Characterization of Low-Temperature Enzymatic Reactions through Heterologous Expression and Functional Analysis of Two Beta-Glucosidases from the Termite Symbiotic Bacterium *Elizabethkingia miricola* Strain BM10

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ABSTRACT

Lower termites need symbiotic microbes for cellulose digestion. *Elizabethkingia miricola strain* BM10 has been proposed as a symbiotic microbe that assists in low-temperature digestion and metabolism of *Reticulitermes speratus* KMT1, a termite on Bukhan Mountain, Seoul, Korea. In *E. miricola* strain BM10, *β*-glucosidase genes expressed at 10℃ were identified, and the psychrophilic enzymatic characteristic was confirmed by heterogeneously expressed proteins. Crude *β*-glucosidase in the culture broth of *E. miricola* strain BM10 showed specific enzymatic properties, and its substrate affinity was 4.69 times higher than that of Cellic CTec2. Among the genes proposed as *β*-glucosidase, two genes, *bglB_1* and *bglA_2*, whose gene expression was more than doubled at 10℃ than at 30℃, were identified. They were heterogeneously expressed in *Escherichia coli* and identified as psychrophilic enzymes with an optimal reaction temperature of about 20℃–25℃. In this study, *E. miricola* strain BM10, a symbiotic bacterium of lower termites, produced psychrophilic *β*-glucosidases that contribute to the spread of the low-temperature habitat of a lower termite, *R. speratus* KMT1.

Keywords: heterogeneous gene expression, high substrate affinity, psychrophilic *β*-glucosidase, symbiotic bacterium, termite

1. INTRODUCTION

Termites are wood-eating pests (Arinana *et al.*, 2022; Su and Scheffrahn, 2000). Since termites damage wooden buildings, many studies have been conducted to control them in various ways (Adfa *et al.*, 2020, 2023; Arsyad *et al.*, 2020; Cahyono *et al.*, 2020; Ella Nkogo *et al.*, 2022; Im and Han, 2020; Lee *et al.*, 2020; Oramahi *et al.*, 2022; Priadi *et al.*, 2023). However, they also serve as ecological suppliers providing various nu-trients, including nitrogen compounds (Govorushko, 2019; Lobry de Bruyn and Conacher, 1990). They are widely distributed worldwide (Bourguignon *et al.*, 2016), including Korea with freezing temperatures in winter (Cho *et al.*, 2010b; Kim *et al.*, 2012). In Korea, a representative lower termite species is *Reticulitermes speratus* (Cho *et al.*, 2010b; Kim *et al.*, 2012). It requires symbiotic microbes for the digestion of woody cellulose (Peterson and Scharf, 2016). Therefore, in the digestive tract of *R. speratus*, symbiotic microbes that aid in digestion must

exist at low temperatures. In a previous study, a microorganism that produces β-glucosidase at 10℃ was proposed among the symbiotic microorganisms in the gut of termites (Lee *et al.*, 2018). In this study, we aim to show that the proposed low-temperature-produced β-glucosidases are not simply enzymes produced at low temperature, but that their enzymatic properties also have low-temperature enzymatic reaction characteristics.

Reticulitermes flavipes has been reported to survive at low temperatures. In the range of 8.1℃ to 5.7℃, it enters a cold coma, and half of individuals have been reported to die (LT50) after 1 hour at −5.1℃ (Clarke *et al.*, 2013). *Porotermes adamsoni* and *Stolotermes victoriensis* were active near 0℃, and half of individuals dying at −5.66℃ and −7.32℃, respectively (Lacey *et al.*, 2010). For *R. speratus*, half of the individuals died at −8.0℃, −8.0℃, −6.6℃, and −6.4℃ after a 2-hour incubation of kings, queens, workers, and soldiers, respectively (Takata *et al.*, 2023).

Cellulose, the main nutritional source found in wood, is efficiently broken down by three types of enzymes known as cellulases: endo-β-1,4-glucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (Kumar *et al.*, 2008). When termites crush cellulose, it undergoes degradation into smaller molecules through the action of EG, which is secreted by their salivary glands. In the case of the lower termite such as *R. speratus*, the cellulose is further converted into glucose with the additional CBH and β-glucosidase enzymes produced by symbiotic microorganisms in the termite's gut. It has been suggested that *R. speratus* possesses three EG enzymes (Ahn and Kim, 2021), and certain symbiotic microorganisms also possess an EG enzyme (Otagiri *et al.*, 2013). Significant reduction in cellulase activity within the hindgut was observed when bacteria were eliminated by ampicillin in *Nasutitermes takasagoensi*, indicating the involvement of symbiotic microorganisms in cellulose digestion (Tokuda and Watanabe, 2007). Symbiotic microbes found in termites produce CBH and

β-glucosidase, but not EG, which are responsible for the initial breakdown of cellulose into smaller molecular weights (Cho *et al.*, 2010a). This observation may be attributed to symbiotic adaptation, considering that the initial reduction in cellulose molecular weight occurs by EG produced in the termite's mouth, and EG is not required for the subsequent stages of digestion. The population of the symbiotic bacterial community is conserved across different termites due to adaptations resulting from common dietary habits (Arora *et al.*, 2022; Waidele *et al.*, 2019).

The β-glucosidase production of bacteria isolated from the gut of *R. speratus* KMT1 shows various temperature specificities (Lee *et al.*, 2018). In particular, *Elizabethkingia miricola* strain BM10 produces the maximum β-glucosidase activity at 10℃ and exhibits a sharp decrease in β-glucosidase activity when the growth temperature is increased to 20℃. Therefore, *E. miricola* strain BM10 likely provides a decomposition mechanism of symbiotic microbes necessary for cellulose catabolism under low-temperature growth conditions of *R. speratus* KMT1.

In this study, the enzymatic characteristics of β-glucosidase produced by *E. miricola* strain BM10 at low temperature were analyzed. The psychrophilic β-glucosidase genes were identified by increased gene expression level by shifting the cell culture temperature from 30℃ to 10℃. Two selected β-glucosidase genes were produced by heterologous expression in *Escherichia coli* and shown to be psychrophilic enzymes by characterizing the optimal reaction temperature.

2. MATERIALS and METHODS

2.1. Strain and chemicals

E. miricola strain BM10 was isolated from the digestive tracts of *R. speratus* KMT1 (Lee *et al.*, 2015).

The following media were prepared using the given components: tryptone yeast extract (TYE) medium with

1% tryptone (REF: 211705, Becton Dickinson, Seoul, Korea) and 0.5% yeast extract (REF: 212750, Becton Dickinson); yeast extract peptone (YEP) medium with 0.2% yeast extract and 0.8% peptone (REF: 211677, Becton Dickinson) in 0.1 M phosphate buffer (pH 7.0) made with sodium phosphate monobasic dihydrate (Product#: 1429, Duksan, Ansan, Korea) and sodium phosphate dibasic anhydrous (Product#: 1490, Duksan); Luria–Bertani medium with 1% LB broth (REF: 244620, Becton Dickinson); and lysis buffer with 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole. Agar plates were made with the corresponding medium and 1.5% Bacto agar (REF: 214010, Becton Dickinson). Carboxymethyl cellulose (CMC, Catalog number: R7257) was purchased from Sigma-Aldrich (Seoul, Korea).

2.2. β-glucosidase activity of cell free culture broth

E. miricola strain BM10 stored at −80℃ was streaked on agar plates with the TYE medium, 1% CMC, and 1.5% Bacto agar. Cells were incubated at 26℃ for 2 days. A single colony was inoculated in 5 mL of TYE medium with 1% CMC and incubated at 26℃ for 2 days while shaking at 250 rpm. The precultured cells were inoculated into 100 mL of YEP medium with 2% CMC and cultured at 10℃ for 3 days while shaking at 250 rpm. After 3 days in the main culture, 0.002% sodium azide was added, and the cells were removed by centrifugation with 12,000×g at 4℃ for 10 min. The cell free supernatant of the main culture was used to measure the β-glucosidase activity at 30℃ and pH 7 (Lee et al., 2018). The 20 μL of enzyme was mixed with 160 μL of 0.1 M potassium phosphate buffer (pH 7.0) and 20 μL of 10 mM ρ-nitrophenyl-β-D-glucopyranoside, incubated at 30℃ for 30 min, and the reaction was stopped by mixing 20 μL of 2 M calcium carbonate. Absorbance was measured at a wavelength of 405 nm and the amount of ρ-nitrophenol produced was calcula-

ted using a ρ-nitrophenol standard curve. One unit of activity was defined as the amount of β-glucosidase activity that generated 1 μmol ρ-nitrophenol/min. The Cellic CTec2 (Novozymes A/S, Bagsværd, Denmark) was used as a positive control after 1/40,000 dilution. The Lineweaver–Burk equation was obtained using 1/[S] (1/mM) for χ value and 1/V (mL · min/μmol-ρ-nitro-phenol) for y values.

2.3. β-glucosidase genes in *Elizabethkingia miricola* strain BM10 and measurement of gene expression levels via real-time polymerase chain reaction (RT-PCR)

The complete chromosomal DNA sequence of E. miricola strain BM10 is available at the National Center for Biotechnology Information (https://ncbi.nlm.nih.gov/; GenBank number: CP011059). The six proteins with β-glucosidase in the description were selected, and their closest proteins in the protein database were identified using protein BLAST.

E. miricola strain BM10 was cultured in accordance with the procedures described in the section "β-glucosidase activity of cell free culture broth". Cells were harvested by centrifugation with 12,000×g at 4℃ for 10 min. TRIzol® (1 mL; Catalog number: 15596026, Thermo Fisher Scientific, Seoul, Korea) was added and incubated at 4℃ for 5 min. The cells were rapidly frozen and disrupted using TissueLyser LT (Qiagen, Seoul, Korea). After centrifugation at 12,000×g for 10 min, the supernatant was separated and mixed well with 200 μL of chloroform. The mixture was incubated at 23℃ for 3 min. After another centrifugation at 12,000×g for 15 min, the supernatant was separated and mixed with 500 μL of isopropanol. The mixture was incubated at 23℃ for 10 min, and the precipitated pellet was harvested by centrifugation at 12,000×g for 15 min. The pellet was rinsed with 1 mL of 75% cold ethanol. The extracted

RNA was dried and dissolved in pure water treated with 0.1% (v/v) of diethyl pyrocarbonate. For cDNA synthesis, a mixture was made using M-MLV Reverse Transcriptase (Catalog number: M2410, GenDEPOT, Dawinbio, Hanam, Korea) in accordance with the manufacturer's protocol. cDNA was synthesized at 42℃ for 60 min, and the enzyme was inactivated by boiling for 10 min.

For RT-PCR, seven primer pairs of 6 β-glucosidase candidate genes and glyceraldehyde 3-phosphate dehydrogenase A gene as a reference gene of *E. miricola* strain BM10 were designed: 0821F (5'-GAAGGATTGG GACTGGGAAT-3') and 0821R (CAAATTTCTGCACC AGCTCA) for *bglX_1*; 1398F (CTGGTTTCCGGTAAT GCTGT) and 1398R (CCATCTGATGAGCTGCGTTA) for *bglB_1*; 3284F (AGCCAAGATGGGGAAGAGTT) and 3284R (CTTCCGGTGCTCCATACAGT) for *bglX_2*; 3419F (ACCATCCAATAATCCGGTGA) and 3419R (TTTTCTGAGGGTTCCACAGG) for *bglX_3*; 3735F (GCCACTGCTTTTCCTGTAGC) and 3735R (CATTTT CCTGAACGCCATTT) for *bglB_2*; 3904F (CCCGGCA GAAAAAGTATTGA) and 3904R (CGGTTAAGCAAA AGGTCTGC) for *bglA_2*; and G3PDHF (CACGGGC AATTTAAAGGAGA) and G3PDHR (GCGTTAATGT GAGCTTGTGC) for *gapA*.

RT-PCR was performed with 10 μL of iQ^{TM} SYBR$^{®}$ Green Supermix (Catalog number: 1708880, Bio-Rad Laboratories, Hercules, CA, USA), 1 μL of forward primer, 1 μL of reverse primer, 1 μL of cDNA, and 7 μL of deionized water under the following reaction conditions: 1 cycle at 95℃ for 5 min; 40 cycles at 95℃ for 45 s, 55℃ for 30 s, and 72℃ for 30 s; and 1 cycle at 72℃ for 10 min. The specificity of the RT-PCR products was confirmed through melting curve analysis between 60℃ and 95℃. RT-PCR was performed thrice.

2.4. Heterogeneous expression of *bglB_1* and *bglA_2* in *Escherichia coli* TOP10

The expression plasmid was constructed and trans-formed into *E. coli* TOP 10 by Cosmogentech (Seoul, Korea). *bglB_1* and *bglA_2* were amplified through PCR. Since *bglB_1* was difficult to amplify by a single PCR experiment, internal primers were used to amplify *bglB_1* in two segments. Subsequently, the resulting PCR products were combined and used as the template for a subsequent PCR reaction to amplify the complete *bglB_1* gene. The primers for *bglB_1* were BGLB1-NdeIF (CGCGCGGCAGC<u>CATATG</u>CTGAAGAGAATT ATCTTAGCC), BGLB1-XhoIR (GGTGGTGGTG<u>CTC GAG</u>TTACTGAAGTGTAAACTTCAC), BGLB1-intP1 (GAATTCCGCCATACTAGC), and BGLB1-intP2 (TAAGATGATGACTGTTGG). The primers for *bglA_2* were BGLA2-NdeIF (CGCGCGGCAGC<u>CATATG</u>TTA TTGACAAAGGAAGCC) and BGLA2-XhoIR (GGTGG TGGTG<u>CTCGAG</u>TTATTTAATAAAATCGGCATACC). The restriction enzyme sites of NdeI in BGLB1-NdeIF and BGLA2-NdeIF and XhoI in BGLB1-XhoIR and BGLA2-XhoIR were underlined. PCR was performed with 1 μL of cDNA template, 5 μL of the forward primer, 5 μL of the reverse primer, 1 μL of Pfu DNA polymerase, 4 μL of dNTP mixture, 10 μL of 5× reaction buffer, and 24 μL of distilled water under the following reaction conditions: 1 cycle at 95℃ for 5 min; 35 cycles at 95℃ for 60 s, 55℃ for 60 s, and 72℃ for 60 s per kilobase; and 1 cycle at 72℃ for 7 min.

The vector, pET28a, and the PCR products were cut by NdeI and XhoI. The cut pET28a with 4 μL was mixed with 1 μL of the PCR-amplified gene, 1 μL of ligase, 1 μL of 10× reaction buffer, and 3 μL of distilled water. After incubation at 23℃ for 150 s, the mixture was cooled down at 4℃ for 10 min. The ligated recombinant pET28a was transformed into *E. coli* Top10 via the heat shock method at 42℃ for 90 s. The successful transformant produced colonies on LB agar plates with 50 μg/mL kanamycin. The recombinant plasmid was confirmed through colony PCR.

pET28a with the inserted gene was transformed into *E. coli* BL21 strain via the heat shock method, and the

transformed cells were spread on LB agar plates with 50 μg/mL kanamycin. After colony selection, single colonies were obtained by streaking on LB agar plates with 50 μg/mL kanamycin again. The cells were cultured in LB broth at 37℃ overnight and stored with 25% glycerin at −80℃.

For protein production, the cells were streaked and grown on LB agar plates with 50 μg/mL kanamycin. A single colony was inoculated in 5 mL of LB medium test tubes and inoculated at 37℃ overnight. The precultured cells was inoculated into 100 mL of LB broth and cultured at 17℃ for 18 h. Protein expression was induced by 0.01 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Catalog number: I0355, GenDEPOT).

Cells were harvested by centrifugation at 4,000×g for 20 min. The cell pellet was resuspended in 5 mL of lysis buffer and mixed with 25 μg/mL lysozyme. After incubation for 30 min, the cells were disrupted through sonication. Cellular extracts were centrifuged at 10,000×g for 20 min to separate the supernatant (soluble protein) from the pellet (insoluble protein). For protein analysis, proteins were denatured at 95℃ for 5 min and separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% polyacrylamide. Proteins were stained with Coomassie brilliant blue R (Sigma-Aldrich).

2.5. Determination of the optimal reaction conditions of heterologous expressed β-glucosidases

The β-glucosidase activity of heterologous expressed β-glucosidases was measured using the supernatant of cell-free extracts in accordance with previously described methods (Lee *et al.*, 2018) with some modifications. For enzyme activity measurement, the conditions used other than the conditions to be determined in each experiment were 20℃ and pH 7.

Temperature-related changes in β-glucosidase activity were measured in the range of 5℃ to 45℃ at 5℃

intervals to evaluate the psychrophilic enzymatic characteristics. The effect of pH on the β-glucosidase activity was determined with 100 mM sodium citrate buffer (pH 3-6), 100 mM sodium phosphate buffer (pH 6-8), and 100 mM Tris-HCl buffer (pH 8-9). For the thermostability test, enzyme activities were examined at the indicated temperature after incubation for the indicated time in each experiment. Ion-induced changes in β-glucosidase activity were examined with 1 mM of $CuSO_4$, $MnCl_2$, $CoCl_2$, $MgCl_2$, $BaCl_2$, KCl, $FeSO_4$, $ZnSO_4$, $FeCl_3$, $CaCl_2$, and NaCl in order to evaluate the inhibitory effect of ions (Singh *et al.*, 2023; Yan and Lin, 1997).

3. RESULTS and DISCUSSION

3.1. Enzymatic kinetic analysis of β-glucosidase activity in the cell-free culture broth

In a previous study, the maximum activity of β-glucosidase from *E. miricola* strain BM10 was detected at 10℃, and less than 20% of β-glucosidase activity was observed at above 20℃ compared with that at 10℃ (Lee *et al.*, 2018). Enzyme kinetic analysis was performed to reveal the enzymatic characteristics of β-glucosidase expressed at a low temperature (Fig. 1). Since the β-glucosidase activity of Cellic CTec2, a positive control, was too high, it was diluted 40,000 times to resemble the β-glucosidase activity of *E. miricola* strain BM10. Enzyme activity affects V_{max} (the maximal reaction velocity when the enzyme is saturated with its substrate) but not K_m (the substrate concentration at which half of the maximum reaction velocity is reached) because of the unique nature of the enzyme. K_m is an index indicating the affinity of an enzyme to its substrate; the lower the K_m, the higher the substrate affinity. *E. miricola* strain BM10 and diluted Cellic CTec2 had K_m of 1.01 and 4.74 mM, respectively. The K_m of β-glucosidase from Cellic CTec2 was 4.69 times higher

than that of β-glucosidase from *E. miricola* strain BM10. This relatively low K_m indicated that the β-glucosidase from *E. miricola* strain BM10 had a 4.69-fold higher substrate affinity than Cellic CTec2. Enzymes generally react more rapidly with increasing temperature until they are inactivated through denaturation. High substrate affinity is a way to increase the reaction rate of enzymes, allowing them to maintain their activity even at low temperatures. Fig. 1 suggested that the reaction rate of β-glucosidase from *E. miricola* strain BM10 could be maintained at low temperatures by increasing substrate affinity. Many β-glucosidases have been reported to have low K_m (Bockwoldt and Ehrmann, 2022; DelPozo *et al.*, 2012; Pyeon *et al.*, 2019; Zhou *et al.*, 2011), and their optimum reaction temperature is 35℃–55℃. Crude enzymes do not accurately represent the properties of specific enzymes. However, the high substrate affinity of the crude enzyme confirmed the existence of psychrophilic β-glucosidases, and it was necessary to identify psychrophilic β-glucosidases to support the low-temperature habitat of *R. speratus* KMT1.
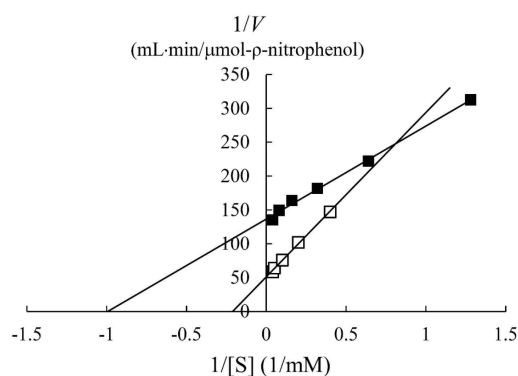
Fig. 1. Lineweaver–Burk plot showing the results of the enzymatic kinetic analysis of the β-glucosidase activity of the cell culture broth of *Elizabethkingia miricola* strain BM10 (■) and Cellic CTec2 (□). The substrate was ρ-nitrophenyl-β-D-glucopyranoside.

3.2. β-glucosidase genes in *Elizabethkingia miricola* strain BM10

The complete chromosomal sequence of *E. miricola* strain BM10 was registered with accession number CP011059 at the National Center for Biotechnology Information (Lee *et al.*, 2015). Six β-glucosidase genes were found in the entire chromosome of *E. miricola* strain BM10 (Table 1). The most homologous non-*Elizabethkingia* proteins, along with the names and amino acid numbers of the six genes, were displayed in the protein database. The percent identity of the six genes with the most homologous protein ranged from 67% to 85%. This low percent identity range with the closest proteins suggested that the β-glucosidases from *E. miricola* strain BM10 may have unique enzymatic characteristics that differ from those of other currently reported species.

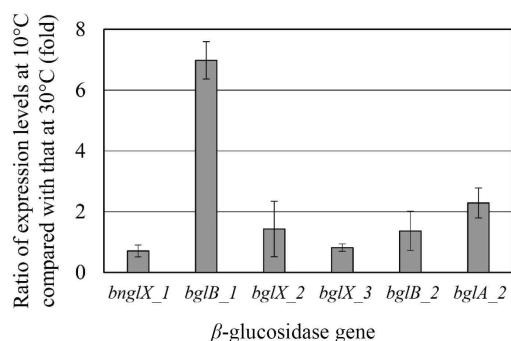## 3.3. Expression level of β-glucosidase genes from *Elizabethkingia miricola* strain BM10 at 10℃

Since *E. miricola* strain BM10 showed a maximum β-glucosidase activity when grown at 10℃ (Lee *et al.*, 2018), β-glucosidase would be expressed at 10℃. The expression levels of the genes proposed as β-glucosidase at 10℃ and 30℃ were compared through RT-PCR (Fig. 2). The genes whose expression levels were more than doubled at 10℃ than at 30℃ were *bglB_1* (6.98-fold) and *bglA_2* (2.28-fold). These results suggested that *bglB_1* and *bglA_2* were responsible for the high β-glucosidase activity at 10℃ in *E. miricola* strain BM10.

3.4. Heterologous expression of *bglB_1* and *bglA_2* in *Escherichia coli*

bglB_1 and *bglA_2* were independently inserted into the pET28a vector, and the recombinant plasmids were

Table 1. Relative *β*-glucosidase (EC 3.2.1.21) genes of *Elizabethkingia miricola* strain BM10 (GenBank: CP011059) and their highest "percent identity" protein outside of *Elizabethkingia* in the protein database of the National Center for Biotechnology Information

| Protein ID	Description	Gene name	Amino acid number	The protein with the highest percent identity in the protein database			
				Accession number	Scientific name of strain having protein	Query coverage (%)	Percent identity (%)
AJW62307	Periplasmic *β*-glucosidase precursor	*bnglX_1*	766	WP_073290053	*Chryseobacterium polytrichastri*	100	82.51
AJW62880	Thermostable *β*-glucosidase B	*bglB_1*	753	WP_103290166	*Chryseobacterium lactis*	100	85.13
AJW64715	Periplasmic *β*-glucosidase precursor	*bglX_2*	773	OJX30905	*Chryseobacterium* sp. 36-9	100	78.71
AJW64849	Periplasmic *β*-glucosidase precursor	*bglX_3*	827	WP_223581078	*Sphingobacterium* sp. GVS05A	95	67.64
AJW65161	Thermostable *β*-glucosidase B	*bglB_2*	216	MCG2793578	*Weeksellaceae bacterium*	98	82.63
AJW65329	*β*-glucosidase A	*bglA_2*	444	WP_115171992	*Sphingobacterium spiritivorum*	100	84.23

Fig. 2. Comparison of gene expression levels at 10℃ and 30℃ through RT-PCR. Six putative *β*-glucosidase genes from Table 1 were tested. The experiment was repeated thrice and the average and SD are shown. RT-PCR: real-time polymerase chain reaction.

obtained from *E. coli* TOP10. The inserted gene in the recombinant plasmids was confirmed through sequencing analysis (data not shown). The confirmed recombinant plasmids were introduced in the expression strain *E. coli*

BL21. The expression of both genes was induced with 0.01 mM IPTG at 17℃ for 18 h. Large amounts of proteins with the desired size were produced in the cells by IPTG for each protein compared with those in the cells without the expressed genes (Fig. 3). Most of the produced proteins were observed in the inclusion bodies, and only a small amount of the protein was produced in a soluble state.

After the proteins were produced, their *β*-glucosidase activities were measured using the soluble fraction. The *β*-glucosidase activities of AJW62880 (the protein of *bglB_1*) and AJW65329 (the protein of *bglA_2*) at 17℃ were 1.582 ± 0.013 and 0.350 ± 0.011 U/mL, respectively. The *β*-glucosidase activities were 7.5-fold higher for *bglB_1*-expressing cells and 1.6-fold higher for *bglA_2*-expressing cells than those of 0.210 U/mL in the cell-free culture medium of *E. miricola* strain BM10. Although many expressed proteins were insoluble, a sufficient enzyme activity was observed in soluble proteins to characterize the enzymes.

A.

B.
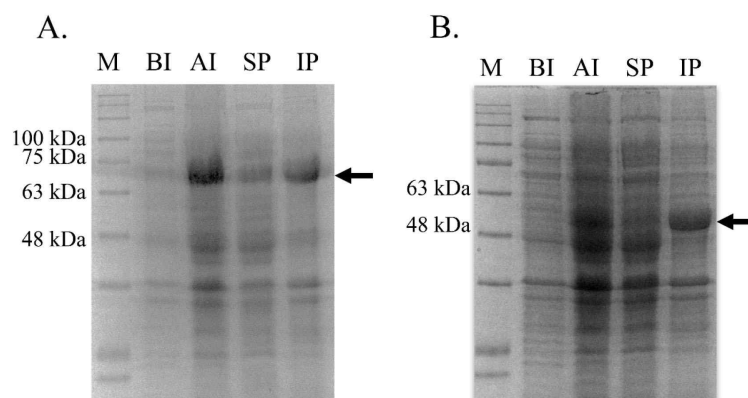
Fig. 3. Heterologous expression of *bglB_1* (A) and *bglA_2* (B) in *Escherichia coli*. Proteins were analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins in the cells without induction (BI) and with induction (AI) were analyzed to verify the protein expression. After cell extract separation, soluble proteins (SP) in the cell-free supernatant and insoluble proteins (IP) in the pellet were examined to measure protein partition. The arrows on the right side of the picture indicated the expected position of the expressed proteins. The protein size marker (M) showed the expected protein size.

3.5. Enzymatic characteristics of heterologous expressed β-glucosidases

The enzymatic characteristics of β-glucosidase were evaluated with the heterologous expressed soluble enzyme. In the case of AJW62880, the optimum reaction temperature and pH were 20℃ (Fig. 4A) and 6.5 (Fig. 4B), respectively. In the thermostability test (Fig. 4C), most of the β-glucosidase activity remained at 30℃ for 2 h, but the enzyme activity rapidly disappeared within 15 min at 40℃. No significant ion-induced changes in enzyme activity were observed (Fig. 4D). Even when the enzyme activity was slightly reduced by some ions, the enzyme activity was not significantly changed, considering that the enzyme activity was not changed by the same ion provided as a different type of salt.

In the case of AJW65329, the optimum reaction temperature and pH were 25℃ (Fig. 5A) and 7.0 (Fig. 5B), respectively. In the thermostability test (Fig. 5C), similar to that observed in AJW62880, β-glucosidase activity was mostly maintained at 30℃ for 2 h. However, at

40℃, the enzyme activity of AJW65329 disappeared more slowly than that of AJW62880. The β-glucosidase activity of AJW65329 was severely affected by ions (Fig. 5D). Particularly, $CuSO_4$, $FeSO_4$, and $ZnSO_4$ strongly inhibited the enzyme activity by more than 60%.

The enzymatic kinetics of two heterologous expressed β-glucosidases were analyzed (Fig. 6). AJW62880 and AJW65329 had K_m of 48.53 and 17.92 mM, respectively. The substrate affinities of these two β-glucosidases were approximately 10 and 4 times lower than that of Cellic CTec2, which had K_m of 4.74. This result is contrary to the high substrate affinity of β-glucosidase in the cell culture broth. This contrary K_m value suggested that the enzyme structure was partially modified according to heterologous expression; thus, enzymatic characteristics changed, or a specific substance present in the cell culture broth increased the substrate affinity of β-glucosidase.

In a previous study, a low-temperature-active β-glucosidase was obtained from symbiotic *Serratia* sp. TN49 isolated from the gut of longhorned beetle (*Batocera horsfieldi*) larvae (Zhou *et al.*, 2011), and its optimum
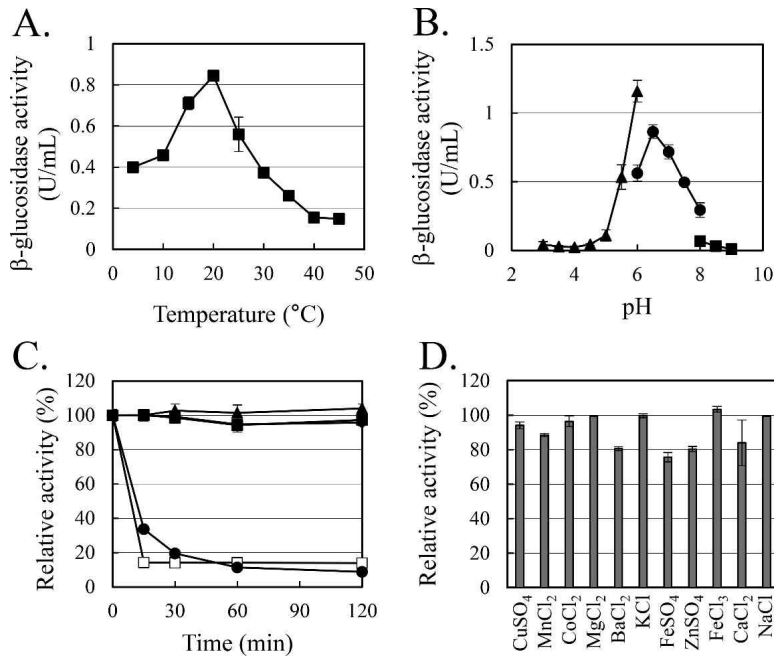
Fig. 4. Effect of reaction conditions on β-glucosidase activity of AJW62880. The effect of reaction temperature (A) and pH (B), thermostabilities (C), and the inhibitory activity of ions (D) on the β-glucosidase activity were evaluated. In the panel B, citrate buffer (▲) for pH 3–6, phosphate buffer (●) for pH 6–8, and tris buffer (■) for pH 8–9 were used. In the panel C, temperatures at 10℃ (■), 20℃ (◆), 30℃ (▲), 40℃ (●), and 50℃ (□) were tested. In the panel D, salt concentration was 1 mM. The experiment was repeated thrice and the average and SD are shown.

reaction temperature is 35℃. In the present study, the optimal reaction temperature of two heterologous expressed β-glucosidases was 20℃–25℃, and the expression of *E. miricola* strain BM10 psychrophilic β-glucosidases at 10℃ (Lee *et al.*, 2018) suggested that AJW62880 and AJW65329 were β-glucosidases more optimized for low temperatures. *R. speratus* KMT1 feeds on wood and requires the help of commensal microorganisms in the gut to digest wood. To survive in regions with sub-zero temperatures in winter, *R. speratus* KMT1 needs symbiotic microorganisms that help in digestion in the intestines. Therefore, the results of this study provided an ecological basis for the spread of termite habitats in low-temperature regions.

Further studies may reveal the structural features of

psychrophilic β-glucosidases with a high substrate affinity and identify the regulatory mechanism of cold gene expression specifically at 10℃.

4. CONCLUSIONS

E. miricola strain BM10, a termite-symbiotic bacterium, produced β-glucosidase with high substrate affinity at 10℃ to have an efficient enzymatic reaction even at low temperatures. Two β-glucosidase genes, *bglB_1* and *bglA_2*, expressed by *E. miricola* strain BM10 at low temperatures were identified. The heterologous expression of the identified genes in *Escherichia coli* confirmed that they were psychrophilic β-glucosidases with an optimal reaction temperature of 20℃–25℃. These results
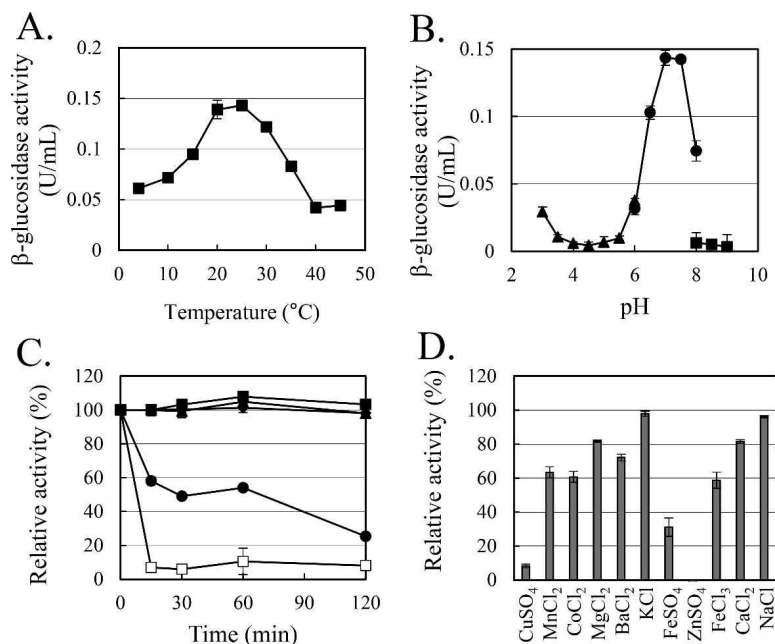
Fig. 5. Effect of reaction conditions on β-glucosidase activity of AJW65329. The effect of reaction temperature (A) and pH (B), thermostabilities (C), and the inhibitory activity of ions (D) on the β-glucosidase activity were evaluated. In the panel B, citrate buffer (▲) for pH 3−6, phosphate buffer (●) for pH 6−8, and tris buffer (■) for pH 8−9 were used. In the panel C, temperatures at 10℃ (■), 20℃ (◆), 30℃ (▲), 40℃ (●), and 50℃ (□) were tested. In the panel D, salt concentration was 1 mM. The experiment was repeated thrice and the average and SD are shown.
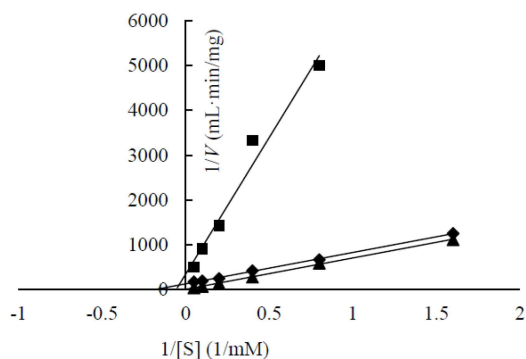
Fig. 6. Lineweaver−Burk plot of the results of the enzymatic kinetic analysis of the β-glucosidase activities of heterologously expressed AJW62880 (▲), heterologously expressed AJW65329 (■), and CellicCTec2 (◆).

demonstrated that symbiotic microbes provided low-temperature-specific enzymes necessary for the survival of termites at low temperatures. Such symbiotic microbes allow termites to expand their habitats to low-temperature regions.

CONFLICT of INTEREST

No potential conflict of interest relevant to this article was reported.

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