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A comparative analysis of β-mannanases of bacteria from Antarctica and Malaysia

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Abstract β-mannanase is an enzyme that is commonly expressed in environmental bacteria. It degrades hemicellulose found in plant material and recycles nutrients back into the environment. Because this enzyme significantly contributes to biodegradation and has recently been applied in industry, we conducted a comparative analysis of bacterial isolates found in soil samples from Schirmacher Oasis, Antarctica, and Sabah, Malaysia that were capable of degrading mannan. A total of 9 bacterial isolates from Antarctica and 30 bacterial isolates from Malaysia exhibited β-mannanase activity. These bacteria were differentiated and clustered using their random amplified polymorphic DNA (RAPD) profiles, and the β-mannanase activity of these isolates was tested at different temperatures and pH. Five out of 9 Antarctica isolates and seven out of 30 Malaysian isolates were identified based on their 16S rDNA sequences. Identified bacterial isolates from Antarctica were: MP1 (*Bacillus amyloliquefaciens*), MP2 (*Bacillus pumilus*), MP5 (*Bacillus pumilus*), A40 (*Arthrobacter* sp.), and C27 (*Arthrobacter oxydans*). Identified bacterial isolates from Malaysia were: Y1 (*Paenibacillus* sp.), Y2 (*Bacillus* sp.), Y16 (*Paenibacillus* sp.), Y18 (*Paenibacillus* sp.), A7 (*Paenibacillus* sp.), B26 (*Streptomyces* sp.), and D4 (*Paenibacillus amylolyticus*). β-mannanases produced by the Antarctica bacterial isolates MP1 (*Bacillus amyloliquefaciens*) and A40 (*Arthrobacter* sp.) were active at 5℃ and 20℃, respectively, while the β-mannanase produced by the bacterial isolate from Malaysia, A7 (*Paenibacillus* sp.), was active at 35℃.

Keywords **Antarctic regions, guar gum, locust bean gum, hemicellulose and hemicellulase**

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1 Introduction

Hemicellulose is the second most abundant polysaccharide found in nature and mannan is one of its primary constituents[1]. Mannan acts as a cell wall structural component in algae and as a storage carbohydrate in bulbs and plant endosperms. It also provides mechanical resistance in seeds and beans[2]. In addition, mannan is a major component of the hemicellulose fraction in soft woods[3]. It can be degraded to mannose by β-mannanases, and these enzymes are usually produced by plants, bacteria, and fungi[4].

Hemicellulases have garnered increased research interest because of their possible industrial applications. For instance, β-mannanases are used in several industrial processes including extracting vegetable oil from leguminous seeds, increasing yield and processing performance during extraction and clarification of fruit and vegetable juice, and reducing viscosity of coffee extracts during the manufacturing of instant coffee[3,5-6]. In the pulp and paper industry, β-mannanases and xylanases act synergistically as biological pre-bleaching agents for softwood kraft pulp, allowing a significant reduction of environmental pollutants when compared with the use of chlorine-based chemical agents[7]. Recently, the introduction of β-mannanases into detergents has improved the removal of various food stains containing guar gum, a commonly used stabilizer and thickening agent in food products[8]. In poultry and animal agriculture, β-mannanases have been added to animal feed containing mannan to improve dietary nutrient utilization. It has been reported that β-mannanase improves energy metabolism, growth, and feed conversion[9].

β-mannanase has been found in environmental bacteria isolated from various regions worldwide[2,10-11]. However,

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there are few reports of β-mannanase-producing bacteria from Schirmacher Oasis, East Antarctica, which is extremely cold and dry, and from Sabah, Malaysia, which has a tropical climate. This study was performed to analyze and compare β-mannanases from psychrophiles and mesophiles from these two contrasting environments. The objectives of this work were to: (i) Determine the general population of β-mannanase-producing bacteria in Antarctica and tropical soils; and (ii) determine β-mannanase enzyme stability at different incubation pH and temperatures.

2 Materials and Methods

2.1 Isolation of Bacteria

Soil samples were aseptically collected from Schirmacher Oasis, Antarctica (70°45′42.11″S, 11°43′33.08″E) and Sabah, Malaysia (6°01′52.94″N, 116°07′15.57″E) using sterilized spatulas and tubes. The samples were stored at −20℃ until used. Luria-Bertani (LB) agar (Sigma), nutrient agar, *Streptomycetes* agar, and media described previously by Abe et al. were used for bacterial isolation[12]. The *Streptomycetes* agar contained: 0.5% glycerol, 0.2% NaCl, 0.1% KNO_3, 0.053 4% Na_2HPO_4, 0.05% $MgSO_4$, 0.027 2% KH_2SO_4, and 1 mL of trace elements solution (0.1% $FeSO_4$, 0.1% $MnCl_2$, and 0.1% $ZnSO_4$), with 2% BactoTMAgar (BD Difco, USA). The isolation medium described by Abe et al.[12] was modified slightly: 0.15% glucose, 0.1% yeast extract, 0.1% polypepton, 0.1% NH_4NO_3, 0.14% KH_2PO_4, and 0.02% $MgCl_2$. Approximately 1 g of each soil sample was inoculated in 10 ml sterile saline solution, serially diluted, and plated on the agar media. The agar plates were incubated at 4℃, 20℃, 24℃, and 37℃ for 1 to 3 d. Bacterial colony-forming units (CFU) were counted and purified with multiples rounds of re-streaking onto fresh agar media.

2.2 β-mannanases screening

Single CFU from the agar plates were transferred separately onto LB and *Streptomyces* agar media containing either 0.15% Remazol brilliant blue (RBB)-guar gum, RBB-locust bean gum, or screening medium described by Abe et al.[12] with minor modifications: 0.15% dye-labeled mannan, 0.1% yeast extract, 0.1% polypepton, 0.1%$NH4NO_3$, 0.14% KH_2PO_4, and 0.02% $MgCl_2$. The dye-labeled mannan (RBB-guar gum and RBB-locust bean gum) was prepared according to Fulop and Ponyi[13]. Screening was carried out at 4℃, 20℃, 24℃, and 37℃.

2.3 Randomly amplified polymorphic DNA polymerase chain reaction

All isolated bacteria were differentiated or clustered by random amplified polymorphic DNA (RAPD) analyses. Bacterial chromosomal DNA was extracted using the boiling lysis method[14]. Nine different primers were used for RAPD analysis (Table 1). Polymerase chain reaction (PCR) reactions were performed in 20 μL and they contained 2.5 mM $MgCl_2$, 250 μM dNTPs, 200 pmol each primer, 2.5 U *Taq* polymerase (Promega, USA), 0.5 X PCR amplification buffer, and 10 μL template DNA. PCR conditions were as follows: initial denaturation at 96℃ for 5 min, followed by 15 cycles of denaturation at 96℃ for 30 s, annealing at 36℃ for 45 s, and extension at 72℃ for 45 s, followed by 20 cycles of denaturation at 96℃ for 30 s, annealing at 45℃ for 45 s, extension at 72℃ for 45 s, and a final extension for 10 min at 72℃.

Table 1 Primer used in the RAPD analyses

RAPD Primers	Sequences
Pri1	5’-tgtacgtgac-3’
Pri3	5’-gacgagacgg-3’
Pri4	5’-tccactcctg-3’
Pri5	5’-cagcatggtc-3’
Pri6	5’-agaagcgatg-3’
Pri10	5’-gtgatcgcag-3’
Pri11	5’-caatcgccgt-3’
Pri13	5’-cagcacccac-3’
Pri17	5’-gaccgcttgt-3’

2.4 16S rDNA gene sequencing and phylogenetic analysis

Chromosomal DNA extraction was performed as described previously by Vaquero et al.[15]. PCR amplification of 16S rDNA was carried out using primers BSF8/20 5’-AGAGTTTGATCCTGGCTCAG-3’ and BSR1541/20 5’-AAGGAGGTGATCCAGCCGCA-3’. PCR reactions for 16S rDNA amplification was carried out in 20 μL containing 1 μL DNA sample, 300 μM dNTPs, 1.5 mM $MgCl_2$, 50 pmol forward primer, 50 pmol reverse primer, and 2 U *Taq* polymerase (Promega,USA) in 1X PCR buffer. The PCR conditions were: pre-denaturation for 5 min at 95℃, followed by 30 cycles of denaturation at 95℃ for 30 s, annealing at 52℃ for 1 min, extension at 72℃ for 40 s, ending in a final extension step at 72℃ for 10 min. DNA amplicons were cloned into plasmid pCR2.1 (Invitrogen, USA) according to the manufacturer’s instructions. Plasmid DNA was extracted and the 16S rDNA insert was sequenced using an Automated Biosystems Sequencer AB3100. The 16S rDNA sequences obtained (437–806 base pairs [bp] range) were identified when used as probes with the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih. gov/blast) software[16].

2.5 β-mannanase assays

β-mannanase assays were conducted using dinitrosalicylic acid (DNSA) and a method adapted for the detection of reducing sugar according to Stalbrand et al[17]. The enzyme assay reaction contained: 0.5% locust bean gum, 50 mM sodium citrate buffer, and 0.3 $\mu g \cdot mL^{-1}$ crude enzyme. The β-mannanase assays were carried out at pH ranging from 4

to 9 and temperatures ranging from 0℃ to 40℃. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent D-mannose per min. Three replicates were prepared for each reaction.

3 Results

3.1 Bacterial isolation

A total of 300 bacterial isolates each from Antarctica and Malaysia were analyzed. Several incubation temperatures were used to grow the 600 isolates. Isolates from Antarctica were tested for growth on nutrient, Luria, *Streptomycetes*, and modified agar media described by Abe et al. at 4℃, 20℃, 24℃, and 37℃[12]. The Antarctica bacterial isolates formed yellow, pink, white, gray, or orange colonies that grew well at 20℃ or lower temperatures. In contrast, all the Malaysian bacterial isolates tested formed either white or cream-colored colonies that grew rapidly on nutrient, Luria, *Streptomycetes*, and modified agar media described by Abe et al. at 37℃ and slower at 4℃, 20℃, and 24℃[12].

3.2 β-mannanase activity screening

Three hundred bacterial isolates each from Antarctica and Malaysia were screened for β-mannanase activity using agar medium that contained mannan. Only 30 Malaysian isolates (10%) showed evidence of β-mannanase activity under the conditions tested. Of these, 25 isolates grew on modified screening medium of Abe et al., four isolates grew on LB-RBB-guar gum medium, and one isolate grew on Streptomycetes medium[12]. They produced clear halos on RBB-guar gum agar at 20℃ and 37℃ (Figure 1). These isolates were designated Y1–Y19, A3–A7, B24–B26, and C25–C27.

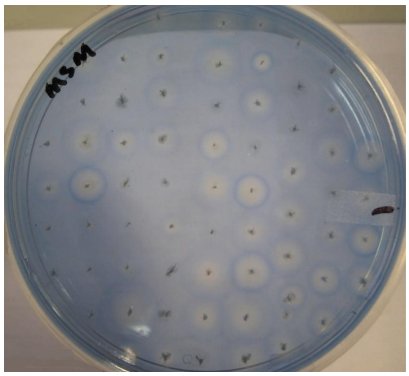

Figure 1 A representative agar plate showing hydrolysis halos produced by the bacterial isolates taken from Sabah, Malaysia. Guar gum was used as the carbon source.

In contrast, only nine (3%) Antarctic bacterial isolates exhibited β-mannanase activity at 20℃. These nine isolates, designated MP1–MP6, A40, A42, and C27, were isolated from the modified agar medium described by Abe et al.[12]. None of the Antarctic bacterial isolates that grew on the nutrient or LB agar media exhibited β-mannanase activity, suggesting that the enzyme produced by these bacteria was either inactive at 20℃, or the β-mannanase activity was too low or suppressed by the rich media to be detected. Seven Antarctic strains (A5, A7, A8, A9, MP1, MP2, and MP5) showed β-mannanase activity at 20℃ but not at higher temperatures, while strong β-mannanase activity was observed at 4℃ for the two other isolates, A40 and C27. Both of these exhibited weak activity at 20℃ and no activity at 24℃ or 37℃.

3.3 RAPD analysis

RAPD-PCR is widely used to detect sequence polymorphisms exhibited by identical or nearly identical bacterial genomes, and it is frequently applied to distinguish subgroups of closely related organisms[18]. To differentiate and cluster the β-mannanase-producing bacterial isolates, RAPD fingerprinting was performed using nine different RAPD primers (Table 1). A total of 30 DNA banding profiles were produced for the Malaysian bacterial isolates using primers Pri3 and Pri6. A representative agarose gel DNA profile of these isolates is shown in Figure 2. We were unable to differentiate the 30 bacterial cultures with the other seven primers as the RAPD-PCR gave very few or no amplified DNA band (data not shown). After comparing the DNA profiles and excluding all overlapping isolates, we obtained seven different Malaysian isolates designated Y1, Y2, Y16, Y18, A7, B26 and D4, and five Antarctic isolates designated MP1, MP2, MP5, A40 and C27.

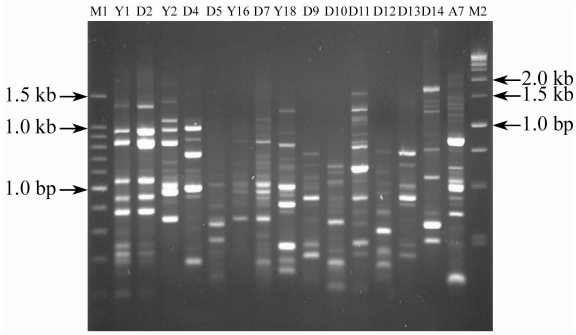

Figure 2 Representative agarose gel analysis of the RAPD-PCR profiles. The amplicons were generated using primer Pri3 for the Malaysian bacterial isolates (M1:100 bp ladder and M2:1 kb ladder).

3.4 Identification of bacteria by 16S rDNA

Seven Malaysian bacterial isolates (Y1, Y2, Y16, Y18, A7, B26, and D4) and five Antarctic bacterial isolates (MP1, MP2, MP5, A40, and C27) were identified by their 16S rDNA sequences. One segment of the 16S rDNA gene of the 12 bacterial isolates were sequenced. These sequences were deposited into the GenBank database with the following accession numbers: Y1 (FJ209090), Y2 (FJ209093), Y16 (FJ209094), Y18 (FJ209091), A7 (FJ209095), B26

(DQ396881), D4 (DQ396883), MP1 (FJ209096), MP2 (FJ209092), MP5 (FJ209097), A40 (DQ396880), and C27 (DQ396882).

Alignment of these 12 16S rDNA sequences revealed that four Malaysian isolates (Y1, Y16, Y18, and A7) had 95%–98% sequence similarity to the known genus *Paenibacillus*, while strain D4 was closest to *Paenibacillus amylolyticus*. The other two Malaysian isolates, Y2 and B26, were 97%–99% similar to the *Bacillus* genus and *Streptomyces* species, respectively. Antarctic isolates MP2 and MP5 were closest to *Bacillus pumilus* while MP1 was closest to *Bacillus amyloliquefaciens* with 97%–99% similarity. Antarctica isolates A40 and C27 were closest to the *Arthrobacter* genus and *Arthrobacter oxydans*, with 94% and 91% sequence similarity, respectively.

3.5 β-mannanase assays

The β-mannanase activity of three representative isolates with enzyme activity at different temperatures was analyzed. The isolates were the Malaysian isolate A7 (*Paenibacillus* sp.), and two Antarctic isolates, MP1 (*Bacillus amyloliquefaciens*) and A40 (*Arthrobacter* sp.). The optimal pH for β-mannanase activity of MP1 was pH 6, while a pH of 7 was determined optimal for A7and A40 (Figure 3). The optimal temperatures for β-mannanase activity for A40, MP1, and A7 were 5℃, 20℃, and 35℃, respectively (Figure 3). Only A7 retained approximately 65% of its activity at 25℃, while A40 and MP1 were inactivated at 30℃ and 35℃, respectively (Figure 3).

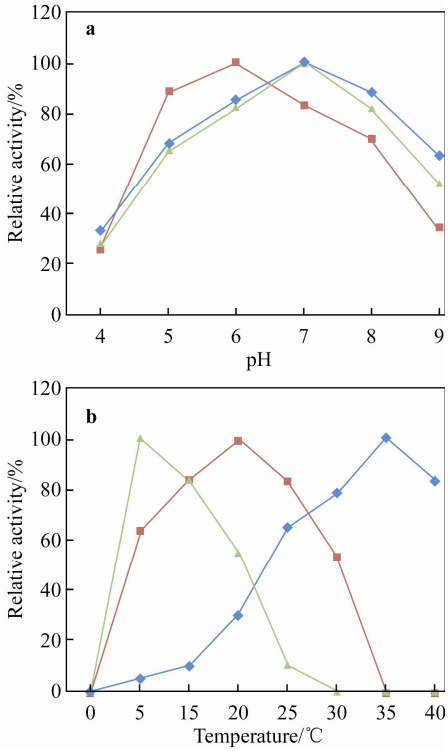

Figure 3 β-mannanase production by the bacterial isolates A7 (*Paenibacillus* sp., ♦), MP1(*Bacillus* sp., ■), and A40 (*Arthrobacter* sp., ▲) at different pH (**a**) and temperatures (**b**).

4 Discussion

β-mannanase-producing bacteria have been isolated from various regions around the world. They are microorganisms that degrade hemicellulose and return essential nutrients to the environment. In addition, their β-mannanase activity has been recently industrially harnessed. We performed an analysis of bacteria isolated from two very different climates, Antarctica and Malaysia, to compare bacterial growth, β-mannanase activity, and its relationship to temperature and pH.

Many Antarctic bacterial isolates analyzed in this study grew well at 20℃, suggesting that psychrotolerant bacteria are abundant on this continent. It has been previously suggested by O'Brien et al. that psychrotolerant bacteria, which grow optimally at approximately 20℃, may possess excellent temperature-coping mechanisms and grow rapidly during the summer[19]. In addition, most of the Antarctic bacterial isolates we identified in this study had pigmented CFU. These observations are consistent with the predominance of pigmented Antarctic bacteria reported by Shivaji et al., and they contrasted with the isolates from Malaysia that had either white or cream CFU[20].

We discerned that the screening medium used in our analysis, containing either 0.15% RBB-guar gum or RBB-locust bean gum, was most suitable for assaying β-mannanase activity compared with other media tested. In this assay, halos around the CFU indicated that β-mannanase had cleaved the dye-labeled substrates[22]. In general, bacteria typically produce β-mannanase in the agar medium described by Abe et al.[12]. This is a relatively low nutrient medium when compared with LB or *Streptomyces* agar media with mannan as the sole carbon source. Zakaria et al. and Stoll et al. found that β-mannanases were not produced in media containing monosaccharides or disaccharides such as glucose, mannose, galactose, xylan, or glycerol as a carbon source[2,11]. In this study, however, we identified five bacterial isolates that exhibited β-mannanase activity on LB and *Streptomyces* agar media that contained simple sugars and glycerol, respectively. These observations suggest that the β-mannanase activity in some Malaysian bacterial isolates was not inhibited by the sugar sources present in these media. A similar observation was reported by Valentine and Salyers, in which *Bacteroides ovatus* was able to produce β-mannanase in the presence of simple sugars such as glucose[21].

Environmental microorganisms are well known for their ability to degrade organic compounds, and in particular, polysaccharides[22]. In this study, we identified 39 mannan-degrading bacterial isolates from Antarctica and Malaysia. However, the majority of these β-mannanase-producing bacteria (76.9%) were recovered from soil samples taken from the tropical climate. This result concurs with those of Doi et al. who indicated that carbohy-

drate-hydrolyzing bacteria are usually found around plant material such as rotting plants and humus that are plentiful in tropical soils[23]. This also suggests that not many bacteria from Schirmacher Oasis, Antarctica can degrade mannan and this is most likely because hemicellulosic material such as plants are largely absent on this continent. Additionally, there was likely no hemicellulosic material from seaweed in the Schirmacher Oasis, as it is located far from the sea.

When the 16S rDNA of the bacterial isolates was sequenced, one bacterial isolate from Malaysia (Y2) and three from Antarctica (MP1, MP2 and MP5) were identified as *Bacillus* species. These results show that *Bacillus* species are common mannan-degrading bacteria that can be found in soil from both extremes of the climate spectrum. *Paenibacillus* spp. (Y1, Y16, Y18, A7 and D4) seemed to be the major mannan-degrading bacteria that are confined to the tropical region while the *Arthrobacter* spp. (A40 and C27) are confined to the Antarctica. Bacteria from the genera *Paenibacillus*, *Bacillus* and *Streptomyces* all have well-characterized polysaccharide-hydrolyzing activity. Mendoza et al. and Ma et al. demonstrated that *Bacillus* species, and in particular *Bacillus subtilis*, exhibit high β-mannanase activity[10,24]. They have suggested that the enzyme's activity may be induced by locust bean gum. Several *Paenibacillus* species, including *Paenibacillus polymyxa*, also produce β-mannanase[25-26]. Similarly, several members in the order *Actinomycetales* (e.g. *Streptomyces* species) express β-mannanases, and the β-mannanase gene manA from *Streptomyces lividans* has been cloned and characterized[3,27]. Last, β-mannanase-producing *Arthrobacter* species have also been reported[28-29].

The β-mannanase activity exhibited by the Malaysian isolate A7 (*Paenibacillus* sp.) was stable at 35℃, but it was not so for the Antarctic isolates (MP1 and A40), indicating that the β-mannanase produced by these Antarctic isolates is heat labile. The A40 isolate (*Arthrobacter* sp.) exhibited β-mannanase activity at 5℃, while optimal MP1 (*Bacillus amyloliquefaciens*) β-mannanase activity was noted at 20℃. For both, enzymatic activity was unnoticeable at 35℃. These data suggest that β-mannanase enzymatic expression and activity may be strongly influenced by environmental factors.

The Antarctic isolates A40 (*Arthrobacter* sp.) and MP1 (*Bacillus amyloliquefaciens*), as well as the Malaysian isolate A7 (*Paenibacillus* sp.), produced β-mannanase with more than 50% relative activity at pH ranging from 5–8. Interestingly, A40 and MP1 exhibited optimal β-mannanase activity at pH 6 (20℃), and pH 7 (5℃), respectively, despite being isolated from the same soil sample. This indicates that Antarctic soil probably harbors a pool of bacteria that produce β-mannanases that are active at different pH and temperatures. This suggests that overall mannan degradation may not be affected and can be carried out during fluctuations in temperature or pH.

In this study, we identified β-mannanase-producing bacterial isolates from both Antarctica and the tropical country of Malaysia. These isolates may exhibit novel β-mannanase enzymatic properties that have adapted to different temperatures. β-mannanases that are active at normal and low temperatures are useful for the treatment of industrial products such as pulp and paper. During the treatment process, temperatures in the bioreactor fluctuate often, and will allow for both heat stable and heat labile β-mannanases to optimally degrade their substrates. Future studies involving cloning, comparative sequence analysis, and three-dimensional protein structure analysis of the β-mannanases expressed in these new isolates will allow for further characterization of these potentially useful enzymes.

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