for corn cobs, rice husks, and wheat bran, respectively. Whilst, reasonable values of glucose were found to be 0.18 and 0.25 mg/g, during SSF of rice husks by *A. glaucus* and *A. oryzae*, respectively. The different ratios of glucose concentration, which were recorded during SSF of different cellulosic wastes, suggest that the synthesis of individual cellulase enzymes is regulated independently; furthermore, it suggests that enzyme quality and quantity also, substantially, depend upon the type of inducers used (Trivedi and Rao, 1980; Margartis and Merchant, 1986). Likewise, wheat bran was found to maximize the yield production of cellulase enzymes by *A. glaucus* xc9; whereas, maximum growth and yield of cellulases have been obtained by *A. niger* kk2 during growth on ground rice straw (Kang *et al*., 1999; Chang *et al*., 2006). Furthermore, Abul Hossain *et al*. (2004) stated that biohydrolysis of rice straw has been achieved by dual inoculum of *T. harzianum* with *Mucor hiemalis* or *Phanerochaete chrysosporium*. Saccharification of agro-wastes has been conducted during growth of *Penicillium* and *Aspergillus* strains under solid-state fermentation conditions (Considine *et al*., 1988; Kang *et al*., 2004). Production of cellulose and hemicellulose-degrading enzymes, by filamentous fungi cultivated on wet oxidized wheat straw, and their applications in biotechnology has previously been reported (Lutzen *et al*., 1983; Kuhad and Singh, 1993; Bajpai, 1999; Thygesen *et al*., 2003). Generally, the enzymatic hydrolysis of cellulosic wastes by fungal enzymes has been suggested as a feasible alternative procedure for the conversion of cellulosic wastes into fermentable sugars and ethanol (Oksanen *et al*., 2000; Shin *et al*., 2000).

Time course study of saccharification : The time course profile of saccharification of selected cellulosic wastes by the aforementioned fungi was studied (Table 1). Data clearly show that a 10 day incubation period was the optimum for highest glucose production during SSF of the wastes under study. Moreover, no correlation was found among the three tested parameters: glucose production, pH values, and weight

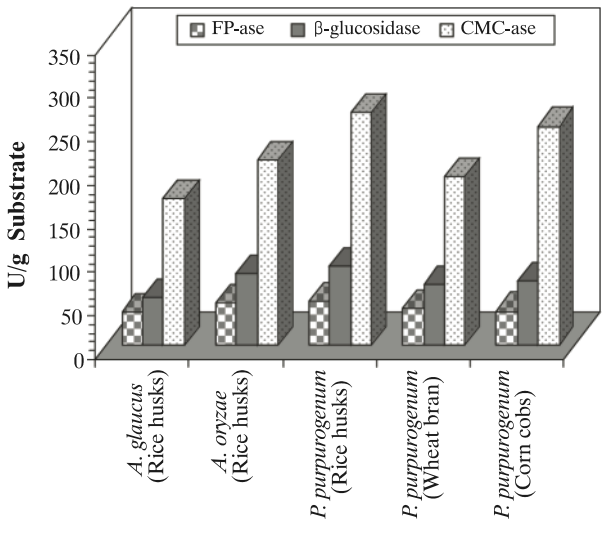

Fig. 2. Cellulolytic activity of the tested fungi during SSF of different wastes

Table 2. Correlation coefficient among the tested parameters during course of the experiment

	Incubation period	Final pH	Loss in weight
Final pH	-0.243 ^{ns}		
Loss in weight	-0.063 ^{ns}	-0.249 ^{ns}	
Glucose	-0.098 ^{ns}	-0.149 ^{ns}	-0.059 ^{ns}

^{ns} not significant

loss. It is the combined action of FP-ases, which represent overall cellulolytic activity (Margaritis and Merchant, 1986); endoglucanases (CMC-ase), which perform the first, and an essential, step in the decomposition process of cellulolytic substrates (Lynd *et al*., 2002); and β-glucosidase activity, that results in the formation of glucose as the major product. A major product that is directly converted into different organic molecules. The difference in values of glucose production, evident in this report, may be due to the difference in structure and characteristics of cellulase produced by fungal strains (Chang *et al*., 2006).

Earlier investigation of Kang *et al*. (2004) studied the saccharification of rice straw and wheat bran by *A. niger* KK2. The maximum FP-ase activity obtained in the study was 19.5 IU/g, after 4 days. Whereas, maximum CMC-ase (129 IU/g), β-glucosidase (100 IU/g), and xylanase (5,070 IU/g) activities were found after 5 to 6 days of fermentation. Moreover, Liua and Ørskovb (2000) found that *P. funicalosum* produces cellulase during saccharification of stem pre-treated rice straw, after fermentation for one to three weeks. 24 days was the optimum incubation interval for cellulase production and biodegradation of wheat straw by white-rot fungi (Dinis *et al*., 2009); whereas, 14 days (Sridevi *et al*., 2009) and 45 days (Lakshmikant, 1990) were reported for maximum production of cellulase during SSF of wheat straw.

All combinations of correlation coefficient analysis, among incubation period, final culture pH, glucose concentration, and weight loss of the tested materials, were not significant (Table 2); although the process obviously resulted in the production of glucose, as well as a marked reduction in the weight of the substrate. This may be related to the consumption of the resulting glucose through the biosynthesis of different biological compounds; e.g., organic acids (Saber *et al*., 2010)

Cellulase enzymes activity : The data plotted in Fig. 2 illustrate the relative activity of FP-ase, CMC-ase, and β-glucosidase enzymes, produced during the saccharification of rice husks, wheat bran, and corn cobs by the cellulytic fungi studied. The data obtained show that *P. purpurogenum* exhibited the highest FP-ase activity (50.5 U/g) during SSF of rice husks. The highest values of CMC-ase and β-glucosidase, 267.8 and 90.2 U/g, respectively, were produced during SSF of rice husks by *P. purpurogenum*; but, *A. glaucus* produced less FP-ase (38.6 U/g) during SSF of rice husks.

Lakshmikant (1990) studied the biodegradation of cellulosic wastes, the maximum activity of endoglucanase, exo-glucanase, and FP-ase was associated with *C. globosum*, whereas β-glucosidase activity was maximal in *A. niger*, *T. konningii*, and *T. roseum*. Furthermore, Badhan *et al*. (2007) found that rice straw supported maximal production of β-glucosidase (7.48 IU/g), FP-ase (2.44 IU/g), and endoglucanase (32 IU/g) by *Myceliophthora* sp. The production of cellulase enzyme by *T. viride*, *Trichoderma* sp., and *A. niger* has also been studied (Esterbauer *et al*., 1991; Kim *et al*., 1997; Yan and Zhang, 1999). In general, the efficiency of fungi in degrading cellulosic materials depends upon the presence of complete cellulase activity in an adequate quantity (Canevascini and Gattlen, 1981).

Amylase and invertase activities : Amylolytic activity of *A. glaucus*, *A. oryzae*, and *P. purpurogenum*, during SSF of the

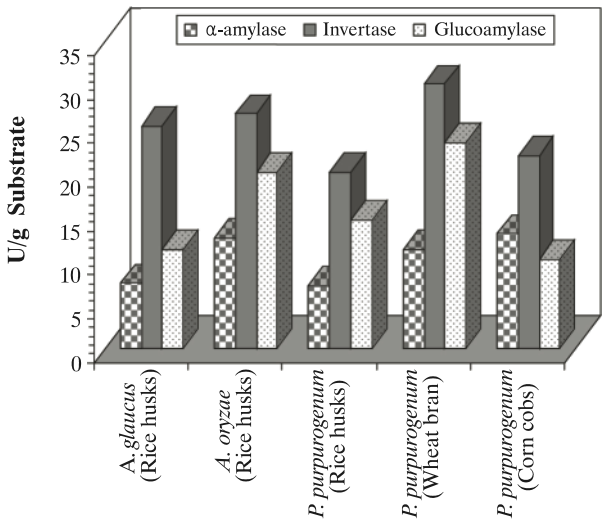

Fig. 3. Amylase, glucoamylase and invertase activities of cellulytic fungi, during SSF of lignocellulosic materials.

Table 3. Phytase activity and soluble P concentration as a function of RP (75 mg P_2O_5) addition

Fungus/substrate	Final pH	Phytase (Unit/g)	Soluble P (μg/g)
A. glaucus/ Rice husks	4.0	10.3	109.2
A. oryzae/ Rice husks	4.1	21.2	123.1
P. purpurogenum/ Rice husks	3.1	7.1	114
P. purpurogenum/ Wheat bran	3.2	17.2	100.3
P. purpurogenum/ Corn cobs	3.2	16.1	92.2

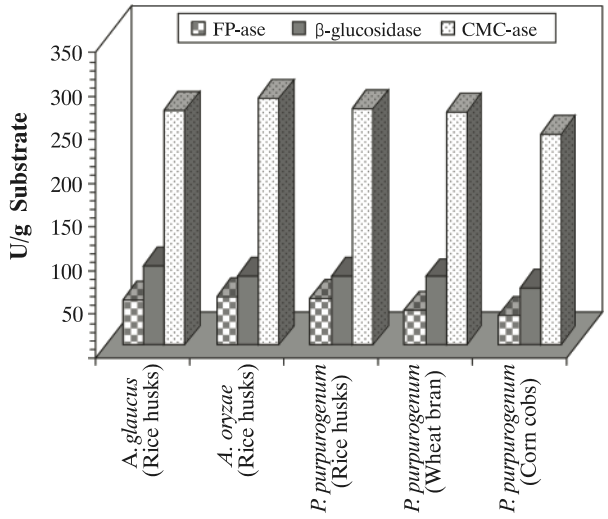

Fig. 4. Cellulase activity as a function of RP (75 mg P_2O_5) addition.

selected wastes is summarized in Fig. 3. The data obtained show that the highest yield of α-amylase, 13.1 U/g, was achieved during SSF of corn cobs by *P. purpurogenum*. *A. oryzae* exhibited the highest value of α-amylase activity (12.6 U/g) during SSF of rice husk. Whereas, highest glucoamylase and invertase activities, 23.5 and 30.2 U/g, were achieved during SSF of wheat bran by *P. purpurogenum.* The production of invertase using agro-industrial residues has also been reported using *A. ochraceus*; highest enzyme production was achieved with sugar cane bagasse and under SSF conditions (Guimaraes *et al*., 2007). *A. caespitosus* was grown in wheat bran or soybean bran, and produced 117.4 U/g and 28.3 U/g, of invertase, respectively (Alegre *et al*., 2009).

Cellulases and phytase activities as a function of RP

The pH values, cellulase and phytase activities, and soluble P concentrations, as a function of RP, are presented in Table 3 and Fig. 4. pH values were found to decreased during SSF. FP-ase, CMC-ase and β-glucosidase activities were high (50.5, 267.8, and 90.2, respectively), during the SSF of rice husks by *A. glaucus*; whereas, they were comparatively reduced during SSF of corn cobs by *P. purpurogenum*, with values of 32.3, 240.9, and 64.3 U/g, respectively. The phytase enzyme exhibited high activity (21.2 U/g) during SSF of rice husks by *A. oryzae*; soluble P concentration was highest under these conditions. Contrarily, soluble P concentration was lowest in the case of SSF of corn cobs by *P. purpurogenum*.

Biosolubilization of RP was found to be dependent upon RP structure complexity, particle size, phytase and organic acids secreted by microorganisms. Biosolubilization is dependent upon the kind of organic acids mainly citric, oxalic and succinic, not the quantity and is not always accompanied by a drop in pH (Singh and Amberger, 1998; Vassilev *et al*., 2007; Kumari *et al*., 2008).

Cellulases and phytase activities as a function of omitting $(NH_4)_2SO_4$

In this report, the omission of $(NH_4)_2SO_4$ from SSF media, to promote lignocellulosic degradation by the tested fungi and minimize production costs, was studied. The influence of removing $(NH_4)_2SO_4$ from the culture media on pH values, cellulase and phytase activities, and soluble P concentrations is shown in Table 4 and Fig. 5. The data obtained show that the activities of the cellulases (FP-ase, CMC-ase, and β-glucosidase) increased in the absence of $(NH_4)_2SO_4$ during SSF of rice husks by *A. glaucus* or *A. oryzae*; moreover, both FP-ase and β-glucosidase activities increased during SSF of rice husks by *P. purpurogenum*. These data coincide with the findings of Chang *et al*. (2006), who found that peptone provided the maximum production of cellulase by *A. glaucus* CX9, among the N sources studied; followed by organic nitrogen, nitrate and ammonium compounds. Conversely, Kesker (1992) studied cellulase (CMC-ase, FPC-ase, and β-glucosidase) production by *P. janthinellum* during fermentation of cellulose powder

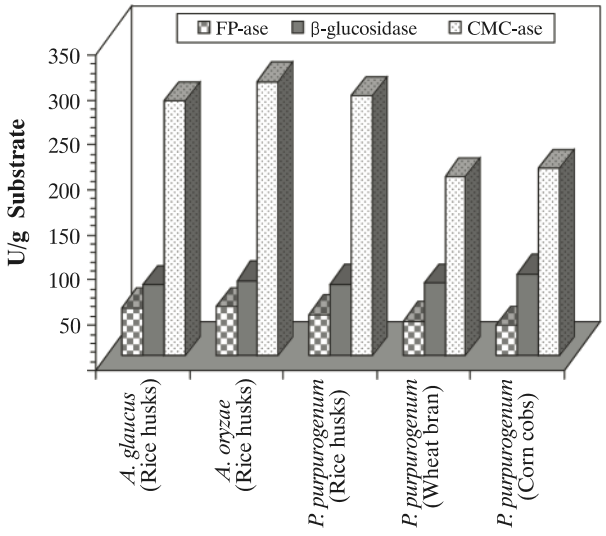

Fig. 5. Cellulase activity as a result of removing $(NH_4)_2SO_4$.

Table 4. Phytase activity and soluble P concentration as a function of omitting $(NH_4)_2SO_4$

Fungus/substrate	Final pH	Phytase (Unit/g)	Soluble P (μg/g)
A. glaucus/ Rice husks	4.3	18.2	123.1
A. oryzae/ Rice husks	4.2	26.4	136.1
P. purpurogenum/ Rice husks	3.9	21.2	130.6
P. purpurogenum/ Wheat bran	3.5	18.5	112.1
P. purpurogenum/ Corn cobs	3.7	20.8	121.6

Table 5. Simple correlation coefficient test results for phytase, soluble P concentration and final pH during the composting of the tested substrates with RP

	Final pH	Phytase
Phytase	0.525^{ns}	
Soluble P	0.735*	0.571^{ns}

^{ns} not significant
* significant at $P \leq 0.05$

and wheat bran; growth and enzyme production were comparatively poor if no inorganic nitrogen source was included in the medium. NH_4^+ salts, with or without urea, provided good cellulase activities, but nitrate was poorer, and urea alone was not effective in inducing enzyme production. $(NH_4)_2SO_4$, at 0.7% (w/v), induced optimum enzyme activity. Another study pointed out that corn steep liquor, as nitrogen source, maximized β-glucosidase activity (8.3 IU/ml) within 6-8 days of the fermentation process of lignocellulosic wastes by *A. niger* kk2 (Kang *et al*., 1999). Moreover, the values for phytase activity decreased in the presence of $(NH_4)_2SO_4$, during SSF of tested wastes by cellulytic fungi, and consequently lower concentrations of soluble P were obtained.

Analysis of correlation coefficients (Table 5) revealed that RP solubilization was significantly related to pH, but phytase production was not. Reddy *et al*. (2002) reported that phosphate solubilization was not always accompanied by a drop in pH, but always showed the largest production of acids. Phosphate-solubilizing microorganisms have been distinguished by their relative abilities to dissolve complex phosphates [e.g., rock phosphate (RP)], this activity is frequently attributed to the production of organic acids, which, are also reported to be the end products of cellulosic hydrolysis by fungi (Singh and Amberger, 1998).

Quantitative determination of organic acids

Saccharification and/or biodegradation of most lignocellulosic wastes are associated with organic acids. So, organic acids were detected in fermented products, as a result of hydrolysis and biochemical activity of fungi; i.e. citric, itaconic, oxalic, succinic, levulinic, and maleic acids were produced during fermentation process of cellulosic wastes by *A. niger*, *A. terreus*, and *P. chrysogenium* (Andersson and Hedlund, 1983; Singh and Amberger, 1998; Kurbanoglu and Kurbanoglu, 2004; Magnuson and Lasure, 2004; Saber *et al*., 2010).

The hydrolysates of saccharified lignocellulosic wastes were examined for associated organic acids by HPLC (Table 6). Levulinic (46.15 mg/g) and oxalic (43.2 mg/g) acids were detected during SSF of corn cobs and rice husks by *P. purpurogenum*, respectively. Likewise, succinic acid was detected (16.20 mg/g) during SSF of wheat bran by *P. purpurogenum*. Itaconic acid was detected, 8.42 and 4.21 mg/g, during SSF of rice husks by *A. glaucus* and *A. oryzae*, respectively. Contrarily, acetic and citric acids were detected at lower concentrations, 1.29 and 1.35 mg/g, respectively, during SSF of rice husks by *A. glaucus.* Additionally, formic (1.21 mg/g) and maleic (0.014 mg/g) acids were detected only during SSF of rice husks by *P. chrysogenium.* These results are comparable with previous studies that showed oxalic and citric acids are major components of the organic acids produced by *P. bilaii*. Citric acid production is promoted under nitrogen-limited conditions, while oxalic acid production is promoted under carbon-limited conditions (Cunningham and Kuiack, 1992). Itaconic acid was produced during the fermentation process, by *A. terreus* (Magnuson and Lasure, 2004). Fumaric, succinic and maleic acids were also detected during the biohydrolysis of rice straw with RP (Kumari *et al*., 2008). Generally, the linear regression analysis, comparing total organic acids and loss in dry weight of the tested substrates (Fig. 6), did not exhibit a significant correlation (r=0.08, ns).

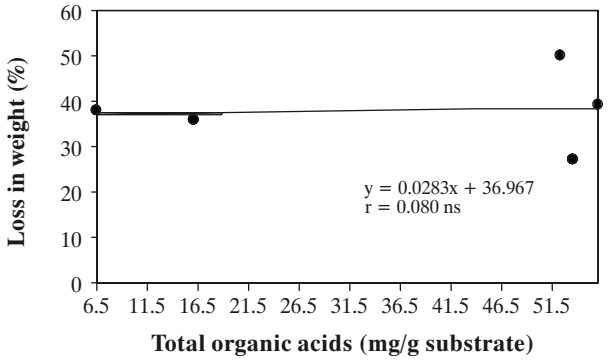

Fig. 6. Linear regression showing the relation between total organic acids and loss of dry weight of the tested substrates.

Table 6. HPLC determination of organic acids (mg/g substrate) associated with SSF of cellulosic wastes

Organic acid	*A. glaucus* on rice husks	*A. oryzae* on rice husks	*P. purpurogenum* on rice husks	*P. purpurogenum* on wheat bran	*P. purpurogenum* on corn cobs
Acetic	1.290	UD	2.448	0.810	UD
Ascorbic	0.837	0.251	0.018	0.050	0.100
Citric	1.358	0.179	UD	0.266	UD
Formic	UD	UD	1.210	UD	UD
Itaconic	8.420	4.210	4.250	2.200	UD
Levulinic	4.070	1.720	2.480	8.120	46.150
Maleic	UD	UD	0.014	UD	UD
Oxalic	UD	UD	43.200	24.200	UD
Succinic	UD	0.142	1.820	16.200	6.820
Total	15.975	6.502	55.440	51.846	53.070

Conclusion

The fungal strains studied (*A. glaucus* MN1, *A. oryzae* MN2 and *P. purpurogenum* MN3) demonstrated the ability to saccharify some lignocellulosic waste products during SSF. Rice husks, wheat bran and corn cobs were the best inducers for enzyme activity in the tested fungi. FP-ase, CMC-ase, and β-glucosidase were produced in appreciable amounts during SSF. The highest concentration of levulinic acid (46.15 mg/g) was produced during SSF of corn cobs by *P. purpurogenum* MN3. Likewise, oxalic acid was detected at a concentration of 43.2 mg/g.

SSF technique was found to be more economical, and could be a feasible procedure for commercial production of organic acids by cellulytic fungi through the process of cellulosic waste product fermentation.

Acknowledgements

The authors would like to express about their sincere thanks to Prof. Dr. Wesam Ismail Saber, Department of Microbiology, Soils, Water and Environment Research institute, ARC, Giza, Egypt for providing the fungal strains and critical review of the manuscript.

References

Abul Hossain, M., F.R Ahmadun, and M.Z. Alam. 2004. Evaluation of solid-state bioconversion of domestic waste water sludge as a promising environmental-friendly disposal technique. *Water Res*. 38, 4143-4152.

Alegre, A.C.P., M.L.T.M. Polizeli, H.F. Terenzi, J.A. Jorge, and L.H.S. Guimarães. 2009. Production of thermostable invertases by *Aspergillus caespitosus* under submerged or solid state fermentation using agroindustrial residues as carbon source. *Brazil J. Microbiol*. 40, 612-622.

Andersson, R. and B. Hedlund. 1983. HPLC analysis of organic acids in lactic acid fermented vegetables. *Z Lebensm Unters Forsch*. 176, 440-443.

Badhan, A.K., B.S. Chadha, J. Kaur, H.S. Saini, and M.K. Bhat. 2007. Production of multiple xylanolytic and cellulolytic enzymes by thermophilic fungus *Myceliophthora* sp. IMI 387099. *Bioresour. Technol*. 98, 504-510.

Bajpai, P. 1999. Application of enzymes in the pulp and paper industry. *Biotechnol. Prog*. 15, 147-157.

Bhat, M.K. and S. Bhat. 1997. Cellulose degrading enzymes and their potential industrial applications. *Biotechnol. Adv*. 15, 583-630.

Canevascini, G. and C. Gattlen. 1981. A comparative investigation of various cellulase assay procedures. *Biotechnol. Bioeng*. 23, 1573-1590.

Chang X., L. Minnan, W. Xiaobing, X. Huijuan, C. Zhongan, Z. Fengzhang, and X. Liangshu. 2006. Screening and characterization of the high-cellulase-producing strain *Aspergillus glaucus* XC9. *Front. Biol. China* 1, 35-40.

Considine, P.J., A. O'Rorke, T.J. Hackett, and M.P. Coughlan. 1988. Hydrolysis of beet pulp polysaccharides by extracts of solid state cultures of *Penicillium capsulatum*. *Biotechnol. Bioeng*. 31, 433-438.

Cunningham, J.E. and C. Kuiack. 1992. Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. *Appl. Environ. Microbiol*. 58, 1451-1458.

Dinis, M.J., R.M.F. Bezerra, F. Nunes, A.A. Dias, C.V. Guedes, L.M.M. Ferreira, J.W. Cone, G.S.M. Marques, A.R.N. Barros, and M.A.M. Rodrigues. 2009. Modification of wheat straw lignin by solid state fermentation with white-rot fungi. *Bioresour. Technol*. 100, 4829-4835.

El-Sawah, M.M.A., H.H. El-Rafey, and W.I.A. Saber. 2001. Reduction of phytic acid content in soy bean by *A. niger* phytase immobilized into gelatin film by cross-linking. *J. Agric Sci. Mansoura Univ*. 26, 5165-5175.

Esterbauer, H., W. Steiner, I. Labudova, A. Hermann, and M. Hayn. 1991. Production of *Trichoderma* cellulase in laboratory and pilot scale. *Bioresour. Technol*. 36, 51-65.

Guimaraes, L.H.S., H.F. Terenzi, M.L.T.M. Polizeli, and J.A. Jorge. 2007. Production and characterization of a thermostable extracellular β-D-fructofuranosidase produced by *Aspergillus ochraceus* with agroindustrial residues as carbon sources. *Enzyme Microb. Technol.* 42, 52-57.

Jackson, M.L. 1967. Soil chemical analysis. Prentice Hall of India, New Delhi, India.

Kang, S.W., E.H. Ko, J.S. Lee, and S.W. Kim. 1999. Over-production of β-glucosidase by *Aspergillus niger* mutant from lignocellulosic biomass. *Biotechnol. Lett*. 21, 647-650.

Kang, S.W., Y.S. Park, J.S. Lee, S.I. Hong, and S.W. Kim. 2004. Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. *Bioresour. Technol*. 91, 153-156.

Kesker, S.S. 1992. High activity xylanase from thermotolerant *Streptomyces* T7, cultural conditions and enzyme properties. *Biotechnol. Lett*. 14, 481-486.

Kim, S.W., S.W. Kang, and J.S. Lee. 1997. Cellulase and xylanase production by *Aspergillus niger* KKS in various bioreactors. *Bioresour. Technol*. 59, 63-67.

Kuhad, R.C. and A. Singh. 1993. Lignocellulose biotechnology: current and future prospects. *Crit. Rev. Biotechnol*. 13, 151-172.

Kumari, A., K.K. Kapoor, B.S. Kundu, and R.K. Mehta. 2008. Identification of organic acids produced during rice straw decomposition and their role in rock phosphate solubilization. *Plant Soil Environ*. 54, 72-77.

Kurbanoglu, E.B. and N.I. Kurbanoglu. 2004. Ram horn peptone as a source of citric acid production by *Aspergillus niger*, with a process. *J. Ind. Microbiol. Biotechnol*. 31, 289-294.

Lakshmikant. 1990. Cellulose degradation and cellulase activity of five cellulytic fungi. *World J. Microbiol. Biotechnol*. 6, 64-66.

Liua, J.X. and E.R. Ørskovb. 2000. Cellulase treatment of untreated and steam pre-treated rice straw effect on *in vitro* fermentation characteristics. *Animal Feed Sci. Technol*. 88, 189-200.

Lutzen, N.W., M.H. Nielsen, K.M. Oxiboell, M. Schiilein, and B.S. Olessen. 1983. Cellulase and their application in the conversion of lignocellulose to fermentable sugar. *Philos. Trans. R. Soc. London.* 300, 283.

Lynd, L.R., P.J. Weimer, W.H. van Zyl, and I.S. Pretorius. 2002. Microbial cellulose utilization: Fundamental and biotechnology. *Microbiol. Mol. Biol. Rev.* 66, 506-577.

Magnuson, J.K. and L.L. Lasure. 2004. Organic acid production by filamentous fungi, pp. 307-340. *In* Jan and L. Lange (eds.), Advances in fungal biotechnology for industry, agriculture and medicine. Kluwer Academic, Plenum Publishers.

Margartis, A. and R.F. Merchant. 1986. Optimization of fermentation conditions for thermostable cellulase production by *Thielavia terrestris*. *J. Ind. Microbiol. Biotechnol*. 1, 149-156.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem*. 31, 426-428.

Oksanen, T., J. Pere, L. Paavilainen, J. Buchert, and L. Viikari. 2000. Treatment of recycled kraft pulps with *T. reesei* hemicellulases and cellulases. *J. Biotechnol*. 78, 39-48.

Reddy, M.S., S. Kumar, K. Babita, and M.S. Reddy. 2002. Biosolubilization of poorly soluble rock phosphates by *Aspergillus tubingensis* and *Aspergillus niger*. *Bioresour. Technol*. 84, 187-189.

Saber, W.I.A., N.El. El-Naggar, and S.A. AbdAl-Aziz. 2010. Bioconversion of Lignocellulosic wastes into organic acids by cellulolytic rock phosphate-solubilizing fungal isolates grown under solid-state fermentation conditions. *Research J. Microbiol*. 5, 1-20.

Shin, C.S., J.P. Lee, J.S. Lee, and S.C. Park. 2000. Enzyme production of *Trichoderma reesei* Rut C-30 on various lignocellulosic substrates. *Appl. Biochem. Biotechnol*. 84-86, 237-245.

Singh, C.P. and A. Amberger. 1998. Organic acids and phosphorus solubilization in straw composted with rock phosphate. *Bioresour. Technol*. 63, 13-16.

Sridevi, A., G. Narasimha, and B.R. Reddy. 2009. Production of cellulase by *Aspergillus niger* on natural and pretreated lignocellulosic wastes. The *Internet J. Microbiol*. Vol. 7(1). Available online at: http://www.ispub.com

Thygesen, A., A.B. Thomsen, A.S. Schmidt, H. Jfrgensen, B.K. Ahring, and L. Olsson. 2003. Production of cellulose and hemicellulose degrading enzymes by filamentous fungi cultivated on wet-oxidized wheat straw. *Enzyme Microb. Technol.* 32, 606-615.

Trivedi, L.S. and K.K. Rao. 1980. Cellulase induction in *Aspergillus fumigatus* M 216. *Indian J. Exp. Biol*. 18, 240-242.

Vassilev, N., M. Vassileva, V. Bravo, M.F. Serrano, and I. Nikolaeva. 2007. Simultaneous phytase production and rock phosphate solubilization by *Aspergillus niger* grown on dry olive wastes. *Ind. Crops Prod*. 26, 332-336.

Yan, Y.Q. and S.Q. Zhang. 1999. Studies on the cellulase producer. *Trichoderma viride* T-99. *Appl. Environ. Biol*. 5, 200-203.

Zhu, S., Y. Wu, Z. Yu, X. Zhang, H. Li, and M. Gao. 2006. The effect of microwave irradiation on enzymatic hydrolysis of rice straw. *Bioresour. Technol*. 97, 1964-1968.