Enzymatic Characterization of Pectinex XXL, a Pectinase Produced by *Aspergillus niger*, and Its Application in Fruit Juice Production

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bstract: Pectinex XXL, a commercially prepared pectinase, was investigated for its potential application in the fruit juice industry. Polygalacturonic acid was used as the substrate for determining the enzymatic properties of Pectinex XXL using the DNS method. According to the results, the optimal pH for Pectinex XXL activity was 4.5, and the enzyme was stable in the pH range of 3.0~4.5. The optimal pH and pH stability range are consistent with those of some tropical and subtropical fruits. The optimal temperature for Pectinex XXL activity was 60 ℃ , and the enzyme remained stable after one hour in a water bath set at 40 ℃ . Additionally, the enzymatic activity was not inhibited in the presence of 1 mmol/L of Na^{+}, Mg^{2+}, Ba^{2+}, Co^{2+}, Zn^{2+}, and Fe^{2+}, whereas it was slightly inhibited in the presence of 2 mmol/L of K^{+} and Fe^{2+} and partially inhibited in the presence of 1 and 2 mmol/L of Ca^{2+} and Mn^{2+}, demonstrating its good stability in acids and excellent thermal catalytic performance. Based on the above experimental results, depectinization experiments were performed on plantain and cherry tomato juices using different amounts of Pectinex XXL. After one hour reaction with 16 U/mL of the enzyme, the yields of the plantain and cherry tomato juices were substantially increased by 119.03% and 15.97%, respectively, while their light transmittance was remarkably enhanced by 37.65% and 12.35%, respectively. Furthermore, the enzyme reduced the viscosity of the plantain and cherry tomato juices by 88.29% and 29.50%, respectively. The juice production experiments confirmed that this enzyme can significantly improve the yield and light transmittance of plantain juice, while effectively reducing its viscosity. These findings indicate the potential of Pectinex XXL in the industrial production of plantain juice.

Key words: plantain; cherry tomato; fruit juice; *Aspergillus niger*; pectinase

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黑曲霉Pectinex XXL菌株产果胶酶的酶学特性及其在果汁生产中的应用

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摘要：该研究旨在探讨商业果胶酶制剂 Pectinex XXL 的酶学性质及其在果汁工业生产中的应用潜力。该实验

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以聚半乳糖醛酸为底物，使用 DNS 法对 Pectinex XXL 的酶学特性进行了表征，结果表明：其最适 pH 值为 4.5，且在 pH 值 3.0~4.5 稳定。该酶最适 pH 值和 pH 稳定范围与一些热带和亚热带水果的 pH 值一致。其最适温度为 60 ℃，且在 40 ℃水浴 1 h 后保持稳定。此外该酶活性不受 1 mmol/L Na^+、Mg^{2+}、Ba^{2+}、Co^{2+}、Zn^{2+}、Fe^{2+} 抑制，受 2 mmol/L K^+ 和 Fe^{2+} 轻微抑制，受 1 mmol/L 和 2 mmol/L Ca^{2+} 和 Mn^{2+} 部分抑制，证明其具有良好的酸稳定性和热催化特性。在以上实验基础上探讨不同酶量对芭蕉果汁和圣女果汁进行脱胶实验，反应 1 h，该酶（16 U/mL）能显著提高芭蕉果汁和圣女果汁的产量和透光率，分别为 119.03%、15.97% 和 37.65%、12.35%，并使芭蕉果汁和圣女果汁粘度分别下降 88.29% 和 29.50%。果汁生产实验证明，该酶能显著提高芭蕉果汁出汁率和透光率，并能有效降低芭蕉果汁粘度。因此 Pectinex XXL 在芭蕉果汁工业生产中具有应有潜力。

关键词：芭蕉；圣女果；果汁；黑曲霉；果胶酶

Pectin is a type of heteropolysaccharide widely present in the primary wall and inner layer of plant cell walls, which promotes the formation of plant morphology through cross-linking with cellulose and hemicellulose[1]. Pectin molecule is composed of a main chain constructed of galacturonic acids linked by *α*-1,4-glycosidic bonds, and methanol and 12 monosaccharides are linked to the main chain[2]. Pectinase is a kind of enzyme that can degrade pectin, which belongs to the complex enzyme. Pectinase is mainly composed of pectinesterase and pectin depolymerase[3]. Pectinesterase can remove the methoxyl groups from pectin substances and generate pectinic acid and methanol. Pectin depolymerase includes lyase and polygalacturonase. Lyase plays a decomposition role by trans elimination of pectin acid polymer, while polygalacturonase can cleave the *α* 1-4 glycosidic bond by (endo-or exo-) hydrolysis. In the pectinase family, endo-polygalacturonase can randomly hydrolyzes the inner *α*-1,4 linkages of pectin, which leads to a rapid decline in substrate viscosity and ultimately improves the production efficiency and yield of downstream products[4]. Therefore, endo-polygalacturonase is the most important component of commercial pectinase.

Plantain (*Musa basjoo* Siebold) is a kind of world-wide famous fruit[5]. However, plantain is a perishable fruit. The metabolic enzymes of harvested plantain are highly active and cause rapid deterioration of quality. And the highly active plant cell wall component degrading enzymes lower the intensity of fruit peel and increase the risk of being attacked by microorganism and damage in the transport process[6]. Due to the local policy, market, climate and management, the global plantain yield suffer loss of 10%~30% every year, and even as high as 40% in some major producing countries[7].

Developing juice and wine using plantain as material can reduce the loss of yield and bring additional value of plantain[8]. Recently, some less-known fruits were used as material for juice production[9]. These fruit juices showing different flavors and nutrition component from common seen fruit juices gave more choices for the public. Due to the especially high starch content (up to 34.5% of dry solid) of plantain[5], it is used as staple food and ranged among the top staple foods in some tropical and subtropical countries[12]. However, plantain is a good candidate as material for industrial fruit juice production, because it is rich in aromatic compound, vitamins, K^+ and other nutritional materials[5,11]. However, there is still lack a commercial trial case report for plantain juice production.

Endo-polygalacturonase play an important role in fruit juice production[13]. It was reported that endo-polygalacturonases PoxaEnPG28A[14] and PoxaEnPG28A-Pp[15] have been used in plantain juice production, but PoxaEnPG28A and PoxaEnPG28-Pp were produced by recombinant *Pichia pastoris* GS115, the cost for producing enzyme was high and were not suitable for industrial used. Some *Aspergillus niger* strains are excellent commercial plant cell wall component degrading enzymes producers and their commercial enzyme reagents are global-available. Among them, the pectinase reagent Pectinex XXL has been reported can be applied for laboratory scale extraction of bioactive compounds from fruit pomaces[16] and edible film extraction[17]. But the

application of this reagent in fruit juice is rare[18]. Thus, estimating the performance of Pectinex XXL in plantain juice production was attractive for industry.

Cherry tomato (*Lycopersicon esculentum*) is rich in vitamin and is favorite for many people[19]. However, it is a perishable fruit and suffered from cost lost due to its highly active internal plant cell wall component degrading enzymes[20]. Producing fruit juice and wine are good resolutions.

In this study, the enzymatic characteristics of the pectinase reagent Pectinex XXL was determined. And based on its enzymatic characteristics, the conditions of juice production were set. Finally, Pectinex XXL was found functioned well in plantain and cherry tomato juices production.

1 Materials and methods

1.1 Materials and chemicals

Newly harvested fresh plantain and cherry tomato were fully mature but not over ripen, and they were purchased from local agricultural market of Yongning region, Nanning city, China. *Aspergillus niger* pectinase reagent Pectinex XXL was purchased from Novozymes (Bagsvaerd, Denmark). Polygalacturonic acid (PGA), carboxyl methyl cellulose (CMC), beechwood xylan and soluble starch were from Megazyme (Wicklow, Ireland). Other used chemicals were all of analytical pure grade.

1.2 Enzyme activities determination

All enzyme activities were determined in our laboratory at pH values 4.5 and 50 ℃ for 10 minutes. Pectinase activity was determined using method reported previously[16]. Cellulase, xylanase and amylase activities were determined according to previous reports using CMC[21], xylan[22] and starch[23] as substrate, respectively. One unit of enzyme activity was defined as the enzyme amount needed to produce one micro mole product in one minute. Galacturonic acid, xylose and glucose were used as substrate to construct standard curves for pectinase, xylanase and cellulase/amylase activities calculation, respectively.

1.3 Effect of pH values and temperature on the enzyme activity and stability of Pectinex XXL

The pectinase activity of properly diluted Pectinex XXL was determined at pH values 3.0 to 6.0, and the pH values responding to the highest activity was the optimal pH values of the pectinase. The pectinase activity was also determined at 30 ℃ to 75 ℃, and the temperature responding to the highest activity was the optimal temperature of the pectinase. All enzyme activities at other pH values (or temperature) were calculated as relative activities according to the optimal pH values (or temperature) as 100%, respectively.

One volume of properly diluted Pectinex XXL was mixed with nine volumes of 0.1 mol/L buffers with different pH values and incubated at 4 ℃ for 24 hours, and then the residual enzyme activity was determined. Properly diluted Pectinex XXL was incubated at 40 ℃ to 60 ℃ for 15, 30, 45 and 60 minutes, and the residual enzyme activities were determined. The residual enzyme activity of enzyme mixed with super pure water and incubated at 4 ℃ was set as 100%, and all enzyme activities of incubated enzyme samples (at different pH values or temperatures) were calculated as relative activities.

1.4 Effect of metal ions on the activity of Pectinex XXL

Inorganic salt NaCl, KCl, $MgCl_2$, $CaCl_2$, $CuCl_2$, $MnCl_2$, $BaCl_2$, $CoCl_2$, $FeCl_2$ and $ZnCl_2$ were respectively added into the enzymatic determination system of Pectinex XXL. Enzyme activity in enzymatic determination system without extra inorganic salt was setted as 100%, and other enzyme activities were calculated as relative activities.

1.5 Effect of Pectinex XXL on plantain and cherry tomato juice extraction

Peeled fresh plantain or cherry tomato was chopped and mixed with equal weight of ultra pure water, then pulped. Different doses of pectinase were added and incubated at 45 ℃ for 60 minutes (played in triplicate), and then were centrifuged at 3 500 g for 3 minutes. The yield (or light transmittance and viscosity) of the set (mean value of data from triplicate-set) with 0 U pectinase addition were set as 100%, and values after pectinase addition were calculated as relative values. Data was measured according to methods reported previously[14].

Table 1 Enzyme activities of Pectinex XXL

Enzyme	Pectinase	Cellulase	Xylanase	Amylase
Activity/(U/mL)	36 670.27 ± 5.73	1 236.00 ± 3.24	5 366.00 ± 7.11	1 321.00 ± 1.08

1.6 Statistical analysis

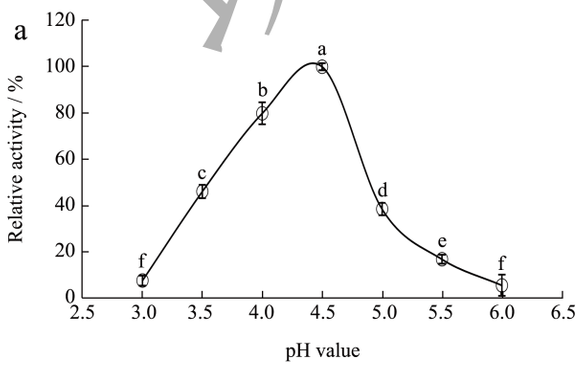
All experiments were carried out for three times, and data were expressed in means±SD. The comparisons between groups were analyzed by Waller-Duncan test of one-way variance (ANOVA). All statistical methods were performed by the statistical software Statistical Package for Social Sciences 22.0. Values of $P < 0.05$ were regarded as statistically significant.

2 Results and discussion

2.1 Enzyme activities of Pectinex XXL

Pectinex XXL showed pectinase of 36 670.27 U/mL at pH values 4.5 and 60 ℃. And it also contained cellulase (1 236 U/mL), xylanase (5 366 U/mL) and amylase (1 321 U/mL) activities (Table 1). *Aspergillus niger* was reported to produce many plant cell wall macro molecules degrading enzymes like pectinase[24], cellulase[25] and xylanase, etc. However, due to the high starch content of plantain, amylase activity might be necessary, because it can pre-degrade the starch and increase the sweetness of juice. Nevertheless, other plant cell wall component degrading enzymes also contributed to the de-pectinization process[26]. Pectinex XXL showed potential for plantain degradation.

2.2 Effect of pH values and temperature on the enzyme activity and stability of Pectinex XXL



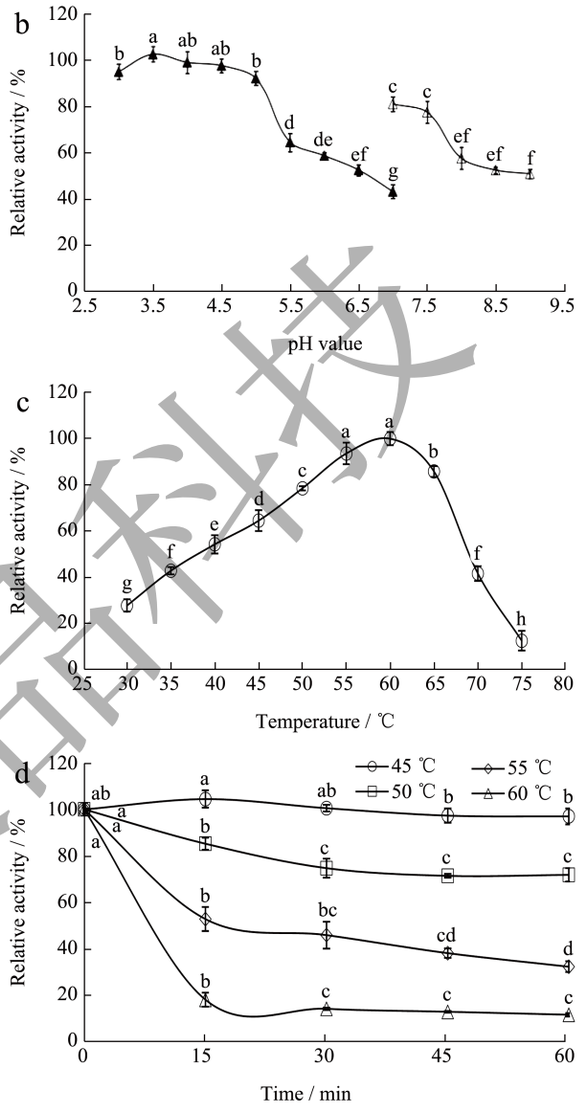


Fig.1 Effects of pH value and temperature on the enzyme activity and stability of the purified Pectinex XXL

Explanatory note: (a) Effect of pH values on enzyme activity. (b) Effect of pH values on enzyme stability. At pH values 3.0~7.0, citric acid-Na_2HPO_4 (○); At pH values 7.0~9.0, Na_2HPO_4-NaH_2PO_4 (□). (c) Effect of temperature on enzyme activity. (d) Effect of temperature on enzyme stability. 40 ℃ (○), 45 ℃ (□), 50 ℃ (◊), 55 ℃ (△). Error bars present the standard deviation of three repeats (n=3).

The pectinase activity of properly diluted Pectinex XXL was most active at acidic pH values 4.5 (Fig.1a)

and more than 80% enzyme activity was retained after incubation at pH values 3.0 to 4.5 (Fig.1b). Similarly, some endo-polygalacturonases with optimum pH values of 3.8~4.3[24] and 4.0[27] were reported. Most major tropical and subtropical fruits show slightly acidic pH values, it can be seen that Pectinex XXL might be suitable for these fruit juice production [14,28].

The optimal temperature of Pectinex XXL was found to be 60℃ , and it showed 90% enzyme activity at 55 ℃ ; however, the enzyme activity dropped sharply at 65 ℃ (Fig.1c). The optimal temperature of Pectinex XXL was similar to some reported endo-polygalacturonases[29-33]. It was reported that A. niger produced multiple kinds of pectinase[34], so the optimum temperature of Pectinex XXL might not depended by the optimum temperature of a single endo-polygalacturonase[35]. Several endo-polygalacturonases from A. niger have been reported, but their optimum temperatures were all lower than 60 ℃[16]. Endo-polygalacturonase, exo-polygalacturonase and lyase can degrade the backbone of polygalacturon, and pectin esterase removes methoxyl groups of polygalacturon and provide more action sites for pectin depolymerases[36], the change of the activity of these enzymes alongside the change of temperature might affect the polygalacturon degrading enzyme activity of Pectinex XXL[37]. Pectinex XXL was quite stable at 40 ℃ and lost 80% enzyme activity after 15-minute incubation at 55 ℃ (Fig.1d).

2.3 Effect of metal ions on the activity of Pectinex XXL

Metal ions are can not avoided in the working environment of pectinase. because they are one kind of component of fruit[38]. At 1 mmol/L, Na^+, Mg^{2+}, Ba^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} did not inhibit the enzyme activity of Pectinex XXL (Table 2). When the molar concentration of metal salt increased to 2 mmol/L, K^+ and Fe^{2+} slightly inhibited the activity of Pectinex XXL. 1 mmol/L and 2 mmol/L Na^+ did not inhibited the enzyme activity of Pectinex XXL, this enzymatic characteristic guaranteed the potential use of Pectinex XXL in fruit juice production using plantain as material, since plantain is rich in Na^+ [5,11].1 mmol/L and 2 mmol/L Ca^{2+} , Fe^{2+} and Mn^{2+} partially inhibited the enzyme activity of Pectinex XXL. Some pectinases were reported to be inhibited by Mn^{2+}, Ba^{2+} and Co^{2+}. However, the pectinases in Pectinex XXL were more tolerant to heavy metal ions. The pectinases activity of Pectinex XXL maintained 77%, 101% and 100% enzyme activities in the present of 1 mmol/L Mn^{2+}, Ba^{2+} and Co^{2+}, respectively; while PG2 was completely inhibited by 1 mmol/L Mn^{2+}, and can only maintained 19.25% enzyme activity in the present of 1 mmol/L Co^{2+} [39].

Table 2 Effect of metal ions on the activity of Pectinex XXL

Metal ions	Relative activity[a]/%	
	1 mmol/L	2 mmol/L
Control	100 ± 0.04	100 ± 0.04
NaCl	106 ± 0.04	102 ± 0.05
KCl	96 ± 0.05	85 ± 0.06
$MgCl_2$	102 ± 0.02	93 ± 0.04
$CaCl_2$	94 ± 0.03	90 ± 0.07
$MnCl_2$	94 ± 0.09	87 ± 0.07
$BaCl_2$	100 ± 0.04	94 ± 0.01
$CoCl_2$	100 ± 0.01	96 ± 0.01
$ZnCl_2$	98 ± 0.03	95 ± 0.01
$FeCl_2$	97 ± 0.07	90 ± 0.02
$CuCl_2$	108 ± 0.03	101 ± 0.02

Explanatory note: [a] All experiments were performed in triplicate and mean values were presented. The experiments were repeated three times and similar results were obtained. The same as in the following table.

2.4 Effect of Pectinex XXL on plantain and cherry tomato juice extraction

The pH values of plantain juice was 4.6, which was near to previous reported 4.71[14]. And the pH values of used plantain juice was quite near to the optimal pH values of Pectinex XXL (pH values 4.5). Pectinex XXL in 4 U/mL significantly increased the yield by 90.27%, increased the light transmittance by 24.50% and reduced the viscosity by 81.69% (Table 3). The enhancement effect increased when the enzyme doses were increased to 8 U/mL. However, the increment was less significant when the enzyme dose keep increasing. And the enzyme dose of 16 U/mL was near to saturation.

Table 3 Effect of Pectinex XXL on plantain juice extraction

Enzyme mass concentration/(U/mL)	Increment of yield[a]/%	Increment of light transmittance[a]/%	Reduction of viscosity[a]/%
0	0.00 ± 0.00	5.45 ± 2.05	0.00 ± 0.00
4	90.27 ± 9.77	24.50 ± 0.14	81.69 ± 0.34
8	103.54 ± 5.1	33.05 ± 1.34	85.84 ± 0.4
12	110.18 ± 18.39	34.70 ± 1.56	88.00 ± 0.59
16	119.91 ± 7.38	37.74 ± 0.35	88.42 ± 0.79

Table 4 Effect of Pectinex XXL on cherry tomato juice extraction

Enzyme mass concentration/(U/mL)	Increment of yield[a]/%	Increment of light transmittance[a]/%	Reduction of viscosity[a]/%
0	0.00 ± 0.00	2.50 ± 0.14	0.00 ± 0.00
4	11.65 ± 1.86	4.60 ± 0.71	19.06 ± 1.72
8	12.65 ± 3.74	6.7 ± 0.28	24.82 ± 0.2
12	12.31 ± 3.27	9.4 ± 0.14	28.06 ± 0.73
16	15.97 ± 0.43	11.5 ± 0.57	29.5 ± 0.72

It was reported that two endo-polygalacturonases from Penicillium oxalicum CZ1028 increased the light transmittance of plantain juice by-10.3% (PoxaEnPG28A)[14] and 47.3% (PoxaEnPG28B-Pp)[16], respectively. In theory, using Pectinex XXL should obtain better performance than using endo-polygalacturonase alone, because cellulase and xylanase accelerated the extraction process of fruit juice by playing synergistic effect with endo-polygalacturonase[16]. Other plant cell wall component degrading enzymes in Pectinex XXL should help to obtain a better effect than only endo-polygalacturonase was used, but the performance in this study was inferior to that using PoxaEnPG28B-Pp alone[16]. The difference of performance obtained from references and this study might caused by the difference of plantain, because the component of plantain from different cultivars in different ripen and storage periods might different[16]. To our best knowdelgement, Pectinex XXL was first time used for fruit juice production, and plantain was first time used for fruit juice production using a commercial pectinase reagent.

The addition of Pectinex XXL also enhanced the extraction of cherry tomato juice. The boost effect no longer increased when 16 U/mL pectinase was used, and the performance of enzyme was increment of yield: 15.97%, increment of light transmittance: 11.50%, reduction of viscosity: 29.50% (Table 4). It seems that the performance on cherry tomato was inferior to that on plantain, but this might be due to the deference of fruit. The boost effect of pectinase on banana juice extraction was also seemed limited. For example, the performances of PoxaEnPG28A (increment of yield: 8.76%, increment of light transmittance: 6.2%)[14] and the pectinase produced by *Sporotrichum thermophile* Apinis on banana juice (increment of yield: 2%)[40] were limited. The difference of ripeness degree and cultivar of fruit also contributed to the difference of juice production[41].

3 Conclusion

In conclusion, the enzymatic characteristics of a commercial *A. niger* pectinase Pectinex XXL were first time determined and the enzyme reagent was applied for plantain and cherry tomato juice extraction. The enzyme increased the extraction effect at low enzyme mass concentration of 4 U/mL fruit pulp and reached the peak effect at high enzyme dose of 16 U/mL fruit fresh. Our study showed the potential of both pectinase Pectinex XXL and plantain and cherry tomato for industrial fruit juice production.

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