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Degradation Characteristics of A Novel Multi-Enzyme-Possessing *Bacillus licheniformis* TK3-Y Strain for the Treatment of High-Salinity Fish Wastes and Green Seaweeds

Kyeong Hwan Kang and Joong Kyun Kim*

Department of Biotechnology, Pukyong National University, Busan 48513, Korea

Abstract

To reutilize fisheries waste, we isolated a bacterial strain from a coastal area located in Busan. It was identified as *Bacillus licheniformis* TK3-Y. Using plate assay and 500-mL flask experiments, we found that the isolate simultaneously possessed cellulolytic, proteolytic, and lipolytic activities with salt tolerance. 10% (v/v) inoculums, were used to examine the biodegradation characteristics of the TK3-Y strain on carboxymethylcellulose, skim milk, and olive oil media. The optimum conditions for pH, temperature, agitation speed, and NaCl concentration on each 1% substrate were 6, 50°C, 180 rpm, and 17.5%, respectively. Under optimal conditions, the TK3-Y strain showed 1.07 U/mL cellulolytic, 1,426 U/mL proteolytic, and 6.45 U/mL lipolytic activities. Each enzyme was stable within a range of 17.5–35% NaCl. Therefore, the salt tolerance ability of strain TK3-Y was superior to other related strains. In degradation of a mixed medium containing all three substrates, both the cellulolytic and proteolytic activities were somewhat lower than those on each single substrate, while the lipolytic activity was somewhat higher. From the above results, the TK3-Y strain appears to be a good candidate for use in the efficient treatment of fisheries waste in which components are not collected separately.

Key words: *Bacillus licheniformis* TK3-Y, Multiple enzymes, Salt tolerance, Fish waste, Biodegradation

Introduction

Globally, the consumption of seafood, such as fish and seaweed, has increased every year. To accommodate this demand, large amounts of seafood are processed industrially and fisheries waste is accordingly generated during seafood processing. This trend also has been observed in South Korea, and approximately 2,100 tons of fisheries waste was reported to be generated every day from numerous seafood processing plants and restaurants (Kim et al., 2010). Fisheries waste, including seafood by-products, the components of which are normally not separated, are a source of environmental concerns. So far, fisheries waste has not been efficiently utilized, significantly affecting the local environments (Kim et al., 2014). In Jeju Island, green seaweeds, such as *Ulva pertusa* and *Enteromorpha prolifera*, often drift over the beach, ruining the appeal of the beach by causing a bad odor.

Fisheries waste is usually disposed of by landfill, incineration, or ocean dumping (Gwon and Kim, 2012). Each disposal method, however, has some problems, and ocean dumping of fisheries waste has been prohibited in South Korea since 2014 according to the London Convention (IMO, 2006). Therefore, efficient reutilization of fisheries waste by biodegradation was recently suggested. Biodegradation is less dangerous and simpler than physicochemical reactions and has some advantage, such as the re-utilization of wasted materials and the

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***Corresponding Author**
E-mail: junekim@pknu.ac.kr

production of various useful materials, by using microorganisms (Kim, 2011). For the biodegradation of fisheries waste, intact cells orpurified enzymes are used. When the purified enzymes are used, undesirable side reactions can be avoided and high catalytic activity can be achieved. However, purification of target enzymes is likely to be expensive and the purified enzymes may be more sensitive to inactivation than those present within an intact cell (Kim et al., 2013). Economically, bacterial cells are recommended for use in the treatment of fisheries waste.

The major components of fisheries waste are carbohydrates, proteins, and lipids (NFRDI, 2009), which are converted to useful compounds, such as bioenergy resources or bioactive substances, by diverse microbial strains, including cellulolytic *Acremonium strictum* (Goldbeck et al., 2013), alginate- and laminarin-degrading *Microbacterium oxydans* (Kim et al., 2013), proteolytic *Bacillus pseudofirmus* (Raval et al., 2014), and lipolytic *Aneurinibacillus thermoaerophilus* HZ (Masomian et al., 2013). Conversion of fishery waste into liquid fertilizer by mixed microbes (Kim et al., 2007; Kim et al., 2010), conversion of brown-seaweed waste by *Microbacterium oxydans* (Kim et al., 2013), and conversion of red-seaweed waste by *Bacillus alcalophilus* (Kang and Kim, 2014) have also been reported.

When fisheries waste is discarded, it is not practical to separate its components (carbohydrates, proteins, and lipids). This means that various microbes specific for each component are required for efficient biodegradation. This could cause difficulties in optimization of biodegradation, because each strain might have different optimal reaction conditions. Particularly in the fish sauce industry, a high concentration of salt (20–25%) is added during processing (Cho et al., 2014). Therefore, salt-tolerant microbes are required, especially for the degradation of fish sauce waste. To satisfy the above requirements, a salt-tolerant strain that possesses multiple enzymes involved in the degradation of carbohydrates, proteins, and lipids is essential. In this study, we isolated a novel strain exhibiting these characteristics. The degradation of high-salinity fisheries waste containing green seaweed by this strain was characterized for industrial use.

Materials and Methods

Screening and isolation of potential fisheries-waste-degrading microbes

Potential fisheries-waste-degrading microbes were isolated from a marsh located near a coastal area in Busan. One gram of marsh sample was added to a sterile 250-mL flask that contained (per L): 10 g of green seaweed powder, 1 g of NH_4Cl, and 35 g of NaCl (pH 7). The flask was incubated at 37°C and 180 rpm for 3 weeks. After 3 weeks, 10 mL of liquid suspension were transferred to fresh medium and incubated again under the same conditions. Subsequently, the cells cultivated in the flask were spread with a platinum loop onto plates solidified with 1.5% nutrient agar. Purified isolates were obtained by repeated streaking onto fresh agar plates. Each strain was maintained on an agar plate at 4°C and transferred to fresh agar plates every 2 weeks.

Tests for diverse degradation abilities of isolates

Each isolate was spread on carboxymethylcellulose (CMC) agar (2 g/L peptone, 5 g/L yeast extract, 1 g/L carboxymethylcellulose sodium salt, and 15 g/L agar, pH 6.8), skim milk agar (10 g/L skim milk powder, 8 g/L nutrient broth, and 15 g/L agar, pH 6.8), and spirit blue agar (31.25 g/L spirit blue agar and 10 g/L tributyrin). The chemicals used in this study were purchased from Sigma Aldrich Company (St. Louis, MO, USA). All agar plates were incubated at 37°C for 24 h. After incubation, each colony that formed on the CMC, skim milk, and/or spirit blue agar plates was used in tests of degradation abilities on diverse substrates. To determine the cellulose-degrading ability of each isolate, 10 mL of Gram's iodine solution was poured onto a CMC agar plate on which a colony was formed (Kanasa et al., 2008). A clear zone was generated around the colony when the isolate possessed cellulose-degrading ability. Protein-degrading ability was determined when the isolate generated a clear zone around a colony forming on skim milk agar. The lipid-degrading ability of an isolate was determined when the colony was tinged with blue on spirit blue agar.

Identification of the isolate

After the tests for diverse degradation abilities of isolates, one bacterium simultaneously possessing cellulose-, protein-, and lipid-degrading abilities was screened. Colony morphology, Gram-staining, catalase test, malachite-green staining to test for spore formation, and microscopic examinations were performed. Next, the specific identification of the screened isolate was determined by 16S rRNA gene sequence analysis. DNA was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea), according to the manufacturer's instructions. PCR amplification of DNA using the primers 518F (50-CCAGCAGCCGCGGTAATACG-30) and 800R (50-TACCAGGGTATCTAATCC-30 was performed using a DICE model TP600 PCR thermal cycler (Takara, Japan). The 50-mL reaction mixture contained primers (10 ρmol/mL), 2.5 mM dNTPs, 10x reaction buffer, 2.5 U Taq polymerase (Takara, Japan), 1 mg template DNA, and sterilized water. The PCR reactions were carried out under the following conditions: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min. The DNA sequencing was carried out by Macrogen, Ltd. (Seoul, South Korea). The 5'- and 3'-ends of the constructs were sequenced using the

M13 primers that flanked the cloning sites. These sequences were compared with GenBank (National Center for Biotechnology Information; NCBI, Rockville Pike, Bethesda, MD, USA) entries using the Advanced Basic Local Alignment Search Tool (BLAST) similarity search option, which is accessible from the NBCI (http://www.ncbi.nlm.nih.gov/). The BioEdit Sequence Alignment Editor (version 5.0.9) was used to check the alignment and remove all positions with gaps before calculating distances with the DNAdist program in PHYLIP (version 3.5c, University of Washington, Seattle, WA, USA). Phylogenetic analysis of the given sequences and their close relatives was conducted using the neighbor joining method with 1000 bootstrap replicates in the MEGA version 5.2 software (Tamura et al., 2011).

Degradation characteristics of the isolate

To optimize the culture conditions for the degradation of cellulose, proteins, and lipids by the isolate, experiments were carried out in 500-mL flasks (with a 125-mL working volume). Each flask was incubated for 5 days in parallel at various pHs (5, 6, 7, 8, and 9), temperatures (30, 37, 45, 50, and 55°C), agitation speeds (100, 120, 150, 180, and 200 rpm), and NaCl concentrations (0, 3.5, 7, 10, 14, 17.5, and 20%). Stability of enzyme activity was also tested at various NaCl concentrations (0, 3.5, 7, 10, 14, 17.5, 20, 25, 30, and 35%). The basic culture medium for this experiment contained (per L): 5 g yeast extract, 2 g peptone, 1 g K_2HPO_4, 1 mL mineral solution, and 1 mL vitamin solution. To characterize the cellulolytic, proteolytic, or lipolytic activity during biodegradation, 10 g/L of an individual substrate (carboxymethylcellulose sodium salt, skim milk, or olive oil) were added to the basic culture medium. The mineral solution contained (per L): 3 g $FeSO_4 \cdot 7H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g $MnSO_4 \cdot H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $ZnSO_4$, and 0.5 g ethylenediaminetetraacetic acid (EDTA). The vitamin solution contained (per L): 0.2 g nicotinic acid, 0.4 g thiamine-HCl, 0.2 g nicotinamide, and 0.008 g biotin. To characterize enzyme activity on a mixed substrate during biodegradation, biodegradation of the isolate was also carried out in basic culture medium containing all three substrates. The pH of the culture medium was adjusted to 6.0 before autoclaving. All culture media, excluding the vitamin solution, were sterilized at 121°C for 15 min. The vitamin solution was separately added to the autoclaved medium after filtration through a 0.2-mm membrane. Samples were taken every 24 h from each flask to measure changes in cell concentration (optical density or number of colonies), reducing sugar concentration, viscosity, pH, and enzyme activity.

Analytical methods

Cell growth of the isolate was measured by both optical density at 600 nm using a VIS/UV spectrophotometer and the number of colonies on the agar plate, presented as colony forming units (cfu/mL). The viscosity of the culture broth, measuring shear stress in a 0.3–100 rev/min range, was determined by a Brookfield (Middleboro, MA, USA) Series LVDV-II + Pro Viscometer equipped with an SC4 chamber and 31/13R spindle. A standardized single value was obtained for each sample by interpolation or extrapolation on a log-log scale to a standard rate of shear at 100 rev/min.

The depolymerized products from cellulose biodegradation were verified by thin-layer chromatography (TLC). According to a modified method by Kim et al. (2013), supernatants of culture broth (1 μL) were first spotted on silica gel 60 TLC plates (E. Merck, Darmstdt, Germany). Then, an n-butanol:isopropanol:ethanol:water (2:3:3:2, v/v/v/v) solution was prepared and used to develop the depolymerized products. The spots were visualized by spraying with 12 N H_2SO_4 in ethanol, followed by baking at 105°C for 15 min. The standard markers used in this assay were glucose, cellobiose, cellotriose, and cellotetraose. The reducing sugars were quantified according to the 3,5-dinitrosalicylic acid (DNS) method (Ghose, 1987) in which glucose is used as the standard substrate for cellulose.

The cellulolytic, proteolytic, and lipolytic activities were analyzed according to the methods described by Ghose (1987), Meyers and Ahearn (1977), and Sharma et al. (2012), respectively. All analyses were performed using a VIS/UV spectrophotometer and measured at 540, 660, and 420 nm. One unit of cellulolytic, proteolytic, or lipolytic activity was defined as the concentration of enzyme needed to release 1 μmol of glucose, tyrosine, or ρ-nitrophenol per min per mL under standard conditions, respectively.

Results

Isolation of potential fisheries-waste-degrading microbes

From the plate assays, six different types of colonies showed various reactions on CMC, skim milk, and/or spirit blue agar plates. However, only one colony showed reactions on all three media. This isolate formed clear zones around its single colony on the CMC agar (3.6-cm diameter) and skim milk agar (3.0-cm diameter zone) plates. The colony was also tinged with blue on spirit blue agar (Fig. 1). After repeated streaking on an agar plate, we purified the strain and named it TK3-Y.

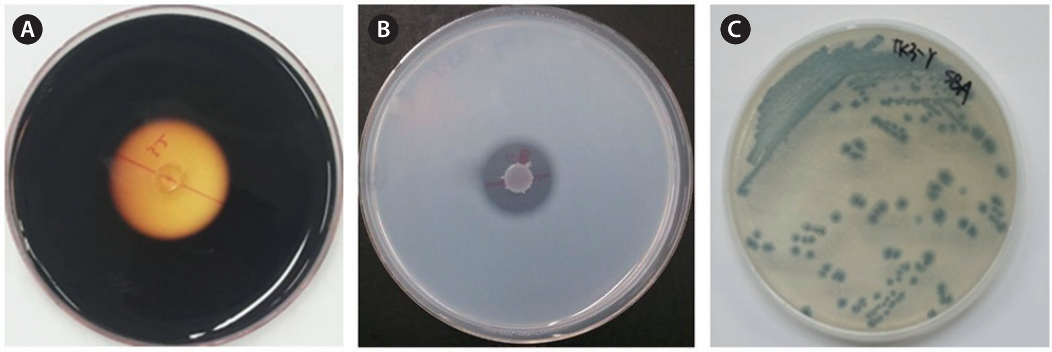

Fig. 1. Plate assay for the identification of cellulolytic activity on CMC (A), proteolytic activity on skim milk (B) and lipolytic activity on spirit blue agar (C).

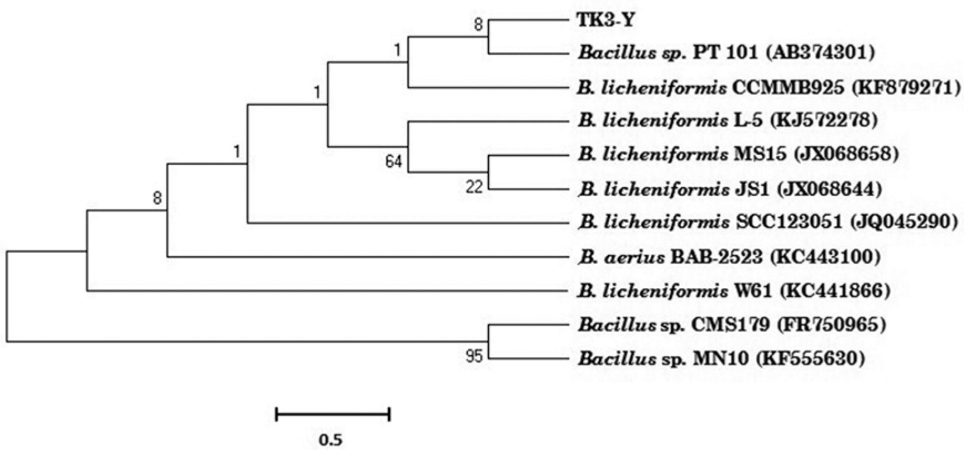

Fig. 2. Phylogenetic tree based on the partial 16S rRNA gene sequence of *Bacillus licheniformis* TK3-Y and other related *Bacillus* species.

Identification of the isolate

Based on microscopic observation, strain TK3-Y was very motile in the vegetative state and had Gram-positive rods measuring 0.5–1 μm in width and 3–4 μm in length. It occurred mostly in random groups and was catalase-positive and formed endospores. During 16S rRNA gene sequence analysis for species-specific identification, a 1662-bp fragment of the 16S rRNA gene of the isolate was amplified and sequenced. The sequence analysis of the 16S rRNA gene by BLAST confirmed that the isolate was *Bacillus* sp. PT 101 (GenBank Accession No. AB374301.1), with a sequence identity of 98%. Furthermore, the phylogenetic tree based on the partial 16S rRNA gene sequence revealed the relationships between strain TK3-Y and other related strains (Fig. 2). Strain TK3-Y was closely related to other *Bacillus licheniformis* strains. Hence, we designated the isolate as *Bacillus licheniformis* TK3-Y.

Biodegradation characteristics of the isolate

The biodegradation characteristics of the isolate on various substrates were determined in 500-mL flasks. The cultures of strain TK3-Y in 1% CMC, skim milk, and olive oil showed equally maximum cell growth and enzyme activity at pH 6, 50°C, 180 rpm, and 17.5% NaCl (Table 1). From the test of salt effect on enzyme activity, it was found that enzyme activity was stable within a range of 17.5–35% NaCl (Fig. 3). Under optimal conditions, the enzyme activities of strain TK3-Y were examined for 5 days on 1% CMC, skim milk, olive oil, and a mixture of these three substrates. During the CMC degradation, the optical density (OD_{600}) increased to 0.93 within 4 days, with a steady increase in pH (Fig. 4). The pH reached a maximum of 7.6 after 5 days. The maximum concentration of reducing sugar (0.98 mg/mL) and cellulolytic activity (1.07 U/mL) were achieved at day 4 of cultivation. As CMC was degraded, the viscosity of the culture broth decreased steadily. To obtain further evidence

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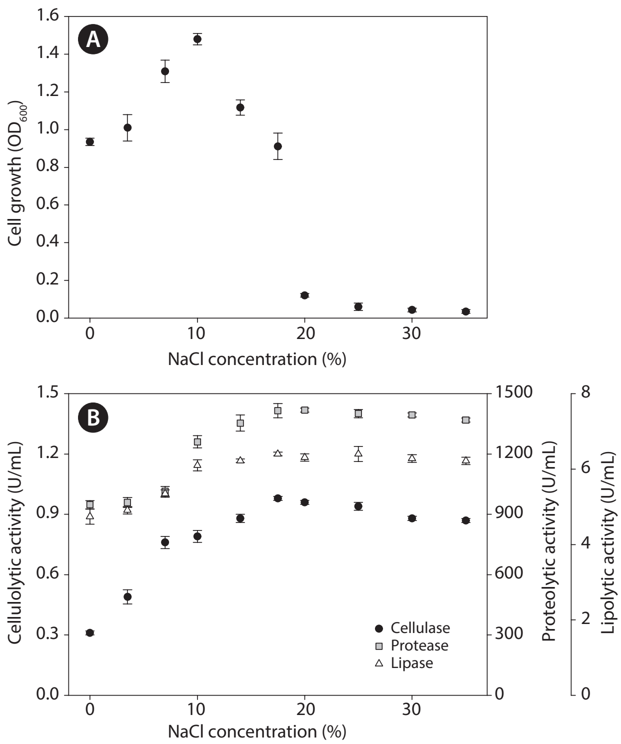

Fig. 3. Cell growth (A) and enzyme stability (B) on CMC, skim milk and olive oil media by *Bacillus licheniformis* TK3-Y cultivated at various NaCl concentrations.

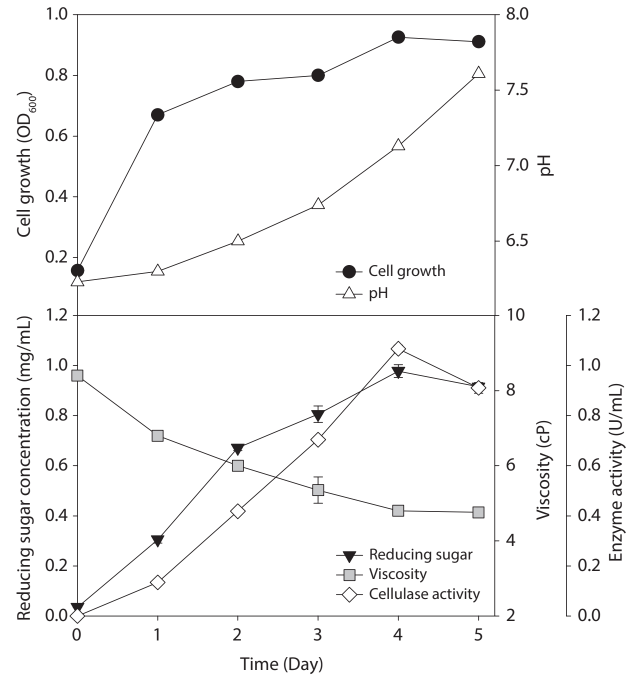

Fig. 4. Profiles of the reaction parameters for CMC degradation by *Bacillus licheniformis* TK3-Y under optimum conditions. Error bar: mean ± S.D. of the three replicates.

Table 1. Results of cell growth and enzyme activities under various culture conditions

Parameter		Cell growth (OD_{600})	Cellulolytic activity (U/mL)	Proteolytic activity (U/mL)	Lipolytic activity (U/mL)
pH	5	0.11 ± 0.01	0.01 ± 0.00	83 ± 4	0.11 ± 0.02
	6	0.72 ± 0.04	0.27 ± 0.01	730 ± 11	1.85 ± 0.01
	7	0.66 ± 0.02	0.22 ± 0.02	711 ± 6	1.61 ± 0.02
	8	0.55 ± 0.01	0.17 ± 0.03	655 ± 8	1.40 ± 0.01
	9	0.50 ± 0.02	0.13 ± 0.01	548 ± 9	0.99 ± 0.02
Temp (˚C)	30	0.53 ± 0.03	0.10 ± 0.01	512 ± 21	1.13 ± 0.01
	37	0.62 ± 0.01	0.20 ± 0.02	688 ± 13	1.99 ± 0.02
	45	0.83 ± 0.02	0.31 ± 0.02	779 ± 18	2.41 ± 0.03
	50	0.91 ± 0.10	0.36 ± 0.01	897 ± 22	3.01 ± 0.01
	55	0.85 ± 0.04	0.34 ± 0.04	852 ± 10	2.88 ± 0.02
Agitation speed (rpm)	100	0.73 ± 0.04	0.25 ± 0.04	802 ± 24	2.10 ± 0.01
	120	0.79 ± 0.03	0.30 ± 0.01	841 ± 16	2.54 ± 0.06
	150	0.88 ± 0.04	0.33 ± 0.03	886 ± 17	2.88 ± 0.04
	180	0.91 ± 0.01	0.36 ± 0.01	897 ± 22	3.01 ± 0.01
	200	0.89 ± 0.02	0.35 ± 0.02	871 ± 13	2.95 ± 0.02
NaCl concentration (%)	0	0.91 ± 0.01	0.36 ± 0.01	897 ± 22	3.01 ± 0.01
	3.5	0.94 ± 0.01	0.50 ± 0.02	911 ± 7	3.12 ± 0.04
	7	1.18 ± 0.02	0.55 ± 0.04	1014 ± 14	4.83 ± 0.01
	10	1.41 ± 0.02	0.81 ± 0.04	1148 ± 20	5.35 ± 0.07
	14	1.10 ± 0.07	0.95 ± 0.01	1365 ± 16	6.11 ± 0.01
	17.5	0.90 ± 0.04	1.07 ± 0.06	1426 ± 34	6.45 ± 0.05
	18	0.31 ± 0.01	0.51 ± 0.03	728 ± 11	3.07 ± 0.07
	19	0.10 ± 0.01	0.10 ± 0.01	97 ± 2	0.26 ± 0.01
	20	0.09 ± 0.02	0.02 ± 0.01	88 ± 4	0.13 ± 0.02

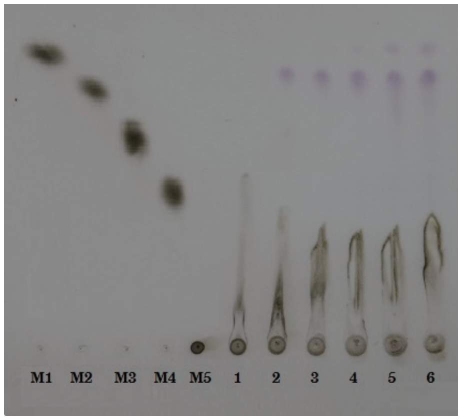

Fig. 5. TLC of the CMC degradation products in the CMC culture broth. M1 - M5 indicate standard markers. M1, glucose; M2, cellobiose; M3, cellotriose; M4, cellotetraose; M5, CMC; lane 1, day 0; lane 2, day 1; lane 3, day 2; lane 4, day 3; lane 5, day 4 and lane 6, day 5 of cultivation.

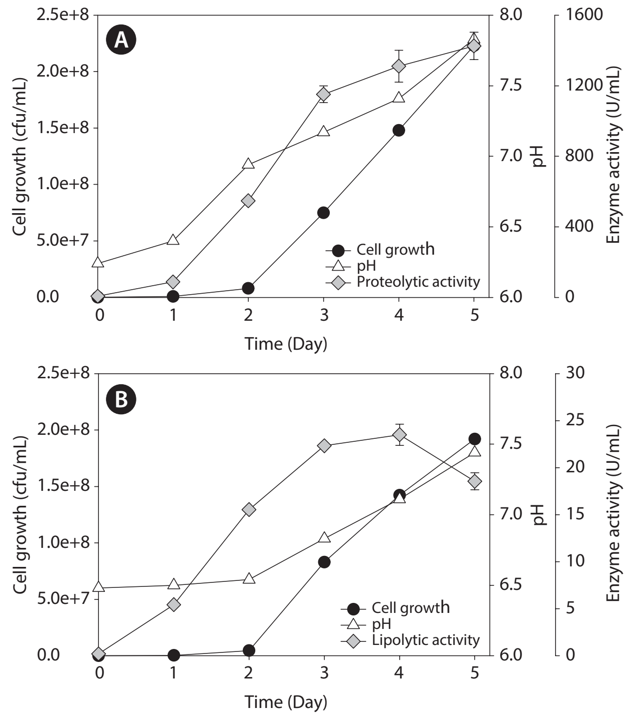

Fig. 6. Profiles of the reaction parameters for degradation of skim milk (A) and olive oil (B) by *Bacillus licheniformis* TK3-Y under optimum conditions. Error bar: mean ± S.D. of the three replicates.

of the biodegradation ability of strain TK3-Y over time in culture, culture supernatant was analyzed by TLC for 5 days. The degradation products migrated on the TLC plate as CMC was degraded over time in culture (Fig. 5). As strain TK3-Y degraded CMC, cellobiose and glucose started to appear in TLC analysis after 1 and 3 days, respectively. After 4 days, their bands were clearly visible by TLC. During the degradations of skim milk and olive oil, the growth of cells increased up to 2.3×10^8 and 2.0×10^8 cfu/mL, respectively, within 5 days (Fig. 6). Like the pH profile during CMC degradation, pH increased steadily in the degradations of both skim milk and olive oil, and after 5 days the pHs ended at 7.83 and 7.44, respectively. The maximum proteolytic and lipolytic activities were measured at 1426 U/mL (at day 5) and 6.45 U/mL (at day 4), respectively.

The biodegradation characteristics of strain TK3-Y were also determined on a simulated medium of fisheries waste in which the three substrates (CMC, skim milk, and olive oil) were added together. As shown in Fig. 7, the cells grew to a maximum of 6.2×10^7 cfu/mL and pH increased steadily, similar to the patterns of the degradations of CMC, skim milk, and olive oil. The maximum cellulolytic, proteolytic, and lipolytic activities were observed at 0.83, 1394, and 7.12 U/mL, respectively, at day 4, and pH ended at 7.79 after 5 days. Both the cellulolytic and proteolytic activities on the mixed substrate were somewhat lower than those on each single substrate. However, lipolytic activity showed the opposite tendency.

Discussion

For the efficient treatment of fisheries waste in which the components are not collected separately, a fisheries-waste-degrading microbe was newly isolated from a marsh. This isolate showed reactions on CMC, skim milk, and spirit blue agars during plate assays, indicating that it simultaneously possessed cellulolytic, proteolytic, and lipolytic enzymes. From the 16S rRNA gene sequence analysis, the isolate was designated as *Bacillus licheniformis* TK3-Y. Among *Bacillus* strains, *B. licheniformis* strains were reported to be multi-functional and multi-enzyme-producing bacteria that can degrade diverse substrates and grow under various environmental conditions (Ghani et al., 2013; Parrado et al., 2014). Several *B. licheniformis* strains possessing multiple enzymes are shown in Table 2. A *B. licheniformis* KJ-9 strain (Seo et al., 2010) could synthesize cellulase and protease, while both *B. licheniformis* VSG1 (Sangeetha et al., 2010) and *B. licheniformis* ATCC 21415 (Parrado et al., 2014) could synthesize protease and lipase. So far, however, there has been no report of a *B. licheniformis* strain simultaneously possessing cellulolytic, proteolytic, and lipolytic activities. Lin et al. (2011) reported that a mixed-cell culture could utilize a mixture of multi-substrates with various compositions, while a single-cell culture had some limits in utilizing various substrates. In addition, a mixed-cell culture may have some benefits if there is an amicable interaction between the culture conditions and microbial communities. However, the optimal culture conditions for each microbe or related enzyme in a mixed-cell culture can often differ. On the other hand, cultivation of single-cell cultures possessing multiple enzymes has some advantages: easy control of culture conditions and

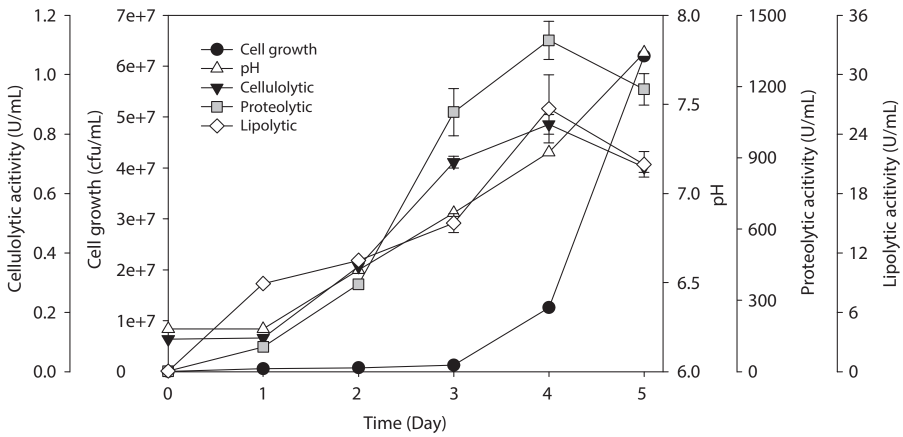

Fig. 7. Profiles of the reaction parameters for degradation of mixed substrate by *Bacillus licheniformis* TK3-Y under optimum conditions. Error bar: mean ± S.D. of the three replicates.

avoidance of poor biodegradation by variances in cell populations of mixed-cell cultures due to non-ideal culture conditions. Accordingly, strain TK3-Y is a potential bacterium for use in the efficient treatment of fisheries waste containing a mixture of cellulose, proteins, and lipids. Mixed-type fisheries waste is what is normally collected, because segregated fisheries-waste collection is not easy in practice. It is known that cellulose is relatively abundant in green seaweeds (10–13%) and also present in other seaweeds (approximately 6.6% in *Undaria* and 4.2% in *Porphyra*) (NFRDI, 2009). Accordingly, this isolate could be fit for the treatment of mixed waste containing fish and seaweed.

Some *Bacillus* strains possessing tolerance within a range of NaCl concentrations have been reported: *Bacillus flexus* (3.5–10%; Trivedi et al., 2011), *Bacillus megaterium* (4–12%; Mishra et al., 2011), *B. licheniformis* (7–12%; Ghani et al., 2013), and *Bacillus ligniniphilus* (0–10%; Zhu et al., 2014). It was also reported that a *Bacillus* sp. and *B. licheniformis*

Table 2. *Bacillus licheniformis* strains possessing multiple enzymes

Strains	Enzymes					References
	Amylase	Cellulase	Xylanase	Protease	Lipase	
B. licheniformis NH1	+	-	-	+	-	Hmidet et al. (2009)
B. licheniformis KJ-9	-	+	-	+	-	Seo et al. (2010)
B. licheniformis VSG1	-	-	-	+	+	Sangeetha et al. (2010)
B. licheniformis KIBGE-IB3	+	-	-	+	-	Ghani et al. (2013)
B. licheniformis JK7	-	+	+	-	-	Seo et al. (2013)
B. licheniformis ATCC 21415	-	-	-	+	+	Parrado et al. (2014)
B. licheniformis TK3-Y	-	+	-	+	+	Present study

Table 3. Comparison of each enzyme activity between TK3-Y and other microbes

Enzyme	Strain	Enzyme activity (U/mL)	References
Cellulase	TK3-Y	1.07 (on CMC)	Present study
		0.83 (on mixture substrate medium)	
	Enterobacter cloacae	0.20	Vasan et al. (2011)
	Aneurinibacillus thermoaerophilus WBS2	0.43	Acharya and Chaudhary (2012)
	B. licheniformis MVS1	0.54	Acharya and Chaudhary (2012)
	Geobacillus sp.	0.80	Rastogi et al. (2010)
Protease	TK3-Y	1426 (on skim milk)	Present study
		1394 (on mixture substrate medium)	
	Bacillus sp. MPTK 6	1450	Veerabadran et al. (2012)
	Bacillus firmus CAS7	2478	Annamalai et al. (2014)
Lipase	TK3-Y	6.4 (on olive oil)	Present study
		7.1 (on mixture substrate medium)	
	B. licheniformis MTCC-10498	2.0	Sharma et al. (2012)
	B. subtilis	5.0	Song et al. (2013)

RKK-04 isolated from fish sauce possess a NaCl-tolerant protease (Kim et al., 2009; Toyokawa et al., 2010). In addition, a cellulase synthesized by *B. flexus* exhibited ~70% of its maximum activity at 15% NaCl (Trivedi et al., 2011). Furthermore, both a cellulase synthesized by *B. licheniformis* AU01 and a protease synthesized by *Bacillus firmus* CAS7 have been reported to show high activities even on 30% NaCl (Annamalai et al., 2011, 2014). In this study, a newly isolated TK3-Y strain could grow on 17.5% NaCl, and its enzymes had maximum activities at 17.5% NaCl. In addition, enzyme activities were maintained in the range of 17.5–35% NaCl. The results obtained from this study imply that the salt-tolerance ability of strain TK3-Y is superior to the strains mentioned above. These results could increase the value of strain TK3-Y for use in the reutilization of fisheries waste.

During the biodegradation of CMC, the viscosity of the culture medium decreased as CMC was degraded by strain TK3-Y, accompanied by the production of reducing sugars. This result was also observed in a study of biodegradation of red-seaweed waste (Kang and Kim, 2014). The extent of CMC degradation could be verified by TLC analyses of samples taken at various culture intervals. The migration of degraded oligosaccharides was revealed as CMC was degraded by strain TK3-Y over time in culture.

The increase in pH coincided with cell growth, showing a similar tendency to the cultivation of *B. licheniformis* SVD1 (van Dyk et al., 2009). In Table 3, the activity of enzyme synthesized by strain TK3-Y is compared with enzyme activities from other strains. Cellulolytic and lipolytic activities of strain TK3-Y on both single substrates and mixed substrate were higher than those of enzymes synthesized by other strains. However, strain TK3-Y-synthesized protease showed lower activity than other proteases. It was reported that there was no effect on the production of lipase synthesized by *Candida rugosa* when sugar or mannitol was additionally added to fatty-acid culture medium (Dalmau et al., 2000); however, the production of lipase synthesized by *Burkholderia cepacia* could be increased three fold by addition of glucose to mustard-oil culture medium (Rathi et al., 2001). In this study, the lipolytic activity of strain TK3-Y on the mixed substrate was somewhat higher than that on the single substrate, olive oil. It was confirmed by TLC analysis that strain TK3-Y produced glucose even under mixed substrate conditions, due to its cellulolytic activity. The glucose produced could have affected the increase in lipolytic activity.

This study presents the possibility of reutilization of mixed-type fisheries waste through biodegradation by a multi-enzyme-possessing bacterium. The degradation characteristics of a newly isolated bacterium appear to be applicable for the reutilization of high-salinity fishery waste, and food waste as well. This biodegradation method would provide a favorable solution to critical problems regarding the prohibition of ocean dumping, reutilization of fisheries waste, and preservation of coastal environments. Further research will focus on direct degradation of non-segregated high-salinity fisheries waste by *B. licheniformis* TK3-Y.

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