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for the Degree of Doctor of Philosophy

Characterization, Improvement and Utilization of *Bacillus licheniformis* 1,4-β-Endoglucanase from Korean Native Goat

한국재래산양으로부터 분리한 *Bacillus licheniformis* 1,4-β-Endoglucanase 의 특성규명, 역가 향상 및 이용에 관한 연구

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농 학 박 사 학 위 논 문

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SUMMARY

A series of experiments were conducted to isolate a proper *Bacillus* sp. from the rumen of Korean native goats and characterize its endoglucanase. In the subsequent experiment activity of the enzyme was improved by various DNA technologies and DFMs were prepared by solid-state-fermentation method to study effects of feeding DFM on rumen fermentation and feed digestion in Holstein steers.

Exp. 1. Characterization of Cellulolytic and Xylanolytic Enzymes of *Bacillus licheniformis* JK7 Isolated From the Rumen of a Native Korean Goat

A facultative bacterium producing cellulolytic and hemicellulolytic enzymes was isolated from the rumen of a native Korean goat. The bacterium was identified as a *Bacillus licheniformis* on the basis of biochemical and morphological characteristics and 16S rDNA sequences, and has been designated *Bacillus licheniformis* JK7. The optimum temperature for the enzymes of *Bacillus licheniformis* JK7 was 70℃ for endoglucanase (0.75Units/ml) and 50℃ for β-glucosidase and xylanase (0.63Units/ml, 0.44Units/ml, respectively). The optimal

pH for the three enzymes was 5.0, at which their activity was 1.46, 1.10, and 1.08 Units/ml, respectively. Endoglucanase activity was increased 113% by K^+, while K^+, Zn^+, and tween20 enhanced β-glucosidase activity. Xylanase showed considerable activity even in presence of selected chemical additives, with the exception of Mn^{2+} and Cu^{2+}.

Exp. 2. Genome Shuffling to Improve β-1, 4-Endoglucanase Activity of *Bacillus licheniformis* JK7

Three strains (GS2-18, GS3-8 and GS3-20) which had higher endoglucanase production than those of wild type strain(WT) have been selected after genome shuffling of *Bacillus licheniformis* JK7. Genome shuffled strains showed higher growth performance than those of WT strain after 4 hrs up to final incubation. Endoglucanase production increased rapidly from hr 6 up to hr 16 in all strains including, WT. The increase in enzyme production was associated with an increase in cell growth.

Exp. 3. *In situ* Degradation Characteristics of *Bacillus licheniformis* Based DFM

The objective of this study was to develop *Bacillus licheniformis* sp. -based DFM using solid-state fermentation (SSF), then to evaluate the difference between non-fermented meal (NFM) and fermented meal (FM) in ruminal disappearance in an *in situ* study. FM had higher CP, EE and ash contents but had lower DM, CF, NDF, ADF and lignin

compared to NFM. FM showed significantly higher DM, CP, NDF and ADF disappearance rate at 3, 6 and 24 hrs incubation. FM had microbial count (1×10^9 CFU/g) and it had cellulolytic and xylanolytic (9.90 units/g for endoglucanase; 1.67 units/g for β-glucosidase; 1.52 units/g for xylanase) enzymes, which were not found in NFM.

Exp. 4. Effects of *Bacillus licheniformis* -Based DFM on Rumen Fermentation and Microbial Population under *in vitro* Condition

The objective of this experiment was to investigate the effects of *Bacillus* sp.-based DFM on rumen fermentation and rumen microbial population in the *in vitro* experiment.

1. *In vitro* experiment 1

Solid-state-frmentation significantly increased *in vitro* DM digestibility of ($p<0.05$). There were no significant differences between NFM and FM in total VFA and butyrate at all time points except 3 hrs incubation ($p>0.05$). In contrast, NFM had significantly higher portion of acetate (0, 3hrs incubation and mean value) as well as propionate (12, 24hrs incubation and mean value) than FM ($p<0.05$). FM reduced the number of. *flavefaciens*, *R. amylophilus* and *S. bovis* in at 3, 6 and 12hr ($p<0.05$), which might be due to some antimicrobial substances produced by *Bacillus licheniformis* sp during solid-state-fermentation.

2. *In vitro* experiment 2

Second *in vitro* study was conducted to study effects of DFM on in vitro fermentation pattern and substrate digestion. All treatments had timothy as a main substrate. NFM (10%, DM basis) (Con) , 5% of FM (T5) or 10% of FM (T10) was added to incubation medium. The addition of FM resulted in higher DM digestibility than those of Con ($p<0.05$), indicating that FM gave beneficial effect on fiber digestibility. T5 or T10 showed significantly higher production of total, acetate, propionate, isobutyrate, butyrate, isovalerate and n-valerate than Con ($p<0.05$). Log copy numbers of fibrolytic (*R. albus, R. flavefaciens, E. ruminantium*), starch using bacteria (*R. amylophilus, S. bovis*) and proteolytic bacteria (*P. ruminicola*) increased significantly by addition of FM ($p<0.05$).

Exp. 5. **Effects of Supplementation of *Bacillus licheniformi*s Based DFM on Rumen Fermentation and Microbial Population in Holstein Steers**

An *in vivo* study was conducted to confirm the beneficial effects of *Bacillus*-based DFM in steers. Total tract digestibility of DM, CP, EE, NDF and ADF were investigated but there was no significant difference between treatments ($p>0.05$). Ruminal pH variation, NH_3-N concentration and microbial N also showed no difference between treatments ($p>0.05$). Total VFA and acetate concentrations were not different significantly between treatments at all incubation time points. Only *R. flavefaciens* at 6hrs and *B. licheniformis* at 0, 6, 12hrs were

significantly different ($p<0.05$). The significant difference of *B. licheniformis* population was also detected in fecal samples. The endoglucanase, β-glucosidase and xylanase activity in the rumen also analyzed but concentration of all enzymes was not influenced by treatments.

Key words: *Bacillus licheniformis*, DFM, cellulase, xylanase, genome shuffling, rumen microbial population

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LIST OF ABBREVIATIONS

AA: Amino acid

ADF: Acid detergent fiber

ADG: Average daily gain

BHB: β-hydroxybutyrate

BW: Body weight

CFU: Colony-forming unit

CMC: Carboxy methyl cellulose

CP: Crude protein

CTAB: Cetyltrimethyl Ammonium Bromide

DFM: Direct-fed microbials

DM: Dry matter

DMI: Dry matter intake

DNA: Deoxyribose nucleic acid

DNS: 3, 5-dinitrosalicylic acid

ED: Effective degradability

EE: Ether extract

EMS: Ethyl methane sulfonate

ETBR: Ethidium bromide

FCM: Fat-corrected milk

FM: Fermented meal

GIT: Gastro intestinal tract

GH5: Glycosil hydrolase family 5

GS: Genome shuffled

LAB: Lactic acid producing bacteria

LB: Luria-Bertani

LUB: Lactic acid utilizing bacteria

MMS: Methyl methane sulfonate

MPS: Microbial protein synthesis

NCBI: National center for biotechnology information

NDF: Neutral detergent fiber

NEFA: Non esterified fatty acid

NFM: Non fermented meal

NTG: N-Methyl-N'-nitro-N-Nitroguanidine

OD: Optical density

OM: Organic matter

PAB: Penassay broth

PBMC: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

PD: Purine derivatives

PEG: Poly ethylene glycol

SBM: Soy bean meal

SMF: Submerged fermentation

SMM: Sucrose, maleic acid and $MgCl_2$

SSF: Solid-state fermentation

RAPD: Randomly amplified polymorphic DNA

rDNA: Ribosomal DNA

RM: Regeneration media

TMR: Total mixed ration

UV: Ultraviolet

VFA: Volitile fatty acid

WB: Wheat bran

WT: Wild type

UNITS AND MARKS

℃: Degree(s) Celcius

g: Gram(s)

hr: Hour(s)

µl: Microliter(s)

mg: Milligram(s)

ml: Milliliter(s)

mM: Millimol per liter

min: Minute(s)

nm: Nanometer(s)

%: Percent

pH: Potential of Hydrogen

vol: Volume

wt: Weight

INTRODUCTION

Improvements of feed utilization, animal production and health, and animal food safety are the goals of rumen microbial studies. These goals can be achieved by facilitating desirable rumen fermentation, minimizing ruminal disorders, and excluding pathogens. The use of antibiotics like monensin, plant extracts, essential oils, rumen protected nutrients, prebiotics and Direct-Fed Microbials (DFM) were some of classical methods to modify rumen microbial ecosystem and to improve nutrient digestion and utilization by animals as well as hind-gut health. Among them, DFM have received much interests from researchers since the use of DFM might give beneficial effects on rumen (modification of fermentation, the improvement of fiber digestion and antimicrobial effects) and hind-gut (pathogen exclusion by competition, stimulation of immune response) (McAllister et al., 2011).

In an effort to develop DFM that can produce high cellulolytic activities, *Bacillus licheniformis* JK7 was isolated from Korea native goat's rumen **(Experiment 1)** as a candidate DFM strain because this strain is known as spore forming bacteria which can survive in harsh

conditions such as acidic stomach and high bile acid concentration in hind-gut. Then this strain was undergone reculsive protoplast fusion, called genome shuffling to enhance endoglucanase production potential of this microorganism **(Experiment 2)**

After developing strain with improved endoglucanase production, DFM was prepared by using solid state fermentation technique and the characteristics of developed DFM were studied in the *in situ* trial. The enzyme production potential and cell counts of DFM were also investigated **(Experiment 3)**. Finally, the efficacy of DFM was assessed in the *in vitro* **(Experiment 4)** and *in vivo* experiment **(Experiment 5)** to investigate effects of DFM on rumen fermentation characteristics, microbial population in the rumen or intestine, and feed digestion.

LITERATURE REVIEW

Microorganisms used in DFM products

Microorganisms used in DFM for ruminants include species of *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus, Bacillus* and *Propionibacterium,* all of which are commonly used as probiotics for human and mono-gastric animals or as inoculates for dairy product processing (Table 1). Other distinctive bacterial species such as *Megasphaera elsdenii* and *Prevotella bryantii* have also been used as DFM to stabilize or improve rumen function. These bacterial DFM strains may be classified as lactic acid producing, lactic acid utilizing, or other microorganisms. In ruminant animals, the rumen is the first organ that DFM reach upon ingestion. DFM grow in the rumen and beneficially modify its microbial ecosystem and/or fermentation characteristics. The intestinal tract may also provide a habitat for DFM. Lactic acid production and utilization in the rumen is closely related to feed efficiency and animal health. Although bacterial DFM are emphasized, fungal DFM are also commonly added to ruminant diets

(Kung Jr, 2001). Most commercial yeast products contain species of *Saccharomyces* and *Aspergillus*.

Table 1. Microorganisms used as DFM for ruminants

Genus	Species
Lactic acid producing bacteria	
Lactobacillus	*Lactobacillus acidophilus*
	Lactobacillus plantarum
	Lactobacillus casei
	Lactobacillus gallinarum
	Lactobacillus salivarius
	Lactobacillus reuteri
	Lactobacillus bulgaricus
Bifidobacterium	*Bifidobacterium pseudolongum*
	Bifidobacterium thermophilium
	Bifidobacterium longum
	Bifidobacterium lactis
Streptococcus	*Streptococcus bovis*
	Streptococcus faecium
Enterococcus	*Enterococcus faecium*
	Enterococcus faecalis
Lactic acid utilizing bacteria	
Megasphaera	*Megasphaera elsdenii*
Propionibacterium	*Propionibacterium shermanii*
	Propionibacterium freudenreichii
	Propionibacterium acidipropionici
	Propionibacterium jensenii
Other bacteria	
Prevotella	*Prevotella bryantii*
Bacillus	*Bacillus subtilis*
	Bacillus licheniformis
	Bacillus coagulans
Yeast	
Saccharomyces	*Saccharomyces cerevisiae*
	Saccharomyces boulardii
Fungi	
Aspergillus	*Aspergillus oryzae*
	Aspergillus niger

Modes of DFM action

1) Ruminal effects

Bacterial DFM intended to have beneficial effects on the post-ruminal gastrointestinal tract, but certain bacterial DFM were recently found to play a beneficial role in the rumen itself. The modes of action of different DFM sources in the rumen are summarized in Table 2. Lactic acid producing bacteria (LAB) have been proposed to have beneficial effects in the intestinal tract. However, some researchers have suggested that LAB might also have positive effects in the rumen. LAB such as lactobacilli and *Enterococci* might prevent ruminal acidosis in dairy cows (Nocek *et al*., 2002) by facilitating the growth of ruminal microorganisms adapted to the presence of lactic acid in the rumen (Yoon and Stern, 1995) and by stimulating lactic acid utilizing bacteria (LUB).

LUB have also been proposed as DFM and have been used successfully to decrease concentrations of lactate and maintain ruminal pH. *Megasphaera elsdenii* may utilize lactate and prevent drastic pH drops caused by accumulation of lactate in the rumen when fed a highly fermentable diet (Kung and Hession, 1995), and the supplementation of *M. elsdenii* was proposed as a means of preventing acute acidosis in transition animals.

Table 2. Modes of action of DFM in the rumen

Proposed mechanisms
Lactic acid producing bacteria
1. Provision of a constant lactic acid supply
2. Adaptation of overall microflora to the lactic acid accumulation
3. Stimulation of lactate utilizing bacteria
4. Stabilization of ruminal pH
Lactic acid utilizing bacteria
1. Conversion of lactate to VFA (e.g., *Megasphaera elsdenii*)
2. Production of propionic acid rather than lactic acid (e.g., *Propionibacterium spp.*)
3. Increase of feed efficiency
4. Decrease of methane production
5. Increase of ruminal pH
Fungal DFM
1. Reduction of oxygen in the rumen
2. Prevention of excess lactic acid in the rumen
3. Provision of growth factors such as organic acid and vitamin B
4. Increase of rumen microbial activity and numbers
5. Improvement of ruminal end products (e.g., VFA, rumen microbial protein)
6. Increase of ruminal digestibility

Propionibacteria ferments lactate to propionate, and propionate is the major precursor for gluconeogenesis in early lactation dairy cows (Reynolds *et al.*, 2003). Increments of propionate production in the rumen result in increases of hepatic glucose production (Stein et al., 2006), providing more substrates for lactose synthesis, improving energetic efficiency and reducing ketosis (Weiss et al., 2008). In addition, increased propionate may reduce hydrogen available for methane production in the rumen (Stein et al., 2006). Certain species of

propionibacteria were reported to modify rumen fermentation and increase the molar portion of ruminal propionate (Stein et al., 2006).

Fungal DFM have been extensively used in ruminants for improving performance and normalizing rumen fermentation. Increases in bacterial numbers recovered from the rumen are the most reproducible effects of dietary yeast supplementation. Rose (1987) suggested that yeasts removed oxygen in the rumen. Yeast cells in the rumen use available oxygen on the surfaces of freshly ingested feed to maintain metabolic activity. Jouany *et al*. (1999) observed a significant decrease in redox potential, up to -20mV, in the rumen with yeast supplementation. This change created better conditions for the growth of strict anaerobic cellulolytic bacteria, stimulated their attachment to forage particles (Roger *et al*, 1990), and increased the initial rate of cellulolysis. In addition, *S. cerevisiae* was able to compete with other starch utilizing bacteria for fermentation of starch (Lynch and Martin, 2002) leading to the prevention of lactate accumulation in the rumen (Chaucheyras *et al*., 1995). Chaucheyras *et al.* (1995) also reported that *S. cerevisiae* had the ability to provide growth factors, such as organic acids or vitamins, thereby stimulating ruminal populations of cellulolytic bacteria and LUB.

2) Post-ruminal GIT effects

As noted, previous inquiries regarding feeding bacterial DFM to ruminant animals focused on its potential beneficial effects on the post ruminal gastro intestinal tract (GIT). Some suggested mechanisms are summarized in Table 3. Proposed roles of beneficial DFM are to:

a. attach to the intestinal mucosa and prevents potential pathogen establishment
b. maintain lower pH in the GIT thereby inhibiting growth of pathogens
c. produce antibacterial compounds such as bacteriocins and hydrogen peroxide
d. modulate immune cells and stimulate immune function
e. modulate microbial balance in the GIT
f. prevent illness caused by intestinal pathogens or stress

Enterotoxin-producing strains of *E. coli* attach to intestinal epithelial cells and mucus to induce diarrhea (Jones and Rutter, 1972). Lee *et al.* (2003) discovered that *L. rhamonsus* GG could attach to epithelial cells via hydrophobic interactions and limited pathogens from attaching to the enterocytic receptor. Steric hindrance displaces pathogens, which eventually detach from the enterocytic receptor. In addition, *L. rhamonsus* (Lcr35) decreases adhesion of enteropathogenic and

enterotoxigenic *E. coli* and *Klebsiella pneumonia* (Forestier *et al*., 2001). In other experiments, LAB was able to adhere to the intestinal tracts of mice, protecting animals against *Salmonella Dublin* DSPV 595T (Frizzo et al., 2010). LAB produces lactate and acetate as main metabolic end products. These acids have critical roles in penetrating microbial cells and interfering with essential cell function to reduce intracellular pH (Holzapfel *et al*., 1995).

Table 3. Modes of action of DFM in the post-rumen GIT

Proposed mechanisms
1. Production of antibacterial compounds (acids, bacteriocins, antibiotics)
2. Competition with pathogens for colonization of mucosa and/or for nutrients
3. Production and/or stimulation of enzymes
4. Stimulation of immune response by host
5. Metabolism and detoxification of desirable compounds

Hydrogen peroxide and several bacteriocins produced by LAB are also important compounds due to their competitive exclusion and probiotic characteristics. Hydrogen peroxide can oxidize, on the bacterial cell, sulfhydryl groups of cell proteins and membrane lipids (Dicks & Botes, 2010), thereby blocking glycolysis due to the oxidation of sulfhydryl groups in metabolic enzymes such as glucose transport enzymes, hexokinase, and glycerol aldehyde-3-phosphate dehydrogenase (Carlsson *et al*., 1983). Holzapfel *et al*. (1995) suggested that LAB produced hydrogen peroxide, which effectively inhibited *S. aureus* and *Pseudomonas* spp.

LAB bacteriocins were well documented by Cotter *et al*. (2005). Reuterin, produced by *L. reuteri* when grown anaerobically in the presence of glucose and glycerol (Dicks & Botes, 2010), inhibited the binding of substrates to the subunit of ribonucleotide reductase so that interfering with DNA-synthesis of target microorganisms (Dobrogosz *et al*., 1989). *Lactobacillus* GG, isolated from humans, was able to produce unidentified antimicrobial compounds that limited the growth of *Staphylococcus* spp., *Streptococcus* spp., and *Pseudomonas* spp. in *in vitro* (Silva *et al*., 1987).

Modulation of host immune function is another mode of action of DFM. In the GIT, there are various immune cells such as dendritic cells, natural killer cells, macrophages, neutrophils, and T and B lymphocytes that are aggregated in Peyer's patches, lamina propria, and intraepithelial regions (Krebiel *et al*., 2003). After DFM are administered to the GIT, they are directly taken up by intestinal epithelial cells via transcytosis. Antigen presenting cells, macrophages or dendritic cells engulf them, finally stimulating an immune response (Dicks & Botes, 2010). Various strains of LAB activated macrophages to produce cytokines that stimulate immune response. Matsuguchi *et al*. (2003) suggested that *L. casei* Shirota and *L. rhamnosus* Lr23 stimulated macrophages to secrete TNF-α or promote development of regulatory dendritic cells. Miettinen *et al*. (1996) also reported that LAB could induce the production of proinflammatory cytokines, TNF-

α, and interleukin-6 from human peripheral blood mononuclear cells (PBMC), thereby stimulating non-specific immunity.

Effects on performance of ruminants

1) Calves

Since young calves have to digest a significant amount of ration nutrients in their intestines, they may be at risk of intestinal proliferation of detrimental organisms. Neonatal calves are often stressed in new environments, such as transport, weaning, vaccination, and dehorning (Krehbiel et al., 2003). In intensive farm systems, calves are rapidly separated from cows before their intestinal microbiota have completely colonized. This situation might increase the possibility of diarrhea and weight loss. The administration of large amounts of beneficial microorganisms may allow stressed intestinal environments to be colonized and return GIT function to normal more quickly in scouring calves (Kung Jr, 2001). Therefore many studies have been conducted to evaluate the effects of DFM on young calves (Table 4).

Table 4. The effects of various DFM on calf performance

Strains	Dose	Effects	References
Aspergillus oryzae	5 x 10^7cfunits/ml	Higher total VFA, propionate, and acetate concentrations in the rumen. Cellulolytic bacterial counts tended to be higher than controls.	(Beharka et al., 1991)
Lactobacillus acidophilus	5 x 10^7cfunits/ml	Calves receiving *L. acidophilus* maintained initial BW, and control calves lost BW until 2 wk of age.	(Cruywagen et al., 1996)
Bifidobacterium pseudolongum *Lactobacillus acidophilus*	3 x 10^9cfunits/ml	Both strains improved ADG, feed efficiency and reduced diarrhea incidence.	(Abe et al., 1995)
Lactobacillus acidophilus	Not noted	Incidence of diarrhea decreased after week 1 in calves fed DFM containing *Lactobacillus.* Lactobacilli increased in feces of calves fed a liquid diet treated with *L. acidophilus* 27SC.	(Abu-Tarboush et al., 1996)
Lactobacillus plantarum	Not noted		
Lactobacillus acidophilus 27SC	1.85x 10^7cfunits/ml		
Lactobacillusa acidophilus *Propionibacterium freudenreichii*	from 1 x 10^6cfunits/ml to 1 x 10^9cfunits/ml	Calves fed DFM showed lower fecal shedding of *E.coli.*	(Elam et al., 2003)
Propionibacterium jensenii 702 (PJ702)	1.1 x 10^8cfunits/ml 1.2 x 10^9cfunits/ml	Calves fed PJ 702 exhibited successful gastrointestinal transit of the bacterium.	(Adams et al., 2008)
Lactobacillus acidophilus *Saccharomyces cerevisiae*	1 x 10^9 3 x 10^9 cfu/flask/kg	ADG and feed efficiency were higher in calves receiving probiotics plus enzyme supplements.	(Malik & Bandla, 2010)
Lactobacillus casei DSPV 318T *Lactobacillus salivarius* DSPV 315T *Pediococcus acidilactici* DSPV 006T	3 x 10^9 cfu/kg live weight	Inocula stimulated earlier consumption of starter and earlier development of the rumen.	(Frizzo et al., 2010)

Many studies indicated that LAB could regulate diarrhea incidence as well as improve weight gain and feed efficiency when used as a DFM source. Holstein calves supplemented with *L. acidophilus* 27SC had significantly higher colony counts in feces compared to calves fed a control diet. As a result, calves fed *L. acidophilus* 27SC showed significant differences in scour index during weeks 5, 7 and 8 compared with calves fed a control diet and during weeks 7 and 8 compared with calves fed a mixed lactobacilli diet (Abu-Tarboush et al., 1996). Abe *et al*. (1995) investigated the effects of oral administration of *Bifidobacterium pseudolongum* or *L. acidophilus* on newborn calves. Oral administration of the two types of LAB improved body weight (BW) gain and feed efficiency, and reduced frequencies of diarrhea occurrence compared calves that did not receive LAB. The BW gain was different between treated and control groups, but not between groups fed *Bifidobacteria* and *Lactobacilli*. Dicks and Botes (2010) suggested that *bifidobacteria* produce acetic and lactic acids at a ratio of 3:2, and that these acids might be more effective for the control of gram-negative pathogens and yeasts in the GIT than *Lactobacillus* spp. because acetate was more effective against gram-negative bacteria, moulds and yeasts (Gilliland, 1989).

In recent experiments, LAB were also inoculated into young calves to improve growth performance (Frizzo et al., 2010). Young calves were fed milk replacer and a large quantity of spray-dried whey powder to

generate an intestinal imbalance. Under these conditions, calves fed probiotics had higher average daily gain (ADG), total feed intake, and starter diet intake as well as lower fecal consistency index, indicating that diarrhea incidence was reduced (Frizzo et al., 2010).

Adams *et al*. (2008) examined the effect of a novel bacterial strain, *Propionibacterium jensenii* 702 isolated in Australia on growth performance. Most bacterial DFM for young calves contain LAB, whereas dairy *Propionibacteria* are rarely used. *Propionibacteria* can increase propionate and butyrate concentration in the rumen thereby stimulating rumen development. Fecal recovery of *P. jensenii* 702 from the treatment groups from week 2 indicated successful gastrointestinal transit of the bacterium and these calves exhibited higher weight gain during pre-weaning and post-weaning periods.

2) Adult ruminants

During transition periods, defined as 3 weeks prior to calving to 3 weeks after calving (Grummer, 1995), dairy cows are stressed due to calving, changing diets to rapidly fermentable carbohydrate sources, and lactation. Sudden changes that occur during this time may cause metabolic disorders such as sub-acute acidosis in dairy cows (Chiquette et al., 2008; Oetzel et al., 2007). In finishing beef cattle, it is also very important to prevent ruminal acidosis caused by highly fermentable feeds. Both dairy and beef cattle fed DFM showed improved growth

performance, milk and meat production, and feed efficiency in many experiments (Ghorbani et al., 2002; Krehbiel et al., 2003; Nocek et al., 2002; Stein et al., 2006).

LAB with yeast or LUB has been used as DFM to improve performance of dairy cows. *Enterococcus faecium* with yeast was top dressed in a supplement during both pre- and postpartum periods. DFM increased dry matter intake, milk yield, and milk protein content during the postpartum period. Blood glucose and insulin levels were higher and non esterified fatty acid (NEFA) levels were lower for cows receiving DFM during the postpartum period (Nocek *et al*., 2003). In another study (Nocek and Kautz, 2006), cows supplemented with *E. faecium* with yeast had higher ruminally available dry matter (DM), consumed more DM during both the pre- and postpartum periods, and produced more milk/cow per day. There were no differences in 3.5% fat-corrected milk (FCM) between cows supplemented with DFM and controls. There were no differences in milk fat yield or milk protein percentage and yield. Cows consuming DFM had higher blood glucose postpartum, as well as lower beta-hydroxybutyrate (BHB) levels both pre-partum and on day 1 postpartum. Oetzel *et al*. (2007) reported that *E. faecium* plus *S. cerevisiae* increased milk fat percentages when used as DFM for first lactation cows and increased milk protein percentages for second and greater lactation cows during the first 85 DIM. Second-lactation cows receiving DFM also received fewer antibiotic treatments before 85 DIM than cows receiving placebo.

Table 5. The effects of various strains of DFM on adult ruminant performance

Strains	Dose	Effects	References
Enterococcus faecium *Lactobacillus plantarum*, *Sacchromyces cerevisiae*	from 1×10^5cfunits/ml to 1×10^7cfunits/ml	Sustained a higher nadir pH than cows fed 10^6 or 10^7 and had a higher digestion rate of high moisture ear corn (HMEC) dry matter.	(Nocek et al., 2002)
Propionibacterium P15 *Enterococcus faecium* EF212	1×10^9cfu/g	DMI and ruminal pH were not different. DFM resulted in numerically lower blood CO_2 concentrations and reduced risk of metabolic acidosis.	(Ghorbani et al., 2002)
Enterococcus faecium Yeast	5×10^9cfu/g 5×10^9cfu/g	Cows fed DFM consumed more DM, and produced 2.3kg more milk/cow per day.	(Nocek & Kautz, 2006)
Propionibacterium P169	6×10^{10}cfu/cow 6×10^{11}cfu/cow	Cow fed high doses and low doses of P169 exhibited 7.1 and 8.5% increases above controls in daily 4% FCM, respectively.	(Stein et al., 2006)
Lactobacillus acidophilus LA747 *Propionibacteria freudenreichii* PF24 *Lactobacillus acidophilus* LA45	1×10^9cfu/cow 2×10^9cfu/cow 5×10^8cfu/cow	No differences in average DMI, yield of 4% FCM, ruminal pH and total VFA concentration in the rumen were observed.	(Raeth-Knight et al., 2007)
Enterococcus faecium *Saccharomyces cerevisiae*	5×10^9cfu/cow/d 5×10^9cfu/cow/d	First lactation cows fed DFM produced more milk fat % and second lactation cows fed DFM received fewer antibiotic treatments.	(Oetzel et al., 2007)
Saccharomyces cerevisiae subspecies *boulardii* CNCM I-1079	0.5g of yeast /steer/d	Treatments did not affect DMI, ADG, or feed efficiency during the experimental period.	(Keyser et al., 2007)
Prevotella bryantii	2×10^{11}cfu/dose	*Prevotella bryantii* treatment increased milk fat %, concentration of acetate, butyrate, and decreased lactate concentration 2 to 3 hour after feeding.	(Chiquette et al., 2008)
Propionibacterium strain P169	6×10^{11}cfu/d	Cows fed P169 had lower concentrations of acetate, greater concentrations of propionate, and higher energetic efficiency.	(Weiss et al., 2008)

Raeth-Knight *et al*. (2007) evaluated the effects of the combination of *L. acidophilus* LA747 and *P. freudenreichii* PF24 on 84 d dairy cattle performance and 28 d periods ruminal characterizations. DFM was top dressed on the total mixed ration (TMR) once daily. DFM did not affect performance including DM intake (DMI), 4% FCM, percentage or yield of milk components, feed efficiency, apparent digestibility of DM, crude protein (CP), neutral detergent fiber (NDF), starch, rumen pH or concentrations of ammonia or total volatile fatty acids (VFA).

DFM effects in the rumens of dairy cows have been studied in a feeding trial, in which mixtures of *E. faecium, L. plantarum,* and *S. cerevisiae* at a level of 10^5, 10^6, or 10^7 cfu/ml rumen fluid were directly administered via rumen cannula to cows in early lactation once daily for 21 d. Cows fed 10^5 cfu sustained a higher nadir pH than cows fed 10^6 or 10^7 cfu. Cows fed 10^5 cfu had a higher digestion rate of high moisture ear corn dry matter. Corn silage digestion was higher for cows fed 10^5 cfu and 10^6 cfu compared to those receiving 10^7 cfu (Nocek *et al*., 2002). Weiss *et al*. (2008) supplemented dairy cows from 2 wk before anticipated calving to 119 d in milk with *Propionibacterium* strain P169. Cows fed P169 had lower concentrations of acetate, greater concentrations of propionate and butyrate. Plasma and milk glucose or plasma BHB levels were not affected by DFM. Cows fed P169 had greater concentrations of plasma NEFA on d 7 of lactation. Cows fed P169 during the first 17 wk of lactation produced similar amounts of milk with similar composition as cows fed a control diet.

Calculated net energy use for milk production, maintenance, and body weight change were similar between treatments, but cows fed the P169 consumed less DM, which resulted in a 4.4% increase in energetic efficiency.

Ruminal anaerobic bacteria were also studied as DFM sources for dairy cows. *Prevotella bryantii* 25A was used as a DFM to dairy cows in early lactation (Chiquette et al., 2008). Six cows were given 2 x 10^{11} cells/dose of *P. bryantii* 25A, inoculated directly with a syringe through the rumen cannula. Administration of *P. bryantii* 25A did not change milk yield, but tended to increase milk fat in accordance with increased acetate and butyrate concentrations in the rumen. *P. bryantii* 25A also decreased lactate concentration after 2-3h feeding compared with control treatments, thereby exhibiting the potential to prevent acidosis (Chiquette et al., 2008). Exogenous cellulolytic bacteria have been studied as DFM to improve ruminal fermentation (Chiquette *et al*., 2007). *Ruminococcus flavefaciens* NJ, isolated from the rumen of a wild moose, was supplemented into the rumens of non-lactating dairy cows fed either a high concentrate or a high forage diet daily. NJ modified the abundances of other cellulolytic bacterial populations, and improved *in sacco* digestibility of timothy hay in the rumen when fed as part of a high concentrate diet. The presence of *Aspergillus oryzae* or *S. cerevisiae*, or a change of concentrate to forage ratio in the diet did not succeed in establishing the new strain in the rumen. In an early study, genetically marked *Ruminococcus albus* was inoculated into the

rumen of a goat and the extent of bacterial survival in the rumen was measured (Miyagi *et al*., 1995). *R. albus* persisted in the rumen for 14 d at 10^2 cells/mL of rumen contents.

Strategies of DFM application for ruminant animals

1) Aero-tolerant microorganisms

As discussed above, microbials for DFM must be:

a. viable during preparation and delivery to animals
b. able to survive in digestive environments

Cultivation and preparation of ready-to-use strict anaerobes may be cost-prohibitive. Any dosing method other than adding DFM to the diet is unlikely to be acceptable as a general on-farm practice (Nagaraja *et al*., 1997), especially for daily dosing. Individual administration may be labor and time-intensive and prohibitive for large feedlots. DFM studies of strict anaerobic bacterial species generally focus on establishment of exogenous or genetically modified strains after short-term administration (Jones and Megaritty, 1986; Robinson *et al*., 1992; Miyagi *et al*., 1995; Gregg *et al*., 1998; Chiquette *et al*., 2007), while studies of facultative or aero-tolerant anaerobic bacterial species include long-term daily supercharging in the rumen (Swinney-Floyd *et al*., 1999; Ohya *et al*., 2000; Elam *et al*., 2003; Krehbiel *et al*., 2003).

Synergesties jonesii (Jones and Megaritty, 1986) and *B. fibrisolvens* (Gregg *et al*. 1998) established populations in the rumen, while *R. albus* strain A3 (Miyagi *et al*., 1995) and *R. flavefaciens* NJ (Chiquette *et al*., 2007) did not persist in the rumen at effective population sizes. However, repeated dosing increased cell numbers of *R. flavefaciens* NJ in the rumen. The chance to succeed as a DFM with one-time administration may be limited to only a few strains. Therefore, innate or acquired aero-tolerance may be an important criterion for DFM to be useful to supercharge populations daily or establish populations in the rumen.

An experiment was conducted to evaluate potentiality of aero-tolerant rumen LUB (Kim, 2007). Ruminal contents were collected from dairy cattle and enriched in lactic acid media anaerobically via two transfers (N2), and then used as inocula for further enrichments. A strict anaerobic preparation (N6) was enriched through four additional anaerobic subcultures. An aero-tolerant preparation (N2A2N2) was passed through two aerobic subculturing and then two anaerobic enrichments. An aerobic preparation (N2A4) passed 4 aerobic enrichments. When these enrichments were added to acidosis-inducing *in vitro* ruminal fermentation, N2A4 completely inhibited lactate accumulation, yielded greater total VFA and maintained higher pH than N6 or N2A2N2. Aerobic enrichment may increase the chances to isolate aero-tolerant lactic acid-utilizers by reducing strict anaerobes in the culture. The current study also supports the potential use of aero-

tolerant rumen microorganisms as DFM for cattle. However, there are only a few species of aero-tolerant microorganisms. Aero-tolerance is required only during delivery to the rumen, and does not guarantee that a microorganism will be effective as DFM.

2) Spore forming bacteria

Tolerance of microorganisms to heat is also important for DFM since they have to survive processing during feed production. In general, most yeast and LAB are destroyed by heat during pelleting (Kung Jr, 2001). Spore forming bacteria have advantages as probiotics for humans and animals (Ripamonti et al., 2009). Ripamonti *et al*. (2009) suggested that the ability to form spores might provide probiotics (DFM) with higher resistance to stresses during production and storage processes (Hyronimus *et al*., 2000) and also higher resistance to gastric and intestinal environmental conditions (Hong et al., 2005; Sanders et al., 2003).

Several recent studies demonstrated the probiotic (DFM) effects of *Bacilli*, spore forming bacteria, on ruminant performance (Table 6). *Bacillus* species have specific mechanisms that inhibit gastrointestinal infection by pathogens or producing antimicrobials.

Table 6. Effects of DFM containing bacilli on ruminant performance

Strains	Animals	Effects	References
Bacillus licheniformis *Bacillus subtilis*	Sheep and lambs	Control group tended to have higher mortality than the DFM treated group and produced significantly more milk.	(Kritas et al., 2006)
Bacillus licheniformis *Bacillus subtilis*	Holstein cows	Milk yield and protein were increased by supplementation of *Bacilli*. *Bacillus licheniformis* increased ruminal digestibility and total VFA concentration.	(Qiao et al., 2009)
Bacillus subtilis	Holstein calves	Fecal shedding of presumptive *Clostridium perfringens* at day 7 was reduced in scouring calves treated with electrolytes plus DFM compared to scouring calves treated with electrolytes alone.	(Wehnes et al., 2009)
Bacillus licheniformis *Bacillus subtilis*	Holstein calves	Cows fed DFM had a higher ADG, final live weight.	(Kowalski et al., 2009)
Bacillus cereus var. Toyoi *Saccharomyces boulardii*	Sheep	Both probiotics enhanced humoral immunity.	(Roos et al., 2010)
Bacillus subtilis strain 166	Cattle	There were no dignificant differences observed between treatments for either hide or fecal prevalence of *E. coli* O157:H7.	(Arthur et al., 2010)

Kritas *et al*. (2006) examined the effects of DFM containing *Bacillus licheniformis* and *B. subtilis* on young lambs and milking ewes under field conditions. The addition of DFM tended to reduce the mortality of young lambs and increased the daily milk yield of ewes. Another experiment regarding bacilli DFM was conducted in China (Qiao *et al*., 2009), and yields of 4% FCM, FCM/DMI, and milk protein percentages were increased after *B. licheniformis* supplementation. Total VFA and acetate concentrations were higher with *B. licheniformis* than in the other two groups, *B. subtilis* or animals that received no supplements.

In addition to the practical advantages of spore forming DFM, strong cellulolytic activity may support the potential of bacilli as DFM for ruminant or nonruminant animals by improving fiber digestion in the rumen and/or in the GIT by supplying oligosaccharides to beneficial microorganisms.

Enzymes for ruminant

The improvement of fiber digestion is one of main tasks in ruminant nutrition since the major part of feed for ruminant is forage which has approximately 30-70% of NDF based on DM. Furthermore, the total tract digestibility of NDF might be less than 65% when rumen condition was optimal for cellulolytic microorganisms (Van Soest, 1994). This digestibility could decrease by 35 to 50% when rumen condition was abnormal due to high concentrated diets (Beauchemin et al. 2001). Therefore, the improvement of fiber digestion could bring some beneficial effects such as the increment of energy utilization, DMI, ADG, feed conversion efficiency, increasing milk yield, and meat production. Oba and Allen (1999) also reported that 1% increment of NDF degradability in the rumen brought the 0.17kg/day of DMI as well as 0.25kg/day of milk yield higher than before. Because of above reasons, many researchers used fibrolytic enzymes as feed additives to improve fiber digestion.

Fibrolytic enzyme is mostly consisted to cellulase and hemicellulase. Cellulase is the enzyme which can degrade cellulose materials. Cellulase which is produced by fungi or bacteria, can be divided into three types; endoglucanase (endo-1,4-β-D-glucanase, EC 3.2.1.4), cellobiohydrolase (exo-1,4-β-D-glucanase, EC 3.2.1.91) and β-glucosidase (1,4-β-D-glucosidase EC 3.2.1.21) (Hong et al., 2001). Endoglucanase hydrolyze internal β-1,4-glysidic bonds of cellulose chains randomly so that new chain ends were produced. While, cellobiohydrolase cleave cellulose chains at the end site to produce cellobiose or glucose. β-glucosidase can attack only cellobiose and release glucose units (Kumar et al., 2008; Percival Zhang et al., 2006). These cellulolytic enzymes work together to degrade cellulosic materials quickly. Endoglucanase make new chain site which cellobiohydrolase can work. Then the end product is degraded by β-glucosidase. The hemicellulase system which is more complicated than cellulase system, include various enzymes such as β-1,4-endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α -L-arabino furanosidase (EC 3.2.1.55), β-mannosidase (EC 3.2.1.25) α-glucuronidase (EC 3.2.1.139) and acetyl xylan esterase (EC 3.1.1.72) (Wong et al., 1988).

As mentioned above, the administration of fibrolytic feed enzyme has beneficial effects on ruminant energy utilization. First of all, the administration of exogenous fiber degrading enzyme can induce synergistic effect with ruminal fibrolytic enzyme. Morgavi et al. (2000)

reported that exogenous fibrolytic enzymes combined with rumen microbial enzymes increased cellulose and xylan degradation up to 35, 100%, respectively. Secondly, the use of exogenous enzyme has beneficial effects under low pH rumen condition since some commercial enzyme additives from fungal or bacterial have lower optimal pH than those of rumen microbial cellulolytic enzyme (Beauchemin et al., 2004). The other advantage of fibrolytic enzyme additives for ruminant fiber digestion is that the increase of rumen bacterial attachment and the stimulation of bacterial growth. The administration of fibrolytic enzyme can modify the surface of forage sources thereby helping bacterial colonization. Morgavi et al (2000b) suggested that exogenous fiber degrading enzymes may remove physical barrier which inhibits bacterial attachment.

Classical strategies for improvement of microbial production

Microorganisms have been receiving strong interests from many biotechnologists due to their capability of producing various compounds which are beneficial for human being. Primary metabolites, second metabolites, antibiotics and various enzymes produced by microorganism have been applied at industrial scale. However, some limiting steps must be solved before applying to industrial production. Microorganisms only produce ideal materials as much as they need for their growth, maintenance and reproduction (Adrio & Demain, 2006). They do not overproduce beneficial metabolites and have complex processing step. Strain improvement can provide the opportunity for reducing costs without any additional processing equipment or production system. For example, if microbes are modified to consume lower cost carbohydrate sources, then production cost will be significantly lower than wild type microorganisms which utilize pure glucose as an energy source. Therefore, Improvement of strain is prerequisite condition for industrial mass production.

Table 7. Mutagens employed for strain development (Parekh et al., 2000)

Mutagen	Mutation induced	Impact on DNA	Relative effect
Radiation			
Ionizing radiation			
1. X-rays, γ-rays	Single or double strand breakage of DNA	Deletions, structural changes	High
Short wavelengths			
2. Ultra violet (UV) rays	Pyrimidines diamerization and cross link in DNA	Transversion, deletion, frame shift, transitions from GC -> AT	Medium
Chemicals			
Base analogs			
3. 5-Chlorouracil	Results in faulty pairing	AT -> GC, GC -> AT transition	Low
5-Bromouracil		AT -> GC, GC -> AT transition	Low
4. 2-Aminopurine deaminating agents	Errors in DNA replication		Low
5. Hydroxylamin (NH_2OH)	Deamination of cytosine	GC -> AT transition	Low
6. Nitrous acid (HNO_2)	Deamination of A, C and G	Bi-directional translation, deletion AT -> GC and / or GC -> AT transition	Medium

(continue)

Mutagen	Mutation induced	Impact on DNA	Relative effect
Alkylating agents			
7. N-Methyl-N'-nitro	Methylation, high pH	GC -> AT transition	High
N-Nitroguanidine (NTG)	Alkylation of C and A	GC -> AT transition	High
8. Ethyl methanesulfonate (EMS)	Alkylation of bases C and A	GC -> AT transition	High
9. Mustards di-(2-chloroethyl)-sulfide			
Intercalating agents			
10. Ethidium bromide (ETBR) Acridinedyes	Interaction between two base pairs	Frame shift, loss of plasmids and microdeletions	Low
Biological			
11. Phage, plasmid, DNA transposing	Base substitution, breakage	Deletion, duplication, insertion	High

Artificially induced mutation has been widely used in commercial to improve the production of industrial strains. The most widely used method to acquire mutant strains which have desired characteristics is to adjust a population using mutagenic sources until a specific survivor is discovered. Mutation induces altering at least one nucleotide at a certain site throughout the DNA strand permanently. Almost part of mutations cause harmful incidence to host microorganisms, but certain mutation can lead the target microorganisms to adapt to harsh environment or improve metabolites production without any negative performances. Genetic alteration or improvement of strains along with mutation can be classically achieved by a diversity of physical as well as chemical mutagens which are listed in Table 7 (Pareke et al., 2000). N-Methyl-N'-nitro-N-Nitroguanidine (NTG), ethyl methanesulfonate (EMS), methylmethansulfonate (MMS), hydroxylamine and ultraviolet (UV) irradiation were the mostly used for mutation as a mutagen. These mutagens affect DNA condition by ways of nucleotides deletion, addition, base alteration and breakage of DNA strands. The success of mutagenesis is dependent upon how much we can improve the frequency of desired strains after mutation process because most mutagenesis cause negative effects on productivity of target strain, especially in the highly developed strains (Parekh et al., 2000). NTG have been known for an excellent chemical reagent for mutation since this can cause the DNA mutation at multi points and provide the highest generation rate of mutant among survivors (Baltz, 1986). In

addition to types of mutagen, the culture type of target strain, dosing amount of mutagen, incubation time with mutagen, the methods of treatment or post treatment can affect the frequency of successful mutagenesis (Vinch and Byng, 1998). The Improvement of microbial cellulase production was also achieved by above mutagens (Table 8). Many microorganisms including fungal and bacterial strain were mutated experimentally with various chemical, physical mutagens.

Table 8. Research related with strain improvement for cellulase production using classical method

Wild type strain	Mutagen	Effects	References
Fusarium oxysporum DSM841	NTG UV rays	Mutant NTG-19 had enhanced production cellulase	(Kuhad et al., 1994)
Cellulomonas flavigena PN-120	EMS NTG	Mutant M9-82 had 2.7 fold higher xylanase activity	(Reyes & Noyola, 1998)
Bacillus pumilus	EMS	Catabolite repression-insensitive mutant released more cellulase(11.4mg/g of cell mass) than those (6.2mg/g of cell mass) of wild type	(Kotchoni & Shonukan, 2002)
Bacillus pumilus	Chemical mutagen	Mutant type, BpCRI6 has ability to produce cellulase efficiently under catabolite repression condition	(Kotchoni et al., 2003)
Fungi (not identifyed)	NTG ETBR UV rays	Increase cellulase and endoglucanase activity	(Chand et al., 2005)
Penicillium echinulatum sp.	Hydrogen peroxide	Mutant strain showed 1.35 fold higher cellulase activity than those of wild type	(Dillon et al., 2006)

Wild type strain	Mutagen	Effects	References
Penicillium janthinellum NCIM 1171	EMS UV rays	Mutant showed enhanced cellulase production and clearance zone on Avicel containing agar plate	(Adsul et al., 2007)
Acremonium cellulolyticus C-1	NTG UV rays	strain CF2612(mutant) showed higher filter paperase(17.8Units/ml) and β-glucosidase activity	(Fang et al., 2009)
Penicillium janthinellum JU-A10	EMS UV rays	Mutant showed enhanced clear halo zone on cellulose containing agar plate	(Cheng et al., 2009)
Trichoderma reesei Rut C-30	NTG UV rays	Mutant strain exhibited increased cellulase production, clear halo zone in agar media and an efficient growth on Avicel plate	(Jun et al., 2009)
Trichoderma reesei ATCC66589	UV rays	Mutant produced cellulase in media which had glucose as a sole carbon source	(Ike et al., 2010)
Trichoderma reesei KCTC6950	proton beam rays	Mutant exhibited enhanced Fpase (165%), CMCase (146%), and β-glucosidase (313%) compared with the wild type	(Jung et al., 2012)

Current strategies for development of improved cellulase activity

Currently, there are two strategies which are available for improving the performance of cellulase: 1) Rational design and 2) Directed evolution.

1) Rational design

Rational design, which is one of approaches of protein engineering, was developed after the introduction of DNA recombination and site-directed mutagenesis technology. Rational design has three steps (Percival Zhang et al., 2006)

1) Ideal enzyme selection
2) Identification of the amino acid sites to be replaced depending on their structure visualized by computer model
3) Mutant characterization

The achievement of successful rational design needs detailed information on selected protein structure and relationship between protein structure and function. The choice of the certain site of protein to be altered to other amino acids requires knowledge of original

function as well as expected function when this region will be changed. Site-directed mutagenesis for point mutation has been used for rational design. In addition, the modification of secondary structure or whole domain exchange was also used for rational design (Percival Zhang et al., 2006). Even though a lot of enzyme's structures and their function were reported, still many enzymes need to be identified yet including cellulase. This is the reason why site-directed mutagenesis is used without any conventional rules and remains in a trial-testing procedure recently (Maki et al., 2009).

2) Directed evolution

Still limited information on protein structure and their function have restricted the use of rational design for improve enzyme activity, stability and catalytic properties. In this reason, directed evolution has emerged as another method to develop ideal enzymes because this strategy does not require any knowledge of enzyme structure or function. Directed evolution was achieved by the following steps:

1) Target gene selection
2) Induce of random mutagenesis such as error-prone PCR or DNA shuffling
3) Looking for improved enzyme through appropriate selection or screening method

4) Repeat 1) to 3) until desired gene is developed

Directed evolution was typically started from target gene selection so called parent gene. This gene is mutated randomly using genetic recombination method such as error-prone PCR or DNA shuffling. The library of candidate gene was used to make proteins which will undergo selection or screen process to search specific genes having enhanced properties (Bloom & Arnold, 2009). Thus, the development of appropriate screening or selection method that is able to detect improved genes rapidly is most important step rather than figuring out detailed knowledge about target protein structure and their function. Mutants that show enhanced performances are applied again to next round of random mutation as parent genes while the mutants not showing enhanced properties were discarded. Directed evolution have been extensively used for generation of improved characteristics of cellulase including activity, stability, catabolite repression sensitivity (Percival Zhang et al., 2006). Screening method conventionally used for improved cellulase gene includes carboxy methyl cellulose (CMC) containing agar plate with chromogenic dye such as Congo red, Trypan blue or chromogenic cellulosic substrates (Maki et al., 2009).

3) Genome shuffling

Many industrial strains have been improved by classical mutagenesis so far and this successful result makes it still to be used for development of performance enhanced industrial strains at present. In nature, sexual breeding allows that entire genomes are recombined, but only two parents are possible to participate this mating. In the mean time, DNA shuffling for directed evolution induce recombination between multiple parents per each generation, but this technology are used for only DNA fragments (Zhang et al., 2002). The development of genome shuffling has been originated from the experiment which tries to combine both advantages of classical mating and DNA shuffling together, thereby the recombination between entire genomes from multiple parents can reduce the time to get a desired phenotype efficiently.

Zhang et al. (2002) reported that just two rounds of genome shuffling processed for 1 year were enough to get the results which were achieved by classical random mutagenesis for 20years. GS2 strain undergone two rounds of genome suffling showed 9 fold higher tylosin production than that of wild type strain (*Streptomyces fradiae*) and this status was similar with that of SF2l strain which was developed after 10 years of classical strain improvement trials using NTG, UV irradiation and HNO_2.

Genome shuffling generally was achieved by three steps:

1) The development of parent library
2) Repeated trial of protoplast fusion
3) Screening or selection for desired mutant

The construction of parent strains is typically preliminary step for genome shuffling. Both of quantity and quality of parent strain is criteria for successful genome shuffling since the increment of diversity of parental strain is the important power for next step which is recursive protoplast fusion based on parental strains. To construct parental library, the selected microorganism should be randomly mutated using classical chemical or physical mutagen. Classical strain improvement is needed for generation of various parental strains.

Basically, protoplast fusion which has been accomplished before 30 years approximately is fundamental technology for genome shuffling. Protoplast fusion was used to improve the characteristics of prokaryotic as well as eukaryotic cells and showed successful results even though this technique was applied to different species or kingdoms (Hopwood et al., 1977; Iwata et al., 1986; Rassoulzadegan et al., 1982; Scheinbach, 1983). Protoplast fusion exhibited a higher gene transfer or recombination efficiency than those of other genetic transfer technique such as conjugational transformation of genetic materials (Petri & Schmidt-Dannert, 2004). If the parental strains were prepared as protoplast types, these cells were undergone next steps; mixing, fusion and regeneration. Entire steps including the preparation of protoplast to

regeneration are repeated until desired strain is developed and regenerated strains were used as parental strains for next steps of genome shuffling. Diverse parental strain and recursive protoplast fusion is the basement tools of genome shuffling for the objective of strain improvement. There are some examples using genome shuffling for strain improvement (Table 9).

As mentioned above, genome shuffling has both advantages from classical breeding and directed evolution such as DNA shuffling. This technique does not need any detailed knowledge about genome sequence data, genetic information, protein structure and their function. Genome shuffling also showed rapid improvement of selected strains than other classical technology. Furthermore, genome shuffling has another advantage that shuffled strain is not classified as genetically modified organism since this technique does not use gene sources originated from other organisms. Therefore shuffled strain can be used for food industry such as probiotics for human or animals. This is the reason why genome shuffling was used for this experiment for the development of probiotics strain which can be used to ruminant animals

Table 9. Recent studies on genome shuffling for strain improvement

Wild type strain	Mutation method to prepare parent strain	Protoplast fusion type	Results	References
Candida versatilis	EMS	Conventional protoplast fusion	Mutant showed stronger resistance of salts, higher production of aroma compounds	(Cao et al., 2009)
Bacillus subtilis	UV irradiation He-Ne irradiation	Protoplast inactivation fusion	Mutant exhibited enhanced antagonistic activity against *Fusarium oxysporum* f. sp. *Melonis* and chemical fungicides	(Chen & Chen, 2009)
Penicillium decumbens JU-A10	EMS UV irradiation N^+ ion implanting	Protoplast inactivation fusion	Mutant had enhanced cellulase activity	(Cheng et al., 2009)
Clostridium diolis DSM 15410	NTG	Conventional protoplast fusion	Mutant developed by 4 rounds of shuffling exhibited 80% improved 1,3-propandiol production	(Otte et al., 2009)
Streptomyces pristinaespiralis	UV irradiation NTG	Protoplast inactivation fusion	Mutant produced 11.4-fold pristinamycin than that of the wild type strain	(Xu et al., 2009)
Diasporangium sp. *Aspergillus niger*	-	Protoplast fusion between different strains	shuffled *Diasporangium* sp. showed enhanced arachidonica acid production	(Zhao et al., 2009)
Pichia stipitis	UV irradiation	Conventional protoplast fusion	Mutant had improved tolerance against hardwood spent sulphite liquor	(Bajwa et al., 2010)

Wild type strain	Mutation method to prepare parent strain	Protoplast fusion type	Results	References
Zygosaccharomyces rouxii	EMS	Conventional protoplast fusion	Mutant showed stronger resistance of salts, potassium chloride and lithum chloride	(Cao et al., 2010)
Propionibacterium shermanii	NTG EMS UV irradiation	Protoplast inactivation fusion	Mutant exhibited higher vitamin B12 production and up regulated riboflavin synthase expression in 2D analysis	(Zhang et al., 2010)
Sporolactobacillus inulinus ATCC 15538	UV irradiation Diethyl sulfate	Conventional protoplast fusion	Mutant had higher acid tolerance and 119% higher D-lactate production than that of original strain	(Zheng et al., 2010)
Nocardia sp. ALAA 2000	EMS UV irradiation	Conventional protoplast fusion	Mutant exhibited 19 fold higher ayamycin production than that of wild type strain	(El-Gendy & El-Bondkly, 2011)
Aureobasidium pullulans	EMS UV irradiation	Protoplast inactivation fusion	Mutant showed improved pullulans production	(Kang et al., 2011)

EXPERIMENT I

Characterization of Cellulolytic and Xylanolytic Enzymes of *Bacillus licheniformis* JK7 Isolated from the Rumen of a Native Korean Goat

Introduction

Lignocellulosic materials are the most abundant resource for the production of renewable bioenergy and fermented products. Cellulosic materials need to be first hydrolyzed into fermentable sugars since they are not useful in their polysaccharide form (Li et al., 2009). The biohydrolysis of cellulose through the use of cellulolytic microorganisms is an attractive approach since the degradation of cellulose by chemical agents produces environmental pollution (Rizzatti et al., 2001). Cellulase, which is produced by fungi and bacteria, can be divided into three major types: endoglucanase (endo-1,4-β-D-glucanase, EC 3.2.1.4), cellobiohydrolase (exo-1,4-β-D-glucanase, EC 3.2.1.91), and β-glucosidase (1,4-β-D-glucosidase EC 3.2.1.21) (Hong et al., 2001). Endoglucanases randomly hydrolyze the internal β-1,4-glysidic bonds of cellulose chains so that new chain ends are produced. In contrast, cellobiohydrolases cleave cellulose chains at the ends to produce cellobiose or glucose. β-glucosidase only hydrolyzes cellobiose, and releases glucose units (Kumar et al., 2008; Percival Zhang et al., 2006).

Fungal species have been primarily used commercially for cellulase production because of their capacity to secrete cellulolytic enzymes into their medium, which allows for easy purification and extraction (Maki et al., 2009). Among the cellulolytic fungi, *Trichoderma* spp.

and *Aspergillus* spp. have been extensively investigated since they can produce all three types of cellulose-degrading enzymes (Wang et al., 2008). However, bacterial cellulases have several advantages. First, bacteria have faster growth rates than fungi and can easily grow to high cell densities in inexpensive nutrient sources (Maki et al., 2009). Second, the enzyme expression system of bacteria is more convenient. Third, bacteria can not only survive harsh conditions but can also excrete enzymes that are stable under extreme conditions of high temperature and low or high pH.

Several bacterial genera show cellulolytic activity, including *Bacillus, Clostridium, Cellulomonas, Rumminococcus, Alteromonas, Acetivibrio,* and *Bacteriodes* (Roboson &Chambliss, 1989). Among these, *Bacillus* species produce a variety of extracellular cellulolytic enzymes. *Bacillus licheniformis* is a facultative and a Gram-positive endospore-forming bacterium (Sneath et al., 1986) which is used extensively in large-scale commercial enzyme production since it can excrete proteins in large quantities of up to 20-25g/l (Schallmey et al., 2004).

Many cellulolytic or xylanolytic *Bacillus* species have been isolated from compost (Archana & Satyanarayana, 1997; Rastogi et al., 2010), milled paper (Geetha & Gunasekaran, 2010), swine waste (Liang et al., 2009), and hot springs (Mawadza et al., 2000). However, the isolation of cellulolytic and xylanolytic *Bacillus* sp. from the rumen of goats has

not previously been reported as the rumen environment is a strictly anaerobic environment, which can make it difficult for aerobic bacteria to survive. In this study, the facultative anaerobic bacteria *Bacillus licheniformis* JK7, which can secrete endoglucanase, β-glucosidase, and xylanase was isolated from the rumen of a native Korean goat. *Bacillus licheniformis* is expected to survive on harsh condition such as provision of low quality roughage as a sole feed source (Son, 1999). The objectives of this study were 1) to isolate and identify the microorganism responsible for degrading cellulose and xylan, and 2) to characterize the endoglucanase, β-glucosidase, and xylanase released by selected *Bacillus* sp.

Materials & Methods

1) Materials

All chemicals, media components and reagents used in these experiments were purchased from Sigma (Sigma & Aldrich, St. Louis, USA) and Difco laboratories (Sparks, USA). Azo-CM-Cellulose (Megazyme co. Ltd., Ireland) was used as a substrate to screen cellulolytic bacteria.

2) Isolation and screening of cellulose-degrading bacteria

The ruminal fluid of goats was collected before morning feeding by rumen fistulas. The rumen fluid was diluted with modified Dehority (MD) medium (Scott and Dehority, 1965) using 1% CMC as the sole carbon source and anaerobically cultured overnight at 37℃. The fluid was then spread onto MD agar plates containing 1% Azo-CMC and anaerobically cultured overnight at 39℃ to screen for bacteria with endoglucanase activity. The colonies forming clear zones were then carefully picked and re-streaked onto Azo-CMC agar plate to check for enzyme activity and isolate single strains. The strains which showed consistent endoglucanase activity were transferred to aerobic conditions and cultured in Luria-Bertani (LB) medium overnight at 37℃. Surviving strains which were facultative anaerobic cellulolytic bacteria were selected. The isolated strain was analyzed by Gram staining as

described by Moaledj (1986). Spore formation was examined using phase-contrast microscopy (Nikon Optiphot-2, Japan).

3) 16s rDNA sequencing for strain identification

A total of 1.5 ml of LB culture was centrifuged (10,000g x 1min.) to obtain a cell pellet for DNA extraction, which was performed using a DNeasy Blood & Tissue Kit (Qiagen, Seoul, South Korea). PCR amplification of the 16s rDNA gene fragments was performed using the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3'). The amplified PCR product was visualized by gel electrophoresis. The 16s rDNA band was cut and purified using a Gel DNA extraction kit (Qiagen, Seoul, South Korea). The purified PCR product was then cloned using pGEM-T Easy Vector and transformed into *E.coli* top10 competent cells (Promega, USA) as per the manufacturer's protocol. Plasmids were isolated using a plasmid extraction kit (Bioneer, Korea). A sequence similarity search was carried out using BLAST with the NCBI database (http://www.ncbi.nlm.nih.gov) and alignment was carried out using V-NTI (Life Science Technology, Co. Ltd., USA).

4) Biochemical analysis of strain identification

Exponentially growing cells were biochemically analyzed using the API 50 CHB Kit (Biomeriux, USA) according to the manufacturer's instructions.

5) Growth curve

The culture medium used in this experiment was liquid LB medium containing 1% CMC. The seed culture was developed prior to measurement of growth phase using same media. The culture media (100ml) in 500ml shake flasks was inoculated with 1% of seed culture having 0.5 of OD_{600} value. Aliquots of the bacterial cultures were taken from the growth medium at two hour intervals, and absorbance was measured at 600nm. Growth curves were plotted as absorbance vs. time. Enzyme activity was also calculated at the two hour intervals.

6) Enzyme assays

Cellulase and xylanase activities were measured by spectrometric determination of reducing sugars by the 3, 5-dinitrosalicylic acid (DNS) method (Ghose, 1987). Briefly, a mixture of the enzyme and a 1% CMC solution (1:1) was prepared in 50 mM phosphate buffer (pH 6). Endoglucanase activity was assayed using CMC as a substrate. β-glucosidase activity was determined using salicin (2-hydroxymethyl-phenyl-β-D-glucopyranoside) as a substrate and xylanase activity was determined by measuring the release of xylose from birch wood xylan. For crude enzyme preparation, *Bacillus licheniformis* JK7 was cultured in the basal medium (g/l, 2.5 KH_2PO_4, 2.5 K_2HPO_4, 0.1 NaCl, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, 0.007 $MnSO_4 \cdot 7H_2O$, 0.05 $CaCl_2 \cdot 2H_2O$, 1.0 $(NH_4)_2SO_4$, 2.5 yeast extract, 5.0 CMC, 5.0 birchwood xylan) at 37 ℃ for 24h. The cultures were centrifuged at

13,000g x 10min at 4℃ and the supernatant was used for the enzyme assay. The reaction mixture was incubated at 37℃ for 30 min. After incubation, 300 µl of DNS reagent was added and the mixture was heated to 99℃ for 5 min in a boiling water bath. The release of reducing sugars was calculated from the OD measured at 546 nm. One unit of enzymatic activity was defined as the amount of enzyme that released 1 µmol of reducing sugar per minute. All assays were performed in triplicate and average values are reported.

7) Optimum pH and temperature of cellulase and xylanase and their stability

The optimum pH for crude enzyme preparations was measured in different buffers (50 mM acetate buffer for pH 3-5, 50 mM phosphate buffer for pH 6-8) at 37℃. The stability of the enzymes at different pH values was determined by pre-incubating crude enzyme in various pH buffer solutions for 4 hr at 4℃ (Dong et al., 2010). Relative activity was expressed as the percentage of enzyme activity that remained after incubation in comparison to the maximum observed activity at each pH. To determine the optimum temperature for cellulolytic and xylanolytic enzymes, crude enzyme preparations were incubated at a range of temperatures (20-80℃) in 50 mM phosphate buffer (pH6). Thermal stability was determined by incubating crude enzyme at selected temperatures (20-80℃) for one hour. The relative activity was calculated in comparison to the maximum observed activity at

respective temperature. All assays were carried out in triplicate, and average values are reported.

8) Effects of ions and detergents on enzyme activity

The effect of various metal ions and detergents on the activity of the crude enzyme preparations was investigated. The additives used in this study were 5 mM of nine different metal ions ($CaCl_2$, $CoCl_2$, KCl, $MnCl_2$, $NiCl_2$, $MgCl_2$, $FeCl_2$, $CuCl_2$, $ZnCl_2$) and 0.25% detergent (TritonX-100, Tween20). The reaction mixtures were incubated with respective additives for 60 min at 37℃ and pH 6, and enzyme activities were assayed as described previously. Residual activity was calculated as relative (%) value to control. All assays were performed in triplicate.

9) Statistical analysis

Data from the characterization of the enzymes at different temperatures and pH values were analyzed statistically using the MIXED procedure in SAS (SAS, 1996). The effects of enzymes, treatments, and the interactions between enzymes and treatments were considered fixed. Significant differences ($p<0.05$) in treatment least square means were reported only if the Tukey-test (SAS, 1996) for treatments was also significant ($p<0.05$). The relative enzyme activities of different chemical additives were analyzed using the GLM procedure (SAS, 1996). Differences between treatments were considered significant if $p<0.05$.

Results & Discussion

1) Isolation and identification of cellulolytic bacteria

The majority of rumen bacteria are anaerobic as the rumen maintains an obligate anaerobic environment. Representatives of many *Bacillus* strains are known to grow even under anaerobic conditions (Williams & Withers, 1983), but there have been few reports of the isolation of *Bacillus* spp. from the rumen ecosystem. In this study, ten spore-forming facultative microorganisms were screened on LB agar plates containing 1% Azo-CMC. Of these, bacteria JK7 showed maximum endoglucanase activity (data not shown). This strain was found to be a facultative, spore forming, Gram-positive bacteria. The physiological and biochemical characteristics of this organism are listed in Table 10.

This bacteria was found to be able to hydrolyze various carbohydrates, including L-arabinose, galactose, fructose, mannose, α-methyl-D-glucoside, N-acethyl-glucosamine, D-turanose, salicin, cellobiose, β-gentiobiose, and D-xylose (Table 10), but did not utilize D-arabinose, erythritol, sorbose, dulcitol, inositol, α-methyl-D-mannoside, Lactose, D, L-arabitol, 2-keto-gluconate, or 5-keto-gluconate (Table 10). Based on these results, JK7 was preliminarily identified as *Bacillus licheniformis*. Strain JK7 was found by 16S rDNA sequence alignment to be closely related to the *Bacillus* genus, with the highest similarity with *Bacillus licheniformis* ATCC14580 (99%). Therefore, this strain

was identified as a *Bacillus licheniformis* and designated to *Bacillus licheniformis* JK7 on the basis of biochemical and morphological characteristics and 16S rDNA sequences.

Table 10. Physiologic and biochemical characteristics of Bacillus licheniformis JK7

Characteristics	result	Characteristics	result
Gram stain	+	Esculine	+
Spore formation	+	Salicin	+
Glycerol	+	Cellobiose	+
Erythritol	-	Maltose	+
D-arabinose	-	Lactose	-
L-arabinose	+	Melibiose	-
Ribose	+	Sucrose	+
D-xylose	+	Trehalose	+
L-xylose	-	Inuline	-
Adonitol	-	Melezitose	-
β-methyl-D-xylose	-	D-raffinose	-
Galactose	+	Starch	+
Glucose	+	Glycogen	+
Fructose	+	Xylitol	-
Mannose	+	β-Gentiobiose	+
L-sorbose	-	D-turanose	+
Rhamnose	+	D-lyxose	-
Dulcitol	-	D-tagatose	+
Inositol	-	D-fucose	-
Mannitol	+	L-fucose	-
Sorbitol	+	D-arabitol	-
α-methyl-D-mannoside	-	L-arabitol	-
α-methyl-D-glucoside	+	Gluconate	+
N-acethyl-glucosamine	+	2-keto-gluconate	-
Amygdaline	+	5-keto-gluconate	-
Arbutine	+		

2) Growth curve

The endoglucanase production and cell growth of *Bacillus licheniformis* JK7 was measured by culturing in a 500 ml shake flask with 100 ml of LB media containing 1% CMC at pH6 and 37℃ (Figure 1).

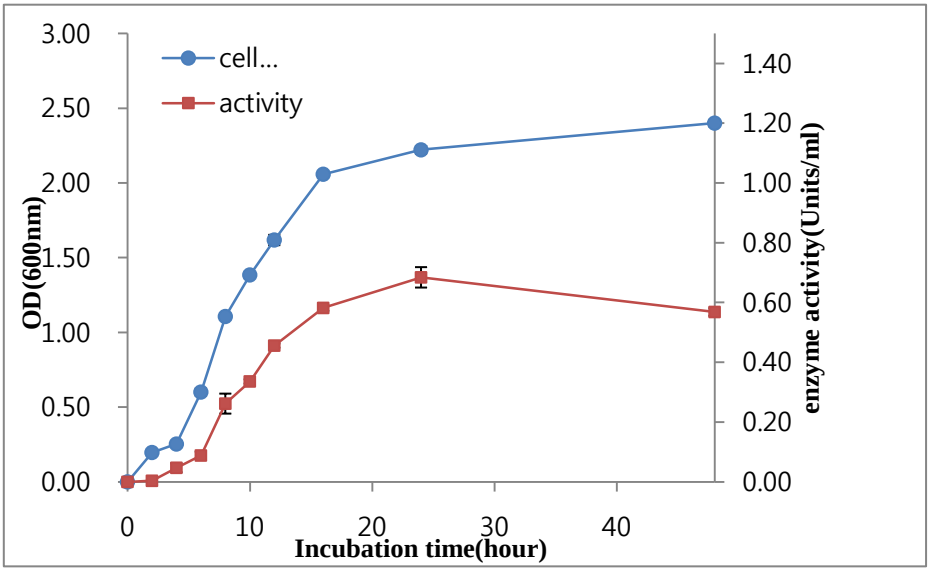


Figure 1. Bacterial growth curve (●) and endoglucanase activity (■) of *Bacillus licheniformis* JK7. The cell growth was determined by measuring the OD600 of the cell culture. Enzyme activity was determined using the culture supernatants. All experiments were performed in triplicates. The data points and error bars indicate the average values and standard errors.

The growth phase of *Bacillus licheniformis* JK7 started at time zero (0 hour) and has grown to sixteen hours later (16hour). It started faster than previously studied *Bacillus sp.* (Peixoto et al., 2011; Samiullah et al., 2009; Yang et al., 1995). The difference is possibly due to different culture condition and the amount of inoculums population. The stationary began at hour sixteen, which was similar to another *Bacillus sp.* (Peixoto et al., 2011) and *Geobacillus thermoleovorans* (Sharma et al., 2007). Bacterial growth was maintained up to hour 48. The OD_{600} values were around 2.1 at the stationary phase, and maximum values reached around 2.3 at hour 48. The stationary phase started faster than seen in the growth curves of the previously described *Bacillus licheniformis* 77-2 (Damiano et al., 2003) and *Bacillus licheniformis* SVD1(van Dyk et al., 2009) and lasted for 30 hours, which was longer than previously described *Bacillus sp.* (Samiullah et al., 2009) and *Bacillus sp.* V1-4 (Yang et al., 1995).

Endoglucanase production increased rapidly from hour 6 up to hour 16, with a maximum value of 0.68 units/ml at 24 hours and a steady decrease thereafter. The increase in enzyme production was associated with an increase in cell growth, which indicated that cellulose was actively utilized by *Bacillus licheniformis* JK7 during the growth phase. There have been several previous studies on endoglucanase production which reported similar patterns as our results (Ariffin et al., 2008; Ko et al., 2011; Rastogi et al., 2010; Saratale & Oh, 2011). For example,

Rastogi et al. (2010) showed that *Bacillus sp.* DUSELR13 had maximum CMCase activity (0.12Units/ml) at day 9, when the culture had reached the dying phase. The *Geobacillus* strain WSUCF1 also produced maximum CMCase activity (0.13Units/ml) on day 7 at the end of stationary phase (Rastogi et al., 2010). Saratale and Oh (2011) reported that the decrease in cellulolytic enzyme production at the stationary phase was caused by metabolite repression by molecules released after the hydrolysis such as glucose or cellobiose.

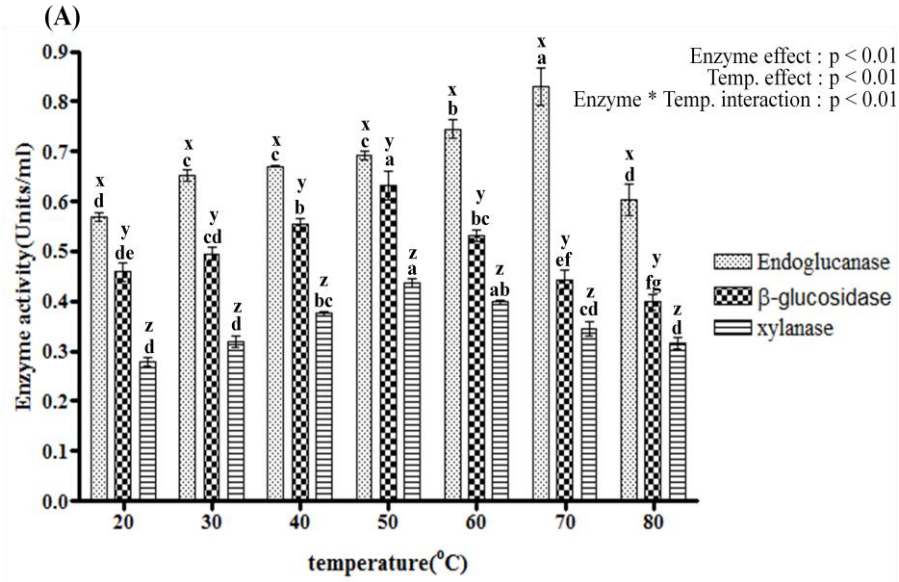
3) Effect of temperature on endoglucanase, β-glucosidase and xylanase activity and stability

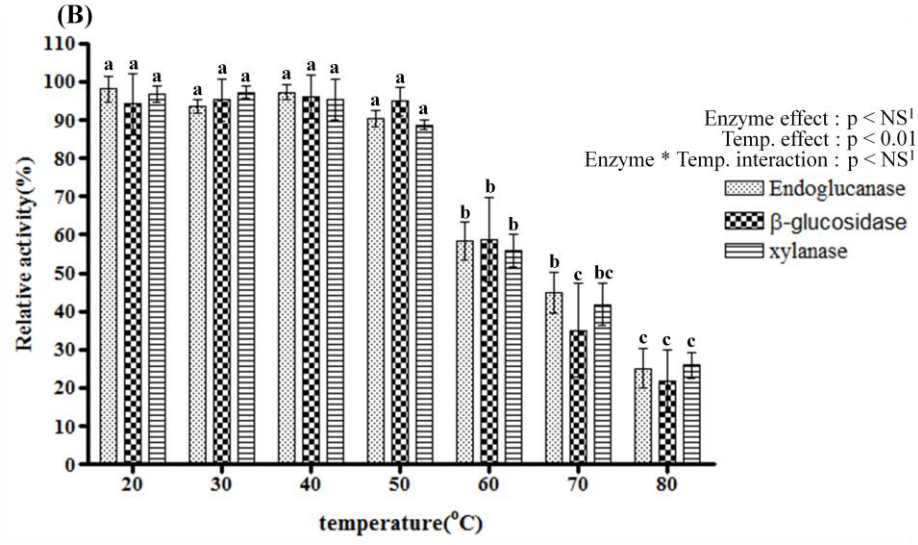
The effect of temperature on endoglucanase, β-glucosidase, and xylanase activity of the crude enzyme was determined over a temperatures range of 20 to 80℃ at pH 6.0 (Figure 2(A)). At all temperatures, endoglucanase activity was higher than that of either β-glucosidase or xylanase. In the present study, the xylanase activity of *Bacillus licheniformis* JK7 was lower than those reported by others at all temperatures. According to van Dyk et al. (2009), *Bacillus licheniformis* SVD1 predominantly produced xylanase, and showed minimal production of mannanase, CMCase and avicelase. This difference might be due to differences in culture conditions (Geetha and Gunasekaran, 2010; Saratale and Oh, 2010). Van Dyk et al. (2009) also used complex media containing 1% xylan, which could induce increased xylanase production.

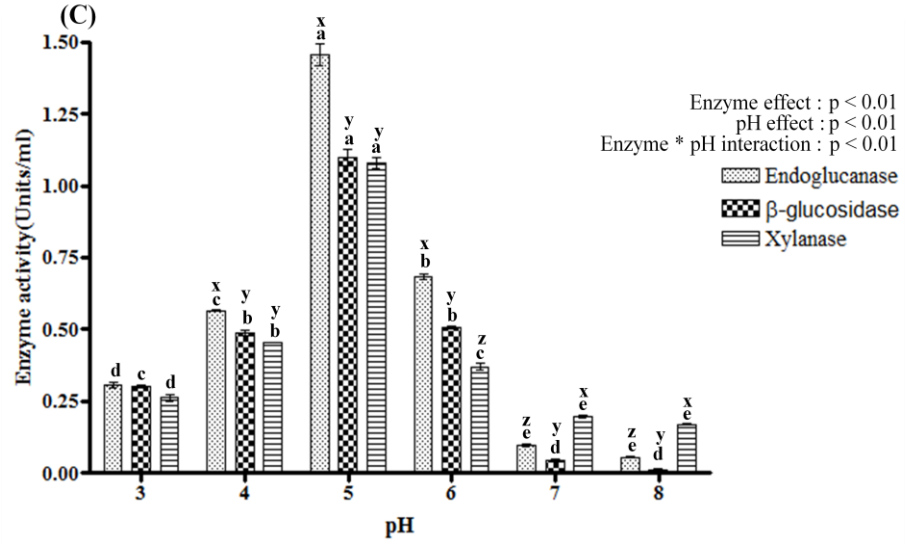
The optimum temperature for *Bacillus licheniformis* JK7 endoglucanase activity was 70℃, at which activity was 0.75 Units/ml. Activity increased linearly with increased temperature up to 70℃, and declined thereafter. The previously described *Bacillus* sp. CH43 (Mawadza et al., 2000) showed a similar optimal temperature for endoglcucanase. In another study, *Bacillus* DUSELR13 (Rastogi et al., 2009) also showed maximum endoglucanase activity at 75℃. However, in that study, endoglucanase activity was very low at low temperatures (20-40℃). In comparison, *Bacillus licheniformis* JK7 showed endoglucanase activity at broad range of temperatures in our study. Thermophilic cellulose degrading enzymes have great potential for the biofuel, leather, textile, food and agriculture industry since high temperatures are often required in these processes (Rastogi et al., 2009; Trivedi et al., 2011).

Bacillus licheniformis JK7 showed maximum β-glucosidase and xylanase activity (0.63 Units/ml, 0.44 Units/ml respectively) at 50℃ (Figure 2(A)). This is similar to that of *Bacillus licheniformis* KCTC1918 (Choi et al., 2009), which had a r optimal temperature of 47℃. The optimum temperature of various *Bacillus* sp. xylanases have also been reported in the literature with similar results (Archana & Satyanarayana, 1997; Ko et al., 2010; Ko et al., 2011; Yang et al., 1995; Yin et al., 2010).

The thermo-stability of endoglucanase, β-glucosidase, and xylanase was assessed at selected temperatures ranging from 20-80℃ as shown Figure 2(B). The relative activity was calculated as the relative enzyme activity compared to the maximum value observed across the range of temperatures. All three enzymes were stable at a range from 20 to 50℃. At 50℃, endoglucanse, β-glucosidase and xylanase had 90.29, 94.80, and 88.69% residual activity, respectively. However, the residual activity of endoglucanse, β-glucosidase and xylanase declined at higher than 50℃. In the case of endoglucanase, maximum activity was observed at 70℃, but it maintained 44.68% residual activity after one hour of pre-incubation.




(B)
Relative activity(%)
temperature(°C)
Enzyme effect : p < NS¹
Temp. effect : p < 0.01
Enzyme * Temp. interaction : p < NS¹
Endoglucanase
β-glucosidase
xylanase


(C)
Enzyme activity(Units/ml)
pH
Enzyme effect : p < 0.01
pH effect : p < 0.01
Enzyme * pH interaction : p < 0.01
Endoglucanase
β-glucosidase
Xylanase

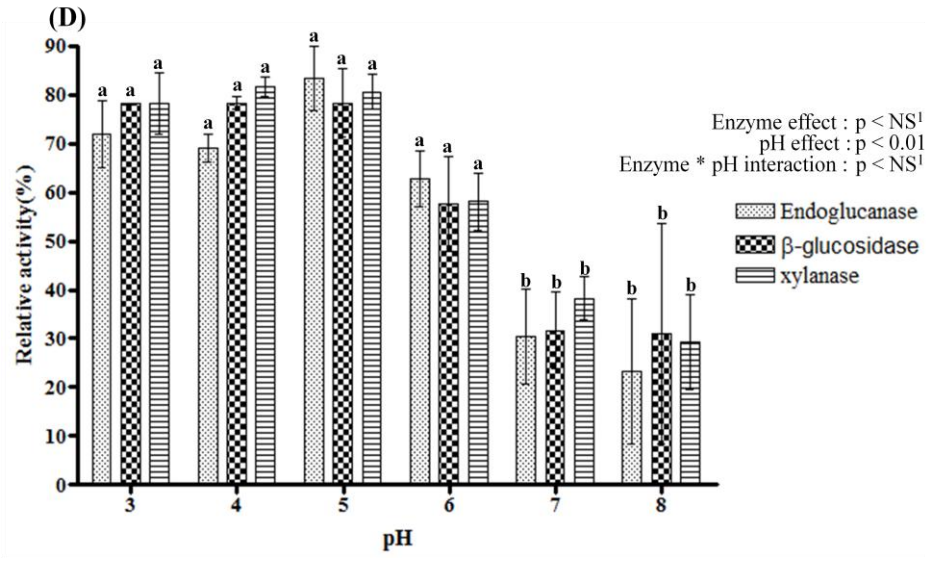


Figure 2. Temperature and pH effects on endoglucanase, β-glucosidase and xylanase of *Bacillus licheniformis* JK7 activity (A and C) and their stability (B and D). Enzyme activity of the culture supernatants was determined at 24 hours. All assays were performed in triplicates. The data points and error bars indicate the average values and standard errors.

^{a,b,c,d,e,f,g} indicates a significantly ($p < 0.05$) different activity influenced by temperature or pH in the same enzyme group.

^{x,y,z} indicates a significantly ($p < 0.05$) different activity between different enzymes within same temperature or pH.

[1] NS means not significant

4) Effect of pH on endoglucanase, β-glucosidase and xylanase activity and stability

The effect of pH on enzyme activity was investigated at various pH levels ranging from a pH of 3.0 to 8.0 as shown Figure 2(C). The optimal pH for all three enzymes was 5.0 and their activity at that pH was 1.46, 1.10 and 1.08 Units/ml, respectively. Endoglcucanase retained 39% and 46% of its maximum activity across the pH range of 4.0 to 6.0 and β-glucosidase retained more than 45% residual activity in the same range. Xylanase maintained 41% and 34% of its maximum activity between pH 4.0 and 6.0. This is consistent with Bishoff et al. (2007), who reported a cloned glycoside hydrolase family 5 endoglucanase gene from *Bacillus licheniformis* B-41361 had maximum endoglucanase activity at pH5.5. The optimum pH of xylanase in the multi enzyme complex of *Bacillus licheniformis* SVD1 was also 5.0 (van Dyk et al., 2010).

Many industrial cellulase producing systems need to use extreme pH conditions to reduce contamination by other bacteria (Dong et al., 2010). Since these processes often require acidophilic enzymes to degrade fiber efficiently under low pH conditions, the relatively high acidophilic nature of the enzymes examined in this study might be considered beneficial for industrial application. All three enzymes were strongly inhibited at pH 7.0-8.0. Figure 2(D) shows the pattern of pH stability of selected enzymes. Relative activity was calculated as the

percentage of the maximum observed activity for each enzyme. Endoglucanase, β-glucosidase and xylanase activities were found to be stable in the pH range of 3 to 6. They maintained more than 58% of their maximum activity at selected pH after four hour pre-incubation at 4℃. At pH 7 and 8, relative enzyme activity of all three enzymes dramatically declined, with remaining endoglucanase, β-glucosidase and xylanase activity of only 23, 30 and 29% of their maximum activity, respectively.

5) Effects of various chemicals on endoglucanase, β-glucosidase and xylanase activity

The effects of various chemicals on endoglcucanase, β-glucosidase, and xylanase were investigated by the DNS assay method. Crude enzyme was incubated for one hour with 5 mM of each of the metal ions and 0.25% of TritonX-100 and Tween20 before determining the residual activity of the three enzymes. Residual activity was expressed as the relative amount (%) compared to control (100%). Understanding the effect of various metal ions and reagents on enzyme activity is important since many industrial applications need to increase enzyme activity, which is often accomplished by addition of chemical additives at various stages of the process (Dong et al., 2010). The majority of *Bacillus* spp. which produces cellulase showed responses ranging from stimulation to inhibition depending on the specific cation (Christakopoulos et al., 1999).

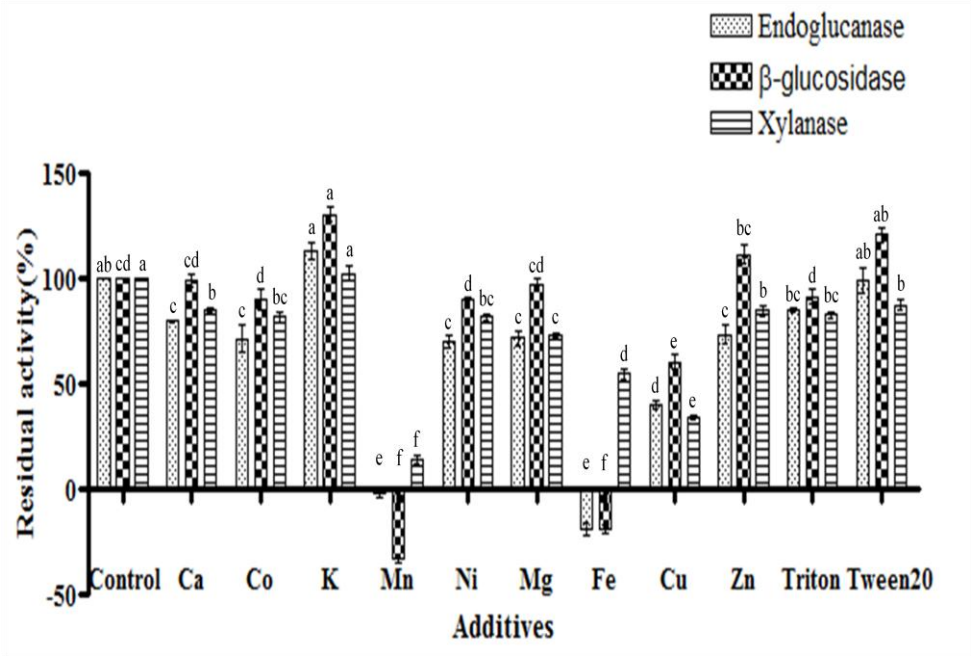


Figure 3. Effect of chemical additives on endoglucanase, β-glucosidase, xylanase activity of *Bacillus licheniformis* JK7. Residual activity was calculated as relative (%) activity considering control as 100%. All assays were performed in triplicates. The data points and error bars indicate the average values and standard errors.

^{a,b,c,d,e,f} indicates a significantly ($p < 0.05$) different enzyme activity compared to control.

In this study, endoglucanase activity was stimulated by K^+ by 113% over control. Glycosil hydrolase family 5 (GH5) endoglucanase from *Martelella mediterranea* (Dong et al., 2010) was previously reported to show increased relative activity when K^+ was added. K^+ may stimulate enzyme activity by altering the structure of the enzyme itself (Kui et al.,

2009). On the other hands, the enzyme activity was inhibited by Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, and TritonX-100. In particular, Mn^{2+} and Fe^{2+} both strongly inhibited endoglucanase activity (2.18 and 19.0%, respectively). The strong inhibitory effect of Mn^{2+} on endoglucanase activity is consistent with previous reports of *Bacillus amyloliquefaciens DL-3* and *Bacillus flexus* (Lee et al., 2008; Trivedi et al., 2011). An inhibitory effect on enzyme activity by metal ions usually suggests the presence of a sulfhydryl group in the active site, where oxidation by the metal ions destabilizes the conformational folding of the enzymes (Karnchanatat et al., 2007).

In present study, the relative activities of β-glucosidase with 5 mM Ca^{2+}, Co^{2+}, K^{+}, Mn^{2+}, Ni^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, and 0.25% of TritonX-100 and tween20 were 99, 90, 130, -33, 90, 96, -19, 60, 111, 91 and 120%, respectively. Detergents such as tween20 have been implicated in altering the conformational or structural characteristics of selected enzymes (Bajaj et al., 2009). Xylanase was not influenced by selected chemical additives, with the exception of Mn^{2+} and Cu^{2+}. The strong inhibition of Mn^{2+} on the xylanase activity of *Bacillus* species was also reported in a previous study (Mamo et al., 2006). The slight stimulatory effect of K^{+} and the inhibition of the xylanase enzyme activity of *Bacillus licheniformis* by tritonX-100 is also similar to what was observed by Archana et al. (2003).

EXPERIMENT II

Genome Shuffling to Improve β-1, 4-Endoglucanase Activity of *Bacillus licheniformis* JK7

Introduction

Microorganisms have been receiving strong interests from many biotechnologists due to their capability of producing various compounds which are beneficial for human beings. Primary metabolites, second metabolites, antibiotics and various enzymes produced by microorganism have been applied at industrial scale. Strain improvement of microorganism is prerequisite process for applying to industrial production since all microorganisms which have been used for industry recently have some limitations for production of ideal materials.

Many industrial strains have been improved by classical mutagenesis so far and this successful result makes it still to be used for development of performance enhanced industrial strains at present (Gong et al., 2009). However, these methods require a lot of manpower, time as well as repeated rounds of same experiment to pick desirable strains. The development of genome shuffling has been originated from the experiment which tried to combine both advantages of classical mating and DNA shuffling together and therefore the recombination among entire genomes from multiple parents can reduce the time to get a desired phenotype efficiently.

After development of genome shuffling, many kinds of strains were improved using this method for a lot of different purposes (Table 4 in Chapter I) but only a few studies (Cheng et al., 2009) genome shuffling in enhancing cellulose activity. The objective of this experiment was to evaluate the effect of genome shuffling on cellulase production of *Bacillus licheniformis* JK7 isolated from Korean native goat's rumen.

Materials & Methods

1) Strains and materials

Facultative bacteria, *Bacillus licheniformis* JK7 which has endoglucanase activity was isolated from Korea native goat's rumen and used as the wild type (WT) strain. All chemicals, media components, reagents, lysozyme and mutanolysin used in this experiment were purchased from Sigma-aldrich (Electrophoresis grade, ACS reagents, Ultrapure) and Difco laboratories (Sparks, USA). Azo-CM-Cellulose (Megazyme co. Ltd., Ireland) was used as a substrate to screen cellulolytic bacteria. Pharmalyte(pH3.5-10) was from Amersham Biosciences and IPG DryStrips(pH4-10NL, 24cm) were Genomine Inc. Modified porcine trypsin(sequencing grade) was from Promega.

2) The composition of media and solutions for genome shuffling

Penassay broth (PAB) containing 1% of CMC (10g of peptone, 1.5g of yeast extract, 1.5g of beef extract, 3.5g of NaCl, 10g of CMC, 1.32g of KH_2PO_4 and 3.68g of K_2HPO_4 in 1liter distilled water adjusted pH 7.0) was used as basal media.

Regeneration media (RM, 5g of glucose, 5g of casein acid hydrolysate, 3.5g of K_2HPO_4, 1.1.5g of KH_2PO_4, 1.9g of $MgCl_2$, 0.1g of L-tryptophan, 250ml of 2M sodium succinate, 15g of polyvinyl

pyrrolidone and 12g of agar in 1liter distilled water) (Akamatsu & Sekiguchi, 1984) was used for genome shuffling basically. RM1 media containing 1% of glucose, 0.8% of CMC and 0.2% of AZO-CMC were used, while 5g glucose was used for 1st genome shuffling to isolate strains having higher endoglucanase activity. RM2 media had 2% of glucose and RM3 media had 3% of glucose as well as 1% of cellobiose. SMM solution (0.5M sucrose, 0.02M maleic acid and 0.02M $MgCl_2$) and PEG solution (40g of Polyethylene glycol 6000 in 100ml of SMM solution) were used for protoplast fusion.

3) Mutagenesis

UV irradiation method was used for development of first mutant strains. Uncovered PAB agar plates having wild type strain were irradiated under UV lamp (20W, 250nm, Sankei Co. Ltd. Japan) at the distance of 30cm for 15min and incubated for 2 days at 37 ℃. The survived colonies were isolated and analyzed for enzyme activity. The strains showing higher endoglucanase production were selected as parental strains for genome shuffling.

4) Protoplast formation

Parental colonies were inoculated and cultured at 37 ℃ overnight in 10ml of PAB in a 150ml shake flask. Seed culture was inoculated in 40ml of PAB in a 250ml shake flask repeatedly and incubated until OD value of 0.5. Then, cells were harvested by centrifuge for 4000g ×

10min at 4℃. Pellets were suspended in 4ml of SMM solution containing lysozyme and mutanolysin (Romero et al., 2006) and incubated for 30min at 37℃ on a rotary shaker 100rpm. The presence of protoplasts was confirmed by light microscopy. Then protoplasts were carefully centrifuged at 3000g for 5min at 4℃ and washed twice with SMM solution.

5) Genome shuffling

Protoplast fusion was performed as suggestedin previous study (Wang et al., 2007). Protoplast suspensions from different strains were mixed and divided into two parts. One part was inactivated with heat at 70℃ for 60 min. and the other was irradiated with UV for 20min. Differently treated protoplasts were mixed again in a ratio of 1:1, harvested by centrifugation and re-suspended in 1ml of PEG6000 solution. After incubation at 37℃ for 30min with PEG solution, the fused protoplasts were washed with 1ml of SMM solution twice. Finally fused protoplast was diluted serially and spread on RM1 plate for 3days at 37℃. The strains which showed improved endoglucanase activity were obtained and named GS1, which were applied for 2^{nd} round of genome shuffling by repeated protoplast fusion as mentioned above using RM2. RM3 media was also used for next rounds of protoplast fusion.

6) Randomly amplified polymorphic DNA (RAPD) PCR

A total of 1.5ml of the PAB culture was centrifuged (10,000g x 1min.) to obtain the cell pellet for DNA extraction using DNeasy Blood & Tissue Kit (Qiagen, Seoul, South Korea). Each DNA from selected strains was extracted according to the manufacturer instruction. RAPD amplification was conducted using PCR pre mixture (Bioneer Co. Ltd. Korea) with 1μl of primer. PCR was carried out with following condition; an initial denaturation phase at 94 ◦C for 4min, followed by 45 cycles of denaturation at 94 ◦C for 30sec, annealing at 36 ◦C for 30sec, and extension at 72 ◦C for 2min, and a final extension at 72 ◦C for 8 min. The amplified PCR product was visualized by gel electrophoresis using 1.5% agarose gels with ETBR in 0.5× TBE buffer. The oligo primers used in this experiment are listed in Table 11.

Table 11. Oligo-primers used for RAPD PCR

Name	Sequence	GC contents(%)	Tm(℃)
B1	5' TACAACGAGG 3'	50.0	30.0
B2	5' TGGATTGGTC 3'	50.0	30.0
B3	5' TCGGTCATAG 3'	50.0	30.0
B4	5' TACCTAAGCG 3'	50.0	30.0
B5	5' GATCATAGCG 3'	50.0	30.0
B6	5' AACGCGTAGA 3'	50.0	30.0
B7	5' TACGATGACG 3'	50.0	30.0
B8	5' TAGAGACTCC 3'	50.0	30.0
B9	5' CACATGCTTC 3'	50.0	30.0

7) Growth curve

The culture medium used in this experiment was PAB containing 1% of CMC. An aliquot of bacterial cultures were taken from growth medium at an interval of 2hr and absorbance was taken at 600nm. The growth curve was plotted with absorbance vs. time and enzyme activity was also calculated at an interval of 2hr.

8) Enzyme assays

The endoglucanase activity was measured by spectrometric determination of reducing sugars by DNS method (Ghose, 1987). Briefly, the mixture of enzyme and 1% CMC solution (1:1) was prepared in 50mM phosphate buffer (pH6). Endoglucanase activity was assayed with CMC as a substrate. For crude enzyme preparation, WT

and genome shuffled strains were cultured in PAB at 37 ℃ for 16h. The cultures were centrifuged at 13,000g for 10min at 4℃ and supernatant was subjected to enzyme assay. The reaction mixture was incubated at 37℃ for 30 min. After incubation 300ul of DNS reagent was added and the mixture was heated to 99℃ for 5 min in a boiling water bath. The release of reducing sugar was calculated from OD measured at 546nm. One unit of enzyme activity was defined as the amount of enzyme that released 1umol of reducing sugar per minute. All the assays were performed in triplicates.

9) Optimum pH and temperature of cellulase and xylanase, and their stability

Optimum pH of crude enzyme preparations was measured in different buffers (50mM acetate buffer for pH 3-5, 50mM phosphate buffer for pH 6-8) at 37℃. The enzyme stability at different pH was determined by pre-incubating crude enzyme in various pH buffer solutions for 4h at 4℃(Dong et al., 2010). The relative activity was expressed as percentage of enzyme activity remained after incubation in comparison to the maximum observed activity at respective pH. To determine the optimum temperature for cellulolytic and xylanolytic enzymes crude enzyme preparations were incubated at a range of temperatures (10-80℃) in 50mM phosphate buffer (pH6). The thermal stability was determined by incubating the crude enzyme at selected temperatures (10-80℃) for 1hour. The relative activity was calculated in comparison

to the maximum observed activity at respective temperature. All assays were carried out in triplicates.

10) Statistical analysis

The data for characterization of enzymes at different temperature and pH were analyzed statistically by MIXED procedure of SAS (SAS, 1996). The effects of enzymes, treatments and enzymes × treatments interaction were considered fixed. Treatment least square means showing significant differences at the probability level of $p<0.05$ were reported only if the Tukey-test (SAS, 1996) for treatments was significant ($p<0.05$). The data for enzyme activity of different strains were analyzed using GLM procedure (SAS, 1996). Significant difference between treatments were considered at $p<0.05$.

Results & Discussion

1) Development of parent strains for protoplasts fusion

Success of genome shuffling requires the diversity of parent strains at the starting point (Patnaik et al., 2002). Therefore, UV irradiation method was used to secure first mutant strains. The enzyme production of wild type and mutant strains were illustrated on Figure 4. UV3, UV9, UV33 and UV50 exhibited 0.76, 0.65, 0.78 and 0.78 Units/ml of endoglucanase respectively, which were higher than that of WT (0.60 Units/ml), were selected for parent strains for genome shuffling.

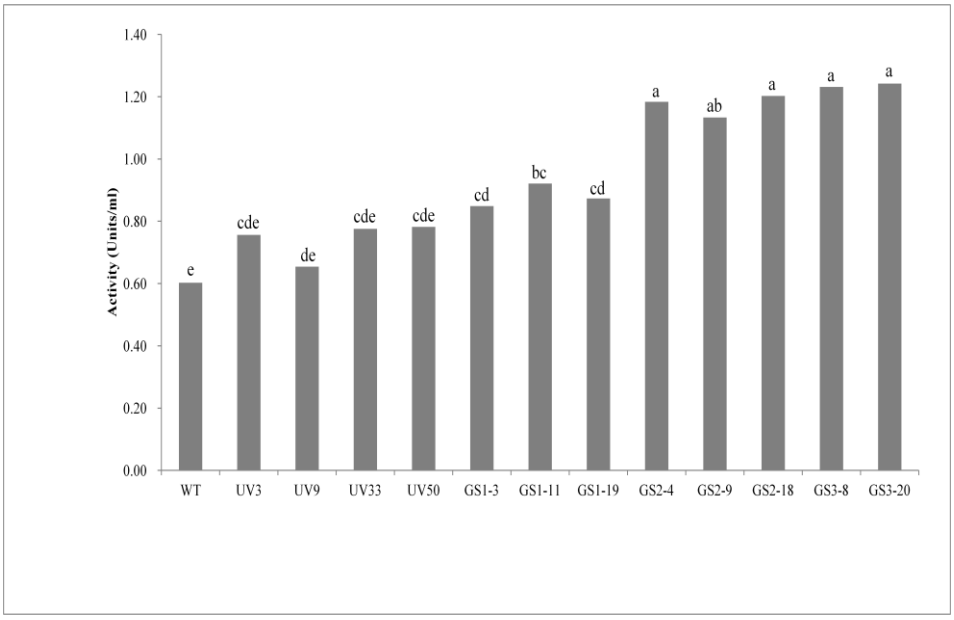


Figure 4. Improvement of endoglucanase production by UV mutation and genome shuffling.

2) Genome shuffling of *Bacillus licheniformis* JK7

Four mutant strains (UV3, UV9, UV33 and UV50) were processed to form protoplasts and then inactivated using UV irradiation or heat treatment. The inactivation was used in this experiment since starting strain might not be survived after inactivation treatment (Wang et al., 2007; Cheng et al., 2009). If the differently inactivated mutants could survive, they have to be fused each other at the protoplast condition. GS1-3, GS1-11 and GS1-19 strains were picked after the first round of genome shuffling. All three strains exhibited higher endoglucanase activity (0.85, 0.92 and 0.87 units/ml respectively) than WT and parental strains. GS1 strains were selected and used for starting strains for next round of genome shuffling. The three isolates at second round of genome shuffling, GS2-4, GS2-9 and GS2-18 showed endoglucanase activities of 1.18, 1.13 and 1.20 units/ml, respectively (Figure 4.) which were significantly higher ($p<0.05$) compared to WT, UV irradiated strains and GS1 strains.

Finally, GS3-8 and GS3-20 together with GS2-18 were selected as final candidate isolates after three rounds of shuffling. Cellulase producing fungi have repression of cellulase production by end product like glucose commonly (Chandra et al., 2009). Cheng et al (2009) also demonstrated that the regeneration media having more glucose has good potential to improve cellulase production by searching shuffled strains which have more resistance to glucose repression. Proper

amount of glucose and cellobiose was added to RM to find catabolite repression resistant strains at each generation of shuffling and this media gave good contribution to the improvement of endoglucanase production.

3) Growth curve and enzyme production of WT and genome shuffled strains

The growth curve and endoglucanase activity was checked at each incubation time point. The growth phases of all strains started at 0 hr and have grown to 12 hrs. The stationary phases began at 12 hrs. The interesting finding was that genome shuffled strains showed faster growth than WT strain after 4 hrs up to final incubation (Figure 5). Endoglucanase activity increased rapidly from hr 6 up to hr 16, with a maximum activity at 16 hrs. The increase in enzyme production was associated with an increase in cell growth, which indicated that cellulose was actively utilized by *Bacillus licheniformis* JK7 during the growth phase. In consistent with growth data, endoglucanase productions of shuffled strains were also higher than that of WT from 8 hrs to 16 hrs incubation (Figure 6.).

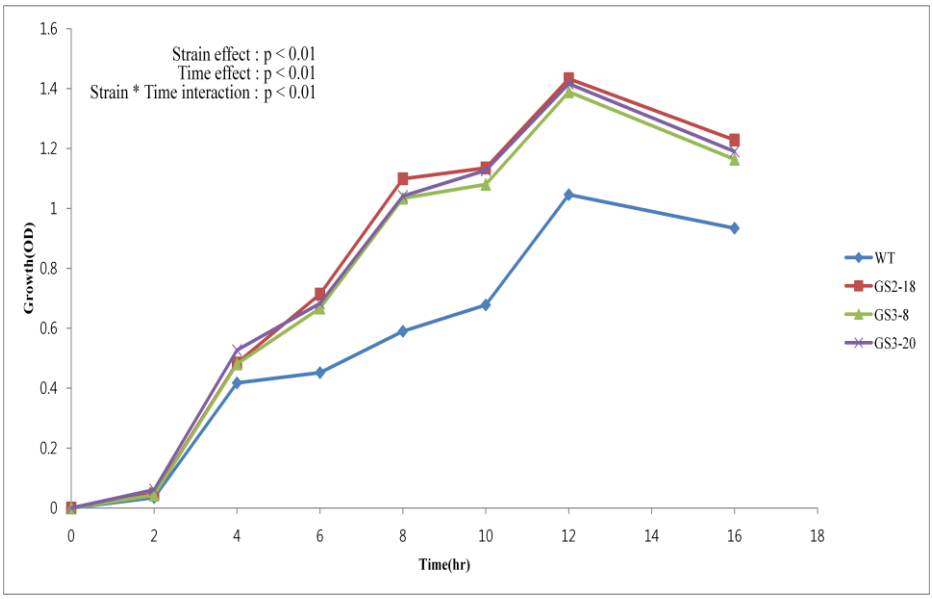


Figure 5. Time courses growth curve of WT, GS2-18, GS3-8 and GS3-20 strains.

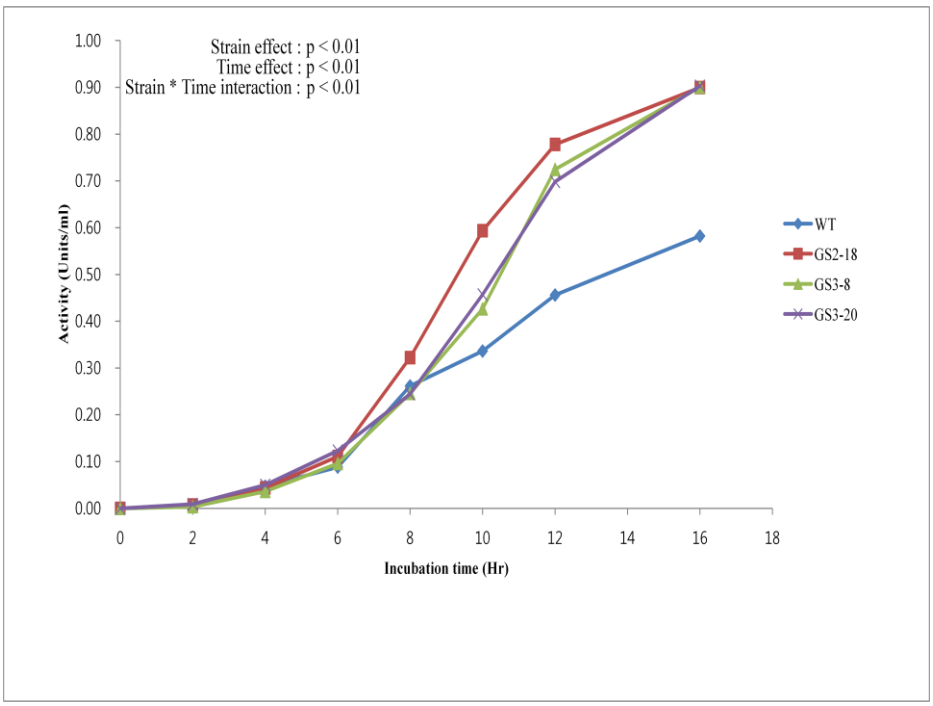


Figure 6. Time courses endoglucanase production of WT, GS2-18, GS3-8 and GS3-20 strains.

Cheng et al. (2009) reported that shuffled strains showed higher growth rate, biomass and extra cellular production. Zhang et al. (2010) also reported that shuffled *Propionibacterium shermanii* F2-3 have grown rapidly and produced more Vitamin B12 compared to WT. It might be suggested that the increased secretion of extracellular protein by the improvement of growth phase is the one of the reasons for increment of endoglucanase production.

4) Effects of temperature and pH on endoglucanase activity and stability of WT and genome shuffled strains

The effect of temperature on endoglucanase activity of the crude enzyme was determined over a temperatures range of 20 to 80℃ at pH 6.0 (Figure 7(A)). The optimum temperature for endoglucanase activity of all strains was 70 ℃, at which activities were 1.09, 1.63, 1.70 and 1.42 units/ml, respectively. The effect of pH on enzyme activity of all strains was investigated at various pH levels ranging from a pH of 3.0 to 8.0 as shown in Figure 7(C). The optimal pH for endoglucanase of all strains was 5.0 and their activity at that pH was 1.44, 2.15, 2.38 and 1.93units/ml, respectively. These results indicated that genome shuffling did not influence characteristics of endoglucanase but the enzyme production was influenced by genome shuffling.

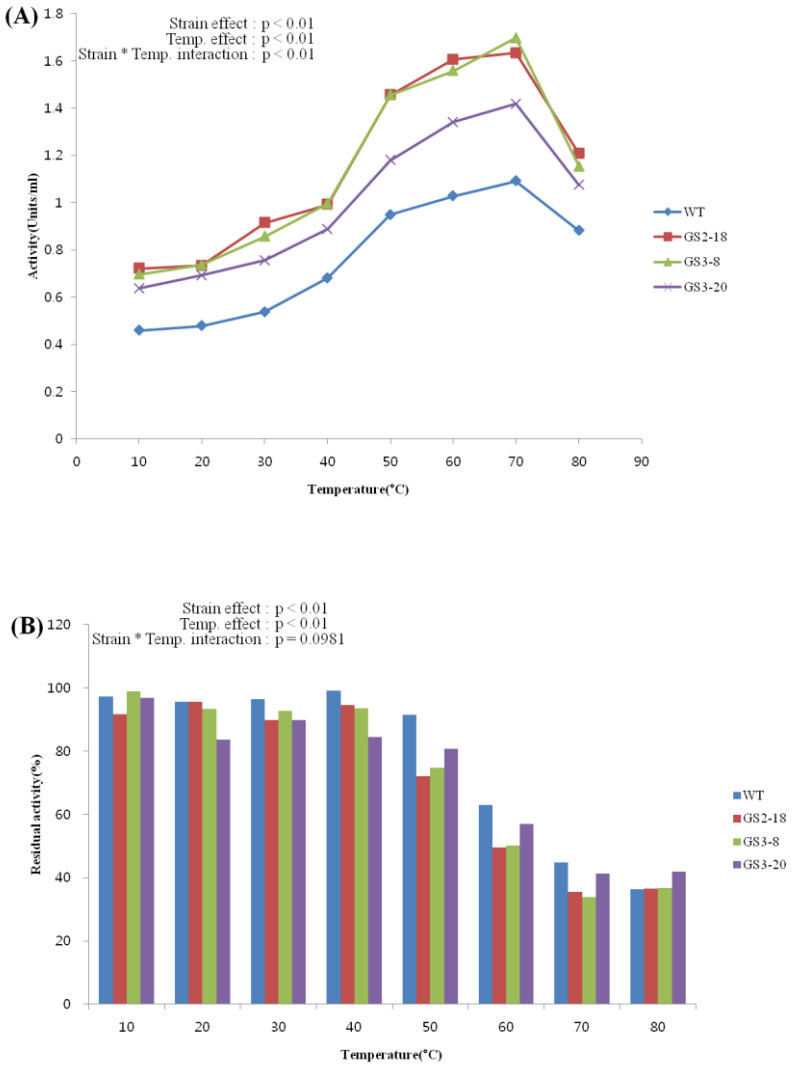


Figure 7. Temperature and pH effects on endoglucanase of WT and genome shuffled strains activity (A and C) their stability (B and D). Enzyme activity of the culture supernatants was determined at 16 hours.

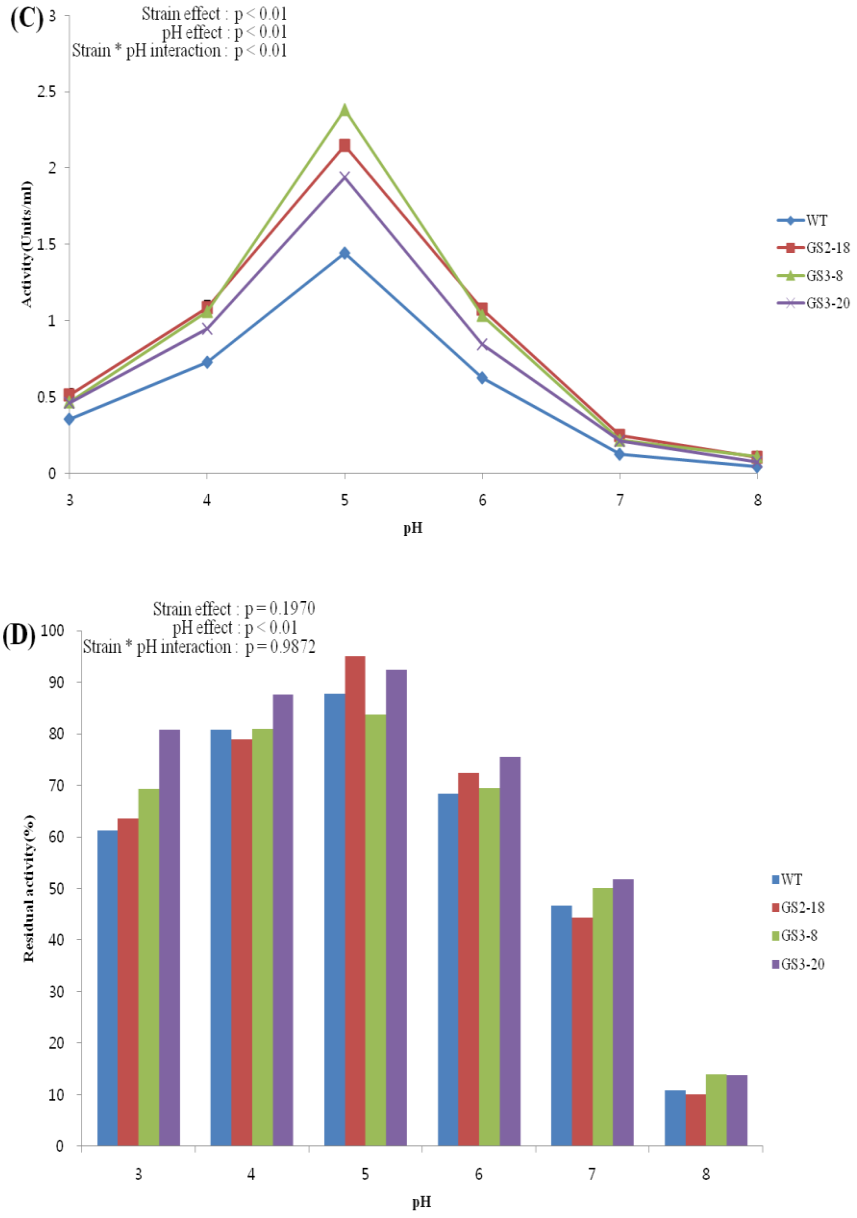


Figure 7. (continued) Temperature and pH effects on endoglucanase of WT and genome shuffled strains activity (A and C) their stability (B and D). Enzyme activity of the culture supernatants was determined at 16 hours.

5) RAPD PCR assays

RAPD PCR assays was used to confirm the genetic diversity between WT and shuffled strains of *Bacillus licheniformis* sp. B4 primer was used for RAPD PCR among nine primers (Table 11) since this primer showed most variable fragments. As shown in Figure 5, GS strains had specific fragment with about 2kbp, suggesting that there was increasing genetic diversity in shuffled strains. There were needed to be further study for sequencing the specific fragment.

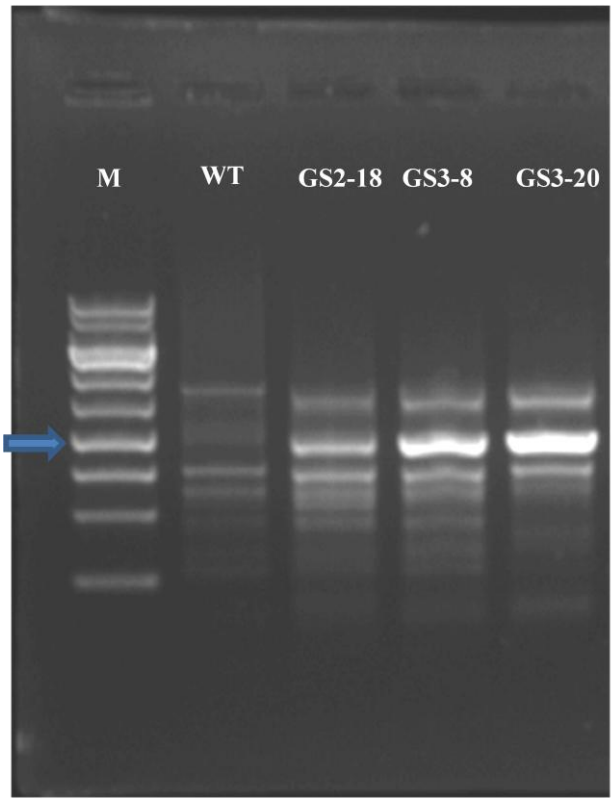


Figure 8. RAPD fragments pattern of WT and GS strains using B4 primer. Arrow indicated 2kbp of band size which was not shown in WT DNA templates. M; marker for DNA size.

EXPERIMENT III

***In situ* Degradation Characteristics of *Bacillus licheniformi*s Based DFM**

Introduction

Several feed additives have been used to improve animal performance and feed efficiency and to prevent disease. Antibiotics, probiotics (direct-fed microbials, DFM) and prebiotics (microbial growth promoters) have been studied to manipulate the microbial ecosystem and fermentation characteristics in the rumens and intestinal tracts of livestock animals. Many livestock producers have explored alternative strategies to enhance animal performance and health. Recently, DFM have been increasingly evaluated to replace or facilitate reductions in the use of antibiotics. In the previous study, *Bacillus licheniformis* JK7 having endoglucanase, β-glcucosidase and xylanase activity was isolated from Korean native goat's rumen. Furthermore, Genome shuffling was used to enhance their cellulolytic enzyme activity. The objective of this study was to develop *Bacillus licheniformis* sp. -based DFM using solid-state fermentation (SSF), and then to evaluate the efficacy of *Bacillus licheniformis* sp. -based DFM in *in situ* ruminal disappearance.

Materials & Methods

1) Preparation of inoculums

B. licheniformis which was stored at -80℃ was inoculated and seed cultured at 37℃ for overnight using nutrient broth (Sigma-Aldrich, St. Louis, Mo, USA). Seed culture was inoculated again to larger flask and *B. licheniformis* was grown up until the OD value reached 0.5. This sub culture was used as inoculums for solid state fermentation.

2) Raw materials and procedure for solid state fermentation

Wheat bran (WB) and soy bean meal (SBM) were used as ingredients for SSF. Two ingredients were ground in a Wiley mill (Arthur H. Thomas, Philadelphia, PA) through a 1mm screen and dried at 65℃ for 2days. WB and SBM were mixed at the ratio of 4:6. Solid state fermentation was performed in 3 Liter shake flasks which had 300g of mixed diet. Appropriate amount of distilled water was added to flasks to maintain moisture level of substrates at 45%. The flasks were sterilized by autoclaving at 121℃ for 20min and taken UV irradiation until flask was cooled at room temperature.

Then, 1% (vol/wt of substrate DM) of *B. licheniformis* culture was inoculated to solid media while, control group flasks were inoculated with the same volume of distilled water. The flasks containing substrates and inoculums were thoroughly mixed and incubated at 37℃

for 8days. Samples were taken at respective incubation time to estimate the enzyme activity, colony forming units (CFU) and nutrient composition.

3) Enzyme assays

The cellulase and xylanase activity was measured by spectrometric determination of reducing sugars by DNS method (Ghose, 1987). Briefly, the mixture of enzyme and 1% CMC solution (1:1) was prepared in 50mM phosphate buffer (pH6). Endoglucanase activity was assayed with CMC as a substrate. β-glucosidase activity was determined using salicin (2-hydroxymethyl-phenyl-β-D-glucopyranoside) as a substrate and xylanase activity was determined by measuring the release of xylose from birch wood xylan.

For crude enzyme preparation, collected samples were suspended in distilled water at a rate of 10% (wt/vol) and shaken at 100rpm at 37℃ for 30min. The mixture was squeezed through 4 layers of cheese cloth and filtrates were centrifuged at 13,000g for 5min. The supernatants were used for enzyme assays. The reaction mixture was incubated at 37℃ for 30 min. After incubation 300ul of DNS reagent was added and the mixture was heated to 99℃ for 5 min in a boiling water bath. The release of reducing sugar was calculated from OD measured at 546nm. One unit of enzymatic activity was defined as the amount of enzyme that released 1umol of reducing sugar per minute.

4) *In situ* experiment

An *In situ* trial was conducted to examine the degradation kinetics of each experimental diet. Ruminal DM, CP, NDF and ADF disappearance rates of two diets were measured using the nylon bag technique as suggested by Ørskov et al. (1980). Three rumen cannulated Holstein steers, weighing 450±30 kg, were used for the *in situ* experiment. Experimental animals were offered twice daily (at 8 am and pm) timothy hay and a commercial concentrate feed (Cargill Agribrand Purina Korea Co. Ltd.) at the ratio of 4:6. Total amount of feed offered to animals was 2% of body weight. The chemical composition of the commercial concentrate was 87.7% of DM, 14.8% of CP, 4.13% of ether extract (EE), 5.22% of ash and 71.5% of total digestible nutrient DM basis, respectively. Fresh water and a mineral mixture were available to each animal at all times.

A total of 15g of FM and NFM were weighed and allotted per nylon bag having 53±10 μm (Bar Diamond, Idaho, USA) pore size. The bags were placed into the ventral sac of the rumen of each Holstein steer through rumen cannula. The incubation started at 8:30 am after feeding. All feed samples were incubated for 0, 3, 6, 12, 24 and 48h. After incubation for respective time, bags were removed from the rumen, and washed by hand under cold tap water to remove feed particle attached to the surface of the bags until the rinse water became clear. Zero time incubation bags were washed in the same way without incubation in the

rumen. After washing, all nylon bags were dried at 65℃ for 48h and weighed to estimate the DM residues. Contents of the bags were stored at 4℃ until chemical analysis.

5) Evaluation of the characteristics of rumen degradation

Disappearance rates of DM, CP, NDF and ADF were calculated as the difference between the weight of the content before and after incubation of each sample. The degradability data obtained for each treatment were fitted to the following equation (Ørskov and Mcdonald, 1979):

$$P = a + b\,(1-e^{-ct})$$

using the PROC NLIN (SAS, 1996) program, where P is the degraded feed at time 't'; a is the rapidly soluble fraction; b is the insoluble but potentially degradable fraction; c is the rate of degradation of fraction b; t is the incubation time.

The effective degradabilities (ED) of DM, CP, NDF and ADF were calculated using the above parameters (a, b, c) and ruminal outflow rates of 0.05%/hr by the following equation:

$$P = a + \left(\frac{bc}{c+k}\right)$$

Where P is the effective degradability of DM, CP, NDF and ADF and k is the estimated rumen outflow rate (%/hr).

6) Chemical analysis

Collected samples were ground in a Wiley mill (Arther H. Thomas, Philadelphia, PA) and then analyzed for DM, ash and CP (AOAC, 1990). NDF and ADF were analyzed by the method of Van Soest et al. (1991) with Ankom 200 fiber analyzer (Ankom Technology, NY, USA).

7) Statistical analysis

The data for in situ trial and enzyme production were analyzed using GLM procedure (SAS, 1996). Tukey's test was used for comparison of difference. Significant difference between treatments were considered at $p<0.05$.

Results & Discussion

1) Development of FM using SSF

SSF has some advantages compared to submerged fermentation (SMF). This process requires cheap equipment as well as substrates such as agricultural by products(Zhao et al., 2008). Hence it does not produce any wastes after fermentation (Adams et al., 2000). Therefore SSF method was applied to the preparation of DFM using *Bacillus lichemiformis* sp.

The mixture of WB and SBM was inoculated by selected microbial and cultured for 8 days. WB is widely used and suitable substrate for inducing cellulolytic or hemicellulolytic enzymes because it contains variety of nutrients for microbes and provides a large surface so that cellulolytic microorganism can produce enzymes quickly and effectively (Archana & Satyanarayana, 1997). Furthermore, WB is major by-product of the wheat processing industry and commonly available. Thus it has potential as industrial fermentation materials.

Figure 9 shows the growth curve and endoglucanase production of *Bacillus licheniformis* sp. when it was grown on solid state. The maximum growth of bacteria was obtained after 96 hrs incubation which was longer than for SMF in previous study. The endoglucanase production was also peaked at 96hrs. It might suggest that our strain

requires cellulose for growth thereby enzyme production also increased simultaneously. Based on these results 96hrs was dertermined as optimal incubation time for development of DFM. FM showed higher endoglucanase, β-glucosidase and xylanase activity (9.90, 1.67 and 1.52 units/g respectively) compared to NFM (0.87, 1.13 and 1.00 units/g respectively) indicating that target bacteria can grow in this media and secrete cellulolytic enzymes suitably (Figure 10).

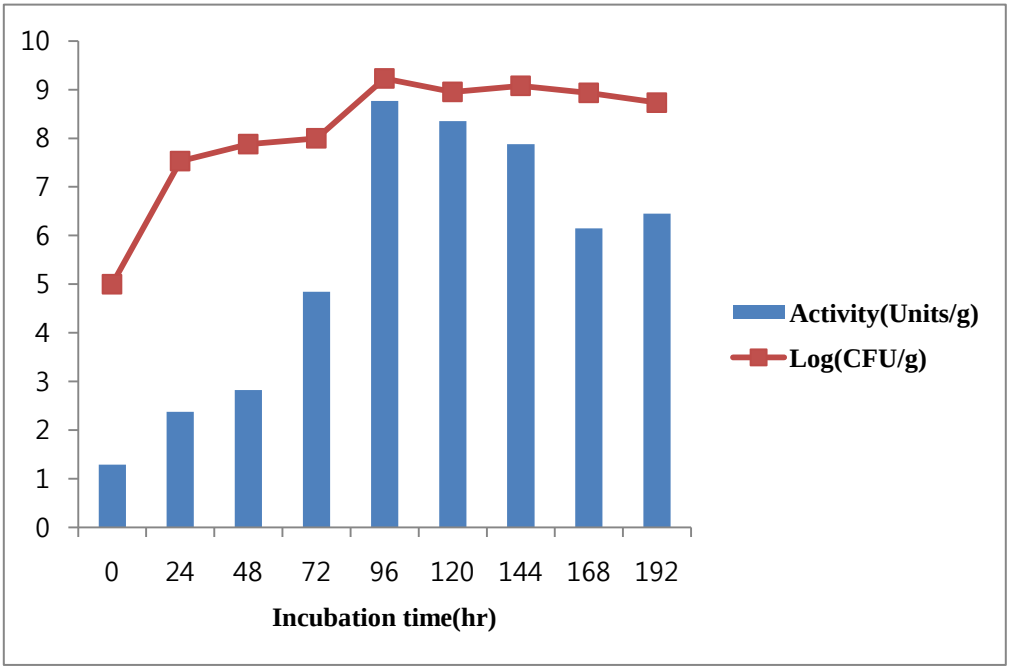


Figure 9. The growth and endoglucanase production of FM.

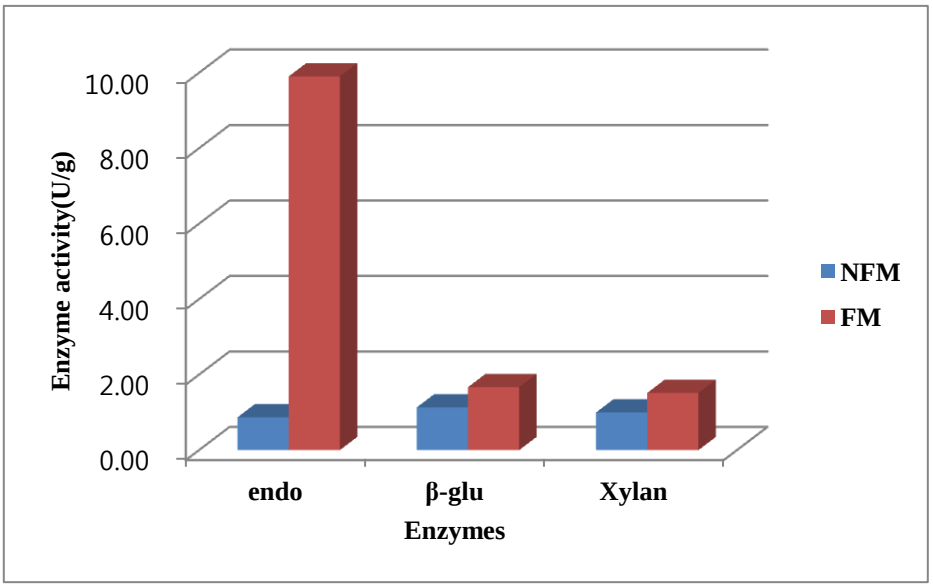


Figure 10. Endoglucanase, β-glucosidase and xylanase activity of NFM and FM (units/g).

2) The difference of chemical composition between NFM and FM

After SSF, the chemical composition and AA profiles between NFM and FM (Table 12 and 13) was evaluated. As shown in Table 12, FM had higher CP, EE and ash contents but had lower DM, CF, NDF, ADF and lignin compared to NFM. *Bacillus licheniformis* used in SSF has cellulolytic enzymes indicating the cellulosic materials in the substrate might be degraded by secreted enzymes. That is the reason why FM had lower NDF and ADF contents than those of NFM.

However, CP and EE of FM were higher compared to NFM. This is probably due to bacterial growth. Selected bacteria used fiber component in substrates for growth and therefore, increased protein and

fat contents came from the growth of *Bacillus licheniformis* sp.. Increased AA (Table 13) can be explained by the same reason. SSF increased total and several AA (asparagine, threonine, glutamine, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, methionine and cysteine).

Table 12. Chemical composition of NFM and FM (DM basis %)

Item	NFM	FM
DM	92.45	90.65
CP	39.74	43.54
EE	2.09	3.27
CF	7.21	7.02
Ash	6.50	6.87
ADF	11.57	8.76
NDF	49.72	26.29
Hemicellulose	38.15	17.53
Lignin	2.54	1.23
Ca	0.38	0.26
P	0.82	0.84

Table 13. Total amino acid composition of NFM and FM(%)

	NFM	FM
Total (%)	27.84	31.94
Asparagine	3.28	3.41
Threonine	1.22	1.32
Serine	1.55	1.5
Glutamine	6.01	7.88
Glycine	1.37	1.43
Alanine	1.36	1.63
Valine	1.19	1.44
Isoleucine	1.12	1.29
Leucine	2.25	2.53
Tyrosine	0.87	1.18
Phenylalanine	1.33	1.72
Lysine	1.65	2.06
Histidine	0.7	0.81
Arginine	2.1	1.91
Proline	0.96	0.76
Methionine	0.43	0.53
Cysteine	0.45	0.54

3) *In situ* experiment

Since FM had cellulolytic and hemicellulolytic enzymes, *in situ* degradation study was conducted to if SSF modified degradation characteristics of substrate used for SSF. FM showed significantly higher DM, CP, NDF and ADF disappearance rate at 3, 6 and 24 hrs incubation (Table 14).

Table 14. DM, CP, NDF and ADF disappearance rate of NFM and FM (%)

		Incubation time (hr)					Mean	P-value		
		0	3	6	12	24		Diet	Time	Diet * Time
DM										
	NFM	7.76[b]	22.96	26.31[b]	33.92[b]	42.18[b]	26.63[b]	P<0.01	P<0.01	P<0.01
	FM	13.01[a]	22.78	29.75[a]	39.78[a]	57.13[a]	32.49[a]			
CP										
	NFM	8.62	16.99[b]	18.27[b]	23.32[b]	27.55[b]	18.95[b]	P<0.01	P<0.01	P<0.01
	FM	10.98	22.58[a]	28.58[a]	38.61[a]	57.17[a]	31.58[a]			
NDF										
	NFM	8.42[b]	13.94	16.47[b]	23.67[b]	29.36[b]	18.37[b]	P<0.01	P<0.01	P=0.0107
	FM	13.69[a]	19.58	22.31[a]	34.54[a]	39.69[a]	25.96[a]			
ADF										
	NFM	10.38[b]	11.15[b]	16.71[b]	18.32[b]	24.44[b]	16.20[b]	P<0.01	P<0.01	P<0.01
	FM	15.05[a]	16.52[a]	19.25[a]	25.40[a]	34.23[a]	22.09[a]			

[a, b] indicates significant difference between NFM and FM at the same time points.

Based on this result we calculated degradation kinetics of DM, CP, NDF and ADF of the experimental diets (Table 15). Fraction a and b of DM and CP increased by SSF indicating potentially degradable part was increased by fermentation. Since the value of a+b fraction of FM were higher than those of NFM, effectively degradable value was also higher in case of FM treatment although NFM had bigger value of 'c' which means constant rate of degradation of 'b' fraction of NFM was higher. Likewise, all degradation kinetics of CP showed same pattern as for DM in two diets. As shown Table 15, only a fraction and ED value of NDF and ADF was significantly different between NFM and FM ($p<0.05$). The b fraction and value of c were significantly different between the treatments ($p<0.05$).

Based on nutrient disappearance rate and their kinetics results, it could be postulated that the mixture of WB and SBM was fermented well by selected bacteria and nutrients in the mixture of WB and were converted to more degradable form by SSF. FM had abundant microbial count which makes it excellent candidate for DFM. The fact that FM contains live microbial, enzymes and more degradable nutrients can be good characteristics of a suitable feed additive as a DFM for ruminants.

Table 15. DM, CP and OM degradation kinetics of the feeds *in situ* study

Items	Feeds	a[1]	b[1]	c[1]	a+b[1]	ED(k=0.05)[2]
DM						
	NFM	9.00[b]	33.62[b]	0.13[a]	42.62[b]	33.35[b]
	FM	13.73[a]	66.66[a]	0.05[b]	80.39[a]	44.70[a]
SEM[3]		1.0884	7.8661	0.0189	8.8279	2.5731
CP						
	NFM	9.38[b]	19.00[b]	0.12[a]	28.38[b]	22.76[b]
	FM	12.28[a]	70.45[a]	0.04[b]	82.73[a]	44.28[a]
SEM[3]		0.7794	12.2319	0.0182	12.7913	4.8453
NDF						
	NFM	8.48[b]	26.50[a]	0.07[a]	34.99[b]	23.61[b]
	FM	13.08[a]	33.45[a]	0.08[a]	46.53[a]	32.66[a]
SEM[3]		1.1475	2.2847	0.0061	2.9582	2.0364
ADF						
	NFM	9.97[b]	34.92[a]	0.04[a]	44.89[a]	20.73[b]
	FM	14.01[a]	56.00[a]	0.02[a]	70.02[a]	28.95[a]
SEM[3]		1.0353	9.1386	0.0123	9.9645	1.9082

[1] a= readily soluble fraction, b= insoluble but potentially degradable fraction,
c= constant rate of degradation of b (%/h).
[2] ED = Effective degradability. Rumen passage rate (k) was considered to 0.02, 0.05 and 0.08.
[3] SEM means Standard error for means.
[a,b] Within the same column, means without a common superscript are significantly different ($p<0.05$).

EXPERIMENT IV

Effects of *Bacillus licheniformis* Based DFM on Rumen Fermentation and Microbial Population under *in vitro* Condition

Introduction

There have been numerous attempts to improve production performance of ruminant animals for last decades. The use of antibiotics like monensin, plant extracts, essential oils, rumen protected nutrients, prebiotics and DFM were some of options used to modify rumen microbial ecosystem for the improvement of ruminal digestion animal performances as well as hind-gut health.

Among them, DFM have received much interests from researchers since the use of DFM might give beneficial effects on rumen (modification of fermentation, the improvement of fiber digestion and antimicrobial effects) and hind-gut (pathogen exclusion by competition, stimulation of immune response) (McAllister et al., 2011). Mostly used microbial additives for ruminants were yeast(Nocek & Kautz, 2006; Nocek et al., 2003) , lactic acid producing bacteria(Nocek et al., 2002; Raeth-Knight et al., 2007) and lactic acid utilizing bacteria(Stein et al., 2006).

Recently *Bacillus* sp. like *Bacillus subtilis* and *Bacillus licheniformis* have risen as candidates for DFM source because of some beneficial characteristics of these species. *Bacillus* sp. can develop spore which is helpful not only for survival in harsh conditions (such as low pH in stomach or heat during feed pelleting process) but also for improved

immune response in the intestine without harmful action (Hong et al., 2005; Sanders et al., 2003). *Bacillus licheniformis* JK7 was isolated from Korea native goat's rumen, endoglucanase activity was enhanced by genome shuffling and then DFM was prepared by using SSF in the previous study. The objective of this experiment was to investigate the effects of *Bacillus* sp. -based DFM on rumen fermentation and rumen microbial population under *in vitro* condition.

Materials & Methods

1) *In vitro* experiment procedure

WB and SBM were used as ingredients for SSF. WB and SBM were mixed in the ratio of 4:6. Non-fermented mixed diet (NFM, 0.5g/incubation bottle) and fermented with *Bacillus licheniformis* (FM, 0.5g/incubation bottle) were used for the first in vitro experiment. Two diets were ground in a Wiley mill (Arthur H. Thomas, Philadelphia, PA) through a 1mm screen. Rumen fluid was collected 2 to 3hr after morning feeding from three Holstein steers that were fed timothy hay and a commercial concentrate feed (Cargill Agribrand Purina Korea Co. Ltd.) in the ratio of 4:6 and mixed before *in vitro* experiment. The strained rumen fluid was filtered through 8 layers of cheesecloth and transported to the laboratory in a pre-warmed thermo-flask, and then mixed with artificial buffer (Mcdougall, 1948, Table 16) in the ratio of 1:4 under an O_2 free CO_2 gas. The 25ml of rumen fluid and buffer mixture was dispensed anaerobically into 50ml serum bottles. Then the bottles were filled with CO_2 gas and capped with a butyl rubber stopper and aluminum caps. The bottles were anaerobically incubated for 0, 3, 6, 12 and 24 hr in a 39℃ incubator.

Table 16. The composition of Macdougall buffer

Chemicals	Amount (/ 1.5L)
$NaHCO_3$	11.76 g
$Na_2HPO_4.2H_2O$	5.544 g
KCl	0.684 g
NaCl	0.564 g
$MgSO_47H_2O$	0.045 g
4% $CaCl_2$ solution	1.2 ml

In 2nd *in vitro* trial, 0.5g of timothy (Con), 0.5g of timothy supplemented with 5% (T5) and 10% FM (T10) were used to investigate effects of DFM supplementation on forage digestibility, rumen fermentation and microbial population.

2) Chemical analysis

After fermentation, bottle contents were filtered through Whatman No. 541 paper (Fisher Scientific Company, Pittsburgh, PA, USA), and the residues were dried at 65℃ for 48hr to measure DM digestibility. The filtrate was centrifuged at 10,000g for 20min at 4℃, and the supernatant was collected to determine NH_3-N and VFA concentration. Filtrate pH was measured immediately after centrifugation using pH meter (Mettler Delta 340, Mettler Toledo, Essex, UK). After filtering the supernatant through a Millipore filter (0.22μm pore size), VFA concentration was measured by gas chromatography using a Hewlett Packard 5880A gas chromatograph (Hewlett Packard, Palo Alto, Ca, USA) employing a method described by Erwin (1961). Before

measuring VFA concentration, 1ml of rumen fluid which was mixed with 200μl of metaphosphoric acid (25%wt/vol) was stored at -20℃. Concentration of NH_3-N was determined using a colorimetric method (Chaney & Marbach, 1962). Briefly, phenol color reagent and alkali–hypochlorite reagent were mixed with rumen fluid samples and incubated at 37 ◦C water bath for 15 min. Then, the absorbance was measured using a spectrophotometer (Simadzu, Kyoto, Japan) at 630 nm. All samples were ground in a Wiley mill (Arther H. Thomas, Philadelphia, PA) and then analyzed for DM, ash, CP contents per AOAC (1990). NDF and ADF were analyzed by the method of Van Soest et al. (1991) with Ankom 200 fiber analyzer (Ankom Technology, NY, USA).

3) Real time PCR

The incubation of same set of batch culture was conducted to obtain whole bacterial sample associated with liquid and solid part. Representative rumen bacteria and *bacillus licheniformis* in culture samples were quantified to evaluate the effect of *bacillus* based DFM on rumen fermentation. Collected sample was frozen using liquid nitrogen and ground to disrupt bacterial cell wall. Then, genomic DNA was extracted by Cetyltrimethyl Ammonium Bromide (CTAB) method (Walker et al., 1991). The purity and integrity of genomic DNA extracted was checked using NanoDrop 200c spectrophotometer (Thermo Fisher 194 Scientific, Waltham, MA). The 16s rDNA primers

specific for *F. succinogens, R. albus, R. flavefaciens, E. ruminantium*, methanogen, *P. ruminicola, R. amylophilus, S. bovis* as well as 23s rDNA primers for *Bacillus licheniformis* were used for real time PCR.

Real-time PCR amplification and detection was carried out using LightCycler system (Roche, Penzberg, Germany). The reaction was performed with 20µl of final reaction volume which included 10µl of FastStart DNA Master SYBR Green I reaction kit (Roche, Penzberg, Germany), 1µl of forward primer, 1µl of reverse primer, 2µl of distilled water (DNAse & RNAse free water, Bioneer Co. Ltd., Korea) and 2µl of template DNA sample. Real time primers specific for selected microorganisms are listed in Table 17. Melting curve were performed by slow increase of temperature from 65 to 95℃ at a rate of 0.1℃ /sec to determine the specificity of each PCR amplification products. Absolute quantification based on standard curves of respective genes and their preparation using plasmid DNA containing specific genes were conducted according to Koike et al. (2007).

Table 17. Primer sequence for real time PCR

Species	Forward primer	Reverse primer	Size (BP)	References
F.succinogens	GTT CGG AAT TAC TGG GCG TAA A	CGC CTG CCC CTG AAC TAT C	121	(Denman,2006)
R. albus	CCC TAA AAG CAG TCT TAG TTC G	CCT CCT TGC GGT TAG AAC A	176	(Koike & Kobayashi, 2001)
R. flavefaciens	TCT GGA AAC GGA TGG TA	CCT TTA AGA CAG GAG TTT ACA A	295	(Koike & Kobayashi, 2001)
E. ruminantium	GCA TAA GCG CAC AGT ACC GCA	GAG ATA CTT CAT CAC TCA CGC G	248	This manuscript
Methanogen	GTC TCT GGT GAA ATC CTG TAG CTT A	GCA GCT CAA AGC CAC CCA ACA	247	This manuscript
P. ruminicola	CGA TGG ATG CCC GCT GTT TG	GAC AAC CAT GCA GCA CCT CC	243	This manuscript
R. amylophilus	GCG ACG ATC TCT AGC TGG TC	CGG AGT TAG CCG GTG CTT CTT	240	This manuscript
S. bovis	GCT CAC CAA GGC GAC GAT AC	GTA GTT AGC CGT CCC TTT CTG G	250	This manuscript
B. licheniformis	GAA ACC CAC TGC TCG TAA TGG	CGT TCT TTT GCA ACT CCG TAC A	210	This manuscript

4) Statistical analysis

All data including rumen fermentation characteristics as well as rumen microbial population profile were analyzed using GLM procedure (SAS, 1996). Tukey's test was used for comparison of difference. Significant difference between treatments were considered at $p<0.05$.

Results & Discussion

1) *In vitro* experiment I

1-1) Rumen fermentation characteristics

Table 18shows DM digestibility, pH variation and NH_3-N concentration at each time points. FM had significantly higher DM digestibility after 3 to 24 hrs incubation ($p<0.05$). This result is similar to results of previous *in situ* study. The effects of diet, time and their interaction on DM digestibility were also significant.

Bacillus licheniformis sp. Is known to degrade the substrate effectively since this microorganism has traditionally strong protease and α-amylase (Rojo et al., 2005). Furthermore, the strain used in SSF had endoglucanase, β-glucosidase and xylanase which can hydrolyze cellulosic materials effectively. Proteolytic and amylolytic enzymes released by *Bacillus licheniformis* sp. might be used to extend the potentially degradable portion in SBM. Hence, WB in FM could be degraded partially by improved cellulolytic enzyme from *Bacillus licheniformis* .

Table 18. DM digestibility, pH variation and NH_3-N concentration as influenced by treatments

	Incubation time (hr)					Mean	P-value		
	0	3	6	12	24		Diet	Time	Diet * Time
DM digestibility, %									
NFM	16.97	18.21^{b}	23.54^{b}	30.17^{b}	38.75^{b}	25.53^{b}	P<0.01	P<0.01	P<0.01
FM	16.71	29.08^{a}	39.69^{a}	48.60^{a}	58.52^{a}	38.52^{a}			
pH									
NFM	6.93^{b}	6.41^{b}	6.12^{b}	5.68^{b}	5.49^{b}	6.13^{b}	P<0.01	P<0.01	P<0.01
FM	7.02^{a}	6.74^{a}	6.56^{a}	6.42^{a}	6.41^{a}	6.63^{a}			
NH_3-N concentration, mg/ml									
NFM	1.41^{b}	3.17	1.47^{b}	7.29^{b}	15.97^{b}	5.86^{b}	P<0.01	P<0.01	P<0.01
FM	6.21^{a}	4.49	6.90^{a}	13.43^{a}	28.04^{a}	11.82^{a}			

NH_3-N concentration of FM was significantly higher than those of NFM at the incubation of 0, 6, 12, 24 ($p<0.05$). FM showed significantly higher pH values at all incubation time points than NFM and Mean pH was also higher when FM was incubated compared to NFM, which is presumed to be caused byhigher concentration of NH_3-N from FM.

1-2) VFA concentration

There were no significant differences between NFM and FM in total VFA and butyrate at all time points except 3 hrs incubation ($p>0.05$). In contrast, NFM had significantly higher portion of acetate (0, 3hrs incubation and mean value) as well as propionate (12, 24hrs incubation and mean value) than FM ($p<0.05$). FM showed higher isobutyrate, isovalerate and n-valerate concentration compared to NFM but their portion among total VFA concentration was small. Total VFA, butyrate and A:P ratio had only time effect but acetate, propionate, isobutyrate, isovalerate as well as n-valerate showed significant effect of diet, time and interaction between diet with time. As shown in Table 19, the relationship between VFA concentration and NH_3-N did not conform to the generally accepted one. Generally, highly degradable diets produce more end-products such as short chain fatty acids, NH_3-N and lower ruminal pH. However, our data show higher NH_3-N production did not lower VFA release. Therefore, rumen microbial population which could influence on rumen fermentation characteristics was investigated.

Table 19. VFA profiles as influenced by treatments

		Incubation time (hr)					Mean	P-value		
		0	3	6	12	24		Diet	Time	Diet * Time
Total VFA, mM										
	NFM	29.51	48.65[a]	73.37	100.61	133.27	77.08	P=0.11	P<0.01	P=0.2031
	FM	22.55	35.57[b]	72.22	101.31	135.38	73.41			
Acetic acid										
	NFM	20.85[a]	32.79[a]	43.65	51.64	65.87	42.96[a]	P<0.05	P<0.01	P=0.0569
	FM	14.47[b]	22.27[b]	40.67	53.14	66.16	39.34[b]			
Propionate										
	NFM	4.46	9.97	21.68	36.55[a]	46.10[a]	23.75[a]	P<0.01	P<0.01	P<0.05
	FM	3.43	7.46	22.29	32.49[b]	39.41[b]	21.01[b]			
Isobutyrate										
	NFM	0.27	0.30[b]	0.24[b]	0.43[b]	0.96[b]	0.44[b]	P<0.01	P<0.01	P<0.01
	FM	0.39	0.47[a]	0.80[a]	0.97[a]	3.37[a]	1.20[a]			
Butyrate										
	NFM	2.87	4.65[a]	6.97	10.66	16.98	8.43	P=0.0653	P<0.01	P=0.1925
	FM	2.48	3.58[b]	6.52	11.16	16.50	8.05			
Isovalerate										
	NFM	0.50[b]	0.31[b]	0.28[b]	0.46[b]	1.44[b]	0.60[b]	P<0.01	P<0.01	P<0.01
	FM	1.09[a]	1.28[a]	1.48[a]	2.76[a]	6.79[a]	2.68[a]			
n-valerate										
	NFM	0.55	0.63	0.53	0.87	1.91[b]	0.90[b]	P<0.01	P<0.01	P<0.01
	FM	0.68	0.52	0.45	0.79	3.15[a]	1.12[a]			
A:P ratio										
	NFM	4.67[a]	3.29[a]	2.01	1.41	1.43	2.56	P=0.0999	P<0.01	P<0.01
	FM	4.22[b]	2.99[b]	1.82	1.64	1.68	2.47			

1-3) Rumen microbial population

Rumen microbial population was investigated using real-time PCR (Table 20). The log copy numbers of *F. succinogens* in FM was significantly higher than those of NFM at 12hrs but lower at 3 and 6hrs including mean value ($p<0.05$). The counts of *R. flavefaciens*, *R. amylophilus* and *S. bovis* in FM were also lower than those of NFM at 3, 6 and 12hr as well as overall mean ($p<0.05$).

It is not easy to explain the current result of reduced major rumen microbial population. One possiblility is *Bacillus licheniformis* sp. might have produced some type of antibacterial substances during SSF. Pattnaik et al. (2001) isolated *Bacillus licheniformis* sp. from water buffalo and purified antimicrobial compound called 'lichenin' from this bacteria. They reported that lichenin had antagonistic activity against *S. bovis*, *R. flavefaciens*, *R. albus* and *E. ruminantium* in strictly anaerobic condition (Pattnaik et al., 2001). In present study fermented feed containing high number of *Bacillus licheniformis* sp. was used as a sole substrate for *in vitro* experiment without other nutrient sources. So, antimicrobial compounds induced by FM might have affected rumen microbial ecosystem negatively. The other interesting observation was that *Methanogen* was also reduced by FM treatment (Table 3, $p<0.05$) at 3 and 6hrs. The mean value of *R. albus*, *E. ruminantium* and *P. ruminicola* showed no significant difference between treatments ($p>0.05$).

Table 20. Rumen microbial population as influenced by treatments

	Incubation time (hr)				Mean	P-value		
	0	3	6	12		Diet	Time	Diet * Time
F.succinogens ,log copy numbers/ml								
NFM	5.94	7.22[a]	7.30[a]	7.45[b]	6.98[a]	P<0.01	P<0.01	P<0.01
FM	6.03	6.39[b]	6.63[b]	7.91[a]	6.74[b]			
R. albus								
NFM	4.81[b]	5.12	5.30	5.50[a]	5.18	P=0.6624	P<0.01	P<0.01
FM	5.24[a]	5.10	5.17	5.31[b]	5.21			
R. flavefaciens								
NFM	7.60	8.23[a]	8.23[a]	8.27[a]	8.08[a]	P<0.01	P<0.01	P<0.01
FM	7.47	7.61[b]	7.71[b]	7.77[b]	7.64[b]			
E. ruminantium								
NFM	6.50	7.25[a]	7.57	7.65	7.24	P=0.2462	P<0.01	P<0.05
FM	6.54	6.99[b]	7.52	7.75	7.20			
Methanogen								
NFM	5.81	6.39[a]	6.70[a]	6.51	6.35[a]	P<0.01	P<0.01	P<0.01
FM	5.86	6.04[b]	6.39[b]	6.44	6.18[b]			
P. ruminicola								
NFM	7.25	8.15[a]	8.50	8.53	8.11	P=0.1016	P<0.01	P=0.1756
FM	7.35	7.91[b]	8.36	8.44	8.02			
R. amylophilus								
NFM	6.70	8.23[a]	8.53[a]	8.80[a]	8.07[a]	P<0.01	P<0.01	P<0.01
FM	6.72	7.64[b]	8.32[b]	8.60[b]	7.82[b]			
S. bovis								
NFM	5.61[b]	7.94[a]	7.65[a]	7.65[a]	7.21[a]	P<0.05	P<0.01	P<0.01
FM	6.15[a]	7.42[b]	7.38[b]	7.44[b]	7.10[b]			

2) *In vitro* experiment II

2-1) Rumen fermentation characteristics

In the first *in vitro* experiment, there was some indication that FM might bring some negative effects on rumen fermentation when FM was used for sole nutrient source possibly due to some antimicrobial activity by *Bacillus licheniformis* in FM. Also it seems that considerable amounts of nutrient in substrate would be used by DFM firstly and therefore more fermented products such as VFA were actively consumed by DFM itself. That might be the other reason why FM showed similar VFA production although FM treatment had higher DM digestibility compared to NFM in previous study.

Therefore, second *in vitro* experiment which used DFM as a feed additive was conducted. All treatments had timothy as a main substrate. We added 10% of NFM (DM basis) (Con), 5% of FM (T5) and 10% of FM (T10) to main substrate. Table 21 shows DM digestibility, pH variation and NH3-N production in second experiment. The addition of FM resulted in higher DM digestibility ($p<0.05$) indicating that FM gave beneficial effect on fiber digestibility possibly by the action of FM cellulolytic enzymes. T5 and T10 exhibited lower pH value which was different from previous experiment although these treatment had higher NH3-N concentration than those of Con ($P<0.05$). That was understandable result considering VFA production (Table 22). All

parameters in Table 21 had the significant effect of diet, time and their interaction.

Table 21. DM digestibility, pH variation and NH_3-N concentration as influenced by treatments

	Incubation time (hr)					Mean	P-value		
	0	3	6	12	24		Diet	Time	Diet * Time
DM digestibility, %									
Con	12.53	13.10^{b}	15.83^{b}	22.17^{b}	34.93	19.71^{b}	P<0.01	P<0.01	P<0.01
T5	13.70	16.41^{a}	19.66^{a}	27.26^{a}	39.75	23.36^{a}			
T10	13.52	17.08^{a}	19.20^{a}	29.54^{a}	37.95	23.46^{a}			
pH									
Con	6.85	6.76	6.73^{a}	6.62^{a}	6.37^{a}	6.67^{a}	P<0.01	P<0.01	P<0.01
T5	6.85	6.72	6.67^{ab}	6.54^{b}	6.26^{b}	6.61^{b}			
T10	6.84	6.69	6.64^{b}	6.50^{c}	6.16^{c}	6.57^{c}			
NH_3-N concentration, mg/ml									
Con	2.08^{b}	4.92	5.62	4.64^{b}	5.77^{b}	4.61^{c}	P<0.01	P<0.01	P<0.01
T5	3.12^{ab}	5.34	5.87	5.72^{b}	9.49^{a}	5.91^{b}			
T10	3.62^{a}	5.64	7.01	8.09^{a}	11.76^{a}	7.22^{a}			

2-2) VFA concentration

T5 or T10 showed significantly higher concentration of total VFA (6, 12, 24hrs and mean value), acetate (6, 12, 24hrs and mean value), propionate (3, 6, 12, 24hrs and mean value), isobutyrate (3, 6, 12, 24hrs and mean value), butyrate (6, 12, 24hrs and mean value), isovalerate (3, 6, 12, 24hrs and mean value) and n-valerate (6, 12, 24hrs and mean value) than Con(Table 22, $p<0.05$).

All VFA including total VFA were affected significantly by diet, time and their interaction ($p<0.01$). This result indicated that FM gave positive effect on rumen microorganism when it used as feed additive having appropriate amount. Several researches were also reported the effect of *Bacillus sp.* DFM source on VFA production which was similar with our result. Qiao et al. (2010) reported that *Bacillus licheniformis* enhanced nutrient digestibility, bacterial-N production and VFA production in the rumen thereby improving milk yield. The propionate production was also increased when early lactation dairy cows were fed *Bacillus subtilis natto* fermentation product (Peng et al., 2012).

Table 22. VFA profiles as influenced by treatments

	Incubation time (hr)					Mean	P-value		
	0	3	6	12	24		Diet	Time	Diet * Time
Total VFA, mM									
Con	17.04	29.81	36.04^{c}	44.41^{b}	59.18^{c}	37.30^{c}	P<0.01	P<0.01	P<0.01
T5	18.27	27.98	38.34^{b}	49.96^{a}	69.75^{b}	40.86^{b}			
T10	18.65	31.33	41.15^{a}	53.82^{a}	76.07^{a}	44.20^{a}			
Acetic acid									
Con	11.64	19.46	23.49^{b}	29.02^{b}	37.53^{b}	24.23^{c}	P<0.01	P<0.01	P<0.01
T5	12.52	18.06	24.82^{ab}	32.8^{a}	44.65^{a}	26.57^{b}			
T10	12.88	20.15	26.25^{a}	34.41^{a}	47.62^{a}	28.26^{a}			
Propionate									
Con	3.42	7.43^{ab}	8.92^{b}	10.6^{b}	14.42^{c}	8.96^{c}	P<0.01	P<0.01	P<0.01
T5	3.67	7.1^{b}	9.65^{b}	11.9^{b}	16.82^{b}	9.83^{b}			
T10	3.67	8.1^{a}	10.68^{a}	13.45^{a}	19.14^{a}	11.01^{a}			
Isobutyrate									
Con	0.20	0.26^{b}	0.27^{c}	0.33^{c}	0.52^{c}	0.32^{c}	P<0.01	P<0.01	P<0.01
T5	0.22	0.26^{b}	0.32^{b}	0.39^{b}	0.65^{b}	0.37^{b}			
T10	0.22	0.31^{a}	0.38^{a}	0.45^{a}	0.75^{a}	0.42^{a}			
Butyrate									
Con	1.38	2.15	2.72^{b}	3.52^{b}	5.29^{b}	3.01^{c}	P<0.01	P<0.01	P<0.01
T5	1.47	2.04	2.82^{ab}	3.8^{b}	5.96^{ab}	3.22^{b}			
T10	1.48	2.21	3.03^{a}	4.24^{a}	6.58^{a}	3.51^{a}			
Isovalerate									
Con	0.24	0.26^{b}	0.25^{c}	0.31^{c}	0.61^{c}	0.33^{c}	P<0.01	P<0.01	P<0.01
T5	0.25	0.28^{ab}	0.3^{b}	0.38^{b}	0.72^{b}	0.39^{b}			
T10	0.25	0.31^{a}	0.37^{a}	0.47^{a}	0.85^{a}	0.45^{a}			
n-valerate									
Con	0.16	0.25	0.39^{b}	0.62^{c}	0.82^{c}	0.45^{c}	P<0.01	P<0.01	P<0.01
T5	0.15	0.24	0.43^{ab}	0.69^{b}	0.94^{b}	0.49^{b}			
T10	0.16	0.24	0.45^{a}	0.81^{a}	1.13^{a}	0.56^{a}			
A:P ratio									
Con	3.4^{b}	2.62^{a}	2.64	2.74	2.65	2.81^{a}	P<0.05	P<0.01	p=0.1686
T5	3.42^{ab}	2.54^{ab}	2.57	2.76	2.65	2.79^{a}			
T10	3.51^{a}	2.49^{b}	2.46	2.56	2.49	2.7^{b}			

2-3) Rumen microbial population

Rumen microbial population was estimated by using real-time PCR to confirm the interaction between rumen fermentation and rumen microbial population (Table 23). Interestingly, fibrolytic (*R. albus, R. flavefaciens, E. ruminantium*), starch using bacteria (*R. amylophilus, S. bovis*), proteolytic bacteria (*P. ruminicola*) numbers are increased significantly by addition of FM (Table 23, $p<0.05$). Overall mean of *F. succinogens* was not different significantly ($p>0.05$) but, T5 and T10 had significantly higher copy numbers than those of Con at 12hrs incubation ($p<0.05$). The addition of FM increased copy numbers of *B. licheniformis* significantly at all time points and mean value indicating that *B. licheniformis* in FM could adapt to the rumen ecosystem. Qiao et al. (2010) suggested that *B. licheniformis* supplementation might stimulate fibrolytic bacteria since the culture of *B. licheniformis* provided vitamins, glucose, short chain fatty acids such as malate, formate, succinate and aspartate which were required for rumen bacterial growth. Once *Bacillus* based DFM was ingested, it seems to stimulate the growth of rumen bacteria by their beneficial end-products (Qiao et al., 2010) which can induce the improvement nutrient digestibility, VFA production (Sun et al., 2012) and microbial protein synthesis in dairy cows. Present results also showed effects of *Bacillus* culture supplementation on rumen fermentation as in previous studies. The number of methanogens was reduced significantly when 10% of FM was added to substrate (Table 23, $p<0.05$). Methane emission by

methanogens in the rumen is considered to cause the greenhouse effect. The mechanism how *Bacillus* based DFM reduced the methanogen requires further study but, this methanogen repression effect can be a good merit of this organism and can become a good candidate for animal feed industry for reducing greenhouse gas emission and improving the efficiency of energy utilization by ruminants.

Table 23. Rumen microbial population as influenced by treatments

	Incubation time (hr)				Mean	P-value		
	0	3	6	12		Diet	Time	Diet * Time
*F.succinogenes,*log copy numbers/ml								
Con	6.83	6.57	6.47	7.19c	6.77	p=0.4111	P<0.01	p=0.0523
T5	6.87	6.51	6.63	7.28b	6.82			
T10	6.86	6.40	6.47	7.42a	6.79			
R. albus								
Con	4.63b	4.35	4.13	4.64	4.44b	p=0.0656	P<0.01	p=0.1039
T5	4.83a	4.30	4.22	4.67	4.56a			
T10	4.78a	4.15	4.45	4.73	4.47b			
R. flavefaciens								
Con	7.20	7.17	7.02b	7.27b	7.17b	P<0.01	P<0.01	P<0.01
T5	7.31	7.18	7.38a	7.43a	7.33a			
T10	7.31	7.04	7.13b	7.42a	7.23b			
E. ruminantium								
Con	6.11b	6.10	6.11	6.33b	6.16b	P<0.05	P<0.01	P<0.05
T5	6.23a	6.07	6.30	6.38ab	6.25a			
T10	6.18ab	5.97	6.10	6.44a	6.17b			
Methanogen								
Con	3.99	4.07a	4.2a	4.41a	4.17a	P<0.01	P<0.01	p=0.3211
T5	4.22	3.98ab	4.32a	4.41a	4.23a			
T10	4.02	3.78b	3.87b	4.24b	3.98b			
P. ruminicola								
Con	6.67	7.18	7.05c	7.21b	7.03b	P<0.01	P<0.01	P<0.01
T5	6.75	7.14	7.38a	7.35a	7.15a			
T10	6.69	7.07	7.19b	7.45a	7.1a			
R. amylophilus								
Con	6.49b	6.87	7.06c	7.17c	6.90b	P<0.01	P<0.01	P<0.01
T5	6.6a	6.81	7.29a	7.29b	7.00a			
T10	6.53ab	6.74	7.17b	7.46a	6.98a			
S. bovis								
Con	4.06b	5.03	4.93b	5.15b	4.79b	P<0.01	P<0.01	P<0.05
T5	4.16a	5.10	5.21a	5.24ab	4.93a			
T10	4.13a	5.08	5.11a	5.39a	4.93a			

	Incubation time(hr)				Mean	P-value		
	0	3	6	12		Diet	Time	Diet * Time
B. lichniformis								
Con	0.00b	0.00c	0.00b	2.12b	0.53b	P<0.01	P<0.01	P<0.05
T5	3.57a	3.75b	3.88a	3.86a	3.77a			
T10	3.65a	3.97a	4.01a	4.27a	3.98a			

EXPERIMENT V

Effects of Supplementation of *Bacillus licheniformi*s Based DFM on Rumen Fermentation and Microbial Population in Holstein Steers

Introduction

Previously, the effects of *Bacillus* based DFM on rumen fermentation and microbial population were investigated in the *in vitro* experiment. Some beneficial effect was found in digestibility, VFA production and bacterial growth stimulation as well as methane production when FM was added to substrate. Therefore, *in vivo* experiment was conducted to confirm the beneficial effects of *Bacillus* based DFM by using steers. In this experiment, *in situ* ruminal fiber digestibility, total tract digestibility, rumen fermentation characteristics, rumen microbial protein synthesis (MPS), and rumen microbial population were also investigated. The population of *Bifidobacterium, E.coli* and *B. licheniformis* was also investigated in the fecal sample to confirm the effects of *Bacillus* based DFM on intestinal microbial population.

Materials & Methods

1) Development of DFM using solid state fermentation

WB and SBM were used as ingredients for solid state fermentation. Two ingredients were ground in a Wiley mill (Arthur H. Thomas, Philadelphia, PA) through a 1mm screen and dried at 65℃ for 2days. WB and SBM were mixed at the ratio of 6:4. Solid state fermentation was conducted in mass fermentation system. Appropriate amount of distilled water was added to fermentable tray to maintain moisture level of substrates at 45%. The trays were sterilized by autoclaving at 121℃ for 20min and taken UV irradiation until substrates were cooled at room temperature. Then, 1% (vol/wt of substrate DM) of *B. licheniformis* culture was inoculated to solid media while, control group trays were applied with same volume of distilled water. The trays containing substrates and inoculums were thoroughly mixed to distribute the inoculums on substrates, thereafter whole mixture was incubated at 37℃ for 4days.

2) Preparation of experimental diets

Timothy hay, commercial concentration feed, NFM and FM were used in this study. Timothy hay was chopped in size of 10cm and mixed with concentration feed at a ratio of 4:6 before feeding. NFM and FM were ground in a Wiley mill (Arthur H. Thomas, Philadelphia, PA)

through a 1mm screen. The chemical composition of timothy and concentration feed are presented in Table 24.

Table 24. Chemical composition of experimental diet

Item (%)	Timothy	Concentrate	Experimental diet
Dry matter	90.42	86.61	88.13
Crude protein	8.13	14.09	11.71
Ether extract	1.7	5.14	3.76
Crude fiber	37.54	6.94	19.18
Crude ash	6.02	4.77	5.27
ADF	41.82	10.29	22.90
NDF	71.49	20	40.60
Hemicellulose	29.67	9.71	17.69
Lignin	0.48	0.44	0.46
Silica	1.02	0.05	0.44
Ca	0.9	0.75	0.81
P	0.66	0.35	0.47

3) Animals and experimental procedure

Three Holstein steers, weighing 563±50 kg, were fitted with a rumen cannula and were fed three different diets in order to evaluate effects of *B. licheniformis* based DFM supplementation on rumen fermentation, MPS and rumen microbial population. Steers were allocated to one of three dietary treatment regimens and were housed in individual pens. All diets had same basal diet which included timothy hay and commercial feed at a ratio of 4:6 except that two test group received additional supplementation of DFM. Control group (Con) were fed basal diet with 0.5% of NFM, while T1 and T2 group were given basal

diet with 0.1%, 0.5% of FM supplementation. The animals were fed at 2.0% of their body weight in two equal portions, one at 8:00 am and the other at 8:00 pm. A clean water and mineral mixture was available to each animal at all times in unlimited amounts. The experimental feeding scheme was based on a 3×3 Latin square, and each of the three feeding periods was 18 days long, with 10 days for adaptation four days for fecal sampling and the last four days for *in situ* trial, in which effects of DFM supplementation on forage digestion in the rumen was examined.

4) Sample collection

Rumen fluid was collected via the cannula on the 14 day of each period at 0, 3, 6 and 12 h after the 8:00 a.m. feeding. The ruminal fluid was centrifuged at 8,000 rpm for 10 min, and the supernatant withdrawn from each tube was transferred to a 50 ml Corning centrifuge tube. Ruminal pH was measured immediately after sampling using a pH meter (Mettler Delta 340, Mettler Toledo, Essex, UK). Each supernatant was acidified with 5 ml 6 N HCl and stored at -20℃ for subsequent determination of ammonia and volatile fatty acid concentrations.

Urine was collected for three consecutive days (day 11 to day 13) of each period using a plastic receptacle, which contained 500 ml 4 N H_2SO_4 to prevent N losses. Total urine output was determined by

weight each day, prior to the morning feeding. The urine was sampled at an output rate of 10% and was frozen at -20℃ after collection. Total feces were collected at 7:30 am during each sampling period. A 10% of aliquot of total feces was sampled and pooled for each animal. These samples were dried in a 60℃ dry oven for 72 h and ground to pass through a 2 mm screen.

5) *In situ* study

An *in situ* trial was conducted to examine the ruminal degradation kinetics of timothy hay when steers were fed different type and amount of bacterial DFM. Ruminal DM, organic matter (OM) and CP disappearance rates of each treatment were measured using the nylon bag technique as suggested by Ørskov et al. (1980). Experimental animals were offered twice daily (at 8 am and 8pm) Con, T1 and T2 diets. Total amount of feed offered to animals was 2% of BW. Fresh water and a mineral mixture were available to each animal at all times.

A total of 15g of timothy hay which were ground in a Wiley mill (Arthur H. Thomas, Philadelphia, PA) through a 1mm screen, were weighed and allotted per nylon bag having 53±10 μm (Bar Diamond, Idaho, USA) pore size. The bags were placed into the ventral sac of the rumen of each Holstein steer through rumen cannula. The incubation started at 8:30 am after feeding. All feed samples were incubated for 0, 3, 6, 12, 24 and 48hr. After incubation for respective time, bags were

removed from the rumen, and washed by hand under cold tap water to remove feed particle attached to the surface of the bags until the rinse water became clear. Zero time incubation bags were washed in the same way without incubation in the rumen. After washing, all nylon bags were dried at 65℃ for 48hr and weighed to estimate the DM residues. Contents of the bags were stored at 4℃ until chemical analysis.

6) Analysis

Stored rumen fluid was thawed and centrifuged at 10,000g for 20min at 4℃. After filtering the supernatant through a Millipore filter (0.22μm pore size), VFA concentration was measured by gas chromatography using a Hewlett Packard 5880A gas chromatograph (Hewlett Packard, Palo Alto, Ca, USA) employing a method described by Erwin (1961).

Before measuring VFA concentration, 1ml of rumen fluid which was mixed with 200μl of metaphosphoric acid (25%wt/vol) was stored at 20℃. Concentration of NH_3-N was determined using a colorimetric method (Chaney & Marbach, 1962). Briefly, phenol color reagent and alkali–hypochlorite reagent were mixed with rumen fluid samples and incubated at 37 ◦C water bath for 15 min. Then, the absorbance was measured using a spectrophotometer (Simadzu, Kyoto, Japan) at 630 nm.

All feed and fecal samples, urine and *in situ* residues were ground in a Wiley mill (Arther H. Thomas, Philadelphia, PA) and then analyzed for DM, ash, CP contents per AOAC (1990). NDF and ADF were analyzed by the method of Van Soest et al. (1991) with Ankom 200 fiber analyzer (Ankom Technology, NY, USA).

Urine was analyzed for purine derivatives (PD) according to the modified method (George et al., 2006) of Chen et al. (1990) for allantoin and uric acid using HPLC (HP1100, Hewlett Packard, Palo Alto, Ca, USA). The daily output of PD in the urine was calculated, and this value was used to estimate the amount of microbial N absorbed, using the method of Chen et al. (1992).

The cellulase and xylanase activity was measured by spectrometric determination of reducing sugars by DNS method (Ghose, 1987). Briefly, the mixture of enzyme and 1% CMC solution (1:1) was prepared in 50mM phosphate buffer (pH6). Endoglucanase activity was assayed with CMC as a substrate. β-glucosidase activity was determined using salicin (2-hydroxymethyl-phenyl-β-D-glucopyranoside) as a substrate and xylanase activity was determined by measuring the release of xylose from birch wood xylan. For crude enzyme preparation, collected rumen fluids were centrifuged at 13,000g for 5min. The supernatants were used for enzyme assays. The reaction mixture was incubated at 37℃ for 30 min. After incubation

300ul of DNS reagent was added and the mixture was heated to 99℃ for 5 min in a boiling water bath. The release of reducing sugar was calculated from OD measured at 546nm. One unit of enzymatic activity was defined as the amount of enzyme that released 1umol of reducing sugar per minute.

7) Real time PCR

Representative rumen bacteria and *bacillus licheniformis* in collected rumen fluid samples were quantified to evaluate the effect of *bacillus* based DFM on rumen fermentation. Collected samples were frozen using liquid nitrogen and ground to disrupt bacterial cell wall. Then, genomic DNA was extracted by CTAB method (Walker et al., 1991). The purity and integrity of Genomic DNA extracted was checked using NanoDrop 200c spectrophotometer (Thermo Fisher 194 Scientific, Waltham, MA). The specific 16s rDNA primers for total bacteria, *F. succinogens, R. albus, R. flavefaciens, E. ruminantium*, methanogen, *P. ruminicola, R. amylophilus, S. bovis* as well as 23s rDNA primers for *Bacillus licheniformis* were used for real time PCR.

Real-time PCR amplification and detection was carried out using LightCycler system (Roche, Penzberg, Germany). The reaction was performed with 20μl of final reaction volume which included 10μl of FastStart DNA Master SYBR Green I reaction kit (Roche, Penzberg, Germany), 1μl of forward primer, 1μl of reverse primer, 2μl of distilled

water (DNAse & RNAse free water, Bioneer Co. Ltd., Korea) and 2μl of template DNA sample. Specific real time primers for selected microorganisms are listed in Table 25. Melting curves were obtained by increment of temperature from 65 to 95℃ at a rate of 0.1℃ /sec to determine the specificity of each PCR amplification products. Absolute quantification based on standard curves of respective genes and their preparation using plasmid DNA containing specific genes were conducted according to Koike et al. (2007).

Table 25. Primer sequence for real time PCR

Species	Forward primer	Reverse primer	Size (BP)	References
F.succinogens	GTT CGG AAT TAC TGG GCG TAA A	CGC CTG CCC CTG AAC TAT C	121	(Denman,2006)
R. albus	CCC TAA AAG CAG TCT TAG TTC G	CCT CCT TGC GGT TAG AAC A	176	(Koike & Kobayashi, 2001)
R. flavefaciens	TCT GGA AAC GGA TGG TA	CCT TTA AGA CAG GAG TTT ACA A	295	(Koike & Kobayashi, 2001)
E. ruminantium	GCA TAA GCG CAC AGT ACC GCA	GAG ATA CTT CAT CAC TCA CGC G	248	This manuscript
Methanogen	GTC TCT GGT GAA ATC CTG TAG CTT A	GCA GCT CAA AGC CAC CCA ACA	247	This manuscript
P. ruminicola	CGA TGG ATG CCC GCT GTT TG	GAC AAC CAT GCA GCA CCT CC	243	This manuscript
R. amylophilus	GCG ACG ATC TCT AGC TGG TC	CGG AGT TAG CCG GTG CTT CTT	240	This manuscript
S. bovis	GCT CAC CAA GGC GAC GAT AC	GTA GTT AGC CGT CCC TTT CTG G	250	This manuscript
B. licheniformis	GAA ACC CAC TGC TCG TAA TGG	CGT TCT TTT GCA ACT CCG TAC A	210	This manuscript

8) Statistical analysis

The data for *in situ* trial, enzyme production, rumen fermentation characteristics and rumen microbial population were analyzed using GLM procedure (SAS, 1996). Tukey's test was used for comparison of difference. Significant difference between treatments were considered at $p<0.05$.

Results & Discussion

Total tract digestibility of DM, CP, EE, NDF and ADF are presented inTable 26. Nutrient digestibility was not significantly influenced by different diets ($p>0.05$). Dietary treatment did not affect ruminal pH, NH_3-N concentration and microbial N synthesis either ($p>0.05$).

Table 26. Total tract digestibility of nutrients, pH variation, NH_3-N concentration and microbial N as influenced by treatments

	Con	T1	T2	SEM	P value
Digestibility (%)					
DM	67.04	66.46	66.15	0.73	0.9069
CP	65.02	63.49	64.72	0.68	0.6827
EE	69.13	75.37	76.51	2.29	0.4208
ADF	44.60	45.66	43.80	1.96	0.9445
NDF	51.48	52.52	51.25	1.54	0.9527
pH variation					
0h	6.49	6.13	6.31	0.11	0.4858
3h	6.30	6.02	6.07	0.07	0.3134
6h	6.17	6.13	6.33	0.06	0.3712
12h	6.44	6.26	6.48	0.07	0.4108
NH_3-N concentration (mg/100ml)					
0h	5.49	5.21	4.29	0.38	0.4678
3h	5.89	7.83	6.76	0.60	0.4824
6h	2.61	3.14	2.81	0.53	0.9376
12h	4.36	4.42	4.55	0.35	0.9819
microbial N(g/d)					
	103.30	103.50	100.62	6.17	0.9832

Table 27. VFA production as influenced by treatments

	Con	T1	T2	SEM	P value
Total VFA(mM)					
0h	157.55	115.01	115.02	9.60	0.0938
3h	182.20	136.06	155.93	10.40	0.2025
6h	151.53	133.95	125.86	6.53	0.2918
12h	141.80	100.58	135.46	8.14	0.0549
Acetic acid					
0h	103.96	84.17	89.10	5.77	0.3959
3h	129.38	99.80	111.75	7.15	0.2612
6h	107.25	99.77	91.72	4.47	0.4192
12h	97.99	75.26	100.31	5.38	0.0903
Propionate					
0h	29.78^{a}	17.15^{b}	20.88^{b}	2.08	0.0066
3h	31.40	20.73	27.53	2.03	0.0692
6h	26.09	20.12	21.84	1.32	0.1666
12h	26.56^{a}	15.72^{b}	21.79^{ab}	1.88	0.0280
Butyrate					
0h	19.71^{a}	13.23^{a}	4.84^{b}	2.29	0.0015
3h	20.29	14.84	16.20	1.41	0.2941
6h	16.69	13.80	11.94	1.00	0.1412
12h	16.37^{a}	9.45^{b}	12.82^{ab}	1.15	0.0142
n-valerate					
0h	4.10^{a}	0.45^{b}	0.27^{b}	0.75	0.0290
3h	1.13	0.69	0.44	0.16	0.2093
6h	1.51^{a}	0.25^{b}	0.36^{b}	0.22	0.0089
12h	0.88	0.16	0.54	0.16	0.1771
A:P ratio					
0h	3.49^{c}	4.90^{a}	4.24^{b}	0.21	0.0008
3h	4.12	4.86	4.08	0.18	0.1516
6h	4.12	4.94	4.26	0.18	0.1104
12h	3.71^{b}	4.79^{a}	4.64^{a}	0.18	0.0038

As seen in Table 27, total VFA and acetate concentration were not different significantly among treatments at all time points. However, Con had significantly higher portion of propionate and butyrate than those of other groups which had FM as a feed additive at 0 and 12hrs ($p<0.05$). N-valerate of Con was also higher than those of T1 and T2 at 0 and 6 hrs.

Ruminal fiber digestion was also evaluated by *in situ* experiment using timothy as a substrate (Table 28). All groups showed increased DM disappearance rate as incubation time passed but there was no significant difference between treatments ($p>0.05$).

Table 28. DM disappearance rate as influenced by treatments. Timothy was used for *in situ* experiment to investigate ruminal fiber digestion.

	Con	T1	T2	SEM	P value
DM disappearance(%)					
0h	9.14	7.79	7.77	0.51	0.5211
3h	17.20	16.26	16.20	0.38	0.5500
6h	20.08	19.47	19.89	0.48	0.8992
12h	27.44	25.54	25.93	0.77	0.6279
24h	38.30	36.85	36.13	0.73	0.5318
48h	49.87	50.59	51.60	0.36	0.1425

The rumen microbial population was investigated to find interaction between rumen fermentation and rumen microbial population (Table 29). Only *R. flavefaciens* at 6hrs and *B. licheniformis* at 0, 6, 12hrs were significantly higher ($p<0.05$) in T1 and T2 feeding steers than in Con group. Fecal samples from steers fed T1 and T2 contained higher *B. licheniformis* population. The addition of *Bacillus* based DFM did not affect the population of other bacteria such as beneficial (*Bifidobacterium* sp.) and harmful (*E. Coli* O157) ones significantly but the treatments having DFM showed higher log copy numbers of *B. licheniformis* compared to Con (Table 30).

Table 29. Rumen microbial population profiles by real-time PCR

	Con	T1	T2	SEM	P value
F.succinogens					
0h	8.71	8.91	8.83	0.12	0.8288
3h	8.16	8.68	8.49	0.21	0.6621
6h	8.16	8.76	8.73	0.20	0.4179
12h	8.76	8.84	8.95	0.09	0.7573
R. albus					
0h	6.40	6.59	6.54	0.17	0.9117
3h	5.76	6.34	5.18	0.35	0.4567
6h	5.82	6.51	6.48	0.18	0.2191
12h	5.23	6.81	6.71	0.41	0.2220
R. flavefaciens					
0h	7.75	8.27	8.32	0.16	0.3752
3h	7.29	8.28	8.05	0.20	0.0919
6h	7.43^{b}	8.27^{ab}	8.37^{a}	0.18	0.0286
12h	7.90	8.51	8.50	0.14	0.1226

	Con	T1	T2	SEM	P value
E. ruminantium					
0h	8.47	8.61	7.06	0.48	0.3901
3h	7.80	8.41	8.28	0.19	0.4291
6h	7.80	8.10	8.43	0.15	0.2728
12h	8.44	8.58	8.54	0.06	0.6788
Methanogen					
0h	8.62	8.52	8.46	0.08	0.7554
3h	6.76	7.98	7.80	0.55	0.6988
6h	7.12	8.35	8.52	0.32	0.1480
12h	8.28	7.45	8.59	0.42	0.5863
P. ruminicola					
0h	9.85	10.13	9.99	0.13	0.7396
3h	9.32	10.10	9.85	0.24	0.4506
6h	9.31	10.03	9.88	0.20	0.3529
12h	9.91	10.22	10.04	0.11	0.5756
R. amylophilus					
0h	8.11	5.31	8.29	1.03	0.4705
3h	7.72	7.95	8.12	0.14	0.5557
6h	5.09	7.38	5.42	1.14	0.7343
12h	7.63	5.20	8.29	0.90	0.3813
S. bovis					
0h	8.13	7.83	7.59	0.10	0.0667
3h	6.14	7.64	7.67	0.53	0.4523
6h	6.00	7.30	7.52	0.49	0.4579
12h	7.75	7.72	7.76	0.08	0.9813
B. licheniformis					
0h	4.69^{b}	5.52^{ab}	5.85^{a}	0.22	0.0519
3h	4.66	3.58	6.08	0.65	0.3239
6h	4.34^{b}	5.56^{a}	5.96^{a}	0.27	0.0062
12h	4.29^{b}	5.31^{a}	5.77^{a}	0.23	0.0017

Table 30. *Bifidobacterium, E. Coli* and *B. licheniformis* sp. in fecal sample

	Control	T1	T2	SEM	P value
Bifidobacteria	8.47	9.00	8.44	0.20	0.5013
E. coli	7.30	7.04	4.61	0.83	0.3983
B. licheniformis	6.33^{b}	6.77^{a}	6.78^{a}	0.08	0.0110

The endoglucanase, β-glucosidase and xylanase activity in the rumen fluid was analyzed using DNS method (Table 29). Regardless of treatments, all enzymes at 0 and 12hrs showed higher activity compared to other rumen fluid which was collected at 3 and 6hrs after feeding but there was no significant difference among treatments ($p>0.05$).

Table 31. enzyme activities as influenced by treatments (units/ml).

	Control	T1	T2	SEM	P value
Endoglucanase					
0h	0.46	0.29	0.53	0.06	0.2559
3h	0.27	0.31	0.30	0.02	0.6930
6h	0.32	0.21	0.32	0.03	0.2161
12h	0.43	0.33	0.46	0.04	0.3110
Xylanase					
0h	0.32	0.34	0.40	0.05	0.8424
3h	0.28	0.26	0.25	0.01	0.7538
6h	0.26	0.23	0.26	0.01	0.6768
12h	0.34	0.30	0.38	0.03	0.6471
b-glucosidase					
0h	0.36	0.18	0.41	0.05	0.0761
3h	0.20	0.22	0.20	0.02	0.8494
6h	0.19	0.19	0.23	0.02	0.4584
12h	0.26	0.26	0.34	0.04	0.6737

Although some beneficial effects were found when the DFM was used in case of *in vitro* study, similar results at *in vivo* experiment were not observed. There are several reasons why the beneficial effects do not occur in the *in vivo* study. It is well known that there are many factors involved in effectiveness of DFM in ruminants and some of factors suggested are: differences in microbial organisms (bacterial or fungal), strains and combination of various microorganisms (Qiao et al., 2010). Hence, the inclusion level of DFM in the feeds, diet composition, the level of feed intake, the frequency of feeding and animal factors like the species, age, the status of health, stress condition and physiological stage could affect on the effects of DFM on ruminant (Wagner et al., 1990). DFM used in present study could have resulted in more positive response if highly susceptible ruminants such as neonatal or weaning calves, early lactating dairy cows and finishing beef having such metabolic disorder by fed excessive amounts of grain diets might be a suitable model for DFM experiment. In this experiment, we used fully grown steers having no metabolic diseases and more research under diverse experimental conditions is warranted in the future.

However, the high copy number of *B. licheniformis* found in the rumen fluid and fecal samples after feeding T1 and T2 diet may indicates that this microorganism suitably adapted and maintained its population in the rumen as well as in hind-gut environment, which is one of the most important factors for DFM. Selected strains of DFM must survive in digestive systems of animals to provide intended beneficial effects such

as the improvement of nutrient utilization, the modulation of rumen microbial ecosystem, the stimulation of immune response and competitive exclusion of pathogen as a probiotics.

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SUMMARY IN KOREANS

생균제는 대상 동물의 하부장관의 위해 미생물의 감염을 막고 유익 균총을 유지시키기 위한 목적으로 사용된다. 현재 가축에게 항생제 사용이 금지됨에 따라 생균제는 항생제 대체용으로서 그 중요성이 대두되고 있다. 생장이 어려운 환경에서 포자를 형성할 수 있는 바실러스 균주는 지금까지 널리 사용되어 온 생균제용 균주인 유산균과 더불어 근래 생균제용 균주로서 각광받고 있다. 바실러스가 만들어내는 포자 자체가 하부장관 내 면역 반응을 활성화 시킬 수 있을 뿐만 아니라 장내 환경 및 사료 제조 공정에서도 다른 균주에 비해 생존율이 더욱 높기 때문이다. 또한 섬유소 분해효소는 반추위뿐만 아니라 하부장관에서도 섬유소 분해를 도와줘 소화율 및 이용성을 증진시킬 수 있고, 섬유소 분해 산물인 다당류는 prebiotics 로서 하부장기에서 이용될 수 있을 것이다. 본 연구에서는 생균제의 본래 역할을 함과 동시에 효소제로서도 이용될 수 있는 균주를 선발하고 선발된 균주의 섬유소 분해역가를 높이려 시도하였으며 최종적으로 *in vitro* 와 *in vivo* 실험을 통해서 개발된 생균제의 효과를 확인하려 하였다.

실험 1 에서는 한국 재래 산양에서 섬유소 분해능력이 뛰어난 *Bacillus licheniformis* JK7 균주를 선발하고 상기 균주가 가지고 있는 섬유소 분해효소 (endoglucanase, β-glucosidase, xylanase)의 특성을 규명하였다.

실험 2 에서는 genome shuffling 기법을 적용하여 선발 균주의 endoglucanase 의 생산을 증가시키려 하였다. 총 3 회의 genome shuffling 을 시도하였으며 이를 통해 선발된 GS2-18, GS3-8 및 GS3-20 균주는 WT 에 비해 유의적으로 2 배이상 높은 endoglucanase 생산을 보였고 성장곡선에서도 유의적으로 높은 생산속도를 보였다. Endoglucanase 최적 pH 와 온도는 WT 과 GS 균주사이에 차이를 보이지 않았다. 이를 통해 endoglucanase 자체의 유전적 재조합이 이루어졌기 보다 균주의 성장능력이나 단백질 생산능력 부분에 재조합이 이루어진 것으로 추정된다.

실험 3 에서는 실험 2 에서 선발된 균주를 이용하여 고상발효를 통한 생균제를 개발하였다. 밀기울과 대두박이 4:6 의 비율로 혼합된 기질에 균주를 접종하여 35℃에서 배양하였고 생균제는 배양 4 일차에서 가장높은 균수와 endoglucanase 역가를 보였다. 발효제품(FM)은 비 발효제품(NFM)에 비해 높은 CP 함량과 낮은 NDF, ADF 함량을 보였는데 이는 균주가 배지내에서 성장함에 따라 CP 의 함량을 높이고 섬유소 분해효소를 지속적으로 분비함으로서 기질 내의 섬유소 함량을 떨어뜨린 것으로 사료된다.

실험 4 에서는 FM 의 급여가 반추동물에게 미치는 영향을 *in vitro* 실험을 통해 평가하였다. FM 과 NFM 을 단독 기질로서 사용한 in vitro 실험에서 FM 처리구는 대조구에 비해 높은 소화율을 보였으나 VFA 생산성 및 미생물 균총변화에서는 긍정적인 효과를 보이지 않았다. 그러나 조사료에 FM 과 NFM 을 첨가한 두번째 *in vitro* 실험에서는 FM 처리구가 높은 소화율 및 VFA 생산성을 보였고 반추위 주요 미생물의 수 또한 FM 처리구에서 유의적으로 높았다($p<0.05$).

실험 4 에서 FM 첨가의 긍정적인 효과를 in vivo 에서 확인하기 위해 실험 5 를 진행하였다. FM 첨가구 (T1; 0.1% FM 첨가, T2; 0.5%FM 첨가)는 대조구 (0.5% NFM 첨가)와 소화율 및 반추위 발효성상 그리고 반추 미생물 균총변화에서 유의적인 차이를 보이지 않았다. 오히려 VFA 생산은 대조구에서 수치적으로 더욱 높았다. 생균제의 급여는 전반적으로 반추동물의 소화율 및 미생물 성장에 큰 영향을 미치진 않았으나 위액 및 분 내 균총에서 유의적으로 높은 *Bacillus licheniformis* 수치를 보였으며 이는 본 실험에서 급여된 균주가 반추동물의 장내에서 원만히 정착한 것으로 추정된다.

주요어: *Bacillus licheniformis,* DFM, cellulase, xylanase, genome shuffling, 반추위 미생물 균총
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학부생 시절 지도교수님이시자, 심사위원장으로서 학위 논문을 지도해주신 최윤재 교수님께, 감사의 말을 전하고 싶습니다. 아침 9 시 수업에 언제나 10 분전에 오셔서 강의 준비를

하시던 교수님의 근면하신 모습은 저에게 깊은 인상을 안겨주었습니다. 제가 기억하지 못하던 저와의 사소한 일도 추억으로 기억하고 계시던 김유용 교수님께 역시 감사드립니다. 축산학도로서 양축농가를 먼저 생각하라는 교수님의 가르침 잊지 않겠습니다. 연구실 선배님이자 심사위원을 맡아주셨던 장종수 교수님, 미국에서 한달 간 계실 때 저 혼자 산다고 점심식사도 챙겨주시고, 주말에는 맛있는 저녁도 해주셨던 교수님의 모습이 아직도 생생합니다. 그 때 해주셨던 많은 얘기들 잊지 않고 저도 후배들에게 나눠줄 수 있도록 노력하겠습니다. 박사과정동안 같은 연구과제를 진행하였던 진바이오텍의 이찬호 대표님, 과제를 수행하는 동안 가졌던 세미나에서 박사님이 보여주셨던 열린 시각은 같은 주제를 가지고도 다르게 접근할 수 있는 법을 저에게 가르쳐주었습니다. 심사과정 동안 모자란 점도 가르쳐 주셔서 정말 감사드립니다.

그리고 연구실 선배님이신 고종열 박사님, 고영곤 박사님, 곽병오 박사님께 감사드립니다. 연구실 행사때문에 많이 귀찮게 해드림에도 불구하고 언제나 참석하셔서 아낌없는 조언 베풀어 주신 점, 정말 감사합니다. 아울러 백명기 교수님, 최연호 박사님, 이성실 교수님, 김창현 교수님, 이왕식 교수님, 김창현 교수님, 김현진 박사님, 성하균 박사님, 최낙진 교수님, 이상석 교수님, 학위 기간 중에 많이 뵙지 못해 송구합니다. 앞으로 살면서 자주 뵐 수 있는 기회 만들고 많은 얘기 나눌 수 있도록 노력하겠습니다. 지금 미국에 계신 김선우 박사님, 박사님이 잠깐 한국에 계실 때

저한테 남겨주신 메모 아직도 제 자리에 붙여놓고 있습니다. 올 해, 다시 나가면 그 때는 꼭 찾아뵙고 감사하다는 말 직접 전하고 싶습니다. 어려운 마음으로 조언을 구할 때, 왜 진작 얘기하지 않았느냐며 따뜻하게 하지만 날카로운 내용으로 충고해주셨던 서성원 교수님, 정말 감사합니다. 기회가 된다면 꼭 실험을 같이 해보고 싶습니다. 회식 자리에서는 유쾌하지만, 일을 할 때는 완벽주의를 추구하던 감동근 박사님, 후배를 생각하는 마음으로 솔직하게 조언해주셔서 정말 감사드립니다. 앞으로도 자주 찾아뵙겠습니다. 석사로 처음 들어왔을 때, 연구실 최고참으로 있으면서 학문적으로, 인성적으로 본보기를 보여주셨던 이세영 교수님, 제가 처음에는 많이 답답하셨죠? 교수님과 같이 생활하면서 정말 많이 배웠습니다. 그리고 감사드립니다. 학회에서 가장 빛을 발하는 송재용 박사님, 같은 연구실에서 생활하진 않았지만 학위 과정동안 서로 나눴던 얘기들이 많이 기억이 납니다. 앞으로 제가 힘이 될 수 있도록 정진하겠습니다. 김민석 박사님, 제가 연수갈 때 이것저것 자꾸 여쭤봐서 많이 귀찮으셨죠? 논문 그리고 미국 생활에 대한 박사님의 조언이 없었다면 그렇게 빨리 적응할 수 없었을 것입니다. 정말 감사드립니다. 회사 생활 때문에 많이 바쁘지만 기회가 되면 어떻게든 챙겨주고 싶어하셨던 박성호 선배님의 따뜻한 마음을 잊을 수가 없습니다. "회사 놀러오라니까 왜 안오냐?"라는 투박하지만 정감어린 말에 제대로 응하지 못해 죄송하구요. 졸업 논문 나오면 들고 가겠습니다. 나이는 같지만 실험실 한참 선배님인 박민아 박사님, 처음 미생물 실험하면서 모르는 게

많았는데 그럴 때 마다 항상 힘이 돼줘서 정말 고마워. 회사 다니느라 많이 바쁘지만 힘내고 화이팅이야.

이찬희 박사님, 술자리에서는 안그런데 이렇게 글로 표현하려니 많이 쑥스럽네요. 항상 열심히 사는 형님의 모습이 정말 자랑스럽고 제가 석사할 때부터 실험에 관해 많이 가르쳐 줘서 정말 고맙습니다. 기쁜 일과 슬픈(?) 일을 함께 겪었던 양지영 박사님, 제가 버릇없게 굴 때도 너그러이 받아주시던 그 모습 꼭 배우고 싶습니다. 학부생 때부터 절친이었던 민기형, 저는 형이 연구생 선배라는 걸 입학하고 알았답니다. 실험실 잠깐 두달 정도 있을 때 저랑 인혁이 아침에 소들 밥주는 거 대신한다고 했을 때 정말 고마웠어요. 학부생 때부터 아끼는 후배라고 밥도 많이 사주고..앞으로 제가 잘 할게요. 지금 미국에서 한창 공부하고 있는 내 동기 명후랑 인혁이 내가 어떻게 너희들 얘기를 안할 수 있겠니..지금 생각해 보면 우리 세명이 연구실에 있을 때 싸우기도 싸웠지만 참 재밌었고 좋았었어. 가장 먼저 어른이 된 우리 인혁이, 힘들어도 잘 지내리라고 믿어 의심치 않는다. 연수기간동안 내가 많이 귀찮게 해서 미안하고 또 고맙다. 차로 두시간 넘게 걸리는 데도 불구하고 직접 차를 끌고 보러와 준 명후야. 난 자리는 안다고 너랑 석사 때부터 쭉 붙어있다가 너 미국가니까 많이 생각나더라. 우리 앞으로도 항상 서로 전화하고 연락 많이 하고 지내자. 그리고 연구실 계실 동안 제 옆자리에 있었던 종근이형, 항상 밤에 실험하던 동석이형, 실험실 있을 때 제가 많이 까불었죠? 이제는 두 분 다 결혼하셔서 가정을 꾸리고 있는 모습을 보니 신기하기도

하고 정말 대단한 거 같아요. 그리고 정말 고마웠습니다. 조금은 늦게 공부를 시작했지만 꿈을 잃지 않고 유학까지 나가신 준표형님, 미국 학회 때 찬희형이랑 같이 봐서 정말 반가웠습니다. 석사 할 때, 제가 실장이라고 좀 까탈스러웠을 텐데 잘 이해해 주시고 정말 고맙습니다. 지금 성호 형님이랑 열심히 일하고 있는 형일아. 작년에 결혼한 거 진심으로 축하한다. 그리고 연구실에서 준표형이랑 같이 많이 도와줘서 정말 고맙다. 어린 나이에 유학와서 고생 많이 한 민우야, 힘들어도 묵묵히 일하고 수원에서의 여러 일을 너에게 맡기다시피 했는데도 열심히 하는 너의 모습에 형이지만 느낀 게 많았다. 앞으로 백명기 교수님과도 열심히 공부하고 앞으로 어려운 점 있으면 꼭 같이 얘기하도록 하자. 시크한 박은혜양, 네가 졸업하고 나서 더 많이 친해진 거 같아 괜히 미안하고 그렇다. 지금 회사에서 일 열심히 하는 네 모습 너무 보기 좋고, 앞으로 멋진 커리어 우먼이 되길 바랄게. 실험실 막내로 들어와서 이제 유학가는 탄솔아. 너가 없었으면 내가 맘 놓고 연수 갔다오지 못했을 거야. 형이 논문 무사히 마무리 할 수 있게 도와줘서 정말 고맙고 굳이 실험실 일 뿐만 아니라 너랑은 맞는 부분이 많았던 거 같아. 너 유학가기 전까지 우리 계획했던 건 다 할 수 있도록 하자꾸나. 그리고 지수야, 석사 생활 하는 동안 내가 많이 간섭해서 힘들었지? 미안하고 지금 네가 준비하는 거 꼭 이룰 수 있길 바랄게. 대학원 입학 때 부터 물적으로 심적으로 많이 도와주신 안혜정누님, 정말 감사드립니다. 항상 소녀같으신 모습 잃지 않으셨으면 좋겠어요.

다른 실험실이지만 항상 모범적인 모습을 보여주셨던 은배형님, 연구와 가정을 두루 챙기시는 형님의 모습에 정말 놀랐고, 저도 그리할 수 있도록 노력하겠습니다. 그리고 퍼듀에서 명후랑 같이 공부하고 계신 창수형님, 어찌 그리 하나도 안 변하셨는 지...형님 댁에서 먹은 저녁식사 정말 고마웠습니다. 인디애나에서 학회 열릴 때 꼭 뵈었으면 좋겠어요. 지금 휴스턴에서 열심히 공부하고 있는 동현아, 작년에 퀄 본다고 많이 힘들었을텐데 정말 고생했고 그리고 고맙다. 연수 기간에 만났지만 정말 좋은 말 많이 해주시고 돌봐주셨던 송민호 교수님, 사람을 대하는 예에 대해서 많은 가르침을 받았던 거 같습니다. 정말 감사합니다.

그리고 학부 때부터 저 많이 이뻐해 주고 후배로서 많이 아껴주신 기현이형, 정현이형에게도 감사의 마음을 표하고 싶습니다. 저 군대간다고 기현이형이랑 같이 논산훈련소까지 따라가주었던 세원이형, 재훈이형, 남경이형, 자주 연락 드리지 못해 죄송하네요. 그래도 항상 맡은 자리에서 열심히 살고 계시리라 믿어 의심치 않고 그리고 저 많이 아껴주셔서 감사합니다. 형님들이랑 같이 살았던 그 원룸은 잊지 못할거에요. 그리고 우리 00 학번 동기들, 경남에 있으면서도 동기모임이라면 늘 참석했던 동률이, 엉뚱한 매력이 있는 재헌이, 동기회장 윤하, 애들한테 장난으로 많이 놀림 받아도 늘 의연한 상욱이, 영양학실에서 같이 공부하던 필승이, 멋쟁이 영규, 무뚝뚝하지만

정이 많은 태호 다들 정말 고맙고 앞으로도 지금처럼 항상 연락하고 지냈으면 좋겠다.

감사의 글을 쓰면서 보니, 지금까지 저를 도와주시고 아껴주신 분들이 정말 많다는 것을 새삼 느끼게 되었습니다. 이 글에서 미처 제가 언급하지 못한 분이 계신다면 넓으신 아량으로 이해해주시길 바라며 이만 줄이겠습니다. 앞으로 맺어진 인연의 소중함을 가슴속 깊이 간직하고 정말 열심히 살겠습니다. 저에게 베풀어 주셨던 깊은 사랑과 진심어린 충고들을 항상 기억하며 다른 사람들에게도 이런 따뜻한 마음을 전할 수 있는 사람이 되겠습니다. 다시한번 감사드립니다.

2013 년 2 월 4 일

서자겸 올림.