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Evaluate the Capability of Some Local Fungal Isolates to Produce Pectinase Enzyme using Some Fruit Peels

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ABSTRACT

Background: The process of producing enzymes from microorganisms is one of the most current approaches with a bright future. Pectinase is an acidic polysaccharide that is produced by microorganisms, accounts for a large proportion of commercially marketed enzymes and is extremely important because they are used in a variety of industrial, food and medical applications. This study aimed to use the peels of some fruits as a basic material in the production of pectinase enzyme by some fungi isolated from some fruits and vegetables via both solid-state and submerged fermentation methods.

Methods: The current study included the isolation and identification of some fungi from fruits and vegetables in the local markets of Al-Diwaniyah City/Iraq, the detection of their capability to produce pectinase enzyme using the standard medium and the possibility of using the peels of some fruits as basic materials.

Result: The results indicated the isolation and identification of 178 fungal isolates belonging to 11 genera at different frequency rates and it was found that the isolate *P. expansum* isolated from Apples was the most efficient isolate in producing pectinase enzyme and the use of Apple peels gave the highest production of the enzyme from the fungal isolate understudy compared to other fruit peels used.

Key words: Fruits peels, *P. expansum*, Pectinase enzyme, Solid-state fermentation, Submerged fermentation.

INTRODUCTION

In the field of biotechnology, the process of producing enzymes from microorganisms is one of the most current approaches with a bright future (Prasanthi *et al*., 2019; Sharma *et al*., 2021). Pectinase is an acidic polysaccharide with a basic structure that includes D-galacturonic acid molecules in the main chain connected by a (1-4) bond and Arabinose and Xylose molecules in the side chain (Mohnen, 2008). Pectinase enzymes such as Polygalacturonase, Pectin Lyase, Pectolase and Pectinesterase break the glycosidic bond of the long carbon chain and they are considered a class of enzymes that degrade pectin materials, these enzymes are produced by microorganisms, account for a large proportion of commercially marketed enzymes and are extremely important because they are used in a variety of industrial, food and medical applications (Amin *et al*., 2019; Haile and Ayele, 2022). They are used to enhance and accelerate the extraction and leaching processes and purify fruit juices, extract dyes from materials and textile industries and the paper industry, as well as for oil extraction and gum removal from plant fibers, make bio-detergents, waste treatment and animal feed (Sankalpa *et al*., 2017; Nadar *et al*., 2018; Patidar *et al*., 2018). For the ease of microorganism development and handling, there has been an increased interest in producing pectinase enzymes from microorganisms as an alternative to plant and animal enzymes (Raveendran *et al*., 2018). Some bacteria and fungi isolated from different materials are characterized by their capability to produce pectinase enzymes such as *Bacillus* spp., *Clostridium* spp., *Pseudomonas* spp., *Aspergillus* spp., *Alternaria alternata*, *Penicillium* spp. and *Fusarium* spp. (Ahmadi *et al*., 2020). Various industrial and agricultural wastes and by-products of some industries such as fruit and vegetable peels, sugar cane residues, wheat bran and other manufacturing processes residues are highly perishable and lead to major problems in manufacturing processes or as pollutants when thrown into the environment (Esparza *et al*., 2020). Therefore, the use of such wastes in the production of enzymes and other valuable materials such as biogas, ethanol, citric acid, flavoring compounds, fatty acids and biomass using fermentation is an important achievement from an economic point of view using the appropriate microorganism (Venkatesh *et al*., 2009; Arabhanvi *et al*., 2015). So, this study aimed to use the peels of some fruits (apples, bananas, oranges and tangerines) as a basic material in the production of pectinase enzyme by fungi isolated from fruits and vegetables *via* both solid-state and submerged fermentation methods.

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MATERIALS AND METHODS

Isolation of fungi

A group of fruits and vegetables (apples, oranges, strawberries, cucumbers, potatoes, tomatoes and Onions) were used to isolate the fungi in the local markets of Al-Diwaniyah City/Iraq, which showed symptoms of fungal infection in August 2022. The samples were brought to the laboratory, washed with distilled water and 1 cm^2 from the fungus-infected area superficially sterilized for three minutes with 1% Sodium hypochlorite solution, then washed with sterile distilled water and dried between two sterile filter papers, then cultured in Petri dishes containing sterile (PDA) medium with the antibiotic streptomycin at a concentration of 50 mg/liter of three pieces for each type of fruits and vegetables, the dishes were incubated in the incubator for 7 days at 28°C, with three replications for each treatment (Saleh and Al-Thani, 2019).

Identification of fungi

After the growth of fungi on the PDA medium, small parts were taken using a culture needle from the edge of each fungal colony and were planted individually on Petri dishes containing a sterile PDA medium to obtain pure cultures of the fungi and their identification and the dishes were incubated in the incubator for 3-4 days at 28°C and after the appearance of growth was diagnosed morphologically and microscopically based on the taxonomic keys of fungi (Robinson, 2011; Watanabe, 2018). Then the diagnosed and isolated fungi were stored purely in test tubes containing sterile tilted PDA medium in the fridge at 4°C until use, the percentage of fungi frequency was then calculated using the following equation:

$$\text{Frequency \%} = \frac{\text{The number of isolates belonging to the same genus or species}}{\text{The total number of isolates}} \times 100$$

Preparation of suspended spores

The spores of the isolated and most frequent fungi that were diagnosed in the previous step were prepared by preparing the sterile PDA culture medium in sterile Petri dishes and then inoculating by streak plate method from the pure isolates under study. After 3 days of incubation at 30°C, 5 mL of sterile distilled water containing 1% Tween 80 was added to the dishes under sterile conditions and the spores were washed and transferred to sterile tubes, which were stored in the fridge at 4°C until use.

Pectinase enzyme production test from isolated fungi using standard medium

To test the capability of the isolated and most frequent fungi to produce pectinase enzyme, a modified Czapek-Dox agar medium was used, replacing the carbon source with citrus pectin, after sterilizing the medium in an autoclave for 20 minutes at 121°C, it was poured into sterile Petri dishes and allowed to cool before drilling holes of equal diameters in each dish, three duplicates of each isolate were inoculated with 1 ml of suspended spores at a concentration of 10^6 spores/ml in each hole, then for 5 days, the dishes were incubated at 30°C and the clear zones formed were determined using a regular ruler (average of two perpendicular diameters) by adding Lugol's Iodine solution, as the appearance of a clear zones halo around the fungal colony indicates the production of the enzyme and the greater the diameter of the halo indicates an increase in the activity of the isolate in the production of pectinase enzyme (Aboaba, 2009). Then the isolate with the highest enzyme production was determined to carry out other experiments.

Pectinase production test using peels of some fruits

After determining the most productive isolate of the pectinase enzyme on the standard medium, which is *Penicillium expansum* (Apple), it is the capability to produce the enzyme was tested using the peels of some fruits. Rangarajan *et al*. (2010) methods were used to prepare the peels of fruits (Apples, Bananas, Oranges and Tangerines) a sufficient amount of peels were collected and cut into roughly equal sizes before drying in an oven at 55°C until the weight was stabilized, then the samples were ground with an electric mill and stored in glass containers away from moisture. The ground peels were added to the pectinase production medium, which is the modified Czapek-Dox agar medium by replacing the carbon source with Apple peels, the medium was then sterilized and poured into sterilized Petri dishes, which were then allowed to cool before the holes of equal diameters were drilled in each dish, each hole in the dishes was inoculated with 1 ml of suspended spores at a concentration of 10^6 spores/ml for the fungal isolate under study and with three replicates, then for 5 days the dishes were incubated at 30°C, the same experiment was repeated by replacing the Apple peels with peels (banana, orange and tangerine) separately. The clear zones formed were determined using Lugol's Iodine Solution, as the appearance of a clear zone halo around the fungal colony was evidence of the enzyme production, determining the peels of fruits that gave the highest production of the enzyme to carry out other experiments.

Assay for pectinase enzyme production using solid-state and submerged fermentation methods

Apple peels were used, as they produced the most enzymes in the previous experiment using the fungal isolate *P. expansum* (Apple). The method of Martin *et al*. (2004) was used to produce the enzyme by solid-state fermentation (SSF) method, with a weight of 10 g of pre-prepared Apple peels, placed in 250 ml glass flasks and sterilized with an autoclave for 40 minutes at 121°C, then, 10 ml of the mineral nutrient solution was added to the peel solution and in sterile conditions, containing (0.1% NH_4NO_3, 0.1% $NH_4H_2PO_4$ and 0.1% $MgSO_4.7H_2O$), the medium was sterilized in an autoclave for 15 minutes at 121°C before being inoculated with 10 ml of spore suspension at a concentration of

10^6 spores/ml in three replicates, then the flasks were incubated at 30°C in a shaker incubator at 200 rpm for 5 days. The enzyme was also produced by submerged fermentation (SmF) method by preparing Apple peel extract after weighing 100 gm of Apple peels in flasks and adding 1600 ml of distilled water, placing it on a hot plate at 100°C for 3 hours and keeping the extract in the fridge at 4°C until use, 100 ml of the extract was taken and 10 ml of the mineral nutrient solution mentioned above was added to it under sterile conditions, then the medium was inoculated with 10 ml of suspended spores at a concentration of 10^6 spores/ml and in three replications. The flasks were then shaken for 5 days at 30°C at 200 rpm in a shaker incubator.

Crude pectinase enzyme extraction

Crude pectinase enzyme was extracted from solid cultures by adding 100 ml of buffer solution sodium acetate with pH 5.5 and a concentration of 0.05 M to the components of the fermented flasks. The medium was mixed well, then a filtration process was carried out under a vacuum and the centrifugation process was then carried out at 10,000 rpm for 20 minutes, with the filtrate representing the crude enzyme being collected. In the submerged cultures, the centrifugation process was conducted directly to the liquid culture and the filtrate was collected and the enzymatic activity of all samples was estimated (Garg *et al*., 2016).

Estimation of pectinase activity

The effectiveness of pectinase was estimated using citrus pectin as a base material, as the mixture contained equal amounts of 1% pectin solution prepared in a buffer solution of sodium acetate with pH 5.5, the concentration of 0.05 M and a suitable dilution of the crude enzyme, after 30 minutes in a water bath at 50°C, 1 ml of 3,5-Dinitrosalicylic acid (DNS) was added and the mixture was heated for 10 minutes before being cooled, the optical absorption at a wavelength of 540 nm was determined using a Spectrophotometer, the efficacy was estimated based on the standard curve of pre-prepared galacturonic acid and was found the unit of activity, which is the amount of enzyme required in experimental conditions to release 1 micromole of galacturonic acid per minute (Sengupta *et al*., 2000).

Statistical analysis

Significant differences between the means were determined using Duncan's polynomial test at a probability level of 5% using an analysis of variance (ANOVA) (Armitage *et al*., 2008).

RESULTS AND DISCUSSION

Isolation and identification of fungi from some fruits and vegetables

The results in Table (1) indicated the isolation and identification of 178 fungal isolates from some different fruits and vegetables belonging to 11 fungal genera and these genera are: (*Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Rhizoctonia* sp., *Cladosporium* sp., *Botrytis* sp., *Stemphylium* sp., *Cephalosporium* sp., *Rhizopus* sp. and *Mucor* sp.) and the results indicated that the most frequent fungi, according to the isolated source, were *P. expansum* and *Al. alternata* isolated from apples with a frequency of 34.78% and 21.73%, respectively, *P. italicum* and *P. digitatum* isolated from oranges with 30.76% and 26.92%, respectively, *Al. alternata* and *Botrytis cinerea* isolated from Strawberries with 36.0% and 24.0%, respectively, *Al. alternata* and *F. solani* isolated from Cucumbers with 30.30% and 21.21%, respectively, *A. niger* and *F. oxysporum* isolated from potatoes with 30.0% and 25.0%, respectively, *Al. alternata* and *F. solani* isolated from tomatoes at 34.61% and 19.23%, respectively, then *A. niger* and *P. expansum* isolated from onions at 44.0% and 24.0%, respectively. These findings support prior studies that show it is possible to isolate various fungi from fruits and vegetables after harvesting, as *A. niger* and *F. oxysporum* were isolated from apples (Oelofse *et al*., 2006; Chatanta *et al*., 2008). As well as *A. niger* from tomatoes and oranges (Yıldız and Baysal, 2006; Reddy *et al*., 2008). *Fusarium* sp. is a moisture-loving fungus found on fruits high in moisture content such as Oranges (Tournas *et al*., 2005). As well as *Alternaria* sp., *Penicillium* sp. and *Rhizopus* sp (Bukar *et al*., 2009). *Alternaria* sp. and *Rhizopus* sp. from cucumber (Hoque and Shamsi, 2011). The high moisture content of fruits and vegetables and the different transportation and storage conditions that may increase the humidity and temperature of fruits and vegetables are among the most important factors that encourage the growth of fungi on them, as well as external environmental conditions such as insect intrusion and wounds that facilitate the entry and spread of fungi as well as the presence of foreign materials such as sand and dust and the remnants of other damaged fruits and vegetables among them (Rawat, 2015).

Pectinase enzyme production test from isolated fungi using standard medium

The results indicated that the fungal isolates of *Penicillium* spp were one of the most efficient isolates in producing pectinase enzyme using the standard medium, the isolate of *P. expansum* isolated from Apples was the most efficient isolate in producing the enzyme, reaching a clear zones diameter of 5.9 cm, followed by isolating *P. expansum* isolated from onions with 4.7 cm, then the two isolates *P. italicum* and *P. digitatum* with 4.2 cm and 4.0 cm, respectively, with significant differences compared to the other fungal isolates under study (Table 2). These findings are consistent with previous studies that found that the species of *Penicillium* spp. it can produce pectinase enzymes, including *P. italicum*, *P. viridicatum*, *P. roqueforti*, *P. expansum* and *P. griseoroseum* (Alaña *et al*., 1990; Silva *et al*., 2002; Perièin *et al*., 2007; Cardoso *et al*., 2007). Therefore, the fungal isolate *P. expansum* isolated from apples was chosen to carry out other experiments in this study.

Pectinase production test using peels of some fruits

The results in Fig (1) indicated that the use of Apple peels gave the highest production of pectinase enzyme, as it reached a clear zones diameter of 7.2 cm, followed by orange peels with 6.3 cm, tangerine peels with 5.8, then banana peels with 5.6. The majority of developed countries use agricultural and industrial waste to produce high-value raw materials, juice factories have used waste products such as citrus peels and Apple waste and these wastes contain percentages of pectic substances that reach 15-25% in citrus peels and 15-30% in Apple peels, which helps the growth of fungi that can produce pectinase enzyme (Ravindran and Jaiswal, 2016; Marić *et al*., 2018). So Apple peels were selected to carry out other experiments in this study.

Pectinase enzyme production assay using SSF method and SmF method

The results in Fig (2) indicated the superiority of the solid-state fermentation method over the submerged fermentation method in enzyme production, as the efficiency reached 760 and 425 units/mg, respectively, which could be due to a variety of factors, including lower catabolic repression in SSF compared to SmF and lower microorganism diffusion in SSF, as well as increased growth rates due to higher oxygen levels in the solid-air phase (Doriya *et al*., 2016).

Table 1: Fungi isolated from fruits and vegetables and their frequency.

Fruits and vegetables	Scientific name	Fungal isolates	Number of isolates	Frequency of fungi (%)
Apple	*Malus domestica*	*Alternaria alternata*	5	21.73
		Aspergillus niger	4	17.39
		Fusarium oxysporum	3	13.04
		Penicillium chrysogenum	3	13.04
		P. expansum	8	34.78
Orange	*Citrus sinensis*	*Al. alternata*	6	23.07
		Cladosporium sp.	2	7.69
		F. oxysporum	3	11.53
		P. italicum	8	30.76
		P. digitatum	7	26.92
Strawberry	*Fragaria chiloensis*	*Al. alternata*	9	36.0
		Botrytis cinerea	6	24.0
		Cladosporium sp.	3	12.0
		Rhizoctonia solani	4	16.0
		Stemphylium sp.	3	12.0
Cucumber	*Cucumis sativus*	*Al. alternata*	10	30.30
		Cephalosporium sp.	5	15.15
		Cladosporium sp.	3	9.09
		F. solani	7	21.21
		R. solani	5	15.15
		Rhizopus stolonifer	3	9.09
Potato	*Solanum tuberosum*	*A. niger*	6	30.0
		Al. alternata	3	15.0
		F. oxysporum	5	25.0
		F. solani	4	20.0
		Rh. stolonifer	2	10.0
Tomato	*Lycopersicon esculentum*	*A. niger*	2	7.69
		Al. alternata	9	34.61
		Cladosporium sp.	1	3.84
		F. oxysporum	2	7.69
		F. solani	5	19.23
		P. italicum	3	11.53
		R. solani	4	15.38
Onion	*Allium cepa*	*A. niger*	11	44.0
		F. oxysporum	2	8.0
		Mucor sp.	3	12.0
		P. expansum	6	24.0
		Rh. stolonifer	3	12.0

Table 2: Production of pectinase enzyme from the most frequent fungi using standard medium.

Most frequent fungal isolates	Clear zones diameter (cm)	Source of isolates
Penicillium expansum	5.9±0.11a	Apple
Alternaria alternata	3.5±0.12c	
P. italicum	4.2±0.15b	Orange
P. digitatum	4.0±0.21b	
Al. alternata	1.8±0.17f	Strawberry
Botrytis cinerea	2.2±0.09e	
Al. alternata	3.1±0.15d	Cucumber
F. solani	3.7±0.13c	
A. niger	2.9±0.24d	Potato
F. oxysporum	3.0±0.16d	
Al. alternata	2.3±0.09e	Tomato
F. solani	1.7±0.12f	
A. niger	2.8±0.21d	Onion
P. expansum	4.7±0.17c	

-The results are the average of three replicates with standard error.

-For vertical comparisons, averages with the same letters do not differ significantly according to Duncan's polynomial test at the 5% probability level.

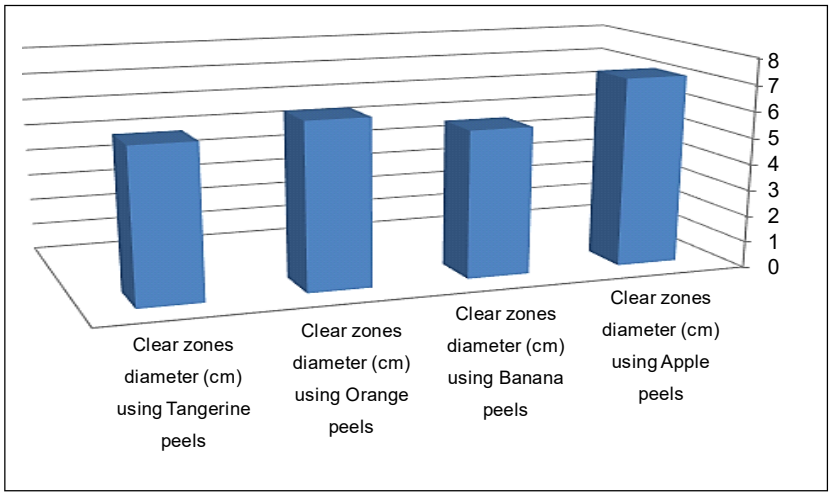

Fig 1: Production of pectinase enzyme using peels of some fruits.

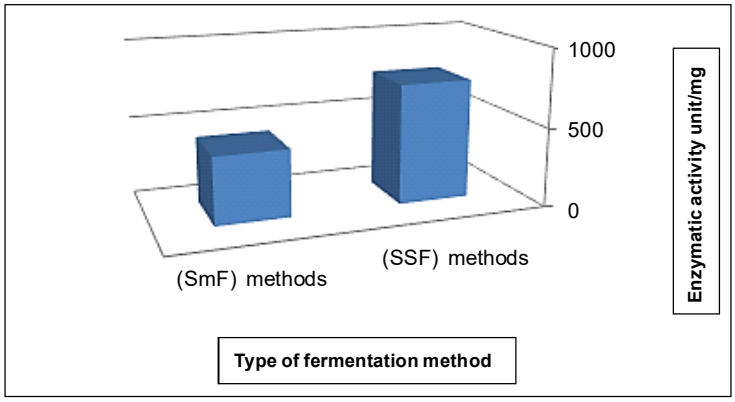

Fig 2: SSF and SmF methods to produce the pectinase enzyme.

CONCLUSION

The pectinase enzyme has been extracted from Apple fruit with great efficiency in solid-state fermentation using cheap Apple peels rather than being disposed of as polluting waste to the environment using *P. expansum*, a local fungal isolate and this enzyme can be used for a variety of industrial applications.

Conflict of interest: None.

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