Die Untersuchung der Feststofffermentation von Maniokabfällen für die Tierernährung

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vorgelegt von

Catur Sriherwanto

aus Surabaya, Ost-Java, Indonesien

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List of Abbreviations

ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
DAMO	Diacetylmonoxime
DMSZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DNS	3,5-dinitrosalicylic acid
DW	dry weight
FAO	Food and Agriculture Organisation
g	gram(s)
GCBA	gelatinised cassava bagasse agar
GCBM	gelatinised cassava bagasse mash
GRAS	generally recognised as safe
h	hour(s)
HCN	hydrogen cyanide
HPLC	high pressure liquid chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	kilogram(s)
L	litre(s)
m	metre(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
MW	molecular weight
mV	millivolt(s)
mV.min	millivolt.minute(s)
µg	microgram(s)
µl	microlitre(s)
µmol	micromole(s)
N	number of analysis
NA	not available

ng	nanogram(s)
NPN	non protein nitrogen
Ø	diameter
pg	picogram(s)
ppm	parts per million
RCBA	raw cassava bagasse agar
rpm	revolutions per minute
sp.	species
spp.	species (plural)
ssp.	subspecies
SSF	solid state fermentation
TCA	trichloroacetic acid
TNM	total nitrogenous matter
TRNM	total real nitrogenous matter
TSC	thiosemicarbazide
USP-U	United States Pharmacopedia-Unit
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
WHO	World Health Organisation
WW	wet weight

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1 Introduction

Solid state fermentation (SSF), as the term implies, is a living microbial cultivation system involving moistened solid substrate on which microorganisms grow in the absence of free flowing water, but where enough moisture is available to allow microbial growth and metabolism, with air as the continuous phase (Rahardjo et al. 2006). SSF has been gaining increasing attention from researchers as evidenced by numerous reviews published in recent years on topics such as SSF bioreactor design (Durand 2003; Robinson and Nigam 2003), physico-chemical and environmental factors affecting SSF (Krishna 2005), advantages of SSF over submerged fermentation (Viniegra-González et al. 2003; Hölker et al. 2004; Hölker and Lenz 2005; Bhargav et al. 2008), modelling of microbial growth in SSF (Mitchell et al. 2000; Mitchell et al. 2004; Rahardjo et al. 2006), sensors and measurement techniques in SSF systems (Bellon-Maurel et al. 2003), and advances in SSF research and development (Wang et al. 2007). Among important applications of SSF are those in the production of industrially important enzymes (Bhargav et al. 2008), food and feed (Couto and Sanromán 2006; Longo et al. 2008).

SSF has been practically applied for centuries in different countries for the preparation of traditional fermented foods. One of these is tempe, or its alternative spelling “tempeh", a well known traditional food originating from Indonesia, which is produced on an industrial scale (Figure 1-1). Consumed for centuries as a protein-rich alternative to meat, tempe is made of peeled cooked soybeans fermented with an edible filamentous fungus of the genus *Rhizopus*. Decades of research on tempe have generated a wealth of information on the physico-chemical alterations brought about through the metabolic activities of the growing fungi, resulting in a fermented food that is organoleptically, nutritionally, and functionally superior to unfermented soybeans. As described by Hesseltine (1985), Nout and Rombouts (1990), Nout and Kiers (2005), as well as Babu et al. (2009) in their reviews, the beneficial changes associated with the tempe fermentation process include the removal of the bitter beany flavour; the reduction of antinutritional factors; the enhancement of flavour, aroma, texture, and digestibility; improved nutritional values as seen in higher concentrations of free amino acids, free fatty acids, water-soluble solids, and many water-soluble vitamins; the conferment of antioxidative capacity and antimicrobial properties, especially those against potentially pathogenic bacteria such as *Staphylococcus, Streptococcus, Leuconostoc, Bacillus,* and *Clostridium*.

Figure 1-1: Today, tempe is commercially produced in European countries, for example in the Netherlands, and sold in a plastic packaging (A). When sliced across the length, the Dutch tempe exhibits a cross-sectional view of cooked dehulled soybeans bound tightly together by cottony white *Rhizopus* mycelium (B).

The various nutritional quality and functional properties of soybean tempe have triggered researchers around the world to apply this knowledge to the production of non-soybean tempe by substituting soybeans with other agricultural crops such as fava beans (Randhir et al. 2004; Ahmad et al. 2008), mungbeans (Randhir and Shetty 2007), chickpeas (Reyes-Moreno et al. 2004), yambeans (Marshall et al. 2007), wheat (Wang et al. 1968), fingermillet (Mugula and Lyimo 1999), lupine (Fudiyansyah et al. 1995), sorghum (Mugula and Lyimo 2000), oats (Nowak 1992), grasspea seeds (Starzynska-Janiszewska et al. 2008), groundnuts (Amadi et al. 1999), barley (Feng et al. 2007), rapeseed meal (Pal Vig and Walia 2001), and cassava (Soccol et al. 1994a). In other studies, agroindustrial wastes, which are considered less desirable for human consumption, have also been utilised for potential use in animal diets through the enrichment of their nutritional contents. These substances include cocoa pod husks, cassava peels, palm kernel cakes (Lateef et al. 2008), corn cobs, rice bran, cowpea husks (Oduguwa et al. 2008), sweet potato residue (Yang et al. 1993), and cassava bagasse (Soccol et al. 1995c).

The tempe-like fermentation of cassava bagasse using *Rhizopus* spp. has been considered as a way of generating value-added products for animal feed that is otherwise poor nutritionally (Soccol et al. 1995a; Soccol et al. 1995c; Soccol et al. 1995b; John 2009). Although success has been reported in the production of cassava bagasse tempe, studies on its nutritional and functional properties are extremely rare and narrowly limited to its protein content and growth reduction of unwanted microbial contamination (Soccol et al. 1995c). Thus, there is still a considerable scope of other such wide-ranging aspects to explore as has already been extensively done for soybean tempe. In this context, the present work is an effort to contribute knowledge on the extent of fungal protein enhancement as well as water-soluble vitamin enrichment in cassava bagasse by SSF using *Rhizopus* spp. In addition, the physico-chemical and biological phenomena occurring during this tempe-like fermentation are compared with previous findings on soybean tempe as well as other non-soybean tempe.

1.1 Cassava bagasse

1.1.1 Cassava plant

Cassava (Figure 1-2), also commonly called tapioca, manioc, mandioca, yuca, aipim, castelinha and macaxeira in different languages, is classified as a member of the dicotyledonous *Euphorbiaceae* family (Alves 2002; Soccol and Pandey 2004) and has the scientific name

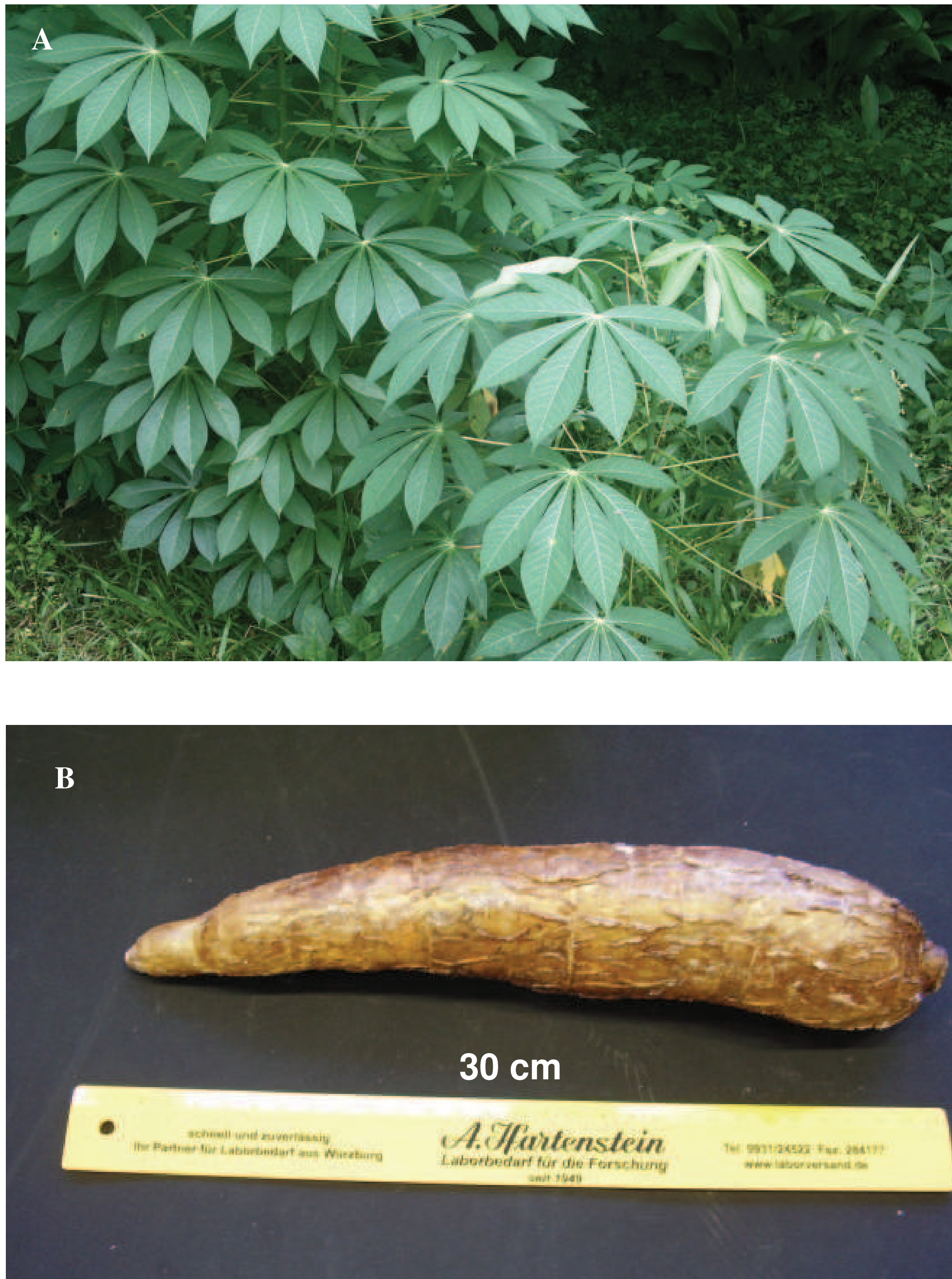


Figure 1-2: The cassava plant (A) and the starch storage root (B).

Manihot esculenta Crantz, or its synonym *Manihot utilissima* Pohl (Nassar 1978; cited in Nassar 2007). Cultivated mainly for its starchy roots, this perennial shrub can reach heights of 1-4 m, assuming either an upright form with or without branching at the top, or a spreading form. The leaf of the cassava is formed by the lamina and petiole and is lobed with palmated veins, generally consisting of an odd number of lobes. Although the main starch storage organ of the cassava is its roots, but not all of these undergo enlargement and elongation to become

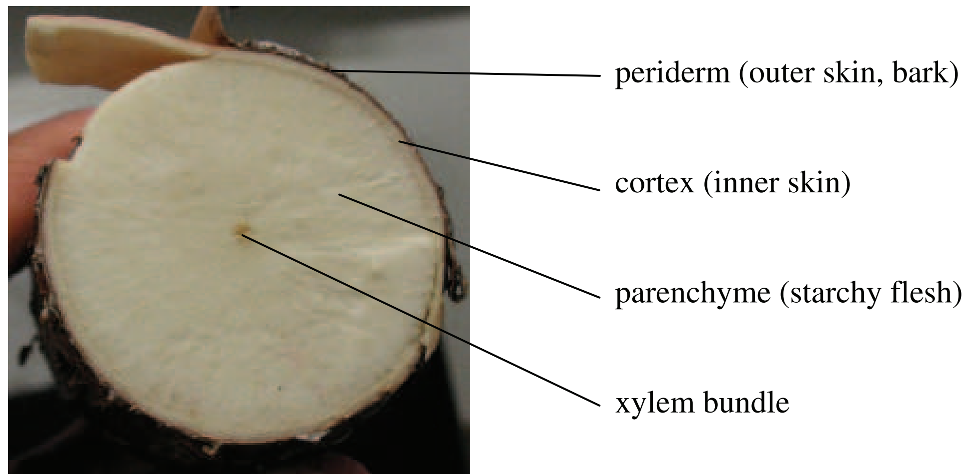


Figure 1-3: Cross sectional view of cassava.

the tapered, bark-brown skinned tubers that function as storage roots. Most fibrous roots remain thin and perform their task in the transport of water and nutrients from the soil (Alves 2002; Barceloux 2009). In this work, the term "cassava" is used to refer exclusively to the edible starchy tuber part of the plant (Figure 1-2B, Figure 1-3).

Considered to be a native species of Central and South America, the plant had spread by the 17th century beyond its continental habitat to Africa and the Indian subcontinent, and is widely cultivated today in tropical and subtropical regions (Barceloux 2009). More intensive cassava cultivation seems to be found outside of its countries of origin. This is clearly indicated by the fact that according to FAO (2009), Brazil is the only South American country, ranked second after Nigeria, that has made the list of the world's five largest cassava producers in the last several years; the top two are followed by Thailand, Indonesia and Congo (Figure 1-4).

Cassava contains toxic compounds called cyanogens which are endogenously present in cassava as cyanogenic glucosides (5% lotaustralin and 95% linamarin (Siritunga and Sayre 2003)), cyanohydrins, and free cyanide. The fresh roots of the cassava varieties normally used in the starch extraction industry contain cyanogens in concentrations of 237.5–339.4 mg HCN/kg DW (Piyachomkwan et al. 2005). When the cyanogens are hydrolytically broken down, poisonous hydrogen cyanide is liberated and can have detrimental effects on human and animals (Vetter 2000; Barceloux 2009; Chauynarong et al. 2009). Fortunately, various

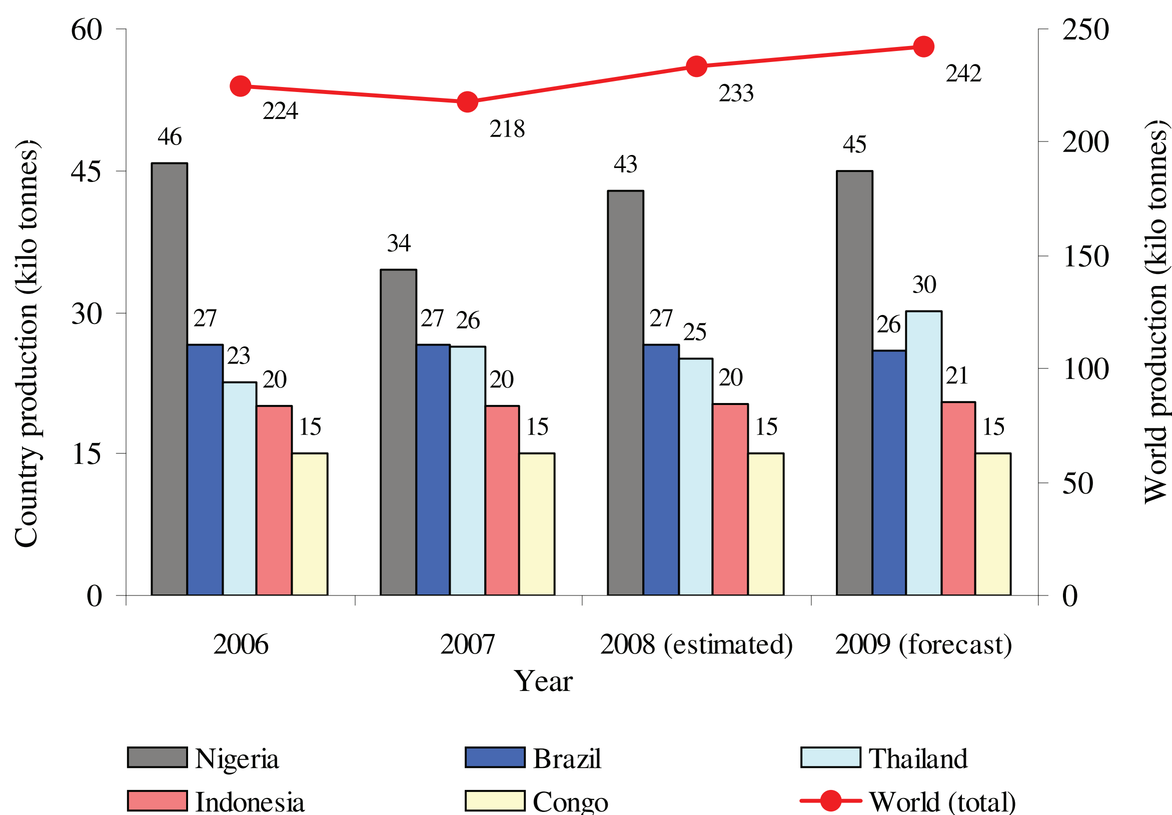


Figure 1-4: World cassava production according to FAO (2009).

techniques normally employed to process raw cassava into ready-to-eat cassava products as well as during starch extraction, are able to remove considerable amounts of these toxic compounds, making the products safe for dietary purposes. Crushing or pounding is considered the best method of removing the cyanogenic glucoside, since the procedure destroys cell compartments, allowing the mixing of linamarin and the enzyme linamarase, which facilitates the hydrolytic degradation of the cyanogens. The combination of crushing the cassava roots followed by drying under the sun in the preparation of cassava flour reduces the total cyanogens content to as low as 1–4% of the original value (Montagnac et al. 2009b).

Phytate (inositol hexakisphosphate) is a regulator of signalling between cells and a phosphate storage compound found in plants. In cassava, its concentration is present at 95–135 µg/kg (Charles et al. 2005). It is classified as an antinutritient due to its ability to form complexes with minerals such as iron and zinc in the gastrointestinal tract, leading to mineral-associated deficiencies in humans (Montagnac et al. 2009b; Kumar et al. 2010). Additionally, it has a negative influence on the utilisation of lipids and proteins. However, phytate consumption

also has various positive benefits, including anticancer effects, pH reduction, the promotion of DNA repair, the enhancement of the activity of natural killer cells, etc (Kumar et al. 2010).

Other antinutrients found in cassava roots belong to the group of polyphenol compounds. As reviewed by Montagnac et al. (2009b), various polyphenols have been identified in cassava, including gallocatechin, catechin, catechin gallate, hydroxycoumarins, rutin and kaempferol-3-rutinoside.

1.1.2 Production and use of cassava

Occupying the sixth highest position in production amongst world food crops (Muthoni and Nyamongo 2010), approximately 233 million tons of cassava were estimated to be globally produced in 2008 (Figure 1-4). The production has been predicted to increase to 242 million tons in 2009 (FAO 2009). Cassava is one of the most important food staples in tropical regions, where it is considered the fourth most significant energy source (Alves 2002). Its global consumption comprises a staple for approximately 600 million people (Sautter et al. 2006). Used mainly for human consumption (Nassar 2007), the utilisation of cassava as animal feed in the form of pellets and dried chips is also practiced, mostly in countries like Brazil, Colombia, Nigeria, China, in the Caribbean and in the Republic of Korea. Uses for human consumption and animal feed industries account for about 60% and 33% of the total world production, respectively (Soccol and Pandey 2004). Other non-dietary uses of cassava include its implementation as a raw material for ethanol production and in the cassava starch industry (Figure 1-6) (FAO 2009). Cassava starch is widely used as a non-food material in the textile, pharmaceutical, oil drilling, chemical, adhesive, paper, rubber, and detergent industries (Tonukari 2004; FAO 2006).

Cassava starch, also commonly called tapioca starch, is produced by the extraction of the starch stored in root parenchyma tissues (Figure 1-3), which contain 65–91% starch (Sánchez et al. 2009). To produce 1 ton of cassava starch (12% moisture), 4.21 tons of cassava tuber (60% moisture) and 18 m^3 water are required. The extraction process (Figure 1-5) generates 19.1 m^3 waste water, 0.38 tons of sand and peel (70% moisture), and 1.4 tons of solid fibrous residue (35–40% moisture) (Chavalparit and Ongwandee 2009). This starch solid residue (Figure 1-7) is referred to by various names in scientific literatures including cassava bagasse (Teixeira et al. 2009), cassava pulp (Kosugi et al. 2009), cassava solid waste (Raupp et al. 2004), cassava starch residue (Aro 2008), cassava fibrous residue (Ray et al. 2009), cassava

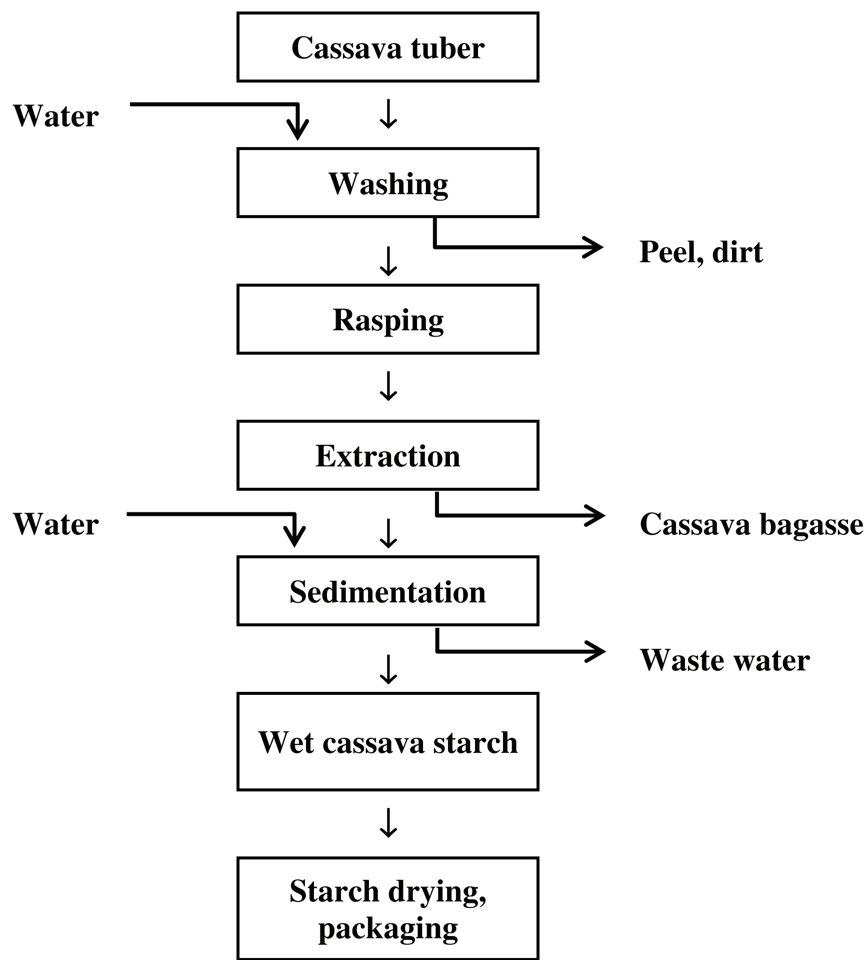


Figure 1-5: Basic procedure for tapioca starch production.

dregs (Hong et al. 2001), onggok (Djazuli and Bradbury 1999), cassava starch pomace (Aro 2008), and so on.

Around 6 million tons of cassava starch are estimated to be produced annually throughout the world (FAO 2006). This means that about 8.4 million tons of cassava bagasse is globally generated each year. Apart from its use as animal feed (Chauynarong et al. 2009) for feedlot cattle (Yimmongkol et al. 2009), lactating cows (Lima et al. 2008), swine, and broilers (Khempaka et al. 2009), this massive organic solid waste is generally discarded into the environment as landfill. This poses the serious problems of environmental pollution and aesthetic nuisance (Pandey et al. 2000; Ubalua 2007). Thus, the utilisation of this organic solid residue for the production of valuable products could be an option for alleviating the ecological problems.

Figure 1-6: A heap of cassava tubers (A) and the extracted tapioca starch (B) in the tapioca starch factory "Tapioka 35" in Lampung, Indonesia.

Figure 1-7: Industrial processing of tapioca starch such as in the "Tapioka 35" factory, Lampung, Indonesia, results in the mass production of solid by-products: cassava peels (A) and cassava bagasse (B).

1.1.3 Bioconversion of cassava bagasse

Cassava bagasse is fibre-rich residue consisting of 40-70% starch on a dry weight basis. Ash, lipids and proteins are each present in very low concentrations, usually less than 2% (Soccol and Pandey 2004). The residue also contains minerals such as copper, zinc, manganese, iron, and magnesium. An initial analysis by Chauynarong et al. (2009) indicated that cassava bagasse from several different sources in Thailand contains lysine, methionine, alanine, leucine, isoleucine and threonine in amounts of up to 64, 7.0, 106, 82, 57, and 70 g/kg of protein, respectively. The residues were also found to contain (mg/kg dry matter) calcium (3.15-6.5), phosphorous (0.27-0.63), sodium (0.21-1.20), and potassium (2.28-3.86).

The toxic cyanogenic compounds originally present in fresh cassava was found to be absent in cassava bagasse (Soccol and Pandey 2004; John 2009). In contrast, Piyachomkwan et al. (2005), who studied the balance and distribution of cyanogens during the cassava starch manufacturing process, found 56.4–94.7 mg HCN/kg dry basis in cassava starch residues obtained from seven production runs in a factory with a daily production capacity of circa 100 tons of starch. These different results might be due to the former corresponding to sun- or air-dried samples, with the latter being residues sampled freshly for immediate cyanogen measurement. It is known that freshly produced cassava bagasse loses its cyanogen content upon oven and sun drying (Tewe and Iyayi 1989; cited in Tewe 1992). In any case, a determination of the cyanogen level is necessary if cassava bagasse is intended for dietary purposes in order to make sure that the value falls below the safety limit of 10 ppm set by the FAO/WHO (JECFA 2009).

Numerous studies have been carried out in order to explore potential applications of cassava bagasse in various fields. One of these is its bioconversion into commercially important products including organic acids (such as citric acid (Prado et al. 2005), fumaric acid (Carta et al. 1998), and lactic acid (John et al. 2007)), enzymes (such as α-amylase (Ray and Kar 2009), phytase (Hong et al. 2001), and cellulase (Pothiraj et al. 2006)), ethanol (Kosugi et al. 2009), aroma compounds (Medeiros et al. 2001), xanthan gum (Woiciechowski et al. 2004), mushroom (Barbosa et al. 1997), compost (Kamolmanit and Reungsang 2006), and nutritionally enriched animal feed (Wizna et al. 2009a). Its application as animal feed has the advantage that the fermented product can be directly fed to animals without prior separation and purification steps.

The microbial biotransformation of cassava bagasse into nutritionally superior products destined for animal feed has been studied for over two decades using filamentous fungi and bacteria as inocula. Cassava bagasse with a protein enrichment of 5.09% compared to the initial level of 1.26% was reported after an 18-day fermentation using *Trichoderma pseudokonigii* Rifai (Balagopalan and Padmaja 1988; cited in Aro 2008). Employing *Aspergillus niger* as the biotransforming microorganism, the protein content of cassava bagasse was increased from 1.65 to 7.7% after a 5-day fermentation (Manilal et al. 1987; cited in Chauynarong et al. 2009). Cultivating the same mould species on cassava bagasse, Supriyati (2003) achieved an improved 18.4% protein content in the fermented product compared to the starting value of 2.2%. A subsequent feeding trial by including 10% of the fermented bagasse into the ration given to 3-day old chicks for 4 weeks did not influence body weight gain, the feed conversion ratio, feed consumption, or the weight of the liver or giblets. Wizna et al. (2009a) developed poultry feed based on cassava bagasse through bioconversion using the bacterial strain *Bacillus amyloliquefaciens.* They found that the fermentation led to a 32% reduction in crude fibre and 360% increase in crude protein content in the fermented product, which ultimately contained 65.95% nitrogen retention, 2190 Kcal/kg metabolic energy, 7.9% crude protein, 2.75% crude fat, 11.55% crude fibre, 0.26% calcium and 0.17% phosphorous. In following experiments on broilers and ducklings, the authors showed that substituting corn meal with the fermented cassava bagasse into the birds' diets had no effect on the broilers' feed consumption, carcass percentage, average gain of body weight, or feed conversion. However, feed conversion was significantly decreased and body weight gain was significantly increased in the ducklings when the diets consisted of up to 60% fermented cassava bagasse. Shortly, the *B. amyloliquefaciens*-fermented cassava bagasse could be supplemented at up to 60% in diets (replacing 100% of the corn meal) for ducklings and as much as 40% in broilers' rations (replacing 67% of the corn meal) (Wizna et al. 2009b).

Thus, the results mentioned above suggest a strong potential for the practical utilisation of cassava bagasse in animal nutrition to replace, at least partially, conventional feedstuffs such as maize. This is highly relevant today and perhaps even in the future, bearing in mind the insufficient supply of cereal grains like maize, their increasing prices, and their diversion from use as animal feed towards their utilisation as raw materials for biofuels (Chauynarong et al. 2009; FAO 2009). In countries like Indonesia, a national policy has been established by the Ministry of Agriculture (2009) to reduce dependency on imported feedstuffs. Although the animal feed industry's demand for maize in 2009 could be met locally due to production sur-

plus, other dietary components such as soybean meal, rapeseed meal, corn gluten meal, calcium phosphate, feed additives and vitamins are still supplied entirely from overseas. Feed constituents such as fish meal can be supplied partly (5-20%) by domestic production, but the rest still must be imported. Thus, the utilisation of fermented cassava by-products could be an option to substitute the imported feedstuffs, given that Indonesia is the world's fourth largest producer of cassava (FAO 2009).

1.2 Tempe mould

1.2.1 Nutritional properties

Tempe mould is taxonomically classified as a member of the genus *Rhizopus* with three major groups *R. microsporus*, *R. stolonifer* and *R. oryzae* (Abe et al. 2006). It has been used for centuries in preparing the Indonesian fermented soybean-based food "tempe", leading to the acceptance of fermentation using mould as safe (GRAS) (Holzapfel 1997). Beneficial nutritional, functional, and chemical modifications to the fermented soybeans as a result of the metabolic activities of the growing fungus have been thoroughly investigated scientifically by researchers worldwide, triggering its acceptance, industrial production, and consumption in the USA and Europe as well as Japan. Continental Asia and Africa are also potential markets for tempe (Nout and Kiers 2005; Babu et al. 2009). Although domestically grown on soybeans, which provide good nutrient sources, *Rhizopus* spp. can also be cultivated on other growth media containing various simple or complex organic or inorganic compounds (Sorenson and Hesseltine 1966; Seaby et al. 1988; Graffham et al. 1995).

Nutritionally, the *Rhizopus* mycelial biomass contains nearly 50% protein. In studies using soluble starch as the main substrate component, Jin et al. (1999; 2001; 2002) cultivated *Rhizopus* spp. in liquid fermentation systems and obtained mycelial biomass containing 42.8–49.7 g protein/100 g DW mycelial biomass. The authors showed that the *R. oligosporus* fungal biomass contained significant amounts of amino acids, most of which were superior to those of the FAO reference protein (Jin et al. 2002). In a similar study, Omar and Li (1993) isolated *Rhizopus arrhizus* and grew it in a shake culture containing palm oil as the major substrate constituent. They produced a fungal biomass with a protein content of 42.8%, with amino acid profiles comparable to those of the FAO reference values. The authors also reported that the fungal biomass contained lipids (20.6%), carbohydrates (17.7%), ash (6.6%), crude fibres (5.2%), and nucleic acids (2.2%).

Nucleic acid content is a major limiting factor in the use of microbial protein as food (Anupama and Ravindra 2000; Bijl and Kruyssen 2003). This is because humans lack the uricase enzyme, which breaks down relatively insoluble uric acid to release highly soluble allantoin and hydrogen peroxide (Pay and Terkeltaub 2003). Thus, when too much nucleic acids enter the human body, this leads to uric acid precipitation, triggering health problems like kidney stone formation and gout (Parajó et al. 1995; Anupama and Ravindra 2000). The fact that *Rhizopus* biomass contains a low nucleic acid content therefore lends it to use as a component in animal diets (Omar and Li 1993).

1.2.2 Mycotoxins related to *Rhizopus*

In their experiments more than two decades ago, Rabie et al. (1985) cultivated 104 isolates from 14 species of *Rhizopus* for 3 weeks on heat sterilised whole yellow maize. Following the feeding of the fermented maize to ducklings, 47 of the isolates were found to be toxigenic. Two of these toxigenic isolates, namely *R. microsporus* and *R. chinensis*, were shown to be acutely toxic after a subsequent test involving rats, while all seven isolates belonging to the species *R. oryzae* slowed the growth of the animals. Their work was an example demonstrating the potentially detrimental effects of consuming feed contaminated or fermented with *Rhizopus* spp.

Rhizonin and rhizoxin have previously been described as mycotoxins associated with the genus *Rhizopus.* Rhizoxins are strong toxins with severe antimitotic activity, while rhizonins are cyclopeptides with hepatotoxic activity (Jennessen et al. 2005; Rohm et al. 2010). Believed for many years to be metabolites produced by *Rhizopus*, it has been recently found that the two toxins are in fact biosynthesised by bacteria from the genus *Burkholderia* living as an endosymbiont in the cytoplasm of the fungi (Partida-Martinez and Hertweck 2005; Scherlach et al. 2006; Partida-Martinez et al. 2007a; Partida-Martinez et al. 2007b).

Rohm et al. (2010) demonstrated the occurrence of toxigenic bacteria in a starter inoculum used for sufu production. Using metabolic profiling of the fungus cultivated according to the standard conditions employed for the preparation of sufu and tempe, the authors detected the presence of the rhizoxin complex synthesised in critical quantities. Thus, the endosymbiont bacteria might also be present as contaminants in the starter. Jennessen et al. (2005) previously warned of the production of the toxin during tempe fermentation, stressing the importance of finding methods that ensure the use of pure starter cultures of *R. oligosporus*. The

authors additionally reported that barley tempe was successfully made in their laboratory without the detection of rhizoxins and rhizonins.

Traditionally, soybean tempe is made in non-sterile conditions, in which external microbial contaminations are likely to occur (Han et al. 1999). Thus, it is very possible that the cultivated *Rhizopus* fungi harbour rhizonin- and rhizoxin-producing endosymbiont bacteria during the course of tempe preparation in Indonesia. However, soybean tempe has been consumed at all stages of life for many years without any known detrimental effects, indicating that it presents no serious safety concern at the known consumption levels observed in Central Java, Indonesia (Nout and Kiers 2005).

1.2.3 Cereal and tuber tempe

Tempe made by the *Rhizopus* spp. fermentation of non-soybeans substrate with lower protein contents such as cereals and dietary tubers have been reported, and were demonstrated in some cases to have developed superior nutritional and functional properties compared to the unfermented substrates. For example, Hesseltine (1985) reported that *Rhizopus* grew well on wheat, oats, rye, rice and barley, producing tempe cakes similar to those made from soybeans, with white mycelium connecting the cereal grains, and with no considerable sporulation being observed. Many years later, a new, improved process was patented for producing cereal-based tempe that was microbiologically safe and stable, had a good appearance as well as an acceptable consistency and flavour, and lacked or had minimal sporulation (Berg et al. 2004). Tempe made from whole-grain barley and whole-grain oats were produced using *Rhizopus oligosporus* and, after subsequent trials in humans, were demonstrated to have a low glycemic index and insulin index (Alminger and Eklund-Jonsson 2008). Handoyo et al. (2006) studied the fermentation of buckwheat with *Rhizopus oligosporus* for 48 hours. The authors found that the fungal growth lead to the increased formation of amino acids, some of which were increased 50-fold, compared to unfermented buckwheat. In addition, the fungus *R. oligosporus* was highly effective in lowering levels of allergenic proteins in buckwheat.

Unlike soybeans, which have a high protein content (37.5–44.6%) (Padgette et al. 1996), or cereals with average protein levels (8–18%) (Fontaine et al. 2002), starch rich tubers like cassava are poor protein sources, with protein contents ranging from 0.95-6.42%, with an average value of 3.24% (Ceballos et al. 2006). Tempe-like fermentation using cassava products (such as cassava tubers, cassava peels, cassava bagasse, and cassava starch) as substrates have

been studied with the main purpose of increasing the protein content. Since cassava products lack the nitrogen required by the cultivated *Rhizopus* fungi, they must be supplemented with external nitrogen sources such as urea and ammonium salts prior to fermentation. *Rhizopus* spp. have been successfully grown on differently pre-treated cassava roots, resulting in cassava tempe with protein contents increased from the initial value of 1.0–2.4% to 9.4–14.1% (Daubresse et al. 1987; Soccol et al. 1993a; Soccol et al. 1993b; Soccol et al. 1994a; Soccol et al. 1995c).

Some authors have investigated other aspects of fermented cassava products beyond protein enrichment. For instance, Oboh and Elusiyan (2007) biotransformed cassava flour made from low- and medium-cyanide cassava varieties using *R. oryzae* as an inoculum and found that levels of antinutrients such as tannin, cyanide and phytate initially present in the flour were considerably reduced after fermentation. Soccol et al. (1995c) demonstrated that it was possible to obtain cassava bagasse tempe both free of undesirable bacteria, namely *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella,* as well as having reduced population of mesophiles bacteria and yeasts. The influence of *Rhizopus* fermentation on the fat composition of cassava flour was reported by Harris (1970), who identified a reduction in the total lipid of the fermented product. He observed the disappearance of glycolipids normally found in plants, which were then replaced by fungal phospholipids. In addition, fermentation triggered the formation of γ-linolenic acid as well as changes in the relative amount of other acids. Daubresse et al. (1987) obtained protein enriched cassava via *Rhizopus* fermentation and analysed, amongst others, amino acid composition, residual urea and cyanide as well as vitamin B_1. Their results showed an almost complete disappearance of urea, a reduction in cyanide level, and a considerable increase in the amount of total and individual amino acids, as well as vitamin B_1. The other vitamins were not determined by the authors, and no work has been published thus far which measures the contents of other vitamins in cassava products fermented with *Rhizopus* spp.

1.2.4 *Rhizopus* and animal feed

Feeding trials of *Rhizopus* biomass in animal diets have been reported. Omar and Li (1993) cultivated *R. arrhizus* using liquid fermentation in a palm oil medium. The harvested fungal mycelial biomass was then fed to test animals to investigate its digestibility and general acceptability. They concluded that the fungal biomass, being non-pathogenic and non-toxic, could be potentially used as a protein source. Edebo (2009) used autoclaved fungal biomass of

the species *R. oryzae* isolated from human food in fish diets. The mycelial biomass, produced using the wastewater of paper pulp as the growth substrate, was substituted for the standard fish meal ingredient commonly used in fish diets. The fungal biomass was incorporated into the feed in order to achieve a protein content equivalent to that of standard feed. When given to young rainbow trout, the fungi-containing feed was eaten well. Moreover, these fish had a higher specific growth rate than those fed with standard feed. Other similar findings related to the utilisation of *Rhizopus* mycelial biomass in animal feed preparation have been patented (Hogan and Gierhart 1989) or submitted for patent applications (Bijl and Kruyssen 2003; Power 2006).

Other studies employed *Rhizopus* spp. for the fermentation of substrates intended for animal diets. In experiments aimed at developing composite rabbit feed, Oduguwa et al. (2008) carried out the fermentation using *R. oligosporus* on substrates from several feedstuffs including corn cobs, rice bran and cowpea husks. After analysing the fermented products, they found that the feedstuffs had undergone significant fibre degradation. Nutritional analyses of cassava peels fermented with *Rhizopus* sp. were conducted by Ofuya and Obilor (1993), indicating a superiority in terms of digestibility, protein content, and total amino acid composition over unfermented peels. In addition, 20-day feeding trials were performed with young poults, with the results revealing that the animals fed with unfermented cassava peels underwent severe growth retardation with higher (100%) mortality rate than those fed with the fermented feed (12.5%). Both cyanide poisoning as well as nutritional deficiency symptoms were observed following the autopsy of the birds fed with the unfermented peels.

Rhizopus spp. also play positive roles in reducing antinutrients and toxigenic compounds endogenously present in plant products. Cyanide content was considerably reduced by 90.6% and 95% when cassava peels were fermented using *R. stolonifer* for 120 hours (Lateef et al. 2008) or *Rhizopus* sp. for 96 hours (Ofuya and Obilor 1994), respectively. Padmaja and Balagopal (1985) as well as Oboh and Elusiyan (2007) found *R. oryzae* to be capable of degrading cyanogenic compounds in cassava tubers. Another antinutrient commonly found in plants, phytic acid, was also reduced during the *Rhizopus oligosporus* fermentation of soybeans (Sudarmadji and Markakis 1977; Van der Riet et al. 1987), cowpeas, groundbeans (Egounlety and Aworh 2003), and rapeseed meal (Pal Vig and Walia 2001). The latter authors additionally reported a reduction of glucosinolates, which were known to negatively affect the feeding quality of rapeseed meal (Halkier and Du 1997).

The detoxification of mycotoxins produced by other microorganisms is another advantageous quality of *Rhizopus* spp. In a study involving the mycotoxin-producing fungus *Aspergillus flavus*, *Rhizopus oligosporus* was demonstrated to reduce the level of aflatoxin B1 in chicken feed by 64.5% (Kusumaningtyas et al. 2006). A much greater inhibition (89.6–99.7%) of aflatoxin B1 synthesis was exhibited by *Rhizopus* spp. in liquid culture medium (Shantha 1999). In another study involving *Rhizopus* and *Mucor* fungal isolates, Varga et al. (2005) found several isolates that broke down ochratoxin A, zearalenone and patulin. The successful degradation of ochratoxin A in a liquid medium was achieved by culturing *Rhizopus stolonifer*, *R. microsporus, R. homothallicus*, two *R. oryzae* isolates, and four unidentified *Rhizopus* isolates. *Rhizopus* isolates were found to destroy over 95% of ochratoxin A within 16 days. An effective degradation of ochratoxin A was exhibited by a *R. stolonifer* isolate on moistened wheat.

1.2.5 Vitamin formation by *Rhizopus* spp.

The formation of vitamins by *Rhizopus* spp. during soybean tempe fermentation has been extensively studied. When previously published results are taken collectively, water-soluble vitamins such as riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folate and ascorbic acid were all found to be increased by fungal growth in unfermented soybean substrates (Roelofsen and Talens 1964; Murata et al. 1967; Robinson and Kao 1977; Kao and Robinson 1978; Van der Riet et al. 1987; Bisping et al. 1993; Keuth and Bisping 1993; Wiesel et al. 1997; Arcot et al. 2002). In contrast, a reduction of thiamine was also reported by all of the authors, although Murata et al. (1967) showed an initial increase at 24 hours, followed by its decrease at 48 and 72 hours. Physiologically active vitamin B_{12} was not produced by tempe moulds, but rather biosynthesised by contaminating bacteria during the tempe production process. For example, some vitamin B_{12}–synthesising bacteria have been isolated during traditional tempe preparation and have been identified as *Klebsiella pneumoniae* and *Citrobacter freundii* (Keuth and Bisping 1994). Preparing tempe using traditional methods or by deliberately co-fermenting with vitamin B_{12}-producing bacteria resulted in an increase in vitamin B_{12} content, whereas the use of pure *Rhizopus* culture did not (Liem et al. 1977; Wiesel et al. 1997).

Changes in the contents of water-soluble vitamins also occurred during the *Rhizopus* fermentation of cowpeas (Prinyawiwatkul et al. 1996) and bambara groundnuts (Fadahunsi 2009). Soaking and boiling cowpeas decreased thiamine, folate, niacin, and riboflavin contents, all of

which were recovered during the fermentation with the exception of thiamine, which underwent continuous reduction. The biotin level, also measured in the bambara groundnut fermentation, was also enhanced. Wang and Hesseltine (1966) produced wheat tempe with a smaller thiamine content but higher niacin and riboflavin concentrations than unfermented wheat. Chickpea and horse bean tempe were produced by Robinson and Kao (1977). Compared to the unfermented substrates, both tempe contained higher amounts of thiamine, riboflavin, pyridoxine, cobalamin, niacin, pantothenic acid, and ascorbic acid. Working on cooked cassava inoculated with *R. oryzae*, Daubresse et al. (1987) found an increase in thiamine during fermentation.

Vitamins in soybean tempe has been extensively studied, contributing valuable nutritional information about the food. However, much less is known regarding vitamins in non-soybean tempe, including that made from cassava bagasse. Thus, the investigation of the vitamin content of *Rhizopus* spp.-fermented cassava bagasse is necessary, especially if the product is intended for use as animal feed. An optimal supply of vitamins constitutes one of the most crucial factors in modern animal nutrition for the maintenance of animal health during animal farming (Albers et al. 2002a).

1.3 Aims of the study

The present work was aimed at investigating the nutrient enrichment, specifically that of proteins and water-soluble vitamins, of cassava bagasse brought about by SSF using edible filamentous fungi *Rhizopus* spp. The study consisted of the following stages (Figure 1-8):

A. Proximate analysis and pretreatment of cassava bagasse

Information about the major physico-chemical composition of cassava bagasse were investigated to help not only in determining the necessary nutritional supplementation for the cultivation of the mould but also to facilitate the interpretation of the results. Substrate pretreatment was carried out mainly to homogenise as well as to pregelatinise the bagasse.

B. Selection of the best growing *Rhizopus* strains

Rhizopus strains used in this work were previously domesticated in tempe fermentation using soybeans as nutritionally rich growth substrate. Thus, selection was necessary in order to determine which strains grow well on cassava bagasse substrate, which is poor in nutrients.

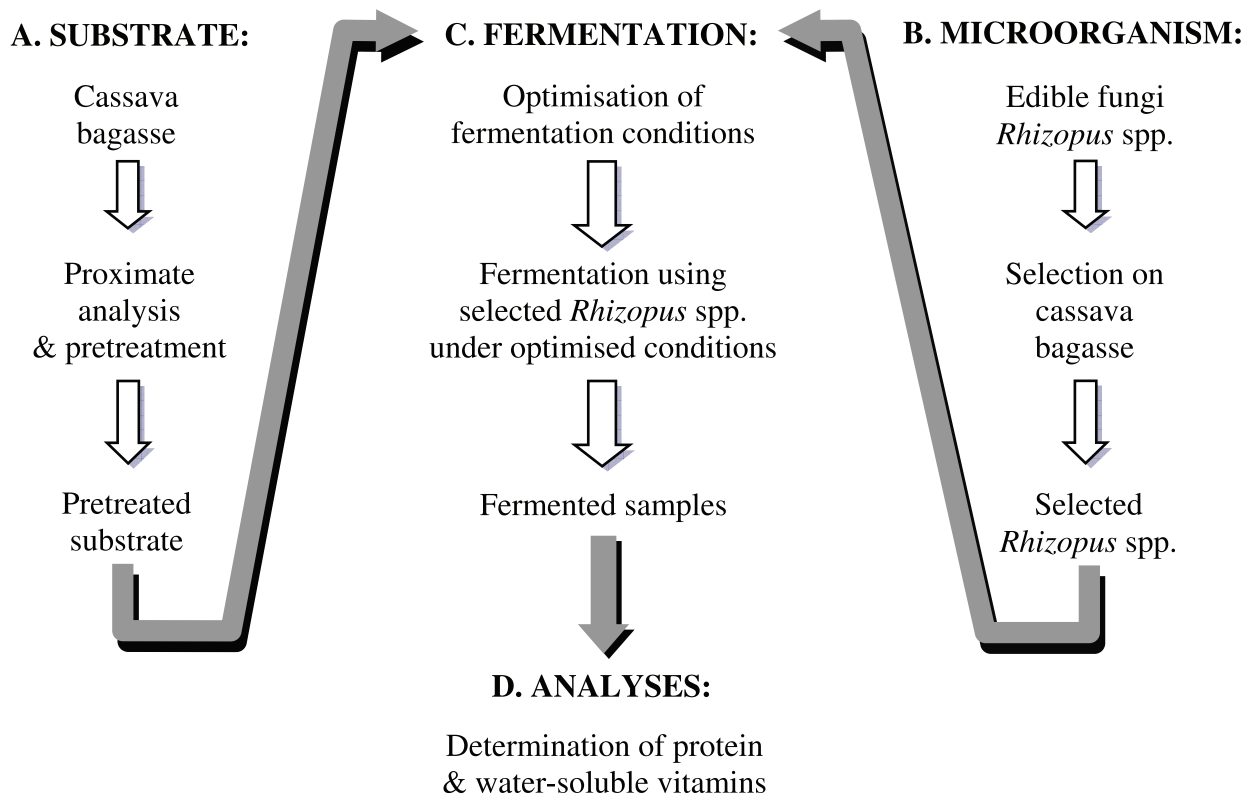


Figure 1-8: General outline of the present work

C. Optimisation of the fermentation conditions

Environmental conditions during fungal growth needed to be optimised in order to ensure the maximal utilisation of the substrate for fungal biomass formation. A number of parameters considered to be important were optimised.

D. Analysis of protein and water-soluble vitamins

Protein is not only important nutritionally in the fermented product, but also quantitatively as an indirect measurement of fungal growth, bearing in mind that the direct quantification of fungal mycelia in SSF is practically impossible. In addition, six water-soluble vitamins were determined.

2 Materials and Methods

All values are given on a dry weight (DW) basis, unless stated otherwise. Additional related information regarding materials and methods are included in the appendix (Section 7.2-7.8, and 7.15).

2.1 Substrate

2.1.1 Origin of cassava bagasse

Cassava bagasse used as the fermentation substrate in this project was obtained from the tapioca starch factory “Tapioka 35” situated in the Pekalongan subdistrict, Lampung Timur regency, Lampung, Indonesia. The plant has the capacity to process 140 tons of fresh cassava tubers per day, producing 20% tapioca starch and 20% dried cassava bagasse. The cassava bagasse was sun-dried in an open environment around the factory for 2–3 days.

2.1.2 Pretreatment

Before the cassava bagasse was used as the fermentation substrate, a pretreatment was carried out which included milling and pregelatinisation.

2.1.2.1 Milling

The cassava bagasse was brought from Lampung to Germany in the form of dried lumps and granules with heterogeneous physical dimensions, rendering homogenisation necessary. This was done by using an ultra-centrifugal mill (Retsch ZM 100, Haan, Germany) until the product could pass through a 1.00 mm sieve. The obtained cassava bagasse flour was subsequently subjected to proximate analysis and pregelatinisation.

2.1.2.2 Pregelatinisation

Milled cassava bagasse (200 g) was moistened with 300 mL distilled water and mixed in a household mixer (Braun Type KM 32, Frankfurt am Main, Germany). Each 200 g portion of this moistened bagasse was distributed into a 31 x 22 cm perforated polypropylene plastic bag (Brand, Wertheim, Germany), sealed, and autoclaved at 121°C for 15 minutes (Systec 2540 EL, Wettenberg, Germany). The resulting brown, gelatinised cassava bagasse was dried overnight at 60-70°C in an incubator (Hereaus, Hanau, Germany), and then ground mechanically

(Retsch ZM 100, Haan, Germany) to pass through a 1.00 mm sieve. Autoclaving at 121°C for 15 minutes was carried out to obtain a sterile substrate ready for fermentation experiments.

2.1.3 Proximate Analysis

Proximate analysis was performed on milled cassava bagasse to determine the contents of moisture, crude protein, crude lipids, fibre, starch, ash, and cyanogens.

2.1.3.1 Moisture and ash

The moisture content of cassava bagasse was determined according to a method modified from Windham (1998d). A 2-g sample was weighed to the nearest 0.001 g in a preweighed ceramic crucible on an analytical balance (Sartorius- BP211D, Göttingen, Germany) and dried to a constant weight at 100°C for 5 hours in an oven (M104, Heraeus, Hanau, Germany). The sample was allowed to cool to room temperature in a desiccator and was accurately weighed to determine the dry weight. The loss in weight was attributed to moisture content. The dried sample was subsequently pre-ashed and then ashed at 600°C for 2 hours (Windham 1998a) in the oven. Afterward, the crucible containing the white ash was carefully transferred to the desiccator to cool to room temperature before weighing on the analytical balance to the nearest 0.001 g. Moisture and ash were expressed as percentage or g/100 g, and calculated as follows:

$$\%\ Moisture\ (\%M) = \left(\frac{WW - DW}{WW}\right) \times 100\%$$

$$\%\ Ash\ (\%A) = \left(\frac{AW}{DW}\right) \times 100\%$$

WW = Wet weight, weight of sample before drying, in g

DW = Dry weight, weight of sample after drying, in g

AW = Ash weight, in g

2.1.3.2 Crude protein

Crude protein was determined using the Kjeldahl method as recommended by AOAC (Thiex 2000). The digestion and distillation of the sample were carried out using the K-424 Digestion

Unit and K-314 Distillation Unit (Büchi, Flawil, Switzerland) according to the procedure recommended by the manufacturer.

Reagents

Sodium hydroxide solution: for an 8 M solution, 320 g NaOH pellets (Carl Roth, Karlsruhe, Germany) were added with 1000 mL distilled water and stirred until a clear homogenous solution was obtained.

Boric acid solution: for a 2% solution, 20 g boric acid (Merck, Darmstadt, Germany) were added with 1000 mL distilled water.

Procedure

Samples (0.5-2 g) were weighed to the nearest 0.001 g, transferred into digestion vessels (or an empty digestion vessel for a blank), and added with 2 Kjeldahl tablets (Carl Roth, Karlsruhe, Germany) and with 15-25 mL of 98% sulphuric acid (Carl Roth, Karlsruhe, Germany). The samples were digested on the preheated digestion unit at heat level 10 for 60 minutes, and allowed to cool at room temperature.

The digested samples were added with 50 mL distilled water, placed into the distillation unit, added with 70 mL of 8 M NaOH, and distilled for 3-4 minutes. The distilled ammonia was collected in 60 mL boric acid solution (2%), and titrated against 0.1 N H_2SO_4 solution (Carl Roth, Karlsruhe, Germany) using an automatically operated titrator unit (TitroLine Alphaplus, Schott, Mainz, Germany). To obtain the nitrogen and crude protein values, the following formulae described by the manufacturer of the Büchi distillation unit were used:

$$\%\ Nitrogen\ (\%N) = \frac{[(V_S - V_B)] \times F \times c \times f \times M(N)}{DW \times 1000}$$

$$\%\ Crude\ Protein\ (\%CP) = \%N \times 6.25$$

V_S = volume of the standard H_2SO_4 titrant required to titrate the sample, in mL

V_B = average volume of the standard H_2SO_4 titrant required to titrate the blank, in mL

F = molar reaction factor

c = concentration of the standard H_2SO_4 titrant, in mol/L

f = factor of the standard H_2SO_4 titrant

M(N) = molecular weight of nitrogen, 14.007 g/mol

DW = Sample dry weight, in g

2.1.3.3 Crude lipids

Crude lipids were determined according to a modified method originally recommended by AOAC (1998) and Galyean (1997). In this procedure, crude lipids in the sample were extracted with diethyl ether in a soxhlet apparatus.

Procedure

Folded filter papers (Ø 125 mm, 595½, Whatman Schleicher & Schuell, Dassel, Germany), which would be used in the subsequent step to contain the test samples, were put into a thimble, and defatted by refluxing with 200 mL diethyl ether (Carl Roth, Karlsruhe, Germany) contained in a 250 mL round (boiling) beaker. Lipid extraction was performed for 4 hours at a condensation rate of 4-6 drops per second. The defatted thimble and papers were then dried at 105°C for 24 hours, followed by cooling to room temperature.

Samples (2 g) were weighed to the nearest 0.001 g in the defatted filter papers, as prepared above, dried at 105°C for 24 hours, and then put into a desiccator to cool to room temperature. Next, the sample was washed with 5 x 20 mL of 40°C distilled water to remove water-soluble components such as soluble carbohydrates, urea, lactic acid, glycerol, and others that might interfere with the lipid extraction (Windham 1998c). The sample was then dried overnight in a 35°C ventilated incubator (MIR-153, Sanyo, Osaka, Japan), followed by drying at 105°C for 24 hours.

The dried sample was then defatted by soxhlet refluxing using the aforementioned method using a defatting thimble and filter paper. After 4 hours, the thimble containing the sample was removed. The round beaker containing a mixture of the diethyl ether and the extracted lipid was then disconnected from the soxhlet apparatus and reconnected to a rotating distillation unit to remove the diethyl ether. The distilled diethyl ether was collected into a collecting flask until the round beaker was almost dry. The round beaker, containing the extracted lipid, was then placed in a 100°C oven for 30 minutes, cooled in a desiccator to room temperature, and weighed on the analytical balance. The weight difference of the round beaker before and after the ether extraction represented the crude lipid content of the sample (Galyean 1997). The following formula was used to calculate the crude lipid content in the sample:

$$\% \ Crude \ Lipid \ (\%CL) \ = \left(\frac{EL}{WW}\right) \times 100\%$$

EL = Extracted crude lipid, in g

WW = Wet weight, in g

2.1.3.4 Crude fibre

Crude fibre was determined according to the method of Galyean (1997) and Windham (1998b) with modifications. Dried, defatted samples (Section 2.1.3.3) were sequentially boiled in dilute acid and dilute base. The remaining sample was dried, and burned into ash in a furnace. The weight difference of the sample before and after ashing constituted the crude fibre content (Wright and Lackey 2008).

Reagents

Ceramic suspension: for a 7.5% suspension, 7.5 g finely ground boiling chips (Merck, Darmstadt, Germany) were suspended in 100 mL distilled water.

Sulphuric acid solution: for a 1.25% (w/v) solution, 1.25 g sulphuric acid (98%, Carl Roth, Karlsruhe, Germany) were dissolved in 1000 mL distilled water.

Sodium hydroxide solution: for a 1.25% (w/v) solution, a 1.25 g sodium hydroxide pellets (Merck, Darmstadt, Germany) was dissolved in 1000 mL distilled water.

Procedure

Two grams of dried, defatted sample were weighed to three decimal points on an analytical balance (Sartorius- BP211D, Göttingen, Germany). Then, 20 mL of the 7.5% aqueous ceramic suspension, 200 mL boiling 1.25 % sulphuric acid, and 5-8 boiling chips were added. The mixture was boiled for 30 minutes in a 600 mL glass beaker and rotated periodically to prevent solids from adhering to the sides. The content was subsequently filtered through a Buchner funnel precoated with 10 mL of the 7.5% aqueous ceramic suspension. The beaker was rinsed with 50-75 mL of boiling-hot distilled water, washed through the funnel, and sucked dry. This step was repeated with 3 x 50 mL boiling-hot distilled water, washed through the funnel, and sucked dry. The fibre mat with the residue was then returned to the beaker, to which 200 mL of boiling-hot 1.25% NaOH were added, and boiled for 30 minutes.

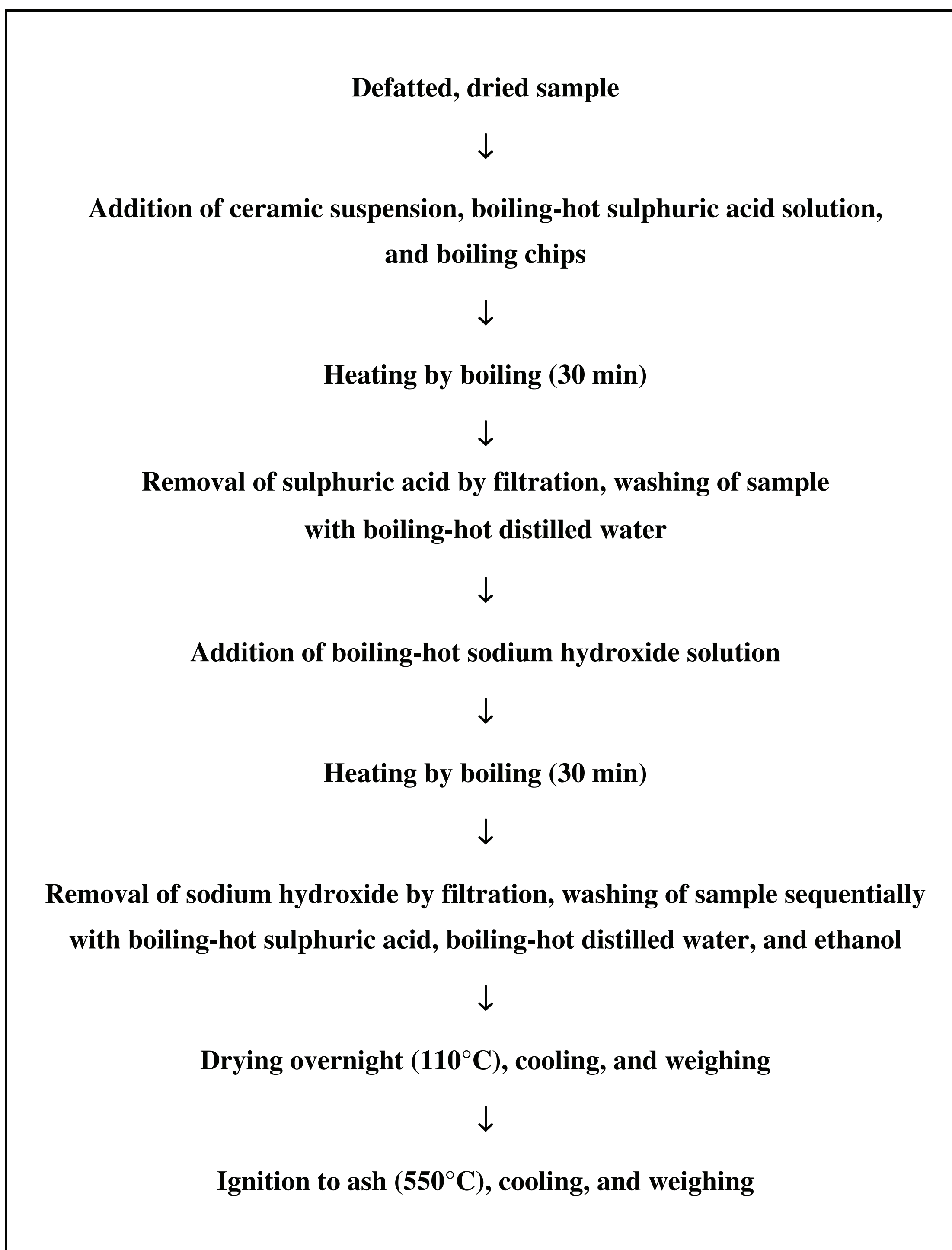


Figure 2-1: Crude fibre determination procedure.

The beaker was then removed and filtered as before. Next, the funnel was washed sequentially with 25 mL of boiling-hot 1.25% sulphuric acid, 3 x 50 mL of boiling-hot distilled water, and 25 mL of 96% ethanol (Carl Roth, Karlsruhe, Germany). The ceramic mat and the residue were subsequently removed and transferred into an ashing crucible, dried overnight at

100°C, cooled in a desiccator, and weighed on the analytical balance. Finally, the sample was pre-ashed, followed by ashing by ignition at 550°C for 2 hours, and cooled in a desiccator to room temperature to obtain a constant weight. Crude fibre was calculated using the following formula:

$$\% \ Crude \ Fibre \ (\%CF) \quad = \quad \left(\frac{CF}{WW} \right) \times 100\%$$

CF = Extracted crude fibre (= loss in weight on ignition), in g

WW = Wet weight, in g

2.1.3.5 Starch

The starch content of cassava bagasse was determined indirectly by quantifying the glucose residues released after the hydrolysis of the starch. The starch in the sample was first solubilised, and then hydrolysed enzymatically to release glucose residues, which were in turn quantified spectrophotometrically (U-2000, Hitachi, Tokyo, Japan) as total reducing sugars using the dinitrosalicylic acid (DNS) method.

Reagents

Hydrochloric acid solution: to produce an 8 M solution, 20 mL of 37% hydrochloric acid (Merck, Darmstadt, Germany) was added with distilled water, mixed, and filled to 30 mL.

DNS reagent: to make 100 mL reagent, two separate solutions first had to be made. Solution 1 was prepared by dissolving 1 g DNS (Merck, Darmstadt, Germany) in 20 mL of 2 N sodium hydroxide solution, warming with vigorous stirring to facilitate dissolution. Solution 2 was made by dissolving 30 g sodium potassium tartrate tetrahydrate (Rochelle salt) (Carl Roth, Karlsruhe, Germany) in distilled water, making up to 50 mL. The DNS reagent was prepared by combining these two solutions, making up the volume to 100 mL with distilled water, and mixing homogenously.

Glucose standard solution: to produce a 1000 µg/mL standard stock solution, 16.5 mg of α-D(+)-glucose monohydrate (Carl Roth, Karlsruhe, Germany) was dissolved in 15 mL blank solution (see paragraph below under “Solubilisation of starch”). Appropriate dilutions were

made with the blank solution to produce a series of standard solutions with concentrations between 100–1000 µg/mL.

Procedure

Starch in the cassava bagasse was solubilised according to the method described in the technical bulletin of Sigma's Starch Assay Kit (SA-20, Sigma, Saint Louis, Missouri, USA). The sample (500 mg) was added with 20 mL dimethyl sulphoxide (DMSO) (Carl Roth, Karlsruhe, Germany) and 5 mL of 8 M HCl. The flask was covered and incubated for 30 min at 60°C in a 250 rpm shaking water bath (Gyrotory G76, New Brunswick Scientific, New Jersey, USA). Then, 50 mL of deionised water was added and the pH was adjusted to 4.0-5.0 with 5 N NaOH. After cooling to room temperature, the whole volume was made up to 100 mL with deionised water. A blank was prepared similarly in the absence of sample to obtain a blank solution.

To hydrolyse the solubilised starch, an amyloglucosidase solution (50U/mL) from *Aspergillus niger* (Starch Assay Reagent S 9144, Sigma, Saint Louis, Missouri, USA) was used. A series of solubilised sample tests and blanks were prepared as below:

Tube designation	Mixture component (mL)			Total volume (mL)
	Glucoamylase	Sample	Deionised water	
Glucoamylase blank	0.5	0	0.5	1
Sample blank	0	0.5	0.5	1
Sample test	0.5	0.5	0	1

Samples were incubated at 60°C for 30 minutes with 1000 rpm rotary shaking (Mastercycler Personal, Eppendorf, Hamburg, Germany), cooled to room temperature, and centrifuged at 13,000 rpm for 15 minutes (Biofuge Pico, Heraeus, Hanau, Germany). The clear supernatant was pipetted into new microtubes, and stored at -2°C until use.

The DNS method developed by Bittman (1974) for the spectrophotometric quantification of reducing sugars was used to quantify the glucose released from the hydrolysed starch. One

millilitre sample (diluted when necessary) was mixed with 1 mL DNS reagent and 3 mL deionised water. After heating for 5 minutes in boiling water and cooling promptly under tap water to stop the reaction, the volume was adjusted to 10 mL with deionised water. The absorbance was then read spectrophotometrically at 540 nm (U-2000, Hitachi, Tokyo, Japan).

The absorbance values of the glucoamylase blank and the sample blank were subtracted from those of the test samples. The total reducing sugar concentration was then determined from the linear regression of the standard curve of the absorbance versus the known concentration of the α-D(+)-glucose standard solution (Appendix 7.2). The starch content was expressed as the glucose equivalent multiplied by 0.9 (Cruz et al. 2003).

2.1.3.6 Cyanide

Total cyanide and cyanide potential in cassava bagasse were determined spectrophotometrically using a cyanide test kit based on the modified König reaction which has been described by several authors (Lambert et al. 1975; Bradbury et al. 1991; Walker et al. 2008)

Reagents

Phosphoric acid: for a 0.1 M solution, 1.4 mL of 85% phosphoric acid (Carl Roth, Karlsruhe, Germany) were dissolved in 200 mL distilled water.

Cyanide standard solution: to produce a 1 mg/mL stock standard solution, cyanide standard solution ($K_2[Zn(CN)_4]$ in H_2O at a concentration of 1000 mg CN^-/L, Merck, Karlsruhe, Germany) was diluted 1000 times with deionised water. Further appropriate dilutions were made with blank solution to produce a series of standard solutions with concentrations of 0.002, 0.006, 0.01, 0.02, 0.04, 0.05, 0.06, 0.08, 0.10, and 0.20 mg/L.

Procedure

Total cyanide was extracted with phosphoric acid solution according to a previously described method (Bradbury et al. 1991) with modifications. Five grams of samples were homogenised in 50 mL of 2°C-cold 0.1M H_3PO_4 using ultraturrax for 2 minutes at medium speed. The suspension was then centrifuged at 10,000 rpm for 15 minutes (Centrikon H-401, Kontron Hermle, Eching Munich, Germany) at 4°C to extract the supernatant.

Table 2-1: *Rhizopus* strains used for fermentations in the present study.

No.	Species	Strain	Collection No.	Origin (Location, Country)
1.	*R. oligosporus*	Bali	312T	Denpasar, Bali, Indonesia
2.	*R. oligosporus*	Hepla	2009T	Bandung, Java, Indonesia
3.	*R. oligosporus*	Tebo	290T	Bogor, Java, Indonesia
4.	*R. oligosporus*	CN	1143T	Bogor, Java, Indonesia
5.	*R. oligosporus*	East jba	2038T	Jakarta, Java, Indonesia
6.	*R. oligosporus*	East jbp	2039T	Jakarta, Java, Indonesia
7.	*R. oligosporus*	L2/1	2013T	Lampung, Sumatra, Indonesia
8.	*R. oligosporus*	Balu 1	2007T	Lampung, Sumatra, Indonesia
9.	*R. oligosporus*	MS3	2019T	Medan, Sumatra, Indonesia
10.	*R. oligosporus*	MS5	2020T	Medan, Sumatra, Indonesia
11.	*R. oligosporus*	Pon	2022T	Pontianak, Kalimantan, Indonesia
12.	*R. oligosporus*	Purwo	2023T	Purwokerto, Java, Indonesia
13.	*R. oligosporus*	Sama	2026T	Samarinda, Kalimantan, Indonesia
14.	*R. oligosporus*	Tegal	2031T	Tegal, Java, Indonesia
15.	*R. oligosporus*	Q1	2024T	Tulungagung, Java, Indonesia
16.	*R. oligosporus*	Q2	2025T	Tulungagung, Java, Indonesia
17.	*R. oligosporus*	Tup	2033T	Tulungagung, Java, Indonesia
18.	*R. oligosporus*	Uju	2034T	Ujung Pandang, Sulawesi, Indonesia
19.	*R. oligosporus*	CD	1133T	Bandung, Java, Indonesia
20.	*R. oligosporus*	Puda	20S	Yogyakarta, Java, Indonesia
21.	*R. oligosporus*	Serp	2027T	Serpong, Java, Indonesia
22.	*R. oryzae*	Fi	10S	Bogor, Java, Indonesia
23.	*R. oryzae*	EN	1134T	Enschede, Netherlands
24.	*R. oryzae*	L2	2036T	Jakarta, Java, Indonesia
25.	*R. oryzae*	Mala	11S	Malang, Java, Indonesia
26.	*R. oryzae*	ZB	18S	Zoersel, Belgium
27.	*R. stolonifer*	GT	1136T	Jakarta, Java, Indonesia
28.	*R. chinensis*	Sur	277T	Surabaya, Java, Indonesia

Free and readily liberated cyanides were determined photometrically using the Spectroquant cyanide test kit (Merck, Karlsruhe, Germany). The aqueous samples obtained above were subjected to a procedure carried out according to the manufacturer's instructions, including a number of steps: digestion to liberate the cyanide, the reaction of the cyanide ions with a chlorinating agent to form cyanogen chloride, and the addition of 1,3-dimethylbarbituric acid to form a purple solution (pyridine-free König reaction). The standard solutions were treated as with the samples, but without the digestion step. Finally, a spectrophotometric measurement was taken at 585 nm (U-2000, Hitachi, Tokyo, Japan). The absorbance values obtained were used to calculate cyanide concentrations using the linear regression of standard curves constructed with a series of cyanide standard solutions (Appendix 7.2).

2.2 Microorganisms

2.2.1 *Rhizopus* strains

The twenty-eight *Rhizopus* strains used in this study were obtained from the strain collection of the Food Microbiology and Biotechnology Division, Institute of Food Chemistry, Department of Chemistry, Universität Hamburg, Germany (Table 2-1). The strains originated from tempe cakes and tempe inocula sold commercially in Indonesia, Belgium, and the Netherlands. About two-thirds of the strains had already been used in previous studies on proteolysis (Baumann and Bisping 1995), changes in fatty acid composition (Hering et al. 1991), and vitamin formation (Keuth and Bisping 1993) during soybean tempe fermentation.

2.2.2 Culture maintenance

Prior to selection on cassava bagasse growth media (Section 2.2.3), all of the *Rhizopus* strains were maintained on malt extract agar (MEA). After five best-growing *Rhizopus* strains were selected, these strains were subsequently maintained on cassava bagasse agar.

2.2.2.1 Culture maintenance before selection

Malt extract agar (MEA) was prepared by dissolving 48 g of MEA powder (Merck, Darmstadt, Germany) in 1000 mL distilled water and homogenised by incubation in a 100°C water bath with occasional stirring until a brownish yellow solution was obtained. The medium was then sterilised by autoclaving at 121°C for 15 minutes.

Rhizopus strains were regenerated from the -80°C stock culture and cultivated on slant MEA at 30°C for 4 days or until sporulating. Subsequent maintenance subculture was carried out in 4- to 7-week intervals on the same medium at 30°C for 7 days or until sporulating. The cultures were stored at 2-4°C.

2.2.2.2 Culture maintenance after selection

Cassava bagasse agar (CBA) was prepared according to Mitchell et al. (1990), with components modified as detailed in Table 2-2. All of the components were mixed homogenously by heating in boiling water with occasional stirring until the cassava bagasse was homogenously gelatinised. Sterilisation was then carried out in an autoclave at 121°C for 15 minutes (Systec 2540 EL, Wettenberg, Germany). On this medium, the selected *Rhizopus* strains were subcultured at 30°C for 7 days or until sporulation. The cultures were stored at 2-4°C for up to 24 months.

2.2.3 Selection of *Rhizopus* strains

The ability of the *Rhizopus* strains in the culture collection (Table 2-1) to grow well on starch rich, protein poor substrates like cassava bagasse has not yet been studied. Therefore, strain selection was necessary. For this purpose, three types of media were prepared in 9-cm-diameter Petri dishes, namely raw cassava bagasse agar (RCBA), gelatinised cassava bagasse agar (GCBA), and gelatinised cassava bagasse mash (GCBM). No agar was added to GCBM.

Table 2-2: Composition of the cassava bagasse maintenance medium

Composition	Quantity
$(NH_4)_2SO_4$ (Carl Roth, Karlsruhe, Germany)	10 g
KH_2PO_4 (Fluka, Steinheim, Germany)	1 g
K_2HPO_4 (Carl Roth, Karlsruhe, Germany)	1 g
Cassava bagasse	15 g
Agar-agar powder (Kobe I, Carl Roth, Karlsruhe, Germany)	20 g
Distilled water	1000 mL

2.2.3.1 Preparation of selection media

The mineral base E solution of Owens and Keddie (1969) had already been used to prepare synthetic growth media for *Rhizopus* spp. in previous studies (Graffham et al. 1995; Sparringa and Owens 1999c). In this experiment, the mineral base solution, with modifications (Table 2-3), was prepared and included in the selection media, as the mineral content of cassava bagasse had not been analysed. This mineral supplementation was included to ensure that *Rhizopus* growth performance would not be due to lack of essential minerals but was rather determined solely by the individual strain's ability to utilise cassava bagasse as a carbon and energy source.

To prepare GCBA medium, 15 g of agar (Kobe I powder, Carl Roth, Karlsruhe, Germany), 10 g of $(NH_4)_2SO_4$ and 1.4 g of $K_2HPO_4{\cdot}3H_2O$ were dissolved in 1000 mL of the mineral solution (Table 2-3) by homogenous stirring and heating in boiling water. Then, 20 g of cassava bagasse were added and mixed homogenously until gelatinisation occurred, followed by autoclaving at 121°C for 15 minutes.

RCBA medium was made using a procedure similar to that described for the preparation of GCBA above, with the exception that the cassava bagasse (100 g) was autoclaved separately. When the autoclaved agar medium had cooled to 50°C, the sterilised cassava bagasse was added and mixed homogenously with magnetic stirring. At a temperature of 50°C, the agar had not yet solidified and the starch contained in the added cassava bagasse had not gelatinised. The bagasse precipitates easily in aqueous suspension and settles at the bottom. Therefore, a higher concentration of cassava bagasse was used than in GCBA medium in order to ensure that enough cassava bagasse would remain in the upper layer after the agar had set.

To prepare GCBM medium, 350 mL of mineral solution were prepared according to the composition described in Table 2-3, but was slightly modified to contain 2% $(NH_4)_2SO_4$ and 0.025% $K_2HPO_4{\cdot}3H_2O$. The solution was mixed with 52.5 g cassava bagasse. After homogenising and gelatinising in boiling water with occasional stirring, the medium was autoclaved at 121°C for 30 minutes. After cooling to 80-50°C, this thick and viscous mash was poured into Petri dishes (20 g/dish).

Table 2-3: Composition of mineral solution.

Ingredient	Amount
KH_2PO_4 (Fluka, Steinheim, Germany)	750 mg
$MgSO_4 \cdot 7H_2O$ (Fluka, Steinheim, Germany)	200 mg
NaCl (Carl Roth, Karlsruhe, Germany)	100 mg
$CaCl_2 \cdot 2H_2O$ (Fluka, Steinheim, Germany)	34 mg
$EDTANa_2 \cdot 2H_2O$ (Merck, Darmstadt, Germany)	19.1 mg
$ZnSO_4 \cdot H_2O$ (Fluka, Steinheim, Germany)	4.1 mg
$MnSO_4 \cdot H_2O$ (Merck, Darmstadt, Germany)	1.3 mg
$FeSO_4 \cdot 7H_2O$ (Carl Roth, Karlsruhe, Germany)	1.5 mg
$CuSO_4$ (Fluka, Steinheim, Germany)	0.3 mg
$Na_2MoO_4 \cdot 2H_2O$ (Merck, Darmstadt, Germany)	0.45 mg
Distilled water	filled to 1000 mL

During the preparation of the selection media, no pH adjustment was made. To measure the initial pH of the media before use, 5 g medium were homogenised in 50 mL distilled water using the Ultra-Turrax (TP 18/10, IKA Werk, Staufen, Germany) at the lowest speed for 60 seconds, and the pH values were then measured. The pH values of GCBA, RCBA, and GCBM were found to be 4.7, 6.2, and 4.8, respectively.

2.2.3.2 Preparation of the spore inoculum

The spore inoculum was prepared using a sterile 0.9% saline solution. This solution was made by dissolving 9 g NaCl (Carl Roth, Karlsruhe, Germany) in 1000 mL distilled water, and autoclaved at 121°C for 15 minutes.

Rhizopus strains were cultured on malt extract agar (Section 2.2.2.1) or cassava bagasse agar (Section 2.2.2.2) at 30°C for 7 days or until sporulation. The spores were harvested by adding 3 mL of sterile 0.9% saline containing 0.001% Tween 80, vortexing briefly, and rubbing us-

ing an inoculating needle to facilitate spore removal. The spore concentration was determined using a 0.100 mm depth counting chamber (Assistent Neubauer, Sondheim, Germany) and adjusted by dilution with the saline solution to achieve the required concentrations.

2.2.3.3 Selection on cassava bagasse media

Twenty microlitres of spore inoculum (10^6 spores/mL) were used for single-point inoculation on the centre of the RCBA and GCBA media. (Single-point inoculation allowed the fungi to form a single mycelial colony growing radially as a circular mat from the point of inoculation outward toward the edge of the media). Each strain was inoculated in duplicate, and allowed to grow at 30°C for 56 hours.

Single-point inoculation on GCBM was carried out by pipetting 10 µl of spore inoculum (10^6 spores/mL) onto the centre of the medium contained in a Petri dish. Duplicate samples of each strain were made and the incubation was carried out at 30°C for 44 hours.

Growth performance was evaluated by measuring the diameter of the mycelial colony formed at the end of incubation period. Brand et al. (2000) showed that *Rhizopus* strains that produced a higher quantity of biomass corresponded to those showing a faster spreading growth rate on coffee-husk extract agar medium.

The perpendicular diameters of the circular fungal mat formed by each strain on the agar selection media were measured and the average diameter was calculated. On the mash medium, the fungal mat did not develop as regular circular shape, making the measurement of the circular diameter practically impossible. Therefore, the growth of each *Rhizopus* strain on the latter medium was qualitatively evaluated based on whether or not it was able to grow over the entire surface of the medium within the given incubation period.

The cassava mash plates overgrown by the *Rhizopus* mycelial mats were subjected to further physico-chemical analyses. These included soluble protein (Section 2.4.2.1), true protein (Section 2.4.2.2.1) as well as residual carbohydrate (Section 2.4.3) analyses. pH values were determined after fermentation by suspending 3 g of fermented mash, vortexing vigorously for 10 seconds, and then measuring on a pH meter (WTW, Weilheim, Germany).

2.3 Fermentation

Pregelatinised cassava bagasse (Section 2.1.2.2) was used as both a substrate as well as a growth medium for the cultivation of the selected *Rhizopus* strains (Section 2.2.3). To facilitate better growth, substrate utilisation, and biomass formation of these strains, an optimisation of the fermentation conditions was carried out.

2.3.1 General fermentation procedure

During optimisation and subsequent experiments, a general fermentation procedure was carried out as described in Figure 2-2 and Figure 2-3, with each sample in duplicate. To every 10 g of substrate, warm salt solution was added, hand mixed with a spatula, and then allowed to cool to room temperature. Next, 1 mL of the spore suspension was added, and hand mixed

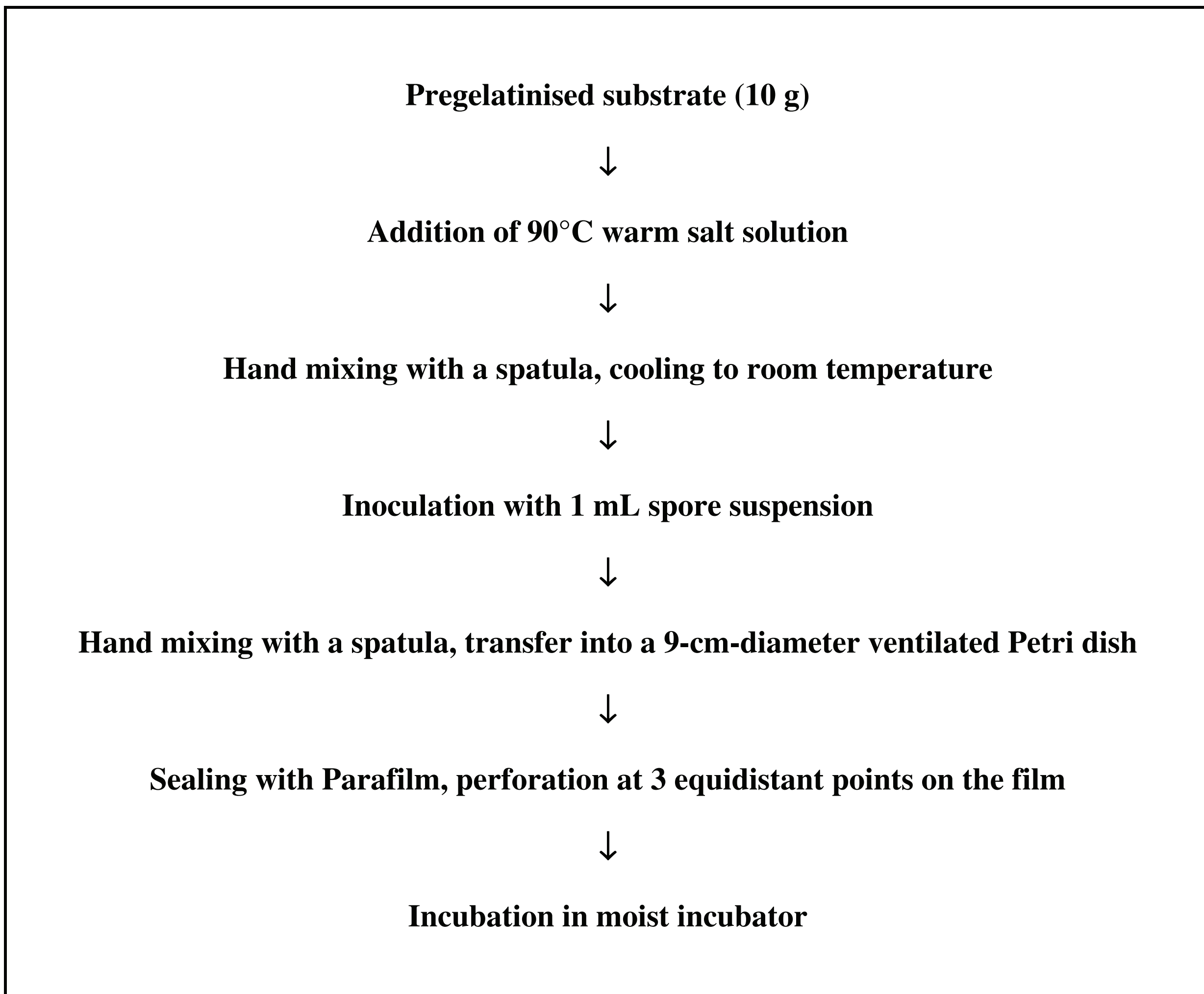


Figure 2-2: General fermentation procedure.

again with a spatula. The whole mixture was subsequently transferred into a 9-cm-diameter ventilated Petri dish. The ventilated dish had three venting ribs into the underside of the lids to allow air circulation in and out of the dish. To limit aeration, Parafilm (Pechiney, Chicago, USA) was then applied to seal the dish along the interface between the lid rim and the dish side. On the seal, three equidistant points were perforated using a 200-µl yellow pipette tip. The incubation was carried out in an incubator with moist air created by placing 4 round glass dishes (14 cm diameter) containing water at the bottom inside the incubator. At the end of the incubation period, the fermented samples were taken out and stored at -20°C or -80°C until use.

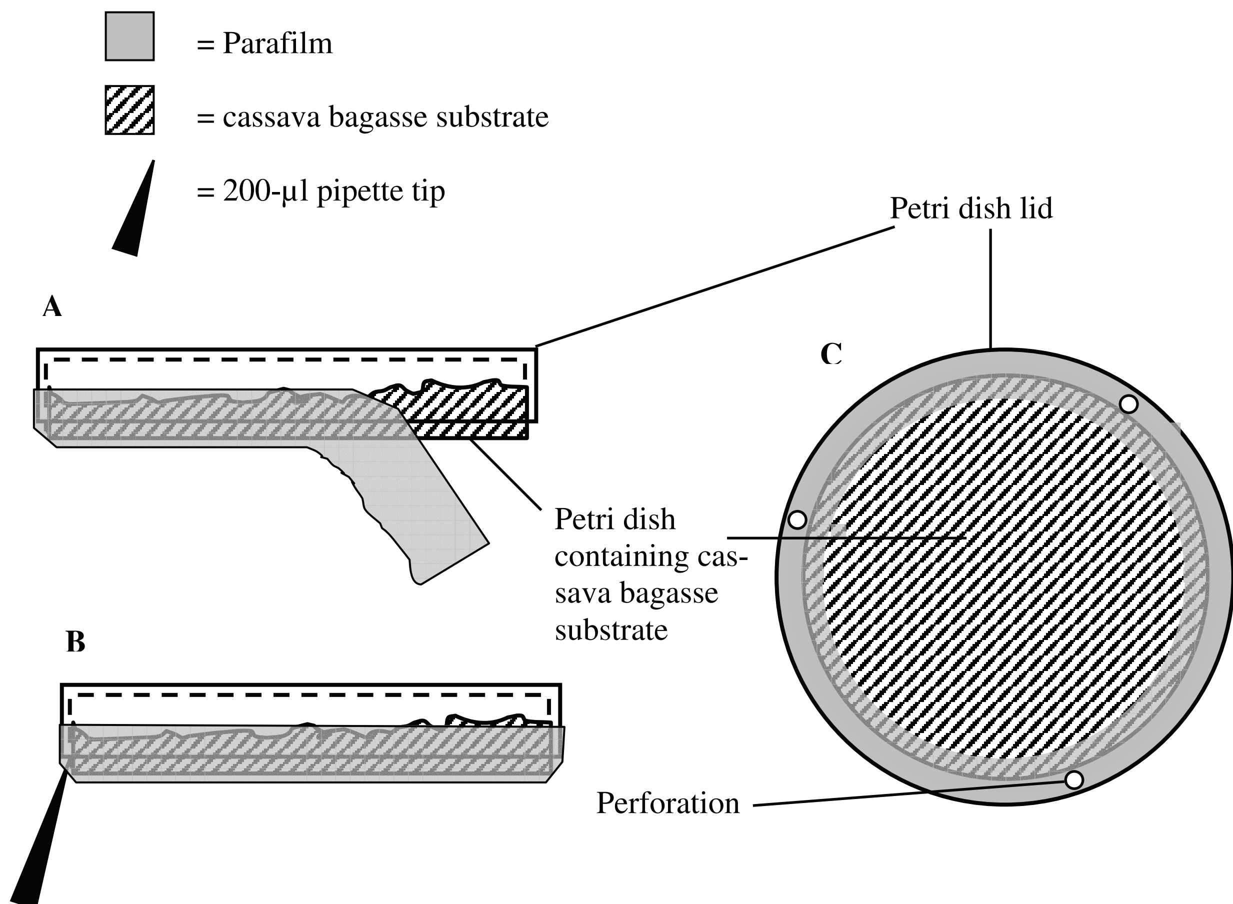


Figure 2-3: Schematic representation of the moistened cassava bagasse in a Petri dish being sealed with Parafilm (A) and perforated using a 200-µl pipette tip (B) at three equidistant points on the underside (C).

2.3.2 Optimisation of the fermentation conditions

An optimisation of fermentation conditions was carried out using the one-factor-at-a-time (OFAT) approach. The parameters optimised were inoculum concentration, ammonium sulphate concentration, moisture content, substrate initial pH, incubation temperature, the composition of the mineral solution, and nitrogen sources.

Unless otherwise stated, pregelatinised cassava bagasse was used as the fermentation substrate. The general fermentation procedure was carried out under sterile conditions. During the optimisation, *R. oryzae* ZB spores were exclusively used as the inoculum, since this strain was found to produce the highest biomass when grown on GCBM selection medium (Section 2.2.3.3). The effects of the optimised parameters on fungal growth were measured indirectly by determining the amount of soluble fungal protein of the fermented product (Section 2.4.2.1).

2.3.2.1 Inoculum concentration

The inoculum concentration was optimised by varying the concentration of the *R. oryzae* ZB spore suspension used as the inoculum. Seven different concentrations of the spore suspension ranging from 10^1-10^7/mL were prepared.

The mineral solution was prepared according to the composition described previously (Table 2-3), with 2% $(NH_4)_2SO_4$. This mineral solution was diluted with distilled water at a ratio of 6:5 (v/v), mixed homogenously, and sterilised at 121°C for 15 minutes.

The fermentation procedure was performed as previously described (Section 2.3.1). To every 10 g of substrate, 12.2 mL of the salt solution and 1 mL inoculum with calculated concentrations of 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, or 10^7 spores per mL were added. The spore inoculum was prepared as described previously (Section 2.2.3.2). The moistened substrate had a calculated initial moisture content of 55% and an initial substrate pH of 4.8. The cultures were grown for 93 hours at 30°C.

2.3.2.2 Ammonium sulphate concentration

The ammonium sulphate was optimised by changing its concentration in the salt solution used to moisten the cassava bagasse substrate. Six different salt solutions containing different concentrations of ammonium sulphate were prepared.

Table 2-4: Salt solutions with varying concentrations of ammonium sulphate.

Treatment	Volume of 40% ammonium sulphate added (mL)	Concentration of ammonium sulphate in:		initial pH of moistened substrate
		100 mL salt solution (%)	moistened fermentation substrate (% DW)	
1	45	18	18	4.6
2	33	13.1	13.8	4.5
3	21	8.2	9.1	4.7
4	8	3.3	3.8	4.7
5	4	1.6	2	4.8
6	0	0	0	5.3

Six salt solutions were prepared as indicated in Section 2.3.2.1. Each consisted of 55 mL of mineral solution added to a precalculated volume of 40% ammonium sulphate solution to produce salt solutions with different concentrations of ammonium sulphate (Table 2-4). The final volume was then filled to 100 mL with distilled water. In addition, the concentrations of $K_2HPO_4{\cdot}3H_2O$ and KH_2PO_4 in the original mineral solution (Table 2-3) were increased to 2720 and 1500 mg/L, respectively, to increase buffering strength. Fermentation was carried out as before (Section 2.3.1), with a spore inoculum concentration of 10^3 spores/mL. Incubation was carried out at 30°C for 67 hours.

2.3.2.3 Moisture content

The initial moisture content of the moistened cassava bagasse substrate at the start of fermentation was optimised by varying the volume of the salt solutions added to the substrate. Six different volumes of the same salt solution were prepared to obtain initial moisture contents ranging from 55–73%.

Table 2-5: Variation of the moisture content in the cassava bagasse substrate.

Treatment	Salt solution added per 10 g substrate (mL)	Initial moisture content of the moistened substrate (% WW)	Ammonium sulphate concentration in the moistened substrate (% DW)	Initial pH of the moistened substrate
1	12.2	55	3.9	4.7
2	15	60	4.7	4.7
3	18	64.3	5.6	4.8
4	21	67.7	6.5	4.8
5	24	70.6	7.3	4.9
6	27	73	8.2	4.9

The salt solution (Treatment 4 in Table 2-4) optimised in Section 2.3.2.2 was used. This time, the volume of the salt solution added to the cassava bagasse was varied. This caused the moisture content and concentration of the compounds constituting the salt solution, notably ammonium sulphate, to change as well (Table 2-5). The previously described fermentation procedure (Section 2.3.1) was used with an incubation time of 68 hours.

2.3.2.4 Initial pH

The initial pH of the moistened cassava bagasse substrate was optimised by preparing six salt solutions with different pH values. The pH of each solution was adjusted prior to heat sterilisation so as to have values ranging from 2.0 to 8.0.

Based on the previously optimised parameters, the salt solution containing 3.3% ammonium sulphate was used and added to the substrate to yield an initial moisture content of 67.7% (Section 2.3.2.3). This was equivalent to 21 mL salt solution per 10 g dry substrate.

To prepare similar salt solutions with different pH values, 16.4 g ammonium sulphate were mixed with 276.4 mL mineral solution (prepared as in Section 2.3.2.3), and filled to 400 mL with distilled water. Forty millilitres of this solution was adjusted to different pH values (Table 2-6) by the stepwise addition of 1 M HCl or 1M Na_2CO_3 before making the final volume

up to 50 mL with distilled water. The fermentation procedure was carried out as previously described (Section 2.3.1) with an incubation time of 61 hours.

Table 2-6: pH of the salt solutions and moistened substrates.

Sample	pH of salt solution	pH of moistened substrate
1	2.0	3.5
2	3.5	4.5
3	6.5	5
4	7.0	5.3
5	7.4	6
6	8.0	6.6

2.3.2.5 Incubation temperature

The incubation temperature was optimised by varying the temperature of the incubator used to grow the fungus on the cassava bagasse substrate. Substrate was mixed with salt solution prepared as described in Section 3.3.2.4 so as to achieve a moistened substrate with an initial pH of 4.5–6.5. The fermentation procedure was performed as previously described (Section 2.3.1), but at varying incubation temperatures of 27, 30, 33, 36, and 39°C. Samples were taken at 36, 48, and 61 hours.

2.3.2.6 Composition of mineral solution

So far, the cassava bagasse substrate was always moistened with a complete mineral solution (Table 2-3). To find out whether all of the salts added to the mineral solution were in fact necessary for the growth of *R. oryzae* ZB, the experiment described below was carried out.

A series of salt solutions was prepared with different compositions of minerals (Table 2-7). The subsequent procedure for the preparation of the salt solutions was described before (Section 2.3.2.5). The fermentation procedure was performed as described in Section 2.3.1 with an incubation temperature of 30°C for 48 hours.

2.3.2.7 Nitrogen sources

The complete mineral solution (Table 2-3) was no longer used because prior experiment (Section 2.3.2.6) had shown that the supplementation of only KH_2PO_4 and ammonium sulphate was enough to support good fungal growth. In this step, however, urea was supplemented to the salt solution as an additional nitrogen source. This was because an earlier study had indicated that the optimisation of the ratio of urea to ammonium sulphate as nitrogen sources was shown to enhance the growth of *Aspergillus niger* (Raimbault and Alazard 1980). Nitrogen sources were then optimised by varying the concentration ratio of urea to ammonium sulphate in the salt solution.

Table 2-7: Different compositions of the mineral solution.

Component	Concentration (mg/L)	Mineral solution[1]							
		1	2	3	4	5	6	7	8
K_2HPO_4	2720	+	+	+	+	+	+	–	–
KH_2PO_4	1500	+	+	+	+	+	–	–	+
$MgSO_4{\cdot}7H_2O$	200	+	+	+	+	–	–	–	–
NaCl	100	+	+	+	–	–	–	–	–
$CaCl_2{\cdot}2H_2O$	34	+	+	–	–	–	–	–	–
$EDTANa_2{\cdot}2H_2O$	19.1	+	–	–	–	–	–	–	–
$ZnSO_4{\cdot}H_2O$	4.1	+	–	–	–	–	–	–	–
$MnSO_4{\cdot}H_2O$	1.3	+	–	–	–	–	–	–	–
$FeSO_4{\cdot}7H_2O$	1.5	+	–	–	–	–	–	–	–
$CuSO_4$	0.3	+	–	–	–	–	–	–	–
$Na_2MoO_4{\cdot}2H_2O$	0.45	+	–	–	–	–	–	–	–
$(NH_4)_2SO_4$	33	+	+	+	+	+	+	+	+

[1] + : supplemented, – : not supplemented

Five salt solutions were made by adding different volumes of 10% ammonium sulphate solution to 225 mL of 1.5 g/L KH_2PO_4 solution, mixed well, adjusted to a pH between 7.0-7.5 with 1 M Na_2CO_3, autoclaved at 121°C for 15 minutes, and then allowed to cool to room

temperature. Filter sterilised (0.45 µm) 10% urea (Carl Roth, Karlsruhe, Germany) solution was added to achieve appropriate ratios (v/v) of ammonium sulphate to urea in the solution. The volume was finally filled to 400 mL with sterile distilled water. The total combined concentration (w/v) of both nitrogen sources were kept the same, namely 3.3% in the salt solution and 6.5% (DW) in the moistened substrate (Table 2-8). The calculated concentration of KH_2PO_4 in the salt solution was 0.8%.

Table 2-8: Different ratios of ammonium sulphate to urea in the salt solution.

Sample	Concentration in salt solution (% w/v)			Concentration of ammonium sulphate + urea in 67.7%-moistened substrate (% DW)
	Ammonium sulphate	Urea	Total ammonium sulphate + urea	
1	3.3	0	3.3	6.5
2	2.5	0.8	3.3	6.5
3	1.7	1.6	3.3	6.5
4	0.9	2.4	3.3	6.5
5	0	3.3	3.3	6.5

The fermentation procedure was performed as described previously (Section 2.3.1), but 10^5 spores/mL inoculum was used, and the incubation was done at 30°C for 120 hours. Samples were taken at 0, 24, 48, 72, and 120 hours.

2.3.3 Influence of some other factors

Further experiments were carried out to investigate the influence of substrate pretreatment, sulphur source, the temperature of the salt solution, and different *Rhizopus* strains on fungal growth. The general fermentation procedure described in Section 2.3.1 was applied using fermentation parameters optimised in the previous experiments (from Section 2.3.2.1 until Section 2.3.2.7) as shown in Figure 2-4.

A salt solution containing 0.8% potassium dihydrogen phosphate, 1.7% ammonium sulphate and 1.6% urea, with a pH of 7.0-7.5 was prepared as described in Section 2.3.2.4. The subsequent procedure was described in Figure 2-4.

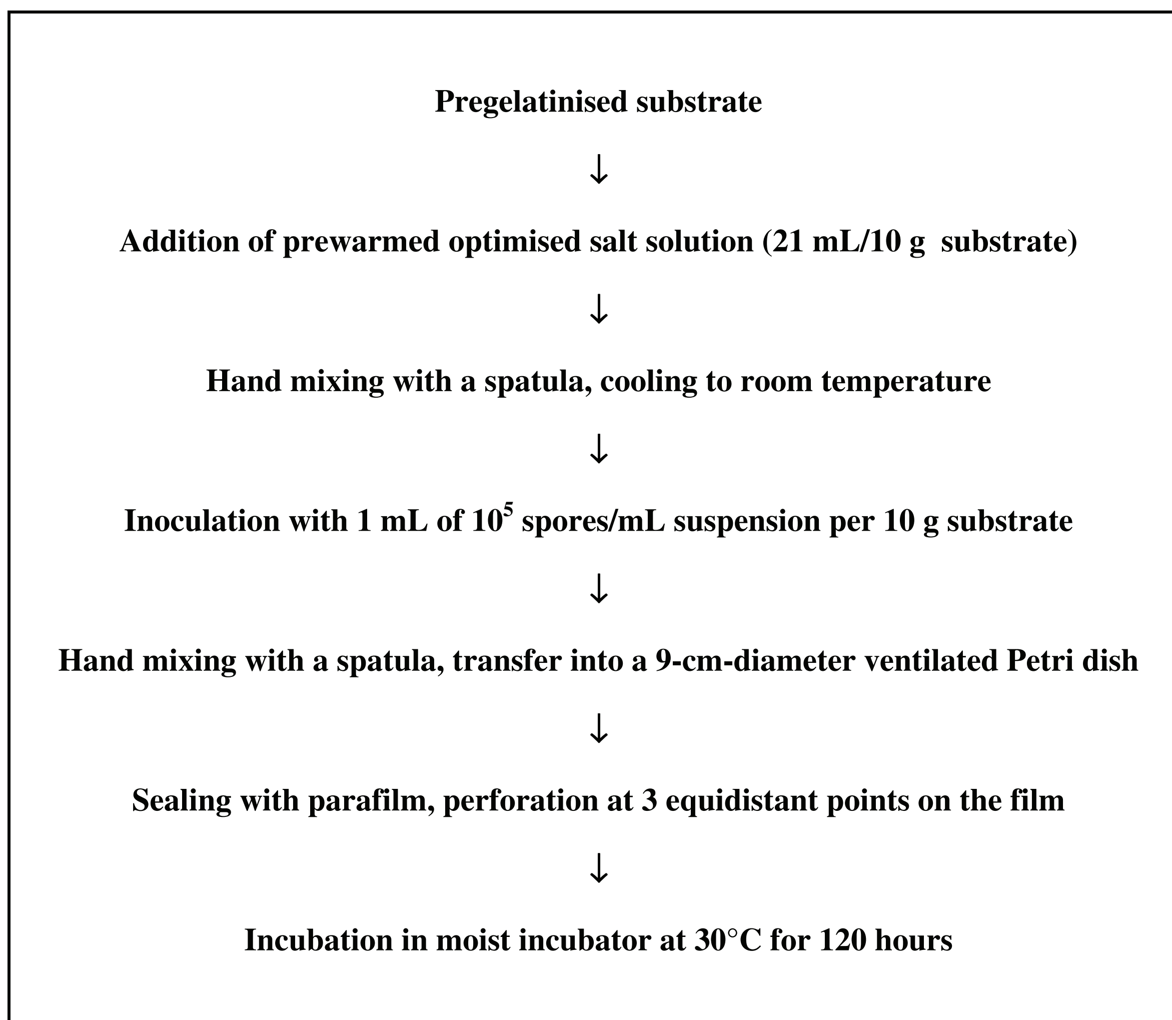


Figure 2-4: Fermentation procedure using optimised parameters.

2.3.3.1 Substrate pretreatment

Three different substrates were used: cassava bagasse flour (prepared as described in Section 2.1.2.1) without pregelatinisation, pregelatinised cassava bagasse (prepared as described in Section 2.1.2.2), and pregelatinised cassava tuber. The latter was prepared from fresh cassava tuber obtained from Lampung, Indonesia. The tuber was peeled, cut into small cubes (ca. 0.5-1.0 cm^3), and autoclaved at 121°C for 15 minutes. After overnight drying at 65°C, the dried cubes were milled to pass through a 1 mm sieve (Retsch ZM 100, Haan, Germany).

To make 100 mL of the previously optimised salt solution (Section 2.3.2.7), 1.7 g ammonium sulphate was dissolved in 56.3 mL of 1.5 g/L of KH_2PO_4 solution, adjusted to a pH of 7.0-7.5 with 1 M Na_2CO_3, and sterilised at 121°C for 15 minutes. After cooling to room temperature, 16 mL of 0.45µm-filter sterilised 10% urea solution was added, and the solution was filled to

100 mL with sterile distilled water. Subsequent steps were carried out as described in Figure 2-4.

2.3.3.2 Temperature of the salt solution

This experiment was conducted to determine whether it was necessary to use 90°C-prewarmed salt solution to moisten the pregelatinised cassava bagasse substrate. Therefore, two salt solutions with different temperatures (90°C and ambient temperature) were used. The substrate was added to prewarmed or cool salt solutions, and mixed. The moistened substrates were then left to rest for 45 minutes before inoculation. Subsequent steps were carried out as described in Figure 2-4.

2.3.3.3 *Rhizopus* strain inoculum

To compare the influence of different fungal strains on the contents of true protein and water-soluble vitamins in the fermented products, the five previously selected best growing *Rhizopus* strains (Section 2.2.3) were used as inocula. These strains were *R. oryzae* EN, *R. oryzae* Fi, *R. oryzae* Mala, *R. oligosporus* Tebo, and *R. oryzae* ZB. Five duplicates were prepared for each strain using a procedure slightly different from that described in Section 2.3.1. The procedure as described in Figure 2-4 was used with the following modifications: the salt solution was not prewarmed, and the inoculum was added after the substrate had been transferred to the Petri dish.

2.3.3.4 Sulphur source

Ammonium sulphate can also act as a sulphur source. Therefore, an experiment was carried out to investigate the influence of substituting ammonium sulphate with other sulphur-containing compounds on fungal growth.

Salt solution

In the optimised salt solution, ammonium sulphate was present at a concentration of 1.7%, which was equivalent to 0.41% of sulphur element. Thus, each of the sulphurous compounds used to replace ammonium sulphate were supplemented in such a way as to provide 0.41% sulphur element in the salt solution. To prepare a 100 mL salt solution, an appropriate amount of a given sulphur-containing compound (Table 2-9) was dissolved in 56.3 mL of 1.5 g/L KH_2PO_4 solution, adjusted to a pH of 7.0 ± 0.1 with 1 M Na_2CO_3, and then sterilised at 121°C

for 15 minutes. After cooling to room temperature, 16 mL of the 0.45 µm-filter sterilised 10% urea solution were added to the solution, and made up to 100 mL with sterile distilled water. As an exception, the salt solution containing $MgSO_4 \cdot 7H_2O$ as the sulphur source was sterilised by filtration (Ø 0.45 µm, Type AC, Sartorius, Göttingen, Germany) to avoid precipitation.

Table 2-9: Sulphur-containing compounds as substitutes for ammonium sulphate.

Sulphur-containing compound[1] (Purity)	Molecular weight of sulphur constituents (g/mol)	Amount dissolved in 100 mL salt solution (g)
$MgSO_4 \cdot 7H_2O$ (≥ 99%)	32.06	3.17
Na_2SO_4 (≥ 99%)	32.06	1.83
$(NH_4)_2SO_4$ (≥ 99%)	32.06	1.7
L-Methionine (99%)	32.06	1.92
L-Cystine[2] (≥ 98.5-101.1%)	64.12	1.55
DMSO (≥ 95%)	32.06	1.01

[1] $MgSO_4 \cdot 7H_2O$ was from Fluka, Steinheim, Germany, and the other sulphur-containing compounds were from Carl Roth, Karlsruhe, Germany.

[2] L-cystine is a dimer consisting of two cysteine amino acid monomers joined by a disulfide bond (Kwanyuen and Burton 2010).

2.3.4 Soybean tempe

Soybean tempe was prepared in order to serve as a positive control for good *Rhizopus* growth on its natural substrate. The procedure was carried out as described previously by Nout and Kiers (2005). Soybeans were obtained from a local Asian shop in Hamburg, Harburg, Germany. They originated from Canada, and were imported by Heuschen & Schrouff OFT B.V., Landgraaf, The Netherlands. Two hundred grams of soybeans were soaked in 400 mL distilled water for 90 minutes, followed by dehulling by manual hand rubbing and removing the hulls by floatation. The dehulled soybeans were soaked overnight in 500 mL distilled water. The water was then drained, 600 mL fresh distilled water were subsequently added, and boiled for 20 minutes. The beans were subsequently dried at 60°C for 2 hours and autoclaved

at 121°C for 15 minutes. After cooling to room temperature, the beans were transferred into Petri dishes, with each containing 31 g soybeans, and inoculated with *R. oryzae* ZB spores (1 mL of 10^5 spores/mL). The dishes were sealed with Parafilm, perforated at three equidistant points as in Figure 3-3, and incubated at 30°C for 48 hours.

2.4 Analysis

2.4.1 Sample pretreatment

Fermented and unfermented samples were freeze dried (Alpha 1-4, Martin Christ, Osterode am Harz, Germany) and homogenised mechanically using an ultracentrifugal mill (Retsch ZM 100, Haan, Germany) to pass through a 0.5 mm sieve. The pulverised, lyophilised samples were stored at -20°C until use, and are referred to in the following simply as “samples”, unless otherwise stated.

2.4.2 Protein

2.4.2.1 Soluble fungal protein

Growth measurements via direct mycelium biomass recovery and quantification were impossible in SSF due to the inseparable association between substrate and filamentous fungal mycelium (Mitchell et al. 1991). To estimate the mycelium biomass formed during optimisation (Section 2.3.2), an indirect method as developed by Córdova-López et al. (1996) was used. The method is based on the quantification of solubilised protein according to Bradford (1976). It allows the accurate estimation of mycelium biomass without the interference of solid culture medium, metabolite constituents, or spore formation. The method was also simple to carry out, and was suitable in this context, in which the cassava bagasse substrate contained a very low protein level. Thus, any increase in protein content during fermentation can be attributed to fungal growth.

Reagents

<u>Phosphoric acid solution</u>: for a 0.25 M phosphoric acid solution, 2.9 g of 85% phosphoric acid (Carl Roth, Karlsruhe, Germany) were dissolved in 100 mL deionised water.

<u>Bradford reagent</u>: to produce 100 mL reagent, 10 mg Coomassie Brilliant Blue G-250 (Fluka, Steinheim, Germany) were added to 5 mL absolute ethanol (Merck, Darmstadt, Germany), and mixed by agitating. Ten millilitres of 85% phosphoric acid (Carl Roth, Karlsruhe, Ger-

many) were then added, mixed thoroughly, and the total volume was in turn made up to 100 mL with deionised water.

<u>Protein standard solution</u>: to produce a 100 µg/mL stock standard solution, 15 mg of bovine serum albumin (BSA) (Carl Roth, Karlsruhe, Germany) was dissolved in 15 mL of 0.25 M H_3PO_4 , mixed homogenously, and then diluted 10 times. Appropriate dilutions were carried out to produce a series of standard solution with concentrations of 10, 20, 30, 40, 50, 60, 70, 80, and 90 µg/mL.

Procedure

Samples (0.1-0.5 g) were weighed to the nearest 0.001 g, added with 10 mL 0.25M H_3PO_4, and vortexed briefly until the entire solid matrix was thoroughly suspended. The suspension was heated in boiling water for 10 minutes, cooled to room temperature, and centrifuged at 13,000 rpm for 10-15 minutes (Biofuge Pico, Heraeus, Hanau, Germany) to remove the insoluble matrix. Two hundred microlitres of the supernatant, diluted if necessary, were then added to 800 µl Bradford reagent. The absorbance was measured spectrophotometrically within 10 minutes at 595 nm (U-2000, Hitachi, Tokyo, Japan). The absorbance values were used to calculate the soluble protein concentrations in the samples using a second-order polynomial regression equation obtained by plotting a curve of the absorbance versus the known concentration of the protein standard solution (Appendix 7.2).

2.4.2.2 True protein

The true protein of a sample is the protein as determined by the Kjeldahl procedure of precipitated protein after non-protein nitrogen (NPN) compounds have been removed. In other words, it is crude protein minus NPN compounds such as free amino acids, ammonia, urea, nucleic acid, chitin, and chitosan.

In this study, treating fermented samples with trichloroacetic acid (TCA) or cupric hydroxide caused a precipitation of protein in the sample. The soluble residual urea and ammonium sulphate that might have still been present in the sample after the fermentation process, as well as other low molecular mass nitrogenous compounds, were removed.

2.4.2.2.1 TCA method according to Rajoka et al. (2004)

For samples containing large amounts of starch (more than 60%), the method previously described by Rajoka et al. (2004) for determining the single cell protein content of rice fermented using *Candida utilis* was used. High concentrations of residual starch were found when the substrate utilisation of the growing *Rhizopus* fungi was poor. This occurred during strain selection on the cassava bagasse mash medium (Section 2.2.3.3). The high starch content caused clogging during the filtration of the water-suspended samples which could be alleviated by solubilising the starch in the samples at a high temperature and filtering while hot.

Reagent

TCA solution: for a 5% solution, 25 g TCA (Carl Roth, Karlsruhe, Germany) were dissolved in 500 mL distilled water. To produce 2.5% solution, a measured volume of 5% TCA solution was added and mixed with the same volume of distilled water.

Procedure

Samples (0.5 g) were weighed to the nearest 0.001 g, added with 20 mL of 5% TCA solution, vortexed vigorously until all solid materials were mixed thoroughly, and left at room temperature for about 4 minutes. After brief vortexing, the suspension was placed in a 90°C water bath for 15 minutes, and occasionally mildly vortexed. While hot, the sample was filtered through a Buchner funnel lined with filter paper (Macherey-Nagel, 640 w, Ø 90 mm), and rinsed with 90°C hot water three times (30mL). The nitrogen content in the precipitate was determined by the Kjeldahl procedure (Section 2.1.3.2), and then multiplied by 6.25 to give the true protein value.

2.4.2.2.2 TCA method according to Marais and Evenwell (1983)

For samples containing low concentrations of starch, the tricholoroacetic acid (TCA) method developed by Marais and Evenwell (1983) for the determination of true protein in feed samples was used. This method was used to determine the true protein content of fermented samples with low starch (less than 40%), where no clogging of the paper filter occurred. Low starch content was found in the fermented samples obtained in experiments described in Section 2.3.3, in which the already optimised fermentation conditions were employed.

Reagent

TCA solutions were prepared as described in Section 2.4.2.2.1.

Procedure

Samples (0.5 g) were weighed to the nearest 0.001 g, added with 15 mL distilled water, and mixed by rigorous vortexing until all of the solid matter was suspended. After heating in boiling water for 10 minutes and cooling to room temperature, 15 mL of 10% TCA was added. The suspended sample was then mixed well by rigorous vortexing, and left for at least 2 hours to allow for precipitation. The precipitate was collected on a Buchner funnel lined with filter paper (Macherey-Nagel, 640 w, Ø 90 mm), washed with 50 mL of 2.5% TCA, and dried at 65°C for at least 4 hours. The nitrogen content in the precipitate was determined by the Kjeldahl procedure (Section 2.1.3.2), and multiplied by 6.25 to give the true protein value.

2.4.2.2.3 Cupric hydroxide method

This method is based on the determination of albuminoid nitrogen (Horwitz 1965) by protein precipitation using cupric hydroxide. Gheysen et al. (1985) used this procedure to quantify the actual protein content of cassava fermented with filamentous fungi *Rhizopus* spp. in both SSF and liquid fermentation. They proved that the protein contents measured using the procedure were not significantly different from those determined by calculating the total amino acid residues, which is the method recommended by FAO (Maclean et al. 2003). The former method is preferred over the latter based on convenience, simplicity, and cost. This method was employed in this study in addition to the TCA methods described above with the aim of comparing the present results with those published by previous authors who had also used the cupric hydroxide method.

Reagents

Sodium hydroxide solution: for a 10% solution, 10 g sodium hydroxide pellets (Carl Roth, Karlsruhe, Germany) were dissolved in 100 mL distilled water.

Alkaline cupric hydroxide: one hundred grams of $CuSO_4 \cdot 5H_2O$ (Carl Roth, Karlsruhe, Germany) was dissolved in 5 L distilled water to which 2.5 mL of 86% glycerol had previously been added (Carl Roth, Karlsruhe, Germany). A sodium hydroxide solution (10%) was added gradually while stirring until a pale green suspension with an alkaline pH of 8.0 ± 0.2 was

obtained. The precipitate was collected by filtration (Whatman 595½ filter paper, Ø 185 mm, Dassel, Germany) and the residue was homogenously resuspended with distilled water containing 5 mL glycerol/L distilled water. The suspension was then washed by decanting and filtering until the washing liquid was no longer alkaline. Finally, the residue was resuspended homogenously in 10% (w/v) glycerol in order to obtain a uniform gelatinous mass that could be measured using a pipette.

To determine the approximate quantity of the suspended solid $Cu(OH)_2$, 5 mL of the suspension were diluted to 50 mL with distilled water, mixed thoroughly, and filtered (Whatman 595½ 185 mm, Dassel, Germany). The precipitate on the filter paper was washed twice with 50 mL distilled water, and then ignited at 600°C (2 hours). After cooling to room temperature in a desiccator, the CuO ash was weighed on an analytical balance to three decimal places.

Procedure

Samples (0.7 g) were weighed to the nearest 0.001 g, added with 100 mL distilled water, mixed thoroughly, and heated in boiling water for 10 minutes. While hot, 23 mL cupric hydroxide suspension, which was equivalent to 0.53 g $Cu(OH)_2$, were immediately added to the sample, and mixed thoroughly. After cooling to room temperature, the suspension containing the sample was filtered (Rotilabo filter paper, qualitative, 125 mm, Karlsruhe, Germany), and the precipitate was washed twice with 50 mL distilled water. The residue and the filter paper were dried overnight at 70°C for the subsequent determination of the nitrogen content using the Kjeldahl method as described in Section 2.4.2.2. The resulting nitrogen values were multiplied by 6.25 to yield the true protein content.

2.4.3 Reducing sugars

Total reducing sugars were determined according to the method of Bittman (1974), and were used in the present study to quantify (1) total free reducing sugar liberated in the fermented substrate not metabolised by the fungi, and (2) total residual carbohydrate not metabolised by the fungi. The latter necessitated the prior acidic hydrolysis of the residual starches to release glucose residues. This method was already described in Section 2.1.3.5 as part of the procedure to determine the starch content of the cassava bagasse. However, some aspects of the procedure needed modifications, which are described below.

Reagents

Sulphuric acid solution: for a 1.5 M solution, 82 mL sulphuric acid (98%, Carl Roth, Karlsruhe, Germany) were dissolved in 1000 mL distilled water.

Sodium hydroxide solution: to produce a 20% sodium hydroxide solution, 100 g sodium hydroxide pellets (Carl Roth, Karlsruhe, Germany) were dissolved in 500 mL distilled water.

Dinitrosalycilic acid (DNS) reagent: prepared as previously described (Section 2.1.3.5).

Glucose standard solution 1: to produce a 1000 µg/mL stock standard solution for determination of total residual carbohydrate, 22.5 mg of α-D(+)-glucose monohydrate (Carl Roth, Karlsruhe, Germany) was dissolved in 15 mL of blank sample solution (see below under "Total carbohydrate residue"). Appropriate dilutions were made to produce a series of standard solution with concentrations of 300, 600, 900, 1200 and 1500 µg/mL.

Glucose standard solution 2: to produce a 730 µg/mL stock standard solution for the determination of total free reducing sugar, 1.825 g of α-D(+)-glucose monohydrate was dissolved in 50 mL distilled water. The appropriate dilutions were made to produce a series of standard solutions with concentrations between 40 and 400 µg/mL.

Procedure

To determine total residual carbohydrate, samples (0.01 mg) were weighed to the nearest 0.001 g, added with 10 mL of 1.5 M H_2SO_4, and heated in boiling water for 20 minutes to hydrolyse the residual starch. A tube without sample as a blank was also treated similarly. After immediate cooling under tap water, 6 mL of 20% NaOH were added, mixed, and diluted with distilled water to obtain 20 mL. Following the removal of solid residue by filtration (Ø 125 mm, Whatman, Schleicher & Schuell, Dassel, Germany), 1 mL of the filtrate was mixed with 1 mL DNS reagent and 3 mL distilled water. After heating in boiling water for 5 minutes and cooling promptly under tap water to stop the reaction, the volume was adjusted to 15 mL, mixed well, and measured spectrophotometrically at 540 nm (U-2000, Hitachi, Tokyo, Japan). Similarly, 1 mL glucose standard solution was mixed with 1 mL DNS reagent and 3 mL of distilled water. After boiling for 5 minutes in a water bath and cooling promptly under tap water to stop the reaction, the mixture volume was adjusted to 10 mL, and then measured spectrophotometrically as above.

To determine total free reducing sugar, 1 g samples were weighed to the nearest 0.01 g, added with 20 mL distilled water, and vortexed vigorously for 20 seconds until the entire solid was suspended. One millilitre of the suspension was heated at 90°C with shaking at 800 rpm for 5 minutes (Thermomixer Comfort, Eppendorf, Hamburg, Germany) to stop any remaining amylolytic activity, followed by centrifugation at 13,000 rpm for 10 minutes (Biofuge Pico, Heraeus, Hanau, Germany). Two hundred millilitres of the supernatant were taken and added to 4 mL of twice-diluted DNS reagent, mixed, and heated in boiling water for 5 minutes, followed by immediate cooling under tap water. The sample was then filled to 10 mL with distilled water, mixed homogenously, and measured spectrophotometrically at 540 nm (U-2000, Hitachi, Tokyo, Japan). The absorbance values obtained were used to calculate reducing sugar concentrations using the linear regression of standard curves as described in Section 2.1.3.5.

2.4.4 Ammonium

Reagent

Ammonium standard solution: to produce a series of concentrations between 0.06–3.33 mg/L ammonium, an NH_4Cl standard solution (1000 ± 2 mg NH_4^+/L, Merck, Darmstadt, Germany) was diluted appropriately with distilled water.

Procedure

Samples (1.0 g) were added with 20 mL distilled water, and vortexed vigorously for 20 seconds until the entire solid was suspended. The insoluble part was separated by centrifugation (Biofuge Pico, Heraeus, Hanau, Germany) at 13,000 rpm for 10 minutes. The supernatant was diluted so that the ammonium concentration fell within the recommended measuring range (0.06–3.86 mg NH_4^+/L). Ammonium determination was carried out photometrically using an ammonium test kit (Spectroquant, Merck 114752, Darmstadt, Germany) according to the manufacturer's instructions. The absorbance was measured using a spectrophotometer (U-2000, Hitachi, Tokyo, Japan) at 692 nm with a 10 mm cuvette. Spectrophotometric absorbance values were used to calculate ammonium concentrations in the samples by use of a linear regression equation of the standard graph of absorbance versus the known concentration of ammonium solution (Appendix 7.2).

2.4.5 Urea

Urea concentration was quantified according to the method of Rahmatullah and Boyde (1980), which allows for the spectrophotometric determination of urea without deproteination. The method used a chromogenic reagent consisting of diacetylmonoxime (DAMO) and thio-semicarbazide (TSC).

Reagents

Urease solution: for a concentration of 2 mg/mL, 40 mg urease (Merck, Darmstadt, Germany) were dissolved in 40 mL distilled water while stirring vigorously. The solution was then filtered (Ø 125 mm, Whatman, Schleicher & Schuell, Dassel, Germany) to remove insoluble materials.

Brij-35 solution: to produce a 20% solution, 4 g of Brij-35 (Merck, Darmstadt, Germany) were dissolved in 20 mL distilled water.

Chromogenic reagent: acid ferric solution and DAMO-TSC solution were two components of the chromogenic reagent. To produce 100 mL acid ferric solution, 30 mL of 98% sulphuric acid (Carl Roth, Karlsruhe, Germany), 10 mL of 85% phosphoric acid (Carl Roth, Karlsruhe, Germany), and 60 mL distilled water were mixed homogenously. The resulting solution was then used to dissolve 16.7 mg ferric chloride hexahydrate (Carl Roth, Karlsruhe, Germany). For a 100 mL DAMO-TSC solution, 500 DAMO (Carl Roth, Karlsruhe, Germany) and 10 mg TSC (Sigma, Saint Louis, Missouri, USA) were dissolved in 100 mL distilled water. The chromogenic reagent was freshly made immediately before use by mixing two volumes of the acid ferric solution with one volume of the DAMO-TSC solution.

Urea standard solution: for a 30 µmol/mL solution, 180 mg urea (Carl Roth, Karlsruhe, Germany) were dissolved in 100 mL distilled water. From this stock, appropriate dilutions were carried out to obtain a series of urea standard solutions with concentrations of 2.5, 5, 10, 20, 30, 40, and 50 nmol/ 900 µl.

Procedure

Samples (1 g) were weighed to the nearest 0.001 g, added with 20 mL distilled water, vortexed vigorously for 2 minutes, and the volume was made up to 50 mL with distilled water.

The insoluble material was then removed by filtration (595½, Whatman Schleicher & Schuell, Dassel, Germany) and the clear supernatant was diluted, if necessary, prior to determination of the urea content. A sample blank was prepared for each sample. To a supernatant sample prepared as above (900 µl) was added 100 µl of 0.2% urease solution, and incubated at 37°C for 30 minutes. To keep the colour stable, the samples were protected from exposure to light during the procedure.

Samples, sample blanks, or standards (0.9 mL) were mixed with 2 mL chromogenic reagent and 0.1 mL of 20% (w/v) Brij-35 solution, and heated for 10 minutes in boiling water. After cooling to room temperature, absorbance was read spectrophotometrically (U-2000, Hitachi, Tokyo, Japan) at 525 nm. The spectrophotometric absorbance was used to calculate the urea concentrations of the samples by use of the linear regression equation of a standard graph of absorbance versus the known concentration of the urea standard solution (Appendix 7.2).

2.4.6 pH value

To 1 g of sample was added 20 mL distilled water, and mixed by vigorous vortexing for 20 seconds. The pH of the suspension was measured using a pH meter (Schott, Mainz, Germany)

2.4.7 Increase in moisture and loss of dry weight

During fermentation, carbohydrate metabolism resulted in an increase in moisture content due to the release of metabolic water and a loss of dry matter as carbon dioxide. The increase in moisture content and the loss of dry weight were calculated using the formulae given below after the moisture content of freeze-dried samples had been determined using the previously described formula (Section 2.1.3.1):

$$\%\ Increase\ in\ moisture\ (\%IM) = \left(\frac{M_t - M_o}{M_t}\right) \times 100\%$$

$$\%\ Loss\ of\ dry\ matter\ (\%LDM) = \left(\frac{DW_o - DW_t}{DW_o}\right) \times 100\%$$

$$\%\ Dry\ matter\ (\%DM) = \left(\frac{DW_t}{DW_o}\right) \times 100\%$$

M_t = Moisture content of the fermented substrate after 120 hours fermentation, in g

M_o = Moisture content of the substrate before fermentation (at 0 hours), in g

DW_o = Dry weight of the substrate before fermentation (at 0 hours), in g

DW_t = Dry weight of the substrate after 120 hours fermentation, in g

2.4.8 Water-soluble vitamins

Six water-soluble vitamins were extracted from the samples and determined using either HPLC (thiamine and riboflavin) or microbiological methods (pyridoxine, niacin, biotin, and folic acid). Samples and vitamin standard solutions were protected from light by using amber glass flasks or transparent flasks covered by aluminium foil, and by carrying out the vitamin extraction and determination procedures under subdued light.

To avoid erroneous results, all clean glassware were heated at 250°C for at least 1 hour to burn off any contaminating organic residues. Clean plastic wares were soaked in 3N HCl solution for at least 6 hours or overnight, rinsed with distilled water three times, and dried at 65°C.

Freeze-dried samples (1-1.6 g) were weighed on an analytical balance (Type BP211D or Handy H110, Sartorius, Göttingen, Germany) to at least three decimal places. Following an extraction procedure, the aqueous extract sample was filtered through a sterile disposable filter with a 0.22 µm pore size (PES, Carl Roth, Karlsruhe, Germany), with strictly sterile conditions applied during work with samples to be used in the microbiological assays.

2.4.8.1 Vitamin extraction

2.4.8.1.1 Extraction of thiamine, riboflavin and pyridoxine

Reagents

Hydrochloric acid solution: to produce a 0.1 M solution, 8.3 mL of 37% hydrochloric acid (Merck, Darmstadt, Germany) were added to distilled water, mixed, and filled to 1000 mL.

Sodium acetate solution: for a 2.0 M solution, 16.4 g anhydrous sodium acetate (Fluka, Steinheim, Germany) were dissolved in 100 mL deionised water.

Takadiastase solution: to produce 6% solution, 6 g Takadiastase (Fluka, Steinheim, Germany) was added with deionised water, and filled to 100 mL.

Papain solution: for a 0.4% solution, 80 mg papain (>30,000 USP-U/mg, Carl Roth, Karlsruhe, Germany) were dissolved in 20 mL deionised water.

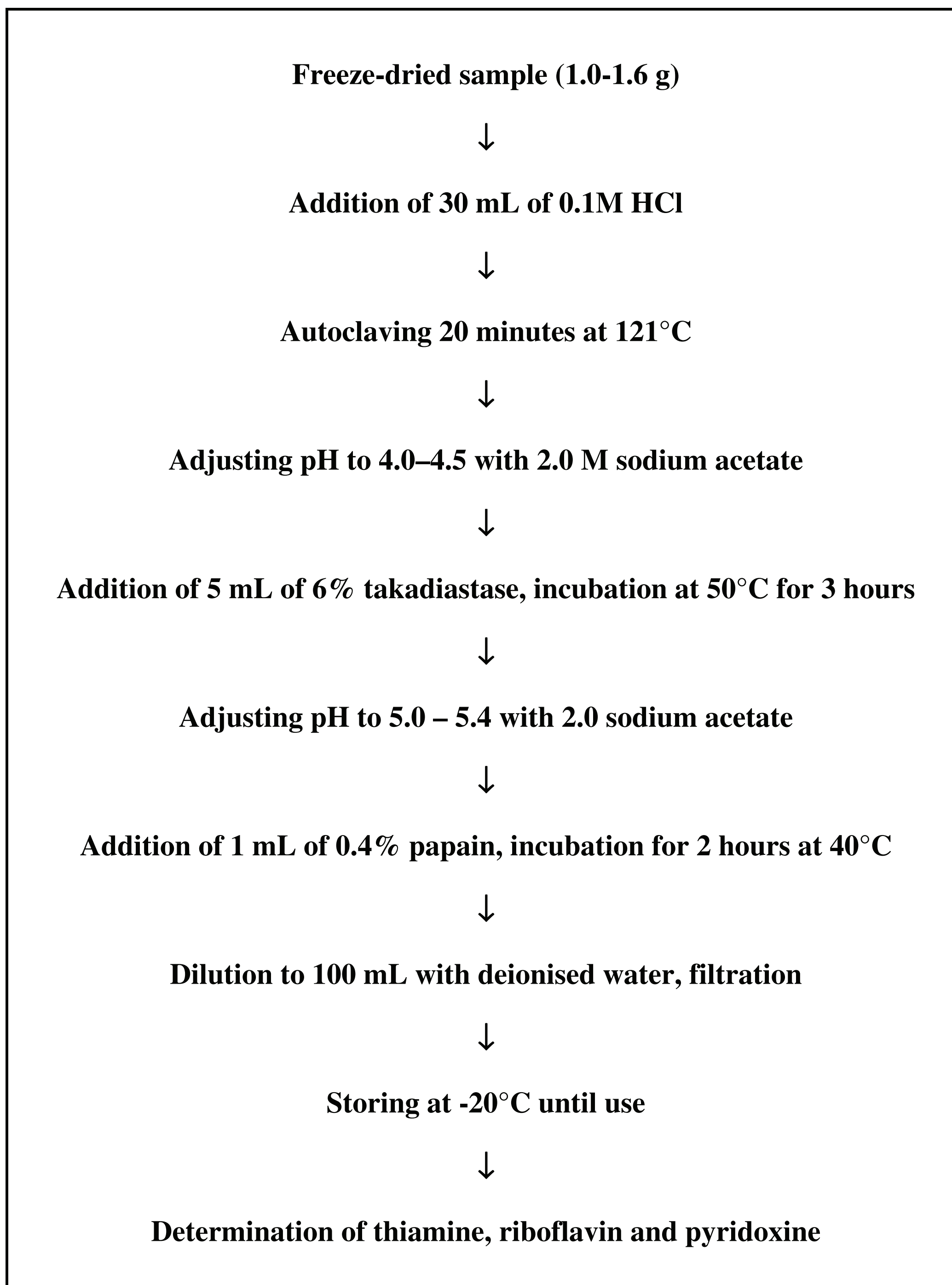


Figure 2-5: Procedure for thiamine, riboflavin, and pyridoxine extraction.

Procedure

Thiamine, riboflavin and pyridoxine were extracted together according to a modified method based on Hagg (1994) and Ndaw et al. (2000), and were described in Figure 2-5. Samples (1-1.6 g) were subjected to acid digestion by adding 30 mL 0.1 M hydrochloric acid, and autoclaving at 121°C for 20 minutes. After cooling to room temperature, the pH of the digested samples were adjusted to 4.0-4.5 with 2.0 M sodium acetate, followed by an incubation with 5 mL of 6% takadiastase for 3 hours at 50°C. Afterward, the pH values were adjusted to 5.0-5.4 with 2.0 M sodium acetate, and the samples were incubated with 1 mL of 0.4 % papain for 2 hours at 40°C. Finally, the whole volumes were diluted to 100 mL with deionised water, mixed well, and the suspended materials were removed by filtration (595½, Whatman Schleicher & Schuell, Dassel, Germany). The clear filtrates were sterilised by filtration through 0.22 µm filters, and stored at -20°C until use. A blank extraction (without sample) was carried out simultaneously, and processed in the same steps as those used for the samples.

To calculate the recovery rate for each vitamin, the following vitamin standards were added to the samples prior to the acid hydrolysis step, yielding final standard vitamin concentrations in the aqueous extracted samples of 1.2 µg/mL thiamine chloride hydrochloride (Merck, Darmstadt, Germany), 1.1 µg/mL pyridoxamine dihydrochloride, 1.5 µg/mL pyridoxal hydrochloride (Merck, Darmstadt, Germany), 1.3 µg/mL pyridoxol hydrochloride (Merck, Darmstadt, Germany) and 1.2 µg/mL riboflavin (Merck, Darmstadt, Germany).

2.4.8.1.2 Extraction of niacin

Reagents

Sulphuric acid solution: to produce 0.5 M solution, 27 mL sulphuric acid (98%, Carl Roth, Karlsruhe, Germany) were dissolved in 1000 mL deionised water.

Sodium hydroxide solution: for a 5 M solution, 20 g sodium hydroxide pellets (Carl Roth, Karlsruhe, Germany) were dissolved in 100 mL deionised water.

Procedure

Niacin extraction was carried out according to method recommended by the AOAC official method 944.13 (AOAC 1995) (Figure 2-6). A sample was suspended in 30 mL of 0.5M H_2SO_4, agitated vigorously, added with 10 mL of 0.5M H_2SO_4 to wash down the sides of the

flask, and finally autoclaved at 121°C for 30 minutes. After cooling to room temperature, pH was adjusted to 6.7 with 5M NaOH, and then with 1M NaOH. The whole volume was subsequently filled to 100 mL with deionised water, followed by paper filtration (595½, Whatman Schleicher & Schuell, Dassel, Germany) to remove the insoluble matrix. The clear filtrate was sterilised through a 0.22 µm filter and stored at -20°C until use. A blank extraction (without sample) was performed at the same time using the same procedure. To calculate the recovery, nicotinic acid (Merck, Darmstadt, Germany) was added to the samples prior to the acid hydrolysis step to yield a final standard niacin concentration of 13 µg/mL in the extracted samples.

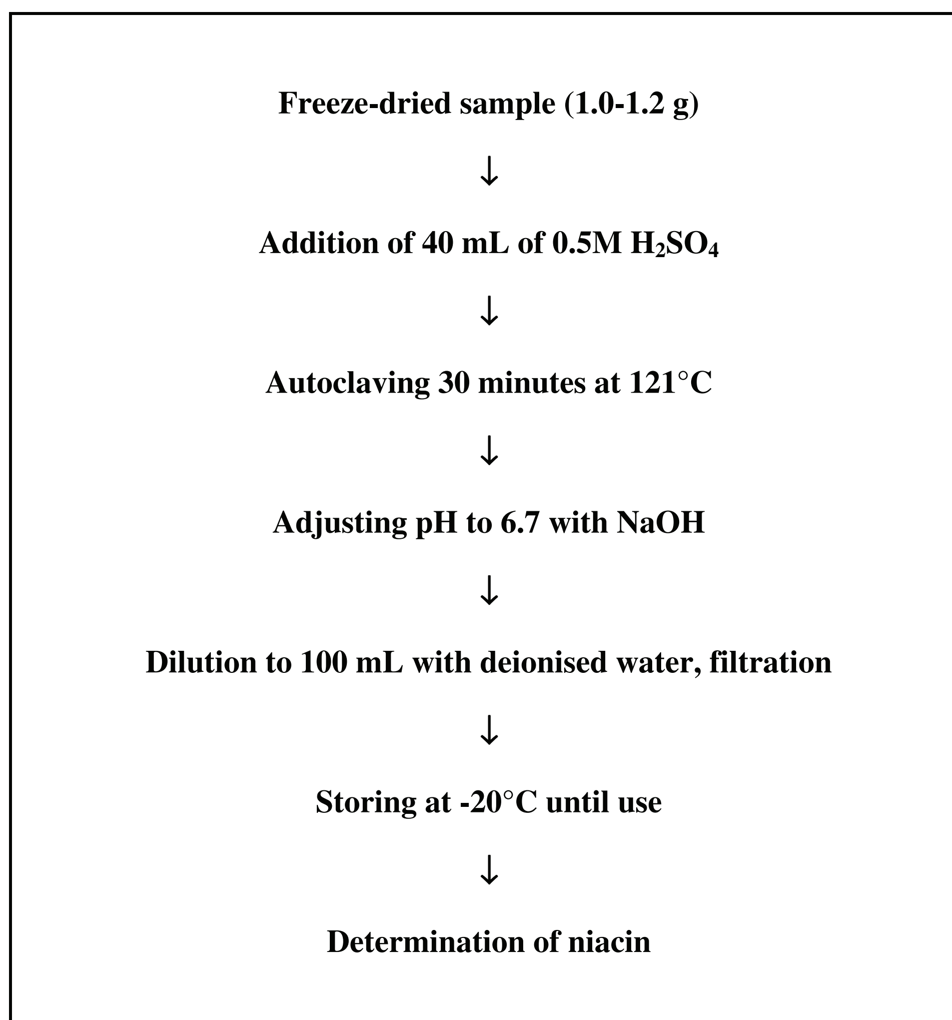


Figure 2-6: Procedure for niacin extraction.

2.4.8.1.3 Extraction of biotin

Reagents

Sulphuric acid solution: for a 1 M solution, 27 mL of 98% sulphuric acid (Carl Roth, Karlsruhe, Germany) were dissolved in 500 mL deionised water.

Sodium hydroxide solution: to produce an 8 M solution, 16 g sodium hydroxide pellets (Carl Roth, Karlsruhe, Germany) were dissolved in 50 mL deionised water. For a less concentrated solution, eight dilutions were made to obtain a 1 M solution.

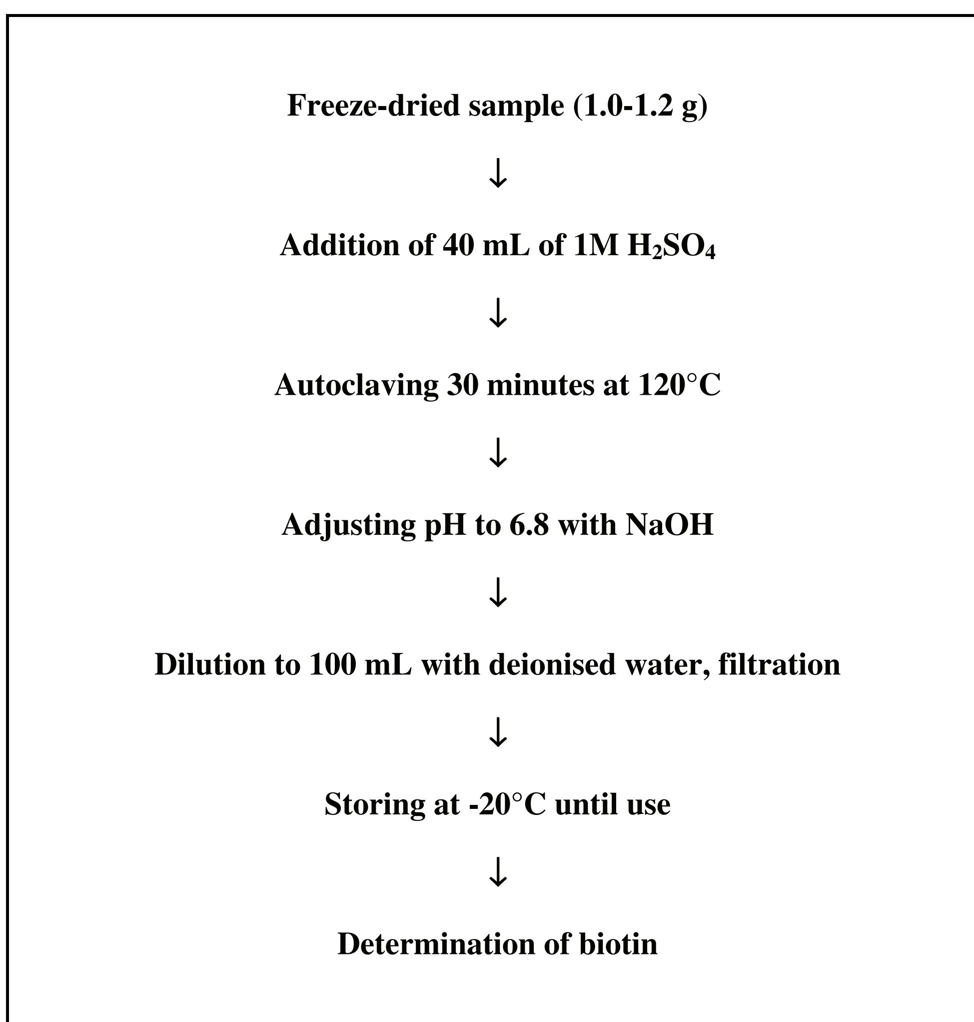


Figure 2-7: Procedure for biotin extraction.

Procedure

Biotin extraction was done according to the modified method of Höller et al. (2006) (Figure 2-7). Samples (1-1.2 g) were suspended in 30 mL of 1 M H_2SO_4, and mixed thoroughly. Ten millilitres of 1M H_2SO_4 were added to wash the sides of the flasks containing the samples, and finally autoclaved for 30 minutes at 120°C. After cooling to room temperature, the pH values were adjusted to 6.8 with 8M NaOH, and then with 1M NaOH. The whole volumes were made up to 100 mL with deionised water, mixed well, and filtered to remove the suspended solid. The filtrates were finally sterilised by filtration through 0.22 µm filters and stored at -20°C until use. A blank extraction (without sample) was done using the same complete procedure. To calculate the recovery rate, D(+)-biotin (Carl Roth, Karlsruhe, Germany) were added to the samples prior to the acid hydrolysis step to yield a final standard biotin concentration of 240 ng/mL in the extracted samples.

2.4.8.1.4 Extraction of folic acid

Reagents

Dipotassium hydrogen phosphate solution: for a 1 M solution, 17.4 g dipotassium hydrogen phosphate (Carl Roth, Karlsruhe, Germany) were dissolved in 100 mL deionised water.

Solution of potassium dihydrogen phosphate: for a 1 M solution, 13.6 g potassium dihydrogen phosphate (Fluka, Steinheim, Germany) were dissolved in 100 mL deionised water.

Phosphate buffer: to produce a 0.1 M phosphate buffer pH 6.2, 20 mL solution of dipotassium hydrogen phosphate and 80 mL solution of potassium dihydrogen phosphate were added to 10 g ascorbic acid (Merck, Darmstadt, Germany). After mixing homogenously, the resulting solution was added with deionised water to achieve a total volume of about 900 mL. The pH was then adjusted to 6.2 with the buffer components, and the volume was made up to 1000 mL with deionised water. Final calculated concentration of the ascorbic acid was 57 mM.

Papain solution: for a 0.2 mg/mL solution, 4 mg papain (>30,000 USP-U/mg, Carl Roth, Karlsruhe, Germany) were dissolved in 20 mL deionised water. The resulting papain solution had an enzyme activity of approximately 6000 USP-U/mL.

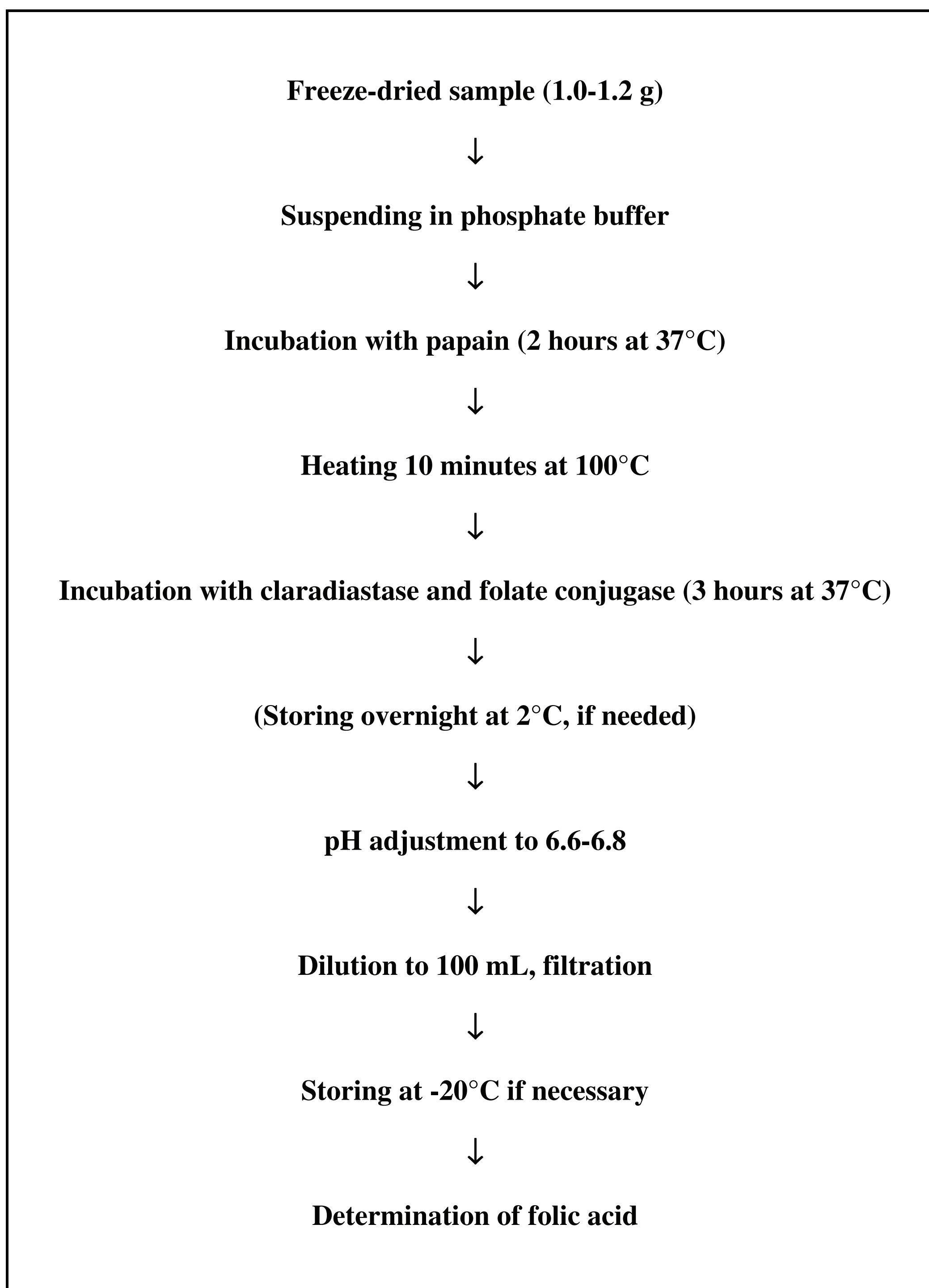


Figure 2-8: Procedure of folic acid extraction.

Carbon treated claradiastase solution: to produce a solution with a calculated amylolytic activity of 2350 U/mL, 1.25 g claradiastase (Crude preparation, 47.0 U/mg, Fluka, Steinheim, Germany) were dissolved in 25 mL distilled water, mixed by vigorous vortexing for 2 minutes, and cooled on ice for approximately 1 hour. To remove indigenous folate that might be

present in the enzyme, 1 g activated carbon (Carl Roth, Karlsruhe, Germany) was added to every 10 mL of this enzyme solution, and vortexed vigorously while on ice for 10 minutes. The suspended carbon was removed by filtration through a filter paper (595½, Whatman Schleicher & Schuell, Dassel, Germany), followed by filtration through a 0.22 µm filter.

Rat serum: as a source of folate conjugase, rat serum containing indigenous folate conjugase (Neat, liquid 0.2 µm filtered, Biozol, Eching, Germany) was used based on previous works by Freisleben (2003) and Rychlik (2003).

Procedure

A method based on a trienzyme extraction procedure (Tamura 1998; Hyun and Tamura 2005) was employed to extract folic acid from the sample (Figure 2-8). Samples (1-1.2 g) were suspended in 20 mL phosphate buffer, followed by the addition of 10 mL buffer to wash the wall of the flasks containing the samples. At this point, the pH values were found to range from 5.9-6.2. An incubation with 1 mL papain solution was subsequently carried out at 37°C with shaking at 160 rpm (Gyrotory G76, New Brunswick Scientific, New Jersey, USA) for 2 hours, followed by heating 10 minutes at 100°C to deactivate the papain. After cooling to room temperature, 1 mL of claradiastase solution and 120 µl of rat serum were added, and incubated at 37°C in the water bath shaker (160 rpm) for 3 hours. (The sample was stored in 2°C overnight, if necessary). The pH values were then adjusted to 6.6-6.8 with 1 M K_2HPO_4, followed by dilution to 100 mL with deionised water and paper filtration (595½, Whatman Schleicher & Schuell, Dassel, Germany) to remove the insoluble matrix. The filtrates were finally filter sterilised through 0.22 µm filters and stored at -20°C until use. A blank extraction (without sample) was carried out simultaneously and processed using the same procedure. For the calculation of folic acid recovery, 100 µl of 100µg/mL folic acid (Sigma, St. Louis, USA) were added to some samples before the acid hydrolysis step.

2.4.8.2 Vitamin determination

2.4.8.2.1 HPLC methods for determination of thiamine and riboflavin

Both thiamine and riboflavin were quantified individually using high performance liquid chromatography (HPLC) to separate the vitamins from the other components. Using a fluorescent detector, the vitamins contents were then calculated based on the standard curve of the peak area plotted against the known concentration of an external standard.

Thiamine was determined according to the method of Arella et al. (1996), with a precolumn derivatisation of thiamine into its fluorescent derivative thiochrome (Lynch and Young 2000). On the other hand, no derivatisation was needed for riboflavin, as it already has a fluorescence group (Silva Jr et al. 2005).

Equipment

HPLC unit: the LaChrom HPLC System (Merck Hitachi, Tokyo, Japan) was used and consisted of the following parts: a fluorescence detector (L-7485), a pump (L-7100), a programmable autosampler (L-7250), an interface module (D-7000), and a system manager software (D-7000).

HPLC column: to determine thiamine and riboflavin levels, Lichrocart (125 × 4 mm, 5-µm particle size) RP-18 and Lichrocart (250×4 mm, 5-µm particle size) RP-18 columns were used, respectively. Both were from Merck (Darmstadt, Germany).

Reagents

Phosphate buffer: the buffer solution was prepared according to the method of Li and Chen (2001). For a 0.1 M potassium dihydrogen phosphate solution, 13.6 g KH_2PO_4 (Fluka, Steinheim, Germany) were dissolved in 1000 mL deionised water. To produce a 0.1 M potassium hydroxide solution, 5.6 g KOH (Merck, Darmstadt, Germany) were dissolved in 1000 mL deionised water. The required buffer (0.1 M phosphate buffer pH 7.0) was made by gradually mixing the two solutions while stirring until a pH of 7.0 was reached.

Thiamine oxidising reagent: first, two component solutions were made: Solution A and B. To prepare solution A, 15 g NaOH were dissolved in 100 mL deionised water to obtain a 3.75 M sodium hydroxide solution. Solution B was prepared by dissolving 0.1 g potassium ferricyanide (Carl Roth, Karlsruhe, Germany) in 10 mL deionised water to obtain a 1% potassium ferricyanide solution. The required oxidising reagent alkaline hexacyanoferrate (III) was freshly prepared prior to use by homogenously mixing 24 mL solution A and 1 mL solution B.

Phosphoric acid solution: for a 7 M solution, 46 g of phosphoric acid (85%, Carl Roth, Karlsruhe, Germany) were dissolved in 100 mL deionised water.

Thiamine standard solution: for a 500 µg/mL stock standard solution, 50 mg thiamine chloride hydrochloride (Merck, Darmstadt, Germany) was dissolved in 100 mL deionised water. A series of standard solution with concentrations of 0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 ng/mL were prepared from the stock solution by appropriate dilution with deionised water.

Riboflavin standard solution: to produce a 60 µg/mL stock standard solution, 60 mg riboflavin (Merck, Darmstadt, Germany) were dissolved in 1000 mL phosphate buffer prepared as described above. A series of riboflavin standard solutions with concentrations of 5, 10, 20, 40, 60, 80, 100, 130, 160 and 200 ng/mL were made by dilution with the buffer.

Mobile phase for thiamine determination: two mobile phases were used, namely deionised water and methanol (HPLC Grade, Carl Roth, Karlsruhe, Germany), and run with a gradient elution program for 15 minutes per injected sample (Table 2-10).

Mobile phase for riboflavin determination: phosphate buffer (0.1 M, pH 7.0) was added with methanol (HPLC Grade, Carl Roth, Karlsruhe, Germany) in a ratio of 30:70 (v/v) to obtain an isocratic mobile phase. The analysis was run at a flow rate of 1 mL/minute for 15 minutes per injected sample.

Table 2-10: Gradient elution program for HPLC determination of thiamine.

Time (min)	Mobile phase		Flow rate (mL/min)
	Methanol	Water	
0	10	90	1
5	40	60	1
8	90	10	1
12	90	10	1
14	35	65	1
15	10	90	1

Procedure

For thiamine determination, 4 mL of sample (or thiamine standard) were added with 2 mL of the oxidising reagent, mixed well, and left at room temperature for 1 minute. The reaction was then stopped by adding 600 µl of 7 M phosphoric acid, mixed, and diluted to 10 mL with deionised water. Twenty microlitres of the derivatised sample or standard were injected into the HPLC, and the eluted thiochrome fraction was detected using a fluorescence detector (355 nm excitation/425 nm emission).

For riboflavin determination, no derivatisation was required. Ten microlitres sample were injected directly into an HPLC with a fluorescence detector set to excitation and emission wavelengths of 522 and 422 nm, respectively (Arella et al. 1996).

The area under the peak shown on the chromatogram as intensity (mV) versus retention time (min) was used to calculate the thiamine or riboflavin concentrations in the injected sample. This was carried out using a linear regression equation for a standard graph of peak area versus the known concentration of the thiamine or riboflavin standard solutions (Appendix 7.2).

2.4.8.2.2 Microbiological methods for determination of niacin, biotin, pyridoxine and folic acid

Microbiological assay techniques were employed to individually determine the contents of niacin, biotin, pyridoxine, and folic acid by using an appropriate assay microorganism which is auxotrophic for the vitamin in question. An auxotrophic microbial strain was grown in a microtiter plate containing test samples (or vitamin standard) with base medium but lacking the vitamin that was to be measured. Turbidity developed as the microorganism grew and was correlated to the quantity of the given vitamin in the test sample (or vitamin standard). Spectrophotometric measurements on a microtiter plate reader gave absorbance values which were then converted into vitamin concentrations by referring to the standard curve of absorbance plotted against the concentration of a vitamin standard. The method described below for the assay of individual vitamins was based on the Difco and BBL manual book (Zimbro and Power 2003a; 2003b; 2003c; 2003d) provided by the producer of the vitamin assay media. The entire procedures were performed under strictly sterile conditions.

Culture media

Maintenance medium: to produce agar maintenance medium, 9.6 g dehydrated Lactobacilli Agar AOAC (Difco, BD, Sparks, USA) were added with 200 mL deionised water, and dissolved completely by heating in boiling water. Sterilisation was done at 121°C for 15 minutes.

Inoculum medium: Lactobacilli Broth AOAC (Difco, BD, Sparks, USA) was prepared according to the manufacturer's manual. Dehydrated medium (3.8 g) was completely dissolved in 100 mL deionised water by heating in boiling water. The liquid broth was dispensed into glass tubes in 10 mL amounts, followed by autoclaving for 15 minutes at 121°C.

Double strength assay media: a given assay medium contained all of the elements necessary for the growth of an assay microorganism, but lacked the vitamin to be assayed. A corresponding assay microorganism cannot grow in this medium except when externally supplemented with vitamin standard or a test sample containing the vitamin. All assay media used in this study were obtained from Difco (DB, Sparks, USA) and prepared according to manufacturer's instructions at double strength concentration, as these media would be twice diluted in microtiter plate wells during the assay. Dehydrated Niacin Assay Medium (7.5 g), Biotin Assay Medium (7.5 g), Folic Acid Casei Medium (9.4 g) or Pyridoxine Y Medium were each dissolved separately in 100 mL deionised water by heating in boiling water for 2-3 minutes with occasional agitation. Subsequently, all media, with the exception of the Pyridoxine Y Medium, were sterilised by autoclaving at 121° for 5 minutes. The Pyridoxine Y Medium was heat sterilised in boiling water for 15 minutes.

Single strength assay media: these media were used to prepare glycerol cryoprotected inocula, and were prepared individually with the same dehydrated media as used for the double strength assay media, but with single strength concentration (i.e. half of the amount of the required dehydrated medium). Each medium was supplemented with the vitamin it lacked to achieve a concentration of 0.6 ng/mL. Each medium was then dissolved by heating in boiling water for 2-3 minutes with agitation. Sterilisation was done by filtration (Ø 0.22 µm, disposable filter PES, Carl Roth, Karlsruhe, Germany).

Reagents

Sterile saline solution: to make a 0.9% solution, 9 g NaCl (Carl Roth, Karlsruhe, Germany) were dissolved in 1000 mL deionised water, and autoclaved at 121°C for 15 minutes.

<u>Concentrated glycerol solution</u>: 16 mL of glycerol (86%, Carl Roth, Karlsruhe, Germany) were added with 4 mL distilled water, mixed homogenously, and sterilised by autoclaving at 121°C for 15 minutes. The final glycerol concentration obtained was 71% (w/w).

<u>Niacin standard solution</u>: for a stock solution of 500 ng/mL, 50 mg nicotinic acid (Merck, Darmstadt, Germany) was dissolved in 100 mL deionised water, stored at -20°C if necessary, and used within 2 weeks. Further serial dilutions (50 times and then 100 times) were conducted to obtain a lower concentration of 100 ng/mL, which was subsequently filter-sterilised through a 0.22 µm filter. From this solution, a series of niacin standard solutions with concentrations of 5, 10, 15, 20, 25, 30 and 35 ng/mL was freshly prepared by dilution with sterile deionised water.

<u>Biotin standard solution</u>: for a stock solution of 1000 ng/mL, 0.050 g of D(+)-biotin (Carl Roth, Karlsruhe, Germany) were dissolved in 500 mL of 25% (v/v) alcohol solution, which had been prepared previously by mixing one volume of ethanol (Rotisolv, Carl Roth, Karlsruhe, Germany) with three volume of deionised water. One millilitre of the resulting solution was further diluted 100 times with deionised water to obtain a concentration of 1000 ng/mL, stored at -20°C if necessary, and used within 2 weeks. After sterilisation through a 0.22 µm filter, this stock solution was used to freshly prepare a series of standard solutions with concentrations of 20, 50, 80, 100, 150, 200, and 300 pg/mL by dilution with sterile deionised water.

<u>Pyridoxine standard solution</u>: to produce a 100 µg/mL solution, 50 mg of pyridoxine hydrochloride (Merck, Darmstadt, Germany) were dissolved in about 100 mL 25% (v/v) ethanol. The ethanol was further added and homogenously mixed to achieve a total volume of 500 mL. The resulting stock solution was stored at -20°C and used within 1 week. Subsequent dilutions were made with deionised water to obtain a 10 ng/mL solution, and sterilised through a 0.22 µm filter. From this solution, a series of pyridoxine standard solutions with concentrations of 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ng/mL was made by dilution with sterile deionised water.

<u>Folic acid standard solution</u>: for a 100 µg/mL solution, 50 mg folic acid (Sigma, St. Louis, USA) were dissolved in 30 mL 0.01 N sodium hydroxide solution and 200-300 mL deionised water, followed by a pH adjustment to 7.5 ± 0.5 with 0.1M hydrochloric acid before the

whole volume was filled to 500 mL with deionised water. The resulting stock solution was stored at -20°C and used within two weeks. To prepare a series of folic acid standard solutions, the stock solution was further diluted with deionised water to obtain a concentration of 10 ng/mL, and sterilised through a 0.22 µm filter. From this sterile solution, standard solutions with concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 ng/mL were made by dilution using sterile deionised water.

Microorganisms

Assay microorganisms: the auxotrophic microbial strains were used as recommended (Blake 2007) and taken from the American Type Culture Collection (ATTC) or from their equivalents from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Table 2-11).

Table 2-11: Microorganisms used in microbiological assays of the vitamins.

Vitamin assayed	Auxotrophic microorganism used for vitamin assay	ATCC equivalent microorganism
Pyridoxine	*Saccharomyces cerevisiae* DSM 70424	*S. cerevisiae* ATCC 9080
Niacin	*Lactobacillus plantarum* DSM 20205	*L. plantarum* ATCC 8014
Biotin	*Lactobacillus plantarum* DSM 20205	*L. plantarum* ATCC 8014
Folic acid	*Lactobacillus casei* ssp. *rhamnosus* ATCC 7469	--

The *L. casei* ssp. *rhamnosus* ATCC 7469 was taken from a frozen culture stock of the strain collection from the Food Microbiology Division, Institute of Food Chemistry, Department of Chemistry, Universität Hamburg, Germany and stabbed into MRS agar. *L. plantarum* DSM 20205 and *S. cerevisiae* DSM 70424 were obtained from the DSMZ as dried cultures in ampoules. The dried cultures were then rehydrated and cultured according to the instruction sheet from the provider of the assay microorganisms. The *L. casei* ssp. *rhamnosus* ATCC 7469 and *L. plantarum* DSM 20205 were maintained for one to two weeks by overnight subculturing in stab Lactobacilli AOAC Agar at 37°C.

Glycerol cryoprotected inoculum: glycerol-cryoprotected inocula of the assay microorganisms were prepared according to the method described by Horne and Patterson (1988) (Figure 2-9). The assay microorganisms were subcultured in 7-10 mL of AOAC broth medium at 30°C. After 24 hours, the cultures were briefly vortexed to homogenise the suspended cells. One hundred microlitres of the suspension were withdrawn for the subculture and transferred into single strength assay broth medium. Incubation was carried out at 30°C for 20 hours for *L. casei* ssp. *rhamnosus* ATCC 7469 and *L. plantarum* DSM 20205, and at 35°C for 18 hours for *S. cerevisiae* DSM 70424. The cultures were then cooled on ice, mixed with equal volumes of an ice-cold concentrated glycerol solution, and stored in aliquots at -80°C until use.

Procedure

The method was adapted from the procedure described by the manufacturer of the assay media (Zimbro and Power 2003a; 2003b; 2003c; 2003d). However, instead of tubes with a total culture volume of 10 mL per tube, sterile 96 well microtiter plates (Nunc, Roskilde, Denmark) with much smaller volumes of 260 µl per well were used, thus saving time and medium. In addition, the clear flat bottom of the microtiter plates allowed for direct photometric reading using the microtiter plate reader, avoiding the need for cuvettes.

Frozen samples obtained in the extraction step (Section 2.4.8.1) were taken from -20°C storage, thawed, and shaken to homogenise. When necessary, dilutions with sterile deionised water were carried out so that the values of the vitamins being measured fell within the standard range.

Inoculum pretreatment and preparation was carried out according to the method described in the Difco & BBL Manual Handbook (Zimbro and Power 2003a; 2003b; 2003c; 2003d) without needing directly count the number of cells in the inoculum suspension. A glycerol cryoprotected inoculum was thawed from -80°C frozen culture, and 100 µl were transferred into 7-10 mL of Lactobacilli Broth AOAC medium. After 18-24 hours incubation at 35°C, the culture was vortexed briefly to homogenise the suspension, and 1–1.5 mL was centrifuged at 10,000 rpm for 4 minutes (Biofuge Pico, Heraeus, Hanau, Germany) to remove the supernatant. The obtained cell pellet was washed three times by resuspending in the same volume

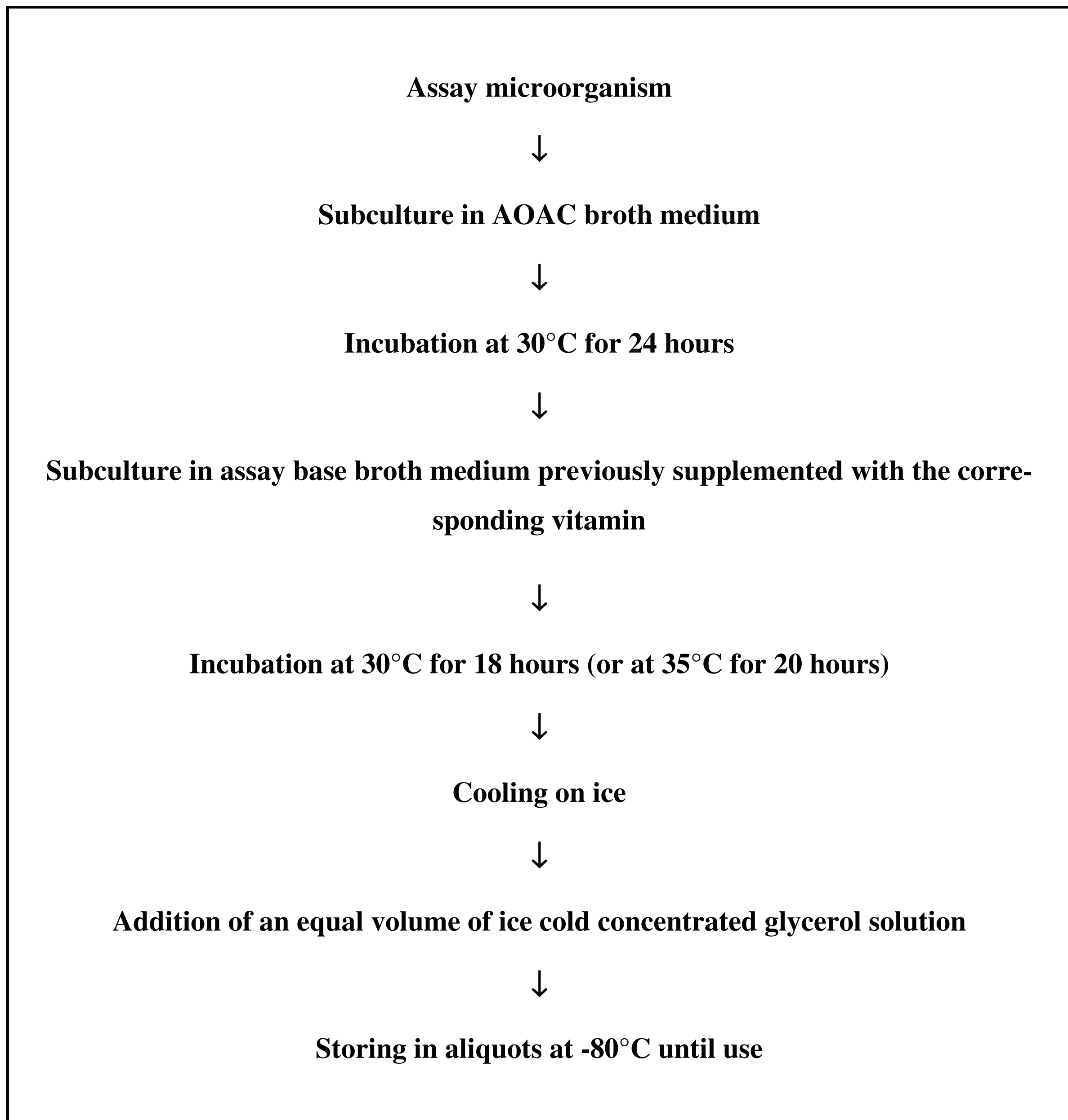


Figure 2-9: Preparation of glycerol cryoprotected inoculum.

of 0.9% saline, followed by centrifugation at 10,000 rpm for 4 minutes to discard the washing saline solution. After the third wash, the cells were resuspended in the same volume of 0.9% saline. From this step onwards, slightly different treatments were performed dependent on the assay microorganism. For *L. casei* ssp. *rhamnosus* ATCC 7469 and *L. plantarum* DSM 20205, the cleaned cell suspensions were simply diluted 1:100 with sterile saline (0.9%). For *S. cerevisiae* DSM 70424, the cell suspension was diluted with 0.9% sterile saline following the third wash so as to achieve a transmittance value of 0.5 when read spectrophotometrically at 660 nm (U-2000, Hitachi, Tokyo, Japan).

Table 2-12: Distribution of assay components in a microtiter plate.

Column	Assay medium	Sample extract	Vitamin standard solution	Inoculum of assay microorganism	Deionised water	0.9% saline solution	Total working volume
1: Blank	130 µl	-	-	-	65 µl	65 µl	260 µl
2-4: Standard	130 µl	-	65 µl	65 µl	-	-	260 µl
5-12: Test	130 µl	65 µl	-	65 µl	-	-	260 µl

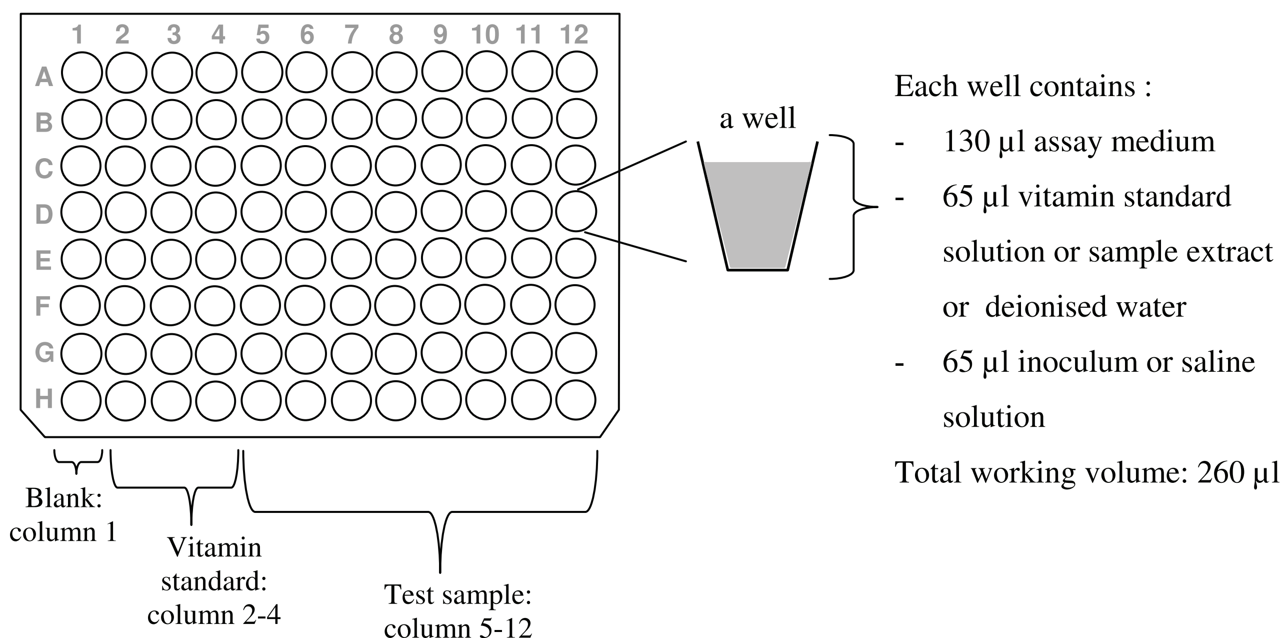


Figure 2-10: Microbiological assay of vitamins using a 96-well microtiter plate.

The assays were performed in 96 well microtiter plates with a total working volume of 260 µl per well, consisting of assay medium, the test sample (or vitamin standard or deionised water) and the assay microorganism inoculum (or saline solution). A series of standards was provided on each plate, from which a standard curve was constructed. The volume and the distribution of the wells for blanks, vitamin standard solutions, as well as test samples are described in Table 2-12 and Figure 2-10.

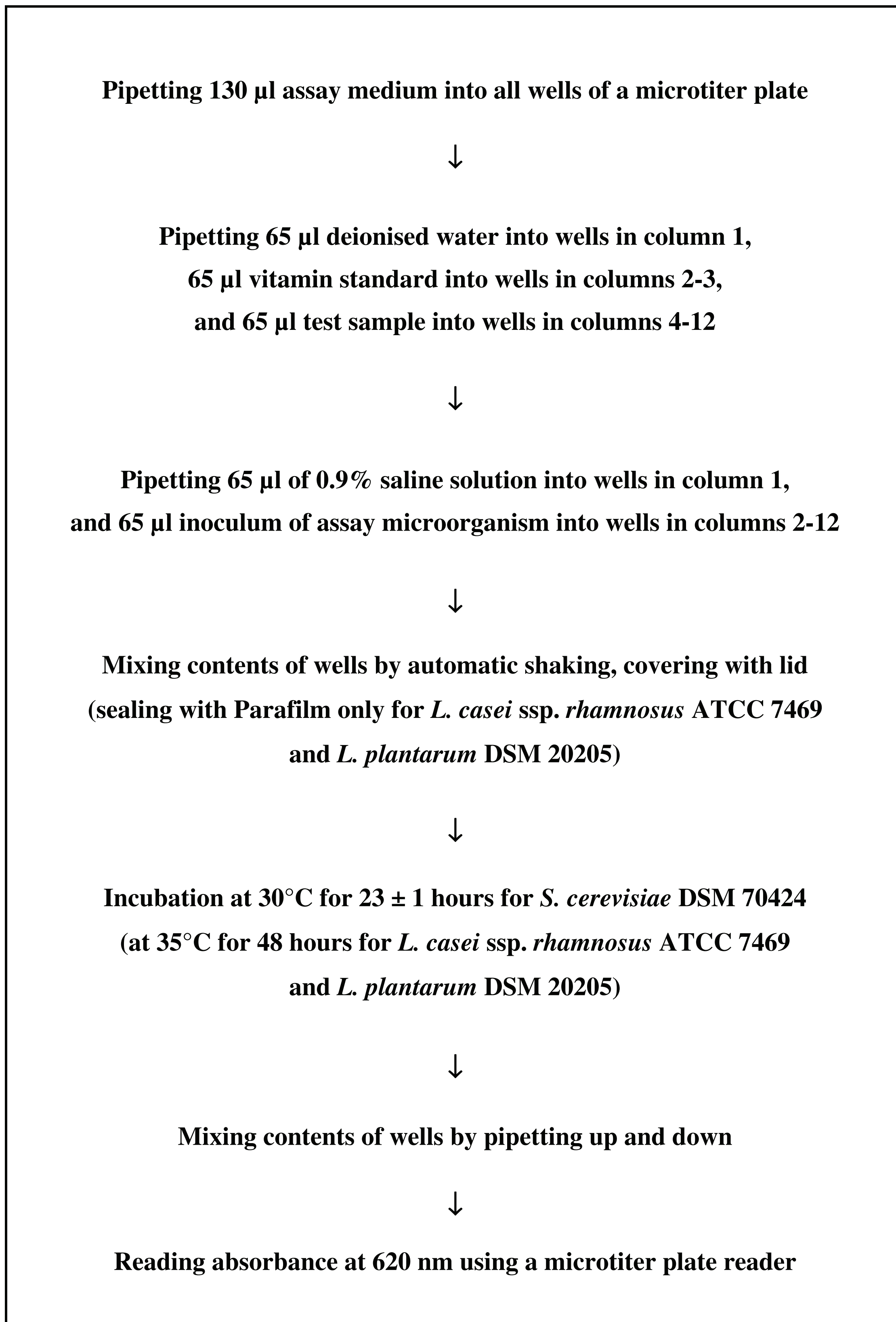


Figure 2-11: General procedure for the microbiological assay of the vitamins.

All of the components were pipetted into the appropriate wells, followed by automatic mixing on a microtiter plate reader (ATTC 340, SLT Labinstruments, Salzburg, Austria) in normal shake mode for 90 seconds (Figure 2-11). The plates were then sealed with Parafilm (not necessary for *S. cerevisiae* DSM 70424) and incubated in a humidified incubator at 30°C for 23 ± 1 hours for *S. cerevisiae* DSM 70424 or at 35°C for 48 hours for *L. casei* ssp. *rhamnosus* ATCC 7469 and *L. plantarum* DSM 20205. After the incubation, the contents of the microtiter plate wells were pipetted up and down using a multichannel pipette to homogenise the cell suspensions. Photometric reading at 620 nm was then carried out using the microtiter plate reader.

The concentration of the vitamin in a given extract sample was determined by the interpolation of the absorbance value using a second-order polynomial regression equation obtained by plotting the absorbance curve against the known concentration of the vitamin standard solution (Appendix 7.15).

3 Results

Results of the measurements and calculations are expressed as ($\overline{X}$ ± SD), that is the mean or average value plus-minus standard deviation (Appendix Section 7.5). Standard deviations were used to plot the error bars in the graphics. All content values were expressed on the dry weight basis of the sample, unless stated otherwise. Values with different alphabetical super-scripts were significantly different ($P<0.05$) based on the statistical analysis of variance (ANOVA) (Appendix Section 7.5.3).

3.1 Cassava bagasse substrate

3.1.1 Proximate composition

Table 3-1 shows that the cassava bagasse consisted mostly of starch and fibres, around 70% and 11% on a dry weight basis, respectively. The other components like protein, lipids, and ash combined for a total of less than 3%. The bagasse was acidic with a pH value of 4.0 ± 0.0 (the average of three measurements). The total amount of free and readily liberated cyano-genic compounds was very low, being 0.08 ± 0.01 mg CN^- equivalent/kg sample.

Table 3-1: Proximate composition of cassava bagasse.

Component (g/100g)	Based on	
	dry weight	wet weight
Moisture	-	9.91 ± 0.21
Crude Protein	1.21 ± 0.15	1.09 ± 0.14
Crude Lipids	0.18 ± 0.09	0.16 ± 0.08
Fibres	11.24 ± 1.28	10.14 ± 1.15
Starch	70.17 ± 0.07	63.21 ± 0.98
Ash	1.24 ± 0.07	1.11 ± 0.07

3.1.2 Pregelatinised cassava bagasse

Cassava bagasse pre-treatment, which included milling and pregelatinisation, resulted in more uniformly sized substrate granules with a brown colour (Figure 3-1).

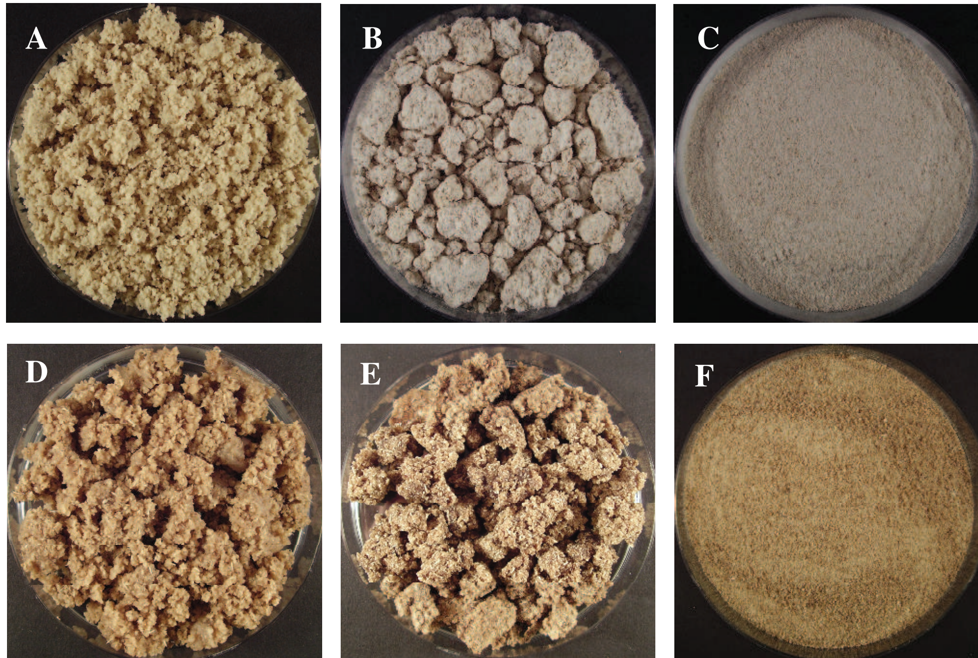


Figure 3-1: Wet fresh cassava bagasse (A) was dried under the sun, resulting in solid granules with various sizes and shapes (B), which were then homogenised into powder (C). After moistening and heating, a brown sticky gelatinised mass was obtained (D), which was then dried (E) and milled into finer granules (F).

3.2 Selection of *Rhizopus* strains

3.2.1 Growth of mycelial colonies on the selection media

On the RCBA and GCBA media, the *Rhizopus* strains showed different growth rates as measured by the diameters of their circular mycelial colonies. *R. oligosporus* Hepla, CN, L2/1, Tegal, Q1, Q2, *R. oryzae* L2 and *R. chinensis* Sur grew with larger colony diameters on RCBA than on GCBA, whereas the opposite was true for *R. oligosporus* East jbp, MS3, MS5, Pon and *R. stolonifer* GT. The rest of the strains formed circular mycelial mats with significantly similar diameters on both selection media. Five *Rhizopus* strains (highlighted in Table 3-2) reached the largest average colony diameter of 85 mm, which was in fact the maximal possible value, since this was the inner diameter of the Petri dish used. They could possibly have grown to greater mycelial colony diameters, had a dish with a larger inner diameter been

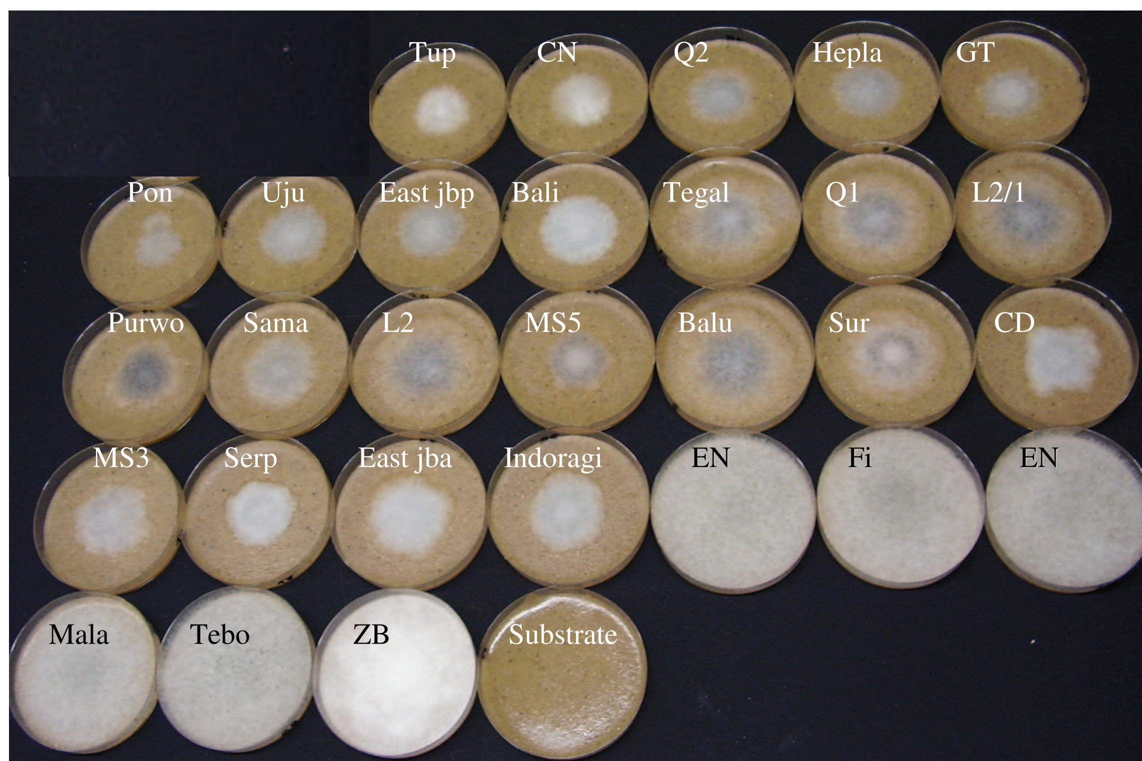


Figure 3-2: Selection of the *Rhizopus* strains on the gelatinised cassava bagasse mash (GCBM) medium.

used. For this reason, the mathematical symbol "≥" was placed in front of the 85 mm, as indicated in Table 3-2. Despite the difference in radial growth behaviour, all of the strains shared the common characteristic of sparser mycelial growth on RCBA than on GCBA.

On the mash selection medium, colony diameters were not measured, as the shapes of the mycelial colonies of most strains were not circular (Figure 3-2). For this reason, growth was qualitatively determined by visually observing whether the mycelial colony was able to cover the entire medium surface.

As seen in Figure 3-2, most of the fungi failed to completely colonise the entire surface within the given fermentation time. Overgrowth was observed exclusively for those same five strains previously found to achieve the largest mycelial colony diameter on the agar selection media. These five strains, namely *R. oligosporus* Tebo, *R. oryzae* Fi, *R. oryzae* EN, *R. oryzae* Mala, and *R. oryzae* ZB, were considered the best growing strains and were selected for subsequent experiments.

Table 3-2: Growth of *Rhizopus* spp. on selection media.

No	Species	Strain	Collection no.	Colony diameter[1] on		Overgrowth
				RCBA	GCBA	on GCBM
1	*R. oligosporus*	Bali	312T	69 ± 2^a	65 ± 2^a	no
2	*R. oligosporus*	Hepla	2009T	60 ± 1^a	57 ± 2^b	no
3	*R. oligosporus*	Tebo	290T	$\geq 85 \pm 0^a$	$\geq 85 \pm 0^a$	yes
4	*R. oligosporus*	CN	1143T	63 ± 3^a	59 ± 2^b	no
5	*R. oligosporus*	East jba	2038T	80 ± 0^a	80 ± 1^a	no
6	*R. oligosporus*	East jbp	2039T	58 ± 3^a	65 ± 2^b	no
7	*R. oligosporus*	L2/1	2013T	72 ± 0^a	70 ± 0^b	no
8	*R. oligosporus*	Balu 1	2007T	73 ± 2^a	72 ± 2^a	no
9	*R. oligosporus*	MS3	2019T	72 ± 1^a	78 ± 1^b	no
10	*R. oligosporus*	MS5	2020T	68 ± 2^a	72 ± 2^b	no
11	*R. oligosporus*	Pon	2022T	42 ± 0^a	62 ± 0^b	no
12	*R. oligosporus*	Purwo	2023T	70 ± 1^a	70 ± 1^a	no
13	*R. oligosporus*	Sama	2026T	71 ± 2^a	70 ± 0^a	no
14	*R. oligosporus*	Tegal	2031T	73 ± 2^a	69 ± 2^b	no
15	*R. oligosporus*	Q1	2024T	72 ± 1^a	69 ± 1^b	no
16	*R. oligosporus*	Q2	2025T	62 ± 1^a	56 ± 1^b	no
17	*R. oligosporus*	Tup	2033T	50 ± 1^a	51 ± 1^a	no
18	*R. oligosporus*	Uju	2034T	57 ± 1^a	59 ± 2^a	no
19	*R. oligosporus*	CD	1133T	76 ± 3^a	78 ± 3^a	no
20	*R. oligosporus*	Puda	20S	80 ± 0^a	80 ± 1^a	no
21	*R. oligosporus*	Serp	2027T	75 ± 0^a	75 ± 0^a	no
22	*R. oryzae*	Fi	10S	$\geq 85 \pm 0^a$	$\geq 85 \pm 0^a$	yes
23	*R. oryzae*	EN	1134T	$\geq 85 \pm 0^a$	$\geq 85 \pm 0^a$	yes
24	*R. oryzae*	L2	2036T	80 ± 1^a	72 ± 1^b	no
25	*R. oryzae*	Mala	11S	$\geq 85 \pm 0^a$	$\geq 85 \pm 0^a$	yes
26	*R. oryzae*	ZB	18S	$\geq 85 \pm 0^a$	$\geq 85 \pm 0^a$	yes
27	*R. stolonifer*	GT	1136T	51 ± 2^a	62 ± 1^b	no
28	*R. chinensis*	Sur	277T	76 ± 1^a	73 ± 2^b	no

[1] Average of 4 diameter measurements. Different alphabetical superscripts indicate a significant difference ($P<0.05$) between the average colony diameters of a *Rhizopus* sp. grown on RCBA and on GCBA. An analysis of variance was not performed between colony diameters of different strains.

3.2.2 Comparison of the five selected *Rhizopus* strains

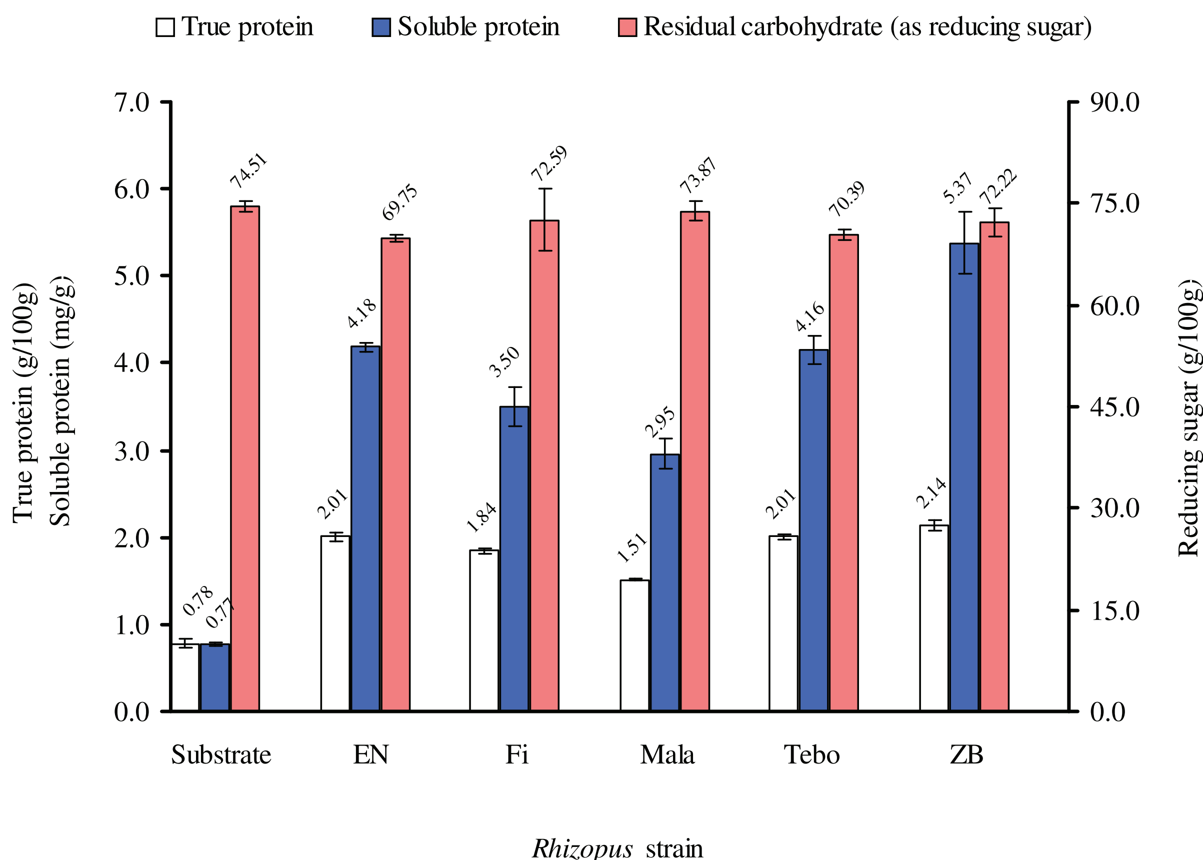


Figure 3-3: Carbohydrate utilisation as well as protein formation of the selected *Rhizopus* strains.

To measure the individual ability of the five selected strains to utilise the cassava bagasse substrate to produce mycelial biomass, protein contents and residual, unassimilated carbohydrates were determined. It was found that the soluble protein values were significantly different for all samples (P<5%, Appendix Table 7-4), except for *R. oryzae* EN and *R. oryzae* Tebo, which had the same value of 4.2 mg/g (Figure 3-3). *R. oryzae* ZB showed the highest value of 5.37 mg/g.

Although soluble protein level proved sufficient for estimating growth performance differences between the strains, true protein was also determined to provide the real, absolute protein content of the fermented substrate. True protein values were also useful for comparing the values obtained in the present study with those reported in previous studies. Figure 3-3 showed that all *Rhizopus* strains increased the true protein content of the cassava bagasse

from 0.8 g/100 g to 1.5-2.1 g/100 g. As with soluble protein, these values all differed significantly from each other, with the exception of *R. oryzae* EN and *R. oryzae* Tebo (Appendix Table 7-12). The two fungal strains enhanced the fermented substrate with protein content of 2.0 g/100 g. *R. oryzae* ZB was noted as having the highest true protein content as well as the least degree of sporulation (Table 3-3). Therefore, this strain was considered the best and used in subsequent experiments to optimise the fermentation conditions.

Only 0.6 – 5.7 g of carbohydrate in the cassava bagasse was utilised by the fungi, which was equivalent to less than 8% of the original amount in the substrate. This implied a very low amount of carbohydrate metabolised by the fungi. Further, the ANOVA test revealed that the residual carbohydrate contents of all of the strains showed almost no significant difference ($P>0.05$) compared to the unfermented substrate, with the exception of *R. oryzae* EN and *R. oligosporus* Tebo (Appendix Table 7-18).

In general, the results indicated low protein production as well as a poor utilisation of the substrate. Therefore, an optimisation of the fermentation conditions was subsequently carried out in order to improve substrate utilisation and mycelial biomass formation by *R. oryzae* ZB.

Table 3-3: Characteristics of cassava bagasse substrate after fermentation using the selected *Rhizopus* strains.

Rhizopus strain	Final pH	Spore formation
Substrate	4.9	–
R. oryzae EN	3.0	Dense
R. oryzae Fi	3.1	Dense
R. oryzae Mala	3.2	Dense
R. oligosporus Tebo	3.0	Dense
R. oryzae ZB	2.9	Hardly seen

3.3 Optimisation of the fermentation conditions

3.3.1 Inoculum concentration

Fungal growth was barely influenced by the difference in inoculum density. Increasing spore concentration from 10^1 to 10^7 spores/10 g substrate had virtually no effect on the soluble protein values (Figure 3-4), all of which showed almost no significant difference (Appendix Table 7-5). However, lower inoculum densities resulted in substrates covered with denser mycelial mats than those with higher spore densities (Figure 3-5). Indeed, fermentation with the highest inoculum density of 10^7 spores/10 g substrate resulted in what appeared to be no fungal growth at all, and was hardly distinguishable from the unfermented substrate. An inoculum density of 10^3 – 10^5 spores/10 g substrate was chosen for subsequent studies, considering that a lower density would increase the probability of overgrowth by contaminating microorganisms, whereas a concentration that was too high might not support the formation of dense mycelia.

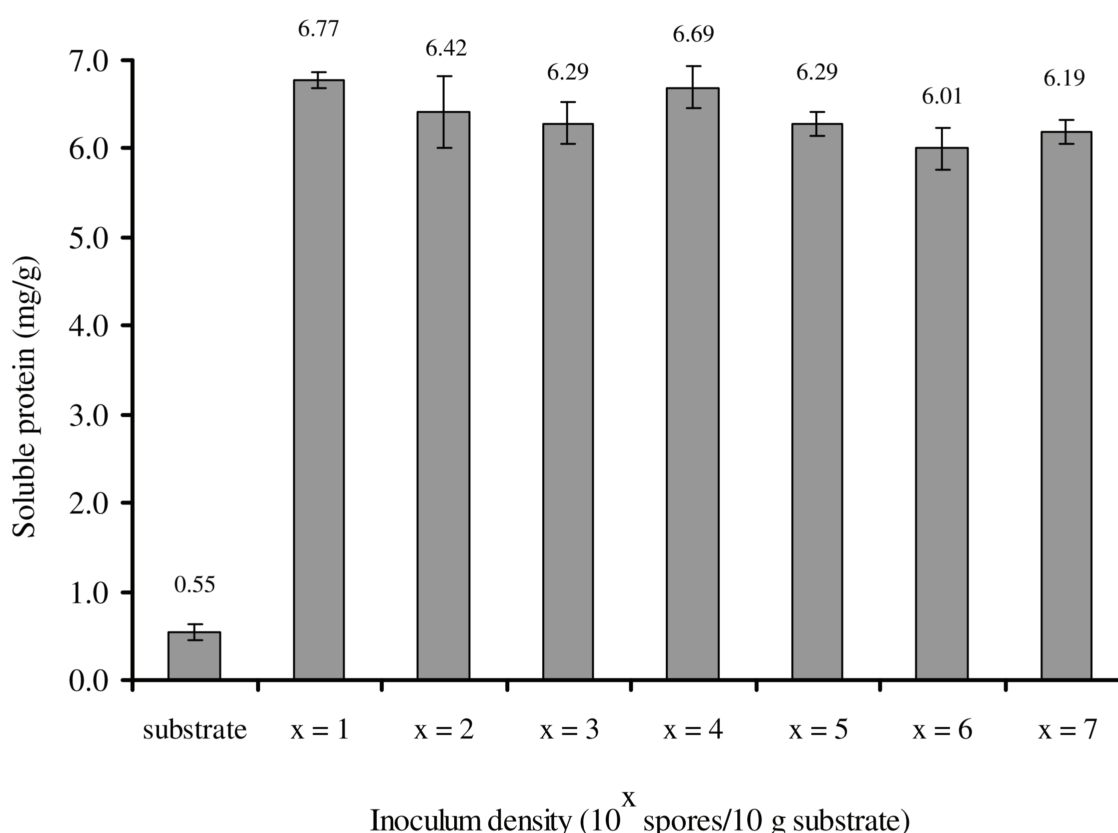


Figure 3-4: Influence of inoculum density on the growth of *R. oryzae* ZB.

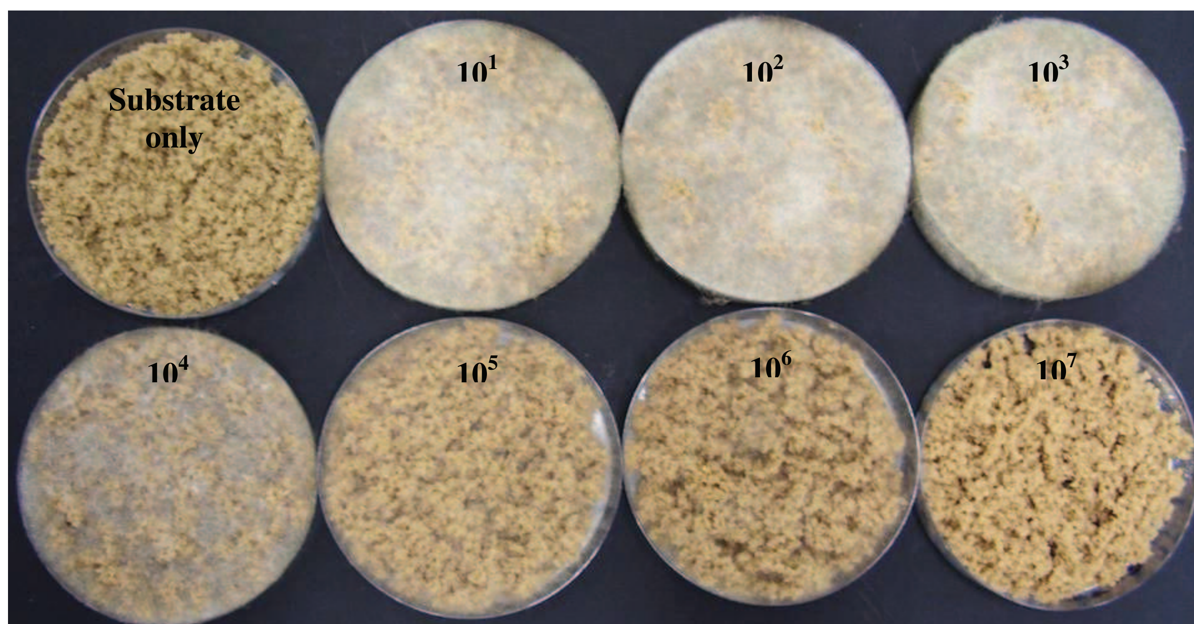


Figure 3-5: Increasing spore inoculum density caused decreasing mycelial density. (Numerical values indicate inoculum density per 10 g substrate).

3.3.2 Ammonium sulphate

Supplementing with too little or too much ammonium sulphate as a nitrogen source resulted in poor growth of *R. oryzae* ZB. As shown in Figure 3-6, the best growth, indicated by soluble protein concentration of 5.18 ± 0.06 mg/g, was achieved through the supplementation of 3.8 g ammonium sulphate per 100 g substrate. This value was used in subsequent experiments, although significantly similar results (P>5%) were achieved with 2.0% ammonium sulphate (Appendix Table 7-6).

3.3.3 Moisture content

Since increasing moisture content was achieved by adding more salt solution to the substrate (Section 3.3.2), higher moisture meant also higher salt content, notably of ammonium sulphate, which was present at a concentration of 3.3%. As seen in Figure 3-7, a small increase in soluble protein content occurred up to 64% moisture, beyond which the value decreased.

The soluble protein values resulting from initial moisture contents of 60%, 64% and 68% were not significantly different (P>5%, Appendix Table 7-7). However, since higher moisture would theoretically provide more nitrogen for the fungus to synthesise more protein, 68% was

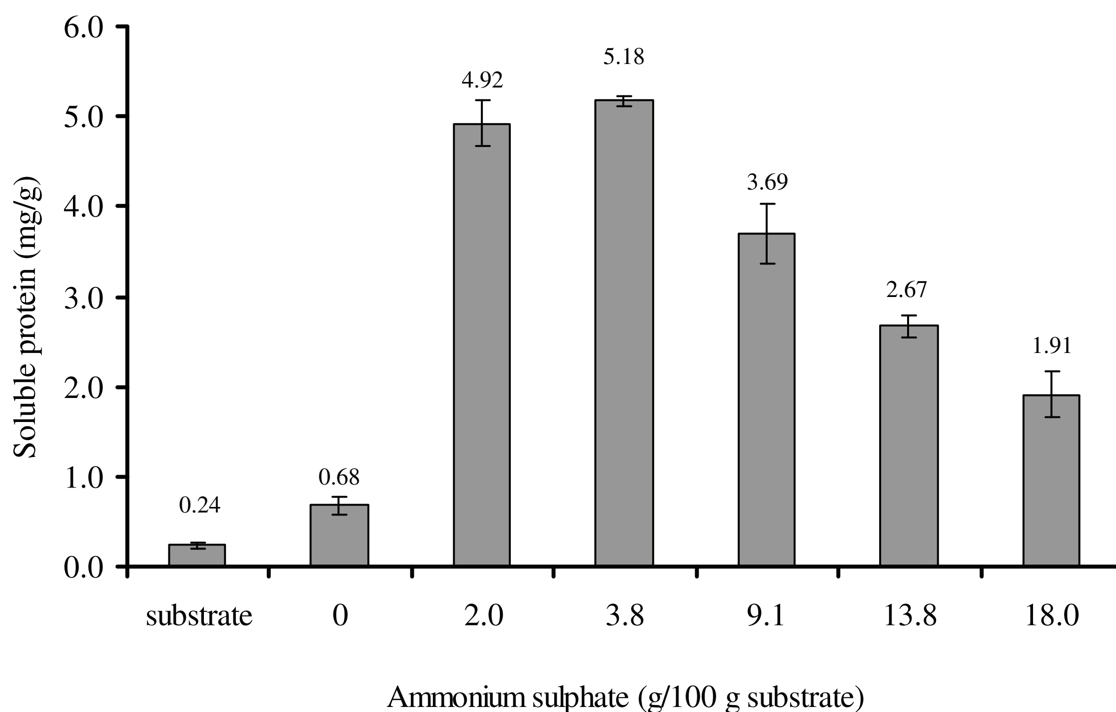


Figure 3-6: Influence of ammonium sulphate on the growth of *R. oryzae* ZB.

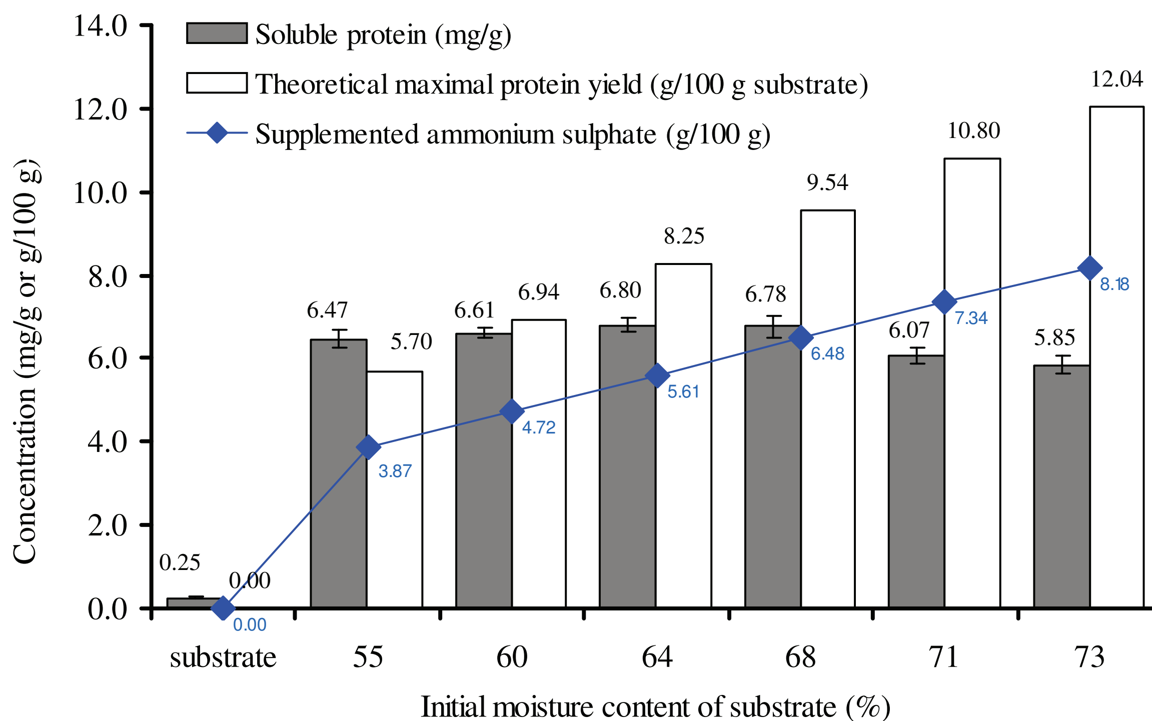


Figure 3-7: Influence of moisture content on the growth of *R. oryzae* ZB.

chosen for the next optimisation step. The theoretical maximal protein value as plotted in Figure 3-7 was the sum of the substrate's crude protein and fungal protein, assuming that all of the ammonium sulphate nitrogen added would be used by the fungus to synthesise protein.

3.3.4 Initial pH of substrate

Adjusting the pH value of the substrate to 5.3-6.6 before adding the inoculum was found to result in higher soluble protein concentrations than when lower pH values were used (Figure 3-8). No significant difference (P>0.05) was found amongst the means of the soluble proteins for substrates with an initial pH of 5.3, 6.0 and 6.6 (Appendix Table 7-8).

Higher pH values were not tested, since a pH around 7.0 increases the risk of unwanted contaminating bacterial growth. In addition, an alkaline salt solution would tend to cause the ammonium in the salt solution to be released as free ammonia, which was known to inhibit the growth of the tempe mould (Sparringa and Owens 1999c).

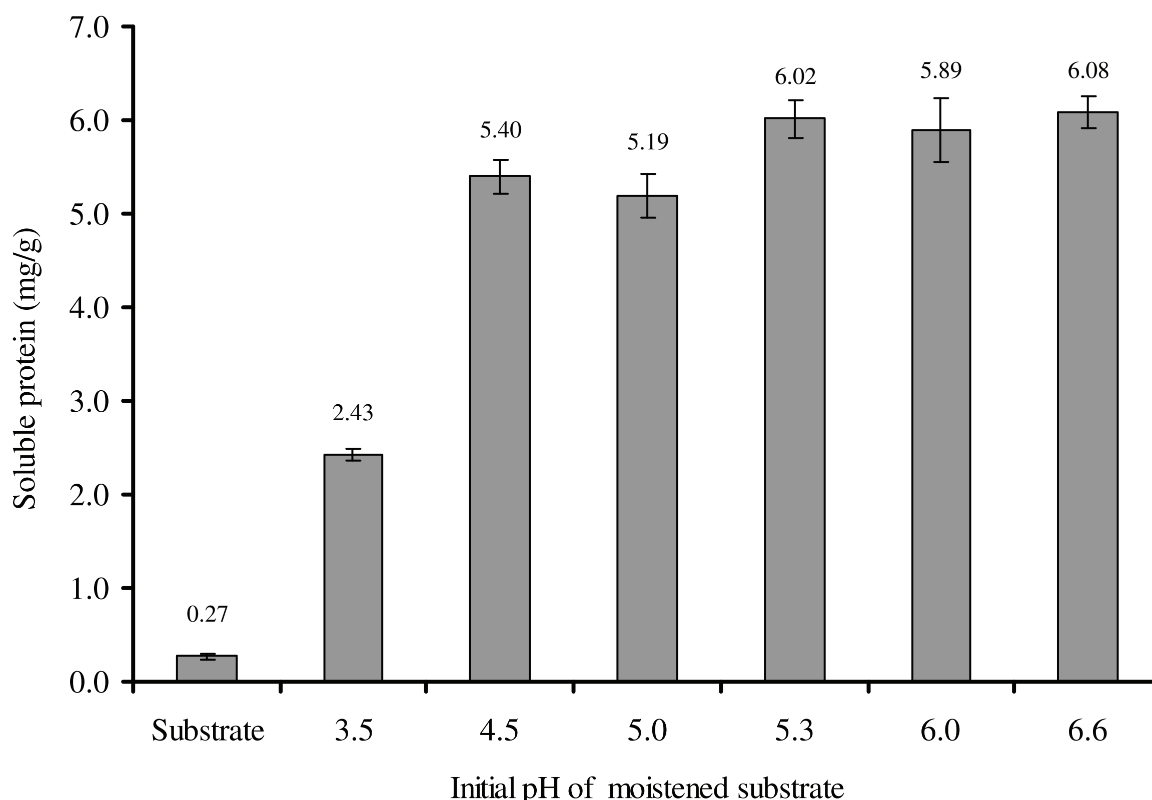


Figure 3-8: Influence of initial pH of the substrate on the growth of *R. oryzae* ZB.

3.3.5 Incubation temperature

As can be seen in Figure 3-9, the highest soluble protein content obtained for each given temperature showed a slowly decreasing trend with the increase in incubation temperature. The analysis of variance gave no indication of a significant difference (P>5%) between the highest soluble protein concentrations achieved by fermentation at 33°C for 36 hours, 30°C for 48 hours, and 27°C for 61 hours. Therefore, a temperature of 30°C was used for subsequent studies.

3.3.6 Composition of the mineral solution

Each type of mineral solution (described in Table 2-7) altered fungal growth in a different manner, as indicated by the soluble protein measurement (Figure 3-10). The supplementation of the substrate with ammonium sulphate alone (mineral solution 7 in Table 2-7) resulted in the poorest fungal growth, which improved significantly when either dipotassium hydrogen phosphate or potassium dihydrogen phosphate was added. The combination of ammonium sulphate and potassium dihydrogen phosphate led to the highest soluble protein content (Figure 3-10, Appendix Table 7-10). The supplementation of these two compounds was considered sufficient, since additional elements, or even a complete mineral solution, did not result in better growth.

3.3.7 Nitrogen source

Combining urea with ammonium sulphate drastically improved the growth of *R. oryzae* ZB. The soluble protein profile (Figure 3-11) clearly indicated that the presence of both nitrogenous compounds in the substrate significantly extended fungal growth beyond 48 hours. Growth of the *Rhizopus* stopped at 72 hours when 24% of the total nitrogen source was urea. When the proportion of the urea supplementation was increased to either 48% or 73%, the fungus grew until 120 hours. In contrast, when either urea or ammonium sulphate alone was provided, the fungus stopped growing after 24 hours. The poorest growth occurred with urea alone, as indicated by the extremely low soluble protein profile throughout the fermentation period. Macroscopical examination of the samples made at 0, 24, 48, 72, and 120 hours demonstrated clearly that the simultaneous presence of urea and ammonium sulphate allowed for the formation of very dense, compact and firmly interwoven *Rhizopus* mycelia covering the substrate's topmost surface (Figure 3-12).

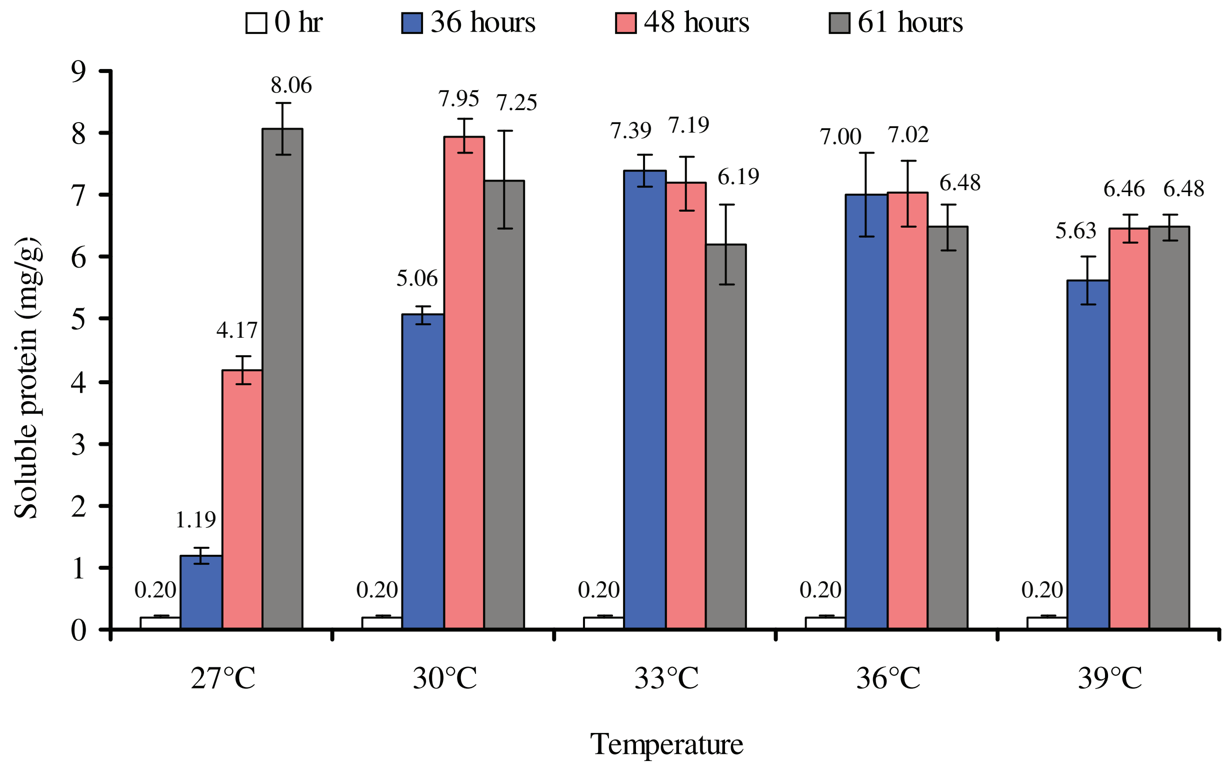


Figure 3-9: Influence of incubation temperature on the growth of *R. oryzae* ZB

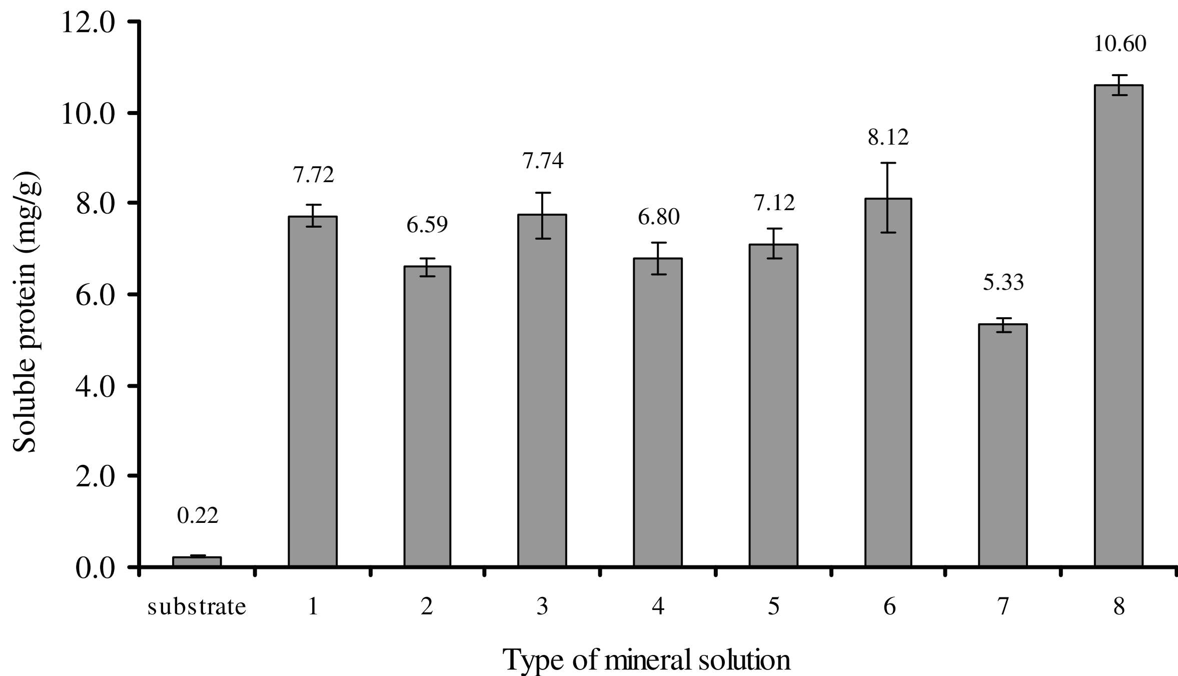


Figure 3-10: Influence of mineral composition (Table 2-7) on the growth of *R. oryzae* ZB.

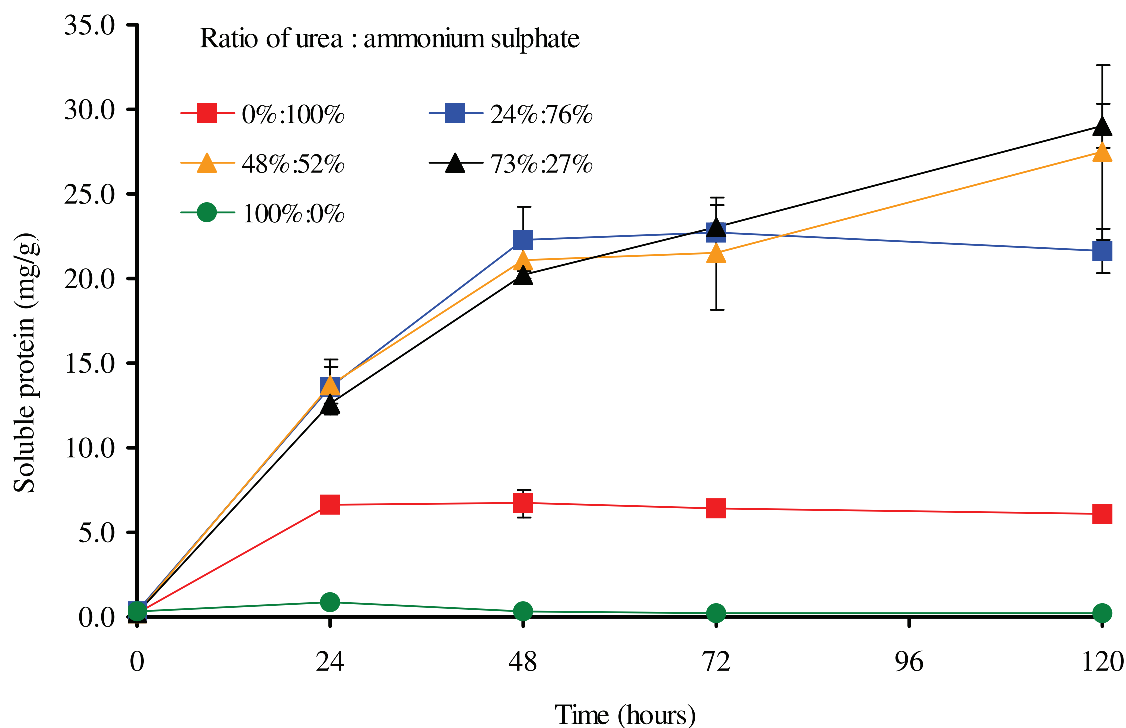


Figure 3-11: Growth profile of *R. oryzae* ZB in the presence of urea and/or ammonium sulphate as nitrogen sources.

At the end of fermentation (120 hours), a mycelial mat filled with numerous tiny dark spores was found only to cover the top surface of the sample supplemented with ammonium sulphate as the sole nitrogen source. When this layer was dissected from the underlying substrate, pre-gelatinised cassava bagasse granules with a very similar physical appearance to the original unfermented substrate were observed (Figure 3-13A). The presence of fungal mycelia was hardly seen in the space between the substrate granules, indicating poor growth in this area.

Similarly, when urea was used as the sole nitrogen source, a superficial layer of fungal growth was also found lying on top of the substrate. This layer was, however, tightly associated with the substrate underneath, which no longer retained its initial granular shape (Figure 3-13B). The fungal fermentation not only caused a loss of the substrate's original granular texture, but also resulted in a watery mash, indicating liquefaction. More mycelia were found growing inside the void space between the substrate aggregates compared to the sample without urea.

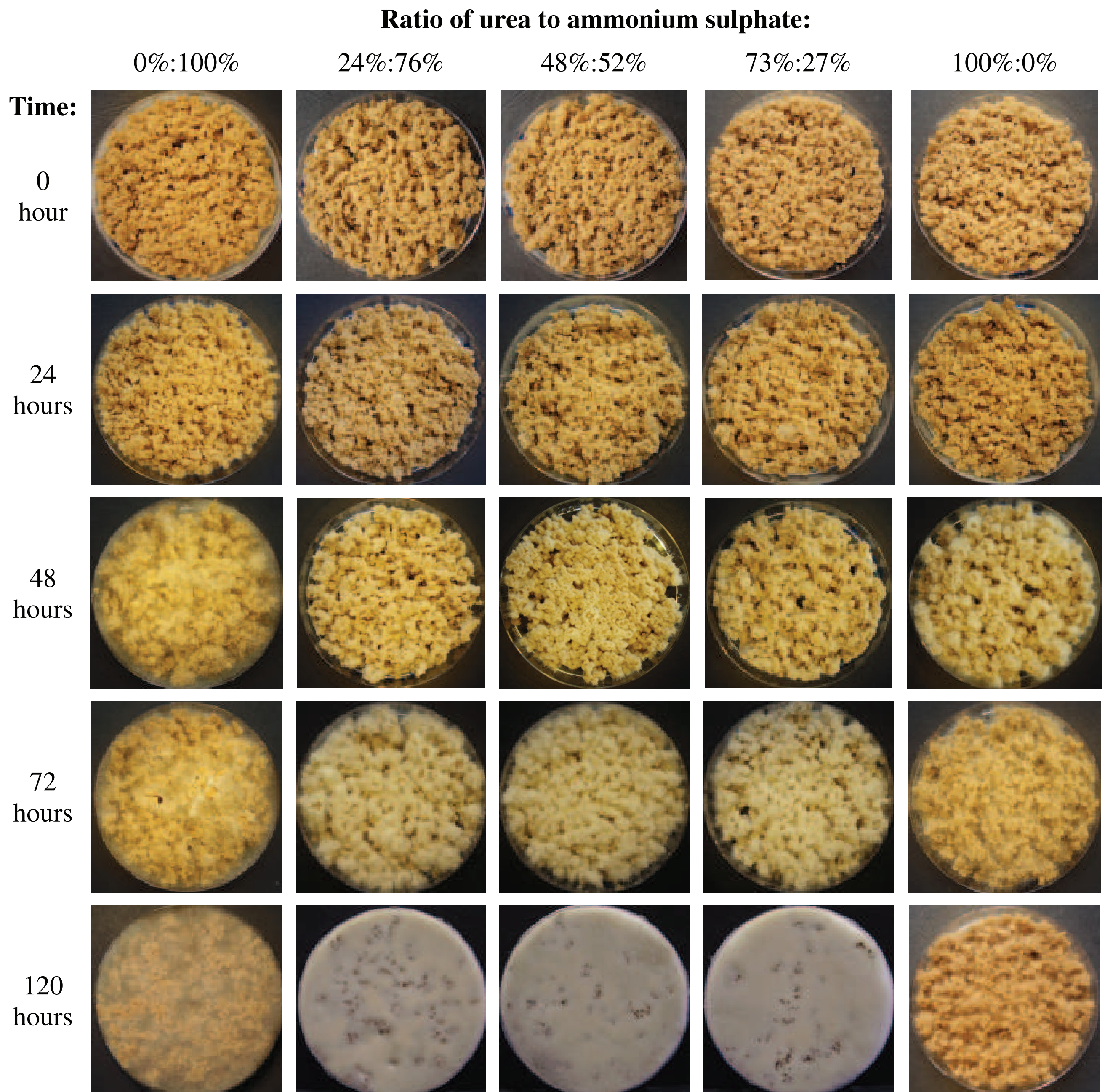


Figure 3-12: Macroscopic appearance at different fermentation times of cassava bagasse fermented with *R. oryzae* ZB supplemented with different ratios of urea to ammonium sulphate.

Very good fungal growth was observed only when both urea and ammonium sulphate were added together to the substrate (Figure 3-13C). The fungus grew massively, forming very compact, dense, cottony white mycelial structures filling up the space between the substrate aggregates, which had lost their original grainy texture. Substrate aggregates were bound

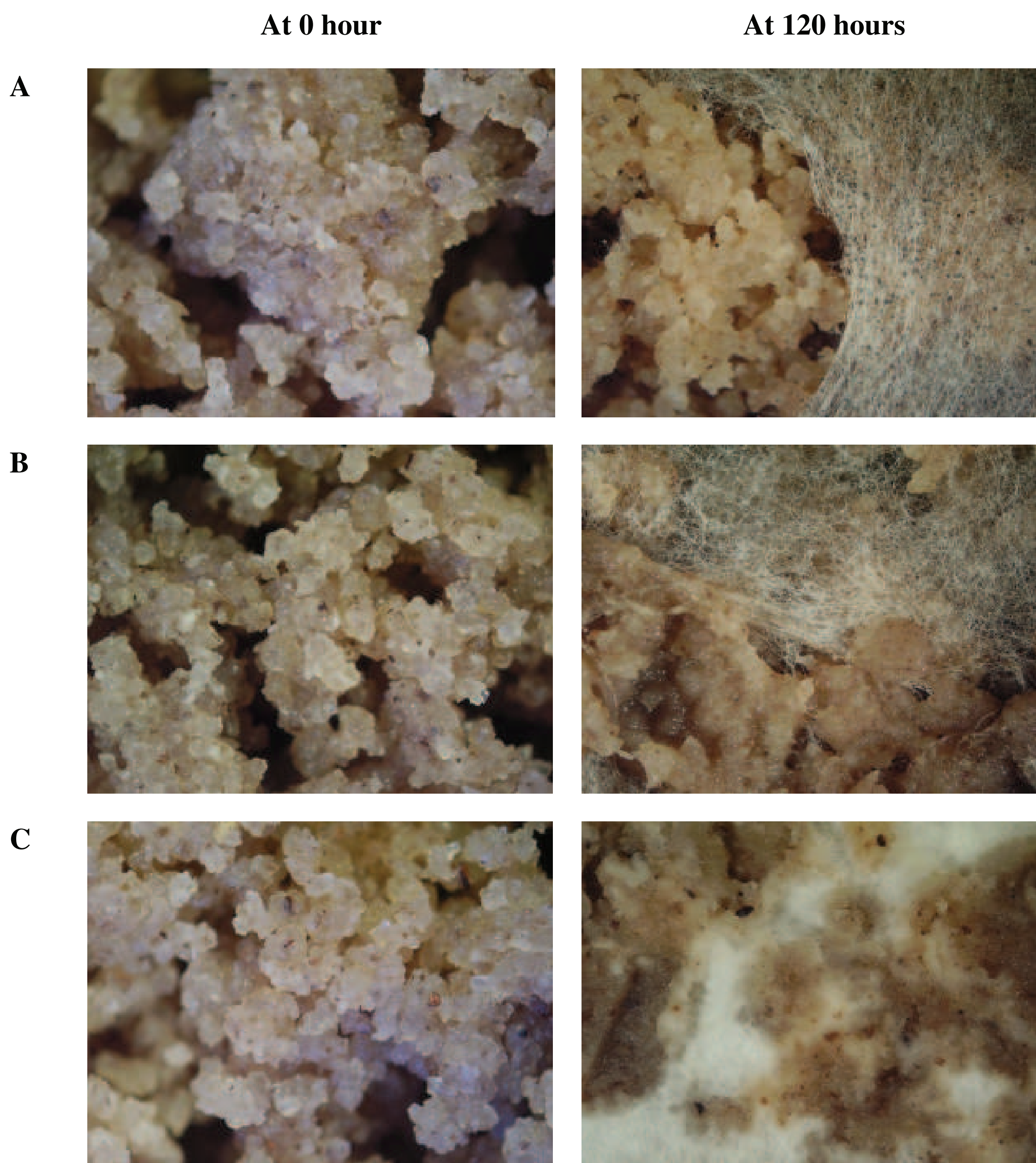


Figure 3-13: Morphological changes to the substrate caused by the growth of *R. oryzae* ZB when grown with a nitrogen source consisting of urea only (A), ammonium sulphate only (B), or both urea and ammonium sulphate (C).

tightly by extremely thick mycelia, forming a virtually inseparable substrate-mycelia aggregate, and producing a solid, sliceable cake (Figure 3-14), just like soybean tempe (Figure 3-15). At 120 hours, spore formation was observed, and the fermentation was therefore discontinued.

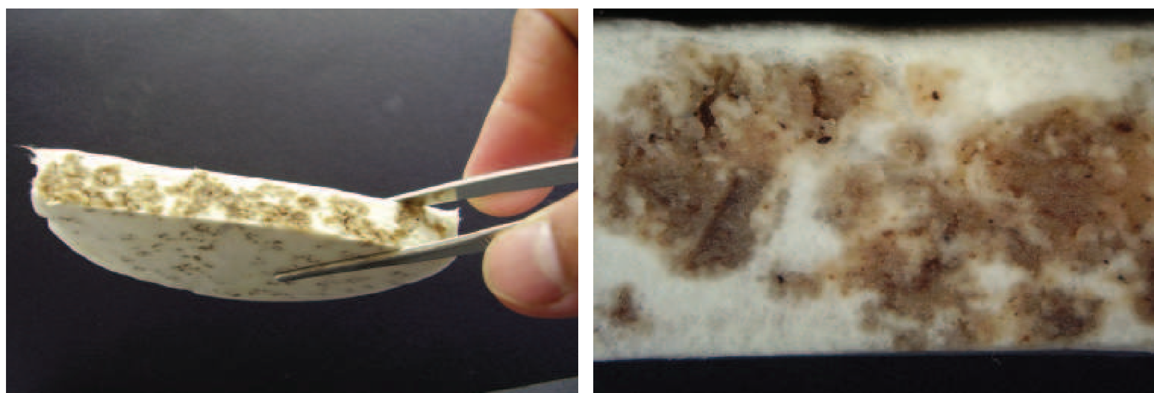


Figure 3-14: A solid, sliceable cake texture was obtained after 120 hours fermentation with *R. oryzae* ZB when urea and ammonium sulphate were supplemented together to the cassava bagasse substrate.

This fermentation with *R. oryzae* ZB increased the true protein content significantly from 0.9 g/100 g to more than 7.5 g/100 g in the samples containing both urea and ammonium sulphate (Figure 3-16). The highest true protein content of 9.0 g/100 g was achieved when urea and ammonium sulphate were added in almost equal quantities. In the absence of urea, the increase in protein content was only 1.6 g/100 g, whereas urea as the sole nitrogen source caused almost no fungal protein enrichment. The simultaneous addition of urea and ammonium sulphate caused the fungus to enhance the substrate, with a significantly ($P<0.05$) high protein content being observed after 120 hours fermentation (Appendix Table 7-13).

Residual starch (Figure 3-16), measured as total reducing sugars, indicated the extent of starch metabolism by the fungus and was heavily influenced by the presence of urea. Slightly more than 50 percent of the substrate's starch was metabolised when both nitrogenous compounds were present. In contrast, starch utilisation was very poor when either urea or ammonium sulphate was supplemented singly. It is worth noting, however, that when only urea was added to the substrate, a significantly high amount of total reducing sugars (Appendix Table 7-22) was liberated and remained in the substrate, without any indication of its being metabolised by the fungus for fungal growth.

As clearly shown in Figure 3-17, in the absence of urea, free reducing sugars were no longer released beyond 24 hours. From this time on, the sugar level stayed essentially constant until 120 hours, similar to the growth profile (Figure 3-11). This therefore suggests that fungal growth, sugar uptake, and amylolytic activity were all discontinued at 24 hours. On the other

hand, the sample with urea as the sole nitrogen source contained the smallest amount of free reducing sugars in the first 24 hours. The sugar level then rose sharply, however, and continued to be released for even up to 120 hours, when the level far exceeded that of the other samples. This clearly shows that the amylolytic activity continued hydrolysing starch throughout the fermentation period for 120 hours in spite of the extremely poor fungal growth, which had already come to a halt at 24 hours. The simultaneous addition of urea and ammonium sulphate not only led to the release of reducing sugars, but also to their dramatic decrease in the period between 48-72 hours, followed by less sharp decline until 120 hours, suggesting the sugar uptake by *R. oryzae* ZB. Interestingly, with 24% urea, sugar uptake was not accompanied by further fungal growth (Figure 3-11).

The ratio of urea to ammonium sulphate was related to the pH profile during fermentation (Figure 3-18). Relative to all of the samples supplemented with urea, the growth of *R. oryzae* ZB with ammonium sulphate as the sole nitrogen source led to a rapid fall in pH to 3.0 after 24 hours fermentation. It decreased even further until 120 hours, where the pH dropped as low as 2.6. On the other hand, in the samples where urea was present, a sharp increase in pH values to around 7.0 or more was noted in the first 24 hours, followed by a dramatic decline until 48 hours. After 48 hours, overall pH values slowly became more and more acidic in samples with 100% or 24% urea, reaching pH values of 3.7 and 3.5, respectively. When urea was present at either 48% or 73%, however, the pH continued to decline slightly until 72 hours, followed by an increase until the end of the fermentation, at which point the overall pH values never fell below 4.4. Overall, a higher percentage of urea led to a higher pH profile in all of the samples supplemented with both nitrogen sources.

Levels of ammonium present in the substrates during fermentation also depended on the ratios of urea to ammonium sulphate (Figure 3-19). For all samples supplemented with urea, the levels increased in the early phase of fermentation, followed by slow decrease toward the end. Overall, the ammonium contents for these samples at the termination of fermentation exceeded their initial values. The only decreasing trend occurred in the sample with ammonium sulphate only. Residual ammonium was higher in samples with higher initial urea supplementation, with the exception of the sample supplemented only with urea. This sample had the lowest level of residual ammonium, indicating that only a very small portion of the urea was converted to ammonium.

Before fermentation

A **B**

Top view

After fermentation

A **B**

Top view

Cross-sectional view

Figure 3-15: *R. oryzae* ZB grew equally well on pregelatinised cassava bagasse (A) and on cooked, dehulled soybeans (B) for 120 hours and for 48 hours, respectively.

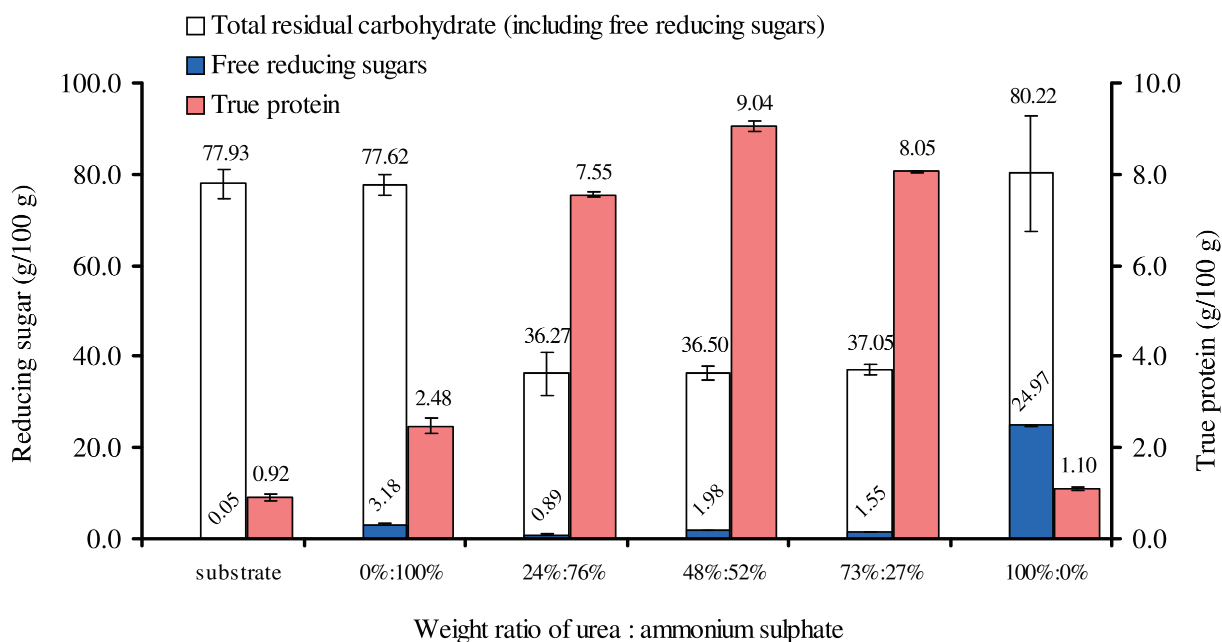


Figure 3-16: True protein content and carbohydrate utilisation by *R. oryzae* ZB as functions of the ratio of urea to ammonium sulphate after 120 hours fermentation. Free reducing sugars indicated the total, not-metabolised reducing sugar residues.

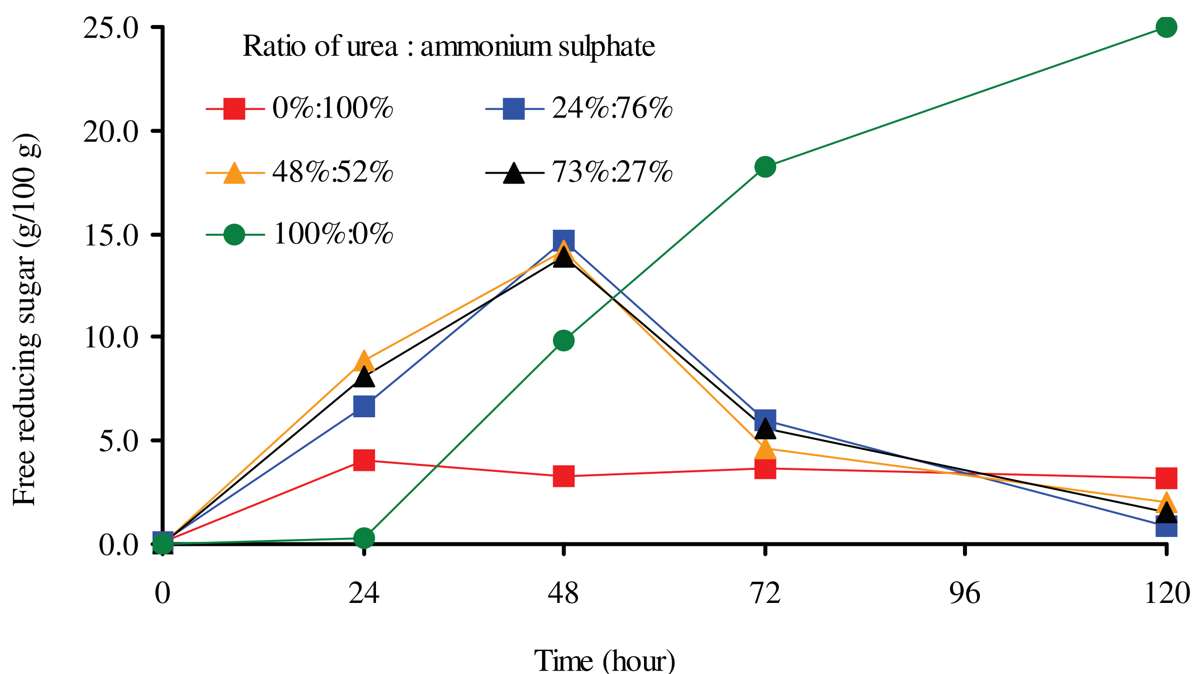


Figure 3-17: Influence of the ratio of urea to ammonium sulphate on the free reducing sugars released due to fungal amylolytic activity.

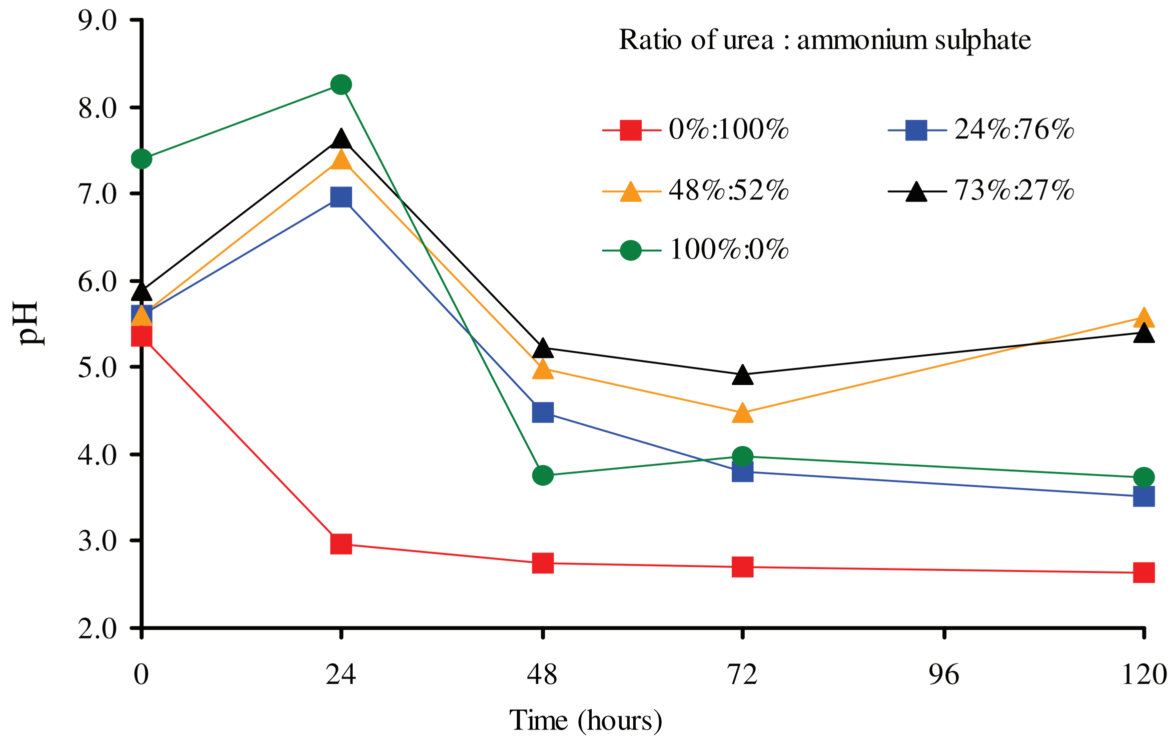


Figure 3-18: Profiles of pH during fermentation with *R. oryzae* ZB supplemented with different ratios of urea to ammonium sulphate.

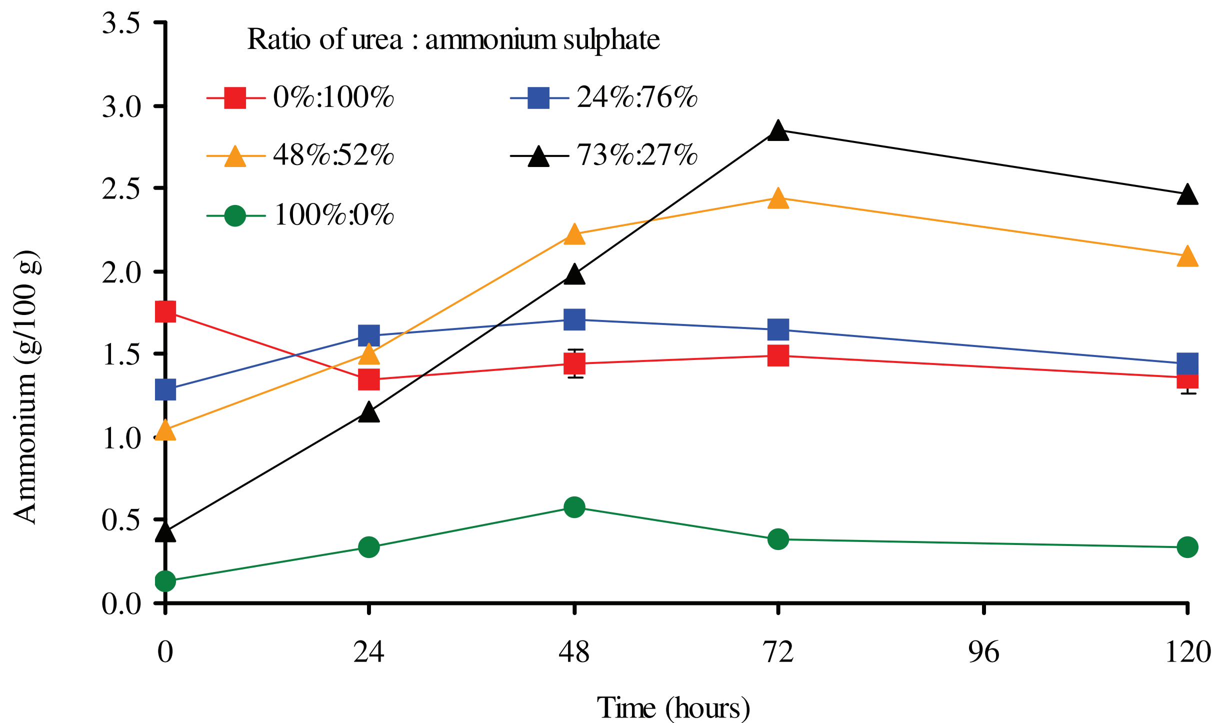


Figure 3-19: Profiles of free ammonium in the substrate during fermentation with *R. oryzae* ZB supplemented with different ratios of urea to ammonium sulphate.

3.4 Influence of some other factors

3.4.1 Substrate pretreatment

With the exception of pregelatinised cassava bagasse, which retained its grainy structure, mixing prewarmed salt solution with either raw cassava bagasse or pregelatinised cassava tuber resulted in a clumpy, paste-like texture. As clearly seen in Figure 3-20, 120 hours fermentation led to the dense mycelial colonisation of the entire surface of both the pregelatinised and raw cassava bagasse substrates. In contrast, sparser, poor fungal growth was observed on the pregelatinised cassava tuber.

The growth of *R. oryzae* ZB after 120 hours fermentation changed the texture of the pregelatinised cassava tuber into a liquefied mash. This did not happen to the other two substrates, where the extremely dense mycelium network bound the substrate aggregates very tightly together, forming a solid structure made up of virtually inseparable mycelium-substrate aggregates. Upon taking a closer look, additional features were distinguished between the last two fermented samples. Starting at the outer layer and working towards the inner layer of the pregelatinised cassava bagasse aggregates, a gradual change in colour from pale whitish brown to darker brown was observed (Figure 3-21). The inside of the aggregates was solid.

On the contrary, no such colour pattern was observed when raw cassava bagasse was used as the substrate (Figure 3-22). Instead, the interior portions of the fermented substrate aggregates were somewhat hollow and filled with irregularly shaped particulates. In this empty space, very thin white threads were observed, which could be the penetrating hyphae of the *R. oryzae* ZB. However, the hyphae were not observed to develop into dense mycelium to fill the void space.

No significant difference ($P>0.05$, Appendix Table 7-14) was found in true protein content after fermentation between raw or pregelatinised cassava bagasse (Figure 3-23). Both substrates allowed for very good mycelial development and protein enrichment up to around 9 percent. In contrast, pregelatinised cassava tuber led to poor fungal growth, with the development of a sparse and thin mycelial mat without any sign of sporulation. Growth was observed on the substrate's surface with the protein content having increased only slightly, from 0.6 to mere a 1.8 g/100 g. The final pH value indicated no sign of adverse acidification (Figure 3-23).

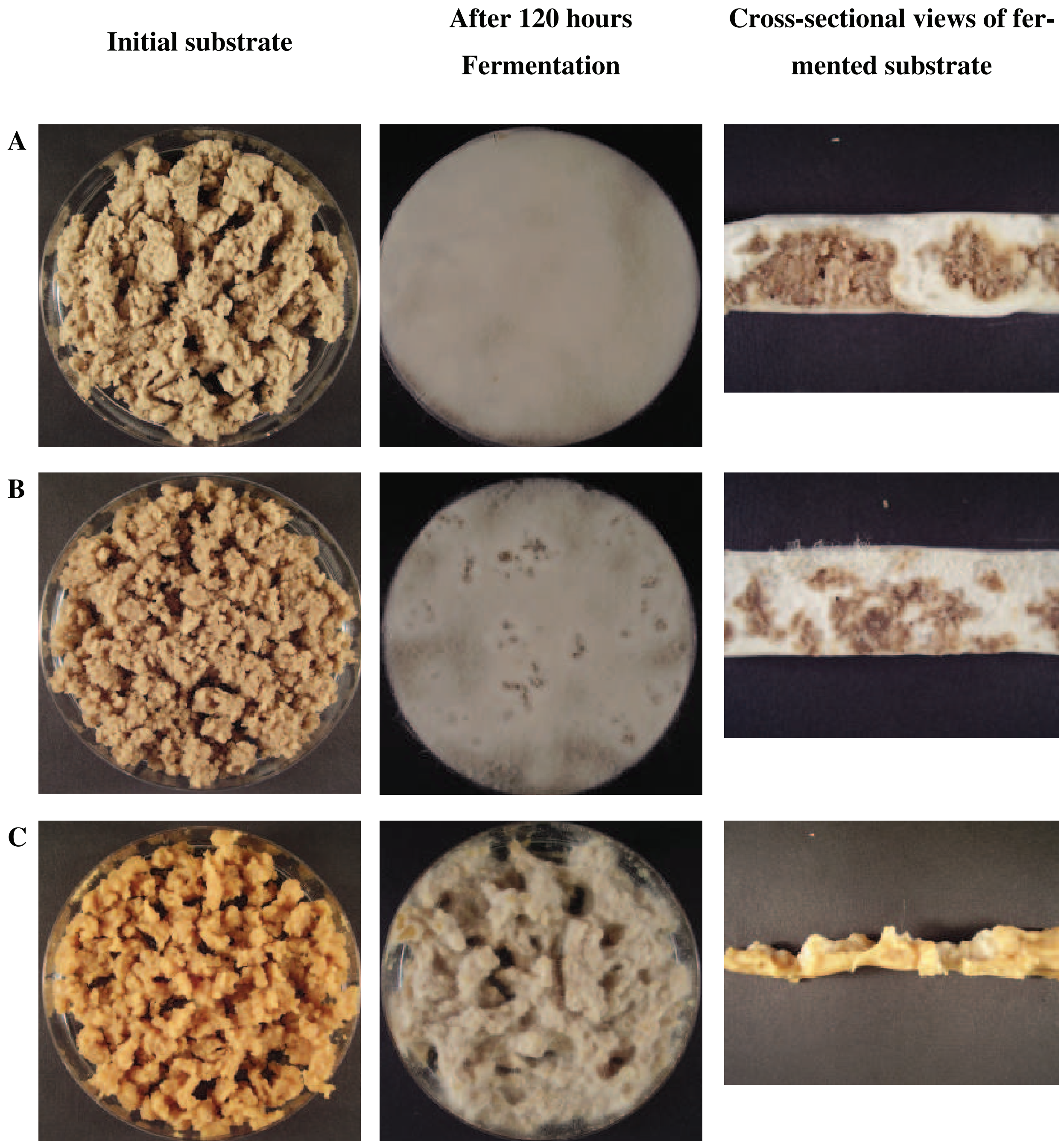


Figure 3-20: Top overview and cross-sectional views of *R. oryzae* ZB fermentation on three different substrates: raw cassava bagasse (A), pregelatinised cassava bagasse (B), and pregelatinised cassava tuber (C).

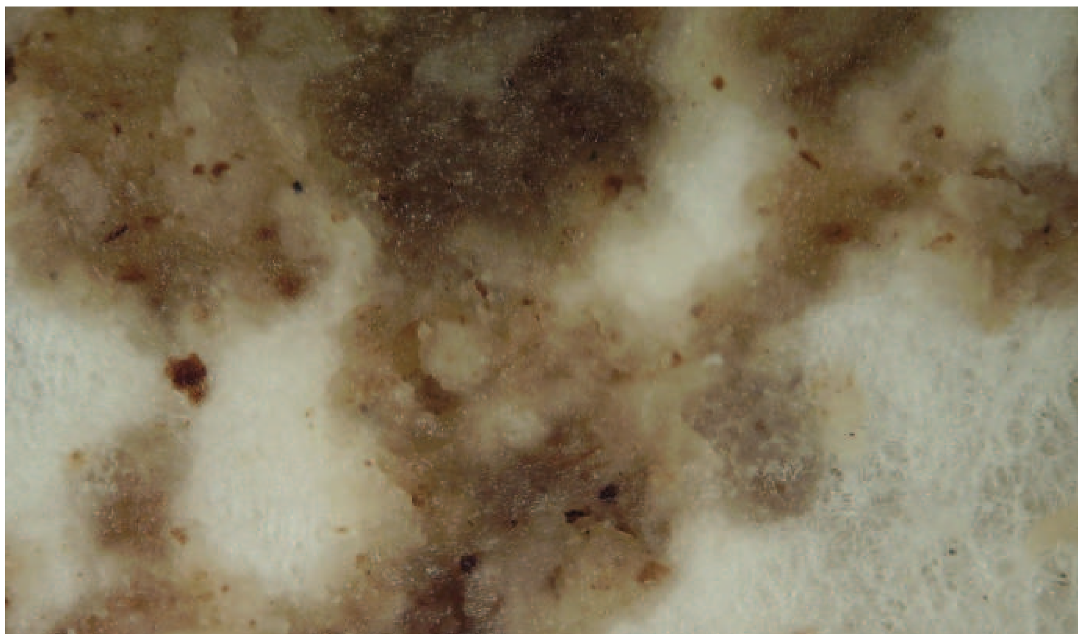


Figure 3-21: Close-up picture of the cross-sectional views of pregelatinised cassava bagasse after 5 days fermentation with *R. oryzae* ZB. The substrate showed a colour gradient from pale whitish brown to dark brown from the outer layer to the inner layer.

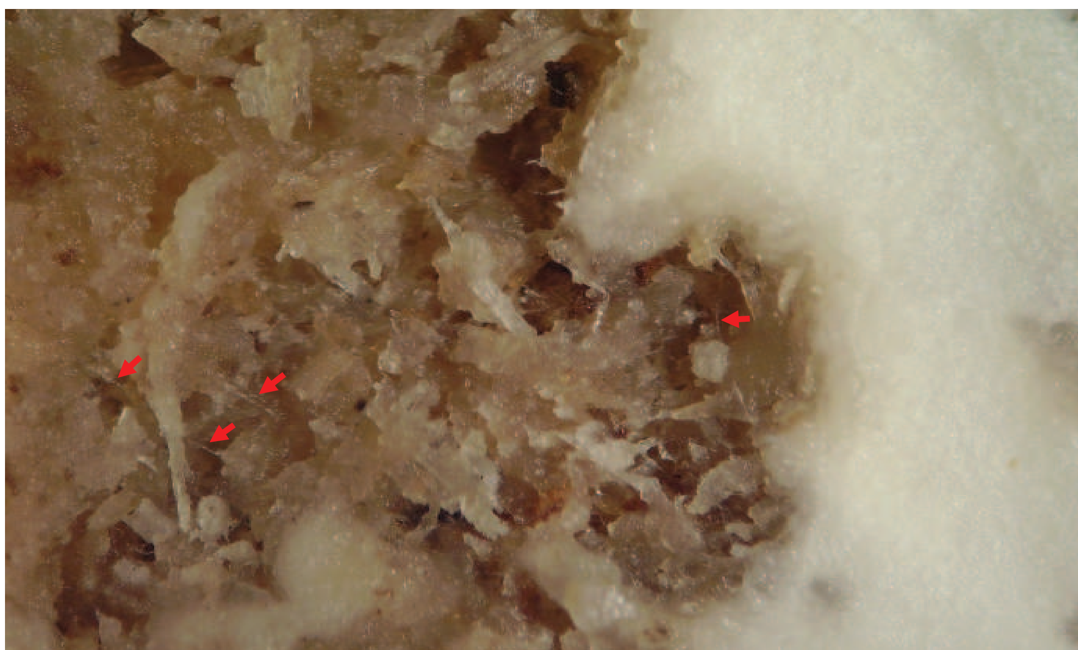


Figure 3-22: Close-up picture of the cross-sectional views of raw cassava bagasse after 5 days fermentation with *R. oryzae* ZB. Some putative penetrating hyphae are indicated with red arrows.

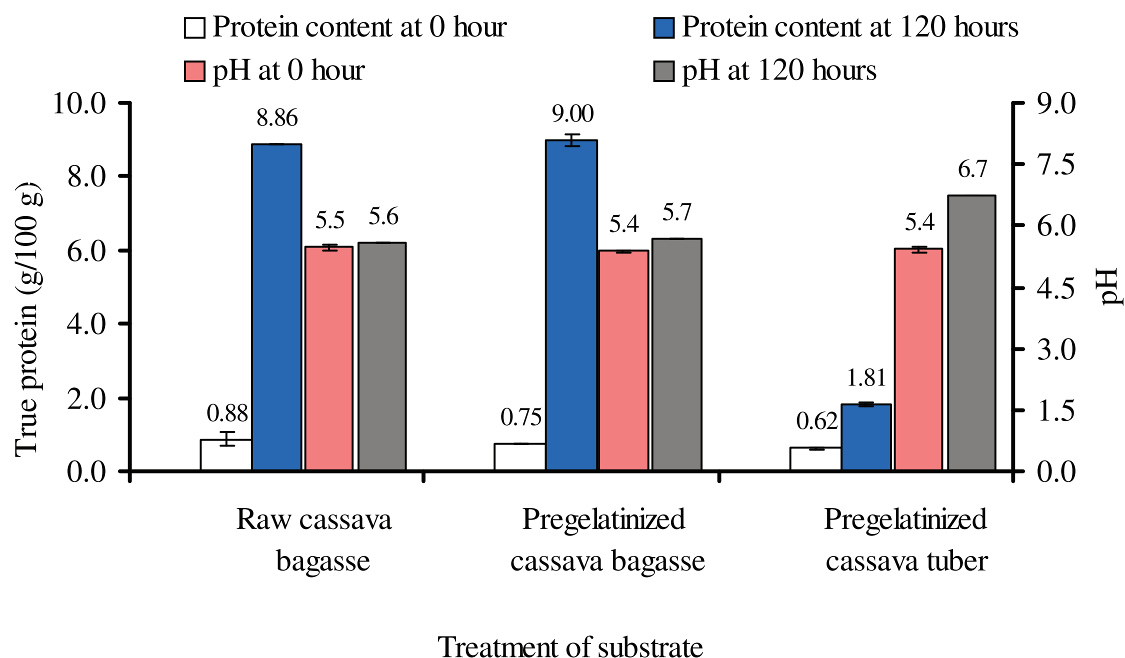


Figure 3-23: Effect of substrate pretreatment on true protein content after 5 days fermentation with *R. oryzae* ZB.

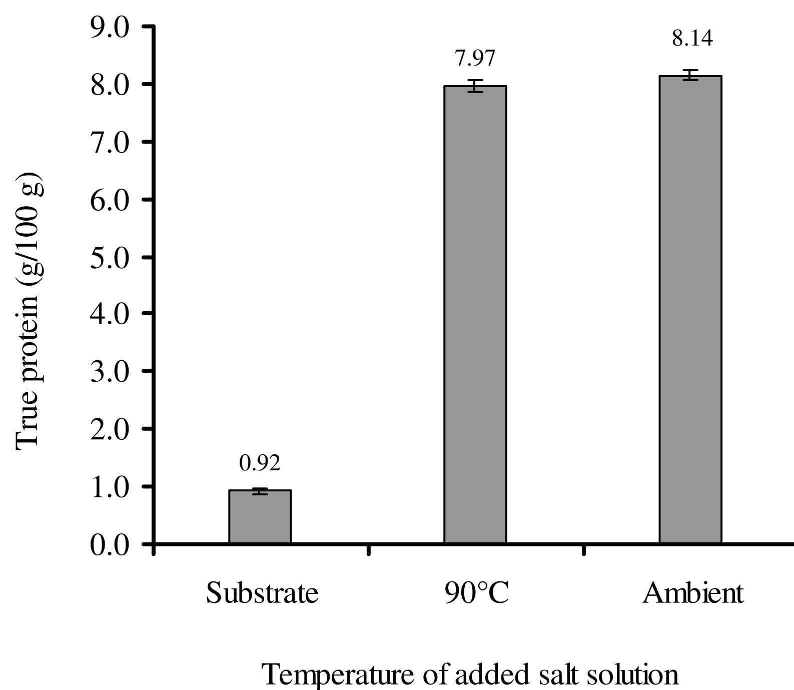


Figure 3-24: Influence of the temperature of the salt solution on true protein content formed by *R. oryzae* ZB after 120 hours fermentation.

3.4.2 Temperature of salt solution

Prewarming the salt solution to 90°C promoted a faster swelling of the pregelatinised substrate than using room-temperature solution. However, it had no influence on the protein content of the fermented products. Both hot and cool salt solutions increased the protein content to 8%, about 9 times higher than the original value (Figure 3-24). No significant difference ($P>5\%$) was found between both treatments (Appendix Table 7-15).

3.4.3 Different *Rhizopus* strains

All of the five selected *Rhizopus* strains grew well on the cassava bagasse substrate and exhibited different patterns of mycelial formations (Figure 3-25). All of the strains were able to utilise more than 60% of the carbohydrates in the initial substrate, and significantly reduced them from the initial amount of 82.5 g/100 g to 27.9–32.6 g/100 g (Figure 3-26A, Appendix Table 7-20). True protein content reached the highest value of 9.2 g/100 g when *R. oryzae* ZB was used as the inoculum, followed by *R. oryzae* EN and *R. oligosporus* Tebo, both of which led to a protein enrichment of 7.7 g/100 g. The lowest true protein content of 6.6 g/100 g was produced with *R. oryzae* Mala as the inoculum. However, net protein enrichment as the result of fungal growth was merely 2.7–4.0 g/100 g DW initial substrate, with *R. oryzae* ZB being the most prolific protein producer (Figure 3-26B).

Only minute amounts of residual urea were detected at the end of fermentation (Table 3-4). This signified that practically all of the supplemented urea had disappeared, probably being hydrolysed to release ammonium, much of which still remained in the fermented substrate. This residual ammonium was measured to be 2.25–2.72 g/100 g, much higher than the ammonium level in the initial substrate (0.9 g/100 g). However, when the increase in moisture content (10.1–11.5%) and the considerable loss of dry matter (36.0–42.7%) were also taken into account, the unassimilated ammonium was calculated to be 1.33–1.74 g/100 g.

The total amount of urea and ammonium sulphate initially supplemented per 100 g DW cassava bagasse substrate was calculated and found to be equivalent to 2.24 g nitrogen (Table 3-5). After 120 hours fermentation, the remaining, unassimilated nitrogen was 1.03–1.35 g/100 g DW initial substrate. Subtracting this from the initial nitrogen content yielded 0.89–1.21 g, which represents the nitrogen metabolised by the fungi. Part of this amount (i.e. 19.2–

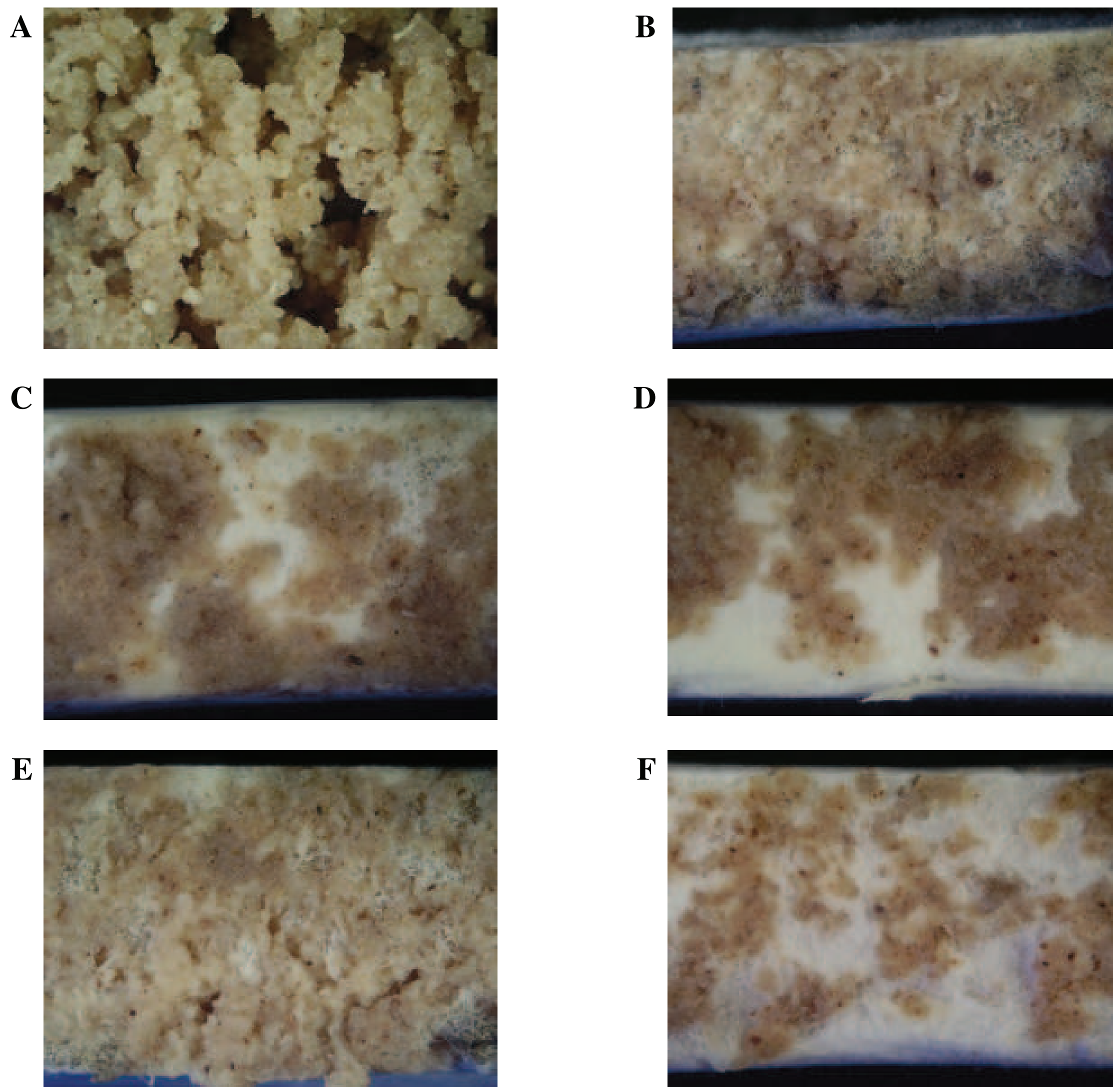


Figure 3-25: Cross-sectional views of cassava bagasse substrate (A) after fermentation for 120 hours with different *Rhizopus* strains: *R. oryzae* EN (B), *R. oryzae* Fi (C), *R. oryzae* Mala (D), *R. oligosporus* Tebo (E), and *R. oryzae* ZB (F).

28.6% of the initially supplemented nitrogen) was used to synthesise 2.71–3.98 g protein/100 g DW initial substrate (Figure 3-26B).

Previous studies have measured the protein contents of *Rhizopus* spp. mycelial biomass and have obtained 13 results ranging from 42.81 to 49.7% protein (Omar and Li 1993; Jin et al. 1999; Jin et al. 2001; Jin et al. 2002). By using the average of these values, which is 47.4%, the fungal biomass produced in the present study was estimated to be 5.72-8.40 g/g DW ini-

tial substrate. A relatively significant amount of nitrogen remained unaccounted for or missing. As shown in Table 3-5, this amounted to 20-26% of the initially supplemented nitrogen.

Across all strains, the utilisation of digestible carbohydrates for protein formation in cassava bagasse was below 25%, whereas 42-53% of the supplemented nitrogen was utilised for protein synthesis (Table 3-6). *R. oryzae* ZB had the highest values for both conversion rates compared to the other strains. Despite having the lowest rate of conversion of digestible carbohydrate to protein, *R. oryzae* Mala had the conversion rate closest to that of *R. oryzae* ZB. This indicates that the *Rhizopus* strain better at assimilating carbohydrates was not necessarily better at assimilating nitrogen from urea and ammonium sulphate for protein formation.

Table 3-4: The physicochemical characteristics of samples after 120 hours fermentation with the selected *Rhizopus* strains.

Rhizopus strain	Residual urea (mg/100 g)[1,4]	Residual ammonium (NH_4^+) expressed as g/100 g DW of		Increase of moisture content (g/100 g)[3,4]	Loss of dry matter (g /100 g)[2,4]
		fermented sample[1,4]	initial substrate[2,4]		
Initial substrate	3292.01 ± 34.82[a]	-	0.90 ± 0.03[a]	-	-
EN	2.73 ± 0.07[b]	2.62 ± 0.01[a]	1.50 ± 0.01[b]	10.6 ± 0.6[ab]	42.7 ± 1.1[a]
Fi	0.12 ± 0.05[b]	2.54 ± 0.07[a]	1.56 ± 0.05[b]	10.1 ± 0.5[a]	38.7 ± 0.6[b]
Mala	0.09 ± 0.07[b]	2.72 ± 0.24[a]	1.74 ± 0.15[c]	10.1 ± 0.4[a]	36.0 ± 1.0[c]
Tebo	1.21 ± 0.11[b]	2.72 ± 0.24[a]	1.58 ± 0.14[b]	11.1 ± 0.9[ab]	42.1 ± 0.8[a]
ZB	0.36 ± 0.29[b]	2.25 ± 0.08[b]	1.33 ± 0.08[d]	11.5 ± 1.3[b]	40.6 ± 1.4[d]

[1] Expressed on the basis of DW sample.

[2] Expressed on the basis of DW initial substrate (before fermentation).

[3] Expressed on the basis of WW initial moistened substrate (before fermentation).

[4] Values with different alphabetical superscripts within the same column are significantly different ($P<0.05$).

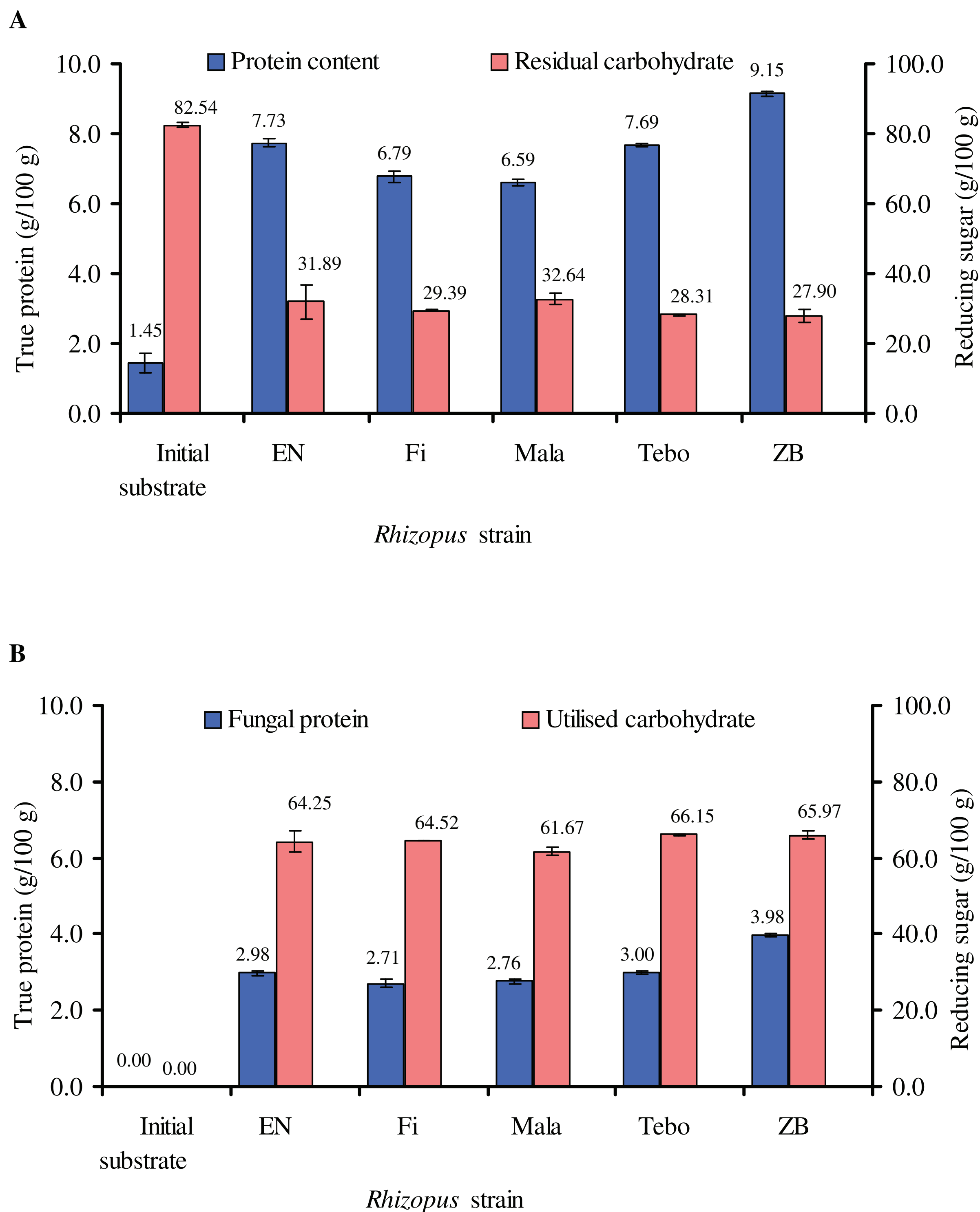


Figure 3-26: Influence of different *Rhizopus* strains on true protein content and residual carbohydrate (expressed on the basis of DW sample) (A), and on net fungal protein increase and carbohydrate utilisation as the result of *Rhizopus* growth (expressed on the basis of DW initial substrate) (B).

Table 3-5: Supplemented-nitrogen utilisation and fungal biomass formation in the 120 hours fermentation of cassava bagasse with the selected *Rhizopus* strains.

Nitrogen	In the initial substrate	In the sample fermented with *Rhizopus* strain:				
		EN	Fi	Mala	Tebo	ZB
Residual nitrogen concentration (g/100 g DW sample)[1,2]	2.24	2.04	1.98	2.12	2.12	1.75
Residual amount of nitrogen (g/100 g DW initial substrate)[2]	2.24 (100%)	1.17 (52.2%)	1.21 (54.0%)	1.35 (60.3%)	1.23 (54.9%)	1.04 (46.4%)
Nitrogen assimilated in fungal protein (g/100 g DW initial substrate)[2,3]	0.00 (0%)	0.48 (21.4%)	0.43 (19.2%)	0.44 (19.6%)	0.48 (21.4%)	0.64 (28.6%)
Unidentified or missing nitrogen (g/100 g DW initial substrate)[4]	0.00 (0%)	0.59 (26.3%)	0.59 (26.3%)	0.44 (19.6%)	0.53 (23.7%)	0.56 (25.0%)
Mycelial biomass (g/100 g DW initial substrate)[5]	0	6.27	5.72	5.82	6.33	8.40

[1] Nitrogen value in the initial substrate was estimated based on the nitrogen constituents of urea and ammonium sulphate supplemented before the fermentation. Nitrogen values in the fermented samples were estimated based on the nitrogen constituent of the residual ammonium alone since the residual urea was found in an extremely low quantity after the fermentation and was, therefore, insignificant (Table 3-4). The nitrogen value from the crude protein content of the unfermented substrate was not included throughout the calculation.

[2] Values in parentheses represent percentage based on the initial amount of nitrogen constituents of urea and ammonium sulphate supplemented to the substrate before fermentation.

[3] Nitrogen constituent of mycelial protein was calculated by dividing the values of the net protein increase (as presented in Figure 3-26B) with the protein conversion factor of 6.25.

[4] Nitrogen which was the constituent of neither mycelial protein nor residual ammonium, and was the difference values obtained after substracting nitrogen amount in the fermented substrate from that of initial unfermented substrate.

[5] Estimated based on an average conversion factor of 47.4 g protein/100 g DW *Rhizopus* sp. biomass (Appendix 7.6, Appendix Table 7-2).

Table 3-6: Conversion rate of substrate to protein using various *Rhizopus* strains.

Rhizopus strain	Conversion rate of digestible carbohydrate to protein[1] (%)	Conversion rate of supplementary nitrogen to fungal protein nitrogen[2] (%)
EN	16.3	44.9
Fi	15.0	41.7
Mala	13.2	50.0
Tebo	18.3	47.5
ZB	24.0	53.3

[1] Expressed as the increase (g) of the net fungal protein per 100 g of the starch metabolised by the fungi.

[2] Expressed as the amount of fungal protein nitrogen (g) per 100 g of the total amount of nitrogen constituents of urea and ammonium sulphate metabolised by the fungi.

3.4.4 Sulphur source

R. oryzae ZB grew well on the cassava bagasse substrate supplemented with all of the sulphur-containing compounds used, with the exception of DMSO (Figure 3-27). Substituting ammonium sulphate with either sodium sulphate or L-cystine did not significantly ($P>0.05$) change the protein content of the fermented products (Figure 3-28, Appendix Table 7-16). However, the values were slightly but significantly lower when magnesium sulphate or L-methionine was used instead. DMSO supplementation led to poor fungal growth, just as when no sulphur compound was added, resulting in virtually no increase in protein content in the original substrate.

Replacing ammonium sulphate with sodium sulphate, magnesium sulphate, L-methionine or L-cystine as the sulphur source significantly reduced the amount of residual ammonium from over 2.0 g/100 g to 0.8–1.2 g/100 g (Table 3-7). This substitution reduced the urea level to below 2.5 mg/100 g at the end of the fermentation. In the absence of a sulphur compound or with DMSO as the sulphur source, a considerable amount of unmetabolised urea remained (3.2 g/100 g).

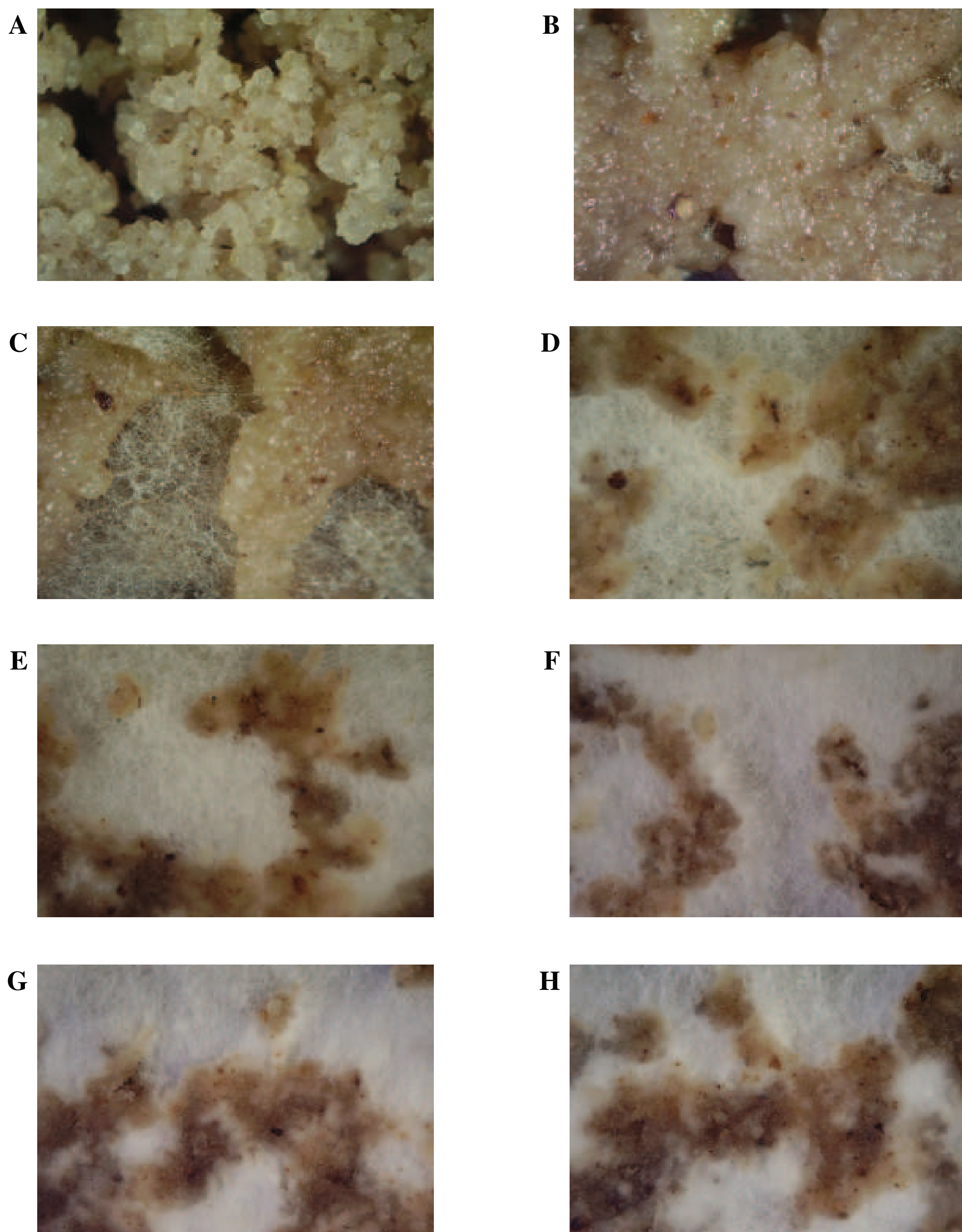


Figure 3-27: Cross-sectional views of cassava bagasse substrate (A) supplemented with different sulphur sources after fermentation with *R. oryzae* ZB for 120 hours: no sulphur source (B), DMSO (C), L-cystine (D), L-methionine (E), magnesium sulphate (F), sodium sulphate (G), and ammonium sulphate (H).

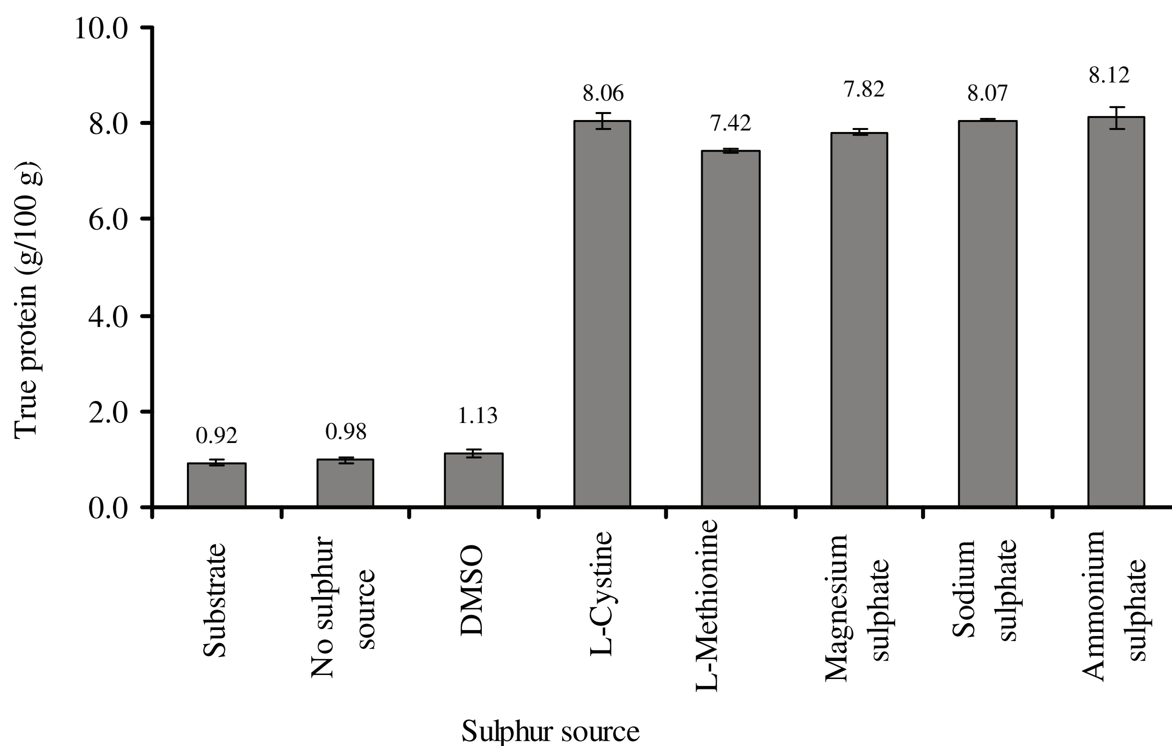


Figure 3-28: Effect of the sulphur source on the true protein content of substrate fermented with *R. oryzae* ZB for 120 hours.

Table 3-7: Residual urea and ammonium in the fermented substrate supplemented with various sulphur sources after fermentation with *R. oryzae* ZB for 120 hours.

Sulphur source	Residual urea[1] (mg/100 g)	Residual ammonium[1] (NH_4^+ mg/100 g)
No sulphur source	3253.3 ± 53.3[a]	729.0 ± 12.0[a]
DMSO	3182.8 ± 118.0[b]	966.7 ± 36.7[b]
L-Cystine	1.0 ± 0.1[c]	1201.8 ± 69.0[c]
L-Methionine	2.1 ± 0.5[c]	1571.7 ± 3.2[d]
Magnesium sulphate	0.4 ± 0.1[c]	883.2 ± 26.9[e]
Sodium sulphate	1.5 ± 0.4[c]	792.3 ± 14.0[f]
Ammonium sulphate	6.2 ± 1.4[c]	2286.5 ± 71.1[g]

[1]Values with the same alphabetical superscripts within the same column are not significantly different ($P<0.05$)

3.5 Water-soluble vitamins

The profiles of six water-soluble vitamins following fermentation with the selected *Rhizopus* strains are shown in Figure 3-29, Figure 3-30 and Figure 3-31. They are presented as two charts with two different units, one in total vitamin quantity per dry weight of fermented sample, and the other in net changes in vitamin content contributed by fungal growth per dry weight of initial substrate. The latter represents the quantity of the vitamins that was actually biosynthesised by the fungi alone, excluding the vitamins endogenously present in the initial cassava bagasse substrate. It also takes the loss of dry matter during fermentation into account. The plots of the vitamin histograms were grouped based on the respective concentration ranges of the vitamins.

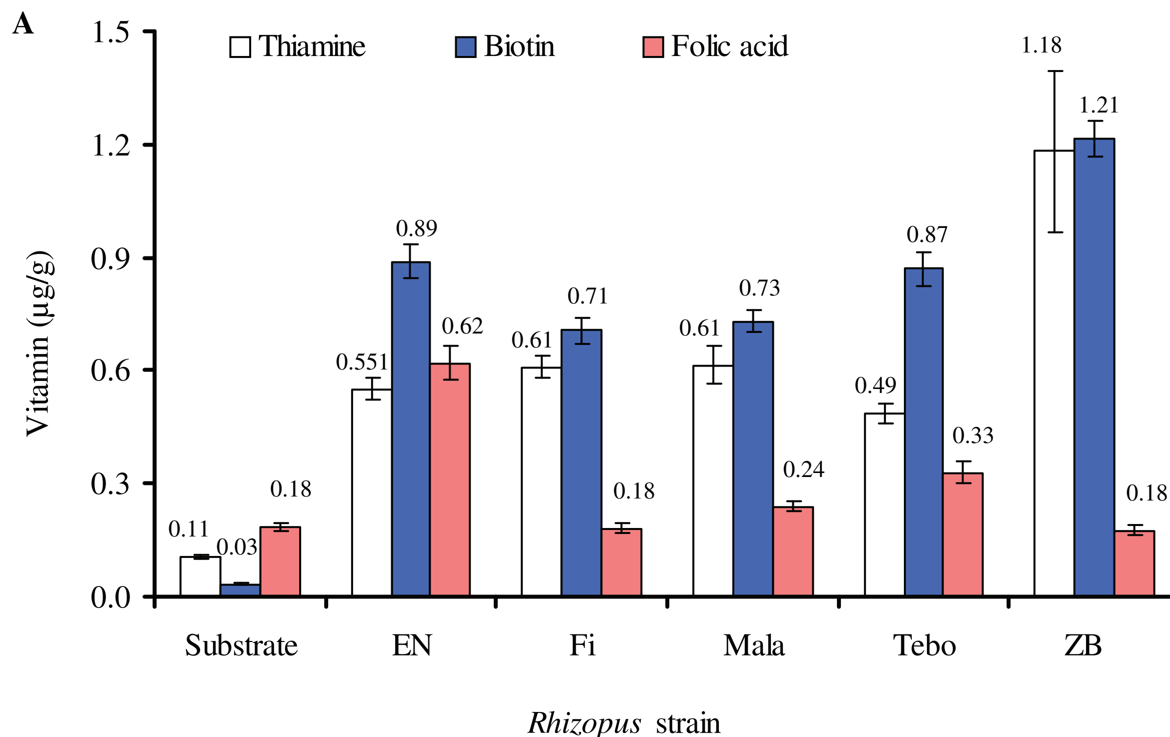
3.5.1 Thiamine, biotin and folic acid

All of the five *Rhizopus* strains increased the thiamine and biotin contents of the fermented cassava bagasse (Figure 3-29A). The highest levels of both thiamine (1.18 µg/g) and biotin (1.21 µg/g) were achieved by *R. oryzae* ZB, whereas the greatest concentration of folic acid (0.62 µg/g) was obtained when *R. oryzae* EN was used as the inoculum. Cultivating *R. oligosporus* Tebo resulted in the enrichment of thiamine and biotin, each being found at a concentration similar to that obtained by culturing *R. oryzae* EN. Likewise, comparable histogram profiles for the two vitamins were shared by *R. oryzae* Fi and Mala, both with significantly same levels of thiamine (0.6 µg/g) and biotin (0.7 µg/g).

The growth of *R. oryzae* Fi, ZB and Mala either increased the folic acid content in the fermented products very slightly or not at all. When the loss of dry matter was considered (Figure 3-29B) and the net change in folic acid content contributed by the fungal growth was calculated, however, negative values were obtained for *R. oryzae* Fi, ZB and Mala, suggesting a reduction in the initial substrate's folic acid.

The growth of *R. oligosporus* Tebo had no influence on the folic acid component in the initial substrate. *R. oryzae* EN was the only strain that was able to synthesise folic acid, producing 0.17 µg folic acid/g DW initial substrate.

A



B

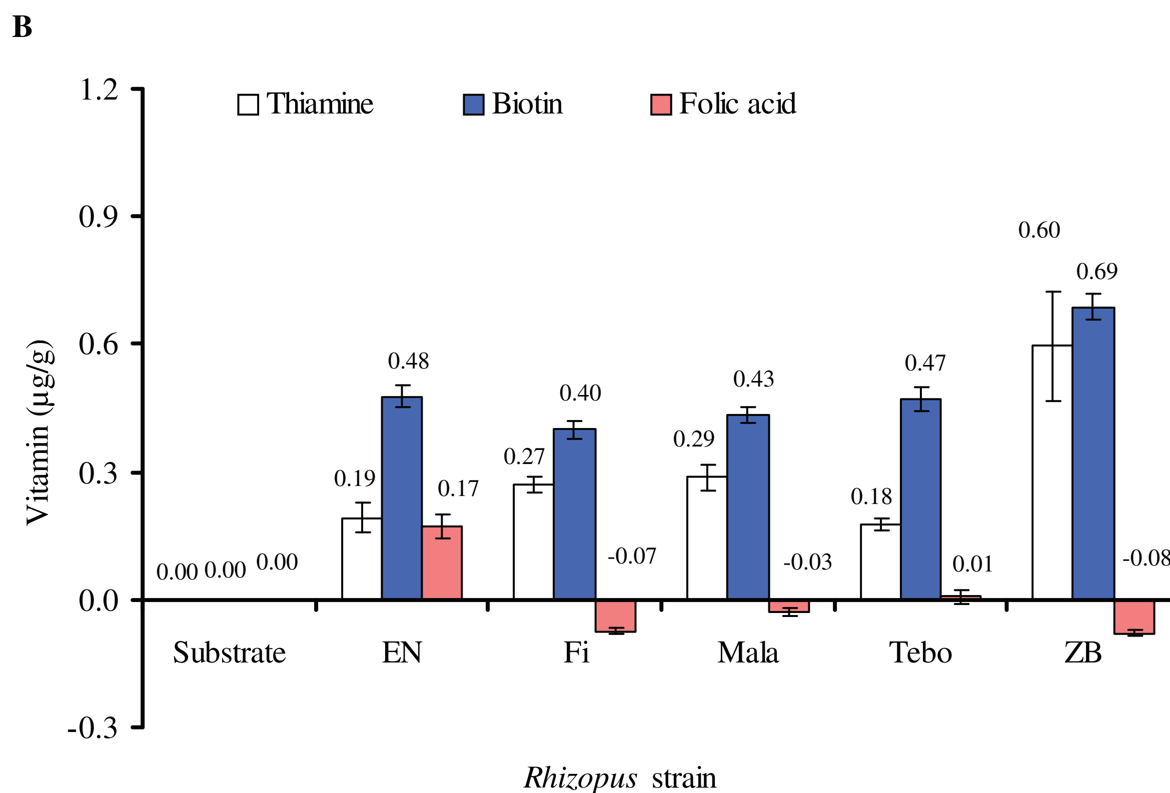
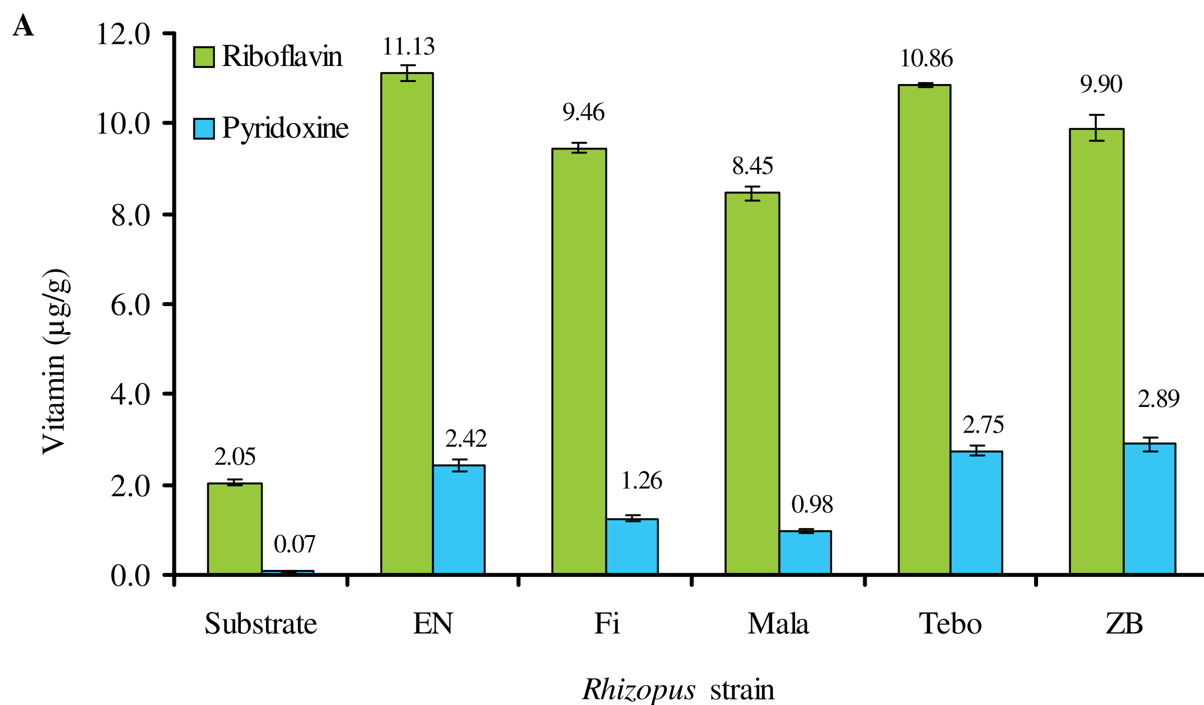


Figure 3-29: Concentrations of thiamine, biotin and folic acid in the cassava bagasse substrate after 120 hours fermentation expressed as µg vitamin/g DW sample (A), and as the net change in vitamin content due to fungal growth in µg/g DW initial substrate (B).



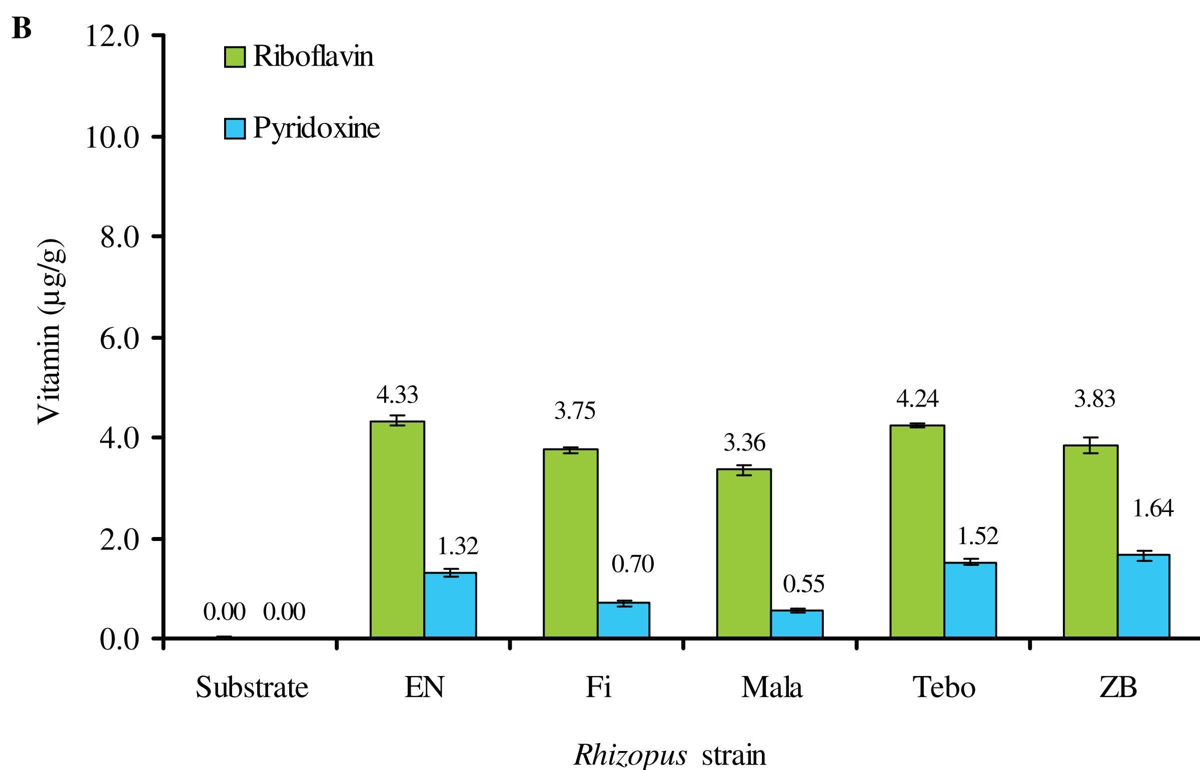


Figure 3-30: Concentrations of riboflavin and pyridoxine in the cassava bagasse substrate after 120 hours fermentation expressed as µg vitamin/g DW sample (A), and as the net change in vitamin content due to fungal growth in µg/g DW initial substrate (B).

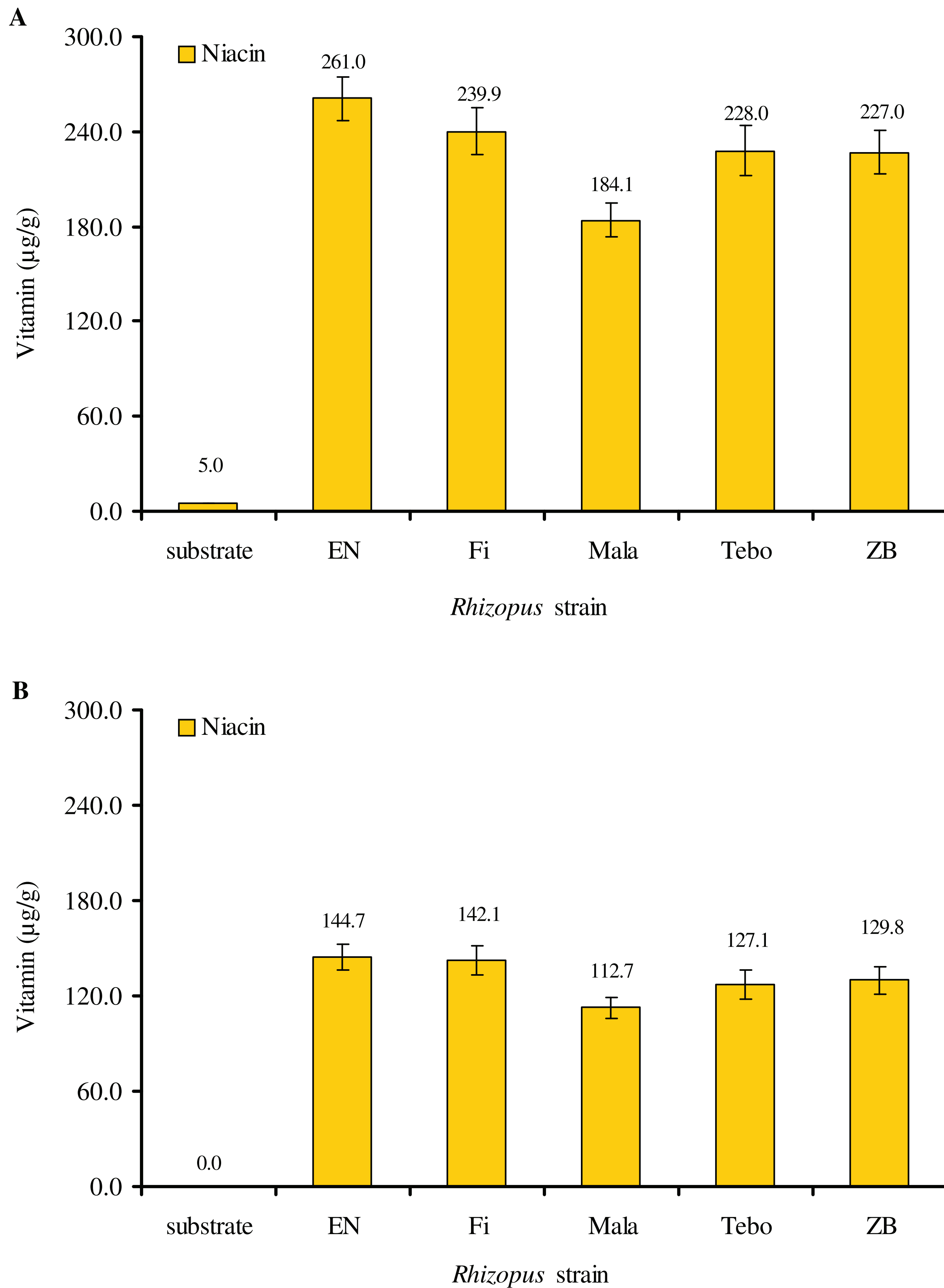


Figure 3-31: Concentrations of niacin in the cassava bagasse substrate after 120 hours fermentation expressed as µg vitamin/g DW sample (A), and as the net change in vitamin content due to fungal growth in µg/g DW initial substrate (B).

3.5.2 Riboflavin and pyridoxine

Across the strains, the enrichment of riboflavin in the fermented cassava bagasse did not exceed 12 µg/g, whereas pyridoxine added up to less than 4 µg/g (Figure 3-30A). Considering the net vitamin productions by the fungi, it was clear that *R. oryzae* EN and ZB were the best producers of riboflavin (4.33 µg/g) and pyridoxine (1.64 µg/g), respectively. In contrast, *R. oryzae* Mala produced the smallest amount of both vitamins.

3.5.3 Niacin

Of all of the water-soluble vitamins measured, niacin was the most abundantly synthesised vitamin in the fermented substrates (Figure 3-31A). *R. oryzae* EN caused the greatest increase in niacin content (261 µg/g), while *R. oryzae* Mala showed the lowest (184 µg/g). Fermentation with *R. oryzae* Tebo did not bring about an amount of niacin significantly different from that obtained by using *R. oryzae* ZB. Although *R. oryzae* EN led to the highest level of niacin in the final fermented product, the real niacin increase made by the strain was not significantly different ($P>0.05$) from that of *R. oryzae* Fi (Figure 3-31B, Appendix Table 7-27). Similarly, niacin was biosynthesised by both *R. oryzae* ZB and *R. oligosporus* Tebo in significantly similar quantities of 127 and 130 µg/g DW initial substrate, respectively. *R. oryzae* Mala, as in the case of riboflavin and pyridoxine, produced the least amount of niacin during fermentation.

4 Discussion

4.1 Cassava bagasse

4.1.1 Chemical composition of cassava bagasse

Cassava bagasse from Lampung, Sumatra, Indonesia, was found to consist mainly of fibres and carbohydrates. It is nutritionally inferior to soybeans, which are the natural substrate on which the *Rhizopus* strains used in this study had been domesticated. However, the proximate composition of the cassava solid waste was comparable to previously reported values (Table 4-1). The variation between the individual components, especially starch and fibres, was suggested to be the result of varying cassava processing technologies used to extract the starch (Soccol and Pandey 2004; John 2009). In an investigation by Guillaume et al. (2008) in three different cassava processing communes in the Hoai Duc district, the Ha Tay province of Vietnam, revealed that three different processing types produced cassava bagasse with varying contents of starch, fibre and ash. In another research project conducted in Thailand (Chavalparit and Ongwandee 2009), the eight tapioca starch plants examined were all found to suffer starch loss in fibrous residues and effluent in varying magnitudes, despite the fact that the processing of the tapioca starch was similar between the plants. The authors suggested, however, that the manufacturers might employ different techniques and machines in each stage of production.

The rest of the components, namely proteins, lipids, and ash were present in much lower levels. This was not surprising, as their original amounts in the cassava tuber were also low, each accounting for quantitatively less than 3.5% (Charles et al. 2005; Rojas et al. 2007; Rodríguez-Sandoval et al. 2008; Dongmeza et al. 2009).

Contrary to previous reports stating that cyanide was absent in cassava bagasse (Soccol and Pandey 2004; John 2009), 0.08 mg/kg cyanide was still detected in the present study in the tapioca residue from Lampung, Indonesia. This was lower than the cyanide level of 9 mg/kg reported by Djazuli and Bradbury (1999) for cassava bagasse from Indonesia, and 17.3-26.7 mg/kg from Nigeria (Tewe and Iyayi 1989; cited in Tewe 1992). In any case, the cassava bagasse from Indonesia used in this study contained cyanide concentrations below the maximum safety level of 10 mg/kg as recommended by the FAO/WHO for edible cassava flour (JECFA 2009).

Table 4-1: Physicochemical composition of cassava bagasse from cassava processing industries in Brazil (Soccol and Pandey 2004), Vietnam (Guillaume et al. 2008), Thailand (Sriroth et al. 2000) and Indonesia (present study). The composition of soybeans (Padgette et al. 1996) is presented for comparative purposes.

Component	Origin of cassava bagasse (region, country):				Soybean
	Paraná, Brazil	Ha Tay, Vietnam	Chonburi, Thailand	Lampung, Indonesia	
Moisture (g/g WW)	5.02–11.20	NA	NA	9.91±0.21	7.55–8.73
Protein (g/100 g DW)	0.32–1.61	NA	1.55±0.03	1.21±0.15	37.5–44.6
Lipids (g/100 g DW)	0.53–1.06	NA	0.12±0.01	0.18±0.09	14.10–18.63
Fibres (g/100 g DW)	21.10–50.55	14.9–21.4	27.75±0.20	11.24±1.28	5.91–7.89
Carbohydrate (g/100 g DW)	40.50–63.85 (mainly starch)	41.2–58.7 (starch)	68.89±4.00 (starch)	70.17±0.07 (starch)	33.9–41.3
Ash (g/100 g DW)	0.66–1.50	1.3–2.2	1.70±0.01	1.24±0.07	4.29–5.34

4.1.2 Pregelatinised cassava bagasse

In this study, preliminary fermentation was carried out using a gelatinised substrate (data not presented) prepared by mixing cassava bagasse with salt solution and followed by heat sterilisation. The resulting moist substrate was sticky and thus posed problems during its mixing with the spore inoculum. The stickiness also caused substrate granules to clump together, forming a large mass, and decreasing the surface area available for fungal growth. This situation was previously described by Mitchell et al. (1988b), who attributed the poorer growth of *R. oligosporus* on mashed cassava as compared to chipped cassava to the stickiness and clumping of the former substrate.

In order to avoid this problem while at the same time retaining the gelatinised quality, the cassava bagasse was subjected to pregelatinisation. The resulting pregelatinised substrate was less sticky but retained a discrete granular structure when mixed with the prewarmed salt solution. The pregelatinisation also changed the originally light brownish white colour of the raw substrate to brown. This contrasted nicely with the white cottony colour of the growing *Rhizopus* mycelium, allowing for easier macroscopic observation of the growing fungal mycelium.

4.2 Selection of *Rhizopus* strains

Mycelial growth on GCBA was sparser than on RCBA for all the strains tested, confirming previous finding (Mitchell et al. 1988a). This sparse mycelial network was said to be due to minimal hyphal branching resulting from its growth on poor-nutrient medium (Mitchell et al. 1988a; Boswell et al. 2007). However, the two media in the present study were prepared to have essentially the same composition, differing merely in the amount and treatment of the cassava bagasse added. It would perhaps be more appropriate to suggest that nutrients were less accessible to the fungi cultured on RCBA than on GCBA. The reason could lie in the method used in preparing the first medium. When mixed with agar solution, the resulting raw (ungelatinised) cassava bagasse suspension was unstable. The solid bagasse precipitated while the agar was in the process of setting, possibly creating a vertical nutrient gradient, with the surface of the solidified agar containing fewer nutrients. However, this was deemed unlikely, as large amounts of raw cassava bagasse, five times more than were added in GCBA, were added in anticipation of this issue.

Another possible explanation could be related to the physico-chemical properties of the raw starch in the ungelatinised cassava bagasse used to prepare the RCBA. Raw cassava bagasse might be hydrolysed less readily by fungal amylolytic enzymes than the gelatinised one, releasing free sugars for uptake by the fungi at a slower rate. Raw ungelatinised starch is also called native granular starch and is known to be hydrolysed very slowly by amylolytic enzymes. This is because in its native semi-crystalline granular form, starch molecules are packed inside the granules, restricting access to attacking enzymes (Tester et al. 2004). Gelatinisation causes the semi-crystalline structure of the starch granules to become amorphous, thus making the starch more easily digestible for amylases (Tester and Sommerville 2001; Tester et al. 2006; Noda et al. 2008)

Only five out of twenty-eight *Rhizopus* strains screened on the cassava bagasse selection media were able to grow well. Four of these strains belonged to *R. oryzae*. Similarly, in earlier studies using raw cassava flour and cassava bagasse as selecting media, Soccol et al. (1994a; 1995a) screened 19 *Rhizopus* strains and found that three of these grew well on the substrate, two of these being of the *R. oryzae* species. It is not known whether a specific correlation exists between growth performance on starch rich substrates and the *Rhizopus* species used. However, previous reports established that amylase is produced both by *R. oryzae* as well as *R. oligosporus*, with the latter producing amylase only after an extended duration of fermentation (Hesseltine 1965; cited in Sukara and Doelle 1989). In addition, Hesseltine (1985) noticed that *R. oligosporus* produced little amylase late in its growth, which distinguishes it from *R. oryzae* known to form a considerable amount of amylase. On the contrary, in protein-rich soybean fermentation using 36 *Rhizopus* strains, Baumann and Bisping (1995) obtained seven proteolytically most active strains, six of which were *R. oligosporus* and one *R. oryzae*.

Soccol et al. (1995a) suggested that the good growth performance of the *Rhizopus* strains on cassava bagasse was due to the ability to hydrolyse starch and synthesise protein. *Rhizopus* spp. are known to be able to produce the starch hydrolysing enzyme glucoamylase in large quantities in SSF (Ashkari et al. 1986). The enzyme has been produced commercially, amongst other reasons, for its ability to obtain near 100 percent yields of glucose from starch (Mertens and Skory 2007).

4.3 Optimisation of the fermentation conditions

The optimised fermentation conditions obtained in this study were comparable to previous published results (Table 4-2).

4.3.1 Influence of inoculum density

Greater inoculum density evidently led to thinner mycelium formation. However, it had little influence on the soluble protein content of the fermented samples. Substrates inoculated with the highest inoculum density (10^6 spores/g substrate) were seen to show hardly any mycelium formation, but the soluble protein levels differed insignificantly or were slightly lower than those with lower spore densities were. Compared with previous studies, the optimum spore concentration seems to be species specific (Table 4-2). In a similar study using raw cassava bagasse, Soccol et al. (1995c) found 10^5 spores/g substrate to provide the best conditions for

the biomass formation of *R. oryzae* 8627, whereas increasing the inoculation density to 10^6, 10^7 and 10^8 spores/g substrate had a progressively negative effect on fungal growth. The same authors also experimented on raw cassava flour cultured with *R. delemar* ATCC 34612 and found that an inoculation density of 2 x 10^7 spores/g DW substrate supported the best growth, while lower or higher inoculation densities led to poorer fungal growth (Soccol et al. 1994a).

These results indicate a maximum limit for spore density, beyond which fungal growth is inhibited, possibly due to overheating and/or self-inhibition. It has been reported that uncontrollable fermentation associated with the excessive rise in temperature and premature death of *Rhizopus* mould might occur when soybeans are inoculated with an overdose ($\geq 10^6$ Colony-Forming Unit/g) of inoculum (Nout and Kiers 2005). The self-inhibition phenomenon has been demonstrated in *Rhizopus oligosporus* by Breeuwer et al. (1997). The authors found that nonanoic acid, a self-inhibitor produced by different fungi, inhibited the germination of *R. oligosporus* sporangiospores. It is not yet known, however, which self-inhibitory compounds are endogenously produced by the genus *Rhizopus*. Other fatty acids such as octanoic acid, decanoic acid, and acetic acid were also found to have an inhibitory effect. Poor germination at high spore concentrations, also termed the crowding effect, has previously been observed for other filamentous fungi and is considered to be caused by inhibitory biochemicals produced by the organism itself (Hobot and Gull 1980; Lax et al. 1985; Barrios-González et al. 1989; Inoue et al. 1996; Chitarra et al. 2004; Chitarra et al. 2005).

4.3.2 Importance of mineral supplementation

R. oryzae ZB demonstrated the best growth with simultaneous supplementation with ammonium sulphate and potassium dihydrogen phosphate, and without the further addition of other elements, confirming the results of previous studies (Table 4-2). The present results also suggest that these two compounds are necessary for the growth of the fungi on cassava bagasse substrate. The external supplementation of ammonium sulphate was vital considering the very low amount of crude protein, and hence total nitrogenous compounds, in cassava bagasse. The genus *Rhizopus* is known to be able to assimilate nitrogen from organic as well as inorganic compounds, including ammonium salts (Sorenson and Hesseltine 1966; Graham et al. 1976; Seaby et al. 1988; Graffham et al. 1995). *R. oryzae* ZB could still grow, albeit poorly, on

Table 4-2: Fermentation conditions reported in some SSF studies involving cassava product as the substrate and *Rhizopus* spp. as the inoculum.

Reference	Substrate and *Rhizopus* strain	Fermentation condition[1]	Supplemented compound[2] , and Bioreactor used[3]
Present study	Pregelatinised cassava bagasse, *R. oryzae* ZB	IT: 27-33 ID: 10^2-10^4 IM: 68 IP: 5.3-6.6 FP: 120	Potassium dihydrogen phosphate (1.7), ammonium sulphate (3.6), urea (3.4). 9-cm-diameter Petri plate (10).
Soccol et al. (1995c)	Raw cassava bagasse, *R. oryzae* 28627	IT: 30 ID: 10^5 IM: 71-77 IP: 4.3-4.9 FP: 30	Potassium dihydrogen phosphate (1.7), ammonium sulphate (3.4), urea (0.83). Petri plate (40), little tray (1020), big tray (4000), little column (140), big column (4000).
Soccol et al. (1994a)	Raw cassava flour, *R. delemar* ATCC 34612	IT: 35 ID: $2x10^7$ IM: 50-52 IP: 5.0 FP: 48	Potassium dihydrogen phosphate (4.75), ammonium sulphate (9.30), urea (2.3). Perforated polypropylene container with 15.5 cm diameter and 3 cm depth (20).
Daubresse et al. (1987)	Gelatinised cassava, *R. oryzae* MUCL 26486	IT: 27 ID: 10^7 IM: 60 IP: 3.5 FP: 65	Urea (3.4), potassium dihydrogen phosphate (1.5), magnesium sulphate heptahydrate (0.8). 12.2 kg cassava spread out uniformly as a 2 cm thick layer on a tray.

[1] IT: incubation temperature (°C), ID: inoculum density (spores/g DW substrate), IM: initial moisture (%), IP: substrate initial pH, and FP: fermentation period (hours).

[2] Numbers in parentheses indicate quantity in % or g/100 g DW substrate.

[3] Numbers in parentheses indicate quantity in g DW substrate.

cassava bagasse with only supplementary ammonium sulphate and without phosphate, indicating the existence of an insufficient amount of metabolisable phosphorous compounds in the bagasse.

One of the phosphorous compounds present in the cassava bagasse could be the organic phosphate phytic acid, known to be endogenously present in cassava (Nzigamasabo and Hui Ming 2006; Favaro et al. 2008; Montagnac et al. 2009b). Phytic acid liberates metabolisable inorganic phosphates upon hydrolysis by the enzyme phytase known to be produced by *Rhizopus* spp. (Sutardi and Buckle 1988; Sabu et al. 2002; Casey and Walsh 2004). Phytase from *Rhizopus* sp. seems to be secreted only when the fungi were grown in complex substrate such as agroindustrial wastes without further addition of simple carbon, nitrogen, and phosphor sources. Using coconut oil cake Sabu et al. (2002) found that the phytase yield from *R. oligosporus* was no better when the substrate was supplemented with various carbon sources such as maltose, glucose, mannitol, sorbitol, lactose, and sucrose. Many of these simple carbohydrates were even demonstrated by the authors to inhibit the production of the enzyme. Moreover, additional inorganic and organic nitrogen sources like potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate, beef extract, malt extract, yeast extract, and peptone were shown to decrease phytase production. This could provide another explanation for the earlier finding by Graffham et al. (1995) that none of their *R. oligosporus*, *R. arrhizus*, *Amylomyces rouxii*, *R. oryzae* and *R. stolonifer* strains grew with phytic acid as the only phosphate source in synthetic defined liquid media with ammonium salt and glucose as nitrogen and carbon sources.

The supplementation of inorganic phosphate salts has been shown to be indispensable for the cultivation of *Rhizopus* spp. (Graffham et al. 1995; Zhou et al. 1999; Riscaldati et al. 2000). A number of authors have indicated the crucial role of phosphate compounds during the germination of *Rhizopus* spp. Ekundayo and Carlile (1964) found that PO_4^{3-} and K^+ (or Na^+) were required for maximal spore swelling during the germination process of *R. arrhizus*, whereas Medwid and Grant (1984) found that phosphate, together with glucose, optimised the proline-stimulated germination of *R. oligosporus* sporangiospores. Similar findings were made by Thanh et al. (2005), who observed hardly any stimulating effect of glucose, either alone or together with single amino acids, on *R. oligosporus* germination and colony outgrowth when phosphate was absent. The combined addition of proline and phosphate was found to be effective in inducing the germination of spores of *R. arrhizus* Fisher (Weber and Ogawa 1965; cited in Thanh et al. 2005) and *R. stolonifer* Lind (Weber 1962; cited in Thanh et al. 2005).

Beyond the germination phase, the availability of potassium dihydrogen phosphate in sufficient amount was also shown to be important. Daubresse et al. (1987) demonstrated a decrease in the protein content of *R. oryzae*-fermented cassava as a result of reduced fungal

growth when the amount of supplemented phosphate salt was lowered by more than 50%. The potassium ions from the added potassium dihydrogen phosphate might also play crucial role in the growth of *Rhizopus* spp. on cassava bagasse, as previous studies have revealed a linear relationship between the biomass formation of *R. oligosporus* and K^+ concentration in liquid medium (Peñaloza et al. 1991).

Other essential elements required by the fungus in much smaller quantities were likely already present in the cassava bagasse, as indicated by approximately 1% of the ash content (Table 4-1). Cassava bagasse might also obtain such elements as external contaminants during cassava processing. It might also retain them, despite the vigorous tapioca extraction process, from the original cassava tuber, which contains calcium, iron, potassium, magnesium, copper, zinc, and manganese in comparable amounts to those of many legumes (Montagnac et al. 2009a).

4.3.3 Role of urea and ammonium sulphate

The use of both urea and ammonium sulphate allowed for the prolongation of the fermentation period for up to 120 hours. At this time, a cassava bagasse tempe cake was obtained with morphological and physical characteristics resembling those of soybean tempe fermented for 32 hours and considered mature by Ruiz-Teran and Owens (1996): soybean cotyledons bound in a solid cake by fungus mycelium.

Previous studies on the SSF of cassava using the filamentous fungi *Aspergillus niger* (Raimbault and Alazard 1980) and *Rhizopus oligosporus* (Daubresse et al. 1987) demonstrated the important roles of urea as a nitrogen source as well as in counteracting acidification due to ammonium (NH_4^+) uptake during fungal growth. Ammonium can originate from ammonium salts and from the hydrolysis products of urea, both of which were added to the substrate. As an ammonium molecule is taken up intracellularly by the fungus, a proton (H^+) is released into the extracellular space (Raimbault and Alazard 1980; Nagel et al. 1999), causing a steep drop in pH. This represents a proton extrusion phenomenon which was also observed with *Penicillium* spp. (Roos and Luckner 1984; Franz et al. 1993) and *Aspergillus* sp. (Papagianni et al. 2005) as ammonium was taken up by the fungi. The rapid acidification was neutralised through alkalinisation by the ammonia produced by the further hydrolysis of urea. This buffering role was shown in the present study to work well only when the urea was supplemented in appropriate amounts and together with ammonium sulphate, as also demon-

strated in earlier study (Raimbault and Alazard 1980). Otherwise, as the present results indicate, a urea dose that is too low leads to a weak buffering capacity against the acidification, causing a considerable drop in pH to values that inhibit further growth (Mitchell et al. 1988a). On the contrary, urea supplementation exceeding the optimum value released more ammonia and produced a lower fungal protein concentration. This might be due to the accumulation of liberated ammonia reaching a level inhibitory or toxic to fungal growth. Using chemically-defined media, Sparringa and Owens (1999c) demonstrated that certain concentrations of ammonia reduced and even stopped the hyphal extension rate of *Rhizopus oligosporus* NRRL 2710. This strain causes a liberation of ammonia, a major cause of alkalinisation in soybean fermentation (Sparringa and Owens 1999a), and it was suggested as a result of the utilisation of the soybean protein as a carbon and energy source (Ruiz-Teran and Owens 1996).

Could urea play other growth promoting roles beyond providing nitrogen source and preventing acidification? So far, no such studies involving *Rhizopus* sp. have been conducted. It is interesting to note, however, that in the present work, the hydrolysis of urea to release ammonia occurred in an early phase of growth when the supplemented ammonium sulphate was still available in adequate amounts. This raised the question of why the fungus liberated ammonia from urea to meet its nitrogen requirement if there was in fact still enough nitrogen from the ammonium sulphate. A similar question has been posed in previous published works as to why, when growing on soybean, the moulds concomitantly utilise amino acids as additional source of energy, despite their metabolism of lipids as the major source of energy (Ruiz-Teran and Owens 1996; Sparringa and Owens 1999a; Sparringa and Owens 1999d). Alternative plausible explanations might be that both urea and certain amino acids play essential roles during the germination of *Rhizopus* spp. Amino acids have been demonstrated to have different influences on the germinating spores of *R. oligosporus,* from stimulating or slightly inducing, to neutral or even counteracting. Alanine, in particular, is taken up by the germinating spores of *R. oligosporus* and is shown to stimulate the germination of dormant spores, meeting the requirements of both carbon and nitrogen for spore germination (Thanh et al. 2005). Likewise, it could be suggested that some of the urea might have escaped hydrolysis and been transported intact intracellularly by the germinating *Rhizopus* spores to perform similar functions. This hypothetical possibility is put forward while bearing in mind that both urea and amino acids share some common physico-chemical properties such as being organic compounds made up of carbon, hydrogen, oxygen and nitrogen; having small molecular masses (89 and 60 g/mol, respectively); and possessing amine (-NH_2) as well as carbonyl groups (-

C=O). Indeed, the intracellular uptake of urea and its subsequent utilisation as a nitrogen and carbon source for spore germination has already been demonstrated for the fungus *Geotrichum candidum* (Shorer et al. 1972).

The present study and others (Daubresse et al. 1987; Soccol et al. 1994a) have shown an increase in pH in the final period of cassava and cassava bagasse fermentation using *Rhizopus* spp. In soybean tempe fermentation, the liberation of ammonia due to oxydation of amino acids also contributed to a progressive increase in pH (Davey et al. 1991; Ruiz-Teran and Owens 1996; Sparringa and Owens 1999a; Handoyo and Morita 2006). In this study, in which an optimised ratio of urea and ammonium sulphate was employed, the increase in the ammonium level as a result of urea hydrolysis was accompanied by the dramatic increase in pH from 5.6 to 7.4. This occurred within the first 24 hours of the cassava bagasse fermentation. Similarly, during soybean fermentation, an increase in ammonia has been detected as early as 12 hours after the start of incubation. A rapid rise in pH from the initial value of 3.6 to 6.9 was also observed at 26 hours (Ruiz-Teran and Owens 1996). Afterwards, the pH courses of the cassava bagasse and soybean fermentations progressed differently. The former underwent a rapid pH decrease to 5.0 and 4.5, at 48 and 72 hours, respectively, followed by an increase to 5.6 at 120 hours. In contrast, the latter showed a continuous increase in pH, albeit slowly, reaching 7.9 at 180 hours.

As stated previously, the fall in pH in cassava bagasse fermentation was suggested to be due to the proton extrusion accompanying ammonium uptake. Additional acidification might also be contributed by organic acids such as lactic acid, malic acid, acetic acid, propionic acid and fumaric acid, known to be produced by the genus *Rhizopus* (Soccol et al. 1994a; Oda et al. 2003). On the other hand, acids were also released in considerable amounts during the fermentation of soybeans. However, these are long-chain, high molecular weight fatty acids, which are relatively water insoluble and therefore have little influence on the aqueous pH values of the soybean tempe (Ruiz-Teran and Owens 1996).

When ammonium sulphate was used as a nitrogen source, adding urea was considered by Nagel et al. (1999) ineffective in manipulating pH values. This is because urea generated a large fluctuation in pH of more than one unit throughout fermentation, as also evidenced by this study. To get around this problem, alternative buffering agents were tried by Nagel et al. (1999). They found citric acid to be the most suitable buffer for growing *R. oligosporus* in a

model system of synthetic agar medium in the presence of ammonium sulphate. Unfortunately, no published research was found that reported whether the proposed buffer worked equally well outside the modelled environments.

Lacking ammonium sulphate, substrate supplemented with urea alone as a nitrogen source supported only the very poor growth of *R. oryzae* ZB. This suggested the growth-limiting role of the sulphur rather than of the nitrogen element of the added ammonium sulphate (discussed further in Section 4.4.2). The absence of ammonium sulphate, however, did not stop fungal amylolytic activity. Up to 25% of the free reducing sugars were released even when the pH had dropped to as low as 3.7-4.0 and in the presence of urea as a known protein denaturant. The amount of added urea, calculated to be 3.1 g in 100 g DW initial substrate (or approximately 0.017 mol/100 g DW initial substrate), might be too low to exert any negative effect on the activity of the enzyme. However, it might even have the opposite effect of enhancing the activity. It has been demonstrated that when present in certain concentrations and/or in appropriate settings, urea has a positive influence on enzyme activities (Lin et al. 1971; Barash et al. 1972; Deshpande et al. 2001; Kumar et al. 2003; Mukherjee and Banerjee 2006; Lei et al. 2007; Negi and Banerjee 2009). A work was even published reporting that glucose isomerase, entrapped in functionalised mesoporous silica, remained active in a denaturing solution containing 8.0 M urea. Moreover, its measured specific activity was above the highest specific activity of the enzyme in solution without entrapment (Lei et al. 2007). A solid substrate environment could play a role in microorganisms producing enzymes of better quality than those in liquid culture. In some studies, enzymes released by *Rhizopus* spp. (Mateos Diaz et al. 2006; Sun and Xu 2009) and by other filamentous fungi (reviewed by Hölker et al. 2004) in solid substrate cultures were found to be superior to those obtained through liquid or submerged fermentations. The enzymes produced by SSF were found to exhibit higher activity, low or no catabolite repression, no substrate inhibition, and greater pH and heat stability than those produced by submerged fermentations.

4.4 Influences of some other factors

4.4.1 Substrate pretreatment

Cultivating *R. oryzae* ZB on either raw or pregelatinised cassava bagasse resulted in significantly similar protein contents. This indicated that the fungus grew equally well on both substrates, regardless of the difference in pretreatment. This result was rather unexpected, as dur-

ing the selection step using cassava agar media (Section 4.2), the *Rhizopus* strains had been predicted to grow better on the pregelatinised cassava bagasse than on the raw cassava bagasse. This finding also failed to correspond with the results published by Soccol et al. (1994a), who showed that protein content was higher when using cooked, and hence gelatinised, cassava root than raw cassava root after fermentation with *R. oryzae* MUCL 28168, *R. delemar* ATCC 34612 or *R. oryzae* MUCL 28627. The difference in fermentation substrate, *Rhizopus* strain, and other fermentation conditions might account for this contradiction.

The larger particle size in the raw substrate did not prevent the starchy material inside the substrate lumps from being attacked by fungal amylolytic enzymes. This assault probably being facilitated by the penetrative *Rhizopus* hyphae. Similar penetration had already been substantiated microscopically during *Rhizopus* spp. growth on other substrates such as κ-carrageenan gel (Nopharatana et al. 2003a), potato dextrose agar (Nopharatana et al. 2003b), barley (Noots et al. 2003), quinoa (Penaloza et al. 1992), soybean (Jurus and Sundberg 1976; Varzakas 1998), and defatted soybean flour (Varzakas 1998). Microscopically, Jurus and Sundberg (1976) observed that the hyphae of *R. oligosporus* could reach deep down to a depth of 742 µm below the surface of a soybean cotyledon. This was equivalent to around 25% of the average width of the cotyledon. In other studies (Varzakas 1998) involving soybean cotyledons fermented for 40 hours, *R. oligosporus* were seen to infiltrate the cotyledons even deeper, circa 2 mm. An even deeper penetration (~5-7 mm) was further achieved during the same incubation period when defatted tempe flour was used instead. The deeper penetration in this example might be attributed to the cells in the flour not being associated with each other (Varzakas 1998), which could be also the case with the raw cassava bagasse. Thus, in the context of the present study the particle size of the cassava bagasse substrate was shown not important, as noted earlier by Perez-Guerra et al. (2003) in their review.

Rhizopus spp. were shown to produce glucoamylase, which can bind to raw starch and is highly active in hydrolysing it (Abe et al. 1985). This could perhaps provide an additional explanation for why pregelatinising the substrate prior to fermentation provided no advantage in terms of achieving a higher protein content. The fungal ability to utilise raw cassava bagasse, as also demonstrated by previous studies (Soccol et al. 1995c), would considerably simplify the substrate pretreatment process as well as reduce energy input.

4.4.2 Sulphur source

The present results show that ammonium sulphate could be replaced by other sulphur-containing compounds like L-cystine and sodium sulphate without any negative effect on the protein content of the *Rhizopus*-fermented samples. L-methionine and magnesium sulphate also supported the growth of the fungus. This indicated that it was the sulphur element of ammonium sulphate that was vital for *Rhizopus* growth. The sulphur element is so important to the fungi that a *R. oryzae* strain isolated from rotten cassava tubers was even shown to oxidise elemental sulphur (S^0) to $S_2O_3^{2-}$, $S_4O_6^{2-}$ and SO_4^{2-}, as a result of fungal rhodanase activity (Ray et al. 1991). This also explained why very poor growth occurred when neither ammonium sulphate nor any other appropriate sulphur source was provided to *R. oryzae* ZB.

Sulphur is also important for *Rhizopus* spp. in their natural soybean substrate. It has been suggested that the sulphur-containing amino acids methionine and cysteine (or its dimeric form, cystine), known to be limiting (Zarkadas et al. 1999; Zarkadas et al. 2007) or deficient (Kwanyuen and Burton 2010) in soybeans, are metabolised by *Rhizopus* spp. to meet their sulphur needs in the course of soybean tempe fermentation (Sorenson and Hesseltine 1966). The crucial requirement of sulphur might also be the reason why the content of thiamine, a sulphur-containing B-vitamin, was lower in fermented soybean than in the initial soybeans. Some of the soy thiamine might be metabolised to satisfy the *Rhizopus'* sulphur needs. Thiamine was already demonstrated to be the sole sulphur source for growth in microorganisms such as *Rhodococcus rhodochrous* (Kayser et al. 1993).

As with soybeans, cassava roots are also deficient in methionine and cysteine (or its dimeric form, cystine), two sulphur-containing amino acids (Montagnac et al. 2009a). Despite this fact, the important requirement of a sulphur source has not been dealt with by previous authors studying *Rhizopus* spp. cultivation on cassava derived substrates, even when they supplemented the substrates with ammonium sulphate (Mitchell et al. 1988b; Soccol et al. 1994a; Soccol et al. 1995b; Soccol et al. 1995c). Perhaps the authors attached a much more important role to ammonium sulphate as a nitrogen source than as sulphur source. Alternatively, the latter role might not have been considered at all. These authors referred to the previous work of Raimbault and Alazard (1980), who recognised an advantage in optimally combining urea and ammonium sulphate as nitrogen sources as urea hydrolysis releases ammonia which counteracts extreme acidification during fermentation.

At least one study has been conducted on the fermentation of cassava with *Rhizopus oryzae* in which urea was used as the sole nitrogen source, and not in combination with ammonium sulphate (Daubresse et al. 1987). The authors also supplemented the substrate with potassium dihydrogen phosphate, magnesium sulphate and an acid. They carried out tests to determine the effects of reducing potassium dihydrogen phosphate, omitting magnesium sulphate, as well as replacing sulphuric acid with citric acid. Without describing the experimental designs and the combinations of the supplementary minerals and acids used, the authors stated to have obtained conflicting results. That is, the authors found that the protein content of the fermented products was not affected in some cases, while in others, removing magnesium sulphate led to the cessation of mycelial growth, accompanied by heavy sporulation after 24 hours. The latter description was interestingly in line with the findings of the present study when ammonium sulphate or any other sulphur source was absent. Unfortunately, the authors blamed the inconsistency on the endogenous magnesium content of the cassava substrate being variable depending on the cassava plant variety and the soil where the cassava was cultivated. They failed to mention any possible growth-limiting role of the sulphur element from the added magnesium sulphate or the acidifier sulphuric acid. Thus, based on the present findings, the aforementioned contradictory results might be resolved by suggesting that the absence of magnesium sulphate led to poor growth only when the substrate was acidified using citric acid and not sulphuric acid. Otherwise, the fungus could still meet its sulphur requirement from the added sulphuric acid and grew well, despite being deprived of magnesium sulphate. In short, it is likely sulphur, and not magnesium, which is the limiting factor for the growth of *Rhizopus* spp. on cassava products.

The use of sulphur sources other than ammonium sulphate helped decrease the residual ammonium by circa 65% from 2.3 to the lowest value of 0.8 g/100 g (with sodium sulphate as the sulphur source). This unassimilated ammonium must have come from the hydrolysed urea, which was reduced to extremely low concentration (4-21 ppm) at the end of the fermentation. A similar urea depletion had also been previously reported after 65 hours fermentation with *Rhizopus oryzae* MUCL 28627 (Daubresse et al. 1987). This might be due to urease activity, which was shown to be exhibited by *Rhizopus* spp. (Farley and Santosa 2002; Geweely 2006).

4.4.3 Different *Rhizopus* strains

In the present study, the five selected strains all grew very well on the cassava bagasse substrate under the optimised fermentation conditions. The fermented products showed the same physical characteristics as those described for mature soybean tempe (Ruiz-Teran and Owens 1996). The fermented products contained different protein contents depending on the strains used. When these strains are listed from highest to lowest in terms of the protein contents of their fermented products (Figure 3-26A), the following ranking is obtained: *R. oryzae* ZB > (*R. oryzae* EN = *R. oligosporus* Tebo) > *R. oryzae* Fi > *R. oryzae* Mala. Interestingly, this ranking is not different from those based on the true protein and soluble protein contents of the fermented cassava bagasse mash before the optimisation was carried out (Figure 3-3). Thus, the relative growth performance between the *Rhizopus* strains were not affected by the fermentation conditions before or after optimisation, indicating strain-specific differences in the ability to utilise the cassava bagasse substrate for the fungal growth.

4.4.3.1 Mycelium biomass

All of the five selected *Rhizopus* strains formed very compact mycelial biomasses of 5.72–8.40 g/100 g DW initial cassava bagasse. This value is higher than the result estimated by Sparringa and Owens (1999d) for mature soybean tempe fermented for 46 hours, which was 5.4 g mycelium of *R. oligosporus* NRRL 2710 per 100 g initial dry soybean cotyledons. However, an absolute comparison is impossible, since both results are estimations. The former was estimated using fungal protein content, while the latter was taken from the glucosamine component of the mould. Nonetheless, the comparison serves the additional purpose of showing quantitatively that the fungi, which were originally domesticated to grow on nutritionally rich soybean, could still grow very well on their non-natural, nutritionally poor cassava bagasse supplemented with non-organic minerals.

4.4.3.2 Protein and residual starch contents

The protein contents of the fermented cassava bagasse produced in the present study were related to earlier results and presented in Table 4-3. The cited authors determined protein content using either the cupric hydroxide precipitation technique or the method developed by Lowry et al. (1951). The latter method was shown to overestimate the actual protein content (Gheysen et al. 1985), while the former yielded results very close to that actual proteins values when determined according to the method recommended by the FAO (Maclean et al.

2003). Thus, a direct comparison between the results obtained using the different protein assay methods was impossible. To allow for an approximate comparison, the values of the protein contents determined using Lowry's method must first be converted to values as would have been determined according to the cupric hydroxide method. This was done by multiplying the Lowry's values by a conversion factor of 0.83. The conversion factor was estimated from the work of Gheysen et al. (1985), who measured the protein contents of cassava substrates fermented with different *R. oryzae* strains in solid and liquid systems using both methods (Appendix 7.7).

As clearly seen in Table 4-3, the protein and the residual starch contents of the fermented product obtained in this study, particularly when using *R. oryzae* ZB, are comparable to previous reported results. The values are also similar to the endogenous protein contents of cereal feedstuffs including (given in range) wheat (7.9-17.6%), barley (8.4-15.6%), triticale (8.4-9.2%), wheat bran plus wheat middling (8.7-9.3%), rice bran (10.1-18.3%), sorghum (5.7-14.6%), and corn (6.2-11.0%) (Fontaine et al. 2002). However, the protein contents of the fermented cassava bagasse are certainly below those of protein-rich animal feed ingredients like soybean meal and full-fat soybeans (31.5-53.1%), rapeseed meal (31.7-40.1%), sunflower meal (25-49%), field peas (16.9-26.4%), fish meal (45.5-78.0%), meat meal products (35.3-66.5%), and poultry by-product meal (49.5-70.3%) (Fontaine et al. 2001). Hence, as far as protein content is concerned, the cassava bagasse tempe could potentially be used to substitute for cereals as components of animal feed.

The protein content in the fermented cassava bagasse discussed so far does not represent the net protein increase that is the actual fungal protein quantity contributed solely by the *Rhizopus* growth on the cassava bagasse substrate. When the net protein increases due to mycelial formation were considered, lower values of 2.7–4.0% were obtained. Thus, the seemingly high protein contents in the fermented products were in fact contributed to a large proportion by the 36–43% loss of substrate dry matter, which was released as carbon dioxide (Stuart and Mitchell 2003).

Table 4-3: Protein enrichment of cassava products through SSF with *Rhizopus* spp.

Reference, fermentation substrate, fermentation period	Strain[1]	Protein content of fermented product		Net protein increase [5]	Residual starch[6]
		as stated in the reference[2,3]	equivalent CH value[2,4]		
Present study, pregelatinised cassava bagasse, 120 hours	*Ro* EN	7.73 (CH)	-	2.98	31.89
	Ro Fi	6.79 (CH)	-	2.71	29.39
	Ro Mala	6.59 (CH)	-	2.76	32.64
	Rl Tebo	7.69 (CH)	-	3.00	28.31
	Ro ZB	9.15 (CH)	-	3.98	27.9
Soccol et al. (1995c), raw cassava bagasse, 24 hours	*Ro* 28627	9.4–13.7 (CH)	-	NA	27.9–31.0
Soccol et al. (1993a), differently pretreated cassava, 26 hours	*Ra* MUCL 28168	10.9–13.9 (L)	9.05–11.54	5.02–7.11	NA
Soccol et al. (1994a), raw and cooked cassava, 48 hours	*Ra* MUCL 28168	10.96–12.30 (L)	9.10–10.21	NA	NA
	Rd ATCC 34612	10.93–14.10 (L)	9.07–11.70	NA	NA
	Ro MUCL 26827	10.53–13.80 (L)	8.74–11.45	NA	NA
Daubresse et al. (1987), gelatinised cassava, 65 hours	*Ro* MUCL 28627	10.77 (CH)	-	6–7	49.74

[1] *Ro*: *Rhizopus oryzae*, *Rl*: *Rhizopus oligosporus*, *Ra*: *Rhizopus arrhizus*, *Rd*: *Rhizopus delemar*.

[2] Expressed as g protein/100 g DW sample.

[3] CH: determined using the cupric hydroxide method, L: determined using Lowry method.

[4] Estimated by multiplying the values determined using the Lowry's method with the conversion factor of 0.83 (Appendix 7.7).

[5] Protein increase to the fungal growth alone, expressed as g protein/100 g DW initial substrate.

[6] Expressed as g reducing sugar/100 g DW sample.

4.4.3.3 Loss of dry matter

The loss of substrate dry matter during fermentation was also partly contributed by the 62–66% reduction in carbohydrates, which were metabolised by the growing fungus as a carbon and energy source. This amount was somewhat higher than earlier published results. In similar studies involving cassava substrates fermented with *R. oryzae* MUCL 28627, Daubresse et al. (1987) reported an approximately 32% loss of dry matter and circa 30% carbohydrate utilisation during 65 hours fermentation. These discrepancies could lie in the longer fermentation duration, the different *Rhizopus* strains used, the substrate's origin from different cassava processing industries and cassava varieties, the bioreactor designs employed or other fermentation aspects that were not optimised in these studies.

Loss of dry matter also occurred during *Rhizopus* sp. cultivation on its natural substrate, soybeans. It amounted (on the percentage basis of the dry weight of the initial cotyledons) to 2% at 28 hours, 9% at 46 hours and 16.5% at 72 hours (Sparringa and Owens 1999d). When the incubation was extended to 180 hours, Ruiz-Teran and Owens (1996) found the over-fermented soybean tempe to have lost nearly 22% of the initial dry material. This value was nearly 50% lower than the results of the present studies. However, unlike in the fermentation of starchy cassava and cassava bagasse, where up to 66% of the utilised starch serves as the main energy source, lipids are considered to be the primary energy source during soybean tempe fermentation (Ruiz-Teran and Owens 1996; Sparringa and Owens 1999d), whose oxidation and utilisation account for the greater part of the total dry matter loss. This can represent up to 70% and 80% of the total dry matter loss at 46 and 72 hours, respectively (Sparringa and Owens 1999d). Other sources of dry matter loss come from proteins as well as other unidentified compounds in considerable quantities (Ruiz-Teran and Owens 1996). The unknown compounds might have been carbohydrates, which make up around 40% of the soybean total dry matter (Table 4-1). The reported decrease in starch content following soybean fermentation (Van der Riet et al. 1987; Olanipekun et al. 2009) might indicate this possibility. Van der Riet et al. (1987) additionally suggested that the observed reduction in soy starch level due to *Rhizopus* growth may be nutritionally important; a question which deserves further research, given the low content of starch in soybeans, which is less than 1 percent (Reddy et al. 1984).

In the 120-hour cassava bagasse fermentation, virtually all of the supplemented urea was depleted. About 19–29% of the initial nitrogen content, externally added in the form of urea and

ammonium sulphate (totalling 2.2 g nitrogen/100 g DW initial cassava bagasse), was converted to fungal protein. During the fermentation of soybeans, which contain 40% protein (equivalent to approximately 7.2 g protein nitrogen/100 g initial dry cotyledon), only a fourth of the initial protein content was hydrolysed within 46 hours. Of this hydrolysed protein (about 1.8 g nitrogen/100 g initial dry soybean cotyledons), only 25% was assimilated into fungal biomass, mostly as fungal protein (Sparringa and Owens 1999d). Thus, in *Rhizopus* fermentation using the two different substrates, the moulds were estimated to have utilised similar amounts of nitrogen per 100 g of initial dry substrate. This suggests that the fungi could assimilate nitrogen from soy protein (peptides, amino acids), as well as from ammonium sulphate and urea in their biomass well. This confirms previous findings about the ability of *Rhizopus* in utilising both organic and inorganic nitrogenous compounds as nitrogen source (Sorenson and Hesseltine 1966; Graham et al. 1976; Seaby et al. 1988; Graffham et al. 1995).

4.4.3.4 Residual ammonium

The fermented cassava bagasse obtained here contained ammonia at higher concentrations than found in soybean tempe as reported by previous authors (Table 4-4). Sparringa and Owens (1999d) found in their studies on soybean tempe that ammonia accumulation reached 0.10, 0.22 and 0.45 g/100 g initial dry soybeans after incubation periods of 28, 46 and 72 hours, respectively. The authors stated their results to be similar to those obtained by Murata et al. (1967), van Buren et al. (1972) and Ruiz-Teran and Owens (1996). However, after careful examination of the cited literature (Table 4-4), their claim seems to be only partially true. As indicated in the table, in mature tempe fermented for 36-48 hours, the ammonia contents reported by Sparringa and Owens (1999d) were indeed close to that reported by Van Buren et al. (1972), but higher than that reported by (Murata et al. 1967; Ruiz-Teran and Owens 1996). The lower ammonia content obtained by the latter authors could be caused by ammonia disappearance during freeze-drying, a method which was not employed by Van Buren et al. (1972) and Sparringa and Owens (1999d). Ammonia removal from experimental samples using freeze-drying is a technique that has been employed in various studies (Spiro 1967; Spiro 1969; Bauriedel et al. 1971; Hetenyi et al. 1984; McConnell et al. 1991). Thus, similar situation might also account for the unidentified loss of nitrogen from the samples used in the present experiments, where the loss amounted to 20-26% of initially supplemented nitrogen or 0.44-0.59 g/100 g DW initial substrate.

Assimilation in other fungal non-protein nitrogenous biomolecules such as chitin, chitosan, and nucleic acids might also account for the nitrogen loss. The nitrogen content of chitin and chitosan in the *Rhizopus* spp. cell walls can be estimated from the content of their principal component glucosamine ($C_6H_{13}NO_5$, MW 179.17 g/mol). Based on previous studies (Farley 1991; Sparringa and Owens 1999b), glucosamine content of *R. oligosporus* was determined to be 5.1-11.1 g/100 g dry fungal biomass. It means that 0.40-0.89 g nitrogen was assimilated in glucosamine per 100 g DW fungal biomass. Since in the present studies 5.67–8.33 g *Rhizopus* biomass was produced per 100 g DW initial substrate, the nitrogen content assimilated in the glucosamine is estimated to be 0.023-0.074 g/100 g DW initial substrate. Using similar calculation and basing on the 2.2% nucleic acid content of *Rhizopus arrhizus* as determined by Omar and Li (1993), *Rhizopus* biomass obtained in this study is estimated to contain 0.125-0.183 g nucleic acid/100 g DW initial substrate. By considering guanine ($C_5H_5N_5O$, MW 151 g/mol), which is the nucleic acid with the highest proportion of nitrogen content, this is equivalent to 0.058-0.085 g nucleic-acid nitrogen/100 g DW initial substrate. Thus, the total amount of nitrogens assimilated in the glucosamine and nucleic acid components of the *Rhizopus* spp. used in the present study is estimated to be 0.081-0.159 g/100 g DW initial substrate. This is still much below the value of the unidentified nitrogen loss, which is 0.44-0.59 g/100 g DW initial substrate.

A clue coming from the pH and free ammonium profiles (Figure 3-18 and Figure 3-19) might provide a third explanation for such a large nitrogen loss. As seen in the graphs, a rise in ammonium concentration and a dramatic increase in pH to above 7 were observed during the first 24 hours of incubation. This might suggest a phase in which a very active hydrolysis of urea occurred, producing considerable amounts of ammonia. During this period, the rate of ammonium uptake by the growing *Rhizopus* mycelium might still have occurred slowly. This could lead to a rapid accumulation of free ammonia and the subsequent abrupt rise in pH to above 7.0, causing a significant portion of the ammonia to be released into the atmosphere.

A fourth alternative explanation for the missing nitrogen is that it might represent ammonium that was already converted into free amino acids. This unfortunately cannot be verified, since free amino acid content was not quantified in this study. However, a similar situation was reported by Sparringa and Owens (1999d), who calculated nitrogen mass balance in soybean tempe fermentation. The authors termed the unidentified nitrogenous compounds as filterable nitrogen other than ammonia, and believed it to be amino acids and small peptides. Although

Table 4-4: Ammonia content in soybean and cassava bagasse tempe.

Reference, (Fermentation substrate)	Treatment of fermented sample before ammonia extraction	Ammonia extraction method	Ammonia concentration in dried fermented sample (g/100g)	Net increase of ammonia in dried initial substrate (g/100g)
Murata et al. (1967) (soybean)	Freeze drying, grinding to powder	NA	0.023 (0 hr) 0.143 (48 hr) 0.148 (72 hr)	0.000 (0 hr)[1] 0.108 (48 hr)[1] 0.100 (72 hr)[1]
Van Buren et al. (1972) (soybean)	Air drying, grinding to powder	NA	0.05 (0 hr) 0.47 (36 hr) 0.59 (72 hr)	0.00 (0 hr)[1] 0.40 (36 hr)[1] 0.44 (72 hr)[1]
Ruiz-Teran and Owens (1996) (soybean)	Freeze drying, grinding to powder	Homogenising with purified water, heating 10 minutes in 60°C-warm water	NA	0.06 (36 hr)
Sparringa and Owens (1999d) (soybean)	Fresh sample	Homogenising with perchloric acid, adjustment to pH 7.	0.005 (0 hr)[2] 0.219 (48 hr)[2] 0.449 (72 hr)[2]	0.000 (0 hr) 0.214 (48 hr) 0.444 (72 hr)
Present study (cassava bagasse)	Freeze drying, grinding to powder	Homogenising with purified water.	0.12 (0 hr) 1.26-1.64 (120 hr)	0.000 (0 hr) 1.14-1.52 (120 hr)

[1] Calculated using the formula as described in Appendix 7.3. Loss of dry matter was not provided by the corresponding authors and was therefore estimated using the results of similar studies by other authors (Appendix 7.8).

[2] Estimated by the ammonia nitrogen x 17/14 (17 and 14 represent the molecular weights of ammonia and nitrogen, respectively).

the proteolytic activities of *Rhizopus* were considered responsible for the release of free amino acids in the fermentation of protein-rich soybeans (Baumann and Bisping 1995), the same explanation cannot be applied to the fermentation of cassava bagasse, which contains only a very low quantity of protein. If the great proportion of the missing nitrogen was in fact truly present as free amino acids, how was it then produced by the fungi grown on cassava ba-

gasse? A concrete explanation should be provided here, namely that the superfluous, toxic concentration of intracellular ammonium might have forced the fungi to conduct detoxification by excreting amino acids. This mechanism was already demonstrated in yeasts by Hess et al. (2006). The authors found that in yeast cells, extremely high levels of external ammonium can lead to an influx of ammonium through potassium channels, increasing the intracellular ammonium level. Detecting the toxic level of ammonium, yeast cells react by releasing amino acids extracellularly in abundant quantities approximately equivalent to the extent of ammonium toxicity. The nitrogen in the expelled amino acids is not taken up via the potassium channels and the cell is in this sense detoxified.

Trevelyan (1974) warned about the residues of unassimilated ammonium salts in *Rhizopus* fermentation involving cassava as a substrate. The issue seems not to be addressed by later authors, with the exception of Daubresse et al. (1987). The latter authors found a minute amount of urea after 65 hours fermentation of cassava initially supplemented with 3.4% urea. When a higher urea dose (4.5%) was used, which was equivalent to 2.1 g nitrogen/100 g DW initial substrate, an increase in protein content was observed by the authors. However, they also reported that 19-20% of the added nitrogen remained unassimilated. Assuming this all to be in the form of ammonium, this residual nitrogen is equivalent to about 1.10–1.16 g ammonium/100 g DW initial substrate. Their findings regarding urea and residual nitrogen were confirmed by the results of the present study in which 3.4% urea and 3.6% ammonium sulphate, which was equivalent to 2.35 g nitrogen/100 g DW initial substrate, were supplemented to the substrate prior to the fermentation. Using five different *Rhizopus* strains, 120-hour fermentation depleted the initial amount of urea almost completely to below 30 ppm, while at the same time producing a residual ammonium level of 1.33–1.74 g/100 g DW initial substrate (or 2.25–2.72 g ammonium/100 g DW fermented sample).

The unassimilated ammonium that is present in similar high amounts in the fermented cassava bagasse in the present study and in the fermented cassava obtained by Daubresse et al. (1987) has already been mentioned above. It is likely to be contributed partly by the ammonium from the supplemented ammonium sulphate. When ammonium-free sulphur sources such as sodium sulphate or magnesium sulphate were used instead, the residual ammonium was reduced to as low as 0.8-0.9%. Although supplementing cassava substrate with magnesium sulphate in their fermentation using *R. oryzae*, Daubresse et al. (1987) emphasised the compound's role as a magnesium and not as a sulphur source. Nonetheless, the fermented product they ob-

tained contained 10.77% total real nitrogenous matter (TRNM) and 15.08% total nitrogenous matter (TNM). These two values are used synonymously in the present work with true protein content and crude protein content, respectively. The difference between TRNM and TNM is 4.31%, and is assumed to represent the unassimilated nitrogen in the form of residual ammonium. When this value is divided by the conversion factor 6.25 and multiplied by 18/14 (molecular weight of ammonium/molecular weight of nitrogen), it yields 0.89% (or 0.89 g ammonium/100 g DW fermented sample). This value is close to 0.88% and 0.79%, obtained in the present study when either magnesium sulphate or sodium sulphate was used as the sole sulphur source, respectively (Table 3-8).

Published literatures did not report any negative health effect caused by ammonia through the consumption of soybean tempe in the course of its dietary use for hundreds of years. Soybean tempe intake rate in Indonesia is 19-34 g daily per person (Sayogyo in Hermana et al. 1990; cited in Nout and Kiers 2005). Assuming the rate is given on the basis of DW sample, and that there is 0.22% ammonium in mature tempe fermented for 48 hours (Sparringa and Owens 1999d), then the daily intake of ammonium is equivalent to about 42-75 mg ammonia (or 44-79 mg ammonium) per person. This is equivalent to 0.7– 1.3 mg ammonium per kg body weight, based on the 63 kg average body weight of Indonesian adults (21–25 years old) (Sumirtapura et al. 2002). This very low amount of ammonia as well as its possible reduction through cooking might explain why it poses no health risk to the consumers. Toxicological problems associated with ammonia also have not been specifically reported for tempe fed to experimental animals including piglets (Kiers et al. 2003; Kiers et al. 2006), growing pigs (Zamora and Veum 1979b), and rats (Zamora and Veum 1979a; Watanabe et al. 2006; Watanabe et al. 2008). However, it would be worth investigating in future studies whether ammonia has a detrimental effect on animals fed with diets containing the cassava bagasse tempe; a maximum safety limit should also be determined. This is important since the ammonia in the cassava bagasse tempe could reach a level that is much higher than that in mature soybean tempe, as demonstrated in the present study (Table 4-4).

4.4.3.5 Extended fermentation period

An investigation was not carried out in the present study regarding the reasons why the cassava bagasse fermentation took 120 hours to attain such a maturity as described for soybean tempe (Ruiz-Teran and Owens 1996). This fermentation period is much longer than the 24-65 hours reported in previous similar studies (Table 4-3). This relatively slow growth of the

fungi might result from those fermentation parameters not optimised in the present study. The carbon dioxide and oxygen composition of the fermentation chamber is an example which has been shown to be growth limiting for *Rhizopus* (Soccol et al. 1994b; De Reu et al. 1995; Han and Nout 2000). Forced or active aeration was also not employed during the investigation, meaning that volatile compounds released during fermentation, such as ammonia, were not actively removed. This might have caused an accumulation of ammonia to a level high enough to slow spore germination and/or fungal growth of the *Rhizopus* spp., thus prolonging the maturation period. Negative effects of ammonia on fungi have often been reported, and its removal by, for instance, forced air flow has been shown to alleviate the problem (Leal et al. 1970).

4.5 Water-soluble vitamins

It is not possible to compare the individual values of the water-soluble vitamins measured here to those from previously published results due to possible differences in the analytical procedures used. It is known that various vitamin analytical procedures may yield highly variable results for a given sample. For example, results that were 38–55% lower were obtained for folic acid determination in soybean tempe using HPLC compared to values determined via a microbiological assay using *Lactobacillus casei* (Ginting and Arcot 2004). Similarly, Kall (2003) used both the HPLC and microbiological methods to quantify vitamin B_6 in foods. His results showed that the HPLC values were circa 20% higher for fruits and vegetables and 70% higher for animal foodstuffs, but approximately 20% lower for grain products than those determined using the microbiological method. Considering this fact, the results presented here are compared to the previous values without intending to make any absolute comparison. However, it is interesting to note the difference and similarities in vitamin enrichment brought about by the *Rhizopus* fermentation of different substrates.

4.5.1 Enrichment of water-soluble vitamins

The results of the analyses of water-soluble vitamins done on samples fermented with the five selected *Rhizopus* strains showed that no single strain produced all of the vitamins at the highest level. The opposite is also true, that no single strain was found to be the poorest producer of all the vitamins. Each strain has its own vitamin profile, indicating that different *Rhizopus* strains have different abilities to synthesise different vitamins. Similar findings were reported previously when *Rhizopus* spp. were grown on soybeans (Keuth and Bisping 1993).

As shown in Table 4-5, the values of the six water-soluble vitamins measured in the fermented cassava bagasse were comparable to those seen in mature soybean tempe. This indicates that the vitamins were able to be synthesised de novo by *Rhizopus* spp. on cassava bagasse equally as well as on soybeans. However, when the vitamin values after fermentation are compared to those of the initial unfermented substrates, it is clear that not all of the vitamins were increased by the growth of the fungi. Comparing the individual value of thiamine in the fermented product to that of the corresponding unfermented substrate, a difference was observed; both fermented cassava and fermented cassava bagasse contained higher levels of thiamine than the unfermented ones. In contrast, all of the fermented soybeans have lower thiamine levels than the unfermented soybeans, probably indicating thiamine metabolism by the fungi. As already discussed previously in Section 4.4.2, the thiamine in the soybeans might be utilised by the fungi as a sulphur source.

Table 4-5 clearly indicates that the thiamine level decreased during soybean tempe fermentation in previously published studies. A decrease in thiamine during soybean fermentation with *Rhizopus* sp. was also reported by Kao and Robinson (1978) as well as by Keuth and Bisping (1993), who used 14 *Rhizopus* strains from the species of *R. oryzae*, *R. oligosporus* and *R. stolonifer*. Murata et al. found that the thiamine level increased in the first 24 hours of soybean fermentation, and then decreased at 48 and 72 hours to levels below that of the unfermented soybeans. A lower concentration of thiamine was also reported following *Rhizopus* fermentation to produce cowpea tempe (Prinyawiwatkul et al. 1996), bambara groundnut tempe (Fadahunsi 2009), and wheat tempe (Wang and Hesseltine 1966).

In contrast, either unchanged or increased levels of thiamine were reported in tempe made from chickpeas, horse beans, and cassava. Robinson and Kao (1977) claimed that the thiamine contents of their chickpea and horse bean tempes were 1.01 and 1.06 times higher than those of the unfermented substrates, respectively. In other words, there was virtually no change in the thiamine content. The authors obtained the values by calculating the ratio between the vitamin content after and before fermentation, based on mg thiamine per 100 g organic matter. Their claim is hardly verified, since the absolute values of the vitamin contents were not presented in their published paper; nor was the loss of dry matter during fermentation. If the latter parameter had been numerically significant, thiamine levels would have possibly been lower in the fermented samples. Daubresse et al. (1987) found higher thiamine

Table 4-5: Contents of water-soluble vitamins in soybean and cassava tempe.

Reference, FS, IP[1]	Vitamin content (µg/g DW fermented substrate)[2]					
	Thiamine	Riboflavin	Niacin	Pyridoxine	Biotin	Folic acid
Present study, cassava bagasse, 120 hr	0.49–1.18 (0.11)	8.45–11.13 (2.05)	184.1–261.0 (5.0)	0.98–2.89 (0.07)	0.71–1.21 (0.03)	0.18–0.33 (0.18)
Daubresse et al. (1987), cassava, 65 hr	2.75 (2.48)	NA	NA	NA	NA	NA
Roelofsen and Talens (1964), soybean, 48 hr	3.8 (5.5)	10 (3.4)	NA	NA	NA	NA
Murata et al. (1967), soybean, 48 hr	1.3–1.6 (2.2–2.6)	4.9–14.1 (0.3–0.6)	43.9–43.1 (2.1–9.0)	3.5–5.8 (0.4–0.8)	NA	NA
Wiesel et al. (1997), soybean, 36 hr	0.8 (1.1)	2.6–10.4 (1.0)	170–230 (10.7)	7.1–10.0 (7.1)	0.51–0.53 (0.18)	0.30–0.55 (0.2)
Van der Riet et al. (1987), soybean, 48 hr	NA	2.2–2.4 (0.6)	28.2–29.7 (26.5–29.0)	NA	NA	NA

[1] FS: fermentation substrate, IP: incubation period.

[2] Values in parentheses represent vitamin contents of the unfermented substrate.

concentrations in their *Rhizopus* fermented cassava than in cassava before fermentation. They also reported a 32% loss in the dry matter of the fermented cassava. Thus, taking this dry matter loss into account to recalculate their net thiamine gain, which is the actual quantity of the thiamine contributed solely by the growth of the fungi, and expressed per initial substrate dry weight, the result would be 1.87 ppm compared to the 2.48 ppm before fermentation. In another word, there was a reduction of 0.61 ppm. All of these results were in contrast with those of the present study, where a net increase in thiamine content was observed across all of the *Rhizopus* strains used.

The decrease of thiamine in soybean tempe fermentation might indicate the metabolism of thiamine endogenously present in soybeans by the growing fungi as a sulphur source. Sul-

phur-containing amino acids methionine and cystine (a dimer consisting of two cysteine amino acid molecules), as the current work has demonstrated, could also be used by *Rhizopus* spp. as a sulphur source in the absence of inorganic sulphate salts. These two sulphurous amino acids are, together with typtophan, known to be the most limiting in soybean (Krishnan 2005), amounting to 0.55 and 0.60 g/100 g, respectively (Padgette et al. 1996). Only about 10% of the protein in soybeans was hydrolysed by the mould during the 46 hour tempe fermentation (Sparringa and Owens 1999d), lowering its total availability as a sulphur source for fungal growth to approximately 28 mg sulphur atom/100 g (assuming that other metabolisable sulphur-containing biomolecules in soybeans are qualitatively insignificant). Thus, this low availability of sulphur might restrict thiamine biosynthesis by the fungi, as the sulphur atom of thiamine has been shown to originate from L-cysteine (Tazuya et al. 1987; Velíšek and Cejpek 2007). The insufficient supply of sulphur atoms from methionine and cysteine might even force the fungi to degrade and utilise the thiamine in soybeans as an additional sulphur source for other metabolic activities. The breakdown and metabolism of thiamine has been reported in the bacterium *Rhodococcus rhodochrous* (Kayser et al. 1993) and the mould *Phycomyces blakesleeanus* (Lowry and Chichester 1971). In addition, the thiamine-degrading enzyme thiaminase has been found in bacteria and fungi (Kelleher et al. 1995; Kurtzman Jr 2005; Richter et al. 2009).

In the works of Daubresse et al. (1987), the cassava substrate, containing a combined 0.05% methionine sulfone ($C_5H_{11}NO_4S$, MW 181.21 g/mol) and cysteic acid ($C_3H_7NO_5S$, MW 169.16 g/mol), was supplemented with magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$, MW 246.47) to achieve a final concentration of 0.8%. Combining these three sulphurous compounds together gives an estimated value of 113 mg equivalent elemental sulphur per 100 g DW cassava substrate. In contrast, the sulphur content in the cassava bagasse supplemented with ammonium sulphate prepared in this study (Table 2-9) corresponds to 873 mg/100 g. Thus, the latter value is 31 times higher than that in soybeans and 7 times higher than that in the cassava substrate prepared by Daubresse et al. (1987). This much higher amount of sulphur might be the reason behind the increase in thiamine content during the fermentation of cassava bagasse in the present study and not of the other aforementioned substrates in the previously reported studies (Table 4-5). Sulphate as a source of sulphur has been shown to be used efficiently by the yeast *Saccharomyces cerevisiae* for the biosynthesis of thiamine (Hitchcock and Walker 1961).

Table 4-6 shows the net changes in water-soluble vitamins contributed solely by the metabolic activities of *R. oryzae* EN during the fermentation of soybeans and cassava bagasse. It is clear that the fungi, previously domesticated on soybeans, could synthesise all six water-soluble vitamins using their non-natural, nutritionally poor, cassava bagasse substrate. All of the vitamins measured, with the exception of niacin, were biosynthesised by *R. oryzae* EN in higher quantities when grown on the cassava bagasse than on soybeans. This is possibly because the latter substrate already initially contained the vitamins in higher quantities than the former prior to fermentation (values in parentheses in Table 4-6). The smaller quantities of the vitamins in cassava bagasse might have forced the fungus to produce the necessary vitamins in higher quantities to meet its own growth requirements. In contrast, since the vitamins were already endogenously present in soybeans in sufficient amounts and were readily utilisable for the growing *R. oryzae* EN, the fungus might have had no reason to synthesise additional vitamins. It is not known, however, why the fungus produced more niacin during soybean fermentation than during cassava bagasse fermentation.

Table 4-6: The net changes in water-soluble vitamin contents of cassava bagasse and soybeans fermented with *R. oryzae* EN.

Vitamin (µg/g)[1]	Cassava bagasse fermented for 120 hours (Present study)[2]	Soybeans fermented for 36 hours (Wiesel et al. 1997)[2, 3]
Thiamine	0.19 (0.11)	-0.34 (1.1)
Riboflavin	4.33 (2.05)	1.47 (1.0)
Niacin	144.7 (5.0)	207.7 (10.7)
Pyridoxine	1.32 (0.07)	-0.36 (7.1)
Biotin	0.48 (0.03)	0.30 (0.18)
Folic acid	0.17 (0.18)	0.08 (0.2)

[1] Expressed on the basis of DW initial substrate.

[2] Values in parentheses are vitamin contents in the unfermented substrate.

[3] Estimated using the formula described in Appendix 7.3 with a percentage of dry matter content of 94.97% (estimated as in Appendix 7.8).

4.5.2 Potential alternative feedstuff

Regardless of the *Rhizopus* strain used in this study, the final fermented cassava bagasse contained water-soluble vitamins in amounts comparable to those found in common feedstuffs (Table 4-7). In addition, Table 4-8 shows that cassava bagasse fermented with *R. oryzae* EN could theoretically be used as a feedstuff which could partly or entirely meet the requirements of thiamine, riboflavin, niacin, pyridoxine, biotin and folic acid in the diets of chickens, turkeys, quails, and fish. The "cassava bagasse tempe", when used as the sole feedstuff, is clearly not sufficient to supply the prescribed amount of dietary thiamine, whereas only small fraction of it is needed to meet the recommended dosage of biotin. The use of only fermented cassava bagasse without additional ingredients should suffice to fulfil the need for riboflavin, niacin, and biotin in the diets of domesticated birds and almost all of the fish mentioned in Table 4-8. The cassava bagasse tempe obtained in this study may therefore constitute an alternative feed that contains high contents of certain vitamins.

Table 4-7: Vitamin contents of feedstuffs (Albers et al. 2002b; Combs Jr. 2008a) compared to the fermented cassava bagasse obtained in this study.

Feedstuff	Vitamin (average value per kg of feedstuff)					
	Thiamine (mg)	Riboflavin (mg)	Niacin (mg)	Pyridoxine (mg)	Biotin (mg)	Folic acid (mg)
Corn and cob meal	NA	1.1	20	5	0.05	0.3
Corn germ meal	NA	3.7	42	NA	3	0.7
Corn gluten feed	2	2.2	66	NA	0.3	0.2
Corn gluten meal	NA	1.5	50	8	0.15	0.7
Corn gluten meal, 60% protein	NA	1.8	60	9.6	0.2	0.84
Soybean meal	4	3.3	27	8	0.32	3.6
Soybean meal, dehulled	NA	3.1	22	8	0.32	3.6
Soybeans, full-fat, processed	NA	2.6	22	11	0.37	2.2
Fish meal, anchovetta	NA	6.6	64	3.5	0.26	0.2
Fish meal, herring	NA	9	89	3.7	0.42	0.24
Fish meal, menhaden	NA	4.8	55	3.5	0.26	0.2
Fish meal, pilchard	NA	9.5	55	3.5	0.26	0.2
Fish meal, redfish waste	NA	NA	NA	3.3	0.08	0.2
Fish meal, whitefish waste	NA	9	70	3.3	0.08	0.2
Rapeseed meal	3	3.7	155	NA	NA	NA
Rice bran	NA	2.6	300	NA	0.42	NA
Coconut oil meal (copra meal)	NA	3.5	24	4.4	6.6	0.3
Wheat bran	8	3.1	200	10	0.48	0.78
Fermented cassava bagasse[1]	0.69 (0.49-1.18)	9.96 (8.45-11.13)	228 (184-261)	2.06 (0.98-2.89)	0.88 (0.71-1.21)	0.31 (0.18-0.33)

[1] Values in parentheses represent a range obtained after cassava bagasse was fermented for 120 hours with five different *Rhizopus* strains: *R. oryzae* EN, Fi, Mala, ZB, and *R. oligosporus* Tebo.

Table 4-8: Estimated vitamin requirements of selected domestic animals according to the Committee on Animal Nutrition of the U.S. National Research Council (Combs Jr. 2008b).

Species		Vitamin, units per kg of diet[1]					
		Thiamine (mg)	Riboflavin (mg)	Niacin (mg)	Pyridoxine (mg)	Biotin (µg)	Folic acid (mg)
Chickens	Growing chicks	1.8 *(31%)*	3.6 *(309%)*	27 *(967%)*	2.5-3 *(81%)*	0.1-0.15 *(592,120%)*	0.55 *(113%)*
Chickens	Laying hens	0.8 *(69%)*	2.2 *(506%)*	10 *(2,610%)*	3 *(81%)*	0.1 *(888,180%)*	0.25 *(248%)*
Chickens	Breeding hens	0.8 *(69%)*	3.8 *(293%)*	10 *(2,610%)*	4.5 *(54%)*	0.15 *(592,120%)*	0.25 *(248%)*
Quails	Growing coturnix	2 *(28%)*	4 *(278%)*	40 *(653%)*	3 *(81%)*	0.3 *(296,060%)*	1 *(62%)*
Quails	Breeding coturnix	2 *(28%)*	4 *(278%)*	20 *(1,305%)*	3 *(81%)*	0.15 *(592,120%)*	1 *(62%)*
Turkeys	Growing poults	2 *(28%)*	3.6 *(309%)*	40-70 *(373%)*	3-4.5 *(54%)*	0.1-0.2 *(444,090%)*	0.7-1 *(62%)*
Turkeys	Breeding hens	2 *(28%)*	4 *(278%)*	30 *(870%)*	4 *(61%)*	0.15 *(592,120%)*	1 *(62%)*
Fishes	Carp	NA	7 *(159%)*	28 *(932%)*	5-6 *(40%)*	NA	NA
Fishes	Catfish	1 *(55%)*	9 *(124%)*	14 *(1,864%)*	3 *(81%)*	20 *(4,441%)*	5 *(12%)*
Fishes	Coldwater spp.	10 *(6%)*	20 *(56%)*	150 *(174%)*	10 *(24%)*	NA	NA

[1]Numbers in parentheses represent the percentage weight of each vitamin requirement that would theoretically be met if a diet consisted of 100% cassava bagasse fermented with *R. oryzae* EN.

5 Summary (Zusammenfassung)

5.1 Summary

The present work studies the protein and water-soluble vitamin enrichment of cassava bagasse by solid substrate fermentation (SSF) using edible filamentous fungi *Rhizopus* spp. Both the substrate cassava bagasse and the microorganisms *Rhizopus* spp. were first subjected to proximate analysis and strain selection, respectively, before both were used in combination in a fermentation system. Cassava bagasse, a solid waste of the tapioca starch processing industry, was found to be nutritionally poor, consisting (in g/100 g) mainly of starch (70.17) and fibres (11.24), with very low contents of crude protein (1.21), crude lipids (0.18), and ash (1.24). After screening on cassava bagasse media, five out of twenty-eight *Rhizopus* strains were selected as the best growing strains, namely *R. oryzae* Fi, Mala, EN, ZB and *R. oligosporus* Tebo. An optimisation of the fermentation conditions using *R. oryzae* ZB was subsequently carried out and the following optimal conditions were obtained: incubation temperature of 27-30°C, inoculum density of 10^2–10^4 spores/g initial substrate, initial moisture content of 68%, initial substrate's pH of 5.3–6.6, fermentation period of 120 hours, and mineral supplementation (in g/100 g initial substrate) with potassium dihydrogen phosphate (1.7), ammonium sulphate (3.6), and urea (3.4). The resulting fermented product, termed cassava bagasse tempe, had physical characteristics very similar to its mature soybean tempe counterpart. Depending on the *Rhizopus* strain used, the initial protein content was increased from 1.45 to 6.6–9.2 g/100 g. The fermentation also resulted in the enrichment (in µg/g) of thiamine (from 0.11 to 0.49–1.18), riboflavin (from 2.05 to 8.45–11.13), pyridoxine (from 0.07 auf 0.98-2.89), niacin (from 5.0 to 184.1–261.0), and biotin (from 0.03 to 0.71–1.21). No increase in folic acid was measured except when *R. oryzae* EN was used as the fermenting fungus, where the concentration increased from 0.18 to 0.62 µg/g. Since these protein and water-soluble vitamin contents were comparable to those of certain conventional feedstuffs, cassava bagasse tempe can thus be viewed as a potential alternative for animal feed.

5.2 Zusammenfassung

Die vorliegende Arbeit untersucht die Anreicherung von Protein und wasserlöslichen Vitaminen in Maniokbagasse durch Feststofffermentation mit dem essbaren Schimmelpilz *Rhizopus*. Zunächst wurden das Maniokbagasse-Substrat und die *Rhizopus* spp. Pilze jeweils kurzcharakterisiert und ausgewählt, bevor die beiden in einem Fermentations-System kombiniert

verwendet wurden. Die Maniokbagasse ist ein fester nährstoffarmer Abfall der Maniokstärke verarbeitenden Industrie, und durchschnittlich bestehend aus (in g/100 g) Stärke (70,17) und Ballaststoffen (11,24) mit sehr niedrigen Gehalten an Protein (1,21), Lipiden (0,18) und Asche (1,24). Nach dem Screening auf den Maniokbagasse-Medien wurden fünf von achtundzwanzig *Rhizopus* Stämmen als die am besten wachsenden Stämme ausgewählt, nämlich *R. oryzae* Fi, Mala, EN, ZB und *R. oligosporus* Tebo. Optimierungen der Fermentationsbedingungen wurden mit *R. oryzae* ZB durchgeführt und die folgenden optimalen Fermentationsbedingungen erreicht: Eine Inkubationstemperatur von 27-30°C, eine Inokulum-Dichte von 10^2-10^4 Sporen/g Anfangssubstrat, ein anfänglicher Feuchtigkeitsgehalt von 68%, ein Anfangs-pH-Wert im Substrat von 5,3-6,6, eine Fermentationsdauer von 120 Stunden und eine mineralische Supplementierung (in g/100 g des ersten Substrates) mit Kaliumdihydrogenphosphat (1,7), Ammoniumsulfat (3,6), und Harnstoff (3,4). Das daraus resultierende fermentierte Produkt, Maniokbagasse-Tempe genannt, hatte sehr ähnliche physikalische Eigenschaften wie reifes Sojabohnen-Tempe. Abhängig von dem verwendeten *Rhizopus*-Stamm wurde der ursprüngliche Gehalt des Proteins von 1,45 auf 6,6-9,2 g/100 g erhöht. Die Fermentation führte ebenfalls zu einer Anreicherung (in µg/g) bei Thiamin (von 0,11 auf 0,49-1,18), bei Riboflavin (von 2,05 auf 8,45-11,13), bei Pyridoxin (von 0,07 auf 0,98-2,89), bei Niacin (von 5,0 auf 184,1-261,0), und bei Biotin (von 0,03 auf 0,71-1,21). Eine Erhöhung der Folsäure wurde nicht beobachtet, ausser wenn *R. oryzae* EN als fermentierender Pilz verwendet wurde, in denen die Konzentration von 0,18 auf 0,62 µg/g erreicht wurde. Da diese Gehalte an Proteinen und wasserlöslichen Vitaminen mit einigen konventionellen Futtermitteln vergleichbar waren, kann Maniokbagasse Tempe somit eine mögliche Alternative für die Tierernährung sein.

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7 Appendix

7.1 Hazardous chemicals

7.1.1 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

Chemicals	Hazard symbol	Risk phrase	Safety phrase
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
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	S26, S30, S45
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

7.1.2 Abbreviation and description of hazard

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 levels 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

7.1.3 Risk phrases and description of risk

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

Abbreviation	Description of risk
R41	Risk of serious damage to eyes
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 long-term adverse effects in the aquatic environment
R51/53	Toxic to aquatic organisms, may cause long-term 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

7.1.4 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

7.2 Calculation of an analysed substance from the standard curve

Standard curves were constructed from absorbance values (or the area under the HPLC chromatogram peaks for thiamine and riboflavin) plotted against known concentrations of standard solutions using a scatter plot (Excel 2002 Software, Microsoft, USA). A trend line and an equation for each analysis were then generated as linear or second order polynomial regressions (Table 7-1, Figure 7-1).

Table 7-1: Regressions applied to the standard curves used for analyses.

Substance analysed	Regression for standard curve
Reducing sugar, residual carbohydrate, residual urea, residual ammonium, total free cyanide, thiamine, and riboflavin.	Linear
Soluble protein, niacin, biotin, pyridoxine and folic acid	Second order polynomial

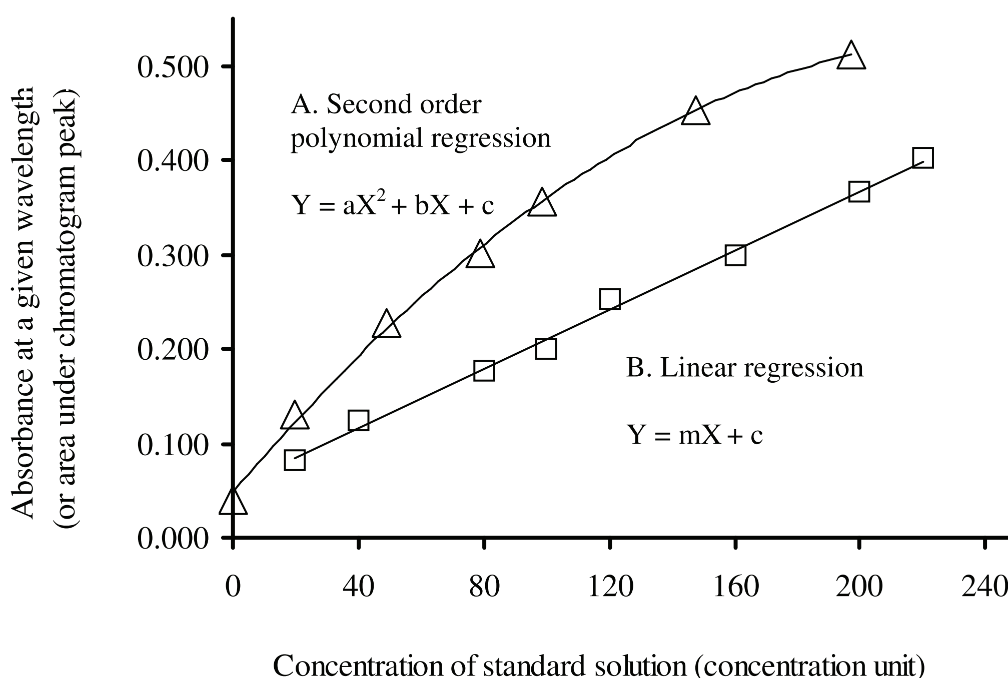

Figure 7-1: Second order polynomial (A) and linear standard curve (B).

Using the equations described below, the concentration of an analysed substance was calculated:

Linear standard curve:

$$Y = mX + c$$

thus

$$X = \frac{(Y - c)}{m}$$

Second order polynomial curve:

$$Y = aX^2 + bX + c$$

thus

$$X = \frac{-b + \sqrt{b^2 + 4a(Y - c)}}{2a}$$

y	=	absorbance at a given wavelength, absorbance unit (in case of thiamine and riboflavin the area under the chromatogram peak, mV.min)
x	=	concentration of the measured substance in the liquid test sample, concentration unit
a, b, c, m	=	real numbers

Final concentration in the initial solid sample is given by:

$$C_S = \frac{X \times V \times DF \times CF}{M \times R}$$

C_S	=	concentration of the analysed substance, expressed on the basis of DW sample
X	=	concentration of the measured substance in the liquid test sample, concentration unit
V	=	volume of the aqueous liquid, in which the analysed substance was dissolved, volume unit
DF	=	dilution factor
CF	=	conversion factor, to obtain C_s in the desired concentration unit
M	=	weight of the initial solid, freeze dried sample, weight unit
R	=	recovery rate, relevant only for calculating water-soluble vitamins (Appendix 7.15). For the other measurements, it was assumed that the measured substances were 100% recovered in the aqueous extracting liquid (hence, R = 1).

7.3 Net changes in protein, vitamins and ammonium

During fermentation, a considerable loss of substrate dry weight occurred. Thus, at the end of the fermentation, any changes in protein or vitamins expressed as the dry weight of the fermented sample (Appendix 7.2) could be, at least to a certain extent, attributed to the loss of substrate dry matter and to the protein and vitamins already initially present in the substrate prior to fermentation. To calculate the quantity of protein and vitamins that was contributed by fungal growth alone, the following equation was used:

$$C_T = (C_S \times \%DM) - C_0$$

C_T	=	net change in protein or vitamin as a result of fungal growth, expressed as g/100 g or µg/g DW initial substrate, respectively.
C_S	=	total concentration of protein or vitamin, expressed as g/100 g or µg/g DW fermented substrate, respectively.
C_0	=	concentration of protein or vitamin in the initial unfermented substrate expressed as g/100 g or µg/g DW initial substrate, respectively.
%DM	=	percentage of dry matter, calculated as in Section 2.4.7

7.4 Carbohydrate utilisation as a result of the growth of *Rhizopus* spp.

During fermentation, carbohydrates, especially starch, were metabolised by the fungi as a source of energy and carbon. To calculate the amount of carbohydrates metabolised by the fungi, the following equation was used:

$$S_T = S_0 - [S_S \times (1 - \%LDM)]$$

S_T	=	amount of carbohydrate utilised by the fungi, expressed as g/100 dry weight of initial substrate.
S_0	=	initial amount of carbohydrate present in the unfermented substrate, expressed as g/100 g DW initial substrate.
S_S	=	residual, unassimilated carbohydrate present in the fermented substrate, expressed as g/100 g DW fermented substrate.
%LDM	=	percentage loss of dry matter, calculated as in Section 2.4.7

7.5 Statistics

Numerical results were presented as

$$\overline{X} \pm S$$

$\overline{X}$	=	mean value
S	=	standard deviation

Measurements and calculations were statistically analysed according to the formulae described in the next subsections. Means and standard deviations were calculated using the statistical functions of Excel 2002 (Microsoft Corp., USA).

7.5.1 Mean (Average value)

$$\overline{X} = \frac{\sum X_i}{N}$$

$\overline{X}$	=	mean value
Xi	=	individual sample value
N	=	number of samples

7.5.2 Standard deviation

$$SD = \sqrt{\frac{\sum \left(X_i - \overline{X} \right)}{N-1}}$$

SD	=	standard deviation
X_i	=	individual sample value
$\overline{X}$	=	mean value
n	=	number of samples

7.5.3 ANOVA (Analysis of Variance)

To analyse the difference between the mean values, an analysis of variance (ANOVA) with a 5% significance level followed by a Fischer-LSD Post-hoc mean comparison test were carried out using STATISTICA software version 8 (Statsoft, Tulsa, USA). When P > 5%, there was

no significant difference between any of the mean values analysed, but when $P < 5\%$, at least one of the means was significantly different from the others. Those mean values found to be significantly different were additionally noted with different alphabetical superscripts.

7.6 Conversion factor to determine fungal biomass of *Rhizopus* spp.

Some authors have published works expressing the protein content of *Rhizopus* sp. mycelium on a dry weight basis of *Rhizopus* sp. mycelium biomass. The biomass was produced using liquid fermentation, which, unlike solid state fermentation, enables the recovery of a virtually substrate-free mycelial biomass of the fungi. The individual values as well as the average value of the protein contents in the dried mycelial biomass of the fungi *Rhizopus* spp. are listed in Table 7-2.

Table 7-2: Protein contents of the *Rhizopus* sp. biomass.

Reference and fermentation characteristic[1]		*Rhizopus* strain	Protein[2]
Omar and Li (1993), culture shake with palm oil medium		*R. arrhizus*	42.81
Jin et al. (1999), starch processing water as substrate, supplemented nutritionally with various compounds listed in the adjacent column on the right.	none	*R. oligosporus* DAR 2710	45.56
	$(NH_4)_2SO_4$	*R. oligosporus* DAR 2710	46.58
	Urea	*R. oligosporus* DAR 2710	46.34
	NH_4NO_3	*R. oligosporus* DAR 2710	47.64
	$NaNO_3$	*R. oligosporus* DAR 2710	45.54
	K_2HPO_4	*R. oligosporus* DAR 2710	48.87
	KH_2PO_4	*R. oligosporus* DAR 2710	47.88
Jin et al. (2001), different culture modes described in the adjacent column on the right.	batch	*R. arrhizus* DAR 2602	49.6
	SC–V_{out}/V_t 0.90[3]	*R. arrhizus* DAR 2602	49.2
	SC–V_{out}/V_t 0.70[3]	*R. arrhizus* DAR 2602	48.8
	SC–V_{out}/V_t 0.50[3]	*R. oligosporus* DAR 2710	47.8
Jin et al. (2002), comprehensive pilot plant system.		*R. oligosporus* DAR 2710	49.7
Average value of protein content from the above 13 individual values:			47.4 ± 2.0

[1] Only main distinct characteristics are cited here. Detailed descriptions of the fermentation systems can be found in the references cited.

[2] Protein content in the *Rhizopus* biomass, expressed as g protein/100 g DW mycelial biomass.

[3] SC: semi continuous, V_{out}: volume drawn out, and V_t: total culture volume.

7.7 Conversion factor for protein content based on Lowry's procedure

The determination of protein content in food based on the sum of individual amino acid residues is recommended by FAO (Maclean et al. 2003). However, the method is lacking in simplicity and practicality.

Table 7-3: Protein contents of cassava substrates cultivated with *R. oryzae* strains in liquid and solid state fermentation obtained by Gheysen et al. (1985).

Culture mode	*Rhizopus* strain	N	Protein content (g/100 g)	
			Folin phenol (Lowry's) assay	Cupric hydroxide precipitation assay
Solid state fermentation	*R. oryzae* MUCL 28627	22	11.65	10.52
	R. oryzae MUCL 28486	3	11.23	9.79
	R. oryzae MUCL 28486	3	15.87	13.73
Liquid fermentation	*R. oryzae* MUCL 9667	4	55.51	40.45
	R. oryzae MUCL 28486	5	48.62	42.48
	R. oryzae MUCL 28627	5	54.44	43.87
	R. oryzae MUCL 49151	3	48.94	44.13
	R. oryzae MUCL 20415	4	46.78	41.39
	R. oryzae MUCL 16179	4	44.55	38.76
	R. oryzae MUCL 28627	4	57.66	47.3
	R. nigricans MUCL 28169	4	48.81	33.9
	Rhizopus sp. MUCL 28627	3	28.83	30.61

Two other commonly employed methods of protein assay are those involving the Folin phenol reagent developed by Lowry et al. (1951) and protein precipitation using cupric hydroxide (Vervack 1973; cited in Gheysen et al. 1985). The latter, which is also described as the AOAC standard method (Horwitz 1965) and closely correlates with the sum of amino acid residues, was used in the present study. To obtain a mathematical relationship between the protein values determined using the cupric hydroxide precipitation method and those from Lowry's method, the results reported by Gheysen et al. (1985) were used (Table 7-3).

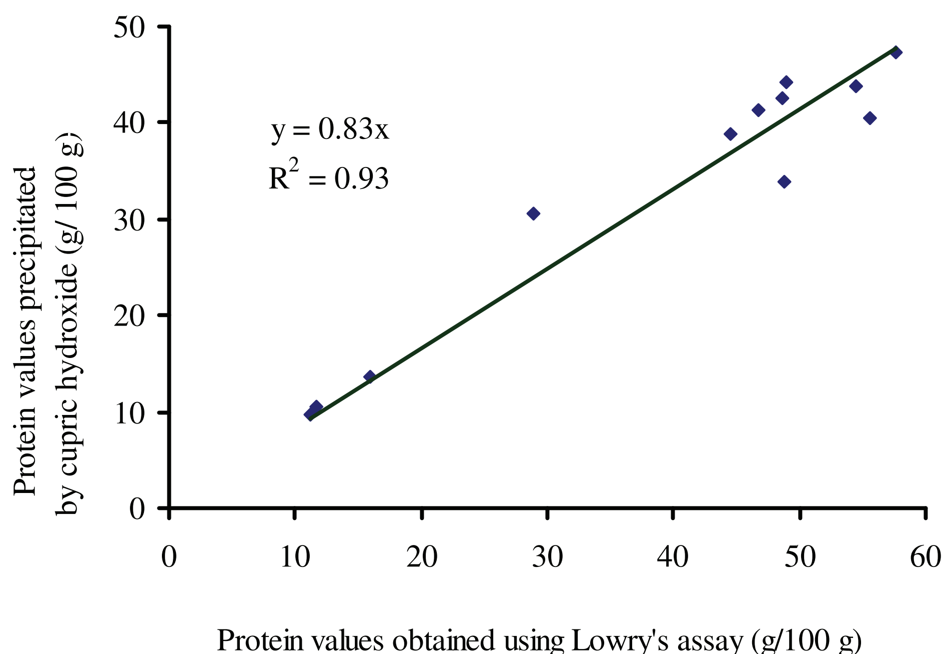

Figure 7-2: Mathematical relationship between protein values obtained by Lowry's method and the cupric hydroxide method (Gheysen et al. 1985), in which a conversion factor of 0.83 was produced.

The Pearson coefficient of correlation (r) between the two series of protein values (Table 7-3) was generated using Excel 2002 software (Microsoft, USA). The value r = 0.97 was obtained, indicating a strong correlation. When the data were plotted and a linear regression was applied, the conversion factor of 0.83 and the mathematical function shown in (Figure 7-2) were obtained, where x = protein values determined using Lowry's method, and y = protein values determined using the cupric hydroxide method.

7.8 Estimation of dry matter content in soybean tempe

The fermentation of soybeans using *R. oligosporus* resulted in the reduction of its dry matter to 98 ± 9 g/(100 g of initial dry cotyledons) at 28 h, 91 ± 11 g at 46 h, and 83.5 ± 15 g at 72 h (Sparringa and Owens 1999d). Plotting these reduction values against the fermentation time, a linear equation is obtained which can be used to estimate the amount of soybean dry matter lost within a given fermentation time (Figure 7-3).

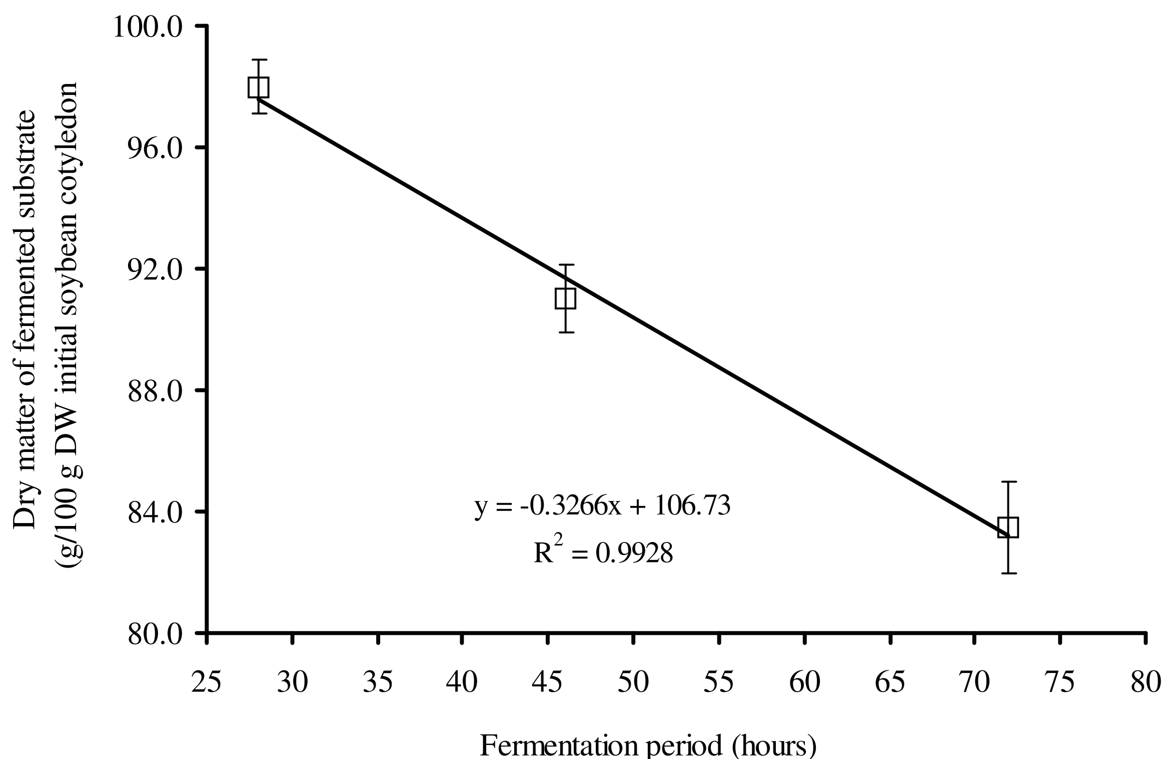

Figure 7-3: Quantity of soybean tempe dry matter as a function of fermentation period, indicating a loss of substrate dry matter due to *Rhizopus* fermentation (Sparringa and Owens 1999d).

The equation shown in the graph was used to estimate the dry matter of the fermented soybeans (*y*) at any given fermentation time (*x*), provided that the time falls within the range of 28-72 hours.

7.9 Results of the soluble protein determination

Table 7-4: Selection on cassava bagasse mash medium.

Rhizopus strain	Soluble protein after 44 hours fermentation[1,2]
Unfermented substrate	0.77 ± 0.02[a]
R. oryzae EN	4.18 ± 0.06[b]
R. oryzae Fi	3.50 ± 0.22[c]
R. oryzae Mala	2.95 ± 0.17[d]
R. oligosporus Tebo	4.16 ± 0.16[b]
R. oryzae ZB	5.37 ± 0.36[e]

[1] For all mean values, N = 4;

[2] Values expressed as mg soluble protein/g DW sample.

Table 7-5: Optimisation of inoculum concentration.

Inoculum density (10^x spores/mL)	Soluble protein after 93 hours fermentation[1,2]
Unfermented substrate	0.55 ± 0.09[a]
X = 1	6.77 ± 0.09[b]
X = 2	6.42 ± 0.40[bc]
X = 3	6.29 ± 0.23[cd]
X = 4	6.69 ± 0.23[b]
X = 5	6.29 ± 0.13[cd]
X = 6	6.01 ± 0.23[d]
X = 7	6.19 ± 0.14[bc]

[1] For all mean values, N = 3.

[2] Values expressed as mg soluble protein/g DW sample.

Table 7-6: Optimisation of ammonium sulphate concentration.

Ammonium sulphate (g/100 g substrate)	Soluble protein after 67 hours fermentation[1,2]
Unfermented substrate	0.24 ± 0.03[a]
0	0.68 ± 0.11[b]
2.0	4.92 ± 0.25[c]
3.8	5.18 ± 0.06[c]
9.1	3.69 ± 0.34[d]
13.8	2.67 ± 0.12[e]
18.0	1.91 ± 0.25[f]

[1] For all mean values, N = 3.

[2] Values expressed as mg soluble protein/g DW sample.

Table 7-7: Optimisation of moisture content.

Moisture content (%)	Soluble protein after 68 hours fermentation[1,2]
Unfermented substrate	0.25 ± 0.03[a]
55	6.47 ± 0.21[b]
60	6.61 ± 0.11[bc]
64	6.80 ± 0.16[c]
68	6.78 ± 0.27[bc]
71	6.07 ± 0.17[d]
73	5.85 ± 0.23[d]

[1] For all mean values, N = 3.

[2] Values expressed as mg soluble protein/g DW sample.

Table 7-8: Optimisation of initial pH.

Initial pH of substrate	Soluble protein after 61 hours fermentation[1,2]
Unfermented substrate	0.27 ± 0.03[a]
3.5	2.43 ± 0.06[b]
4.5	5.40 ± 0.18[c]
5.0	5.19 ± 0.23[c]
5.3	6.02 ± 0.20[d]
6.0	5.89 ± 0.34[d]
6.6	6.08 ± 0.17[d]

[1] For all mean values, N = 3.

[2] Values expressed as mg soluble protein/g DW sample.

Table 7-9: Optimisation of incubation temperature.

Incubation temperature (°C)	Fermentation period (hr.)	Soluble protein[1,2]
27	36	1.19 ± 0.14[a]
27	48	4.17 ± 0.22[b]
27	61	8.06 ± 0.41[j]
30	36	5.06 ± 0.15[c]
30	48	7.95 ± 0.28[ij]
30	61	7.25 ± 0.78[hi]
33	36	7.39 ± 0.26[hij]
33	48	7.19 ± 0.44[gh]
33	61	6.19 ± 0.64[de]
36	36	7.00 ± 0.68[fgh]
36	48	7.02 ± 0.53[fgh]
36	61	6.48 ± 0.37[ef]
39	36	5.63 ± 0.40[cd]
39	48	6.46 ± 0.22[ef]
39	61	6.48 ± 0.21[efg]

[1] For all mean values, N = 3.

[2] Values expressed as mg soluble protein/g DW sample.

Table 7-10: Optimisation of the composition of the mineral salt solution.

Type of mineral solution[1]	Soluble protein after 48 hours fermentation[2,3]
Unfermented substrate	0.22 ± 0.03[a]
1	7.72 ± 0.23[b]
2	6.59 ± 0.19[c]
3	7.74 ± 0.51[b]
4	6.80 ± 0.36[c]
5	7.12 ± 0.33[c]
6	8.12 ± 0.75[b]
7	5.33 ± 0.16[d]
8	10.60 ± 0.23[e]

[1] The mineral composition of each type of solution salt is listed in Table 2-3.

[2] For mean values of the unfermented substrate, N = 3; for all other mean values, N = 4.

[3] Values expressed as mg soluble protein/g DW sample.

Table 7-11: Optimisation of nitrogen source (ratio of urea to ammonium sulphate).

Ratio of urea to ammonium sulphate (%)	Soluble protein after a fermentation period of[1,2]				
	0 hr	24 hr	48 hr	72 hr	120 hr
0:100	0.25 ± 0.06	6.62 ± 0.31	6.70 ± 0.79	6.40 ± 0.14	6.09 ± 0.07
24:76	0.28 ± 0.16	13.59 ± 1.59	22.32 ± 1.92	22.69 ± 1.64	21.66 ± 1.31
48:52	0.18 ± 0.06	13.71 ± 1.07	21.05 ± 1.07	23.32 ± 3.31	24.50 ± 5.18
73:27	0.26 ± 0.12	12.56 ± 0.46	20.20 ± 0.31	22.99 ± 0.28	29.01 ± 1.31
100:0	0.38 ± 0.12	0.88 ± 0.17	0.34 ± 0.11	0.20 ± 0.08	0.27 ± 0.02

[1] For all mean values, N = 3.

[2] Values expressed as mg soluble protein/g DW sample.

7.10 Results of the true protein determination

Table 7-12: Selection on cassava bagasse mash medium.

Rhizopus strain	True protein based on the TCA method of Rajoka et al. (2004) after 44 hours fermentation[1,2]
Unfermented substrate	0.78 ± 0.05[a]
R. oryzae EN	2.01 ± 0.05[b]
R. oryzae Fi	1.84 ± 0.03[c]
R. oryzae Mala	1.51 ± 0.01[d]
R. oligosporus Tebo	2.01 ± 0.03[b]
R. oryzae ZB	2.14 ± 0.06[e]

[1] For all mean values, N = 3.

[2] Values expressed as g protein/100 g DW sample.

Table 7-13: Optimisation of nitrogen source (weight percentage ratio of urea to ammonium sulphate).

Ratio of urea to ammonium sulphate (%)	True protein based on the TCA method of Marais and Evenwell (1983) after 120 hours fermentation[1,2]
Unfermented substrate	0.92 ± 0.07[a]
0:100	2.48 ± 0.16[b]
24:76	7.55 ± 0.07[c]
48:52	9.04 ± 0.11[d]
73:27	8.05 ± 0.01[e]
100:0	1.10 ± 0.04[f]

[1] For all mean values, N = 3.

[2] Values expressed as g protein/100 g DW sample.

Table 7-14: Influence of substrate pretreatment on fungal protein.

Substrate pre-treatment	True protein based on the TCA method of Marais and Evenwell (1983) after 12 hours fermentation[1,2]	
	at 0 hour	at 120 hours
Raw cassava bagasse	0.88 ± 0.18[a]	8.86 ± 0.01[a]
Pregelatinised cassava bagasse	0.75 ± 0.02[ab]	9.00 ± 0.17[b]
Pregelatinised cassava tuber	0.62 ± 0.05[b]	1.81 ± 0.06[b]

[1] For the mean values of the pregelatinised cassava bagasse at 120 hours, N = 2; for all other mean values, N = 3.

[2] Values expressed as g protein/100 g DW sample.

Table 7-15: Influence of salt solution temperature.

Temperature of added salt solution (°C)	True protein based on theTCA method of Marais and Evenwell (1983) after 120 hours fermentation[1,2]
Unfermented substrate	1.63 ± 0.02[a]
90	8.13 ± 0.19[b]
Ambient	7.97 ± 0.21[b]

[1] For the mean values of the unfermented substrate, N = 3; for all other mean values, N = 4.

[2] Values expressed as g protein/100 g DW sample.

Table 7-16: Influence of sulphur source.

Sulphur source	True protein based on theTCA method of Marais and Evenwell (1983) after 120 hours fermentation[1,2]
Unfermented substrate	0.92 ± 0.07[a]
No sulphur	0.98 ± 0.06[ab]
DMSO	1.13 ± 0.09[b]
L-Cystine	8.06 ± 0.16[c]
L-Methionine	7.42 ± 0.04[d]
Magnesium sulphate	7.82 ± 0.07[e]
Sodium sulphate	8.07 ± 0.04[c]
Ammonium sulphate	8.12 ± 0.22[c]

[1] For the mean values of DMSO and ammonium sulphate, N = 4; for all other mean values, N = 3.

[2] Values expressed as g protein/100 g DW sample.

Table 7-17: Influence of *Rhizopus* strain inoculum.

Rhizopus strain	True protein based on thecupric hydroxide method after 120 hours fermentation[1]	
	Total protein content of the fermented substrate[2]	Net protein increase contributed by fungal growth alone[3]
Unfermented substrate	1.45 ± 0.28[a]	0.00 ± 0.00[a]
R. oryzae EN	7.73 ± 0.12[b]	2.98 ± 0.07[cd]
R. oryzae Fi	6.79 ± 0.16[c]	2.71 ± 0.10[b]
R. oryzae Mala	6.59 ± 0.10[c]	2.76 ± 0.06[bc]
R. oligosporus Tebo	7.69 ± 0.06[b]	3.00 ± 0.03[d]
R. oryzae ZB	9.15 ± 0.07[d]	3.98 ± 0.04[e]

[1] For the means values of Tebo and ZB, N = 4; for all other mean values, N = 3.

[2] Expressed as g protein/100 g DW sample.

[3] Expressed as g protein/100 g DW initial substrate.

7.11 Results of the residual carbohydrate determination

Table 7-18: Selection on cassava bagasse mash medium.

Rhizopus strain	Residual carbohydrate after 44 hours fermentation[1,2]
Unfermented substrate	74.51 ± 2.36[a]
R. oryzae EN	69.75 ± 0.55[b]
R. oryzae Fi	72.59 ± 4.51[abc]
R. oryzae Mala	73.87 ± 1.51[ac]
R. oligosporus Tebo	70.39 ± 0.79[bc]
R. oryzae ZB	72.22 ± 2.11[abc]

[1] For all mean values, N = 4.

[2] Expressed as g reducing sugar/100 g DW sample.

Table 7-19: Optimisation of nitrogen source.

Weight ratio of urea to ammonium sulphate (%)	Residual carbohydrate after 120 hours fermentation[1,2]
Unfermented substrate	77.93 ± 3.22[a]
0:100	77.62 ± 2.30[a]
24:76	36.27 ± 4.67[b]
48:52	36.50 ± 1.56[b]
73:27	37.05 ± 1.16[b]
100:0	80.22 ± 12.72[a]

[1] For all mean values, N = 4.

[2] Expressed as g reducing sugar/100 g DW sample.

Table 7-20: Influence of *Rhizopus* strain inoculum.

Rhizopus strain	Residual carbohydrate after 120 hours fermentation[1]	
	Residual carbohydrate in the fermented substrate[2]	Carbohydrate utilisation by fungi[3]
Unfermented substrate	82.54 ± 0.81[a]	0.00 ± 0.00[a]
R. oryzae EN	31.89 ± 4.87[bc]	64.25 ± 0.10[c]
R. oryzae Fi	29.39 ± 0.17[bd]	64.52 ± 1.04[cd]
R. oryzae Mala	32.64 ± 1.63[c]	61.67 ± 0.06[b]
R. oligosporus Tebo	28.31 ± 0.26[d]	66.15 ± 0.15[e]
R. oryzae ZB	27.90 ± 2.06[d]	65.97 ± 1.22[de]

[1] For the mean values of the unfermented substrate, N = 4; for all other mean values, N = 6.

[2] Expressed as g reducing sugar/100 g DW sample.

[3] Expressed as g reducing sugar/100 g DW initial substrate.

7.12 Results of the free reducing sugar determination

Table 7-21: Optimisation of nitrogen source.

Weight ratio of urea to ammonium sulphate (%)	Free reducing sugar released[1,2]				
	0 hr	24 hr	48 hr	72 hr	120 hr
0:100	0.07 ± 0.02	4.08 ± 0.06	3.25 ± 0.01	3.65 ± 0.12	3.18 ± 0.04
24:76	0.05 ± 0.01	6.66 ± 0.22	14.67 ± 0.40	5.99 ± 0.22	0.89 ± 0.09
48:52	0.04 ± 0.01	8.90 ± 0.22	14.16 ± 0.26	4.67 ± 0.07	1.98 ± 0.05
73:27	0.04 ± 0.01	8.14 ± 0.38	13.94 ± 0.57	5.60 ± 0.11	1.55 ± 0.11
100:0	0.01 ± 0.03	0.32 ± 0.02	9.84 ± 0.32	18.28 ± 0.11	24.97 ± 0.18

[1] For all mean values, N = 4.

[2] Expressed as g reducing sugar/100 g DW sample.

Table 7-22: Free reducing sugar profile at 120 hours during the optimisation of nitrogen source.

Weight ratio of urea to ammonium sulphate (%)	Free reducing sugar after 120 hours fermentation[1,2]
Unfermented substrate	0.05 ± 0.02[a]
0:100	3.18 ± 0.04[b]
24:76	0.89 ± 0.09[c]
48:52	1.98 ± 0.05[d]
73:27	1.55 ± 0.11[e]
100:0	24.97 ± 0.18[f]

[1] For all mean values, N = 4.

[2] Expressed as g reducing sugar/100 g DW sample.

7.13 Results of pH measurement

Table 7-23: Influence of substrate pretreatment.

Substrate pretreatment	pH[1]	
	0 hr	120 hr
Pregelatinised cassava bagasse	5.46 ± 0.07	5.59 ± 0.01
Raw cassava bagasse	5.37 ± 0.03	5.68 ± 0.01
Pregelatinised cassava tuber	5.42 ± 0.08	6.74 ± 0.01

[1] For all mean values, N = 3

Table 7-24: Optimisation of nitrogen source.

Weight ratio of urea to ammonium sulphate (%)	pH[1]				
	0 hr	24 hr	48 hr	72 hr	120 hr
0:100	5.35 ± 0.03	2.97 ± 0.01	2.74 ± 0.03	2.69 ± 0.03	2.64 ± 0.04
24:76	5.60 ± 0.03	6.96 ± 0.02	4.49 ± 0.01	3.80 ± 0.03	3.50 ± 0.02
48:52	5.59 ± 0.03	7.39 ± 0.05	4.98 ± 0.02	4.47 ± 0.01	5.58 ± 0.01
73:27	5.89 ± 0.11	7.64 ± 0.07	5.24 ± 0.02	4.92 ± 0.01	5.41 ± 0.08
100:0	7.40 ± 0.19	8.26 ± 0.23	3.75 ± 0.01	3.97 ± 0.01	3.73 ± 0.01

[1] For all mean values, N = 3

7.14 Results of the residual ammonium determination

Table 7-25: Optimisation of nitrogen source.

Weight ratio of urea to ammonium sulphate (%)	Residual ammonium[1,2]				
	0 hr	24 hr	48 hr	72 hr	120 hr
0:100	1.76 ± 0.05	1.35 ± 0.11	1.44 ± 0.07	1.49 ± 0.01	1.36 ± 0.01
24:76	1.28 ± 0.04	1.62 ± 0.01	1.71 ± 0.04	1.65 ± 0.02	1.45 ± 0.11
48:52	1.05 ± 0.08	1.51 ± 0.01	2.22 ± 0.02	2.44 ± 0.16	2.10 ± 0.06
73:27	0.44 ± 0.04	1.15 ± 0.02	1.98 ± 0.12	2.85 ± 0.01	2.46 ± 0.17
100:0	0.13 ± 0.01	0.33 ± 0.05	0.57 ± 0.06	0.39 ± 0.03	0.33 ± 0.08

[1] For all mean values, N = 4.

[2] Expressed as g ammoniuim/100 g DW sample

7.15 Water-soluble vitamin determination

7.15.1 Recovery rate

The mean recovery rate was calculated using the following equation as previously presented by Arella et al. (1996):

$$R\% = \frac{2(X_3 + X_4) - \left(\frac{X_1}{m_1} + \frac{X_2}{m_2}\right)(m_3 + m_4)}{4X_0}$$

R%	=	mean recovery of the added vitamin
m_1, m_2, m_3, m_4	=	weight of samples 1, 2, 3 and 4 (g)
X_1, X_2	=	vitamin content in samples 1 and 2 (µg/mL)
X_3, X_4	=	vitamin content in the spiked samples 3 and 4 (µg/mL)
X_0	=	vitamin content added (spiked) to samples 3 and 4 (µg/mL)

Table 7-26: Recovery rate in the vitamin assay methods.

Water-soluble vitamin	Recovery rate (%)[1]
Thiamine	86.9%
Riboflavin	88.0%
Niacin	107.7%
Biotin	78.0%
Pyridoxine	156.9%[2]
Folic acid	75.3%

[1] Determined according to the formula described above (under Appendix 7.15).

[2] Recovery rates much higher than 100% for microbiological assay of pyridoxine have been previously reported by different authors (Atkin et al. 1943; Hodson 1956) and suggested to be due to the presence of a growth promoting factor for the assay microorganism (Andon et al. 1989).

7.15.2 Representative HPLC chromatograms for thiochrome and riboflavin

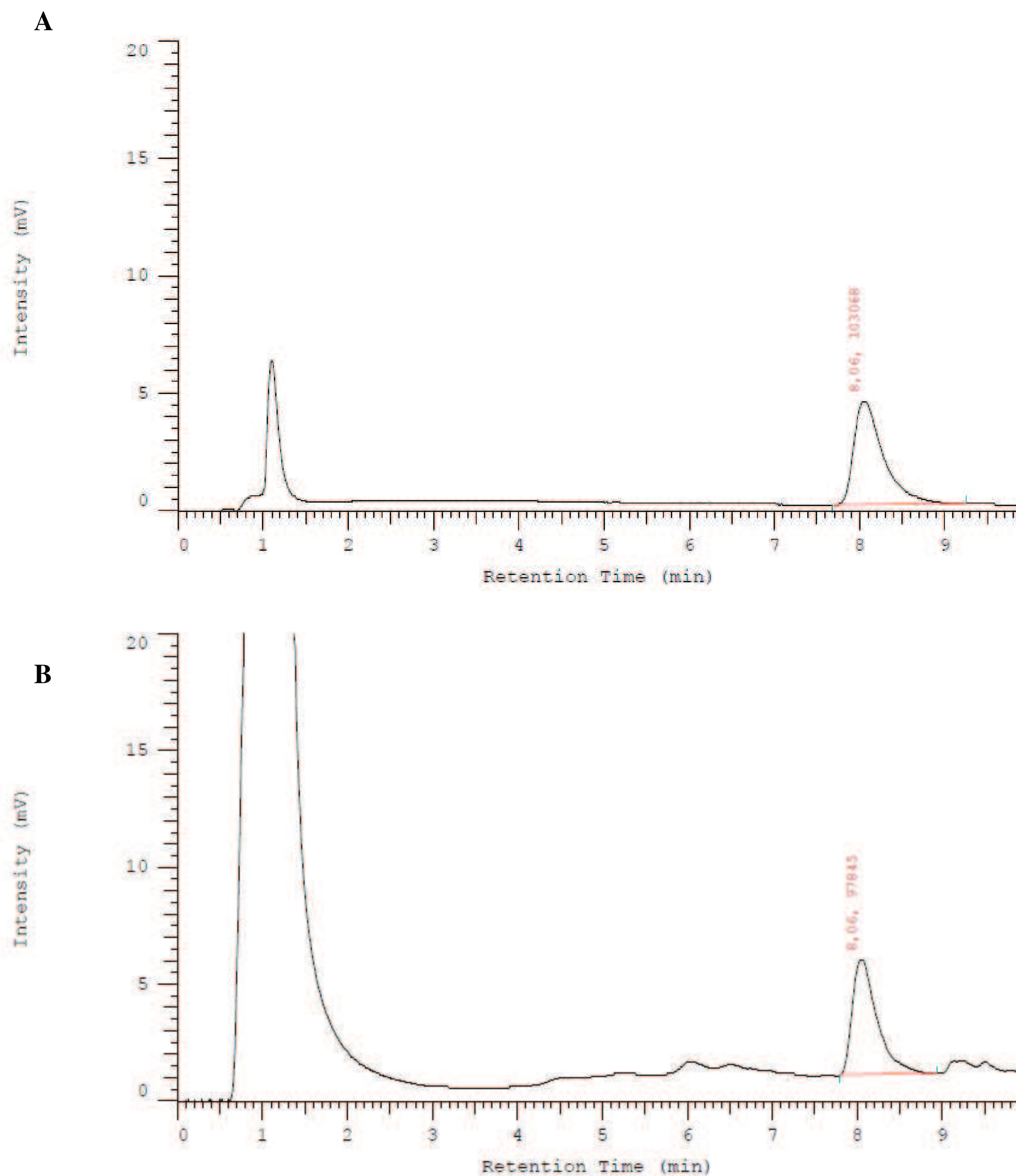

Figure 7-4: HPLC chromatogram representations of thiochrome, which is an oxidised form of thiamine, for 50 pg thiamine standard (A), and for a sample extracted from the cassava bagasse substrate after 120 hours fermentation with *R. oligosporus* Tebo (B). Peaks occurred at a retention time of 8.1 minutes.

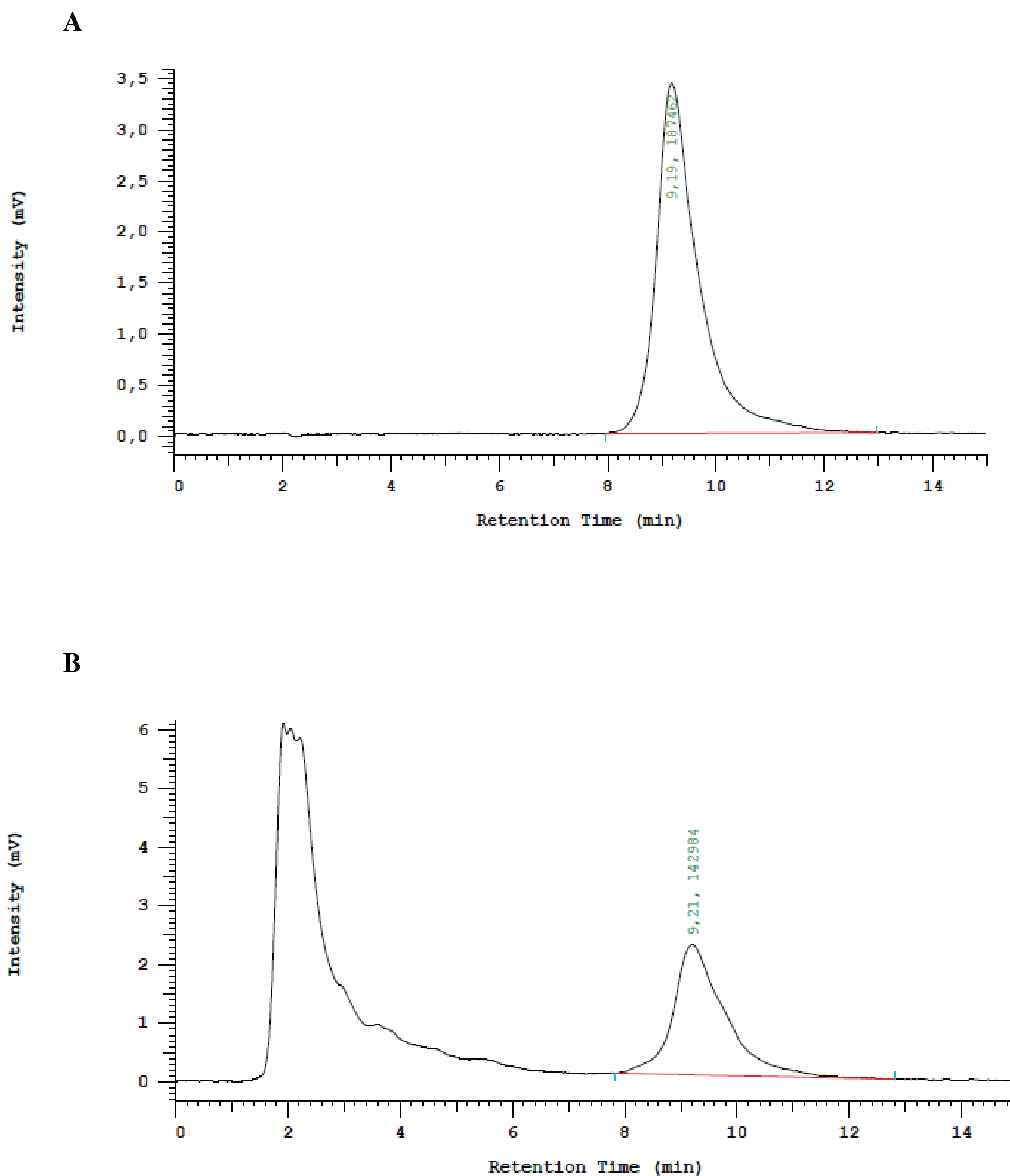

Figure 7-5: HPLC chromatogram representations of riboflavin for 3 ng riboflavin standard (A), and for a sample extracted from the cassava bagasse substrate after 120 hours fermentation with *R. oryzae* Mala (B). Peaks occurred at a retention time of 9.2 minutes.

7.15.3 Representative standard curves for thiamine and riboflavin

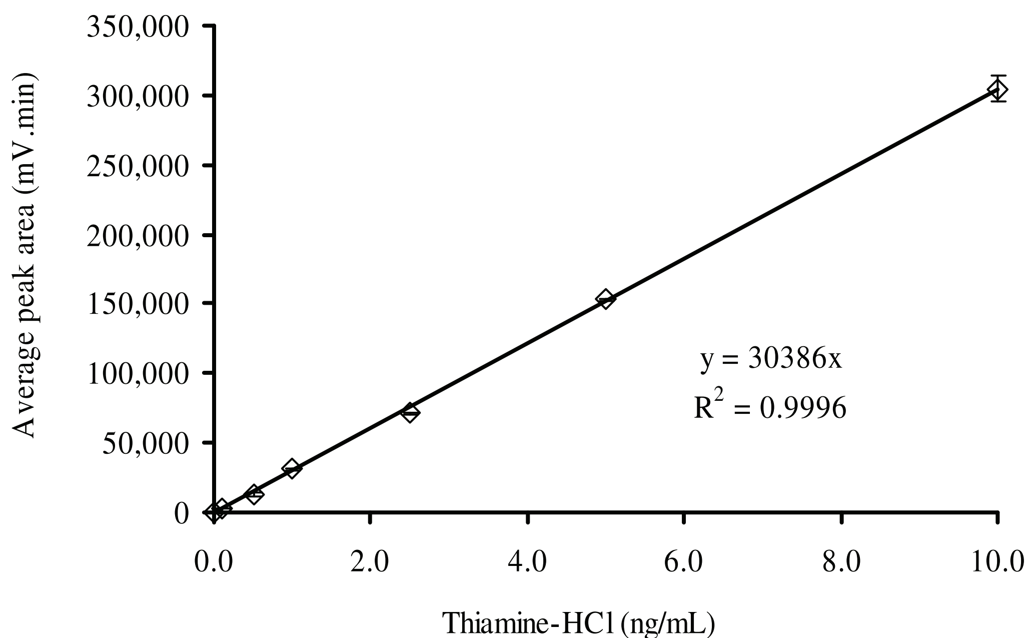

Figure 7-6: A representative standard graph for thiamine.

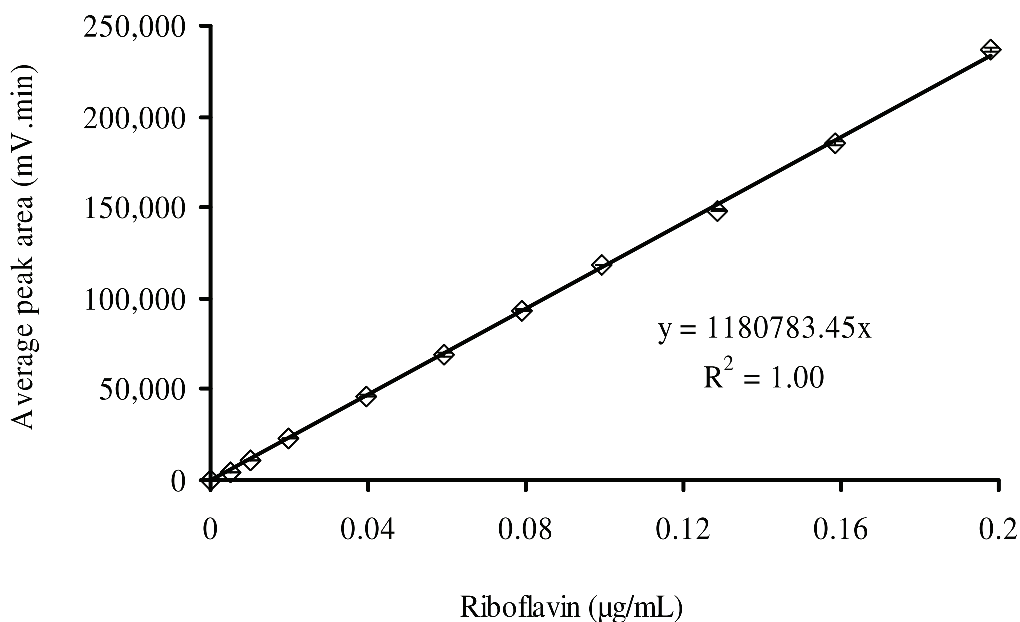

Figure 7-7: A representative standard graph for riboflavin.

7.15.4 Representative standard curves for niacin, pyridoxine, biotin and folic acid

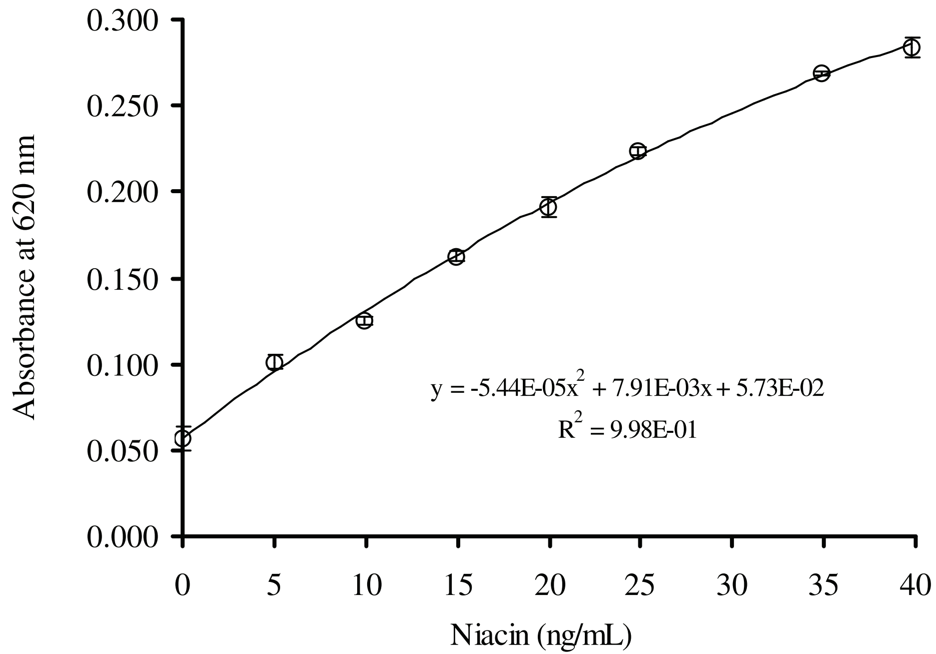

Figure 7-8: A representative standard graph for niacin.

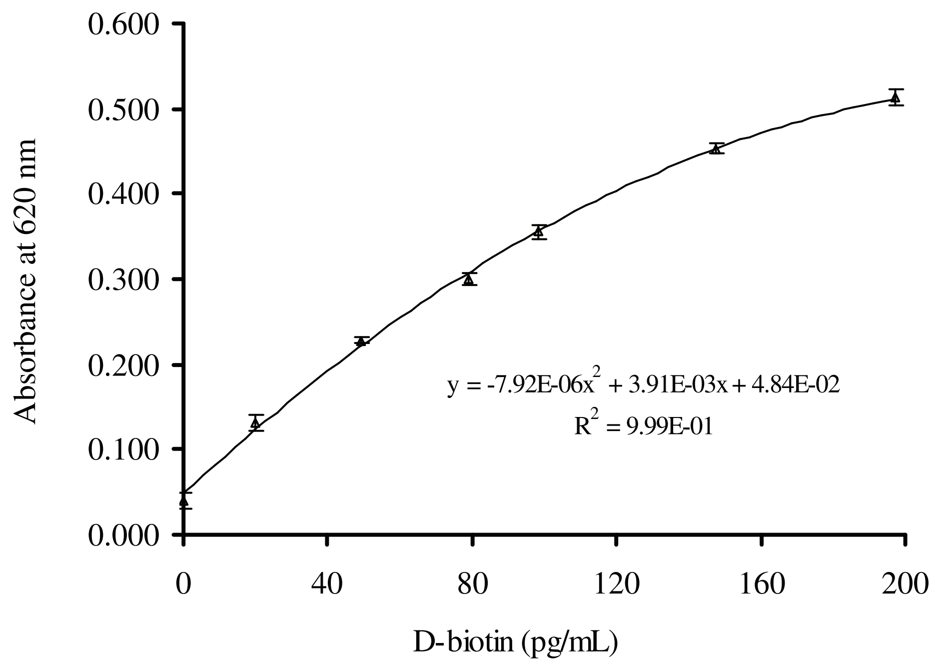

Figure 7-9: A representative standard graph for biotin.

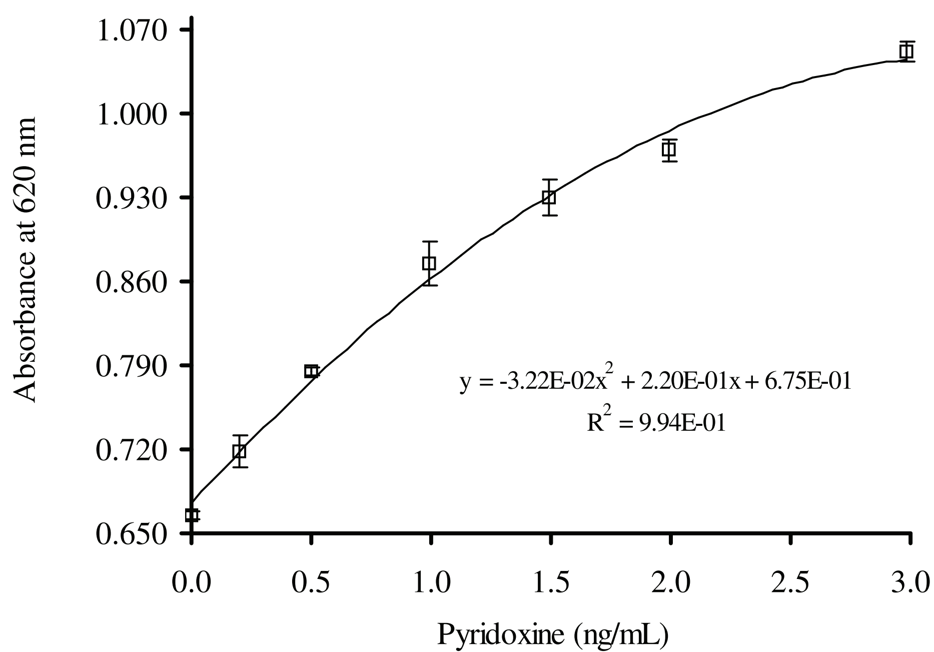

Figure 7-10: A representative standard graph for pyridoxine.

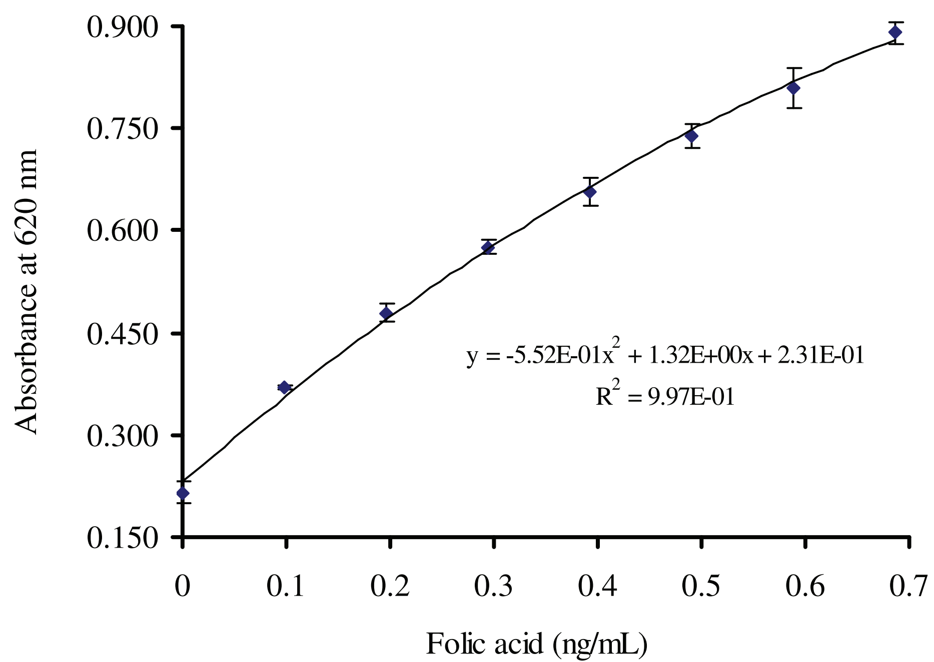

Figure 7-11: A representative standard graph for folic acid.

7.15.5 Results of the analyses of water-soluble vitamins

Table 7-27: Water-soluble vitamin contents of substrates fermented with the five selected *Rhizopus* strains.

Rhizopus strain	Water-soluble vitamin (µg/g)[1,2]					
	Thiamine		Riboflavin		Niacin	
	$\bar{X} \pm S$	N	$\bar{X} \pm S$	N	$\bar{X} \pm S$	N
Unfermented substrate	0.11 ± 0.01^{a} (0.00 ± 0.00^{m})	6	2.05 ± 0.05^{a} (0.00 ± 0.00^{m})	6	5.02 ± 0.28^{a} (0.00 ± 0.00^{m})	18
EN	0.55 ± 0.03^{bc} (0.19 ± 0.03^{n})	6	11.13 ± 0.17^{b} (4.33 ± 0.10^{p})	6	261.01 ± 13.82^{b} (144.67 ± 7.93^{p})	20
Fi	0.61 ± 0.03^{c} (0.27 ± 0.02^{o})	6	9.46 ± 0.10^{c} (3.75 ± 0.06^{o})	6	239.94 ± 14.93^{c} (142.12 ± 9.16^{p})	18
Mala	0.61 ± 0.05^{c} (0.29 ± 0.03^{o})	6	8.45 ± 0.15^{d} (3.36 ± 0.10^{n})	6	184.10 ± 10.43^{d} (112.75 ± 6.67^{n})	18
Tebo	0.49 ± 0.02^{b} (0.18 ± 0.01^{n})	6	10.86 ± 0.05^{e} (4.24 ± 0.03^{p})	6	227.98 ± 15.96^{e} (127.06 ± 9.25^{o})	22
ZB	1.18 ± 0.22^{d} (0.60 ± 0.03^{p})	6	9.90 ± 0.27^{f} (3.83 ± 0.16^{o})	6	227.02 ± 14.06^{e} (129.82 ± 8.35^{o})	19

Rhizopus strain	Water-soluble vitamin (µg/g)[1,2]					
	Biotin		Pyridoxine		Folic acid	
	$\bar{X} \pm S$	N	$\bar{X} \pm S$	N	$\bar{X} \pm S$	N
Unfermented Substrate	0.03 ± 0.00^{a} (0.00 ± 0.00^{m})	14	0.07 ± 0.02^{a} (0.00 ± 0.00^{m})	7	0.18 ± 0.01^{a} (0.00 ± 0.0^{m})	9
EN	0.89 ± 0.04^{b} (0.48 ± 0.03^{p})	16	2.42 ± 0.12^{b} (1.32 ± 0.07^{n})	10	0.62 ± 0.05^{b} (0.17 ± 0.03^{p})	15
Fi	0.71 ± 0.03^{c} (0.40 ± 0.02^{n})	15	1.26 ± 0.08^{c} (0.70 ± 0.05^{o})	6	0.18 ± 0.01^{a} (-0.07 ± 0.01^{o})	7
Mala	0.73 ± 0.03^{c} (0.43 ± 0.02^{o})	16	0.98 ± 0.05^{d} (0.55 ± 0.03^{p})	8	0.24 ± 0.01^{c} (-0.03 ± 0.01^{n})	8
Tebo	0.87 ± 0.05^{b} (0.47 ± 0.03^{p})	16	2.75 ± 0.10^{e} (1.52 ± 0.06^{q})	10	0.33 ± 0.03^{d} (0.01 ± 0.02^{m})	12
ZB	1.21 ± 0.05^{d} (0.69 ± 0.03^{q})	13	2.89 ± 0.16^{f} (1.64 ± 0.10^{r})	12	0.18 ± 0.01^{a} (-0.08 ± 0.01^{o})	8

[1] Values are expressed on the basis of DW sample.

[2] Values in parentheses represent the net vitamin changes due to *Rhizopus* growth alone, and are expressed on the basis of DW initial substrate. Values in parentheses are not compared statistically with those outside parentheses, and negative values indicate a decrease in the vitamin contents.

8 Curriculum vitae

Personal Data

Name:	Catur Sriherwanto
Place, date of birth:	Surabaya, Indonesia, May 11, 1975

Primary & secondary education

1981 - 1987	Primary school:	SD Hang Tuah VIII Surabaya, Indonesia
1987 - 1990	Junior high school:	SMP Negeri 24 Surabaya, Indonesia
1990 - 1993	Senior high school:	SMA Negeri 6 Surabaya, Indonesia
1994 - 1995	A level:	Tresham Institute Kettering, Northamptonshire, UK

Graduate education

1995 - 1998	Bachelor of Science:	Biochemistry & Biotechnology, Sheffield University, The United Kingdom
1998 - 2000	Master of Science:	Biotechnology (4 Semesters Joint Program), Bandung Institute of Technology, Indonesia with 2 Semesters in New South Wales University, Australia
2005 - 2010	PhD student:	at The Department of Food Chemistry, University of Hamburg, Germany

Professional jobs

2001 - 2002	Research assistant:	Plant cell culture laboratory, Biotech Centre, Centre for the Assessment and Application of Technology (BPPT), Serpong, Indonesia
2002 - 2005	Research assistant:	Veterinary microbiology laboratory, Biotech Centre, Centre for the Assessment and Application of Technology (BPPT), Serpong, Indonesia

Academic work

1998	Bachelor of Science thesis:	Site-Directed Mutagenesis of the Ce3 Domain of Human Immunoglobulin E
2000	Master of Science thesis:	Development of Fed-Batch Strategy for Recombinant CHO Cells Adapted to Serum-Free Conditions

Language skills

Indonesian	Fluent, mother language
English	Proficient in writing and speaking
German	Advanced level

List of Publications

Poster publications

Sriherwanto C, Koob C, Bisping B (2007) Studies on the role of tempe mould *Rhizopus* sp. in nutrition enhancement of solid cassava waste for potential use as animal feed (Poster), presented at The 8th VAAM Symposium of "Molecular Biology of Fungi", MBF 2007, 23-26 September 2007, Biozentrum Klein-Flottbek, Hamburg, Germany.

Sriherwanto C, Koob C, Bisping B (2008) Roles of urea and ammonium sulphate in the fermentation of cassava bagasse with *Rhizopus oryzae* (Poster), presented at The 21st International ICFMH Symposium "Evolving Microbial Food Quality and Safety," 1-4 September 2008, Aberdeen, United Kingdom.

Sriherwanto C, Koob C, Bisping B (2009) Cassava bagasse fermented by *Rhizopus* spp. for potential use as animal feed (Poster), presented at The 14th European Congress on Biotechnology, 13-16 September 2009, Barcelona, Spain.

Journal publication

Water-soluble vitamin contents of cassava bagasse fermented by *Rhizopus* spp. for potential use as animal feed (Scientific paper). In preparation.

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbständig verfasst habe und die angegebenen Quellen und Hilfsmittel verwendet habe. Ich habe vorher weder die vollständige Dissertation noch Teile der Dissertation an anderer Stelle eingereicht. Dies ist mein erster Promotionsversuch, um den Doktorgrad zu erlangen.

Declaration

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.

Catur Sriherwanto