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Product optimization, purification and characterization of a novel polygalacturonase produced by *Macrophomina phaseolina*

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Abstract

Introduction: Production of a novel polygalacturonase (PG) active at pH = 3.0 and suitable to be used in fruit juice industries from *Macrophomina phaseolina* was evaluated. Suitable carbon, nitrogen and phosphorous forms were determined and the condition was optimized for higher PG production.

Materials and methods: *Macrophomina phaseolina* was cultured in so called production medium. The secretome was separated from fungal cells and polygalacturonase was isolated via column chromatography. The biochemical activity of both secretome and isolated polygalacturonase were assayed calorimetrically. The production of polygalacturonase was optimized via changes in culture medium in terms of contents, pH and temperature and Taguchi analysis of data. Enzyme kinetics was partially performed followed by the determination of pH and temperature stability.

Results: A range of sugars except glucose and chitin demonstrated to improve the PG production. Ammonium sulfate and peptone demonstrated to be suitable nitrogen sources. Amongst phosphorous sources, dipotassium hydrogen phosphate had the greatest effect. Taguchi's orthogonal array demonstrated that pH, temperature, ammonium sulfate and trace elements had significant effect on PG production. Furthermore, mean comparisons showed that the optimum condition achieved at pH 6.0, 35 °C, 2 $g.l^{-1}$ ammonium sulfate and 2 $mg.l^{-1}$ trace elements.

Discussion and conclusion: The purified PG with a relative molecular mass of 70 kDa was demonstrated its highest activity at pH = 3, and 30 °C. Amongst tested cations and chemicals, Fe^{2+} improved the enzyme activity by 2 fold, while the secretome responded differently.

Key words: *Macrophomina phaseolina*, polygalacturonase, fruit juice industry, Taguchi orthogonal method, purification and characterization

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Introduction

The plant cells are bound by a rigid structure called cell wall that is made from cellulose microfibrils and matrix polysaccharides with relatively equal amount that are embedded in a gel of pectin with 40-60% of wall content (1). Although pectins have certain advantages in food industry specifically as gelling agents, stabilizers and thickeners (2), in beverage industry it has been considered as nuisance trapping some of juice, making the juice look murky and further leading to inefficiencies within the filtering systems. Pectinases next to other wall hydrolytic enzymes, *i.e.*, cellulases, glucanases and xylanases, improve the clarification and liquefaction of fruits, improving the quality and yield of beverages and concentrates in fruit juice factories, eventually reducing the waste product (3).

Amongst many forms of pectinases, polygalacturonases (PG) hydrolyze the α-1,4-D-galacturonan backbone and appear in two forms of endo- (E.C. 3.2.1.15), and exo- (E.C. 3.2.1.67) hydrolases (4); with greater contribution of endopolygalacturonases in pectin hydrolysis through random breakage of glycosidic linkages between galacturonic acid residues. Plants, bacteria and fungi are the known sources of organisms producing PGs for fruit ripening and softening (5), abscission (6), growth (7) and microbial infection (8, 9). Additionally, some industrial applications have been considered for polygalacturonases when they have been used alone or in combination of other lytic enzymes (10). Producing oligogalacturonides as functional food components (11), extraction of vegetable oils (12), recovering more must/wine in oenology and improving the wine quality, *i.e.*, color and flavor (13), biobleaching of kraft pulp to improve the paper quality (14, 15), producing high quality linen fibers (16), and bioethanol production (17) next to the fruit and vegetable maceration in fruit juice industries (18- 22) are some of these applications.

Since 1930s commercial enzymes have been widely used within every industry transforming fruit juice. However, looking for enzymes with greater potentiality from other microorganisms seems to be inevitable (23- 25). In addition to find new enzymatic sources, one needs to optimize the condition towards obtaining more functional enzymes with fewer resources. This can be achieved through factorial experiments in which many factors with a range of levels are considered simultaneously (26). However as the number of factors grows, the number of experiments will increase. In contrast, Taguchi (27) developed a rather cost-effective approach to reduce the number of experiments via implementing orthogonal arrays that appreciates the signal-to-noise (S/N) ratio (28).

In the current study, fungal phytopathogens (9) were screened for primarily polygalcturonase activity and further the fungus with functional PG at lower pH and higher thermal stability was selected (data not shown). Amongst fungal strains, *Macrophomina phaseolina* was chosen and the production of PG was analyzed to determine the best time-course, temperature, carbon, nitrogen, and phosphorous sources. Following the initial determination of the suitable condition, Taguchi's statistical method was applied to determine the best levels of each factor. Finally, for the first time *Macrophomina phaseolina* PG was purified and analyzed for its biochemical characteristics.

Materials and Methods

Chemicals and fungal strains

Polygalcturonic acid, α-D-galacturonic acid, and 3,5-dinitrosalisylic acid (DNS) were purchased from Sigma (St. Louis, MO, USA). DEAE-Sepharose was obtained from Pharmacia (Stockholm, Sweden). All other chemicals were obtained from Merck (Darmstadt, Germany). *Macrophomina phaseolina*, *Bipolaris* sp., *Alternaria raphini*, *Alternaria brassicicola*, *Fusarium graminearum*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Phytophtora* sp. were kindly provided by Dr. Sarpele at Phytopathology Research Institute, Tehran, Iran. Primary colorimetric screening of the fungal strains using rhetinum red (Sigma Chemical Co, St Louis, USA) was carried out according to Torres et al. (29) (data not shown).

Culture media

The fungal strain was cultured on potato dextrose agar plates and transferred into production medium. The production medium (1 l) was contained 5 g polygalcturonic acid, 3 g ammonium sulfate, 10 g potassium dihydrogen phosphate, 2 g magnesium sulfate, 0.7 mg sodium borate, 0.5 mg ammonium molybdate, 10 mg iron sulfate, 0.3 mg copper sulfate, 0.11 mg manganese sulfate, 17.6 mg zinc sulfate and 100 mg amoxyciline at pH 7.2. Cultures were incubated for 24-144 h at 30 °C and passed through a filter paper were in 24 h intervals. The filtrate was centrifuged at 10,000 ×g for 20 min at 4°C and the resulting supernatants were collected and used for subsequent polygalcturonase assay according to Chaplin and Kennedy (30). In each step the protein concentration was measured using a commercial assay kit (Bio-Rad Protein Assay kit) with bovine serum albumin as standard, following the procedure described by Bradford (31).

Polygalacturonase activity assay

In this colorimetric assay the increase of reducing end of galacturonic acid residues during the depolymerization of substrate, 1% (w/v) polygalacturonic acid in 20 mM sodium acetate buffer (pH = 6.0), was determined in the presence of 1% 3,5-dinitrosalicylic acid (DNS) (32).

The substrate (200 µl, 1% polygalacturonic acid) was added to the supernatant (200 µl) and incubated at 35 °C for 15 min. DNS reagent was added to the reaction mixture and boiled at 100 °C for 5 min. The absorbance was read at 530 nm using a UV spectrophotometer (Beckman DU530, USA). One unit (U) of polygalacturonase activity was defined as the amount of enzyme required to produce 1 micromole/min of reducing sugar once galcturonic acid is being used as standard.

Determination of factors required for optimal PG production

The optimization of medium constituents to improve production of PG by the fungus was carried out. Sugars (1% w/v) including monosaccharides (glucose, fructose, galactose, mannose, arabinose), disaccharaides (lactose, sucrose, maltose), and polysaccharides (starch, pectin, chitin) were used as carbon source in the medium. The sugar molecules were filter-sterilized and added to the production medium lacking polygalcturonic acid. The cultures were aerated at 160 rpm at 30 °C for 72 h. Similar conditions for nitrogen source were applied using 1% (w/v) of each pepton, $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, $CaNO_3.4H_2O$, $C_4H_{11}NO_3$, $(NH_4)_2HPO_4$, trypton, and yeast extract following the

removal of ammonium sulfate from the production medium. KH_2PO_4, K_2HPO_4, Na_2HPO_4, NaH_2PO_4 (1% w/v) were used separately as phosphate source. The best sources of the carbon, nitrogen, and phosphate were determined and used in combination with other factors in the following experiment.

Statistical optimization of growth condition

The PG production was measured in L27 in Taguchi's orthogonal arrays considering combinatorial effects of seven factors with three levels (Table 1). On the basis of L27, 27 experiments were carried out in triplicate (Table 2) and the enzymatic assay was conducted as stated above. The results were analyzed using MiniTab software (version 14, USA). The mean values of data were analyzed with Qualiteck-4^{TM} (Nuteck Inc., MI, USA). It is worth stating that in the full-factorial experimental design, a total of 343 (7^3) experiments needed to be carried out to provide the same conclusions as the Taguchi's array method with 27 experiments. In order to determine the statistically significant factors influencing the PG production, analysis of variance was performed.

Table 1- Factors and their levels used to evaluate polygalacturonase production by *M. phaseolina*

No.	Factors	Level 1	Level 2	Level 3
1	Temperature (°C)	25	30	35
2	pH	4.5	6.0	7.5
3	Polygalacturonic acid % (w/v)	0.1	0.5	0.9
4	Ammonium sulfate ($g.l^{-1}$)	2	3	4
5	Dipotassium hydrogen phosphate ($g.l^{-1}$)	5	10	15
6	Magnesium sulfate ($g.l^{-1}$)	1	2	3
7	Trace elements% (v/v)	0.1	0.2	0.3

Trace elements were 0.7 mg sodium borate, 0.5 mg ammonium molibdate, 10 mg iron sulfate, 0.3 mg copper sulfate, 0.11 mg manganese sulfate and 17.6 mg zinc sulfate per liter.

Isolation and purification of *M. phaseolina* polygalacturonase via anion exchange chromatography

M. phaseolina secretome, grown in the availability of polygalacturonic acid, was precipitated by 85% (w/v) ammonium sulfate. The precipitate was dialyzed against 20 mM sodium acetate buffer (pH = 7) at 4 °C for 24 h and fractionated by DEAE-Sepharose column. The column was equilibrated with 20 mM Tris buffer (pH = 8.5) and following loading the dialyzed solution, 2 mL fractions were eluted from the column by 20 mM Tris buffer (pH = 8.5) in a gradient of 0 to 1 M NaCl over a 16.5 h period. The fractions were checked at 280 and 530 nm for the presence of protein and polygalacturonase activity (as stated above), respectively. The fractions with the enzyme activity were further purified with the same column, but with a different gradient of NaCl (0-0.5 M). Samples of fractions with enzyme activity were visualized on SDS-PAGE following silver nitrate staining.

Table 2- Variable factors (7), their relative levels (3) and assignment of experimental factors of the L27 Taguchi's array and relative enzyme activities

Test	pH	Temp (°C)	PGA (%)	AS ($g.l^{-1}$)	DPHP ($g.l^{-1}$)	TE ($mg.l^{-1}$)	MS ($g.l^{-1}$)	PG activity (relative%)
1	4.5	25	0.1	2	5	1	1	70.68
2	4.5	25	0.1	2	10	2	2	71.84
3	4.5	25	0.1	2	15	3	3	62.1
4	4.5	30	0.5	3	5	1	1	73.43
5	4.5	30	0.5	3	10	2	2	70.97
6	4.5	30	0.5	3	15	3	3	76.84
7	4.5	35	0.9	4	5	1	1	80.32
8	4.5	35	0.9	4	10	2	2	65.9
9	4.5	35	0.9	4	15	3	3	77.55
10	6	25	0.5	4	5	2	2	86.82
11	6	25	0.5	4	10	3	3	83.73
12	6	25	0.5	4	15	1	1	62.41
13	6	30	0.9	2	5	2	2	94.43
14	6	30	0.9	2	10	3	3	85.4
15	6	30	0.9	2	15	1	1	90.23
16	6	35	0.1	3	5	1	2	100
17	6	35	0.1	3	10	2	3	97.05
18	6	35	0.1	3	15	3	1	97.68
19	7.5	25	0.9	3	5	1	3	13.37
20	7.5	25	0.9	3	10	2	1	52.11
21	7.5	25	0.9	3	15	3	2	66.30
22	7.5	30	0.1	4	5	1	3	15.49
23	7.5	30	0.1	4	10	2	1	62.57
24	7.5	30	0.1	4	15	3	2	65.19
25	7.5	35	0.5	2	5	1	3	63.76
26	7.5	35	0.5	2	10	2	1	87.06
27	7.5	35	0.5	2	15	3	2	69.71

Temp: temperature, PGA: poly galacturonic acid, AS: ammonium sulfate, DPHP: dipotassium hydrogen phosphate, TE: trace element, MS: Manganese sulfate

pH profile

Purified polygalcturonase was used in assay reactions in the presence of 50 mM mixed buffer (glycine, sodium phosphate and sodium acetate) prepared in a range of pH from 3 to 12. To determine the pH profile, the enzyme solution incubated for 90 min at any particular pH was added to the substrate, prepared at certain pH (3-12), and the mixture was incubated at 35 °C for 15 min. The enzyme activity was assayed colorimetrically at 530 nm.

In order to determine the pH stability, the enzyme fraction was incubated in 5 mM mixed buffer for 90 min and substrate was added to the reaction mixture. The enzyme stability was assessed in the same buffering condition, but in a time point manner for 180 min in 30 min intervals. At each time point the substrate, prepared in three pHs (3, 7, 12), was added and enzyme assay was conducted.

Temperature profile

To establish the temperature profile, 200 µl of each of substrate (1% w/v) and enzyme were incubated individually at the particular temperature (10 to 90 °C) for 5 min and combined with each other at the same temperature and incubated for 15 min. Subsequently, DNS reagent (400 µl) was added, the mixture was boiled at 100 °C for 5 min. The mixture was centrifuged at 6000×g for 15 min and the absorbance was measured at 530 nm.

Enzyme kinetics

The polygalacturonase enzyme kinetics was assayed in the presence of various concentrations of polygalcturonic acid as the substrate and plotted using Michaelis-Menton after measuring the catalytic parameters by Lineweaver-Burk curve. The assay reaction was repeated trice.

The effects of cations, chelators and chemicals on enzyme activity

A range of cations (NaCl, $NiSO_4$, $MnCl_2$, KCl, $BaCl_2$, $MgSO_4$, LiCl, $FeSO_4$, $CaCl_2$, $CuSO_4$, $CoCl_2$, $ZnCl_2$) in 1, 5, 10, and 15 mM, and 1% of detergents (Urea, Tween 80, Tween 20, Triton X100), chemicals (glycerol, citric acid, ascorbic acid, Tris-HCl, naphtaline acetic acid, iodoacetamid, iodoacetic acid, PMSF) and EDTA as chelator were prepared in 20 mM acetate buffer (pH = 4) and enzyme activity were recorded and compared with control with no extra additives.

Results

Preliminary optimization of PG production

M. phaseolina was cultured in production medium for six days and in 24 h intervals samples were collected and assayed for PG activity. The results demonstrated that at 72 h the highest production of the enzyme was achieved (Fig. 1). Polygalcturonase production started to decline after 72 h. This decline may be explained either by diminishing the nutrients within the culture medium or through PG turnover via the hydrolytic activity of proteases possibly after 72 h (30). Analysis of carbon sources (Fig. 2a) revealed that in the availability of glucose and chitin, PG had the lowest activity that is an indication of lower production. Since glucose is the preferred choice of carbon by relatively all microorganisms, the production decline can easily be explained. However in the presence of chitin, the only reason that may explain the reduced PG production is its complex structure that may have trapped the enzyme, inhibiting it from properly performing its hydrolytic activity. In the presence of other sugars, polygalacturonase production was high. Although the highest production was noted when the polygalacturonic acid was included in the reaction, the production was indifferent from the other mono-, di-, and polysaccharides, presenting an unspecific response (Fig. 2a).

Amongst nitrogen sources that used in this experiment, the highest polygalacturonase production was seen in the presence of ammonium sulfate and peptone (Fig. 2b). Ammonium sulfate was chosen as the nitrogen source and used for Taguchi analysis, which also caused a stabilizing effect on the enzymes (33). From the different phosphate sources that used, dipotassium hydrogen phosphate was demonstrated a significant effect on PG production (Fig. 2c).

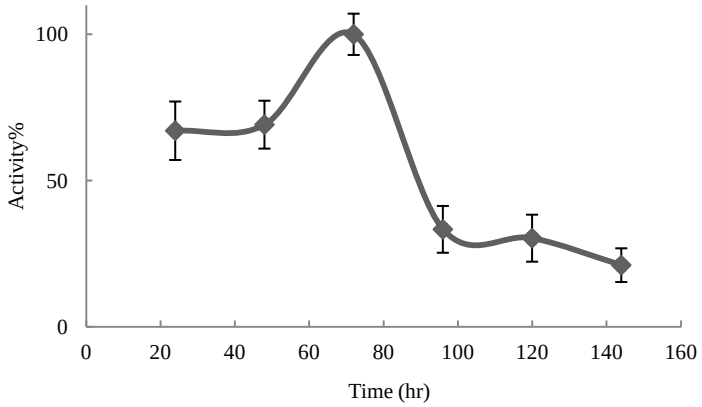

Fig. 1- Determination of the best incubation period to achieve the highest PG production. The highest production was noticed after 72 h. The experiment was performed in three replicates

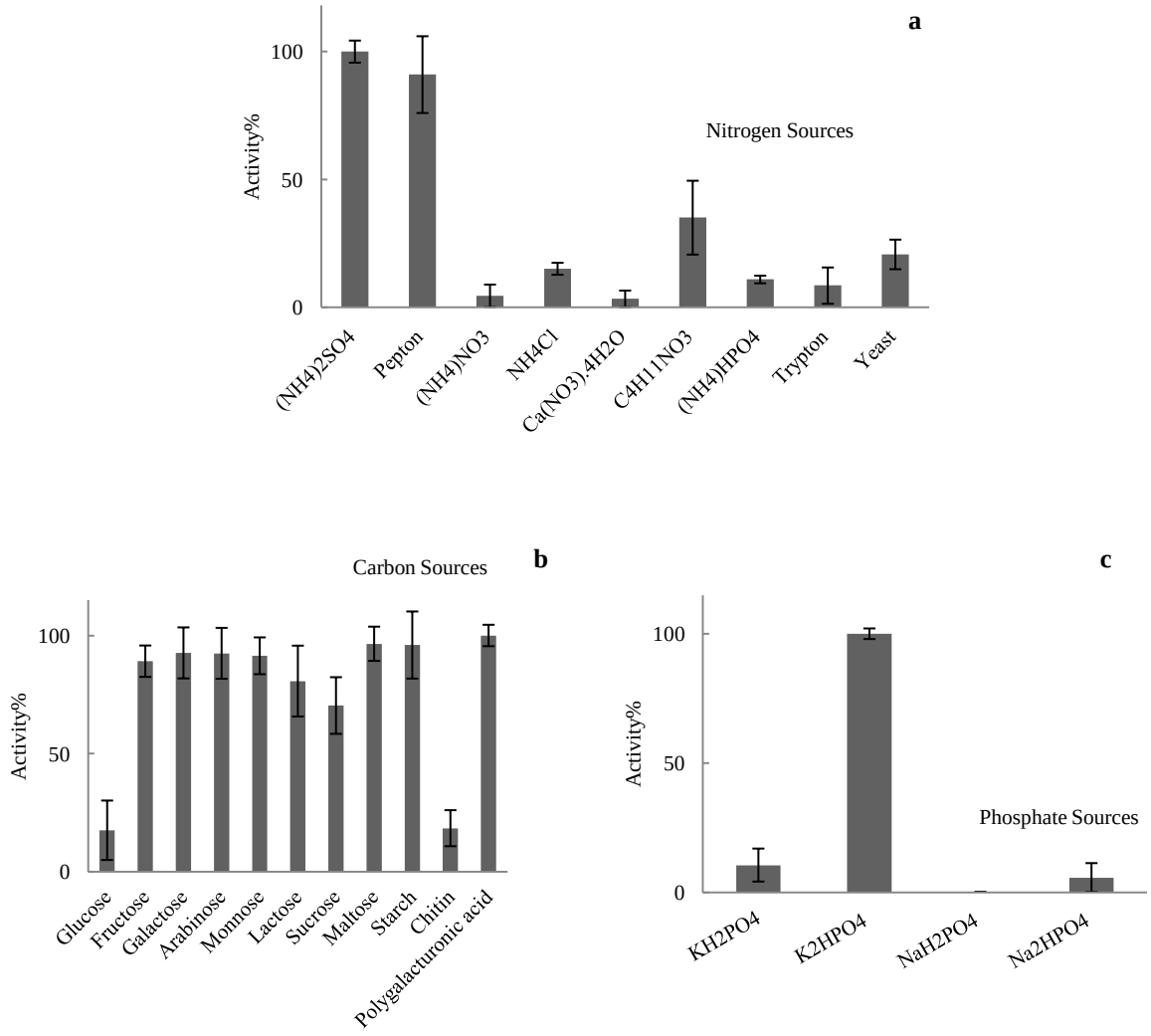

Fig. 2- Determination of the best carbon **(a)**, nitrogen **(b)** and phosphate **(c)** sources for PG production. Each experiment was carried out in three replicates

Taguchi's analysis of variance

In order to provide the best condition for *M. phaseolina* to produce more polygalacturonase, seven factors including temperature, pH, polygalcturonic acid as substrate, trace elements, ammonium sulfate, dipotassium hydrogen phosphate and magnesium sulfate in three levels were analyzed using Taguchi's L27 orthogonal array method (Tables 1 and 2). The PG activity within each experiment was measured as stated above (Table 2). Analysis of variance was showed that temperature (*P value <0.01*), pH ($P< 0.01$), ammonium sulfate ($P < 0.05$) and trace elements ($P < 0.05$) had significant effects on polygalacturonase production (Table 3). Response values of means were compared to determine the best level of each factor. The data was demonstrated that the highest production rate for polygalacturonase can be achieved at pH = 6.0, 35 °C, in the presence of 2 $g.l^{-1}$ ammonium sulfate and either 0.1% or 0.2% (v/v) trace elements. For small scale product optimization, the enzyme activity that is an indication of enzyme production improved by almost 100% (from 0.537 enzyme unit to 1.116; Table 2).

Isolation of polygalcaturonase and partial characterization

Anion exchange chromatography in two steps was used to isolate the ammonium sulfate precipitated secretome of *M. phaseolina* polygalacturonase. Following the second chromatography, two peaks (fractions 47 and 56) with polygalacturonase activity were evident (Fig. 3a). The purified fractions were separated on SDS-PAGE side by side to the ammonium sulfate precipitated secretome. The presence of two purified polygalcturonases was apparent with molecular weights of 120 and 60 kDa (Fig. 3b). The enzyme with relative molecular mass of 60 kDa (fraction 56) was used for further analysis for its higher purity.

Table 3- Analysis of variance of seven factors to determine their significant effect on polygalacturonase production by *M. phaseolina.*

SOV	df	SS	MS	F
Temperature	2	0.0126	0.00699	123.8^{**}
pH	2	0.0047	0.00266	47.10^{**}
Polygalacturonic acid%	2	0.0002	0.00006	1.17^{ns}
Ammonium sulfate ($g.l^{-1}$)	2	0.0008	0.00056	99.47^{**}
Dipotassium hydrogen phosphate ($g.l^{-1}$)	2	0.0002	0.00002	0.39^{ns}
Magnesium sulfate ($g.l^{-1}$)	2	0.0001	0.00028	5.00^{**}
Trace elements ($ml.l^{-1}$)	2	0.0016	0.00082	14.50^{**}
Residual	66	0.0037	0.00005	-
Total	80	0.0241	-	-

SOV: sources of variance, df: degrees of freedom, SS: sum of squares, MS: mean of squares, F: Fischer's Test. *: significant level at 0.05%, **: significant level at 0.01%, ns: non-significant.

The purified polygalacturonase of *M. phaseolina* grown in the availability of polygalacturonic acid was analyzed to determine the pH optima. The optimum pH was appeared to be 3 (Fig. 4a), while the polygalcturonase activity was appeared to be stable in a relatively wide range of pH (2-8, data not shown). Enzyme was stable at pH 4 and 7 for 180 min without a significant decline in its activity. The enzyme was assayed for polygalacturonase activity at different temperatures and the

highest activity was achieved at 30 °C (Fig. 4b).

Based on substrate concentrations, Michaelis-Menton curve (Fig. 5) was plotted and the enzyme catalytic parameters were calculated with Lineweaver-Burk standard line (Fig. 5, right panel: y = 0.425x + 4.55, R^2 = 0.904). Accordingly V_{max} and k_m were 0.219 U/mL and 0.093 mg/ml, respectively.

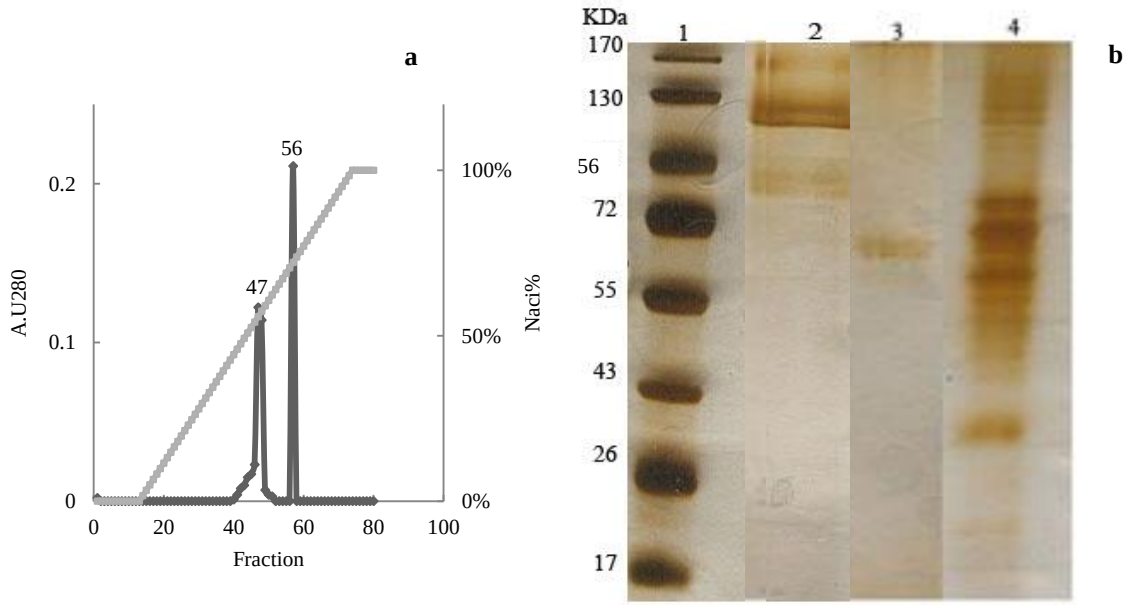

Fig. 3- Anion exchange chromatography to isolate secreted polygalacturonases from *M. phaseolina*. a) DEAE-Sepharose was used to separate secreted proteins using NaCl gradient (0-0.5 M) in a period of 80 min, fractions 47 and 56 demonstrated polygalacturonase activity **(a)**. SDS-PAGE separation of fractions 47 (lane 2), 56 (lane 3), and ammonium sulfate precipitated secretome prior to column chromatography (lane 4) **(b)**.

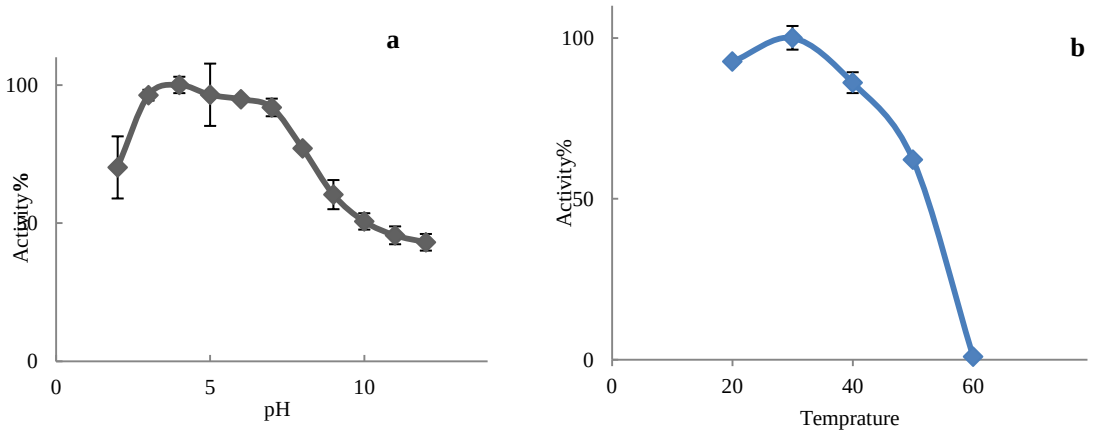

Fig. 4. Temperature and pH profile of purified polygalacturonase with relative molecular mass of 60 kDa. The PG was active at pH range of 2-6 and the highest activity was observed at pH = 3 **(a)**. PG was active at temperatures ranging from 20 to 50 °C with the highest activity at 30 °C **(b)**. Each experiment performed in three replicates.

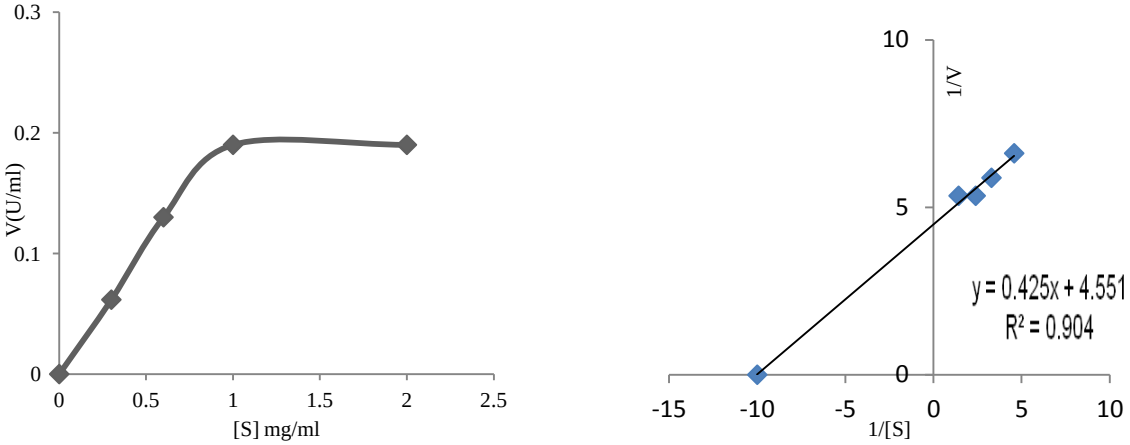

Fig. 5. The curve represents the Michaelis-Menton equation in different substrate concentrations. The linear graph represents the Lineweaver-burk equation. The calculated V_{max} and K_m for PG with MW = ~ 72 kDa are 0.219, and 0.093, respectively. The data were collected from three replicates (R^2 = 0.904).

The effect of chemicals on PG production

Amongst tested cations 15 mM $MnCl_2$, 1 mM $BaCl_2$, and 1 mM $CuSO_4$ improved enzyme production, while other cations and chemicals reduced the enzyme production. Tween 20 improved the enzyme production by ~ 50%. Citric acid, ascorbic acid and naphtalene acetic acid (NAA) inhibited the enzyme production almost completely.

For the purified polygalcturonase, the sole cation that improved the enzyme activity was 1 mM $FeSO_4$ with about 100%. Other cations and chemicals had inhibitory effects on the enzyme activity.

Discussion and conclusion

Use of enzyme technology seems to open a wide variety of doors to human need from bioconversion of fruits to juice, to catalysis of feedstock to produce biofuel. Nevertheless, our findings are mostly dependent on natural resources to isolate the suitable enzymes and to optimize the conditions for higher enzyme production. The goal of this research was to determine a fungal polygalacturonase (s) active at a relatively wide range of acidic pH suitable for fruit juice industries. Amongst a few fungi that we looked at, *Macrophomina phaseolina* was demonstrated to be the suitable choice, particularly on the basis that no prior report was available on its PG activity. Therefore, it has been decided to optimize the culture condition in a way to yield higher novel PG and at the same time the enzyme was isolated for its biochemical characterization. *Macrophomina phaseolina* genome sequence has been sequenced more recently (34)[1]. Earlier analysis of genome data demonstrates that *Macrophomina* has seven polygalacturonases (407923909, 407922771, 407917170, 407925949, 407924329, 407921266, and 407920911) belonging to GH28 superfamily of hydrolases and a much smaller protein sequence (407925004) that appears to be a duplicated pseudogene of 407923909. Nevertheless, at time that the project was started we did not have access to such data and following the screening of fungal phytopathogens, *Macrophomina* was stood up as the sole fungus with polygalacturonase active at acidic pH.

The highest production of PG by *M. phaseolina* was achieved following 72 h of incubation and started to decline thereafter. In regards to highest production time of

polygalcturonases, different fungal generally behave differently. Some of which such as *Cryptococcus* (35), *Penicillium* (36) and *Byssochlamys* (37) reach the highest production rate after 72 h similar to *Macrophomina*, while for some others it takes longer periods of time (38, 39).

Generally the carbon sources are important in fungal survival and earlier reports are indicative that in most cases pectin is the greatest source of carbon in producing polygalacturonase by the fungi (35, 37), while some other fungi prefer polygalacturonic acid over pectin (40). In our research when a variety of carbon sources were used, they did not demonstrate significant difference to each other and equally enhanced the PG production (Fig. 2a). The carbon sources that diminished PG production were glucose and chitin (Fig. 2a). Furthermore, changing the concentration of polygalacturonic acid in Taguchi analysis did not result in a significant change in enzyme production. It may indicate that *M. phaseolina* irrespective to carbon source can produce polygalacturonase and from the industrial point of view it can be considered as a benefit since the culture medium can rely on cheap sources of carbon.

For the nitrogen source, a range of organic and inorganic sources were examined (Fig. 2b). Amongst which $(NH_4)_2SO_4$ and peptone showed to have a greater role in enzyme production. The results showed that $(NH_4)_2SO_4$ concentration has a significant role in enzyme production and the highest PG production was obtained at 2 $g.l^{-1}$ (Table 3).

Enzymes usually use divalent cations to establish enzyme-substrate bonds. Some metal cations have inhibitory effects on some polygalacturonases (37, 38) and few others might be necessary for enzyme activity (41). For instance in *Penicillium soiltum* isolated from apple, magnesium increased the polygalacturonase activity (42), while in another strain of this fungus isolated from pear fruit it had inhibitory role (43). In *Aspergillus niger* MTCC 3223, PG activity was improved in the presence of Mg^{2+} and Cu^{2+} (21). Here, the use of magnesium sulfate demonstrated that Mg^{2+} has a significant role in enzyme activity and production even at low concentrations (Table 3).

Trace elements are constituents of micronutrients that in lower amounts are beneficial to growth and development of organisms and in greater quantities appear to be toxic. Here, our data was suggestive that *M. phaseolina* perform better at the availability 0.1-0.2% (v/v) of trace elements, but by increasing to 0.3% (v/v) the enzyme production diminishes significantly (Table 3). The highest enzyme production by the fungus was achieved at pH = 4.0 and remained stable in a pH range of 3-7 and at 40 $^{\circ}$C. Although the optimum temperature for enzyme production and activity was between 35-40 $^{\circ}$C, the enzyme retained most of its activity at 50 $^{\circ}$C for almost an hour. It highlights the enzyme tolerance to higher temperatures, something that often occurs in industry.

Current experiments were not limited to the optimization of enzyme production by the fungus, but the PG was purified and characterized for its biochemical properties. Temperature and pH of the enzyme are generally determining factors that suggest an enzyme is suitable for certain biotechnological applications or particular industry. Fungal polygalacturonases are commonly stable in a wide range of acidic pH (36). The highest enzyme activity followed by purification was at pH = 3 (Fig. 4a) and 30 $^{\circ}$C (Fig. 4b), somewhat different from the production condition (pH = 6.0 and 35 $^{\circ}$C). This is indicative that the activity condition per se may not be the same as the optimum production state.

Thus, it can be said that the determination of how the enzyme behave in its native milieu and in its purified state might be necessary for higher production and then higher enzyme stability and activity. This may lead to greater efficiencies, leading to better outcomes. In contrast to previously reported polygalacturonases (44), isolated PG from *M. phaseolina* was demonstrated to have a relatively very low K_m (0.093) mg/ml) indicating higher affinity towards the substrate and possibly greater potential in industry.

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بهینه سازی تولید، خالص‌سازی و شناسایی پلی گالاکتوروناز از قارچ عامل بیماری ساق سیاه لوبیا

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چکیده

مقدمه: در این مطالعه، تولید یک پلی گالاکتوروناز از قارچ بومی عامل بیماری ساق سیاه لوبیا، فعال در اسیدیته ۳ و مناسب برای استفاده در صنایع آب میوه‌گیری بررسی شد. اشکال مناسب کربن، نیتروژن و فسفر شناسایی و برای تولید بهینه آنزیم به کار گرفته شد.

مواد و روش‌ها: قارچ عامل بیماری ساق سیاه لوبیا در محیط تولید کشت شد. سکروتوم قارچی جدا شد و از آن پلی‌گالاکتوروناز به کمک کروماتوگرافی ستونی به خلوص نسبی رسید. فعالیت بیوشیمیایی آنزیم و سکروتوم هر دو به روش کالریمتری تحلیل شد. تولید پلی‌گالاکتوروناز با تغییر در محتوی محیط کشت، اسیدیته و دما از طریق آزمایشات فاکتوریل ناقص موسوم به تاگوچی بهینه شد. سینتیک آنزیمی و پایداری دمایی و اسیدیته آزمایش شد.

نتایج: گستره ای از قندها به غیر از گلوکز و کیتین تولید را افزایش دادند. سولفات آمونیوم و پپتون نشان دادند که منابع مناسب ازتی هستند. در بین منابع فسفاتی، دی پتاسیم هیدروژن فسفات بیش‌ترین تأثیر را نشان داد. تحلیل به کمک روش آرایه‌های متعامد تاگوچی نشان داد که اسیدیته، دما، آمونیوم سولفات و عناصر میکرو اثر معنی داری در تولید پلی گالاکتوروناز داشتند. به علاوه، مقایسات میانگین گویای این بود که حالت بهینه تولید در اسیدیته ۶، دمای ۳۵ درجه سانتی‌گراد، ۲ گرم در لیتر آمونیوم سولفات و ۲ میلی‌گرم در لیتر عناصر میکرو به دست آمد.

بحث و نتیجه‌گیری: آنزیم خالص شده، وزن مولکولی برابر ۷۰ کیلو دالتون داشت که بیش‌ترین فعالیت خود را در اسیدیته ۳ و دمای ۳۰ درجه سانتی‌گراد نشان می داد. در بین کاتیون های مورد آزمون، آهن دو ظرفیتی فعالیت آنزیم را تا ۲ برابر افزایش داد ولی عصاره قارچی پاسخ متفاوتی نشان داد.

واژه‌های کلیدی: قارچ ساق سیاه لوبیا، پلی گالاکنورونـاز، صنایع آب میوه‌گیری، روش متعامد تاگوچی، خالص‌سازی و شناسایی

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