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Characterization of Yeast and Bacterial Type Strains with Food and Agricultural Applications by MALDI-TOF Mass Spectrometry Biotyping

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Various microorganisms play important roles in food fermentation, food spoilage, and agriculture. In this study, the biotype of 54 yeast and bacterial strains having high potential for utilization in food and agriculture, including *Candida* spp., *Lactobacillus* spp., and *Acetobacter* spp., were characterized by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS). This characterization using a fast and robust method provides much-needed information on the selected microorganisms and will facilitate effective usage of these strains in various applications. Importantly, the unique protein profile of each microbial species obtained from this study was used to create a database of fingerprints from these species. The database was validated using microbial strains of the same species by comparing the mass spectra with the created database through pattern matching. The created reference database provides crucial information and is useful for further utilization of a large number of valuable microorganisms relevant to food and agriculture.

Keywords: **MALDI-TOF Mass Spectrometry, food, agriculture, biotyping, microorganisms**

Introduction

Precise characterization and identification of microbial strains wtih a reliable and fast method of biotyping are highly beneficial. Of particular interest, biotyping of microbial strains employed in food and agricultural applications is vital for the economy, since the food and agricultural industries generate several billion dollars a year in economic values, and they greatly affect people's way of life. For example, accurate biotyping of microbial strains involved in food processing can be used for fast strain identification in food fermentation or for quality control of microbial strain used in industrial purpose, especially that food quality and safety are increasingly important concerns for human health with the globalization of food trade and the complexity of food ingredients. In addition, fermentation is a popular ancient technique for food processing and preservation of natural food ingredients. Most fermented products have long-tern stability and are of low cost relative to other foods with similar characteristics [1]. Especially, many microorganisms encompassing major species of bacteria and fungi play an important role in food fermentation and have been associated with health benefits, ranging from better digestion to stronger immunity [2]. Moreover, they provide favorable flavor, texture, and color to the food. Thus, bacteria and yeasts acting as probiotics in certain fermented food are of great interest in food, beverage,

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and pharmaceutical industries. Despite this interest and the potential public health benefits of these foods, there is still considerable confusion regarding the types and roles of these microbes during the fermentation and their influence on the gut microbiome. In particular, the characterization of microorganisms in traditional fermented food has been largely underperformed, leading to incomplete information of the microorganisms, even though the fermentation most likely involves many microorganisms with a symbiotic relationship. For example, even though kefir (fermented milk beverage) has been shown to contain a complex symbiosis between yeast and bacteria [3], the activity of each organism and how they interact have not been studied in depth. Another concern for the fermented products is the possibility of food contamination with other microorganisms that can cause food spoilage or food poisoning. Rapid and accurate detection of contaminated organisms is of utmost importance to alleviate health and economic problems associated with food spoilage, as food product contamination presents a problem of global concern with possibilities of foodborne diseases and food wasting. In addition, informative biotyping of microorganisms involved in biofertilizer and bioremediation will be most useful for regional agriculture. As microorganisms are essential for decomposing organic matter and recycling old plant material, an advanced understanding of microbial activities in agricultural settings and plant-microorganism relationship can be used to enhance agricultural sustainability.

Biotyping (intraspecific discrimination) has been an important aspect of the identification of microorganisms of interest. Most of the potential applications rely on distinguishing microorganisms at the level of species or strains for the discovery of the effective strain, maintenance of the functional strain, detection of contaminants, verification of the desired strain, and protection of patented strains. Previously, the gold standard for biotyping includes morphological, biochemical testing analysis, along with molecular identification at the DNA and RNA levels, such as PCR amplification of repeated DNA regions and restriction fragment length polymorphism (RFLP). The abovementioned methods are reliable in identifying and characterizing strains of interest. However, those processes remain time-consuming and labor-intensive with the requirement of expertise and high cost. Thus, accurate and reliable methods for the early detection and rapid biotyping of industrial production strains, clinical pathogens, contaminated species, or quality control in the industrial process to monitor batch quality are considered to be of high advantage.

Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a relatively fast, reliable, and cost-effective tool for the identification and classification of microorganisms [4, 5]. The method was first employed mainly for bacterial analysis, particularly for clinical diagnostics and protein characterization [6]. Currently, it has been expanded to the analysis of fungi in clinical medicine, biotechnology, and industry [7, 8]. It operates with unique mass spectrometric profiles (fingerprints) acquired by the desorption of specific peptide biomarkers from the cell of microorganisms [5]. A characteristic mass-to-charge (m/z) spectrum called protein/peptide mass fingerprint (PMF) is then generated for analytes in the sample. Finally, the m/z peaks are evaluated in accordance with the available reference database, and the identity of microbial species is revealed.

Currently, the most utilized protein/peptide fingerprint databases (such as Bruker Daltonics' MALDI Biotyper IVD library, VITEK MS IVD library, or Shimadzu's SARAMIS library) are not extensive, since they mostly focus on pathogenic microorganisms found in medical diagnostics. Thus, there is a large gap in the database lacking characteristic information on large numbers of microorganisms. Increasing the wealth of information in the database to include more microorganisms is thus required, because it will promote further utilization of those microorganisms in more diverse applications. This created reference database will expand the commercially available database already existing and facilitate the accurate identification of a large number of valuable microorganisms, especially in food and agricultural applications.

Materials and Methods

Yeast and bacterial strains

Yeast and bacterial strains used in this study were obtained from Thailand Bioresource Research Center (TBRC). Yeast strains were maintained on YPD (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) agar

plates, whereas bacterial strains were maintained on LB (5 g/l yeast extract, 10 g/l peptone, and 5 g/l NaCl), MRS (10 g/l peptone, 10 g/l meat extract, 5 g/l yeast extract, 10 g/l glucose, 1 g/l Tween-80, 2 g/l K_2HPO_4, 5 g/l sodium acetate, 2 g/l ammonium citrate, 0.2 g $MgSO_4$, and 0.05 g/l $MnSO_4$), or GECA (25 g/l glucose, 5 ml/l ethanol, 3 g/l yeast extract, 5 g/l peptone, and 7 g/l $CaCO_3$) agar plates. All yeast strains were cultivated on YPD agar medium at 30℃ for 48 h. Bacterial strains were cultivated on LB or MRS agar medium at 37℃ for 48 h.

Peptide extraction by ethanol/formic acid

Peptide extraction was performed *via* ethanol/formic acid extraction procedure as described by Pavlovic *et al.* [9] with minor modifications. Briefly, microbial cells were collected from the agar plate or culture broth and suspended in 1 ml distilled water. Cell concentration was measured by absorbance at 600 nm (OD_{600}) using a UV-visible spectrophotometer (UV-2502, Labomed, Inc., USA). A cell population of approximately 1×10^8 cells were obtained and transferred to an Eppendorf tube. Yeast or bacterial cells were then harvested by centrifugation at 13,000 × *g* for 2 min. The cell pellet was resuspended in 300 µl of distilled water by vigorous vortexing, and 900 µl of absolute ethanol was added to the cell suspension. The suspension was mixed and centrifuged at 13,000 × *g* for 2 min, and the supernatant was then discarded. Centrifugation was repeated for the complete remove of all residual liquid. The ethanol-exposed cell pellet was air-dried in a laminar-flow hood at room temperature for 5 min. For peptide extraction, 50 µl of 70% formic acid (v/v) was added to the pellet, and the contents were thoroughly mixed by vortexing for 2 min. Thereafter, 50 µl of 100% acetonitrile was added to the solution and mixed thoroughly by vortexing. The suspension was centrifuged at 13,000 × *g* for 2 min, and the supernatant containing peptide extracts will then be collected.

MALDI-TOF MS

Protein extract (1 µl) was poured to a specified spot on a ground-steel MALDI target plate and air-dried in a laminar-flow hood at room temperature for 5 min. The applied sample was then overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid (HCCA) solution and air-dried. When the sample was dry, the target plate was analyzed by MALDI-TOF MS. All samples were prepared in triplicate. For microbial identification, eight spots per sample were used. For database construction, 29 spots were applied on the target plate.

Protein samples were subjected to Ultraflex III MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany). Mass spectra were recorded in the linear mode in the range of m/z 2000–20000 for peptides. Spectra accumulation of at least 500 shots for each spot was performed to obtain high-quality data from different optimal spot positions. Mass spectra were calibrated using the exact mass values of defined protein mixtures according to the manufacturer's instruction. Data was processed (i.e., smoothing, baseline subtraction, normalization, and peak picking) and analyzed using the FlexAnalysis 3.0 software provided by Bruker Daltonics. To validate the authenticity of mass spectral data generated by the MALDI-TOF MS instrument, each batch of samples contained the Bruker bacterial standard (Bruker). The Bruker Daltonics MALDI Biotyper software applies pattern matching to compare unknown mass spectra with reference data stored in the Bruker Daltonics mass spectral database to routinely identify microorganisms.

Principle component analysis (PCA)

PCA was employed to statistically evaluate MALDI-TOF mass spectra. PCA is a mathematical formula for reducing the dimensionality of multivariate data, while preserving most of the variance, with calculations performed using a software package available commercially (Bruker). All spectra were normalized before PCA analysis. These models allowed the determination of the similarities or dissimilarities between samples.

Acquisition of in-house database

For each microbial strain, protein extracts were spotted on the target plate at 29 spots. After m/z data peaks are obtained, the data peaks from 29 spots were collected and analyzed using the ClinProTools software provided by the manufacturer of the MALDI-TOF MS machine and used as representative m/z peaks for subsequent comparisons with unknown samples. A representative PMF contains the average mass and the average intensity of the selected peaks (representing the most reproducible and typical for a certain strain), as

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well as the frequency of the peaks in multiple measurements. The peak matrix was exported for further analysis using software tools to generate dataset with standard formats, such as mzXML, in order to facilitate data exploration and utilization. The database was generated and maintained using the sMOL Explorer tool [10], enabling it to be openly accessible.

Results

In this work, MALDI-TOF MS approach was used for biotyping of bacteria and yeast type strains involved in food and agriculture. Bacterial strains in the study include both gram-positive (e.g., *Lactobacillus pentosus*, *Komagataeibacter swingsii*, and *Pediococcus siamensis*) and gram-negative (e.g., *Kozakia baliensis*, *Gluconacetobacter liquefaciens*, and *Komagataeibacter hansenii*) strains. Yeast strains used in the study encompassed many species, such as *Candida* sp., *Hanseniaspora* sp., *Lipomyces* sp., and *Wickerhamomyces* sp. In order to obtain more consistent results for all microbial strains, similar numbers of cells, approximately 10^8 cells, were used.

Proteins were extracted from bacterial and yeast strains with the ethanol/formic acid method. A range of proteins up to >116 kDa were found when separated on SDS-PAGE and stained with Coomassie Blue staining (data not shown). The extracted proteins were spotted onto the target plate with the HCCA matrix as four technical replicates and analyzed by MALDI-TOF MS in order to generate protein mass fingerprints (PMF), which are unique for each microbial species. Concurrently for some strains, direct PMF acquisition from the intact cells was also used to confirm the result. All microbial strains exhibited spectra with a substantial number of peaks in the mass range of 2,000 to 20,000 Da. These results revealed that most of the resulting PMF spectra were generated effectively. Each microbial strain exhibited a unique and representative PMF spectrum differentiable from that of other strains, thereby confirming the specificity of the MALDI-TOF MS identification.

The obtained PMFs for 24 bacterial strains and 30 yeast strains were then made into our in-house MALDI-TOF MS database. These strains encompassed bacterial and yeast species important for food and agriculture that did not yet contain PMFs in Bruker's database. They included *Lactobacillus* spp., *Acetobacter* spp., *Gluconacetobacter* spp., *Komagataeibacter* spp., *Candida* spp., *Hanseniaspora* spp., *Lipomyces* spp., and *Wickerhamomyces* spp. (Supplementary Table 1). For each strain, a PMF was generated through the accumulation of ≥5,000 laser shots from 29 technical replicates of the same spectrum (Figs. 1 and 2). PCA was employed to statistically evaluate MALDI-TOF mass spectra to collect spectra with 95% similarity. The spectra were then used to generate representative PMF for each strain and stored as in-house database (Fig. 3).

In order to establish the confidence of the created database, the resulting mass spectra in the created database were used as reference spectra for matching with PMFs generated by other microbial strains of the same species. It was found that when the spectra were compared, the match between PMF spectra of the same species generated highly similar score, according to the analysis settings from Bruker's system. The scores were in the range of 1.98–2.39 (Supplementary Table 2), demonstrating the high specificity and reliability of the database to distinguish microbial species. In order to further test the suitability of the in-house database, additional PMFs in the in-house database were generated from strains that already have PMFs in the commercially available Bruker's database, such as *Candida catenulata*, *G. liquefaciens*, *L.*, *Lactobacillus versmoldensis*, and *Wickerhamomyces anomalus*. The resulting PMFs in the in-house database were compared with those in the available Bruker's database by matching an independent sample to these two databases. The results revealed that, for these microbial strains, the in-house database was comparable to Bruker's database, as the matching scores with the two databases were quite similar in the range of 1.96 ± 2.34 (Supplementary Table 3). Therefore, our in-house database has high potential for further usage for microbial biotyping.

The PMF spectra for these stains were additionally converted into mzXML format and made into a publicly available database using the sMOL Explorer tool. This web-based, in-house database can be accessed at http://web3a/smol_explorer. Our database will be made available for researchers or for people interested in the utilization of these microorganisms in food or agriculture applications.

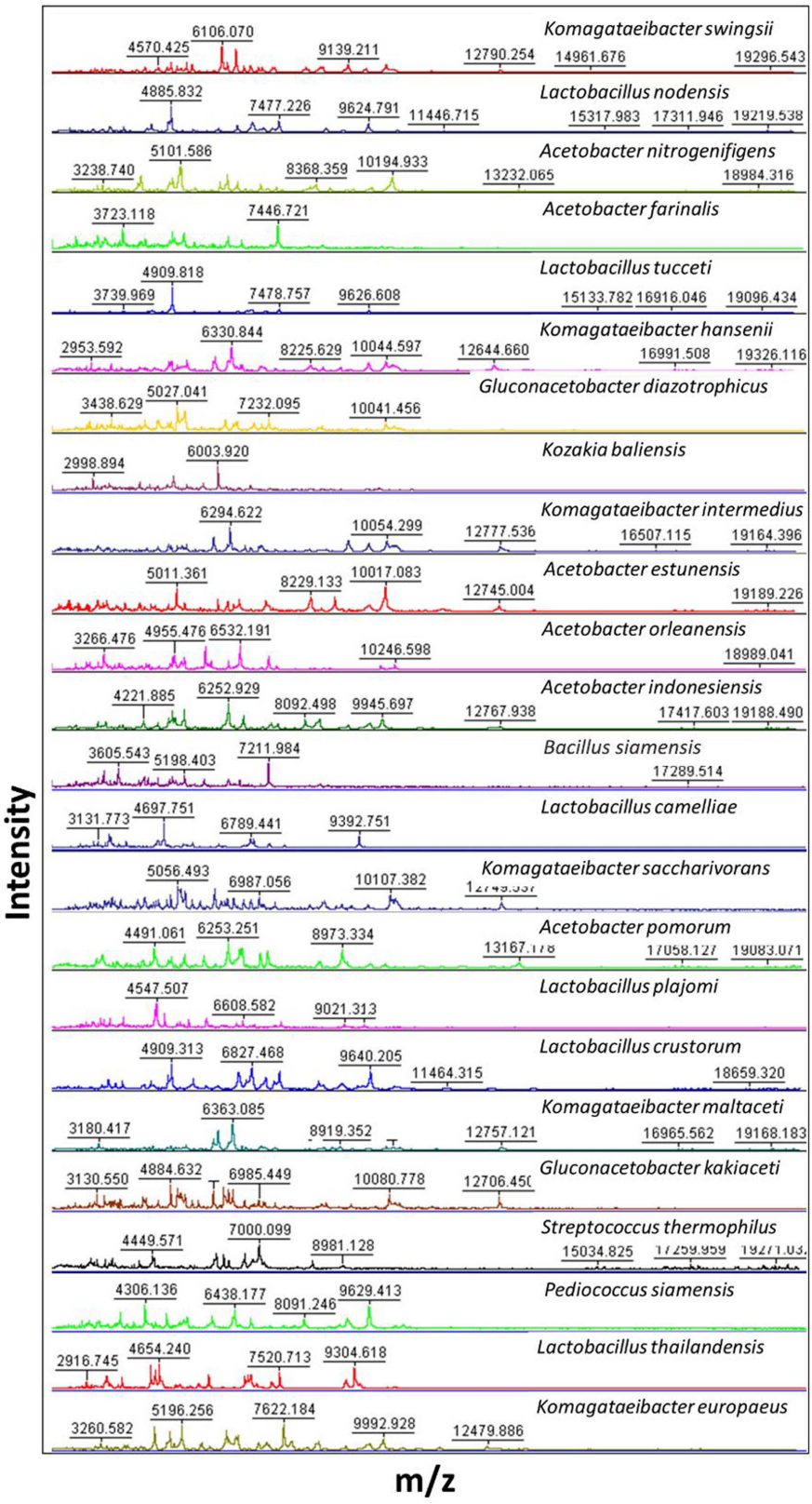

Fig. 1. Representative spectra of 28 bacterial strains with food and agricultural applications used in this study.

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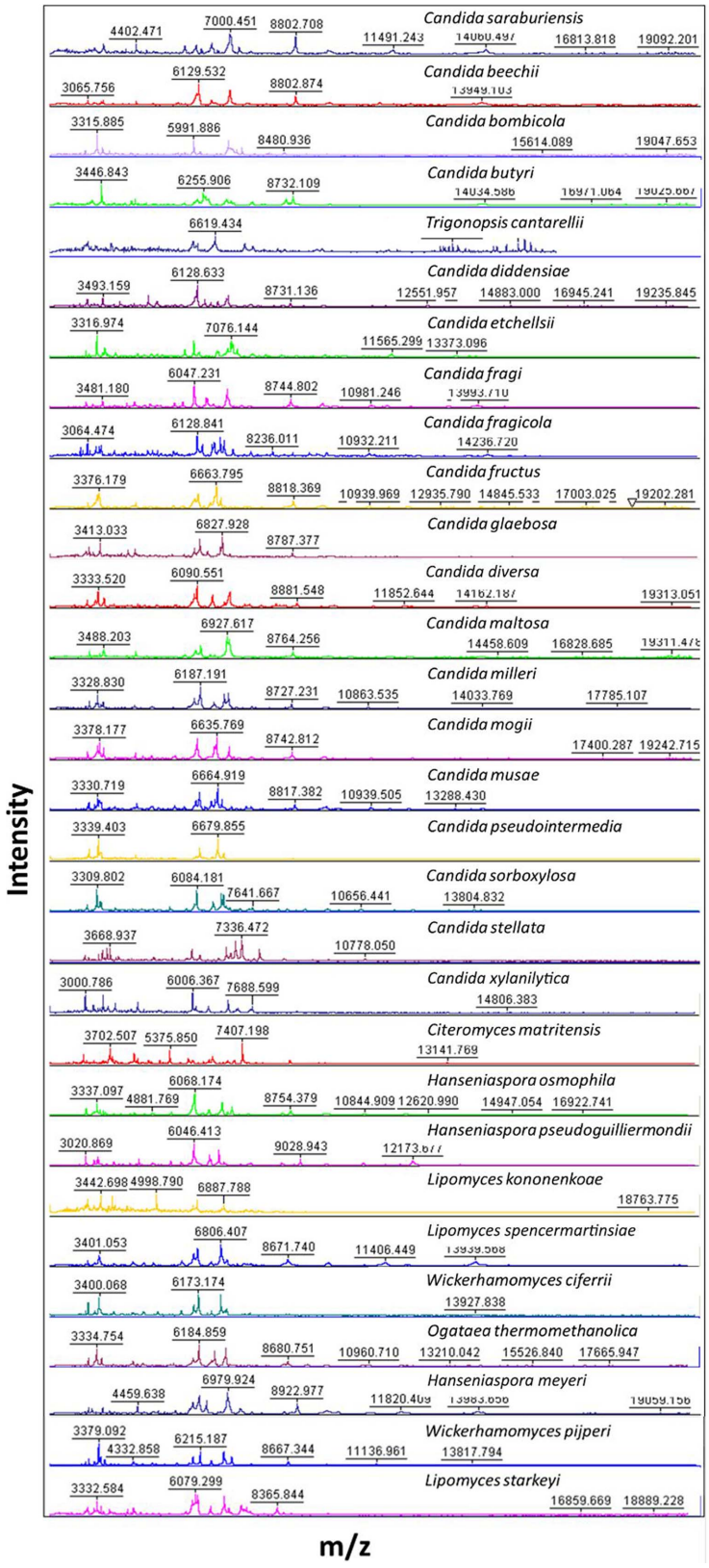

Fig. 2. Representative spectra of 32 yeast strains with food and agricultural applications used in this study.

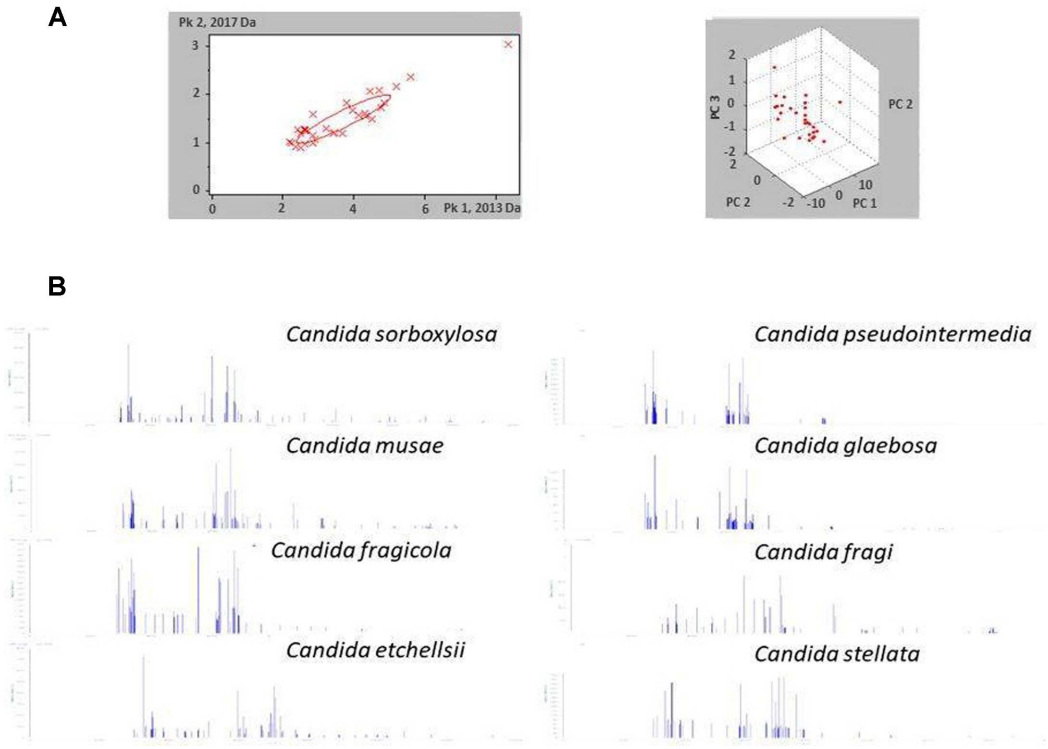

Fig. 3. In-house database acquisition. (A) PCA analysis was conducted to group 29 technical replicates, and data with 95% similarity were further used to obtain representative spectrum for each strain during in-house database construction. (B) Examples of unique protein mass fingerprints of bacterial and yeast strains deposited in the web-based, in-house database accessible at http://web3a/smol_explorer

Discussion

MALDI-TOF MS has been implemented in microbiology as a rapid and effective identification technique [11]. Our results revealed the efficacy of profiling of protein spectra from the cell extract or intact cells over the mass range of 2,000 to 20,000 m/z, which represents mainly small ribosomal proteins along with a few housekeeping proteins. Mass spectrometry analysis of these proteins forms the basis for a very sensitive biotyping assay since the proteins are present in great abundance (up to 60–70% dry weight in *E. coli* and *Bacilli*). Each microbial species exhibit a unique protein profile, resulting in identifiable PMF [12].

The present work has confirmed that MALDI-TOF MS may represent an efficient and inexpensive alternative tool for accurate identification of microorganisms. After standard cultivation steps, sample processing and analysis procedures are both rapid (<1 h) and labor-effective relative to molecular techniques, such as PCR and rRNA hybridization. More importantly, data analyses are robust, simple, and rapid compared with morphological and molecular methods [13]. The analyses can additionally be scaled up, such as acquisition performed on 384 sample MALDI target plates, lending to the high-throughput capability. The cost-effective nature (low consumables and labor requirements) of the MALDI-TOF MS platform offers another advantage over traditional techniques, even if the initial price of the MALDI-TOF MS device could be expensive. The cost of bacterial identification was estimated to represent only 17–32% of the costs of conventional identification methods in the study performed by Seng *et al.* [14], which is supported by two other prospective studies by Bizzini and Greub [11] and Dhiman *et al.* [15].

Microbial biotyping with MALDI-TOF MS has revolutionized microbiology with regard to the routine identification of bacteria, archaea, and some fungi [11, 14, 16] with its economical and accurate nature. This proteomic tool was first successfully applied to routine microbial

identification in clinical settings, where the fast characterization of pathogens is of great advantage. Since then, this technique has been expanding for the identification and characterization of microorganisms in other applications [17–19]. However, the uses for those applications are still limited. The success of microbial identification employing the MALDI-TOF MS technique is dependent on the reference strains found in the mass spectral database, but the current main limiting factor of MALDI-TOF MS analysis is the small diversity of species included in the available MS spectra reference database, especially reference spectra for nonclinical microbial strains [17, 20]. Misidentification or the inability of MALDI-TOF MS to identify particular microorganisms most often resulted from the absence of a corresponding reference spectrum for that species in the database.

It has been urgently necessary for researchers to establish custom reference databases of beneficial microbial strains that are not available in the commercially available, pre-established PMF databases [21–23]. In order to expand the database and allow the rapid identification of isolates, such as these additional species with food and agricultural applications, PMF reference spectra of these species should be created for inclusion into in-house database. This in-house database would further allow effective analysis of these species for further utilization of microbial species in a more comprehensive manner.

Considering that various microorganisms have been routinely used in food and agricultural applications, a comprehensive characterization of these microbial strains should highly facilitate the fast strain identification in the food fermentation process or in controlling the quality of microbial strain used in industrial purposes. In addition, it can allow rapid identification of microorganisms that may cause food spoilage or food poisoning as well as microorganisms involved in biofertilizer and bioremediation that are useful for regional agriculture. It is crucial to construct a PMF database to include the abovementioned microorganisms in order to accelerate spectral matching for the identification. In this study, the reference database can be readily extended and updated to include newly obtained PMFs. Storing and re-analyzing these spectra will facilitate the periodic screening of previously unidentified strains against an updated database. Microorganisms in this study include many *Lactobacillus* spp. that are producers of lactic acid and are utilized extensively for fermented food and animal feed production, as they are considered as probiotics. *Lactococcus lactis* is also a common, potential beer-spoilage bacteria and has implications in the beer industry. Other microbial strains, including *Acetobacter* spp., can convert ethanol to acetic acid in the presence of oxygen and have been isolated from industrial vinegar fermentation processes. *Acetobacter nitrogenifigens* was isolated from kombucha tea beverage and contains nitrogen fixation ability. *Komagataeibacter maltaceti* was isolated from malt vinegar production. *Citeromyces matritensis* was isolated from fermented fruits in syrup. Some *Candida* strains, such as *Candida fragi*, are common, potential spoilage species. Most importantly, some of these yeasts and bacteria are isolated locally. *Lactobacillus plajomi* was isolated from Thai traditional fermented fish, Pla Jom. *Candida saraburiensis* was isolated from digested corn cobs in Saraburi Province in Thailand and exhibited an ability to utilize xylose. Cataloging and characterizing these strains can have great impacts on food and agricultural industries, as well as the export economy. Once PMF database has been established for these species, biotyping with MALDI-TOF MS can be further used for fast and accurate strain identification in food, quality control, and spoilage detection in the future. It was observed that even for species that exhibit very closely related profiles, their PMFs can be distinguished from each other to effectively discriminate different species. The in-house database can be used successfully to identify microorganisms with the identification score of 1.98–2.39 based on Bruker's classification score. As it has been recommended by the company that a score of ≥2.00 signifies high confidence level of species identification, our results revealed the effectiveness of our in-house database in species identification. In fact, the lower threshold for species identification of ≥1.70 can also be acceptable, as shown by Normand and colleagues [24].

As mentioned above, since the commercially available databases are usually inaccessible, proprietary, and often costly for other researches to access, there is a need to construct a database platform that can share information among interested parties. In this case, data was converted from the commercially specific platform into the mzXML platform, which is a standard format for the

database. sMOL explorer was then used to allow access to the data *via* Internet. In the future, the database may be connected to other web-based databases such as MicrobeNet from CDC. This will allow easy access and sharing between interested researchers. With a plan to continuously update this reference library with more representative spectra, the continual addition of type strains to the custom database will allow for increased confidence in identifying taxonomic group members. This custom database will aid in the detection of potentially novel species within our archived microbial collection. Therefore, in the future, microbial strains with high potential in food and agricultural applications, such genera as *Debaryomyces*, *Pichia*, *Torulaspora*, *Kluyveromyces*, *Hanseniaspora*, *Rhodotorula*, *Wickerhamomyces*, *Candida*, and *Williopsis* [25], can be further characterized to expand the database to enable more effective usage of these strains in industry or research.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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