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THE PRESENCE OF INDIGENOUS YEASTS WITH PROTEOLYTIC ACTIVITY ISOLATED FROM HOMEMADE-MOZZARELLA WHEY

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Abstract

Whey, by product from cheese production, having high content of protein, can be a source of indigineous yeast with proteolytic activity. This research aims to identify indigenous yeasts with proteolytic activity from homemade-mozzarella cheese whey. There were 2 kinds of whey collected throughout the making of mozzarella, the first whey was collected after the coagulation process and the second whey was collected after mozzarella curdling process. Each types of whey were inoculated into Potato Dextrose Agar (PDA) media modified with 3% yeast extract and 10 ppm Amoxicillin and were further incubated for 48 hours in room temperature. Each colony formed were then differentiated macroscopically and purified in a separate modified PDA media for 4 times. Purified colonies were further identified under microscope and only the colonies having yeasts morphology were tested for its proteolytic activity with paving block method using Nutrient Agar plus 3% Skim Milk, with formation of clear zone were measured as proteolytic activity. Yeasts with proteolytic activity were identified using RapID yeast plus system. The results showed 7 yeasts colonies found and proteolytic activity was only found in 1 isolate with ±9.5 mm diameter of clear zone. The isolate was further brought to identification and revealed as Trichosporon beigelii.

Key words**:** *Indigenous yeast, mozzarella, proteolytic activity, Trichosporon beigelii, whey*

INTRODUCTION

Whey is a byproduct produced in cheese making and often causes environmental problems due to improper handling. Whey is formed from the separation between casein and milk fat when the process of adding acid to coagulate casein.

The nutrients contained in whey are lactose, fats such as triglycerides, diglycerides, fatty acids and phospholipids and minerals such as calcium, magnesium, phosphorus, potassium, chlorine, sodium, zinc, iron, iodine and copper, vitamins B5, B2, C , and B6, as well as minor proteins such as immunoglobulin, lactoferrin, lactoperoxidase, and lysozyme [8]. As a source of nutrients, whey is a really good environment for microorganisms to inhabit, such as yeast.

Natural whey starter, a traditional starter for the ripening of traditional Italian cheese, microbiota is mainly composed of thermophilic lactic acid bacteria but also contained some yeasts such as *Candida parapsilosis, Candida rugosa, Debaromyces hansenii, Kluyveromyces lactis, Kodamaea ohmeri, Torulaspora delbrueckii,* and *Zygosaccharomyces rouxii* [4].

Another natural whey starter collected from 9 factories of Tandil, Argentina showed to contain *Kluyveromyces marxianus, Saccharomyces cerevisiae, Clavispora lusitaniae,* and *Galactomyces geotrichum* [3]. Previous research has also showed the occurrence of *Candida lambica* from mozzarella whey [29].

A traditional Greek fermented whey product consists of *Zygosaccharomyces rouxii, Torulaspora delbrueckii, Debaromyces hansenii, Pichia farinosa, Candida mogii, Candida intermedia,* and *Saccharomyces cerevisiae* [16].

High protein that's contained in whey, indicates that whey can contain indigenous micoorganisms especially that have proteolytic activity.

Therefore, the current study had the purpose to identify indigenous yeasts with proteolytic activity from homemade mozzarella whey.

MATERIALS AND METHODS

Materials

Fresh unpasteurized cow milk, purchased from Ciparanje Dairy Farm organized by Faculty of Husbandry, University of Padjadjaran, Indonesia, used for making of mozzarella cheese whey. Citric acid (Brataco Chemika), rennet tablet, salt (Cap Kapal) and ice were also used as the materials for mozzarella cheese. Agar media used were Potato Dextrose Agar/PDA (Oxoid Ltd.) modified with 3% of yeast extract (Kraft Foods) and 10 ppm of Amoxicillin (Kimia Farma) for isolation and purification of yeasts and Nutrient Agar/NA (Oxoid Ltd.) with 3% of Skim Milk (Prolac, Pendairy) for proteolytic activity assay. Nutrient Broth (Oxoid Ltd.) and NaCl 0,85% were also used. Identification of yeast with proteolytic activity done by RapID yeast plus system (Remel Thermo Scientific).

Methods

Making of Mozarella Cheese Whey

The making of mozzarella were done according to Seth & Bajwa (2015) [25] with modification. 1 liter of fresh unpasteurized cow milk was pasteurized at 60^{o}C for 3 minutes and brought to cool until 35-40^{o}C. 200 ml of citric acid added to milk and stirred followed by incubation for 5 minutes to let the milk acidify. 0.02 g/l of rennet tablet, crushed, added to milk, and stirred, followed by another incubation for 1 hour to let the milk coagulate and form curd. The curd were cut and drained to separate from all whey. This whey is collected and described as Whey 1. In a separate container, cold water with addition of 2 tablespoons of salt and 2 tablespoons of Whey 1 was prepared and described as cooling agent.

The next step is the cooking of mozzarella, where a big pot, half full with water, was brought to boil and another smaller size pot filled with full water was put inside the big pot. The pots were heated until the water inside small pot reached 75^{o}C. A strainer was then put above the small pot until soaked and the curd was put on the strainer. Curd was pressed and folded using a spatula for approximately 10 minutes and then collected, leaving the remaining liquid as Whey 2. The elastic curd formed after the folding was soaked inside the cooling agent until harden and formed mozzarella cheese.

Isolation and Purification of Indigenous Yeast from Mozarella Cheese Whey

Isolation of indigenous yeast(s) were done by pour plate method using modified PDA, incubated for 48 hours at room temperature. Colonies appeared on the surface (aerobic) or at the base of media (anaerobic) were observed macroscopically for its characteristics and further differentiated from each other. Each colony were then purified by streak method on modified PDA for 4-5 times. Purified isolates were observed under microscope and only isolates having the morphological characteristics of yeasts were tested for proteolytic activity.

Proteolytic Activity of Indigenous Yeasts and Identification

Proteolytic activity assay done by paving block method as described by Putranto et al. (2015) [21]. Each isolates of indigenous yeasts were identified for its proteolytic activity with Na+3% skim milk. Each isolates was propagated by swabbing 1 loopful on the surface of modified PDA, incubated for 48 hours at room temperature. Meanwhile, NA with 3~~1~~% skim milk was pour into a petri dish, let harden, and a hole was punched on the agar. Isolate formed on the modified PDA were collected by the same way as the hole formed in NA and the collected agar was put inside the hole of NA skim milk. Incubation was done for 48 hours at room temperature and proteolytic activity was described as the formation of clear zone, which later being measured. Indigenous yeast showing proteolytic activity was identified using RapID yeast plus system.

RESULTS AND DISCUSSIONS

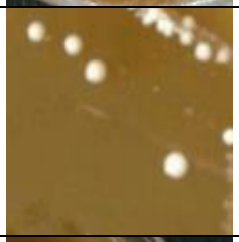
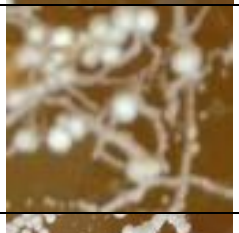
Morfological Identification of Yeast

Isolation of indigenous yeasts done with 2 type of mozzarella cheese whey.

Whey 1 attained from curdling process, while whey 2 attained after the curd was cooked and folded. Each of the whey inoculated and incubated in room temperature for two days.

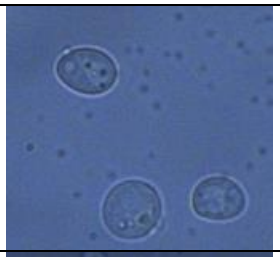
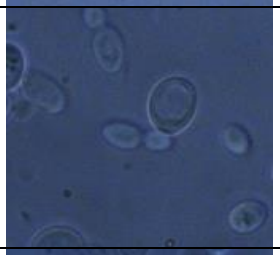
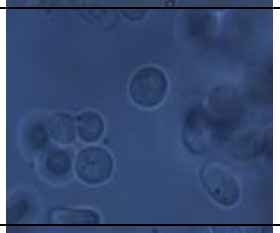
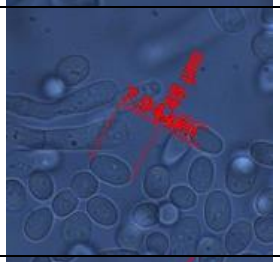
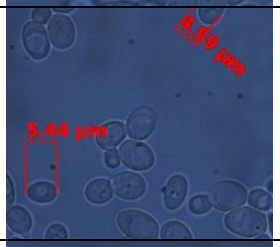
There were seven colonies formed based on macroscopic morphology that shown in Table 1. The colonies were further identified in microscope, which can be seen in Table 2.

Table 1. Macroscopic morphology of colonies

Colo-nies	Image	Morphology
A		Round; concave; yellowish colony
C		Round-shape; flat; white colony
D		Round-shape; yellowish
F		Round-shape; small; transparent
G		Round-shape; yellowish
H		Round-shape; yellowish
M		Oval shape; flat; white colony; small

Source: Own results.

Table 2. Microscopic morphology of colonies

Colo-nies	Image	Morphology
A		Round; oval; size ± 7.00 µm
C		Round; pseudomycelium; size ± 7.00 µm
D		Round; pseudomycelium; size ± 5.00 µm
F		Oval; pseudomycelium
G		Oval; pseudomycelium
H		Round/ oval
M		Long, cylindric

Source: Own results.

All seven colonies are categorized in to yeast according where yeast cell has length around 1-5 µm until 20-50 µm, and width sized 1-10 µm. Mostly all seven colonies unicellular and have oval, round and long shaped, and the other shaped resemblance to pseudomycelium shaped [11]. Colonies formed can be classified as yeast, because they were all grown in media with addition of antibiotics (amoxycillin), according to [18]

Proteolytic activity of yeast

Positive proteolytic activity was only shown in isolate D, as shown below (Fig. 1).

Fig 1. Proteolytic Activity
Source: Own results.

The appearance of clear zone is a result of yeasts activity which degrades the casein added as skim milk [23]. Though generally yeasts have proteolytic activity such as caseinolytic, aminopeptidase, and carboxypeptidase, the types of proteases as well as its activity among species or strains can be very different [7]. Secretion of proteases also dependent towards some factors such as the composition of the medium. For instance, supplementation of medium with protein increased the acidic protease activity secreted by *Candida humicola,* while supplementation of amino acid and ammonium sulfate resulted in low activity of the mentioned protease [22]. Enzyme also known to work specifically and have different degree of specificity. Proteases specificity on cleaving peptide bonds depend on the two amino acids that are bounded to each other [15].

Proteolytic Yeast RapID Identification

The resulting of biochemical activity of the tested isolates were shown on table 4. Biochemical activity has been compared with Electronic RapID Compendium (ERIC) database and concluded the isolate tested was *Trichosporon beigelii.* The sugar reduction activity of tested isolate showed some similarity towards another *Trichosporon* species, *Trichosporon moniliiforme,* which was isolated from curdling milk (table 3) [2014].

Table 3. Biochemical activity of tested isolates and sugar reduction activity of *Trichosporon moniliiforme.*

Substrate	**Tested Isolate**	***Trichosporon moniliiforme***
Glucose	-	-
Maltose	-	-
Sucrose	-	-
Trehalose	-	-
Raffinose	-	-
Lipid	-	unknown
NAGA	-	unknown
αGlucoside	+	unknown
bGlucoside	+	unknown
ONPG	-	unknown
αGalactoside	+	unknown
bFucoside	+	unknown
PHS	-	unknown
PCHO	-	unknown
Urea	+	unknown
Prolyne	+	unknown
Histidine	+	unknown
Leucyl-Glycine	+	unknown
Yeast Name	***Tri.beigelii***	

Trichosporon beigelii is a basidiomycetous and yeast-like organisms that are often considered as an opportunistic pathogen that cause *trichosporon*osis in immune-compromised people [13, 17]. *T. beigelii* is classified into family of Cryptococcaceae, under genus of *Trichosporon*, and it is normal flora found in respiratory and digestive tracts of human and animal [12]. *T. beigelii* is also a saprophyte in soil, water and other substrate as well [26]. This species of yeast are also found in the cuticule of freshwater crayfish *(Astacus astacus)* in Amsterdam [26] and on Spanish fermented sausages [10].

Though considered as opportunistic pathogen, *T. beigelii* is also found in some food product and so far no reports have ever mentioned that *T. beigelii* could cause foodborne illnesses. *T.*

beigelii is often found in dairy based products, both fermented and not. *T. beigelii* are common yeast found in raw milk along with other kind of yeast : *D. hansenii* and *K. marxianus* [14]. Beside raw milk, *T. beigelii* were also found in Armada cheese which made from unpasteurized goat's milk [28]. Armada cheese is a variety of cheese that is made without any addition of starter cultures, so it is likely that the ripening is caused by the activity of indigenous yeast that live in goat's milk. *T. beigelii* is also found even in cheese brines, a byproduct of cheese [24]. This explain why *T. beigelii* could be detected in whey, because whey are byproduct of cheese.

The proteolytic activity detected was considered low, as it only showed a faded color of the agar and not totally creating a clear zone. But, the result agreed with other research that showed the presence of proteolytic activity among *Trichosporon* spp. Research by Bentubo & Gompertz (2014) revealed that out of 44 clinically isolated *Trichosporon* spp., 22 isolates showed to possess proteolytic activity and 41% of which have a strong proteolytic activity [2]. However, it disagreed with Anitha et al. (2015) where none of 45 isolates of *Trichosporon* spp. collected from various clinical samples showed proteolytic activity [1].

The disagreement among results are caused by several factors that can interfere with the identification of proteolytic activity. Research by Anitha et al. (2015) mentioned that the absence of proteolytic activity might be caused by the Bovine Serum Albumin Agar method used, that lack of sensitivity when it comes to a very small proteolytic activity [1]. Therefore, it should be considered that the method used to identify proteolytic activity, is among factors that will define proteolytic activity among *Trichosporon* or any other yeasts.

Incubation temperature is also considered to be a determinant factor in identifying *in vitro* proteolytic activity. *T. asahii* and *T. inkin*, favored 37^{o}C over 25^{o}C for its proteolytic activity to be well detected [2]. Species of *Trichosporon* spp. will also define the degree of proteolytic activity observed. Furthermore, *T. beigelii* included a wide range of species and now it has been replaced by 51 accepted *Trichosporon* species [17]. Therefore, it is possible that *T. beigelii* observed in this research should have been a more specific *Trichosporon* species. *Trichosporon* species that has been observed and showed positive proteolytic activity are *T. asahii*, *T. mucoides*, *T. ovoides*, and *T. inkin* [2, 19]. Proteolytic activity was present in 66,7% (12 out of 18) isolates of *T. asahii*, 50% (2 out of 4) of *T. mucoides*, 50% (5 out of 10) of *T. inkin*, and 25% (3 out of 12) of *T. ovoides*. Strong proteolytic activity existed on 33,3% of *T. asahii*, 16,7% *T. ovoides*, and 10% of *T. inkin* [2]. Another study by Montoya et al. (2015) resulted the same, where 3 isolates of *T. asahii* Genotype I showed strong proteolytic activity and 6 isolates of showed very strong proteolytic activity [19].

Proteolytic activity in yeast such as *T. beigelii* which considered as a pathogen is very common. Proteolytic activity is included as a virulence factor, which defines the growth of pathogen inside the host and the establishment of the disease it causes [9]. According to Mariné et al. (2015), proteolytic activity is needed to penetrate the host's immunological barriers [17]. However, it is important to remember that proteolytic activity of the same yeast species could not be considered the same if collected from different sources due to the different nutrients availability [5]. Though the proteolytic activity of clinical *T. beigelii* and other *Trichosporon* spp. have been well documented, there is still a few observation that determines the proteolytic activity of *Trichosporon* spp. collected from food source, including a research conducted by Cardoso et al. (2015) that revealed positive proteolytic actvity in 1 out of 3 isolates of *Trichosporon* spp. and 2 out of 2 isolates of *T. montevideense* collected from Brazillian Serro Minas Cheese [6].

CONCLUSIONS

The isolation and identification of indigenous yeast with proteolytic activities in home-made mozzarella cheese whey were later indentified as *Trichosporon beigelii. T. beigelii* is not a

common yeast found in food product, and usually classified as pathogenic type of yeast. *T. beigelii* shows a weak proteolytic activity, as it only showed a faded color of the agar and not totally creating a clear zone. Though the proteolytic activity showed in agar, can vary depending on the substrate presence in the media.

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