A DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Isolation of cellulolytic *Bacillus* species indigenous to Jeju Island, comparison of their cellulase genes and application to cucumber seedling growth

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Department of Food Science and Engineering

GRADUATE SCHOOL

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제주지역 토착 섬유소 분해성 바실러스 균주의 분리, 섬유소 분해효소 유전자의 비교 및 오이 육묘에의 응용

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Isolation of cellulolytic *Bacillus* species indigenous to Jeju Island, comparison of their cellulase genes and application to cucumber seedling growth

YuKyoung Kim

(Supervised by Professor Young Hwan Ko**)**

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The bioconversion of cellulose and hemicellulose to soluble sugars is important for global stabilization and a sustainable human society. Here, hundreds of cellulolytic bacteria were screened and isolated from soil, compost, and animal waste slurry. Among the isolates, three strains, SL9-9, C5-16, and S52-2, showing higher potential for practical uses, were purified on carboxymethylcellulose (CMC) agar plates and identified as *Bacillus subtilis* strains by morphological, physiological, and biochemical characterization and 16S rRNA gene analysis. The production patterns of cellulose or hemicellulose-degrading enzymes were investigated during cell culture. All three isolated strains produced carboxymethylcellulase (CMCase), avicelase, β-glucosidase, and xylanase enzymes, which suggested synergic cellulolytic systems in *Bacillus subtilis*. The enzymes showing CMCase, avicelase, and xylanase activities existed in cell-free culture supernatant, meanwhile β-glucosidase activity was detected in cell debris suggesting that three of the enzymes, including CMCase, avicelase, and xylanase, were extracellular, and β-glucosidase was cell membrane-bound. The three isolates, SL9-9, C5-16, and S52-2, were not the same strains, presenting slight differences in biochemical characteristics, 16S rRNA gene sequences, and enzyme activities. The CMCase of three isolates were characterized and *Bacillus subtilis* KACC10111 was used as a reference for enzyme activity comparisons. Therefore, both of the enzyme of SL9-9, S52-2 and KACC10111 were most active at pH 5 and 60℃. The enzyme of C5-16 was most active at pH 5 and 50℃ and the optimum reaction time of CMCase is considered between 15 to 20 min. Also, studies on the optimization of conditions for CMCase production of three *Bacillus subtilis* strains were examined and the optimal culture conditions were determined as follows: as substrate medium containing 1% CMC, 0.4% soytone and 0.4% yeast extract; and initial medium pH of 5.0, temperature of 30～35℃, shaking speed of 150 rpm and

aeration of 1vvm. Furthermore, the effect of the addition of various agricultural wastes as a carbon source to the growth medium on cellulolytic enzymes production of *B. subtilis* SL9-9 was studied. We selected the rice bran as a carbon source because it was a more economical and easily available material for mass culture among various agricultural wastes. As a result, 0.5% rice bran was optimal for the production of cellulase from *B. subtilis* SL9-9.

Three *B. subtilis* strains were isolated from soil, compost, and animal waste slurry in Jeju Island, Korea and their endo-β-1,4-glucanase genes cloned. Cellulase genes from *B. subtilis* SL9-9, C5-16, and S52-2 encoded proteins of 480, 470, and 499 amino acid residues, respectively. DNA sequences of the genes were compared with those of other known cellulase genes. The deduced amino acid sequences of the cellulase genes from the isolates matched quite well the modern concepts of multidomain cellulolytic enzymes. The enzymes were composed of three discrete domains: catalytic domains (CD) of glucosyl hydrolase family 5/A2 and interdomain linker and cellulose-binding domains (CBD) of family IIIa. Similar to the modular organization of many *Bacillus* endoglucanases, the CDs of these enzymes were located in the N-terminal region and CBDs in the C-terminal region.

The effects of exogenous cellulase/cellulolytic bacteria application to bedsoils incorporated with or without organic liquid manure as a nurient source (especially in N, P, and K) on bedsoil fertility and seedling growth of cucumber were determined. The inoculation of *B. subtilis* SL9-9 had significantly greater impact on seedling growth of cucumber. Supplementation of *Bacillus* cells increased shoot dry weight (up to 79%), root dry weight (up to 71%) and leaf area (up to 108%), respectively compared to control (without inoculation of *Bacillus* cells). And, the supplementation of cellulolytic bacteria, *B. subtilis* SL9-9, promoted the bedsoil fertility and microbial activity. As a result, we supposed that these results came from mainly the acceleration in decomposition of organic matter *i.e.* organic liquid manure, peatmoss and cocopeat by *B. subtilis* SL9-9. Also, *B. subtilis* isolates had various plant growth promoting activities as well as antimicrobial activity against various pathogens. And

thus, we supposed that these activitises had a good impact on growth of plants. Therefore, the cellulolytic bacteria application has the potential to be an environment-friendly approach to manage crops and soils.

CONTENTS

PART Ⅱ. Comparison of cellulase genes from isolated *Bacillus subtilis* strains

PART Ⅲ. Evaluation of *Bacillus subtilis* SL9-9 as a biofertilizer in cucumber seedling growth

ABBREVIATION

ACC	1-aminocyclopropane-1-carboxylate
B1,3G	Endo-β-1,3-glucanase
B1,6G	Endo-β-1,6-glucanase
BCA	2,2'-Bicinchroninate
BG	β-glucosidase
BTB	Bromothymol blue
C	Carbon
CB	Commercial bedsoil
CBD	Cellulose-binding domain
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
CD	Catalytic domain
CD	Cell debris
CEL	Cellulase
CFS	Cell-free culture supernatant
CHI	Chitinase
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcellulase
CrI	Crystallinity index
DHA	Dehydrogenase
DNS	Dinitrosalicylic acid
DOB	Developed organic bedsoil
DP	Degree of polymerization
EG	Endo-β-1,4-glucanase
EXP	Expansin
FFLM	Fish-fermented liquid manure
FPA	Filter paper assay
HC	Hemicellulase
HCA	Hydrophobic cluster analysis
HEC	Hydroxyethyl cellulose
INT	2-(p-iodophenyl)-3-(pnitrophenyl)-5-phenyltetrazoliumchloride
K	Potassium
KACC	Korean Agricultural Culture Collection
KCTC	Korean Collection for Type Cultures

LM	Liquid manure
N	Nitrogen
NAGLU	N-Acetylglucoaminidase
ρNPG	ρ-nitrophenyl-β-D-glucopyranoside
P	Phosphorus
PAHBAH	4-hydroxybenzoylhydrazine
PCR	Polymerase chain reaction
PT	Pectinase
R	Root
S	Shoot
TEM	Transmission electron microscope
TNP-CMC	Trinitrophenyl-carboxymethylcellulose
XET	Xyloglucan endotransglycosylase
XGH	Xyloglucan hydrolase

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LITERATURE REVIEWS

1. Cellulose

Cellulose is the primary product of photosynthesis in terrestrial environments, and the most abundant renewable bioresource produced in the biosphere (~100 billion dry tons $year^{-1}$) (Holtzapple, 1993; Jarvis, 2003; Zhang and Lynd, 2004). Of great scientific importance is access to cellulose using enzymatic and chemical methods, respectively, developed during the last decade.

Cellulose is a linear condensation polymer consisting of D-anhydro glucopyranose joined together by β-1,4-glycosidic bonds with a degree of polymerization (DP) from 100 to 20,000 (O'Sullivan, 1997; Zhang and Lynd, 2004). Anhydrocellobiose is the repeating unit of cellulose (Fig. 1). Coupling of adjacent cellulose chains and sheets of cellulose by hydrogen bonds and van der Waals forces results in a parallel alignment and a crystalline structure with straight, stable supra-molecular fibers of great tensile strength and low accessibility (Notley *et al*., 2004; Zhbankov, 1992) as shown in Fig. 2. The cellulose molecule is very stable, with a half life of 5~8 million years for β-glucosidic bond cleavage at 25℃ (Wolfenden and Snider, 2001), while the much faster enzyme-driven cellulose biodegradation process is vital to return the carbon in sediments to the atmosphere (Berner, 2003; Cox *et al*., 2000).

The main source of cellulose is the occurrence of this polysaccharide in different types of plants often combined with other biopolymers. The primary occurrence of cellulose is the existing lignocellulosic material in forests, with wood as the most important source. Other cellulose-containing materials include agriculture residues, water plants, grasses and other plant substances. Besides cellulose, they contain hemicelluloses, lignin and a comparably small amount of extractives (Klemm, 2005). Commercial cellulose production concentrates on harvested sources such as wood or on naturally highly pure sources such as cotton (Table 1).

Cellulose biodegradation by cellulases (CELs) and cellulosomes, produced by numerous microorganisms, represents a major carbon flow from fixed carbon sinks to atmospheric CO_2 (Berner, 2003; Falkowski *et al*., 2000; Melillo *et al*., 2002), is very important in several agricultural and waste treatment processes (Angenent *et al*., 2004; Das and Singh, 2004; Haight, 2005; Hamer, 2003; Schloss *et al*., 2005), and could be widely used to produce sustainable biobased products and bioenergy to replace depleting fossil fuels (Demain *et al*., 2005; Galbe and Zacchi, 2002; Hoffert *et al*., 2002; Kamm and Kamm, 2004; Moreira, 2005; Reddy and Yang, 2005; Wyman, 2003).

2. Cellulase

Cellulase (CELs) is a collective term referring to enzymes able to hydrolyze cellulose (Bhat and Bhat, 1997). Although cellulose is a homopolymer of repeated units of cellobiose, the β-1,4-glycosidic linkages make the structural organization highly ordered and tightly packed (crystallinity), with few amorphous regions. To achieve complete hydrolysis of cellulose, three categories of CELs are required. Firstly, endoglucanases (EG; endo-1,4-β-D-glucanase, EC 3.2.1.4), preferably, attack amorphous regions and randomly cleave the internal bonds of the glycan chains, thus providing reducing or nonreducing ends of cellooligosaccharides for cellobiohydrolases (CBH; or exoglucanase, 1,4-β-D-glucan-cellobiohydrolase, EC 3.2.1.91) to attack. CBH then hydrolyzes those chain ends in the processive manner, yielding cellobiose as the major product. Lastly, β-glucosidase (BG; cellobiase, β-D-glucoside -glucanohydrolase, EC 3.2.1.21) further hydrolyzes cellobiose to glucose and also releases glucose from the nonreducing ends of soluble cellooligosaccharides (Fig. 3) (Jørgensen *et al*., 2007; Lynd *et al*., 2002).

Unlike soluble substrates that can diffuse the active sites of enzymes, cellulose is insoluble; thus, CELs, on the contrary, have to diffuse, attach, and move the segment of the cellulose polymer to their active sites (Wilson, 2011). Most CELs are modular

proteins comprising discrete catalytic modules that typically appended one or more carbohydrate-binding modules (CBMs) joined by a flexible linker (Shoseyov *et al.*, 2006). The CBM functions as a cellulose probe, in which the main responsibility is binding the enzyme to the cellulose and increasing the effective concentration of enzymes on the surface of the cellulose (Araki *et al.*, 2010). In addition, some CBMs are known to possess the ability to disrupt crystalline cellulose (Shoseyov *et al.*, 2006). Therefore, the presence of CBMs appears to be important in enhancing the enzymatic activity toward insoluble polysaccharides, as well as crystalline cellulose.

Classification of cellulases and hemicellulases according to the structural features of their CDs was first introduced in the end of the 1980s and beginning of the 1990s (Gilkes *et al.*, 1991). It was based on hydrophobic cluster analysis (HCA) and later spread to all glycosyl hydrolases. According to HCA methodology, protein amino acid sequence is represented as two-dimensional longitudinal section of a cylinder formed by a hypothetical helical folding of polypeptide chain. Current classification of cellulase-hemicellulase enzymes is partially reproduced in the Table 2.

Corresponding CDs are grouped in 15 families: 5/A, 6/B,7/C, 8/D, 9/E, 10/F, 11/G, 12/H, 26/I, 44/J, 45/K, 48/L, 51, 60, and 61, three of which (5/A, 9/E, and 45/K) being additionally divided in subfamilies. Separated by the slash lettering designations correspond to the initial structural nomenclature of cellulases introduced in 1991. Besides cellulases, other types of glycosyl hydrolases involved in plant cell wall polysaccharide degradation are also included (Rabinovich *et al.*, 2002).

CBD is the second important and the most wide spread element of cellulase structure involved in cellulose transformation. In fact, CBD often plays the role of a recognition factor, which is used by enzyme producing microorganism to address secreted polysaccharide hydrolases to the plant cell wall to be decomposed. The first evidence of their existence was obtained more than two decades ago (Rabinovich, 1977). Since then, the number of CBDs identified as the elements of cellulases, xylanases, mannanases, and other enzymes now exceeds 150 and constantly increases.

Current structural classification of CBDs includes at least thirteen different families (Mattinen *et al*., 1997). Variable localization of CBDs (at the C or N terminus, or in the middle of polypeptide chain) as well as the presence of some identical or even different CBDs in one protein molecule, and finally, the existence of non hydrolytic proteins containing only CBD repeats suggest an independent function of these structural modules and high level of their autonomy. Therefore, their coupling with the enzyme CDs may be a recent evolutionary event. Table 3 illustrates the CBD classification developed by the scientists of the University of British Columbia (Vancouver). It aims to overview the spread of CBDs among different species as well as the diversity of their structural forms and localization in the molecules of different proteins. As seen from this table, bacterial CBDs significantly differ in their polypeptide chain length and form nearly a decade of families (II to X), large families II and III being additionally divided in subfamilies (a) and (b). Contrary to that, known eucaryotic CBDs are (with minor exceptions) highly homologous and are grouped in one family I. Apart from CBDs originating from the enzymes of basidiomycetous, filamentous, and anaerobic fungi, this family also includes CBDs of the polysaccharide binding protein from the alga *Porphyra purpurea*, which is, in fact, the fourfold repeat of fungal CBD. However, family does not include any procaryotic CBDs (Tomme *et al*., 1995).

In most cases CDs and CBDs are separated in the molecules of cellulases and other enzymes by linker sequences. Usually, linkers comprise flexible disordered chains rich in proline and hydroxy amino acid residues (serine and threonine), as well as glycine and alanine (so-called PT-linkers similar with extensins of plant cell walls) (Table 4) (Gilkes *et al*., 1991). Their length can vary from 5~6 to 100 residues, although most often is limited by the range 20 to 50 residues. In many linkers repeated motifs of 4~7 residues can be identified, where some positively, or negatively charged, or hydrophobic residues are inserted within PTS-rich sequence.

Periodicity with high proline content can result in collagen-like secondary structure typical for extensins (Tomme *et al*., 1995). However, their crystal structure

cannot be obtained because numerous threonines serve as O-glycosylation sites, while glycines provide increased flexibility. Because of this, linker sequences along with attached CBDs are usually removed by specific proteolysis before crystallization. Proteolysis occurs in the most flexible part of linker usually adjacent to one the functional domains, where G and P residues are localized. More rigid linker parts containing numerous hydroxyamino acids are supposed to be protected from proteolysis by glycosylation (Rabinovich *et al.*, 2002).

Linkers are believed to provide spatial separation of CDs from CBDs to allow their autonomous function on the surface of insoluble substrate. Rigid glycosylated part is responsible for spatial separation, whereas flexible part provides autonomous domain function and is supposed to play a role of a hinge. Reduction of linker's length has almost no effect on binding and within the certain limits only slightly decreases enzyme activity towards insoluble substrate. However, mutant fungal cellobiohydrolases I with deleted hinge and rigid parts of linker demonstrated reduced activity towards ordered cellulose while had almost the same affinity for the insoluble substrate (Srisodsuk *et al*., 1993). Therefore, the role of linkers may not be restricted by the solely spatial separation of functional domains; rather, they provide a concerted action of all parts of the enzymatic molecular machine on the cellulose surface. It is interesting to note in this respect, that extensins, the proteoglycans of primary cell walls of higher plants, whose amino acid sequence reveals similarity with linkers, play, according to one of hypotheses, a principal role in the shift of protein matrix along cellulose fibers during expansion growth of plant cells (Rabinovich *et al*., 2002).

Rabinovich *et al*. (1984) suggested that It was obvious that the mode of action of a certain enzyme on cellulose surface was defined by a number of parameters: length and structure of its active site, the strength of fixation of the polypeptide loops forming active site, the probability of free rotation of the segments of polymeric substrate molecule within the active site, affinities of different subsites for monomeric units of polymer, location of CBD with respect to CD and its affinity for cellulose

surface, length, and degree of conformational elasticity of the interdomain linker, the presence of other domains in the enzyme structure, which can form protein-protein aggregate on cellulose surface. Because of strictly topochemical character of the overall process, structural data should be considered in the context of heterogenous kinetics and thermodynamic, taking into account evolution of the enzyme-substrate interactions during hydrolysis.

3. Cellulolytic microorganisms

Cellulolytic microorganisms are found among extremely variegated taxonomic groups as shown in Table 5. Most belong to eubacteria and fungi, but anaerobic, cellulose-degradation protozoa have also been identified in the rumen (Coleman, 1978). Cellulolytic microorganisms can be found in all biota where cellulosic waste accumulates. They usually occur in mixed populations comprising cellulolytic and non cellulolytic species, which often interest synergistically. These interactions lead to the complete degradation of cellulose. which is ultimately converted into carbon dioxide and water under aerobic conditions, and into carbon dioxide, methane, and water under anaerobic conditions (Zhang *et al*., 2005).

Unlike non-complexed fungal cellulase, anaerobic microorganisms possess complexed cellulase systems, called cellulosomes (Bayer *et al*., 2004; Demain *et al*., 2005; Doi and Kosugi, 2004; Schwarz, 2001). Leschine (1995) estimated that anaerobic cellulose degradation could account for only 5~10% of total cellulose biodegradation, but it could be underestimated because anaerobic cellulose hydrolysis is responsible for considerable carbon recycling in the anoxic zones of ponds, lakes, oceans, and intestines of ruminants and guts of termites.

4. Substrates for cellulase activity assays

Substrates for cellulase activity assays can be divided into two categories, based on their solubility in water (Table 6).

Soluble substrates. Soluble substrates include low DP cellodextrins from 2 to 6 sugar units and their derivatives, as well as long DP cellulose derivatives (ca. several hundreds of sugar units). They are often used for measuring individual cellulase component activity. Long DP cellulose derivatives can be dissolved in water because of their chemical substitutions. Ionic substituted carboxymethylcellulose (CMC) is often used for determining endoglucanase activity, called CMCase, because endoglucanases cleave intramolecular β-1,4-glucosidic bonds randomly, resulting in a dramatic reduction in the DP (ie, specific viscosity) of CMC. Also, the viscosity of ionic CMC is influenced by pH, ionic strength, and polyvalent cation concentration. Therefore, it is recommended to use nonionic substituted celluloses, such as hydroxyethylcellulose (HEC), for determining endoglucanase activity (Wood and Bhat, 1988).

Insoluble substrates. Insoluble cellulose-containing substrates for cellulase activity assays include nearly pure celluloses (cotton linter, Whatman No. 1 filter paper, bacterial cellulose, microcrystalline cellulose, and amorphous cellulose) and impure cellulose-containing substrates (dyed cellulose, α-cellulose, and pretreated lignocellulose). Native cellulose, referred to as cellulose I, has two distinct crystallite forms, Iα which is dominant in bacterial and algal cellulose, and Iβ, which is dominant in higher plants (Atalla and Vanderhart, 1984). The crystallinity index (CrI) of cellulose, quantitatively measured from its wide range X-ray diffraction pattern (Ramos *et al*., 2005; Zhang and Lynd, 2004), is not strongly associated with hydrolysis rates (Mansfield *et al*., 1999; Zhang and Lynd, 2004). Nevertheless, it is still a convenient indicator representing the change in cellulose characteristics for one material before and after treatment. Cotton, bacterial cellulose, and the Valonia ventricosa algal cellulose are examples of highly crystalline cellulose (Fierobe *et al*., 2002), whereas amorphous cellulose is at the other extreme. Microcrystalline cellulose, filter paper, α -cellulose, and pretreated cellulosic substrates have modest CrI values, and can be

regarded as a combination of crystalline fraction and amorphous fraction, but there is no clear borderline between two fractions. Whatman No.1 filter paper is made from long fiber cotton pulp with a low CrI=～45% (Dong *et al*., 1998). Microcrystalline cellulose, called hydrocellulose or avicel (the commercial name), can be purchased from several companies, such as FMC, Merck, and Sigma. Avicel is a good substrate for exoglucanase activity assay, because it has a low DP and relatively low accessibility (*i.e*., the highest ratio of FRE/Fa) (Table 7). Therefore, some researchers feel that "avicelase" activity is equivalent to exoglucanase activity (Wood and Bhat, 1988). However, some endoglucanases can release considerable reducing sugars from avicel (Zhang and Lynd, 2004). α-Cellulose contains major cellulose and a smallamount of hemicellulose. The commercial Sigma α-cellulose is often used as a reference cellulosic material to evaluate the hydrolysis ability of total cellulase (Kim *et al*., 2003).

5. Quantitative assays

All existing cellulase activity assays can be divided into three types: (1) the accumulation of products after hydrolysis, (2) the reduction in substrate quantity, and (3) the change in the physical properties of substrates (Zhang *et al*., 2006).

The majority of assays involve the accumulation of hydrolysis products, including reducing sugars, total sugars, and chromophores. The most common reducing sugar assays include the dinitrosalicyclic acid (DNS) method (Ghose, 1987; Miller, 1959), the Nelson-Somogyi method (Breuil *et al*., 1985a; Nelson, 1944; Somogyi, 1952), the 2,2′-bicinchroninate (BCA) method (Waffenschmidt and Janeicke, 1987), the 4-hydroxybenzoylhydrazine (PAHBAH) method (Lever *et al*., 1973), and the ferricyanide methods (Kidby and Davidson, 1973). Total soluble sugars, regardless of their chain lengths, can be measured directly by the phenol-H_2SO_4 method (Zhang and Lynd, 2005) or the anthrone-H_2SO_4 method (Roe, 1955). Glucose can be measured by an enzymatic glucose kit using coupled hexokinase and

glucose-6-phosphate dehydrogenase (Zhang and Lynd, 2004), or HPLC after post-hydrolysis conversion to glucose.

The DNS and Nelson-Somogyi methods are two of the most common assays for measuring reducing sugars for cellulase activity assays because of their relatively high sugar detection range (*i.e.*, no sample dilution required) and low interference from cellulase (*i.e.*, no protein removal required). However, the primary drawback for this method is the poor stoichiometric relationship between cellodextrins and the glucose standard (Coward-Kelly *et al.*, 2003). For example, the results may suffer from an underestimation of cellulase activity when glucose is used as the standard and β-glucosidase is not in excess (Schwarz *et al.*, 1988). The ferricyanide, PAHBAH, and BCA methods, having higher sensitivity to reducing sugar, can detect as little as several micrograms per sample, but suffer from non-specific interference from protein.

Total carbohydrate assays, including the phenol-H_2SO_4 method and the anthrone-H_2SO_4 method, offer two obvious advantages as compared with reducing sugar assays: a strict stoichiometric relationship between cellodextrins (glucose equivalent) and the glucose standard, and little or no interference from protein. But they are limited for application to pure celluloses, because any carbohydrates and their derivatives can have strong interference readings. Using an enzymatic glucose assay kit or HPLC can overcome nonspecific readings from other sugars, but this requires an extra step—conversion of longer cellodextrins to glucose (Zhang *et al.*, 2006).

The two basic approaches to measuring cellulase activity are (1) measuring the individual cellulase (endoglucanases, exoglucanases, and β-glucosidases) activities, and (2) measuring the total cellulase activity. In general, hydrolase enzyme activities are expressed in the form of the initial hydrolysis rate for the individual enzyme component within a short time, or the end-point hydrolysis for the total enzyme mixture to achieve a fixed hydrolysis degree within a given time. For cellulase activity assays, there is always a gap between initial cellulase activity assays and final hydrolysis measurement (Sheehan and Himmel, 1999). To be most meaningful,

individual cellulase component assays must also be based on a reliable estimation of the amount of individual enzyme component present in the assay. This information permits the calculation of specific activity, *i.e.*, bonds broken per milligram enzyme per unit time.

Endoglucanases. Endoglucanases cleave intramolecular β-1,4-glucosidic linkages randomly, and their activities are often measured on a soluble high DP cellulose derivative, such as CMC with the lowest ratio of FRN/Fa (Table 7). The modes of actions of endoglucanases and exoglucanases differ in that endoglucanases decrease the specific viscosity of CMC significantly with little hydrolysis due to intramolecular cleavages, whereas exoglucanases hydrolyze long chains from the ends in a processive process (Teeri, 1997; Zhang and Lynd, 2004). Endoglucanase activities can be measured based on a reduction in substrate viscosity and/or an increase in reducing ends determined by a reducing sugar assay. Because exoglucanases also increase the number of reducing ends, it is strongly recommended that endoglucanase activities be measured by both methods (viscosity and reducing ends). Because the carboxymethyl substitutions on CMC make some glucosidic bonds less susceptible to enzyme action, a linear relationship between initial hydrolysis rates and serially diluted enzyme solutions requires (1) dilute enzyme preparation, (2) a short incubation period (*e.g.*, 2~4min) or a very low enzyme loading, (3) a low DS CMC, and (4) a sensitive reducing sugar assay. Many workers agree that the BCA method for reducing sugar assay is superior to the DNS method (Carcia *et al.*, 1993). For example, the modified BCA method, which is conducted at 75°C to avoid β -glucosidic bond cleavage during the assay, delivers a strict stoichiometry for the reducing ends of cellodextrins regardless of sugar chain lengths and offers a much higher sensitivity as shown in Table 6 (Zhang and Lynd, 2005). Soluble oligosaccharides and their chromophore-substituted substrates, such as p-nitrophenyl glucosides and methylumbelliferyl-β-D-glucosides, are also used to measure endoglucanase activities based on the release of chromophores or the formation of shorter oligosaccharide fragments, which are measured by HPLC or TLC (Zverlov *et*

al., 2005). Endoglucanase activities can also be easily detected on agar plates by staining residual polysaccharides (CMC, cellulose) with various dyes because these dyes are adsorbed only by long chains of polysaccharides (Jang *et al*., 2003; Kim *et al*., 2000; Ten *et al*., 2004). These methods are semi-quantitative, and are well suited to monitoring large numbers of samples. Precision is limited because of the relationship between the cleared zone diameters and the logarithm of enzyme activities. For example, differences in enzyme activity levels less than 2-fold are difficult to detect by eye (Sharrock, 1988). Unfortunately, most exoglucanase activities are not detected by these methods, since the processive action of exoglucanases is blocked by carboxymethyl substitutions, which prohibits cellulose chain from shortening. The lack of efficient exoglucanase plate screening method explains some of the difficulty in detecting exoglucanase genes cloned from *C. thermocellum* (Demain *et al*., 2005).

Exoglucanases. Exoglucanases cleave the accessible ends of cellulose molecules to liberate glucose and cellobiose. *T. reesei* cellobiohydrolase (CBH) I and II act on the reducing and non-reducing cellulose chain ends, respectively (Teeri *et al*., 1998; Zhang and Lynd, 2004). Avicel has been used for measuring exoglucanase activity because it has the highest ratio of FNR/Fa among insoluble cellulosic substrates (Table 7).

During chromatographic fractionation of cellulase mixtures, enzymes with little activity on soluble CMC, but showing relatively high activity on avicel, are usually identified as exoglucanases. Unfortunately, amorphous cellulose and soluble cellodextrins are substrates for both purified exoglucanases and endoglucanases. Therefore, unlike endoglucanases and β-glucosidases, there are no substrates specific for exoglucanases within the cellulase mixtures (Sharrock, 1988; Wood and Bhat, 1988). Claeyssens and his coworkers (van Tilbeurgh *et al*., 1982) found that 4-methylumbelliferyl-β-D-lactoside was an effective substrate for *T. reesei* CBH I, yielding lactose and phenol as reaction products, but it was not a substrate for *T. reesei* CBH II (van Tilbeurgh and Claeyssens, 1985) and some endoglucanases (van

Tilbeurgh *et al*., 1982). *T. reesei* EG I, structurally homologous to CBH I, also cleaves 4-methylumbelliferyl-β-D-lactoside, yet these enzymes can be differentiated by adding cellobiose, an inhibitor that strongly suppresses cellobiohydrolase activity (Claeyssens and Aerts, 1992). *T. reesei* CBH II does not hydrolyze 4-methylumbelliferyl-β-D-aglycones of either glucose or cellobiose units, but does cleave 4-methylumbelliferyl-β-D-glycosides with longer glucose chains (van Tilbeurgh *et al*., 1985). Deshpande *et al*. (1984) reported a selective assay for exoglucanases in the presence of endoglucanases and β-glucosidases. This assay is based on the following: (1) exoglucanase specifically hydrolyzes the aglyconic bond of p-nitrophenyl-β-D-cellobioside to yield cellobiose and p-nitrophenol, (2) β-glucosidase activity is inhibited by D-glucono-1,5-δ-lactone (Holtzapple *et al*., 1990) and (3) the influence of exoglucanase hydrolysis activities must be quantified in the assay procedure in the presence of added purified endoglucanases. However, this technique has its own limitations: (1) CBH II activity cannot be measured using p-nitrophenyl-β-D-cellobioside, (2) the specific activity of the available purified endoglucanases may not be representative of all existing endoglucanases in the mixture, and (3) the product ratio from endoglucanase actions may be influenced by the presence of exoglucanases.

β-D-Glucosidases. β-D-Glucosidases hydrolyze soluble cellobiose and other cellodextrins with a DP up to 6 to produce glucose in the aqueous phase. The hydrolysis rates decrease markedly as the substrate DPs increase (Zhang and Lynd, 2004). The term "cellobiase" is often misleading due to this key enzyme's broad substrate specificity beyond a DP of 2. β-D-glucosidases are very amenable to a wide range of simple sensitive assay methods, based on colored or fluorescent products released from p-nitrophenyl-β-D-1,4-glucopyranoside (Strobel and Russell, 1987), β-naphthyl-β-Dglucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside (Polacheck *et al*., 1987), and 4-methylumbelliferyl-β-D-glucopyranoside (Setlow *et al*., 2004). Also, β-D-glucosidase activities can be measured using cellobiose, which is not hydrolyzed by endoglucanases and exoglucanases, and using longer cellodextrins,

which are hydrolyzed by endoglucanases and exoglucanases (McCarthy *et al*., 2004).

Total cellulase. The total cellulase system consists of endoglucanases, exoglucanases, and β-D-glucosidases, all of which hydrolyze crystalline cellulose synergically. Total cellulase activity assays are always measured using insoluble substrates, including pure cellulosic substrates such as Whatman No. 1 filter paper, cotton linter, microcrystalline cellulose, bacterial cellulose, algal cellulose; and cellulose-containing substrates such as dyed cellulose, α-cellulose, and pretreated lignocellulose. The heterogeneity of insoluble cellulose and the complexity of the cellulase system cause formidable problems in measuring total cellulase activity. Experimental results show that the heterogeneous structure of cellulose (filter paper and bacterial cellulose) gives rise to a rapid decrease in the hydrolysis rate within a short time (less than an hour), even when the effects of cellulase deactivation and product inhibition are taken into account (Valjamae *et al*., 1998; Zhang *et al*., 1999). In an attempt to clarify this situation, a functionally based model has been developed to demonstrate that the degree of synergism between endoglucanase and exoglucanase is influenced by substrate characteristics, experimental conditions, and enzyme loading/composition ratio. This model clearly suggests the complexity of total cellulase activity assays and infers that it is nearly impossible to apply the results of the total cellulase activity assay measured on one solid substrate to a different solid substrate. The most common total cellulase activity assay is the filter paper assay (FPA) using Whatman No. 1 filter paper as the substrate, which was established and published by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). This assay requires a fixed amount (2 mg) of glucose released from a 50 mg sample of filter paper (*i.e.*, 3.6% hydrolysis of the substrate), which ensures that both amorphous and crystalline fractions of the substrate are hydrolyzed. A series of enzyme dilution solutions is required to achieve the fixed degree of hydrolysis. The strong points of this assay are (1) it is based on a widely available substrate, (2) it uses a substrate that is moderately susceptible to cellulases, and (3) it is based on a simple procedure (the removal of residual substrate is not necessary

prior to the addition of the DNS reagent). However, the FPA is reproduced in most laboratories with some considerable effort and it has long been recognized for its complexity and susceptibility to operators' errors (Coward-Kelly *et al*., 2003; Decker *et al*., 2003). Reliability of results could be influenced by (1) the β-D-glucosidase level present in the cellulase mixture (Schwarz *et al*., 1988; Sharrock, 1988), because the DNS readings are strongly influenced by the reducing end ratio of glucose, cellobiose, and longer cellodextrins (Kongruang *et al*., 2004; Zhang and Lynd, 2005); (2) the freshness of the DNS reagent, which is often ignored (Miller, 1959); (3) the DNS reaction conditions, such as boiling severity, heat transfer, and reaction time (Coward-Kelly *et al*., 2003); (4) the variations in substrate weight based on the area size (1×6 cm a strip), because this method does not require substrate excess (*i.e.*, substrate amounts strongly influence enzyme activity) (Griffin, 1973); and (5) filter paper cutting methods, because the different papercutting methods such as paper punching, razoring, or scissoring could lead to different accessible reducing ends of the substrate (Zhang and Lynd, 2005). Cotton fiber, microcrystalline cellulose, bacterial cellulose, and algal cellulose are several other common pure cellulosic substrates. Powder microcrystalline cellulose could become a preferred substrate to replace filter paper because (1) it can be rapidly dispensed volumetrically as a slurry and thus permits robotics methods; (2) it can be easily pelleted by centrifugation, and the total sugars released are measured more exactly by the phenol-H_2SO_4 method than by the DNS assay; (3) it is a more recalcitrant substrate, yielding a more stringent substrate for total cellulase activity than does filter paper; and (4) activities measured on microcrystalline cellulose could more accurately represent hydrolysis ability on pretreated lignocellulose, because its characteristics are closer to those of pretreated lignocelluloses, based on cellulose accessibility to cellulase and the degree of polymerization (Zhang and Lynd, 2004). Sigmacell-20, a readily available microcrystalline cellulose powder, could also be a good alternative substrate for a total cellulase activity assay, replacing Whatman No. 1 filter paper. Keep that in mind, some of the pretreated lignocellulose still contains significant amounts of

hemicellulose and lignin, while microcrystalline cellulose does not contain hemicellulose and lignin. α-Cellulose and pretreated lignocellulose are often used to evaluate the digestibility of commercial cellulase or of a reconstituted cellulase mixture for a prolonged reaction. The primary difference, as compared to cellulase activity assays using model cellulosic substrates, is the time required for assays, which ranges from several minutes to hours for model substrates (initial hydrolysis rate) to several days for pretreated lignocellulose to obtain the final digestibility (cellulose conversion). Clearly, the presence of hemicellulose and even lignin results in more complexity. Again, the desired outcome of the experiment must indicate the substrate chosen, especially in the case of total cellulase performance.

In conclusion, the measurement of isolated individual cellulase activity is relatively easy, but it is still challenging to measure *T. reesei* CBH I and CBH II activities specifically in the presence of endoglucanases. There is no clear relationship between the hydrolysis rates obtained on soluble substrates and those on insoluble substrates, mainly because of huge differences in substrate accessibility and DP. For insoluble cellulose, it is highly unlikely that any substantial solubilization of crystalline or semicrystalline cellulose will proceed linearly with time, due to varying β-glucosidic bond accessibilities and chain end availability for different regions of fibers. Researchers must state clearly all parameters of their assay conditions, and resist temptation to compare their results to those of other researchers using different substrates, assay methods, *etc*.

6. Application of cellulase and/or microorganisms

Recently, many researchers have discussed the potential applications of CELs and related enzymes and/or microorganisms in agriculture, biotechnology, and bioenergy as shown in Table 8. Supplementation of CELs to accelerate decomposition of plant residues in soil results in improved soil fertility (Han and He, 2010a, b; Henriksen and Breman, 1999; Novotny *et al*., 2009; Xu *et al*., 2006b). So far, applying

CELs/antagonistic cellulolytic fungi to crops has shown to promote plant growth performance, including enhanced seed germination and protective effects (Brummel and Harpster, 2001; Catala *et al*., 2000; Cosgrove, 2005; Ding *et al*., 2008b; Payasi *et al*., 2009). Their actions are believed mainly to trigger plant defense mechanisms and/or to act as biocontrol agents that mediate disease suppression (Cotes *et al*., 1996; Inbar *et al*., 1994; Moreno *et al*., 2009). However, the exact interaction between the enzymes/fungi and plants has not been clearly elucidated. CELs have recently shown great potential in enzyme aid extraction of bioactive compounds from plant materials before selective extraction through enhancing release of target molecules, especially those associated with the wall matrix (Barzana *et al*., 2002; Ishida and Chapman, 2009; Kapasakalidis *et al*., 2009; Kim *et al*., 2005; Sun *et al*., 2005). To date, attempts have been made to formulate CEL preparation for cellulosic-based bioethanol production (Lim and Lian, 2001). The high cost of CELs has created a bottleneck, resulting in an uneconomic production process. The utilization of low-cost carbohydrates, strain improvement, and gene manipulations has been alternatively aimed at reducing the cost of CEL production.

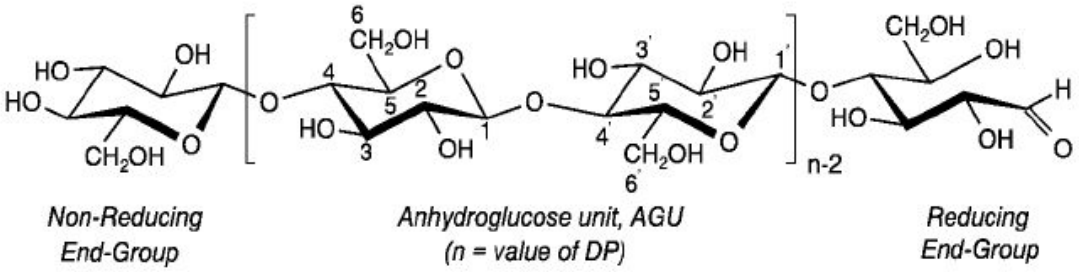

Fig. 1. Molecular structure of cellulose (Redrawn from Klemm *et al*., 2005).

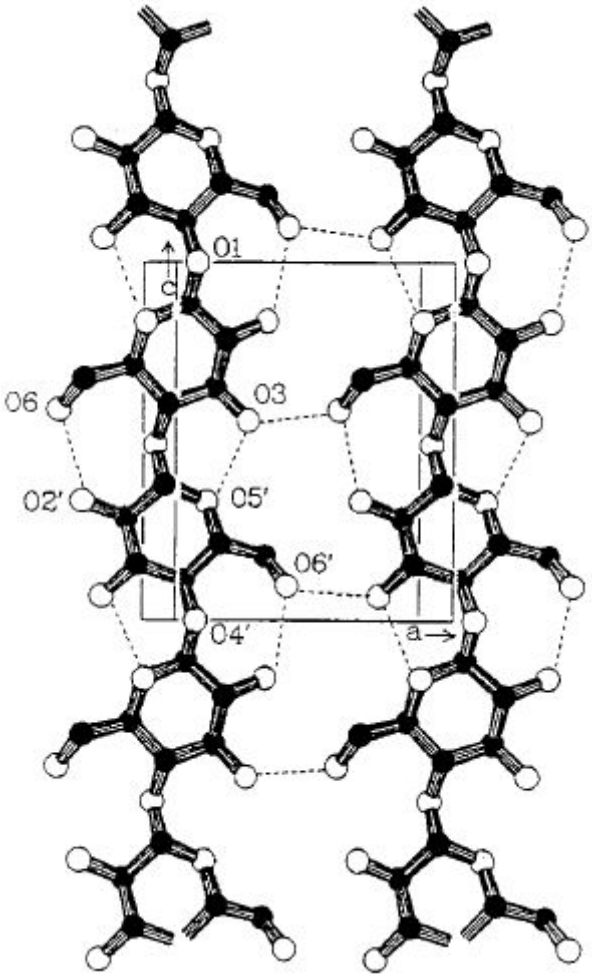

Fig. 2. Most probable bond pattern of cellulose (Redrawn from Klemm *et al.*, 2005).

Table 1. Chemical composition of some typical cellulose-containing materials[a]

Source	Composition (%)			
	Cellulose	Hemicellulose	Lignin	Extract
Hard wood	43~47	25~35	16~24	2~8
Soft wood	40~44	25~29	25~31	1~5
Bagasse	40	30	20	10
Coir	32~43	10~20	43~49	4
Corn cobs	45	35	15	5
Corn stalks	35	25	35	5
Cotton	95	2	1	0.4
Flax(retted)	71	21	2	6
Flax(unretted)	63	12	3	13
Hemp	70	22	6	2
Henequen	78	4~8	13	4
Istle	73	4~8	17	2
Jute	71	14	13	2
Kenaf	36	21	18	2
Ramie	76	17	1	6
Sisal	73	14	11	2
Wheat straw	30	50	15	5

[a]Adapted from Klemm *et al*., 2005.

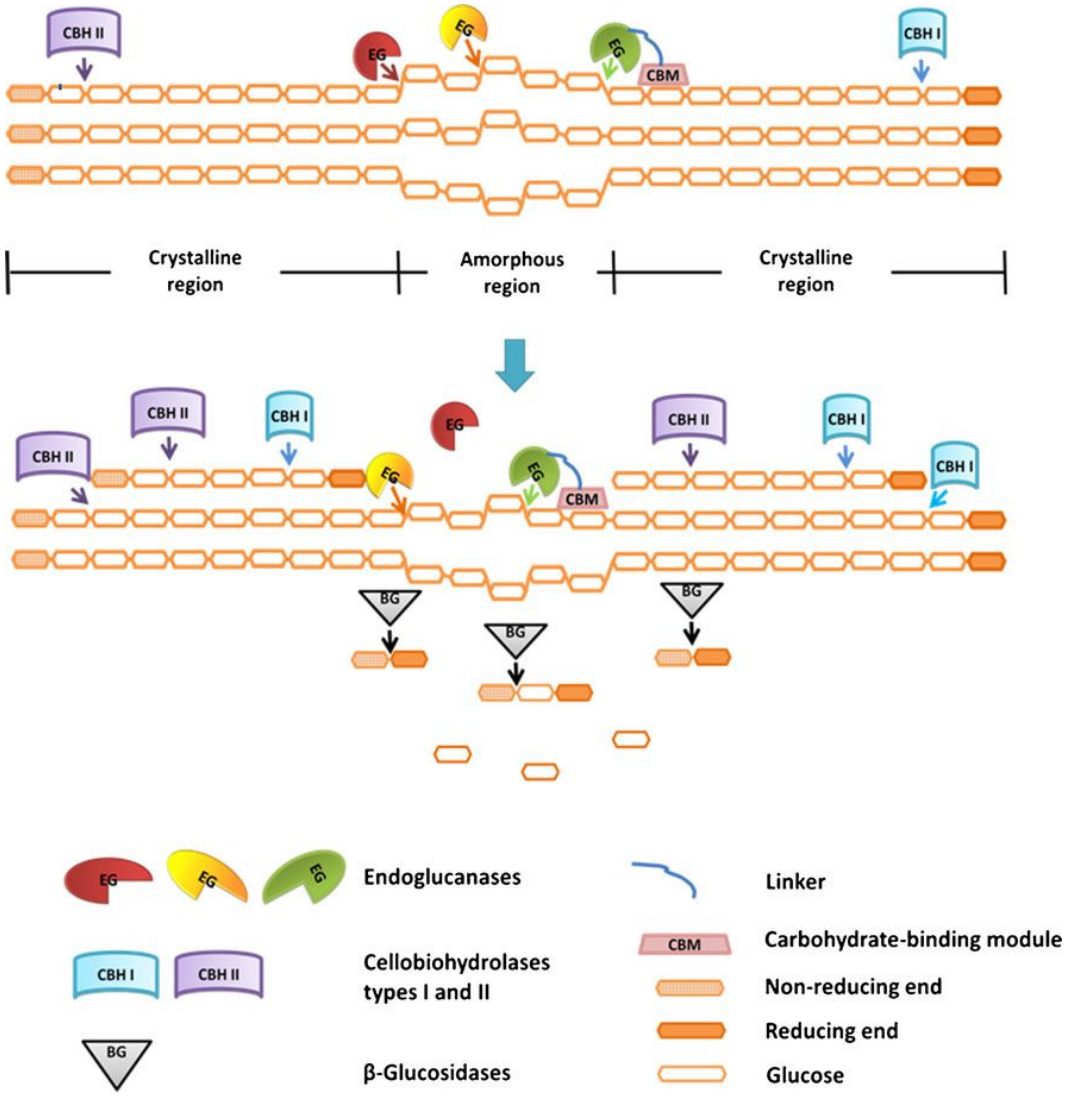

Fig. 3. A simplified model of enzymatic hydrolysis of cellulose.

EGs are presumed to first cleave amorphous regions of the cellulose polymer, thus providing reducing or nonreducing ends of cellooligosaccharides for CBHs to attack and processively hydrolyze those chain ends. BGs further hydrolyze the resulting products, cellobiose, to glucose and also release glucose from the nonreducing ends of the higher oligomers (Redrawn from Lynd *et al.*, 2002).

Table 2. Systematic positions of the enzymes capable of splitting 1,4-β-glucosidic bonds in the general structural classification of glucosyl hydrolases[a]

Family	Type and origin of the enzyme	EC	Clan	Fold	Type of catalysis
1	2	3	4	5	6
5/A1	fungal endo-1,4-β-mannanases; endo glucanases of aerobic and anaerobic bacteria	3.2.1.78 3.2.1.4	GHA	TIM-barrel = (α/β)8 barrel = (β/α)8 barrel; active site includes nucleophilic glutamate at the C-terminus of β-strand 7 and general acidbase asparagine-glutamate at C-terminus of β-strand 4	retain configuration of the anomeric carbon in the reaction products
5/A2	endoglucanases of ctinomycetes and aerobic and anaerobic bacteria, as well as animals (nematodes)	3.2.1.4	same	same	same
5/A3	exo-1,3-β-glucanases; endoglucanases/1,3-1,4-β -glucanases; 1,3-βglucanases (yeast, Clostridium)	3.2.1.58 (cryst.) 3.2.1.4/73 3.2.1.39	same	same; insertion of an additional helical subdomain near active site	same
5/A4	endoglucanases and mannanases of actinomycetes, aerobic and anaerobic bacteria, and anaerobic fungi	3.2.1.4 3.2.1.78	same	same	same
5/A5	endoglucanases of filamentous fungi and aerobic bacteria	3.2.1.4	same	same	same
5	endo-1,6-β-glucanases	3.2.1.75 3.2.1.123	same	same	same
6/B	endoglucanases and cellobiohydrolases of aerobic bacteria, actinomycetes, and anaerobic and filamentous fungi	3.2.1.91 (cryst.) 3.2.1.4 (cryst.)		incomplete TIM barrel	inverse configuration
7/C	cellobiohydrolases and endoglucanases of filamentous fungi	3.2.1.91 (cryst.) 3.2.1.4	GHB	concanavalin A type β-sandwich	retain configuration
8/D	1,31,4βglucanases and endoglu canases of aerobic and anaerobic bacteria	3.2.1.73 3.2.1.4 (cryst.)	not defined	(α,α)6-barrel	inverse
9/E1	endoglucanases of actinomycetes and aerobic and anaerobic bacteria; cellobiohydrolases of anaerobic bacteria	3.2.1.4 (cryst.) 3.2.1.91	same	same	same
9/E2	endoglucanases of aerobic and anaerobic bacteria, plants, and insects (termites); endo/exoglucanase of actinomycetes	3.2.1.4 (cryst.) 3.2.1.4/91	same	same; additional strongly fixed type III CBD	same

Table 2. (contd.)

1	2	3	4	5	6
10/F	xylanases and xylanases/endoglucanases of actinomycetes, aerobic bacteria, anaerobic archaeand eubacteria, and aerobic and anaerobic fungi; bacterial xylanase /exoglucanase	3.2.1.4/8 3.2.1.8/91 (cryst.) 3.2.1.8 (cryst.)	GHA	TIM-barrel	retain
11/G	strictly specific xylanases of aerobic and anaerobic fungi, aerobic and anaerobic bacteria, and actinomycetes	3.2.1.8 (cryst.)	GHC	"ribcage"like β-sandwich	same
12/H	endoglucanases of aerobic fungi, actinomycetes, aerobic bacteria, and anaerobic archaebacteria	3.2.1.4 (cryst.)	same	same; two β-sheets of six and nine strands, and α-helix across	same
26/I	endoglucanases; endoglucanases /xylanases; endomannanases of aerobic and anaerobic bacteria	3.2.1.78 3.2.1.4 3.2.1.4/8	GHA	TIM-barrel	same
44/J	endoglucanases of aerobic and anaerobic bacteria	3.2.1.4	not defined	not defined	inverse
45/K1	endoglucanases of filamentous and anaerobic fungi and aerobic bacteria	3.2.1.4	not defined	6-β-barrel	same
45/K2	endoglucanase of mollusks				
48/L	endoglucanases; cellobiohydrolases; endoglucanases/cellobiohydrolases of actinomycetes and aerobic and anaerobic bacteria	3.2.1.4 3.2.1.91 3.2.1.4/91	not defined	(α,α)6-barrel	same
51	α-L-arabinofuranosidases of ascomycetes and actinomycetes; endoglucanase of F. succinogenes	3.2.1.55 3.2.1.4	GHA	TIM-barrel	retain
60	Clostridium endoglucanases	3.2.1.4	not defined	not defined	not defined
61	endoglucanases of ascoand basidiomycetes	3.2.1.4	not defined	not defined	same

Note: EC, enzyme classification according to IUB; (cryst.), proteins with resolved crystalline structure.
[a]Adapted from Tomme *et al*., 1995 & Gilkes *et al*., 1991.

Table 3. Classification of cellulose binding domains[a]

Family	Organism	Enzyme	Location[b]	Length[c]	Fold	CD family	Source[d]
1	2	3	4	5	6	7	8
I	*Agaricus bisporus*	Cel1	C	36	wedgelike,	61	M86356
	Fusarium oxysporum	Xyn	N	36	triple-stran	10/F	L29380
	Neocallimastix patriciarum	XylB	C	33	ded antiparallel	10/F	S71569
	Humicola insolens	EGY	C	33	β-sheet	45/K	
	Neurospora crassa	CBHI	C	33		7/C	X7778
	Penicillium janthinellum	CBHI	C	33			X59054
	Phanerochaete chrysosporium	CBHI	C	34			M22220
	Porphyra purpurea	PBP	×4	33		polysaccharide binding protein	U08843
	Trichoderma reesei	CBHI	C	33		7/C	P00725
		CBHII	N	36		6/B	M16190
		EGI	C	33		7/C	M15665
		EGII	N	36		5/A5	M19373
		EGY	C	36		45/K	Z33381
		Man	C	34		5/A1	L25310
	Myceliophthora thermophila	CDH	I			cellobiose dehydroge-nase	
IIa	*Butirivibrio fibrisolvens*	End1	C	95	ten antiparallel β-strands, nine of them	5/A4	X17538
	Cellulomonas fimi	CenA	N	106	forming	6/B	M15823
		CenB	C	103	β-barrel	9/E2	M64644
		CenD	C	105		5/A1	L02544
		CbhA	C	106		6/B	L25809
		CbhB	C	104		48/L	L29042
		Cex	C	106		10/F	L11080
	Clostridium cellulovorans	EngD	C	108		5/A4	M37434
	Dictiostelium discoideum	SGSP	C,I	98/106			M33861
	Microbispora bispora	CelA	C	100		6/B	P26414
	Pseudomonas fluorescens	CelA	C	100		9/E1	X12570
		CelB	N	102		45/K	X52615
		CelC	N	99		5/A1	X61299
		CelE	C	100		5/A2	X86798
		XynA	N	101		10/F	X15429
	Streptomyces lividans	CelA	N	108		5/A2	M82807
		CelB	C	106		12/H	U04629
		ChiC	N	105		18/chitinases	D12647

Table 3. (contd.)

1	2	3	4	5	6	7	8
	Thermomonospora fusca	E2	C	96		6/C	M73321
		E3	N	103		6/C	U18978
		E5	N	103		5/A2	L01577
IIb	*Cellulomonas fimi*	XynD	×2C	90	skew β-sandwich	11/G	X76729
	Streptomyces lividans	Axe	C	86		acetylxylan esterase	
	Thermomonospora fusca	XynA	C	86		11/G	U01242
IIIa	*Bacillus lautus*	CelA	C	150	nine-stranded β-sandwich binding Ca^{2+}	44/J	M76588
	Bacillus subtilis	Cel	C	133		5/A2	X67044
	Caldicellulosiruptor	CelA	×2I	172		9/E2//48/L	L32742
	(Caldocellum)	CelB	I	172		10/F//5/A1	X13602
	saccharolyticum	CelC	×2I	172		9/E2//5/A4	
		ManA	×2I	172		5/A4//44/J	L01257
	Clostridium cellulovorans	CbpA	N	161		scaffoldin	M73817
	Clostridium stercorarium	CelZ	C	133		9/E2	X55299
	Clostridium thermocellum	Cbh3	C	132		9/E1	X80993
		CipA	I	156		scaffoldin	L08665
		CelI	C	150		9/E2	L04735
	Erwinia carotovora	CelV	C	156		9/E2	X76000
IIIb	*Cellulomonas fimi*	CenB	I	138		9/E2	M64644
	Clostridium stercorarium	CelZ		144			X55299
	Clostridium thermocellum	CelI		137			L04735
IV	*Cellulomonas fimi*	CenC	×2N	148	"jelly roll" of two β-sheets each containing five (or five and six) strands	9/E1	X57858
	Clostridium cellulolyticum	CelE	N	168			M87018
	Streptomyces reticuli	Cel1		125			L04735
	Thermomonospora fusca	E1		141			L20094
	Myxococcus xantus	CelA		139		6/B	X76726
V	*Erwinia chrysanthemi*	EgZ	C	63	"ski boot", βsheet of three antiparallel β strands with perpendicular flexible loop	5/A2	Y00540
VI	*Bacillus polymyxa*	XynD	C	90			X57094
	Clostridium stercorarium	XynA	×2C	87/92		11/G	D13325

Table 3. (contd.)

1	2	3	4	5	6	7	8
	Clostridium thermocellum	XynZ	I	92		10/F	M22624
	horseshoe crab	G-α	×2C	87		16 (coagulation factor)	D16623
	Microbispora bispora	BglA	C	85			L06134
VII	*Clostridium thermocellum*	CelE	I	240		5/A4	M22759
VIII	*Dictiostelium discoideum*	CelA	N	152		9/E2	M33861
IX	*Clostridium thermocellum*	XynX	×2C	174/189		10/F	M67438
	Thermoanaerobacterium sp.	XynA		174/187			M97882
	Thermotoga maritima	XynA		170/180			Z46264
X	*Cellvibrio mixtus*	XynA	C	51	two βsheets of	11/G	Z48925
	Pseudomonas fluorescens	CelA	I	55	two and three	9/A1	X12570
		CelB		55	antiparallel	45/K	X52615
		CelC		53	strands, α-helix	5/A1	X61299
		CelE	C	53	across larger	5/A2	X86798
		XynA		53	sheet	10/F	X15428
		XynE		55		11/G	Z48927

[a]Adapted from Tomme *et al*., 1995; [b]Localization of CBD at C, N terminus, or in the middle of protein chain; ×4 or ×2 indicates the number of CBD repeats; [c]Approximate length of CBD polypeptide; [d]Codes of amino acid sequences from SWISSPROT, EMBL/GenBank, or PIR as given in corresponding reviews.

Table 4. Linker sequences of some cellulolytic enzymes and their comparison with central parts of family I CBD sequences[a]

Organism	Enzyme	Sequence	Length[b]	Pro	Tre+Ser
1	2	3	4	5	6
Bacillus spp.	CelA	$T_2P_2SDPTP_2SDPDGEPGPDPGEPDPTP_2SDP$	33	15	7
	CelB	$(1)P_2SDPTP_2SDPDPGEPDPTP_2SDPGEYP$	28	13	5
	Egl1	$(2)P_2SEPSDP_4SEPE(PDPGE)_3PDPTP_2SDPEYP$	42	20	5
Butyrivibrio spp.	End1	$T_2EPVEPEPVDPGE_2TP_2$	18	6	3
	Xyn	$(PDPTPVD)_4PDPQPVDPTP$	38	17	5
Caldicellulosiruptor	CelB	$PGSFTPQPTITPQ(PT)_2PSGQT$	26	7	8
(Caldocellum)		$(1)T_2S_2(PT)_4(VT)_2(PT)_5VTAT(PT)_3PVSPAT$	43	14	23
saccharolyticum		$(2)PAPTMTVAPTAT(PT)_2LSPTV(TP)_2APTQTAI(PT)_2LTPN(PT)_2$	44	14	17
Cellulomonas fimi	CenA	$PT_2S(PT)_4T(PT)_7VTPQPT$	33	14	17
	CenB	$(1)PTGT_3DT_2P_2T_2PGTP$	17	5	9
		$(2)T_2DT_2GETEP_2T_2PGTP$	17	4	8
		$(3)T_2A_2PVDTVAPTVPGTP$	17	4	5
		$(4)S_2PVTFT_2LPVTSTPS$	16	3	8
	CenC	$(1)SLT_2SATP_3$	10	3	5
		$(2)PVPTAP$	6	3	1
	Cex	$(PT)_3T(PT)_3T(PT)_3S$	21	9	12
Clostridium spp.	Egl	$T_2PTS_3PVYTSPITISKT_3$	21	3	13
	CelA	$PLSDLSGQPTP_2SNPTPSLP_2$	21	8	6
	CelB	$TPSVT(PS)_2ATPSPT_2ITAP_2T$	22	7	11
	CelE	$PLVS(PT)_3LMPTPSPTVT$	20	7	8
	CelH	$(1)(PT)_3WTSTP_2S_3P$	16	6	9
		$(2)PGTYPSYSPKPSPTPRPTKP_2VTP$	24	10	7
	XynZ	$(1)TPVPTPSPKP$	10	5	3
		$(2)TPNPSVTPTQTPIPT$	15	6	6
Erwinia chrysanthemi	CelZ	$T_2DPSTDT_2MTP_2LTNRPQPT$	21	5	9
Fibrobacter succinogenes	CelC	$PVS_3DMSPTS_2DAVIDPTS_3A_2V_2DPST$	30	4	13
Microbispora bispora	CelA	$P_2TYSPSPTPST(PS)_3QSDPGS(PS)_3$	30	12	14

Table 4. (contd.)

1	2	3	4	5	6
Xanthomonas campestris	EngA	$T_2(PT)_{11}$	24	11	13
Streptomyces sp.	CasA	(1)$PRT_2(PT)_2P$	9	4	4
		(2)$PA_2TGA(SP)_2AP_2ASPAPSADS$	22	7	6
Pseudomonas fluorescens	CelA	(1)$S_{11}VPVS_7I_2PS_6IQPS_6MPS_8V_2AS_5VS$	59	4	44
		(2)$S_4ASNINS_{12}AIVS_5V_2S_6$	37	0	28
	CelB	(1)$S_2APS_2VAS_7V_2S_2TPRS_5VS_3VPGTS_7$	42	3	30
		(2)$STS_3TPLS_6RS_2VAS_4LS_2ATS_3AS_2VS_2$	37	1	28
	XynA	(1)$S_3APAS_2VPS_2IAS_3PS_2VAS_2VIS_2MAS_3PVS_4VAS_2TPGS_3$	49	5	28
	XynB	(2)$S_6LS_4V_2S_2IRS_9$	26	0	21
		(1)$SAT_2S_2VAS_4TPT_2S_4AS_2VAS$	26	1	19
		(2)$SVS_5VQS_9A_2S$	21	0	16
Trichoderma reesei	EGI	PPPPPASSTTFSTTRRSSTTSSSPS	25	6	14
	EGII	PGATTITTSTRPPSGPTTTTRATSTSSSTPPTSS	34	6	21
	CBHI	GNPSGGNPPGGNRGTTTTRRPATTTGSSPGPT	34	6	11
	CBHII	$PGAAS_5TRAASTTSRVSPTTSRSSSATPPPGST_2RVPPVG$	44	7	22
		Fragments 15-26 of family I CBDs			
Trichoderma reesei	EGI	SGcKTcTSGTTc	12(3)	0	6
	EGII	SGPTNcAPGSAcST	14(2)	2	5
	CBHI	SGPTVcASGTTc	12(2)	1	5
	CBHII	SGPTccASGSTc	12(3)	1	5
Agaricus bisporus	Cel3a	TGPTTcASGSTc	12(2)	1	6
Humicola insolens	EgY	SGcTTcVAGSTcT	13(3)	0	6
Neurospora crassa	CBHI	SGPTTcPEPYTc	12(2)	3	4

[a]Adapted from Tomme *et al.*, 1995 & Gilkes *et al.*, 1991; [b]In parentheses, the numbers of Cys residues which are not present in the linkers.

Table 5. Microorganisms having cellulolytic activities

Fungi	**Soft rot fungi**
	Aspergillus niger; *A. nidulans; A. oryzae; A. terreus; Fusarium solani; F. oxysporum; Humicola insolens; H. grisea; Melanocarpus albomyces; Penicillium brasilianum; P. occitanis; P. decumbans;* ***Trichoderma reesei;*** *T. longibrachiatum; T. harzianum; Chaetomium cellulyticum; C. thermophilum; Neurospora crassa; P. fumigosum; Thermoascus aurantiacus; Mucor circinelloides; P. janthinellum; Paecilomyces inflatus; P. echinulatum; Trichoderma atroviride*
	Brown rot fungi
	Coniophora puteana; Lanzites trabeum; Poria placenta; Tyromyces palustris; Fomitopsis sp.
	White rot fungi
	Phanerochaete chrysosporium; Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotus ostreatus; Phlebia gigantea
Bacteria	**Aerobic bacteria**
	Acinetobacter junii; A. amitratus; Acidothermus cellulolyticus; Anoxybacillus sp.; ***Bacillus subtilis; B. pumilus; B. amyloliquefaciens; B. licheniformis; B. circulan;*** *B. flexus; Bacteriodes sp.; Cellulomonas biazotea; Cellvibrio gilvus; Eubacterium cellulosolvens;Geobacillus sp.; Microbispora bispora; Paenibacillus curdlanolyticus; Pseudomonas cellulosa;Salinivibrio sp.; Rhodothermus marinus*
	Anaerobic bacteria
	Acetivibrio cellulolyticus; Butyrivibrio fibrisolvens; ***Clostridium thermocellum;*** ***C. cellulolyticum; C. acetobutylium; C. papyrosolvens;*** *Fibrobacter succinogenes; Ruminococcus albus*
Actino -mycetes	*Cellulomonas fimi; C. bioazotea; C. uda; Streptomyces drozdowiczii; S. lividans; Thermomonospora fusca; T. curvata*

Table 6. Substrates containing β-1,4-glucosidic bonds hydrolyzed by cellulases and their detections[a]

Substrate	Detection	Enzyme
Soluble		
Short chain(low DP)		
Cellodextrins	RS, HPLC; TLC	Endo, Exo, BG
Radio-labeled cellodextrins	TLC plus liquid scintillation	Endo, Exo, BG
Cellodextrin derivatives		
β-methylumbelliferyl-oligosaccharides	Fluorophore liberation, TLC	Endo, Exo, BG
p-nitrophenol-oligosaccharides	Chromophore liberation, TLC	Endo, Exo, BG
Long chain cellulose derivatives		
carboxymethylcellulose(CMC), hydroxyethyl cellulose(HEC)	RS; viscosity	Endo
Dyed CMC	Dye liberation	Endo
Insoluble		
Crystalline cellulose		
Cotton. microcrystallin cellulose(Avicel),	RS, TSS, HPLC	Total, Endo, Exo
Valonia cellulose, bacterial cellulose	RS, TSS, HPLC	Total, Endo, Exo
Amorphose cellulose-PASC, alkali-swollen cellulose RAC	RS, TSS, HPLC, TLC	Total, Endo, Exo
Dyed cellulose	Dye liberation	Total, Endo
Fluorescent cellulose	Fluorophore liberation	Total
Chromogenic and fluorephoric derivatives		
Trinitrophenyl-carboxymethylcellulose (TNP-CMC)	Chromophore liberation	Endo
Fluram-cellulose	Fluorophore liberation	Endo, Total
Practical cellulose-containing substrates		
α-cellulose, pretreated lignocellulosic biomass	HPLC, RS	Total

[a]Adapted from Zhang *et al*., 2006.

Table 7. Summary of typical values of model celluloses for crystallinity index(CrI), the fraction of β-glucosidic bond accessible to cellulase(Fa), which is estimated by maximum cellulase adsorption capacity(Zhang and Lynd, 2004), the number average of degree of polymerization(DPN), the fraction of reducing ends(FRE), and relative ratio of FRE/Fa[a]

Substrate	CrI	F_a (%)	DP_N	$F_{RE(\%)}$		F_{RE}/F_a	
				Low	High	Low	High
Soluble							
Cellodextrines and their derivatives	N.A.	100	2~6	16.67	50	0.167	0.5
CMC	N.A.	100	100 ~2000	0.05	1	0.0005	0.01
Insoluble							
Cotton	0.8 ~0.95	0.2	1000 ~3000	0.033	0.1	0.167	0.5
Whatman No.1 filter paper	~0.45	1.8	750 ~2800	0.036	0.133	0.0198	0.741
Bacterial cellulose	0.8 ~0.95	6	600 ~2000	0.05	0.167	0.0083	0.0278
Microcrystalline cellulose(Avicel)	0.5 ~0.6	0.6	150 ~500	0.2	0.667	0.333	1.11
PASC	0	12	100 ~1000	0.1	1	0.00833	0.0833
Pulp(Solka Floc)	0.4 ~0.7	1.8	750 ~1500	0.067	0.133	0.0370	0.0741
Pretreated cellulosic substrataes	0.4 ~0.7	0.6	400 ~1000	0.1	0.25	0.167	0.417

[a]Adapted from Zhang *et al*., 2006.

Table 8. Potential applications of CELs and related enzymes and/or microorganisms in agriculture, biotechnology and bioenergy[a]

Application	Enzyme/microorganism	Function	Outcome/advantage	Limitation/disadvantage	Reference
Soil fertility	CELs, protease	Accelerated decomposition of plant residue incorporated with soil	Recovery and increases soil available N, microbial activity, and organic matter-buffered soil pH	Increase in nutrients and final soil pH depending on plant residues used and soil properties Cost-expensive	Han and He(2010a, b), Henriksen and Breman(1999), Novotny *et al*.(2009), Xu *et al*.(2006b)
Growth promoting agents					
Cell growth	CELs and relevant enzymes, particularly XETs, XGXs, and PTs	Synergistic interaction with: Expansin proteins during cell extensibility, by degradation or modification of the plant cell wall polysaccharides	Enhanced cell loosening and enlargement during cell growth and developmental process	Vulnerability of biotic infection, susceptibility of diseases, and reduction of pathogenic resistance	Brummel and Harpster(2001), Catala *et al*.(2000), Cosgrove (2005), Ding *et al*.(2008b), Payasi *et al*.(2009)
Seed germination and protective effects	CELs, B1,3,Gs, B1,6Gs, CHIs, peroxidases *Trichoderma spp., T. harzianum, T.kiningii, T. koningiopsis, T. longibranchiatum*	Seed colonization	Enhanced seed germination and shortened nursery period	Variation in conditions used	Cotes *et al*.(1996), Inbar *et al*.(1994), Moreno *et al*.(2009)
		Degradation of fungal cell walls	Improved self-defense mechanisms	Requiring field experiments/ conditions	
		Solubilization of some active compounds acting as fungicides or elicitors from plant cell walls	Enhanced nutrient solubilization and improved root environment		
		Root colonization			

Table 8. (contd.)

Application	Enzyme/microorganism	Function	Outcome/advantage	Limitation/disadvantage	Reference
Biocontrol of pathogens and diseases	CELs, *Pythium oligandrum, Trichoderma longibrachiatum*	Disruption and degradation of fungal cell walls	Facilitated host cell penetration	Environmental factors particularly other soil microbes probably involving in defense response in situ	Benhamou *et al.*(1999). Haran *et al.*(1996), Picard *et al.*(2000)
		Induction of abnormal morphology of pathogens	Improved plant growth	Genomic and proteomic analyses required for unraveling systematic mechanisms upon antagonism	
		Mycoparasitism	Self-biofertilizer Environmentally friendly process		
Bioactive compound extraction	CELs, HCs, PTs, BGs	Breakdown of plant cell walls	Increased liberation of desired bioactive molecules, yield, and preserved antioxidant activity	Requiring suitable organic solvents to extract target molecules	Barzana *et al.*(2002), Ishida and Chapman(2009), Kapasakalidis *et al.*(2009), Kim *et al.*(2005), Sun *et al.*(2005)
			Facilitated release of cell wall-associated biomolecules	Loss of pigment and antioxidant activity due to side-enzymatic activity	
			Improved degradation of rigid and thick cell walls		
			Reduced chemicals(ie, acids) used in cell wass degradation and severe condition		
			Reduction in waste management		

Table 8. (contd.)

Application	Enzyme/microorganism	Function	Outcome/advantage	Limitation/disadvantage	Reference
Plant protoplast production	CELs, HCs, PTs, BGs	Removal of plant cell polysaccharides	Reduced protoplast damage by chemicals used		Lim and Lian(2001)
			Increased protoplast viability and yield	Optimized enzyme formulation required for different plant tissues used	Takebe *et al.*(1986), Tamura *et al.*(2002)
			Increased subsequent cell division and plant regeneration		
Bioethanol production	CELs, HCs, PTs, BGs	Degradation of plant cell wall	Increased release of fermentable sugars	Requiring pretreatment to alter structure	Beukes and Pletschke(2010), Jeya *et al.*(2010)
			Reduced sugar loss and toxic compound formation	Requiring lignin removal	Tayer *et al.*(2008), Yu *et al.*(2007)
				High cost of enzymes	
				Optimized enzyme formulation required for different plant residues	

[a]Adapted from Phitsuwan *et al.*, 2006.

PART Ⅰ

Isolation and identification of cellulolytic microorganisms

Ⅰ. Introduction

The bioconversion of cellulose to soluble sugars and glucose is catalyzed by a group of enzymes called cellulases (CELs) that are produced by microorganisms (Wood *et al.*, 1990). These cellulolytic microorganisms play an important role in the biosphere by recycling cellulose, the most abundant and renewable biopolymer on earth. The demand for microbial CELs and related enzymes is growing more rapidly than ever before (Li *et al.*, 2009).

CELs are relatively costly enzymes, and a significant reduction in cost will be important for their commercial use in biorefineries. Cellulase-based strategies that will make the biorefinery processing more economical include: increasing commercial enzyme volumetric productivity, producing enzymes using cheaper substrates, producing enzyme preparations with greater stability for specific processes, and producing cellulases with higher specific activity on solid substrates. Currently, most commercial cellulases (including β-glucosidase) are produced by *Trichoderma* species and *Aspergillus* species (Cherry and Fidantsef, 2003; Kirk *et al.*, 2002). The demand for microbial cellulases and related enzymes is growing more rapidly than ever before (Li *et al.*, 2009).

Fungal cellulases are produced in large amounts, which include all the components of a multi-enzyme system with different specificities and modes of action, *i.e.* endoglucanases (or CMCase), exoglucanases (cellobiohydrolase), and β -glucosidases (or cellobiases), acting in synergy for the complete hydrolysis of cellulose (Bhat *et al.*, 1997). Synergy between cellulase components during the

hydrolysis of cellulose in *Trichoderma viride* was first demonstrated by Giligan and Reese (Giligan, 1954).

Synergic multi-enzyme systems are also expected in bacterial cellulase complexes. Cellulolytic bacteria include aerobes such as *Pseudomonas* and Actinomycetes, facultative anaerobes such as *Bacillus* and *Cellulomonas*, and strict anaerobes such as *Clostridium*. Most of these bacteria produce mainly endoglucanases (Wood, 1985). A variety of *Bacillus* species secrete cellulases, including strains of *B. cereus* (Thayer *et al*., 1978), *B. subtilis* (Robson *et al*., 1984), *B. licheniformis* (Dhillon *et al*., 1985), *Bacillus sp*. KSM-330 (Ozaki, K. and S. Ito., 1991), and alkaliphilic *Bacillus* (Horikoshi, 1997). In addition, a fairly common observation has been that *bacilli* lack a complete cellulase system, with primary activity being on carboxymethylcellulose (CMCase, endoglucanase), and which do not hydrolyze crystalline cellulose (Robson, L. M. and G. H. Chambliss, 1984). However, in contrast, there are reports of certain *Bacillus* endoglucanases (CMCase) that have shown detectable activity on microcrystalline cellulose (Aa, 1994; Kim *et al*., 1995).

In this work, we isolated cellulase-producing *B. subtilis* strains from soil, compost, and animal waste slurry *etc*, and studied their cellulolytic enzymes. This study reports the occurrence of these cellulolytic enzymes from *B. subtilis* strains isolated from different habitats. In addition, the optimized cultivation conditions for the production of cellulase of isolated *B. subtilis* strains and mass culture conditions for scaling up were determined.

Ⅱ. Materials and Methods

1. Bacterial strains

Three strains, *B. licheniformis* KACC10476, *B. pumilus* KACC10917, and *B. subtilis* KACC10111, were obtained from KACC (Korean Agricultural Culture Collection, RDA, Korea). Four strains, *B. amylolicheniformis* KCTC2105, *B. licheniformis* KCTC3045, *B. pumilus* KCTC3348, and *B. subtilis* KCTC3560, were obtained from KCTC (Korean Collection for Type Cultures, KRIBB, Korea). Three bacterial isolates, *B. subtilis* SL9-9, C5-16, and S52-2, were acquired in this study.

2. Isolation of cellulolytic microorganisms

A total of 176 samples were collected from soil, compost, and animal waste slurry *etc.,* in Jeju Island, Korea, and were screened for cellulolytic microbes. The samples were stored at 4℃ in the dark until use. After appropriate dilutions with sterile water, 1 mL each of the sample dilutions was spread onto CMC (carboxymethylcellulose) agar plates that consisted of carboxymethylcellulose, 10.0; yeast extract, 1.0; $(NH_4)_2SO_4$, 2.5; $K_2HPO_4 \cdot 3H_2O$, 0.25; NaCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.125; $FeSO_4 \cdot 7H_2O$, 0.0025; $MnSO_4 \cdot 4H_2O$, 0.025; and agar, 10(g L^{-1}, each), and then the plates were incubated at 28℃ for 2 days. The initial medium pH was adjusted to 7.0 if not specified. A few bacterial colonies were harvested and transferred to fresh CMC agar plates containing trypan blue. The plates were incubated at 28℃ for 2 days and the cellulolytic clones were detected by clear halos around the colonies (Wood, 1980).

Three clones were finally chosen based on their relatively higher cellulolytic activities among 279 cellulase-positive clones that showed good colonial development and visible clearing zones, and were maintained on CMC agar.

Table 9. CMC basal medium

Ingredient	Concentration (g L^{-1})
Carboxymethylcellulose	10
Yeast extract	1.0
$(NH_4)_2SO_4$	2.5
$K_2HPO_4 \cdot 3H_2O$	0.25
NaCl	0.1
$MgSO_4.7H_2O$	0.125
$FeSO_4 \cdot 7H_2O$	0.0025
$MnSO_4 \cdot 4H_2O$	0.025
Agar	10
pH	7

3. Identification of bacterial isolates

The three isolates were morphologically and physiologically characterized, and identified up to genus level according to Bergey's Manual of Determinative Bacteriology (8th ed.). An API 50CHB kit (BioMeriuex, France) was also used for the identification of Gram-positive bacteria. API strips were inoculated with 24 hr-grown cultures and then incubated at 28℃. The results were read according to the manufacturer's instructions and compared with other known *B. subtilis* strains obtained from KACC and KCTC. Standard procedures (Gibson *et al*., 1974) were used to analyze the clones for motility, sporulation, catalase activity, and gram reaction *etc*.

Genes of 16S rRNA were sequenced and compared for identification of the bacterial isolates. The bacterial cells grown on CMC agar were harvested and used for chromosomal DNA isolation according to the protocols (Ausubel *et al*., 1993). The chromosomal DNA was used as a template for amplification of 16S rRNA *via* the polymerase chain reaction (PCR). The primers used were 27F: 5'-AGAGTTTGATCATGGCTCAG-3' as a forward primer and 1522r:

5'-AAGGAGGTGATCCARCCGCA-3' as a reverse primer. The PCR reaction mixture was composed of 5 μL of template (50 ng μL^{-1}), 5 μL of 10× reaction buffer (100 mM Tris-HCl, 400 mM KCl, 500 μg mL^{-1} BSA, pH 8.3), 5 μL of deoxynucleoside triphosphtes (2.5 mM, each), 1 μL each of primer (10 pmol μL^{-1}), 0.5 μL of Taq DNA polymerase and distilled sterile water to make final total volume of 50 μL. The reaction mixture was incubated in a thermocycler (MJ Research, USA) programmed to run 30 cycles repeatedly (1 min at 94℃ for denaturation, 1 min at 55℃ for annealing, 1.5 min at 72℃ for polymerization), and finally, further incubated at 72℃ for 10 min for DNA amplification. The molecular sizes of the resulting PCR products were analyzed on 1.0% agarose gel to confirm 1.5 kb 16S rRNA. This 16S rRNA was purified using a DNA purification kit (QIAGEN) and its nucleotide sequences were determined by the dideoxy chain-termination method using a BigDyeTM Terminator v3.0 Sequencing Kit (Amersham Pharmacia Biotech, USA). The 16S rRNA sequences were confirmed and compared through a BLAST nucleotide search provided by the National Center for Biotechnology Information (NCBI) GenBank (U.S. National Library of Medicine, Bethesda, Maryland). The nucleotide sequence similarity of each isolate was obtained using the Gendoc program.

4. Enzyme activity assay

4-1. Preparation of cellulolytic enzyme solution

Starter cultures were prepared by transferring cells with an inoculation loop from the CMC agar plates to 100 mL of CMC liquid medium, the initial pH of which was adjusted to 7 if not specified, in 500 mL Erlenmeyer flasks. Two days after shaking incubation at 28℃, aliquots of 2 mL starter cultures were seeded into 200 mL of CMC liquid medium in 500 mL flasks. The flasks were further incubated on a shaker at 150 rpm for 7 days at 28℃. Culture samples were taken every 24 hr

during incubation and their cell-free supernatants (CFS) were obtained by centrifugation (10,000 x g, 5 min) and analyzed for cellulolytic activities. Meanwhile, the precipitated cells were suspended, washed in 5 mL of 0.05 M phosphate buffer (pH 6.5), and disrupted by sonication (150 mA, 20 min). The intracellular milieu (ICM) was obtained by centrifuging (12,000 x g, 30 min) the sonicated samples at 4℃ and the remaining cell debris (CD) was resuspended in 1 mL of 0.05 M phosphate buffer (pH 6.5). Also, CDs were assayed for cellulolytic enzyme activities.

4-2. Carboxymethylcellulase activity

Carboxymethylcellulase (CMCase) activity was measured by incubating 0.2 mL of enzyme solution with 0.5 mL of 1% (w/v) CMC, prepared in 0.1 M sodium acetate buffer (pH 5.0), and 0.3 mL of 0.1 M sodium acetate buffer (pH 5.0) for 10 min at 50℃. The reducing sugars liberated were estimated by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The enzyme reaction was stopped by the addition of 3 mL DNS solution to the above 1 mL reaction mixture, boiled in capped glass tubes for 5 min, cooled in cold water, and then optical density was measured at 540 nm. The CMCase activity was determined using a calibration curve for D-glucose. One unit of CMCase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars as glucose equivalents min^{-1}.

4-3. Avicelase activity

Avicelase activity was measured by incubating 0.5 mL of enzyme solution with 1 g of Avicel, a microcrystalline cellulose, as substrate and 1.5 mL of 0.1 M sodium acetate buffer (pH 5.0) for 1 hr at 50℃. After incubation, the reaction mixture was centrifuged at 10,000 x g for 5 min and then 1 mL of the supernatant was taken to determine reducing sugars by the DNS method. One unit of avicelase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars as glucose equivalents min^{-1}.

4-4. β-Glucosidase activity

β-Glucosidase (or cellobiase) activity was measured by using ρ-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate. The hydrolysis of pNPG releases ρ-nitrophenol, a pigmented substance that can be measured spectrophotometrically at 400 nm. The reaction mixture, containing 0.5 mL of 1 mg mL^{-1} pNPG, 0.5 mL of 0.05 M sodium acetate buffer (pH 5.0), and 0.5 mL of enzyme solution, was incubated at 50℃ for 1 hr. The enzyme reaction was stopped by adding 2 mL of 1 M Na_2CO_3, and the absorbance was measured at 400 nm. One unit of β-glucosidase activity was defined as the amount of enzyme that released 1 μmol of para-nitrophenol mL^{-1} hr^{-1}.

4-5. Xylanase activity

Xylanase activity was measured by using Beechwood xylan as a substrate (Bailey *et al.*, 1992). The reaction mixture containing 0.2 mL of crude enzyme, 0.5 mL of 1% xylan solution in 0.05 M phosphate buffer (pH 6.0), and 0.3 mL of buffer (pH 6.0) was incubated at 50℃ for 10 min. The enzymatic reaction was stopped by adding 3 mL of DNS reagent, boiled in capped glass tubes for 5 min, and cooled in cold water for color stabilization. The resulting optical density was measured at 520 nm. D-xylose was used as a standard for the preparation of a calibration curve. Enzyme units were expressed as μmol of reducing sugars released per mL of enzyme per minute.

5. Characterization of cellulase

5-1. Effect of pH

The optimum pH of the cellulase was determined by incubating the mixture of the crude enzyme and 1% (w/v) CMC dissolved in the presence of appropriate buffers; citric acid-Na_2HPO_4 buffer (pH 3 to 5), Na_2HPO_4-NaH_2PO_4 buffer (pH 6 to

8), and sodium carbonate-sodium bicarbonate buffer (pH 9 to 10). The reaction mixtures in various pH buffers were incubated for 60 min at 50℃ and the cellulase activity was assayed by DNS method.

5-2. Effect of temperature

The optimum temperature of the enzymes for hydrolyzing of CMC in 50 mM sodium acetate buffer (pH 5.0) was determined by incubating the mixture of the crude enzyme and 1% CMC (w/v) for 60 min at different temperature in the range of 30~90℃.

5-3. Effect of reaction time

The optimun reaction time of the enzymes for hydrolyzing of CMC at 50 mM sodium acetate buffer (pH 5) was determined by incubating the mixture of the crude enzyme and 1% CMC (w/v) for the different reaction time ranging from 10 to 60 min and assaying the reducing sugars released.

6. Optimization of culture conditions for cellulase production

6-1. Effect of incubation temperature

To enhance the production efficiency of the *Bacillus* strains, the effect of incubation temperature on cell growth was examined in the range of 5℃ to 60℃. The CMC-S medium used in this experiment, containing CMC, 10.0; soytone, 4.0; yeast extract, 4.0; $CaCl_2 \cdot 2H_2O$ 2.0; NaCl, 0.2; K_2HPO_4 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; and agar, 20 (g L^{-1}, each). The initial pH of medium were adjusted to 7 with 0.1 N HCl or 0.1 N NaOH. The plates were used 24 hole culture plates and prepared by adding with 1 mL of agar medium and then 50 μL of starter cultures were inoculated onto the plates. The plates were further incubated at respective temperatures for 24 hr. And then the plates were scored by the degree of growth. Also, The response of cell

growth and CMCase activity at the different culture temperatures *i.e.* 15℃, 25℃ and 35℃ were determined periodically. 200 μL of starter cultures were seeded into 10 mL of CMC-S liquid medium in 50 mL tube. The tube were further incubated in a shaker of 150 rpm at 25℃, pH 7 for 5 days. Culture samples were analyzed for cell growth and CMCase activity, respectively.

6-2. Effect of initial pH

To enhance the production efficiency of the *Bacillus* strains, the effects of initial pH of medium on cell growth and production of CMCase were investigated. The medium used for the production of cellulase by isolated bacteria hydrolyzing CMC, contained with CMC-S liquid medium. The initial pH of medium were adjusted from 4 to 10 with 0.1 N HCl and 0.1 N NaOH. 200 μL of starter cultures were seeded into 10 mL of CMC-S liquid medium in 50 mL tube. The tube were further incubated in a shaker of 150 rpm at 25℃ for 3 days. Culture samples were analyzed for cell growth and CMCase activity, respectively.

6-3. Effect of nitrogen sources

The production of cellulase enzyme was determined by performing the assay with some nitrogen sources: tryptone, peptone, soytone, casein, yeast extract, beef extract, urea, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, NH_4Cl, NH_4NO_3, $NaNO_3$, and KNO_3. The medium used for production of the cellulase by isolated bacteria hydrolyzing CMC, contained with various nitrogen sources by 0.4%, respectively. 200 μL of starter cultures were seeded into 10 mL of CMC liquid medium containing 1% of CMC and 0.4% of different nitrogen sources in 50 mL tubes, respectively. The tubes were further incubated in a shaker at 150 rpm, pH 5, 25℃ for 5days. Culture samples were analyzed for cell growth and CMCase activity, respectively.

Table 10. Some nitrogen sources

No.	Ingredient	Concentration (g L^{-1})
1	Tryptone	4
2	Peptone	4
3	Soytone	4
4	Casein	4
5	Yeast extract	4
6	Beef extract	4
7	Urea	4
8	$(NH_4)_2SO_4$	4
9	$(NH_4)_2HPO_4$	4
10	NH_4Cl	4
11	NH_4NO_3	4
12	$NaNO_3$	4
13	KNO_3	4

6-4. Effect of carbon sources

The substrate specificity of the enzyme was determined by performing the assay with different substrates: avicel (microcrystalline cellulose), cellulose, starch and carboxymethylcellulose. 200 μL of starter cultures were seeded into 10 mL of liquid basal medium containing soytone 4.0; yeast extract, 4.0; $CaCl_2 \cdot 2H_2O$ 0.2; NaCl, 0.2; K_2HPO_4 1.0; $MgSO_4 \cdot 7H_2O$, 0.2 (g L^{-1}, each) and 1% of different substrates as the sole carbon sources in 50 mL tubes. The tubes were further incubated in a shaker at 150 rpm, pH 5, 25℃ for 5 days. Culture samples were analyzed for cell growth and CMCase activity, respectively. And effect of the additive concentrations of CMC as a carbon source on the producton of CMCase were determined by incubating on CMC-S liquid medium containing 0.1, 0.3, 0.5, 1, 1.5 and 2% of CMC, respectively. 200 μL of starter cultures were seeded into 10 mL of CMC-S liquid medium in 50 mL tubes. The tubes were further incubated in a shaker at 150 rpm, pH 5, 25℃ for 5 days. Culture samples were analyzed for cell growth and

CMCase activity, respectively.

6-5. Effect of metal ions

The response of the activity of CMCase to metal ions were determined by incubating on CMC-S liquid medium with 1 mM and 0.2 mM metal salts such as $MnSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $FeSO_4 \cdot 7H_2O$. 200 μL of starter cultures were seeded into 7 mL of CMC-S liquid medium in 50 mL tubes. The tubes were further incubated in a shaker at 150 rpm, pH 5, 25℃ for 5 days. Culture samples were analyzed for cell growth and CMCase activity, respectively.

Table 11. Additive concentration of metal ions

Metal ion	Additive concentration (g L^{-1})	
	1 mM	0.2 mM
$MnSO_4 \cdot 5H_2O$	0.2410	0.05493
$ZnSO_4 \cdot 7H_2O$	0.2875	0.06637
$CuSO_4 \cdot 5H_2O$	0.2497	0.06354
$FeSO_4 \cdot 7H_2O$	0.2780	0.05585

7. Scale-up conditions for cellulase production

7-1. Production of cellulase from different agricultural wastes

The efforts have been made to study the production cellulase by *B. subtilis* on low cost cellulogic biomass materials. The production of cellulase was determined by performing the assay with some agricultural wastes as a carbon source; rice bran, citrus press cake, potato powder, bone dust, fish meal, rapeseed press cake, soybean meal, sudan grass meal, hairyvetch meal, and rye meal. 5 mL of starter cultures were seeded into 200 mL of liquid medium sterilized at 121℃ for 30 min in 500 mL bellco-culture flasks. The medium consisted of each carbon sources 1% on the

basic medium instead of CMC. The flasks were further incubated in a shaker at 150 rpm, pH 5, 28℃ for 5 days. Culture samples were analyzed for the cellulase production. To determine the mass cultivation condition of *B. subtilis* SL9-9, rice bran was used as a carbon or nitrogen source. A 2 g of CMC or rice bran was mixed with 200 mL of basal liquid medium with or without soytone as a nitrogen source, respectively. 5 mL of starter cultures were seeded into 200 mL of the liquid medium in 500 mL bellco-culture flasks. The flasks were further incubated in a shaker at 150 rpm, pH 5, 28℃ for 5 days. Culture samples were analyzed for the cellulase production.

Table 12. Conditions of mass culture medium

Ingredient	Concentration ($g\ L^{-1}$)	Conditions[a]			
		CMC+S	RB+S	CMC-S	RB-S
$CaCl_2 \cdot 2H_2O$	0.2	○	○	○	○
NaCl	0.2	○	○	○	○
K_2HPO_4	1	○	○	○	○
$MgSO_4 \cdot 7H_2O$	0.2	○	○	○	○
Yeast extract	4	○	○	○	○
Soytone	4	○	○		
CMC	10	○		○	
Rice bran	10		○		○

[a]CMC, carboxymethylcellulose; RB, rice bran; S, soytone.

7-2. Bioreactor and cultivation condition for cellulase production

This experiments were carried out using a 5 L bioreactor (Liflus GM-4, Biotron, Korea). The medium used for cellulase production by a isolated bacteria hydrolyzing rice bran contained the following components: 0.5% rice bran, $CaCl_2 \cdot 2H_2O$ 0.2, NaCl 0.2, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.2, Yeast extract 4 ($g\ L^{-1}$, each) at pH 5. Cell culture for the production of cellulase was carried out in 5 L bioreactor with the 3 L

working volume. Bioreactors filled with the substrate and the inert support, were sterilized in autoclave at 121℃ for 30min. After inoculation, cultivation proceed at 30℃, 150 rpm for 120 hr. Starter cultures were prepared by transferring cells from agar plates to 150 mL of medium in 500 mL bellco-culture flasks. The resulting cultures were inoculated after incubation for 2 days at 28℃, 150 rpm. Each starter culture was used as an inoculum for 3 L of medium in a 5 L bioreactor. The inoculum size was 5% (v/v). The aeration rate was 1.0vvm or non, respectively. Samples were periodically withdrawn from the cultures to examine cell growth and cellulase production.

7-3. Stability test of *B. subtilis* SL9-9 as a biofertilizer

The stability of *B. subtilis* SL9-9 was tested in the liquid and solid-state cultivation conditions as a biofertilizer. The liquid and solid biofertilizers of *B. subtilis* SL9-9 were made and stored at normal temperature, respectively. The samples were taken periodically every months and the CMCase activity of the cells were investigated during about 33 weeks. The liquid biofertilizer of *B. subtilis* SL9-9 was prepared by incubating in the liquid medium containing with 0.5% rice bran. Each 15 mL of the cultures were divided into several test tubes, respectively and used to test the stability. The solid biofertilizer was prepared using perlite as a biocarrier. Distilled water 5 mL was added into vinyl bags with perlite 50 mL. The bags were sterilized at 121℃ for 30 min. The seed cultures were prepared by transferring cells from agar plates to 200 mL of medium in 500 mL bellco-culture flasks. The resulting cultures were inoculated after incubation for 24 hr at 28℃, 150 rpm. Each 3 mL of seed cultures were added into the several bags as an inoculum for 50 mL of perlite media. Samples were periodically withdrawn to examine the production of cellulase. Each 0.1 mL or 0.1 g of samples were seeded into 7 mL of CMC liquid medium at the optimized condition for 5 days. Culture samples were analyzed for the cellulase production.

Ⅲ. Results

1. Screening of cellulolytic microorganisms

Cellulolytic bacteria were sought among 176 different samples collected from various environments such as soil, compost, animal waste slurry and cultured native microbes in Jeju Island. Samples collected for screening of cellulolytic microbes were listed in Table 13.

The selection of cellulolytic microorganisms were proceeded step by step. In the first round screening, a total of 848 cellulolytic clones were detected by clear halos around the clones with the trypan blue staining method on CMC agar medium (Table 14). The clones detected by clear halos were isolated in pure through the successive transfer cultures.

Table 13. Samples collected for screening of cellulolytic microbes

Source	Samples collected (ea)
Total	176
Soils	67
Composts	50
Animal waste slurry	22
Cultured native microbes	37

Table 14. The cellulolytic clones detected by clear halos in the first round

Source	Cellulolytic clones (ea)
Total	848
Soils	204
Composts	183
Animal waste slurry	165
Cultured native microbes	296

And then 279 clones were chosen based on their relatively good colonial development and visible clearing zones in the second round. Among 279 clones, the distributions of bacteria, actinomycetes and fungi were 70.6%, 27.7% and 1.7%, respectively (Fig. 4). There were 38, 45, 165 and 61 clones derived from soils, composts, animal waste slurry and cultured native microbes, respectively. In the third round, 69 clones were selected based on their relatively higher activities in a wide range of pH (Table 15~19). And then the 69 isolates were cultured on CMC agar medium in the 3 to 10 range on the pH and observed of the degree of growth after 24 culture (Table 20~23).

Finally, each one clone showing relatively higher cellulolytic activity and broader pH optimum in cell growth was selected in which animal waste slurry, compost, and soil, respectively. The three isolated bacteria were designated as SL9-9, C5-16, and S52-2 based themselves on the animal waste slurry, compost and soil, respectively (Fig. 5).

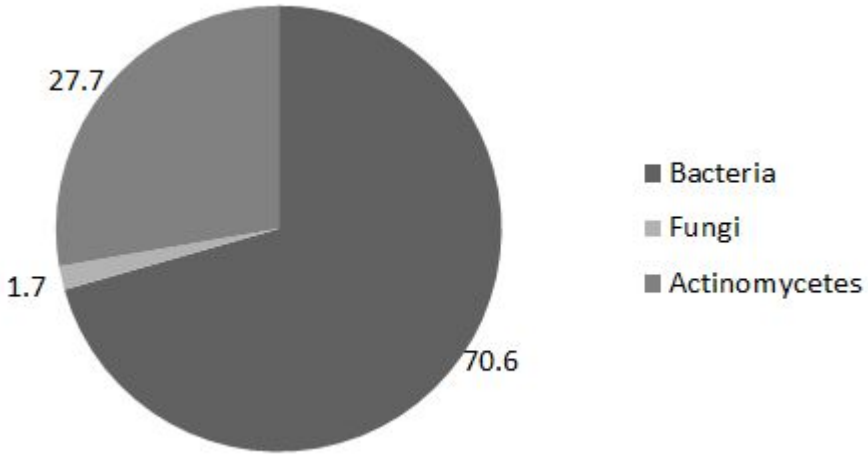

Fig. 4. Distribution of microbes selected in the second round.

Table 15. Selection of some clones from soils based on clear zone diameters of cellulolytic activities on trypan blue agar plates

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
1	S09-3	20.49	21.06	17.02	
2	S09-4	26.83	26.52	24.58	○
3	S19-1	26.03	20.28	14.99	
4	S24-4	26.20	18.69	15.78	○
5	S33-1	11.99	24.32	24.80	
6	S33-13	10.25	24.86	25.69	
7	S33-6	11.17	24.77	25.13	○
8	S34-3	26.18	25.81	23.17	○
9	S35-5	25.31	24.17	19.63	○
10	S37-1	14.37	-	13.43	
11	S37-2	17.23	17.77	15.51	
12	S37-3	10.97	14.27	15.39	
13	S38-1	11.18	24.21	25.06	
14	S38-7	19.16	26.00	25.66	○
15	S40-5	24.75	19.82	15.38	
16	S43-4	23.27	24.40	24.99	
17	S44-5	26.19	17.11	15.37	○
18	S44-8	10.64	25.12	24.70	
19	S45-1	-	20.65	21.49	
20	S45-4	12.19	24.53	23.91	
21	S45-8	16.87	20.75	17.19	
22	S46-13	22.65	20.74	15.66	
23	S46-8	23.74	24.86	23.52	
24	S48-7	10.57	23.86	23.10	
25	S49-1	-	23.43	24.28	
26	S49-7	22.02	23.38	23.81	
27	S51-10	11.32	23.94	25.30	
28	S52-2	27.69	27.20	26.41	○
29	S52-3	10.70	25.23	25.25	○
30	S53-5	23.76	25.74	25.04	○
31	S55-1	21.05	19.63	17.23	
32	S55-3	24.95	19.41	15.02	
33	S60-13	23.22	19.50	15.00	
34	S60-2	9.57	-	-	
35	S60-7	22.69	25.12	25.27	
36	S60-9	21.68	22.13	23.93	
37	S63-2	13.75	10.77	9.89	○
38	S65-9	20.57	15.68	17.45	○

Table 16. Selection of some clones from composts based on clear zone diameters of cellulolytic activities on trypan blue agar plates

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
1	C01-4	10.08	22.14	22.11	○
2	C01-18	30.97	27.54	22.66	○
3	C01-23	20.76	26.25	25.19	○
4	C01-4(a)	22.42	28.27	24.84	
5	C02-10	29.75	30.31	25.44	○
6	C02-16	28.09	28.12	29.41	○
7	C03-14	25.06	25.42	23.52	
8	C03-26	24.89	27.20	23.91	
9	C03-50	26.11	26.24	23.08	
10	C03-51	24.78	26.18	22.49	
11	C03-7	-	22.45	21.37	
12	C05-08	27.80	26.99	25.81	
13	C05-16	28.57	27.87	24.33	○
14	C05-17	27.58	27.80	25.63	
15	C05-19	28.48	23.21	25.37	
16	C05-3	28.56	22.83	24.28	
17	C05-9	30.33	27.34	23.48	○
18	C07-17	13.94	12.81	11.50	
19	C07-19	29.00	30.12	25.12	○
20	C08-23	28.45	25.30	23.61	
21	C08-24	27.75	28.15	25.04	○
22	C08-25	28.29	24.87	20.66	
23	C09-22	26.09	26.62	22.93	○
24	C09-29	28.31	27.27	2.94	○
25	C10-2	39.33	25.84	24.15	○
26	C10-9	24.30	27.35	24.62	○
27	C11-6	26.49	2.73	22.44	
28	C12-14	32.96	27.65	23.88	○
29	C12-15	26.54	27.82	25.41	
30	C12-16	26.28	28.09	24.66	○
31	C12-3	26.54	26.63	23.94	
32	C12-5(a)	31.81	22.69	26.89	○
33	C12-5(b)	24.22	26.98	19.38	○
34	C12-7	30.43	25.64	16.28	○
35	C13-18	23.68	22.59	26.30	

Table 16. (Contd.)

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
36	C17-10	25.67	26.13	24.91	
37	C18-11	25.77	25.48	23.68	
38	C18-5	31.40	25.46	24.14	
39	C20-11	9.48	12.44	12.92	
40	C24-7	11.46	19.50	21.20	
41	C25-2	10.16	26.00	23.21	
42	C38-2	22.95	21.68	16.45	
43	C41-6	24.49	23.42	20.35	
44	C44-3	12.81	22.44	19.88	
45	C47-2	14.28	23.74	23.01	

Table 17. Selection of some clones from slurry based on clear zone diameters of cellulolytic activities on trypan blue agar plates at neural pH

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
1	SL2-1	13.61	20.55	17.25	
2	SL2-8	22.87	25.20	23.19	○
3	SL2-9	23.28	25.52	21.24	○
4	SL2-11	11.95	26.67	23.94	
5	SL3-1	18.52	24.79	19.44	
6	SL3-3	11.07	18.44	18.16	
7	SL3-5	10.62	19.15	18.63	
8	SL3-7	13.35	26.04	20.87	
9	SL4-1	24.84	26.42	23.42	
10	SL4-4	18.50	20.75	17.15	
11	SL5-2	14.87	20.71	15.51	
12	SL5-3	11.03	17.30	11.47	
13	SL5-6	18.00	20.13	16.29	
14	SL5-7	12.13	21.85	20.49	
15	SL5-9	26.11	25.22	24.33	○
16	SL6-1	11.32	10.31	-	
17	SL6-3	23.98	23.95	22.53	
18	SL6-4	11.65	13.50	12.15	
19	SL2-14	10.49	25.44	24.16	
20	SL9-1	26.62	26.95	23.65	○
21	SL9-2	14.91	23.00	19.86	
22	SL9-3	10.85	22.08	14.57	
23	SL9-4	19.44	18.47	18.93	
24	SL9-5	12.27	16.02	10.77	
25	SL9-6	10.33	18.09	13.04	
26	SL9-7(a)	11.10	24.91	17.75	
27	SL9-7(b)	13.08	23.17	18.05	
28	SL9-8	13.37	17.94	22.23	
29	SL9-9	24.95	27.60	27.99	○
30	SL9-10	18.93	15.8	17.85	
31	SL10-1	26.71	25.69	24.89	○
32	SL10-2	17.37	15.32	11.00	
33	SL10-3	17.60	16.46	12.48	
34	SL10-4	16.99	10.14	-	
35	SL10-5	23.66	21.29	16.30	

Table 17. (Contd.)

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
36	SL10-6	10.87	22.22	16.96	
37	SL10-7	22.52	22.05	19.91	
38	SL10-8	21.94	19.45	14.71	
39	SL11-1	26.08	26.73	24.81	○
40	SL11-5(a)	15.65	-	-	
41	SL11-5(b)	22.92	21.00	16.10	
42	SL11-7	24.51	24.01	24.36	○
43	SL11-8(a)	22.64	24.89	20.85	
44	SL11-8(b)	22.61	20.80	18.48	
45	SL11-10	20.47	24.73	18.86	
46	SL12-2	20.00	21.11	20.28	
47	SL12-3(a)	11.92	27.84	20.90	○
48	SL12-3(b)	26.73	26.61	17.10	○
49	SL12-6	24.90	26.53	23.06	
50	SL14-1	11.25	22.12	18.94	
51	SL15-1	13.42	19.66	16.45	
52	SL15-2	26.03	26.40	24.60	
53	SL16-1	21.16	21.21	12.18	
54	SL16-2	24.14	26.50	19.32	
55	SL17-1	19.98	24.90	18.41	
56	SL17-2	15.56	23.87	21.12	
57	SL17-3	11.90	23.21	16.35	
58	SL18-1	13.37	25.19	20.56	
59	SL18-3	11.22	23.07	18.42	
60	SL18-4	25.01	25.47	23.94	○
61	SL19-1	13.03	20.53	16.87	
62	SL19-2	14.67	22.10	19.33	
63	SL19-3	10.99	21.88	17.27	
64	SL19-4	13.06	20.35	15.58	
65	SL19-5	9.85	19.43	12.73	
66	SL19-6	24.85	28.04	24.15	○
67	SL19-7	10.94	21.29	17.85	
68	SL19-8	12.20	21.21	17.41	
69	SL20-1	13.02	25.70	19.70	
70	SL20-2	11.18	23.79	20.57	

Table 17. (Contd.)

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
71	SL20-3	11.70	27.95	20.30	
72	SL20-4	10.68	15.91	13.42	
73	SL20-5	11.59	22.94	18.16	
74	SL22-1	12.28	26.89	19.57	
75	SL22-2	23.04	16.28	10.97	
76	SL23-1	17.84	24.85	21.07	
77	SL23-2	26.76	28.69	24.58	○
78	SL23-3	21.55	23.04	19.42	
79	SL23-4	26.25	27.78	23.27	○
80	SL23-5	10.15	23.91	16.94	
81	SL23-6	23.94	28.87	24.28	○

Table 18. Selection of some clones from slurry based on clear zone diameters of cellulolytic activities on trypan blue agar plates at alkali pH

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
1	SL1-3(a)	-	23.82	22.59	
2	SL1-3(b)	10.36	26.85	26.52	
3	SL1-4	16.62	20.39	15.80	
4	SL2-1	26.09	18.43	12.37	
5	SL2-2	22.57	19.68	14.30	
6	SL2-4	9.97	18.49	16.47	
7	SL3-4	23.11	23.11	22.51	
8	SL3-12	10.32	23.12	20.43	
9	SL4-1	12.45	19.78	15.34	
10	SL4-2	25.90	20.88	15.92	
11	SL4-3	11.87	20.71	12.26	
12	SL5-1	10.58	17.64	17.37	
13	SL5-2	10.58	18.60	18.47	
14	SL5-3	10.95	23.41	23.86	
15	SL5-4	17.77	19.23	17.45	
16	SL9-7	11.94	24.26	19.74	
17	SL9-9	-	19.22	17.69	
18	SL9-12	29.46	27.29	26.49	○
19	SL10-1	18.17	20.33	17.90	
20	SL10-2	11.87	-	13.44	
21	SL11-1	13.60	23.21	19.46	
22	SL11-2	13.73	24.32	21.17	
23	SL11-3	13.38	24.56	20.42	
24	SL11-5	12.69	22.26	20.21	
25	SL14-1	14.08	18.72	14.96	
26	SL15-1	20.08	18.48	20.79	
27	SL15-2	16.60	-	-	
28	SL15-3(a)	24.43	25.32	23.77	
29	SL15-3(b)	22.87	26.18	21.96	
30	SL16-1	13.29	20.91	16.40	
31	SL16-2	26.26	27.64	25.14	○
32	SL16-3	17.14	-	-	
33	SL16-4	18.19	20.43	22.00	
34	SL17-1	13.11	19.12	16.51	
35	SL17-2	23.77	29.86	23.10	

Table 18. (Contd.)

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
36	SL17-3	23.84	24.84	21.22	
37	SL17-4	16.24	29.66	23.25	
38	SL17-5	10.88	20.04	18.50	
39	SL17-6	25.47	23.53	26.49	
40	SL17-7	18.91	21.14	17.58	
41	SL17-8	9.91	19.07	17.21	
42	SL17-9	15.07	20.53	17.80	
43	SL17-10	22.92	27.17	26.11	
44	SL18-1	20.65	20.59	19.98	
45	SL18-2	24.89	27.26	23.52	○
46	SL18-3	19.43	19.86	19.07	
47	SL18-4	22.81	24.17	20.41	
48	SL18-5	23.20	20.75	17.02	○
49	SL18-6	24.50	15.23	9.97	
50	SL18-7	18.46	18.29	16.24	
51	SL19-1	11.71	20.62	16.02	
52	SL19-2	16.25	18.19	14.82	
53	SL19-3	10.98	18.28	13.62	
54	SL19-4	10.98	19.35	15.63	
55	SL19-5	11.44	19.84	14.09	
56	SL19-6	10.18	16.34	12.44	
57	SL19-7	11.68	20.63	11.94	
58	SL19-8	11.51	27.36	22.09	
59	SL19-9	19.32	25.86	21.23	○
60	SL19-10	22.84	26.20	19.78	○
61	SL20-1	13.55	23.20	19.57	
62	SL20-2	14.49	25.39	22.19	
63	SL20-3	11.20	16.90	13.64	
64	SL20-4	22.68	24.17	19.41	
65	SL20-5	10.84	21.54	15.19	
66	SL20-6	13.77	22.98	16.62	
67	SL20-7	26.16	29.89	25.13	○
68	SL20-8	9.90	21.60	19.53	
69	SL20-9	13.92	24.05	19.88	
70	SL21-1	14.42	-	-	

Table 18. (Contd.)

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
71	SL22-1	23.12	15.14	13.87	
72	SL22-2	21.77	18.27	12.09	
73	SL23-1	28.06	26.58	18.85	○
74	SL23-2	12.05	26.93	23.62	
75	SL23-3	27.18	26.08	24.07	○
76	SL23-4	21.73	12.87	13.49	
77	SL23-5	26.03	28.62	26.60	○
78	SL23-6	26.09	27.58	24.13	
79	SL23-7	26.86	30.22	25.80	
80	SL23-8	9.78	21.33	17.42	
81	SL23-9	21.19	18.49	9.68	
82	SL23-10	26.10	29.38	25.64	○
83	SL23-11	23.32	26.46	24.46	○
84	SL23-12	14.59	21.92	19.22	

Table 19. Selection of some clones from cultured native microbes based on clear zone diameters of cellulolytic activities on trypan blue agar plates

No.	Clones	Diameter of clear zone (mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
1	M02-1	29.78	29.47	26.36	
2	M02-3	29.52	27.95	23.80	○
3	M02-8	28.48	28.43	25.21	○
4	M02-9	28.23	29.04	25.98	
5	M03-1	44.34	28.13	24.58	○
6	M03-2	28.62	28.13	23.01	
7	M03-3	25.41	28.93	24.27	
8	M03-5	21.41	27.30	24.68	
9	M05-1	14.07	22.70	19.43	
10	M05-3	14.00	26.98	21.90	
11	M06-1	26.83	26.90	22.82	
12	M09-2	11.96	23.79	16.46	
13	M10-1	29.71	32.15	26.32	○
14	M11-8	25.94	28.30	23.79	
15	M15-4	25.21	28.85	26.46	
16	M16-7	29.73	29.20	24.82	
17	M19-2	20.43	28.30	22.52	
18	M19-3	17.27	27.00	22.98	
19	M19-4	35.28	28.99	25.05	○
20	M19-8	28.67	28.36	24.59	
21	M23-18	29.58	27.99	24.86	
22	M23-8	27.01	27.82	21.31	
23	M23-9	21.83	31.18	23.27	
24	M25-8	29.14	27.26	22.83	
25	M26-1	28.66	28.24	25.19	
26	M26-13	29.12	29.98	26.62	○
27	M26-14	27.98	29.54	24.91	
28	M26-15	30.20	28.39	23.47	
29	M26-4	28.10	28.33	22.70	
30	M26-6	30.75	30.18	25.12	○
31	M26-7	27.73	28.21	24.68	
32	M26-8	28.08	32.05	24.76	
33	M26-9	29.78	29.00	24.15	
34	M27-14	28.39	29.13	24.52	
35	M27-2	23.85	-	-	

Table 19. (Contd.)

No.	Clones	Diameter of clear zones(mm)			Selection
		pH 4.0	pH 7.0	pH 9.0	
36	M27-6	29.47	28.70	25.12	
37	M28-1	29.82	29.81	25.80	
38	M28-2	28.01	29.36	24.64	○
39	M29-3	28.38	28.03	26.10	
40	M29-6	27.01	29.05	23.95	
41	M30-1	29.43	31.82	25.61	
42	M30-5	32.02	31.26	26.46	○
43	M30-6	30.83	29.39	25.20	
44	M30-9	29.51	26.12	24.69	
45	M31-5	29.81	27.99	24.61	
46	M33-1	30.05	29.73	26.11	
47	M33-12	27.79	29.11	26.03	
48	M33-4	30.87	30.03	24.95	
49	M33-5	30.19	28.09	24.56	
50	M33-9	29.30	28.85	24.89	○
51	M34-2	28.38	28.58	24.45	
52	M35-3	30.80	28.76	26.10	
53	M35-6	28.07	29.92	26.74	
54	M36-1	28.55	30.30	25.64	
55	M37-1	29.22	21.55	20.01	
56	M37-1	28.06	29.41	25.64	
57	M37-10	28.67	29.10	24.51	○
58	M37-2	27.93	29.27	24.68	○
59	M37-3	26.80	28.66	23.97	
60	M37-4	27.85	30.00	25.32	
61	M37-5	29.52	28.15	23.85	

Table 20. Cell growth of the clones selected from soils in the pH range of 3~10

No.	Clones	pH of CMC agar medium							
		3	4	5	6	7	8	9	10
1	S09-4	-	-	+	++	+++	+++	-	-
2	S24-4	-	-	-	+	++	++	-	-
3	S33-6	-	-	-	+	++	++	-	-
4	S34-3	-	-	+	+++	+++	++	+	-
5	S35-5	-	-	-	++	+++	+++	-	-
6	S38-7	-	-	++	+++	+++	+++	-	-
7	S44-5	-	-	-	++	+++	+++	-	-
8	S52-2	-	-	++	+++	+++	+++	+	-
9	S52-3	-	-	-	++	++	+++	+	-
10	S53-5	-	-	-	++	++	+++	+	-
11	S63-2	-	-	-	+++	+++	+++	+	-
12	S65-9	-	-	-	++	++	+++	-	-

Note : - , non; +, poor; ++, medium; +++, good growth.

Table 21. Cell growth of the clones selected from composts in the pH range of 3~10

No.	Clones	pH of CMC agar medium							
		3	4	5	6	7	8	9	10
1	C01-4	-	+	+	+++	+++	+++	-	-
2	C01-18	-	+	+	+++	+++	+++	-	-
3	C01-23	-	+	+	++	+++	+++	-	-
4	C02-10	-	-	+	+++	+++	++	-	-
5	C02-16	-	-	++	+++	+++	+++	-	-
6	C05-16	-	+	++	+++	+++	+++	+	-
7	C05-9	-	-	+	+++	+++	++	-	-
8	C07-19	-	+	+++	+++	+++	+++	-	-
9	C08-24	-	+	+	+++	+++	+++	-	-
10	C09-22	-	-	+	+++	+++	+++	-	-
11	C09-29	-	+	+	+++	+++	+++	+	+
12	C10-2	-	+	+	+++	+++	+++	-	-
13	C10-9	-	+	++	+++	+++	+++	-	-
14	C12-14	-	-	+	+++	+++	+++	-	-
15	C12-16	-	-	+	+++	+++	+++	-	-
16	C12-5(a)	-	-	+	+++	+++	+++	+	+
17	C12-5(b)	-	+	+	+++	+++	+++	-	-
18	C12-7	-	-	+	+++	+++	+++	+	+

Note : - , non; +, poor; ++, medium; +++, good growth.

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Table 22. Cell growth of the clones selected from cultured native microbes in the pH range of 3~10

No.	Clones	pH of CMC agar medium							
		3	4	5	6	7	8	9	10
1	M02-3	-	-	-	++	+++	+	-	-
2	M02-8	-	-	-	+	+	+	-	-
3	M03-1	-	-	+	+	+	+	-	-
4	M10-1	-	-	-	++	+	++	-	-
5	M19-4	-	-	+	+	+	+	-	-
6	M26-13	-	-	-	+	+	+	-	-
7	M26-6	-	-	-	+	+	+	-	-
8	M28-2	-	-	-	+	++	+	-	-
9	M30-5	-	-	-	+	+	++	-	-
10	M33-9	-	-	-	+	++	++	-	-
11	M37-10	-	-	+	++	++	++	-	-
12	M37-2	-	-	+	++	++	++	-	-

Note : - , non; +, poor; ++, medium; +++, good growth.

Table 23. Cell growth of the clones selected from animal waste slurry in the pH range of 3~10

No.	Clones	pH of CMC agar medium							
		3	4	5	6	7	8	9	10
1	SL2-8	-	-	-	++	+++	+	+	+
2	SL2-9	-	-	-	+++	++	++	-	-
3	SL5-9	-	-	-	+++	++	+	+	-
4	SL9-1	-	-	+	+++	++	++	-	-
5	SL9-9	-	-	+	+++	+++	+++	+	+
6	SL10-1	-	-	+	+++	++	++	-	-
7	SL11-1	-	-	+	++	+++	+++	-	-
8	SL11-7	-	-	+	++	++	+++	+	-
9	SL12-3(a)	-	-	+	++	++	++	+	-
10	SL12-3(b)	-	-	+	+++	+++	++	-	-
11	SL18-4	-	-	-	+	+	++	+	-
12	SL19-6	-	-	-	+	+	++	-	-
13	SL23-2	-	-	+	++	+++	++	-	-
14	SL23-4	-	-	+	+	++	+++	-	-
15	SL23-6	-	-	+	++	++	++	-	-
16	SL9-12	-	-	+	+++	+++	+++	-	-
17	SL16-2	-	-	+	++	+++	++	-	-
18	SL18-2	-	-	+	+++	+++	++	+	-
19	SL18-5	-	-	+	+++	+++	++	+	-
20	SL19-9	-	-	+	++	++	++	+	-
21	SL19-10	-	-	+	++	+++	++	+	-
22	SL20-7	-	-	+	++	+++	++	+	-
23	SL23-1	-	-	+	+++	+++	++	-	-
24	SL23-3	-	-	-	++	++	++	+	+
25	SL23-5	-	-	-	++	+++	++	+	+
26	SL23-10	-	-	-	++	+++	+++	+	-
27	SL23-11	-	-	+	++	+++	+++	+	-

Note : - , non; +, poor; ++, medium; +++, good growth.

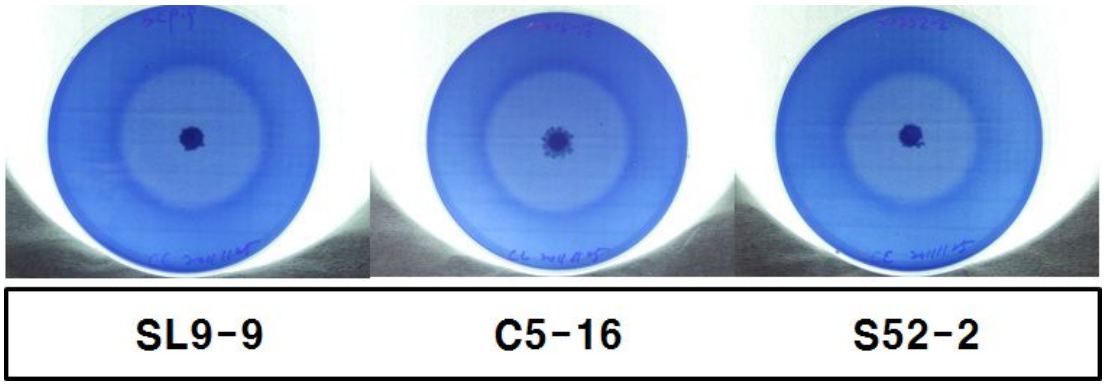

Fig. 5. Carboxymethylcellulase (CMCase) activities of isolated cellulolytic bacteria.

2. Identification of isolated cellulolytic bacteria

Morphological and cultural studies revealed that all the clones were Gram-positive and rod-shaped bacteria (Table 24 & Fig. 6). They were also catalase-positive, aerobic, moderate thermophiles. Their biochemical properties were further examined with an API kit and compared with other *B. subtilis* strains, namely *B. subtilis* KACC10111 and *B. subtilis* KCTC3560 (Table 25). The three bacterial isolates showed slight differences from each other in such biochemical properties as methyl-α -D-glucopyranoside, amygdalin, salicin, D-maltose, D-lactose, inulin, glycogen, gentiobiose, and D-turanose utilization when the API 50CHB kit was used.

Table 24. Morphological and physiological properties of isolated cellulolytic bacteria

Characteristic	Bacterial isolates		
	SL9-9	C5-16	S52-2
Gram staining	positive(+)	positive(+)	positive(+)
Motility	+	+	+
Catalase	+	+	+
Cell shape	rod	rod	rod
Size(L, ㎛)	2.5~3.0	2.5~3.0	2.5~3.0
Colony appearance			
Shape	round	round	round
Margin	entire	entire	undulate
Elevation	umbonate	umbonate	umbonate
Growth temp range(℃)[a]	15~50	15~55	15~50
Growth pH range[b]	5~10	4~9	5~9

[a]Incubated for 72 hr; [b]Incubated for 48 hr.

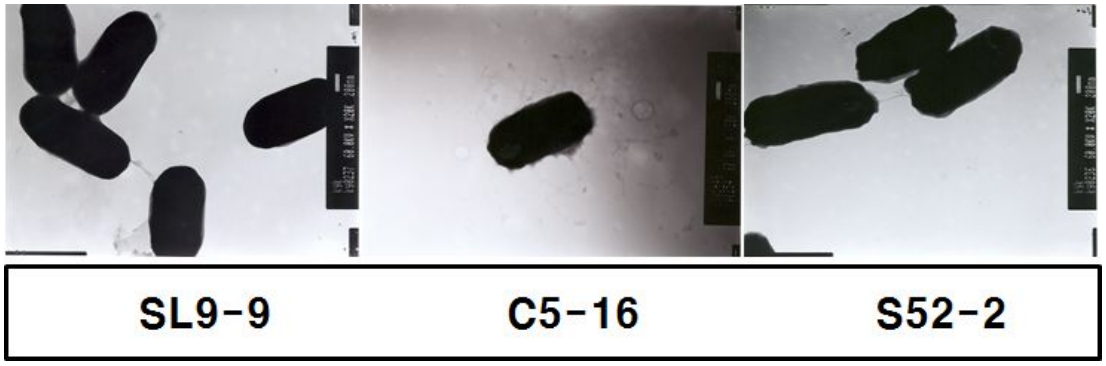

Fig. 6. TEM photos of isolated cellulolytic bacteria.

Table 25. Biochemical properties of isolated cellulolytic bacteria

Test[a)]	Strains				
	SL9-9	C5-16	S52-2	*B. subtilis* KACC 10111	*B. subtilis* KCTC 3560
Control	-	-	-	-	-
Glycerol	+	+	+	+	+
Erythritol	-	-	-	-	-
L-Arabinose	+	+	+	+	+
D-Ribose	+	+	+	+	+
D-Xylose	+	V	V	+	V
L-Xylose	-	-	-	-	-
D-Adonitol	-	-	-	-	-
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
L-Sorbose	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Dulcitol	-	-	-	-	-
Inocitol	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Sorbitol	+	+	+	+	+
Methyl-α-D-Glucopyranoside	+	+	-	+	+
N-Acetyl-glucosamine	-	-	-	-	-
Amygdalin	+	+	-	+	V
Arbutin	+	+	+	+	+
Esculin ferric citrate	+	+	+	+	+
Methyl-β-Xylopyranoside	-	-	-	-	-
D-Lactose (bovine origin)	+	-	+	-	-
D-Arabinose	-	-	-	-	-

Table 25. (Contd.)

Test[a]	Strains				
	SL9-9	C5-16	S52-2	*B. subtilis* KACC 10111	*B. subtilis* KCTC 3560
Salicin	+	+	-	+	+
D-Cellobiose	+	+	+	+	+
D-Maltose	+	+	-	+	+
D-Melibiose	+	+	+	+	∨
D-Saccharose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
Inulin	-	+	-	+	∨
D-Melezitose	-	-	-	-	-
Glycogen	+	+	-	+	+
Xylitol	-	-	-	-	-
Gentiobiose	-	∨	-	-	∨
D-Turanose	-	+	-	+	∨
D-Lyxose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
D-Arabitol	-	-	-	-	-
Potassium gluconate	-	+	-	+	-
Potassium 2-ketogluconate	-	-	-	-	-
D-Galactose	-	-	-	-	-
D-Raffinose	+	+	+	+	∨
Potassium 5-ketogluconate	-	-	-	-	-
Methyl-αD-Mannopyranoside	-	-	-	-	-
AmiDon (Starch)	+	+	-	+	+
L-Arabitol	-	-	-	-	-

[a]API 50CHB Kit (BioMeriuex, France)was used to determine positive (+), negative (-) or variable(∨).

These three bacterial isolates were finally identified by 16S rRNA gene sequence analysis (Fig. 7~9). Their sequences were entered into the nucleotide-nucleotide BLAST (NCBI) system and percentage identities were established (Table 26). The highest identity for the isolate SL9-9 (accession No. HQ236379) was 99% with the *B. subtilis* strain BFAS (accession No. AY775778.1). The isolates C5-16 (accession No. HQ236380) and S52-2 (accession No. HQ236381) showed the highest identity at 99% with *B. subtilis* strain CE1 (accession No. JQ435698.1) and at 99% with *B. subtilis* isolate C9-1 (accession No. EU257446.1), respectively. Based on their morphological, physiological, and genetic data, the three bacterial isolates were designated as *B. subtilis* SL9-9, C5-16, and S52-2, respectively.

```
1    AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGG 70
71   GAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGAT 140
141  AACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTT 210
211  CGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACG 280
281  ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG 350
351  CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT 420
421  CGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCT 490
491  AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA 560
561  TTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAG 630
631  GGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG 700
701  TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC 770
771  GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGG 840
841  TTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAA 910
911  CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 980
981  CTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGT 1050
1051 GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT 1120
1121 CTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG 1190
1191 ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCG 1260
1261 AAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGT 1330
1331 GAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1400
1401 GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTAAGGAGCCAGCCGCCGAA 1470
1471 GGTGGGACAGATGATTGGGGTGAAGTCG 1498
```

Fig. 7. Nucleotide sequence of 16S rRNA gene of SL9-9.

```
1    AGAGTTTGACCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGG 70
71   AGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAATGACTGGGAT 140
141  AACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTT 210
211  CGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACG 280
281  ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG 350
351  CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT 420
421  CGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCT 490
491  AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA 560
561  TTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAG 630
631  GGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG 700
701  TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGARGAGCGAAAGC 770
771  GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGG 840
841  TTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAA 910
911  CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC 980
981  CTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGT 1050
1051 GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT 1120
1121 CTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG 1190
1191 ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCG 1260
1261 AAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGT 1330
1331 GAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC 1400
1401 GCCCGTCACTCCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAA 1470
1471 GGTGGGACAGATGATTGGGGTGAAGTCGTAAAAGG 1505
```

Fig. 8. Nucleotide sequence of 16S rRNA gene of C5-16.

```
1    AGAGTTTGACCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGG 70
71   AGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATA 140
141  ACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTC 210
211  GGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGA 280
281  TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC 350
351  AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC 420
421  GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTA 490
491  ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAAT 560
561  TATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG 630
631  GTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT 700
701  AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCG 770
771  TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGT 840
841  TTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAAC 910
911  TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC 980
981  TTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTG 1050
1051 GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATC 1120
1121 TTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA 1190
1191 CGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGA 1260
1261 AACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTG 1330
1331 AAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG 1400
1401 CCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTATGGAGCCAGCCGCCGAAG 1470
1471 GTGGGACAGATGATTGGGGTGAAGT 1495
```

Fig. 9. Nucleotide sequence of 16S rRNA gene of S52-2.

Table 26. Comparison of 16S rRNA gene sequences with those of other bacteria

Isolated strain	Identity (%)	Nucleotide different /Nucleotide compared	Reference strain
SL9-9 (HQ236379)[a]	99	1/1498	*Bacillus subtilis* BFAS (AY775778.1)
C5-16 (HQ236380)	99	5/1505	*Bacillus subtilis* CE1 (JQ435698.1)
S52-2 (HQ236381)	99	4/1495	*Bacillus subtilis* C9-1 (EU257446.1)

[a]NCBI GenBank accession No.

The CMCase activity of three isolates was compared with those of other known *Bacillus* species obtained from KACC and KCTC by CMCase activity assay using cell-free supernatant obtained from the liquid cultures (Fig. 10). Their CMCase activities remained quite high around pH 5~8 compared to other *Bacillus* species.

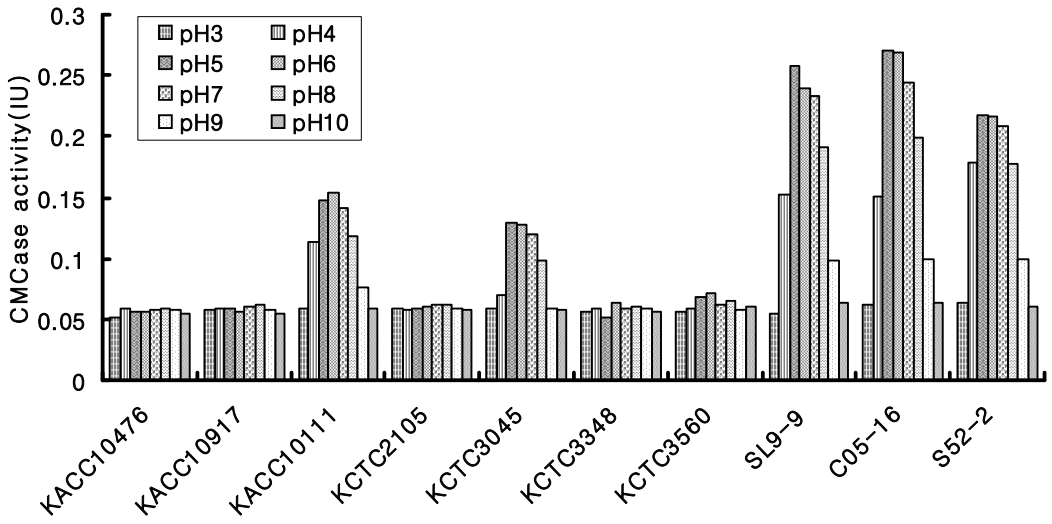

Fig. 10. Comparison of carboxymethylcellulase (CMCase) activity of *Bacillus* species at various cultivation pH.

KACC10476, *B. licheniformis*; KACC10917, *B. pumilus*; KACC10111, *B. subtilis*; KCTC2105, *B. amylolicheniformis*; KCTC3045, *B. licheniformis*; KCTC3348, *B. pumilus*; KCTC3560, *B. subtilis.*

3. Production of cellulolytic enzymes by isolated *B. subtilis* strains

The three isolates were examined for CMCase, avicelase, β-glucosidase, and xylanase production after cultivation in 200 mL of CMC liquid medium. *B. subtilis* KACC10111 (Fig. 10), which showed higher CMCase activity than the other 6 *Bacillus* species obtained from KACC and KCTC, was used as a reference for enzyme activity comparisons.

SL9-9, C5-16 and S52-2 presented similar behavior towards reducing sugar liberation and consumption (Fig. 11), as a peak of sugar concentration was observed in 24 hr of cultivation, followed by sharp decline, which indicates carbon source consumption. While KACC10111 which showed very little variation during cultivation, showed different behavior. KACC10111 liberated the lowest amount of reducing sugar whose concentration increased a litter up to 96 hr and decrease a little up to the end of the process.

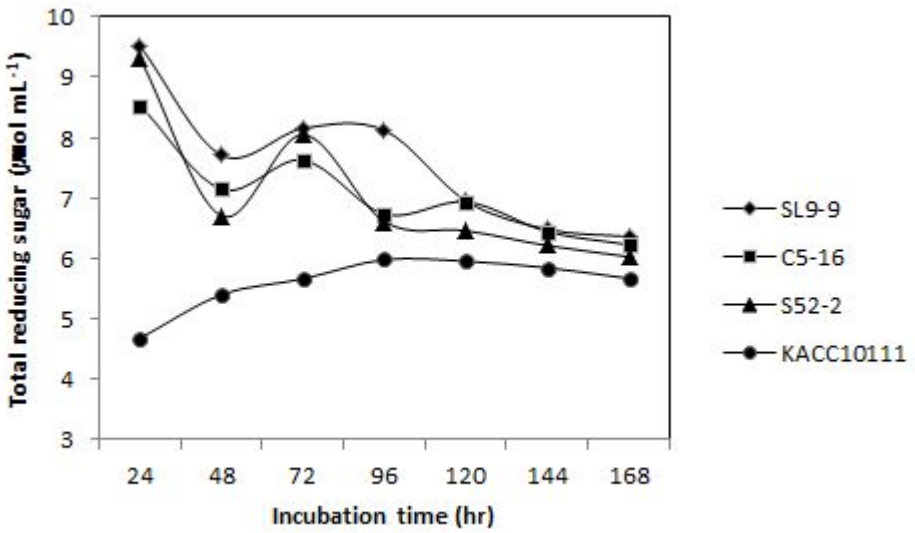

Fig. 11. Total reducing sugar in cell-free culture supernatant of isolated *B. subtilis* strains.

Fig. 12. shows the CMCase activity profiles obtained during shaking incubation for 7 days with 10 g L^{-1} of CMC as a carbon source. In the cell-free supernatant, both strains of SL9-9 and C5-16 showed considerable CMCase activity, reaching their maxima after 72 hr of cultivation (0.9 IU and 0.8 IU, respectively), while the other two strains, S52-2 and KACC10111, presented relatively lower activities. The CMCase activities decreased slightly after 120 hr of cultivation. In the cell debris fraction there was no observable CMCase activity (Fig. 12B).

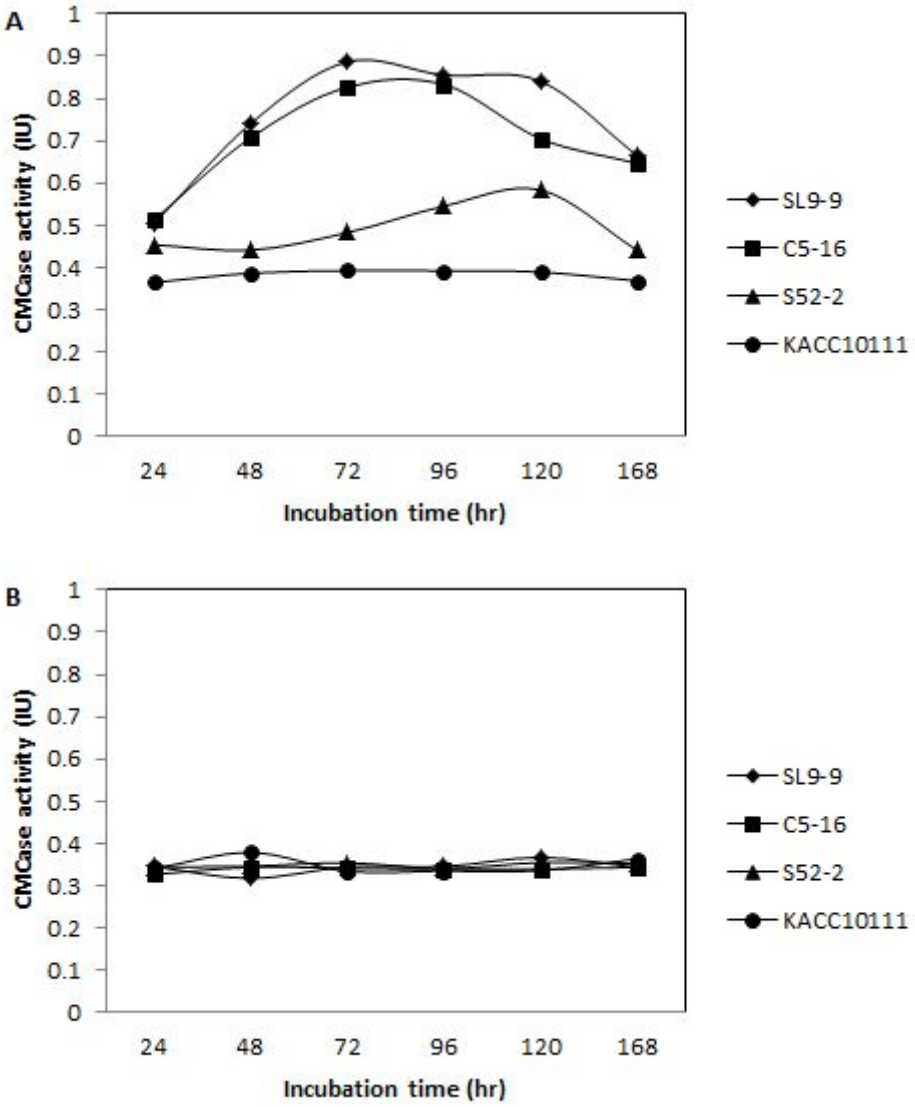

Fig. 12. Carboxymethylcellulase (CMCase) activity in cell-free culture supernatant (A) and cell debris (B) of isolated *B. subtilis* strains.

Fig. 13. shows the avicelase activity profiles obtained during shaking incubation for 7 days with 10 g L^{-1} of CMC as a carbon source. In the cell free supernatant, all the strains produced considerable avicelase activity and maintained maximum activity after 72~96 hr of cultivation, although C5-16 showed a slight drop after 144 hr of cultivation. On a whole, SL9-9 presented higher activity than the other isolates from the beginning of cultivation. In the cell debris fraction, there was no definite avicelase activity.

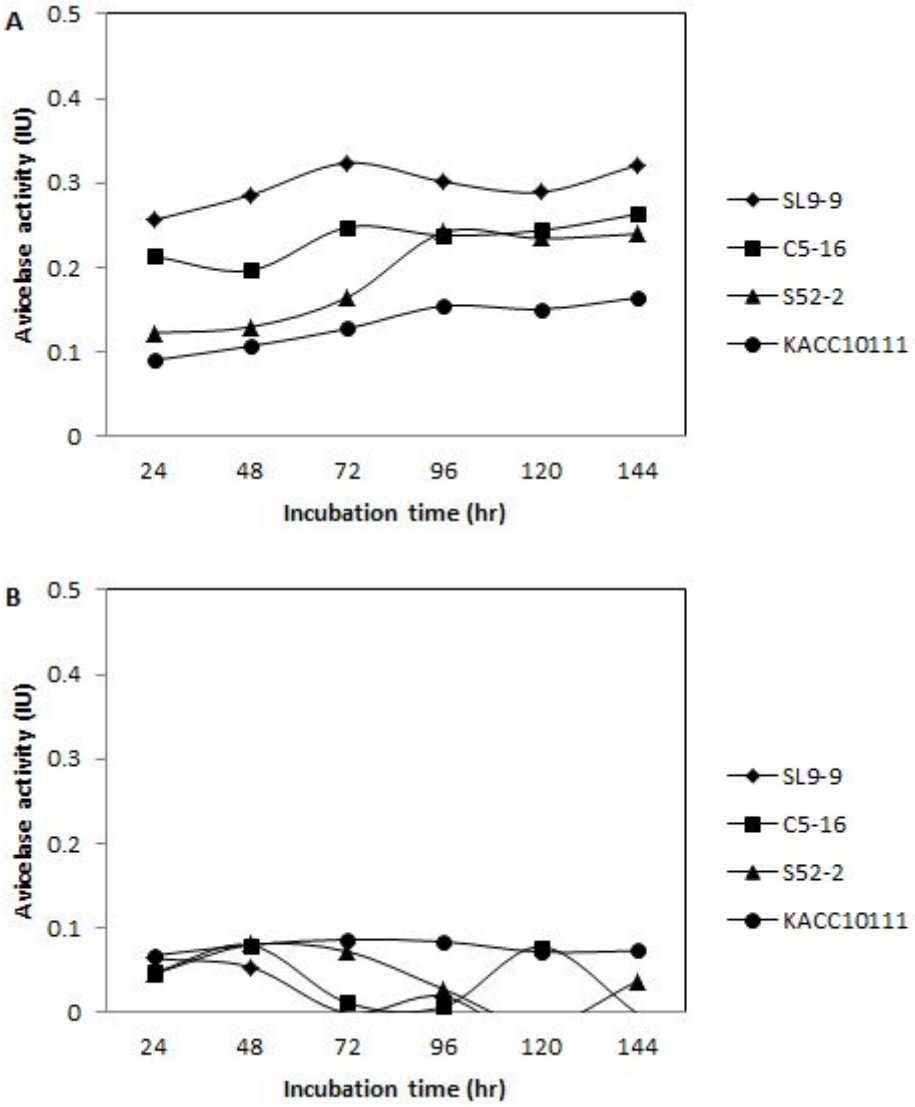

Fig. 13. Avicelase activity in cell-free culture supernatant (A) and cell debris (B) of isolated *B. subtilis* strains.

Fig. 14. shows the β-glucosidase activity profiles obtained during shaking incubation for 7 days with 10 g L^{-1} of CMC as a carbon source. In contrast to CMCase and avicelase, no β-glucosidase activity was observed in the cell-free supernatant. However, all the strains showed considerable β-glucosidase activity in the cell debris fraction. Maximum activities (1.0, 1.0, 0.6, and 1.2 IU for SL9-9 C5-16, S52-2, and KACC10111, respectively) were detected early after cultivation for 24 hr. The enzyme activities subsequently decreased after reaching maximum values, and then second rises and falls were observed.

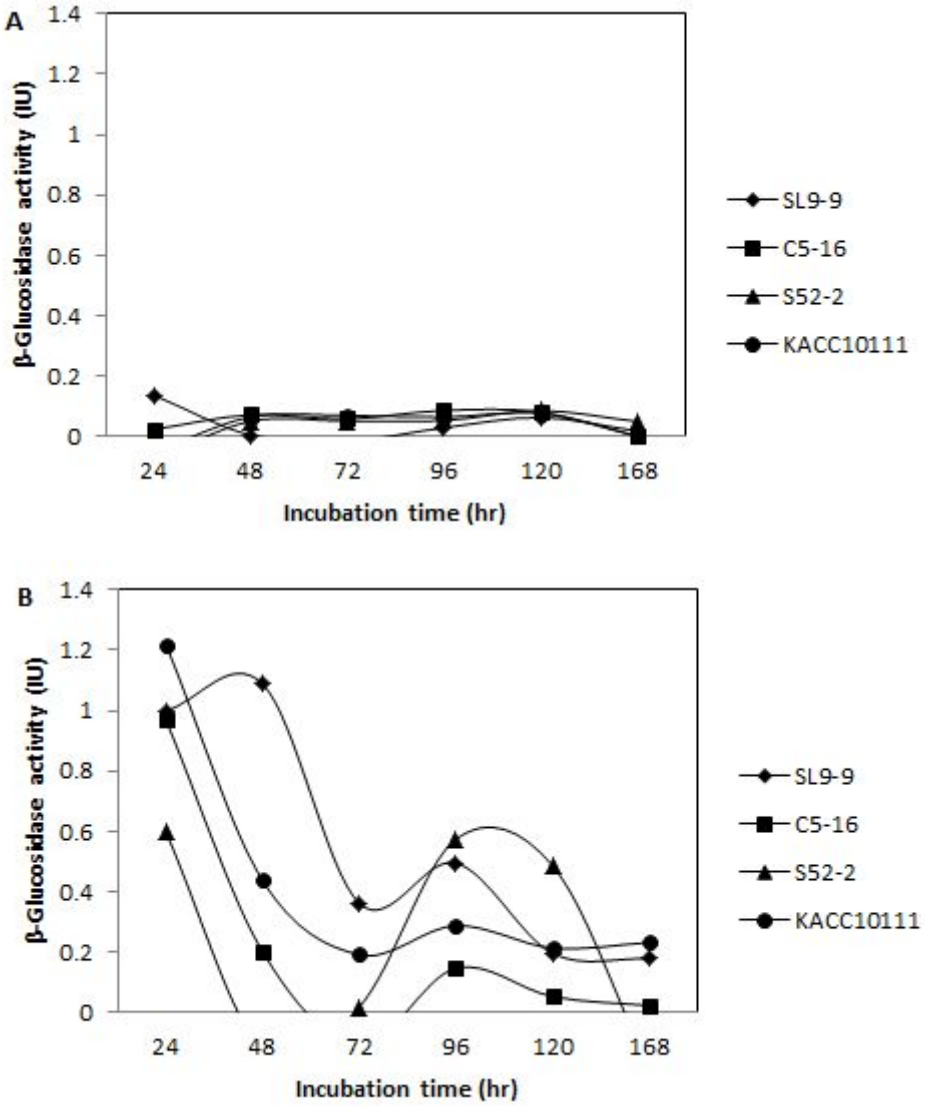

Fig. 14. β-Glucosidase activity in cell-free culture supernatant (A) and cell debris (B) of isolated *B. subtilis* strains.

The *Bacillus* strains SL9-9 and S52-2 showed considerable xylanase activity in the cell-free culture supernatant, and their activities reached maximum values (12.0, and 11.5 IU respectively) after shaking culture for 96 hr with 10 g L^{-1} of CMC as a carbon source, as shown in Fig. 15. The other two strains, C5-16 and KACC10111, presented lower overall activity from the beginning of cultivation. No xylanase activity was detected in the cell debris fraction.

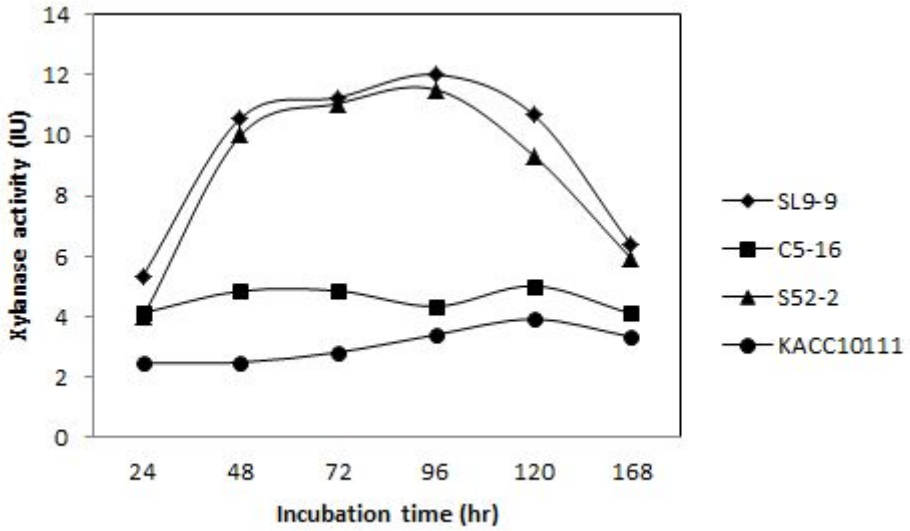

Fig. 15. Xylanase activity in cell-free culture supernatant of isolated *B. subtilis* strains.

4. Characterization of cellulase from isolated *B. subtilis* strains

The four strains of SL9-9, C5-16, S52-2 and KACC10111 were used to characterize their CMCase. Effects of pH, temperature and reaction time on the activity of CMCase were investigated and the optimum conditions were determined.

4-1. Effect of pH on the activity of CMCase

The effect of pH on CMCase activity of four *Bacillus* strains was examined at various pHs ranging from pH 3 to 10 as shown in Fig. 16. Four *Bacillus* strains CMCase of SL9-9, C5-16, S52-2 and KACC10111 exhibited optimal activity in the pH range of 5~7, with the highest activity at pH 5, while the KACC10111 enzyme at pH 6. The SL9-9, C5-16, S52-2 and KACC10111 enzymes had about 47%, 44%, 76% and 58% of the maximal level, respectively at pH 4 and none at pH 3. At pH 8 and 9, the SL9-9, C5-16, S52-2 and KACC10111 enzymes expressed over 64% and 19% of the maximal level, respectively, while were inactive at pH 10.

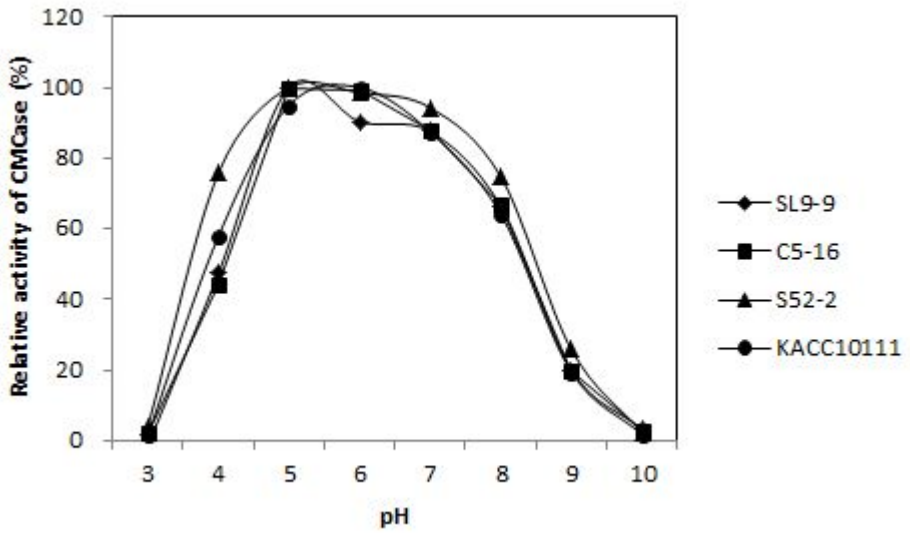

Fig. 16. Effect of pH on carboxymethylcellulase (CMCase) activity of *B. subtilis* strains.

Relative activity is expressed as percentage of the maximum.

4-2. Effect of temperature on the activity of CMCase

The effect of temperature on the CMCase activity of isolated bacteria was determined at various reaction temperatures in the range of 30~90℃ by measurement of residual activity after incubation in sodium acetate buffer (pH 5) for 1 hr at a particular temperature (Fig. 17). The enzymes of SL9-9, S52-2, and KACC10111 showed a thermal optimum at 60℃ and the enzyme of C5-16 was at 50℃. A rapid decrease in activity was seen above 60℃. Relative activities of CMCase of *B. subtilis* SL9-9 at 30℃, 40℃, 50℃, 60℃, 70℃, 80℃, and 90℃ were 60%, 81%, 96%, 100%, 25%, 11%, and 4%, respectively. Relative CMCase activities of *B. subtilis* C5-16 at 30℃, 40℃, 50℃, 60℃, 70℃, 80℃, and 90℃ were 61%, 83%, 100%, 88%, 16%, 10%, and 4%, respectively. Relative activities of CMCase of *B. subtilis* S52-2 at 30℃, 40℃, 50℃, 60℃, 70℃, 80℃, and 90℃ were 45%, 61%, 81%, 100%, 31%, 12%, and 4%, respectively. And relative CMCase activities of *B. subtilis* KACC10111 at 30℃, 40℃, 50℃, 60℃, 70℃, 80℃, and 90℃ were 45%, 65%, 88%, 100%, 15%, 10%, and 7%, respectively.

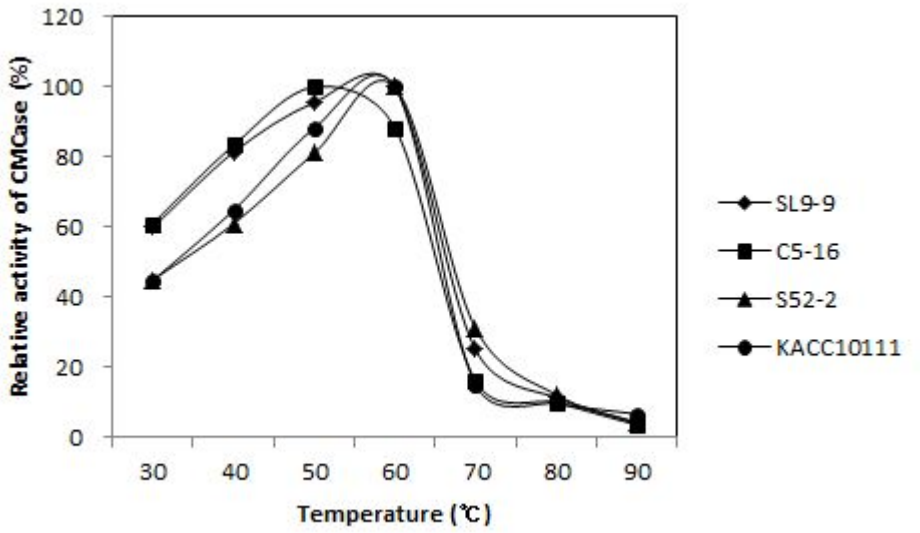

Fig. 17. Effect of temperature on carboxymethylcellulase (CMCase) activity of *B. subtilis* strains.

Relative activity is expressed as percentage of the maximum.

4-3. Effect of reaction time on the activity of CMCase

The effect of reaction time on the CMCase activity was determined at various reaction time in the range of 10~60 min by measurement of residual activity after incubation in sodium acetate buffer (pH 5) for reaction time at 50℃ (Fig. 18). The values of absorbance of CMCase increased steadily up to 60 min with a linear movement at 540 nm. On the contrary to this, the activity of CMCase decreased little by little. Hence, the optimum reaction time of CMCase is considered between 15 to 20 min.

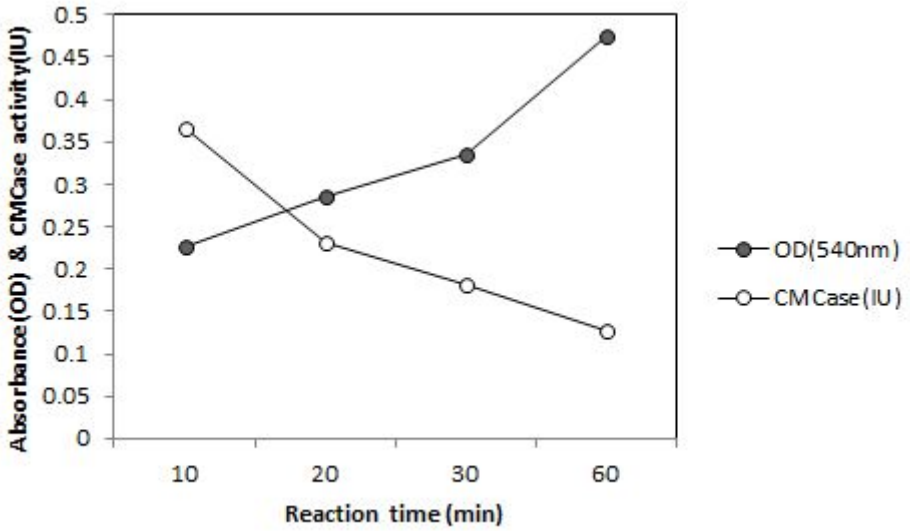

Fig. 18. Effect of reaction time on carboxymethylcellulase (CMCase) activity of *B. subtilis* strains.

5. Optimization of culture conditions for cellulase production

5-1. Effect of incubation temperature

As shown in Table 27, the cell growth of SL9-9 and S52-2 were available between 15℃ to 50℃ and showed the good growth ranging from 25℃ to 45℃. The cell growth of C5-16 was available between 15℃ to 55℃ and showed the good growth ranging from 25℃ to 50℃.

Furthermore, the response of cell growth and CMCase activity at the different culture temperature *i.e.* 15℃, 25℃, 35℃ were determined periodically. Fig. 19. shows the microbial growth profiles obtained during shaking incubation for 5 days at three different temperatures. There were few changes in the cell growth of three *Bacillus* strains at 15℃. At 25℃, the strain of SL9-9 increased rapidly up to 72 hr since then increased gradually in the cell growth and the strain of C5-16 increased rapidly up to 48 hr more than SL9-9 and S52-2 since then increased gradually. While the strain of S52-2 increased very slowly up to 120 hr. At 35℃, both strains of SL9-9 and C5-16 increased exponentially up to 48 hr, the strain of S52-2 up to 96 hr in the cell growth. The variation of CMCase activity were similar to that of the cell growth. At 15℃, there were few changes in the activity of CMCase, just like the cell growth. At 25℃, the CMCase activity of three *Bacillus* strains increased gradually up to 120 hr, while at 35℃, the enzyme activity increased exponentially up to 48 hr and then increased slightly up to 120 hr. The S52-2 presented relatively lower activities (Fig. 20).

Table 27. Effect of incubation temperature on cell growth of isolated *B. subtilis* strains

Strain	Incubation temperature (℃)									
	5	10	15	20	25	35	45	50	55	60
SL9-9	-	-	+	++	+++	+++	+++	++	-	-
C5-16	-	-	+	++	+++	+++	+++	+++	++	-
S52-2	-	-	+	++	+++	+++	+++	++	-	-

Note : - , non; +, poor; ++, medium; +++, good growth

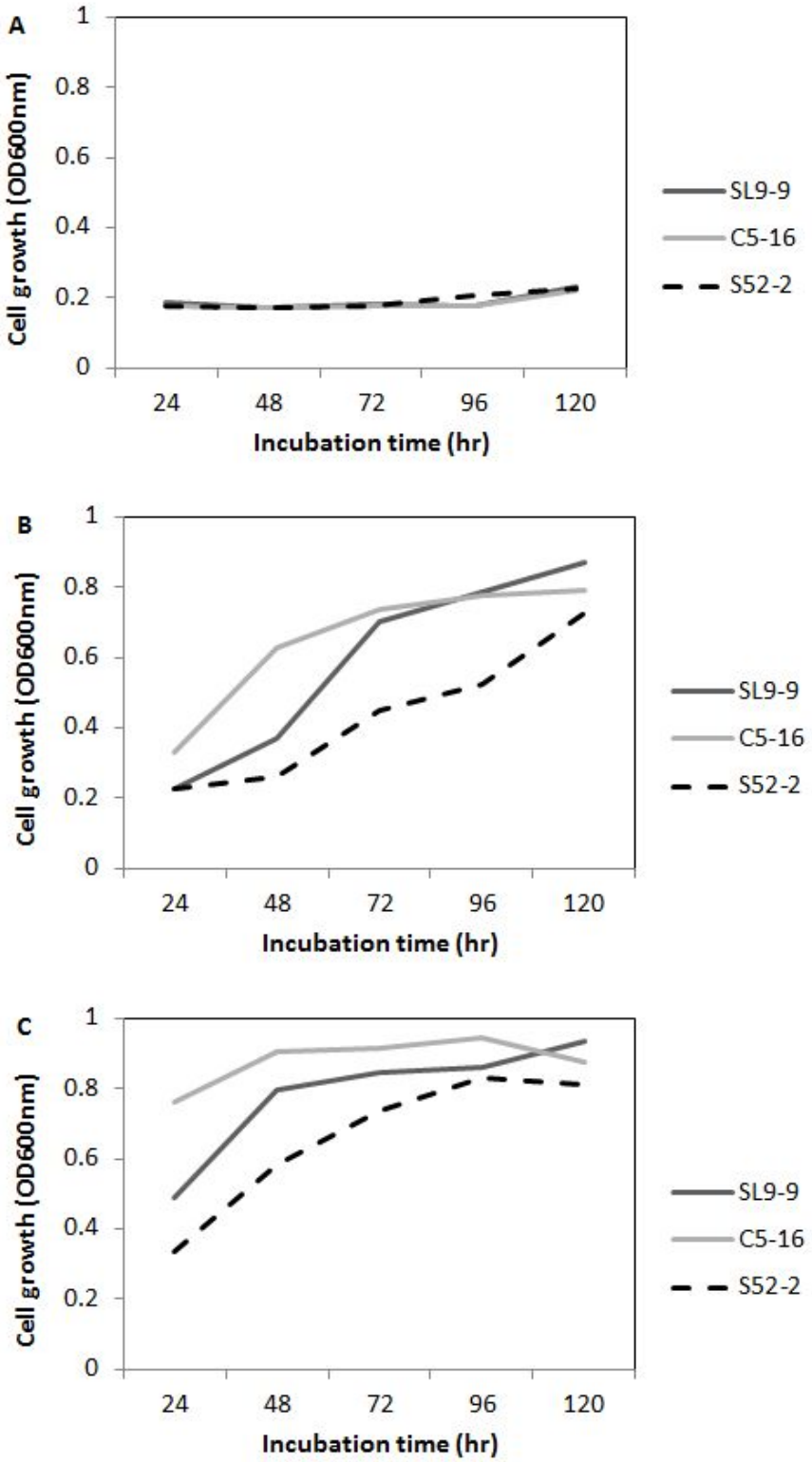

Fig. 19. Microbial growth of isolated *B. subtilis* strains at 15℃ (A), 25℃ (B), and 35℃ (C) for 5 days.

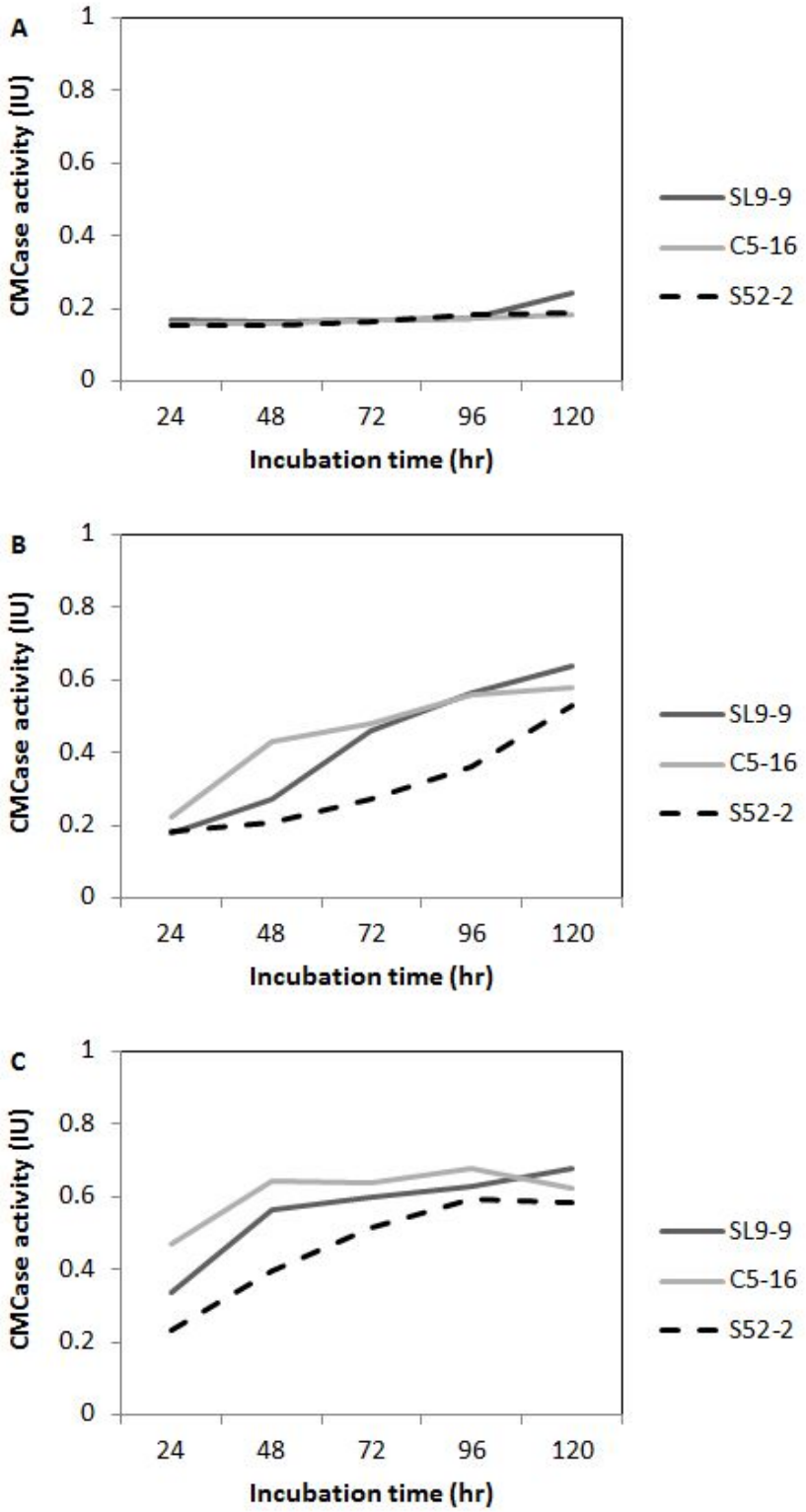

Fig. 20. Carboxymethylcellulase (CMCase) activity of isolated *B. subtilis* strains at 15℃ (A), 25℃ (B) and 35℃ (C) for 5 days.

5-2. Effect of initial pH of medium

The effect of initial pH of medium on the cell growth and the production of CMCase were examined as shown in Fig. 21. The cell growth of *Bacillus* strains SL9-9 and S52-2 exhibited good in the pH range of 5 to 9, with the highest growth at pH 5, while C5-16 exhibited good growth in the pH range of 7 to 9, with the highest at pH 7. The CMCase activity of *Bacillus* strains SL9-9 and S52-2 were the highest in pH 5 while the C5-16 was in pH 6. After pH 5 and 6, the CMCase activity decreased gradually. Also, Changes of diameter of clear halos on CMC agar medium adjusted initial pH in the range of 4 to 10 by *B. subtilis* SL9-9, C5-16, S52-2 and KACC10111 were investigated (Fig. 22~25). As a result, diameter of clear halos was biggest at pH 5 or 6 and smallest at pH 4 or 10 in most of strains. These results seemed to be similar tendency with Fig. 21(B).

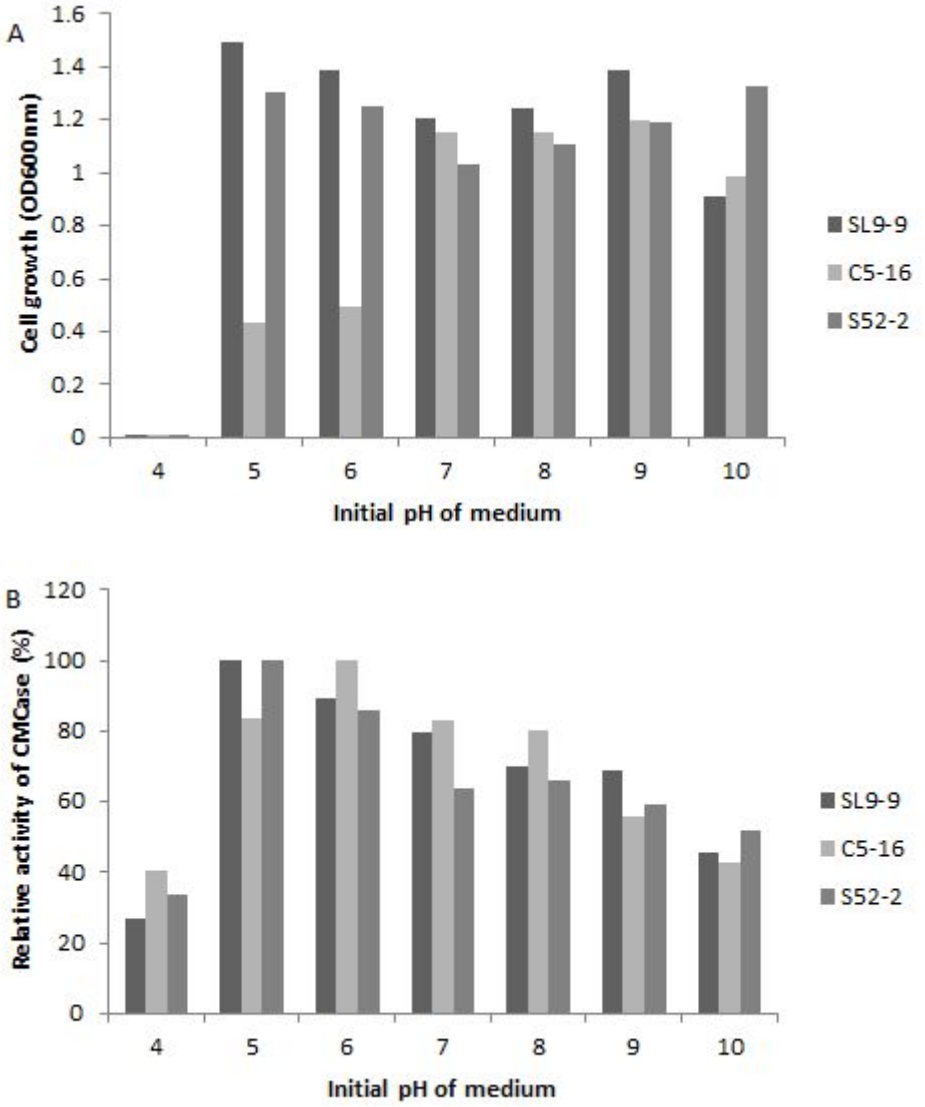

Fig. 21. Effect of initial pH on cell growth (A) and relative activity of CMCase (B) of isolated *B. subtilis* strains.

Rrelative activity is expressed as percentage of the maximum.

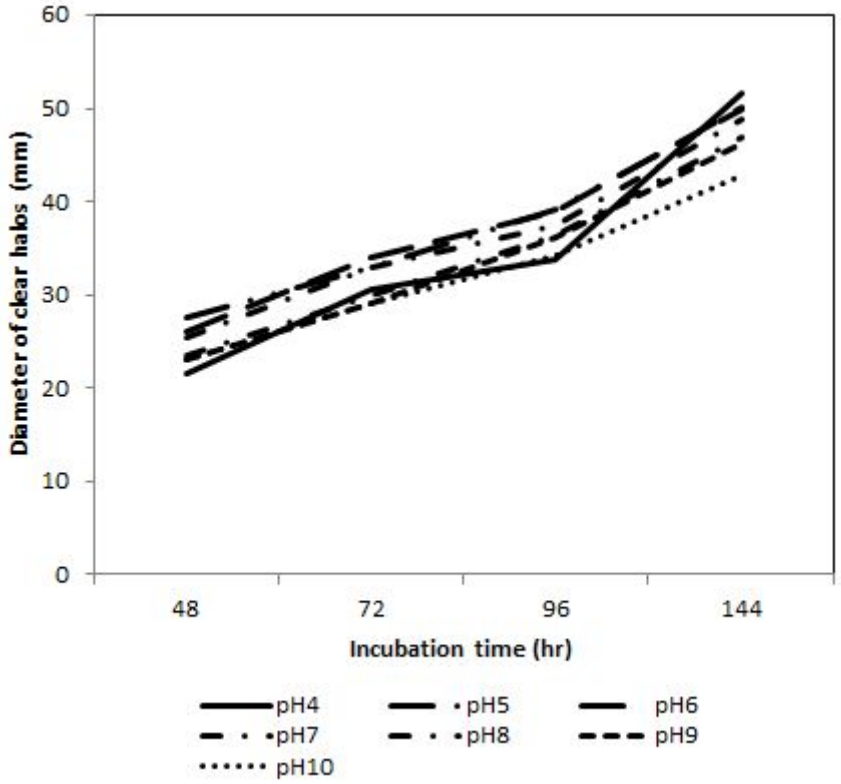

Fig. 22. Change of diameter of clear halos by *B. subtilis* SL9-9 on CMC agar medium adjusted initial pH in the range of 4 to 10.

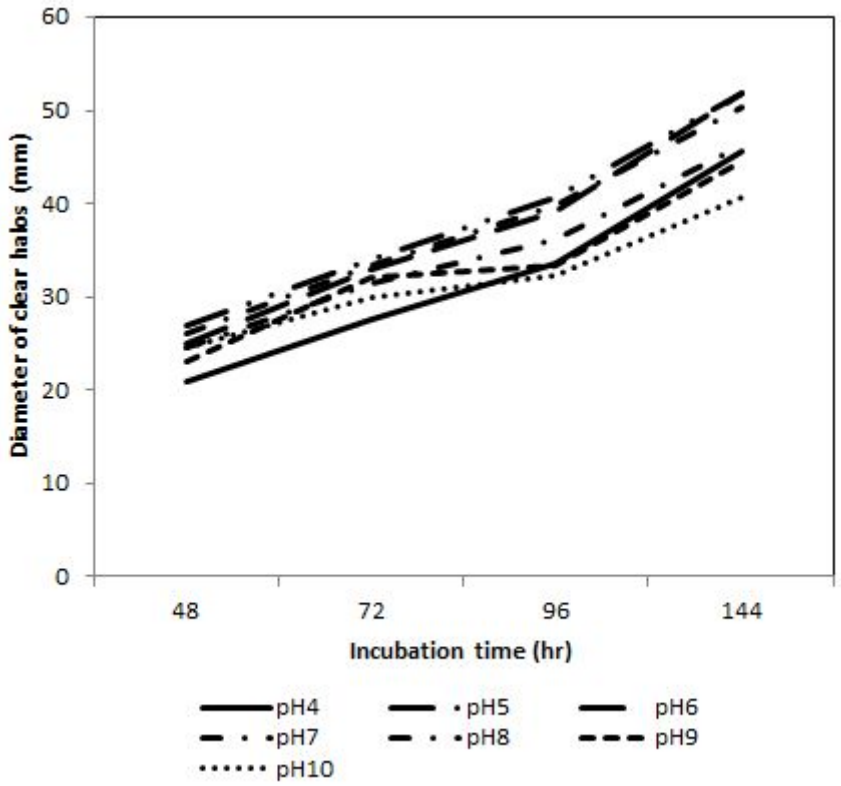

Fig. 23. Change of diameter of clear halos by *B. subtilis* C5-16 on CMC agar medium adjusted initial pH in the range of 4 to 10.

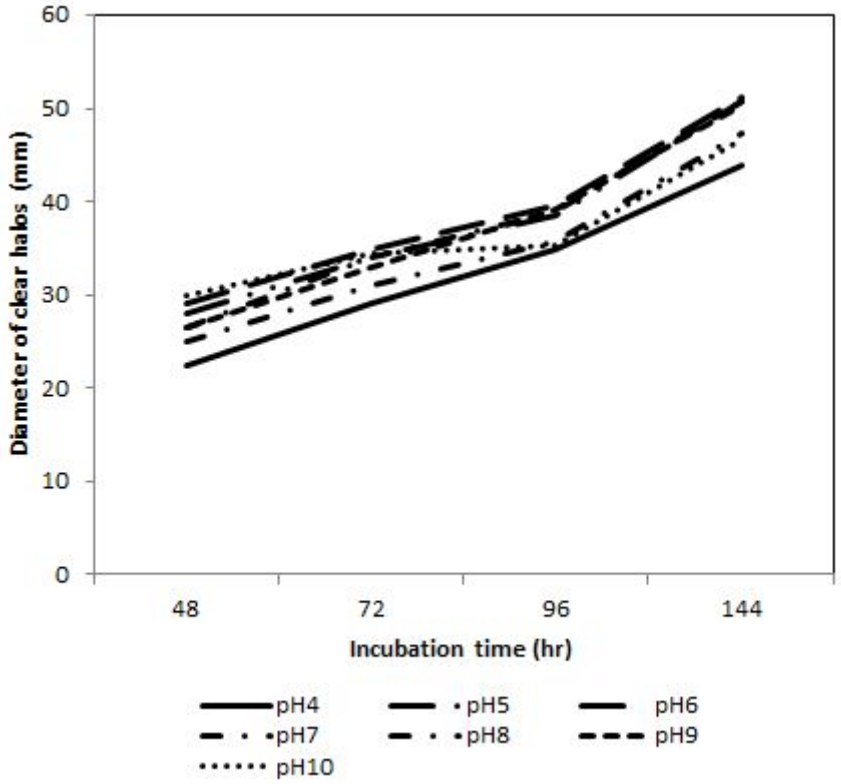

Fig. 24. Change of diameter of clear halos by *B. subtilis* S52-2 on CMC agar medium adjusted initial pH in the range of 4 to 10.

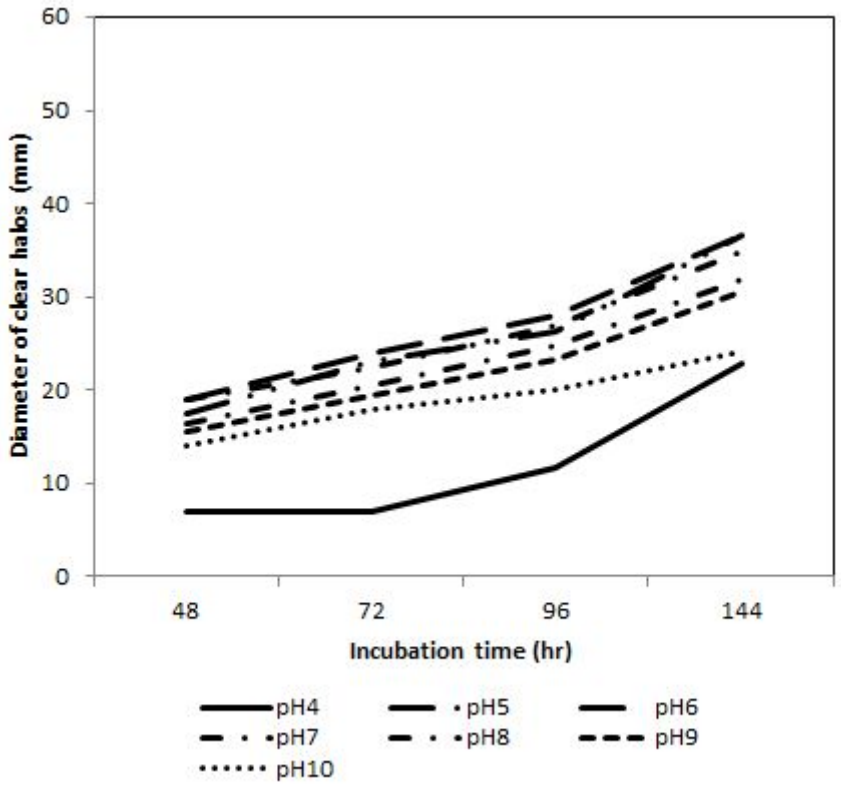

Fig. 25. Change of diameter of clear halos by *B. subtilis* KACC10111 on CMC agar medium adjusted initial pH in the range of 4 to 10.

5-3. Effect of nitrogen sources

The effect of different nitrogen sources on cell growth and production of CMCase of *Bacillus* strains were determined by incubating on 1% CMC liquid medium added with 0.4% of nitrogen sources, respectively. As shown in Fig. 26, the cell growth and enzyme activity of three *Bacillus* strains showed difference in response to some of the nitrogen sources. The cell growth of the three *Bacillus* strains were maximum in the presence of yeast extract as the sole nirtogen source, while, the production of CMCase of the three *Bacillus* strains were maximum in the presence of soytone as the sole nitrogen source. And the production of the enzyme were relatively higher in the presence of yeast extract compared to other sources.

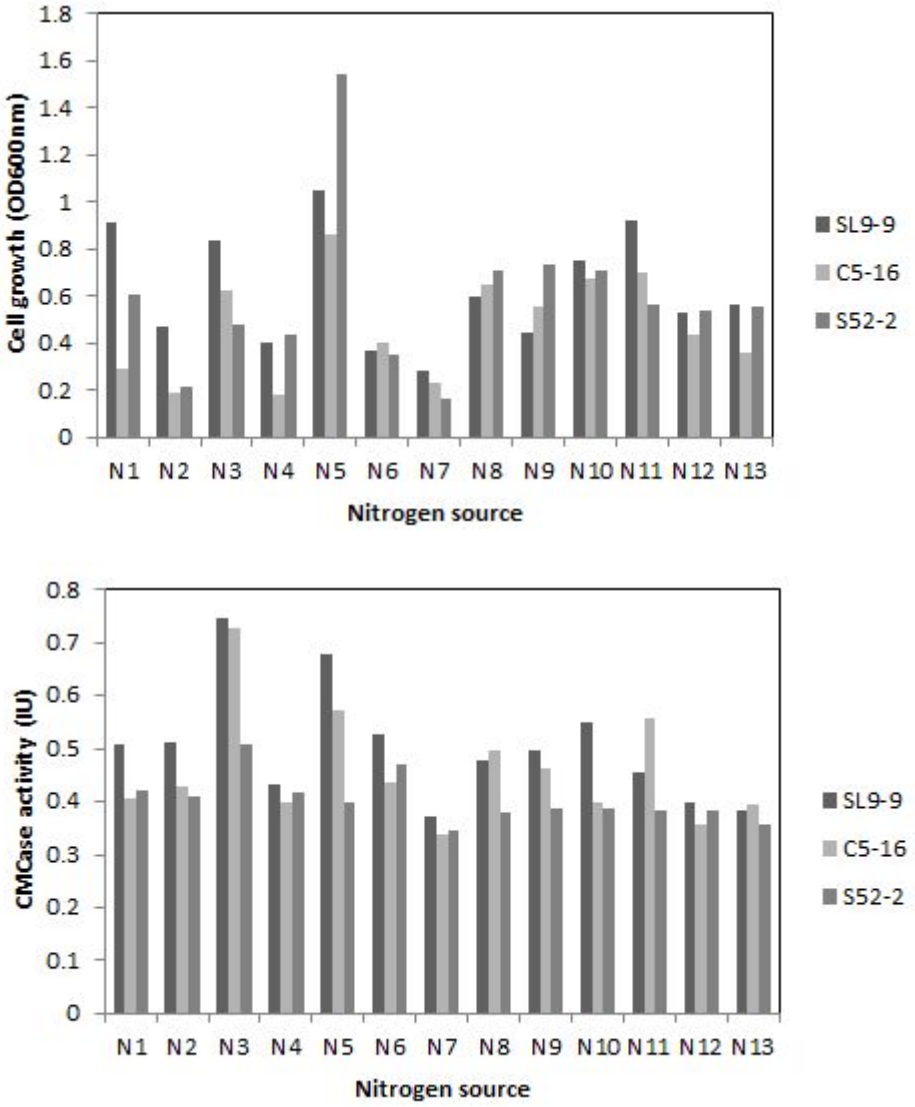

Fig. 26. Effect of nitrogen sources on cell growth and CMCase activity of isolated *B. subtilis* strains.

Nitrogen sources were added with 0.4%, respectively. N1, tryptone; N2, peptone; N3, soytone; N4, casein; N5, yeast extract; N6, beef extract; N7, urea; N8. $(NH_4)_2SO_4$; N9, $(NH_4)_2HPO_4$; N10, NH_4Cl; N11, NH_4NO_3; N12, $NaNO_3$; N13, KNO_3.

5-4. Effect of carbon sources

The effect of different carbon sources on cell growth and production of CMCase of *Bacillus* strains were determined by incubating on liquid medium added with 1% of carbon sources, respectively. As shown in Fig. 27, the cell growth and CMCase activity of three *Bacillus* strains showed difference in response to carbon sources. The cell growth of three *Bacillus* strains were maximum in the presence of CMC as a sole carbon source, while the production of CMCase of the two *Bacillus* strains SL9-9 and C5-16 were maximum in the presence of starch as the sole carbon source.

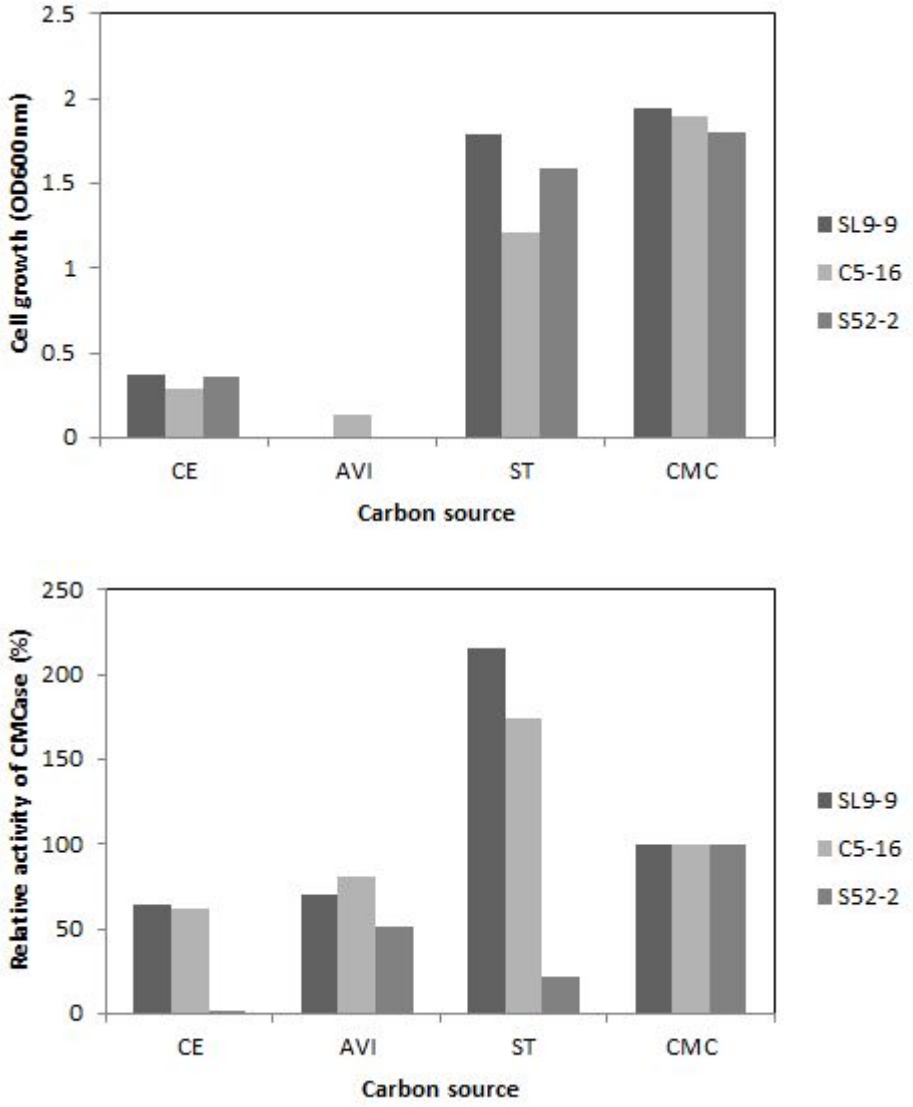

Fig. 27. Effect of carbon sources on cell growth and CMCase activity of isolated *B. subtilis* strains.

Carbon sources were added with 1%, respectively; CE, cellulose; AVI, avicel; ST, starch; CMC, carboxymethylcellulose; Relative activity is expressed as percentage of the maximum.

5-5. Effect of carboxymethylcellulose (CMC) concentration

The response of cell growth and CMCase activity to the additive concentration of CMC was determined by incubating in liquid medium added with 0.1%, 0.%3, 0.5%, 1%, 1.5% and 2% of CMC as a carbon source, respectively. As shown in Fig. 28, the cell growth of *B. subtilis* SL9-9 and S52-2 showed no difference in response to the concentration of CMC. The cell growth of the C5-16 decreased gradually according to the increase of concentration of CMC after 0.5% of CMC. The CMCase of *B. subtilis* SL9-9 increased a little according to the increase of concentration of CMC. The C5-16 ans S52-2 showed no differences in concentration of CMC.

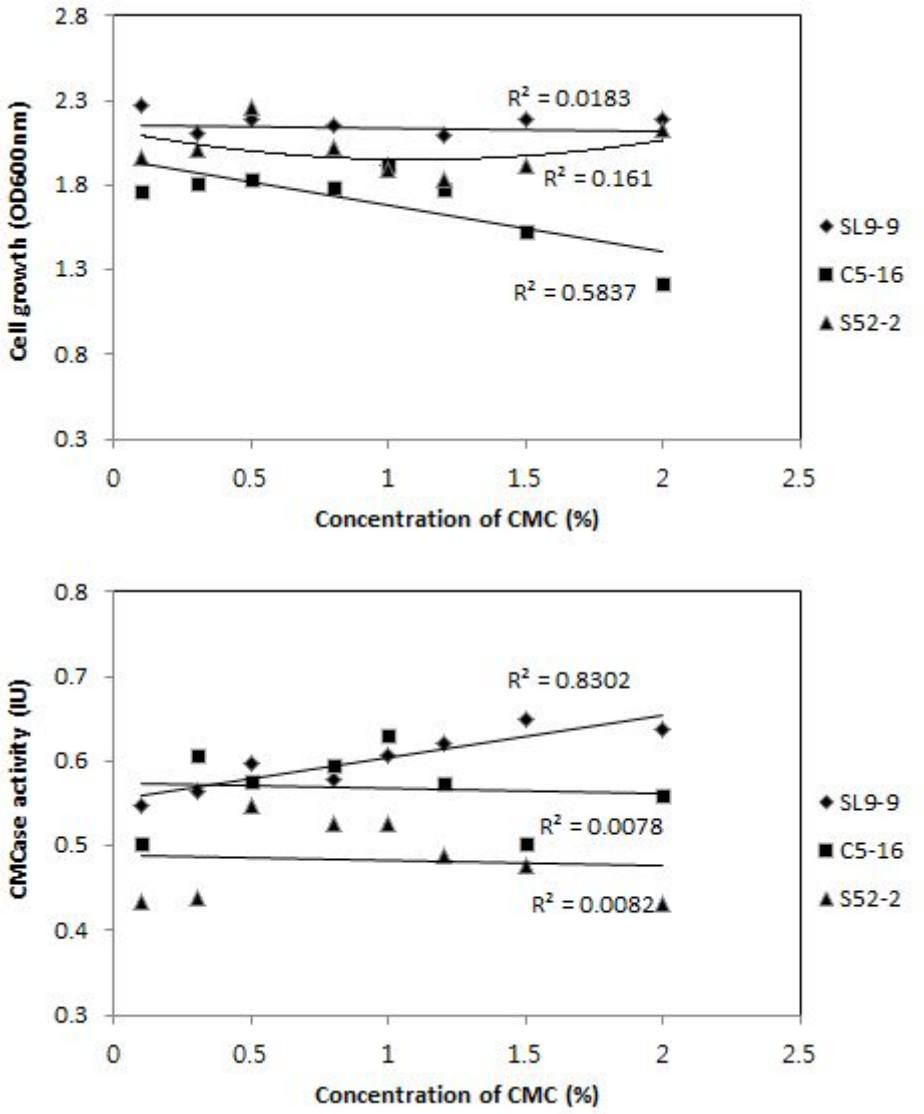

Fig. 28. Effect of CMC concentration on cell growth and CMCase activity of isolated *B. subtilis* strains.

5-6. Effect of shaking speed

Fig. 29. shows the cell growth of *B. subtilis* strains, obtained at the various shaking speed ranging from 100 to 200 rpm at pH 5, 25℃ for 5 days. The both of *B. subtilis* SL9-9 and S52-2 strains were similar at 100, 150 and 200 rpm in the cell growth, while the cell growth of C5-16 was lower at 200 rpm, especially.

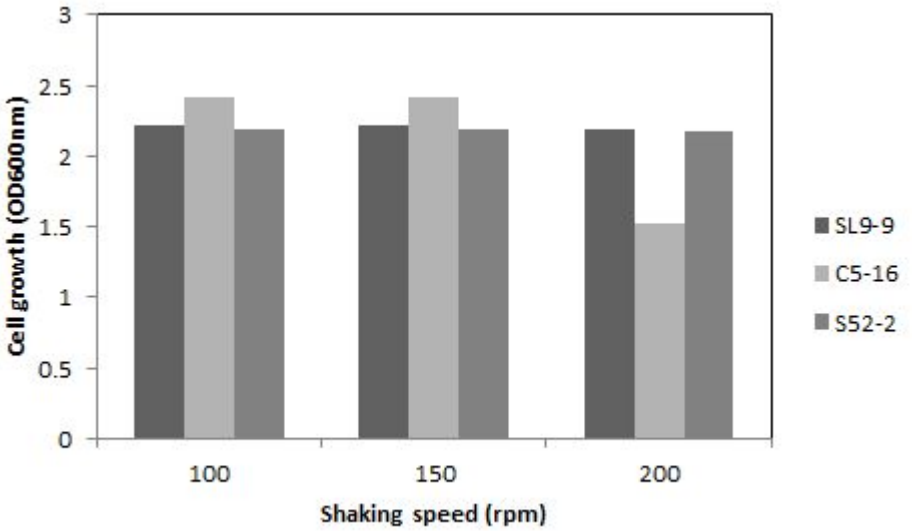

Fig. 29. Effect of shaking speed on cell growth of isolated *B. subtilis* strains.

5-7. Effect of metal ions

It is well known that the decomposition of soil organic matter can be strongly inhibited by heavy metal, in particular by copper. Therefore, the response of the cell growth and CMCase activities to metal ions were determined by incubating on CMC liquid medium added with 1 mM and 0.2 mM metal salts such as $MnSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$. As shown in Table 28, Cu and Zn ions cause a significant decrease of cell growth of three *Bacillus* strains, while Mn and Fe caused a increase in the cell growth at 0.2 and 1 mM. However, three enzymes of *Bacillus* strains showed difference in response to some of the metal ions excepting Cu. In all of them, Cu ion cause also a serious damage in the CMCase activity. As shown in Table 29, SL9-9 and S52-2 enzyme was inhibited by Zn, Mn and Fe both 0.2 and 1 mM concentration. In comparison, only 1 mM of Fe caused loss in C5-16 enzymes activity to a lower extent, *i.e.* 30%.

Table 28. Effect of metal ions on cell growth of isolated *B. subtilis* strains

Metal ion	Concentration (mM)	Relative cell growth (%)		
		SL9-9	C5-16	S52-2
$CuSO_4 \cdot 5H_2O$	0.2	0.3	0.2	0.6
	1	0.3	0.8	0.7
$ZnSO_4 \cdot 7H_2O$	0.2	30.0	52.5	42.7
	1	20.1	29.9	26.8
$MnSO_4 \cdot 5H_2O$	0.2	125.5	120.0	153.3
	1	122.3	90.2	118.8
$FeSO_4 \cdot 7H_2O$	0.2	125.9	129.6	162.1
	1	130.6	119.2	113.2
Control[a]		100.0	100.0	100.0

[a]Control, non-additive.

Table 29. Effect of metal ions on CMCase activity of isolated *B. subtilis* strains

Metal ion	Concentration (mM)	Relative activity (%)		
		SL9-9	C5-16	S52-2
$CuSO_4 \cdot 5H_2O$	0.2	7.1	3.7	15.5
	1	N.D.[b]	N.D.	N.D.
$ZnSO_4 \cdot 7H_2O$	0.2	44.8	206.8	50.8
	1	29.1	113.8	46.1
$MnSO_4 \cdot 5H_2O$	0.2	78.8	166.6	63.2
	1	86.5	94.6	57.0
$FeSO_4 \cdot 7H_2O$	0.2	79.2	127.8	66.7
	1	87.1	69.9	56.8
Control[a]		100.0	100.0	100.0

[a]Control, non-additive; [b]N.D., not detected.

5-8. Cultural properties of *B. subtilis* SL9-9 in the optimized condition

B. subtilis SL9-9 was grown in optimized conditions. Fig. 30 shows that the cell growth and activity profile of enzymes produced by *B. subtilis* SL9-9 during growth under the optimized conditions of 1% CMC as a carbon source and 0.4% soytone and 0.4% yeast extract as nitrogen sources on a shaker at 150 rpm, pH 5. 28℃, for 10 days. The enzyme were released in the medium during exponential phase and maximum activities were observed at the stationary phase. The cell growth peaked on 24 hr and CMCase production peaked on 120 hr, respectively. The pH and EC increased rapidly up to 96 hr and then increased slightly up to the end of cultivation.

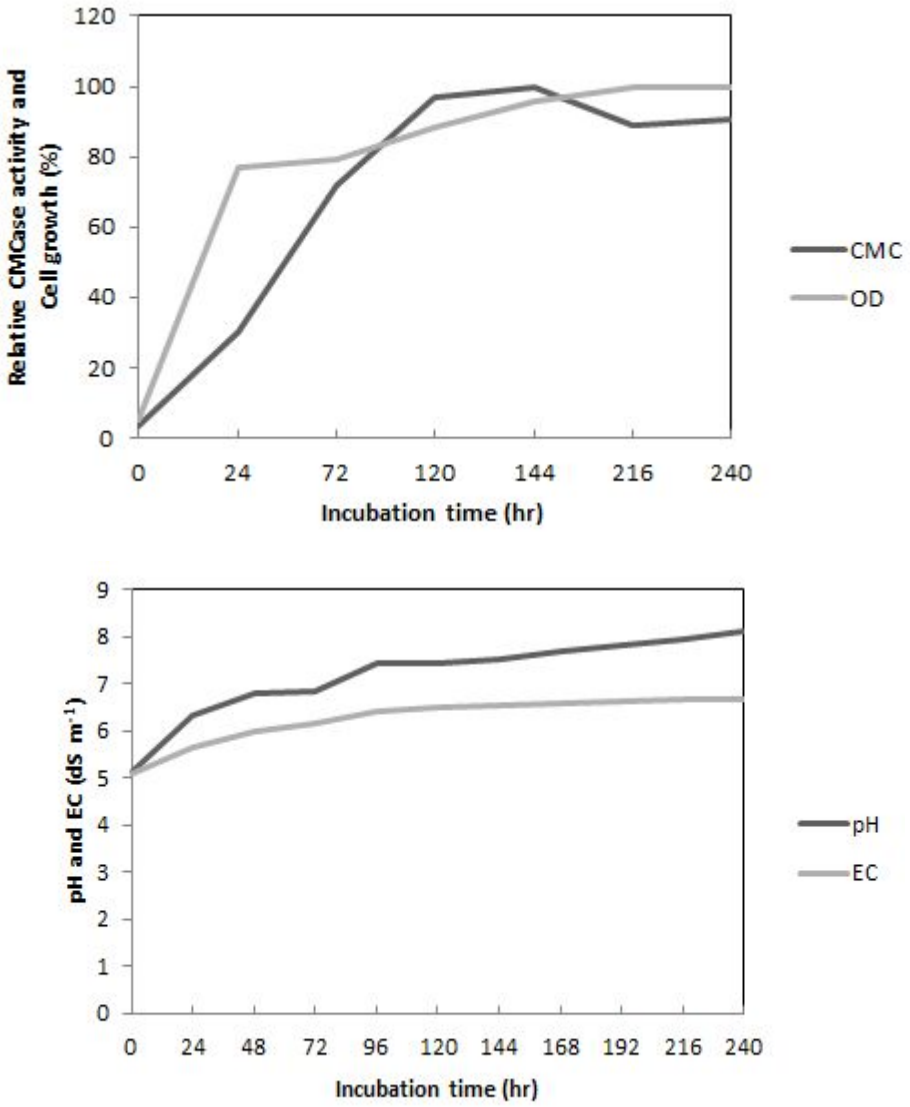

Fig. 30. Cultural properties of *B. subtilis* SL9-9 under optimized conditions.

6. Scale-up conditions for cellulase production by *B. subtilis* SL9-9

6-1. Production of cellulase from different agricultural wastes

In this studies, the efforts have been mae to study the production cellulase by *B. subtilis* on low cost cellulogic biomass materials. The maximum cellulase activity was detected in potato powder as a carbon source followed by rice bran and increased by 65% and 19% respectively compared to CMC (Table 30). Thus, we selected rice bran as a carbon source because it was more economical and easily available material for mass culture.

Table 30. Production of cellulase from different agricultural wastes by *Bacillus* isolates

Carbon source	Relative productivity (%)			
	SL9-9	C5-16	S52-2	Mean
Carboxymethyl -cellulose	100	100	100	100
Rice bran	160	93	104	119
Citrus press cake	130	96	125	117
Potato powder	222	124	151	165
Bone dust	88	68	52	69
Fish meal	128	94	130	117
Rapeseed press cake	129	101	43	91
Soybean	109	82	84	91
Sudan grass	92	74	76	80
Hairyvetch	117	64	68	83
Rye	99	57	59	71

The production of CMCase and avicelase by *B. subtilis* SL9-9 was tested in 5 L Bioreactor using rice bran as a carbon source for scaling up. The CMCase production was much higher by 12-13% in the rice bran medium added with soytone compared than CMC medium with or without soytone. and the avicelase was also highest in the rice bran medium added with soytone (Fig. 31).

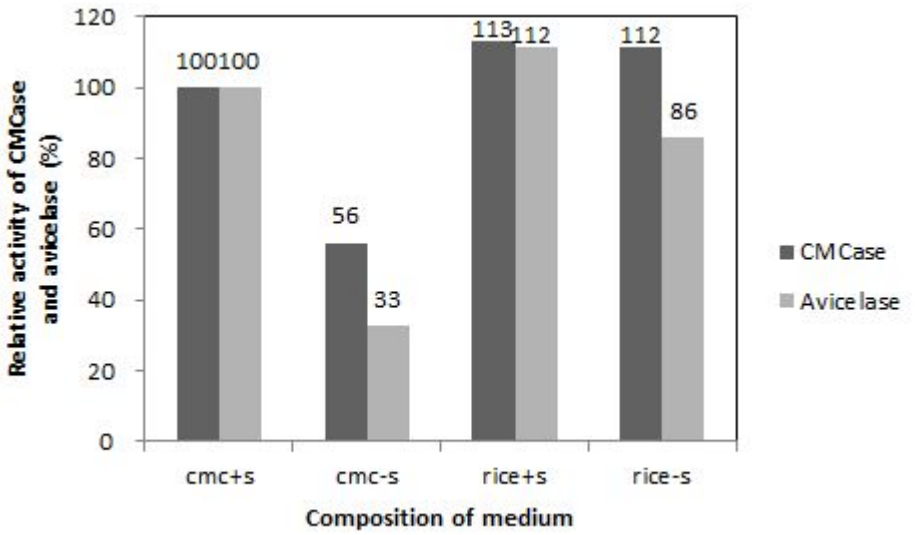

Fig. 31. CMCase and avicelase activities of *B. subtilis* SL9-9 when using rice bran.

cmc+s, 1% CMC with 0.4% soytone; cmc-s, 1% CMC without soytone, rice+s, 1% rice bran with 0.4% soytone; rice-s, 1% rice bran without soytone.

6-2. Bioreactor and cultivation conditions for scale-up optimization

Fig. 32 shows that the activity profile of enzymes produced by *B. subtilis* SL9-9 during growth under the conditions of aeration or none with 0.5% rice bran as a carbon source in 5 L bioreactor. As can be observed, under 1vvm of aeration the production of CMCase was higher than non-aeration, especially after 96 hr. CMCase production peaked on 216 hr under 1vvm treatment and peaked on 360 hr under non-aeration treatment, respectively.

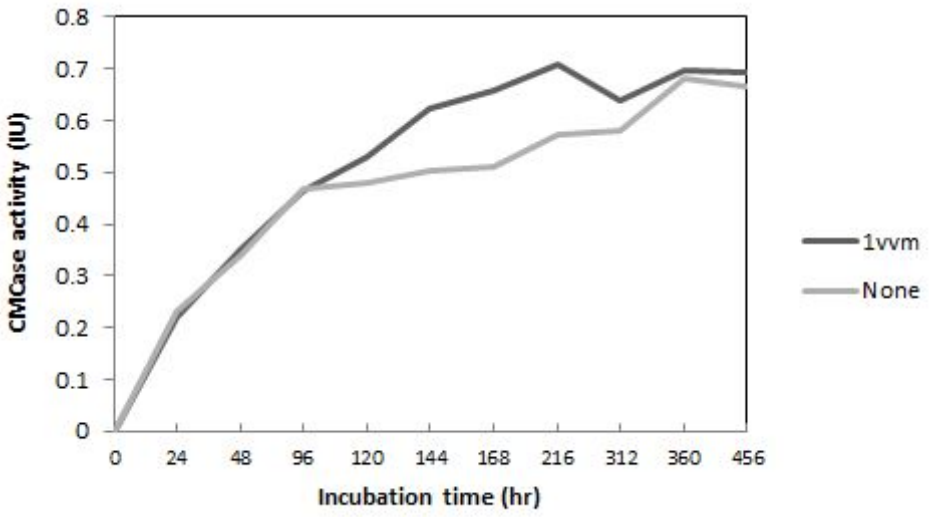

Fig. 32. CMCase activity during growth of *B. subtilis* SL9-9 under 1vvm-aeration or non-aeration condition in 5 L bioreactor.

6-3. Stability test of *B. subtilis* SL9-9 as a biofertilizer

Fig. 33 shows that the CMCase activity of *B. subtilis* SL9-9 was maintained during 33 weeks the liquid and solid phase at normal temperature. From this results, we concluded that *B. subtilis* SL9-9 secreting the extracellular CMCase was made good use as a inoculant.

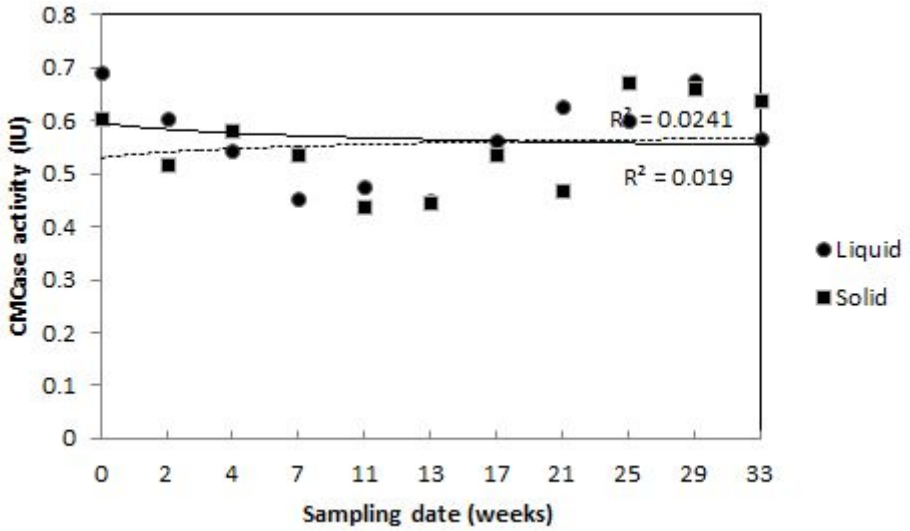

Fig. 33. CMCase activity of *B. subtilis* SL9-9 during cultivation in liquid and solid media.

Table 31. Scale-up culture condition for cellulase production by *B. subtilis* SL9-9

Condition	Seed culture	Mass culture
Initial pH	5	5
Incubation temperature	30~35℃	30℃
Shaking speed	100~150 rpm	150 rpm
Carbon source	1% CMC	0.5% rice bran
Nitrogen source	0.4% Soytone 0.4% Yeast extract	0.4% Yeast extract
Culture time	120 hr	120 hr
Aeration		1.0 vvm

Ⅳ. Discussion

1. Screening of cellulolytic bacteria

Cellulolytic bacteria were sought among 176 different samples collected from various environments such as soil, compost, animal waste slurry and cultured native microbes in Jeju Island (Table 13).

The selection of cellulolytic microorganisms were proceeded step by step. In the first round screening, a total of 848 cellulolytic clones were selected by clear halos around the clones with the trypan blue staining method on CMC agar medium (Table 14). And then 279 clones were chosen based on their relatively good colonial development and visible clearing zones in the second step. Among them, there were 38 clones were derived from soils, 45 clones from composts, 165 clones from animal waste slurry, and 61 clones from cultured native microbes. In the third round, 69 clones were chosen based on their relatively higher activities in a wide range of pH (Table 15~19). Finally, each one clone showing relatively higher cellulolytic activity and broader pH optimum was selected in which animal waste slurry, compost, and soil, respectively. The three isolated bacteria were designated as SL9-9, C5-16 and S52-2 based themselves on the animal waste slurry, compost and soil, respectively.

2. Identification of isolated cellulolytic bacteria

Morphological and cultural studies revealed that all the clones were Gram-positive and rod-shaped bacteria (Table 24). They were also catalase-positive, aerobic, moderate thermophiles. The three bacterial isolates showed slight differences from each other in such biochemical properties as methyl-α-D-glucopyranoside, amygdalin, salicin, D-maltose, D-lactose, inulin, glycogen, gentiobiose, and D-turanose utilization when the API 50CHB kit was used (Table 25).

These three bacterial isolates were finally identified by 16S rRNA gene sequence analysis. Their sequences were entered into the nucleotide-nucleotide BLAST (NCBI) system and percentage identities were established (Table 26). Based on their morphological, physiological and genetic data, the three bacterial isolates were designated as *Bacillus subtilis* SL9-9, C5-16, and S52-2, respectively.

Their CMCase activities were examined and compared with those of other known *Bacillus* species obtained from KACC and KCTC by CMCase activity assay using cell-free supernatant obtained from the liquid cultures (Fig. 10). Their CMCase activities remained quite high around pH 5~8 compared to other *Bacillus* species.

3. Production of cellulolytic enzymes by isolated *B. subtilis* strains

The three isolates were examined for CMCase, avicelase, β-glucosidase and xylanase production after cultivation in CMC liquid medium. *B. subtilis* KACC10111, which showed higher CMCase activity than the other 6 *Bacillus* species obtained from KACC and KCTC, was used as a reference for enzyme activity comparisons.

As shown in Fig. 12, in the cell-free supernatant, both strains of SL9-9 and C5-16 showed considerable CMCase activity, while the other two strains, S52-2 and KACC10111, presented relatively lower activities. In the cell debris fraction there was no observable CMCase activity (Fig. 12B). Thus, CMCase was suggested as an extracellular enzyme. As shown in Fig. 13, in the cell free supernatant, all of the strains produced considerable avicelase activity and maintained maximum activity after 72~96 hr of cultivation. On a whole, SL9-9 presented higher activity than the other isolates from the beginning of cultivation. In the cell debris fraction, there was no definite avicelase activity. In addition, when the same *Bacillus* strains were examined for CMCase activity using Whatman No.1 filter paper as a substrate, low hydrolytic activity levels (0.025~0.030 IU) were observed, and slightly increased as cultivation continued like the avicelase activity profiles (data not shown). It is highly possible that both the avicelase and CMCase (endo-β-1,4-glucanase) activity resulted from the

same enzyme protein. Our results show some contrast to a previous report (Robson *et al*., 1987) in which the endo-β-1,4-glucanase produced by *B. subtilis* DLG was not able to significantly degrade crystalline cellulosic substrates. Fukumori *et al*.(1985) also reported that endo-β-1,4-glucanases from alkalophilic *B. subtilis* strains 1139 and N-4 were capable of hydrolysing CMC, but could not degrade avicel significantly. Hamamoto *et al*. (1991) suggested that a synergistic function of the NH_2-terminus and COOH-terminus of the endoglucanase in *Clostridium cellulovorans* is essential for the hydrolysis of crystalline cellulose. These phenomena suggest that crystalline cellulose-hydrolyzing activity does not depend on the same catalytic site of endo-β-1,4-glucanase.

In contrast to CMCase and avicelase, no β-glucosidase activity was observed in the cell-free supernatant (Fig. 14). However, all the strains showed considerable β-glucosidase activity in the cell debris fraction. Maximum activities (1.0, 1.0, 0.6, and 1.2 IU for SL9-9 C5-16, S52-2, and KACC10111, respectively) were detected early after cultivation for 24 hr. The enzyme activities subsequently decreased after reaching maximum values, and then second rises and falls were observed. A reason for the rise and fall in β-glucosidase activity might the negative regulation of β-glucosidase gene expression by glucose level (catabolite repression) in the cells. β-Glucosidase activities are inferred to be related to membrane-associated enzymes. So far, β-glucosidase has scarcely been reported in *Bacillus* strains, although its production by other bacteria like *Clostridium thermocellum* (Ait *et al*., 1979) and *Alcaligens faecalis* (Han *et al*., 1969) has been documented. Bartley *et al*. (1984) reported that β-glucosidase in actinomycete *Microbispora bispora* was cell membrane-bound. Pajni *et al*. (1989) reported that all 34 strains of cellulolytic *Bacillus* species isolated from soil produced xylanase and 82.4% of them also produced β-glucosidase. On the other hand, Dhillon *et al*. (1985) found that *B. licheniformis* could grow in minimal media containing cellobiose, but failed to show the presence of cellobiase in either the cellular fraction or culture supernatant. It was hypothesized that the utilization of cellobiose even in the absence of cellobiase

involved the enzyme cellobiose phosphorylase (Robson *et al*., 1984).

The *Bacillus* strains SL9-9 and S52-2 showed considerable xylanase activity in the cell-free culture supernatant, and their activities reached maximum values (12.0, and 11.5 IU respectively) after shaking culture for 96 hr, as shown in Fig. 15. The other two strains, C5-16 and KACC10111, presented lower overall activity from the beginning of cultivation. No xylanase activity was detected in the cell debris fraction. Xylanase production has been previously reported in *Bacillus* strains (Heck *et al*., 2002). Pajni *et al*. (1989) reported that all examined cellulolytic *Bacillus* species were also xylanase positive, and units of xylanase activity were found to be much higher as compared to corresponding CMCase activity units. Xylans, with a linear backbone of β-1,4-linked xylose residues, form the major group of hemicelluloses. Endoxylanases hydrolyze xylan to xylooligosaccharides and xylose residues, while β-xylosidases catalyze the release of xylosyl residues by the terminal attack of xylooligosaccharides. It is highly possible that the xylanase activities of our *B. subtilis* strains came from the combined actions of independent endoxylanase and β-xylosidase enzymes.

Three cellulolytic bacterial strains, SL9-9, C5-16, and S52-2, were isolated and identified as *B. subtilis* in this study. The isolates were not the same strains, showing slight differences in biochemical characteristics, 16S rRNA gene sequences, and production patterns of cellulases and xylanases. These strains are presently being employed in organic agriculture as a biofertilizer supplement.

4. Characterization of cellulase from isolated *B. subtilis* strains

The carboxymethylcellulases (CMCases) of three isolates were characterized and *B. subtilis* KACC10111 was used as a reference for enzyme activity comparison. The CMCase of SL9-9, C5-16, S52-2 and KACC10111 exhibited optimal activity in the pH range of 5~7, with the highest activity at pH 5, while the KACC10111 enzyme at pH 6. This is in the same range as the isoelectric point of the enzymes, as was

the case for *B. subtilis* CK-2 endoglucanase with a pI of 5.3 and pH optimum of 5.6~5.8 (Aa *et al*., 1994).

However, most *Bacillus* endoglucanases have pH optima far removed from their respective pI values; examples of enzymes with low pI /high optimum pH (Fukumori *et al*., 1985; Yoshimatsu *et al*., 1990) and high pI /low pH optimum (Ozaki and Ito, 1991) have been reported. Robson *et al*.(1989) reported that the optimal pH for cellulase activity of *Bacillus* is usually within the range pH 5.0~6.0. Mawadza *et al*. (2000) reported that *Bacillus sp*. CH43 cellulase exhibited optimal activity in the pH range of 5~7, with the highest activity at pH 6.5 in phosphate buffer and the enzymes expressed about 40% and 50% activity at pH 4 and 9, respectively.

Also, the pH optimum can be interpreted as suggested by Campbell(1988). According to his model, hydrolysis of the glucosic bound is catalysed only if the two key groups, as aspartic and a glutamic carboxyl group at the reactive centre of the enzyme, are in the appropriate protonation state. The model assumes that enzymatic activity is lost if either the aspartic carboxyl group is deprotonated or the glutamic carboxyl group is protonated.

The SL9-9, C5-16, S52-2 and KACC10111 enzymes had about 47%, 44%, 76% and 58% of the maximal level, respectively at pH 4 and none at pH 3. At pH 8 and 9, the SL9-9, C5-16, S52-2 and KACC10111 enzymes expressed over 64% and 19% of the maximal level, respectively, while were inactive at pH 10. The pH stability of the cellulase produced by *B. subtilis* strains over a broad pH range seems to be a common characteristic of many *Bacillus* endoglucanases.

The enzymes of SL9-9, S52-2, KACC10111 showed a thermal optimum at 60℃ and the enzyme of C5-16 was at 50℃. A rapid decrease in activity was seen above 60℃ (Fig. 17). Robson *et al*.(1989) reported that the endo-β-1,4-glucanases of *B. subtilis* strains generally exhibit thermal stabilities up to 50℃±5℃, although maximal activity in frequently observed at 60℃. Therefore, both of the enzyme of SL9-9 and S52-2 were most active at pH 5 and 60℃. The enzyme of C5-16 was most active at pH 5 and 50℃ and the optimum reaction time of CMCase is considered between

15 to 20 min (Fig. 18).

5. Optimization of culture conditions for cellulase production

Study on the optimization of conditions for CMCase production of the three *B. subtilis* strains was examined. The carbon source and nitrogen source were 1% CMC, 0.4% soytone and 0.4% yeast extract. The optimum conditions for the production of the enzyme of *B. subtilis* strains were pH 5.0, temperature 30~35℃ and 150 rpm.

B. subtilis SL9-9 was grown in optimized conditions; carboxymethylcellulose (CMC) liquid medium (pH 5.0) in a shaker at 150 rpm for 10 days at 28℃. The CMCase was released in the medium during exponential phase and maximum activities were observed at the stationary phase. The cell growth peaked on 24 hr and CMCase production peaked on 120 hr, respectively. The pH and EC increased rapidly up to 96 hr and then increased slightly up to the end of cultivation. Cell lyses, causing the liberation of basic amino acids and peptides into the medium, were probably responsible for a strong pH and EC variation of cultures (Heck *et al.*, 2002). The high values of pH and EC obtained at the end of cultivation. Our results point to the need of pH control during cultivation of *Bacillus* when enzymatic activities may be halted by high pH values.

Also, it is well known that the decomposition of soil organic matter can be strongly inhibited by heavy metal, in particular by copper. For example, Watson *et al*. (1976) and Geiger *et al*.(1993) found accumulation of undecomposed plant litter on heavy metal polluted soils near a metal smelter. Various studies have shown that the activities of many enzymes are inhibited by trace metals (Wenzel *et al*., 1995). While the impairment of enzyme activity in soil by heavy metals is in general reviewed as an indirect consequence of the impairment of soil microbial function. Mathur *et al*. (1983) found that soil organic matter mineralization in organic soils can be inhibited by copper despite no negative effect on the survival or proliferation of added *Escherichia coli* was observed. Thus, a direct inactivation of the

extracellular enzymes such as cellulase was postulated.

Therefore, the response of the cell growth and CMCase activities to metal ions were determined by incubating on CMC liquid medium added with 1mM and 0.2mM metal salts such as $MnSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$. As shown in Table 28, Cu and Zn ions cause a significant decrease of cell growth of three *Bacillus* strains, while Mn and Fe caused a increase in the cell growth at 0.2 and 1 mM. However, three enzymes of *Bacillus* strains showed difference in response to some of the metal ions excepting Cu. In all of them, Cu ion cause also a serious damage in the CMCase activity. As shown in Table 29, SL9-9 and S52-2 enzyme was inhibited by Zn, Mn and Fe both 0.2 and 1 mM concentrations. In comparison, only 1 mM of Fe caused loss in C5-16 enzymes activity to a lower extent, *i.e.* 30%. Chen *et al*. (2000) reported that cellulase production of neutral cellulase by *Bacillus sp.* Y106 could be improved by Fe^{2+}, Fe^{3+}, Na^{+} and Ca^{2+}, while inhabited by Cu^{2+}, Ag^{+}, Co^{2+} and Hg^{2+}. Geiger *et al*. (1998) investigated that the activities of cellulase and β-glucosidase were found to be inhibited at copper concentrations above 200 μM and the influence of copper was strongest in the pH range 5~5.5. Thus, inhibition and activation patterns with cellulase of *Bacillus sp*. were different depending upon types of cations. These results agree well with those found by Hakamada *et al*. (1997).

6. Scale-up conditions for cellulase production by *B. subtilis* SL9-9

Very few reports are available on production of cellulase enzymes on low cost renewable sources (Moo-Young, 1992). Hence, in this studies, efforts have been made to study the production cellulase by *B. subtilis* on low cost cellulogic biomass materials. The enzyme production for cellulases was carried out at optimized experimental conditions *i.e.* 28℃, pH 5.0, 150rpm for 5 days. After that, the contents were centrifuged and the extracellular enzyme activity was determined in the supernatant. The maximum cellulase activity was detected in potato powder as a

carbon source followed by rice bran and increased by 65% and 19%, respectively compared to CMC (Table 30).

Most of the workers in the past have used purified carbon sources as substrate for growth of organisms and subsequent enzyme production. Very few reports are available on production of enzyme from low cost renewable resources (Grajek, 1987). Brown *et al*. (1987) have reported production of cellulase (2.85~72.2U mL^{-1}), and xylanase (3.21~8.39U mL^{-1}) from hammer milled barely straw by using different strains of *Penicillium pinophilum* and *Trichoderma reesei*. Bhalla and Joshi (1993) have also reported cellulase (5.0U mL^{-1}) and xylanase (4.2U mL^{-1}) by growing *T. viride* and *A. niger* on dried apple pomace. According to Moo-Young (1992), the action of cellulase was synergistic over substrate, especially for microorganisms isolated from environments where wood and agro-residues are biodegraded. Our results confirmed these observations. Another important aspect for industrial applications of enzyme is the need for reduced costs of production.

In these studies. we selected the rice bran as a carbon source because it was a more economical and easily available material for mass culture. The CMCase production was much higher by 12~13% in the rice bran medium added with soytone compared than CMC medium with or without soytone. And the avicelase was also highest in the rice bran medium added with soytone (Fig. 31). This results indicated that the use of rice bran as substrate for the CMCase production proved to be possible, with good perspectives for scaling up, thereby minimizing the high costs when other chemicals are used for cellulase production.

Ⅴ. Conclusion

The bioconversion of cellulose to soluble sugars is important for global stabilization and a sustainable human society. Here, hundreds of cellulolytic bacteria were screened and isolated from soil, compost, and animal waste slurry. Among the isolates, three strains, SL9-9, C5-16, and S52-2, showing greater potential for practical uses were purified on carboxymethylcellulose (CMC) agar plates and identified as *B. subtilis* strains by morphological, physiological, and biochemical characterization and 16S rRNA gene analysis. The production patterns of cellulose or hemicellulose-degrading enzymes were investigated during cell culture. All three isolated strains produced CMCase, avicelase, β-glucosidase and xylanase, which suggested synergic cellulolytic systems of *B. subtilis*. The enzymes showing CMCase, avicelase and xylanase activities existed in cell-free culture supernatant, meanwhile β-glucosidase activity was detected in cell debris suggesting that three of the enzymes, including CMCase, avicelase, and xylanase, were extracellular, and β-glucosidase was cell membrane-bounded. The three isolates, SL9-9, C5-16, and S52-2, were not the same strains, presenting slight differences in biochemical characteristics, 16S rRNA gene sequences and enzyme activities. The CMCase of three isolates were characterized and *B. subtilis* KACC10111 was used as a reference for enzyme activity comparisons. Therefore, both of the enzyme of SL9-9, S52-2 and KACC10111 were most active at pH 5 and 60℃. The enzyme of C5-16 was most active at pH 5 and 50℃ and the optimum reaction time of CMCase was considered between 15 to 20 min. Studies on the optimization of conditions for CMCase production of the three *B. subtilis* strains were examined and the optimal culture conditions were determined as follows: as substrate medium containing 1% CMC, 0.4% soytone and 0.4% yeast extract; and initial medium pH of 5.0, temperature of 30~35℃, shaking speed of 150 rpm and aeration of 1vvm. Furthermore, the effect of

the addition of various agricultural wastes as a carbon source to the growth medium on the production of cellulolytic enzymes of *B. subtilis* SL9-9 was studied. And we selected the rice bran as a carbon source because it was a more economical and easily available material for mass culture among various agricultural wastes. As a result, 0.5% rice bran was optimal for the production of cellulase from *B. subtilis* SL9-9. From these results, we conclude that *B. subtilis* SL9-9 secreting the extracellular CMCase is made good use not only as a inoculant for decomposing the agricultural organic wastes into liquid and solid fertilizers but also as a biofertilizer promoting the growth of plants. Furthermore, it is deserved codsideration of a study on the applicability of cellulolytic bacteria to the recalcitrant biomass utilization and environmental conservation.

PART Ⅱ

Comparison of cellulase genes from isolated *Bacillus subtilis* strains

Ⅰ. Introduction

Cellulolytic microbes have been investigated for many years (Sukumaran *et al*., 2005). Much recent study has focused on the cellulolytic enzyme systems present among certain isolates of the genus *Bacillus*. The study of *Bacillus* cellulases has been greatly aided by the ease of genetic manipulation in the genus and because the genomes of several species have been relatively well characterized (Robson *et al*., 1989).

A variety of species secrete cellulases, including strains of *B. subtilis* (Koide, *et al*., 1986), *B. licheniformis* (Dhillon, *et al*., 1985), *B. stearothermophilus* (Kim *et al*., 1997), *B. amyloliquefaciens* (Lee *et al*., 2008), *B. polymyxa* (Fogarty *et al*., 1973), *B. cereus* (Thayer, 1978) and many other species (Lynd *et al*., 2002, see review). In addition to isolating cellulolytic microbes, reduction of enzyme production costs and enhancement of cellulase activity have become major areas of research (Li, *et al*., 2009).

The cellulase genes of many microorganisms have been cloned, and the nucleotide sequences of endo-β-1,4-glucanase genes from several *Bacillus* strains have been determined (Mackay *et al*., 1986; Nakamura *et al*., 1987; Ozaki *et al*., 1991; Park, *et al*., 1991; Robson *et al*., 1987). The study of cellulolytic enzymes at the molecular level has revealed some of the modular features that contribute to their catalytic activity (Rabinovich *et al*., 2002, see review). Despite considerable diversity, sequence comparisons show that the catalytic cores of cellulases belong to a restricted number of families (Beguin *et al*., 1994).

Here, we describe the properties and DNA gene sequences of cellulases (endo-β -1,4-glucanases) from *B. subtilis* strains isolated from soil, compost, and animal waste slurry in Jeju Island, Korea. The sequences of the genes were compared with those of other known cellulase genes, and their modular structures were defined.

Ⅱ. Materials and Methods

1. Bacterial strains and media

Hundreds of cellulolytic bacteria were screened and isolated from soil, compost, and animal waste slurry. Among the isolates, three strains, SL9-9, C5-16, and S52-2, were chosen and identified as *B. subtilis* (Kim *et al.*, 2011) and deposited in the Korean Agricultural Culture Collection (KACC, Rural Development Administration, Korea). They were maintained and subcultured in CMC-S agar medium containing carboxymethylcellulose (CMC), 10.0; soytone 4.0; yeast extract, 4.0; $CaCl_2 \cdot 2H_2O$, 0.2; NaCl, 0.2; K_2HPO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; agar, 20 (g L^{-1}, each).

2. Cloning and DNA sequence analysis of cellulase genes

The full-length β-1,4-glucanase genes from the three bacterial strains were cloned from the genomic DNAs of each bacterial strain by polymerase chain reaction (PCR) using primers based on the DNA sequences of previously cloned *Bacillus* cellulase genes. The primers used were BS cel up : 5'-ATG AAA CGG TCA ATC TCT ATT TTT-3' as a forward primer and BS cel dn : 5'-ACT AAT TTG GTT CTG TTC CCC AAA-3' as a reverse primer. Molecular sizes of the resulting PCR products were analyzed on 1.0% agarose gel to confirm the 1.2 kb β-1,4-glucanase gene. These PCR products were purified with a DNA purification kit (QIAGEN, Valencia, California, USA), after which the purified PCR products were cloned in top GEM-Tvector and sequenced. The nucleotide sequences of the cellulase genes were confirmed and compared through a BLAST nucleotide search provided by the National Center for Biotechnology Information (NCBI) Gene bank (U.S. National Library of Medicine, Bethesda, Maryland, USA).

Ⅲ. Results

1. Isolation of cellulase gene of *B. subtilis* SL9-9

A DNA of cellulase gene of *B. subtilis* SL9-9 was 1441bp corresponding to 480 amino acids (Fig. 34). Its deduced amino acid sequences showed 99% similarity of that of *B. subtilis* C-36 (GenBank accession number ABG78039), 93% similarity of that of *B. subtilis* C5-16 (GenBank accession number JQ346089) and 96% similarity of that of *B. subtilis* S52-2 (GenBank accession number JQ346091) (Table 32).

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1      ATGAAACGGTCAATC TCTATTTTTATTACG TGTTTATTGATTGCG GTATTGACAATGGGC GGCTTGCTGCCTTCG
1       M  K  R  S  I   S  I  F  I  T   C  L  L  I  A   V  L  T  M  G   G  L  L  P  S
76     CCGGCTTCAGCAGCA GAGACAAAAACGCCG GCAGCCAAGAATGGT CAGCTTAGCATAAAA GGTACACAGCTCGTA
26      P  A  S  A  A   E  T  K  T  P   A  A  K  N  G   Q  L  S  I  K   G  T  Q  L  V
151    AACCGAGACGGTAAA GCGGTACAACTGAAA GGGATCAGTTCACAT GGATTGCAATGGTAT GGAGATTTCGTCAAT
51      N  R  D  G  K   A  V  Q  L  K   G  I  S  S  H   G  L  Q  W  Y   G  D  F  V  N
226    AAAGACAGCTTAAAA TGGCTGAGAGACGAT TGGGGCATAACCGTT TTCCGCGCGGCGATG TATACGGCAGATGGC
76      K  D  S  L  K   W  L  R  D  D   W  G  I  T  V   F  R  A  A  M   Y  T  A  D  G
301    GGTTATATTGACAAC CCGTCCGTGAAAAAT AAAGTAAAAGAAGCG GTTGAAGCGGCAAAA GAACTTGGGATATAT
101     G  Y  I  D  N   P  S  V  K  N   K  V  K  E  A   V  E  A  A  K   E  L  G  I  Y
376    GTCATCATTGACTGG CATATTTTAAATGAC GGCAACCCAAACCAA AATAAAGAGAAGGCA AAAGAATTTTTCAAG
126     V  I  I  D  W   H  I  L  N  D   G  N  P  N  Q   N  K  E  K  A   K  E  F  F  K
451    GAGATGTCAAGTCTT TACGGAAACACGCCG AACGTCATTTATGAA ATTGCAAACGAACCA AACGGTGACGTGAAC
151     E  M  S  S  L   Y  G  N  T  P   N  V  I  Y  E   I  A  N  E  P   N  G  D  V  N
526    TGGAAGCGTGATATT AAACCGTATGCGGAA GAAGTGATTTCCGTT ATCCGCAAAAATGAT CCAGACAACATCATC
176     W  K  R  D  I   K  P  Y  A  E   E  V  I  S  V   I  R  K  N  D   P  D  N  I  I
601    ATTGTCGGCACCGGT ACATGGAGCCAGGAT GTGAATGATGCTGCC GATGACCAGTTAAAA GATGCAAACGTCATG
201     I  V  G  T  G   T  W  S  Q  D   V  N  D  A  A   D  D  Q  L  K   D  A  N  V  M
676    TACGCGCTTCATTTT TATGCCGGCACACAC GGGCAATTTTTACGG GATAAAGCAAACTAC GCACTCAGCAAAGGA
226     Y  A  L  H  F   Y  A  G  T  H   G  Q  F  L  R   D  K  A  N  Y   A  L  S  K  G
751    GCGCCTATTTTCGTG ACGGAATGGGGAACA AGCGATGCGTCTGGA AATGGCGGTGTATTC CTAGACCAGTCGCGG
251     A  P  I  F  V   T  E  W  G  T   S  D  A  S  G   N  G  G  V  F   L  D  Q  S  R
826    GAATGGCTGAATTAT CTCGACAGCAAGAAC ATCAGCTGGGTGAAC TGGAATCTTTCTGAT AAGCAGGAATCATCT
276     E  W  L  N  Y   L  D  S  K  N   I  S  W  V  N   W  N  L  S  D   K  Q  E  S  S
901    TCGGCTTTAAAGCCG GGAGCATCTAAAACA GGCGGCTGGCCGCTT ACAGATTTAACTGCT TCAGGAACATTCGTA
301     S  A  L  K  P   G  A  S  K  T   G  G  W  P  L   T  D  L  T  A   S  G  T  F  V
976    AGAGAAAACATTCGC GGTACTAAAGATTCA ACGAAGGACGGCCCT GAAACGCCAGCACAA GATAACCCCACACAG
326     R  E  N  I  R   G  T  K  D  S   T  K  D  G  P   E  T  P  A  Q   D  N  P  T  Q
1051   GAAAAAGGCGTTTCT GTACAATACAAAGCA GGGGATGGGCGTGTG AACAGCAATCAAATC CGCCCGCAGCTTCAC
351     E  K  G  V  S   V  Q  Y  K  A   G  D  G  R  V   N  S  N  Q  I   R  P  Q  L  H
1126   ATAAAAAATAATGGC AATGCGATGGTTGAT TTAAAAGATGTCACT GCCCGTTACTGGTAT AACGCGAAAAACAAA
376     I  K  N  N  G   N  A  M  V  D   L  K  D  V  T   A  R  Y  W  Y   N  A  K  N  K
1201   GGCCAAAACGTTGAC TGTGACTACGCGCAG ATGGGATGCGGCAAT CTGACCCACAAGTTT GTGACGCTGCATAAA
401     G  Q  N  V  D   C  D  Y  A  Q   M  G  C  G  N   L  T  H  K  F   V  T  L  H  K
1276   CCTAAGCAAGGTGCA GATACCTATCTGGAA CTGGGGTTTAAAACA GGAACGCTGTCACCG GGAGCAAGCACAGGG
426     P  K  Q  G  A   D  T  Y  L  E   L  G  F  K  T   G  T  L  S  P   G  A  S  T  G
1351   AATATTCAGCTTCGT CTTCACAATGATGAC TGGAGCAATTATGCA CAAAGCGGCGATTAT TCCTTTTTCCAATCA
451     N  I  Q  L  R   L  H  N  D  D   W  S  N  Y  A   Q  S  G  D  Y   S  F  F  Q  S
1426   AATACGTTTAAAACA
476     N  T  F  K  T
```

Fig. 34. Nucleotide and deduced amino acid sequences of cellulase gene of *B. subtilis* SL9-9.

2. Isolation of cellulase gene of *B. subtilis* C5-16

A DNA of cellulase gene of *B. subtilis* C5-16 was 1452bp corresponding to 470 amino acids (Fig. 35). Its deduced amino acid sequences showed 99% similarity of that of *B. subtilis* AH18 (GenBank accession number ABK63475), 93% similarity of that of *B. subtilis* SL9-9 (GenBank accession number JQ346090) and 93% similarity of that of *B. subtilis* S52-2 (GenBank accession number JQ346091) (Table 32).

```
1       ATGAAACGGTCAATC TCTATTTTTATTACG TGTTTATTGATTACG TTATTGACAATGGGC GGCATGCTGGCTTCG
1        M  K  R  S  I   S  I  F  I  T   C  L  L  I  T   L  L  T  M  G   G  M  L  A  S
76      CCGGCATCAGCAGCA GGGACAAAAACGCCA GTAGCCAAGAATGGC CAGCTTAGCATAAAA GGTACACAGCTCGTT
26       P  A  S  A  A   G  T  K  T  P   V  A  K  N  G   Q  L  S  I  K   G  T  Q  L  V
151     AACCGAGACGGTAAA GCGGTACAGCTGAAG GGGATCAGTTCACAC GGATTGCAATGGTAT GGAGAATATGTCAAT
51       N  R  D  G  K   A  V  Q  L  K   G  I  S  S  H   G  L  Q  W  Y   G  E  Y  V  N
226     AAAGACAGCTTAAAA TGGCTGAGGGACGAT TGGGGTATCACCGTT TTCCGTGCAGCGATG TATACGGCAGATGGC
76       K  D  S  L  K   W  L  R  D  D   W  G  I  T  V   F  R  A  A  M   Y  T  A  D  G
301     GGTTATATTGACAAC CCGTCCGTGAAAAAT AAAGTGAAAGAAGCG GTTGAAGCGGCAAAA GAGCTTGGGATATAT
101      G  Y  I  D  N   P  S  V  K  N   K  V  K  E  A   V  E  A  A  K   E  L  G  I  Y
376     GTCATCATTGACTGG CATATCTTAAATGAC GGTAATCCAAACCAA AATAAAGAGAAGGCA AAAGAATTCTTCAAG
126      V  I  I  D  W   H  I  L  N  D   G  N  P  N  Q   N  K  E  K  A   K  E  F  F  K
451     GAAATGTCAAGCCTT TACGGAAACACGCCA AACGTCATTTATGAA ATTGCAAACGAACCA AACGGTGATGTGAAC
151      E  M  S  S  L   Y  G  N  T  P   N  V  I  Y  E   I  A  N  E  P   N  G  D  V  N
526     TGGAAGCGTGATATT AAACCGTATGCGGAA GAAGTGATTTCCGTT ATCCGCAAAAATGAT CCAGACAACATCATC
176      W  K  R  D  I   K  P  Y  A  E   E  V  I  S  V   I  R  K  N  D   P  D  N  I  I
601     ATTGTCGGAACCGGT ACATGGAGCCAGGAT GTGAATGATGCTGCC GATGACCAGCTAAAA GATGCAAACGTTATG
201      I  V  G  T  G   T  W  S  Q  D   V  N  D  A  A   D  D  Q  L  K   D  A  N  V  M
676     TACGCACTTCATTTT TATGCCGGCACACAC GGCCAATTTTTACGG GATAAAGCAAACTAT GCACTCAGCAAAGGA
226      Y  A  L  H  F   Y  A  G  T  H   G  Q  F  L  R   D  K  A  N  Y   A  L  S  K  G
751     GCACCTATTTTTGTG ACAGAGTGGGGAACA AGCGACGCGTCTGGC AATGGCGGTGTATTC CTTGATCAATCGAGG
251      A  P  I  F  V   T  E  W  G  T   S  D  A  S  G   N  G  G  V  F   L  D  Q  S  R
826     GAATGGCTGAAATAT CTCGACAGCAAGACC ATCAGCTGGGTGAAC TGGAATCTTTCTGAT AAGCAGGAATCATCC
276      E  W  L  K  Y   L  D  S  K  T   I  S  W  V  N   W  N  L  S  D   K  Q  E  S  S
901     TCAGCTTTAAAGCCG GGGGCATCTAAAACA GGCGGCTGGCGGTTG TCAGATTTATCTGCT TCAGGAACATTCGTT
301      S  A  L  K  P   G  A  S  K  T   G  G  W  R  L   S  D  L  S  A   S  G  T  F  V
976     AGAGAAAACATTCTC GGCACCAAAGATTCG ACGAAGGACATTCCT GAAACGCCAGCAAAA GATAAACCCACACAG
326      R  E  N  I  L   G  T  K  D  S   T  K  D  I  P   E  T  P  A  K   D  K  P  T  Q
1051    GAAAACGGTATTTCT GTACAATACAGAGCA GGGGATGGGAGTATG AACAGCAACCAAATC CGTCCGCAGCTTCAA
351      E  N  G  I  S   V  Q  Y  R  A   G  D  G  S  M   N  S  N  Q  I   R  P  Q  L  Q
1126    ATAAAAAATAACGGC AATACCACGGTTGAT TTAAAAGATGTCACT GCCCGTTACTGGTAT AACGCGAAAAACAAA
376      I  K  N  N  G   N  T  T  V  D   L  K  D  V  T   A  R  Y  W  Y   N  A  K  N  K
1201    GGCCAAAACGTTGAC TGTGACTACGCGCAG CTTGGATGCGGCAAT GTGACATACAAGTTT GTGACGTTGCATAAA
401      G  Q  N  V  D   C  D  Y  A  Q   L  G  C  G  N   V  T  Y  K  F   V  T  L  H  K
1276    CCAAAGCAAGGTGCA GATACCTATCTGGAA CTTGGATTTAAAAAC GGAACGCTGGCACCG GGAGCAAGCACAGGG
426      P  K  Q  G  A   D  T  Y  L  E   L  G  F  K  N   G  T  L  A  P   G  A  S  T  G
1351    AATATTCAGCTTCGT CTTCACAATGATGAC TGGAGCAATTATGCA CAAAGCGGCGATTAT TCCTTTTTCAAATCA
451      N  I  Q  L  R   L  H  N  D  D   W  S  N  Y  A   Q  S  G  D  Y   S  F  F  K  S
1426    AATACGTTTAAAACA ACGAAAAAAATC
476      N  T  F  K  T   T  K  K  I
```

Fig. 35. Nucleotide and deduced amino acid sequences of cellulase gene of *B. subtilis* C5-16.

3. Isolation of cellulase gene of *B. subtilis* S52-2

A DNA of cellulase gene of *B. subtilis* S52-2 was 1497bp corresponding to 499 amino acids (Fig. 36). Its deduced amino acid sequences showed 99% similarity of that of *B. subtilis* WRD-2 (GenBank accession number AAX54913), 96% similarity of that of *B. subtilis* SL9-9 (GenBank accession number JQ346090) and 93% similarity of that of *B. subtilis* C5-16 (GenBank accession number JQ346089) (Table 32).

```
1       ATGAAACGGTCAATC TCTATTTTTATTACG TGTTTATTGATTACA GTATTGACAATGGGC GGCTTGCAGGCTTCC
1        M  K  R  S  I   S  I  F  I  T   C  L  L  I  T   V  L  T  M  G   G  L  Q  A  S
76      CAGGCATCTGCAGCA GGGACAAAAACGCCA GCAGCCAAGAATGGG CAGCTTAGCATAAAA GGAACACAGCTCGTA
26       Q  A  S  A  A   G  T  K  T  P   A  A  K  N  G   Q  L  S  I  K   G  T  Q  L  V
151     AACCGGGACGGCAAA GCGGTACAATTGAAA GGGATCAGTTCACAT GGATTGCAATGGTAT GGCGATTTTGTCAAT
51       N  R  D  G  K   A  V  Q  L  K   G  I  S  S  H   G  L  Q  W  Y   G  D  F  V  N
226     AAAGACAGCTTAAAA TGGCTGAGAGACGAT TGGGGCATAACCGTT TTCCGCGCGGCGATG TATACGGCAGATGGC
76       K  D  S  L  K   W  L  R  D  D   W  G  I  T  V   F  R  A  A  M   Y  T  A  D  G
301     GGTTATATTGATAAT CCGTCCGTGAAAAAT AAAGTAAAAGAAGCG GTTGAAGCGGCAAAA GAACTTGGGATATAT
101      G  Y  I  D  N   P  S  V  K  N   K  V  K  E  A   V  E  A  A  K   E  L  G  I  Y
376     GTCATCATTGACTGG CATATCTTAAATGAC GGCAACCCAAACCAA CATAAAGAGAAGGCA AAAGAATTTTTTAAG
126      V  I  I  D  W   H  I  L  N  D   G  N  P  N  Q   H  K  E  K  A   K  E  F  F  K
451     GAAATGTCAAGTCTT TACGGAAACACGCCA AACGTCATTTATGAA ATTGCAAACGAACCA AACGGTGATGTGAAC
151      E  M  S  S  L   Y  G  N  T  P   N  V  I  Y  E   I  A  N  E  P   N  G  D  V  N
526     TGGAAGCGTGATATT AAACCGTATGCGGAA GAAGTGATTTCCGTT ATCCGCAAAAATGAT CCAGACAACATCATC
176      W  K  R  D  I   K  P  Y  A  E   E  V  I  S  V   I  R  K  N  D   P  D  N  I  I
601     ATTGTCGGAACCGGT ACATGGAGCCAAGAT GTGAATGATGCAGCC GATGATCAGCTAAAA GATGCAAACGTCATG
201      I  V  G  T  G   T  W  S  Q  D   V  N  D  A  A   D  D  Q  L  K   D  A  N  V  M
676     TACGCGCTTCATTTT TATGCCGGCACACAC GGCCAATCTTTACGG GATAAAGCAAACTAT GCACTCAGTAAAGGA
226      Y  A  L  H  F   Y  A  G  T  H   G  Q  S  L  R   D  K  A  N  Y   A  L  S  K  G
751     GCGCCTATTTTCGTG ACGGAATGGGGAACA AGCGACGCGTCTGGA AATGGCGGTGTATTC CTTGACCAGTCGCGG
251      A  P  I  F  V   T  E  W  G  T   S  D  A  S  G   N  G  G  V  F   L  D  Q  S  R
826     GAATGGCTGAATTAT CTCGACAGCAAGAAC ATCAGCTGGGTGAAC TGGAATCTTTCTGAT AAGCAGGAATCATCC
276      E  W  L  N  Y   L  D  S  K  N   I  S  W  V  N   W  N  L  S  D   K  Q  E  S  S
901     TCAGCGTTAAAGCCG GGAGCATCTAAAACA GGCGGCTGGCCGCTT ACAGATTTAACTGCT TCAGGAACATTCGTA
301      S  A  L  K  P   G  A  S  K  T   G  G  W  P  L   T  D  L  T  A   S  G  T  F  V
976     AGAGAAAACATTCTC GGCAACAAAGATTCA ACGAAAGAACGCCCT GAAACGCCAGCACAA GATAACCCCGCACAG
326      R  E  N  I  L   G  N  K  D  S   T  K  E  R  P   E  T  P  A  Q   D  N  P  A  Q
1051    GAAAACGGCATTTCT GTACAATACAAAGCA GGGGATGGGGGTGTG AACAGCAACCAAATC CGCCCGCAGCTTCAC
351      E  N  G  I  S   V  Q  Y  K  A   G  D  G  G  V   N  S  N  Q  I   R  P  Q  L  H
1126    ATAAAAAATAACGGC AATGCGACGGTTGAT TTAAAAGATGTCACT GCCCGTTACTGGTAT AACGCGAAAAACAAG
376      I  K  N  N  G   N  A  T  V  D   L  K  D  V  T   A  R  Y  W  Y   N  A  K  N  K
1201    GGCCAAAACTTTGAC TGTGACTACGCGCAG ATTGGATGCGGCAAT CTGACCCACAAATTT GTGACGCTGCATAAA
401      G  Q  N  F  D   C  D  Y  A  Q   I  G  C  G  N   L  T  H  K  F   V  T  L  H  K
1276    CCTAAGCAAGGTGCA GATACCTATCTGGAA CTGGGTTTTAAAACA GGAACGCTGTCACCG GGAGCAAGCACAGGG
426      P  K  Q  G  A   D  T  Y  L  E   L  G  F  K  T   G  T  L  S  P   G  A  S  T  G
1351    AATATTCAGCTTCGT CTTCACAATGATGAC TGGAGTAGTTATGCA CAAAGCGGTGATTAT TCCTTTTTTCAATCA
451      N  I  Q  L  R   L  H  N  D  D   W  S  S  Y  A   Q  S  G  D  Y   S  F  F  Q  S
1426    AATACGTTTAAAACA ACGAAAAAAATTACA TTATATCATCAAGGA AAACTGATTTGGGGA ACAGAACCAAAT
476      N  T  F  K  T   T  K  K  I  T   L  Y  H  Q  G   K  L  I  W  G   T  E  P  N
```

Fig. 36. Nucleotide and deduced amino acid sequences of cellulase gene of *B. subtilis* S52-2.

4. Comparison of cellulase genes of *B. subtilis* strains

The nucleotide sequences of the cloned genes were compared with those of the cellulase genes registered in the BLAST database using the Gendoc program (Table 32).

We compared the deduced amino acid sequences of the cellulases from the three *B. subtilis* isolates with those of *B. subtilis* PAP115 (GenBank accession number CAA28392) (Mackay *et al*., 1986), *B. subtilis* DLG (GenBank accession number P07983) (Robson *et al*., 1987) and *B. subtilis* C-36 (GenBank accession number ABG78039) (Guan *et al*., 2009). The high degree of sequence similarity observed between the endoglucanases from the different *B. subtilis* strains revealed a close evolutionary relationship between the isolates (Fig. 37). Amino acid sequences of cellulase proteins from different sources were aligned and compared using the COBALT (Constraint-based Multiple Alignment Tool) program provided by NCBI (Fig. 38).

Table 32. Homology among nucleotide sequences of cellulase genes from *Bacillus* strains

Bacterial isolate	Homology(%)	Source of cellulase gene	GenBank accession No.
B. subtilis SL9-9	99%	*B. subtilis* strain C-36 endo-β-1,4-glucanase	ABG78039
	93%	*B. subtilis* C5-16	JQ346089
	96%	*B. subtilis* S52-2	JQ346091
B. subtilis C5-16	99%	*B. subtilis* strain AH18 cellulase	ABK63475
	93%	*B. subtilis* SL9-9	JQ346090
	93%	*B. subtilis* S52-2	JQ346091
B. subtilis S52-2	99%	*B. sp.* WRD-2 cellulase	AAX54913
	96%	*B. subtilis* SL9-9	JQ346090
	93%	*B. subtilis* C5-16	JQ346089

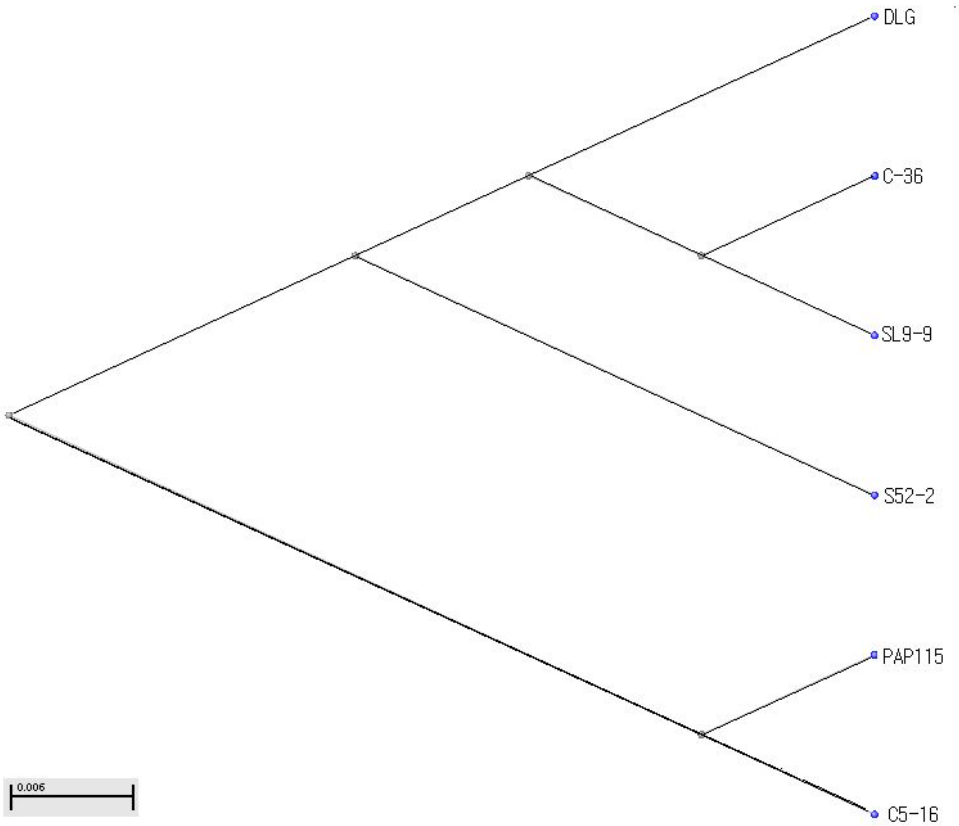

Fig. 37. Phylogenetic tree resulting from complete sequencing of cellulase genes.

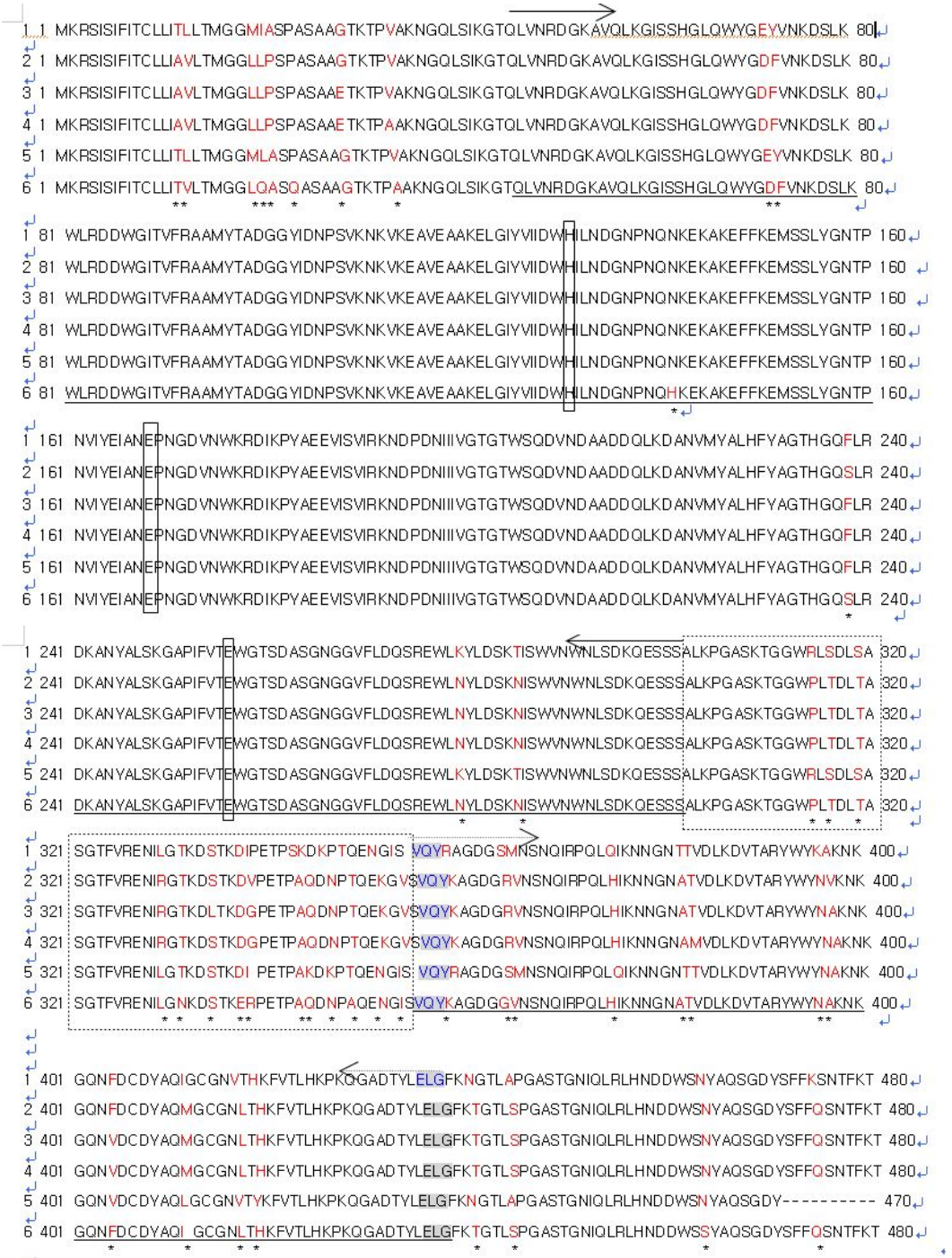

Fig. 38. Alignment of deduced amino acid sequences of endo-β-1,4-glucanases from three isolates with those from other *B. subtilis* strains.

1, B. subtilis PAP115 endo-β-1,4-glucanase gene; 2, B. subtilis DLG endo-β-1,4-glucanase gene; 3, B. subtilis C-36 endo-β-1,4-glucanase gene; 4, B. subtilis SL9-9; 5, B. subtilis C5-16; 6, B. subtilis S52-2. Region 1-29, signal peptide; Region 48-301, cellulase catalytic domain (glucosyl hydrolase family 5/A2); Region 302-355, linker region; Region 356-437, cellulose binding domain (CBD family IIIa); Site 131-H, 169-E, and 257-E, active sites (proton donor or nucleophile).

Ⅳ. Discussion

The nucleotide sequences of the cloned genes were compared with those of the cellulase genes registered in the BLAST database using the Gen doc program (Table 32). The *B. subtilis* SL9-9 gene showed the highest identity with the *B. subtilis* strain C-36 endo-β-1,4-glucanase gene with 99% identity. The *B. subtilis* C5-16 gene showed the highest identity with the *B. subtilis* strain AH18 cellulase gene with 99% identity. The *B. subtilis* S52-2 gene showed the highest identity with the *B. subtilis* WRD-2 cellulase gene with 100% identity. The *B. subtilis* SL9-9 gene showed 93% identity with the *B. subtilis* C5-16 gene and 96% identity with the *B. subtilis* S52-2 gene. Further, the C5-16 and S52-2 genes shared 93% identity. Based on these results, it is reasonable to conclude that all three of the cloned genes from our *Bacillus* strains code for cellulase protein.

We compared the deduced amino acid sequences of the cellulases from the three *B. subtilis* isolates with those of *B. subtilis* PAP115 (GenBank accession number CAA28392) (Mackay *et al*., 1986), *B. subtilis* DLG (GenBank accession number P07983) (Robson *et al*., 1987), and *B. subtilis* C-36 (GenBank accession number ABG78039) (Guan *et al*., 2009).

Amino acid sequences of cellulase proteins from different sources were aligned and compared using the COBALT (Constraint-based Multiple Alignment Tool) program provided by NCBI. The high degree of sequence similarity observed between the endoglucanases from the different *B. subtilis* strains revealed a close evolutionary relationship between the isolates (Fig. 37).

According to the modern concept of cellulases (Rabinovich, 2002, see review), most cellulolytic enzymes are comprised of modular multidomain proteins containing at least three separate structural elements with different functions, *i.e.,* catalytic domain (CD), interdomain linker and cellulose binding domain (CBD). Currently,

CDs of polysaccharases are grouped into at least 15 of the more than 80 known glycosyl hydrolase families, whereas CBDs fall into at least 13 families.

The cellulase genes identified in the nucleotide sequences of *B. subtilis* SL9-9, C5-16 and S52-2 encoded proteins of 480, 470, and 499 amino acid residues, respectively (Fig. 38). Computer analysis of the deduced amino acid sequences of the cellulases from our three isolates using the CDART program of NCBI revealed a modular enzyme composed of two discrete domains in the following order: CD (Q-48 through S-301) of the glucosyl hydrolase family 5/A2 (endoglucanase, EC 3.2.1.4) and CBD (V-356 through G-437) of family IIIa. Similar to the modular organization of many *Bacillus* endoglucanases, the CDs of these enzymes were located in the N-terminal region and CBDs in the C-terminal region.

When the homologous sequences were suitably aligned, a total of 45 amino acid substitutions among the six *Bacillus* strains were identified (Fig. 5). There were 5 to 6 amino acid residue substitutions in the signal peptide region (1 through 29), and 23 amino acid residues were conserved among all of the cellulases, resulting in 79% homology. The leader region (30 through 47) consisted of 18 residues and 16 amino acid residues among them were conserved among the cellulases, resulting in 89% homology. The CD consisted of 254 residues (48 through 301), and 248 amino acid residues were conserved among the cellulases, resulting in 97.6% homology. In the linker region (302 through 355) consisting of 54 residues, 40 amino acid residues were conserved among the cellulases, resulting in 74% homology. In the CBD (356 through 437) consisting of 83 residues, 71 amino acid residues were conserved among the cellulases, resulting in 85.5% homology. The CD showed the highest 97.6% homology while the regions of signal, leader, linker and CBD regions showed low homology.

The endo-β-1,4-glucanase gene encoded a signal peptide at the N-terminal end of the protein. Two basic residues at positions 2 and 3 (lysine and arginine) in the hydrophilic leader region were followed by a hydrophobic core of 18 amino acid residues rich in leucine and isoleucine, which is in agreement with the findings of

Mezes and Lampen (Mezes *et al*., 1985). They found that *Bacillus* signal peptides are characterized by a short, hydrophilic, basic region along with a subsequent, long, hydrophobic region. The N-terminal residues of the endoglucanase enzymes from the isolates indicate that the cleavage sites for signal peptide removal were between amino acids A-29 and A-30. This is in agreement with the findings of Mackay *et al*. (1986) and Robson and Chambliss (1987) regarding the enzymes from *B. subtilis* PAP115 and *B. subtilis* DLG, respectively.

Henrisatt *et al*. (1989) classified fungal and bacterial cellulases into six families on the basis of hydrophobic cluster analysis. Specifically, amino acid residues His-131 and Glu-169 of endoglucanase were predicted to form the active site in the CD domain. When Park *et al*.(1993) changed these amino acids by site-directed mutagenesis, a mutation at His-131 resulted in complete abolishment of enzyme activity, whereas a mutation at Glu-169 resulted in significant loss of enzyme activity. Robson and Chambliss (1987) also reported that there are two active sites in the endo-β-1,4-glucanase from *B. subtilis* DLG. One is located at Glu-169 as a proton donor and the other at Glu-257 as a nucleophile. We confirmed that three of the amino acid residues, H-131 (histidine), E-169 (glutamic acid), and E-257 (glutamic acid), were conserved at the same CD sites in our *Bacillus* species.

Hamamoto *et al*.(1992) suggested that synergistic function of the NH_2-terminus and COOH-terminus of the endoglucanase from *C. cellulovorans* is essential for hydrolysis of crystalline cellulose. The truncated endoglucanase from *B. subtilis* is 4-fold more active on CMC but 5-fold less active on insoluble cellulose upon C-terminal mutation (Hefford *et al*., 1992). In a previous study, when the CBD of cellobiohydrolase I from *T. reesei* was removed by protease, the activity towards Avicel was completely lost, and decreased adsorption of the enzyme onto this microcrystalline cellulose was observed, whereas activity towards chromogenic oligosaccharides remained unchanged (Tilbeurgh *et al*., 1986). Park *et al*. (1993) reported that the cellulose-binding ability of *B. subtilis* endoglucanase is independent of its catalytic activity to hydrolyze CMC, as a mutation in the CD active site does

not affect its cellulose-binding ability. All of these reports strongly support that cellulases are composed of physically separate domains and that CBDs play crucial roles in the hydrolysis of crystalline cellulose by binding to the cellulose surface (Liu *et al*., 2011).

Usually, linkers comprise flexible disordered chains rich in proline and hydroxy amino acid residues (serine and threonine), as well as glycine and alanine (Gilkes *et al*., 1991). Their lengths can vary from 5~6 to 100 residues, although most often limited to a range of 20 to 50 residues. They are believed to provide flexibility and spatial separation of CDs from CBDs, which allows their autonomous function on the surfaces of insoluble substrates (Rabinovich *et al*., 2002). In this study, there were many G (glycine), S (serine), T (threonine), K (lysine), and P (proline) residues in the linker region as shown in Fig. 38.

Our comparison of the deduced amino acid sequences of cellulase genes cloned from the three isolates, *B. subtilis* SL9-9, C5-16, and S52-2, found that although these enzymes were not extraordinary, they matched quite well with the modern concepts of multidomain cellulolytic enzymes.

Ⅴ. Conclusion

Three *B. subtilis* strains were isolated from soil, compost and animal waste slurry in Jeju Island, Korea and their cellulase genes cloned. Cellulase genes from *B. subtilis* SL9-9, C5-16 and S52-2 encoded proteins of 480, 470 and 499 amino acid residues, respectively. DNA sequences of the genes were compared with those of other known cellulase genes. The deduced amino acid sequences of the cellulase genes from the isolates matched quite well the modern concepts of multidomain cellulolytic enzymes. The enzymes were composed of three discrete domains: catalytic domain (CD) of glucosyl hydrolase family 5/A2, interdomain linker and cellulose binding domainns (CBD) of family Ⅲa. Similar to the modular organization of many *Bacillus* endo-β-1,4-glucanases, the CDs of these enzymes were located in the N-terminal region and CBDs in the C-terminal region.

PART Ⅲ

Evaluation of *Bacillus subtilis* SL9-9 as a biofertilizer in cucumber seedling growth

Ⅰ. Introduction

Biofertilizers for plant growth promoting, in strict sense, are not fertilizers, which directly give nutrition to crop plants. These are cultures of microorganisms like bacteria, fungi, packed in a carrier material. Thus, the critical input in biofertilizer is the microorganisms (Boraste *et al*., 2009). They help the plants indirectly through better nitrogen (N) fixation or improving the nutrient availability in the soil. The term "Biofertilizer" or more appropriately a "Microbial inoculants" can generally be defined as preparation containing live or latent cells of efficient strains of nitrogen fixing, phosphate-solublizing or cellulolytic microorganisms used for application to seeds, soil or composting areas with the objective of increasing the number of such microorganisms and accelerate those microbial process which augment the availability of nutrients that can be easily assimilated by plants. Microorganisms, which can be used as biofertilizer, include bacteria, fungi and blue green algae. These organisms are added to the rhizosphere of the plant to enhance their activity in the soil. Sustainable crop production depends much on good soil health. Soil health maintenance warrants optimum combination of organic and inorganic components of the soil. Repeated use of chemical fertilizers destroys soil biota. In nature, there are a number of useful soil microorganisms that can help plants to absorb nutrients. Their utility can be enhanced with human intervention by selecting efficient organisms, culturing them and adding them to soils directly or through seeds. The cultured microorganisms packed in some carrier material for easy application in the

field are called biofertilizers.

Cellulose is the major part of plant biomass. Therefore, the wastes generated from forests, agricultural fields and agro industries contain a large amount of unutilized or underutilized cellulose. Agricultural and industrial wastes are among the causes of environmental pollution (Milala *et al*., 2005). Nowadays, these so called wastes are judiciously converted into valuable products such as enzymes (Ray *et al*., 1994), sugar (Ghosh and Ray, 2010), biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds, plant and human nutrients (Howard *et al*., 2003), which is accomplished by cellulase. Especially, in agrosystem, composting is a process essentially meant to utilize soil waste of animal-plant origin. Composting procedure can be hastened by inoculating the residue with cellulolytic and lignolytic microorganisms such as *Trichoderma viridae*, *Aspergillus niger*, *Aspergillus terrus etc*. The improvement in soil fertility due to sludge application was reflected through population diversity of soil microbial community.

Cellulase (CEL) mostly found in plants, insects and microorganisms, of which bacteria and fungi appear to be major sources (Watanabe and Tokuda, 2010). In nature, bacteria and fungi play a vital role as decomposers that accelerate the decay of plant biomass (Sánchez, 2009). CEL presently constitutes a major group of industrial enzyme based on its diverse ranges of utilization. Apart from such current and well-established applications - as in cotton processing, paper recycling, detergent formulation, juice extraction, and animal feed additives - their uses in agricultural biotechnology and bioenergy have been exploited (Phitsuwan *et al*., 2012).

Supplementation of CELs to accelerate decomposition of plant residues in soil results in improved soil fertility. So far, applying CELs/antagonistic cellulolytic fungi to crops has shown to promote plant growth performance, including enhanced seed germination and protective effects (Phitsuwan *et al*., 2012). Their actions are believed mainly to trigger plant defence mechanisms and/or to act as biocontrol agents that mediate disease suppression. However, the exact interaction between the enzymes/fungi and plants has not been clearly elucidated.

The present study was proposed with an objective to access the species of cellulase producing bacteria, *B. subtilis* SL9-9 isolated from Jeju, for cucumber seedling growth and to evaluate their efficacy to bedsoil fertility.

Ⅱ. Materials and Methods

1. Bacterial strain

B. subtilis SL9-9 originally selected for their cellulase properties(Kim, 2011; Kim, 2012) was investigated in this study. The strain was maintained by long-term storage in nutrient broth with 15% glycerol at -70℃ prior to test.

2. Inoculation of *B. subtilis* SL9-9

For this experiment, pure culture was grown in 0.5% rice bran medium in the bioreactor (150 rpm, 30℃, 1 vvm) for 5 days. *Bacillus* cells were then harvested and measured the density of cells. The biocarrier of the strain was prepared mixing peatmoss, cocopeat, perlite, and vermiculite in the ratio of four, four, one to one in advance. The bacterial inoculation involved spraying the diluted solutions onto the biocarriers.

3. Preparation of bedsoils

For the organic seedling, the bedsoils were made without chemical materials. Therefore, as a source of nutrient, fish-fermented liquid manure was added into the bedsoils. To achieve our goal, sixteen media were formulated by adjusting blending ratio of organic liquid manure (LM, fish fermented liquid manure) and cell density of the *Bacillus*. The amended ratios of LM were 0%, 1%, 2% and 3% (v/v), (L0, L1, L2, L3), respectively and the amended density of *Bacillus* cells were 0, 1×10^5, 1×10^6, 1×10^7 (cfu mL^{-1}) (T1, T2, T3, T4), respectively. The LM was manufactured by fermenting the by-product of mackerel for more than six months. Commercial bedsoil (SukSuki, Nongwoo Bio. C., Korea) was used as a control. Usually,

commercial bedsoils contain chemical materials like chemical fertilizer *etc*. and so they weren't used in organic farming.

4. Plant growth promotion

For this experiment, the variety of cucumber "Jeongseon-Samcheok" (Dongbu Hitek C., Korea) was used. A seed germination test was conducted in various media for 6 and 10 days after sowing. Seeding characteristics were investigated at 30 days after sowing. To investigate the seedling properties, the shoot length, root length, stem diameter, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, leaf area, and chlorophyll content were measured. Data collected were analyzed using SAS and compared with DMRT($p<0.05$).

5. Field trial

Field experiment was carried out in the vinyl house managed with organic farming in Guja, Jeju Island, in order to evaluate the yield and growth of cucumber in relation to the inoculation of *Bacillus* cells and addition of LM. This experiment was conducted using a completely randomized factorial design. Treatments with three replicated (each having 15 plants) were as follows ; (1) commercial bedsoil, (2) developed organic bedsoil with the *Bacillus* inoculation. The seedling of cucumber raised in the commercial and organic bedsoil were planted in 3rd September, 2008. The growth and yield of cucumber were recorded periodically from 11th October to 14th November. Data collected were analyzed using SAS and compared with DMRT($p<0.05$).

6. Analysis of chemical properties of bedsoils

6-1. pH and electrical conductivity (EC)

The bedsoil samples were prepared by air-dried. To a 20 mL bedsoil sample in a flask, 100 mL of distilled water is added and the bedsoil was agitated with 200 rpm for one hour. And the shaken suspension was filtered by No.2 filter paper. The pH and EC values is then determined within 60 seconds after the pH/EC electrode(Orion 3-star) was immersed in the suspension.

6-2 Available phosphate concentration

Available phosphate of bedsoil was determined by Lancaster Method. The extraction solution was prepared by dissolution of NH_4F 4.44 g, $(NH_4)_2SO_4$ 26.66 g and NaOH 34 g in 4 L distilled water dissolved with CH_3COOH 80 mL and lactic acid 60 mL (pH 4.25±0.05). A 20 mL sample of bedsoil was placed in a 100 mL flask and extracted with 40 mL of the extraction solution for 20 min with 180 rpm in a rotary shaker. The suspension was filtered by No.6 filter paper and then the supernatant solution was discarded. And T-P was determined by inductively coupled plasma-optical emission spectroscopy (PerkinElmer, Optima 7300, USA).

6-3. Exchangeable cations

The extraction solution was prepared by complex of NH_4OH 272 mL in 2 L distilled water and CH_3COOH 226 mL in 2 L distilled water (pH 7.0). A 20 mL sample of bedsoil was placed in a 100 mL flask and extracted with 50 mL of the extraction solution for 30 min with 180 rpm in a rotary shaker. The suspension was filtered by No.6 filter paper and then the supernatant solution was discarded. And potassium, calcium and magnesium were determined by inductively coupled plasma-optical emission spectroscopy(PerkinElmer, Optima 7300, USA).

7. Analysis of dehydrogenase activity of bedsoils

Microbial activity of bedsoil was estimated by dehydrogenase activity (DHA). Measurements were made according to the iodonitrotetrazolium chloride (INT) method (Neto *et al*., 2007). Sub samples were weighed approximately 1~2 g portions of naturally moist sample into each of test tubes. 0.8 ml of 0.4% INT solution (INT, Iodonitrotetrazolium chloride 0.2 g in 50 mL tris-HCl buffer solution) were added to the sample tubes and 0.8 ml of tris-HCl buffer solution instead of the substrate solution into blank tubes. Test tubes were sealed and incubated at 25±1 °C in the dark for 24 hr. Developed iodonitrotetrazolium formazan (INTF) was extracted using 10 ml of methanol. The tubes were shaked well. The liquid phase was poured carefully or pipetted into centrifugation tubes and centrifuged the extract at 4°C and 4500 rpm for 10 min. The INTF was measured spectrophotometrically at 490 nm (Varian, Cary-500, Australia) and the result was expressed as microgram of INTF per gram of sample.

8. Plant growth-promoting activity of *B. subtilis* strains

8-1. Nitrogen fixation

The nitrogen fixation of the strain was determined by measuring the color and white band formed by ammonification of atmosphere N_2. Nfb medium, nitrogen free malate media, containing bromothymol blue (BTB) as an indicator, was used for determining and incubated at 28ºC up to 7 days. The blue coloured media producing isolates were marked as nitrogen fixers in the semi-solid culture conditions (Gothwal *et al*., 2007).

Table 33. Nfb medium contents

Ingredient	Concentration (g L^{-1})
Malic acid	5.0
K_2HPO_4	0.6
KH_2PO_4	0.4
$MnSO_4$	0.01
$MgSO_4$	0.05
NaCl	0.02
Na_2MoO_4	0.002
Bromothymol blue (0.5% in alcohol)	2mL
Agar	1.75
KOH	4.0
pH	6.6~7.0

8-2. Phosphate solubilization

The phosphate-solubilizing activity of the strain was determined by measuring the zone size formed by solubilization of insoluble phosphate on Pikovskaya's agar plates (in g L^{-1}: $(NH_4)_2SO_4$, 0.5; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; $MnSO_4$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.002; Yeast extract, 0.5; glucose, 10.0; $Ca_3(PO_4)_2$, 5.0; agar, 20.0; pH 7.0). A sterilized paper disc placed on the agar plate. Individual samples were suspended in 1 mL of sterilized distilled water in 1.5 mL e-tube and then 40 μL of each sample inoculated on the disc and the plates were incubated at 30℃ for 14 days.

8-3. ACC deaminase activity

DF minimal salts media was used for ACC deaminase activity. The amount of ACC added to DF salts minimal medium was 30 μM. Except for heat-labile ACC, all media were sterilized by autoclaving for 15 minutes at 121°C. The heat-labile ACC was filtered-sterilized using 0.2 μm membrane filter (Millipore) before added to

the sterilized medium. One single colony was streaking on the plate and incubated at 28℃ for 3~4 days. And then the ACC deaminase activity was determined by observing the growth of strains.

Table 34. DF minimal salts media

Ingredient	Concentration (g L^{-1})
KH_2PO_4	4.0
Na2HPO4	6.0
$MgSO_4$7H2O	0.2
Glucose	2.0
Gluconic acid	2.0
Citric acid	2.0
Trace metal solution (Sol. A)[a]	0.1mL
Sol. B[b]	0.1mL
Bacto Agar (N-free)	20
ACC (30 μM $plate^{-1}$)	

[a]Sol. A (in mg 100 mL^{-1}, each : H_3BO_3, 10.0; $MnSO_4 \cdot H_2O$, 11.19; $ZnSO_4 \cdot 7H_2O$, 124.6; $CuSO_4 \cdot 5H_2O$, 78.22; MoO_3, 10.0; D.W., 100mL)

[b]Sol. B (in mg 10 mL^{-1}, each : $FeSO_4 \cdot 7H_2O$, 100; D.W., 10mL)

8-4. Antimicrobial activity

Antimicrobial activity was determined by Dual culture method. PDA, V8 and ISP2 were used as medium for bacterial, fungal and actinomycetical strains respectively. Positive control experiment was carried out under the similar condition. The assessment of antimicrobial activity was based on the measurement of diameter of inhibition zone formed.

Table 35. Test pathogens and culture conditions

Pathogen	Culture conditions		
	Media	Temperature (℃)	Time (hr)
Rhizoctonia solani	PDA	28	3
Phytophthora capsici	V8		10
Fusarium oxyporum	PDA		5
Botrysis cinerea	PDA		10
Colletotrichum acutatum	PDA		15
Streptomycetes scabies	ISP2		7
Streptomycetes acidiscabies	ISP2		7
Streptomycetes turgidiscabies	ISP2		7

Ⅲ. Results

1. Effect of *B. subtilis* SL9-9 on cucumber seedling growth

For organic seedling, effects of *B. subtilis* SL9-9 and organic liquid manure (LM, fish-fermented liquid manure) on the growth of cucumber seedlings were investigated. Germination rates of cucumber were in the 59~100 percent range at the 6 days after sowing. Especially, the germination rate was lowest in L3 treatments (bedsoils amended 3% (v/v) of LM) as shown in Table 36. At the 10 days after sowing, the germination rates of L3 treatments increased also up to 97.9~98.6%, but were still lower compared with other treatments. As a result, the *Bacillus* had no effect on germination rate of cucumber, whereas the concentration of organic liquid manure may affect on germination rate. Thus, the additive level of LM was proper less than 2%.

Table 36. Effect of organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure on the germination of cucumber seedlings

Treatment	Amended ratio of LM[a] (%, v/v)	Amended density of *B. subtilis* SL9-9 (cfu mL^{-1})	Germination rate (%)	
			6 days[b]	10 days
Control[c]	-	-	99.1a[e]	100.0a
L0-T1	0	DW[d]	99.5a	99.5ab
L0-T2		1×10^5	98.1a	98.6abc
L0-T3		1×10^6	99.1a	99.5ab
L0-T4		1×10^7	98.1a	99.1abc
L1-T1	1	DW	97.7a	99.1abc
L1-T2		1×10^5	99.5a	99.5ab
L1-T3		1×10^6	97.7a	99.5ab
L1-T4		1×10^7	96.8a	97.7c
L2-T1	2	DW	97.7a	98.1abc
L2-T2		1×10^5	97.2a	99.5ab
L2-T3		1×10^6	97.7a	98.6abc
L2-T4		1×10^7	100.0a	100.0a
L3-T1	3	DW	65.7bc	98.1bc
L3-T2		1×10^5	59.0c	97.9bc
L3-T3		1×10^6	93.1a	98.6abc
L3-T4		1×10^7	76.9b	98.1abc

[a]LM, fish fermented liquid manure; [b]days after sowing; [c]Control, commercial bedsoil; [d]DW, distilled water; [e]Data within a column accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 39, shoot length was greater in L2 (2% of LM) and decreased adversely in L3 (3% of LM). In the T2, T3 and T4 supplemented *Bacillus* cells of L1, L2 and L3, shoot length increased significantly compared with T1, respectively. Especially, in L2-T4 (2% of LM and 1×10^7 of *Bacillus* cells), the shoot length was greatest. The density of the *Bacillus* did not cast a long shadow to shoot length, but the shoot length was mostly greatest in T4 (1×10^7 of *Bacillus* cells). The bacterial inoculation of T4 increased average of shoot length in L1, L2 and L3 respectively, by 44, 86 and 64% compared with T1 (without *Bacillus* cells). However, in L0, the inoculation of T2~T4 did not differ from T1 in term of shoot length. As a result, shoot length of L2-T4 was similar to control (commercial bedsoil).

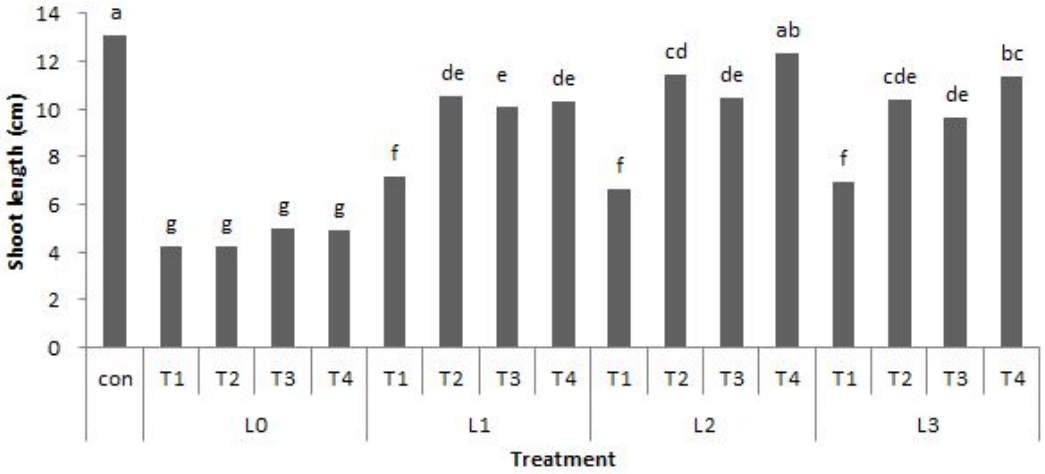

Fig. 39. Shoot length of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 40, root length was greatest in L0-T4 (0% of LM and 1×10^7 of *Bacillus* cells). Root length decreased significantly with the increases of amended ratio of LM on the contrary to shoot length. The density of the *Bacillus* had no direct effect on root growth. These results probably come from the EC content of bedsoils. The EC contents increased with the increases of amended ratio of LM. Thus, root length may restrain for reasons of that.

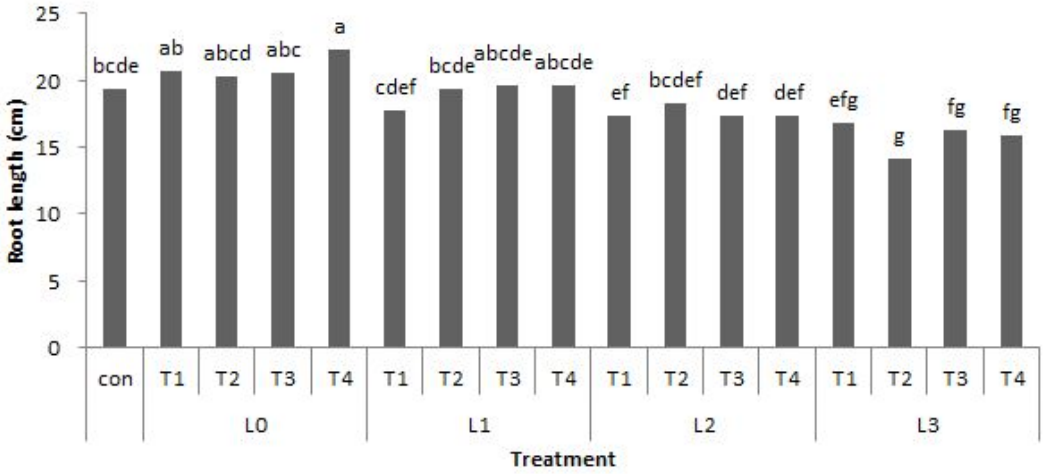

Fig. 40. Root length of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 41, shoot diameter was greatest in L3-T2 (3% of LM and 1×10^5 of *Bacillus* cells) and increased gradually with the increase of LM. In the T2, T3 and T4 supplemented *Bacillus* cells of L1, L2 and L3, shoot diameter increased significantly compared with T1, respectively. The density of the strain did not cast a long shadow to shoot diameter. The bacterial inoculation of T4 increased average of shoot diameter in L1, L2 and L3 respectively, by 21%, 26% and 21% compared with T1 (without *Bacillus* cells). However, in L0, the inoculation of T2-T4 did not differ from T1 in term of shoot diameter.

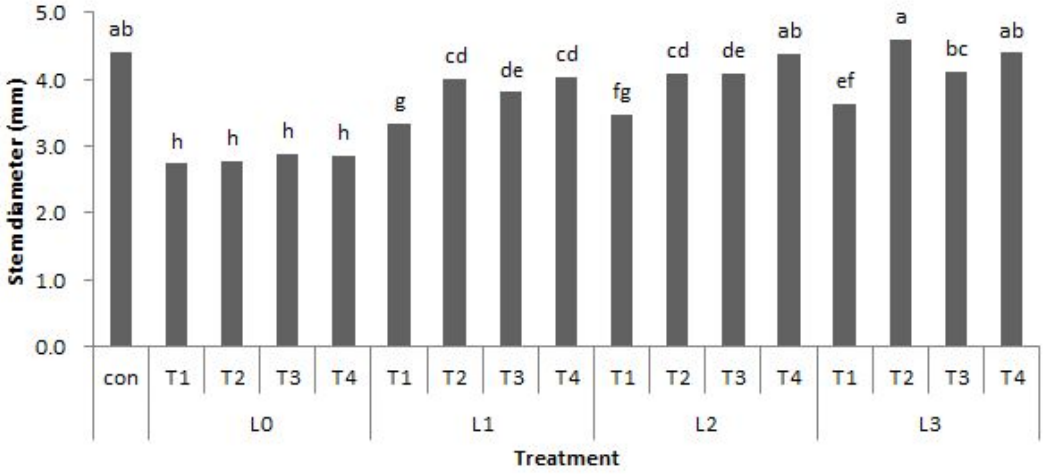

Fig. 41. Stem diameter of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 42, shoot fresh weight was greatest in control (commercial bedsoil) and L2-T4 (2% of LM and 1×10^7 of *Bacillus* cells) and showed a tendency to increase gradually with the increase of LM. In the T2, T3 and T4 supplemented *Bacillus* cells of L1, L2 and L3, shoot fresh weight increased significantly compared with T1, respectively. Shoot fresh weight was mostly greatest in T4 (1×10^7 of *Bacillus* cells). The bacterial inoculation of T4 increased average of shoot fresh weight in L1, L2 and L3 respectively, by 81%, 139% and 107% compared with T1 (without *Bacillus* cells). However, the inoculation of T2-T4 did not differ from T1 in term of shoot fresh weight in L0.

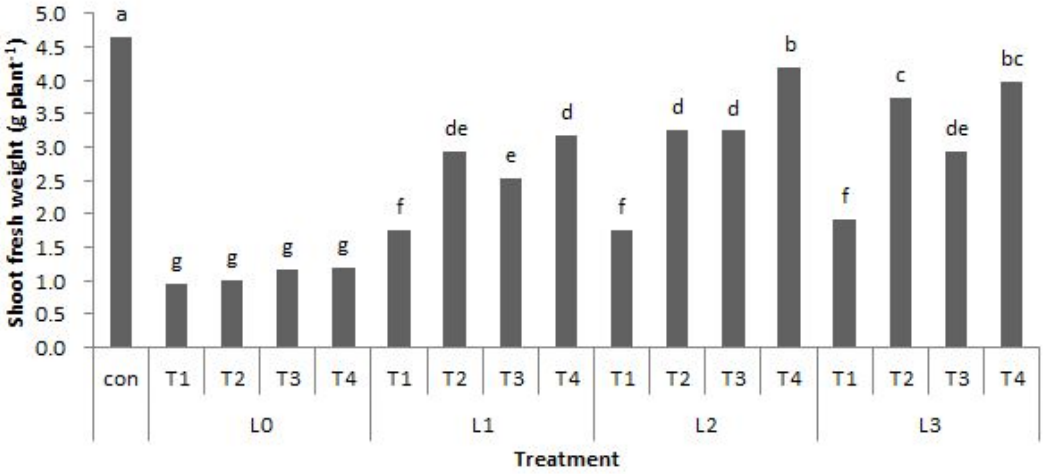

Fig. 42. Shoot fresh weight of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 43, root fresh weight was greatest in L2-T4 (2% of LM and 1×10^7 of *Bacillus* cells), L3-T2 (3% of LM and 1×10^5 of *Bacillus* cells), and L3-T4 (3% of LM and 1×10^7 of *Bacillus* cells) and showed a tendency to increase gradually with the increase of LM. This tendency was similar to shoot fresh weight and opposite to root length. In the T2, T3 and T4 supplemented *Bacillus* cells of L1, L2 and L3, root fresh weight increased significantly compared with T1, respectively. Root fresh weight was mostly greatest in T4 (1×10^7 of *Bacillus* cells). The bacterial inoculation of T4 increased average of root fresh weight in L1, L2 and L3 respectively, by 51%, 79% and 63% compared with T1 (without *Bacillus* cells). Also, in L0, the inoculation of T3-T4 were a little higher than T1 and T2 in term of root fresh weight.

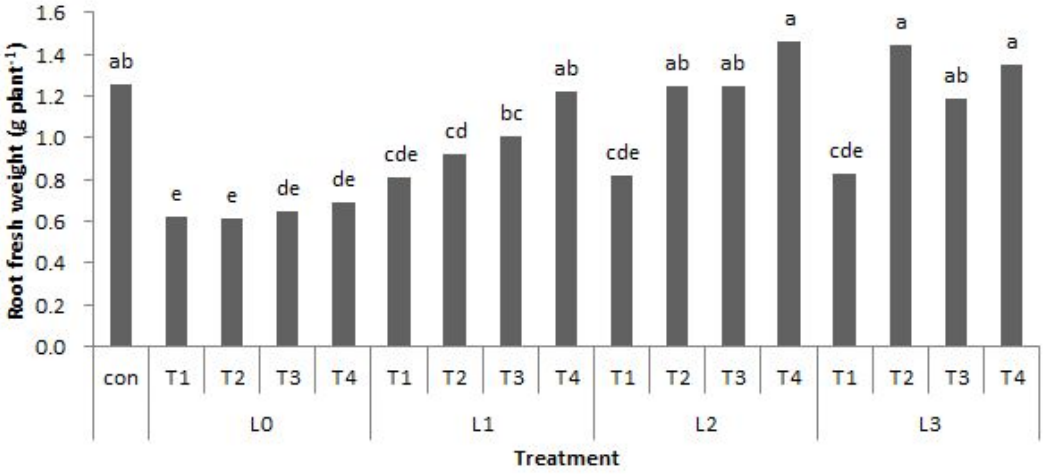

Fig. 43. Root fresh weight of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 44, shoot dry weight was greatest in control and L2-T4 (2% of LM and 1×10^7 of *Bacillus* cells) and showed a tendency to increase slightly with the increase of LM. This tendency was similar to shoot fresh weight. Shoot dry weight was mostly greatest in T4 (1×10^7 of *Bacillus* cells). The bacterial inoculation of T4 increased average of shoot dry weight in L1, L2 and L3 respectively, by 54%, 63% and 79% compared with T1 (without *Bacillus* cells). Also, in L0, the inoculation of T2-T4 were a little higher than T1 in term of shoot dry weight.

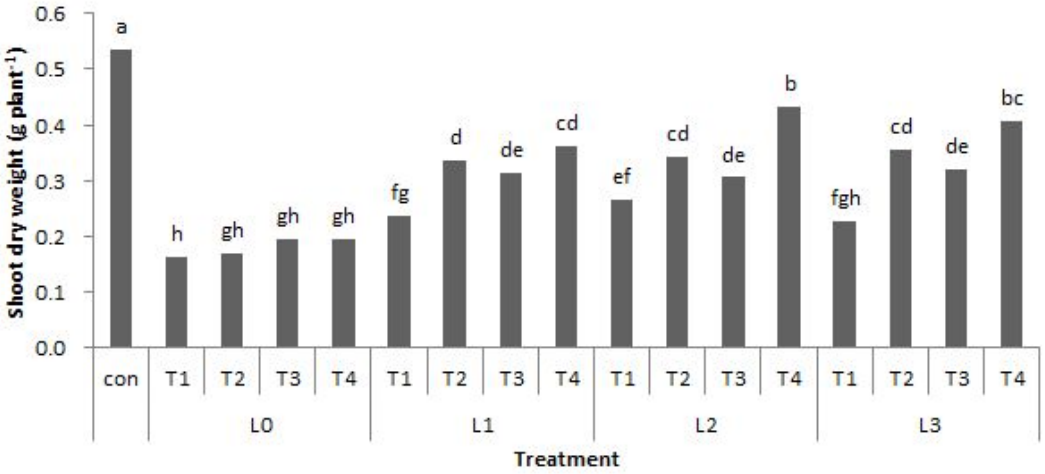

Fig. 44. Shoot dry weight of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 45, root dry weight was greatest in L2-T4 (2% of LM and 1×10^7 of *Bacillus* cells) and showed a tendency to increase gradually with the increase of LM. This tendency was similar to root fresh weight. In the T2, T3 and T4 supplemented *Bacillus* cells of L1, L2 and L3, root dry weight increased significantly compared with T1, respectively. Root dry weight was mostly greatest in T4 (1×10^7 of *Bacillus* cells), except in L3. The bacterial inoculation of T4 increased average of root dry weight in L1, L2 and L3 respectively, by 32%, 71% and 61% compared with T1 (without *Bacillus* cells). Also, in L0, the inoculation of T3-T4 were a little higher than T1 and T2 in term of root dry weight.

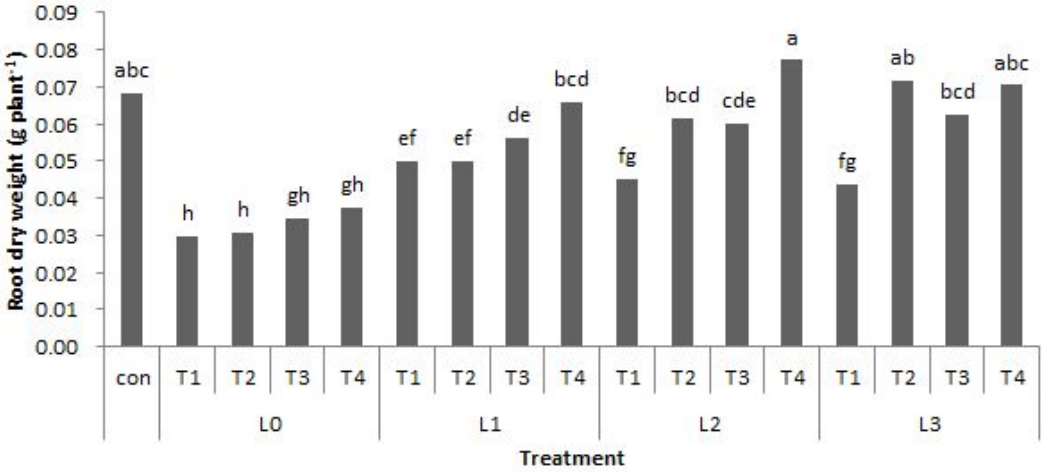

Fig. 45. Root dry weight of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 46, Leaf area was measured with a CI 202 portable digital area meter. Leaf area was greatest in control (commercial bedsoil) and L2-T4 (2% of LM and 1×10^7 of *Bacillus* cells) and showed a similar tendency with shoot fresh weight. The bacterial inoculation of T4 increased average of leaf area in L1, L2 and L3 respectively, by 70%, 108% and 89% compared with T1 (without *Bacillus* cells). However, in L0, the inoculation of T2-T4 did not differ significantly from T1 in term of leaf area.

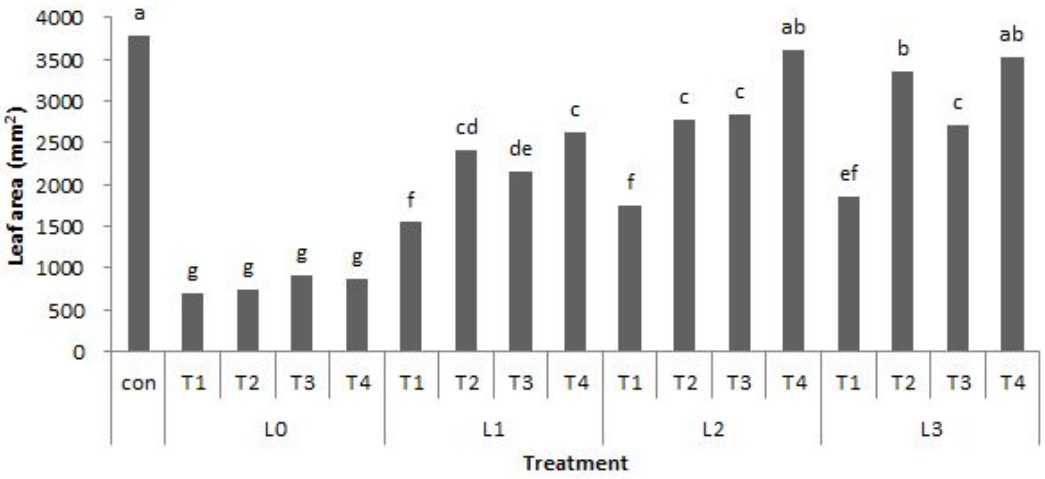

Fig. 46. Leaf area of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 47, chlorophyll content was measured with SPAD 502 chlorophyll meter (Minolta. Japan). Chlorophyll contents were greatest in L3 treatments (3% of LM) and increased significantly with the increase of LM. The bacterial inoculation didn't make much difference to the chlorophyll content.

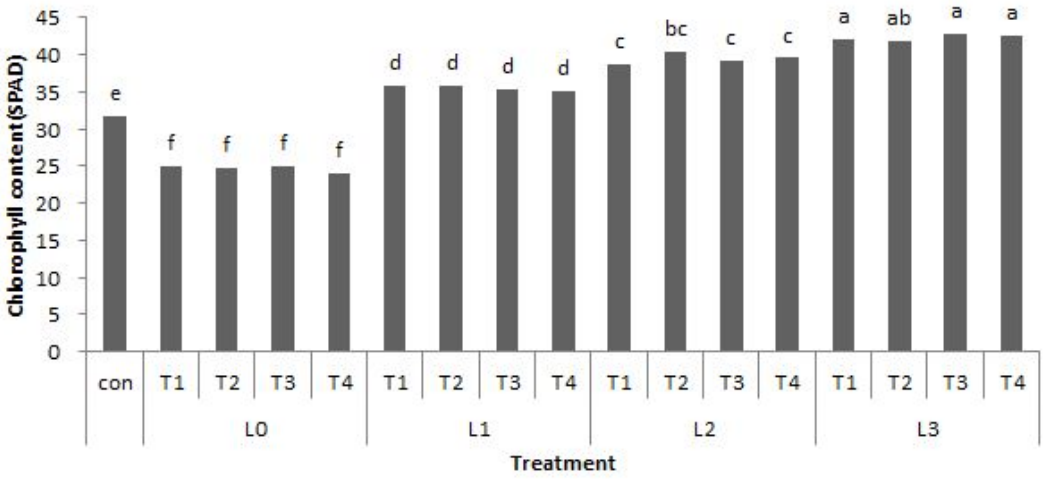

Fig. 47. Chlorophyll content of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

As shown in Fig. 48, S/R (Shoot/Root) ratio was measured by the dry weight basis of shoot and root of cucumber. S/R ratios were mostly lower in organic media supplemented with *Bacillus* cells and LM, whereas it was highest in control as 7.85 of S/R ratio compared with others. This result showed that the shoot growth of commercial bedsoil was better than developed organic bedsoil, whereas the root growth of organic bedsoil was better. Thus, the fidelity of seedling was higher in organic bedsoil, supplemented with *Bacillus* cells and LM.

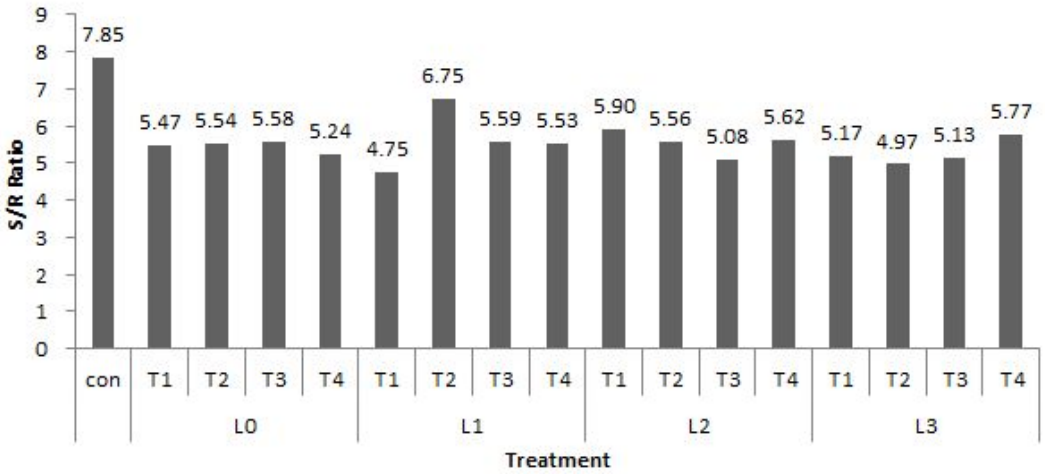

Fig. 48. S/R ratio of cucumber in organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure in cucumber seedlings for 30 days.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

2. Effect of *B. subtilis* SL9-9 on the chemical properties of bedsoils

Effect of inoculation of *B. subtilis* SL9-9 on chemical fertility of bedsoil was determined. As shown in Fig. 49, pH of bedsoil ranged from 5.9~6.6 and was relatively lower in L0 (0% of LM) and the bacterial inoculation didn't make much difference to the pH of bedsoils. EC (electronic conductivity) of bedsoils ranged from 0.12~0.47 (dS m^{-1}) and showed a tendency to increase with the increase of LM. The values of EC were mostly greatest in T4 (1×10^7 of *Bacillus* cells). The bacterial inoculation of T4 increased average of EC in L0, L1 and L2, respectively, by 15%, 16% and 22% compared with T1 (Fig. 50). As shown in Fig. 51, available phosphate of bedsoils ranged from 16~221 mg kg^{-1} and showed a tendency to increase with the increase of LM. Especially, the values of available phosphate were mostly greatest in T4 and the inoculation had an greater effect on the contents of available phosphate of bedsoils in the level of 1×10^7 of *Bacillus* cells. Perhaps, these results come from the phosphate-solubilizing activity of *B. subtilis* SL9-9. Ex-K of bedsoils ranged from 0.35~0.91 $cmol^+$ kg^{-1} and showed a tendency to increase with the increase of LM. The values of Ex-K were mostly greatest in T4 (1×10^7 of *Bacillus* cells). Thus, we supposed that the bacterial inoculation affected a little to the content of Ex-K (Fig. 52). But Ex-Ca of bedsoil ranged from 1.65 and 1.94 and Ex-Mg of bedsoil ranged from 1.21 and 1.31 and then there were no significantly differences in treatments (Fig. 53~54).

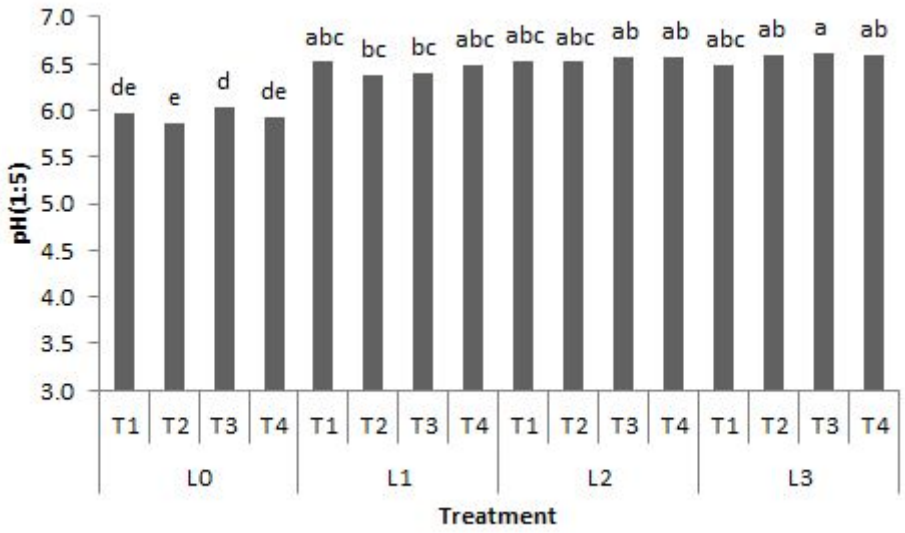

Fig. 49. pH of organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

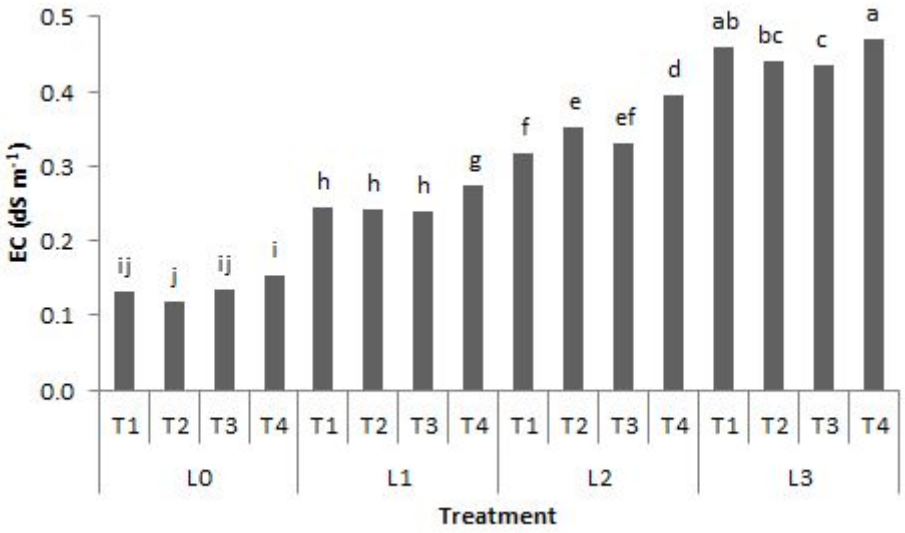

Fig. 50. Electronic conductivity (EC) of organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

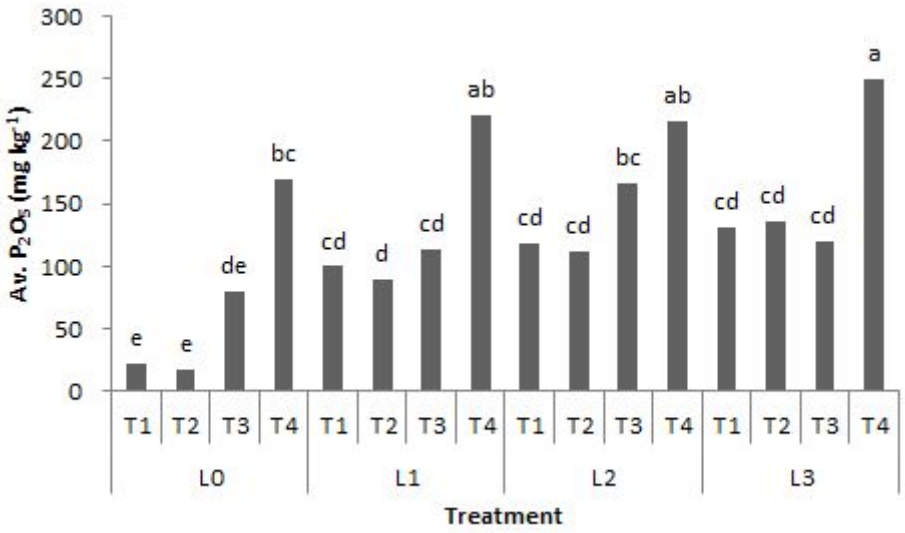

Fig. 51. Available phosphate of organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

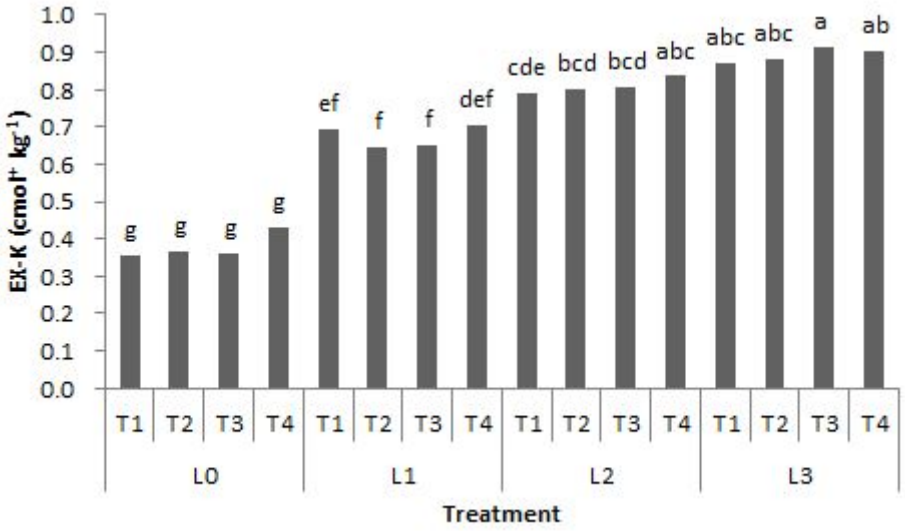

Fig. 52. Exchangeable potassium of organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

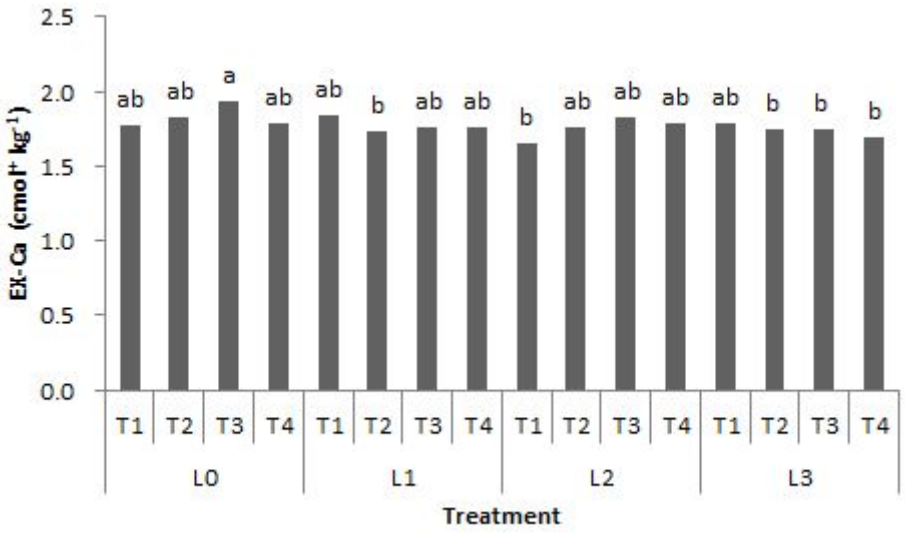

Fig. 53. Exchangeable calcium of organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

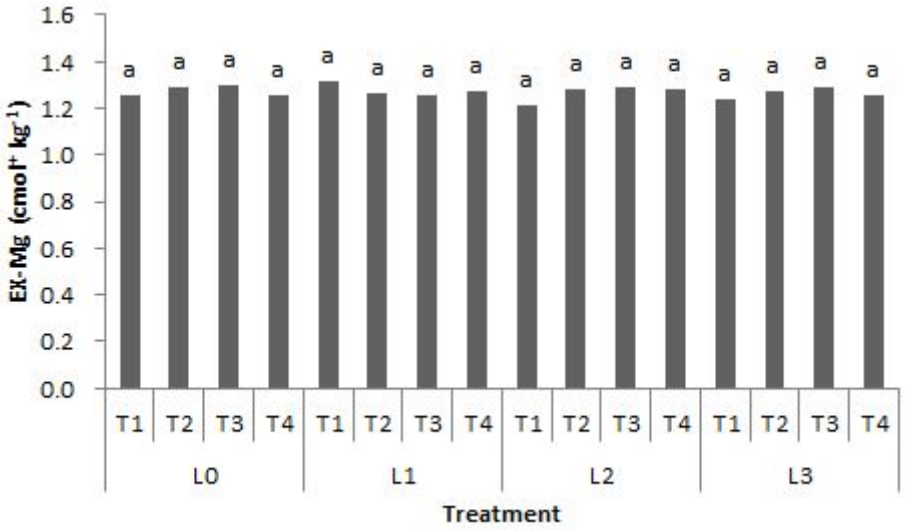

Fig. 54. Exchangeable magnesium of organic bedsoils with different ratios of *B. subtilis* SL9-9 and organic liquid manure.

Treatment, see Table 36; Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

3. Survival of *B. subtilis* SL9-9 during seedling growth

To determine the stability of the inoculated *B. subtilis* SL9-9 during cucumber seedlings, the change of population of the *Bacillus* cells was investigated for 33 days. The inoculation methods onto carrier were used by spray and absorption. Before planting, the density of *Bacillus* cells in bedsoils inoculated with spray and absorption were 1.85 and 2.84×10^{6} (cfu g^{-1}, dry weight), respectively. At the end of seedling, the density were 2.45 and 2.62×10^{6} (cfu g^{-1}, dry weight), respectively (Fig. 55). Thus, the amended *Bacillus* cells were stable in bedsoils during seedling. And so, we selected the spraying method to formulate bedsoils for an inoculation. Because It was easier to make formulations and more efficient.

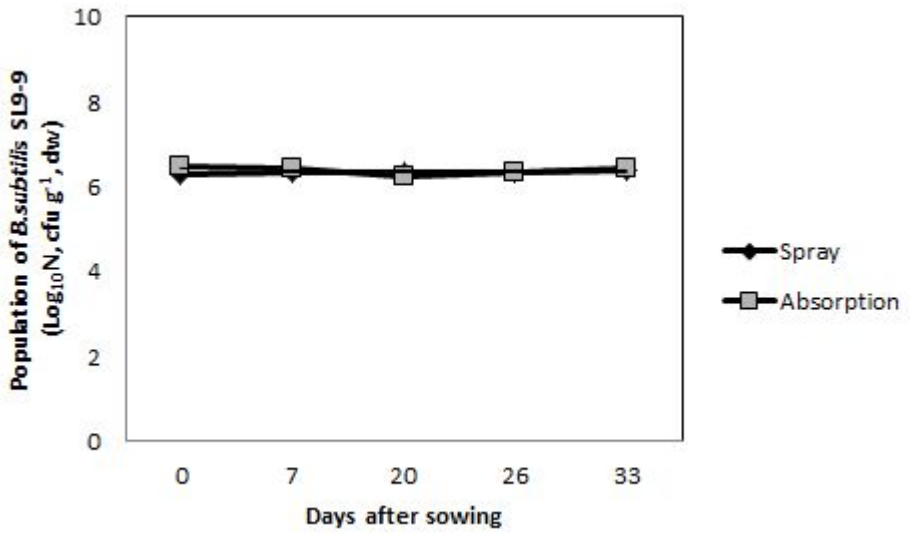

Fig. 55. Population of *B. subtilis* SL9-9 in bedsoils during cucumber seedling for 33 days.

4. Dehydrogenase activity of bedsoils

Dehydrogenases (DHA) are essential components of the enzyme systems of microorganisms. DHA activity can therefore be used as an indicator of biological redox systems and as measure of microbial activity in soil. Thus, dehydrogenase activity was determined to test the microbial activation of bedsoils in this study. In this experiment, *B. subtilis* SL9-9 was supplemented by 2% (v/v) to adjust the density of *Bacillus* cells in bedsoil to be 1×10^6 cfu g^{-1}(dw). DHA activities of control (commercial bedsoil), L1 (1% (v/v) of LM), L1-M (1% (v/v) of LM and 2% (v/v) of *B. subtilis* SL9-9), L2 (2% (v/v) of LM), L2-M (2% (v/v) of LM and 2% (v/v) of *B. subtilis* SL9-9) were 1.1, 6.4, 10.5, 56.4 and 79.8 (μg INTF g^{-1}), respectively. The activity was greatest in L2-M. The bacterial inoculation increased average of DHA in L1 and L2 by 64% and 41% compared with non-inoculation, respectively (Fig. 56). Also, the supplemented *Bacillus* cells were stable in L2-M (Fig. 57) and the cucumber growth of L2-M was greater than that of L2 (Fig. 58).

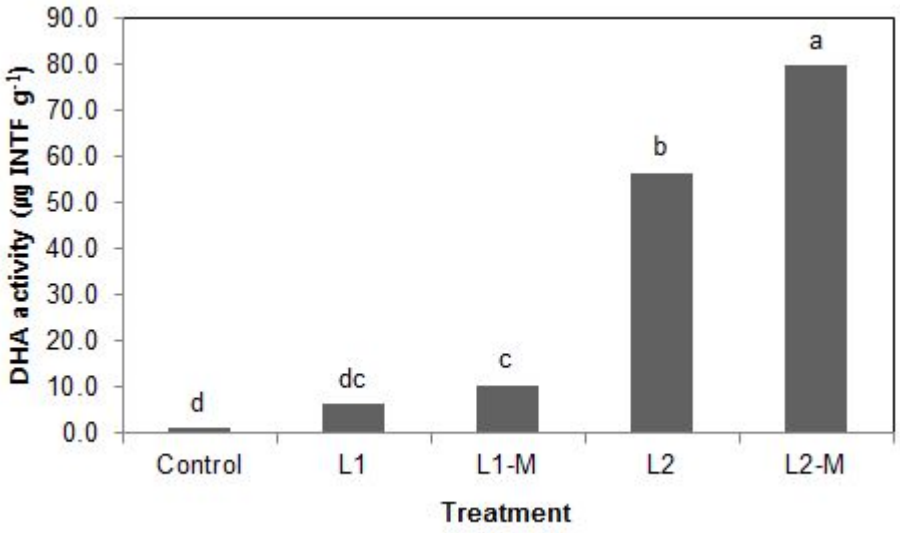

Fig. 56. Dehydrogenase (DHA) activity of bedsoils with *B. subtilis* SL9-9 and organic liquid manure.

CON, commercial bedsoil; Ll, 1% of LM; L1-M, 1% (v/v) of LM and 2% (v/v) of *B. subtilis* SL9-9; L2, 2% (v/v) of LM; L2-M, 2% (v/v) of LM and 2% (v/v) of *B. subtilis* SL9-9 : Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

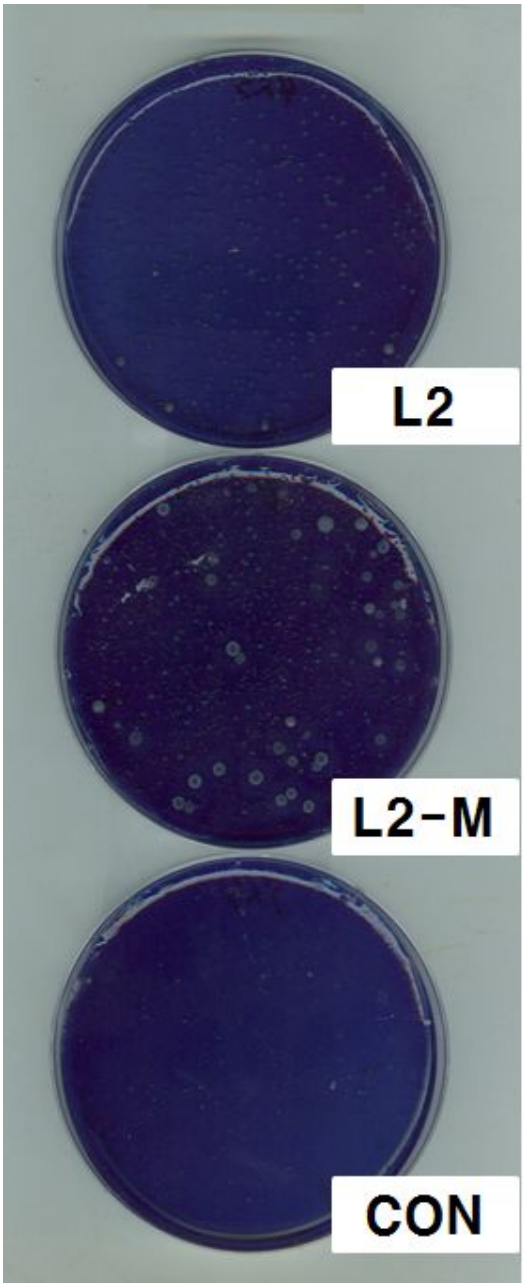

Fig. 57. Survival of *B. subtilis* SL9-9 after seedling for 33 days.

L2, bedsoil amended with organic liquid manure by 2% (v/v); L2-M, bedsoil amended with *B. subtilis* SL9-9 by 2% (v/v) and organic liquid manure by 2% (v/v); CON, commercial bedsoil.

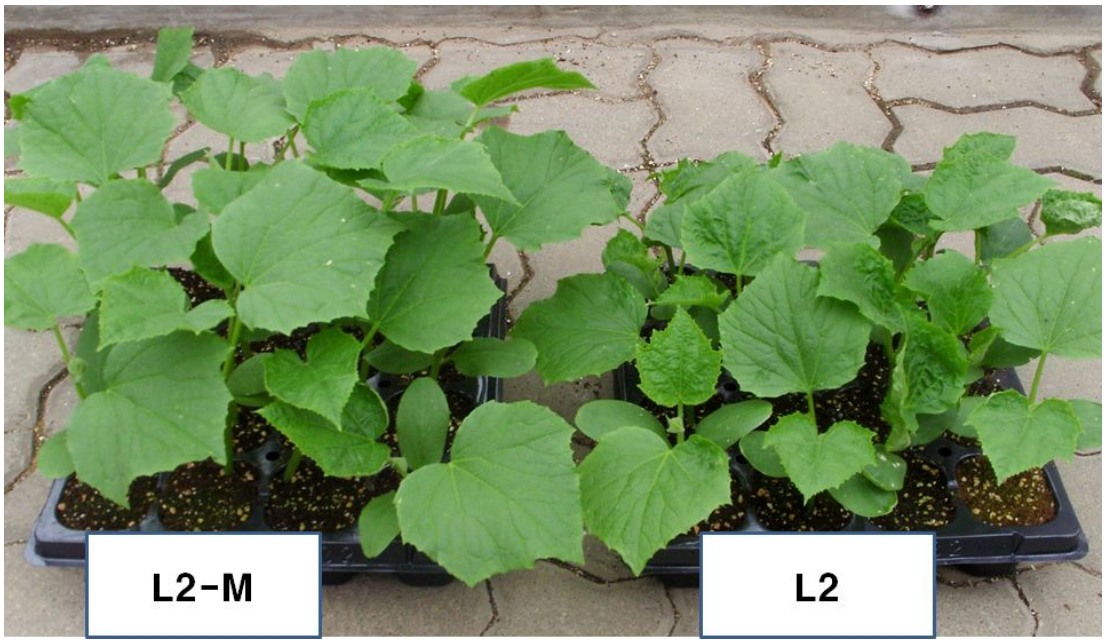

Fig. 58. Photo of cucumber seedling.

L2-M, bedsoil amended with *B. subtilis* SL9-9 by 2% (v/v) and organic liquid manure by 2% (v/v); L2, bedsoil amended with organic liquid manure by 2% (v/v).

5. Field trial

In field trial, there were no significantly difference in the yield of cucumber between commercial bedsoil (CB) and developed organic bedsoil (DBO, bedsoil with 2% (v/v) of *B. subtilis* SL9-9 and 1% (v/v) of LM). Instead, the yield of DOB was higher than that of CB. Therefore, *B. subtilis* SL9-9 and fish-fermented organic liquid manure can be used as bedsoil amendments of plant growth media for seedling in organic farming (Fig. 59).

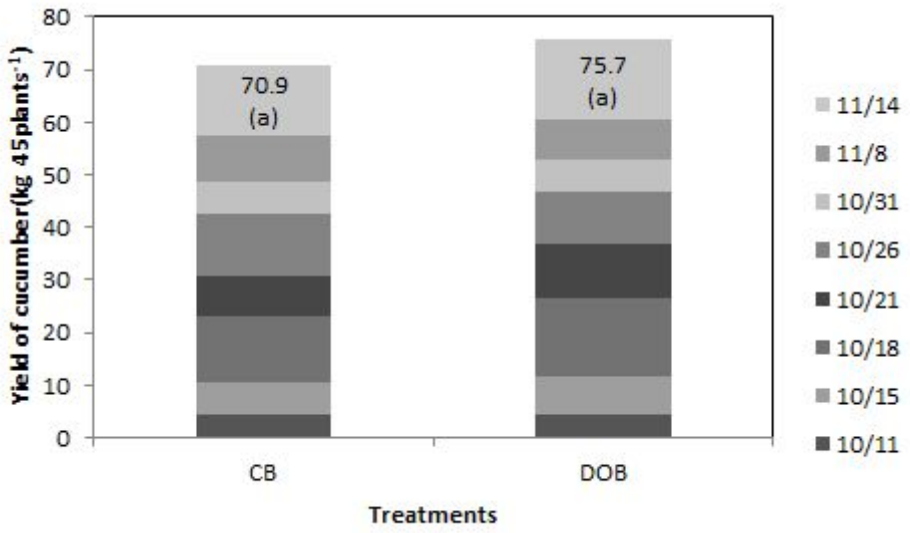

Fig. 59. Yield of cucumber in field trial.

CB, commercial bedsoil; DOB, developed organic bedsoil amended with 1% of LM and 2% of *B. subtilis* SL9-9 : Date of harvest (month/day), 10/11, 10/15, 10/18, 10/21, 10/26, 10/31, 11/8 and 11/14 : Data accompanied by the same letter do not significantly differ according to Duncan's multiple range test at $p<0.05$.

6. Plant growth-promoting activity of *B. subtilis* isolates

Nitrogen fixation, phosphate solubilization, ACC (1-aminocyclopropane-1-carboxylate) deaminase and antimicrobial activity were examined for determining the plant growth promoting activity of three *B. subtilis* isolates besides cellulase production. As shown in Table 37, all of them had various plant growth promoting activity as well as antimicrobial activity against various pathogens. Thus, we supposed that these activities had a good impact on the growth of plants (Fig. 60~63).

Table 37. Plant growth-promoting activities of *B. subtilis* isolates

Plant growth-promoting activity	*Bacillus* strains		
	B. subtilis SL9-9	*B. subtilis* C5-16	*B. subtilis* S52-2
Nitrogen fixation	+	+	+
Phosphate solubilization	+	+	+
ACC(1-aminocyclopropane-1-carboxylate) deaminase activity	+	+	+
Antimicrobial activity	+	+	+
Rhizoctonia solani	+	+	+
Phytophthora capsici	+	+	+
Fusarium oxyporum	+	+	+
Botrysis cinerea	+	+	+
Colletotrichum acutatum	+	+	+
Streptomycetes scabies	+	+	+
Streptomycetes acidiscabies	+	+	+
Streptomycetes turgidiscabies	+	+	+

Note : positive (+) or negative (-).

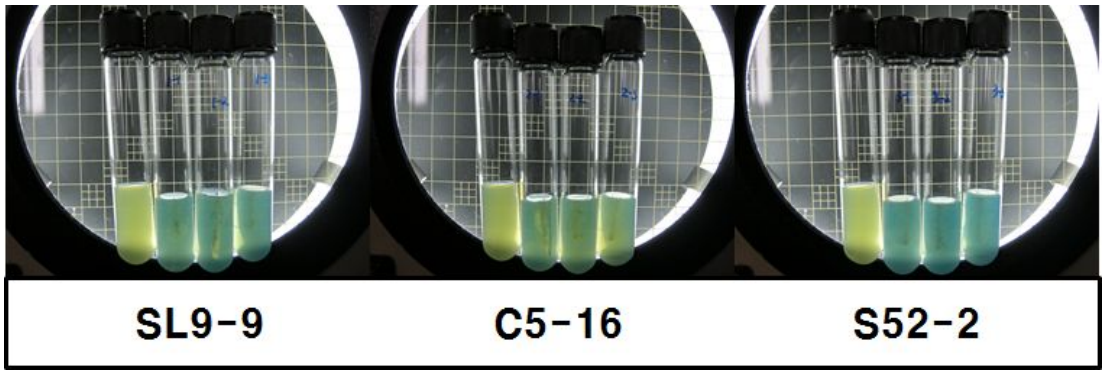

Fig. 60. Nitrogen fixation of isolated *B. subtilis* strains.

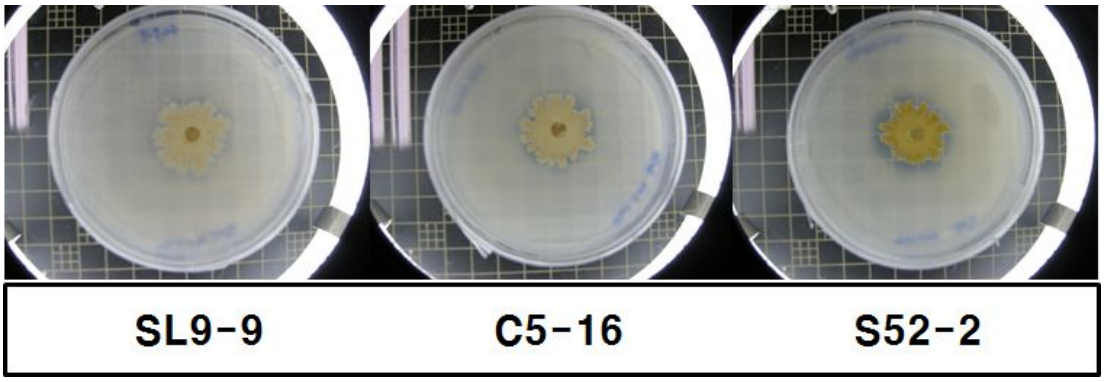

Fig. 61. Phosphate solubilization of isolated *B. subtilis* strains.

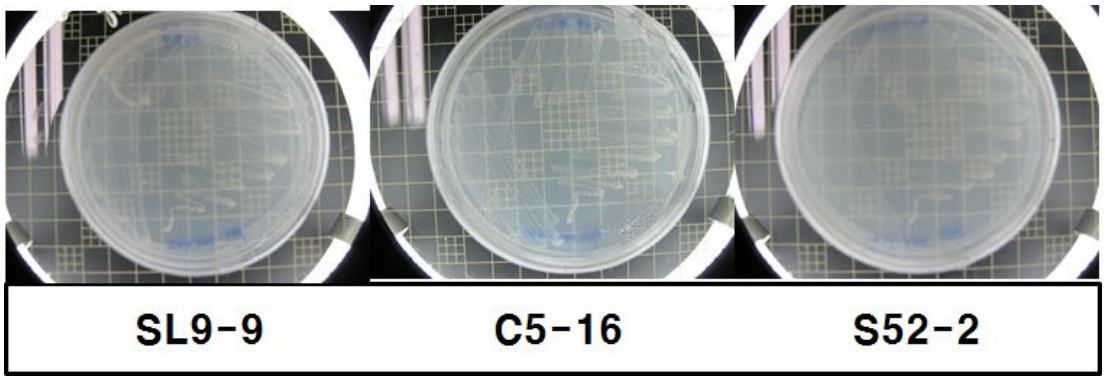

Fig. 62. ACC deaminase activity of isolated *B. subtilis* strains.

ACC, 1-aminocyclopropane-1-carboxylate.

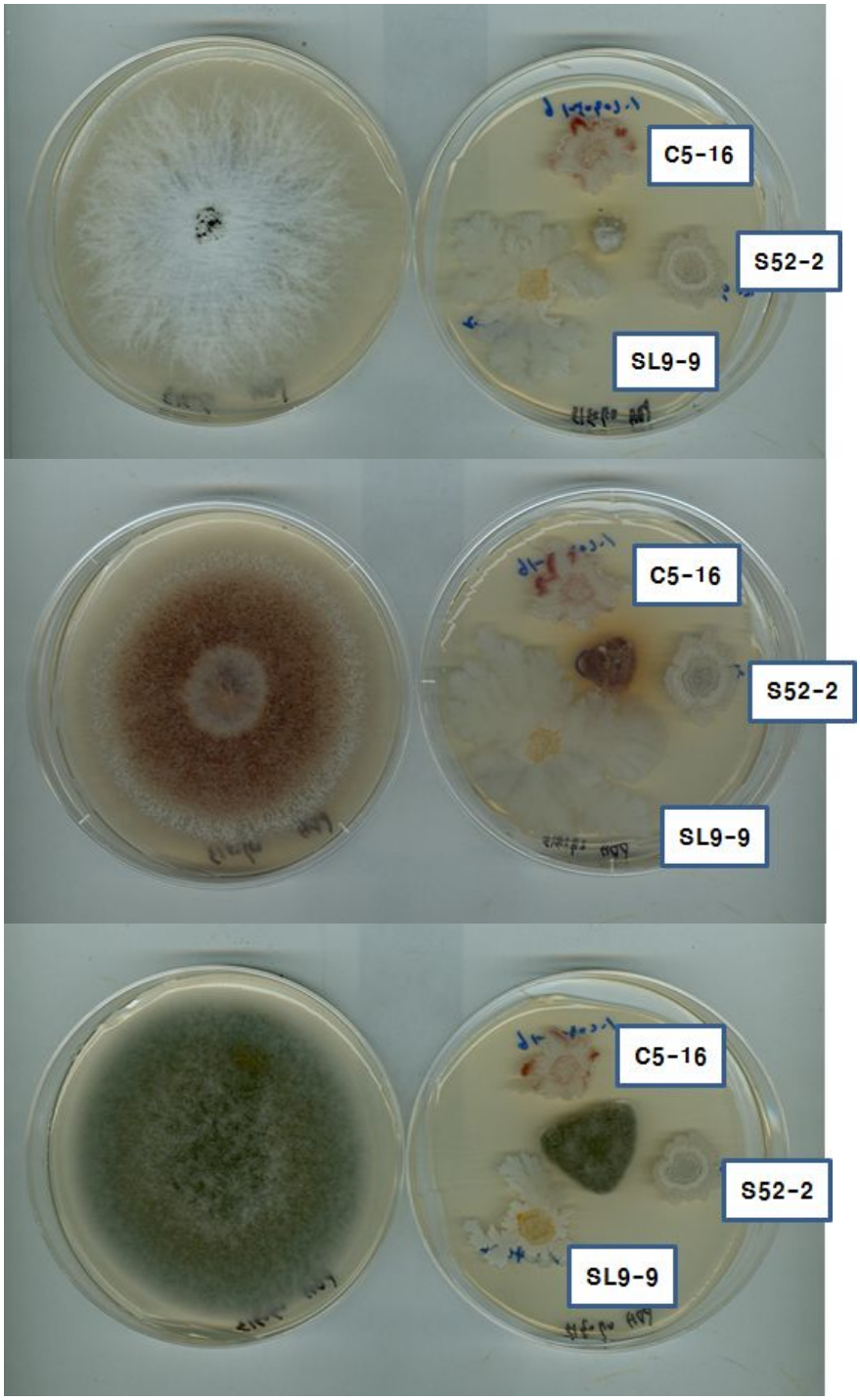

Fig. 63. Antimicrobial activity of isolated *B. subtilis* strains.

Ⅳ. Discussion

In this study, we investigated the effects of *B. subtilis* SL9-9 producing cellulase application to bedsoils incorporated with or without organic liquid manure (LM, fish fermented liquid manure) as a nutrient source (especially in N, P, and K) on bedsoil fertility and seedling growth of cucumber.

It was found that addition of *Bacillus* cells to LM-added bedsoils enhanced seedling growth and yield of cucumber under greenhouse and field conditions (Fig. 39~48). Supplementation of *Bacillus* cells increased significantly the shoot length (up to 86%), shoot diameter (up to 26%), shoot fresh weight (up to 139%), root fresh weight (up to 79%), shoot dry weight (up to 79%), root dry weight (up to 71%) and leaf area (up to 108%, respectively) compared with control (without inoculation of *Bacillus* cells). Comparison of our developed organic bedsoil (DOB) with other commercial bedsoils (CB) revealed that the root weight of cucumber was greater, which led to the production of high quality plug seedlings. In field trial, there were no significant difference in the yield of cucumber. Instead, the yield of cucumber grown in developed organic bedsoil was a little higher than that in commercial bedsoil Therefore, *B. subtilis* SL9-9 and fish-fermented liquid manure can be used as bedsoil amendments of plant growth media for seedling in organic farming.

Cellulase and nonpathogenic microbes, especially *Trichoderma spp.*, appear to be involved in diverse biological events that may be related to plant growth, including cell expansion, and seed germination and protection. However, their roles are not clearly understood, though they have been studied extensively (Bhat, 2000; Vinale *et al*., 2008). To achieve proper growth, plants need to weaken their cell walls using hydrolytic enzymes, such as CELs and pectinases (PTs); also, proteins are reported to play a vital role in cell wall extensibility. Expansins (EXPs) are small extracellular plant proteins involved in cell wall enlargement and in developmental processes that

require wall loosening (Cosgrove, 2005). Although EXPs themselves have no hydrolytic activities towards polysaccharides, the proteins are able to weaken the mechanical strength of cellulose (Li and Cosgrove, 2001). Therefore, the main action of EXPs is not considered as cell wall degradation but a disruption of noncovalent-bonding between cellulose microfibrils and matrix polymers, thereby leading to cell wall loosening. In addition, the CBMs of EXPs are also believed to collapse the crystalline structure of cellulose (Cosgrove, 2005).

Although Cosgrove and Durachko (1994) showed that application of CELs and PTs to the native cell wall of cucumber enhanced cell wall extensibility, several cell wall-degrading enzymes, including CELs, PTs, and xyloglucan endotransglycosylase/ hydrolase (XET/XGH), are, to date, reported to coexpress with EXPs (Payasi *et al.*, 2009), suggesting the occurrence of a series of controlled biochemical events acts upon expansive growth. The correlation between those enzymes and EXPs likely occurs during wall loosening and growth. For example, to achieve expansive growth, EXPs are assumed to loosen the tight association of glucan–xyloglycan chains in the cell wall by disrupting the hydrogen bonds between those two polymers, thus allowing accessibility for CELs to attack (Brummell and Harpster, 2001). PTs are believed to promote wall extension by removing pectins that limit accessibility of EXPs to cellulose–xyloglucan (Wei *et al.*, 2010), while XETs may be involved in xyloglucan modification in the cell wall hemicelluloses (Cosgrove, 2005). Additionally, some phytohormones, such as auxin, were reported to characteristically regulate coexpression of CELs, XETs, and EXPs during cell extension (Catalá *et al.*, 2000; Sharova, 2007).

It is notable that although loosening the cell walls is a key for plant growth, it may, in turn, make the plant prone to infection by virulent invaders. Some pathogenic bacteria produce auxin analogs in order to induce expression of EXPs to weaken plant cell walls (Navarro *et al.*, 2006), and may, simultaneously, secret extracellular enzymes, such as CELs and PTs, to degrade host tissues (Laine *et al.*, 2000). The loosening cell walls may be susceptible to hydrolytic action, thus

enhancing pathogenic colonizing ability and nutrient utilization (Balestrini *et al*., 2005; Laine *et al*., 2000). Ding *et al*. (2008b) reported that overexpression of the auxin-responsive gene GH3-8 increased disease resistance by reducing auxin accumulation. However, the phenotype of plant traits showed abnormal growth, owing to the suppression of EXPs via auxin signaling.

Hydrolytic activities are necessary for protective effects during seed germination. Cotes *et al*. (1996) studied the correlation between hydrolytic activities of *Trichoderma* colonized seeds and protection against pathogens. The pregerminated seeds dressed with *Trichoderma kiningii* TH-11 for 24 hr showed the highest degree of seed colonization and 100% protection level towards *Pythium splendens* with the increase of CEL, β-1,3-glucanase (B1,3 G) and chitinase (CHI) activities found in seed tissue. B1,3 G and CHI activities were suggested as defense mechanisms, since they degraded the cell walls of *P. splendens* based on the liberation of reducing sugars from the mycelium of *Pythium*, whereas CEL activity was believed to originate from the fungi, presumably necessary for colonizing the seeds (Cotes *et al*., 1996). The hypothesis on CEL-aided colonization was supported by the fact that the application of commercial CELs to pregerminated seeds increased the level of colonization of the inferior colonizing strain, *Trichoderma longibranchiatum* TH-13, with an improved protective effect (Cotes *et al*., 1996). Besides facilitating *Trichoderma* seed colonization, the seeds pretreated with CELs alone also exhibited increased protection. It is possible that CELs might have direct effects on *P. splendens*, perhaps by liberating some toxic chemicals from the seeds that inhibited germination of *P. splendens*, or by releasing some glucans that might act as plant endogenous elicitors (Cotes *et al*., 1996). According to the protective effects mentioned above, the application of *Trichoderma spp.* in seed priming, and at early stages of growth, also showed an enhanced rate of germination and development during the nursery period, thereby shortening required nursery time, which is important for economic reasons (Inbar *et al*., 1994; Moreno *et al*., 2009).

Also, we found that the supplementation of *Bacillus* cells/CELs in LM-amended

bedsoils mixed with peat and cocopeat increased significantly the values of EC, available phosphate and potassium (Fig. 50~52). Especially, the EC and K contents showed a tendency to increase as amended ratios of *Bacillus* cells and LM increases and available P was significantly higher in T4 treatments (amended with 1×10^7 density of *Bacillus* cells). Also, the dehydrogenase activity (DHA) of bedsoils was significantly higher in L2-M (bedsoil amended with 2% (v/v) of LM and *Bacillus* cells) and the bacterial inoculation increased average of DHA in L1 (1% (v/v) of LM) and L2 (2% (v/v) of LM) by 64% and 41% compared with non-inoculation, respectively (Fig. 57).

Han and He (2010a) studied the effects of CEL application to soils incorporated with or without straw on N and P release. It was found that addition of CELs to rice and wheat straw-amended soils increased the release of N and P content, whereas there were no significant differences in N and P concentrations in the control (straw-amended soil). Therefore, available soil nutrients are mainly dependent on the action of CELs that degrade cellulose in straw-amended soils. Soil microbiota help improve fertility and stability of soil, and their growth is likely associated with soil nutrients, particularly N. Henriksen and Breland (1999) found that when the concentrations of available N (organic N from straw and soil inorganic N) were below 1.2% of straw dry matter, it significantly reduced the rate of carbon (C) mineralization from straw residues and the growth of total soil microbial biomass, especially fungal growths. In addition, one possible explanation is that CELs degrade cellulose structures, thus releasing into the soil organic and inorganic N associated with cellulosic material. As a result, the sufficiency of available N supports fungi to synthesize lignocellulolytic enzymes to utilize growth substrates (Novotný *et al*., 2009). Unlike fungi, bacteria and actinomycetes likely require an easily mineralizable fraction of organic matters in soils (Gryndler *et al*., 2003; ŘezáČová *et al*., 2007).

Dehydrogenase activity was determined to test the microbial activation of bedsoils. A dehydrogenase (also called DHO in the literature) is an enzyme that oxidise a substrate by a reduction reaction that transfers one or more hydrides(H^-) to an

electron acceptor, usually $NAD^+/NADP^+$ or a flavin coenzyme such as FAD or FMN. Dehydrogenases, as respiratory chain enzymes, play the major role in the energy production of organisms. They oxidize organic compounds by transferring two hydrogen atoms. Dehydrogenases are essential components of the enzyme systems of microorganisms. Dehydrogenase activity can therefore be used as an indicator of biological redox systems and as measure of microbial activity in soil (Skujins, 1973). Haiyan *et al*. (2007) reported that in a long-term (16 years) field experiment, Organic manure had a significantly greater ($P<0.05$) impact on the biomass C and the activity, compared with mineral fertilizers. Microbial metabolic activity (dehydrogenase activity per microbial biomass C) was significantly higher ($P<0.05$) under balanced fertilization than under nutrient-deficiency fertilization. Phylogenetic analysis showed that the change of bacterial community in organic manure-fertilized soil might not be because of the direct influence of the bacteria in the compost, but because of the promoting effect of the compost on the growth of an indigenous Bacillus sp. in the soil. We emphasize the importance of balanced-fertilization, as well as the role of P, in maintaining soil organic matter, and promoting the biomass and activity of microorganisms.

Also, the three isolates, SL9-9, C5-16, and S52-2, had various plant growth promoting activity such as nitrogen fixation, phosphate solubilization, ACC (1-aminocyclopropane-1-carboxylate) deaminase as well as antimicrobial activity against various pathogens (Table 36). Especially, the 1-aminocyclopropane-1-carboxylate (ACC) deaminase (E.C.4.1.99.4) is a cytoplasmically localized enzyme produced by some soil bacteria to catalyze the degradation of ACC, a precursor of ethylene, as their source of nitrogen (Jacobson *et al*., 1994; Glick, 1995). The importance of ACC deaminase-producing bacteria for plant growth is to control ethylene biosynthesis in the plants. The dual function of ACC deaminase for plant growth, *i.e.* as plant growth promotion and defense against plant pathogens, puts this enzyme as one of the important traits among various beneficial characters of plant growth-promoting bacteria (Cattelan *et al*., 1999; Shaharoona *et al*., 2007).

Thus, it is obvious that *B. subtilis* isolates producing cellulase involved in plant growth promotion, as well as process developments for enzyme production. Demand for these enzymes is growing, particularly in agriculture. However, at the present time, the cost of enzymes is still relatively high compared to the agricultural products themselves. Therefore, research and development should pay attention to low-cost enzyme production with optimized processes. Utilization of CELs in the form of microbial cells may offer benefits for plants in terms of plant protection and biological controls. However, the positive effects may not be as predictable as chemical use (fungicide) and may result in complications after long-term cultivation. Our expectation is that the obtained knowledge will shorten the period of plantation, improve product yield and quality, reduce environmental damage, and improve the quality of life for farmers.

Ⅴ. Conclusion

The effects of exogenous cellulase/cellulolytic bacteria application to bedsoils incorporated with or without organic liquid manure as a nurient source (especially in N, P, and K) on bedsoil fertility and seedling growth of cucumber were determined. The inoculation of *Bacillus* cells had significantly greater impact on seedling growth of cucumber. Supplementation of *Bacillus* cells increased shoot dry weight (up to 79%), root dry weight (up to 71%) and leaf area (up to 108%), respectively compared to control (without inoculation of *Bacillus* cells). And, the supplementation of cellulolytic bacteria, *B. subtilis* SL9-9, promoted the bedsoil fertility and microbial activity. As a result, we supposed that these results came from mainly the acceleration in decomposition of organic matter *i.e*. organic liquid manure, peatmoss and cocopeat by *B. subtilis* SL9-9. Also. *B. subtilis* isolates had various plant growth promoting activities as well as antimicrobial activity against various pathogens. And thus, we supposed that these activities had a good impact on the growth of plants. Therefore, the cellulolytic bacteria application has the potential to be an environment-friendly approach to manage crops and soils.

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국 문 요 약

본 연구는 제주지역에 서식하고 있는 유용한 미생물자원 중에서 섬유소 분해효소 생산능이 우수한 균주를 개발하기 위하여 수행되었다. 섬유소는 식물 세포벽을 구성하고 있는 주요성분으로 자연계에 널리 분포하고 있는 중요한 자원이며, 섬유소의 분해는 지구상에서 탄소의 순환 등 물질순환에 매우 중요한 위치를 차지하고 있다. 그리고 섬유소를 분해하는 효소는 대부분 미생물에 위해 생산된다.

본 연구에서는 토양, 퇴비 그리고 가축분뇨 등에서 섬유소 분해효소를 생산하는 균주를 탐색하고 848 균주를 분리하였다. 그리고 각각의 분리원에서 섬유소 분해효소 생산능이 가장 우수한 균주인 SL9-9, C5-16, 그리고 S52-2 등 3종을 선발하였다. 최종 선발한 3종의 균주는 형태학적, 생리·생화학적 특성 그리고 16S rRNA를 분석한 결과 모두 *Bacillus subtilis*로 동정되었다.

그리고 선발한 *B. subtilis* SL9-9, C5-16 그리고 S52-2 균주의 섬유소 분해에 관여하는 여러가지 효소의 생산 특성을 조사하였다. 선발한 3종 모두 섬유소 분해에 관여하는 효소인 endoglucanase (carboxymethylcelluase, CMCase), exoglucanase (avicelase), 그리고 β-glucosidase 효소를 생산하였으며, 이중에서 endoglucanase 활성이 가장 높았다. SL9-9 및 S52-2 균주는 hemicellulose를 분해하는 효소인 xylanase 생산능도 우수하였다. 그리고 CMCase, avicelase 그리고 xylanase 효소의 활성은 배양 상등액에서 그리고 β-glucosidase 효소의 활성은 균체내에서 검출되어, CMCase, avicelase 그리고 xylanase는 세포외 효소, β-glucosidase는 세포내 효소임을 확인하였다. 또한 선발한 3종의 *Bacillus* 균주가 생산하는 CMCase의 효소학적 특성을 조사하였는데, *B. subtilis* SL9-9, S52-2 균주의 CMCase는 pH 5 및 온도 60℃에서 최적 활성을 보였으며, *B. subtilis* C5-16 균주의 CMCase는 pH 5 및 온도 50℃에서 최적의 활성을 보였다.

선발한 *Bacillus* 균주 3종의 섬유소 분해효소 생산능을 높이기 위한 배양조건을 선발하였다. 최적 배양조건은 탄소원, 1% CMC, 질소원, 0.4% soytone 및 0.4% yeast extract, 배지 pH, 5.0, 배양온도, 30~35℃, 회전속도, 150 rpm, 그리

고 공기압은 1vvm이었다. 그리고 섬유소 분해효소 대량생산 비용을 줄이기 위하여 여러 가지 농산부산물의 이용 가능성을 검토하였는데, 미강을 이용하였을때 CMCase 활성이 12~13% 증가하여 선발 *Bacillus* 균주의 대량배양을 위한 탄소원으로 선발하였다.

분리원이 다른 환경조건에서 선발한 3종의 *Bacillus* 균주는 섬유소 분해효소 활성에서도 균주간 차이를 보였는데, 이러한 차이를 유전학적으로 비교 검토하기 위하여 섬유소 분해효소 생합성 유전자를 분리하고 동정하였다. *B. subtilis* 균주 3종의 cellulase 생합성에 관여하는 유전자가 클로닝되었으며, 그들의 염기 및 아미노산 서열을 분석한 결과 *B. subtilis* endo-β-1,4-glucanase 유전자와 99%의 유사성을 보였다. 그리고 선발 균주간에는 93% ~ 96% 정도의 유사성을 보였다. 그리고 분리한 *B. subtilis* 균주의 cellulase 생합성 유전자의 아미노산 서열을 이미 보고되어 있는 다른 *B. subtilis* 균주의 유전자와 비교 분석하여 유전자의 특성을 파악하였는데, 분리한 *B. subtilis* 균주의 cellulase 생합성 유전자는 3개의 domain으로 구성되어 있었으며, catalytic domain (CD)은 glucosyl hydrorase family 5/A2 그룹으로, cellulose-binding domain (CBD)는 family IIIa로 분류되었고 그리고 domain을 연결하고 있는 linker 부분으로 구분되었다.

선발한 *Bacillus* 균주 중에서 다양한 효소활성을 보이며 pH에 안정하고 제제화가 용이한 *B. subtilis* SL9-9 균주 1종을 최종 선발하고 미생물비료로서 농업적 이용가능성을 검토하였다. *B. subtilis* SL9-9 균주가 첨가된 유기상토 개발을 위하여 화학비료 대신 영양원으로 유기성 액비를 첨가하여 시험을 수행하였는데, *B. subtilis* SL9-9 균주의 첨가로 지상부 및 지하부 건물중 그리고 엽면적이 무처리구 대비각각 79%, 71% 및 108% 증가하고 상토의 비옥도 또한 증가함을 확인하였다. 또한 선발한 *Bacillus* 균주는 섬유소 분해효소를 생산할 뿐만아니라 작물생육촉진과 관련된 질소고정, 인산가용화, 그리고 항균활성 등 다양한 농업적 기능성을 보유하고 있는 것으로 나타났다.

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우선 지난 10여년간 부족한 학문의 깊이를 채워주시고 바른 길을 갈 수 있도록 아낌없는 격려와 가르침을 주신 고영환 교수님께 깊은 감사를 드립니다. 바쁘신 중에도 미흡한 논문의 틀을 바로잡아 주시고 꼼꼼하게 내용을 검토하여 보완하여 주신 식품생명공학과 박은진 교수님과 김현정 교수님, 한국생명공학연구원 김창진 박사님 그리고 제주특별자치도농업기술원 이신찬 과장님께 감사드립니다. 그리고 석사과정부터 지도하여 주시면서 조언과 격려를 해주신 강영주 교수님, 하진환 교수님 그리고 임상빈 교수님께 감사를 드립니다.

본 학위 논문이 완성될 수 있도록 배려해주시고 항상 따뜻하게 격려해 주시는 제주특별자치도농업기술원 이상순 원장님과 강성근 국장님께 감사의 인사를 올립니다. 그리고 오한준 실장님, 홍순영 실장님 등 직장 동료 여러분께도 깊은 감사를 드립니다. 특히 실험실과 사무실에서 분석과 자료정리를 도와주신 조영윤님, 김순애님, 강순복님, 김창선님과 그동안 같이 근무하면서 도움을 주신 모든 분들께도 고맙다는 말씀을 전합니다. 또한 본 논문이 완성되기까지 큰 힘이 되어준 오현정 박사님과 식품생명공학과 선후배님들에게도 감사를 드립니다.

언제나 한결같은 마음으로 곁에서 든든하게 지켜주시는 아버지, 어머니 사랑합니다. 그리고 늘 가족들 걱정으로 한평생 살아오시면서 오늘도 당신 손자들 돌보는데 여념이 없는 시부모님 죄송하고 고맙습니다. 이 논문이 부모님 은혜에 조금이나마 보답할 수 있는 선물이 되었으면 합니다. 그리고 옆에서 조언과 격려를 해주는 언니들, 형부, 오빠, 동생, 조카들 그리고 시댁식구 모두에게도 고마움을 전합니다.

어느새 훌쩍 커서 오히려 엄마, 아빠를 염려해주고 응원해주는 승훈이와 정훈이 그리고 나의 반쪽 종욱씨 오랫동안 믿어주고 기다려줘서 너무 고맙고 사랑해요.

돌이켜보면 이 논문이 완성되기까지 수많은 분들의 도움이 없었다면 불가능했을 것입니다. 도움을 주신 분들에게 부끄럽지 않도록 좀 더 배려하고 베풀 수 있는 사람이 되도록 노력하겠습니다.