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A selected β−mannanase producing bacilli capable of miang extract tolerant isolated from traditional fermented tea leaf from north thailand

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Abstract

A total 95 presumptive bacilli were isolated from Miang samples collected from north Thailand and all isolates were screened for β−mannanase activity on locust bean gum agar plate supplemented with 20% (v/v) Miang extract (ME). Fifty isolates showed the capability of growth on LBG agar supplemented 20% ME, but only 38 isolates showed the clear zone of mannan degrading activity surrounding the colony. The isolate K9.1 showed the highest β−mannanase activity at 14.5 U/mL after cultivated in LBG medium at 37°C for 24 h and was selected for further study. It was identified to be *Bacillus tequilensis* based on 16S rRNA gene sequence analysis. An extracellular β−mannanase from *B. tequilensis* K9.1 was partially purified 52.2-fold by ammonium sulphate precipitation and anion exchange chromatography. The molecular weight of the enzyme was suggested to be 35 kDa by SDS−PAGE and activity staining. The partial purified β−mannanase showed its maximum activity at 60°C and pH 7.0, and its thermal and pH stability were 30 to 55°C and 6.0 to 7.0, respectively. The enzyme activity was markedly activated by Co^{2+}, Mn^{2+} and Fe^{3+}. Moreover, the β−mannanase activity from *B. tequilensis* K9.1 showed the stability against Miang extract at 10 and 20% ME at 4°C for 24 h.

Key words: β−mannanase, Fermented tea, Miang, *Bacillus* strains

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1. Introduction

Miang is a traditional fermented food product of north Thailand made from Assam tea leaves (*Camellia sinensis* var. *assamica*) through a unique fermentation process *(*Kawakami *et al.,* 1987; Khanongnuch *et al.,* 2017). It has been part of dietary cultural make–up and socio–economy of Lanna people in north Thailand since several hundred years ago and is commonly served at all ceremonial celebrations (Kanpiengjai *et al.,* 2016). The fermentation processes are different depending on geographical region and the diverse ethnicity of Miang producers. However, overall process can be categorized into two major processes; (1) filamentous fungi process (FFP) or two-step fermentation process and (2) non–filamentous fungi (NFP) or single–step fermentation process (Khanongnuch *et al.,* 2017). Lactic acid bacteria and yeast are suggested to play as the key microbes in Miang fermentation process (Khanongnuch *et al.,* 2017; Okada *et al.,* 1986; Unban *et al.,* 2019). The recent research on microbial population changes during six months of Miang fermentation by the NFP process also confirmed that yeast and lactic acid bacteria are the main microbial population (Khatthongngam, 2019). Beside the lactobacilli and yeast, the endospore forming bacteria or *Bacillus* spp. from all collected Miang samples were found up to 40–45% of total bacterial counts, which is a significant high number (Unban *et al.,* 2019). Regarding the toxicity of tannin (Scalbert, 1991) and other polyphenolic compounds (Daglia, 2012) against various microbes, the growth and survival of those yeast, lactic acid bacteria and Bacilli is very attractive as they have to survive against the high tannin and polyphenolics environment in steamed tea leaves. In addition, the antimicrobial activities of Miang water–extracted solution against some pathogenic bacteria such as *Salmonella* sp., *Vibrio* sp. and also *Streptococcus mutans* was also recently reported (Unban *et al.,* 2019).

In general, carbon source is the most important requirement for microbial growth particular in yeast and common heterotrophic bacteria as lactic acid bacteria and *Bacillus* spp. (Eiler *et al.,* 2003). We have investigated for the enzyme activity of glycosyl hydrolase including cellulases (endoglucanase or CMCase), xylanases, β–mannanase, α–amylase and pectinase during Miang fermentation for 7 days and found that the β–mannanase was found in the highest up to 6 U/mL with the runner up of 1.5 U/mL of pectinase while cellulases and xylanases were found in the small quantity whereas no amylase activity was detected (unpublished data). β–Mannanase (1,4–β–D–mannan mannohydrolase, EC 3.2.1.78) is an endo–acting enzyme that catalyzes the random hydrolysis of the 1,4–β–D–mannosidic linkages in the main chain of β–1,4–mannans, glucomannans and galactomannans *(*Khanongnuch *et al.,* 1998). The enzyme transforms the abundant heteromannans to

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manno–oligosaccharides and a small amount of mannose, glucose and galactose (Chauhan *et al.,* 2012).

This enzyme is produced by a number of plants, bacteria, fungi, and has found a number of applications in different sectors including food, feed, pharmaceutical, and pulp/paper industries as well as pre–treatment of lignocellulosic biomass for the production of second generation biofuel (Dhawan and Kaur, 2007). There is a number of bacilli produced β–mannanase such as *Bacillus subtilis* 5H (Khanongnuch *et al.,* 1998) *B.subtilis* MR10 (Wongputtisin *et al.,* 2014), *B.amyloliquefaciens* (Cho, 2009; Mabrouk and El Ahwany, 2008), *B. pumilus* (M27) (Adiguzel *et al.,* 2015), and *B. licheniformis* (Ge *et al.,* 2016; Zhang *et al.,* 2000) while rare of yeast produced the extracellular β–mannanase and none of β–mannanase activity has been reported from lactic acid bacteria. It would be interesting to explore the mannan degrading enzyme produced from the tannin tolerant Bacilli and also investigate for the property of mannanolytic enzyme.

This work describes on the screening of *Bacillus* spp. isolated from Miang samples with high capability in β–mannanase production. The strain identification and characterization of the partial purified β–mannanase from the selected strain are also described.

2. Materials and Methods

2.1 Sample collection and bacterial isolation

Miang samples were collected from different locations in Upper-Northern Thailand including Chiang Mai, Chiang Rai, Nan and Phare province. Ten grams of each sample was mixed with 90 mL of sterile 0.85% (w/v) NaCl solution by using stomacher for 1 min. One milliliter of each mixed sample was transferred to 9 mL of sterile 0.85% (w/v) NaCl solution. The diluted solutions were incubated in water bath at 80ºC for 12 min, and then these solutions were spread on nutrient agar (NA) medium, incubated at 37ºC for 18 h. The single bacterial colony formed were assumed to be *Bacillus* sp. (Santana *et al.,* 2008). The different morphological colonies were randomly picked up and kept on NA slant for further studies.

2.2 Screening of β–mannanase producing strain capable of Miang extract tolerance

To prepare Miang extract (ME), 100 g of fresh young tea leaves were washed and steamed for 1 h. The steamed tea leaves were mixed with 300 mL of distilled water and then filtered by cotton sheet to discard tea leaves residue. The filtrate was centrifuged at 6,000 rpm for 20 min, concentrated using rotary evaporator at 40°C for 20 min to obtain the final volume of 200 mL. A single colonies of *Bacillus* spp. were transferred to NA containing 0.5% (w/v) locust bean gum (LBG) and 20% ME solution by replica plating technique and incubated at 37ºC for 24 h. The hydrolyzing zones on LBG agar were visualized by flooding with 0.5% (w/v)

iodine solution (Rattanasuk and Ketudat Cairns, 2009). The isolates produced clear zones on LBG agar were assumed to be the mannan hydrolyzing enzyme producing strains and were selected for further experiments. The clear zone surrounded colonies were selected to further cultivation in nutrient broth (NB) containing 0.5% (w/v) LBG on 180 rpm rotary shaker at 37°C for 24 h and the cell–free culture supernatants were separated and determined for β–mannanase activity. The strain of *Bacillus* sp. produced the highest activity of β–mannanase was selected as the β–mannanase producer.

2.3 Identification of microorganism and phylogenetic analysis

Cell morphology of the isolate was determined using a microscope. Gram staining was investigated as described by Morlon–Guyot *et al.* (1998). Spore formation and colony formed pattern were observed by spreading bacterium cell on LBG agar and cultivated at 37ºC for 24 h. Genomic DNA was extracted as described by method of Sambrook and Russell (2001). Amplification of the 16S rRNA gene was performed by polymerase chain reaction (PCR) using two universal primers; 27F (5'–AGAGTTTGATCCTGGCTCAG–3') and 1525R (5'–AAGGAGGTGWTCCARCC–3'). The PCR products were purified by GF–1 PCR clean-up gel extraction Kits (Vivantis, Malaysia) and visualized by electrophoresis on 1% (w/v) agarose gels. The 16S rDNA sequence was analyzed and determined using the BLAST algorithm of GenBank (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.,* 1997). Multiple alignments were performed using BioEdit 7.0 and the phylogenetic tree was created based on the neighbor–joining method by MEGA version 4.0 software (Tamura *et al.,* 2007).

2.4 Effect of Miang extract on growth of *B. tequilensis* K9.1

The selected strain of *B.tequilensis* K9.1 was transferred to sterile basal medium containing gram per liter of 5.0 LBG, 0.5 yeast extract, 1.0 K_2HPO_4, 1.0 $NaNO_3$, 1.0 KCl and 0.5 $MgSO_4$ supplemented with 10 and 20% (v/v) ME adjusted to initial pH values of 7.0 and incubated with shaking at 180 rpm and 37^{o}C for 48 h. The culture broth was sampled at 6 h intervals for the determination of a viable cell count on NA medium. The pH was measured using a pH meter and β–mannanase activity was assayed as described below.

2.5 Purification of β-mannanase from *B. tequilensis* K9.1

Crude enzyme was precipitated by 80% ammonium sulfate. The ammonium sulfate was gradually added into crude enzyme solution and stirred at 4°C until the completely dissolve. The precipitated proteins were harvested by centrifugation at 10,000 rpm, 4°C for 20 min and dissolved with 20 mM sodium phosphate buffer pH 7.0. This solution was then dialyzed against the same buffer at 4°C for at least 12 h. The equilibrium solution was applied into HiTrap DEAE–Sepharose FF 5 mL column (GE Healthcare, UK) equilibrated with 20 mM sodium phosphate buffer pH 7.0. The enzyme was eluted with 20 mM sodium phosphate

buffer pH 7.0 with NaCl gradient elution (0 to 1.0 M) operated with AKTA purification system (GE Healthcare, UK). Protein from each fraction was monitored by absorbance at 280 nm and the β−mannanase activity was determined. The active fraction was evaluated for their purity by SDS−PAGE. The purified enzyme was stored at −20°C for further characterization.

2.6 Enzyme assay and protein determination

β−Mannanase activity was determined by measuring the amount of reducing sugars released by using the dinitrosalicylic acid (DNS) method (Miller, 1959). Briefly, the reaction mixture containing 0.125 mL of the desired dilution of enzyme and 0.125 mL of 0.5% (w/v) LBG in 0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 10 min. The reaction was stopped by addition of 0.25 mL of DNS (Sigma−Aldrich, St. Louis, MO, USA), then boiled for 10 min, and 5 mL of distilled water was added. The absorbance was measured at 540 nm. One unit of β−mannanase activity was defined as the amount of enzyme that liberated 1 μmole of reducing sugar per minute under the assay condition.

Protein concentration was determined by the dye binding method (Bradford, 1976) using bovine serum albumin (BSA) as standard protein. The protein concentration was determined by mixing 0.015 mL of sample with 0.6 mL of Bradford solution, and then incubated for 10 min at room temperature. Afterwards, the sample absorbance was measured at 595 nm.

2.7 Characterization of the partially purified β−mannanase

2.7.1 Optimum pH and stability

The optimum pH of the partially purified β−mannanase activity was examined at pH values ranging from 4.0 to 9.0. A locust bean gum solution (0.5%, w/v) in the appropriate buffers, each at 50 mM citrate phosphate (pH 4.0−5.0), sodium phosphate (pH 6.0−7.0), and Tris−HCl (pH 8.0−9.0) was used to determine β−mannanase activity. The residual enzyme activity was determined as described above conditions and was calculated compared to that of the highest activity. Enzyme stability was determined using the same buffer systems in the range of 4.0 to 9.0 by incubating partially purified β−mannanase in the various buffer solutions at 4°C for 24 h. Then, the remaining enzyme activity was measured using locust bean gum as substrate under standard assay conditions.

2.7.2 Optimum temperature and stability

The effect of temperature on β−mannanase activity was determined by performing the standard activity assay at temperatures ranging from 30 to 90°C. The relative activity was calculated compared to that of the highest activity. Determination of thermal stability was investigated by incubation the partially purified β−mannanase at various temperatures for 1 h.

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Thereafter, the remaining enzyme activity was measured using the standard assay conditions previously and presented as the relative activity.

2.7.3 Effect of metal ion

The effects of EDTA well as various metal ions (K^{+}, Na^{+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+}), each at a concentration of 1 mM, on partially purified β−mannanase activity were determined under standard assay conditions. The remaining activity was calculated compared to that without the presence of metal ion and chemicals.

2.8 Effect of Miang extract on partially purified β−mannanase

The partially purified β−mannanase from *B. subtilis* MR10 (Wongputtisin *et al.,* 2014) and *B.subtilis* TISTR 1460 were prepared as same as the method in purification of β−mannanase from *B.tequilensis* K9.1. The effect of ME on the β−mannanase activity was determined by incubating the partially purified enzymes in sodium phosphate buffer (pH 7.0) supplemented with 10 and 20% (v/v) of ME at 4°C for 24 h. The residual β−mannanase activity was determined under standard assay conditions.

3. Results and Discussion

3.1 Isolation, screening and identification of β−mannanase producing *Bacillus* strain

A total 95 isolates of *Bacillus* sp. were isolated from 25 Miang samples. Plate screening on LBG agar supplemented with 20% ME solution revealed that 50 isolates showed the capability of growth on LBG agar supplemented 20% ME solution, but only 38 isolates gave the clear zone surrounding colony. These selected 38 isolates were cultured in the liquid LBG medium for the confirmation of β−mannanase producing strain. Among 38 β−mannanase producing isolates, the isolate K9.1 produced the highest enzyme activity at 14.5 U/mL after cultivated in LBG medium at 37°C for 24 h (Fig 1). Therefore, the *Bacillus* sp. K9.1 was selected for further studies as β−mannanase producer.

The single colony of the isolate K9.1 on LBG agar medium is creamy, slightly convex shape with irregular edges after incubated at 37°C for 24 h (Fig 2a). Molecular taxonomic identification was carried out by 16S rRNA gene sequence analysis. The 16S rDNA fragment was amplified by PCR using chromosomal DNA of the isolate K9.1 as a template and the nucleotide sequence of 16S rRNA gene was compared for similarities against the bacterial 16S rDNA sequence database in GenBank. The 16S rRNA gene sequence was deposited in GenBank under accession number MH889121. It was clearly demonstrated that the isolate K9.1 showed the high similarity of 99% to *Bacillus tequilensis.* The phylogenetic tree showing the relationships between the isolate K9.1 to other bacterial strains were presented in Fig 2c.

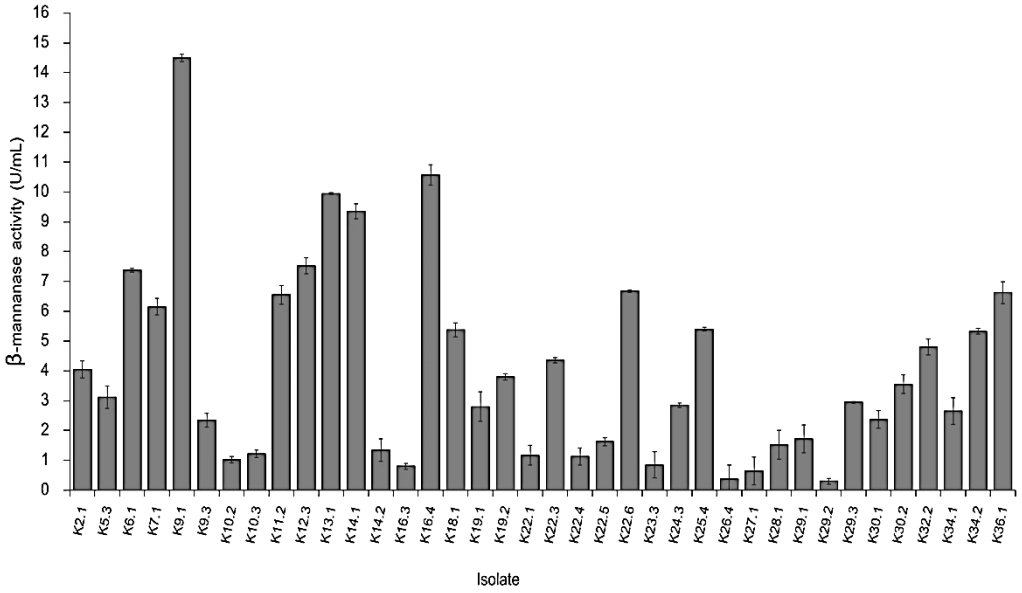

Fig 1 β−Mannanase activity of 50 isolates incubated in LBG medium at 37ºC for 24 h

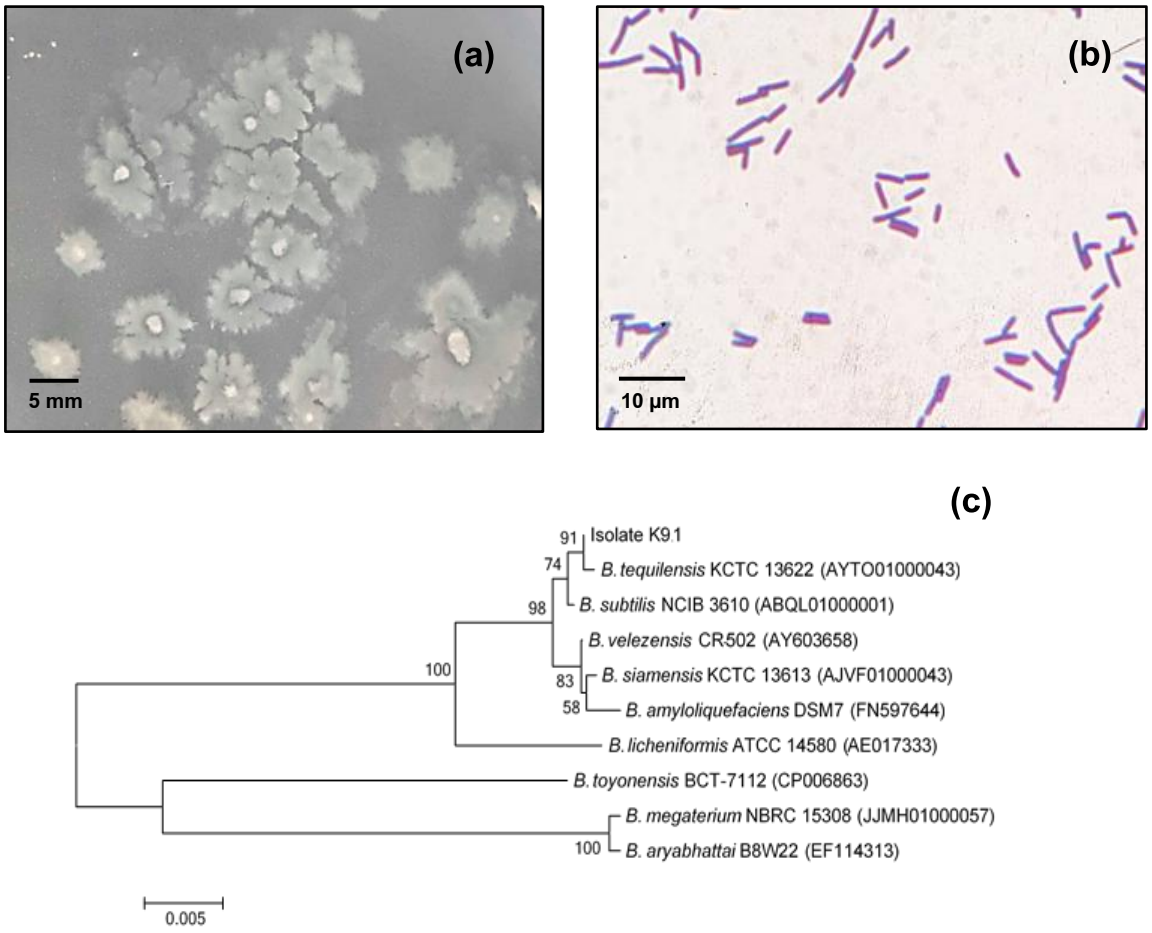

Fig 2 Colony formation (a), Gram staining (b) and phylogenetic tree of isolate K9.1 and its related species based their 16s rRNA genes (c)

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3.2 Effect of Miang extract on β-mannanase production by *Bacillus tequilensis* K9.1

The effect of ME on β-mannanase production was carried out using LBG supplemented with 10% and 20% (v/v) of ME and the result were presented in Fig 3. The maximum β-mannanase activity was around 15.6 U/mL at 30 h found from LBG medium and decreased to around 13.7 U/mL at 30 h when cultivated in LBG supplemented with 10% ME, whereas, the activity from 20% ME was around 2.0 U/mL and stable until the end of fermentation. The viable cell number of *B.tequilensis* K9.1 in LBG medium and LBG with 10% ME was approximately 10^9 cfu/mL at 6 h and be stable until 48 h, while, viable cell of culture with 20% ME was decreased from 10^7 at 6 h to approximately 10^6 cfu/mL at 48 h. The result showed that *B.tequilensis* K9.1 is able to grow in LBG supplemented with 10% ME, whereas, 20% ME had inhibition effect on cell growth. Interestingly, β-mannanase activity from *B.tequilensis* K9.1seem to be tolerate with 20% ME while growth of organism was influenced by ME. Therefore, the β-mannanase were purified and characterized to elucidate the enzyme property.

3.3 Characterization of the partially purified β-mannanase

Crude extracellular β-mannanase produced by *B.tequilensis* K9.1 was separated from culture broth by centrifugation at 8,000 rpm at 4°C. Then, the enzyme solution was purified by ammonium sulfate precipitation followed by HiTrap DEAE-Sepharose FF anion exchange column. Partially purified enzyme exhibited specific activity of 466 U/mg protein which corresponds to 52.2 purification folds and 25.3% yield (Table 1). The results of SDS-PAGE (Fig 4) indicated that the partially purified enzyme showed protein band of around 35 kDa. Low molecular weight β-mannanase has also been found to be present in *B.subtilis* KU-1 (39 kDa) (Zakaria *et al.,* 1998), *B.subtilis* SA-22 (38 kDa) (Yu *et al.,* 2003). *B.subtilis* B36 (38 kDa) (Li *et al.,* 2006) and *B.subtilis* BCC 41051 (38 kDa) (Summpunn *et al.,* 2011).

Table 1 Partial purification steps of β-mannanase from B.tequilensis K9.1

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery yield (%)	Purification (fold)
Crude enzyme	12,120	1,360.0	8.9	100.0	1.0
80% Ammonium sulfate	3,348	38.6	86.7	27.6	9.7
HiTrap DEAE-Sepharose FF	3,075	6.6	466.0	25.3	52.2

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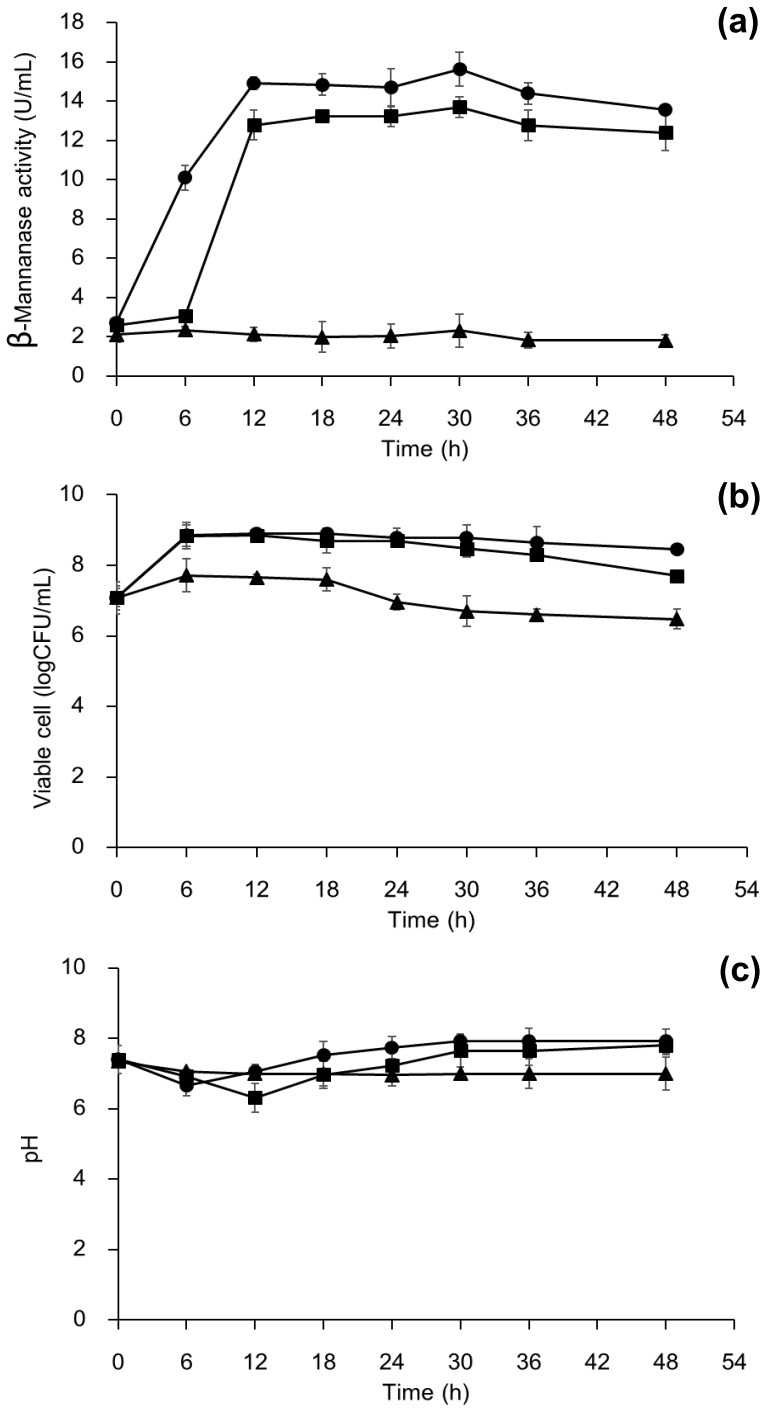

Fig 3 Profile of β−mannanase activity (a), viable cell (b) and pH (c) by *B. tequilensis* K9.1 in LBG medium (●) and LBG medium supplemented with 10% (■) and 20% (v/v) (▲) of Miang extract at 37°C for 48 h

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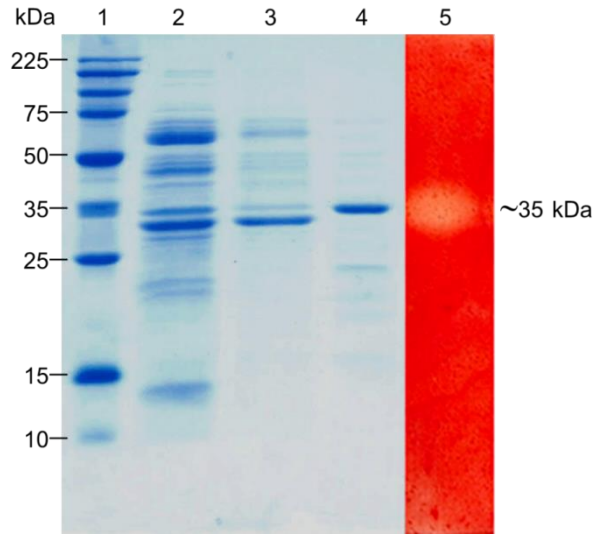

Fig 4 SDS–PAGE and zymogram of purified β–mannanase from *B.tequilensis* K9.1. Lane 1, molecular mass markers; Lane 2, SDS–PAGE of crude enzyme; Lane 3, SDS–PAGE of salt precipitation step; Lane 4, SDS–PAGE of ion exchange step; Lane 5, zymogram of purified enzyme

3.4 Effect of temperature on partially purified β–mannanase activity and stability

The temperature dependence of the β–mannanase activity was studied at pH 7.0 in the rage of 30–90°C and the result is shown in Fig 5a. The optimum temperature for the partially purified β–mannanase was found to be 60°C. Corresponding to optimum temperature, the enzyme was stable at 30–55°C by retaining above 80% of original activity. Beyond 60°C, the relative activity declined rapidly and retained on only 10% of its activity at 80°C and was completely inactivated at 90°C. The properties of partially purified β–mannanase from *B.tequilensis* K9.1was interesting with high temperature stability.

3.5 Effect of pH on partially purified β–mannanase activity and stability

The effect of pH rage of 3.0 to 9.0 on the activity of enzyme was studied. As shown in Fig 5b, the optimum pH for the partially purified β–mannanase was around 6.5 and the enzyme activity was retained at more than 80% of residual activity at the pH rage of 6.0 to 7.0 at 4°C for 24 h. The results were similar to those of the β–mannanase from *B.subtilis* KU–1 (Zakaria *et al.,* 1998), *B.subtilis* SA–22 (Yu *et al.,* 2003).

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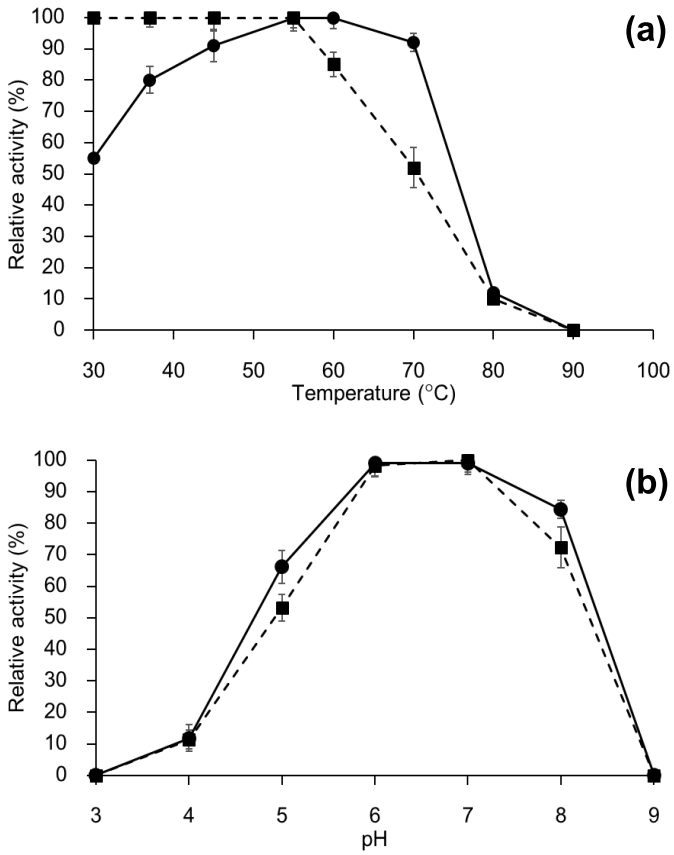

Fig 5 The effect of temperature (a) and pH (b) on partially purified β−mannanase activity (●) and stability (■)

3.6 Effect of metal ion

The effect of metal ions on the partially purified β−mannanase was investigated with various metal ions at a final concentration of 1 mM, and the results were presented in Fig 6. It was found that the enzyme was highly activated by Co^{2+}, Mn^{2+} and Fe^{3+} in the range of 120−160%. Additionally, others metal ions slightly enhanced enzyme activity and no inactivation by any metal ions was observed at the concentration of 1 mM. It is quite interesting as all of metal ions investigated in this experiment show the activation effect on β−mannanase activity while EDTA is not influence, this means the metal ions does not participate in the structure of enzyme protein.

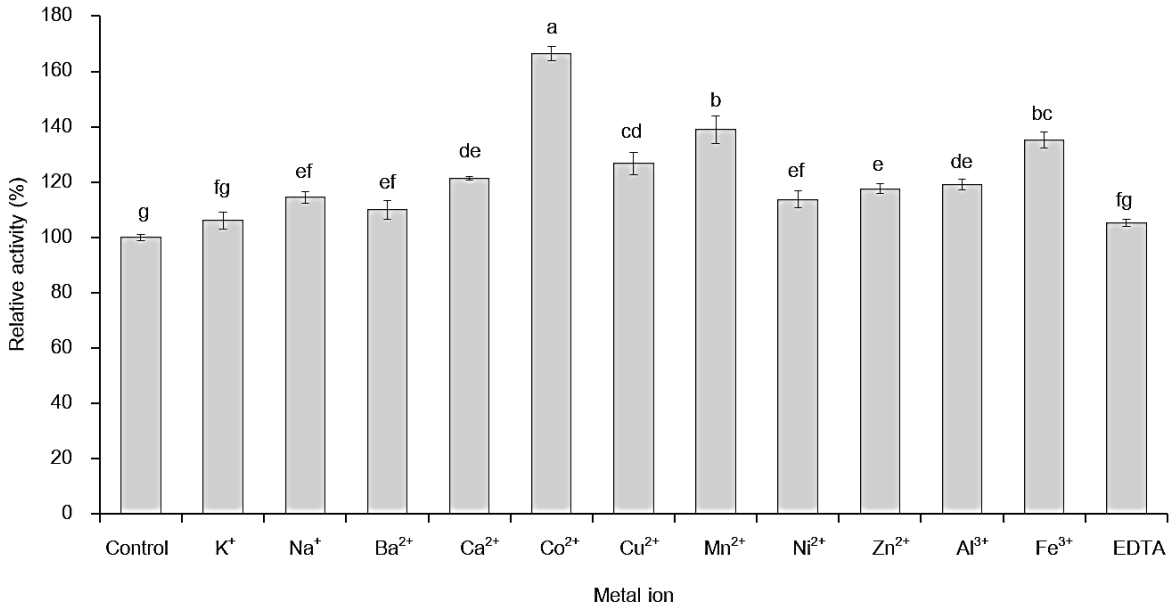

Fig 6 The effect of metal ion on partially purified β−mannanase activity

3.7 Effect of Miang extract on β−mannanase activity

The stability against Miang extract of partially purified β−mannanase from *B.tequilensis* K9.1 was compared with the partially purified β−mannanase from *B.subtilis* MR10 and *B.subtilis* TISTR 1460, and the results were presented in Fig 7. Residual activity was measured versus incubation time in different ME concentration at 0, 10 and 20%. The result shows that the β−mannanase activities from *B.subtilis* MR10 and *B.subtilis* TISTR 1460 were declined when the incubation was prolonged, while the enzyme from strain K9.1 remained stable during incubation in the presence of 10 or 20% ME at 4°C for 24 h. The retained activity against 20% ME for 24 h of K9.1, MR10 and TISTR 1460 were 92.8, 60.5 and 41.2%, respectively. This confirms that the β−mannanase from *B.tequilensis* K9.1 isolated from Miang had ability to tolerate tannin and other phenolic compounds presented in ME. Even the general properties of the β−mannanase from *B.tequilensis* K9.1 are similar to those of other *Bacillus* spp., but it shows the difference in stability against ME which may cause from the difference in the primary structure of β−mannanase. This finding could explain why the β−mannanase activity is main activity of glycosidase found during the fermentation of Miang reported by Khatthongngam (2019).

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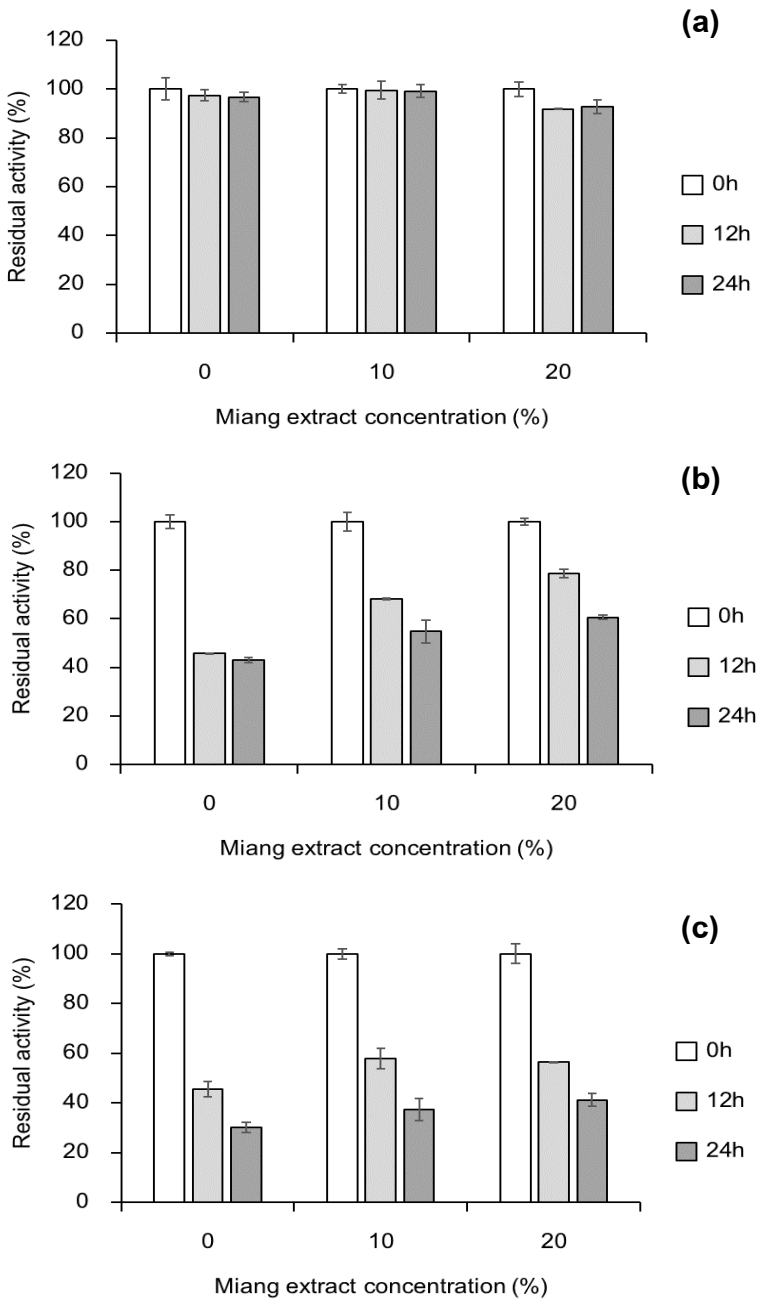

Fig 7 The stability of partially purified β−mannanase from *B.tequilensis* K9.1 (a), *B.subtilis* MR10 (b) and *B.subtilis* TISTR 1460 (c) against 0−20% (v/v) of Miang extract at 4°C for 24 h

4. Conclusions

According to the findings, it can be concluded that the partially purified β−mannanase from *B.tequilensis* K9.1 was resisted to metal ions, stable in high temperature, and was tolerated to Miang extract which is a special characteristic of microorganism isolated from high tannin containing resource.

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References

Adiguzel, A., Nadaroglu, H. and Adiguzel, G. 2015. Purification and characterization of beta-mannanase from *Bacillus pumilus* (M27) and its applications in some fruit juices. Journal of Food Science and Technology. 52: 5292-5298.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 25: 3389-3402.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72: 248-254.

Chauhan, P.S., Puri, N., Sharma, P. and Gupta, N. 2012. Mannanases: microbial sources, production, properties and potential biotechnological applications. Applied Microbiology and Biotechnology. 93: 1817-1830.

Cho, S.-J. 2009. Isolation and characterization of mannanase producing *Bacillus amyloliquefaciens* CS47 from horse feces. Journal of Life Science. 19: 1724-1730.

Daglia, M. 2012. Polyphenols as antimicrobial agents. Current Opinion in Biotechnology. 23: 174-181.

Dhawan, S. and Kaur, J. 2007. Microbial mannanases: an overview of production and applications. Critical Reviews in Biotechnology. 27: 197-216.

Eiler, A., Langenheder, S., Bertilsson, S. and Tranvik, L.J. 2003. Heterotrophic bacterial growth efficiency and community structure at different natural organic carbon concentrations. Applied and Environmental Microbiology. 69: 3701-3709.

Ge, Y., Li, K., Li, L., Gao, C., Zhang, L., Ma, C. and Xu, P. 2016. Contracted but effective: production of enantiopure 2, 3-butanediol by thermophilic and GRAS *Bacillus licheniformis*. Green Chemistry. 18: 4693-4703.

Kanpiengjai, A., Chui-Chai, N., Chaikaew, S. and Khanongnuch, C. 2016. Distribution of tannin-'tolerant yeasts isolated from Miang, a traditional fermented tea leaf (*Camellia sinensis* var. *assamica*) in northern Thailand. International Journal of Food Microbiology. 238: 121-131.

Kawakami, M., Chairote, G. and Kobayashi, A. 1987. Flavor constituents of pickled tea, miang, in Thailand. Agricultural and Biological Chemistry. 51: 1683–1687.

Khanongnuch, C., Asada, K., Tsuruga, H., Ooi, T., Kinoshita, S. and Lumyong, S. 1998. β-Mannanase and xylanase of *Bacillus subtilis* 5H active for bleaching of crude pulp. Journal of Fermentation and Bioengineering. 86: 461–466.

Khanongnuch, C., Unban, K., Kanpiengjai, A. and Saenjum, C. 2017. Recent research advances and ethno–botanical history of miang, a traditional fermented tea (*Camellia sinensis* var. *assamica*) of Northern Thailand. Journal of Ethnic Foods. 4: 135–144.

Khatthongngam, N. (2019). Changes of microbial population and bioactive compounds during Miang fermentation process, Chiang Mai University.

Li, Y.N., Meng, K., Wang, Y.R. and Yao, B. 2006. A β–mannanase from Bacillus subtilis B36: purification, properties, sequencing, gene cloning and expression in *Escherichia coli*. Zeitschrift für Naturforschung C. 61: 840–846.

Mabrouk, M.E. and El Ahwany, A.M. 2008. Production of 946-mannanase by *Bacillus amylolequifaciens* 10A1 cultured on potato peels. African journal of biotechnology.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31: 426–428.

Morlon-Guyot, J., Guyot, J., Pot, B., De Haut, I.J. and Raimbault, M. 1998. *Lactobacillus manihotivorans* sp. nov., a new starch-hydrolysing lactic acid bacterium isolated during cassava sour starch fermentation. International Journal of Systematic Bacteriology. 48: 1101–1109.

Okada, S., Daengsubha, W., Uchimura, T., Ohara, N. and Kozaki, M. 1986. Flora of lactic acid bacteria in miang produced in northern Thailand. The Journal of General and Applied Microbiology. 32: 57–65.

Rattanasuk, S. and Ketudat Cairns, M. 2009. Chryseobacterium indologenes, novel mannanase-producing bacteria. Songklanakarin Journal of Science & Technology.

Sambrook, J. and Russell, D.W. (2001) Molecular clonging: a laboratory mannual. 3rd/Ed. Cold Spring Harbor Press, New York

Santana, M.A., Moccia–V, C.C. and Gillis, A. 2008. *Bacillus thuringiensis* improved isolation methodology from soil samples. Journal of Microbiological Methods. 75: 357–358.

Scalbert, A. 1991. Antimicrobial properties of tannins. Phytochemistry. 30: 3875–3883.

Summpunn, P., Chaijan, S., Isarangkul, D., Wiyakrutta, S. and Meevootisom, V. 2011. Characterization, gene cloning, and heterologous expression of β–mannanase from a thermophilic *Bacillus subtilis*. The Journal of Microbiology. 49: 86–93.

Unban, K., Khatthongngam, N., Shetty, K. and Khanongnuch, C. 2019. Nutritional biotransformation in traditional fermented tea (Miang) from north Thailand and its impact on antioxidant and antimicrobial activities. Journal of Food Science and Technology. 56: 2687–2699.

Wongputtisin, P., Khanongnuch, C., Kongbuntad, W., Niamsup, P., Lumyong, S. and Sarkar, P. 2014. Use of *Bacillus subtilis* isolates from Tua–nao towards nutritional improvement of soya bean hull for monogastric feed application. Letters in Applied Microbiology. 59: 328–333.

Yu, Y.H., Sun, M.Y., Wang, J.W., Yang, Y.S. and Yang, H.Y. 2003. Purification and properties of *Bacillus subtilis* SA22 Endo–1, 4–β–D mannanase. Chinese Journal of Biotechnology. 19: 327–331.

Zakaria, M., Yamamoto, S. and Yagi, T. 1998. Purification and characterization of an endo–1, 4–β–mannanase from *Bacillus subtilis* KU–1. FEMS Microbiology Letters. 158: 25–31.

Zhang, J., He, Z. and Hu, K. 2000. Purification and characterization of β–mannanase from *Bacillus licheniformis* for industrial use. Biotechnology Letters. 22: 1375–1378.