Microbiological and Biochemical Investigations of Cocoa Bean Fermentation

Dissertation

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by

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The following work was conducted during the time period from April 2011 – October 2014 in the research group of Professor Dr. Bernward Bisping at the Division of Food Microbiology and Biotechnology, Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Hamburg, Germany.

1. Gutachter / Reviewer: Prof. Dr. Bernward Bisping
2. Gutachter / Reviewer: Prof. Dr. Sascha Rohn

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I hereby declare under oath that I have worked on this dissertation independently and have used sources and equipments as specified in this work. This dissertation has not been previously submitted in part or in total to any other institution. This is my first attempt to submit a dissertation in order to obtain a doctorate degree.

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Fahrurrozi

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List of Publications

Poster publications

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Fahrurrozi, Bahmann C, Lieberei R, Bisping B (2013) Antifungal activity in seed coat extracts of *Theobroma cacao* L. Poster presentation at VAAM International Conference on Microbiology, Bremen, Germany, 10 - 13 March 2013.

Fahrurrozi, Bahmann C, Niemenak N, Lieberei R, Bisping B (2014) Antifungal protein of seed coat extracts of *Theobroma cacao* L. during fermentation. N Biotechnol 31: S178.

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1   Introduction

1.1   History of cocoa and chocolate

The term 'cocoa' is a corruption of the word 'cacao' that is taken directly from Mayan and Aztec languages. Chocolate is derived from cocoa beans, found in the fruit of cocoa tree, *Theobroma cacao*, which is indigenous to South America and believed to have originated from the Amazon and Orinoco valleys. *Theobroma cacao* (food of the gods) belongs to the family *Sterculiaceae* and includes four principal types: Criollo, which accounts for about 5% of world cocoa production; the more common Forastero, with smaller, flatter and purple beans; and Nacional with a fine flavour, grown in Ecuador. The fourth variety, Trinitario, a more disease-resistant hybrid of Criollo and Forastero is regarded as a high flavour bean (Fowler 2009).

Wild cocoa trees originate from the Amazon, but the cultivated crop is found around the world on the tropical belt, where the humid and hot climate allows for economically lucrative cocoa production. *Theobroma cacao* grows between the tropics of Cancer and Capricorn, with varieties originating in forest areas of South America. Forastero basic cocoa grows mainly in Brazil and West Africa, whilst flavour cocoas are largely hybrids and are cultivated in Central and South America (Afoakwa 2010).
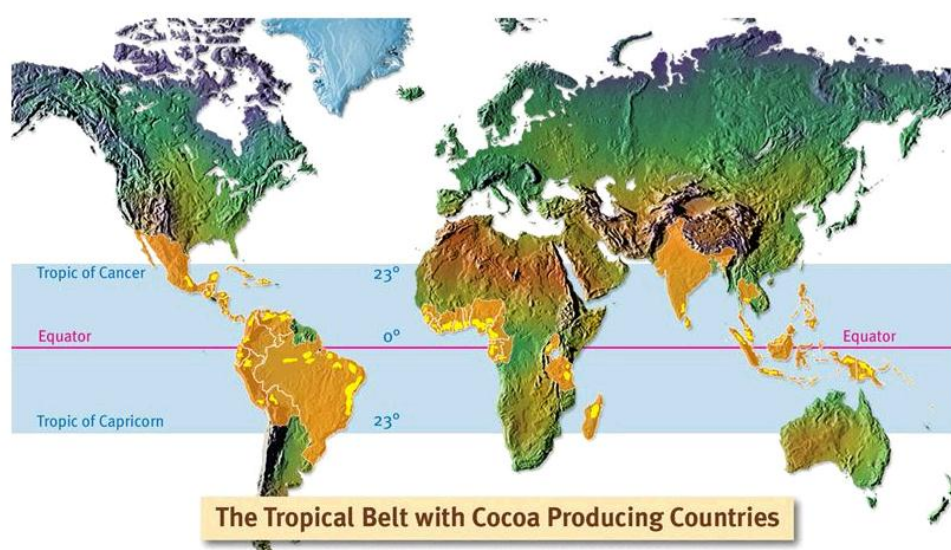
Figure 1.1 Cocoa producing countries (Rohsius et al. 2010)

The distribution of cocoa throughout the world started from Mexico with descendants of the Criollo group. Later, mainly Forasteros from the Eastern part of South America were transported to West Africa (known as the West African Amelonados). Although cocoa breeding programs have been enforced since the early 1960s, the ancient distribution pattern of fine and flavour vs. bulk cocoas can still be observed in most of the countries. Fine and flavour Criollo and Trinitario descendants still predominate in e.g. Java, Papua New-Guinea and Madagascar, whereas bulk cocoa (Forastero) is prevalent in West Africa (Rohsius et al. 2010).

The use of cocoa beans dates back at least 1400 years (Rössner 1997), when Aztecs and Incas used the beans as currency for trading or to produce the so-called "chocolatl", a drink made by roasting and grinding cocoa nibs, mashing them with water, and often adding other ingredients such as vanilla, spices or honey. In the 1520s, the drink was introduced into Spain (Minifie 1989) although Coe and coworkers emphasize that the European arrivals in the new world, including Christopher Columbus and Hernan Cortes, were unimpressed with the Mayan beverage (Coe et al. 1996). Nevertheless, the conquistadors familiarized Europe with the chocolate beverage, and being expensive, it was initially reserved for consumption by the highest social classes. It was only in the seventeenth century that the consumption of chocolate spread throughout Europe (Afoakwa 2010).

Progress in cocoa and chocolate production together with industrialization during the 18th and 19th century made it possible to give chocolate creative and innovative shapes that would forever change its appearance from a drink to pressed blocks and bars. Amongst others, the cocoa beans grinder (invented by the Frenchman Doret in 1778), the cocoa press (discovered by the Dutchman Van Houten in 1828) and the conche (invented by the Swiss Lindt in 1879) were introduced, and the first solid 'eating' chocolates were created by incorporating sugar and cocoa butter into the paste (created by the English Fry in 1848) (Afoakwa 2010). Milk chocolate was first created by the Swiss Peter in 1875 after Nestlé is discovery of how to manufacture milk powder in 1867 (Afoakwa 2010).

In today's Europe, the leading chocolate producers are Belgium, Switzerland, France, Germany, and the United Kingdom. Whereas Belgium is world famous for its extremely fine, excellent tasting and high-quality dark chocolate, French chocolate became renowned in

French gastronomy; Swiss chocolate is appreciated worldwide for its seductive silky smoothness and purity. Today, Cadbury, Nestlé, Mars, the Ferrero Group, and Hershey Foods represent 60% of the world market of chocolate (www.candyindustry.com).

1.2 World cocoa production

Theobroma cacao originated in the Amazon Basin, where the optimal growth conditions of 20 – 30 °C, 1500–2500 mm of annual rainfall and 2000 hours of sunshine per year are found. Table 1.1 shows that the density of production is centered within West Africa, accounting for approximately 71% of world cocoa production in the 2012 – 2013 growing season. West African countries are ideal in terms for climate for growing cocoa as a cash crop. However, as a consequence, natural or man-made problems can potentially have a disproportionately large impact on the cocoa trade. Small holders in West Africa have dominated world production since the 1930s. In the 1980s, the emergence of Malaysian and Indonesian productions led to a more balanced geographical spread of production (Afoakwa 2010).

Table 1.1 World cocoa production

Countries	Thousand tones 2011/12	%	Thousand tones 2012/2013	%	Thousand tones 2013/2014*	%
World total	4085	100.0	3929	100.0	4162	100.0
Africa	**2919**	**71.5**	**2823**	**71.9**	**2981**	**71.6**
Cameroon	207	5.1	225	5.7	205	4.9
Ivory Coast	1486	36.4	1449	36.9	1610	38.7
Ghana	879	21.5	835	21.3	850	20.4
Nigeria	235	5.8	225	5.7	230	5.5
Others	113	2.8	89	2.3	86	2.1
America	**655**	**16.0**	**622**	**15.8**	**676**	**16.2**
Brazil	220	5.4	185	4.7	210	5.0
Ecuador	198	4.8	192	4.9	210	5.0
Others	237	5.8	245	6.2	256	6.2
Asia & Oceania	**511**	**12.5**	**484**	**12.3**	**505**	**12.1**
Indonesia	**440**	**10.8**	**410**	**10.4**	**425**	**10.2**
Papua New Guinea	39	1.0	37	0.9	40	1.0
Others	32	0.8	37	0.9	40	1.0

Source: ICCO Quarterly Bulletin of Cocoa Statistics, Vol. XL, No. 2, Cocoa year 2013/14, *) forecasts

1.3   Cocoa varieties and the effect of genotype on cocoa bean flavours

The oval, leathery cocoa fruits vary among varieties in size, shape, external color, and appearance (Fig. 1.2). Criollo pods are small and elongated, soft, thin-skinned and are typically red or yellow, sometimes green or white. The pods have a bumpy (five to ten deep furrows) or warty skin with pointed tips. Forastero pods are moderately sized, thick-walled, green, and relatively smooth with a more or less bulbous (no pointed tips) or, sometimes round shape (type Amelonado). The woodier hull makes the pods harder to open. Trinitario pods can be red or yellow as well as orange or purple, large, elongated but not pointed, moderately ribbed, sometimes warty or with a relatively smooth skin. Nacional pods are large, green, thick walled, deeply bumped, and oval, slightly narrowed at the basis and with

a blunted point. At harvest time, cocoa pods change color from green or dark red-purple to yellow, orange, or red, depending on the cocoa variety (Wood and Lass 2008).

a) Criollo   b) Nacional

c) Forastero   d) Trinitario
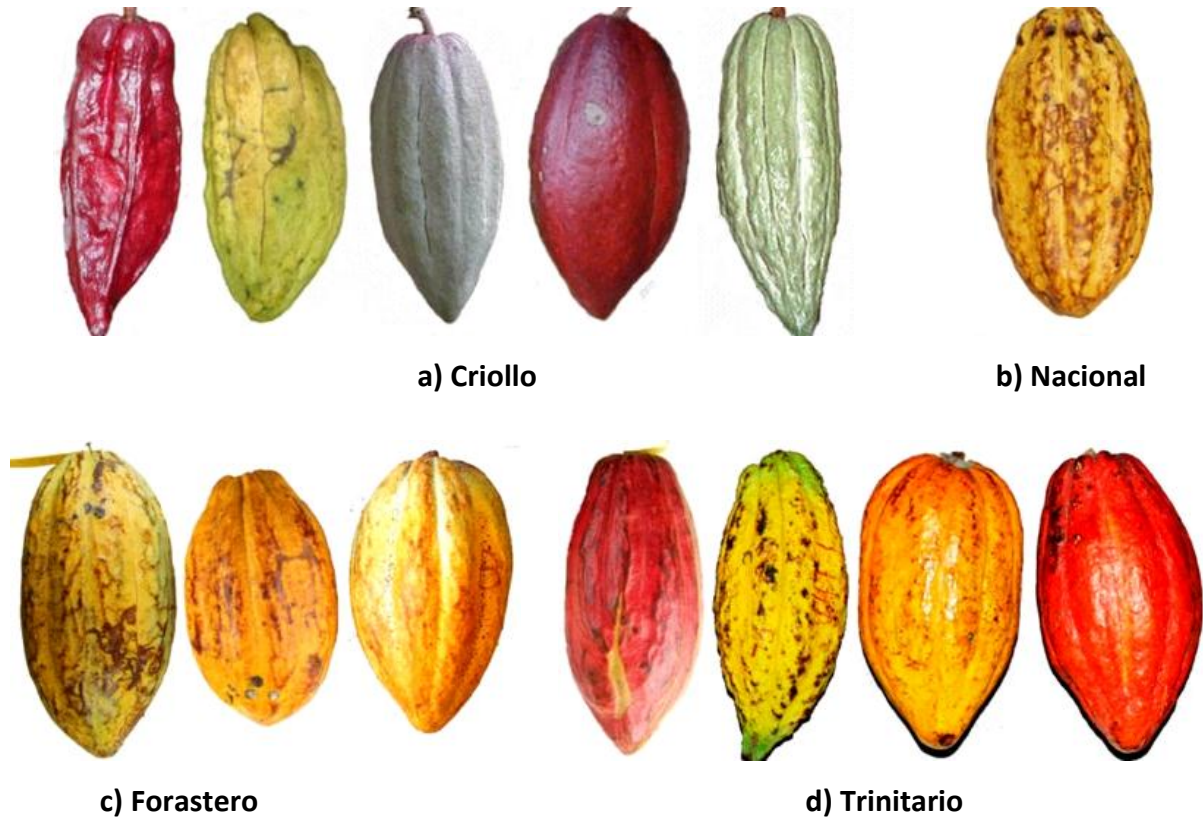
Figure 1.2 Cocoa varieties: a) Criollo, b) Nacional, c) Forastero, d) Trinitario

Criollo, Trinitario and Nacional are fine flavoured cocoas with different flavour attributes; Criollo are nutty and floral, Trinitario are acidic and fruity, while Nacional are floral, fruity, raw/beany/green. Forastero is bulk grade cocoa with the flavour attributes of cocoa, bitter and astringent (Afoakwa 2010; Beckett 2009). Bulk cocoa is usually used for milk chocolates and cocoa butter, while fine or flavour cocoas are usually used for premium plain dark chocolates and couvertures (Rohsius et al. 2010).

1.4 The cocoa pulp and beans

1.4.1 Cocoa pulp

Cocoa pulp is the raw material which serves as the basis for fermentation, as it is a rich medium for microbial growth. This mucilage constitutes about 10% of the mass of the cocoa fruit. It is composed of 82 - 87% water, 10 - 15% sugars, 1 - 5% pectin, 1 - 3% citric acid, 0.1 - 0.4% other non-volatile acids (malic acid and tartaric acid), 0.5 - 0.7% proteins and 8 - 10% minerals and trace elements (Schwan et al. 1995). Of the sugars present, about 60% is sucrose and 39% is a mixture of glucose and fructose. The concentration of sucrose, glucose, and fructose is a function of cultivar and fruit age, with unripe pods containing a higher proportion of sucrose and ripe pods containing mainly fructose and glucose (Thompson et al. 2001). The amino acids, aspartic acid, glutamic acid, and asparagine, have been reported to be present at high levels in the pulp compared to other amino acids. Vitamin C is the most important vitamin in cocoa pulp. Potassium and sulfate are the major cations and anions, respectively. Cocoa pulp contains a higher concentration of sulfate than chloride or phosphate. The pH of the pulp is relatively low (pH 3.0 to 4.0), mainly due to the citric acid content. The high content of pectin and other polysaccharides (cellulose, hemicellulose, lignin) makes the pulp viscous, sticky, and cohesive.

1.4.2 Cocoa beans

Each cocoa pod contains 20 - 30 (Criollo) or 30 - 40 (Forastero and Trinitario) beans embedded in a sweet, white, mucilaginous pulp that represents approximately 40% of the seed's fresh weight. The beans are arranged in five rows, derived from the five locules of the ovary around and loosely attached to a central placenta (Camu et al. 2007).

Criollo beans are large, rather round and white (or slightly pale violet in pigment, possibly due to the presence of alleles of Forastero origin) in cross-section, ferment quickly (2 - 3 days), and in the past were reported to have a highly regarded (caramel, nut, or honey flavour), slightly bitter, but usually weak, chocolate flavour. Compared to Criollo, Forastero beans are smaller and flatter and the cotyledons are violet. They ferment slowly (5 - 7 days) and their flavour produced upon proper processing is stronger (bitter and cocoa flavour).

In addition, the Forastero beans possess a higher fat content than Criollo beans. Trinitario beans provide a less intense but fine taste, constituting an intermediary between the Criollo and Forastero beans. The Arriba-type beans (Nacional) are large, flat, and deep purple, and produce cocoa with a 'fine' flavour, i.e. an intense cocoa flavour, which is very aromatic and with a slight bitterness. This typical, highly appreciated flavour is determined largely by genetic factors, but the fermentation process and further processing play a role, too. Finally, a different and fruitier flavour can be processed in much the same way as chocolate in order to produce 'cupulate' from beans of *Theobroma grandiflorum* or cupuaçu (Rohsius et al. 2006; Reisdorff et al. 2004).

Fresh, unfermented cocoa beans basically consist of two parts (Fig. 1.3 a): an outer part comprising the testa (seed coat) surrounding the bean, and an inner part comprising two cotyledons contained within the testa and uniformed at a small embryonic axis and the embryo (germ). The cotyledons are referred to as the nibs in the cured bean. The testa of the cocoa seed is impermeable to larger molecules, whereas smaller volatile molecules such as ethanol and acetic acid can easily penetrate the testa. In this way, the testa provides a natural barrier against organic acid (citric acid) penetrating from the pulp into the bean as well as discouraging the outward diffusion of theobromine, caffeine, and polyphenols, hence controlllings the kinetics of fermentation and the concomitant diffusion processes (see Section 1.9.2).

The cotyledons are basically made up of different types of storage cells, such as white lipid/protein/starch and purple polyphenol-containing cells, with parenchyma plasma forming a grid between the two types of cells (Fig. 1.3 b). Whereas the storage cells of starch granules, aleurone grains, and fat droplets constitute the reserve material for the embryo and lipids form a natural barrier around the water-soluble compounds (enzymes, substrates, and inhibitors), the larger, vacuolated, polyphenol storage cells contain unique cocoa bean components encompassing purine alkaloids (theobromine and caffeine) and polyphenols, both possibly involved in the stress resistance responses of the plant.

The fat of the cocoa bean is important for chocolate production, which depends on cocoa butter as an essential ingredient. Fat makes up slightly more than 50% of the mass of the dry unfermented cocoa bean cotyledons. The cocoa butter present in the bean is a relatively

simple fat comprised primarily of palmitic acid (C16:0, saturated, 25%), stearic acid (C18:0, saturated, 35%), and oleic acid (cis-C18:1, mono-unsaturated, 35%), with small amounts of myristic, linoleic, linolic, and arachidic acids (5%). The chemical composition of an unfermented bean is shown below (Tab. 1.2).

Table 1.2 Bean composition of unfermented West African (Forastero) cocoa

Constituents	Dried bean (%)	Fat-free materials (%)
Cotyledons	89.60	—
Shell	9.63	—
Germ	0.77	—
Fat	53.05	—
Water	3.65	—
Ash (total)	2.63	6.07
Nitrogen		
Total nitrogen	2.28	5.27
Protein nitrogen	1.50	3.46
Theobromine	1.71	3.95
Caffeine	0.085	0.196
Carbohydrates		
Glucose	0.30	0.69
Sucrose	1.58	3.86
Starch	6.10	14.09
Pectins	2.25	5.20
Fibre	2.09	4.83
Pentosans	1.27	2.93
Mucilage and gums	0.38	0.88
Polyphenols	7.54	17.43
Acids		
Acetic (free)	0.014	0.032
Oxalic	0.29	0.67

Sources: (Afoakwa 2010; Reineccius et al. 1972; Rohan 1963)

a

Cotyledons

Radicle

Pulp

Testa

b

Lipid/protein/starch
storage cells

Polyphenol
storage cells

Fat vacuoles

Plasma

Nucleus

Mitochondria

Starch grains

Protein granules

Polyphenols
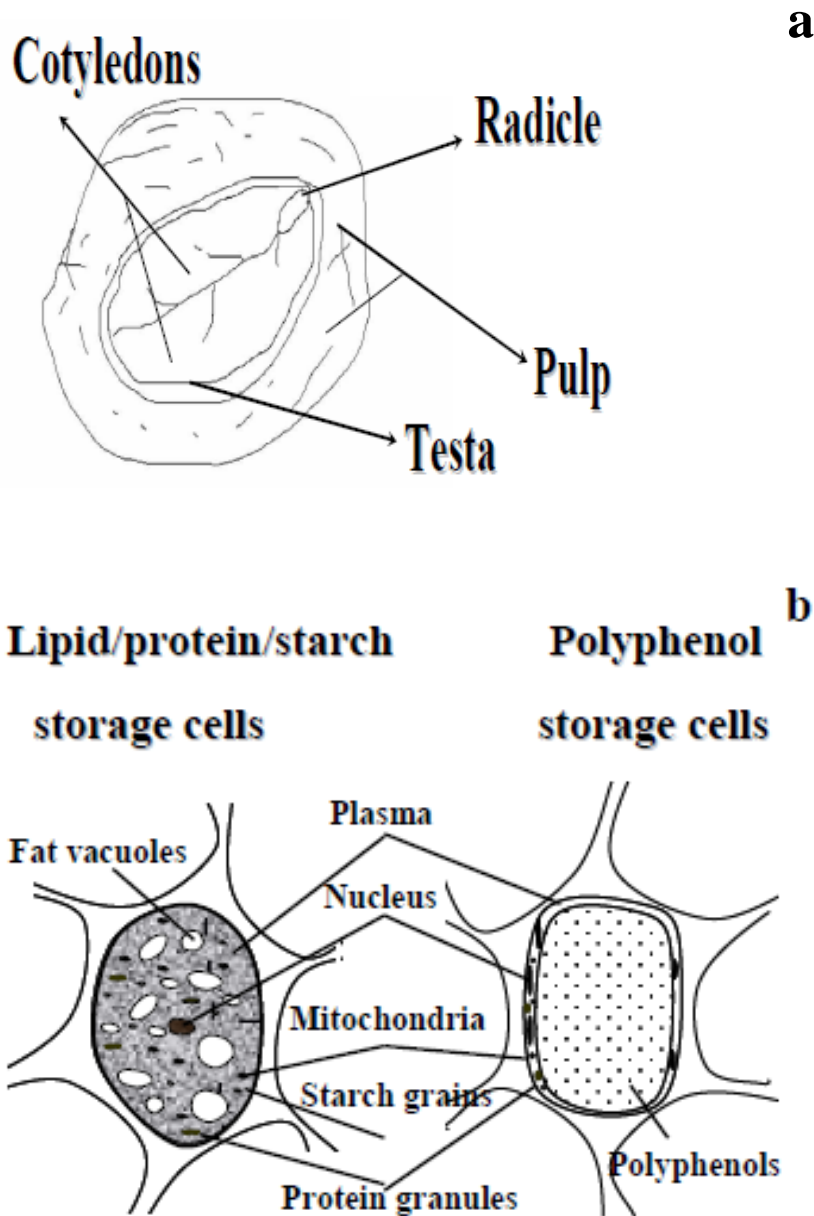
Figure 1.3 a) Cocoa bean, b) Cocoa bean storage cells

1.5 Fermentation of cocoa pulp and beans

1.5.1 Cocoa pulp inoculation

The pulp is microbiologically sterile when healthy, undamaged pods are opened aseptically. The interior of ripe pods may contain a few hundred microorganisms (yeasts) per gram (Jespersen et al. 2001). When the pulp and bean are manually removed from the opened

pods, the pulp is accidentally contaminated with a variety of microorganisms, prevalent in the surrounding environment. Contamination can occur through cocoa pod surfaces, banana and plantain leaves used to construct and cover the heaps, boxes, and baskets, knives used for opening the pods, the workers' hands, unwashed baskets used for transient seed transport, dried mucilage left on the walls of receptacles from previous fermentations, and fruit flies and other insects (Ostovar and Keeney 1973; Jespersen et al. 2001). Many but not all of these microorganisms contribute to the subsequent fermentation (Schwan and Wheals 2004).

1.5.2   Cocoa bean fermentation

The batch process of cocoa bean fermentation forms the basis of the entire chocolate production and plays a significant role in determining the flavour and health properties of chocolate and chocolate-related products. Cocoa bean fermentation is a spontaneous fermentation process. It takes two to eight days, depending on the variety of cocoa and local practice, with five to six days being most common, and is most often carried out in heaps or wooden boxes with 100 - 1000 kg bean. The bean may be mixed or turned once daily (usually after 24 and 48 h, and sometimes after 96 and 144 h of fermentation) or not at all during the fermentation process (Wood and Lass 2008).

Critical process parameters for cocoa bean fermentation are the type of cocoa bean, the health status of the cocoa pods, the ripeness of the pods, post-harvest pod storage, the quantity of bean and pulp, bean packing or spreading, fermentation method, batch size, the duration of fermentation, turning, seasonal variations and weather conditions, etc. For instance, storing the harvested cocoa pods for a number of days before opening is considered beneficial for fermentation, as it results in a faster fermentation, because the pulp sucrose is already converted into glucose and fructose by cotyledon invertase activity (Schwan and Wheals 2004; Tomlins et al. 1993, Schwan 1998). Pod ripeness at harvest is also of crucial importance. Fresh beans can vary considerably in the ratio of pulp to bean and in the amount of sugars per bean, depending on cocoa cultivar, growing conditions, and post-harvest storage of the pods. For example, pulp and beans can contain more water during the wet season, which influences fermentation by affecting the aeration of the fermenting mass

and beans acidity. Indeed, beans with more pulp restrict gas exchange and make the bean mass more anaerobic, so that a higher bean sugar content may lead to greater amounts of acids in the bean cotyledons at the end of fermentation. Up to 20% of the pulp of the total fresh weight of the bean plus pulp can be removed, either through a natural 'sweatings' drain with mechanical depulpers, or through the addition of pectinolytic enzymes, which enables a reduction of fermentation time from seven to four days, and hence lowers the acidity and enhances the flavour of the final cocoa (Schwan et al. 1995; Schwan and Wheals 2004). As an example, in Malaysia harvested cocoa pods are stored for up to 15 days before breaking and removal of the beans, or the beans are pressed or pre-dried to reduce the pulp volume before fermentation (Lehrian and Patterson 1983). Unfortunately, Malaysian cocoa produced on a large scale is considered to be poor quality cocoa.

1.6   Methods of cocoa bean fermentation

Various cocoa bean fermentation systems have been developed worldwide. The actual methods of fermentation vary in different cocoa-producing countries and regions and even from one grower to another. The different fermentation methods include heap, box, basket, tray, and platform fermentation. Of these, two methods of fermentation have been commonly used for many years worldwide, namely the heap and box methods. Approximately one-half of the world cocoa crops is fermented in some type of box, and the remaining half is fermented by using heaps or other traditional methods that are slowly disappearing.

1.6.1   Heap fermentation

The heap fermentation is the most simple and most commonly used method of fermentation on smaller farms (Fig. 1.4, 1.5). The equipment required is simple and is available at practically no cost, so that this type of fermentation can be run by a family. In heap fermentation, the wet beans are piled onto banana or plantain leaves, spread out in a circle on the ground, or sometimes raised above soil level to allow for easy pulp drainage. When the heap is complete, it is covered with more leaves, which are often held in place by small logs. The cover protects the fermenting mass against surface mold growth and keeps

the heat inside. In general, sweatings are allowed to flow away and penetrate into the ground. The size of the heaps varies widely; heaps from 25 - 2000 kg are common. This method is used throughout West-Africa and almost exclusively in Ghana, where farmers ferment their beans in heaps of 200 - 500 kg for six days with care, explaining the higher quality of Ghanaian cocoa compared with Nigerian or Ivorian (Baker et al. 1994). To ensure uniform fermentation, to enhance the growth of beneficial microorganisms, and to discourage surface mold growth, the heap should be mixed every two days, which is done practically by forming another heap. However, turning the heaps is tedious and most Ghanaian farmers only turn large heaps or do not turn their heaps at all (Baker et al. 1994).
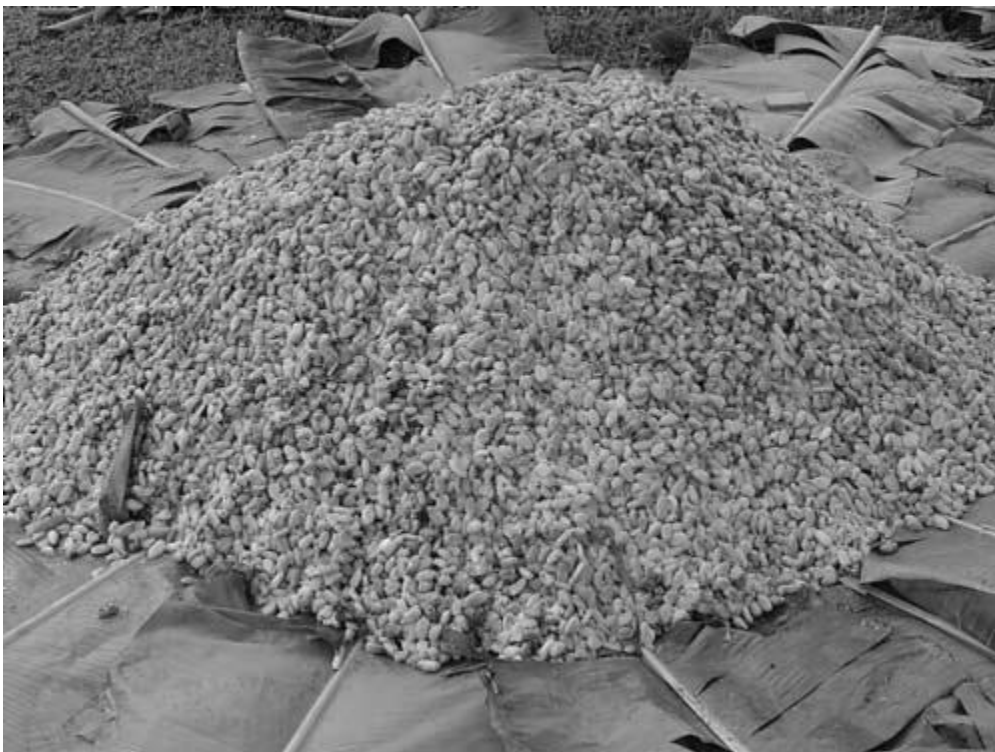
Figure 1.4 Heap of cocoa beans prior to fermentation on a West African farm (Afoakwa 2010)

Figure 1.5 Cocoa beans covered with plantain leaves in heap fermentation (Afoakwa 2010)

1.6.2   Box fermentation

Box fermentations are carried out in large, perforated, open, wooden boxes, made of local hardwoods in a wide variety of shapes and sizes. A typical box measures 1.2 x 1.2 x 0.9 m and holds just over a ton of wet beans when loaded to a depth of 0.75 m (Fig. 1.6). The box fermentation method is generally used on larger farms or plantations in for instance Brazil, Trinidad, Indonesia, and Malaysia. The floor of the boxes is usually made of similar wood, in which 15-mm holes are drilled at intervals of 10 - 15 cm. These holes are essential to provide drainage for 'sweatings' and allowing air to enter the boxes. Therefore, the boxes are always raised above ground level and placed over a drain. A box may be a single unit or a large box with either fixed or movable internal partitions to create a number of compartments. In the latter case, mixing is achieved by simply removing a dividing wall and shoveling the beans into the next compartment. In the former case, the boxes are arranged in cascades making use of a slope (tier design), so that mixing is achieved by simply moving beans from a higher box to a lower one. The objective of these arrangements is to reduce the labor involved in

the turning of the beans. The fermenting boxes are usually housed in a barn to protect them from rain.

Following pod breaking, the beans are placed in the top box or compartment. The wet beans are then covered by banana leaves or jute sacks to retain heat and prevent the surface beans from drying. During the course of fermentation, the beans are moved from one box or compartment to another to ensure uniform conditions. This turning aerates the bean mass and as a result, the temperature falls immediately after mixing and later rises as fermentation proceeds. Typical box fermentation in Indonesia lasts for four to five days, during which time the beans are mixed every 24 h, when the beans are moved to the middle and lowest box, respectively.

Figure 1.6 Box fermentation, capacity 800 − 1000 kg Nusantara Plantation XII (PTPN XII), East Java, Indonesia

1.7   The succession of microorganisms during fermentation

1.7.1   The three-phase cocoa bean fermentation process

Successful cocoa bean fermentation requires a succession of microorganisms, or better, of microbial activities (Schwan and Wheals 2004). The initial microbial population is variable in numbers and type and so are the microbial population dynamics. They vary between countries, regions, and fermentation techniques. Cocoa bean fermentations carried out in Africa (Ghana, Ivory Coast, Madagascar), Latin-America (Brazil, Dominican Republic, Mexico, Trinidad, Ecuador), and South-East Asia (Malaysia, Indonesia) have been studied and different species of yeasts, bacteria, and/or fungi have been isolated (Ardhana and Fleet 2003; Camu et al. 2007; Nielsen et al. 2007; Ostovar and Keeney 1973; Papalexandratou et al. 2011 c; Papalexandratou et al. 2013; Schwan et al. 1995). Although it is believed that most of the microorganisms present are not essential, the key groups of microorganisms active during cocoa bean fermentation are yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB).

Three phases can be considered during the actual cocoa bean fermentation process, reflecting the environmental factors (temperature, pH, and oxygen tension) and the metabolism of substrates available in the cocoa pulp (Fig. 1.7 a): phase 1 or the anaerobic development of yeasts; phase 2 or the development of LAB; and phase 3 or the development of AAB (Schwan et al. 1995; Ardhana and Fleet 2003; Schwan and Wheals 2004; Thompson et al. 2001). In practice, there is considerable overlap between these phases. In general, the total count of microorganisms present in the pulp during fermentation increases during the first 24-36 h, and then stabilizes or gradually reduces. The acidity and high temperatures that develop in the fermenting mass, the diffusion of important components in and out of the bean cotyledons during fermentation, and enzymatic conversions of constituents within the cotyledons during fermentation and drying have been attributed to the metabolism of these microorganisms (Fig. 1.7 b).
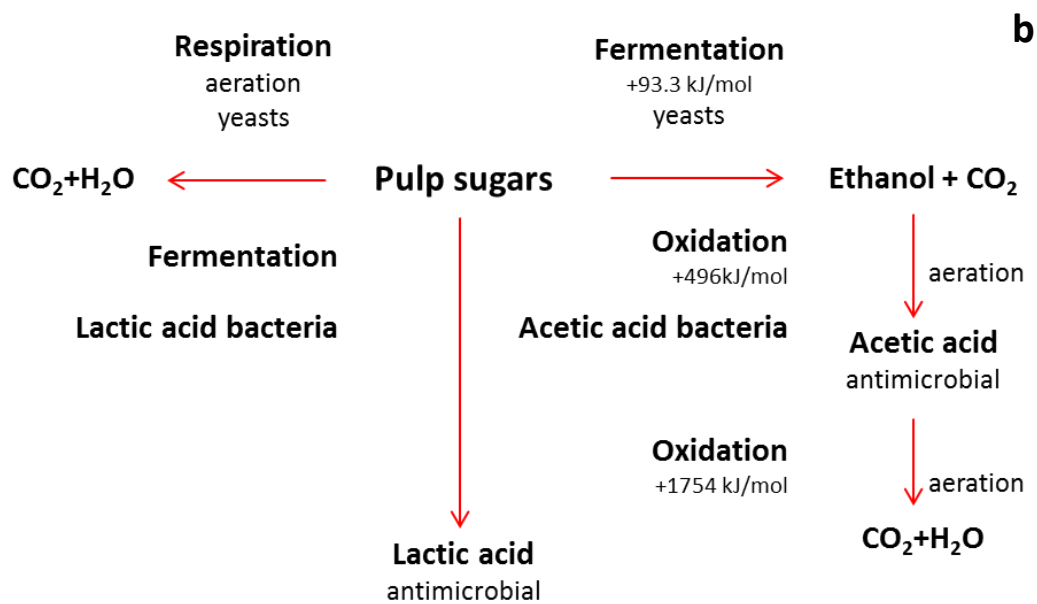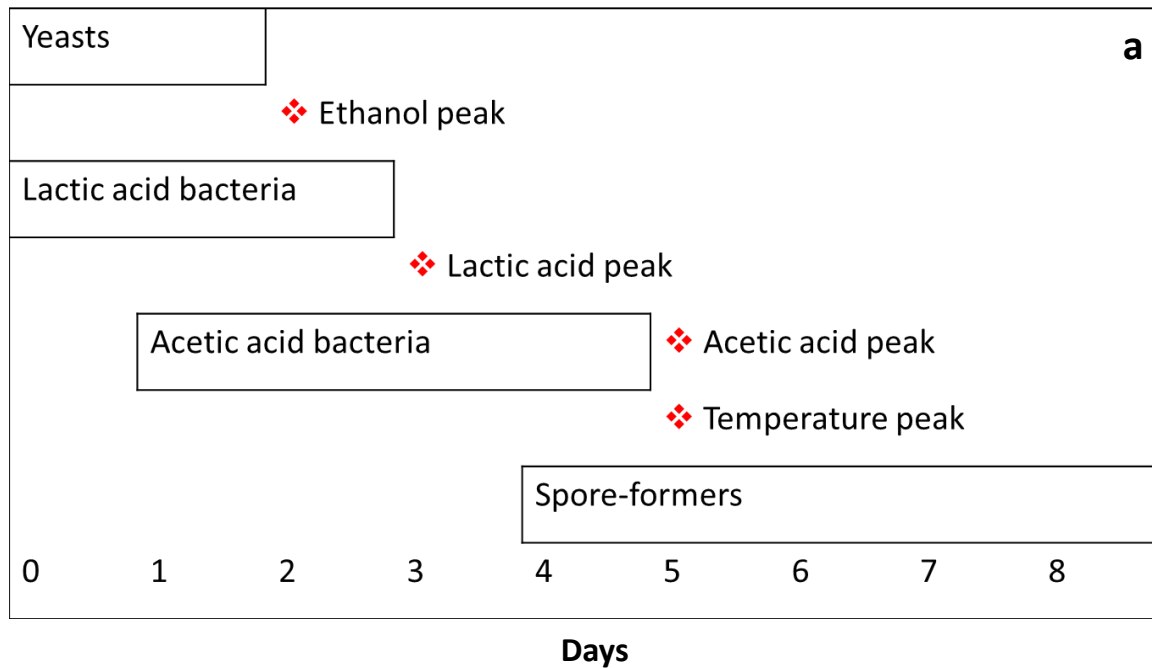
Figure 1.7 a) Schematic diagram of microbial succession during cocoa bean fermentations, along with peak of temperature and metabolite concentrations, b) Microbial activities during cocoa bean fermentation

1.7.2 Yeast fermentation

During phase 1, yeasts convert sugars (sucrose, glucose, and/or fructose) into alcohol (ethanol) under conditions with high carbohydrate concentrations (characteristic for fresh cocoa pulp), limited oxygen availability (due to tight packing of the beans), and a pH of below 4.0 (due to the relatively high content of citric acid in cocoa pulp). Sucrose is the preferred energy source for yeasts, which is converted by yeast invertase into glucose and fructose, releasing 18.8 kJ of heat per mol of sucrose hydrolyzed. However, as mentioned above, sucrose concentrations depend on pod ripeness and hence cotyledon invertase activity. Further, glucose is preferentially converted into ethanol by anaerobic yeast fermentation, leaving fructose to a large extent unfermented. Pulp ethanol concentrations resulting from yeast fermentation may be as high as 6.5% or as low as 1% or less. The production of ethanol is accompanied by a moderate temperature increase, as the conversion of sugars into ethanol (and carbon dioxide) is an exothermic process producing 93.3 kJ per mol of glucose or fructose and thus elevating the temperature of the fermenting mass from an ambient temperature of 25 - 30 °C to 35 – 40 °C within 48 h. The exhaustion of the appropriate energy sources, the production of toxic ethanol and a small amount of heat and further conversion of ethanol into toxic acetic acid by AAB later in the fermentation are responsible for a rapid decline in the dominance of yeasts during fermentation.

The most important roles of the yeasts are the following (Schwan et al. 1995, Ardhana and Fleet 2003): (i) the production of ethanol under low-oxygen and high-sugar conditions. Ethanol disappears upon fermentation through its oxidation to acetic acid by AAB (see below) or alternatively through oxidative consumption by aerobically growing yeasts or through sweating and evaporation; (ii) the breakdown of citric acid in the pulp, which together with losses in the sweatings leads to an increase in the pH of the pulp allowing growth of bacteria (see below); although a few yeasts assimilate citric acid, citric acid-consuming yeasts, such as *Pichia fermentans* and certain isolates of *Candida krusei* have been isolated from the cocoa bean fermenting mass (Roelofsen 1958; Jespersen et al. 2005); (iii) the production of organic acids (acetic, malic, oxalic, phosphoric, succinic, and tartaric acids), which have a buffering capacity and tend to reduce fluctuations in pH; (iv) the production of some volatile organic compounds, principally fusel alcohols, fatty acids, and fatty acid esters, which may contribute either to cocoa flavour or, more likely, to precursors

of cocoa flavour. Yeasts such as *Kloeckera apiculata* and *Saccharomyces cerevisiae* var. *chevalieri* produce large amounts of aroma compounds (Schwan and Wheals 2004); (v) the secretion of pectinases, which reduces the viscosity of the pulp and causes its drainage. Some of the yeasts produce pectinolytic enzymes that break down the walls of cells in the pulp, among which *Kluyveromyces marxianus* and *S. cerevisiae* var. *chevalieri* show substantial activity (Sanchez et al. 1984; Schwan et al. 1995, 1997; Jespersen et al. 2005}. The spaces formed between the beans due to the collapse of parenchyma cells in the pulp between beans allow air to enter; the aeration of the pulp mass is important for the growth of AAB (see below). To speed up the fermentation process and enhance the quality of the final product, pectinases may be added to the pulp or strains over-producing pectinolytic enzymes may be used (Schwan and Wheals 2004).

1.7.3 Lactic acid bacteria (LAB) fermentation

During phase 2, LAB grow rapidly, though large numbers may be present for only a brief period. Although LAB are present at the start of the fermentation, yeasts are dominant at this point. As more pulp drains away, more air penetrates the fermenting mass, ethanol is still produced through yeast activity, and both temperature and pH increase up to 40 °C and pH 4.0, respectively. These conditions are favorable for the growth of lactic acid and acetic acid bacteria. Around 24-72 h, conditions are more favorable for microaerophilic acid tolerant LAB, which then dominate. This coincides with a rapid decline in the dominance of the yeast population due to exhaustion of the appropriate energy sources and inhibition by an increasingly toxic ethanol concentration, heat production, increasing pH, and greater aeration. During the later stages of fermentation, LAB numbers decrease as well.

LAB convert a wide range of sugars (mainly glucose and fructose) and some organic acids (e.g. citric acid and malic acid) into lactic acid and – depending on the strain of LAB – into lactic acid, acetic acid, ethanol, mannitol, and carbon dioxide (Axelsson 2004). Their relative proportions thus change the composition of the pulp and consequently may influence microbial succession. The lactic acid concentration peaks around 0.2%. Most LAB prefer glucose as an energy source. Some LAB species use fructose as an alternative external electron acceptor, which is then converted into mannitol. Another important function of the

LAB during cocoa bean fermentation is the metabolism of citric acid. Citric acid is metabolized via oxaloacetate (and acetate) into either succinic acid (via malic acid and fumaric acid) or pyruvic acid, which in turn is converted into end-products of pyruvate metabolism (lactic acid, acetic acid, ethanol, etc.) (Axelsson 2004). The assimilation of citric acid causes the pH of the pulp to increase from 3.5 to 4.2, allowing for the growth of other bacteria. Aerobic spore-forming bacteria such as *Bacillus* species, may dominate the fermentation by the fourth day. Lactic acid may be consumed by yeasts as well (Camu et al. 2007).

1.7.4 Acetic acid bacteria (AAB) fermentation

During phase 3 from 24 - 112 h of fermentation, aerobic AAB, which grow very early and survive the initial steps of fermentation, persist until the end, when the conditions for AAB growth are optimal. When more of the pulp is metabolized and drained away, aeration increases, and the temperature rises above 37 °C, AAB grow rapidly. Moreover, turning of large heaps and moving of the bean during box fermentation favor AAB growth (Nielsen et al. 2007).

The main activity of AAB is the oxidation of ethanol, initially formed by the yeasts, into acetic acid, which provides them with the necessary energy. This exothermic reaction, producing 496 kJ per mol of ethanol oxidized, is responsible for the rise in temperature of the fermenting mass, which can reach 45 - 50 °C or higher in some fermentations. The decline in ethanol concentration coincides with a decline in lactic acid concentration, indicating its simultaneous oxidation by AAB to acetate and carbon dioxide and water, respectively. The acetic acid concentration peaks around 2%, followed by a decline likely due to evaporation at the high temperature of the fermenting mass and its further metabolism. Indeed, some AAB are responsible for the further oxidation of acetic acid into carbon dioxide and water, in turn liberating 1754 kJ per mol of acetic acid over oxidized. The oxidation of lactic acid and acetic acid may result in a further pH increase.

1.7.5 Bean death

As mentioned above, ethanol and acetic acid diffuse into the bean, which in combination with the heat produced in the fermenting bean mass leads to the death of the bean embryo. Bean death is a prerequisite for the biochemical reactions responsible for the formation of chocolate flavour precursors. Indeed, changes in the internal cellular structure of the bean due to penetrating acetic acid (the lowering of the pH leads to the formation of fat globules surrounded by an aqueous phase of water-soluble components that, after being liberated, can undergo hydrolysis), the mixing of compounds (enzymes, substrates, and inhibitors) in the bean, and hence biochemical changes involving flavour and color development lead to well-fermented cocoa beans. Bean death usually occurs on the second day of fermentation and is mainly caused by the internal acetic acid concentration, the rise in temperature of the bean being relatively unimportant. However, high temperatures in combination with ethanol and acetic acid limit microbial growth in the cocoa pulp and can even kill a microbial cell, which halts the cocoa bean fermentation process. The production of ethanol during the anaerobic growth of yeasts correlates very closely with the death of the seed embryo as well, with seeds being unable to germinate 24 h after maximum concentrations of ethanol are attained within the bean cotyledons (Thompson et al. 2001). Thanks to all of these processes, events associated with germination and certain quality defects, such as the utilization of valuable bean components (for instance cocoa butter) and the opening of the testa by hypocotyl extensions do not occur, leading to a more stable, desirable end-product. Alternatively, lactic acid remains in the bean indefinitely due to its limited volatility, and hence compromises the final quality of the bean (Camu et al. 2007).

1.7.6 Optimal fermentation course and end of fermentation

An optimal course of fermentation requires appropriate microbial population dynamics such as a correct succession of microorganisms and concomitant activities as the basis for the development of flavour precursors within the bean and the characteristic coloration of the bean.

As cocoa beans vary in their degree of fermentation, because of the unpredictable fermentation step, a fermentation index has been introduced as a quantitative measure for

the degree of cocoa bean fermentation. Cocoa bean can be under-fermented (fermentation too short), over-fermented (fermentation too long), or optimally fermented (exact fermentation time), with corresponding sensory attributes. The fermentation index is determined colorimetrically as the ratio of the absorbance of oxidation products of cocoa bean polyphenols at 460 nm to that at 530 nm (Gourieva and Tserevitinov 1979). A fermentation index value of > 1 would indicate that the cocoa bean mass is sufficiently fermented (Shamsuddin and Dimick 1986).

A key decision involved in obtaining fermented cocoa beans of high quality is when to remove the beans from their fermentation environment to start the drying process. As mentioned above, when all of the ethanol is oxidized into acetic acid and then further into carbon dioxide and water, the fermentation stops, as the energy sources for microbial growth are no longer available, and the temperature of the bean mass starts to decrease. Extending the fermentation can result in undesirable microbial activity, leading to putrefaction and the production of off-flavours. The following factors collectively indicate optimal fermentation: (i) decrease in temperature of the fermenting mass; (ii) smell of the fermenting mass (distinct smell of alcohol and acetic acid during the early and late stages of cocoa bean fermentation, respectively); (iii) visual appearance of molds at the surface of the fermenting mass; (iv) plumping or swelling of the bean (the water content of fermenting bean increases from about 35% at harvest time to about 40% after bean death); (v) external colour of the bean; (vi) internal colour of the bean making use of the bean cut test; and (vii) internal pH of the bean. However, other factors may influence the decision to end fermentation, in particular when certain components of the cocoa bean have to be valorized (see Section 13.3).

1.8 Drying of fermented cocoa beans

1.8.1 Drying process

After fermentation, the beans are removed from the heaps or boxes and dried in the sun on the ground (Fig. 1.8) until fully dried, usually occurring within 5 – 10 sunny days. The fermentation must be stopped completely, the moisture content of the beans must be

brought from an initial 40 - 60% down to 6 - 7% to avoid the growth of molds within the beans during storage, and the major part of acetic acid formed during fermentation must be eliminated (Thompson et al. 2001). The drying process relies on air movement to remove water. This results in a pronounced reduction in the number of viable microorganisms (Schwan and Wheals 2004). Furthermore, biochemical processes important for the flavour and color development of the cocoa beans, initiated during fermentation, continue during drying. A slow migration of moisture throughout the beans will transport flavour precursors formed during fermentation. Hence, some oxidation will occur and some excess acids, in particular acetic acid, may volatilize through the shell, both of which are beneficial. Non-volatile lactic acid is partly transported by the water from the bean to the shell (Nganhou et al. 2003; Thompson et al. 2001; Wood and Lass 2008).

In the case of artificial drying (Fig. 1.9a, b), the rate of drying has an important bearing on the flavour and quality of the dried beans. If drying is too slow, there is a danger that molds will develop and penetrate the testa and that off-flavours may arise. Rapid drying may cause case hardening (fast drying on the bean surface with moisture retention inside the bean), which may prevent oxidative changes, resulting in excessive acidity. It has been established that artificial drying should take at least 48 h to allow for proper flavour development.

Figure 1.8 Sun drying in Nusantara Plantation XII (PTPN XII), Jember, East Java, Indonesia

1.8.2   Drying apparatus

Plastic covers, raised bamboo mats, wooden platforms, or concrete terraces are generally used for sun-drying. It is important to ensure uniform drying by mixing the beans regularly (for instance four times per day), thereby breaking up clumps of beans that may form and discouraging mold growth. Sun-drying can take from 5 to 10 days, but in the case of bad weather conditions can last up to 4 weeks, increasing the risk of mold development and spoilage. In general, sun-drying is employed on small farms, whereas large estates may resort to both natural and artificial drying.

Several methods of mechanical drying exist which can save time and man-power but necessitate adapted equipment and energy supply. Generally, hot air dryers fueled by wood or oil are employed. Numerous designs have been developed, including direct contact with the flow gasses, but normally indirect heating using heat exchangers is preferred. In the latter case, the disadvantageous case hardening by hot air conduction is reduced, as heat convection also reaches the inside of the beans. A temperature gradient between beans and the surrounding air as well as the use of a closed and controlled atmosphere encourages the

evaporation of water and acetate (Afoakwa 2010). In the case of artificial drying, the temperature should be kept at a maximum of 60 °C for at least 48 h and the beans should be mixed regularly. The beans are considered dry when they are crunchy. Elevated temperatures tend to produce cocoa with brittle shells and cotyledons that crumble during handling, which is not desirable. Smoke contamination due to badly constructed, poorly maintained or improperly used equipment may give smoky or hammy off-flavours, which is characteristic for bean from some countries (Wood and Lass 1985; Thompson et al. 2001).

Figure 1.9 Artificial drying facility with a capacity of 4500 kg, a) view from outside, b) view from inside in Nusantara Plantation XII (PTPN XII), Jember, East Java, Indonesia

1.9 Biochemical changes in the cocoa beans during fermentation and drying

1.9.1 Creating an environment for flavour precursor development during fermentation

As described above, the fermentation of cocoa pulp provides the conditions necessary for the development of cocoa flavour and colour precursors within the cotyledons of the bean, namely (Fig. 1.10): (i) the initiation of bean germination, which leads to water uptake by the protein vacuoles within the cells; (ii) the death of the bean embryo (germ), mainly caused by acetic acid and ethanol penetrating the testa and entering the bean (accumulating to levels of about 3.5% and 0.4%, respectively), whereby the biological barriers (membranes) between the cells (storage and pigment cells) within the bean cotyledons break down. This in turn allows various enzymes and substrates to mix freely, because ethanol, acids and water diffusing into the cotyledons act as solvents so that components are transported to sites of enzyme activity and vice versa. The subsequent biochemical reactions produce the flavour and colour precursors; (iii) the conditions of pH and temperature. The pH, determined mainly by the diffusion of acetic acid into the bean, is important, and the reaction rates are also increased by the warm temperatures during fermentation and drying. The penetrating acetic acid causes the bean pH to drop from an initial value of 6.3 - 6.8 to 4.0 - 4.8. Microbial activity contributes to the increasing temperature of the fermenting bean mass; (iv) the penetration of oxygen. When oxygen begins to enter the bean around 96 h of fermentation, a series of enzymatic, oxidative reactions are initiated. These result in a browning of the cotyledon.
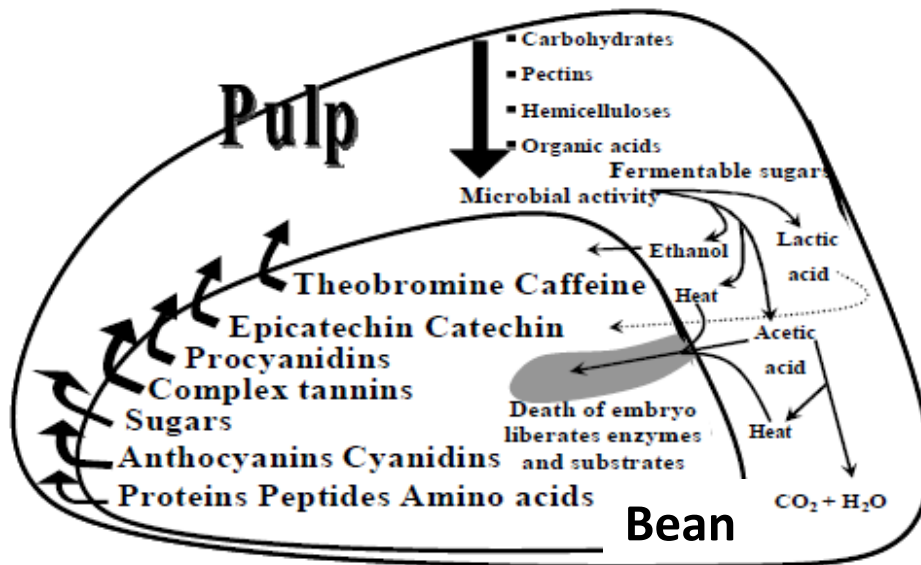
Figure 1.10 Biochemical changes in pulp and beans during cocoa bean fermentation (Lopes, 1986)

1.9.2 Enzymatic and diffusion processes in the beans cotyledons during fermentation and drying

Biochemical processes within the beans include enzymatic and diffusion processes influenced by several environmental factors, such as pH, temperature, and moisture (Thompson et al. 2001). Enzymatic reactions within the bean cotyledons encompass the breakdown of proteins to peptides and amino acids and of sucrose to fructose and glucose (Hansen et al. 1998). The (non)-enzymatic reactions involving polyphenols are considered to be pivotal in the development of chocolate flavour and colour. These enzymatic reactions are of short duration, as optimal conditions of pH (decreasing in the bean) and temperature (increasing in the bean) are transient upon fermentation. In addition, a certain amount of moisture (increasing during fermentation and decreasing during drying) is necessary to allow enzymes and their substrates to react to form the desirable products. Significant changes in pH, temperature, and moisture occuring during cocoa bean fermentation and drying influence the type and quantity of flavour precursor compounds produced by endogenous enzymes.

Moisture content within the cotyledon during fermentation is usually greater than 35% and allows for the adequate migration of enzymes and substrates for enzymatic activities. However, once the drying process begins, moisture content gradually decreases, making it increasingly difficult for enzymes and substrates to react. When a moisture content of 6 - 8% is achieved upon drying, virtually all enzyme activities cease. An increase in temperature of more than 20 °C during fermentation can also have a profound impact on enzyme activity. If the temperature fails to change significantly, enzymatic activity is reduced, resulting in fewer flavour precursors and subsequently poor cocoa flavour. Likewise, if inadequate amounts of organic acids are produced during fermentation, the pH of the cotyledons will not be suitable for optimal enzyme activities and the flavour of the resulting cocoa will be affected. On the other hand, too much acid produces excessive sourness that can mask the chocolate flavour. Consequently, there is a need to achieve balance between the length of fermentation, environmental factors, and microbial activities that influence enzymatic activities within the bean cotyledons.

Biochemical and physical changes within the bean cotyledons can be divided into two general phases, namely the anaerobic-hydrolytic phase and the aerobic-oxidative phase. In practice, there is a considerable overlap of both phases, as even though oxygen penetrates the surface of the bean cotyledons, parts of the interior remain anoxic. The cotyledons are initially anaerobic and hydrolytic reactions involving proteins, sugars, and polyphenols take place during the anaerobic-hydrolytic phase. This occurs through the absorption of water and diffusion of substrates in the tissue during fermentation after seed death occurs. The hydrolysis of bean cotyledon substrates contributes to the formation of aroma precursor molecules necessary for cocoa flavour development. During the aerobic-oxidative phase, oxygen begins to penetrate the testa and causes oxidative changes to polyphenols and protein material on the surface of the cotyledon from the fourth day onwards. These reactions (browning of the surface of the cotyledon), which occur towards the end of the fermentation and are to some extent responsible for inhibiting enzyme activities, contribute to the removal of astringency and tanning of proteins. The oxidative phase also contributes to the formation of ancillary (e.g. fruity) flavours. Oxygen continues to penetrate the bean during the drying process, allowing some enzymatic activities to continue until rising temperatures and insufficient moisture become inhibiting factors.

Cocoa bean hydrolytic enzymes, such as invertase, glycosidases, and proteases, display highest activities during the anaerobic phase of cocoa bean fermentation. After seed death, the proteolysis of vicilin-like globular storage proteins in the bean cotyledons is catalyzed by aspartic endoprotease, carboxypeptidase, and aminopeptidase enzymes, which are particularly active at pH 3.0 - 3.5, 5.8 - 6.0, and 6.8 - 7.0, respectively. This in turn, indicates full activity, partial activity during 1 – 2 days of fermentation, and partial activity during 3 – 4 days of fermentation, respectively (Biehl et al. 1982; Hansen et al. 1998; Hashim et al. 1998a; Hashim et al. 1998b; Voigt et al. 1994).

The cocoa bean aspartic endoprotease cleaves the protein substrate preferentially at hydrophobic amino acid residues to produce oligopeptides displaying hydrophobic amino acid residues at their carboxy terminal ends. The endogenous carboxypeptidase plays an important role in converting hydrophobic oligopeptides to hydrophilic oligopeptides and hydrophobic free amino acids, which are aroma precursors required for the formation of typical cocoa aroma components in the presence of reducing sugars upon roasting (Amin et al. 2002; Biehl et al. 1982; Voigt et al. 1994).

Sucrose is the major sugar in unfermented cocoa beans. It is not a reducing sugar and, therefore, does not participate in non-enzymatic browing reactions occuring during roasting. However, sucrose is converted to glucose and fructose by cocoa bean invertase during fermentation. Hansen et al. (1998) reported the existence of both a cotyledon and pulp invertase. Total sugar content decreases throughout fermentation. About 70% of the total sugars remain after fermentation and are of value for flavour development during roasting (Lehrian and Patterson 1983).

The polyphenols and alkaloid contents of the beans decrease slightly during fermentation due to the diffusion of these molecules out of the beans. The polyphenols (including the anthocyanidins) are oxidized and polymerize to insoluble, characteristically coloured, high molecular-mass compounds (tannins) during fermentation and drying. These reactions are non-enzymatic or are catalyzed by the cotyledon enzyme polyphenol oxidase. This enzyme possesses optimal activity at pH 4.5 - 7.0 and hence is strongly inactivated during the first days of fermentation, retaining only 50 and 6% of its enzymatic activity after 1 and 2 days, respectively (Hansen et al. 1998). Polyphenol oxidase converts the polyphenols, mainly

epicatechin (the main cocoa bean polyphenol) and free anthocyanidins, to quinones. Polyphenols and quinones complex with other polyphenols, proteins, peptides, and amino acids to form tannins, which on the one hand decreases their solubility, astringency, and bitterness, and gives rise to the brown colouration of the bean that is typical of well-fermented cocoa beans. A dramatic drop (70%) in the content of soluble polyphenols occurs between the first and fourth day of fermentation (Wollgast and Anklam 2000). The occurrence of condensation reactions is confirmed by the sharp decrease in epicatechin content between the second and third day of fermentation. The epicatechin and soluble polyphenols content is reduced to approximately 10 - 20% during fermentation and drying. Polyphenol oxidase is also sensitive to drying, so that the remaining enzyme activity after fermentation and drying of the bean is only about 2%. In addition to the oxidation process and diffusion of polyphenols into fermentation sweatings, it is believed that the non enzymatic oxidation of polyphenols could also be important during the drying process (Hansen et al. 1998; Jinap et al. 2002).

Apparently, no change in starch and fat content occurs during fermentation and drying (Roelofsen 1958; Lehrian and Patterson 1983). However, as the non-fat portion of the cocoa bean is the source of polyphenols, the polyphenol and anti-oxidant contents of chocolate products are determined by their levels in the cocoa solid contents of these products. Therefore, the flavour-enhancing properties of cocoa bean fermentation must be balanced against the polyphenol health effects of fermentation, when polyphenol-enriched chocolate products are to be produced. The availability of cocoa bean with various degrees of fermentation provides the chocolate manufacturer with the opportunity to produce chocolate products that meet the diverse needs of the modern consumer (Thompson et al. 2001).

1.10 Use of starter cultures for the fermentation of cocoa beans

As mentioned above, cocoa flavour is determined by several factors, particularly the cocoa beans variety, the fermentation and drying process, and the subsequent processing of well fermented cocoa beans. Therefore, technological challenges at the level of the natural cocoa bean fermentation have an impact on the quality of the cocoa beans and hence on the

quality of the cocoa and chocolate made thereof. Such influences include a possible impact on colour and flavour development during fermentation, on sugar and fat contents, on polyphenol and alkaloid contents, etc. As cocoa bean fermentations may be performed in an artisan manner on small scale or under non-optimal large scale conditions, the results are variable in quality. In addition, there might be problems of acidity, lack of cocoa flavour (due to incomplete fermentation) and presence of off-flavours (due to over-fermented beans and spoilage), all of which lead to low crop value for the farmer. Therefore, it would be desirable to change the fermentation from a wholly natural and unpredictable process to a controlled process, initiated with an appropriate starter culture, in which fermentation occurs more quickly. However, one of the reasons why chocolate quality has not been a priority for farmers is that there is no financial incentive to produce high-quality fermented cocoa beans. In the last 25 years, multinational companies have put most of their efforts into two areas: encouraging farmers to maximize production and to improve processing of the fermented cocoa beans. The fermentation process itself has been largely neglected.

The dairy, meat, and alcoholic beverage industries have largely replaced traditional natural fermentations with defined inocula of high-quality raw materials, the strict control of the fermentation processes, the better treatment of the final products, and the diversification of the market (Leroy and De Vuyst 2004). Cocoa bean fermentations have a long way to go before they reach that stage of development, but preliminary experiments using a defined starter culture in such a complex fermentation show promising results. For instance, Schwan (1998) used a defined microbial cocktail inoculum consisting of a yeast, *Saccharomyces cerevisiae* (var. *chevalieri*), two LAB species, *Lactobacillus delbrueckii* ssp. lactis (formerly named *Lactobacillus lactis*) and *Lactobacillus plantarum*, and two AAB species, *Acetobacter aceti* and *Gluconobacter oxydans* (ssp. *suboxydans*) to perform fermentations in 200 kg wooden boxes with aseptically prepared cocoa beans, inoculated at different points of time. The fermentation process exactly mimicked the conditions in 800 kg boxes on Brazilian farms. Using the zero-time inoculum, the fermentation proceeded almost identically to the natural one. The fermentation with a phased-addition inoculum was similar, but slower and less pronounced, which led to a slightly poorer end-product. Although preliminary, these data show that the many common species of microorganisms found in natural cocoa bean fermentations can be replaced through judicious selection and concentration of members of

each physiological group important to the process. There is of course need for improvement in the choice of species and the way of inoculation and fermentation when this technology is to be used commercially. For instance, independent studies on the pectinolytic enzyme endopolygalacturonase (Schwan et al. 1997), which is produced by the yeast *Kluyveromyces marxianus* isolated from spontaneous cocoa bean fermentations in Bahia (Schwan et al. 1995), suggest that this yeast species would be a better source of pectinase. Therefore, Schwan (1998) proposed that a combination of *K. marxianus* as pectinase producer and the naturally vigorous yeast *S. cerevisiae* as an ethanol producer might be a better choice of yeasts for future defined inocula.

One of the ultimate purposes of controlled cocoa bean fermentations will be to speed up the fermentation process and to influence the flavour of chocolate products through fermentation. Faster fermentations will be necessary to allow controlled, large-scale fermentation and to increase production capacity in order to respond to the growing demand for cocoa. 'Flavour'-controlled fermentations will be necessary to produce marketable, tailor-made cocoa. Demonstrating a reduction in fermentation time and obtaining well-fermented cocoa bean with desirable sensory attributes would represent a scientific step forward. However, to scale up the initiation of cocoa bean fermentation with a defined inoculum, whether applied to small farm holdings or large estates, would be a great challenge, especially considering costs, culture production and maintenance, and inoculation method (Thompson et al. 2001).

1.11 Chocolate flavour development and chocolate production

1.11.1 Chocolate flavour

The most notable attribute of chocolate is its unique flavour, being an extremely complex mixture of more than 550 compounds (Nijssen et al. 1996). The mix and balance of these numerous compounds contributing to the final cocoa flavour depends on plant genetics (cultivar), environmental conditions of crop cultivation (climate, soil quality and richness, water management, shade canopy management, pollination, etc.) and fermentation (country and region, method, weather conditions), pod harvesting and storage (fruit

ripeness), primary processing (fermentation, drying, and roasting), and final storage. With chocolate, the chemical complexity of flavour development is evident when one realizes these numerous parameters that may influence its production. Its complexity is equally obvious when considering that, even today, this desirable flavour has yet to be duplicated by the flavour chemist (Dimick and Hoskin 1999).

1.11.2 Chocolate production

Chocolates are semisolid suspensions of fine solid particles of sugar and cocoa (and milk, depending on type), resulting in about 70% in a continuous fat phase. Primary chocolate categories are dark, milk and white, which differ in their contents of cocoa solid, milk fat and cocoa butter. The outcomes include varying proportions of carbohydrates, fats and proteins (Tab. 1.3).

Table 1.3 Dark, milk and white chocolate: major constituents

Product	Carbohydrate (%)	Fat (%)	Protein (%)
Dark chocolate	63.5	28.0	5.0
Milk chocolate	56.9	30.7	7.7
White chocolate	58.3	30.9	8.0

Source: (Afoakwa et al. 2007)

Chocolate manufacturing processes (Afoakwa 2010; Beckett 2009) differ due to variation in national consumer preferences and company practices. The various steps in these processes are presented schematically in Fig. 1.11 (Wood and Lass 1985).
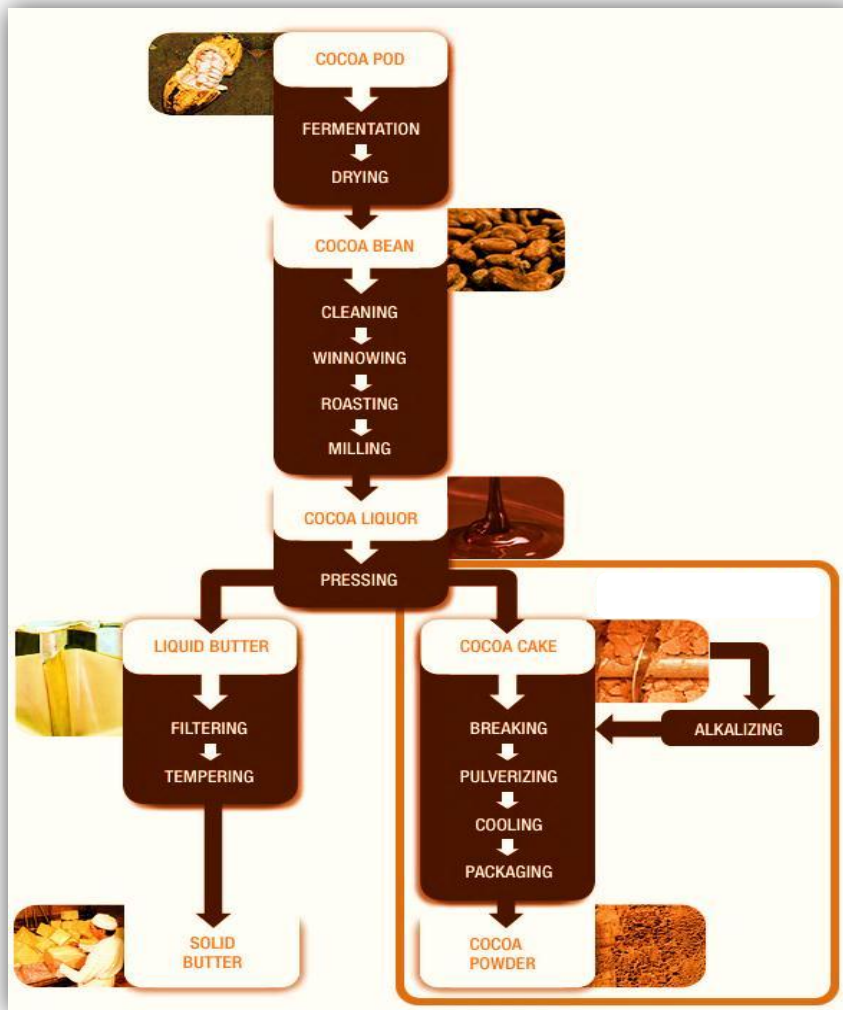
Figure 1.11 Schematic diagram of cocoa bean processing

In a first step before processing, the quality of the bean is evaluated using the following indicators: degree of fermentation, moisture content, bean count, colour, uniformity of bean size, etc. Before processing, cocoa beans are next cleaned, broken and winnowed to obtain nibs of consistent quality. These processes also ensure that the nibs are cleaned (free from dirt and infestation), well broken and properly deshelled. The kernels (nibs) obtained after the process must be of uniform size to achieve constant quality. First, the beans are sieved to remove all extraneous materials such as stones, strings, coins, wood pieces, soil particles and nails. The cleaned beans are then broken to loosen the shells from the nibs using multiple steps to avoid an excess of fine particles. The products obtained are then sieved

into smaller numbers of fractions to obtain optimal separation during subsequent winnowing. The fractions are then transported to the winnowing cabinet, where the lighter broken shells are removed by a stream of air (Afoakwa 2010).

After cleaning and breaking, the next step is roasting. Cocoa beans are roasted to further develop the original cocoa flavour that exists in the form of precursors generated during the fermentation and drying of the beans. During the roasting of the dried fermented beans, several physical and chemical changes take place, including the loosening of the shells and moisture, the changing of nib colour, the reduction of the number of microorganisms, the degradation of protein and amino acids, and the losses of volatile acids and other substances that contribute to acidity and bitterness. The roasting temperature varies between 90 and 170 °C. Three main roasting methods are employed in the cocoa processing industry: 1) whole bean roasting, in which the bean are roasted before winnowing, 2) nib roasting, in which the shells are removed before roasting and 3) liquor roasting, in which thermal pre-treatment is often used before winnowing (Afoakwa 2010).

After roasting, cocoa nibs need to be milled to form cocoa liquor. The purpose of this step is to produce as low a viscosity as possible in order to obtain smooth cocoa powder and chocolate taste during the subsequent use of the liquor. The nib has a cellular structure containing about 55% cocoa butter in solid form locked within the cells. Grinding the nib cells releases the cocoa butter with a particle size up to 30 μm, into the liquor, with fine grinding being particularly important for the production of cocoa powder. The viscosity of the liquor is related to the degree of roasting preceding the grinding and to moisture content of the nib. Many machines are used for reducing the nibs into liquor, including stone mills, disc mills, pin or hammer mills and bead or ball mills. The grinding is performed in a multistage process, and the heat treatment generated during the grinding process causes the cocoa butter in the nib to melt, forming the cocoa liquor. The refined cocoa liquor is then heated in storage tanks at a temperature of about 90 – 100 °C for aging and microbial destruction, after which the liquor is packaged for sale (Afoakwa 2010; Beckett 2009).

In the next step, the liquor is pressed to produce cocoa butter and cocoa cake. Cocoa butter constitutes about half the weight of the cocoa nib. This fat is removed from the cocoa liquor by means of hydraulic presses applying pressures as great as 520 kg/cm^2, with the larger

presses processing to 113.4 kg per pressing cycle. Depending on the pressing time and the settings of the press, the resulting cake has a fat content of between 10 and 24%. Two kinds of cocoa cakes can be obtained by the process: 1) high-fat cakes containing between 22 - 24% residual fat and 2) low-fat cakes containing between 10 - 12% residual fat (Afoakwa 2010; Beckett 2009).

After liquor pressing, two types of cocoa form, namely cocoa butter and the cocoa cake which will later become cocoa powder. Prior to this, the cocoa cake needs to be alkalized and ground. Alkalisation consists of treating the cocoa nibs with an alkali solution such as potassium or sodium carbonate. The alkali is used to raise the pH of the bean or nibs from 5.2 - 5.6 to near neutrality at 6.8 – 7.5 depending on the alkali used. The purpose of this step is primarily to modify the colour and flavour of the cocoa powder or cocoa liquor, and also to improve the dispersibility or suspension of the cocoa solids in water. The cakes released after grinding are quite big and are therefore passed through kibbling machines to be broken down into smaller pieces, known as kibbled cake. The kibbled cake obtained is stored according to fat content and degree of alkalisation, and may be blended before pulverisation to obtain the desired type of cocoa powder (Afoakwa 2010).

The final step is the production of cocoa powder. The powder grinding lines usually employ hammer-and-disc or pin mills, which pulverize cocoa cake particles into the defined level of fineness for cocoa powder. The powder is then cooled so that the fat in the cocoa powder crystallises into its stable form (Afoakwa 2010).

1.12 Seed coat (testa) of *Theobroma cacao* L., and plant antifungal protein

1.12.1 Seed coat (testa) of *Theobroma cacao* L.

The testa is the outer shell surrounding the bean. The testa of the cocoa seed is impermeable to larger molecules, whereas smaller volatile molecules such as ethanol and acetic acid can easily penetrate the testa. The testa thus provides a natural barrier against organic acid (citric acid) penetration from the pulp into the bean and the outward diffusion of theobromine, caffeine and polyphenols; hence controlling the kinetics of fermentation and the concomitant diffusion processes (Camu 2007).
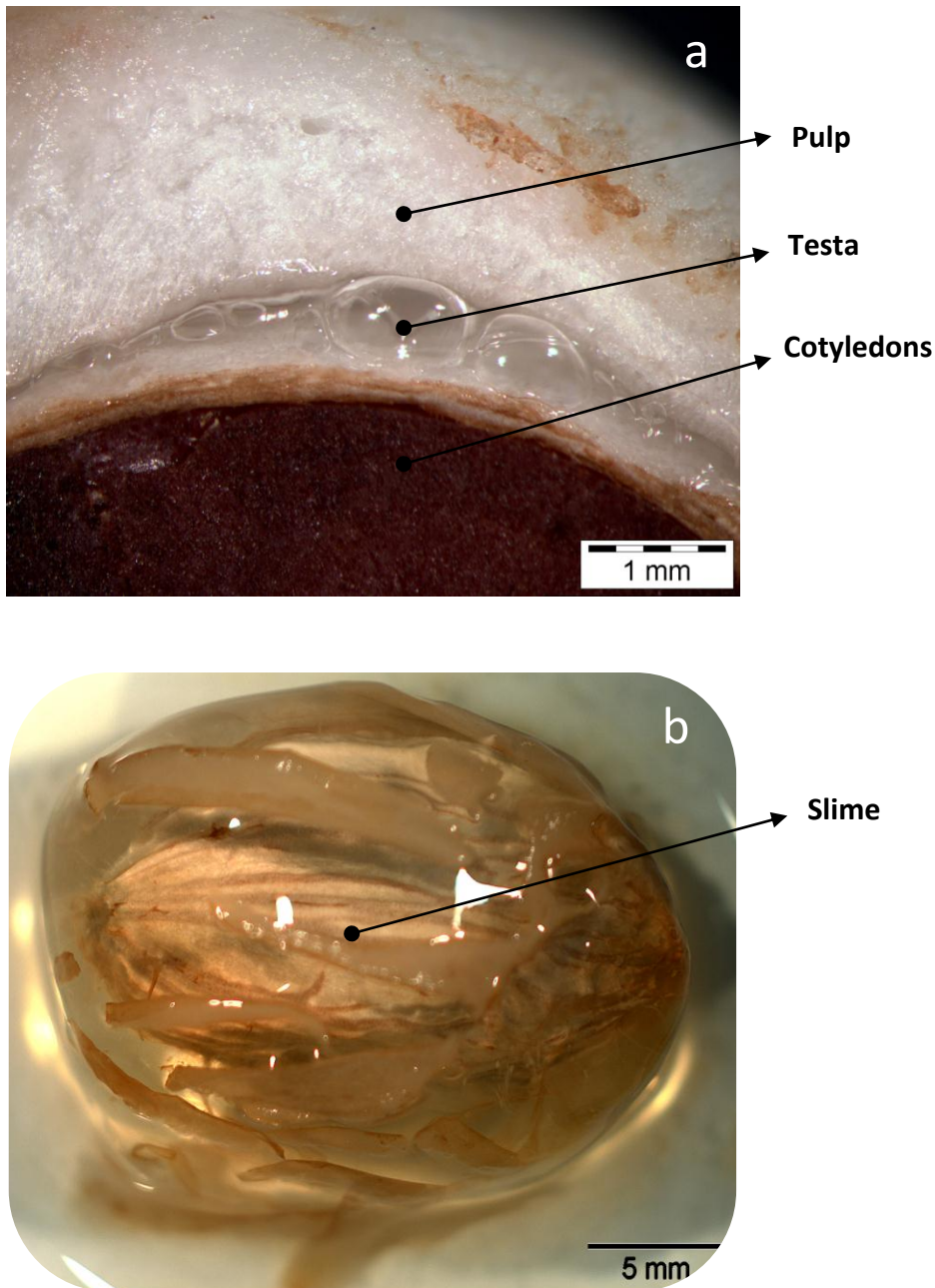
Figure 1.12 a) Appearance of testa under microscope analysis, b) Testa after removal of pulp and soaked 24 h in water (Bahmann, 2014)

In the testa of *Theobroma cacao* L., a layer of slime is located directly below the outer epidermis. This slime has yet to be described in detail in the literature and its chemical composition was previously unknown. Bahmann (2014) reported that cocoa seeds produce this slime for three days after removal of the pulp. This slime is composed out of sugars and different kinds of proteins including chitinase, glucanase, and osmotin (Bahmann 2014).

The seed coat is an important tissue for the regulation of imbibition and the maintenance of seed integrity (Bewley and Black 1994). It is also the first seed barrier encountered by pests and pathogens (Moise et al. 2005). However, this tissue has previously been considered to be an exclusively physical barrier and the involvement of seed coat molecules in this defensive role has not been considered. Seed cotyledons contain an array of proteins that may be involved in the protection of quiescent seeds against fungi (Santos et al. 2008).

1.12.2 Plant antifungal proteins

Plants are exposed to a large number of pathogenic fungi; although they do not have an immune system, plants have evolved a variety of potent defense mechanisms including the synthesis of low-molecular-weight compounds, proteins, and peptides with antifungal activity (Caruso et al. 1996; Grenier et al. 1993; Kitajima and Sato 1999; Linthorst and Van Loon 1991; Selitrennikoff 2001).

Several classes of antifungal proteins are involved in the inhibition of the synthesis of the fungal cell wall or disrupt cell wall structure and/or function; others perturb fungal membrane structure, resulting in fungal cell lysis. Plant antifungal proteins have been classically divided into five groups, PR-1, -2, -3, -4, and -5, based on serological and amino acid sequence analyses (Van Loon 1985). The mechanisms of antifungal action have only been clearly identified for the PR-2 and PR-3 groups of proteins.

PR-1 proteins. PR-1 proteins have been found in rice, wheat, maize, tobacco, *Arabidopsis thaliana,* barley, and many other plants (Agrawal et al. 2000; Bryngelsson et al. 1993). PR-1 proteins have antifungal activity at the micromolar level directed against a number of plant pathogenic fungi, including *Uromyces fabae, Phytophthora infestans*, and *Erysiphe graminis*. PR-1 proteins have molecular masses of 15 - 17 kDa and show homology with the superfamily of cysteine-rich proteins. Although the precise mechanism of antifungal activity is not understood, the PR-1-like protein helothermine from the Mexican banded lizard interacted with membrane-channel proteins of target cells, thus inhibiting the release of Ca^{2+}. Whether antifungal plant PR-1 proteins act by this mechanism is not known yet but is suspected (Selitrennikoff 2001).

PR-2 proteins (β-glucanases). PR-2 proteins have (1,3) β-endoglucanase activity in vitro and have been grouped into three classes on the basis of amino acid sequence analysis. Class I glucanases are basic proteins of 33 kDa and are found in the plant vacuole. Classes II and III include acidic extracellular proteins of about 36 kDa. The major structural difference between class I proteins and the other two classes is that class I proteins are synthesized as preproteins that are processed prior to being enzymatically active. PR-2 proteins have been found in a wide variety of plants, including tobacco, *A. thaliana*, peas, grains, and fruits (Cote et al. 1991; Kim and Hwang 1997). The proteins are active in vitro at micromolar levels (~50 μg mL^{-1}) against a wide number of fungi, including human and plant pathogens (e.g. *Rhizoctonia solani, Candida albicans* and *Aspergillus fumigatus*). The antifungal activity of plant (1,3) β-glucanases is thought to occur by PR-2 proteins hydrolyzing the structural (1,3) β-glucan present in the fungal cell wall, particularly at the hyphal apex of filamentous molds where glucan is most exposed, resulting in a weakened cell wall. This weakened cell wall results in cell lysis and cell death (Selitrennikoff 2001).

PR-3 proteins (chitinases). A number of enzymatic assays have shown PR-3 proteins to have in vitro chitinase activity. Most PR-3 proteins have molecular masses of between 26 and 43 kDa (Nielsen et al. 1997; Watanabe et al. 1999). Chitinases (both plant PR-3 chitinases and chitinases from other sources) have been divided into five groups. Class I chitinases contain an N-terminal cysteine-rich domain of ~40 amino acids (also known as the wheat germ agglutinin domain), a chitin-binding hevein-like domain, a highly conserved central portion, and a hinge region; most class I proteins have molecular masses of ~32 kDa. Class II proteins are similar in amino acid sequence to class I proteins, but they lack the N-terminal cysteine-rich domain and have molecular masses of 27 to 28 kDa. Class IV proteins resemble class I chitinases but are significantly smaller due to four major deletions. Class III proteins do not share amino acid sequence homology to any other class and have molecular masses of ~28 to 30 kDa. Class V chitinases show sequence similarities to bacterial exochitinases and have molecular masses of 41 to 43 kDa. In addition to chitinases, a chitosanase (chitosan is deacetylated chitin) from *Streptomyces* sp. strain N174 with antifungal activity has been isolated (Money and Harold 1992), and its X-ray structure has been determined.

Chitinases have been isolated from fungi, tobacco, cucumber, bean, peas, grains, among others, and exhibit potent antifungal activity against a wide variety of human and plant

pathogens including *Trichoderma reesei, Alternaria solani, Alternaria radicina, Fusarium oxysporum, Rhizoctonia solani, Guignardia bidwellii, Botrytis cinerea*, and *Coprinus comatus*. Using analogy of β-glucanases, the mode of action of PR-3 proteins is relatively straightforward: PR-3 proteins are endochitinases that cleave cell wall chitin polymers in situ, resulting in a weakened cell wall and rendering fungal cells osmotically sensitive. Not surprisingly, PR-2 (β-glucanases) and PR-3 (chitinases) proteins act synergistically in inhibiting fungal growth, both in vitro and in planta (Jach et al. 1995; Selitrennikoff 2001).

PR-4 (chitin-binding) proteins. PR-4 proteins are chitin-binding proteins, have molecular masses of 13-14.5 kDa, and have been classified into two groups. Class I proteins exibit amino acid sequence similarities to hevein (a chitin-binding polypeptide) and belong to the superfamily of chitin-binding lectins. Class II proteins lack the chitin-binding domain. PR-4 proteins have been isolated from potato, tobacco, barley, tomato, and many other plants. Both classes of proteins have potent antifungal activity against a wide variety of human and plant pathogens (e.g. *Trichoderma harzianum, Fusarium culmorum, Fusarium graminearum*, and *Botrytis cinerea*). The antifungal activity of class I proteins is likely the result of protein binding to nascent fungal cell wall β-chitin in the nascent fungal cell wall. By mechanisms not yet understood these results in disrupted cell polarity and subsequent growth inhibition. The mechanism of action of class II proteins (which lack the chitin-binding hevein domain but are antifungal nonetheless) is not understood (Selitrennikoff 2001).

PR-5 (TL) proteins. PR-5 proteins share significant amino acid homology with thaumatin (a sweet-tasting protein [to humans] from the South African ketemfe berry bush) and are known as TL proteins. TL proteins have been isolated from *Arabidopsis thaliana*, corn, soybean, rice, wheat, tobacco, tomato, pumpkin, bean, barley, flax, and many other plants. The majority of PR-5 proteins haves molecular masses of ~22 kDa and are stabilized by eight disulfide bonds. This highly stabilized structure allows PR-5 proteins to be very resistant to protease degradation. The X-ray structures for two PR-5 proteins and thaumatin have been determined (Kolbe et al. 1998; Ogata et al. 1992).

Although the precise mechanism of action of PR-5 proteins is not completely understood, a number of interesting observations have been made that may eventually lead to a unified hypothesis for how these proteins function in killing fungi. First, several TL proteins cause

cell permeability changes in fungal cells with a cell wall but have little or no effect on protoplasts (Roberts and Selitrennikoff 1990). For example, zeamatin (a TL protein from corn) caused very rapid cell lysis in *Neurospora crassa*, even at 4 °C; lysis occurred primarily at subapical regions (Roberts and Selitrennikoff 1990). Second, osmotin, a TL protein from tobacco, causes perturbations in the regulation of fungal cell wall assembly (Yun et al. 1998; Yun et al. 1997). Regardless of the precise mode of action of TL proteins, they are fungicidal against a wide number of plant and human pathogens in vitro. Importantly, one protein, zeamatin, has shown efficacy in a murine vaginal model of *C. albicans* infection (White et al. 2002). It is therefore possible that certain PR-5 proteins may be developed into human therapeutics.

1.13 Structure of the fungal cell wall

A schematic diagram of a typical fungal cell wall is shown in Fig. 1.13. It is important to note that fungi possess significant internal turgor pressure, so that even a slight perturbation of the cell wall results in fungal cell lysis (Kaminskyj et al. 1992).
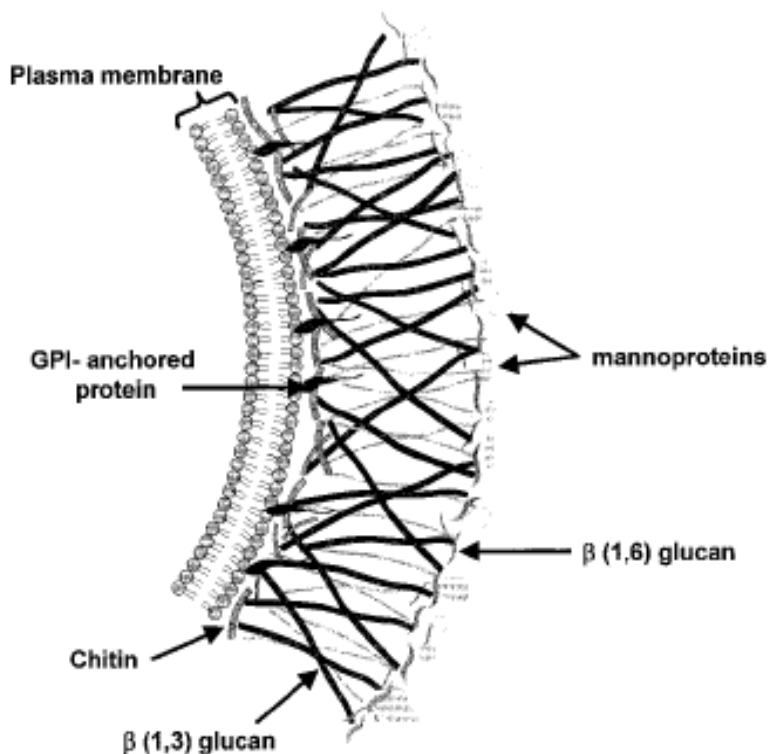
Figure 1.13 Schematic diagram of the fungal cell wall, GPI, glycophosphatidylinositol (Selitrennikoff 2001)

2 Aims of the thesis

The aim of this thesis was to investigate microbiological and biochemical aspects of the cocoa bean fermentation process. In particular, this study aimed to develop a starter culture for a controlled cocoa bean fermentation process of potential use for the production of cocoa products. In addition, this study aimed to investigate the antifungal proteins in the seed coat slime of *Theobroma cacao* which have not been previously described in order to understand their influence on fermentation of cocoa bean. The study consists of the following stages:

A first stage was to investigate the population dynamics of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) and the investigation of important fermentation parameters such as temperature, pH, sugars, organic acids and ethanol during spontaneous box fermentation of cocoa bean in a commercial plant in Nusantara Plantation XII (PTPN XII), East Java, Indonesia.

In the second stage, the microbial diversity of cocoa bean fermentations in three different cocoa producing regions, namely East Java (Indonesia), Mocache (Ecuador) and Santo Domingo (Trinidad and Tobago) was compared in order to physiologically characterize dominant species for applications as starter culture in the cocoa bean fermentation process.

In stage three, the results from in stages 1 and 2 were employed to develop a laboratory fermentation process (aerobic and anaerobic fermentation conditions) using a starter culture with the goal of understanding its influence during cocoa bean fermentation and to control the quality of cocoa bean fermentation products.

The next stage aimed to study the antifungal activity of the seed coat slime of *Theobroma cacao* L. found during cocoa bean fermentation and to determine which proteins play a role in inhibitory process as well as their inhibitory capacities.

The final goal of this work was to perform cocoa bean fermentation after removal of the pulp using a starter culture under controlled and sterile conditions. Further, the inhibitory activity of the seed coat slime of *Theobroma cacao* during fermentation process was investigated

and its influence on biochemical and metabolic product changes during cocoa bean fermentation was monitored.

3 Material and Methods

3.1 Cocoa bean fermentation samples

In order to compare the microbial diversity of cocoa bean fermentations carried out in three different cocoa producing regions, samples from cocoa bean fermentations were collected in Jember, East Java, Indonesia, after 0, 24, 48, 72 and 96 h of fermentation, in Mocache (Ecuador) after 0, 12, 24, 96 and 120 h of fermentation, and in Santo Domingo (Trinidad & Tobago) after 0, 12, 24, 48 and 72 h of fermentation.

3.2 Fermentation protocol for commercial fermentation plant in Nusantara Plantation XII (PTPN XII)

Cocoa beans were fermented in the context of commercial operations using box fermentations at PTPN XII (PT. Perkebunan Nusantara XII: Nusantara Plantation XII, Ltd. Co.), Jember, East Java, Indonesia. The beans were harvested from plantations by traditional methods and used in fermentations within 6 h after harvest. Typically, cocoa pods were harvested from the tree and opened within 30 min for removal of the beans. The pods were cut with a machete by plantation workers and the beans plus surrounding pulp were scooped out by hand and placed in piles on a plastic mat. The beans were collected from the mat, transferred in to plastic or hessian sacks and transported to the fermentary. At each fermentary, the beans (1000 kg approximately) were placed into wooden boxes (150-200 cm long x 100 cm wide x 75-100 cm deep), where natural fermentation started. After 12–16 h, the beans were mixed by transferring them to another box. Such transfers were repeated every 20–24 h until fermentation was complete (after 4 days). The wooden boxes were not cleaned between operations, and contained holes drilled into the base and sides to allow for drainage of liquids (sweatings) generated by the fermentation (Ardhana and Fleet 2003). Following fermentation, the beans were sundried for 4–5 h before being artificially dried for 20 h at 40-70 °C. Approximately 100 g beans were sampled with a sterile plastic bag at 24 h intervals. Samples were taken approximately 15 cm from the surface.

3.3 Fermentation protocol for laboratory level

3.3.1 Materials

Cocoa pod samples were provided by ICCRI (Indonesia Coffee and Cocoa Research Institute), East Java, Indonesia. Cocoa bean pods of Forastero varieties were surface sterilized using 3% sodium hypochlorite. Subsequently, the pods were opened in laminar airflow under sterile conditions.

3.3.2 Starter culture strains and preparation

All of the microorganisms in the defined cocktail used had been isolated previously from cocoa bean fermentations from Indonesia (*Saccharomyces cerevisiae* IDI-Y001, *Acetobacter pasteurianus* IDI-A019) and Ecuador (*Lactobacillus fermentum* IDE-L007). All of the strains used in this study had been physiologically characterized for heat, acid and ethanol tolerance. To inoculate the fermentary Erlenmeyer flasks, the three strains were first grown in 100-mL Erlenmeyer flasks in standard media (yeast cells were grown in malt broth, lactic acid bacteria were grown in MRS broth and acetic acid bacteria were grown in GYP broth). The cells were then centrifuged in an Eppendorf 5804R centrifuge (Eppendorf, Hamburg, Germany) for 5 min at 4.000 x g, and resuspended in 10 mL of Ringer solution. The cells were counted (*S. cerevisiae* IDI-Y001 10^7 CFU g^{-1}, *L. fermentum* IDE-L007 10^6 CFU g^{-1}, *A. pasteurianus* IDI-A019 10^7 CFU g^{-1}) and then sprayed over the cocoa beans.

3.3.3 Design of the cocoa bean fermentation

Two types of fermentation, aerobic and anaerobic, were carried out to determine the influence of microbes on the quality of fermented cocoa bean. The fermentation was performed under sterile conditions. The cocoa pods used in this study were Forastero varieties obtained from the fields of the Indonesia Coffee and Cocoa Research Institute (ICCRI) Jember in East Java, Indonesia. Mature pods were harvested by farmers and transported to Hamburg the following day. Cocoa pods were opened under sterile conditions as described (Section 3.3.1). Shortly after opening, about 250 g cocoa beans were

put into each fermentary flask. Each fermentation method (aerobic and anaerobic) was repeated two times. The fermentation was conducted under sterile conditions in a Cytoperm™ 2 CO_2 Gassed Incubator (Thermo Scientific™, St. Leon-Rot, Baden-Württemberg, Germany), as follows:

Table 3.1 Fermentation design

Fermentation time (h)	Starter culture	Aerobic fermentation				Anaerobic fermentation			
		°C	CO_2	O_2	rH	°C	CO_2	O_2	rH
0	Yeast, LAB	30	5	10	90	30	5	10	90
24	Yeast, LAB	30	5	10	90	30	5	10	90
48	Yeast, LAB	35	0.04	20	90	35	5	10	90
72	Yeast, LAB, AAB	40	0.04	20	90	40	5	10	90
96	Yeast, LAB, AAB	45	0.04	20	90	45	5	10	90
120	Yeast, LAB, AAB	45	0.04	20	90	45	5	10	90

Add: yeast (*S. cerevisiae* IDI-Y001), LAB (*L. fermentum* IDE-L007), AAB (*A. pasteurianus* IDI-A019), sampling every 24 h.

Figure 3.1 Laboratory fermentation (Thermo Scientific Heraeus® Cytoperm® 2. CO_2 and CO_2 /O_2 Incubator)

3.4 Fermentation protocol for fermentation with only 10% of pulp

Cocoa pod samples were provided by ICCRI (Indonesia Coffee and Cocoa Research Institute), Jember, East Java, Indonesia. Five pods of SCA 6 varieties were surface sterilized using 3% sodium hypochlorite. Subsequently, pods were opened in laminar airflow Herasafe (Heraeus, Hanau, Germany) under sterile conditions. The beans and pulp were manually separated using sterile surgical blades. Shortly after, approximately 200 g separated beans were placed into sterile Erlenmeyer flasks for fermentation. Afterwards, beans with 10% pulp were fermented as described above (Section 3.3).

3.5 Determination of physical and chemical changes during fermentation

3.5.1 Determination of temperature value

The temperature of fermentation masses was recorded at each sampling site by inserting a thermometer into the fermenting mass.

3.5.2 Determination of pH value

For pH determinations, the pulp was separated from the beans following the protocol of Ardhana and Fleet (2003). Beans and pulp were physically separated by adding 50 g of distilled H_2O (dH_2O) to 50 g of sample in a plastic bag and massaging the bag for 5 min. The pulp fraction was recovered by decanting. Twenty grams of the pulp and bean fraction was added to 80 g of dH_2O each and, mixed in a laboratory stomacher (type BA 7021, Seward Medical, London, UK). The homogenate was centrifuged at 11.953 x g at 10 °C for 15 min in a Sorvall RC 5C Plus (Kendro Laboratory, Newtown, CT, USA) and the supernatant retained. The sediment was washed one time with 20 mL of distilled water and the washings combined with the first supernatant. This extract for analysis was filtered through a 0.45 µm filter (Macherey-Nagel, Düren, Germany). The pH of the filtrate was determined using a pH meter (pH 21, Hanna, Rhode Island, USA).

3.5.3 Determination of water activity (a_W) value

The water activity (a_w) value of the fermented cocoa bean samples was measured by Hygromess Labo 4701 (Hygrocontrol, Hanau, Germany) every 24 h (0, 24, 48, 72, 96, 120 h).

3.6 Microbiological analyses

3.6.1 Media and reagents

3.6.1.1 Media

Table 3.2 Composition of malt extract agar (MEA) and malt extract broth (MEB)

MEA		MEB	
Substance	**g L^{-1}**	**Substance**	**g L^{-1}**
Peptone	3	Malt extract	17
Malt extract	30	pH	4.8
Agar-agar	15		
pH	5.6		

Table 3.3 Composition of DeMan-Rogosa-Sharpe agar (MRSA) and DeMan-Rogosa-Sharpe broth (MRSB)

MRSA		MRSB	
Substance	**g L^{-1}**	**Substance**	**g L^{-1}**
Peptone	10	Peptone	10
Meat extract	10	Meat extract	8
Yeast extract	4	Yeast extract	4
D(+)-glucose	20	Tween 80	1
Dipotassium hydrogen phosphate	2	Di-ammonium hydrogen citrate	2
Tween 80	1	Sodium acetate	5
Di-ammonium hydrogen citrate	2	Magnesium sulfate	0.2
Sodium acetate	5	Manganese sulfate	0.04
Magnesium sulfate	0.2	pH	5.7
Manganese sulfate	0.04		
Agar-agar	14		
pH	5.7		

Table 3.4 Composition of glucose yeast extract peptone calcium carbonate agar (GYPA) and glucose yeast extract peptone broth (GYPB)

GYPA		GYB	
Substance	**g L^{-1}**	**Substance**	**g L^{-1}**
D-glucose	20	D-glucose	20
Yeast extract	5	Yeast extract	5
Peptone	5	Peptone	5
Calcium carbonate	3	pH	5.6
Agar-agar	15		
pH	5.6		

Table 3.5 Composition of Acetobacter agar and plate count agar (PCA)

Acetobacter agar		PCA	
Substance	**g L^{-1}**	**Substance**	**g L^{-1}**
Yeast extract	10	Peptone	5
Ethanol	15	Yeast extract	2.5
Bromocresol purple	0.04	D(+)-glucose	1
Agar-agar	25	Agar-agar	14
pH	6.5	pH	7

Table 3.6 Composition of yeast extract glucose chloramphenicol agar (YGCA) and yeast extract glucose chloramphenicol bromophenol blue agar (YGCBA)

YGCA		YGCBA	
Substance	**g L^{-1}**	**Substance**	**g L^{-1}**
Yeast extract	5	Yeast extract	5
D(+) glucose	20	D(+) glucose	20
Chloramphenicol	0.1	Chloramphenicol	0.1
Agar-agar	14.9	Bromophenol blue	0.01
pH	6.6	Agar-agar	14.9
		pH	6.6

3.6.1.2   Reagents

TE buffer

Tris	0.606 g
EDTA	0.146 g
H_2O_{bidest}	ad 500 mL
pH	8

TE-sucrose buffer

20% sucrose dissolved in 1x TE buffer

Lysis buffer

10% [wt/vol] sodium dodecyl sulfate	2.5 mL
1 N NaOH	5 mL
H_2O_{bidest}	92.5 mL

3.6.2   Enumeration, isolation and purification of yeasts, lactic acid bacteria, and acetic acid bacteria

Ten grams of samples of cocoa beans and adhering pulp were added to 90 ml of Ringer's solution (Merck, Darmstadt, Germany) and mixed for 2 min in a laboratory blender stomacher 400 (Seward Medical, London, UK) at medium speed. From this, 10-fold dilutions were prepared in 1 × Ringer's solutions. Yeasts were enumerated by spreading on malt extract agar (Merck, Darmstadt, Germany) containing 100 mg L^{-1} chloramphenicol (Sigma, St. Louis, Missouri, USA) and were incubated for 3 days at 28 °C. Lactic acid bacteria were enumerated by spreading on MRS agar (Merck, Darmstadt, Germany) containing 0.1% cycloheximide (Merck, Darmstadt, Germany) to suppress the growth of yeasts. Incubation was done in anaerobic jars BBL GasPak system (BD, New Jersey, USA) and supplementation with Anaerocult A (Merck, Darmstadt, Germany) to obtain anaerobic conditions during incubation for 3 – 4 days at 30 °C. Acetic acid bacteria were enumerated by spreading on

GYP agar, containing 0.1% cycloheximide (Merck, Darmstadt, Germany) to inhibit growth of yeasts and incubated for 3 – 5 days at 30 °C (Lisdiyanti et al. 2003). Acetobacter agar containing 0.1% cycloheximide (Merck, Darmstadt, Germany) to inhibit growth of yeasts and incubated for 3 – 5 days at 30 °C was also used. Aerobic mesophilic bacteria and *Bacillus* spp. were enumerated by spreading on plate count agar (Merck, Darmstadt, Germany) containing 0.1% cycloheximide (Merck, Darmstadt, Germany) and incubated for 3 days at 30 °C. Following incubation, the number of colony forming units (CFU) was recorded.

Subsequently, the morphological characteristics of each colony type were recorded and counts were made for each type. For each colony type a number of colonies corresponding to the square root of the number of colonies of that type were restreaked and purified. Purified isolates were stored at −80 °C. Yeasts were stored in YGP broth (yeast extract 5 g L^{-1}, glucose 10 g L^{-1}, peptone 10 g L^{-1}, pH 5.6) containing 20% (w/w) glycerol; LAB were stored in 10% skim milk, containing 20% (w/w) glycerol; AAB were stored in GYP broth (glucose 20 g L^{-1}, yeast extract 5 g L^{-1}, peptone 5 g L^{-1}, pH 5.6) containing 20% glycerol.

3.6.3 Gram staining

Gram staining was carried out according to the methods of Hucker and Conn (1923). Further on the KOH test was performed using the reaction with 0.3% KOH (Powers 1995). Cultures were incubated in a basal slant medium at 30 °C for 18 - 24 h. In the former method, a loop-full of culture was placed on a clean slide, spread using the loop, and allowed to dry, forming a smear. The smear was fixed by passing it quickly through a Bunsen flame twice. A heat-fixed smear was stained for 1 min with crystal violet before being rinsed briefly under water, treated for 1 min with Lugol's iodine (a solution of iodine and potassium iodine in water), and briefly rinsed again. Decolorization was then conducted by treating the stained smear with 95% ethanol and, rinsing in water. The smear was counterstained for 1 min with safranine. After a brief rinse, the smear was blotted dry and examined under the oil-immersion objective of the Zeiss axiostar plus microscope (Carl Zeiss Microscopy**,** Thornwood, NY, USA) (magnification approximately 1000×). Cells that stained red were Gram-negative, while those that stained blue were Gram-positive (Hucker and Conn 1923). In the second method, colonies grown on a basal agar plate were placed on a slide. A drop of

3% KOH was added and the cells were mixed using a loop. The appearance of a viscous thread-like slime when the loop was raised for about 1 cm from the slide indicated Gram-negative strains.

3.6.4 Catalase activity

Catalase is an enzyme which is present in all cells with aerobic metabolism. It contains iron protoporphyrin (haemin) as a coenzyme. Catalase cleaves toxic hydrogen peroxide produced in metabolic processes into oxygen and water. The presence or absence of catalase activity is a taxonomic property of microorganisms and can be used for their differentiation or identification. Part of the colony to be examined was picked up with a loop and placed onto a dry glass slide. A drop of the catalase reagent 'Bactident Catalase' (Merck, Darmstadt, Germany) contening 3% aqueous solution of hydrogen peroxide was placed on the bacteria. Gas formation indicated positive reaction.

3.6.5 Oxidase test

Cytochrome oxidase is a very widespread enzyme belonging to the group of iron porphyrins. It oxidizes reduced cytochrome c and is itself converted to its reduced inactive form. The reduced cytochrome oxidase is reconverted to its oxidized active form via the transfer of electrons to molecular oxygen. In the presence of molecular oxygen the electrons can be removed by the cytochrome oxidase/cytochrome c system from a number of organic compounds, e.g. the so-called Nadi reagent (1-naphthol + dimethylparaphenylenediamine) with formation of the condensation molecule indophenol blue. This reaction is utilized for the classification and identification of bacteria. The oxidase test was done by using a platinum loop to apply single colonies from the plate medium to the reactive zone of the oxidase strip 'Bactident Oxidase' (Merck, Darmstadt, Germany) containing N,N-Dimethyl-1,4-phenylenediammonium chloride; 1-naphthol. After 20 - 60 seconds, the test strip was compared with the color scale provided. Cytochrome oxidase-positive bacteria are present when the reactive zone exhibits a blue to purple color.

3.7 Carbohydrate assimilation test using the API kit

3.7.1 Carbohydrate assimilation test for yeasts using API ID 32 C

API ID 32 C (bioMérieux, Marcy-L'Etoile, France) was used for the assimilation test of yeasts. The application of these ID 32 C assimilation kits was performed according to manufacturer's instructions.

3.7.2 Carbohydrate assimilation test for lactic acid bacteria using API 50 CHL

API 50 CHL (bioMérieux, Marcy-L'Etoile, France) was used for the assimilation test of *Lactobacillus*. The application of these API 50 CHL assimilation kits was performed according to manufacturer's instructions.

3.8 FT-IR spectroscopy for the identification of yeasts

Because of the large number of isolates, a high throughput method was necessary for the identification of yeast in cocoa bean fermentation. This was accomplished using Fourier-transfom infrared (FT-IR) spectroscopy (Bruker, Ettlingen, Baden-Württemberg, Germany), which allows for the processing of a large number of isolates. This spectroscopy method is based on the isolation of pure cultures. After standardized cultivation on YGCA (22 g ± 1 g) for 24 h ± 0.5 h at 27 °C, the middle infrared range of 4000-500 wave length/cm^2 (25.000 - 2.500 nm) was used to shift atoms in molecule bonds into vibration. By absorption of light, a spectrum can be measured, calculated and constructed. This specific absorption can then be attributed to several cell components (polysaccharides, fatty acids, proteins, mixed region, fingerprint-region) which are used for species identification using an appropriate comparative database. This database includes approximately 215 yeast species. By hierarchical cluster analysis (HCA), a differentiation on strain level is possible and can be used for choosing several important species for strain typing by random amplified polymorphic DNA (RAPD) and δ-PCR (Naumann et al. 1991; Kümmerle et al. 1998) (Fig. 3. 2).
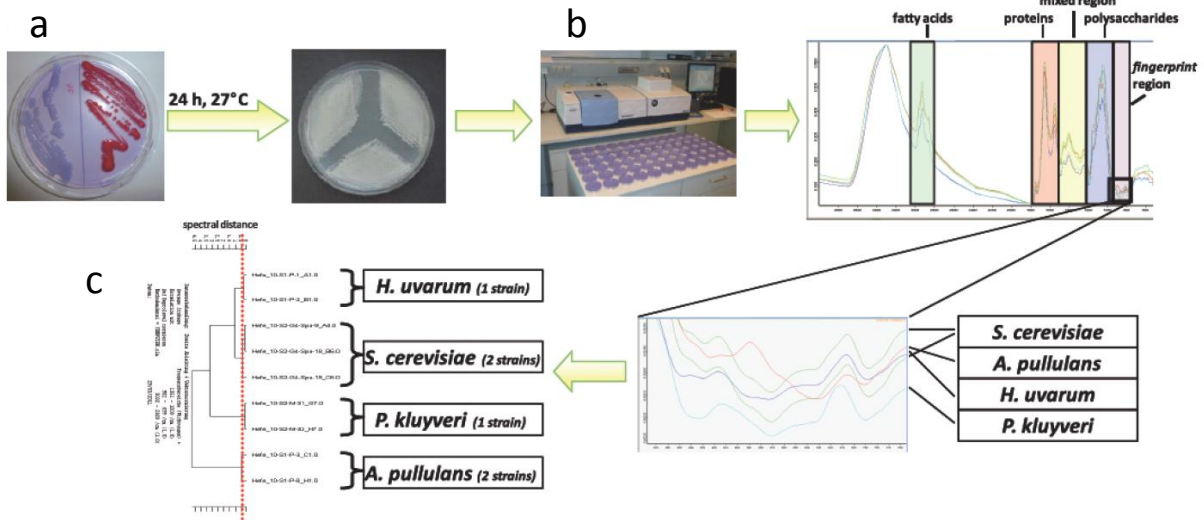
Figure 3.2 a) Pure yeast culture on YGCBA, b) Measurement on a ZnSe microtiterplate (96-well format) in a FT-IR (Bruker), comparison of the absorbance pattern with a database and identification of the isolate, c) Differentiation by hierarchical cluster analysis (HCA) up to strain level (strain range 0.3 spectral distances)

3.9 Identification using molecular methods

3.9.1 Isolation of genomic DNA and purification

3.9.1.1 Isolation of genomic DNA using classical methods

A preculture of LAB and AAB was grown in 30 mL of MRSB and GYPB medium at 30 °C for 2-3 days. Then 5 - 10 mL of this preculture was centrifuged in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 30 sec at 13.000 x g. To remove excess medium, the pellet was resuspended in 1 mL NaCl. Then, cells were harvested in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 30 sec at 13.000 x g and resuspended in 250 µL TE-sucrose buffer. After adding 250 µL TE buffer containing lysozyme (10 mg mL^{-1}) and RNAase (1 mg mL^{-1}), the mixture was incubated for 1 h at 37 °C. Proteinase K (1 mg mL^{-1}) was added to the suspension and incubated for at least 1 h. After adding 250 µL phenol/chloroform (1:1), the suspension was mixed well until a white emulsion appeared. The emulsion was centrifuged in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 20 min at 17.000 x g and the supernatant was carefully transferred into a new

eppendorf cup (Eppendorf, Hamburg, Germany). The phenol/chloroform step was repeated twice. Then, 250 µL chloroform were added to the supernatant, mixed well and centrifuged in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 2 min at 17.000 x g. The supernatant was then transferred into a fresh eppendorf cup (Eppendorf, Hamburg, Germany) and precipitated with 2.5 volumes of 96% EtOH and 0.3 M NaOAc. The eppendorf cup was inverted several times and incubated at -70 °C for 10 min. After centrifugation for 20 min at 17.000 x g, the supernatant was decanted and mixed with 1 mL 70% EtOH and centrifuged in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 2 min at 17.000 x g. This step was repeated once more. The DNA pellet was then dried, resuspended in 100 µL 1x TE buffer or H_2O_{bidest} and incubated for 1 h at 60 °C or at room temperature overnight.

3.9.1.2   Isolation of genomic DNA using the peqGOLD Fungal DNA kit

Highly pure fungal DNA was isolated using the peqGOLD Fungal DNA Kit (PEQLAB Biotechnologie, Erlangen, Germany). Culture volumes of 5 mL were sufficient for the isolation of yeast DNA. The application of these DNA isolation kits was done according to manufacturer's instructions.

3.9.1.3   Isolation of genomic DNA using the peqGOLD Bacteria DNA kit

Highly pure bacteria DNA was isolated using peqGOLD Bacteria DNA Kit (PEQLAB Biotechnologie, Erlangen, Germany). Culture volumes of 5 mL were sufficient for the isolation of bacteria DNA. The application of these DNA isolation kits was done according to manufacturer's instructions.

3.9.2   Determination of DNA concentration and purity

The quantity and purity of DNA was measured using a photometer SmartSpecTM Plus Spectrophotometer (BIO RAD, Hercules, CA, USA). DNA concentration was measured in a disposable micro UV cuvette (Plastibrand®, Brand, Wertheim, Germany) at 260 nm against H_2O_{bidest}. An OD_{260} of 1.0 corresponds to 50 µg mL^{-1} of double-stranded DNA. The purity was determined by calculating the ratio of the extinction values at 260 and 280 nm. Pure DNA solutions have a ratio OD_{260}:OD_{280} of 1.8 - 2.0 (Sambrook 2001).

3.9.3 Agarose gel electrophoresis

The size, quality and quantity of DNA were analyzed by agarose gel electrophoresis. DNA is negatively charged and moves through a 1.0 % (w/v) agarose gel. The run time of the DNA fragments is dependent on their size. Smaller fragments move through the matrix faster, and thus fragments are separated according to their size. The electrophoresis was performed at 100 V for at least 35 min with a PowerPac^{TM} HC (BioRad, Munich, Germany) power supply in an electrophoresis gel chamber Sub-Cell® GT Agarose Gel (BioRad, Munich, Germany) filled with TAE buffer. Samples (1-5 µL) were mixed with 1 µL of 6X DNA loading dye (Fermentas, St. Leon-Rot, Baden-Württemberg, Germany) before they were applied to the gel.

10X TAE Buffer		6X DNA loading dye	
Tris	48.5 g	Tris-HCl (pH 7.6)	10 mM
Glacial acetic acid	11.4 mL	Tris-HCl (pH 7.6)	10 mM
0.5M EDTA (pH 8.0)	20 mL	bromophenol blue	0.03%
H_2O_{bidest} to	1000 mL	xylene cyanol FF	0.03%
		glycerol	60%
		EDTA	60 mM

3.9.4 Visualization and documentation of DNA

After performing the electrophoresis, the DNA was stained in an ethidium bromide solution (10 µg mL^{-1}) for 15 min and visualized under UV light of 254 nm in a Molecular Imager® (GelDoc^{TM} XR+ Imaging System, BioRad, Munich, Germany). The gel was documented with Quantity One 1-D analysis software version 4.6.9 (BioRad, Munich, Germany). The size of the DNA fragments was estimated by comparison with marker bands of a GeneRuler™ 1 kb DNA Ladder (Fermentas, St. Leon-Rot, Baden-Württemberg, Germany) which was also loaded onto the gel.

3.9.5 Amplification of DNA by polymerase chain reaction (PCR)

Table 3.7 Primers used in this study

Primers	Sequence 5' – 3'	Length [bp]	GC content [%]	Tm [°C]
NL1	GCA TAT CAA TAA GCG GAG GAA AAG	24	41.7	59.3
NL4	GGT CCG TGT TTC AAG ACG G	19	57.9	58.8
27 F	AGA GTT TGA TCM TGG CTC AG	20		58.6
1492 R	TAC GGY TAC CTT GTT ACG ACT T	22		59.1
FD1	AGA GTT TGA TCC TGG CTC AG	20		61.0
RD1	AAG GAG GTG ATC CAG CC	17		60.2
20 F	GAT TTT GAT CCT GGC TCA G	19	47.4	54.5
1500 R	GTT ACC TTG TTA CGA CTT	18	38.9	49.1

Oligonucleotide primers were ordered from Operon Biotechnologies (Eurofins MWG, Ebersberg, Germany).

3.9.5.1 Standard PCR

To set up parallel reactions and to minimize the possibility of pipetting errors, DreamTaq PCR Master Mix (Fisher Scientific, Schwerte, North Rhine-Westphalia, Germany) was used for standard PCR. The application of these DreamTaq PCR Master Mix kits was done according to manufacturer's instructions.

Table 3.8 PCR master mix reaction for standard PCR

Reaction components	Final concentration	Per 50 µL
10X DreamTaq Buffer		5 µL
dNTP Mix (2 mM each)	0.2 mM of each dNTP	5 µL
Forward primer (10 µM)	0.5 µM	2.5 µL
Reverse primer (10 µM)	0.5 µM	2.5 µL
Template DNA	10pg-1 µg	2 µL
DreamTaq DNA Polymerase	1.25 u	1.25 u
Water, nuclease-free		To 50 µL

Table 3.9 PCR cycling program for yeasts

Cycling step	Temperature and time
Initial denaturation	95 °C, 5 min
Denaturation	95 °C, 1 min
Annealing	52 °C, 15 sec
Extension	72 °C, 2 min
Cycles	35 cycles
Final extension	72 °C, 7 min

Modified PCR cycling (Kurtzman and Robnett 1998)

Table 3.10 PCR cycling program for LAB

Cycling step	Temperature and time
Initial denaturation	95 °C, 5 min
Denaturation	95 °C, 1 min
Annealing	56 °C, 1.15 min
Extension	72 °C, 1.15 min
Cycles	35 cycles
Final extension	72 °C, 7 min

Modified El-Ghaish (El-Ghaish et al. 2011)

Table 3.11 PCR cycling program for AAB

Cycling step	Temperature and time
Initial denaturation	95 °C, 5 min
Denaturation	95 °C, 18 sec
Annealing	55 °C, 18 sec
Extension	72 °C, 1.0 min
Cycles	30 cycles
Final extension	72 °C, 7 min

(Lisdiyanti et al. 2001)

3.9.5.2   Yeasts direct colony PCR

For yeasts colony PCR, yeasts were grown on malt agar (Merck, Darmstadt, Germany) for 24-48 h at 28 °C or in 5 mL malt broth (Merck, Darmstadt, Germany) for 24 h at 25 °C on a rotary shaker GFL 3015 (Burgwedel, Hanover, Germany) at 150 rpm and harvested by centrifugation in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 5 min at 13.000 x g. PCR template was prepared using 50 µL 0.02 M NaOH for pure yeasts colony or 40 µL 0.1 NaOH for overnight culture. The culture solutions were incubated for 5 min at 37 °C. Yeast DNA templates were amplified using KAPA2G Robust PCR Kits (PEQLAB Biotechnologie, Erlangen, Germany). The application of PCR kits was done according to manufacturer's instructions. The PCR cycling programs used are described above (Tab. 3.9), PCR master mix conditions are given below.

Table 3.12 PCR master mix reaction for yeasts

Reaction component	Final concentration	Per 25 µL
PCR grade water		14.3 µL
5x KAPA2G Buffer B	1x	5.0 µL
dNTP mix (10 mM each dNTP)	0.2 mM each dNTP	0.50 µL
Forward primer NL-1 (10 µM)	0.5 µM	1.25 µL
Reverse primer NL-4 (10 µM)	0.5 µM	1.25 µL
Template DNA		2.5 µL
KAPA 2G Robust HotStart (5 U/µL)	1 unit per 25 µL	0.20 µL
Total		25 µL

3.9.5.3   Lactic acid bacteria direct colony PCR

For LAB colony PCR, LAB were grown on MRSA (Merck, Darmstadt, Germany) for 24 - 48 h at 25 °C or in 5 mL MRSB (Merck, Darmstadt, Germany) for 24-48 h at 37 °C anaerobically and were harvested by centrifugation in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 5 min at 13.000 x g. A small portion of a single colony was resuspended in 20 µL lysis buffer (2.5 ml of 10% [wt/vol] sodium dodecyl sulfate, 5.0 mL of 1 N NaOH, and 92.5 mL of ultrapure water). The mixture was heated at 95 °C for 15 min and cooled immediately on ice. After centrifugation in an Eppendorf 5417R centrifuge for 30 sec at

13.000 x g, 180 µL of sterile ultrapure water were added. Subsequently, the mixture was centrifuged in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 5 min at 13.000 x g and stored at -20 °C (Van der Meulen et al. 2007). If this treatment method was not able to produce a high quality DNA template, the peqGOLD Bacterial DNA Kit was used. LAB DNA template was amplified using KAPA2G Robust PCR kits (PEQLAB Biotechnologie, Erlangen, Germany). The application of PCR kits was done according to manufacturer's instructions. The PCR cycling program used are described above (Tab. 3.10), PCR master mix conditions are given below.

Table 3.13 PCR master mix reaction for lactic acid bacteria

Reaction component	Final concentration	Per 25 µL
PCR grade water		15.9 µL
5x KAPA2G Buffer B	1x	5.0 µL
dNTP mix (10 mM each dNTP)	0.2 mM each dNTP	0.50 µL
Forward primer fD1 (10 µM)	0.5 µM	1.25 µL
Reverse primer rD1 (10 µM)	0.5 µM	1.25 µL
Template DNA		1 µL
KAPA 2G Robust HotStart (5 U/µL)	1 unit per 25 µL	0.10 µL
Total		25.0 µL

3.9.5.4 Acetic acid bacteria direct colony PCR

For AAB colony PCR, AAB were grown on GYPA (Lisdiyanti et al. 2003) for 24-48 h at 30 °C aerobically or in 5 mL GYPB (Lisdiyanti et al. 2003) for 24-48 h at 30 °C aerobically on a rotary shaker GFL 3015 (Burgwedel, Hanover, Germany) at 150 rpm and harvested by centrifugation in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 5 min at 13.000 x g. A small portion of a single colony was resuspended in 20 µL of ultrapure water. The mixture was heated at 95 °C for 5 min and cooled immediately on ice. After centrifugation in an Eppendorf 5417R centrifuge for 30 sec at 13.000 x g, 180 µL of sterile ultrapure water were added. Subsequently, the mixture was centrifuged in Eppendorf 5417R (Eppendorf, Hamburg, Germany) for 5 min at 13.000 x g and stored at -20 °C. If this treatment method was not able to produce a high quality DNA template, the peqGOLD

Bacterial DNA Kit was used. LAB DNA template was amplified using KAPA2G Robust PCR Kits (PEQLAB Biotechnologie, Erlangen, Germany). The application of PCR kits was done according to manufacturer's instructions. The PCR cycling programs used are described above (Tab. 3.11). PCR master mix conditions are given below.

Table 3.14 PCR master mix for acetic acid bacteria

Reaction component	Final concentration	Per 25 µL
PCR grade water		15.9 µL
5x KAPA2G Buffer B	1x	5.0 µL
dNTP mix (10 mM each dNTP)	0.2 mM each dNTP	0.50 µL
Forward primer 20f (10 µM)	0.5 µM	1.25 µL
Reverse primer 1500r (10 µM)	0.5 µM	1.25 µL
Template DNA		1 µL
KAPA 2G Robust HotStart (5 U/µL)	1 unit per 25 µL	0.10 µL
Total		25.0 µL

3.9.6 Purification of PCR product

Prior to sequencing, enzymes, primers and remaining nucleotides had to be removed from the PCR products. The purification was carried out by using the GeneJET[TM] PCR Purification kit (Fermentas, St. Leon-Rot, Baden-Württemberg, Germany) according to the manufacturer´s instructions.

3.9.7 Sequencing analyses of DNA

Sequencing was carried out by the commercial DNA sequencing service Eurofins MWG Operon (Ebersberg, Germany). Sample preparation for sequencing: 100 ng µL^{-1} purified PCR products in 15 µL ddH$_2$O, including 15 pmol primer. This premixed DNA sample was sent to Eurofins MWG Operon (Ebersberg, Germany) for sequencing. An ABI 3730XL DNA analyzer (Applied Biosytems/Life technologies, Darmstadt, Germany) was used based on the Sanger technique (Sanger et al. 1977).

3.9.8   BLAST analyses

Sequences results were corrected and aligned using Bio Edit software. The corrected sequences were subsequently aligned to 16S rRNA gene sequences for bacteria and 26S rRNA gene sequences for yeasts in the GenBank Database using the BLAST algorithm (Altschul et al. 1997).

3.10 Physiological adaptation to fermentation environment (acid, ethanol and heat tolerance)

3.10.1 Physiological adaptation of yeasts

Malt extract broth (MEB) (Merck, Darmstadt, Germany) was used for the characterization of the physiological adaptation of yeasts. To test pH tolerance, MEB was adjusted to pH 2.5, 3.5, and 5.0 with 5M HCl. To test ethanol tolerance, MEB was supplemented with 5%, 10%, or 15% (v/v) ethanol. To test heat tolerance, MEB was adjusted to pH 5.5, and was incubated at 25, 35, and 45 °C. All tubes were inoculated with 1% (v/v) of a yeast culture grown at 30 °C for 24 h. Growth was determined by measurements of OD$_{600nm}$ after incubation at 25 °C for 5 days.

3.10.2 Physiological adaptation of lactic acid bacteria

De Man, Rogosa and Sharpe broth (MRSB) (Merck, Darmstadt, Germany) was used for the characterization of the physiological adaptation of LAB. To test pH tolerance, MRSB was adjusted to pH 2.5, 3.5, and 5.0 with 5M HCl. To test ethanol tolerance, MRSB was supplemented with 5%, 10%, or 15% (v/v) ethanol. To test heat tolerance, MRSB was adjusted to pH 5.5 and was incubated at 25, 35, and 45 °C. All tubes were inoculated with 1% (v/v) of a yeast culture grown at 30 °C for 24 h. Growth was determined by measurement of OD$_{600nm}$ after incubation at 30 °C for 5 days.

3.10.3 Physiological adaptation of acetic acid bacteria

Glucose yeast extract peptone broth (GYPB) containing 2.0% D-glucose, 0.5% yeast extract and 0.5% peptone (Lisdiyanti et al. 2002) was used for the characterization of the physiological adaptation of AAB. To test pH tolerance, GYPB was adjusted to pH 2.5, 3.5, and 5.0 with 5M HCl. To test ethanol tolerance, GYPB was supplemented with 5%, 10%, or 15% (v/v) ethanol. To test heat tolerance, GYPB medium was adjusted to pH 5.5 and was incubated at 25, 35, and 45 °C. All tubes were inoculated with 1% (v/v) of a yeast culture grown at 30 °C for 24 h. Growth was determined by measurement of OD_{600nm} after incubation at 25 °C for 5 days.

3.11 Analyses of metabolites with HPLC

3.11.1 Measurement of sugars and ethanol

The HPLC system utilized in this study consisted of a Merck Hitachi L-6200 pump (Merck Hitachi, Darmstadt, Germany), an ERC-3512 degasser (Erma, Tokyo, Japan), an AS-2000A sample injector (Merck Hitachi, Darmstadt, Germany) and a Merck Hitachi Lachrom RI detector L-7490 (Darmstadt, Germany). The software, D-7000 HPLC-System-Management HSM, Version 4.1 (Hitachi, Tokyo, Japan), was used to manage the HPLC system and to evaluate and quantify the results. The column used was an 850 BP-OA H+ 300*7.8mm organic acid column (Benson polymeric, Sparks, NV, USA) for organic acid and glycerol and a Rezex RCM-Monosaccharide column (Phenomenex, Torrance, CA, USA) for sugar and ethanol. All solvents used were of analytical grade purchased from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

3.11.1.1 Preparation of the samples

Bean and pulp were physically separated by adding 50 g distilled H_2O (dH_2O) to 50 g sample in a plastic bag and massaging the bag for 5 min. The pulp fraction was recovered by decanting. Twenty grams of the pulp and bean fraction was added to 80 mL dH_2O and mixed

in a laboratory blender stomacher 400 (Seward Medical, London, UK). The homogenate was centrifuged at 11.953 x g at 10 °C for 15 min in a Sorvall RC 5C plus (Kendro Laboratory, Newtown, CT, USA) and the supernatant retained. Sediment was washed one time with 20 mL distilled water and the washings were combined with the first supernatant. This extract for analysis was filtered through a 0.45 µm filter (Macherey-Nagel, Düren, Germany).

3.11.1.2 Sugars and ethanol standards

Sucrose, glucose, fructose, mannitol and ethanol were used as standards. All standards were diluted with distilled water. The range of the standards was (0.2 − 2 g 100 mL^{-1}). Different concentrations of each standard solution were injected into the Rezex RCM-Monosaccharide column (Phenomenex, Torrance, CA, USA) as a solid phase and were eluted with water as mobile phase at 60 °C, and a flow rate of 0.6 mL min^{-1}. The injection volume was 10 µL and the column eluate was monitored by Lachrom RI Detector. The calibration curves were obtained by plotting peak area (mV min^{-1}) versus amount injected (range covered, 0.2 − 2 g 100 mL^{-1}).

3.11.1.3 Organic acids and glycerol standards

Citric acid, lactic acid, acetic acid, succinic acid and glycerol were used as standards. All standards were diluted with distilled water. The range of the standards was (0.1 − 2.5 g 100 mL^{-1}). Different concentrations of each standard solution were injected into organic acid column 850 BP-OA H+, 300*7.8mm (Benson Polymeric, Sparks, NV, USA) as a solid phase. All of the chromatographic separations were carried out at 60 °C. A flow of 0.6 mL min^{-1} with 26 mM sulfuric acid was used as mobile phase. The injection volume was 10 µL and the column eluate was monitored by Lachrom RI Detector. The calibration curves were obtained by plotting peak area (mV min^{-1}) versus amount injected (range covered, 0.1 − 2.5 g 100 mL^{-1}).

3.11.1.4 Measurement of sugars and ethanol

The concentrations of sucrose, glucose, fructose, mannitol, and ethanol were determined by high performance liquid chromatography (HPLC) (Merck Hitachi, Darmstadt, Germany) with a Rezex RCM-Monosaccharide column (Phenomenex, Torrance, CA, USA) as a solid phase

and were eluted with water as a mobile phase at 60 °C, and a flow rate of 0.6 mL min^{-1}. The injection volume was 10 µL and the column eluate was monitored by Lachrom RI Detector.

3.11.2 Measurement of organic acids and glycerol

Organic acids (citric acid, lactic acid, acetic acid, succinic acid) and glycerol were determined by HPLC with an organic acid column 850 BP-OA H+, 300*7.8mm (Benson Polymeric, Sparks, NV, USA) as a solid phase. All of the chromatographic separations were carried out at 60 °C. A flow of 0.6 mL min^{-1} with 26 mM sulfuric acid was used as a mobile phase. The injection volume was 10 µL and the column eluate was monitored by Lachrom RI Detector.

3.11.3 Measurement of amino acids

Amino acid contents were analysed according to the method described by Rohsius et al. (2006)

3.11.3.1 Preparation of samples

Two grams of freeze-dried fermented cocoa bean were milled to a powder with a particle size of ca. 1 µm^3 in a Retsch MM 200 (Haan, North Rhine-Westphalia, Germany) laboratory mill using 10 mL n-hexane for fat removal. The pulverised sample then was rinsed twice with 50 mL petroleum in a Buchner funnel to reduce the residual fat content to ≤5%. Subsequently, the defatted powder was dried in a vacuum oven (Heraeus, Hanau, Germany) at room temperature and 100 mbar.

3.11.3.2 Preparation of standard for amino acids

Amino acid standards were prepared by injecting of mixtures containing 1 – 10 pmol µL^{-1} of each amino acid into the column.

3.11.3.3 Measurement of amino acids

Free amino acid contents were analysed according to the method described by Rohsius et al. (2006). One-hundred milligram defatted cocoa powder were stirred at 4 °C for 1 h with 300 mg polyvinyl-polypyrrolidon (PVPP) and 5 - 10 mL distilled water. Immediately after adding the water, the pH was adjusted to pH 2.5 with 10% aqueous trifluoroacetic acid. The

homogenate was centrifuged in Biofuge 11R (Heraeus, Hanau, Germany) for 10 min at 2.683 x g. The clear supernatant solution was filtered through a 0.45 µm filter (CS-Chromatography, Düren, North Rhine-Westphalia, Germany). About 30 µL of each sample were lyophilized in Christ Alpha 1-4 (SciQuip, Shrewsbury, West Midlands, UK) for 1 h at -20 °C, 0.05 mbar directly into the vial and kept at -20 °C until analysis.

Free amino acids were derivatized with *O*-phthalaldehyde (OPA) prior to HPLC analysis. Chromatographic separation was performed with a LiChroCART 250-4 (Merck, Darmstadt, Germany) provided with precolumn LiChrospher 100 RP-18 (5 µm) (VWR international, Radnor, PA, USA). Chromatographic analyses were conducted with a reverse phase binary gradient [A: 1.6 L sodium acetate solution/glacial acetic acid (50 mmol L^{-1}; pH 6.2), 50 mL MeOH (Lichrosolv®, gradient grade, Merck, Darmstadt, Germany), 20 mL tetrahydrofuran (Lichrosolv®; gradient grade, Merck, Darmstadt, Germany); B: 200 mL sodium acetate solution/glacial acetic acid (50 mmol L^{-1}; pH 6.2), 800 mL MeOH (Lichrosolv®, gradient grade, Merck, Darmstadt, Germany)] at a flow rate of 1.3 mL min^{-1}.

The OPA derivatization procedure was performed according to Rohsius et al. (2006). Twenty microliters of the derivative samples were injected into the column for separation and were subsequently detected with the Hitachi F-1050 Fluorescence Spectrophotometer (Triad Scientific, Manasguan, NJ, USA) (λex = 334 nm, λem = 425 nm). The column temperature was 30 °C. The autosampler was from Merck-Hitachi AS-4000 (Darmstadt, Germany). The solution degasser consisted of the Degassex DG-4400 from Phenomenex (Torrance, California, USA). Quantification was carried out by calculating the peak area of chromatograms from standard mixtures containing 1–10 pmol $µL^{-1}$ of each amino acid. Total free amino acid concentrations were obtained by summation of the individual amino acid concentrations.

3.11.4 Measurement of polyphenols and anthocyanins

3.11.4.1 Preparation of the samples

Polyphenol and anthocyanins contents were analysed according to the method described by Niemenak et al. (2006) and Elwers et al. (2009). Two grams of freeze-died fermented cocoa

beans were milled to a powder with a particle size of ca. 1 μm^3 in a Retsch MM 200 (Haan, North Rhine-Westphalia, Germany) laboratory mill using 10 mL n-hexane for fat removal. The pulverised sample was then rinsed 2 twice with 50 mL petroleum in a Buchner funnel to reduce the residual fat content to ≤5%. Subsequently, the defatted powder was dried in a vacuum oven (Heraeus, Hanau, Germany) at room temperature and 100 mbar (Niemenak et al. 2006; Elwers et al. 2009).

3.11.4.2 Preparation of the standards

Epicatechin and quercetin were obtained from Sigma (St. Louis, Missouri, USA). Protocatechiuc acid and catechin were from Aldrich (St. Louis, Missouri, USA) and Fluka (St. Louis, Missouri, USA), respectively. 3-a-L-arabinosyl cyanidinin and 3-b-D-galactosyl cyanidin were purchased from Polyphenols AS (Sandnes, Rogaland, Norway). All solvents used were of analytical grade purchased from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

3.11.4.3 Measurement of polyphenols and anthocyanins

For RP-HPLC analysis, 100 mg of the defatted cocoa powder were weighed in a centrifuge tube (16 x 100 mm^2), 3 mL of methanol were added and the mixture stirred for 20 – 30 s with an ULTRA-TURRAX® T25 (Ika Labortechnik, Staufen, Germany) agitator. The agitator was then rinsed with 2 mL methanol and the solutions were combined. The centrifuge tube was cooled for 15 min at 0 °C and then centrifuged in Megafuge 11R (Heraeus, Hanau, Germany) for 10 min at 2.683 x g. The methanolic supernatant containing phenolic compounds was decanted into a 50 mL pear-shaped flask. The extraction was repeated 3 additional times, with a cooling phase of only 2 min.

The methanol was removed from the combined extracts by rotary evaporation under partial vacuum at 40 °C and 100 mbar. Subsequently, the residue was dissolved in 3.0 mL methanol (Lichrosolv®, Merck, Darmstadt, Germany). The sample was transferred into an HPLC vial through a 0.45 µL syringe filter PTFE Multoclear® (CS-Chromatographie, Langerwehe, North Rhine-Westphalia, Germany). The vial was sealed hermetically and the sample stored at -20 °C until further analysis.

Chromatographic analyses were carried out on a HPLC system equipped with an AS-4000 (Merck Hitachi, Darmstadt, Germany) automatic injector, two Knauer (Berlin, Germany) HPLC pumps 64, a Knauer HPLC programme 50 solvent controller and, a Waters (Eschborn, Hesse, Germany) 996 Photodiode Array Detector (PDA) and were analysed by means of Millennium TM 3.2 software (Millipore, Milford, MA, USA). The separation of the polyphenols was performed on a Waters Novapac C18 (3.9 x 300 mm^2; endcapped) column at 26 °C. The binary mobile phase (Tab. 3.15) consisted of 2% acetic acid in water (A) and a mixture of acetonitrile, water and concentrated acetic acid (400:90:10 v/v/v) (B).

Twenty microliters of sample was injected onto the column. The separation of polyphenols was monitored using a PDA detector at 280nm and anthocyanins were recorded at 520 nm.

The identity of each peak was confirmed by comparing the retention time and coelution with authentic standards for protocatechiuc acid, catechinhydrate, epicatechin, quercetin, cyanidin-3-galactoside and cyanindin-3-arabinoside.

Table 3.15 RP-HPLC gradients used for the separation of cocoa seed polyphenoles and anthocyanins present in cocoa beans

Time (min)	Flow rate (mL min^{-1})	A (%)	B (%)
0	1.2	90	10
8	1.2	90	10
38	1.1	77	23
50	1	60	40
70	1	10	90
73	1	10	90
78	1.2	90	10
93	1.2	90	10

3.12 Extraction of the seed coat slime

3.12.1 Preparation of the seed coat slime from different times of fermentation

Five-hundred gram samples of cocoa bean fermentations were obtained at different times during box fermentation in PTPN XII, Jember, East Java, Indonesia specifically after 0 h (D0), 24 h (D1), 48 h (D2), 72 h (D3) and 96 h (D4) of fermentation.

To remove the pulp from the beans, each sample was soaked in pectinase enzyme diluted in an equal amount of water to the cocoa seed sample. Subsequently, the mixture was incubated in a rotary shaker GFL 3015 (Burgwedel, Hanover, Germany) overnight at 200 rpm at room temperature. Afterwards, the solution containing the pulp was removed from the seeds. The seeds without pulp were soaked in water of equal volume overnight at room temperature. Finally, the seed coat slime was harvested from the seeds using a stainless steel flour sieve and kept at -20 °C until use.

3.12.2 Preparation of the seed coat slime from different cocoa varieties

Five-hundred gram samples of cocoa bean fermentations were obtained from different varieties of cocoa Forastero (F) and Trinitario (T) provided by the Indonesia Coffee and Cocoa Research Institute (ICCRI), Jember, East Java, Indonesia.

To remove the pulp from the beans, each sample was soaked in pectinase enzyme diluted in an equal amount of water to the cocoa seed sample. Subsequently, the mixture was incubated in a rotary shaker GFL 3015 (Burgwedel, Hanover, Germany) overnight at 200 rpm at room temperature. Afterwards, the solution containing the pulp was removed from the seeds. The seeds without pulp were soaked in water of equal volume overnight at room temperature. Finally, the seed coat slime was harvested from the seeds using a stainless steel flour sieve and kept at -20 °C until use.

3.13 Antimicrobial activity assays

3.13.1 Microorganisms for assays

Twenty-five of fungal strains (Tab. 3.16), 13 strains of Gram positive bacteria (Tab. 3.17), and 7 strains of Gram negative bacteria were used for antimicrobial assay against seed coat slime extracts. The fungal, gram positive and Gram negative strains were isolated from cocoa bean fermentation and from the laboratory culture collections of Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany).

Table 3.16 List of fungal strains for the antifungal inhibition test

Fungus	Fungus
*Aspergillus niger***	*Candida pulcherrima**
*Aspergillus flavus***	*Candida guilliermondii**
*Aspergillus parasiticus***	*Cryptococcus laurentii**
*Mortierella isabellina***	*Kloeckera apis***
Mucor sp. **	*Phaeococcomyces nigrans***
*Penicillium citrinum***	*Rhodotorula glutinis**
*Penicillium purpurogenum***	*Rhodotorula mucilaginosa**
*Penicilium roquefortii***	*Rhodotorula rubra***
*Rhizopus oligosporus***	*Saccharomyces cerevisiae**
*Rhizopus oryzae***	*Schizosaccharomyces pombe***
*Candida krusei***	*Torulopsis magnolia***
*Candida lipolytica**	*Torulaspora delbrueckii**
*Candida pelliculosa**	

Add: growth medium (malt broth, Merck, Darmstadt, Germany), bioassay agar medium (malt agar, Merck, Darmstadt, Germany)
*: strains from cocoa bean fermentation
**: strain from laboratory culture collections of Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany).

Table 3.17 List of Gram-positive bacteria strains for the antimicrobial test

Gram positive bacteria	Growth medium	Bioassay medium
Bacillus subtilis ATCC 6633	Nutrient broth	Nutrient agar
Micrococcus luteus DSM 1790	Nutrient broth	Nutrient agar
Staphylococcus aureus LMH 5P	Nutrient broth	Nutrient agar
Streptococcus thermophilus LMH 5P	Nutrient broth	Nutrient agar
Lactobacillus plantarum LMH 13P	MRS broth	MRS agar
Lactobacillus plantarum DSM 20205	MRS broth	MRS agar
Lactobacillus acidophilus LMH 16P	MRS broth	MRS agar
Lactobacillus casei ssp. *casei* LMH 17P	MRS broth	MRS agar
Lactobacillus fermentum DSM 20052	MRS broth	MRS agar
Lactobacillus reuteri DSM 20016	MRS broth	MRS agar
Lactobacillus delbrueckii ssp. *lactis* DSM 20355	MRS broth	MRS agar
Propionibacterium freudenreichii DSM 20271	MRS broth	MRS agar
Propionibacterium shermanii DSM 20270	MRS broth	MRS agar

Add: All gram positive bacteria were from laboratory culture collections of Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany).

Table 3.18 List of Gram-negative bacteria strains for the antimicrobial test

Gram-negative bacteria	Growth medium	Bioassay medium
*Eschericia coli***	Nutrient broth	Nutrient agar
*Acetobacter pasteurianus**	GYP broth	GYP agar
*Acetobacter tropicalis**	GYP broth	GYP agar
*Acetobacter pomorum**	GYP broth	GYP agar
*Acetobacter peroxydans***	GYP broth	GYP agar
*Gluconobacter frateurii***	GYP broth	GYP agar
*Acetobacter orientalis**	GYP broth	GYP agar

*: strains from cocoa bean fermentation
**: strain from laboratory culture collections of Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany).

3.13.2 Preparation of the seed coat slime solution for assays

One gram of surface-sterilizied seed coat slime (see Section 3.12.1) was shaken in 10 mL sterile distilled water overnight and the liquid was decanted and filtered through Whatman No. 4 paper (Macherey-Nagel, Düren, Germany). Afterwards, the seed coat slime solution was filtered with microfilter 0.20 µm (Macherey-Nagel, Düren, Germany) to remove microbes which may have contaminated the sample. Thus, the concentration of the final extract was 100 mg mL^{-1}. To obtain a lower concentration the sample was further diluted with sterile distilled water.

3.13.3 Antimicrobial assay against molds

The antifungal assay was carried out using agar diffusion methods as described by Ye et al. (2000) with slight modifications. All mold cultures were grown on malt slant agar (Merck, Darmstadt, Germany) for 48-72 h at 25 °C. After growth of mycelia, 5 mL sterile water supplemented with 0.1% Tween 20 (Merck, Darmstadt, Germany) were added. Then, the conidia were carefully rubbed with a sterile cotton swab and transferred with a pipette to a sterile tube. Afterwards, the suspension was adjusted with sterile distilled water to produce a mold spore suspension of 1–5x10^6 CFU mL^{-1} by counting the conidia in a Haemocytometer chamber (Glaswarenfabrik Karl Hecht, Rhön, Germany). The suspension was loaded onto a sterile cotton swab (Heinz Herenz Medizinalbedarf, Hamburg, Germany) that was rotated several times and pressed firmly against the inside wall of the tube to remove excess inoculum from the swab. The dried surface of a malt agar plate was inoculated by streaking the swab over the entire sterile agar surface (Ye et al. 2000).

This procedure was repeated two more times, rotating the plate approximately 60° each time to ensure a uniform distribution of the inoculum. Subsequently, sterile blank paper discs 6.5mm (Mast Group, Merseyside, UK) were impregnated with 50 µL seed coat slime solution (100 mg mL^{-1}). The discs were allowed to be permeated by the seed coat slime solution at room temperature for 2-3 min and were then placed onto the surface of the agar. The plates were incubated at 25 °C for 48–72 h. The zone diameter was read to the nearest whole millimeter at the point at which a sharp reduction in growth occurs. The control was sterile distilled water.

3.13.4 Antimicrobial assay against yeasts

The antifungal assay was carried out using agar diffusion methods as described by Ye et al. (2000) with slight modifications. Agar diffusion was carried out with malt agar (Merck, Darmstadt, Germany). The inoculum was prepared using 24–48 hour plate cultures. The colonies were suspended in 0.85% saline (Merck, Darmstadt, Germany) and the turbidity was compared with the 0.5 McFarland standard (bioMérieux®, SA, Marcy-l'Étoile, France) to produce a yeast suspension of $1\text{-}5\text{x}10^6$ CFU mL^{-1}. The concentration of the cell suspension for the assay was $1\text{-}5\text{x}10^5$ CFU mL^{-1}. The suspension was loaded onto a sterile cotton swab (Heinz Herenz Medizinalbedarf, Hamburg, Germany) that was rotated several times and pressed firmly against the inside wall of the tube to remove excess inoculum from the swab. The dried surface of a malt agar plate was inoculated by streaking the swab over the entire sterile agar surface (Ye et al. 2000).

This procedure was repeated two more times, rotating the plate approximately 60° each time to ensure a uniform distribution of the inoculum. Afterwards, blank paper discs 6.5mm (Mast Group, Merseyside, UK) were prepared, and the seed coat slime solution was added at a concentration of 50 mg mL^{-1}, with a final volume of 50 µL. The plates were incubated at 25 °C for 24–48 h. The zone diameter was read to the nearest whole millimeter at the point at which a sharp reduction in growth occurs. The control was sterile distilled water.

3.13.5 Antimicrobial activity assay against Gram-positive and Gram-negative bacteria

The antimicrobial assay was carried out using agar diffusion methods as described by Pongtharabfkul and Demirci (2004) with slight modifications. All strains (Tab. 3.17, 3.18) were taken from the laboratory culture collections of the Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany). All stock cultures were maintained at −80 °C in 20% glycerol. The working cultures were maintained on agar slants of the same media at 4 °C.

The bioassay agar plates prepared for Gram-positive and Gram-negative bacteria used the optimal media for each culture (Tab. 3.17, 3.18). In each medium, 0.75% agar-agar (Merck) and 1% Tween 20 (J.T. Baker, Phillipsburg, NJ, USA) were added before boiling and sterilizing.

After autoclaving, the agar medium was cooled down to 40 °C and inoculated with 1% of each 24 h culture. To ensure that the same numbers of cells of each culture were inoculated into the agar medium each time, the inoculum size was adjusted to reflect an optical cell density reading of 1.7 at 600 nm. The corresponding diluted media used for each culture were used as blanks for optical density measurements. The final population of each culture was approximately 10^8 CFU mL^{-1} agar medium. The bioassay agar (25 mL) was aseptically poured into sterile petri dishes (100×15 mm) and allowed to solidify for 3 h. Four holes were bored on each plates using a 7-mm outer diameter stainless steel borer with slight suction applied.

After placing 100 µL of the 100 mg mL^{-1} seed coat slime solution into the holes, the agar plates were stored at 4 °C for 24 h to allow for the pre-diffusion of the seed coat slime solutions and were then incubated at 30 °C for another 24 h. The diameter of the inhibition zone around each well was measured horizontally and vertically using a digital caliper (lsr tools, Hückeswagen, Germany) to the nearest 0.01 mm and averaged. Each culture was replicated and evaluated three times (Pongtharangkul and Demirci 2004).

3.14 Minimum inhibitory concentration against molds and yeasts

The antifungal activity of seed coat slime was investigating using the agar diffusion and broth microdilution methods.

3.14.1 Agar diffusion methods

The seed coat slime (see Section 3.13.2), inoculum (see Section 3.13.3, 3.13.4) and medium assay were prepared as described before (see Section 3.13.3, 3.13.4).

For the agar diffusion method, sterile blank paper discs 6.5mm (Mast Group, Merseyside, UK) were impregnated with 50 µL seed coat slime solution with different concentrations (100, 75, 50, and 25 mg mL^{-1} and 40, 30, 20 and 10 mg mL^{-1}) for molds and yeasts, respectively. The discs were allowed to be permeated by the seed coat slime solution at room temperature for 2-3 min. Subsequently, discs with different concentrations of the seed

coat slime solution were placed onto the surface of the agar inoculated with the test molds and yeasts. The plates were incubated at 25 °C for 48–72 h. The zone diameter was read to the nearest whole millimeter at the point at which a sharp reduction in growth occurs. The control was sterile distilled water.

3.14.2 Broth microdilution methods

3.14.2.1 Broth microdilution for molds

The broth microdilution was performed as described by Scorzoni et al. (2009) with modification. The seed coat slime solution was prepared as described before (see Section 3.13.2). The inoculum was prepared as described above (see Section 3.13.3), the concentration of cells used in this study was $1\text{-}5\text{x}10^5$ CFU mL^{-1}. The medium used in this study was malt broth (Merck, Darmstadt, Germany).

A total volume of 100 µL malt broth (Merck, Darmstadt, Germany) was placed into the microplate reader Nunc™ MicroWell™ 96-Well Microplates (Fermentas, St. Leon-Rot, Baden-Württemberg, Germany). One-hundred microliters of different concentrations of seed coat extract (100, 75, 50, and 25 mg mL^{-1}) were added to the medium in the microplate reader. Afterwards, the microplate was incubated at 35 °C with medium agitation using the Absorbance Microplate Reader ELx808^{TM} (Biotek®Instruments, Montpelier, VT, USA). The optical density of the growing fungi was read every 12 h until 48 h at an absorbance 530 nm using the Absorbance Microplate Reader ELx808^{TM}, (Biotek®Instruments, Montpelier, VT, USA). Data were analyzed using Gen5 2.0 (Biotek®Instruments, Montpelier, VT, USA) (Riesselman et al. 2000; Scorzoni et al. 2009).

One hundred microliter of 2 mg mL^{-1} propionic acid were added to 100 µL malt broth (Merck, Darmstadt, Germany) as a positive control, while 100 µL sterile distilled water were added to 100 µL malt broth (Merck, Darmstadt, Germany) as a negative control.

3.14.2.2 Broth microdilution for yeasts

The broth microdilution was performed as described by Scorzoni et al. (2009) with modifications. The seed coat slime solution was prepared as described above (see Section

3.13.2). The inoculum was prepared as described above (see Section 3.13.4), concentration of cell used in this study was 1-5x10^5 CFU mL^{-1}. The medium used in this study was malt broth (Merck, Darmstadt, Germany).

A total volume of 100 μL malt broth (Merck, Darmstadt, Germany) was placed into the microplate reader Nunc™ MicroWell™ 96-Well Microplates (Fermentas, St. Leon-Rot, Baden-Württemberg, Germany). One-hundred microliters of different concentrations of seed coat extract (40, 30, 20, and 10 mg mL^{-1}) were added to the medium in the microplate reader. Afterwards, the microplate was incubated at 35 °C with medium agitation using the Absorbance Microplate Reader ELx808^{TM} (Biotek®Instruments, Montpelier, VT, USA). The optical density of the growing fungi was read every 12 h until 48 h at an absorbance 530 nm using the Absorbance Microplate Reader ELx808^{TM} (Biotek®Instruments, Montpelier, VT, USA). Data were analyzed using Gen5 2.0 (Biotek®Instruments, Montpelier, VT, USA) (Riesselman et al. 2000; Scorzoni et al. 2009).

One hundred microliter of 2 mg mL^{-1} propionic acid were added to 100 μL malt broth (Merck, Darmstadt, Germany) as a positive control, while 100 μL sterile distilled water were added to 100 μL malt broth (Merck, Darmstadt, Germany) as a negative control.

3.15 Biochemical analyses of seed coat slime proteins

3.15.1 Quantitative determination of protein content

The measurement of the concentrations of protein solutions was carried out using the Bradford protein assay. This method is based on the shift in the absorbance of the Coomassie Brilliant Blue G-250 dye (absorbs at 595 nm), when the previously red form of the Coomassie reagent (absorbs at 465 nm) changes and stabilizes into Coomassie blue (Bradford 1976).

One-hundred microliters of seed coat slime protein were added to 1 mL Bradford solution (see below), mixed well and incubated for 15 min at RT in the dark. The extinction was measured at a wavelength of 595 nm with a SmartSpec™ Plus Spectrometer (BIO RAD, Hercules, CA, USA). H$_2$O$_{bidest}$ was used as a control. The extinctions were compared with a

standard curve made with concentrations of BSA between 10 and 150 mg mL^{-1} as a reference protein and the corresponding concentrations were calculated.

Coomassie Brilliant Blue G-250	100 mg
Ethanol (95%, v/v)	50 mL
H_3PO_4 (85%, w/v)	100 mL
H_2O_{bidest} ad	1000 mL

3.15.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

All chemical reagents for SDS-PAGE were purchased from Bio-Rad (Bio-Rad, Munich, Germany).

The protein solutions were analyzed by SDS-PAGE. SDS is an anionic amphipathic substance that binds to polypeptides. It denatures and confers an almost evenly distributed charge. The denatured proteins can migrate in the electromagnetic field through the matrix. The electrophoretic mobility is a function of the length of a polypeptide chain and its charge. Smaller polypeptides move faster through the matrix than bigger ones. Mini-Protean equipment (BioRad, Munich, Germany) was used for preparing the gels and carrying out the electrophoresis.

3.15.2.1 Preparation of denaturing SDS-polyacrylamide gels

Discontinuous gels with a 7% stacking gel and a 12 or 15% separating gel were prepared. After cleaning both with 70% EtOH, a spacer plate was put together with a short plate and arrested in a casting frame. The glass plate sandwich was clamped in a casting stand and loaded with approximately 5 mL separating gel (Tab. 3.19). The gel was overlaid with a thin wall of water in order to obtain a smooth gel surface and to ensure nearly anoxic polymerization. After ca. 30 min, the water was removed with a tissue and the stacking gel (Tab. 3.19) was applied on the separating gel. A comb with 10 molds was set in the stacking gel before its polymerization. After ca. 20 min, the comb was removed and the gel was ready for electrophoresis. After the glass plates with the SDS-gel were loaded into a Mini-Protean (BioRad, Munich, Germany) inner chamber they were put horizontally into an electrophoresis tank filled with running buffer (Laemmli 1970).

3.15.2.2 Composition of the SDS-PAGE gel

Preparation of the SDS-PAGE stock solution

Acrylamide stock solution			10% SDS solution
Acrylamide		29.2 g	10 g SDS in 100 H_2O_{bidest}
N'N'-bis-methylene-acrylamide		0.8 g	
H_2O_{bidest}	ad	100 mL	

Resolving gel stock solution			10% APS solution
Tris base		18.15 g	0.1 g APS in 1 mL
H_2O_{bidest}	ad	100 mL	
pH 8.8 (with 6 N HCl)			

Stacking gel stock solution			N,N,N',N'-Tetramethylene ethylene diamine
Tris base		6.0 g	(TEMED) supplied by Bio-Rad Laboratories
H_2O_{bidest}	ad	100 mL	(Munich, Germany)
pH 6.8 (with 6 N HCl)			

10x electrophoresis running buffer			2x Laemmli Sample Buffer	#161-0737
Tris base		30.3 g	Tris-HCl, pH 6.8	65.8 mM
Glycine		144.0 g	Glycerol	26.3% (w/v)
SDS		10.0 g	SDS	2.1%
H_2O_{bidest}	ad	1 L	Bromophenol blue	0.01%
pH 8.3 (with Glycine)			2-mercaptoethanol	355 mM

Table 3.19 Composition of the SDS-PAGE gels used in this study

Component	Resolving gel (12%)	Stacking gel (7%)
H_2O	1650 µL	1700 µL
Acrylamide stock solution	2000 µL	415 µL
Resolving gel stock solution	1250 µL	-
Stacking gel stock solution	-	315 µL
10% SDS	75 µL	25 µL
10% APS	75 µL	25 µL
TEMED	3 µL	2.5 µL

3.15.2.3 Preparation of protein samples and SDS-PAGE gel electrophoresis

Aliquots of 15 µL of obtained protein extracts were mixed with 15 µL 2x Laemmmli sample buffer (#161-0737, Bio-Rad, Munich, Germany), mixed well and incubated at 95 °C for 5 min. After centrifugation at 13.000 x g for 2 min in Eppendorf 5417R (Eppendorf, Hamburg, Germany), samples were applied to the wells of the SDS-gel. For detection of molecular masses, protein bands were compared with the bands of a protein molecular weight marker (Precision Plus Protein All Blue Standards, #161-0373, Bio-Rad, Munich, Germany) which was also applied to the SDS-gel (Laemmli 1970).

The chamber was filled with 1x electrophoresis buffer. Aliquots of 20 µL of each sample were then loaded carefully into the gel pockets and the electrophoresis was carried out at 20 mA for the stacking gel and was increased to 40 mA for the resolving gel.

3.15.2.4 Coomassie staining of SDS-PAGE gels

Following electrophoresis, the gel was carefully removed from the glass plates and stained overnight with Coomassie Brilliant Blue R250 under gentle shaking using the Polymax 1040 (Heidolph Instruments, Schwabach, Germany). The gel was destained for about 3 h with destaining solution.

Staining solution (L):

Coomassie Brilliant Blue R250   1 g

Methanol   400 mL

Acetic acid   100 mL

H$_2$O$_{bidest}$ 500 mL

Destaining solution (L)

Methanol   200 mL

Glacial acetic acid   100 mL

H$_2$O$_{bidest}$ 700 mL

3.15.3 Precipitation of seed coat slime proteins

After extraction, 40 mL seed coat slime were precipitated with 80% acetone in a 250 mL centrifuge acetone resistant bottle and mixed well. Subsequently, the mixture was precipitated for 48 hours at -20 °C. Afterwards, the mixture was centrifuged in a Sorvall RC 5C Plus (Kendro laboratory, Newtown, Connecticut, USA) at 25.673 x g for 20 min at 4 °C. Finally, the pellet was dried at room temperature for about 3-4 hours.

3.15.4 Extraction of seed coat slime proteins

Extractions of seed coat slime protein were obtained by using the phenol based procedure as described by Noah et al. (2012) with slight modifications. One-hundred miligrams precipitated seed coat slime protein were vortexed using Vortex Genie®2 (MO BIO Laboratories, Carlsbad, CA, USA) together with glass beads (innuSPEED Lysis Tube J, Analytik Jena, Jena, Germany) for 10 min at full speed. Afterwards, the mixture was dissolved in 0.75 mL extraction buffer and 0.75 mL phenol buffered at 7.5 with Tris-EDTA. The mixture was then incubated for 1 h at 0 °C. After incubation, the mixture was centrifugated in a Eppendorf 5417R (Eppendorf, Hamburg, Germany) at 13.000 x g for 20 min at 4 °C. Finally, the upper surface containing the seed coat slime proteins was collected (Noah et al. 2012).

Precipitations of the seed coat slime protein were performed using an ammonium acetate based procedure described by Colditz et al. (2004). After extraction, the proteins were precipitated with five times their volume in 100 mM ammonium acetate solubilized in methanol at -20 °C overnight, and then centrifuged in a Eppendorf 5417R (Eppendorf, Hamburg, Germany) at 13.000 x g for 20 min at 4 °C. The pellet was washed three times with cold 100 mM ammonium acetate in methanol, centrifuged in an Eppendorf 5417R centrifuge

(Eppendorf, Hamburg, Germany) at 4.000 x g for 4 min at 4 °C and once with cold 80% acetone in a Eppendorf 5417R (Eppendorf, Hamburg, Germany) at 4.000 x g for 4 min at 4 °C. Finally, the protein mixture was dried at room temperature for 1 h and stored at -20 °C until use. Before being used as a SDS-PAGE sample, the protein solution was resuspended in 50 µL SDS-PAGE sample buffer and dissolved by incubation at 37 °C for 1 h (Colditz et al. 2004).

Buffer extraction

Sucrose	700 mM
Tris	500 mM
EDTA	50 mM
KCl	100 mM
β-mercaptoethanol	2% (v/v)
PMSF	2 mM
pH adjusted to 8.0	

3.15.5 Separation of seed coat slime proteins with SDS-PAGE electrophoresis

Protein samples from different fermentation times (0 h (D0), 24 h (D1), 48 h (D2), 72 h (D3) 96 h (D4)) and from different varieties (Forastero (F), and Trinitario (T)) were prepared and electrophoresed using the methods described above (see Section 3.15.2 – 3.15.4).

3.15.6 Identification of seed coat proteins with mass spectrometry

Preparation of solutions

AmBiCa stock solution	Trypsin solution
1.186 g ammonium bicarbonate	dissolve 20 µg Trypsin in 50 µL reconstitution
add 15 mL distilled water (d H_2O)	buffer (kit)

Digest buffer

750 µL AmBiCa stock solution

add 1.5 mL ACN

add 15 mL d H_2O (12.75 mL)

Shrinking solution

750 µL AmBiCa stock solution

add 9 mL ACN

add 15 mL dH_2O (5.25 mL)

Swelling solution

1.5 mL AmBiCa stock solution

add 15 mL distilled water (13.5 mL)

Digest solution

0.4 µL Trypsin solutions add 20 µL digest buffer (19.6 µL) for one sample!

5 % formic acid solution

0.5 mL formic acid (FA) (85 %)

add 10 mL d H_2O (9.5 mL)

Squeeze solution

7.5 mL ACN

add 0.5 mL formic acid (85 %)

30 % ACN solution

3 mL ACN add 10 mL d H_2O (7 mL)

0.2 % formic acid solution

20 µL formic acid (85 %)

add 10 mL (9.98 mL) dH_2O

60 % ACN solution

6 mL ACN add 10 mL d H_2O (4 mL)

Bands of interest were excised, the proteins reduced with 200 µL shrinking solution (1 mM NH_4HCO_3, 9 mL acetonitrile, 5.25 mL dH_2O), samples were incubated at room temperature for 30 min, and 200 µL swelling solution (0.1 mM NH_4HCO_3), incubation at room temperature for 20 min, and the protein digested in-gel with trypsin [20 µg trypsin were dissolved in 50 µL resuspension buffer (50 mM acetic acid), 0.4 µL of the trypsin solution (modified trypsin, Promega, Madison, Wisconsin, USA) add 20 µL digest buffer (50 mM NH_4HCO_3), 37 °C, overnight]. After digestion, the reaction was stopped by adding 1 µL of 5% formic acid solution. The supernatant was transferred into a new cup. The remaining gel pieces were repeatedly extracted (50 % acetonitrile and 5 % formic acid). The combined extracts were then dried down in a vacuum concentrator, redissolved in 30 % acetonitrile and 0.2 % formic acid and desalted on a C18 microZipTip (Millipore, Billerica, Massachusetts, USA), conditioned with 60% acetonitrile and equilibratined with 0.2 % formic acid.

Desorption of the peptides was carried out with 60% acetonitrile. Samples were analysed by nano-electrospray mass spectrometry in a QTOF II instrument (Micromass, Manchester, UK). The MS/MS spectra of the peptides obtained by collision-induced fragmentation were evaluated both manually and by the Mascot MS/MS ion search algorithm (Matrix Sciences, London, UK) (Makrypidi et al. 2012).

3.15.7 Temperature stability of the seed coat slime extract

To study the temperature stability of the seed coat slime proteins, 40 mL of seed coat slime protein mixture were incubated for 24 h at different temperatures (30, 40 and 50 °C). After incubation, seed coat slime proteins were extracted using the methods described above. Then the protein was used as a sample for SDS-PAGE analysis as described above (see Section 3.15.2 – 3.15.4).

4 Results

4.1 Dynamics and biodiversity of the populations of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) involved in the spontaneous box fermentation of cocoa beans in Nusantara Plantation XII (PTPN XII), East Java, Indonesia

4.1.1 Changes in temperature and pH during cocoa bean fermentation

The initial temperature of cocoa bean box fermentation was 28 °C, which gradually increased after 24 h of fermentation. This correlated with the increased numbers of AAB after mixing of the fermenting cocoa beans at 24, 48 and 72 h. The temperature increased from 28 to 34 °C and reached a maximum value of 48 °C at the end of fermentation.
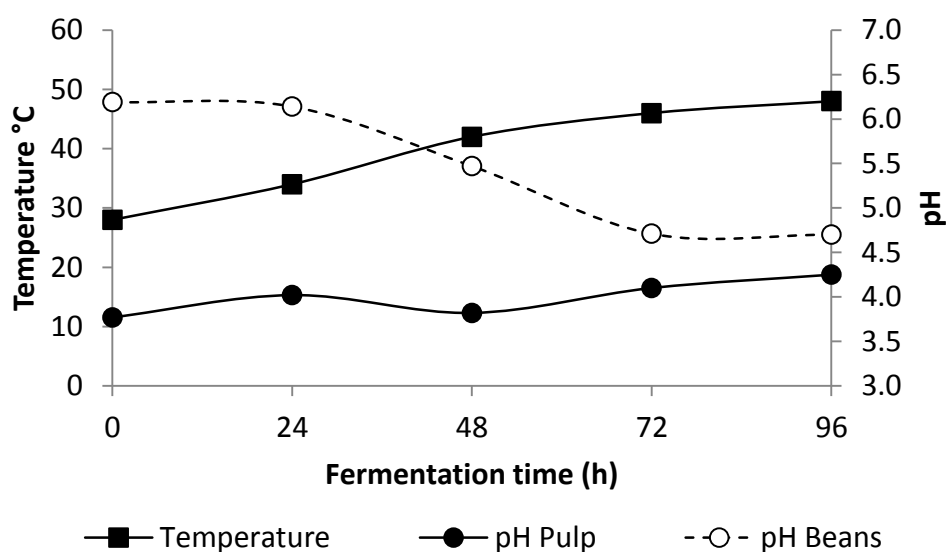
Figure 4.1 Changes in temperature and pH during cocoa bean fermentation

The initial pH of the pulp was 3.8, before increasing after 24 h of fermentation to 4.0, probably due to the degradation of citric acid by yeasts during the first 24 h of fermentation. Afterwards, the pH fell again to 3.8 as a consequence of the production of lactic and acetic acid. At the end of the fermentation, the pH increased to a value of 4.3. In contrast to the

pulp pH, the pH of the beans decreased during fermentation from an initial 6.2 to 4.7 by the end of fermentation (Fig. 4.1).

4.1.2   Microbiological analysis of cocoa bean fermentation

A yeast population count of 10^5 CFU g^{-1} was present in the bean mass at the beginning of fermentation. The size of the yeast population increased during the first 24 h and grew to a maximum population of 10^8 CFU g^{-1}. Upon prolonged fermentation, the yeast population declined to 10^6 CFU g^{-1} (Fig. 4.2).
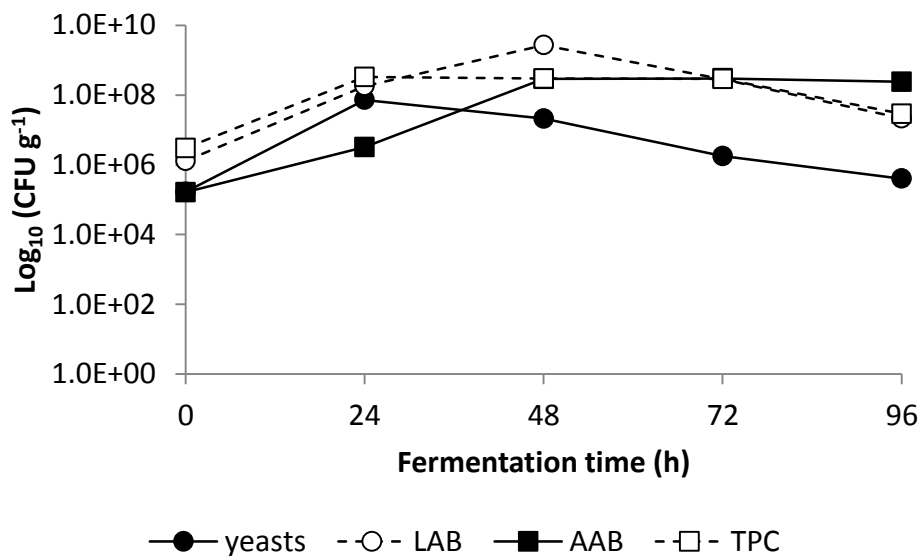
Figure 4.2 Microbiological analysis of cocoa bean fermentation

LAB population count increased during the first 48 h from an initial population of 10^6 CFU g^{-1} to a maximum population 10^9 CFU g^{-1}. Afterwards, the LAB colonies decreased to 10^8 CFU g^{-1} and slightly decreased to 10^7 CFU g^{-1} at the end of fermentation (Fig. 4.2). AAB population count increased slowly from an initial population of 10^5 CFU g^{-1} within the first 24 h of fermentation. Then, beginning with the turning around of the cocoa beans every 20-24 h, growth occurred rapidly, with a maximum population of 10^8 CFU g^{-1} after 72 h. Growth remained at this level up to the end of fermentation (Fig. 4.2). Total bacterial population

count increased from an initial population of 10^6 CFU g^{-1} to a maximum population of 10^9 CFU g^{-1} after 24 h of fermentation. Growth remained at this level up to 72 h of fermentation and decreased after that to 10^7 CFU g^{-1} until the end of fermentation (Fig. 4.2).

4.1.3 Yeast identification

Eighty yeasts isolated were identified based on DI/D2 large subunit (LSU) sequencing, Including *Saccharomyces cerevisiae* (63 isolates; 78.75% of the total number of isolates, isolation during 0 – 96 h fermentation), *Hanseniaspora guilliermondii* (3.75%; isolation from 24 and 48 h of fermentation), *Torulaspora delbrueckii* (3.75%; isolation from 24 and 48 h of fermentation), *Pichia kudriavzevii* (2.5%; isolation from 24 h of fermentation), *Hanseniaspora opuntiae* (1.25%; isolation from 24 h of fermentation), *Candida tropicalis* (1.25%; isolation from 24 h of fermentation), *Hanseniaspora thailandica* (1.25%; isolation from 24 h of fermentation), *Issatchenkia orientalis* (1.25%; isolation from 24 h of fermentation) and *Sporobolomyces roseus* (2.5%; isolation from 96 h of fermentation) (Tab. 4.1).

Table 4.1 Yeasts species diversity

Yeast species	Fermentation time, (h)				
	0	**24**	**48**	**72**	**96**
Saccharomyces cerevisiae	2 (2*)	23 (16*)	20 (14*)	3 (3*)	15 (10*)
Hanseniaspora guilliermondii	1	1	1		
Torulaspora delbrueckii		2	1		
Pichia kudriavzevii		2			
Hanseniaspora opuntiae	1	1			
Candida tropicalis	1	1			
Hanseniaspora thailandica	1	1			
Issatchenkia orientalis		1			
Sporobolomyces roseus					2

*) Number of *S. cerevisiae* identified using 26S rRNA gene sequencing. Other strains of *S. cerevisiae* were identified by API ID 32C. All strains of other yeasts species were identified by 26S rRNA gene sequencing.
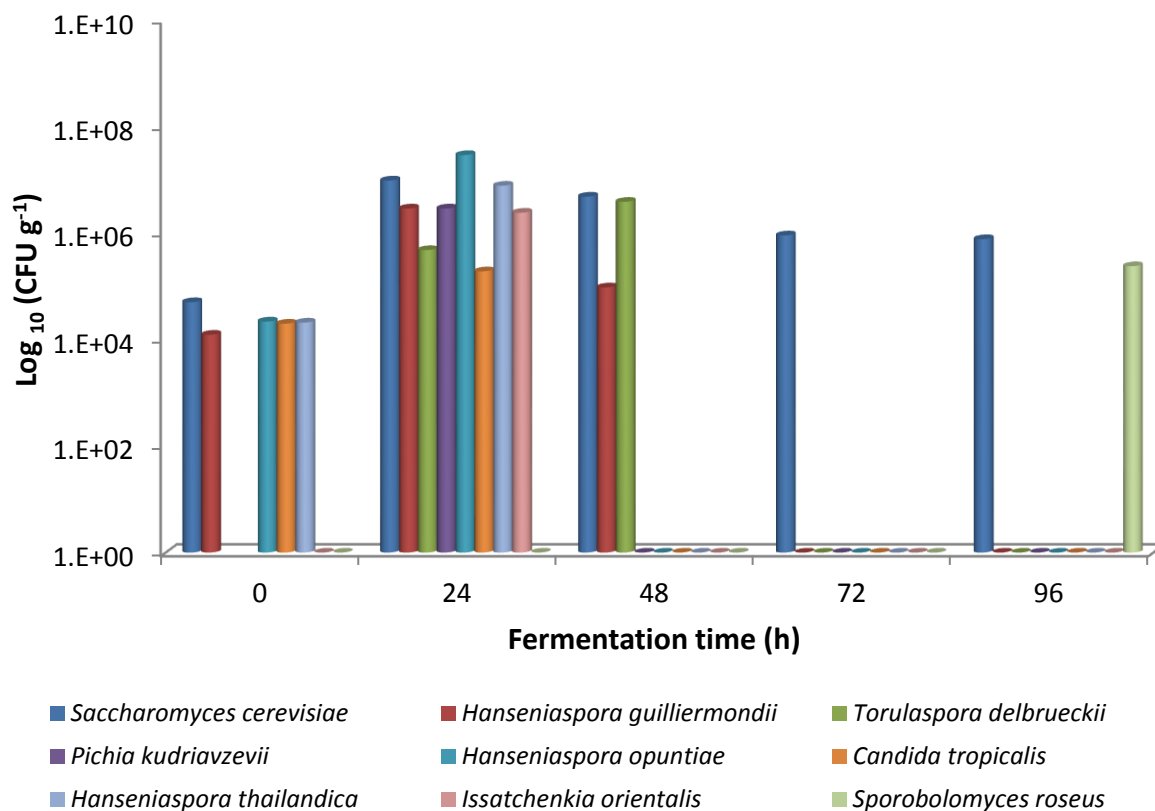
Figure 4.3 Yeast population dynamics during fermentation

Yeasts were dominant during the first 24 h of cocoa bean mass fermentation. Ten different yeasts species were detected. *H. thailandica* (10^7 CFU g^{-1}), *S. cerevisiae* (10^7 CFU g^{-1}), *H. opuntiae* (10^7 CFU g^{-1}), *H. guilliermondii* (10^7 CFU g^{-1}), *C. tropicalis* (10^5 CFU g^{-1}) were the dominant yeast species during the first 24 h of fermentation. Other yeast species such as *P. kudriavzevii* (10^7 CFU g^{-1}) and *I. orientalis* (10^7 CFU g^{-1}) also contributed to the beginning of fermentation, though in smaller numbers. After 24 h, other yeast species such as *T. delbrueckii* (10^7 CFU g^{-1}) and *S. roseus* (10^6 CFU g^{-1}) were found (Fig. 4.3).

4.1.4 Lactic acid bacteria identification

Table 4.2 Lactic acid bacteria species diversity

LAB species	Fermentation time, (h)				
	0	**24**	**48**	**72**	**96**
Lactobacillus plantarum	1 (1*)	34 (9*)	41 (12*)	6 (6*)	22 (10*)
Lactobacillus fermentum	2 (1*)	11 (2*)	5 (3*)	3 (2*)	6 (2*)
Lactobacillus brevis	0	2 (1*)	0	2 (2*)	2 (2*)

*) Number of LAB strains identified using 16S rRNA gene sequencing. The other strains were identified by using API kit 50 CHL.
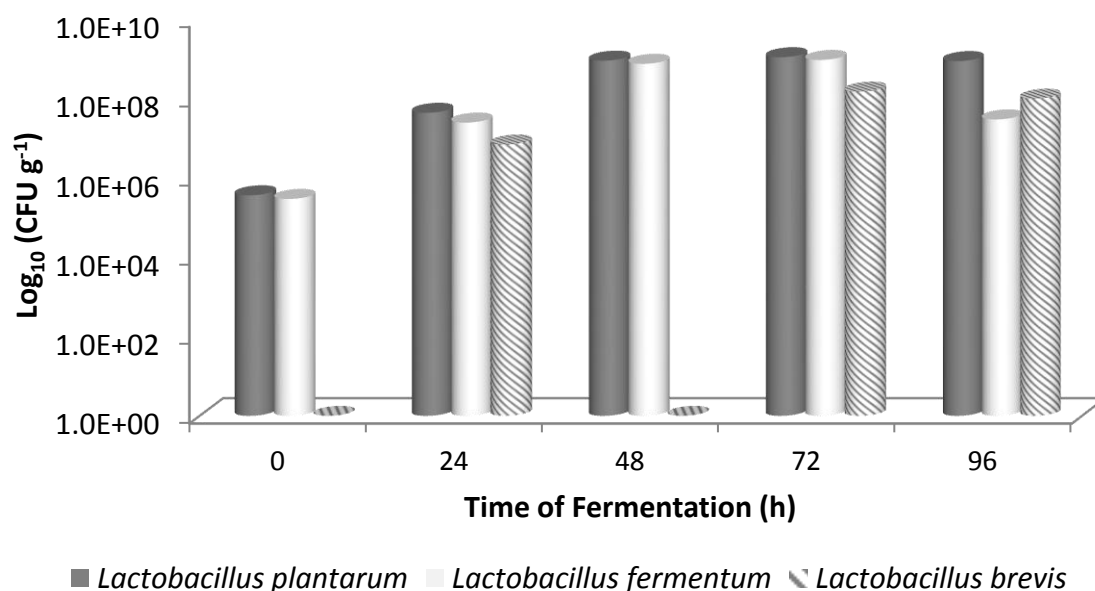

Figure 4.4 Lactic acid baceria population dynamics during fermentation

From a total of 219 isolates from MRS, 137 isolates could be recovered following transport and storage from Indonesia to Hamburg, Germany. All 137 isolates were phenotypically characterized as Gram-positive, catalase-negative. According to API KIT 50 CHL classification and identification and in combination with 16S rRNA gene sequencing, the representative isolates showed two dominant LAB species, namely *L. plantarum* (104 isolates, 74.82%, 38 representative isolates were identified by 16S rRNA gene sequencing) and *L. fermentum* (27 isolates, 19.42%, 10 representative isolates were identified by 16S rRNA gene

sequencing). Other species included *L. brevis* (6 isolates, 4.31%, 5 representative isolates were identified by 16S rRNA gene sequencing).

Both *L. plantarum* and *L. fermentum* could be isolated from the beginning of fermentation until the end of fermentation. The initial number of *L. plantarum* 10^6 CFU g^{-1} increased during fermentation to a maximum of 10^9 CFU g^{-1} at 72 h of fermentation. *L. fermentum* grew form 10^5 CFU g^{-1} initially to a maximum of 10^9 CFU g^{-1} at 72 h of fermentation. *L. brevis* started to grow at 24 h of fermentation with initial number of 10^7 CFU g^{-1}. At 48 h *L. brevis* could not be found. It reached a maximum of of 10^8 CFU g^{-1} at 72 h of fermentation (Fig. 4.4).

4.1.5 Acetic acid bacteria identification

From a total of 145 isolates from GYP and *Acetobacter* medium, 112 isolates could be recovered following transport and storage from Indonesia to Hamburg, Germany. All 112 isolates were phenotypically characterized as Gram-negative, oxidase-negative and catalase-positive. According to API kit 50 CHL classification and identification and combined with 16S rRNA gene sequencing, the representative isolates showed that two AAB species were dominant, namely *Acetobacter pasterianus* (80 isolates, 71.42%, 22 representative isolates were identified by 16S rRNA gene sequencing) and *Acetobacter pomorum* (25 isolates, 22.32%, 20 representative isolates were identified by 16S rRNA gene sequencing). Other species were including *Acetobacter ghanensis* (5 isolates, 4.46%, 4 representative isolates were identified by 16S rRNA gene sequencing), and *Acetobacter tropicalis* (2 isolates, 1.78%, 2 isolates were identified by 16S rRNA gene sequencing) (Tab. 4.3).

Table 4.3 Acetic acid bacteria species diversity

AAB species	Fermentation time (h)				
	0	24	48	72	96
Acetobacter pasteurianus	11 (2*)	8 (3*)	16 (4*)	9 (1*)	36 (12*)
Acetobacter pomorum		3 (2*)	4 (2*)	5 (5*)	12 (11*)
Acetobacter tropicalis					2 (2*)
Acetobacter ghanensis			2 (1*)		3 (3*)

*) Number of AAB strains identified using 16S rRNA gene sequencing. The other strains were identified by using API kit 50 CHL.
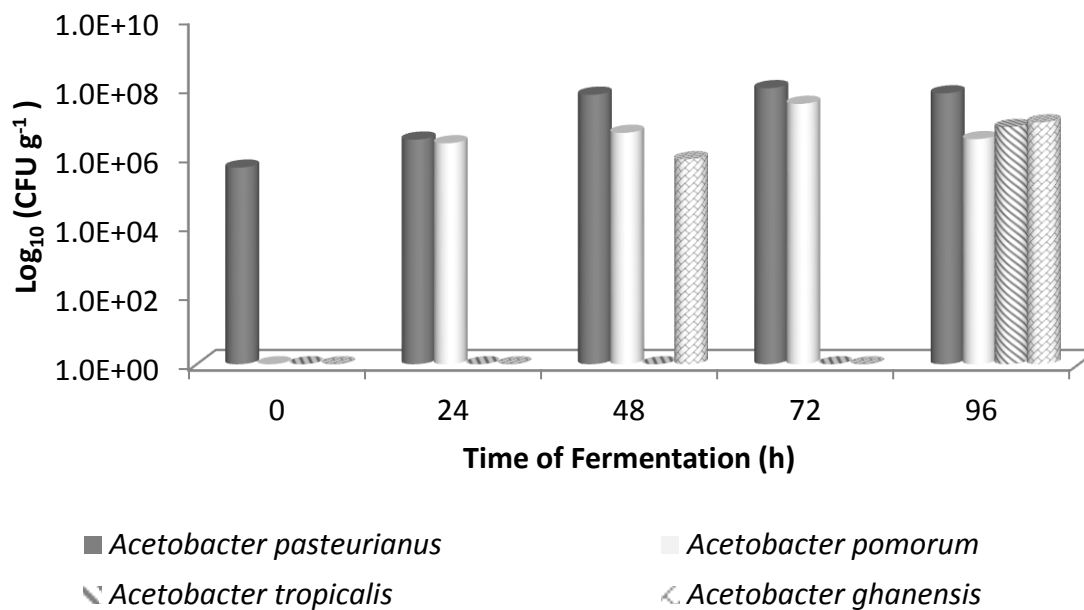
Figure 4.5 AAB population dynamics during fermentation

The most dominant isolates at the beginning of fermentation contained *A. pasterianus* in the amount of 10^6 CFU g^{-1}, which increased during fermentation to a maximum of 10^8 CFU g^{-1} at 72 h of fermentation, followed by *A. pomorum* in the amount of 10^6 CFU g^{-1}, which also increased during fermentation to a maximum of 10^8 CFU g^{-1} at 72 h of fermentation. *A. ghanensis* was found at 48 h and 96 h of fermentation in amounts of 10^6 CFU g^{-1} and 10^7 CFU g^{-1}, respectively. *A. tropicalis* was found at the end of fermentation in the amount of 10^7 CFU g^{-1} (Fig. 4.5).

4.1.6 Carbohydrates (sucrose, glucose, fructose, and mannitol)

Glucose (39.9 mg g^{-1}) and fructose (43.6 mg g^{-1}) were the main sugars found in fresh cocoa pulp, sucrose was not detected in the pulp. From the start of fermentation up to 24 h, glucose (from 39.9 to 5.34 mg g^{-1}), fructose (from 43.6 to 7.8 43.6 mg g^{-1}) were metabolized in box fermentation, coinciding with the increased production of mannitol, ethanol, glycerol, succinic acid, lactic acid and acetic acid (Fig. 4.6, 4.8).

Mannitol was detected in cocoa pulp at 24 h of fermentation with an initial concentration of 5.93 mg g^{-1}. The mannitol concentration reached a maximum of 8.79 mg g^{-1} at 48 h of fermentation. Afterwards, the concentration of mannitol decreased up to the end of fermentation to a final concentration of 1.23 mg g^{-1} (Fig. 4.6).
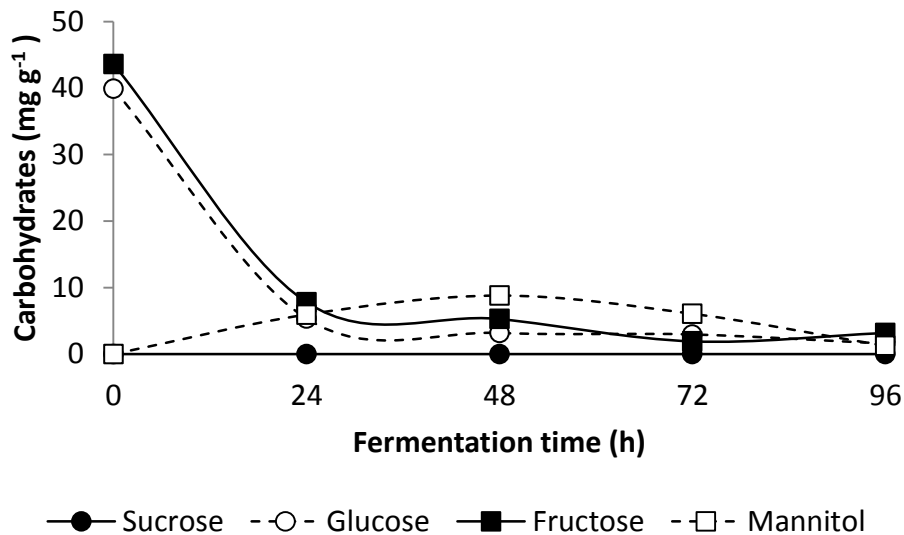
Figure 4.6 Changes in carbohydrates during fermentation in the pulp
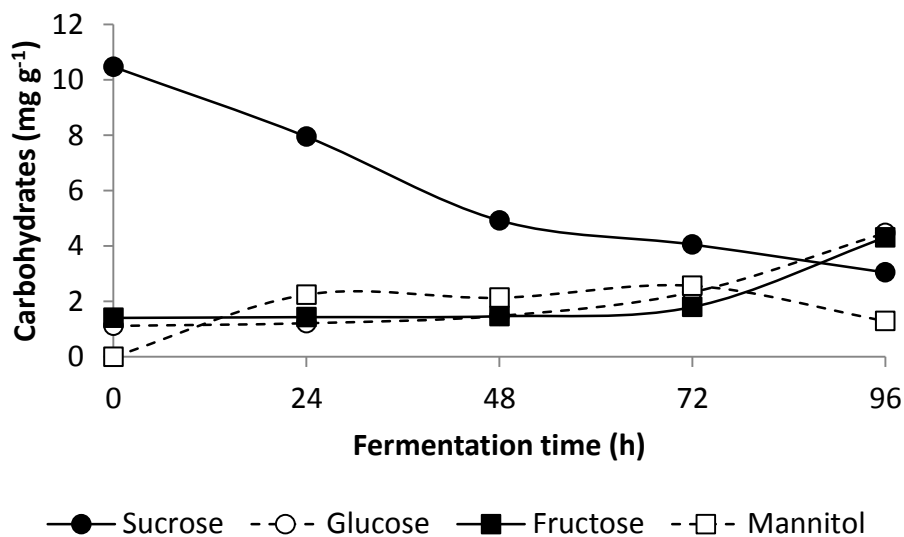
Figure 4.7 Changes in carbohydrates during fermentation in the beans

In contrast to the pulp, sucrose (10.46 mg g^{-1}) was found to be the main sugar of the cocoa beans, and only low concentrations of glucose (1.1 mg g^{-1}) and fructose (1.4 mg g^{-1}) were found in the fresh cocoa beans. Sucrose was slowly metabolized during fermentation and decreased to a concentration of 3.04 mg g^{-1}at the end of fermentation. In contrast to the pulp, the concentration of glucose and fructose increased during fermentation until the end of fermentation. The concentration of mannitol was variable during the course of fermentation (Fig. 4.7).

4.1.7 Organic acids (citric acid, succinic acid, lactic acid, acetic acid)

Citric acid (16.56 mg g^{-1}) was the main organic acid in fresh cocoa pulp; succinic acid, lactic acid, acetic acid, ethanol, and glycerol were not detected in fresh cocoa pulp. From the start of fermentation up to 24 h, citric acid (from 16.56 to 1.56 mg g^{-1}) were metabolized in box fermentation. Succinic acid, lactic acid and acetic acid with initial concentrations of 0.07, 0.9 and 1.9 mg g^{-1}, respectively, where detected in the cocoa pulp after 24 h of fermentation. The concentrations of succinic acid, lactic acid and acetic acid, 3.47, 5.69, and 11.53 mg g^{-1}, respectively, reached maximum concentrations at 72 h of fermentation and then decreased up to the end of fermentation (Fig. 4.8).
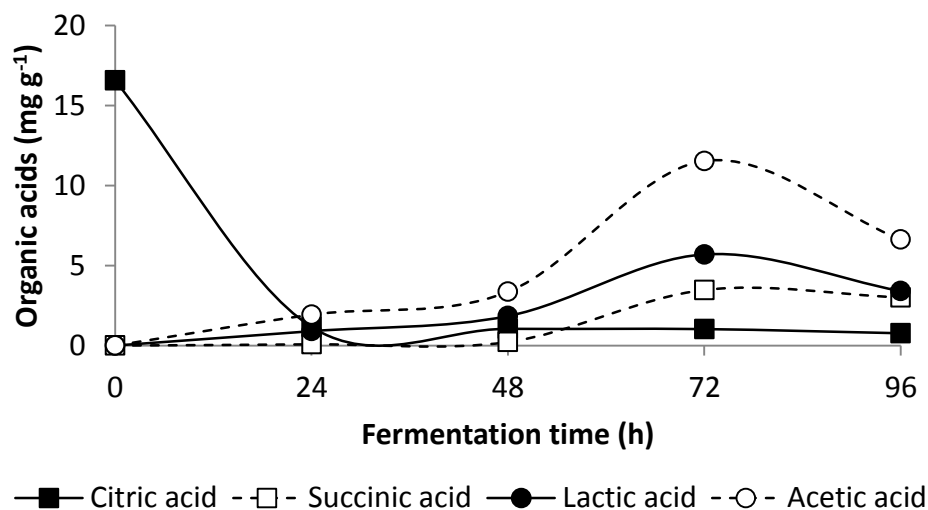
Figure 4.8 Changes in organic acids during fermentation in the pulp
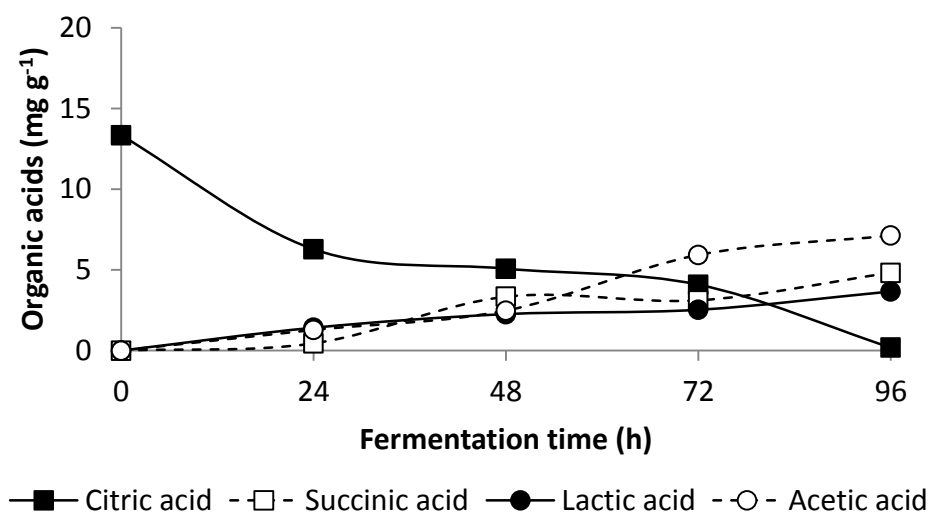
Figure 4.9 Changes in organic acids during fermentation in the beans

Citric acid in cocoa beans was slowly metabolized and decreased until the end of fermentation, paralleled by increasing concentrations of succinic acid, lactic acid and acetic acid with maximum concentrations of 4.8, 3.65 and 7.1 mg g^{-1} at the end of fermentation (Fig. 4.9).

4.1.8 Ethanol and glycerol concentrations

The concentration of ethanol in the pulp reached a maximum concentration of 17.5 mg g^{-1} after 48 h of fermentation. At the end of fermentation, the ethanol concentration had declined to 9.7 mg g^{-1}. In contrast to the pulp, the ethanol concentration in the beans increased continually until the end of fermentation, with a maximum concentration of 11.37 mg g^{-1} found at the end of fermentation (Fig. 4.10).
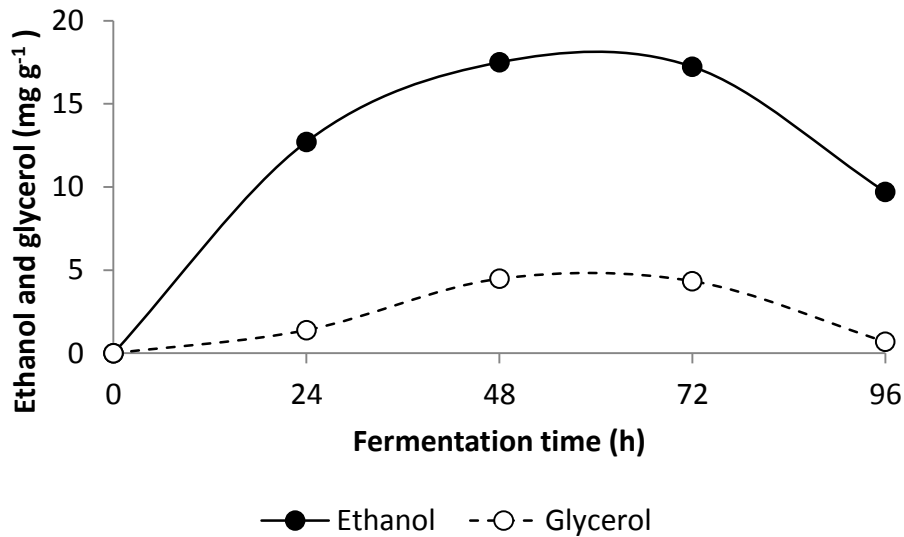
Figure 4.10 Changes in ethanol and glycerol during fermentation in the pulp
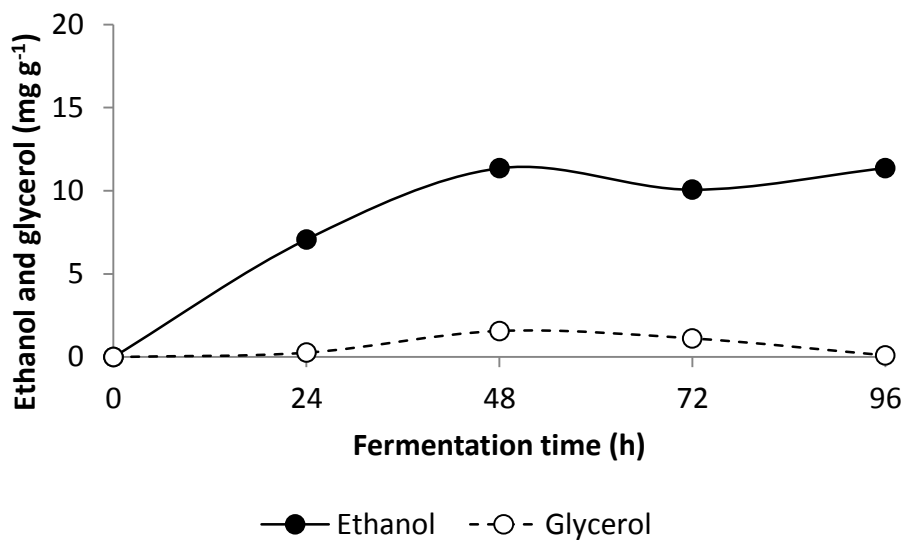
Figure 4.11 Changes in ethanol and glycerol during fermentation in the beans

The concentration of glycerol increased almost simultaneously in the pulp and beans during the course of fermentation. The maximum concentration of glycerol was 4.5 and 1.57 mg g^{-1} in the pulp and beans, respectively. At the end of fermentation, the concentration of glycerol decreased to minimum concentrations of 0.7 and 0.1 mg g^{-1} in the pulp and beans, respectively (Fig. 4.10, 4.11).

4.2 Diversity and characterization of yeasts, lactic acid bacteria and acetic acid bacteria in spontaneous cocoa bean fermentations carried out in Jember, Indonesia; Mocache, Ecuador; Santo Domingo, Trinidad & Tobago

4.2.1 Identification and diversity of yeasts

The diversity of yeasts found during cocoa bean fermentation varies depending on regions and methods of fermentation. Fermented cocoa beans from Ecuador had the highest diversity of yeasts compared with Indonesia and Trinidad & Tobago. For example, species of *Bulleromyces albus*, *Candida parapsilosis*, *Cryptococcus laurentii, Metschnikowia pulcherrima, Rhodotorula glutinis* and *Wickerhamomyces anomalus* were found only in the fermentations from Ecuador. On the other hand species of *Hanseniaspora thailandica, Issatchenkia orientalis, Pichia kudriavzevii* and *Sporobolomyces roseus* were only isolated from cocoa bean fermentations from Indonesia*.* The species of *Candida quercitrusa* was only isolated out of cocoa bean fermentations from Trinidad & Tobago (Tab 4.4).

Start of fermentation by yeasts was found in cocoa bean box fermentation in Indonesia, which has been proved by the isolation of several yeast species such as *Candida tropicalis, Hanseniaspora guilliermondii, Hanseniaspora opuntiae, Hanseniaspora thailandica,* and *Saccharomyces cerevisiae.* Most of the yeasts species in cocoa bean box fermentation from Indonesia could only be isolated between 24 and 48 h of fermentation, while *S. cerevisiae* could be isolated until the end of fermentation.

The fermentation of cocoa beans from Ecuador also started with yeasts. For example, *B. albus*, *C. parapsilosis*, *C. tropicalis*, *C. laurentii*, *M. pulcherrima, Rhodotorula mucilaginosa, Torulaspora delbrueckii* and *W. anomalus* could be isolated at the beginning of fermentation, though some species were no longer found after 24 h of fermentation. A few species of yeasts such as *H. guilliermondii*, *H. opuntiae*, *T. delbrueckii* and *W. anomalus* could be isolated up to 24h after the start of fermentation, while only *S. cerevisiae* could be isolated until the end of fermentation. In contrast to the Indonesian samples, species of

T. delbrueckii, *W. anomalus*, and *H. guilliermondii* were found to be more dominant during early phases of cocoa bean fermentation in the sample from Ecuador (Tab 4.4).

Table 4.4 Diversity of yeasts species in cocoa bean fermentations from Indonesia, Ecuador and Trinidad & Tobago

Yeast species	Fermentation time (h)														
	Indonesia					Ecuador					Trinidad & Tobago				
	0	24	48	72	96	0	12	24	96	120	0	12	24	48	72
Bulleromyces albus						4									
Candida parapsilosis						3									
Candida quercitrusa											1				
Candida sp. VTT C-04530*											1				
Candida tropicalis	1	1				1									
Cryptococcus laurentii						2									
Hanseniaspora guilliermondii	1	1	1				5	9			6				
Hanseniaspora opuntiae	1	1						2							
Hanseniaspora thailandica	1	1													
Hanseniaspora uvarum							1				1				
Issatchenkia orientalis		1													
Metschnikowia pulcherrima						4									
Pichia kudriavzevii		2													
Rhodotorula glutinis							2								
Rhodotorula mucilaginosa						10							1		
Saccharomyces cerevisiae	2	16	14	3	10					16			3		
Sporobolomyces roseus					2										
Torulaspora delbrueckii		2	1			1	4	4							
Wickerhamomyces anomalus						11	8	7							

*) VTT "Valtion Teknillinen Tutkimuskeskus" (State Technical Research Center), VTT Technical Research Centre of Finland

The composition of yeasts from Indonesia and Ecuador proved to be similar. The fermentation of cocoa bean from Trinidad & Tobago was initiated by yeasts, with *H. guilliermondii* and *S. cerevisiae* consistently present at the beginning of fermentation. Only *S. cerevisiae* was found in all regions at consistently stable level until the end of fermentation (Indonesia, Ecuador and Trinidad & Tobago) (Tab 4.4).

4.2.2 Identification and diversity of lactic acid bacteria

Fermented cocoa beans from Ecuador showed a greater diversity of LAB compared to Indonesia and Trinidad & Tobago. For example, species of *Leuconostoc pseudomesenteroides* were only isolated in cocoa bean fermentation samples from Ecuador. On the other hand, species of *Lactobacillus brevis* were only isolated out of cocoa bean fermentation samples from Indonesia (Tab 4.5).

Table 4.5 Diversity of lactic acid bacteria species in cocoa bean fermentations from Indonesia, Ecuador and Trinidad & Tobago

LAB species	Fermentation time (h)														
	Indonesia					Ecuador					Trinidad & Tobago				
	0	24	48	72	96	0	12	24	96	120	0	12	24	48	72
Lactobacillus brevis		2		2	2										
Lactobacillus fermentum	2	2	3	2	2	2	2	2	14	12			1		1
Lactobacillus plantarum	1	9	12	6	10	1		12	3				5		
Leuconostoc pseudomesenteroides								4							
Pediococcus acidilactici									6	4	1		1		2
Pediococcus pentosaceus								2	16	1					

At the onset of lactic acid fermentation, particularly *L. plantarum* and *L. fermentum* were isolated from cocoa bean box fermentations from Indonesia*. L. plantarum* and *L. fermentum* could be consistently isolated during the course of fermentation, while *L. brevis* could only temporarily be isolated during fermentation (Tab. 4.5).

L. plantarum and *L. fermentum* were isolated at the onset of fermentation in cocoa bean fermentations from Ecuador. *L. pseudomesenteroides* was also isolated during the early phases of fermentations, but could no longer be detected after 24 h of fermentation. *L. fermentum* was found consistently during the course of fermentation. *P. acidilactici* and *P. pentosaceus* prevailed during the late phases of the Ecuadorian cocoa bean fermentation (Tab. 4.5).

L. fermentum, L. plantarum and *P. acidilactici* were isolated out of cocoa bean fermentation samples from Trinidad and Tobago, with *L. fermentum* being found consistently during the fermentation.

L. fermentum and *L. plantarum* were the only species of LAB found consistently during fermentations from all regions (Indonesia, Ecuador and Trinidad & Tobago) (Tab. 4.5).

4.2.3 Identification and diversity of acetic acid bacteria

Fermented cocoa beans from Indonesia showed the highest diversity of AAB compared to Ecuador and Trinidad & Tobago. For example, species of *Acetobacter ghanensis, Acetobacter pomorum,* and *Acetobacter tropicalis* were only isolated in cocoa bean fermentations from Indonesia (Tab. 4.6).

Table 4.6 Diversity of acetic acid bacteria species in cocoa bean fermentations from Indonesia, Ecuador and Trinidad & Tobago

AAB species	Fermentation time (h)														
	Indonesia					Ecuador					Trinidad & Tobago				
	0	24	48	72	96	0	12	24	96	120	0	12	24	48	72
Acetobacter ghanensis			1		3										
Acetobacter pasteurianus	2	3	4	1	12				6	6	2		6	3	2
Acetobacter pomorum		2	2	5	11										
Acetobacter tropicalis					2										

A. pasteurianus was the only AAB species isolated from cocoa bean fermentation samples from Ecuador and Trinidad & Tobago. *A. pasteurianus* and *A. pomorum* were found consistently in cocoa bean box fermentations from Indonesia. Only *A. pasteurianus* was observed during the course of fermentation in samples from all regions (Indonesia, Ecuador and Trinidad & Tobago (Tab. 4.6).

4.2.4 Yeasts characterization

To investigate the physiological adaptation of the yeast species to the cocoa bean fermentation enviroment, various physiological characteristics were determined for selected yeasts isolates representing all detected species.

All yeast isolates tested grew at 25 and 35 °C, with the exception of the four isolates of *T. delbrueckii* which only showed weak growth at 35 °C. Two isolates of *P. kudriavzevii*

showed growth at 45 °C. Three isolates of *R. mucilaginosa* and two isolates of *S. cerevisiae* grew weakly at 45 °C (Tab. 4.7).

Table 4.7 Diversity and physiological characterization of yeasts isolates adapted to cocoa bean fermentation

ID	Yeast strains	Temperature, °C			pH			Ethanol, %		
		25	35	45	2.5	3.5	5.0	5	10	15
IDI-Y31	*Candida tropicalis*	+	+	-	+	+	+	+	+	-
IDE-Y62	*Candida tropicalis*	+	+	-	+	+	+	+	+	-
IDI-Y35	*Hanseniaspora guilliermondii*	+	+	-	+	+	+	+	+	-
IDI-Y36	*Hanseniaspora guilliermondii*	+	+	-	+	+	+	+	+	-
IDE-Y19	*Hanseniaspora guilliermondii*	+	+	-	+	+	+	+	+	-
IDT-Y07	*Hanseniaspora guilliermondii*	+	+	-	+	+	+	+	+	-
IDT-Y16	*Hanseniaspora guilliermondii*	+	+	-	+	+	+	+	+	-
IDI-Y30	*Hanseniaspora opuntiae*	+	+	-	+	+	+	+	-	-
IDE-Y25	*Hanseniaspora opuntiae*	+	+	-	+	+	+	+	-	-
IDE-Y27	*Hanseniaspora opuntiae*	+	+	-	+	+	+	+	-	-
IDI-Y34	*Hanseniaspora thailandica*	+	+	-	-	+	+	+	-	-
IDE-Y56	*Hanseniaspora uvarum*	+	+	-	-	w	+	+	w	-
IDT-Y05	*Hanseniaspora uvarum*	+	+	-	-	w	+	+	w	-
IDE-Y40	*Metschnikowia pulcherrima*	+	+	-	-	+	+	+	w	-
IDE-Y48	*Metschnikowia pulcherrima*	+	+	-	-	+	+	+	w	-
IDI-Y51	*Pichia kudriavzevii*	+	+	+	+	+	+	+	+	+
IDI-Y52	*Pichia kudriavzevii*	+	+	+	+	+	+	+	+	+
IDE-Y42	*Rhodotorula mucilaginosa*	+	+	w	-	w	+	+	w	-
IDE-Y87	*Rhodotorula mucilaginosa*	+	+	w	-	w	+	+	w	-
IDT-Y02	*Rhodotorula mucilaginosa*	+	+	w	-	w	+	+	w	-
IDI-Y01	*Saccharomyces cerevisiae*	+	+	w	+	+	+	+	+	+
IDI-Y02	*Saccharomyces cerevisiae*	+	+	-	+	+	+	+	+	+
IDI-Y03	*Saccharomyces cerevisiae*	+	+	w	+	+	+	+	+	+
IDE-Y31	*Saccharomyces cerevisiae*	+	+	-	+	+	+	+	+	+

Table 4.7 Continuation

ID	Yeast strains	Temperature, °C			pH			Ethanol, %		
		25	35	45	2.5	3.5	5.0	5	10	15
IDT-Y06	*Saccharomyces cerevisiae*	+	+	-	+	+	+	+	+	+
IDI-Y43	*Torulaspora delbrueckii*	+	w	-	-	+	+	+	w	-
IDI-Y57	*Torulaspora delbrueckii*	+	w	-	-	+	+	+	w	-
IDE-Y71	*Torulaspora delbrueckii*	+	w	-	-	+	+	+	w	-
IDE-Y13	*Torulaspora delbrueckii*	+	w	-	-	+	+	+	w	-
IDE-Y01	*Wickerhamomyces anomalus*	+	+	-	-	w	+	+	+	-
IDE-Y15	*Wickerhamomyces anomalus*	+	+	-	-	w	+	+	+	-
IDE-Y21	*Wickerhamomyces anomalus*	+	+	-	-	w	+	+	+	-

+ = growth   - = no growth   w = weak growth

All yeast isolates grew at pH 5.0. Nearly all yeasts grew at pH 3.5, with the exception of the isolates of *H. uvarum, R. mucilaginosa,* and *W. anomalus.* All isolates of *C. tropicalis, H. guilliermondii, H. opuntiae, P. kudriavzevii, S. cerevisiae,* grew at pH 2.5. None of the isolates of *H. thailandica, H. uvarum, M. pulcherrima, R. mucilaginosa, T. delbrueckii* and *W. anomalus* grew at pH 2.5 (Tab. 4.7).

All yeast isolates tested grew at 5% ethanol. All isolates of *C. tropicalis, H. guilliermondii, P. kudriavzevii, S. cerevisiae*, and *W. anomalus* grew at 10% ethanol. All isolates of *H. uvarum, M. pulcherrima, R. mucilaginosa*, and *T. delbrueckii* grew weakly at 10% ethanol. Isolates of *H. opuntiae* and *H. thailandica* failed to grow at 10% ethanol. Only very few yeast isolates grew at 15% ethanol. Only *species* of *P. kudriavzevii* and *S. cerevisiae* were able to grow at 15% ethanol (Tab. 4.7).

4.2.5 Lactic acid bacteria characterization

All LAB isolates tested grew at 25 and 35 °C. All isolates of *L. fermentum, L. plantarum* and *Pediococcus acidilactici* grew at 45 °C. Three isolates of *Pediococcus pentosaceus* showed weak growth at 45 °C, while three isolates of *L. brevis* failed to grow at 45 °C. All LAB isolates tested grew at pH 5.0 and 3.5 but not at pH 2.5. All LAB isolates tested grew at 5% and 10%

ethanol, except for three isolates of *L. brevis* which weak growth at 10%. None of the LAB isolates tested showed growth at 15% ethanol (Tab. 4.8).

Table 4.8 Diversity and physiological characterization of lactic acid bacteria species adapted to cocoa bean fermentation

ID	LAB strains	Temperature, °C			pH			Ethanol, %		
		25	35	45	2.5	3.5	5.0	5	10	15
IDI-L03	*Lactobacillus brevis*	+	+	-	-	+	+	+	w	-
IDI-L06	*Lactobacillus brevis*	+	+	-	-	+	+	+	w	-
IDI-L07	*Lactobacillus brevis*	+	+	-	-	+	+	+	w	-
IDE-L07	*Lactobacillus fermentum*	+	+	+	-	+	+	+	+	-
IDE-L11	*Lactobacillus fermentum*	+	+	+	-	+	+	+	+	-
IDE-L36	*Lactobacillus fermentum*	+	+	+	-	+	+	+	+	-
IDE-L38	*Lactobacillus fermentum*	+	+	+	-	+	+	+	+	-
IDE-L42	*Lactobacillus fermentum*	+	+	+	-	+	+	+	+	-
IDI-L16	*Lactobacillus plantarum*	+	+	+	-	+	+	+	+	-
IDI-L17	*Lactobacillus plantarum*	+	+	+	-	+	+	+	+	-
IDI-L18	*Lactobacillus plantarum*	+	+	+	-	+	+	+	+	-
IDT-L46	*Lactobacillus plantarum*	+	+	+	-	+	+	+	+	-
IDT-L45	*Lactobacillus plantarum*	+	+	+	-	+	+	+	+	-
IDE-L54	*Lactobacillus plantarum*	+	+	+	-	+	+	+	+	-
IDE-L59	*Lactobacillus plantarum*	+	+	+	-	+	+	+	+	-
IDE-L87	*Lactobacillus plantarum*	+	+	+	-	+	+	+	+	-
IDE-L23	*Pediococcus acidilactici*	+	+	+	-	+	+	+	+	-
IDE-L09	*Pediococcus acidilactici*	+	+	+	-	+	+	+	+	-
IDE-L13	*Pediococcus acidilactici*	+	+	+	-	+	+	+	+	-
IDT-L37	*Pediococcus acidilactici*	+	+	+	-	+	+	+	+	-
IDT-L40	*Pediococcus acidilactici*	+	+	+	-	+	+	+	+	-
IDE-L44	*Pediococcus pentosaceus*	+	+	w	-	+	+	+	+	-
IDE-L21	*Pediococcus pentosaceus*	+	+	w	-	+	+	+	+	-
IDE-L15	*Pediococcus pentosaceus*	+	+	w	-	+	+	+	+	-

+ = growth   - = no growth   w = weak growth

4.2.6 Acetic acid bacteria characterization

All AAB isolates tested grew at 25 and 35 °C, while only isolates of *A. pasteurianus* showed weak growth at 45 °C. None of the isolates of *A. ghanensis* and *A. pomorum* grew at 45 °C. All AAB isolates grew at pH 5.0 and 3.5, though none did so at pH 2.5. All AAB isolates tested grew at 5% and 10% ethanol, with the exception two isolates of *A. ghanensis* and three isolates of *A. pomorum* which showed weak growth at 10%. None of the AAB isolates grew at 15% ethanol (Tab. 4.9).

Table 4.9 Diversity and physiological characterization of acetic acid bacteria species adapted to cocoa bean fermentation

ID	AAB strains	Temperature, °C			pH			Ethanol, %		
		25	35	45	2.5	3.5	5,0	5	10	15
IDI-A22	*Acetobacter ghanensis*	+	+	-	-	+	+	+	w	-
IDI-A23	*Acetobacter ghanensis*	+	+	-	-	+	+	+	w	-
IDI-A19	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDI-A03	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDI-A04	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDE-A05	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDE-A12	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDE-A14	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDT-A19	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDT-A18	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDT-A21	*Acetobacter pasteurianus*	+	+	w	-	+	+	+	+	-
IDI-A18	*Acetobacter pomorum*	+	+	-	-	+	+	+	w	-
IDI-A20	*Acetobacter pomorum*	+	+	-	-	+	+	+	w	-
IDI-A02	*Acetobacter tropicalis*	+	+	-	-	+	+	+	w	-

+ = growth   - = no growth   w = weak growth

4.3 Development of a starter culture at laboratory level for improved quality of cocoa bean fermentation products

4.3.1 Changes in pH during cocoa bean fermentation

There was no significant difference between the pH observed in the pulp of cocoa beans fermented with aerobic and anaerobic methods. On the other hand, the pH of beans was different, whereas the pH of beans fermented by anaerobic methods was slightly less acid compared to aerobic methods.
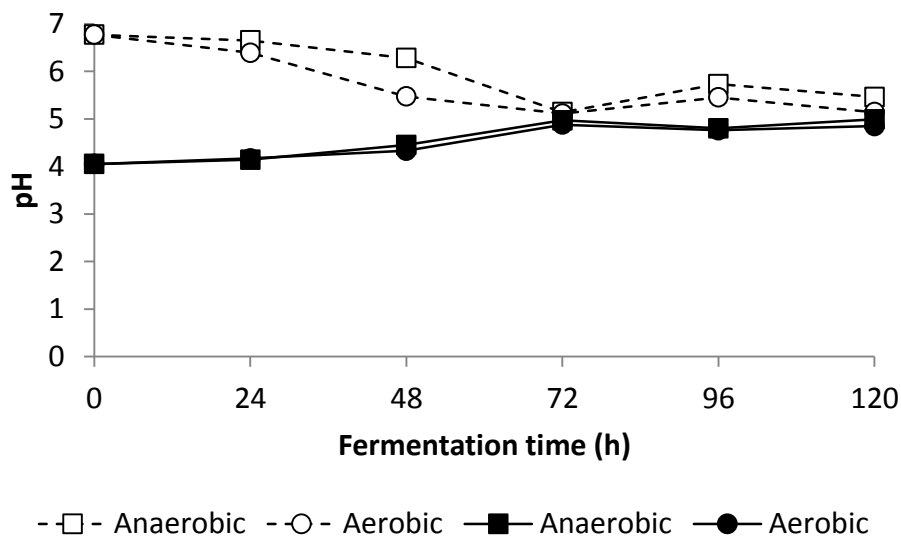
Figure 4.12 Changes in pH during aerobic and anaerobic fermentation, full symbol (pulp), open symbol (beans)

The initial pH of the pulp was 4.05, which then increased to pH 4.88 after 72 h of fermentation, probably due to the degradation of citric acid by *Saccharomyces cerevisiae* and *Lactobacillus fermentum* during the first 24-48 h of fermentation. Afterwards, the pH remained stable until the end of fermentation. In an inverse relationship to the pH of the pulp, the pH of the beans decreased during fermentation from an initial pH of 6.77 to 5.46 by the end of the fermentation. This is reflected in the increased amount of citric, lactic and acetic acid during fermentation. This was partly responsible for the death of the cell cotyledon after 48 h of fermentation.

4.3.2 Growth of the starter culture during fermentation

To investigate the growth of the starter culture during aerobic fermentation, cocoa beans were inoculated with *S. cerevisiae* and *L. fermentum* at the onset of fermentation with an initial cell count of 10^7 and 10^6 CFU g^{-1}, respectively. *A. pasteurianus* was inoculated after 48 h of fermentation with an initial cell count of 10^8 CFU g^{-1}. The cell count of *S. cerevisiae* increased during the first 24 h and grew to a maximum number of 10^9 CFU g^{-1}. Upon prolonged fermentation, the *S. cerevisiae* cell count declined down to 10^4 CFU g^{-1} (Fig. 4.13). The size of the *L. fermentum* cell count increased during fermentation and grew to a maximum population of 10^8 CFU g^{-1} after 72 h of fermentation. Afterwards, the *L. fermentum* cell count decreased to 10^5 CFU g^{-1}, before slightly decreasing until the end of fermentation to 10^4 CFU g^{-1} (Fig. 4.13). *A. pasteurianus* was added to the fermented cocoa beans mass after 48 h of fermentation with an initial number of 10^8 CFU g^{-1}, before increasing rapidly to a maximum cell count of 10^8 CFU g^{-1} after 72 h, followed by a decline until the end of fermentation down to 10^4 CFU g^{-1} (Fig. 4.13).
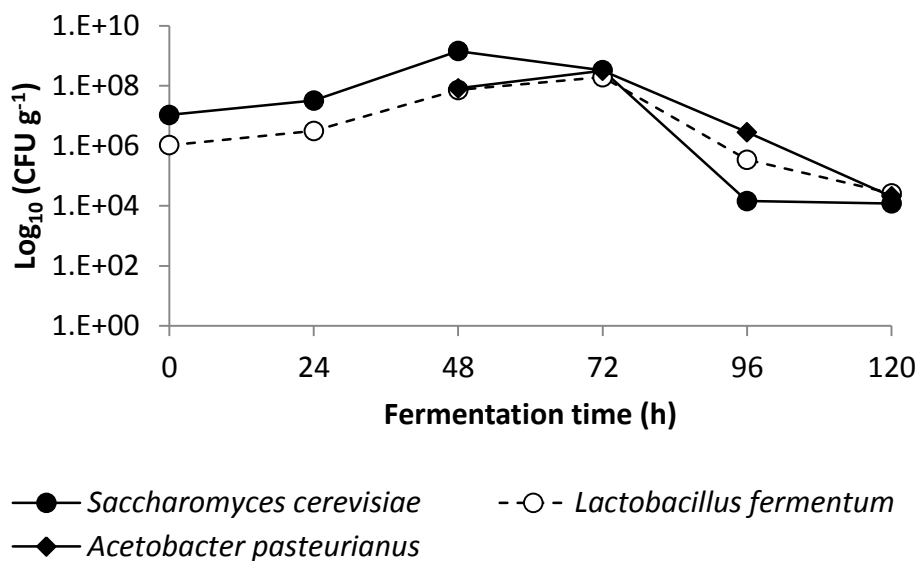
Figure 4.13 Growth of the starter culture during aerobic fermentation

To investigate the growth of the starter culture during anaerobic fermentation, cocoa beans were inoculated with *S. cerevisiae* and *L. fermentum* at the onset of fermentation with an initial population of 10^7 and 10^6 CFU g^{-1}, respectively. *A. pasteurianus* was inoculated after 48 h of fermentation with an initial population of 10^8 CFU g^{-1}. The size of the *S. cerevisiae* population increased during the first 24 h and grew to a maximum population of 10^9 CFU g^{-1}. Upon prolonged fermentation, the *S. cerevisiae* population declined to a undetectable level (Fig. 4.14). The size of the *L. fermentum* population increased during fermentation and grew to a maximum population of 10^8 CFU g^{-1} after 72 h of fermentation. Afterwards, the *L. fermentum* population decreased to 10^5 CFU g^{-1}, before decreasing until the end of fermentation to 10^4 CFU g^{-1} (Fig. 4.14). *A. pasteurianus* was added to the fermenting cocoa beans after 48 h of fermentation with an initial population of 10^7 CFU g^{-1}, before increasing rapidly to a maximum population of 10^8 CFU g^{-1} after 72 h, followed by a decline until the end of fermentations down to undetected level (Fig. 4.14).
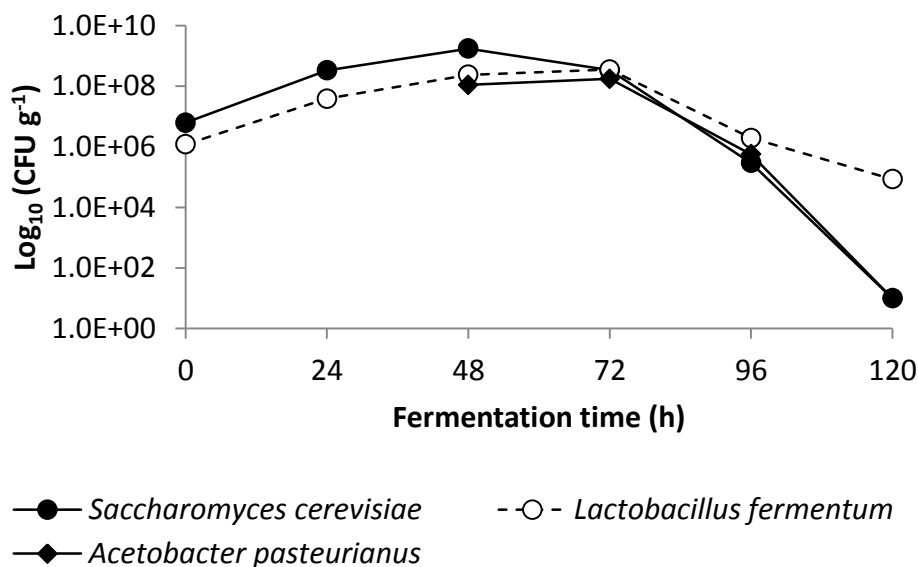
Figure 4.14 Growth of the starter culture during anaerobic fermentation

S. cerevisiae grew until the end of fermentation in the aerobic fermentation, whereas it could not be detected in anaerobic fermentation after 120 h. *A. pasteurianus* grew well during aerobic fermentation and survived until the end of fermentation. In contrast to this in anaerobic fermentation it declined down to undetectable levels at the end of fermentation.

4.3.3 Analysis of metabolites

4.3.3.1 Carbohydrates (glucose and fructose)

Changes in carbohydrates (glucose and fructose) during aerobic fermentation were as follows: glucose (40.1 mg g^{-1}) and fructose (44.5 mg g^{-1}) were the main sugars of the fresh cocoa pulp. Sucrose was not detected in the pulp. From the start until 24 h of fermentation, glucose (from 40.1 to 12.2 mg g^{-1}) and fructose (from 44.5 to 6.65 mg g^{-1}) were metabolized in Erlenmeyer flask fermentations, coinciding with an increase in the production of ethanol, lactic acid and acetic acid. The concentrations of sugars in the beans was relatively stable during fermentation (Fig. 4.15).
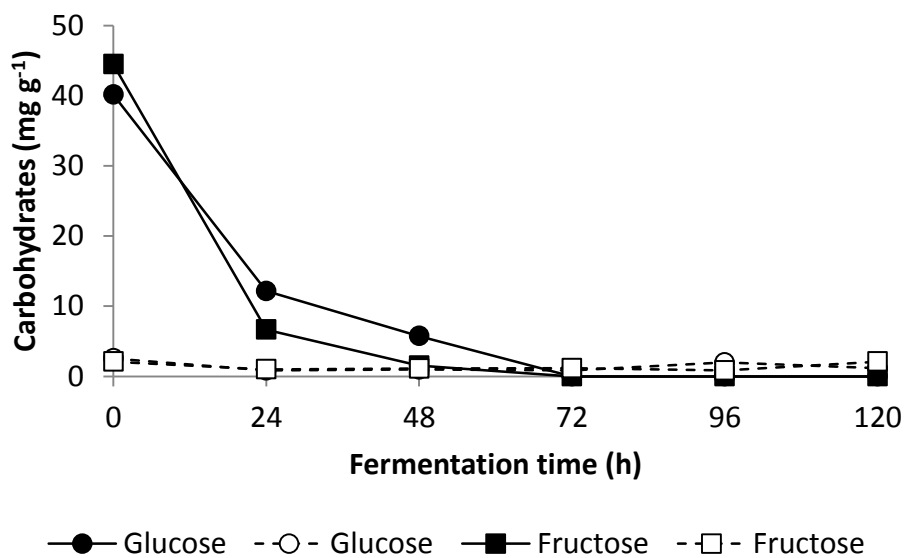
Figure 4.15 Changes in carbohydrates (glucose and fructose) during aerobic fermentation, full symbol (pulp), open symbol (beans)
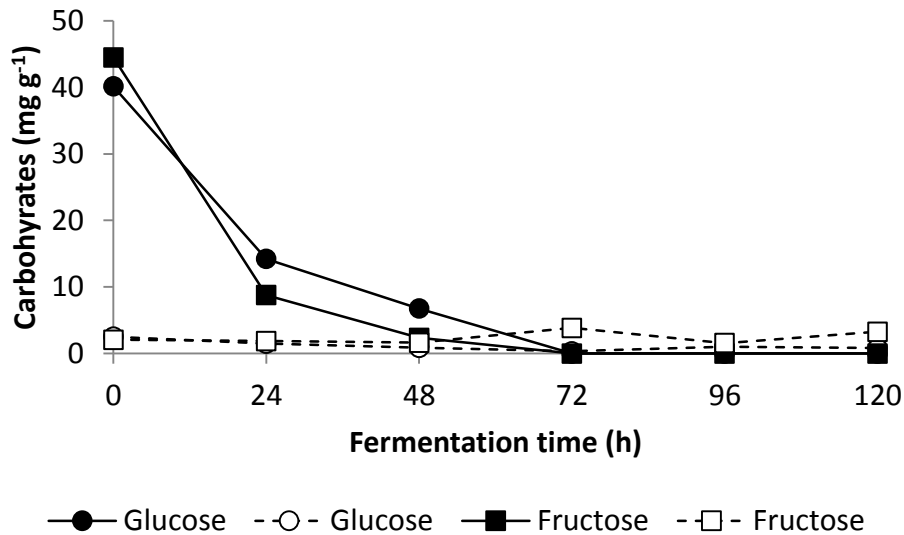
Figure 4.16 Changes in carbohydrates (glucose and fructose) during anaerobic fermentation, full symbol (pulp), open symbol (beans)

Changes in carbohydrates (glucose and fructose) during anaerobic fermentation were as follows: similar to aerobic fermentation, glucose (40.1 mg g^{-1}) and fructose (44.5 mg g^{-1}) were the main sugars found in the fresh cocoa pulp. Sucrose was not detected in the pulp. From the start until 24 h of fermentation, glucose (from 40.1 to 14.2 mg g^{-1}) and fructose (from 44.5 to 8.8 mg g^{-1}) were metabolized during fermentation, coinciding with an increase in production of ethanol, lactic acid and acetic acid. The concentrations of sugars in the beans remained stable during fermentation (Fig. 4.16).

The concentration of sugars in the pulp was always higher than that in the beans, both in the aerobic and anaerobic fermentation. Glucose and fructose in the pulp were consumed more quickly and in greater amounts in the aerobic fermentation.

4.3.3.2   Organic acids (citric acid, lactic acid, acetic acid)

Changes in organic acids (citric acid, lactic acid, acetic acid) during aerobic fermentation were as follows: citric acid was the main organic acid in cocoa pulp. Lactic acid and acetic acid were not detected in cocoa pulp. From the start of fermentation up to 24 h, citric acid (from 6.9 to 1.66 mg g^{-1}) was metabolized during fermentation, coinciding with an increase in the production of ethanol, lactic acid and acetic acid. Lactic acid and acetic acid were detected in the cocoa pulp at 48 h of fermentation, with initial concentrations of 1.55 and 0.8 mg g^{-1}, respectively. Lactic acid reached a maximum concentration of 11.9 mg g^{-1} at 72 h of fermentation and then decreased until the end of fermentation down to a value of 7.58 mg g^{-1}. Acetic acid reached a maximum concentration of 23.1 mg g^{-1} at 96 h of fermentation and then decreased until the end of fermentation down to a value of 17.8 mg g^{-1} (Fig. 4.17). Citric acid in the cocoa beans was slowly metabolized and decreased until the end of fermentation. In contrast to the pulp, the concentrations of lactic acid and acetic acid in the beans increased to maximum concentrations of 3.24 and 3.1 mg g^{-1}, respectively, until the end of fermentation (Fig. 4.17).
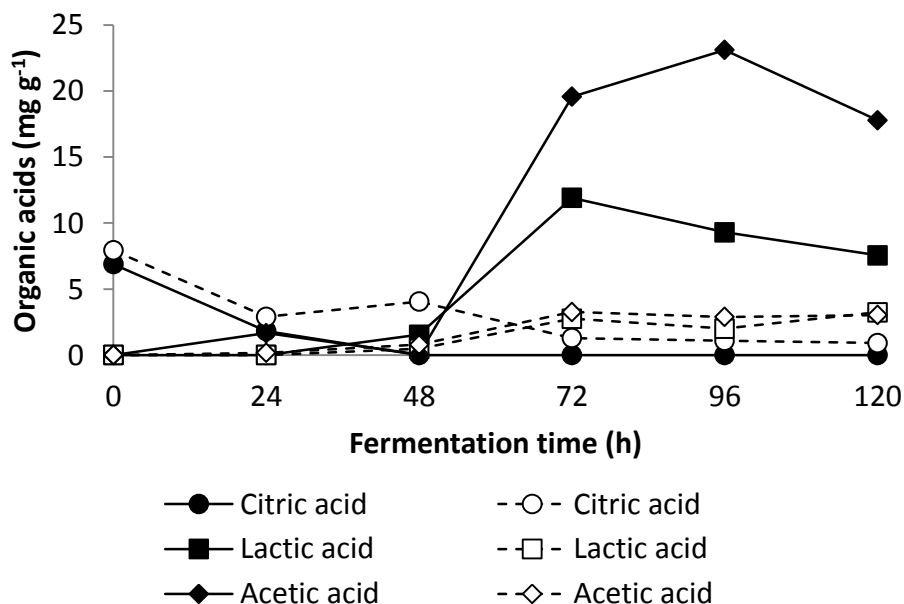
Figure 4.17 Changes in organic acids (citric acid, lactic acid, acetic acid) during aerobic fermentation, full symbol (pulp), open symbol (beans)
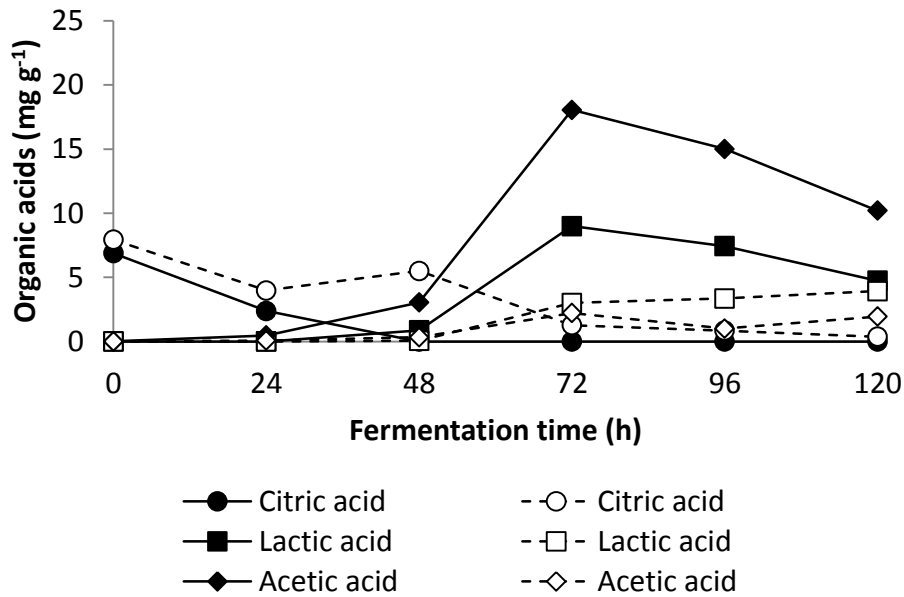
Figure 4.18 Changes in organic acids (citric acid, lactic acid, acetic acid) during anaerobic fermentation, full symbol (pulp), open symbol (beans)

Organic acids (citric acid, lactic acid, acetic acid) changed during anaerobic fermentation as follows: similar to aerobic fermentation, citric acid is the main organic acid found in cocoa pulp. Lactic acid and acetic acid were not detected in the cocoa pulp. From the start of fermentation until 24 h later, citric acid (from 6.9 to 2.38 mg g^{-1}) was metabolized in the Erlenmeyer flasks fermentation, coinciding with an increased production of ethanol, lactic acid and acetic acid. Lactic acid and acetic acid were detected in the cocoa pulp at 48 h of fermentation with initial concentrations of 0.34 and 3.0 mg g^{-1}, respectively. Lactic acid reached a maximum concentration of 9.0 mg g^{-1} at 72 of fermentation, and then decreased until the end of fermentation down to 4.74 mg g^{-1}. Acetic acid reached a maximum concentration of 18.1 mg g^{-1} at 72 h of fermentation and then decreased until the end of fermentation down to 10.2 mg g^{-1} (Fig. 4.18). Citric acid in the cocoa beans was slowly metabolized and decreased until the end of fermentation. In contrast to the pulp, concentrations of lactic acid and acetic acid in the beans increased until the end of fermentation to maximum concentrations of 3.9 and 2.0 mg g^{-1}, respectively (Fig. 4.18). The production of lactic acid and acetic acid was slightly higher in the aerobic fermentation compared to the anaerobic fermentation.

4.3.3.3   Changes in ethanol during aerobic and anaerobic fermentation

The concentration of ethanol in the pulp of the aerobic fermentation reached a maximum concentration of 18.54 mg g^{-1} after 48 h of fermentation. The ethanol concentration declined to 4.0 mg g^{-1} at the end of fermentation. The concentration of ethanol in the pulp of the anaerobic fermentation reached a maximum concentration of 20.16 mg g^{-1} after 48 h of fermentation. At the end of fermentation, the ethanol concentration had declined down to 5.20 mg g^{-1} (Fig. 4.19).
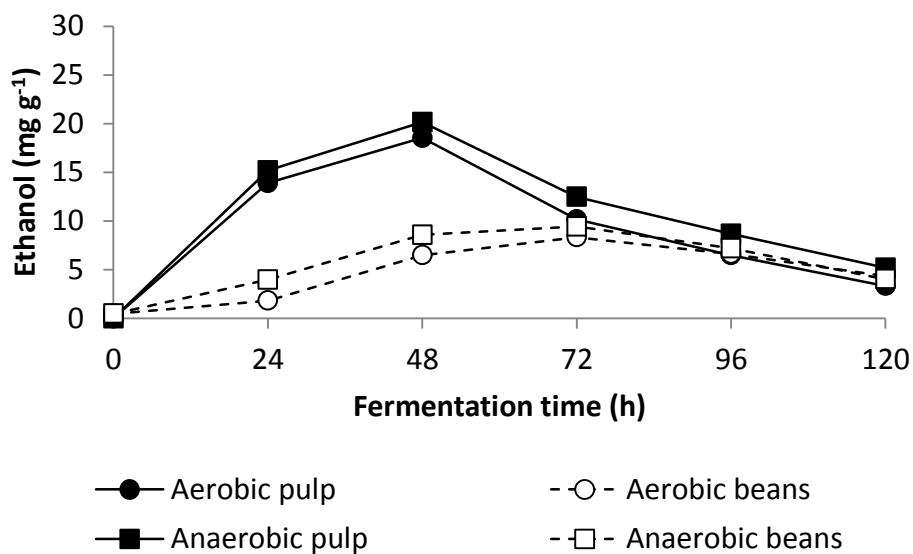
Figure 4.19 Changes in ethanol during aerobic and anaerobic fermentation, full symbol (pulp), open symbol (beans)

In contrast to the pulp, the concentration of ethanol in the beans increased during fermentation before slightly declining until the end of fermentation. The concentration of ethanol in the aerobic and anaerobic fermentations increased to maximum values of 8.33 and 9.44 mg g^{-1} after 72 h of fermentation, respectively. The concentration of ethanol in the anaerobic fermentation was slightly higher compared to the aerobic fermentation (Fig. 4.19).

4.3.3.4   Changes in amino acids during aerobic and anaerobic fermentation

The concentrations of free amino acids increased during fermentation, both in aerobic and anaerobic fermentations. Acidic amino acids decreased after 72 h of fermentation, whereas basic and hydrophobic amino acids increased after 72 h of fermentation. The concentrations of free amino acids was higher in the aerobic fermentation compared to the anaerobic fermentation (Fig. 4.20 and 4.21).
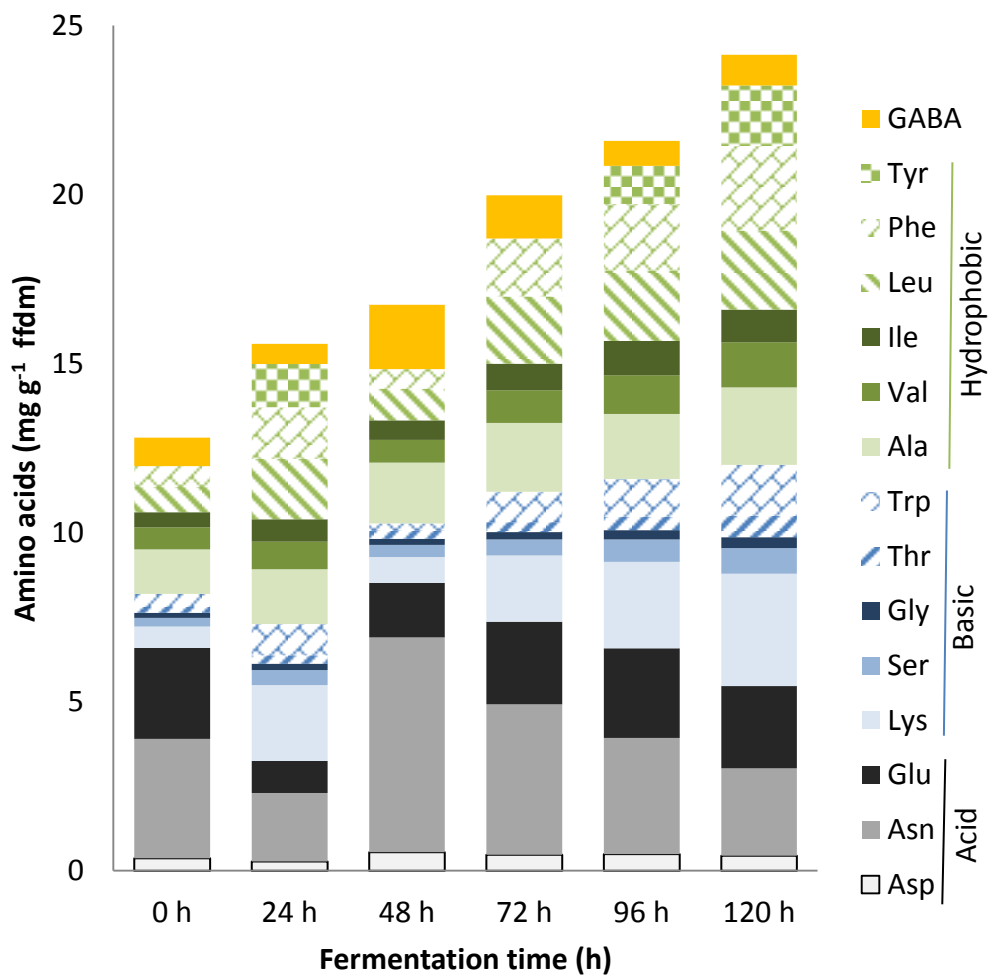
Figure 4.20 Changes in amino acids during aerobic fermentation
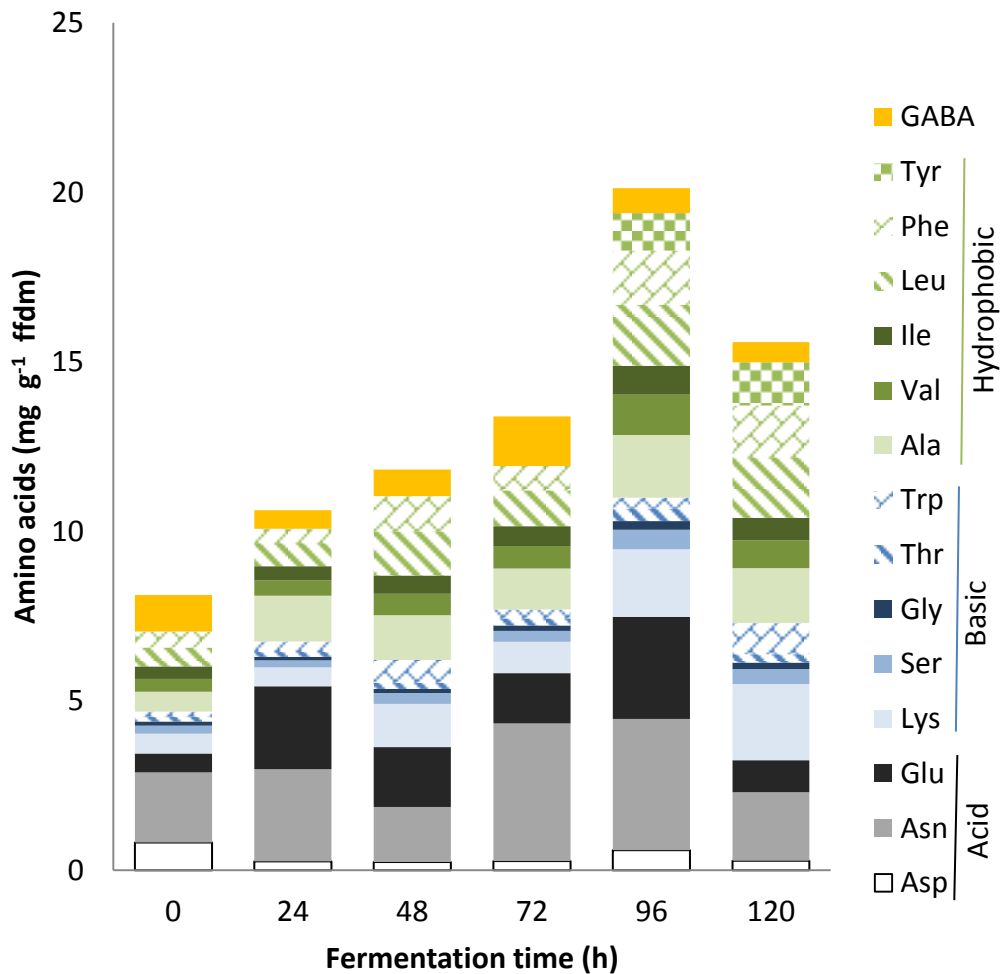
Figure 4.21 Changes in amino acids during anaerobic fermentation

4.3.3.5 Changes in polyphenols during aerobic and anaerobic fermentation

The concentration of polyphenols decreased during fermentation, particularly after 48 and 72 h of fermentation in aerobic and anaerobic fermentation, respectively. More than 50% of polyphenols were lost after 48 and 72 h of fermentation in aerobic and anaerobic fermentation, respectively. The concentration of polyphenols was higher in the anaerobic fermentation compared to aerobic fermentation. The concentration of polyphenols was more stable during the first 48 h of fermentation in the anaerobic fermentation. The polyphenol concentration was found to be extremely low after 48 h of fermentation in aerobic fermentation (Fig. 4.22, 4.23).
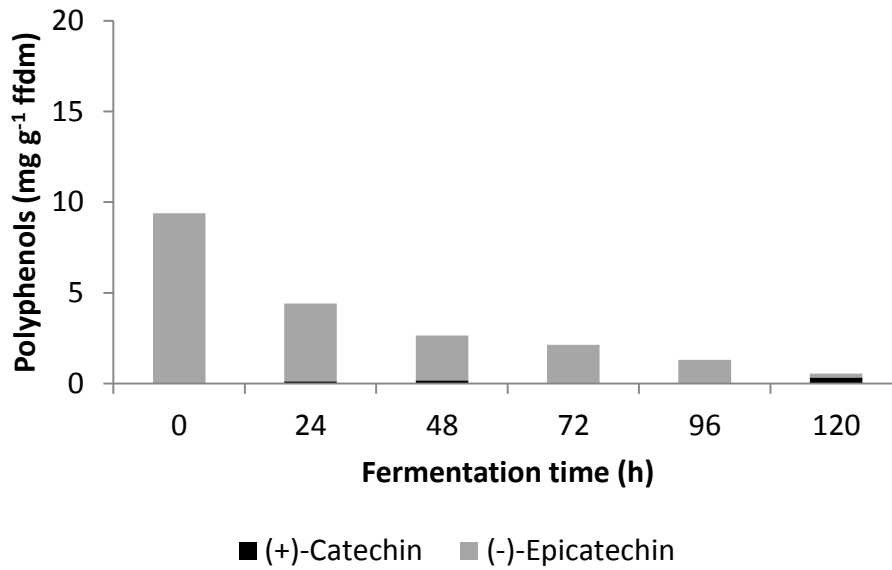
Figure 4.22 Changes in polyphenols during aerobic fermentation
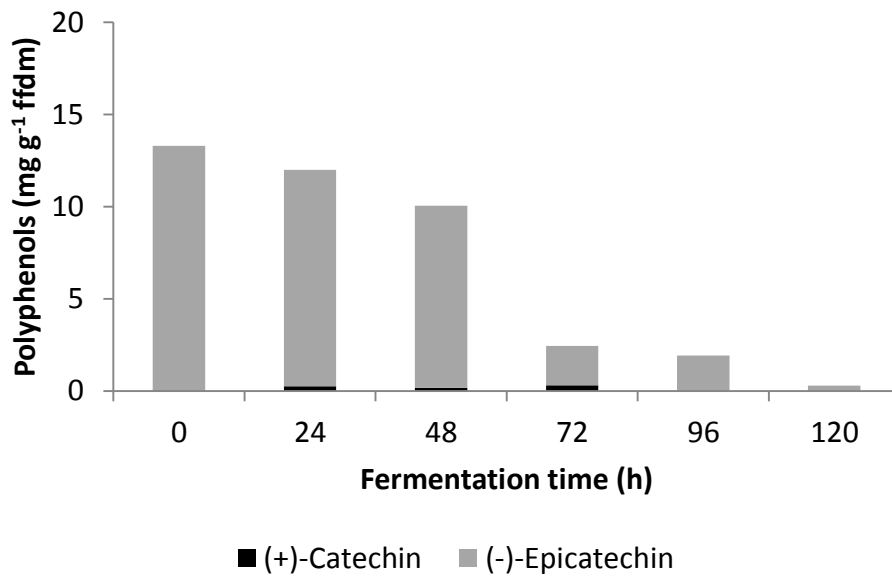
Figure 4.23 Changes in polyphenols during anaerobic fermentation

4.4 Investigation of activity and characterization of antifungal proteins in the seed coat slime of *Theobroma cacao* L.

4.4.1 Antimicrobial activity assay against fungi, Gram-positive bacteria and Gram-negative bacteria

Strains of microorganisms were isolated from cocoa bean fermentations from Indonesia and Ecuador, or strains from the microorganism collection at the Division of Food Microbiology and Biotechnology, Department of Chemistry, University of Hamburg were used (Tab. 4.10).

Table 4.10 Seed coat slime inhibition test against fungi

| Fungus	Zone diameter (mm)	Fungus	Zone diameter (mm)
*Aspergillus niger***	12.55	*Candida pulcherrima**	0
*Aspergillus flavus***	14.49	*Candida guilliermondii**	18.55
*Aspergillus parasiticus***	12.49	*Cryptococcus laurentii**	14.55
*Mortierella isabellina***	11.72	*Kloeckera apis***	0
Mucor sp. **	0	*Phaeococcomyces nigricans***	0
*Penicillium citrinum***	12.72	*Rhodotorula glutinis**	0
*Penicillium purpurogenum***	12.82	*Rhodotorula mucilaginosa**	19.52
*Penicillium roquefortii***	12.52	*Rhodotorula rubra***	19.62
*Rhizopus oligosporus***	0	*Saccharomyces cerevisiae**	19.84
*Rhizopus oryzae***	0	*Schizosaccharomyces pombe***	19.62
*Candida krusei***	16.82	*Torulopsis magnoliae***	0
*Candida lipolytica**	19.62	*Torulaspora delbrueckii**	0
*Candida pelliculosa**	0		

*: strains from cocoa bean fermentation
**: strain from laboratory culture collections of Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany).

Aspergillus flavus   Aspergillus niger   Aspergillus parasiticus

Saccharomyces cerevisiae   Rhodotorula rubra
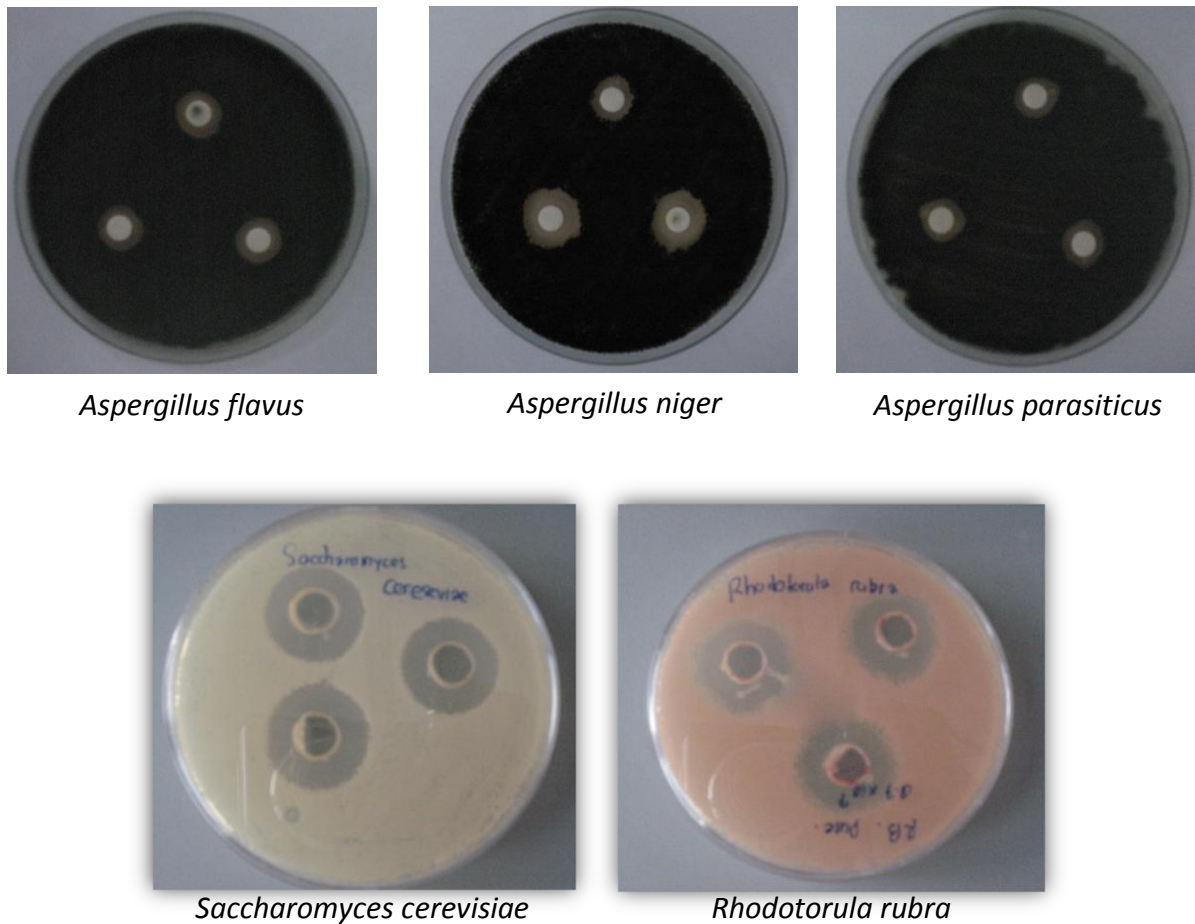
Figure 4.24 Representative images for fungus inhibition test

For each strain of microorganism, three holes in the agar or three sterile blank paper discs of 6.5mm containing of 100 mg mL^{-1} of seed coat slime were applied to determine antimicrobial activity of seed coat slime. Seed coat slime can inhibit the growth of certain fungi (e.g. *Aspergillus niger, Aspergillus flavus, Aspergillus parasiticus, Mortierella isabellina, Penicillium citrinum, Penicillium purpurogenum, Penicillium roquefortii*), though other strains of *Mucor* sp., *Rhizopus oligosporus* and *Rhizopus oryzae* were not inhibited. *A. flavus* was inhibited most strongly by seed coat slime, with a 14.49 mm inhibition zone (Tab. 4.10). A number of yeast strains were also inhibited by seed coat slime (e.g. *Candida krusei, Candida lipolytica, Candida guilliermondii, Cryptococcus laurentii, Rhodotorula mucilaginosa, Rhodotorula rubra, Saccharomyces cerevisiae, Schizosaccharomyces pombe*) while strains of *Candida pelliculosa, Candida pulcherrima, Kloeckera apis, Phaeococcomyces nigricans,*

Rhodotorula glutinis, Torulopsis magnoliae and *Torulaspora delbrueckii* were not inhibited (Tab. 4.10).

No inhibition effect could be detected against Gram-positive and Gram-negative bacteria (Tab. 4.11, 4.12).

Table 4.11 Seed coat slime inhibition test against Gram-positive bacteria

Gram-positive bacteria	Zone diameter (mm)
Bacillus subtilis ATCC 6633	0
Micrococcus luteus DSM 1790	0
Staphylococcus aureus LMH 5P	0
Streptococcus thermophilus LMH 5P	0
Lactobacillus plantarum LMH 13P	0
Lactobacillus plantarum DSM 20205	0
Lactobacillus acidophilus LMH 16P	0
Lactobacillus casei LMH 17P	0
Lactobacillus fermentum DSM 20052	0
Lactobacillus reuteri DSM 20016	0
Lactobacillus delbrueckii DSM 20355	0
Propionibacterium freudenreichii DSM 20271	0
Propionibacterium shermanii DSM 20270	0

Add: All gram positive bacteria were from laboratory culture collections of Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany).

Table 4.12 Seed coat slime inhibition test against Gram-negative bacteria

Gram-negative bacteria	Zone diameter (mm)
Escherichia coli	0
Acetobacter pasteurianus	0
Acetobacter tropicalis	0
Acetobacter pomorum	0
Acetobacter peroxydans	0
Gluconobacter frateurii	0
Acetobacter orientalis	0

*: strains from cocoa bean fermentation
**: strain from laboratory culture collections of Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany).

4.4.2 Minimum inhibitory concentration against molds and yeasts

The antifungal activity of the seed coat slime of *Theobroma cacao* L. was evaluated by the disk diffusion agar and microdilution methods. To determine the minimum inhibitory concentration (MIC) of seed coat slime of *Theobroma cacao* L., a susceptibility test was performed against *A. niger, P. citrinum, S. cerevisiae* and *R. rubra.*

Both the disk diffusion agar and microdilution methods showed good results during testing. MIC test using disk diffusion agar methods showed difficulties to distinguish between different concentrations of seed coat slime, while microdilution methods allowed to distinguish between the concentrations of seed coat slime. The diffusion agar method was thus less sensitive than the microdilution method in these antifungal tests.

A. niger was significantly inhibited by seed coat slime. 25 mg mL^{-1} of seed coat slime inhibited 56.7% and 66.48% of the growth of *A. niger* after 24 and 48 h of incubation, respectively. 100 mg mL^{-1} of seed coat slime inhibited 84.1% and 89.6% of the growth of *A. niger* after 24 and 48 h of incubation, respectively. 25 mg mL^{-1} of seed coat slime was the lowest concentration capable of inhbiting 50% of the growth of *A. niger* after 24 h of incubation (Fig. 4.25).
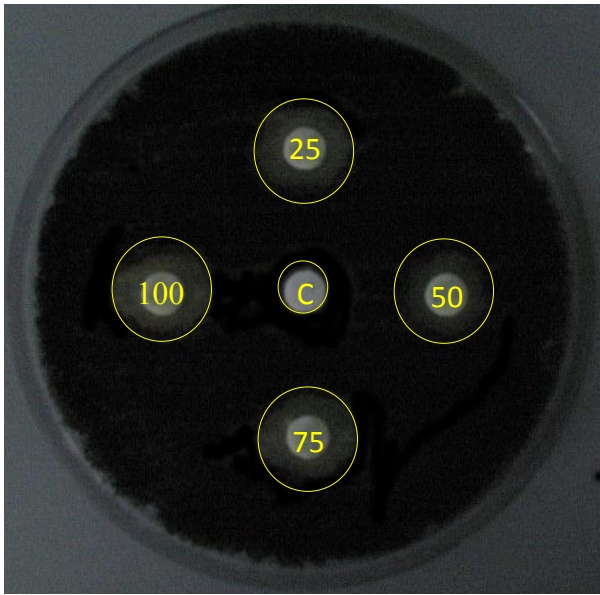
a) Agar diffusion methods, 25, 50, 75, 100 (seed coat slime extract mg mL^{-1}), C: control (sterile H$_2$O)
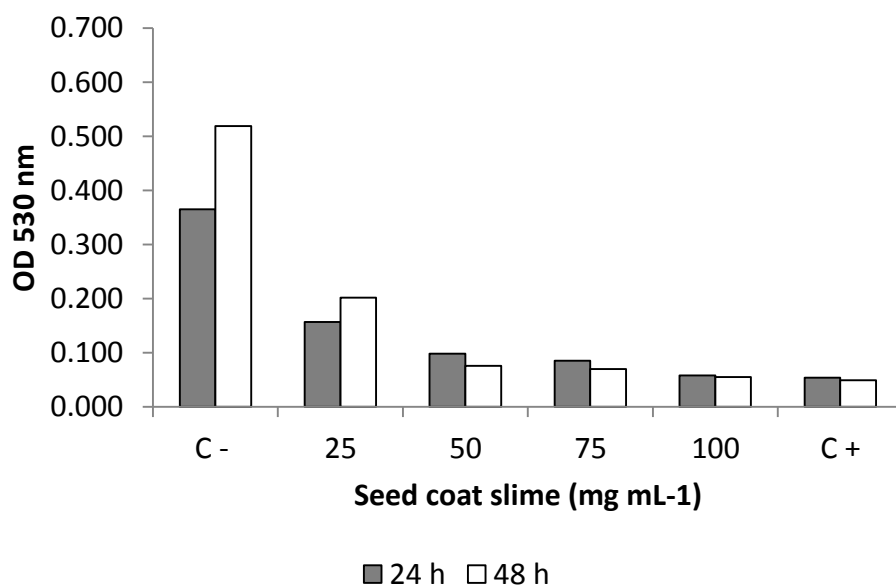
b) Microdilution methods, 25, 50, 75, 100 (seed coat slime extract mg mL^{-1}), C- (control negative, sterile H$_2$O), C+ (control positive, 2 mg mL^{-1} propionic acid)

Figure 4.25 Minimum inhibitory concentration against *Aspergillus niger*, a) agar diffusion methods. b) microdilution methods

P. citrinum was not strongly inhibited by seed coat slime. 25 mg mL^{-1} of seed coat slime inhibited 16.74% and 32.42% of the growth of *P. citrinum* after 24 and 48 h of incubation, respectively.
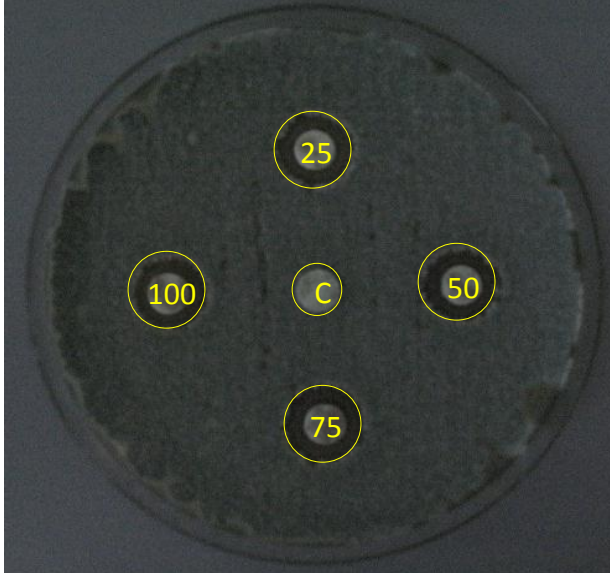
a) Agar diffusion methods, 25, 50, 75, 100 (seed coat slime extract mg mL^{-1}), C: control (sterile H$_2$O)
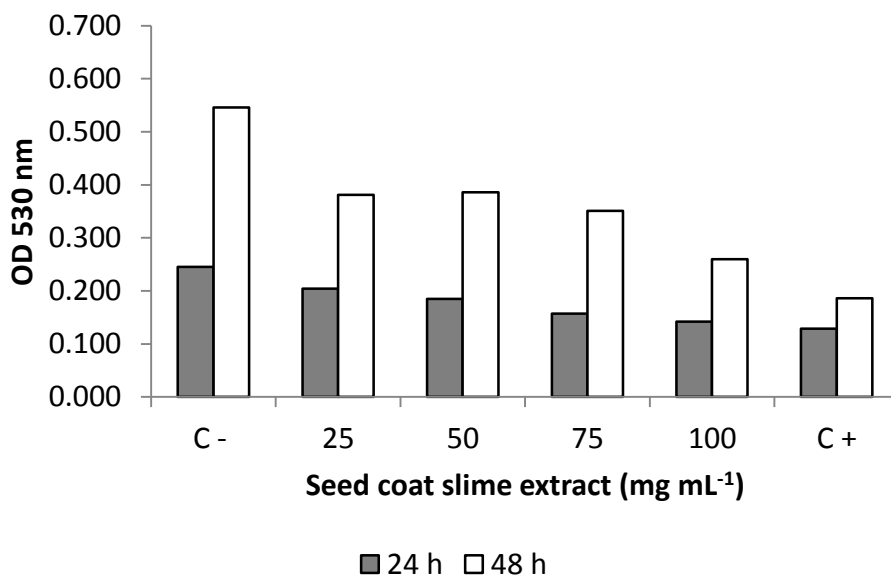
b) Microdilution methods, 25, 50, 75, 100 (seed coat slime extract mg mL^{-1}), C- (control negative, sterile H$_2$O), C+ (control positive, 2 mg mL^{-1} propionic acid)

Figure 4.26 Minimum inhibitory concentration against *Penicillium citrinum*, a) agar diffusion methods. b) microdilution methods

100 mg mL^{-1} of seed coat slime inhibited 42.04% and 55.13% of the growth of *P. citrinum* after 24 and 48 h of incubation, respectively. 100 mg mL^{-1} of seed coat slime was the lowest concentration capable of inhibiting 50% of the growth of *P. citrinum* after 48 h of incubation (Fig. 4.26).

S. cerevisiae was not strongly inhibited by seed coat slime using microdilution methods. 10 mg mL^{-1} of seed coat slime inhibited 6.9% and 2.56% of the growth of *S. cerevisiae* after 24 and 48 h of incubation, respectively. 40 mg mL^{-1} of seed coat slime inhibited 17.86% and 11.6% of the growth of *S. cerevisiae* after 24 and 48 h of incubation, respectively. 40 mg mL^{-1} of seed coat slime could not achieve 50% growth inhibition of *S. cerevisiae* after 48 h of incubation (Fig. 4.27). For *S. cerevisiae*, 24 h of incubation seems to be the best time for the inhibition test.

R. rubra was significantly inhibited by seed coat slime. 10 mg mL^{-1} of seed coat slime inhibited 52.22% and 60.03% of the growth of *R. rubra* after 24 and 48 h of incubation, respectively. 40 mg mL^{-1} of seed coat slime inhibited 78.22% and 87.63% of the growth of *R. rubra* after 24 and 48 h of incubation, respectively. 10 mg mL^{-1} of seed coat slime was the lowest concentration capable of inhibiting 50% of the growth of *R. rubra* after 24 h of incubation (Fig. 4.28).
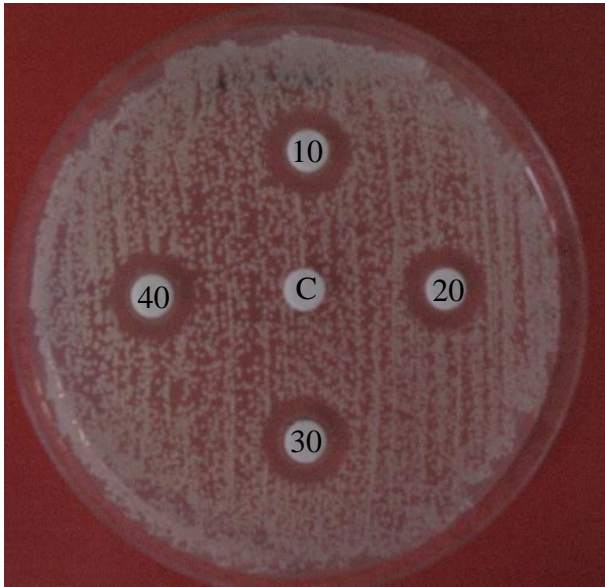
a) Agar diffusion methods, 10, 20, 30, 40 (seed coat slime extract mg mL^{-1}), C: control (sterile H$_2$O)
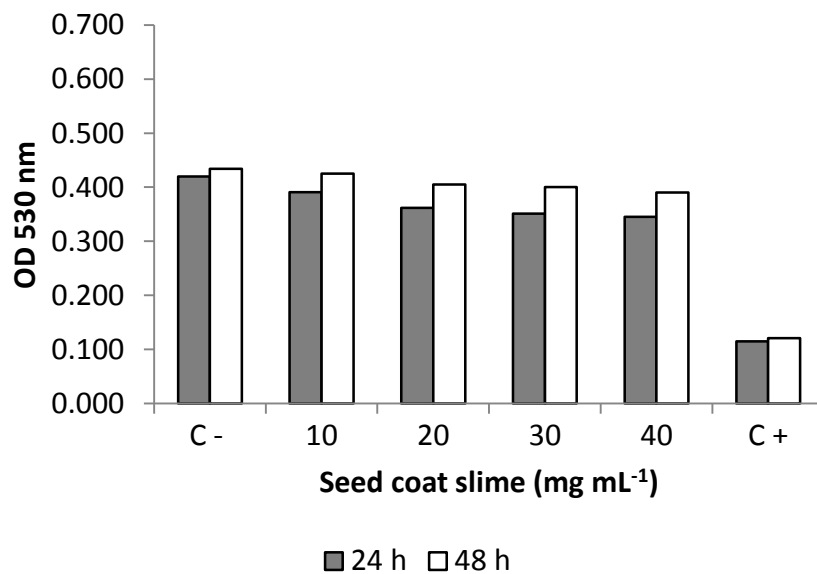
b) Microdilution methods, 10, 20, 30, 40 (seed coat slime extract mg mL^{-1}), C- (control negative, sterile H$_2$O), C+ (control positive, 2 mg mL^{-1} propionic acid)

Figure 4.27 Minimum inhibitory concentration against *Saccharomyces cerevisiae*, a) agar diffusion methods, b) microdilution methods
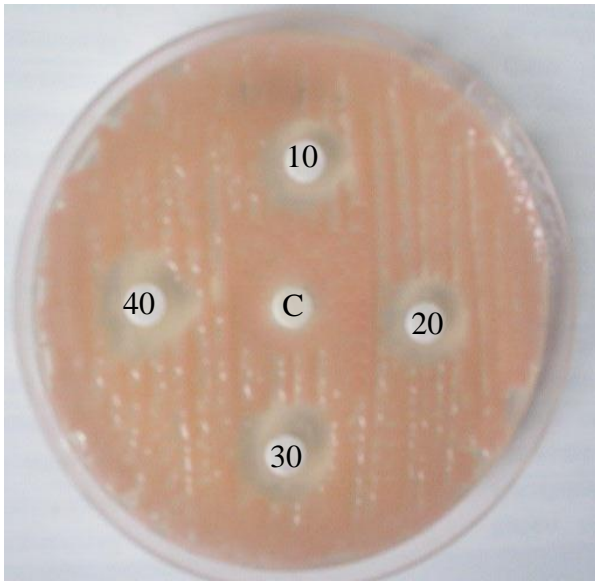
a) Agar diffusion methods, 10, 20, 30, 40 (seed coat slime extract mg mL^{-1}), C: control (sterile H$_2$O)
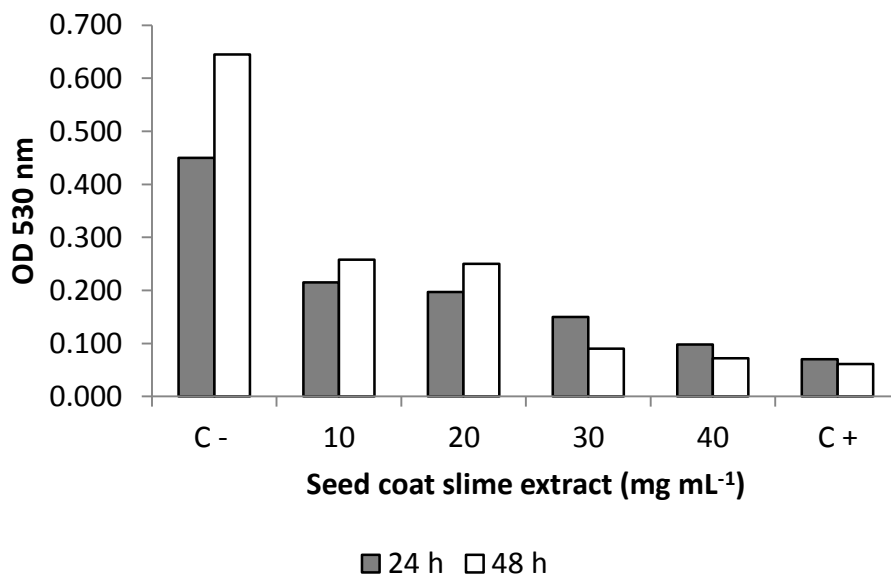
b) Microdilution methods, 10, 20, 30, 40 (seed coat slime extract mg mL^{-1}), C- (control negative, sterile H$_2$O), C+ (control positive, 2 mg mL^{-1} propionic acid)

Figure 4.28 Minimum inhibitory concentration against *Rhodotorula rubra*, a) agar diffusion methods, b) microdilution methods

4.4.3 Protein concentration in the seed coat slime

The protein concentration of seed coat slime differed between varieties of *Theobroma cacao* L., with Forastero (7.0 µg mL^{-1}) showing a higher concentration than Trinitario (5.2 µg mL^{-1}). The concentration of the seed coat slime (Forastero) decreased during fermentation. The concentration of the seed coat slime was 10.75 µg mL^{-1} at the onset of fermentation before decresing by 50% after 96 h of fermentation down to 4.83 µg mL^{-1}. Seed coat slime does not seem to be resistant to high temperature, as the protein concentration of the seed coat slime decreased during heat treatment to 5.12, 4.94 and 2.35 after incubation for 24 h at 30, 40 and 50 °C, respectively (Tab. 4.13). Only 4 and 1 protein band could be seen after 24 h incubation at 40 and 50 °C, respectively (Fig. 4.30).

Table 4.13 Concentration of seed coat slime proteins

Seed coat sample	Protein concentration µg mL^{-1}
Forastero	6.98
Trinitario	5.17
Fermentation, 0 h	10.75
Fermentation, 24 h	10.08
Fermentation, 48 h	9.25
Fermentation, 72 h	6.40
Fermentation, 96 h	4.83
Heated 30 °C, 24 h	5.12
Heated 40 °C, 24 h	4.94
Heated 50 °C, 24 h	2.35

4.4.4 Protein extraction and separation by electrophoresis

It could be seen that seed coat slime proteins degraded during the fermentation process, probably caused by an increase in temperature and by activity of proteases produced by the microbes during the fermentation.
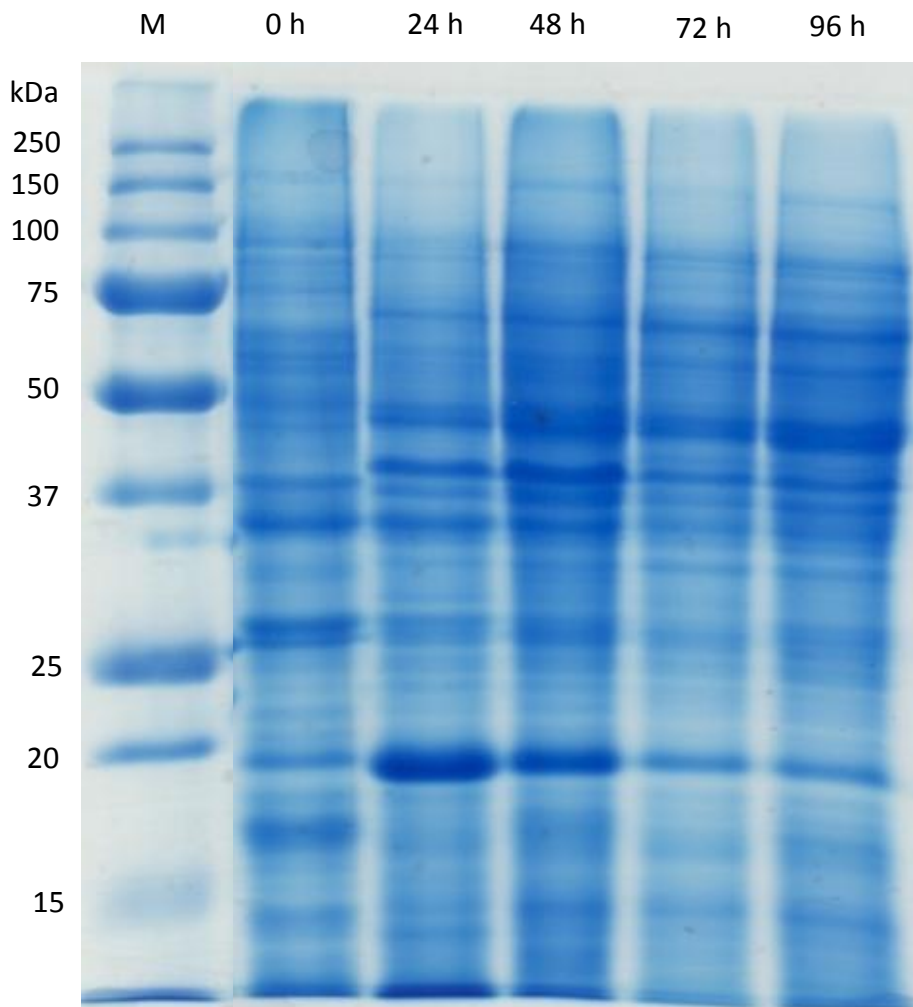
Figure 4.29 Seed coat slime proteins from different fermentation times

4.4.5 Temperature stability of seed coat proteins

To study the stability of seed coat slime proteins at different temperatures, the slime was incubated at various temperatures (30, 40 and 50 °C) for 24 h. The study on temperature stability showed that the slime proteins were stable at 30 °C for 24 h, but not at 40 and 50 °C.
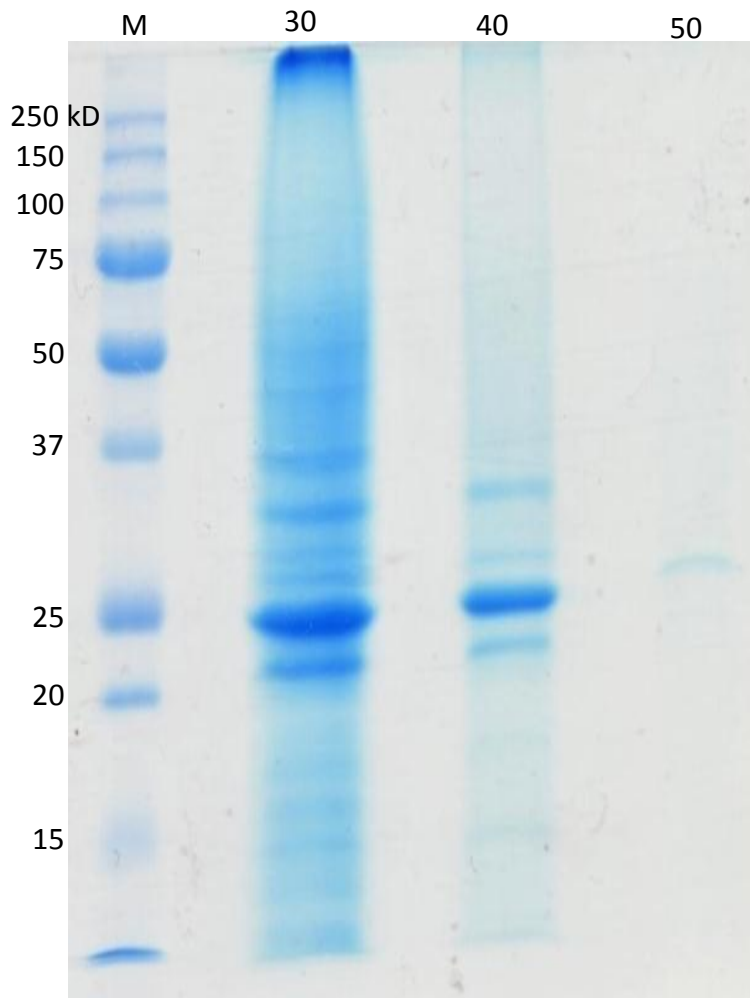
Figure 4.30 Seed coat slime protein from different incubation temperatures (30, 40, 50 °C), 24 h

4.4.6 Identification of seed coat slime proteins

Analyses of proteins using nano-electrospray mass spectrometry in a QTOF II instrument (Micromass, Manchester, UK) showed 3 proteins that could have an antifungal effect, namely osmotin 24.1 kDa (6) chitinase 28.8 and 31.6 kDa (7, 8) and glucanase 33 kDa (9) (Fig. 4.31).
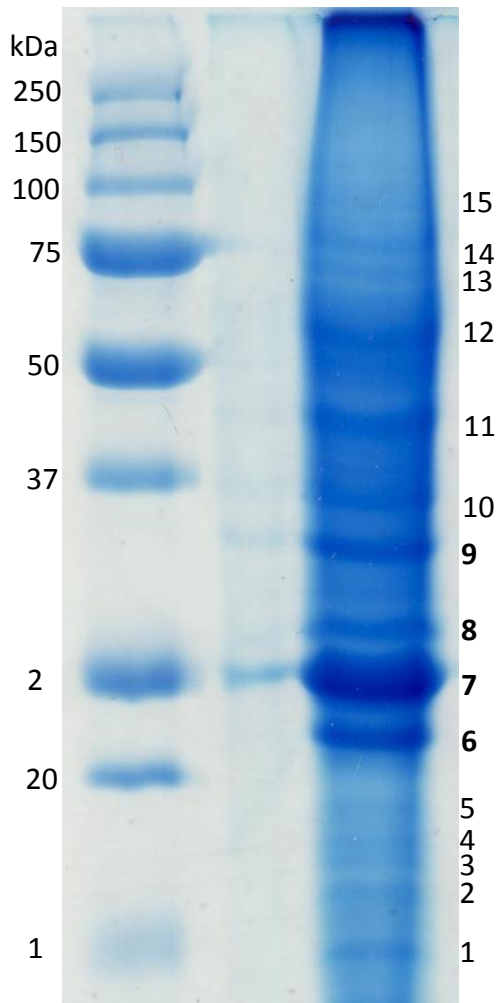
Figure 4.31 Seed coat slime proteins from Forastero

Table 4.14 Protein determination by mass spectrometry

Protein	Identified proteins	Molecular weight (kDa)
1.	Glycoprotein [*Bromus inermis*]	15.5
2.	Ribosomal protein S11 family protein [*Theobroma cacao*]	16.3
3.	17.6 kDa class II heat shock protein [*Theobroma cacao*]	17.5
4.	Ribosomal protein L11 family protein [*Theobroma cacao*]	17.8
5.	21 kDa seed protein [*Theobroma cacao*]	18.7
6.	**Osmotin 34 [*Theobroma cacao*]**	**24.1**
7.	**Basic chitinase [*Theobroma cacao*]**	**28.8**
8.	**Carrot EP3-3 chitinase [*Theobroma cacao*]**	**31.6**
9.	**Glucan endo-1,3-beta-glucosidase [*Theobroma cacao*]**	**33.0**
10.	Lactate dehydrogenase family protein [*Theobroma cacao*]	35.4
11.	Aldolase-type TIM barrel family protein [*Theobroma cacao*]	47.8
12.	ATP synthase alpha [*Theobroma cacao*]	59.7
13.	Vacuolar ATP synthase subunit A [*Theobroma cacao*]	73.2
14.	Hsc70 [*Solanum lycopersicum*]	75.0
15.	Phospholipase D alpha 2 [*Theobroma cacao*]	91.6

4.5 Investigation of the influence of seed coat slime on growth of starter culture and physical and chemical changes during cocoa bean fermentation at laboratory level

4.5.1 Changes in pH during fermentation

No significant differences in pH of the pulp and beans were found between fermentations with pulp (complete pulp) and with only 10% pulp during fermentation.
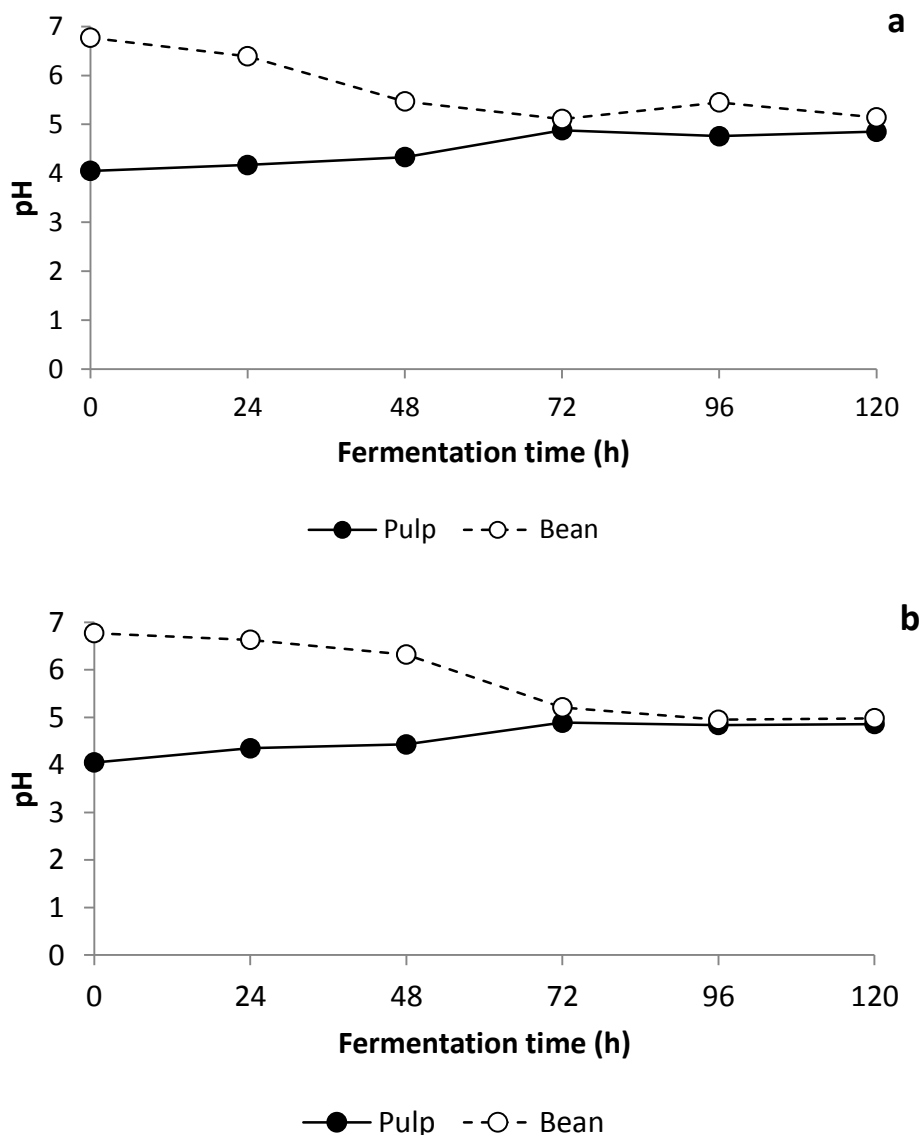
Figure 4.32 Changes in pH during fermentation, a) with pulp, b) with only 10% pulp

4.5.2   Growth of the starter culture during fermentation

To investigate the growth of the starter culture during fermentation with complete pulp, cocoa beans were inoculated with *S. cerevisiae* and *L. fermentum* at the onset of fermentation with an initial cell count of 10^7 and 10^6 CFU g^{-1}, respectively. *A. pasteurianus* was inoculated after 48 h of fermentation with an initial cell count of 10^8 CFU g^{-1}. The cell count of *S. cerevisiae* increased during the first 24 h and grew to a maximum number of 10^9 CFU g^{-1}. Upon prolonged fermentation, the *S. cerevisiae* cell count declined down to 10^4 CFU g^{-1} (Fig. 4.33 a). The size of the *L. fermentum* cell count increased during fermentation and grew to a maximum population of 10^8 CFU g^{-1} after 72 h of fermentation. Afterwards, the *L. fermentum* cell count decreased to 10^5 CFU g^{-1}, before slightly decreasing until the end of fermentation to 10^4 CFU g^{-1} (Fig. 4.33 a). *A. pasteurianus* was added to the fermented cocoa bean mass after 48 h of fermentation with an initial number of 10^8 CFU g^{-1}, before increasing rapidly to a maximum cell count of 10^8 CFU g^{-1} after 72 h, followed by a decline until the end of fermentation down to 10^4 CFU g^{-1} (Fig. 4.33 a).

To investigate the growth of the starter culture during fermentation with only 10% of pulp, cocoa beans were inoculated with *S. cerevisiae* and *L. fermentum* at the onset of fermentation with an initial cell count of 10^7 and 10^6 CFU g^{-1}, respectively. *A. pasteurianus* was inoculated after 48 h of fermentation with an initial cell count of 10^8 CFU g^{-1}. The cell count of *S. cerevisiae* increased during the first 24 h of fermentation to a maximum number of 10^8 CFU g^{-1}. After 24 h of fermentation, the *S. cerevisiae* cell count declined down to a minimum of 10^1 CFU g^{-1} until the end of fermentation (Fig. 4.33 b). The size of the *L. fermentum* cell count increased during fermentation and grew to a maximum population of 10^8 CFU g^{-1} after 72 h of fermentation. Afterwards, the *L. fermentum* cell count decreased to 10^6 CFU g^{-1}, before slightly decreasing until the end of fermentation to 10^5 CFU g^{-1} (Fig. 4.33 b). *A. pasteurianus* was added to the fermented cocoa beans mass after 48 h of fermentation with an initial number of 10^8 CFU g^{-1}, before increasing rapidly to a maximum cell count of 10^9 CFU g^{-1} after 72 h, followed by a decline until the end of fermentation down to 10^6 CFU g^{-1} (Fig. 4.33 b).

Compared to fermentation with complete pulp, the growth of *S. cerevisiae* was lower in cocoa bean fermentation with only 10% pulp, particulary after 48 h of fermentation (Fig. 4.33 b).
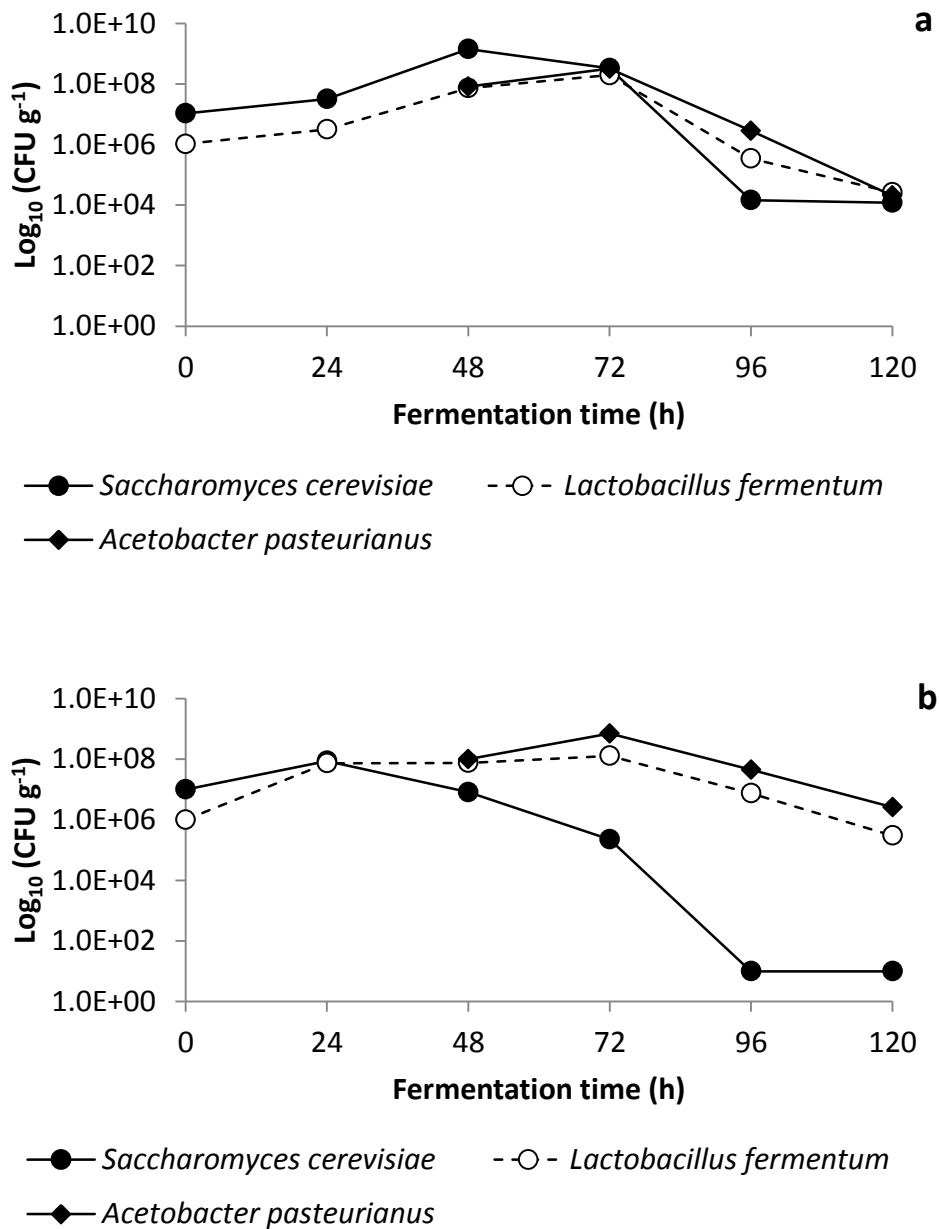

Figure 4.33 Growth of the starter culture during fermentation, a) with pulp, b) with only 10% pulp

4.5.3 Analysis of metabolites

4.5.3.1 Carbohydrates (sucrose, glucose, and fructose)

The concentrations of glucose and fructose in cocoa bean fermentation with only 10% pulp were lower in comparison to fermentation with complete pulp. In the residual pulp the concentrations of glucose and fructose that were found counted only 11.1 and 12.3 mg g^{-1}, respectively (Fig. 4.34 b).
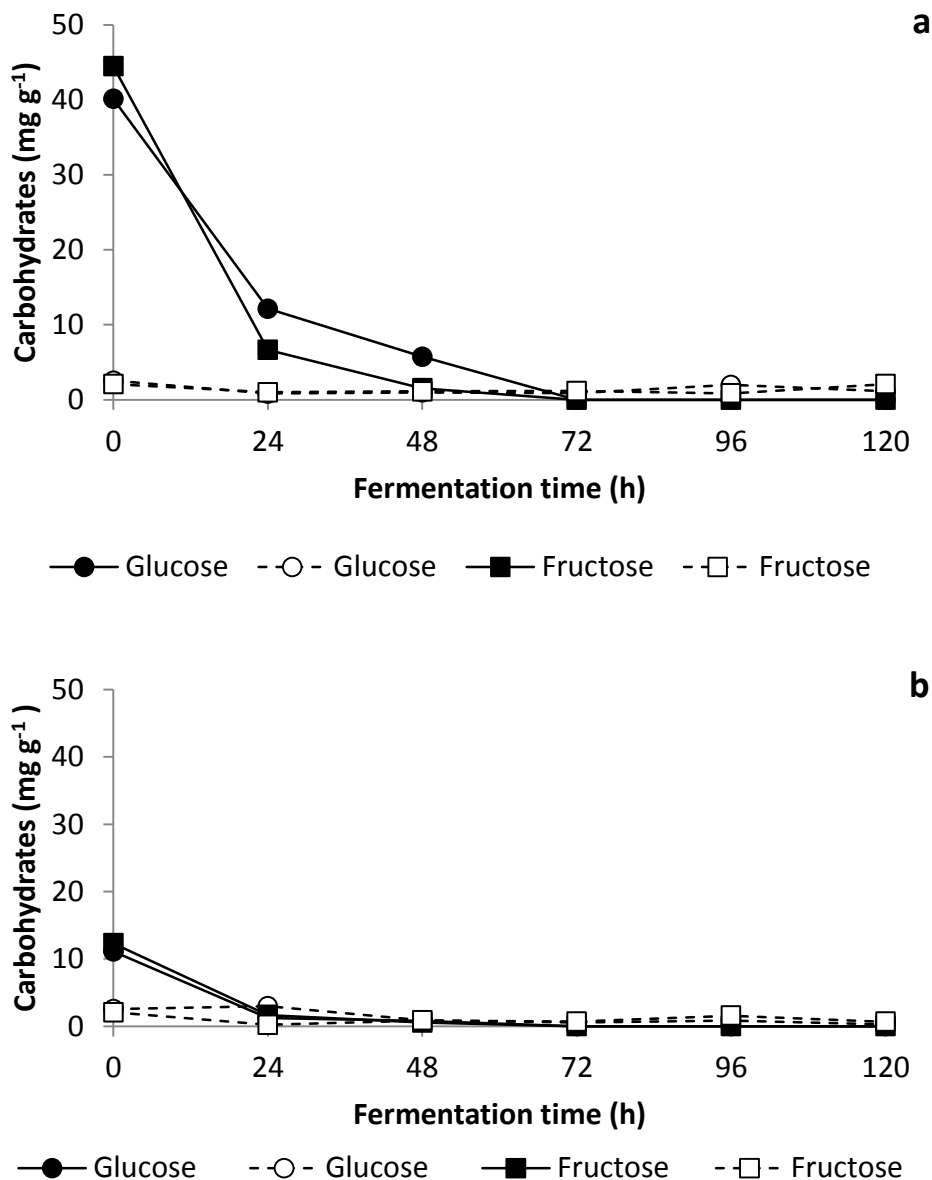
Figure 4.34 Changes in carbohydrates in pulp and beans during fermentation, full symbol (pulp), open symbol (beans), a) with pulp, b) with only 10% pulp

4.5.3.2 Organic acids (citric acid, lactic acid, acetic acid)

Citric acid was utilized during fermentation, whereas lactic acid and acetic acid were produced. No significant differences in concentrations of citric acid, lactic acid and acetic acid between fermentation with complete pulp and with only 10% pulp were to be seen, both in the pulp and and in the beans (Fig. 4.35, 4.36).
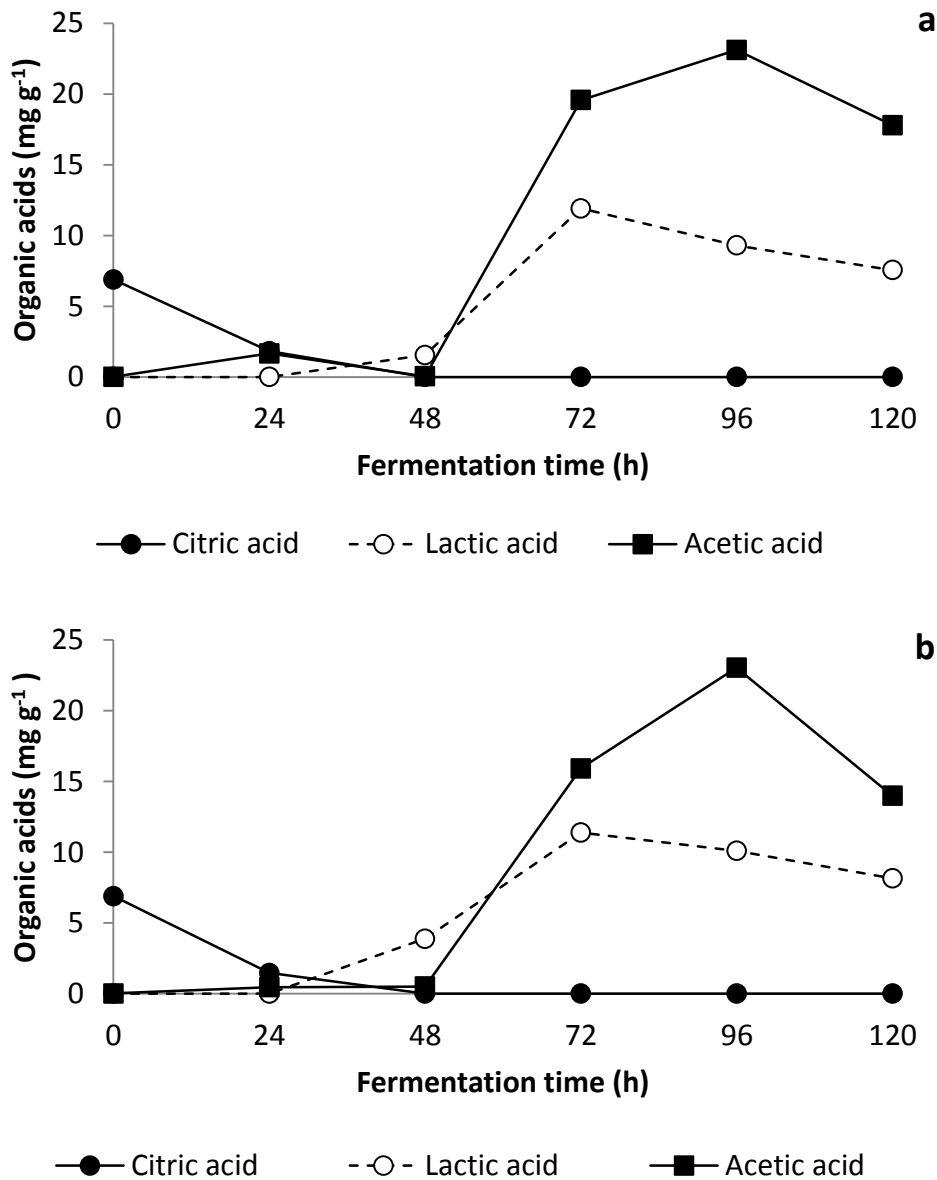
Figure 4.35 Changes in organic acids in the pulp during fermentation, a) with pulp, b) with only 10% pulp
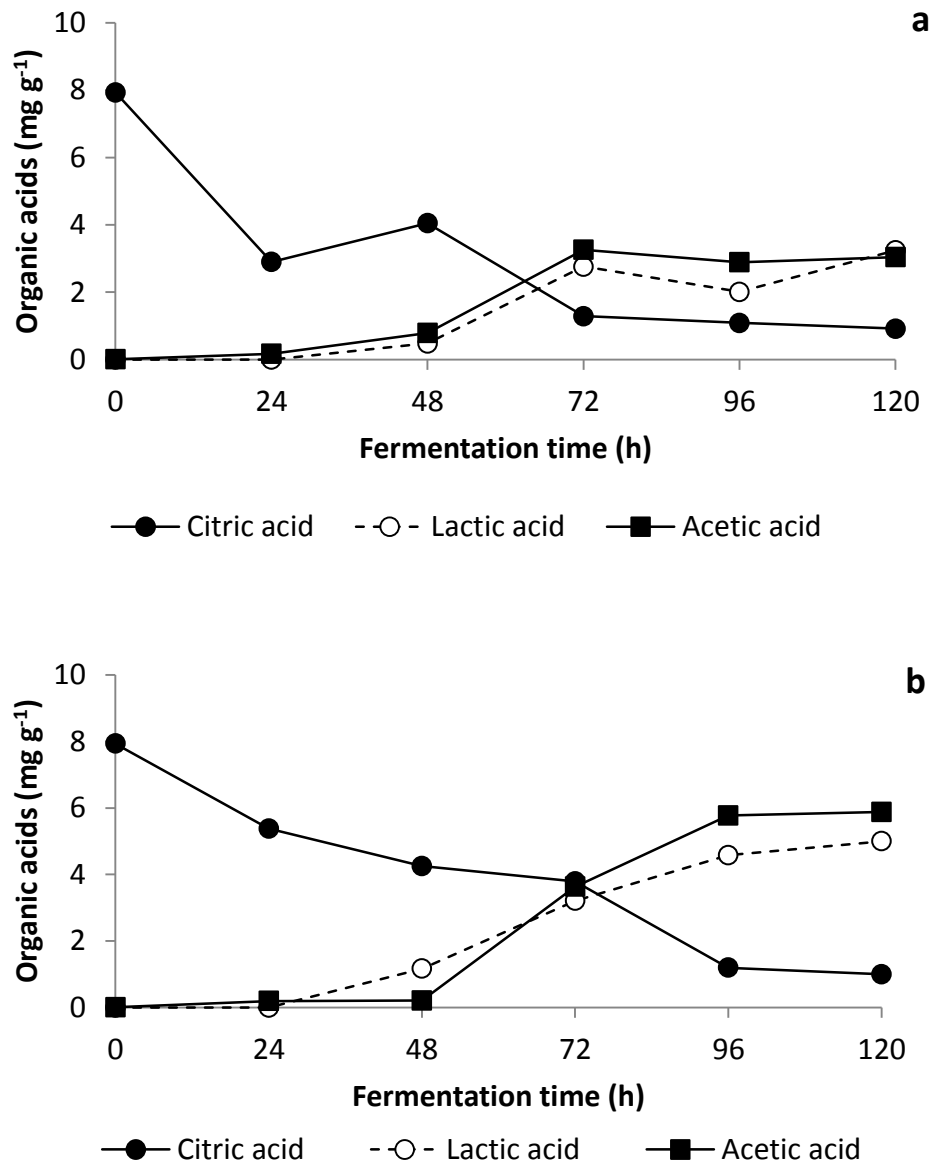
Figure 4.36 Changes in organic acids in the beans during fermentation, a) with pulp, b) with only 10% pulp

4.5.3.3 Ethanol

Compared to the fermentation with complete pulp, the production of ethanol was 2 mg g^{-1} lower in the fermentation with only 10% pulp in the pulp. In contrast with pulp the concentration of ethanol in the beans was $2 - 4$ mg g^{-1} higher in cocoa bean fermentation with complete pulp (Fig. 4.37 a, b).
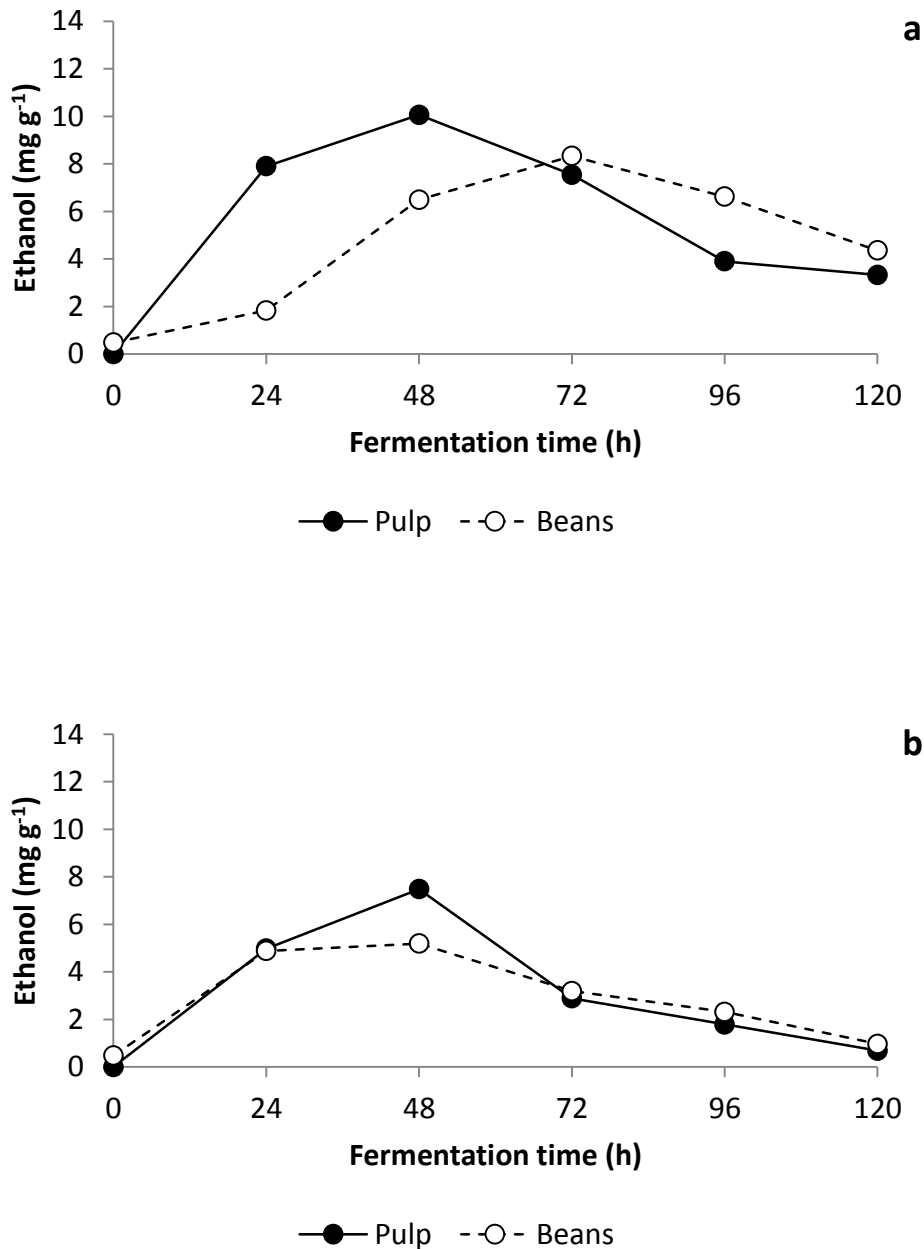
Figure 4.37 Changes in ethanol during fermentation, a) with pulp, b) with only 10% pulp

4.5.3.4 Amino acids

The concentration of free amino acids increased during fermentation. Acidic amino acids decreased after 48 h of fermentation, whereas hydrophobic amino acids increased after 72 h of fermentation (Fig. 4.38 a, b).
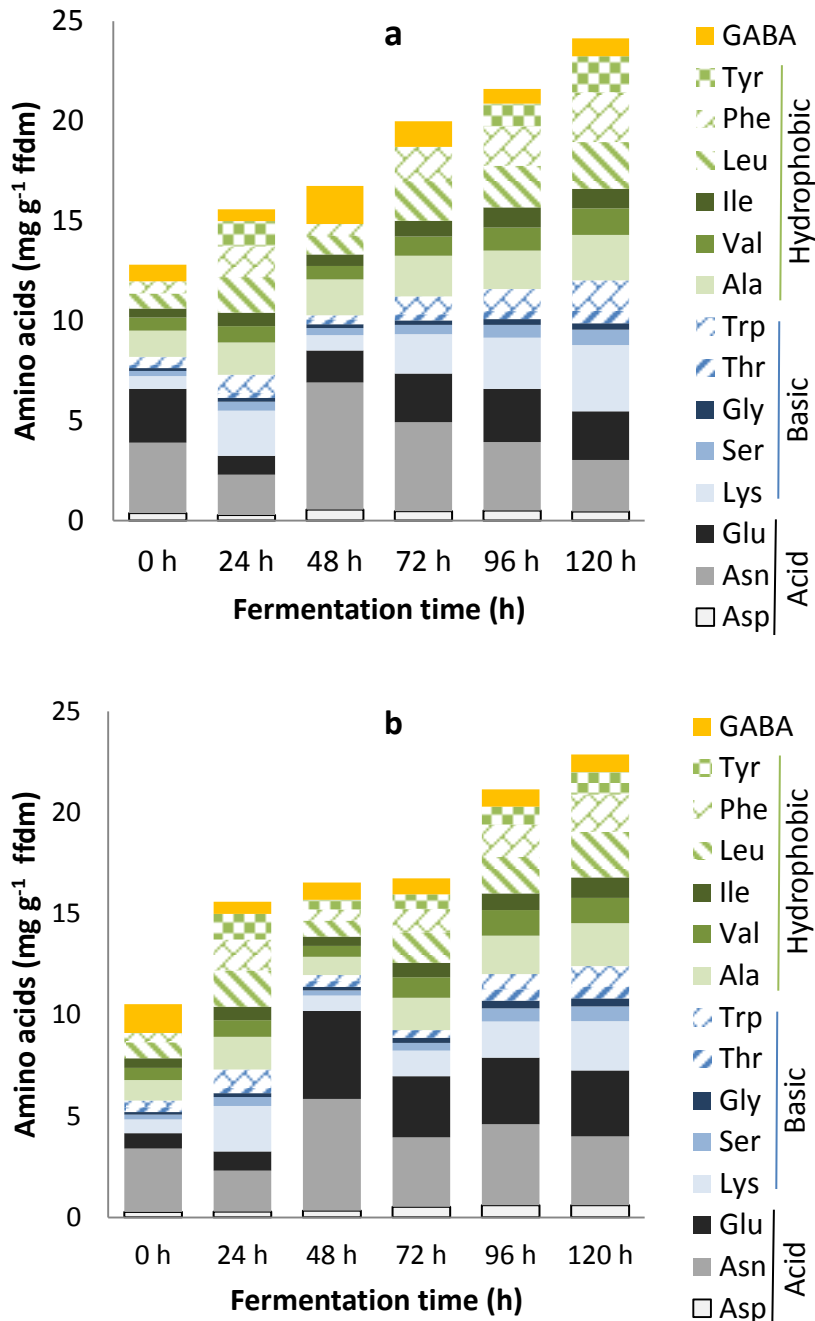
Figure 4.38 Changes in amino acids during fermentation, a) with pulp, b) with only 10% pulp

There is no significant difference in the concentration of free amino acids between fermentation with complete pulp and with only with only 10% pulp.

5 Discussion

5.1 Dynamics and biodiversity of the populations of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) involved in the spontaneous box fermentation of cocoa beans in Nusantara Plantation XII (PTPN XII), East Java, Indonesia

The fact that temperature of cocoa bean mass increased from an initial temperature of 28 °C to a maximum value of 48 °C, is caused by the conversion of pulp sugar into ethanol by yeasts and by the further oxidation of ethanol into acetic acid by AAB which is an exothermal process. This exothermal process produces a lot of energy and causes rise in the temperature of the fermenting mass during fermentation (Camu et al. 2007; Nielsen et al. 2007; Schwan and Wheals 2004; Thompson et al. 2001). Typically cocoa bean box fermentations at PTPN XII are always turned every 24 h during fermentation.

The activity of microbes during cocoa bean fermentation is characterized by the metabolism of pulp sugars in fresh cocoa beans and the production of significant amounts of ethanol, lactic acid, and acetic acid during fermentation (Ardhana and Fleet 2003; Lopez and Dimick 1995). Pulp sugars (glucose, fructose) and organic acid (citric acid) were utilized during fermentation, paralleled by increasing concentrations of ethanol, glycerol, lactic acid and acetic acid. Glucose and fructose were utilized by yeasts to produce ethanol and carbon dioxide. LAB utilized glucose, fructose and citrate to produce lactic acid, acetic acid, ethanol and mannitol. AAB oxidized ethanol into acetic acid. The citric acid metabolized during fermentation caused the pH of the pulp to increase during this process.

In the first 24-48 h, yeasts were the dominant population of the cocoa bean box fermentations. The consistent presence of yeasts during earlier phases of fermentation was also reported by Ardhana and Fleet (2003) for cocoa bean fermentations in Java, Indonesia and even from different countries such as Ghana (Nielsen et al. 2007), Ecuador (Papalexandratou et al. 2011 c), Malaysia (Meersman et al. 2013; Papalexandratou et al. 2013), Brazil and Ivory Coast (Papalexandratou and De Vuyst 2011).

Ten different yeasts species were found during cocoa bean box fermentation in nusantara plantation XII (PTPN XII). This diversity of yeasts species was even greater than that previously reported by Ardhana and Fleet (2003). For example, the species of *H. thailandica, P. kudriavzevii, I. orientalis*, *T. delbrueckii and S. roseus* were not found by Ardhana and Fleet (2003).

H. thailandica, *S. cerevisiae*, *H. opuntiae*, *H. guilliermondii*, and *C. tropicalis* were the dominant yeast species present during the first 24 h of fermentations. However, the population size decreased after about 24 h. Other yeast species such as *P. kudriavzevii* and *I. orientalis* also contributed to the beginning of fermentation but in low numbers. *H. guilliermondii*, *S. cerevisiae*, and *C. tropicalis* were also reported by Ardhana and Fleet (2003) as predominant yeasts species during the early phase of fermentation. In contrast to this *H. thailandica* and *H. opuntiae* were not reported by Ardhana and Fleet (2003). *H. opuntiae* was reported to be the predominante species of yeast during early phase of well-performed Malaysian cocoa bean box fermentation (Papalexandratou et al. 2013), while *H. thailandica, T. delbrueckii* and *P. kudriavzevii* were reported to be present at higher densities during spontaneous cocoa bean box fermentation in Malaysia (Meersman et al. 2013).

After 24 h of fermentation, *T. delbrueckii* and *S. roseus* were also isolated. However, only limited numbers of yeasts species have adapted to the cocoa bean mass environment. Only yeasts with specific characteristics like resistance to lactic acid, growth at 40 °C or in the range of 40 to 50 °C, and tolerance to high ethanol and acid concentrations have been found to persist until the later stages of cocoa bean fermentation (Lima et al. 2011). Overall, only *S. cerevisiae* has been able to survive until the end of fermentation. This is due to the adaptive ability of this species to survive heat and high concentrations of acid and ethanol (see Section 5.2).

Citric acid-consuming yeasts, were also isolated in this study during early phases of fermentation namely, *I. orientalis*. The main activity of this species is to break down the citric acid in the pulp, leading to an increase in the pH of the pulp during fermentation (Jespersen et al. 2005; Schwan and Wheals 2004).

Most of the species reported in this study have also been reported previously in spontaneous cocoa bean fermentations worldwide, but one of these species *Sporobolomyces roseus* has thus far never been associated with spontaneous cocoa bean fermentations. *S. roseus* is belonging to red to pinkish ballistoconidia-forming yeasts (Bai et al. 2002; Nakase 2000). This strain was later described as *Sporidiobolus metaroseus* sp. nov (Valério et al. 2008) and has also been found in other substrates such as air, seawater, freshwater, and phylloplane (Valério et al. 2008).

The production of toxic ethanol and the increase in temperature as well as the further conversion of ethanol to toxic acetic acid by AAB later in the fermentation seem to be responsible for the rapid decline in the dominance of yeasts during fermentation (Camu et al. 2007). Our study shows for the first time that the decline of the yeasts population after 48 h of fermentation was caused by antifungal proteins in the cocoa bean seed coat slime (see Section 5.4).

As pulp drains away, more air penetrates the fermenting mass, ethanol is still produced through the activity of the yeasts, and both temperature and pH increase up to 40 °C and pH 4.0, respectively. These conditions are favorable for the growth of lactic acid and acetic acid bacteria.

LAB grew from the beginning of fermentation and increased to a maximum population size at 48-72 h of fermentation. The primary activity of LAB is to metabolize pulp sugars (glucose, fructose) and some organic acids (citric acid, malic acid) by homofermentative and heterofermentative metabolism. The end products of lactic acid, acetic acid, ethanol, mannitol and carbon dioxide have an impact on the acidity and quality of the beans (Axelsson 2004; Schwan and Wheals 2004; Thompson et al. 2001).

A small number of LAB species was found in this study, including *L. plantarum, L. fermentum* and *L. brevis*. This finding was confirmed by Ardhana and Fleet (2003), who showed only a limited number of LAB species in cocoa bean box fermentations in Indonesia. In contrast to this, other studies in Ghana (Camu et al. 2007; Nielsen et al. 2007), Brazil (Garcia-Armisen et al. 2010; Papalexandratou et al. 2011 a), the Ivory Coast (Papalexandratou et al. 2011 a), Ecuador (Papalexandratou et al. 2011 c), and Malaysia (Papalexandratou et al. 2013) found a wide number of LAB species.

Both *L. plantarum* and *L. fermentum* were counted in high numbers during fermentation (Schwan and Wheals 2004; Thompson et al. 2001). In contrast with findings previously reported by Ardhana and Fleet (2003), *L. plantarum* was more consistent during fermentation than *L. fermentum,* while *L. brevis* was less consistent during fermentation. However, *L. plantarum* and *L. fermentum* are the most dominant LAB species worldwide, for example in Ghana (Camu et al. 2007; Nielsen et al. 2007), Brazil (Garcia-Armisen et al. 2010; Papalexandratou et al. 2011 a), the Ivory Coast (Papalexandratou et al. 2011 a), Ecuador (Papalexandratou et al. 2011 c), and Malaysia (Papalexandratou et al. 2013).

AAB growth was observed during the earlier phases of cocoa bean fermentation and increased until the end of fermentation. The intensive activity of yeasts and LAB metabolized pulp sugars and organic acids together with pectinolytic activity, causing the pulp to drain away, and increasing the temperature to more than 37 °C. This activity corresponds with the turnover of the cocoa beans every 24 h, which can lead to better aeration of the cocoa bean mass. Those aerobic conditions favor the growth of AAB. The main activity of AAB is to oxidize ethanol, initially formed by yeasts, into acetic acid, which provides them with the necessary energy. This exothermic reaction is mainly responsible for the rise in temperature of the fermenting mass.

Several AAB species have been found during fermentation, including *A. pasterianus, A. pomorum*, *A. ghanensis,* and *A. tropicalis.* Compared to Ardhana and Fleet (2003), the AAB species in this study showed greater diversity, with *A. ghanensis,* and *A. tropicalis* for example, not having been found by Ardhana and Fleet (2003). Only *A. pasterianus* and *A. pomorum* were dominant and found consistently until the end of fermentation. This finding was also confirmed by previous studies from Indonesia, Ghana and Malaysia (Ardhana and Fleet 2003; Camu et al. 2007; Meersman et al. 2013). The diversity of AAB species in cocoa bean box fermentation in Indonesia was less than previously reported in studies from Ghana (Camu et al. 2007; Nielsen et al. 2007), Brazil (Garcia-Armisen et al. 2010), Ecuador (Papalexandratou et al. 2011 d) and Malaysia (Meersman et al. 2013; Papalexandratou et al. 2013).

It is generally accepted from previous studies that *S. cerevisiae, L. plantarum, L. fermentum* and *A. pasteurianus* are the key microbiota observed during cocoa bean fermentations

worldwide (Ardhana and Fleet 2003; Camu et al. 2007; Garcia-Armisen et al. 2010; Nielsen et al. 2007; Papalexandratou et al. 2011 a; Papalexandratou et al. 2011 c; Papalexandratou et al. 2013; Papalexandratou and Vuyst 2011). The activity of yeasts (particularly *S. cerevisiae*), LAB (particularly *L. plantarum,* and *L. fermentum*) and AAB (particularly *A. pasteurianus*) is correlating to the sequence of microbial communities during cocoa bean fermentation described in literature (Camu et al. 2007; Lima et al. 2011; Schwan and Wheals 2004).

The intense metabolic activity and secretion of pectinolytic enzymes by yeasts and eventually molds leads to the degradation and drainage of pulp. After 48 h of fermentation, approximately 80% of the pulp was degraded, a condition in which yeasts and molds can come into close contact with the slime of the cocoa bean seed, leading to the death of yeasts and molds. Yeasts producing pectinolytic enzymes (*Kluyveromyces marxianus* and *S. cerevisiae* var. *chevalieri*) were reported before (Schwan et al. 1995; Schwan et al. 1997; Jespersen et al. 2005). Ardhana and Fleet (2003) reported also molds (*P. citrinum*) producing pectinolytic enzymes.

During fermentation, the concentration of citric acid decreased as the result of the assimilation of citrate by *L. plantarum* during the early phases of fermentation (Ardhana and Fleet 2003; Camu et al. 2007; Palles et al. 1998; Schwan and Wheals 2004; Thompson et al. 2001). The consumption of citrate by *L. plantarum* also explains the increase in pH during fermentation (Camu et al. 2007; Schwan and Wheals 2004; Thompson et al. 2001). The concentration of succinic acid also increased after 24 h of fermentation. LAB producing succinic acid from citric acid has been previously reported (Kaneuchi et al. 1988).

The concentration of mannitol was found to be increased after 24 h of fermentation due to the consumption of fructose by *L. fermentum* and its conversion into mannitol. The production of mannitol enables the production of acetic acid and extra ATP, contributing to both volatile acidity and enhanced growth competitiveness, respectively (Camu et al. 2007; Wisselink et al. 2002). Further, the concentration of volatile acids likely influences the quality of fermented cocoa beans (Camu et al. 2007; Holm et al. 1993; Jinap and Zeslinda 1995).

The concentration of acetic acid increased after 48 h of fermentation, paralleled by the increased population of AAB. At the end of fermentation after about 72-96 h, the concentration of acetic acid increased and the temperature was increased to 40-45 °C. Part

of the acetic acid volatilized, while another part penetrated into the cotyledons of the beans and was, together with part of the ethanol and the heat, responsible for certain changes in the subcellular structure of the beans as well as killing the cocoa seed embryo (de Brito et al. 2001), a processes which represents an important end point of fermentation (Camu et al. 2007).

Bean death is a prerequisite for certain biochemical reactions responsible for the formation of chocolate flavour precursors. Bean death usually occurs on the second day of fermentation and is mainly caused by an increase in internal acetic acid concentration (Camu et al. 2007). The production of ethanol during the anaerobic growth of yeasts correlates very closely with the death of the seed embryo resulting in a total inability of the seeds to germinate occurring about 24 h after maximum concentrations of ethanol are attained within the bean cotyledons (Thompson et al. 2001).

5.2 Diversity and characterization of yeasts, lactic acid bacteria and acetic acid bacteria in spontaneous cocoa bean fermentations carried out in Jember, Indonesia; Mocache, Ecuador; Santo Domingo, Trinidad & Tobago

Cocoa bean fermentation has a serious impact on the formation of the characteristic flavour and color precursors of cocoa (De Vuyst et al. 2010; Schwan and Wheals 2004). In addition, fermentation practices such as methods used and the duration of the fermentation may influence the nature and course of local cocoa bean fermentations (Wood and Lass 2008). Therefore, extensive studies have been conducted to determine the influence of external factors on the fermentation process in order to improve traditional practices so that the best final products can be achieved (Camu et al. 2008; Camu et al. 2007; Nielsen et al. 2007; Schwan 1998).

Most of the yeast species isolated from all regions (Tab. 4.4) were only found during the early phase of the fermentation, due to their physiological ability to grow in the cocoa bean environment during fermentation. However, *S. cerevisiae* seems to be more tolerant to acid, ethanol and heat, as it can still be isolated until the end of fermentation, as seen in the

samples from Indonesia and Ecuador. None of the yeast species isolated during the early phases of fermentation were able to grow at 35 °C and 15% ethanol. This explains why these species can not be isolated after 48 h of fermentation. *S. cerevisiae* and *P. kudriavzevii* were able to grow at 45 °C, pH 2.5 and 15% ethanol concentration (Tab. 4.7).

The diversity of yeast species present during cocoa bean fermentation in Ecuador was greater compared to that in Indonesia and Trinidad & Tobago (Tab. 4.4). None of the yeast species isolated during early phases of cocoa bean fermentation from Ecuador including *B. albus, C. parapsilosis* (synonym *C. quercitrusa*), *C. tropicalis, C. laurentii, M. pulcherrima, R. mucilaginosa, T. delbrueckii* and *W. anomalus* were able to grow at 35 °C and 15% ethanol. This explains why these species could not be isolated after 48 h of fermentation (Tab 4.7).

During early phases of fermentation in cocoa bean fermentations from Ecuador, fermentative yeasts such as *H. guilliermondii, T. delbrueckii, C. tropicalis*, and *C. parapsilosis* (synonym *C. quercitrusa*) could be isolated, as well as respiratory yeasts species such as *R. mucilaginosa*.

B. albus, *M. pulcherrima* and *R. mucilaginosa* represented the main yeast species involved in cocoa bean fermentations from Ecuador. The latter has not yet been reported for cocoa bean fermentations. *B. albus* was originally isolated from straw of *Hordeum jubatum* in the USA (Boekhout et al. 1991). *M. pulcherrima* was originally isolated from grapes in California (Mrak and McClung 1940). *C. parapsilosis* (synonym *C. quercitrusa*) and *C. laurentii* were isolated during earlier phases of cocoa bean fermentations from Ecuador, though species had not been previously reported by Papalexandratou et al. (2011 d) in Ecuadorian cocoa bean fermentations.

In contrast to cocoa bean fermentations from Indonesia and Trinidad & Tobago, fermentation in Ecuador was dominanted by *W. anomalus* during the earlier phases of fermentation. *W. anomalus* is frequently associated with spoilage or processing in food and grain products. Its ability to grow on a wide range of carbon sources at low pH under high osmotic pressure and with little or no oxygen enables it to propagate in a wide range of environments (Passoth et al. 2006). It is a non *Saccharomyces* wine yeast that contributes to the wine aroma observed in the production of volatile compounds. In recent years it has

been used as a biocontrol agent against other fungi due to its ability to produce mycocin killer toxins (Naumov et al. 2001; Wang et al. 2009). It has also been studied for its cyanide-resistant alternative oxidase activity (Minagawa et al. 1990; Minagawa and Yoshimoto 1987) and it possesses an active beta-glucosidase that plays a role in wine fermentations (Swangkeaw et al. 2011).

In the present study, *H. thailandica, I. orientalis, P. kudriavzevii,* and *S. roseus* were only isolated out of cocoa bean fermentations from Indonesia. During earlier phases of fermentation, fermentative yeasts such as *S. cerevisiae, H. guilliermondii, T. delbrueckii, C. tropicalis,* and *I. orientalis* could also be found. *I. orientalis,* a citric acid-consuming yeast, could be isolated during early phases of fermentation. The main activity of *I. orientalis* is to break down the citric acid in the pulp, leading to an increase in the pH of the pulp during fermentation (Jespersen et al. 2005; Schwan and Wheals 2004). *S. roseus* was isolated only in the end of fermentation, probably due to contamination from the fermentation box. *P. kudriavzevii* has been reported to be an acid- and ethanol-tolerant yeast species (Okuma et al. 1986; Daniel et al. 2009). It ferments reducing carbohydrates at a slower rate than *S. cerevisiae*, but it is able to use malic acid efficiently, in contrast to *S. cerevisiae* (Kim et al. 2008). Although *P. kudriavzevii* partially reduces citric acid, cocoa-originating *H. opuntiae* and *S. cerevisiae* isolates do not assimilate citric acid, indicating a possible role of *P. kudriavzevii* in citric acid conversion in addition to that of LAB (Camu et al. 2007, 2008 a, b; Daniel et al. 2009).

H. guilliermondii was more prevalent because of its ability to survive in the cocoa bean mass environment (Tab. 4.7). *H. thailandica* represented the main yeast species involved in the Indonesian fermentations and has not yet been reported before for Indonesian cocoa bean fermentations by Ardhana and Fleet (2003). *H. thailandica* was originally isolated from insect frass in Khao-Yai National Park, Thailand (Jindamorakot et al. 2007).

Compared to the microbial diversity in cocoa bean fermentations from Indonesia and Ecuador, fermented cocoa beans from Trinidad & Tobago showed a lower level of yeast diversity but strains of *S. cerevisiae* and *H. guilliermondii* could be isolated. Smaller numbers of yeasts were observed in the fermentation of cocoa beans from Trinidad & Tobago

(Tab. 4.4), due to the high fermentation temperatures, which can reach up to 56 °C (Ostovar and Keeney 1973).

In general, a limited number of yeast species was found in cocoa bean fermentations from different cocoa-producing regions, with *H. guilliermondii* being the prevailing species during the initial phase of fermentation before being replaced by *S. cerevisiae* which dominates until the end of fermentation. In the present study and based on the samples analyzed, *S. cerevisiae* was the predominant yeast species found in most fermentations. *S. cerevisiae* and *P. kudriavzevii* were able to grow at 45 °C, pH 2.5 and 15% ethanol concentration (Tab. 4.7). This explains why *S. cerevisiae* was the dominant species until the end of fermentation.

As pulp drains away, more air penetrates the fermenting mass. Ethanol is still produced through yeast activity, and both temperature and pH increase up to 40 °C and pH 4.0, respectively. These conditions are favorable for the growth of lactic acid and acetic acid bacteria. The primary activity of LAB is to metabolize pulp sugars (glucose, fructose) and some organic acids (citric acid, malic acid) by homofermentative and heterofermentative mechanisms, into lactic acid, acetic acid, ethanol, mannitol and carbon dioxide. These end products have an impact on the acidity and quality of the resulting bean (Axelsson 2004; Schwan and Wheals 2004; Thompson et al. 2001).

The diversity of LAB species found during cocoa bean fermentations from Ecuador was greater than that out of the Indonesian and Trinidad & Tobago samples (Tab. 4.5). During early phases of Ecuadorian cocoa bean fermentation both, *L. plantarum* and *L. fermentum*, as well as *L. pseudomesenteroides* could be isolated. *P. acidilactici* and *P. pentosaceus* also prevailed during the later phase of fermentation. Cocoa beans from Ecuador were fermented by heterofermentative LAB species during the early phase. Afterwards, homofermentative LAB species were found to dominante. Lactic acid can be produced by either homofermentative metabolism, with a yield higher than 85%, or heterofementative metabolism, with a yield of only 50% (Axelsson 2004).

The ability of LAB species to adapt to the conditions of fermenting mass environment explains how they can survive until the end of fermentation. *L. fermentum, L. plantarum,* and *P. acidilactici* grew at 45 °C, while isolates of *P. pentosaceus* showed weak growth at

45 °C. Species of *L. fermentum, L. plantarum, P. acidilactici* and *P. pentosaceus* have the ability to grow at pH 3.5 and 10% ethanol (Tab. 4.8).

LAB grew during the early phase of cocoa bean fermentation in Indonesia. Only a small number of LAB species was found in samples from Indonesia cocoa bean fermentations including *L. plantarum, L. fermentum* and *L. brevis*. Both *L. plantarum* and *L. fermentum* were counted in high numbers during fermentation, while *L. brevis* could be temporarly isolated during fermentation. All of the LAB species involved in cocoa bean box fermentations from Indonesia were heterofermentative species. *L. plantarum* and *L. fermentum* have the ability to grow at 45 °C, pH 3.5 and 10% ethanol, while *L. brevis* did not grow at 45 °C, and 10% ethanol. This explains why only *L. plantarum* and *L. fermentum* can survive until the end of fermentation (Tab. 4.8).

Similar to fermented cocoa beans from Ecuador, fermented cocoa beans from Trinidad & Tobago were fermented by heterofermentative and homofermentative LAB species.

A number of LAB species was found in different cocoa bean fermentations in different cocoa-producing regions, with *L. plantarum* and *L. fermentum* being the prevailing species in fermentations in all regions. Homofermentative LAB species such as *P. acidilactici* and *P. pentosaceus* were found to dominate during late phases of fermentation in cocoa bean fermentations from Ecuador.

The intensive activity of yeasts and LAB in the metabolization of pulp sugar and acid causes the pulp to drain away. This leads to an increase in temperature to above 37 °C, a result of the transfer of the cocoa beans every 24 h, which provides better aeration to cocoa bean mass. This condition favours the growth of AAB. The main activity of AAB is to oxidize ethanol, initially formed by the yeasts, into acetic acid, which provides them with necessary energy. This exothermic reaction is mainly responsible for the rise in temperature of the fermenting mass.

Fermented cocoa beans from Indonesia showed the greatest diversity of AAB species compared to samples from Ecuador and Trinidad & Tobago (Tab. 4.6). *A. pasteurianus* was the only AAB species isolated from cocoa bean fermentations from Ecuador and Trinidad & Tobago. *A. pasteurianus* and *A. pomorum* were consistently isolated during the course of

cocoa bean box fermentations from Indonesia. Only *A. pasteurianus* was isolated consistently from fermentations from all regions (Tab. 4.6).

Only a strain of *A. pasteurianus* showed weak growth at 45 °C. *A. ghanensis* and *A. pomorum* did not grow at 45 °C. *A. pasteurianus, A. ghanensis, A. pomorum,* and *A. tropicalis* showed growth at pH 5.0 and 3.5, but not at pH 2.5. *A. pasteurianus, A. ghanensis,* and *A. tropicalis* grew at 5 and 10% ethanol, while *A. pomorum* grew weakly at 10%. None of these species grew at 15% ethanol. This fact explains why only *A. pasteurianus* prevailed during the course of fermentation and survived until the end of fermentation in all regions (Tab. 4.9).

5.3   Development of a starter culture at laboratory level for improved quality of cocoa bean fermentation products

The metabolic products of cocoa bean fermentation depend on the microbial population present during fermentation. The microbial activity in cocoa bean pulp is carried out by a well-defined succession of microorganisms, led by yeasts, which dominate the total microbial population during the first hours. These are then surpassed by LAB, which in turn decline after 48 h of fermentation in favour of the vigorous development of AAB (Lima et al. 2011). The metabolic activity of yeasts in the cocoa bean pulp leads to the production of ethanol, carbon dioxide, acids, and volatile compounds, with a concomitant increase in temperature. The primary metabolic activity of LAB is to produce lactic acid, ethanol, acetic acid and other organic acids, as well as glycerol, mannitol, carbon dioxide and volatiles. The metabolism of AAB is shifted towards the utilization of ethanol as a main carbon source. AAB can oxidize ethanol to acetic acid, and species from the genus *Acetobacter* have the additional capacity to oxidize acetic acid to carbon dioxide and water (Lima et al. 2011).

Fermentation is the key in developing cocoa and chocolate flavour. Chocolate flavour is an extremely complex mixture of more than 550 compounds, and as analytical methods improve this number increases (Nijssen et al. 1996). Whereas undesirable bitter and astringent tastes decrease during fermentation, desirable fruity, floral and cocoa flavours develop during fermentation and drying. Essential precursors of the cocoa-specific flavour components are generated during the microbial fermentation of the pulp surrounding the

beans as well as by enzymatic processes in the beans (Biehl et al. 1985; Camu et al. 2008; Camu et al. 2007; de Brito et al. 2001; Hansen et al. 1998; Rohan 1964). The optimal activity of the endogenous enzymes is influenced by fermentation temperature and pH, which increase and decrease in the beans during the course of fermentation, as well as by the diffusion of ethanol, acetic acid, and to a lesser extent lactic acid into the beans and polyphenols and alkaloids out of the beans (Camu et al. 2008; Hansen et al. 1998; Wollgast and Anklam 2000).

Based on previous results from this study (Section 4.1) concerning the dynamics and biodiversity of the populations of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) involved in the spontaneous box fermentation of cocoa beans in PTPN XII, East Java, Indonesia a cocoa bean fermentation method (aerobic and anaerobic fermentation) in laboratory scale was developed. A starter culture under controlled and sterile conditions was used to investigate the influence of starter cultures and fermentation methods.

No significant difference was observed between the aerobic and anaerobic fermentations during the first 48 h of fermentation. After 48 h, the growth of *S. cerevisiae, L. fermentum* and *A. pasteurianus* was higher in the aerobic fermentation. Particularly *A. pasteurianus* grew to a higher concentration of cells and longer during fermentation because of the availability of oxygen during fermentation. Conversely, the growth of *S. cerevisiae, L. fermentum* and *A. pasteurianus* was lower in the anaerobic fermentation, particularly *A. pasteurianus* as this species represents an obligate aerobic bacterium.

Glucose, fructose and citric acid were utilized during fermentation, both in the aerobic and anaerobic fermentation. The consumption of glucose, fructose and citric acid in aerobic fermentation was greater than in the anaerobic fermentation, since the growth of *S. cerevisiae, L. fermentum* and *A. pasteurianus* was greater. Further, the production of ethanol, lactic acid and acetic acid in the pulp and beans was also greater in the aerobic fermentation, resulting in larger populations of *S. cerevisiae, L. fermentum* and *A. pasteurianus* during fermentation.

It is known, that well-fermented cocoa contains about 8-14 mg g^{-1} ffdm and unfermented cocoa contains as low as 2-4 mg g^{-1} ffdm free amino acids. Contents as high as 25 mg g^{-1} ffdm occur in fermented cocoas are reported in this study. Fermented cocoa beans from

Madagascar, Papua New Guinea and Java, Indonesia were also reported to contain high amounts of free amino acids (Rohsius et al. 2006).

These results show clearly that the content of free amino acid differs between fermentation methods. In general, the number of free amino acids increased during fermentation, both in the aerobic and anaerobic fermentation. The production of free amino acids was greater in the aerobic fermentation compared to the anaerobic fermentation. The largest amount of free amino acids seen in the aerobic fermentation was 25 mg g^{-1} ffdm after 120 h of fermentation, whereas the largest amount of free amino acids in the anaerobic fermentation was 20 mg g^{-1} ffdm after 96 h of fermentation. This amount of free amino acids was relatively higher, compared to the 8-14 mg g^{-1} ffdm (Rohsius et al. 2006).

The production of hydrophobic amino acids increased during fermentation, both in the aerobic and anaerobic fermentation, reaching a maximum after 72 h of fermentation in the aerobic fermentation and after 96 h in the anaerobic fermentation. Conversely, acidic amino acids decreased during fermentation, both in the aerobic and anaerobic fermentation. Acidic amino acids decreased after 72 h of fermentation, both in the aerobic and anaerobic fermentation. The increase in hydrophobic amino acids and hydrophilic oligopeptides is due to cocoa beans proteolytic activity (Biehl et al. 1993; Camu et al. 2007; Hansen et al. 1998; Voigt et al. 1994). Differences in these enzyme activities exist between cocoa genotypes, but they are not linked to differences in cocoa flavour formation (Hansen et al. 1998; Lefeber et al. 2012).

In general, proteolysis depends primarily on the fermentation conditions, specifically the duration and intensity of acidification, temperature, and aeration (de Brito et al. 2001; Kirchhoff et al. 1989). Oxidation, condensation, and complexation (with polypeptides) of polyphenols can also play a role (de Brito et al. 2001; Jinap et al. 2003). Fermentation conditions determine the amount of free amino acids, oligopeptides, reducing sugars, and polyphenols of fermented, dried cocoa beans, which all play an important role in aroma precursor formation during cocoa processing (de Brito et al. 2001; Thompson et al. 2001). The metabolite results presented in this paper in combination with literature data indicate that a successful fermentation process is reached after about 72 h of fermentation (Camu et al. 2007).

In general, the presence of polyphenols in cocoa beans is dependent on several factors, including degree of pod ripeness, cocoa variety, processing, and storage (Camu et al. 2008; Hammerstone et al. 1999; Nazaruddin et al. 2006; Porter et al. 1991). However, there are no qualitative differences in polyphenol compounds in cocoa beans, in spite of their genetic origin (Camu et al. 2008).

The total polyphenol content decreased throughout fermentation by approximately 10–50% during 48 h of fermentation, both in the aerobic and anaerobic fermentation. The concentration of polyphenols was higher in the anaerobic fermentation. Losses of polyphenols differed between the aerobic and anaerobic fermentation, indicating slight differences in population dynamics during fermentation. The decrease in polyphenol concentration is due to their diffusion out of the bean (through water release) and further oxidation and condensation of the polyphenol compounds (Jinap et al. 2002; Jinap et al. 2003; Nazaruddin et al. 2006; Wollgast and Anklam 2000). Oxidase reactions are both non-enzymatic and catalyzed by polyphenol oxidase. This polyphenol oxidase is strongly inactivated during the first days of anaerobic (yeast) fermentation at a temperature of 28 – 35 °C, retaining only 50 and 6% of initial enzyme activity after 1 and 2 days, respectively (Camu et al. 2008; Hansen et al. 1998).

Although a higher concentration of polyphenols would decrease cocoa flavour (Jinap et al. 2004), efforts are being made to maintain their levels while avoiding taste problems, as there are abundant health benefits ascribed to polyphenols (Schroeter et al. 2006; Sies et al. 2005; Wollgast and Anklam 2000). The polyphenol concentrations have no influence on the acidity, fruity/floral flavour, raw/green, and mouldy/earthy properties of chocolate (Camu et al. 2008; Jinap et al. 2004).

Aerobic fermentation was characterized by a high amino acids and a low polyphenols content in the fermented beans. This type of bean showed better parameters for the production of flavour in chocolate. In contrast, anaerobic fermentation was characterized by high polyphenol content, resulting in healthy raw bean for chocolate production.

5.4 Investigation of activity and characterization of antifungal proteins in the seed coat slime of *Theobroma cacao* L.

Disk diffusion agar and microdilution were both successfully used for the antifungal assay, although microdilution was more sensitive than disk diffusion test. The advantage of the disk-diffusion agar method is its simplicity and low cost as well as the possibility of testing up to six extracts per plate against a single microorganism using small sample volumes (Hadacek and Greger 2000).

The disadvantage of the disk diffusion agar method is that the potency of different samples may not always be proper, mainly because of differences in physical properties, such as solubility, volatility and diffusion in agar. Compounds with a high diffusion coefficient or solubility and low antimicrobial activity may penetrate the agar rapidly, even in small amounts, and give zones like those of active compounds with poor penetration. This problem is encountered when zones of inhibition are compared for different classes of compounds. Additionally, the size of the inhibition zones might be influenced by the volatilization of antimicrobial substances, disk size, the amount of compound added to the disk, adsorption by the disk, the type of agar, agar strength, pH, the volume of agar, and microbial strains used (Pauli 2006; Scorzoni et al. 2009).

In the dilution method, each compound is mixed with an appropriate medium that has previously been inoculated with the fungal strain. It is thus possible to determine the minimal inhibitory concentration (MIC), which is defined as the lowest concentration capable of inhibiting any visible fungal growth. Turbidity due to growth can be estimated visually or more accurately by measuring the optical density at 490nm. The liquid-dilution method can also be used to determine whether a compound or extract has a fungicidal or fungistatic action at a given concentration. This test yielded the most reproducible results for the MIC and was recommended as the general standard method for testing natural products (Hadacek and Greger 2000).

In the case of antifungal activity assay for the seed coat slime of *Theobroma cacao* L., both the disk diffusion agar and microdilution were used successfully, with microdilution being

more sensitive than disk diffusion. A wide variety of fungi was inhibited by antifungal proteins from seed coat slime of *Theobroma cacao* L.

Generally, individual fungi within the same taxon reacted similarly to *Theobroma cacao* L. seed coat slime. The size of the inhibition zones varied for different fungi, indicating differences in sensitivity to the levels of inhibitory substances among the test organisms likely due to a concentration gradient of the substances that presumably developed in the agar around the seeds. Zones of inhibition were very diffuse for certain fungi, suggesting an effect on fungus growth rate rather than total inhibition of growth.

Some species of fungi were inhibited by seed coat slime. The inhibition activity depends on the composition of the fungal cell wall. Fungi with cell walls containing β-glucan and chitin showed greater sensitivity to seed coat slime antifungal proteins such as chitinase and glucanase. Chitinase and glucanase act synergistically to hydrolyze the structural (1,3) β-glucan and chitin present in the fungal cell wall, particularly at the hyphal apex of filamentous fungi where glucan is most exposed. This weakened cell wall results in cell lysis and cell death (Selitrennikoff 2001). On the other hand, fungi with cell walls containing chitosan (e.g. *Mucor* sp., *Rhizopus oligosporus*) are more resistant to seed coal slime, as seed coat slime does not contain chitosanase.

Seed coat slime from *Theobroma cacao* L. inhibited the growth of a number of fungi such as *A. niger, A. flavus, A. parasiticus, M. isabellina, P. citrinum, P. purpurogenum* and *P. roquefortii* as well as a number of yeasts such as *C. krusei, C. lipolytica, C. guilliermondii, C. laurentii, R. mucilaginosa, R. rubra, S. cerevisiae* and *S. pombe*. The minimum inhibitory concentration (MIC) found for seed coat slime was 25 mg mL^{-1} against fungi (e.g. *P. citrinum, A. niger, P. purpurogenum*) and 10 mg mL^{-1} against yeasts (e.g. *S. cerevisiae, R. rubra, C. lipolytica*). These results suggest the opportunity to use seed coat slime for a wide array of applications such as an antifungal agent against fungi causing black pod disease in *Theobora cacao* L. tree, as well as in pharmaceutical or therapeutic applications for combating fungi that are pathogenic for humans. This is the first report of antifungal protein activity from the seed coat slime of *Theobroma cacao* L. Future research is needed for the screening of antifungal activities from different cocoa varieties, the purification and characterization of

antifungal proteins or compounds from seed coat slime and the establishment of methods for MIC test against pathogenic fungal for medical applications.

Analyses of proteins using nano-electrospray mass spectrometry (Q-TOF II) showed the existence of three proteins in the cocoa slime that have antifungal effect, namely osmotin 24.1 kDa (6), chitinase 28.8 and 31.6 kDa (7, 8) and glucanase 33 kDa (9).

Osmotin is an antifungal protein belonging to the PR-5 (TL) family of proteins. TL proteins have been isolated from *Arabidopsis thaliana*, corn, soybean, rice, wheat, tobacco, tomato, pumpkin, bean, barley, flax, and many other plants. The majority of PR-5 proteins have molecular masses of 22 kDa and are stabilized by eight disulfide bonds. This highly stabilized structure allows PR-5 proteins to be very resistant to protease degradation. Although the precise mechanism of the action of PR-5 proteins is not completely understood, a number of interesting observations have been made that may eventually lead to a unified hypothesis for the mechanism how these proteins function to kill fungi. First, several TL proteins cause cell permeability changes in fungal cells with a cell wall, but have no or little effect on protoplasts (Roberts and Selitrennikoff 1990). For example, zeamatin (a TL protein from corn) causes very rapid cell lysis in *Neurospora crassa*, even at 4 °C; lysis occurred primarily at subapical regions (Roberts and Selitrennikoff 1990). Second, osmotin, a TL protein from tobacco, causes perturbations in the regulation of fungal cell wall assembly (Yun et al. 1998; Yun et al. 1997). Regardless of the precise mode of action of TL proteins, they are fungicidal against a wide number of plant and human pathogens in vitro. Most importantly, one protein, zeamatin, has shown efficacy in a murine vaginal model of *Candida albicans* infection (White et al. 2002). PR-5 proteins may be used in the future for the development of human therapeutics.

Chitinase is an antifungal protein belonging to PR-3 family of proteins. A number of enzymatic assays have shown PR-3 proteins to have in vitro chitinase activity. Most PR-3 proteins have molecular masses between 26 and 43 kDa (Nielsen et al. 1997; Watanabe et al. 1999). Chitinases have been isolated from fungi, tobacco, cucumber, beans, peas, grains, and many others, and have potent antifungal activity against a wide variety of human and plant pathogens, including *Trichoderma reesei*, *Alternaria solani*, *Alternaria radicina*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Guignardia bidwellii*, *Botrytis cinerea*, and *Coprinus*

comatus. By analogy with β-glucanases, the mode of action of PR-3 proteins is relatively straightforward: PR-3 proteins are endochitinases that cleave cell wall chitin polymers in situ, resulting in a weakened cell wall and rendering fungal cells osmotically sensitive. Not surprisingly, PR-2 (β-glucanases) and PR-3 (chitinases) proteins act synergistically in inhibiting fungal growth, both in vitro and in planta (Jach et al. 1995; Selitrennikoff 2001).

Glucanase is an antifungal protein belonging to the PR-2 family of proteins (β-glucanases). PR-2 proteins have (1,3) β-endoglucanase activity in vitro and have been grouped into three classes based on amino acid sequence analysis. Class I glucanases are basic proteins of 33 kDa and are found in the plant vacuole. Classes II and III include acidic, extracellular proteins of about 36 kDa. The major structural difference between class I proteins and the other two classes is that class I proteins are synthesized as preproproteins that are processed prior to being enzymatically active. PR-2 proteins have been found in a wide variety of plants, including tobacco, *Arabidopsis thaliana*, peas, grains, and fruits (Cote et al. 1991; Kim and Hwang 1997). The proteins are active in vitro at micromolar levels (~50 μg mL^{-1}) against a wide number of fungi, including human and plant pathogens (e.g., *Rhizoctonia solani, C. albicans*, and *Aspergillus fumigatus*). The antifungal activity of plant (1,3) β-glucanases is thought to occur by PR-2 proteins hydrolyzing the structural (1,3) β-glucan present in the fungal cell wall, particularly at the hyphal apex of filamentous molds where glucan is most exposed. This weakened cell wall results in cell lysis and cell death (Selitrennikoff 2001).

The seed coat proteins seem to be degraded during the fermentation process, probably caused by an increase in temperature and by activity of proteases produced by microbes, particularly *Bacillus* sp., during the fermentation. This was confirmed by the heat stability test of seed coat slime, whereas the seed coat slime was shown to be not stable for 24 h at 40 and 50 °C. The temperature of fermentation mass increased up to 45 – 50 °C for the last 2 days of fermentation, during which time the population of *Bacillus* species increased as well (Ardhana and Fleet 2003; Camu et al. 2007; Nielsen et al. 2007).

5.5 Investigation of the influence of seed coat slime slime on growth of starter culture and physical and chemical changes during cocoa bean fermentation at laboratory level

It is well known that after 2 days of fermentation alcoholic fermentation by yeasts is extremely decreased (Ardhana and Fleet 2003; Camu et al. 2007; Nielsen et al. 2007; Papalexandratou et al. 2011 c; Schwan and Wheals 2004). Until recently, the reason for this extreme decrease was unknown. Many researchers argue that the collapsing of yeasts after 2 days of fermentation is probably caused by the physiological adaptation of yeasts to heat, acid and ethanol concentrations during fermentation.

In this study, it was found that the seed coat slime of *Theobroma cacao* L. inhibits a wide variety of yeasts commonly found during cocoa bean fermentation.

In this treatment, 90% of cocoa bean pulp was reduced from the bean. After removal of 90% of the pulp in fermentations using a defined starter the concentration of substrates, particularly glucose and fructose, decreased by about 75% at onset of fermentation compared to the control. The concentration of organic acids, particularly citric acid was similar to the control. The reduced concentration of sugar at the onset of fermentation affected the growth of the starter culture and hence the metabolic products created during fermentation.

The growth of the starter culture in fermentations with only 10% pulp was lower compared to the control (fermentation with pulp), particularly for *S. cerevisiae* in consequence of the reduction of sugar concentration at the onset of fermentation and the activity of antifungal proteins from seed coat slime.

No significant difference was found between fermentations with complete pulp and with only 10% pulp. 90% of cocoa pulp was reduced, but the concentration of citric acid was similar between fermentation with complete pulp and with only 10% pulp.

Glucose, fructose and citric acid were utilized during fermentation, whereas ethanol, lactic acid and acetic acid were produced during fermentation. The production of ethanol was lower in fermentation with only 10% pulp, due to the lower amount of cells of *S. cerevisiae*.

The concentration of lactic acid and acetic acid was relatively similar to the fermentation with complete pulp. This means there was no significant effect to be seen by reducing cocoa seeds pulp at the onset of fermentations.

The concentration of amino acids was relatively similar to that in the control, no significant effect was found concerning the concentration of amino acids between fermentation with complete pulp and with only 10% pulp. This is because the metabolized amino acids were produced by endogenous enzymes, and the activity of these enzymes depends on pH during fermentation. There was no significant difference in pH during fermentation between fermentation with complete pulp and with only 10% pulp.

6   Summary (Zusammenfassung)

6.1   Summary

Cocoa beans are the principal raw material used in chocolate production. These seeds are derived from the fruit pods of the cocoa tree (*Theobroma cacao* L.), which is cultivated in plantations in the equatorial zone, with Ivory Coast, Ghana and Indonesia as the major producers. Raw cocoa has an astringent unpleasant taste and a spontaneous fermentation is the first step in a process leading cocoa beans to develop their characteristic cocoa flavour and taste. The quality of raw cocoa beans is highly dependent on the post-harvest practice of fermentation. Three main phases occur during cocoa bean fermentation: phase 1 anaerobic fermentation by yeasts, phase 2 microaerophilic fermentation by lactic acid bacteria and phase 3 aerobic fermentation by acetic acid bacteria.

The quality of fermented cocoa beans varies between regions of cocoa producer countries, varieties of cocoa, fermentation methods, drying and cocoa beans storage. The fermentation itself depends on farm practices such as bean selection, mixing of the fermenting mass and the activity of microbes during fermentation. Cocoa bean fermentation is a spontaneous, uncontrolled process that results in end products of variable quality.

It is generally known that after 48 h of fermentation, depectinization of pulp and alcoholic fermentation by yeasts is extremely decreased. Until recently it was not known why this occurs. Up to date, the decreasing of yeasts has been most commonly attributed to the physiological adaptation of yeasts to the fermentation environment during fermentation in terms of heat, acid and ethanol tolerance.

During the first 24 h of fermentation, yeasts are the dominant species, with *Saccharomyces cerevisiae* being stably and consistenly present during fermentation. Lactic acid bacteria show dominant growth during 48-72 h of fermentation with the species of *Lactobacillus plantarum* and *Lactobacillus fermentum* being dominant. Acetic acid bacteria dominate the last stage of fermentation, where only species of *Acetobacter pasterianus* remain consistent and stable. Glucose, fructose and citric acid of the pulp are metabolised during fermentation. The metabolic activity of microbes during fermentation produces ethanol, lactic acid, mannitol, acetic acid, as well as heat. Ethanol, acetic acid, lactic acid and heat

diffuse into the bean, causing the death of the bean embryo.

Further results indicated the presence of microbial diversity between different regions of cocoa bean fermentations. Some species were shown to be present in the cocoa bean fermentations from all investigated regions. Fermented cocoa beans from Ecuador showed the highest diversity of yeasts and lactic acid bacteria compared to Indonesia and Trinidad & Tobago, but the diversity of acetic acid bacteria species was highest in cocoa bean fermentations from Indonesia. Based on the physiological characterization of dominant species from all cocoa bean fermentation regions, the use of *Saccharomyces cerevisiae* IDI-Y001, *Lactobacillus fermentum* IDE-L007, and *Acetobacter pasteurianus* IDI-A019 is suggested as the best combination for use as a starter culture for cocoa bean fermentation process.

The development of laboratory fermentation methods showed differences between aerobic and anaerobic methods. Aerobic fermentation methods were characterized by higher organic acid concentrations at the end of fermentation and higher free amino acid concentrations. This is typical for fermented cocoa beans used for the production of chocolate rich in flavour. The anaerobic fermentation method was characterized by lower levels of organic acids and free amino acids, but higher polyphenol concentrations. This is typical for fermented cocoa beans used for the production of healthy chocolate.

A novel result of this study is the fact that the seed coat slime of *Theobroma cacao* L. possesses the ability to inhibit the growth of yeasts and molds. 25 mg mL^{-1} of seed coat slime inhibit the growth of molds (e.g. *Penicillium citrinum, Aspergillus niger, Penicillium purpurogenum*) and 10 mg mL^{-1} seed coat slime inhibit the growth of yeasts (e.g. *Saccharomyces cerevisiae, Rhodotorula rubra, Candida lipolytica*). Analyses of proteins using nano-electrospray mass spectrometry (Q-TOF II) showed that three proteins have antifungal effect, namely glucanase (33 kDa), chitinase (28.8 kDa) and osmotin (24.1 kDa). The seed coat slime proteins were not stable at 40 and 50 °C for 24 h.

Reduction of 90% of pulp can inhibit the growth of the starter culture, specifically growth of the yeasts after 48 h of fermentation, providing the existence of an inhibition by antifungal proteins from the seed coat slime.

6.2 Zusammenfassung

Kakaosamen sind das Rohmaterial für die Schokoladenproduktion. Diese Samen werden aus den Früchten des Kakaobaumes (*Theobroma cacao* L.) gewonnen, der in Plantagen der Äquatorialzone angebaut wird. Dabei stellen die Elfenbeinküste, Ghana und Indonesien die hauptsächlichen Kakaoproduktionsländer dar. Roher Kakao hat einen unangenehmen adstringierenden Geschmack und spontane Fermentationen stellen die erste Stufe in einem Prozess dar, um den charakteristischen Kakaogeschmack und das charakteristische Kakaoaroma zu entwickeln. Die Qualität der rohen Kakaosamen ist sehr stark von der Nacherntebehandlung, der Fermentation, abhängig. Während der Fermentation der Kakaosamen treten drei Hauptphasen auf: 1) eine anaerobe Fermentation durch Hefen, 2) eine mikroaerophile Fermentation durch Milchsäurebakterien und 3) eine aerobe Fermentation durch Essigsäurebakterien.

Die Qualität der fermentierten Kakaosamen variiert zwischen den Regionen der Kakao produzierenden Länder, den Kakaovarietäten, den Fermentationsmethoden, der Trocknung und der Lagerung der Kakaosamen. Die Fermentation selber hängt von den auf den Farmen durchgeführten Verfahren, wie der Auswahl der Samen, der Umwälzung der fermentierenden Masse und der mikrobiellen Aktivität während der Fermentation, ab. Grundsätzlich ist die Fermentation der Kakaosamen ein spontaner unkontrollierter Prozess, der zu Endprodukten mit unterschiedlicher Qualität führt.

Es ist generell bekannt, dass die alkoholische Gärung und der Pektinabbau durch die Hefen nach 48 Stunden Fermentation nachlässt. Die Ursache dafür war bis jetzt unbekannt. Bis jetzt ist man davon ausgegangen, dass die Reduktion der Hefepopulation durch die Fermentationsbedingungen, das heißt, durch eine geringe Hitze-, Säure- und Ethanoltoleranz der Hefen verursacht wird.

Während der ersten 24 Stunden stellen Hefen die dominanten Spezies dar, wobei *Saccharomyces cerevisiae* durchgehend stetig bei der Fermentation auftritt. Während eines Zeitraumes von 48 bis 72 Stunden zeigen Milchsäurebakterien ein dominantes Wachstum,

wobei die Spezies *Lactobacillus plantarum* und *Lactobacillus fermentum* besonders hervortreten. Essigsäurebakterien überwiegen im letzten Stadium der Fementation wobei die Spezies *Acetobacter fermentum* stetig und durchgehend auftritt. Während der Fermentation werden durch die Stoffwechselaktivität der Mikroorganismen Ethanol, Milchsäure, Mannitol, Essigsäure und Wärme erzeugt. Durch die Diffusion von Ethanol, Essigsäure und Wärme in die Samen hinein wird der Embryo im Samen abgetötet.

Weitere Ergebnisse zeigten die mikrobielle Diversität zwischen unterschiedlichen Regionen auf. Einige mikrobielle Spezies ließen sich in Kakaosamenfermentationen aus allen untersuchten Regionen nachweisen. Fermentierte Kakaosamen aus Ecuador zeigten die höchste Diversität an Hefen und Milchsäurebakterien im Vergleich zu fermentierten Kakaosamen aus Indonesien und aus Trinidad und Tobago.

Demgegenüber war die Diversität der Essigsäurebakterien aus Kakaofermentationen aus Indonesien am höchsten. Auf der Basis der physiologischen Charakterisierung der dominanten Spezies der untersuchten Kakaofermentationen aus allen drei Regionen wird die Kombination der Stämme *Saccharomyces cerevisiae* IDI-Y001, *Lactobacillus fermentum* IDE-L007 und *Acetobacter pasteurianus* IDI-A019 als die beste Kombination für eine Starterkultur für die Kakaofermentation vorgeschlagen.

Die Untersuchung von Laborfermentationsmethoden wies Unterschiede zwischen aeroben und anaeroben Verfahren auf. Aerobe Fermentationen wiesen zum Schluss höhere Konzentrationen an organischen Säuren und an freien Aminosäuren auf. Dieses ist typisch für fermentierte Kakaosamen, die für die Produktion von aromareicher Schokolade benutzt werden. Die anaeroben Fermentationen waren durch niedrige Konzentrationen an organischen Säuren und freien Aminosäuren gekennzeichnet, wiesen aber höhere Gehalte an Polyphenolen auf. Dieses ist typisch für fermentierte Kakaosamen, die für die Herstellung von Schokolade mit gesundheitlichen Effekten benutzt werden.

Ein neues Ergebnis der Arbeit ist die Erkenntnis, dass der Testa-Schleim von *Theobroma cacao* L. die Fähigkeit besitzt das Wachstum von Hefen und Schimmelpilzen zu inhibieren. 25 mg Testa-Schleim mL^{-1} inhibieren das Wachstum von Schimmelpilzen (z.B. *Penicillium citrinum*, *Aspergillus niger*, *Penicillium purpurogenum*) und 10 mg mL^{-1} hemmen das

Wachstum von Hefen (z.B. *S. cerevisiae*, *Rhodotorula rubra*, *Candida lipolytica*). Mit Hilfe der Nano-Electrospray-Massen-Spektroskopie konnte gezeigt werden, dass drei Proteinfraktionen aus dem Testa-Schleim einen antimykotischen Effekt haben, nämlich Glucanase (33 kDA), Chitinase (28,8 kDa) und Osmotin (24,1 kDa). Es konnte gezeigt werden, dass die Testa-Schleim-Proteine bei Lagerungen über 24 Stunden bei 40 ^{o}C und bei 50 ^{o}C nicht stabil waren.

Eine Verminderung der Pulpa um 90% kann das Wachstum der Starterkultur inhibieren. Insbesondere wird das Wachstum der Hefen nach 48 Stunden Fermentationszeit inhibiert, was einen Hinweis auf die Hemmung der Hefen durch die antimykotisch wirkenden Proteine des Testa-Schleimes darstellt.

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8 Figures and Tables

8.1 List of figures

8.2   List of tables

9 Appendix

9.1 Abbreviations

9.1.1 General abbreviations

aw	Water activity
CFU	Colony forming units
AAB	Acetic acid bacteria
ACN	Acetonitrile
APS	Ammonium persulfate
BLAST	Basic local alignment search tool
Da	Dalton (unit of molecular weight)
DMSZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DNA	Deoxyribonucleic acid
dNTP	Desoxyribonucleotide-5'-triphosphate
DTT	Dithiothreitol
E-cup	Eppendorf cup
e.g.	(lat.: *exempli gratia*) for example
EDTA	Ethylen diamin tetra acetate
et al.	(lat.: *et alii*) and others
etc.	(lat.: *et cetera*) and other things
EtOH	Ethanol
FA	Formic acid
ffdm	Fat-free dry mass
FTIR	Fourier-transfom infrared
GABA	gamma-Aminobutyric acid
GC	Guanine and cytosine content

GYPA	Glucose yeasts extract peptone agar
GYPB	Glucose yeasts extract peptone broth
h	Hour
H_2O	Distilled water
H_2O_{bidest}	Double distilled water
HCA	Hierarchical cluster analysis
HCl	Hydrogen chloride
HPLC	High Performance Liquid Chromatography
i.e.	id est/that is
ICCRI	Indonesian coffee and cocoa research institute
kb	Kilo base pairs
kDa	kilo Dalton (unit of molecular weight)
KOH	Potassium hydroxide
L	Liter
LAB	Lactic acid bacteria
LMH	(ger.: *Lebensmittelmikrobiologie Universität Hamburg*)
Log	logarithm
M	Molar concentration (molarity)
mA	Mili ampere
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
max.	Maximum
MEA	Malt extract agar
MEB	Malt extract broth
MeOH	Methanol
mg	Milligram

MIC	Minimum inhibitory concentration
min	Minute
min.	Minimum
mL	Milliliter
mM	Milli mole
mm	Mili meter
mol	Molar
MRSA	de Man Rogosa and Sharpe agar
MRSB	de Man Rogosa and Sharpe broth
MS	Mass spectrometry
Mw	Molecular weight
NA	Nutrient agar
NaCl	Sodium chloride
nt	Nucelotide
OD	Optical density
OD_{530}	Optical density at wavelength 530 nm
OD_{600}	Optical density at wavelength 600 nm
OPA	*O*-phthalaldehyde
PCA	Plate count agar
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
pmol	Picomole
ppm	parts per million
PTPN XII	(ina.: *PT. Perkebunan Nusantara XII*) Nusantara Plantation XII, Ltd. Co.
PVPP	Polyvinyl-polypyrrolidon
RAPD	Random amplified polymorphic DNA

RNA	Ribonucleic acid
RNAse	Ribonuclease
RP	Reverse-phase (a type of HPLC column)
rpm	Rounds per minute
Rt	Retention time
RT	Room temperature
SDS	sodium dodecyl sulfate
sec	Second
sp.	Species
spp.	species (plural)
T_{ann}	Annealing temperature
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TEMED	N,N,N´,N´-Tetramethylene ethylene diamine
T_m	Melting temperature
TPC	Total plate count
Tris	Tris-(hydroxymethylene)-aminoethane
U	Unit
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
YGCA	Yeast extract glucose chloramphenicol agar
YGCBA	Yeast extract glucose chloramphenicol bromophenol blue agar

µ	Micro (10^{-6})		
µg	Microgram		
µL	Microliter		

9.1.2 Abbreviations for amino acids

Ala	Alanine	Met	Methionine
Cys	Cysteine	Asn	Asparagine
Asp	Aspartic acid	Pro	Proline
Glu	Glutamic acid	Gln	Glutamine
Phe	Phenylalanine	Arg	Arginine
Gly	Glycine	Ser	Serine
His	Histidine	Thr	Threonine
Ile	Isoleucine	Val	Valine
Lys	Lysine	Trp	Tryptophan
Leu	Leucine	Tyr	Tyrosine

9.1.3 Abbreviations for microorganisms

9.1.3.1 Fungi

A. flavus	*Aspergillus flavus*
A. niger	*Aspergillus niger*
A. parasiticus	*Aspergillus parasiticus*
B. albus	*Bulleromyces albus*
C. krusei	*Candida krusei*
C. laurentii	*Cryptococcus laurentii*
C. lipolytica	*Candida lipolytica*
C. parapsilosis	*Candida parapsilosis*
C. pelliculosa	*Candida pelliculosa*
C. quercitrusa	*Candida quercitrusa*
C. tropicalis	*Candida tropicalis*
H. guilliermondii	*Hanseniaspora guilliermondii*
H. opuntiae	*Hanseniaspora opuntiae*
H. thailandica	*Hanseniaspora thailandica*
H. uvarum	*Hanseniaspora uvarum*
I. orientalis	*Issatchenkia orientalis*
M. isabellina	*Mortierella isabellina*
M. pulcherrima	*Metschnikowia pulcherrima*
P. citrinum	*Penicillium citrinum*
P. kudriavzevii	*Pichia kudriavzevii*
P. purpurogenum	*Penicillium purpurogenum*
P. roquefortii	*Penicilium roquefortii*

R. glutinis   *Rhodotorula glutinis*

R. mucilaginosa   *Rhodotorula mucilaginosa*

R. oligosporus   *Rhizopus oligosporus*

R. oryzae   *Rhizopus oryzae*

S. cerevisiae   *Saccharomyces cerevisiae*

S. roseus   *Sporobolomyces roseus*

T. delbrueckii   *Torulaspora delbrueckii*

W. anomalus   *Wickerhamomyces anomalus*

9.1.3.2   Gram negative bacteria

A. ghanensis   *Acetobacter ghanensis*

A. orientalis   *Acetobacter orientalis*

A. pasteurianus   *Acetobacter pasteurianus*

A. peroxydans   *Acetobacter peroxydans*

A. pomorum   *Acetobacter pomorum*

A. tropicalis   *Acetobacter tropicalis*

E. coli   *Eschericia coli*

G. frateurii   *Gluconobacter frateurii*

9.1.3.3 Gram positive bacteria

B. subtilis	*Bacillus subtilis*
L. acidophilus	*Lactobacillus acidophilus*
L. brevis	*Lactobacillus brevis*
L. casei	*Lactobacillus casei*
L. delbrueckii	*Lactobacillus delbrueckii*
L. fermentum	*Lactobacillus fermentum*
L. plantarum	*Lactobacillus plantarum*
L. reuteri	*Lactobacillus reuteri*
L. pseudomesenteroides	*Leuconostoc pseudomesenteroides*
M. luteus	*Micrococcus luteus*
P. acidilactici	*Pediococcus acidilactici*
P. pentosaceus	*Pediococcus pentosaceus*
P. freudenreichii	*Propionibacterium freudenreichii*
P. shermanii	*Propionibacterium shermanii*
S. aureus	*Staphylococcus aureus*
S. thermophilus	*Streptococcus thermophilus*

9.2 Hazardous chemicals

9.2.1 List of hazardous chemicals

Chemicals	Hazard symbol	Risk phrase	Safety phrase
1,3-Dimethylbarbituric acid	Xn	R22, R41	S26, S36/39
Boric acid	T	R60, R61	S53, S45
Calcium chloride dihydrate	Xi	R36	S22, S24
Claradiastase	Xn	R20, R36/37/38,R42/43	S22, S26, S36
Copper (II) sulphate	Xn, N	R22, R36/38,R50/53	S22, S60, S61
Diethyl ether	F+, Xn	R12, R19, R22,R66, R67	S16
Dinitrosalicylic acid	Xn	R22, R37/38	S22, S24/25
Ethanol	F	R11	S7, S16
Hydrochloric acid	C	R34, R37	S26, S45
Iron (II) sulphate heptahydrate	Xn	R22	
Kjeldahl tablets (free of Hg and Se)	N	R51/R53	S61
Manganese (II) sulphate monohydrate	Xn, N	R48/20/22, R51/53	S22, S61
Methanol	F, T	R11, R23/24/25,R39/23/24/25	S7, S16, S36/37,S45
Nicotinic acid	Xi	R36	S26
Papain	Xn	R36/37/38, R42	S22, S24, S26,S36/37

Chemicals	Hazard symbol	Risk phrase	Safety phrase
Phosphoric acid	C	R34	S26, S45
Polyoxyethylene lauryl ether	Xn	R22, R38, R41	S26, S36/37/39
Potassium cyanide – zinc cyanide solution	T, N	R23/24/25, R51/53	S36/37, S45, S61
Potassium ferricyanide	Xn	R20, R21, R22, R32	S26, S36
Pyridoxamine dihydrochloride	Xi	R36/37/38	S26, S36
Sodium hydroxide	C	R35	S26, S37/39, S45
Sulphuric acid	C	R35	R35
Takadiastase	Xn	R42	S22, S24, S36/37
Thiosemicarbazide	T+	R28	S22, S26, S36/37,S45
Trichloroacetic acid	C, N	R35, R50/53	S26, S36/37/39,S45, S60, S61
Zinc sulphate monohydrate	Xn, N	R22, R41, R50/53	S22, S26, S39,S46, S60, S61

9.2.2 Abbreviations and description of hazards

Abbreviation	Hazard	Description of hazard
F+	Extremely flammable	Chemicals that have an extremely low flash point and boiling point, and gases that catch fire in contact with air.
F	Highly flammable	Chemicals that may catch fire in contact with air, only need brief contact with an ignition source, have a very low flash point or evolve highly flammable gases in contact with water.
T+	Very toxic	Chemicals that at very low level cause damage to health.
T	Toxic	Chemicals that at low levels cause damage to health.
Xn	Harmful	Chemicals that may cause damage to health.
C	Corrosive	Chemicals that may destroy living tissue on contact.
Xi	Irritant	Chemicals that may cause inflammation to the skin or other mucous membranes.
N	Dangerous for the environment	Chemicals that may present an immediate or delayed danger to one or more components of the environment.

9.2.3 Risk phrases and description of risks

Abbreviation	Description of risk
R11	Highly flammable
R12	Extremely flammable
R19	May form explosive peroxides
R20	Harmful by inhalation
R22	Harmful if swallowed
R23/24/25	Toxic by inhalation, in contact with skin and if swallowed
R28	Very toxic if swallowed
R32	Contact with acids liberates very toxic gas
R34	Causes burns
R35	Causes severe burns
R36	Irritating to eyes
R36/37/38	Irritating to eyes, respiratory system and skin
R36/38	Irritating to eyes and skin
R37	Irritating to respiratory system
R37/38	Irritating to respiratory system and skin
R38	Irritating to skin
R39/23/24/25	Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed
R41	Risk of serious damage to eyes

Abbreviation	Description of risk
R42	May cause sensitisation by inhalation
R42/43	May cause sensitisation by inhalation and skin contact
R48/20/22	Harmful: danger of serious damage to health by prolonged exposure through inhalation and if swallowed
R50/53	Very toxic to aquatic organisms, may cause longterm adverse effects in the aquatic environment
R51/53	Toxic to aquatic organisms, may cause longterm adverse effects in the aquatic environment
R60	May impair fertility
R61	May cause harm to the unborn child
R66	Repeated exposure may cause skin dryness or cracking
R67	Vapours may cause drowsiness and dizziness

9.2.4 Safety phrases and description of safety

Abbreviation	Description of safety
S7	Keep container tightly closed
S16	Keep away from sources of ignition - No smoking
S20	When using do not eat or drink
S22	Do not breathe dust
S24	Avoid contact with skin
S24/25	Avoid contact with skin and eyes
S26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S30	Never add water to this product
S36	Wear suitable protective clothing
S36/37	Wear suitable protective clothing and gloves
S36/37/39	Wear suitable protective clothing, gloves and eye/face protection
S36/39	Wear suitable protective clothing and eye/face protection
S37/39	Wear suitable gloves and eye/face protection
S39	Wear eye/face protection
S45	In case of accident or if you feel unwell seek medical advice immediately (show the label where possible)
S46	If swallowed, seek medical advice immediately and show this container or label
S53	Avoid exposure - obtain special instructions before use
S60	This material and its container must be disposed of as hazardous waste
S61	Avoid release to the environment. Refer to special instructions/safety data sheet

9.3 Representative results of fourier-transfom infrared (FT-IR) spectroscopy for yeast identification
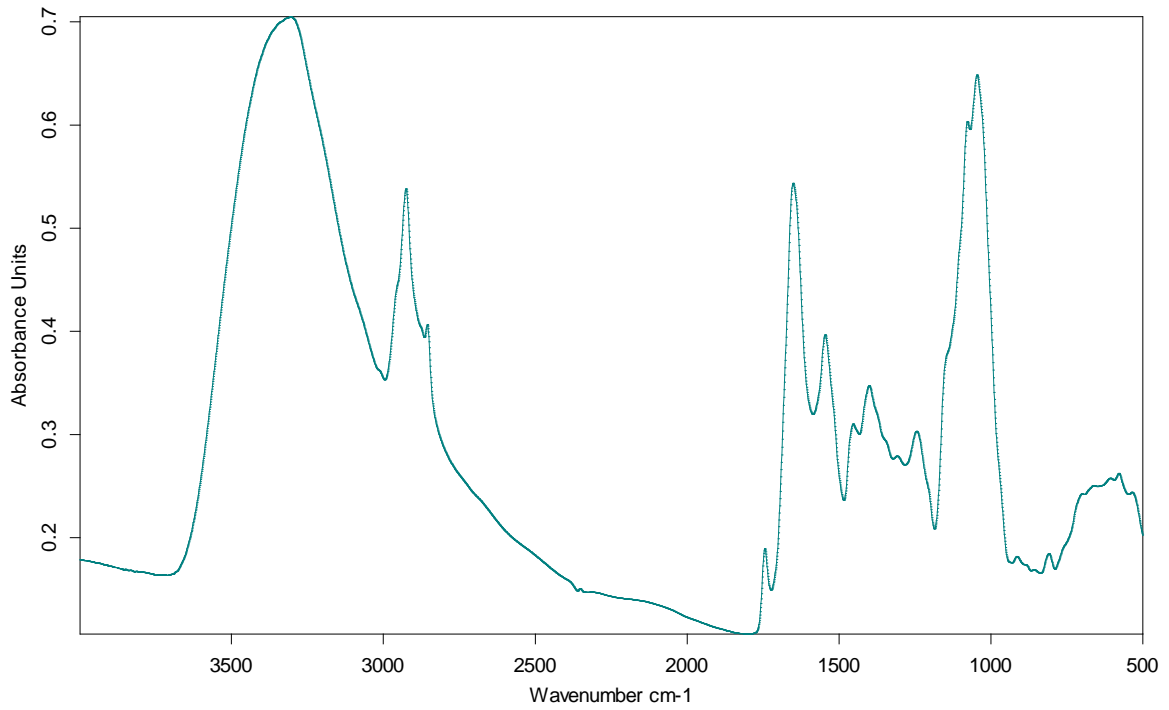
Pichia anomala **IDE-Y001**
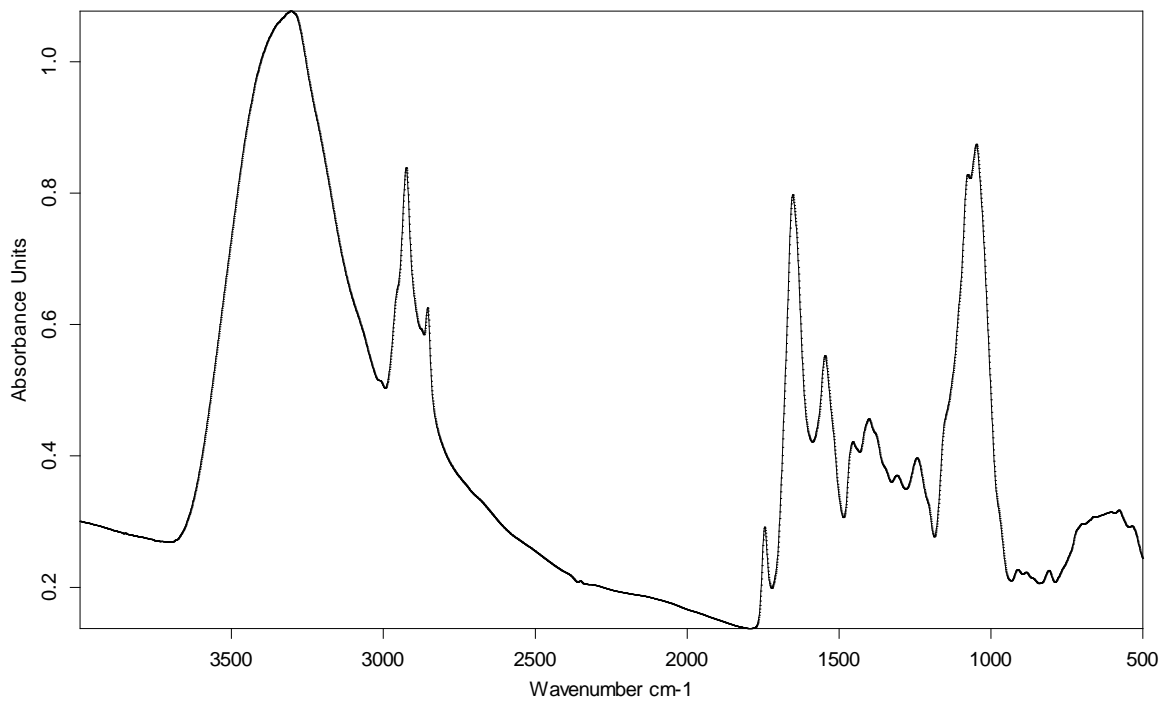
Candida parapsilosis **IDE-Y007**
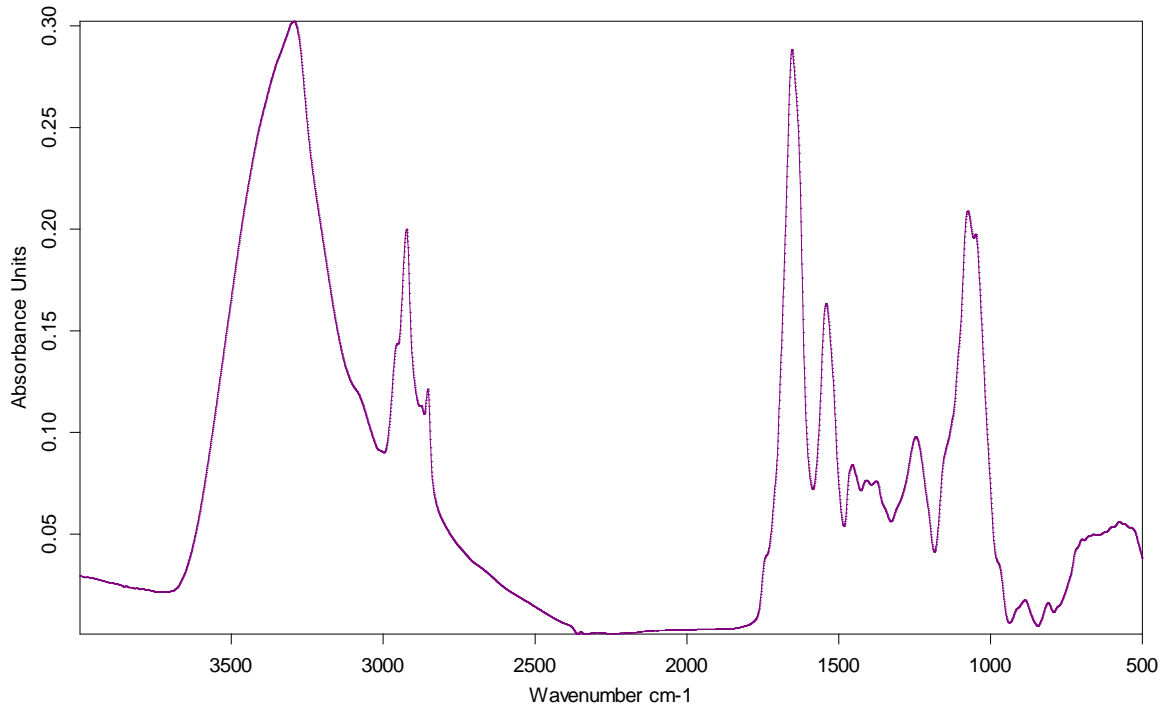
Hanseniaspora guilliermondii **IDE-Y054**

9.4 Representative results of 26S rRNA gene sequencing for yeasts
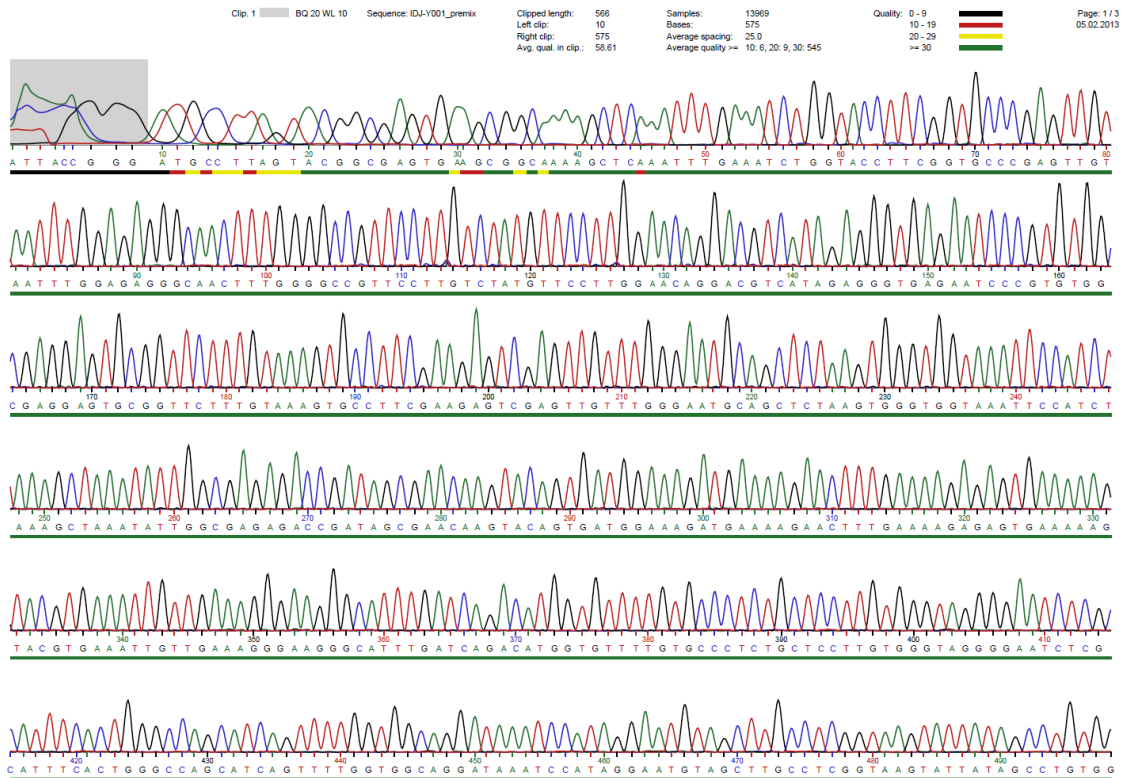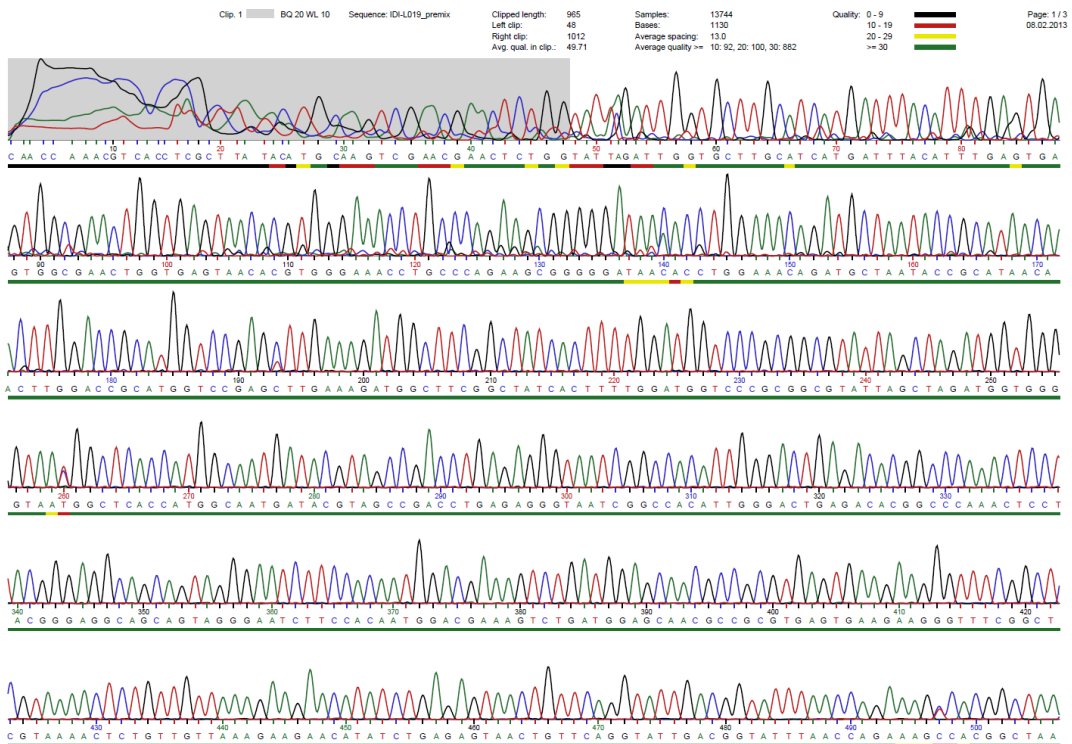

Saccharomyces cerevisiae **IDI-Y001**
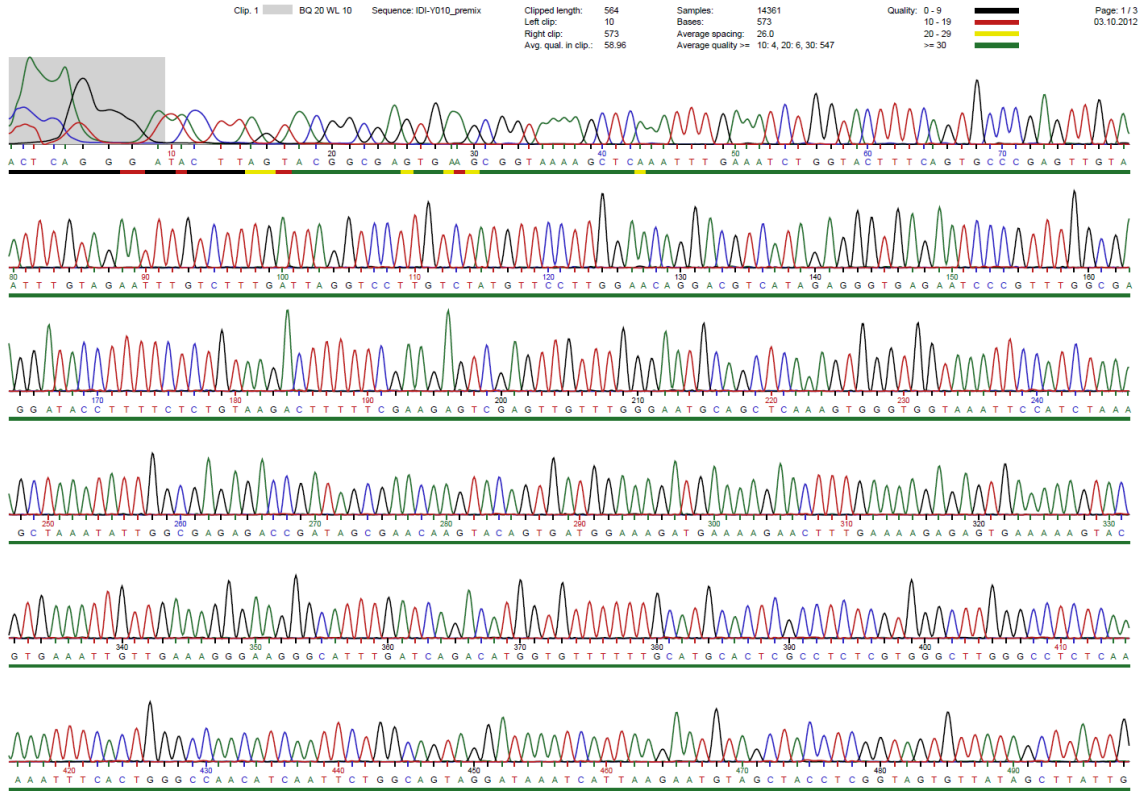
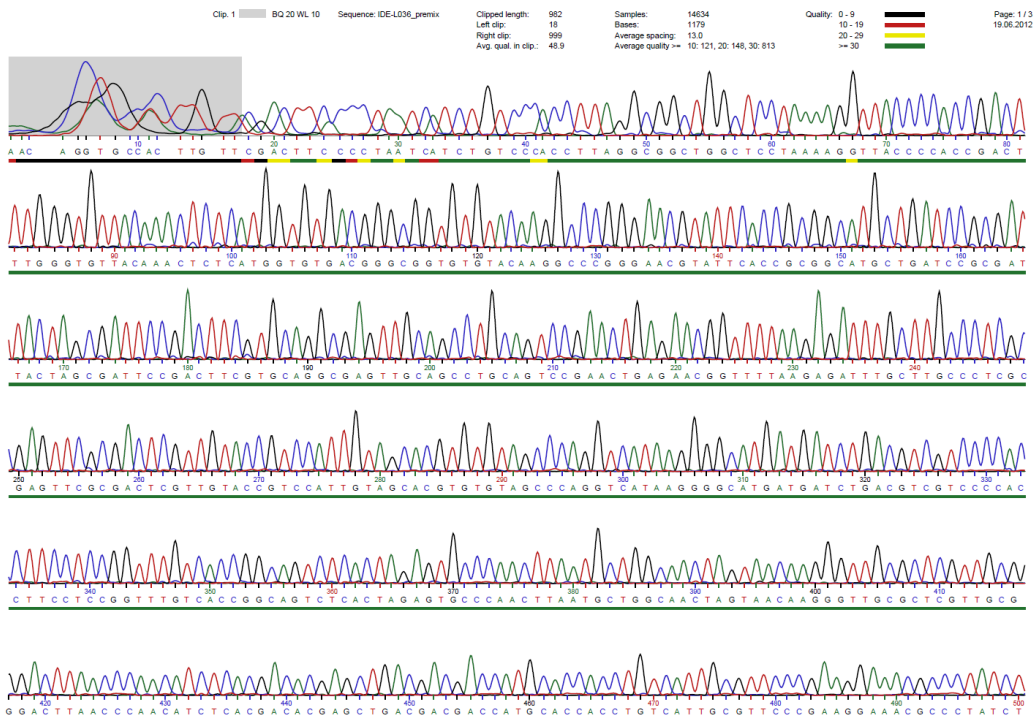*Hanseniaspora guilliermondii* **IDI-Y039**
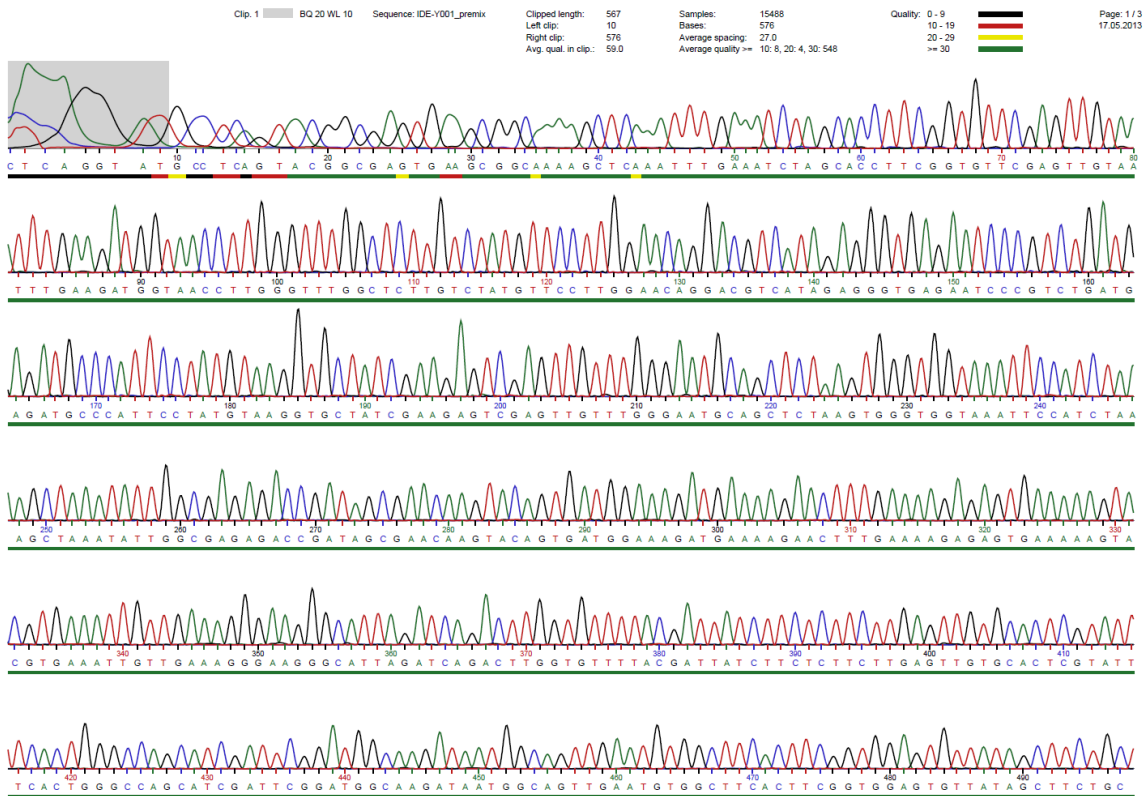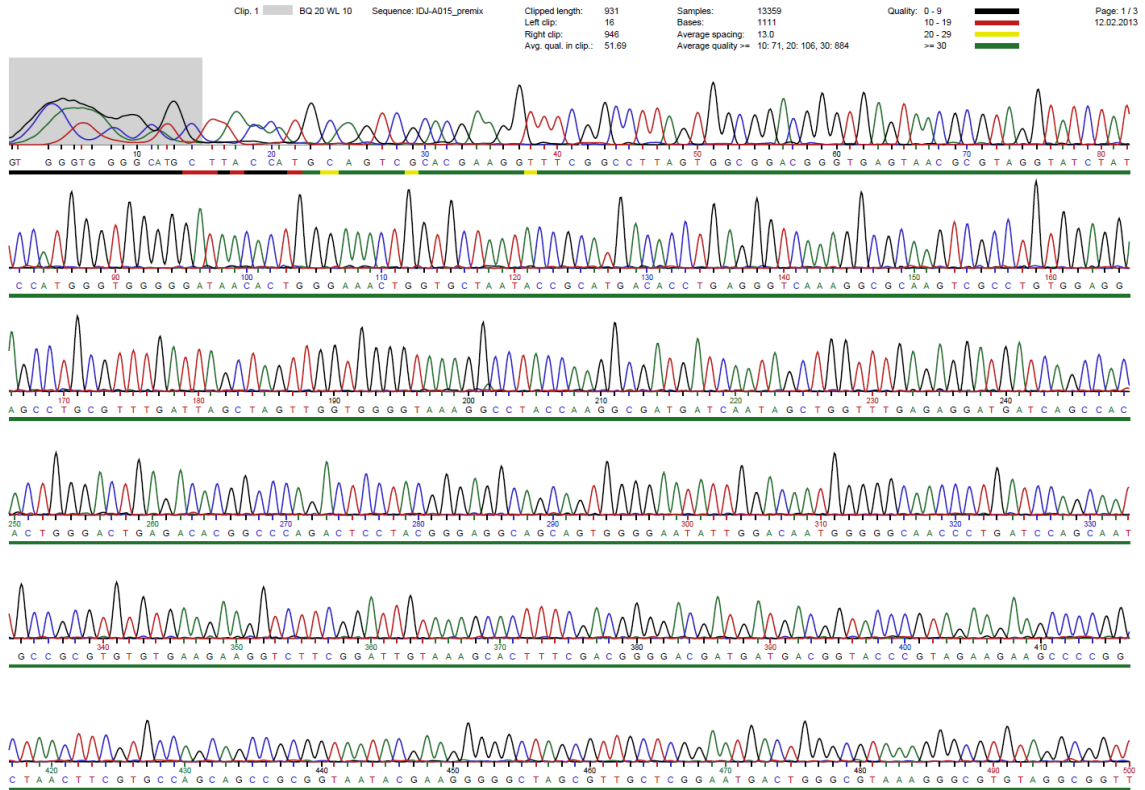

Wickerhamomyces anomalus **IDE-Y001**

9.5 Representative results of 16S rRNA gene sequencing for lactic acic bacteria and acetic acid bacteria

Lactobacillus plantarum IDI-L019

Lactobacillus fermentum IDE-L036

Acetobacter pasteurianus **IDI-A003**

9.6 Representative HPLC chromatogram for carbohydrates analysis
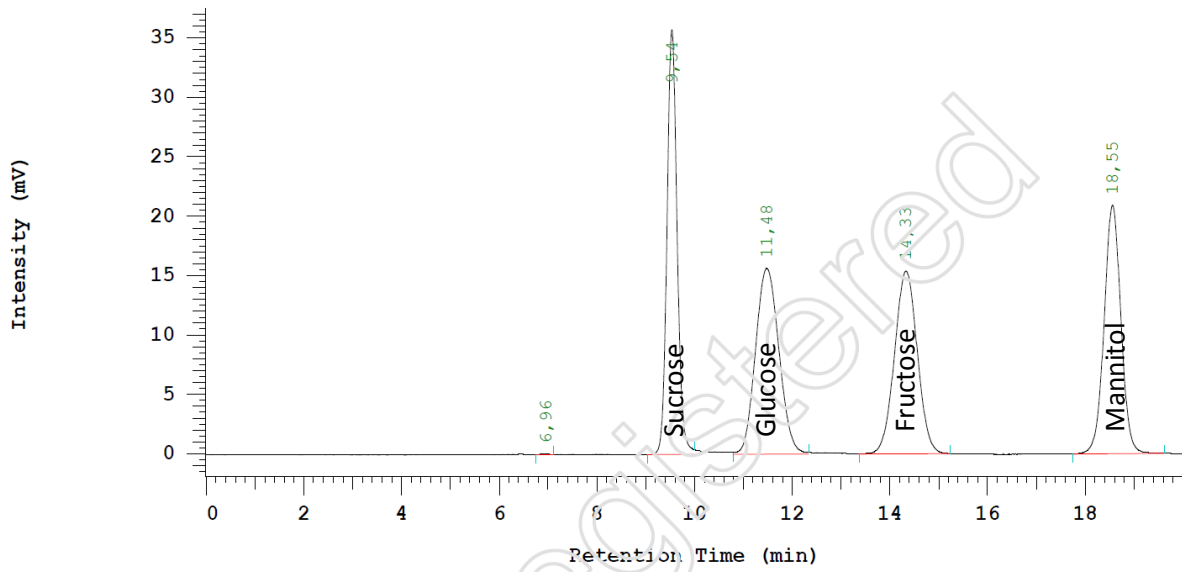
Chromatogram profile of mixed carbohydrates standards at a concentration of 2 mg mL^{-1}
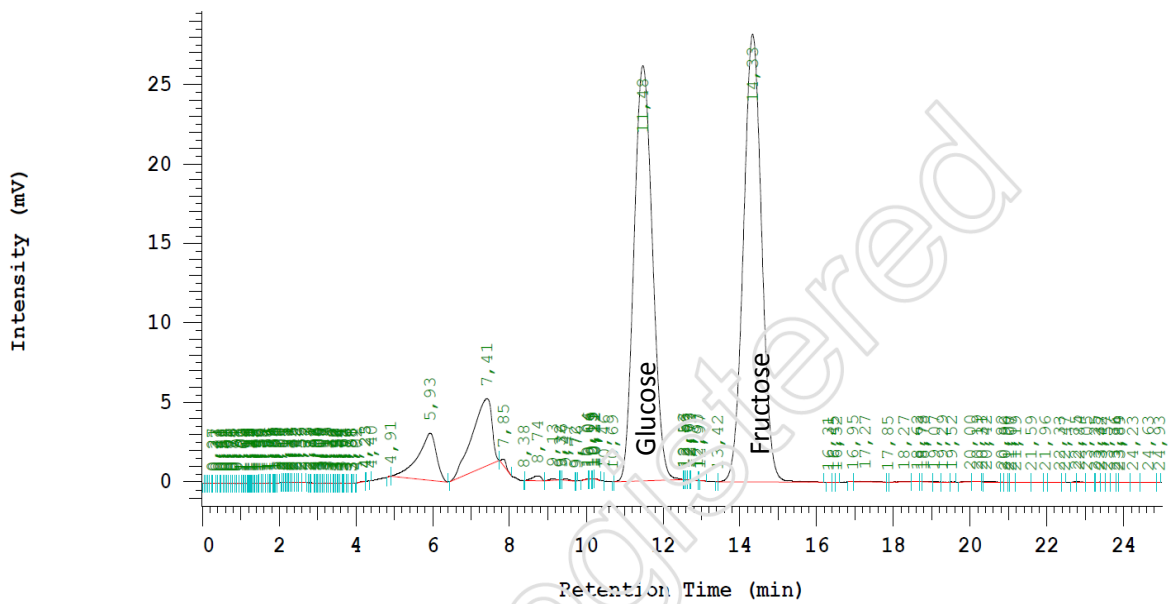
Chromatogram profile of carbohydrates s sample from cocoa bean fermentation

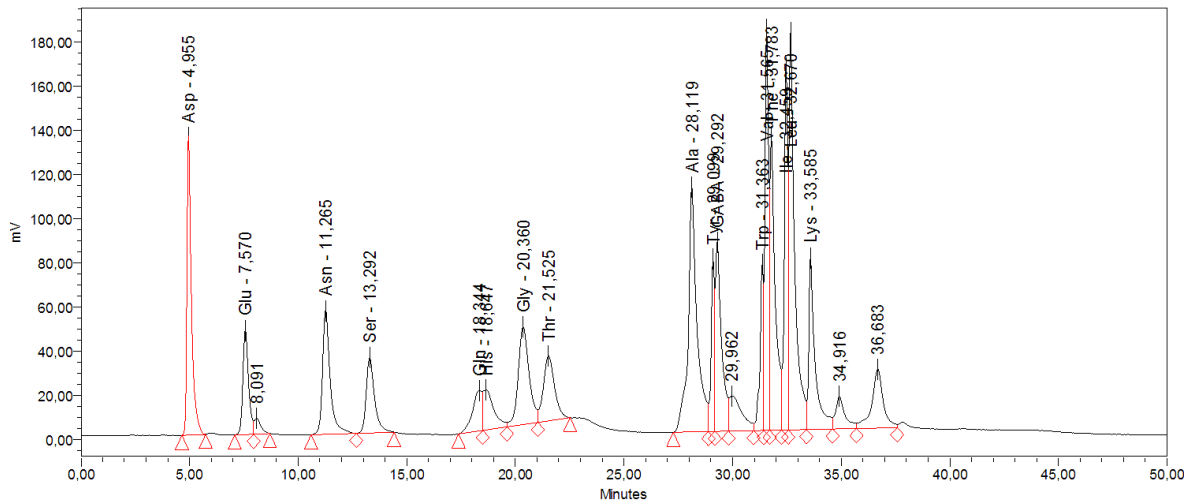## 9.7 Representative HPLC chromatogram for organic acids analysis
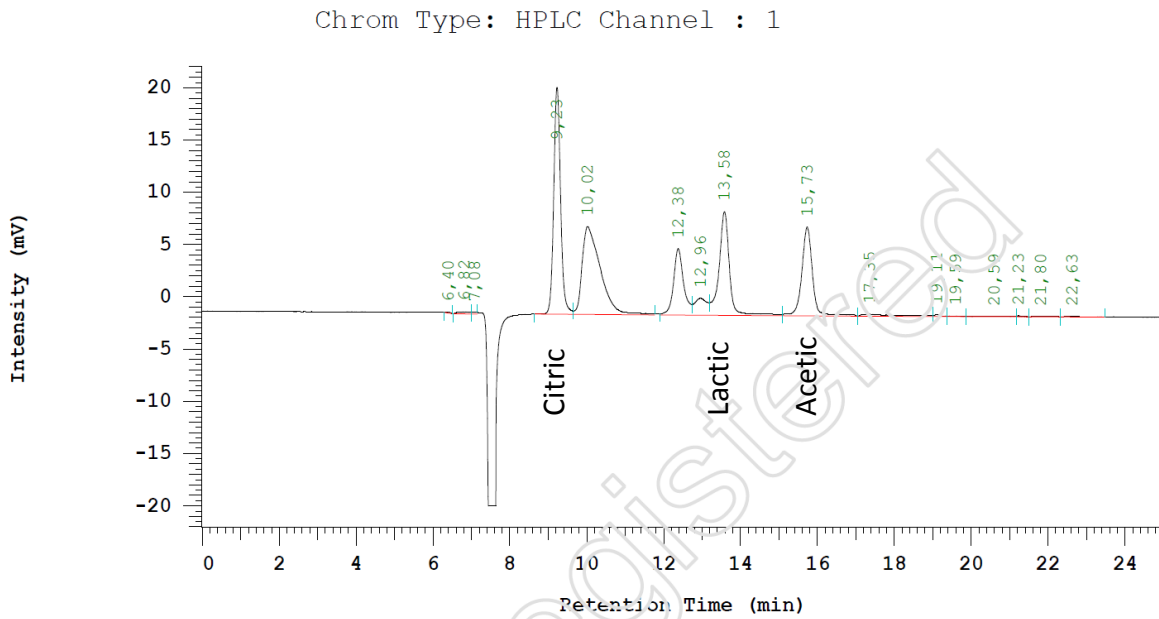
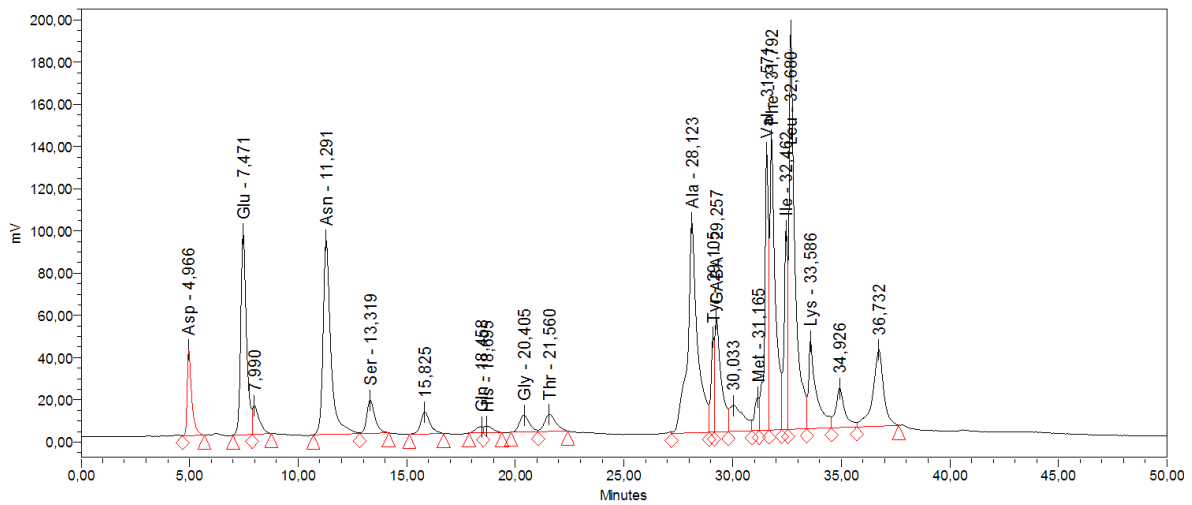Chromatogram profile of mixed organic acids standards at a concentration of 1 mg mL^{-1}
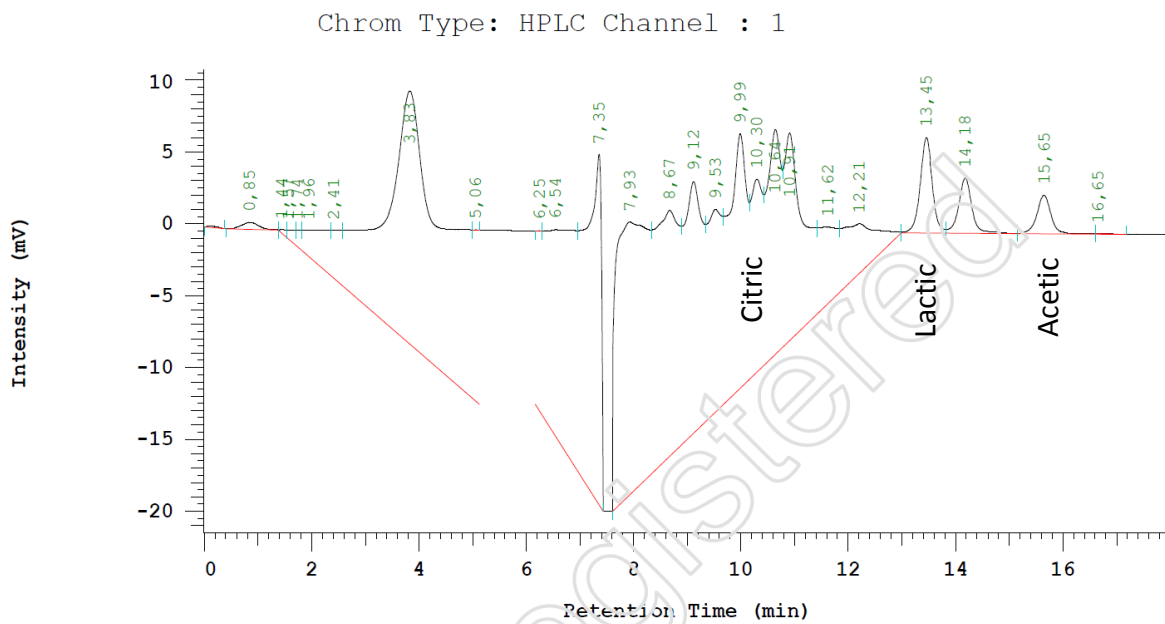
Chromatogram profile of organic acids sample from cocoa bean fermentation

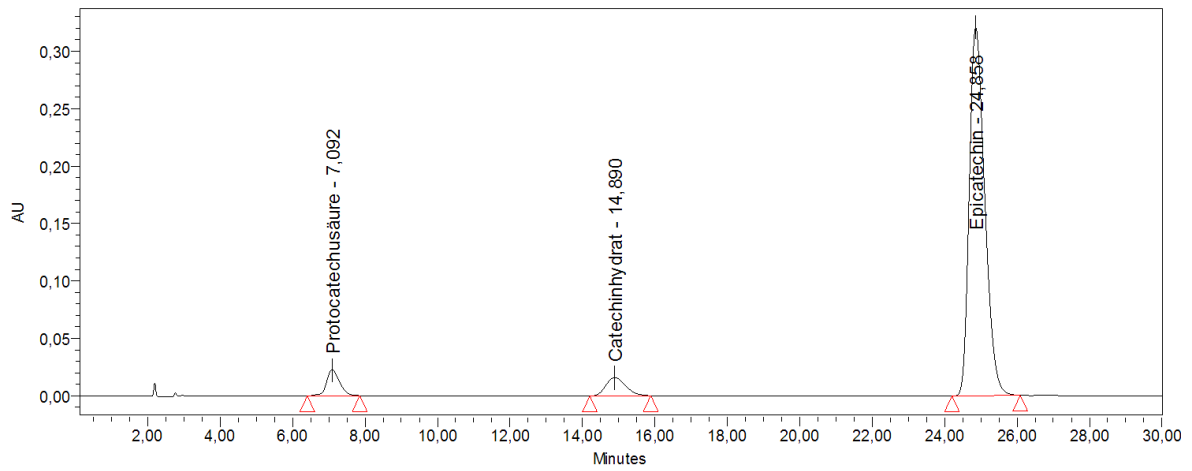## 9.8 Representative HPLC chromatogram for amino acids analysis

Chromatogram profile of amino acids standard at a concentration of 500 ng mL^{-1}
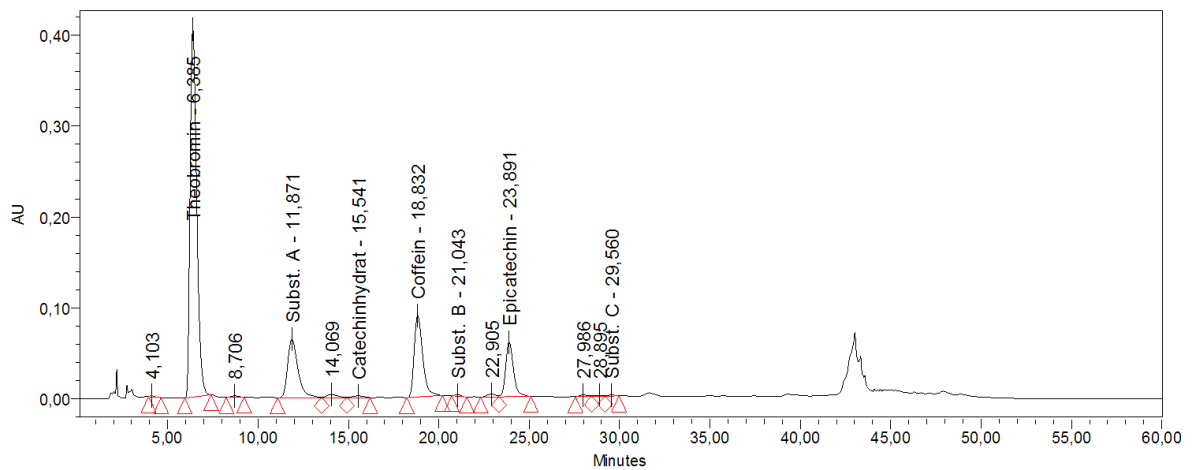

Chromatogram profile of amino acids sample from cocoa bean fermentation

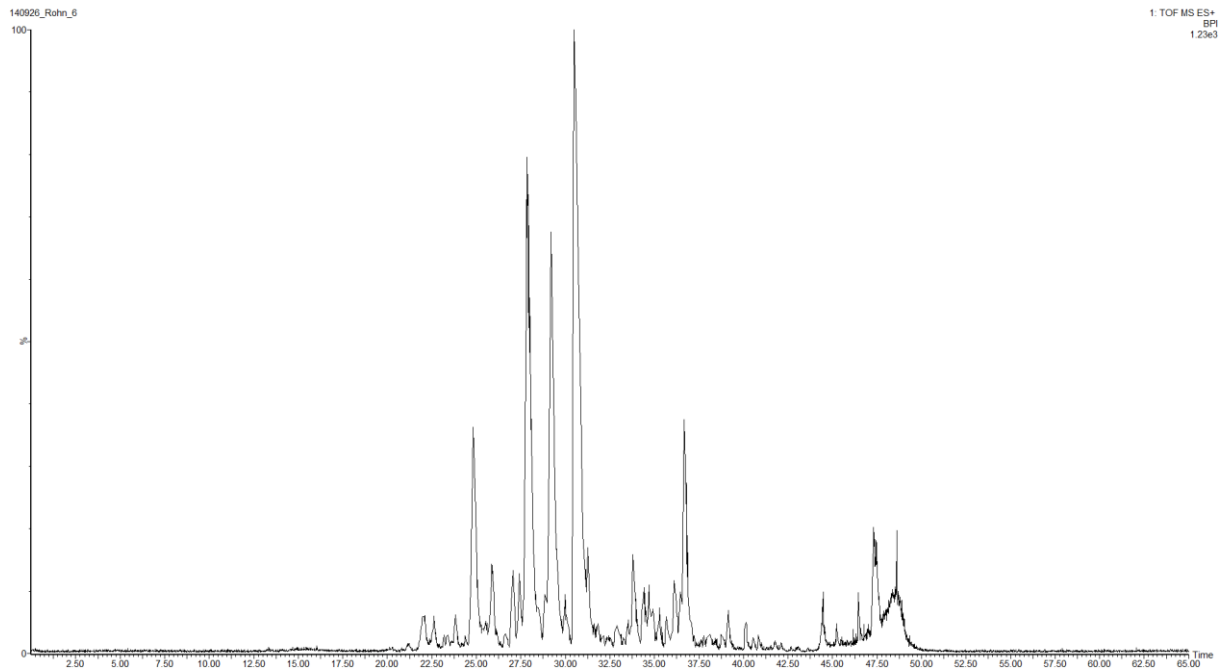## 9.9 Representative HPLC chromatogram for polyphenol analysis

Chromatogram profile of polyphenols standard A
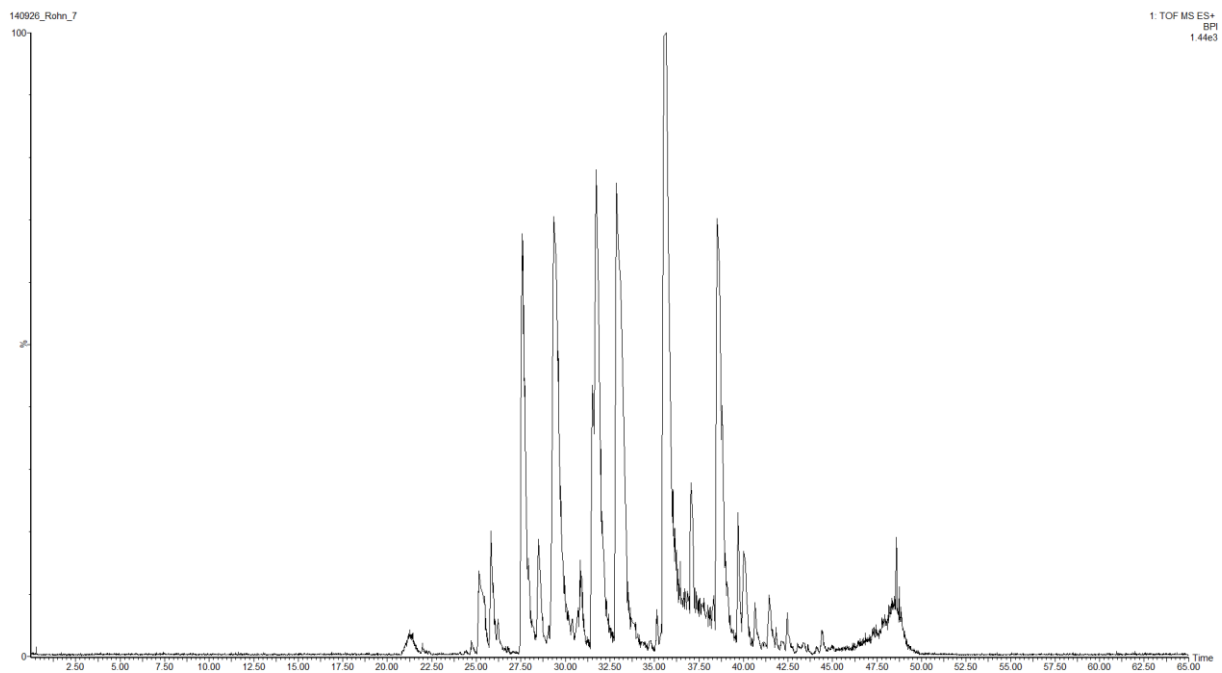

Chromatogram profile of polyphenols sample from cocoa bean fermentation
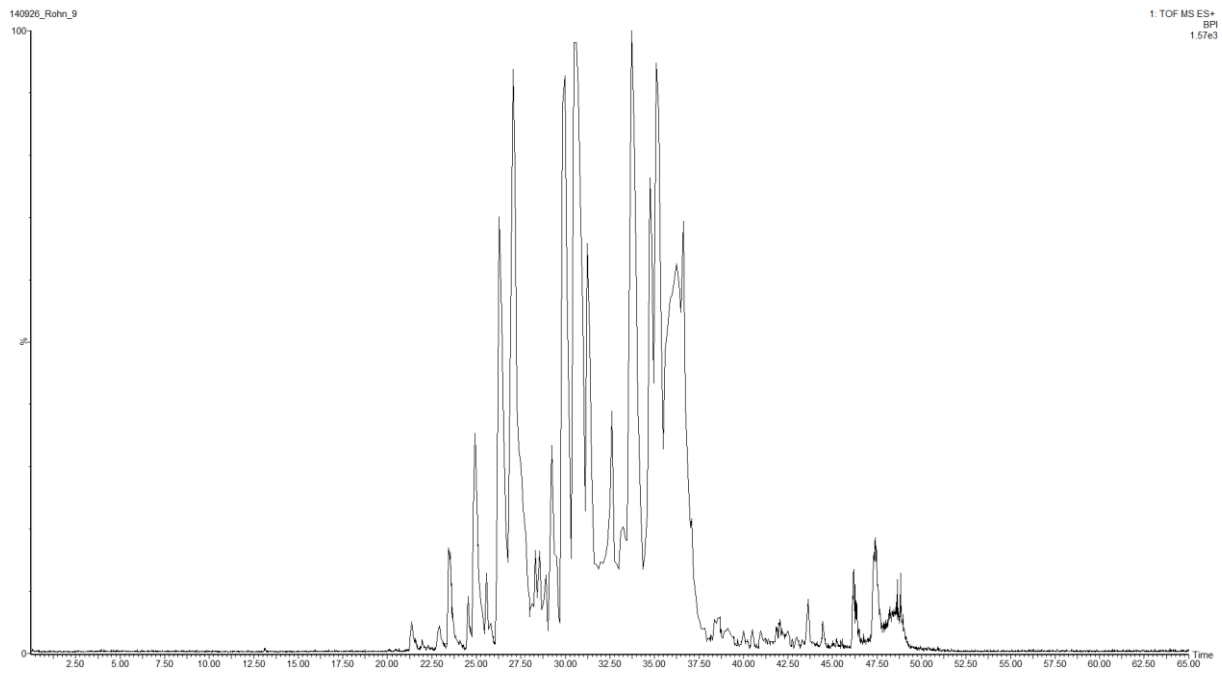
9.10 Representative MALDI TOF MS chromatogram for protein analysis

Chromatogram profile of osmotin protein from *Theobroma cacao* L.

Chromatogram profile of chitinase protein from *Theobroma cacao* L.

Chromatogram profile of glucan endo-1,3-beta-glucosidase protein from *Theobroma cacao* L.

10 Curriculum vitae

Personal Data

Name   : Fahrurrozi

Date of birth   : Mataram, May 3rd 1981

Nationality   : Indonesian

Educational Background

No.	Year	Degree	Field and University
1.	1999 - 2003	Bachelor of Science	Technology for agricultural products, Departement of Agricultural Technology, University of Mataram, Indonesia
2.	2004 - 2007	Master of Science	Protein and enzymology, Department of Biotechnology, Bogor Agricultural Institute, Indonesia
3.	2011 - 2014	PhD student	Food Microbiology and Biotechnology, Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany

Research Experiences

No.	Year	Position	Research
1.	2003	Bachelor of science thesis	Making of soy sauce from the tofu waste
2.	2007	Master of science thesis	Purification and characterization of extracellular xylanase *Streptomyces* 234P-16
3.	2007	Research assistant	Utilization of palm kernel cake waste for production manno-oligosaccharides as functional food components
4.	2007	Research assistant	Studies of specification of mannanase enzyme to produce functional food components
5.	2008	Research assistant	Utilization of indigenous potato (Porang : *Amorphophallus onchophyllus*) for manno-

oligosaccharides production

6.	2010	Research assistant	BIOGROUTING: Utilization of marine microbes carbonate precipitation for beach erosion application
7.	2014	PhD thesis	Microbiological and biochemical investigations of cocoa bean fermentation

Professional jobs

No.	Year	Positions	Field and Institution
1.	2007 - 2008	Research assistant	Laboratory of Biocatalyst and Fermentation, Research Center for Biotechnology, Indonesia Institute of Sciences (LIPI), Cibinong, Indonesia
2.	2008 - 2010	Research assistant	Laboratory of Applied Microbiology Research Center for Biotechnology, Indonesia Institute of Sciences (LIPI), Cibinong, Indonesia

Publications

1. Anja Meryandini, Trio Hendarwin, **Fahrurrozi**, Alina Akhdiya, Deden Saprudin, Yulin Lestari. 2009. Characterization and Purification a Specific Xylanase Showing Arabinofuranosidase Activity from *Streptomyces* spp. 234P-16. *Biodiversitas*, Vol. 10, No. 3. p 115-119.

2. **Fahrurrozi**, Shanti Ratnakomala, Trisanti Anindyawati, Puspita Lisdiyanti, and Endang Sukara. 2010. Rapid Assessment of Diverse *Trichoderma* Isolates of Indonesian Origin For Cellulase Production. *Annales Bogorienses* Vol. 14, No. 1. 2010.

3. **Fahrurrozi**, Shanti Ratnakomala and Puspita Lisdiyanti. 2009. Screening and Characterization of Actinomycetes Degrading Hemicellulose. Proceedings in The First International Symposium of Indonesia Wood Research Society. Bogor, Indonesia. 2nd – 3rd Nopember 2009.

4. Shanti Ratnakomala, **Fahrurrozi**, and Puspita Lisdiyanti. Screening and Characterization of Cellulase Enzyme Producers from Indonesian Actinomycetes. Proceedings in The First International Symposium of Indonesia Wood Research Society. Bogor, Indonesia. 2nd – 3rd Nopember 2009

5. Puspita Lisdiyanti, Kiki Bayu Wardani, **Fahrurrozi**, and Shanti Ratnakomala. Molecular Identification of Actinomycetes Isolated from Termite Mound. Proceedings in

The First International Symposium of Indonesia Wood Research Society. Bogor, Indonesia. 2nd – 3rd Nopember 2009

6. Yopi, Dwi Susilaningsih, Ahmad Thontowi, Awan Purnawan, Apridah C. Djohan, **Fahrurrozi**, and Puspita Lisdiyanti. 2007. Study on Hemicellulytic Bacteria: Production of Oligosaccharides from Palm Kernel Cake using Fermentation. Proceedings in Internasional Seminar "Advances in Biological Science: Contribution Towards a Better Human Prosperity" Faculty of Biology Gajah Mada Universuty, Yogyakarta-Indonesia. September 7-8, 2007, p 111-113

7. Yopi, A. Thontowi, **Fahrurrozi**, A. Purnawan and A.C. Djohan. Utilization of Indigenous potato (Porang: *Amorphophallus onchophyllus*) for manno-oligosaccharides production. Proceedings in ITSF SEMINAR on Science and Technology. Jakarta, 11st February 2009.

8. **Fahrurrozi**, Bahmann C, Lieberei R, Bisping B (2013) Antifungal activity in seed coat extracts of *Theobroma cacao* L. Poster. Dechema Jahrestagung der Biotechnologen, Frankfurt am Main, 4.-5. March 2013.

9. **Fahrurrozi**, Bahmann C, Lieberei R, Bisping B (2013) Antifungal activity in seed coat extracts of *Theobroma cacao* L. Poster presentation at VAAM International Conference on Microbiology, Bremen, Germany, 10 - 13 March 2013.

10. **Fahrurrozi**, Bahmann C, Niemenak N, Lieberei R, Bisping B (2014) Antifungal protein of seed coat extracts of *Theobroma cacao* L. during fermentation. Poster 16th European Congress on Biotechnology, Edinburgh, Scotland, 13-16 July 2014.