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Detection and Identification of Microbial Contaminant in Bakery Products in Yogyakarta City, Indonesia

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Abstract

Bread has been chosen as an alternative food because of its availability. In Indonesia, consumption of breads is increased due to the change of diet pattern into packed and ready-made meals. Therefore, it is important to raise the awareness of bakery products quality. The purpose of this study was to detect and identify the microbial contaminant in bakery products in Yogyakarta City, Indonesia. Bacterial colonies from expired bakery products were isolated into pure isolate then confirmed by API Staph and Polymerase Chain Reaction (PCR) method. The results showed there were five kinds of gram-positive bacteria. Isolated bacteria identified by API were *Bacillus cereus* (52.8%), *Bacillus subtilis* (97.7%), *Staphylococcus aureus* (97.7%), *Staphylococcus epidermidis* (97,9%) and *Staphylococcus saprophyticus* (72,2% and 61,8%).

Keywords: bakery products, microbial contaminant, API Staph, PCR, mold

Introduction

Breads are considered as an important food in Western countries and recognized as starchy food together with rice, potatoes, pasta and breakfast cereals (O'Connor, 2012). It has been chosen as an alternative food because of its availability in the market, furthermore it is easy to reach by everyone. These days, bread has many variations based on its flavor and type, such as sliced bread, pizza and donut that makes bread is delighted by all ages (Edwards, 2007). Ingredients, baking process and environment are the crucial factors that affected the quality and safeness of the bakery products. The chance of contaminants to enter the product increased along with poor baking process and unhygiene environment. Contaminants might caused poisonings which lead into diseases if it is consumed (Saranraj and Geetha, 2012). Bacteria (*Salmonella* sp., *Bacillus* sp., & *Staphylococcus* sp.) and mold (*Rhizopus* sp., & *Aspergillus* sp.) are the common contaminants found in bakery products. One of the examples of the common contaminants found on bakery products is the case that was occurred in May 2013 at Ben Tre, Vietnam. Total of 173 people suffered from gastroenteritis because they consumed the breads that was contaminated. Food Safety Agency (FSA) later found the microbial contaminant was *Salmonella* sp. (Vo *et al*., 2014).

Indonesia, as low and middle-income countries (LMICs), the diets pattern has changed into increasing consumption of packed and ready-made meals (Colozza and Avendano, 2019), including bakery products. There were some cases of food poisoning in Indonesia because of unstandardized quality of bread products. Yet the research about it was so limit thus, inhibited the information of its microbial contaminants toxicity. As a part of Indonesian food consumption, it is necessary to improve the quality of bread from its ingredients, environment and baking process. It is important to know the possibility of contamination during bread making. Based on that fact, this research was conducted as an initial step to give the information about the bread contaminant to prevent poisoning case because of bread consumption. Therefore, the purpose of this study was to detect and identify the microbial contaminant in bakery products.

Materials and Methods

Materials

The samples were 30 pieces of bakery products from 10 types of bread that have been randomly collected from 10 places

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(market, shops and canteens) in Yogyakarta City, Indonesia. Three samples were taken from each places. The condition of samples were fresh bakery product with the time of storage about 0-2 days from production time. The samples were analyzed with 3 replication for each methodologies. Peptone water, malt extract agar (MEA), nutrient agar (NA), mannitol salt agar (MSA) and baird-parker agar (BPA), brain heart infusion agar (BHIA) and potato dextrose agar (PDA) were purchased from Millipore Corp. (Massachusetts, USA). Mannitol yolk polymyxin (MYP) agar was purchased from Hardy diagnostics (California, USA). Gram stain reagent and endospore stain reagent were purchased from Quelab Laboratories Inc. (Montreal, Canada). API Staph was purchased from bioMerieux Company (Marcy-l'Étoile, France) and 100 bp DNA Ladder was purchased from Thermo Fisher Scientific (Massachusetts, USA). All chemicals and reagents were of analytical grade.

Methods

Isolation and identification of bacteria

The sample (10 g) was dissolved into 90 mL of 1% peptone water and incubated for 12 h in room temperature for the resuscitation process, then diluted into 10^{-5}, 10^{-6} and 10^{-7} solution. Each of the solution was inoculated into growth medium in petri disk. Based on the previous studies, mannitol yolk polymyxin (MYP) and baird-parker agar (BPA) were selected to be the growth medium for inoculation and incubation of the solution in 37 °C for 24-48 h. The steps were repeated until the pure culture obtained and selected as the collection sample. This culture then being identified by biochemistry analyses for coloring the bacteria and its endospore. Motility and confirmation analysis was done using API Staph.

Detection of nuc gene of Staphylococcus aureus isolate

Deoxyribonucleic acid (DNA) of *Staphylococcus aureus* was isolated using *The Wizard® Genomic DNA Purification Kit* and the identification was done using polymerase chain reaction (PCR, SureCycler 8800 Thermal cycler, Agilent, CA, USA) with the reference primer from Kim *et al.* (2001) with the fragment length of 270 bp. The amplification was done for 35 cycles with pre-denaturation at 95 °C for 2 min, denaturation at 95 °C for 1 min, annealing at 58 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min.

Detection of sea gene of Staphylococcus sp.

DNA of *Staphylococcus* sp. was isolated using The Wizard® Genomic DNA Purification Kit and the identification was done using PCR with the reference primer from Johnson *et al.* (1991), with the fragment length of 120 bp. The amplification was done for 35 cycles with pre-denaturation at 95 °C for 3 min, denaturation at 94 °C for 2 min, annealing at 58 °C for 90 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min.

Detection of hbl and nhe gene of Bacillus sp. *isolate*

DNA of *Bacillus* sp. was isolated using *The Wizard® Genomic DNA Purification Kit* and the identification was done using PCR with the reference method from Soleimani *et al.* (2018). The primer used were 3 pairs of non-hemolytic enyerotoxin (Nhe) and hemolysin BL (Hbl) toxin with different fragment length. The amplification was done for 35 cycles with pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 49 °C for 1 min and 52 °C for 1 min, extension at 72 °C for 10 min and final extension at 4 °C for 30 min.

Results

Detection of microbial contaminant in bakery product

In this research, the calculation of the colony was done only for predicted *Staphylococcus* sp. in MSA medium with three replications. The result showed that in the sample, there were *Staphylococcus* sp. existed in the range of 1.7×10^7 up to 2.2×10^7 CFU per gram sample as shown in Table 1.

Staphylococcus sp. colony were growth in MSA medium and in this research, two kind of colonies were found. First colony in Fig. 1a shows as yellow color and grow before 24 h of incubation and the second

Table 1. The amount of bacterial colony in the sample

Sample	Colony (CFU/g)
1	spreader
2	3.5×10^7
3	spreader
4	1.7×10^7
5	2.2×10^8
6	spreader
7	spreader
8	1.4×10^8
9	3.7×10^7
10	2.3×10^7

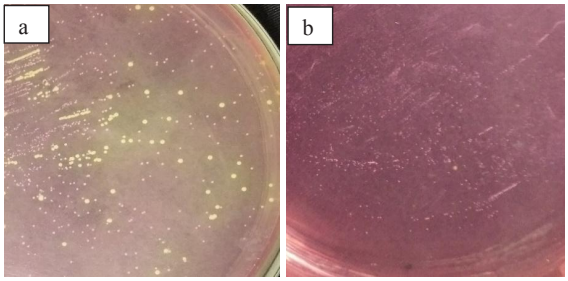

Figure 1. The growth of yellow (a) and pink (b) bacterial colony in MSA medium

Figure 2. Bacteria colony in BPA medium

Figure 3. Bacteria colony in NA medium

colony (Fig. 1b) shows to be pink color. The pink colony changed its color into yellow after 24 h. The yellow colony was predicted as *Staphylococcus aureus* and another species of *Staphylococcus* was predicted for the second colony with pink color.

The colony that has been growth in MSA medium then re-grow in BPA medium. In this step, the result showed that the growth colony has the shiny black color with transparent zone on its surrounding (Fig. 2) and this appearance indicated as coagulase positive bacteria.

In the NA medium, the colony of bacteria, predicted as *Bacillus* sp., was growth as shown in Figure 3.

The predicted *Bacillus* sp. colony then inoculated into MYP medium. In this medium, two kind of colonies were growth. The pink color colony predicted as *Bacillus cereus* (Fig. 4a) and yellow colony with white edge predicted as *Bacillus subtilis* (Fig. 4b).

Mold contamination was also found in some of the samples after storage for 14 days inside the sterile plastic bag. The color of the mold was black (Fig. 5a) or yellow greenish (Fig. 5b) as shown in Figure 5.

Identification of bacterial contaminant in the bakery product

Coloring the bacteria and endospore

Two genera of bacteria that have been found was colored to see the type and shape of the bacteria cell. Under the microscope, all of the isolate bacteria showed to have purple color. The result shows that there were 16 isolates with coccus shape predicted as *Staphylococcus* sp. (Fig. 6a) and 16 isolates with rod shape predicted as *Bacillus* sp. (Fig. 6b).

Under microscope, all isolates showed green color from the *Malachite green* reagent as shown in Figure 7. and can be differentiate from the bacteria cell colored in red from safranin reagent.

Motility analysis

Predicted *Bacillus* sp. isolates were tested for its motility and the result showed that there was isolate which spread out of the initiate area in the semi solid NA medium as shown in Figure 8.

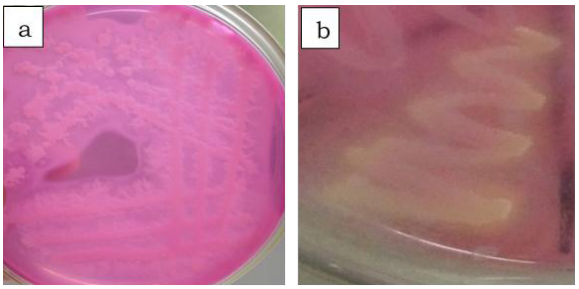

Figure 4. The pink (a) and yellow (b) colony of bacteria in MYP medium

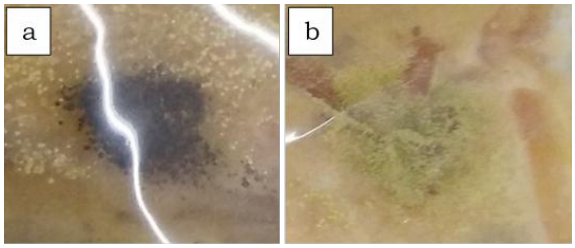

Figure 5. The black (a) and yellow greenish (b) spore of mold occurred in the sample

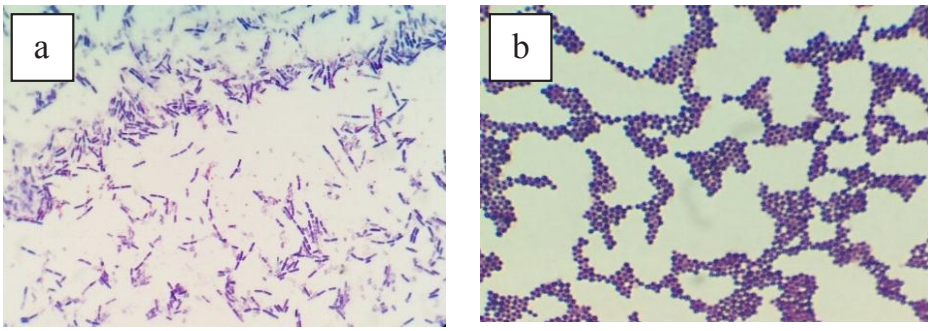

Figure 6. The coccus (a) and rod (b) shape of isolates bacteria

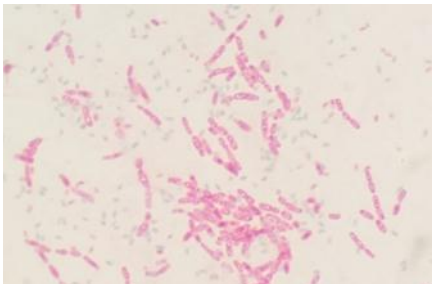

Figure 7. Endospore coloring for predicted *Bacillus* sp.

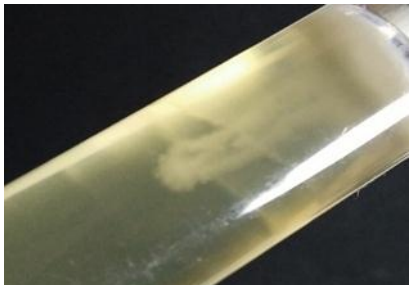

Figure 8. Motility analysis of *Bacillus* sp. in semi solid NA medium

Confirmation of predicted Staphylococcus isolate using API Staph

Confirmation analysis was done by randomly selected six predicted *Staphylococcus aureus* and *Staphylococcus* sp. isolates to confirm the species that already found. This analysis tested the ability of isolate to ferment some kind of carbohydrate, *Voges Proskauer*, nitrate, alcohol dehydrogenases (ADH) and urea. The result showed there were three different species after analyzed by API Staph as shown in Table 2.

Molecular analysis

Figure 9. shows that no nuc gene was detected (270 bp). In other hand, sea gene was detected (120 bp) in the predicted *S. aureus*

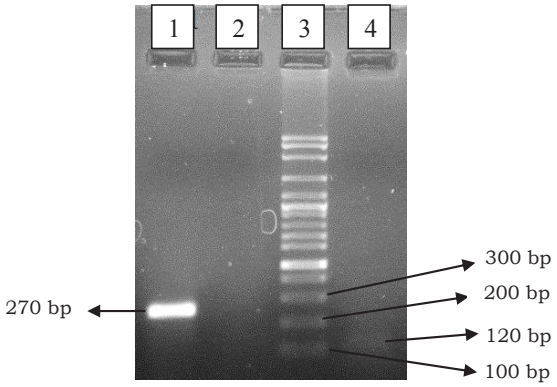

Figure 9. PCR detection of nuc and sea gene for predicted *Staphylococcus aureus* isolate

Note:
Line 1. Comparison isolate (nuc gene 270 bp)
Line 2. Nuc gene isolate test
Line 3. Marker (DNA 100 bp ladder)
Line 4. Sea gene isolate test (120 bp)

Table 2. API Staph result of predicted *Staphylococcus* isolate

Sample	Code	Predicted species	API Staph result	ID (%)
2	S.2.1	*S. aureus*	*S. aureus*	52.8
2	S.2.2	*S. aureus*	*S. saprophyticus*	72.2
4	S.4.2	*S. aureus*	*S. aureus*	97.7
6	S.6.1	*S. epidermidis*	*S. epidermidis*	97.9
7	S.7.2	*S. aureus*	*S. saprophyticus*	61.8
9	S.9.2	*S. aureus*	*S. aureus*	97.7

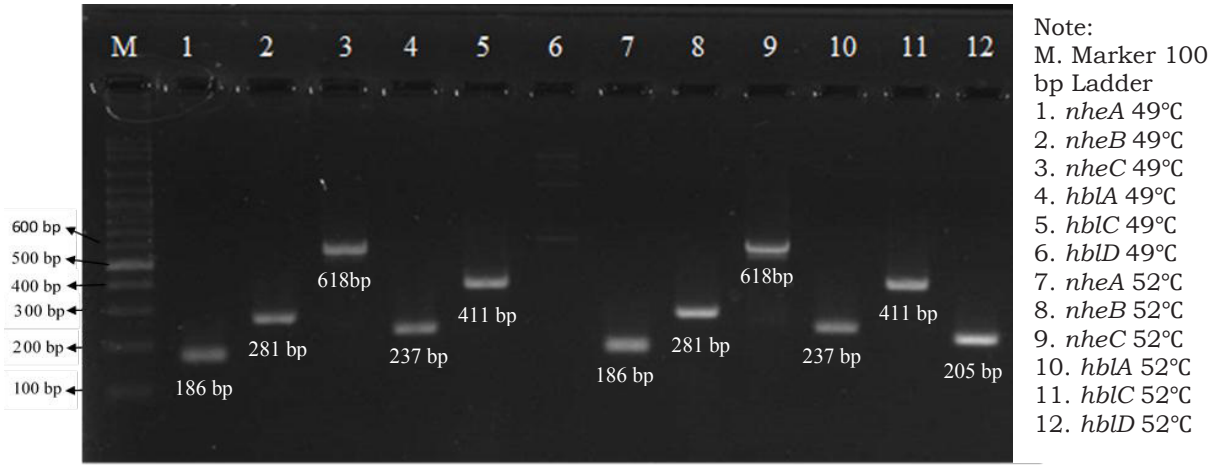

Figure 10. PCR detection of nhe and hbl gene for predicted *Bacillus subtilis* isolate

isolate. Predicted *Bacillus subtilis* isolate was found to be poisonous potency after the molecular analysis was conducted under the annealing temperature of 49 and 52 °C because all of the target gene (*nheA*, *nheB, nheC, hblA, hblC,* and *hblD*) were found in the PCR detection result (Figure 10).

Discussion

Bakery are food products that require several processes before ready to consume. Raw ingredients (eggs, water, cream, and wheat), imperfect baking and fermentation process, equipment, packaging and environment are the important factors affecting the bread quality because the contaminations are mostly happened in those factors. In this study, detection of microbial contaminant started with resuscitation to reactivate the injured microbial because of baking process. The microbial remain alive but could not grow and this is called viable but not culturable (VBNC) condition (Oliver, 2005).

Some of the bakery products used in this research from small bakery shops have the expired date on it. However, almost all the bakery products from market and school canteen did not have the expired date. The range of the bacterial colony in these samples from Table 1. could produce toxin and lead to consumer poisonous. Two genera of bacteria have been detected as the contaminant in the samples with total isolate was 32 consisted of predicted *Bacillus* sp. (16 isolates) and *Staphylococcus* sp. (16 isolates). Those bacteria survived from heat during baking process because of its peptidoglycan wall thus, predicted as gram positive bacteria. Another reason to predict the genus of bacteria was the fact that *Bacillus* sp. formed endospore as its defense mechanism to survive when the environment conditions were not supportive. This result was comparable with the research done by (Stenfors *et al.*, 2008), which found out that the endospore of *Bacillus cereus* will be germinated in the stable environment condition and then the bacteria were reactive. The identification then to see the changing color of the predicted bacteria. The yellow colony (Fig. 1a) was predicted as *Staphylococcus aureus* because

of its abilities to ferment mannitol and grow in the medium contained of 7.5% NaCl. Second colony with pink color (Fig. 1b) was predicted as another species of *Staphylococcus* with the coagulase negative characteristic or *Micrococcus* which could not ferment mannitol but survive in 7.5% NaCl in the medium. These result is similar with (Kateete *et al.*, 2010). Figure 2. was the further step to identify the appeared colony from this study. In this study, *Staphylococcus aureus* was predicted as the black colony with transparent zone and the other colony without transparent zone was predicted as lecithinase negative *Staphylococcus aureus*, another species of *Staphylococcus, Bacillus* spp., *Preoteus* spp., *Enterococci* and *Micrococci.*

Those colony reduced tellurite into tellurium and the transparent zone indicated the activity of lecithinase enzyme as described from (Capita *et al.*, 2001). The predicted *Bacillus* sp. shown in Figure 3 from this study were characterized as white color, contained mucous, irregular size and tend to form group of uncalculated colonies. This results were comparable with the result from (Tallent *et al.*, 2012). The predicted *Bacillus* sp. colony then inoculated into MYP medium which contained of Polymyxin B to inhibit the growth of gram-negative bacteria and showed in Figure 4a and 4b. The pink color colony (Fig. 4a) indicated no mannitol was fermented. In the yellow colony (Fig. 4b), there was fermentation of mannitol and the white edge was precipitant of the egg yolk from the medium. Mold contaminant was also identified in this study by macromorphology and micromorphology. The detected mold were predicted as *Aspergillus niger* and *Aspergillus flavus* because of its characteristics were the same as described by (Silva *et al.*, 2011), and (Gautam and Bhadauria, 2012). Bacterial contaminant was identified by coloring the bacteria and its endospore. The purple color that appeared from two founded genera was because of the peptidoglycan wall in the bacteria cell absorbed violet crystal solution and this indicated the gram-positive bacteria. Coloring the endospore was done only for predicted *Bacillus* sp. because of its ability to form endospore. The green color appeared from all of isolates indicated that they were *Bacillus* sp. Motility analysis for this predicted *Bacillus* sp. isolate showed that the isolate could move around the medium thus, supported the prediction that the isolate was *Bacillus* sp. For founded predicted *Staphylococcus* sp. isolates, confirmation analysis was done to confirm its species. It was found that from this study, there were three species of *Staphylococcus* with the highest percentage of ID (97.9%) was found to be *Staphylococcus epidermidis.* Molecular analysis was done for predicted *Staphylococcus aureus* and *Bacillus subtilis* isolate because those are amongst the dangerous pathogenic bacteria. It produced enterotoxin compound that in a specific amount can be poisonous (Ahmed and Mashat, 2014).

Sea gene detected in the predicted *S. aureus* isolate indicated that this isolate produced enterotoxin A. All of the target gene (*nheA*, *nheB, nheC, hblA, hblC,* and *hblD*) were found in predicted *Bacillus subtilis* isolate. The research from (Soleimani *et al.*, 2018) found out that all of the target gene was produced toxin compound and commonly found in *Bacillus cereus.* Additional bacteria in this research was *Staphylococcus* sp. The total colony was more than the maximum level (10^2 CFU/ mg) of the standard of bakery product from Badan Pengawas Obat dan Makanan Republik Indonesia/ BPOM (Badan Standarisasi Nasional, 2009). Therefore, qualitative examination is required before consumed bakery products.

Conclusion

Five kinds of gram-positive bacteria were identified in this study as the contaminant in the bakery products after their expired date. The colony of pathogenic bacteria, *Staphylococcus* sp., was above the maximum standard level allowed by BPOM and could cause consumer poisonous. Although this study has given the information of bakery product contaminants to consumers, further studies are necessary to understand its pathogenic mechanism. Thus, will improve food safety and raise the producers and consumers awareness of food contaminants.

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