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Optimization of Solid-state Fermentation for Acidophilic Pectinase Production by *Aspergillus niger* Jl-15 Using Response Surface Methodology and Oligogalacturonate Preparation

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ABSTRACT

Polygalacturonases that hydrolyzed the α-(1,4) glycosidic linkages of pectin is one of widely used industrial enzymes and applied in food, feed, paper and pulp, fruit juice and textile industries. To improve the production of extracellular pectinase (PgA) by a newly isolated *Aspergillus niger* JL-15 strain, the conditions of solid-state fermentation (SSF) were optimized by response surface methodology (RSM). The maximum pectinase activity (525.70 IU g^{-1} dry fermentation product) was obtained at 12.10% orange peel powder, 3.20% ammonium sulfate employing wheat bran as the solid substrate, 51.10% moisture content and 75.00 h fermentation and was 4.10 times as high as that of the basic medium (125.80 IU g^{-1}). SDS-PAGE analysis showed that the molecular mass of PgA was about 40.0 kDa. The PgA was optimally active at 45°C and pH 4.0 and was stable over a broader pH range (4.0-8.0). The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of PgA for citrus pectin was 4.04 mg mL^{-1} and 40.16 μmol min^{-1} mL^{-1}, respectively. The enzyme mediates a decrease in the viscosity of pectin associated with a release of small amounts of reducing sugar. High performance liquid chromatography (HPLC) analysis revealed that PgA liberated a series of oligogalacturonate from pectin with the digalacturonate (G2) and trigalacturonate (G3) as major products. The mode of action study showed that the enzyme was an endo-acting polygalacturonase.

Key words: Pectinase, *Aspergillus niger*, solid-state fermentation, oligogalacturonate, enzymatic properties

INTRODUCTION

Pectin is a complex polysaccharide and one of the major components of plant cell wall and fruit lamella. Pectin represents about 1.5-3.0% wet weight of orange peel which is an important by-product of the can and beverage industry (Voragen *et al.*, 2003). Polygalacturonic acid and rhamnogalacturonan are the two fundamental constituents of pectin. The former can possess up to 200 galacturonic acid residues, while the later is a hetero-polysaccharide having arabinans, galactans and highly branched arabinogalactans attached at the C4 position of many of the rhamnosyl residues. The hydrolysis of the pectin involve several enzymes, such as pectate lyase (EC 4.2.2.2), pectin lyase (EC 4.2.2.10) exo-polygalacturonase (EC 3.2.1.67) and endo-polygalacturonase (EC 3.2.2.15) and the later was the most important one (Nikolic and Mojovic,

2007; Wei *et al.*, 2010). Endo-polygalacturonase have attracted considerable research interest in recent years mainly due to their widely application in food, treatment of wastewater, animal feed, paper and pulp, fruit juice, textile industries (Jayani *et al.*, 2005). For example, there are many researches that reported production of POS by enzymatic hydrolysis pectin from pectin-rich materials (Kashyap *et al.*, 2001; Elboutachfaiti *et al.*, 2008; Yadav *et al.*, 2008).

Filamentous fungi belonging to the genus *Aspergillus*, with the specific reproductive and growth characteristics, are well adapted to a large variety of substrates, being excellent decomposers of vegetal material. *Aspergillus niger* is used throughout the world for production of hemicellulase, cellulase, pectinase and other enzymes (Krengel and Dijkstra, 1996; Martens-Uzunova and Schaap, 2009; Heerd *et al.*, 2012). The solid-state fermentation (SSF) that carried out in absence or near-absence of free liquid water between the medium is a complex heterogeneous three-phase (gas-liquid-solid) and generally defined as the growth of microorganisms, on the surface of a porous and moist solid substrate particle in which enough moisture is present to maintain microbial growth and metabolism (Figueroa-Montero *et al.*, 2011). The solid-state fermentation gained renewed interest in recent years in the production of many enzymes due to lower operation costs and energy requirements and simpler plant and equipment projects compared to submerged fermentation (SmF) (Pandey, 2003; Couto and Sanroman, 2006). In SSF process, byproducts of agro-industry are generally considered as suitable substrates for growth of fungi and the production of enzymes (Bayoumi *et al.*, 2008). There has been an increasing trend towards efficient utilization and value-addition of agriculture-industrial residues. In 2009, the total orange production was about 18.5 million tonnes and the orange peel and the orange pomace residue was over 5.2 million tonnes in China. The optimal design of the culture medium and is a very important aspect in the development of SSF processes. Response Surface Methodology (RSM) is a very useful tool for the optimal selection of nutrient, which can provide statistical models aid in understanding the interactions among the process parameters at different levels and calculating the best level of each factor for the given target (Bas and Boyaci, 2007).

The purpose of the present study was to optimize pectinase production by *Aspergillus niger* JL-15, a new enzyme producer, in SSF employing wheat bran as substrate and orange peel powder as an inducer. The pectinase was partially purified and characterized. The hydrolytic products released from citrus pectin and oligogalacturonides by the pectinase were determined and quantified specifically.

MATERIALS AND METHODS

Materials: Citrus pectin, D-(+)-galacturonic acid, the standard oligogalacturonides and Bovine Serum Albumin (BSA) were from Sigma Chemical Company in 2008. The protein molecular weight marker was obtained from Transgen Biotech Company, Beijing in 2008. Sephadex G-25 and SephacrylTM S-100 HR were from Amersham Biosciences (2008). Pellicon-2 Mini Holder and hollow fibre ultra-filtration membrane modules were from Millipore in 2007. All other chemicals used in the present study were of analytical grade.

Microorganism and solid-state fermentation: The enzyme producer, *Aspergillus niger* JL-15 strain, was isolated from Hangzhou Botanical garden soil. For pectinase production *Aspergillus niger* JL-15 was cultivated in solid-state fermentation (SSF). The basic medium contained wheat bran (9.80 g), $MgSO_4$ (0.05 g), KH_2PO_4 (0.15 g) and H_2O (12.00 g) and then sterilized at 121°C for 20 min. *Aspergillus niger* JL-15 was cultivated in a 250 mL flask containing 25.00 g medium at 30°C for 72 h.

Optimization of PgA production via response surface methodology (RSM): Optimization of SSF for PgA production focused on the concentration of orange peel powder (inducer) and ammonium sulfate (added nitrogen source) employing the wheat bran of basic medium, moisture content, fermentation time and their interactions between each other. According to central composite design (CCD, 4-variable, 5-level), total 31 experiments were employed to fit the polynomial model:

$$Y = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{11}X_1^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{22}X_2^2+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{33}X_3^2+\beta_{34}X_3X_4+\beta_{44}X_4^2$$

where, Y is the dependent or response variable, β is the regression coefficient and X is the coded level of the independent variables. Corresponding coefficients of both variables and interaction variables were estimated by SAS 9.0 (SAS Institute Inc., Beijing, China) while their response surface graphs were drawn by MATLAB 6.5 (Math Works, USA) (Dai *et al*., 2011; Bai *et al*., 2011).

Statistical analysis on the significance of coefficient estimations was performed via Student's t-test and Fisher F-test. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface plots.

Partially purification and SDS-PAGE analysis of PgA: Three hundred grams fermentation products were suspended in 3.0 L of McIlvaine's buffer (0.2 M sodium phosphate, 0.1 M citric acid, pH 6.0) with constant stirring. The 3.0 L crude enzyme was concentrated to 500 mL using ultra-filtration module with a polyethersulfone membrane (Millipore Biomax, 100 and 10 kDa cut-off) and the trans-membrane pressure of 0.8 kg cm^{-2}. The 500 mL concentrated sample was treated with ammonium sulfate to 65% saturation to precipitate enzyme. The saturated solution was left overnight at 4°C, centrifuged and precipitate was resuspended in 25 mL of McIlvaine's buffer (pH 6.0). The fraction was loaded onto Sephadex G-25 column (Pharmacia, 20.0×2.4 cm) and eluted with McIlvaine's buffer (pH 6.0). The fractions containing polygalacturonase activity were pooled and concentrated. The concentrated enzyme solution was then applied to SephacrylTM S-100 HR column (Pharmacia, 60.0×1.6 cm) and eluted with the same buffer at a flow rate of 1.0 mL/min. The active fractions were pooled for subsequent assays.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the partially purified sample was carried out by using Bio-Rad electrophoresis system. In the Laemmli system (Laemmli, 1970), the stacking and separating gels consisted of 5 and 12% polyacrylamide, respectively. Proteins were visualized with Coomassie brilliant blue R-250 straining. Molecular weight of test protein was compared with standard protein marker.

Enzyme assays: The pectinase activity was assayed with 0.5% citrus pectin (w/v) as substrate and the liberation of reducing sugars was estimated by the dinitrosalicylic acid (DNS) procedure (Miller *et al*., 1959) using the D-(+)-galacturonic acid as standard. Protein concentration was measured by the dye-binding method of Bradford and Bovine Serum Albumin (BSA) was used as the standard (Bradford, 1976). The kinetic parameters for pectinase were calculated from initial velocities using concentrations ranging from 1 to 10 mg mL^{-1} citrus pectin.

One unit of pectinase activity was defined as the amount of the enzyme that catalyzed the formation of 1.0 μmol of reducing sugar from pectin in 1 min under its optimal conditions (at 45°C, pH 4.0). For each assay in this study, triplication measurements were conducted to obtain a mean value of activity.

Effect of temperature on the activity and thermal stability of PgA: Effect of temperature on pectinase activity was measured from 30 to 80°C at pH 4.5 (McIlvaine's buffer). Thermal stability of xylanase was determined by assaying residual activity after incubation from 30 to 80°C at pH 4.5 for 4 min, respectively.

Effect of pH on the activity and stability of PgA: Effect of pH on pectinase activity was measured over a range of pH 2.2 to 7.0 (McIlvaine's buffer) and 8.0 to 9.0 (Glycine-NaOH buffer) at 45°C. To determine the pH stability, PgA was incubated in various pH buffers at 25°C for 1 h and the residual activities were measured at 45°C and pH 4.5.

Changes in viscosity and reducing sugars concentration during hydrolysis: The relationship between reducing sugars and viscosity of citrus pectin solution in the presence of the PgA was determined as follows: a 250 mL reaction mixture containing 3.0% pectin and purified enzyme (400.0 U) was incubated at 30°C (Liu *et al.*, 2010). The viscosity and the amount of reducing sugar released were estimated at different time intervals.

Oligogalacturonate released by PgA from pectin: The 1.0% (w/v) citrus pectin in McIlvaine's buffer (pH 4.5) was hydrolyzed by the partially purified PgA at 45°C for 2 h with constant shaking (100 rpm). The hydrolysis products were analyzed by HPLC with Sugar-pak™ 1 column (6.5 mm diameter and 300 mm length, Waters), pure water as mobile phase (0.5 mL/min) and injection volumes of 10 µL. The column was maintained at 80°C. Sugar peaks were screened using Waters 2410 refractive index detector (Sun *et al.*, 2007). The hydrolysates were quantified based on their own standard curves.

The mode of action of PgA on oligogalacturonate: The mode of action of PgA was determined using digalacturonate (G2) and trigalacturonate (G3) as substrates. The standard oligosaccharides solutions (water, pH 7.0) were incubated with purified PgA at 30°C. The samples at different intervals were determined and quantified by HPLC. The injection volume was 10 µL.

RESULTS AND DISCUSSION

Optimization of PgA production by RSM: The concentration of added orange peel powder and ammonium sulfate to basic medium, moisture content and fermentation time had a significant effect on the PgA production ($p<0.05$). The Fisher F-test with a very low probability value ($p<0.0001$) showed the high statistical significance of the regression model. The goodness of fit of the model was checked by the determination coefficient ($R^2 = 0.929$), which indicates that the following second order polynomial model could explain 92.9% of the total variation (Table 1, 2). The model can be shown as follows:

$$Y = 529.731+9.711X_1+11.221X_2+8.479X_3+13.539X_4+0.390X_1\times X_2+14.155X_1\times X_3+18.628X_1\times X_4-13.087X_2\times X_3-17.733X_2\times X_4-26.073X_3\times X_4-33.254X_1^2-51.925X_2^2-33.744X_3^2-55.763X_4^2$$

where, Y is the pectinase yield and X_i is the coded independent variables (X_1 and X_2, concentration of added orange peel powder and ammonium sulfate, respectively; X_3, moisture content; X_4, fermentation time). Statistical optimization method for fermentation process could overcome the limitation of classic empirical methods and was proved to be a powerful tool for the optimization of

Table 1: The variables in central composite design (CCD) and the PgA production

Run	X_1 (%)	X_2 (%)	X_3 (%)	X_4 (h)	Pectinase yield (IU/g dry product)
1	0 (10)	0 (3.0)	0 (50)	0 (72)	531.20
2	1 (15)	1 (4.0)	1 (55)	-1 (60)	505.53
3	-1 (5)	-1 (2.0)	-1 (45)	1 (84)	485.10
4	0	0	0	0	520.10
5	0	-2 (1.0)	0	0	468.13
6	-1	-1	1	1	479.84
7	2 (20)	0	0	0	500.62
8	0	0	0	0	528.00
9	0	0	0	0	530.80
10	1	-1	1	-1	492.72
11	-2 (0)	0	0	0	485.56
12	1	-1	-1	1	493.93
13	0	0	0	-2 (48)	444.46
14	1	1	1	1	502.18
15	0	0	0	2 (96)	496.70
16	-1	1	-1	-1	498.89
17	1	1	-1	1	512.63
18	-1	-1	-1	-1	459.65
19	0	2 (5.0)	0	0	480.71
20	1	-1	1	1	496.99
21	1	-1	-1	-1	461.21
22	-1	1	1	1	471.08
23	1	1	-1	-1	480.02
24	0	0	0	0	531.10
25	-1	1	-1	1	489.26
26	-1	-1	1	-1	477.83
27	0	0	2 (60)	0	507.38
28	0	0	-2 (40)	0	477.83
29	-1	1	1	-1	497.16
30	0	0	0	0	529.80
31	0	0	0	0	528.10

Coded levels (+2, +1, 0, -1, -2) and actual values (in parentheses) of the independent variables in CCD. X_1 and X_2, the concentration of orange peel powder and ammonium sulfate added to basic medium, respectively, X_3: moisture content; X_4: fermentation time

the production of enzyme. The application of RSM yielded the regression equation showing positive linear and negative quadratic effect and expressing a relationship between the PgA production and the independent variables.

The relation between factors and response can be understood by examining three-dimensional response surface and contour plots as a function of two factors at a time and holding all other factors at fixed levels. It was evident from the plots that the addition of higher concentration of orange peel powder to wheat bran, the middle levels of the ammonium sulfate and moisture content and the longer fermentation time is responsible for the enhancement of PgA production (Fig. 1). Based on the analysis of regression equation and plots, optimum of the four variables were found to be, the orange peel powder 12.10%, ammonium sulfate 3.20%, moisture content 51.10% and fermentation time 75.00 h, where the production of 531.20 IU g^{-1} (dry fermentation products). The predicted yield was verified by performing an experiment with the optimized variables in basic

Table 2: Analysis of variance and coefficient estimates of central composite design

Source of variation	Degree of freedom	Sum of squares	Mean square	F-value	p-value
Regression	14	15744.0	1124.57	16.46	0.000**
Linear	4	2852.5	713.12	10.44	0.000**
Quadratic	4	11178.4	2794.61	40.90	0.000**
Interaction	6	1713.0	285.51	4.18	0.010**
Lack of fit	10	1072.1	107.21		0.000**
Pure error	6	21.2	3.53		
Total	16	1093.3	68.33		
Factors	Degree of freedom	Coefficient estimate	Standard error	t-value	p-value
Intercept	1	529.731	3.124	169.551	0.0000**
X_1	1	9.711	3.375	2.878	0.011*
X_2	1	11.221	3.375	3.325	0.004**
X_3	1	8.479	3.375	2.513	0.023*
X_4	1	13.539	3.375	4.012	0.01**
$X_1 \cdot X_2$	1	0.390	8.266	0.047	0.963
$X_1 \cdot X_3$	1	14.155	8.266	1.712	0.106
$X_1 \cdot X_4$	1	18.628	8.266	2.253	0.039*
$X_2 \cdot X_3$	1	-13.087	8.266	-1.583	0.133
$X_2 \cdot X_4$	1	-17.733	8.266	-2.145	0.048*
$X_3 \cdot X_4$	1	-26.073	8.266	-3.154	0.006**
X_1^2	1	-33.254	6.183	-5.378	0.000**
X_2^2	1	-51.925	6.183	-8.398	0.000**
X_3^2	1	-33.744	6.183	-5.457	0.000**
X_4^2	1	-55.763	6.183	-9.018	0.000**

*p<0.05, **p<0.005

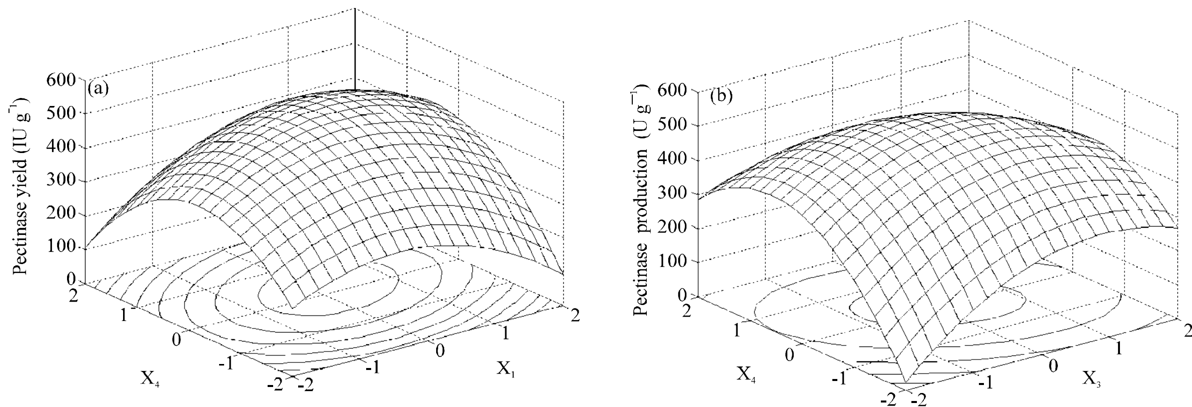

Fig. 1(a-b): Response surface/contour plot showing effect of independent variables (a) Orange peel powder (X_1) and fermentation time (X_4) and (b) Initial moisture content (X_3) and fermentation time (X_4) on PgA production with the other two variables at 0 level

medium and the recovery of pectinase was 525.70 IU g^{-1} (dry fermentation products), which was close to the predicted one and was 4.10 times as high as that of the basic medium (125.80 IU g^{-1}).

The RSM method was used for some similar topic. The *Aspergillus niger* Aa-20 was cultured in solid-state bioreactor using lemon peel pomace as support and carbon source. The maximum pectinase activity obtained at 96 h was 2181 IU L^{-1} maximum biomass (Ruiz *et al*., 2012). The

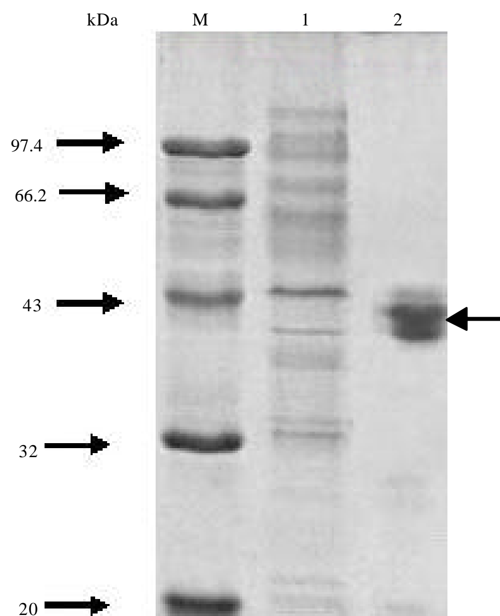

Fig. 2: SDS-PAGE analysis of PgA, Note: Lanes M: standard protein marker; Lane 1: crude enzyme; Lane 2: partially purified PgA after ultrafiltration and chromatography on Sephacryl S-100 HR

optimum operational conditions for maximum pectinase yield by *Bacillus subtilis* RCK in SSF were moistened wheat bran (1:7 solid substrate-to-moisture ratio), 1.5% (v/w) of 24 h old strain, 48 h fermentation and the predicted pectinase value was 1610.6 IU g^{-1} dry substrate (Gupta *et al.*, 2008). Effects of cultivation time, pH and substrate concentration on production of xylanase by *A. niger* AN-3 were studied. The optimal fermentation parameters for enhanced xylanase production were cultivation time (53.3 h), pH (7.92) and wheat bran concentration (54.2 g L^{-1}). Under these conditions the xylanase production was 127.12 IU mL^{-1} (Cao *et al.*, 2008).

Purification and kinetic parameters of PgA: The ultrafiltration, ammonium sulfate precipitation and gel filtration resulted in a 9.10-fold increase in specific activity of pectinase (from 27.60 to 251.20 U mg^{-1}) with 25.20% recovery. SDS-PAGE revealed that the molecular mass of PgA was about 40.0 kDa (Fig. 2). Kinetic parameters that reflect the effect of substrate concentration on the reaction velocity were depicted. The rate dependence of the enzyme reaction on citrus pectin concentration followed the Michaelis-Menten kinetics. The values for K_m an V_{max} were of 4.04 mg mL^{-1} and 40.16 μmol min^{-1} mL^{-1}, respectively, which were lower than those of pectinase from *Aspergillus oryzae* JL-14 (Liu *et al.*, 2010) and higher than those of pectinase from *Bacillus subtilis* JL-13 (Bai *et al.*, 2011). These values are consistent with the reported range of kinetic values for microbial pectinase (Jayani *et al.*, 2005).

Effect of temperature on the activity and stability of PgA: The pectinase activity increased with the rise of temperature, reached the maximum at 45°C and then decreased with the rise of temperature. Over 40°C, the PgA was much less stable (Fig. 3a). The T_m of PgA was 51.9°C, which was lower than many reported pectinase (Celestino *et al.*, 2006).

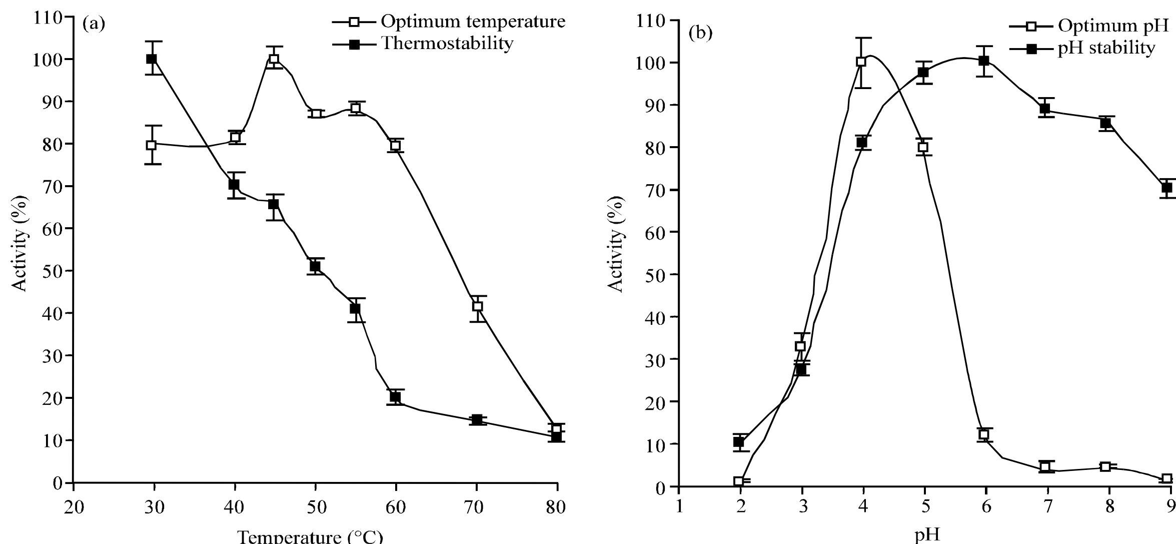

Fig. 3(a-b): Relative and residual activities of optimum and stable (a) Temperature and (b) pH of PgA, respectively

Effect of pH on the activity and stability of PgA: The PgA showed high activity in a pH range of 4.0-5.0, with the optimal pH at 4.0 (Fig. 3b), which suggested it was a acidophilic pectinase. PgA was very stable from pH 4.0 to 8.0. Over 80% of xylanase activity was retained after treatment of the enzyme by preincubation over a pH range of 4.0-8.0 for 1 h at 25°C.

Decrease in viscosity and the release of reducing sugars from citrus pectin: The PgA rapidly decreased the viscosity of pectin solution by 30.2 and 80%, at 5 min and 45 min, respectively, but released a small amount of reducing sugars (Fig. 4), which was similar to the endo-polygalacturonase from the psychrophilic fungus *Mucor flavus* (Gadre *et al.*, 2003). The pectinase decreased the viscosity of pectin associating with a release of only small amounts of reducing sugar.

Oligogalacturonate released by PgA from pectin: Pectin represents about 1.5-3.0% wet matter of orange peel, which is an important by-product of the can and beverage industry (Voragen *et al.*, 2003). The major hydrolysis products released by PgA from citrus pectin were digalacturonate and trigalacturonate (Fig. 5), which was similar to those pectinase from *Mucor flavus* and *Mucor rouxii* NRRL 1894, respectively (Gadre *et al.*, 2003; Saad *et al.*, 2007). After 2 h reaction, the concentration of digalacturonate and trigalacturonate in hydrolysis products from citrus pectin by PgA were 0.375 and 0.575 mg mL^{-1}, respectively.

Oligogalacturonate that used as a functional food in many countries can suppress the activity of entero putrefactive bacteria, shown to inhibit toxicity of Shiga-like toxins from *Escherichia coli* O157:H7 and can selectively be used by the beneficial gastrointestinal microflora, *Bifidobacterium* species. (Tomomatsu, 1994; Sako *et al.*, 1999; Olano-Martin *et al.*, 2003). Reported beneficial effects of *Bifidobacterium* spp. on human health include: preventing the proliferation of pathogenic

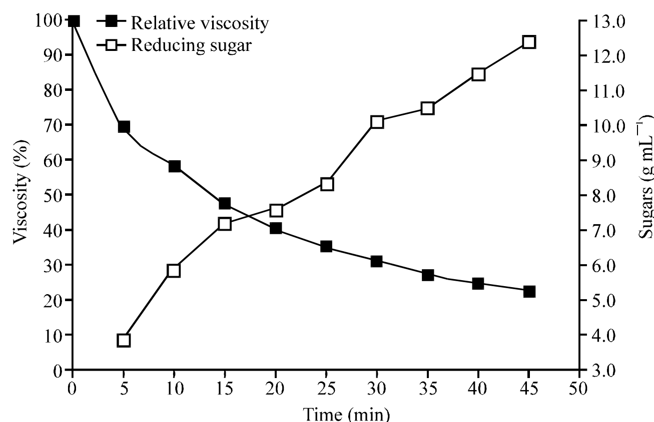

Fig. 4: The changes in viscosity and reducing sugars from pectin solution, The viscosity of reaction mixture was measured at 5 min interval by DV-II prime viscometer (Brookfield, spindle: sp-2, speed: 100 rpm), 1.0 mL reaction mixture withdrawn and the reducing sugars released were quantified using DNS method

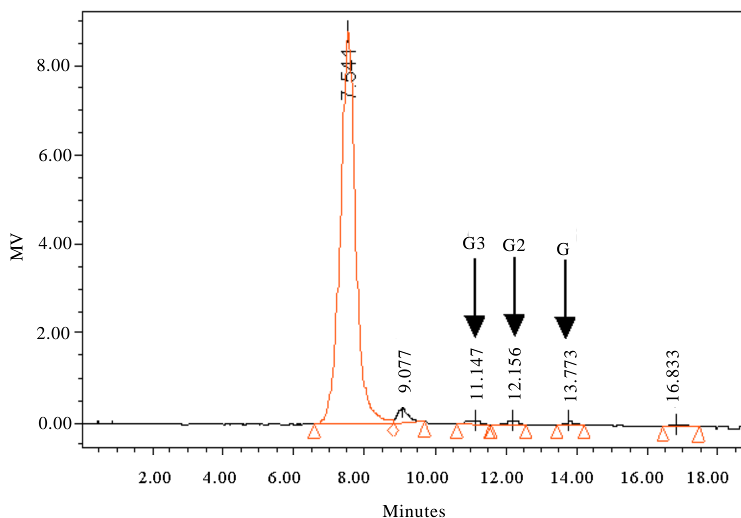

Fig. 5: HPLC profiles of pectin degradation, HPLC analysis of hydrolysis product from citrus pectin released by the PgA for 2 h, The positions of galacturonic acid (G), digalacturonate (G2), trigalacturonate (G3) are shown

intestinal bacteria and facilitating the digestion and absorption of nutrients (Van Loo *et al.*, 1999). Oligogalacturonate can increase the *Eubacterium rectale* population and butyrate levels, which is of potential benefit to the host (Mussatto and Mancilha, 2007).

The mode of action of PgA on oligogalacturonate: The mode of action of PgA was determined using digalacturonate and trigalacturonate as substrate, which could be further hydrolyzed by the pectinase. The PgA showed very low activity on digalacturonate and trigalacturonate (Fig. 6a, b). The presence of trace amounts of galacturonic acid from

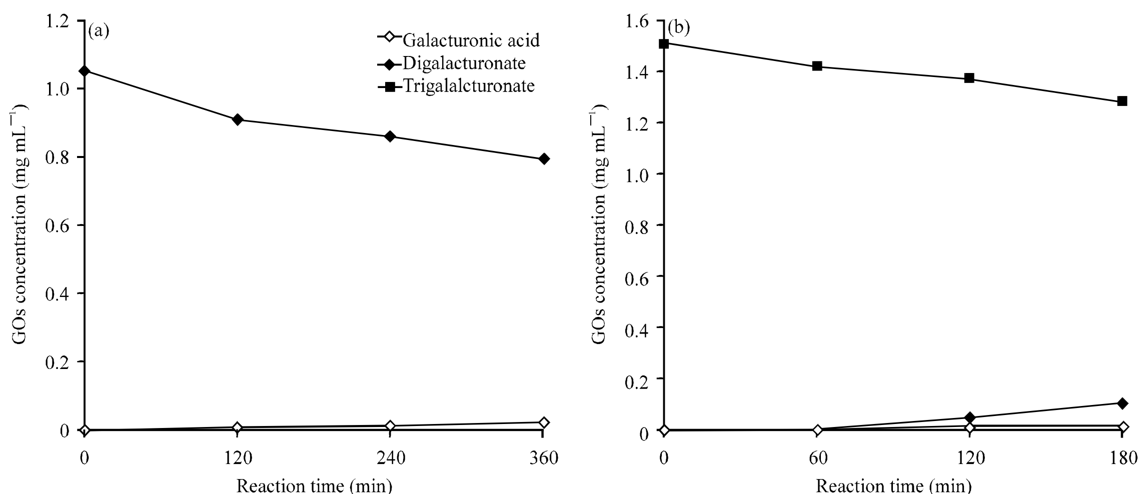

Fig. 6(a-b): Hydrolysis products of PgA incubated with (a) 1.05 mg mL^{-1} digalacturonate and (b) 1.5 mg mL^{-1} trigalacturonate at 40°C for different time, At regular time intervals, aliquots of the reactions were analyzed by HPLC for examining hydrolysis products

digalacturonate and trigalacturonate by PgA revealed that enzyme preferentially cleaved the internal glycosidic bonds of oligogalacturonate and it was an endo-acting pectinase.

CONCLUSION

In the study, the optimum parameters of SSF for pectinase production by *A. niger* JL-15 were obtained using RSM method. The verifiable pectinase yields were 525.70 IU g^{-1} dry fermentation products, which was 3.10 times higher than that of the basic medium (125.80 IU g^{-1}). Digalacturonate and trigalacturonate were main hydrolysis products released from citrus pectin by PgA. These results indicate the present method can be successfully used to improve the pectinase production by *A. niger* Jl-15 and a potentiality of PgA for the production of oligogalacturonate.

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