CLONING AND EXPRESSION OF *AspPecA* PECTINASE GENE FROM *Aspergillus niger* M13 IN *Pichia pastoris* X33

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ABSTRACT

This study aims to investigate the expression gene encoding pectinase (*AspPecA*) from *Aspergillus niger* M13 in *Pichia pastoris* X33 and the application of recombinant pectinase in fruit juice processing. *A. niger* M13 strain isolated from Da Xanh pomelo (*Citrus maxima*) peel was revived by culturing in Potato Dextrose Broth (PDB) at 35 ℃ for 18-20 h. The *AspPecA* gene was cloned into vector pPICZαA and expressed in *P. pastoris* X33 under the control of the AOX1 promoter. Purified rAspPecA had a molecular weight of approximately 42 kDa, as evidenced by SDS-PAGE. *P. pastoris* X33 transformants expressing recombinant pectinase were selected on an agar plate, and their ability to produce the pectinase was evaluated in flask cultures. Recombinant pectinase rAspPecA can clarify apple juice. Compared with controls, rAspPecA at a concentration of 2,400 U/L reduced apple juice turbidity by 29%. The results obtained in this work provide a basis for the development of applications of recombinant pectinase in various biotechnological fields.

TẠO DÒNG VÀ BIỂU HIỆN GEN PECTINASE *AspPecA* TỪ *Aspergillus niger* M13 TRONG *Pichia pastoris* X33

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TỪ KHÓA

Nấm mốc *Aspergillus niger*
Gen *AspPecA*
Làm trong nước quả
Nấm men *Pichia pastori*s
Pectinase tái tổ hợp

TÓM TẮT

Mục tiêu của nghiên cứu này là tạo dòng và biểu hiện gen mã hóa pectinase (*AspPecA*) từ *Aspergillus niger* M13 trong nấm men *Pichia pastoris* X33 và ứng dụng pectinase tái tổ hợp vào quá trình chế biến dịch quả. Chủng *A. niger* M13 phân lập từ vỏ bưởi Da Xanh (*Citrus maxima*) được phục hồi bằng cách nuôi cấy trong Potato Dextrose Broth (PDB) ở 35 ℃, 18-20 giờ. Gen *AspPecA* được tạo dòng vào vector pPICZαA và được biểu hiện trong *P. pastoris* X33 với promoter AOX1. Các thể biến nạp *P. pastoris* X33 biểu hiện pectinase tái tổ hợp được chọn lọc trên đĩa agar và khả năng sản xuất pectinase của chúng được đánh giá trong nuôi cấy flask. Pectinase tái tổ hợp rAspPecA có trọng lượng phân tử xấp xỉ 42 kDa, có khả năng làm trong nước ép quả táo. rAspPecA nồng độ 2.400 U/L đã làm giảm độ đục của nước ép quả táo lên đến 29%. Các kết quả thu được từ nghiên cứu này cung cấp cơ sở khoa học cho việc phát triển ứng dụng pectinase tái tổ hợp trong các lĩnh vực công nghệ sinh học khác nhau.

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1. Introduction

Pectin is a complex acidic heteropolysaccharide with a high molecular weight and is found mainly in the middle lamella and cell walls of plants (vegetables, cereals, fruits, and fibers) [1], [2]. Pectinase is an important enzyme that breaks down pectins through de-esterification and depolymerization reactions [3]. Pectinases come in different forms, such as protopectinases, esterases, and depolymerases, due to the complex structure of pectin. They are used in fruit juice processing, juice clarification, winemaking, refining of vegetable fibers, degumming of natural fibers, and wastewater treatment [2], [4] - [6].

Although various sources exist for producing pectinase, it is essential to develop a microbial pectinase that is highly stable and can tolerate diverse physicochemical conditions for various industrial applications. However, the cost of pectinase production remains high, limiting its applicability. Strain improvement is an important tool in the development of commercial processes for microbial fermentation to produce high-speed enzymes. Recently, the research [7] showed that cloning works using microorganisms successfully expressing a specific pectinase gene. Pectate lyases from *Bacillus* sp., heterologously expressed in *E. coli* by Zhou et al. [8] and Guan et al. [1], exhibited maximum enzymatic activities at optimal pH values of 10.5 and 8.0, and optimal temperatures of 70 °C and 35 °C, respectively. Yuan et al. [9] illustrated the maximum activity of recombinant pectate lyase from *Paenibacillus polymyxa* in *E. coli* at pH 10.0 and 40 °C. Cloning and expression of pectinase genes from microorganisms are important in food technology. The goal is to produce pectinase enzyme in higher quantities than traditional methods, which will improve cost efficiency, especially in food processing, where purity and stability are decisive factors in the quality of the final product. In this study, we conducted cloned and expressed the *AspPecA* pectinase gene from *Aspergillus niger* M13 in *Pichia pastoris* X33. Although heterologous expression of pectinase in *P. pastoris* has been reported in several studies, our focus was on cloning the pectinase gene from *A. niger*, expressing it in *P. pastoris X33*, and further characterizing the potential of the expressed pectinase for application in fruit juice.

2. Methodology

2.1. Strains, plasmids, and reagents

E. coli TOP10 strain was used for plasmid amplification. The pPICZαA plasmid and the *P. pastoris* X33 strain were purchased from Invitrogen (Invitrogen-Thermo Fisher Scientific, USA). The pPICZαA plasmid and the *P. pastoris* X33 strain were used as the recombinant protein expression host. The *E. coli* TOP10 strain was grown in LB medium at 37 °C. *P. pastoris* X33 strain was grown in Yeast Peptone Dextrose (YPD) medium (1% yeast extract (w/v), 2% tryptone (w/v), 2% glucose (w/v), 2% agar (w/v) for plates) at 28 to 30°C. *A. niger* M13 strain was revived by culturing in PDB at 35°C for 18-20 h. Restriction enzymes *Eco*RI (#ER0271), *Xba*I (#ER0681), pUC19 vector, GeneJET Plasmid Miniprep Kit (#K0502), GeneJET Gel Extraction Kit (#K0691), protein marker, and GeneRuler 1 kb DNA Ladder were purchased from Thermo Fisher Scientific (USA).

Table 1. *Primer used for cAspPecA*

Primers	Sequences	Primers	Sequences
cAspPecA-F	5'GAATTCCTGCTCCTCTTGAGAAG3'	AOX1-F	5'GACTGGTTCCAATTGACAAGC3'
cAspPecA-R	5'TCTAGATAATCGCTGCAGGAAGC3'	AOX1-R	5'GCAAATGGCATTCTGACATCC3'

2.2. Cloning into vector pPICZαA

Based on the analyzed complete nucleotide sequence of the *AspPecA* gene (GenBank accession number MT502411), the nucleotide sequence encoding cAspPecA was synthesized by Phu Sa Biotech Co., Ltd., Vietnam. The synthesized sequence was deleted of intron segments, the peptide signal, and the stop codon, and the *Eco*RI and *Xba*I sequences were added. The synthesized nucleotide sequence was sequenced to accurately determine the quality of the synthesized product. After synthesis, the *cAspPecA* sequence was error-free and was attached to the pUC19 vector. The pUC19 vector carrying the *cAspPecA* gene (pUC19/*cAspPecA*) was transformed into *E. coli* TOP10 by heat shock (SBH130D, Stuart, Bibby Scientific Limited, Staffordshire, UK). *E. coli* TOP10 cells carrying the pUC19/*cAspPecA* vector after transformation were selected on solid Luria Bertani (LB) medium supplemented with 25 µg/mL zeocin for 16 h at 37 °C. The recombined plasmid was extracted and checked using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, USA). The *cAspPecA* gene and the pPICZαA vector were each cut with *Eco*RI and *Xba*I restriction enzymes. The *cAspPecA* gene was ligated into the pPICZαA vector using T4 DNA ligase, the mixture was mixed and incubated overnight at 16 °C. The reaction mixture contained: 4 µL pPICZαA vector (100 ng), 4 µL DNA (40 ng), 1 µL ligation buffer (10×), and 1 µL T4 DNA ligase (2.5 U). The ligation reaction mixture was incubated overnight at 16 °C and then transformed into competent *E. coli* TOP10 cells using the heat-shock method. Transformed cells were selected on solid LB medium supplemented with 25 µg/mL zeocin and incubated overnight at 37 °C. A single colony was transferred to a new tube containing 5 mL liquid LB medium with 25 µg/mL zeocin at 37 °C and shaken at 200 rpm for 12 h. After that, the cell culture was harvested, and the pPICZαA recombinant plasmid was extracted from *E. coli* TOP10 cells using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA). The presence of the inserted pPICZαA/*cAspPecA* vector was confirmed by PCR using cAspPecA-F/cAspPecA-R specific primers (Table 1).

2.3. Transformation of AspPecA gene into P. pastoris

The pPICZαA/*cAspPecA* recombinant plasmid was transformed into *P. pastoris* X33 by electroporation according to standard heat shock protocols after linearization of the plasmid with *Pme*I [10]. The transformation reaction mixture consisted of 80 µL of *P. pastoris* X33 cells and 10 µL of the pPICZαA/*cAspPecA* recombinant plasmid solution. The mixture was transferred to a 0.2 cm cuvette and placed on ice for 5 min. Subsequently, this cuvette was inserted into the "Shock Pod" chamber of an electroporation device and pulsed at 1.5 kV for 5 ms. 1 mL of cold 1 M sorbitol was added, and the suspension was transferred to a sterile 15 mL tube and incubated at 30 °C for 1 h without shaking. The transformation mixture was streaked on Yeast Peptone Dextrose Sorbitol (YPDS) agar medium (1% yeast extract, 2% peptone, 2% D-glucose, 1 M sorbitol) supplemented with 100 µg/mL zeocin and incubated at 30 °C for 24 h. The *P. pastoris* transformants containing the expression vector pPICZαA without the *cAspPecA* gene insert were used as the negative control.

To verify the presence of the pectinase genes in the *P. pastoris* X33 transformants, the cells were grown in YPD medium, and the total DNA was extracted. The presence of the pectinase genes was then confirmed by PCR using the AOX1 and cAspPecA primers (Table 1).

2.4. Expression of pectinase-encoding cAspPecA gene

The induction of expression was performed according to the manufacturer's recommendations for *P. pastoris* X33. Selected transformants (10) from YPDS medium containing zeocin were transferred and cultured in 5 mL of YPD medium supplemented with 100 µg/L zeocin and incubated at 30 °C with shaking at 250 rpm for 24 h. The mixture was centrifuged for 5 min at 1,500 rpm at room temperature (RT), and the supernatant was discarded. The pellet was re-suspended in 100 mL of YPD medium supplemented with 1% glycerol. The cells were then incubated for 24 hours at 30 °C and 250 rpm. Cells were harvested by centrifugation for 5 min at RT and 1,500 rpm. The supernatant was removed, and the cell pellet was re-suspended to give an OD_{600} of 1 in the YPD induction medium to induce expression by adding 100% methanol daily to the final concentration of 1% required for maintaining the induction. The resultant sample was used to analyze the expression level of protein production and determine the enzyme activity.

2.5. Application of recombinant pectinase in fruit juice processing

The ability of recombinant pectinase from *P. pastoris* to clarify apple juice at different concentrations: 600 U/L, 1,200 U/L, 1,800 U/L, 2,400 U/L, and 3,000 U/L, and control (without enzyme). The transmittance of the apple juice was measured at an absorbance of 450 nm, and the apple juice clarification efficiency was calculated as a percentage of transmittance after a 4 h incubation period. The formula used to determine the transmittance percentage was as follows:

$$\% \text{ transmittance} = \frac{OD_{control} - OD_{TN}}{OD_{control}} \times 100\% \ [11]$$

OD_{TN}: OD of enzyme-treated fruit juice; $OD_{control}$: OD of the control sample (without enzyme)

2.6. Data analysis

The statistically significant (p-value<0.05) of various factors on enzyme activity was determined using one-way ANOVA (with multiple comparisons).

3. Results and discussion

3.1. Cloning cAspPecA gene

The *cAspPecA* gene from *A. niger* M13 was used as a template to synthesize a gene, which was then inserted into the pUC19 vector and transformed into *E. coli* TOP 10. The transformed *E. coli* with the pUC19/*cAspPecA* vector would be cultured on an LB liquid medium with 25 µg/mL zeocin at 37 °C to extract the plasmid. The pUC19/*cAspPecA* plasmid would then be separated using *Eco*RI and *Xba*I restriction enzymes and analyzed with gel electrophoresis on a 0.8% agarose gel.

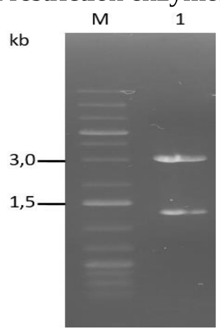

Figure 1. *EcoRI and XbaI digested the product of* the *pUC19/cAspPecA vector. M: GeneRuler 1 kb Plus DNA Ladder. 1: pUC19/AspPecA digested product*

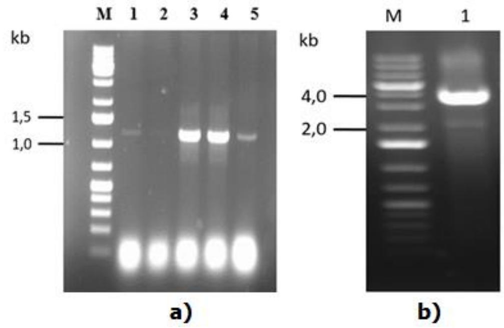

Figure 2. *Transformation of pPICZαA/cAspPecA vector into E. coli TO10. M: GeneRuler 1 kb Plus DNA Ladder. a) PCR products form 5 colonies (1-5), b) pPICZαA/AspPecA vector (1)*

The results of DNA electrophoresis in Figure 1 indicate complete digestion of the recombined plasmid, resulting in DNA fragments of approximately 2.7 kb and 1.1 kb in size. The lengths of these DNA fragments correspond to the size of the pUC19 vector and *cAspPecA* gene, respectively. The DNA fragment corresponding to the *cAspPecA* gene was purified and ligated with the pPICZαA vector that had been linearized with *Eco*RI and *Xba*I enzymes using ligase. The resulting mixture was transformed into competent *E. coli* Top10 cells using the heat shock method and selected on a selective medium supplemented with zeocin at a concentration of 25 µg/mL. The transformation was confirmed by PCR using cAspPecA-F/cAspPecA-R specific primers. Selected bacterial colonies showed a band of approximately 1.1 kb, the same size as the *cAspPecA* gene. Colonies 3 and 4 had high DNA concentrations, indicating that they were colonies containing the recombinant pPICZαA/*cAspPecA* vector (Figure 2a). This result shows that the recombinant pPICZαA/*cAspPecA* vector has been successfully transformed into *E. coli* TOP 10 host cells. The purified plasmid recombinant pPICZαA/*cAspPecA* was observed through 1% agarose gel electrophoresis and had a size of approximately 4 kb with a concentration of approximately 400 µg/µL (Figure 2b). This material was used to transform *P. pastoris*.

3.2. Transformation into P. pastoris X33

To successfully integrate the *cAspPecA* gene into pPICZαA, the recombination vector pPICZαA/*cAspPecA* was transformed into *P. pastoris* X33 by electroporation. Prior to this procedure, the vector was linearized using PmeI. The transformed P. pastoris X33 colonies were selected through screening with zeocin antibiotic at a concentration of 100 µg/mL on YPDS agar medium (Figures 3a and 3b).

The bacteria growing in the YPDS environment were cultured, and the total DNA was extracted to check for the presence of the *cAspPecA* gene in the host cell's genome. The results of the total DNA extraction of *P. pastoris* X33 reconstituted are shown in Figure 3c, indicating that the concentration and quality are suitable for use as raw materials for the process of checking the presence of *cAspPecA* in the genome of *P. pastoris* X33.

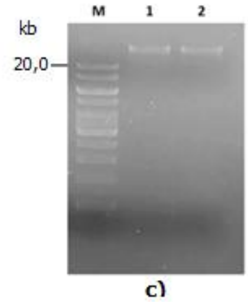

Figure 3. *Transformation pPICZαA/cAspPecA into P. pastoris X33***:** *(A, B)*: *Screening of P. pastoris X33 cells transformed with the pPICZαA/cAspPecA recombinant vector. a) Control, b) Transformed P. pastoris X33 cells, c) Total DNA of P. pastoris X33 carrying the recombinant pPICZαA/cAspPecA vector (M: GeneRuler 1 kb DNA Ladder, 1-2: Total DNA)*

The integration of the recombinant vector pPICZαA/*cAspPecA* into the genome of *P. pastoris* X33 was confirmed by colony PCR reaction using *AOX1F/AOX1R* primer pair. The electrophoresis result in Figure 4 depicts the presence of two different bands, sizes 1.5 kb and 1.0 kb. The 1.5 kb band corresponded to the combination of the *cAspPecA* gene (1,040 bp) and the *AOX1* sequence (400 bp) within pPICZαA. In contrast, the other band had a size of 1.0 kb, which matched the size of the synthesized *cAspPecA* gene. The 600 bp band responds to the *AOX1* gene in the *P. pastoris* X33 genome. Consequently, it can be inferred that the *cAspPecA* gene was successfully integrated into the genome of *P. pastoris* X33.

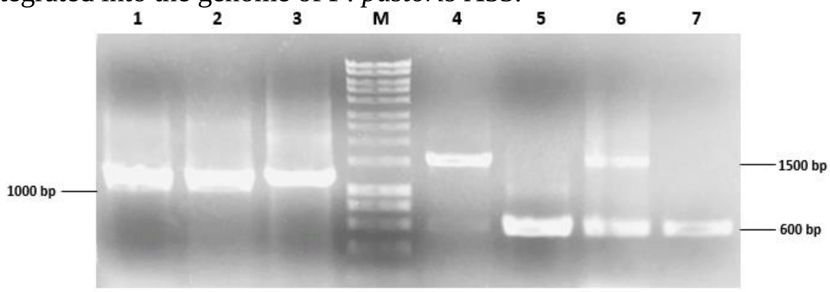

Figure 4. *PCR product of cAspPecA gene was amplified using the AOX1 primer. M: Hypper Ladder 1 kb DNA (Bioline, USA), 1: PCR product from PUC19/cAspPecA vector with cAspPecA primers, 2: PCR product from pPICZαA/cAspPecA vector with cAspPecA primers, 3: PCR product from recombinant P. pastoris X33 genome with cAspPecA primers, 4: PCR product from recombinant P. pastoris X33 genome with AOX1 primers, 5: PCR product from P. pastoris X33 genome with AOX1 primers, 6: PCR product from pPICZαA/cAspPecA vector with AOX1 primers, 7: PCR product from pPICZαA vector with AOX1 primers*

3.3. Expression of cAspPecA pectinase gene in Pichia pastoris X33

The expression level of the transformed *P. pastoris* X33 carrying pPICZαA/*cAspPecA* was evaluated by culturing them in a YP medium supplemented with methanol as an inducing agent. Extracellular enzymes were diffused onto plates containing pectin as a substrate. The results in Figure 5 show that strains 3 and 4 exhibited a stronger pectin clearance zone than the other strains. There was no pectinase activity (clearance zone) in the negative control because *P. pastoris* X33 does not utilize pectin. However, the *P. pastoris* X33 strain carrying the *cAspPecA* gene fragment was able to form a clearance zone on a pectin-containing agar plate. Therefore, strain 3 was selected for further studies on its expression levels, enzyme characteristics, and applications.

Pectinase was expressed using the pPICZαA vector system, and the resulting protein was fused with a His-tag sequence. The fractions with the highest pectinase activity were selected for further testing using SDS-PAGE and additional activity testing. SDS-PAGE analysis of the purified pectinase (Figure 6a) showed a band with a molecular weight of approximately 42 kDa, corresponding to the theoretical weight of pectinase (37.954 kDa). The pectinase band of protein after SDS-PAGE on pectin substrate showed a corresponding band, confirming the presence of pectinase in *P. pastoris* X33 (Figure 6b).

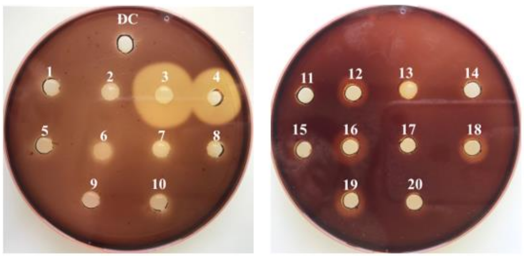

Figure 5. *Pectin degradation zone from P. pastoris X33 strains carrying the cAspPecA gene. 1-10: Extracellular enzyme of single colonies of P. pastoris X33 carrying the cAspPecA gene, 11-20: Extracellular enzyme of single colonies of P. pastoris X33*

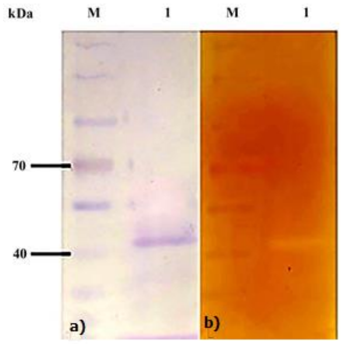

Figure 6. *SDS-PAGE electrophoresis and zymogram of recombined rAspPecA. a) SDS-PAGE (M: PageRuler™ Prestained Protein (Thermo Scientific, USA), 1: rAspPecA biosynthesis from P. pastoris X33 after purification). b) Zymogram (M: PageRuler™ Prestained Protein (Thermo Scientific, USA), 1: Zymogram of recombined rAspPecA)*

3.4. Effect of rAspPecA on the apple juice clarification process

We also evaluated the effectiveness of rAspPecA from *A. niger* M13 in *P. pastoris* X33 for clarifying apple juice at enzyme concentrations of 600-3,000 U/L. The apple juice clarification efficiency was determined by calculating the percentage of transmittance after a 4 h incubation period. The results presented in Figure 7 demonstrate an increase in % transmittance as the enzyme concentration was increased from 600 to 2,400 U/L (from 6% to >29%, respectively).

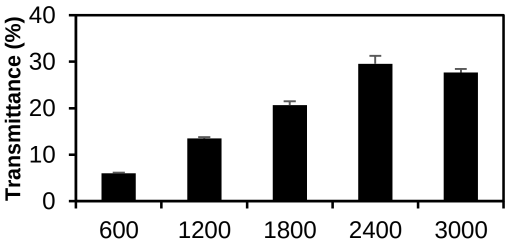

Figure 7. *Effect of rAspPecA on the apple juice clarification process*

However, at a concentration of 3,000 U/L, the transmittance decreased. This is because pectinase breaks down pectin in apple cell walls, which increases its ability to release fruit juice into the external environment. As the enzyme concentration increased, the destruction of cell walls increased, resulting in a significant increase in fruit juice production.

Heterologous expression produces industrial enzymes such as cellulase, xylanase, lipase, pectinase, and protease. *P. pastoris* is a highly efficient expression system for making large quantities of heterologous proteins in a secreted form, resulting in cost-effective enzyme production. Most commercial pectinase products used in the food industry are derived from *A. niger*-a promising producer. In this study, *AspPecA* from *A. niger* M13 has been successfully expressed in *P. pastoris* X33 through the pPICZαA vector. Pectin methylesterase gene *pme-zj5a* was isolated from *A. niger* ZJ5, cloned, and expressed in *P. pastoris*. The *pme-zj5a* nucleotide sequence is 1,088 bp long and encodes a polypeptide chain of 327 amino acids. Pure PME-ZJ5A has a molecular weight of about 37 kDa, still higher than its calculated molecular weight (34.8 kDa). The observed change in the apparent molecular weight of PME-ZJ5A suggests that it has undergone post-translational N-glycosylation modification during its heterologous expression in *P. pastoris* [12].

In 2021, Zhong et al. [13] cloned and successfully expressed the pectin methylesterase gene (*Pxpme*) from *Paenibacillus xylanexedens* SZ 29 in *E. coli*. The PxPME encoding gene codes for a polypeptide chain of 1,005 bp with 334 amino acids. PxPME was reassembled in *E. coli* BL21 as a histidine-tagged protein. After purification by Ni^{2+} affinity chromatography, the specific activity of PxPME was 39.38 U/mg against citrus pectin. Pure PxPME showed a single band on SDS-PAGE with an apparent molecular weight of about 45 kDa, slightly higher than the calculated molecular weight (36.8 kDa). Pectin lyase gene *pni-zj5a* from *A. niger* ZJ5 was successfully expressed in *P. pastoris* GS115. Purification and SDS-PAGE analysis of PNL-ZJ5A showed that the activity of the recovered enzyme was 4.1% and the molecular weight of PNL-ZJ5A was ~54 kDa, higher than the theoretical weight (~47 kDa) [14].

The pectinase-encoding gene is endo-polygalacturonase (*endoPG*) from *Aspergillus aculeatus*, which has been cloned and expressed in *P. pastoris* KM71. The full-length *endoPG* gene (1,029 bp) has a complete molecular weight of 38.7 kDa, consistent with the theoretical molecular weight [15]. According to Famotemi et al. [16], pectinase from *Aspergillus niger* F7-02 strain had optimal activity at 65°C and pH 4.0. It exhibits stability in a pH range of 3.0-6.0 and shows high activity in the presence of Mn^{2+} and Ca^{2+} ions. The molecular weight of pectinase was 40 kDa. A different pectinase-encoding gene is *exopelA*, which has been cloned and expressed in *E. coli* BL21. The *exopelA* gene consists of 1,236 nucleotide sequences, encoding a protein consisting of 425 amino acids, and the molecular weight of pure pectinase is ~50 kDa (higher than the predicted molecular weight of ~48.7 kDa) [17]. In general, the molecular weight of recombinant pectinase from different microorganisms ranges from 25 kDa to 70 kDa. Therefore, *cAspPecA* gene encoding pectinase was successfully expressed in *P. pastoris*, which originates from *A. niger* M13 and is of great importance for studying the biotechnological potential of this enzyme.

Several previous studies have shown that increasing enzyme concentration significantly improves juice clarification. Moharram and Zohri [18] showed that pectinase synthesized from *Cladosporium parasphaerospermum* (10 U/mL) increased the yield of apple, orange, apricot, and peach juice by 17, 20, 13, and 24%, respectively. On the other hand, the clarity and colour of orange juice were improved by 194% and 339%, respectively. Another study by Sudeep et al. [19] showed that the yield of mature ripened orange juice, total soluble solids, and clarity (% transmittance) increased as the concentration of pectinase increased, indicating its potential use in juice processing. According to Xu et al. [14], the *pnl-zj5a* gene produces 1 U pectinase, which reduced apple juice viscosity by 38.8% and increased transmittance by 86.3%. This enzyme shows potential for use in the food industry, particularly in fruit juice processing and plant tissue extraction. In a study by Phan and Nguyen [20], it was found that increasing the amount of enzyme also increased the amount of apricot juice obtained. The highest juice recovery efficiency (52.09%) was achieved at a pectinase concentration of 0.2%. In short, utilizing microorganisms to produce recombinant enzymes has great potential in the fruit juice processing industry.

4. Conclusion

In the present study, *cAspPecA* gene from *A. niger* M13 encoding a pectinase cAspPecA was successfully cloned and expressed in *P. pastoris* X33. The rAspPecA can clarify apple juice. These favourable enzymatic properties of rAspPecA show potential application in the food industry, especially in juice-processing applications. However, further studies must be performed, such topics as determining optimal parameters for pectinase biosynthesis from microbial sources, the cost of the production processes to assure its commercial application in large-scale food formulation and processing, which will be the of interest for our research group in the near future.

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