

Bioconversion of Oranges Wastes for Pectinase Production Using *Aspergillus niger* under Solid State Fermentation

N. Hachemi, A. Nouani, A. Benchabane

Abstract—The influence of cultivation factors such as content of ammonium sulfate, glucose and water in the culture medium and particle size of dry orange waste, on their bioconversion for pectinase production was studied using complete factorial design. A polygalacturonase (PG) was isolated using ion exchange chromatography under gradient elution 0-0,5 m/l NaCl (column equilibrate with acetate buffer pH 4,5), subsequently by sephadex G75 column chromatography was applied and the molecular weight was obtained about 51,28 KDa. Purified PG enzyme exhibits a pH and temperature optima of activity at 5 and 35°C respectively. Treatment of apple juice by purified enzyme extract yielded a clear juice, which was competitive with juice yielded by pure Sigma Aldrich *Aspergillus niger* enzyme.

Keywords—Bioconversion, orange wastes, optimization, pectinase.

I. INTRODUCTION

UTILITIES of enzyme preparation include processing of virtually all biological raw materials formation of product and performance of product quality control [1].

Requirements to be met by enzymes used in the food industry include high catalytic activity (within broad ranges of temperatures, pH, agitation speed, and other variables) and lack of toxicity [1]. The production of enzyme preparation occupies a significant place in modern technology. The value of production in this branch of industry is constantly increasing [1]. Pectinase production occupies about 10% of overall manufacturing of enzymes preparation. These enzymes have an acidic pH optimum and widely used in production of juices, fruit drinks and wines. Most commercially available pectinase preparations are derived from *Aspergillus niger* [1]. Pectinase production is inducible and pectin as carbon source induces this enzymes production. Agriculture waste have large amount of cellulose, hemicelluloses and pectin, which can be used as inducer of pectinase production [2]. Citrus fruit constitute an important group of fruit crops produced all over the world; the family of citrus fruit constitutes of oranges, kinnow, khatta, lime, lemon, grappe fruit, malta, sweet orange etc. [3]. World citrus production has increased significantly

since 1980s, for example in 2010 the orange productions estimated to reach 66,4 million MT, which represents a 14% increase compared to that 1997-1999 about 30,1 million MT of the orange production will be processed to yield juices, essential oils, and other byproducts [4]. Orange peel is the principal by product of the orange processing industry and constitutes about 50% of the fresh fruit weight [5]. The orange processing residues are attractive and potential feedstock for their biological conversion to value added products, as they are rich in both soluble and insoluble's carbohydrates [5]. In our study the production of notable and highly effective pectinase by local fungal strain *Aspergillus niger* utilizing house waste orange peel and pulp scraps, excluded in the orange juice was achieved in 5 days under solid state fermentation culture.

II. MATERIALS AND METHODS

A. Microorganism and Culture Conditions

Aspergillus niger was obtained from Algies Pasteur Institute of Algeria; it was kept on potato dextrose agar (PDA) at 4°C. The inoculums were made after the growth of this microorganism on PDA during 5 days at 30°C until the complete fungal sporulation (Figs. 1 (a) and (b)). The spores were suspended in sterilized aqueous solution with 0,01% tween 80. Spores concentration was determined by direct count in Malassez cell. For polygalacturonase (PG) production orange waste was used for induction of pectinase because of their highly content of pectin (35%) [6]. The waste was ground using a food processor and then washed five times with distilled water. Peels were dried at 50°C for 24 hour before being utilized as substrate for the production of extracellular enzyme. The fermentation was carried in 500 ml Erlenmeyer flasks containing 60 g of orange waste; (NH)₂SO₄; KH₂PO₄; Urea; MgSO₄; FeSO₄ and distilled water was added to humidify medium. The medium was autoclaved at 121°C for 20 min. the flasks were inoculated with 6 10⁶ spore/ml. the flasks containing medium were carried out at 37°C, on rotator shaker at 150 rpm for 144 hours (Fig. 1 (c)). Samples were collected in 24 hours intervals during 144 hours. Enzyme was extracted by adding 100 ml of distilled water in the medium and agitated 20 min. the samples were centrifuged at 10000 rpm at 4°C. The filtrate was used as crude enzyme preparation.

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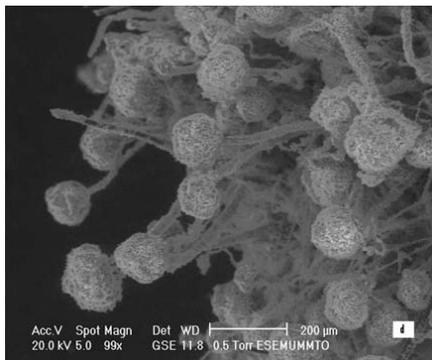
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B. Determination of Extracellular Enzyme Activities in Crude Extract

Pectin lyase activity was assayed spectrophotometrically by measuring the formation of 4-5 unsaturated oligogalacturonate at 235 nm [6]. Polygalacturonase activity was assayed using Somogyi- Nelson method [7], [8] by measuring the content of reducing sugar formed during 60 minutes of incubation crude enzyme with Sigma citrus pectin. Pectin esterase activity was determined by measuring the change in the pH value of medium due to the formation of free carboxyl bonds of Sigma citrus pectin in the presence of crude enzyme.



(a)



(b)



(c)

Fig. 1 (a) Microorganism in petri plate, (b) Show with scanning electron microscopy, (c) In production medium

C. Experimental Design

A 2^4 full factorial design was applied to study the influence of medium composition on PG productivity (Table I), the variables studied are dry orange waste particle size; $(\text{NH}_4)_2\text{SO}_4$, glucose and moisture contained in the medium.

TABLE I
LEVEL OF FACTORS USED IN EXPERIMENTAL DESIGN

Factor	Levels			Step change
	-1	0	+1	
X_1 (mm)	0,2	0,5	0,8	0,3
X_2 (%)	0	5	10	5
X_3 (%)	30	50	70	20
X_4 (%)	0	1	2	1

D. Polygalacturonase Isolation and Chemical Characterization

To separate pectin enzymes complex, ion exchange chromatography was employed on DEAE Sephadex A 25 QTY 10 column (1,5 cm x 30 cm), a sample containing 32, 85 $\mu\text{g}/\text{ml}$ of soluble proteins (determined by Lowry method) was applied to the column equilibrate with acetate buffer pH 4,2. The column was washed with the starting buffer and the bound protein was eluted with NaCl 0,5 M gradient 1,2 ml /min. the fractions exhibited polygalacturase activity were pooled. The same enzyme extract was subject to gel filtration chromatography on sephadex G 75 column (QTY 1,5cm x 30 cm) equilibrated and eluted with sodium acetate buffer pH 5,5. The fractions exhibiting polygalacturonase activities were pooled.

The optimum pH of PG was investigated by using isolated enzyme in reaction mixture containing 1% of Sigma citrus pectin in several buffer solutions (pH 2 to 8), the reaction mixture was incubated of 1 hour at 25°C and enzyme activity was determined. The optimum temperature was investigated by using purified enzyme in reaction mixture containing 1% Sigma citrus pectin in acetate buffer solution pH 5,5, which was incubated at different temperatures, the residual activity was determined.

E. Apple Juices Clarification

This method is based on the ability of enzyme to depectinize non-clarified apple juice at 35°C and natural pH. The cloud degradation was studied by measuring of turbidity in the treated juice in presence of a control sample (no enzymatically treated juice).

1. Preparation of Apple Juice

Fresh apple were purchased from a local market, they were washed with water, peeled, seeded and crushed with blender. The homogenate was divided in three parts, one 1 ml of crude enzyme was added in the first part, one ml of distilled water was added in the second part and one ml of Sigma *Aspergillus niger* enzyme was added in the third part; the three parts were incubated at 35°C for one hour. After this, the homogenate apple were pressed through two layers of gauze, the juice was heated at 90°C for 5 min, next cooled to 35°C and enzymatically treated; the juice was divided in three parts: one part was treated with crude enzyme, one part treated with Sigma *Aspergillus niger* pectinase and one part with distilled water (sample control). The three parts were incubated at 35°C for one hour, and heated at 90°C for 5 min and cooled. The clear juice was obtained by centrifugation at 4500 rpm for 10

minutes; the yield of juice was subject for turbidity, brix, optic density (DO_{420nm}), viscosity and pH determination.

III. RESULTS AND DISCUSSION

A. Pectinases Production

A gradual decrease in in pH is shown in Fig. 1 to a value of 2, 8, this decrease is probably due to release of organic acid in the medium, from 100 hours of fermentation the pH resumes its increasing. Soluble proteins rate increase during the first 72 hours of fermentation time, it goes from 4,8 to 20 mg/ml, this increase can be explained by enzyme synthesis by *Aspergillus niger*, [9] recorded a variation in protein concentration between 4,6 and 8,6 mg/ g during production of pectinase by *Aspergillus niger*, *orysae* and *sojae* spices.

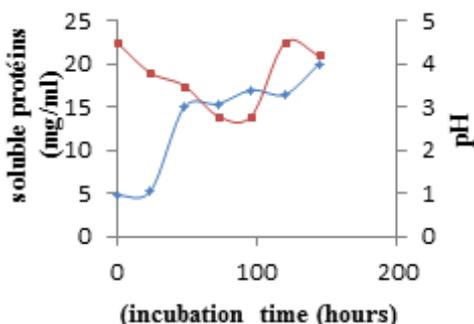
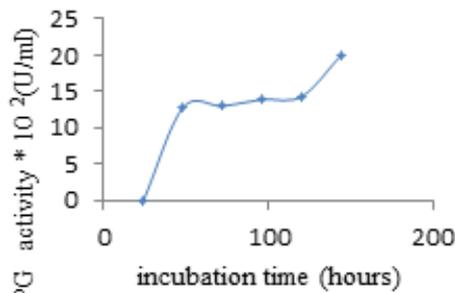


Fig. 2 Soluble proteins (•) and pH (•) in the crude extract during fermentation process

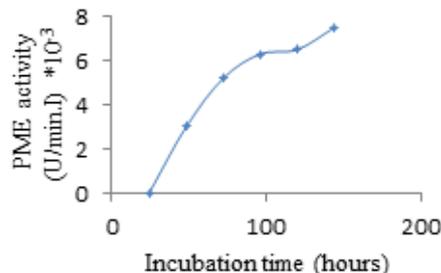
Fig. 3 (a) shows a higher production of PG activity (2000 U/ml). Reference [10] reported values of PG activities between 22, 12 and 162,5 U/ml produced by *Aspergillus carbonarius* in another hand [11] reported an exo PG activity that varies between 0,8 and 13,25 u/ml, whereas [12]-[14], [9] recorded 51,82, 34,12, 142 U/ml and 45 U/ml respectively. Fig. 3 (b) shows also that PME activity is in range of 0,003-0,007U/ml these values are smaller compared to a value of 0,03 U/ml found by [14] produced by *Aspergillus niger* and 0,29 U/ml produced by *Aspergillus oryzae*. In addition, pectin lyase activities values were found smaller that PG activity (Fig. 3 (c)), the values are about 0,021 U/ml, and smaller to that found by [14] (0,432 U/ml produced by *Aspergillus niger* and 0,180 U/ml produced by *Aspergillus oryzae*).

B. Analyze of Factorial Design

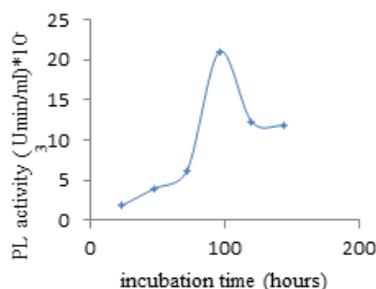
In the normal probability plot of the effects (Fig. 4), points that do not fat near the line usually signal important effect. Important effects are larger and further from the fitted line than unimportant effects. Unimportant effects tend to be smaller and centered around a zero. So, we show that all factors and their interaction are significant: the most significant is the ammonium sulfate followed by particles size, the two factors shows an antagonist effect, while the ammonium sulfate has a positive effect, the particle size has a negative effect, and the interaction between these two factors has a greater effect (Fig. 4).



(a) Polygalacturonase Production



(b) Pectnemethylesterase Production



(c) PL Production

Fig. 3 Pectolytic enzyme production

Normal Probability Plot of the Standardized Effects (response is exoPG, Alpha = .10)

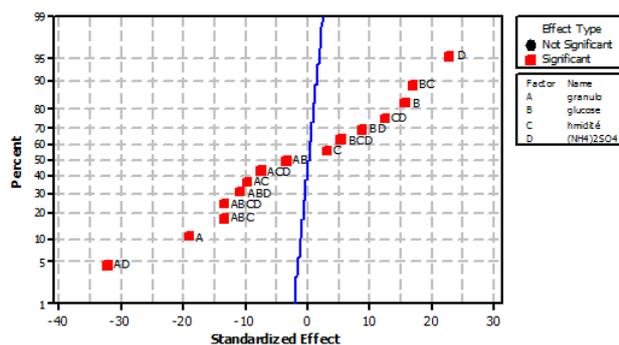


Fig. 4 Normal probability plot

In Fig. 5 we show that PG activity is higher when the particle size is > 0,2 μm. Interaction between humidity and ammonium sulfate contain in the fermentation medium has a positive effect. However, the interaction between humidity

and glucose contain in the medium has a negative effect. In this way, an optimum value of PG activity calculated by *Minitab 14* was about $17,30 \times 10^2$ U/ml under particle size of $0,2 \mu\text{m}$, 10 % Glucose, 1,95% of ammonium sulfate, and 150ml of added water. In the medium, with a factor of desirability (d) of 0,54, were the coefficient of desirability is the weight geometric mean of the individual desirability for responses. The coefficient were estimated with $R^2 = 99,53$ and their p values ($p < 0,05$); the analysis of variance ANOVA is presented in Table II.

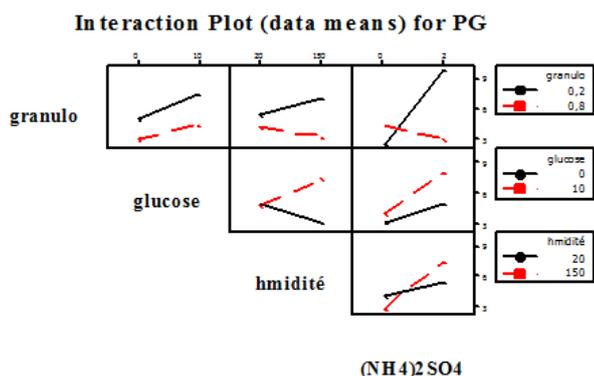


Fig. 5 Interaction plot

TABLE II
ANALYSIS OF VARIANCE ANOVA FOR PG ACTIVITY

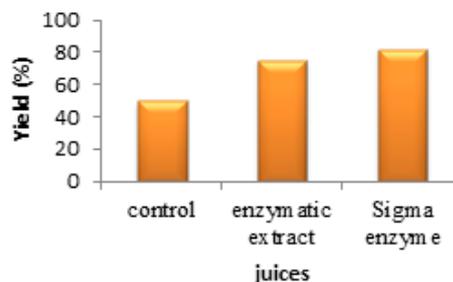
Source	DF	Seq ss	Adj ss	Adj MS	F	P
Main effects	4	159,128	159,128	39,7819	284,41	0,000
2- way effects	6	232,567	232,567	38,766	277,11	0,000
3- way effects	4	55,530	55,530	13,882	99,25	0,000
4- xay effects	1	25,906	25,906	25,905	185,00	0,000
Residual error	16	2,238	2,238	0,1399		
Pure error	16	2,238	2,238	0,1399		
Total	13	475,368				

C. Application of Crude Enzyme in Apple Juice Depectinization

The enzymatic treatment of homogenate apple was much higher in efficiency compared to the control (Fig. 6 (a)) the yield of juice was 50 % in the control 75 % in the treated apple with crude enzyme and 82% when apple was treated with Sigma *A. niger* enzyme.

It has previously been reported that pectinase treatment may result in either increased or decreased turbidity of fruit [15]. This phenomenon may be linked to the paradox that the pectolytic enzyme attack can result in two different events [16]: (1) pectinase catalyze the degradation of high molecular weight pectin structure into small pectin fractions [17]. The resulting small, negatively charged, pectin fractions may be able to stay in suspension and contribute to increase the immediate turbidity due to an increased scatter effect of small particles as compared to larger [17]. (2) The pectinase act on pectin layer encapsulating the protein core of proteinaceous pectin particles in suspension [17]. This action results in an electrostatic agglomeration of oppositely charged particles that

may lead to transient turbidity increase and subsequently results in precipitation of the agglomerated complex resulting in decrease turbidity. In Table III we show that turbidity was decreased from 44,1 UN to 0,5 UN in juice treated with Sigma enzyme, and it decrease to 6,27 in juice treated with crude enzyme, this decrease leads to depectinization of apple juice , so we can estimate that obtained *A. niger* enzyme is competitive with the commercial Sigma pectinase. The optic density (DO) decrease also from 2 to 0,689 in juice (2) and 0,5 in juices (3) of Fig. 6, and this decrease is related to the loss in the clod in juice. No significant change in relative viscosity and brix were observed, but a decrease in pH from 4,5 in the control juice to 3,45 in juice (a) and to 3,38 in juice (b) were observed. This is related probably to organic acid liberation in juice such as galacturonic acid.



(a)



(b)

Fig. 6 (a) Yield of juice apple, (b) enzymatic treatment of apple juice: 1 is a control; 2 is the crude enzyme treatment and 3 is the Sigma *A. niger* PG treatment

TABLE III
CHEMICAL CHARACTERISTICS OF ENZYMATIC CLARIFIED APPLE JUICE COMPARED TO CONTROL

	Brix	pH	Turbidity (UN)	DO (420 nm)	Relative viscosity
Control juice (1)	12,8	4,15	44,1	2	1,1
Juice treated with commercial enzyme (3)	12,5	3,38	0,5	0,528	1,01
Juice treated with crude enzyme (2)	10,65	3,45	6,27	0,689	1,05

D. Polygalacturonase Chromatography

From elution profile of the chromatography DEAE sephadex A25 column, one polygalacturonase PGI was separated, it has an activity of 652,14 U/ml (Fig. 7 (a)). From

the gel filtration, chromatography two polygalacturonases were separated PGI and PGII. This last one has a higher activity 813,31 U/ml and 166,66 U/mg proteins as a specific activity (Fig. 7 (b)). This PG activity is in good agreement with the results of [18] workers, who found PG activity of 276 U/mg protein using *Trichoderma harzianum*, these results were 9- 12 fold higher than that PGs from *Bacillus* sp. [19]. *A. niger* [20], but 3-5 fold lower than that of PGs from *Penicillium capsulatum* [21], *Mucor flavus* [22] and *T. reesei* [23].

PGII separated from sephadex G75 has a relative molecular weight about 51, 28 kDa, determined in reference to proteins markers (γ - globuline, β -lactalbuline and BSA), this proteins mixture solution was eluted in the same column.

Often polygalacturonases are in multiple molecular isoenzyme, [24] obtained two polygalacturonases about 31 kDa, the higher molecular weight were PG of *Bacillus* sp. (115 kDa) [19].

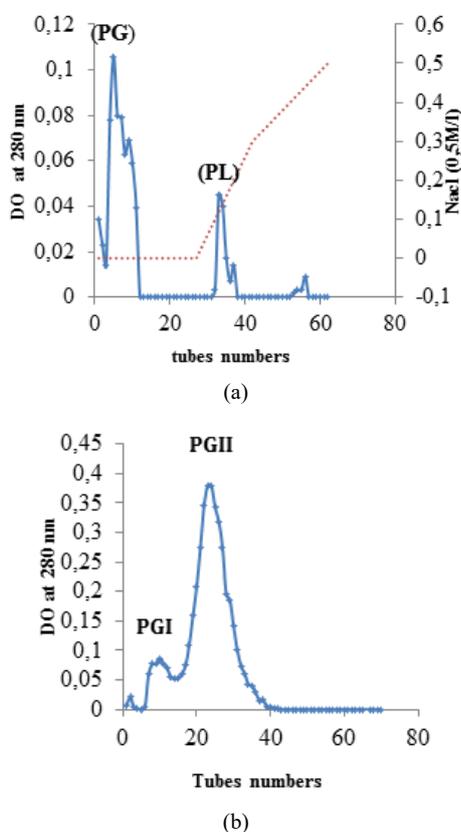
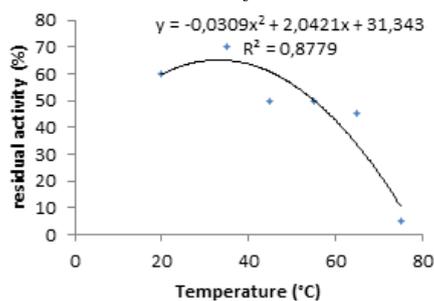
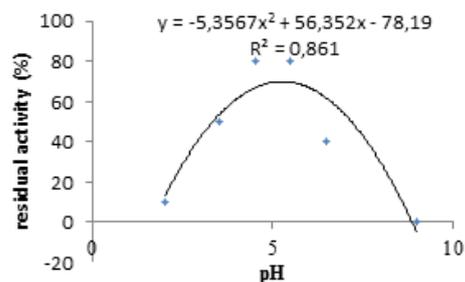


Fig. 7 Polygalacturonase chromatography; (a): DEAE sephadex column (1cmx30cm) eluted tampon acetate (1,2 ml/min) and NaCL (0,5 M/l); (b): sephadex G75 column eluted tampon acetate pH 4,2 (1,2 ml/min)

E. Chemical Characterization of PG II



(a)



(b)

Fig. 8 Temperature Optimum (a) and pH Optimum (b) of *A. niger* PGII

PGII of *A. niger* was found to have temperature optimum at 35°C (Fig. 8 (a)). similarly temperature optima for PG are from most mesophilic mold [25], [26] found an optimum temperature PG activity from mould at 30° and at 45°C was found by [27]; while [18] found an optimum temperature PG activity of *T. harzianum* at 40°C. The effect of pH on PGII activity was studied at 35°C, it was found at pH 5 (Fig. 8 (b)); the same pH optimum was reported for PGs from *Aspergillus niger* [28], *Fusarium moniliforme* [29], *Trichoderma harzianum* [18].

IV. CONCLUSION

Orange waste were found a good substrate for pectinase production by *A. niger* sp. full factorial design was found to be efficient tool for composition fermentation medium study ; ammonium sulfate was the most significant factor influencing PG production, the interactions between this factors and particle size of dry orange waste has a greater effect on the polygalacturonase production. The isolated polygalacturonase has a relative molecular weight of 51,28 kDa and optimum pH of 5, the optimum temperature was at 35°C. The crude enzyme was able to depectinize an apple juice prepared at laboratory scale; decrease in turbidity compared to control juice was efficient and it is comparable to *A. niger* pectinase provided from Sigma Aldrich.

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