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Phylogenetic tree and heat resistance of thermoduric bacteria isolated from pasteurization milk in Indonesia

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Abstract

Milk pasteurization process might result in the emergence of thermoduric bacteria. Medium scale milk industries obtained raw milk from traditional farmers, which may trigger the contamination from many different sources. To ensure the safety of the product, thermoduric bacteria included in pasteurized milk had to be identified. One of the methods to identify thermoduric bacteria was PCR (*Polymerase Chain Reaction*) techniques. The samples used were thermoduric bacteria isolated from pasteurized milk in Indonesia. The results showed that some bacteria which contaminated the pasteurized milk shall be: *Brachybacterium nesterenkovii* strain DSM 9573, *Kocuria varians* G33 strain, *Klebsiella* sp. A4, *Chryseobacterium* sp. PcR 003, *Dermacoccus nishinomiyaensis* strain DSM 20448 and *Pseudomonas otitidis* strains J11N with similar results of each 99.8%, 99.4%, 94.7%, 100%, 100% and 100%. *Chryseobacterium* sp. PCR 003 has a high heat resistance than *D. nishinomiyaensis* strain DSM 20448.

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Introduction

Pasteurization of milk was aimed to destroy pathogenic microbes, microbial spoilage and reduce the activity of the enzyme. The positive impact of pasteurization was to improve safety and extend the shelf life of milk and dairy products. A temperature pasteurization could kill pathogenic bacteria known to most heat resistant and do not form spores as *Mycobacterium tuberculosis* and *Coxiella burnetti*, but some species of thermoduric bacteria, namely thermophilic and some gram-negative rod-shaped microbe still can survive at pasteurization temperatures (Chavarri *et al*., 2000; Lejeune and Rajala-Schultz, 2009; Chavan *et al*., 2011).

Ruegg and Reinemann (2002) and Schelderman *et al.* (2005) found a number of spores forming bacterium that is resistant to high temperatures in fresh milk. Thermoduric bacteria that can grow at temperatures of pasteurization are *Bacillus*, *Clostridium*, *Micrococcus*, *Mycobacterium*, *Lactobacillus* and sometimes also *Streptococcus*. This opinion is reinforced by Hassan *et al*., 2009, that after pasteurization, the microbes identified were: *Bacillus*, *Staphylococcus*, *Micrococcus*, *Enterobacter*, *Pseudomonas*, *Streptococcus*, *Pediococcus* and *Lactobacillus*. Groups of bacterial non spores that can survive pasteurization temperatures are Streptococcus and Lactobacillus group and other groups. This type of bacteria is commonly found in products that have gone through the pasteurization process at a temperature of 63°C for 30 minutes or 72°C for 15 seconds. *Micrococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Bacillus* and *Clostridium* are several types of thermoduric bacteria, *Aspergillus* and *Penicillium* are a type of mold that can grow at pasteurization temperature.

We carried out a preliminary characterization of many thermoduric bacteria isolated from milk pasteurization in Malang Indonesia. Some microbes that often arises, we identified using PCR. PCR-based specific assays are a valuable alternative methods, being far more rapid, specific, and unhindered by the presence of non-target microorganisms. 16S rRNA gene sequences from the thermoduric isolates were compared to those in the GenBank sequence databases to determine the sequences for established species of bacteria to which they were most similar. From the results of PCR we chose two species to be tested heat resistance. Selected thermoduric bacteria is expected to provide information about the adequacy of heat, so it can be used as an alternative in the heat processing of pasteurized milk in Malang, Indonesia. Determination of heat resistant by calculating the value of D and z on selected isolates. The objective of this study was to examine the phylogenetic tree and the heat resistant (D and z value) identities of the thermoduric bacteria. Phylogenetic analyses of 16S rRNA gene sequences were utilized in combination with analyses of heat resistant of thermoduric bacteria in order to address these questions. We present evidence that many of the isolates are novel strains or species of thermoduric bacteria.

Material and methods

Milk samples

The research material used in this experiment is thermoduric bacterial isolates. Thermoduric bacteria isolated from milk pasteurized processed by Dairy Cooperative in Malang, East Java, Indonesia. Sampling was done aseptically using a sterile 50 ml syringe, samples were then transferred to a sterile glass tube 100 ml. Milk samples immediately cooled and transported to the laboratory in a storage container 4°C (Prejit *et al*., 2007).

Isolation of thermoduric bacteria

Milk samples diluted 10^{-1} to 10^{-6}. The sample was grown in a medium *Plate Count Agar* (PCA) using the technique of line / T and incubated at a temperature of 37°C for 24 hours. Colonies identified as thermoduric bacteria will be studied further.

Identification Isolate with 16S rRNA (ribosome-Ribonucleic Acid)

Thermoduric bacterial identification used PCR techniques (Rossi *et al*., 1999). Identification based genotype isolates were grown on nutrient broth at

30°C for 24 hours. Furthermore, 1.5 mL of fermentation broth were centrifuged 10,000 x g for 5 min at room temperature and separated solids. DNA was isolated by using *FastPrep*, a special kit for the isolation of DNA (*Deoxyribo Nucleic Acid*). *Lysis* using lysing matrix sample kit and homogenized using *FastPrep* for 40 seconds at 4500 rpm. DNA amplification is done using PCR with primers 765 R (5'-CTGTTTGCTCCCCACGTTTC-3') and 1141 R (5'-GCCTTGCGCTCGTTGC-3'). PCR primer containing 765 R and 1141 R is added to a solution of DNA, subsequently purified using gel extraction kit / DNA. The 16S rRNA gene sequences obtained DNA is then performed using a 3.1 V Dye® terminator cycle sequencing kit. DNA sequences used equipment is ABI 300 genetic analyzer. Furthermore, sequences obtained were compared with the available databases in NCBI Blast (*Basic Local Alignment Search Tool*) uses BLAST search engines (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic Tree Classification

Procedure the first step of the phylogenetic classification was by isolating and purifying the chromosomal DNA of each species. Furthermore, the gene was amplified by PCR of each of the chromosomal DNA samples. Amplification product was then purified to in sequence. Data obtained gene sequences from each species were then used as the basis for classifying it as phylogenetics. Phylogenetic classification was done through the phylogenetic tree construction. The Phylogenetic tree obtained was the result of classification of phylogenetic relationships of each classified species. To arrange phylogenetic tree based on the gene sequence data, several steps taken should be: Preparation of gene sequence, gene sequence alignment, phylogenetic tree construction, phylogenetic tree visualization, editing phylogenetic tree, presentations phylogenetic tree, construction matrix similarity and difference nucleotides.

Heat Resistance Measurements

The selected isolates were *Chryseobacterium sp.* PcRB003 and *Dermacoccus nishinomiyaensis* strain DSM 20448. Heat Resistant measurement milk containing 1 mL suspension isolate (10^6 – 10^6 cfu/ml) was placed rapidly in a large water bath set at desired temperature (80°C, 85°C, 90°C, and 95°C), and the temperature of the bath was monitored with a precision mercury-in-glass thermometer. A come up time of approximately 0 minutes, 3 minutes, 6 minutes and 9 minutes was observed for 80°C, 85°C, 90°C and 95°C, respectively. Thermal inactivation kinetics of microorganisms is obtained by first establishing a survivor curve, which is a logarithmic (Awuah *et al.*, 2007). Plot of the number of microorganisms surviving is given heat treatment at a given temperature against the heating time. This pre-supposes that microbial destruction generally follows a first order reaction. Two key parameters (*D* and *z* values) are then determined from the survivor and resistance curves, respectively. The *D*-value represents a heating time that results in 90% reduction of the existing microbial population. This is expressed mathematically as follows:

$$\text{D D} \frac{\text{DD D DD}}{\text{DDDDDDDDDDDDD}}$$

where *A* and *B* represent the survivor counts following heating for times t_1 and t_2 minutes. The first order reaction rate constant (*k*) is obtained from the expression $k = 2.303/D$. The temperature sensitivity (*z*-value) which represents the temperature change that results in a 10-fold change in the *D*-value, is represented mathematically as follows:

$$\text{D D} \frac{\text{DD D DD}}{\text{DDDDDDDDDDDDDD}}$$

Where D_1 and D_2 are *D*-values at temperatures T_1 and T_2, respectively.

Results and discussion

Bacteria were microbes that could grow everywhere according to their needs. Some bacteria can adapt quickly to unfavorable environment such as changes in temperature or dropped dramatically and increased suddenly. Results of thermoduric isolation and identification of bacteria found in milk pasteurization largely included in the group *Micrococcus*. PCR results showed that the bacteria

found in pasteurized milk are generally mesophilic bacteria, and some of the new species in the group thermoduric.

Brachybacterium nesterenkovii strain DSM 9573

Results of sequencing using the blast showed that isolates A has a percentage of 99.8% similar to the strain *Brachybacterium nesterenkovii* DSM 9573. Gvozdyak *et al.*, 1992, found the species *B. nesterenkovii* product isolated from milk. *B. nesterenkovii* a family *Micrococcaceae*, aerobic bacteria, gram-positive, kemoorganotropic, catalase negative, the minimum temperature for growth is 28°C, class mesophilic and halophilic. *Brachybacterium* also found in milk that has undergone heating at Kenyans farms and can cause disease. *Brachybacterium* group was also found by Callon *et al.*, (2007) in goat milk. Sarikhan *et al.*, (2011) adds that strain *nesterenkonia sp.* have genes that can respond to the pressure of osmosis and oxidation that can withstand heat stress, cold stress and have a detoxification system. So as expected, the presence of *B. nesterenkovii* strain DSM 9573 in pasteurized milk that is processed in Dairy Cooperative caused by the ability of the gene in bacterial cells. Results phylogenetic tree is shown in Figure 1.

Table 1. Calculated D value for linier of survival curves.

Species	Temperature	Linier D value (min)	z-value (°C)	R^2
Chryseobacterium sp. PcRB003	80°C	35.71	20.41	0.917
	85°C	5.81	(R^2 = 0.636)	0.861
	90°C	9.26		0.880
	95°C	4.67		0.799
D. nishinomiyaensis strain DSM 20448	80°C	22.22	21.28	0.883
	85°C	7.81	(R^2 = 0.841)	0.746
	90°C	4.37		0.936
	95°C	4.41		0.899

Kocuria varians strain G33

Results of sequencing using the blast showed that isolates B and have a percentage of 99.4% similar to the strain *Kocuria variance* G33. According Tremonte *et al.*, (2010) *K. variance* is a gram-positive bacteria, catalase positive, oxidase-positive, aerobic, can use glucose to aerobic conditions, optimum growth of 25-37°C. *K. variance* generally isolated from mammalian skin, but can also be found in water and soil.

O'Mahony *et al.*, (2001) conducted a study and found that *K. varians* could produce variacin which served as an anti-microbe. *K. varians* was able to control the growth of *Bacillus cereus* in cooled milk. It was believed as the cause of *B. cereus* was not found in pasteurized milk from Dairy Cooperative. Total 23% of bacteria in pasteurized milk are *K. varians*. *K. varians* is also found in fresh milk. Colonies of K. varians were not found after the pasteurized milk was stored in the refrigerator at day 14 and 17 (Fromm and Boor, 2004). This is presumably because *K. varians* is bacteria that resistant to temperature pasteurization, but not resistant to refrigerator temperature, so it belongs to the group of thermoduric bacteria. *K. varians* generally non-pathogenic, colonies, which can be found in the skin, mucosa and orofarink. There was a first case reported that *K. varians* are pathogenic and can damage the brain (Tsai *et al.*, 2010).

Klebsiella sp. A4

Results of sequencing using the blast shows that isolates C has a percentage of 94.7% similar to *Klebsiella* sp. A4. Adil *et al.*, (2011) say that *Klebsiella* is a *coliform* group that is found in water, soil, vegetation, plants, animals, insects and human. It has no specific requirements to grow. *Klebsiella* optimum growth is at a temperature of 35-37°C but it can grow well at a temperature between 35-37°C and at pH 7.2. *Klebsiella* species are facultative anaerobes, and most strains can survive with citrate and glucose as the carbon source and ammonia as the source of nitrogen.

Fig. 1. Phylogenetic Tree *Brachybacterium nesterenkovii* strain DSM 9573.

Mdegela *et al*., (2005) found that *Klebsiella* on dairy farms are less hygienic. Abbott *et al.* (2011) add that the use of pasteurization temperature 60-75°C and stored for 0.25 to 4 hours will result in earlier death compared to using temperature 45-50°C then stored for 4-120 hours, then death will be slower. At a pasteurization temperature, the number of vegetative cells *in Klebsiella pneumoniae* will decrease. Some cases have been reported that *Klebsiella* was found in fresh milk at Ghana (Donkor *et al*., 2007), in yoghurt at Abuja-Nigeria (Okpalugo *et al*., 2008), *K. pneumoniae* in evaporated milk (Oladipo and Omo-Adua, 2011), in the fresh milk (Uddin *et al*., 2011) and in the nono, traditional fermented milk from Nigeria, as well as in yoghurt (Obande and Azua, 2013). Donkor *et al*., (2007) adds that *Klebsiella spp* in milk is rarely associated with food borne infections. Most bacteria have been identified in milk is a group of *enterobacteria*. The existence of the group *enterobacteria* in milk indicates that there has been contamination of milk as a result of poor sanitation.

Fig. 2. Phylogenetic Tree *Kucoria varians* strain G33.

Chryseobacterium sp. PcRB003

Results of sequencing using the blast showed that isolates D have a percentage of 100% similar to *Chryseobacterium sp.* PcRB003. Hantsis-Zacharov *et al.*, (2008) revealed that the genus *Chryseobacterium* are family members *Flavobacteriaceae* (phylum *Bacteroidetes*) and currently consists of 21 species. *Chryseobacterium* species is found in water, soil and clinical environments. These bacteria include *psychrotolerant* and *proteolytic* bacteria that can cause a variety of defects in food products such as milk, meat, poultry and fish.

Fig. 3. Phylogenetic Tree *Klebsiella sp.* A4.

Bernardet *et al.*, (2006) reported that many species *Flavobacterium* associated with food spoilage classified as *Cryseobacterium* that cause spoilage of various food products, including fish, meat, poultry and milk and milk products. Bekker (2011) added that some species *Chryseobacterium* have been isolated from raw milk. Other species found in milk include *C. Bovis*, *C. Joostei sp. nov* (Hugo *et al.*, 2003), *C. Haifense sp. nov.* (Hantsis-Zacharov and Halpern, 2007), and *C. oranimense* (Hantsis-Zacharov *et al.*, 2008) were all isolated from fresh milk.

Rate of growth and the level of protease production by bacteria can be an indication of the level and speed of decay. According to Bekker (2011) growth characteristics *Chryseobacterium joostei*, *C. bovis* and *Pseudomonas fluorescens* was found that *C. joostei* have the highest growth rate at temperatures above 7°C. At lower temperatures, *P. fluorescens* showed a higher growth rate than the species *Chryseobacterium*. Arrhenius plot results showed that *C.bovis* is most sensitive to changes in temperature. For protease activity, *Chryseobacterium joostei* showed the highest activity followed by *C. bovis* then *P. Fluorescens*. When considering the level of growth and production of protease, showed that *C. joostei* may have the ability to cause decay than *P. fluorescens* at temperatures above 7°C.

Chryseobacterium sp. PcRB003 in pasteurized milk produced by the Dairy Cooperative allegedly due to previous fresh raw milk is contaminated by *Chryseobacterium*, but the species *Chryseobacterium sp.* PcRB003 still able to survive after the high temperature. *Flavobacterium* has been reported to cause bitterness and fruit pasteurized; the milk is cooled as well as discoloration and mucus production of cheese. *Chryseobacterium* durability in high temperature was reported by Bekker (2011).

Fig. 4. Phylogenetic Tree *Chruseobacterium sp.* PcRB003.

Dermacoccus nishinomiyaensis strain DSM 20448

Results of sequencing using the blast showed that isolates E has the percentage of similar to 100% *Dermacoccus nishinomiyaensis* strain DSM 20448. Njage *et al.*, (2013) found *D. nishinomiyaensis* isolated from fresh camel milk and camel milk fermentation.

Fig. 5. Phylogenetic Tree *Dermacoccus nishinomiyaensis* strain SDM 20448.

Another name of *D. nishinomiyaensis* is *Micrococcus nishinomiyaensis*. *D. nishinomiyaensis* bacteria strain DSM 20448 are a gram-positive bacteria, shaped *cocci*, catalase positive, oxidase-positive, aerobic, optimum growth of 25-37°C, cannot grow in the salt content of 7.5%. In general, it isolated from the skin of mammals and water (Szczerba, 2003).

Pseudomonas otitidis strain J11N

Results of sequencing using the blast showed that the percentage of isolates F has a 100% similarity with *Pseudomonas otitidis* J11N. *Pseudomonas otitidis* species are gram-positive bacteria that can cause otitis in humans. According Mehri *et al.*, (2013) *Pseudomonas* can be isolated from various sources such as soil, plants, mineral water and can grow on minimal nutrient medium. Fromm and Boor (2004) found *Pseudomonas* in pasteurized milk, but in small amounts. *Pseudomonas* colony count of one colony increased to five colonies after pasteurization of milk stored in the refrigerator at day 17. It is suspected that *Pseudomonas* is a bacterium that is resistant to pasteurization temperatures and can grow at refrigerator temperatures, so it belongs to a group of bacteria thermoduric psichrophilic.

Fig. 6. Phylogenetic Tree *Pseudomonas otitidis* strain J11N.

Van Tassel *et al.*, (2012) found the species *Pseudomonas* in pasteurized milk. *Pseudomonas spp* 4,8x103 CFU / mL found in milk that has been pasteurized in Abuja, Nigeria (Okpalugo *et al.*, 2008), the fresh milk (Gunasekera *et al.*, 2003) and on the surface of the cheese (Denis *et al.*, 2001). According Eneroth *et al.*, (2000) *Pseudomonas spp.* regarded as the most important organisms that contribute to spoilage of milk through the production of lipolytic and proteolytic enzymes. Although the cells of *Pseudomonas* can die in the process of pasteurization, thermal enzyme is more stable and can reduce the quality and shelf life of dairy products. *Pseudomonas* also easily contaminates dairy products after pasteurization, so the bacterial genus *Pseudomonas* is categorized as the most important bacteria responsible for the pasteurization of milk spoilage during storage use. Therefore, the detection and enumeration of *Pseudomonas* should be useful to trace the source of contamination and hygienic status of milk.

According to Teh *et al.*, (2011) *Pseudomonas* colonies found in fresh milk tank is believed to be able to survive at a temperature of 7°C and produces an enzyme that is resistant to high temperatures. Aguirre *et al.*, 2009, add that the heat resistance among bacteria *Listeria innocua, Enterococcus faecalis, Salmonella enteric* serovar enteridis are different from *Pseudomonas fluorescens. P. Fluorescent* is more heat-resistant, and become more resistant to heat when it is in the milk, thus using the milk as the

media may affect the heat resistance of bacteria. The number of bacteria in media before heating process also affects the same number of bacteria after heating.

Heat Resistance

Table 1 show that, value of D and z on Chryseobacterium sp. PcRB003 is generally higher than *D. nishinomiyaensis* strain DSM 20448. *D. nishinomiyaensis* strain DSM 20448 shows a higher heat resistance than *Chryseobacterium sp*. PcRB003 at a temperature of 85°C. However, when the temperature was raised to 90°C and 95°C, *Chryseobacterium sp*. PcRB003 more resistant than the *D. nishinomiyaensis* strain DSM 20448. It is alleged that *Chryseobacterium sp* PcRB003 more adapting in high temperature than *D. nishinomiyaensis* stain DSM 20448. *Chryseobacterium sp*. PcRB003 has fat with a melting point higher than *D. nishinomiyaensis* strain DSM 20448.

The increase of branch chain saturated fatty acids, could lead to higher melting point and greater flexibility than the membrane. Composition of foodstuffs in the milk can protect bacteria against suspected heat thus improving the heat resistance. Water content, fat, salt, carbohydrates, protein and other substances can affect the heat resistance of microbes (Jay *et al*., 2005). Bacterial species will show different responses to heat, it is influenced by differences in strains because of differences in environmental factors such as temperature growth, medium growth and exposure to heat (Byrne *et al*., 2006). The number of microbes on the material also affects microbial heat resistance, because the higher number of microbes in food, is needed high temperatures with the longer time. In this study the number of *D. nishinomiyaensis* strains DSM 20 448 less when compared with the number of *Chryseobacterium sp*. PcRB003, so affect the heat resistance.

The conclusion of this research is: Thermoduric bacterial identification results in pasteurized milk in Indonesia by using PCR techniques are: *B. nesterenkovii* strain DSM 9573, *K. variance* G33, *Klebsiella sp*. A4, *Chryseobacterium sp*. PcRB003, *D. nishinomiyaensis* DSM 20 448 and *Pseudomonas otitidis* J11N with similar results respectively 99.8%, 99.4%, 94.7%, 100%, 100% and 100%. *Chryseobacterium sp*. PcRB003 is more resistant than *D. nishinomiyaensis* DSM 20448.

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