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EFFECT OF CULTURE CONDITIONS ON β-1,3-GLUCANASE PRODUCTION BY *Paenibacillus polymyxa* M6

Hoang Ha My A, Vo Thi Ngoc Trai, Nguyen Bao Hung*

Institute of Biotechnology, Hue University, Nguyen Đinh Tu St., Hue, Vietnam

* Correspondence to Nguyen Bao Hung <nguyenbaohung@hueuni.edu.vn>

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Abstract. In this study, we isolated and optimized the culture conditions for β-1,3-glucanase biosynthesis by a highly enzyme-producing bacterial strains isolated in Thua Thien Hue province. Among the three isolates M6, DH2, and T5, strain M6 exhibited the strongest β-1,3-glucanase production with the strongest hydrolysis zone of 19.03±0.05 mm diameter. Strain M6 was identified through nucleotide sequencing by 16S rRNA region, showing a similarity of 99.72% with *Paenibacillus polymyxa* HGPJ-62 in the GenBank database (NCBI). The optimized medium composition and culture conditions for strain *Paenibacillus polymyxa* M6 were determined as follows: the β-1,3-glucanase induction medium comprised the following components: $K_2HPO_4 \cdot 3H_2O$ 1.3 g; $MgSO_4 \cdot 7H_2O$ 1 g; KCl 0.5 g; yeast extract 0.5 g; $NaNO_3$ 1 g; oat flour 5 g; pH = 6, incubation temperature 40–50°C for 24 hours, shaking speed 180 rpm, and inoculum ratio 1%. The β-1,3-glucanase activity reached 1.5 U/mL. The present study highlights the β-1,3-glucanase biosynthesis by strain M6, demonstrating its suitability for development and application for prevent gummosis disease in Thanh Tra pomelo trees as well as in controlling fungal pathogens with glucan-dominant cell walls.

Keywords: β-1,3-glucanase, *Paenibacillus polymyxa, culture conditions, Thua Thien Hue*

1 Introduction

β-1,3-glucanase belongs to the group of polysaccharide hydrolases, capable of breaking β-1,3-glucosidic bonds in β-1,3-glucan chains. β-1,3-glucan is a major component of fungal cell walls and certain other microorganisms. By disrupting the structure, β-1,3-glucanase plays a critical role in morphogenesis, biomass degradation, and environmental protection [1]. β-1,3-glucanase exhibits high specificity and is produced by numerous microorganisms, including bacteria (e.g., *Bacillus* spp., *Paenibacillus* spp., *Pseudomonas* spp.) and fungi (e.g., *Trichoderma* spp., *Aspergillus* spp.). Particularly, bacterial-derived enzymes are highly valued for their rapid production, ease of cultivation, and robust stability under harsh environmental conditions [2].

β-1,3-glucanase plays a pivotal role in biocontrol activities due to its ability to degrade the cell walls of pathogenic microorganisms. This capability has been widely applied in agriculture to protect crops from diseases caused by fungi and other microorganisms [3]. Enhancing β-1,3-glucanase activity in soil ecosystems helps suppress pathogenic fungi such as *Fusarium* spp., *Phytophthora* spp., and *Sclerotinia* spp [4]. Recent studies indicate that this enzyme is also effective

against diseases in short-duration crops (e.g., rice, maize) and citrus fruits (e.g., oranges, lemons). In agricultural practices, microorganisms producing β-1,3-glucanase are often formulated into bioproducts to reduce pathogen density in soil [5].

Soil microorganisms represent a vital resource for β-1,3-glucanase production. Bacterial species such as *P. polymyxa*, *Bacillus subtilis*, and *Pseudomonas fluorescens* have been isolated and demonstrated high efficacy in enzyme production. Among these, *P. polymyxa*, a soil bacterium of particular interest, exhibits antagonistic activity against a wide range of pathogens and produces β-1,3-glucanase under suitable culture conditions [6]. Studies on *B. subtilis* have shown its ability to produce β-1,3-glucanase, contributing to antifungal activity and protecting plants from pathogenic agents [7]. Additionally, the fungus *Trichoderma* has been explored as a promising source of β-1,3-glucanase. The biocontrol indicators of *Trichoderma* include antagonism against pathogenic fungi such as *Rhizoctonia solani* and *F. oxysporum*. However, studies on microbial-based production of β-1,3-glucanase, particularly from bacterial species like *P. polymyxa*, remain highly promising due to their growth efficiency and flexibility in activity under diverse conditions [8].

Research on optimizing culture conditions (e.g., carbon sources, pH, temperature) for β-1,3-glucanase production is being widely conducted, particularly for promising microbial strains such as *P. polymyxa*. These factors play a crucial role in regulating enzyme production efficiency. For example, appropriate carbon sources can significantly enhance enzyme yield, while optimal pH and temperature ensure stable and sustainable enzyme activity [9]. Adjusting these culture conditions not only improves enzyme yield but also minimizes production costs, thereby unlocking broader potential applications of β-1,3-glucanase in agriculture and industry. Notably, in the context of increasing demand for environmentally friendly bioproducts, optimizing microbial enzyme production processes is essential to enhancing the efficiency of β-1,3-glucanase enzyme applications [10]. This is particularly significant as the demand for environmentally friendly biological products continues to grow. Studies have shown that using low-cost carbon sources, such as agricultural by-products or organic waste, can improve the economic viability of enzyme production processes [11].

The current research on β-1,3-glucanase from microorganisms reveals its promising application potential in agriculture and biology. Exploiting and innovating microbial culture processes to optimize enzyme production capacity will remain a key focus in the future.

2 Materials and Methods

2.1 Materials

The bacterial strain was isolated and selected from the rhizosphere soil of healthy Thanh Tra pomelo (*Citrus grandis*) trees in Thua Thien Hue province. Two sampling points were selected around the base of the healthy pomelo trees: one located 30 cm from the trunk, and the other positioned at the outermost edge of the canopy projection onto the soil surface. At each sampling point, 50 g of surface soil, 50 g of soil at a depth of 5 cm, and 50 g of soil at a depth of 20 cm were collected.

2.2 Methods

Isolation and Screening of Bacterial Strains

Approximately 50–100 g of soil was collected from the rhizosphere of healthy Thanh Tra pomelo trees in Thua Thien Hue. From each sample, 1 g of soil was weighed and added to a test tube containing 9 mL of sterile distilled water. The mixture was vortexed to fully dissolve the sample, producing a 10^{-1} dilution. Serial dilutions were then prepared (10^{-3}, 10^{-4}, and 10^{-5}) following the same procedure. From these dilutions, 50 µL was spread onto β-1,3-glucanase induction medium containing 0.5% laminarin. The plates were incubated at 37°C for 24 hours, and the development of bacterial colonies was observed. Strains capable of producing β-1,3-glucanase were identified based on the formation of clear halos around the colonies, following the modified method of Mahasneh AM and Stewart DJ [12]. The bacterial isolates were streaked on LB agar medium at least twice to obtain pure cultures [13].

Identification of Bacterial Strains Producing β-1,3-Glucanase

The bacterial strain was identified through molecular biology techniques by analyzing the 16S rRNA gene sequence. Total DNA was extracted using the TOPURE Genomic DNA Extraction Kit (ABT, Vietnam). The 16S rRNA sequence of the bacterial strain was amplified using the primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction cycle included the following steps: initial denaturation at 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 1 minute, and a final elongation step at 72°C for 10 minutes. Electrophoresis of the total DNA samples was performed on a 1% agarose gel at 80V using the Mini-Sub Cell GT system (Bio-Rad) for 30 minutes. The sequencing results of the bacterial strain were analyzed using BioEdit software, and the DNA sequence identity was determined by comparing it with

previously published sequences using the BLAST search tool available on the NCBI - National Center for Biotechnology Information.

A phylogenetic tree was constructed using the Neighbor-Joining statistical method and the Maximum Composite Likelihood substitution model in MEGA11 software, based on 16S rRNA sequences of *Paenibacillus* spp. and related species. The analysis included 1,000 bootstrap replicates, with sequences from the present study marked as "this study."

Investigation of cultivation conditions affecting β-1,3-glucanase synthesis of bacterial strain

The induction medium composition, modified from Mahasneh AM and Stewart DJ [12], included $K_2HPO_4 \cdot 3H_2O$ (1.3 g/L), $MgSO_4 \cdot 7H_2O$ (1 g/L), KCl (0.5 g/L), yeast extract (0.5 g/L), $NaNO_3$ (1 g/L), and laminarin (5 g/L) at pH 7. To assess the culture conditions, 50 mL of the induction medium was used and β-1,3-glucanase activity measured every 24 hours over a 5-day period. The optimized culture conditions identified from these experiments were then applied to a separate applied experiment.

Effect of inoculum ratio: The spore suspension of *Paenibacillus polymyxa* M6, cultivated for 16 hours, was adjust with sterile distilled water to achieve an optical density (OD) of 1 and used as the initial inoculum. The investigated concentrations were 1%, 5%, and 10% (v/v).

Effect of shaking speed: Using the optimal inoculum ratio identified in the previous experiment, *Paenibacillus polymyxa* M6 was cultivated with fermentation conducted at shaking speeds of 150 rpm, 180 rpm, and 210 rpm.

Effect of nitrogen sources: Following a similar methodology, organic nitrogen sources such as tryptone, yeast extract, and meat extract (0.05% concentration) and inorganic nitrogen sources including NH_4NO_3, $(NH_4)_2SO_4$, $NaNO_3$, and KNO_3 (0.1% concentration) were evaluated.

Effect of carbon sources: The investigated carbon sources included barley, oats, molasses, glucose, wheat yeast, laminarin and rice bran, each at a concentration of 0.5%.

The optimal temperature for enzyme activity was investigated in a range of 20-80°C with an interval of 10. For determination of optimum pH, the enzyme was diluted with 0.1 M sodium acetate buffer with a pH range from pH 3.0 to 5.0 with an interval of 1, potassium phosphate buffer (pH 6.0–7.0), and glycine–NaOH buffer (pH 8.0) [14]. The thermal stability and pH stability for the enzyme were obtained by incubating the enzyme at various temperatures between 20-80°C and pH of 3-8 for 30 min without substrate and measuring the residual activities.

The effect of metal ions and some surfactants on the activity of the β 1,3-glucanase produced by *Paenibacillus polymyxa* M6 was studied through the addition of 5 mM of metal ions (Ca^{2+}, Na^{+}, Cu^{2+}, Mg^{2+}, Al^{3+}, Fe^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}) and chemical agents (EDTA, SDS) to the reaction buffer.

β-1,3-glucanase assay

β-1,3-glucanase activity was determined according to the protocol described by Ueki et al., with minor modifications [15–17].

The activity of β-1,3-glucanase is determined based on the amount of reducing glucose released during the hydrolysis reaction. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol of glucose per minute under standard assay conditions.

200 μL of the enzyme solution were mixed with 300 μL of 0.5% laminarin (prepared in 0.1 M sodium acetate buffer, pH 5), then incubated at 40°C for 30 minutes. Subsequently, 500 μL of DNS reagent was added, and the mixture was heated at 100°C for 10 minutes. After rapid cooling to room temperature, the absorbance was measured at 540 nm.

2.3 Data Analysis Method

All experiments were performed in triplicate. Data were processed using Microsoft Excel and analyzed through ANOVA with Duncan's test, employing the SPSS statistical software at a significance level of $p \leq 0.05$.

3 Results and Discussion

3.1 Isolation and Screening of Bacterial Strains Producing β-1,3-Glucanase

From soil samples collected in Thanh Tra pomelo orchards in Thua Thien Hue, after isolation, screening, and purification, three bacterial strains were obtained: T5, DH2, and M6. These strains demonstrated the ability to produce β-1,3-glucanase, as evidenced by the formation of a clear zone with a diameter from 17.3±0.05 to 19.3±005 mm. Among them, we selected strain M6, which exhibited β-1,3-glucanase activity with a halo diameter of 19.3 ± 0.05 mm, for further study.

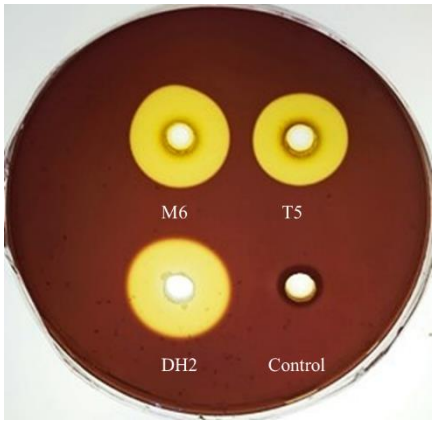

Figure 1. Clear zones of bacterial strains producing β-1,3-glucanase

Table 1. Morphological characteristics of the isolated bacterial strains with β-1,3-glucanase activity

Strain	Colony Morphology	Colony Image
M6	Diamond-shaped, milky white, smooth and glossy, with a flattened center and irregular, serrated edges.	
T5	Diamond-shaped, milky white, with a smooth surface, flattened center, and irregular serrated edges.	
DH2	Circular, opaque white, with a dry and rough surface, raised center, and irregular edges.	

3.2 Identification of the Bacterial Strain Producing β-1,3-Glucanase via Molecular Biology Techniques

Among the three bacterial strains screened, strain M6, which exhibited the strongest β-1,3-glucanase production, was selected for identification through gene sequencing. The 16S rRNA gene sequence of strain M6, with a length of 1422 nucleotides, was analyzed and compared using the BLAST N tool on NCBI. Results showed a high similarity (99.72%) with a bootstrap value of 100% to *Paenibacillus polymyxa* HZH1-92 (Accession No. PQ302227.1). Therefore, strain M6 was classified as *Paenibacillus polymyxa* (Figure 2).

A phylogenetic tree was also constructed to evaluate the relationship between the isolated strain M6 and other *Paenibacillus polymyxa* strains published in the GenBank database. The results demonstrated that strain M6 had a very close genetic relationship with other *Paenibacillus polymyxa* strains, forming a distinct clade separate from Bacillus subtilis, which was included for comparison in the phylogenetic tree (Figure 2).

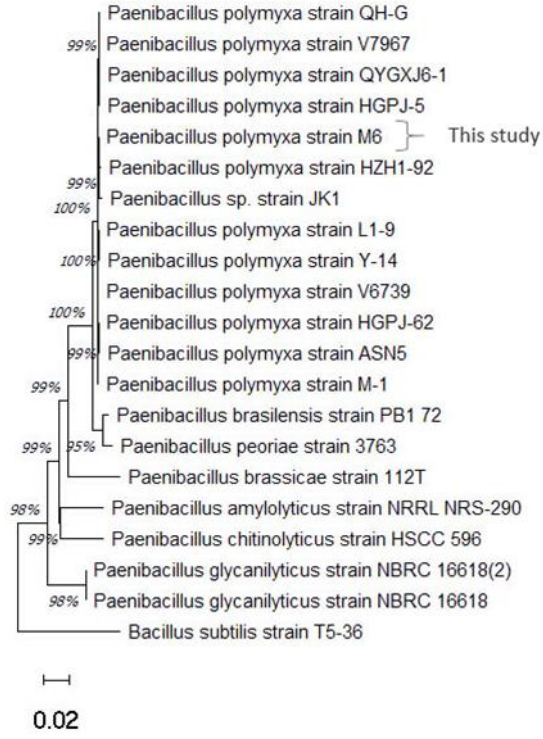

Figure 2. Phylogenetic tree of the M6 bacterial strain

3.3 Influence of cultivation conditions on the β-1,3-glucanase production ability of *Paenibacillus polymyxa* M6

Inoculum ratio

In this investigation, inoculum ratios of 1%, 5%, and 10% (v/v) were evaluated. The results revealed that an initial 1% inoculum led to the highest β-1,3-glucanase activity, reaching 1.5 U/mL after 48 hours of cultivation (Figure 3). In contrast, increasing the inoculum ratio markedly decreased the enzyme activity throughout the cultivation period. This reduction is likely attributable to excessive bacterial density, which rapidly depletes the substrate and essential nutrients required for the β-1,3-glucanase metabolic pathway.

Shaking speed

Shaking speed plays a critical role in modulating enzyme–substrate interactions. The experimental results indicated that *Paenibacillus polymyxa* M6 exhibited optimal growth and β-1,3-glucanase production at a shaking speed of 180 rpm, achieving an enzyme activity of 1.5 U/mL (Figure 4). Furthermore, when β-1,3-glucanase activity was evaluated at various shaking speeds, it showed a pronounced decline as shaking speed increased. Lencki et al. demonstrated that shear forces might enhance chemical or thermal enzyme inactivation, potentially by promoting the coagulation of denatured proteins and reducing their ability to renature [18]. During fermentation using shakers, stirrers, etc., shear forces are often generated; however, shear forces alone rarely damage proteins, as they lack sufficient energy to disrupt protein structures under

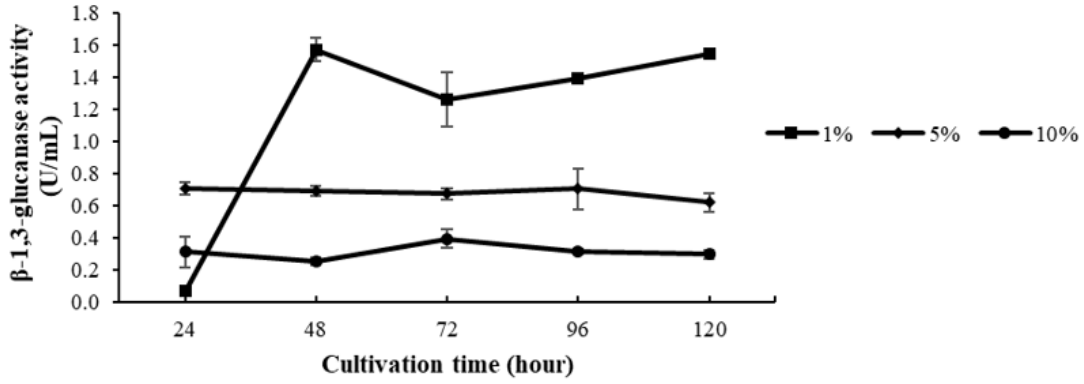

Figure 3. Influence of inoculum ratio on the biosynthesis of β-1,3-glucanase by *Paenibacillus polymyxa* M6

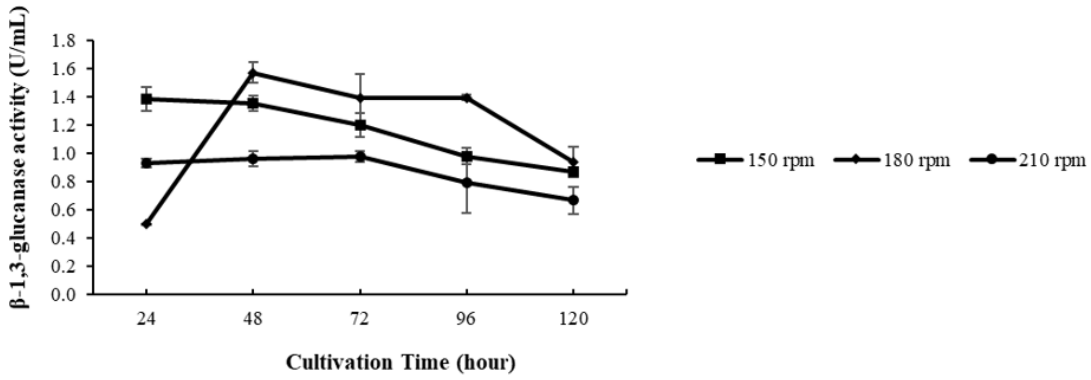

Figure 4. Influence of shaking speed on the biosynthesis of β-1,3-glucanase by *Paenibacillus polymyxa* M6

typical conditions. Nonetheless, they can amplify interfacial effects, which are the primary cause of protein instability [18]. At gas-liquid interfaces, proteins adsorb and unfold, leading to aggregation, as shown by Maa and Hsu with recombinant human growth hormone [19]. Similarly, hydrophobic solid-liquid interfaces exacerbate protein denaturation and aggregation compared to hydrophilic surfaces. Thus, shear indirectly contributes by enhancing these interfacial phenomena [18].

Nitrogen sources

During enzyme production, nitrogen is critical for enzyme synthesis as it supplies amino acids and other essential nutrients. Organic nitrogen sources are often preferred for inducing the synthesis of specific enzymes. The nitrogen sources evaluated in this study included tryptone, yeast extract, NH_4NO_3, $(NH_4)_2SO_4$, $NaNO_3$, KNO_3, and meat extract, all tested as replacements for the nitrogen source in the β-1,3-glucanase induction medium. These nitrogen sources are widely used and frequently reported in academic studies. The findings showed that employing yeast extract, an organic nitrogen source, resulted in the highest β-1,3-glucanase activity at 1.52 U/mL (Figure 5), aligning with the work of Tang and colleagues [20]. Studies on different microbial strains, such as *Moniliophthora perniciosa* and *Penicillium rolfsii*, also highlighted yeast extract as the optimal nitrogen source for culturing to produce β-1,3-glucanase [21, 22].

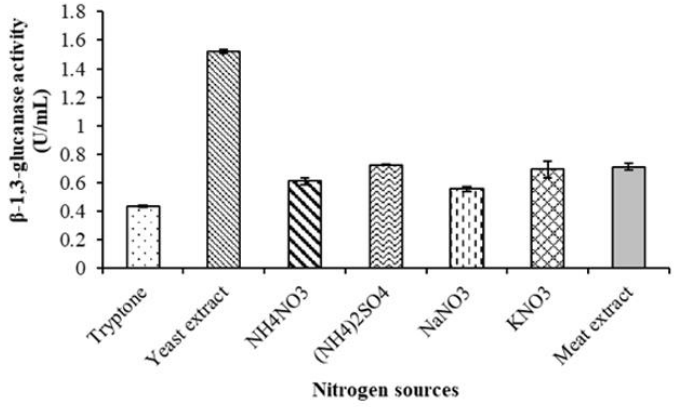

Figure 5. Influence of nitrogen sources on the biosynthesis of β-1,3-glucanase by *Paenibacillus polymyxa* M6

Carbon sources

Paenibacillus polymyxa M6 was cultivated under previously optimized conditions using a range of carbon sources, including barley, oats, molasses, glucose, wheat yeast, laminarin, and rice bran. Among these substrates, molasses and oats supported the highest β-1,3-glucanase activity, reaching 1.5 U/mL (Figure 6). Selecting an appropriate substrate depends on the specific production objectives. Molasses, a low-cost by-product rich in glucose and sucrose, is suitable for generating various enzymes due to its affordability and availability. Conversely, although oats are more expensive, they consist primarily of β-glucan, making them particularly effective for inducing β-1,3-glucanase. In the research by Santana and collaborators on *Rhodotorula oryzicola*,

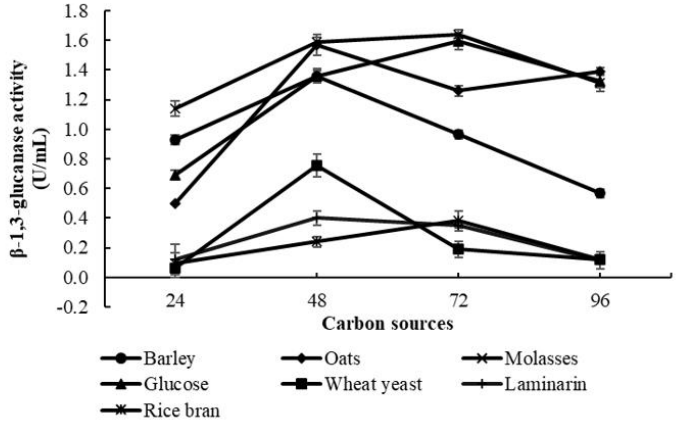

Figure 6. Influence of carbon sources on the biosynthesis of β-1,3-glucanase by *Paenibacillus polymyxa* M6

glucose as a carbon source yielded a β-1,3-glucanase activity of 0.2 U/mL. Although glucose demonstrated the ability to support β-1,3-glucanase production, it was not considered the most optimal carbon source compared to other evaluated substrates [23].

Temperatures

β-1,3-glucanase exhibited enzymatic activity throughout a temperature range of 20–80°C. The data revealed an optimal temperature of 50°C, and although activity declined at higher temperatures, over 50% of the activity was retained (Figure 7). These results agree with those of Zhang and colleagues, who reported that β-1,3-glucanase remains fully active at its optimal temperature of 50°C and retains 84% of its original activity after 1 hour of incubation at 50°C [24]. Additionally, the β-1,3-glucanase synthesized by *Paenibacillus polymyxa* M6 remained highly active in the 20–40°C temperature range, retaining 94.3% of its activity at 40°C. At 60°C, the residual activity markedly declined to 76% (Figure 7). Consequently, the β-1,3-glucanase recovered from this study is capable of efficiently catalyzing glucan hydrolysis at room temperature without denaturation, a characteristic that is advantageous for large-scale industrial applications.

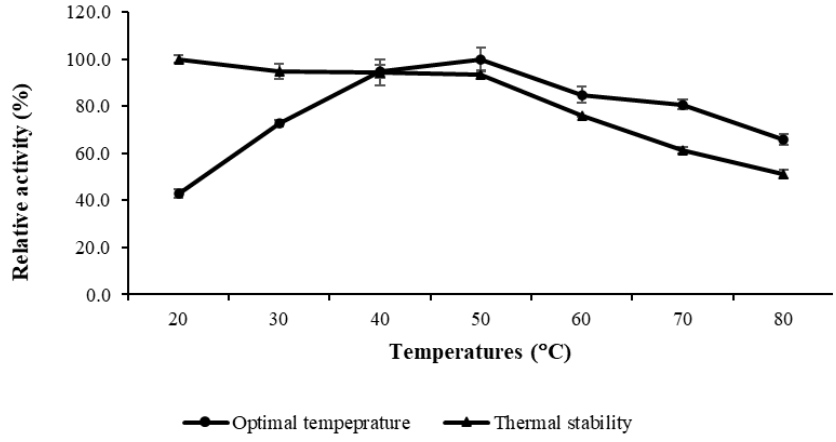

Figure 7. Influence of temperatures on β-1,3-glucanase

pH

Each microorganism has an optimal pH range for growth; excessively high or low pH values can inhibit or even kill the cells. Likewise, enzymes produced during microbial metabolism are influenced by the environmental pH, which can lead to denaturation or reduced enzymatic activity. In this study, the optimal pH and pH stability of β-1,3-glucanase from *Paenibacillus polymyxa* M6 were examined in the range of pH 3 to pH 8. The enzyme remained stable from acidic to near-neutral pH (with residual activity exceeding 60%) and exhibited its highest activity at pH 6 (Figure 8). According to Ueki et al., β-1,3-glucanase from *Clostridium* TW1 operates most effectively at pH 6.5–7.0 [17]. This bell-shaped curve aligns well with the typical characteristics of enzymes.

Metal ions

Metal ions can either enhance or inhibit enzyme activity, depending on their concentration. In this study, metal ions were applied at a concentration of 5 mM, and the results demonstrated that Ca^{2+}, Mn^{2+}, and Co^{2+} increased enzyme activity by 68.17%, 46.55%, and 38.44%, respectively, compared with the control that lacked metal ions (Figure 9). Although SDS is a powerful detergent capable of denaturing enzymes by disrupting non-covalent bonds in their tertiary and quaternary structures, the findings indicated that SDS at 5 mM did not denature β-1,3-glucanase. The residual enzyme activity remained at 98.05% (Figure 9). The effects of the other metal ions on enzyme activity were minimal, with the enzyme retaining more than 80% of its activity in each case. Thus, apart from the enhancements provided by Ca^{2+}, Mn^{2+}, and Co^{2+}, the presence of other

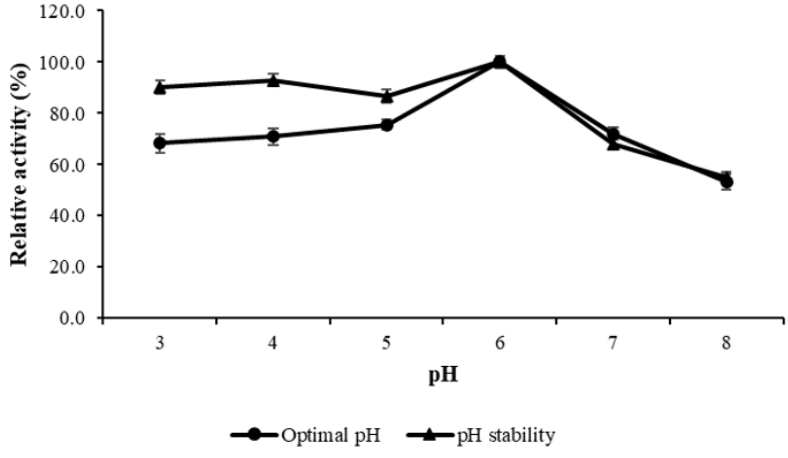

Figure 8. Influence of pH on β-1,3-glucanase

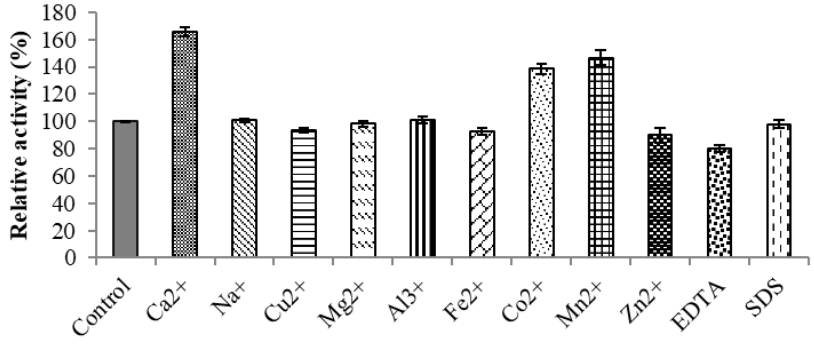

Figure 9. Influence of metal ions on β-1,3-glucanase

metal ions at a concentration of 5 mM did not significantly influence the β-1,3-glucanase activity of *Paenibacillus polymyxa* M6. At the same metal ion concentration of 5 mM, Calloni's study reported that Ca^{2+} had no significant influence on enzyme activity, whereas Co^{2+} and Mn^{2+} exerted pronounced inhibitory effects [25]. Another study on β-1,3-glucanase from *Delftia tsuruhatensis* by Blättel and colleagues demonstrated that Cu^{2+} and Zn^{2+} ions could reduce the enzyme's activity to only 20% of the control at a concentration of 1 mM [26].

4 Conclusions

In this study, the bacterial strain *Paenibacillus polymyxa* M6 was isolated and selected for its ability to synthesize β-1,3-glucanase, achieving an enzyme activity of 1.5 U/mL. The culture conditions were optimized as follows: a shaking speed of 180 rpm, an inoculum ratio of 1%, and an incubation temperature of 40–50°C for 48 hours. The induction medium for β-1,3-glucanase consisted of 1.3 g $K_2HPO_4{\cdot}3H_2O$, 1 g $MgSO_4{\cdot}7H_2O$, 0.5 g KCl, 0.5 g yeast extract, 1 g $NaNO_3$, 5 g oat flour, adjusted to pH 6. The findings indicate that *Paenibacillus polymyxa* M6 has significant potential for use in further investigations aimed at developing biological control products against pathogenic fungi whose cell walls are primarily composed of glucan.

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