Optimization of Cultural Conditions for Pectinase Production by *Diaporthe* Isolate Z1-1N and Its Pathogenicity on Kiwifruit (#112035)

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Optimization of Cultural Conditions for Pectinase Production by *Diaporthe* Isolate Z1-1N and Its Pathogenicity on Kiwifruit

Shu-Dong Zhang [1], **Ling-Ling Chen** [1], **Chao-Yue Li** [1], **Xiao-Qing Long** [1], **Xue Yang** [1], **Xiao-Duo He** [1], **Li-Wen Du** [1], **Heng-Feng Yang** [1], **Li-Zhen Ling** [Corresp. 1]

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Diaporthe Z1-1N, the primary causal agent of soft rot disease in kiwifruit, exhibited higher pectinase activity compared to cellulase activity in both in vitro and in vivo incubation models. To gain deeper insights into the role of pectinases in the pathogenicity of this fungus, we evaluated the effects of incubation temperature (ranging from 18 to 38°C), duration (1 to 7 days), and medium pH (4.0 to 9.0) on the activities of two crucial pectinases: polygalacturonase (PG) and polymethylgalacturonase (PMG). Our single-factor experiments revealed that the optimal conditions for maximizing PMG yield were a pH of 7.5 and a temperature of 28°C, with peak activity occurring after three days of incubation. Notably, PG activity peaked on the fourth day under the same pH and temperature conditions. Under the optimal conditions identified through an orthogonal experimental design, PMG exhibited higher activity than PG. Further analysis showed that temperature was the most influential factor on PMG activity, followed by incubation duration and pH. The purified pectinase extracts exhibited an impact of up to 50% on the lesion size of the fungal mycelium of *Diaporthe* Z1-1N . These findings underscore the significance of PG and PMG as key virulence factors in the pathogenicity of *Diaporthe* Z1-1N, providing a solid scientific basis for future research into the functions of these enzymes.

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Abstract: *Diaporthe* Z1-1N, the primary causal agent of soft rot disease in kiwifruit, exhibited higher pectinase activity compared to cellulase activity in both in vitro and in vivo incubation models. To gain deeper insights into the role of pectinases in the pathogenicity of this fungus, we evaluated the effects of incubation temperature (ranging from 18 to 38° C), duration (1 to 7 days), and medium pH (4.0 to 9.0) on the activities of two crucial pectinases: polygalacturonase (PG) and polymethylgalacturonase (PMG). Our single-factor experiments revealed that the optimal conditions for maximizing PMG yield were a pH of 7.5 and a temperature of 28° C, with peak activity occurring after three days of incubation. Notably, PG activity peaked on the fourth day under the same pH and temperature conditions. Under the optimal conditions identified through an orthogonal experimental design, PMG exhibited higher activity than PG. Further analysis showed that temperature was the most influential factor on PMG activity, followed by incubation duration and pH. The purified pectinase extracts exhibited an impact of up to 50% on the lesion size of the fungal mycelium of *Diaporthe* Z1-1N. These findings underscore the significance of PG and PMG as key virulence factors in the pathogenicity of *Diaporthe* Z1-1N, providing a solid scientific basis for future research into the functions of these enzymes.

Keywords: Kiwifruit; Soft rot disease; *Diaporthe*; Pectinases; Optimization; Orthogonal design

Introduction

Kiwifruit (*Actinidia* spp.), commonly referred to as "mihoutao" (monkey peach) in China, represents a significant genus within the family Actinidiaceae. The most recent taxonomic revision identifies 55 species and 20 varieties within this genus (Li et al. 2007). Among these, *A. chinensis* and *A. deliciosa* are the predominant species cultivated, collectively accounting for

nearly all kiwifruit in international trade (Nishiyama 2007). Currently, *A. arguta* is cultivated on a smaller scale in Europe, New Zealand, and the United States, both commercially and by enthusiastic amateurs (Ferguson 2013). Kiwifruit is renowned for its excellent flavor and is rich in vitamin C, minerals, dietary fiber, phenols, carotenoids, and other essential nutrients (Liu et al. 2019; Ma et al. 2017; Nishiyama et al. 2004; Sivakumaran et al. 2018). However, soft rot disease poses a significant threat to the quality and yield of kiwifruit. Research indicates that fungi from the *Diaporthe* spp. are among the primary agents responsible for this soft rot disease (Díaz et al. 2014; Jiqing et al. 2019; Zhou et al. 2015), resulting in considerable annual economic losses.

The genus *Diaporthe* comprises over 1,300 taxa, with *Phomopsis*, its asexual states, also encompassing more than 1,000 species listed in MycoBank (www.mycobank.org, accessed in December 2024), which exhibit broad host ranges and a global distribution. According to the International Code of Nomenclature for algae, fungi, and plants (Kirk et al. 2015), *Diaporthe* takes precedence for recommendations regarding generic names due to the existence of two or more genera typified by either a sexual or asexual morph (Rossman et al. 2015). Research has shown that species within the *Diaporthe* genus represent some of the most harmful fungal pathogens, capable of causing various diseases, including stem canker, leaf spot blight, and fruit decay (Bai et al. 2016; Chen et al. 2017; Díaz et al. 2017; Wan et al. 2022). Currently, more than 10 *Diaporthe* species have been reported to cause soft rot disease during both the growing season and post-harvest storage of kiwifruit (Ling et al. 2024b). These pathogens can secrete cell wall degrading enzymes (CWDEs) that interact with host cell wall constituents, thereby facilitating pathogen penetration through the loosened host cell walls and middle lamella matrices. The CWDEs synthesized during plant infection by *Diaporthe* spp. include cellulases, xylanases, and pectinases (Chen et al. 2018; Zhang 2010).

Pectinases are pivotal in plant pathogenesis, marking the initial enzymes synthesized by specific fungal and bacterial pathogens that colonize isolated plant cell walls, priming these cell wall components for subsequent degradation by other enzymes (Abbott & Boraston 2008). Typically, pectin degradation is orchestrated by a suite of pectinases, which include pectate lyases (families 1, 2, 3, and 9), polygalacturonases and rhamnogalacturonases (glycoside hydrolase family 28), pectin methylesterases (carbohydrate esterase family 8), and pectin acetylesterases (carbohydrate esterase family 12) [CAZY Database] (http://www.cazy.org/index.html)). These enzymes are hypothesized to contribute to soft rot

infections by dismantling cell wall polysaccharides, leading to fruit softening and a decrease in disease resistance. Polygalacturonase (PG), pectinesterase (PE), and polymethylgalacturonase (PMG) are particularly noted for their roles in facilitating pectin disassembly in postharvest fresh fruits (Lin et al. 2018). Our recent research has shown that kiwifruit inoculated with *Diaporthe* Z1-1N displayed a higher disease index compared to non-inoculated harvested kiwifruit, with two pectinases (PG and PMG) showing increased activities in both in vitro and in vivo incubation models (Chen et al. 2024; Ling et al. 2024a). This highlights the significant role of these enzymes in the pathogenic process and their potential as targets for disease management strategies.

Previous studies have indicated that pectinase activity levels are influenced by factors such as Ca^{2+}, pH, and the culture medium (Pagel & Heitefuss 1990; Qi et al. 2010). To investigate the regulation of these activities in phytopathogenic microorganisms and their potential role as virulence factors under various environmental conditions in the fungi-host plant interaction, it is essential to examine the presence of pectinase activity levels. Therefore, this study aims to utilize the activities of two pectinases, PG and PMG, as indicators to optimize the culture conditions for their production. Specifically, we will investigate the effects of incubation temperature, incubation time, and the initial pH of the culture medium through single-factor and orthogonal optimization experiments. Additionally, the ammonium sulfate fractionation precipitation method will be employed to purify the enzyme solution. This research will also explore the pathogenicity of pectinase in the soft rot of 'Hongyang' kiwifruit, thereby providing valuable insights into the pathogenic mechanisms underlying these conditions.

Materials and Methods

Plant material and pathogenic fungi

Kiwifruit (*A. chinensis* cv. Hongyang) of similar size was purchased from the local market in Liupanshui, Guizhou Province, China. The pathogenic fungi *Diaporthe* spp. Z1-1N were isolated in our laboratory from rotten 'Hongyang' kiwifruit. This isolate was cultured on potato dextrose agar (PDA) medium at 25 °C in the dark for 6 days. ~~Subsequently, it was used to obtain inoculum for the enzyme assays and pathogenicity trials conducted in this study.~~

Optimization of pectinase production in liquid culture

In this study, we employed a modified Marcus liquid medium, as described in our previous

research, to investigate the production of two pectinases: PG and PMG (Ling et al. 2024a). Five millimeter mycelial discs from a 6-day-old PDA culture were inoculated into 100 ml of liquid medium contained in 250 ml flasks, which were then incubated on an orbital shaker. Optimization of pectinase production was conducted through a series of single factor experiments, assessing variables such as incubation periods (1, 2, 3, 4, 5, 6, and 7 days), temperature (18, 23, 28, 33, and 38 °C), and the initial pH of the medium, which ranged from 4 to 9 in 0.5 intervals. Cultures were harvested on the third day, and the contents were filtered using a vacuum to remove the fungal mycelia. The filtrates were subsequently centrifuged at 10,000 g for 15 minutes at 4 °C, and the supernatant was utilized to assess enzyme activity. ~~Finally, the optimal conditions for PMG and PG production were determined from each single factor experiment~~.

Based on the results of the single-factor experiment, a 3-factor, 3-level standard orthogonal table, L_9 (3^3), was selected to optimize the production conditions for two pectinases (PG and PMG) as shown in Table 1. The inoculation temperature (A) was set at 25, 28, and 31 °C; the initial pH of the medium (B) was set at 6.5, 7.0, and 7.5; and the inoculation time was set at 2, 3, and 4 days. These factors and their corresponding levels were incorporated into the L_9 (3^3) orthogonal table, resulting in a total of nine experimental groups with various combinations of the three parameters in this study.

Measurement of pectinase activity

The activities of PMG and PG were determined using the 3,5-dinitrosalicylic acid (DNS) colorimetric method, following the procedures outlined by our previous study (Ling et al. 2024a).

Purification of crude enzyme extracts and inoculation on kiwifruit fruit

Pectinase was induced for production from the liquid culture of the *Diaporthe* isolate Z1-1N using orange pectin as the carbon source. The purification process adhered to the method described ~~by us~~ (Ling et al. 2024a). The pathogenicity of various treatments, including pectinase extract and mycelial plugs from the *Diaporthe* isolate Z1-1N, was assessed. Sterile water and sterile plugs served as controls, respectively. Ten kiwifruit fruits were used in each treatment. The disease is classified based on the diameter of the lesion (Li et al. 2019): grade 0 (0 cm); grade 1 (0 cm-1.5 cm); grade 3 (1.5-3 cm); grade 5 (3-4.5 cm); grade 7 (4.5-6 cm); grade 9 (more than 6 cm). The Disease Index (DI) is calculated as follows:

$$\text{Disease Index (DI)} = \frac{\sum (\text{The rotten fruits of each grade} \times \text{the corresponding grade})}{\text{Total number of tested fruits} \times \text{the topmost grade}} \times 100\%$$

Data and statistical analysis

All experiments were performed in triplicate ~~and data collection was carried out in accordance with this replicate setup. From the gathered data~~, we calculated the mean and standard error to provide a comprehensive analysis. To discern significant differences among the means, we employed one-way analysis of variance (ANOVA) using SPSS version 19.0 for Windows. Following the ANOVA, we applied Duncan's multiple range test to separate the means, establishing statistical significance at the $P < 0.05$ threshold. Additionally, we leveraged the Orthogonal Designing Assistant II V3.1 software for the rigorous evaluation of our statistical experimental design, ensuring the precision of our experimental outcomes.

Results

Effect of initial pH of medium on two pectinase production

The initial pH of the medium is a crucial factor in the production of pectinases, as it influences both the type and quantity of enzymes produced by fungi. In this study, the *Diaporthe* Z1-1N was found to produce pectin methylgalacturonase (PMG) and polygalacturonase (PG), with their activities showing no significant differences from pH 4.0-7.0. When the pH increased from 7.5 to 9.0, PG activity significantly decreased relative to that of PMG. As illustrated in Figure 1, PMG activity remained relatively stable across different pH levels, with a significant peak observed at pH 7.5 (10.14 U/ml). In contrast, PG activity exhibited a notable decline when the pH increased from 4.5 to 5.0, followed by a gradual increase that reached its highest level at pH 7.5 (9.70 U/ml), before dramatically declining from pH 8.0 to 9.0. Therefore, these results indicate that the optimal production of both PMG and PG by *Diaporthe* Z1-1N occurs at pH 7.5.

Effect of incubation temperature on two pectinase production

Temperature is directly related to the metabolic activities of microorganisms and significantly influences both growth and product formation. Figure 2 illustrates the effects of various temperatures on the production of two types of pectinase. The activity of PMG was consistently higher than that of PG across different temperatures, with the exception of 33 °C, where PG exhibited greater activity than PMG. Additionally, both PMG and PG displayed a similar trend, characterized by an initial increase in activity followed by a decline. Our results indicate that maximum pectinase production occurred at 28 °C, yielding 10.14 U/ml for PMG

and 9.70 U/ml for PG. Subsequently, the second most favorable temperature for PMG production was 23 °C (8.87 U/ml), while for PG, it was 33 °C (8.92 U/ml). Conversely, the lowest production levels for both pectinases were observed at 38 °C, with values of 7.32 U/ml for PMG and 6.39 U/ml for PG. Therefore, these findings suggest that the optimal temperature for the growth and production of PMG and PG by *Diaporthe* Z1-1N is 28 °C.

Effect of incubation periods on two pectinase production

The incubation period is crucial for maximizing enzyme yield. Over the course of 1 to 7 days, we monitored the activities of PMG and PG, as illustrated in Figure 3. Throughout this period, PMG generally exhibited higher activity than PG, with the exception of the second and fifth days. Our data further indicated that both pectinases displayed significant fluctuations in activity, albeit with distinct patterns. For PG, activity gradually increased from the start, peaking on the third day at a concentration of 10.52 U/ml, before gradually declining by the seventh day. PMG followed a similar trend with a slight variation; its activity began to rise on the second day, dipped slightly on the third day, and then reached its maximum on the fourth day at 10.43 U/ml. Subsequently, PMG activity decreased, reaching its lowest point at the 7-day mark with a yield of 7.42 U/ml, as depicted in Figure 3. These findings suggest that the optimal times for PMG and PG production are the fourth and third days of incubation, respectively.

Optimization of cultural conditions for pectinase productivity

Culture conditions, such as pH levels, incubation temperatures, incubation duration, and the sources of carbon, nitrogen, and mineral salts, are pivotal in dictating the synthesis and secretion of extracellular enzymes by microorganisms (Jia et al. 2010; Koirala et al. 2014; Patidar et al. 2018). Hence, meticulous adjustment of these parameters is essential when investigating the pathogenicity associated with extracellular enzymes secreted by pathogenic fungi. An orthogonal experiment demonstrated that the highest activity of PMG, at 11.228 U/mL, was attained at a temperature of 28°C, an initial pH of 7.5, and an incubation period of 2 days (Table 2). Under these same conditions, PG achieved its peak activity of 10.056 U/mL (Table 2). This finding highlights that PMG exhibited superior activity compared to PG under identical culture conditions, necessitating a more detailed analysis of PMG activity.

In this study, we examined the impact of three key factors—incubation temperature (A), initial medium pH (B), and incubation duration (C)—on PMG production by *Diaporthe* Z1-1N. By analyzing the magnitude of the extreme R values, we ranked the influence of these factors on

PMG production activity as follows: A > C > B (Table 3). This ranking indicates that incubation temperature had the greatest impact, followed by incubation duration, and then initial pH value. As previously mentioned, the optimal conditions were determined to be an incubation time of 2 days, an incubation temperature of 28°C, and an initial pH of 7.0. To confirm the effectiveness of the optimization approach based on the orthogonal experiment's results, we conducted a verification experiment using the refined parameter combination. The findings revealed that PMG activity increased significantly to 11.50 U/mL (Table 3), surpassing the activity observed in all previous orthogonal experiments. This outcome validates the efficacy of the optimization strategy employed.

Pathogenicity of the pectinase extract on kiwifruit

In this study, we investigated the effects of a purified pectinase extract on kiwifruit. The pectinase was prepared from a three-day shaken lipid culture using orange pectin as the sole carbon source, based on the optimal conditions for PMG production. Our results indicated that the pectinase extract induced significant necrotic lesions, with the disease index (DI) value reaching 47.62% after seven days of treatment (Table 4). Additionally, we observed that the symptoms caused by the pectinase extract were similar to those resulting from infection with *Diaporthe* Z1-1N mycelium. However, the necrotic lesions induced by the pectinase extract were considerably less severe than those caused by the mycelium plug, which exhibited a DI of 94.81%. In contrast, the control fruits inoculated with sterile water or PDA plugs showed no lesions. Therefore, these results suggest that pectinase plays a role in lesion development associated with *Diaporthe* Z1-1N.

Discussion

One of the primary barriers against phytopathogenic fungi is the plant cell wall, which is rich in polysaccharides. Most fungi must breach this barrier to access plant cells, necessitating the secretion of various enzymes capable of degrading cell wall polymers. Pectinases, a group of enzymes that decompose pectic substances, are classified into several categories: polygalacturonase (PG), polymethylgalacturonase (PMG), pectin lyase (PL), pectate lyase (PAL), and pectin methylesterase (PME) (Xue et al. 2018). Our recent study reported that *Diaporthe* Z1-1N exhibited the increased activities of two pectinases (PG and PMG) when cultivated in a kiwifruit infection model (Ling et al. 2024a). Furthermore, the production of these enzymes

secreted by this fungus was biochemically assayed in an *in vitro* system (Ling et al. 2024a). To determine the optimal external conditions for enzyme production, this study aims to investigate the production of these two pectinases by *Diaporthe* Z1-1N under varying incubation temperatures (25-55 °C), incubation durations (1-7 days), and pH levels of the medium (3.6-5.2).

Our findings indicated that during the single-factor experiment, both PMG and PG achieved their maximum activities at the same incubation temperature of 28°C and medium pH of 7.5. However, there was a slight variation in the timing of these peak activities over the incubation period. Specifically, PMG reached its peak on the third day, whereas PG attained its maximum on the fourth day. Previous research has established a sequence for the appearance of cell wall-degrading enzymes in phytopathogenic fungi (Lisker et al. 1975a; Lisker et al. 1975b). Polygalacturonase, recognized as a virulence factor, is known to be induced in the early stages of infection (Lisker et al. 1975a; Vyas et al. 2025). I In a related context, PMG enzyme accumulation was observed early in the infection process of Diaporthe Z1-1N, which is associated with the development of kiwifruit soft rot. Furthermore, the levels of cell wall-degrading enzyme (CWDE) activity have been correlated with the severity of pathogenesis (Gawade et al. 2017; Zhang et al. 2014; Zhou et al. 2016). PG activity is essential for full virulence across a range of host plants (Kubicek et al. 2014; Vyas et al. 2025). In this study, under the same incubation temperature and medium pH, PMG exhibited higher activities compared to PG. However, during the same incubation periods, PG demonstrated more robust production than PMG. The optimal conditions for PMG production by *Diaporthe* Z1-1N, as determined through an orthogonal experimental design, significantly enhanced its activity in an *in vitro* cultivated model. Additionally, the purified pectinase extracts resulted in a 50% lesion size of fungal mycelium. These findings emphasize that both PG and PMG are crucial virulence determinants in the pathogenicity of *Diaporthe* Z1-1N, aligning with observations made in in *Rhizoctonia solani* Kühn (Chen et al. 2006). However, other pectinases, such as PME, PL, and β-galactosidase (β-gal), are also involved in disease development caused by phytopathogenic fungi (Gawade et al. 2017). Consequently, further investigation into the roles of these additional enzymes is planned.

Moreover, varying cultural conditions have led to differences in the activities and types of cell wall-degrading enzymes produced. For instance, PMG exhibited a higher activity when soluble starch was used as a carbon source, while PG demonstrated a greater activity when

utilizing pectin as a substrate during *in vitro* cultivation of *Phomopsis longanae* Chi (Chen et al. 2018). According to the results of our orthogonal experiments, the incubation temperature was identified as the most influential factor for the production of PMG by *Diaporthe* Z1-1N. A previous research has indicated that temperature affects the mycelial growth of *Sclerotium rolfsii* and the severity of rot on potato tubers (Daami-Remadi et al. 2010). Notably, *Diaporthe* Z1-1N was isolated from rotten kiwifruit during cold storage. Therefore, it is essential to investigate the effects of low temperature on the activities of PG and PMG, as well as conduct pathogenesis tests. Additionally, other cultivation factors, such as metal ion concentrations (Ca^{2+}, Fe^{2+}) and shaking conditions, also influence the production of pectinases (Chen et al. 2006; Pagel & Heitefuss 1990). Consequently, the relationship between the cultivation conditions of specific cell wall-degrading enzymes and their virulence requires further investigation.

Conclusions

In this study, we optimized the production of two pectinase enzymes, PG and PMG, from *Diaporthe* Z1-1N, the microorganism responsible for soft rot disease in kiwifruit. This was achieved by evaluating three key factors—pH, temperature, and incubation duration—using an orthogonal experimental design. Our findings revealed that the highest production of PMG occurred at pH 7.0 and 28°C on the third day, whereas maximum PG production was observed at the same pH and temperature but on the fourth day. Notably, PMG demonstrated superior activity compared to PG when each factor was optimized individually. A more detailed analysis of PMG activity, based on the orthogonal design, indicated that incubation temperature was the most influential factor, followed by inoculation time and pH. The purified pectinase extracts, obtained under these optimized conditions, exhibited significant pathogenicity, as demonstrated by a 50% lesion size of the fungal mycelium of *Diaporthe* Z1-1N. These results provide a solid foundation for future research into the functions of PMG and PG.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Figure 1

Figure 1. Effect of pH on PMG and PG production

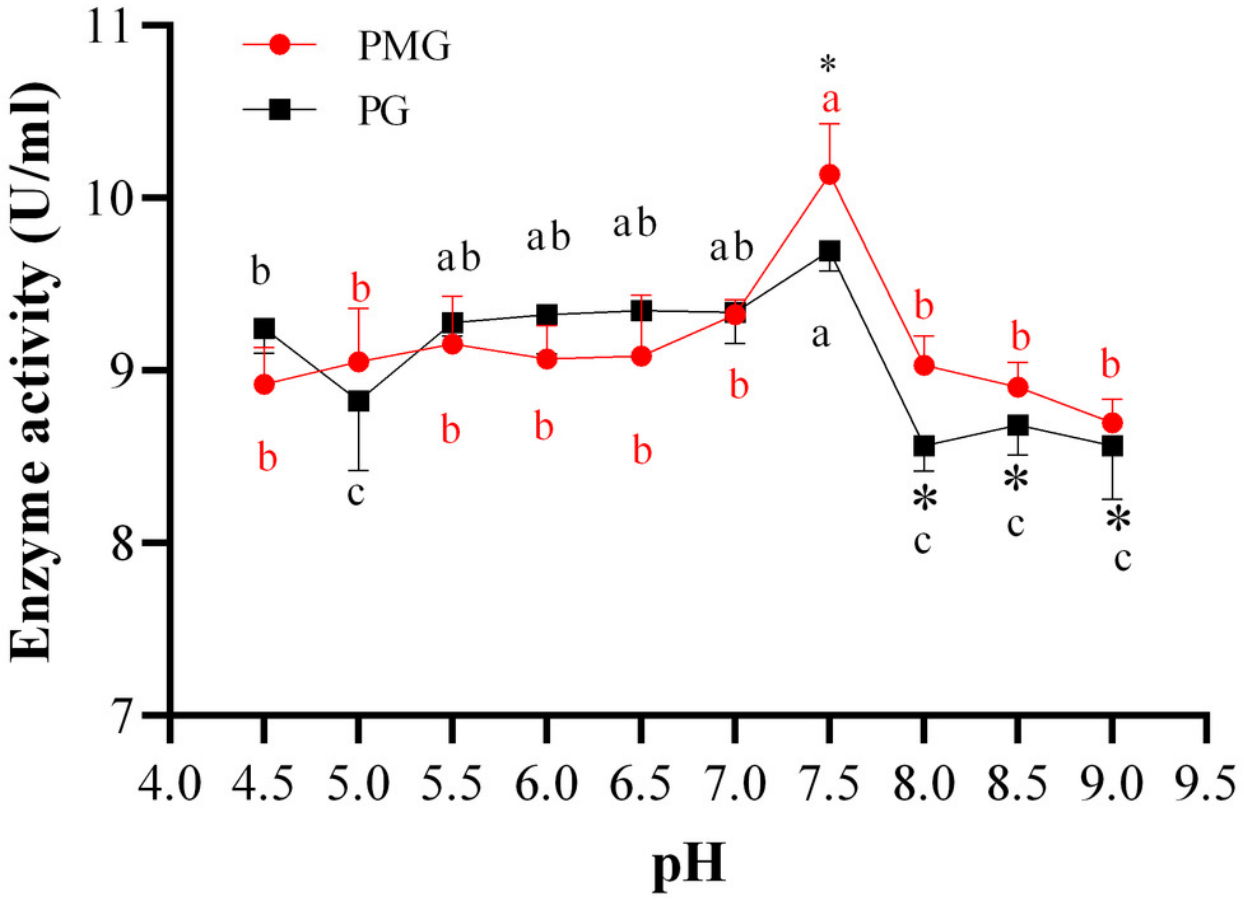

Figure 2

Figure 2. Effect of incubation temperature on PMG and PG production

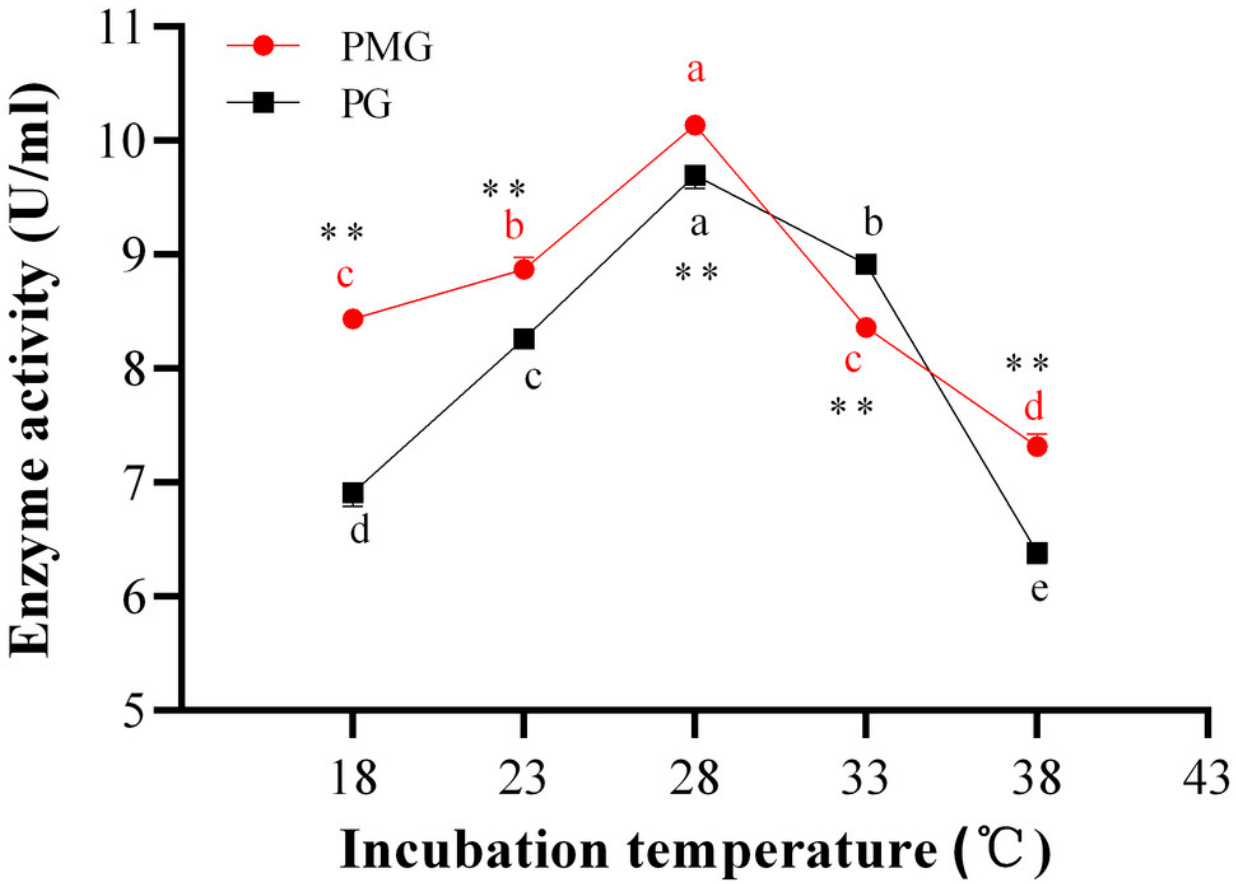

Figure 3

Figure 3. Effect of incubation time on PMG and PG production

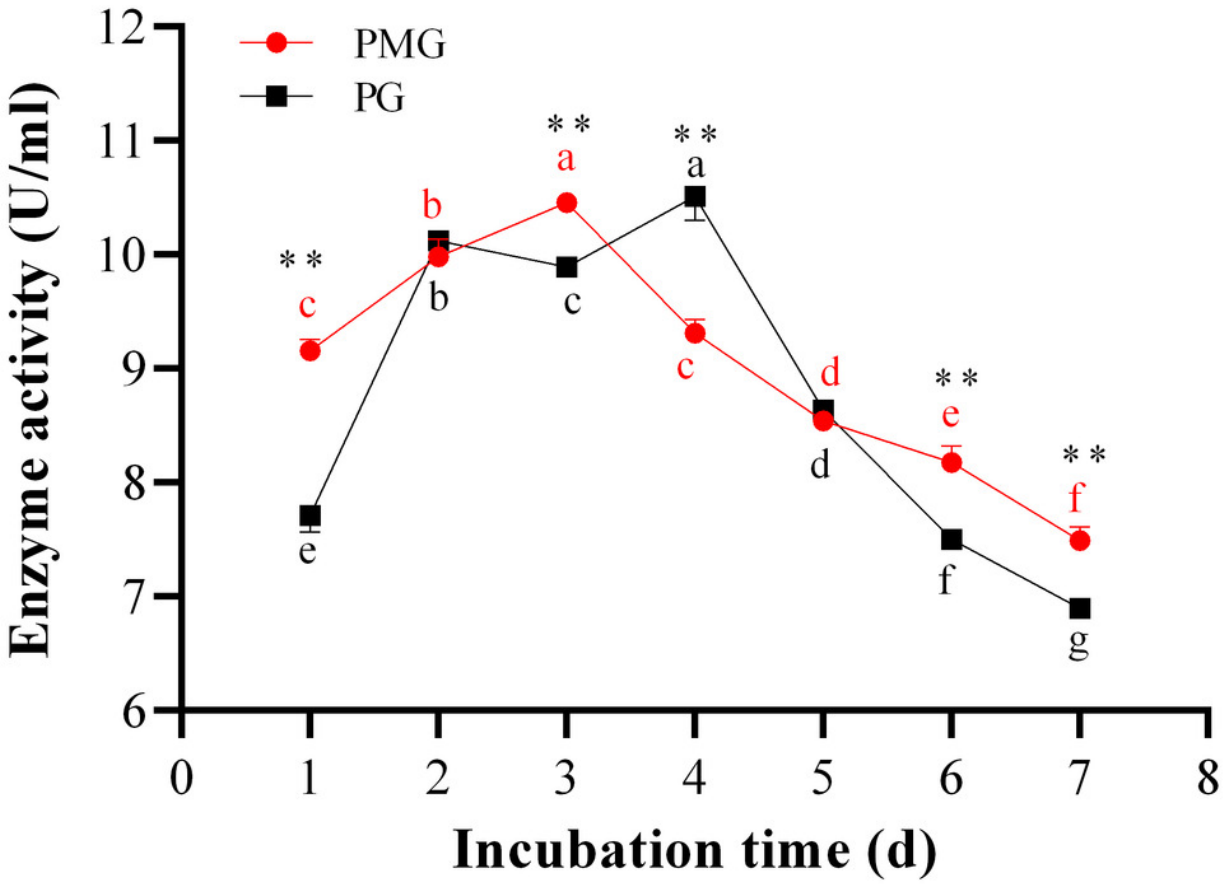

Table 1(on next page)

Table 1. Factors and levels of pectinase production

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Levels	Factors		
	Inoculation temperature (℃)	Initial pH of the medium	Inoculation time (d)
	A	B	C
1	25	6.5	2
2	28	7.0	3
3	31	7.5	4

Table 2(on next page)

Table 2. Results of the orthogonal experimental design

Table 2. Results of the orthogonal experimental design

Tested No.	Factors			Enzymatic activity (U/mL)	
	A	B	C	PMG	PG
1	25	6.5	2	9.702	9.027
2	25	7.0	3	9.755	9.213
3	25	7.5	4	9.306	7.864
4	28	6.5	3	10.949	9.999
5	28	7.0	4	10.002	9.415
6	28	7.5	2	11.228	10.056
7	31	6.5	4	8.730	6.141
8	31	7.0	2	10.661	8.536
9	31	7.5	3	9.467	8.384

Note: Factor A: Inoculation temperature (℃); Factor B: Initial pH of the medium; factor C: Inoculation time (d).

Table 3(on next page)

Table 3. Analysis of orthogonal experiment of PMG activity

Table 3. Analysis of orthogonal experiment of PMG activity

	PMG activity (U/mL)		
	A	B	C
K1	9.59	9.79	10.53
K2	10.73	10.14	10.06
K3	9.62	10.00	9.35
Range (R)	1.14	0.35	0.58
The factor importance	A＞C＞B		
Optimal combination	$A_2B_2C_1$ (11.50 U/mL)		

Note: Factor A: Inoculation temperature (℃); Factor B: Initial pH of the medium; factor C: Inoculation time (d).

Table 4(on next page)

Table 4. Disease degree of 'Hongyang' kiwifruit under different treatments

Table 4. Disease degree of 'Hongyang' kiwifruit under different treatments.

Treatments	DI (%)
Sterile water	0.00
Pectinase extract	47.62
Sterile plug	0.00
Mycelial plug	94.81

Note: the data was obtained after seven days of the different treatment.