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ORIGINAL ARTICLE

Screening and Optimization of pH and Temperature for Enhanced Chitosanase Production by Bacteria Isolated from Terengganu's East Coast

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Abstract

Chitosan is a naturally occurring, linear biopolyaminosaccharide that has gained popularity as a useful biopolymer with uses in food, cosmetics, pharmaceuticals, and agricultural sectors. Chitosanase is an enzyme that can cleave the chain of chitosan polymers and generally hydrolyse chitosan to chitosan oligomers and glucosamine as the product of hydrolysis.They are used as preservatives, additives, drug delivery systems (wound healing), and in plant protection (biopesticides) and growth. Chitosan oligomers were traditionally processed in the industry using chemical processes that would cause environmental harm due to high short-chain oligosaccharide production, low oligosaccharide yields and high separation expenses. Many previous studies revealed that certain bacteria are effective at producing chitosanase, offering benefits such as environmental compatibility, cost efficiency, and consistent reproducibility. Thus, it brings to the objective to isolate, screen and identify potential bacteria chitosanase producers and to study the optimum condition for the production of chitosanase (in term of pH and temperature). Methodology: The samples had been collected from eight different beach coasts near Besut and Kuala Terengganu. The experiments were conducted with four main stages, which were isolation, screening, fermentation, and optimization of potential chitosanase-producing bacteria. Three key analyses were carried out throughout the study, which were the measurement of optical density (OD) to assess bacterial cell growth, determination of colony-forming units (CFU) to evaluate cell viability and culturability, and chitosanase activity assay utilizing the Dinitro Salicylate (DNS) method. Results: The bacteria strain from Pantai Benting Lintang (BL) exhibited the highest chitosanase assay (0.113 U/mL), indicating superior chitosanase production. Optimal conditions for growth and chitosanase production were determined at pH 7.0 (0.127 U/mL) and 28°C (0.19 U/mL). Conclusion: This bacteria strain (BL) proved effectiveness in generating chitosanase, offering a potential alternative to chemical processes in chitosan biodegradation, while minimizing environmental impact. The study's findings contribute valuable insights for future research on environmentally friendly chitosanase-producing bacteria with broader applications in daily life.

Keywords: Chitin; chitosan; chitosanase; chitosanase assay; chitosanase-producing bacteria

Introduction

Chitosan is known for its efficacy as a flocculating agent, antifungal element, and hydrating agent. The anticancer, antibacterial, and antifungal properties of chitosan have been established, and it possesses various agricultural applications. They hold the ability to chelate nutrients and minerals, inhibit pathogens from accessing them, regulate sickness or impede its proliferation, or enhance a plant's natural defences (Kou et al., 2021). Chitosan is derived from the exoskeleton of marine organisms like crustaceans, lobster, shrimp, and crabs. Chitosan, a derivative from chitin hydrolysis, contains D-glucosamine polymer (Kaczmarek et al., 2019), that is either entirely or partially deacetylated, or nonacetylated (Roman et al., 2021).

Chitosanase is a hydrolytic enzyme produced in many microorganisms, including bacteria, actinomycetes, and fungi. Its major function is to cleave the β-1,4-glycosidic linkages in chitosan, a partially deacetylated derivative of chitin, leading to the formation of chitosan oligomers (or COS, chitosan oligosaccharides) and D-glucosamine monomers. These substances of degradation offer various biological functions and have broad applications in agriculture, medicine, and biotechnology. The efficiency of chitosanase, as well as the characteristics of the generated oligomers, are significantly influenced by the degree of acetylation (DA) of the chitosan substrate. The DA regulates the proportion of N-acetyl-D-glucosamine and D-glucosamine units in the polymer chain, which affects its solubility, charge distribution, and interaction with the enzyme. This makes the control of substrate properties and chitosanase crucial for tailoring chitosan degradation processes to specific industrial or biomedical applications.

Chitosanase is crucial to green biotechnology because it permits the conversion of waste chitin (from seafood industries, for instance) into high-value bioactive compounds under mild, environmentally friendly favourable conditions. Unlike methods involving chemicals, enzymatic hydrolysis allows for greater selective, avoids potentially harmful byproducts and can be altered by selecting specific microbial strains or enzyme variants. Moreover, the biological activities of COS and glucosamine vary based on their molecular weight and structure, which are regulated by the chitosanase and the DA of the substrate, making this a highly customisable system for tailored applications. Chemical processing challenges chitosan production from chitin, leading to high costs and environmental issues (Pellis et al., 2022; Arnold et al., 2020). Chitosanase biodegradation and hydrolysis offers a cost-effective, eco-friendly alternative (Riofrio et al., 2021).

Various bacteria and fungi strains like *Bacillus* sp., *Paenibacillus* sp. and *Streptomyces* sp. produce chitosanase (Xu et al., 2021; Zhang et al., 2021; Ma et al., 2020). Several studies have focused on isolating and characterizing microbial strains with high chitosanase-producing capability from varying ecological environments. For example, Huynh et al. (2019) successfully isolated bacterial strains from soil, saltwater, lake water, and lagoon environments, indicating the wide environmental dispersion of chitosanase-producing microorganisms. These isolates have showed promising activity in chitosan degradation, particularly under optimal fermentation conditions. Furthermore, efforts to optimise chitosanase production on a wide scale have focussed on optimizing several factors such as pH, temperature, and nutrient composition during fermentation (Lestari et al., 2020). These optimization efforts are crucial for enhancing enzyme yield and activity, thereby enabling the efficient and cost-effective production of chitooligosaccharides for commercial and biotechnological applications (Aranaz et al., 2021; Reshad et al., 2021; Bandara et al., 2020; Morin et al., 2019).

Materials and Methods

Microorganisms Growth

Bacteria samples were locally isolated from the beaches of Besut and Kuala Terengganu, including Pantai Benting Lintang, Pantai Bukit Kluang, Pantai Pulau Perhentian and Kuala Ibai

lagoon, as well as the lake at UniSZA Besut, resulting in over 10 strains. The screening for chitosanase-producing bacteria involved soil (sand), seawater, brackish water, and shell samples, which were collected in plastic bags and Schott bottles. Direct plating and enrichment methods at 30°C for microbial growth yielded various single colonies exhibiting different colours like white, pink, dull yellow, cream, and peach. The formation of clear zone around the single colonies were also observed. The formation of clear zones surrounding the microbial colonies exhibited positive strains (Zulkifly et al. 2010), which indicate chitosanase activity occurred as the colonies capable of hydrolysing chitosan, showed against an opaque tone of non-hydrolysed medium.

Culture Media

In this study, two types of culture media were utilized for screening chitosanase-producing bacteria: chitosan agar medium (M1) and chitosan liquid medium (M2) (Lestari et al., 2020). M1, used for screening, contained 1% chitosan powder, 0.5% $(NH_4)2SO_4$, 0.2% $K_2HPO_4.3H_2O$, 0.5% NaCl, 0.1% $MgSO_4.7H_2O$, 0.1% yeast extract, 1.5% agar, and 200 mL acetic acid, with the pH adjusted to 7.0. Sterilization at 90°C for 20 minutes eliminated undesirable contaminants. M2, utilised for chitosanase generation screening, differed only by excluding agar. Chitosan solutions were prepared by dissolving 1 g chitosan powder in 70 mL of 0.1 M acetic acid and adjusting pH with 6 M NaOH before topping up to 100 mL with distilled water. Initial pH was adjusted to 7.0 for bacteria isolation (Huynh et al., 2019).

Isolation of Chitosanase-Producing Bacteria

Fresh sand samples were dispersed on chitosan-containing media (M1), while 1 mL of salt and brackish water was evenly distributed on M1. Shells were sprinkled on M1 agar for shell samples. In the enrichment procedure, 2 mL of liquid samples and 2 g of sand were inoculated in 15 mL broth media (M2) and incubated at 30°C for 24 hours with a rotary shaker. Subsequently, 1 mL of the sample from the enrichment flask was spread on agar plate media (M1) and incubated at 30°C for 24 hours. Chitosan-containing mediums were identified by clearing zones.

The spread plate technique transferred 0.1 mL of the sample to chitosan agar, incubating for 24 hours at 30°C, with clearing zones signifying chitosan hydrolysis. Colonies with clear zones were transferred to culture from each plate, following the quadrant streak method for pure culture isolation (Lestari et al., 2020). All 10 samples have been shown in Table 1 with different locations. The sample also has been collected with different types of samples such as sand, seawater, and shell.

Screening of Chitosanase-Producing Bacteria

Using a solid medium with chitosan as the only carbon source (MI), the chitosanase properties of the bacteria isolated from the samples were examined. Bacterial strains were streaked onto a culture medium (M1) and were cultured for 24-48 hours at 30°C. After incubating the colonies, the clear zone around them was examined to indicate that the bacteria could break down the chitosan. Bacterial isolates exhibiting chitosanolytic activity can produce clear zones on chitosan agar plates with colloidal chitosan.

The chitinolytic index is the proportion of the exact zone diameter to the colony diameter. The emergence of the clear zone on the plate can identify the bacteria that produce chitosanase (Lestari et al., 2020). Single colonies with robust development and translucent rings were chosen for further purification. On pre-screening media plates, these colonies were injected using a sterile toothpick and allowed to develop for three days at 30°C (Zhang et al., 2021).

Fermentation of Chitosanase-Producing Bacteria

The bacteria were allowed to grow and produce chitosanase over a defined period. Bacteria were grown at 30°C for 24 hours in conical flask. Various parameters were closely monitored and controlled during this period, including temperature, pH, and agitation. These parameters were adjusted to create an optimal environment for bacterial growth and chitosanase production. The purified chitosanase was characterized to determine its activity, stability, and other properties. It was then stored in appropriate conditions, such as freezing or lyophilization, for long-term use or further analysis. In this study, the parameters that have been examined were temperature and pH. The studies were done in the batch culture phase and were performed in triplicate. Fermentation samples were analyzed for the measurement of cell growth using spectrophotometer (OD_{650}), colony-forming unit (CFU) for the cell culturability and chitosanase assay.

Optimization of Chitosanase Production in Batch Culture Fermentation

Optimization of Temperatures: 28°C, 37°C and 45°C

The effects of different temperatures (28°C, 37°C and 45°C) on the synthesis of chitosanase were investigated using M1 and M2 media for 2-3 days. The pH of 7.0 was adjusted as the starting point, and the experiment was run at 150 rpm.

Optimization of pH: pH 3.0, 7.0, and 9.0

The impact of various pH (pH 3.0, 7.0, and 9.0) on the production of chitosanase were also investigated. The experiment was run at 30°C with 150 rpm, by using M1 and M2 media. Citrate buffer (pH 3.0-4.0), phosphate buffer (pH 6.0-8.0), and boric buffer (pH 8.0-9.0) were used to determine the ideal pH for fermentation (Lestari et al., 2020).

Analytical Methods

For sample analysis, a volume of 5.0 mL was taken aseptically from the conical flask at 3-hour intervals for the first 9 hours and after 24 hours. The samples were put into a falcon tube and spun for 15 minutes at 4000 rpm. Chitosanase activity were performed using the obtained supernatant.

Chitosanase Activity
The amount of reducing sugars created by the soluble chitosan was measured to evaluate the chitosanase activity. Each sample underwent an enzyme experiment to determine each strain's enzyme activity and chitinolytic potential. The enzyme activity was examined by examining the quantity of reducing sugars chitosan produces. The reaction mixture comprises 0.1 mL of diluted enzyme solution, 0.9 mL of 1% chitosan, and 1 mL of 0.1 M sodium acetate buffer (pH 5.8). The reaction tubes were incubated at 50°C for 25 minutes. The reducing sugar produced was quantified in the supernatant using a modified dinitro salicylic acid method.

Dinitro Salicylate (DNS) Method: Detection of N-Acetylglucosamine (NAG)
The detection of enzyme activity was performed using the dinitro salicylic acid (DNS) method, as described in a previous work (Su et al., 2017) with certain modifications. The enzyme activity was determined by quantifying the optical density at a wavelength of 540 nm, which was correlated with the concentration of reducing sugar in the solution using D-glucosamine as a reference standard. An enzyme activity unit (U) in this experiment is defined as the quantity of enzyme needed to generate 1 µmol of glucosamine per minute, or 1 µmol/min. The unit of enzyme activity

per millilitre of fermentation broth (or per milligram of enzyme protein) is denoted as U/mL (or U/mg). A reaction mixture containing 0.5 mL, 10% culture supernatant, 2% chitosan, and 0.2 mm sodium acetate buffer was incubated for 10 minutes at 47°C and pH 5.4 in the DNS methods. The process was stopped by adding 0.5 mL of DNS reagent and heating the mixture for 10 minutes at 100°C. The concentration of reducing sugar in the reaction supernatant was determined by measuring the absorbance at 540 nm with a spectrophotometer. Three identical reactions have been carried out. Chitosanase activity was measured by the enzymatic release of 1 mol of D-glucosamine per minute.

Colony-Forming Units (CFU)

CFU (colony-forming units) is a unit of measurement used to quantify the number of viable bacterial cells in a sample. In the context of measuring chitosanase production in bacteria, CFU is significant because it allows for the quantification of the number of bacterial cells that produce chitosanase (Zhang et al., 2021).

CFU for chitosanase-producing bacteria involved plating of diluted bacterial samples onto solid agar medium and incubating it under appropriate conditions to allow the growth of individual colonies. After incubation, the number of colonies formed were counted, and calculated based on the dilution factor to get the volume of the actual samples. This method was used to quantify the number of viable bacterial cells in a sample and is essential for assessing chitosanase production (Asli et al., 2017).

Statistical Analysis

All experimental conditions in this study were made in duplicates of the replica flasks. Statistical data were analyzed using Microsoft Excel Software 2019 (Microsoft 365) and calculated as the mean of the measurements. The results were presented with ± standard deviation. The difference between the means was tested via T-test and the means of the group were considered significantly different at $p<0.05$.

Results and Discussion

Isolation and Screening of Microbe's Production of Chitosanase

Bacteria from sand, seawater, brackish water, and shells from eight locations around Kuala Terengganu and Besut, Terengganu were successfully isolated and characterized using standard culture methods. Ten colonies with diverse morphological characteristics were selected and transferred to fresh chitosan agar plates (M1) and chitosan liquid medium (M2). From the ten positive strains (Table 1), strain BL from a shell sample at Pantai Benting Lintang exhibited the highest chitosanase activity (0.113 U/mL), thus was chosen for further optimization fermentation (Fig. 1).

The next screening process considered factors such as colour, shape, and sample location, with careful curation of bacterial colonies based on various morphological characteristics. These comprehensive procedures aim to capture a diverse microbial population with potential applications in chitosanase production. Positive colonies were selected based on their ability to grow well and healthy on media containing chitosan as the sole carbon source and their morphology on the media. Besides that, positive colonies were also selected based on their ability to produce chitosanase and biodegrade chitosan by observing the formation of clear zones (Kaur et al., 2012).

Table 1. Screening of 10 strains bacteria

Strain	Morphology	Chitosanase Activity (U/mL)	Source	Isolation Methods
MA	Maroon, small, round	0.088	Pathology Laboratory UniSZA	Direct plating
KB	Red, small, round	**0.093**	Sand from Jeti Kuala Besut	Direct plating
TK	Yellow, small, round	**0.092**	Fresh water from Tasik Kenyir	Enrichment media
PP1	Cream, small, round	0.086	Seawater from Pulau Perhentian	Enrichment media
KI	Pink, small, round	0.084	Sand from Kuala Ibai Lagoon	Direct plating
PP2	Yellow, small, round	**0.091**	Seawater from Pulau Perhentian	Direct plating
BL	Orange yellowish, small, round	**0.113**	Shell from Pantai Benting Lintang	Direct plating
BK1	White, small, round	**0.095**	Sand from Pantai Bukit Keluang	Direct plating
BK2	Light green, small, round	0.086	Seawater from Pantai Bukit Keluang	Enrichment media
TU	Yellow, small, round	0.087	Freshwater from UniSZA Lake, Besut Campus	Direct plating

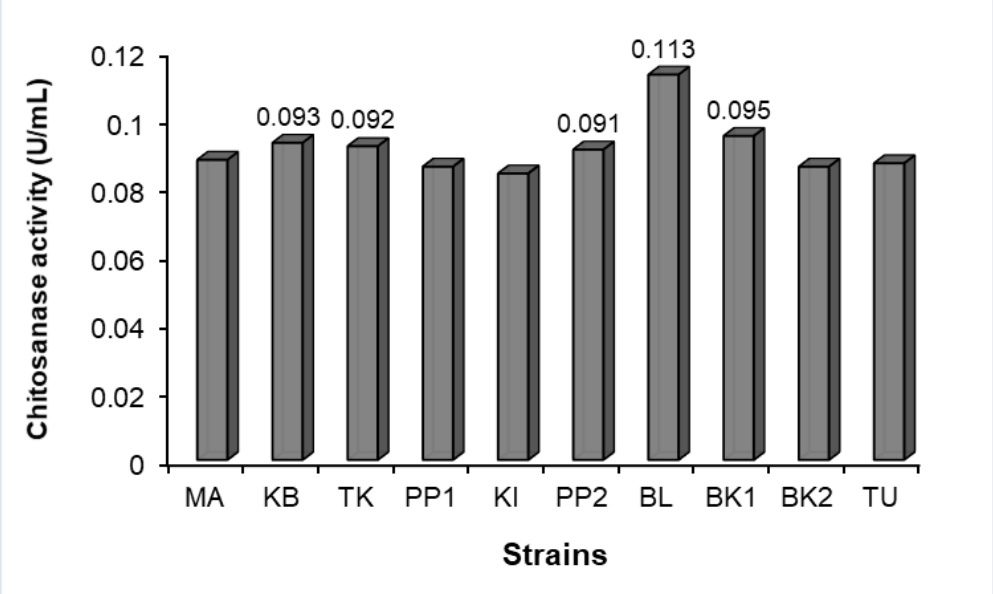

Figure 1. Chitosanase activity for ten strains of bacteria in chitosan biodegradation

Effect of Different pH on Chitosanase Production

The effect of pH on the activity of chitosanase in bacteria is significant, as pH strongly affects chitosanase activity. The optimal pH level for chitosanase activity may vary depending on the bacterial strain and the fermentation conditions. It is crucial to optimize the pH in order to determine the ideal pH level required for isolating bacteria that produce chitosanase. Thus, a range of pH were selected for this parameter optimization, utilising pH 3.0, pH 7.0, and pH 9.0. The initial temperature control in all flasks was adjusted at 30°C (Zhang et al., 2021) and was observed for 24 hours.

Fig. 2(a) showed the growth of the BL strain with three different pH. The measurement value at pH 7.0 demonstrated a notable increase in cell growth, consistent with the findings by Huynh et al. (2019), which focused on the low-cost synthesis of chitosanolytic enzymes from *Lentzea* sp. strain OUR-I1, an actinomycete isolated from environmental resources. Chitosanase activity also exhibited considerable stability at pH 7.0. Fig. 2(b) illustrated a significant difference in chitosanase activity across the three pH levels, with the P value <0.05. Notably, pH 7.0 exhibited a considerable difference in activity compared to the other pH levels evaluated. Huynh et al. (2019) stated that their findings demonstrated complete dissolution of the media at pH 7.0, improving accessibility to microorganisms; consequently, pH 7.0 was chosen as the best condition. The enzyme showed greatest activity at neutral pH (7.0), which is suited for food industry applications where enzymatic stability in gently neutral conditions is desirable, suggesting its usefulness as a bio-preservative or natural food sanitizer.

pH is an essential factor that affecting microbial metabolism and reproduction. Most bacteria favour neutral pH levels around pH 7.0, although some are adapted to flourish in more alkaline or acidic environments. Oh (2011) investigated the extracellular chitosanase from a marine-derived *Bacillus subtilis* strain CH2 which exhibited optimal activity at pH 3.0, indicating a strong acidophilic nature. This characteristic is advantageous for specific applications, such as food preservation or organic acid-based formulations, where the enzyme must remain stable and active in low-pH environments. While Kim et al. (2004) studied a constitutively expressed chitosanase from *Bacillus* sp. MET 1299, demonstrated maximum activity at pH 9.0, classifying it as alkaliphilic. Such enzymes are especially useful in industrial uses requiring alkaline conditions, such as textile processing, waste treatment, and some pharmaceutical formulations.

At neutral pH, the concentration of hydrogen ions (H^+) and hydroxide ions (OH^-) are equilibrated, which is generally the optimal condition for cellular metabolic processes. For numerous bacteria, a pH of 7.0 represents an ideal environment, as the enzymes facilitating metabolic activities in these organisms are frequently most active and stable at neutral pH. Extreme pH levels, whether acidic or alkaline, can alter the structure of enzymes, rendering them inactive or less efficient, thus disrupting protein folding (Zulkifly et al., 2023).

Effect of Different Temperatures on Chitosanase Production

Chitosanase production is also influenced by temperature levels, with potential stimulatory or inhibitory effects, leading to an increase or reduction in chitosanase synthesis and activity. The temperature used as the parameter for this optimization study was 28°C, 37°C and 45°C. The initial pH control was adjusted at pH 7.0 (Huynh et al., 2019) and was observed for 24 hours.

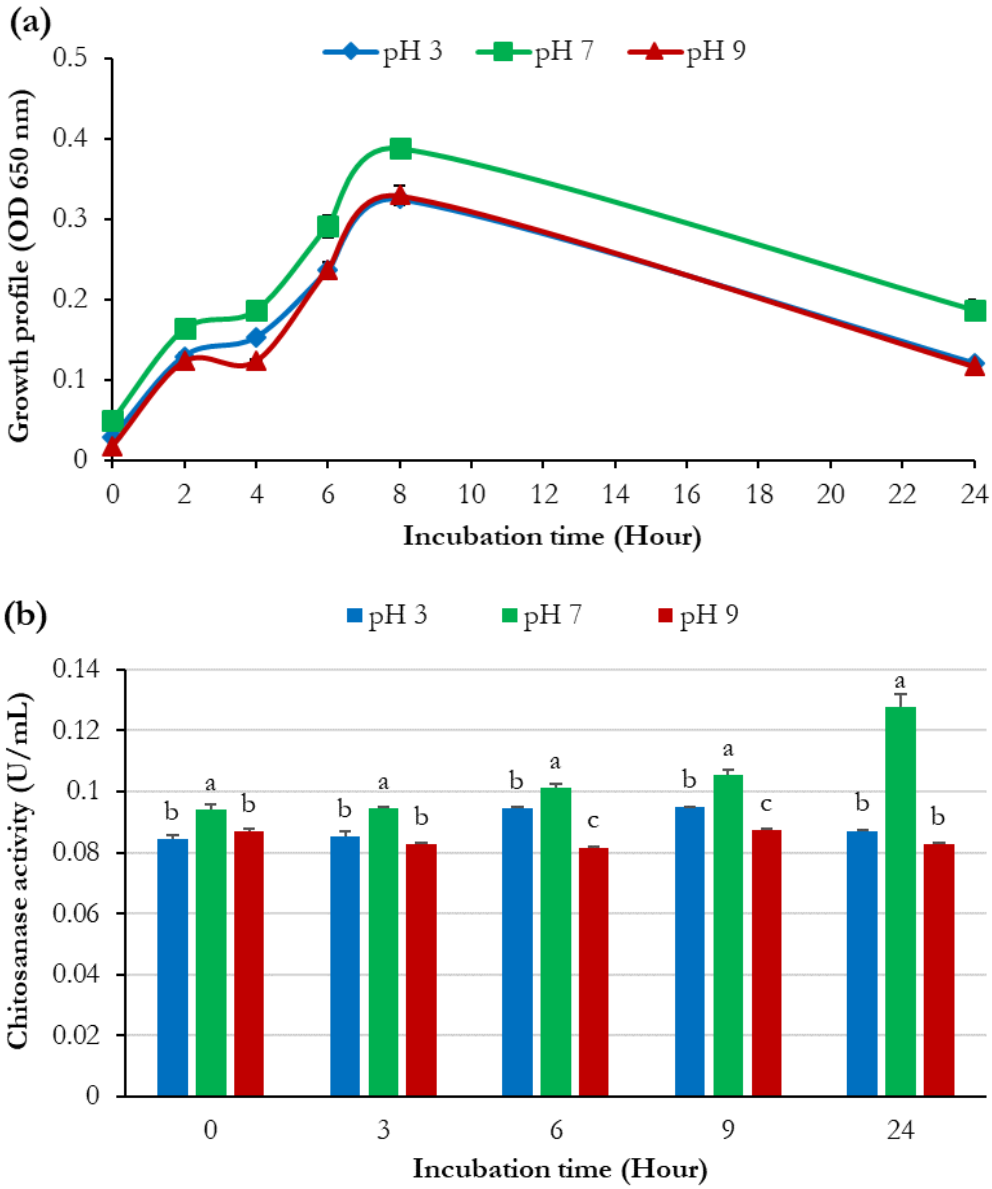

Figure 2. OD and chitosanase activity of BL strain for batch culture with different pH

Fig. 3(a) showed the growth of the BL strain with three different temperatures. Early fermentation measures exhibited uniform cell growth at all three temperatures. The cell growth curves initially showed a steady, predictable pattern under all temperature ranges. After the 6th hour of fermentation, the growth of cells rates began to vary between temperatures. The fluctuations may indicate temperature-sensitive factors affecting cell metabolism and stress responses, which may have influenced cell growth. This growth pattern shift demonstrates that while the cells initially adapted well to all tested temperatures (Su et al., 2017; Doan et al., 2021), the prolonged fermentation period revealed key variances in temperature-dependent responses.

Fig. 3(b) illustrated that there was no statistically significant difference in chitosanase activity throughout the three temperatures (p>0.05). However, after 8 hours of fermentation, there was a notable increase in chitosanase activity at 28°C, compared to the other temperatures. Observation of cell growth and chitosanase activity at all three different temperatures over 24 hours of fermentation, as presented in Figure 3, revealed that 28°C demonstrated considerable

and ideal improvement, yielding the chitosanase activity of 0.19 U/mL. Chasanah et al. (2009) found that 28°C was the ideal temperature as indicated by the chitosanase activity of their bacterial chitosanase from marine environment. This temperature reflects the typical ambient conditions of tropical marine ecosystems, showing that the isolated strains were mesophilic, which often provide unique enzymatic properties due to their adaptive response to saline and variable temperature conditions.

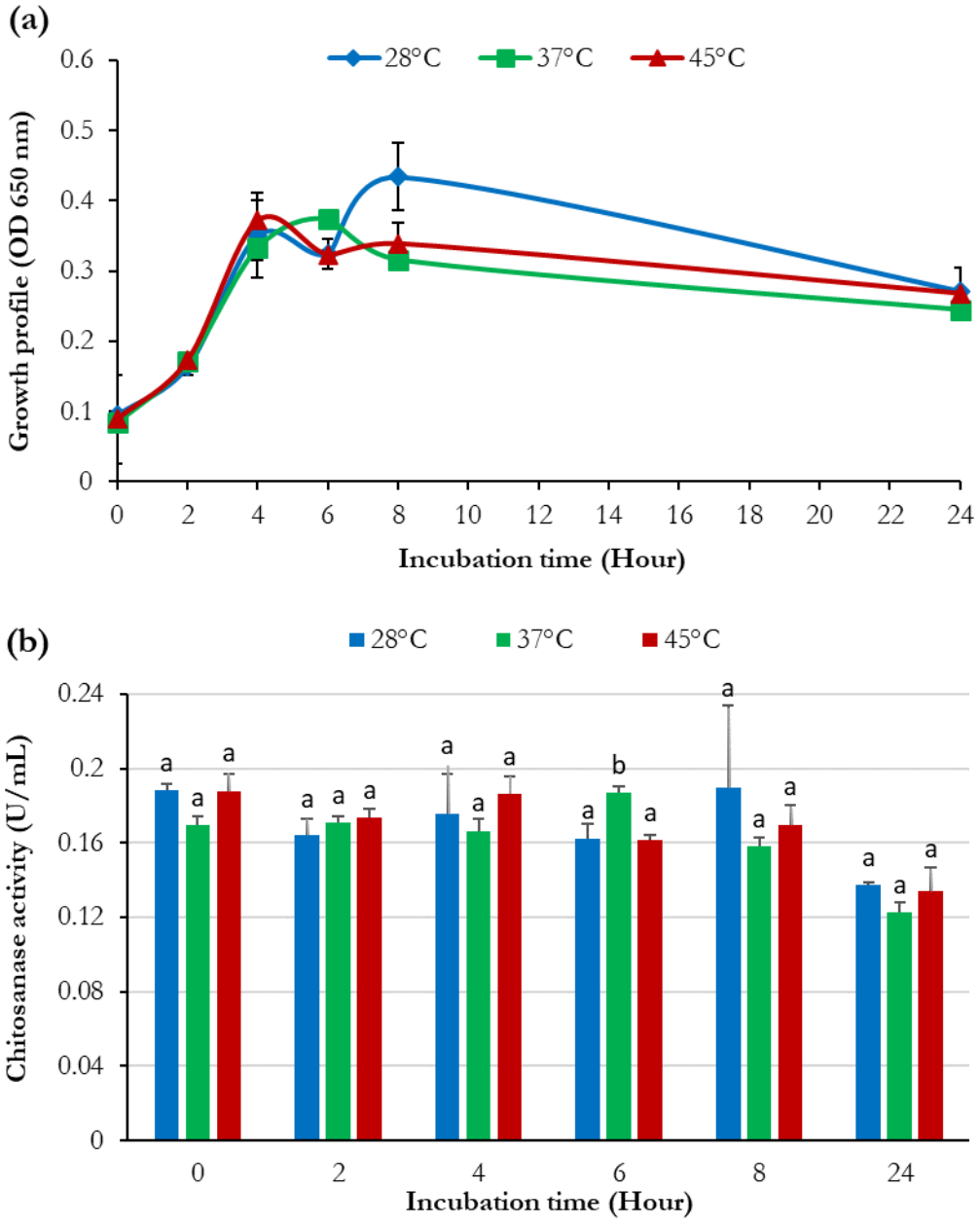

Figure 3. OD and chitosanase activity of BL strain for batch culture with different temperatures

The lower temperature range often results in a higher yield of chitosanase production compared to higher temperatures due to several factors, including enzyme stability, balanced microbial metabolism and optimized gene expression (Yang et al., 2019). Lower temperatures help maintain its structure, promoting proper folding of the chitosanase, ensuring its optimal catalytic activity. At lower temperatures, the metabolic processes of the bacteria may be more balanced, leading to efficient energy utilization and chitosanase synthesis. Chitosanase, at higher temperatures, can be denatured, losing its shape and function. High temperatures can also induce stress responses in bacteria, diverting energy away from chitosanase production and towards survival mechanisms (Larsen et al., 2018). However, according to Chasanah et al. (2009), the enzyme remained relatively stable below 60°C. Su et al. (2017) explored on how *Bacillus subtilis* regulated the secretion of chitosanase at a relatively temperature of 45 °C. This thermotolerant behaviour suggests that the enzyme may be useful for large-scale operations involving high temperatures, including continuous fermentation or bioreactor processes.

Conclusion

Ten colonies were identified to be potential chitosanase-producing bacteria based on their morphology and the clear zones they generated. Strain BL (from shell samples at Pantai Benting Lintang) exhibited considerable chitosanase activity during the screening phase, hence being chosen for the optimization study. Results revealed that optimal conditions for chitosanase production include a pH of 7.0 and a temperature of 28°C, with chitosanase activity of 0.19 U/mL. These findings underscore the important role of controlling pH and temperature conditions to optimize chitosanase production in bacteria. In addition, microbial growth and chitosanase activity are also influenced by the availability of nutrients and other environmental factors.

Thus, different parameter measurements will also be taken into consideration. To further advance the application and understanding of chitosanase-producing microorganisms, future studies should also focus on comprehensive strain identification through molecular techniques such as 16S rRNA sequencing or whole-genome analysis to accurately classify and characterize novel isolates. Additionally, there is significant potential for scaling up enzyme production through larger bioreactor, which would facilitate large-scale industrial use. Future work should also emphasize the purification and biochemical characterization of chitosanase enzymes. These optimization strategies are critical for improving enzyme yield and activity, thus enabling the efficient and cost-effective production of chitooligosaccharides for commercial and biotechnological applications.

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