Screening and Identification of Bacteria that Produce Chitinase Enzymes from Soil in Na Si Nuan Forest, Maha Sarakham, Thailand

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Abstract:

This research aimed to screen the bacteria that produce chitinase enzymes from soil in Na Si Nuan forest located at Kantarawichai District, Maha Sarakham Province, Thailand. The conservation project in response to Plant Genetic Conservation Project under the Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhorn has occupied the community forest of 19.2 ha in this study. Isolation of bacteria was performed from soil on chitin agar. Our results were found two isolates of chitinase-positive bacterial strains. The isolate of 3.2 CT1 was showed the highest of halo: colony ratios with 4.5. The morphology isolate of 3.2 CT1 is gram negative, and rod shaped. The optimum temperature for chitinase activity was 40 ˚C with enzyme activity of 0.40 ± 0.01 U/mL and given a specific enzyme activity of 0.49 ± 0.01 U/mg. protein. The optimum pH was 6.0 with activity of 0.70 ± 0.01 U /mL. This chitinase may be used for agricultural purposes, such as plant pathogen inhibition and as insecticide for insect pest control.

Keywords: Chitinase enzymes, Bacteria, Soil, Na Si Nuan Forest, Agriculture.

Introduction

The conservation forest area, which is a community forest in Na Si Nuan, is located at Maha Sarakham, Thailand, has occupied 19.2 ha. The nature of the soil in this forest area is moderately saline and strongly saline, which was found in the basin area [9]. In addition, many species of edible and inedible mushrooms were also found in this forest. Local people [4] in the nearby area often come to use the bioresources for foods.

Chitinase enzyme is derived enzyme from many types of microorganisms. Qualified to decompose chitin which is a component of fungal cell wall. Chitinase is possible that it can be used in combination with anti-fungal drugs to increase the effectiveness of the drug in inhibiting and destroying fungi. Chitinase enzymes in bacteria were found *Aeromonas* sp. 10s-24 [2][13] and *Bacillus stearothermophilus* CH-34 [10] fungus also found chitinase enzymes is *Trichoderma harzianum* [14] *Aspergillus cameus* [1][12]etc. The mechanism of chitinase found that living organisms produce chitinase enzymes to protect themselves against the invasion of pathogens. Chitinases hydrolysis of chitin, linear homopolymer of p-1,4-linked N-acetyl-D-glucosamine residues. This polysaccharide is attendant in the cell wall of fungal and in exoskeleton of insects. In addition to control of phytopathogens fungal other different applications of chitinase such as mosquito control, Estimation of fungal biomass, target for biopesticides and morphogenesis have been discovered. Biocontrol of plant pathogens furnish an interesting choice for handle of plant disease without the negative impact of chemical fungicides that can cause environmental pollution and usually expensive, and may cause disease resistance [16].

This research purpose this to study of the chitinase enzyme bacteria screening and to identify from soil in Na Si Nuan forest and determine the activity of chitinase enzyme that can inhibit the fungi that destroy plants.

Materials and methods

Soil sampling

Soil samples were collected from Na Si Nuan forest, Maha Sarakham province, Thailand (Fig. 1) at a depth of 10-15 cm from the surface or Rhizosphere area [5][11]. Then the soil samples were placed in sterile polythene bags and kept at room temperature until analysis. The physical properties of the soil were analyzed by measuring electrical conductivity and pH.

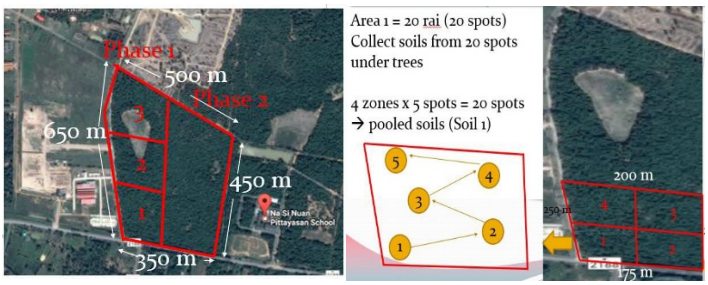


Figure 1 Soil sampling area in Na Si Nuan forest in Maha Sarakham province, Thailand.

Isolation of bacteria from soil samples

Ten grams of soil were weighed, and dissolved in 90 mL of sterile 0.85% NaCl. Soil samples were diluted to obtain serial dilutions at 10-2- 10-6. After that, 0.1 mL of solution was spread on chitin agar [(g/100 mL); 3% (w/v) colloidal chitin; 0.1% KH2PO4; 0.05% MgSO4.7H2O; 50 mM sodium phosphate buffer, (pH 6.0)] [15] and incubated at 37 °C for 3 days. Iodine solution was poured over the agar plates and cleared zones. It was observed around colony in the presence of bacterial chitinase that can hydrolyze chitin agar. These colonies of bacteria that produce chitinase were purified on LB agar using streak plate method and cell morphology, which was expressed under microscope (1,000 X).

Halo: colony ratio

Bacterial isolates were cultured in LB media for 18 h. A toothpick was used to touch the culture and the point inoculation on a solid agar was performed, incubated at 37 °C for 2 days. The ratio of halo: colony was measured as the radius of clear area over the radius of the colony.

Bacterial chitinase activity

Select of pure isolates of chitinase-producing bacteria [6] were cultured and adjusted at 108 CFU/mL equivalent to 0.5 McFarland were inoculated into 10 mL chitin liquid medium (100 ml): 3% w/v colloidal chitin [0.1% KH_2PO_4; 0.05% $MgSO_4.7H_2O$; 50 mM sodium phosphate buffer (pH 6.0)]. They were incubated at 37 ˚C with shaking at 150 rpm for 24, 48 and 72 h, and centrifuged at 12,000 g for 20 min at 4 ˚C as well as kept the supernatant (extracellular enzyme) reducing volume, for chitinase enzyme assay following a method of Mandana Zarei *et al.* [7][8]. The crude enzyme extracted with highest chitinase activity was determined [3].

Enzyme stability for chitinase enzyme activity

pH

The crude extract of the highest chitinase activity from above analysis was used to determine its optimum pH for activity using various pHs in suitable buffers (0.1 M sodium phosphate buffer: pH 6.0, 7.0 and 0.1 M Tris-HCl buffer: pH 8.0, 9.0, 10.0 and 11.0) in a range of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 as well as incubated at 37 °C for 30 min. Then, chitinase activity was recorded (3 repeated).

Temperatures

The crude extract of the highest chitinase activity with optimum pH was used to determine its optimum temperature for activity using various temperatures at 45, 55, 65, 75, 85, 95 ˚C for 0, 5, 10, 15 and 30 °C for 30 min and lipase activity was recorded (3 repeated).

Statistical analysis

Measurements were obtained in triplicates as means ± standard deviation (SD). Statistical analysis was performed using One-way analysis (One-way ANOVA) of variance and Duncan Multiple's Range Test by the software SPSS package version 17.0 at $P < 0.05$.

Results and discussion

Chitinase can also be detected in human blood and possibly cartilage. As in plant chitinases, this may be related to pathogen resistance, which is derived enzyme from many types of microorganisms. Qualified to decompose chitin which is a component of fungal cell wall. In addition control of phytopathogens fungal other different applications of chitinase such as mosquito control, estimation of fungal biomass, target for biopesticides and morphogenesis have been discovered.

Area	Longitude/Latitude	Surrounding	EC (µS/cm)	Temperature (°C)	pH
1.1	N 16 20 17.945/ E 103 12 14.731	Under tree *Azadirachta indica*	36.2	31.6	8.69
1.2	N 16 20 17.945/ E 103 12 14.731	Under tree *Terminalia chebula*	19.9	31.0	7.72
1.3	N 16 20 6.619/ E 103 11 0.985	Unknown	22.3	31.0	7.06
1.4	N 16 20 23.547/E 103 12 35.244	*Tectona grandis*	20.9	31.0	6.40
2.1	N 16 20 36.882/ E 103 12 32.315	The open space was found around tree *Ageratum conyzoides*	28.8	31.0	6.27
2.2	N 16 20 36.863/ E 103 12 32.416	*Dipterocarpus intricatus* forest	27.7	30.9	5.77
2.3	N 16 19 39.152/E 103 12 33.655	*Eucalyptus* nearby *Pomegranate*	184	31.0	4.49
2.4	N 16 20 37.157/E 103 12 33.579	Uvaria hahinii sincl	24.1	31.0	5.39
3.1	N 16 20 36.44/E 103 12 34.072	Under the *Mimosa pudica*	23.4	31.0	5.66
3.2	N 16 20 35.406/E 103 12 35.157	*Catunaregam spathulifolia*	22.4	31.4	5.46
3.3	N 16 20 35.405/ E 103 12 35.154	Near tree *Parinari anamensis Hance*	26.3	32.0	5.40
3.4	N 16 20 35.425/E 103 12 35.178	Under tree *Polyalthia debilis Finet & Gagnep*	24.2	33.8	5.26
3.5	N 16 20 36/E 103 12 36	*Russula virescens* area	23.5	32.5	5.39
3.6	N 16 20 36/E 103 12 36	Soil surface found near *Russula virescens*	30.2	31.3	5.25

In this study, the pH and electrical conductivity of soils were recorded. These factors are related to salinity of soil samples collected from Na Si Nuan forest, Kantarawichai district, Maha Sarakham province. (Table 1).

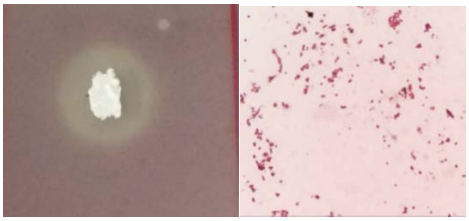
Table 1 Sources of soil samples and physical property analysis of soil.

Separate bacteria that chitinase enzyme

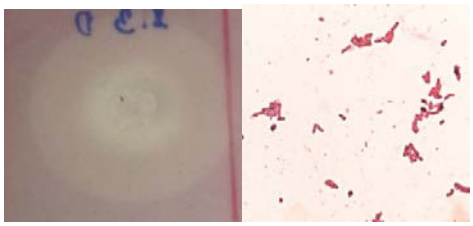
In this study, the pH and electrical conductivity of soils were recorded. These factors are related to salinity of soil samples collected from Na Si Nuan forest, Kantarawichai district, Maha sarakham province. The top 2 bacterial isolates were collected and isolated with highest lipase activity based on highest halo : colony ratios from the soil at locations 2.2 and 3.2 with a pH range 6.0

In this study, two chitinase-positive isolates, selected of pure isolates of chitinase-producing bacterial isolates (3.2CT1), showed the highest degrading zones on 3% chitin agar with different halo : colony ratios. Four bacterial representative strains showed similar colony morphologies and appeared to be gram-

negative and rod-shaped. The results showed that halo: colony ratios ranged from 4.5. (3.2 is the soil, the point 3.2, PS is chitinase, 1 is the first bacteria)



2.2 CT1 (G-, Cocci shaped)
Halo : Colony ratio = 2.74



3.2 CT1 (G-, rod shaped)
Halo : Colony ratio = 4.50

Figure 2 chitinase enzyme bacteria isolates

Chitinase production

The results of the bacterial dissolution of chitinase enzyme bacteria with the highest halo value ratio 3.2 CT1 had the highest bacteria chitinase enzyme activity. After induction bacteria in chitin medium containing colloidal chitin is substrate incubate at 37 ° C for 24, 48 and 72 h. Isolates 3.2 CT1 have enzyme specific activity at 0.32 ± 0.01 U/mg protein after incubation at 37 °C for 72 h (Fig. 3).

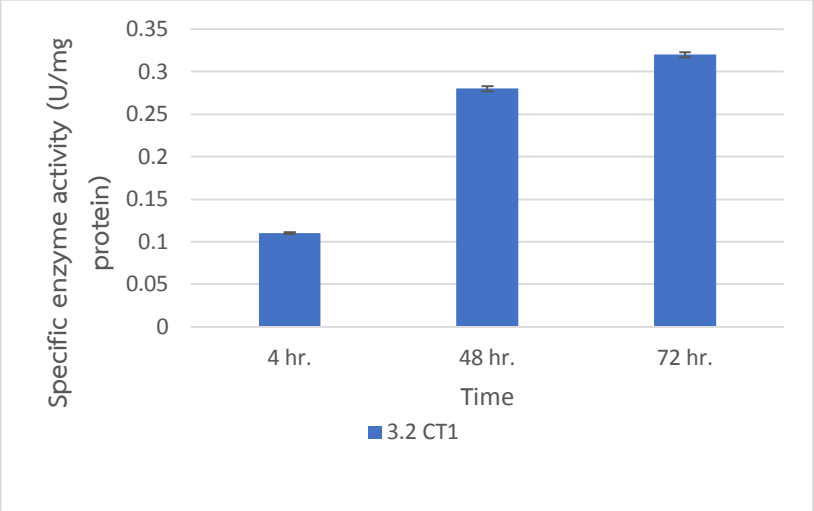


Figure 3 Specific bacteria activity of chitinase enzyme producing by 3.2 CT1

Chitinase optimization

In this study, the optimum conditions for chitinase enzyme were evaluated. Our studied found 3.2 CT1 gave the highest lipase activity at 0.32 ± 0.01 U /mL at 37 °C for 72 h after incubation with 3% colloidal chitin induction at 40 °C, pH 6.0 after curing for 30 min (Fig. 4), and highest enzyme activity with 0.70 ± 0.01 U/mL. It has been mentioned that colloidal chitin was used as substrate for screening of chitinase enzyme bacterial and has been referred as one of the best inductors

Optimum temperature

Substrate for chitinase enzyme our results show that isolate 3.2 CT1 its optimum at 40°C cultured in chitin medium, incubated for 72 h. pH 7, various temperatures from 30, 40, 50, 60, 70 and 80 °C for 30 min. (Fig. 4)

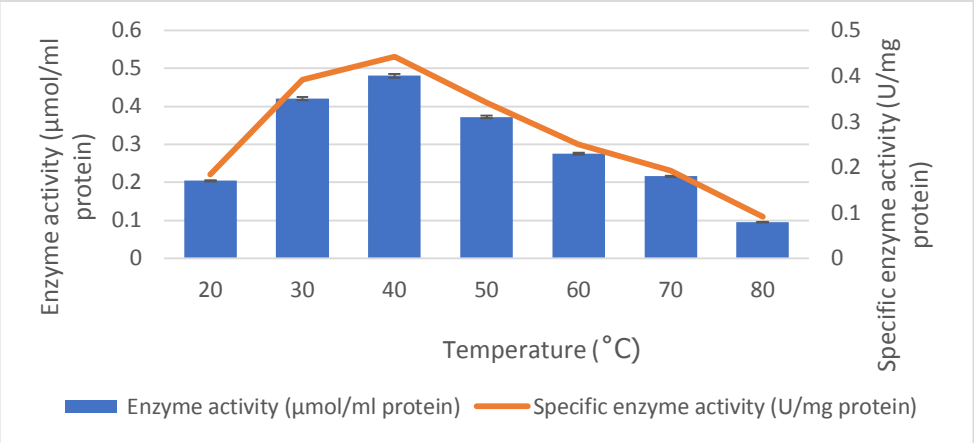


Figure 4 Optimum temperatures

pH

pH determination in various pH buffers were studied from pH 3, 4, 5, 6, 7, 8, 9 and 10, respectively. Phosphate buffer at various pH incubated at 40 °C for 30 min. It was found that pH 6 isolates 3.2 CT1 had the highest enzyme activity value of 0.70 ± 0.01 U/mL, while the enzyme specific activity value was 0.70 ± 0.01 U/mg protein (Fig. 5)

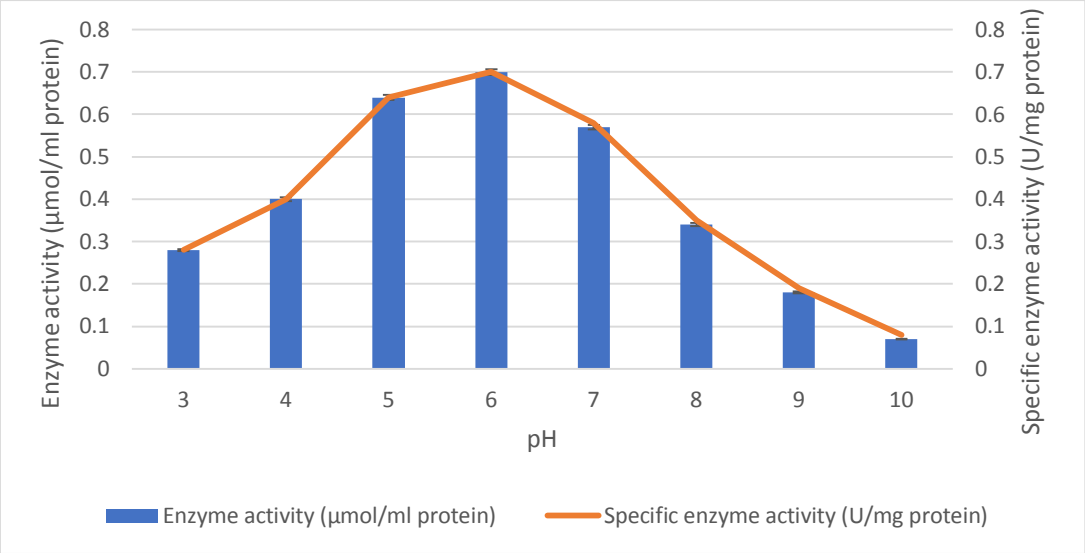


Figure 5 Optimum pH

Conclusions

The conservation forest area, which is a community forest in Na Si Nuan, Maha Sarakham, Thailand, has occupied 19.2 ha. The nature of the soil in this forest area is moderately saline and strongly saline was found in the basin area. In addition, many species of edible and inedible mushrooms were also found in this forest. Local people in the nearby area often come to use the bioresources as foods from this forest. Isolation of bacteria was performed from soil on chitin agar. The results found 2 isolates of bacteria that produce chitinase enzymes. The results showed that 2 isolates select with the highest halo: colony ration is 3.2 CT1 at 4.5 for test the morphology of bacteria is G-, and rod shaped. The optimum temperature for chitinase activity was 40 ˚C with enzyme activity of 0.40 ± 0.01 U/ml and specific enzyme activity was 0.49 ± 0.01 U/mg protein. The optimum pH was 6.0 with activity of 0.70 ± 0.01 U /ml.

Acknowledgement

This research was financially supported by the Plant Genetic Conservation Project under His Majesty the King's initiative Princess Maha Chakri Sirindhorn (Year 2018) awarded to Ketsara Suwunnapukdee.

References

[1] Abdel Naby MA, Mohamed A, Kwon DY. (1992). The production of xylanase and Beta-xylosidase by Aspergillus niger NRC 107. App Microbiol Biotechnol. 20:543–550.

[2] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215, 403–410.

[3] APHA-AWWA-WEF (1995) Standard Methods for the Examination of Water and Wastewater. 19th Edition, Washington DC.

[4] Arbhabhirama, A., D, Phantunvanit, J.Elklngton, Phaitoon Jngkasuwan. (1988). Thailand Natural Resources Profite. Oxford University Press. Oxford, New York, 431 pp.

[5] Arnoldus, H.M.J. (1977).Predicting Soil Loss Due to Sheet and Rill Erosion. Food and Agriculture Organization (FAO) Conservation Guide Volume I, p.88-98

[6] Chang, W. T., Chen, C. S., and Wang, S. L. (2003) An antifungal chitinase produced by Bacillus cereus with shrimp and crab shell powder as a carbon source, Curr Microbiol 47, 102-108.

[7] Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1998), "Protein measurement with theFolin phenol reagent," The Journal of Biological Chemistry, vol. 193, no. 1, pp. 265–275, 1951.

[8] Mendpara J., Parekh V., Vaghela S., Makasana A., Kunjadia P.D., Sanghvi G., Vaishnav D. and Dave G.S. (2013), Isolation and characterization of high salt tolerant bacteria from agricultural soil. European Journal of Experimental Biology, 3(6):351-358

[9] Norman, B.W. (1984), Report on the Comparison of New and Old Development Areas.Thai-Australia-World Bank Land Development Project, Chiang Mai, Thailand 339.

[10] Sakai Y, et al. (1994) High-level ATP production by a genetically-engineered Candida yeast. Biotechnology (N Y) 12(3):291-3

[11] Smith, R.F and R. van den Bosch. (1967), Integrated ontrol. Pest Control. Edited by W. Kilgoreand R. doutte. Academic Prees, 298-299 p.

[12] Tamura, K., Nei, M. (1993). Estimation of the numbr of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Bioloy and Evolution, 10, 512-526.

[13] Ueda, M. and Arai, M (1992) Purification and some properties of chitinase from Aeromonas sp. No. 10S-24. Biosci Biotechnol Biochem 56, 460–464.

[14] Ulhoa, C. J. and Peberdy, J. F. (1992) Purification and some proper ties of the extracellular chitinase produced by Trichoderma harzianum. Enzyme Microb. Technol., 14, 236-240.

[15] Vincy, V., Vinu, S.M., Viveka, S., Mary, V.T., Jasmin, B.R. (2014), Isolation and characterization of chitinase from bacteria of Shrimp pond. Euro. J. Exp.Bio, 4(3): 7882.

[16] Wang, R.F., Cao, W.W., and Cerniglia, C.E. (1996), PCR detection and quantitation of predominantanaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol, 62: 1242-124