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Optimization, Purification and Characterization of Polygalacturonase from Mango Peel Waste Produced by *Aspergillus foetidus*

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Summary

Pectin-rich mango peel from industrial waste was used as a substrate for the production and characterization of novel polygalactauronase enzyme. Its production was optimized with five important factors; KH_2PO_4, pH, peptone, $MgSO_4$ and urea by employing response surface methodology using submerged fermentation with *Aspergillus foetidus*. Total of 50 experimental runs were carried out and the predicted values for optimization were in good agreement with experimental data. The results showed that a satisfactory production of polygalacturonase from the mango peel could be achieved, reaching up to 36.5 U/mL under optimized medium conditions of pH=5.8 and (in %, by mass per volume): KH_2PO_4 0.22, peptone 0.5, $MgSO_4$ 0.02 and urea 0.2 %. The polygalacturonase was partially purified to 3.4-fold and the molecular mass was found to be 34 kDa. The optimum pH and temperature for polygalacturonase activity were 5 and 55 °C, respectively.

Key words: mango peel, *Aspergillus foetidus*, submerged fermentation, polygalacturonase, response surface methodology

Introduction

Mango peel is one of the major by-products from the mango pulp processing industries. During the processing of mango fruit, peel and stone are generated as waste (40–50 % of total fruit mass). Mango processing waste constitutes 20–25 % peel, which was found to be a good source for the extraction of pectin of good quality, with a high degree of esterification and phenolic compounds (*1*). Pectin acts as the inducer for the production of pectinase enzymes by microbial systems (*2*), and pectin-rich mango peel is considered to be a good source for pectinase production and ideal substrate for the decomposition of mango peel by microorganisms (*3*).

Pectinases are produced mainly by plants and microorganisms (*4*). Several genera of fungi like *Aspergillus*, *Penicillium*, *Sclerotium*, *Fusarium* and *Rhizopus* can produce pectinases (*5*). One of the barriers against phytopathogenic fungi is the polysaccharide-rich cell wall of plants. The vast majority of fungi need to breach this barrier to gain access to the plant tissue, and for this purpose secrete a number of enzymes capable of degrading the wall polymers. When fungi are grown on plant cell wall material *in vitro*, pectinases are invariably

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the first enzymes to be secreted, followed by hemicellulases and cellulases. Pectinases degrade the pectin substances of the plant cell wall. Acidic pectinases are widely used in the maceration, solubilization and clarification of fruit juices (*4*), while alkaline pectinases have potential applications in retting and degumming of fibre crops, textile processing, coffee and tea fermentations, paper and pulp industry, and oil extraction (*6*). Pectinases have been developed for numerous applications in fruit juice and winemaking industry; the most well-known are mash treatment of fruits like apples and pears for higher yield of juice, mash treatment of grapes for higher colour release, clarification and depectinization of juice and wine (*7*). Pectinase treatment enhances the juice extraction and decreases the viscosity of juice, which could make the wine production from mango more economical (*3*).

Polygalacturonase (PG) plays an important role in converting protopectin present in fruit cell walls into a soluble form during fruit ripening. It plays a key role in softening of fruits during ripening, by depolymerization of the middle lamella present in the fruit cell wall (*8*). Most of the PGs of fungal origin, either alone or in a complex with other pectinases have several commercial applications in the food and beverage industries (*9*). The synthesis of pectolytic enzymes by microorganisms has been reported to be highly influenced by the components of the growth medium (*10*). Most extracellularly induced enzymes are known to be synthesized in higher quantities when inducers are present in the cultivation medium. Pectolytic enzymes have been reported to be induced by several substances. In many cases, pectin, and in some cases complex media such as beet sugar, wheat bran, groundnut meal and citrus peel were used for pectinase production (*11,12*).

The production and characterization of an enzyme are necessary for its industrial application. The first step in achieving this goal is the establishment of a suitable enzyme production technology. The conventional method for optimization involves changing one independent variable at a time while fixing all the others at a certain level. This method is very time-consuming and requires a large number of experiments to determine the optimum levels (*13*). Cultivation involves many factors, such as temperature, pH, aeration and agitation, which are important and affect the growth and productivity. It is difficult to find the most important factors and to optimize the conditions. Response surface methodology (RSM) is an experimental strategy for seeking the optimum conditions for a multivariable system (*14*). Tari *et al.* (*15*) applied the response surface design techniques in fermentation process development for improving the production of pectinase enzyme from *Aspergillus sojae* ATCC 20235. As a result of this optimization, maximum pectinase activity was achieved. A 1.5-fold increase in pectinase secretion by *Kluyveromyces wickerhamii* was attained when pH, temperature and inoculation period were optimized by RSM (*16*). A 41-fold enhancement in alkaline pectinase production by *Bacillus pumilus* was achieved by using Plackett-Burman design and RSM (*17*).

The present study was undertaken to utilize mango peel from the fruit processing industries as suitable substrate for the production of PG with the optimization of submerged fermentation conditions using RSM by *Aspergillus foetidus*. Furthermore, the separation and purification of PG were carried out with great efficiency and purified PG was characterized.

Materials and Methods

Mango peel pectin

Mango peel (totapuri) was obtained locally from fruit processing industries located around Tirupati (India). The mango peel was individually dried in a hot air oven at 45 °C for 24 h and milled to a particle size of 40 BS (British standard) mesh in a grinding mill (Microteknik, Ambala, Haryana, India). Pectin was extracted by the method of Rao and Maini (*18*). Dried and ground mango peel of 15 g was weighed and 50 mL of 0.05 M HCl were added. Pectin extraction was done by boiling the above mixture at 100 °C for 1 h and filtering after it had cooled. Two volumes of absolute alcohol were added to precipitate the pectin. The pectin content was determined by carbazole method (*19*).

Inoculum preparation

Aspergillus foetidus NCIM 514 was procured from the National Chemical Laboratory (NCL), Pune, India. The culture was maintained on PDA agar slants at 4 °C. The spores were harvested from the 96-hour-old culture in 0.01 % Tween 80 solution.

Production media and fermentation conditions

Fermentation was carried out in a medium containing 2.73 g of mango peel pectin, media components (KH_2PO_4, peptone, $MgSO_4$ and urea) and distilled water up to 100 mL in Erlenmeyer flask (100 mL). The pH was adjusted in each run with diluted NaOH. The flasks were sterilized at 121 °C for 15 min and inoculated with $2 \cdot 10^7$ spores/mL. The cultured flasks were incubated at 30 °C for 5 days under shaking conditions (150 rpm) on a rotary shaker. All the runs were carried out according to the central composite design. After fermentation, the contents of each flask were filtered under vacuum using 0.45 μm membrane filter (Sartorius, Göttingen, Germany). The culture filtrate was used as the enzyme source and stored at 4 °C for further assays.

Experimental design

The experimental design and statistical analysis were made using Design Expert v. 7.1.6 (Stat-Ease Inc, Minneapolis, MN, USA) software. Central composite experimental design (CCD) (*20*) with quadratic model was employed to study the combined effect of five independent variables, namely KH_2PO_4 (X_1 %, by mass per volume), pH (X_2), peptone (X_3 %, by mass per volume), $MgSO_4$ (X_4 %, by mass per volume) and urea (X_5 %, by mass per volume). The dependent variable (Y) measured was PG (U/mL) from mango peel. This dependent variable was expressed individually as a function of the independent variables known as response function. In CCD, the range and the levels of the variables investigated in this study are given in the Table 1. A 2^3-factorial CCD with six ax-

Table 1. Coded and actual values of the factors in central composite design

Factor	Parameter	Low actual	High actual	Low coded	High coded	Mean value
X_1	KH_2PO_4/% (by mass per volume)	0.20	1.0	–1	1	0.619
X_2	pH	3.50	8.0	–1	1	5.750
X_3	Peptone/% (by mass per volume)	0.50	2.0	–1	1	1.271
X_4	$MgSO_4$/% (by mass per volume)	0.02	0.3	–1	1	0.167
X_5	Urea/% (by mass per volume)	0.05	0.2	–1	1	0.127
Response	Parameter	Obs.	Min.	Max.	Mean	S.D.
Y	PG activity/(U/mL)	50	5.1	33.5	16.68	7.68

Obs.=observed run values, S.D.=standard deviation, PG=polygalacturonase

ial points ($a=\sqrt{3}$) and six replications at the centre points (N_0=6) leading to a total number of fifty experiments was employed for the optimization of the fermentation conditions (Table 2). The second degree polynomials were calculated with the statistical package (Stat-Ease Inc) to estimate the response of the dependent variable. The variance for each assessed factor was partitioned into linear, quadratic and interactive components and was represented using the second order polynomial function as follows:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_5X_5+b_{11}X_1^2+b_{22}X_2^2+ \\ +b_{33}X_3^2+b_{44}X_4^2+b_{55}X_5^2+b_{12}X_1X_2+b_{13}X_1X_3+b_{14}X_1X_4+ \\ +b_{15}X_1X_5+b_{23}X_2X_3+b_{24}X_2X_4+b_{25}X_2X_5+b_{34}X_3X_4+ \\ +b_{35}X_3X_5+b_{45}X_4X_5 \quad /1/$$

where Y is the predicted response, X_1, X_2, X_3, X_4 and X_5 are independent variables, b_0 is the offset term, b_1, b_2, b_3, b_4 and b_5 are linear effects, b_{11}, b_{22}, b_{33}, b_{44} and b_{55} are squared effects and b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24}, b_{25}, b_{34}, b_{35} and b_{45} are interaction terms. The significance of all terms in the polynomial functions were assessed statistically using F-value at a probability (p) of 0.001, 0.01 or 0.05. The regression coefficients were then used to generate contour maps by keeping one variable constant at the centre point and varying the other variables within the experimental range.

Table 2. Analysis of variance of the experimental results of the central composite design

Source	Sum of squares	df	Mean square	F-value	p-value
Model	2690.25	20	134.53	19.39	0.0001[c]
X_1	0.34	1	0.34	0.04	0.8271
X_2	30.07	1	30.07	4.33	0.0463[a]
X_3	72.81	1	72.81	10.49	0.0030[a]
X_4	6.96	1	6.96	1.00	0.3249
X_5	139.32	1	139.32	20.08	0.0001[a]
X_1X_2	24.66	1	24.66	3.55	0.0695
X_1X_3	98.32	1	98.32	14.17	0.0008[b]
X_1X_4	29.70	1	29.70	4.28	0.0476[a]
X_1X_5	12.59	1	12.59	1.81	0.1885
X_2X_3	549.54	1	549.54	79.19	0.0001[c]
X_2X_4	189.20	1	189.20	22.26	0.0001[c]
X_2X_5	15.75	1	15.75	2.27	0.1428
X_3X_4	71.37	1	71.37	10.28	0.0033[a]
X_3X_5	663.30	1	663.30	95.58	0.0001[c]
X_4X_5	295.18	1	295.18	42.53	0.0001[c]
X_1^2	30.69	1	30.69	4.42	0.0443
X_2^2	130.19	1	130.19	18.76	0.0002[b]
X_3^2	151.55	1	151.55	21.84	0.0001[c]
X_4^2	26.56	1	26.56	3.83	0.0601
X_5^2	65.33	1	65.33	9.41	0.0046[a]
Residual	201.25	29	6.94		
Lack-of-fit	124.63	20	26.23	13.43	0.0130[a]
Pure error	76.62	9	8.51		
Corr. total	2891.92	49			

[a]$p<0.05$=significant at 5 % level, [b]$p<0.001$=significant at 1 % level, [c]$p<0.0001$=significant at 0.1 % level

The five factors which influence the fermentation strongly, namely KH_2PO_4, pH, peptone, $MgSO_4$ and urea, are considered for the optimization of PG production. Using CCD, a total number of 50 experiments with different combinations of KH_2PO_4, pH, peptone, $MgSO_4$ and urea were performed in triplicate. The encoded values of the initial parameters, as well as the mean values of the triplicate results obtained in the 50 assays performed to determine PG production from the mango peel were recorded.

Validation of the optimized conditions in a bioreactor

Polygalacturonase production was validated in a 3-litre bioreactor (SCIGENICS Pvt. Ltd., Chennai, India) with a working volume of 2 L. The medium was inoculated with $2 \cdot 10^7$ spores/mL and fermentation was carried out at 30 °C, 150 rpm for 72 h with controlled pH=5.8. Dissolved oxygen (DO) and air flow rate were maintained above 20 % saturation and 1.0 vvm, respectively. Samples were analyzed after every 12 h.

Estimation of polygalacturonase activity

The activity was determined by measuring the release of reducing groups from the mango peel substrate. The reaction mixture containing 0.3 mL of crude enzyme sample was added to 1 mL of 1 % pectin substrate and 0.7 mL of 0.1 M acetate buffer (pH=4.5). The samples were incubated at 40 °C for 30 min. The reducing sugars were determined using dinitrosalicylic (DNS) acid method with

galacturonic acid as standard (*21*). One unit of PG activity (U) was defined as the amount of enzyme that liberates 1 μmol of galacturonic acid per min per g of substrate.

Enzyme purification

The cultured medium at the end of fermentation was filtered under vacuum using 0.45-μm membrane filter (Sartorius). The culture filtrate (220 mL) was brought to 80 % saturation with ammonium sulphate and allowed to stand overnight at 4 °C. The precipitated protein was removed by centrifugation at 9503×*g* for 30 min at 4 °C. It was dissolved in 15 mL of sodium acetate buffer (100 mM, pH=5). The protein content of the fraction was determined by the method of Lowry *et al*. (*22*). The precipitated protein was dialysed against 100 mM of sodium acetate buffer, pH=5, overnight. The dialysate was concentrated to 2 mL by polyethylene glycol (PEG).

The vertical glass tube column (1.8 cm×30 cm; Sephadex G-100, Pharmacia, Stockholm, Sweden) was prepared by suspending 5 g of Sephadex G-100 in 100 mM sodium acetate buffer (pH=5) and the column was equilibrated with the same buffer overnight. Concentrated protein (1 mL) was applied to gel filtration column and eluted in 100 mM of sodium acetate buffer, pH=5, at a flow rate of 12 mL/h. Protein absorption was monitored in the eluted fractions at 280 nm, and enzyme activity was assayed.

SDS-polyacrylamide gel electrophoresis

The purified protein sample was analyzed using electrophoresis (SDS-PAGE) in 12 % separation gels to check the homogeneity of the enzyme and determine the molecular mass (*23*). Molecular mass markers (Fermentas, Thermo Scientific, Ottawa, ON, Canada) of 14–116 kDa were used. Protein bands were visualized by silver staining method described by Gromova and Celis (*24*). The identification of the bands with PG activity was carried out on Novex®, NativePAGE™ Bis Tris Gel system (Life Technologies, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) containing 0.1 % pectin (*25*). The gels were washed for 2 h in 10 mM Tris buffer, pH=7.0, then incubated in phosphate buffer, pH=5, for 60 min at 30 °C. The pectinase production was revealed as clear zones after staining with 0.1 % Ruthenium Red.

Characterization of polygalacturonase

The effect of substrate (pectin) concentration on the enzyme activity (PG) was determined by using different concentrations of pectin (1–12 mg/mL) in the reaction mixture and the enzyme activity was estimated. Kinetic parameters were measured with GraphPad Prism v. 4.00 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). For K_m determination, the data were plotted to fit the Michaelis-Menten equation by non-linear regression.

Inactivation temperature was determined by incubating the enzyme for 30 min at a temperature from 30–80 °C in assay buffer and then measuring the remaining activity by standard procedure. The optimal pH for PG activity was evaluated by varying the pH of the reaction mixture between 3.0 and 9.0 at increments of 1.0. Activity was then assessed under standard conditions.

Results and Discussion

Production and optimization of PG from mango peel using RSM

Optimization of experiments was designed to provide in-depth information about the variables identified during screening as having the greatest impact on the performance. Finally, verification of 50 experiments was used to validate the results under specific experimental conditions (*14*). The influence of KH_2PO_4, pH, peptone, $MgSO_4$ and urea on PG production was investigated using RSM and the results are summarized in Table 1. The calculated regression equation for the optimization of fermentation conditions showed that the PG production (U/mL) is a function of KH_2PO_4 (X_1 %, by mass per volume), pH (X_2), peptone (X_3 %, by mass per volume), $MgSO_4$ (X_4 %, by mass per volume) and urea (X_5 %, by mass per volume). By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to represent the PG production adequately:

$$Y=15.22-0.10\,X_1-0.83\,X_2+1.45\,X_3-0.46\,X_4-2.01\,X_5-1\,X_1^2-1.5\,X_2^2+2.22\,X_3^2+0.93\,X_4^2+1.46\,X_5^2+0.88\,X_1X_2+1.75\,X_1X_3+0.96\,X_1X_4+0.63\,X_1X_5+4.14\,X_2X_3-2.43\,X_2X_4-0.70\,X_2X_5+1.49\,X_3X_4-4.55\,X_3X_5-3.04\,X_4X_5 \quad /2/$$

The goodness of fit of the model was checked by different criteria. The R^2 value for the response variable was higher than 0.90, indicating that the regression model explained the reaction well. The analysis of variance (ANOVA) of the quadratic regression model demonstrated that Eq. 2 is highly statistically significant model of PG response, as was evident from the Fisher's test with a very low probability value [(p model>F)=0.0001]. The goodness of fit of the model was checked by determination coefficient (R^2).

The coefficient of determination (R^2) of polynomial model was calculated to be 0.93. This implied that the sample variation of PG production of 93 % was attributed to the independent variables and only 7 % of the total variations were not explained by the model. This ensured a satisfactory adjustment of the quadratic model to the experimental data. The adjusted R^2 (0.8824) was also very high, making the model very significant. The Pred R^2 of 0.7558 for PG production was in reasonable agreement with the Adj R^2 of 0.8824. This indicated a good agreement between the experimental and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared. A lower value of CV (15.79 %) indicates a greater reliability of the experimental performance. The model F-value of 19.39 implies that the model is significant. There is only a 0.01 % chance that a model F-value this large can occur due to noise. Values of Prob>F less than 0.05 indicate that model terms are significant. In this case, X_2, X_3, X_5, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4, X_3X_5, X_4X_5, X_1^2, X_2^2, X_3^2, X_4^2, X_5^2 are significant model terms. Values greater than 0.10 indicate the model terms are not significant (Table 2). If there are many insignificant model terms, model reduction may improve the model.

Response contours are calculated by using the model Eq. 1. Environmental conditions can affect protein production and secretion of pectolytic enzymes in various organisms. The activities of enzymes responsible for polysaccharide synthesis have an optimum pH value so that their efficiency will be modified by changes in the external pH (*26*). The contour plot (Fig. 1) shows increase in PG activity that can be obtained at intermediate levels of KH_2PO_4 (0.6 %) and pH=5.75. Yeast extract and peptone have been reported to stimulate maximum pectinase production from *Kluyveromyces lactis* NRRL 1137 (*27*). Ammonium sulphate and potassium phosphate have been reported to have no influence on the production of pectinases at lower concentrations (*28*). The statistical models predict that the highest values of responses can be obtained at the lowest investigated ratios of KH_2PO_4, peptone, $MgSO_4$ and urea. The enzyme (PG) production increased at lower pH and the lowest ratio of peptone. The contour plot of $MgSO_4$ and urea predicted the maximum PG production with the increase in their ratios. The maximum production of PG of 8.2 U/mL was obtained with the initial fermentation conditions of KH_2PO_4 0.3 %, pH=7, peptone 0.1 %, $MgSO_4$ 0.03 % and urea 0.15 %. For the selection of optimum conditions and ratios of each of the factors, the models were analyzed separately. The maximum response predicted from the model was 36.80 U/mL.

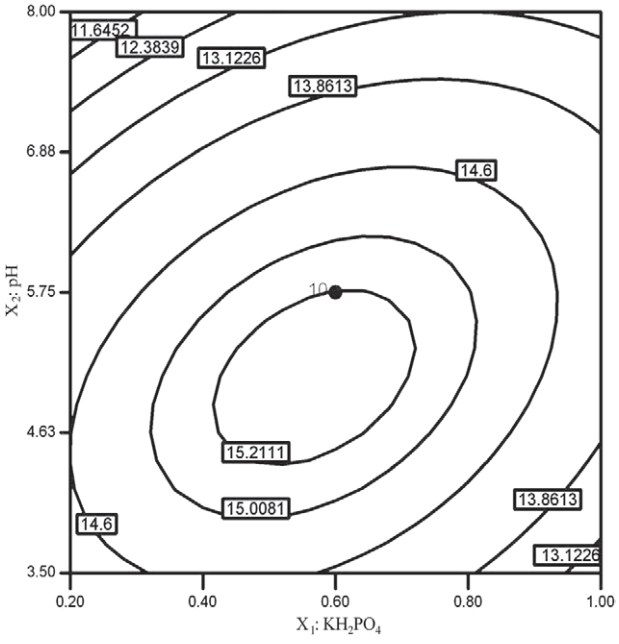

Fig. 1. Contour plot of polygalacturonase production showing the interactive effect of KH_2PO_4 and pH

In addition to optimization, RSM can be successfully used to study the interaction effect among the variables. The interaction between the variables in relation to enzyme production is clear from the data (Table 3). The highest value of PG production was noted in the 16th run where KH_2PO_4, pH, peptone and $MgSO_4$ were at +1 level and urea at –1 level. The lowest rate of enzyme production (4.51 U/mL) was noted in run 27. The values of responses clearly indicate that the selected ranges were appropriate. The values of coefficients obtained by the model equation reflect the interactions of the chosen parameters in relation to enzyme production. The interaction effect among the variables is very significant and cannot be neglected.

Validation of the model

In order to validate the adequacy of the model, a total of three verification experiments were conducted in the bioreactor under various fermentation conditions within the experimental range to verify the predicted optima. The results of three replications were in agreement with the predicted value and the model was proven to be adequate. The final optimized fermentation conditions obtained were KH_2PO_4 0.22 %, pH=5.8, peptone 0.5 %, $MgSO_4$ 0.02 % and urea 0.2 %. Compared to the original value, the PG production increased from 8.2 to 36.5 U/mL. Polygalacturonase production was observed within 24 h and reached a maximum in only 72 h at 150 rpm (Fig. 2). This increase in the production could be due to increased/controlled oxygen transfer rates in the bioreactor, which supported both higher growth as well as enzyme yield. These results indicate that the model was satisfactory and could enhance the enzyme yield considerably.

Purification of PG

The polygalacturonase was purified through gel filtration. Total protein content of the sample decreased from 52.5 mg in the crude sample to 4.24 mg in the final step. The specific activity had a marked increase in every step; from 152.95 U/mg in the crude sample to 525.94 U/mg in the chromatographic step (Table 4). The elution profile shows the protein content in the eluted fractions from gel filtration chromatography and PG activity in the eluted fractions (Fig. 3). Total enzyme activity in the crude sample was 8030 U. The yield of the enzyme was 27.7 % with respect to the starting material.

Pectinases from various microorganisms have also been purified and characterized. Gummadi and Panda (*29*) reviewed the purification and biochemical properties of microbial pectinases, and concluded that many pectinases from various microorganisms were purified to homogeneity with a combination of ion exchange and gel filtration chromatography as main procedures (*30*). Therefore, we used the same technique to obtain the purified PG from *A. foetidus* with a single peak of enzyme activity and single protein peak in the gel. The obtained enzyme activity peak was not a symmetrical one. The final fold and yield were 3.43 and 27.7 %, respectively.

A single band of the purified fraction was observed when subjected to SDS-PAGE. The molecular mass of PG was determined to be 34 kDa (Fig. 4). This study reveals the presence of a single polypeptide in PG of *Aspergillus foetidus*.

Characterization of PG

Degradation of pectin by PG was assayed by measuring the released reducing groups. The degradation rate of the substrate decreased with the increase of the es-

Table 3. Central composite design matrix

Run no.	X_1*	X_2	X_3*	X_4*	X_5*	Y	Predicted
1	–1	–1	–1	–1	–1	18.43	18.22
2	1	–1	–1	–1	–1	9.68	9.97
3	–1	1	–1	–1	–1	14.89	12.77
4	1	1	–1	–1	–1	7.43	8.04
5	–1	–1	1	–1	–1	13.96	15.44
6	1	–1	1	–1	–1	14.14	14.21
7	–1	1	1	–1	–1	25.85	26.57
8	1	1	1	–1	–1	29.72	28.86
9	–1	–1	–1	1	–1	22.58	23.32
10	1	–1	–1	1	–1	17.61	18.93
11	–1	1	–1	1	–1	6.17	8.15
12	1	1	–1	1	–1	9.71	7.27
13	–1	–1	1	1	–1	27.55	26.52
14	1	–1	1	1	–1	33.50	29.14
15	–1	1	1	1	–1	29.50	27.93
16	1	1	1	1	–1	30.00	34.06
17	–1	–1	–1	–1	1	29.00	29.53
18	1	–1	–1	–1	1	25.10	23.80
19	–1	1	–1	–1	1	20.60	21.28
20	1	1	–1	–1	1	17.59	19.06
21	–1	–1	1	–1	1	12.60	8.55
22	1	–1	1	–1	1	8.55	9.83
23	–1	1	1	–1	1	14.14	16.87
24	1	1	1	–1	1	24.74	21.66
25	–1	–1	–1	1	1	23.47	22.49
26	1	–1	–1	1	1	22.72	20.61
27	–1	1	–1	1	1	5.17	4.51
28	1	1	–1	1	1	5.32	6.14
29	–1	–1	1	1	1	5.86	7.48
30	1	–1	1	1	1	11.00	12.61
31	–1	1	1	1	1	7.68	6.07
32	1	1	1	1	1	16.43	14.72
33	0	0	0	0	0	6.96	15.22
34	2.378	0	0	0	0	8.72	9.79
35	0	–2.378	0	0	0	7.32	8.73
36	0	2.378	0	0	0	5.10	4.77
37	0	0	–1	0	0	16.53	15.99
38	0	0	2.378	0	0	30.07	31.24
39	0	0	0	0	0	16.72	15.22
40	0	0	0	2.378	0	18.32	19.39
41	0	0	0	0	–1	19.33	18.68
42	0	0	0	0	2.378	17.51	18.70
43	0	0	0	0	0	16.43	15.22
44	0	0	0	0	0	16.14	15.22
45	0	0	0	0	0	14.72	15.22
46	0	0	0	0	0	16.35	15.22
47	0	0	0	0	0	16.35	15.22
48	0	0	0	0	0	15.58	15.22
49	0	0	0	0	0	16.28	15.22
50	0	0	0	0	0	14.96	15.22

X_1=KH_2PO_4, X_2=pH, X_3=peptone, X_4=$MgSO_4$, X_5=urea, Y=polygalacturonase activity (U/mL)
*in %, by mass per volume

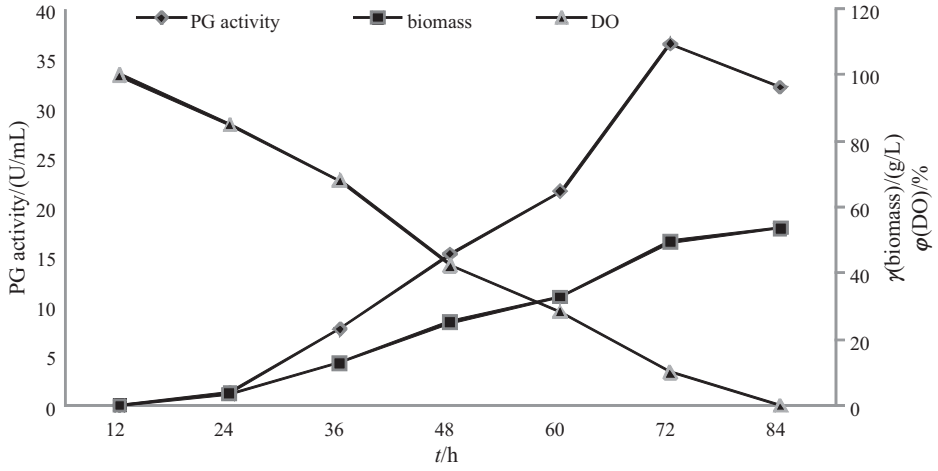

Fig. 2. Fermentation profile of polygalacturonase (PG) produced from *Aspergillus foetidus*; DO=dissolved oxygen

Table 4. Purification of polygalacturonase produced by *Aspergillus foetidus* from mango peel

Purification step	Total activity	*m*(total protein)	Specific activity	Purification fold	Yield
	U	mg	U/mg		%
Crude cell filtrate	8030	52.50	152.95	1.00	100.0
Ammonium sulphate fraction	3600	13.80	260.00	1.69	44.8
Gel filtration	2230	4.24	525.94	3.43	27.7

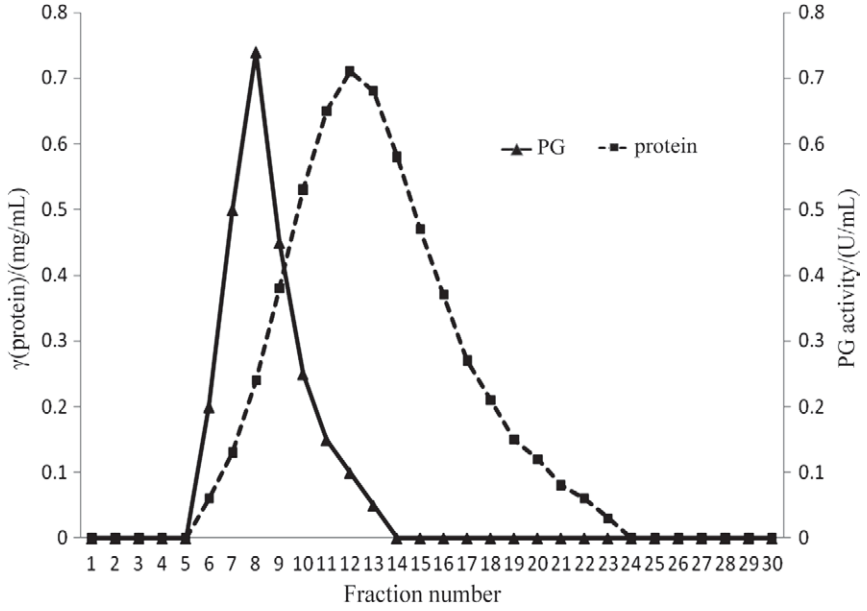

Fig. 3. Protein content and polygalacturonase (PG) activity in the eluted fractions of gel filtration chromatography

terization of the substrate. The same result was reported for PG from different sources, whereas the hydrolysis of pectin decreased with the increase of the degree of esterification (*31*). In this study, kinetic parameters were determined using pectin as a substrate in a range of 1–12 mg/mL. Michaelis-Menten equation was found to fit the reaction of PG from the *Aspergillus foetidus*. The v_{max} of PG was found to be 11.98 mmol/(mL·min) and K_m 7.3 mg/mL (Fig. 5). When the substrate concentration was above 10 mg/mL, the enzyme activity decreased. Previous studies showed multiple forms of PGs produced by different strains of *Aspergillus.* Taskin and Eltem (*32*) purified PGs from two strains of *A. foetidus* with the K_m values on pectic substrate of 4.52 and 4.62 mM, and v_{max} values of 22.62 and 153.84 mmol/(mL·min) for EGEK145 and K635, respectively. In this study a considerable decrease in the enzyme activity was observed at a substrate concentration above 8 mg/mL. Gillespie *et al.*

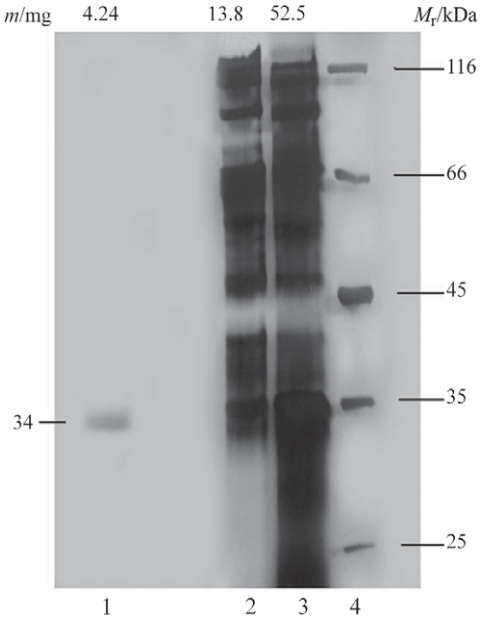

Fig. 4. Characterization of polygalacturonase by SDS-PAGE using silver staining.
Lane 1: purified enzyme, lane 2: ammonium sulphate fraction, lane 3: crude enzyme, lane 4: molecular mass marker proteins

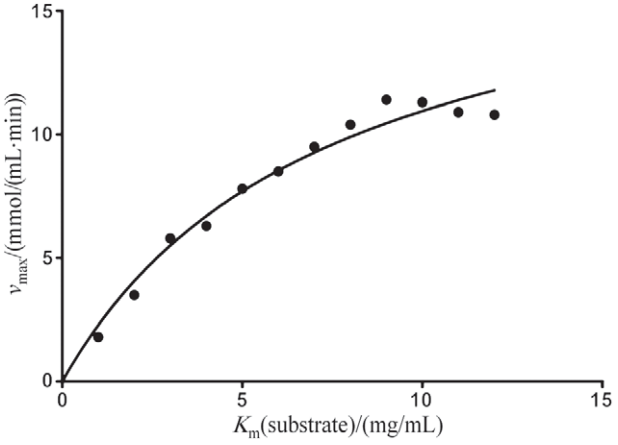

Fig. 5. Michaelis-Menten plot for purified polygalacturonase

(*33*) observed the same phenomenon, but in their case the enzyme activity was high at the substrate concentration of 5 mg/mL. These results showed that the enzyme activity is inhibited by the excess concentration of substrate. According to them, the reason for the decrease of enzyme activity may also be due to the high viscosity of the reaction mixture.

The optimum temperature for the activity of purified polygalacturonase from *A. foetidus* was 55 °C. The PG activity increased with increasing temperature from 30 to 55 °C and decreased from 60 to 80 °C. From 80 °C onwards, the enzyme activity was lost. The PG exhibited the highest enzymatic activity at pH=5. The enzyme had the highest activity between pH=4.5 and 6. Above pH= 5.0, the enzyme activity began to decrease. At pH=8.0, the relative activity was 18.09 % of that of the control. No activity was determined at pH=9.0.

Conclusion

Mango peel, a major waste of mango processing industry, was used to produce polygalacturonase (PG). Response surface methodology (RSM) proved to be an effective method for optimization of fermentation conditions for its production. The average PG production was 36.5 U/mL, which was enhanced by 4.1-fold after optimization of critical parameters using RSM. The enzyme was purified and its molecular mass was 34 kDa. This is the first report on the production of PG from mango peel waste with optimized submerged fermentation using *Aspergillus foetidus*.

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