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PHYSIOLOGY PRODUCTION OF POLYGALACTURONASE BY *Aspergillus niger* AND ITS APPLICATION IN ORANGE JUICE PRODUCTION

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ABSTARCT

Seven cultures of *Aspergillus niger* from local and international sources were screened for their abilities to produce extracellular polygalacturonase (PG) enzyme using two different assay methods, namely, quantitative viscosity reduction (Reyes units) and liberation of reducing moieties (as galacturonic acid) of standard apple pectin solutions. Appreciable levels of extracellular PG were formed by all tested cultures on wheat bran media as sole nutrients source between 1%-10% (w/v) in tap water with the highest enzyme levels obtained at 5%-10% (w/v) final concentrations. Based upon relative thermal stability and high productivity of PG enzyme the culture *Aspergillus niger* F-19 was selected for further studies. The selected culture *Aspergillus niger* F-19 could form PG enzyme on Dox medium supplemented with pectin, wheat bran, orange or lemon peels with the highest enzyme level formed on 5% wheat bran medium supplemented with 4% orange peels and 0.2% ammonium sulfate. The maximum specific enzyme activity and enzyme yield in the culture supernatant were attained after 4-6 days of incubation at 30°C with notable stability in the culture fluid at least up to nine days incubation period. Application studies were carried out on the possible use of the enzyme in orange juice production. Optimum enzyme concentration for juice production was 10.4 mg protein/150 gm of fruits using 30 minutes maceration reaction time at 50°C. Under optimized reaction conditions more than 25% increase in total juice yield could be obtained with excellent chemical, physical and organoleptic properties as compared to those of the control juice (without enzyme treatment). The obtained results were discussed in the light of possible application in orange juice industry in Egypt.

Keywords: Polygalacturonase – Extraction - Orange juice - *Apergillus niger* – Production.

INTRODUCTION

According to Bigelis (1993) pectic enzymes, collectively termed pectinases, are mixtures of enzymes that act on pectic substances that maintain the integrity of cell wall or middle lamella of the plant cells. These pectic substances are acidic heteropolysaccharides with molecular weights ranging between 30.000 to 300.000 and consist mainly of pectin, a polymer of D-galacturonic acid mostly linked in chain with α D (1-4) linkages. At least 75% of the monomers of D-galacturonic acid are esterified mainly with methanol (methoxylated) or with a variety of other polysaccharides. El-Gharably (2000) summarized the main economically important pectic group of enzymes to contain three subgroups polygalacturonases, pectinestrases and pectate lyases. The ploygalacturonases (PG) catalyze the hydrolytic cleavge of α- 1, 4 glycosidic bonds including the exo. PG (EC 3.2.1.67) that cleaves from the non-reducing end and the endo-PG (EC 3.2.1.15) that

attacks the pectic substrate randomly. The second group is pectinestrases (PE), (EC 3.1.1.11) which catalyze the hydrolysis of methyl ester groups results in the deestrification of pectin. The third subgroup is composed of pectate lyases (PEL) that catalyze the cleavage of non esterified polygalacturonate units via β-elimination mechanism. Here are also exo PEL (EC 4.2.2.9) and endo PEL (4.2.2.2) enzymes attacking the non-reducing end of the polygalacturonate chain and at random respectively have been detected.

The pectic enzymes are found in higher plants and also produced by a large number of fungi and bacteria. The plant enzymes are involved in the maturation processes of the fruits and vegetables. On the other hand, pectic enzymes from microbial origin have found wide applications in food processing and fruit juice extraction (Rombouts and Pilnik 1971; Pilnik *et al*. 1975; Cheetham, 1985; Askar *et al*., 1990; Sreenath *et al.*, 1987; 1995; Shata, 1999). According to Pilnik and Voragen (1993) and El-Gharably (2000) the pectic enzymes have been used in fruit juice processing for several purposes; juice extraction, increase juice yield and facilitate color extraction, juice clarification, liquifation and maceration, cloud stabilization in natural juices, and reduction of juice viscosity.

The present work has been devoted to the study of some factors affecting production of polygalacturonase using *Aspergillus niger* and its application in the juice extraction from orange. The results are discussed in the light of possible application feasibility in Egypt.

MATERIALS AND METHODS

Organism used and source of cultures:

Seven cultures of *Aspergillus niger* were used in the present work. Five of these cultures were local isolates originally obtained from local environments, that were identified at the Department of Pests and Plant Protection at the National Research Centre, Dokki, Cairo, Egypt. In addition to cultures namely *Aspergillus niger* NRRL3 and NRRL595 which were originally obtained from Northern Regional Research Laboratory, Peoria, Illinois, USA. The cultures were maintained on agar slants of malt extract-peptone medium and periodically transferred every six weeks on new slants.

Growth conditions and physiology of enzyme production:

i- Screening of cultures of *Aspergillus niger* for polygalacturonase formation:

Seven cultures of *Aspergillus niger* were screened for their ability to produce extracellular polygalacturonase in the growth media. Discs of seven-days old well sporulated cultures grown on malt agar plates were used to inoculate 250 ml conical flasks each containing 25 ml of wheat bran media in varying concentrations ranging between 1-10% w/v as final concentration. The inoculated flasks were then incubated on a rotary shaker for 4-5 days at 30°C. At the end of the incubation period the cultures were centrifugation at

3000 rpm for 10 minutes. The mycelial growth pellets were discarded and the supernatant was assayed for polygalacturonase activity.

b- Effect of type of media on enzyme production:

In this experiment eight different media were tested for their ability to support polygalacturonase enzyme biosynthesis among the high enzyme-producing cultures recorded in the previous experiment. Most of those tested media were based on Czapeck-Dox as a base medium with supplementation with some carbon and nitrogen sources as compared with 5% wheat bran medium.

iii- Thermal stability of enzyme in wheat bran medium:

This experiment was aimed at the selection of *Aspergillus niger* culture that produced more heat-stable enzyme among the high enzyme producing cultures for further studies. Thus aliquots of the cultures supernatants were heated at temperatures ranging between 40-90°C for 10 minutes and the remaining enzyme activities were assayed under standard reaction conditions.

iv- Effect of incubation period on enzyme production:

This experiment was carried out using the most promising culture namely *Aspergillus niger* F19. The enzyme activity were daily measured during nine days of aerobic incubation at 30°C.

v- Effect of aeration levels on enzyme production:

The selected culture namely *Aspergillus niger* F-19 was grown in 5% wheat bran medium under both static and shaking conditions for 5 days at 30°C. The aeration levels in both cases were varied by changing air : medium ratio in the culture experimental flasks. The enzyme activity were assayed at the end of the incubation period.

Assay of polygalacturonase activity:

The enzyme activity was assayed with two different methods:

i- Viscosity reduction method:

The viscosity reduction of 1.2% apple pectin solution dissolved in 0.1 M citrate phosphate buffer pH 4.5 was measured as described by Hancock *et al.* (1964) and Mehta and Mehta (1985). The activity was expressed in units as described by Reyes *et al.* (1984). One unit of polygalacturonase activity is defined as the amount of enzyme which catalyzes 1% reduction in viscosity of substrate under standard reaction conditions (Reyes *et al.*, 1984).

ii- Increase in reducing groups:

This method is based upon following the increase in reducing groups, expressed as galacturonic acid, as a result of the enzyme action on apple pectin substrate. One enzyme unit is defined as the amount of enzyme which releases 1.0 μ mole of galacturonic acid per 10 minutes under standard conditions (Martinez *et al.*, 1982).

Assay of pectin lyase (PL) activity:

The pectin lyase was assayed according to the method of Albersheim (1966), as follows: To 2.0 ml of substrate (0.5% apple pectin from Arabic Laboratory Equipment Co.) dissolved in 0.1 M citrate phosphate buffer, 0.1 ml enzyme source was added. The reaction mixture was incubated for 1, 2, 5 and 10 minutes. Absorbance was measured at 232 nm. One PL unit is defined as the amount of enzyme which librates 1 μ mole unsaturated products (measured as optical increament at 232 nm) under specific assay conditions.

Testing the fungal culture for the presence of mycotoxins:

The presence of mycotoxins were tested according to the method of Shih and Marth (1971). Twenty five ml of the fungal culture supernatant grown on wheat bran medium were transferred into 100 ml separating funnel. Then 25 ml of chloroform were added (3 times), shaked for 3 min., and the chloroform layers were collected and evaporated nearly to dryness over water-steam bath. The residue was transferred quantitatively using chloroform to a clean vial, and the solution was evaporated till nearly dryness. A defined volume, 20 μl of extract was spotted on precoated thin layers of silica gel G-60 plates 20 b 20 con. Merck. The extracts were screened for the presence of aflatoxins and ochratoxin by chromatography. Standards obtained in vials from Sigma Chemical Co., and 2 μg of each were spotted on the same TLC plates using toluene-ethyl acetate-formic acid (6:3:1 v/v/v) for development. The detection was carried out under ultraviolet (UV) light using long wave 366 and short wave 254 nm.

Enzyme production for application studies on juice extraction from orange fruits:

A laboratory 7.5 liters mechanically-driven fermentor (New Brunswick Scientific Co., Inc., Edison New Jersey, USA) was used for this purpose. Active slants five-days old of *Aspergillus niger* F19 were used to inoculate 250 ml Erlenmeyer flasks each containing 25 ml of 5% wheat bran medium and placed on rotary shaker for three days at 30°C. These fast growing cultures were used to inoculate the fermentor jar containing four liters of the same sterile medium at inoculum size rate 5% (w/w). The growth was allowed for four days at 30°C with agitation (200 rpm) and aeration rate 1 V/V/min. At the end of the incubation period the culture was filtered to remove the mycelial biomass. The clear supernatant was then passed through 0.45 μ Millipore microbiological filter to remove the remaining spores and the sterile culture supernatant was used as an enzyme source for application studies.

Maceration of orange fruits using filter-sterilized crude poly-galacturonase of *Aspergillus niger* F-19:

i- Washing and peeling of fruits:

The orange fruits were washed well with tap water and lightly peeled to remove the flavido layer and leaving the albido layer intact.

ii- Fruit cutting, crushing and extraction of juice:

All fruits were cut into four quarters, the seeds were removed then cut fruits were crushed with electric machine for 30 seconds. A series of experiments were conducted to evaluate the possible use of the laboratory prepared polygalacturonase enzyme of *Aspergillus niger* F-19 in the production of orange juice of a local variety (Balady) of orange fruits.

c- Effect of enzyme concentration on juice yield and chemical composition:

Various concentrations of filtered-sterilized polygalacturonase enzyme ranging between 2.6 and 20.8 mg protein/150 gm of crushed fruit were tested with appropriate control run simultaneously. The total volume of juice obtained was measured using 250 ml cylinder and chemical analysis was determined.

d- Effect of maceration reaction time with fungal enzyme on the resulting orange juice yield:

The enzymatic maceration reaction time was allowed to proceed for different periods of time ranging between 15 to 120 minutes. The volume of resulting juice produced after different intervals was measured and representative aliquots were subjected to chemical analysis.

e- Effect of maceration reaction temperature on juice production:

In this experiment the maceration reaction temperature was varied using water bath set at different temperatures ranging between 20°C to 60°C for 30 minutes reaction time, then the total yield and chemical analysis of juice obtained were determined.

f- Effect of initial pH value of maceration reaction on total yield and chemical composition of orange juice:

In this experiment citrate buffer 0.02 M as final concentration was used in the maceration reaction with different pH values ranging between 3.0 and 5.0 with appropriate controls run simultaneously. After 30 minutes of maceration reaction at 50°C the total yield and chemical composition of the juice obtained were determined.

Analytical Methods:

- Protein Determination:

Soluble protein was determined by the method described by Ohnistti and Barr (1978) using a solution of bovine serum albumin as a standard.

- Determination of total and reducing sugars:

Reducing sugars were determined according to Nelson (1944). Total sugars were determined according to Dubois *et al.* (1956).

- Total acidity:

Total acidity was determined according to the method of A.O.A.C. (1990), then calculated as citric acid in the analyzed orange juice.

- Total soluble solids (T.S.S.):

The total soluble solids content of juice samples were determined using Carl Zeiss Refractometer.

- pH value:

The pH value was determined directly using a Beckman glass electrode pH meter.

- Turbidity of orange juice:

Juice turbidity was measured according to the method of Krop and Pilnik (1974).

- Measurement of browning:

Browning was measured by the method reported by Mohamed *et al.* (1973).

- Organoleptic evaluation and statistical analysis:

All samples were evaluated by ten panelists. A scale from 1 to 10 was applied to evaluate color, flavor, taste, appearance and overall acceptability (Notter *et al.*, 1959). The obtained data were statistically analyzed according to Snedecor and Cochran (1967).

- Measurement of color:

Color was assessed using spectrocolorimeter with the CIE Lab Color Scale. This color assessment system is based on the Hunter L*, a* and b* coordinates. L* represents lightness and darkness, + a* redness, - a* greenness, + b* yellowness and – b* blueness (Hunter, Lab Scan XE, USA). The instrument was standardized against a white tile of Hunter Lab Color Standard (Lx No. 16379): X = 77.26, Y = 81.94 and Z = 88.14.

RESULTS AND DISCUSSION

Production of polygalacturonase by *Aspergillus niger* on wheat bran media of different concentrations:

Table (1 and 2) show the yields of polygalacturonase produced by the seven cultures of *Aspergillus niger* on wheat bran media assayed by viscosity reduction (Reyes units/ml) and reducing groups concentrations (determined as galacturonic acid moieties), respectively. Both assay methods for polygalacturonase activity gave a good agreement in their results indicating high degree of reliability and accuracy. The highest yield of enzyme as determined by viscosity reduction method was obtained in media with wheat bran concentration between 3% and 10% (w/v) for most fungal cultures tested. However upon using the assay of reducing groups liberated the highest enzyme activity levels were more pin-pointed to be within using wheat bran concentrations 5% and 10% in the growth medium for all tested cultures. However, the enzyme assay by measuring the release of reducing groups is presumably more accurate due to the enzyme specificity in breaking the α D

(1-4) linkage in the pectin molecules quantitatively. In all cases four cultures of *Aspergillus niger* namely F-16, F-19, F-21 and F-25 produced marked higher levels of the enzymes understudy. Wheat bran was used by many workers for the production of this enzyme from different fungal cultures. Arima *et al.* (1964) produced polygalacturonase enzyme using *Penicillium chrysogenum* mutants and *Penicillium indicum*; Fu-Main *et al.* (1988) used *Aspergillus niger* CP-831 for large scale production of pectinase, and Shata, 1999 studied the physiology of polygalacturonase formation by *Aspergillus oryzae* under solid state fermentation conditions.

Detection of Pectin Lyase Activity:

The crude enzyme filtrates of *Aspergillus niger* were assayed for pectin lyase activity (PL). Low PL activity was detected that would not account significantly in viscosity reduction.

Our results agree in general with those reported by Friedrich *et al.* (1994) who indicated the presence of low activity of PL as compared with the major activity due to polygalacturonase enzyme.

Effect of type of medium on enzyme production:

The results obtained are shown in Table (3). The four tested cultures of *Aspergillus niger* failed to produce any detectable polygalacturonase activity on Czapek's Dox medium as such, thus indicating the inducible nature of this enzyme under the specified conditions. The same medium with 2% apple pectin could induce low enzyme activity in all tested cultures. However, supplementation of the same medium with wheat bran or orange peel yielded high enzyme activity whereas lemon peel addition instead of pectin was not highly promising with respect to enzyme production. On the other hand, very high yield of polygalacturonase were obtained in all cultures upon the use of 5% wheat bran medium. Such high levels of the enzyme produced were further improved as recommended by Fu-Main *et al.* (1988) by the incorporation of orange peel and ammonium sulphate in wheat bran medium at 4% and 0.2% as final concentrations, respectively.

Foda *et al.* (1984) investigated the inducible nature of polygalacturonase produced by *Aspergillus aculeatus* DSM 63261 and *Mucor pusillus* QM 346.

The inducible nature of pectinases was emphasized by Jain *et al.* (1990), and Said *et al.* (1991) for *Penicillium accitanis*, *Penicillium frequentans*, respectively. Working on *Aspergillus niger* No. 36, Mansour (1996) reported that the presence of lemon peel powder plus carrot pomace 1:1 (w/w) as inducer instead of 2% pure pectin in the basal medium gave highest production of polygalacturonase enzyme.

Comparative thermal stability of polygalacturonase enzyme of *Aspergillus niger* group in wheat bran medium:

Thermal stability of polygalacturonase produced by the four tested remaining cultures are shown in Table (4). All enzyme aliquots from different cultures were stable after heating at least up to 50°C. however, upon heating at 60°C marked reduction in enzyme activities could be noticed. The most

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heat stable polygalacturonase activity was that of *Aspergillus niger* culture F-19 whereas the remaining activity amounted 98.6% and 82.5% of the original non heated level upon heating at 60°C and 65°C, respectively. Therefore, *Aspergillus niger* F-19 culture was selected for further studies.

Nevertheless, Murad (1989), working on *Myrothecium verrucaria* reported that polygalacturonase enzyme exhibited thermal stability up to 60°C. While, Mansour (1996) found that the enzyme produced by *Aspergillus niger* No 36 was stable at 40°C for 60 min.

Effect of incubation period on production of polygalacturonase enzyme by *Aspergillus niger* F-19:

From the results in Table (5), it could be concluded that the polygalacturonase levels of the culture increased progressively up to 5-6 days of culture age where it attained maximum activity. Therefore, no further increase could be noticed upon extended incubation period and the attained enzyme level was nearly stable at least up to nine days of incubation.

Table (5): Effect of incubation period on the production of extracellular polygalacturonase by *Aspergillus niger* F19.

Incubation period (days)	Enzyme activity (μmole/ml)	Soluble protein conc. in supernatant (mg)	Specific activity unit/mg protein
2	91.3	4.8	19.0
3	141.6	3.9	36.3
4	181.1	4.3	42.1
5	183.0	4.3	42.5
6	186.5	4.4	42.4
7	185.2	4.3	43.0
9	181.1	4.8	37.7

Different incubation periods were reported for the production of pectolytic enzymes. Marcus *et al.* (1986) incubated culture of *Rhizoctonia solani* on rotary shaker for 12 days, while Blieva and Rodionova (1988); Fu-Mian *et al.* (1988); and Hours *et al.* (1988) produced pectolytic enzymes after incubation period 4, 3 and 2 days respectively. Mikhailova *et al.* (1992) reported optimal incubation period 48-60 hours for pectinases production in *Aspergillus alliceus*. Yao *et al.* (1995) produced PG ase in liquid culture using *Botrytis cinerea* with continuous shaking for 10 days.

Effect of aeration levels on enzyme production by *Aspergillus niger* F-19:

The effect of aeration levels under both shake and static cultures of *Aspergillus niger* F-19 was through varying the ratios of air : medium in the experimental cultures. The results obtained are shown in Table (6). The organism could form high levels of enzyme at high air : medium ratios in the experimental culture under both shaked and/or static conditions. The enzyme yields were reduced progressively by requirement for aeration for enzyme

production. The reduction in enzyme levels was accompanied by notable decrease in the pH value of culture particularly under static incubation conditions. These results may be explained by possible accumulation of organic acids under low aeration levels.

Hours *et al.* (1988) investigated some factors affecting pectinase production by *Aspergillus foetidus* from apple pomace in solid state culture. Murad (1989) stated that higher polygalacturonase levels were produced under static growth conditions on Czapeck-Dox media containing 2% beet pulp. Boccas *et al.* (1994) used solid state fermentation for pectinase production from coffee pulp by a wild strain of *Aspergillus niger*, while Mansour (1996) found that maximum PG production was under shaking conditions after 3 days incubation period. Mikhailova *et al.* (1992) grew *Aspergillus alliceus* using 4:1 air:medium ratio on rotary shaker (180-200 rpm).

Table (6): Effect of aeration level on productivity of extracellular polygalacturonase of *Aspergillus niger* F19.

Aeration Level		Static Culture				Shaked Culture			
Volume of Medium (ml)	Air: Medium ratio	pH at Harvest time	Activity (mole ml)	Protein (mg)	Speciefic activity	pH at Harvest time	Activity (mole ml)	Protein (mg)	Specific activity
12.5	19:1	5.8	388	9.4	41.3	4.7	477.8	10.0	47.7
25	9:1	3.9	321.7	5.6	57.4	4.1	320	4.2	76.2
50	4:1	2.8	217.4	5.6	38.8	3.8	219.2	4.3	50.9
100	1.5:1	2.4	170.2	5.4	31.5	3.9	197.5	6.4	31.0

Examination of crude enzyme of *Aspergillus niger* F-19 for ochratoxins and aflatoxins:

Sample of crude polygalacturonase of *Aspergillus niger* F-19 were examined for the presence of aflatoxins and ochratoxins using standard methods. These results have shown that the crude enzyme preparations were free from any traces of aflatoxins and ochratoxins.

Enzymatic maceration of orange fruits and juice production using polygalacturonase of *Aspergillus niger* F-19:

a- Effect of enzyme concentration on juice yield and chemical composition:

The total juice yield and chemical composition of the obtained juice are shown in Table (7). Increasing the enzyme concentration added led to a progressive increase in juice yield up to 10.4 mg/150 gm of fruits. At higher enzyme concentration no further increase in juice produced could be observed. No notable changes in chemical composition of the juice were evident.

b- Effect of maceration reaction time with fungal enzyme on the resulting orange juice yield:

Results obtained are summarized in Table (8). The results show that the volume of orange juice obtained increased progressively with increasing maceration time up to 30 minutes reaction time. Further increase in the

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maceration period decreased juice yield. Similar results were reported by El-Gharably (2000) working on enzymatic juice production from peaches and tomatoes with commercial enzyme preparation. This observation may be explained by the fact that excessive breakdown of pectic substances as a consequence of extended maceration time would result in the formation of relatively short chain of pectic substance. These short-chained pectic substances are highly hydrophilic in nature thus holding considerable amounts of the juice, which can not be liberated by centrifugation.

c- Effect of maceration reaction temperature on juice production:

Table (9) shows the volume of the obtained juices as well as some of their chemical composition properties. The juice yield increased progressively with increasing incubation temperature of the maceration mixtures up to 50°C. No notable change in the resulting juice yield could be observed upon raising incubation temperature to 60°C.

d- Effect of initial pH value of maceration reaction on total yield and chemical composition of orange juice:

The obtained results have shown that only slight increases could be detected in the yield of the juice determined from maceration reaction mixtures when initial pH values were between 4.0 and 5.0 as compared to maceration reactions carried out with initial pH values less than 4.0.

Comparative yields and chemical analysis of control orange juice obtained with maceration:

With polygalacturonase using *Aspergillus niger* F-19 large volumes of orange juice were obtained by enzymatic maceration of the fruits using the optimized conditions used in the previous experiments. Those included primarily the enzyme concentration, maceration time and maceration temperature. The obtained yield per unit weight of fruits was compared to that obtained without treatment with the fungal enzyme (control). Furthermore, chemical and physical analyses of the resulting juice were carried out.

Table (10) shows the comparative yields as well as chemical composition and physical properties of the obtained juices with and without enzymatic treatments. An average of more than 26% increase in total juice yield was obtained as a result of prior maceration of orange fruits with polygalacturonase enzyme of *Aspergillus niger* F-19. On the other hand, great similarities in both chemical composition and physical properties of both types of juice could be noted with special reference to total solids, pH values, acidity, total sugars, browning and color specifications.

Macerating enzymes had found application in extraction of juice from citrus fruits. Citrus pulp constitutes about 25% of the total fruit weight. Thus enzymatic degradation of the pulp releases entrapped juice during pressing resulting in increased juice yields (Mullins and Knauf, 1988).

From Table (10), the results indicate that the L* values were close in the control and treated orange juice but it was darker in the juice obtained by enzymatic treatment than the control. The a* values were close in the control and the experimental orange juice whereas almost no browning was detected

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in both types of orange juice. The b* values (degree of yellowness) of control and treated orange juice were close to each other although the experimental juice exhibited slightly less value of yellow color as compared to the control. On the other hand, the R-values reached 2.51 and 3.28 in both experimental and control juice respectively.

Organoleptic evaluation of juice obtained with fungal enzyme treatment:

The results shown in Table (11) indicate insignificant differences between experimental and control samples in flavor, appearance, color, taste and overall score. These results along with the remarkable net increase in juice yield to over 25% make the application of the fungal enzyme of *Aspergillus niger* F-19 highly promising and economically feasible.

Table (11): Sensory evaluation of orange juice obtained with fruit maceration with fungal enzyme in comparison with that obtained with ordinary method (Control).

Type of orange juice	Color	Flavor	Taste	Appearance	Overall score
Control	9.2 [a]	8.3 [a]	8.05 [a]	8.6 [a]	8.5 [a]
Treatment with fungal enzyme	9.0 [a]	8.8 [a]	8.00 [a]	8.9 [a]	8.7 [a]
$LSD_{0.05}$	0.857	1.031	1.028	0.807	0.56

The mean scores in column with the same letter are not significantly different at 5%.

In the present work a successful study could be carried out for the production of fungal pectic enzymes from local sources and could be applied for orange juice extraction yielding promising results. However, further studies will be required to reach final conclusions before application on industrial scale.

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فسيولوجيا إنتاج إنزيم البولى جالاكتويورينيز بواسطة الفطر *أسبرجيلس نيجر* وتطبيقه فى إنتاج عصير البرتقال

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أجريت دراسة حصرية لسبعة مزارع محلية وعالمية لفطر *أسبرجلس نيجر* من حيث قدرتها على إنتاج إنزيم البولى جالاكتويورينيز خارج الخلايا باستخدام طريقتين مختلفتين للتحليل هما الإنخفاض الكمى للزوجة باستخدام وحدات رايز والتقدير الكمى للزيادة فى تركيز الوحدات المختزلة فى صورة حمض جالاكتويورونيك المنطلق من مادة التفاعل (محلول بكتين التفاح القياسى) نتيجة للتفاعل الإنزيمى.

وقد تم الحصول على مستويات عالية من إنزيم البولى جالاكتويورينيز خارج الخلايا على كل بيئات الردة المستخدمة كمصدر وحيد للغذاء بتركيزات تراوحت من ١% إلى ١٠% (وزن/حجم) فى ماء الصنبور وكان أعلاها عند ٥% و ١٠% (وزن/حجم) تركيز نهائى للردة.

وبناء على الثبات الحرارى والإنتاجية الأعلى للإنزيم تم إختيار الفطر *أسبرجيلس نيجر* ف ١٩ لمزيد من الدراسة حيث وجد أنه ينتج تركيزات عالية من إنزيم البولى جالاكتويورينيز على البيئات المستخدمة حيث أنتجت أعلى مستويات للإنزيم على بيئة الردة (٥ %) مضاف إليها ٤ % قشر برتقال مطحون و ٠,٢ % سلفات الأمونيوم. بلغ أعلى نشاط نوعى وأعلى تركيز للإنزيم بعد ٤ – ٦ أيام من التحضين عند ٣٠°م مع ثبات ملحوظ للإنزيم فى المزرعة على الأقل لمدة ٩ أيام من التحضين.

وقد تم إجراء دراسات تطبيقية على إمكانية استخدام الإنزيم فى زيادة إنتاجية عصير البرتقال حيث وجد أن تركيز الإنزيم الأمثل لإنتاج العصير ١٠,٤ ملليجرام بروتين/١٥٠ جرام برتقال خلال ٣٠ دقيقة زمن تفاعل عند ٥٥°م. وتحت هذه الظروف المثلى للتفاعل تم الحصول على زيادة تقدر بأكثر من ٢٥ % فى الناتج الكلى للعصير وبخواص كيميائية وطبيعية مناسبة مقارنة بالعصير المنتج بالطرق العادية بدون استخدام الإنزيم الفطرى هذا وقد تمت مناقشة النتائج فى ضوء *إمكانية* تطبيقها فى صناعة إنتاج عصير البرتقال فى مصر.

Table (1): Screening of seven strains of *Aspergillus niger* grown aerobically on different concentrations of wheat bran medium for the production of extracellular polygalacturonase activity. The enzyme activity was assayed by viscosity reduction of apple pectin solution in term of Reyes units.

Strain no.	Wheat bran concentration															
	10%				5%				3%				1%			
	pH at harvest time	Activity Reyes Units/ml	Protein mg/ml	Activity units/mg protein	pH at harvest time	Activity Reyes Units/ml	Protein mg/ml	Activity units/mg protein	pH at harvest time	Activity Reyes Units/ml	Protein mg/ml	Activity units/mg protein	pH at harvest time	Activity Reyes Units/ml	Protein mg/ml	Activity units/mg protein
F16	2.7	933	5.1	183.0	3.9	900	5.1	176	6.5	795	1.7	467.6	7.2	810	0.5	1620
F19	2.5	890	5.3	168.0	5.2	926	5.0	185	5.6	858	1.9	451.6	2.0	800	0.5	1600
F21	2.4	906	5.8	156.2	2.4	915	5.7	160	2.0	906	1.7	533	2.7	810	0.44	1840
F25	2.5	933	6.1	153.0	3.2	936	6.1	153	5.5	837	1.4	598	7.4	850	0.5	1700
K10	1.9	825	4.5	183.3	2.1	874	5.6	156	1.7	785	2.4	327	1.6	780	0.56	1392
NRRL3	3.9	869	5.8	150.0	7.0	880	5.8	152	2.4	900	1.4	643	3.6	846	0.54	1566
NRRL595	1.7	785	4.5	174.0	2.1	800	4.5	178	1.6	800	2.1	381	1.6	652	0.43	1516

Table (2): Screening of seven strains of *Aspergillus niger* grown aerobically on different concentrations of wheat bran medium for the production of extracellular polygalacturonase activity. The enzyme activity was assayed by measuring the liberated reducing moieties from apple pectin substrate and calculated as galacturonic acid under standard reaction conditions.

Strain No.	Wheat Bran Concentration							
	10%		5%		3%		1%	
	Reducing groups M	Specific activity unit/mg protein	Reducing groups M	Specific activity unit/mg protein	Reducing groups M	Specific activity unit/mg protein	Reducing groups ىM	Specific activity unit/mg protein
F16	250	49	235	46	175	106.7	110	239
F19	265	50	235	47	180	97.3	125	250
F21	200	34.5	225	39.5	105	60.3	115	261
F25	250	41	245	40.1	185	137	120	250
K10	175	38.9	175	31	125	51.3	110	197
NRRL3	215	37	200	34.5	150	106.4	115	213
NRRL595	175	39	170	37.7	145	66.8	95	221

Table (3): Production of extracellular polygalacturonase activity by four selected strains of *Aspergillus niger* grown aerobically on various media*.

Type of medium	Strain No.											
	F16			F19			F21			F25		
	pH at harvest	Activity ى mole	Specific activity	pH at harvest	Activity ى mole	Specific activity	pH at harvest	Activity ى mole	Specific activity	pH at harvest	Activity ى mole	Specific activity
Czapeck's-DOX as such	1.8	0.0	0.0	1.5	14.6	4.8	2.1	0.0	0.0	1.6	0.0	0.0
CZ-DOX +2%pectin	2.4	60.4	16.8	2.5	77.7	23.4	2.8	81.7	22.7	2.6	53.2	14.8
CZ-DOX + 2% wheat bran	3.7	150	32.6	3.1	146.4	31.8	3.2	91.3	21.2	3.4	60.0	15.2
CZ-DOX +2%Orange peal	3.8	132.1	12.1	3.25	55.9	8.2	3.3	31.4	5.7	3.5	28.7	5.51
CZ-DOX + 2% lemon peal	6.7	22.7	9.5	5.9	80.4	10.7	4.6	61.3	10.4	6.25	36.8	6.1
Wheat bran only 5%	2.7	227.4	36.1	2.8	225	37.5	2.6	144.3	24.1	3.3	205.6	34.9
Fu-Main *et al.* (1988)	3.2	265.5	19.2	2.6	264.1	15.3	2.6	162.1	10.3	2.9	208.3	13.8
Orange peal 4% + amm. Sulfate 0.2%	2.3	114.5	10.8	2.2	117.1	10.2	2.3	113.0	9.0	2.4	96.7	7.74

*** The cultures incubated on rotary shaker for four days at 30 ˚C. The enzyme activity was assayed by measuring reducing medium liberated from apple pectin substrate and calculated as galacturonic acid under shaking reaction conditions.**

Table (4): Thermal stability of extracellular polygalacturonase produced by four selected strains of *Aspergillus niger* grown on 5% wheat bran*.

Temperature (˚C)	Strain No.							
	F16		F19		F21		F25	
	Activity (Reyes units)		Activity (Reyes units)		Activity (Reyes units)		Activity (Reyes units)	
	unit/ml	per mg protein	unit/ml	per mg protein	unit/ml	per mg protein	unit/ml	per mg protein
30	932	182.7	945	189.0	906	159.0	927	152.0
40	932	182.7	945	189.0	906	159.0	927	152.0
50	932	182.7	945	189.0	906	159.0	927	152.0
60	906	177.6	932	186.4	753	132.1	897	147.0
65	736	144.3	780	156.0	575	101.0	768	126.0
70	355	69.6	438	87.6	362	63.5	319	52.3
80	170	33.3	255	51.0	234	41.0	230	37.7
90	155	30.4	170	34.0	127	22.3	155	25.4

*** The enzyme activity was assayed by viscosity reduction of apple pectin substrate in term of Reyes units.**

Table (7): Effect of polygalacturonase concentration of *Aspergillus niger* F19 on total yield and chemical composition of orange juice obtained by fruit maceration for 30 minutes with the fungal enzyme.

Enzyme Conc. mg protein/150 gm fruit	Juice yield (ml)		Total Soluble Solids (%)	Final pH value	Total acidity per 100ml		Total Sugars gm/100 ml
	Mean of 3 replicates	% increase			(gm)	(%)	
0.0	63.3	-	12.03	3.50	0.08	0.80	6.74
2.6	78.7	24.3	12.30	3.48	0.065	0.65	6.06
5.2	79.0	24.8	11.27	3.42	0.075	0.75	5.36
10.4	83.3	31.6	12.30	3.46	0.082	0.82	5.28
15.6	79.7	25.9	12.20	3.51	0.067	0.67	6.85
20.8	80.3	26.9	11.20	3.51	0.059	0.59	6.34

Table (8): Effect of maceration period using polygalacturonase of *Aspergillus niger* F19 on total yield and chemical composition of orange juice obtained by fruit maceration with the fungal enzyme*.

Maceration Period (min.)	Juice yield (ml)		Total Soluble Solids (%)	Final pH value	Total acidity per 100ml		Total Sugars gm/100 m
	Mean of 3 replicates	% increase			gm	%	
0.0	59	-	12.0	3.80	0.073	0.73	7.23
15	64.7	9.7	12.6	3.60	0.066	0.66	8.06
30	73.0	23.7	12.9	3.68	0.069	0.69	8.04
45	67.0	13.6	12.9	3.60	0.067	0.67	8.74
60	64.0	8.5	13.0	3.60	0.067	0.67	8.53
90	52.3	11.3	12.9	3.63	0.067	0.67	8.07
120	53.7	9.0	12.9	3.85	0.070	0.70	8.55

*** Enzyme concentration: 10.4 mg protein/150 gm fruits.**

Table (9): Effect of maceration temperature using polygalacturonase of *Aspergillus niger* F19 on total yield and chemical composition of orange juice obtained by treatment with the fungal enzyme*.

Temperature C	Juice yield (ml)		Total Soluble Solids (%)	Final pH value	Total acidity per 100 ml		Total Sugars gm/100 ml
	Mean of 3 replicates	% increase			gm	%	
20	48.3	-	12.1	3.64	0.064	0.64	7.60
30	60.7	25.6	12.6	3.62	0.057	0.57	7.28
40	62.0	28.4	12.7	3.62	0.065	0.65	8.80
50	67.7	40.1	12.9	3.88	0.059	0.59	8.84
60	67.7	40.1	13.0	3.83	0.061	0.61	8.88

*** Time of maceration: 30 minutes, Enzyme concentration: 10.4 mg protein/150 gm fruits.**

Table (10): Yield and chemical composition of orange juice obtained by maceration of orange fruit with polygalacturonase enzyme of *Asp. niger* F19 as compared to that of juice obtained by the ordinary method (Control).

Orange Juice	Yield		Total SolubleSolids (%)	pH value	acidity		Total Sugars (%)	Turbidity	Browning**	Hunter Color			
	Mean ml/150g fruit	%. Inc.			(gm)	(%)				L*	a*	b*	R400
Control	54	-	12	3.62	0.077	0.77	5.86	1.703	0.062	55.15	-1.59	43.21	3.28
Maceration with fungal enzyme	68.5	26.85	12.1	3.54	0.078	0.78	6.75	1.389	0.067	51.91	-2.61	39.8	2.51

*** L = degree of lightness, a = degree of redness, b = degree of yellowness. ** Expressed as O.D. at 420 nm in serum.**