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Identification and production improvement of pectinase from a pectinolytic fungus isolated from Da Xanh pomelo (*Citrus maxima*) peel

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Abstract: Pectinases are important enzymes due to their broad potential applications in various industrial processes including food, paper and pulp, textile, medicinal, and environmental treatment. The current work aimed to identify the pectinolytic fungus, named M13 isolate, and determined the effect of various culture conditions on enzyme accumulation. The primary screening indicated the isolate produced high pectinase on both pectin agar and Czapek with supplemented pectin media. Then, the isolate was identified based on the nucleotide sequence of *ITS* region comparison to the NCBI database, showing the isolate shared 100% similarity to *Aspergillus niger* family. Thus, the isolate was named *Aspergillus niger* M13. The fungus isolate produced maximal pectinase activity in the culture conditions, including an incubation time of 48 h, pH 6.5, 0.5% (w/v) maltose, and 0.2% (w/v) yeast extract. Overall, the *A. niger* M13 is a potential candidate for large scales production of pectinase for industrial applications.
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Keywords: *Aspergillus niger* M13; identification; pectinase production; industry

1. Introduction

Pectinases are enzymes that degrade pectic substances such as propectins, pectins, pectic acids, and pectinic acids (Satapathy et al., 2020). Pectinases are known as pectinolytic or pectin enzymes belonging to the polysaccharides hydrolysis enzyme family (Oumer and Abate, 2018; Gurung et al., 2013). Generally, pectinolytic enzymes are classified into three groups: Protopectinases (PPases), pectin methylesterases (PMEs), and polygalacturonases (PGases). PPases degrade insoluble protopectin in unripe fruits and convert them into highly polymerized soluble pectin. PMEs catalyze the removal of methoxyl group linked to pectic units, producing pectic acid and methanol. PGases cleave the backbone of the polygalacturonic acid chain in the presence of water (Haile and Ayele, 2022). Pectinases are secreted by various fungi, bacteria, higher plants, nematodes, protozoans, and insects (Pedrolli et al., 2009; Khairnar et al., 2009).

Pectinases are crucial enzymes for their broad potential applications in different industrial processes and areas. These enzymes have been applied to extract and clarify fruit juice and wine, improvement of winemaking process efficiency; concentration and fermentation of cocoa beans, tea, and coffee; preparation of jellies and jam; extraction of vegetable oil (El-Sheekh et al., 2009; Barman et al., 2015; Abdullah et al., 2018a). In addition, pectinases are found to be involved in the textile process, including degumming of plant fibers, retting, bio-scouring of cotton, and wastewater treatment (El-Sheekh et al., 2009); the paper and pulp industry, protoplast fusion technology, bioenergy production process, livestock feed additives (Gummadi and Panda, 2003); agriculture waste treatment, and medicinal industry (Mulluye and Atnafu, 2022).

Several microorganisms are producing different types of pectinase (Jayani et al., 2010). Most commercial enzymes are obtained through the fungal cultures (Sharma et al., 2012). Up to date, numerous pectinases producing fungi have been reported including *Aspergillus* genera (Satapathy et al., 2021). Several species *Aspergillus* contributed a lot to the efficient production of pectinase, such as *Aspergillus sojae* (Demir and Tari, 2014), *Aspergillus oryzae* (Dange and Harke, 2018), *Aspergillus candidus*, and *Aspergillus flavus* (Naseer et al., 2018), *Aspergillus tamari* (Munir et al., 2019), *Aspergillus fumigatus* (Zehra et al., 2020), *Aspergillus parvisclerotigenus* (Satapathy et al., 2021), *Aspergillus niger* (Khairnar et al., 2009; Abdullah et al., 2018b; Khalil et al., 2020). Among them, *A. niger* is widely applied for production of pectinase on industrial scale (Oumer and Abate, 2018).

There are some factors affecting the pectinase production of microorganisms. The physical conditions

and the sources of essential nutrients are important factors. These factors are reported to enhance enzyme production. The outcomes from numerous previous studies showed the modification of physicochemical parameters resulted in increasing enzyme activity (Jalil et al., 2023). In our previous studies, some highly pectinase-active molds were isolated and screened from peels of some fruits and tubers rich in pectinase such as carrot, potato, banana, apple, mango, dragon, orange, lemon, tangerine, and pomelos (Diem et al., 2018).

In the present study, some cultivation conditions such as cultivation time, pH, carbon source, and nitrogen source were improved to enhance pectinase production by the pectinolytic mold M13 strain, which was successfully isolated from Da Xanh pomelo peels in Hue City, Vietnam.

2. Material and Methods

Medium

The Czapek-pectin medium (g/L) consisting of pectin 5.0 g, $FeSO_4.7H_2O$ 0.1 g, K_2HPO_4 0.2 g, KCl 0.5 g, $NaNO_3$ 2.0 g, $MgSO_4.7H_2O$ 0.1 g, agar 20.0 g, pH 6.5 were used to cultivate the fungus strain.

Fungus strain

The pectinolytic fungus named M13 was isolated from Da Xanh pomelo (*Citrus maxima*) peels in a previous study (Diem et al., 2018). The fungus strain was maintained on a Czapek-pectin medium for pectinase evaluation.

Molecular identification

The total DNA of the M13 strain was isolated according to the method of Sambrook et al. (2001). The *ITS1-4* region was used for the identification of species. Using the genomic DNA as the template, the *ITS1-4* region was amplified using a pair primer of ITS1-F and ITS4-R (Hashem et al., 2019), and the PCR product was purified and sequenced by Nam Khoa Trading and Service Co., Ltd, Vietnam. The *ITS1-4* nucleotide sequence was used for the Basic Local Alignment Search Tool (BLAST) searches against GenBank database, and compared with highest similar sequences. Pectinolytic fungus species was identified based on the nucleotide similarity with the standard species in the database. These sequences were collected and generated a phylogenetic tree was reconstructed using MEGA6.0 software with the neighbor-joining method and the Tamura-Nei model (Tamura et al., 2013). The confidence level of the phylogenetic tree was tested with 1000 replicates of bootstrap.

Screening of pectinase production

M13 mycelium on Czapek-pectin agar medium was transferred into 5 mL liquid Czapek-pectin and cultivated for three days at 30°C. Then, the fungus spore was inoculated into a 250 mL shaking flask containing 50 mL pectin medium. The culture was carried out at 30 °C shaking speed of 120 rpm for four days. The fermentative culture was filtered through a Whatman filter paper. Then, the filtrated supernatant was examined for pectinase activity, whereas dried fungal biomass (g/L) was calculated by drying the fungal at 100°C until a constant weight.

Pectinase activity assay

Pectinase activity was qualified using the 3,5-dinitrosalisylic acid (DNSA) method (Miller, 1959). The reaction mixture (3.0 mL) containing 0.8 mL of pectin solution and 0.2 mL of enzyme solution was incubated with 2.0 mL of 100 mM sodium acetate buffer (pH 5.0) for 10 min at 40°C. After incubation, 1.0 mL NaOH and 1.0 mL DNSA were added to stop the reaction. The released reducing sugar was determined by boiling the reaction mixture for 10 min and measured the optical density at a wavelength of 420 nm. The amount of released reducing sugar by enzymatic hydrolysis was calculated based on a standard curve using galacturonic acid. One unit of enzyme activity was calculated as the required enzyme amount to release 1.0 µmol of galacturonic acid in one minute under the assay conditions.

Effect of incubation time

To evaluate the correlation between pectinase production and cultivation period by the isolate, the fungus was cultured in 50 mL pectin medium at 30 °C, shaking speed of 120 rpm with various incubation times from 24 h to 144 h. The sample fluid was harvested after each 24 h and measured pectinase activity.

Effect of initial pH

The effect of initial pH on enzyme secretion was performed by cultivating the fungus with the best cultivation time using different initial pHs of 4.0-7.0.

Effect of nutrition sources

The effect of carbon sources including glucose, maltose, lactose, fructose, starch, carboxymethyl cellulose (CMC), and molasses was qualified at a concentration of 0.5% (w/v). Meanwhile, different organic nitrogen substrates including gelatine, urea, meat extract, yeast extract, peptone, and inorganic nitrogen substrates including ammonium nitrate and ammonium sulfate were individually supplemented to medium at a concentration of 0.2% (w/v). The culture conditions were used as the best result of above experiments.

Statistical analysis

All the experiments were performed with three replicates. The data from the obtained results

were subjected to statistical analysis. The process was performed using SPSS 20.0 software with one-way analysis of variance (ANOVA). The statistical significance was assessed by Duncan's test. All statistical analyses were carried out on Microsoft Excel v.2010.

3. Results

Identification of pectinolytic fungus

A fragment of 873 bp of the *ITS1-4* region was obtained from the pectinolytic fungus M13 strain. The fragment of the *ITS1-4* region was searched BLAST to NCBI nucleotide sequence database. Phylogenetic analysis was conducted and shown in Figure 1. The data indicated that the M13 isolate grouped with the MG675233.1 *Aspergillus niger* clade. The nucleotide BLAST results indicated that the *ITS1-4* nucleotide sequence was 100% similar to *Aspergillus niger* (MG675233). Therefore, the M13 strain was identified as *Aspergillus niger* M13.

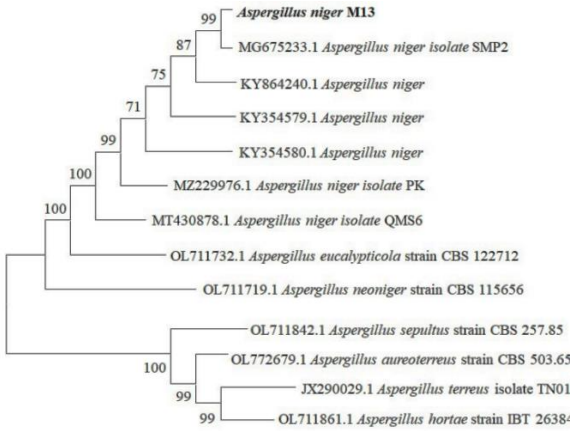

Figure 1. Phylogenetic tree of *Aspergillus* strains based on *ITS* region sequences. The numbers above the branches are bootstrap values in percentage of 1000 replications

Pectinase production

Enzyme production by *A. niger* M13 was screened on pectin agar and Czapek supplemented pectin media. The results are given in Table 1 and Figure 2. The findings showed the diameter of the and pectinase activity were 27.7±0.623 mm and 10.3±1.021 U/mL, respectively.

Table 1. Screening of pectinase production by A. *niger* M13

Strain	Dry biomass (mg/mL)	Diameter of clear zone (mm)	Pectinase activity (U/mL)
A. niger M13	4.8 ± 0.081	27.7±0.623	10.3±1.021

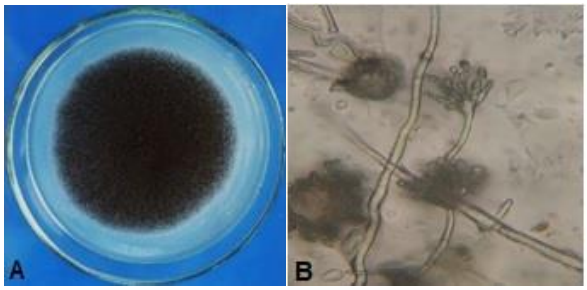

Figure 2. The *A. niger* M13 strain was cultured for four days on the Czapek-pectin medium. A. Colony morphology, B. Microscopy of the *A. niger* M13 strain

Effect of incubation period

The impact of incubation period on enzyme activity, diameter of clear zone, and dry biomass was assessed. The results shown in Table 2 and Figure 3 demonstrate that the best incubation period for the pectinase activity, dry biomass, and clear zone diameter was 48 h of cultivation with their maximum values as 35.6 U/mL, 7.8 g/L, and 32.3 mm, respectively.

Table 2. Effect of different incubation times on pectinase production and mold growth of *A. niger* M13

Strain	Incubation time (h)	Dry biomass (g/L)	Diameter of clear zone (mm)	Pectinase activity (U/mL)
A. niger M13	24	4.2^{d}	22.7^{e}	19.7^{c}
	48	**7.8^{a}**	**32.3^{a}**	**35.6^{a}**
	72	7.3^{b}	30.0^{b}	27.4^{b}
	96	4.8^{c}	27.7^{c}	10.3^{d}
	120	3.9^{e}	25.0^{d}	9.3^{e}
	144	3.1^{f}	21.5^{f}	8.1^{f}

The different letters for each experiment represent that the results are significantly different ($p < 0.05$)

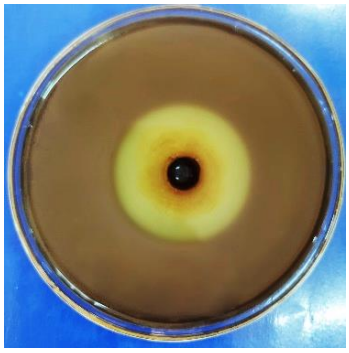

Figure 3. Pectin hydrolytic zone by *A. niger* M13 strain after 48 h of incubation

Effect of pH

The optimal pH for the highest pectinase activity, dry biomass, and diameter of a clear zone by the *A. niger* M13 strain were pH 6.5 with values of 38.4 U/mL, 8.2 g/L, and 33.7 mm, respectively (Table 3 and Figure 4).

Table 3. Effect of pH on pectinase activity and growth of the *A. niger* M13 strain

Mold strain	pH	Dry biomass (g/L)	Diameter of clear zone (mm)	Pectinase activity (U/mL)
A. niger M13	4.0	2.5^{f}	18.3^{g}	5.6^{g}
	4.5	3.6^{e}	20.5^{f}	7.8^{f}
	5.0	4.9^{d}	25.7^{e}	10.9^{e}
	5.5	6.1^{c}	26.7^{d}	25.7^{d}
	6.0	7.4^{b}	27.3^{c}	29.8^{c}
	6.5	**8.2^{a}**	**33.7^{a}**	**38.4^{a}**
	7.0	7.6^{b}	30.3^{b}	32.5^{b}

The different letters for each experiment represent that the results are significantly different ($p < 0.05$)

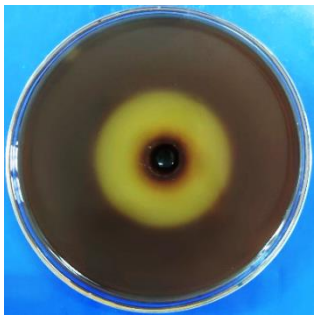

Figure 4. Pectin hydrolytic zone by *A.niger* M13 strain at pH 6.5

Effect of carbon sources

The impacts of glucose, maltose, lactose, fructose, starch, CMC, and molasses were studied. Our results showed that among the carbon sources investigated maltose was the optimal carbon source for pectinase production (Table 4 and Figure 5). Optimal pectinase production obtained when culturing the strain in the medium supplemented with maltose with 34.8 mm and 8.9 g/L of diameter of hydrolytic zone and fungus growth, respectively; followed by starch (32.3 mm and 7.4 g/L of diameter of hydrolytic zone and fungus growth, respectively). On contractor, the remaining carbon sources including fructose, CMC, molasses, and lactose suppressed pectinase production, in which the lowest pectinase accumulation observed in fructose medium (18.2 mm and 3.6 g/L of the diameter of hydrolytic zone and fungus biomass, respectively).

Table 4. Effect of different carbon sources on pectinase production and *A. niger* M13 growth

Mold strain	Source carbon	Dry biomass (g/L)	Diameter of clear zone (mm)
A. niger M13	Glucose	5.7^{c}	26.7^{c}
	Maltose	**8.9^{a}**	**34.8^{a}**
	Lactose	5.2^{d}	26.0^{c}
	Fructose	3.6^{e}	18.2^{f}
	Starch	7.4^{b}	32.3^{b}
	CMC	1.5^{f}	23.0^{e}
	Molasses	3.9^{e}	24.6^{d}

The different letters for each experiment represent that the results are significantly different ($p < 0.05$)

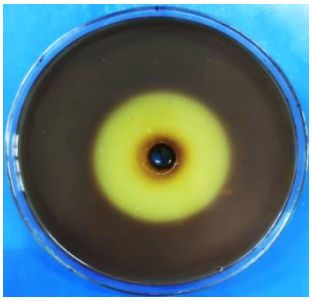

Figure 5. Pectin hydrolytic zone by *A.niger* M13 strain in the medium supplemented with maltose (0.5% w/v)

Effect of nitrogen sources

In the present report, the impact of type of nitrogen sources (inorganic and organic) on pectinase activity was conducted, and the results are shown in Table 5 and Figure 6. The results revealed that yeast extract was optimal nitrogen source for pectinase with the highest diameter of clear zone of 37.3 mm and dried fungal biomass of 9.6 g/L. The results also showed that organic nitrogen including gelatine and meat extract inhibited enzyme production with a value of 25.2 mm, 6.8 g/L; and 25.5 mm, 7.2 g/L of hydrolytic zone and fungus biomass; respectively.

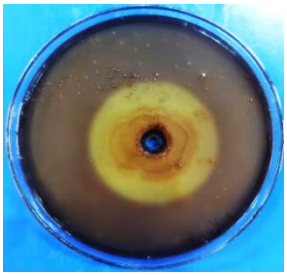

Figure 6. Pectin hydrolytic zone by *A.niger* M13 strain in the medium supplemented with yeast extract (0.2% w/v)

Table 5. Effect of different nitrogen sources on pectinase production and *A. niger* M13 growth

Mold strain	Source nitrogen	Dry biomass (g/L)	Diameter of clear zone (mm)
A. niger M13	Gelatine	6.8[f]	25.2[e]
	Yeast extract	**9.6[a]**	**37.3[a]**
	Meat extract	7.2[e]	25.5[e]
	Peptone	8.4[c]	27.3[d]
	Urea	8.7c	28.6[c]
	Ammonium nitrate	7.7[d]	27.5[d]
	Ammonium sulfate	9.1[b]	29. 2[b]

The different letters for each experiment represent that the results are significantly different ($p < 0.05$)

4. Discussions

In the present study, the pectinolytic fungus named as M13 strain which was isolated from Da Xanh pomelo peels was identified as *A. niger*, and further be used to evaluate pectinase production. Several factors affecting pectinase production including cultivation times, pH, energy sources, and nutrient sources were investigated.

The incubation time for *A. niger* M13 was continuously cultured until 144 h. Meanwhile, the pectinase activity and fungal biomass were measured at an interval of 24 h. The *A. niger* M13 produced the highest enzyme activity at 48 h of the incubation period. This result is in accordance with Khatri et al. (2015) that the optimum pectinase production by *A. niger* MCAS2 reached after 48 h of fermentation. Similarly, Sudeep et al. (2020) found the highest pectinase activity was obtained by *Aspergillus* spp. after 48 h of the incubation period. Enzyme accumulation was gradually decreased after 48 hours of cultivation (Table 2). The cause of this decline might be the lack of essential nutrients and/or toxic metabolites over-accumulation in the culture medium (Manal et al., 2016). The optimum activity of pectinase may alter depending on the fungus isolate, the type of fermentation, the composition of the growth medium, the concentration of the nutrient sources, and the pattern of pectinase production (Jalil et al., 2023). Castilho et al. (2000) observed that the highest pectinase activities by *A. niger* reached after 22 h of the fermentation period. Meanwhile, Patil and Dayanand (2006) reported a gradual increase in pectinase production by *A. niger* after 72 h. Darah et al. (2013) found that pectinase production by *A.niger* grew on pomelo *Citrus grandis* (L.) Osbeck peels in a solid-state system achieved its maximum pectinase activity on day six.

According to Jalil and Darah (2021), the pH is important for the regulation and promotion of enzyme synthesis by fungi. The optimum pH for pectinase production by *A. niger* M13 was 6.5. At the optimal pH, the catalyzed reaction of the enzyme is maximized. As the pH value is higher or lower than the optimum pH the enzymatic activity decreases. The pH of medium culture is affected by the formation, and consumption of organic acids, the release of hydrogen ions, and the absorption of nitrogen sources (Fontana and Silveira, 2012). This result agreed with Debing et al. (2005) who reported that optimal pH for pectinase production by *A. niger* A2.26 strain was 6.5. Meanwhile, the optimum activity of *A. niger* LFP-1 was found at pH 4.5 (Jalil et al., 2023) and that of *Aspergillus* spp. was found at pH 5.8 (Sudeep et al., 2020).

A supplementation carbon source significantly increased fungal biomass and secondary metabolite accumulation. In this study, maltose was the optimal carbon source for pectinase by the strain in comparison to other carbon sources. It was suggested that maltose acts as an inducer and stimulates the biosynthesis of pectinase. A similar observation was reported by Rajmane and Korekar (2012) that maltose promoted the pectinase activity of *Phoma caricae*, *Fusarium oxysporum*, and *Phytophthora nicotiana*. On the other hand, carbon sources such as CMC, fructose, molasses, and lactose inhibited enzyme production. The reason could be due to catabolite repression.

Aside from carbon sources, nitrogen also plays a critical role in the growth and enzyme production of fungi. This study found that yeast extract was the optimal organic nitrogen source. This phenomenon was suggested due to yeast extract maintaining fungi growth better than other residual nitrogen sources. This result was in agreement with the finding of Abdullah et al. (2018b) who reported that yeast extract gave maximum pectinase production. Similarly, Aguilar et al. (1991) showed the optimal organic nitrogen source for exo-pectinases production by *Aspergillus* sp. was yeast extract. In addition, Ire and Vinking (2016) found the supplementation of yeast extract strongly induced pectinase produced by *A. niger*. Meanwhile, Jalil et al. (2023) observed that the optimal inorganic nitrogen source was ammonium nitrate. Our result also showed nitrogen sources such as urea, gelatin, meat extract, ammonium nitrate, and peptone inhibited pectinase production. This result was similar to the report by Jalil et al. (2023) showing that peptone and urea suppressed pectinase production.

In conclution, the pectinolytic *A. niger* M13 from Da Xanh pomelo (*Citrus maxima*) peels produced highest pectinase under cultivation conditions including 48 h of incubation period, pH 6.5, maltose of 0.5% (w/v), and yeast extract of 0.2% (w/v). The hydrolytic zone was 34.8 mm. The present study suggests that the *A. niger* M13 could be used for

pectinase production for further application in food processing.

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