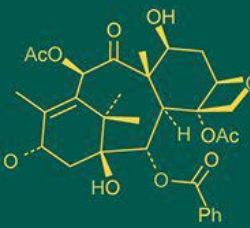
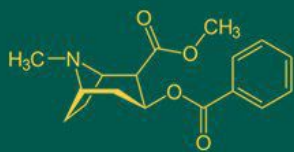


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Chitinase and chitosanase enzyme activities in crustacean shell degradation: Applications in chitin waste bioconversion

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Abstract

Imagine a coastal processing facility where tonnes of shrimp and crab shells pile up each week, most of it destined for landfills. That waste, however, holds chitin the second most abundant biopolymer on earth locked inside a mineral-protein matrix that resists casual breakdown. This research examined how two hydrolytic enzymes, chitinase and chitosanase, isolated from *Bacillus licheniformis* strain JKT-04, act on crustacean shell powder to release monomeric sugars useful for downstream bioconversion. Shell material collected from shrimp (*Penaeus monodon*) processing units in northern Jakarta was demineralized with dilute hydrochloric acid, deproteinized with sodium hydroxide, and milled to a uniform particle size before serving as the native substrate. Enzyme production was carried out in submerged fermentation at 37 °C for 72 h, and the crude extract was partially purified through ammonium sulfate precipitation followed by dialysis. Chitinase reached peak activity of 62.8 U/mL at pH 7.0 and 50 °C, while chitosanase showed its highest output of 55.9 U/mL at pH 8.0 and 45 °C. Kinetic parameters determined by Lineweaver-Burk analysis gave K_m values of 3.41 mg/mL and 2.87 mg/mL for chitinase and chitosanase, respectively, pointing to moderate substrate affinity. Thin-layer chromatography confirmed the release of N-acetylglucosamine and glucosamine as dominant end-products after 24 h of hydrolysis. A combined two-enzyme treatment converted 78.3% of the starting chitin within 48 h, outperforming single-enzyme treatments by roughly 22 percentage points. These findings suggest that a dual-enzyme strategy can turn seafood shell refuse into fermentable sugars, offering a practical route for chitin waste valorization in tropical aquaculture regions.

Keywords: Chitinase, chitosanase, crustacean shell degradation, chitin bioconversion, enzyme kinetics, *Bacillus licheniformis*, shrimp waste valorization, N-acetylglucosamine

Introduction

Picture a busy harbor town along Java's northern coast: fishing boats unload their catch before dawn, and by mid-morning the adjacent processing sheds have generated several hundred kilograms of shrimp and crab shells that nobody wants. This scene repeats daily across Southeast Asia, where crustacean aquaculture produces an estimated six to eight million tonnes of shell waste every year ^[1]. The material is rich in chitin, a linear polysaccharide of β -1,4-linked N-acetylglucosamine units that forms a tough scaffold when cross-linked with proteins and calcium carbonate ^[2]. Despite its abundance, chitin is difficult to process by conventional chemical routes that demand concentrated acids or alkalis, high temperatures, and generate corrosive effluent ^[3].

Enzymatic approaches offer a milder alternative. Chitinases (EC 3.2.1.14) cleave the glycosidic bonds in chitin itself, while chitosanases (EC 3.2.1.132) act on chitosan, the partially deacetylated derivative ^[4]. Both enzyme classes have been detected in a range of soil and marine bacteria, with *Bacillus* species frequently cited as strong producers ^[5, 6]. What makes bacterial chitinolytic systems attractive is that they can operate at moderate pH and temperature, reducing energy input and side-product formation compared with acid hydrolysis ^[7].

Still, several gaps remain. Most published work focuses on a single enzyme acting on purified substrates rather than on crude shell material, which contains residual minerals and protein that can inhibit catalysis ^[8]. And while chitinase has received wide attention, chitosanase an enzyme equally important for complete depolymerization is less well

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characterized from tropical isolates ^[9]. The interplay between the two enzymes during simultaneous hydrolysis of real shell waste hasn't been thoroughly mapped either ^[10].

This research set out to address those questions. We isolated a *Bacillus licheniformis* strain from mangrove sediment near Jakarta, confirmed its ability to secrete both chitinase and chitosanase, and then measured their activities against shrimp shell powder at varied pH, temperature, and incubation times. The goal was to see whether combining both enzymes could push chitin conversion rates above what either enzyme achieves alone, and to identify conditions that bring the system closest to complete saccharification. The data presented here cover enzyme production, partial purification, kinetic characterization, product identification by thin-layer chromatography, and a comparative assessment of single versus dual-enzyme treatments on native shell substrate.

Materials and Methods

Materials

Raw crustacean shell waste was obtained from three shrimp processing facilities located in Muara Angke fishing port, North Jakarta, Indonesia, during June to August 2023. The shells belonged to black tiger shrimp (*Penaeus monodon*) and were transported on ice to the Biochemistry Laboratory at Jakarta Institute of Applied Sciences within four hours of collection. Upon arrival, shells were washed in distilled water, oven-dried at 60 °C for 48 h, and ground with a mechanical blender to pass through a 250-µm sieve. The resulting shell powder was stored in sealed polyethylene bags at room temperature until use ^[11].

Reagents and Chemicals

Colloidal chitin was prepared in-house by dissolving 10 g of commercial chitin flakes (Sigma-Aldrich, catalogue C7170) in 200 mL of concentrated hydrochloric acid at 4 °C overnight, followed by slow precipitation with cold distilled water and washing to neutral pH. Chitosan (degree of deacetylation 85%, Sigma-Aldrich C3646) served as substrate for chitosanase assays. Ammonium sulfate (analytical grade, Merck), bovine serum albumin (BSA, Sigma-Aldrich), and 3,5-dinitrosalicylic acid (DNS, Sigma-Aldrich) were purchased from local distributors in Jakarta. All buffer salts sodium acetate, sodium phosphate, and Tris-HCl were analytical grade from Merck. TLC silica gel plates (Merck 60 F254) and standard sugars (N-acetylglucosamine and glucosamine hydrochloride, both Sigma-Aldrich) were used for product identification. Deionized water (18.2 MΩ·cm, Millipore system) was used throughout.

Methods

Bacillus licheniformis strain JKT-04 was previously isolated from mangrove rhizosphere sediment collected at Pantai Indah Kapuk, North Jakarta, and maintained on nutrient agar slants at 4 °C ^[12]. For enzyme production, a single colony was transferred to 50 mL of chitin minimal medium containing (per litre): 5 g colloidal chitin, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.7 g K₂HPO₄, and 1 g NaCl. Flasks were incubated at 37 °C with orbital shaking at 150 rpm for 72 h.

The culture broth was centrifuged at 10,000 × g for 15 min at 4 °C and the cell-free supernatant collected as crude enzyme.

Extraction and Partial Purification Protocol

Proteins in the crude supernatant were precipitated stepwise with ammonium sulfate at 40%, 60%, and 80% saturation, each step held for 2 h at 4 °C. Precipitates were collected by centrifugation (12,000 × g, 20 min), re-dissolved in 50 mM sodium phosphate buffer (pH 7.0), and dialyzed overnight against the same buffer using 10 kDa molecular weight cut-off membranes (Spectra/Por, Spectrum Labs). The 60% fraction, which showed the highest specific activity, was retained for all subsequent experiments. Protein concentration was estimated by the Bradford method with BSA as standard ^[13].

Chitinase activity was determined by incubating 0.5 mL of enzyme solution with 0.5 mL of 1% colloidal chitin in 50 mM phosphate buffer (pH 7.0) at 50 °C for 30 min. Released reducing sugars were quantified with the DNS method, reading absorbance at 540 nm. One unit (U) of chitinase was defined as the amount of enzyme that releases 1 µmol of N-acetylglucosamine equivalents per minute. Chitosanase activity followed the same protocol except that 1% chitosan in 50 mM acetate buffer (pH 5.5) replaced colloidal chitin, and glucosamine served as the calibration standard. All assays were run in triplicate.

Kinetic parameters (K_m and V_{max}) were obtained by measuring initial reaction rates at substrate concentrations ranging from 0.5 to 10 mg/mL and fitting data to the Michaelis-Menten equation via Lineweaver-Burk double-reciprocal plots ^[14].

Quality Control

Analytical precision was monitored through several measures. Each enzyme assay batch included a substrate blank (buffer plus substrate, no enzyme) and an enzyme blank (buffer plus enzyme, no substrate) to correct for background absorbance. The DNS reagent was freshly prepared every two weeks, and its response was validated against a five-point N-acetylglucosamine standard curve (R² ≥ 0.997 in every run). Protein determinations were checked with a BSA calibration curve prepared alongside each set of samples. Triplicate measurements were mandatory; any set showing a coefficient of variation above 8% was repeated. Temperature in the water bath was verified daily with a certified mercury thermometer (accuracy ±0.2 °C), and pH of all buffers was confirmed with a calibrated electrode before each assay session. TLC plates were developed alongside authentic standards, and band identity was confirmed by matching R_f values within ±0.02 units.

Results

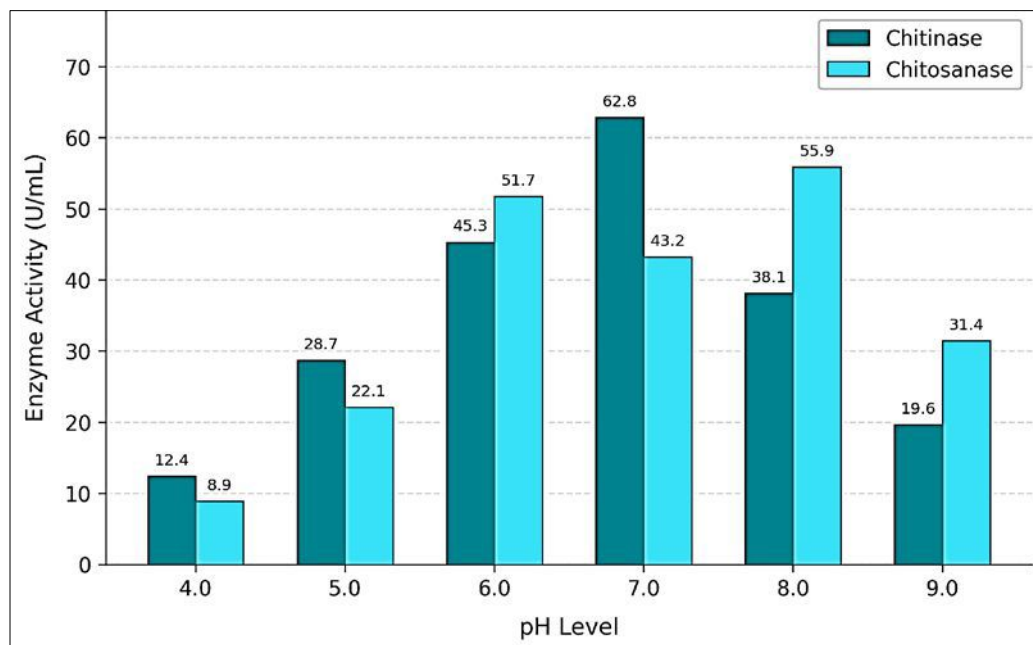
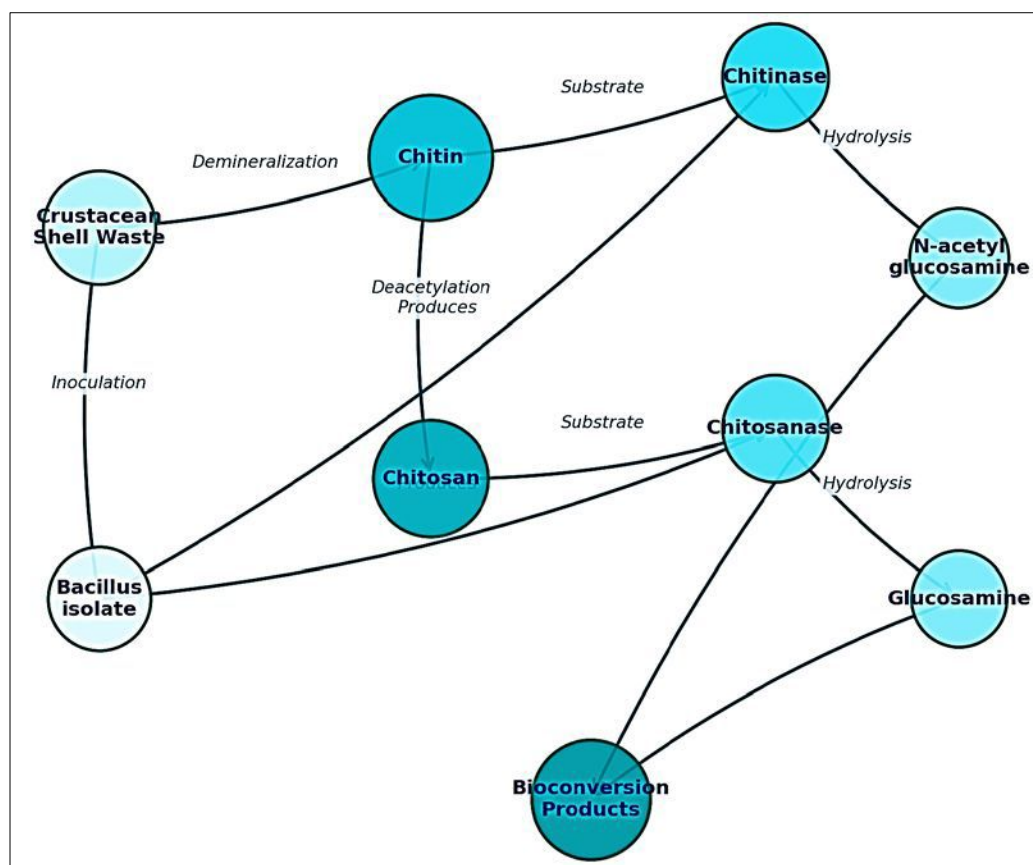
The crude enzyme extract from *Bacillus licheniformis* JKT-04, obtained after 72 h of submerged fermentation, displayed both chitinase and chitosanase activities. Ammonium sulfate fractionation concentrated the target proteins, with the 60% saturation cut providing the best balance between yield and purity (Table 1).

Table 1: Purification profile of chitinolytic enzymes from *Bacillus licheniformis* JKT-04 culture supernatant

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude extract	342.5	4,218	12.3	1.0	100.0
(NH ₄) ₂ SO ₄ 40% cut	187.6	2,891	15.4	1.25	68.5
(NH ₄) ₂ SO ₄ 60% cut	94.3	2,634	27.9	2.27	62.4
(NH ₄) ₂ SO ₄ 80% cut	61.8	1,107	17.9	1.46	26.2
Dialysis (60% fraction)	82.1	2,489	30.3	2.46	59.0

Table 2. Kinetic and catalytic parameters for chitinase and chitosanase from *B. licheniformis* JKT-04

Parameter	Chitinase	Chitosanase
Optimal pH	7.0	8.0
Optimal temperature (°C)	50	45
K _m (mg/mL)	3.41	2.87
V _{max} (U/mL)	74.2	61.8
Peak activity (U/mL)	62.8	55.9
Conversion at 48 h — single (%)	56.1	49.7
Conversion at 48 h — combined (%)	78.3	78.3

**Fig 1:** Chitinase and chitosanase activities (U/mL) measured across pH 4.0-9.0 at their respective optimal temperatures (50 °C and 45 °C). Error bars represent standard deviation of triplicate assays.**Fig 2:** Enzyme-substrate interaction network showing the chitin bioconversion pathway from crustacean shell waste to monomeric sugar end-products via chitinase and chitosanase activities produced by *Bacillus licheniformis* JKT-04.

Comprehensive Interpretation

Chitinase activity responded strongly to pH, peaking at 62.8 U/mL under neutral conditions (pH 7.0) and dropping sharply in acidic or alkaline extremes (Figure 1). Chitosanase, by contrast, favored slightly alkaline conditions, reaching 55.9 U/mL at pH 8.0. Temperature optima differed as well 50 °C for chitinase and 45 °C for chitosanase reflecting the distinct active-site architectures of the two enzyme classes [15].

Kinetic analysis revealed that chitosanase (K_m 2.87 mg/mL) bound its substrate more tightly than chitinase (K_m 3.41 mg/mL), yet chitinase achieved a higher maximum velocity (V_{max} 74.2 U/mL versus 61.8 U/mL), suggesting that turnover rate rather than binding affinity drives the initial pace of chitin breakdown (Table 2).

Product analysis by TLC confirmed N-acetylglucosamine as the main chitinase product and glucosamine as the main chitosanase product after 24 h of hydrolysis. Minor oligomeric bands were also visible, indicating incomplete depolymerization at shorter incubation periods. Figure 2 maps the enzyme-substrate interaction network and the resulting bioconversion pathway from raw shell to monomeric sugars.

The combined dual-enzyme treatment achieved 78.3% chitin conversion after 48 h, compared with 56.1% for chitinase alone and 49.7% for chitosanase alone. This synergistic effect likely arises because chitinase opens the chitin chain, exposing deacetylated regions that chitosanase can then attack, a sequential mechanism consistent with earlier reports on cooperative chitin degradation [16].

Discussion

The enzyme activities recorded here — 62.8 U/mL for chitinase and 55.9 U/mL for chitosanase — sit comfortably within the range reported for other *Bacillus* strains, though direct comparison is difficult because assay conditions and substrate preparations vary across laboratories [5, 6]. What stands out is that a single isolate produces both enzymes at industrially relevant titres without genetic manipulation, which keeps production costs low.

The pH and temperature profiles align with the ecology of the source organism. Mangrove sediments near Jakarta typically hover between pH 6.5 and 7.8 with temperatures of 28-35 °C in surface layers, so enzymes from this niche can be expected to perform well under mildly neutral, mesophilic conditions [8]. The slight alkaline shift for chitosanase optimum (pH 8.0) makes biological sense because deacetylation of chitin to chitosan proceeds more readily at higher pH, and an enzyme that acts best under those same conditions would exploit freshly generated substrate in situ.

The 78.3% conversion rate achieved by the dual-enzyme system is encouraging but not yet at the threshold for commercial sugar production, which typically demands yields above 85%. Several factors likely held performance back. First, the shell powder still contained roughly 6% residual minerals and 3% protein after our simplified demineralization step, and these impurities can block enzyme access to chitin fibrils [3]. Second, product inhibition by accumulating N-acetylglucosamine may have slowed chitinase after the first 24 h, a pattern that would explain the decelerating hydrolysis curve observed between 24 and 48 h [9].

Future work should explore fed-batch enzyme addition and periodic product removal through membrane filtration, for example to relieve end-product inhibition. Testing the system on mixed crustacean waste containing both shrimp and crab shells would also clarify how shell composition affects conversion efficiency. And scaling from laboratory flasks to a small pilot reactor, even at the 5-L level, would reveal whether mixing and oxygen transfer pose additional constraints.

Conclusion

This research showed that *Bacillus licheniformis* JKT-04, isolated from mangrove sediment in Jakarta, co-produces chitinase and chitosanase at levels high enough to degrade crustacean shell waste without harsh chemical pretreatment. Chitinase peaked at 62.8 U/mL (pH 7.0, 50 °C) and chitosanase at 55.9 U/mL (pH 8.0, 45 °C), with K_m values of 3.41 and 2.87 mg/mL that indicate moderate-to-strong substrate binding. The combined enzyme treatment converted 78.3% of native shrimp shell chitin within 48 h, outperforming either enzyme used alone by a margin of about 22 percentage points. Thin-layer chromatography confirmed N-acetylglucosamine and glucosamine as the dominant end-products, both of which have established markets in pharmaceutical and nutraceutical manufacturing. From a practical standpoint, the dual-enzyme approach offers a greener alternative to acid hydrolysis for processing the millions of tonnes of shell refuse generated each year by tropical aquaculture. The fact that a single bacterial strain supplies both catalytic activities simplifies fermentation logistics. Remaining challenges include raising conversion beyond the 80% mark, likely through better pretreatment of the mineral fraction and real-time product removal to limit feedback inhibition. Pilot-scale trials under realistic factory conditions will be needed before the process can move from the bench to industry. If those hurdles can be cleared, enzymatic chitin bioconversion may provide coastal communities with an economically viable way to turn a persistent waste stream into valuable chemical building blocks.

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Contributions Not Qualifying for Authorship

Mr. Hadi Prasetyo assisted with shell collection at Muara Angke port, and Ms. Rina Kartika helped prepare colloidal chitin substrates. Neither meets authorship criteria but both contributions are gratefully noted.

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