



Isolation of Chitinolytic Bacteria From Two Lizard Digestive Tract and Characterization of Their Crude Chitinase

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Abstract : A study on isolation of chitinolytic bacteria from two lizard digestive tract of tokay gecko (*Gekko gecko*) and golden skink (*Mabouya multifasciata*), and characterization of their crude chitinase has been done. Bacterial isolation was conducted on chitin medium incubated at 31°C. Chitinolytic index was measured as ratio of clear zone diameter divided by colony diameter. Crude enzyme activity of the isolates was spectrophotometrically assayed using Schales reagent at 420 nm. Bacterial identification was conducted by using its 16S rRNA gene sequences. Twelve bacterial isolates were isolated from digestive tract of tokay gecko and eight were isolated from digestive tract of golden skink. LK12 and KD5 showed to have relatively high chitinolytic index of 1,551 and 1,098 respectively. Optimum enzyme activity of LK12 and KD5 was observed in 6 days of incubation with activity of 0,205 U/mL and 0,225 U/mL, respectively. Bacterial identification showed that LK12 and KD5 were closed to *Stenothrophomonas maltophilia* strain ATCC 19861 and *Enterobacter tabaci* strain YIM Hb-3 with similarity of 91 and 88%, respectively.

Keywords : chitinase, *Gekko gecko*, *Mabouya multifasciata*.

Introduction

Chitin is a compound easily found on crustacean shells (crabs, shrimps, and lobsters), jellyfishes, exoskeleton of insects, fungal cell wall (22-40%), algae, nematodes, animal skin, and even plants, making it the second biggest biomass compound in the world after cellulose¹. Several bacteria and fungi were known to degrade chitin². Chitin degradation process is highly essential to reduce the accumulation of chitin-containing wastes. Chitinase is a particular enzyme to hydrolyze chitin into its oligomer, i.e. carboxymethyl chitin, hydroxyethyl chitin, N-acetyl-D-glucosamine, and ethyl chitin, which are useful in medical and food industrial purposes. As a product of hydrolysis, N-acetyl-D-glucosamine is utilized as prebiotic, blood sugar controller, anti-inflammation, food supplement, and many others³.

Dwi Suryanto et al //International Journal of ChemTech Research, 2018,11(07): 17-28.

DOI= <http://dx.doi.org/10.20902/IJCTR.2018.110703>

Chitinase is utilised as bioinsecticides and biofungicides on plant pest and disease control⁴, and chitin-containing waste processing in frozen shrimp and crab industries⁵. It produces many beneficial products such as chitosan and other chitin-derived products. Likewise, chitinolytic bacteria was known to be a potential agent to control mosquito larva.

Considering its potential utilisation, exploration of chitinolytic bacteria has been intensively carried out. In this study chitinolytic bacteria from lizard tokay gecko (*Gekko gecko*) and golden skink (*Mabouya multifasciata*) was conducted. Many lizards were known as insect-eater. This makes their digestive tract are potential as chitinolytic microbe source.

Materials and Methods

Isolation and Screening of Chitinolytic Bacteria from Digestive Tract

Surgical preparations of tokay gecko and golden skink were done with ethical clearance where samples were initially anesthetized for 20 minutes and then surged aseptically. The small intestines of each sample were chopped into small pieces and put into 10 mL of sterile physiological saline solution in a test tube. The solution was subjected to serial dilution. A 0.1 mL of the solution was spread on colloidal chitin agar and incubated at ambient temperature for 1-2 days. Morphology of each growing colony was observed. Bacterial isolates was purified using colloidal chitin agar.

Morphological and Biochemical Characterisation

Bacterial isolates were subjected to morphological characterisation including their colony shape, edge, elevation, and color. Gram staining and biochemical characterization including catalase, carbohydrate fermentation, hydrogen sulfide, motility, and citrate utilization test were done.

Chitinolytic Index Measurement

Bacterial cell of sterile physiological saline solution was adjusted to $OD_{600} \approx 0.5$. A 10 μ L of chitinolytic bacteria solution was poured into paper disc. Paper disc of bacterial solution was put on chitin agar at ambient temperature for 5 days. Bacterial ability in degrading chitin was determined as chitinolytic index which was measured as ratio of clear zone diameter divided by colony diameter.

Bacterial Growth Observation

Bacterial isolate was aerobically grown in colloidal chitin solution at 37°C of 120 rpm. Bacterial growth was observed using total plate count method as total colony grew in colloidal chitin agar. Observation was done every day for 7 days.

Total Protein and Chitinase Activity Measurement

Total protein was measured using Bradford method. Chitinase activity was measured as N-acetylglucosamine released using spectrophotometer at 420 nm. One unit activity was defined as amount of enzyme that liberates 1 mmol of N-acetylglucosamine under assay condition.

Chitinase Production and Activity

Bacterial cell solution of sterile physiological saline solution of $OD_{600} \approx 0.5$ was cultured on colloidal chitin broth for 24 hours. Culture was centrifuged at 4 °C of 10,000 rpm for 10 minutes. Supernatant was collected and further examined for its chitinase activity. Chitinase activity was determined quantitatively as previously described⁹. Enzyme was assayed by mixing 150 μ L of supernatant, 150 μ L of phosphate buffer (pH = 7), and 300 μ L of 0.3% colloidal chitin. Suspension was homogenized and incubated at 37°C for 30 minutes and centrifuged at 4°C at 10,000 rpm for 10 minutes. A 300 μ L of the supernatant was collected, and added with 700 μ L aquadest and 1000 μ L Schales reagent (0.5 g of $K_3(FnCn)_6$) in 0.5 M sodium carbonate¹⁰. Solution was heated at 100°C for 10 minutes to stop reaction.

Ammonium Sulfate Precipitation and Enzyme Dialysis

Crude enzyme was obtained by precipitating supernatant of 1 days bacterial culture using ammonium sulfate at 20, 30, 40, 50, 60, and 70% saturation. Ammonium sulfate was added slowly into crude enzyme solution while stirred using magnetic stirrer at 10°C. Solution was subjected to centrifugation at 4°C of 10,000 rpm for 10 minutes. Pellet was dissolved into 10 mL of phosphate buffer at pH 7 for dialysis.

Dissolved enzyme from previous step was dialyzed. Dialysis was done as previously described¹¹. Dialysis membrane was soaked in NaHCO₃ and EDTA solution for 10 minutes. Partially purified enzyme was collected. Its chitinase activity and protein concentration were measured as method previously described.

Effect of pH and Temperature on Chitinase Activity

Effect of pH and temperature was observed as previously described⁹. To know the effect of pH, dialysed enzyme of 150 µL was added with 300 µL of 0.3% colloidal chitin as substrate for 30 minutes in 150 µL of pH buffer solutions from 3 to 8 using acetate buffer for pH 3-6, phosphate buffer for pH 7, and tris-HCl buffer for pH 8. To determine temperature effect, dialysed enzyme and colloidal chitin reaction was subjected to put in different temperature from 20°C to 50°C for 30 minutes. Effect of pH was measured at 37°C, while effect of temperature at chitinase activity was measured at pH 6 every 30 minutes.

Identification of Bacterial Isolates Using Their 16S rRNA Gene

DNA of chitinolytic bacteria was isolated using freeze and thaw method. Microtube of 1.5 mL was aseptically filled with 100 µL of aquabidest. One full loop of pure bacterial culture was put into the tube and suspended. Cell suspension was chilled at -10°C and heated at 90°C for 5 repeated cycles for 10 minutes each¹². DNA was amplified using Sensiquest Labcyler. A 25 µL of PCR reaction solution was prepared by mixing 2 µL of template DNA of previous cell suspension with 12,5 µL of Master Mix 2X GoTaqGreen (Promega), 1 µL of 10 pmol forward primer of 63f (5' CAGGCCTAACACATGCAAGTC-3'), 1 µL of 10 pmol of reverse primer of 1387r (5'-GGCGGWGTGTACAAGGC-3')²⁷, and 8,5 µL of nuclease free water. DNA amplification reaction was done as followed: pre-denaturation at 94°C for 2 minutes, denaturation at 92°C for 30 seconds, annealing at 55°C for 30 seconds, primer elongation at 72°C for 1 minute, and post-PCR at 72°C for 5 minutes. These reaction was run for 40 cycles. DNA amplification product was visualized using gel electrophoresis. DNA was commercially sequenced. DNA sequence was compared to that of *GenBank* of *The National Center for Biotechnology Information* (NCBI), using *Basic Local Alignment Search Tool* (BLAST).

Result

Isolation and Characteristic of Chitinolytic Bacteria

Isolation using colloidal chitin agar showed 12 different bacterial colonies of tokay gecko, and 8 different bacterial colonies were isolated from golden skink of digestive tract. Chitinolytic bacteria was characterized by their clear zone around colony (Figure 1). Bacterial colonies able to grow and produce clear zone around their colony in colloidal chitin agar indicated that they are able to produce chitinase enzyme¹³.

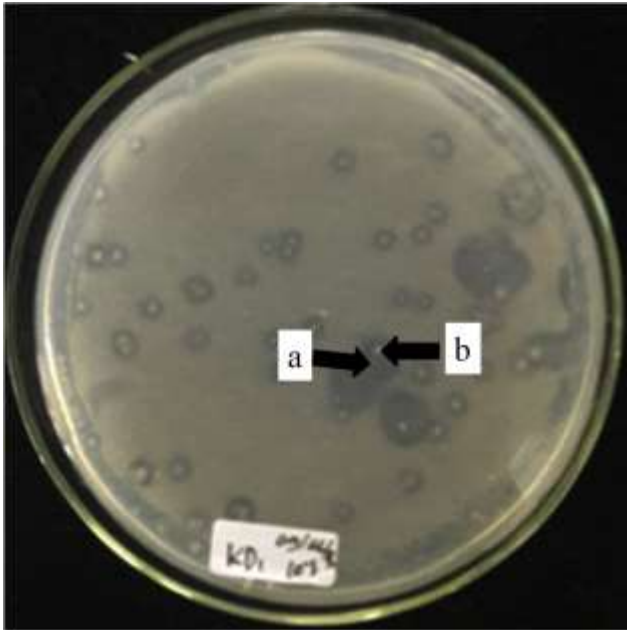


Figure 1. Chitinolytic bacterial ability was shown by (a) clear zone around (b) bacterial colony

Out of 20 bacterial colonies, 13 colonies were observed to have irregular colony shape, while the other 7 have circular shape. Colony edge of the isolated bacteria was dominated by undulate (9 isolates) and entire (9 isolates), while only 2 isolates had lobate colony edge. As for the colony elevation and color, 16 isolates had flat elevation and 4 colonies had raised elevation, while 19 isolates were cream in color and only 1 was white. Microscopic observation revealed that 15 isolates were cocci and 5 isolates were bacilli. Different characteristics of bacterial colony could be due to the different source of isolation and different intracellular bacterial pigment (Tabel 1). Different intracellular pigment could be produced by bacteria from different source of isolation¹⁴. Bacterial pigment can be classified as either karotenoid, antocyanin, melanine, tripirilemethenes, and phenazim. Biochemical activity including starch, gelatin, and citrate utilization, carbohydrate fermentation, motility, and catalase test was varied (Tabel 1), indicating that the bacterial species might be varied.

Table 1. Morphological and biochemical characteristics of Chitinolytic Bacterial Isolates of Digestive Tract of Tokay Gecko and Golden Skink

Source	Isolate code	Colony morphology				Cell Shape	Gram staining	Biochemical traits					
		Shape	Edge	Elevation	Color			Starch metabolism	Gelatin hydrolysis	Citrate metabolism	H ₂ S production		Motility
											Slant	Butt	
Tokay Gecko	LK1	Irregular	Undulate	Flat	Cream	Coccus		+	-	+	Yellow	Yellow	+
	LK2	Circular	Entire	Raised	Cream	Rod	+	-	-	+	Yellow	Yellow	+
	LK3	Irregular	Undulate	Flat	White	Coccus	-	-	-	+	Yellow	Yellow	+
	LK4	Irregular	Undulate	Flat	Cream	Coccus	-	-	-	+	Yellow	Yellow	+
	LK5	Irregular	Undulate	Flat	Cream	Rod	-	-	-	+	Yellow	Yellow	+
	LK6	Circular	Undulate	Raised	Cream	Rod	-	-	-	+	Yellow	Yellow	+
	LK7	Irregular	Entire	Raised	Cream	Coccus	-	+	-	+	Yellow	Yellow	+
	LK8	Irregular	Entire	Flat	Cream	Coccus	-	-	-	+	Yellow	Yellow	+
	LK9	Circular	Entire	Flat	Cream	Coccus	-	+	-	+	Yellow	Yellow	+
	LK10	Irregular	Undulate	Raised	Cream	Coccus	-	-	-	+	Yellow	Yellow	+
	LK11	Circular	Entire	Flat	Cream	Coccus	-	-	-	+	Yellow	Yellow	+
	LK12	Irregular	Entire	Flat	Cream	Coccus	-	-	-	+	Yellow	Yellow	+
Golden Skink	KD1	Circular	Entire	Flat	Cream	Coccus	-	-	+	+	Yellow	Yellow	+
	KD2	Irregular	Lobate	Flat	Cream	Rod	-	-	-	+	Yellow	Yellow	+
	KD3	Irregular	Undulate	Flat	Cream	Rod	-	-	+	+	Yellow	Yellow	+
	KD4	Circular	Undulate	Flat	Cream	Coccus	-	+	+	-	Yellow	Yellow	+
	KD5	Circular	Entire	Flat	Cream	Coccus	-	-	+	+	Yellow	Yellow	+
	KD6	Irregular	Undulate	Flat	Cream	Coccus	-	+	+	+	Yellow	Yellow	+
	KD7	Irregular	Lobate	Flat	Cream	Coccus	-	-	+	+	Yellow	Yellow	+
	KD8	Irregular	Entire	Flat	Cream	Coccus	-	+	+	+	Yellow	Yellow	+

Chitinolytic Index of Bacterial Isolates

All chitinolytic bacterial isolates showed to vary in chitinolytic index (Figure 2). The highest chitinolytic index obtained in this study was from LK12 and KD5 with 1.551 and 1.098 respectively, while the lowest chitinolytic index was from KD2 which was not able to degrade chitin. The high chitinolytic index by LK12 and KD5 indicated that both isolates had high chitinolytic activity and was assumed to be able to produce chitinase with high enzyme activity. The different size of clear zone and chitinolytic index produced on the same growth medium was strongly affected by the difference in amount and activity of chitinase enzyme produced by each bacterial species. This also relates to their ability in producing enzymes to degrade chitin in the medium into its monomer¹⁵. Formation of clear zone on chitin agar medium can be easily observed around the bacterial colony¹³

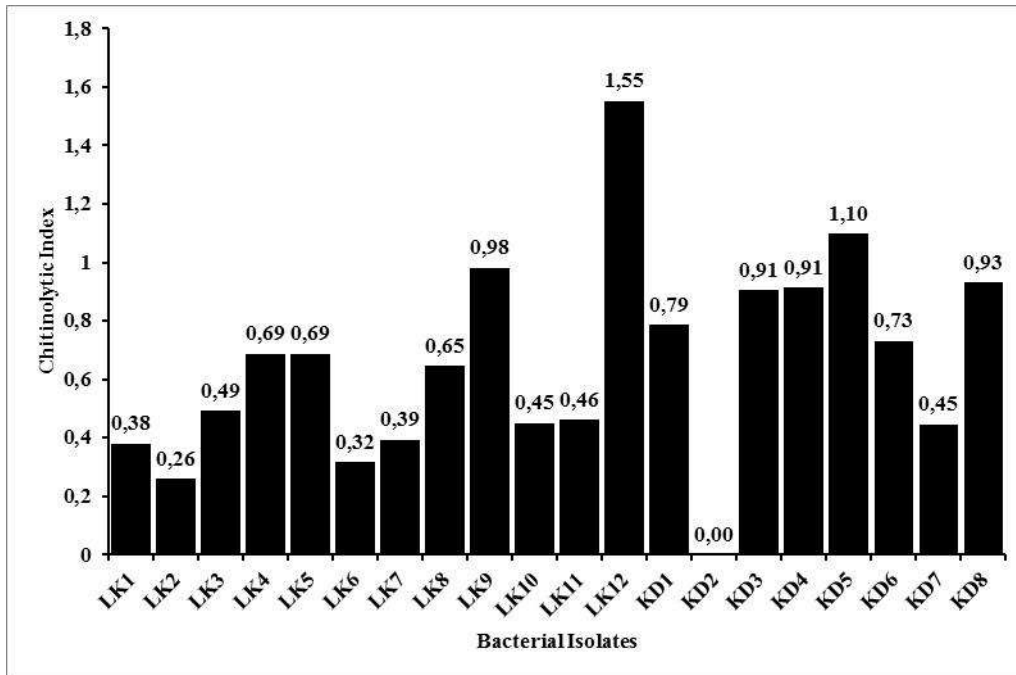


Figure 2. Chitinolytic Index of Bacterial Isolates of Tokay Gecko and Golden Skink Digestive Tract

Bacterial Growth and Production of Crude Enzyme Extract

Growth and chitinolytic activity of two selected isolates was observed for 7 days (Figure 3). Both LK12 and KD5 growth were maximum at 6 days of incubation. Maximum activity of bacterial chitinase was varied among bacteria. Maximum chitinolytic activity of two isolates was observed during stationary phase. *Bacillus licheniformis* A2¹⁶ and *Bacillus subtilis*¹⁷ were at 4 days of incubation similar to that of hot-spring bacterial isolates¹⁸, while *Bacillus licheniformis* A35¹⁶ showed to have maximum activity at 6 days of incubation.

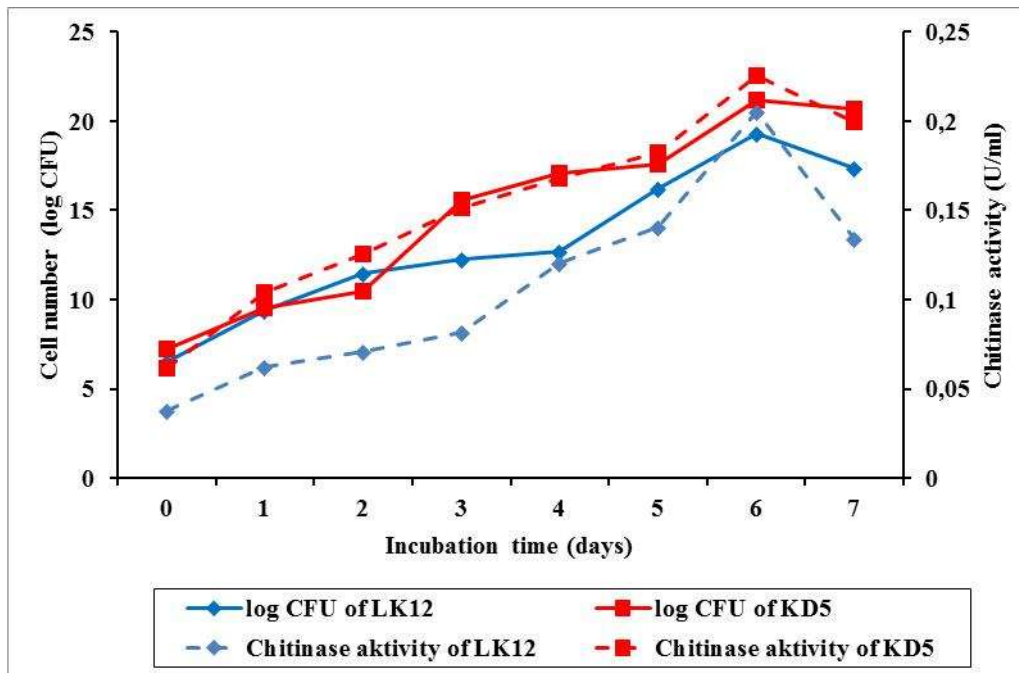


Figure 3. Cell Growth and Chitinolytic Activity of LK12 and KD5

Enzyme, together with other secondary metabolites, were secreted during stationary phase. Lower activity before stationary phase might be due to low number of bacterial cells in medium²⁰. Maximum production of chitinase from *Bacillus licheniformis* MB-2 was observed during stationary phase²². Chitinolytic activity of two isolates showed to have similar pattern with high activity of 0,2255 and 0,2056 U/mL, respectively.

Ammonium Sulfate Precipitation

Precipitated crude enzyme of KD5 and LK12 showed different activity of different fraction as shown on Figure 5 below. Chitinase activity of pellet was gradually increasing together with the increasing concentration of ammonium sulfate, and reached its maximum activity at 60% ammonium sulfate with activity of 0,404 U/mL, while its supernatant chitinase activity was maximum at 30% ammonium sulfate with activity of 0,261 U/mL. Similar pattern was observed in LK12, where crude chitinase activity of pellet was maximum at 50% ammonium sulphate with activity of 0,4001 U/mL, while its supernatant activity was maximum at 30% ammonium sulphate with activity of 0,224 U/mL. Chitinase activity of bacteria isolated from Sibiru-biru hot spring was maximum at 50% ammonium sulphate with activity of 0,451 U/mg¹⁸. Chitinase activity of *Bacillus toyonensis* from digestive tract of *Calotes* sp. was optimum at 50% ammonium sulphate with activity of 0,024 U/mL. Similarly, chitinolytic bacteria *Bacillus* sp., *Bacillus thuringensis*, *Bacillus cereus* and *Stenotrophomonas maltophilia* isolated from soil and *Nephentes* showed to have optimum chitinase activity at 50% ammonium sulphate, with activity of 0,049, 0,027, and 0,026 U/mL respectively¹⁹.

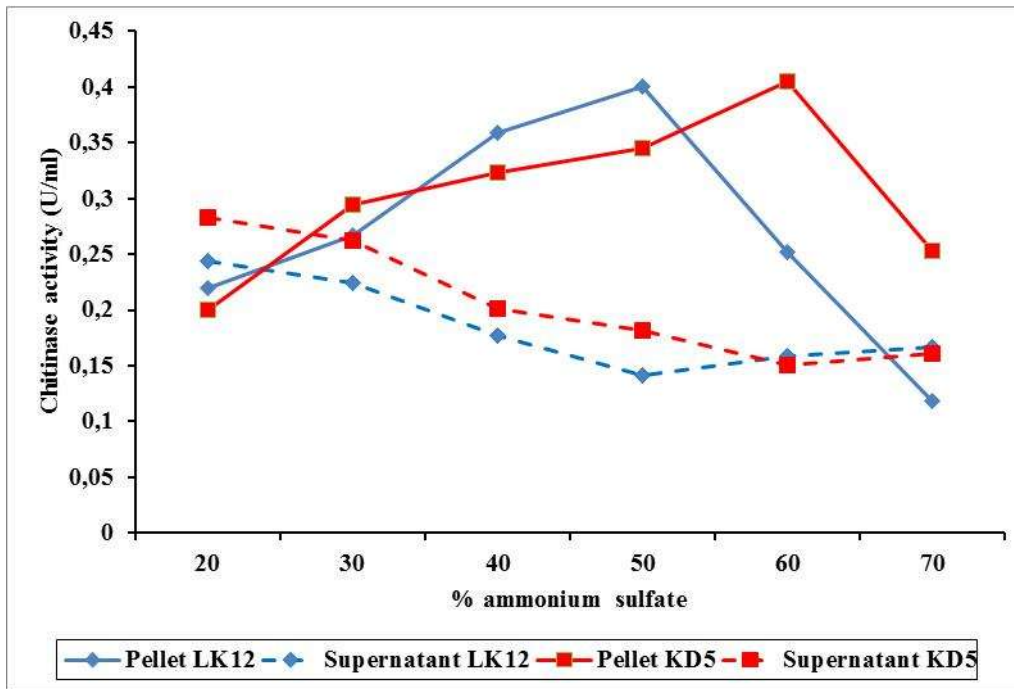


Figure 4. Chitinase Activity of Supernatant and Pellet of LK12 and KD5 in Ammonium Sulphate Precipitation

Activity of Dialysed Chitinase

Chitinase activity after extraction, precipitation, and dialysis was observed to increase. Purified enzyme after dialysis showed to have relatively high activity compared to those of extraction and precipitation. Protein was observed to significantly decrease after the process, as shown in Table 2. below.

Table 2. Bacterial Chitinase Activity of Crude, Ammonium Precipitation, and Dialysis of Chitinase of KD5 and LK12

Isolates	Volume (mL)	Preparation	Total Activity(U)	Total Protein (mg/mL)	Specific activity (U/mg)	Purification fold	Result (%)
KD5	100	Crude Enzyme	22,55	1640	0,013	1	100
	50	Precipitation 60 %	20,253	312,857	0,0647	4,97	89,81
	10	Dialysis	1,0471	6,57	0,1527	11,74	4,6434
LK12	100	Crude Enzyme	20,56	1911,42	0,0107	1	100
	50	Precipitation 50 %	20,007	341,428	0,0585	5,46	97,31
	10	Dialysis	1,6965	21,142	0,0802	7,49	8,25

This result showed that purification of chitinase at 50% and 60% ammonium sulphate increased enzyme activity. Other proteins but chitinase was precipitated using ammonium sulphate²³. Other study reported that specific activity of chitinase isolated from Penen hot spring and precipitated with 50% ammonium sulphate increased after dialysis to 1.040 U/mg with 2.220% purification¹⁸.

Effect of pH and Temperature on Dialysed Chitinase Activity

Optimum pH of chitinase activity partially purified of KD5 and LK12 was at pH 8.0, with activity of 0.1266 U/mL and of 0.2195 U/mL, respectively (Figure 5). Similar result was found in *Bacillus licheniformis* with optimum chitinase activity at pH 8.0²⁴. Chitinase activity of *Aeromonas hydrophila* from soil sample in

India showed optimum activity at pH 8.0²⁵. Enzyme activity was known to be highly affected by the enzyme structure. Any changes of in enzyme structure may affect enzyme activity. pH and temperature change, to some extent, affect enzyme structure and polarity. Substrate availability and medium composition used as source of carbon and nitrogen also affect enzyme activity⁽²¹⁾.

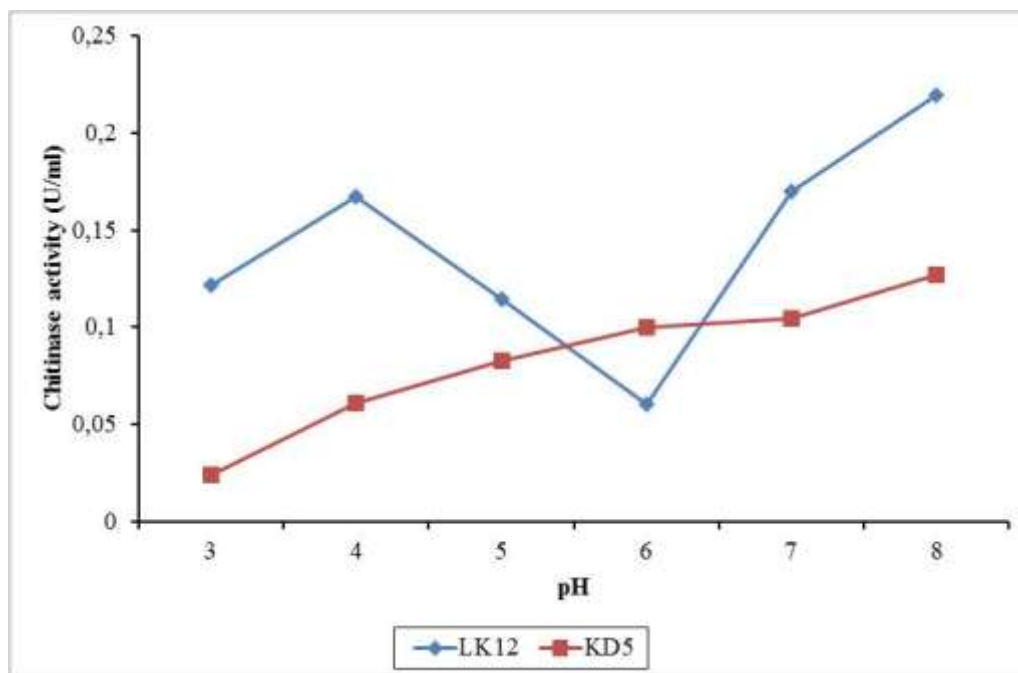


Figure 5. Effect of pH on Dialysed Chitinase Activity of KD5 and LK12

Partially purified chitinase of KD5 and LK12 isolate were assayed to know temperature effect to enzyme activity. KD5 and LK12 chitinase showed to have optimum activity at 35°C with activity of 0.323 U/mL and 0.1886 U/mL, respectively (Figure 6). A study on *Bacillus subtilis* showed that its chitinase was optimum at 35°C¹² that was also shown by other study¹⁹. The enzyme was decreased at 45 °C¹². Increasing of temperature resulted in increasing enzyme activity due to collision between substrate. However, very high temperature may cause enzyme to denature, which makes it to lower enzyme activity.

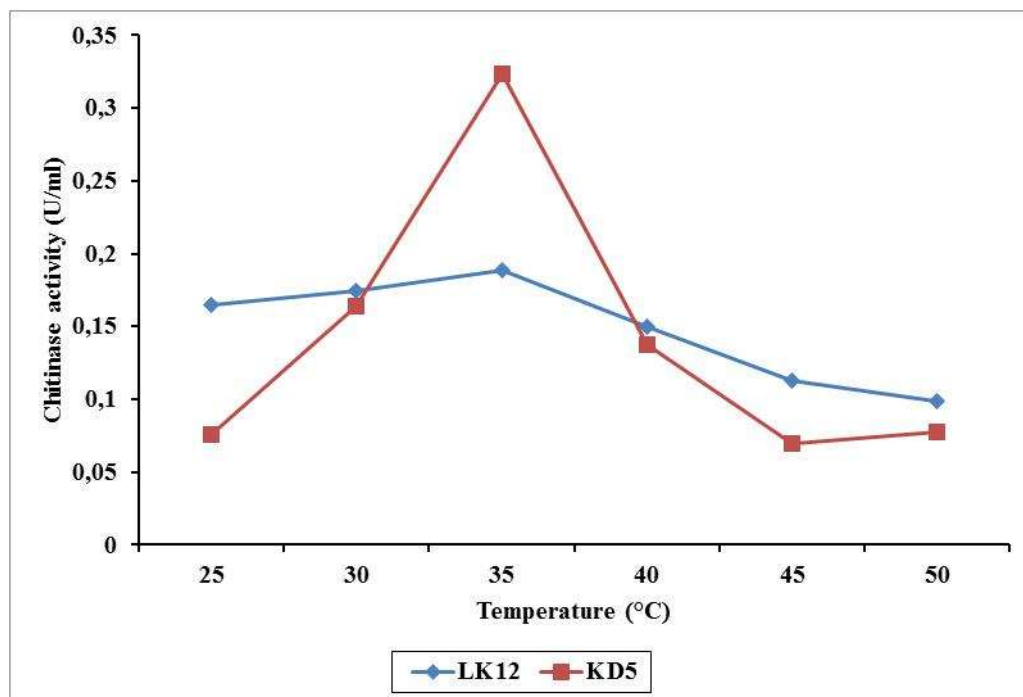


Figure 6. Effect of Temperature on Dialysed Chitinase Activity of LK12 and KD5

Identification of Chitinolytic Bacteria Using 16S rRNA Gene Sequence

Both selected isolates, KD5 and LK12, were identified using 16S rRNA gene sequences. Identification of the gene sequences showed that LK12 was identified as *Stenothrophomonas maltophilia* strain ATCC 19861 with 91% gene similarity, while KD5 was closely related to *Enterobacter tabaci* strain YIM Hb-3 with 88% gene similarity.

Stenothrophomonas maltophilia was previously reported as proteolytic and chitinolytic bacteria. Thermostable chitinase from *S. maltophilia* was reported from this bacteria. Additionally, *S. maltophilia* was isolated from *Nepenthes*, an insect-catch plant. On the other hand, *E. tabaci* a newly isolated species from tobacco belongs to the Enterobacteriaceae group was previously reported as keratinolytic bacteria with high potential in degrading keratin waste. ((by Duan et al (2015)). Therefore, this might be the first report to show that *E. tabaci* is also chitinolytic bacteria.

Conclusion

Twenty bacterial isolates were isolated from the digestive tract of tokay gecko and golden skink. Two potential isolates KD5 and LK12 showed to have crude chitinase activity of 0.013 U/mg and 0.0107 U/mg, respectively. Precipitated with ammonium sulfate of 60% and 50% followed by dialysis increased chitinase activity of LK12 to 0.0585 U/mg and 0.0802 U/mg, and of KD5 to 0.0647 U/mg and 0.1527 U/mg. Both isolates showed to have similar optimum pH of 8 and temperature of 35°C, in which KD5 showed to have chitinase activity of 0.126 U/mL, while LK12 demonstrated its chitinase activity of 0.188 U/mL. Based on their 16S rRNA sequence LK12 was closely related to *Stenothrophomonas maltophilia* strain ATCC 19861 and KD5 was closely related to *Enterobacter tabaci* strain YIM Hb-3 with gene similarity of 91 and 88%, respectively.

Acknowledgment

Special thanks is addressed to University of Sumatera Utara for partially supporting this research.

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