Research Article

Biochemical Characterization of Mannanase from Newly Isolated *Acinetobacter* sp. KUB-ST1-1 and its Hydrolysate Containing Mannooligosaccharides: Potential as Applied Prebiotic for Pet Food

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Abstract
The β-mannanase enzyme derived from isolated *Acinetobacter* sp. KUB-ST1-1 was studied for its potential for mannooligosaccharide production. Extracellular mannanase from *Acinetobacter* sp. KUB-ST1-1, cultured in nutrient broth with 0.5% (w/v) locust bean gum, was purified using ultrafiltration, anion exchange chromatography, and cation exchange chromatography. The enzyme, with a molecular weight of 57 kDa, had high activity in a 50 mM phosphate buffer at pH 6.0 and was stable from pH 4.0 to 7.0. It had high-temperature stability at 40 °C and 50 °C for up to 18 h. This enzyme was highly active toward konjac and galactomannan, especially with locust bean gum and copra meal. The hydrolysis products consisted mainly of the 2 and 3 units of mannooligosaccharide. The mannooligosaccharide exhibited prebiotic properties by promoting increased growth of some beneficial lactic acid bacteria. Furthermore, the defatted copra meal hydrolysate had considerable resistance to pepsin, trypsin, and bile salts under simulated gastrointestinal conditions for dogs. These characteristics highlighted the enzyme's excellent properties and suggested its potential as a promising candidate for applications in the food and bio-industries, particularly in pet food production.

Keywords: *Acinetobacter* sp. KUB ST1-1, β-Mannanase, Mannooligosaccharides, Pet food, Prebiotics

1 Introduction

Copra meal (CM), also known as coconut residual cake, is an abundant and inexpensive byproduct of coconut oil extraction. According to the U.S. Department of Agriculture, the CM production volume in Thailand is projected to be 23,000 tonnes in 2023–2024 [1]. CM contains 15–25% crude protein, 7% lipid, and 7–15% crude fiber, making it a viable option for livestock feed production [2]. However, its

high content of non-starch polysaccharides prevents it from being used as a complete feed ingredient for animals [3]. Research has been conducted to improve the dietary and feeding value of CM through various processing methods and enzyme additions, and to identify the ideal inclusion level that ensures optimal animal performance [4]–[9]. Additionally, it contains 50% total sugars, including mannose (63%) and galactose (7%), due to its high mannan content and a small amount of galactomannan, with a mannan-to-galactose ratio of 14:1 [10]. It can be used as a cost-effective alternative for producing mannooligosaccharides (MOSs) to support sustainable waste management and to add value to inexpensive CM as a biological resource, in line with the Bio-Circular-Green Economy (BCG) model promoted by the Thai government [11]. Extensive studies have been conducted to produce MOS through the microbial enzyme hydrolysis of CM [12]–[17].

MOS is a non-nutritional food additive that provides dietary fiber and prebiotics with the potential for application as a pet food additive. For example, MOS has been reported to have several beneficial uses: to promote the growth of beneficial microbial populations in the host, such as *Bifidobacterium infantis*, *B. longum*, and *Lactobacillus acidophilus* [18], [19]; to modulate the systemic immune characteristics of the host [20]; and anti-cancer, anti-inflammatory and anti-diabetic properties [21]. In addition, it suppresses the growth of pathogens in the colon, such as *Clostridium perfringens, C. difficile*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella typhimurium*, through various mechanisms, including the production of organic acids and antimicrobial compounds and the inhibition of pathogen adhesion by probiotics [22]–[24].

β-Mannanases act on the random β-1,4-mannosidic linkages between the mannose molecules found in mannans and heteromannans as endo-type enzymes, resulting in the production of MOS molecules of varying lengths [25]. β-Mannanases have been widely utilized across various industries. In the food industry, these enzymes are used to clarify fruit juice and wine, as well as to reduce the viscosity of instant coffee during extraction [26], [27]. In animal feed, they promote higher weight gain, more efficient feed conversion, better growth performance, and improved immunity [28]. In the paper industry, they are used for the enzymatic bleaching of softwood pulp [29], [30].

These enzymes are utilized for producing MOS from inexpensive agricultural byproducts such as CM, locust bean gum (LBG), and palm kernel meal. Research has shown that mannanases can be purified from a variety of sources, including bacteria, yeasts, fungi, algae, and the germinating seeds of terrestrial plants. Furthermore, several of these mannanase genes have been successfully cloned and expressed in different host systems [31]–[34]. Researchers have shown interest in microbial mannanases due to their efficient degradation capability, high production capacity, and easily controllable conditions [35], aiming for cost-effectiveness in investment. Most microbial mannanases reported to date exhibit activity across a broad pH range, with optimal conditions spanning from acidic to neutral pH levels, as well as being effective at mesophilic to thermophilic temperatures. The broad pH tolerance range and thermal stability of the enzyme make it suitable for diverse biotechnological applications, particularly in industrial fermentation processes [36]. In addition, several studies have investigated low-temperature-active mannanases, such as *Cryptopygus antarcticus*, *Enterobacter* sp., *Klebsiella oxytoca* KUB-CW2-3, and *Sphingomonas* sp. [37]–[40]. At low temperatures, the presence of active enzymes is crucial, with considerations for energy conservation being necessary. These properties can be extremely useful in various biotechnological applications, especially considering the increased costs and product denaturation caused by heating [41].

The current research investigated the characterization of purified β-mannanase obtained from *Acinetobacter* sp. KUB-ST1-1. The purified mannanase was used for hydrolytic efficiency in MOS production and the prebiotic potential of MOS was investigated to improve the growth rates of some lactic acid bacteria (LAB). Additionally, it inhibited the growth of the pathogens *Enterococcus faecium* and *E. hirae*. The overall procedures followed in this study are shown in Figure 1. The purified mannanase should be highly applicable in various biotechnological fields, particularly in the animal feed market due to its excellent characteristics, such as remarkable mesostability, a diverse substrate range, and significant pH stability.

2 Materials and Methods

2.1 *Reactants*

Alpha-mannan, avicel, birchwood xylan, carboxymethylcellulose (CMC), galactose (Gal), glucose (Glu), guar gum (GG), LBG, mannose (M1), and xylan were obtained from Sigma-Aldrich (USA). Ivory nut mannan, konjac (KGM), and the mannooligosaccharides (M2−M6) were obtained from Megazyme (Ireland).

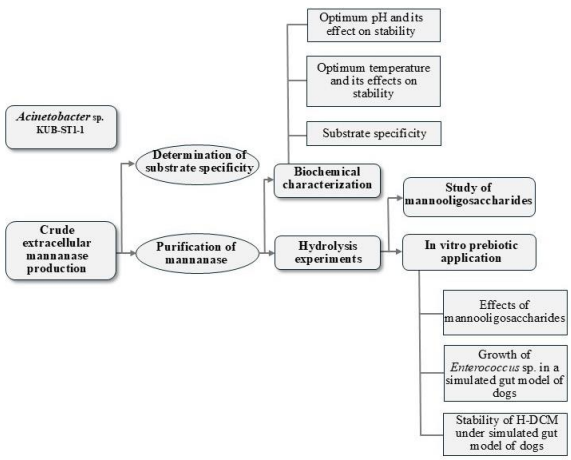

Figure 1: Overview diagram of research.

2.2 *Defatted copra meal*

Copra meal, as an agricultural waste material, was ground into smaller particles using a blender and then sieved through 40-mesh and 60-mesh screens. Then, the sieved material was dried at 55–60 °C to a constant weight. Next, the fat was extracted using the Soxhlet method [42], resulting in defatted copra meal (DCM).

2.3 *Bacterial strains and culture conditions*

Acinetobacter sp. KUB-ST1-1, a strain that produces mannanase, was isolated from fermented coconut waste in Thailand [17] and then grown in a nutrient (NB) medium at 37 °C and shaking at 150 rpm for 24 h.

LAB was cultivated using De Man, Rogosa, and Sharpe (MRS) medium, while the pathogenic bacteria were grown in NB medium at the same temperature for 18 hours to assess their prebiotic properties.

In the early stage of this study, 253 bacteria were isolated from the fresh feces of dogs. Most were Gram-positive coccus and catalase-negative belonging to *Enterococcus*, in particular *Enterococcus faecium* and *E. hirae*. Specifically, *E. faecium* KUB-17, *E. faecium* KUB-22, and *E. hirae* KUB-23, which had been identified as pathogens, were used to assess prebiotic properties and were grown in MRS broth for 18 h at the same temperature as the LAB.

2.4 *Crude extracellular mannanase production*

To induce extracellular mannanase production, a 0.5% (w/v) LBG solution was incorporated into the NB medium, which also contained a 5.0% (v/v) inoculum of *Acinetobacter* sp. KUB-ST1-1. This crude mannanase solution was collected and then used in subsequent procedures. The initial mannanase activity levels for the crude and purified mannanase substrate specificity were 15.08 U/mL and 1.42 U/mL, respectively.

2.5 *Substrate specificity*

A 50 mM phosphate buffer (pH 6.0) was used to prepare each substrate solution, which contained 0.5% (w/v) avicel, CMC, CM, GG, ivory nut mannan, KGM, LBG, alpha-mannan, and xylan. This solution was subsequently used to evaluate substrate specificity at 50 °C for 30 min.

2.6 *Purification*

The crude mannanase solution was acquired using centrifugation at 4 °C, followed by a three-step purification process for the mannanase: 1) ultrafiltration of the mannanase solution was conducted using a 1 kDa NMWL membrane from Merck (Germany); 2) the concentrated proteins were loaded onto an equilibrated DEAE-cellulose 52 column (Whatman; USA) for anion exchange chromatography, followed by elution with 0.2 M NaCl, with the protein fractions measured for activity and then combined, dialyzed, and concentrated using a Vivaspin 20 membrane with a 10 kDa cutoff (GE Healthcare, China); and 3) the partially purified enzyme solution was applied to an equilibrated CM-cellulose 52 (Whatman; USA) for cation exchange chromatography, followed by elution with 0–0.5 M NaCl, after which the activity proteins were combined and subsequently analyzed for protein purity, molecular weight, purified protein activity (zymogram), protein concentration, and mannanase activity [39], [43].

2.7 *Biochemical characterization*

2.7.1 Optimal pH and its effects on stability

The impact of different pH levels (ranging from 3.0 to 10.0) was investigated to determine the optimal pH. Mannanase activity was assessed using a 0.5% (w/v) LBG solution in 50 mM citrate buffer (for pH 3.0–6.0), 50 mM phosphate buffer (for pH 6.0–8.0), and 50 mM glycine-NaOH buffer (for pH 8.0–10.0) at 50 °C for 30 min.

The buffers were incubated at 4 °C for 24 h to test pH stability across the same range. Following incubation, the remaining enzyme activity was measured under identical assay conditions (50 °C for 30 min). The initial mannanase activity at optimal pH was 1.28 U/mL, while the pH stability was 1.45 U/mL.

2.7.2 Optimal temperature and its stability effects

The impact of temperature on mannanase activity was assessed across 30–70 °C in 50 mM phosphate buffer (pH 6.0) for 30 min. Then, the mannanase activity was measured.

Thermostability was assessed based on incubating the enzyme solution in 50 mM phosphate buffer (pH 6.0) at 40 °C, 50 °C, or 60 °C for various durations. Subsequently, the remaining mannanase activity was analyzed. The initial mannanase activity at optimal temperature was 0.92 U/mL, while the temperature stability was 0.88 U/mL.

2.8 *Hydrolysis experiments*

The hydrolytic reaction was conducted using purified mannanase (10 U/reaction) and a 1.0% (w/v) KGM, LBG, and DCM solution at 50 °C and 150 rpm for 6 h. Then, the supernatant was obtained through centrifugation at 12,000 × g and 4 °C for 15 min. Subsequently, the hydrolysates were analyzed.

2.9 *Study of mannooligosaccharides*

The hydrolysates were analyzed using thin-layer chromatography (TLC) after the hydrolytic reaction [39]. The mobile phase was composed of a 2:1:1 mixture of n-butanol, 98% glacial acetic acid, and deionized water. A 10 µL sample was applied to a TLC plate coated with silica gel (Merck; Germany). The plate was developed in the mobile phase, allowed to dry, and then developed again in the same mobile phase. Following the application of 10% sulfuric acid in ethanol to the silica gel plate, the chromatogram was developed at 105 °C for 20 min.

2.10 *In vitro prebiotic application*

2.10.1 Effects of mannooligosaccharides

The potential prebiotic properties of mannooligosaccharides on the growth rate of LAB and pathogens were assessed using a modified method based on the work of Pongsapipatana *et al.* [39]. To generate hydrolysates, a reaction was conducted at 50 °C for 6 h using 1.0% (w/v) substrates (KGM, LBG, and DCM) and 10 U of purified mannanase. Before being added to the medium, the hydrolysates, which had been prepared previously, were passed through a 0.44 µm filter membrane. 0.05% (w/v). Glucose and commercial MOS (Actigen™, USA) served as positive controls, while the basal medium (BM) was used as a negative control. The BM medium was prepared using the following MRS formulation without glucose to determine the growth rate of LAB. The pH was adjusted to 6.8. The LAB cells from overnight cultures were harvested using centrifugation at 12,000 × *g* and 4 °C for 5 min. Then, the cell pellets were resuspended in 0.85% NaCl and adjusted to an optical density at 600 nm of 0.5. A 1.0% (v/v) inoculum of the LAB culture was added to 0.2 mL of BM medium containing 0.05% (v/v) of each hydrolysate. Simultaneously, a 1.0% (v/v) inoculum of the pathogen was transferred into 0.2 mL of NB medium to determine the specific growth rate (µ). Cell density was assessed using a microplate reader (Bio-Rad, USA). The specific growth rate (µ) was calculated as follows

$$\mu\ (\text{hour}^{-1}) = (\ln A_2 - \ln A_1) / (t_2 - t_1)$$

where A_1 and A_2 represent the optical density at times t_1 and t_2, respectively, measured during the logarithmic phase at 600 nm.

The following criteria were used for data analysis: 1) bacterial growth rates under hydrolysate conditions that were statistically similar to or lower than those in the BM medium indicated that the hydrolysate did not promote bacterial growth; and 2) conversely, a growth-promoting effect was indicated if the bacterial growth rates under hydrolysate conditions were statistically higher than those

observed in the BM medium alone, regardless of their comparison to growth rates under the glucose treatment.

2.10.2 Growth of E. faecium and E. hirae in simulated gut model of dogs

The growth rates of *E. faecium* KUB-17, *E. faecium* KUB-22, and *E. hirae* KUB-23 in a simulated canine gut model were evaluated using a method that was slightly modified from those of Pongsapipatana *et al*. [39] and Das *et al*. [44]. The BM medium at pH 8.0 was supplemented with 0.03% (w/v) bile salts and 0.01% (w/v) pancreatin. Hydrolysates at a concentration of 0.05% (v/v) and 1.0% (v/v) bacterial cells were added to the BM medium without added sugar and incubated at 37 °C, and the specific growth rates of each strain were determined.

2.10.3 Stability of H-DCM under a simulated gut model of dog

The gastrointestinal tract stability of the hydrolysates was evaluated using a method that was slightly adapted from Das *et al*. [44]. The artificial gastric juice at pH 3.0 contained 1,000 U/mL of pepsin. The intestinal fluid at pH 8.0 contained 0.5% (w/v) bile salts and 1,000 U/mL of trypsin. The reaction mixture ratio of H-DCM to artificial gastric juice solution was 1:3. The solutions were collected and the contents of reducing sugars [45] and total sugars [46] were determined to calculate the percentage of stability. The stability percentage was determined by measuring the total amount of sugar that remains as follows

$$\text{Stability (\%)} = 100 - [(A - B) * 100] / (C - B)$$

where A represents the reducing sugar at each time point, B is the initial reducing sugar, and C is the total sugar content.

2.11 *Mannanase activity*

The assay, with a total volume of 0.2 mL, consisted of 0.1 mL of 0.5% (w/v) LBG solution in 50 mM phosphate buffer at pH 6.0 and 0.1 mL of mannanase enzyme solution. The reaction was conducted at 50 °C for 30 min. Following incubation, 0.2 mL of 3,5-dinitrosalicylic acid solution was added to the mixture, which was then boiled in hot water for 5 min and allowed to cool for an additional 5 min. The enzyme activity was assessed by measuring the absorbance at 540 nm [17].

One unit of mannanase activity was defined as the quantity of mannanase that generated 1 μmol of reducing mannose per minute.

2.12 *Electrophoresis*

The protein purity and molecular weight (20 μg of protein) were determined using a 12% (w/v) acrylamide gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver stain (Thermo Scientific; USA) [39]. A pre-stained protein ladder (GeneDireX; Taiwan) was utilized as the protein standard. The specific activities of mannanase in the steps involving unpurified protein, ultrafiltration, DEAE-cellulose 52, and CM-cellulose 52 were 0.86, 0.96, 3.82, and 12.55 U/mg, respectively.

The identification of protein bands from Native-PAGE and zymogram analysis followed a modified method based on Pongsapipatana *et al*. [39], using a 12% (w/v) acrylamide gel (native gel) and a MiniPROTEIN® Tetra cell (Bio-Rad; USA). The samples were diluted in Native-PAGE tracking dye solution at a ratio of 1:4 without undergoing heating.

2.13 *Statistical evaluation*

A *p*-value of less than 0.05, as determined using the SPSS version 21.0 software (SPSS Inc., USA), indicated a statistically significant difference across three independent trials.

3 Results and Discussion

3.1 *Evaluating substrate specificity of unpurified extracellular mannanase*

The crude mannanase produced by strain KUB-ST1-1 was assessed against various substrates with different compositions. Based on Figure 2, the crude mannanase from *Acinetobacter* sp. KUB-ST1-1 had 100% relative activity on glucomannan from KGM (15.08 U/mL) and varying activity levels on galactomannan (72.63% on LBG, 21.31% on CM, and 16.38% on GG). However, it exhibited minimal activity towards mannan from ivory nut (16.36%), alpha-mannan (11.55%), xylan (from oat spelts (15.74%) and birchwood (15.74%)), as well as cellulose (carboxymethylcellulose, CMC) (13.08%)

and avicel (12.75%). Based on the substrate specificity screening assay, the crude mannanase produced by *Acinetobacter* sp. KUB-ST1-1 could hydrolyze the β-glycosidic bonds between the glucose and mannose in glucomannan, as well as between the galactose and mannose in galactomannan, in a non-specific manner [47]. Thus, it could potentially be utilized for the production of mannooligosaccharides.

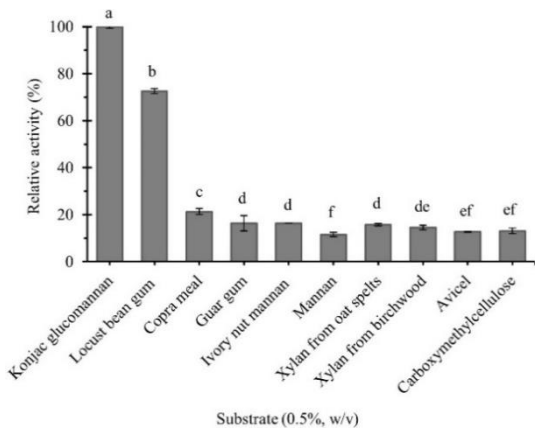

Figure 2: Relative activity of crude mannanase generated by *Acinetobacter* sp. KUB-ST1-1 using different substrates, with relative activity for KGM defined as 100%. Mean values ± standard deviation with different lowercase letters are significantly (*p*-value < 0.05) different.

3.2 *Purification of mannanase*

The purification process for mannanase enzymes from bacteria generally involves several steps. Protein separation techniques are used to remove contaminating proteins and enrich the target enzyme. Common methods include centrifugation, precipitation, and filtration. In the current study, adjusting the pH of the enzyme solution, ammonium sulfate precipitation, and ethanol precipitation resulted in lower levels of specific activity and yield compared to ultrafiltration. Ultrafiltration not only removed the contaminants but also concentrated the enzyme solution. After partial purification, additional purification can be performed using ion-exchange chromatography, which separates proteins based on their charge. This technique is available in two types: a) anion-exchange chromatography, which captures proteins with a positive charge using exchangers with a negative charge, such as diethylaminoethyl (DEAE) or quaternary ammonium sepharose; or b) cation-exchange chromatography, which targets proteins with a negative charge using exchangers with a positive charge, such as carboxymethyl (CM) sepharose or sulfopropyl sepharose [48]–[54].

In the current study, the concentrated mannanase was loaded onto a DEAE-cellulose 52 column (a weak anion exchanger) for anion exchange chromatography. Two elution methods were used: a stepwise NaCl gradient (0–0.5 M) and a linear NaCl gradient (0–0.2 M). Based on the results, proteins with mannanase enzyme activity were negatively charged and the DEAE-cellulose 52 column was bound to the mannanase enzyme. The mannanase showed a single peak using a stepwise gradient of 0.1 M NaCl, followed by a linear gradient from 0.1 to 0.2 M NaCl. Notably, the mannanase purified using the linear gradient had many-fold greater purification than that purified with the stepwise gradient, which aligned with the SDS-PAGE analysis. Following this, 50 mL of the purified mannanase was dialyzed and loaded onto a CM-cellulose 52 column (a weak cation exchanger) for cation exchange chromatography. However, most of the protein and mannanase activity were not retained on the column, with a single peak of mannanase activity that was neither bound to the column nor retained, so it was eluted with the starting buffer.

The culture supernatant with mannanase activity was concentrated via ultrafiltration and subsequently purified using both anion and cation exchange chromatography, as summarized in Table 1. The β-mannanase achieved a 1.82% recovery and a 14.50-fold purification, with a band of protein displaying activity at an approximate molecular weight of 57 kDa (Figure 3). Based on this result, it appeared that the mannanase preparation was notably uniform. The activity of the mannanase was demonstrated using a zymogram (Figure 3).

Table 1: Mannanase purification process.

Process	Total Volume (mL)	Total Protein (mg)	Total Mannanase Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Unpurified protein	1,000	2,611	2,257	0.86	1.00	100.00
Ultrafiltration	100	1,916	1,846	0.96	1.11	81.79
DEAE-cellulose 52	50	11.07	42.29	3.82	4.44	1.87
CM-cellulose 52	24	3.29	41.24	12.55	14.50	1.82

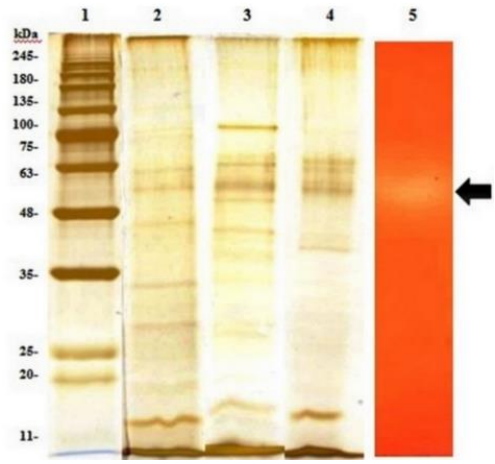

Figure 3: Analysis of protein and active protein. Lane 1, pre-stained protein ladder marker; Lane 2, unpurified protein; Lane 3, protein from DEAE-cellulose 52 column; Lane 4, protein from CM-cellulose 52 column; Lane 5, mannanase zymogram. Each well contains 20 μg of protein. Arrow mark represent mannanase activity.

The mannanase from *Acinetobacter* sp. KUB-ST1-1 had a molecular weight (57 kDa) consistent with that of the mannanases (18–130 kDa) found in other bacterial sources and fungal sources such as *Bacillus* sp. N16-5 (55 kDa), *Bacillus subtilis* KU-1 (39 kDa), *Lactobacillus casei* HDS-01 (37 kDa), *Streptomyces* sp. RDA1496 (63 kDa), *Vibrio* sp. MA-128 (49 kDa), and *Bacillus* sp. strain JAMB-750 (130 kDa) [54]–[59].

3.3 *Biochemical analysis*

3.3.1 Optimal pH and its effects on stability

These results are presented in Figure 4 as % relative activity. The optimal pH was 6.0 (1.28 U/mL). Typically, bacterial mannanases display optimal activity at pH levels ranging from acidic to neutral. As an example, the purified mannanases derived from *L. casei* HDS-01, *Bacillus* sp. SWU60, *Enterobacter ludwigii* MY271, *Paenibacillus thiaminolyticus*, and *K. pneumoniae* SS11 had optimal pH values of 5.0, 6.0, 7.0, 7.0, and 7.0, respectively [57], [60]–[63]. Based on the analysis of the pH stability of purified *Acinetobacter* sp. KUB-ST1-1 mannanase at 4 °C for 24 hours, shows the enzyme maintained 60–95% of its activity at pH 4.0–10.0 (Figure 4). This pH stability was broader than that of mannanases derived from *P. cookii* and *B. subtilis* BE-91 [64], [65].

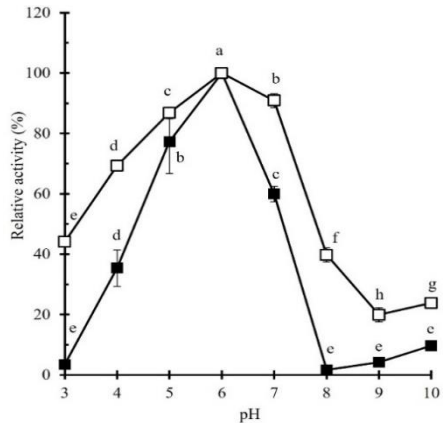

Figure 4: Effect of pH on activity of purified mannanase. (■) Optimal pH, where mannanase activity was assessed using 0.5% (w/v) LBG solution in various buffers—50 mM citrate buffer (pH 3.0–6.0), 50 mM phosphate buffer (pH 6.0–8.0), and 50 mM glycine-NaOH buffer (pH 8.0–10.0) and reactions were conducted at 50 °C for 30 min. (□) pH stability, where the stability of mannanase at different pH levels was evaluated by incubating the enzyme at 4 °C in the same buffer systems for 24 h. Mean values ± standard deviation with different lowercase letters are significantly (p-value < 0.05) different within the same experiment.

3.3.2 Optimal temperature and its stability effects

The temperature optimum exhibited peak activity between 50 and 60 °C (0.92 U/mL), with optimal performance observed at 60 °C for 30 min, displaying more than 80% relative activity (Figure 5(a)). The enzyme had high-temperature stability at 40 and 50 °C, maintaining its activity for up to 18 h. At 60 °C, it retained 80% of its activity for 45 min and had a half-life of 60 min (Figure 5(b)), indicating moderate thermal stability.

In general, the mannanase from *Acinetobacter* sp. KUB-ST1-1 was a mesostable enzyme. The optimal temperature for the purified mannanase was higher than for the fungal mannanases obtained from *Trichoderma harzianum* T4 and *Aspergillus oryzae* NRRL 3448 [49], [66]. Compared to mannanases from other microorganisms, the enzyme in the current study had distinct bacterial enzyme characteristics, including notable thermostability, as it maintained

activity at 50 °C for 9 h. Therefore, the thermostable enzyme should be well-suited for various biotechnological industries, particularly in food and pet food production, where the processes often require moderately high temperatures.

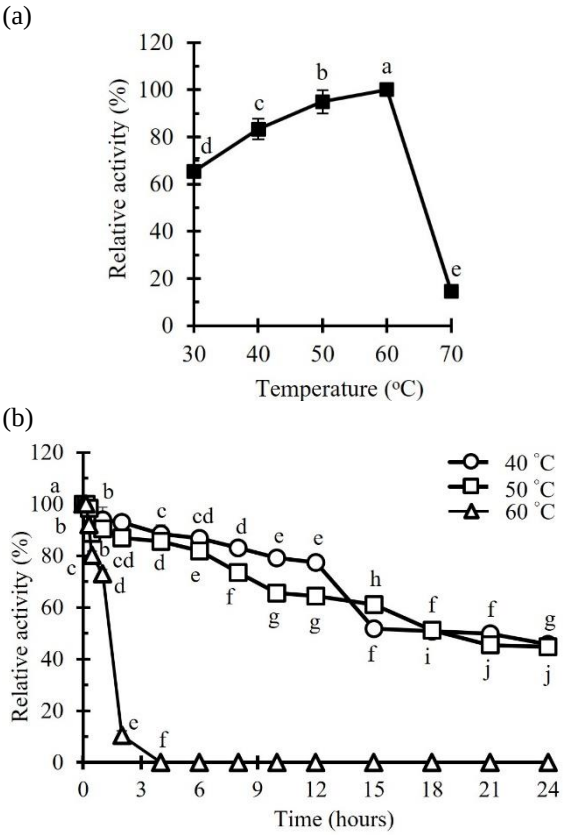

Figure 5: Temperature effects on activity of purified mannanase. (A) Optimal temperature, determined for mannanase activity within 30–70 °C in 50 mM phosphate buffer (pH 6.0) for 30 min. (B) thermostability at 40 °C, 50 °C, and 60 °C over different time periods. Mean values ± standard deviation with different lowercase letters are significantly (p-value < 0.05) different within the same experiment.

3.3.3 Substrate specificity

The substrate specificity assay indicated that the enzyme displayed its highest activity on glucomannan (KGM) and galactomannan (LBG), respectively. In addition, the enzyme effectively hydrolyzed CM and the other mannan substrates, which varied in their side chain composition (Table 2). The purified mannanase achieved its highest activity of 1.42 U/mL on KGM, considered as 100%, with moderate activity on LBG at 85.61%, and low activity on CM at 10.56%. Both the crude and purified mannanases demonstrated activity on a range of mannan substrates, particularly konjac glucomannan and galactomannan. Based on these findings, β-mannanase is highly specific and predominantly active against β-mannan substrates, showing a significant preference for glucomannan and galactomannan and implying that the mannanase preferentially hydrolyzes the D-mannoglucose and D-mannogalactose chains.

Table 2: Characterization of mannanase substrate specificity.

Substrate (0.5%, w/v)	Relative activity (%)*
Glucomannan	
Konjac (KGM)	100.00^{a}±0.02
Galactomannan	
Locust bean gum (LBG)	85.61^{a}±0.01
Copra meal (CM)	10.56^{b}±0.01
Guar gum (GG)	16.38^{c}±0.50
Mannan	
Ivory nut mannan	23.18^{ab}±0.02
α-Mannan	2.66^{c}±0.01
Xylan	
Xylan from oat spelts	2.40^{c}±0.02
Xylan from birchwood	5.50^{c}±0.02
Cellulose	
Avicel	1.97^{c}±0.01
Carboxymethylcellulose (CMC)	1.80^{c}±0.01

*Relative activity for KGM is defined as 100%.
Mean values ± standard deviation with different lowercase superscripts are significantly (p-value < 0.05) different.

3.4 *Analysis of mannooligosaccharide*

The hydrolysis of KGM, LBG, and DCM resulted in reducing sugar concentrations of 4.97, 3.34, and 0.33 mg/mL, respectively, as presented in Table 3. Furthermore, total sugar concentrations of 12.41, 5.72, and 0.66 mg/mL were obtained from KGM, LBG, and DCM, respectively. These findings highlighted the substrate specificity of the mannanase, which had maximum activity on KGM, followed by LBG and DCM, respectively. Consequently, the hydrolysis of KGM produced a greater concentration of both reducing sugars and total sugars compared to the other substrates. The hydrolysis of KGM, LBG, and DCM resulted in a degree of polymerization (DP ≈ 2–3), indicating that the mannanase from *Acinetobacter* sp. KUB-ST1-1 primarily hydrolyzed these substrates into M2 and M3.

Table 3: Degree of polymerization analysis for different substrates.

Type of Substrate	Reducing Sugar (mg/mL)	Total Sugar (mg/mL)	Degree of Polymerization
H-KGM	$4.97^{a} \pm 0.05$	$12.41^{a} \pm 0.30$	≈2.50
H-LBG	$3.34^{b} \pm 0.08$	$5.72^{b} \pm 0.51$	≈1.71
H-DCM	$0.33^{c} \pm 0.01$	$0.66^{c} \pm 0.10$	≈2.00

Mean values ± standard deviation with different lowercase superscripts are significantly (p-value < 0.05) different within the same experiment.

The efficiency of mannanase activity depends on the number of substitutions in sugars other than mannose. For example, the enzyme's efficiency in hydrolyzing glucomannan and galactomannan depends on the amounts of glucose and galactose, respectively. Additionally, the structural differences among KGM, LBG, and CM impact their hydrolysis by mannanase. KGM is a type of glucomannan with a mannose-to-glucose ratio of 1.6:1. LBG and CM are both galactomannans, with mannose-to-galactose ratios of 4:1 and 14:1, respectively [25], [26]. Notably, CM has a unique structure compared to other galactomannans; it is more robust, arranged in a crystalline form, and has a high mannose-to-galactose ratio (14:1) making it more resistant to dissolution in water and digestion [2]. One method to enhance the hydrolysis of CM by mannanase is to remove its fat content. This treatment allows the enzyme to more effectively produce mannooligosaccharides of varying molecular sizes. Mannanase, an endo-type enzyme, acts randomly to cleave the 1,4-mannosidic bonds within the mannan compound. Subsequently, mannosidase (an exo-type enzyme) hydrolyzes the mannooligosaccharides from the reducing end by specifically cleaving the β-1,4-glycosidic bonds, ultimately producing mannose [25], [26].

The types of hydrolysis products were confirmed using TLC with MOS standards (Figure 6). Based on the results, H-KGM and H-LBG (but not H-DCM) produced similar hydrolysis products (M1–M5) but differed in having high molecular weights. The unidentified oligosaccharides of glucomannan and galactomannan, such as M1-M2, M2-M3, and others, could be MOS, substituted with glucose or galactosyl side chains. Consequently, the mannanase produced by *Acinetobacter* sp. KUB ST1-1 could randomly hydrolyze KGM, LBG, and DCM at the β-1,4-glycosidic bonds, yielding various products. This process resulted in the production of M1 in the hydrolyzed reaction mixture as a consequence of β-mannosidase activity [26]. Thus, a mixture of MOS with varying sizes would be produced from the extensive hydrolysis of inexpensive and commercially available LBG, KGM, and DCM derived from agricultural waste using purified mannanase. However, MOS production depends on the enzyme and substrate concentrations, incubation time, and the enzyme's temperature stability.

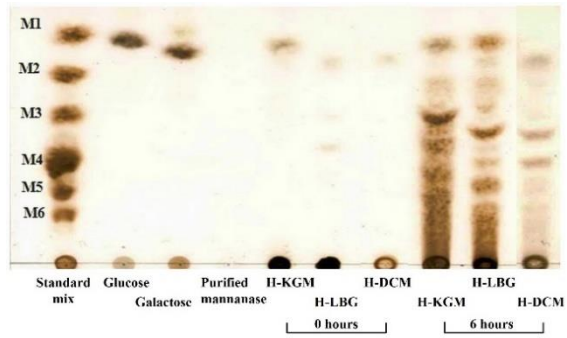

Figure 6: TLC analysis of hydrolysate products . Standard mix, M1–M6; Purified mannanase, reaction of purified mannanase; H-KGM, hydrolysis of KGM; H-LBG, hydrolysis of LBG; and H-DCM, hydrolysis of defatted CM.

3.5 *Prebiotic properties of mannooligosaccharide produced from various mannan sources*

3.5.1 Effects of mannooligosaccharides

The prebiotic properties of various hydrolysates were evaluated by measuring the growth rates of LAB and *E. coli* (as a pathogen) in a BM medium containing 0.05% (v/v) of each hydrolysate. LAB had the highest specific growth rates in the BM medium supplemented with glucose (Figure 7). Commercial MOS significantly promoted the growth of *W. confusa* JCM 1093, although not as effectively as glucose. However, MOS did not significantly enhance the proliferation of *L. citreum* JCM 9698, *L. lactis* subsp. *lactis* JCM 5805, *L. reuteri* KUB-AC5, or *L. johnsonii* KUNN19-2. All the tested hydrolysates markedly increased specific growth rates for LAB, specifically *W. confusa* JCM 1093, *L. citreum* JCM 9698, *L. lactis* subsp. *lactis* JCM 5805, and *L. johnsonii* KUNN19-2. However, they were ineffective in supporting the growth of *L. reuteri* KUB-AC5. Notably, the hydrolysates produced significantly high specific growth rates for *L. lactis* subsp. *lactis* JCM 5805 and *L. johnsonii* KUNN19-2, comparable to those observed in glucose-enriched basal medium.

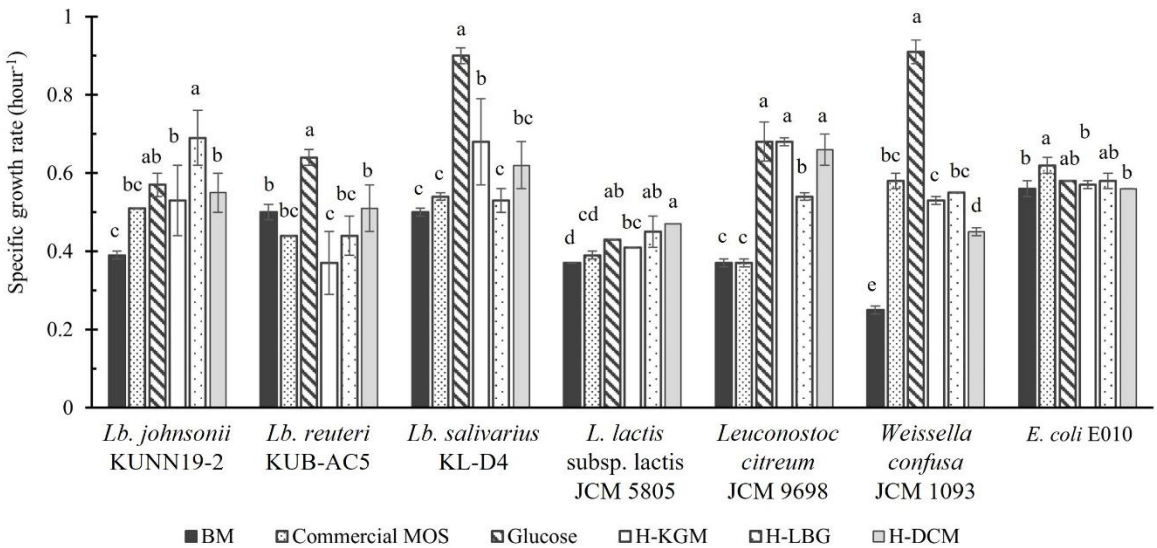

Figure 7: Specific growth rates of LAB and pathogens in different sugars. Mean values ± standard deviation with different lowercase letters are significantly (p-value < 0.05) different within the same experiment.

H-DCM significantly supported the growth of *L. citreum* JCM 9698, similar to the effect observed with the glucose-supplemented BM medium. H-KGM notably enhanced the specific growth rates of *L. citreum* JCM 9698 and *L. salivarius* KL-D4, while H-LBG significantly improved the growth of *L. lactis* subsp. *lactis* JCM 5805 and *L. johnsonii* KUNN19-2. None of the hydrolysates supported the growth of *E. coli* E010. Based on these results, the three hydrolysates promoted the growth of *W. confusa*, *L. citreum*, *L. lactis*, and *L. johnsonii*, while inhibiting the bacterium *E. coli* more effectively than commercial MOS. This suggested that specific LAB could utilize all three types of hydrolysates as carbon sources. Identifying the oligosaccharides in these hydrolysates and conducting further studies will be necessary to fully understand their effects on growth mechanisms. Other research has shown that prebiotic MOS can increase populations of beneficial bacteria, including *Lactobacillus* spp., *B. circulans* NT. 6.7, and *K. oxytoca* KUB-CW2-3, while simultaneously reducing pathogenic bacteria [67], [68]. MOSs, derived from the culture filtrate of *B. circulans* NT. 6.7 grown on CM, have been shown to enhance the growth of *L. reuteri* KUB-AC5, a well-recognized probiotic [69]. MOSs positively impact growth performance, digestibility, and intestinal microflora [70].

LAB and *E. coli* had the highest specific growth rates in the glucose-enriched basal medium, indicating that glucose was the best carbon source for microorganisms, particularly for LAB and pathogenic bacteria. Furthermore, glucose is cheaper than KGM, LBG, and DCM, respectively [71], [72]. Notably, glucose also promotes the growth of pathogens, such as *E. coli*, which can be harmful to host cells. However, the prebiotic substances from KGM glucomannan, LBG, and DCM are used only as carbon sources by bacteria that are beneficial to host cells. Therefore, based on the results of the current study, prebiotic MOS from *Acinetobacter* sp. KUB ST1-1 could be a valuable functional food additive in the pet food industry to enhance beneficial intestinal microflora and inhibit pathogenic bacteria.

3.5.2 Growth of E. faecium and E. hirae in a simulated gut model of dogs

In another study, *E. faecium* and *E. hirae* showed heme hydrolysis, and so are expected to be pathogenic. They can be a major bacterial group living in the gastrointestinal tract of dogs and may cause urinary tract infection, as well as other diseases such as wound infections [73], [74]. Therefore, the growth of pathogens, such as *E. faecium* KUB-17, *E. faecium* KUB-22, and *E. hirae* KUB-23, isolated from fresh dog feces, was assessed for their interaction with MOS in a simulated gut model of dogs. Notably, none of the hydrolysates supported the growth of *E. faecium* KUB-17, *E. faecium* KUB-22, or *E. hirae* KUB-23 as pathogens, except for H-KGM, which significantly enhanced the growth of *E. hirae* KUB-23 (Table 4).

Table 4: Specific growth rate of *E. faecium* and *E. hirae* in a simulated gut model of dog.

Enterococcus sp.	Specific Growth Rate (hour^{-1})				
	BM	Glucose	H-KGM	H-LBG	H-DCM
E. faecium KUB-17	$0.46^{a} \pm 0.04$	$0.44^{a} \pm 0.03$	$0.43^{a} \pm 0.03$	$0.46^{a} \pm 0.02$	$0.35^{b} \pm 0.01$
E. faecium KUB-22	$0.39^{ab} \pm 0.06$	$0.46^{a} \pm 0.02$	$0.35^{ab} \pm 0.03$	$0.34^{b} \pm 0.02$	$0.37^{ab} \pm 0.02$
E. hirae KUB-23	$0.44^{b} \pm 0.03$	$0.68^{a} \pm 0.04$	$0.67^{a} \pm 0.01$	$0.43^{b} \pm 0.01$	$0.47^{b} \pm 0.00$

Mean values ± standard deviation with different lowercase superscripts are significantly (p-value < 0.05) different within the same experiment.

3.5.3 Stability of H-DCM under a simulated gut model of dog

Prebiotics in the colon are fermented by probiotic bacteria. Thus, to be classified as a prebiotic, MOS should be able to tolerate gastric acidity and bile salts solution. H-DCM demonstrated strong resistance to artificial gastric juice at pH 3.0 after 4 h and to artificial intestinal fluid at pH 8.0 for 4 h (Table 5). The maximum hydrolysis was 13.89% after 4 hours. Based on these results, more than 80% of the consumed H-DCM would reach the large intestine in dogs, serving as a carbon source for several health-promoting bacteria, including probiotics. Thus, KUB-ST1-1 mannanase should be a good candidate for functional food and feed.

Table 5: Stability of H-DCM under a simulated gut model of dog at pH 3.0.

Incubation Time (hours)	Hydrolysis of H-DCM (%)	
	Gastrointestinal System	Intestinal System
0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
0.5	$0.00^{a} \pm 0.90$	$8.76^{b} \pm 0.89$
1	$0.00^{a} \pm 0.28$	$11.08^{b} \pm 2.84$
2	$0.00^{a} \pm 0.20$	$12.80^{b} \pm 2.17$
4	$0.00^{a} \pm 0.96$	$13.89^{b} \pm 2.43$

Mean values ± standard deviation with different lowercase superscripts are significantly (p-value < 0.05) different within the same experiment.

4 Conclusions

The research reported the biochemical characterization of a newly isolated *Acinetobacter* sp. KUB-ST1-1 and its potential for mannooligosaccharides production. The purified mannanase protein (MW of 57 kDa) had mannanase activity as demonstrated by a single band on the zymogram. Additionally, this purified mannanase had high specific activity and was effective across a variety of pH levels and temperatures. The hydrolysis products of KGM, LBG, and DCM mainly consisted of M2 and M3. The MOS generated from KGM, LBG, and DCM using mannanase from *Acinetobacter* sp. KUB-ST1-1 had notable prebiotic properties, including the ability to enhance the growth of LAB while inhibiting pathogens, thus having the potential to play an important role in gut health. Furthermore, H-DCM produced significant resistance to pepsin, trypsin, and bile salts, suggesting its potential to positively impact and enhance the gastrointestinal tract of dogs. All these properties indicate that mannanase from *Acinetobacter* sp. KUB-ST1-1 could be a potentially useful candidate in industrial applications, including food and pet food, as well as in other biotechnological processes

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Author Contributions

A.A.: writing the original draft preparation, Z.Z.: methodology, S.M.: methodology, S.N.: making suggestions and editing; W.S.: writing-, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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