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Microbial populations, sensory, and volatile compounds profiling of local cooked rice

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Abstract

Objectives: This study is designed to investigate the microbial populations, sensory, and volatile compounds profiling of locally cooked rice stored at room temperature (30 °C) on days 0, 1, 2, and 3 (D0, D1, D2, and D3) for the determination of specific spoilage organisms (SSOs).

Materials and Methods: Microbiological, physicochemical, sensory evaluation, and volatile organic compounds (VOCs) analyses were conducted for samples of cooked rice stored at room temperature on days 0, 1, 2, and 3. SSO of cooked rice was then identified by linking present organisms with the changes occurring throughout the storage period.

Results: Microbiological analysis revealed the presence of spore-forming bacteria, yeast, and moulds, which survived the cooking process. While total viable count exceeded the microbiological limit at 6.90 log CFU/g on D3, panellists deemed cooked rice unacceptable for consumption on D2 at a total sensory score of 11.00, which indicates that D2 was the day cooked rice spoiled. Through culture-dependent and culture-independent methods, *Bacillus cereus* was identified as the sole bacteria observed throughout the storage period, confirming that the bacteria were SSOs. The VOC analysis proposed several metabolites, 3-eicosene, 1-heptadecene, hexacosane, phenol, 4,4′-(1-methylethylidene)bis-, *n*-nonadecanol-1, and cyclohexanone, as potential spoilage markers of cooked rice.

Conclusions: *B. cereus* is confirmed to be the SSO of cooked rice, in which spoilage occured after 48 h of storage at room temperature. The organoleptic rejection in cooked rice was shown to be linked to the production of various compounds by *B. cereus* which could be proposed as potential spoilage markers for cooked rice.

Keywords: Cooked rice; room temperature; specific spoilage organisms (SSOs); *B. cereus*; sensory analysis; volatile organic compounds (VOCs).

Introduction

Rice is one of the most critical food items consumed by more than half of the world's population. Placing second after maize (corn), paddy rice production reaches 755 million tons annually (Shaheen *et al.*, 2022), with Asia being its largest producer globally. Rice is rich in various nutrients such as carbohydrates, proteins, lipids, minerals and vitamins (Lina and Min, 2022). It provides approximately 21% and 19% of global human per-capita energy and protein, respectively (FAO, 2012). It also accounts for 35%–60% of calorie intake for more than 3 billion people in Asia and approximately one-third of the approximately 1.5 billion people in Africa and Latin America (Fageria *et al.*, 2003). The prediction of an increase in the population of up to 9 billion people by 2050 (FAO, 2014) shows that the increasing demand for rice is undeniable. As rice becomes more critical, it is necessary to ensure the safety of said food from spoilage for consumption.

Owing to its high nutrient content, cooked rice can be a suitable medium for the growth of microorganisms and is therefore highly perishable. However, only a small part of microbial communities is responsible for causing food spoilage, defined as specific spoilage organisms (SSOs). According to Boziaris and Parlapani (2017), these microorganisms can produce metabolites that can lead to organoleptic rejection of food, indicating spoilage. Ketones, alcohols, aldehydes, amines, organic acids and sulphides are among the compounds that cause an off-odour during the spoilage of food products such as meat and seafood (Parlapani *et al.*, 2015). An ongoing problem of cooked rice spoilage due to improper storage conditions has increased the need for SSO establishment, which can contribute to the improvement of food quality by methods of upgrading detection tools, shelf-life predictions, and preservation approaches (Macé *et al.*, 2014). In 2019 alone, various news reported hundreds of people

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becoming victims of food poisoning due to their consumption of rice dishes, such as coconut rice milk and fried rice, which are suspected to spoil (Bernama, 2019; Norazzura, 2019; Sinar, 2019). The determination of SSOs of food products involved identifying microorganisms present during spoilage occurrence in food and establishing their relationships to the development of off-odours and metabolites produced. Various SSOs studies have shown the possibility of SSO microorganisms being both culturable and unculturable under laboratory conditions (Piotrowska-Cyplik *et al.*, 2017; Yang *et al.*, 2017). This highlights the importance of conducting culture-dependent and culture-independent methods to obtain an accurate observation of SSO communities in foods. Culture-based methods can also be used for enumeration, which is crucial for determining spoilage occurrence compared to the recommended microbiological limit of bacteria in food. Sensory analysis is another vital aspect of determining SSOs, as it is utilized to establish the rejection time point of food, as shown in multiple studies (Parlapani *et al.*, 2015; Parlapani *et al.*, 2019b; Antunes-Rohling *et al.*, 2019). The rejection time point is determined when organoleptic rejection occurs, which involves the development of off-odours and off-flavours. Volatile organic compounds (VOCs) have been investigated in multiple studies as potential spoilage markers (Zhu *et al.*, 2015; Mansur *et al.*, 2019). This shows that determining the volatile compounds profile of food products can be useful for determining SSOs and as a potential spoilage marker for the products.

In comparison to the myriad studies conducted on SSOs of chicken, meat, and seafood products (Macé *et al.*, 2014; Ghollasi-Mood *et al.*, 2016), limited investigations have been conducted to determine the occurrence of spoilage in cooked rice, especially for the determination of SSOs. Therefore, this study aims to determine the SSOs of cooked rice by investigating the total sensory scores, physicochemical and microbiological analysis, microbial communities, and VOCs of cooked rice during a three-day storage period at 30 °C. Such investigations are believed to yield valuable information regarding the spoilage occurrence of cooked rice caused by SSOs.

Materials and Methods

Sample preparation (cooking of rice)

Rice grain (*Oryza sativa indica*) was purchased from a local retail grocery store (Serdang, Selangor, Malaysia). After washing the rice twice with distilled water, rice was cooked at a 2:1 water to rice ratio using a rice cooker (Phillips, Amsterdam, the Netherlands) for 20 min. The cooked rice was then cooled in a laminar flow cabinet for 5–10 min to a temperature of approximately 50–60 °C before being placed in a sterile container. The solution was subsequently stored in an incubator at 30 °C and analysed on days 0, 1, 2, and 3. The samples were stored for testing in triplicate at each time point. A temperature of 30 °C was assigned as the storage temperature in this study to imitate the impact of this improper temperature on cooked rice that always occurs during food service establishments.

Microbial enumeration

At every sampling point, 25 g of cooked rice was placed in a stomacher bag with 225 mL of 0.1% peptone water. For the enumeration of spore-forming microorganisms, the cooked rice sample was first heated in a water bath at 80 °C for 12 min before adding 0.1% peptone water. The mixture was then homogenized by a stomacher (LabBlender 400, Seward, Worthing, UK) for 1 min. Aliquots of 0.1 mL were then spread on the following growth media: Plate Count Agar (PCA; Oxoid, Basingstoke, UK), which was incubated at 37 °C for 24 h for total viable count (TVC) and Spore-former Count (SFC); Potato Dextrose Agar (PDA; Oxoid) and chloramphenicol (Calbiochem, San Diego, CA, USA) for yeast and mould count (YMC) incubated at 30 °C for 72 h.

Bacterial identification via culture-dependent and culture-independent methods using Illumina

For the culture-independent method, samples of cooked rice stored on each storage day were used for identification. For the culture-dependent method, colonies from PCA plates of TVC analysis of each storage day were used for identification. All the colonies were scraped and deposited into an Eppendorf tube containing 95% ethanol.

Extraction of DNA was conducted, proceeding with amplification of 16S rRNA V4 region using OneTaq 2X Master Mix (NEB, Ipswich, MA, USA) with primer pair 515F–806R (Walters *et al.*, 2016) that contained a partial Illumina Nextera adapter at the 5′ end. PCR was performed using the following programme: 94 °C for 30 s, 30 cycles of 94 °C for 15 s, 50 °C for 15 s, and 68 °C for 30 s. SPRI magnetic beads were used to clean the PCR products and index PCR to incorporate dual-index barcode and the remaining Illumina adapter. The index PCR products were pooled, beadpurified, and quantified using a fluorescence quantification kit (Denovix, Wilmington, Delaware, USA). The library was sequenced on an ISeq100 (Illumina, San Diego, CA, USA) located at GeneSEQ Sdn. Bhd.

For the data analysis, generated single-end demultiplexed fastq files were trimmed with Cutadapt (verson 1.18; National Bioinformatics Infrastructure Sweden, Uppsala, Sweden) to remove the non-biological forward and reverse primer sequences located on the 5′ and 3′ ends of each read, respectively. The trimmed reads were then used as input for amplicon sequence variant (ASV) generation and abundance table construction using dada2 (http://benjjneb.github.io/dada2/; Callahan *et al.*, 2016), which is part of the QIIME2 (version 2020.8) pipeline (https://qiime2.org/; Bolyen *et al.*, 2019). Taxonomic assignment of the ASVs was performed using the QIIME2 scikit-learn naive Bayes machine-learning classifier (Bokulich *et al.*, 2018).

Bacterial identification via culture-dependent and culture-independent methods using Nanopore

The same samples used in the previous section were also used for bacterial identification using Nanopore. The samples were homogenized with 0.1 mm and 0.5 mm silica beads in sodium dodecyl sulphate (SDS) homogenization buffer (1% SDS, 100 mmol/L NaCl, 50 mmol/L Tris–HCl (pH 8), 50 mmol/L EDTA), followed by deproteination using chloroform extraction. The DNA was precipitated by the addition of 0.6 volume isopropanol followed by centrifugation at 10 000×*g* for 15 min. The DNA pellet was washed twice with 500 µL 70% ethanol and resuspended in 100 µL 0.1×TE buffer (1 mmol/L Tris–HCl (pH 8), 0.1 mmol/L EDTA).

The amplification of the V1–V9 region of 16S rRNA was conducted using 27F and 1496R primers. First, PCR products were visualized on gel and purified using SPRI Bead (Oberacker *et al.* 2019), followed by index PCR using an EXP-PBC001 kit (Oxford Nanopore, Oxford, UK). Next, barcoded libraries were pooled and gel-purified using WizPrep™ Gel/PCR Purification

Mini Kit (WizBio, Seongnam-si, Republic of Korea). The pooled barcoded amplicons were quantified using Denovix high sensitivity, and appropriate amplicons were used as the input for LSK109 library preparation (Oxford Nanopore). Sequencing was then performed on a Nanopore Flongle Flowcell for 24 h.

For data analysis, Guppy (version 4.4.0; high accuracy mode; Oxford Nanopore) was used for the basecalling and demultiplexing of the raw Nanopore reads. The filtered reads were subsequently processed with NanoClust (Rodríguez-Pérez *et al.*, 2021), which performs read clustering, consensus generation, taxonomic assignment and abundance estimation. The NCBI 16S rRNA database was also used to search for consensus sequences. The top BLASTHits from NCBI were aligned with the consensus sequences using MAFFT (version 7; https://mafft.cbrc.jp/alignment/software/; Katoh *et al.*, 2002) and subsequently used to construct a maximum likelihood tree with Fasttree2 (-nt -gtr) (http://www.microbesonline.org/fasttree/; Price et al., 2010). The visualization of the tree used FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Fungal identification using Nanopore

The cooked rice samples of D3 storage used in bacterial identification using Illumina were also subjected to fungal identification using Nanopore. The samples were homogenized with 0.1 mm and 0.5 mm silica beads in SDS homogenization buffer (1% SDS, 100 mmol/L NaCl, 50 mmol/L Tris–HCl (pH 8), 50 mmol/L EDTA) followed by deproteination using chloroform extraction. The DNA was precipitated by the addition of 0.6× volume isopropanol followed by centrifugation at 10,000×*g* for 15 min. The DNA pellet was washed twice with 500 µL of 70% ethanol and subsequently resuspended in 100 µL 0.1×TE buffer (1 mmol/L Tris–HCl (pH 8), 0.1 mmol/L EDTA).

The amplification of partial 18S and full-length ITS1 region was conducted using e515Fngs (GCCAGCAACCGCGGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) primers (Tedersoo *et al.* 2015). The PCR products were visualized on gel and purified using SPRI Bead (Oberacker *et al.*, 2019), followed by index PCR using the EXP-PBC001 kit (Oxford Nanopore). The barcoded libraries were pooled and gel-purified using WizPrep™ Gel/PCR Purification Mini Kit (WizBio). Quantification of the pooled barcoded amplicons was performed using Denovix high sensitivity, and an appropriate amount of the amplicons was used as the input for LSK109 library preparation (Oxford Nanopore). Sequencing was then performed on a Nanopore Flongle Flow cell for 24 h.

For data analysis, Guppy (version 4.4.0; high accuracy mode) was used for basecalling and demultiplexing of the raw Nanopore reads. The filtered reads were subsequently processed with NanoClust (Rodríguez-Pérez *et al.*, 2021), which performs read clustering, consensus generation, taxonomic assignment and abundance estimation. The NCBI Fungal ITS1 database was also used to search for consensus sequences.

Physicochemical analysis

pH

A sample (25 g) of cooked rice was placed in a sterile stomacher bag with 200 mL of distilled water and stomached. The pH value was recorded using a pH meter (Mettler Toledo, Columbus, OH, USA).

Water activity

One gram of cooked rice was placed into a sample cup, covering the bottom and filling only half of the cup. The sample cup was placed into the water activity meter (AquaLab, Pullman, WA, USA), and the value was recorded.

Sensory analysis

Sensory analysis was conducted based on the study of Piotrowska-Cyplik *et al.* (2017). The evaluation was performed by nine semi-trained panellists using a five-point rating scale (from 1 to 5) on cooked rice stored at 30 °C. As the evaluations were required to be done consecutively for four days (D0, D1, D2, and D3 of storage), the commitment of the panellists was very important; thus, only nine panellists were able to be employed. The selected panellists were also very familiar with cooked rice characteristics. During the sensory evaluation session, each panellist was required to remove the cooked rice from the containers and place it in a sterile Petri dish. The texture, aroma, colour, and appearance of the cooked rice were observed. A score of 5 indicated the highest quality of the examined parameter, and a score of 1 indicated the lowest quality. The points were then summed to obtain an overall sensory score (maximum of 20).

Volatile organic compounds analysis

Ten grams of cooked rice samples from each storage day were mixed with 100 mL of 95% ethanol and incubated at 30 °C for 2 h. The mixtures were then filtered using Whatman filter paper (Maidstone, UK) with an 11 µm pore size. The filtrates were then subjected to an evaporation process for 15 min using a rotary evaporator with the following parameters: 38 °C, 7 kPa, and 30 r/min. The remaining filtrates were then transferred to 15-mL centrifuge tubes and dried using nitrogen gas until the liquid evaporated. One millilitre of 95% ethanol was added to each tube and transferred to gas chromatography–mass spectrometry (GC-MS) vials for analysis. An Agilent 7890A instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 5975 mass selective detector was used to carry out GC-MS analysis. The chromatographic column used was 30 m×0.25 mm×0.25 µm film thickness of the HP5MS capillary column. Helium was used as carrier gas at a flow rate of 1.0 mL/min. The injector temperature was set to 250 °C with split injection mode. The column temperature was programmed to be held at 40 °C for 6 min, increased from 40 °C to 150 °C and then increased to 200 °C, after which the temperature was held for 1 min. For mass spectrometry, the conditions were set as follows: ion source temperature, 230 °C; quadrupole temperature, 150 °C; electron energy, 70 eV; transfer line temperature, 280 °C; and a scan range of 20–700 mass units. The obtained compounds were matched with the data provided by the NIST11/s library and were quantified using the area normalization method.

Statistical analysis

The means of microbiological enumeration, physicochemical and sensory analysis were analysed by one-way analysis of variance (ANOVA) using Minitab 18.0 software (State College, PA, USA). The results are given as the standard deviation (SD) of the mean.

Results and Discussion

Enumeration, pH and water activity analysis

Enumeration of cooked rice's TVC, SFC, and YMC were evaluated alongside pH and water activity (a_W) during storage. The results are presented in Table 1. TVC, SFC

Table 1. pH, water activity (a_w), total viable count (TVC), spore-forming count (SFC), and yeast and mould count (YMC) of cooked rice at 30 °C during storage days 0, 1, 2, and 3

Storage day	pH value	a_w value	TVC (log CFU/mL)	SFC (log CFU/mL)	YMC (log CFU/mL)
0	6.37±0.22[a]	0.982±0.02[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
1	6.34±0.18[a]	0.985±0.02[a]	5.07±0.33[b]	4.15±0.85[b]	3.79±0.10[b]
2	6.43±0.03[a]	0.989±0.01[a]	5.55±0.66[b]	5.56±0.86[c]	4.10±0.17[b]
3	6.23±0.10[a]	0.985±0.01[a]	6.90±0.73[c]	6.45±0.88[c]	4.42±0.66[b]

Values are mean±standard deviation. Means in a column followed by different letters differ significantly ($P<0.05$) between the time of storage.

and YMC showed no visible colony (<2 log CFU/g) immediately after cooking. As rice can be contaminated with various microorganisms, the undetectable growth on D0 shows that the cooking process has the ability to eliminate microorganisms present before that. However, the significant increase ($P<0.05$) of TVC, SFC, and YMC on D1 with values of 5.07±0.33 , 4.15±0.85, and 3.79±0.10 log CFU/g, respectively, showed that there are exceptions towards microorganisms such as spore-former, yeast and moulds. Spore-formers are among the most common foodborne microbial pathogens associated with cooked rice, especially *Bacillus* species. These bacteria can contaminate rice even before harvesting and remain due to their ability to endure extreme environments such as heat (Hneini and Nawas, 2019). Fungal presence has also been reported in cooked rice by Tahir *et al.* (2012). Their presence could be linked to the physiology of the contaminating genera, which might have been able to withstand high temperatures during the cooking process.

The sharp increase in TVC, SFC, and YMC in cooked rice after only 24 h of storage highlighted the impact of improper holding temperature on food. The room temperature of 30 °C applied in this study is within the range of optimum temperature for both spore-formers such as *Bacillus* species and fungi (Ehling-Schulz *et al.*, 2015). Temperature not only allows the multiplication of microorganisms but also induces spore germination (Ubong *et al.*, 2019). Further incubation of cooked rice at temperature-abused conditions led to increasing TVC, SFC, and YMC values as observed on D2 and D3, in which the latter obtained the highest values of cooked rice with values of 6.90±0.73, 6.45±0.88, and 4.42±0.66 log CFU/g, respectively. In the case of food safety, the number of microorganisms present in food should be limited to a recommended microbiological limit, where an excess amount will indicate that the food is unsafe for consumption. In this study, the cooked rice was shown to be highly contaminated on D3. It was considered unsafe to be consumed due to the breaching of $>10^6$ CFU/g of the microbiological limit permitted by Food Regulations 1985 (Ahmad *et al.*, 2020).

The pH and a_w values of cooked rice during storage were also observed in the present study (Table 1). On D0, the cooked rice was detected with a pH value of 6.37±0.22, similar to the value reported in freshly cooked rice by Moulavi *et al.* (2018). No significant changes ($P>0.05$) in the values were observed throughout the storage period. According to studies on carbohydrate foods, reduced pH, reportedly due to fermentation, was observed after more than one week of storage (Sang *et al.*, 2015; Montero *et al.*, 2020). Thus, it is assumed that no fermentation occurred in cooked rice in this study due to the three-day storage period. Even so, it is notable that the pH in this study was close to neutral pH, which can support the growth of many microorganisms, making food more susceptible to spoilage. Similar to pH analysis, there was also no significant difference ($P>0.05$) in the a_w on each storage day. This is also observed in Dong's (2013) study on noodles, another high-carbohydrate food. This can be attributed to the nature of water activity being hardly affected by food except by the involvement of external or internal factors. Despite this, water activity still plays a significant role in microbial growth and its relation to chemical and enzymatic reactions in food (Maltini *et al.*, 2003). The value of a_w at 0.98 shown throughout the storage period leads to a high potential of cooked rice in supporting microorganisms.

Microbial community identification using next-generation sequencing

Bacterial identification

Bacteria were identified in cooked rice via culture-dependent (Figure 1) and culture-independent (Figure 2) methods. For the culture-dependent method, Illumina identification was done to the colonies obtained by the TVC plates on all storage days except for D0, where no colonies had grown. An average of 27 867 raw reads were generated for each sample, with minimum and maximum reads being 7062 and 40 917, respectively. Only a single bacterium was present in cooked rice throughout the storage period of D1, D2, and D3, as shown in Figure 1. The bacteria were identified as Firmicutes and Bacilli at phyla and class level, respectively, with abundances of >99%. However, the bacteria could not be identified from order to species level and were only labelled as 'Not Assigned'. By referring to multitudes of studies that reported proper identification up to species level when using at least two primers (Boziaris and Parlapani, 2017; Yu *et al.*, 2018), it is assumed that the use of only one primer (V4 region), as shown in this study, is not enough to obtain complete identification of microbial communities. Through the Nanopore method using the entire length of 16s rRNA as primers, the bacterium could be identified as *Bacillus cereus*. Figure 3A shows the identification of two clusters (OTUs) by the culture-dependent method, which confirmed they belonged to the same species of bacteria, *B. cereus*. This was validated by the tight clustering of both sequences within the *B. cereus* clade shown in the phylogenetic tree in Figure 4. It was determined that the difference in the length of the identified sequences led to the differentiation of the identified bacteria into two clusters according to the database.

In the culture-independent method, Nanopore also identified *B. cereus* as the sole bacterium present in cooked rice. However, this was only observed on D2 and D3 of the cooked rice storage period, which is different from the Illumina result

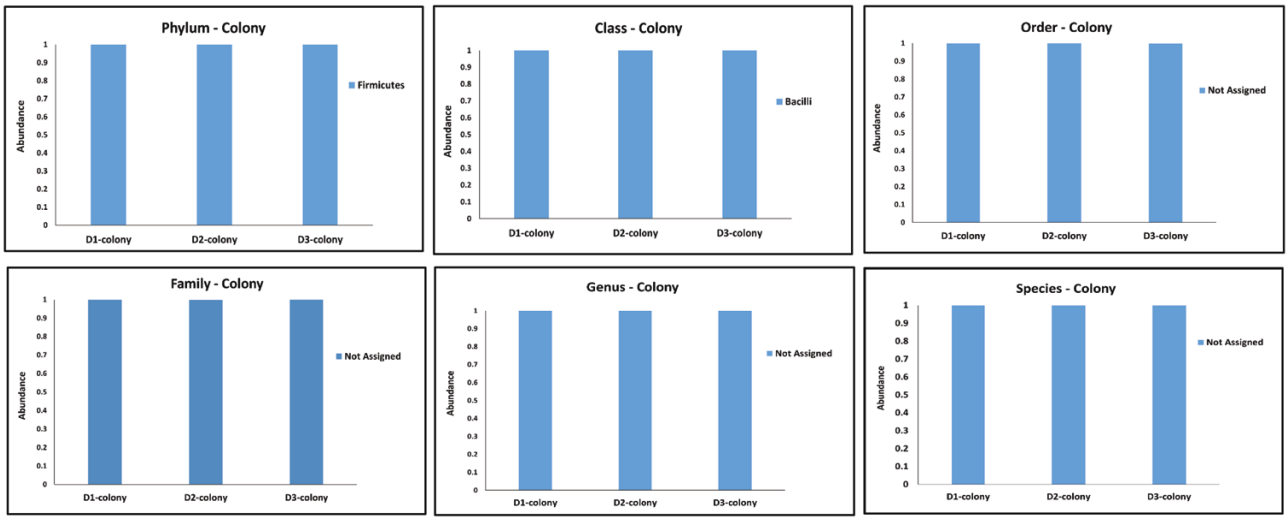

Figure 1. Illumina identification of cooked rice at 30 °C during storage days 0, 1, 2, and 3 by the culture-dependent method.
*Not Assigned refers to bacteria that cannot be identified from order to species level.

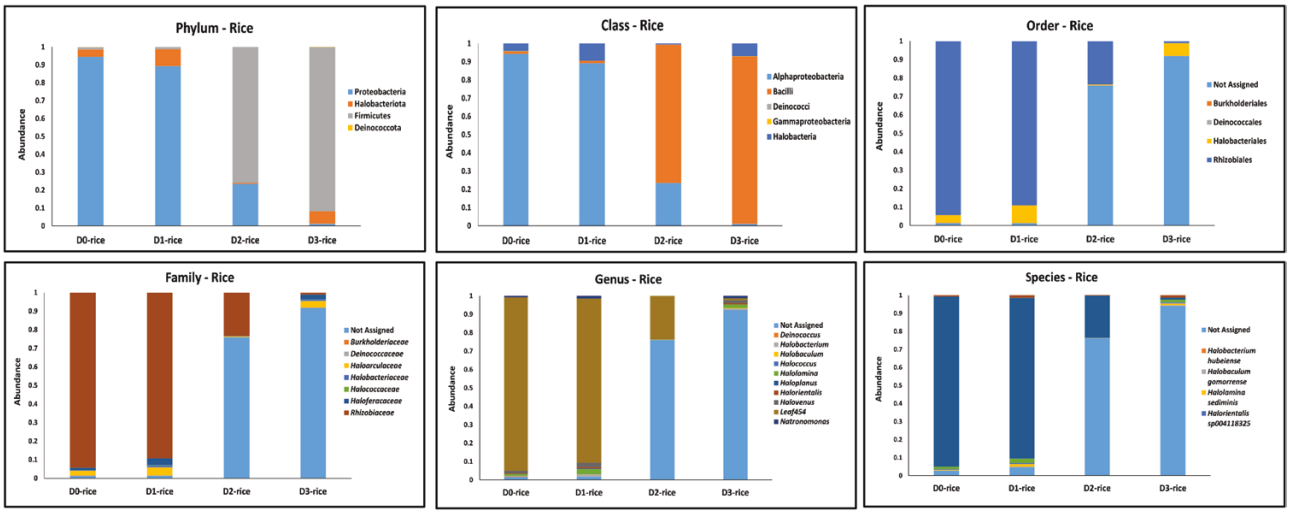

Figure 2. Illumina identification of cooked rice at 30 °C during storage days 0, 1, 2, and 3 by culture-independent method.

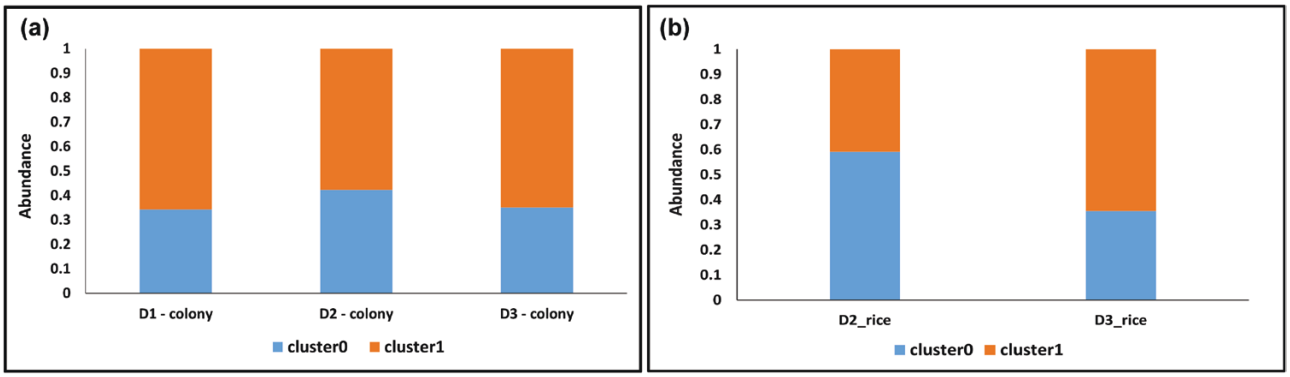

Figure 3. Nanopore identification of cooked rice at 30 °C during storage days 0, 1, 2, and 3 by (A) culture-dependent method and (B) culture-independent method.

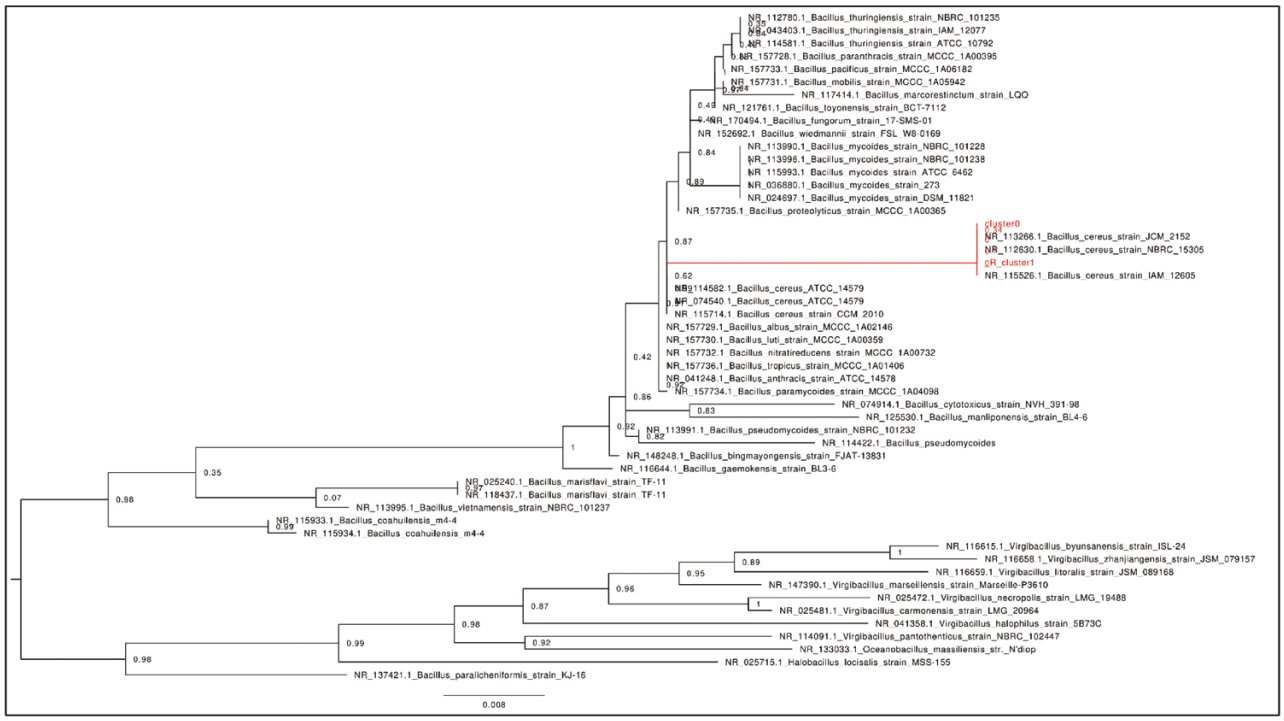

Figure 4. The phylogenetic tree confirming cluster 0 and cluster 1 are from *Bacillus cereus* species.

that detected multiple bacteria presence throughout the storage period, as shown in Figure 2. At D0, Illumina detected the composition of microbiota consisting of Proteobacteria (94.5%), Halobacteriota (4.3%), and Firmicutes (<2%). The dominant bacteria on D0 with phyla Proteobacteria were later identified as *Leaf454_sp001425485* sp. at the species level. Other species observed on D0 were *Halovenus aranensi*, *Halorientalis sp004118325*, *Halolamina sediminis*, *Natronomonas moolapensis,* and an unknown bacterium labelled as 'Not Assigned', all of which were detected at low levels (<3%). As proven by Nanopore, this unknown bacterium was confirmed to be *B. cereus*. *Leaf454_sp001425485* (89.2%) was the dominant species at D1, followed by *B. cereus* (4.3%) and other species; *Halovenus aranensis*, *Halorientalis sp004118325*, *Halolamina sediminis,* and *Natronomonas moolapensis*, which remained at low levels (<2%). On D2 and D3, however, *B. cereus* became the dominant species, with 76% and 91.9% abundance, respectively.

In food, only a tiny proportion of microbial communities known as SSOs contribute to the spoilage process (Bekaert *et al.*, 2015). These are microorganisms that are present in high abundance, which is observed by both species of *Leaf454_sp001425485* and *B. cereus* in cooked rice in this study. As for other bacteria, their low abundance throughout storage suggested that they did not play any crucial roles. *Leaf454_sp001425485* sp., however, was determined to be incorrectly identified due to the lack of identification of the species, even though its supposedly high abundance was detected by Illumina. This is because, compared with Illumina, Nanopore uses full-length 16s rRNA, which allows more accurate identification of bacteria present, which uses only the V4 region. To the best of the author's knowledge, there is also no information on this species found except for it being mentioned in a report by Hay (2020) that investigated the presence of cryospheric bacteria. This lack of information prevents us from connecting this species' finding in cooked rice, further proving the possibility of misidentification by Illumina.

In comparison to *Leaf454_sp001425485* sp., which was detected only through the culture-independent method, *B. cereus* was identified by both methods. However, its presence varies slightly between the two methods for D0 and D1. Identification through the culture-dependent method was unable to proceed due to the lack of growth on PCA, whereas the culture-independent method detected the presence of *B. cereus* on D0. This can be a limitation of the culture-dependent method, which relies on the ability of microorganisms to grow on the agar for further identification, compared to the culture-independent method, where DNA can be directly extracted from food samples and sent for identification. This limitation was also observed in the study of Yang *et al.* (2017), which showed bacteria identified by the culture-independent method being undetected by the culture-dependent method. For D1 of cooked rice storage, *B. cereus* was unable to be detected by Nanopore of the culture-independent method, which contradicted the finding of its presence by Illumina in the culture-independent method and culture-dependent method. This, however, can be assumed due to the limitation of food matrices. Cooked rice contains natural constituents such as carbohydrates, proteins, and fats that can lead to the remains of molecules that may act as PCR inhibitors (Ercolini, 2013), limiting its detection by Nanopore. The differences in these results proved the necessity of conducting both methods for microbial identification to overcome any limitations.

Overall, *B. cereus* was confirmed by culture-dependent and culture-independent methods to be the only bacterium present in cooked rice stored at 30 °C. While the bacteria were in low amounts during the early storage period, their abundance increased on D2 and D3. This growth pattern matches

the description given for SSOs by Boziaris and Parlapani (2017), where SSOs may initially appear small but increase in number, resulting in food spoilage. A study on cooked rice conducted by Yu *et al.* (2018) observed the presence of both *B. cereus* and *B. subtilis* when cooked rice was stored at 25 °C. While this study similarly showed that *B. cereus* was the dominant bacterium, *B. subtilis* was not detected at all, which may be due to the initial contaminant microorganisms of rice prior to cooking. The study also observed a bacterial count of >6 log CFU/g after three days of storage, similar to the TVC result obtained in this study. The result of *B. cereus* acting as the spoilage bacteria of cooked rice confirms it that the SSO is the most commonly associated bacteria with rice, even among *Bacillus* species.

Fungal identification

Using Nanopore, fungal presence was identified in the cooked rice samples after a three-day storage using ITS sequences (Table 2). Overall, *Aspergillus* sp. had the highest abundance of 45%, followed by *Lasiodiplodia* sp. (38%), *Phanerochaete* sp. (11%), and *Pyrrhoderma* sp. (6%). To the best of the author's knowledge, except for *Aspergillus* sp., none of the other fungi have shown any association with rice. Their presence in this study may have been attributed to contamination from soil from which they had been previously reported to be isolated. *Aspergillus* sp. is a commonly found fungi contaminating rice alongside *Penicillium* sp., *Rhizopus* sp. and *Fusarium* sp. (Choi *et al.*, 2014; Krishnan *et al.*, 2018).

The presence of *Aspergillus* sp. in rice is concerning due to its ability to produce aflatoxins. This toxin is produced mainly by *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* (Atungulu *et al.*, 2019). Aflatoxin B1 (AFB1), a toxin mainly produced by *A. flavus*, causes nephrotoxic, immunotoxic, mutagenic, teratogenic, and carcinogenic effects (Songsamoe *et al.*, 2017). These toxins are also highly resistant to heat treatments with a decomposition temperature of >235 °C. However, it has been reported that its production can be affected by water activity and temperature interactions. In the study of Sani and Sheikhzadeh (2017), 24.8% of aflatoxin content was observed when the high temperature was combined with the increasing moisture content during the rice cooking process, which enhanced the destruction of toxins. This shows that the cooking process of rice in this study should reduce the aflatoxin content if it is present, which can otherwise cause adverse health effects upon consumption.

Sensory analysis

The total sensory scores of cooked rice obtained from the summation of texture, aroma, colour, and appearance scores were

Table 2. Fungal identification in cooked rice during three-day storage at 30 °C using Nanopore

Cluster	Fungal genus	Relative abundance (%)	Base pair (bp)	Extracted ITS1 sequence
0	*Aspergillus* sp.	45	1233–1413 (181 bp)	*CCGAGTGTAGGGTTCCTAGCGAGCCCAACCTCCCACCCGTGTTTACTG TACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCT CTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTG ATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTA*
1	*Lasiodiplodia* sp.	38	1241–1376 (136 bp)	*CCGAGTTTTCGAGCTCCGGCTCGACTCTCCCACCCTTTGTGAACGTACC TCTGTTGCTTTGGCGGCTCCGGCCGCCAAAGGACCTTCAAACTCCAGTC AGTAAACGCAGACGTCTGATAAACAAGTTAATAAACTA*
2	*Pyrrhoderma* sp.	6	1242–1486 (245 bp)	*ATGAGTTTTTTAAAGTAAACTTGATGCTGGTCGGTCTCTGGACTTGCATG TGCTCAGTTTGCGCTCATCCATCTCACACCTGTGCACTTACTGAAGAGAG AGAGGGAGAGGGAGAGTGGTTTATTCGTTTATTCATTTATTCGTGTATTC AACTCAAAGTCTTCAATCTCTCTTTTGACTTTATAATAAACAACTATATTGT TTGTGTAGAATGCATTAGCCTCATTGTAGGTGAAATAACTATA*
3	*Phanerochaete* sp.	11	1242–1450 (209 bp)	*ACGAGTTAATTGAACGGGTTGTTGCTGGTATCATTCTATCAAAGGGT GTTCATGTGCACGCCTGGCTCATCCACTCTTCAACCCCTGTGCACTT ATTGTAGGCTGGGTGGAAGGGTCGAGCTCTTCGGGCTTACTTGGAA GCCTTCCTATGTTTTACTACAAACGCTTCAGTTTAAGAATGTAATCTC TGCGTATAACGCATCTATATA*

Table 3. Sensory analysis of cooked rice at 30 °C during storage days 0, 1, 2, and 3

Storage day	Sensory attribute				Total sensory score
	Appearance	Texture	Aroma	Colour	
0	4.86±0.38^{a}	4.57±0.79^{a}	4.71±0.49^{a}	4.86±0.38^{a}	19.00±0.14^{a}
1	4.71±0.49^{a}	4.29±0.49^{a}	4.14±0.69^{a}	4.43±0.53^{a}	17.57±0.24^{a}
2	2.71±0.49^{b}	2.14±0.38^{b}	3.00±0.82^{b}	3.14±0.90^{b}	11.00±0.44^{b}
3	1.14±0.38^{c}	1.00±0.00^{c}	1.00±0.00^{c}	1.71±0.76^{c}	4.86±0.34^{c}

Values of parameters are mean±standard deviation. Values of total sensory scores are the summation of theparameters on each storage day±standard deviation. Means in a column followed by different letters differ significantly (P<0.05) between the time of storage.

observed to decrease with increasing storage time (Table 3). Initially, on D0, cooked rice had a total sensory score of 19.00±0.14, which showed a very high sensory quality that matched the lack of growth of microorganisms on TVC plates as shown in Table 1. A slight reduction in the total sensory score was observed on D1 with a value of 17.57±0.24. Despite having no significant changes in sensory quality to D0, there was a significant increase in microorganism presence on D1 of 5.07 log CFU/g. On D2, the total sensory score decreased significantly ($P<0.05$) to 11.00±0.44. The texture of the cooked rice was observed with the lowest score at 2.14±0.38, which considered mushy and could be attributed to the breakdown of cooked rice by microorganisms as a source of nutrients (Banwart, 2012). This is followed by appearance, aroma, and colour with scores of 2.71±0.49, 3.00±0.82, and 3.14±0.90, respectively.

Despite the significantly lower sensory quality of D2 compared to D1, there was no significant difference ($P>0.05$) between the presence of microorganisms in both cooked rice. This shows that there may be other factors in addition to microbes involved in causing physicochemical changes in the cooked rice (Petruzzi *et al.*, 2017). While the cooked rice on D3 had the lowest total sensory score of 4.86±0.34 out of all the storage days, D2 was deemed the day the cooked rice was spoiled, as the panellists considered the cooked rice to be unacceptable for consumption. This shows the occurrence of organoleptic rejection of cooked rice even before the breaching of the microbiological limit, as observed by the TVC result on D3. This differs from the study of Piotrowska-Cyplik *et al.* (2017), which reported organoleptic changes in cooked ham with microbial growth of 6–7 log 10 CFU/g. The differences may be due to the different food types involved, which have different properties. The sensory method used in this study was to determine the shelf life of cooked rice by analysing the changes in texture, aroma, colour and appearance of cooked rice. Nevertheless, a sensory method based on consumer perception could also be considered for insights from the point of view of the consumer (Cais-Sokolińska *et al.*, 2021; de Alcântara *et al.*, 2023).

Volatile organic compounds analysis

The profiles of 85 VOCs, which consisted of hydrocarbons (48), esters (16), alcohols (14), ketones (5), aldehydes (2), organic acids (2), amine (1), and miscellaneous compounds (disparlure and glycerine), are shown in Table 4. According to the literature, the majority of these compounds had never been reported, which is not surprising considering that this is the first report on VOCs produced by cooked rice during storage, which has also been investigated in relation to spoilage microorganisms. The number of VOCs in the study gradually increased over the storage period with most of the compounds being observed initially on D1 instead of D0. This can be due to the escape of volatiles during the rice cooking process. This study had a different experimental design to the ones shown by Takemitsu *et al.* (2016) and Yang *et al.* (2008), which used a steam rice cooking machine and a specially designed beaker, respectively, both of which prevented the escape of volatiles (cooked rice aroma) captured during the cooking process.

The result showed that hydrocarbons were the major VOCs on all the storage days. Both alkanes and alkenes were commonly linked to the occurrence of lipid oxidation in rice and have been associated with flavours such as grassy, fatty, and soapy (Bryant and McClung, 2011). In addition to hydrocarbons, groups of esters, alcohols and ketones made up most of the compounds obtained from the cooked rice. Dibutyl phthalate and 2,4-bis(1,1-dimethylethyl)-phenol are the only compounds from the ester and alcohol groups, respectively, present throughout storage. Both have been identified in various cooked rice varieties, the latter of which are associated with the aroma of pharmaceuticals/pencils.

According to the study of Parlapani *et al.* (2019a, 2019b), the compounds whose concentrations significantly changed between the initial and rejection time points were proposed as potential spoilage markers. This can be correlated with the properties of SSOs, which can produce metabolites that cause organoleptic rejection as an indication of spoilage (Boziaris and Parlapani, 2017). In the present study, the sensory analysis confirmed D2 as the rejection time point of cooked rice. Therefore, compounds that were found to be present only on D2 and those increasing significantly on D2 compared to D0 and/or D1 were evaluated. The compounds tested were: cycloeicosane, 3-eicosene, 1,2-diethyl-cyclohexadecane, 1-eicosene, 1-heptadecene, 2-butyl-1,1,3-trimethylcyclohexane, 9-octadecene, hexacosane, 2-tridecene, *cis*-2-methyl-7-octadecene, 7-tetradecene, 1-decene, 1-docosene, dichloroacetic acid heptadecyl ester, 9,12-octadecadienoic acid methyl ester, 9,12-octadecadienoic acid methyl ester, trichloroacetic acid tridecyl ester, trichloroacetic acid dodecyl ester, decyl trifluoroacetate, phenol 4,4′-(1-methylethylidene)bis-, chloroxylenol, *n*-nonadecanol-1, 2-furanmethanol, behenic alcohol, 2-methyl-*Z*,*Z*-3,13-octadecadienol, butyrolactone, 2(3H)-furanone, dihydro-4-hydroxy-, 2(5H)-furanone, and cyclohexanone.

Of these compounds, only 3-eicosene, 1-heptadecene, hexacosane, phenol, 4,4′-(1-methylethylidene)bis-, *n*-nonadecanol-1, and cyclohexanone have been previously reported to be products of secondary metabolites of various *Bacillus* species (Munjal *et al.*, 2016; Raza *et al.*, 2016; Panneerselvam *et al.*, 2019; Matloub *et al.*, 2020; Elamin *et al.*, 2021). Among these compounds, 3-eicosene had the highest abundance followed by *n*-nonadecanol-1 and phenol, 4,4′-(1-methylethylidene)bis- with relative areas of 12.03%, 9.03%, and 4.08%, respectively. As *B. cereus* was proven to be the SSO of cooked rice in this study, these compounds are highly likely to be produced by bacteria and therefore can be proposed as potential spoilage markers of cooked rice.

In general, changes in microbiological, sensory, and volatile compounds profiles during the storage of cooked rice at room temperature are important for several practical elements. First, in terms of food safety, there is a need to understand the conditions that promote the growth of bacteria, which in turn ensures food safety. Second, understanding the proper storage conditions for rice can help reduce food waste, and this research can lead to innovation in preservation methods to extend the shelf life of cooked rice at room temperature. Third, these findings can be used to increase public awareness, by educating the public about the importance of proper cooked rice storage, especially for food handlers.

Conclusions

This study investigated the microbial populations, sensory, and volatile compounds profiles of cooked rice during a three-day storage period at 30 °C to determine its SSOs. As

Table 4. Volatile organic compounds (VOCs) and their relative area (%) in cooked rice at 30 °C during storage days 0, 1, 2, and 3

VOCs	Relative area (%)			
	D0	D1	D2	D3
Hydrocarbons				
Cetene	5.53	2.44	4.41	3.08
3-Heptadecene, (*Z*)-	7.43			
1-Nonadecene	14.69	11.25	9.90	0.91
2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-ethylidene-	2.77			
5-Octadecene, (*E*)-	8.56	5.24	3.77	1.88
Octacosane	1.35			
Tetratetracontane	1.58			
9-Eicosene, (*E*)-		7.45	2.95	3.16
3-Tridecene, (*Z*)-		0.83		
3-Octadecene, (*E*)-		1.63	0.87	1.73
Cycloeicosane		2.55	5.56	2.12
3-Eicosene, (*E*)-		3.55	12.03	3.63
Octadecane		1.99	1.83	1.68
3-Hexadecene, (*Z*)-		0.63		6.62
Cyclohexadecane, 1,2-diethyl-		3.02	7.18	3.88
1-Pentadecene		2.17		
10-Methyl-9-nonadecene		0.75		
5-Eicosene, (*E*)-		9.51	6.37	8.18
Butane, 1,1-diethoxy-3-methyl-		0.80		0.49
7-Hexadecene, (*Z*)-		1.24	0.83	1.90
2-Methyl-*E*-7-hexadecene		3.37		
Cyclooctane, methyl-		1.39	0.34	1.37
1-Pentadecene, 2-methyl-		0.47	0.14	0.26
1-Octadecene		7.47	3.43	12.12
1-Tetradecene		0.34		
1-Eicosene		0.70	2.88	3.87
Hexadecane		0.64	0.97	1.48
Cyclotetradecane		0.13	0.29	
Cyclopentadecane		0.13		
8-Heptadecene, 8-methyl-, (*E*)-		0.96		
1-Heptadecene		0.27	1.14	
Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-		0.16	0.31	1.83
Cyclohexane, 2-butyl-1,1,3-trimethyl-		0.93	1.51	0.82
Cyclohexadecane		0.48		
9-Octadecene, (*E*)-			0.80	
Hexacosane			1.79	2.01
2-Tridecene, (*Z*)-			0.42	
cis-2-Methyl-7-octadecene			3.51	
7-Tetradecene			0.37	
1-Decene			0.63	
1-Docosene			1.29	4.53
4-Trifluoroacetoxyhexadecane				1.84
3-Tetradecene, (*E*)-				1.46
Cyclopropane, 1-pentyl-2-propyl-				1.53
1-Tridecene				1.56
6-Tridecene				1.62
Cyclopropane, 1-methyl-1-(1-methylethyl)-2-nonyl-				0.55
Tetradecane				0.18
Esters				
Hexadecanoic acid, methyl ester	6.69	2.05		3.61

Table 4. Continued

VOCs	Relative area (%)			
	D0	D1	D2	D3
Dibutyl phthalate	14.12	40.46	8.29	12.60
Pentafluoropropionic acid, decyl ester		0.59		
1,2-Benzenedicarboxylic acid, butyl octyl ester		2.21		
Heptafluorobutyric acid, hexadecyl ester		0.13		
Trichloroacetic acid, hexadecyl ester		0.06		
Dichloroacetic acid, heptadecyl ester			2.24	5.62
9,12-Octadecadienoic acid, methyl ester			0.74	
9,12-Octadecadienoic acid (*Z*,*Z*)-, methyl ester			0.32	
Trichloroacetic acid, tridecyl ester			0.35	
Trichloroacetic acid, dodecyl ester			0.14	
Decyl trifluoroacetate			0.17	
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester				1.81
Heptafluorobutyric acid, pentadecyl ester				1.38
Sulfurous acid, octadecyl 2-propyl ester				2.07
Trichloroacetic acid, tetradecyl ester				0.18
Alcohols				
Phenol, 2,4-bis(1,1-dimethylethyl)-	15.97	46.69	10.93	14.48
2,3-Butanediol	0.97			
Phenol, *p-tert*-butyl-	0.62			
11-Hexadecyn-1-ol		3.21		
2-Methoxy-4-vinylphenol		0.32		0.24
4-Chloro-2,6-dimethylphenol		0.51		
Phenol, 2,5-bis(1,1-dimethylethyl)-		2.38		0.18
Phenol, 4,4′-(1-methylethylidene)bis-		0.67	4.08	3.48
Chloroxylenol		0.21	0.27	0.53
n-Nonadecanol-1			9.73	
2-Furanmethanol			1.40	0.31
Behenic alcohol			8.78	5.50
2-Methyl-*Z*,*Z*-3,13-octadecadienol			0.96	
2-Ethyl-1-dodecanol				1.32
Ketones				
7,9-Di-*tert*-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione		9.64	3.25	4.12
Butyrolactone			0.29	
2(3H)-Furanone, dihydro-4-hydroxy-			0.44	0.20
2(5H)-Furanone			1.04	
Cyclohexanone			1.61	
Aldehydes				
E-15-Heptadecenal				1.76
Cyclopropaneoctanal, 2-octyl-				0.57
Acids				
Acetic acid	4.40			
9,12-octadecadienoic acid (*z*,*z*)-		0.16	0.60	2.95
Amine				
Methylamine, *n*,*n*-dimethyl-	56.64			
Others				
Disparlure	0.62			
Glycerin	1.72	1.69	0.59	1.42

the organoleptic rejection of food is a marker for spoilage, D2 was deemed indicate that the day cooked rice was spoiled. According to both culture-dependent and culture-independent methods, *B. cereus* was the sole bacteria present throughout the storage period, confirming that it was the SSO of cooked rice. Furthermore, VOCs such as 3-eicosene, 1-heptadecene,

hexacosane, phenol, 4,4′-(1-methylethylidene)bis-, *n*-nonadecanol-1, and cyclohexanone are believed to be responsible for the organoleptic rejection caused by *B. cereus* in cooked rice, suggesting their potential application as spoilage markers.

Author Contributions

Conceptualization: Mohammad Sabri Nur-Shahera and Mahmud Ab Rashid Nor-Khaizura; Performing the experiment: Mohammad Sabri Nur-Shahera; Assisting the experiments: Chong Kah Hui, Jiang Shan, and Kousalya Padmanabhan; Data curation: Mohammad Sabri Nur-Shahera, Han Ming Gan, and Mahmud Ab Rashid Nor-Khaizura; Writing original draft preparation: Mohammad Sabri Nur-Shahera; Review and editing: Mohammad Sabri Nur-Shahera, Nor Ainy Mahyudin, Muhammad Shirwan Abdullah Sani, and Mahmud Ab Rashid Nor-Khaizura. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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