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Isolation of the polysaccharidase-producing bacteria from the gut of sea snail, *Batillus cornutus*

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Abstract−This study was conducted to isolate microorganisms from the gut of the marine turban shell, *Batillus cornutus*, which inhabits the mainland of South Korea and primarily feeds on brown algae. We were interested in isolating such gut bacteria by considering their potential to produce the polysaccharidases required for digestion of brown seaweeds and isolated three different bacteria from the gut of *Batillus cornutus*. The isolated bacteria were identified as *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C. The organisms were evaluated for their ability to produce polysaccharidases such as cellulase, alginate lyase, laminarinase and kelp-lyase. *Bacillus* sp. JMP-A and *Bacillus* sp. JMP-B showed a clear zone of CMC hydrolysis with a radius 1.10 (±0.057) and 3.88 cm (±0.088), respectively, whereas *Staphylococcus* sp. JMP-C showed no zone of CMC hydrolysis. SEM analysis confirmed that the ability of the bacterial isolates to degrade kelp differs and is correlated with kelp-lyase production. The cell free extract of the *Bacillus* sp. JMP-A isolate showed the highest activities of CM-cellulase, α-cellulase, laminarinase and kelp-lyase, which were 22.76, 27.10, 66.59 and 64.36 U/mg, respectively. Meanwhile, the amount of sugars released was higher during the saccharification of kelp by dialyzed intracellular enzymes of the bacterial isolates than when dialyzed extracellular enzyme was used. Experimental results of dialyzed enzymatic saccharification of the kelp demonstrated that use of partially purified enzymes was effective for glucose production.

Key words: *Batillus cornutus*, Gut Bacteria, Polysaccharidases, Kelp-degradation, SEM

INTRODUCTION

Seaweeds are very valuable marine resources that contain a wide variety of useful substances. Cannell [1] outlined potential uses of different algae including wastewater treatment, a source of food and feed, an energy source, and in the production of polysaccharides, lipids, glycerol, pigments, enzymes and novel biologically active compounds. However, the amount of seaweed waste has increased in recent years due to its culture as an industrial resource and eutrophication of seawater. Hence, the disposal and utilization of seaweed wastes has become essential for preservation of the marine environment and recycling of organic substances [2]. Recent widespread and persistent harmful algal blooms (HABs) have had severe impacts on public health and fishery economics along all coasts of Korea. Therefore, many studies have been conducted in the last decade to elucidate the relationship between algal blooms and the marine environment, specifically in the southern coastal regions of Korea [3].

Phaeophyta includes brown seaweeds with a multicellular and branched thallus structure [4]. The division includes *Fucus* species and kelps, which include *Laminaria*, *Saccorhiza* and *Alaria* species [5]. Macroalgae such as *Laminaria japonica* (kombu), *Undaria pinnatifida* (wakame) and *Porphyra* species (nori), are extensively farmed in Asia, especially by China, Japan and South Korea [5]. Kelps are more commonly found in all coastal regions of Korea. Cellulose, alginic acids, laminarans, and fucoidans are the main polysaccharides produced by these brown seaweeds. Cellulose, which is the most abundant carbohydrate in nature, is composed of glucose residues connected by β-(1→4)-linkages [6]. Cellulase is commonly used to release glucose units from cellulosic materials [7], specifically from agricultural, industrial and municipal wastes as inexpensive carbon sources [8]. Alginates are linear unbranched polymers that contain randomly organized blocks of homopolymeric sequences of either β-(1→4)-linked D-mannuronic acid residues (polyM) or α-(1→4)-linked L-guluronic acid residues (polyG) separated by heteropolymeric sequences of polyMG arranged in a nearly alternating fashion with a wide range of molecular weights [9]. Alginate lyase catalyzes the depolymerization of alginate by β-elimination, generating molecules containing 4-deoxy-L-erythro-hex-4-enepyranosyluronate at the non-reducing end [10]. Laminarin, which is also known as callose, is the major storage polysaccharide of brown algae and consists of glucose monomers primarily joined together by β-(1→3)-glycosidic bonds with some β-(1→6)-linkages [11]. Laminarinase is a hemicellulase enzyme that is capable of hydrolyzing laminarin. To date, two laminarinases have been identified, endo-β-(1→3, 4)-glucanase (EC 3.2.1.6), which are capable of hydrolyzing both β-(1→3)- and β-(1→5)-glycosidic bonds, and endo-β-(1→3)-glucanase (EC 3.2.1.39), which is primarily responsible for the hydrolysis of β-(1→3)-glycosidic bonds [11].

Herbivorous marine invertebrates possess various types of polysaccharide degrading enzymes in their digestive fluid [12-14], which catalyze depolymerization of dietary polysaccharides into oligo- and monosaccharides. These oligosaccharides and monosaccharides are considered to be directly assimilated by the animals themselves

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or indirectly through fermentation by intestinal bacteria [15,16]. Bacteria isolated from invertebrate guts and seaweed fronds can digest a wide range of storage and structural carbohydrates; however, the degree of dependence of the invertebrate host on the enzymatic contribution of their enteric bacteria differs [17]. Recently, efforts have been made to isolate polysaccharidase producing bacteria [6,7,9] for the production of commercially important oligosaccharides and monosaccharides from seaweeds. Currently, the main bioethanol feed-stocks are sugarcane, maize, and wheat [18], which are all land-based crops; accordingly, little work has been conducted to evaluate marine biomass as a source of monosaccharides for the production of bioethanol. However, approximately 50% of the global biomass is thought to be generated in a marine environment [19]; therefore, marine biomass has great potential for use as a feed stock for future bioethanol generation [5].

Batillus cornutus is one of the most common species of turban shells inhabiting mainland of South Korea, and this organism feeds mainly on brown algae. In the present study, microorganisms were isolated from the gut of *Batillus cornutus* and evaluated for their ability to produce polysaccharidases such as cellulase, alginate lyase and laminarinase. Degradation of kelp powder using dialyzed enzymes was then conducted to check the saccharification efficiency by measuring the amount of monosaccharides released.

MATERIALS AND METHODS

1. Isolation of Bacteria from the Gut of *Batillus cornutus*

The turban shell, *Batillus cornutus*, was purchased from the local fish market, Jukdo, in Pohang, Korea. The turban shell was washed, cleaned and dissected in a laminar air flow chamber to remove its gut. The homogenized gut was then submerged in 100 ml of nutrient medium containing (g/L) NaCl (5.0), yeast extract (3.0) and peptone (2.0) in a 250 ml Erlenmeyer flask and incubated for 48 h at 30 °C while shaking at 120 rpm. Next, 1.0 ml aliquots of the culture broths were transferred into fresh medium and incubated. The culture broth was transferred repeatedly (three times) under the same conditions until the culture broth showed obvious turbidity. Next, the turbid culture broth was serially diluted, streaked onto nutrient agar plates, and incubated for 24 h at 30 °C. Morphologically different bacterial colonies were then isolated and grown separately on Lauria Berteni (LB) agar slants and preserved at 4 °C.

2. Chemicals and Reagents

All chemicals and reagents were of practical grade and purchased from Samchun Pure Chemical Co. Ltd., Korea. Di-nitro salicylic acid (DNSA) reagent was prepared according to the method described by Ghose (1987). Carboxyl methyl cellulose (CMC) was obtained from Kanto Chemical Co. Inc., Tokyo, Japan. The α-cellulose and sodium alginate were obtained from Sigma Aldrich Co., St. Louis, USA. Laminaran was obtained from TCI (Tokyo Chemical Industry, UK Ltd.), Japan. The *laminaria* lelp powder was generously gifted by Prof. Hee Chul Woo. This powder was passed through a 100 μm pore size sieve and used for subsequent experiments.

3. Gram Staining and Scanning Electron Micrographs of the Bacterial Isolates

The bacterial cells grown (24 h) in LB medium were harvested (2,862 ×g, for 5 min) and used for Gram staining and morphology studies. Gram staining was conducted as described by Dubey and Maheshwari [20] and bacteria were observed under a phase contrast microscope (Nicon ECLIPSE 80i, Nicon instrument Inc., U.S.A.) at 100× magnification. For scanning electron microscopy (SEM), the harvested cells were washed with sodium phosphate buffer (50 mM, pH 7.0) and then fixed by immersion in 2.5% glutaraldehyde at 4 °C overnight. These chemically stabilized cells were dehydrated through an ethanol series, mounted on a silicon chip and air dried in a desiccator. The mounted samples were then coated with 30 nm of Au in a Cressington high-resolution sputter coater 108 Auto, after which they were examined with a JEOL JSM-6510 scanning electron microscope operated at 5 kV.

4. Phylogenetic Analysis of the Bacterial Isolates

Genomic DNA was isolated from 1.0 ml of liquid bacterial culture using a Genomic DNA preparation kit for bacteria (Sol Gent Co. Ltd. Korea). Polymerase chain reaction (PCR) amplification of the 16S ribosomal DNA of the isolated bacteria was conducted using the forward primer (27F) 5′-AGAGTTTGATCCTGGCTCAG-3′ and the reverse primer (1492R) 5′-GGTTACCTTGTTACGACTT-3′. Each vial contained 25 μl of reaction mixture containing 16.7 μl nano pure water, 2.5 μl of 10× EF-Taq Buffer, 0.5 μl of 10 mM dNTP(T), 3.0 μl of template DNA, 0.3 μl (2.5 U) Taq polymerase and 1.0 μl of each primer. The reaction mixture was heated for 15 minutes at 95 °C and amplification was then conducted by subjecting the samples to 30 cycles of 20 seconds at 95 °C, 40 seconds at 50 °C and 90 seconds at 72 °C, with a final extension for 5 min at 72 °C. The PCR product was purified using a Sol Gent PCR purification kit (Sol Gent Co. Ltd. Korea), after which the amplified 16S rDNA fragment (about 1,500 bp) obtained from each isolate (50 ng/μl) was partially sequenced commercially (Sol Gent Co. Ltd. Korea). The gene sequences were compared with other sequences in the GenBank database using NCBI BLAST (www.ncbi.nlm.nih.gov). Gene sequences of the 16S rDNA of the selected organisms were obtained from GenBank and aligned with the sequences of our isolates using the MEGA4 software. The evolutionary history was inferred using the Neighbor-Joining method [21] and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was determined [22]. The evolutionary distances were computed using the Jukes-Cantor method [23] and are in the units of the number of base substitutions per site. The codon positions included were 1st+2nd+3rd+Noncoding. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic analyses were conducted in MEGA4 [24].

5. CMC Hydrolysis by the Bacterial Isolates

The ability of CMC hydrolysis was evaluated for three of the isolates using a plate assay according to the procedure described by Teather and Wood [25]. Briefly, basic mineral medium agar plates containing 1.0% CMC (without supplement of glucose and peptone) were bored and 100 μl of bacterial culture (grown in LB medium for 24 h) was inoculated into the hole, after which the samples were incubated at 37 °C for 72 h. Next, the plates were flooded with Congo red (0.1%), incubated at 37 °C for 30 min and then destained with 1.0 M NaCl solution. Yellow zones around the bored holes were considered to be due to hydrolysis of CMC.

6. Growth Pattern and Enzymatic Studies of the Bacterial Isolates

The enzymatic activity of bacteria was evaluated by growing the

isolates in basic mineral medium [26] containing (g/*l*) NH_4NO_3 (2.5), $Na_2HPO_42H_2O$ (1.0), KH_2PO_4 (0.5), $MgSO_47H_2O$ (0.5), Fe $(SO_4)_3$ $5H_2O$ (0.01), Co $(NO_3)_2$ $6H_2O$ (0.005), $CaCl_22H_2O$ (0.001), $MnSO_4$ $2H_2O$ (0.0001) and $(NH_4)_6Mo_7O_244H_2O$ (0.0001). All of the components were autoclaved separately and used in 100 ml batch culture medium supplemented with glucose (0.1%) and peptone (0.1%). A loop-full bacterial lawn was then inoculated into 100 ml medium in 250 ml Erlenmeyer flasks and incubated at 30 °C while shaking at 120 rpm. Aliquots were withdrawn at certain time intervals and the growth was evaluated in the same medium based on the increase in optical density at 660 nm and the amount of glucose consumed from the medium.

For enzymatic preparation, bacterial cultures were grown for 12 h (30 °C, 120 rpm), centrifuged (2,862 ×g, 5 min) and the culture supernatant was used as the extracellular enzyme. The harvested biomass was dissolved in the assay buffer (equal volume to that of culture), vortex and sonicated (Jaac Ultrasonic 2010, KODO Technical Research Co. Ltd. Korea) five times (5 min each stroke) with a 1 min interval under chilled conditions. The obtained cell free extract was used as intracellular enzyme. Cell lysis was evaluated by SEM analysis (Data not shown). The activity of CM-cellulase and α-cellulose was determined based on the procedure described by Ekperigin [27], with some modifications, using a reaction mixture (2.0 ml) composed of 0.5 ml cellulose (1.0%), 1.0 ml acetate buffer (50 mM, pH 4.8) and 0.5 ml enzyme. The activity of laminarinase was determined by the procedure described by El-Katatny et al. [28] using a reaction mixture (2.0 ml) that contained 0.5 ml laminarin (1.0%), 1.0 ml acetate buffer (50 mM, pH 4.8) and 0.5 ml enzyme. The activity of alginate lyase was determined using a previously described procedure [2,10], with brief modifications. The reaction mixture (2.0 ml) used to determine the alginate lyase activity contained 0.5 ml sodium alginate (1.0%), 1.0 ml Tris-HCl buffer (20 mM, pH 8.0) and 0.5 ml enzyme. For the activity of kelp-lyase, kelp powder (sieved using a 100 µm pore size sieve) dissolved in distilled water was used as the substrate. The reaction mixture (2.0 ml) contained 0.5 ml kelp (1.0%), 1.0 ml sodium phosphate buffer (50 mM, pH 7.0) and 0.5 ml enzyme. The reaction mixtures were incubated at 40 °C for 30 min, after which the reaction was stopped by incubating the reaction mixture at 100 °C for 3 min. This reaction mixture was then centrifuged (2,862 ×g, 1 min) to remove the enzyme/substrate debris and 1.0 ml of the supernatant was used to determine the amount of reducing sugar released due to enzymatic actions using the DNSA method [29]. The protein content was determined by the Lowery method [30] and the enzyme activity was calculated as follows: Activity=Enzyme test−(Enzyme control+ Substrate control). Unit activity was defined as µg of reducing sugars released per min per mg of protein.

7. Scanning Electron Micrograph (SEM) for the Degradation of Kelp

The ability of the isolates to degrade kelp was evaluated by adding 1.0 g of kelp powder (dry autoclaved) into 100 ml of bacterial culture (grown for 12 h at 30 °C, 120 rpm) and then incubating the samples at 30 °C and 120 rpm. After 20 days of incubation, the kelp content in the culture flask was harvested by centrifugation at 2,862 ×g for 5 min, washed with sodium phosphate buffer (50 mM, pH 7.0) and prepared for SEM analysis as described above for the bacterial SEM analysis.

8. Saccharification of Kelp by Dialyzed Enzymes

Saccharification of kelp by dialyzed enzymes was studied in terms of the amount of reducing sugar released and measured by DNSA method [30]. The 10 ml of dialyzed enzyme and 200 mg of kelp powder added to the 125 ml capacity Erlenmeyer flask containing 50 ml sodium phosphate buffer (50 mM, pH 7.0) and incubated (120 rpm) at 30 °C. After a certain interval 1 ml aliquot was withdrawn and the total carbohydrate content of the kelp was determined by using the phenol-sulfuric acid method according to the method described by Sadashivam and Manickam [31].

9. Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) and the Tukey-Kramer Multiple Comparison Test. All values shown represent the standard error of the mean (SEM).

RESULTS AND DISCUSSION

1. Identification of the Bacterial Isolates

Three different bacteria were isolated from the gut of the turban shell, *Batillus cornutus*. Two bacterial isolates were gram positive rods and one was gram positive cocci (data not shown). The scanning electron micrograph (SEM) analysis showed distinct structures of these isolates (Fig. 1). Based on the molecular data analysis, phylogenetic trees for the three isolates were constructed by comparing the nucleotide sequences with available 16S rDNA sequences. Constructed phylogenic trees for these isolates are presented in Fig. 2. Two bacterial isolates were identified as *Bacillus* sp. and one was identified as *Staphylococcus* sp. These organisms were named *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C, respectively. As shown in Fig. 2(a), *Bacillus* sp. JMP-A was most closely related to strains in the *Bacillus cereus* group, whereas the

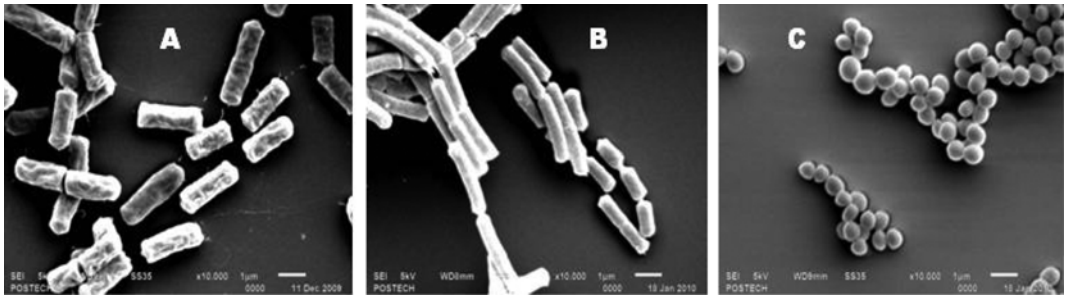

Fig. 1. The SEM images of bacterial isolates *Bacillus* sp. JMP-A (A), *Bacillus* sp. JMP-B (B) and *Staphylococcus* sp. JMP-C (C).

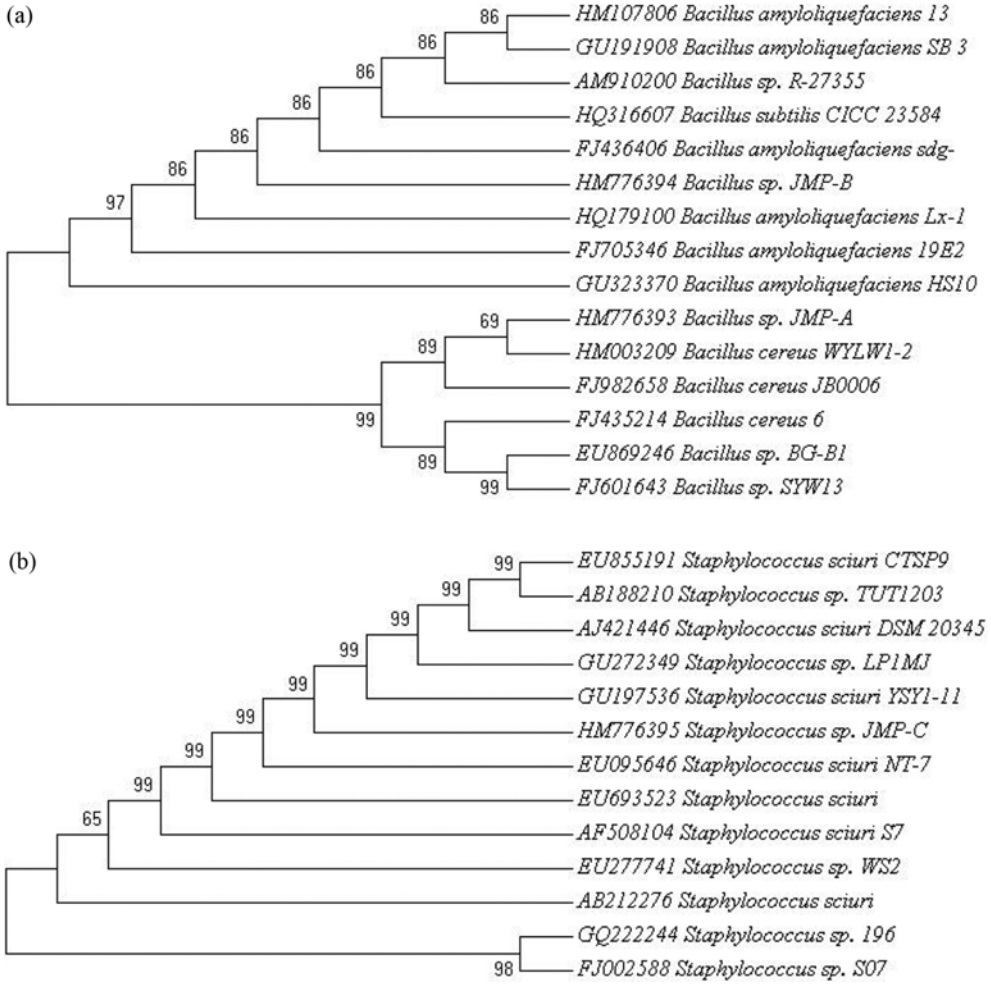

Fig. 2. The phylogenetic tree for *Bacillus* sp. JMP-A (a), *Bacillus* sp. JMP-B (a) and *Staphylococcus* sp. JMP-C (b). The evolutionary history was inferred using the neighbor-Joining method. The optimal tree with the sum of branch lengths shown for *Bacillus* sp. JMP-A, JMP-B and *Staphylococcus* sp. JMP-C was 0.35216521, 0.00758268 and 0.08944095, respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. There were a total of 1578, 1541 and 1554 positions in the final dataset for *Bacillus* sp. JMP-A, JMP-B and *Staphylococcus* sp. JMP-C, respectively. Accession number of each compared bacterial strain is given in the bracket.

results shown in Fig. 2(a) reveal a close resemblance of *Bacillus* sp. JMP-B to the strains of the *Bacillus subtilis* group and the findings presented in Fig. 2(b) show that *Staphylococcus* sp. JMP-A was closely related to members of the genus *Staphylococcus*.

Bacteria from the genus *Bacillus* and *Staphylococcus* have been reported in the literature as soil or rumen inhabitants [32-35]. Bacilli have been reported as predominant bacteria in the gut of invertebrates and play a major role in the degradation of polymeric materials under oxygen limitation conditions [33]. *Bacillus* sp. S17110 and *Staphylococcus* sp. S17111 have previously been isolated from the gut of the turban shell and applied to the production of protease [34]. Additionally, *Staphylococcus epidermidis* CMSTPi 1 has been isolated from the gut of the shrimp *Penaeus indicus* and evaluated for its ability to produce thermostable lipase [35]. Here, we report the isolation of three different bacterial strains, *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C, from the gut of turban shell, *Batillus cornutus*. Recent evidence of *Bacillus* sp. and *Staphylococcus* sp. inhabiting the gut of the sea snail *B. cornutus* has suggested that a symbiotic association developed during the course of evolution that might be based on the concomitant feeding style of this sea snail. Therefore, these bacterial isolates are considered to be novel strains that can be exploited for the production of polysaccharidases specifically for their inability to digest seaweed polysaccharides.

2. Zone of CMC Hydrolysis

The isolated bacteria were studied for their ability to hydrolyze CMC by the plate assay method and Fig. 3 shows the zone of CMC hydrolysis produced by the three isolates. *Bacillus* sp. JMP-A and *Bacillus* sp. JMP-B produced clear zones of CMC hydrolysis with radii of 1.10 (±0.057) and 3.88 cm (±0.088), respectively, whereas *Staphylococcus* sp. JMP-C did not produce a zone of CMC hydrolysis. The cell density (OD_{660}) of *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C culture during inoculation into the plate bore holes was 1.490 (±0.040), 1.473 (±0.052) and 1.937 (±0.050), respectively.

Although the Congo red test is sensitive enough for primary isolation and screening of cellulolytic bacteria, the clear zone width was not implying the amount of CM-cellulase activity, which is simi-

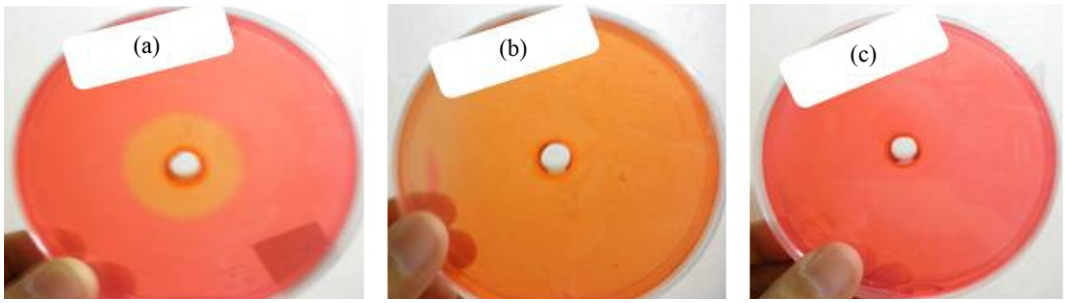

Fig. 3. The zone of CMC hydrolysis produced by *Bacillus* sp. JMP-A (a), *Bacillus* sp. JMP-B (b) and *Staphylococcus* sp. JMP-C (c).

lar to results described by Krairitthichai and Thongwai [36]. In addition, according to Krootdilaganandh [37], among seventy-seven thermo-tolerant bacterial isolates grown on CMC agar, isolate CMU4-4 exhibited the highest enzyme activity, even though its clear zone was smaller than that of other isolates. Fungi generally show higher enzyme activities during solid state fermentation than submerged fermentation [38,39]. In the case of *Aspergillus niger*, strain KKS produce considerable levels of cellulases and xylanases under submerged conditions [40], whereas strain KK2 produce more cellulases and hemicellulases during solid state fermentation [41]. These findings clearly suggest that production of an enzyme under submerged conditions may differ from that by organisms cultured on solid media and that such production may vary among microorganisms. Similarly, the bacterial species mentioned in the present study showed a high enzyme activity when grown in the liquid medium, but showed no zone of clearance when cultured on the solid nutrient medium agar plates.

3. Growth and Enzymatic Status of the Bacterial Isolates

The growth pattern of the bacterial isolates was evaluated during culture in basic mineral medium supplemented with adequate carbon and nitrogen. Fig. 4 shows the cell density and glucose consumption during the growth of the three of the bacterial isolates. At 12 h of incubation, the three isolates were at the beginning of the stationary phase. Glucose consumption by *Bacillus* sp. JMP-A was greatest, whereas *Staphylococcus* sp. JMP-C consumed the least amount of glucose during its exponential growth. Bacteria produce different polysaccharidases during different phases of growth. We studied the enzymatic status at 12 h of growth, keeping in mind that

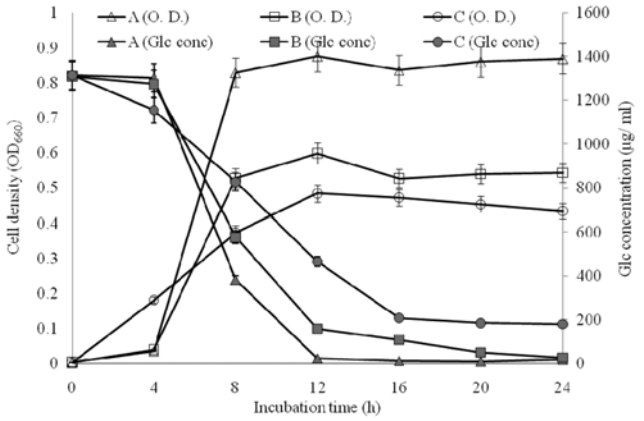

Fig. 4. Cell density and glucose consumption during the growth of *Bacillus* sp. JMP-A (a), *Bacillus* sp. JMP-B (b) and *Staphylococcus* sp. JMP-C (c).

the bacterial isolates will be used for saccharification of kelp. Table 1 shows the enzymatic status of the bacterial isolates *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C. The results indicated that *Bacillus* sp. JMP-A produced a higher activity for all enzymes when compared to the other two isolates, except for alginate lyase. Additionally, the activity of CM-cellulase and α-cellulase was higher in the cell free extract than the extracellular culture supernatant of *Bacillus* sp. JMP-A. The cell free extract of *Staphylococcus* sp. JMP-C showed no CM-cellulase or α-cellulase activity, and the cell free extract of *Bacillus* sp. JMP-B showed no α-

Table 1. The extracellular and intracellular enzymatic status of bacterial isolates *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C at 12 h of growth[$]

Enzyme	Extracellular (culture supernatant)			Intracellular (cell free extract)		
	Bacillus sp. JMP-A	*Bacillus* sp. JMP-B	*Staphylococcus* sp. JMP-C	*Bacillus* sp. JMP-A	*Bacillus* sp. JMP-B	*Staphylococcus* sp. JMP-C
CM-cellulase*	9.632±1.39	4.315±0.648	11.481±0.782	22.758±2.806	1.845±0.927	NA
α-Cellulase*	5.519±.752	0.484±0.243	6.653±1.375	27.103±5.011	NA	NA
Laminarinase*	17.610±0.991	4.937±1.053	33.173±3.754	66.585±7.205	24.172±2.782	22.008±4.417
Alginate lyase*	25.753±4.879	16.031±2.559	46.581±3.370	15.517±2.534	1.845±0.927	4.144±1.118
Kelp-lyase*	18.372±2.353	4.419±1.251	3.222±0.530	64.363±4.293	4.821±1.513	1.479±0.752

[$]Values are the mean of three experiments (standard error of the mean (SEM) [±]). Data were analyzed by one-way ANOVA
*U/ mg of protein
NA=No activity

cellulase activity in the cell free extract. The activity of laminarinase was higher in the cell free extract of *Bacillus* sp. JMP-A and *Bacillus* sp. JMP-B than in the extracellular culture supernatant. Conversely, the activity of laminarinase produced by *Staphylococcus* sp. JMP-C was higher in the extracellular culture supernatant than the cell fee extract. In the case of alginate lyase, the activity in the extracellular culture supernatant was higher than that of the cell free extract for all three isolates. The maximum activity of kelp lyase was observed in the cell free extract of *Bacillus* sp. JMP-A. The activity of kelp-lyase, indicating the ability to degrade kelp, was highest in *Bacillus* sp. JMP-A, followed by *Bacillus* sp. JMP-B and then *Staphylococcus* sp. JMP-C.

Few studies have been conducted to evaluate the use of marine bacteria for the production of polysaccharidases. *A. anitratus* and *Branhamella* sp. showed the maximum cellulase production during the exponential growth phase [27], whereas *Ochrobactrum* sp. produced alginate lyase during the stationary phase and the decline phase of growth [9]. These findings suggest that the status of polysaccharidase enzymes also depends on the physiological state of the cells and the cell density. We evaluated the enzymatic status during the early stationary phase of bacterial growth. Specifically, we evaluated polysaccharidases that would be correlated with the ability of the bacterial isolates to degrade kelp. Table 1 shows the efficiency with which the isolated bacteria produced polysaccharidases that play a major role in the saccharification of kelp. Higher kelp lyase activity in the cell free extract of *Bacillus* sp. JMP-A suggested that this strain can more effectively degrade the versatile polysaccharides present in the kelp. Despite the kelp lyase activity, the status of other polysaccharidases and their combined actions on the constituents of kelp may lead to saccharification of kelp. The maximum cellulase activity of organisms isolated from *Archachatina marginata* was observed in the culture supernatant of two bacteria species, *Acinetobacter anitratus* and *Branhamella* sp., being 0.48 and 2.56 U/ml, respectively [27]. The laminarinase activity of the gastropodean marine mollusk, *Haliotis tuberculata*, was 2.63 U/mg; however, this increased to 86.66 U/mg following purification to homogeneity by four step purifications [42]. The extracellular alginate lyase secreted by marine *Vibrio* sp. YWA isolated from decayed *Laminaria japonica* was 0.17 U/mg, which increased to 56.40 U/mg after two step purification [43]. Additionally, the production of laminarinase has been studied in several *Bacillus* sp., including *Bacillus polymyxa* [44] and *Bacillus* sp. X-b [45]. However, there have been almost no reports on the production of polysaccharidases such as cellulase, alginate lyase or laminarinase by members of the genus *Staphylococcus*. Moreover, the present study is the first to report the production of different polysaccharidases from bacteria isolated from the gut of *Batillus cornutus*.

4. Degradation and Dialyzed Enzymatic Saccharification of the Kelp

The comparative ability of the bacterial isolates to degrade kelp was evaluated with respect to the enzymatic profile. Fig. 5 shows the SEM images of the control kelp and the kelp that was degraded by the bacterial isolates. SEM revealed that the highest level of kelp degradation was produced by *Bacillus* sp. JMP-A and *Bacillus* sp.

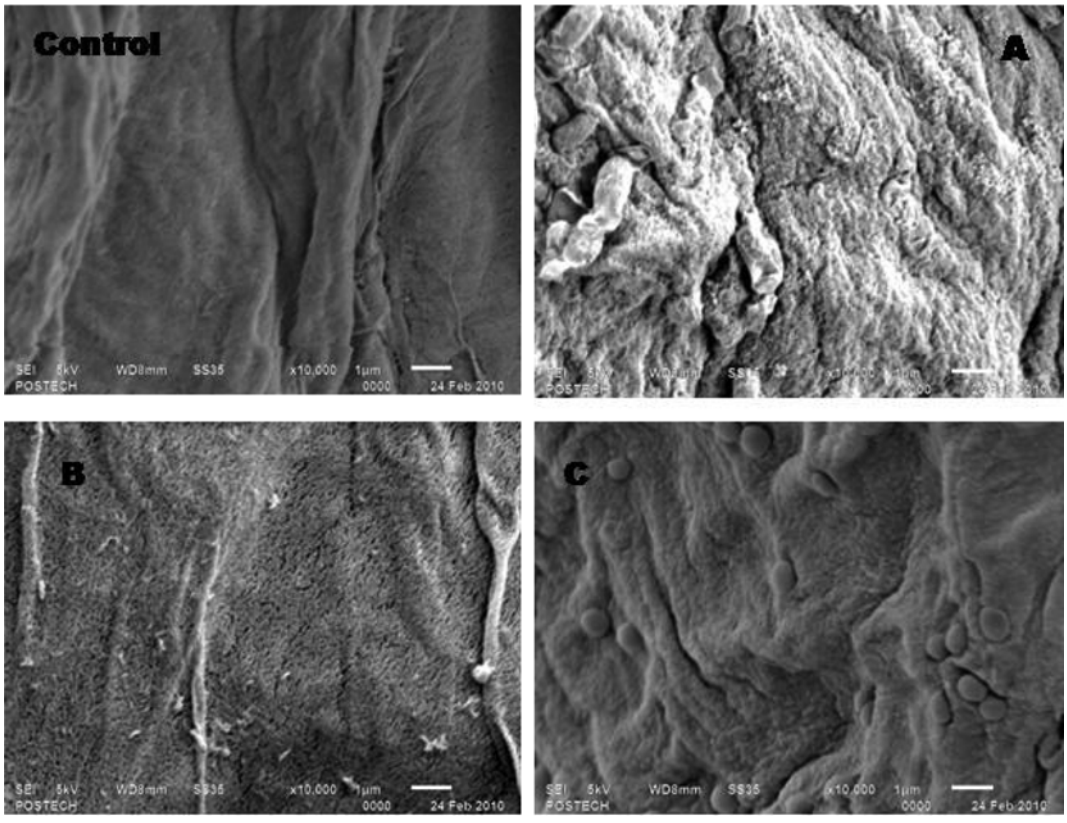

Fig. 5. The SEM images of kelp (control) after its degradation by *Bacillus* sp. JMP-A (A), *Bacillus* sp. JMP-B (B) and *Staphylococcus* sp. JMP-C (C).

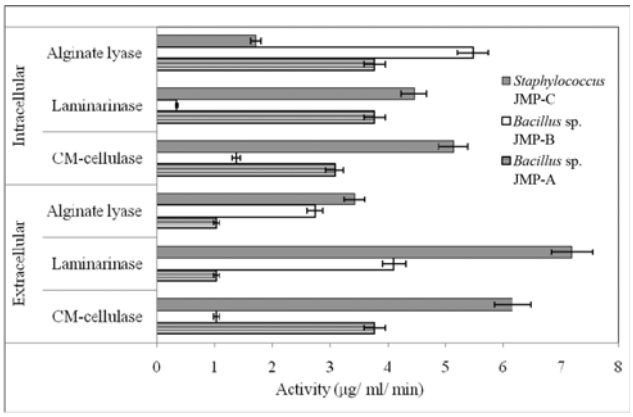

Fig. 6. Activity profile of the dialyzed enzyme.

JMP-B. We also evaluated dialyzed enzymatic saccharification of the kelp to check the amount of sugars released after kelp degradation.

Fig. 6 indicates the status of CM-cellulase, laminarinase and alginate lyase after the dialysis. Total protein obtained from *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B, and *Staphylococcus* sp. JMP-C as the extracellular enzymes in 10 ml volume was 826.99, 1,363.60 and 1,126.10 µg, respectively, whereas that of the intracellular enzymes was 61.58, 114.37 and 114.37 µg, respectively. Extracellular CM-cellulase, laminarinase and alginate lyase activity present in the 10 ml volume of enzyme obtained from *Bacillus* sp. JMP-A was 45.92, 12.52 and 12.52 U/mg, whereas that in intracellular enzyme was 513.50, 627.66 and 627.66 U/mg, respectively. In the case of *Bacillus* sp. JMP-B, extracellular CM-cellulase, laminarinase and alginate lyase activity was 7.55, 30.20 and 20.13 U/mg, whereas that of intracellular enzyme was 124.45, 31.09 and 498.00 U/mg, respectively. Meanwhile, extracellular CM-cellulase, laminarinase and alginate lyase activity obtained from *Staphylococcus* sp. JMP-C was 55.02, 64.19 and 30.56 U/mg, whereas that of intracellular enzyme was 466.81, 404.63 and 155.54 U/mg, respectively. Fig. 7 indicates the amount of sugar released from kelp by the action of dialyzed extracellular and intracellular enzymes obtained from *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C. The amount of sugars released was higher during the saccharification of kelp by dialyzed intracellular enzymes of the bacterial isolates except *Staphylococcus* sp. JMP-C than when dialyzed extracellular enzyme was used. The total carbohydrate concentrations released from kelp during saccharification by dialyzed intracellular enzymes of *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C were accumulated up to 440 µg/mL, 230 µg/mL and 60 µg/mL at 24 hours, respectively.

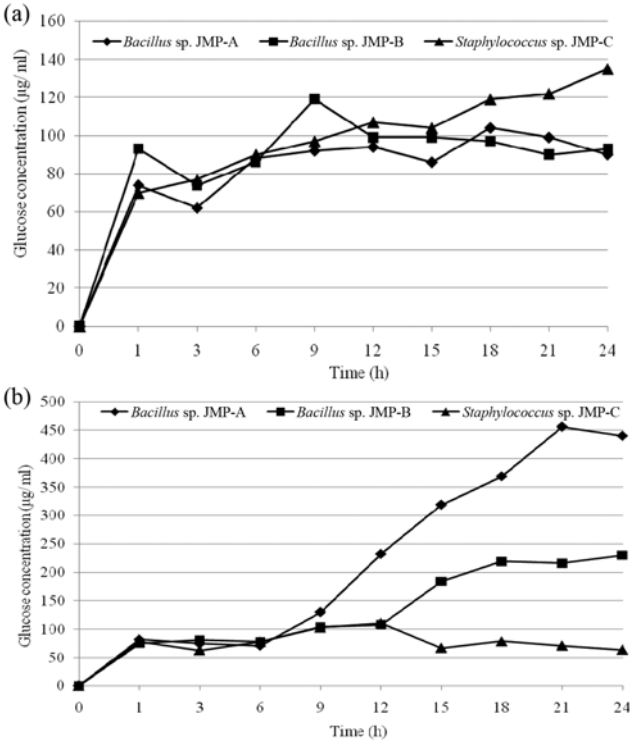

Fig. 7. Amount of released sugar concentration during the kelp degradation using dialyzed extracellular enzyme (a) and intracellular enzyme (b).

Seaweeds have long been investigated as a potential source of different polysaccharides than the crop-based mass or lignocellulosic polymers for the bioethanol production. Recently, enzymatic saccharification of marine algal biomass has been preferably studied rather than the exploitation of different microorganisms. Enzymatic methods do not put the risk of environmental contamination and work for longer time of incubation without undergoing the death phase. The saccharification of raw seaweed using liquozyme, dextrozyme, viscozyme, and rapidase with ascorbic acid has been reported as a feasible method of sugar production [46]. A seaweed Ceylon moss (Gelidium amansii) was hydrolyzed using cellulase, β-glucosidase and Xylanase, and suggested the efficiency of sodium chlorite pretreated enzymatic hydrolysis of this mass with the potential saccharification for bioethanol production [47]. Literature survey reveals that there are no reports on the production of polysaccharidases such as cellulase, laminarinase and alginate lyase, and their utilization for the saccharification of algal biomass. Hence, present studies report the vast potential of these polysaccharidases for kelp saccharification that may open a new set of processes in the field of bioethanol production.

CONCLUSIONS

Three different bacteria are isolated from the gut of *Batillus cornutus*. Two bacterial isolates are gram positive rods and one is gram positive cocci. Based on the 16S rDNA sequences analysis, isolates were identified as *Bacillus* sp. JMP-A, *Bacillus* sp. JMP-B and *Staphylococcus* sp. JMP-C. Three of the bacterial isolates showed distinct capability for the production of polysaccharidases such as cellulase, alginate lyase, laminarinase and kelp-lyase. *Bacillus* sp. JMP-A produces higher amount of extracellular as well as intracellular cellulase than that of the other two isolates. The α-cellulase activity was not found in the cell free extracts of *Bacillus* sp. JMP-B as well as *Staphylococcus* sp. JMP-C. Activity of laminarinase is greater in cell free extract of both *Bacillus* sp. JMP-A and *Bacillus* sp. JMP-B than that of extracellular culture supernatant. In contrast, *Staphylococcus* sp. JMP-C has more laminarinase activity in extracellular culture supernatant than the cell fee extract. However, alginate lyase activity was higher in the extracellular culture supernatant than the cell free extracts of the three of the isolates. For the saccharification

of kelp culture, supernatant/cell free extract seems to be more efficient than the bacterial culture. The study of dialyzed enzymatic saccharification of the kelp indicated that use of partially purified enzymes (to remove undesirable components) gave effective saccharification.

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