ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 13 (4): 152-158, 2014
ISSN 1682-296X / DOI: 10.3923/biotech.2014.152.158

Screening of Inulinolytic Potentialities of some Fungi Isolated from Egyptian Soil

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Abstract: Forty five soil samples from different sites of non-cultivated soil, salt marshes and rhizosphere of some wild and cultivated soils collected from seven governorates of Egypt were used as local source for isolation of some inulinolytic fungi. Forty six fungal species belonging to three sub-divisions of Zygomycotina, Ascomycotina and Deutromycotina were identified. Eight unknown filamentous fungi characterized by their black reverse color on PDA medium and Czapek agar plates were recorded. Frequency of occurrence indicates that *Aspergillus foetidus* var. *pallidus* (frequency 52.2%) was the most dominant followed by *A. sclerotiorum* (39.1%). All fungi were able to grow on medium containing sugar cane bagasse and Jerusalem artichoke tubers powder (1:1). No significant correlation observed between the fungal growth, liberated soluble protein and inulinase activities. Inulinase activity indicate that *Aspergillus foetidus* var. *pallidus* (564.71±1.22 $Ugds^{-1}$), *A. sclerotiorum* (534.78±1.37 $Ugds^{-1}$), *Emericella nidulans* (495.73±3.85 $Ugds^{-1}$) and *A. aculeatus* (444.37±2.37 $Ugds^{-1}$) were the most active fungal species able to produce a considerable amount of enzyme activity.

Key words: Inulinase, *Aspergillus foetidus*, *Aspergillus sclerotiorum*, solid state fermentation

INTRODUCTION

Inulin is a widespread naturally occurring polyfructan, occurs as a reserve carbohydrate in bulbs, tubers and roots of many plant families including; Liliaceae, Amaryllidaceae and Asteraceae (Carpita *et al*., 1989; Chi *et al*., 2011). It consists of linear chains of β 2, 1-D fructofuranose molecules terminated with a glucose residue at the reducing end (Lopez-Molina *et al*., 2005). Such inulin sources have received a great interest as they represent relatively inexpensive and abundant substrates for the microbial production of high fructose syrup which has gained importance in food, drink and pharmaceutical industries (Zhao *et al*., 2010). Fructose syrup has beneficial effects in diabetic patients, increases the iron absorption in children, has high sweetening capacity so it can be used in the diet of obese persons, stimulates calcium absorption in postmenopausal women, stimulates growth of bifidobacteria in large and small intestine, prevents colon cancer (Rocha *et al*., 2006).

The use of microbial inulinase (β-D-fructan fructanohydrolase) for hydrolysis of inulin containing extracts for the production of high fructose syrup has been reported by Chen *et al*. (2011a). Microorganisms including, *Penicillium*, *Aspergillus* and *Kluyveromyces* are the most industrial strains that used as inulinase producers (Pandey *et al*., 1999). Their inulinases are classified into exo-inulinases (EC 3.2.1.80) that split terminal fructose from the non-reducing end and endo-inulinases (EC 3.2.1.7) that hydrolyze the internal β-(2→1)-fructofuranosidic linkages of inulin (Chi *et al*., 2011). Traditionally, inulinases are produced by submerged fermentation (Gill *et al*., 2003). Inulinase can also be produced by solid-state fermentation (SSF) (Xiong *et al*., 2007).

Increasing potential of microbial inulinase applications for the production of ultra-high-fructose syrup, inulo-oligosaccharide, bioethanol, single-cell protein, citric acid, 2, 3 butanediol, lactic acid and sugar alcohols (Yun *et al*., 1997; Saha, 2006; Ricca *et al*., 2009; Liu *et al*., 2010; Lim *et al*., 2011; Chi *et al*., 2011) are promoted us to screen for new inulinase-producing microorganisms. In the present study, an attempt try to isolate and identify some inulinase-producing filamentous fungi from the soil and rhizosphere of some plants for further screening of their inulinase producing potentialities by solid state fermentation technique.

MATERIALS AND METHODS

Collection of soil samples: Forty five soil samples were collected from different sites of non-cultivated soil, salt

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marshes and rhizosphere of wild and cultivated soils were during 2009 from seven governorates of Egypt, Dakahlia, Kafr El-Sheikh, Beheira, Alexandria, Fayoum, Monofeya and South Sinai.

Isolation and purification of inulinolytic fungi: The fungal species were isolated according to method of Warcup (1950). The 25 g of each soil sample was suspended in 225 mL of sterilized distilled water (1:10; 10^{-1} dilution) and subsequently 10 mL of this suspension were added to 990 mL of sterilized distilled water. Petri-dishes containing inulin agar medium according to Nakamura *et al*. (1997) plus chloranphenicol (100 mg L^{-1}) were inoculated with 1.0 mL of the 1:1000 diluted soil suspensions. The inoculated plates were incubated at 28±2.0°C for 3 days. Morphological appearances of the plates were observed and fragments of distinct colonies were transferred separately to the same inulin agar medium to obtain pure isolates which were then maintained and stored at 4°C.

Identification of the fungi: Pure isolated fungi were confirmed for identification through Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt, by observing its macroscopic characteristics (colour, texture appearance and diameter of the colonies) and microscopic (microstructures), according to Domsch *et al*. (1993): For soil fungi, Zycha and Siepmann (1973) for families, genera and species of Mucorales, Baijal and Mehrotra (1980) for genus *Cunninghamella*, Schipper and Stalpers (1984) for genus *Rhizopus*, Raper and Fennell (1965, 1973) and Samson (1992) for the genus *Aspergillus* and its teleomorphs, Pitt (1979) for the genus *Penicillium* and its teleomorphic states of *Eupencillium* and Talaromyces, Barron (1977) for genera of Hyphomycetes from soil, Booth (1971) for *Fusarium* species and Ellis (1971, 1976) for dematiaceous Hyphomycetes.

Basal medium: The basal medium (free of carbon) used in experiments was contained the following (g L^{-1} of acetate buffer, 0.1 M, pH 4.8): peptone, 5.0, $NH_4H_2PO_4$, 8.0, $(NH_4)_2HPO_4$, 4.0, KCl, 0.5, $MgSO_4.7H_2O$, 0.5 and $FeSO_4.7H_2O$, 0.01 according to Nakamura *et al*. (1997).

Inoculum preparation: Fungal cultures were sub-cultured on modified agar media containing 1.0% inulin as a sole carbon source, at 30°C for 5.0 days and maintained at 4°C. Induced slant was mixed with 10 mL sterile basal medium for preparing spore suspension. The spore count in the suspension was about 2.0×10^7 spore mL^{-1}.

Solid state fermentation: Fermentation was carried out in 250 mL Erlenmeyer flask with 0.1 g mixed substrate of sugar cane bagasse and Jerusalem artichoke tubers powder by a ratio of 1:1, mixed with 2.0 mL of freshly prepared basal medium (0.1 M acetate buffer, pH 4.8). The flasks were autoclaved at 121°C at 15 lbs for 20 min after cooling each flask was inoculated under aseptic conditions and distributed carefully with 2.0 mL spore suspension previously prepared (final moisture content was 80%) and incubated for 5.0 days at 30°C under static conditions.

Extraction of inulinase: The fermented substrates were mixed and homogenized well with 50 mL of 0.1 M acetate buffer (pH 4.8). The mixture was shaken thoroughly on a rotary shaker (150 rpm) at room temperature (20±2°C) for 60 min. The mixtures were filtered off through muslin cloth; then centrifuged at 5000×g for 10 min. After centrifugation, the supernatant was collected and stored in a deepfreezer (-20°C) until its use as a crude inulinase (Chen *et al*., 2011b).

Inulinase assay: The inulinase activity was determined by measuring the reducing sugars released by the hydrolysis of inulin according Smogyi (1952) method. The assay mixture for inulinase containing 0.1 mL of crude enzyme and 0.1 mL of 0.1% (w/v) inulin (freshly prepared in 0.1 M acetate buffer; pH 4.8). The reaction mixture was incubated at 40°C for 30 min, then, liberated reducing sugars were estimated at 700 nm using Spectro UV-Vis RS spectrophotometer. One unit (U) of inulinase was defined as the amount of enzyme that released one μmol of fructose per minute from inulin at 40°C and other assay conditions.

Estimation of soluble protein: The soluble protein concentration was determined by the method of Bradford (1976), by measuring the optical density of the color against blank at 595 nm using spectro UV-Vis RS spectrophotometer. The protein concentration was calculated using μg standard curve of bovine serum albumin (BSA).

Statistical analysis: The analysis of data was done by using the statistical software Graph-Pad Prism 4. Each experiment was repeated three times (minimum) and the data are means of triplicate determinations. Results with probability levels greater than 5% were regarded as non-significant.

RESULTS AND DISCUSSION

The increased potential of inulinase applications (production of high fructose syrups, fructooligosaccharides, ethanol and inulo-oligosaccharides which are extensively used in

pharmaceutical and food industry), promoted us to search for new sources of inulinases and hence microorganisms are the best sources for commercial production of inulinases because of their easy cultivation and high yields of the enzyme, our attempt was made to isolate and identify inulinase-producing filamentous fungi from the soil and rhizosphere of some plants and screening their inulinase producing potentialities.

In this study approximately 380 filamentous fungal isolates (Table 1) were isolated by dilution plate method on inulin agar medium, the number of isolates and its biodiversity are restricted by inulin (1.0%) used in isolation medium (sole carbon source). The rhizospheres of inulin rich plants including; Jerusalem artichoke (*Helianthus tuberosus* L.) (Abuzahran site, Beheira) and *Allium cepa*, (Sinbillawain; Dakahlia) were the richest soils (24, 22 isolates, respectively) compared with the others. This high fungal occurrence may be attributed to the bioactive materials of root exudates (sugars, organic acids and amines…) which released into the plant rhizosphere during their life cycle which enhance plant growth and fungal populations (Westover *et al*., 1997) furthermore this may also due to the release of root fragments that represent potential substrates for fungi, being therefore, a way of these microorganisms to obtain inulin as a carbon source. In this connection, De Souza-Motta *et al*. (2003) reported the distribution of about 50 fungal species from rhizosphere of Asteraceae plant. By contrast, the least number of isolates (3 isolates) were obtained from salt marshes soils of Baltim (Kafr El-Sheikh) and Burj Al Arab (Alexandria), due to high salt concentrations which interfere or prevent the fungal population as well as plant growth. Furthermore, decreasing the number of isolates in the non cultivated salt marshes may be also prohibited by the presence of inulin in isolation plats as sole carbon source.

The isolates were classified and identified as 46 isolates by Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Nasr City, Cairo, Egypt. Results (Table 2) showed 38 fungi were identified to the species level while, 8 species (not completely identified) belonging to deuteromycotina were recorded by their isolation code numbers. The well identified species belonged to three sub-division called Zygomycotina (4 species), Ascomycotina (29 species) and Deutromycotina (5 species). This predominance of Deuteromycetes and Ascomycetes are in agreement with results obtained by Garrett (1976) and De Souza-Motta *et al*. (2003) where, they isolated about 49 fungal species belonging to Ascomycota, Deuteromycota and Zygomycota from the rhizospheres of sunflower cultivated in field and in greenhouse. On the other hand Santos *et al*. (1989) reported the predominance of Deuteromycota in sugar cane rhizosphere, by analyzing 142 samples obtained from 22 plantation sites, spread over several regions of the Pernambuco State (Brazil).

The frequency of fungal occurrence showed that *Aspergillus foetidus* var. *pallidus* (frequency 52.2%) was the most prevalent followed by *Aspergillus sclerotiorum* (39.1%), *Aspergillus aculeatus* (34.8%) and

Table 1: Occurrence of inulinase producing fungal isolates from soils and rhizosphere of some wild and cultivated crops collected from different sites of seven Egyptian governorates, Dakahlia, Kafr El-Shaikh, Beheira, Alexandria, Fayoum, Monofeya and South Sinai

Governorates	Site	Cultivated plant	N	Percentage	Governorates	Site	Cultivated plant	N	Percentage
Dakahlia	Si	*Cichorium intybus*	10	2.63	Beheira	AZ	*Helianthus tuberosus* L.	24	6.32
		Trifolium sp.	5	1.32			*Helianthus tuberosus* L.	12	3.16
		Triticum sp.	8	2.11		10,000	*Vicia faba*	6	1.58
		Allium cepa	12	3.16			*Triticum* sp.	4	1.05
		Allium cepa	11	2.89			*Phaseolus vulgaris*	7	1.84
		Allium cepa	22	5.79			*Solanum lycopersicum*	4	1.05
	Ma	*Citrus* sp.	7	1.84		Ga	*Triticum* sp.	4	1.05
		Citrus sp.	8	2.11			*Phaseolus vulgaris*	21	5.53
		Brassica sp.	9	2.37			*Xanthium spinosum*	8	2.11
		Lactuca sativa	10	2.63			*Cucurbita pepo*	8	2.11
	BO	Non cultivated soil	5	1.32			*Aptenia cordifolia*	4	1.05
Kafr El-Shaikh	Ba	*Solanum lycopersicum*	5	1.32			*Urtica* sp.	16	4.21
		Avena sativa	4	1.05	Alexandria	BuA	*Limbarada crithmnides*	16	4.21
		Salt marshes soil	3	0.79			*Olea* sp.	8	2.11
		Aegilops bicornis	14	3.68			*Allium cepa*	10	2.63
	Ha	*Saccharum* sp.	7	1.84			*Peganum harmala*	16	4.21
		Trifolium sp.	4	1.05			*Lalium perenne*	8	2.11
		Vicia faba	9	2.37			*Chrysanthemum coronarium*	4	1.05
Fayoum	MA	*Sorghum* sp.	4	1.05			*Ficus* sp.	7	1.84
		Allium cepa	7	1.84			*Ononis serata*	6	1.58
		Trifolium sp.	16	4.21			Salt marshes soil	3	0.79
Monofeya	Kh	Non cultivated soil	8	2.11			*Onopordum alexandrinum*	4	1.05
South Sinai	RM	Non cultivated soil	4	1.05					

N.B: Total isolates (T) = 380, No. of isolates (N) and Percentage: (N/T)×100, Si: El- Sinbillawain, Ma: Mansoura, BO: Bani Obeid, Ba: Baltim, Ha: Hamoul, MA: Minshat Abdullah, Kh: Elkhatatba, RM: Ras Mohamed, protected area, AZ: Abu Zahran, Ga: Ganaklis, BuA: Burj Al Arab

Table 2: List of identified inulinase producing fungi and their frequencies after isolation from soils and rhizosphere of some wild and cultivated crops from different sites of seven Egyptian governorates of El-Dakahlia, Kafr El-Shaikh, El-Beheira, Al-Alexandria, El-Fayoum, El Monofeya and South Sinai

Isolate name	R	FI	FT	Isolate name	R	FI	FT
Aspergillus aculeatus	16	34.78	4.21	*Emericella quadrilineata*	9	19.57	2.37
Aspergillus alliaceus	9	19.57	2.37	*Fusarium poae*	3	6.52	0.79
Aspergillus auricoma	9	19.57	2.37	*Fusarium solani*	3	6.52	0.79
Aspergillus brevipes	15	32.61	3.95	*Fusarium tabacinum*	6	13.04	1.58
Aspergillus carneus	6	13.04	1.58	*Mucor circinelloides*	12	26.09	3.16
Aspergillus duricaulis	9	19.57	2.37	*Penicillium citrinum*	6	13.04	1.58
Aspergillus flavus	12	26.09	3.16	*Penicillium fellutanum*	3	6.52	0.79
Aspergillus foetidus var. *pallidus*	24	52.17	6.32	*Penicillium janthinellum*	6	13.04	1.58
Aspergillus fumigatus var. *ellipticus*	9	19.57	2.37	*Penicillium purpurogenum*	3	6.52	0.79
Aspergillus japonicas	6	13.04	1.58	*Penicillium simplicissium*	3	6.52	0.79
Aspergillus niveus	3	6.52	0.79	*Penicillium wortmannii*	12	26.09	3.16
Aspergillus oryzae	6	13.04	1.58	*Pseudallescheria ellipsoidea*	9	19.57	2.37
Aspergillus petrakii	9	19.57	2.37	*Rhizopus azygosporus*	6	13.04	1.58
Aspergillus peyronelii	6	13.04	1.58	*Rhizopus microsporus*	3	6.52	0.79
Aspergillus pseudo-niger	6	13.04	1.58	*Trichoderma koningii*	9	19.57	2.37
Aspergillus sclerotiorum	18	39.13	4.74	F1 (1-43)	12	26.09	3.16
Aspergillus sydowii	6	13.04	1.58	F2 (1-10)	9	19.57	2.37
Aspergillus tamari	9	19.57	2.37	F3 (1-19)	12	26.09	3.16
Aspergillus terreus	9	19.57	2.37	F4 (1-38)	9	19.57	2.37
Aspergillus terreus var. *aureus*	9	19.57	2.37	F5 (4-41)	6	13.04	1.58
Aspergillus ustus	6	13.04	1.58	F6 (1-16)	6	13.04	1.58
Cunninghamella echinulata	3	6.52	0.79	F7 (1-33)	3	6.52	0.79
Emericella nidulans	16	34.78	4.21	F8 (3-35)	9	19.57	2.37

N.B: Total isolates (T) = 380, Identified isolates (I): 46, Repetition of isolates (R), Frequency (FI): (Repetition/Identified isolates)×100, Frequency (FT): (Repetition/Total isolates)×100

Table 3: Inulinase activities and protein conc. produced by different isolated fungi

Fungal strains	Protein (mg gds^{-1})	Inulinase (U gds^{-1})	Fungal strains	Protein (mg gds^{-1})	Inulinase (U gds^{-1})
Aspergillus aculeatus	1.84±0.019	444.37±2.37	*Emericella quadrilineata*	2.11±0.044	126.49±0.56
Aspergillus alliaceus	1.79±0.020	39.29±2.13	*Fusarium poae*	0.59±0.043	42.88±2.83
Aspergillus auricoma	0.97±0.023	104.22±2.98	*Fusarium solani*	0.63±0.021	18.13±1.27
Aspergillus brevipes	2.40±0.045	297.51±2.94	*Fusarium tabacinum*	0.69±0.015	84.80±1.52
Aspergillus carneus	1.15±0.015	60.31±3.73	*Mucor circinelloides*	0.91±0.078	0.00±0.00
Aspergillus duricaulis	0.72±0.018	000.00±0.00	*Penicillium citrinum*	1.16±0.035	138.99±2.43
Aspergillus flavus	3.20±0.029	121.41±1.62	*Penicillium fellutanum*	0.62±0.007	16.89±1.35
Aspergillus foetidus var. *pallidus*	2.46±0.032	564.71±1.22	*Penicillium janthinellum*	0.67±0.012	45.09±0.68
Aspergillus fumigatus var. *ellipticus*	2.02±0.046	220.19±3.86	*Penicillium purpurogenum*	1.53±0.038	45.71±0.55
Aspergillus japonicas	2.26±0.064	132.67±1.49	*Penicillium simplicissium*	0.57±0.076	7.12±0.39
Aspergillus niveus	2.06±0.017	85.36±6.34	*Penicillium wortmannii*	1.46±0.026	304.00±1.22
Aspergillus oryzae	2.79±0.023	82.89±3.05	*Pseudallescheria ellipsoidea*	0.88±0.057	30.62±0.93
Aspergillus petrakii	0.85±0.015	191.12±1.20	*Rhizopus azygosporus*	1.18±0.010	70.50±0.91
Aspergillus peyronelii	1.19±0.045	53.22±1.77	*Rhizopus microsporus*	1.19±0.043	27.03±1.46
Aspergillus pseudo-niger	2.23±0.042	252.36±1.78	*Trichoderma koningii*	1.25±0.023	5.15±0.28
Aspergillus sclerotiorum	1.58±0.042	534.78±1.37	F1 (1-43)	0.57±0.017	000.00±0.00
Aspergillus sydowii	1.78±0.017	111.95±0.61	F2 (1-10)	0.79±0.038	000.00±0.00
Aspergillus tamarii	2.16±0.048	130.19±3.14	F3 (1-19)	0.69±0.033	006.49±0.20
Aspergillus terreus	1.82±0.018	157.11±1.42	F4 (1-38)	0.75±0.026	000.00±0.00
Aspergillus terreus var. *aureus*	0.65±0.040	000.00±0.00	F5 (4-41)	0.66±0.015	000.00±0.00
Aspergillus ustus	0.79±0.015	001.84±0.04	F6 (1-16)	0.66±0.023	000.00±0.00
Cunninghamella echinulata	0.90±0.042	062.92±1.95	F7 (1-33)	0.70±0.021	000.00±0.00
Emericella nidulans	2.40±0.026	495.73±3.85	F8 (3-35)	0.74±0.023	000.00±0.00

Emericella nidulans (34.8). Dominance of *Aspergillus* species in the rhizosphere of inulin rich plants were reported by Bonciu *et al*. (2010).

The results (Table 3) showed that, all fungi were able to grow on the selective medium with different levels. However, no significant correlation between the fungal biomass (data not recorded) and inulinase activities in such studied fungi. *Aspergillus foetidus* var. *pallidus* (564.71±1.22 Ugds^{-1}), *A. sclerotiorum* (534.78±1.37 Ugds^{-1}), *Emericella nidulans* (495.73±3.85 Ugds^{-1}), *A. aculeatus* (444.37±2.37 Ugds^{-1}), *Penicillium wortmannii* (304.00±1.22 Ugds^{-1}), *A. brevipes* (297.51±2.94 Ugds^{-1}), *A. pseudo-niger* (252.36±1.78 Ugds^{-1}) and *A. fumigatus* var. *ellipticus*

(220.19±3.86 $Ugds^{-1}$) were the most active fungal species able to produce a considerable amount of active inulinase. While, moderate or little inulinase activities were detected by some fungi (001.84±0.04-191.12±1.20 $Ugds^{-1}$). About 10 species were negative results for inulinase productivity under the studied fermentation conditions. These results are in agreement with that obtained by fungi isolated from Asteraceae rhizosphere with some different behavior (De Souza-Motta *et al*., 2003); this behavior may attribute to the genetical and physiological characteristics among strains of the same fungal species. AbdAl-Aziz *et al*. (2012) reported that *Aspergillus* and *Penicillium* species isolated from rotten Jerusalem artichoke tubers are the most potent inulinase producers.

Among 46 isolated species more than 78% (36 from 46 species) were inulinolytic fungi. The most common genera were *Aspergillus*, *Emericella* and *Penicillium*. In this connection Onodera and Shiomi (1992) and Kumar *et al*. (2005) reported *P. trzebinskii* and *A. niger* as inulinase producers. In the present study, *A. foetidus* var. *pallidus* and *A. sclerotiorum* are recorded as the highest inulinase producer as compared with the other species after 4.0 incubation days. Jing *et al*. (2003) showed that natural inulin from Jerusalem artichoke tuber powder is believed to be an inducer for inulinase production, it induces much more inulinase over that observed with fructose. Therefore, our results reported that inulinase is an inducible enzyme; it induced by its substrate as reported by Saber and El-Naggar (2009) and El-Hersh *et al*. (2011). The advantage of using natural inulin degrading fungi isolated from nature habitats over the genetically manipulated technique is the easier adaptation and succession, such isolate can be effectively used for biotransformation of natural inulin to mono-sugars. So, we used both *A. foetidus* var. *pallidus* and *A. sclerotiorum* as inulinase producers for further studies.

CONCLUSION

In this study, approximately 380 filamentous fungal isolates belonging to three sub-divisions, Zygomycotina, Ascomycotina and Deutromycotina were screened for their inulinolytic potentialities, the results revealed that *Aspergillus foetidus* var. *pallidus* (564.71±1.22 $Ugds^{-1}$), *A. sclerotiorum* (534.78±1.37 $Ugds^{-1}$), *Emericella nidulans* (495.73±3.85 $Ugds^{-1}$) and *A. aculeatus* (444.37±2.37 $Ugds^{-1}$) were the most active fungal species able to produce a considerable amount of enzyme activity.

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