CHAPTER 2

LITERATURE REVIEW

2.1 Black glutinous rice

Black glutinous rice is also known as black sticky rice (In Thai words, *kao* is rice; *niow*, sticky; *dahm*, black). Purple rice is the seed of *Oryza sativa* Linne, a type of ancient rice originated in Hanzhong, Shanxi in China and is also called purple-black rice or black rice. Unpolished purple rice has a hard surface and a white inner area, and contains purple black pigments (anthocyanin) in the unpolished seed coat (Brouillard and Delaporte, 1977; Abdel-Aal et al., 2006). Pigmented rice (*O. sativa* L.*)* has been consumed for a long time in Asia, especially China, Japan, Korea and many countries in Southeast Asia. Several varieties of pigmented rice, particularly red and black rice, have been cultivated in Thailand. Among these varieties, black glutinous rice is the most famous one, generally used as an ingredient in snacks and desserts. Nowadays, whole grain pigmented rice has been categorized as one of the potent functional foods since it contains high amounts of phenolic compounds, especially anthocyanin in pericarp. Study reports have demonstrated antioxidant activity and radical scavenging ability of the pigmented rice and/or its extract both in vitro and in vivo models as well as other biological effects of the extracts, including antimutagenic and anticarcinogenic activities, reduction of the atherosclerotic plague formation, aldose reductase inhibitory activity and attenuation of some metabolic abnormalities associated with high fructose diets, such as glucose intolerance and hyperlipidemia. These researches scientifically displayed the health benefits of the pigmented rice (Tananuwong and Tewaruth, 2010).

Purple rice contains anthocyanin glycosides such as chrysanthemin (cyanidin-3-glucoside) that are not contained in other types of rice. The rice is used as a material for edible vinegar and alcohol and added to flour and kneaded to make bread, fine noodles,

and noodles (Brouillard and Delaporte, 1977; Abdel-Aal et al., 2006). Glutinous rice has properties genetically different from the non-glutinous variety, such as its lack of amylose in the starch. It does not contain dietary gluten (glutenin and gliadin), and should be safe for gluten-free diets. The glutinous rice contains high amounts of amylopectin and no or low amylose. Amylopectin is responsible for the sticky quality of the rice. The rice can be used either milled or unmilled (with the bran removed or not removed). Milled rice is white in color, whereas the bran can give unmilled glutinous rice a purple or black color. However black and purple glutinous rice are distinct strains from white glutinous rice. Both black and white glutinous rice can be cooked as grains or ground into flour and cooked as a paste. In traditional preparation method, cooked Thai glutinous rice is produced by steam cooking after overnight soaking. This prolonged soaking is needed to soften the grain, which facilitates water uptake by the starch during cooking. The soaking time can be reduced by employing slightly elevated temperatures, since higher rates of water uptake are associated with higher temperatures (Ahromrit et al., 2006).

Starches are obtained from cereal grain seeds, particularly from corn, waxy corn (waxy maize), high-amylose corn, wheat, and various rice. Starch is a major carbohydrate component isolated from green plants. This carbohydrate component consists of mixture of amylopectin and amylose. Amylose is a linear polymer made of α-D-glucopyranose units linked through α(1-4) linkage. Amylopectin is a large branched molecule with side chain grafted to the linear α(1-4) polymer by a single α(1-6) junction (BeMiller and Whistler, 1996; Chough et al., 2006).

Soaking is an essential step in wet-milling of rice flour. The effects of soaking duration and temperature (at 5 and 25°C) on the properties of rice flour have been investigated. The moisture content after soaking appeared to be a key factor on loosening the structure of rice kernels, which resulted in the production of small particle flours with little starch damage. The uptake of water by rice kernels increased with temperature and reached a plateau at about 30–35%. Protein, lipid, and ash leached out during soaking (Chiang and Yeh, 2001). Wongkhalaung and Boonyaratanakornkit (2000) soaked

Jasmine rice in water at 1:2.5 ratio for about 30 min before a saccharification process to produce rice milk. In this study, the ratio of soaking water of 1:2.5, 1:5, and 1:10 for black glutinous rice powder and water, respectively, and the soaking time of 30 min, 1 h, and 2 h for each ratio of soaking water were investigated to find the optimum soaking water ratio and soaking time.

Sutharut and Sudarat (2012) studied the effect of germination on total anthocyanin content (TAC) and antioxidant activity of coloured rice (*Oryza sativa* L). The normal, non-waxy rice variety Phitsanulok 2 was used as a control and two pigmented rice including black glutinous rice (local name *Niew Dam*), and black non-waxy rice (local name *Hom Nil*) were used in this study. Rough rice and dehulled rice kernels were steeped in water for 12 and 6 h, respectively, before further left to germinate for 0, 6, 12, 18, and 24 h. Rice samples were evaluated for TAC, trolox equivalent antioxidant capacity (TEAC), and ferric reducing antioxidant power (FRAP). Rice grains without husk showed higher germination rate compared to those of rough rice. After germination, the results showed that TAC and TEAC of black glutinous rice were significantly higher than that of the other two samples. Black rice germinated from rough rice showed higher TAC content and TEAC than that germinated from grains without husk. It was suggested that rice with husk intact should be employed for the preparation of germinated pigmented rice to protect TAC loss during germination process. Chen et al. (2006) investigated black rice anthocyanins to inhibit cancer cells invasion via repressions of MMPs and u-PA expression. Anthocyanins are natural colourants belonging to the flavonoid family, and are wildly used for their antioxidant properties. The molecular evidence associated with the anti-metastatic effects of peonidin 3-glucoside and cyanidin 3-glucoside, major anthocyanins extracted from black rice (*Oryza sativa* L. *indica*), showed a marked inhibition on the invasion and motility of SKHep-1 cells. This effect was associated with a reduced expression of matrix metalloproteinase (MMP)-9 and urokinase-type plasminogen activator (u-PA). Peonidin 3-glucoside and cyanidin 3-glucoside also exerted an inhibitory effect on the DNA binding activity and

the nuclear translocation of AP-1. Furthermore, these compounds also exerted an inhibitory effect of cell invasion on various cancer cells (SCC-4, Huh-7, and HeLa). Finally, anthocyanins from *O. sativa* L. *indica* were evidenced by its inhibition on the growth of SKHep-1 cells in vivo.

For pigmented rice, the level of pigments decreased from the surface to the endosperm and yellow and red pigments were concentrated in bran and outer endosperm. Colour pigments were uniformly distributed in the middle and core endosperm. Colour measurements of cooked rice indicated that water uptake during cooking, leaching of pigments in the cooking water (excess water was used in the cooking experiment) and diffusion of pigments from the rice surface to the inner layer were responsible for differences in colour between raw and cooked rices (Lamberts et al., 2007).

Yafang et al. (2011) examined total phenolic content and antioxidant capacity of rice. Whole cereal grains have been received increasingly attention by consumers due to their potential health benefits because of their antioxidant capacity, which is probably derived from their high contents of phenolics, flavonoids and other phytochemicals. This study investigated the contents of phenolics and flavonoids in different rice genotypes with grain size ranging from extremely small to normal size. The smaller grains had higher phenolic content, flavonoid content and antioxidant capacity than the normal and larger grains. The phenolic content had positive correlation with the flavonoid content ($P<0.001$) and the antioxidant capacity ($P<0.01$). The phenolic and flavonoid content had negative correlation with grain length, grain length to width ratio and 100-grain weight ($P<0.01$), but had no relationship with grain width and grain thickness. Thus, the phenolic content could be indirectly predicted by grain length and 100-grain weight. New rice varieties high in antioxidant levels could be obtained by breeding for extremely small grain rice.

2.2 Enzymes

Enzymes are biological catalysis. They increase the rate of chemical reactions taking place within living cells without themselves suffering any overall change. The reactants of enzyme-catalyzed reactions are termed substrates and each enzyme is quite specific in character, acting on a particular substrate or substrates to produce a particular product or products. The fact that enzymes act as catalysts in key biochemical process known as metabolism, is characterized by chemical and physical changes continuously going on in what we call life. The living cell is the site of tremendous biochemical activity called metabolism. This is the process of chemical and physical change which goes on continually in the living organism. The greatest majority of these biochemical reactions do not take place spontaneously. The phenomenon of catalysis makes possible biochemical reactions necessary for all life processes. Catalysis is defined as the acceleration of a chemical reaction by some substance which itself undergoes no permanent chemical change. The catalysts of biochemical reactions are enzymes and are responsible for bringing about almost all of the chemical reactions in living organisms (Bennett and Frieden, 1969; Naz, 2002).

All enzymes are protein of high molecular weight (10,000 to 2,000,000), made up primarily of chain of amino acids linked together by peptide bonds. However, without the presence of a non-protein component called a cofactor, many enzyme proteins lack catalytic activity. When this is the case, the inactive component of an enzyme is termed the apoenzyme, and the active enzyme, including cofactor, the holoenzyme. The cofactor may be an organic molecule, when it is known as a coenzyme, or it may be a metal ion. Some enzymes bind cofactors more tightly than others. When a cofactor is bond so tightly that it is difficult to remove without damaging the enzyme, it is sometimes called a prosthetic group. Substances that are able to increase the activity of an enzyme in a non-specific manner are called activator. They are part of the activating system and are required before the enzyme can activate its substrate. An activator is not a coenzyme because a coenzyme is a part of the reaction system and plays no role in the activation of

the substrate. Many inorganic radicals such as chloride, potassium, calcium, magnesium and phosphates are activators. Any compound which reduces the activity of an enzyme is an inhibitor. Inhibition of enzyme activity by a compound may be a result of its reaction with apoenzyme, necessary cofactors, activators, intermediates in the pathway, or the essential groups of the enzyme. The exceptional features of enzymes that make them different from non-biological catalysts include structural feathers, specificity towards a particular reaction or type of reaction, and mechanism of catalysis (Naz, 2002).

2.2.1 Amylolytic enzymes

Amylases are carbohydrases that catalyse the hydrolysis of α-D-1,4 glycoside of starch and related oligo- and polysaccharides by the transfer of a glycosyl residue (donor) to H_2O (acceptor). Amylolytic enzymes form a large group of enzymes operating on starch and related oligo- and polysaccharides. The two best known amylases are amylase and glucoamylase (rarely γ-amylase). Since the starch or pullulan and glycogen belong to the important sources of energy for microorganisms, plants and animals, amylolytic enzymes are produced by a great variety of living systems (Vihinen and Mäntsäla, 1989).

2.2.1.1 α-Amylase

α-Amylase (EC. 3.2.1.1, 1,4-α-D-glycan glucano-hydrolase) is an endo-glucoside that cleaves the α-1,4-glycosidic bond of the substrate at internal positions to yield dextrins and oligosaccharides with the C1-OH in the α-configuration. Although the endo-enzyme implies random cleavage, numerous experiments have suggested that the enzyme action followed a definite pattern depending on the source of the enzyme. An α-1,4 linkage neighbouring an α-1, 6 branching point in the substrate is resistant to attack by

the enzyme. α-Amylase is found almost universally distributed in plants, animals, bacteria and fungi. The *Aspergillus oryzae* enzyme consists of 478 amino acid residues folded into two domains. Domain A, which contains the N-terminal 380 amino acid residues, consists of a $(\beta/\alpha)_8$ barrel with eight β-strands alternating with eight helices joined by loops. The loop linking the β strand 3 and helix 3 contains an extended chain of three β strands. Domain B is an 8-stranded antiparallel β sheet linked to domain A via a single polypeptide chain. Four disulphide bonds are located at residues 30-38, 150-164, 240-283, and 439-474. The enzyme is N-glycosylated at Asn 197 (Naz, 2002).

The place where catalysis by an enzyme from the α-amylase family is performed is located at the C-terminal end of the parallel β-barrel of domain A in a cleft (Matsuura et al., 1984). However, comparison of known three-dimensional models of different enzymes from the family along with a multiple sequence alignment have suggested that the diversity in specificity arises by variation in substrate binding at the β→α loops (Svensson, 1994). Also the active-site cleft is not always of the same shape, e.g., in neopullulanase it was found wider and shallower than the cleft of other α-amylases. The core of the catalytic cleft is built up of three catalytic residues, Asp 206, Glu 230 and Asp 297 at strands β4, β5 and β7, plus of a few additional residues depending on concrete enzyme specificity. The α-amylase-type of the active site, accommodating many related specificities, offers good opportunity to tailor the specificity by combination of suitable mutations of the active site residues (Matsuura et al., 1984 and Svensson, 1994).

Most of the starch products are used in the food and beverage industries. Amylases, which are starch hydrolyzing enzymes, are the most important industrial enzymes and are used in different processes in food, textile, pharmaceutical industries, etc. α-Amylase is known to attack both insoluble starch and starch granules held in aqueous suspension. It is also recognised that both proteins and hydrolysis products can inhibit the action of α-amylase on starch (Apar and Ozbek, 2005).

(1) Reaction mechanism

As shown in Figure 2.1, the enzyme contains a carboxyl and a nitrogen group (imidazole group) in the active site. The substrate forms an adsorptive complex with the enzyme which positions the susceptible glucosidic bond in juxta position with the carboxyl anion and imidazolium group. In the proposed scheme the carboxyl anion serves as an attacking nucleophile on the C (1) position of the substrate and this attack is aided by protonation of the linkage by the general acid (imidazolium). A glucosyl-enzyme intermediate involving covalent bonds is formed as a result. In the deglucosylation reaction, the imidazole group (unprotonated) assists as a general base to remove a proton from water leaving the OH- to attack at the C(1) position of the

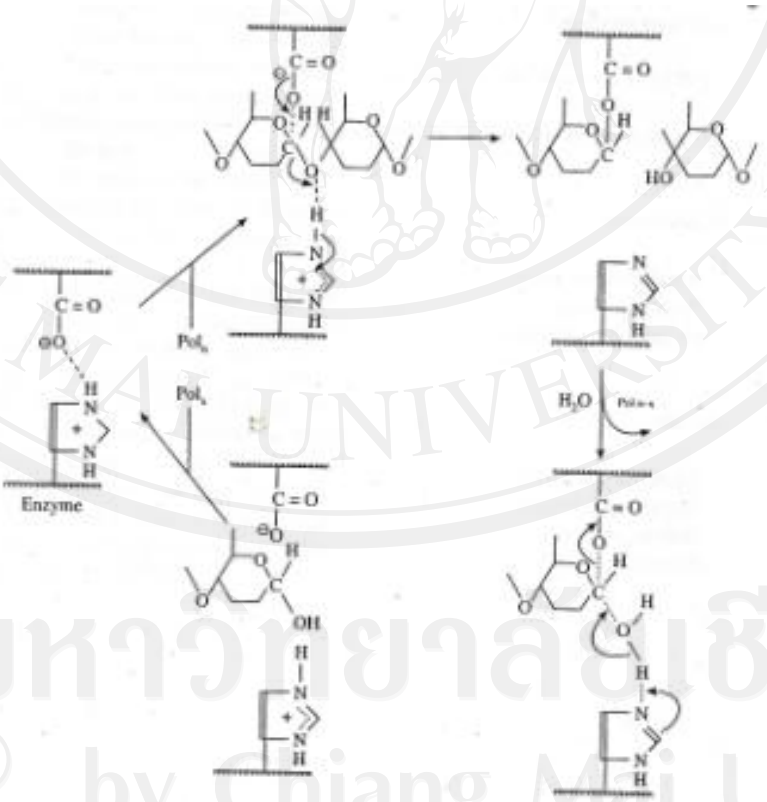

Figure 2.1 A proposed mechanism of α-amylase (Naz, 2002).

glucosyl-enzyme. The configuration about C(1) is not changed by this double displacement mechanism so the production of reaction is of α-configuration (Naz, 2002).

(2) Optimum pH and temperature

The pH optimum of α-amylase varies depending on the enzyme source (6.0-7.0 for mammalian, 4.8-5.8 for *A. oryzae*, 5.86-6.0 for *Bacillus subtilis*, 5.5-7.0 for *Bacillus lichemiformis*). Temperature optimum for the enzyme activity varies, for example at 70-72°C for α-amylase produced by *B. subtilis*, and at 90°C for the enzyme from *B. lichemiformis*. The applications of α-amylase in starch processing are at temperatures $\geq$ 105°C (Naz, 2002).

Apar and Ozbek (2005) studied α-amylase inactivation during rice starch hydrolysis. The effects of operating conditions on the enzymatic hydrolysis of rice starch were investigated using a commercial α-amylase produced by *Bacillus* spp. in a stirred batch reactor. The degree of starch hydrolysis (%) and residual α-amylase activity (%) were investigated versus process variables including pH, temperature, viscosity, impeller speed, processing time and some materials added such as hydrolysate, maltose, glucose, ethanol and $CaCl_2$ using a stirred batch reactor. Mathematical models depending on the operating conditions were also derived using the experimental data of residual starch concentration. Some models were tested for understanding of the relationship between the process variables and enzyme stability during the hydrolysis process. The process conditions and added materials mentioned above have a significant role to play in the starch hydrolysis process. The stability behavior observed was different for each process condition. Thus, the operational parameters should be identified and optimised for the enzyme used to obtain higher degrees of hydrolysis as these parameters could cause inactivation or activation of the enzyme during the hydrolysis process.

2.2.1.2 Amyloglucosidase

Amyloglucosidase, also occasionally called glucoamylase or γ-amylase in some literature, is an exoglucosidase that catalyses the hydrolysis of both α-D-1, 4- and α-D-1,6-glucosidic linkage at the branch point, although hydrolysis of the latter occurs at a slower rate. Amyloglucosidase is an exo-enzyme that liberates glucose units from a non-reducing end of starch, amylose, amylopectin, amylodextrin chains, produce glucose as the sole end-product from starch and related polymers. The enzyme removes glucose unites successively from the surrounding and of the substrate. The rate of hydrolysis increases with the chain length of the substrate. The end product is exclusively glucose in the β-configuration. Amyloglucosidase is a microbial enzyme found only in molds and yeasts. The enzyme is commercially produced from *Aspergillus niger* (Naz, 2002; Auto et al., 2006; Chough et al., 2006).

Amyloglucosidase is a glycoprotein containing 5-20 percent carbohydrate, mainly mannose, glucose, galactose and glucosamine. The molecular weight varies depending on the source of the enzyme, and may exist in multiple forms. Fungal amyloglucosidase generally exists in multiple forms. For example, *A. niger* amyloglucosidase exists in two forms, GAI and GAII. The GAI form (616 amino acids, MW 71 KD) consists of a catalytic site region (1-440), a Thr- and Ser-rich linker region (442-512) that is heavily glycosylated, and a C-terminal region (513-616). The latter is missing in the GAII form (MW 61 KD), and is referred to as the granular starch binding domain (Naz, 2002).

Optimum pH and temperature

The amyloglucosidase enzyme has pH optimum of 4.0-4.4 with stability over a pH range of 3.5-5-5. The enzyme is stable at a temperature range of 40-65°C with an optimum temperature range of 58-65°C in the hydrolysis of starch at pH 4.2 (Naz, 2002).

Amyloglucosidase (glucoamylase) production has been investigated by solid-state fermentation of agro-industrial wastes generated during the processing of paddy to rice flakes (categorized as coarse, medium and fine waste), along with wheat bran and rice powder by a local soil isolate *Aspergillus* spp. HA-2. Highest enzyme production was obtained with wheat bran (264 ± 0.64 U/gds) followed by coarse waste (211.5 ± 1.44 U/gds) and medium waste (192.1 ± 1.15 U/gds) using 10^6 spores/ml as inoculum at 28 ± 2ºC, pH 5. A combination of wheat bran and coarse waste (1:1) gave enzyme yield as compared to wheat bran alone. Media supplementation with carbon source (0.04 g/gds) as sucrose in wheat bran and glucose in coarse and medium waste increased enzyme production to 271.2 ± 0.92, 220.2 ± 0.75 and 208.2 ± 1.99 U/gds respectively. Organic nitrogen supplementation (yeast extract and peptone, 0.02 g/gds) showed a higher enzyme production compared to inorganic source. Optimum enzyme activity was observed at 55ºC, pH 5. Enzyme activity was enhanced in the presence of calcium whereas presence of EDTA gave reverse effect (Auto et al., 2006).

2.2.2 Application of amylases in food processing

The main application of amylase in food industries is;

Starch processing

The food industry uses amylase enzymes as processing aids to convert starch bearing materials to starches, starch derivatives and starch saccharification products. Industrial processes for starch hydrolysis to glucose rely on inorganic acid or enzyme catalysis. The use of enzyme is currently preferred and offers a number of advantages associated with improved yields and favourable economics. Enzymatic hydrolysis allows greater control over amylolysis, the specificity of the reaction, and the stability of the generated products. The milder reaction conditions involve lower temperature and near neutral pH, thus reducing unwanted side reactions. Fewer off-flavour and off-colour

compounds are produced, especially 5-hydrozyl-2-methylfurfuraldehyde, anhydroglucose compound and undesirable salts. Enzymatic methods are favoured because they also lower energy requirements and eliminate neutralization steps. Starch derivatives obtained via the liquefaction process have commercial value as food ingredients. The products have a DE (dextrose equivalent) value of about 10 and are sold as maltodextrins, which are carbohydrate preparations composed of 3 percent maltose, 4 percent maltriose and 93 percent tetroses or larger polysaccharides (Naz, 2002).

(1) Gelatinization and liquefaction of starch

Current starch processing methods require the gelatinization of corn starch after wet milling. This pretreatment is essential before an extend period of enzymatic hydrolysis. The starch which is present as 5-25 μm particles, is exposed to temperatures above 60°C that swell and disrupt the granules. The addition of heat-stable α-amylase from strains of *B. subtilis* at this stage causes random hydrolysis of glucosidic bonds and significantly thins the starch slurry. After gelation of the starch, process of liquefaction begins. The thinned starch slurry composed of 30-40 percent solids is treated with a thermostable α-amylase that is able to function at pH 6.5. The starch is contained in a steam jet cooker operated in a continuous mode for 5-10 min at 103-107°C. Afterward, a 1-2 h treatment at lower temperature of about 95°C permits hydrolysis of the thinned starch to a syrup of 0.5-1.5 DE. Digestion is allowed to continue until a DE unit of 10-15 is attained. The net yield of dextrose after complete enzymatic digestion of the corn starch with bacterial amylase is 95-97 percent if hydrolysis is coupled with a fungal amylase treatment (Naz, 2002; Apar and Ozbek, 2005).

(2) Saccharification of starch

The saccharification of starch, that is, the hydrolysis of starch to glucose, is

accomplished with fungal amyloglucosidase. The enzyme is effective since it has both exoamylase and debranching activity and is important for the production of a corn syrup of high DE or of crystalline dextrose. A debranching enzyme such as pullulanase may also be used. Fungal amylase can hydrolyse starch to glucose almost completely after a treatment time of 3-4 days at pH 4.0 and 60°C. The low pH optimum of fungal amylolytic enzymes permits the convenient use of acid conditions for the saccharification. Such conditions reduce unwanted isomerization reactions to fructose and other sugars that may reduce the glucose yield. Moreover, acid conditions restrict the growth of contaminating microorganism in the saccharification reactions (Naz, 2002).

Wongkhalaung and Boonyaratanakornkit (2000) made an experimental development of a yoghurt-type product from saccharified rice. They saccharified Jasmine rice with amylolytic enzymes at 55°C. Park et al. (2005) determined characteristics of yogurt-like products prepared from the combination of skim milk and soymilk containing saccharified-rice solution. α-Amylases and amyloglucosidase were used to do the saccharification at 60°C for 1 h. α-Amylase inactivation during rice starch hydrolysis was determined by Apar and Ozbek (2005). They reported that pH, temperature, viscosity, impeller speed, processing time, and materials added could affect the activity of α-amylases.

2.3 Yoghurt

Yoghurt is probably the most popular fermented milk. It is made in a variety of compositions (fat and dry matter content), either plain or with added substrates: fruits, gelling agents. The essential flora of yoghurt consists of the thermophiles *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. For a satisfactory flavour to develop, approximately equal numbers of both species should be present. They have a stimulating effect on each other's growth. Volatile compounds produced by the yoghurt bacteria include small amounts of acetic acid, diacetyl, and most importantly,

acetaldehyde. Beverages and edible ices derived from yoghurt are also produced. Yoghurt and yoghurt-like products are made widely in the Mediterranean area, Asia, Africa, and central Europe. Zabady is traditionally made from ewes' milk. For the production of dahi and dadih, buffaloes' milk is often used, sometimes in combination with bovine milk (Walstra et al., 1999). The principal metabolic products of the yoghurt microorganisms are lactate, aroma compounds, and sometimes exopolysaccharides. The symbiotic relationship between the 2 lactic acid bacteria species during milk acidification by mixed cultures results in greater bacterial concentration, acid production, flavour development, and improved texture, than do single culture growth (Xanthopoulos et al., 2001). In the production of yoghurt, pH is reduced by fermentation with the yoghurt bacteria that convert milk sugar (lactose) into lactic acid. Commercial yoghurts are produced in two different forms; set yoghurt and stirred yoghurt. Stirred yoghurt, where fermentation is carried out in large tanks and the acid gel is then disrupted by stirring and sieving to give a more fluid product, is often used as a base for inclusion of fruit before packaging (Haque et al., 2001).

2.3.1 Yoghurt types

The traditional production of set yoghurt is made of concentrated milk. The milk was heated on an open fire until one third of the water had evaporated. The milk was then allowed to cool, and when a temperature of about 50°C was reached, the milk was inoculated with a little yoghurt. After fermentation, a fairly firm gel was obtained. A similar process is still being used, but either the milk is evaporated under vacuum or milk powder is added. One may use the same process for making set yoghurt from non-concentrated milk (see Figure 2.2). The yoghurt obtained is less rich in flavour, far less firm, and prone to syneresis (wheying off). Generally, some gelling agent is added to prevent syneresis and to enhance firmness, especially if pieces of fruit are added. Another difference between both these products is their titratable acidity. Because a satisfactory

flavour and texture are only obtained at a pH below 4.5 and concentrated milk has a greater buffering capacity, the latter is fermented to an acidity of about 130 mM, as against 90 to100 mM for non-concentrated milk (Tamime and Robinson, 1999; Walstra et al., 1999).

Another type is stirred yoghurt, almost always made from non-concentrated milk. After a gel is formed, it is gently stirred to obtain a smooth and fairly thick, but still pourable, product (see Figure 2.2). There are other differences in the manufacturing process. Set yoghurt is fermented after being packaged, implying that final cooling has to be achieved in the package. Stirred yoghurt is almost fully fermented before it is packaged. Another difference is that only certain strains of yoghurt bacteria produce the correct consistency or thickness after stirring, and only so when incubating at a fairly low temperature. However, the bacteria make less of the desired flavour compounds at lower temperatures. In order to ensure that stirred yoghurt has a distinct yoghurt flavor, it is necessary that the starter be propagated under the same conditions as for set yoghurt, i.e., at about 45°C and with such an inoculum size and incubation time as to reach about equal numbers of cocci and lactobacilli. The rate of acidification greatly differs in set and stirred yoghurt due to the differences in inoculum size and incubation temperature. Gelation is seen to start when the pH reaches about 4.7 and the stiffness then rapidly increases, to reach a fairly high value in about 20 min. When making stirred yoghurt, gelation begins at about the same pH, but it takes a longer time before the gel has become sufficiently firm for the stirring to be started. Acidification will go on, albeit slowly, after the product has been cooled. To minimize or even prevent ongoing acidification, stirred yoghurts or yoghurt-like products are sometimes pasteurised; this also prevents the growth of any yeasts and moulds present. To allow pasteurisation without the product becoming inhomogeneous, it is necessary to add specific thickening agents (pectins, modified starch, gelatin) (Walstra et al., 2006).

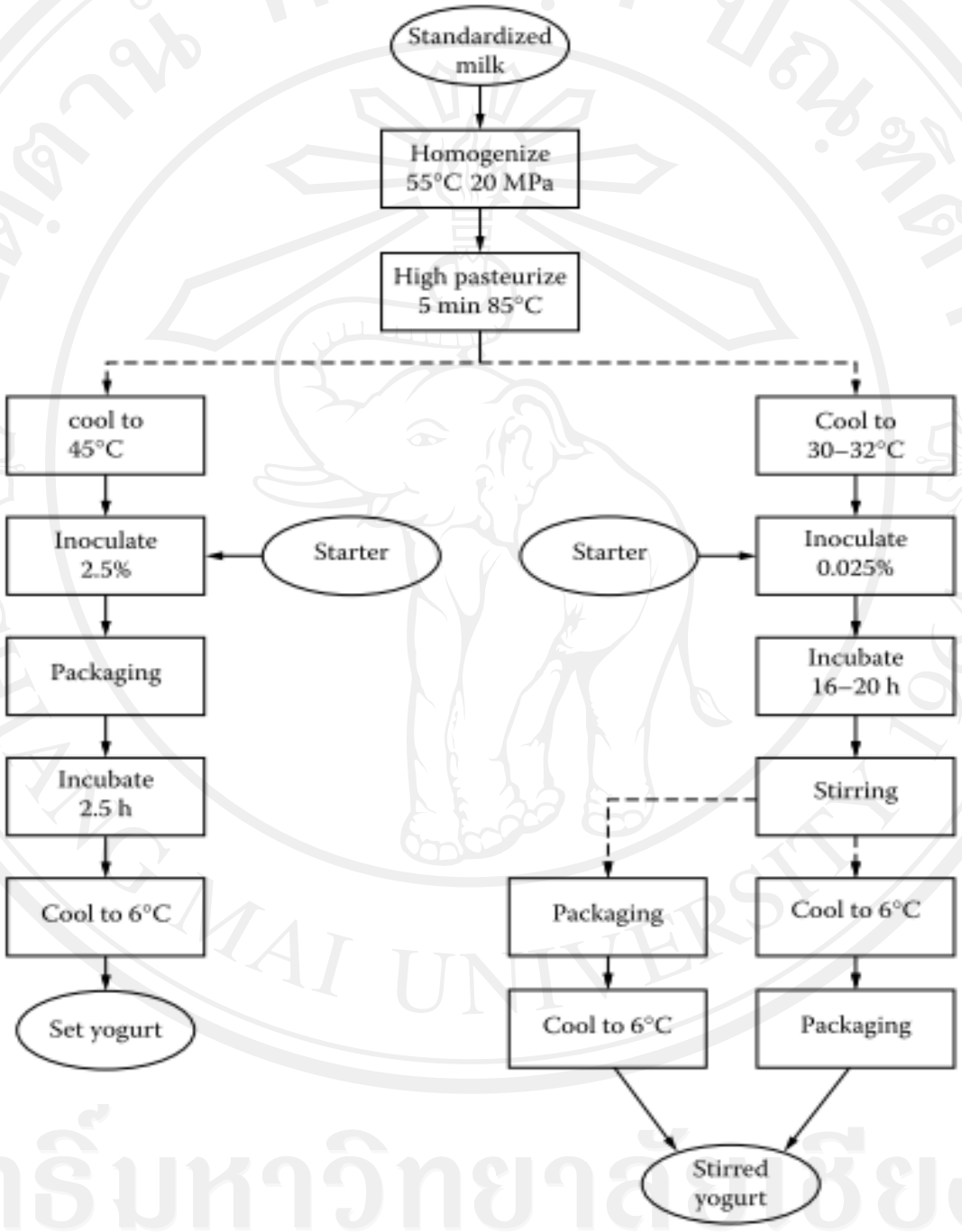

Figure 2.2 Examples of the manufacture of set yoghurt and of stirred yoghurt from whole milk. Set yoghurt is often made from concentrated milk (Q ≈1.4) (Walstra et al., 2006).

2.3.2 Yoghurt bacteria

Yoghurt is produced by fermentation of milk. Two of the bacteria found in yoghurt are *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus.* These are lactose fermenting bacteria that produce lactic acid from the lactose milk. The lactic acid they produce gives yoghurt its taste. These bacteria are microaerophiles; they grow best in the low or absence of oxygen. They are also thermophiles so they grow best at fairly high temperatures of 40 – 42°C. The bacterial strain *S. thermophilus* was known to promote gastrointestinal health. The yoghurt bacteria ferment the sugar in the milk, also known as lactose. The lactose is transformed into lactic acid, which is highly effective in preventing lactose intolerance. The production of the lactic acid reduces pH and results in the milk curdling and turning tart. This also prevents the growth of other bacteria that causes food poisoning (Thiel, 1999).

The yoghurt bacteria, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, grow in milk better when present together than each alone (protocooperation). The proteolytic rods enhance growth of the streptococci by forming small peptides and amino acids, the main amino acid being valine. Milk contains too little of these amino acids and the cocci, which are weakly proteolytic, form the acids too slowly. The cocci enhance the growth of the rods by forming formic acid out of pyruvic acid under anaerobic conditions and by a rapid production of CO_2. The stimulatory effect of formic acid remains unnoticed in intensely heated milk because in this milk formic acid has been formed by decomposition of lactose. The production of formic acid by the cocci is, however, essential in industrial practice, where more moderate heat treatments of yoghurt milk are applied, e.g., 5 to 10 min at 85°C. Due to mutual stimulation during combined growth of the yoghurt bacteria in milk, lactic acid is produced much faster than would be expected on the basis of the acid production by the individual pure cultures. Some antibiosis also occurs in yoghurt in that the cocci cannot grow after a certain acidity has been reached. The rods are less susceptible to acid and continue to grow. Protocooperation and antibiosis are of great importance in the growth of the yoghurt

bacteria as well as for the quality of yoghurt (see Figure 2.3) (Walstra et al., 2006).

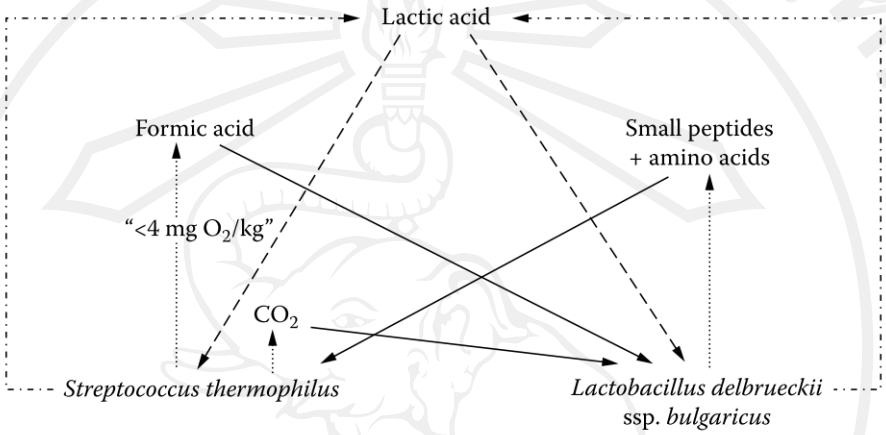

Figure 2.3 Outline of the stimulation and the inhibition of the growth of yoghurt bacteria in milk (Walstra et al., 2006).

The cocci as well as the rods contribute significantly to the properties of yoghurt. The properties of the bacterial strains used should be matched to each other because not every combination of strains is suitable. Furthermore, both species should be present in large numbers in the product, and hence in the starter. The mass ratio of the two species depends on the properties of the strains and is often approximately 1:1. This ratio between the yoghurt bacteria is best maintained if the inoculum percentage is 2.5, the incubation time is 2.5 h at 45°C, and the final acidity is approximately 90 to 100 mM (pH ≈ 4.2). During incubation, the ratio between the species keeps changing. Initially, the streptococci grow faster due to the formation of growth factors by the rods and probably also due to the latter compounds being added via the inoculum (especially in the manufacture of set yoghurt). Afterwards, the cocci are slowed down by the acid produced. Meanwhile, the rods have started to grow faster because of the growth factors (CO_2 and formic acid) formed by the cocci. As a result, the original ratio is regained. The

yoghurt should then have attained the desired acidity. Continuing incubation or inadequate cooling caused the rods to become preponderant (Walstra et al., 2006).

Varying the aforementioned conditions during incubation changes the ratio between the rods and the cocci as follows:

(1) *Incubation time*: A shorter incubation time, which means a lower acidity, will cause too high a proportion of cocci. Transferring a yoghurt starter repeatedly after short incubation times during the production of the starter may also cause the rods to disappear from the culture. Conversely, long incubation times will cause an increasing preponderance of the rods.

(2) *Inoculum percentage*: Increasing the inoculum percentage will enhance the rate of acid production. The acidity at which the cocci are slowed down will thereby be reached earlier, resulting in an increased number of rods (incubation time being the same). At a smaller inoculum percentage, the ratio between the bacteria will shift in favour of the cocci.

(3) *Incubation temperature*: The rods have a higher optimum temperature than the cocci. Incubation at a slightly higher temperature than 45°C will shift the ratio in favour of the rods; incubation at a lower temperature will enhance the cocci (Chandan, 2006; Walstra et al., 2006).

Obviously, a correct ratio between the species in the starter can be maintained, or be recovered if need be, by proper selection of the propagation conditions. Currently, concentrated starters are increasingly used, ensuring a correct bacterial composition of the starter (Tamime and Robinson, 1999; Chandan, 2006).

2.3.3 Quality of yoghurt

The commercial yoghurt is usually made by fermenting milk with a mixed

culture of *L. bulgaricus* and *S. thermophilus*. Each of these organisms acidifies milk and produces specific yoghurt flavour and aroma. The addition of probiotic bacteria is made because of certain claimed health-promoting effects in the intestinal tract. These beneficial health effects included enhancement of the immune system, reduction of lactose intolerance, control of diarrhea and reduction of low-density lipoprotein cholesterol. Product quality and consumer satisfaction are important for the acceptance of various types of yoghurt products. Quality assessment encompasses specifications, sampling, testing procedures and recording or reporting. Yoghurt quality is difficult to standardise because of many forms, varieties, manufacturing methods, ingredients and consumer preferences that exist (Hussain et al., 2009). The quality of the product depends on the production control of lactic acid formed by fermentation. Lactic acid provides the tart flavour and the destabilization of milk protein forms the gel structure. The pH measurement monitors lactic acid production and aids in the quality control of yoghurt's ingredients (Tamime and Robinson, 1999).

Physical properties of yoghurt

The physical structure of yoghurt is a network of aggregated casein particles onto which part of the serum proteins have been deposited due to their heat denaturation. The network encloses fat globules and serum. The largest pores of the network are on the order of 10 μm. The existence of a continuous network implies that yoghurt is a gel, a viscoelastic material characterized by a fairly small yield stress (around 100 Pa). If the gel is broken up, as in the making of stirred yoghurt, a fairly viscous non-Newtonian liquid can be formed; it is strongly shear rate thinning and thus has an apparent viscosity. Set and stirred yoghurt have markedly different textures (Walstra et al., 2006).

(1) Firmness of set yoghurt

Firmness of set yoghurt is often estimated by lowering a probe of a given

weight and dimensions into the product for a certain time. The reciprocal of the penetration depth then is a measure of firmness. Firmness is not closely related to an elastic modulus but rather to a yield stress. Its value depends on the method of measurement, especially the timescale, and on several product and process variables:

Casein content of the milk: Firmness is approximately proportional to the crude of the casein content. Natural variation in casein content can thus have a marked effect. Evaporating the milk, adding skim milk powder, or partial ultrafiltration increase firmness.

Fat content: The higher the fat content, the weaker the gel because the fat globules interrupt the network.

Homogenising: Homogenisation of the milk leads to a much enhanced firmness because the fat globules then contain fragments of casein micelles in their surface coat by which they can participate in the network upon acidification. The volume fraction of casein is thus effectively increased. Homogenisation of skim milk makes no difference.

Heat treatment: Heat treatment of the milk considerably enhances firmness. The deposition of denatured serum proteins increases the volume fraction of aggregating protein; it may also alter the number and the nature of the bonds between protein particles. Milk is generally heated for 5 to 10 min at 85 to 90°C.

Yoghurt cultures: These vary in the firmness they produce (at a given acidity), but as a rule, the differences are small.

Acidity: Generally, the yoghurt is firmer at a lower pH. The preferred pH is between 4.1 and 4.6.

Incubation temperature: The lower it is, the longer it takes before a certain pH, and thereby certain firmness is reached, but the finished product is much firmer.

Temperature of the yoghurt: For the same incubation temperature, a lower measuring temperature gives a greater firmness. The effect is quite strong. The explanation is, presumably, that the casein micelles swell when the temperature is lowered (and vice versa); because the particles are essentially fixed in the network and the network cannot swell, this would imply that the contact or junction area between any two micelles is enlarged, by which a greater number of bonds are formed per junction (Tamime and Robinson, 1999; Walstra et al., 2006).

(2) Syneresis

Syneresis of casein gels is discussed for rennet-induced gels. Briefly, syneresis is due to a rearrangement of the network, leading to an increase in the number of particle–particle junctions. The network then tends to shrink, thereby expelling interstitial liquid. Acid casein gels are not very prone to syneresis. In yoghurt, syneresis is undesirable. The tendency to exhibit syneresis greatly depends on the incubation temperature. If milk is incubated at 20°C (with a mesophilic starter because yoghurt bacteria hardly grow at that temperature) so that the gel is formed at that temperature, absolutely no syneresis occurs, whereas when incubating at 32°C, syneresis is possible. When incubating at 45°C, syneresis can only be prevented if the milk has been intensively heated, if its casein content has been increased, and the storage temperature is low. However, if the package containing the product is even slightly shaken at a time when gel formation has just started and the gel is still weak, it may fracture locally with copious syneresis occurring subsequently. If the top surface of the set yoghurt is wetted, possibly because water is condensed on the inside of the lid of the package and a few drops fall off, whey separation may be induced. If the pH of the yoghurt has fallen below 4, some syneresis may also occur, especially if the temperature is fairly high and the package is shaken. Containers made of a material to which the formed gel does not stick will readily induce whey separation between the wall and the product. In the manufacture of stirred yoghurt, significant syneresis will lead to a poor product. The stirring breaks the gel into lumps, which then would immediately exhibit syneresis. An inhomogeneous

mixture of lumps in whey is formed; further stirring would break down the lumps and make a smoother product, but it would then become insufficiently viscous. To prevent this, it is necessary to incubate the milk at a low temperature, e.g., 32°C or even lower, if the casein content of the milk is small (Walstra et al., 2006; Hussain et al., 2009).

(3) Viscosity of stirred yoghurt

Stirred yoghurt should be smooth and fairly viscous. A good product also gives the impression of being 'long' or 'stringy'; when slowly pouring it, a fairly thin thread readily forms that behaves somewhat elastically when it breaks. Viscosity is most easily determined by means of a Ford cup; a given amount of yoghurt is allowed to flow from an opening at the conical lower end of a cup, and the time needed for that is a measure for the viscosity. The product is strongly shear rate thinning. After a high shear rate is applied, the apparent viscosity at lower shear rates is permanently decreased, and the viscous behaviour becomes closer to Newtonian. This implies a lasting breakdown of structure. This is all in agreement with the behaviour of a liquid containing gel fragments. The viscosity increases with the viscosity of the continuous liquid ('solvent' or 'whey') and with the volume fraction φ of gel fragments. The latter is larger than the volume fraction of casein particles because the fragments contain a lot of interstitial solvent. More intensive stirring (a higher shear rate) further breaks down the gel fragments and also gives them a more rounded shape, thereby decreasing the effective φ. Vigorous agitation of stirred yoghurt during further processing must be avoided to prevent the product from becoming too thin. Packaging machines can be especially damaging (Tamime and Robinson, 1999; Walstra et al., 2006).

(4) Flavour defects and shelf life

A main quality problem with yoghurt is that souring tends to go on after delivery to the retailer, and the product may be too acidic when consumed; the acid flavour tends to be more pronounced in low-fat yoghurt. Moreover, the yoghurt may become bitter due to excessive proteolysis; this would also depend on the starter strains

used. The development of these defects generally determines the shelf life. Even though the product is cooled to slow down acidification, but it is difficult to cool it fast enough. Set yoghurt is acidified in a package and cannot be stirred; stirred yoghurt should not be stirred too vigorously because it would then become too thin. And even at refrigerator temperatures, acidification and other changes caused by the enzyme systems go on, albeit slowly. Other defects may be caused by contaminating organisms, mainly yeasts and moulds. The off-flavours may be characterized as yeasty, fruity, musty, cheesy, or bitter, and occasionally, soapy-rancid. A flavour threshold is generally reached at a count of about 10^4 yeasts and moulds per ml. The growth of these microbes is largely determined by the amount of oxygen available, and hence by the headspace volume and the air permeability of the container (Tamime and Robinson, 1999; Walstra et al., 2006).

Another defect is insufficient characteristic flavour due to a low acetaldehyde formation (which is of less importance in yoghurts with added fruits). It may be due to a low incubation temperature, an excessive growth of the streptococci, or the lactobacilli being weak aroma producers. Insufficient acidification, e.g., because the milk is contaminated with penicillin, also leads to a bland product. Finally, off-flavours in raw milk used for manufacture may naturally cause flavour defects in the product (Walstra et al., 2006).

Total solid of yoghurt

Hussain et al. (2009) reported the quality comparison of probiotic and natural yoghurt, the average total solids content of probiotic yogurt was 17.75 with a standard deviation of 0.006. While the average total solids content of natural yogurt was of 19.2 with a standard deviation of 0.035. As regards the probiotic yogurt these results are in line with findings of Muhammad et al. (2005) who reported the highest range of total solids in yogurt was17.1%. The average of total acidity probiotic yogurt was 1.41 percent with a standard deviation of 0.03. The average total acidity of natural yogurt was 1.44

with a standard deviation of 0.01. These results are in line with findings Tarakci and Erdogan (2003) in which acidity increased over the storage period. Guler and Mutlu (2005) also observed an increase in total acidity during the storage period.