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Original Article

Isolation and Identification of the Highly Cellulolytic and P-Solubilizing Fungi

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Abstract

Rice Straw (RS) is one of the most important agrowaste worldwide. There are variations in mycobiota inhabiting RS in cellulolytic profile. This study aims for isolation and screening of some fungal species from rice straw, and test them for the production of cellulose and P-solubilization. In this work, 14 fungal species from different localities in Dakhalia Province in Egypt were recovered. The initial screening of fungal growth on the carboxy-methyl cellulose (CMC) as a carbon source, showed that 12 isolates were able to grow and degrade CMC substrate with different degrees, where they showed maximum zones of hydrolysis with the highest enzymatic indices. Therefore, they were tested for the production of cellulases. Cellulase activity was determined by using carboxy-methly cellulase assay (CMC-ase) and filter paperase assay (FP-ase). *Penicillium purpurgnium* and *Aspergillus niger* were the most active strains for producing CMC-ase with values: 180.00 U mL^{-1} RS and 172.40 U mL^{-1} gm RS and FP-ase with values: 38.12U m^{-1} gm RS and 20.21 U mL^{-1} gm RS, respectively. Furthermore, the solubilization of Phosphate Rock (PR) was carried out by using 25 mg P_2O_5 from PR and 5% (v/w) fungal inoculum. The results showed that, *Aspergillus niger* has the maximum phosphorus solubilization with value of 40.37 mg mL^{-1} followed by *Penicillium purpurgnium* with value of 37.11 mg mL^{-1} . Therefore, they were the most active cellulolytic strains for degrading stored RS and PR- solubilization.

1. Introduction

Rice straw (RS) is a renewable lignocellulosic biomass, with global production of 600 to 900 million tons $year^{-1}$ (Karimi *et al.*, 2006). RS is considered as one of the most abundant lignocellulosic waste products in the world, which may create numerous environmental prob-

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lems, if not consumed very well. In most countries, including Egypt; RS stored for domestic uses for several years, while a huge amount of it is directly burning in the open field, causing negative impact on the health of all living organisms (Kim *et al.,* 2010).

The chemical composition of RS is cellulose (32-47%), hemicellulose (19-27%) and lignin (5-24%) (Kim *et al.,* 2010). Cellulose has enormous potential as a renewable source of energy. Subsequently, an important aspect of the carbon cycle within the biosphere is the degradation of cellulosic biomass. These processes are more efficient by using cellulolytic microorganisms (Béguin and Aubert 1994). This bioconversion of cellulosic biomass to fermentable sugars will reduce the usage of biofuel and reduce the environmental pollution (Kumar *et al.,* 2009).

Filamentous fungi are perfect examples of non-pathogenic microorganisms, due to their capability for production of useful extracellular enzymes (Soccol *et al.,* 1994). Wide ranges of *Aspergillus*, *Fusarium, Penicillium* and *Trichoderma* species have been identified to possess all components of cellulases complex (Azzaz 2009). Three types of cellulase enzymes are involved in the cellulose hydrolysis process including; exogluconase or filterpaperase (FP-ase), endoglucanase or carboxymethylcellulase (CMC-ase) and β-glucosidase (Saber *et al.,* 2010).

Screening for cellulase producing microorganisms can be carried out by agar plates with a cellulosic substrate; such as amorphous cellulose called CMC for microorganisms' growth, as carbon source, which has a low viscosity. Congo red dye can be used as an indicator (Teather and Wood, 1982; Sazci *et al.,* 1986; Petersonr *et al.,* 2009).

Phosphate-solubilizing microorganisms (PSMs) have been distinguished by their relative abilities to dissolve complex phosphates; e.g., phosphate rock (PR). This activity attributes in the production of organic acids as end products (Singh and Amberger, 1998; Jha *et al.,* 2014). Phosphate Rock is the main source of phosphate fertilizers "P-fertilizers" (Van Kauwenbergh, 1997).

Applying solid-state fermentation (SSF) in the industry have more attention in the last few years. It has various advantages as; low wastewater output, lower production cost, reduced energy requirement, high rate of productivities, easier aeration, simple fermentation media, flexible pH condition, reduced occurring of bacterial condition, etc. (Raimbault, 1998).

In addition to the usage of RS with CMC and PR under SSF condition, this work aims to study the most active cellulolytic and PR-solubilzing fungal isolates associated with one of the most abundant agrowaste, rice straw, in the world. Furthermore, determining which fungal species have the ability to produce cellulases, by measuring FP-ase, CMC-ase and p-solubilisation, to apply the usage of them for the biodegradation of nonconventional lignocellulosic substrates i.e. rice straw with PR.

2. Materials and Methods

2.1. Preparation of different RS samples for isolation of fungal strains:

Samples of rice straw were collected from different storages in Dakhalia Province, Egypt, during March to May 2012, representing three different periods of rice storage (1-2, 3-5, and over 7 years periods).

The air-dried samples were cut into 0.5-1 cm. The fragments of RS were transferred to plates with Potato Dextrose Agar medium (PDA). For obtaining pure isolates, hyphal tip and single spores' isolation techniques were carried out, followed by maintenance on pure PDA slants for identification and further studies

2.2. Samples of rice straw were collected from dif

Fungal genera and species identification was carried out through their cultural properties, morphological and microscopical characteristics. This was according to Raper and Thom (1949), Ellis (1971), Booth (1977), Domsch (1980), Klich and Pitt (1988), Pitt (1988) and Moubasher (1993).

2.3. Screening for cellulolytic isolates:

The pure isolates were cultivated and maintained on CMC-Agar medium at 30 ^{o}C for 6 days. Then, cultures were incubated at 50 ^{o}C for 18 hr. (which is the opti-

mum temperature for cellulases activity), to accelerate the action of extracellular cellulases. Clearing zone around the cellulase-producing colonies was rapidly developed (Pragya *et al.,* 2012).

Cellulolytic activity was recorded by staining the plates with 2.5 g/L Congo red dye, for 0.5-1hr., and then followed by addition of 1M NaCl solution for 15-20 min (Apun *et al.,* 2000). The experiments were carried out independently for 3 times under the same conditions. Unstained areas (clear zone of hydrolysis) mentioned to the CMC which broken down to produce β (1→ 4) glucans, that contains seven or fewer glucose residues (Montenecourt and Eveleigh, 1977).

The appearance of a pale halo zone, with orange edges "areas of hydrolysis", indicates the cellulase production. This halo zone was measured for subordinate calculation of the enzymatic index (EI) using the equation:

EI= Diameter of hydrolysis zones / Diameter of colony

Fungal species that having EI more than 1.40 are considered as potential producers of cellulases (Florencio 2012).

2.3.1. Preparation of inocula

Isolates were inoculated on pure PDA slants and incubated for 7 days at 28 °C. Spore suspensions were made by adding 5 mL of sterilized distilled water to these slants then, followed by gently probing to the surface with the tip of Pasteur pipette; this was considered as the standard inoculum, that was adjusted around 75 X 109 mL-1spores for all screening experiments.

2.3.2. Screening of lignocellulosic isolates for cellulase production

The obtained fungal isolates were screened for the production of cellulase on the broth medium (Sohail et al., 2009), using 10 gL^{-1} CMC as a sole source of carbon. Before autoclaving, the pH of the medium was adjusted to 5.5. 250-mL Erlenmeyer flasks containing 50 mL of sterilized medium were inoculated with five percent (v/v) of previously prepared inoculum of each isolate, and then all flasks were incubated under shaking condition (120 rpm), at 28 °C, for one week. The filtrates were centrifuged for 10 min, at 4000 rpm to obtain enzyme solutions for further tests.

2.3.3. Cellulases assay

CMC-ase and FP-ase activities were estimated by incubating of 0.5 mL enzyme solution and 0.5 mL citrate buffer at 0.05 M, pH 4.8, with 1 % salicin, carboxymethyl cellulose and 50 mg Whatman No. 1 filter paper at 50 °C for 30 and 60 min, respectively (Mathur, 1990). Reducing sugars, released in assay mixture, were measured by Nelson (1944) and Somogyi (1952).

International Unit (IU) is the terms for determination of enzyme activity. One unit of CMC-ase or FP-ase was appointed as the amount of enzyme, which released μ mole of reducing sugar per mL per minute under the assay conditions.

2.4. Screening of the most active fungal isolates for PR solubilization

PR containing 7.97% phosphorus (P) was obtained from Soil, Water and Environment Research Institute, Agriculture Research Center, Giza, Egypt. The Nautiyal, (1999) broth medium was used for screening the most active cellulolytic fungal isolates for PR solubilization efficiency where PR was incorporated in the medium as the sole source of P (25 mg P_2O_5 /50 mL broth media).

The flask containing 50 mL of the sterile broth medium was inoculated with 5% (v/v) of the previously prepared inoculum of each isolate. All flasks were incubated at 28 °C, on a rotary shaker (160 rpm) for 7 days. After an incubation period, the supernatant was collected by centrifugation at 4000 rpm, for 10 min to determine the final pH and titratable acidity (TA) by the method of Cerezine *et al.* (1988), as well as for determining of soluble free phosphorous by the method of Jacson (1958).

2.5. Solid-state fermentation (SSF)

The modified medium of Xu *et al.* (2006) for optimizing of both cellulase production and PR solubilization, under SSF conditions was applied. This medium

was composed of 1 g of ground rice straw and 3 mL of salt solution (4.0 KH_2PO_4., 1.6 $(NH_4)2SO_4$ and 1.0 $MgSO_4$, gL^{-1}, but in this solution, KH_2PO_4 was replaced by 25 mg P_2O_5 from PR, as the sole P source, which added separately to each flask before autoclaving. Then, the sterile flasks were inoculated with 5% (v/w) spore suspensions (75 X 109 mL^{-1}) of the two most active tested fungi. All flasks were incubated at 28 ℃, for 7 days, followed by the addition of 50 mL distilled water, then shakened at 140 rpm on a rotary shaker for 30 min and filtered through Whatman No. I filter paper (Kumari *et al.,* 2008). Finally, the amount of free phosphorous was determined according the method of Jacson (1958).

3. Results

3.1. Isolation of the fungal isolates from rice straw samples

Fourteen fungal species were isolated from the collected RS samples. The highest number of fungal species was recovered in the time range of 1-2 year (6 fungal species) as compared to 3-5 year (3 fungal species) and over 7 years (5 fungal species) time periods. Stored period of RS plays major role in ability and activity of different fungi growing on it.

3.2. Initial screening for cellulolytic activity of the fungal isolates

The isolated fungi were screened for their ability to degrade cellulose for determining their enzymatic activities. Therefore, all species were grown on agar plates containing CMC, as the only carbon source, then the hydrolysis of cellulosic substrate were recorded by the determination of the clear zone. *Penicillium* sp2. and Aspergillus niger were able to hydrolyze it and have clear zones of hydrolysis with diameter 3.17 and 2.23 cm, respectively.

3.2.1. Applying of Congo red Test

The activities of selected fungal species were tested using Congo red dye. This test is based on the observation of the outset growth of the hydrolysis halo zone and measurement of the accurate length of it that is used for calculation of the (EI).

Table (1) shows the results of EI values that obtained after cultivation of the fungal species on synthetic medium containing CMC as a sole carbon source, after 4 days of incubation period at 30 ℃.

Table 1. Enzymatic index (EI) of isolated fungi on CMC- agar plates, using Congo red test.

Duration period	Name of isolates	Clear zone (cm)	Colony diameter (cm)	Enzyme Index (EI)
1st Duration	*Aspergillus flavus*	2.27	1.47	1.54
	Aspegillus oryzae	3.6	2.33	1.55
	Curvularia sp.	3.4	2.47	1.40
	Fusarium incarnatum	3.2	2.13	1.50
	Fusarium solani	2.1	1.63	1.29
	Tricoderma viride	3.67	2.4	1.53
2nd Duration	*Alternaria alternata*	2.27	1.47	1.54
	Pencillium sp1.	3.5	2.4	1.46
	Ulocladium alternariae	2.9	2.13	1.36
3rd Duration	*Aspergillus fumigatus*	2.13	1.43	1.49
	Aspergillus niger	2.23	1.27	1.76
	Aurobasidium sp.	2.8	2.2	1.27
	Pencillium sp2.	3.17	1.83	1.73
	Rhizopus sp.	2.1	1.37	1.53

The twelve species that showed EI above 1.40 were taken into our consideration for the next test (Screening of fungal isolates for cellulase production of liquid media). However, the other two species Aurobasidium sp. and Ulocladium altelnaria which have EI equal1.3 and 1.36, respectively, were discarded at the next test.

3.3.1. Determination of fungal biomass

Aspergillus niger and Penicillium sp2. recorded the minimum pH values of broth media, however it was adjusted to a value 5.5 at the beginning of the experiment. Decreasing of pH is referring to degradation of amorphous cellulose (CMC) and production of cellulose degrading complexes that have acidic character. On the other hand, Fusarium incarnatum and Trichoderma viride recorded the maximum values of pH, which refer to little degraded amount of CMC Table (2).

3.3.2. Cellulases activity of the isolated species

The results in Table (2) indicated that the isolated fungal species showed variations in cellulases activity. The most active isolate was *Penicillium* sp2. for FP-ase (38.12 U mL^{-1}) followed by *Aspergillus fumigatus* (32.7 U mL^{-1}). While, *Pencillium* sp1., *Aspergillus orazae* and *Aspergillus niger*, showed moderate activities (23.40, 21.44 and 20.21 U mL^{-1} gm RS respectively). On the other hand, *Curvularia* sp. and *Fusarium solani* showed very weak activities (8.59 and 7.66 U mL^{-1} gm RS, respectively).

The most active isolates for CMC-ase production were *Penicillium* sp2, *Aspergillus niger* and *Aspergillus flavus* (180.00, 172.40 and 160.91 U mL^{-1} gm RS, respectively). *Curvularia* sp. have showed a very weak activity of CMC-ase 30.85 U m L^{-1} gm RS.

3.4. Screening of the isolates for PR solubilization

The most six active cellulolytic fungi obtained from the previous screening, were further screened on the base of PR solubilization (Table 3). For this purpose, the fungal isolates were grown on PR solubilizing medium. Generally, the final culture pHs were reduced to the acidic side for all isolates, which accompanied with the consuming of NaOH for the titration of the resulted acids (TA) in the cultural filtrates. Additionally, measuring the released soluble P from PR. The released soluble P in descending order, were 41.91, 39.02, 38.43, 38.00, 37.09 and 36.88 mg/ mL^{-1} in the culture filtrates of *Aspergillus niger, Penicillium* sp2., *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspegillus oryzae* and *Penicillium* sp1, respectively.

Table 2. Comparative evaluation of cellulolytic fungal activities by means of biomass (final culture pH and cellulases activities)

Isolate species	pH	CMC-ase Unit mL^{-1}	FP-ase Unit mL^{-1}
Alternaria alternata	4.94	122.13	14.61
Aspergillus flavus	4.9	160.91	12.23
Aspergillus fumigatus	4.79	144.12	32.70
Aspergillus niger	4.06	172.40	20.21
Aspegillus oryzae	4.99	154.20	21.44
Curvularia sp.	4.81	30.85	8.59
Fusarium incarnatum	5.09	100.60	9.02
Fusarium solani	4.74	111.12	7.66
Penicillium sp1.	4.80	128.50	23.40
Penicillium sp2.	4.36	180.00	38.12
Rhizopus sp.	4.83	89.58	11.65
Trichoderma viride	5.21	149.08	12.89

Table 3. Phosphate Rock solubilization efficiency by the most active cellulolytic fungi grown on broth medium.

Isolate species	Final culture pH	TA (mg NaOH mL)	Soluble P (µg/ mL)
Aspergillus flavus	4.51	0.75	38.00
Aspergillus fumigatus	3.96	1	38.43
Aspergillus niger	3.45	5.75	41.91
Aspergillus oryzae	4.22	1.25	37.09
Penicillium sp1.	4.04	0.5	36.88
Penicillium sp2.	3.61	4.5	39.02

3.4. Solid-State Fermentation (SSF)

SSF experiment illustrated that; *Aspergillus niger* has higher cellulase enzymatic activity (252.94 Unit mL^{-1}) and P-solubilization (40.51 mg/L) than *Penicillium* sp2. (200.66) and (37.11), as shown in Table (4), Therefore, *Aspergillus niger* is the most active cellulolytic and P-solubilization species.

From the previous results we can concluded that, *Aspergillus niger* and *Penicillium* sp2. were the most active cellulolytic species in degrading stored RS and solubilization of PR.

Table 4. Solid-State fermentation results of the most active cellulolytic and P-solubilization species.

Isolate species	CMC-ase Unit mL^{-1}	Soluble P (µg/mL)
Aspergillus niger	252.94	40.51
Penicillium sp2.	200.66	37.11

4. Discussion

RS has a high rate of production not only in Egypt, but also all over the world as agricultural residues. Therefore, it is ready available substrate for conversion into fungal cellulases production (Berka *et al.,* 1991).

In many countries, including Egypt, there is a common habit as storing of RS for domestic uses. In the present work, the long storage period revealed a remarkable variation of the growing mycobiota. According to Gooday (1979), there are many mycobiota have the capacity to degrade cellulose. The result revealed that, the capability of fungi to grow on stored RS differs between different species of fungi.

Screening of fungi on Agar Plates containing CMC, followed by applying Congo red test considered as an easily method to calculate the enzymatic index (EI). According to Ten *et al.* (2004); selection of strains that have effcient ability in degrading some types of polysaccharides as cellulose, xylan and amylose can be determined by calculation of the diameter of the halo zone accurately. Moreover, Ruegger and Tauk-Tornisielo (2004) applied the enzymatic index method as a simple and rapid method for selection of strains that have the same potential for production of enzymes within the same genus.

According to Lamb and Loy (2005) after the test, there are two zones, which were observed on plates. First zone is the halo zone resulted due to hydrolysis of cellulose in the media, which is directly related to the region of proceeding of cellulolytic enzymes. Since the second zone is the region where the dye only stay attached to it, where there is β−1, 4-D-glucanohydrolase bonds. The pale halo "hydrolysis zone" around the colonies, which identifying to the zone of CMC degradation, was observed in all species but differ in diameter.

The EI value can be used for the selection of isolates within the same genus. According to Kasana *et al.* (2008), the Congo red test was a simple test with low hydrolysis zone intensities. Furthermore; the earlier results reported by Sazci *et al.* (1986) are similar to our results, that indicate the using of Congo red dye decoloration for determination of the cellulase activity of fungal

cultures activation, which isolated from stored RS.

Results demonstrated that fungi *Aspergillus niger* and *Penicillium purpurginium* have been most active cellulolytic strains and the main source of cellulase. These results supported the studies obtained by Hanif *et al*. (2004); Kang *et al*. (2004) and Chandra *et al*. (2007). Regarding to Mathur (1990); Kubicek (1992), the efficiency of fungi in degradation of crystalline cellulosic materials based on the presence of complete cellulase activities in appropriate quantity, i.e., FP-ase, CMC-ase and β-glucosidase activities.

For all tested fungal isolates, cellulase levels of CMC-ase are higher than the FP-ase, our results agree with the previous results recorded by Kubicek (1992); Wen *et al*. (2005); Crisp (2013). The results showed that, isolates of *Aspergillus* sp. and *Penicillium* sp. are the most active cellulolytic species based on their high yield of FP-ase and CMC-ase.

Biosolubilization of PR was a complex process; due to PR has a complex structure with definite particle size. Reduction of pH values is attached with raising in the TA value that is due to the consuming of NaOH during the titration of the resulted complexes in the solution. According to Rashid *et al*. (2004) and Saber *et al*. (2010), these complexes may contribute to biosolubilization of PR. There is a weak or poor correlation between the dropping of pH and the amount of solubilizied P. Some microbes as soil fungi, predominantly of genera *Aspergillus* sp. and *Penicillium* sp. have the ability to solubilize springily soluble phosphates in vitro by secreting of inorganic or organic acids (Whitelaw, 1999).

Applying of these experiments in large scale in industry may give an attention because they will provide natural products such as organic acids and natural consuming of lignocellulolosic wastes including rice straw and wheat straw.

5. Conclusion

The obtained results in this study showed that, storage period plays an essential role in some physiological and morphological changes, which make RS as a natural microbial incubator, differ in availability of microbes in degrading its components. In addition, the superiority of *Aspergillus niger* and *P. purpurgnium* over the other tested fungal cultures, for production of cellulase and solubilization of complex phosphate components was investigated. Therefore, the commercial production of cellulases and P-solublization enzymes, which imported for use in Egypt at a high cost, should be encouraged for the utilization of agricultural wastes as substrates, which may be promising for the production of cellulases.

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الملخص العربى

عزل وتعريف لبعض السلالات الفطرية السيليلوزيه والأكثر اذابه للفوسفور

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قش الأرز (RS) هو واحد من أهم agrowaste "الفضلات الزراعيه" في جميع أنحاء العالم. هناك اختلاف في نوعيه الفطريات التي تقطن RS . وتهدف هذه الدراسة إلى عزل وفرز بعض الأنواع الفطرية من قش الأرز (RS)، لإنتاج السليلوز واذابه الفوسفور الصخرى في هذا العمل، تم عزل 14 نوعا فطرا مختلف من عينات قش الأرز تم جمعها من مناطق مختلفة في محافظة الدقهلية في مصر. أظهر الفحص الأولي لنمو الفطريات على كربوكسي ميثيل السليلوز (CMC) كمصدر الكربون 12، عزله كانت قادرة على النمو على CMC ، بدرجات مختلفة. ولذا تم اختبارها لإنتاجانزيم السليلوليز. تم تحديد النشاط السيلولوزى باستخدام (CMC-ase) و (FP-ase) كانت البنسليوم بيؤبيرجينيوم والاسبيرجيلس النيجر السلالات الأكثر نشاطا لإنتاج CMCase: 180 وحده في الميليمتر المحتوى على 1جراو من القش و FPase بقيم 38.12 وحده في المليمتر و 20.21 وحده/مليمتر على التوالي. وعلاوة على ذلك، تم تنفيذ ذوبان الفوسفات (PR) من خلال استخدام 25 ملجم P_2O_5. وأظهرت النتائج أن النيجر الأقصى ذوبان الفوسفور مع قيمة 346 ملجم / مل-1 متبوعا البنسليوم purpurgnium بقيمة 316.5 ملغ / مل- 1. لذلك، هاتان السلالاتان الأكثر نشاطا في اذابه RS المخزن و PR وذلك من بين كل الأنواع المعزولة في هذه الدراسة.

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JOESE 5

Isolation and Identification of the Highly Cellulolytic and P-Solubilizing Fungi

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