Wang et al. Bioresources and Bioprocessing (2015) 2:33
DOI 10.1186/s40643-015-0061-9

Bioresources and Bioprocessing
a SpringerOpen Journal

RESEARCH Open Access

Preliminary investigations on a polygalacturonase from *Aspergillus fumigatus* in Chinese Pu'er tea fermentation

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Abstract

Background: Polygalacturonase is one kind of pectinases which hydrolyze the alpha-1,4 glycosidic bond between galacturonic acid residue. Polygalacturonase has been widely used in the fields of food, biofuel, and textile industries, in which thermostable polygalacturonase is often demanded at high temperatures of 50–60 °C. Herein, we reported a thermostable polygalacturonase producing from *Aspergillus fumigatus* isolated from the pile fermentation of Pu'er tea in China.

Results: The thermophilic polygalacturonase-producing strain was identified as *A. fumigatus* L45 on basis of its morphology, physicochemical properties, and 18S rDNA analysis. The crucial fermentation parameters affecting polygalacturonase activity were optimized by response surface methodology (RSM); the optimum fermentation parameters were the following: inoculums concentration of 0.07 % (v/v), fermentation time of 36 h, pH of 5.0, and temperature of 45 °C. Under the optimized conditions, the highest polygalacturonase activity of 359.1 ± 10.1 U/mL was obtained. The polygalacturonase showed good thermostability and pH stability. The enzyme was activated by metal ions Zn^{2+} and Mg^{2+}, but inhibited by K^{+}. However, Na^{+} and Ca^{2+} showed little effects on its activity. K_m and V_{max} values were estimated to be 35.0 mg/mL and 7.69 μmol/mL/min, respectively.

Conclusions: A polygalacturonase from *A. fumigatus* L45 was preliminarily investigated, the crucial fermentation parameters were optimized by RSM, and the properties of polygalacturonase was examined. The polygalacturonase showed good thermostability and pH stability, which suggested the enzyme has potential applications in the biofuel and textile industries.

Keywords: Polygalacturonase; *Aspergillus fumigatus*; Identification; Response surface methodology (RSM); Enzymatic properties

Background

Pectinases, also called pectinolytic enzymes, are a group of enzymes hydrolyzing pectin substrates in plant cell [1]. Pectinases are mainly composed of polygalacturonase (E.C.3.2.1.15), pectin lyase (E.C.4.2.2.10), pectate lyase (E.C.4.2.2.2), and pectinesterase (E.C.3.1.1.11) [2]. The main function of pectinases is to destroy plant tissue and cell wall structure to allow other depolymerases accession to enhance the productivity [3]. Nowadays, pectinases have been widely used in the fields of food, oils, flavors and pigments extraction, cellulose fibers preparation, coffee and tea fermentations, and oligogalacturonides production [4]. So, it is crucial to screen novel pectinases with remarkable characteristics from native environments [5, 6]. Pectinases are generally produced from bacteria, fungi, and plants; however, most of the commercial pectinases are from fungal sources [7], which optimal pH was between 3.0 and 5.5 [8]. *Aspergillus* species, especially *Aspergillus niger*, is most commonly used for commercial pectinases production [8–10], followed by *Penicillium* species [11–13]. Pectinases from bacteria are mainly alkaline with the optimal pH over 7.0 and mostly used in pulp and paper processing [14–16]. Apart from

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Bacillus species, little information on pectinases from bacteria has been available to date.

In literature, the optimum temperature of pectinases was mostly within the ranges of 30–50 °C. For instance, pectinases from *A. niger* reported by Kittur et al. showed the maximal activity at 47 °C [17]. Singh and Appu Rao [18] claimed that two endo-polygalacturonases from *A. niger* could achieve their maximal activities at 43 and 45 °C. A polygalacturonase from *Saccharomyces cerevisiae* isolated from marsh grape fruit pulp possessed the maximal activity at 45 °C [19]. As well known, high temperatures of 50–60 °C are potentially useful for application in the fabric industry to separate cotton fabrics and polish enzymes, in the paper industry to remedy the retention problems in mechanical pulp bleaching, and in the food industry to clarify juice [20, 21]. Therefore, thermostable pectinases are highly demanded in these modern industries.

Among the aforementioned four pectinases, polygalacturonase is the most commonly used in food industries. So many researchers have been focusing on the isolation and characterization of thermostable polygalacturonases. For instance, a polygalacturonase from thermophilic fungus *Thermoascus aurantiacus* had a high thermostability at 60 °C [1]. The polygalacturonase from *Penicillium* SPC-F 20 isolated from citrus fruit was found to show the highest activity at 60 °C [22]. Maller et al. [23] revealed that the polygalacturonase from *Aspergillus niveus* showed maximum activity at 55 °C and pH 4.0, and the enzyme was stable at 60 °C for 90 min. Martins et al. [24] found that the polygalacturonase from *T. aurantiacus* achieved its highest activity at 60–65 °C and pH 4.5–5.5 and was stable at 50 °C for 1 h.

In this work, a novel thermostable polygalacturonase produced from the microorganism in the pile fermentation of Pu'er tea in Yunnan province, China, is reported for the first time. The microorganism was then identified on basis of its morphology, physicochemical properties, and 18S rDNA analysis. After that, the crucial fermentation parameters affecting polygalacturonase activity were optimized by response surface methodology (RSM). Finally, the enzymatic characterization of the thermostable polygalacturonase was investigated. It is hoped that the findings in this work could facilitate the application of polygalacturonase in biofuel and textile industries.

Methods

Materials

Pu'er tea was gifted from Yunnan Pu'er Tea (Group) Co. (Yunnan province, China). The reason of screening the thermophilic strain from Pu'er tea fermentation pile was the fact that the temperature of the pile fermentation of Pu'er tea could always reach more than 60 °C. The strain was isolated, purified, and identified in our lab and kept in −80 °C fridge. Pectin from citrus peel (galacturonic acid ≥74.0 % (dried basis)) with an item number of P9135 was brought from Sigma-Aldrich (Beijing, China). D-(+)-galacturonic acid was also purchased from Sigma-Aldrich (Beijing, China). DNeasy plant Mini Kit with a production number of 69104 was bought from Qiagen Co. (Beijing, China). TaKaRa Ex Taq DRR001A, 10× Ex Taq Buffer DRR001A, DL2000 DNA Marker SN125-1, dNTPs, and primers were from Takara Co., Ltd (Dalian, China). All other reagents were of analytical grade and bought from local market in Beijing, China.

Compositions of Luria-Bertani (LB) medium are the following: peptone 1 g, yeast extract 0.5 g, NaCl 1 g, agar 1.5 g, and deionized water 100 mL. Compositions of yeast extract peptone dextrose (YEPD) medium are the following: peptone 2 g, yeast extract 1 g, glucose 2 g, agar 1.5 g, and deionized water 100 mL. Compositions of Congo red medium are the following: pectin 0.1 g, sucrose 2 g, $MgSO_4$ 0.05 g, KCl 0.05 g, $Fe_2(SO_4)_3$ 1 mg, K_2HPO_4 0.1 g, $NaNO_3$ 0.3 g, agar 1.5 g, Congo red 0.02 g, and deionized water 100 mL. Compositions of submerge medium are the following: KCl 0.05 g, $MgSO_4$ 0.1 g, $NaNO_3$ 0.1 g, KH_2PO_4 0.1 g, $FeSO_4$ 0.01 g, yeast extract 0.1 g, peptone 0.1 g, sucrose 1.0 g, and deionized water 100 mL. The pH of the medium was adjusted to 5.0 with 1.0 N HCl.

Isolation and identification of thermophilic strain

Isolation of thermophilic strain

Sample preparation of 20 kg of Pu'er tea was fermented for 43 days as optimized previously. The pile of the tea was artificially divided into upper (1.0–10.0 cm), middle (10.0–30.0 cm), and lower (>30.0 cm) layers. The middle layer, with the temperature of 55–65 °C and pH of 4.5–6, was most suitable for the screening of thermophilic strains. So, in this layer, four monitoring points were set for sample collection. Every 3 days, 10 g of tea samples were obtained from each monitoring point at eight o'clock in the morning. Each tea sample was added with 90 mL normal saline and homogenized for 3 min. The homogenate was used for primary screening.

Primary screening: The homogenate was divided into two parts. Each part was respectively incubated in LB and YEPD media at 45 °C for 2 days. After cultivation and crossed purification, the strains were stored in the tube culture at 4 °C. The crossed purification on the solid medium was processed until the strains with the same morphology were examined by microscope. Then the strains were numbered and incubated on Congo red medium using pectin as the solo carbon source. The diameters of the pectin degradation halos (D_p) and the potency index (defined as the ratio of D_p to the diameters of the colonies (D_c)), D_p/D_c) were calculated and used as the standard to screen the pectinase-producing strain.

Secondary screening: After primary screening test, seven microbes with higher potency indexes and D_p values were further tested by submerged fermentation at 45 °C. At regular intervals (12, 24, and 36 h), aliquots of fermentation broth were withdrawn and centrifuged at 12,000 rpm and 4 °C. The supernatant was served as the crude pectinase. The pectinase activity was assayed from the aspect of polygalacturonase activity, i.e., the enzyme activity was determined by measuring the release of reducing groups using the method of 3,5-dinitrosalicylic acid (DNS) [25]. 0.2 mL of crude enzyme solution was mixed with 0.8 mL of 1 % (w/v) pectin and incubated at 50 °C for 10 min following by adding 1 mL DNS reagent. The mixture was heated in boiling water bath for 5 min. After cooling, the solution was diluted to 10 mL and centrifuged at 6000 rpm for 10 min. The supernatant was collected to determine enzyme activity by measuring absorbance at 540 nm. One "polygalacturonase unit" was defined as the amount of the enzyme in 1 mL fermentation broth that hydrolyzed 1 μg reducing sugar (herein, it is galacturonic acid) per minute under standard assay conditions.

Morphology and culture characters of the strain

The morphological and cultural characteristics of the strain were observed by naked eyes after incubated on LB, YEPD, and Congo red media for 24 h. The spores of the strain were examined by SU1510 Hitachi scanning electron microscope (SEM) (Hitachi City, Japan). Before observation, the strain cell was first fixed by glutaraldehyde (2.5 %, pH 7.4) and osmic acid solution (1 %) and then dehydrated by ethanol followed by being dried in a vacuum freeze dryer for 12 h. The specimen was sputter-coated with gold in an ion coater for 2 min.

Physicochemical properties of the strain

A total of 15 tests were conducted for physiological characterization experiments, including 9 types of carbon sources and 6 types of nitrogen sources utilization in submerged fermentation medium. The tested carbon sources were sucrose, pectin, fructose, glucose, maltose, lactose, galactose, and soluble starch. The tested nitrogen sources were beef extract, peptone, $NaNO_3$, $(NH_4)_2SO_4$, urea, and yeast extract. The culture stability of the strain growth on submerged fermentation medium was also examined at 45 °C for 48 h.

18S rDNA identification and phylogenetic analysis of the strain

The methods of 3,5-dinitrosalicylic acid (DNA) isolation, 18S rDNA amplification, and phylogenetic analysis of the strain were in accordance with the methods described by Lin and Liu [26]. The nucleotide sequence coding for the 18S rDNA gene of strain (607 bp) was deposited in the GenBank database with accession number of KJ818296. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.0 software.

Optimization of fermentation parameters using RSM

The single factor experiments revealed that inoculums concentration (0.04–0.08 %), pH (4.5–5.5), and temperature (40–50 °C) were three crucial variables affecting the production of polygalacturonase from *Aspergillus fumigatus* (data not shown). Therefore, the three aforementioned parameters were optimized using RSM. A three-level-three-factor center composite rotation design (CCRD) with three star points and 17 trials (also known as the Box-Behnken (BB) design) was employed to fit a second-order response surface [27, 28]. To reduce the error, experimental designs were carried out at random. A second-order polynomial equation was obtained from the BB design and shown in Eq. 1:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j \qquad (1)$$

where Y represented polygalacturonase activity (dependent variable); β_0 was the constant coefficient; β_i, β_{ii}, and β_{ij} were the coefficients for the linear, quadratic, and interaction terms, respectively; and X_i and X_j represented the independent variables. Subscript i and j were the numbers 1 to 3.

Coefficient of determination (R^2) and analysis of variance (ANOVA) were used to evaluate the fit of the second-order polynomial equation (Eq. 1). The contour plots and three-dimensional response surfaces were attained by holding one of the independent variances constant while changing the level of other variables.

Enzymatic characterization of the polygalacturonase

The purification of polygalacturonase

The polygalacturonase-producing strain *A. fumigatus* was incubated in submerged medium for 36 h. The fermentation broth was centrifuged at 12,000 rpm and 4 °C for 10 min. The supernatant was filtered with a polyethersulfone membrane (0.22 μm) followed by being dialyzed with a dialysis bag (molecular weight cut off, 1 kDa) at 4 °C for 24 h. Then the solution was transferred into another dialysis bag with a molecular weight cut off of 25 kDa to be concentrated by 10 % (m/v) polyethylene glycol (PEG) at 4 °C for 24 h. Mixture solutions of trichloroacetic acid/acetone (v/v, 1:4) were put into the concentrated enzyme solution to precipitate the enzyme at −20 °C for 12 h. The volume ratio of enzyme solution to mixture organic solvents was 1:4. After precipitation, the enzyme solution was centrifuged at 12,000 rpm for 10 min. The protein pellet was obtained and washed by 100 % acetone for three times and dried as the crude enzyme for the following experimental use. The enzyme protein content was monitored

by Bradford protein assay method using Bradford reagent from BioRad at a wavelength of 595 nm with a standard calibration curve of BSA. The purity and molecular weight of polygalacturonase were measured by SDS-PAGE [8].

Effects of pH, temperature, and metal ions on polygalacturonase activity

The effects of pH (pH = 2.0 to 8.0), temperature (20 to 80 °C), and metal ions (K^+, Na^+, Ca^{2+}, Zn^{2+}, and Mg^{2+}) on polygalacturonase activity were investigated. The polygalacturonase activity was assayed according to the method depicted in "Isolation of thermophilic strain" section. The relative polygalacturonase activity was estimated by Eq. 2:

$$\text{Relative enzyme activity (\%)} = \frac{\text{Enzyme activity after treatment}}{\text{Enzyme activity before treatment}} \times 100\% \quad (2)$$

Besides, the pH stability and thermostability of polygalacturonase were also evaluated. For pH stability, the enzyme was incubated at a certain pH (pH 2–8) for 24 h before activity measurement. In view of thermostability, the enzyme was incubated at a certain temperature (20–80 °C) for 2 h before activity measurement.

Determination of K_m and V_{max} values of enzyme kinetics

The kinetics parameters of polygalacturonase were fitted to the Michaelis-Menten Eq. 3:

$$v = \frac{V_{\max} \times [S]}{K_m + [S]} \quad (3)$$

where v is the reaction velocity (dependent variable), $V_{\max}$ is the maximum velocity, K_m is the Michaelis-Menten constant, and $[S]$ is the concentration of substrate (independent variable). To obtain the values of K_m and $V_{\max}$, Eq. 3 was usually converted into Lineweaver-Burk equation by taking the reciprocal:

$$\frac{1}{v} = \frac{K_m + [S]}{V_{\max} \times [S]} = \frac{K_m}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (4)$$

Therefore, the values of K_m and $V_{\max}$ can be obtained from the intercept and slope of the $1/v$ vs. $1/[S]$ plot [29]. Herein, the substrate (pectin) concentrations used were in the range of 0.2 to 1.8 % (w/v). Experiments were conducted at pH 5 and 45 °C.

Table 1 Primary selection of polygalacturonase-producing isolates on Congo red medium

Potency index (D_p/D_c)	Number of isolates
$D_p/D_c > 3.5$	7
$3.5 > D_p/D_c > 2.0$	58
$2.0 > D_p/D_c$	78
$D_p/D_c = 0$	57

Results and discussion

Isolation and identification of thermophilic strains

Isolation of thermophilic polygalacturonase-producing strains

To deeply survey the thermophilic polygalacturonase-producing strains, 84 bacteria and 116 fungi were separated from the pile fermentation of Pu'er tea. The total 200 isolates were subjected to primary selection via Congo red medium using pectin as the sole source of carbon. The results showed that 57 isolates (28.5 %) could not grow on the Congo red medium, while 143 isolates (71.5 %) showed different diameters of pectin degradation halos, indicating that they have extracellular pectinolytic activities. Among the 143 pectinolytic isolates, the potency indexes (D_p/D_c) were estimated to screen the strains producing extracellular pectinolytic activity [4]. As shown in Table 1, 7 isolates had a potency index above 3.5, which were categorized as high pectinase-producing strains. Fifty-eight isolates presenting the potency index between 2.0 and 3.5 were classified into moderate pectinase-producing strains. While other 78 isolates with the potency index less than 2.0 were relative to low pectinase-producing microbes. Seven high pectinase-producing strains were further screened

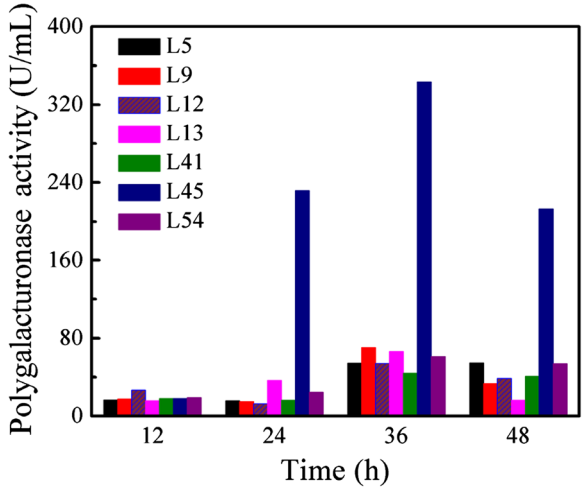

Fig. 1 Secondary selection of the seven high polygalacturonase-producing isolates using submerged fermentation. The serial numbers of the isolates in the total 200 isolates were given in the figure

through submerged fermentation by quantitative estimation of pectinase activities.

In the submerged fermentation, the strains were screened in the aspect of polygalacturonase activity using the DNS method (Fig. 1). It was demonstrated that strain L45 presented the highest polygalacturonase activity of 343 U/mL after 36 h of fermentation. Furthermore, the propagating stability of strain L45 was evaluated, and the results showed that the polygalacturonase activity could maintain *ca*. 94 % after five generations. It indicated that the strain L45 showed good genetic stability. Hence, it was selected as the potential source of polygalacturonase in the following experiments.

Identification of the strain L45

The strain L45 was identified according to its characteristics of morphology, physicochemical properties, and 18S rDNA. It can be seen from Fig. 2 that the colony of strain L45 was circular regardless of the growth media types, i.e., LB, YEPD, and Congo red media. At the early growth stage, the colony of the strain was white and grew mycelium. SEM analysis results (Fig. 3) showed that the length of the mycelium was about 2.5 nm, and the diameter of the spore was about 15 μm. In the maturing stage, the colony of the strain displayed smoky green in the center and white around with spores on the surface. Simultaneously, the strain could grow on the medium using sucrose, pectin, fructose, glucose, maltose, lactose, galactose, or soluble starch as the sole carbon source and beef extract, peptone, $NaNO_3$, $(NH_4)_2SO_4$, urea, or yeast extract as the sole nitrogen sources, respectively.

18S rDNA molecular identification, coding for 18S ribosomal RNA, is widely used in molecular analysis to

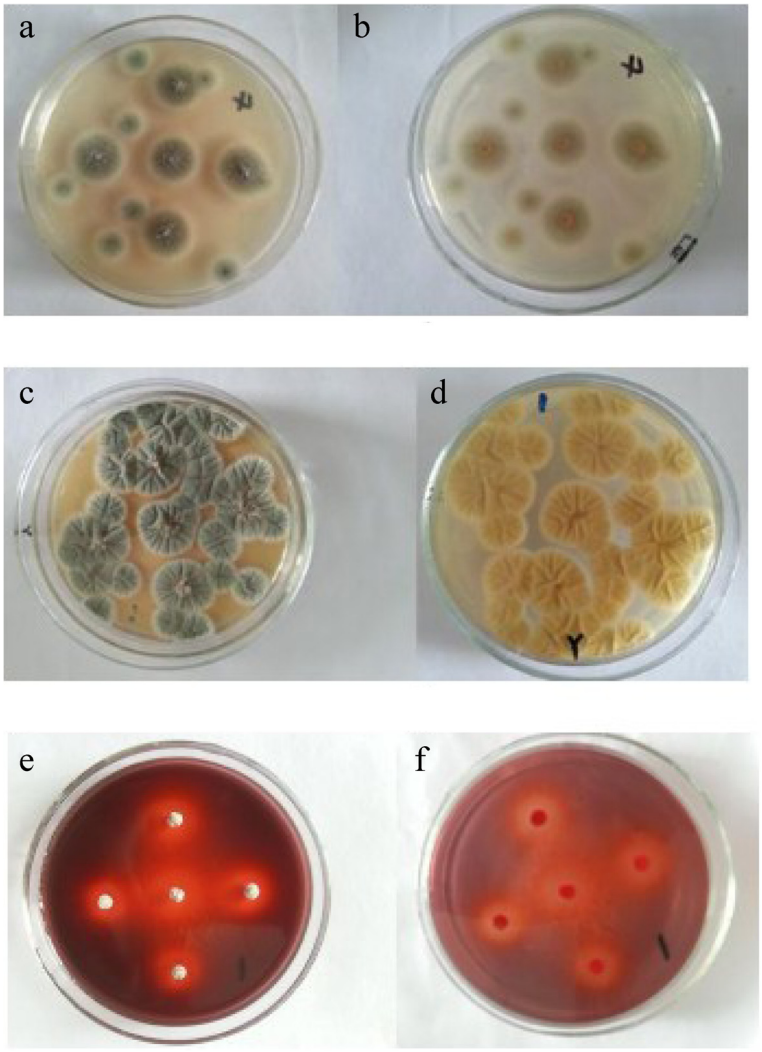

Fig. 2 Morphology of *Aspergillus fumigatus* L45 cultivated on LB (**a** and **b**), YEPD (**c** and **d**), and Congo red media (**e** and **f**)

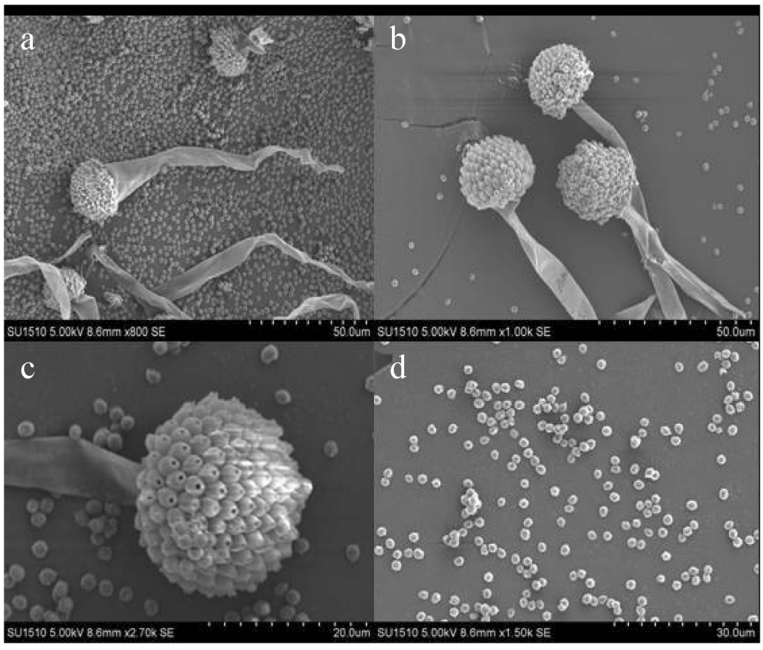

Fig. 3 SEM images of *Aspergillus fumigatus* L45. **a**, **b**, and **c** stand for the SEM images of mature strain; **d** stands for the SEM image of immature strain

reconstruct the evolutionary history of organisms due to its slow evolutionary rate [30, 31]. Herein, the 18S rDNA of the strain L45 was firstly extracted, amplified, and sequenced. Then the 607 bp sequence, as presented in Fig. 4a, was registered in the GenBank database with an accession number of KJ818296. The sequence was compared with previously published sequences of microorganisms in GenBank database using the BLAST program for species identification (http://blast.ncbi.nlm.nih.gov/Blast.cgi). As shown in Fig. 4b, a phylogenetic tree was obtained by applying the neighbor joining method. It can be seen that the L45 strain was 96 % similar to *A. fumigatus* strain NTGMP02. So the strain L45 belongs to *A. fumigatus* genus. However, the phylogenetic analysis indicates that strain L45 would represent a distinct phyletic line suggesting a new genomic species. Therefore, the strain was identified as *A. fumigatus* L45.

Fermentation parameters optimization by RSM

Single factor experiments on effects of fermentation time, carbon source, nitrogen source, metal ions, inoculum concentration, temperature, and pH on polygalacturonase activity were conducted to determine the main effective factors (data not shown). Results revealed that the main effective factors were inoculum concentration, temperature, and pH for the polygalacturonase activity produced from the fermentation of *A. fumigatus* L45. On the basis of these results, the crucial parameters affecting polygalacturonase activity were optimized using RSM through BB design. The optimized experimental designs and results were shown in Table 2.

Through Design Expert 8.0 analysis, a polynomial quadratic equation (Eq. 5) was obtained in terms of coded factors from the experimental data in Table 2:

$$\begin{aligned} Y = {} & 360.12 + 7.58 \times X_1 - 2.52 \times X_2 - 2.39 \times X_3 \\ & + 1.51 \times X_1 \times X_2 - 0.94 \times X_1 \times X_3 + 5.22 \\ & \times X_2 \times X_3 - 12.78 \times {X_1}^2 - 66.28 \\ & \times {X_2}^2 - 46.48 \times {X_3}^2 \end{aligned} \quad (5)$$

From the ANOVA analysis in Table 3, the R^2 was 0.9494, and the lack of fit tests of model was 0.96. These values indicated that model equation was highly significant and fitted the response surface regression analysis of the data.

When one variable was set to zero, the contour plots and the three-dimensional response surfaces of the other two variables were graphed in Fig. 5. The figure clearly interpreted the optimal combination of variables and showed the interactions between the two varying factors and their effects on polygalacturonase activity. Therefore, it can be deduced from the figure that the optimal conditions were inoculums concentration 0.07 % (v/v), temperature 44.91 °C, and pH 4.99. Under the optimized condition, the polygalacturonase activity was predicted to be 361.3 U/mL.

In order to verify the reliability of the derived model (Eq. 5), experiments were carried out in triplicate under optimized conditions. The results showed that the practical polygalacturonase activity of 359.1 ± 10.1 U/mL

a 18S rDNA sequences

ACTGGGTGCTACCTGATCCGAGGTCACCTTAGAAAAATAAAGTTGGGTGTC

GGCTGGCGCCGGCCGGGCCTACAGAGCAGGTGACAAAGCCCCATACGCTC

GAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGAG

AGGGGGACGGGGGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGAC

GCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAGATTGCGTTCA

AAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC

TGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAA

CTGATTACGATAATCAACTCAGACTGCATACTTTCAGAACAGCGTTCATGTT

GGGGTCTTCGGCGGGCGCGGGCCCGGGGGCGCAAGGCCTCCCCGGCGGCC

GTCGAAACGGCGGGCCCGCCGAAGCAACAAGGTACGATAGACACGGGTG

GGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGTTC

ACCTACGGAAACCTTGTTACGACTTTTACTTCCTCAATGTGGACCAAGAA

b phylogenetic analysis

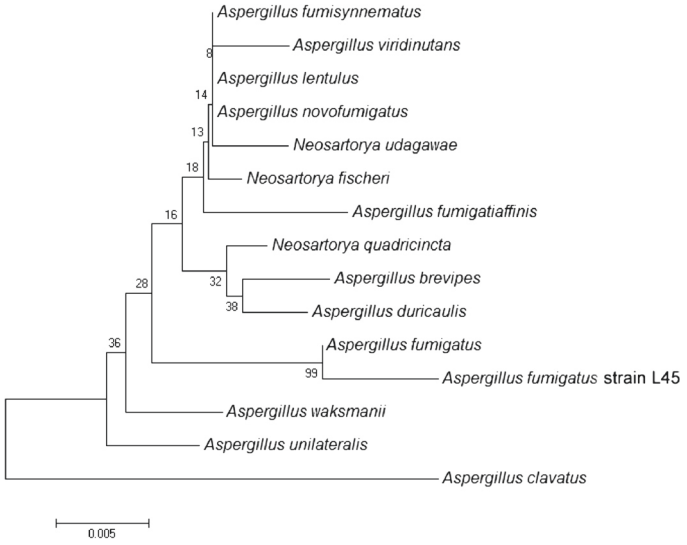

Fig. 4 18S rDNA gene sequence (**a**) and phylogenetic analysis (**b**) of *Aspergillus fumigatus* L45. The numbers above (*under*) the nodes represent bootstrap values

was in highly agreement with the predicted value of 361.3 U/mL. As a result, the validity of the regression model was satisfactory.

In literature, many studies referred to the optimal fermentation parameters for the production of polygalacturonase. For instance, a polygalacturonase produced from *A. fumigatus* Fres. MTCC 4163 showed maximum activity of 1270 U/g when fermented at 50 °C and pH 4.0–5.0 for 2–3 days [4]. A polygalacturonase activity was the highest (ca. 2 U/mL) when the strain *T. aurantiacus* was cultivated at 45 °C and pH 5.5 for 5 days in submerged fermentation with pectin as the substrate [1]. *Penicillium*

Table 2 RSM design and experimental results

No.	Inoculums concentration (%)	Temperature (°C)	pH	Polygalacturonase activity (U/mL)
1	0.08	50	5.0	278.3
2	0.04	40	5.0	286.7
3	0.06	45	5.0	364.9
4	0.08	40	5.0	305.8
5	0.06	50	4.5	250.2
6	0.04	50	5.0	253.3
7	0.08	45	5.5	297.1
8	0.06	50	5.5	264.8
9	0.06	40	4.5	240.3
10	0.06	40	5.5	234.0
11	0.06	45	5.0	360.0
12	0.06	45	5.0	364.9
13	0.06	45	5.0	351.5
14	0.08	45	4.5	312.8
15	0.04	45	5.5	290.8
16	0.04	45	4.5	302.6
17	0.06	45	5.0	359.0

SPC-F 20 produced the maximum polygalacturonase activity of ca. 1.8 U/mL when the fungus was cultivated at 30 °C and pH 5.0 for 3 days with pectin as the substrate [22]. *A. niger* DMF 27 produced the maximum polygalacturonase activity (3.8 U/mL for endo-polygalacturonase and 11.1 U/mL for exo-polygalacturonase) when fermented at 30 °C and pH 5.0 for 72 h using pectin as the substrate [32]. *A. foetidus* var. pallidus Ege-K-730 produced the highest polygalacturonase activity of 122 U/mL when incubated at pH 2.3 for 72 h [33]. By comparison, these results with ours (maximum activity of 359.1 U/mL at 45 °C and pH 5 for 36 h), it is obviously seen that the optimal fermentation temperature and pH in this study were moderate, while the fermentation time was much shorter than that in literature. Besides, since the activity could be greatly affected by different source of substrates, the polygalacturonase activities in literature could just be roughly compared. In summary, *A. fumigatus* L45 would be a promising fungus to produce thermostable polygalacturonase in the future.

Enzymatic properties of the polygalacturonase

Polygalacturonase produced by *A. fumigatus* L45 was purified according to the method described by Fachin et al. [34]. Pooled fractions of polygalacturonase were applied on a gel filtration column (Hi Load 16/60 Superdex 75). The activity and molar mass of purified polygalacturonase were estimated. From Fig. 6, the molecular weight of polygalacturonase was about 45 kDa according to SDS-PAGE image, which is almost in agreement with the data reported in the literature [34–37] and the activity was 25,500 U/mg after purification. Furthermore, the enzymatic properties of polygalacturonase, such as pH effect and stability, temperature effect and thermostability, metal ions effect, and kinetic parameters (K_m and V_{max}), were investigated. The results were shown in Fig. 7.

Table 3 The main effective analysis of variance (ANOVA) of RSM data

Source	Sum of squares	DF	Mean square	*F* value	*P* value
Model	31248.93	9	3472.10	14.59	0.0009**
X_1	459.44	1	459.44	1.93	0.2073
X_2	50.73	1	50.73	0.21	0.6583
X_3	45.77	1	45.77	0.19	0.6742
X_1 X_2	9.08	1	9.08	0.04	0.8507
X_1 X_3	3.56	1	3.56	0.01	0.9062
X_2 X_3	108.99	1	108.99	0.46	0.5203
X_1^2	687.33	1	687.33	2.89	0.1331
X_2^2	18494.31	1	18494.31	77.70	<0.0001**
X_3^2	9095.86	1	9095.86	38.21	0.0005**
Residual	1666.17	7	238.02		
Lack of fit	1545.40	3	515.13	17.06	0.96
Pure error	120.77	4	30.19		
Total	32915.09	16			
R^2	0.9494				

DF degree of freedom, R^2 the coefficient of determination
**means significance

pH effect and pH stability

The effect of pH on polygalacturonase activity and its pH stability were investigated, and the results were depicted in Fig. 7a. It was observed that the polygalacturonase showed the maximal enzyme activity at pH 5.0. Beyond that pH value, the polygalacturonase activity presented a decreasing tendency. This phenomenon was in accordance with those results in literature [24, 10, 38]. pH stability of polygalacturonase showed that the residual activity was maintained over 80 % after 24 h of incubation from pH 2.0 to 7.0. By comparing the pH stability with other polygalacturonase listed in Table 4, it can be indicated that the polygalacturonase from *A. fumigatus* L45 showed high pH stability within neutral pH values.

Temperature effect and thermostability

Experiments were conducted to investigate the effect of temperature on polygalacturonase activity and its thermostability, and the results were shown in Fig. 7b. It was revealed that the optimum temperature for polygalacturonase activity was 60 °C. Even at 80 °C, the polygalacturonase could still keep 63 % catalytic activity. The thermostability

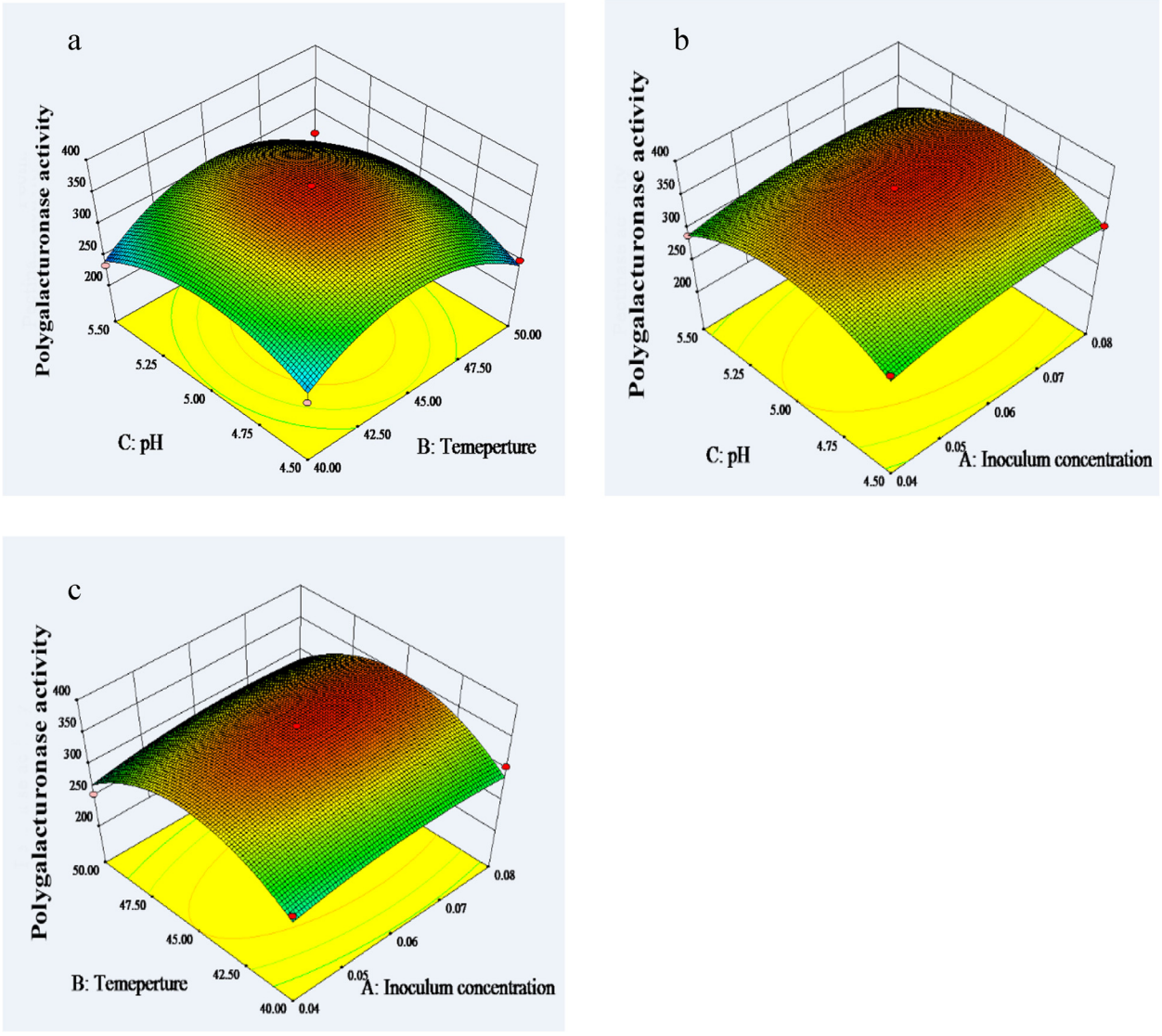

Fig. 5 The contour plots and three-dimensional response surfaces. **a** Effects of pH and temperature on polygalacturonase activity; **b** effects of pH and inoculum concentration on polygalacturonase activity; **c** effects of temperature and inoculum concentration on polygalacturonase activity

of the polygalacturonase showed that the residual activities of 100, 80, and 54 % were obtained at 50, 60, and 80 °C for 2 h incubation, respectively.

In literature, a polygalacturonase from *A. fumigatus* strain 4 showed the highest activity at 65 °C. However, the enzyme was only stable at 4–6 °C. When stored at 26–30 °C, its activity was reduced by 24 % [39]. Another pectinase from *A. fumigatus* showed maximal activity at 40 °C and pH 5.5 and was stable up to 40 °C [40]. A polygalacturonase produced by *Penicillium* strain presented a residual activity of 48 % after 2 h incubation at 60 °C [22]. A polygalacturonase producing from *T. aurantiacus* showed a residual activity of 13 % after 1-h incubation at 60 °C [1]. Other related data were shown in Table 4. Through comparison of characters of polygalacturonase reported in literature, it was confirmed that the polygalacturonase from *A. fumigatus* L45 showed good thermostability.

Effects of metal ions on enzyme activity

The effects of different metal ions on the polygalacturonase activity were evaluated, and the results were shown in Fig. 7c. It can be seen that metal ions Na^+ and Ca^{2+} showed almost noninfluence on the polygalacturonase activity, K^+ inhibited the polygalacturonase activity by *ca*. 25 %, Mg^{2+} enhanced the polygalacturonase activity by 25 %, and Zn^{2+} could increase the polygalacturonase activity to 1.80-fold. The activity of a polygalacturonase

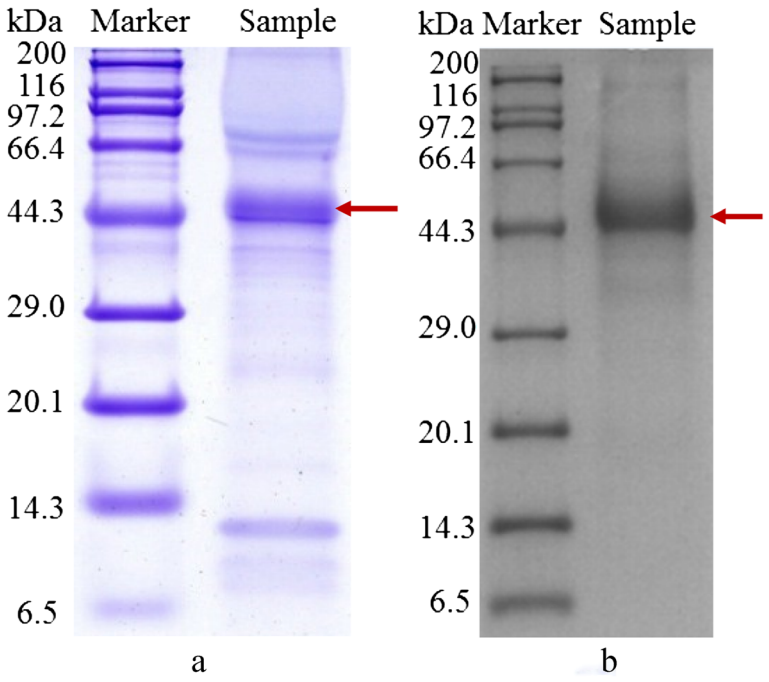

Fig. 6 SDS-PAGE for purification of polygalacturonase from *Aspergillus fumigatus* L45 fermentation broth. **a** The PAGE image of broth after primary concentration; **b** the PAGE image of enzyme after dialyzing purification

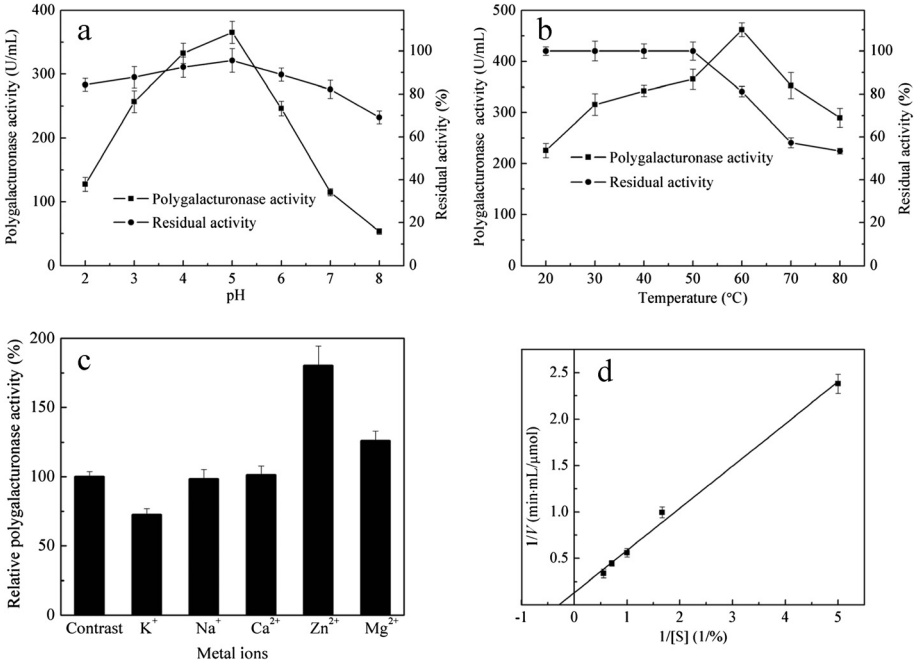

Fig. 7 Characteristics of the polygalacturonase. **a** Effect of pH on the polygalacturonase activity and pH stability for 24-h incubation; **b** effect of temperature on the polygalacturonase activity and thermostability for 2-h incubation; **c** effects of metal ions on the polygalacturonase activity; **d** Lineweaver-Burk plot for polygalacturonase activity

Table 4 Comparisons of enzymology properties of polygalacturonase reported in the literatures

No.	Strains	Optimal temperature (°C)	Optimal pH	K_m (mg/mL)	Stability	Refs.
1	*Sckrotinia sclerotiorum*	45	5.0	0.83	Mostly inactive at 65 °C,	[42]
2	*Aspergillus fumigatus* strain 4	65	3.5–4.5	-	Stable between 3.0 and 9.0 and at 4–6 °C; activity reduce by 24 % at 26–30 °C	[39]
3	*Aspergillus niger*	43	3.8–4.3	0.12	$T_m = 43$ °C	[18]
4	*Aspergillus niger*	45	3, 4.6	0.72	$T_m = 46$ °C	[18]
5	*Penicillium viridicatum* Rfc3	55	5	–	Stable in neutral pH range and at 40 °C for 1 h	[43]
6	*Thermoascus aurantiacus* 179-5	65	5	–	Stable in the acidic to neutral pH range at 60 °C for 1 h	[44]
7	*Penicillium* SPC-F 20	60	5.5	–	A residual activity of 48 % after 2 h of incubation at 60 °C	[22]
8	*Rhizopus microsporus var. rhizopodiformis*	65	3.5		Activity decreased by 28 and 39 at 60 and 65 °C, respectively, for 90-min incubation	[45]
9	*Thermoascus aurantiacus*	60	5.0	-	Stable in pH 3.0–4.5 at 55 °C for 1 h	[1]
10	*Rhizomucor pusilis*	55	5.0	0.22	Stable up to 50 °C for 120 min at pH 4.0–5.0; decreased rapidly above 60 °C and above pH 5.0	[8]
11	*Penicillium oxalicum SX6*	50	5.0	–	Stable at pH 3.5–6.0 and 40 °C	[13]
12	*Aspergillus fumigatus*	60	5.0	35	Stable at pH 2.0–7.0 and 60 °C for 2 h	In our work

from *A. niger* was inhibited by K^+, Zn^{2+}, and Ca^{2+} to 55.83, 43.11, and 73.02 %, respectively, while promoted by Mg^{2+} by 11.55-fold [8]. The activity of another polygalacturonase from thermophilic fungus *T. aurantiacus* was inhibited 59, 77, and 100 % by Zn^{2+}, Mn^{2+}, and Hg^{2+}, respectively [24].

Kinetic parameters (K_m and V_{max})

The enzyme kinetic parameters K_m and V_{max} from Michaelis-Menten equation were determined through the Lineweaver-Burk plot (seen in Fig. 7d). V_{max} represents the maximum rate the system can achieved at saturating substrate concentrations. K_m is the substrate concentration at which the reaction rate is half of V_{max}; therefore, K_m is negatively related to the affinity of the enzyme to the substrate. The K_m and V_{max} values of polygalacturonase were 35.0 ± 2.8 mg/mL (or 3.5 ± 0.28 %) and 7.69 ± 0.59 μmol/mL/min, respectively, in this work. Further, we obtained the K_{cat} value of 1.38 min^{-1} following the equation of $K_{cat} = V_{max}$/enzyme concentration. So K_{cat}/K_m was 0.04 mg/mL/min.

Kant et al. [8] demonstrated that the K_m and V_{max} values of a purified polygalacturonase from *A. niger* MTCC 3323 were 0.083 mg/mL and 18.21 μmol/mL/min, respectively, using polygalacturonic acid as a substrate. Rashad et al. [41] confirmed that K_m and V_{max} values of a purified polygalacturonase from *Pleurotus ostreatus* were 1.33 mg/mL and 28.6 μmol/mL/min, respectively, using pectin as a substrate. Other K_m values from references were presented in Table 4. The high K_m and lower V_{max} values of our polygalacturonase mainly stemmed from the fact that the enzyme was just primarily purified and had a relatively low purity.

Conclusions

A thermophilic polygalacturonase-producing strain *A. fumigatus* L45 was screened from the pile fermentation of Pu'er tea in China. The fermentation parameters of the strain were optimized by RSM, and the result showed that the polygalacturonase achieved a maximum activity of 359.1 ± 10.1 U/mL under the optimized conditions (inoculum concentration of 0.07 % (v/v), temperature of 45 °C, and pH 5.0). The optimal temperature and pH for polygalacturonase were 60 °C and pH 5.0. The enzyme maintained 100, 80, and 54 % of its activity after incubated at 50, 60, and 80 °C for 2 h, respectively. The good thermostability and pH stability suggested that the enzyme has potential applications in the biofuel and textile industries. The polygalacturonase gene cloning, protein structural biology, and applications are ongoing in our lab.

Abbreviations

ANOVA: analysis of variance; D_c: the diameter of the colony; DNA: 3,5-dinitrosalicylic acid; D_p: the diameters of the pectin degradation halos; LB: Luria-Bertani; R^2: coefficient of determination; RSM: response surface methodology; SEM: scanning electron microscope; YEPD: yeast extract peptone dextrose; BB design: Box-Behnken design.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SW wrote the draft manuscript. ZL performed all the experimental works. LW checked the figures and referecnes formation. XY confirmed the SDS-page experimental data. and YL designed the experiments and proofread the manuscript. All authors read and approved the final manuscript.

Received: 26 March 2015 Accepted: 30 June 2015
Published online: 21 July 2015

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