Sys Rev Pharm 2021;12(1):1358-1366
A multifaceted review journal in the field of pharmacy

Mozzarella Whey Indigenous Yeasts and their Potential in Amino acid and Peptide Production through Fermentation

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ABSTRACT

One of the methodologies that can be applied in whey utilization is fermentation. The products or metabolites that can be produced from whey fermentation include amino acids and peptides. Amino acids and peptides are metabolites that have broad benefits both for the food industry and for human health. Amino acids and peptides can be produced through the breakdown of proteins by protease and peptidase enzymes secreted by microorganisms. The use of microorganism's types in fermentation will greatly affect the profile of amino acids and peptides produced, because each microorganism has a different proteolytic system. Indigenous yeast is one of the microorganisms that naturally live on whey and has the potential to be reapplied in whey fermentation to produce amino acids and peptides. Indigenous yeast has several advantages over other microorganisms, such as better adaptability to fermentation conditions and the ability to grow and dominate the substrate more easily and quickly. Some of the yeasts with proteolytic activity that have been isolated from whey are *Candida lambica, Candida parapsilosis, Candida rugosa, Debaromyces hansenii, Kluyveromyces lactis, Kodamaea ohmeri, Torulaspora delbrueckii, Zygosaccharomyces rouxii, Candida ethanolica, Candidium pseudolasci, Pichia farinosa, Candida mogii, Candida intermedia, Saccharomyces cerevisiae, Kluyveromyces marxianus, Clavispora lusitaniae, and Galactomyces geotrichum*. *Candida tropicalis*, *Trichosporon beigelii*, and *Blastoshizomyces capitatus* were reported as three indigenous yeasts isoalted from mozzarella whey. The yeasts are able to release the aspartate protease enzyme and produce peptides and amino acids that show various benefits including health benefits.

Keywords: Amino Acid, Fermentation, Indigenous Yeasts, Peptides, Mozzarella Whey

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INTRODUCTION

Whey is the liquid fraction obtained after the clumping and separation of casein during the cheese production process and often causes pollution, both on soil and water (1,2). This problem arises because there is no further processing before disposal of whey into the environment. The lactose content in whey can reach 70-72% of the total solids present, so the need for oxygen to break down lactose is very high (3). As a result, the BOD and COD values of whey are large, namely 40,000-60,000 mg / l and 50,000-80,000 mg/l (4).

Apart from the large risk of pollution, the problems caused by whey are also caused by the large volume of whey production. Whey production during cheese production can reach 80-90 liters of whey for every 100 liters of milk used (5). According to Illanes (6), world whey production is estimated at 150 to 200 million tonnes/year with an annual average increase of approximately 2%. Therefore, whey processing is a very important thing to do. Unfortunately, according to Slavov (7), as much as 47% of whey produced in the world is directly discharged into the environment without further treatment.

Various weaknesses in the waste treatment method cause the industry to be less sympathetic in treating whey waste. For example, the high cost of reagents and the low efficiency of COD removal in the physicochemical method commonly used to process whey (4). In addition, other methods such as biological methods, both through anaerobic and aerobic digestion, also have several drawbacks, such as the accumulation of volatile fatty acids and sludge formation which causes process delays, thus increasing maintenance costs (8). Therefore, disposal of whey waste to the environment without further treatment has a very dangerous effect on the surrounding environment, so we need an alternative method or technology to treat the whey waste.

One alternative method that can be applied in whey processing is fermentation. Fermentation itself can be defined as any production process of a product with the help of microorganism culture (9). Fermentation can be applied to whey because of the complete nutritional content of whey, namely 4-5% lactose, 0.6-1% protein, 0.4-0.9% minerals and vitamins, so that it can support the life of microorganisms (10). Fermentation has advantages compared to other processing technologies, namely that it can improve product quality due to nutrient enrichment through certain metabolites produced, such as vitamins, amino acids, and proteins, as well as the release of certain components that are beneficial to humans (11).

One of the products or metabolites that can be produced from whey fermentation are amino acids and peptides. Amino acids and peptides are metabolites that have broad benefits both for the food industry and for human health. Some amino acids have a function as food additives, for example cysteine as a dough conditioner, glutamic acid as a flavoring, phenylalanine as a flavoring and aspartame sweetener precursor, and so on (10,12). Amino acids, both essential and non-essential, also play a role in various biological activities of the body, for example in the immune system, neurology, antioxidative responses, protein synthesis, reproduction, and growth, which will affect human health (13). Likewise, several peptides that have biological activity such as antimicrobials, antioxidants, antihypertensives, immunomodulators, and antidiabetic (2,14).

The enrichment of nutrients during fermentation, including the production of amino acids and peptides, can occur due to the metabolic activity of microorganisms, which causes the formation of certain new metabolites or products. These metabolites or products can be formed through various mechanisms, for example through the hydrolysis of sugars to produce simple acids, alcohol, or carbon dioxide, or through biotransformation reactions such as removing residual glycol groups to form new compounds that have health benefits (15).

Meanwhile, amino acids and peptides can also be produced through protein breakdown by protease and peptidase enzymes secreted by microorganisms (16). The breakdown of complex molecules into simple molecules during fermentation is one of the main mechanisms for microorganisms to meet their nutritional needs. Complex molecules have a large size so that they cannot enter the cell membrane of microorganisms, therefore microorganisms secrete certain enzymes to simplify complex molecules, such as proteins, into simple molecules, such as peptides or amino acids, which are smaller in size (17).

The use of types of microorganisms in fermentation will greatly affect the profile of amino acids and peptides produced, because each microorganism has a different proteolytic system, for example the type and amount of protease enzymes that can be secreted (18). Indigenous yeast, namely yeast that naturally lives in a material, has the potential to be applied to whey fermentation to produce these amino acids and peptides (10). Indigenous yeast even has several advantages over other microorganisms, such as better adaptability to fermentation conditions and the ability to grow and dominate the substrate more easily and quickly (19).

Several studies have succeeded in isolating yeasts with proteolytic activity in whey and whey-based products such as *Kluyveromyces marxianus, Clavispora lusitaniae, Galactomyces geotrichum, Candida parapsilosis, Cryptococcus albidus, Kodamaea ohmeri, Zygosaccharomyces rouxii* (20–22). However, so far the research that has investigated the profile of amino acids and peptides produced by indigenic yeast during whey fermentation is still limited. Therefore, this article is expected to provide new insights regarding the biodiversity of yeast contained in whey waste and the potential of these indigenic yeasts in terms of fermentation to produce useful metabolites, especially amino acids and peptides.

Potential of Whey as a Fermentation Substrate in Producing Amino Acids and Peptides

Whey contains 55% of the nutrients contained in milk, so it is a waste that is rich in nutrients and can still be further utilized for various needs (20). In general, whey is classified into 2 types based on the coagulation technique, namely acidic whey and sweet whey. Acidic whey is whey resulting from making cheese which is coagulated using mineral acids, acetic acid, or through fermentation of microorganisms, and has a low pH of 4.3-4.6 (23). Meanwhile, sweet whey is produced from making coagulated cheese using rennet enzymes and has a pH of 5.9-6.6 (24).

Table 1 shows that the nutritional content of sweet whey and acidic whey has slight differences, one of which is the mineral content, especially calcium. Acidic whey's higher mineral content is due to different coagulation techniques used during cheese making. As is well known, sweet whey is produced from cheese coagulated using rennet which works by cutting and inactivating kappa casein so that it becomes para-kappa-casein. This para-kappa casein does not have the ability to stabilize the structure of casein micelles, causing calcium-micelle casein to precipitate and form a curd (25).

Meanwhile, in acidic whey, the addition of acid and low pH causes colloidal calcium phosphate (CCP) to dissociate from the neutralization of the negative micelle charge (26). This causes CCP to become insoluble and casein is released into the serum, until finally casein forms an aggregate and coagulates at isoelectric pH (27).

Table 1. Nutritional Content of Sweet Whey and Acidic Whey (4)

Compound	*Sweet Whey* (g/L)	*Acidic Whey* (g/L)
Total Solid	63-70	63-70
Lactose	46-52	44-46
Protein	6-10	6-8
Calcium	0.4-0.6	1.2-1.6
Phosphate	1-3	2-4.5
Lactate	2	6,4
Chloride	1.1	1.1

The difference in the mechanism between rennet and acid shows that coagulation using acid causes the release of calcium into whey, while rennet can maintain calcium to bind to casein (26). Therefore, the calcium content in acidic whey is higher than sweet whey. The use of mineral acids such as hydrochloric or sulfuric acid can also cause high chlorides and sulfates.

Another difference in the content of the two types of whey is in the protein content. This is due to differences in the degree of casein hydrolysis or proteolysis during cheese making. The degree of proteolysis can be seen from the remaining protein content and the amino acid and peptide content formed after coagulation. During coagulation, a number of proteins will be converted into amino acids or peptides, so that the protein content will decrease while amino acids and peptides will increase. The higher the protein content, the lower the amino acid and peptide content, which means that the degree of proteolysis is low and vice versa.

In general, the protein content in acidic whey is lower than sweet whey, especially acidic whey which is produced through fermentation of microorganisms (10). Therefore, the high protein in rennet indicates that the proteolytic activity of the enzyme is not as high as the proteolytic activity of microorganisms. This is in line with the free amino acid content in sweet whey which is only 4 times higher than milk, while acidic whey contains 10 times higher free amino acids than milk (28). In addition, the peptide content in acidic whey also tends to be more complete than sweet whey (29).

Not only limited to coagulation techniques, other factors, such as the type and quality of milk, coagulation time and temperature, and so on, also cause the nutritional content of whey to vary (4). The type of milk used will affect the composition of the whey produced, for example Cheddar cheese whey which is made from a mixture of Jersey milk and Holstein-Friesian milk. The use of Jersey milk causes whey to have a low protein content, but a high lactose and total solid content (30). Cheese that was coagulated at 33 ºC and 36 ºC also produced whey which was higher in fat than coagulation at 27 ºC and 30 ºC. This is due to the fact that the gel and curd tissue structure formed is irregular and less continuous, and the fat globules are thinner so that fat mobility is high (31).

Each process unit involved in the production of a type of cheese can produce different types of whey. An example is Mozzarella cheese which produces two types of whey during processing. The first whey is the liquid fraction that remains after separating from the coagulated (curd) casein. Meanwhile, the second whey, or commonly referred to as stretchwater, is formed during stretching of the curd formed, so that the curd has a stretchable or elastic texture (32). This stage is done by cooking while stretching the curd in water. The remaining water from the stretching process is known as the second whey.

The two types of whey have different compositions as shown (Table 2). This is because the treatment experienced by the two types of whey is different. The lower lactose content in whey 2 can be caused by heat treatment so that the lactose transforms into lactulose and acid (33). Meanwhile, whey 2 contains higher casein than whey 1. The casein content in whey 2 is caused by the presence of casein from curd which releases again during curd stretching. This is directly proportional to whey 1 which contains almost no whey 2, because most of the casein in whey 1 has coagulated into curd. This shows that the treatment received by each whey during processing will result in different compositions, even though the whey comes from the production of the same type of cheese.

Table 1. The composition of whey and Stretchwater (34)

Compound	Whey	Stretchwater
Lactose (g/100 g)	4.13	3.54
Mineral (mM)		
Cl	28.62	43.21
Ca	16.26	41.47
K	41.10	37.03
Mg	3.97	5.82
Na	18.59	49.10
Nnitrogen Fraction (g/100 g)		
Non protein Nitrogen	0.18	0.20
Casein	0.06	0.21
Whey protein	0.68	0.32
Total N	0.91	0.73

Even though each type of whey has a different nutritional composition, whey is still a waste that is rich in nutrients so that it can be a substrate for fermentation by microorganisms. The nutrients needed by microorganisms are generally divided into two, namely macronutrients, such as carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^{+} which are needed in amounts above 10^{-4}M, and the micronutrients Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Na^{+}, vitamins, growth hormones, and certain precursors are needed in amounts less than 10^{-4}M (35). All the nutritional requirements of these microorganisms are contained in whey, so that whey can be a fermentation substrate and has the potential to produce useful metabolites, especially peptides and amino acids.

Yeasts Biodiversity in Whey and Its Potential in Producing Amino Acids and Peptides

Food products and their derivatives contain various types of microorganisms naturally or commonly known as indigenic microorganisms. Indigenous microorganisms can be used as a starter in fermentation to produce a variety of new products, including amino acids and peptides. One type of microorganism that can be used is indigenous yeasts. Indigenous yeasts has advantages compared to modified yeasts from the laboratory, such as better adaptability to fermentation conditions and the ability to grow and dominate the substrate more easily and quickly (36). Yeasts also has several advantages that make it superior to other microorganisms, for example, the living temperature range of yeast, which is between 20ºC to 30ºC or room temperature, so it doesn't require complex temperature settings during fermentation. Yeasts can also live optimally on a slightly acidic substrate with a pH of 4.5 to 5.5, so it can live in both sweet and acidic whey (37). Some other advantages are the ability of yeasts to deal with stressful conditions such as high.

Indigenous yeasts have been isolated from milk-based products, including cheese. The presence of indigenic yeast in cheese is caused by the high tolerance of yeast to extreme conditions such as low pH and Aw, high salt concentrations, and low temperatures during ripening (38). Types of yeasts commonly found in traditional cheeses are *Debaromyes hansenii, Yarrowia lipolytica, Geotrichum candidum, Kluyveromyces marxianus, Kluyveromyces lactis, Candidum zeylanoides, Candida utilis, Candida kefyr,* and *Saccharomyces cerevisiae* (39). Several other types of yeasts isolated from various types of cheese can be seen in table 3. Table 3 shows that the yeasts biodiversity in cheese is very diverse and different for each type of cheese. One type of cheese does not necessarily contain the same type of yeasts. This shows that there are factors that affect the presence of types of yeasts, especially factors related to production, ranging from raw material preparation, processing, to storage, which can affect the biodiversity of yeasts found in cheese.

One of the factors affecting yeasts biodiversity in cheese is the use of a starter. A starter added to cheese is not always a commercial starter, but it can also be a natural starter such as the natural whey starter (NWS). Traditional cheeses from Italy are usually fermented using NWS. NWS itself is whey produced from cheese on the previous day and left to stand, so that it contains certain indigenous microorganisms that play a role in ripening the cheese (20). Several types of yeasts, namely *Candida parapsilosis, Candida rugosa, Debaromyces hansenii, Kluyveromyces lactis, Kodamaea ohmeri, Torulaspora delbrueckii,* and *Zygosaccharomyces rouxii* are yeasts found in both NWS and Canastra cheese.

Table 2. Yeasts Biodiversity in Different Types of Cheese

Cheese Type	Yeasts
Cottage	*Geotrichum candidum; Yarrowia lipolytica; Kluyveromyces marxianus; Candida lusitaniae; Kluyveromyces lactis; Pichia membranifaciens; Pichia fermentans; Pichia kluyverii; Candida sake; Candida glabrata; Dekkera bruxellensis; Pichia kluyverii; Candida milleri; Candida rugose*
Camembert	*Yarrowia lipolytica; Debaromyces hansenii; Candida catenulate; Candida parapsilopsis; Trichosporon Inkin; Metschnikowia reukaufii; Cryptococcus laurentii;*
Blue-Veined	*Debaryomyces hansenii; Yarrowia lipolytica; Candida mesenterica; Rhodotorula mucilaginosa; Cryptococcus curvatus*
Soft Cheese	*Geotrichum candidum; Debaryomyces hansenii*
Goat's-milk	*Geotrichum candidum; Kluyveromyces marxianus*
Trappist	*Debaryomyces hansenii; Debaryomyces hansenii; Kluyveromyces lactis; Candida maltose; Torulaspora delbrueckii*
Ementhaler	*Debaryomyces hansenii*
Sweet Cottage	*Candida catenulate; Saccharomyces exiguus*
Pecorino Crotonese	*Candida inospicua; Candida intermedia; Pichia carsonii; Debaromyes hansenii; Yarrowia lipolytica; Kluyveromyces lactis; Saccharomyces cerevisiae*
Canastra	*Kluyveromyces lactis; Torulaspora delbrueckii; Kodamaea ohmeri; Candida intermedia*
Feta	*Debaromyces hansenii; Candida famata; Kluyveromyces lactis; Candida sphaerica; Saccharomyces cerevisiae*
Asiago	*Debaromyces hansenii; Candida famata*
Montasio	*Kluyveromyces marxianus; Candida kefyr*
Monte Veronese	*Debaromyces hansenii; Candida famata; Torulaspora delbrueckii; Candida colliculosa*
Caprino	*Yarrowia lipolytica; Kluyveromyces lactis; Candida sphaerica*
Mozzarella	*Saccharomyces cerevisiae; Kluyveromyces marxianus; Candida kefyr; Yarrowia lipolytica*
Gubbeen	*Debaromyces hansenii; Candida catenulata; Candida lusitaniae; Trichosporon ovoides; Candida parapsylosis; Candida intermedia; Pichia guillermondii*
Taleggio	*Debaryomyces hansenii, Kluyveromyces lactis, Kluyveromyces marxianus, Yarrowia lipolytica, Pichia Guilliermondii; Torulaspora delbrueckii; Candida sake; Candida etchellsii*
Salers	*Kluyveromyces lactis*; *Kluyveromyces marxianus; Saccharomyces cerevisiae*; *Candida zeylanoides; Debaryomyces hansenii;Candida parapsilosis; Candida silvae; Candida intermedia; Candida rugosa; Saccharomyces unisporus; Pichia guilliermondii*

Sources: (20,40–46)

However, not all yeasts found in the NWS are also found in the resulting Canastra cheese. *Candida ethanolica, Candida pseudolambica, Candida zeylanoides,* and *Cryptococcus albidus* are yeasts found in NWS, but not found in Canastra cheese (42). The difference between yeast in cheese and NWS is caused by during ripening events, such as the development of a particular species, lysis of certain cells, and DNA degradation, which lead to changes in yeasts population (46).

On the other hand, the use of whey as a starter in cheese production also indicates that whey contains an indigenous yeast. However, so far, the research on indigenous yeasts in whey is still limited. One of them is research by Utama et al. (47) who isolated *Candida lambica* from Mozzarella cheese whey. Manouri, a typical Greek cheese made from naturally fermented whey, contains yeasts such as *Pichia membranefasciens, Zygosaccharomyces rouxii, Torulaspora delbrueckii, Debaromyces hansenii, Pichia farinosa, Candida mogii, Candida intermedia,* and *Saccharomyces cerevisiae* (48). Another NWS collected from 9 cheese factories in Tandil, Argentina, contains *Kluyveromyces marxianus, Saccharomyces cerevisiae, Clavispora lusitaniae,* and *Galactomyces geotrichum* (38). Therefore, the biodiversity of yeasts in whey and cheese is influenced by various factors, such as the quality of the milk used, the handling and heat treatment of milk, the starter culture used, the temperature and humidity during curing, the amount and method of adding salt, the equipment used, the intervention. workers and cheese exposure to outside microorganisms during and after processing (22).

Indigenous yeast found in whey and cheese has the potential to be used as a starter in whey fermentation to produce amino acids and peptides. Therefore, in terms of fermentation to produce amino acids and peptides, the proteolytic activity of indigenous yeasts is an important parameter. Yeast has proteolytic activity such as caseinolytic, aminopeptidase, and carboxypeptidase which varies from one species to another, even between different strains under the same species (49).

Some indigenous yeasts found in cheese or whey that have proteolytic activity are *Kluyveromyces marxianus, Clavispora lusitaniae, Galactomyces geotrichum, Candida catenulata, Candida parapsilosis, Cryptococcus albidus, Issatchenkia orientalis, Rhodotorula glutinis, Rhodacotorula mucilaginosa, Zyrowspora Candida intermedia, Galactomyces candidum, Kodamaea ohmeri, Trichosporon sp.,* and *Trichosporon montevideense* (38,50). Research by Chaves-López et al., (51) showed proteolytic activity as well as increased amino acid and peptide production by *Torulaspora delbrueckii*, *Rhodotorula mucilaginosa, Clavispora lusitaniae*, and *Kluyveromyces marxianus* in skim milk fermentation. *Torulaspora delbrueckii* demonstrated the ability to produce anti-ACE bioactive peptides with an inhibition value of 74% (52). *Clavispora lusitaniae* has a total protein activity of 1.35-1.99 mg / ml and produces leucine free amino acid concentrations of 0.11-0.17 mM. Meanwhile, *Rhodotorula mucilaginosa* with a total proteolytic activity of 0.66-1.22 mg / ml can produce the highest increase in free amino acid concentrations compared to *Kluyveromyces marxianus* and *Torulaspora delbrueckii*, namely 0.16-0.22 mM leucine (53).

The fermentation of skim milk by *Kluyveromyces marxianus* shows the ability of the yeast to hydrolyze milk protein into bioactive peptides which have ACE inhibitory activity with an ACE inhibition value of 80% (51). In addition, *Kluyveromyces marxianus* was also shown to be able to hydrolyze α-lactalbumin and β-lactoglobulin in whey by up to 33.7% and produce a peptide chart area of 1.45 AU (54).

Fermentation of milk by *Candida catenulata* at 10ºC and 25ºC produced the highest total amino acids than *Debaromyces hansenii* and *Kluyveromyces marxianus*, with the dominance of the amino acid's leucine, phenylalanine, lysine, arginine, and glutamic acid. Meanwhile, the dominant amino acids in milk fermented by *Debaromyces hansenii* are glutamic acid, proline, glycine, alanine, and arginine, while the amino acids in fermentation by *Kluyveromyces marxianus* are leucine, valine, alanine, isoleucine, and phenylalanine (55). Another Candida species, namely *Candida parasilopsis*, can also produce peptides with biological activity. Co-culture of *Candida parapsilosis* and *Lactobacillus paracasei* isolated from Comte cheese can produce Trp-Leu-Ala-His-Lys (α-la f (104–108)) peptide which has ACE (antihypertensive) activity in goat whey fermentation (56).

Fermentation of milk by *Galactomyces geotrichum* was also proven to produce a total amino acid of 0.196 mM leucine after 48 hours of fermentation. This yield is even higher than milk fermentation by *Lactobacillus plantarum*, *Enterococcus faecalis* and coculture (57).

Mozzarella Whey Indigenous Yeast and the Potential of Peptide and Amino Acid Production

There are three indigenous yeasts in whey mozzarella, namely *Candida tropicalis*, *Trichosporon beigelii*, and *Blastoshizomyces capitatus* (58). The three yeasts are reported to be found in food and non-food sources and have been shown to have proteolytic activity.

Candida tropicalis has been found in cheese products and other milk-based products, such as Serro Minas cheese (Brazil), Kumis (Colombian fermented milk), and Amasi (Zimbabwean fermented milk) (50,51). *Trichosporon beigelii* was isolated in various types of cheese such as Crescenza (Italy), Serro Minas, Graukase (Austrian cheese), and cheese brine (50,59). *Geotrichum capitatum*, another name for *Blastoschizomyces capitatus*, is found in milk, curd during cheese production, Suucuk (a typical Turkish fermented sausage), cheese brine, and from membrane reverse osmosis apparatus in milk processing plants (60,61).

Candida tropicalis has been shown to have proteolytic activity, one of which is by secreting the aspartate protease enzyme. Zaugg et al. (62) showed that five of the six *Candida tropicalis* strains tested had proteolytic activity on BSA (bovine serum albumin) medium and contained the SAPT1 gene, one of the genes that allows the secretion of aspartate protease in microorganisms. *Candida tropicalis* isolated from Kumis, fermented milk typical of Colombia, showed proteolytic activity of 1.45-1.91 mg / ml and caused an increase in free amino acids by 0.08-0.18 mM of leucine in fermented milk. A total of 2 isolates from 11 *Candida tropicalis* isolated even showed the ability to produce bioactive peptides with Angiotengsin Converting Enzyme (ACE) inhibition activity of a minimum of 8.69% and a maximum of 10.11% (63).

Trichosporon beigelii, which has been reclassified into 51 other *Trichosporon* species, has various proteolytic activities (64). The proteolytic activity of *Trichosporon beigelii* has been widely described in various studies. As many as 50% of *Trichosporon cutaneum* isolates isolated from cow's milk, goat, buffalo, sheep, and cheese have proteolytic activity on gelatin (65). In fact, optimization of the production of the protease enzyme from one of the *Trichosporon species*, namely *T. japonicum* has been carried out with the production of 4758.4 U / mg of protease at its optimum conditions (66).

T. ovoides, isolated from salt solution in the production of Limburger and Munser cheese, showed proteolytic activity which was characterized by increasing concentrations of water soluble peptides and amino acids (WSN) and non-protein nitrogen (NPN). The WSN concentration in the blank was 22.7%, while the WSN concentration in the cheese curd slurry inoculated with *T. ovoides* was 27.6%. Meanwhile, the NPN concentration in the cheese curd slurry was 6.3% greater than the blank which was only 5.1% (21).

T. asahii and *T. insectorum* isolated from Kumis were shown to have proteolytic activity. *T. asahii* and *T. insectorum* were respectively found in Kumis with a percentage of 1.08% and 2.1%. *T. asahii* has proteolytic activity of 1.62-1.82 mg / ml and produces free amino acids of 0.14-0.18 mM leucine. Meanwhile, *T. insectorum* has a proteolytic activity of 1.28-2.1 mg / ml and produces free amino acids of 0.12-0.26 mM leucine (63).

T. asahii isolated from Manteca, a fermented whey product from Italy, has proteolytic activity on gelatin and casein. The caseinolytic activity of *T. asahii* can produce 100 mg of free amino acids per 100 ml of sample, for 7 days of fermentation. Some strains even produce 700 mg of free amino acids per 100 ml of sample. The fermentation of Manteca by *T. asahii* also produces biogenic amines such as phenylethylamine, putrescine, cadaverine, histamine, and spermidin, as a result of its proteolytic activity. The highest production of biogenic amines was spermidin which reached 3.54-4.97 mg / kg for 2 days of fermentation and 14.6-15.4 mg / kg for 7 days of fermentation (67).

Other studies have shown that *Geotrichum capitatum* is the best producer of protease enzymes. However, there are several factors such as carbon source supplementation, nitrogen source and incubation temperature that can affect protease production. Maltose supplementation as the only carbon source resulted in maximum protease production, which was 75.9 U / mg protein. Peptone supplementation as a nitrogen source can also encourage protease production up to 88.6 U / mg protein. The resulting protease has a maximum activity at an incubation temperature of 30º C (68).

The amino acid composition produced by yeast will be influenced by the type of protease it secretes. This is because enzymes work specifically and have different degrees of specification. These specifications are determined by the peptide bond between the two amino acids that the protease can cut. Therefore, the peptides or amino acids produced by each protease will be different (14).

Candida tropicalis, *Trichosporon beigeilii*, and *Blastoschizomyces capitatus* can secrete the enzyme aspartate protease. Dinika et al. (5) showed that *Candida tropicalis* can secrete aspartate protease with different proteolytic activity, depending on the temperature and incubation time. *Trichosporon beigelii*, specifically *Trichosporon asahii*, can secrete aspartate peptidase with an atomic mass of 30 kDa (69). Meanwhile, research by Pontieri et al. (70) showed that 3 out of 16 strains of *Blastoschizomyces capitatus* tested could secrete aspartate protease. Several enzymes classified as aspartate proteases, such as pepsin, catepsin, and chymosin, have been shown to be able to produce peptides (71). The hydrolysis of whey and casein goat milk by pepsin has been shown to produce bioactive peptides with residues of leucine, proline, histidine, serine and threonine which contribute to the antioxidant activity of the hydrolyzates produced (72). Pepsin can also produce bioactive peptides from whey with sequences of KVAGT, VRT, IRL, PEGDL, LPMH, EKF, LKPTPEGDL, LKPTPEGDLEIL, IPAVFKIDA, WLAHKAL, WLAHKALCSEKLDQ, LAHKALCSEKL, LKPTPEGDLEIL, IPAVFKIDA, WLAHKAL, WLAHKALCSEKLDQ, LAHKALCSEKL, LAHKALCSEKL (1,2,10,18). Therefore, *Candida tropicalis, Trichosporon beigelii* and *Blastoschizomyces capitatus* have the potential to produce peptides with the amino acid composition above.

Apart from aspartate protease, *Candida tropicalis* can also secrete serine protease. Research by Portela et al., (73), showed that *Candida tropicalis* produces serine proteases with atomic masses of 52, 56, and 62 kDa. The resulting serum protease can also hydrolyze bovine serum albumin, human serum albumin, laminin, and fibrinonectin.

As many as one third of all proteases are serine proteases, such as trypsin (Di Cera, 2009). Trypsin has been shown to be able to produce peptides from whey protein with various sequences, namely VAGTWY, ALPMHIR, VFK, LAMA, LDAQSAPLR, WLAHK, VGINYWLAHK, AASDISLLDAQSAPLR, IPAVFK, VLVDTDYK, EQLTK, IPAVFD, with the potential activity of anti-AIDS, anti-hypertension and anti-HIV (1,2,10,73). Therefore, *Candida tropicalis* with its serine protease secretion also has the potential to produce peptides with the above composition.

Concluding Remarks

The studies show that some indigenous yeasts in whey and cheese have been shown the ability in producing certain peptides and amino acids during fermentation. Therefore, the use of indigenous yeasts as a starter in whey fermentation has the potential to increase the production of peptides and amino acids. Three indigenous yeasts such as *Candida tropicalis, Trichosporon beigelii* and *Blastoschizomyces capitatus* were found in mozzarella cheese whey and shown the potential in producing peptides and amino acids with the highest protein hydrolysis occurred at the mesophilic temperature (10-25ºC). However, the production of peptides and amino acids during fermentation are both influenced by certain factors depend on the yeast's species. These factors affect the lifecylce and metabolic activity of yeasts so that it will have an impact on the amount and composition of amino acids and peptides produced. Further, some sequences of amino acids and peptides produced were shown functional and health effects such as ACE inhibitory activities so that need to be measured in future study.

ACKNOWLEGDMENT

Authors would like to thank the Deputy for Research and Development Strengthening, Ministry of Research and Technology / National Research and Innovation Agency of Indonesia who has funded this research through the scheme of Penelitian Dasar Unggulan Perguruan Tinggi with grant number 1827/UN6.3.1/LT/2020.

REFERENCES

1. Brandelli A, Daroit DJ, Corrêa APF. Whey as a source of peptides with remarkable biological activities. Food Res Int. 2015 Jul 1;73:149–61.
2. Dinika I, Verma DK, Balia R, Utama GL, Patel AR. Potential of cheese whey bioactive proteins and peptides in the development of antimicrobial edible film composite: A review of recent trends. Trends Food Sci Technol. 2020 Sep 1;103:57–67.
3. Isfari D, Gemilang Lara U. Cheese whey as potential resource for antimicrobial edible film and active packaging production. Foods Raw Mater. 2019 Oct 31;229–39.
4. Chatzipaschali AA, Stamatis AG. Biotechnological Utilization with a Focus on Anaerobic Treatment of Cheese Whey: Current Status and Prospects. Energies. 2012 Sep 10;5(9):3492–525.
5. Dinika I, Nurhadi B, Masruchin N, Utama GL, Balia RL. The Roles of Candida tropicalis Toward Peptide and Amino Acid Changes in Cheese Whey Fermentation. Int J Technol. 2019 Dec 9;10(8):1533.
6. Illanes A. Whey upgrading by enzyme biocatalysis. Electron J Biotechnol [Internet]. [cited 2020 Dec 6];14(6). Available from: http://www.bioline.org.br/abstract?ej11069
7. Slavov AK. General Characteristics and Treatment Possibilities of Dairy Wastewater – A Review. Food Technol Biotechnol. 2017 Mar;55(1):14–28.
8. Poh PE, Gouwanda D, Mohan Y, Gopalai AA, Tan HM. Optimization of Wastewater Anaerobic Digestion Using Mechanistic and Meta-heuristic Methods: Current Limitations and Future Opportunities. Water Conserv Sci Eng. 2016 Jun 1;1(1):1–20.
9. Stanbury PF, Allan Whitaker, Hall SJ. Principles of fermentation technology. 3rd ed. Oxford [Oxfordshire] ; New York: Pergamon Press; 2017. 255 p. (Pergamon international library of science, technology, engineering, and social studies).
10. Mazorra-Manzano MA, Robles-Porchas GR, González-Velázquez DA, Torres-Llanez MJ, Martínez-Porchas M, García-Sifuentes CO, et al. Cheese Whey Fermentation by Its Native Microbiota: Proteolysis and Bioactive Peptides Release with ACE-Inhibitory Activity. Fermentation. 2020 Mar;6(1):19.
11. Melini F, Melini V, Luziatelli F, Ficca AG, Ruzzi M. Health-Promoting Components in Fermented Foods: An Up-to-Date Systematic Review. Nutrients [Internet]. 2019 May 27 [cited 2020 Dec 6];11(5). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6567126/
12. Bicas J, Maróstica Jr. M, Pastore G, editors. Biotechnological Production Of Natural Ingredients For Food Industry [Internet]. BENTHAM SCIENCE PUBLISHERS; 2016 [cited 2020 Dec 6]. Available from: http://www.eurekaselect.com/143636/volume/1

13. Wu G. Functional Amino Acids in Growth, Reproduction, and Health12. Adv Nutr. 2010 Nov 16;1(1):31–7.
14. Jakubczyk A, Karaś M, Rybczyńska-Tkaczyk K, Zielińska E, Zieliński D. Current Trends of Bioactive Peptides—New Sources and Therapeutic Effect. Foods [Internet]. 2020 Jun 29 [cited 2020 Dec 6];9(7). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7404774/
15. Phang-Lyn S, Llerena VA. Biochemistry, Biotransformation. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 [cited 2020 Dec 6]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK544353/
16. Nguyen TTH, Myrold DD, Mueller RS. Distributions of Extracellular Peptidases Across Prokaryotic Genomes Reflect Phylogeny and Habitat. Front Microbiol [Internet]. 2019 [cited 2020 Dec 6];10. Available from: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00413/full
17. Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A, et al. Microbial Proteases Applications. Front Bioeng Biotechnol [Internet]. 2019 Jun 12 [cited 2020 Dec 6];7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6584820/
18. Daliri EB-M, Oh DH, Lee BH. Bioactive Peptides. Foods. 2017 May;6(5):32.
19. Varela C, Sundstrom J, Cuijvers K, Jiranek V, Borneman A. Discovering the indigenous microbial communities associated with the natural fermentation of sap from the cider gum Eucalyptus gunnii. Sci Rep. 2020 Sep 7;10(1):14716.
20. Andrade RP, Melo CN, Genisheva Z, Schwan RF, Duarte WF. Yeasts from Canastra cheese production process: Isolation and evaluation of their potential for cheese whey fermentation. Food Res Int. 2017 Jan;91:72–9.
21. Fröhlich-Wyder M-T, Arias-Roth E, Jakob E. Cheese yeasts. Yeast. 2019;36(3):129–41.
22. Banjara N, Suhr MJ, Hallen-Adams HE. Diversity of Yeast and Mold Species from a Variety of Cheese Types. Curr Microbiol. 2015 Jun;70(6):792–800.
23. Lievore P, Simões DRS, Silva KM, Drunkler NL, Barana AC, Nogueira A, et al. Chemical characterisation and application of acid whey in fermented milk. J Food Sci Technol. 2015 Apr;52(4):2083–92.
24. León-López A, Pérez-Marroquín XA, Campos-Lozada G, Campos-Montiel RG, Aguirre-Álvarez G. Characterization of Whey-Based Fermented Beverages Supplemented with Hydrolyzed Collagen: Antioxidant Activity and Bioavailability. Foods. 2020 Aug;9(8):1106.
25. Broyard C, Gaucheron F. Modifications of structures and functions of caseins: a scientific and technological challenge. Dairy Sci Technol. 2015 Nov 1;95(6):831–62.
26. Sinaga H, Bansal N, Bhandari B. Effects of milk pH alteration on casein micelle size and gelation properties of milk. Int J Food Prop. 2017 Jan 2;20(1):179–97.
27. Huppertz T, Chia LW. Milk protein coagulation under gastric conditions: A review. Int Dairy J. 2021 Feb 1;113:104882.
28. Božanić R, Barukčić I, Lisak K, Tratnik L. Possibilities of Whey Utilisation. 2014;7.
29. Minj S, Anand S. Whey Proteins and Its Derivatives: Bioactivity, Functionality, and Current Applications. Dairy. 2020 Dec;1(3):233–58.
30. Bland JH, Grandison AS, Fagan CC. Effect of blending Jersey and Holstein-Friesian milk on Cheddar cheese processing, composition, and quality. J Dairy Sci. 2015 Jan 1;98(1):1–8.
31. Luo J. Colloidal interactions in an alternate make cheese. Massey University (Manawatū), New Zealand; 2017.
32. Gernigon G, Schuck P, Jeantet R. Processing of Mozzarella cheese wheys and stretchwaters: A preliminary review. Dairy Sci Technol. 2010 Jan 1;90(1):27–46.
33. Fox PF, Uniacke-Lowe T, McSweeney PLH, O'Mahony JA. Heat-Induced Changes in Milk. In: Dairy Chemistry and Biochemistry [Internet]. Cham: Springer International Publishing; 2015 [cited 2020 Dec 6]. p. 345–75. Available from: http://link.springer.com/10.1007/978-3-319-14892-2_9
34. Gernigon G, Piot M, Beaucher E, Jeantet R, Schuck P. Physicochemical characterization of Mozzarella cheese wheys and stretchwaters in comparison with several other sweet wheys. J Dairy Sci. 2009 Nov;92(11):5371–7.
35. Shuler ML, Kargi F. Bioprocess Engineering: Basic Concepts. Subsequent edition. Upper Saddle River, NJ: Pearson College Div; 2001. 553 p.
36. Walker G, Stewart G. Saccharomyces cerevisiae in the Production of Fermented Beverages. Beverages. 2016 Nov 17;2(4):30.
37. Peña A, Sánchez NS, Álvarez H, Calahorra M, Ramírez J. Effects of high medium pH on growth, metabolism and transport in Saccharomyces cerevisiae. FEMS Yeast Res [Internet]. 2015 Mar 1 [cited 2020 Dec 6];15(2). Available from: https://academic.oup.com/femsyr/article/15/2/fou005/534737
38. Binetti A, Carrasco M, Reinheimer J, Suárez V. Yeasts from autochthonal cheese starters: technological and functional properties. J Appl Microbiol. 2013 Aug;115(2):434–44.
39. Yalçin SK, Ozbas ZY. Yeasts from Traditional Cheeses for Potential Applications. In: Barbosa-Cánovas GV, María Pastore G, Candoğan K, Medina Meza IG, Caetano da Silva Lannes S, Buckle K, et al., editors. Global Food Security and Wellness [Internet]. New York, NY: Springer New York; 2017 [cited 2020 Dec 6]. p. 277–93. Available from: http://link.springer.com/10.1007/978-1-4939-6496-3_14
40. Vasdinyei R, Deák T. Characterization of yeast isolates originating from Hungarian dairy products using traditional and molecular identification techniques. Int J Food Microbiol. 2003 Sep 1;86(1–2):123–30.
41. Gardini F, Tofalo R, Belletti N, Iucci L, Suzzi G, Torriani S, et al. Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. Food Microbiol. 2006 Oct;23(7):641–8.
42. Borelli BM, Ferreira EG, Lacerda ICA, Franco GR, Rosa CA. Yeast populations associated with the artisanal cheese produced in the region of Serra da Canastra, Brazil. World J Microbiol Biotechnol. 2006 Nov 1;22(11):1115–9.
43. Andrighetto C, Psomas E, Tzanetakis N, Suzzi G, Lombardi A. Randomly amplified polymorphic DNA

(RAPD) PCR for the identification of yeasts isolated from dairy products. Lett Appl Microbiol. 2000 Jan;30(1):5–9.

44. Rea MC, Görges S, Gelsomino R, Brennan NM, Mounier J, Vancanneyt M, et al. Stability of the Biodiversity of the Surface Consortia of Gubbeen, a Red-Smear Cheese. J Dairy Sci. 2007 May 1;90(5):2200–10.
45. Giannino ML, Buffoni JN, Massone E, Feligini M. Internal transcribed spacer as a target to assess yeast biodiversity in Italian Taleggio PDO cheese. J Food Sci. 2011 Sep;76(7):M511-514.
46. Callon C, Delbès C, Duthoit F, Montel M-C. Application of SSCP–PCR fingerprinting to profile the yeast community in raw milk Salers cheeses. Syst Appl Microbiol. 2006 Mar 1;29(2):172–80.
47. Utama GL, Kurnani TBA, Sunardi -, Balia RL. The Isolation and Identification of Stress Tolerance Ethanol-fermenting Yeasts from Mozzarella Cheese Whey. Int J Adv Sci Eng Inf Technol. 2016 Apr 30;6(2):252-257–257.
48. Lioliou K, Litopoulou-Tzanetaki E, Tzanetakis N, Robinson RK. Changes in the microflora of manouri, a traditional Greek whey cheese, during storage. Int J Dairy Technol. 2001;54(3):100–6.
49. Schlander M, Distler U, Tenzer S, Thines E, Claus H. Purification and Properties of Yeast Proteases Secreted by Wickerhamomyces anomalus 227 and Metschnikovia pulcherrima 446 during Growth in a White Grape Juice. Fermentation. 2016 Dec 26;3(1):2.
50. Cardoso VM, Borelli BM, Lara CA, Soares MA, Pataro C, Bodevan EC, et al. The influence of seasons and ripening time on yeast communities of a traditional Brazilian cheese. Food Res Int. 2015 Mar 1;69:331–40.
51. Chaves-López C, Serio A, Paparella A, Martuscelli M, Corsetti A, Tofalo R, et al. Impact of microbial cultures on proteolysis and release of bioactive peptides in fermented milk. Food Microbiol. 2014 Sep;42:117–21.
52. Amorim FG, Coitinho LB, Dias AT, Friques AGF, Monteiro BL, Rezende LCD de, et al. Identification of new bioactive peptides from Kefir milk through proteopeptidomics: Bioprospection of antihypertensive molecules. Food Chem. 2019 Jun;282:109–19.
53. Lario LD, Pillaca-Pullo OS, Durães Sette L, Converti A, Casati P, Spampinato C, et al. Optimization of protease production and sequence analysis of the purified enzyme from the cold adapted yeast Rhodotorula mucilaginosa CBMAI 1528. Biotechnol Rep [Internet]. 2020 Oct 22 [cited 2020 Dec 6];28. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7653053/
54. Diniz RHS, Silveira WB, Fietto LG, Passos FML. The high fermentative metabolism of Kluyveromyces marxianus UFV-3 relies on the increased expression of key lactose metabolic enzymes. Antonie Van Leeuwenhoek. 2012 Mar;101(3):541–50.
55. Roostita R, Fleet GH. Growth of yeasts in milk and associated changes to milk composition. Int J Food Microbiol. 1996 Aug 1;31(1):205–19.
56. Hamme V, Sannier F, Piot J-M, Didelot S, Bordenave-Juchereau S. Crude goat whey fermentation by *Kluyveromyces marxianus* and *Lactobacillus rhamnosus*: contribution to proteolysis and ACE inhibitory activity. J Dairy Res. 2009 May;76(2):152–7.
57. Chaves-López C, Serio A, Rossi C, Pepe A, Compagnone E, Paparella A. Interaction between Galactomyces geotrichum KL20B, Lactobacillus plantarum LAT3 and Enterococcus faecalis KE06 during Milk Fermentation. Fermentation. 2017 Dec;3(4):52.
58. Utba F, Balia RL, Utama GL. THE PRESENCE OF INDIGENOUS YEASTS WITH PROTEOLYTIC ACTIVITY ISOLATED FROM HOMEMADE-MOZZARELLA WHEY. 2018;18(1):8.
59. Alinovi M, Rinaldi M, Mucchetti G. Spatiotemporal Characterization of Texture of Crescenza Cheese, a Soft Fresh Italian Cheese [Internet]. Vol. 2018, Journal of Food Quality. Hindawi; 2018 [cited 2020 Dec 6]. p. e5062124. Available from: https://www.hindawi.com/journals/jfq/2018/5062124/
60. A. Hayaloglu A, H. Ozer B, F. Fox P. Cheeses of Turkey: 2. Varieties ripened under brine. Dairy Sci Technol. 2008;88(2):225–44.
61. Ozturk I, Sagdic O. Biodiversity of yeast mycobiota in "sucuk," a traditional Turkish fermented dry sausage: phenotypic and genotypic identification, functional and technological properties. J Food Sci. 2014 Nov;79(11):M2315-2322.
62. Zaugg C, Borg-Von Zepelin M, Reichard U, Sanglard D, Monod M. Secreted aspartic proteinase family of Candida tropicalis. Infect Immun. 2001 Jan;69(1):405–12.
63. Chaves-López C, Tofalo R, Serio A, Paparella A, Sacchetti G, Suzzi G. Yeasts from Colombian Kumis as source of peptides with Angiotensin I converting enzyme (ACE) inhibitory activity in milk. Int J Food Microbiol. 2012 Sep 17;159(1):39–46.
64. Mariné M, Brown NA, Riaño-Pachón DM, Goldman GH. On and Under the Skin: Emerging Basidiomycetous Yeast Infections Caused by Trichosporon Species. PLOS Pathog. 2015 Jul 30;11(7):e1004982.
65. Corbo MR, Lanciotti R, Albenzio M, Sinigaglia M. Occurrence and characterization of yeasts isolated from milks and dairy products of Apulia region. Int J Food Microbiol. 2001 Sep 19;69(1):147–52.
66. Venkat Kumar S, Rao A, Nazareth JR. Screening, MeSCREENING, MEDIA OPTIMIZATION AND PARTIAL PURIFICATION OF PROTEASE BY TRICHOSPORON JAPONICUM VITVK1dia Optimization and Partial Purification of Protease by Trichosporon japonicum VITVK1. Int J Pharm Pharm Sci. 2015;187–91.
67. Suzzi G, Schirone M, Martuscelli M, Gatti M, Fornasari ME, Neviani E. Yeasts associated with Manteca. FEMS Yeast Res. 2003 Apr 1;3(2):159–66.
68. Al-Falih AM. Proteolytic Activity of Some Local Isolates of Yeasts as Affected by Cultural Conditions. Pak J Biol Sci. 2001 Jun 1;4(6):718–21.
69. Valle RS, Ramos LS, Reis VJ, Ziccardi M, Dornelas-Ribeiro M, Sodré CL, et al. Trichosporon asahii secretes a 30-kDa aspartic peptidase. Microbiol Res. 2017 Dec;205:66–72.
70. Pontieri E, Caracciolo C, Ceddia T, Oliva B, Ferrini A, Girmenia C, et al. Genetic Variability among Blastoschizomyces Capitatus Isolates from Different Clinical Sources. Int J Immunopathol Pharmacol. 2005 Jul 1;18(3):531–9.
71. Hayes M, Tiwari BK. Bioactive Carbohydrates and Peptides in Foods: An Overview of Sources, Downstream Processing Steps and Associated

Bioactivities. Int J Mol Sci. 2015 Sep 17;16(9):22485–508.
72. Ahmed AS, El-Bassiony T, Elmalt LM, Ibrahim HR. Identification of potent antioxidant bioactive peptides from goat milk proteins. Food Res Int. 2015 Aug 1;74:80–8.
73. Portela MB, Souza IPR, Abreu CM, Bertolini M, Holandino C, Alviano CS, et al. Effect of serine-type protease of Candida spp. isolated from linear gingival erythema of HIV-positive children: critical factors in the colonization. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 2010 Nov;39(10):753–60.