Production of cell wall polysaccharide-degrading enzymes by fungi isolated from spoilage fruits using solid state fermentation

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Abstract: In this study, we isolated and identified some of spoilage fungi from local fruits specially date. Date (*Phoenix dactylifera* L.) is one of the most consumed fruit in Saudi Arabia. Nine spoilage fruit fungi ere isolated and identified as follows *Aspergillus niger* (Rabea-1 dates)**,** *spergillus parasiticus* (Rabea-2 dates), *Aspergillus awamori* (Mabrooma dates), *Aspergillus wentii* (Safawi dates), *spergillus japonicus* (Rutab dates), *Aspergillus niger* **(**Cantaloupe), *Aspergillus foetidus* (Quince), *Mucor racemosum* **(**Mango), *Pythium* sp. (Avocado). The results reported that Aspergillus spp. caused the spoilage for all dates tested. The isolated fungi were cultured on their peels of spoilage fruits Rabea-2 dates, Rabea-2 dates, Mabrooma dates, Safawi Dates, Rutab Dates, Cantaloupe, Quince, Mango, and Avocado using solid state fermentation. Cell wall polysaccharide-degrading enzymes: xylanase, polygalacturonase, cellulase and α-amylase were detected in the crude extract of all tested fungi. Xylanase and polygalacturonase had highest level of activities as compared to the cellulase and α-amylase. The results indicated that xylanase and polygalacturonase of fungi had important role for spoilage of fruits.
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1. Introduction

Worldwide, post harvest losses have been estimated at 50% and much of this is due to fungal and bacterial infections (Magro *et al.,* 2006). Moulds are ubiquitous biological agents that are able to colonize foods because of their potential to synthesize a wide diversity of hydrolytic enzymes. They cause pathologic disorders in plants bringing considerable economic losses for food producers. Fruits and vegetables are highly susceptible to fungal spoilage, both in the field and during postharvest storage. Significant genera include Pythium, Phytophthora, Fusarium, Penicillium, Alternaria, Botrytis, Geotrichum, Sclerotinia and Rhizoctonia spp. Fungal growth on fresh fruits and vegetables is responsible for food spoilage and numerous plant diseases, which lead to significant economic losses. Mould growth depends on abiotic factors such as pH, water activity (aw), solute concentration, temperature, atmosphere, time, etc. However conditions of temperature and aw are the main variables determining the development of fungi. Grain crops are also vulnerable to fungal contamination, with Aspergillus, Penicillium, Fusarium and Alternaria being the most frequent genera. In this matrix, moulds are responsible for off-flavor formation and contribute to heating and loss in dry matter in grains through the utilization of carbohydrates as an energy source, degradation of lipids and proteins, production of volatile metabolites and production of allergenic compounds. This causes a reduction in the quality of animal feed and seed (Magan and Aldred, 2007; Cabral *et al.,* 2013). These events can take place even before the fungal growth is evident (Lee *et al.,* 2007).

Fruit ripening is accompanied by an ethylene synthesis peak during the onset of the respiratory climacteric (Rhodes, 1980). Rapid ripening results in excessive softening, which affects the sensory quality, and lead to loss of pathogen resistance and reduction of shelf-life. Progressive loss of firmness or softening of fruit is a consequence of decomposition of cell wall components and structure. Pectin is an abundant component in the cell walls of plants, constituting approximately a third of the structure (Ridley *et al.,* 2001). It participates in cell-to-cell adhesion, which is accomplished largely by calcium cross-linkage between partially de-methyl esterified homogalacturonans in the middle lamella (Jarvis *et al.,* 2003; Vincken *et al.,* 2003). The pectin in immature fruit is water-insoluble protopectin which decomposes into water-soluble pectin during maturation (Inari *et al.,* 2002). Thus the softening of the fruit flesh is largely attributed to the solubilization of the protopectins (Prasanna *et al.,* 2007). Cellulose combined with pectin and hemicelluloses makes up the primary cell wall and keeps fruit firmness (Pirrello *et al.,* 2009).Cell wall decomposition involves a number of cell wall enzymes (Brummell and Harpster, 2001; Giovannoni, 2001). Polygalacturonase (PG; EC3.2.1.15), and pectin methylesterase (PME; EC 3.1.1.11) are considered as

the primary hydrolysis enzymes involved in the softening process (King and O'Donoghue, 1995). Barka *et al.* (2000) found that not only PME and PG, but also other enzymes were involved in cell wall degradation, such as cellulase (EC3.2.1.4), xylanase (EC3.2.1.8), β-d-galactosidase (EC3.2.1.23), and protease.

The aim of this study is to produce cell wall polysaccharide-degrading enzymes by fungi isolated from spoilage fruits using their fruit peels in solid state fermentation.

2. Materials and methods

Fruit materials

Eight types of various fruits, Rabea dates, Mabrooma dates, Safawi dates, Rotab dates, cantaloupe (Balady), quince (imported), mango (Balady) and Avocado (imported) were purchased from local markets in Jeddah Province in their individual packages weighing approximately 3 kilo's each.

Isolation of fruit spoilage fungi

Several methods were carried out individual for fungi isolation, by incubation of the whole fruits at 28°C, incubation of intact fruits after injuring to their surfaces at 28°C and washing off the surfaces of intact fruits. The washing off method give the maximum growth of fungi compared to the other methods. Therefore, we choose the wash off method for isolation of fungi. The fruits were washed with sterile water then sub-culturing the fungi washed off water. The sub-culturing was carried out by using a sterile fresh medium of potato dextrose agar (PDA) and incubated at 28°C until fungal proliferation on medium surface. The isolation of pure fungal colony in culture medium was performed by using slants of a sterile fresh medium of PDA and incubated at 28ºC for 5-7 days. The isolated fungi were maintained at 4°C.

Identification of the isolated fungi

The pure isolated fungi were identified according to the most documented keys in fungal identification (Domsch *et al.,* 1993; Klich, 2002; Samson and Varga, 2007). The fungal isolates were subjected to certain morphological studies by an Image Analysis System using Soft-Imaging GmbH software (analysis Pro ver.3.0) as well as using the newly introduced RCMB Database Management System for *Aspergilli* identification at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt. The gross morphology viz. the rate of growth, colony diameter, colony texture, colony color and reverse pigmentation as well as the measurements of the diagnostic structures that characterized the species were taken.

Fruit Peels

Fruit peels were dried in an oven at 60ºC for 48 h. The solid was then milled in a commercial mill and sieved. The mean diameter of the solid was 0.7 mm.

Production of cell wall degrading enzymes by solid state fermentation

Cell wall degrading enzymes as pectinases, xylanases, cellulases and amylases from the isolated fungi were produced using their spoilage fruit peels as culture media in solid state fermentation. Fungi were inoculated under aseptic conditions in 50-ml Erlenmeyer flasks contained sterilized fruit peels (1g/1 ml distilled water). The inoculated flasks were incubated at 28ºC for 5 days. Then add 5 ml distilled water to the flask, which subjected to rotary shaker at 180 rpm/min overnight. The suspension is then centrifuged at 7000 rpm for 10 min and the supernatant is designated as a crude extract. The crude extract was subjected to dialysis against 20 mM Tris-HCl buffer, pH 7.2 over night. The dialyzate was centrifuged at 10,000 rpm for 12 min and the supernatant was designed as crude extract.

Enzyme assays

Polygalacturonase (EC 3.2.1.15), cellulase (EC 3.2.1.21), xylanase (EC 3.2.1.8), and α-amylase (EC 3.2.1.1) activities were assayed by determining the liberated reducing end products using galacturonic acid, glucose, xylose and maltose as standards, respectively (Miller, 1959). The reaction mixture (0.5 ml) contained 1% substrate, 0.05 M sodium acetate buffer pH 5.5 and a suitable amount of crude extract. Assays were carried out at 37°C for 1 h. Then 0.5 ml dinitrosalicylic acid reagent was added to each tube. The tubes were heated in a boiling water bath for 10 min. After cooling to room temperature, the absorbance was measured at 560 nm. Substrates used were polygalacturonic acid, CM-cellulose, xylane and starch for polygalacturonase, cellulase, xylanase, and α-amylase, respectively. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of reducing sugar per h under standard assay conditions.

3. Results and Discussion

In this study, we isolated and identified some of fungi from spoilage local fruits specially date palm. Date (*Phoenix dactylifera* L.) is one of the most consumed fruit in Saudi Arabia, mainly because of its high content of carbohydrates (70–80%), dietary fibre (6.40–11.50%), minerals (0.10–916 mg/100 g dry weight), vitamins (C, B1, B2, B3 and A) and antioxidant compounds. During field production, handling, transportation and storage, dates are susceptible to damage and to colonization by spoilage fungi (Jowkar *et al.,* 2005), which may result in economic losses, especially for exporting

countries. However, it is estimated that more than 50% of the total production of dates is lost due to fungal spoilage (Atia, 2011). Aspergillus spp. had been reported to be the most common fungal species infecting dates (Ahmed *et al.,* 1997). In the present study, nine fruit spoilage fungi were isolated and identified as follows *Aspergillus niger* (Rabea-1 dates)**,** *spergillus parasiticus* (Rabea-2 dates), *Aspergillus awamori* (Mabrooma dates), *Aspergillus wentii* (Safawi Dates), *spergillus japonicus* (Rutab Dates), *Aspergillus niger* **(**Cantaloupe), *Aspergillus foetidus* (Quince), *Mucor racemosum* **(**Mango), *Pythium* sp. (Avocado) (Figs. 1, 2). The results proved also that Aspergillus spp. caused the spoilage for all dates tested. For mango, the fungi species isolated were *A. niger, Alternaria* sp. *Botryodiolodia theobromae* and *Colletotrichum gloeosporioides. Fusarium* sp, *A. flavus* and *Phoma* sp. were also isolated but could not prove pathogenicity when inoculated into healthy mango fruits. *A. niger* was responsible for brown round shaped spots showing a depression (Okereke *et al.,* 2010).

Solid-state fermentation (SSF), whereby an insoluble substrate is fermented with sufficient but no free moisture (Chahal, 1985), typically uses agricultural residues such as wheat bran, wheat straw, rice bran, etc. for production of larger amounts of microbial metabolites at a lower cost (Smits *et al.,* 1996; Lequart *et al.,* 1999; Jecu, 2000; Waites and Morgan, 2001), although, normally the production of industrial enzymes, like xylanase is performed by submerged culture (Adamsen, Lindhagen and Ahring 1995; Beg *et al.,* 2001). Optimal environmental condition is a prerequisite for promotion of maximum growth and production of enzymes where SSF is used (Deschamps and Hute 1985; Gessesse and Mamo 1999). Therefore, in this study, the isolated fungi were cultured on their peels of spoilage fruits Rabea-2 dates, Rabea-2 dates, Mabrooma dates, Safawi Dates, Rutab Dates, Cantaloupe, Quince, Mango, and Avocado using solid state fermentation. Xylanase, polygalacturonase, cellulase and α-amylase were detected in the crude extract of all tested fungi. Xylanase and polygalacturonase had highest level of activities as compared to the cellulase and α-amylase (Tables 1). The highest activities levels of polygalacturonase (250 units/g fruit peel), cellulase (111 units/g fruit peel) and α-amylase (74 units/g fruit peel) were detected in *Aspergillus awamori* isolated and grown on Mabrooma and dates. *Aspergillus wentii* isolated and grown on Safawi dates produced the highest activity of xylanase (198 units/g fruit peel). The lowest activities levels of xylanase (80 units/g fruit peel), polygalacturonase (68 units/g fruit peel), cellulase (6.4 units/g fruit peel) and α-amylase (3.5 units/g fruit peel) were detected in *Pythium sp.* isolated and grown on avocado. Using solid state fermentation, high activity of polygalacturonase and xylanase were produced from *Penicillium decumbens* (Yang *et al*., 2001), *A. niger (*Couri *et al*., 2000), *A. oryzae* (Yamane *et al*., 2002) and *A. awamori* (Botella *et al*., 2007).

Table 1. Cell wall degrading enzymes from spoilage fungi cultured on fruit peels using solid state fermentation.

Fruit peel	Fungi	Units/g fruit peel			
		Cellulase	Polygalacturon-ase	α-amylase	Xylanase
Rabea-1 dates	*Aspergillus niger*	35.64	161.04	31.46	117.7
Rabea-2 Dates	*Aspergillus parasiticus*	55.5	198	35.7	156.9
Mabrooma dates	*Aspergillus awamori*	111.32	244.86	74.14	167.2
Safawi Dates	*Aspergillus wentii*	92.16	247.68	63.68	198.72
Rutab Dates	*Aspergillus japonicus*	32.8	101.6	16.6	96
Cantaloupe	*Aspergillus niger*	39.6	159.06	25.96	121.22
Quince	*Aspergillus foetidus*	70.84	198.66	46.2	168.96
Mango	*Mucor racemosum*	71.4	170.94	41.58	156.66
Avocado	*Pythium* sp.	6.4	68.64	3.52	80.96

Fig. 1. Fungi isolated from different spoilage fruits.

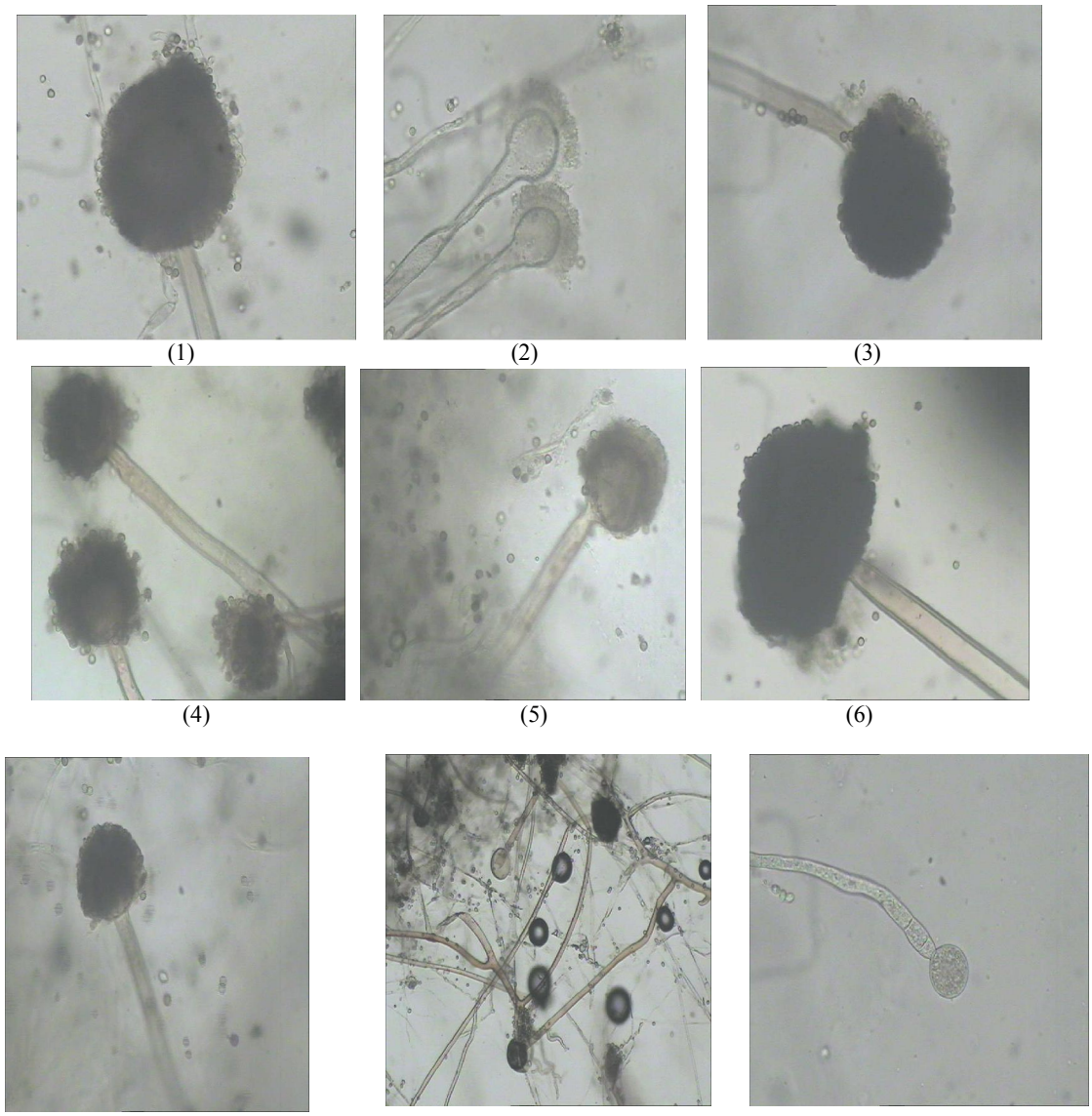

Fig. 2. Images of *Aspergillus niger* from Rabea-1 dates (1), *A. parasiticus* from Rabea-2 Dates (2), *A. awamori* from Mabrooma dates (3), *A. wentii* from Safawi Dates (4), *A. japonicus* from Rutab Dates (5), *A. niger* from Cantaloupe (6), *A. foetidus* from Quince (7), *Mucor racemosum* from Mango (8) and *Pythium* sp. from Avogado (9).

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