Symbiotic Relationship between *Microbacterium* sp. SK0812 and *Candida tropicalis* SK090404

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A bacterium growing inside yeast cytoplasm was observed by light microscope without staining. The bacterium was separately stained from yeast cell by a fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI). The bacterium actively moved inside yeast cytoplasm and propagated in company with the yeast growth. The bacterium was separated from the yeast cytoplasm by selective disruption of yeast cells and the yeast without the intracellular bacterium (YWOB) was obtained by selective inactivation of bacterial cells. The yeast and the intracellular bacterium were identified as *Candida tropicalis* and *Microbacterium* sp., respectively. The length of *Microbacterium* sp. and *C. tropicalis* measured with SEM image was smaller than 0.5 μm and was larger than 5 μm, respectively. The yeast with the intracellular bacterium (YWIB) grew in a starch-based medium but the YWOB was not *C. tropicalis* has neither extracellular nor intracellular saccharification enzyme. Glucose was produced from starch by the extracellular crude enzyme (culture fluid) of *Microbacterium* sp. YWIB produced significantly more ethanol from glucose than YWOB but did not from starch. Conclusively, *C. tropicalis* is thought to catabolize starch dependent upon *Microbacterium* sp. growing in its cytoplasm and furnish stable habitat for the *Microbacterium* sp.

Keywords: *Microbacterium* sp., *C. tropicalis*, starch-hydrolysis, saccharification enzyme, intracellular bacterium

All eukaryotes interact with microbes in relationships that can be benign, malign, beneficial, or detrimental to one or both organisms (Vivas and Goodrich-Blair, 2001). Especially, intracellular symbiosis which has been studied is found in the relationship of *Rhizobium*-legume (Valera and Alexander, 1965; Kuydendall and Elkan, 1976; Shantharam and Wong, 1982) and several insect orders (Finlay and Falkow, 1997; Goebel and Gross, 2001; Gross *et al.*, 2003). In fact, facultative or obligate intracellular bacteria can be found throughout the three of life from protists to plants and animals (Houk and Griffiths, 1980; Ishikawa, 1989; Corsaro *et al.*, 1999). Moreover, the first stable intracellular symbiotic association of one prokaryote within another prokaryotic cell was recently describes (Von Dohlen *et al.*, 2001). An ectoparasite *Bdellovibrio* (Guerrero *et al.*, 1986) and the facultative intracellular pathogen *Daptobacter* were known to be thriving within the cytosol of other bacteria (Martin, 2002; Rendulic *et al.*, 2004). Within an animal host cell, the bacteria can reside in two different compartments. Either they can be localized to a vacuole which may be derived from a phagosome formed during engulfment of the bacteria, or they may colonize the host cell cytosol (Goebel and Gross, 2001; Ochman and Moran, 2001). The mutualistic association between *Vibrio fischeri* bacteria and *Euprymna scolopes* squid was reported to be a striking resemblance to the interactions between pathogens and immune systems (McFall-Ngal and Ruby, 1998). This intracellular location may be aimed mainly at the exploitation of host metabolites in order to support bacterial multiplication in a relatively safe host compartment devoid of several potent host defense mechanisms (Lee *et al.*, 1999). Moreover, the intracellular state may contribute to the dissemination of the bacteria within the host and, after evading the host cells, their release into the environment of direct transmission to another host organism (Finlay and Falkow, 1997; Gross *et al.*, 2003).

Some bacteriocyte endosymbionts were reported to be descendents of free-living *Enterobacteriacea*; however, the relationship between host bacteria and the intracellular bacteria is still under debate (Canback *et al.*, 2004). Symbiotic associations between single-cell prokaryotes and single-cell protists have been studied based on molecular sequences (Berchtold *et al.*, 1999). A prokaryotic symbiont belonging to the order *Bacteroidales* was identified as an intracellular endosymbiont of the protist *Pseudotrichonympha grassii* (Noda *et al.*, 2005). Some gut flagellates are regularly colonized by endosymbionts located in the cytoplasm or in the nucleus (Ohkuma, 2003; Stingl *et al.*, 2005). The symbiotic interaction between single cell organisms is not general phenomenon and its physiological mechanism is not definitely examined. We found a moving particle in the yeast cytoplasm and isolated that from the yeast cell, but on the other hand the *Microbacterium* sp. was cured from the cytoplasm of *C. tropicalis*.

In this study, we characterized the *Microbacterium* sp. and *C. tropicalis* to estimate the possibility that the *Microbacterium* sp. may be an endosymbiont and *C. tropicalis* may

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be a host. The *C. tropicalis* produced ethanol from glucose like other yeast strain and the *Microbacterium* produced glucose from starch by an extracellular crude enzyme *in vitro* test; however, *C. tropicalis* with the intracellular *Microbacterium* sp. did not produce ethanol from starch. This is the first discover that a bacterium species is growing inside yeast cell.

Materials and Methods

Isolation of yeast
A yeast species was isolated from sediment of Jungrangcheon located in Sanggye-dong (Korea). The yeast was cultivated in a medium containing 10 g/L of glucose and 1 g/L of yeast extract. A moving-particle inside yeast cytoplasm was observed under a light microscope, which was separated from yeast cell by selective disruption of yeast.

Separation of moving particle
In order to separate the bacterium growing inside the yeast cytoplasm, yeast cells were disrupted by a mini bead beater (Biospec, USA). The 0.5 mm beads were used for selective disruption of yeast cells. Tubes and beads were autoclaved to protect contamination and all procedures for bacterial separation were performed under aseptic condition. The bead beater was operated at 4°C and 2,500 strokes for 10 min, and then the disrupted cell suspension was diluted and spread on agar plate containing 2 g/L of yeast extract. After incubated at 30°C for 48 h, the morphologically different colonies from yeast cells were transferred to broth medium composed of 2 g/L of yeast extract, which was specified as the intracellular bacterium.

Curing of intracellular bacterium
All procedures to cure the intracellular bacterium from yeast cytoplasm were aseptically performed to protect contamination. 100 ml of yeast culture was centrifuged at $3,000 \times g$ and 4°C for 30 min and the precipitant was suspended in 50 ml of saline and then divided into 5 ml in test tubes. The cell suspensions were treated with microwave range (Samsung, Korea) for 5, 10, 15 sec. One hundred microliters of the treated cell suspension was spread on agar plate containing 2 g/L of yeast extract. After 48 h incubation, the emerged colonies were transferred to broth medium composed of 2 g/L of yeast extract. The colonies without the intracellular bacterium were selected using a light microscope, which was specified as the yeast without intracellular bacterium (YWOB) by contrast with the yeast with intracellular bacterium (YWIB).

Growth on carbohydrates
The YWOB and intracellular bacterium were cultivated on starch and sugar-base carbohydrates to compare the basic catabolism. Medium composed of 0.1 g/L of yeast extract and 5 g/L of individual carbohydrate was prepared in test tubes with the Durham tube. Pre-cultivated cells in a medium containing 5 g/L of yeast extract were washed with saline by centrifugation at $5,000 \times g$ and 4°C for 30 min. Suspended cells in 2 volumes of saline was used as an inoculum and inoculation size was adjusted to 5% (v/v). The growth of

bacterium or yeast was determined with gas collected in the Durham tube or based on turbidity increase.

DAPI staining of yeast and bacterium
The culture of YWIB and YWOB were filtered through black polycarbonate filter (pore size, 0.22 μm, Millipore, USA), which was rinsed twice with 10 ml of double distilled water by vacuum suction. One hundred microliters of DAPI solution (0.5 μg/ml) was dropped on the filter and then incubated at room temperature for 60 min. The filter was rinsed with 50 ml of double distilled water by vacuum suction and immersed with a drop of buffered glycerin, and covered with a cover glass. The oil immersion objective was examined under a fluorescence microscope (Karl Zeiss, Axioskop 50, German) with UV light (Saby *et al.*, 1997).

Preparation of SEM image
The yeast and bacterial cells were fixed in 4% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4). The procedures for dehydration and sample preparation were performed by the general method (Corsaro *et al.*, 1999). The scanning electron micrograph was prepared in the Korea Basic Science Institute (KBSI) located in Daegu metropolitan city.

Identification
Bacterial 16S-rDNA was amplified via direct PCR using the following universal primers: forward; 5'-GAGTTGGATCCT GGCTCAG-3' and reverse; 5'-AAGGAGGGGATCCAGCC-3'. PCR reaction mixture (50 μl) was consist of 2.5 U Taq polymerase, 250 μM of each dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 100 ng template, 50 pM primer, and 1.5 mM $MgCl_2$. Amplification was conducted for 30 cycles of 1 min at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C using a PCR machine (T Gradient model, Biometera, German). The PCR products were directly sequenced with an ABI Prism 3700 genetic analyzer upon request to a professional company (Macrogen Inc., Korea). The 16S rDNA sequences were analyzed using the GenBank database, and identification was performed on the basis of 16S rDNA sequence homology.

Meanwhile, 18S ribosomal DNA of yeast was amplified by direct PCR using a yeast forward primer (yfor2); 5'-GCA GTAAAAAGCTCGTAGTTGAAC-3', and a reverse primer (yrev2); 5'-CTTACTAGGAATTCCTCGTTGAAGA-3' (Berchtold *et al.*, 1999). All of other procedures were completely same to those for amplification of bacterial 16S-rDNA except the annealing temperature (54.7°C). The 18S rDNA sequences were analyzed using the GenBank database, and identification was performed on the basis of 16S rDNA sequence homology.

Growth on starch
The YWIB, YWOB and the intracellular bacterium were cultivated in a medium containing 0.1 g/L of yeast extract and 10 g/L of starch. During cultivation, 1 ml of culture was sampled from each culture and diluted with saline by 10-folded dilution method. The diluted cultures were spread on agar plate containing 2 g/L of yeast extract. After 3 days of cultivation, the emerged colonies were counted and de-

Table 1. Primers prepared based on extracellular glucoamylase gene STAI of *Saccharomyces diastaticus.* One product was obtained from PCR performed with chromosomal DNA of *C. tropicalis* SK090404 (FJ889131)

Source of primer	Primer pair	Nucleotide sequences (F, Forward; R, Reverse)	Location	Annealing temp. (°C)
Extracellular glucoamylase gene STA1 in *S. diastaticus*	I	F: 5'-AACTCCATTCAGCTCTGC-3' R: 5'-TGTTGTAACAGGGGCGGTAGC-3'	160-795	No product
	II	F: 5'-GGTGAAACTACCTCTGGATGC-3' R: 5'-TCCGTGAAAGCCGTGTTGTCG-3'	691-1391	54.8
	III	F: 5'-CGTTGGGACCTGAGGTTC-3' R: 5'-ATCGTGGACGGTGTTCGCAGC-3'	1537-1866	No product
	IV	F: 5'-GATGTTGCACATGCGTTCG-3' R: 5'-TGTTCCCACGTGGCTTTCAGC-3'	1938-2323	No product

scribed as a colony-forming unit (CFU).

Crude enzyme preparation
The YWIB and *Microbacterium* sp. were cultivated in a medium containing 5 g/L of starch and 0.1 g/L of yeast extract for 7 days. Microorganisms were separated from culture fluids by centrifugation at 5,000×g and 4°C for 40 min. The cell-free culture fluid was more than 500 times concentrated with an ultra filtration apparatus (Amicon, USA) equipped with YM 30 membrane filter (Millipore, USA), which were used as the extracellular crude enzymes. The harvested YWIB and *Microbacterium* sp. were suspended in 50 mM Tris-HCl buffer (pH 7.5) and disrupted by bead beater with 0.5 mm and 0.1 mm beads, respectively. The bead beater was regularly stopped and operated at the intervals of 30 sec at 4°C for 60 min to maintain cooling temperature of samples. The suspension of disrupted cells was centrifuged at 8,000×g and 4°C for 40 min to separate cell-free extract, which was used as the intracellular crude enzyme. Protein concentration was determined with Bradford reagent (Bio-Rad, USA) and bovine serum albumin.

Crude enzyme activity
5 g/L soluble starch in 25 mM phosphate buffer (pH 7) was mixed with same volume of crude enzyme and then incubated at 30°C for 3 h. After incubation, the enzyme-substrate mixture was analyzed with HPLC to estimate the glucose production from starch. Specific activity of crude enzyme was determined by glucose production per min and mg protein of crude enzyme (mg glucose/min/mg protein).

Ethanol production
The YWIB and YWOB were cultivated in medium composed of 100 g/L of glucose or 50 g/L of starch and 5 g/L of yeast extract to estimate ethanol production from glucose and starch. The ethanol production was analyzed with HPLC.

Analysis
The glucose and ethanol contained were analyzed via HPLC with an Aminex HPX-87H ion exchange column (Bio-Rad, USA) and a refractive index detector. The column and detector were adjusted to a temperature of 35°C. The mobile phase was sulfuric acid (0.008 N) and the flow rate was 0.6 ml/min. The samples prepared via 30 min of centrifugation at 12,000×g and 4°C were filtrated with a membrane filter with a pore size of 0.22 µm in order to remove the microparticles. The filtrate was then injected into the HPLC in-

jector, and the injection volume was controlled automatically with a 20 µl loop. The concentrations of glucose and ethanol were calculated on the basis of the peak area in the chromatograms obtained with standard materials.

Glucoamylase-coding gene
The glucoamylase-coding gene was amplified by direct PCR using four pairs of primers, which were designed using the full sequence (2,334 bp) of the *S. diastaticus* gene encoding extracellular glucoamylase (GenBank accession no. X02649) (Douglas *et al.*, 1989), as shown in Table 1 (Yamashita *et al.*, 1985). Amplification was conducted by same method and procedures used for the 16S rDNA amplification, although the annealing temperature was modified. The annealing temperature used for amplification was programmed at a gradient from 46.5°C to 60°C at intervals of 1.5°C in the consideration of the optimal annealing temperature (54.3 and 57.6°C). The PCR products were directly sequenced by a professional company (Macrogen Inc., Korea) with an ABI Prism 3700 genetic analyzer upon request. The product sequences were analyzed using the GenBank database.

Electrophoresis
SDS-PAGE technique used in the present study was adapted from Laemmli (1970). Samples were prepared from cell-free culture fluid of *Microbacterium* sp. The culture fluid was more than 1,000 times concentrated with ultra-filtration
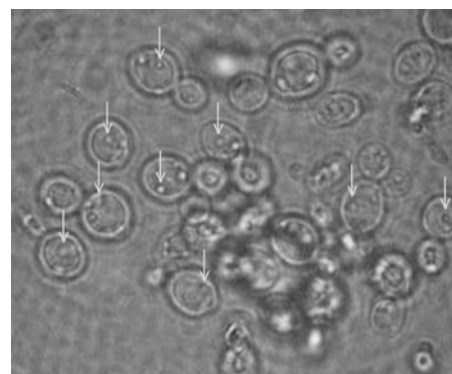

Fig. 1. A light microscopic image of yeast cells captured from a dynamic imaging. Arrow marks indicate the inner bacterial cell growing inside of the yeast cell. The bacterial cells are frequently out of focus by moving.
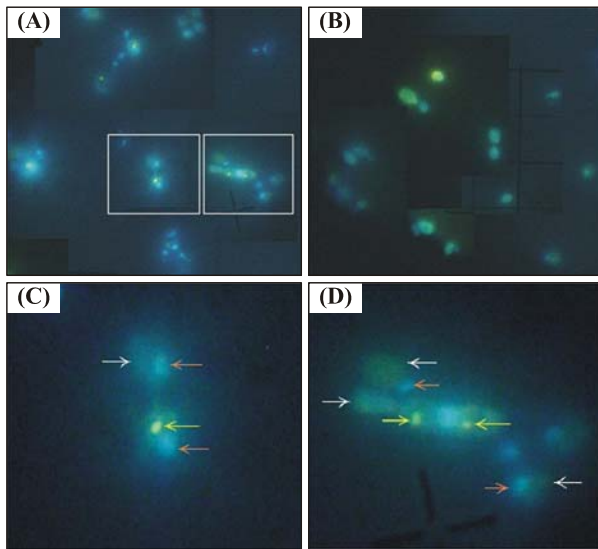
Fig. 2. Yeast cells made fluorescent by staining with DAPI. DAPI works by penetrating cells and binding to DNA, which typically makes prokaryotic cells fluorescence uniformly. The bright spots in the yeast cells are bacterial cells growing within yeast cells (A), which was contrast with the yeast cell without the inner bacterial cell (B). Figure C is an enlarged image of left box and Fig. D is that of right box in Fig. A. White arrows indicate yeast cells, pink ones do yeast nucleus and yellow ones do the inner bacterial cell.

system (Amico, USA) and YM-30 membrane (Millipore, USA). Native PAGE for activity-staining of saccharification enzyme band was performed by same technique with SDS-PAGE, in which SDS was substituted by distilled water and protein sample was not treated with SDS and mercaptoethanol and by boiling. After the concentrated culture fluid was resolved by PAGE, the acrylamide gel was soaked in 3 g/L of starch solution in 25 mM Tris-HCl buffer (pH 7.5) at 30°C for 30 min. The acrylamide gel was quickly washed with distilled water and an iodine solution was spread on the acrylamide gel to induce a starch-iodine reaction, by which the gel was converted to dark blue except the band of saccharification enzyme.

Results

Morphological characterization
In the 1,500 times enlarged micrograph, a particle was observed as shown in Fig. 1. The particles were frequently out of focus by continuous moving but converted to a photo image by image-capturing. In fluorescence image, the particle (yellow arrow marks) was more intensively stained than yeast cells as shown in Fig. 2A, which was significantly differentiated from the yeast nucleus (red arrow marks). No specific intensive image inside yeast cells were observed in the YWOB picture as shown in Fig. 2B.

SEM image
The isolated *Microbacterium* sp. from yeast cell and the *C. tropicalis* were separately cultivated and morphologically compared using SEM image. Mixture of two microorganisms
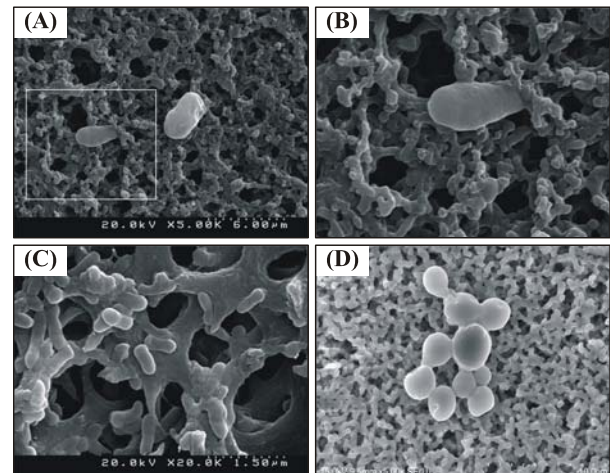

Fig. 3. SEM pictures of bacterial cells growing within the yeast cell and yeast cells. (A) was prepared to compare the size of bacteria and yeast at a glance. (B) was an enlarged image of box in (A), by which bacterial image can be discriminated. (C) was the bacterial cells separately prepared from yeast cells, in which bacterial size is greatly smaller than the pore size (0.22 μm) of membrane filter. (D) was the yeast cells separately prepared from bacterial cells, in which yeast size is greatly larger than the pore size (0.45 μm) of the lattice-type membrane filter.

was examined by SEM to compare the size in one picture as shown in Fig. 3A. In the SEM image showing two microorganisms, size of *Microbacterium* sp. was more than 10 times lower than *C. tropicalis* as shown in Fig. 3B. In a detailed SEM image, the *Microbacterium* sp. was smaller than the pores size (0.22 μm) of membrane filter as shown in Fig. 3C; however, the *C. tropicalis* was significantly larger than the pores size (0.45 μm) of membrane filter as shown in Fig. 3D.

Growth on carbohydrates
The YWOB and intracellular bacterium were cultivated on different sugar-based carbohydrates to compare the sugar-

Table 2. Growth of YWOB, YWIB and *Microbacterium* sp. on various carbohydrates. The growth was determined with gas collected in Durham tube or turbidity variation.

Carbon sources	YWOB	YWIB	Microbacterium sp.
Starch	X	O	O
D-Glucose	O	O	O
D-Fructose	O	O	O
D-Mannose	O	O	O
Maltose	O	O	O
Lactose	X	X	X
Trehalose	X	O	O
D-Mannitol	X	O	O
D-Melibiose	O	O	O
Sucrose	O	O	O
Xylose	O	O	O

O, growth; X, no growth

Table 3. Viable cell number of *C. tropicalis* SK090404 and *Microbacterium* sp. SK0812 cultivated in a medium composed of 5 g/L starch and 0.1 g/L yeast extract. The *Microbacterium* sp. growing inside of yeast cytoplasm was cured or maintained to estimate the effect of the bacterial cell on yeast growth

Microorganisms	CFU/Incubation time (day)				
	1	2	3	4	5
YWOB	0	2	2	40±8	200±42
YWIB	10±2	1,310±45	2,950±350	34,800±2,200	200,800±8,500
Microbacterium sp.	61,500±1,640	343,000±23,000	1,542,000±68,000	1,145,000±85,000	954,000±52,000

Table 4. Ethanol production by YWOB and YWIB in a medium containing 100 g/L of glucose or 50 g/L of starch and 5 g/L of yeast extract under anaerobic condition for 2 days

Yeast strains	Glucose (g/L)	Starch
YWIB	41.3±2.5	Not detected
YWOB	24.2±4.1	Not detected

utilizing character. As shown in Table 2, the YWOB did not utilize starch but the intracellular bacterium utilized. On the basis of this result, the growth of YWOB, YWIB, and intracellular bacterium on the starch-based medium containing 5 g/L of starch and 0.1 g/L of yeast extract was analyzed by the viable cell count method.

Growth on starch
As shown in Table 3, the YWIB and intracellular bacterium grew on starch-based medium; however, the viable cell number of YWOB was significantly lower than the YWIB and intracellular bacterium. A few number of YWOB grown on the starch-based medium might utilize the yeast extract (0.1 g/L) contained in the medium as substrate.

Identification
16S-rDNA of the intracellular bacterium and 18S-rDNA of the YWOB was analyzed by GenBank database system. The intracellular bacterium was 92% identified with *Microbacterium* sp. and the YWOB was 98% identified with *Candida tropicalis*. GenBank accession no. of the *Microbacterium* sp. and the *C. tropicalis* was FJ562096 and FJ889131, respectively.

Saccharification of starch
Both extracellular and intracellular crude enzyme of YWIB and intracellular crude enzyme of the *Microbacterium* sp. did not catalyze the saccharification of starch. Experimentally, 1.86±0.35 mg/L of glucose was produced from starch by mg of extracellular crude enzyme of the *Microbacterium* sp. for 1 min.

Ethanol production
YWIB grew in the starch-based medium but did not produce ethanol from starch as shown in Table 4. YWIB produced 41.3±2.5 g/L of ethanol and YWOB did 24.2±4.1 g/L of ethanol for 2 days.

Amplification of glucoamylase-coding gene
Both intra- and extra-cellular crude enzyme obtained from YWIB (*C. tropicalis*) did not catalyze glucose production from starch. One DNA product (Table 1) was obtained from PCR of four primers and chromosomal DNA of *C. tropicalis* as shown in Fig. 4. The DNA product was not identified with any glucoamylase-coding gene in GenBank database system but 66% homologous with *Candida dubliniensis* CD36 chromosome 7.

Extracellular proteins of *Microbacterium* sp.
As shown in left part of Fig. 5, three protein bands were observed in SDS-PAGE. One of the three proteins hydrolyzed starch on acrylamide gel as shown in right part of Fig. 5. The difference of protein band position between SDS-PAGE and native PAGE was caused by the structural difference of proteins used in the PAGE. Natural proteins were used for activity straining of the Saccharification enzyme in the native-PAGE.

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1GGTGACTAGCATCTAATCCTGTTGGAG28CTTTTCCAAACGGATTAATCAACCCAGTGACCGCTA
ATAAATCAATTTGAAGAATAAACTCCTCTGCAGAAGCATCGGCCGGATAACCACTAATTGCAGAAA
CGGGAACATACCAGTTTAAATAACCGTCCAAGAAATTGATCTCTCATGCTCCATAGGCATTACAAG
ATAATTGGCTGAAAAATTCAAACTCACAAGGGCTATCTCCCATACAATTTGAACGTTTGATTTTAT
AACATGAAGAAATGATTTCTTAGATGCTAATTTATACATTTTAAATCACCCCCTTGAATTATCATA
TCAACGTCTTGATGTTTGGGAGAGGAGTTATTTCTTCAAAAGTTTTCCACGCCACACCCCCACGAG
TTGAATCGTTTTGATAAACACCAACATGATTGTCAGATGATAGTTGCACTCGGATGACATCGGGAC
CTGATTCTATA470TTTATCCCCTATCCGCCTGATTATAAACAATTTAAAGCGACCCATCTCCGAT
TTCGGCGACAACCCTACCATCCATAGCACCCTCTACTTTGGCTGCCCTATTAGCAAGTTGGGGAGT
CAATCGGTAACACGGCTTTCACGTAAAATCAGTCAGCGGTTATGCTTCGCGTGCTACATGNTGAGC
AGGCGCCCACCAGTATTTT700
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Fig. 4. PCR product obtained from primer II (Table 1) and template that is chromosomal DNA of *C. tropicalis*. 443 base pair of the PCR product (700 bp) was 66% homologous with *C. tropicalis* CD36 chromosome 7 (heavy character) in the GenBank database system.
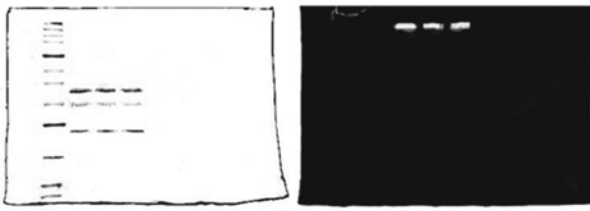
Fig. 5. SDS-PAGE (left) and active-staining PAGE (right) of the extracellular protein of *Microbacterium* sp. grown on starch-base medium for 10 days. The cell-free culture fluid was 1,000 times concentrated by ultra-filtration system and YM-30 membrane filter. Lane 1 indicates protein marker that are 212, 158, 116, 97.2, 66.4, 55.6, 42.7, 34.6, 27, 20, 14.3, 6.5, 3.4, and 2.3 kDa from upper.

Discussion

The morphological character of *C. tropicalis* SK090404 and *Microbacterium* sp. SK0812 examined by a light microscope, DAPI staining technique and SEM presented a crucial clue that *Microbacterium* sp. can grow both inside and outside cytoplasm of *C. tropicalis*. A relationship between the intracellular *Microbacterium* sp. and *C. tropicalis* is possible to be a symbiosis based on the *in vitro* and *in vivo* growth of *Microbacterium* sp. and the viable cell number of YWIB and YWOB cultivated on starch. The physiological function of *Microbacterium* sp. *in vivo* may be to produce glucose from starch for *C. tropicalis*, meanwhile, *C. tropicalis* is supposed to furnish more stable habitat for the small-sized *Microbacterium* sp. than natural environments (Zientz *et al.*, 2004; Wanner *et al.*, 2008). The extracellular saccharification enzyme secreted by the intracellular *Microbacterium* sp. may function as if an intracellular enzyme for *C. tropicalis* growing on starch; however, the saccharification enzyme is conditionally effective dependent upon the physiological function of *C. tropicalis* capable of transporting starch from outside to cytoplasm. The physiological ability of *C. tropicalis* for starch uptake was verified by the viable cell number of YWIB and YWOB cultivated on the starch-base medium. During separation of the *Microbacterium* sp. from *C. tropicalis*, the YWIB grew on agar plate containing starch as a sole carbon source but did not degrade starch around colonies (data not shown), which is a visual evidence that extracellular saccharification enzyme was not secreted by YWIB. The *C. tropicalis* was biochemically and genetically confirmed not to produce the saccharification enzyme by enzymatic reaction and DNA sequence homology of glucoamylase-coding gene.

At least one of three extracellular proteins produced by *Microbacterium* sp. may be a saccharification enzyme, of which specific activity was 1.86±0.35 mg/L of glucose/min/mg protein. The concentration of glucose produced in cytoplasm of *C. tropicalis* may be arithmetically estimated based on the specific activity. On the basis of the specific activity of Saccharification enzyme, minimal 5.36 g/L of glucose can be produced in the cytoplasm of *C. tropicalis* by mg of extracellular saccharification enzyme for 2 days. Theoretically, ethanol can be fermented from intracellular glucose gen-

erated by saccharification of starch or extracellular glucose supplied as a medium ingredient. However, YWIB did not produce ethanol from starch but produced significantly more ethanol from the extracellular glucose than YWOB. This is a clue that the intracellular *Microbacterium* sp. may produce some physiological factors to activate ethanol fermentation of YWIB under anaerobic condition.

The relationship between *Microbacterium* sp. SK0812 and *C. tropicalis* SK090404 may be not syntrophism based on the growth pattern of *Microbacterium* and YWOB on sugar-base carbohydrates (Johannes *et al.*, 2000; De Bok *et al.*, 2002). In the syntrophic relationship, the one organism is absolutely dependent upon the metabolite produced from other organism whose metabolism has to be stopped without consumption of its metabolite by the syntrophic mate. The *Microbacterium* sp. may be an intracellular symbiont under only a specific growth condition in consideration of the growth difference of YWIB and YWOB on starch; however, the function of *C. tropicalis* for the *Microbacterium* sp. may be a host itself.

Conclusively, the physiological function of the *Microbacterium* sp. SK 0812 may be limited to produce glucose from starch when *C. tropicalis* SK090404 grows on starch-rich environment, but on the other hand *C. tropicalis* may be limited to furnish a stable habitat to the *Microbacterium* sp. in compensation for glucose production from starch.

Now, we are analyzing the effect of nutritional factors on growth and ethanol production of *C. tropicalis* SK 090404 with and without the intracellular *Microbacterium* sp. using various metabolic intermediates and amino acids. Another two proteins excreted by *Microbacterium* sp. will be biochemically analyzed using two-dimensional SDS-PAGE and protein identification technique.

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