Studies on novel high-temperature fermentation for L-lactic acid production

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Summary

The sustainable production of fuels, chemicals and polymers from the biomass resources within integrated biorefinery is very important for minimizing the dependency on limited fossil fuels. Waste biomass such as food waste generated from every household is a big burden to our community, especially in their recycle system. Utilization of available biomass resources via the microbial fermentation process is one solution for developing environmental-friendly and sustainable society. In this study, a noble mixed culture system (MCS) and its microorganisms in high temperature fermentative production of L-lactic acid (LA), a well-known material for the synthesis of fine biodegradable bioplastics, was investigated.

In chapter II, new LA fermentation of food waste with MCS, named as 'meta-fermentation' was investigated. When temperature was set as a primary controlling factor, high L-LA yield with 100% optical purity was achieved at temperatures 50–55ºC. The microbial community structure at 50–55ºC, analyzed by denaturing gradient gel electrophoresis, showed the existence of six major bacteria (*Bacillus coagulans*, *B. smithii*, *B. humi*, *B. thermoamylovorans B. thermocloaceae* and *Corynebacterium sphenisci*) in the MCS. In chapter III, to further control and understand such MCS, a systematic isolation method of all the major bacteria involved in efficient production of L-LA was designed and named as systematic feedback isolation technique. This includes feedback of literature informations and high throughput colony screening by direct colony MALDI-TOF-MS. As a result, six major targeted bacteria and other several unexpectedly isolates were successfully obtained. In chapter IV, identification of the unexpectedly isolated thermotolerant bacterial strain MO-04 was performed with comparing to the closest bacterial species *Bacillus thermolactis* R-6488^{T}. Other than their DNA-DNA relatedness (44.5%), the strain MO-04 showed many distinguishable characteristics

from strain R-6488^T, particularly in sugar assimilation, growth pH and growth temperature. Based on its morphological feature, novel species *Bacillus kokeshiiformis* MO-04^T was proposed. In chapter V, characterization of one of the targeted major bacteria in MCS, isolate MC-07 (99.2% 16S rRNA gene similarity with *B. thermoamylovorans* LMG18084^T) was carried out. The isolate MC-07 showed abilities to make a clear halo zone in starch containing media, and to produce optically pure L-LA directly from starch. In particular, isolate MC-07 successfully produced L-LA with 100% optically purity and yield of 0.977 g/g at 50°C under pH-swing control at 7.0 in a simple mineral salt medium without external enzymatic liquefaction of starch.

In conclusion, a new concept, meta-fermentation was proposed for efficient utilization of food waste by mixed culture seed. To elucidate this kind of MCS, whole systematic isolation technique was developed. During the studies on MCS, a novel thermotolerant species *Bacillus kokeshiiformis* MO-04^T was identified and proposed, and characterized a thermotolerant bacterial species directly fermenting starch without enzymatic liquefaction. These new findings would be helpful for sustainable utilization of waste biomasses to value added fine chemicals.

Contents

CHAPTER I: General Introduction

This chapter is a modified version of the submitted review manuscript (Poudel P., Tashiro Y., Sakai K. New application of *Bacillus* strains for optically pure L-lactic acid production: General overview and future prospect) to Bioscience, Biotechnology and Biochemistry, 2015.

1.1. Bio-refinery concept: Future sustainable society

The sustainable production of energy, fuels and chemicals and materials from the biomass resources within integrated bio-refinery is very important for minimizing the dependency on limited fossil resources (Cherubini, 2010). There are several environmental issues, especially the emission of greenhouse gases by the usage of non-renewable fossil resources. To mitigate the environmental issues and conserving future alternate source of energy, chemicals and materials, the bio-refinery concept should be improved by developing technologies that avoid the emission of greenhouse gases. For instance, biological conversion of renewable biomass to value added fine chemical, is considered to be an environmental-friendly sustainable production system (Cherubini, 2010). Besides, recent, increase in bio-refining of available renewable resources to harmless useful fine chemicals such as lactic acid (LA), a material for synthesizing biodegradable and biocompatible fine polymers, by microbial technology, would obviously be a part to promote and create future environmental-friendly and sustainable society indeed.

1.2. Application of lactic acid

LA has been found in application by agricultural, medicinal, food, pharmaceutical and chemical industries, in particular, there is much interest in the usage of optically pure (L or D-isomer) LA for the synthesis of high-quality polylactic acid (PLA) (Hofvendahl & Hahn, 2000; Wee *et al.,* 2006 and Castillo *et al.,* 2013). PLA are used for the production of biodegradable and environmental friendly bioplastics and are assumed to substitute the petrochemical-based plastics (John *et al.,* 2007). Due to the several applications as the materials, it has been estimated that global LA production demand would be is around 130,000–150,000 tons per year (Ghaffar *et al.,* 2014).

1.3. Lactic acid producers

Nowadays, LA is mainly produced by microbial fermentation process rather than traditional chemical synthesis. The traditional chemical synthesis of LA is based on the hydrolysis of lactonitrile derived from acetaldehyde and hydrogen cyanide, which should lead to the formation of racemic mixtures of DL-LA (Hofvendahl & Hahn, 2000). Optically pure D-LA or L-LA can only be produced by microbial fermentation process using the specific microbes (De Boer et al., 1990; Sakai and Ezaki, 2006; Chen *et al.,* 2013). Furthermore, another significant advantage over the traditional LA chemical synthesis is that microbial fermentation can use various plant derived renewable biomass as a substrate for LA production.

In general, fermentative LA productions are mostly accomplished by members in domain bacteria. A few microorganisms in domain Archaea (*Scenedesmus* sp. and *Nannochlorum* sp.) and eukaryota (basically fungi, *Rhizopus oryzae*) have been known to produce LA (Abdel-Rahman *et al.,* 2013). Within domain bacteria, all the wild type LA producers belong to phylum Firmicutes and class Bacilli, but 2 orders Lactobacillales (8 genera) or Bacillales (3 genera). Order Lactobacillales contains all the lactic acid bacteria (LAB), particularly known as great LA producers including genera *Lactobacillus* (L-, D- or DL-LA), *Enterococcus* (L-LA), *Tetragenococcus* (D-LA), *Carnobacterium* (D-LA), *Pediococcus* (L- or DL-LA), *Leuconostoc* (D-LA), *Streptococcus* (L-LA) and *Weissella* (D- or DL-LA). On the other hand, within the order Bacillales and family Bacillaceae (total 48 genera, till date), mostly genus *Bacillus* has been reported as potent L-LA producers. Only two species of genus *Geobacillus* (*G. stearothermophilus*) (Danner *et al.,* 1998) and *Halolactibacillus* (*H. halophilus*) (Calabia *et al.,* 2011) are reported as L-LA producers. In the domain Eukaryotes, wild type fungi especially *Rhizopus oryzae* (Zhang *et al.,* 2007) and *Rhizopus microspores* (Kitpreechavanich *et al.,* 2008)

have been known as the potent L-LA producers. In addition, some genetically modified bacteria and yeast have been also developed for either D-/L-LA production depending upon the genetic manipulations (Litchfield, 2009). In future, the approaches to screen new wild type of LA-producers and to modify LA producers genetically suited for targeted processes will be also performed actively and continuously.

Recently, *Bacillus* strains are attracted in L-LA production due to their interesting features such as high growth rate, tolerance to high temperature, growth in a simple nutritional condition at relatively low pH (~4), ability to ferment wide ranges of sugars including pentoses and hexoses, and to produce optically pure L-lactic acid (up to 100%) recently become more attractive.

1.4. Special features of LA-producing *Bacillus* strains in comparison with other LA producers

1.4.1. Nutrients in the media

Bacillus strains are commonly isolated from nutritionally poor environmental samples such as soil under various climatic conditions (Vilain *et al.,* 2006). LAB are isolated only from the nutritionally rich environment that contained high amount of amino acids, vitamins and nucleotides in addition to carbon source. Yeast extract and peptone are rich in nitrogen, vitamins and other nitrogenous growth stimulating factors. The culture media for LAB require supplementations of organic nitrogen sources such as yeast extract, peptones and other proteinous substances, which ultimately increases the cost in the medium formulation (John *et al.,* 2007; Rosenberg *et al.,* 2005). Altaf *et al.,* (2007) investigated that yeast extract was found to account for ca. 38% of total medium cost in LA production by LAB. So, it is necessary to use

cheap substitutes for these expensive proteinous substances or to employ LA producers which can grow well under low concentrations of organic nitrogen sources.

Interestingly, *Bacillus* strains profusely grow and produce L-LA in a simple mineral salt media containing very low yeast extract (Heriban, 1993; Poudel *et al.,* 2015). Many researchers have already mentioned the minimal nutritional requirements for the growth and LA production by *Bacillus* strains (Poudel *et al.,* 2015; Wang *et al.,* 2011). So far, in case of *Bacillus* strains, ammonium sulphate (Gao and Ho, 2013), NH_4Cl (Gao *et al.,* 2012), low grade peanut meal (Meng *et al.,* 2012), corn steep liquor (Ou *et al.,* 2011), excess sludge hydrolysate (Ma *et al.,* 2014), yeast autolysate (Heriban, 1993) are already reported as alternative cheaper nitrogen supplements in LA fermentation. In addition, Meng *et al.,* (2012), reported that strain *Bacillus* sp. WL-S20 efficiently produced L-LA by using inexpensive peanut meal as nitrogen source which accounted for 20% higher in LA concentration than that using yeast extract. Ma *et al.,* (2014) reported that the performance of LA production (98.1 g/L of L-LA) using 10 g/L of excess sludge hydrolysate was competitive to that (98.9 g/L L-LA) using 2 g/L of yeast extract by strain *B. coagulans* NBRC12583^T. Therefore, it is better to develop microbial strains that can produce LA in a simple and nutritionally low media in future.

1.4.2. Tolerance to high temperature

LA-producing *Bacillus* strains show optimum growth temperatures range between 45–60°C and growth limitation up to 70°C, whereas optimum growth temperatures by most of LAB range from 30°C to 43°C (Sakai *et al.,* 2012). As the exceptions, thermotolerant D-LA producer *Lactobacillus delbrueki* subsp. *lactis* QU 41 (Tashiro *et al.,* 2011) and L-LA producer *Enterococcus faecium* QU 50 (Abdel-Rahman *et al.,* 2015) are reported to be tolerant even at

50°C. Many thermotolerant LA-producing *Bacillus* strains have been isolated from various environments. For instance, Poudel *et al.,* (2015) isolated thermotolerant *Bacillus* sp. MC-07 from the compost and produced L-LA directly from starch at 50°C by MC-07 strain. Basically, high temperature LA fermentation has several advantages compared to mesophilic fermentation.

First, high temperature fermentation would significantly reduce the cooling cost compared to mesophiles where temperature should be controlled by additional cooling systems to maintain the growth of mesophiles during the fermentation (Abdel-Rahman *et al.,* 2013). Second, representing high temperature fermentation is advantageous in minimizing the contamination; *B. coagulans* JI12 produced 137.5 g/L of L-LA with the high yield of 0.98 g/g and productivity of 4.4 g/L·h from D-xylose at 50°C even under non-sterile condition (Ye *et al.,* 2013a). Third, fermentation temperature should affect the microbial activity, and substrate conversion rate (Ramos *et al.,* 2000). Heriban *et al.,* (1993) reported that high temperature was beneficial for the synthesis of LA due to the improvement of the rate of biochemical reactions and the higher activity of microorganisms. Fourth, high temperature is beneficial for simultaneous saccharification and fermentation (SSF) process because several hydrolytic enzymes exhibit higher optimum temperatures than ca. 50°C (Ou *et al.,* 2009). According to Ou *et al.,* (2009) thermophilic *B. coagulans* 36D1 (ca. 26.99 g/L of LA and 0.903 g/g LA yield) showed better LA production performance compared to mesophilic LAB (*Lactococcus lactis* subsp. *lactis*) (ca. 20.34 g/L of LA and 0.709 g/g LA yield) when SSF of 40 g/L of crystalline cellulose was carried out using 20 FPU/g cellulose at 50°C and 40°C respectively. In addition, the activity of amylase enzyme for the SSF of starch is also enhanced at thermophilic condition (Ma *et al.,* 2014). Practically, high temperature fermentation minimizes cooling cost, exhibits no need of sterilization, and improves the enzymatic hydrolysis efficiency in SSF.

1.4.3. Tolerance to pH

pH is one of the key parameters in LA fermentation. As indicated in LAB (Abdel-Rahman *et al.,* 2013), *Bacillus* strains also show their optimum pH at neutral, but are sensitive to acidic pH (under pH 5.0). The pH for LA production by *Bacillus* strains ranged 5.0–9.0, whereas pH 4.0–6.0 is reported in LAB. The ability of *Bacillus* strains to tolerate alkaline pH can minimize the contamination (Calabia *et al.,* 2011). Under pH control at 9.0, *Bacillus* sp. WL-S20 (Meng *et al.,* 2012) and *Halolactibacillus halophilus* (Calabia *et al.,* 2011) efficiently produced L-LA (225 g/L, optical purity >99%; 65.8 g/L, optical purity of 98.8%) from D-glucose, respectively. On the other hand, fermentation at acidic pH around pKa of LA (3.86) can reduce the amount of agents to neutralize LA produced although most of LA-producing *Bacillus* strains are more sensitive to acidic condition than LAB (pH ~4.0) and fungi (pH ~3.0). Within the genus *Bacillus*, *Bacillus acidicola* was reported to tolerate pH up to 3.5 lower than the pKa value of LA, while the efficiency of LA fermentation under acidic condition by this strain has not been studied well (Albert *et al.,* 2005). Therefore, much more studies are required to improve the L-LA fermentation under acidic conditions for *Bacillus* strains such as genome shuffle technique, where the pH tolerance of *Lactobacillus rhamnosus* ATCC 11443 has been enhanced up to 3.6 (Wang *et al.,* 2007).

1.4.4. Optical purity of LA produced

Optical purity of LA produced in fermentation is mainly dependent on the gene-expression levels and enzyme activities of L-lactate dehydrogenase (L-LDH), D-lactate dehydrogenase (D-LDH), and lactate racemase. L-LDH and D-LDH catalyze the conversions of pyruvic acid to L-LA and D-LA, respectively, and almost all the LAB possess both D-LDH and L-LDH (Wang *et al.,* 2014)

On the other hand, a few LAB are reported to possess lactate racemase for the catalysis of transforming D-LA to L-LA (Sakai *et al.,* 2006). Therefore, due to these properties, the LA optical purities are considerably variable among LAB (Abdel-Rahman *et al.,* 2013). Contrary to LAB, most of the LA-producing *Bacillus* strains possess both L-LDH (encoded by *ldhL* gene) and D-LDH (encoded by *ldhD* gene) enzymes responsible for the production of L-LA and D-LA, respectively, and lack lactate racemase enzyme (Wang *et al.,* 2014), even though they produce L-LA with optical purity of more than 97%. Furthermore, it is reported that the optical purities of L-LA by *Bacillus* strains are variable even within same species such as the potent L-LA producer *B. coagulans* strains with the ranges of 97-100% (Peng *et al.,* 2013; Tongpim *et al.,* 2014). However, information regarding the synthesis mechanism of high optical purity of LA in *Bacillus* strains has not been studied well.

1.5. Available biomass for LA production by *Bacillus* species

LA production by *Bacillus* strains are mainly investigated using expensive refined sugars (e.g. D-glucose) as a sole carbon source. However, to substitute the expensive refined sugars, cheap and widely abundant biomass resources such as lignocellulose (eg. wood and crop residues), sucrose (eg. sugarcane molasses and beet molasses) and starch (eg. tapioca starch, cornstarch) are rapidly gaining its importance for the production of LA (John *et al.,* 2007).

1.5.1. Lignocellulose

Lignocelluloses consists of cellulose (insoluble fibers of β-1,4-glucan), hemicellulose (non-cellulosic polysaccharides of xylan, arabinose, glucan and mannan) and lignin (complex aromatic polymer) (Abdel-Rahman *et al.,* 2010). The compositions of cellulose (35–50%), hemicellulose (20–40%) and lignin (10–30%) depends upon the types of plants (Anuj *et al.,*

2011; Saha, 2003; Neureiter *et al.,* 2004; Pandey *et al.,* 2000) Upon enzyme hydrolysis of lignocelluloses, cellulose mainly liberates D-glucose and cellobiose while several sugars are obtained from hemicellulose including hexoses (D-mannose and D-galactose, D-glucose) and pentoses (D-xylose, L-arabinose). D-xylose is the dominant sugar in hemicellulose. So far, various lignocelluloses including wheat straw (Maas *et al.,* 2008), paper sludge (Budhavaram and Fan, 2009), corn fiber hydrolysate (Bischoff et al., 2010), corncob molasses (Wang *et al.,* 2010),empty fruit bunches (EFB) of palm oil (Ye *et al.,* 2013b; 2014), Jerusalem antichoke powder (Wang et al., 2013), Siberian larch (Walton *et al.,* 2010) have already been efficiently used as raw materials for LA production by *Bacillus* strains. For example, *B. coagulans* JI12 utilized EFB hydrolysate (containing 4.7 g/L of D-glucose, 48.8 g/L of D-xylose and 9.6 g/L of L-arabinose) and produced 59.2 g/L of L-LA with L-LA yield of 0.97 g/g and the productivity of 6.2 g/L·h (Ye *et al.,* 2013b).

However, one of the major challenges in a fermentative production process using lignocelluloses as the substrate exist in their pretreatment process for generating fermentable sugars and efficient LA production. The pretreatment of lignocelluloses also liberates phenolic compounds (furfural and 5-hydroxy methyl furfural (HMF)), furan derivatives (ferulic acid and vanillin) and weak organic acid (acetic acid) (Palmqvist and Hahn, 2000). These compounds derived from various pretreatment methods are known to inhibit the microbial growths and LA productions at the concentrations ranging from 0.08 g/L to 5.3 g/L (Palmqvist and Hahn, 2000). Mainly three approaches have been investigated to improve LA production from pretreated linocelluloses containing inhibitors: usage of LA-producing *Bacillus* strains with the tolerance for inhibitors, introduction of removal process before fermentation process, and performance of

conversion process from inhibitors to less-toxic substances by LA-producing *Bacillus* strains. Some *B. coagulans* strains resistant to certain level of inhibitors are reported, (Peng *et al.,* 2013; Bischoff *et al.,* 2010; Wang *et al.,* 2013; Zhang *et al.,* 2014), for instance, *Bacillus* sp. P38 with tolerance for 6 g/L 2-furfural in corn stover hydrolysate produced 180 g/L of L-LA with the productivity of 2.4 g/L·h and yield 0.96 g/g under fed-batch fermentation process (first approach) (Peng *et al.,* 2013). On the other hand, to the best of our knowledge, the removal process using chemicals such as overliming and activated charcoal (Zheng *et al.,* 2015) prior to fermentation process has not been reported to remove inhibitors from pretreated lignocelluloses in LA fermentation using *Bacillus* strains (second approach). As the third approach, simultaneous detoxification, saccharification and fermentation process has been succeeded using *B. coagulans* JI12 by conversing furfural (inhibitor) to 2-furoic acid (less inhibitor), producing 73.9 g/L of L-LA with L-LA yield of 1.09 g/g from oil palm empty fruit bunch (Ye *et al.,* 2014). This process has become attracting in terms of not only overcoming the crucial issue from pretreated lignocelluloses but also simplifying the overall processes by an environmental friendly and less-energy required method (Anuj *et al.,* 2011).

1.5.2. Sucrose

Sugarcane molasses and beet molasses are sucrose-rich biomasses obtained from sucrose-based industries as waste byproducts. Sugarcane molasses contains 40 to 60% sucrose that can be utilized by microorganisms to produce LA (Dumbrepatil *et al.,* 2008). A few researchers have investigated the feasibility of sucrose as raw material for LA production using *Bacillus* strains (Payot *et al.,* 1999; Xu and Xu, 2014). Payot *et al.,* (1999), reported that *B. coagulans* TB/04 produced 55 g/L of LA from 60 g/L of sucrose derived from sugarcane molasses. On the other hand, beet molasses containing 47% sucrose, 0.5% nitrogen and 3% betaine has been shown to

be used as not only a fermentation substrate but also an enhancer in LA fermentation by *B. coagulans* H-1 (Xu and Xu, 2014). The addition of beet molasses in glucose solution increased the L-LA productivity by 22% compared to the fermentation using only D-glucose as a sole carbon source. Unlike other carbohydrates (cellulose and starch), these sucrose-containing molasses have a significant advantage that direct utilization for LA production without any requirements of saccharification because many *B. coagulans* strains can convert sucrose to LA (Tongpim *et al.*, 2014).

1.5.3. Starch

Starch is a polysaccharide consisting of D-glucose with α-1,4- or α-1,6-bonds. It is usually found in cereal-based products (rice, corn, wheat) and tuber-plants (potato, cassava). Starchy biomasses also represent cheap alternative to refined sugars. Inexpensive starch, starch-containing wastes and starch-derivatives obtained from many starchy industries, would provide an attractive carbon source for LA production even though starch requires saccharification by enzymes including α-amylase and glucoamylase before fermentation process (Shimizu-Kadota *et al.*, 2014; Im *et al.*, 2012). Several enzymatic hydrolysates of corn starch (Zhou et al., 2013) and tapioca starch (Ma *et al.*, 2014) have been used as substrates for LA production by *Bacillus* strains. Similarly, considerable amount of starch-containing wastes such as food waste has been also utilized for LA production by *B. coagulans* and *Bacillus licheniformis* strains (Sakai, 2008).

On the other hand, direct fermentation from starch should show the advantage of skipping saccharification process by using amylolytic LA-producers. Although some amylolytic LAB (John *et al.*, 2007) and fungi (*R. oryzae*) (Zhang *et al.*, 2007) are reported to produce LA from starch without any enzymatic hydrolysis, there are still the several problems regarding low optical purity of LA (<98%), low yield (for *Rhizopus*), mesophilic conditions (≤45°C), and

requirement of abundant nutrients (for LAB) (Abdel-Rahman *et al.*, 2013). Recently, Poudel *et al.* (2015) have established direct starch fermentation to optically pure L-LA (purity 100%, yield 0.977 g/g) by *Bacillus* sp. MC-07. Furthermore, the fermentation process using strain MC-07 could be performed in a simple mineral salt medium containing only 0.001% yeast extract of organic nitrogen sources at the high temperature of 50°C. This finding shows that some low-cost starchy biomasses can be directly fermented to L-LA with a high yield and optical purity by omitting the addition of enzymes and nitrogen sources, which lead to a relatively simple methodology for the process of LA fermentation.

1.6. *Bacillus* strains in fermentative production of LA

1.6.1. Bacillus strains

As shown in Table 1, *B. coagulans*, *B. licheniformis*, *Bacillus subtilis*, *Bacillus thermoamylovorans*, *Bacillus laevolacticus* and some not yet identified *Bacillus* species have been reported as potent L-LA producers. All the *Bacillus* strains accumulated optically pure L-LA (optical purity ranged 97–100%) except for D-LA-producing *B. laevolacticus* (De Boer *et al.*, 1990). Among the reported *Bacillus* species, *B. coagulans* accumulated higher amount of L -LA and showed the thermotolerant behaviors (Table 1).

Tongpim *et al.*, (2014), recently proposed that L-LA-producing thermotolerant *B. coagulans* strains formed a three distinct cluster in phylogenetic tree based on their 16S rRNA gene sequence and showed heterogeneity in sugar metabolism. Although it is reported that a few LAB utilize xylose as the carbon sources, 8 strains of the tested 11 *B. coagulans* strains were able to metabolize xylose (one of the major sugar component in lignocelluloses) (Tongpim *et al*., 2014). In addition, De Clerck *et al*., (2004), also reported half of the *B. coagulans* strains (15

strains) metabolize D-xylose. These phenomena suggest the feasibility of efficient L-LA production from various types of lignocelulloses using *B. coagulans* strains.

B. coagulans can produce L-LA as a major fermentative product either under strict anaerobic or high oxygen supply conditions, one of the most interesting findings. In particular *B. coagulans* strain M21, accumulated 100% optically pure L-LA under high oxygen supply condition from D-glucose (Tongpim *et al.*, 2014). On the other hand, it has been reported that LAB (*Lactobacillus plantarum*, *L. rhamnosus*) degrades LA especially in presence of oxygen as an electron accepter and convert initially formed LA from sugars (glucose and cellobiose) to acetic acid, H_2O_2 and CO_2 (Quatravaux *et al*., 2006).

1.6.2. Fermentation modes

During LA fermentation, high LA yield, productivity and the concentration of final accumulated product are the important factors to evaluate the fermentation efficiency. To achieve such economically important parameters, numerous investigations on LA fermentations have been conducted under batch, fed-batch and continuous fermentation process (Tables 1 and 2).

Batch fermentation mode is the most frequently used in LA production (Mazzoli *et al.*, 2014). The advantage of using batch fermentation is low risk of contamination compared with other fermentation approaches because it is a closed system in which no nutrients are added during the fermentation period (Qin *et al.*, 2009 & 2010). High LA production of 210 g/L from D-glucose (Zhou *et al.*, 2013) and 140.9 g/L from D-xylose (Ye *et al*., 2013a) has been obtained by *Bacillus* strains in batch fermentation under non-sterile condition (Table 1). These LA concentrations are the highest among the published literatures using *Bacillus* strains under batch fermentation. However, problems such as substrate inhibition due to initial high concentration of

sugar, carbon catabolite repression (CCR) in mixed sugar fermentation caused by consumption of more preferable sugars and lagging the less preferable sugars, and product inhibition cause by LA accumulation are still existing in batch fermentation.

In fed-batch fermentation mode, fresh medium or nutrients is continuously or sequentially fed to fermentation broth without the removal of culture broth mainly to overcome the problem of substrate inhibition and nutrient deficiencies as occurred in batch fermentation (Abdel-Rahman *et al.,* 2010). Fermentative LA production by *Bacillus* strains under fed-batch fermentation mode is illustrated in Table 2. Several feeding strategies including pulse feeding, constant feeding and exponential feeding have been developed to increase the LA concentration in fed-batch fermentation mode. Among these, pulse feeding strategy is considered easier to operate and suitable for industrial scale (Qin *et al.,* 2009). When pulse feeding strategy was adopted, the highest L-LA concentration of 225 g/L was obtained by using strain *Bacillus* sp. WL-S20 under fed-batch fermentation mode (Meng *et al.,* 2012). Meantime, repeated batch fermentation is also being investigated for the better performance in LA yield and productivity by *Bacillus* strains. In repeated batch mode, microbial cells can be repeatedly used for the subsequent batch fermentation processes (Zhang *et al.,* 2014). This method led to improve in LA productivity, reduction in fermentation time and avoid the inoculum preparations. *B. coagulans* strain 2-6 accumulated highest L-LA concentration of 107 g/L, optical purity of 99.8% under open repeated batch fermentation from D-glucose, in which the yield and productivity was improved up to 0.95 g/g and 2.9 g/L·h respectively compared to batch fermentation (63 g/L of LA, yield up to 0.94 g/g, productivity of 1.4 g/L·h) (Zhao *et al.,* 2010).

Compared to batch and fed-batch fermentation modes, continuous fermentations have been less studied in *Bacillus* strains. Continuous fermentation mode is adopted to overcome the

issue of product inhibition that should occur in batch and fed-batch fermentation processes. In continuous fermentation, products as the inhibitors are constantly diluted by adding fresh medium at the same rate with outflow of the broth under the steady volume in the fermentor. Besides, cells can be maintained at a stable physiological state and a constant growth rate thereby attaining the steady maximum productivity. *B. laevolacticus* NCIB 10269 (De Boer *et al.*, 1990), *B. coagulans* TB/04 (Payot *et al.*, 1999) and *B. subtilis* MUR1 (Gao and Ho, 2013) have been investigated under continuous fermentation mode. Gao and Ho (2013), reported average LA productivity of 16.8 g/L·h at the dilution rate of 0.4 h^{-1} using glucose under continuous fermentation mode that resulted in 4.8-fold increase in productivity compared to fed-batch fermentation mode (Gao and Ho, 2013; Gao *et al.*, 2012). In continuous mode, selection of optimum dilution rate for attaining the maximum cell growth and LA productivity is important in *Bacillus* strains. The LA productivity was highly improved for long period in continuous fermentation process compared to batch and fed-batch fermentation processes.

Under non-controlled pH conditions, *Bacillus* strains are not able to accumulate high concentration of LA because of intolerance to acidic pH. Under controlled pH, LA concentration and productivity are found to be improved in batch fermentation by *Bacillus* strains. pH of the fermentation broth is controlled by various neutralizing agents such as $CaCO_3$ (Peng *et al.*, 2013), $Ca(OH)_2$ (Peng *et al.*, 2013),NaOH (Ye *et al*., 2013a) KOH (Zhao *et al.*, 2010) and NH_3 (Sakai and Ezaki, 2006). Among these neutralizing agents, $CaCO_3$ and $Ca(OH)_2$ have been widely used. However, the disadvantage using them is the formation of calcium salt as the calcium lactate in the fermentation broth, which requires the additional acidification procedure by H_2SO_4 to obtain free LA in recovery process (Ye *et al*., 2014). This disadvantage can be minimized using NH_3 as neutralizing agent because calcium lactate is not formed and can be recovered in the supernatant

of culture broth. Sakai and Ezaki (2006), reported L-LA fermentation (concentration, 40 g/L; productivity, 2.5 g/L·h) by *B. licheniformis* TY7 using NH_3 as neutralizing agent to control the pH at 7.0 from food waste of the LA fermentation in which maximum L-LA concentration of 40 g/L with productivity of 2.5 g/L·h. Furthermore, total recycle poly-L-LA production process has been proposed, where NH_3 is recycled and utilized as the neutralizing agent for the subsequent LA fermentation after recovery of the free L-LA (Sakai *et al.,* 2003).

Bacillus strains have been also employed to produce LA from mixed sugars derived from lignocellulses (Peng *et al.,* 2013; Ye *et al.,* 2014; Walton *et al.,* 2010; Patel *et al.,* 2004; Patel *et al.,* 2006). However, major obstacle using mixed sugar is difficulty in simultaneous utilizations of each sugar during fermentation. Several researchers have reported on the carbon catabolite repression in *B. coagulans* strains, i.e. initial consumption of more preferable sugars like glucose and lagging the utilization of less preferable sugars like D-xylose and L-arabinose behind (Ou *et al.,* 2009; Budhavaram and Fan, 2009; Ye *et al.,* 2014, Zhang *et al.,* 2014). The CCR in mixed sugars is mainly reported under batch fermentation mode in LA-producing *Bacillus* strains (Ye *et al.,* 2014). It was indicated that repeated batch fermentation by *B. coagulans* strains showed simultaneous utilization of mixed sugars (D-glucose, D-xylose and L-arabinose) to some extent (Ye *et al.,* 2013b; Patel *et al.,* 2006). Zhang *et al.,* (2014), investigated the repeated batch fermentation using substrates (glucose, xylose and arabinose) by *B. coagulans* IPE22 for up to six consecutive batches. In first batch, fermentation exhibited a lag phase of 5 h and then pentose sugars (xylose and arabinose) were utilized to produce 54.14 g/L of LA in 24 h, whereas in case of sixth batch, lag phase was totally eliminated and all sugars were simultaneously utilized to produce 56.27 g/L of LA in 17 h. However, complete avoidance of CCR has not been solved by using LA-producing *Bacillus* strains, which requires us to isolate more potent CCR negative LA-

producers, or obtain it by genetic manipulations, or optimize fermentation conditions.

1.7. Specific metabolic pathway for lactic acid production in *Bacillus* strains

LA-producing *Bacillus* strains metabolize pentose and hexose sugars by pentose phosphate pathways (PPP) and Embden-Meyerhof-Parnas pathway (EMP) to produce major end product as LA homofermentatively, respectively (Patel *et al.*, 2006). In EMP, hexose sugar such as glucose (six carbons) in the presence of ATP is phosphorylated to glucose 6-phosphate by hexokinase, and then isomerized to fructose 6-phosphate (F6P) by phosphoglucose isomerase. F6P is further phosphorylated to fructose 1,6-bisphosphate (FBP) by phosphofructokinase with a consumption of ATP. FBP splits to glyeraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), which is catalyzed by fructose bisphosphate aldolase. Furthermore, GAP is converted to pyruvate via two substrate-level phosphorylation. Ultimately, pyruvate is reduced by NADH to produce L-LA or D-LA catalyzed by L-LDH or D-LDH, respectively. In some strains of LAB, lactate racemase that catalyzes interconversions of L-LA/D-LA is reported to result in the formation of racemic mixture of LA in EMP (Sakai *et al.*, 2006). During EMP, the theoretical yield of LA from glucose is 1 g/g (2 mol/mol) in homo-lactic fermentation.

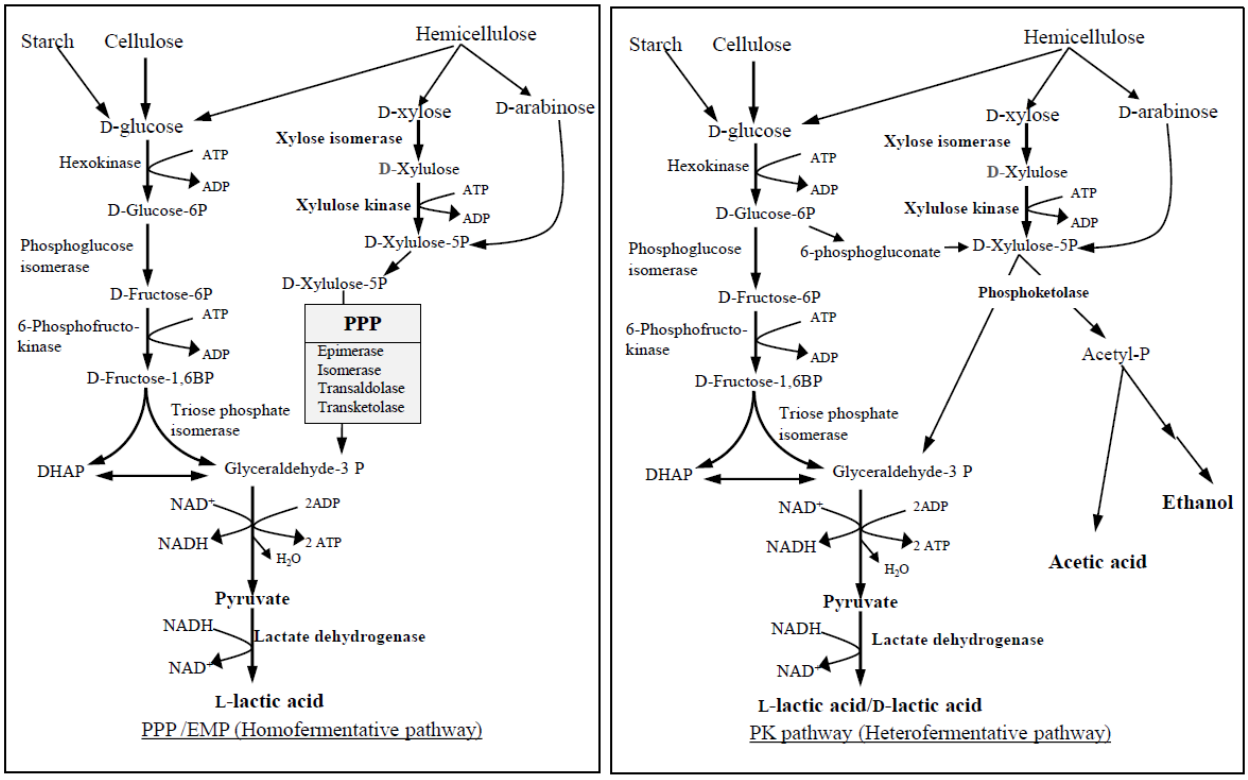

Fig.1-1.Metabolic pathway for LA production from lignocellulose derived sugars (xylose, glucose, arabinose) and starch via homofermentative and heterofermentative pathways

In PPP, pentose sugar such as D-xylose is converted to D-xylulose by xylose isomerase and is further phosphorylated to xylulose 5-phosphate (X5P) by xylulose kinase. X5P is subsequently metabolized to form GAP via key enzymes of transaldolases and transketolases. Thus, obtained GAP is converted to pyruvate and further to LA via EMP. In case of PPP, 3 moles of D-xylose is converted to 5 moles of LA with the theoretical yield of 1 g/g (1.67 mol/mol) (Patel *et al.*, 2006) and the highest yield (0.98 g/g) of LA production by *B. coagulans* JI12 was reported in the literatures (Ye *et al.*, 2013a) In contrast, a few LAB are able to metabolize pentose sugar (D-xylose) (Abdel-Rahman *et al.*, 2013). Most of pentose-utilizing LAB have been utilize phosphoketolase (PK) pathway mainly to convert pentose sugar to LA and byproducts (acetate or ethanol) with the theoretical yield of only 0.6 g/g (1 mol/mol), which leads to heterofermentation

(Tanaka *et al.,* 2002). As the exceptions among the LAB, *Enterococcus mundtii* QU 25 (Abdel-Rahman *et al.,* 2013), *Enterococcus faecium* QU 50 (Abdel-Rahman *et al.,* 2015), and some metabolically engineered LAB such as *Lactobacillus plantarum* (Okano *et al.,* 2010) are reported to produce L-LA from D-xylose homofermentatively via PPP/EMP with yields of 0.904 g/g, 0.89 g/g and 0.89 g/g, respectively. Similarly, in *Rhizopus* sp., the fate of D-glucose metabolism results in the formation of various byproducts such as ethanol and fumaric acid that drastically reduced the final LA yield (<0.79) (Zhang *et al.,* 2007). Therefore, EMP and PPP are much better suited for increasing the LA yield, which is common in LA producing *Bacillus* strains.

Additionally, much progress has been made in understanding the LA metabolism in *Bacillus* strains. The draft genome sequence of potent L-LA-producing *B. coagulans* strains (2-6, 36D1, DSM1, H-1, XZL4, XZL9) showed the presence of xylose utilizing genes encoding the enzymes xylose isomerase, ribulokinase, and ribulose 5-phosphate 4-epimerase involved in PPP (Su and Fu, 2014) while the genes involved in PK pathway were absent in all the tested *B. coagulans* strains, which may result in high LA yield from xylose. Actually, Ye *et al.,* (2013a), reported *B. coagulans* C106 produced 83.5 g/L of L-LA from 85 g/L of D-xylose with L-LA yield of 0.98 g/g via PPP.

1.8. Metabolic engineering in *Bacillus* species for LA production

Metabolic engineering is being applied to improve the cellular properties through the alteration of specific biochemical reactions or introduction of new genes by means of DNA recombinant tools (Kovacs *et al.,* 2010; Zhang *et al.,* 2011; Mazumdar *et al.,* 2010; Ishida *et al.,* 2005; Wang *et al.,* 2011b). However, attempts of metabolic engineering with the genus *Bacillus* for LA production are much fewer than those with LAB (Okano *et al.,* 2010), *Escherichia coli*

(Mazumdar *et al.,* 2010) and fungi (Ishida *et al.*, 2005) so far. This section focused on the recent studies on establishment of genetically modified *Bacillus* strains for improvement of LA fermentation.

Among the LA-producing *Bacillus* strains, the approaches on the genetic engineering is being widely studied in *B. subtilis* for its good performance and established easy method (Romero *et al.,* 2007). Zhang *et al.,* (2011), reported the direct fermentation of cellulose to L-LA by recombinant cellulolytic *B. subtilis* XZ7. In this case, overexpression of β-endoglucanase-coding genes by two-round directed evolution was performed, and the gene encoding α-acetolactate synthase gene (*alsS*) in 2, 3-butanediol pathway was knocked out to increase the L-LA yield. As the result, *B. subtilis* XZ7 could digest the up to 92% insoluble regenerated amorphous cellulose and produced L-LA with the yield up to 0.63 g/g. This study demonstrated that *B. subtilis* is one of the hosts for genetic manipulation to breed more excellent LA-producing *Bacillus* strains for the desired processes in future.

The development of genetic tools for L-LA-producing *B. coagulans* has been gradually developing even though highly efficient genetic tools are still lacking as compared to *B. subtilis*. Basically wild type *B. coagulans* strains produce L-isomer efficiently from several sugars while D-LA-producers have been also required for synthesis of stereocomplex PLA consisting of PLLA and PDLA (Nishida *et al.,* 2004; Hirata *et al.,* 2008). For the fermentative production of D-LA, some *B. coagulans* strains have been genetically modified using specific genetic tools (Wang *et al.,* 2011b; Kovacs *et al.,* 2010). Kovacs *et al*., (2010) have successfully applied the widely used *Cre-lox* system for genomic modifications and removal of specific genes. In this case, the native *ldhL* gene of *B. coagulans* DSM1 (potent L-LA producer) was disrupted and the D-lactate dehydrogenase (*ldhD*) gene was overexpressed to generate D-LA. The engineered strain

could produce 16.9% D-LA and 83.1% L-LA. However, optically pure D-LA could not be achieved by the engineered strain, which requires further improvements. On the other hand, Wang *et al.* (2011b), engineered thermotolerant *B. coagulans* strain P4-102B (a potent L-LA producer, optical purity >99%) targeting the genes encoding the enzyme D-LDH for D-LA production with high yield by deleting the native *ldhL* (L-lactate dehydrogenase) and *alsS* (acetolactate synthase) genes. The engineered strain produced optically pure D-LA (90 g/L of D-LA, optical purity >99%) from D-glucose at 50 °C and can be expected to reduce the cost of LA production for biopolymers.

Aside from genetic engineering, another approach has been investigated to breed thermotolerant L-LA producing *B. licheniformis*, whose wild type BL1 strain could not ferment xylose to LA efficiently. Wang *et al*., (2011a), transferred BL1 strain in the medium containing xylose as the sole carbon source, and evolved BL2 strain, after 13 transfer, improved L-LA production (concentration, 24.5 g/L, yield, 79.5%, maximum LA productivity, 7.0 g/L·h) from 30 g/L xylose compared with the parent strain (0.6 g/L, 60.0%, and 0, respectively). Furthermore, transcriptional analysis revealed 5.1- and 6.2-folds higher mRNA expression levels of *xylA* (xylose isomerase) and *xylB* (xylulose kinase), respectively, in BL2 strain than those in BL1 strain using xylose. Although further work is needed to understand the mechanism including other genes and enzymes in this strain, this method is also a new and easy technique to modify the metabolisms of LA-producing *Bacillus* strains.

In future, the progress in more efficient and easy methods for genetic manipulation and evolutionary technique will activate the studies on LA-producing *Bacillus* strains in the field of metabolic engineering.

1.9. Mixed culture in LA fermentation

Besides LA production in pure culture (Tables 1-1 and 1-2), efficiency of mixed culture in LA fermentation have been investigated using more than one strain recently. Table 1-3 summarizes the LA production by mixed culture originated from various sources. Liang *et al.,* (2014) investigated LA production from potato peel waste by undefined mixed culture (waste water plant treatment sludge as a mixed culture seed) and produced 14.2 g/L of DL-LA with yield of 0.22 g/g. Even though yield and optical purity of LA are comparatively lower than those in LA production in pure culture, recently, LA production in mixed culture are attracted due to its potential to produce LA under non-sterile conditions and ability to utilize a wide range of cheap substrates including industrial wastes and agricultural wastes (Liang *et al.,* 2014). However, much fewer investigations are reported on LA production in mixed culture compared to LA production in pure culture. It is important to improve LA yield, optical purity and LA selectivity in mixed culture fermentation system by further investigating.

To establish the efficient mixed culture system for LA production, we proposed a novel concept of "meta-fermentation", meaning fermentative production of pure chemicals and fuels such as lactic acid by controlled mixed culture system. It is assumed that meta-fermentation have several benefits: lower susceptibility to contamination, no need to sterilize the media, feasibility under open conditions, low product costs and so on. Several environmental factors including pH, temperature could be the useful factors to control the mixed culture system because controlling the pH and temperature have the significant effects on final product formation (Zhou *et al.,* 2013). In parallel, to further control the meta-fermentation, estimation and isolation of major members of mixed culture system is also equally important.

1.10. Systematic isolation of major microorganisms from mixed culture system

It is well known that isolation of bacteria from environmental samples has led to discovery of many novel and useful secondary metabolites. However due to failure of isolation of vast majority of bacteria (more than 99%) in the artificially designed solid media from the environments, much more existing useful applications of bacteria remain unexplored (Vartoukian *et al.*, 2010). One possible reason behind the difficulties in isolation might be due to insufficient information on growth conditions (specific nutrients, pH conditions, incubation temperatures, level of oxygen and many more) (Kaeberlein *et al*., 2002). Therefore, new strategies to isolate the bacteria from the certain environmental samples are continuously being investigated.

Recently, molecular analyses from direct environmental samples have explored the phylogenetic information of many not yet isolated bacteria. In addition, based on the information obtained from the molecular techniques (cultivation-independent information), isolation of several physiologically active bacteria have been also performed (Dunders *et al.*, 1999; Kawai *et al.*, 2002; Tamaki *et al.*, 2005, Pedro *et al.*, 2003, Cho *et al.*, 2008). For instance, Pedro *et al*., (2003) targeted the some dominant bacteria (*Propionibacterium acnes*, *Methylobacterium radiotolerans* and *Bacillus thermocloacae*) of composting which were detected in denaturing gradient gel electrophoresis (DGGE). They could successfully isolate bacteria *P. acnes* and *B. theermocloacae*. We assumed that, isolation of multiple bacteria based on information obtained from molecular technique is more effective than the traditional isolation methods. To accomplish the isolation of major members of mixed culture system, firstly we proposed feedback isolation strategy from already known meta-fermentation system and obtained useful information on isolation (specific nutrients in the media, pH conditions, incubation temperatures, level of

oxygen) of major bacteria from the previous literatures. And further to minimize many similar colonies, we adopted direct colony matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) technique (Freiwald and Sauer, 2009) because direct colony MALDI-TOF MS has already confirmed its robustness in screening large set of bacteria in short interval of time (Welker and Moore, 2011). Therefore, here the systematic isolation of bacteria that covers feedback isolation and screening the similar clones obtained from different isolation media will obviously be beneficial to discover novel bacteria. Besides, the physiological characterization of all the isolated major members could be beneficial in understanding the mixed culture system.

1.11. Outline of present study

Currently industrial production of optically pure LA is becoming an important because of its application is many sectors such as food, chemical, pharmaceutical industries. Besides, LA can be a raw material for the systhesis of PLA, biodegradable and biocompatible biopolymer. Many researchers are investigating and looking for novel, most efficient and cost effective means for producing optically pure LA. In this regards, high temperature fermentation has become an alternative for LA production. The main aim of this study is:

- To establish the novel high temperature LA fermentation from food waste biomasses and its sugar derivatives by mixed culture. The results obtained from this study will satisfy the requirements for efficient LA production.

In the chapter II, high temperature L-LA fermentation from food waste using mixed culture seed (MAR compost) was investigated.

In chapter III, systematic isolation major bacteria of mixed culture system which were involved in efficient L-LA production was investigated by adopting feedback isolation technique and characterized.

In chapter IV, taxonomic characterization of novel thermotolerant *Bacillus* sp. MO-4 unexpectedly isolated from mixed culture seed.

In chapter V, direct starch fermentation to L-LA by thermophilic *Bacillus* sp. MC-07, one of the targeted major bacteria of mixed culture system.

In chapter VI, general conclusions and future perspectives were summarized.

Table 1-1 Lactic acid production efficiencies among the *Bacillus* strains under batch fermentation mode.

Fermentation substrate	Strains	Lactic acid				Fermentation parameters	References
		C (g/L)	*Y* (g/g)	*P* (g/L·h)	*OP* (%)†		
Glucose	*B. thermoamylovorans* CNCM I-1378^{T}	11.8	nd	ca. 0.49	100	pH control at 7.0; Ferm.temp. 50°C; Yeast extract, 20 g/L.	Combet-Blanc et al., 1995
Molasses	*B. coagulans* TB/04	55.0	0.93	nd	99	pH control at 6.4; Ferm.temp. 52°C; Yeast extract, 2 g/L.	Payot et al., 1999
Soft wood hydrolysate	*B.coagulans* BS121	13.7	nd	ca. 0.19	99	pH control at 6.0; Ferm.temp. 60°C; Yeast extract, 4 g/L.	Neureiter et al., 2004
Acid hydrolysis sugarcane baggase	*B.* coagulans 17C5	55.8	ca. 0.93	ca. 0.80	99.5	pH control at 5.0; Ferm.temp. 50; Corn steep liquor, 5 g/L.	Patel et al., 2004
Acid hydrolysis of sugarcane	*B. coagulans* 36D1	36.0	nd	nd	99.0	pH control at 5.0; Ferm.temp. 50;	Patel et al., 2006
Saccharified food waste	*B. coagulans* NBRC 12583^{T}	86.0	0.97	0.72	97.0	pH control at 6.5; Ferm.temp. 55°C;	Sakai and Ezaki, 2006
Saccharified Food waste	*Bacillus licheniformis* TY7	40.0	1.35	2.50	97.0	pH control at 6.5; Ferm.temp. 55°C;	Sakai and Yamanami, 2006
Paper sludge hydrolysates	*B. coagulans* 36D1	92.8	0.77	0.96	99.5	pH control at 5.0; Ferm.temp. 50; Corn steep liquor 5 g/L	Budhavaram and Fan, 2009

Table 1-1..continue

Fermentation substrate	Strains	Lactic acid				Fermentation parameters	References
		C (g/L)	*Y* (g/g)	*P* (g/L·h)	*OP* (%)†		
Corn fiber hydrolysate	*B. coagulans* MXL-9	40.2	0.58	0.54	99.5	pH control at 6.0; Ferm.temp. 50°C; Yeast extract 10 g/L	Bischoff et al., 2010
Hot water-extracted Siberian larch	*B. coagulans* MXL-9	33.0	0.94	0.55	99	pH control at 7.0; Ferm.temp. 50°C; Yeast extract 5 g/L and tryptone 10 g/L	Walton et al., 2010
Glucose	*B.* coagulans 2-6	107.0	0.95	2.90	99.8	pH control at 6.0; Ferm.temp. 50°C; Yeast extract 10 g/L	Zhao et al., 2010
Cellobiose	*B. licheniformis* BL1	4.4	0.86	0.30	>99.0	pH control at 7.0; Ferm.temp. 50°C; Yeast extract 5 g/L	Wang et al., 2011a
Corn stover hydrolyzate	*B. coagulans* XZL4	81.0	0.98	1.86	99.5	pH control at 6.0; Ferm.temp. 50;	Xue et al., 2012
Corn Stover hydrolysate/Xylose	*B. coagulans* NL01	75.0	0.75	1.04	99.8	pH control at 6.5; Ferm.temp. 50°C; Yeast extract 2.5 g/L	Ouyang et al., 2013
Oil palm empty fruit bunch hydrolysate	*B. coagulans* JI12	59.2	0.97	6.2	99.6	pH control at 6.0; Ferm.temp. 50°C; Yeast extract 10 g/L	Ye et al., 2013b
Xylose	*B. coagulans* C106	83.6	0.98	7.5	99.6	pH control at 6.0; Ferm.temp. 50; Yeast extract 20 g/L	Ye et al., 2013a
		140.9	0.98	4.8			

Table 1-1..continue

Fermentation substrate	Strains	Lactic acid				Fermentation conditions	References
		C (g/L)	*Y* (g/g)	*P* (g/L·h)	*OP* (%)†		
Glucose	*B. coagulans* WCP10-4	210.0	0.99	3.50	100	pH control at 6.0; Ferm.temp. 50°C; Yeast extract 20 g/L	Zhou et al., 2013
Oil palm empty fruit bunch hydrolysate	*B. coagulans* JI12	80.6	ca. 1.19	3.40	99.6	pH control at 6.0; Ferm.temp. 50°C; Yeast extract 10 g/L	Ye et al., 2014
Glucose [a]/ Food waste Wheat straw hydrolysate	*B. coagulans* M21	12.5/35.1	0.62/0.86	0.31/0.58	100/100	pH control at 7.0; Ferm.temp. 50°C; Yeast extract 5 g/L	Tongpim et al., 2014
Wheat straw hydrolysate	*B. coagulans* IPE22	56.4	0.96	2.35	100	pH control at 6.0; Ferm.temp. 52°C; Yeast extract 4 g/L	Zhang et al., 2014
Soluble starch	*Bacillus* sp. MC-07	16.6	0.977	0.70	100	pH control at 7.0; Ferm. Temp. 50°C; Yeast extract 0.01 g/L	Poudel et al., 2015

Symbol: C, concentration; Y, yield; P, productivity; OP, †optical purity (only L-isomer); [a], under aerobic condition (K_La=1.43 min^{-1} refer to Tongpim *et al.*, 2014); The optical purity (%) of L-LA was defined as ([L]−[D]) × 100/([L]+[D]), where [L] and [D] denote the concentrations of L-LA and D-LA, respectively.

Table 1-2 Lactic acid production in fed-batch and continuous fermentation processes

Fermentation substrate	Strains	Lactic acid				Fermentation mode and feeding strategy	Fermentation parameters	References
		C (g/L)	*Y* (g/g)	*P* (g/L·h)	*OP* (%)†			
Glucose	*Bacillus laevolacticus* NCIB 10269*	45.0	1.0	ca 13.1	D-isomer, >99	Continuous	pH control at 6.0, dilution rate 0.07 h-1,; Ferm. Temp. 30°C; Yeast extract 2 g/L; Anaerobic condition by sparging N_2 gas	De Boer et al., 1990
Sucrose	*B. coagulans* TB/04*	20.1	0.93	6.1	>99	Continuous	pH control at 6.4, dilution rate 0.03 h-1, Ferm. Temp. 50°C, Yeast extract, 4 g/L	Payot et al., 1999
Wheat straw hydrolysate	*B. coagulans* DSM2314	40.7	0.43	0.74	97.2	Fed-batch with constant feeding	pH control at 6.0; Ferm.temp. 50°C; Yeast extract 10 g/L	Neureiter et al., 2004
Cellulose	*B. coagulans* 36D1	80.0	0.88	ca. 0.84	99.0	Fed-batch with constant feeding	pH control at 5.0; Ferm.temp. 50°C; Corn steep liquor 7.5 g/L	Ramos et al., 2000
Glucose	*Bacillus sp.* WL-S20	225.0	0.99	1.04	100	Fed-batch with pulse feeding	pH controlled at 9.0; Ferm.temp. 45°C; Peanut meal 20 g/L	Meng et al., 2012
Xylose	*B. coagulans* C106	215.7	0.95	4.0	99.6	Fed-batch	pH control at 6.0; Ferm.temp. 50; Yeast extract 20 g/L	Ye et al., 2013a

Fermentation substrate	Strains	Lactic acid				Fermentation mode and feeding strategy	Fermentation parameters	References
		C (g/L)	Y (g/g)	P (g/L·h)	OP (%)			
Corn Stover hydrolysate	*B. coagulans* NL01	75.0	0.75	1.04	>99	Fed-batch with pulse feeding	pH controlled at 6.5; Ferm.temp. 50°C; Yeast extract 2.5 g/L	Ouyang et al., 2013
Acid treated corn stover hydrolysate	*B. coagulans* P38	180.0	0.98	2.40	100	Fed-batch with pulse feeding	pH controlled at 4.8–5.0; Ferm.temp. 42°C;	Peng et al., 2013
Jeruselem antichoke hydrolysate	*B. coagulans* XZL4	134.0	0.96	2.50	>99.0	Fed-batch with pulse feeding	pH controlled at 5.2–5.5; Ferm.temp. 50°C; Corn steep powder 10 g/L	Wang et al., 2013
Glucose	*B. coagulans* 2-6	122.0	ND	2.50	99.2	Fed-batch with exponential feeding	pH controlled at 5.6; Ferm.temp. 50°C for 36 h and increased up to 55°C at 0.2°C/h; Yeast extract 5 g/L	[74)] Qin et al 2009
Mixture of untreated Cane molasses and Glucose	B. coagulans H-1	168.3	0.88	2.10	>99	Fed-batch with constant feeding	pH controlled at 6.2; Ferm.temp. 52°C; Yeast extract 10 g/L	Xu and Xu, 2014

C, concentration; Y, yield; P, productivity; OP, optical purity (only L-isomer unless indicated); ND, not determined.

Table 1-3 Lactic acid production from mixed culture

Source of mixed culture	Temperature (°C)	Substrate	*C* (g/ L)	*Y* (g/g)	OP (%)	S (%)	Reference
Waste water treatment plant sludge	35	Potato peel waste	14.2	0.22	ND	ca.67	Liang et al. 2014
MAR compost	50	Kitchen refuse	34.2	0.540	100	91.6	Tashiro et al., 2013
Anaerobic digestion sludge	37	Glucose	4.17	0.429	ND	67.4	Itoh et al., 2012
Anaerobic digestion sludge	50	Glucose	23	0.92	ND	>90	Kim et al., 2012
Excess sludge	50	Sucrose	8.5	1.06	ND	ND	Maeda et al., 2009
Naturally inhabiting bacteria in garbage	55	Garbage	37.7	0.58	95.0	ND	Akao et al., 2007
Naturally inhabiting bacteria in garbage	37	Kitchen refuse	45	ND	ND	94	Sakai et al., 2000
Naturally inhabiting bacteria in garbage	35	Kitchen refuse	64	0.62	ca.60	ND	Zhang et al., 2008
Naturally inhabiting bacteria in garbage	45	Kitchen waste	<27.5	ND	ND	ND	Wang, et al., 2011

1.12. References

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CHAPTER II: A novel high temperature L-lactic acid fermentation from kitchen refuge using mixed culture

This chapter is a modified version of the paper (Tashiro Y., Matsumoto H., Miyamoto H., Okugawa Y., Poudel P., Miyamoto H., Sakai K. A novel production process for optically pure L-lactic acid from kitchen refuse using a bacterial consortium at high temperatures) published in Bioresource Technology 146: 672–681 (2013).

2.1. Abstract

In this study, L-lactic acid production of kitchen refuse using a mixed culture seed at temperatures from mesophilic (30°C) to thermophilic (65°C) range was investigated. At low temperatures butyric acid was accumulated, whereas at higher temperatures L-lactic acid was produced. In particular, fermentation at 50°C produced 34.5 g/L L-lactic acid with 90% lactic acid selectivity and 100% optical purity. Change in microbial community during the entire fermentation period at different thermophilic temperatures was analyzed using denaturing gradient gel electrophoresis (DGGE). 16S rRNA gene sequence retrieved from DGGE analysis revealed that, *Bacillus coagulans* was predominant strain throughout the fermentation period at 45, 50 and 55°C at which higher concentration of L-lactic acid were accumulated. This is the first report to show the 100% optical purity of L-lactic acid using a mixed culture of bacteria.

2.2. Introduction

Food waste is an organic waste disseminated from varieties of sources such as domestic kitchens, cafeteria and restaurants, commercial food industries. According to FAO (2011) nearly 1.3 billion tonnes per annum of food waste that includes fresh vegetables, fruits, meat, bakery and dairy products, are generated. In Asian countries, food waste is expected to increase from 278 to 416 million tonnes from 2005 to 2025 (Melikoglu *et al.,* 2013). In Japan, the total amount of food waste generated amounted to ca. 20 million tonnes per annum. It contained 17% from food production industry (industrial waste), 28% from food supply and food catering industries (general waste from business) and 55% million ton from general households. Only 3% has been recycled from the household waste (http://www.asiabiomass.jp/) which indicates that more can be utilized as a biomass for the production of valuable products.

Traditional practices for food waste treatment include the incineration or landfill disposal leading to environmental pollution. It contains high moisture and is reported to produce dioxins during combustion. In addition, incineration of food waste can lead to air pollution by releasing greenhouse gases such as CO_2. Therefore, management of food waste should be strongly needed. Alternatively, food waste is being utilized as a source of biofuel production so called second generation fuel. It mainly consists of starch, non-starch polysaccharides (cellulose, hemicellulose), proteins, lignin, lipids, organic acids, and inorganic substances (Sakai et al 2006). In particular, saccharification of polysaccharides generates fermentable sugars which are feasible for microbial fermentation. Food wastes are utilized to generate various valuable bio-products such as methane gas (Sasaki *et al.,* 2009), ethanol (Ma *et al.,* 2009), hydrogen, lactic acid (Sakai *et al.,* 2000), and so on.

Lactic acid is mainly produced by pure culture and much few reports have been investigated using mixed culture seed. The mixed culture seed used in this study; marine-animal-resource (MAR) composts are produced from coffee-extracted residues of MAR such as small fishes, shrimps, and crabs by marine repeated fed-batch aerobic fermentation, where the fermentation temperature reaches ca. 75°C without heating. A bacterial community structure analysis of mixed culture seed showed relatively stable bacterial community structure with several *Bacillus* strains (Niisawa *et al.,* 2008). Because some *Bacillus* strains are known to be potent L-lactic acid producers, we expected that optically pure L-lactic acid could be produced with this mixed bacterial strains existed in MAR compost as the seed. To date, there are no reports on optically pure L-lactic acid production from kitchen refuse as the substrate by mixed bacterial strains of compost.

This study aims to investigate the effects of temperatures on optically pure L-lactic acid production from kitchen refuse using mixed culture seed, and to analyze the bacterial community structures of their fermentations. This process could produce high L-lactic acid at

an extremely high optical purity under thermal conditions. Denaturing gradient gel electrophoresis (DGGE) analysis revealed that *Bacillus coagulans* was the predominant throughout the fermentation which contributed to high L-LA production

2.3. Materials and Methods

2.3.1. Mixed culture seed and media preparation

MAR compost was used as mixed culture seed in this experiment. A previous study indicated that bacteria belonging to the Firmicute phylum were dominant in this compost (Niisawa et al., 2008). One gram of compost was suspended in 9 ml of sterilized water and used as a seed.

Model kitchen refuse (MKR) medium was used for the fermentation experiments as follows, and contained the following compounds per liter of tap water: vegetables (66.7 g of carrot peel, 66.7 g of cabbage, and 66.7 g of potato peel), fruit (50 g of banana peel, 50 g of apple peel, and 50 g of orange peel), 70 g of baked fish, 50 g of rice, and 30 g of used tea leaves. After homogenization of the above materials, the medium was sterilized at 121°C for 15 min. Industrial glucozyme (#200000, Nagase ChemteX, Osaka, Japan) was added to the sterilized MKR medium at 300 ppm and saccharification was performed at 50°C for 2 h at pH 5.8. After saccharification, the pH of the MKR medium was adjusted to 7.0 with 10% ammonia solution, and thereafter, we directly used it as the fermentation medium without separation of the solid fraction.

2.3.2. Fermentation in MKR medium

1 mL of mixed culture seed was inoculated into 30 ml of MKR medium in a 50-mL plastic tube, and fermentation was performed statically at temperatures of 30, 37, 40, 45, 50, 55, 60, and 65°C. The pH was adjusted every 24 h to 7.0 using 10% ammonia solution. Samples

were periodically withdrawn for analysis of products, substrate, and bacterial community structure.

2.3.4. Chemical analysis

Chemical analyses of fermented samples were carried out by following the previously described methodology (Sakai and Yamanami, 2006). Concentrations of organic acids such as total lactic acid (sum of D- and L-lactic acids), formic acid, acetic acid, propionic acid, and butyric acid were determined using a special high-pressure liquid chromatography system (Organic acid Analyzer, Shimadzu, Kyoto, Japan). Selective production of lactic acid (lactic acid selectivity) was defined as the weight percentage of total lactic acid in the sum of organic acids analyzed. D- and L-lactic acids were analyzed by using a high-pressure liquid chromatography system equipped with a MCL Gel CRS10w column (Mitsubishi Chemical Co., Japan). The total sugar concentration was determined using the phenol-sulfuric acid assay (Dubois *et al.*, 1956).

2.3.5. Calculations

The optical purity of L-lactic acid ($OP_{L\text{-}LA}$) was calculated as follows:

$$OP_{L\text{-}LA}\ (\%) = (C_{L\text{-}LA} - C_{D\text{-}LA}) \times 100/(C_{L\text{-}LA} + C_{D\text{-}LA})$$

where $C_{L\text{-}LA}$ and $C_{D\text{-}LA}$ are the respective concentrations (g L^{-1}) of L and D-lactic acid produced.

Selectivity for lactic acid (S_{LA}) and selectivity for butyric acid (S_{BA}) were calculated as follows:

$$S_{LA}\ (\%) = (C_{L\text{-}LA} + C_{D\text{-}LA}) \times 100 / (C_{L\text{-}LA} + C_{D\text{-}LA} + C_{AA} + C_{BA} + C_{PA} + C_{FA})$$

$$S_{BA}\ (\%) = C_{BA} \times 100 / (C_{L\text{-}LA} + C_{D\text{-}LA} + C_{AA} + C_{BA} + C_{PA} + C_{FA})$$

where C_{AA}, C_{BA}, C_{PA}, and C_{FA} are the respective concentrations (g L^{-1}) of acetic acid, butyric acid, propionic acid, and formic acid produced.

2.3.6. DNA extraction and PCR amplification of 16S rRNA gene

Total DNA was extracted from centrifugation pellets of 1-ml fermentation broth using a PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's instructions. The partial 16S rRNA gene (V3 region) was amplified from the extracted DNA with universal primer pairs for eubacteria 357f-GC and 518r in a total volume of 50 µl containing 1.25 U of Ex Taq DNA polymerase (Takara Bio, Shiga, Japan), 0.2 mM dNTPs, 0.8 µM both primers, and ca. 0.2 ng/µl DNA template. The PCR cycling conditions were as follows: 94°C for 3 min, 52°C for 1 min, 72°C for 1 min, followed by 30 cycles, and final extension at 72°C for 10 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3.7. DGGE analysis and 16S rRNA gene sequencing

DGGE analysis was performed on the DCode™ universal mutation detection system (BioRad, CA, USA). 8% polyacrylamide gels with denaturing gradient of 30%–60% of urea plus formamide (100% denaturant corresponds to 7 M urea and 40% formamide [v/v]) were prepared and ca. 300 ng of purified PCR products and 5 µL of DGGE marker II (Nippon Gene, Tokyo, Japan) as a reference marker were applied to the gels (16 × 16 cm). DGGE runs were performed for 5 h at 200 V using Tris-acetate-EDTA buffer under a constant temperature of 60°C. After electrophoresis, the gels were stained with SYBR Green I (Lonza Rockland, ME, USA) for 30 min, visualized under UV light, and photographed using the Gel Doc ™ XR+ (BioRad, USA), where the UV exposure time was appropriately adjusted for each gel to compensate for a few strong bands appearing in any lanes.

Selected DGGE bands were excised from gels and suspended in TE buffer at 4°C overnight to elute the DNA from the bands. Reamplification was performed using the eluted DNA as a template with the corresponding primers described above. PCR products were

purified as above and were subjected to DGGE analysis to confirm the single band. PCR products that exhibited a single band in DGGE analysis were sequenced. Sequence homology was compared with 16S rRNA gene sequences available in the DDBJ/EMBL/GenBank DNA database using the FASTA algorithm (http://www.ddbj.nig.ac.jp/) and all reference sequences were obtained through the Ribosomal Database Project II (http://rdp.cme.msu.edu). Sequences were aligned using CLUSTAL W ver.2.01 (http://clustalw.ddbj.nig.ac.jp).

2.4. Results and discussion

2.4.1. L-LA fermentation at different temperatures

Fermentation was conducted at 30, 37, 40, 45, 50, 55, 60, and 65°C with the pH adjustments at 7.0 every 24 h by 10% NH_3. As shown in Figs. 2-1 and 2-2, the concentration behavior of lactic acid (L-, D-, and total), acetic acid, and butyric acid were quite different at various fermentation temperatures. L- and D-lactic acids were accumulated until 72 h and 96 h, respectively, and then butyric acid production was observed with lactic acid consumption at 30 and 37°C, [Figs. 2-1(a), (b) and 2-2(a), (b)]. Although 32 g/L L-lactic acid was accumulated after 120 h of culture, L-lactic acid decreased to 16.6 g L^{-1} with production of acetic acid (5.70 g L^{-1}) and butyric acid (6.51 g L^{-1}) at 40°C [Figs. 2-1(c) and 2-2(c)]. This finding suggested that lactic acid produced in initial phases was converted to butyric acid and acetic acid during the late phase at 30–40°C. In contrast, large amounts of L-lactic acid were produced without butyric acid production and lactic acid consumption at 45, 50, and 55°C [Figs. 2-1(d–f) and 2-2(d–f)]. Fermentations at 60 and 65°C exhibited much lower acid production than those at lower temperatures.

Table 2-1 shows the summary of the final product concentrations in MKR fermentation at each temperature for 168 h of culture. Butyric acid concentrations and selectivities reached 24.8 g L^{-1} and 26.8 g L^{-1}, and 80.5% and 75.5% with production of

acetic acid (2.27 g L^{-1} and 7.06 g L^{-1}) and propionic acid (2.41 g L^{-1} and 1.63 g L^{-1}) with little or no lactic acid production at 30°C and 37°C, respectively. Although fermentations at >40°C produced L-lactic acid, relatively low L-lactic acid concentrations (16.6 g/L, 9.51 g/L and 7.93 g/L) were obtained with low optical purities (86.2, 72.6, and 71.7%) and selectivity (58.3, 57.3, and 73.2%) at 40, 60, and 65°C, respectively. In contrast, high L-lactic acid (>21 g L^{-1}) and low acetic acid (<2.8 g L^{-1}) were produced with very high optical purity (>98%) and lactic acid selectivity (ca. 90%) at 45, 50, and 55°C. In particular, the batch fermentation at 50°C gave the best values [L-lactic acid concentration; 34.5 g L^{-1}, lactic acid selectivity; 91.6%; optical purity of L-lactic acid; 100%, by-products production (butyric acid, propionic acid, and formic acid); 0].

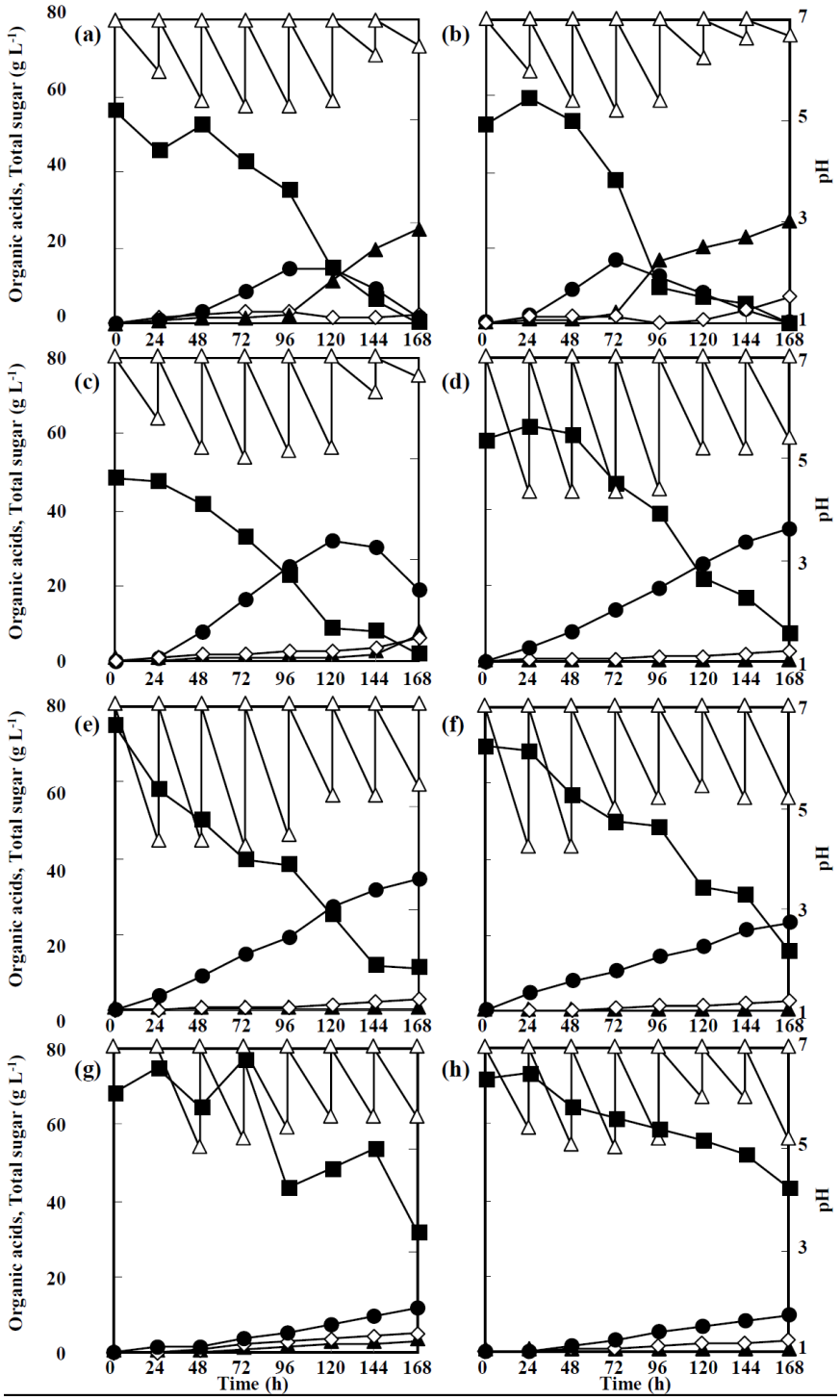


Fig. 2-1. Time-courses of fermentation profiles in model kitchen refuse media using MAR composts as seeds at various temperatures: (a), 30°C; (b) 37°C; (c) 40°C; (d) 45°C; (e) 50°C; (f) 55°C; (g) 60°C; (h) 65°C. ●, lactic acid; ▲, butyric acid; ■, total sugar; ◊, acetic acid; △, pH.

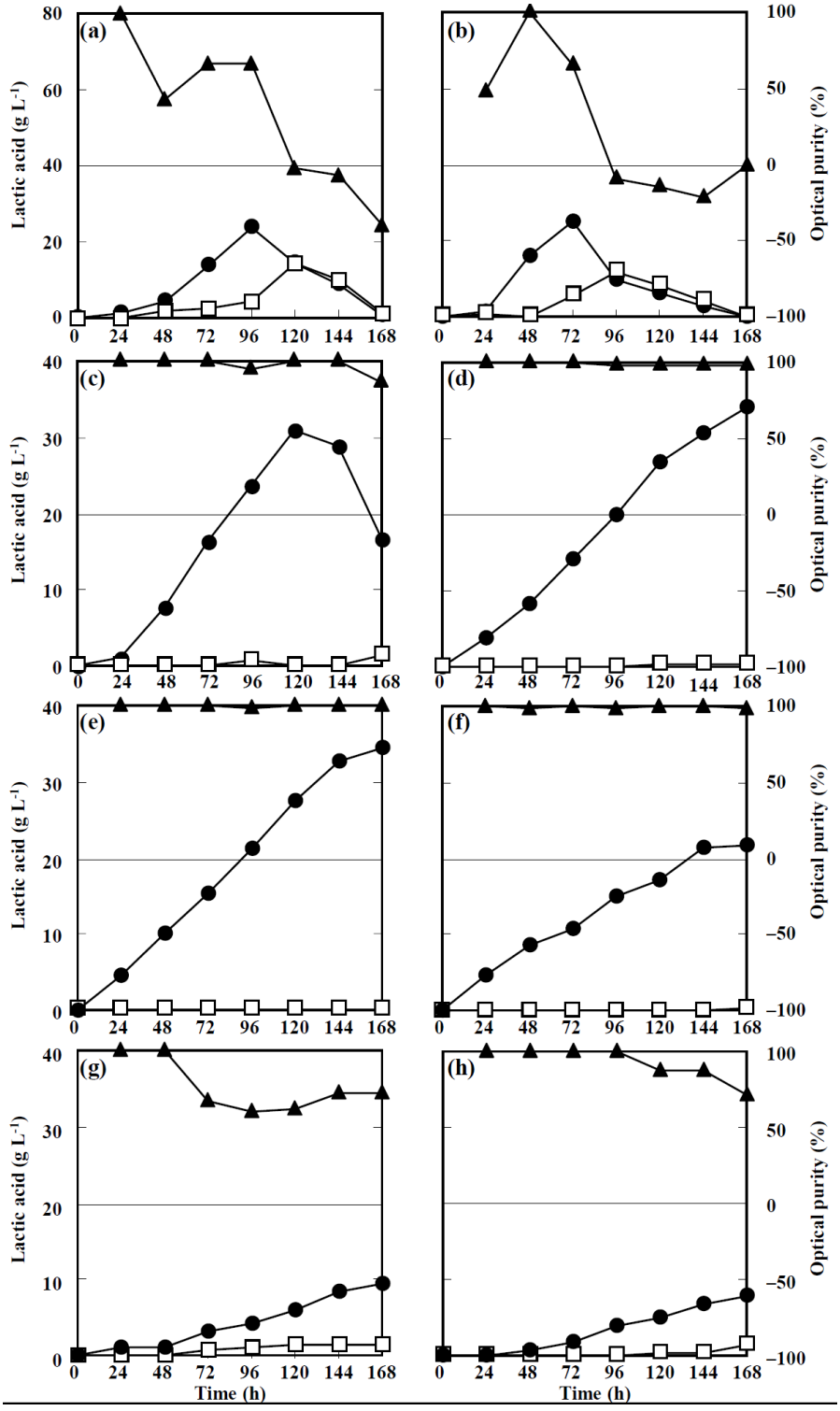


Fig. 2-2 Time-courses of fermentation profiles in model kitchen refuse media using MAR composts as seeds at various temperatures: (a), 30°C; (b) 37°C; (c) 40°C; (d) 45°C; (e) 50°C; (f) 55°C; (g) 60°C; (h) 65°C. ●, L-lactic acid; □, D-lactic acid; ▲, optical purity of L-lactic acid.

These results indicated that one could change the main products from MKR by controlling only fermentation temperature using MAR compost as the mixed culture seed, achieving high L-lactic acid production with 100% optical purity in batch fermentation at 50°C. This also suggested that the bacterial community structures during fermentation would change depending on temperature, resulting in varying fermentation behaviors.

Table 2-1 Effect of temperature on fermentation in MKR medium using mixed culture seed

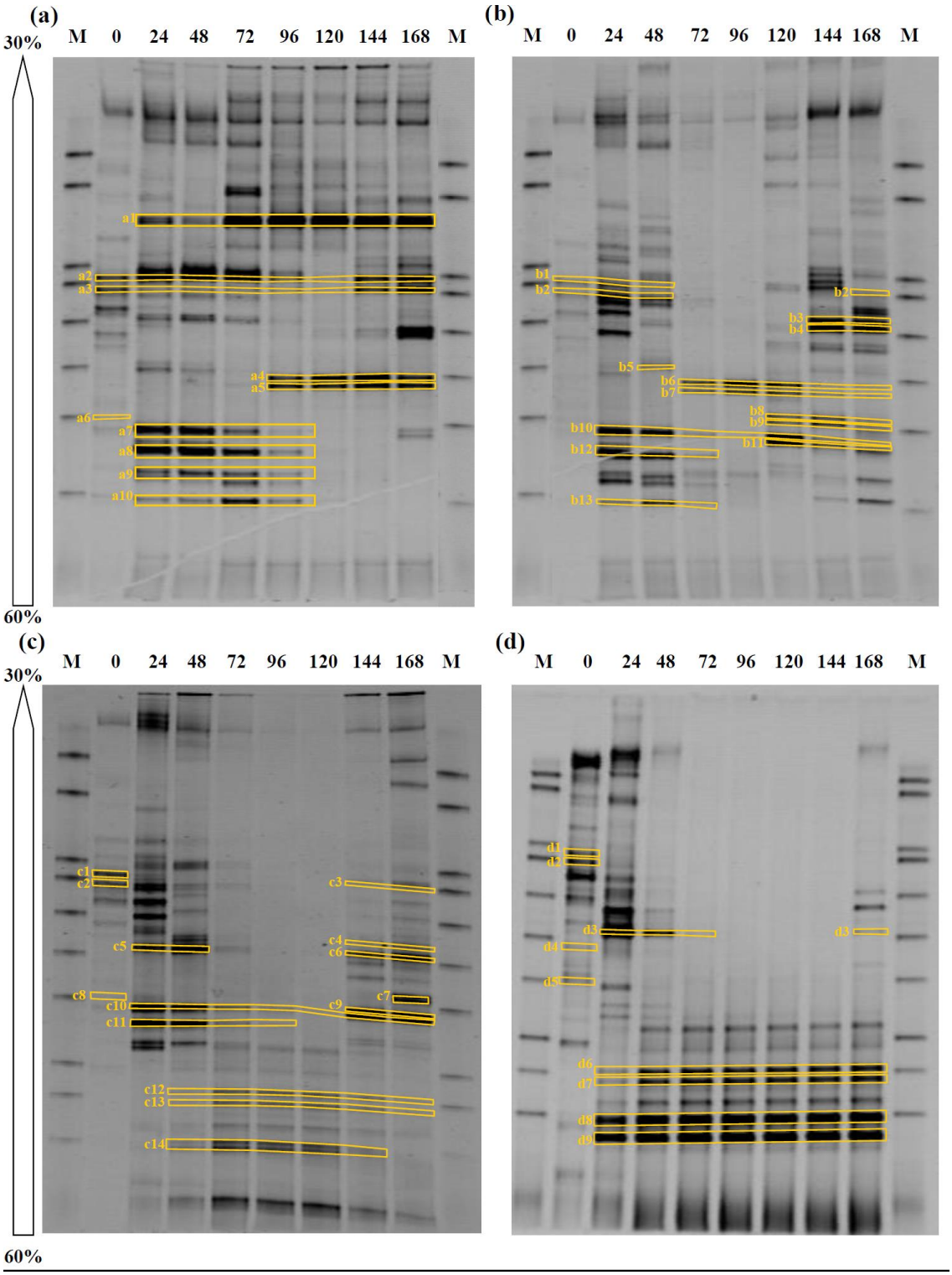
Temperature (^{o}C)	$C_{L\text{-}LA}$ (g L^{-1})	$C_{D\text{-}LA}$ (g L^{-1})	C_{AA} (g L^{-1})	C_{BA} (g L^{-1})	C_{PA} (g L^{-1})	C_{FA} (g L^{-1})	$OP_{L\text{-}LA}$ (%)	S_{LA} (%)	S_{BA} (%)
30	0.330	0.755	2.27	24.8	2.41	0.219	–39.2	3.69	80.5
37	0	0	7.06	26.8	1.63	0.010	-	0	75.5
40	16.6	1.23	5.70	6.51	0.090	1.00	86.2	58.3	20.9
45	34.2	0.224	2.57	0	0	0.143	98.7	91.3	0
50	34.5	0	2.80	0	0	0	100	91.6	0
55	21.7	0.185	2.30	0	0	0	98.3	89.7	0
60	9.51	1.51	5.24	3.00	0	0	72.6	57.3	15.6
65	7.93	1.31	2.73	0	0.166	0.586	71.7	73.2	0

$C_{L\text{-}LA}$, $C_{D\text{-}LA}$, C_{AA}, C_{BA}, C_{PA}, and C_{FA} represent concentrations of L-Lactic acid, D-Lactic acid, acetic acid, butyric acid, propionic acid, and formic acid after 168 h of culture, respectively. $OP_{L\text{-}LA}$, S_{LA}, and S_{BA} indicate optical purity of L-lactic acid, selectivity of lactic acid, and selectivity of butyric acid, respectively.

Although there are several reports on lactic acid production from kitchen refuse and food waste using several microorganisms and mixed culture (Wang *et al.,* 2011; Ohkouchi and Inoue, 2006; Sakai and Yamanami, 2006; Zhang *et al.,* 2008; Sakai *et al.,* 2000), ours is the first report of fermentation of L-lactic acid of 100% optical purity from kitchen refuse using a mixed culture. Compared with the 64 g L^{-1} of lactic acid reported by Zhang *et al.,* (2008), the lactic acid concentration of 34.5 g L^{-1} was not high in this study because the total sugars were mostly consumed after 168 h of culture (Fig. 2-1). Continuous pH-controlled fermentation was reported to produce higher lactic acid from kitchen refuse than intermittent pH-controlled fermentation (Sakai and Yamanami, 2006). To improve lactic acid production from kitchen refuse, we plan to investigate batch fermentation with higher initial total sugars or fed-batch fermentation by feeding the substrates under continuous pH control.

2.4.2. DGGE analysis of bacterial community structure during fermentation at different temperatures

In order to monitor changes in bacterial community structures and identify the dominant bacteria, we performed DGGE analysis targeting the partial 16S rRNA gene during fermentations at various temperatures (Fig. 2-1) from kitchen refuse using mixed culture seed (Fig. 2-3). At all fermentation temperatures except 30°C (fragments bands a2 and a3), most of the major fragments initially detected were diminished after 24 h of cultivation, and new fragments produced at specific fermentation temperatures appeared. Typically, more DGGE fragments were detected, with appearance and disappearance, at low fermentation temperatures depending on fermentation phase, whereas DGGE analysis at high fermentation temperatures showed fewer but more stable bands. Thus, bacterial community structure affected the time-course results (Figs. 2-1 and 2-2).


(a)
30%
M 0 24 48 72 96 120 144 168 M
a1
a2
a3
a4
a5
a6
a7
a8
a9
a10
60%
(b)
M 0 24 48 72 96 120 144 168 M
b1
b2
b2
b3
b4
b5
b6
b7
b8
b9
b10
b11
b12
b13
(c)
30%
M 0 24 48 72 96 120 144 168 M
c1
c2
c3
c4
c5
c6
c7
c8
c9
c10
c11
c12
c13
c14
60%
(d)
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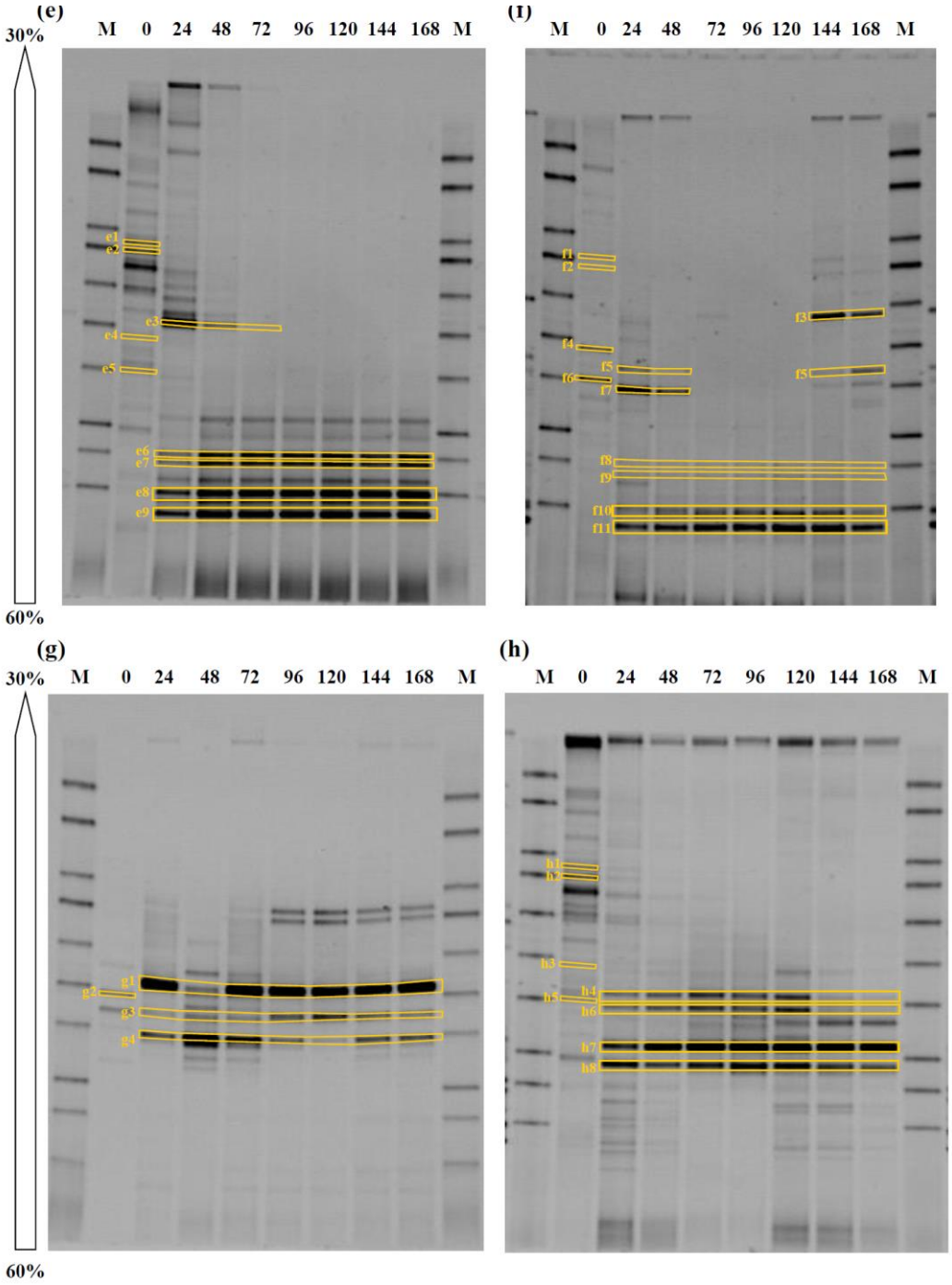

Fig. 2-3 DGGE profiles of fermentations in model kitchen refuse media using MAR composts as seeds at various temperatures: (a), 30°C; (b) 37°C; (c) 40°C; (d) 45°C; (e) 50°C; (f) 55°C; (g) 60°C; (h) 65°C. As revealed by positions of markers and detected bands, several band pairs showed similar sequences among different fermentation temperatures: [P1] a2, b1, c1, d1, e1, f1, and h1; [P2] a3, b2, c2, d2, e2, f2, and h2; [P3] a4, and b6; [P4] a5 and b7; [P5] a6, c8, d5, e5, f6, and h5; [P6] a7, b10, and c10; [P7] a8, b12, and c11; [P8] a10, and b13; [P9] b2, and c3; [P10] b8 and c7; [P11] c5, d3, and e3; [P12] c12, d6, e6, and f8; [P13] c13, d7, e7, and f9; [P14] c14, d8, e8, and f10 ; [P15] d4, e4, f4, g2, and h3; [P16] d9, e9, and f11.

Several fragment bands in the lower parts of gels were detected during 24–72 h, when lactic acid production was observed at 30°C (bands a7, a8, a9, and a10) and 37°C (bands b10, b12, and b13). In contrast, after 96 h and 72 h of culture, new bands appeared (at 30°C; bands a4 and a5 and 37°C; bands b3, b4, b6, b7, b8, b9, and b11), which we presumed contributed to the utilization of lactic acid and production of butyric and acetic acids. In the fermentation at 40°C, b and c14 appeared during lactic acid production (until 144 h) in addition to bands c10 and c11. Several bands (c3, c4, c6, c7, c9) appeared at late fermentation period (after 144 h), which should be responsible for butyric acid and acetic acid production with the consumption of lactic acid. At temperatures of 45, 50, and 55°C, the band pairs P12, P13, P14 and P16 were dominant throughout the fermentations, with several minor bands such as d3, e3, f3, f5, f5, and f7. These bands indicated that high L-lactic acid concentration (>21 g L^{-1}) with high optical purity (>98%) and selectivity (ca. 90%) would be produced by these dominant bacteria. In contrast, distinct bands at clearly different positions from the bands at 45, 50, and 55°C were dominant at 60°C (g1–g3) and 65°C (h4, h6–8) throughout the fermentations, although lactic acid concentrations produced were lower than 10 g L^{-1}.

These results suggested that various mesophilic bacteria showing various types of organic acid fermentation grew at relatively low temperatures (≤40°C), whereas the limited thermophilic bacteria likely to be enriched at higher temperatures than 45°C. In conclusion, the fermentation temperature exerted drastic effects not only on fermentation behaviors but also on bacterial community structures.

2.4.3. Sequencing and retrieval of DGGE dominant bands

We determined the sequences of 16 bands and band pairs derived from DGGE profiles, including major bands at various temperatures in fermentations from kitchen refuse using MAR composts, although all bands were not sequenced (Table 2-2).

The sequences of DGGE bands detected at 0 h were assigned to major members of *Cellulosilyticum ruminicola* (P1 and P2), *Bacillus thermocloacae* (P15), and *Corynebacterium sphenisci* (P5) (Fig. 2-3). A previous study of bacterial community structures of MAR composts by clone analysis targeting different region (V3–V5) of partial 16S rRNA gene revealed the presence of the same genera, *Bacillus* and *Corynebacterium*, but not genus *Cellulosilyticum* (Niisawa et al., 2008). Because we diluted the MAR composts more than 300-fold during preparation and inoculation of the seed (Materials and methods), the difference is attributable to the dilution of the 16S rRNA genes in this study.

The band pairs of P6–P7, and the pairs of P10 and band c9 detected in DGGE profiles at 30, 37, and 40°C exhibited high similarities to sequences from *Clostridium sartagoforme* and *Clostridium sporogenes*, respectively (Fig. 2-3, Table 2-2). As described above, these bands appeared to be associated with the utilization of lactic acid and production of butyric and lactic acids. Some Clostridia are known as butyric acid producers (Zhang *et al.*, 2009), and the band pairs of P10 and band c9 are expected to have been responsible for butyric acid production during the late phase at 40°C (Fig. 2-3). In addition, Clostridia are reported to possess metabolic pathways for assimilation and production of lactic acid (van Gylswyk and van der Toorn, 1987; Yoshida *et al.*, 2012).

The sequences of the dominant band pairs P14 and P16 detected in L-lactic acid fermentations at 45, 50, and 55°C showed high similarities to sequences of *Bacillus coagulans* with RDPII scores of 1.0 (Fig. 2-3, Table 2-2). *B. coagulans* has been reported to be thermotolerant at ca. 50°C and to produce L-lactic acid with high optical purity (Ye *et al.*, 2013). In previous studies of lactic acid production with bacterial consortia, mesophilic bacteria such as lactic acid bacteria and clostridia were considered to be main lactic acid producers in fermentation at 35°C (Zhang *et al.*, 2008) and 37°C (Sakai *et al.*, 2000), whereas the fermentations at high temperatures of 50°C and 55°C contained dominant strains of *B.*

coagulans by pyrosequencing analysis (Kim *et al.*, 2012) and clone analysis (Akao *et al.*, 2007), respectively. Although *B. coagulans* was minor not only in the fermentation broth at 0 h in this study (Fig. 2-3) but also in the MAR composts in the previous study (Niisawa *et al.*, 2008), high temperatures, around 45–55°C, would be suitable for the growth of *B. coagulans* strains and would promote L-lactic acid production even in a mixed culture system. Although an industrial fermentation process generally shows a difference in the temperatures of ±5°C, this property is advantageous for the industrial production of L-lactic acid because L-lactic acid production (concentration and yield) was not drastically different (Table 2-1).

The method for L-lactic acid production using MAR composts as mixed culture seed from kitchen refuse studied in this study has several advantages and feasibilities as follows: [1] only one pretreatment process, with simple homogenization of kitchen refuse without any separation of liquid and solid fractions; [2] direct usage of MAR compost without a seed culture; [3] open fermentation, with insusceptibility to contamination as a result of the relatively high fermentation temperatures; [4] no saccharified enzymes because of the detection of starch- (*B. thermoamylovorans*; Combet-Blanc et al., 1995) and cellulose- (*C. ruminicola*; Combet-Blanc et al., 1995) degrading bacteria by DGGE analysis (Table 2-2); and [5] the safety of MAR composts without pathogenic bacteria (Niisawa et al., 2008). These advantages and feasibilities can simplify the overall processes from garbage collection to L-lactic acid production as well as decrease the costs in terms of the equipments and saccharified enzymes used. Based on the above considerations, it would be possible to perform industrial L-lactic acid production during garbage collection in a truck, with a homogenizer and fermentor, by only adding MAR composts to the kitchen garbage. Furthermore, it is very important to efficiently utilize of the solid fraction of fermentation residues collected from the overall process which is under investigations in our laboratory.

The number of studies of fermentative production of valuable products by mixed culture systems using more than two microorganisms is much lower than that of studies describing pure culture systems using sole microorganisms. In this mixed culture system, fermentation temperature played a significant role in final LA concentration and optically purity of L-LA. At 50ºC and 55ºC, comparatively high L-LA with 100% optical purity was obtained. Fermentation temperature will be a primary factor in the control of a fermentation process by mixed culture systems. To further to understand and control the meta-fermentation process, we tried to isolate the major bacteria that contributed for efficient L-lactic acid production, which will be discussed in chapter III.

Table 4. Phylogenetic affiliation of major DGGE bands in batch fermentations in MKR medium using MAR composts as seed

Closest relative based on partial sequence homology	Accession No.	Band (Pairs) No.	RDP II score
Cellulosilyticum ruminicola	EF382648	P1	0.872
		P2	0.803
Corynebacterium sphenisci	AJ440964	P5	1.00
Clostridium sartagoforme	Y18175	P6	0.823
		P7	0.799
Oceanobacillus caeni	AB275883	P9	0.824
Clostridium sporogenes	X68189	P10	0.799
		c9	0.882
Bacillus thermoamylovorans	L27478	P11	0.858
		c4	0.761
Bacillus coagulans	AB271752	P14	1.00
		P16	1.00
Bacillus humi	AJ627210	f3	0.892
Bacillus thermocloacae	Z26939	P15	0.866
Bacillus smithii	Z26935	f5	0.882
		f7	0.973

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CHAPTER III: Systematic isolation of major bacteria from mixed culture system by feedback isolation technique

This chapter is a modified version of the submitted manuscript (Poudel P., Tashiro Y., Miyamoto H., Okugawa Y, Sakai K. Systematic isolation of major bacteria from mixed culture system by feedback isolation technique and colony MALDI TOF-MS analysis) to Journal of Applied Microbiology, 2015.

3.1. Abstract

Major bacteria corresponding to *Corynebacterium sphenisci*, *Bacillus thermocloacae*, *Bacillus thermoamylovorans*, *Bacillus smithii*, *Bacillus humi* and *Bacillus coagulans*, involved in mixed culture fermentation system detected by denaturing gradient gel electrophoresis (DGGE) in chapter 2, were targeted for the isolation. Based on these results, preferable cultivation conditions (media, pH, temperature etc.) for the major bacteria were obtained from several literatures. Appeared colonies from mixed culture seed and fermentation broth were firstly screened by MALDI-TOF MS direct colony method and further compared by 16S rRNA gene sequencing and found that numerical threshold value of 0.55 for similar bacterial isolates. Total 21 bacterial species including six major bacteria were successfully isolated. We succeeded in systematic isolation of major bacteria involved in mixed culture fermentation system by adopting feedback isolation technique and direct colony MALDI-TOF MS. The study reveals that multiple targeted bacterial strains can be isolated by adopting systematic feedback isolation technique. It provides novel efficient and simple methodology to screen total bacterial members in a certain mixed culture fermentation system.

3.2. Introduction

Isolations of bacteria by forming colonies on solidified media from an intended sample have been not only traditional but also fundamental and essential. This method has contributed to great developments in the field of microbiology because we can obtain information on phylogenetic taxonomy and function of the isolates by further analysis. However, it is estimated that only approximately 1% of bacteria on Earth have been isolated using solidified media at a laboratory and a number of remaining bacteria are not yet to be isolated (Vartoukian *et al*., 2010). Different strategies for isolating not yet isolated bacteria such as an

enrichment of bacteria on the selective conditions, development of growth media reflecting the habitat environments, and addition of other bacterial extract as the aids to growth have been traditionally applied (Kaeberlein *et al*. 2002). Nevertheless, most of them failed to isolate the targeted bacteria because a solidified medium that mimic the material and conditions of the isolation source sometimes are not sufficient to isolate the targeted bacteria (Kaeberlein *et al*. 2002). In addition, even though several colonies could be obtained on the solidified media, 16S rRNA gene analysis of those isolates sometimes reveals the same species, which results in time-consuming, labor, and costly procedures with increasing the number of the colonies. To further develop the isolation strategy, we need to establish systematic methodologies for more efficient isolation, i.e. high rate of colony formation and rapid screening of representative colonies.

One of the reasons why all the bacteria cannot form colonies on solidified medium has been considered to be attributed to the inadequate culture conditions such as medium compositions, temperature, pH, oxygen atmosphere, and so on (Vartoukian *et al*., 2010). Even though it is possible that more colonies can be obtained under the suitable culture conditions, the investigations and optimizations of them should not be easy. On the other hand, researchers sometimes determine the targeted bacteria for isolations from several samples based on the results by molecular biological techniques, and information on culture conditions or physiological properties in terms of the closest relatives with the targets has been available in literatures. Therefore, we propose “feedback isolation” strategy to isolate the colonies of the targets on the solidified medium by selection of the specific culture condition from the available information, which would increase the rate of colony formation.

Recently, direct colony matrix assisted laser desorption ionization time of flight mass spectrometry (“colony-MALDI-TOF MS”) technique (Freiwald and Sauer, 2009) has been developed as robust technique for rapid and effective screening of bacteria (Mellamann *et al*.,

2008). Colony-MALDI-TOF MS analysis can select the candidates by comparing the protein spectra of an isolate and calculating similarity with the closest relatives (e.g. the type strain) and the other isolates at the colony level for several minutes. Thus, this technique may decrease the number of colony, time, labor, cost before the application of 16S rRNA gene analysis, and can improve the efficiency of screening from a lot of colonies rapidly. So far, because the researchers can use only commercial statistical software to calculate the similarity from the protein spectra obtained by colony-MALDI-TOF MS analysis, there are no reports on application of generalized and readily-available software for this approach.

In chapter II, major bacteria (*C. sphenisci, B. thermocloacae, B. thermoamylovorans, B. humi, B. smithi* and *B. coagulans*) involved in the mixed culture system were detected by denaturing gradient gel electrophoresis (DGGE). In order to analyze the individual or interactive functions of the bacteria in mixed culture system, an isolation of major bacteria was required. Therefore, the main aim of this study is to isolate the above 6 bacteria according to "systematic isolation" approach: "feedback isolation" and "colony-MALDI-TOF MS" by using a generalized algorithm.

3.3. Material and Methods

3.3.1. Reference strains used in this study

In this studies, following reference strains were used; *Bacillus thermolactis* DSM 23332^T (Coorevits et al., 2011), *Bacillus thermoamylovorans* LMG 18084^T (Combet-Blanc *et al.*, 1995 & *Coorevits et al, 2011*), *Bacillus humi*, DSM 16318^T (Heyrman et al, 2005), *B. ruris* DSM 17057^T (Heyndrickx *et al.*, 2005), *Bacillus thermocloacae* DSM 5250^T (Demharter and Hensel, 1989), *Bacillus coagulans* NBRC 12583^T (De Clerk et al., 2004), *Paenibacillus barengoltzii* DSM 22255^T (Osman *et al.,* 2006) and *Corynebacterium sphenisci* CECTT

(Goyache *et al.,* 2003). Strains *B. thermolactis* DSM 23332^T, *B. thermoamylovorans* LMG 18084^T were maintained on TSA at 50°C, strains *B. humi* DSM 16318^T and *B. ruris* DSM 17057^T were maintained at 30°C on TSA. *Bacillus thermocloacae* DSM 5250^T was maintained at 55°C on Ottow medium. *B. coagulans* NBRC 12853^T was maintained at 50°C in nutrient agar. *P. barengoltzii* DSM 22255^T was maintained on TSA at 37 °C. *C. sphenisci* CECT^T was maintained on Columbia blood agar at 37 °C.

3.3.2. Isolation source

Mixed culture seed (compost) (Niisawa *et al.,* 2008) and the fermented broth were used as isolation sources. The mixed culture fermentation was performed as described previously (chapter 2). The compost extract used in this study was prepared as follows: Compost was mixed with deionized water (1:3) with thorough shaking for 1 min and kept overnight at room temperature statically. The mixture was then centrifuged at 15,000 × g for 10 min to obtain supernatant and was added into the medium as the extractant. The fermented broth (fermented at 50°C and 55°C) extract was prepared as follows: fermented broth was mixed in deionized water (1:1), centrifuged at 15,000 × g for 10 min and obtained supernatant was added into the medium as the extractant only after sterilization at 121°C for 20 min.

Table 3-1 Feed-back information from literature for the cultivation of major bacteria involved in meta-fermentation

Target strain	Temp. (°C)	pH	Isolation medium	O_2 requirement	Incubation time (h)	Representative isolates	Reference
Corynebacterium sphenisci	37*, 50	7.2	Fosfomycin-Columbia blood agar	aerobic	48	CPh-1	Goyache et al., 2003; Graevenitz et al., 1998
		7.2	Corynebacterium medium†	aerobic	48		Atlas, 1997
Bacillus thermocloacae	55*, 60	8.5	Ottow + compost extract	aerobic	72	24-3	Demharter and Hensel, 1989
Bacillus humi	30, 50*	7.4	CASO	aerobic	48	MO-14	Heyrmann et al., 2005
Bacillus coagulans	50	6.8	Nutrient	aerobic	24	MN-07	De Clerck et al., 2004
Bacillus thermoamylovorans	50	7.0	Tryptone Soy, CASO (supplemented with 2% soluble starch)	aerobic	24	MC-07	Combet–Blanc et al., 1995
Bacillus smithii	50*, 55	6.8	Tryptone soy, CASO	aerobic	48	MC-17	Nakamura et al., 1988

*isolated temperature; †not able to form the colonies of targeted strain

3.3.3. Colony formation by feedback isolation from meta-fermentation

In this study, *C. sphenisci*, *B. thermocloacae*, *B. humi*, *B. coagulans*, *B. thermoamylovorans*, and *B. smithii* were targeted candidates and their information for isolations were listed in Table 3-1. For the isolation, each of 1 g mixed culture seed and 1 mL fermented broth, respectively, were enriched in 9 mL of respective sterilized liquid medium at given temperatures (Table 3-1) for 4 h under aerobic condition without shaking. The enrichment culture broths were serially diluted in saline solution (0.85% NaCl), spread onto respective media, and then the plates were incubated to form the colonies under indicated conditions as shown in Table 3-1. All the media were sterilized at 121°C for 15 min.

For the isolation of *C. sphenisci* from the compost, Corynebacterium agar (12 g Tryptic digest of casein [Difco^{TM}; Becton-Dickinson, Franklin Lakes, NJ, USA], 5 g NaCl, 5 g yeast extract [Becton-Dickinson], 15 g agar per 1 L of deionized water, pH 7.2) (Graevenitz et al., 1998) and Fosfomycin-Columbia blood agar (12 g pancreatic digest of casein [Becton-Dickinson], 5 g NaCl, 3 g beef extract [Nacalai Tesque, Kyoto, Japan], 3 g yeast extract [Becton-Dickinson], 1 g soluble starch [Wako Pure Chemicals Industries, Osaka, Japan], 1 g tween 80, 100 mL compost extract, and 15 g agar per 1 L of deionized water) (Graevenitz *et al*. 1998) were used. Filter sterilized fosfomycin disodium salt (Sigma St. Louis, MO, USA) solution and sheep blood (Nippon Biotest Laboratories Inc. Tokyo, Japan) were added to Fosfomycin-Columbia blood agar (final concentrations: 50 mg/L and 50 mL/L, respectively) when the media was cooled to 50°C.

For the isolation of *B. thermocloaceae* from the compost, Ottow medium (1 g glucose, 7.5 g peptone, 5.0 g meat extract, 2.5 g yeast extract, 2.5 g casamino acids, 5.0 g NaCl, and 20 g agar in 1 L deionized water, pH 8.5, 100 mL mixed culture seed extract) was used (Demharter and Hensel, 1989).

To isolate *B. smithi* and *B. thermoamylovorans*, CASO (15.0 g peptone from casein, 5.0 g peptone from soy-meal [Nacalai Tesque], 5.0 g NaCl per 1 L of deionized water) and Tryptone soy agar (15 g pancreatic digest of casein (Becton-Dickinson), 5 g Papaic digest of soybean meal [Nacalai Tesque], 5 g NaCl, 15 g agar, per 1 L of deionized water) were used. In case of isolating *B. thermoamylovorans*, 2% soluble starch (Wako) was added into CASO medium (Combet–Blanc *et al.*, 1995). To isolate *B. humi*, CASO medium was used. For isolating *B. coagulans*, nutrient agar (3 g beef extract [Nacalai Tesque], 5 g enzymatic digest of gelatin [Wako], 5 g NaCl, 15 g agar per 1 L of deionized water) with 0.001% $MnSO_4$ was used (De Clerck *et al.*, 2004). Ten percentages of the media (CASO, tryptone soy agar and nutrient agar) contained the sterilized fermented broth extractant as growth aid for targeted bacteria. Incubation temperature, pH and time for the targeted strains are listed in Table 3-1.

3.3.4. Screening isolates by direct colony MALDI-TOF MS

Bacterial isolates were screened using direct colony MALDI-TOF MS method (Freiwald and Sauer, 2009). Bacterial isolates were cultured onto the respective isolation media and incubated for 20 h at appropriate temperature. Matrix solution was prepared by mixing 500 µl of 50% acetonitrile and 500 µl of 2.5% tri-flouroacetic acid solution followed by addition of 10 mg of α–cyano-4-hydroxycinnamic (Freiwald and Sauer, 2009). Single colony was spread on MALDI target plate using sterile tooth-pin and dried at room temperature for 20 min. Two microliters of freshly prepared matrix solution was overlaid on each target and dried at room temperature for 30 min, and the crystallized samples were analyzed with MALDI-TOF MS (Axima plus, Shimadzu, Kyoto, Japan), at Center for Advanced Instrument and Educational Supports, Faculty of Agriculture, Kyushu University, in linear positive ion mode with constant potential of 17 kV. Each spectrum was the sum of the ions from ca. 200 laser shots for the different regions of the same well. Similarly, the optimal mass-spectrometer was set in the range from m/z 1 kDa to 20 kDa to find differences. Calibration of machine was based

on the precise mass values of well-known *E. coli* DH5α cells (inoculated in nutrient agar and incubated at 35°C for 20 h before preparations of the sample for MALDI-TOF MS analysis) (Freiwald and Sauer, 2009). MALDI-TOF MS data (mass molecular weight and area of each peak) of each strain was first analyzed by free software of SpecAlign (Wong *et al*., 2005) to calculate proximity matrices, and then commercially available XLSTAT ver. 2014 (http://www.xlstat.com/en/) computed similarity distance and Pearson correlation coefficient, and made a dendrogram from the matrices.

3.3.6. 16S rRNA gene sequencing of isolates and correlation to MALDI-TOF MS clustering

DNA was extracted by using Qiagen DNA extraction kit (Qiagen, Hilden, Germany). Amplification of the 16S rRNA gene was performed using the following universal primer sets: 8F (5′ AGA GTT TGA TCC CTC AG 3′) and 1492R (5′ GGT TAC CTT GTT ACG ACTT 3′). The amplification conditions were as follows: 30 cycles of DNA denaturation at 98°C for 10 s, primer annealing at 55°C for 5 s, and elongation at 72°C for 1 min. Polymerase chain reaction products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The sequencing of amplified pure DNA was performed by Dragon Genomics Center, (TaKaRa, Japan). The similarity score of nearly full length sequence was calculated by EzTaxon-e server (Kim *et al.,* 2012). 16S rRNA gene sequences of closely related taxa were obtained from GenBank/EMBL/DDBJ DNA database and Ribosomal Database Project (Cole *et al*., 2009) and edited by BioLign ver 4.0.6 program (Hall, 1999). Sequences were aligned using CLUSTAL W ver. 2.01 (http://clustalw.ddbj.nig.ac.jp) (Thompson *et al*., 1997), and the phylogenetic analysis (neighbor-joining) was conducted using MEGA ver.6 with bootstrap values calculated from 1,000 replications (Tamura *et al.,* 2013). The assigned DDBJ/EMBL/GenBank sequence accession numbers for isolated strains of MCS used in this study are AB849115–AB849118, JX848632–JX848635, LC011486–LC011524 and LC012004.

3.3.6. Characterizations of targeted isolates of meta-fermentation

Cell morphology was observed by light microscopy (Olympus BX50 [Olympus Optical Co. Ltd, Tokyo, Japan]). Endospore formation was detected by using malachite green, as described by Schaeffer and Fulton (1933) and observed by light microscopy (Olympus BX50). Motility was observed on semi-solid agar by using the method described by Tittsler and Sandholzer (1936). Oxidase activity was determined using an oxidase reagent (BioMérieux, Marcy l'Etoile, France) and catalase activity was tested by adding 3% (v/v) H_2O_2 solution to a bacterial colony and observing immediate O_2 gas production. Hydrolysis of casein, starch, and cellulose was also examined after 48 h of incubation at each temperature (Smibert & Krieg, 1994). Growth at different temperatures (30, 35, 37, 40, 42, 45, 50, 55, 60, 64, 68, 70.2°C) was investigated in the respective liquid medium after 48 h of incubation and measuring the OD at 610 nm (UV-1600 spectrometer, Shimadzu) in a Advantec TN-2148 gradient temperature incubator (Advantec, MFS inc., Dublin, US). Other biochemical properties were performed using commercially available API kits (BioMérieux) according to the manufacturer's description.

3.3.7. Lactic acid production by meta-fermentation candidate

Medium for lactic acid production and the chemical analysis process were performed as described in chapter II.

3.4. Results and Discussion

3.4.1. Feedback isolation of major bacteria from meta-fermentation

As previously reported in the DGGE analysis of meta-fermentation, six major bands at 50°C and 55°C were assigned to *B. thermocloacae, C. sphenisci, B. humi, B. thermoamylovorans, B. smithii,* and *B. coagulans* sharing 96.6%, 99.8%, 96%, 99.02%, 99.8% and 100% 16S rRNA gene similarity respectively (chapter II).

By implementing feedback isolation technique, altogether 136 bacterial colonies (14 colonies were recovered from nutrient agar, 21 colonies from CASO, 6 colonies from tryptone soy agar, 14 colonies from fosfomycin-Columbia blood agar, 50 colonies from Ottow medium and 28 colonies from Corynebacterium agar plate) were picked-up from different isolation media and subjected to screening. 136 colonies were purified by repetitive streaking on the respective agar plates, and further were analyzed below.

3.4.2. Availability of direct colony MALDI-TOF MS technique and screening of representative isolates

Picked-up many colonies should be screened by removing the very closest relatives (e.g. same species strains) among the isolates and by selecting the candidate close to the targeted type strain, followed by 16S rRNA gene analysis. To confirm the availability of direct colony MALDI-TOF MS technique as an efficient screening method, we compared the results of selected 25 strains including isolates and type strains, having similar mass spectral patterns, between direct colony MALDI-TOF (dendrogram) by using commercial statistical software (XLSTAT) and 16S rRNA gene (phylogenetic tree) analyses (Fig. 3-1). In the both analyses, 6 clusters were formed by almost the same strains based on the similarity scores of >ca.98% (16S rRNA gene) and the similarity distances of >ca.0.55 (direct colony MALDI TOF-MS). On the other hands, although *Bacillus thermolactis*^{T} and *Bacillus kokeshiiformis*^{T}

were included in the same cluster with the high similarity score (99.4%) together with the other 5 isolates by 16S rRNA gene analysis (Fig. 3-1(A)), *B. thermolactis*^{T} was excluded from the cluster in direct colony MALDI TOF-MS dendrogram due to the low similarity distance of <0.42 (Fig. 3-1(B)). From these results, even when 16S rRNA gene analysis may not discriminate bacteria at the species level, it was sometimes possible to distinguish bacteria more clearly by direct colony MALDI TOF-MS analysis than 16S rRNA gene analysis. Therefore, direct colony MALDI-TOF MS could be valuable tool for the rapid screening of a representative isolate by comparing the similarity distance (>ca. 0.55) with the targeted type strains and by selecting one strain in the cluster.

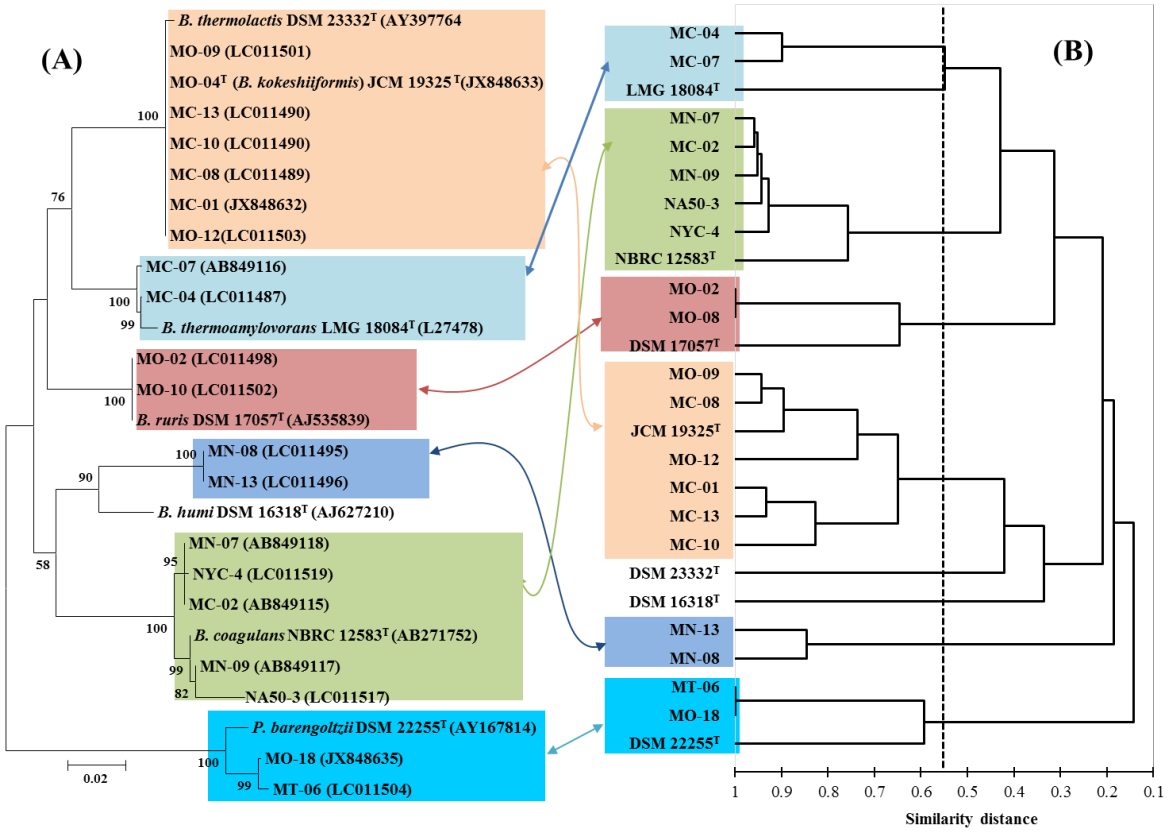
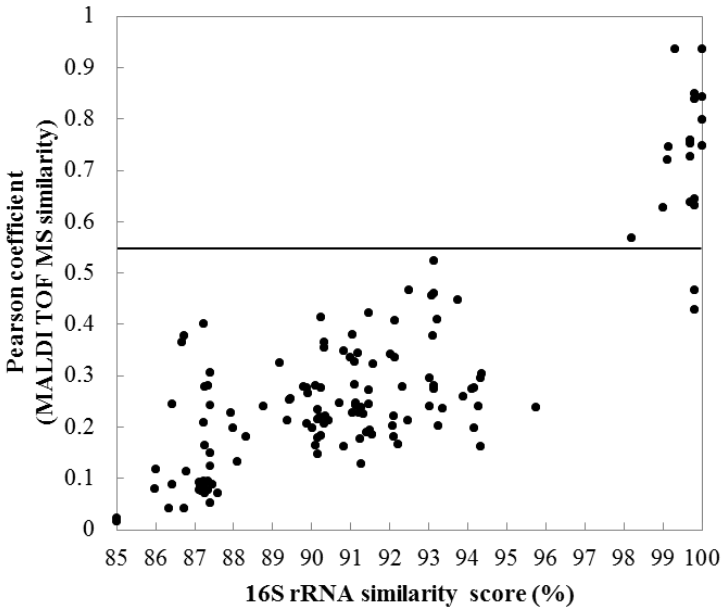


Fig. 3-1 Comparison of 16S rRNA gene sequences phylogenetic tree (A) and MALDI-TOF MS dendrogram (B) with some representative isolates including type strains. Dotted line indicates the threshold value for similar isolates

To further confirm the relationship between direct colony MALDI-TOF MS and 16S rRNA gene sequence analyses in more detail, Pearson correlation coefficient (direct colony MALDI-TOF MS data) calculated by using commercial statistical software (XLSTAT) and similarity scores (16S rRNA gene sequence data) were investigated in terms of each pair of different 2 strains including isolates and type strains. Figure 3-2 indicated the good correlation among them clearly (Pearson correlation coefficient = 0.844, *p*-value <0.0001). In general, most of bacteria with the similarity scores of >ca. 98% by 16S rRNA gene sequence analysis have been considered to be identical at the species level , although an exception has been already reported such as *B. thermolactis*^{T} and *B. kokeshiiformis*^{T} regardless of 99.4% similarity score (Poudel *et al.,* 2014). In this study, all the pairs with higher Pearson correlation coefficient than 0.55 also showed similarity score 98.2%. Note that the threshold values of 0.55 in direct colony MALDI-TOF MS analysis should be same between similarity distance and Pearson correlation coefficient calculated for all the strains and each pair (2 strains), respectively. As described at the previous paragraph, thus, direct colony MALDI-TOF MS analysis can be effective for rapid screening as well as identification.



Mantel test / Two-tailed test:	
r(AB)	0.844
p-value (Two-tailed)	< 0.0001
alpha	0.05

Fig. 3-2 Correlation between the MALDI-TOF MS data and 16S rRNA gene sequence similarity data. Mantel test for MALDI-TOF MS proximity matrix values and 16S rRNA gene sequence similarity score 27 isolates.

Direct colony MALDI-TOF MS technique has been actively investigated for the application of the identification (Mellamann *et al*. 2008; Welker and Moore, 2011). For the identification, currently, the spectral mass of a strain has been analyzed by the specific software, in particular SARAMIS and MALDI Biotyper sold by Shimadzu (Japan) and Brucker Daltonics (Germany), respectively, with own algorithms, and then has been compared with known strains available in the stored database published by the respective companies. However, the specific software is not cheap and should be essential to identify the isolates by this technique, and the numbers of the known strains available in both database have been still limited so far (Shimadzu, more than 500 microbial species; Brucker Daltonics, more than 1800 bacterial species) (Böhme *et al.*, 2012). In addition, the researchers using a company's software can use only the database of the same companies. Although there were few reports on the usage of the other software rather than SARAMIS and MALDI Biotyper

(Hsieh et al. 2008), our results by the analysis method using commercially available and cheap software (XLSTAT) also could show the feasibility of direct colony MALDI-TOF MS technique for identification of strains (Figs. 3-1 and 3-2). Note that all the researchers can compute the similarities by our same method from MALDI-TOF MS data (mass molecular weight and area of each peak) obtained from any MALDI-TOF MS instruments, and can compare them. Therefore, if a database is established based on the similarities calculated by our method, the significance and availability would improve drastically in the field of microbiology like 16S rRNA gene analysis.

Pearson correlation similarities were clearly good interrelated with similarity scores obtained by 16S rRNA gene analysis (Fig. 3-2). To the best of our knowledge, this is the first report to compare the values in terms of each pair (two strains) although the correlations have been showed from dendrograms (direct colony MALDI-TOF MS analysis) and phylogenetic trees (16S rRNA gene analysis) in not only this study (Fig. 3-1) but also literatures (Tani et al., 2012). Based on the concept of our feedback isolation, we can apply the type strains for direct colony MALDI-TOF MS analysis together with isolates because the targeted strains have been decided previously. As proposed by Koubek et al. using 9 strains belonging to *Achromobacter*, *Arthrobacter*, *Gordonia*, *Pseudomonas* genera (Koubek *et al*., 2012), due to the quite good performances (accuracy, reliability, cost, rapidity), this strategy should contribute to an establishment of the systematic isolation strategy for bacteria including *Bacillus* genus.

3.4.3. Phylogenetic affiliation based on 16S rRNA gene sequence

Figure 3-3, demonstrates the phylogenetic position of isolates including their closest relative type strains and 16S rRNA gene similarity score. Each representative isolates formed a clade with closely related type strains which was supported by high bootstrap values (100%). 16S rRNA gene sequence of six targeted bacteria MC-07, MO-14, MC-17, 24-3, CPh-1 and MN-07 shared 99.2%, 99.2%, 99.02%, 98.2%, 99.5% and 100% similarity with related type strains *B. thermoamylovorans*, *B. humi*, *B. smithii*, *B. thermocloacea*, *C. sphenisci* and *B. coagulans* respectively. 16S rRNA gene similarity score of the isolated targeted bacteria matched with those obtained by DGGE analysis (Chapter 2).

On the other hand, some unexpectedly isolated bacteria were closely affiliated to *Bacillus licheniformis, Bacillus subtilis, B. ruris, B. kokeshiiformis, B. isronensis, Lysisnibacillus fusiformis, Bacillus ginsengihumi, Ureibacillus thermosphaericus, Cohnella laeviribosi, Paenibacillus elgii, Paenibacillus cookie, Paenibacillus pabuli, Paenibacillus phoenisci, Paenibacillus barengoltzii* and *Brevibacillus borstelensis* (Fig. 3-3). All these non-targeted strains were isolated from mixed culture seed. Altogether 21 different species including the targeted bacteria were successfully isolated in this study. Some of these non-targeted isolates were also detected in minor amounts by pyrosequencing of this mixed culture seed (unpublished data).

Besides, some of the isolates resembling to genus *Bacillus, Lysinibacillus* and *Paenibacillus* showing 16S rRNA gene sequence similarity less than 98.7% were also found indicating possible existence of novel bacterial genus or species as well. For instance, isolate CORY-14 (Genbank accession number LC012004) isolated from mixed culture seed demonstrated low similarity to *Paenibacillus elgii* B69^{T} (89.8% pairwise 16S rRNA gene identity, Fig. 3-3) that could be allocated to new genus in the order Bacillales.

Meantime, it is interesting to say that, some of isolated but non-targeted strains have been widely practiced in varieties of biotechnological applications. For instance, *B. licheniformis* and *B. subtilis* are used for varieties of enzyme as well as L-lactic acid production (Sakai *et al.,* 2012). *L. fusiformis* is applicable in detoxification of waste water (Chromium III to chromium IV less toxic ion) and hydrolyze urea to produce ammonia and CO_2 (He *et al.,* 2011). *U. thermosphaericus* is applicable in production of meso-diaminopimelate dehydrogenase (Akita *et al.,* 2011). Similarly, *B. ginsengihumi* (high phytase activity) (Akhmetova *et al.,* 2013), *C. laeviribosi* (xylanase and amylase producers) (Kim *et al.,* 2010), *P. cookie* (high endoglucanase producer) (Shinoda *et al.,* 2012), *P. pabuli* (chitinolytic enzyme producer) (Wang *et al.,* 2008), *B. borstelensis* (polyethylene degrading) (Hadad *et al.,* 2005) also have their biotechnological applications too. Therefore, the selective combinations of microbes including the targeted and non-targeted isolated strains would be effective mixed culture for food waste treatments.

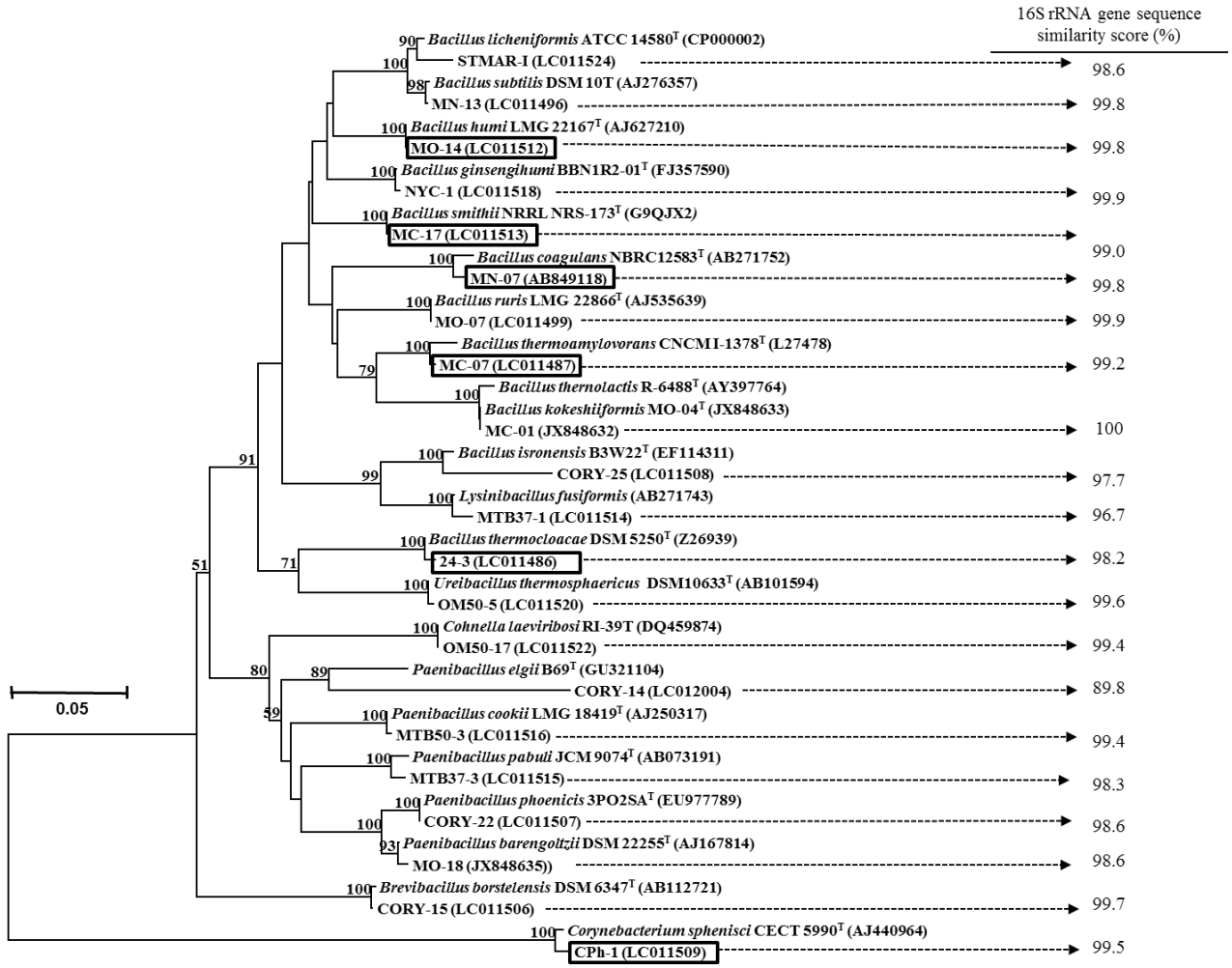


Fig. 3-3 Neighbour-joining tree based on the 16S rRNA sequences, showing the position of isolated strains, the closely related species. Sequence accession numbers used are shown in parentheses. Bootstrap values (expressed as percentages of 1000 replications) above 50% are shown. Bar 0.05 substitutions per nucleotide position. Isolates encoded with the squares were the major candidates of meta-fermentation.

3.4.4. Characterization of the targeted major isolates in meta-fermentation

For characterization of six targeted major bacteria, each representative isolate sharing the highest similarities was compared with their closest type strains. The phenotypic properties of targeted strains along with their closest relatives are depicted in Table 3-2. All the major targeted bacteria showed quite similar phenotypic characteristics with each type strains. Some sugar assimilation profile of isolate MC-07 and strain LMG 18084^T slightly differed. Isolate MC-07 produced acid from D-galactose, L-rhamnose, D-maltose, D-lactose and turanose, while strain LMG 18084^T could not produce acid from them. Interestingly all the targeted bacteria were thermophilic in nature except isolate CPh-1. Wide varieties of sugar were assimilated by isolates MC-07 and MN-07 compared to other isolates.

Previously, the molecular information showed the existences of *Corynebacterium* species and thermophilic *Bacillus* in this mixed culture seed (Niisawa *et al*., 2008). However, their isolations of the targeted bacteria were not successful completely. Major bacteria of meta-fermentation in particular, *B. coagulans* and *B. thermoamylovorans* are reported to produce optically pure L-lactic acid (Sakai *et al*., 2006, Combet-Blanc *et al.*, 1995). Tongpim *et al*., (2013) reported that strain (*Bacillus* sp. L50S4) related to *B. humi* produced L-lactic acid from glucose. However, the role of other major member related *C. sphenisci*, *B. smithii* and *B. thermocloacae* in mixed culture system are yet to be investigated because of much few published information on these bacteria. *B. thermocloacae* was previously isolated from sewage sludge treatment (Demharter and Hansel, 1989) and composting (Pedro *et al*., 2003). It is very important to note that the role of bacteria in mixed culture system can be assessed only when they are successfully isolated and characterized.

3.4.5. L-Lactic acid production from meta-fermentation candidates

To check the efficiency of selective combination of mixed culture, first trial was performed using two major bacteria of meta-fermentation, MN-07 and MC-07 closely related to *B. coagulans* (potent L-LA producer) (Tongpim *et al.*, 2014) and *B. thermoamylovorans* (potent amylase producer) (Combet-blanc *et al.*, 1995) respectively for the fermentative production of LA from food waste. This reconstructed mixed culture could produce 16.42 g/L of L-LA with a yield of 0.721 g/g and productivity of 0.225 g/L· h under non-sterile condition. The optical purity of L-lactic acid was 100% (Fig. 3-4). Even though the concentration of LA was lower than that produced using the original mixed culture seed (described in chapter 2), LA yield was slightly improved.

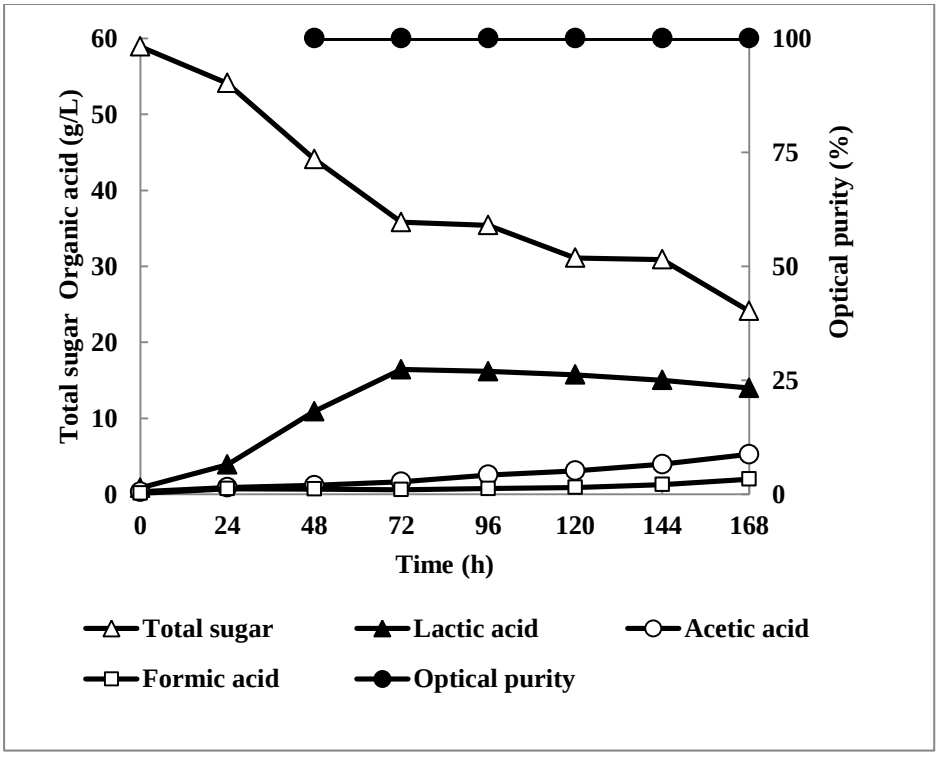


Fig. 3-4 Time course of L-lactic acid production by mixed culture of isolates MN-07 and MC-07 closely related to *B. coagulans* and *B. thermoamylovorans* using food waste at 50ºC under non-sterile condition.

Systematic isolation of major bacteria involved in mixed culture fermentation system by adopting feedback isolation technique and rapid screening by direct colony MALDI-TOF MS was succeeded. The study reveals that multiple targeted bacterial strains can be isolated by adopting systematic isolation technique. It provides novel efficient and simple methodology to screen total bacterial members in a certain mixed culture fermentation system. Furthermore, the reconstruction of a stable community with isolated bacteria for efficient L-Lactic acid production from waste biomasses are under investigation.

Table 3-2 Summary of phenotypic characteristics of major candidates of mixed culture system

Characteristics	1	1*	2	2*	3	3*	4	4*	5	5*	6	6*
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+
Spore former rod	+	+	+	+	+	+	+	+	–	–	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	+	+	–	–	–	–	–	–
Anaerobic growth	+	+	+	+	+	+	–	+	+	+	+	+
Casein hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–
Starch hydrolysis	+	+	–	–	–	d	–	–	–	–	+	+
Cellulose hydrolysis	+	+	–	–	–	–	–	–	–	–	+	–
Motility	+	+	+	+	+	+	+	+	–	–	+	+
Acid is produced from												
Glycerol	–	–	–	–	–	d	–	–	–	–	+	+
D-arabinose	–	–	–	–	–	–	–	–	–	–	–	–
L-arabinose	+	+	–	–	–	–	–	–	–	–	+	+
D-ribose	+	+	–	–	+	d	w	–	+	+	+	+
D-galactose	+	–	–	–	–	–	–	–	–	+	+	+
D-glucose	+	+	+	–	+	+	–	–	+	+	+	+
D-fructose	+	+	–	–	–	+	w	–	+	+	+	+
D-mannose	+	+	–	–	–	–	–	–	+	+	+	+
L-sorbose	–	–	–	–	–	–	w	–	–	–	–	–
L-rhamnose	+	–	–	–	d	d	–	–	–	–	+	+
Myo-inositol	–	+	–	–	–	–	–	–	–	–	–	–
D-xylose	–	–	–	–	–	–	–	–	–	–	+	–

Continue….

Characteristics	1	1*	2	2*	3	3*	4	4*	5	5*	6	6*
Acid is produced from												
D-xylose	–	–	–	–	–	–	–	–	–	–	+	–
Methyl D-glucoside	+	+	–	–	–	–	–	–	–	–	–	–
D-mannitol	–	+	–	–	–	–	–	–	–	–	–	–
D-sorbitol	–	–	–	–	–	d	–	–	–	–	–	–
N-acetyl glucosamine	+	+	–	–	+	+	–	–	–	–	+	+
Amygdalin	+	+	–	–	–	–	–	–	–	–	+	+
Salicin	+	+	+	+	+	–	–	–	–	–	+	+
D-cellobiose	+	+	–	–	–	–	–	–	–	–	+	+
D-maltose	+	–	–	–	+	+	–	–	+	+	+	+
D-lactose	+	–	–	+	–	–	–	–	–	–	+	+
D-melibiose	+	+	–	–	–	–	–	–	–	–	+	+
D-sucrose	+	+	–	–	–	–	–	–	–	–	+	d
D-trehalose	+	+	–	–	+	+	–	–	+	+	+	+
D-raffinose	–	–	–	–	–	–	–	–	–	–	+	+
Starch	+	+	–	–	–	–	–	–	–	–	+	+
Gentibiose	+	+	–	–	–	–	–	–	–	–	–	+
Esculin	+	+	+	+	+	+	–	–	–	–	+	+
Arbutin	–	+	+	+	+	–	–	–	–	–	+	+
D-turanose	+	–	–	–	–	–	–	–	–	–	–	d
D-tagatose	+	+	–	–	–	–	w	–	–	–	–	–
Growth temp. (°C)												
Optimum	50	50	50	30	55	55	55	60	37	37	50	50
Maximum	62	59	55	53	68	69	68	70	50	45	64	65

Strains: 1, isolate MC-04; 1*, *B. thermoamylovorans* LMG 18084^{T}; 2, isolate MO-14; 2*, *B. humi* DSM 16318^{T}; 3, isolate MC-17; 3*, *B. smithii* DSM 4216^{T}; 4, isolate 24-3; 4*, *B. thermocloacea* DSM 5250^{T}; 5, isolate CPh-1; 5*, *Corynebacterium sphenisci* CCUG 46398^{T}; 6, isolate MN-07; 6*, *B. coagulans* NBRC 12583T, None of the strains produced acid from Erythritol, D-arabinose, Methyl D-xyloside, Dulcitol, Methyl D-mannoside, Inulin, D-fucose, L-fucose, D-arabitol, L-arabitol, D-lyxose, L-xylose, D-adonitol, D-melezitose, Xylitol, 2-Keto gluconate and 5-Keto gluconate in API test. d, variables and w, weak

3.5. References

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CHAPTER IV: Taxonomic characterization of thermotolerant *Bacillus* strain MO-04 isolated from mixed culture seed

This chapter is a modified version of the paper (Poudel P., Miyamoto H., Okugawa Y., Tashiro Y., Sakai K. Thermotolerant *Bacillus kokeshiiformis* sp. nov. isolated from marine animal resources compost) published in International Journal of Systematic and Evolutionary Microbiology, 64 (8): 2668–2672 (2014).

4.1. Abstract

A novel Gram-staining-positive, endospore forming rod-shaped, facultatively anaerobic, thermotolerant bacterium, designated strain MO-04^T, was isolated from a marine animal resources (MAR) compost. The 16S rRNA gene sequence of strain MO-04^T showed 99.4% similarity with *Bacillus thermolactis* R-6488^T, 94.1% similarity with *Bacillus thermoamylovorans* CNCM I-1378^T, 93.3% similarity with *B. humi* LMG 22167^T, 93.2% similarity with *B. niacini* IFO 15566^T and others were < 93%. DNA-DNA relatedness between strain MO-04^T and *B. thermolactis* DSM 23332^T was 45%. The DNA G+C content of strain MO-04^T was 33.4 mol%, comparatively lower than that of *B. thermolactis* R-6488^T (35.0 mol%). Strain MO-04^T grew at 35–61°C (optimum 50°C), pH 4.5–9.0 (optimum 7.2), and tolerated up to 8.0% (w/v) NaCl (optimum 2%). The MO-04^T cell wall peptidoglycan type was *meso* 2,6-diaminopimelic acid, and the major fatty acids were $C_{16:1}$, $C_{14:1}$, $C_{17:0}$, and $C_{17:1}$. The major polar lipids were represented by diphosphatidylglycerol and phosphatidylglycerol, and two unidentified phospholipids. The analyzed polyphasic data presented here clearly indicate that the isolate MO-04^T is considered to be a novel species within the genus *Bacillus* for which the name *Bacillus kokeshiiformis* sp. nov. is proposed. The type strain of *B. kokeshiiformis* is MO-04^T (= JCM 19325^T = KCTC 33163^T).

4.2. Introduction

Composting is a biological process of degrading organic waste to produce effective fertilizer under high temperatures (Beffa *et al*., 1996 & Blanc *et al*., 1997). The marine animal resources (MAR) compost used in this research as the source of isolation of the novel thermophilic

Bacillus species has been used for more than 20 years in Japan (Niisawa *et al.*, 2008). Field trial of the MAR compost resulted in an increased crop yield and suppression of plant pathogens.

The genus *Bacillus* represents a heterogeneous group of Gram-staining-positive or Gram-staining-positive only in early stages of growth, or Gram-staining-negative, aerobic, or facultatively anaerobic endospore-forming rod-shaped bacteria widely distributed in nature. *Bacillus* cells may occur singly or in pairs, chains and as filaments. Most *Bacillus* species are reported to grow on the routine media such as nutrient agar or trypticase soy agar. A *meso*-diaminopimelic acid (DAP) is the commonest peptidoglycan type in *Bacillus* species. DNA G+C content of *Bacillus* ranges from 32–66 mol% (Logan and de Vos, 2009). The optimum growth temperature for most of the *Bacillus* species is within mesophilic temperature (Logan and de Vos, 2009). However, some authors have also reported several thermophilic *Bacillus* species in a variety of composts (Sakai *et al*., 1998 & Tashiro *et al*., 2013). This genus comprised more than 300 species including subspecies (www.bacterio.org).

The MAR compost, a degradative by-product of the food industry based on marine animal resources, including small fishes, shrimps, and crabs, is produced under high temperatures by Miroku Co., Japan (Niisawa *et al.*, 2008). Strain MO-04^T was obtained during the isolation of L-lactic acid-producing bacteria from the MAR compost.

4.3. Materials and Methods

4.3.1. Isolation of bacterial strains and culture conditions

Strain MO-04^T was isolated by diluting a MAR-compost sample in sterile 0.85% (w/v) NaCl solution; plating on Ottow medium that contained (per liter) 1 g glucose, 7.5 g peptone, 5.0 g

meat extract, 2.5 g yeast extract, 2.5 g casamino acids, 5.0 g NaCl, and 20 g agar (pH 8.5); and incubating aerobically at 50°C. The isolate was further purified by sub-culturing (three times) and maintained on the same medium. The frozen stock culture was prepared using the same isolation medium supplemented with 20% (v/v) glycerol and stored at –80°C. For phenotypic and genotypic studies, the strain was cultured aerobically on Trypticase Soy Agar (TSA) and in Trypticase Soy Broth (TSB; BBL^{TM}, BD), pH 7.2, at 50°C. For the comparative studies, following reference strains were used; *Bacillus thermolactis DSM 23332^{T} (Coorevits et al, 2011)*, *Bacillus thermoamylovorans* LMG 18084^{T} (Combet-Blanc et al., 1995 & *Coorevits et al, 2011*), *Bacillus humi* DSM 16318^{T} (Heyrman *et al.,* 2005), *B. ruris* DSM 17057^{T} (Heyndrickx *et al*, 2005), and *Bacillus niacini* DSM 2923^{T} (Nagel and Andreesen, 1991). Strains *B. thermolactis* DSM 23332^{T}, *B. thermoamylovorans* LMG 18084^{T} were maintained on TSA at 50 °C, *B. niacini* DSM 2923^{T} was maintained on TSA at 37°C, and strains *B. humi* DSM 16318^{T} and *B. ruris* DSM 17057^{T} were maintained at 30°C on TSA. Strain MO-04T is deposited in Korean Collection for Type Cultures (KCTC 33163^{T}) and Japan Collection of Microorganisms (JCM 19325^{T}).

4.3.2. Phenotypic and Biochemical characteristics

Colony morphology was observed on TSA after incubation at 50°C for 24 h. Gram-staining was examined in exponentially growing cells after 24 h of incubation at 50°C by using a BX50 Olympus microscope. Cell morphology was observed after 24 h of incubation at 50°C by scanning electron microscopy (SEM) using the JSM-6701F microscope (JEOL). Endospore formation was detected in TSB cultures after 3 d at 50°C by the endospore-staining method by using malachite green, as described by Schaeffer & Fulton (1933) and observed by light microscopy (Olympus BX50) and SEM. Motility was observed on semi-solid agar by using the

method described by Tittsler & Sandholzer (1936). Oxidative or fermentative utilization of glucose was determined on the Hugh Leifson's medium (Hugh & Leifson, 1953). Oxidase activity was determined using an oxidase reagent (BioMérieux) and catalase activity was tested by adding 3% (v/v) H_2O_2 solution to a bacterial colony and observing immediate O_2 gas production. Biochemical tests, including those for acid production from carbohydrates, citrate utilization, gelatinase, urease formation, Voges–Proskauer reaction, nitrate reduction, and H_2S production were carried out using commercially available systems (API 50 CHB and 20 E) from BioMérieux by incubating at 50°C for 24 h. Growth at a temperature range from 20°C to 70°C (20 to 70°C) was investigated in TSB after 48 h of incubation by using an Advantec TN-2148 gradient incubator (Japan), and growth difference was monitored by measuring turbidity at OD_{600} by using a UV-1600 spectrometer (Shimadzu, Japan). Growth at different NaCl concentrations was examined in TSB containing 0.5–15% (0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 15) NaCl (w/v). The growth pH range (4, 4.5, 5, 6, 7, 7.2, 7.4, 7.5, 7.8, 8, 8.5, 9, 9.5 and 10) was determined in TSB at 50°C after 48 h; pH was adjusted to acidic, neutral, and alkaline pH by sodium acetate, Tris-HCl, and sodium hydroxide respectively. Hydrolysis of casein, starch, and cellulose was also examined after 48 h incubation at 50°C (Smibert & Krieg, 1994). Similarly, DNA hydrolysis activity was routinely determined using DNase test agar medium (Merck) incubating at 50°C for 72 h. Sole carbon source or carbon and nitrogen source utilization test were performed by using a medium (NH_4Cl, 3.06 g; KH_2PO_4, 3.15 g; $MgCl_2.6H_2O$, 0.47 g; NaCl, 0.3 g; $FeSO_4.7H_2O$, 5 mg; $CaCl_2.2H_2O$, 0.4 mg; yeast extract, 0.3 g per liter of deionized water) to which 0.5% (w/v) substrate was added and growth turbidity (at 600 nm) was observed after 7 d of incubation at 50°C. Inoculated medium without addition of substrate was used as control. Nitrite reduction (KNO_2), Tween 20, Tween 80 tests were performed according to the protocol described by Ventosa et al., (1982). For the hydrolysis of tyrosine and DL-phenylalanine (Atlas,

1997), plates were incubated up to 7 d. All the physiological tests were performed incubating at 50°C unless indicated. Characterization of a novel isolate was performed according to the minimal standards recommended by Logan *et al*., (2009).

4.3.3. Chemotaxonomic analysis

Determination of cell wall *meso*-DAP was performed using 1 g (wet weight) bacterial cells (incubated at 50°C for 24 h on TSA plate) as described by Staneck & Roberts (1974) using *meso* 2,6-DAP and DL-2,6-DAP (Sigma) as standards. For polar lipid analysis, cells were grown in 200 ml of TSB for 24 h at 50°C and extracted using chloroform/methanol/0.3% aqueous NaCl mixture (1:2:0.8 v/v) (Minnikin *et al.*, 1984). Polar lipids were detected by two-dimensional thin layer chromatography (silica gel 60 F254, Merck) by spraying with phosphomolybdic acid reagent (Sigma). Menaquinone was analyzed as previously described (Minnikin *et al.*, 1984). For the cellular fatty acid analysis, biomass was harvested from several colonies grown on TSA for 24 h at 50°C, and methylated according to the protocol developed by Sasser (1990). The methylated solution was analyzed using a GC-2014 gas chromatography (Shimadzu) equipped with an SP^{TM}-2380 column (Supelco), and identified by comparing the retention times with those in the standard Supelco^{TM} 37 fatty acid mixture (Supelco). Fatty acid analysis was repeated five times with reproducible results. The proportions (%) of the methylated fatty acids were estimated by calculating the retention time and peak area for each component relative to the total fatty acid methyl esters (Daron, 1970 & Kates, 1972).

4.3.4. 16S rRNA gene sequencing and phylogenetic analysis

DNA was extracted by using Qiagen DNA extraction kits (Qiagen, Hilden, Germany). The 16S rRNA gene was amplified by PCR by using universal primers 8F (5′-AGA GTT TGA TCC CTC

AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACTT-3′), purified with QIAquick PCR purification kit (Qiagen) according to the manufacturer's instruction, and sequenced by Dragon Genomics Center (TaKaRa, Japan). The similarity score of nearly full length sequence was calculated by EzTaxon-e server (Kim et al., 2012). 16S rRNA gene sequences of closely related taxa were obtained from GenBank/EMBL/DDBJ DNA database and Ribosomal Database Project (Cole *et al*., 2009) and edited by BioLign ver 4.0.6 program (Hall, 1999). Sequences were aligned using CLUSTAL W ver. 2.01 (http://clustalw.ddbj.nig.ac.jp) (Thompson *et al*., 1997), and the phylogenetic analysis (neighbor-joining, maximum likelihood and maximum parsimony) was conducted using MEGA ver.6 with bootstrap values calculated from 1,000 replications (Tamura *et al.*, 2013) (Fig. 4-1).

4.3.5. Genomic analysis

Large-scale DNA extraction to determine the G+C content and Δ*T*m (difference between the melting temperatures of the homologous DNA and the hybrid DNA) was performed according to Marmur *et al*., (1961). The DNA G+C content was analyzed by HPLC as previously described (Tamaoka *et al*., 1984). In order to confirm the novelty of strain MO-04^{T}, it was compared with the closest relative type strain by the DNA–DNA relatedness determined using the fluorimetric method (Moreira *et al.*, 2011 & Gonzalez *et al*., 2005) based on thermal denaturation rate (De Ley *et al*., 1970). Double-stranded DNA was selectively bound to a fluorescent SYBR Green I dye (1:100,000; (Invitrogen), and its thermal denaturation was assessed by decrease in fluorescence at increasing temperatures using CFX Connect^{TM} Real-time PCR (RT-PCR) detection system (BioRad). The melting temperature (*T*m) and the thermal denaturation midpoint Δ*T*m (the difference between the *T*m of the homologous genomic DNA and the *T*m of the hybridized DNA) were calculated after decrease in fluorescence by 50% using the accompanying

software. DNA–DNA relatedness was deduced based on the Δ*T*m value as described by Moreira *et al.*, (2011).

4.4. Result and Discussion

4.4.1. Morphological and phenotypic characteristics

Strain MO-04^T was found to be a Gram-staining-positive, endospore-forming, facultative anaerobic, motile rod-shaped bacterium that was 4–5 µm long and 0.4–0.5 µm wide, positive for catalase and oxidase. Strain MO-04^T formed ellipsoidal endospores at central position when grown in TSB for 3 d at 50°C. When examined by SEM, endospore formation resembled *Kokeshii*-shaped dolls (traditional Japanese dolls with a long trunk and cuboid head) (Fig.4-2). Colonies formed at 50°C after 24 h were 4 mm in diameter, irregular, flat, slightly raised, non-translucent of pale yellow color. Typical phenotypic characteristics of strain MO-04^T were similar to those of the genus *Bacillus*.

4.4.2. Phylogenetic analysis

16S rRNA gene (1454 bp) sequence of strain MO-04^T was compared with those of closely related type strains. The phylogenetic tree constructed by neighbor-joining method shows that strain MO-04^T belong to the genus *Bacillus* (Fig. 4-1).

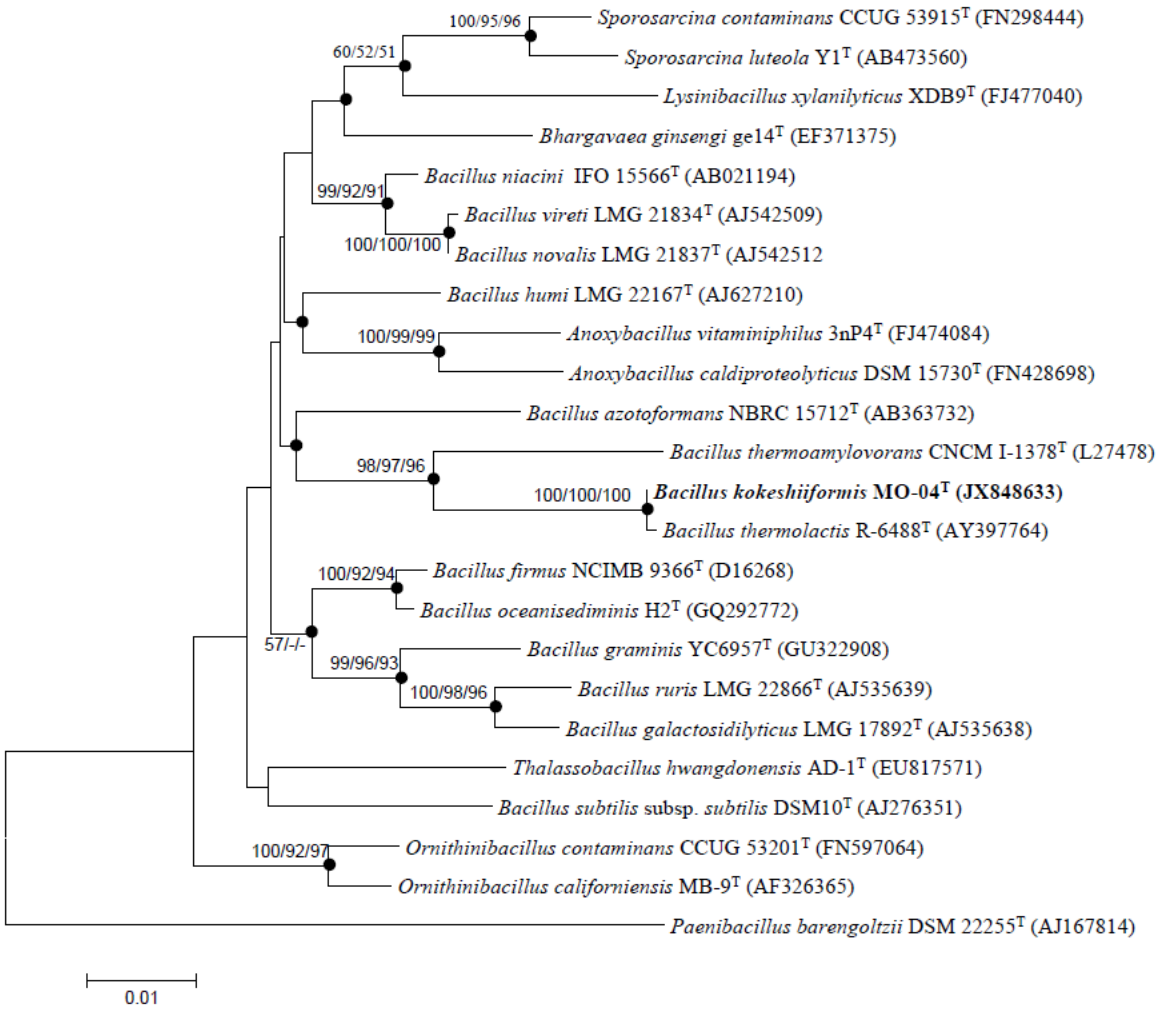

Fig.4-1. Neighbour-joining tree based on the 16S rRNA sequences, showing the position of strain MO-04^T, the closely related species of the genus *Bacillus* and some closely related genera of *Bacillus*. Sequence accession numbers used are shown in parentheses. Branches with filled circles were also found in both maximum-likelihood and maximum-parsimony trees. Bootstrap values (expressed as percentages of 1000 replications) above 50% are shown. Bar 0.01 substitutions per nucleotide position.

The strain MO-04^T showed 99.4% sequence similarity with *B. thermolactis* R-6488^T, 94.1% with *B. thermoamylovorans* CNCM I-1378^T, 93.2 % with *B. humi* LMG 22167^T, 93.2% with *B. niacini* IFO 15566^T and others were below 93%. The 16S rRNA gene sequence similarity higher than 97% require that the new isolate should be further studied with respect to the type strain of *B. themolactis* by DNA–DNA hybridization experiments (Stackebrandt & Goebel, 1994).

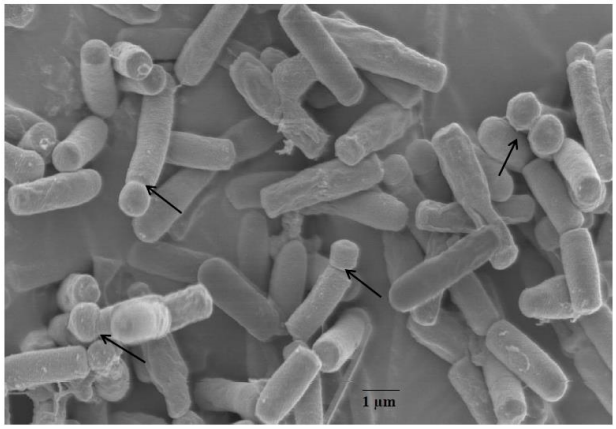

Fig.4-2. Scanning electron microscopy of *Bacillus kokeshiiformis* MO-04^T. Arrows showing endospore as *Kokeshii* (traditional Japanese dolls with a long trunk and cuboid head) shaped.

4.4.3. Genomic characteristics

The mean Δ*T*m value between strain MO-04^T and *B. thermolactis* DSM 23332^T, and *B. thermoamylovorans* LMG 18084^T were 8.2°C (DNA–DNA relatedness value of 45%) and 8.6°C (DNA–DNA relatedness value of 42%), respectively (Fig.4-3). According to Moreira *et al.* (2011), mean Δ*T*m values higher than 8.24°C and lower than 2.2°C obtained by RT-PCR correspond to a strain pair with DNA–DNA relatedness of <60% and >80%, respectively. In addition, Wayne *et al*. (1987) also indicated that Δ*T*m less than 5°C should be considered a

threshold value for delineation of genospecies. Based on previous numerous studies, the results for Δ*T*m and DNA–DNA relatedness are well correlated (Rosselló-Mora & Amann 2001). The 16S rRNA gene sequence similarity score of strain MO-04^T was high (99.4%) with the related type strain *B. thermolactis* R-6488^T, but their DNA–DNA relatedness value (45 %) is much lower than the threshold value (70%) for species delineation. These data were clearly sufficient for distinguishing strain MO-04^T from *B. thermolactis* DSM 23332^T. In addition, the MO-04^T DNA G+C content was 33.4 mol%, which was within the range of 32–69 mol% for the genus *Bacillus* (Logan & de Vos, 2009) but lower than 35.0 mol% for strain *B. thermolactis* R-6488^T (Coorevits *et al*., 2011).

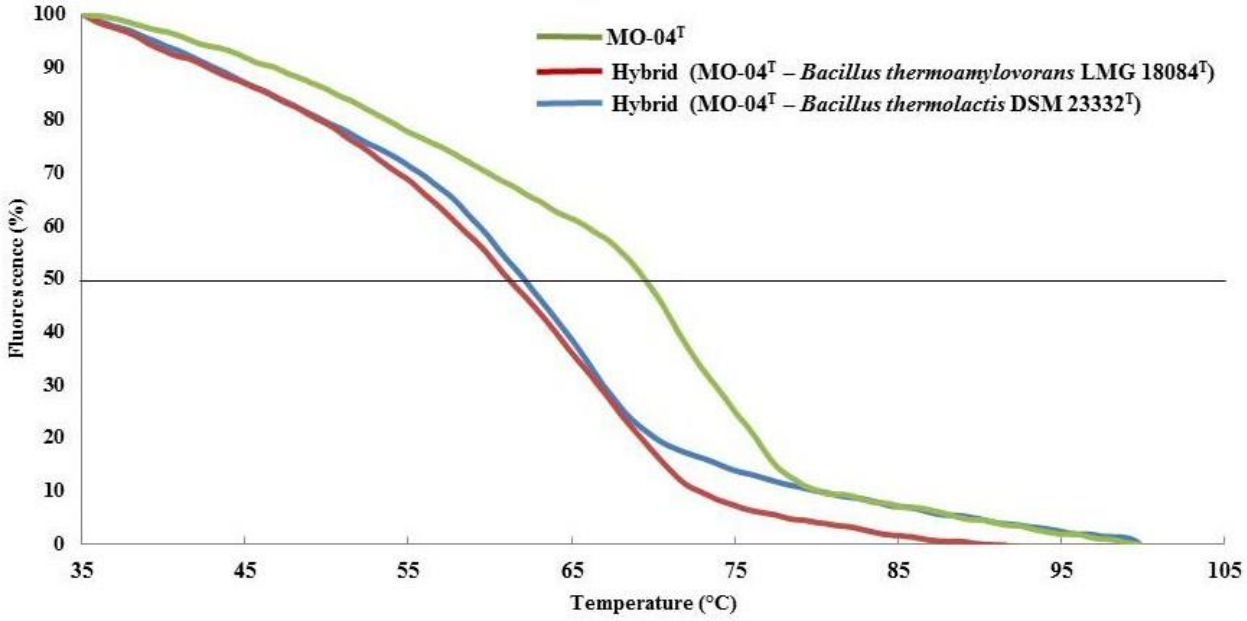

Fig.4-3. Melting curve of genomic DNA obtained from strain MO-04^T (green line) and the MO-04^T and *B. thermolactis* DSM 23332^T hybrid DNA mixture (blue line) and MO-04^T and *B. thermoamylovorans* LMG 18084^T hybrid mixture (red line). Mean Δ*T*m value between strain MO-04^T and *B. thermolactis* DSM 23332^T and *B. thermoamylovorans* LMG 18084^T were 8.2 °C (DNA–DNA relatedness 45 %) and 8.6 °C (DNA–DNA relatedness 42 %) respectively

4.4.4. Chemotaxonomic characteristics

Strain MO-04^T contained major cellular fatty acids as $C_{16:1}$ (mean value, 21.5%), $C_{14:1}$ (mean value, 15.8%), $C_{17:0}$ (mean value, 12.0%), and $C_{17:1}$; (mean value, 10.1%). In addition, traces of $C_{13:0}$; (mean value, 0.3%) and $C_{18:2n6t}$ (mean value, 0.2%) were also detected. The fatty acid composition of MO-04^T was almost similar to the closely related reference strains with small variation in the proportion. The MO-04^T major cell wall peptidoglycan was *meso*-2,6-DAP, which is typical for the peptidoglycan composition of the *Bacillus* species cell wall (Schleifer & Kandler, 1972). Polar lipids diphosphatidylglycerol and phosphatidylglycerol were identified, and two unknown phospholipids were also detected by two-dimensional thin layer chromatography. Strain MO-04^T contained only MK-7 as a menaquinone, whereas strains *B. thermolactis* DSM 23332^T contained MK-7 as well as traces of MK-8. Strain MO-04^T was able to tolerate NaCl concentrations of up to 8%, whereas strain *B. thermolactis* DSM 23332^T could only grow in 1% NaCl. Minimal growth pH for strain MO-04^T was 4.5, whereas for strain *B. thermolactis* DSM 23332^T was 6.0. Sugar assimilation profile using API 50CHB and API 20E gallery is included in species description section. Many other differentiating parameters are presented in Table 1.

4.4.5. Taxonomic conclusion

The differences in phenotypic and genotypic characteristics such as colony morphology, endospore position and shape, acid production from carbohydrates, maximum growth temperatures, tolerance to NaCl concentration, fatty acid composition as well as the genomic DNA G+C content and the DNA–DNA relatedness value can be used to distinguish MO-04^T strain from the closely related strain *B. thermolactis* DSM 23332^T. On the basis of the phenotypic, chemotaxonomic, and genotypic properties, strain MO-04^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus kokeshiiformis* sp. nov. is proposed.

Table 4-1. Differential phenotypic and genotypic characteristics of strain MO-04^T from other closest relatives. Strains: 1, MO-04^T; 2, *B. thermolactis* DSM 23332^T; 3, *B. thermoamylovorans* LMG 18084^T; 4, *B. niacini* DSM 2923^T; 5, *B. humi* DSM 16318^T; 6, *B. ruris* DSM 17057^T. +, positive; v, variable; –, negative; w, weak; +/–, grown but no hydrolysis activity; –/–, not grown and no hydrolysis activity. For endospore position: C, central; ST, subterminal; T, terminal. All the results were obtained from this study performed under the same conditions unless otherwise indicated. [*]Data obtained from Nagel and Andreesen, (1991); [†]data obtained from Heyrman *et al*., (2005); [‡]data obtained from Heyndrickx *et al*., (2005); [**]data obtained from Coorevits *et al*., (2011); [#]data obtained from Combet-Blanc *et al*., (1995).

Characteristics	1	2	3	4	5	6
Cell size	0.4–0.5×4–5 µm	0.7–0.9×4–10 µm[**]	0.45–0.5×3–4 µm[#]	0.9–1.4×3–5.6 µm[*]	0.7–0.9×4–7 µm[†]	0.5–0.8×1–2 µm[‡]
Colony diameter	3–4 mm	1–4 mm[**]	0.5–4 mm	3–5 mm[*]	1 mm[†]	1 mm[‡]
Motility	+	–	+	–	+	+
Endospore position	C	ST[**]	ST[**]	C[*]	ST[†]	ST[‡]
Hydrolysis of						
Starch	–	+	+	–	–	–
Casein	–	+	–	–	+	–
Tween20	+	+/–	+	–/–	–/–	–/–
Tween80	+	+/–	+	–/–	–/–	–/–
Tyrosine	–	+/–	+	–/–	–/–	–/–
Acid production from						
D-xylose	+	w	v	+	–	+
D-tagatose	+	–	–	–	–	–
D-turanose	w	–	–	+	–	–
Arbutin	+	–	–	+	–	–
D-cellobiose	w	–	+	+	–	v
D-maltose	+	–	+	+	–	v
D-sucrose	–	w	+	+	–	+
D-trehalose	+	w	+	+	–	+

Table 4-1 continue

Characteristics	1	2	3	4	5	6
Acid is produced from						
Glycogen	+	–	–	–	–	–
D-galactose	+	–	+	+	–	v
D-mellibiose	+	–	+	+	–	+
Growth at pH						
4.5	+	–	–	–	–	–
9.0	+	–	+	–	+	+
Growth at/in:						
35°C	+	–	+	+	+	+
61°C	+	–	–	–	–	–
2% NaCl	+	–	+	–	+	+
8% NaCl	+	–	–	–	–	–
DNA G+C content (mol%)	33.4	35.0**	37.0	37.0	37.5	39.2
Isolation source	MAR compost	Raw milk	Palm wine	Soil	Soil	Soil

4.4.6. Description of Bacillus kokeshiiformis sp. nov.

Bacillus kokeshiiformis (ko.ke.shi.i.for'mis. N.L. n. kokeshi Japanese, a Japanese doll with a long trunk and a cuboid-shaped head; formis L. adj. suff. (from L. n. forma, figure, shape, appearance), -like, in the shape of; N.L. masc. adj. kokeshiiformis, with the shape of a Japanese kokeshi doll.

Gram-staining-positive rod-shaped bacteria (4–5 × 0.4–0.5 μm) occur singly or in chains. Endospores are ellipsoidal at central position when grown in TSB for 3 days at 50°C. When cultured at 50°C for 24 h, the bacteria form colonies that are usually 3–4 mm in diameter, irregular, flat, slightly raised and convex (some may appear round), non-translucent, with pale yellow color and sticky consistency when manipulated with an inoculating loop. This strain is facultatively anaerobic, catalase- and oxidase-positive. In the API 50CHB gallery, high quantities of acid, not gas, is produced from L-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, arbutin, D-salicin, D-melibiose, D-maltose, D-trehalose, D-tagatose and glycogen. Low amount of acid without gas is produced from D-cellobiose and D-turanose. Acid is not produced from glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, α-methyl-D-mannoside, α-methyl-D-glucosamine, amygdalin, lactose, D-sucrose, inulin, melezitose, L-raffinose, xylitol, gentibiose, D-fucose, L-fucose, D-arabitol, gluconate, 2-keto-gluconate, and 5-keto-gluconate. Aesculin is hydrolyzed. In the API 20E system, gelatin, nitrate reduction and 2-nitrophenyl-β-D-galactopyranoside (ONPG) tests are positive whereas, L-arginine, L-lysine, L-ornithine, trisodium citrate, sodium thiosulfate, urea, tryptophane deaminase, Voges–Proskauer (acetoin production), and indole reactions are negative. Nitrite (KNO_2) is not reduced to N_2 or NH_3 gas and H_2S production is negative. Methyl red test is positive, and Tween 20 and Tween

80 are hydrolyzed. DNA, casein, cellulose, phenylalanine, starch and tyrosine are not hydrolyzed. D-glucose, D-xylose, D-fructose, D-galactose, L-arabinose, D-cellobiose, D-maltose, mannose, trehalose gluconate, formate, pyruvate, acetate, L-arginine, L-glutamic acid, L-histidine, L-isoleucine are utilized as sole carbon or carbon and nitrogen and energy source. On the other hand, sucrose, D-arabinose, starch, inulin, lactate, oxalate, propionate, succinate, D-tartrate, L-alanine, L-aspartic acid, L-cysteine, L-isoleucine, L-lysine, L-threonine and L-valine cannot be utilized as sole carbon or carbon and nitrogen and energy source. D-glucose (20 g/L) is fermented to L-lactic acid (3.1 g/L). The cell wall peptidoglycan is *meso*-DAP. Only menaquinone MK-7 is detected. The major fatty acid (>10%) are $C_{16:1}$, $C_{14:1}$, $C_{17:0}$, and $C_{17:1}$. The major polar lipids are diphosphatidylglycerol and phosphatidylglycerol, and two phospholipids remain unidentified. Growth occurs at 35–61°C (optimum 50°C) and pH 4.5–9.0 (optimum 7.2). Strain MO-04^T shows tolerance to NaCl concentrations up to 8% (w/v) (optimum 2%). The DNA G+C content is 33.4 mol%. Strain MO-04^T (= JCM 19325^T=KCTC 33163^T) was isolated from a marine animal resources compost available in Japan.

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CHAPTER V: Direct fermentation starch to L-lactic acid by a newly isolated thermophilic strain, *Bacillus* sp. MC-07

This chapter is a modified version of the paper (Poudel P., Miyamoto H., Okugawa Y., Tashiro Y., Sakai K. Direct starch fermentation to L-lactic acid by a newly isolated thermophilic strain, *Bacillus* sp. MC-07) published in Journal of Industrial Microbiology and Biotechnology 43 (1): 143–149 (2015).

5.1. Abstract

A newly isolated *Bacillus* sp. MC-07 showed 99.2% 16Sr RNA gene sequence similarity with the *Bacillus thermoamylovorans* LMG 18084^T. It demonstrated optimum and maximum growth temperatures of 50 and 62°C, respectively. The ability of MC-07 to produce optically pure L-lactic acid via direct fermentation of starch without enzymatic hydrolysis was investigated at different pH values (6.0–8.0) by intermittent adjustments every 12 h. During batch fermentation in mineral salt medium containing 0.001% yeast extract at pH 7.0, 20 g/L of soluble starch was utilized to produce 16.6 g/L L-lactic acid at 50°C within 24 h of fermentation, with 100% optical purity, 92.1% lactic acid selectivity, and an L-lactic acid yield of 0.977 g/g. Direct starch fermentation at pH 6.0, 6.5, 7.5, and 8.0 resulted in considerably lower concentrations of lactic acid than did pH 7.0. Compared with *B. thermoamylovorans* LMG 18084^T, the ability of strain MC-07 to produce L-lactic acid was superior.

5.2. Introduction

Lactic acid (LA) has been widely applied in food, pharmaceutical, textile, cosmetic, and some chemical industries (Sakai and Ezaki, 2006). The demand for LA has been increasing considerably because of its use as a monomer for the synthesis of polylactic acid (PLA), a promising and environmentally friendly bio-plastic (Sakai and Ezaki, 2006).

Currently, starchy and lignocellulosic materials are considered as feasible raw material for industrial production of LA. However, compared to lignocellulosic materials the pretreatment cost for starchy material is comparatively lower (John *et al.*, 2009). In addition, lignocellulosic materials contain cellulose and hemicelluloseas the main components, and their degradation requires pretreatments including physical or physico-chemical approaches followed by hydrolysis using multiple enzymes. In general, pretreated starchy materials are easily hydrolyzed

into fermentable sugars such as glucose or oligosaccharides by the addition of enzymes (α-amylase and glucoamylase) before fermentation (John *et al.*, 2009). Furthermore, amylolytic LA producer can skip the hydrolysis process without a dose of any commercial enzymes. So far, some amylolytic LAB like *Lactobacillus paracasei* (Bomrungnok *et al.,* 2012), *Lactobacillus amylovorans, Lactobacillus amylophilus, Enterococcus faecium*, and *Streptococcus bovis* have been already reported to produce LA from starch without any enzymatic hydrolysis under mesophilic conditions (30–40°C) (John *et al.,* 2009). Recently, *Lactobacillus plantarum* SW14 was reported to produce LA directly from the cassava starch at 45°C, which is the highest temperature in direct LA fermentations among the literatures published so far (Petrova *et al.,* 2012). On the other hand, *Bacillus* species have not been reported to produce LA directly from starch at high temperatures to date.

Thus, a thermophilic LA producer, which can convert starch to optical pure LA directly in cheap medium containing a low amount of organic nitrogen sources, should be suitable. In this study, we reported the highly efficient production of LA from starch with high optical purity by the newly isolated thermophilic *Bacillus* sp. MC-07. To our knowledge, this is the first report of direct starch fermentation to LA at high temperatures by a *Bacillus* species.

5.3. Materials and Methods

5.3.1. Isolation and identification

Isolation and identification were described in chapter 3, material and method section. Amylolytic activities of the bacterial isolates after 24 h of incubation at 50°C were screened by the iodine staining method (Guyot & Calderon, 2000) on Tryptone soy agar (15 g pancreatic digest of casein, 5 g Papaic digest of soybean meal, 5 g NaCl, 20 g of soluble starch, 15 g agar, per liter of

deionized water at pH 7.0). Based on the size of the halo formation after iodine staining, strain MC-07 was selected for direct starch fermentation to LA.

5.3.2. Direct starch fermentation by strain MC-07

For direct fermentation of starch to LA, the frozen stock culture of strain MC-07 was refreshed by inoculating 500 µl of stock culture in 5 mL of Tryptone soy broth and incubated at 50°C for 24 h. Then, strain MC-07 was streaked onto fresh TSA plates and incubated as described above. The seed culture was prepared by as follows: a loopful of cells from the fully grown TSA plate was inoculated in 30 mL of Tryptone soy broth containing 20 g/L soluble starch in a 100 mL Erlenmeyer flask, and incubated at 50°C for 16 h with shaking (140 rpm). A 1% seed culture was used for LA fermentation. Preliminary studies on direct starch fermentation by strain MC-07 was conducted in 100 mL of Erlenmeyer flask containing 30 ml of sterilized or non-sterilized MSM (3.06 g NH_4Cl, 3.15 g KH_2PO_4, 0.47 g $MgCl_2 \cdot 6H_2O$, 0.3 g NaCl, 5 mg $FeSO_4 \cdot 7H_2O$, 0.4 mg $CaCl_2 \cdot 2H_2O$, 0.01 g Bacto^{TM} yeast extract [Difco^{TM}; Becton Dickinson, Franklin Lakes, NJ, USA] per liter of deionized water) supplemented with 20 g/L of commercially available soluble starch (Wako pure chemicals, Richmond, USA) as the sole carbon source under non-anaerobic (without sparging oxygen-free nitrogen gas) and anaerobic conditions (with sparging oxygen-free nitrogen gas) at pH 7.0 and 50°C. The pH was adjusted by 10% NH_3 every 12 h of fermentation. Main batch fermentation was carried out in a 500 mL Erlenmeyer flask containing 200 mL of MSM supplemented with 20 g/L of commercially available soluble starch as the sole carbon source. The starch in the MSM was heated at 100°C to allow gelatinization before sterilization at 120°C for 10 min. For the pH optimization, the pH of the medium was adjusted to the desired values (6.0–8.0) with 10% NH_3 before seed culture inoculation. Anaerobic conditions were maintained by sparging with oxygen-free N_2 gas for 20 min. Fermentation was conducted

at 50°C with shaking at 140 rpm under closed conditions to prevent airsupply. The pH values of the fermentation broth were manually adjusted to the respective initial pH values (6.0–8.0) by the addition of 10% NH_3 every 12 h of fermentation, and anaerobic conditions were maintained again as described above. For comparative studies of LA production from starch, the closely related type strain *Bacillus thermoamylovorans* LMG 18084^T (Combet *et al.*, 1995), obtained from the Belgian Coordinated Collection of Microorganisms (Ledeganckstraat, Belgium), was also investigated as the same methods with strain MC-07.

5.3.3. Chemical analysis

Concentrations of organic acids such as total LA (the sum of D-LA and L-LA), formic acid, acetic acid, propionic acid, butyric acid, and pyruvic acid were determined using a high-pressure liquid chromatography system (Organic Acid Analyzer, Shimadzu, Kyoto, Japan) as described previously (chapter 2, material and method). D- and L-LA were analyzed using a high-pressure liquid chromatography system equipped with a MCL Gel CRS10w column (Mitsubishi Chemical Co., Japan).The optical purity (%) of L-LA was defined as ([L] − [D]) × 100/([L] + [D]), where [L] and [D] denote the concentrations of L-LA and D-LA, respectively (Sakai and Ezaki, 2006). The total sugar was determined using the phenol-sulfuric acid assay (Dubois *et al.*, 1956). LA selectivity was defined as the percentage of total LA by weight in the sum of the total organic acids analyzed. In this experiment, LA selectivity (%) was calculated as: $(C_{L\text{-}LA} + C_{D\text{-}LA}) \times 100/(C_{L\text{-}LA} + C_{D\text{-}LA} + C_{AA} + C_{FA})$, where, $C_{L\text{-}LA}$, $C_{D\text{-}LA}$, C_{AA} and C_{FA} are the respective concentrations (g/L) of L-lactic acid, D-lactic acid, acetic acid and formic acid produced.

5.4. Results and Discussion

5.4.1. Isolation and identification of strain MC-07

Strain MC-07 was isolated from compost as an amylolytic strain on Tryptone soy agar, showing a clear halo after iodine staining. The strain MC-07 was Gram-staining-positive with endospore-forming rods, and was oxidase-positive and catalase-positive, which indicated the classification in the family Bacillaceae. The 16S rRNA gene sequence of the MC-07 isolate showed the highest similarity with *B. thermoamylovorans* LMG 18084^T (99.2%) (Combet *et al.*, 1995).The phylogenetic tree constructed using strain MC-07 and other closely related type strains including *Bacillus coagulans* NBRC12583^T, the most well-known thermophilic LA-producing *Bacillus* species, is depicted in Fig. 5-1. The phylogenetic tree showed that strain MC-07 clustered with *B. thermoamylovorans* LMG 18084^T (GenBank accession number L27478) with 100% bootstrap support, indicating strain MC-07 should be assigned to the genus *Bacillus*. The optimum and maximum growth temperatures for strain MC-07 were 50°C and 62°C, the same or slightly higher than those (50°C and 60°C) for LMG 18084^T, respectively, demonstrating a thermophilic property (Fig. 5-2). Based on the API 50CHB gallery, strain MC-07 produced acids from L-arabinose, D-ribose, D-glucose, D-galactose, D-fructose, D-mannose, D-rhamnose, D-maltose, D-lactose, D-cellobiose, D-salicin, D-mellibiose, sucrose, D-trehalose, starch, gentibiose, turanose, and D-tagatose, while strain LMG 18084^T could not produce acid from D-galactose, D-rhamnose, D-maltose, D-lactose, and D-turanose but produced acid from myo-inositol, D-mannitol, and arbutin. Based on its distinct properties, strain MC-07 could not be identified to the species level, although the similarity of 16S rRNA gene sequences was high. Species level identification is under further investigation. Therefore, we described the newly isolated strain as *Bacillus* sp. MC-07.

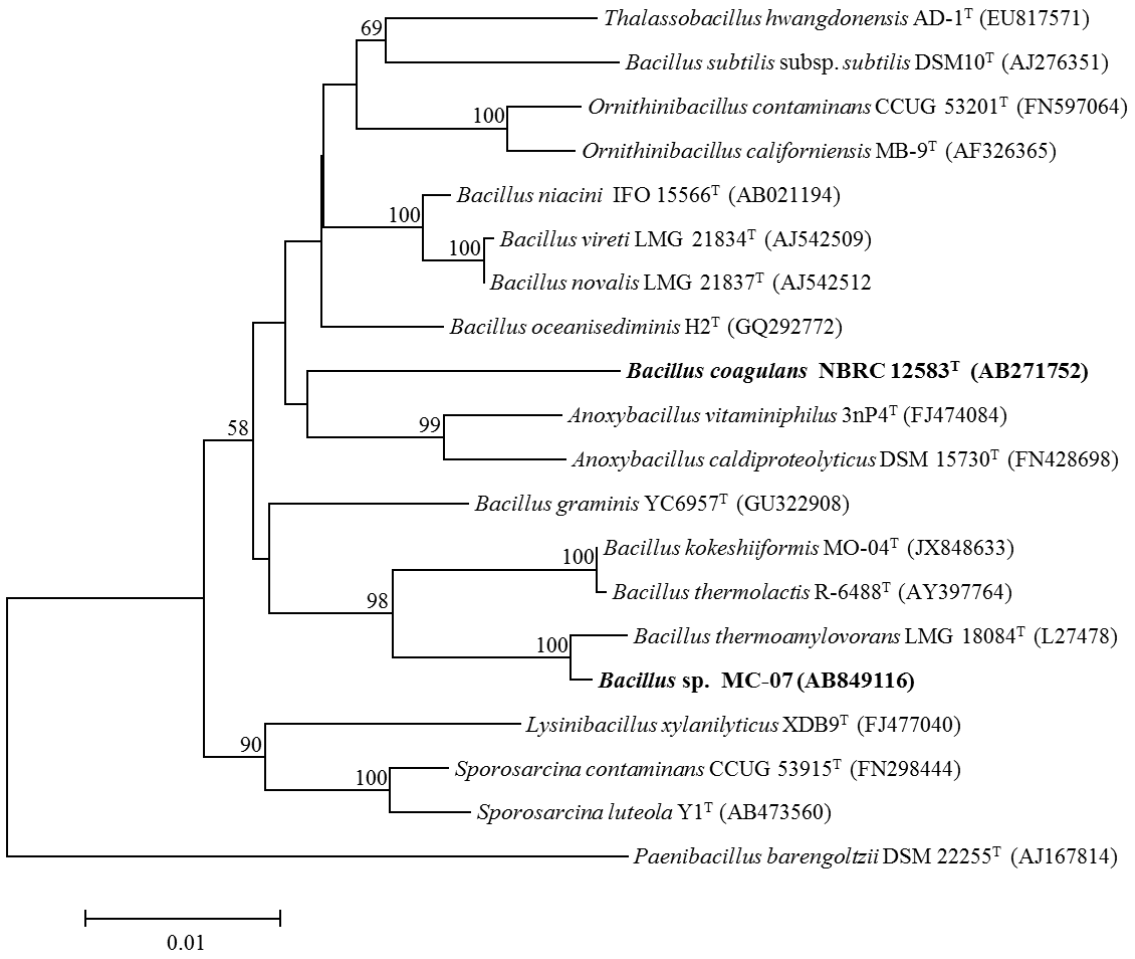


Fig 5-1. Neighbour-joining tree based on the 16S rRNA sequences, showing the position of strain MC-07, its closely related type strains inclusive of *Bacillus coagulans* NBRC 12583^{T}. Sequence accession numbers used are shown in parentheses. Bootstrap values (expressed as percentages of 1000 replications) above 50% are shown. Bar 0.01 substitutions per nucleotide position.

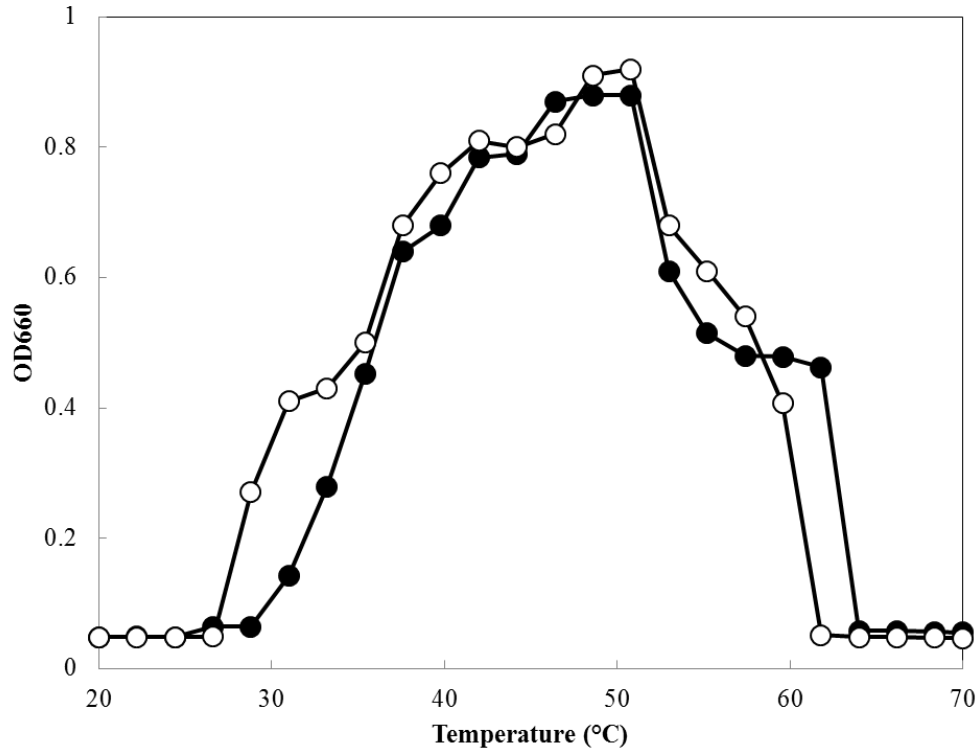
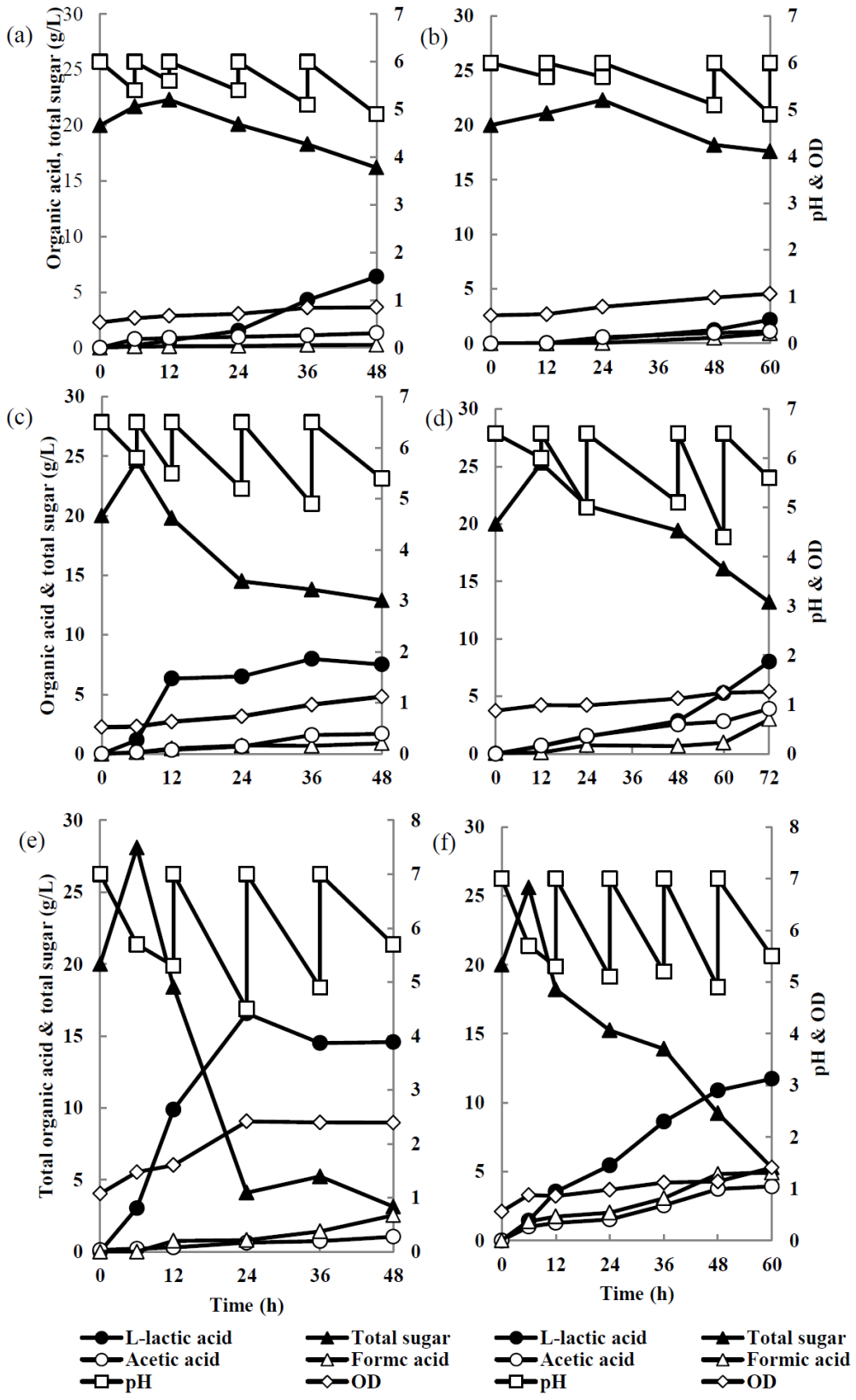


Fig 5-2. Effect of temperature on the growth of *Bacillus* sp. MC-07 (close circles) and *B. thermoamylovorans* LMG 18084^{T} (open circles). Optical density (OD660) values were measured after 48 h of fermentation at 50°C.


(a)
(b)
(c)
(d)
(e)
(f)
Organic acid, total sugar (g/L)
pH & OD
Organic acid & total sugar (g/L)
Total organic acid & total sugar (g/L)
Time (h)
L-lactic acid
Total sugar
Acetic acid
Formc acid
pH
OD
L-lactic acid
Total sugar
Acetic acid
Formic acid
pH
OD

Continue

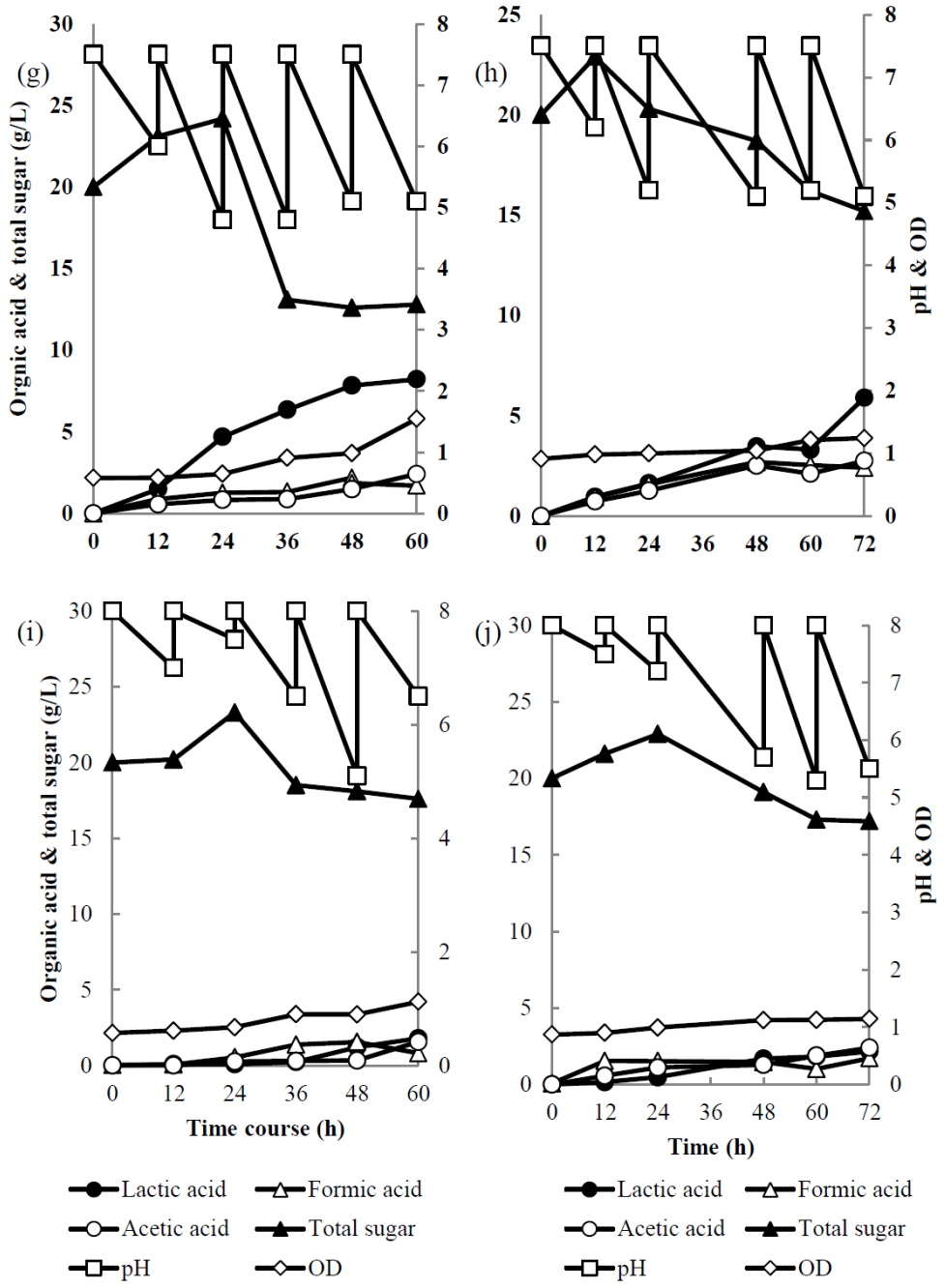


Fig 5-2. Time course of direct starch fermentation to L-LA by *Bacillus* sp.MC-07 (a, c, e, g, i) and *Bacillus thermoamylovorans* LMG 18084^{T} (b, d, f, h, j) at temperature 50°C under intermittent pH adjustment at 6.0, 6.5, 7.0, 7.5, 8.0 respectively.

5.4.2. Effect of pH values on direct starch fermentation to LA by strain MC-07 and LMG 18084^T

In the preliminary experiments, direct starch fermentations to LA were performed by strain MC-07 at 50°C and pH 7.0 by an intermittent adjustment of pH in sterilized or non-sterilized MSM under anaerobic condition by sparging oxygen-free nitrogen gas or non-anaerobic condition without gas-sparging. As the results, the LA selectivity of 90.9% was much higher in the sterilized MSM under anaerobic condition than 47.3% in the sterilized MSM under non-anaerobic condition or 60.7% in non-sterilized MSM under anaerobic condition. Although non-sterilized medium (Zhao et al., 2010) and non-anaerobic condition (Saowanit *et al.,* 2014) have been considered to be suitable for industrial LA production, in this study, we selected sterilized MSM and anaerobic condition in further experiments.

The effect of pH values (6.0–8.0) of culture broth on LA production using strains MC-07 and LMG 18084^T was investigated by an intermittent adjustment of pH every 12 h. Figure 5-3 shows the fermentation profiles at pH of 6.0, 6.5, 7.0, 7.5 and 8.0 for strain MC-07 (5-3a, c, e, g, i) and strain LMG18084^T (5-3b, d, f, h, j). Under and below the pH 7.0, growth during the direct starch fermentation was much lower for both the strains MC-07 (Fig 5-3a, c, g, i) and LMG 18084^T (Fig. 5-3b, d, h, j). Although, both the strains grew in direct starch fermentation in MSM with consumption of starch, strain MC-07 exhibited the higher maximum optical density (OD; 2.42) and maximum specific growth rate (0.0488 h^{-1}) than strain LMG18084^T (1.41 and 0.0416 h^{-1}, respectively) at pH 7.0. At pH 7.0, strain MC-07 rapidly utilized starch after 6 h of fermentation and the highest L-LA (16.6 g/L) was produced within 24 h with a small amount of byproduct (0.802 g/L formic acid and 0.622 g/L acetic acid) formation (Fig. 5-3c). On the other hand, strain LMG18084^T accumulated the highest L-LA titer of only 11.7 g/L after 60 h of fermentation with high byproducts of formic acid (4.93 g/L) and acetic acid (3.92 g/L) (Fig. 5-

3d) at pH. 7.0. These results indicated that strain MC-07 was more able to ferment starch within a short time period than was strain LMG 18084^T. Furthermore, the results suggested that the behaviors of direct starch fermentations should be quite different between strain MC-07 and strain LMG18084^T under the same fermentation conditions.

As shown in Table 5-1, under the intermittent adjustments of pH at 6.0, 6.5, 7.0, 7.5, and 8.0, strain MC-07 produced L-LA concentrations of 6.40 g/L, 7.98 g/L, 16.6 g/L, 8.22 g/L, and 1.79 g/L, respectively, which are relatively higher than those produced by strain LMG 18084^T for each pH value except for 2.16 g/L at a pH of 8.0. In addition, these results indicated that the highest L-LA concentrations were achieved at pH 7.0 for both strains under the intermittent adjustments. At each of the tested pH values, L-LA yield, productivity, and selectivity were superior in strain MC-07 compared to strain LMG 18084^T (Table 5-1).The L-LA yields for strain MC-07 ranged between 0.364–0.977 g/g with a maximum at pH 7.0, while in strain LMG 18084^T they ranged between 0.352–0.877 g/g with a maximum at pH 6.5. On the other hand, the highest L-LA selectivity of 92.1% was attained at pH 7.0 for strain MC-07and was much higher than strain LMG 18084^T (57.0%).The maximum L-LA productivity of 0.701 g/L·h at pH 7.0 for strain MC-07 was significantly higher than that of strain LMG 18084^T (0.195 g/L·h). Low accumulation of L-LA below and above pH 7.0 might be due to poor growth of strains MC-07 and LMG 18084^T because cell growth in the fermentation broth is positively correlated with the accumulation of LA (Guyot and Calderon, 2000).These results indicated that pH 7.0 is optimum for LA production in direct starch fermentation by strain MC-07 under intermittent adjustment, and that the capability of strain MC-07 to ferment starch to L-LA was much better than it was for strain LMG 18084^T under thermophilic conditions. Furthermore, we obtained the reproducible results (15.7 g/L L-LA with LA selectivity of 91.8% and optical purity of L-lactic acid of 100%,

0.58 g/L acetic acid, and 0.82 g/L formic acid) in direct starch fermentation using the strain MC-07 by the intermittent adjustment of pH at 7.0 every 12 h.

The pH values under continuous adjustment during fermentation are a significant factor for LA production by *Bacillus* strains (Zhou *et al.*, 2013). On the other hand, we have previously reported on the better performance of LA fermentation by LAB using the food waste under intermittent pH adjustment than under controlled pH (Sakai *et al.*, 2000). In our results under intermittent pH adjustment, surprisingly, our results suggested that the pH values drastically affect LA production from starch by both *Bacillus* strains, MC-07 and LMG 18084^T (Table 5-1, Fig. 5-3). To our knowledge, however, there are no published reports on effect of pH values on LA fermentation using *Bacillus* strains under intermittent pH adjustment. Therefore, the mechanism underlying such effects is not known and further studies are required.

To date, only some LAB, including *Lactobacillus* species, *Lactococcus* species, *Enterococcus* species, and *Streptococcus* species, have been reported to produce LA from starch directly without the addition of commercially available amylolytic enzymes under mesophilic conditions at ≤45°C (Table 5-2). To the best of our knowledge, this is the first report of direct starch fermentation to LA using *Bacillus* species at high temperatures (50°C and over). In addition, LAB are thought to require the addition of relatively expensive organic nitrogen sources such as yeast extract at more than 0.5% (John *et al.*, 2009). However, our isolate, strain MC-07 fermented starch to L-LA in MSM containing quite little yeast extract (0.01%) and relatively cheap inorganic nitrogen of 3.06 g NH_4Cl. In particular, we are the first to achieve 100% L-LA optical purity using strain MC-07, and demonstrate the highest yield of LA (0.977 g/g) among published studies (99.0% L-LA optical purity (Guyot *et al.*, 2000) and 0.93 g/g yield of LA (Petrova and Petrov, 2012) at maximum). Nevertheless, at higher concentrations of starch

(more than 25 g/L), strain MC-07 did not accumulate higher LA and demonstrated poor growth. This might be due to the substrate inhibition property of bacterial growth (Görke and Stülke, 2008). To further improve LA production, additional research approaches such as fed-batch fermentation could be an alternative.

In this study, direct starch fermentation to produce optically pure L-LA by *Bacillus* sp. MC-07 under anaerobic and thermophilic temperature conditions using MSM containing a small amount of expensive yeast extract was investigated. These findings show that some low-cost starchy substrates can be directly fermented to L-LA with a high yield and optical purity by omitting the addition of enzymes for simultaneous saccharification, and require a relatively simple methodology for the process of LA fermentation. Therefore, our findings demonstrate an efficient means of LA production directly from starch under thermophilic temperatures and could be valuable for industrial scale.

Table 5-1 Direct fermentation of starch to L-LA by strains MC-07 and LMG 18084^T at 50°C and various pHs by intermittent adjustment.

Strains	pH	Time (h)	OD_{660}	μ_{max} (h^{-1})	C_{LA} (g/L)	Y_{LA} (g/g)	S_{LA} (%)	$OP_{L\text{-}LA}$ (%)	P_{LA} (g/L·h)	Residual total sugar (g/L)	C_{AA} (g/L)	C_{FA} (g/L)
MC-07	6.0	48	1.56	0.0324	6.40	0.653	66.7	100	0.133	16.3	1.33	0.262
	6.5	36	1.81	0.0454	7.98	0.851	78.2	100	0.221	12.9	1.57	0.668
	7.0	24	2.42	0.0488	16.6	0.977	92.1	100	0.701	3.02	0.622	0.802
	7.5	48	1.54	0.0375	8.22	0.944	66.8	100	0.171	12.8	2.41	1.70
	8.0	60	1.12	0.0175	1.79	0.364	43.3	100	0.029	17.6	1.56	0.799
LMG 18084^T	6.0	60	1.17	0.0247	3.25	0.644	57.7	100	0.054	16.8	1.13	1.30
	6.5	72	1.26	0.0389	8.01	0.877	56.3	100	0.111	13.2	3.92	3.01
	7.0	60	1.41	0.0416	11.7	0.789	57.0	100	0.195	5.29	3.97	4.93
	7.5	72	1.24	0.0125	5.90	0.771	53.6	100	0.082	15.2	2.75	2.40
	8.0	72	1.14	0.0115	2.16	0.352	33.8	100	0.030	17.2	1.71	2.40

OD: Optical density, μ_{max}: Maximum specific growth rate (calculated by the slope of linear regression of the natural log of the OD and fermentation time), *C*: Concentration, *Y*: Yield, *S*: Selectivity, $OP_{L\text{-}LA}$: Optical purity of L-lactic acid, *P*: Productivity, *LA*: Lactic acid, *AA*: Acetic acid, *FA*: Formic acid

Table 5-2 Comparison of direct starch fermentation to lactic acid by various strains so far published.

Strains	FT (°C)	YE (%)	Starch (g/L)	$OP_{L\text{-}LA}$ (%)	C_{LA} (g/L)	Y_{LA} (g/g)	References
Lb. amylophilus JCM 1125	28	0.5	50	92.5	30.0	ca. 0.60	Yumoto & Ikeda (1995)
Lb. amylovorus ATCC 33620	40	3.0	10	nd	4.2	0.42	Xiaodong *et al.*, (1997)
Lb. plantarum C5	30	0.5	20	nd	13.5	0.71	Sanni *et al.*, (2002)
Lb. amylophilus GV6	30	0.5	100	nd	75.7	0.90	Vishnu *et al.*, (2002)
Lb. manihotivorans LMG 18010^T	30	0.5	17.5	99.0	12.6	0.67	Guyot & Calderon (2000)
Lb. paracasei B41	45	0.5	40.0	92.5	37.3	0.93	Petrova & Petrov (2012)
Lb. plantarum SW14	30	0.5	nd	40.0[a]	20.0	nd	Bomrungnok *et al.*, (2012)
Lc. lactis subsp. *lactis* B84	30	2.0	18	nd	5.5	ca. 0.48	Petrov *et al.*, (2008)
E. faecium No. 78	37	0.5	20	98.6	15.4	0.78	Shibata *et al.*, (2007)
S. bovis 148	37	1.0	20	95.6	14.7	0.88	Narita *et al.*, (2004)
B. thermoamylovorans LMG 18084^T	50	0.01	20	100	11.7	0.789	This study
Bacillus sp. MC-07	50	0.01	20	100	16.6	0.977	This study

Lb, Lactobacillus; Lc, Lactococcus; E, Enterococcus; S, Streptococcus; B, Bacillus; YE, yeast extract; *FT*, Fermentation temperature; nd, not determined, ca. calculated value; LA, lactic acid; C, concentration; Y, Yield; $OP_{L\text{-}LA}$, Optical purity of L-lactic acid; [a]Optical purity of D-lactic acid was 60%.

5.5. References

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CHAPTER VI: General conclusions and future prospective

Lactic acid (LA) is an important platform chemicals widely applicable in food, pharmaceutical and chemical industries. Importantly LA is used as a precursor for poly-lactic acid (PLA) a material for biodegradable environmentally friendly bioplastic. Optically pure LA used for synthesizing high grade PLA can only be achieved by microbial (pure or mixed culture forms) fermentation. Although microbial production of LA is of potential interests, controlling the fermentation process for efficient production of LA is still a challenge because yield, optically purities are highly variable with the substrates, seed culture and operational conditions. Recently, high temperature LA fermentation is becoming beneficial in terms of minimizing contaminations, and low energy of coolant water, compared to mesophilic LA fermentation. In addition, to minimize the substantial usage of pure sugar, alternative biomasses such as food waste and its derived sugars like starch, lignocelluloses can be efficiently utilized for LA fermentation. Therefore, this study was focused on the establishment of efficient high temperature fermentation of LA from food waste and starch. and achieved many interesting results. The essences of each chapter included in this thesis are briefly summarized as the followings:

Chapter I described the general introduction of this thesis. The application of LA and its production process especially using *Bacillus* strains and mixed culture were introduced to emphasize its importance in industrial scales. Besides, some limitations of LA fermentation using *Bacillus* strains and the possible overcoming processes were also summarized.

In chapter II, LA production with mixed culture fermentation of kitchen refuses under mesophilic (30ºC) to thermophilic (65ºC) condition was performed. The pH of the fermentation broth was intermittently adjusted at 7.0 every 24 h by 10% NH_3 up to 168 h. Fermentation at the lower temperatures (30, 37 and 40°C), racemic mixture of L and D-lactic acid as well as other organic acids (acetic acid and butyric acid) were accumulated. On the other hand, only L-lactic acid was selectively accumulated at 45, 50 and 55°C with 100% optical purity. In particular, highest L-LA (34.5 g/L) with 90% LA selectivity was produced

at 50°C. At higher temperatures (60 and 65°C) comparatively low organic acid was produced. In addition, the microbial succession in mixed culture fermentation analyzed by denaturing gradient gel electrophoresis showed the predominance of various *Bacillus* strains. At temperature 45, 50 and 55°C, a potent L-LA producer *B. coagulans* was distributed throughout the entire fermentation. These results indicated that mixed culture fermentation at higher temperature (45–55°C) was efficient for utilization of food waste biomasses. Here, the mixed culture system for LA fermentation was successful for producing 100% optically pure L-LA by simply controlling the temperature and pH. To further control the mixed culture fermentation, isolation of major bacteria involved in efficient L-LA production was investigated as described in chapter III.

In chapter III, feedback isolation technique was adopted for systematic isolation of six major bacteria (*Bacillus coagulans*, *B. smithii*, *B. humi*, *B. thermoamylovorans B. thermocloaceae* and *Corynebacterium sphenisci*) MCS. For this, cultivation information (media, pH, temperature, incubation time) of all the major bacteria of MCS detected by DGGE, were fed-back referring to various literatures. Several colonies recovered from different cultivation media were screened by direct colony MALDI-TOF MS. Altogether 136 colonies were obtained and subjected to screening. By comparing the MALDI-TOF MS fingerprint and 16S rRNA gene sequence results of isolates, we found that numerical threshold value of 0.55 for similar bacterial isolates. Total 21 bacterial species including six major bacteria were successfully selected. We succeeded in systematic isolation of major bacteria involved in mixed culture fermentation system by adopting feedback isolation technique and direct colony MALDI-TOF MS. The study reveals that multiple targeted bacterial strains can be isolated by adopting systematic feedback isolation technique. It provides novel efficient and simple methodology to screen total bacterial members in a certain mixed culture fermentation system. Besides, selective combination (reconstruction) of

mixed culture from the isolated strains could be possible to further control the meta-fermentation system.

In chapter VI, one of the unexpectedly isolated strains MO-04 from mixed culture seed by was taxonomically characterized and proposed as novel strain within genus *Bacillus*. Strain MO-04 showed several differential features compared to closely related type strain *B. thermolactis* R-6488T. Strain MO-04 showed 99.4% 16S rRNA gene sequence similarity with *B. thermolactis* R-6488^T. DNA-DNA relatedness between strain MO-04 and *B. thermolactis* DSM 23332^T was 45%, much lower than the threshold value (≥70%) for similar species. The DNA G+C content of strain MO-04^T was 33.4 mol%, comparatively lower than that of *B. thermolactis* R-6488^T (35.0 mol%). Strain MO-04 grew at 35–61°C (optimum 50°C), pH 4.5–9.0 (optimum 7.2), and tolerated up to 8.0% (w/v) NaCl (optimum 2%). The cell examined under SEM showed a *Kokeshii*-shaped morphology. Based on its morphology, the named was proposed. The Proposed name of a novel species is *Bacillus kokeshiiformis* MO-04^T (= JCM 19325^T = KCTC 33163^T). This novel finding encourages us to further investigate and establish the effective MCS for highly efficient L-LA production.

In chapter V, one of the major candidate of meta-fermentation isolate MC-07 (shared 99.4 % 16S rRNA gene sequence similarity with type strain *Bacillus thermoamylovorans* LMG 18084^T) showed high halo formation in tryptone soy agar containing 1% soluble starch (after 24 h) after iodine staining. Optimum growth temperature for the strain MC-07 was investigated at 50°C. In batch fermentation at pH 7.0, 20 g/L of soluble starch in mineral salt medium containing 0.001% yeast extract, 16.6 g/L L-LA was produced at 50 °C within 24 h of fermentation with a yield of 0.977 g/g , optical purity of 100 % and LA selectivity of 92.1 %. We are the first to establish direct starch fermentation to L-LA without saccharification at 50ºC by *Bacillus* strains.

In conclusion, a new concept, meta-fermentation was proposed for efficient utilization of food waste by mixed culture seed. To elucidate this kind of MCS, whole systematic isolation technique was developed. During the studies on MCS, we identified and proposed new thermotolerant bacteria *Bacillus kokeshiiformis* MO-04^{T}, and identified thermotolerant *Bacillus* sp. MC-07 having the ability to ferment starch without enzymatic liquefaction. These new findings would be useful for sustainable utilization of waste biomasses to value added fine chemicals indeed.