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Research article

Isolation and characterization of cellulolytic and hemicellulolytic fungi from salt affected soils and compost

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Abstract

Lignocellulosic materials are hugely abundant agricultural biomass, which their bioconversion occurs by various microorganisms. Environmental conditions greatly affect the biological processes and the diversity of living organisms. The aim of this study was to isolate and characterize cellulolytic and hemicellulolytic fungi and clearlying thier diversity and efficiency in salt affected soils and compost. Soil samples were collected from saline soils and municipal solid waste (MSW) compost. Cellulolytic fungi were isolated, then purified to single isolate. The isolates were identified morphologically. Cellulase, xylanase, glucanase, and mannanase were determined in plate assays using AZCL-substrates, while CMCase and FPase were evaluated by DNS method. Sixteen fungal isolates were directly isolated. The morphological identification of isolated fungi revealed *Aspergillums* and *Penicillium* genera were the dominant genera in saline environments. All the selected isolates exhibited cellulase and xylanase activity regardless their origin of isolation. Whereas some of *Aspergillus* isolated revealed ß-Glucanase activity, 56 % of isolates the produced mannanase. The mannanase activities among *Aspergillus sp.* isolates were based on isolate as the production depended on the isolated itself irrespective to the species but may be related to isolation source. All isolated fungi have been confirmed to produce CMCase and FPase. The highest CMCase and FPase activity was displayed by *A. glaucus,* SS6 and Com3 isolated from Sinai saline soil in Gilbana and MSW compost, respectively. Some isolates showed high efficiency for bioconversion of lignocellulosic material, according their enzymatic system, and could mitigate salt and alkalinity stress which suggesting an importance in biotechnological applications.

Introduction

Lignocellulosic biomass contains 40–50% cellulose, 25–30% hemicellulose and 15–20% lignin [1]. The cellulose, hemicellulose, and lignin are the backbone of agricultural biomass materials, which its percentage in organic wastes differ depending on the source [2]. The most abundant biomass is cellulose, which represents approximately 180 billion tons of the annual biomass produced through photosynthesis [3]. Furthermore, hemicelluloses, which comprise of a heterogeneous group of polysaccharides that contains xylans, β-glucans, and mannans, are also main components of plant cell walls [4].

Due to their large quantity, the bioconversion of lignocellulosic biomass is indispensable for the carbon cycle and being achieved through several time-consuming processes, which include mechanical, chemical, thermal, and biological treatments [5, 6]. The bioconversion of lignocellulosic materials occurs by the action of various microbial consortiums which yield a series of enzymes that work together to accomplish the bioconversion process [7]. The produced enzymes comprise of cellulases, hemicellulases, and pectinases, etc. which function synergistically to breakdown complex molecules in plant cell-wall [8].

Cellulolytic microorganisms are the major players in the deterioration process converting and hydrolyzing lignocellulosic biomass, through saccharification, to form simple sugar monomers to be fermented into bioethanol for the second generation biofuel production [9, 10]. Among these microbial groups, Fungi are the major cellulase-producing microorganisms, although a few species of bacteria and actinomycetes have also been described to produce cellulolytic enzymes [11, 12].

The degradability of fungal cellulosic enzymes toward cellulosic materials is very common, including that

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produced by species of *Trichoderma*, *Penicillium*, *Aspergillus*, *Phanerochaete*, *Humicola*, *Fusarium* and *Melanocarpus* as reported by Sukumaran *et al.* [11].

The cellulases and xylanases are the major enzymes utilized for the biodegradation of lignocellulosic materials, particularly agricultural and forestry residues and wastes for producing valuable products such as ethanol fuel and other chemicals [13]. Whereas xylans, β-glucans, and mannans are the most important constituents of hemicelluloses in plant cell walls [4], xylanase, β-glucanase, and galactomannanase is considered with a grate importance for the bioconversion of hemicellulose content of organic biomass. Accordingly, determination of cellulase, xylanase, β-glucanase, and mannanase activities of specific microorganism could indicate its cellulolytic and hemicellulolytic degradative potential.

Various plate screening techniques have been described to identify polysaccharide-degrading microorganisms [14], which are mainly based on either the formation of complexes between polysaccharides and dyes [15, 16]. Recently, commercially available insoluble azurine cross-linked (AZCL) substrates as chromogenic substrates were utilized as substrates in various enzyme assays. By these AZCL substrates, a clear correlation between the diameters of the generated blue color zone and enzymes activity was proved [15].

Environmental conditions greatly affect their vital processes and the diversity of living organisms. Microorganisms must possess adaptation mechanisms in order survive through stress in harsh environments including particular kinds of enzymes and resistance to extreme environmental parameters (pH, temperature, pressure and salinity) [17]. Recognition of microorganisms inhabiting such harsh ecosystems is essential for the biotechnological exploration of biodiversity [18, 19].

The current study was carried out to investigate the cellulolytic and hemicellulolytic fungi inhibiting three different environments under stress conditions. Two soil ecosystems, from Kafrelsheikh and North Sinai Governorate, representing high salinity and alkalinity stress condition as well as municipal solid waste compost during its active stage to demonstrate high salinity and high temperature conditions. Exploration of the enzymatic system of isolated fungi toward biodegradation of lignocellulosic materials was among the aims of this study.

Experimental

Sampling

Samples were collected from three Egyptian's harsh environments for fungal isolation. Two soil samples were collected, the first from Northern Sidi-Salim distract, Kafrelsheikh Governorate (31° 36' N, 30° 78' E) and the second from Glbanah area in Northern Sinai (30°56' N, 32°25' E). Both areas are salt affected soils as they are located few kilometers from the Mediterranean Sea. The third sample was collected from an active municipal solid waste (MSW) composting pile from Sidi-Salim MSW composting plant, Kafrelsheikh Governorate. The collected samples were characterized chemically and biologically (Table 1).

Isolation of fungi

Fungi were directly isolated from soils and compost using serial dilution plate technique. A 10 g of each sample was suspended in 90 mL of sterile distilled water and were shaken (150 rpm) for 30 min at room temperature. Then, ten-fold serial dilutions were prepared in sterilized distilled water. One hundred microliters of the 10^{-5} dilution was spreaded on Martin's medium (glucose, 10 g L^{-1}; peptone, 5 g L^{-1}; KH_2PO_4, 1 g L^{-1}; $MgSO_4$. $7H_2O$, 0.5 g L^{-1}; Rose Bengal, 0.033 g L^{-1}; and streptomycin, 0.03 g L^{-1}; pH 5.5-6.0) and plates were then incubated at 30°C for 5-7 days. Colonies with different morphological shapes were selected and transferred several times on the same medium and further incubated at 30°C for 7 days to obtain pure cultures. All fungal isolates were maintained on potato dextrose agar (PDA) slants at 4°C and re-cultured on proper intervals.

For morphological identification, the isolated fungi were grown aseptically on the PDA drop located on the surface of sterile glass microscope slides place in sterile petri dish. Fungal isolates were then identified conventionally according to their morphological features to determine their genera. The macroscopic (colour, texture, appearance, and diameter of colonies) and microscopic (microstructures) characteristics of isolates were observed for identification according to Barnett *et al.*[20], Barnett *et al.*[21], Domsch *et al.*[22], Lieckfeldt *et al.*[23].

Table 1. Some characterization of soil and compost sample

Some characteristics of isolation sources						
	EC	**pH**	**Total Bacteria**	**Total Fungi**	**Actinomycetes**	**Cellulose-degraders**
	dSm^{-2}		CFU 10^6	CFU 10^5	CFU 10^5	MPN
MSW compost	9.85	8.6	115.0	6.3	10.2	140
Gilbana (Sinai) soil	17.40	8.8	6.517	18.25	3.7	17
Sedisalim (Kafrelsheikh) soil	11.10	8.3	95.5	0.475	3.3	26

Enzymes production and extraction

For enzyme production, all fungal isolates were inoculated onto wheat bran agar (WBA) plates (wheat bran, 30 g L^{-1}; agar, 15 g L^{-1}; $ZnSO_4$. $7H_2O$, 0.01 g L^{-1}; and $CuSO_4$. $5H_2O$, 0.005 g L^{-1}; pH 7.4; [5]. The inoculated plates were then incubated for 7 days at 25°C.

The produced enzyme complexes of the isolated fungi were extracted following the method described by Pedersen *et al.* [24]. In brief, twenty agar plugs (6 mm in diameter) with mycelia were excised from the plate of each isolate. The agar plugs were conveyed to a 14-mL vial, then 2.5 mL of distilled water was added, and the vial was then shaken at 150 rpm for 2 h at room temperature. The extracted solution was centrifuged at 6000 rpm for 20 minutes to remove solids. The extracted enzyme solutions were kept at -20°C until used.

Enzyme plate assays

Four different AZCL substrates (AZCL-HE-cellulose, -arabinoxylan, -barley β-glucan, and –galactomannan (Megazyme, Bray, Ireland)) were used for cellulolytic and hemicellulolytic enzyme assays. The method described by Pedersen *et al,* [24] and Eida *et al*., [5] was followed for enzyme assay. The assay suspension containing agarose and each AZCL substrate was poured and allowed to solidify in Petri dishes, then 4 wells with a 4-mm diameter were made in the agarose plates, and 20 µL of enzyme extract was added to each well. The plates were incubated for 24 h at 30°C in the case of HE-cellulose and 12 h for all other substrates. Enzymatic activities were determined by measuring the diameter of the blue zone formed around each well.

CMCase activity

The method described by Mandels *et al*., [25] was followed to determine carboxymethyl cellulase (CMCase) activity. One ml of 1% carboxymethyl cellulose (CMC) in 0.1 M citrate buffer pH 5.6 was placed in a test tube and 0.5 ml of extracted enzyme solution was added. The test tubes were incubated at 50°C in a water bath with shaking for 30°C min. The reaction was stopped by adding 3.0 ml of 3.5–dinitrosalicylic acid (DNS) reagent to the reaction mixture, and boiled for 5 minutes [26]. The absorbance of the appropriately diluted reaction mixture was read at 540 nm using a spectrophotometer. One unit of cellulase was defined as the amount of enzyme that released 1µmol reducing sugars as glucose equivalent per min in the reaction mixture under the specified assay conditions. All enzyme assays were performed in triplicates.

Filter paper activity (FPase)

Filter paper assay (FPA) for total cellulase activity in the cultural filtrate was measured according to the method of Mandels *et al.* [25]. Aliquots of appropriately diluted extracted enzyme were added to Whatman No.1 filter paper strip (1 X 6 cm; ≈ 50 mg) immersed in 1 mL of 0.05 M sodium citrate buffer of pH 5.0. The reducing sugars released were estimated using dinitrosalicylic acid (DNS) according to the method described by Miller [26], after incubation at 50°C. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µ mole of reducing sugar from filter paper per mL per min.

Results and Discussion

Samples were collected from different harsh environments for isolation and characterization of cellulolytic fungi. The MSW compost was collected from active composting piles with high temperature (around 76°C). All samples showed high salinity and high pH levels. Table (1) shows the salinity and alkalinity as well as microbiological characteristics of examines ecosystems.

Isolation of cellulolytic fungi

Three slate affected environments (soils and compost) were explored for their cellulolytic and hemicellulolytic fungi. MSW compost was expected to contain heat as well as salt tolerant microorganisms. From all investigated samples, sixteen fungal isolates were selected, purified and identified. Six isolates were obtained from MSW compost sample while 5 isolates were isolated from soil sample collected from Gilbana area North Sinai Governorate. The last 5 isolates were picked-up from salt affected soil in Said-Salim district, Kafrelsheikh Governorate.

The isolates were identified by morphological features such as color of the colony and growth patterns. Microscopic characteristics were examined under the microscope include spore formation and color. The obtained data of the morphological identification of isolated fungi revealed that members belonging to *Aspergillums* and *Penicillium* genera inhabited all isolation sources. Different species of both genera were dominant in compost samples (Table 2).

The Ascomycota is known to inhabit a wide diverse of environments. Le Goff *et al.* [27] reported the dominance of *Thermomyces lanuginosus*, *Aspergillus*, *Penicillium* during the active phase of composting process. The members of *Aspergillus*, *Penicillium* were among the isolated cellulolytic fungi from different types of compost [5]. Gautam *et al.* [28] isolated and evaluated the cellulolytic activity of different species of *Aspergillus* and *Penicillium* from municipal waste compost.

The dominance of these genera and species may owing to their tolerance capability to several abiotic stress including high temperature, pH and high salt concentrations. Several reports documented the tolerance ability of numerous species of *Aspergillus and Penicillium* to several stressing parameters including drought, temperature degrees, pH value, salinity and acidity degree as well as aluminum and iron concentrations [29-31]. Additionally, Srinivasan *et al.* [32] found that *Aspergillus and Penicillium* were the most

dominant fungi in salt affected soils when studying the Phosphate dissolving microorganisms.

The compost isolated *Aspergillums sp.* was identified morphologically as *A. niger* (Com1), *A. terreus* (Com2 and Com5) and *A. glaucus* (Com3 and Com6), while the only *Penicillium* isolate (Com4) was identified to genus level (Table 2). In accordance with our results, *Aspergillus niger, Aspergillus terreus* and several *Penicillium sp.* had been isolated and identified by Anastasi *et al.* [33]. They isolated several fungi and studied their qualitative and quantitative composition and diversity during thermophilically produced compost and a vermicompost. Similar results have been obtained from both soil samples. *A. niger* (SS1 and KFS2), *A.terreus* (SS5 and KFS5), *A. glaucus* (SS2 and SS6) and *Penicillium sp.* (SS3, KFS1 and KFS3) were isolated and identified from both salt affected soils while last *Aspergillus* isolate was identified to genus level (Table 2). Afza *et al.* [34] isolated and identified several *Aspergillums* species including *Aspergillus ficcum, Aspergillus flavus, Aspergillus flavus var. columnaris, Aspergillus terreus* var. *aureus, Aspergillus fumigatus, Emericella nidulans, Emericella rugulosa and Apergillus terricola* var.*americana* from Pakistani soils. The existence of *Aspergillus glaucus* in soil and compost may refer to its extremely halophilic performance of this fungus as described by Liang *et al.* [35].

Cellulolytic and hemicellulolytic enzymes activities

Cellulose and hemicellulose are most abundant of biological renewable resource for bioconversion process and utilizations. The maximization of cellulose and hemicellulose added value can be done through improving their hydrolysis to glucose and other fermentable sugars which can be biologically converted to ethanol. The bioconversion of biomass materials is achieved by complex enzymatic processes, which required a consortium of microbial enzymes that differed according to constitutes of the raw materials. Cellulases, xylanase, ß-Glucanase and mannanase are major hydrolytic enzymes, produced by many microorganisms, capable of degradation of cellulose for production of variety of valuable products. Evaluation and comparing the activities of these enzymes could facilitated the characterization, selection and further applications of cellulolytic and hemicellulolytic microorganisms.

Cellulase plate assay

All the selected fungal isolates exhibited cellulase activity regardless their origin of isolation. Figure (2) Shows an example for the plate assay results with different AZCL substrates. The highest cellulase activity, expressed as diameter of blue color zone on the plate assay (Figure 1 & 2), was revealed by *Penicillium sp.* isolate (KFS1) and *Aspergillus terreus* isolated (KFS5) which were isolated from salt affected soils in Northern Delta (Kaferelsheikh Governorate). While the following isolates were Com5 (*terreus*), SS1 (*A. niger*) and Com3 (*A. glaucus*). This results are accordance with former findings of Jahangeer *et al.* [36] and de Siqueira *et al.* [37], who stated cellulase production by *Penicillium* sp. and *Aspergillus* sp.

Table 2. Morphological characterization of fungal isolates

Isolate No	Isolation source	Morphological identification	
Com1	MSW compost	*Aspergillus sp.*	*A. niger*
Com2	MSW compost	*Aspergillus sp.*	*A. terreus*
Com3	MSW compost	*Aspergillus sp.*	*A glaucus*
Com4	MSW compost	*Penicillium sp.*	*Penicillium sp.*
Com5	MSW compost	*Aspergillus sp.*	*A. terreus*
Com6	MSW compost	*Aspergillus sp.*	*A glaucus*
SS1	Soil from Sinai	*Aspergillus sp.*	*A. niger*
SS2	Soil from Sinai	*Aspergillus sp.*	*A glaucus*
SS3	Soil from Sinai	*Penicillium sp.*	*Penicillium sp.*
SS5	Soil from Sinai	*Aspergillus sp.*	*A. terreus*
SS6	Soil from Sinai	*Aspergillus sp.*	*A glaucus*
KFS1	Soil from Kafrelsheikh	*Penicillium sp.*	*Penicillium sp.*
KFS2	Soil from Kafrelsheikh	*Aspergillus sp.*	*A. niger*
KFS3	Soil from Kafrelsheikh	*Penicillium sp.*	*Penicillium sp.*
KFS4	Soil from Kafrelsheikh	*Aspergillus sp.*	*Aspergillus sp.*
KFS5	Soil from Kafrelsheikh	*Aspergillus sp.*	*A. terreus*

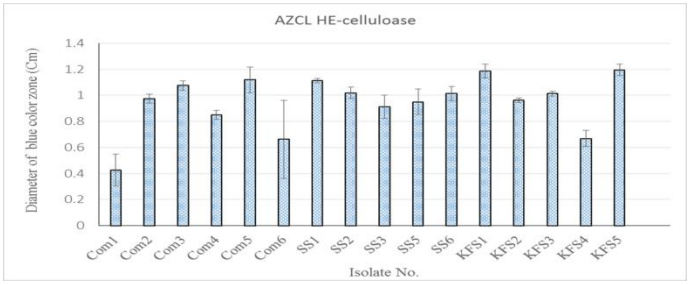


Figure 1. Cellulase activity of isolated fungi from different harsh environments. Error bars present standard deviation.

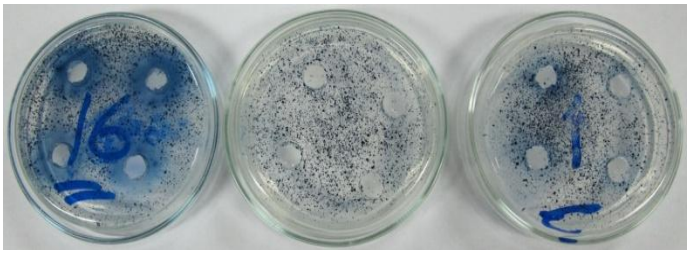


Figure 2. The Enzymes assay using Azurine cross linked substrates. The plate in the middle represent the control without enzyme while the plates to lift and to right represent the high and low enzyme activity, respectively.

Xylanase plate assay

All obtained isolates showed xylanase activities toward degradation of AZCL-arabioxylan as a substrate (Figure 3). Com6 (*A. glaucus*) exhibited the highest xylanase activity followed by *A. niger* (Com1) among compost isolates. Additionally, *A. niger* isolate (SS1) and *Penicillium* sp. isolates were the highest xylanase activities isolated from Sinai soils. With regard the isolated fungi from Kafrelsheikh soils, *Penicillium* sp. were the most active toward xylenes degradation although *A. terreus* (KFS5) showed high xylanase activities. The xylanase activities recorded for some members of *Aspergillus* and *Penicillium* genera was reported by Chàvez *et al.* [38] and Gawande and Kamat [39], respectively.

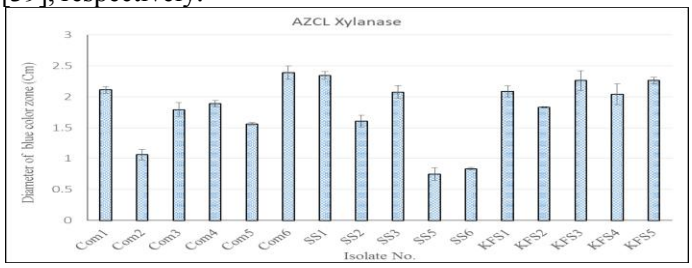


Figure 3. Xylanase activity of isolated fungi from different harsh environments. Error bars present standard deviation.

ß-Glucanase plate assay

The ß-Glucanase activity, as one of imprtant enxymes for degradation of hemicellulose, showed a different trend compared to cellulase and xylanase activities. The activity was isolate dependent regardless the isolation source or the isolated genera (Figure 4). Some of *Aspergillus* isolated revealed ß-Glucanase activity while the other were negatively responded to the assy. *A. terreus* and *A. glaucus* (Com2) and (Com3) and (SS5) and (SS6) among compost and Sinai isolates, respectively, showed the highest activity. Moreover, *A. niger* (KFS2) and *Aspergillus* sp. (KFS4) isolated from kafrelsheikh soil were the only positive isolates. On the other hand, none of *Penicillium* isolates displayed ß-Glucanase activity. The production of ß-Glucanase by *Aspergillus* sp. was described by Eida *et al.* [5] although, in contrast to this result, they stated the production of ß-Glucanase by *Penicillium* sp. This difference may owing to the difference in isolated spieces. This result is in agreement with those of Pederson *et al.* [28], who revealed that enzymatic activities of isolates differed amongst species and also individual strains of the same species. Additionally, they found that the isolation source is one of the major factors affecting enzyme production in individual strains.

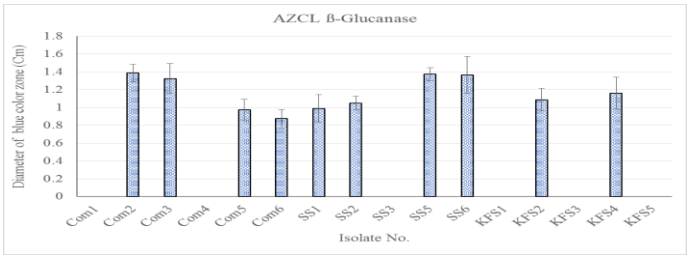


Figure 4. ß-Glucanase activity of isolated fungi from different harsh environments. Error bars present standard deviation.

Mannanase plate assay

Only 56 % (nine out of the 16) of isolates the were able to produce mannanase activity (Figure 5). On the contrary to ß-Glucanase activity, *Penicillium* sp. isolates produced manganese except one isolate from Kafresheikh (KFS3).

The mannanese activities among *Aspergillus* sp. isolates were based on isolate itself irrespective the species but it may be related to isolation source. For instance, *A. terreus* isolated from compost gave a positive activity while isolates from the two soils under investigation were mannanese negative. In general, there were no large difference among the all positive isolates.This results could be supported by those of Eida *et al.* [5, 6] and Pederson *et al.* [28] which explained the variations in enzymatic activities according to the isolated strain. These results clarify that the enzymatic activities is a strain-dependent phenomenon.

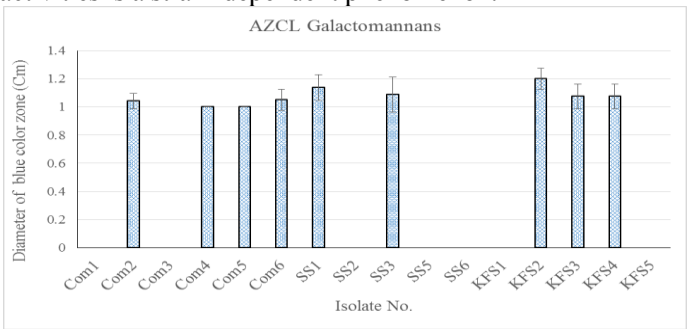


Figure 5. Mannanase activity of isolated fungi from different harsh environments. Error bars present standard deviation.

Carboxymethylcellulase (CMCase)

All isolated fungi have been confirmed to produce CMCase as one of the cellulase enzyme complex (Figure 6). The highest CMCase activities (1.302 and 1.292 U/ml) were displayed by both *A. glaucus* (SS3 and Com3, respectively) isolated from Sinai saline soil in Gilbana area and from MSW compost, respectively, then the *Aspergillus sp.* isolated from Ksfriskeikh soil. Other isolates showed relatively high CMCase activity, higher than 1 U/l, including KFS4 (*Aspergillus* sp.; 1.186 U/ml), SS2 (*A. glaucus*; 1.068 U/ml) and SS1 (*A. niger*; 1.024 U/ml). Whereas the lowest activities (0.496 and 0.534 U/ml) were recorded for Com1 *(A. niger)* and Com4 *(Penicillium* sp.*),* respectively*,* however, there were no big difference between other isolates. Several reports confirmed the cellulolytic activities of *Aspergillus* species which are known to use a broad range of lignocellulosic substrates [40, 41]. Moreover, in accordance with our results, Gautam *et al.* [28] documented the production of CMCase by several fungal species including *Aspergillus* and *Penicillium* isolated from MSW compost as well as soils. Although all isolates showed CMCase activity, the recorded results was lower than found by other researches which could reach up to 15 U/ml for isolates belonging to genera of *Aspergillus* and *Penicillium* [42].

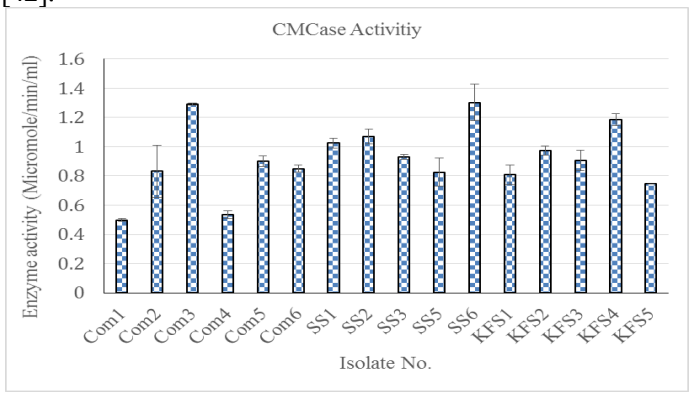


Figure 6. Carboxymethylcellulas activity of isolated fungi from different harsh environments. Error bars present standard deviation.

Filter paperase (FPase)

Filter paper enzyme assay is a famous parameter for evaluating extracellular cellulolytic activity of an organism. Figure 7 illustrates the FPase unites of the fungi isolated from different stressed ecosystems. Among the isolated fungi in the current study, *A. glaucus* (SS6) isolated from Sinai saline soil, which revealed very high CMCase activity, presented the highest FPase (0.856 UFPase/ml) as well. Whereas the KFS4 isolate which was identified as *Aspergillus* sp. displayed the highest FPase activities (0.726 U/ml) among Kafrelsheikh isolates, *A. terreus* isolate no. Com5 exhibited the maximum FPase activity (0.722 U/ml) amongst compost isolates with very low difference. All other isolated revealed close activity units except KFS5 isolate (*A. terreus*) which showed relatively low FPase activity (0.243 U/ml). The cellulolytic activities of *Aspergillus* species are well documented toward broad range of lignocellulosic substrates. The enzymatic activaties of the microbes generally expressed as CMCase and FPase [40, 41]. Moreover, in agreement with results of this study, Gautam *et al.* [28] reported the production of Fpase and CMCase by different fungal species including *Aspergillus* and *Penicillium* isolated from MSW compost as well as soils. Moretti *et al.*[43] reported the difference in FPase activity according to isolates types as well as isolation source for several fungal genera including *Aspergillus* and *Penicillium*. The FPase activities revealed by effective isolates obtained from this study were relatively high (0.726-0.856 U/ml) compared to the FPase activities (less than 0.5 U/ml) of *Aspergillus*, *Penicillium* and *Trichoderma* isolated from soils and plants by Damaso *et al.* [42].

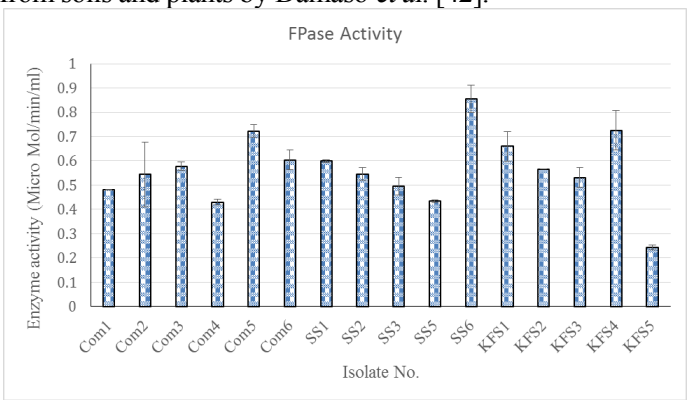


Figure 7. Filter paperase enzyme activity of isolated fungi from different harsh environments. Error bars present standard deviation.

Conclusion

Although several known cellulolytic and hemicellulolytic fungi have been isolated from a wide diverse of environments, the current work exhibited the dominancy of members of *Aspergillus* and *Penicillium* as the major cellulolytic and hemicellulolytic fungi in salt affected environments. In the present study, some isolates showed high efficiency for bioconversion of lignocellulosic material, according their enzymatic system, and could mitigate salt and alkalinity stress. The enzyme activities of isolated fungi were found to be strain dependent. *A. glaucus* (SS6 and Com3) and *A. terreus* (Com5) revealed high CMCase, FPase, with relatively high activities toward other enzyme assays suggesting their importance for biotechnological applications. Further studies need to be conducted to evaluate the characteristic of produced enzymes by selected isolates.

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