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Bioconversion of Lignocellulosic Wastes into Organic Acids by Cellulolytic Rock Phosphate-Solubilizing Fungal Isolates Grown under Solid-State Fermentation Conditions

[1]W.I.A. Saber, [2]Noura El-Ahmady El-Naggar and [3]Samia AbdAllah AbdAl-Aziz
[1]Department of Microbiology, Soils, Water and Environment Research Institute, Agricultural Research Center, Giza, Egypt
[2]Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt
[3]Department of Nucleic Acids Research, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt

Abstract: Two sequenced screening trials were carried out on 21 fungi isolated from decayed rice straw. The aim was to obtain fungal isolates with cellulolytic activity and rock phosphate (RP) solubilization ability for employing them in organic acid production from rice or wheat straw. *Penicillium*, *Aspergillus* and *Stachybotrys* were the predominant isolated genera. On the base of cellulase (filter paperase, carboxymethyl cellulase and β-glucosidase) production, RP solubilization efficiency and antagonism tests, two fungi were selected and molecularly identified as *A. niger* GU 295947 and *P. chrysogenum* GU 295948. Under solid-state fermentation (SSF) conditions of rice straw with RP, the maximum cellulase production and RP solubilization were recorded after 4 weeks incubation in the presence of 75 mg P_2O_5 from RP and 7.5% (v/w) fungal inoculum. Applying these fermentation conditions on rice and wheat straw, using individual or dual inoculation of both fungi led to more than 40% loss in the weight of fermented straw, this was accompanied with the releasing of glucose and soluble phosphorus in the hydrolysate of fermented straw. Using high-performance liquid chromatography, the resulted organic acids by *A. niger* GU 295947 and/or *P. chrysogenum* GU 295948 in the hydrolysate of SSF were investigated. Acetic, ascorbic, citric, formic, itaconic, levulinic, maleic, oxalic and succinic acids were detected. Their presence and concentrations were varying according to the substrate and the microorganism. The most noticeable thing was the broadspectrum varieties and the higher concentrations of produced organic acids when rice or wheat straw was used in combination with RP. Oxalic acid was the highest (40.0 mg g^{-1} straw) detected organic acid in the hydrolysate of fermented straw with RP by *P. chrysogenum* GU 295948. This study suggests production of organic acids especially, oxalic and succinic acids as the major organic molecules by such fermentation.

Key words: Cellulase, *Aspergillus niger*, *Penicillium chrysogenum*, organic acids, rock phosphate, molecular identification

Corresponding Author: Dr. Noura El-Ahmady Ali El-Naggar, Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt

INTRODUCTION

Lignocellulosic materials is one of the most abundant natural complex organic carbons in form of plant biomass, which is highly renewable natural resource in the world, the annual production of lignocellulosic materials is over 150 billion tons on the earth (Zhu *et al*., 2006). It consists of three major components; cellulose, hemicellulose and lignin. Cellulose is linear homopolysaccharide of β-1,4 linked D-glucose residue, whereas hemicellulose exhibits an amorphous, branched structure and contains both five- and six-carbon sugars (i.e., xylose, mannose, galactose, arabinose and glucose). Lignin is an aromatic polymer synthesized from phenylpropanoid precursors, in general, lignin contains three aromatic monomers (coniferyl, sinapyl and paracoumaryl alcohols), in addition to large amounts of phenolic acids such as paracoumaric and ferulic acid, which are esterified to alcohol groups of each other and to other alcohols, lignin is further linked to both hemicelluloses and cellulose (Howard *et al*., 2003; Chena *et al*., 2006; Badhan *et al*., 2007). The full utilization of this natural resource may be important in maintaining sustainable social development. The most promising approach is to convert these wastes by enzymatic hydrolysis into useful chemicals (e.g., polyphenol and organic acids) and solvents (e.g., acetone and butanol) (Kuhad and Singh, 1993; Kang *et al*., 2004). Extensive researches have been carried out in this area in last two decades. The conversion includes two sub-processes; hydrolysis of lignocellulosic materials to fermentable sugars and then fermentation of sugars to target products (Curreli *et al*., 2002; Sun and Cheng, 2002; Zhu *et al*., 2006). The hydrolysis is usually catalyzed by cellulase and the fermentation is carried out by suitable microorganisms.

Nature is abound with microorganisms that can produce cell wall degrading enzymes to solubilize these complex components to simple molecules for completing the carbon cycle. The hydrolysis of cellulose is accomplished by components of cellulase including (1) randomly acting endoglucanse or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4) that cleaves the internal β-1,4-glucosidic bonds, (2) exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), which act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products, exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure and (3) β-glucosidase or β-glucoside glucohydrolases (EC 3.2.1.21) that hydrolyze soluble cellodextrins and cellobiose into glucose units (Kang *et al*., 1999; Lynd *et al*., 2002; Badhan *et al*., 2007).

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 as the end products of cellulosic hydrolysis by fungi (Asea *et al*., 1988; Cunningham and Kuiack, 1992; Singh and Amberger, 1998). There are many applications of organic acids, e.g., citric acid is used in the pharmaceutical, food and beverage industries as an acidifying and flavor enhancing agent, oxalic acid's main applications include cleaning and bleaching, about 25% of produced oxalic acid is used as a mordant in dyeing processes, meanwhile, itaconic acid is used exclusively in the polymer industry where it is employed as a co-monomer for certain products, an ingredient for the manufacture of synthetic fibers, coatings, adhesives, thickeners and binders (Magnuson and Lasure, 2004). To date, the largest commercial quantities of fungal organic acids (citric acid in especial) are prepared by fermentation of glucose or sucrose by *A. niger*. Conventional substrates based on starch or sucrose are not available in sufficient

quantities and at reasonable prices to provide the feedstock for the production of chemical bulk products. Lignocellulosic materials are an attractive alternative since, they are abundant and usually low-priced (Andersson and Hedlund, 1983). However, there is scanty information on types of organic acids produced during decomposition of organic wastes (Kumari *et al*., 2008).

The present study was to find out efficient cellulolytic RP-solubilizing fungal isolates in order to engage them in the biodegradation of nonconventional substrates i.e., rice and wheat straw during fermentation with RP for employing them in practical bioconversion of lignocellulosic wastes into organic acids. The most efficient isolates were molecularly identified.

MATERIALS AND METHODS

Straw and Rock Phosphate (RP)

Rice and wheat straw were collected from Tag El-Ezz Agric. Res. Station, Dakahlia, Egypt. Wastes were dried at 70°C over night and ground in an electric grinder. RP, containing 7.97% phosphorus (P), was kindly obtained from Soil, Water and Environment Research Institute, Agric. Res. Center, Giza, Egypt.

Isolation of Cellulolytic Fungi from Rice Straw

Samples of decayed rice straw stalks were collected from different farms of Dakahlia governorate. Decayed straw were mashed, added to normal saline solution and shaken for 10 min. One milliliter of the suspension was then inoculated in microcrystalline cellulose agar medium (Barbosa *et al*., 2001) and incubated at 28°C. The colonies on the plate with surrounding clear zones were picked for isolation. Single well-isolated colonies were obtained. Primary identification was carried out according to Domsch *et al*. (1980).

Inocula Preparation

Isolates 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, this was considered as the standard inoculum for all screening experiments, in case of solid-state fermentation inoculum was adjusted to 3×10^7 spore mL^{-1}.

Screening of Fungal Isolates for Cellulase Production

Fungal isolates obtained from the previous isolation trail were screened for the production of cellulase on the broth medium of Fadel (1994) using 20 g L^{-1} microcrystalline cellulose as a sole source of carbon. The pH of the medium was adjusted to 5.5 before autoclaving. Five percent (v/v) of previously prepared inoculum of each isolate was used to inoculate 250-mL Erlenmeyer flasks containing 50 mL of sterilized medium. Flasks were incubated under shaking (120 rpm) at 28°C for one week, flasks were then, centrifuged for 10 min at 4000 rpm. The culture filtrate was tested for filter paperase (FP-ase), carboxymethyl cellulase (CMC-ase) and β-glucosidase.

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 1% salicin, carboxymethyl cellulose and 50 mg Whatman No. 1 filter paper for 60, 30 and 15 min, respectively, at 50°C (Lakshmikant, 1990). Reducing sugars released in assay mixture were measured by

dinitrosalicylic acid method (Miller, 1959). One unit of FP-ase, CMC-ase or β-glucosidase was defined as the amount of enzyme, which released μmole of reducing sugar measured as glucose per min under the assay conditions.

Screening of Fungal Isolates for RP Solubilization

The broth medium of Nautiyal (1999) was used for screening the most active cellulolytic fungal isolates for their RP solubilization efficiencies; RP was incorporated in the medium as the sole P source (50 mg P_2O_5 100 mL^{-1}). Two hundred and fifty milliliter Erlenmeyer flask with 50 mL of the sterile broth medium was inoculated with the 5% (v/v) of previously prepared inoculum of each isolate and incubated at 28°C on a rotary shaker (160 rpm) for 7 days. After incubation, the supernatant was collected by centrifugation at 4000 rpm for 10 min to be tested for pH and Titratable Acidity (TA) by the method of Cerezine *et al.* (1988) as well as for soluble P (Jackson, 1967) and phytase production using sodium phytate as substrate (El-Sawah *et al*., 2001). One unit of phytase activity was defined as the amount of enzyme releasing 1 μmole of inorganic P mL^{-1} min^{-1}. It was planned to use mixed culture during organic acids production therefore, antagonism between each pairs of the selected fungi were performed on plates of PDA medium.

DNA Extraction, Amplification and Sequencing of Fungal 18S

The most active cellulolytic RP-solubilizing fungi obtained from the previous two screening tests were molecularly identified. The DNA of the selected fungi was isolated by a modified method of Edwards *et al.* (1991). For amplification using Polymerase Chain Reaction (PCR), the fungal specific primer 18S rRNA as forward and reverse primers was of the sequence; NS1, (5` GTAGTCATATGCTTGTCTC3`) and NS2, (5`GGCTGCTGGCACCAGACTTGC3`). The product was analyzed by electrophoresis on 1% agarose gel, the PCR product was purified using PCR purification kit-FERMETAS k 0701 and finally sequenced. Sequencing of PCR 18S rRNA was carried out using the ABI PRISM dye cycle sequencing ready reaction kit (Perkin Elmer) and an ABI Prism 377 sequencer according to the manufactures protocol (USA) using the forward 18S primer. DNA sequencing and chain-terminating inhibitors was achieved as described by Sanger *et al.* (1977). The obtain sequence (550 bp) has been submitted, deposited into GenBank to obtain similarity of the target sequence and the closely related fungi sequences. The obtained sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) at NCBI database (http://blast.ncbi.nlm.nih.gov). The phylogenetic analysis was accomplished by using the Mega 4 software program ver. 4.1.

Solid-State Fermentation (SSF)

The medium of Chang *et al.* (2006) was used for optimizing both cellulase production and RP solubilization under SSF conditions. The medium composed of 5 g of ground rice straw and 15 mL of salt solution (4.0 g L^{-1} KH_2PO_4, 1.6 g L^{-1} $(NH_4)_2SO_4$ and 1.0 g L^{-1} $MgSO_4$). The 50 mg P_2O_5 from RP was used as the sole P source instead of KH_2PO_4 and added separately to each flask before autoclaving. Inoculation was carried out using 5% (v/w) from the spore suspension (3×10^7 mL^{-1}) of the tested fungi. The initial moisture content was adjusted to 65%. 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 filtrate was examined for both cellulase enzymes and soluble P. This medium was used for studying time course (1 to 7 weeks), concentrations of P (25 to 150 mg P_2O_5 from RP) and inoculum ratio (2.5 to 12.5%, v/w).

Organic Acids Production

After optimization trails on SSF, another set of experiment was carried out on SSF medium prepared from either rice or wheat straw supplemented with RP for organic acid production by single and dual cultivation of the selected fungi. The optimum fermentation conditions from the previous investigation were applied. After incubation for 4 weeks, the residues of the fermented rice straw and wheat straw were dried in an oven at 80°C to constant weight for the determination of residual dry weight. The distilled water extracts a of decomposing straw were used for the determination of pH, TA, soluble P and cellulase activity by the methods described above, as well as for the determination of released glucose using glucose oxidase kit (Spainreact Co., Spain). Finally, the content of the organic acids was detected using high-performance liquid chromatography.

High-Performance Liquid Chromatography (HPLC)

Organic acids analysis in the aqueous extracts of the decomposed rice or wheat straw was performed with HPLC (Agilent 1100 HPLC system) using an HyperREZ XP carbohydrate H, 8 μm column 300×7.7 mm (Part Number, 69008-307780) with a mobile phase of 5 mM H_2SO_4, a flow rate of 0.6 mL min^{-1} and column temperature of 55°C. Injection volume of samples used for HPLC analysis was 5 μL of the final extract. Detection was by UV absorbance at 280 nm. Identities of the acids were established by comparison of retention times with known acids. Detection and quantification of ascorbic acid was accomplished using Hypersil GOLD PFP 5 μm column, 150×4.6 mm (Part Number, 25405-154630), the analysis was carried out isocratically at a flow rate of 1.0 mL min^{-1}, mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The column was thermostated at 25°C; injection volume was 20 μL. Ascorbic acid was detected by fluorescence detector operating at an emission wavelength of 410 nm with an excitation wavelength of 250 nm.

Standards and Quantification of Organic Acids

The organic acids were quantified by reference to the peak areas and retention times obtained for the authentic standards for nine organic acids (acetic, ascorbic, citric, formic, itaconic, levulinic, maleic, oxalic and succinic acids) in mobile phase which analyzed individually and mixed in concentration of 10 mg mL^{-1}. Acetic acid concentration of the aqueous standard solution was 0.5 mg mL^{-1} (Fig. 1).

RESULTS AND DISCUSSION

The environmental-friendly, non-hazardous management and ultimate disposal of wastes is of great concern. Currently, researchers have focused their attention on natural and sustainable techniques through bioremediation, i.e., biological based treatment for wastes and production of some benefit substances e.g., reducing sugars and organic acids. Therefore, a laboratory experiment was conducted on bioconversion of straw into organic acids through four main steps; (1) isolation and screening of cellulolytic fungi (2) screening of the isolated fungi for RP solubilization efficiency, (3) optimization of SSF conditions of the selected fungi on straw-RP medium and finally (4) production of organic acids.

Isolation and Screening of the Isolated Fungi for Cellulase Production

Different fungi were isolated from decayed rice straw on microcrystalline cellulose agar plates. Colonies surrounded by clear zone were selected. Single well-isolated colonies with

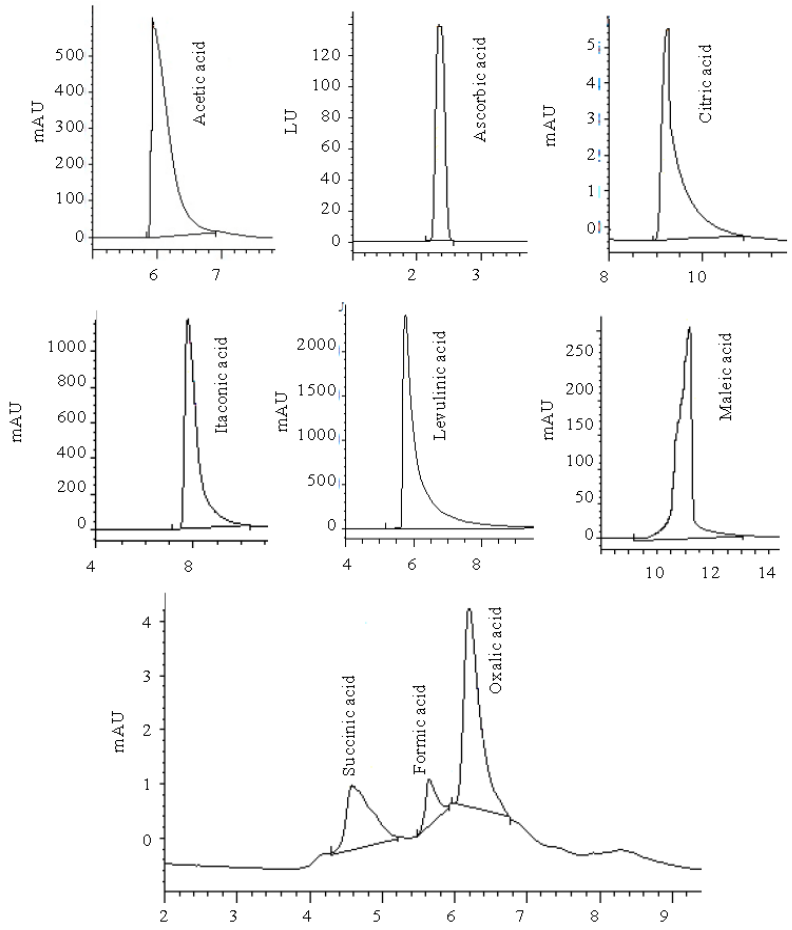

Fig. 1: HPLC chromatograms of standard organic acids, X-axis: Retention time (min), Y-axis: Observed peak area (mAU = Milli-absorbance units; LU = Luminescence units)

cellulase activity were obtained. Accordingly, twenty-one fungal isolates belong to thirteen genera were selected and identified to the genus level only. As presented in Table 1, *Penicillium* was the most frequent genus (19.06%). Meanwhile, *Aspergillus* and *Stachbotrys*

Table 1: Genera, number of species and frequency of cellulolytic fungi isolated from decayed rice straw

Genus	No. of species	Frequency (%)
Alternaria	1	4.76
Aspergillus	3	14.29
Bipolaris	1	4.76
Chaetomium	1	4.76
Cladosporium	2	9.52
Epicoccum	1	4.76
Fusarium	1	4.76
Penicillium	4	19.06
Stachybotrys	3	14.29
Stemphylium	1	4.76
Trichoderma	1	4.76
Trichothecium	1	4.76
Ulocladium	1	4.76
Total	27	100.00

came in the second order of frequency (14.29%). Only *Cladosporium* occupied the third order (9.52%). The other genera of the isolated fungi came later.

Cellulase production was quantitatively determined for the 21 fungal isolates grown in broth medium, the average values ± standard error are presented in Table 2. As shown, the FP-ase, CMC-ase and β-glucosidase activities of *Aspergillus* sp. S2, *Aspergillus* sp. S4, *Penicillium* sp. S13, *Penicillium* sp. S14 and *Trichoderma* sp. S19 were higher than those of the other isolates, suggesting that these five isolates which have appreciable cellulolytic activity are valuable in the bioconversion process of cellulolytic materials.

Table 2: Activity of cellulase enzymes in the filtrate of different isolated fungi grown in broth medium

	Cellulase activity (Unit mL^{-1})*		
Fungal isolate	FP-ase	CMC-ase	β-glucosidase
Alternaria sp. S1	14.2±0.3	122.0±2.0	0.1±0.0
Aspergillus sp. S2	21.3±0.8	161.7±3.9	99.0±3.8
Aspergillus sp. S3	12.2±0.6	111.3±2.2	0.0
Aspergillus sp. S4	32.7±4.3	161.0±11.8	117.6±12.6
Bipolaris sp. S5	0.0	16.3±0.2	0.0
Chaetomium sp. S6	3.9±1.1	38.8±2.2	1.5±0.4
Cladosporium sp. S7	8.1±0.3	52.2±0.2	0.0
Cladosporium sp. S8	3.6±0.4	26.6±0.4	7.0±0.1
Epicoccum sp. S9	12.4±0.4	113.6±2.2	14.3±0.5
Fusarium sp. S10	23.9±0.6	100.6±3.5	0.0
Penicillium sp. S11	20.8±1.2	118.2±2.2	0.0
Penicillium sp. S12	15.0±0.7	109.4±4.6	7.3±0.6
Penicillium sp. S13	23.9±1.0	114.0±1.5	88.1±1.6
Penicillium sp. S14	36.3±2.5	177.7±10.4	112.0±5.1
Stachybotrys sp. S15	4.0±0.6	96.8±1.3	11.4±0.1
Stachybotrys sp. S16	20.1±1.6	94.9±0.7	18.0±3.5
Stachybotrys sp. S17	3.9±0.1	49.9±0.7	0.0
Stemphylium sp. S18	10.9±0.4	87.6±3.1	12.0±0.1
Trichoderma sp. S19	18.4±0.5	152.7±5.4	42.4±5.3
Trichothecium sp. S20	1.1±0.1	50.2±2.6	0.0
Ulocladium sp. S21	3.8±0.2	50.6±0.2	0.0

*Mean of three replicates±standard error

A variety of cellulolytic fungi inhabit different types of agricultural wastes and are involved in their degradation (Lakshmikant, 1990). From rotten branches, mildewy materials and soil samples, Chang *et al.* (2006) isolated forty-one strains that can decompose cellulose, the cellulase activity of *A. glaucus* isolated from mildewy maize cob was higher than the isolates of the other materials. Several fungi have been reported to produce cellulase, e.g., Keskar (1992) found that the maximum yields of FP-ase, CMC-ase and β-glucosidase were obtained by *P. janthinellum*, whereas, *A. niger* KK2 was found to produce high yield of cellulase enzymes (Kang *et al.*, 2004). However, fungal efficiency to degrade the crystalline cellulosic materials depends upon the presence of complete cellulase activities in adequate quantity, i.e., endo-glucanase, exo-glucanase and β-glucosidase activities (Lakshmikant, 1990).

Screening of the Isolated Fungi for RP Solubilization

The most five active cellulolytic fungi, obtained from the previous screening, were further screened on the base of RP solubilization. For this purpose, the fungal isolates were grown on RP solubilizing medium (Table 3). Generally, all fungi reduced the final culture pHs to the acidic side, which, in turn, accompanied with the consuming of NaOH for the titration of the resulted acids (TA) in the culture filtrate. Additionally, the five fungi showed phytase activity and released soluble P from RP. The released soluble P in descending order were

Table 3: Rock phosphate solubilization efficiency by the most active cellulolytic fungi grown in broth medium

Fungus	Final culture pH	TA (μg NaOH mL^{-1})	Phytase (U mL^{-1})	Soluble P (μg mL^{-1})
Aspergillus sp. S2	5.2	61	16.3	39.41
Aspergillus sp. S4	4.5	79	18.2	41.99
Penicillium sp. S13	4.4	77	17.1	40.13
Penicillium sp. S14	5.7	78	17.0	40.65
Trichoderma sp. S19	5.1	74	16.1	40.01

41.99, 40.65, 40.13, 40.01 and 39.41 μg mL^{-1} in the culture filtrates of *Aspergillus* sp. S4, *Penicillium* sp. S14, *Penicillium* sp. S13, *Trichoderma* sp. S19 and *Aspergillus* sp. S2, respectively, which means that these fungi have efficient system for RP solubilization. Anyhow, the variation among the five tested isolates did not reach the significant level.

Biosolubilization of RP was found to be dependent upon the production of phytase and acid phosphatase, RP structure complexity, particle size and organic acids secreted by microorganisms, the biosolubilization is not always accompanied by a drop in pH, but always show production of organic acids mainly citric, oxalic and succinic acids, that is to say, biosolubilization is dependent upon the kind of organic acids not the quantity, however, organic acids may play an important role, but are not the only possible for solubilizing RP, which is confirmed by the weak or poor correlation between the drop in pH and the amount of solubilized P (Asea *et al*., 1988; Singh and Amberger, 1998; Rashid *et al*., 2004; Pradhan and Sukla, 2005; Achala *et al*., 2007; Vassilev *et al*., 2007; Kumari *et al*., 2008).

Antagonism Between Fungal Isolates

It was planned to use single and mixed culture of the most active cellulolytic RP-solubilizing fungi in organic acids production. The selected fungi include two species from each of *Aspergillus* and *Penicillium* and one from *Trichoderma*. Members of the genus *Penicillium* are known for their antibiotic production. Species of *Trichoderma* have also been reported as biocontrol agents due to its ability to successfully antagonize other fungi. The knowledge of such data is especially important when considering mixed fermentation systems. Therefore, antagonism between every pairs of the five fungi was carried out (Table 4) to select the fully compatible fungi in growth under mixed culture. *Trichoderma* sp. S19 and *Penicillium* sp. S13 showed strong to moderate antagonism with all other fungi, so both fungi were excluded from the selection. *Aspergillus oryzae* S2 was also, excluded from further studies, since, this fungus recorded the lowest efficiency in RP solubilization compared with the other fungi. On the other hand, *Aspergillus* sp. S4 and *Penicillium* sp. S14 did not show any antagonism, conversely, they grew together in complete harmony on dual culture. Two microorganisms each lacking in a complimentary cellulase complex can be cultivated in the same bioreactor to produce a culture filtrate complete in all the cellulase components, however, this may only be possible if both organisms have similar growth requirements (Margaritis and Merchant, 1986). From the previous observations, *Aspergillus* sp. S4 and *Penicillium* sp. S14 were found to be the best organisms in both cellulolytic activity and solubilization of RP and may suitably be employed in cellulose bioconversion processes in single and in mixed cultures.

Table 4: Antagonism between each pairs of selected fungi on PDA plates

Fungus	*Aspergillus* sp. S2	*Aspergillus* sp. S4	*Penicillium* sp. S13	*Penicillium* sp. S14
Aspergillus sp. S4	(-)			
Penicillium sp. S13	(±)	(±)		
Penicillium sp. S14	(-)	(-)	(-)	
Trichoderma sp. S19	(+)	(+)	(+)	(±)

(-): No antagonism; (±): Moderate antagonism; (+): Strong antagonism

Molecular Identification of the Isolated Fungi Using 18S rRNA Gene

Aspergillus sp. S4 and *Penicillium* sp. S14 showed cellulolytic RP-solubilizing activities, so, both fungi were molecularly identified. The obtained sequence of 550 bp of amplified 18S rRNA fragment on agarose gel (Fig. 2) showed similarity of the target sequence to the closely related fungi sequences to both *A. niger* and *P. chrysogenum*. Phylogenetic analysis of the 550 bp aligned nucleotide sequences of the amplified 18S rRNA fragment of the 2% isolates are shown in Figs. 3 and 4. The phylogenetic analysis of the available sequence of the isolated *Aspergillus* sp. S4 with the related sequences obtained from the GenBank (Fig. 3) show that the tree is divided into two main groups (I, II) each group is divided into two sub groups. In group I the two sub groups are divided into 6 sub orders, the isolate was found in the sub group1, in the fourth sub order. It is clear that the isolate (*Aspergillus* sp. S4) is closely similar to *A niger* strain KBS4 with the accession number GQ228449 of the same sub group and to *Aspergillus* sp. HD86-9, *P. chrysogenum* Wis and *P. chrysogenum* Wis with accession numbers EU853156, XM 002558241 and XM 002558269, respectively. From the same group it is similar to some extent to AY 227763 of the second sub group of group I. On the other hand, it is near to AY227769, GQ382274 and GQ382273 *A niger* of the second sub group of group II.

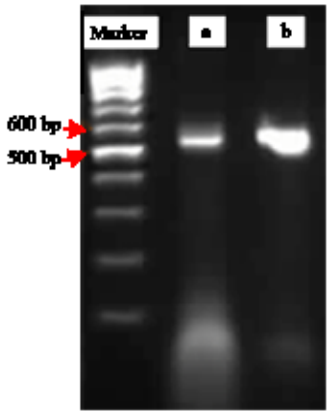

Fig. 2: Agarose gel electrophoresis of a band about 550 bp of the PCR product of the amplified 18S rRNA fragment for (a) *Aspergillus* sp. S4 and (b) *Penicillium* sp. S14

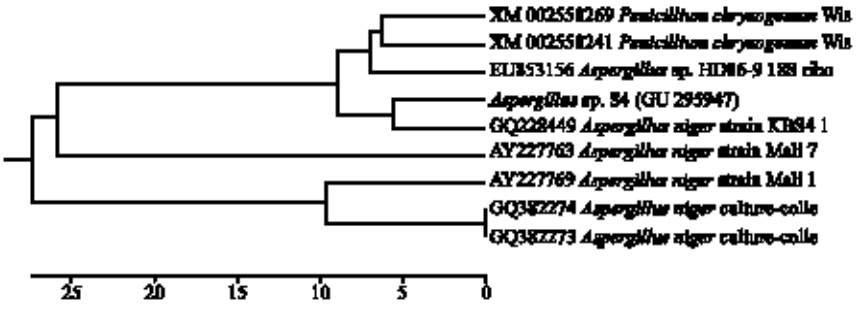

Fig. 3: Molecular phylogenetic tree of the partial sequence of 18S rRNA of *Aspergillus* sp. S4 with respect to the closely related sequences available in GenBank

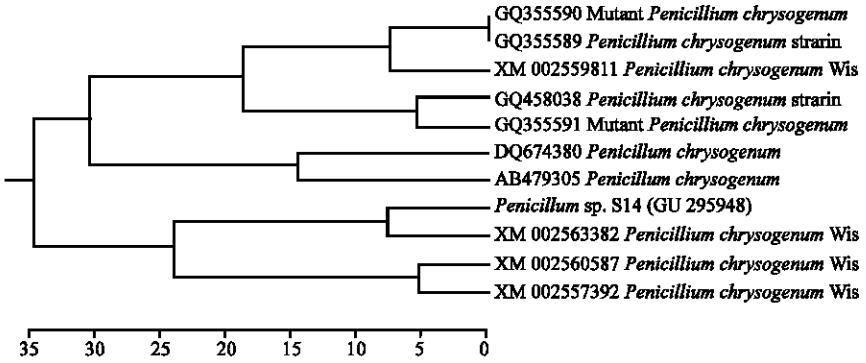

Fig. 4: Molecular phylogenetic tree of the partial sequence of 18S rRNA of *Penicillium* sp. S14 with respect to the closely related sequences available in GenBank

The molecular phylogenetic analysis of the available sequence of the isolated *Penicillium* sp. S14 with the related sequences obtained from the GenBank (Fig. 4) show that, the tree is divided into two groups (I, II) each group has two sub groups. The present isolate is related to group II and sub group I and appears to be closely similar to the GenBank strain XM 002563382 and XM 002560587, XM 002557392 *P. chrysogenum* of the group II. The isolate is nearly similar to the strain AB479305, DQ674380 *P. chrysogenum* of the GenBank of group I and very far from GQ355590, GQ355589, XM 002559811, GQ458038, GQ355591 and DQ674380 of group I. The two tested isolates were identified as *A. niger* GU 295947 and *P. chrysogenum* GU 295948.

Molecular identification techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels. These techniques, which based on the PCR amplification of genes coding for rRNAs and sequence comparison, offer a new rapid tool for identification of filamentous fungi using two specific PCR primers sets. The sequence that is exemplified in the detailed description covers the 18S rRNA gene, through the sequences of internal transcribed spacers (ITS1 and ITS2) and into the 28S rRNA gene, each species has a unique nucleotide sequence through this stretch of genes (Turenne *et al*., 1999; Sette *et al*., 2006).

Cellulase Production and RP Solubilization under SSF Conditions

According to the previous two screening trials, antagonism test and molecular identification, two fungal strains (*A. niger* GU 295947 and *P. chrysogenum* GU 295948) were obtained. Both fungi were grown under SSF conditions to optimize cheap medium for cellulase production in parallel with RP solubilization in the presence of minimal interference from medium components. For this purpose, medium containing rice straw and RP was used to identify cellulase production and released soluble P.

Time Course Profile

It seems that the time course profile of FP-ase, CMC-ase and β-glucosidase production during SSF period were identical and these enzymes secreted under the same condition by both fungi (Table 5). The productivity of the tested enzymes increased greatly in the first 3 weeks and reached the maximum in the fourth week. After 4 weeks, the productivity decreased and only about half of the maximum activities could be detected on the seventh week. The enzymatic profiles showed higher production of cellulase enzymes by *A. niger* than *P. chrysogenum*. Variable results were recorded by many investigators who

Table 5: Time course of cellulase production during SSF of rice straw with RP by *A. niger* and *P. chrysogenum*

	A. niger			*P. chrysogenum*		
Time (week)	FP-ase	CMC-ase	β-glucosidase	FP-ase	CMC-ase	β-glucosidase
	(U g^{-1} straw)			(U g^{-1} straw)		
1	34.7	177.6	75.3	29.0	94.2	47.8
2	37.1	189.1	79.4	33.3	120.5	49.4
3	43.6	233.1	90.3	27.4	160.0	52.0
4	47.5	255.1	98.8	40.2	171.4	55.8
5	44.4	200.5	89.5	28.1	163.9	40.2
6	41.3	212.5	83.3	27.9	151.2	51.3
7	21.2	122.1	55.6	24.6	92.8	37.1

worked on rice and/or wheat straw, for example, Badhan *et al.* (2007) recovered the highest yield of FP-ase, CMC-ase and β-glucosidase from *Myceliophthora* sp. grown on rice or wheat straw under almost identical condition and Liua and Ørskovb (2000) found that *P. funicalosum* produces cellulase during saccharification of stem pre-treated rice straw after fermentation for 1 to 3 weeks. On wheat straw, 28 days of incubation was the best for both biodegradation and cellulase production under SSF with white-rot fungi (Dinis *et al.*, 2009). Additionally, 14 days (Sridevi *et al.*, 2009) and 45 days (Lakshmikant, 1990) were reported for maximum yield of cellulase on wheat straw. On the other hand, Kang *et al.* (2004) reported lower incubation period (4 to 5 days) for optimum production of celluases and hemicelluases on rice straw by *A. niger*.

When *A. niger* and *P. chrysogenum* were grown on rice straw as a solid support in SSF supplemented with RP, the time course profile of RP biosolubilization was as shown in Fig. 5. It was found that RP solubilization process was online with cellulase production during the initial 4 weeks, in which the maximum soluble P was obtained after 4 weeks of incubation, afterwards, the biosolubilization of RP slightly decreased by both fungi. Generally, there was no dramatic variations in RP solubilization after the 4th week of fermentation. *Aspergillus niger* showed higher solubilization efficacy than *P. chrysogenum*. These data reflect the importance of incubation period during SSF of rice with RP, especially, for cellulase production. Thus, 4 weeks was found to be the optimal cultivation period for both cellulase production and RP solubilization. Singh and Amberger (1998) found that organic acid produced in the composted wheat straw with RP play important role in its solubilization, the concentration of organic acids was very high at 30 days, thereafter they disappeared at a very fast rate with composting time.

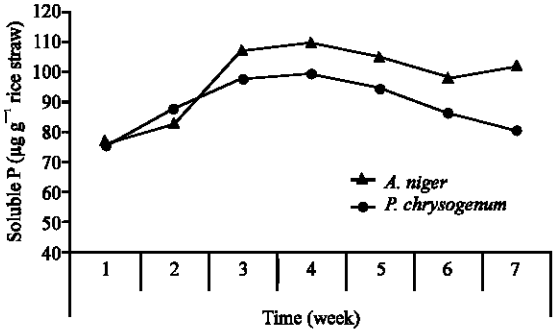

Fig. 5: Time course of RP solubilization during SSF of rice straw with RP by *A. niger* and *P. chrysogenum*

Concentration of RP

The initial concentration of RP plays an important role during SSF of rice straw. Data (Table 6 and Fig. 6) of the present test show that along with cellulase production by both fungi, released P increased with the increment of RP concentration up to 75 mg P_2O_5 from RP to each flask. β-glucosidase of *A. niger* was the only exception which reached to the maximum production at P concentration of 50 mg P_2O_5 from RP. As the concentration of RP increased, there was a slight decrease in cellulase production and RP solubilization capability. Xiao *et al.* (2008) found that the maximum content of soluble P was recorded when 2.5 g L^{-1} RP was added in the medium. However, the content of P in RP must be taken into account when calculating the amount of RP concentration. Since, P content varies according to the kind of RP. Actually, there are many factors controlling the solubilization process including organic acids that characterized by the possession of one or more carboxyl groups, depending on the dissociation properties and number of these carboxyl groups, organic acids can carry varying negative charges, thereby allowing the complexation of metal cations in solution and the displacement of anions (Jones, 1998). Other factors for RP solubilization were discussed earlier in this paper.

Table 6: Cellulase production by *A. niger* and *P. chrysogenum* under SSF of rice straw as affected by the RP concentration

RP conc. (mg P_2O_5)	*A. niger*			*P. chrysogenum*		
	FP-ase	CMC-ase	β-glucosidase	FP-ase	CMC-ase	β-glucosidase
	(U g^{-1} straw)			(U g^{-1} straw)		
25	44.5	214.7	90.9	27.4	161.3	54.0
50	47.5	255.1	98.8	40.2	171.4	55.8
75	47.8	303.3	95.3	45.7	180.3	57.3
100	42.7	272.5	90.0	40.4	162.5	55.6
125	31.3	252.1	88.0	39.0	145.3	55.6
150	30.3	210.4	86.3	26.7	131.2	40.6

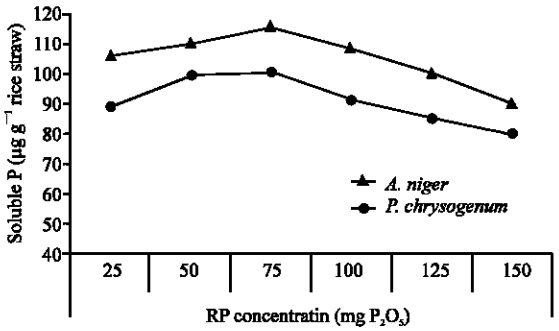

Fig. 6: Biosolubilization of RP by *A. niger* and *P. chrysogenum* under SSF of rice straw as affected by different concentrations of RP

Inoculum Ratio

The activity of different inoculum ratios of the tested fungi on fermented rice straw with RP in the form of cellulase production and RP solubilization is shown in Table 7 and Fig. 7. Productivity of cellulase increased with the gradual increase of inoculum ratio up to 7.5% (v/w), at this ratio, FP-ase, CMC-ase and β-glucosidase recorded 50.8, 343.2 and 99.2 and 47.9, 184.9 and 59.2 U g^{-1} rice straw by *A. niger* and *P. chrysogenum*, respectively. The enzymatic system of *A. niger* was more efficient than that of *P. chrysogenum*. Biosolubilization of RP was greatly influenced by inoculum ratio. Figure 7 shows that the

Table 7: Effect of inoculum ratio on cellulase production by *A. niger* and *P. chrysogenum* grown on rice straw and RP

Inoculum ratio (%, v/w)	*A. niger*			*P. chrysogenum*		
	FP-ase	CMC-ase	β-glucosidase	FP-ase	CMC-ase	β-glucosidase
	(U g^{-1} straw)			(U g^{-1} straw)		
2.5	46.4	253.9	82.3	39.8	171.9	48.5
5.0	47.8	303.3	95.3	45.7	180.3	57.3
7.5	50.8	343.2	99.2	47.9	184.9	59.2
10.0	46.9	301.8	88.4	40.6	177.4	51.1
12.5	44.9	261.0	84.3	37.9	163.5	47.3

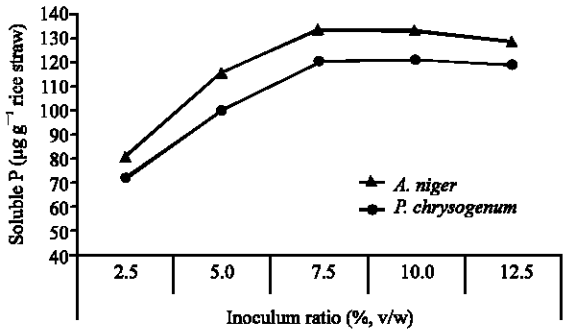

Fig. 7: Effect of inoculum ratio on biosolubilization of RP by *A. niger* and *P. chrysogenum* grown on rice straw

maximum RP solubilization was in accordance with cellulase production, the optimum inoculum ratio for the maximum releasing of soluble P was 7.5% (v/w), out of this point, both fungi showed reduction in both cellulase production and RP solubilization. It is worthy to mention that, in concordant with cellulase production, *A. niger* was more active in solubilization of RP than *P. chrysogenum*.

Biosolubilization of RP was found to be dependent upon the size of inoculum; when the inoculum ratio is lower, superfluous nutrients resulted in the excessive growth of mycelia and delaying enzyme production and solubilization process, higher amounts of fungal inoculum leads to rapid growth of mycelia and higher amounts of fungal biomass accumulation which inhibits enzyme production as a result of insufficient nutrients in the latter growth period, this in turn lowers enzymes production and phosphate solubilization (Chang *et al*., 2006; Vassilev *et al*., 2007).

Organic Acids Production on SSF of Rice or Wheat Straw with RP

Analysis of the Hydrolysate of SSF

Because of the promising data on rice straw, it was found to extent the biodegradation process to another additional cellulosic material i.e., wheat straw, using *A. niger* GU 295947, *P. chrysogenum* GU 295948 and their dual culture. The optimum fermentation conditions obtained from the previous trials were applied. After 4 weeks incubation period, the resulted hydrolysates from SSF media of rice or wheat straw containing RP were analyzed for fungal growth, loss in weight of fermented straw, final pH, TA, soluble P, released glucose and the produced cellulase (Table 8). These tests represent the basic indicators for the biodegradation of both straw and solubilization of RP by the two tested fungal strains. Both of mixed culture and *A. niger* showed very good growth on both wastes, whereas individual *P. chrysogenum* showed lower growth on both straw compared with the other

Table 8: Basic decomposition indicators in the hydrolysate of rice and wheat straw as a result of the activity of *A. niger*, *P. chrysogenum* or their dual culture

Tested parameter	*A. niger*	*P. chrysogenum*	Dual culture
Fungal growth*			
Rice straw	++	+	++
Wheat straw	++	+	++
Loss in dry weight (%)			
Rice straw	42.1	40.5	45.4
Wheat straw	43.0	41.3	43.0
Final pH			
Rice straw	5.7	7.5	6.1
Wheat straw	5.6	5.7	5.4
TA (μg NaOH g^{-1} straw)			
Rice straw	49	-	46
Wheat straw	45	43	52
Soluble P (μg g^{-1} straw)			
Rice straw	133.3	120.3	144.6
Wheat straw	136.1	121.6	143.2
Released glucose (μg g^{-1} straw)			
Rice straw	332	312	259
Wheat straw	169	166	249

* + Good growth; ++ Very good growth

treatments. There was a remarkable decrease in dry weight of the fermented substrates, reached to more than 40% reduction from the original weight. A measure of organic matter loss is one of the methods generally used for the determination of microbial activity during SSF. The loss in organic matter in the presence of RP most probably due to its high $CaCO_3$ content that induces microbial growth during organic matter decomposition in which organic acids are produced that help in mobilization of insoluble P (Singh and Amberger, 1998). Nevertheless, the residual fermented organic matter after organic acids extraction could be added to the soil as RP-enriched compost without worrying from the presence of both fungi since, neither *A. niger* nor *P. chrysogenum* is plant pathogens. This by-product biomass can be also used as a supplement for animal feed (Magnuson and Lasure, 2004).

The final pH of the hydrolysate of all samples (Table 8) was reduced to the acidic side except that of *P. chrysogenum* on rice straw (pH, 7.5); this treatment in special recorded the lowest value of soluble P. This reduction in pH values was accompanied with increment in TA, that is because of the consuming of NaOH for the titration of the resulted acids. However, there was an evidence for RP solubilization, especially, in dual cultivation, in which the released P reached 144.6 and 143.2 μg g^{-1} rice and wheat straw, respectively. It has been stated that pH decreased gradually during fermentation, which resulted in the release of organic acids, the reduction in pH may also due to release of bases from dissolution of insoluble phosphate (Asea *et al*., 1988; Rashid *et al*., 2004). The lower pH value in RP containing treatments after 30 days of composting confirms the production of organic acids (Singh and Amberger, 1998). The presence of RP as a sole source of P in SSF media induced the fungi towards the solubilization, assimilation and utilization of RP-phosphorus, this is achieved by the production of phytase and acid phosphatase and/or the secretion of organic acids (Achala *et al*., 2007; Vassilev *et al*., 2007; Kumari *et al*., 2008) the later is responsible for lowering the pH of the hydrolysate.

Another two evidences for organic matter decomposition were emerged; the first was represented by the released glucose that was detected in the hydrolysate, especially those of *A. ni*ger on rice straw (Table 8), the released glucose in the hydrolysate of the tested samples recorded a range from 166 to 332 μg g^{-1} straw. Comparing the released glucose of the present study with other works, leads to the conclusion that the released glucose in the

present study is less than that reported in the other literatures, e.g., Liua and Ørskovb (2000) and Sridevi *et al.* (2009). This may be due to the rapid and direct bioconversion of the released glucose into organic molecules such as enzymes and organic acids. So, this kind of fermentation could be used in RP solubilization as well as saccharification of lignocellulolytic wastes for glucose production. The second evidence was the obvious productivity of cellulase on both straw (Fig. 8), in which, the combined action of FP-ase that represents the overall cellulolytic activity (Margaritis and Merchant, 1986), endoglucanases (CMC-ase) which is the first and essential step in the decomposition of cellulolytic substrates (Lynd *et al.*, 2002) and the reasonably good β-glucosidase activity favors the formation of glucose as the major product, which directly converted into different organic molecules.

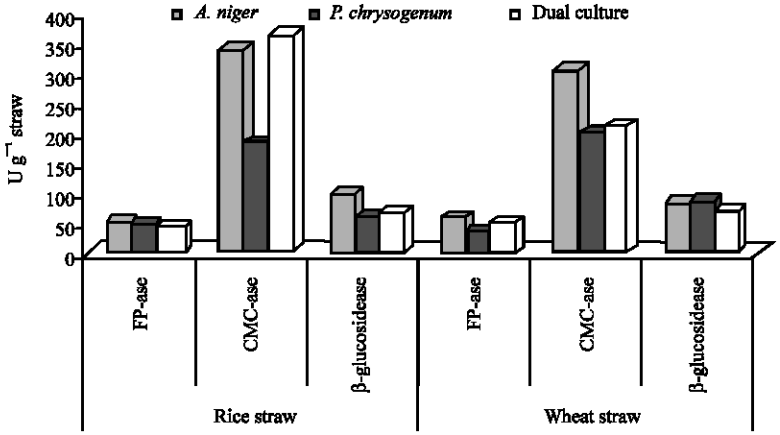

Fig. 8: Cellulase profiles of *A. niger*, *P. chrysogenum* and their dual culture on rice or wheat straw supplemented with RP

Cellulase of *Penicillium* and *Aspergillus* grown under SSF have been used for the saccharification of agro-wastes (Considine *et al.*, 1988; Kang *et al.*, 2004). The enzymatic degradation of waste cellulose by fungal enzymes has been suggested as a feasible alternative for the conversion of lignocellulosics into fermentable sugars and fuel ethanol (Oksanen *et al.*, 2000; Shin *et al.*, 2000). Another study of Abul Hossain *et al.* (2004) found that bioconversion of rice straw has been achieved by a dual inoculum of *T. harzianum* with *Mucor hiemalis* or *Phanerochaete chrysosporium*. However, Margaritis and Merchant (1986) stated that wheat straw itself represents the economical choice for cellulase production amongst all different inducers tested, their survey did not include rice straw. Anyhow, the hydrolysates of SSF were analyzed for organic acids content.

HPLC Analysis of Organic Acids

In the present study, the reduction in pH and RP solubilization are evidences for the bioconversion of fermented straw into organic acids. This hypothesis was previously reported by Singh and Amberger (1998). Many methods are used for analysis of organic acids. However, because of simplicity, accuracy and speed of analysis, the HPLC technique is an attractive method (Andersson and Hedlund, 1983). The resulted organic acids in the hydrolysate of SSF by the cellulolytic RP-solubilizing fungi; *A. niger* GU 295947 and *P. chrysogenum* GU 295948 as well as their dual culture were identified and quantified using HPLC (Table 9). There were nine organic acids in the 30 days-old straw fermented with RP. Acetic, ascorbic, citric, formic, oxalic, itaconic, levulinic, maleic and succinic acids were detected in a water extract of SSF. Results indicate that oxalic, succinic and formic acids were

Table 9: Organic acids production (mg g^{-1} straw) during SSF of rice or wheat straw with RP by *A. niger* and/or *P. chrysogenum*

	Rice straw			Wheat straw		
Organic acid	*A. niger*	*P. chrysogenum*	Dual culture	*A. niger*	*P. chrysogenum*	Dual culture
Acetic acid	UD	0.591	0.409	0.655	UD	0.068
Ascorbic acid	0.095	0.123	0.098	0.092	UD	UD
Citric acid	3.614	0.268	0.451	5.356	0.256	3.220
Formic acid	6.040	5.270	7.450	3.460	4.810	4.600
Oxalic acid	UD	16.915	UD	UD	40.000	UD
Itaconic acid	0.309	0.132	0.230	0.330	0.194	0.219
Levulinic acid	0.014	0.046	0.018	0.013	0.010	0.018
Maleic acid	0.010	UD	0.018	UD	UD	UD
Succinic acid	11.302	6.892	12.562	9.164	10.670	8.120
Total (mg g^{-1})	21.384	30.237	21.236	19.070	55.941	16.245

The SSF period lasted for 4 weeks supplemented with 75 mg P_2O_5 from RP and 7.5% (v/w) inoculum; UD: Undetectable

the three major acids produced. Oxalic acid was produced in the highest quantity when *P. chrysogenum* was used as single inoculum in SSF of rice or wheat straw. In this respect, the recovered oxalic acid from the fermented wheat straw recorded the highest value (40 mg g^{-1} straw). Succinic acid came in the second order after oxalic acid, it was found to be produced on all combination of treatments. Similar to succinic acid, formic, citric, itaconic and levulinic were also produced by the all combinations of treatments. Acetic, itaconic ascorbic, levulinic and maleic acids were detected in lower quantities compared with the other organic acids. Maleic acid produced only on SSF of rice straw and was not detected on any treatment of SSF of wheat straw. With respect to the tested fungi, *P. chrysogenum* was found to be more potent than *A. niger* and the mixed culture in the total organic acid produced on both straw with RP.

Organic acids occur in fermented products as a result of hydrolysis, biochemical metabolism and microbial activity (Andersson and Hedlund, 1983). For example, citric acid is produced during surface and submerged fermentation by *A. niger* (Kurbanoglu and Kurbanoglu, 2004), oxalic and citric acids are the major acidic metabolites 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 is biosynthesized by *A. terreus* (Magnuson and Lasure, 2004) and fumaric, succinic and maleic acids were detected during the decomposition of rice straw with RP (Kumari *et al*., 2008). In general, each organic acid has its own production pathway, but some organic acids share the same pathway. For instance, the biochemistry and physiology of citrate and oxalate fermentations share similar physiology and each product is no more than one enzymatic step from the primary pathway of D-glucose and D-fructose metabolism, likewise, the mechanism leading to malate production is the same as the pathway leading to fumarate, abbreviated by one step, whereas, there are three possible metabolic mechanisms for production of succinate i.e., the oxidative portion of the tricarboxylic acid (TCA) cycle, the reductive portion of the TCA cycle, or the glyoxylate bypass (Magnuson and Lasure, 2004). Moreover, acetic acid originating from acetylated functionalities on lignin and hemicelluloses, formic acid is derived from decomposition of both sugars and lignin, and levulinic acid is produced upon further decomposition of 5-hydroxy-methylfurfural (Palmqvist and Hagerdal, 2000; Chena *et al*., 2006).

The mathematical relationship, represented by the linear regression and correlation coefficient, between the total organic acids from one side and either released glucose or soluble P from the other side showed non-significant relationship (Fig. 9). Likewise, as reported by Vyas and Gulati (2009), no relationship could be ascertained between the quantity of organic acids produced and the solubilization of RP. But the nature of organic

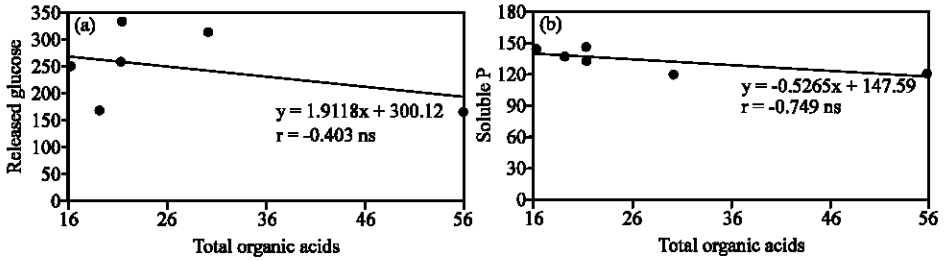

Fig. 9: The mathematical relationship between total organic acids and either (a) released glucose or (b) soluble P

acids has a considerable effect on the solubilization of insoluble phosphates, the position and type of functional group within each acid seems to be a dominant factor that influences the amount of released P (Kumari *et al*., 2008).

Following the culture conditions reported above, the production of organic acids by the molecularly identified; *A. niger* GU 295947 and/or *P. chrysogenum* GU 295948 in SSF was performed. The presence and concentrations of organic acids were different according to the straw and the fungus. The most noticeable thing was the broad-spectrum organic acids and their higher concentrations when rice or wheat straw was used as the carbon source in combination with RP. This is a significant advantage from the viewpoint of practical saccharification reaction. Since, the released glucose converted directly into organic acids. The resulted organic acids will have applications in several fields. However, variability encountered in the composition of wheat or rice straw with seasonal changes and age of material must be taken in consideration, although, the changes in composition varying in a narrow range. Based on the previous study and according to HPLC results, both fungal strains can be directed to the production of organic acids especially, oxalic and succinic acids. In addition, both fungi produce good levels of cellulase enzymes, which together with the high ability to RP solubilization, makes these fungi good candidates for obtaining cellulase enzymes for the biodegradation of lignocellulosic wastes as well as solubilizing complex phosphates.

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