

Polygalacturonase production enhancement by *Piriformospora indica* from sugar beet pulp under submerged fermentation using surface methodology

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Abstract

This study proposed a novel and cost-effective approach to enhance and optimize the polygalacturonase from *P. indica*. In current investigation, the impact of ammonium sulfate, sugar beet pulp (SBP) and glucose as variables on induction of polygalacturonase from *P. indica* was optimized using the central composite design (CCD) of response surface methodology (RSM) under submerged fermentation (SmF). Additionally, partial polygalacturonase purification and in situ analysis were performed. The optimal reaction conditions, which resulted in the highest enzyme activity were observed as the following conditions: ammonium sulfate (4 g/L), SBP (20 g/L), glucose (60 g/L). Under the optimized condition, the maximum enzyme activity reached to 19.4 U/ml (127 U/mg) which increased by 5.84 times compared to non-optimized conditions. The partial purified polygalacturonase molecular weight was estimated 60 kDa. In line with the bioinformatic analysis, exo-polygalacturonase sequence of *P. indica* showed similarity with *Rhizoctonia solani's* and *Thanateporus cucumeris*. These results indicated that SBP act as a cheap and suitable inducer of polygalacturonase production by *P. indica* in a submerged cultivation. The outcome of this study will be useful for industries to decrease environmental pollution with cost-effective approaches.

1. Introduction

Nowadays, considering the global sensitivity about the environment, enzyme production from wastes, helped to overcome the problem of high-cost production in the industry and prevent environmental pollution (Tepe & Dursun 2014). With the increasing application of pectinase, decreasing cost production has become one of the most important targets. Previous researches have mentioned that pectin-containing agro-wastes, including sugar beet pulp, citrus pulp pellets, apple pomace, henequen pulp, lemon pulp and other related materials as carbon source could induce pectinase production by many microorganisms (Bai et al. 2004). Sugar beet pulp (SBP) as the by-product of the beet sugar industry, is produced annually in large quantities. On the other hand, SBP could be an important renewable resource and its bio-conversion appears to be a great biotechnological advantage. The lignocellulosic portion of dried SBP is consist of 22–30% cellulose, 24–32% of hemicellulose (essentially arabinan), 24–32% of pectins substances and 3–4% of lignin (Hutnan et al. 2000). Due to high pectin content of SBP, it could be used for pectinolytic enzymes production without adding any pectinaceous materials as enzyme inducer (Nigam & Pandey 2009).

Pectinases or pectin depolymerases known as a very important industrial enzymes have a broad range of applications in food, pharmaceutical, textile, processing and paper and pulp industries (John et al. 2020). The pectinolytic enzymes are produced by higher plants, bacteria, fungi, yeasts under submerged and solid-state fermentation conditions. They are classified regarding to their mechanism of action: pectin esterase, pectinase (polygalacturonase) and pectin lyase; and caused production of galacturonic acid (Patidar et al. 2018). Like many other depolymerizing enzymes, they are usually inducible by the polymer they could degrade. Among them, fungal pectinases have the greatest significance with extensive applications (Patil & Dayanand 2006). The choice of source, substrate, reaction conditions and reactor

design are the important factors in upstream pectinase production processing (John et al. 2020). Pectin as the acidic heteropolysaccharide is mainly composed of galacturonic acid which present the major components of middle lamella and primary cell wall of plants (Satapathy et al. 2020).

The induction of pectinase production by various organisms from agricultural by-products were described; by *Penicillium fellutanum* from wheat bran (Amin et al. 2021), by *Bacillus pumilus* from mixture of banana and orange peel (Viayaraghavan et al. 2019), by *Aspergillus niger* DMF 27 and DMF 45 from deseeded sunflower head (Patil & Dayanand 2006), by *Aspergillus niger* from citrus waste peel (Ahmed et al. 2016), by *Aspergillus sojae* from agricultural and agro-industrial residues (Heerd et al. 2014), by *Aspergillus niger* and *Bacillus gibsoni* from sugar beet pulp (Jacob 2009), by *T. reesei* Rut C-30 from sugar beet pulp (Olsson et al. 2003), and by *Bacillus pumilus* from sugar beet pulp and wheat bran (Tepe & Dursun 2014).

In the current investigation, the production of exo-polygalacturonase from *P. indica* by sugar beet pulp (SBP) as an inducer was optimized by response surface methodology (RSM) and its molecular characteristics evaluated by partial purification and in situ analysis.

2. Material And Methods

2.1. SBP preparation

SBP was prepared from Moghan Agro-Industry & Livestock Co., dried at 60 °C for 24 h, dried SBP grinded and stored in air tight container. The particles with mesh sieve size adjusted to 500 µm were used for submerged fermentation.

2.2. Microorganism, media and culture conditions

The *P. indica* fungus was selected for production of polygalacturonase and obtained from the Department of Plant Pathology, School of Agriculture, Tarbiat Modares University (Iran). The fungi were cultured on modified Kaefer medium (Käfer 1977) and glucose was replaced with SBP. SBP⁺ represents the SBP-containing medium.

For submerged cultivation of *P. indica*, 10 mm of agar discs were transferred to 250 mL flasks containing 50 mL of modified Kaefer medium supplemented with 10 g/L SBP and incubated in shaker (200 rpm) at 29°C for 10 days. Medium without sugar beet pulp (SBP⁻) was used as control. The samples were assayed for pectinase activity and fungal growth measurement.

2.3. Measurement of cell fresh and dry weight, growth yield and specific growth rate

At the end of each incubation time, the culture broth was filtered through Whatman No. 1 paper and growth parameters including; growth yield ($Y_{X/S}$), specific growth rate (μ) and spore yield fresh and medium pH were determined (Kumar et al. 2011).

2.4. Total protein determination

Protein content and pectinase activity were determined in the cell-free supernatant after centrifugation of culture broth at 12880 rcf at 4 °C for 15 min. Determination of total protein content was performed according to the Bradford's method and bovine serum albumin used as the standard (Bradford 1976).

2.5. Pectinase activity

Polygalacturonase activity was evaluated by measuring the released reducing end products, using 3, 5-dinitrosalicylic acid (DNS) and expressed as galacturonic acid equivalent (Miller 1959). The enzymatic reaction mixture included 0.25 ml of cell-free supernatant and 0.75 ml of 1% pectin in 0.2 M phosphate buffer pH 6.5 as substrate. The mixture was incubated at 60°C for 5 min. One unit (U) was expressed in term of the enzyme quantity which would yield 1 μmol galacturonic acid per minute during the standard assay condition.

2.6. Identification of the significant variables using experimental design

In order to maximize enzyme production and understand the role of interacting variables, optimization of the medium constituents was done by central composite design (CCD). Three variables including; glucose (A), ammonium sulfate (B) and SBP (C) were selected to find the optimized condition for the production of pectinase and twenty experimental runs with three center points generated including the response surface plot by using the statistical software package Design-Expert 7.0.0 (Stat Ease Inc., Minneapolis, USA). The range and the levels of the variables are given in Table 1. The recommended 20 experiments by using different composition of independent variables was shown in Table 2. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by determination coefficient R^2 , and its statistical significance was determined by F-test.

Table 1
Levels of independent variables used in CCD design.

Variables	Units	Symbol code	Levels		
			-1	0	1
Glucose	g/L	A	60	80	100
Ammonium sulfate	g/L	B	6	4	8
Sugar beet pulp (SBP)	g/L	C	10	15	20

Table 2
CCD design matrix for pectinase production factors and corresponding results.

Run	Variables			DW (g/L)	FW (g/L)	pH	Protein (mg/ml)	Observed activity (U/ml)	Specific activity (U/mg)
	A	B	C						
1	60	4	20	62.56	417.85	4.09	0.25	19.41	77.64
2	60	6	15	55.5	323.36	3.72	0.18	14.74	81.88
3	80	6	15	62.06	332.6	3.55	0.157	12.33	78.53
4	80	6	10	57.53	205.86	4.7	0.151	10.46	69.27
5	100	4	20	60.85	380.85	3.78	0.196	14.16	72.24
6	80	6	15	47.77	306.98	5.09	0.148	12.13	81.95
7	100	6	15	64.39	245.88	5.76	0.135	10.76	79.70
8	80	8	20	55.94	373.6	4.85	0.433	10.12	23.37
9	80	8	15	55.335	369.56	5.06	0.217	10.67	49.17
10	60	4	10	66.20	188.61	5.29	0.145	14.03	96.75
11	80	4	15	57.11	381.42	4.28	0.148	14.03	94.79
13	80	6	15	69.22	336.53	3.88	0.105	13.33	126.95
14	60	8	20	53.35	253.26	5.60	0.252	12.35	49
15	100	8	10	52.09	176.76	4.99	0.192	8.07	42.02
16	100	4	10	56.19	251.20	4.69	0.133	9.78	73.53
17	80	6	15	61.75	332.77	4.15	0.19	12.87	67.73
18	80	6	15	73.40	323.33	4.84	0.26	12.54	48.23
19	60	8	10	51.71	266.44	3.6	0.16	10.75	67.19
20	80	6	15	58.99	393.97	5.17	0.11	12.37	111.45

2.7. Pectinase partial purification

To purify the pectinase, the fungus was cultured in 250 mL shake flasks with 100 mL of optimized fermentation medium. The fermentation broth was separated by centrifugation at 10,000 g for 15 min at 4°C and the cell-free supernatant was saturated with ammonium sulphate to 90% saturation. The saturated solution was left overnight at 4°C with gentle agitation, centrifuged at 10000 g for 20 min at 4°C, the precipitate solubilized in minimal amount of 10 mM sodium acetate buffer (pH 5.75) and dialyzed against the same buffer for 24 h at 4°C. The obtained-dialyzed proteins were used for enzyme characterization. The protein content and enzyme activity were determined as described in earlier part.

2.8. Gel electrophoresis

The molecular mass and purity of polygalacturonase were determined by SDS-PAGE (12.5% running gel and 5% stacking gel) Laemmli (1970) (Laemmli 1970). The protein samples were denatured by heating at 100°C with the sample buffer for 5 min before loading and the gel was stained by silver staining method of Merrill et al (Merrill et al. 1981).

2.9. Phylogenetic tree simulation

We applied Molecular Evolutionary Genetics Analysis (MEGA X) software as a powerful tool for constructing sequence alignments, gathering phylogenetic histories, and performing molecular evolutionary analysis. This software can be used for comparing DNA and protein sequences. Firstly, we aligned the DNA sequences of more than fifty different strain types of extracellular polygalacturonases and then a phylogenetic tree was constructed for those data by the maximum likelihood method. In this approach, an initial phylogenetic tree was constructed using a Neighbor-Joining, and its branch lengths are modified to maximize the likelihood of the data set for that tree topology under the desired model of evolution. Then the NNI (nearest neighbor Interchange) approach was used for creating the variants of the topology. NNI tries to search for topologies that are in good shape with the data better. The search is repeated until no greater likelihoods are found. Finally, the Neighbor-joining tree of different extracellular polygalacturonase strains was constructed after 500 iterations and the bootstrap confidence values were calculated and shown on node in Fig. 4. The protein sequences were gathered from Uniprot and GenBank (NCBI) (Kumar et al. 2018) (Chen et al. 2014) (<https://academic.oup.com/nar/article/22/22/4673/2400290?login=true>). (Verma et al. 1998)

3. Results

3.1 Growth of fungus

Production of pectinase by *P. indica* was evaluated for 10 days (Fig. 1). The production of pectinase on both medium, reached its maximum rate on day 6th of culture and then decreased. The production of enzyme on SBP⁻ and SBP⁺ medium was determined 2.2 and 3.32 U/ml, respectively. Also, the highest amount of dry and fresh weight on both medium was detected on day 6 of culture (Fig. 1a and 1b). As shown in Table 3, the lowest and highest dry cell weight were detected on unmodified Kaefler medium and SBP⁻ and glucose-containing medium, respectively. Furthermore, the highest amount of growth yield and specific growth rate were measured on medium containing ammonium sulfate, glucose and SBP, 0.62 and 1.61 respectively.

Table 3
Effect of variables on growth of *P. indica*.

Culture medium	Maximum DCW (g/L)	$Y_{x/s}$ (g/g)	μ (d ⁻¹)
Medium	3.7±0.1414		
Medium + SBP	6.125±0.1768	0.61	0.30
Medium + Ammonium sulphate	3.95±0.21215	0.28	1.27
Medium + Ammonium sulphate + SBP	7.675±0.1768	0.54	0.61
Medium + Glucose	15.625±1.096	0.26	0.45
Medium + Glucose + SBP	26.45±1.202	0.44	0.54
Medium + Ammonium sulphate + Glucose + SBP	73.40	0.62	1.61

3.2 Optimization of the pectinase production by RSM

Three factors that have the maximum effect on the polygalacturonase production were determined by one-factor-at-a-time method and the interaction between various selected factors on polygalacturonase production (glucose, ammonium sulfate and SBP concentration) were investigated by RSM.

Then the results were analyzed by standard analysis of variance (ANOVA) and the CCD design was fitted with the second-order polynomial equation:

$$Y \text{ (U/ml)} = +14.44655 - 0.25632 \times A - 0.050858 \times B + 1.78328 \times C + 0.010096 \times A \times B - 2.36660E-003 \times A \times C - 0.094896 \times B \times C + 8.28012E-004 \times A^2 - 0.031692 \times B^2 - 0.024311 \times C^2$$

Eq. (1) polygalacturonase activity (Y) as a function of Glucose (A), Ammonium sulfate (B) and Sugar beet pulp (C).

The software suggested 20 experiments and the predicted and experimental values for enzyme production are presented in Table 2. The sufficiency of the model was checked using correlation coefficient (R^2) and the closer the value of R^2 to 1, the better the correlation between the observed and the predicted values. The correlation coefficient (R^2) which shows the relationship between the experimental and predicted responses was 0.9866 and thus the model could explain more than 98.66% of the variability in the responses (Table 4).

Table 4
ANOVA for response surface quadratic model of polygalacturonase production.

Source	Sum of squares	df	Mean squares	F value	P – value	
Model	106.59	9	11.84	81.76	<0.0001	significant
A	29.23	1	29.23	201.82	<0.0001	
B	38.49	1	38.49	265.70	<0.0001	
C	19.13	1	19.13	132.08	<0.0001	
AB	0.92	1	0.92	6.35	0.0304	
AC	0.32	1	0.32	2.18	0.1705	
BC	6.14	1	6.14	42.37	< 0.0001	
A ²	0.31	1	0.31	2.15	0.1733	
B ²	0.047	1	0.047	0.32	0.5829	
C ²	1.07	1	1.07	7.40	0.0215	
Residual	1.45	10	0.14	-	-	
Lack of Fit	0.50	5	0.10	0.53	0.7483	Not significant
Pure error	0.95	5	0.19			
Cor total	108.04	19	-	-	-	
Note: R ² (0.9866), Adj R ² (0.9745), Pred R ² (0.9404), Adeq precision 41.209.						
P-values < 0.05 indicate significant differences.						

Moreover, R² values were in reasonable agreement with adjusted R² values of 0.9745 (polygalacturonase production). Values greater than 0.1000 indicate the model terms are not significant. The "Pred R-Squared" of 0.9404 is in reasonable agreement with the "Adj R-Squared" of 0.9745. Table 4 represents the obtained results of the quadratic response surface model fitting in the form of ANOVA.

The model F-value is 81.76 which indicates the model significance. The (B) had the highest F-value of 265 implying that it had the most significant influence on the enzyme activity in comparison to glucose (A) and SBP (C). Moreover, the lack of fit F-value was 0.53 which is nonsignificant relative to the pure error. The model is geared toward perfect fitness.

3.3 Interaction between operating factors

According to the ANOVA Table 4, the significance of the independent variables and interaction between them was determined by F-values and p-values. As seen in Table 4, A, B, C, AB and BC with a very small p-value (p<0.05) were significant while, AC was insignificant on enzyme production.

Due to the positive linear coefficient of SBP, by increasing the SBP concentration within the range assayed the enzyme production increased. Also the negative quadratic coefficients of ammonium sulfate and SBP explain the maximum enzyme production at these levels. Subsequently, the enzyme production decreased out of this point. In accordance with the coefficients, SBP is determined as the factor which has the most positive impact on enzyme production.

Furthermore, the interaction among variables was confirmed by the 3-D response surface plots which is used to identify the optimum levels. The interaction between variables were also evaluated in pectinase production.

The 3-D plots represent the interaction between the two factors, while the other factor was fixed at its optimum level for maximum enzyme production (Fig. 2). Fig. 2a exhibits the interaction between ammonium sulfate and glucose, reveals an increase in pectinase production by increasing in ammonium sulfate and glucose concentrations. The response between glucose and SBP indicated that increasing the SBP content and decreasing glucose concentration led to higher enzyme production (Fig. 2.b). The plot for the interaction between SBP and ammonium sulfate (Fig. 2c) showing an increase in enzyme production at low level of ammonium sulfate and high level of SBP.

The maximum enzyme production was occurred at high levels of SBP and low levels of glucose and ammonium sulfate.

Effect of 3 factors on the response were significant ($P > 0.05$) which showed higher and effective contribution to the enzyme production in SmF (Table 4).

Further, the optimum condition for pectinase production obtained at 60 g/L of glucose, 4 g/L of ammonium sulfate and 20 g/L of SBP. At the optimum condition, the enzyme activity increase to 19.41 U/ml which is 5.58-fold more than unoptimized condition.

3.4 Partial purification of pectinase

For purification, the supernatant was precipitated by ammonium sulfate. The partial purification results were shown in Table 5. Then the specific activity and fold purification was calculated 63.77 and 0.82 (U/mg) respectively. The molecular mass of pectinase was found around 60 kDa by SDS-PAGE (Fig. 3).

Table 5
Partial purification process of pectinase.

Purification step	activity (U/ml)	protein (mg/ml)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude extraction	19.40	0.25	77.6	100	1
Ammonium sulfate precipitation	11.48	0.18	63.77	59.05	0.82

3.5 Simulation results

As shown in Fig. 4, the phylogenetic tree gathered from the concatenated rDNA and TEF alignment by applying heuristic ML analysis with the bootstrap values are shown on nodes. We compared the phylogenetic relationships of *P. indica* with *Rhizoctonia solani*'s extracellular polygalacturonases in NCBI data bank. In Fig. 5, *Rhizoctonia solani*'s and *Thanateporus cucumeris* revealed exo-polygalacturonases similar sequences. Also in Fig. 6 we showed the alignment of the predicted amino acids sequences of *P. indica* polygalacturonase with 8 similar sequences.

4. Discussion

Nowadays, over the global there is a great interest to obtain pectinase from cheap and suitable substrates like agricultural residues.

Here the efficiency of *P. indica* in biodegradation of SBP has been evaluated. Subsequently, the polygalacturonase activity on SBP⁺ and SBP⁻ media has been reported for 10 days (Fig. 1). The polygalacturonase production and fungal growth reached the maximum level simultaneously on both medium. It's established that the components of the medium have great effect on the induction of pectolytic enzymes. Several substances play an inducer role on these enzymes synthesis (Nair &Panda 1997). The utilization of agro-industrial residues, like apple pomace and sugar beet pulp, further could solve environmental problems of the by-products (Heerd et al. 2014). The choice of perfect agricultural residue to induce enzyme production depends on several factors, such as cost and availability of the substrates. It is known that duration of fermentation depends on the medium composition, organism, concentration of nutrients and the physiological conditions (Patil &Dayanand 2006).

SBP has been used as raw material to induce pectinase production by *Aspergillus niger*. Additionally it has been used as carbon source as well as pectinase inducer to produce extracellular alkaline pectinase, by *Bacillus gibsoni*, under SSF (Jacob 2009). Cultivation of *T. reesei* Rut C-30 on sugar beet pulp (50 g/L), the protein content, pectinase activity and specific activity reached their maximum value after 60 h of fermentation (0.43 g/l, 0.82 U/ml and 1.9 U/mg respectively) (Olsson et al. 2003). The highest enzyme activity by *A. sojae* on 30% sugar beet pulp as an inducer and wheat bran as medium wetted agent attained after 8 days (Heerd et al. 2014).

However our results show the highest activity of exo-pectinase in medium containing sugar beet pulp determined 3.4 U/mL on 96 h of fermentation and following optimization it reaches to 19.41 U/ml.

The greatest endo- and exo-pectinase activity by *A. niger* from sunflower head in SSF (5.1U/g and 17.1U/g) and SmF (4.5 U/ml and 16.0 U/ml) were measured on 96 h. According to the different studies about the fermentation time, it exhibited wide range 40–120 h and 90–120 h in submerged and solid state fermentations, respectively (Patil &Dayanand 2006).

The *Colletotrichum* isolated from Argentinian soybean, yielded high amount of the PG (1.08 U/ml) after 7–10 days of incubation and coincide with maximum growth. In medium involving glucose as a sole carbon source decreased polygalacturonase production was monitored (Ramos et al. 2010).

Environmental and nutritional factors are known as two essential factors which affect enzyme production by microorganisms. The pectin and polygalacturonic acid applied as only source of carbon in the medium which induced synthesis of pectinolytic enzymes by *A. niger* and there was no pectolytic activity in medium containing glucose as an only carbon source. Production of pectin degrading enzymes in the presence of pectin and high glucose concentrations were inhibited although glucose in low concentrations promoted their production. The observed low pectinolytic activity in media with high glucose concentrations is possibly due to provide growth need of organism by the glucose consumption and caused to decrease the pectin lysis. Furthermore, at low glucose concentrations, high pectolytic activity were observed (Fawole & Odunfa 2003). In agreement with Fawole et al, Our results showed the highest pectinolytic activity was attained at the lowest glucose concentration (60 g/L).

Pectinolytic activity by *A. niger* on medium containing pectin, poly galacturonic acid and glucose at 30°C for 5 days was 17.2, 13.8 and 0 U/ml, respectively (Fawole & Odunfa 2003).

As it is shown by Aguilar and Huitron (1987) high exogenous glucose and galacturonic acid could be influenced endo-PG enzyme production by catabolite repression, whereas glucose had no effect on the exo-PG. The Glucose concentration above 10% (w/w) in the SSF, decreased noticeably the activity of endo and exo-PG (Aguilar & Huitrón 1990).

In Solis-Pereyra et al study, exo-PG/gdm and endo-PG/gdm activity by *A. niger* on medium containing 16% (w/w) citric pectin, were 281 U and 152 U. Moreover, inhibited enzyme production and growth were detected on 20- 30% (w/w) pectin concentration (Fontana et al. 2005).

Ammonium sulphate was introduced as the favourable nitrogen source for pectinase production by *A. niger* (Fawole & Odunfa 2003). Our results are in concurrence with the observations of Sapunova who also demonstrated that ammonium salts could act as stimulator of the pectinase. It has been described that nitrogen limitation decreases the production of polygalacturonase (Thakur et al. 2010).

Bai and *et al*, examined the impact of different nitrogen sources on pectinase induction and great enzyme activity measured with ammonium sulfate, yeast extract, soya peptone, soya pulp and MGW (Bai et al. 2004).

Patil and *et al* examined the impact of ammonium phosphate and sulphate on pectinase production by *A. niger* from sunflower head in both SSF and SmF. As their study revealed, ammonium phosphate and sulphate could increases the production of pectinase in both fermentation conditions. However, this increase was very less with ammonium phosphate in comparison to ammonium sulphate. The maximum production of endo-pectinase and exo-pectinase by DMF 27 were recorded in SmF condition 18.9 U/ml and 30.3 U/ml respectively (Patil & Dayanand 2006).

As stated in many studies the average molecular mass of polygalacturonase are in the range of 35-80 KDa (John et al. 2020). Different microbial species produced different molecular mass of pectinase enzyme. Different factors such as the substrate, nature of microorganism, host cell wall and analytical

methods results in different mass of pectinase (Oyede, 1998). The molecular weight of *P. indica* polygalacturonase in our study was comparable with previous reports.

5. Conclusion

The present study showed that optimized conditions by RSM, yielded high polygalacturonase activity by *P. indica* and SBP was established as a significant enzyme inducer substrate. In situ analysis confirmed the similarity of exopolygalacturonase of *P. indica* with *R. solani*'s enzyme. Application of agricultural and agro-industrial wastes could be great source for enzyme production, because economically is valuable and decrease environmental pollution. The outcome of the proposed research will open up future pathways for using raw wastes materials to produce valuable products with cost-effective and eco-friendly approaches.

Declarations

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Availability of data and materials

All data and materials are available.

Authors Contributions

Conceptualization, formal analysis, writing - review and editing, project administration, supervision: Parisa Fathi Rezaei. Material preparation, data collection and analysis, writing–original draft preparation: Somayyeh Kiani. Bioinformatic analysis: Sina Jamalzadegan.

All authors read and approved the final manuscript.

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Ethical Approval

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs Signed by all authors as follows:

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Consent to Participate

The authors are consent to participate the article.

Consent to Publish

The authors are consent to publish the article.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

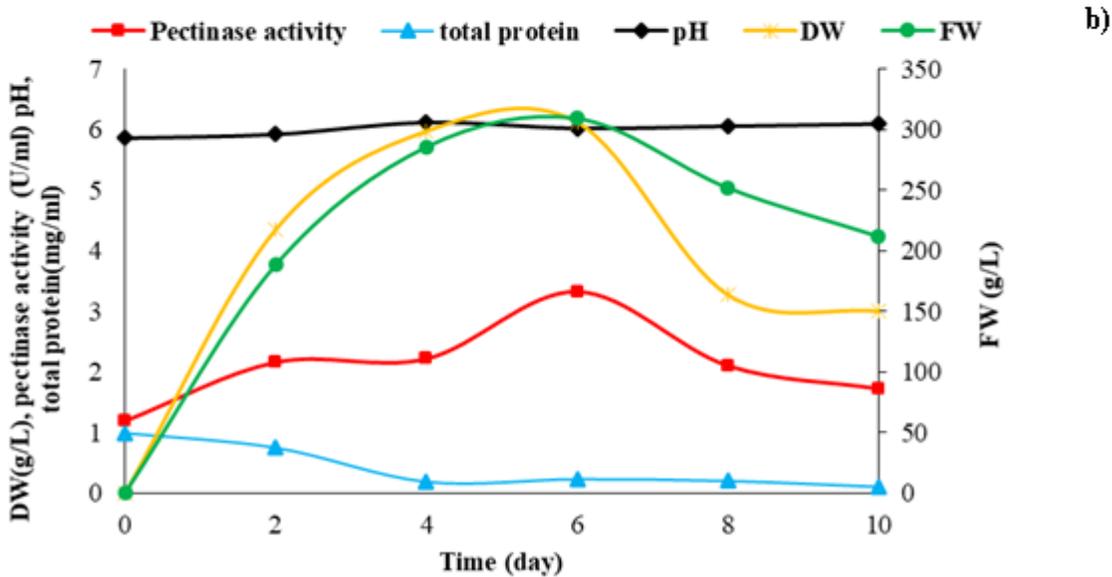
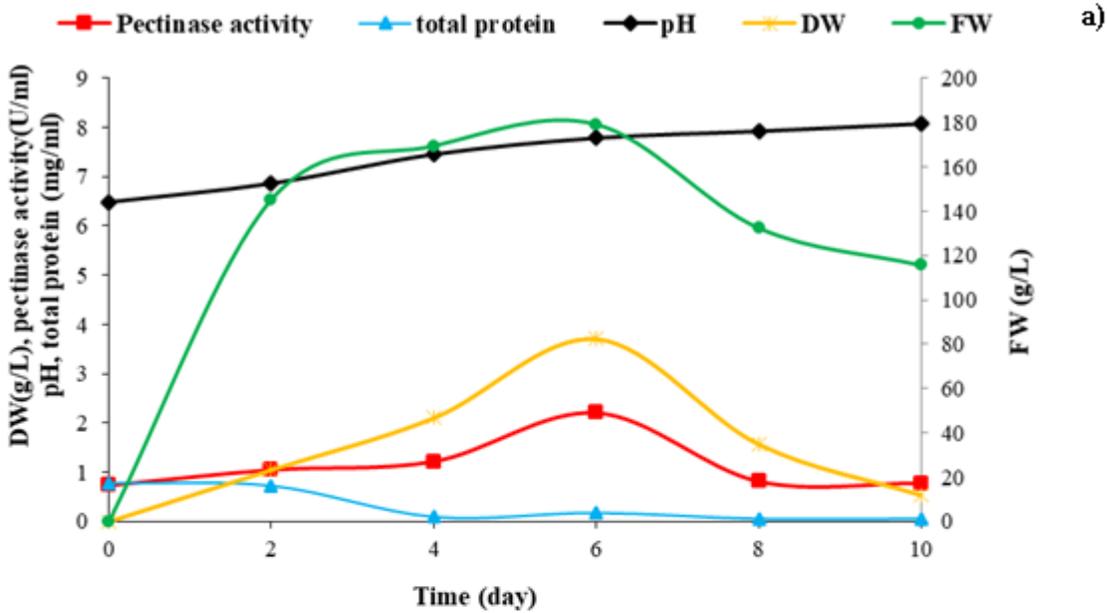
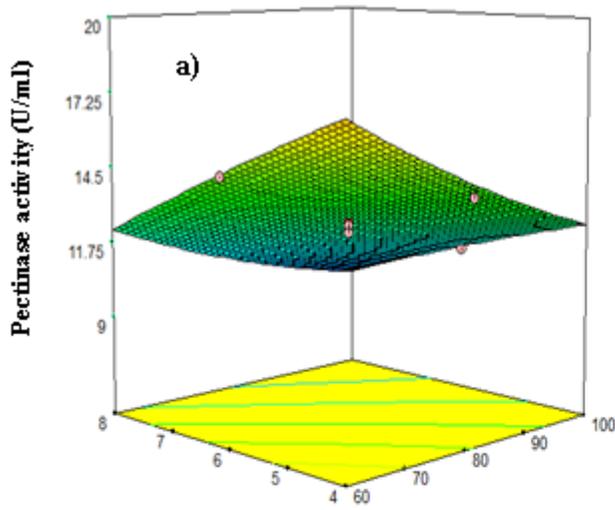


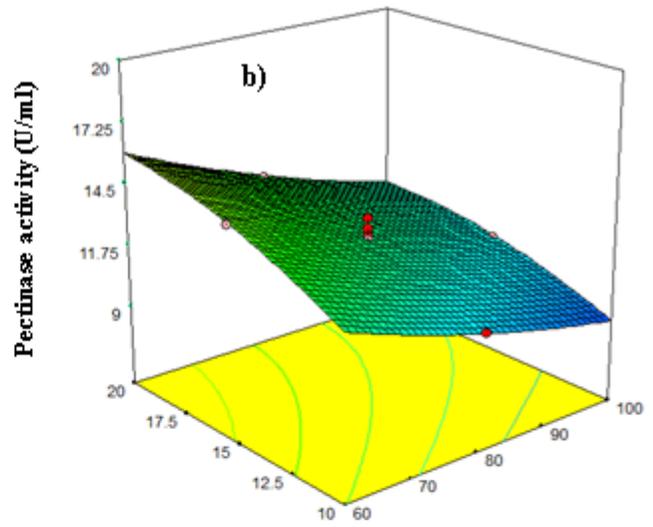
Figure 1

The effect of SBP on growth parameters and pectinase activity of *P. indica*. Time course profile of *P. indica* on Kaerfer medium (a) and supplemented with SBP (b) in SmF. Data are shown as mean \pm SD of three independent experiments in triplicate layout. SBP⁺: medium containing SBP, SBP⁻: medium without SBP, FW: Fresh weight; DW: dry weight.



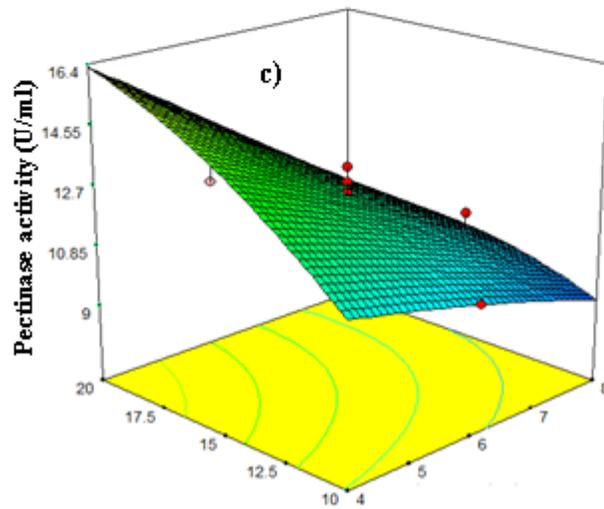
B: Ammonium sulphate (g/L)

A: Glucose (g/L)



C: Sugar beet pulp (g/L)

A: Glucose (g/L)



C: Sugar beet pulp (g/L)

B: Ammonium sulphate (g/L)

Figure 2

Response surface plot of polygalacturonase yield under optimal conditions and interaction between variables. a) interaction between ammonium sulfate and glucose; b) interaction between sugar beet pulp and glucose; and c) interaction between sugar beet pulp and ammonium sulfate.

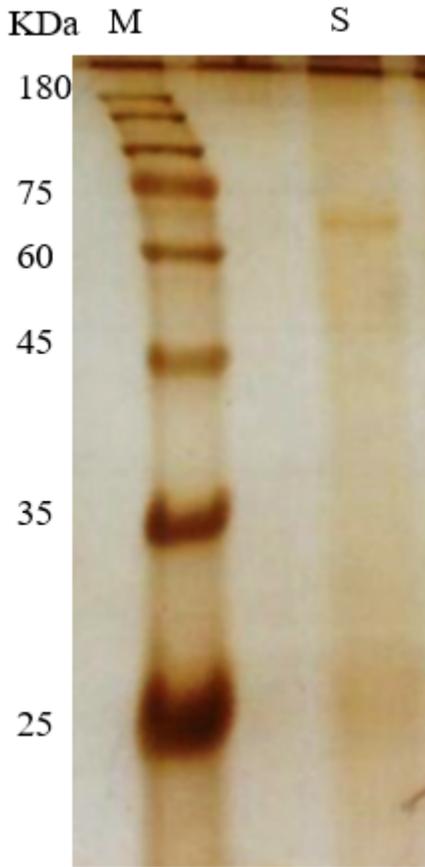


Figure 3

SDS-PAGE analysis of polygalacturonase. M: protein molecular weight marker; S: the sample after partial purification.

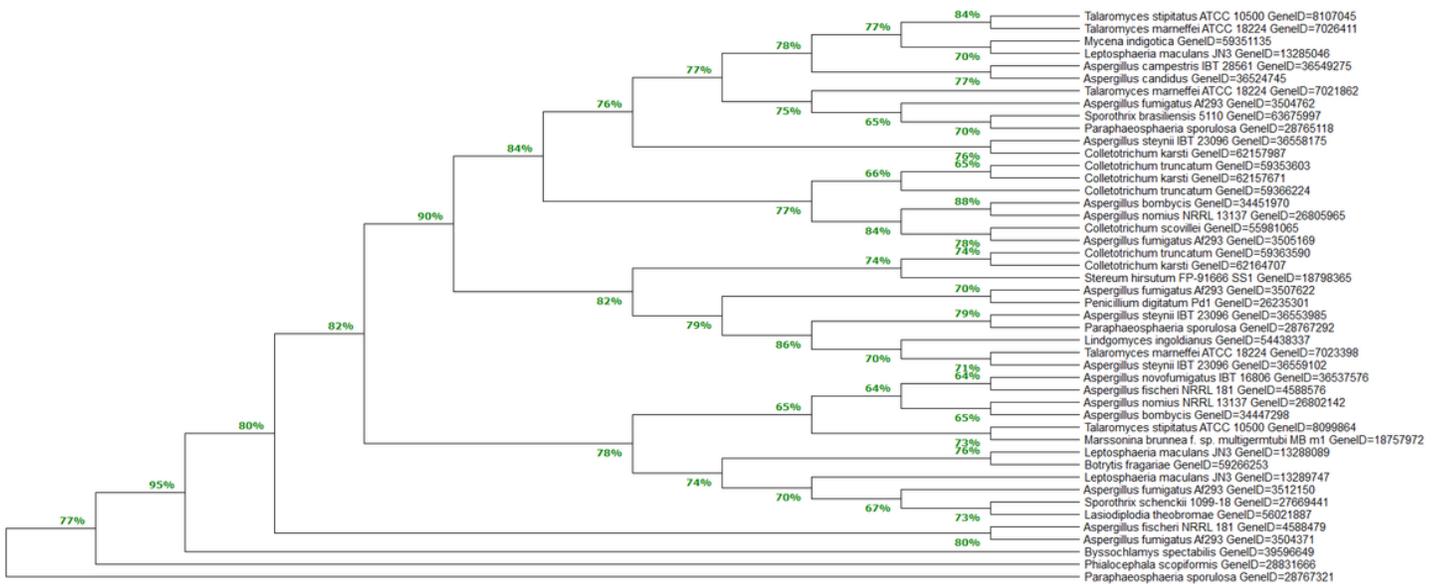


Figure 4

Phylogenetic tree of the *P. indica* and closely related proteins created using the neighbor-joining method. Bootstrap confidence values (500 repetitions) are shown on nodes. The numbers at each node marked the percentage of supporting bootstrap samples.

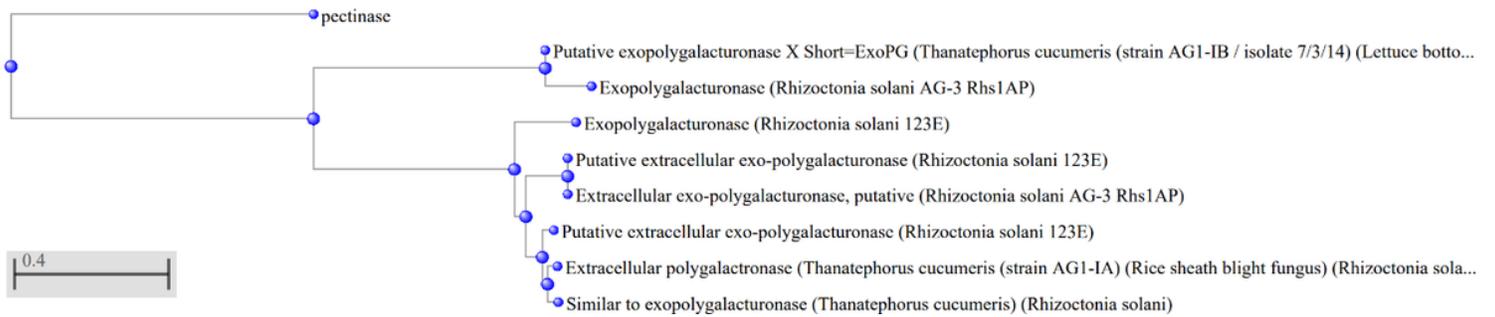


Figure 5

The phylogenetic tree represents similar DNA sequences to the polygalacturonase gene of *P. indica*. The protein sequences were retrieved from Uniprot and GenBank (NCBI).

```

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Query_10002 1 --MLS LFFAALGATSAVALVDSVERYTNPQTCIVP SYGNLNKSDTPAIHAAFKKCGK--GGRIFIKENTTYA 68
Query_10003 1 --MLS[1]FFTAALGATTVASVLDLAERYSNPQTCIIP SYGDLNKSADTPAIHAAFKKCGK--GGRIFIKENTTYA 69
Query_10004 1 --MFS[4]IFTAILGLVTTAVASLGLTERQSTTTTCIVP SRGDLTKSDTPAIHAAFKKCGH--NGRIIFQKNTIYA 72
Query_10005 1 --MFS[4]IFTAILGLVTTAVASLGLTERQSTTTTCIVP SRGDLTKSDTPAIHAAFKKCGH--NGRIIFQKNTIYA 72
Query_10006 1 --MLS[1]FFAALGATTVASVLDLAERYSNPQTCIVP SYGNLNKSDTPAIHAAFKKCGK--GGRIFIKENTTYA 68
Query_10007 -----
Query_10008 1 mk1QLA[1]-IALALSPCVIADSKQNAKWTD-----IK SRGK-DKDDAPYLAKALKS-----NARVRVPKGTILN 61
Query_10009 1 --MLS[1]LLTALGLVTAVALASKGGSK----PQTCIVP SHNNVNVSDASAVHAAFKKCGK--GGRIVFSENTNYT 65

Query_10001 64 -----VFLSGALRLKSNVELHLAQGAIVKFSQNPEDYLPVLLTRFEGVELVNYSPLIYayEAENIAITGGK 129
Query_10002 69 LNELITLTPCKSCTVELEGLRLSDNIYMLKNAT-NPANLTSATYPNL-----DwK--KSSLVSKTGKG 130
Query_10003 70 LNELITLTPCKSCTVELEGLRLSDNIAYMLKNAT-NPANLTSATYPNLVYYPFQDTFAYLILKDWK--KSSLVSKTGKG 146
Query_10004 73 LNELITFTPCCKGCTVELEGLRLSDNMKYMLRNAT-DTNLSSATYPNLVYYPFQDTVAYLILKDWK--NASLVSNTGKG 149
Query_10005 73 LNELITFTPCCKGCTVELEGLRLSDNMKYMLRNAT-DTNLSSATYPNLVYYPFQDTVAYLILKDWK--NASLVSNTGKG 149
Query_10006 69 LNELITMTPCKSCTVELEGLRLSDNIYMLKNAT-NPANLTSATYPNLVYYPFQDTSYLLKDWK--KSSLVSKTGKG 145
Query_10007 1 ----MNTGLFGTHLREGTLRVKPDFAYWAGNAF-----AVPYQKNSAIWLF-----GGENIVLDGGG 55
Query_10008 62 IATAMNTGLFGTHLREGTLRVKPDFAYWAGNAF-----AVPYQKNSAIWLF-----GGENIVMDGGG 120
Query_10009 66 LGELTMAPICGTVLEGLIQLSDNLTYMLKNETMNPNIATFPHLVYYPFQDTVAYLILKDWK--HSTLISKTGKG 143

Query_10001 130 TLDGQGDdeHMPWKR[25]ERQFGKGHYLRPNFIQPYRCKDILIQGVPLNSPMWQHPVLCENVTVDGKIVIGHG-- 228
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Query_10005 150 LIDGAGQ--LWNAFAG QEILSPGSLRRPVLFTVDNANRVLIDNITLKNPAMFNWVDSKYLNYTNIRLSALSTN 223
Query_10006 146 LIDGAGQ--LWNAFAG QEALDPGSLRRPVLFTVDGADKVSIDNVAMKNPAMFNWVDSKYTYTNIRLSALSAN 219
Query_10007 56 TIDGSGQ--SMWDRPQ N-----ASLIPPLTLVIHQAHNARVSNITFYKTPKIANLVQESSDVVEYIYTVNSVNS 124
Query_10008 121 TIDGSGQ--SMWDRPQ N-----ASLIPPLTLVHQSHNVRISNITFYKTPKIANLVQESSDVVEYIYTVNSVNS 189
Query_10009 144 LIDGLGQ--AWMDAAVG QQILIPGTLRRPVLFTLDGANVTVDNVAMRNAPAMFNWVDSKNVLYKNIRLSALSSN 217

Query_10001 229 ----PNTDGVNPECKWVVIKGFHDSGDCCIAVKSGRN[11]NIVIEHNEKMGHGVTIGSEISGG----VKNVIAE 306
Query_10002 205 KNPPKNAAGDIDTYRSHFVLRGAHVVSDDCFDFKGNST YVTVEDVYQCNSHG--VSVGLAQYRQVLDVVEHVKVR 279
Query_10003 221 KNPPKNAAGDIDTYRSHFVLRGAHVVSDDCFDFKGNST YVTVEDVYQCNSHG--VSVGLAQYRQVLDVVEHVKVR 295
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Query_10005 224 KNPPKNAAGDIDTYRSHFVLRGAHVVSDDCFDFKGNST YITVEDVYQCNSHG--VSVGLAQYRQVLDVVEHVKVR 298
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Query_10007 125 TADMRETGDIDTYRSDGIIIRDSIIHNGDDCVSFKPNST NIVVRNLQCTESHG--ISVGLGEIPGVKDIVRNIYVD 199
Query_10008 190 TADMRETGDIDTYRSDGIIIRDSIIHNGDDCVSFKPNST NVVVRNLQCTESHG--ISVGLGEIPGVKDIVRNIYVD 264
Query_10009 218 KNPPANAGDIDTYRSHFELRDSHVVSDDCFDFKGNST YVTIENVYQCNSHG--ISVGLAQYRQVLDVVEHVKVR 292

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Query_10004 299 NITFVNGDRSSNGARIKWAGPV-GSAIVNDVHYEDITVDNVA-NPLVVDSCYFSQAYCATvrGKPVAS-ITNVTVTNV 375
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Query_10006 295 NITFVNGDSSNGARIKWAGPV-GAAIVNDIHYENITVDNVT-NPLVVDSCYFSQAYCAT--GKPVAS-ITDVTVTNV 369
Query_10007 200 NIWMNK----SENGVRIKTFAGQNRGyGTVDNIIYTNFHNWNSD-YPIIDNCKYTSVANCT--TYP5GIkIDNVL---- 268
Query_10008 265 NIWMSD----SENGVRIKTFAGQNRGyGIVDNIIYTNFHNWNSD-YPIIDNCKYTSVANCT--AYPSGIqINNVLRLNI 337
Query_10009 293 NITFVNGDSSNGARIKWAGPV-GSARVNDVHYEDISVNVNT-NPLVVDSCYFSQAYCAT--GKPVAS-ITNVTVTNV 367

Query_10001 376 KSMGGQY---GIRVLAYDHSPTVGLKVTDSIEI---DGVDIPMELkhVkpDPVFSNLYInGKRVDSHKA----- 436
Query_10002 355 RGNSTGA---VVSSIICPEGSTCDIKFNWNIKP-KNGAAPVYRC--F-SVTSEDLGV-NCTYPTIVNGTFKwP[360] 781
Query_10003 371 RGNSTGA---VVSSIICPEGSTCDIKFNWNIKP-KTGAAPVYRC--F-SVTSEELGV-NCTYPTIVNGTFKwPA 437
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Query_10005 376 RGNSTGA---VVSSVICPKGSICDIFKNWNIKP-KTGAAPVHRC--F-SVTSEELGV-NCTYPTIVNGTFKwRA 442
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Query_10007 -----
Query_10008 338 TGTSSGkynsTVASLVCSPGACSPVYLQDINLSPpSNYSAASTYClnL-NVTGpSAGL FNCTSGDIIGSLYPPA- 410
Query_10009 368 TGTSTGK---VVSSIICPEGSTCDIKFNWNIKP-RTGAPVYRC--F-SVKSEELGV-NCTYPTIVNGTFKwP- 433

```

Figure 6

Multiple alignment of the amino acid sequence of *P. indica* polygalacturonase and *Rhizoctonia solani*. The dashes exhibit gaps to improve the alignment. Conserved residues, no gaps, gaps are represented in red, blue and gray, respectively. Query_10001: pectinase, Query_10002: Extracellular polygalacturonase (*Thanatephorus cucumeris* (strain AG1-IA) (Rice sheath blight fungus) (*Rhizoctonia solani*)), Query_10003: Putative extracellular exo-polygalacturonase (*Rhizoctonia solani* 123E), Query_10004:

Putative extracellular exo-polygalacturonase (*Rhizoctonia solani* 123E), Query_10005: Extracellular exo-polygalacturonase, putative (*Rhizoctonia solani* AG-3 Rhs1AP), Query_10006: Similar to exo-polygalacturonase (*Thanatephorus cucumeris*) (*Rhizoctonia solani*), Query_10007: Putative exo-polygalacturonase X Short=ExoPG (*Thanatephorus cucumeris* (strain AG1-IB / isolate 7/3/14) (Lettuce bottom rot fungus) (*Rhizoctonia solani*), Query_10008: Exo-polygalacturonase (*Rhizoctonia solani* AG-3 Rhs1AP), Query_10009: Exo-polygalacturonase (*Rhizoctonia solani* 123E).

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