Influence of cultural conditions on enhancing chitinase produced by *Paenibacillus polymyxa* M6 strain isolated from soil at Thanh Tra pomelo (*Citrus grandis* (L.) Osbeck) farm in Vietnam

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Abstract
In this study, we investigated the culture conditions to optimize the production of extracellular chitinase from a Paenibacillus sp. strain isolated from rhizosphere of pomelo orchards affected by gummosis. The enzyme exhibited maximum activity (56.68 U/ml) under conditions where soy flour served as the nitrogen source. Colloidal chitin concentration was maintained at 0.3%, initial pH was set to 3 and static culture was maintained at 30°C for 72 hours, with the inoculum continuing at 5% (v/v).

The results demonstrate the strain's potential for chitinase production, suggesting its suitability for further development and application in products aimed at preventing gum disease in Thanh Tra pomelo.

Keywords: Chitinase, *Paenibacillus polymyxa*, Thanh Tra pomelo.

Introduction

Microbially-produced chitinase (EC 3.2.1.14) assumes pivotal roles across diverse fields such as biotechnology, waste management, pharmaceuticals, biomedical applications, single-cell proteomics, protoplast isolation from fungi and yeast, drug delivery and the enzyme industry[1]. These applications collectively contribute to transforming chitin, a polymer composed of N-acetyl-β-D-glucosamine (GlcNAc), into valuable products. The primary function of chitinase is to break down chitin by cleaving glycosidic bonds involving endochitinases, responsible for hydrolyzing internal β-1,4-glycoside; exochitinases, which process chitooligosaccharides, releasing diacetyl chitobiose or N-Acetyl glucosamine from the non-reducing end of chitin and β-N-acetylglucosaminidase which cleaves GlcNAc units sequentially from the non-reducing end of the substrate[9].

In the soil and rhizosphere, bacteria effectively utilize chitin from insects and fungi as a source of carbon and nitrogen[17]. With their proficiency in exploiting chitin from these sources, chitinolytic microorganisms are poised to play a crucial role as biocontrol agents and antagonists against pathogens [1]. For example, *Bacillus amyloliquefaciens* V656 displayed peak inhibition of fungal growth (0.4 U ml^{-1}) at 24 hours, *Burkholderia gladioli* CHB101's chitinase impeded various fungal pathogens and *Aeromonas caviae* managed infections by *Rhizoctonia solani*, *Fusarium oxysporum* in cotton and *Sclerotium rolfsii* in beans through chitinase production[9].

Chitinase is renowned for its ability to degrade the cell walls of fungi where chitin serves as the predominant component. Compared to fungi, oomycetes display unique characteristics. Research on their cell wall structure indicates that oomycete cell walls contain minimal chitin, constituting less than 0.5% of the total cell wall substance [10]. In the case of *Saprolegnia* sp., 0.7% of the cell wall is comprised of true crystalline chitin N-acetyl glucosamine, a component non-essential for hyphal growth. Conversely, Aphanomyces spp. relies on 10% of its cell wall being composed of non-crystalline chitosaccharides, essential for hyphal growth[10].

Particularly for *Phytophthora* sp., whose chitin levels undergo changes throughout their growth stages, the use of chitinase to inhibit it proves exceptionally significant. Cheng et al study[5] demonstrated the involvement of chitin and putative chitin synthase in the vegetative growth and asexual reproduction of *Phytophthora* sp., contributing to disease during host plant infection as observed through WGA-FITC labeling methods. Specifically, chitin is predominantly present in zoospores rather than fungal hyphae.

In this study, a *Paenibacillus polymyxa* strain, isolated from the rhizosphere of Thanh Tra pomelo (*Citrus grandis* Osbeck) orchards with symptoms of gummy cankers and antagonistic to *Phytophthora* sp., was investigated for optimized culture conditions to maximize chitinase production, with the aim of potentially inhibiting the growth of *Phytophthora* sp.

Material and Methods

Bacterial strains and cultures: *Paenibacillus polymyxa* M6 and *Phytophthora citrophthora* TB4 strains were previously isolated and identified by our team in a separate study, with the current investigation inheriting this strain for its subsequent study. To do the experiment, the bacterial strain was revived from glycerol stock by steaking onto an LB agar plate and incubating at 37°C overnight. To stimulate chitinase activities, chosen isolates were introduced onto solid Czapek-Dox medium devoid of glucose, supplemented with 1% (w/v) colloidal chitin [16] and incubated at 37°C for

24 hours. The hydrolysis of colloidal chitin was identified using Lugol's solution[15]. The fungal colonies displayed robust growth on potato dextrose agar plate incubated at 30°C.

Determination of antagonistic ability: Potential bacterial strains were evaluated for their ability to resist fungal pathogens using the co-culture method. The bacterial strain was co-cultured with the fungal pathogen *Phytophthora* sp. on the PDA medium plate in a symmetrical streak pattern on both sides, with a distance of 2 cm from the edge of the plate and the fungal strain was inoculated at the center position between the two lines. The control plate was inoculated with only the fungal pathogen *Phytophthora* sp. The plates were incubated at 30°C for 3-9 days and data on the size of the fungal inhibition zone were collected[7].

The efficiency of inhibiting the growth of fungal mycelium by bacteria is calculated according to the formula:

PIRG (%) = (R-r)/R x 100

where PIRG (percentage of inhibition of radial growth) is the percentage of radial inhibition of bacteria (%), R is the radius of the mycelium of *Phytophthora* sp. control (cm) and r is the radius of the mycelium on the plate with the antagonistic bacterial strain.

Colloidal chitin preparation: Five grams of chitin from Shrimp shells (HIMEDIA) were added to 100 ml of HCl (35.5%) and kept in a refrigerator (4°C) for 24 hours. Thereafter, 2 liters of tap water were added and the gelatinous white material thus formed was separated by centrifugation. The precipitate was washed with tap water until the supernatant had a pH of 6.0. The obtained colloidal chitin had a soft, pasty consistency[13].

Chitinase Assay: The method of measuring chitinase activity was adapted from Hoang Anh Nguyen's research and modified. The reaction mixture consisted of 250 µl of a 2%-solution of colloidal chitin in 50 mM sodium phosphate buffer (pH 6) and 250 µl of appropriately diluted enzyme solution. After incubation for 30 min at 40°C, the reaction was stopped by heating at 100°C for 10 minutes and then centrifuged at 10,000 rpm for 5 minutes. The concentration of reducing sugars in the supernatant was determined based on the dinitrosalicylic acid (DNS) method using GlcNAc as the standard. One unit of chitinase activity is defined as the amount of enzyme releasing one µmol of reducing sugar per minute under the specified assay conditions[14].

Optimization of culture conditions: In the process of optimizing cultural conditions, growth occurred in 50 ml of LB medium (1% NaCl, 1% peptone, 0.5% yeast), agitated at 150 revolutions per minute in a shaker at room temperature. After 24 hours, 1 ml of this young seed culture was inoculated in 50 ml of chitin medium containing colloidal chitin, a nitrogen source, 0.5% (w/v) NaCl, 0.05% (w/v) $MgSO_4.7H_2O$, 0.09% (w/v) $K_2HPO_4.3H_2O$, 0.03% KH_2PO_4, 1% (w/v) $(NH_4)_2SO_4$. Following incubation, the supernatant was separated by centrifuging the culture broth at 10,000 rpm for 5 minutes, which was then used as the crude enzyme. To explore the impact of additional nitrogen sources, we tested three simple nitrogen sources (peptone, yeast and soy flour) at a concentration of 0.3% w/v.

After that, we investigated the influence of substrate concentration by assessing chitinase activity. This entailed culturing the isolate with varying concentrations of colloidal chitin (ranging from 0.1% to 1.5%) to determine the most favorable substrate concentration. Furthermore, we investigated the influence of different agitation speeds, temperatures, pH levels, inoculation times and bacterial concentrations. The experiments were conducted sequentially in the mentioned order. The optimized conditions will be consistently applied when examining the next set of variables.

Statistical Analysis: All the optimization parameters were conducted in triplicate and the data was analyzed using single-factor Analysis of Variance (ANOVA) and Duncan's test in IBM SPSS Statistics 20. All the data are graphically presented as mean ± S.D of triplicates. A significant level of $P \leq 0.05$ was considered statistically significant.

Results

Determination of activity of chitinase and its effectiveness in antagonizing the *Phytophthora* sp.: As mentioned above, *Paenibacillus polymyxa* M6 was used to study its ability to produce chitinase enzyme. Figure 1 shows the detection of chitinase activity on an assay plate containing 1% (w/v) colloidal chitin. The diameter of the chitin substrate dissolution ring is about 19 mm. Additionally, experiments assessing its ability to antagonize the *Phytophthora* sp., which causes gummosis disease on Thanh tra pomelo, demonstrated clear antagonism. The results indicated that the inhibition effect on disease strain after 9 days reached 41.97 %.

Influence of nitrogen sources on chitinase production: The effect of additional nitrogen sources on chitinase production is presented in figure 1. The study was carried out using three organic nitrogen sources: peptone, yeast and soy flour, added to the medium containing colloidal chitin as the sole carbon source. Among the nitrogen sources studied, soy flour is considered the best nitrogen source for chitinase production. The measured chitinase activity was 82.908 U/ml, which is twice the activity measured for peptone as a nitrogen source.

Influence of colloidal chitinase concentrations on chitinase production: After selecting soy flour as the nitrogen source, colloidal chitin was investigated at different concentrations (0.1%, 0.3%, 0.5%, 1.0%, 1.5%). The results showed that the *Paenibacillus polymyxa* strain exhibited strong chitinase activity when the substrate concentration in

the culture medium was 0.3%. However, enzyme activity tended to decrease as the substrate concentration increased (Figure 2).

Influence of agitation speed on chitinase production: Culturing the strain in a medium containing 0.3% colloidal chitin and soy flour as the nitrogen source at different shaking speeds showed that the bacterial strain is suitable for static culture to produce extracellular chitinase. Chitinase activity measured during static culture was 53.29 U/ml (Figure 3).

Influence of temperature on chitinase production: The temperature ranges used were 25°C, 30°C, 37°C and 40°C. The strain can produce chitinase at all these temperatures, indicating its activity across a wide temperature range. However, the strains exhibit the strongest chitinase activity at 30°C, reaching 41.63 U/ml, compared to the other temperatures (Figure 4).

Influence of pH on chitinase production: Based on the findings depicted in figure 5, chitinase activity peaks are observed in the culture medium at pH 3 (107.09 U/ml) (Figure 5). As pH levels increase, activity gradually diminishes. This indicates the strain's preference for a highly acidic environment to facilitate chitinase production.

Influence of time on chitinase production: The duration of strain cultivation for chitinase production is a crucial factor. Through observation periods of 24, 48, 72, 96 and 105 hours, it was determined that the optimal culture time for maximum chitinase activity was 72 hours, resulting in a peak activity of 61.73 U/ml (Figure 6). After this period, there is a trend of decreasing measured chitinase activity.

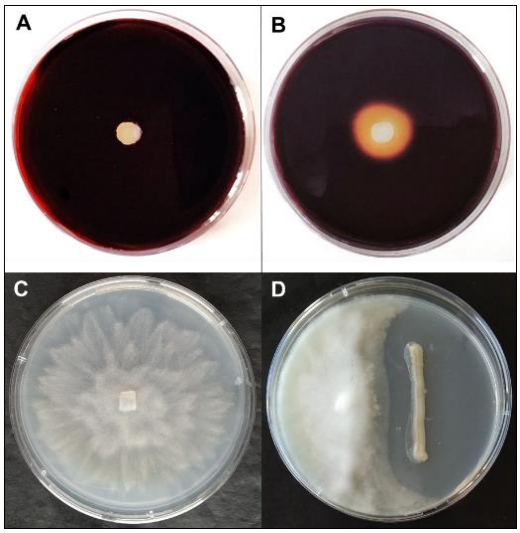

Figure 1: Determination of chitinase activity from *Paenibacillus polymyxa* M6 on an assay plate containing 1% (w/v) colloidal chitin and its effectiveness in antagonizing the *Phytophthora* sp., which causes gummosis disease on Thanh tra pomelo.
A. Control plates stained with Lugol's solution, B. Clear zone observed on the chitinase-containing plate (strain M6), stained using the same method, indicating chitin degradation, C. *Phytophthora citrophthora* TB4 on PDA plate, D. The effectiveness of strain M6 in antagonizing TB4 strain.

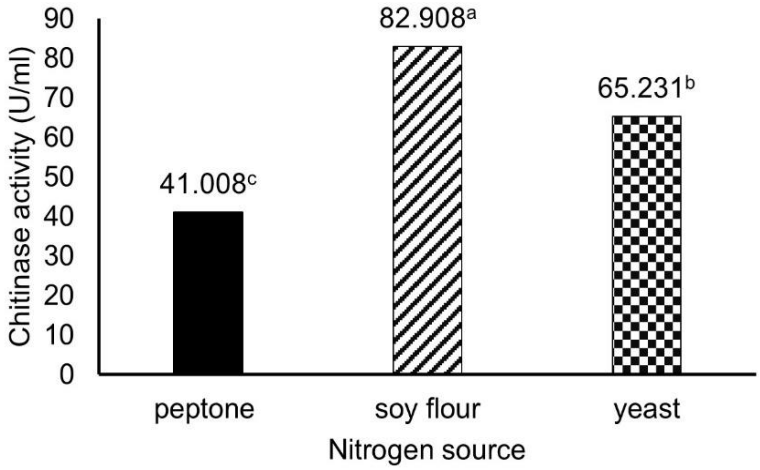

Figure 2: Effect of different nitrogen sources on production of chitinase by *Paenibacillus polymyxa* M6

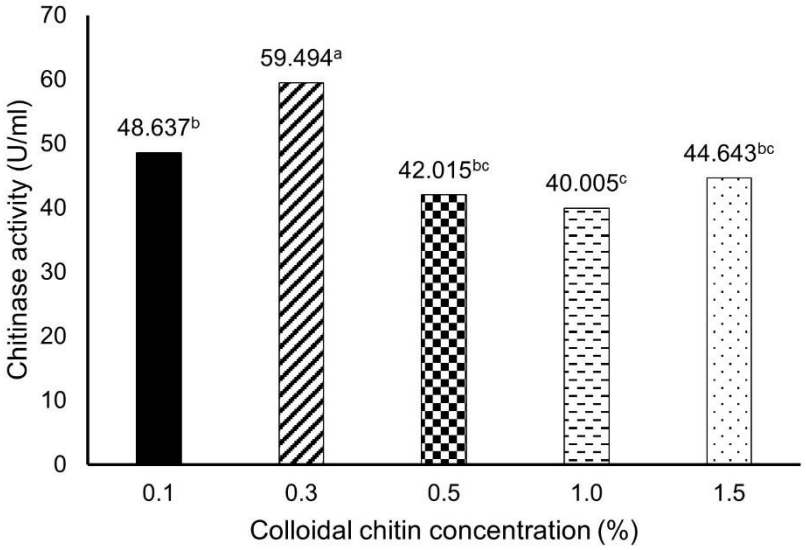

Figure 3: Effect of colloidal chitin concentration on production of chitinase by *Paenibacillus polymyxa* M6

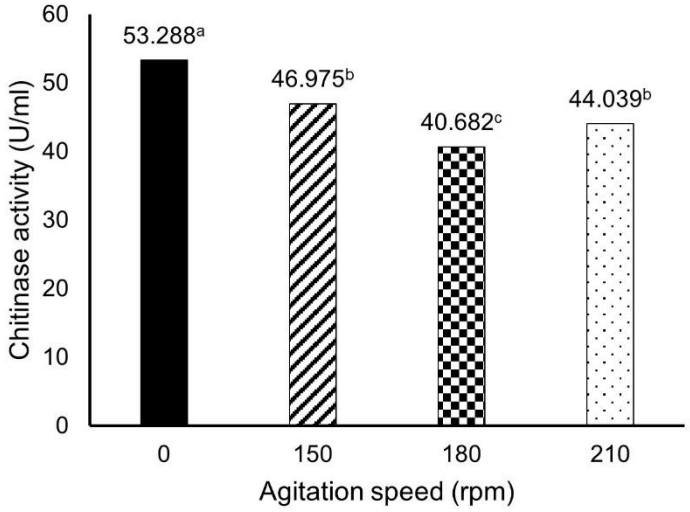

Figure 4: Effect of agitation speed on production of chitinase by *Paenibacillus polymyxa* M6

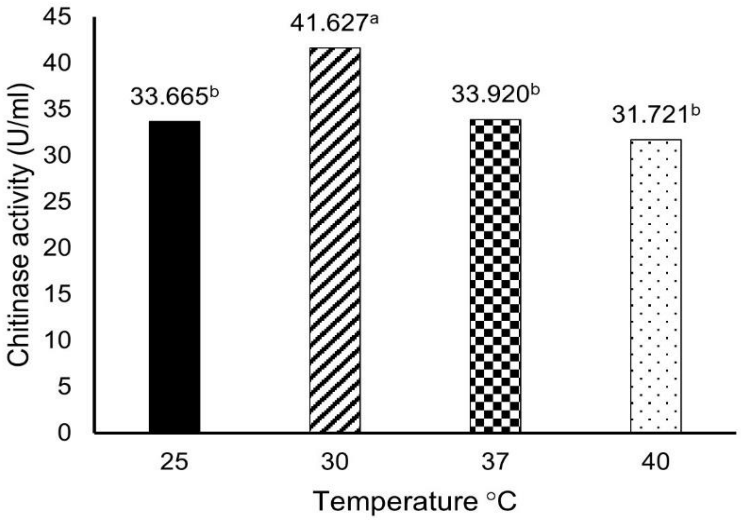

Figure 5: Effect of temperature on production of chitinase by *Paenibacillus polymyxa* M6

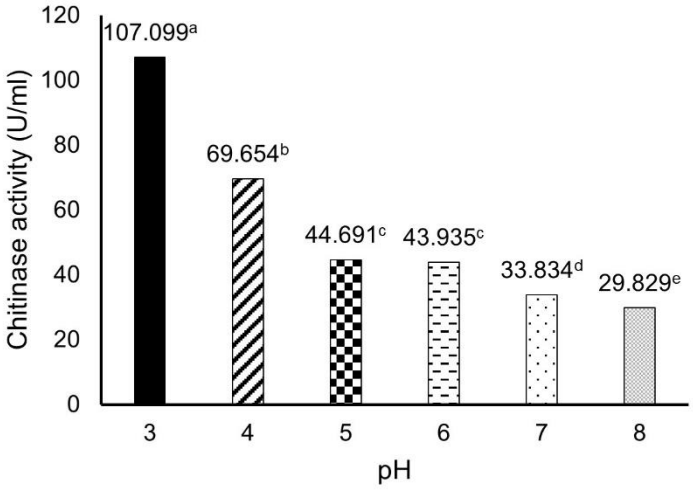

Figure 6: Effect of different pH on production of chitinase by *Paenibacillus polymyxa* M6

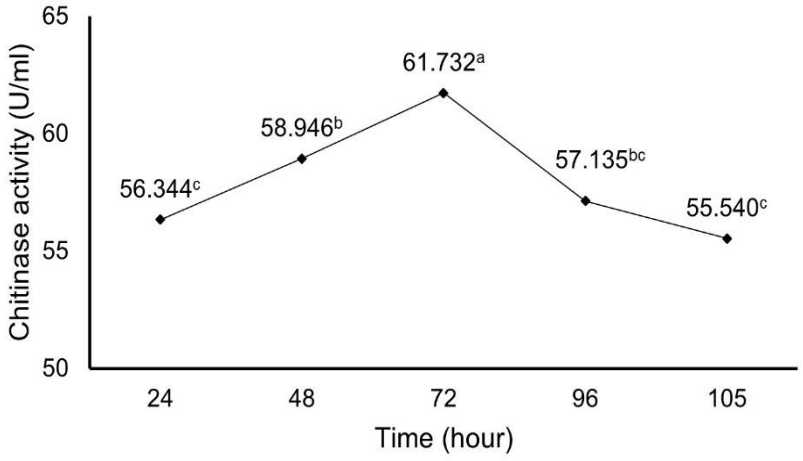

Figure 7: Effect of time on production of chitinase by *Paenibacillus polymyxa* M6

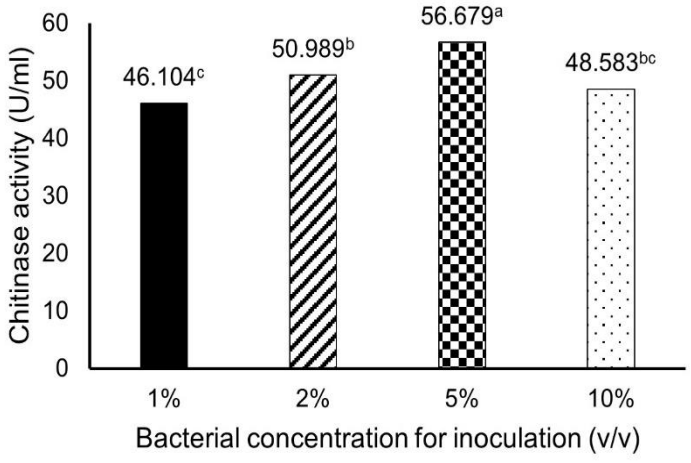

Figure 8: Effect of bacterial concentration for inoculation on production of chitinase by *Paenibacillus polymyxa* M6

Influence of bacterial concentration for inoculation on chitinase production: Optimal bacterial concentration for inoculation also contributes to maximizing the ability to hydrolyze colloidal chitin in the culture medium. The seeding results at 5% demonstrated the highest chitinase activity at 56.68 U/ml (Figure 7). The 2% bacterial concentration for inoculation ranked second. However, if the seeding rate is increased to 10%, the measured activity begins to decrease**.**

Discussion

Indeed, the results indicating a high potential for chitinase production and resistance to pathogens in the isolated microbial strain suggest promising avenues against gummosis disease. Chitinase have been recognized for their ability to degrade chitin, a major component of fungal cell walls and arthropod exoskeletons. By utilizing microorganisms capable of producing chitinase, it is possible to target and to degrade the chitin present in the biofilms formed by pathogenic bacteria associated with gum disease, thereby disrupting their structural integrity and aiding in their elimination.

Moreover, the observed resistance to pathogens in the isolated microbial strain suggests that it may possess inherent mechanisms to compete against or inhibit the growth of other harmful microorganisms commonly implicated in gum disease. This trait could be leveraged to develop probiotic formulations or therapeutic interventions aimed at restoring microbial balance in the oral microbiome and preventing the proliferation of pathogenic species.

Paenibacillus polymyxa M6, isolated from pomelo root soil, can hydrolase chitin. We optimized the culture medium for optimal chitinase production. While glucose, sucrose, or GlcNAc do not induce chitinase production, colloidal chitin is commonly used[6,8,12]. However, the optimal concentration varies. Our research found that M6 produces the most chitinase at 0.3% colloidal chitin. Increasing to 0.5% or more reduces chitinase production. At substrate concentrations greater than 0.5%, the substrate concentration can exceed K_M (Michaelis-Menten constant) significantly and chitinase may not be saturated with colloidal chitin.

When most of the enzyme's active sites are unoccupied due to a high substrate concentration ([S] > K_M), the enzyme activity decreases[11]. This occurs because a smaller fraction of the enzyme molecules is actively catalyzing the reaction, resulting in a reduced rate of product formation.

Despite the presence of ample substrate, the enzyme cannot achieve its maximum catalytic capacity due to the limited availability of active sites for substrate binding[11]. Therefore, the enzymatic rate is lower under these conditions compared to the substrate concentration closer to K_M[11]. The strain is capable of producing chitinase at incubation temperatures of 25-40^{o}C and does not require an oxygen supply through stirring. This is considered advantageous for large-scale applications, where the installation of stirrers, temperature sensors and oxygen supply sensors may not be necessary in large fermentation vessels. Additionally, M6 strain could utilize soy flour, which is an easily accessible and popular source of nitrogen outside the laboratory.

The initial pH of the medium significantly affects cell growth, membrane permeability, enzyme biosynthesis, secretion, as well as activity and stability[4]. Among the culture factors investigated, pH exhibited a significant influence on the ability of strain M6 to produce chitinase. The effectiveness of initial pH is evident in that it further deviates from the optimum pH, the faster the inactivation occurs[3]. This means that while M6 produces chitinase at a strong acid pH of 3, inactivation practically occurs at pH above 8.

Research conducted on various strains has revealed different optimal conditions. For instance, *Paenibacillus* sp. A1 thrives best at a pH of 4.5 and a temperature of 50^{o}C[6]. On the other hand, *Paenibacillus barengoltzii* CAU904 prefers a pH of 3.5 and a temperature of 60^{o}C[8] . *Paenibacillus pasadenensis* NCIM 5434 shows optimal growth at a pH of 10.0 and a temperature of 37^{o}C[8]. Lastly, *Paenibacillus* sp. D1 exhibits optimal conditions at a pH of 5.0 and a temperature of 50^{o}C[2].

After identifying the suitable culture conditions listed above, we determined the initial seeding rate and the required incubation time to establish the optimal seed dose for the chitinase induction medium of strain M6 over a specific period. The ideal incubation time for chitin hydrolysis to achieve maximum chitinase activity is 72 hours, with the inoculum amount capped at 5% (v/v). If incubated for 72 hours, the optimal inoculum size is lower than 5% due to the increase in size, leading to inadequate nutrient availability for the large biomass.

Based on the results obtained from the experiment, the high chitinase activity produced by strain M6 is capable of hydrolase chitin. Meanwhile, we investigated the production of 1,3-beta-glucanase from strain M6 (unpublished). Given that *Phytophthora* sp. spores are highly mobile during heavy rains and in poorly drained pomelo orchards, our objective is not only to antagonize fungal mycelium but also to combine chitinase and 1,3-beta-glucanase to inhibit their spores in the upcoming experiment.

Conclusion

Paenibacillus polymyxa M6 produces chitinase in an acidophilic environment at pH 3, cultivated statically at 30^{o}C for 72 hours. A suitable carbon source is colloidal chitin at 0.3%. The strain can utilize nitrogen sources from soy flour. The inoculum size should not exceed 5%. The chitinase activity from strain M6 was measured at an average of 56.68 U/ml. These findings highlight the potential of harnessing microorganisms and their enzymes such as chitinase as a natural and effective strategy for combating gummosis disease. The potential of the strain can also be exploited to develop microbial preparations with other strains or in combination with other enzymes produced by M6 strain.

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