Isolation and characterization of thermophile lactic acid bacteria from Thailand tropical fruits

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Introduction

Lactic acid bacteria (LAB) are Gram-positive and do not form spore. Their shapes are divided into “cocci” or “rod”. Almost all LAB lack catalase, whereas a few strain has pseudo-catalase. The dominant characteristic of LAB is to produce lactic acid as end product from glucose. Base on the difference of the glucose utilization, LAB are classified into two groups, called as homo-fermentative and hetero-fermentative. The former strains convert glucose to lactic acid as the only or major end product *via* Embden-Meyerhof pathway, whereas the latter ones produce lactic acid together with additional products, like acetate, ethanol, CO_2, formate, or succinate (Salminen and Wright, 1998). LAB are found in fermented foods, such as dairy products, fermented meat, sour dough, fermented vegetables, silage, and beverages. In addition, they are also found in the genital, intestinal, and respiratory tracts of man and animals (Hammes *et al.*, 1991).

Based on sequence data of the 16S or 23S rRNA-encoding genes, the Gram-positive bacteria form two lines of descent. One phylum consists of gram-positive bacteria with a DNA base composition of less than 50 mol% guanine plus cytosine (G+C), the so-call *Clostridium* branch, whereas the other branch (actinomycetes) comprises organisms with a G+C content that is higher than 50 mol%. The typical LAB, such as the genus *Carnobacterium*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, have a G+C content of less than 50 mol% (Wood and Holzapfel, 1995).

In the work of Orla-Jensen (1919), an important character used in the differentiation of the LAB genera is the mode of glucose fermentation under standard conditions, i.e. non-limiting concentration of glucose and growth factors (amino acids, vitamins, and nucleic acid precursors) and limited oxygen available. However, the optimum temperature for growth is mainly employed to distinguish between some strains of the cocci. A genus *Enterococcus* can grow during 10°C and 45°C. However, although genus *Lactococcus* and *Vagococcus* can grow at 10°C, but not at 45°C. A genus *Streptococcus* generally do not grow at 10°C.

The salt-tolerant characteristic of LAB in the presence of 6.5 (w/v) % NaCl may be used to distinguish between enterococci, lactococci/vagococci, and streptococci. The genus *Tetragenococcus* species exhibits tolerance even to 18 (w/v) % NaCl. Tolerance to acid and/or

alkaline conditions may be also useful characteristics. Genus *Aerococcus, Carnobacterium*, *Enterococcus*, *Tetragenococcus*, and *Vagococcus* can grow under the condition of higher pH, but not at pH 9.6 (Wood and Holzapfel, 1995; Salminen and Wright, 1998).

Although some of Orla-Jensen's concepts are still viable, the proper classification of LAB to species is relied on molecular biology method. Now, to identify species of a LAB candidate, the nucleotide sequence of 16S rRNA-encoding gene is determined and compared with the DNA database in DDBJ, EMBL or GenBank. In addition, the presence of diamino acid consisting of the peptidoglycan, the type of teichoic acid, the presence and type of menaquinone, the guanine and cytosine (G+C) content in the genome DNA, the composition of fatty acids, and electrophoretic mobility of the lactate dehydrogenase (LDH) are also necessary for the taxonomical identification (Wood and Holzapfel, 1995; Salminen and Wright, 1998).

Profit to human health of LAB

LAB, which are generally recognized as safe (GRAS), are used as a starter for fermented food products, because they produce lactic acid to change food matrix to low pH condition result the advantage modification of food flavor and texture. Some LAB strains produce antimicrobial polypeptides "bacteriocin", for example "nisin A". The substance inhibits the growth of pathogenic and corruption bacteria for foods. Furthermore, some strains of LAB are useful for pharmaceutical industry which produces medicine and healthcare supplement. Living cells of some LAB strains are more effective to maintain gastro intestinal balance. It has been known that exopolysaccharide (EPS) produced by some strains of LAB is effective to increase the human immunity against both of virus and tumor cell.

Representative of probiotics is LAB, which have been defined by the World Health Organization (WHO) as follows: probiotics are the ingested living microorganisms bring benefit to humans and animals. The beneficial effect of probiotics to human health is occurred by the colonization into gastrointestinal tract. Probiotics have been utilized for prevention of mucosal surface infections (*e.g.* gut and vagina), however the discovery of antibiotics decreased the use of probiotics for clinical treatment. In recent years, since many kinds of multi-drug resistant bacteria are increased to diffuse into the world, the reduction of the

resistant bacteria and the treatment cost are indispensable. The use of probiotics is now considered as an alternative solution method to antibiotics (O'May and Macfarlane, 2005). The LAB strain, which are commercially utilized as probiotics, are mainly *Lactobacillus* (*Lb.*), *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. reuteri*, *Lb. casei*, *Lb. plantarum* (Krishnakuma and Gordon, 2001; Playne *et al.,* 2003; Shah, 2007).

Function of probiotics

The main function of probiotic bacteria is to improve mucosal defense of the gastrointestinal tract. Probiotics can block the colonization of pathogenic bacteria by decreasing pH of gastrointestinal tract. As a result, probiotics inhibit invasion and adhesion of pathogenic bacteria to epithelial cell. LAB produce antimicrobial substances such as bacteriocins and lactic acid. The interaction between LAB and the mucosal epithelial cell in the gastrointestinal tract increases the gut immune reaction against pathogenic bacteria (Bourlioux *et al.*, 2003; Mazahreh and Ershidat, 2009).

The probiotics conflict with pathogen to epithelial binding sites and inhibit the colonization of pathogenic bacteria directly or indirectly (O′Shea *et al.*, 2012). The activation of mucosal barrier function may be a significant mechanism by which probiotics benefit the host in some diseases, like diabetes (Watts *et al.*, 2005; Medding, 2008).

It is pointed out that probiotics affect the epithelial cell, the dendritic cell, the macrophages, and the lymphocytes directly or secondarily (Walker, 2008; Ng *et al.*, 2009). Probiotics are significant to eliminate neoplastic host cells (Soccol *et al.*, 2010). In addition, as the effects of probiotics on B-lymphocytes and antibody production, the secretion of IgA is increased and the response to vaccination is also enhanced (Ng *et al.*, 2009). It has recently been demonstrated that probiotics give a plus effect to the respiratory system by preventing and decreasing the susceptibility of respiratory infections, because the IgA production is increased in the bronchial mucosa (Perdigon *et al.*, 1999).

Exopolysaccharide (EPS) produced by LAB

EPS is long chain polysaccharides that are secreted mainly by microorganism into their surroundings during growth. The substance is secreted as two forms: as a capsular (cell bond)

polysaccharides which associated with the cell surface, and slime EPS which usually secrete as free polymers to the environment (Ruas-Madiedo *et al.*, 2006; Whitfield, 1998). The major physiological function of EPS is biological defense against various such as phage attack, toxic metal ion, and desiccation (Ruas-Madiedo *et al.*, 2002). The chemical structure of EPS has been studied in details (Robijin *et al.*, 1996; Górsk *et al.*, 2010). Base on the structure, EPS is classified into 2 groups: homo-EPS, consisting of a single type of monosaccharide; and hetero-EPS, composed of different types often three to eight of monosaccharides, mainly *D*-glucose, *D*-galactose, *L*-rhamnose, and their derivatives (Mayo *et al.*, 2010). The composition and nature of EPS is altered by environmental conditions, biosynthetic pathway or rate of microbial growth.

1. Homo-EPS

The differences between the homo-EPS are correlated with the features of their primary structure such as the pattern of main chain bonds, molecular weight, and branch structure. Two important groups of homo-EPS which produced by LAB; (i) α-glucans mainly composed of α-1,6 and α-1,3-linked glucose residues, namely dextrans, produced by *Leuconostoc* (*Leu.*) *mesenteroides* subsp. *mesenteroides* and *Leu. mensenteroides* subsp. *dextranicum*, and (ii) fructans, mainly composed of β-2,6-linked fructose molecule, such as levan produced by *Streptococcus* (*S.*) *salivarius* (Cerning, 1990).

Leu. mesenteroides subsp. *mesenteroides* produces dextran from sucrose. However, the dextran-producing ability is disappeared when serial transfers are made in media containing a high concentration of salt. Almost all dextran consist of α-1,6-linkages with branch points 2,3 or 4 (Cerning, 1990). Some strain of *Leu. amelibiosum* (Dellaglio *et al.*, 1995) and *Lb. curvatus* (Minervini *et al.*, 2010) are known to be dextran-producing strains.

Mutan, which is a glucan synthesized by *S. mutans*, differs from dextran in that it contains a high percentage of α-1,3-linkages. Differences in solubility result from the productions of different types of linkages; water-soluble glucans are rich in α-1,6-linkages, while water-insoluble glucans are rich in α-1,3-linkages (Cerning, 1990). The ingestion of mutan has been associated with dental caries as an insoluble substance can adhere to teeth.

Alternan has alternate α-1,6-linkages and α-1,3-linkages, and this structure is thought to be responsible for its distinguish physical properties, such as the high solubility and low viscosity. *Leu. mesenteroides* NRRL B-1355 has been shown to produce alternan (Cote and Robyt, 1982).

Levan, which is an EPS produced from sucrose, is a fructan composed of β-2,6-linked fructose molecules and some β-2,1-linked branches. Inulin is a fructan which is composed of β-2,1-linked fructose molecules and some β-2,6-linked branches. *S. salivarius, Leu. mesenteroides* and *Lb. reuteri* are known to produce levan (Uchida 1996; Van Geel Schutten *et al.*, 1999). In addition, the EPS produced by *Lb. sanfranciscensis* TMW 1.392 has been also demonstrated to be fructan (Koraki *et al.*, 2002).

2. Hetero-EPS

Hetero-EPS has polymerized repeating units mainly composed of *D*-glucose, *D*-galactose, and *L*-rhamnose. The composition of the monosaccharide subunits is considered be not species-specific, except in case of *Lb. kefiranofaciens* subsp. *kefiranofacieins*. The microbial mixture consisting of LAB strains and yeasts, which are isolated from kefir grain as a fermented dairy product made in North Caucasus, produces large amount of polysaccharides. As hetero-EPS-producing LAB strains, *S. thermophilus*, *Lactococcus* (*Lc.*) *lactis, Lb. delbrueckii*, and *Lb. helveticus* have been known (Degeest and de Vuyst, 2000; Knoshaug *et al.*, 2007; Petry *et al.*, 2000; Staaf *et al.*, 2000). EPS production by *S. thermophilus* is 50–350 mg/L. The EPSs by *Lc. lactis* subsp. *cremoris* and *Lb. delbrueckii* subsp. *bulgaricu*s are 80–600 mg/L and 60–150 mg/L, respectively (Cerning, 1990).

The improvement of chemically defined medium containing a carbohydrate source, mineral salts, amino acid, vitamins, and nucleic acid base is in progress to investigate the influence of different nutrients on LAB growth and EPS biosynthesis. The total yield of EPS produced by LAB depends on the composition in carbon and nitrogen sources in the medium. Of course, evaluation of temperature, pH of the medium and incubation time suitable for the growth of the LAB strain is necessary to produce EPS at high level.

With respect to the growth of *Lb. delbrueckii* subsp. *bulgaricu*s NCFB 2772 in milk, it has been shown that high temperatures and slow growth increase the productivity of the EPS per cell (Garcia and Marshall, 1991). The optimum culture condition for the EPS production by *Lb.*

delbrueckii subsp. *bulgaricus* R in semi-defined medium has been investigated. As the result, it has been determined that the optimum temperature and pH condition for EPS production are 36–39°C and pH 4.5–5.5, respectively. The optimal temperature for the EPS production by the growth of the thermophile and mesophile LAB strains was 40°C and around 25°C, respectively (Gamar *et al.*, 1997).

The effect of the nitrogen and carbon sources for EPS production has also been evaluated. It has been reported that neither LAB growth nor EPS production was specifically associated with the presence of casein or whey proteins in the growth medium. A research group has shown that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 increases the EPS production at early growth phase in the presence of the hydrolyzed milk casein, whereas the addition of hydrolyzed casein to MRS medium is not effective for the EPS production (Garcia *et al.*, 1991). The productivity of EPS in LAB strain was 25 mg/L, when grown in a defined medium containing fructose. Interestingly, the production of the EPS was up to 80 mg/L by the addition of glucose instead of fructose (Grobben *et al.*, 1998).

When *Lb. plantarum* was grown in whey, the addition of yeast extract was more effective as nitrogen source for the EPS production than soybean peptide, tryptone, peptone, and Lab-Lemco powder. Moreover, the addition of glucose as a carbon source was more effective than that of galactose, maltose, sucrose, fructose, and raffinose (Tsuda and Miyamoto, 2010). The increased addition of Mg, Mn, and Fe ions has been also reported to stimulate the EPS production to a synthetic medium (Gamar *et al.*,1997).

Biosynthesis of EPS

Homo-EPS is secreted to outside cell by a specific enzyme, glycosyltranferase (GTF) or fructosyltransferase (FTF), which is generally called glucansucrase or fructansucrase. The LAB strain, which produces homo-EPS, also uses the extracellular GTF to form the high molecular mass glucan from sucrose. The energy requirement for the process comes from sucrose hydrolysis. There is no energy requirement for EPS-production other than for enzyme biosynthesis because of EPS synthesis by GTF or FTF does not involve the active transport process or the use of activated carbohydrate precursors. Therefore, the large amount of sucrose

can be easily converted to EPS. *L. sanfranciscensis* produces up to 40 g/L levan and 25 g/L *L*-kestose in the present of 160 g/L sucrose (Korakli *et al.*, 2003).

Glucan-synthesizing reaction by GTF is as below:

sucrose + H_2O→ glucose + fructose

sucrose + acceptor carbohydrate → oligosaccharides + fructose

sucrose + glucan (n) → glucan (n+1) + fructose

The relative molecular weight of glucan from lactobacilli range from 1×10^6 to 5×10^7 Da (Kralj *et al.*, 2004).

However, the fructan-synthesizing reaction by FTF is as follows:

sucrose + H_2O→ fructose + glucose

sucrose + acceptor carbohydrate → oligosaccharides + glucose

sucrose + fructan (n) → fructan (n+1) + glucose

Fructans generally have a relative molecular weight exceeding 5×10^6 Da.

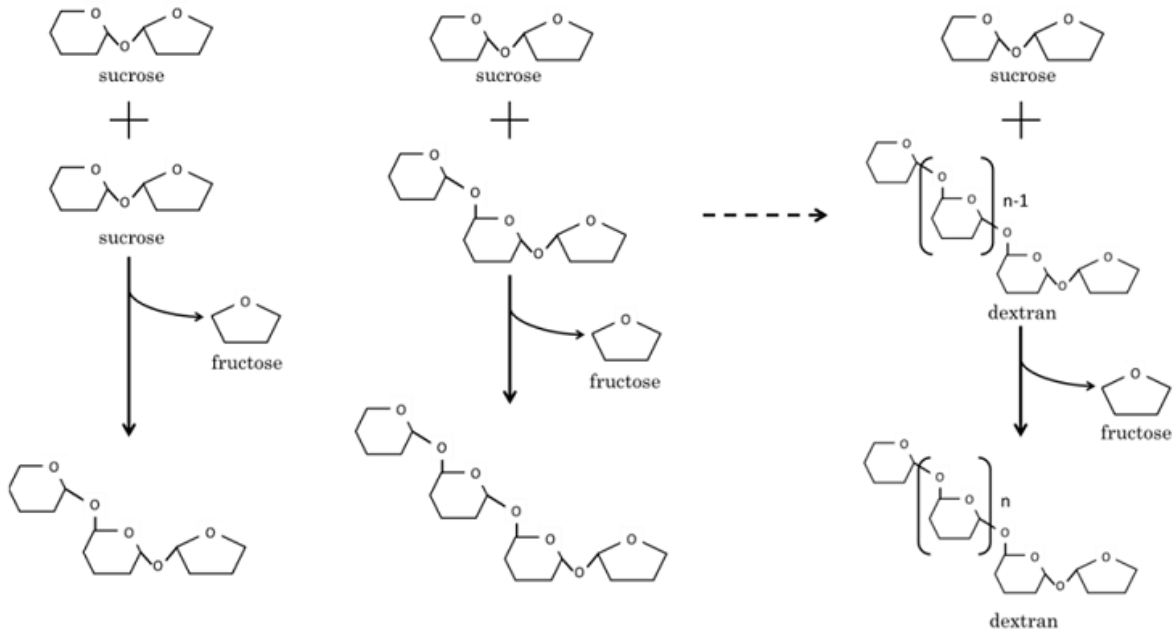

Fig. 1. The dextran synthesis by GTF (dextran sucrase) (Tsuda, 2013).

Biosynthesis of hetero-EPS on LAB

No hetero-EPS is formed by extracellular enzymes, but are formed by a complex sequence of interactions with respect to intracellular enzymes. The EPS are made by polymerization of repeating units, and these repeating units are built by a series of addition of sugar nucleotides at the cytoplasmic membrane.

LAB strains utilize various monosaccharides and disaccharides as energy sources, *via* some well-studied sugar uptake systems include the primary transport-specific ATPase. Polysaccharides must be hydrolyzed before uptake. For example, starch is hydrolyzed by α-amylase, and the resulting products are subsequently hydrolyzed by the enzyme described above.

The biosynthetic pathway of hetero-EPS is broken down into four separate reaction sequences. These are the reactions involved with sugar transport in to the cytoplasm, the synthesis of sugar-1-phosphates, activation of and coupling of sugar, and the process involved in the export of the EPS.

The movement of carbon feed, mainly monosaccharides and disaccharides, from the surrounding growth medium into the cytoplasm is a carefully related process. A number of different proteins control the internalization of sugar. A research group (Postma *et al*., 1993) has shown that the most frequently encountered sugar transport machinery is the bacterial PEP-PT (phosphoenolpyruvate-dependent phosphotransferase) system. The PEP-PT system contains a group of protein that are responsible for binding, transmembrane transport, and phosphorylation of a variety of sugar substrates.

The fate of the carbon feed is determined by the state of phosphorylation of the sugar: sugar-6-phosphates are consumed in catabolic pathways, whereas sugar-1-phosphates can participate in polysaccharides synthesis. Sugar, which is transported into the cytoplasm by PEP-PT system, generates sugar-6-phosphates. It has been recently reported that the pathway converting sugar-6-phosphate to sugar-1-phosphate are altered by the difference of carbon source and of growth condition.

The genes encoding the proteins required for EPS biosynthesis are divided into two groups: that is, genes required for the synthesis of sugar nucleotides and EPS-specific genes. The two groups are physically separated in the genome. The first group consists of the genes encoding

enzymes and proteins required for the synthesis of sugar nucleotides from which the repeat unit is constructed. The sugar nucleotides are needed for the synthesis of a range of polysaccharides and are not specific to EPS biosynthesis.

The sugar nucleotides required for the construction of the majority of EPS structure are UDP-glucose, UDP-galactose, and dTDP-rhamnose. The coding gene or the enzyme needed for the synthesis of the sugar nucleotides from glucose-1-phosphate (*galU*, *galE*, *rfbA, rfbB*, *rfbC*, and *rfbD*) have been identified and cloned from *Lc. lactis* MG 1363 (Boels *et al.*, 1988; Kleerebezem *et al.*, 1999). The first enzyme in the sequence is GalU, that is, a UDP-glucose pyrophosphorylase. It has been reported that the intracellular amounts of UDP-glucose are determined by the activity of the enzyme GalU; the overexpression of the *Lc. lactis galU* gene results in much larger UDP-glucose levels in *Lc. lactis*. The production of UDP-galactose may be derived principally from UDP-glucose *via* the action of GalE that catalyses the interconversion of the two UDP-sugars. The requirement for the GalE biosynthesis in *Lc. lactis* NIZO B40 has been demonstrated by Kleerebezem *et al.*, (1999). The *galE* mutant produces EPS when grown in a galactose-containing medium, but did not when grown in glucose-containing one. The result implied that, in the absence of galactose, UDP-galactose required for EPS synthesis is derived solely from UPD-glucose. Details of the characteristics of the enzymes need to produce dTDP-rhamnose were established in Gram-negative bacteria where rhamnose is a key constitute of the O-antigens of lipopolysaccharides. Four enzymes, RfbA, RfbB, RfbC and RfbD, convert α-glucose-1-phosphate initially to dTDP-glucose then to 4-keto-6-deoxymannose and finally to dTDP-rhamnose.

The next step in the EPS biosynthesis is to use the EPS-specific enzyme. The first gene clusters for production of secreted EPSs has been identified and characterized in *S. thermophilus* Sfi 6 (Stingele *et al.*, 1996) and for *Lc. lacti*s NIZO B40 (van Kranenburg *et al.*, 1997). The organization of the gene clusters is similar among both species and consists of four separate domains. A central core gene encoding the glycosyltransferase is flanked at the ends by gene coding for proteins having a strong homology with enzymes used for polymerization and export. A regulatory domain is present at the start of the gene cluster. Stingele *et al.*, (1999) have demonstrated that the EPS was produced in the heterologous host when the gene cluster

containing EPS-specific enzyme was introduced into the EPS-nonproducing *Lc. lactis* MG1363 as a host.

In vitro experiment using ^{14}C-labelled sugar nucleotides have provided evidence that the monosaccharide repeating unit is assembled on a lipid carrier, which is attached to the cytoplasmic membrane (van Kranenburg *et al.*, 1997; van Kranenburg *et al.*, 1999). The assembly by the repeating unit on a lipid carrier is a process, which is used for the synthesis of excreted polysaccharides, for cell wall peptidoglycans and for cell surface oligonucleotides and polysaccharides. There is an evidence to suggest that the various oligosaccharide and polysaccharides syntheses use the same building blocks sugar nucleotides and scaffolding (lipid carrier). The latter may account for the close relationships between rates of EPS synthesis and cell growth that have been observed by a number of authors (Garcia-Garibay and Marshall, 1991; Cerning *et al.*, 1992).

The genes for polymerization and export of the EPS succinoglycan in *Rhizobium meliloti* has been studied by Gonzalez *et al.*, (1998): concluding that the subunit is constructed on an undecaprenol lipid carrier on the cytoplasmic face of the plasma membrane, which are polymerized in a block fashion. Details of the mechanism by which blocks are polymerized in EPS biosynthesis are not known at this moment. In O-antigen synthesis, three gene products are required for polymerization and export (Whitfield and Valvano, 1993). These gene products encode three types of enzymes: (i) flippase or translocase that catalyze the movement of the lipid-bound material from the cytoplasmic face of the membrane to the periplasmic face, (ii) polymerase that catalyzes the polymerization of the blocks, and (iii) an enzyme involved in chain length determination.

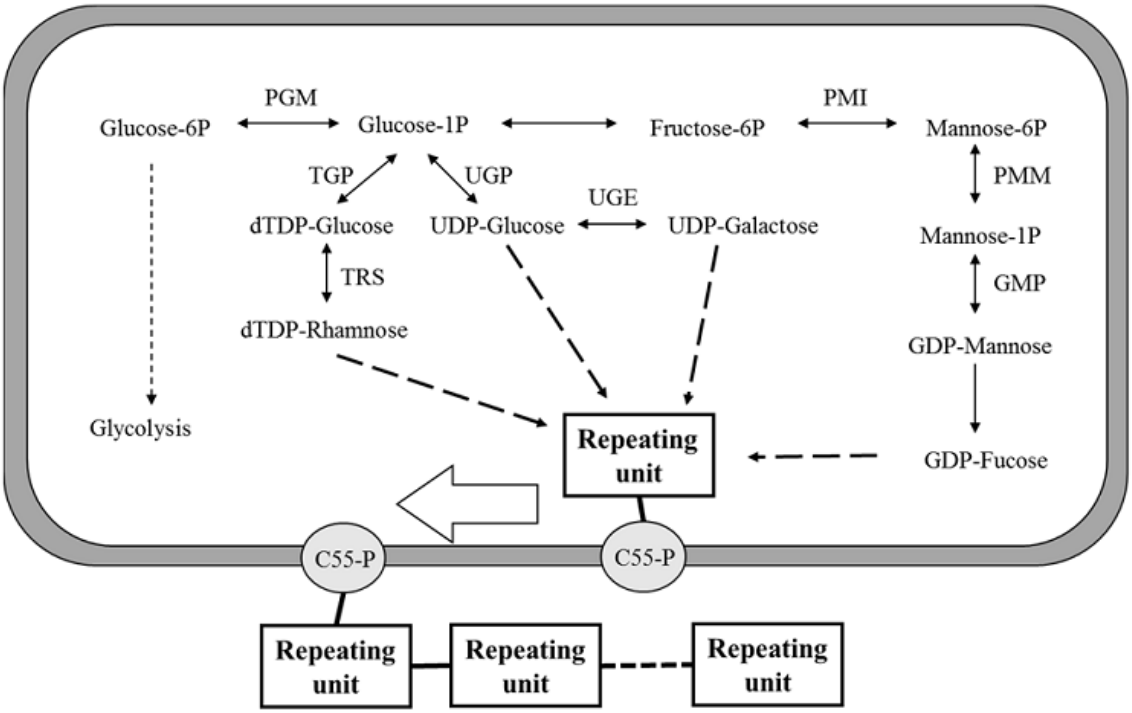

Fig. 2. Outline of biosynthesis of EPS (Tsuda, 2013).

PGM: α-phosphoglucomutase; UGP: UDP-glucose pyrophosphorylase; UGE: UDP-galactose 4-epimerase; TGP: dTDP-glucose pyrophosphorylase; TRS: dTDP-rhamnose synthetic enzyme systm; PMI: phosphomannoisomerase; PMM: phosphomannomutase, GMP: GDP-mannose pyrophosphorylase.

Instability of EPS production.

de Vuyst *et al.*, (2001) have reviewed about the instability of hetero-EPS production: that is, a loss in the ability producing slime must be caused by repeated subculture of bacterial strains or incubation at high temperature. The loss of plasmid in mesophile LAB strains is a reason for loss of slime production. On the other hand, thermophile LAB strains namely *Lb. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*, have been showed to lack a plasmid which encodes components required for slime production. These LAB strains can recover the ability to produce slime following loss due to culture condition. Thus, genetic instability could be a consequence of the action of mobile genetic elements, like insertion sequences. The EPS gene cluster in *Lb. fermentum* TDS030603 has been recently shown to be located on chromosomal DNA (Dan *et al.*, 2009).

Health benefit of LAB-derived EPS

The health benefit of LAB has been attributed to the production of EPS (Ruas-Madiedo *et al.*, 2006). EPS produced by LAB has been claimed to exhibit immuno-stimulatory activity (Vinderola *et al.*, 2006), anti-tumor effect (Furukawa *et al.*, 2000), or blood pressure and cholesterol lowering activity (Maeda *et al.*, 2004; Nakajima *et al.*, 1992). EPS reduces symptoms of lactose intolerance and prevent diarrhea (Grandy *et al.*, 2010). They have been reported that sugar polymer has anti-microbial activity and to heal wound (Rodrigues *et al.*, 2005; Wu *et al.*, 2010). It has been also shown that some EPSs induce cytokine production, act like lymphocytes B mitogens or change functions of splenocytes (Chabot *et al.*, 2001; Bleau *et al.*, 2010). EPSs reduce the symptoms of collagen-induced arthritis or diminish arteriosclerosis in mice. Orally administrated EPS-producing LAB attenuate severity of colitis and may be a promising agent in therapy of inflammatory bowel disease (Sengül *et al.*, 2010).

Objective

1. Isolation of thermophile LAB and its exopolysaccharaide (EPS)-producing strains from Thiland tropical fruits
2. The culture optimization to increase the production of EPS
3. Analysis of the monosaccharides constituting the EPS

Materials and Methods

1. Isolation of lactic acid bacteria to obtain thermophile strains

The pieces of peeled tropical fruits were kept in a test tube containing MRS broth (Merck) at temperature 30, 37, 45, and 50°C for 3 days. The culture was diluted by the sterilized PBS. One hundred μL of the diluted culture broth was spread on a MRS agar medium and incubated under the given temperature for 48 h. The resulting colony was picked up and re-streaked to obtain the single colony. To confirm whether the purified strains are lactic acid bacteria (LAB), Gram-straining was done and checked for catalase production. The strains confirmed as LAB were stored at -80°C in 30 (v/v) % glycerol until use. The method to isolate LAB strain is summarized in Fig. 3.

[PBS]

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.42 g
KH_2PO_4	0.24 g

Buffer components were dissolved with 800 mL of distilled water. Final pH was adjusted to 7.4 with HCl.

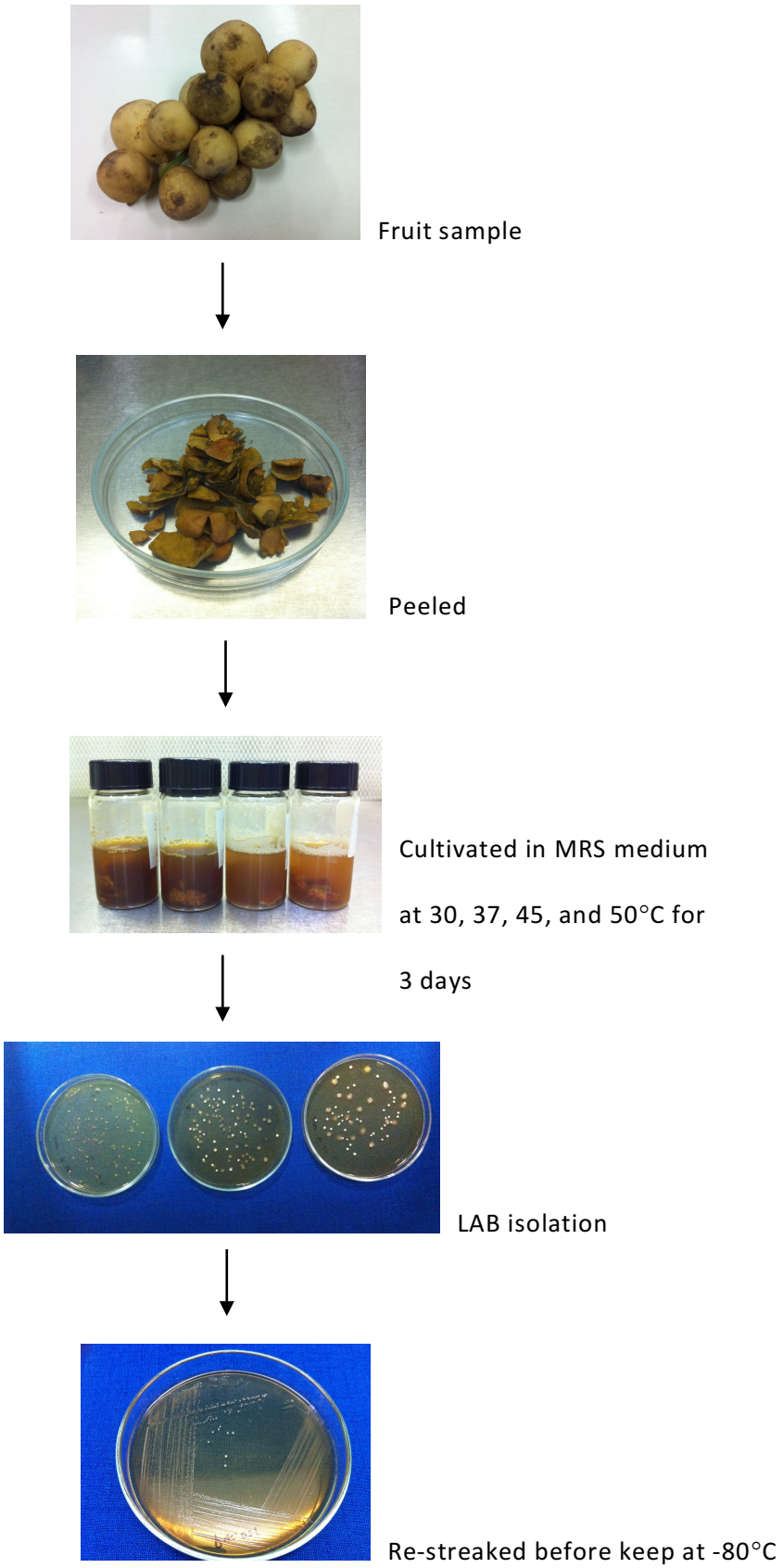

Fig. 3. The process for LAB isolation from Thailand tropical fruits.

2. Growth determination of the selected thermo-tolerant strains

The thermophile strains were designated as LAB candidates growing vigorously at 45°C, and stored until use at -80°C. A portion of from the stored culture was inoculated in MRS broth and incubated at 45°C. After the 18 h-cultivation, the culture broth was used as a seed culture for growth. Growth of individual strain was monitored as follows: A 100-μL portion of the seed culture was inoculated into 3 mL of fresh MRS medium and mixed. A 100-μL portion of the resulting broth was used to determine the living cell numbers by the colony-counting method using a plate. The plate which generates colony was incubated at 45°C for 3 days. The living cell numbers were determined at 24 h intervals. Growth profile of an individual thermo-tolerant strain was compared each other to confirm as the thermopile strains.

3. Identification of thermophile strains.

The strains judged thermopile bacteria were identified at species level by sequencing the 16S rRNA encoding gene: chromosomal DNA of thermophile LAB was isolated by using CloneSaver Card (Whatman, BioScience). Briefly, a 10-μL portion of the 16 h cell suspension was loaded into the CloneSaver Card and allowed to dry at room temperature. Sample was removed from the disk by punched a disk out of the sample area, placed in the PCR tube, and washed twice with 200 μL of FTA purification reagent. The disk was washed twice with TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and dried at 55°C for 10 min before amplification of 16S rRNA-encoding gene. Compositions of the PCR mixture are follows;

Template DNA	Disk
10 × PCR buffer	10.0 μL
dNTP mixture (2.5 mM each)	8.0 μL
Taq polymerase (5U/μL)	0.5 μL
Primer:Forward (F27)	0.5 μL
Reverse (R1492)	0.5 μL
UL water	30.5 μL
Total reaction volume	50 μL

[PCR primers for 16S rRNA encoding gene amprification]

F27 5′-AGAGTTTGATCCTGGCTCAG-3′

R1492 5′-GGCTACCTTGTTACGACTT-3′

[PCR condition]

94 °C (4 min) → 94 °C (45 sec) / 55 °C (1 min) / 72 °C (45 sec) × 30 times → 72 °C (10 min) → 4°C

The nucleotide sequence of amplified DNA was determined and compared with the bacterial 16S ribosomal DNA (rDNA) database of the DNA Data Bank of Japan (DDBJ) by using the BLAST algorithm utilizing the non-redundant database provided by National Center for Biotechnology Information (NCBI).

4. Optimal growth temperature determination

Growth profile of the thermopile LAB strain was determined at 28, 37, 45, 50, and 55°C in MRS broth. Incubation time was operated from 0 to 72 h. Growth index of each strain was determined by counting living cell number (by total plate count) as cfu/mL and monitoring the pH change of the cultured broth.

5. EPS production and effect of carbon source on EPS production

5.1. Media and growth conditions

De Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA, Germany) was used for the growth of all LAB. A semi-defined medium (SDM) lacking yeast nitrogen base, but supplemented with 0.2 (v/v) % vitamin solution and 0.1 (v/v) % trace element solution was named as modified-SDM and was mainly used to evaluate EPS productivity. For cell growth monitoring, pH and cell numbers (cfu/mL) of culture broth were measured at each 3 h intervals.

[modified semi-defined medium (g/L)]

Glucose	20
Tween 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
$MgSO_4 \cdot 7H_2O$	0.1
$MnSO_4 \cdot 5H_2O$	0.05
K_2HPO_4	2.0
Bacto casitone	10.0
Vitamine Soln.†	2 mL
Trace element Soln.‡	1 mL

[†Vitamine Soln. (g/l)]

4-aminobenzoic acid	0.05
Biotin	0.001
Folic acid	0.025
Lipoic acid	0.025
Nicotinic acid	0.1
Pantothenic acid	0.05
Pyridoxamin-HCl	0.25
Vitamine B_{12}	0.05
Pyridoxine	0.025
Riboflavin	0.05
Thiamine	0.1

[‡Trace element Soln. (g/L)]

25% HCl	10 mL
$FeCl_2 \cdot 4H_2O$	1.5
$CoCl_2 \cdot 6H_2O$	0.19
$MnCl_2 \cdot 4H_2O$	0.1
$ZnCl_2$	0.07
H_3BO_3	0.006
$Na_2MoO_4 \cdot 2H_2O$	0.036
$NiCl_2 \cdot 6H_2O$	0.024
$CuCl_2 \cdot 2H_2O$	0.002

5.2. Culture conditions for the EPS production of LAB

For the seed culture, a portion of a freeze-stock culture of LAB was inoculated into an MRS media and incubated as standing culture at 45°C for 48 h. For EPS production by the LY45 strain and PY45 strains, seed culture at 0.2 (v/v) % was inoculated into the modified-SDM medium and incubated at 45°C for 2–5 days. To evaluate the effect of carbon source on EPS production, inoculum at 0.2 (v/v) % of the LY45 and PY45 strains were transferred into the modified-SDM medium supplemented with the given sugar. The sugars, which may be evaluated carbon source suitable for high production of EPS, were mannose, sucrose, maltose, lactose, fructose, and galactose. Bacterial cell inoculated into the modified-DSM medium supplemented with each sugar was grown at 45°C for 2–5 days and followed by purification of EPS.

5.3. Purification of EPS from LAB culture broth

Purification of EPS is described as follows: A 81 mL portion of 100 (w/v) % trichloroacetic acid (TCA) was added and mixed into the 2 L of culture broth. After resulting mixture was kept at 4°C for 30 min, the cells debris and protein was removed by centrifugation at 12,000 × *g* for 10 min at 4°C. A 2 L of acetone was added into the supernatant fluid, the mixture was stand for overnight at 4°C. After centrifugation at 12,000 × *g* for 10 min at 4°C, the crude EPS precipitate was washed by 100 mL of 70

(v/v) % ethanol and dried up *in vacuo*. The washed precipitate was dissolved into a 30 mL of 50 mM Tris-HCl buffer (pH 8.0) with gentle agitation at 4°C for 1 h, and the debris was removed by centrifugation at 20,000 × *g* for 30 min at 4°C. A portion of 300 μL of 1 mg/mL deoxyribonuclease I (Worthington Biochemical Corporation, NJ) and 1 mg/mL ribonuclease A (Nacalai Tesque, Japan) solution dissolved into the same buffer was added to the supernatant fluid. and the mixture was incubated at 37°C for 8 h. A 300 μL portion of 2 mg/mL proteinase K (Wako Pure Chemical Industries, Japan) dissolved into the same buffer was added to the mixture and followed by incubation at 37°C for 16 h. After the mixture was kept on ice for 1 h, a 81 mL portion of 100 (w/v) % TCA was added and kept on ice 1 h. The protein and debris were removed by centrifugation at 20,000 × *g* for 30 min at 4°C, and 105 ml of 100 (v/v) % ethanol was added to the supernatant fluid. After mixing and centrifugation at 15,000 × *g* for 5 min at 4°C, the resulting precipitate was washed by 20 mL of 70 (v/v) % ethanol and dried up *in vacuo*. The precipitate was completely dissolved into a 10 mL of distilled water, and dialyzed against the distilled water by using a dialysis membrane (MWCO = 10 kDa). The concentration of EPS was determined by the phenol-sulfate method (Dubois *et al.*, 1956), and followed by lyophilization to obtain the purified EPS.

6. Elucidation of composition of EPS monosaccharide

Analysis of the sugar composition of EPS was performed by the alditol acetate derivatization procedure using the gas chromatography–mass spectrometry (GC–MS). The derivatization reaction was performed as follows; five mg of purified EPS was acid-hydrolyzed by treatment with 1 mL of 2 M trifluoroacetic acid (TFA) into individual monosaccharides (120°C for 1 h) in the test tube (screw-capped). After cooling the sample, one ml of isopropanol was added and then dried up. A 0.5-mL portion of [5 mg sodium borohydride / 0.5 mL 1 M ammonia (aq)] solution was added to the dried sample, and stand for at RT for 1 h and kept on ice for 1 h. The sample was mixed with 0.1 mL of 5 M acetate, and 1 mL of [acetone:methanol = 1: 9] was added. After repeat 3 times mixing and drying up by evaporator, the sample was mixed with 1 mL of methanol and dried up by evaporator. After this procedure was repeat 3 times, a 0.5-mL portion of

[pyridine: acetic anhydride = 1: 1] was added to the dried sample, and reacted at 120°C for 20 min. The reaction mixture was cooled on ice, and mixed with 1.5 mL of [water : methanol : toluene = 1: 4 : 1] and dried up by evaporator. The sample was further mixed with 1 ml of methanol and dried up by evaporator. This step was repeated 3 times. A 3-mL of [dichloromethane : water = 2 : 1] was added to the dried sample, and the alditol acetate derivative was extract in dichloromethane layer. One mL of water was added to the collected dichloromethane layer to remove the water soluble impurities. This procedure was repeated 2 times. The resulting dichloromethane layer was transferred to a new tube, completely dried up, dissolved into appropriate volume of acetone and provided the sample for GC–MS analysis.

The resultant derivative was analyzed by GC–MS on a JMS-T100GCV "AccuTOF GCv 4G" gas chromatograph-high resolution time-of-flight mass spectrometer (JEOL, Japan) equipped with a source of ions for electron ionization (EI), using a DB-WAX capillary column (0.25 mm × 0.25 μm × 30 m) (Agilent, CA). The GC conditions were as follows: split injection mode (10 : 1); 1 μL injection; injection port temperature 230°C; and column oven temperature programmed from 50 to 230°C at 10°C/min. The MS conditions were as follows: electron ionization mode (EI+, ionization energy 70 eV, ionization current 300 μA); ion source temperature 280°C, and m/z range 29–800. The derivatives of monosaccharides as an internal standard were also prepared and followed by analysis. The identification of each peak was confirmed by comparing its retention time and mass spectrum profile with those of the standard samples.

Results and Discussion

1. Isolation of lactic acid bacteria (LAB) from Thailand tropical fruits

Table 1 is a list of the LAB candidates isolated from the Thailand tropical fruits such as pine apple and lychee *etc*. Thirty-seven kinds of the tropical fruits, which have been collected from 16 provinces in Thailand, were used for isolation of plant-derived LAB. Finally, 327 strains of LAB candidates were stocked as a plant-derived library. One hundred-twenty in the 327 LAB candidates grew vigorously at 45°C, but not at 50°C. To characterize the LAB candidates physiologically and morphologically, it was examined that whether they are gram-positive or negative, rod or coccus shape, spore formation or not, together with the absence of catalase. Judging from the growth curve of 120 strains, 28 strains cultivated at 45°C reached to exponential phase of growth without time lag. The living cell number in the culture broth of each strain was between 1×10^8–10^9 colony-forming unit (cfu) /mL. Thus, the 28 strains were judged as thermophile LAB.

Table 1. Number of LAB candidates isolated from the Thailand tropical fruits.

Source	30°C	37°C	45°C	Source	30°C	37°C	45°C
Rambutan	3	4	2	Banana	4	6	6
Lychee	6	2	7	Guava	3	2	2
Longkang	4	3	3	Spodilla	4	0	0
Jew's plum	5	4	5	Pomelo	2	1	0
Sugar palm	3	3	4	Watermelon	1	1	5
Papaya	2	3	4	Muskmelon	1	0	0
Satol	2	3	9	Longan	0	1	3
Pine Apple	8	9	10	Pomegranate	0	3	1
Sugar Apple	6	5	8	Madagascar plum	2	0	1
Dragon fruit	3	2	1	Jujube	2	9	5
Salak	1	1	5	Lantern Tree	0	0	3
Mak mouv	2	2	0	Jambolan Plum	0	2	0
Nom maew	4	4	0	Marium Plum	0	2	0
Emblic mylablan	2	0	4	Tamarind	0	3	0
Kiffir lime	3	4	0	Madras thorn	4	4	7
Star fruit	8	4	5	Rose apple	2	4	4
Durian	4	3	8	Star gooseberry	0	2	0
Mango	6	7	5	Mongosteen	0	0	1
Jack fruit	3	4	2				
				Total	100	107	120

2. Identification of thermophile LAB candidates

Identification of the thermophile LAB candidates was done by determining the nucleotide sequence of the 16S rRNA-encoding gene and comparing with those in the DNA data bank. Table 2

lists the genus and species names of 28 strains which could grow at 45°C. As shown in this table, these strains were classified into three kinds of *Enterococcus, Pediococcus*, or *Lactobacillus*. Seven species of LAB, which display thermo-tolerant characteristics, were isolated from the fruit samples and identified as *Enterococcus* (*E.*) *faecium*, *E. duran, E. faecalis, E. hirae, Pediococcus* (*P.*) *acidilactici, P. loli, P. pentosaceus* and *Lb. amylovorus.* The present study suggests that no relationships are found between the LAB species and a kind of the fruit.

Table 2. Numbers of thermophile LAB isolates.

Species	Number
E. faecium	9
E. durans	2
E. faecalis	1
E. hirae	1
P. acidilactici	9
P. loli	3
P. pentosaceus	1
Lb. amylovorus	2
Total	**28**

According to the reports of Müll *et al.* (1996) and Doi *et al.* (2013), the genus *Enterococcus, Pediococcus*, and *Lactobacillus* can be generally found from plant sources. The plant-derived LAB stains may have high ability to live in harsh environment than the animal-derived ones.

3. Optimum temperature for the growth of *P. pentosaceus* LY45

Since *P. pentosaceus* LY45 can grow vigorously at 45°C, the strain was judged as a thermopile LAB strain. As described in the legend of the Fig. 4, the LY45 strain isolated from lychee was cultured in MRS medium for 72 h under the temperature from 28 to 55°C. In the experiment, the living cell number and pH in the culture broth were monitored, demonstrating that the strain grew vigorously at 45°C, and the lag phase of growth was shortened to less than 6 h. The living cell number was 1×10^{10} cfu/mL after the 6 h-incubation. However, according to decrease of pH in the culture broth, the cell growth was decreased. The living cell number was also decreased rapidly after reached to the highest peak of growth. The growth curve at 37°C was almost the same as that at 45°C, but the cell number at 37°C until the incubation for 42 h was slowly decreased than that at 45°C. The LY45 strain could grow even at 55°C, although the cell growth was obviously slow. The cell growth of the strain was surely observed even when cultured at 50°C. The bacterial cell number at 50°C increased near 10 to the power 2 when compared to initial cell number, and was maintained after the 36 h-incubation. The growth profile suggests that *P. pentosaceus* LY45 is a thermophile strain because of rapid growth even at 50°C.

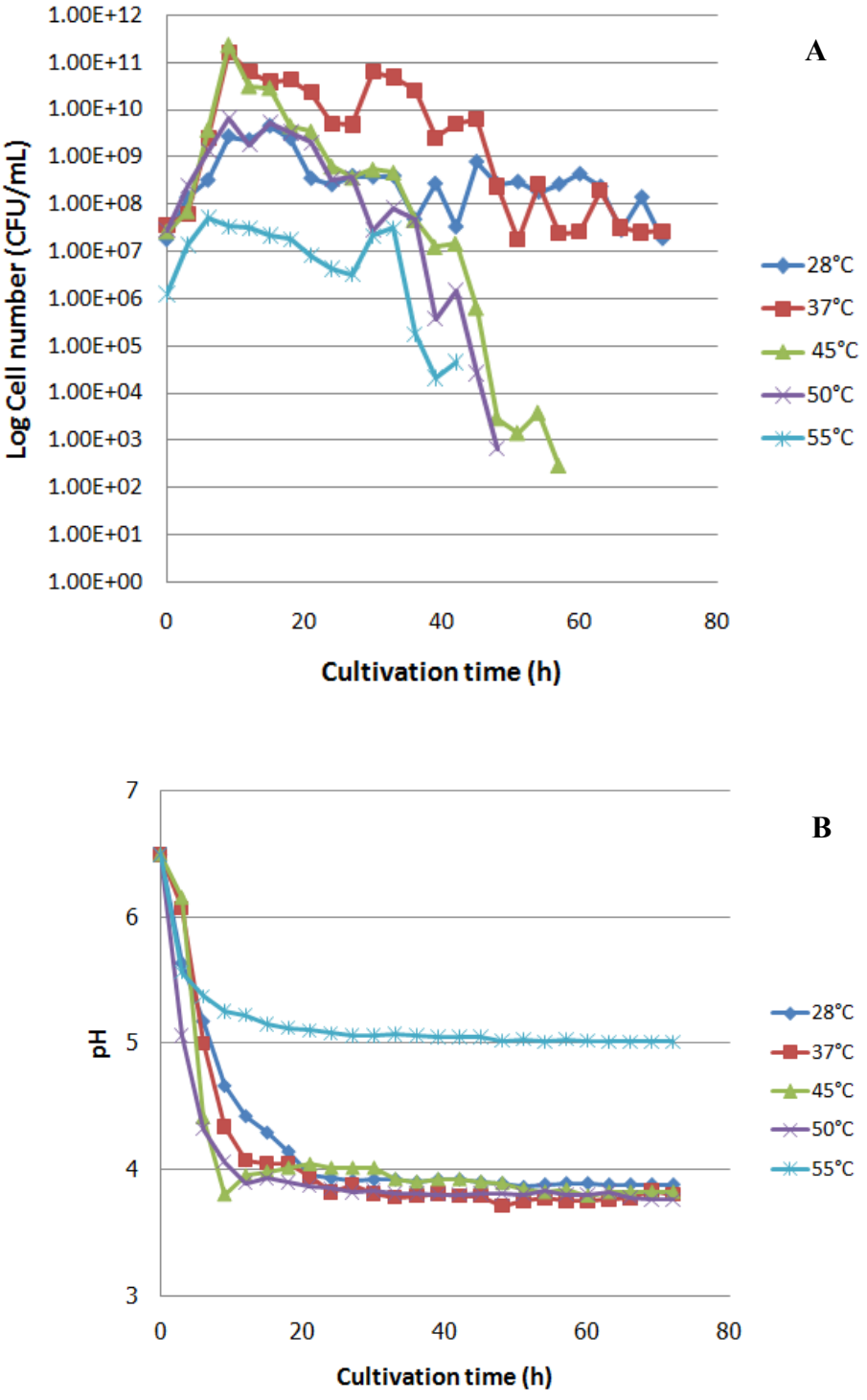

Fig. 4. Thermo-tolerance (A) and acid production (B) of *P. pentosaceus* LY45 at the given temperature.

With regard to *Lb. amylovorus* PY45, Fig. 5 shows that the strain cultured at different temperature can grow rapidly at 45 °C. The lag phase of growth was shortened to less than 6 h, as same as *P. pentosaceus* LY45. The survival cell number reached to 1×10^{11} cfu/mL after the 6 h-incubation. The growth rate at 37°C was almost the same as that at 45°C, but the cell number showed slowly

decreased until 60 h. Although the LY45 strain could grow very slowly at 55°C, the cell number scarcely detected after incubated for 24 h.

As described above, *P. pentosaceus* LY45 and *Lb. amylovorus* PY45 showed the same growth profile, but the former strain shows more rapid growth at every condition. The optimal temperature of both LAB strains for growth is 45°C.

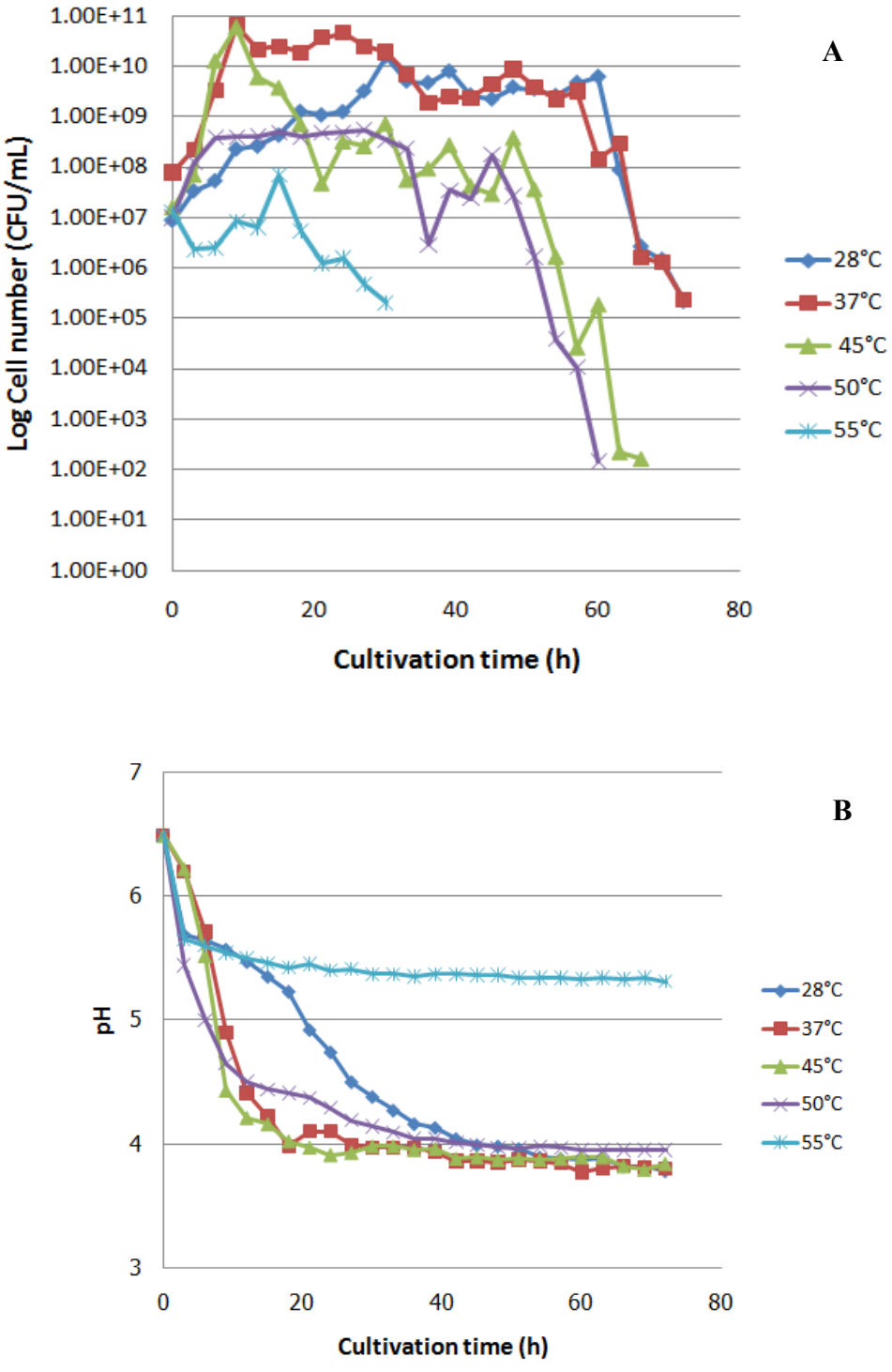

Fig. 5. Thermo-tolerance (A) and acid production (B) of *Lb. amylovorus* PY45 at the given temperature.

5. EPS production by the thermophile LAB

5.1. Effect of carbon source on EPS production by the thermophile LAB

In the present study, it was investigated that the EPS productivity of the PY45 and LY45 strains is altered by the addition of sugar as a carbon source into the culture medium at the given temperature. Figure 6 shows the EPS productivity at 45°C by each strain at a 40 mL culture scale. To clarify whether additional sugar as a carbon source improves the EPS productivity, in the resent study, the productivity in the modified-SDM medium containing 2 (w/v) % glucose and supplemented with 1 (w/v) % of mannose, sucrose, maltose, lactose, fructose, or galactose instead of glucose was measured. As the result, it was shown that the EPS productivity by the LY45 strain after the 2-days cultivation was improved by the addition of maltose to 1.2-fold. On the other hand, fructose slightly improved (1.1 folds) the yield of EPS by the PY45 strain after the 5-days-cultivation. Thus, the addition of maltose and fructose was effective as carbon source for EPS production by *P. pentosaceus* LY45 and *Lb. amylovorus* PY45, respectively.

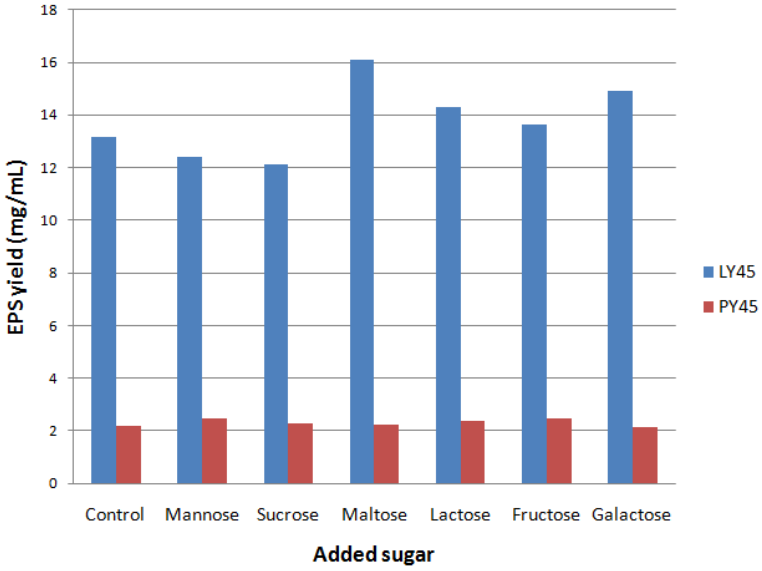

Fig. 6. EPS production by *P. pentosaceus* LY45 and *Lb. amylovorus* PY45 cultured in different carbon source; Glucose (control), Mannose, Sucrose, Maltose, Lactose, Fructose, and Galactose.

5.2. Influence of different concentrations of sugar and cultivation time on EPS production

The concentration of the added sugar to the EPS production by the LY45 and PY45 strains was investigated to find sugar effective to the high production. As described in the section 5.1, kind of sugar effective to high productivity of EPS by each strain was preliminary determined. The effect of the initial concentration of sugar, which is added to the modified-SDM at a 40 mL culture scale, was also determined. As shown in Table 3, the addition of maltose improved the EPS productivity on the LY45 strain. The yield of the EPS production was increased by the addition of maltose up to 5 (w/v) %. However, since excess amount of the added sugar was considered to induce the Maillard

Table 3. Optimal sugar concentration on EPS production by *P. pentosaceus* LY45 and *Lb. amylovorus* PY45.

LY45	EPS yield (mg)		PY45	EPS yield (mg)	
Maltose (%)	45 mL	2 L	Fructose (%)	45 mL	5 L
0	0.78		0	0.23	
1	0.92		1	0.26	34.1
2	1.12	45.9	2	0.23	
4	1.42		4	0.22	

reaction. Therefore, in the present study, the sugar concentration supplemented to the culture medium was decided to be 2 (w/v) % (data not shown). On the other hand, the adding of fructose increased slightly the EPS production by the PY45 strain, however, the addition of fructose over 2 (w/v) % decreased the yield of EPS. In the case of the PY45 strain, the suitable concentration of the added fructose seemed to be 1 (w/v) %. The yield of EPS from each strain decreased gradually with the length of cultivation period.

As a following step, the author expected the high production of the EPS by using a modified-SDM supplemented with the given sugar at a flask scale (Table 3). When the LY45 strain was cultured in 2 L of the medium containing 2 (w/v) % maltose, the yield of EPS purified from the 48 h-culture was 22.5 mg/L. On the other hand, the yield of EPS purified from the culture broth (5 L) of the PY45 strain was only 6.8 mg/L. In this case, the cultivation for 120 h was done using modified-SDM medium supplemented with 2 (w/v) % fructose. The cell viabilities of the LY45 and PY45 strains were 1.8×10^8 and 3.7×10^5 cfu/mL, respectively. In fact, the growth of the PY45 strain was quite weak in the modified-SDM (data not shown).

The optimum growth of *P. pentosaceus* LY45 is 45–50°C and produced large amount of EPS when compared with a mesophile strain *P. pentosaceus* LP28 (data not show). Thus, the kinds of monosaccharide contained in EPS produced by the thermophile LAB strain, which is synthesized at high temperature, is of interest. On the other hand, *Lb. amylovorus* PY45 produced small amounts of EPS (6.8 mg/L) when compared with *Lb. amylovorus* DU-21which produces highest amount (18.71 g/L) of EPS (Jung *et al.*, 2008). In this case, the main difference of the EPS production might be generated by differences of culture medium and the purification method of EPS. In fact, the EPS productivity of *Lb. amylovorus* DU-21 cultured in MRS broth was low. Interestingly, *Lb. amylovorus* PY45 can utilize starch as a carbon source for the growth, suggesting that the strain may be used to produce EPS from a low-cost carbon source such as biomass.

6. Molecular elucidation of EPS

The anion-exchange column chromatography profile indicates that each EPS purified from the culture broth of the *P. pentosaceus* LY45 and *Lb. amylovorus* PY45 strains contains neutral and acidic EPSs, but the content ratio between the two EPS types is quite different. The LY45-derived EPS contains mainly neutral EPSs (about a 4.8-fold higher yield than that of the acidic EPS), whereas the PY45-derived EPS is composed of both neutral and acidic EPSs with a 1.7:1 ratio (Fig. 7).

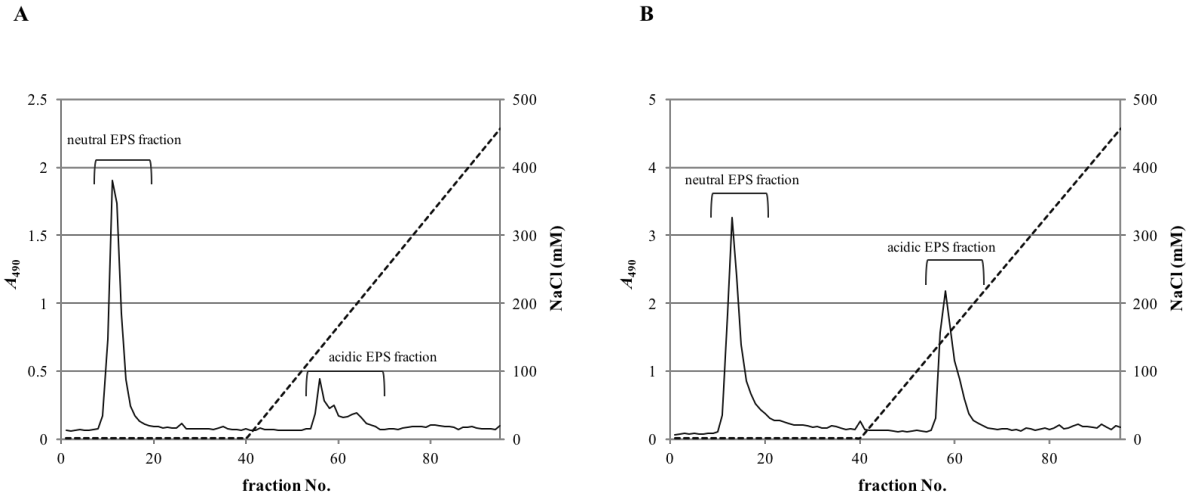

Fig. 7. Fractionation profiles of the EPS purified from *P. pentosaceus* LY45 (A) and *Lb. amylovorus* PY45 (B) by anion-exchange chromatography using a TOYOPEARL DEAE-650M column. The EPS eluted in each fraction was monitored at 490 nm by a phenol-sulfate method (solid line). A dashed line indicates the NaCl concentration in the eluate.

Furthermore, the neutral and acidic EPS of each strain were analyzed by GC/MS using standard sugar. The GC–MS profile of the LY45-derivedd EPS indicates that the neutral EPS consists of glucose and mannose as monosaccharides. On the other hand, the acidic EPS contains mannose, glucose, and galactose (Fig. 8A). The EPS produced by *Lb. amylovorus* PY45 also divided into the neutral and acidic EPS. These EPSs are composed of mainly mannose, together with a small amount of glucose. (Fig. 8B).

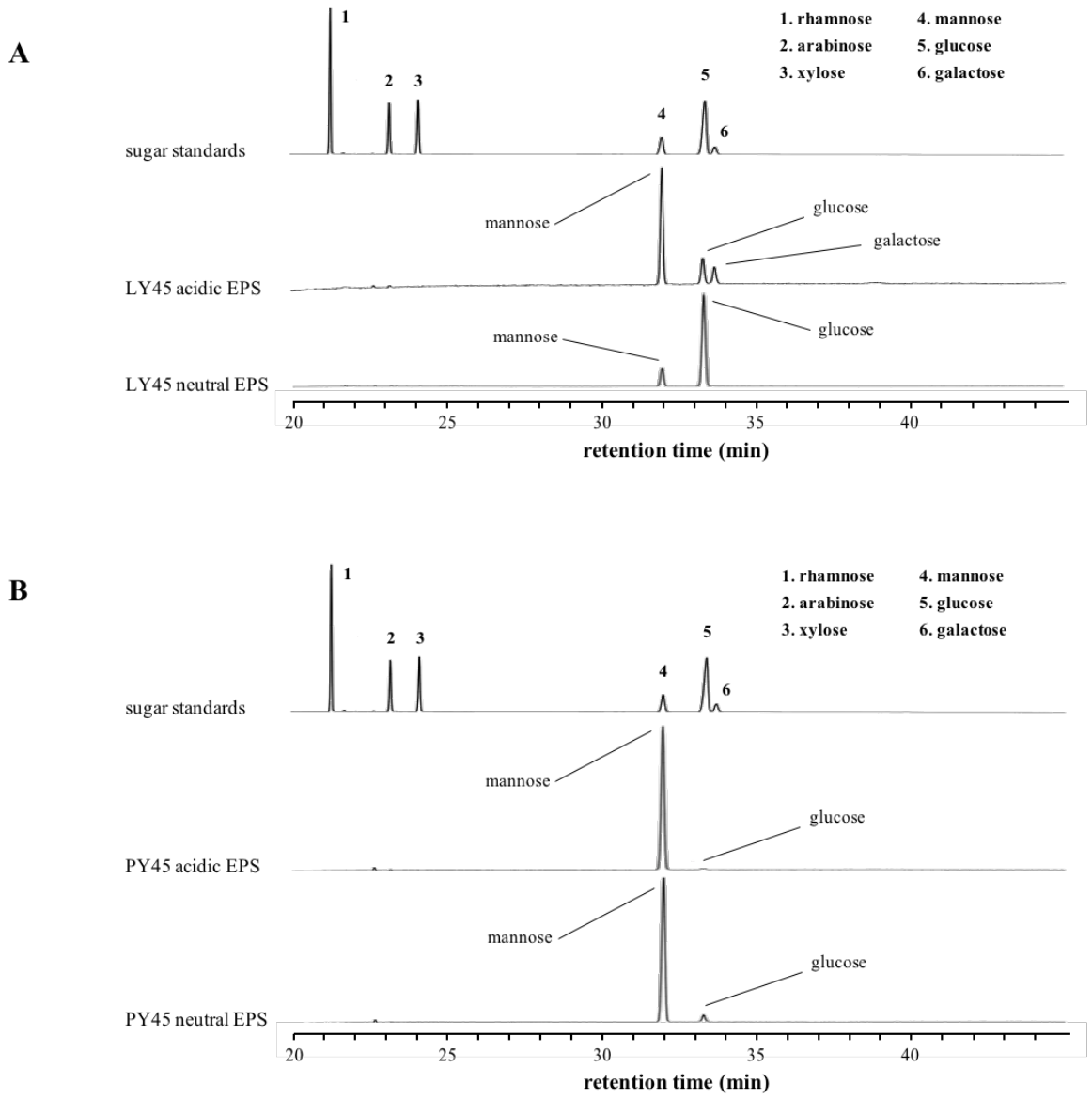

Fig. 8. The GC–MS profiles of EPS purified from *P. pentosaceus* LY45 (A) and *Lb. amylovorus* PY45 (B). The EPS component monosaccharides are detected as an alditol acetate derivative. The identity of each peak was confirmed by retention time and mass spectrometry.

Conclusion

In the present study, 327 strains of LAB were isolated from tropical Thailand fruits. Twenty-eight strains in 327 ones, which were judged as thermophile LAB, were classified into 3 genera and 7 species: that is, *E. faecium* (9 species), *E. durans* (2 species), *E. faecalis* (1 species), *E. hirae* (1 species), *P. acidilactici* (9 species), *P. loli* (3 species), *P. pentosaceus* (1 species), and *L. amylovorus* (2 species).

P. pentosaceus LY45 and *L. amylovorus* PY45, which are thermophile LAB strains, produce EPS. Optimum temperature of both strains for growth is 45 to 50°C in MRS broth medium.

The optimum carbon source for EPS production by each of two strains was investigated. As the result, it was shown that the addition of 2 (w/v) % maltose and 1 (w/v) % fructose to the modified-SDM medium was effective for the EPS production by the LY45 and PY45 strains, respectively. EPS amount of 22.9 and 6.8 mg/mL was produced by strain LY45 and PY45, respectively.

EPS purified from the LY45 and PY45 strains consist of neutral and acidic EPSs, but the ratio between the two types of EPS is quite different: the LY45-derived EPS contains mainly neutral EPSs, whereas the PY45-derived EPS contains both of neutral and acidic EPSs. The neutral EPS from the LY45 strain constitutes of glucose and mannose as monosaccharides. On the other hand, the acidic EPS contains mannose, glucose, and galactose.

The neutral and acidic EPSs from the PY45-derived EPS are composed of mainly mannose, with small amount of glucose.

Since *P. pentosaceus* LY45 is a thermophile LAB which produces high amount of EPS, the strain will be expected as an industrially important LAB strain.

Although *Lb. amylovorus* PY45 produced fewer amount of EPS, the strain can utilize starch as a carbon source for growth. Therefore, the strain may produce EPS from low cost carbon source such as biomass.

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